（H30-化学―一般-001）厚生労働科学研究費補助金（化学リスク研究事業）
(総合)研究報告書

化学物質の動物個体レベルの免疫毒性データ集積とそれに基づくMulti-ImmunoTox assay（MITA）による予測性試験法の確立と国際標準化　（　30210101　）

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研究要旨
本課題においては，1)我々が開発した多項目免疫毒性評価系 Multi-ImmunoTox Assay (MITA)の免疫毒性化学物質評価法としての OECD テストガイドライン化に向けて国際 validation 試験ならびに 2)免疫毒性化学物質のデータベース作成を行ってきた。1）においては，MITA を構成する試験法の一つである IL-2 Luc assay に関して validation 試験、validation report の作成、peer review panel の評価を終了し 2020 年 11 月に SPSF を OECD に提出した。また IL-1 Luc assay に関しても phase I, phase II の validation 試験を終了し，2020 年海外からの liaison 委員を交えた validation management team (VMT) 会議の意見を参考にして validation report を作成し現在 liaison 委員からの意見をとりまとめている。一方，2）においては，上記 validation 試験にて評価した 50 化学物質，validation report 作成にあたり MITA にて評価した 60 化学物質に関して免疫毒性データを収集し免疫毒性データベースを構築した。また MITA の OECD テストガイドライン申請に向けて作成中の in vitro 免疫毒性試験法の現状と MITA の有用性に関する detailed review paper 作成に協力するとともに Section VIII にて MITA の概略を紹介した。

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A．研究目的

研究背景：

アレルギー、自己免疫、免疫抑制など、人体に有害な影響を及ぼす化学物質による免疫毒性は、消費者、生産者はもとより厚生労働行政にとっても重大な課題となっている。現在、免疫毒性評価のゴールドスタンダードは動物実験であるが、数万ともいわれる化学物質を網羅的に評価、管理するには、*in vitro* high throughput 評価系や *in silico* 評価系の構築が不可欠である。そのためには、化学物質のアレルギー発症、易感染性など個体レベルの免疫毒性データの集積、その分子メカニズムの解析、さらにはそれらに基づいた adverse outcome pathway の作成が不可欠である。

我々は，平成18–22年NEDO「高機能簡易型有害性評価手法の開発」プロジェクトにおいて、化学物質の免疫毒性多項目評価システム(Multi-ImmunoToxicity assay；MITA)を構築し国内外の特許を取得している。

また平成24年度から平成26年度の3年間にわたる厚生労働科学研究費補助金事業「多色発光細胞を用いたhigh-throughput免疫毒性評価試験法の開発」においては、作用機序の明らかな種々の免疫抑制剤をMITAにより評価するなかで､化学物質免疫毒性評価におけるMITAのプロトコールを作成し、そのプロトコールに基づいて薬剤の免疫毒性評価を行った。その結果、代表的な免疫抑制剤であるデキサメサゾン(Dex)、サイクロスポリン(CyA)、タクロリムス（Tac）のT細胞とマクロファージ/樹状細胞に対する薬理効果をMITAが予測できることを明らかにした(Kimura et al. 2014)。

さらに平成27年度以降は、皮膚感作性試験法IL-8 Luc assayとMITAを組み合わせたmodified MITAを構築し60種類の化学物質を評価しdata setを作成した。また、そのdata setを基に化学物質のclusteringを行い、化学物質が免疫毒性のprofileの違いにより6つのグループに分類できることを示した(Kimura et al. 2018)。さらに、研究期間中にIL-8 Luc assayをOECDテストガイドライン化することができた(OECD442E) (OECD 2018)。

計画全体の目的：

1）既にOECD テストガイドライン(442E)に承認されている IL-8 Luc assay に加え、MITA を構成する IL-2 転写活性抑制評価試験(IL-2 Luciferase reporter assay; IL-2 Luc assay)と IL-1β転写活性抑制評価試験(IL-1 luciferase reporter assay; IL-1 Luc assay)の国際 validation study を行い、MITA の多項目免疫毒性評価系として OECD テストガイドライン化を目指す。

2)National Toxicology Program (NTP) の Dori Germolec 博士とミラノ大学の Emanuela Corsini 博士の協力を仰ぎ、NTP ならびに European Centre for Ecotoxicology and Toxicology of Chemicals のデータベースおよび PubMed を利用した文献検索に基づき免疫毒性のデーターベースを構築する。

3)上記データベースに基づき，MITA（図 1）を用いた化学物質の免疫毒性別クラスター分類における各クラスター免疫毒性の特性を明らかにする。

2018 年度

① 免疫毒性化学物質の毒性データーベースの構築
② MITA による免疫毒性 clustering の有用性の検討
③ IL-2転写活性抑制試験 (IL-2 Luc assay)に関するvalidation 試験の最終評価ならびにOECD提出用validation report作成
④ IL-1β転写活性抑制試験(IL-1 Luc assay)に関するPhase 0ならびにPhase I validation試験.
⑤ MITA を用いた免疫毒性評価系国際化へ向けての国際評価会議の kick-off meeting の開催

2019 年度

① IL-2転写活性抑制試験 (IL-2 Luc assay)に関するvalidation reportに対するpeer review panelによる評価とそれに対する対応

② IL-1β転写活性抑制試験(IL-1 Luc assay)に関する Phase I, Phase II validation 試験と Validation management teamによる最終評価
③ IL-1 Luc assay，IL-2 Luc assay により多種類の化学物質を評価し data set を作成する。
④ 免疫毒性化学物質のデータベース作成
⑤ MITA による免疫毒性 clustering の有用性の検討
⑥ MITA を用いた免疫毒性評価系国際化へ向けて，detailed review paper 作成を目的とした国際会議の開催

2020 年度
① IL-2転写活性抑制試験 (IL-2 Luc assay) のOECDテストガイドライン化に向けて, SPSFを提出し申請手続きを開始する。
② IL-1β転写活性抑制試験(IL-1 Luc assay)に関しては，validation試験を終了しVMT会議における意見を参考にvalidation reportを作成し，review panelによる評価を受ける。
③ MITA の有用性を確認する目的で，MITA による化学物質の評価を引き続き行い data set の拡充を図る。
④ MITA では評価できない，骨髄抑制，リンパ球増殖抑制をきたす化学物質を評価する IL-2 依存性細胞株を用いた試験法の開発を行う。
⑤ MITA の複数項目に関して効果発現最低濃度 (Lowest observed effect level ; LOWEL)を基にクラスター分類を行い, それにより化学物質の免疫毒性特性を明らかにする。
⑥ MITA の OECD テストガイドライン化にむけて in vitro 免疫毒性試験に関する detailed review paper の作成に協力する。
⑦ IL-8 Luc assay の特異性を改善するためのプロトコールの改変と OECD への修正ガイドラインの提案、ならびに THP-G8 細胞をより長期安定性を確保する目的での人工染色体を用いた新たな細胞株を樹立する。

B．研究方法

2018 年度

① IL-2 Luc assayに関するvalidation 試験の最終評価ならびにOECD提出用validation report作成

既にOECD テストガイドライン(442E)に承認されているIL-8 Luc assayに加え、MITAを構成するIL-2 Luc assay(国際validation phase I、IIが既に終了)の最終結果の総括とvalidation reportを作成する。

② IL-1 Luc assayに関するPhase 0ならびにPhase I validation試験

IL-1β 転写活性抑制評価系の国際 validation study を行い、MITA を多項目免疫毒性評価系として OECD テストガイドライン化を目指す。

③ 免疫毒性物質データベースの作成

National Toxicology Program (NTP)の Dori Germolec 博士とミラノ大学の Emanuela Corsini 博士の協力を仰ぎ、NTP ならびに European Centre for Ecotoxicology and Toxicology of Chemicals のデータベースおよび PubMed を利用した文献検索に基づき個体レベルの免疫毒性の網羅的データーベースを構築する。

④ MITA による免疫毒性 clustering の有用性の検討

一方、我々はこれまでに多項目免疫毒性評価系 (MITA)を開発し、その data set の作成、有用性の検討、国際標準化へむけての validation 等を行ってきた。その中で、60 種類の化学物質を MITA の複数項目に関して効果発現最低濃度 (Lowest observed effect level ; LOWEL)を基にクラスター分類することにより、免疫毒性物質が 6 種類のクラスターに分類できることを明らかにした。そこで、本課題では個体レベルの免疫毒性が明らかな化学物質を MITA による上記 6 種類のクラスターに分類し、クラスターごとの個体レベル免疫毒性発現の特性を明らかにする。

⑤ MITAを用いた免疫毒性評価系国際化へ向けての国際バリデーション実行委員会

平成30年度：2018年10月4-6日、神戸にて第5回国際バリデーション実行委員会会議を行った。

2019 年度

① IL-2 Luc assay validation reportに対するpeer review panelによるコメントとそれに対する対応

以下の会議を開催し，peer review panelからIL-2 Luc assay validation reportに対するコメントが提出され，それらに対応した。

1. 1st International peer review panel meeting on Multi-Immunotoxicity Test Assay (MITA))

2019年2月27-28日，品川

Peer review panel: Henk van Loveren, Haley LaNef Ford, Barbara Kaplan, Sang-Hyun Kim, Fujio Kayama, Takao Ashikaga,

Xingchao Geng
参加者：Hajime Kojima, Yutaka Kimura, Setsuya Aiba
2. 2nd International peer review panel meeting on Multi-Immunotoxicity Test Assay (MITA)(Webex)
2019年10月1日（火）
Peer review panel: Henk van Loveren, Haley Neff-LaFord, Barbara Kaplan, Fujio Kayama, Takao Ashikaga
参加者：Hajime Kojima, Yutaka Kimura, Setsuya Aiba
3. 3rd International peer review panel meeting on Multi-Immunotoxicity Test Assay (MITA) (Webex)
2019年11月18日（月）
Peer review panel: Henk van Loveren, Haley Neff-LaFord, Barbara Kaplan, Lin Shi, Xingchao Geng, Fujio Kayama, Takao Ashikaga
参加者：Hajime Kojima, Yutaka Kimura, Setsuya Aiba

② IL-1 Luc assay Phase IならびにPhase II validation試験

Phase I試験においては，国際バリデーション実行委員会 (VMT)にて選定された５化学物質をコード化し、東北大学，産業技術総合研究所バイオメディカル研究部門，産業技術総合研究所工学研究部門の参加3施設においてMulti-ImmunoTox Assay protocol for TGCHAC-A4 ver. 008Eにのっとり各物質3回繰り返し1セットの試験を３セットと実施した。
Phase II試験においては，VMTにより選定された20化学物質をコード化し、東北大学，産業技術総合研究所バイオメディカル研究部門，産業技術総合研究所工学研究部門の参加3施設においてMulti-ImmunoTox Assay protocol for TGCHAC-A4 ver. 008Eにのっとり各物質3回繰り返し1セットを実施した。
また，validation試験を遂行にあたり以下のVMT会議を行った。

1./ 2019年度第1回MITAバリデーション電話会議 （スカイプ）
2019年4月5日（金）9:30-11:00
参加者：大森、髙木、小島、足利、相場、木村
2./ 2019年度第2回MITAバリデーション電話会議 （スカイプ）
2019年5月2日（木）10:00-12:00
参加者：大森、小島、安野、中島、相場、木村、藤村
3./ Conference call for the MITA assay (Webex)
2019年6月26日（水）20:00-
参加者：Corsini, E., Roggen, E., Germolec, D., Inoue, T., Aiba, S., Kimura, Y., Omori, T., Kojima, H.
4.5th meeting for the MITA Validation study
2020年1月30日（水）10：00-17：00
2020年1月31日（金）10：00-13：00
参加者：Corsini, E., Germolec, D., Inoue, T., Aiba, S., Kimura, Y., Omori, T., Kojima, H., Yasuno, R., Nakajima, Y.

③ IL-2 Luc assay, IL-1 Luc assayのdata set作成

Validation試験で評価した化学物質以外の化学物質もIL-1 Luc assay、IL-2 Luc assayにて評価し，これらの試験法のdata setを作成した。

④ 免疫毒性物質データベースの作成

National Toxicology Program (NTP) の Dori Germolec 博士とミラノ大学の Emanuela Corsini 博士の協力を仰ぎ、NTP ならびに European Centre for Ecotoxicology and Toxicology of Chemicals のデータベースおよびPubMed を利用した文献検索に基づき，validation 試験で用いた化学物質，data set に際して評価した化学物質を中心に免疫毒性データベースを構築した。

⑤ MITA による免疫毒性 clustering の有用性の検討

一方、我々はこれまでに 60 種類の化学物質を MITA の複数項目に関して効果発現最低濃度 (Lowest observed effect level ; LOWEL)を基にクラスター分類することにより、免疫毒性物質が 6 種類のクラスターに分類できることを明らかにした[3]。そこで、さらに改訂された上記データベースを参考に MITA によりクラスター分類を再検討する。

⑥ MITA を用いた免疫毒性評価系国際化へ向けての国際評価会議の開催

皮膚感作性試験法を除いては，in vitro 免疫毒性試験法は OECD テストガイドラインに存在しない。そこで，OECD 免疫毒性試験評価者の in vitro 免疫毒性評価系の現状と MITA の有用性の理解の促進を図る目的で，in vitro 免疫毒性評価法に関する detailed review paper (DRP)の作成を計画し以下の会議を開催した。

1.1st call for DRP in vitro immunotoxicity (Webex)
2019 年 9 月 18 日（水）、20 時
Emanuela Corsini, Erwin Roggen, Dori Germolec, Henk van Loveren, Barbara Kaplan, Setsuya Aiba, Yutaka Kimura, Takayuki Yoshimoto, Hajime Kojima, Steve Venti

2nd call for DRP in vitro immunotoxicity (Webex)

2019 年 10 月 28 日（水）、20 時
Emanuela Corsini, Erwin Roggen, Dori Germolec, Henk van Loveren, Barbara Kaplan, Setsuya Aiba, Yutaka Kimura, Takayuki Yoshimoto, Hajime Kojima, Steve Venti

3.3rd meeting for OECD DRP on in vitro immunotoxicity.
2020 年 1 月 28 日 9:00-17:30
2020 年 1 月 29 日 9:00-15:00
Emanuela Corsini, Dori Germolec, Henk van Loveren, Barbara Kaplan, Setsuya Aiba, Yutaka Kimura, Takayuki Yoshimoto, Hajime Kojima, Steve Venti

2020 年度

① SPSF提出に向けてIL-2 Luc assay validation reportを完成する。

以下の会議を行い、対応策を検討した。
2020 年 6 月 10 日
International peer review meeting on Multi-Immunotoxicity Test Assay (MITA) (Webex)
Emanuela Corsini, Dori Germolec, Haley Neff-LaFord, Henk van Loveren, Barbara Kaplan, Sang-Hyun Kim, Lin Shi, Xingchao Geng, Fujio Kayama, Takao Ashikaga, Setsuya Aiba, Yutaka Kimura, Hajime Kojima

2020 年 6 月 12 日
Conference call for the MITA assay validation study (Webex)
Corsini, E., Roggen, E., Germolec, D., Inoue, T., Aiba, S., Kimura, Y., Omori, T., Nakajima, Y. Yasuno, R., Kojima, H.

2020 年 7 月 21 日
Conference call for the MITA assay validation study (Webex)
Corsini, E., Roggen, E., Germolec, D., Inoue, T., Aiba, S., Kimura, Y., Omori, T., Nakajima, Y., Yasuno, R., Kojima, H.

2020 年 9 月 8 日
Conference call for the MITA assay validation study (Webex)

Corsini, E., Roggen, E., Germolec, D., Inoue, T., Aiba, S., Kimura, Y., Omori, T., Nakajima, Y., Yasuno, R., Kojima, H.

2020 年 9 月 14 日
IL-2 Luc assay SPSF 提出についての打ち合わせ (Webex)
相場、木村（東北大）、足利、小島（国立衛研）

2020 年 10 月 29 日（東北大学皮膚科医局、仙台）
IL-2 Luc assay SPSF 提出についての打ち合わせ
足利（国立衛研）、相場、木村（東北大）

② IL-1 Luc assay validation report作成

前年度に行ったIL-1 Luc assay validation試験の結果をまとめ、Validation management team (VMT) 委員との検討を重ねvalidation reportを作成した。以下に、行った会議を記載する。
2020 年 6 月 10 日
International peer review meeting on Multi-Immunotoxicity Test Assay (MITA) (Webex)
Emanuela Corsini, Dori Germolec, Haley Neff-LaFord, Henk van Loveren, Barbara Kaplan, Sang-Hyun Kim, Lin Shi, Xingchao Geng, Fujio Kayama, Takao Ashikaga, Setsuya Aiba, Yutaka Kimura, Hajime Kojima

③ IL-1 Luc assay, IL-2 Luc assayのdata set作成

これまでの60のdata setに、IL-1 Luc assayにより特異的に評価できる3化学物質を加えて、IL-1 Luc assay, IL-2 Luc assayおよびIL-8 Luc assay に関して，それぞれの試験法の最終判定基準に則りdata setを作成する。

④ IL-1 Luc assay, IL-2 Luc assayのdata set作成

IL-1 Luc assay および IL-2 Luc assay の validation report 作成にあたり、validation 試験で用いた化学物質（各試験法あたり２５化学物質）および lead laboratory の in-house データベース 63 化学物質に関して免疫毒性情報を収集しデータベースを作成した。

⑤ 細胞分裂を抑制することにより免疫毒性をきたす化学物質スクリーニング系の開発

IL-2 Luc assayにはT細胞の細胞増殖や代謝活性を阻害する免疫抑制物質を検出できないという問題点が存在した。そこで、この問題を解決するため、２H4細胞を化学物質と24時間反応させた後に、PMAとIonomycinの混合物(PMA/Io)で刺激し化学物質で処理していないコントロールに比較してIL-2レポーター活性が維持または増加しているにも関わらずGAPDHプロモーター活性が低下、言い替えるとGAPDHで補正したIL-2レポーター活性が上昇することを指標にそのような免疫抑制物質を検出できないかを試みた。

[方法] Jurkat 由来の IL-2 レポーター細胞である 2H4 細胞を 96 ウェルプレートに播種し化学物質を加え 24 時間培養した。その後 PMA/Io で刺激し 6 時間培養後ルシフェラーゼ活性を測定した。
また開発した方法に関して、東北大学を lead laboratory として、国立医薬品食品衛生研究所安全性生物試験研究センター薬理部、国立研究開発法人産業技術総合研究所・健康工学研究部門、国立研究開発法人産業技術総合研究所・バイオメディカル研究部門、神戸大学医学部附属病院・臨床研究推進センターが validation management team を結成し、Liaison member に Emanuela Corsini (Milan Univ., Italy), Erwin L. Roggen (3Rs Management and Consulting ApS, Denmark), Dori Germolec (NTP/NIEHS, USA), Tomoaki Inoue (Chugai Pharmaceutical Co., Ltd.)を迎えて validation 試験を開始した。

⑥ MITA による免疫毒性 clustering の有用性の検討

一方、我々はこれまでに 60 種類の化学物質を MITA の複数項目に関して効果発現最低濃度 (Lowest observed effect level ; LOEL)を基にクラスター分類することにより、免疫毒性物質が 6 種類のクラスターに分類できることを明らかにした。そこで、さらに改訂された上記データベースを参考に MITA によりクラスター分類を再検討行った。

⑦ 試験管内免疫毒性試験法に関する detailed review paper 作成への参加協力

皮膚感作性試験法を除いては，in vitro 免疫毒性試験法は OECD テストガイドラインに存在しない。そこで，昨年度から OECD 免疫毒性試験評価者の in vitro 免疫毒性評価系の現状と MITA の有用性理解の促進を図る目的で， in vitro 免疫毒性評価法に関する detailed review paper (DRP) の作成が厚生労働科学研究法除菌小島肇班を中心に行われている。そこで DRP 作成に協力するとともに Section VIII にて MITA の概略を紹介する。関連して以下の会議に参加した。

2020 年 4 月 15 日
MITA DRP meeting (Webex)
Emanuela Corsini, Erwin Roggen, Dori Germolec, Haley Neff-LaFord, Barbara Kaplan, Setsuya Aiba, Takayuki Yoshimoto, Hajime Kojima, Steve Venti

2020 年 5 月 18 日
MITA DRP meeting (Webex)
Emanuela Corsini, Erwin Roggen, Dori Germolec, Haley Neff-LaFord, Henk van Loveren, Barbara Kaplan, Setsuya Aiba, Yutaka Kimura, Takayuki Yoshimoto, Hajime Kojima, Steve Venti

2020 年 9 月 17 日
Expert group on immunotoxicity testing in OECD (Webex)
Setsuya Aiba　他

2020 年 10 月 14 日
MITA DRP meeting (Webex)
Emanuela Corsini, Erwin Roggen, Dori Germolec, Haley Neff-LaFord, Henk van Loveren, Barbara Kaplan, Setsuya Aiba, Yutaka Kimura, Takayuki Yoshimoto, Hajime Kojima

2020 年 11 月 12 日
MITA DRP meeting (Webex)
Emanuela Corsini, Erwin Roggen, Dori Germolec, Haley Neff-LaFord, Henk van Loveren, Barbara Kaplan, Setsuya Aiba, Yutaka Kimura, Takayuki Yoshimoto, Hajime Kojima

2020 年 12 月 14 日
MITA DRP meeting (Webex)
Emanuela Corsini, Erwin Roggen, Dori Germolec, Haley Neff-LaFord, Henk van Loveren, Barbara Kaplan, Setsuya Aiba, Yutaka Kimura, Takayuki Yoshimoto, Hajime Kojima

⑧ 修正IL-8 Luc assayの提案

厚労科研で開発したIL-8 Luc assay (OECD442E)では、培地に20 mg/mlの濃度で溶解しない化学物質のうち、IL-8 luciferase 活性を上昇させない物質は判定不能と分類される。そのため他の評価系に比べて判定できる化学物質が制限されるという欠点があった。そこで、その問題を改良する目的で、培地に溶解せずIL-8 luciferase 活性を上昇させない物質のうち、GAPDH luciferase活性を低下させる物質に関してGAPDH活性とPropidium iodide排除率等を比較検討した。また、併せてTHP-G8細胞の替わりとなるIL-1βプロモーター下流にSLGルシフェラーゼを含む人工染色体を導入したTHP-1由来細胞株の樹立を目指し実験を行った。

（倫理面への配慮）

ヒト、動物を対象にした研究は含まれていない。

C. 研究結果

2018 年度

① IL-2 Luc assayプロトコールならびにクライテリアの改訂

国際validation委員会にて，昨年度に策定されたクライテリア5を記載したプロトコール(Multi-Immuno Tox Assay protocol Ver.011E) Appendix 1(IL-2 Luc assay validation report draft Appendix)を作成した。このクライテリアを用いバリデーション研究を再評価したところPhase Iでは施設間再現性、施設内再現性はそれぞれ80.0 % (4/5)、86.7 % (13/15)であり、Phase IIでは施設間再現性が80 % (16/20)と良好な結果が得られた。そこで、IL-2 Luc assayのOECDガイドライン化を目指しバリデーションレポートを作成した。

② IL-1 Luc assayプロトコールならびにクライテリアの設定

昨年度、MITAとIL-8 Luc assayの結果を用い免疫毒性物質を６つのカテゴリーに分類する方法を提案した[2]。 既にIL-8 Luc assayは、OECD test guideline (442E)に承認され、またIL-2転写活性抑制評価系は、上述のように国際バリデーションphase I、IIが完了している。MITAのもう一つの構成因子として、THP-1細胞をベースとしたIL-1βレポーター細胞であるTHP-G1b細胞を用いた国際バリデーション試験を開始した。

1) IL-1 Luc assay Phase 0

国際バリデーション実行委員会にて選定したDapson, Diethanolamine, p-Nitroanilineについて参加3施設、産総研つくば、食薬センター、産総研高松においてMulti-Immuno Tox Assay protocol for TGCHAC-A4 ver. 007E (Appendix 3)にのっとり各物質3回繰り返し1セットの試験を2セット行った。%suppressionの閾値を20%と設定した場合、産総研つくば、産総研高松においてはリードラボと同様の結果が得られた。食薬センターについてはLPSによるFInSLG-LAの数値が得られない、再現性が得られない等の問題が認められた。食薬センターを含めた際の施設間再現性は83.3% (5/6)であった。

食薬センターについてはその後LPSによるFInSLG-LAの数値が得られない原因を検討し、FCSの非動化の方法、細胞へのLPSへの添加方法を再確認しアッセイしたところリードラボと同様の結果を得られた。

2) IL-1 Luc assay Phase 1

国際バリデーション実行委員会にて選定した５化学物質をコード化し、参加3施設、東北大学、産総研つくば、産総研高松においてMulti-Immuno Tox Assay protocol for TGCHAC-A4 ver. 008Eにのっとり各物質3回繰り返し1セットの試験を３セット実施した。次年度, Phase 1試験の結果をValidation management teamにて評価し今後の対応を決定する。

③ 免疫毒性物質データベース作成

免疫毒性分野では皮膚感作性試験におけるLLNAのようなゴールドスタンダードが存在せずpredictivity (accuracy)の算出ができない。っそこでValidation management team (VMT) のLiaison membersであるGermolec博士らによる化学物質の免疫機能に対する影響をまとめたレポート(添付資料１：Appendix 7)の提供をうけた。

2019 年度

① IL-2 Luc assay validation reportに対するpeer review panelによるコメントとそれに対する対応

今回IL-2 Luc assay validation reportを作成するにあたり，施設内，施設間再現生は試験開始前の目標値であった80%を達成した。しかし予測性に関しては，そもそも医薬品を除く多くの化学物質の免疫毒性評価が必ずしも定まっていないため確定できないでいた。またpeer review pane会議にて，IL-2 Luc assayは免疫毒性一般を評価する試験系ではなく, T細胞を一次標的として免疫毒性を惹起する免疫毒性物質の評価系であり，それを加味して予測性を決定するように指導された。そこで, 本試験において, NTPのLusterら(Luster et al. 1988; Luster et al. 1992a; Luster et al. 1993; Luster et al. 1992b)が51種類の化学物質の免疫毒性を動物実験を用いて評価した際の判定基準を参考にT細胞を標的とした化学物質の免疫毒性を評価する分類法を提案し，peer review panelにより了承された。これによりIL-2 Luc assayの予測性が決定した。それに基づきvalidation reportを修正し再度提出した。

我々が提出したvalidation reportに対して, International peer review panel meeting on Multi-Immunotoxicity Test Assay (MITA))から３度にわたり修正コメントを受け取り、それに対して適切に対応した。

② IL-1 Luc assay Phase Iならびに Phase II validation試験

IL-1 Luc assay Phase I試験を実施しwithin laboratory reproducibility, between laboratory reproducibility いずれも100%と

極めて良好な結果が得られた。この結果に関して以下の会議を開催した。
2019年度第1回MITAバリデーション電話会議　(スカイプ)
2019年4月5日（金）9:30-11:00
参加者：大森、髙木、小島、足利、相場、木村

2019年度第2回MITAバリデーション電話会議　(スカイプ)
2019年5月2日（木）10:00-12:00
参加者：大森、小島、安野、中島、相場、木村、藤村

第１回VMT会議 Conference call for the MITA assay (Webex)
2019年6月26日（水））20:00-
参加者：Corsini, E., Roggen, E., Germolec, D., Inoue, T., Aiba, S., Kimura, Y., Omori, T., Kojima, H.
以上の会議で，予測性に関しての最終評価は定まっていないが，さらに20化学物質を用いて施設間再現性を評価するPhase II 試験を行う事が了承された。そこで，3施設でPhase II 試験を実施し2019年12月までに全ての施設が試験を完了した。そこで以下の会議で試験結果が検討された。その結果，施設間再現性はPhase II 試験のみの結果で80%，Phase I，II試験を統合した結果で84%となり，Phase Iの施設間再現性と共に試験開始前に想定していた採択基準をクリーアした。しかし，IL-1 Luc assayの再現性に関しては更に議論が必要と言うことになり，最終結論は次回のMVT会議に持ち越された。
第２回VMT会議
2020年1月31日（水））
会場：国立医薬品食品衛生研究所
参加者：Corsini, E., Roggen, E., Germolec, D., Inoue, T., Aiba, S., Kimura, Y., Omori, T., Kojima, H.

③ IL-1 Luc assay, IL-2 Luc assayのdata set作成
IL-1 Luc assay, IL-2 Luc assayおよびIL-8 Luc assay に関して，それぞれの試験法の最終判定基準に則り現時点でのdata setを作成した。

④ 免疫毒性物質データベースの作成
IL-2 Luc assayのvalidationに用いた25化学物質，IL-2 Luc assayのdata set作成に用いた化学物質に関して免疫毒性データベースを作成した。（添付資料1:Appendix Table 1 and 2）データベースでは，化学物質の毒性データをin vivo、ex vivo、in vitroデータの3種類に分類した。具体的には、in vivo データの中には、免疫臓器の重量変化，遅延型過敏症，易感染性，移植腫瘍に対する抵抗性が、ex vivo データには、化学物質を投与された個体から採取した免疫担当細胞を用いてin vitroで化学物質の影響を評価するサイトカイン産生試験，T細胞依存性性抗体産生試験 (T-cell dependent antibody response; TDAR)が、in vitroデータには、個体から採取した免疫担当細胞に、in vitroで化学物質を加えてそのサイトカイン産生能の変化を評価するサイトカイン産生試験，T細胞の増殖能を評価する細胞増殖試験などを含めた。この作成に当たっては，National Toxicology Program (NTP)の協力を仰いだ。

⑤ MITA による免疫毒性 clustering の有用性の検討
あらたに得られたデータセットをもとに IL-8 Luc assay と組み合わせた MITA により化学物質の clustering を実施した。その結果を添付資料 13 に示す。しかし，IL-1 Luc assay，IL-2 Luc assay，IL-8 Luc assay の組み合わせでは，以前論文で報告した IL-2 Luc assay，IL-8 promoter assay，IL-8 Luc assay の組み合わせで行ったようには綺麗に clustering できなかった。また残念ながら、MITA では、一部の DNA 合成、細胞増殖抑制機序に基づく免疫毒性物質が評価できないことも明らかになった。

⑥ 試験管内免疫毒性試験法に関する detailed review paper の作成の参加協力
MITA のテストガイドライン化に向けて in vitro 免疫毒性評価法に関する detailed review paper (DRP)の作成に協力し以下の会議を開催した。
1.1st call for DRP in vitro immunotoxicity (Webex)
2019 年 9 月 18 日（水）、20 時
2. 2nd call for DRP in vitro immunotoxicity (Webex)
2019 年 10 月 28 日（水）、20 時
Emanuela Corsini, Erwin Roggen, Dori Germolec, Henk van Loveren, Barbara Kaplan, Setsuya Aiba, Yutaka Kimura, Takayuki Yoshimoto, Hajime Kojima, Steve Venti
上記会議において，以下の様な項目と執筆担当者が決定した。さらに下記の会議にて draft 案が提案され，それの修正を行った。
3.3rd meeting for OECD DRP on in vitro immunotoxicity.

2020 年 1 月 28 日 9:00-17:30
2020 年 1 月 29 日 9:00-15:00
Emanuela Corsini, Dori Germolec, Henk van Loveren, Barbara Kaplan, Setsuya Aiba, Yutaka Kimura, Takayuki Yoshimoto, Hajime Kojima, Steve Venti

2020 年度

①IL-2 Luc assay validation reportのSPSF提出

2020年11月13日に、改定したIL-2 Luc assay のvalidation report（添付資料１）ならびにSPSF（添付資料２）をWorking group of the National Coordinators for the Test Guidelines Program (OECD)に提出した。またvalidation試験の詳細に関しては、Toxicol in Vitroに報告した(Kimura et al. 2020)。

② IL-1 Luc assay validation report作成

昨年度に終了したIL-1 Luc assay Phase I, Phase II試験の結果ならびにlead laboratoryのin-houseデータを元にVMT委員と討議を重ねvalidation reportを完成させた（添付資料３）。現在、VMT委員と議論している。その結果を踏まえてpeer review panelの評価を受ける予定である。昨年度からに問題になっていたIL-1 Luc assayの予測性に関しては、Toll-like receptorやIL-1受容体の下流にある受容体のうちIRAK4、Myd88などIL-1 Luc assay以外には評価できないシグナル伝達物質が存在することを証明し、IL-1 Luc assayの有用性が確認できた。

③IL-1 Luc assay, IL-2 Luc assayのdata set作成

これまでの60のdata setに、IL-1 Luc assayにより特異的に評価できる3化学物質を加えて、IL-1 Luc assay, IL-2 Luc assayおよびIL-8 Luc assay に関して，それぞれの試験法の最終判定基準に則りdata setを作成した(添付資料４)。

④免疫毒性物質データベースの作成

IL-1 Luc assay作成の過程で、validationで用いた化学物質、data setに含まれる化学物質に関する免疫毒性データを収集しデータベースを作成した(添付資料３：Appendix 14 & 15)。データベースでは，化学物質の毒性データをin vivo、ex vivo、in vitroデータの3種類に分類した。具体的には、in vivo データの中には、免疫臓器の重量変化，遅延型過敏症，易感染性，移植腫瘍に対する抵抗性が、ex vivo データには、化学物質を投与された個体から採取した免疫担当細胞を用いてin vitroで化学物質の影響を評価するサイトカイン産生試験，T細胞依存性性抗体産生試験 (T-cell dependent antibody response; TDAR)が、in vitroデータには、個体から採取した免疫担当細胞に、in vitroで化学物質を加えてそのサイトカイン産生能の変化を評価するサイトカイン産生試験，T細胞の増殖能を評価する細胞増殖試験などを含めた。

⑤細胞分裂を抑制することにより免疫毒性をきたす化学物質スクリーニング系の開発

代表的なDNA合成阻害剤、代謝阻害剤であるgemcitabine hydrochloride、cytarabine、bleomycin sulfate、5-fluorouracilでは、従来のサイトカイン産生抑制を指標とするIL-2 Luc assayでは免疫抑制物質として検出されなかったが、24時間後の反応ではノーマライズしたIL-2レポーター活性の上昇、生存率の低下が認められた。一方dexamethasone、cyclosporine A、tacrolimusのサイトカイン産生抑制を機序とする免疫抑制物質では24時間の培養でもIL-2レポーター活性が抑制された。以上の結果からこの新しい方法 (IL-2 Luc leukocyte toxicity test (IL-2 Luc LTT))ではT細胞の細胞増殖や代謝活性を阻害する免疫抑制物質を検出しうることが示され、将来IL-2 Luc assayと組み合わせ幅広く免疫毒性物質をスクリーニングできる可能性が示唆された。
そこで添付のIL-2 Luc LTT protocolを作成し(添付資料５)、種々の免疫抑制剤、非免疫抑制剤を評価しIL-2 Luc LTT の免疫毒性試験法としての可能性を検討した。その結果、IL-2 Luc assay では評価できなかった細胞分裂、代謝活性に作用して免疫毒性を発現する化学物質を評価できることが分かり、現在国際的validation試験を行っている。また、本試験法に関しては、既に国際特許であるPCT出願を終了した。Validation試験のphase 1の結果を添付する(添付資料６)。Phase 1の結果は、VMTのliaison委員により承認された。

⑥ MITA による免疫毒性 clustering の有用性検討

あらたに得られたデータセットをもとに IL-8 Luc assay と組み合わせた MITA により化学物質の clustering を実施した。その結果を添付資料 13 に示す。しかし，IL-1 Luc assay, IL-2 Luc assay, IL-8 Luc assay の組み合わせでは，以前論文で報告した IL-2 Luc assay, IL-8 promoter assay, IL-8

Luc assay の組み合わせで行ったようには綺麗に clustering できなかった。また残念ながら、MITA では、一部の DNA 合成、細胞増殖抑制機序に基づく免疫毒性物質が評価できないことも明らかになった。

⑦ 試験管内免疫毒性試験法に関する detailed review paper の作成の参加協力

MITA のテストガイドライン化に向けて in vitro 免疫毒性評価法に関する DRP が小島班を中心に作成された。またそれに基づき、第 1 回の OECD expert group meeting が開催され、DRP に対するコメントがよせられている。上記 DRP 作成に協力するとともに Section VIII In vitro immunotoxicological assessments using the combination of cell lines の執筆を担当した。現在、それらのコメントに対し対応を検討している。

⑧ 修正IL-8 Luc assayの提案

厚労科研で開発したIL-8 Luc assay (OECD442E)では、培地に20 mg/mlの濃度で溶解しない化学物質のうち、IL-8 luciferase 活性を上昇させない物質は判定不能と分類される。そのため他の評価系に比べて判定できる化学物質が制限されるという欠点があった。そこで、その問題を改良する目的で、培地に溶解せずIL-8 luciferase 活性を上昇させない物質のうち、GAPDH luciferase活性を低下させる物質は、化学物質が培地中に溶解していることを証明し、陰性と判断することにした。具体的には、GAPDH活性とPropidium iodide排除率等を比較することで明らかにし、Arch Toxicolに掲載された「7」。そこで、改良IL-8 Luc assayに関してもSPSFを提出した(添付資料７)。THP-G8細胞の代替となる人工染色体技術を応用した新規IL-8レポーター細胞を樹立した（添付資料８）。

E. 考察

臨床的に使われる免疫抑制剤を除くと，化学物質の免疫毒性，特にヒトに対する免疫毒性の評価は定まっていない。確かに，個々の化学物質に関して，幾つかの免疫毒性評価試験を行った報告は多数存在するが，それらを総括して化学物質の免疫毒性の有無を総括した報告は我々が調べた限り存在しない。この問題は，免疫毒性試験法のvalidation試験を行う際に大きな障害となった。

そこで本課題において，化学物質の免疫毒性に関する文献資料を基に免疫毒性の有無を判定するクライテリアを提案した。幸い，本課題においてはvalidation試験と並行して行ってきた免疫毒性データベースが存在し，それをもとに分類することを検討した。その際に，Lusterら(Luster et al. 1988; Luster et al. 1992a; Luster et al. 1993; Luster et al. 1992b)が報告した免疫毒性分類法を参考にした。この方法では， 51種類の化学物質をマウスに投与し，その動物を種々の免疫毒性試験法で評価し免疫毒性の有無を判定するクライテリアを提案している。またそのクライテリアの判定結果とマウス感染実験から得られた易感染性の有無との相関も検討している。IL-2 Luc assayの予測性の評価においても，ほぼLusterらのクライテリアを参考に，作成した化学物質免疫毒性データベースをもとに評価化学物質の免疫毒性の有無を決定した。この妥当性は，peer review panelからも承認され、またToxicol in vitroにも掲載された(Kimura et al. 2020)。

これまで行ってきたMITAのvalidation試験の内、IL-2 Luc assayのPhase I、Phase II試験が終了した。これらの試験を通して、IL-2 Luc assayの施設間、施設内再現性が十分にOECDガイドライン化に必要な基準を満たしていることが明らかになった。予測性に関しては、上記化学物質の免疫毒性分類を参考にしつつ、IL-2 Luc assayが免疫毒性評価のなかでも、T細胞を標的にした免疫毒性を評価する試験であるを考慮することにした。具体的には、ex vivo、in vitroのT細胞由来サイトカイン産生能に影響を与える物質ないしは各化学物質のmode of actionにT細胞への作用が明記されている化学物質を陽性物質のリファレンスとした。

その結果、Phase I、IIをまとめたpredictivityは約67%となった。この値は、必ずしも十分な値ではないが、化学物質の免疫毒性の有無が必ずしも明確ではないこと、またT細胞を標的とした免疫毒性にもIL-2転写活性以外を標的とした作用が存在することは容易に想像できることから、他の免疫毒性評価系との組み合わせを前提にOECD TGに申請することにした。

同様にIL-1 Luc assayに関しても、validation試験を実施し、高い施設内、施設間再現生を示す結果が得られた。しかし、予測性に関しては、50%程度と満足のいく結果は得られなかった。加えて、陽性化学物質の大半がIL-2 Luc

assayの重複しており、IL-1 Luc assayの有用性が示せなかった。しかし、IRAK-4やTLR4阻害薬などIL-2転写に関わるシグナル経路に含まれない分子に対する阻害剤の効果を感度良く検出できることが示せた。

また、本研究課題のもう一つのテーマである化学物質の免疫毒性データの集積をNTPの協力を得て行った。IL-2 Luc assay、IL-1 Luc assayのvalidation試験に用いた化学物質、63種類のデータセットの化学物質に関して入手可能な免疫毒性データを網羅し、それらをin vivo，ex vivo、in vitroデータに分類し表にまとめた。その結果、各化学物質の大凡の免疫毒性profileが俯瞰可能となった。

免疫毒性クラスター解析では、IL-1 Luc assayとIL-2 Luc assayの組み合わせでは必ずしも有意義なクラスター分類は不可能であった。今後あらたな免疫毒性試験法との組み合わせを検討し、より有意義なクラスター分類を検討する必要性が明らかとなった。

最後に、本厚労科研の最終目標は、MITAを構築する免疫毒性試験のうちIL-2 Luc assayとIL-1 Luc assayを試験管内免疫毒性試験法としてOECD test guidelineとして承認を目指すことにあった。現在、IL-2 Luc assayに関しては、validation reportのpeer reviewが終了し、OECDのテストガイドライン化に向けて、Working Group of National Coordinators of the Test Guidelines Program (WNT) にSPSFを提出した。一方、IL-1 Luc assayは本厚労科研でvalidationから行いvalidation reportの作成が終了し、現在、validation management teamのexpertにより検討が行われている。また最終年度には、IL-2 Luc assayが判定できなかった細胞分裂、代謝活性に働き免疫抑制を示す免疫毒性物質の評価系を新たに開発し、現在validationを開始している。さらに、これまでの厚労科研で開発したIL-8 Luc assay (OECD TG 442E)の改良版のSPSFをWNTに提出した。またIL-1 Luc assay、IL-2 Luc assayの一連のvalidationを行うなかで、100近い化学物質に関する免疫毒性データベースを作成した。

E. 結論

本課題においては、当初の目標どおり、1)IL-2 Luc assay は OECD に SPSF を提出済み、2)IL-1 Luc assay に関しては Validation report を作成済み、3)約 100 の化学物質に関して免疫毒性に関するデータベースを作成した。4)免疫毒性物質の clustering に関しては、あらたに IL-2 Luc LTT を開発し、これらと IL-2 Luc assay、IL-1 Luc assay との組み合わせにより新しい知見を得ている。

引用文献

Kimura Y, Fujimura C, Ito Y, Takahashi T, Aiba S (2014) Evaluation of the Multi-ImmunoTox Assay composed of 3 human cytokine reporter cells by examining immunological effects of drugs. Toxicol In Vitro 28(5):759-68 10.1016/j.tiv.2014.02.013

Kimura Y, Fujimura C, Ito Y, Takahashi T, Terui H, Aiba S (2018) Profiling the immunotoxicity of chemicals based on in vitro evaluation by a combination of the Multi-ImmunoTox assay and the IL-8 Luc assay. Arch Toxicol 92(6):2043-2054 10.1007/s00204-018-2199-7

Kimura Y, Yasuno R, Watanabe M, et al. (2020) An international validation study of the IL-2 Luc assay for evaluating the potential immunotoxic effects of chemicals on T cells and a proposal for reference data for immunotoxic chemicals. Toxicol In Vitro 66:104832 10.1016/j.tiv.2020.104832

Luster MI, Munson AE, Thomas PT, et al. (1988) Development of a testing battery to assess chemical-induced immunotoxicity: National Toxicology Program's guidelines for immunotoxicity evaluation in mice. Fundam Appl Toxicol 10(1):2-19 10.1016/0272-0590(88)90247-3

Luster MI, Pait DG, Portier C, et al. (1992a) Qualitative and quantitative experimental models to aid in risk assessment for immunotoxicology. Toxicol Lett 64-65 Spec No:71-8 10.1016/0378-4274(92)90174-i

Luster MI, Portier C, Pait DG, et al. (1993) Risk assessment in immunotoxicology. II. Relationships between immune and host resistance tests. Fundam Appl Toxicol 21(1):71-82

Luster MI, Portier C, Pait DG, et al. (1992b) Risk assessment in immunotoxicology. I. Sensitivity and predictability of immune tests. Fundam Appl Toxicol 18(2):200-10 10.1016/0272-0590(92)90047-l

OECD (2018) OECD Test Gudeline for the Testing of Chemicals No.442E: In vitro skin sensitisation assays addressing the key event on activation of dendritic cells on the adverse outcome pathway for skin sensitisation. https://doi.org/10.1787/9789264264359-en.

F.研究発表

１．論文発表

●研究代表者：相場　節也

1. Kimura, Y., Fujimura, C., Ito, Y., Takahashi, T., Terui, H., Aiba, S. Profiling the immunotoxicity of chemicals based on in vitro evaluation by a combination of the Multi-ImmunoTox assay and the IL-8 Luc assay. Arch. Toxicol, 2018; 92: 2043-2054
2. Kimura, Y., Watanabe, M., Suzuki, N., Iwaki, T., Yamakage, K., Saito, K., Nakajima, Y., Fujimura, C., Ohmiya, Y., Omori, T., Kojima, H., Aiba, S. The performance of an in vitro skin sensitisation test, IL-8 Luc assay (OECD442E), and the integrated approach with direct peptide reactive assay (DPRA). J Toxicol Sci, 2018; 43: 741-749
3. Hidaka, T., Fujimura, T., Aiba, S. Aryl hydrocarbon receptor modulates carcinogenesis and maintenance of skin cancers. Frot Med, 2019; 6: 1-7
4. Kimura Y, Yasuno R, Watanabe M, Kobayashi M, Iwaki T, Fujimura C, Ohmiya Y, Yamakage K, Nakajima Y, Kobayashi M, Mashimo N, Takagi Y, Omori T, Corsini E, Germolec D, Inoue T, Rogen EL, Kojima H, Aiba S. An international validation study of the IL-2 Luc assay for evaluating the potential immunotoxic effects of chemicals on T cells and a proposal for reference data for immunotoxic chemicals. Toxicol in Vitro, 2020: 66: 104832
5. Kimura, Y., Fujimura, C., Aiba, S. The modified IL-8 Luc assay, an in vitro skin sensitisation test, can significantly improve the false-negative judgment of lipophilic sensitizers with logKow values > 3.5. Arch Toxicol, 2021:95: 749-758
6. Terui, H., Kimura, Y., Fujimura, C., Aiba, S. The IL-1 promoter-driven luciferase reporter cell line THP-G1b can efficiently predict skin-sensitising chemicals. Arch Toxicol, 2021: in press

●研究分担者：小島　肇

1. 小島　肇: 皮膚・粘膜毒性, トキシコロジー第３版, 朝倉書店, pp.279-286.
2. 小島　肇: 動物実験代替法, トキシコロジー第３版, 朝倉書店, pp.320-325.
3. Dent M, Amaral RT, Da Silva PA, Ansell J, Fanny Boisleve, Hatao M, Hirose A, Kasai Y, Kern P, Kreiling R, Milstein S, Montemayor B, Oliveira J, Richarz A, Taalman R, Vaillancourt E, Verma R, Posada N.V.O.C, Weiss C, Kojima H: Principles underpinning the use of new methodologies in the risk assessment of cosmetic ingredients, Computational Toxicology, 2018(7), 20–26.
4. 中村和昭, 諫田泰成, 山崎大樹, 片岡 健, 青井貴之, 中川誠人, 藤井万紀子, 阿久津英憲, 末盛博文, 浅香 勲, 中村幸夫, 小島 肇, 伊藤弓弦, 関野祐子, 古江－楠田美保：「培養細胞の観察の基本原則」の提案, 組織培養研究, 2018; 37(2), 123-131.
5. 小島　肇：化学物質や医薬品などの安全性評価に用いる動物実験代替法の技術開発の現状と展望, イルシーJapan, 2018;136, 23-31.
6. 小島　肇, 西川秋佳:日本動物実験代替法評価センター平成 29 年度報告, AATEX-JaCVAM, 2018;7(1): 65-70.
7. Kimura Y, Watanabe M, Suzuki N, Iwaki T, Yamakage K, Saito K, Nakajima Y, Fujimura C, Ohmiya Y, Omori T, Kojima H, Aiba S: The performance of an in vitro skin sensitisation test, IL-8 Luc assay (OECD442E), and the integrated approach with direct peptide reactive assay (DPRA), J Toxicol Sci. 2018; 43(12):741-749.
8. Mitachi T, Kouzui M, Maruyama R, Yamashita K, Ogata S, Kojima H, Itagaki H: Some non-sensitizers upregulate CD54 expression by activation of the NLRP3 inflammasome in THP-1 cells. J Toxicol Sci. 2019;44(3):213-224.
9. Fujita M, Yamamoto Y, Watanabe S, Sugawara T, Wakabayashi K, Tahara Y, Horie N, Fujimoto K, Kusakari K, Kurokawa Y, Kawakami T, Kojima K, Kojima H, Ono A, Katsuoka Y, Tanabe H, Yokoyama H, Kasahara T : Cause of and countermeasures for oxidation of the cysteine-derived reagent used in the amino acid derivative reactivity assay, J Appl Toxicol. 2019;39(2):191-208.
10. 小島　肇: 化学物質の毒性評価方法の現状と今後, 化学物質と環境, エコケミストリー研究会, 2019;154, 1-3.
11. Kobayashi-Tsukumo H, Oiji K, Xie D, Sawada Y, Yamashita K, Ogata S, Kojima H, Itagaki H: Eliminating the contribution of lipopolysaccharide to protein allergenicity in the human cell-line activation test (h-CLAT), J Toxicol Sci. 2019;44(4):283-297.

12. Fujita M, Yamamoto Y, Watanabe S, Sugawara T, Wakabayashi K, Tahara Y, Horie N, Fujimoto K, Kusakari K, Kurokawa Y, Kawakami T, Kojima K, Sozu T, Nakayama T, Kusao T, Richmond J, Nicole K, Kim BH, Kojima H, Kasahara T, Ono A: The within- and between-laboratory reproducibility and predictive capacity of the in chemico amino acid derivative reactivity assay: Results of validation study implemented in four participating laboratories. J Appl Toxicol. 2019 Nov;39(11):1492-1505.
13. Kojima H, Sakai Y, Tanaka N: Japanese Contributions to the Development of Alternative Test Methods, The History of Alternative Test Methods in Toxicology, Elsevier, Netherlands, 2019, pp.79-85.
14. Kojima H: Use of non-animal test methods in the safety assessment of chemicals,Translat Regulat Sci., 2019;1(2): 66–72.
15. 小島 肇, 足利 太可雄, 平林 容子: 日本動物実験代替法評価センター(JaCVAM) 平成 30 年度報告書 ,AATEX-JaCVAM,2019;8(1): 35-41.
16./ Akimoto M, Yamamoto Y, Watanabe S, Yamaga H, Yoshida K, Wakabayashi K, Tahara Y, Horie N, Fujimoto K, Kusakari K, Kamiya K, Kojima K, Kawakami T, Kojima H, Ono A, Kasahara T, Fujita M: Oxidation of a cysteine-derived nucleophilic reagent by dimethyl sulfoxide in the amino acid derivative reactivity assay. J Appl Toxicol.2020;40(6),843-854.
17. Marx U, Akabane T, Andersson TB, Baker E, Beilmann M, Beken S, Brendler-Schwaab S, Cirit M, David R, Dehne EM, Durieux I, Ewart L, Fitzpatrick SC, Frey O, Fuchs F, Griffith LG, Hamilton GA, Hartung T, Hoeng J, Hogberg H, Hughes DJ, Ingber DE, Iskandar A, Kanamori T, Kojima H, Kuehnl J, Leist M, Li B, Loskill P, Mendrick DL, Neumann T, Pallocca G, Rusyn I, Smirnova L, Steger-Hartmann T, Tagle DA, Tonevitsky A, Tsyb S, Trapecar M, Van de Water B, Van den Eijnden-van Raaij J, Vulto P, Watanabe K, Wolf A, Zhou X, Roth A: Biology-inspired microphysiological systems to advance patient benefit and animal welfare in drug development. ALTEX. 2020 Feb 28. doi: 10.14573/altex.2001241.
18./ Kimura Y, Yasuno R, Watanabe M, Kobayashi M, Iwaki T, Fujimura C, Ohmiya Y, Yamakage K, Nakajima Y, Kobayashi M, Mashimo N, Takagi Y, Omori T, Corsini E, Germolec D, Inoue T, Rogen EL, Kojima H, Aiba S: An international validation study of the IL-2 Luc assay for evaluating the potential immunotoxic effects of chemicals on T cells and a proposal for reference data for immunotoxic chemicals. Toxicol In Vitro. 2020;66:104832.
19. 山田 隆志, 足利 太可雄, 小島 肇, 広瀬明彦: AOP (Adverse Outcome Pathway ; 有害性発現経路) に基づいた化学物質の安全性評価へ向けたチャレンジ. Yakugaku Zasshi. 2020;140(4): 481-484.
20. 小島 肇:OECD 試験法ガイドライン開発における CERI の国際貢献. CERI NEWS, 2020;90:2-3.
21. 小島 肇:AOP 及び IATA に基づく安全性評価手法の進捗. JETOC 40 周年記念誌, 2020;71-101.
22. 足利太可雄, 小島肇, 平林容子:日本動物実験代替法評価センター（JaCVAM)令和元年度報告書. AATEX-JaCVAM, 2020;9(1), 58-64.
23. Imamura M, Wanibuchi S, Yamamoto Y, Kojima H, Ono A, Kasahara T, Fujita M:Improving predictive capacity of the Amino acid Derivative Reactivity Assay test method for skin sensitization potential with an optimal molar concentration of test chemical solution, J Appl Toxicol.2021;41(2):303-329.

●研究分担者： 中島　芳浩

1. Sato, D., Abe, S., Kobayashi, K., Nakajima, Y., Oshimura, M., Kazuki, Y. Human and mouse artificial chromosome technologies for studies of pharmacokinetics and toxicokinetics. Drug Metab. Pharmacokinet, 2018; 33: 17-30
2. Kimura, Y., Watanabe, M., Suzuki, N., Iwaki, T., Yamakage, K., Saito, K., Nakajima, Y., Fujimura, C., Ohmiya, Y., Omori, T., Kojima, H., Aiba, S. The performance of an in vitro skin sensitisation test, IL-8 Luc assay (OECD442E), and the integrated approach with direct peptide reactive assay (DPRA). J Toxicol Sci, 2018; 43: 741-749
3. Saiki, P., Nakajima, Y., Van Griensven, L., Miyazaki, K. Real-time monitoring of IL-6 and IL-10 reporter expression for anti-inflammation activity in live RAW 264.7cells. Biochem. Biophys. Res. Commun., 2018; 505: 885-890
4. Uno, K., Mutoromi, K., Kazuki, Y., Oshimura, M., Nakajima, Y. Bioluminescence-based cytotoxicity assay for simultaneous evaluation of cell viability and membrane damage in human hepatoma HepG2 cells. Luminescence, 2018; 33: 616-624

5./ Saiki, P., Kawano, Y., Nakajima, Y., Van Griensven, Ljld, Miyazaki, K. Novel and stable dual-color IL-6 and IL-10 reporters derived from RAW 264.7 for anti-inflammation screening of natural products. Int. J. Mol. Sci., 2019; 20: 4620
6./ Suzuki, S., Ohta, K., Nakajima, Y., et al. Meganuclease-Based Artificial Transcription Factors. ACS Synth Biol, 2020: 9: 2679-2691
7./ Suyama, A., Murotomi, K., Arai, S., et al. Brain degeneration suspected of hepatic encephalopathy associated with early nonalcoholic steatohepatitis in TSOD mice. Kyusyu J Exp Animals, 2020: 36: 13-22
8./ Hashimoto, M., Yokota, A., Kajimoto, K., et al. Highly sensitive and rapid quantitative detection of Plasmodium falciparum using an image cytometer. Microorganisms, 2020: 8: 1769
9./ 中島芳浩、人工染色体ベクターを用いた発光安定細胞の樹立と利用、細胞、2020: 52: 416-419
10./ Kimura Y, Yasuno R, Watanabe M, Kobayashi M, Iwaki T, Fujimura C, Ohmiya Y, Yamakage K, Nakajima Y, Kobayashi M, Mashimo N, Takagi Y, Omori T, Corsini E, Germolec D, Inoue T, Rogen EL, Kojima H, Aiba S. An international validation study of the IL-2 Luc assay for evaluating the potential immunotoxic effects of chemicals on T cells and a proposal for reference data for immunotoxic chemicals. Toxicol in Vitro, 2020: 66: 104832

●研究分担者： 安野　理恵

1./ Mitani Y., Yasuno R., Futahashi R., et al. Luciferase gene of a Caribbean fireworm (Syllidae) from Puerto Rico. Scientific reports 2019: 9, 1
2./ Gabriel GV., Yasuno R., Mitani Y., et al. Novel application of Macrolampis sp2 firefly luciferase for intracellular pH-biosensing in mammalian cells. Photo. Photo. Sci. 2019: 18: 1212
3./ Mitani Y., Yasuno R., Isaka M., et al. Novel gene encoding a unique luciferase from the fireworm Odontsyllis undecimdonta. Scientific reports, 2018: 8: 1
4./ Kimura Y, Yasuno R, Watanabe M, Kobayashi M, Iwaki T, Fujimura C, Ohmiya Y, Yamakage K, Nakajima Y, Kobayashi M, Mashimo N, Takagi Y, Omori T, Corsini E, Germolec D, Inoue T, Rogen EL, Kojima H, Aiba S. An international validation study of the IL-2 Luc assay for evaluating the potential immunotoxic effects of chemicals on T cells and a proposal for reference data for immunotoxic chemicals. Toxicol in Vitro, 2020: 66: 104832

●研究分担者： 大森　崇

1./ Sugiyama, M., Akita, M., Alépée, N., Fujishiro, M., Hagino, S., Handa, Y., Ikeda, H., Imai, N., Jitsukawa, S., Katoh, M., Kurihara, K., Kyotani, D., Nomura, S., Okamoto, Y., Okumura, H., Omori, T., Sugibayashi, K., Todo, H., Toyoda, A., Ohno, Y. Comparative assessment of 24-hr primary skin irritation test and human patch test data with in vitro skin irritation tests according to OECD Test Guideline 439 (for quasi-drugs in Japan). J Toxicol Sci, 2018; 43: 751-768
2./ Kimura, Y., Watanabe, M., Suzuki, N., Iwaki, T., Yamakage, K., Saito, K., Nakajima, Y., Fujimura, C., Ohmiya, Y., Omori, T., Kojima, H., Aiba, S. The performance of an in vitro skin sensitisation test, IL-8 Luc assay (OECD442E), and the integrated approach with direct peptide reactive assay (DPRA). J Toxicol Sci, 2018; 43: 741-749
3./ Kimura Y, Yasuno R, Watanabe M, Kobayashi M, Iwaki T, Fujimura C, Ohmiya Y, Yamakage K, Nakajima Y, Kobayashi M, Mashimo N, Takagi Y, Omori T, Corsini E, Germolec D, Inoue T, Rogen EL, Kojima H, Aiba S. An international validation study of the IL-2 Luc assay for evaluating the potential immunotoxic effects of chemicals on T cells and a proposal for reference data for immunotoxic chemicals. Toxicol in Vitro, 2020: 66: 104832

●研究分担者： 木村　裕

1./ Kimura, Y., Watanabe, M., Suzuki, N., Iwaki, T., Yamakage, K., Saito, K., Nakajima, Y., Fujimura, C., Ohmiya, Y., Omori, T., Kojima, H., Aiba, S. The performance of an in vitro skin sensitisation test, IL-8 Luc assay (OECD442E), and the integrated approach with direct peptide reactive assay (DPRA). J Toxicol Sci, 2018; 43: 741-749
2./ Kimura, Y., Fujimura, C., Ito, Y., Takahashi, T., Terui, H., Aiba, S. Profiling the immunotoxicity of chemicals based on in vitro evaluation by a combination of the Multi-ImmunoTox assay and the IL-8 Luc assay. Arch. Toxicol, 2018; 92: 2043-2054
3./ Watabe, A., Yamasaki, K., Asano, M., Kanbayashi, Y., Nasu-Tamabuchi, M., Terui, H., Furudate, S., Kakizaki, A., Tsuchiyama, K., Kimura, Y., Ito, Y., Kikuchi, K., Aiba S. Efficacy of oral cholecalciferol on rhododendrol-induced vitiligo: A blinded randomized clinical trial. J Dermatol, 2018; 45: 456-462

4. Kimura Y, Yasuno R, Watanabe M, Kobayashi M, Iwaki T, Fujimura C, Ohmiya Y, Yamakage K, Nakajima Y, Kobayashi M, Mashimo N, Takagi Y, Omori T, Corsini E, Germolec D, Inoue T, Rogen EL, Kojima H, Aiba S. An international validation study of the IL-2 Luc assay for evaluating the potential immunotoxic effects of chemicals on T cells and a proposal for reference data for immunotoxic chemicals. Toxicol in Vitro, 2020: 66: 104832
5. Kimura, Y., Fujimura, C., Aiba, S. The modified IL-8 Luc assay, an in vitro skin sensitisation test, can significantly improve the false-negative judgment of lipophilic sensitizers with logKow values > 3.5. Arch Toxicol, 2021:95: 749-758
6. Terui, H., Kimura, Y., Fujimura, C., Aiba, S. The IL-1 promoter-driven luciferase reporter cell line THP-G1b can efficiently predict skin-sensitising chemicals. Arch Toxicol, 2021: in press

２．学会発表

●研究代表者： 相場　節也

1. 木村 裕、安野　理恵、渡辺　美香、小林　美和子、岩城　知子、藤村　千鶴、近江谷　克裕、山影　康次、中島　芳浩、小林 眞弓、大森 崇、足利 太可雄、小島 肇、相場 節也: Multi-ImmunoTox Assay（MITA）：バリデーション研究の結果 日本動物実験代替法学会 第31回大会（熊本）（2018. 11）
2. 15th International Congress of Toxicology, Hawaii convention center, July 15, 2019. Immunotoxicological Profiling of Chemicals Using Novel In Vitro Assays. Setsuya Aiba
3. 木村 裕、安野 理恵、渡辺 美香、小林 美和子、岩城 知子、藤村 千鶴、近江谷 克裕、山影 康次、中島 芳浩、真下　奈々、高木 佑実、大森 崇、小島 肇、相場 節也：Multi-ImmunoTox Assay（MITA）の予測性評価に必要な文献に基づく化学物質免疫毒性分類の試み 日本動物実験代替法学会 第32回大会 つくば（2019.11）
4. 木村 裕、藤村 千鶴、相場 節也 IL-8 Luc assay（OECD442E）のクライテリアの改変とLogKowが3.5を超える化学物質における偽陰性率の改善（ポスター）日本動物実験代替法学会 第３３回大会 Web開催（2020.11）
5. 木村 裕、安野 理恵、渡辺 美香、小林 美和子、岩城 知子、藤村 千鶴、近江谷 克裕、山影 康次、中島 芳浩、真下 奈々、岡山 昂祐、高木 佑実、大森 崇、小島 肇、相場 節也 Multi-ImmunoTox Assay（MITA）：IL-1 Luc assay バリデーション試験の結果（ポスター）日本動物実験代替法学会 第３３回大会 Web開催（2020.11）
6. 木村 裕、藤村 千鶴、相場 節也 IL-8 Luc assay（OECD442E）の改良とLogKow >3.5物質への対応（ポスター） 細胞アッセイ研究会 Web開催（2021.1）
7. 木村 裕、藤村 千鶴、照井 仁、相場 節也 新たな皮膚感作性試験法 IL-1 Luc assay の特性（ポスター） 細胞アッセイ研究会 Web開催（2021.1）

●研究分担者： 小島　肇

1. New trend on alternative to animal testing in Japan, Kojima H, OpenTox 2018, 2018/5/24, 国内.
2. Alternative Test Methods Developed in Japan and South Korea for Regulatory Use, Kojima H, 8th Conference of Alternative Methods, 2018/6/12, 国外.
3. Recent Activities for safety assessment, Kojima H, International Symposium on Cosmetic Regulation, 2018/7/13, 国内.
4. 教育講演：ガイドライン化を目指したin vitro試験系導入の具体的な留意点, 小島 肇, 第45回日本毒性学会学術年会, 2018/7/20, 国内.
5. 皮膚・粘膜毒性、光毒性、代替試験法, 小島 肇, 第21回日本毒性学会基礎教育講習会, 2018/8/7, 国内.
6. New Approach on Alternative to Animal Test Methods in JaCVAM and Japanese projects, , Kojima H, The 15th Annual meeting of Korean Society of Alternative to Animal Experiments, 2018/8/24, 国外.
7. Introduction and research status of AAT in JaCVAM, Kojima H, The 2nd International Conference on Cosmetics Alternative Methods in NIFDC, 2018/9/20, 国外.
8. New methods validation and AAT regulatory acceptance in Japan, Kojima H, The 2nd International Conference on Cosmetics Alternative Methods in NIFDC, 2018/9/21, 国外.
9. Japanese Strategy on Alternative to Animal Test Methods for Systemic Toxicology, Kojima H, 20th International Congress on In Vitro Toxicology, 2018/10/16, 国外.
10. h-CLAT における NLRP3 インフラマソームの影響, 丸山 諒, 洪水麻衣, 三田地隆史,

小島 肇, 板垣 宏, 日本動物実験代替法学会第 31 回大会, 2018/11/24, 国内.

11./ タンパク質のアレルギー性を評価する in vitro 試験法の開発 試薬中 LPS の影響除外に関する検討（第 1 報）, 小林（九十九）英恵, 生地加奈実, 山下邦彦, 小島 肇, 板垣 宏, 日本動物実験代替法学会第 31 回大会, 2018/11/24, 国内.

12./ タンパク質のアレルギー性を評価する in vitro 試験法の開発 薬中 LPS の影響除外に関する検討（第 2 報）, 生地加奈実, 小林英恵, 山下邦彦, 小島 肇, 板垣 宏, 日本動物実験代替法学会第 31 回大会, 2018/11/24, 国内.

13./ Multi-Immuno Tox Assay（MITA）: バリデーション研究の結果, 木村 裕, 安野理恵, 渡辺美香, 小林美和子, 岩城知子, 藤村千鶴, 近江谷克裕, 山影康次, 中島芳浩, 小林眞弓, 大森 崇, 足利太可雄, 小島 肇, 相場節也, 日本動物実験代替法学会第 31 回大会, 2018/11/24, 国内.

14./ OECD AOPプロジェクト, 小島 肇, 第一回医薬品毒性機序研究会, 2019/1/10, 国内.

15./ 毒性評価系の国際標準化に向けた戦略, 小島 肇, 毒性評価研究会, 2019/1/31, 国内.

16./ ヒト健康影響を予測するための非動物実験の開発動向, 小島 肇, Translational and Regulatory Science Symposium, 2019/2/7, 国内.

17./ AOP（Adverse Outcome Pathway; 有害性発現経路）に基づいた化学物質の安全性評価へ向けたチャレンジ, 山田隆志, 足利太可雄, 小島 肇, 広瀬明彦, 日本薬学会第 139 年会, 2019/3/23, 国内

18./ The Japanese Strategy on Chemical Risk Assessment with New Approaches, Kojima H, International Symposium for EDCs Testing & Assessment, 2019/5/31, 国外, 口頭.

19./ Use of new approach methods (NAM) in next generation risk assessment (NGRA), Kojima H, International Symposium for EDCs Testing & Assessment, 2019/6/4, 国外, 口頭.

20./ In vitro から in vivo の予測、ヒト外挿性向上への期待, 小島 肇, 第 46 回日本毒性学会学術年会, 2019/6/26, 国内, 口頭.

21./ 実験動物を用いた安全性・リスク評価に携わる人材育成の必要性, 小島 肇, 小川 久美子, 西川 秋佳, 若林 敬二, 鰐渕 英機, 林 真, 福島 昭治, 遠山 千春, 第 46 回日本毒性学会学術年会, 2019/6/27, 国内, 口頭.

22./ 皮膚感作性試験代替法を行政的に受け入れるための国際動向, 小島 肇, 第 46 回日本毒性学会学術年会, 2019/6/27, 国内, 口頭.

23./ OECD AOP プロジェクトにおける日本の対応, 小島 肇, 第 46 回日本毒性学会学術年会, 2019/6/28, 国内, 口頭.

24./ 21st Century Toxicology and Regulatory Testing: An Update from East Asia, Kojima H, The 15th International Congress of Toxicology (ICTXV) , 2019/7/16, 国外, 口頭.

25./ OECD AOP プロジェクト, 小島 肇, 第 26 回日本免疫毒性学会学術年会, 2019/9/10, 国内, 口頭.

26./ 培養組織モデルの国際標準化の状況, 小島 肇, LbL-3D 組織シンポジウム, 2019/9/12, 国内, 口頭.

27./ Multi-ImmunoTox Assay（MITA）の予測性評価に必要な文献に基づく化学物質免疫毒性分類の試み, 木村 裕, 安野 理恵, 渡辺 美香, 小林 美和子, 岩城 知子, 藤村 千鶴, 近江谷 克裕, 山影 康次, 中島 芳浩, 真下 奈々, 高木 佑実, 大森 崇, 小島 肇, 相場節也,日本動物実験代替法学会第 32 回大会, 2019/11/20, 国内, ポスター.

28./ ADRA における DMSO 溶媒中での NAC の酸化と感作性予測精度に与える影響, 秋元美由紀, 吉田浩介, 渡辺真一, 山鹿宏彰, 若林晃次, 田原 宥, 堀江宣行, 藤本恵一, 草苅啓, 神谷孝平, 河上強志, 小島幸一, 寒水孝司, 小野 敦, 小島 肇, 藤田正晴, 山本裕介, 笠原利彦, 日本動物実験代替法学会第 32 回大会, 2019/11/21, 国内, ポスター.

29./ 安全性評価試験法の OECD 等における国際動向と課題, 小島 肇:日本動物実験代替法学会第 32 回大会, 2019/11/22, 国内, 口頭.

30./ 安全性評価における Replacement の概要, 小島 肇, 第 47 回日本毒性学会学術年会, 2020/6/29, web 開催, 国内, 口頭.

31./ OECD におけるコンピューターモデルの行政的な受け入れ, 小島 肇, CBI 学会 2020 年大会, 2020/10/28, web 開催, 国内, 口頭.

32./ 皮膚感作性試験代替法 Epidermal Sensitization Assay (EpiSensA)の Validation 研究(施設内再現性 Phase I), 水町秀之, 渡辺美香, 生悦住茉友, 梶原三智香, 安田美智代, 水野 誠, 今井教安, 佐久間めぐみ, 芝田桃子, 渡辺真一, 上野順子, Basketter

D, Eskes C, Hoffmann S, Lehmann D, 足利太可雄, 寒水孝司, 武吉正博, 宮澤正明, 小島 肇, 日本動物実験代替法学会 第 33 回大会, 2020/11/12, web 開催, 国内, ポスター.

33./ Multi-ImmunoTox Assay (MITA) : IL-1 Luc assay バリデーション試験の結果, 木村 裕, 安野 理恵, 渡辺 美香, 小林 美和子, 岩城 知子, 藤村 千鶴, 近江谷 克裕, 山影 康次, 中島 芳浩, 真下 奈々, 岡山 昂祐, 高木 佑実, 大森 崇, 小島 肇, 相場 節也, 日本動物実験代替法学会 第33回大会, 2020/11/12, web 開催, 国内, ポスター.

34./ Non-animal Alternative Toxicology and Regulatory Testing, Kojima H, An Update from Japan. Virtual International Conference to Mark the Launch of the Society for Alternatives to Animal Testing in Sri Lanka (SAAT-SL), 2020/2/7, web 開催, 国外, 口頭.

●研究分担者： 中島　芳浩

1./ 木村 裕、安野 理恵、渡辺 美香、小林 美和子、岩城 知子、藤村 千鶴、近江谷 克裕、山影 康次、中島 芳浩、真下　奈々、高木 佑実、大森 崇、小島 肇、相場 節也：Multi-ImmunoTox Assay (MITA)の予測性評価に必要な文献に基づく化学物質免疫毒性分類の試み 日本動物実験代替法学会 第 32 回大会 つくば (2019.11)

2./ 木村 裕、安野 理恵、渡辺 美香、小林 美和子、岩城 知子、藤村 千鶴、近江谷 克裕、山影 康次、中島 芳浩、真下 奈々、岡山 昂祐、高木 佑実、大森 崇、小島 肇、相場 節也 Multi-ImmunoTox Assay (MITA) : IL-1 Luc assay バリデーション試験の結果 (ポスター) 日本動物実験代替法学会 第３３回大会 Web開催 (2020.11)

●研究分担者： 安野　理恵

1./ 木村 裕、安野 理恵、渡辺 美香、小林 美和子、岩城 知子、藤村 千鶴、近江谷 克裕、山影 康次、中島 芳浩、真下　奈々、高木 佑実、大森 崇、小島 肇、相場 節也：Multi-ImmunoTox Assay (MITA)の予測性評価に必要な文献に基づく化学物質免疫毒性分類の試み 日本動物実験代替法学会 第 32 回大会 つくば (2019.11)

2./ 木村 裕、安野 理恵、渡辺 美香、小林 美和子、岩城 知子、藤村 千鶴、近江谷 克裕、山影 康次、中島 芳浩、真下 奈々、岡山 昂祐、高木 佑実、大森 崇、小島 肇、相場 節也 Multi-ImmunoTox Assay (MITA) : IL-1 Luc assay バリデーション試験の結果 (ポスター) 日本動物実験代替法学会 第３３回大会 Web開催 (2020.11)

研究分担者： 大森　崇

3./ 木村 裕、安野 理恵、渡辺 美香、小林 美和子、岩城 知子、藤村 千鶴、近江谷 克裕、山影 康次、中島 芳浩、真下　奈々、高木 佑実、大森 崇、小島 肇、相場 節也：Multi-ImmunoTox Assay (MITA)の予測性評価に必要な文献に基づく化学物質免疫毒性分類の試み 日本動物実験代替法学会 第 32 回大会 つくば (2019.11)

4./ 木村 裕、安野 理恵、渡辺 美香、小林 美和子、岩城 知子、藤村 千鶴、近江谷 克裕、山影 康次、中島 芳浩、真下 奈々、岡山 昂祐、高木 佑実、大森 崇、小島 肇、相場 節也 Multi-ImmunoTox Assay (MITA) : IL-1 Luc assay バリデーション試験の結果 (ポスター) 日本動物実験代替法学会 第３３回大会 Web開催 (2020.11)

●研究分担者： 木村　裕

1./ 木村 裕、安野　理恵、渡辺　美香、小林　美和子、岩城　知子、藤村　千鶴、近江谷　克裕、山影　康次、中島　芳浩、小林 眞弓、大森 崇、足利 太可雄、小島 肇、相場 節也：Multi-ImmunoTox Assay (MITA) ：バリデーション研究の結果 日本動物実験代替法学会 第31回大会 (熊本) (2018. 11)

2./ 木村 裕、安野 理恵、渡辺 美香、小林 美和子、岩城 知子、藤村 千鶴、近江谷 克裕、山影 康次、中島 芳浩、真下　奈々、高木 佑実、大森 崇、小島 肇、相場 節也：Multi-ImmunoTox Assay (MITA)の予測性評価に必要な文献に基づく化学物質免疫毒性分類の試み 日本動物実験代替法学会 第32回大会 つくば (2019.11)

3./ 木村 裕、藤村 千鶴、相場 節也 IL-8 Luc assay (OECD442E) のクライテリアの改変とLogKowが3.5を超える化学物質における偽陰性率の改善(ポスター) 日本動物実験代替法学会 第３３回大会 Web開催 (2020.11)

4. 木村 裕、安野 理恵、渡辺 美香、小林 美和子、岩城 知子、藤村 千鶴、近江谷 克裕、山影 康次、中島 芳浩、真下 奈々、岡山 昂祐、高木 佑実、大森 崇、小島 肇、相場 節也 Multi-ImmunoTox Assay (MITA) : IL-1 Luc assay バリデーション試験の結果（ポスター）日本動物実験代替法学会 第３３回大会 Web開催 (2020.11)
5. 木村 裕、藤村 千鶴、相場 節也 IL-8 Luc assay (OECD442E) の改良とLogKow >3.5物質への対応 (ポスター) 細胞アッセイ研究会 Web開催 (2021.1)
6. 木村 裕、藤村 千鶴、照井 仁、相場 節也 新たな皮膚感作性試験法 IL-1 Luc assay の特性（ポスター） 細胞アッセイ研究会 Web開催 (2021.1)

H. 知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得

1. 相場節也 齋藤るみ子 木村裕 近江谷克裕 中島芳浩 西井重明 山崎友実 安田真琴 ; 特許第 5999644 号(2016) ; 多色発光細胞を用いた免疫毒性評価システム
2. 相場節也 木村裕 近江谷克裕 西井重明 ; 特開 2014-3939 ; 免疫毒性評価細胞を用いたＴＮＦ–α 阻害活性を定量化するシステム
3. 木村裕 相場節也 ; 特開 2016-208851 ; ＴＬＲ刺激物質の検出方法
4. 近江谷克裕, 三谷恭雄, 安野理恵 ; 特許第 6441534 号(2018) ; 発光酵素タンパク質
5. 近江谷克裕, 三谷恭雄, 安野理恵 ; WO2017-155036 : Light-emitting enzyme protein
6. 相場節也、木村裕: PTC 出願 (2021): 多色発光細胞を用いた化学物質評価システム

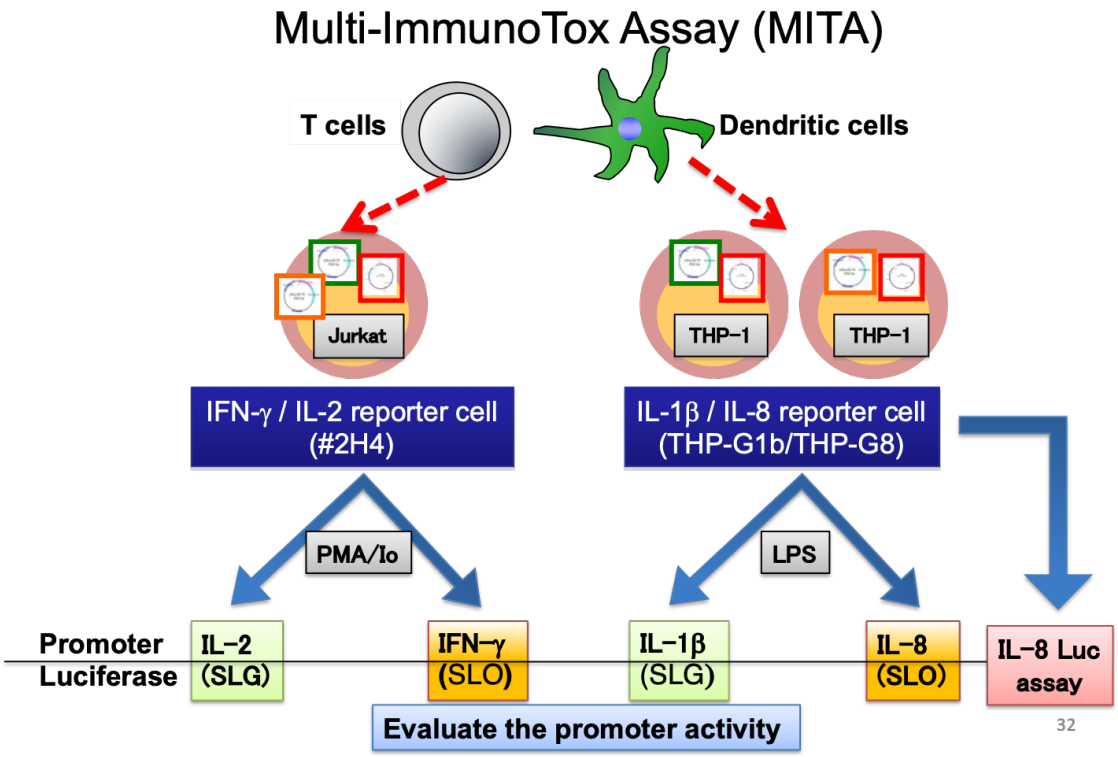

図１． Multi-ImmunoTox Assay (MITA)の概略図

添付資料１． IL-2 Luc assay 修正 validation report

添付資料２． IL-2 Luc assay 修正 SPSF

添付資料３． IL-1 Luc assay validation report.

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添付資料５． IL-2 Luc LTT protocol

添付資料６． IL-2 Luc LTT Phase 1 結果

添付資料７． IL-8 Luc assay 修正提案の SPSF

添付資料８． ジーピーシー研究所からの報告書

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1. Summary

The IL-2 luciferase reporter assay (IL-2 Luc assay) was developed as one of 3 luciferase reporter assays in the Multi-ImmunoTox assay (MITA), a high-throughput screening system that our group had developed to evaluate chemical immunotoxicity. Although our final long-term goal is to officially validate the MITA for within- and between- laboratory reproducibility and predictivity, in this study, we conducted the validation for IL-2 Luc assay as the initial step.

In the MITA, we used 3 stable lines of reporter cells transfected with luciferase genes under control of the IL-2, IFN-γ, IL-8, and IL-1β promoters: 2H4 cells derived from Jurkat cells containing stable luciferase green (SLG) regulated by the IL-2 promoter, stable luciferase orange (SLO) regulated by the IFN-γ promoter, and stable luciferase red (SLR) regulated by the GAPDH promoter; THP-G8 cells derived from THP-1 cells containing SLO regulated by the IL-8 promoter and SLR regulated by the GAPDH promoter; and THP-G1b cells derived from THP-1 cells containing SLG regulated by the IL-1β promoter and SLR regulated by the GAPDH promoter. We selected these 4 cytokines because IL-2 and IFN-γ are primarily produced by T cells (a type of adaptive immune cells), whereas IL-8 and IL-1β are primarily produced by monocytes and dendritic cells (types of innate immune cells).

Using these 3 cell lines, the MITA can evaluate the effects of chemicals on the IL-2 and IFN-γ luciferase activity of 2H4 cells stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Io), those on the IL-1β and IL-8 luciferase activity of THP-G1b and THP-G8 cells, respectively, stimulated by lipopolysaccharide (LPS).

In the validation study of the IL-2 Luc assay, the preliminary test trial, Phase 0, was performed by the participating laboratories following explicit explanations of the Multi-ImmunoTox Assay protocol Ver. 008.1E proposed by the lead laboratory, Tohoku University. Three laboratories participated in the Phase 0 study of the IL-2 Luc assay using 5 open labeled chemicals (2-aminoantracene, citral, chloroquine, dexamethasone and methyl mercuric chloride), in which they conducted 1 set composed of 3

experiments for each chemical. Most response patterns for the 5 chemicals were similar among the 3 laboratories, except for 2 early experiments conducted by the naïve laboratory. Based on these results, the Validation Management Team (VMT) judged that technical and protocol transfer of the IL-2 Luc assay is acceptable.

In the Phase I study, a total of 5 coded chemicals (4 T cell targeting and 1 non-T cell targeting) were evaluated by 3 experimental sets based on the Multi-ImmunoTox Assay protocol Ver. 011E made by the lead laboratory, Tohoku University. The average within-laboratory reproducibility was 86.7% (13/15). The between-laboratory reproducibility was 80.0% (4/5). The average predictivity was 93.3% (14/15).

In the Phase II study, between-laboratory reproducibility and predictivity using a total of 20 coded chemicals (13 T cell targeting, 6 non-T cell targeting, and 1 undetermined) were evaluated by 1 experiment set based on the Multi-ImmunoTox Assay protocol Ver. 009.1E. The between-laboratory reproducibility was 80% (16/20) and the average predictivity was 70.2% (40/57).

In the combined results of the Phase I and II studies, the average within-laboratory reproducibility was 86.7% (13/15). The between laboratory reproducibility was 80% (20/25). The average predictivity was 75.0% (54/72).

Although the within- and between-laboratory reproducibilities could satisfy the acceptance criteria for the validation study, the predictivity was below 80%. We considered several possible reasons for this unsatisfactory predictivity.

Since the 2H4 cell line used in the IL-2 Luc assay is derived from Jurkat cells, the IL-2 Luc assay cannot evaluate immunotoxic effects of immunosuppressive compounds whose mode of action is the inhibition of DNA synthesis leading to myelotoxicity. Thus, these chemicals should be outside the defined applicability domain for the assay. To overcome this limit, the IL-2 Luc assay requires combination with assays capable of detecting myelotoxicity, such as the conventional 28-day repeat dose toxicity test or *in vitro* myelotoxicity tests (Pessina et al., 2003). In addition, chemicals that need metabolic activation or poor water soluble need to be outside the applicability domain.

Even though these applicability domains are taken into consideration, the IL-2 Luc assay alone cannot cover all the effects of chemicals on human immune system. Therefore, it is indispensable to develop other *in vitro* systems to detect the effects of chemicals on different aspects of immune response. By accumulating and combining various approaches to detect chemical immunotoxicity, the *in vitro* assays can cover the effects of chemicals on the broad range of human immune system. The IL-2 Luc assay can be the first step.

2. Objective of the study

The objective of the present validation study was to determine the usefulness and limitations of the IL-2 Luc assay in MITA as a non-animal screening method to detect and assess the immunotoxicity of chemicals.

The specific objectives of the study were to establish:

1) “Transferability”, i.e., the extent to which a laboratory can adapt and easily implement the IL-2 reporter assay;

2) “Between or inter-laboratory reproducibility”, i.e., the extent to which results agree among different laboratories;

3) "Within or intra-laboratory reproducibility", i.e., the extent to which results agree in the same laboratory; and

4) “Predictivity”, i.e., the extent to which the *in vitro* results agree with the known immunological profiles of the chemicals.

3. Background

3-1. What is immunotoxicity?

A well-functioning immune system is essential for maintaining the integrity of an organism. Immune dysregulation can have serious adverse health consequences, ranging from reduced resistance to infection and neoplasia to allergic and autoimmune conditions. Environmental contaminants, food additives, and drugs can target the immune system, resulting in immune dysregulation. Accordingly, the potential for immunotoxicity, which is defined as the toxicological effects of xenobiotics on the function of the immune system, has raised serious concerns from the public as well as regulatory agencies. Currently, the assessment of chemical immunotoxicity relies mainly on animal models and assays that characterize immunosuppression and sensitization. However, animal studies have many drawbacks, such as high cost, ethical concerns, and questionable relevance to risk assessment for humans.

3-2. The current status of *in vitro* approaches to detect immunotoxicants

Now the worldwide vision is promoting alternative testing methods and assessment strategies to reduce the use of laboratory animals and, if possible, replace animals used in scientific studies (Adler et al., 2011). The workshop hosted by the European Centre for the Validation of Alternative Methods (ECVAM) in 2003 focused on state-of-the-art *in vitro* systems for evaluating immunotoxicity (Galbiati et al., 2010; Gennari et al., 2005; Lankveld et al., 2010). In the ECVAM workshop, a tiered approach was proposed. Since useful information can be obtained from regular 28-day general toxicity tests, pre-screening for direct immunotoxicity would begin with the evaluation of myelotoxicity in the proposed tiered approach (Corsini and Roggen, 2017). Compounds that are capable of damaging or destroying bone marrow will most likely have immunotoxic effects. If compounds are not potentially myelotoxic, they are tested for leukotoxicity. Compounds are then tested for immunotoxicity using various approaches such as the human whole-blood cytokine release assay (HWBCRA), lymphocyte proliferation assay, mixed lymphocyte reaction, NK cell assay, T cell–dependent antibody response, dendritic cell maturation assay, and fluorescent cell chip (FCP) assay. Among these assays, the HWBCRA has undergone formal pre-validation, although other techniques are being examined or have been examined in a rigorous pre-validation effort by the ECVAM and other groups. (Fig. 1) However, these assays require fresh rodent or human immune cells, in conflict with animal protection goals. The need for primary cells may decrease reproducibility and makes the assay unsuitable for high-throughput approaches

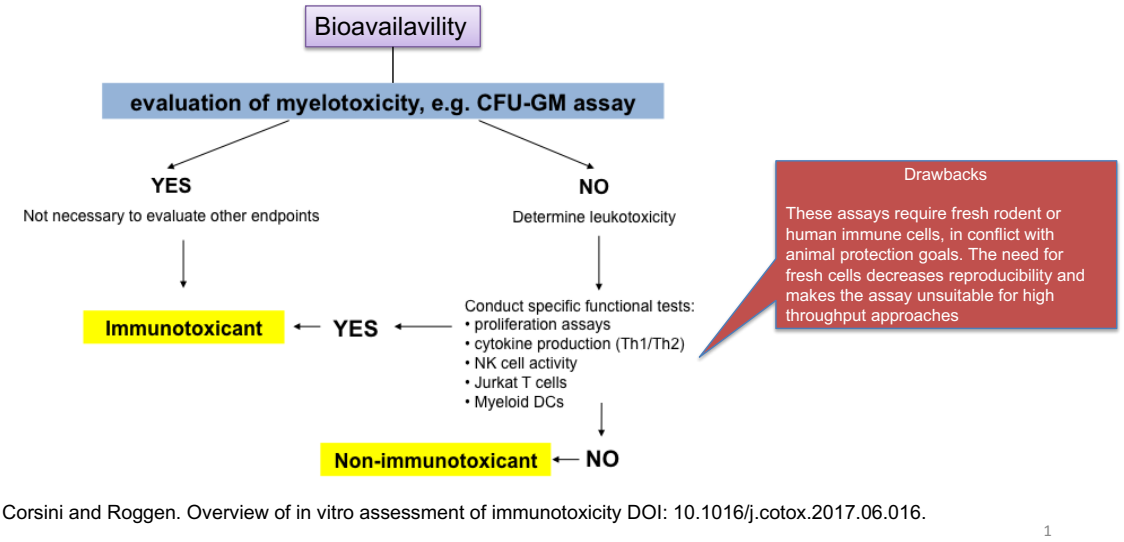

Fig. 1. Decision tree approach for *in vitro* assessment of chemical-induced immunosuppression.

3-3. *In vitro* immunotoxicity tests in principle should evaluate effects on both innate and acquired immunity

The immune system comprises innate and adaptive immunity (Fig. 2). Both arms of the immune response function differently and are driven by different populations of cells. In innate immunity, pathogens are recognized through various pattern recognition molecules, such as C-type lectin receptors, toll-like receptors, nod-like receptors, and retinoic acid–inducible gene-I (RIG-I)-like receptors. In addition, a variety of different cells are involved in this type of response, including neutrophils and other types of granulocytes, macrophages, natural killer (NK) cells, innate lymphoid cells, and mast cells. Adaptive immune responses involve specific antigen receptors encoded by rearranged genes, and T cells and B cells play critical roles in these responses.

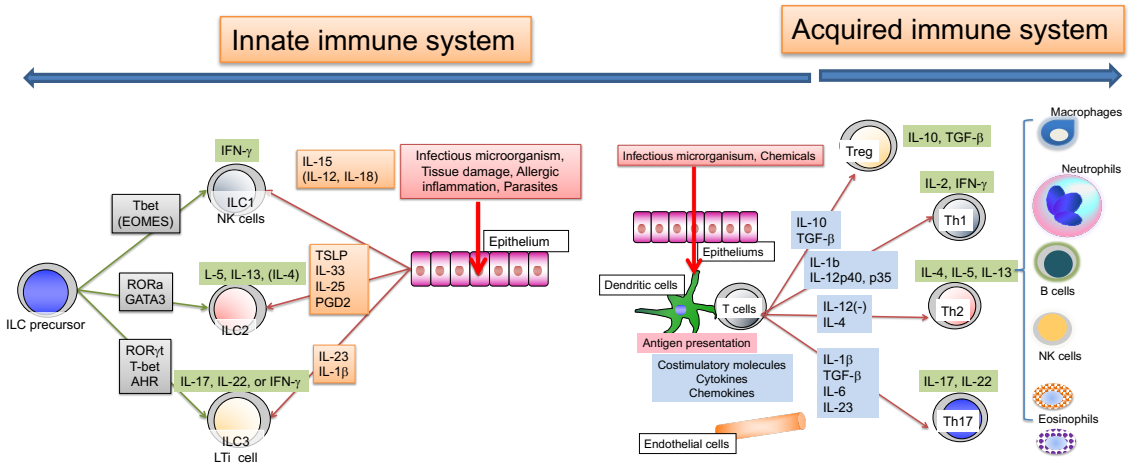

Fig2. Schematic representation of the innate immune system and acquired immune system.

Macrophages and dendritic cells (DCs), which act as antigen-presenting cells (APCs), link the innate and adaptive immune responses because they can present antigens to T lymphocytes in the context of major histocompatibility complex (MHC) class I or II molecules and stimulate their proliferation and effector functions after being stimulated via pathogen recognition receptors (Fig. 3). To induce optimal immune responses to various pathogens and minimize autoreactivity, innate and adaptive immune cells produce a vast array of cytokines, chemokines, and chemical mediators and present the molecules required for direct cell-cell interaction on their surface. A variety of intracellular signaling pathways also play roles in innate and adaptive immune responses.

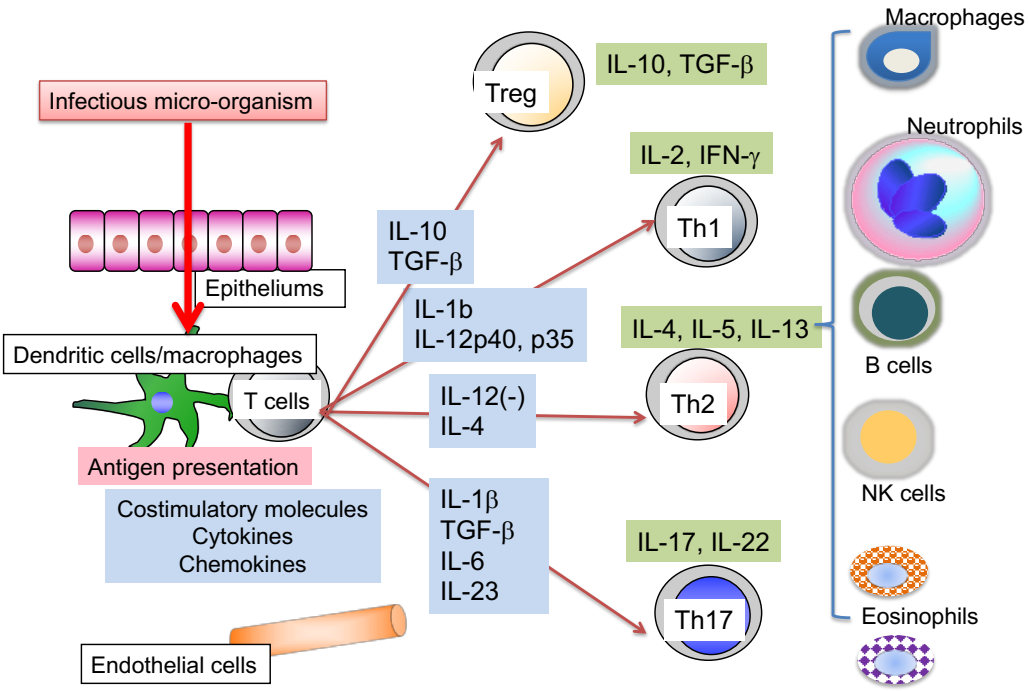

Fig. 3. Dendritic cells link the innate immune response to the acquired immune response.

Theoretically, chemicals can affect the immune system by targeting either the innate immune system or the acquired immune system (Fig. 2 and Fig. 3). Therefore, novel *in vitro* test methods are needed to adequately assess the immunotoxic effects of chemicals on both arms of immune system.

3-4. Mechanism for the induction of immunotoxicity by chemicals

Given the complexity of the immune system, it is unlikely that a single *in vitro* method will be able to detect all immunotoxicants. The mechanisms underlying the immunotoxicity of chemicals can be classified into 3 main categories: 1) killing of immune cells caused by bone marrow toxicity, 2) interference with general or immune-specific signaling leading to changes in the expression of cell surface molecules, cytokines or chemokine production, cell differentiation, and activation, and 3) binding to proteins forming complete antigens or exposing cryptic self-proteins (Fig. 4).

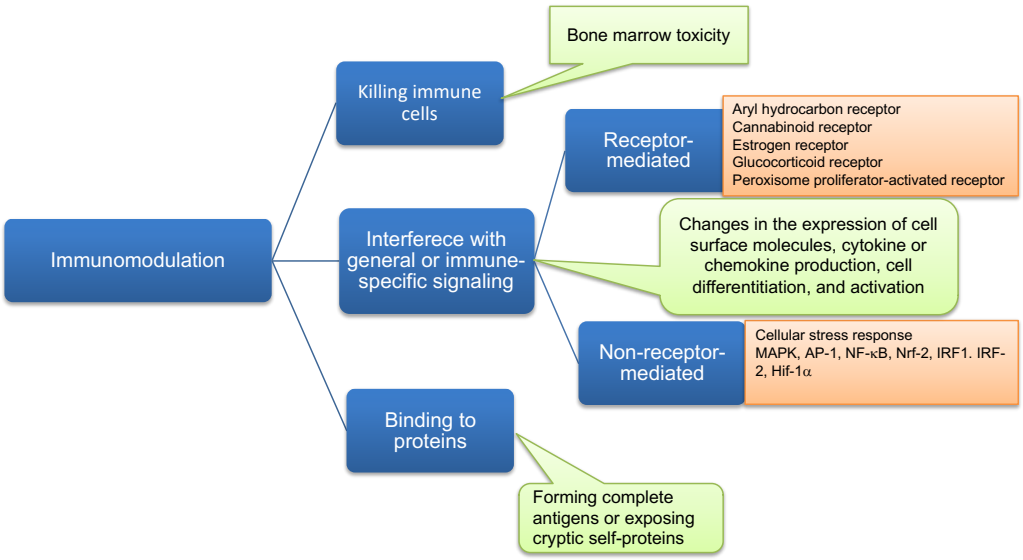

Fig. 4. Main mechanisms of immunotoxicity

Chemicals can interfere with immune-related cell signaling through receptor-mediated pathways using xenobiotic receptors such as the aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), or pregnane X receptor (PXR) (Elentner et al., 2018; Hidaka et al., 2017), cannabinoid receptor, estrogen receptor, glucocorticoid receptor or peroxisome proliferator-activated receptor or through non-receptor-mediated ways. Without specific receptors, it has been demonstrated that so-called cellular stress response can cause immunotoxicity (Fulda et al., 2010; Kultz, 2005). In essence, as long as stress stimulus does not cross a certain threshold, a cell can cope and survive by mounting an appropriate protective response. Conversely, the failure to activate or maintain a protective response (e.g., when the stressor is too strong) results in activation of stress signaling cascades that eventually activate cell death pathways. Depending on the type of stress and its severity, a cell's response can be manifold. However, most cellular protective responses induced by chemicals can be classified into one of several

categories, such as heat shock, unfolded protein, DNA damage, and oxidative stress responses, in addition to the response to danger signals (Gallucci and Matzinger, 2001). These responses are independent of the chemical species (Fig. 5). In addition, these cellular stress responses can affect immune function because they share the same cellular signaling pathways, e.g., MAP kinase, NF-κB, and mTOR, used by the immune response (Milisav, 2011). Indeed, although sensitizers that induce allergic contact hypersensitivity include numerous compounds with different molecular structures, it has become clear that their ability to sensitize is based simply on their reactivity to cysteine residues, which induces a response to oxidative stress (Sasaki and Aiba, 2007). Therefore, although it is assumed that there may be many chemicals with the potential to produce immunotoxicity, only a limited number of assay systems may be required to detect their effects.

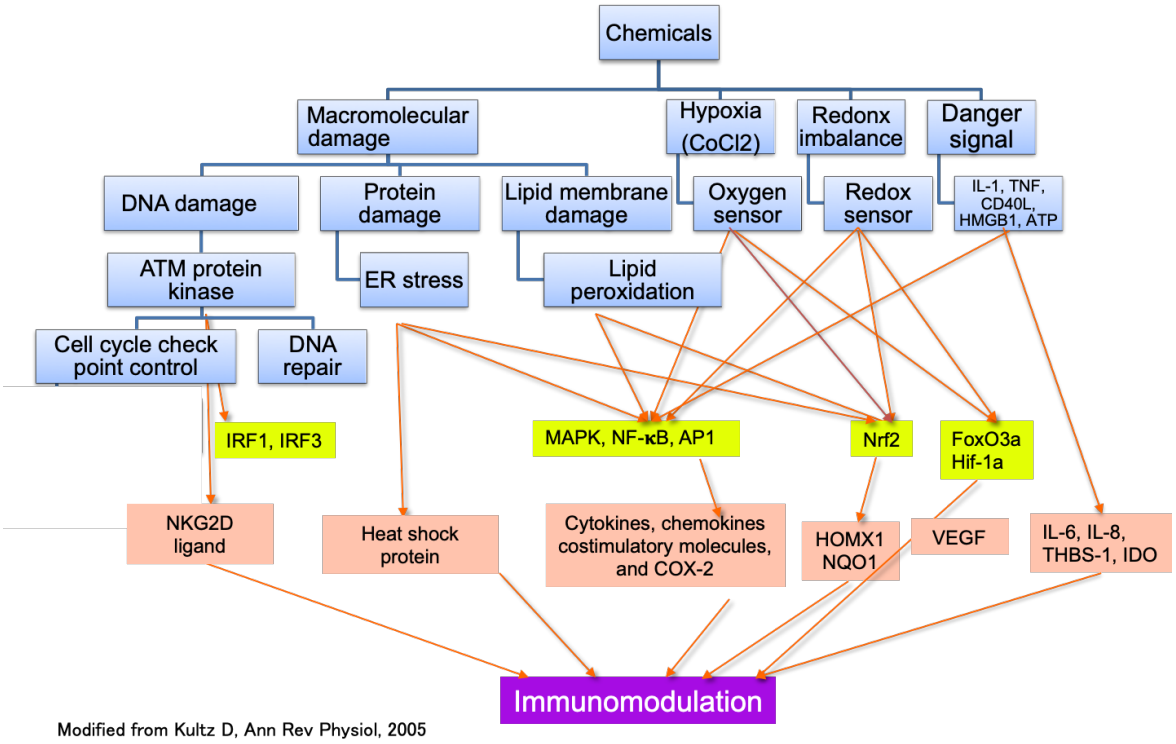

Fig. 5. Cellular stress response and danger signals.

3-5. Multi-ImmunoTox assay (MITA)

Our group developed a high-throughput screening system to evaluate chemical immunotoxicity. We first established 3 stable reporter cell lines transfected with luciferase genes under control of the IL-2, IFN-γ, IL-8, and IL-1β promoters: 2H4 cells derived from Jurkat cells containing stable luciferase green (SLG) regulated by the IL-2 promoter, stable luciferase orange (SLO) regulated by the IFN-γ promoter, and stable luciferase red (SLR) regulated by the GAPDH promoter (Saito et al., 2011); THP-G8 cells derived from THP-1 cells containing SLO regulated by the IL-8 promoter and SLR regulated by GAPDH promoter (Takahashi et al., 2011); and THP-G1b cells derived from THP-1 cells containing SLG regulated by the IL-1β promoter and SLR by the GAPDH promoter (Kimura et al., 2014). These 4 cytokines were selected because IL-2 and IFN-γ are primarily produced by T cells (adaptive immune cells), whereas IL-8 and IL-1β are primarily produced by monocytes and dendritic cells (innate immune cells). Using these 3 cell lines, we established the Multi-ImmunoTox assay (MITA). This assay identifies the effects of chemicals on the IL-2 and IFN-γ luciferase activity in 2H4 cells in the presence of the stimulants phorbol 12-myristate 13-acetate (PMA) and ionomycin (Io), and on the IL-1β and IL-8 luciferase activities in THP-G1b and THP-G8 cells, respectively, in the presence of the stimulant lipopolysaccharide (LPS) (Fig. 6).

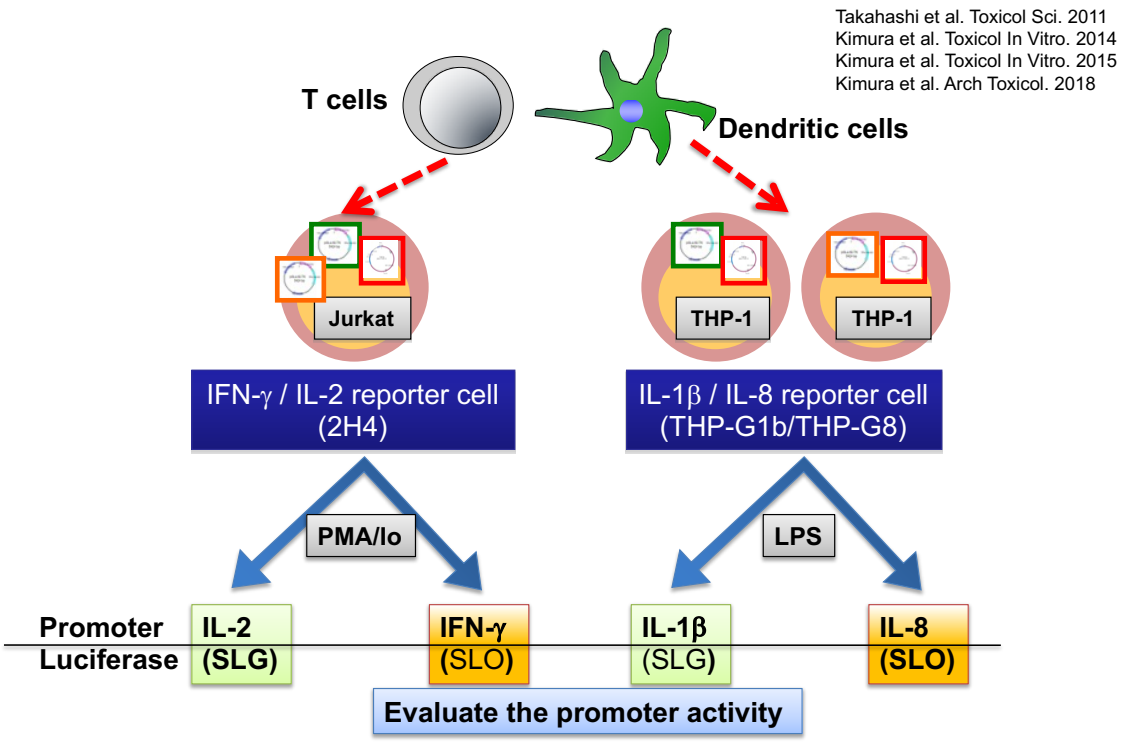

Fig. 6. The Muti-ImmunoTox assay (MITA)

3-6. The luciferase activities of the three MITA cell lines correspond with mRNA expression in the wild type cell lines or in human whole blood cells when stimulated with PMA/Io or LPS in the presence of 3 representative immunosuppressive drugs

After establishing the MITA, we first compared the effects of dexamethasone, cyclosporine, and tacrolimus on the 3 MITA cell lines with those on mRNA expression in the wild type cell lines or in human whole-blood cells stimulated with PMA/Io or LPS. The results confirmed that the MITA correctly reflects changes in mRNA expression in the mother cell lines and whole-blood cells (Kimura et al., 2014).

3-7. The MITA can evaluate the immunotoxicity profiles of well-known immunosuppressive drugs

We next evaluated the performance of the MITA by examining immunosuppressive or immunomodulatory drugs with well-known clinical effects on the human immune system (Kimura et al., 2014). The results obtained with immunosuppressive drugs classified by their principal mechanism of action are shown in Table 1, in which the classification of drugs is based on the review by Allison (Allison, 2000).

The MITA demonstrated that dexamethasone (Dex) significantly suppressed IL-2, IL-1β, and IL-8 reporter activities, while cyclosporine A (CyA) and tacrolimus (Tac) suppressed IL-2 and IFN-γ reporter activities but had no effect on IL-1β and IL-8 reporter activities. However, the MITA could not detect the immunosuppressive effects of the alkylating agent cyclophosphamide, of the inhibitors of de novo purine synthesis azathioprine (AZ), mycophenolic acid (MPA) and mizoribine (MZR), and of the inhibitor of pyrimidine and purine synthesis, methotrexate (MT). These data suggest that the MITA correctly evaluates the effects of chemicals on cytokine expression but cannot detect immunotoxicity associated with the inhibition of DNA synthesis and cell division. This drawback has also been reported for other assays, such as the human whole-blood cytokine release assay (HWBCRA) (Langezaal et al., 2002) and the FCP assay (Wagner et al., 2006). On the other hand, the MITA has the advantage that it can discriminate the effects of chemicals on T cells from those on macrophages/dendritic cells.

Table 1. The MITA can detect immunosuppressive effects of representative immunosuppressive drugs

Principal mechanism of action	Drugs	The effects of transcriptional activity			
		IL-2	IFN-γ	IL-1β	IL-8
Immunosuppressing drugs					
Regulation of gene expression	Dexamethasone (Dex)	S	N	S	S
Kinase and phosphatase inhibitors	Cyclosporin A (CyA)	S	S	N	N
	Tacrolimus (Tac)	S	S	N	N
	Rapamycin (RPM)	A	N	N	N
Alkylation	Cyclophosphamide (CP)	N	N	N	N
Inhibition of de novo purine synthesis	Azathioprine (AZ)	N	N	N	N
	Mycophenolic acid (MPA)	A	A	N	N
	Mizoribine (MZR)	N	N	A	A
Inhibition of pyrimidine and purine synthesis	Methotrexate (MTX)	N	A	N	N
Off-label immunosuppressing drugs					
	Sulfasalazine (SASP)	S	S	S	S
	Colchicine	S	N	A	N
	Chloroquine (CQ)	S	N	N	N
	Minocycline (MC)	S	S	N	N
	Nicotinamide (NA)	S	N	S	S
Non-immunomodulatory drugs					
	Acetaminophen (AA)	N	N	N	N
	Digoxin	S	S	N	N
	Warfarin	N	N	S	S

Kimura et al. Toxicol in Vitro 28: 759-769, 2014

*S and A indicates that drugs showed statistically significant suppression in triplicate experiments for each parameter, while N indicates that drugs did not show significant effects.

3-8. The process of validation of the MITA

Although our final goal is to officially validate the MITA for within- and between-laboratory reproducibility and predictivity, in this study, we conducted the validation study for the IL-2 Luc assay as the initial step. Since 2H4 cells used in this validation study is derived from Jurkat cells that contain SLG regulated by the IL-2 promoter, SLO

regulated by the IFN-γ promoter, and SLR regulated by the GAPDH promoter (Saito et al., 2011), this cell line can simultaneously evaluate the effects of chemicals on IL-2 and IFN-γ transcription. However, our previous study demonstrated the significant correlation between the Lowest Observed Effect Levels (LOELs) for the effects of chemicals on the IL-2 luciferase assay and those on the IFN-γ luciferase assay (Kimura et al, 2014). Therefore, we decided to conduct the validation study of only IL-2 Luc assay. Recently, the process of this validation study has been published (Kimura et al., 2020)

3-9. The proposed Adverse Outcome Pathway (AOP) of chemicals that affect IL-2 transcription

Immune dysregulation may have serious impacts on human health, ranging from reduced resistance to infection and neoplasia to allergic and autoimmune conditions. Pivotal immune elements of these diseases are the development of antigen-specific effector T-helper type (Th2) cells, Th1 cells, Th17 cells, and regulatory T cells (Treg cells) that are associated with clinical features and disease progression. Consequently, identifying the immunotoxicity of chemicals requires clarifying their effects on the development of these T cells (reviewed by (Kaiko et al., 2008)).

IL-2 exerts pleiotropic actions on CD4+ T cell differentiation via its modulation of cytokine receptor expression. IL-2 promotes Th1 differentiation by inducing IL-12Rb2 (and IL-12Rb1), promotes Th2 differentiation by inducing IL-4Ra, inhibits Th17 differentiation by inhibiting gp130 (and IL-6Ra), and drives Treg differentiation by inducing IL-2Ra. IL-2 also potently represses IL-7Ra, which decreases survival signals that normally promote cell survival and memory cell development (reviewed by (Liao et al., 2011)). It is therefore conceivable that chemicals that affect IL-2 release by T cells could significantly impact immune function; consequently, we focused on the regulation of IL-2 transcription and attempted to construct an AOP with transcriptional dysregulation of IL-2 as a central key event.

IL-2 mRNA is transcribed after T cell receptor stimulation. Therefore, chemicals that affect any pathway leading to IL-2 transcription after T cell activation can induce dysregulation of IL-2 mRNA and protein expression by T cells. In antigen presentation, T cells are stimulated by T cell receptor (TCR) with co-receptor CD4 or CD8 and CD28. The TCR with CD4 or CD8 recognizes the major histocompatibility complex (MHC)–peptide complex, which results in activation of the SRC kinase Lck and subsequent phosphorylation of immunoglobulin family tyrosine (Y)-based activation motifs (ITAMs) in the CD3 complex (Y-p). This leads to recruitment and phosphorylation of ζ-chain-associated protein (ZAP70), which phosphorylates adaptor proteins, resulting in activation of phospholipase Cγ1 (PLCγ1) and the guanine triphosphatase RAC. PLCγ, in turn, promotes Ca^{2+} mobilization and RAS activation. The combination of these upstream events leads, by complex signaling cascades, to activation of the mitogen-activated protein (MAP) kinases: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, as well as phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB/Akt). Together, these signals promote different events, including the activation of transcription factors, which result in gene expression and, presumably, T-cell function. On the other hand, CD28 might associate, in its unphosphorylated state, with the serine/threonine phosphatase protein phosphatase 2A (PP2A). Upon T-cell stimulation, CD28 undergoes phosphorylation on its intracellular tyrosine residues (Y), presumably resulting in dissociation from PP2A and recruitment of phosphatidylinositol 3-kinase (PI3K) and growth-factor-receptor-bound protein 2 (GRB2). Activation of PI3K, which induces phosphorylation of phosphatidylinositol (PI) into phosphatidylinositol 3-phosphate (PIP3), might promote activation of protein kinase B (PKB/Akt), followed by activation of nuclear factor-κB (NF-κB), resulting in BCL-XL upregulation that favors T-cell survival. Akt activation might also promote interleukin-2 (IL-2) production. PI3K is negatively regulated by phosphatase and tensin homologue (PTEN). The carboxy-

terminal proline (P)-rich region might promote IL-2 production and proliferation, perhaps by recruiting and activating Lck (reviewed by (Alegre et al., 2001)).

Many chemicals have been reported to affect IL-2 transcription or production. Any component of these signaling cascades can be a potential target of these chemicals, but the mechanism by which they affect IL-2 transcription or production remains largely unknown.

Based on recent advances in immunology, we tentatively propose the following AOP for immunosuppression focusing on IL-2 transcription. Figure 7 shows the AOP with representative chemicals that affect IL-2 transcription. From 2001 to 2017, 54 chemicals were reported to augment IL-2 gene or protein expression in human and 60 chemicals had this effect in mice, while 65 chemicals in human and 47 chemicals in mice were reported to decrease IL-2 gene or protein expression, as determined by a PubMed search.

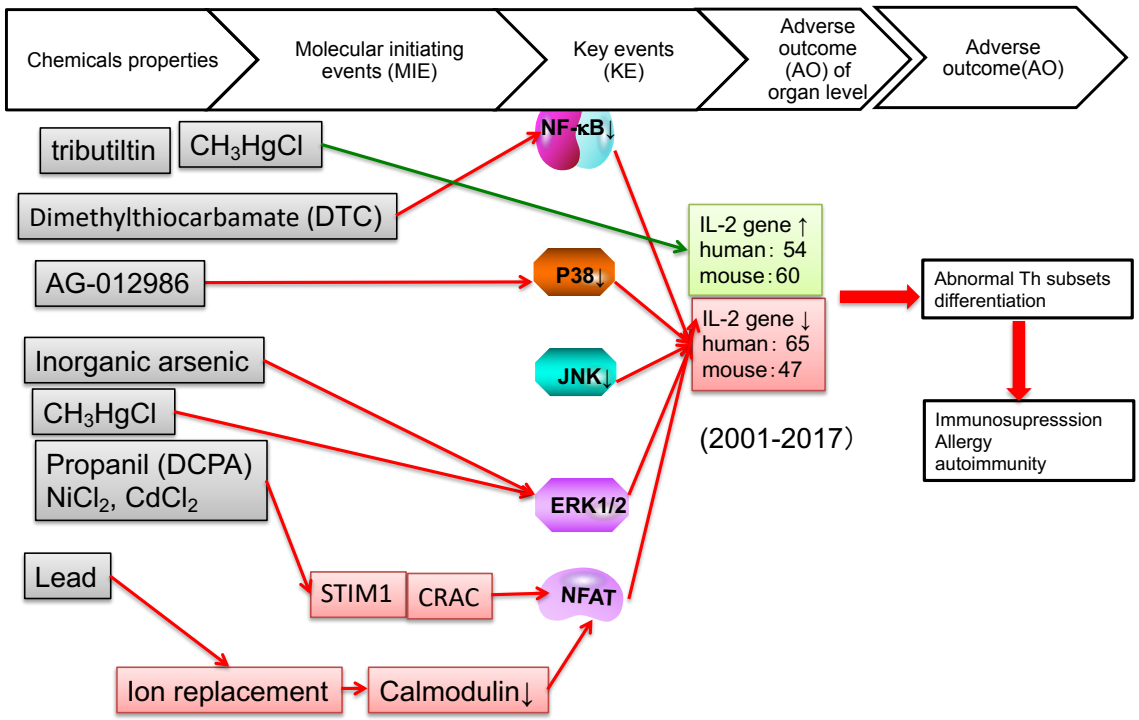

Fig. 7. The proposed AOP for dysregulation of Th subset differentiation triggered by disrupted IL-2 transcription.

4. Test method and modification

4-1. IL-2 reporter cell, 2H4

The Jurkat human acute T lymphoblastic leukemia cell line kindly provided by Professor Kazuo Sugamura, Department of Microbiology, Tohoku University School of Medicine, was cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO) containing Antibiotic-Antimycotic (Invitrogen) and 10% Hyclone^{TM} fetal calf serum (Thermo Fisher Scientific, Waltham, MA) (Jurkat growth medium) at 37°C with 5% CO_2. The luciferase reporter assay system was constructed using 3 luciferases that emit green light (Stable luciferase green; SLG), orange light (Stable luciferase orange; SLO), and red light (Stable luciferase red; SLR) using a single substrate. Namely, we constructed three luciferase vectors, pSLG-test/Hyg^r, pSLO-test/Neo^r, and pSLR-test/Pur^r, by ligating the *Bam*HI/*Sac*I site of resistant gene vectors containing one of three resistant genes, hygromycin (SLG), neomycin (SLO) or puromycin (SLR), SV40 promoter, and HSVtk polyA into luciferase gene vectors, pSLG-test, pSLO-test and pSLR-test (Toyobo, Osaka, Japan), respectively. The activities of the luciferases can be measured simultaneously and quantitatively with optical filters. This system can rapidly and easily monitor the expression of multiple genes (Nakajima et al., 2005; Noguchi et al., 2008).

4-2. Chemical treatment of 2H4 cells and measurement of luciferase activity

Based on previous reports(Saito et al., 2011; Takahashi et al., 2011), 2H4 cells (2×10^5 cells/50 μl/well) in 96-well black plates (Greiner Bio-One GmbH, Frickenhausen, Germany) were pretreated with different concentrations of individual chemicals for 1 h. The 2H4 cells were then stimulated with 25 nM PMA and 1 μM ionomycin (PMA/Io) for 6 h. Three luciferase activities (SLG luciferase activity (SLG-LA), SLO luciferase activity (SLO-LA), and SLR luciferase activity (SLR-LA)) were simultaneously determined using a microplate-type luminometer with a multi-color detection system (Phelios; Atto Co., Tokyo, Japan) and Tripluc luciferase assay reagent (TOYOBO Co., Ltd., Osaka, Japan) according to the manufacturers' instructions. Use of

the 2H4 cell line enabled measurement of SLO-LA driven by the IL-2 promoter (IL2LA), SLG-LA driven by the INF-γ promoter (IFNLA), and SLR-LA driven by GAPDH (GAPLA) in 2H4 cells. In this validation study, however, we just used the IL2LA and GAPLA and ignored IFNLA because there was a significant correlation between LOELs for the effects on the IL2LA and those on the IFNLA (Kimura et al., 2018). We accounted for the variation in cell number and cell viability after chemical treatment by normalizing the data for IL2LA (nIL2LA) by dividing IL2LA with GAPLA in the 2H4 cells. In addition, we calculated % suppression, % augmentation, and Inh-GAPLA as follows:

% suppression = (nIL2LA of 2H4 cells treated with chemicals/nIL2LA of non-treated 2H4 cells) x 100;

% augmentation = (1-(nIL2LA of 2H4 cells treated with chemicals/nIL2LA of non-treated 2H4 cells)) x 100;

Inh-GAPLA = GAPLA of 2H4 cells treated with chemicals/GAPLA of untreated cells.

Definitions of these terms are provided in Table 2.

Table 2. Definition of the parameters in the IL-2 Luc assay.

Abbreviations	Definition
IL-2 Luc assay	IL-2 luciferase assay
GAPLA	SLR luciferase activity reflecting GAPDH promoter activity
IL2LA	SLO luciferase activity reflecting IL-2 promoter activity of 2H4 cells
IFNLA	SLG luciferase activity reflecting IFN-γ promoter activity of 2H4 cells
nIL2LA	IL2LA/GALA of 2H4 cells
nIFNLA	IFNLA/GALA of 2H4 cells
% suppression	(nIL2LA of 2H4 cells treated with chemicals/ nIL2LA of non-treated 2H4 cells) x 100
% augmentation	(1-(nIL2LA of 2H4 cells treated with chemicals/ nIL2LA of non-treated 2H4 cells)) x 100
CV05	The lowest concentration of the chemical at which Inh-GAPLA becomes < 0.05.
Inh-GAPLA	GAPLA of 2H4 cells treated with chemicals /GAPLA of untreated cells.

4-3. Criteria to determine the effects of chemicals on T cells

During the validation study, we modified the criteria to determine the effects of chemicals on T cells to determine the criteria for the MITA.

We used the following Criteria 1 in our first publication describing the MITA. Three independent experiments were conducted for each chemical. For each experiment, a one-way ANOVA test followed by Dunnett's post hoc test was used to evaluate statistical significance. If chemicals showed statistically significant immunosuppression or immunostimulation in 3 experiments, they were judged as immunosuppressive or immunostimulatory drugs, respectively. If chemicals showed statistically significant immunosuppression or immunostimulation in only 2

independent experiments, they were judged as potential immunosuppressive or immunostimulatory drugs, respectively. If not, they were judged as ineffective. Then, for potential immunosuppressive or immunostimulatory drugs, we selected their percent suppression or percent augmentation (negative percent suppression) in 3 experiments that showed the most significant change, calculated their percent suppression or percent augmentation, and statistically compared suppression or augmentation by the chemicals with that of the vehicle control in 3 different experiments by the Student's t-test. Only when chemicals demonstrated statistical significance were they judged as immunosuppressive or immunostimulatory, respectively(Kimura et al., 2014).

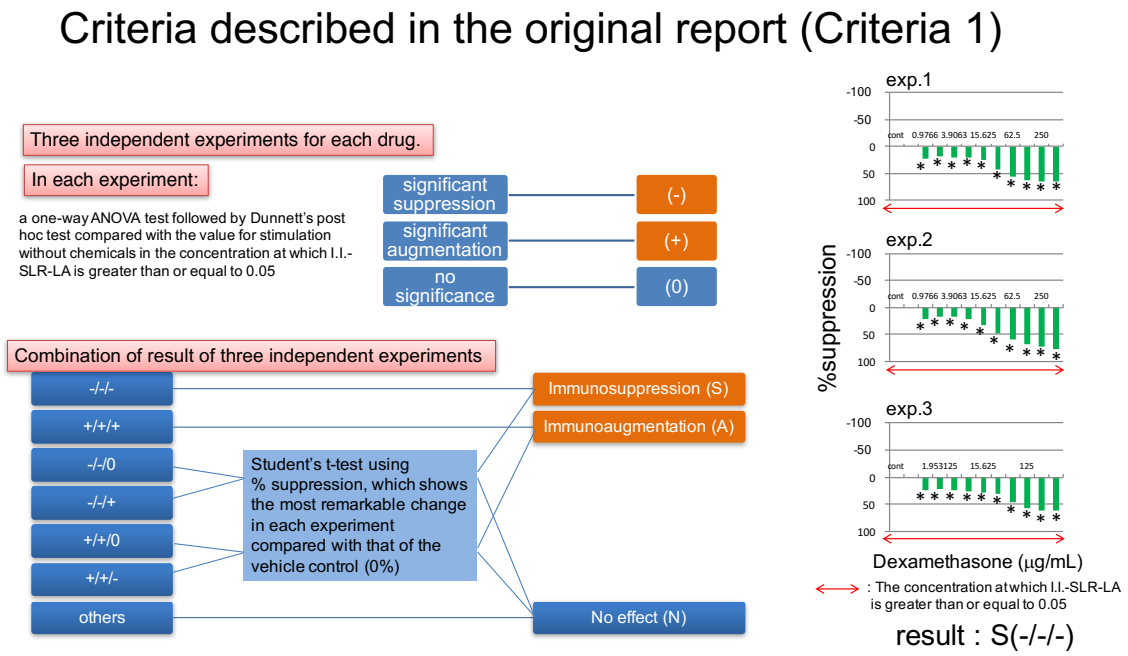

Fig.8 Criteria 1 in the original report

After the pre-validation study, in addition to the original criteria (Criteria 1, Fig.8), two new criteria were proposed by the statistician (Criteria 2, Criteria 3). These 3 criteria were used temporarily and one of these criteria would be adopted after the Phase I validation study.

4-4. Bioluminescence system

In a typical dual-reporter assay, firefly luciferase from *Photinus pyralis* (FLuc) is used as the experimental reporter and Renilla luciferase is used as the internal control reporter. This internal control reporter connects to a constitutively expressed promoter, such as the herpes simplex virus thymidine kinase promoter, cytomegalovirus (CMV) immediate-early promoter, or simian virus 40 (SV40) promoter. This assay system is commercialized as a Dual-Luciferase Reporter Assay System by Promega Corporation. In this system, both luciferase activities are measured sequentially from single extracts on the basis of their bioluminescent substrate specificity. Firefly luciferase activity is measured first by adding firefly D-luciferin, and then Renilla luciferase activity is measured by adding coelenterazine (another name for Renilla luciferin), with concomitant quenching of firefly luciferase luminescence. Finally, firefly luciferase activity is normalized by Renilla luciferase activity as the promoter activity (Michelini et al., 2014; Nakajima and Ohmiya, 2010; Roda et al., 2004).

An alternative chemical test using a cell-based assay requires the analysis of a large number of samples. It is therefore preferable to use an improved assay system whereby gene expression can be monitored simultaneously in a one-step reaction in single extracts. Beetle luciferases emit red luminescence during reaction, compared to the green emitted by firefly D-luciferin. The two colors can be divided using an optical filter. The dual color-reporter assay is based on the color difference between beetle and firefly luciferases and is sold commercially as the Tripluc Reporter Assay System by TOYOBO (Nakajima et al., 2004; Nakajima et al., 2005).

In the IL-2 Luc assay, the multicolor luciferase assay system (Nakajima et al. 2005) consisted of a green-emitting luciferase (SLG; lmax = 550 nm) for the gene expression of the IL-2 promoter, an orange-emitting luciferase (SLO; lmax = 580 nm) for the gene expression of the IFN-γ promoter, and a red-emitting luciferase (SLR; lmax = 630 nm) for the gene expression of the internal control promoter, GAPDH.

The three luciferases emit different colors upon reacting with firefly D-luciferin and

their luminescence is measured simultaneously in a one-step reaction by dividing the emission from the assay mixture using an optical filter (Nakajima et al., 2005). First, the total relative light units (F0) are measured in the absence of the filters. Then, the F1 and F2 values that passed through the R56 filter (>560-nm long-pass filters) or the R60 filter (>600-nm long-pass filters), respectively, is measured. The three luciferase activities are calculated using the simultaneous equation shown below by substituting the F0, F1 and F2 values. In this equation, G, O and R are the activities of the green-, orange- and red-emitting luciferases, respectively, κG_{R56}, κO_{R56} and κR_{R56} are the transmission coefficients of the green-, orange- and red-emitting luciferases of the R56 filter, respectively, κG_{R60}, κO_{R60} and κR_{R60} are the transmission coefficients of the green-, orange- and red-emitting luciferases of the R60 filter, respectively.

$$\begin{pmatrix} F0 \\ F1 \\ F2 \end{pmatrix} = \begin{pmatrix} 1 & 1 & 1 \\ \kappa G_{R56} & \kappa O_{R56} & \kappa R_{R56} \\ \kappa G_{R60} & \kappa O_{R60} & \kappa R_{R60} \end{pmatrix} \begin{pmatrix} G \\ O \\ R \end{pmatrix}$$

Luminescence activity is measured using a filtered 96-well microplate luminometer (for example, Phelios (ATTO, Tokyo, Japan), Tristan 941 (Berthold, Bad Wildbad, Germany), and the ARVO series (PerkinElmer, Waltham, MA). It is necessary to calibrate the luminometer in each experiment to ensure reproducibility (Niwa et al., 2010). Recombinant green-, orange- and red-emitting luciferases are available for this calibration.

5. Validation Management Structure

5-1. Validation Management Team (VMT)

Trial Coordinator:	Hajime Kojima (Japanese Center for the Validation of Alternative Methods (JaCVAM), National Institute of Health Sciences (NIHS), Kawasaki, Japan), VMT trial coordinator, Chemical supplier and Management of quality control
Lead laboratory:	Setsuya Aiba (Tohoku University, Miyagi, Japan), Developer of this assay, Test method, expertise underlying science Yutaka Kimura (Tohoku University, Miyagi, Japan)
International expert members	
EU liaison:	Emanuela Corsini (Milan Univ., Italy), Test system expertise, validation expertise, immunotoxicity expertise Erwin L. Roggen (3Rs Management and Consulting ApS, Denmark), Test system expertise, validation expertise, immunotoxicity expertise
ICCVAM liaison:	Dori Germolec (NTP/NIEHS, USA), Immunotoxicity expertise
JSIT liaison:	Tomoaki Inoue (Chugai Pharmaceutical Co., Ltd.), Immunotoxicity expertise
Data management team:	Takashi Omori (Kobe University, Kobe, Japan), Data analysis, biostatistics dossier
Chemical Selection Committee	Setsuya Aiba (Tohoku University) Yutaka Kimura (Tohoku University Hajime Kojima (JaCVAM) Emanuela Corsini (Milan Univ)

	Erwin L. Roggen (3Rs Management and Consulting ApS) Dori Germolec (NTP/NIEHS) Tomoaki Inoue (Chugai Pharmaceutical Co., Ltd.)
Participating Test Facilities	Test Facility 1: Hatano Res. Inst., FDSC, Study Director (SD): Kohji Yamakage Test Facility 2: AIST, Tsukuba, SD: Rie Yasuno Test Facility 3: AIST, Takamatsu, SD: Yoshihiro Nakajima

5-2. Management office

Hajime Kojima (JaCVAM)
3-25-26 Yodomimati Kawasaki, Kawasaki, 210-9501
TEL: +81-44-270-6600
h-kojima@nihs.go.jp

5-3. Meetings

27-28/1/2016 (Mitoya, Sendai, Japan)
1st International VMT Meeting
Subjects: Kick-off meeting for the MITA assay
VMT members: Corsini, E., Roggen, E., Germolec, D.(telephone), Inoue, T., Kageyama, S., Aiba, S., Kimura, Y., Yamakage, K., Watanabe, M., Kobayashi, M., Yasuno, R., Ohmiya, Y., Omori, T., Kojima, H., Tanabe, S., Venti, S.
Participating laboratories: AIST(Tsukuba), HRI

13/9/2016 (Skype-meeting)
Meeting by Skype
Subjects: Result of the phase 0 study and proposal of the revised protocol

VMT members: Corsini, E., Roggen, E., Germolec, D., Inoue, T.,
Aiba, S., Kimura, Y., Omori, T., Kojima, H.

4-5/2/2017 (Nayamachi community hall, Kyoto, Japan)
2nd International VMT Meeting
Subjects: Validation results, discussion and suggestion
VMT members: Corsini, E., Roggen, E., Germolec, D., Inoue, T.,
Aiba, S., Kimura, Y., Yamakage, K., Watanabe, M., Kobayashi, M.,
Yasuno, R., Nakajima, Y., Omori, T., Mori, A., Kobayashi, M.,
Kojima, H., Venti, S.
Participating laboratories: AIST(Tsukuba), HRI, AIST(Takamatsu)

18-19/11/2017 (Umeda Center Building, Osaka, Japan)
3rd International VMT Meeting
Subjects: Validation results, discussion and suggestion
VMT members: Corsini, E., Roggen, E., Germolec, D., Inoue, T.,
Aiba, S., Kimura, Y., Yamakage, K., Watanabe, M., Kobayashi, M.,
Yasuno, R., Nakajima, Y., Omori, T., Mori, A., Kobayashi, M.,
Kojima, H., Venti, S.
Participating laboratories: AIST(Tsukuba), HRI, AIST(Takamatsu)

29/3/2018 (Skype-meeting)
Meeting by Skype
Subjects: Proposal of the revised protocol
VMT members: Corsini, E., Roggen, E., Germolec, D., Inoue, T.,
Aiba, S., Kimura, Y., Omori, T., Kojima, H.

10/4/2018 (telephone-meeting)

Meeting by telephone

Subjects: Understanding the unexpected results in the IL-2 Luc assay

VMT members: Corsini, E., Roggen, E., Germolec, D., Inoue, T., Aiba, S., Kimura, Y., Omori, T., Kojima, H.

4-6/10/2018 (Kobe Univ., Kobe, Japan)

4th meeting for the MITA Validation study

Subjects: Validation report for the IL-2 assay

VMT members: Corsini, E., Roggen, E., Germolec, D., Inoue, T. Aiba, S., Kimura, Y., Yamakage, K., Watanabe, M., Yasuno, R., Nakajima, Y., Omori, T., Takagi, Y., Mashimo, N., Kado, Y., Kojima, H., Venti, S.

Participating laboratories: AIST(Tsukuba), HRI, AIST(Takamatsu)

6. Study Design (Appendix 12)

The aim of this phase is to (pre)validate the IL-2 Luc assay method to assess transferability and inter-laboratory variability so that this test can be used to screen for immunotoxic chemicals.

The validation study (Phase I and Phase II trials) was conducted by 3 laboratories, based on the study design and schedule shown in Tables 3 and 4 and using the test chemicals shown in Tables 5 and 6. The methods were described above in section 4: ’Test Method 4.1 IL-2 Luc assay’, and the precise protocol is described below in section 8: ‘Protocol 8.2 Protocol for the IL-2 Luc assay’ in Tables 7-9.

Table 3. The number of chemicals analyzed in the validation study

Studies	Within-Laboratory	Between-laboratories	Predictivity
I	5	5	5
II		20	20
Total	5	25	25

7. Test Chemicals

The selection process for the test chemicals for the IL-2 Luc assay validation study is described below.

In addition, the chemical categories or physical state and chemical properties (e.g., solid, liquid, etc.) are included in the tables of these test chemicals in order to investigate the applicable domain.

Table 4. Breakdown of the IL-2 Luc assay validation study

Phase	The number of the test substances	The number of the repetitions	Examination	Date of experiment start
Pre	5	1	Between- laboratory transferability (Non-coded)	July, 2016
I	5	3	Within- and between- laboratory reproducibility (Coded)	September, 2016
II	20	1	Between- laboratory reproducibility and predictivity (Coded)	May, 2017

7-1. Basic rule for chemical selection

The selection of test chemicals by the Chemical Selection Committee (CSC) in the VMT was based on published papers on *in vivo* immunotoxicity tests and validation studies for *in vitro* alternative assays on immunotoxicity test methods.

7-1-1. The applied selection criteria

- information on mode/site of action
- coverage of a range of relevant chemical classes and product classes
- quality and quantity of reference data (*in vivo* and *in vitro*)
- high-quality data derived from animal and (if available) human studies
- information on interspecies variations (for example: variability with regard to the uptake of chemicals, metabolism, etc.)
- coverage of a range of toxic effects/potencies
- chemicals that do not require metabolic activation
- appropriate negative and positive controls

- physical and chemical properties (feasibility of use in the experimental set-up as implicated by the CAS No.)
- single chemical entities or formulations of known high purity
- availability
- cost

In the first phase of the selection procedure, the CSC identified and collected several existing lists of potential chemical immunotoxicants, such as NTP IMMUNOTOX, EPA candidate list. An extensive literature search was performed by the CSC in order to ensure that all the pre-selected chemicals fulfilled the selection criteria described above. In addition, it was decided that at least 20% of the total chemicals to be tested should provide negative results (i.e., not immunotoxic) in order to increase the statistical power of the data analysis.

7-1-2. Chemical Acquisition, Coding and Distribution

Laboratory transferability, and within- and between-laboratory reproducibility and predictivity, in all test facilities were assessed using coded chemicals. Coding was supervised by JaCVAM, in collaboration with CSC. CSC was responsible for coding and distributing the test chemicals, references, and controls for the validation study.

7-1-3. Handling

The chemical master at each test facility received complete information considered essential regarding the test chemicals (physical state, weight or volume of sample, specific density for liquid test chemicals, and storage instructions) by JaCVAM. Moreover, the test facility chemical master stored each chemical at conditions in accordance with the storage instructions and received sealed safety information such as the Material Safety Data Sheet (MSDS) describing hazards identification and exposure controls/personal protection for each chemical. The test chemicals were delivered directly to the study director and the study director was not shown the MSDSs. The study director was to refer to the MSDSs only in the event of an accident. If the study director referred to the MSDS,

he/she was not to reveal the content of the MSDS to the test facility technicians.

No accidents occurred during the course of the validation study, and all test facilities returned the MSDSs for the test chemicals to JaCVAM in their sealed envelope upon completion of the validation study. All test chemicals were disposed of in compliance with the rules and regulations of the test facilities upon completion of the validation study.

7-2. Pre-validation study

Transferability of this assay was checked using five non-coded chemicals (2-aminoanthracene, citral, chloroquine diphosphate salt, dexamethasone and methylmercury(II) chloride) (Appendix 1) in 4 test facilities, including the lead laboratory. These chemicals were selected by the CSC.

7-3. Validation study -Phase I trial

Within- and between-laboratory reproducibility of this assay was checked using 5 coded chemicals in 3 test facilities. These chemicals were selected by CSC based on the in-house data set of the lead laboratory. The chemicals were coded by JaCVAM as shown in Table 5 (Appendix 2) and distributed to the test facilities.

Table 5. Chemical code list on the phase I validation trial for IL-2 Luc assay

No.	Chemical	CASRN	MW	Supplier	Catalog No.	Content	Physical characteristics	Lot	Storage	Purity	LabA	LabB	LabC	LabD
											TOHOKU univ.	AIST-TSUKUBA	FDSC	AIST-SHIKOKU
1	Dibutyl phthalate	84-74-2	278.34	Wako	021-06936	500mL	Liquid	TLN0112	RT	98.0+% (Capillary GC)	MIA003A	MIB014A	MIC027A	MID036A
											MIA004B	MIB017B	MIC026B	MID033B
											MIA007C	MIB016C	MIC023C	MID034C
2	Hydrocortisone (for Cell Culture)	50-23-7	362.46	Wako	080-10194	50g	Solid	SAH3714	RT	97% (HPLC)	MIA005A	MIB017A	MIC029A	MID038A
											MIA007B	MIB019B	MIC028B	MID035B
											MIA009C	MIB018C	MIC025C	MID037C
3	Lead(II) acetate trihydrate (Deleterious substances)	6080-56-4	379.33	Sigma-Aldrich	316512-100G	100g	Solid	09901TS	RT	99.999% trace metals basis	MIA007A	MIB018A	MIC021A	MID310A
											MIA008B	MIB011B	MIC210B	MID037B
											MIA001C	MIB110C	MIC027C	MID038C
4	Zinc dimethyldithiocarbamate (DMDTC)	137-30-4	305.82	Kanto Chemical	48028-31	25g x2	Solid	403N2204	RT	>95.0% (T)	MIA009A	MIB110A	MIC023A	MID037A
											MIA010B	MIB013B	MIC027B	MID039B
											MIA003C	MIB017C	MIC029C	MID310C
5	Nickel (II) sulfate hexahydrate	10101-97-0	262.85	Wako	146-01171	100g	Solid	LKQ2263	RT	99.0-102.0% (as NiSO4・6H2O) (Titration)	MIA001A	MIB012A	MIC025A	MID034A
											MIA002B	MIB015B	MIC024B	MID031B
											MIA005C	MIB014C	MIC021C	MID032C

7-4. Validation study -Phase II trial

Between-laboratory reproducibility of this assay was checked using 20 coded chemicals in 3 test facilities. The chemicals were coded by JaCVAM as shown in Table 6 (Appendix 3) and distributed to the test facilities.

Table 6. Chemical code list on the phase II validation trial for IL-2 Luc assay

	Chemical	Cas.no.	LabA	LabB	LabC	LabD	Note	State	Storage	Supplier	Lot
			TOHOKU univ.	AIST-TSUKUBA	FDSC	AIST-SHIKOKU					
1	2,4-Diaminotoluene	95-80-7	MIA401	MIB515	MIC618	MID702	Deleterious	S	RT	Wako	CDF0347
2	Benzo(a)pyrene	50-32-8	MIA413	MIB516	MIC601	MID703		S	RT	TCI	M8DFD
3	Cadmium chloride	10108-64-2	MIA403	MIB502	MIC602	MID714	Deleterious	S	RT	Wako	DEE3332
4	Dibromoacetic acid	631-64-1	MIA406	MIB518	MIC610	MID720		S	RT	ALDRICH	BCBR5175V
5	Diethylstilbestol	56-53-1	MIA420	MIB509	MIC611	MID711		S	RT	SIGMA	BCBR9766V
6	Diphenylhydantoin	630-93-3	MIA412	MIB510	MIC615	MID704		S	RT	SIGMA	SLBB3874
7	Ethylene dibromide	106-93-4	MIA407	MIB507	MIC605	MID705	Deleterious	L	RT	Wako	KWG5479
8	Glycidol	556-52-5	MIA408	MIB505	MIC607	MID712		L	2-8℃	ALDRICH	MKBX5752V
9	Indomethacin	53-86-1	MIA409	MIB508	MIC609	MID715		S	RT	SIGMA	122K0718
10	Isonicotinic Acid Hydrazide (Isoniazid)	54-85-3	MIA411	MIB517	MIC612	MID707		S	RT	Fluka	SLBF8371V
11	Nitrobenzene	98-95-3	MIA402	MIB519	MIC603	MID701	Deleterious	L	RT	Sigma-Aldrich	SHBG5577V
12	Urethane, Ethyl carbamate	51-79-6	MIA415	MIB520	MIC604	MID719		S	RT	Sigma-Aldrich	WXBC3505V
13	Tributyltin chloride	1461-22-9	MIA404	MIB506	MIC613	MID713	Deleterious	L	RT	TCI	2442A-IQ
14	Perfluorooctanoic acid	335-67-1	MIA414	MIB514	MIC614	MID718		S	RT	TCI	O3U70
15	Dichloracetic acid	79-43-6	MIA416	MIB511	MIC606	MID716	Deleterious	L	RT	Sigma-Aldrich	SHBH3492V
16	Toluene	108-88-3	MIA417	MIB512	MIC616	MID706	Deleterious	L	RT	Sigma-Aldrich	J5136
17	Acetonitril	75-05-8	MIA405	MIB501	MIC617	MID708	Deleterious	L	RT	Wako	KWH4805
18	Mannitol	69-65-8	MIA418	MIB503	MIC619	MID717		S	RT	Wako	LKP4362
19	Vanadium pentoxide	1314-62-1	MIA419	MIB504	MIC608	MID709	Deleterious	S	RT	Wako	SAE6958
20	o-Benzyl-p-chorolophenol	120-32-1	MIA410	MIB513	MIC620	MID710		S	RT	Wako	KPQ0988

7-5. Acceptance criteria

The within-laboratory reproducibility for the all test facilities was done by an independent biostatistical analysis using coded five chemicals, under the VMT. The proportion of concordance should be equal or more than 80% as tentative acceptance criteria for phase I study.

Twenty-five coded test items have been selected to confirm the between-laboratory reproducibility in the phase I and II study. At the end of the testing, the test facilities will submit a QC certified copy of whole study dossier to the trial coordinator (study plan in GLP principle, raw data, records and data analysis, study report in GLP principle). The proportion of concordance between-laboratory reproducibility should be equal or more than 80% as acceptance criteria.

8. Protocols

Overview of the IL-2 Luc assay

An overview of the IL-2 Luc assay is shown in Fig. 9. In addition, the final protocol of the present test (version 011.1E) is provided as attached Appendixes 4 and 5, and the procedures are described in detail below.

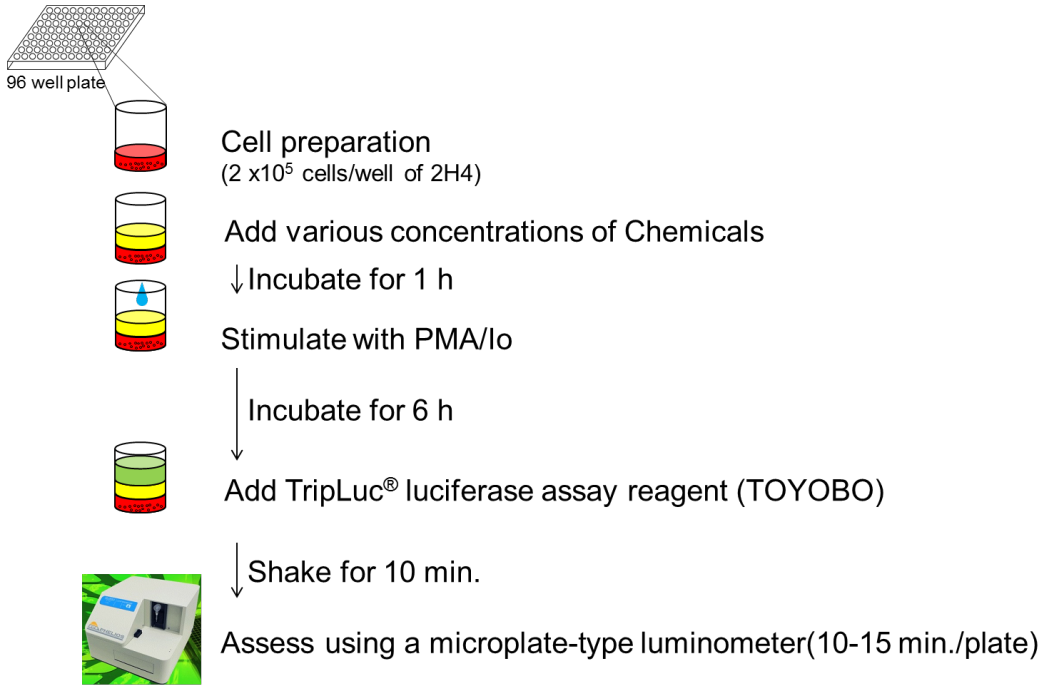

Fig. 9. Overview of the IL-2 Luc assay

8-1 Cells

・/ 2H4 (IL2-SLG、IFNγ-SLO、GAPDH-SLR)

The human acute T lymphoblastic leukemia cell line Jurkat was obtained from the ATCC. A Jurkat-derived IL-2 and IFN-γ reporter cell line, 2H4, that harbors the SLG, SLO and SLR luciferase genes under the control of the IL-2, IFN-γ and GAPDH promoters, respectively, was established by Tsuruga Institute of Biotechnology, TOYOBO Co. Ltd., Fukui, Japan. (Saito et al. 2011)

8-2. Protocol for the IL-2 Luc assay

8-2-1. Reagents and equipment (Appendix 6)

The following reagents and equipment were used.

For maintenance of 2H4 cells

・/ RPMI-1640 (GIBCO Cat#11875-093, 500 mL)

・/ FBS (Biological Industries Cat#04-001-1E Lot: 715004)

・/ Antibiotic-Antimycotic (GIBCO Cat#15240-062)

・/ HygromycinB (CAS:31282-04-9, Invitrogen Cat#10687-010)

・/ G418 (CAS:108321-42-2, Nacalai Tesque Cat#16513-84)

・/ Puromycin (CAS:58-58-2, InvivoGen Cat#ant-pr-1)

For chemical exposure, stimulation and solvents

・/ Ionomycin (CAS:56092-82-1, Sigma Cat#I0634)

・/ Phorbol 12-myristate 13-acetate (PMA) (CAS:16561-29-8, Sigma Cat#P8139)

・/ Ethanol (e.g., Wako Cat#057-00456)

・/ Dimethyl sulfoxide (DMSO) (CAS:67-68-5, Sigma Cat#D5879)

・/ Distilled water (GIBCO Cat#10977-015)

For measurement of luciferase activity

・/ Tripluc® Luciferase assay reagent (TOYOBO Cat#MRA-301)

8-2-2. Culture medium

Various culture media were used depending on the purpose of the cell culture.

Table 7. A medium: for maintenance of 2H4 cells (500 mL, stored at 2-8°C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	440 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	50 mL
Antibiotic-Antimycotic	GIBCO #15240-062	100×	1×	5 mL
Puromycin	InvivoGen #ant-pr-1	10 mg/mL	0.15 μg/mL	7.5 μL
G418	Nacalai Tesque #16513-84	50 mg/mL	300 μg/mL	3 mL
HygromycinB	Invitrogen #10687-010	50 mg/mL	200 μg/mL	2 mL

Table 8. B medium: for luciferase assay (30 mL, stored at 2-8°C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	27 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	3 mL

Table 9. C medium: for thawing 2H4 cells (30 mL, stored at 2-8°C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	26.7 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	3 mL
Antibiotic-Antimycotic	GIBCO #15240-062	100×	1×	0.3 mL

8-2-3. Cell line

The Jurkat human acute T lymphoblastic leukemia cell line (ATCC, Manassas, VA, USA), was cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) with Antibiotic-Antimycotic (Invitrogen, Carlsbad, CA, USA) and 10% Hyclone™ fetal calf serum (Thermo Fisher Scientific, Wilmington, NC, USA) (Jurkat growth medium) at 37 °C with 5% CO_2. The luciferase reporter assay system was constructed using three different luciferases, SLG, SLO and SLR, that emit green, orange, and red light, respectively, with a single substrate. In brief, we constructed three luciferase vectors, pSLG-test/Hygr, pSLO-test/Neor, and pSLR-test/Purr, by ligating the BamHI/SacI site of resistant gene vectors containing one of the three resistant genes, hygromycin (SLG), neomycin (SLO) or puromycin (SLR), the SV40 promoter, and HSVtk polyA into the luciferase gene vectors, pSLG-test, pSLO-test and pSLRtest (Toyobo, Osaka, Japan), respectively. The activities of the luciferases can be measured simultaneously and quantitatively using optical filters. This system can rapidly and easily monitor multiple gene expression (Nakajima et al., 2005; Noguchi et al., 2008). Promoter cloning was carried out as follows. The IL-2 promoter construct containing nt －3006 to +286, the

IFN-γ promoter construct containing nt －4971 to +111, and the GAPDH promoter construct containing nt －1373 to +128 from transcription initiation sites that were identified using DBTSS (http://dbtss.hgc.jp/), were amplified from genomic DNA by PCR using KOD-Plus- ver. 2 (Toyobo) for the IL-2 promoter or KOD-Plus- (Toyobo) for the IFN-γ and GAPDH promoters and specific primers. The IL-2 promoter, IFN-γ promoter, or GAPDH promoter was ligated into pSLG-test/Hygr, pSLOtest/Neor or pSLR-test/Purr vectors that had been digested with MluI and XhoI, MluI and SalI, or MluI and EcoRI, respectively. Before transfection, we confirmed the sequence of the 5' and 3' regions of each promoter using a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). IL-2, IFN-γ and GAPDH reporter plasmids (1 μg) were transfected into Jurkat T cells (5x10^5 cells) using SuperFect (Qiagen, Valencia, CA, USA). After transfection, cells were cultured in Jurkat growth medium containing 200 μg/ml hygromycin (Invitrogen), 300 μg/ml G418 (Nacalai tesque, Kyoto, Japan) and 0.15 μg/ml puromycin (InvivoGen, San Diego, CA, USA) for selection. After repeated limiting dilution, we established a stable cell line (2H4 cells) in Fig.10.

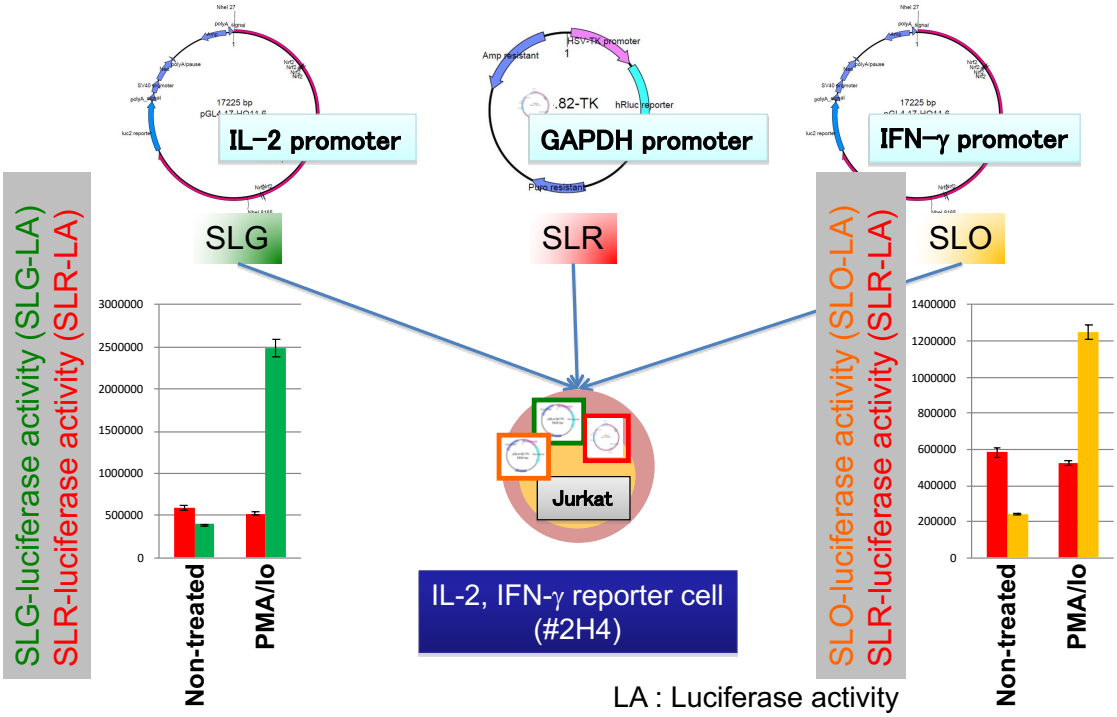

Fig. 10. IL-2 reporter cell, 2H4

8-2-4. Thawing of 2H4 cells

Pre-warm 9 mL of C medium in a 15 mL polypropylene conical tube in a 37°C water bath (for centrifugation) and 15 mL of C medium in a T-75 flask at 37°C in a 5% CO_2 incubator (for culture).

Thaw frozen cells ($2x10^6$ cells/0.5 mL of freezing medium) in a 37°C water bath, then add to a 15 mL polypropylene conical tube containing 9 mL of pre-warmed C medium. Centrifuge the tube at 120-350 x *g* at room temperature for 5 min, discard the supernatant, and resuspend in 15 mL of pre-warmed C medium in a T-75 flask. Cells are incubated at 37°C, 5% CO_2.

8-2-5. Maintenance of 2H4 cells

Pre-warm the A medium in a T-75 Flask at 37°C in a 5% CO_2 incubator. The culture medium should be changed to the pre-warmed A medium 3 or 4 days after thawing. At that time, count the number of cells, centrifuge the tube at 120-350 x *g* at room temperature for 5 min, discard the supernatant, and resuspend in pre-warmed the A medium in a T-75 Flask. Cells are passaged at $3x10^5$/mL and incubated at 37°C, 5% CO_2.

The interval between subcultures should be 3~4 days. Cells can be used between one and six weeks after thawing.

The lead laboratory has examined how long 2H4 cells could be cultured without losing their reactivity to PMA/Io. 2H4 cells maintained their response to PMA/Io up to 16 weeks or 35 passages.

8-2-6. Preparation of cells for assay

A cell passage should be done 2-4 days before the assay.

Use cells between 1 and 6 weeks after thawing.

Pre-warm the B medium in a 37°C water bath. Count the number of cells and collect the number of cells needed (2.0×10^7 cells for two chemicals are required, but to have

some leeway, 3.0 x 10^7 cells for two chemicals should be prepared), centrifuge the tube at 120-350 x *g*, 5 min. Resuspend in pre-warmed the B medium at a cell density of 4×10^6/mL. Transfer the cell suspension to a reservoir (Thermo Scientific), and add 50 µL of cell suspension to each well of a 96 well µclear black plate (flat bottom) using an 8 channel or 12 channel pipetman (Gison, Inc, Middleton, WI, USA). (cf. Figure 11)

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL
B	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL
C	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL
D	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL
E	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL
F	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL
G	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL
H	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL

Fig. 11. Components in each well of 96-well plates after cell preparation.

8-2-7. Preparation of chemicals and cell treatment with chemicals

In Fig. 12, water soluble chemicals were dissolved in distilled water at a concentration of 25 mg/mL. If the chemicals were soluble at 25 mg/mL, then 50 mg/mL solutions were prepared if their solubility was sufficient. If they were not soluble at 50 mg/mL, then 25 mg/mL was judged the highest soluble concentration. If the chemicals were soluble at 50 mg/mL, then 100 mg/mL solutions were prepared if their solubility was sufficient. If they were not soluble at 100 mg/mL, then 50 mg/mL was judged the

highest soluble concentration. If they were soluble at 100 mg/mL, then 100 mg/mL was judged the highest soluble concentration.

Chemicals not soluble in water were dissolved in DMSO at 500 mg/mL. If they were not soluble at 500 mg/mL, the highest soluble concentration was determined by diluting the suspension from 500 mg/mL by a factor of 2 with DMSO. Sonication and vortex mixing were used if needed and the attempt to dissolve the chemical continued for at least 5 minutes. All dissolved chemicals were used within 4 hours of being dissolved in distilled water or DMSO.

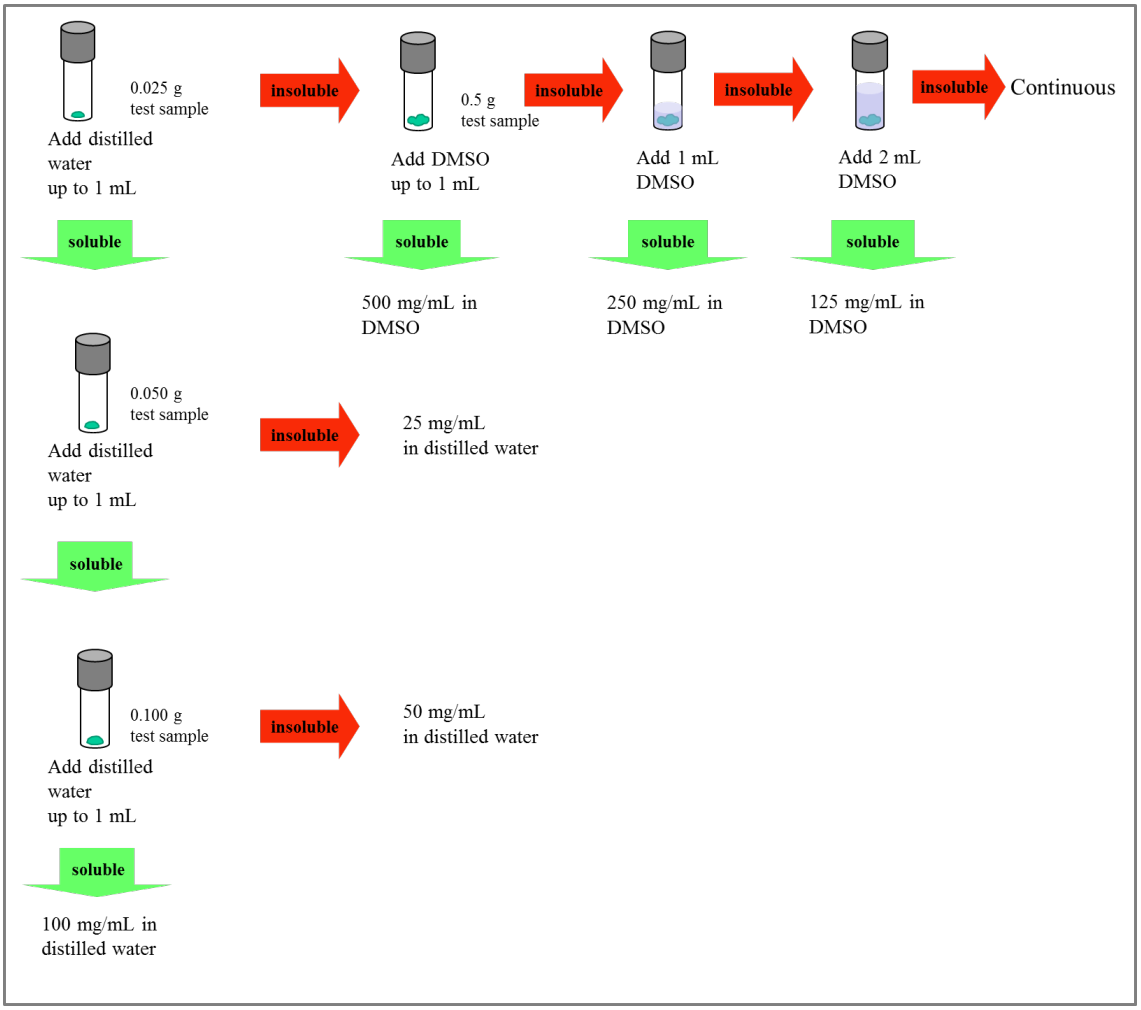

Fig. 12. Dissolution by vehicle

8-2-8. Dilution of chemicals

For water soluble chemicals, 11 serial dilutions were conducted using B medium, diluting by a factor of 2, in the 1st experiment. In the 2nd, 3rd, or 4th experiment, 11 serial dilutions were conducted, diluting by a factor of 1.5. For water insoluble chemicals, 11 serial dilutions were conducted using DMSO as the solvent, diluting by a factor of 2 in the 1st experiment and by a factor of 1.5 in the 2nd, 3rd, and 4th experiments. The diluted chemicals are added to 2H4 cells in a 96 well plate. After one-hour incubation at 37°C in a 5% CO_2 incubator, 2H4 cells are added 10 μL of PMA/Io solution and incubated again at 37°C in a 5% CO_2 incubator for 6 hours.

8-2-9. Measurements

After incubation with the chemical and PMA/Io for 6 h at 37°C in a 5% CO_2 incubator, 100 μL of pre-warmed Tripluc is added to each well in the plate containing reference samples using a pipetman and the plate is shaken for 10 min at room temperature (about 25°C) using a plate shaker. Surface bubbles are removed if present and bioluminescence in each well is measured using a microplate-type luminometer with a multi-color detection system (Phelios; Atto Co., Tokyo, Japan) for 3 sec each in the absence (F0) and presence (F1, F2) of the optical filter. The F0, F1 and F2 data (values are expressed as counts) are processed using an Excel-based data sheet (Appendix 10). SLG-LA, SLO-LA and SLR-LA are calculated for each well based on the algorithm to calculate SLG-LA, SLO-LA and SLR-LA from the raw luminescence data reported previously (Nakajima et al., 2005; Noguchi et al., 2008). In addition to being used to calculate SLG-LA, SLO-LA and SLR-LA, this data sheet can automatically generate final graphs showing the correlation between %suppression and the concentration of chemicals, and between II-SLR-LA and the concentration of the chemical.

8-2-10. Luminometer apparatus

Multi-color detection systems such as microplate-type luminometers are available and include Phelios (ATTO, Tokyo, Japan), Tristan 941 (Berthold, Bad Wildbad, Germany), and the ARVO series (PerkinElmer, Waltham, MA). The luminometer detectors must have high sensitivity and low background noise and are usually equipped with optical filters, such as sharp-cut (long-pass) filters and band-pass filters. The transmission coefficients of these filters for each bio-luminescence signal color must be calibrated prior to all experiments following the manufacturer's recommended protocol because the transmittance of the optical filter or the sensitivity of the detector are dependent on the measurement conditions.

8-2-11. Positive control

In each experimental set, dexamethasone and cyclosporine A are used as positive controls.

8-2-12. Calculation and definition of parameters for the IL-2 Luc assay

In the IL-2 Luc assay, the lead laboratory defined nIL2LA to represent IL-2 promoter activity by the SLG luciferase activity (IL2LA) normalized by SLR luciferase activity (GAPLA). The suppression index of GAPLA (Inh-GAPLA) was obtained by dividing GAPLA of 2H4 treated with chemicals with GAPLA of non-treated 2H4. % suppression reflects the effect of chemicals on IL-2 promoter. (Table 10).

Table 10. Abbreviations used in the 2H4 luciferase assay protocol

Parameter	Definition
IL2LA	Luciferase activity of stable luciferase green (Under the control of IL-2 promoter)
IFNLA	Luciferase activity of stable luciferase orange

	(Under the control of IFN-γ promoter)
GAPLA	Luciferase activity of stable luciferase red (Under the control of GAPDH promoter)
Normalized IL2LA (nIL2LA)	= (IL2LA) / (GAPLA)
Normalized IFNLA (nIFNLA)	= (IFNLA) / (GAPLA)
Inhibition index of GAPLA (Inh-GAPLA)	= (GAPLA of 2H4 treated with chemicals) / (GAPLA of untreated 2H4) (The cytotoxic effect of chemicals)
% suppression	= (1-(nIL2LA of 2H4 treated with chemicals) / (nIL2LA of non-treated 2H4)) x 100 (The effect of chemicals on IL-2 promoter)

8-2-13. Acceptance criteria

The following acceptance criteria should be satisfied when using the IL-2 Luc Assay method.

If Fold induction of nIFNLA of PMA/Io wells without chemicals (=(nIFNLA of 2H4 cells treated with PMA/Ionomycin) / (nIFNLA of non-treated 2H4 cells)) demonstrate less than 3.0, the results obtained from the plate containing the control wells should be rejected.

8-2-14. Prediction model

The experiments are repeated until 2 consistent suppressive (or stimulatory) results or 2 consistent “no effect results” are obtained. When 2 consistent results are obtained, the chemicals are judged as indicated by the obtained consistent results.

An immunotoxicant is identified by the mean of %suppression and its 95%

simultaneous confidence interval.

In each experiment, when the chemicals clearly meet the following 3 criteria, they are judged as suppressive or stimulatory. Otherwise, they are judged as 'no effect' chemicals.

1. The mean of %suppression is ≥35 (suppressive) or ≤-35 (stimulatory) with statistical significance. The statistical significance is judged by its 95% confidence interval.
2. The result shows 2 or more consecutive statistically significant positive (negative) data points or 1 statistically significant positive (negative) data point with a trend in which at least 3 consecutive data points increase (decrease) in a dose-dependent manner. In the latter case, the trend can cross 0, as long as only 1 data point shows the opposite effect without statistical significance.
3. The results are judged using only data obtained at the concentration at which I.I.-SLR-LA is ≥0.05.

8-3. Data collection

8-3-1. Operating procedure

The detail of operating procedure in this assay is describe to the protocol version 011E. The version of protocols was updated during the validation studies, but for the operating procedure, the descriptions of operating procedure described in these protocols are same through the 2 validation studies.

8-3-2. Chemicals

For phase I study, in which the main aim was to evaluate intra- and inter-laboratory reliability, a total of 15 coded chemicals, for 3 rounds of 5 chemicals, were distributed to all the 3 laboratories. Because the different code between rounds was used, the technician in each laboratory did not identify the same chemicals. For the phase II study, in which the main aim of phase I was to evaluate inter-laboratory reliability, 20 coded chemicals were distributed.

In this document the codes for the chemicals were re-coded. To indicate the round, the suffix is used such like P101_R1 for the first chemical of the first round in Phase I study:

P1 means Phase I; 01 means the first chemical; _R1 means first round.

The Table 11 shows the chemical coded through this document.

Table 11. The chemical coded through this document

Phase	Chemical code	Lab A	Lab B	Lab C
I	P101_R1, P101_R2, P101_R3, P102_R1, P102_R2, P102_R3, P103_R1, P103_R2, P103_R3, P104_R1, P104_R2, P104_R3, P105_R1, P105_R2, P105_R3	3 rounds	3 rounds	3 rounds
II	P201, P202, P203, P204, P205, P206, P207, P208, P209, P210, P211, P212, P213, P214, P215, P216, P217, P218, P219, P220	1 round	1 round	1 round

8-3-3. Data handling

The developed Excel data sheet for this study was distributed to the laboratories. We had received data files from the 3 laboratories.

From JaCVAM we received files listed the chemical codes for the distributed 5 chemicals for the phase I study, and 20 chemicals for the phase II study.

For the data analysis, these files were combined and some datasets were constructed for the analysis. The SAS ver. 9.4 and Microsoft Excel was used for the data analysis described in this report.

Since the Excel data sheet is able to display a concentration-response plot for %suppression with its 95% confidence interval, we were able to judge “Suppressive”, “Stimulatory” or “Negative” for each experiment by seeing the plot.

8-3-4. Index from each experiment and decision criteria for judgment

The j-th repetition (j = 1 to 4) of the i-th concentration (j = 0 to 11) is measured for IL2LA and GAPLA respectively. The normalized IL2LA is referred as nIL2LA, and is

defined as

$nIL2LA_{ij} = IL2LA_{ij} / GAPLA_{ij.}$

This is the basic unit of measurement in this assay.

8-3-4-1. %suppression

The %suppression is an index for the averaged nIL2LA for the repetition on the i-th concentration compared with it on the 0 concentration, it is the primary measure of this assay. The %suppression is able to write by the following formula,

$$\%\ \text{suppression}_i = \left\{1 - \frac{\left(\frac{1}{4}\right)\sum_i \text{nIL2LA}_{ij}}{\left(\frac{1}{4}\right)\sum_i \text{nIL2LA}_{0j}}\right\} \times 100 \qquad (1)$$

The lead laboratory has proposed that ± 35 of the value suggests suppressive and stimlatory for a tested chemical. This value is based on the investigation of the historical data of the lead laboratory. Data management team followed to use the value through all the phase of present validation study.

The primary outcome measure, % suppression, is basically the ratio of 2 arithmetic means of nIL2LA as shown in equation (1). The 95% confidence interval (95% CI) of the % suppression for the i-th concentration can be estimated.

The lower limit of the 95% CI above 0 is interpreted as that the nIL2LA with the i-th concentration is statistical-significantly greater than it with the 0-concentration, whereas the upper limit of the 95% CI blow 0 is interpreted as that the nIL2LA with the i-th concentration is statistical-significantly lesser than it with the 0-concentration.

There are several ways to construct the 95% CI. We used the method kwon as the Delta method in this study. This 95% confidence interval theorem is obtained from the following formula.

$$\%\text{suppression} \pm 100 \times \left\{z_{0.975} \times \sqrt{\frac{\text{sd}_i^2}{\text{mean}_0^2} + \frac{\text{mean}_i^2 \times \text{sd}_0^2}{\text{mean}_0^4}}\right\},$$

where mean_i is the mean of nIL2LA at the i-th concentration, mean_0 is the mean of

nIL2LA at 0 concentration, sd_i is the standard deviation of nIL2LA at the i-th concentration and sd_0 is the standard deviation of nIL2LA at 0 concentration. $z_{0.975}$ is 97.5 percentile of the standard normal distribution.

8-3-4-2. Inh-GAPLA

The Inh-GAPLA is a ratio of the averaged GAPLA for the repetition of the i-th concentration compared with it of the 0 concentration, and this is written by

$$\text{Inh} - \text{GAPLA}_i = \{(1/4)\text{x}\sum_j \text{GAP LA}_{ij}\}/\{(1/4)\text{x}\sum_j \text{GAP LA}_{0j}\}$$

Since the GAPLA is the denominator of the nIL2LA, the extremely smaller value of this is considered to cause the large variation of the nIL2LA. Therefore, the i-th %suppression value with extremely smaller value of the Inh-GAPLA might be poor precision.

8-3-4-3. Judgment for "Suppressive", "Stimulatory" or "No effect" in each experiment

In each experiment, when the following 3 criteria are satisfied, they are judged as "suppresive" or "stimulatory". Otherwise, they are judged as no effect chemicals.

1. % suppression is ≥ 35 (suppressive) or ≤ -35 (stimulatory) at any dose and statistically significant.
2. The result shows two or more consecutive statistically significant positive (negative) data or one statistically significant positive (negative) data with a trend in which at least 3 consecutive data increase (decrease) in a dose dependent manner. In the latter case, the trend can cross 0, as long as only one data point shows the opposite effect without statistical significance.
3. The results are judged using only data obtained in the concentration at which Inh-GAPLA is ≥ 0.05

For 1, 2, the statistically significant is judged by the lower limit of 95% confidence interval of %suppression is over 0 or the upper limit of it is under 0.

8-3-4-4. Final judgment for “Suppressive” “Stimulatory” or “No effect” using this assay

In this assay, “Suppressive”, “Stimulatory” or “No effect” is defined as in case that the 2 same judgments were found in a set of experiments.

8-3-5. Reliability

8-3-5-1. Within-laboratory reproducibility for 5 common chemicals

Within-laboratory reproducibility was determined by whether or not tables of 3 sets for the final judgment for each chemical by each laboratory were concordant. The concordance rate was then calculated as a proportion of the concordance of each laboratory.

The concordance rate for within-laboratory reproducibility was based on the results of 3 sets.

To summarize, the concordance rate for within-laboratory reproducibility from the 3 laboratories were used to calculate the averaged concordance rate.

8-3-5-2 Between-laboratory reproducibility

Between-laboratory reproducibility was determined using the results from the final judgment from the 3 laboratories for 25 chemicals, this is, 5 chemicals in Phase I study and 20 chemicals in Phase II study. These judgements were tabulated, then the concordance rate was calculated as a proportion of the concordance in each laboratory.

To summarize, the concordance rate for between-laboratory reproducibility from the 3 laboratories were used to calculate the averaged concordance rate.

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8-3-6. Predictivity

In the evaluation of predictivity, we did not distinguish suppression and stimulation, because both of these indicate modulation of immune function. Then, we dealt as “Positive (P)” in case of “suppression” or “stimulation”, and “No effect (N)” in case of

no significant effects for each chemical judgement.

The concordance, sensitivity and specificity were estimated as the indexes of predictivity. These indexes were estimated using the frequency results obtained from the 2 by 2 contingency table for T cell targeting. The definitions of these indexes are summarized in Table 12 below. This calculation was based on the results decided by a majority for the between-laboratory results for each chemical.

Table 12. Definition of the concordance, sensitivity and specificity

Judgment from the IL-2 Luc assay	Chemical category		Total
	Positive	Negative	
Positive	a	b	a+b
Negative	c	d	c+d
Total	a+c	b+d	N

Sensitivity = 100 × a / (a+c)

Specificity = 100 × d / (b+d)

Accuracy = 100 × (a+d) / N

8-4. Quality assurance

Assays and quality assurance were carried out in the spirit of GLP, although not all the participating laboratories routinely worked under GLP certification. The participating laboratories conducted the experiments in accordance with the protocol provided by the VMT. All raw data and data analysis sheets were pre-checked for quality by each laboratory and then were reviewed by the VMT quality assurance team. The results accurately reflect the raw data.

9. Results

We conducted Phase I and II studies in this validation. The assay procedure and criteria used to judge immunotoxicants in the validation studies are summarized in Fig. 13.

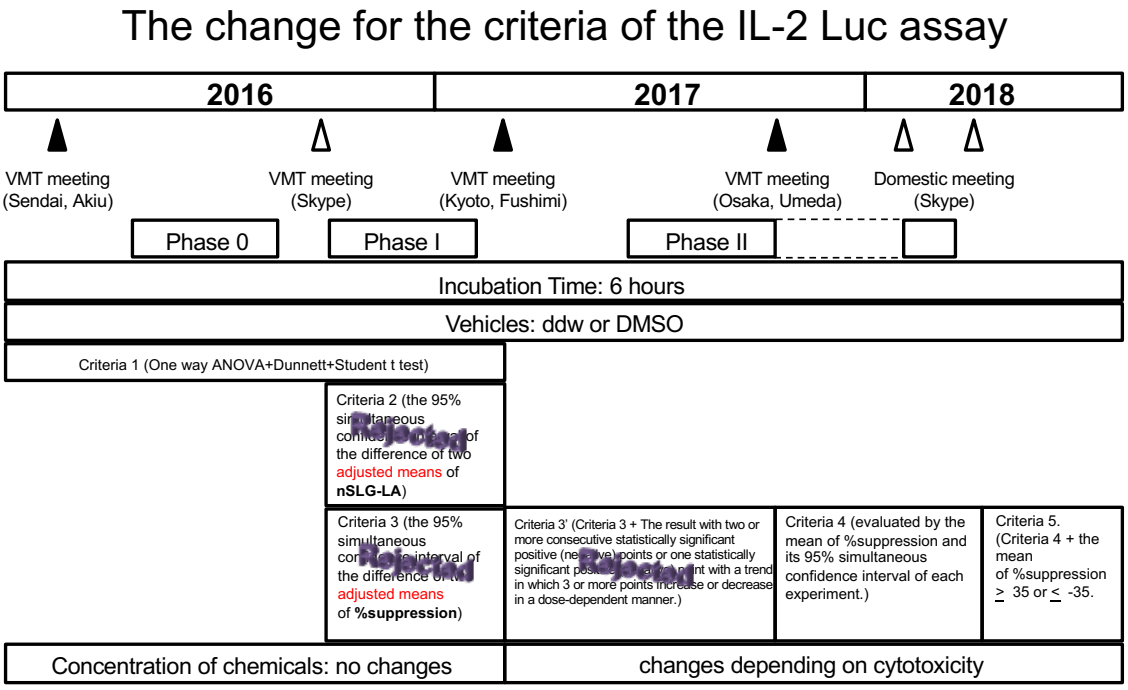


Fig. 13. The modification of the protocols of the IL-2 Luc assay.

9-1. The final criteria

9-1-1. Acceptance criteria

The following acceptance criteria should be satisfied when using the MITA method. In each time of the experiments, a control experiment examining nIL2LA of 2H4 cells treated with PMA/Io and nIL2LA of non-treated 2H4 cells must be conducted. Then, the fold induction of nIL2LA of PMA/Ionomycin wells without chemicals (= (nIL2LA of 2H4 cells treated with PMA/Ionomycin)/(nIL2LA of non-treated 2H4 cells)) is calculated. If the fold induction is less than 3.0, the results obtained from these experiments should be rejected.

9-1-2. Prediction model

The experiments are repeated until 2 consistent suppressive (or stimulatory) results or 2 consistent “no effect results” are obtained. When 2 consistent results are obtained, the chemicals are judged as indicated by the obtained consistent results.

An immunotoxicant is identified by the %suppression and its 95% simultaneous confidence interval.

In each experiment, when the chemicals clearly meet the following 3 criteria, they are judged as suppressive or stimulatory. Otherwise, they are judged as ‘no effect’ chemicals.

1. The mean of % suppression is ≥35 (suppressive) or ≤-35 (stimulatory) with statistical significance. The statistical significance is judged by its 95% confidence interval.
2. The result shows 2 or more consecutive statistically significant positive (negative) data points or 1 statistically significant positive (negative) data point with a trend in which at least 3 consecutive data points increase (decrease) in a dose-dependent manner. In the latter case, the trend can cross 0, as long as only 1 data point shows the opposite effect without statistical significance.
3. The results are judged using only data obtained at the concentration at which Inh-GAPLA is ≥0.05.

9-1-3. Predictivity

To determine the performance of the IL-2 Luc assay, it is crucial to understand the immunotoxicological characteristics of the chemicals used in the study. Since the IL-2 Luc assay focuses on the effects of chemicals on IL-2 transcription by T cells, we attempted to classify the chemicals into two categories: (i) immunotoxic chemicals which target T cells (TTCs), which include chemicals that directly affect T cell viability, T cell proliferation or T cell function and (ii) others (NTTCs), which include chemicals that do not directly affect T cell viability, T cell proliferation or T cell function. In this assay, to define TTCs, we first surveyed the literature and collected the following six findings regarding each of the chemicals proposed for use in the study in Table 13.

Table 13. The immunotoxicological data obtained from the literature.

Endopoints	Information
Endpoint 1	Decreased thymus weight
Endpoint 2	Increased or decreased IL-2, IFN-γ, IL-4 or other T cell-specific cytokine mRNA expression or protein production by T cells in *ex vivo.*
Endpoint 3	Increased or decreased IL-2, IFN-γ, IL-4 or other T cell-specific cytokine mRNA expression or protein production by T cells *in vitro.*
Endpoint 4	Suppressed T cell proliferation
Endpoint 5	Suppressed cytotoxic T cell response
Endpoint 6	The NTP data clearly indicate that one of the immunotoxic mechanism of chemicals are attributed to its effect on T cells.

Then, according to the rationale for classifying immunotoxic chemicals reported by Luster et al (Luster et al., 1992b), we defined TTCs as chemicals that satisfy one of the following criteria and then, made the reference data on immunotoxicity of chemicals in Table 14.

Table 14. The criteria to classify immunotoxic chemicals by affecting T cells.

Criteria	Definition
Criterion 1	Decreased thymus weight with additional one or more findings among endpoints 2 to 5
Criterion 2	Increased or decreased mRNA expression or protein production in one or more cytokines in Endpoints 2 or 3 in multiple reports
Criterion 3	Increased or decreased mRNA expression or protein production in two or more cytokines in Endpoints 2 or 3
Criterion 4	The presence of data suggesting that one of the immunotoxic mechanisms of the chemical was attributed to an effect on T cells in Endpoint 6

Then, by comparing the results of the IL-2 Luc assay (positive or no effect) with the classification of the chemicals (TTC or NTTC), we calculated the accuracy, sensitivity and specificity of the IL-2 Luc assay in the validation study.

To classify 25 chemicals used in the Phase I and II studies, we used the chemical information kindly provided by the National Toxicology Program (NTP). The immunotoxic characteristics of each chemical are shown in Appendix 7. The summarized data of the NTP data and the data collected by the VMT member are shown in Appendix 19. The list of references is in Appendix 8. As already described, IL-2 exerts pleiotropic actions on CD4+ T cell differentiation via its modulation of cytokine receptor expression. Indeed, IL-2 promotes Th1 and Th2 differentiation, while it also drives Treg differentiation. Therefore, it suggests that the augmentation of IL-2

transcription can lead to either immunostimulation or immunosuppression depending on surrounding tissue environment *in vivo.* Therefore, in this assay, if chemicals were judged as either stimulation or suppression, they were both considered as positive (P) and if not, they were judged as negative (N).

9-2. Phase 0 study (for technical transfer)

The preliminary test trial (Phase 0) was performed by the participating laboratories following explicit explanations of the Multi-ImmunoTox Assay protocol Ver. 008.1E by the lead laboratory, Tohoku University. Three laboratories participated in the Phase 0 study of the IL-2 Luc assay using 5 open labeled chemicals, 2-aminoantracene, citral, chloroquine, dexamethasone, methyl mercuric chloride and conducted 1 set (3 experiments) for each chemical. Most response patterns for the 5 chemicals were similar among the 3 laboratories except for 2 early experiments conducted by the naïve laboratory. Based on these results, VMT judged that technical and protocol transfer of the IL-2 Luc assay is acceptable.

After the Phase 0 study, we amended the protocol as follows:

- We changed the speed of centrifugation of the cells, and the preparation method for the selection antibiotics and PMA/Io.
- We set nIFNLA >3 as an acceptance criterion.
- Because nIL2LA is dependent on the properties of the specific luminometer used, we expressed the results of the data by %suppression, which is determined by dividing nIL2LA of the chemically treated cells by nIL2LA of the vehicle-treated cells.
- Volatile chemicals were to be sealed.
- We determined the criteria to judge chemicals from a statistical standpoint (Criteria 2).

9-3. Phase I study (for within and between-laboratory reproducibility)

9-3-1. Test conditions

A total of 5 coded chemicals (4 T cell targeting and 1 non-T cell targeting) were evaluated by 3 experimental sets in the Phase I study based on the Multi-ImmunoTox Assay protocol Ver. 011E.

In each experimental set, 3 or more experiments were conducted for each chemical.

Chemicals that satisfied criteria 5 were judged as positive. Chemicals that provided 2 positive results were judged as immunotoxicants in Tables 15 and 16.

9-3-2. Within-laboratory variation assessments in the Phase I study

Lab A	80.0% (4/5)
Lab B	100% (5/5)
Lab C	80.0% (4/5)
Average	86.7% (13/15)

9-3-3. Between-laboratory variation assessments in the Phase I study

Between-Lab reproducibility (Based on Majority)

80.0% (4/5)

9-3-4. Predictivity in the Phase I study (Based on Majority)

Accuracy of Lab A	80.0% (4/5)
Accuracy of Lab B	100% (5/5)
Accuracy of Lab C	100% (5/5)
Average	93.3% (14/15)

Table 15. Results of the Phase I study

Chemical	CAS	Set	Lab. A	Lab. B	Lab. C	Concordance	T cell targeting	Rationale
Dibutyl phthalate	84-74-2	1st	P	P	P	1	Yes	2, 3
		2nd	P	P	P			
		3rd	P	P	P			
Hydrocortisone	50-23-7	1st	P	P	P	0	Yes	1, 2
		2nd	N	P	P			
		3rd	N	P	N			
Lead(II) acetate	6080-56-4	1st	P	P	P	1	Yes	1, 2, 3
		2nd	P	P	P			
		3rd	P	P	P			
Nickel(II) sulfate	10101-97-0	1st	P	P	P	1	Yes	1, 2, 3
		2nd	P	P	P			
		3rd	P	P	P			
Zinc dimethyldithiocarbamate (DMDTC)	137-30-4	1st	N	N	N	1	No	
		2nd	N	N	N			
		3rd	N	N	N			
Within-laboratory reproducibility (%)			80.0 (4/5)	100 (5/5)	80.0 (4/5)			
			Average 86.7 (13/15)					
Between-laboratory reproducibility (%) (Based on Majority)						80 (4/5)		
Sensitivity (%) (Based on			75.0	100	100			

Majority)	(3/4)	(4/4)	(4/4)
	Average		
	91.7 (11/12)		
Specificity (%) (Based on Majority)	100 (1/1)	100 (1/1)	100 (1/1)
	100 (3/3)		
Accuracy (%) (Based on Majority)	80.0 (4/5)	100 (5/5)	100 (5/5)
	Average		
	93.3 (14/15)		

P: Positive, N : No effect

9-3-5. Contingency tables for the Phase I study

Table 16. Contingency tables for the Phase I study

Lab A		IL-2 Luc assay		Total
		+	-	
T cell targeting	+	10	2	12
	-	0	3	3
Total		10	5	15

Sensitivity : 83.3% (10/12)

Specificity : 100% (3/3)

Accuracy : 86.7% (13/15)

Lab B		IL-2 Luc assay		Total
		+	-	
T cell targeting	+	12	0	12
	-	0	3	3
Total		12	3	15

Sensitivity : 100% (12/12)

Specificity : 100% (3/3)

Accuracy : 100% (15/15)

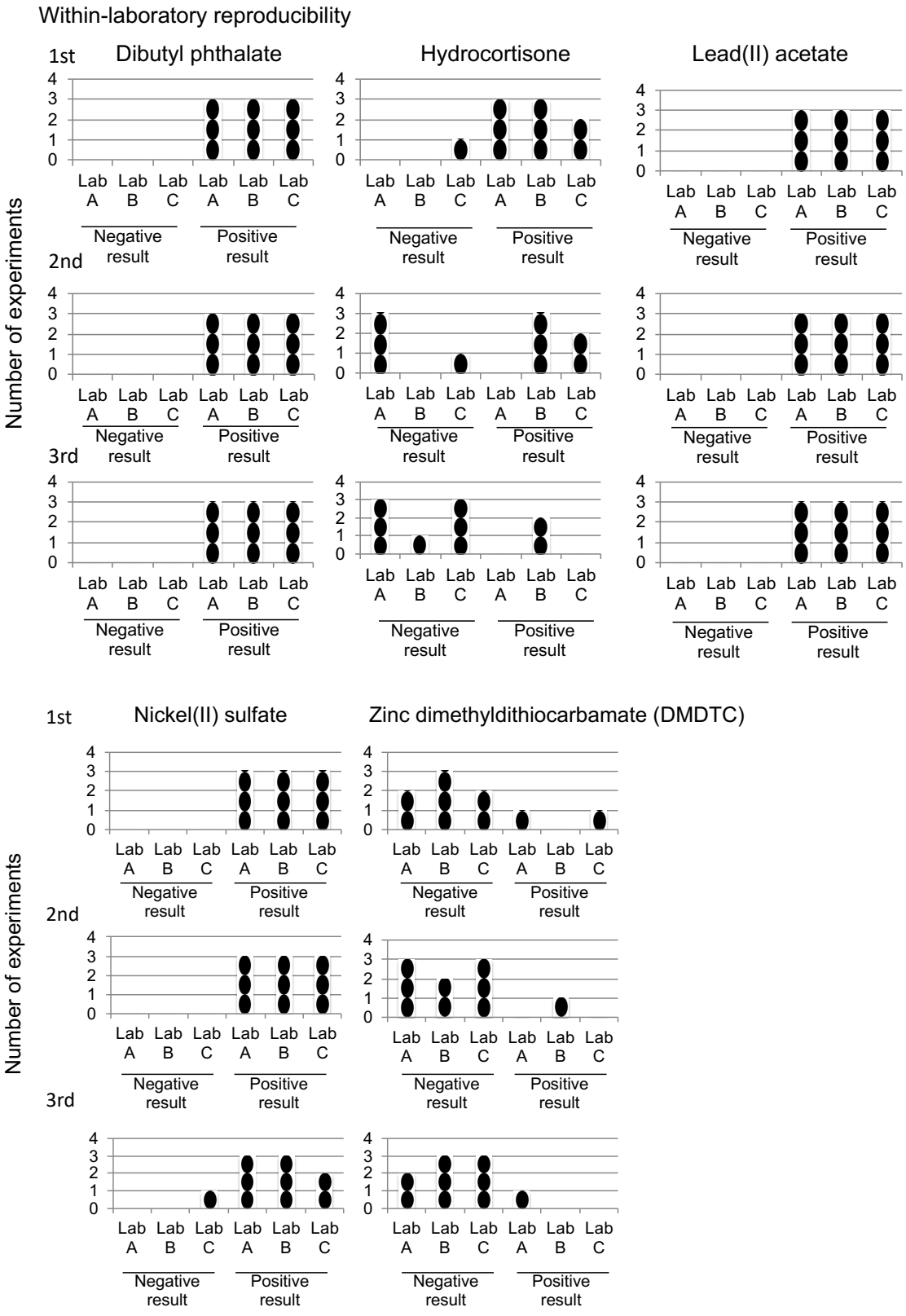
Lab C		IL-2 Luc assay		Total
		+	-	
T cell targeting	+	11	1	12
	-	0	3	3
Total		11	4	15

Sensitivity : 91.7% (11/12)

Specificity : 100% (3/3)

Accuracy : 93.3% (14/15)

A graphical presentation of between- and within-laboratory variation in Phase I study is shown in Fig. 14.


Within-laboratory reproducibility
1st
Dibutyl phthalate
Hydrocortisone
Lead(II) acetate
2nd
3rd
Nickel(II) sulfate
Zinc dimethyldithiocarbamate (DMDTC)
Number of experiments
0
1
2
3
4
Lab A
Lab B
Lab C
Negative result
Positive result

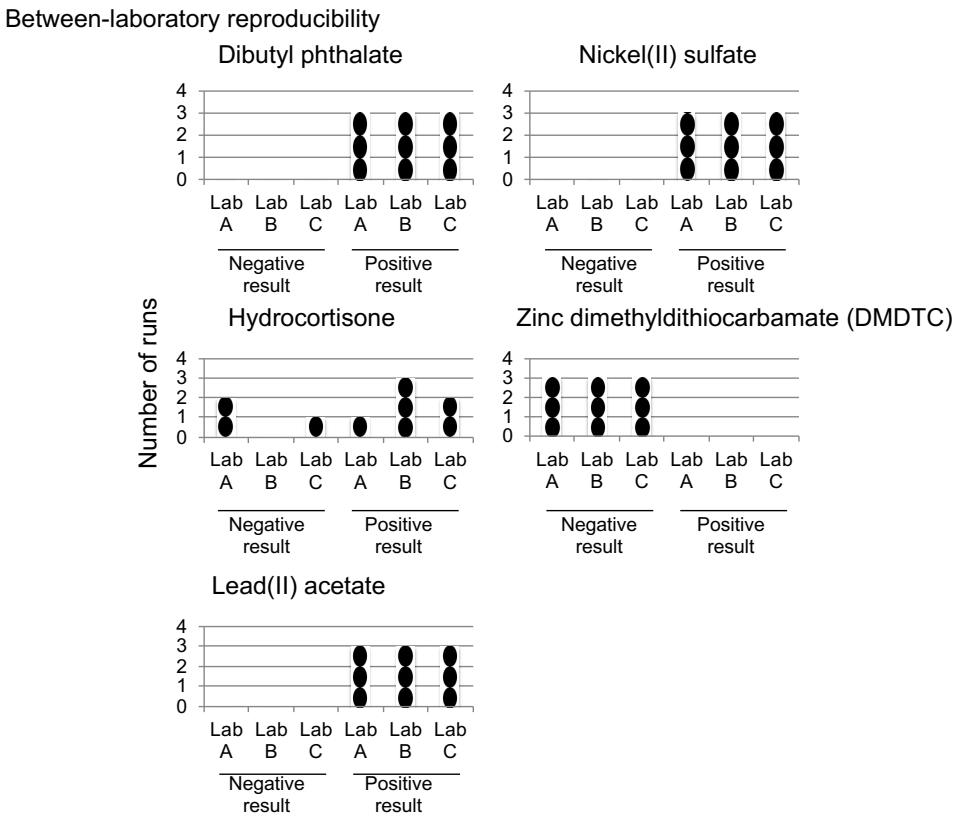


Fig. 14. Between- and within- laboratory variation assessments in the Phase I study

The Phase I study examined within-and between-laboratory reproducibilities using a total of 5 coded chemicals (4 T cell targeting and 1 non-T cell targeting) evaluated by 3 experimental sets based on the MITA protocol Ver. 008.5E. Closed circles represent the judgments for individual experiments for within-laboratory reproducibility or the judgments in individual experimental sets for between-laboratory reproducibility.

9-4. Phase II study (for between-laboratory reproducibility and predictivity)

9-4-1. Test conditions

The Phase II study for between-laboratory reproducibility and predictivity was conducted using a total of 20 coded chemicals (12 T cell targeting, 7 non-T cell targeting and 1 undetermined) and evaluated by 1 experiment set based on the Multi-ImmunoTox Assay protocol Ver. 011E in Tables 17 to 19.

9-4-2. Between-laboratory variation assessments in the Phase II study

Between-Lab reproducibility 80% (16/20)

9-4-3. Predictivity in the Phase II study

Accuracy of Lab A	73.7 (14/19)
Accuracy of Lab B	68.4% (13/19)
Accuracy of Lab C	68.4% (13/19)
Average	70.2% (40/57)

Table 17. Results of the Phase II study

Chemical	CAS	Lab.A	Lab.B	Lab.C	T cell targeting	Rationale
2,4-Diaminotoluene	95-80-7	N	N	N	No	
Benzo(a)pyrene	50-32-8	P	P	P	Yes	2), 3)
Cadmium chloride	10108-64-2	N	N	N	Yes	2), 3)
Dibromoacetic acid	631-64-1	P	P	N	Yes	1), 4)
Diethylstilbestol	56-53-1	P	P	P	Yes	1), 2), 4)
Diphenylhydantoin	630-93-3	N	N	N	Yes	2), 3), 4)
Ethylene dibromide	106-93-4	N	N	N	Yes	1)
Glycidol	556-52-5	P	P	P	No	
Indomethacin	53-86-1	P	P	P	Yes	3), 4)
Isonicotinic Acid Hydrazide	54-85-3	P	N	P	Yes	2)
Nitrobenzene	98-95-3	N	P	N	Undetermined	
Urethane, Ethyl carbamate	51-79-6	P	P	P	Yes	1)
Tributyltin chloride	1461-22-9	P	P	P	Yes	1)
Perfluorooctanoic acid	335-67-1	P	P	P	Yes	1)
Dichloracetic acid	79-43-6	P	P	P	Yes	2), 3)
Toluene	108-88-3	N	N	N	No	
Acetonitril	75-05-8	N	N	N	No	
Mannitol	69-65-8	N	N	N	No	
Vanadium pentoxide	1314-62-1	N	N	N	No	
o-Benzyl-p-chorolophenol	120-32-1	P	P	P	No	

Table 18. Reproducibility of the Phase II study

Between-laboratory reproducibility(%) 80 (16/20)			
Sensitivity (%)	75.0 (9/12)	66.7 (8/12)	66.7 (8/12)
Specificity (%)	71.4 (5/7)	71.4 (5/7)	71.4 (5/7)
Accuracy (%)	73.7 (14/19)	68.4 (13/19)	68.4 (13/19)

P: Positive, N : No effect

9-4-4. Contingency tables for the Phase II study

Table 19. Contingency tables for the Phase II study

Lab A		IL-2 Luc assay		Total
		+	-	
T cell targeting	+	9	3	12
	-	2	5	7
Total		11	8	19

Sensitivity	75.0 (9/12)
Specificity	71.4 (5/7)
Accuracy	73.7 (14/19)

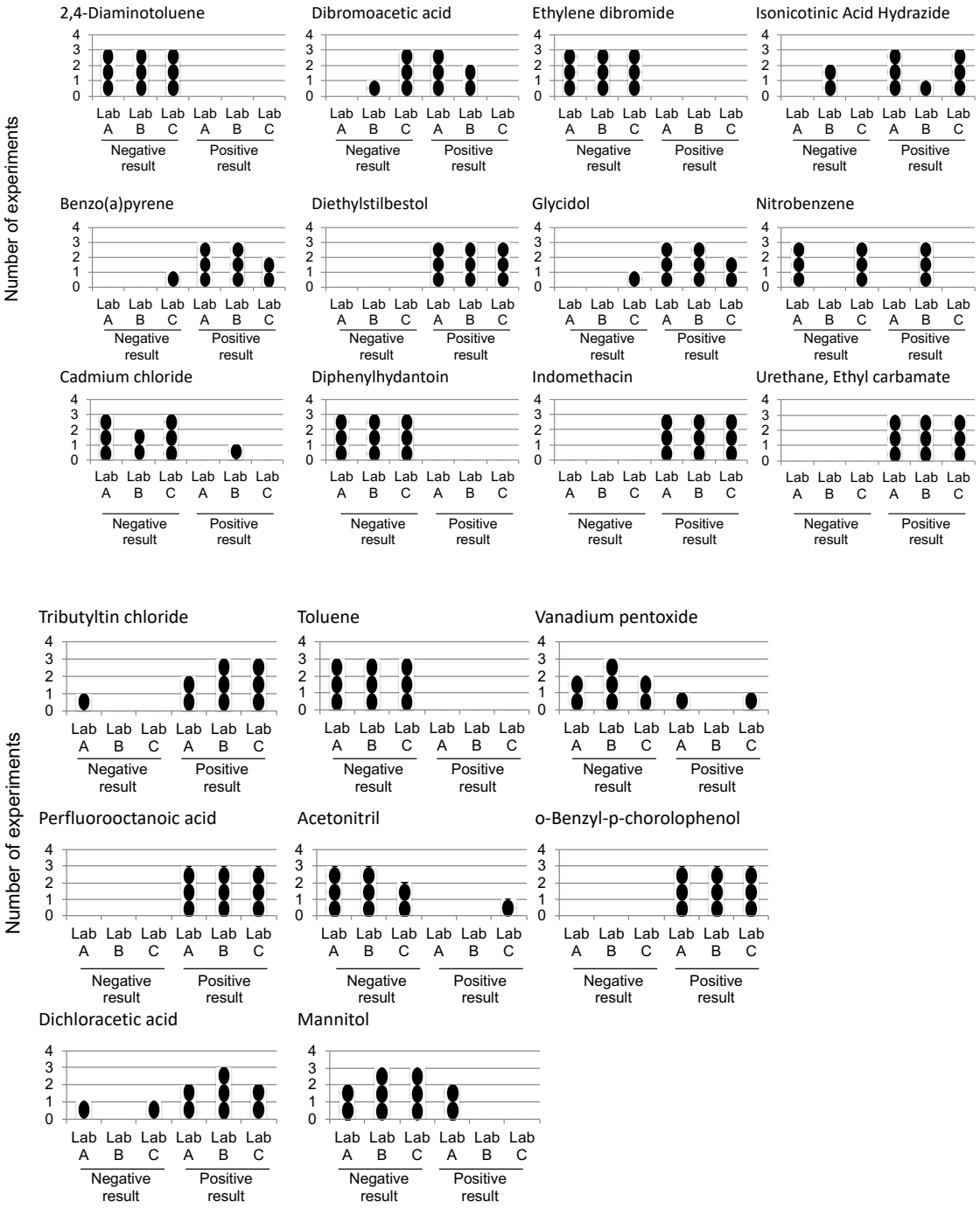
Lab B		IL-2 Luc assay		Total
		+	-	
T cell targeting	+	8	4	12
	-	2	5	7
Total		10	9	19

Sensitivity	66.7 (8/12)
Specificity	71.4 (5/7)
Accuracy	68.4 (13/19)

Lab C		IL-2 Luc assay		Total
		+	-	
T cell targeting	+	8	4	12
	-	2	5	7
Total		10	9	19

Sensitivity	66.7 (8/12)
Specificity	71.4 (5/7)
Accuracy	68.4 (13/19)

The graphical presentation of between- and within-laboratory variation in Phase II study is Fig 15.


2,4-Diaminotoluene
Dibromoacetic acid
Ethylene dibromide
Isonicotinic Acid Hydrazide
Benzo(a)pyrene
Diethylstilbestol
Glycidol
Nitrobenzene
Cadmium chloride
Diphenylhydantoin
Indomethacin
Urethane, Ethyl carbamate
Tributyltin chloride
Toluene
Vanadium pentoxide
Perfluorooctanoic acid
Acetonitril
o-Benzyl-p-chorolophenol
Dichloracetic acid
Mannitol
Number of experiments
0
1
2
3
4
Lab A
Lab B
Lab C
Negative result
Positive result

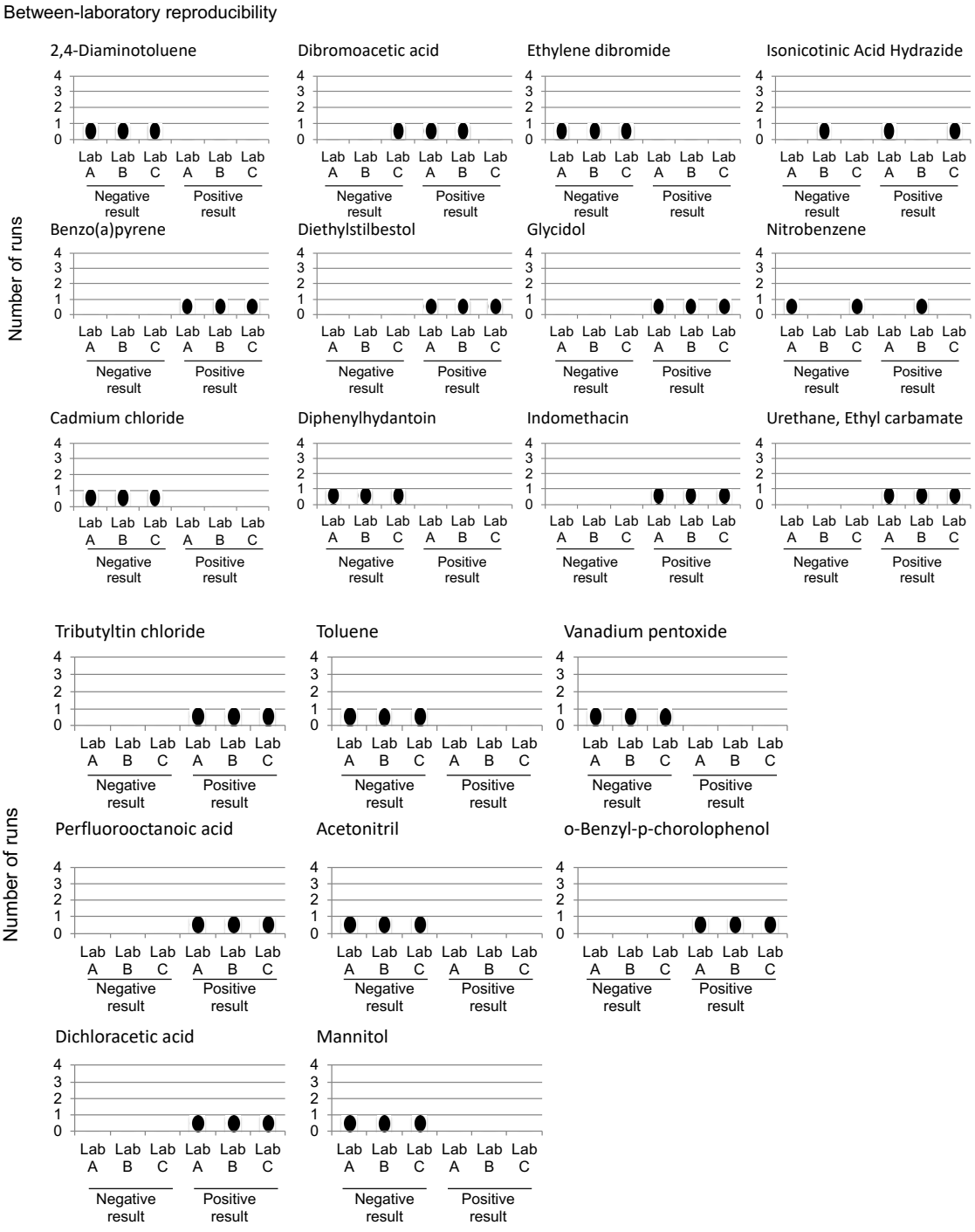


Fig. 15. Between variation assessments in the Phase II study

The Phase II study examined between-laboratory reproducibility using a total of 20 coded chemicals (13 T cell targeting, 6 non-T cell targeting and 1 undetermined) evaluated by 1 experiment sets based on Multi-ImmunoTox Assay protocol Ver. 011E. Closed circles represent the judgments for individual experiments in within-laboratory reproducibility or represent the judgments in individual experimental sets for between-laboratory reproducibility.

9-5. Quality assurance

9-5-1. Chemical Acquisition, Coding and Distribution

The assessment of laboratory transferability, and within- and between-laboratory reproducibility and predictivity, in all test facilities were performed with the coded chemicals. The coding was supervised by JaCVAM (Appendix 14). JaCVAM was responsible for coding and distributing the test chemicals for the validation study.

9-5-2. Handling

The chemical master at each test facility received complete information considered essential regarding the test chemicals (physical state, weight or volume of sample, specific density for liquid test chemicals, and storage instructions) by JaCVAM. Moreover, the test facility chemical master stored each chemical at conditions in accordance with the storage instructions and received sealed safety information such as the Material Safety Data Sheet (MSDS) describing the hazards identification and exposure controls/personal protection for each chemical. The test chemicals were delivered directly to the study director and the study director was not shown the MSDSs. The study director was to refer to the MSDSs only in the event of an accident. If the study director referred to the MSDS, he/she was not to reveal the content of the MSDS to the test facility technicians.

No accidents occurred during the course of the validation study, and all test facilities returned the MSDSs for the test chemicals to JaCVAM in their sealed envelope upon completion of the validation study. All test chemicals were disposed of in compliance with the rules and regulations of the test facilities upon completion of the validation study.

9-5-3. Independent analysis by the biostatistician

All data sheets from the participating laboratories were collected and checked by Dr. Takashi Omori, Kobe univ., the independent biostatistician and JaCVAM. Dr. Omori and his colleagues summarized the data (Appendix 11) and the concentration-response plot for each experiment in phase I (Appendix 17) and phase II (Appendix 18).

9-5-4. Quality assurance by JaCVM

All the record sheets from the participating laboratories were also checked and JaCVAM (Appendix 13). The record sheets mean “Reagent records, solubility test, Cell culture records, Test records and data sheets”. They are total more than 300 pages and available at JaCVAM website (http:// http://jacvam-jp.check-xserver.jp/validation08-login.html). Testings performed as part of a validation study were carried out in accordance with the principles of GLP (OECD, 1998) and necessarily include, without being limited to, the use of protocol and adequate recording of data as well as suitable reporting of results and archival record keeping.

The culture of the cells, the preparation and application of test chemicals and data sheets were completed and the results accurately reflect the raw data. Unfortunately, the record sheets on the maintenance of measuring instruments had not collected before the validation study. JaCVAM considered these records had concerns on quality of data in the validation study. However, JaCVAM checked carefully all the results and judged all data within acceptable ranges.

At least, the reliability of measuring instruments would be checked by an independent organization before the validation study. JaCVAM recommend the validation management team the formal validation study participated with GLP laboratories will be done.

9-6. Combined results of the Phase I and II studies (for between- and within-laboratory reproducibility and predictive capacity)

9-6-1. Test conditions

The within- and between-laboratory reproducibilities, and the predictivity of the IL-2 Luc assay, were evaluated using all the results from Phases I and II in Tables 20 to 22.

9-6-2. Within- and between-laboratory variation assessments from the Phase I and II studies.

Between-Lab reproducibility 80% (20/25)

Within-Lab reproducibility Lab. A 80.0% (4/5)

Lab. B 100% (5/5)

Lab. C 80.0% (4/5)

Average 86.7% (13/15)

9-6-3. Predictivity in the Phases I and II studies

Accuracy of Lab. A 75.0% (18/24)

Accuracy of Lab. B 75.0% (18/24)

Accuracy of Lab. C 75.0% (18/24)

Average 75.0% (54/72)

Table 20. Combined results of the Phase I and II studies

Chemical	CAS	Lab.A	Lab.B	Lab.C	concordance	T cell targeting
Phase I						
Dibutyl phthalate	84-74-2	PPP	PPP	PPP	1	Yes
Hydrocortisone	50-23-7	PNN	PPP	PPN	0	Yes
Lead(II) acetate	6080-56-4	PPP	PPP	PPP	1	Yes
Nickel(II) sulfate	10101-97-0	PPP	PPP	PPP	1	Yes
Zinc dimethyldithiocarbamate (DMDTC)	137-30-4	NNN	NNN	NNN	1	No
Phase II						
2,4-Diaminotoluene	95-80-7	N	N	N	1	No
Benzo(a)pyrene	50-32-8	P	P	P	1	Yes
Cadmium chloride	10108-64-2	N	N	N	1	Yes
Dibromoacetic acid	631-64-1	P	P	N	0	Yes
Diethylstilbestol	56-53-1	P	P	P	1	Yes
Diphenylhydantoin	630-93-3	N	N	N	1	Yes
Ethylene dibromide	106-93-4	N	N	N	1	Yes
Glycidol	556-52-5	P	P	P	1	No
Indomethacin	53-86-1	P	P	P	1	Yes
Isonicotinic Acid Hydrazide	54-85-3	P	N	P	0	Yes
Nitrobenzene	98-95-3	N	S	N	0	Undetermined
Urethane, Ethyl	51-79-6	P	P	P	1	Yes

carbamate						
Tributyltin chloride	1461-22-9	P	P	P	1	Yes
Perfluorooctanoic acid	335-67-1	P	P	P	1	Yes
Dichloracetic acid	79-43-6	P	P	P	1	Yes
Toluene	108-88-3	N	N	N	1	No
Acetonitril	75-05-8	N	N	N	1	No
Mannitol	69-65-8	N	N	N	1	No
Vanadium pentoxide	1314-62-1	N	N	N	1	No
o-Benzyl-p-chorolophenol	120-32-1	P	P	P	1	No

Table 21 Reproducibility of the Phase I and II studies

Within-laboratory reproducibility (%)	80 (4/5)	100 (5/5)	80 (4/5)
	Average 86.7 (13/15)		
Between-laboratory reproducibility (%) (Based on majority for Phase I) 80 (20/25)			
Sensitivity (%)	75.0 (12/16)	75.0 (12/16)	75.0 (12/16)
	Average 75.0 (36/48)		
Specificity (%)	75.0 (6/8)	75.0 (6/8)	75.0 (6/8)
	Average 75.0 (18/24)		
Accuracy (%)	75.0 (18/24)	75.0 (18/24)	75.0 (18/24)
	Average 75.0 (54/72)		

P: Positive, N : No effect

9-6-4. Contingency tables for the Phase I and II studies

Table 22. Contingency tables for the Phase I and II studies

Lab A		IL-2 Luc assay		Total
		+	-	
T cell targeting	+	12	4	16
	-	2	6	8
Total		14	10	24

Sensitivity : 75.0 % (12/16)

Specificity : 75.0 % (6/8)

Accuracy : 75.0 % (18/24)

Lab B		IL-2 Luc assay		Total
		+	-	
T cell targeting	+	12	4	16
	-	2	6	8
Total		14	10	10

Sensitivity : 75.0 % (12/16)

Specificity : 75.0 % (6/8)

Accuracy : 75.0 % (18/24)

Lab C		IL-2 Luc assay		Total
		+	-	
T cell targeting	+	12	4	16
	-	2	6	8
Total		14	10	10

Sensitivity : 75.0 % (12/16)

Specificity : 75.0 % (6/8)

Accuracy : 75.0 % (18/24)

10. Discussion

10-1. Reliability

The IL-2 Luc assay is based on the modulation of PMA + ionomycin-induced luciferase activity in the IL-2 reporter cell line, 2H4. Therefore, it is crucial that 2H4 cells maintain their ability to induce luciferase activity following stimulation by PMA/Io. Before and during this validation study, the response of 2H4 cells to PMA/Io was carefully observed. We confirmed that a frozen stock of 2H4 cells can be cultured without losing luciferase activity for at least 16 weeks or 35 passages.

The culture of 2H4 cells is relatively simple and does not require the use of trypsin or EDTA because 2H4 cells do not adhere to the culture dishes. First, cells adjusted to the optimum concentration are seeded into each well of a 96-well culture plate. Then, chemicals at graded concentrations are added to the wells. After 6 h incubation, 100 μL of pre-warmed Tripluc is added to each of the 96 wells. The subsequent process is completely automated, except for calculating the results using the predesigned Excel spreadsheet. Therefore, the IL-2 Luc assay is a test method that can significantly reduce human error.

Moreover, the IL-2 Luc assay does not require the determination of cell viability after chemical treatment. 2H4 cells can present IL-2 promoter activity as well as promoter activity of GAPDH, a well-known housekeeping gene; therefore, information regarding the effects of the chemical on both IL-2 induction and cell viability is obtained in each experiment. Furthermore, a single experiment takes only 8 h, including the time required for chemical preparation and cell plating, making the IL-2 Luc assay a true high-throughput method.

10-2. Between- and within-laboratory reproducibility

We examined within-laboratory reproducibility in the Phase I study. Lab A, Lab B, and Lab C demonstrated 80%, 100%, and 80% reproducibility, respectively. On the other

hand, the between-laboratory reproducibility of Lab A, Lab B, and Lab C demonstrated 80% in the combined data of the Phase I and Phase II studies. These results satisfied the acceptance criteria for the validation study with a within-laboratory reproducibility of at least 80% and a between-laboratory reproducibility of at least 80%.

10-3. Predictivity

10-3-1. Rationale to determine the predictivity of the IL-2 Luc assay by the concordance between positive effects and the immunotoxic effects targeting T cell response

Reference data showing which chemicals are immunotoxic are essential for determining the performance of the IL-2 Luc assay. However, such reference data are lacking for most chemicals and thus we attempted to create reference data for the chemicals used in this study. Although there is no gold standard to date for classifying immunotoxic chemicals, Luster et al. (Luster et al., 1992b) proposed a rationale for immunotoxic classification, when they presented a screening battery using a 'tier' approach for detecting potential immunotoxic compounds in mice (Luster, 1998). Their proposal was that a positive reference chemical would either produce a significant dose-response effect in the immune test or significantly alter two or more immune test results at the highest dose of the chemical tested. They classified chemicals based on the 1results obtained in 12 immune tests according to this rationale and found a significant correlation between the judgment of immunotoxic chemicals and host resistance (Luster et al., 1993). Therefore, we used this rationale and classified chemicals based on the published previously immunotoxicological information for each chemical.

When immunotoxic information of chemical is collected from the literature, however, most of the published data are not focusing on whether immunotoxicity of chemicals is caused either by their direct effects on T cell or not. To overcome this problem, in this study, the predictivity was evaluated by the criteria whether chemicals affect T cell functions, namely T cell targeting, or not. To determine T cell targeting chemicals (TTCs), we defined the criteria described in 9-1-3.

10-3-2. The predictivity of the Phase I and Phase II studies

To classify 25 chemicals used in the Phase I and II studies, we used the chemical information kindly provided by the National Toxicology Program (NTP) and those collected by the VMT members. The immunotoxic characteristics of each chemical are shown in Appendix 7 and their summarized data are shown in the Appendix 19. Based on the criteria, the 25 chemicals were classified into 16 TTCs, 8NTTCs, and 1 unclassified chemicals that could not be classified because of insufficient data. According to this classification, the sensitivities of the assays as conducted by Lab A, Lab B, Lab C, and their average in the combined data of the Phase I and II studies are 75.0%, 75.0%, 75.0% and 75.0%, respectively. The specificities of the assays as conducted by Lab A, Lab B, Lab C, and their average are 75.0%, 75.0%, 75.0%, and 75.0%, respectively. The accuracies of the assays conducted by Lab A, Lab B, Lab C, and their average are 75.0%, 75.0%, 75.0%, and 75.0%, respectively.

10-4. IL-2 Luc assay data set for 60 chemicals

Based on the Multi-ImmunoTox assay protocol Ver. 011E and the Criteria 5, the lead laboratory reevaluated the data of 60 chemicals reported previously (Kimura et al. 2018) (Table 23). These 60 chemicals were also classified by the criteria described in 9-1-3. The classification of chemicals and their immunotoxic information were summarized in the Appendix 20. The list of references is in the Appendix 9. There were 34 TTCs, 6 NTTCs, and 20 chemicals that were either those without any immunotoxic information or with insufficient information. Similar to the classification by the criteria used in our published paper (Kimura et al., 2018), TAC, CyA, and Dex significantly suppressed IL-2 luciferase activity (IL-2LA), although the average LOEL of TAC and CyA was significantly lower that of DEX. The off-label immunosuppressive drugs, chloroquine, minocycline, and dapsone significantly suppressed IL-2LA. Anti-cancer drugs, actinomycin D and cisplatin also significantly suppressed IL-2LA. In addition,

azathioprine and colchicine were demonstrated to suppress IL-2LA by the Criteria 5. Again, the suppressive effects on the IL-2LA was not demonstrated by some of immunosuppressants the mechanism of which is inhibition of DNA synthesis or anti-proliferative effects on T cells, such as mitomycin C, cyclophosphamide, methotrexate or mizoribine by the Criteria 5.

If we calculated the predictivity of 60 chemicals evaluated by the IL-2 Luc assay based on the classification of chemicals defined in 9-1-3, the sensitivity, specificity and accuracy (predictivity) are 82.4% (28/34), 83.3% (5/6), and 82.5% (33/40), respectively.

Table 23. Data set of the IL-2 Luc assay based on Criteria 5.

Chemical name	Immunotoxicity classification		IL-2 Luc assay	Ave.LOEL(35%)	Ave.LOEL(-35%)
	Classification	Rationale[#]			
FK506	TTC	1,3	P	0.0002	
Cyclosporine A	TTC	1,3	P	0.0041	
Actinomycin D	TTC	3	P	0.0156	
Digoxin	TTC	2, 3	P	0.0686	
Colchicine	TTC	2, 3	P	0.2743	
FR167653	Undetermined	2, 3	P	1.3021	
Benzethonium chloride	Undetermined	1	P	1.6276	
Mercuric chloride	TTC	1,3	P	1.9531	
Chlorpromazine	TTC	1,3	P	1.9531	
Amphotericin B	Undetermined	1	P	2.6042	
Dibutyl phthalate	TTC	3	P	2.6042	
2-Aminoanthracene	Undetermined		P	5.8594	
Formaldehyde	TTC	2,3	P	7.8125	
Pyrimethamine	Undetermined		P	7.8125	
Isophorone diisocyanate	Undetermined		P	15.6250	
Cisplatin	TTC	1,2,3	P	16.9271	
Cobalt chloride	TTC	1, 3	P	16.9271	
Chloroquine	TTC	1,3	P	17.8326	
Minocycline	TTC	3	P	18.5185	
Mitomycin C	Undetermined		P	20.0000	
Hydrogen peroxide	TTC	3	P	23.4375	
Citral	Undetermined	1	P	25.0000	
Dexamethasone	TTC	1,3	P	41.1692	
Pentamidine isethionate	TTC	3	P	52.0833	
Lead(II)acetate	TTC	1, 3	P	57.2917	
Azathioprine	TTC	1, 2, 3	P	58.4778	
Diesel exhaust particle	TTC	1, 3	P	62.5000	
Sodium dodecyl sulfate	TTC	3	P	62.5000	
Dapsone	TTC	3	P	72.9167	
Nitrofurazone	NTTC		P	83.3333	
p-Nitroaniline	TTC	1,3	P	83.3333	
Sulfasalazine	TTC	1,3	P	92.9444	
Aluminium chloride	TTC	1,3	P	104.1667	
Nickel sulfate	TTC	1, 3	P	104.1667	
Hydrocortisone	TTC	1,3	P	125	
Diethanolamine	Undetermined	1	P	250.0000	
Chloroplatinic acid	Undetermined		P	250.0000	
Sodium bromate	Undetermined	1	P	500.0000	
Histamine	TTC	3	P	750.0000	
Isoniazid	NTTC	1	N		
Triethanolamine	Undetermined		N		
Magnesium sulfate	Undetermined		N		
Rapamycin	TTC	1, 3	N		
Mizoribine	Undetermined		N		
Warfarin	TTC	3	N		
2,4-Diaminotoluene	NTTC		N		
Cyclophosphamide	TTC	1	N*		
Dibenzopyrene	Undetermined		N		
Ethanol	TTC	1, 3	N		
Hexachlorobenzene	Undetermined		N		
Lithium carbonate	TTC	1,3	N		
Methanol	NTTC		N		
Methotrexate	TTC	3	N		
Dimethyl sulfoxide	NTTC		N		
Trichloroethylene	NTTC		N		
Mycophenolic acid	Undetermined		P		0.395061728
2-Mercaptobenzothiazole	Undetermined		P		16.11328125
Ribavirin	TTC	1, 3	P		26.04166667
Nicotinamide	Undetermined		P		288.0658436
Acetaminophen	Undetermined		P		288.0658436

P : Positive, N : No effect,

Blue color: accurate, Red color: false, yellow color: Undetermined because of insufficient reported data.

#: The criterion number used to define immunotoxicity

*: cyclophosphamide needs metabolic activity to demonstrate the activity

10-5. Factors responsible for false negative results in the IL-2 Luc assay

Although the within- and between-laboratory reproducibility satisfied the acceptance criteria for the validation study, the predictivity was less than 80%. We considered at least 2 reasons for the poor predictivity of the assay.

1)/ We collected immunotoxic information on the chemicals as much as possible and determined whether the chemicals exhibited T-cell dependent immunotoxicity or not using the criteria we proposed. The information used for classification were the effects of the chemicals on thymus weight, the production of cytokines predominantly produced by T cells, *in vitro* or *ex vivo*, T cell proliferation, and their reported mode of action on T cell function. However, the information available was very limited for most chemicals and very little data had been reproduced by different laboratories. The classification of some chemicals may not be correct.

2)/ The IL-2 Luc assay does not cover every aspect of the effects of the chemicals on T cell function. Other assays targeting T cell functions may be mandatory.

10-6. The applicability domain and the imitations of the IL-2 Luc assay

The IL-2 Luc assay evaluates the effects of chemicals on IL-2 transcription by T cells. Therefore, its applicability domain is immunotoxic chemicals the toxicity of which is caused by the direct effects of chemicals on T cells.

On the other hand, since the 2H4 cell line used in the IL-2 Luc assay is derived from Jurkat cells, a human acute T lymphoblastic leukemia cell line, it is conceivable that this cell line is more resistant to the cytotoxic effects of chemicals than bone marrow cells.

Therefore, the IL-2 Luc assay cannot evaluate the immunotoxic effects of some immunosuppressive drugs the mechanism of which is inhibiting DNA synthesis leading to myelotoxicity (Kimura et al., 2014). Thus, these chemicals in addition to chemicals that need metabolic activation should be outside the applicability domain. To overcome this drawback at present, the IL-2 Luc assay must be combined with assays capable of detecting myelotoxicity, such as *in vitro* myelotoxicity tests (Pessina et al., 2003). Similar to other *in vitro* test methods, poor water soluble chemicals are not suitable for this assay.

10-7. Potential of the IL-2 Luc assay

The IL-2 Luc assay evaluates the effects of chemicals on IL-2 transcription by Jurkat T cells stimulated with PMA and CI. The simultaneous stimulation of PMA and calcium ionophore or ionomycin surrogates the stimulation by T cell receptor (TCR) and CD28 (Kumagai et al., 1987; Truneh et al., 1985). The downstream signaling after the stimulation by TCR/CD28 is shown in Fig. 16. It indicates that the signaling required for IL-2 transcription after TCR/CD28 or PMA/CI stimulation involves the pathways leading the activation of AP1/2, mTOR, NF-κB, and NFAT. The immune system is composed of innate immune system and acquired immune system at least. The innate immune systems are activated by pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patters via Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), Nod-like receptors (NLRs), or cytokine receptors for IL-1 family or TNF family. Most of the downstream signaling after the stimulation of these receptors involves NF-κB and AP1/2 pathways (Newton and Dixit, 2012). In the acquired immune system, in addition to the process of T cell activation, B cell activation after B cell receptor stimulation and the signaling of various cytokines also involves NF-κB pathway (reviewed by Zhang and Sun (Zhang and Sun, 2015). Therefore, it is conceivable that the effects of chemicals on quite a few aspects of immune responses can be detected by the IL-2 Luc assay.

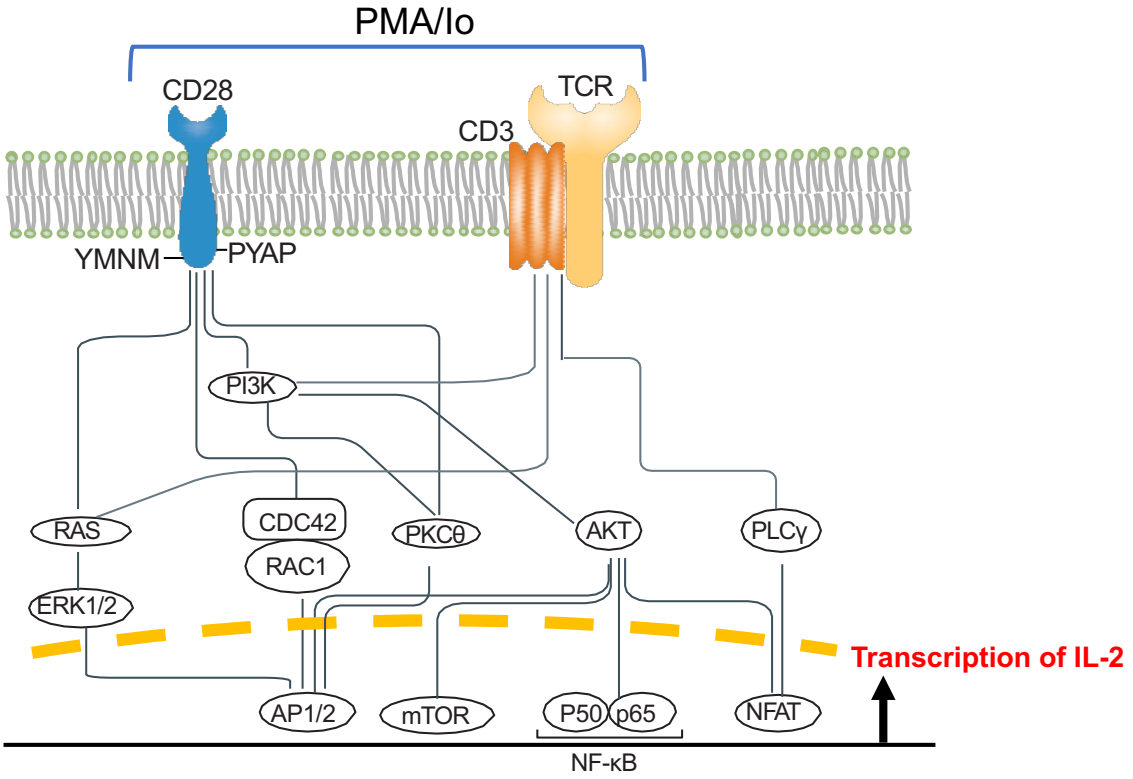


Fig. 16. The schematic presentation of cellular signaling after TCR/CD28 or PMA/Io stimulation.

Luster et al (Luster, 1988) proposed a screening battery using a 'tier' approach for detecting potential immunotoxic compounds in mice. Then, they defined criteria to classify immunotoxic chemicals using several parameters comprising the ‘tier approach’ and then, classified 51 chemicals into immunotoxic compounds or not (Luster et al., 1992b). Furthermore, they examined the ability of various immune tests to predict increased susceptibility in the host resistance classification (Luster et al., 1992a). Their final results demonstrated the following. 1. a number of the immune tests provided a relatively high association with changes in host resistance (i.e. > 70%) such as IgM plaque forming cell (PFC) response to sheep red blood cells, T cell mitogen response,

delayed hypersensitivity response (DHR), surface markers and spleen cellularity while several of the tests, such as leukocyte counts and lymphoproliferative response to LPS, were poor predictors with concordance values of approximately 50%. 2. The combinations of two immune tests compared with the host resistance classification increased the concordance from that obtained using individual tests. Pair-wise combinations which included either the PFC response, surface markers or DHRs gave consistently higher concordances.

When the IL-2 Luc assay examined 31 of the 51 chemicals evaluated by Luster et al. (1992b), its performance was similar to that of mixed lymphocyte reaction (MLR), DHR, and spleen cellularity and better than leukocyte counts or LPS response. Moreover, among 7 chemicals judged as false negative by the IL-2 Luc assay, 5 chemicals was judged as positive by Luster et al. (1992b) based on their suppressive effects on T cell mitogen response. Since our previous study demonstrated the inability of the IL-2 Luc assay to detect immunosuppressive effects of chemicals which are dependent on their suppressive effects on T cell proliferation, these 5 chemicals are out of applicability domain. Taking this into account, the sensitivity, specificity and accuracy of the IL-2 Luc assay was 76.5% (13/17), 44.4% (4/9), and 65.4% (17/26).

The HWBCRA, previously used in a rigorous prevalidation effort by ECVAM and other groups, is an immune test to examine the effects of chemicals on IL-4 or IL-1β production stimulated by staphylococcal enterotoxin B (SEB) or LPS, respectively (Langezaal et al., 2002). Although this study uses human whole blood cells, it examines the production of IL-4 by T cells and of IL-1 by monocytes. This concept is similar to that of the MITA, in which the effects of chemicals on T cells and monocytes are examined using Jurkat cell-derived 2H4 and THP-1-derived THP-G1b cells. Interestingly, the evaluation of chemicals by IL-4 production in the HWBCRA was almost identical to the results of the IL-2 Luc assay: both detected strong immunosuppression by FK506, cyclosporin A, dexamethasone and actinomycin D, which are more potent that chloroquine and azathioprine. Cyclophosphamide and

mizoribine require metabolic activation and thus are not considered as immunosuppressive by both assays. On the other hand, the cardiac glycoside digoxin is classified as an immunotoxic chemical by both assays. These data suggest that the IL-2 Luc assay may be an alternative method to the HWBCRA for examining the effects of chemicals on T cells. In addition, the IL-2 Luc assay has the following advantages over the HWBCRA. 1) The IL-2 Luc assay does not require primary cells, 2) it does not require cytokine quantification using ELISA, and 3) the time required for the IL-2 Luc assay is less than 8 h.

Finally, The performance of the IL-2 Luc assay to examine only immunosuppressive drugs whose effects on human are well established (reviewed by Allison (Allison, 2000)) showed that tacrolimus (TAC), cyclosporine A (CyA) and dexamethasone (Dex) significantly suppressed IL-2 luciferase activity (IL-2LA), although the average Lowest Observed Effect Levels (LOELs) of TAC and CyA were significantly lower that of DEX. The off-label immunosuppressive drugs chloroquine, minocycline and dapsone significantly suppressed IL-2LA. The anti-cancer drugs actinomycin D and cisplatin also significantly suppressed IL-2LA. In addition, azathioprine and colchicine were demonstrated to suppress IL-2LA. No suppressive effects on IL-2LA were demonstrated by several immunosuppressants which inhibit DNA synthesis or anti-proliferative effects on T cells, such as rapamycin, mizoribine, cyclophosphamide, methotrexate and mycophenolic acid.

10-8. Evaluation of the immunotoxicity of 60 chemicals by the modified MITA (mMITA)

Regulatory authorities worldwide require testing for allergic contact dermatitis (ACD) and appropriate hazard labeling to minimize exposures. Thus, we combined the MITA with an *in vitro* sensitization test, the IL-8 Luc assay, recently approved as an OECD test guideline for *in vitro* skin sensitization testing (OECD TG442E)(OECD, 2017). We designated this combined assay ‘modified MITA’ (mMITA). We established a

data set of 60 chemicals by referring to the publication by Wagner et al. (Wagner et al., 2006) in which they examined 46 chemicals characterized to different degrees for their immunotoxic and immunomodulatory properties using the Fluorescent Cell Chip (FCP) assay. In addition, we also evaluated the chemicals listed in the case studies in the Guidance for Immunotoxicity Risk Assessment for Chemicals published by World Health Organization (WHO)/ and Meeting, 2012. Since there were several overlaps between the chemicals we examined in our previous publication and those examined by the FCP, our final data set comprised 60 chemicals evaluated by the mMITA (Kimura et al., 2018) (Table 24). Table 25 lists the chemicals that affected the normalized IL-2 luciferase activity in increasing order of their Lowest Observed Effect Level (LOEL), the results of the MITA evaluation (suppression (S), augmentation (A) or no effect (N)), the LOEL for each parameter of each chemical, and the results of the IL-8 Luc assay evaluation (sensitiser (S) and non-sensitiser (N)).

Table 24. Classification of chemicals by the mMITA in increasing order of the LOEL of the IL-2 Luc assay.

Chemicals	IL-2		IFN-γ		IL-1β		IL-8		IL-8 Luc
	Judge	LOEL	Judge	LOEL	Judge	LOEL	Judge	LOEL	Judge
FK 506	S	0.00	S	0.00	A		N		N
Cyclosporine A	S	0.00	S	0.00	N		N		N
Actinomycin D	S	0.00	S	0.01	N		S	0.00	S
Digoxin	S	0.01	S	0.02	N		N		S
Dexamethasone	S	0.01	N		S	0.01	S	0.01	N
Dibenzopyrene	S	0.01	S	0.03	N		N	0.00	N
Pyrimethamine	S	0.04	N		N		N		N
Chloroquine	S	0.05	S	0.02	S	10.00	S	30.00	S
Cisplatin	S	0.24	S	1.22	N		N		S
Hydrocortisone	S	0.34	A	6.27	S	0.34	S	0.34	N
Mitomycin C	S	0.36	N		N		N		S
Citral	S	0.36	S	1.37	N		N		S
Nitrofurazone	S	0.37	A	3.91	A		A	62.50	S
FR167653	S	0.49	S	0.49	S	145.83	S	125.00	N
Amphoterycin B	S	0.78	S	2.08	A	3.13	A	7.82	S
2-Aminoanthracene	S	0.81	S	5.86	S	2.03	N		S
Lithium carbonate	S	0.98	A	116.67	S	0.39	S	0.39	S
Isophorone diisocyanate	S	0.98	N		S	0.98	S	0.98	S
p-Nitroaniline	S	0.98	S	1.95	S	1.47	S	2.45	N
Dibutyl phthalate	S	0.98	S	1.95	S	39.07	S	31.25	N
Formaldehyde	S	1.71	N		S	15.63	S	15.63	S
Benzethonium chloride	S	1.95	S	1.95	S	3.91	N		S
Isoniazid	S	1.97	N		N		S	800.00	N
Chlorpromazine	S	3.91	S	3.91	S	7.81	S	7.81	S
Cobalt chloride	S	3.91	S	9.12	S	3.91	S	125.00	S
Pentamidine isethionate	S	3.91	S	32.55	S	3.91	S	3.91	N
Aluminum chloride	S	3.91	S	62.50	N		N		N
Lead(II) acetate	S	3.91	S	3.91	N		N		N
Hydrogen peroxide	S	7.82	S	31.25	N		N		S
Minocycline	S	8.33	S	5.00	N		N		S
Histamine	S	9.12	A	5.86	N		S	3.91	S
Diethanolamin	S	9.12	N		N		N		S
Nickel sulfate	S	14.32	S	32.55	S	250.00	S	250.00	S
Sulfasalazine	S	36.00	S	1.20	S	7.80	S	1.20	N
Diesel exhaust particles	S	39.07	A	47.53	N		S	62.50	S
Dapsone	S	45.01	S	55.14	S	46.88	S	134.75	N
Sodium bromate	S	125.00	N		N		N		S
Triethanolamine	S	187.50	S	1416.67	N		N		S
Mercuric chloride	N		A	3.91	S	1.95	S	1.95	S
Chloroplatinic acid	N		N		N		S	15.63	S
2-Mercaptobenzothiazole	N		N		N		S	125.00	S
Cyclophosphamide	N		A	168.00	N		N		S
Magnesium sulfate	N		N		S	15.63	N		S
Sodium dodecyl sulfate	N		N		N		N		S
2,4-Diaminotoluene	N		A	62.50	N		S	0.98	N
Ethanol	N		N		N		N		N
Methanol	N		N		N		N		N
Hexachlorobenzene	N		N		N		N		N
Trichloroethylene	N		N		N		N		N
Azathioprine	N		A	40.01	A	9.23	N		N
Mizoribine	N		N		A	5.20	A	7.45	N
Rapamycin	A	0.00	N		A	0.91	N		S
Nicotinamide	A	0.10	A	110.03	S	3.00	S	10.00	N
Colchicine	A	0.29	A	0.06	A	0.02	A	20.00	S
Mycophenolic acid	A	0.38	A	6.24	N		N		S
Methotrexate	A	0.45	A	0.09	N		N		N
Dimethyl sulfoxide	A	3.91	A	625.00	S	66.41	S	3.91	N
Ribavirin	A	15.63	A	187.50	A	5.86	N		N
Warfarin	A	23.33	N		S	30.00	S	0.00	N
Acetaminophen	A	33.33	A	33.33	A	166.67	A	100.00	N

Table 25. The group by LOEL

Groups	Suppression of IL-2 promoter activity (LOEL μg/ml)
Group 1	LOEL<0.1
Group 2	0.1≤LOEL<1.0
Group 3	1.0≤LOEL<10
Group 4	10≤LOEL<1000
Group 5	None
Group 6	Augmentation

0.0/ of the LOEL means less than 0.001.

Using this data set, we first demonstrated a significant correlation between LOELs for the effects on the IL-2 luciferase assay and those on the IFN luciferase assay, and between LOELs for effects on the IL-1β luciferase assay and those on the IL-8 luciferase assay (Kimura et al., 2018) (Fig. 17). These results indicated that evaluations of the effects of chemicals on the IL-2 and IL-8 luciferase assays can provide immunotoxicological information almost equivalent to the evaluation of these chemicals using the IL-2, IFN-γ, IL-1β, and IL-8 luciferase assays.

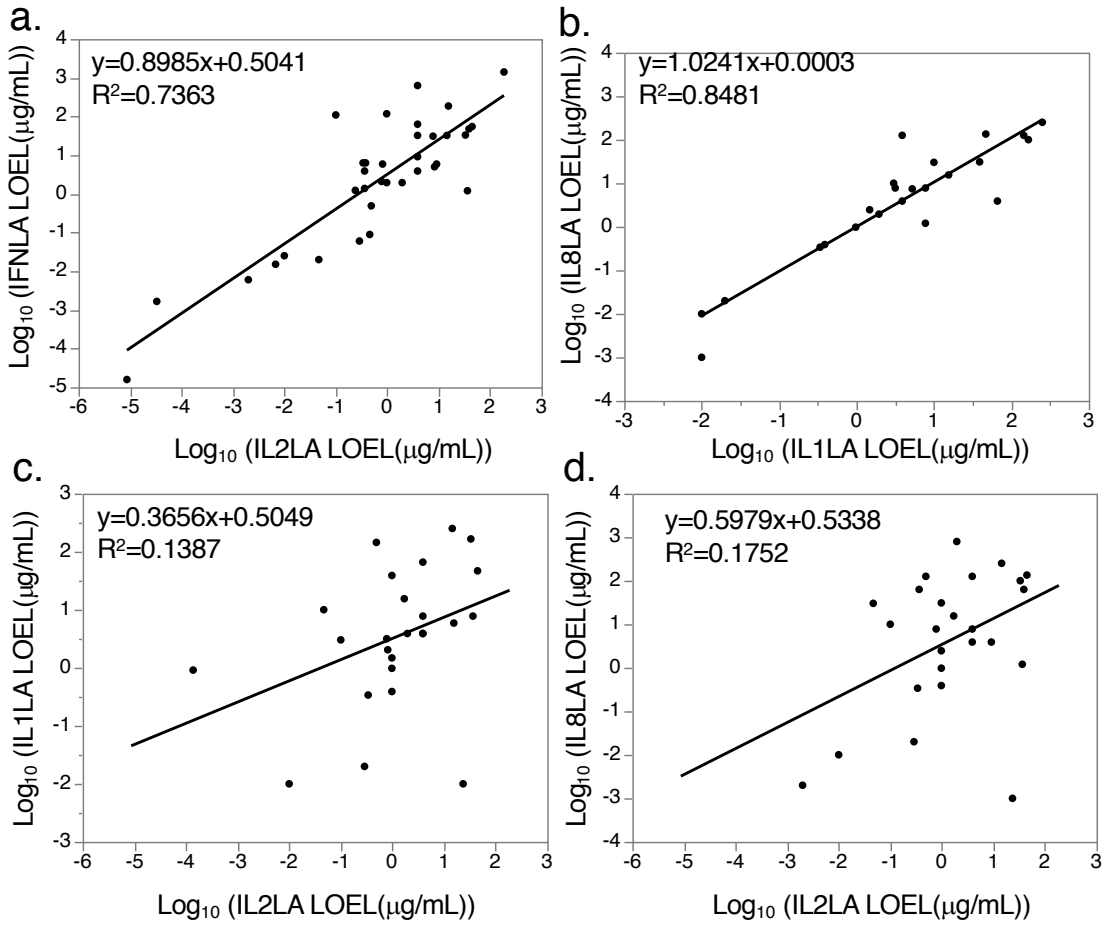
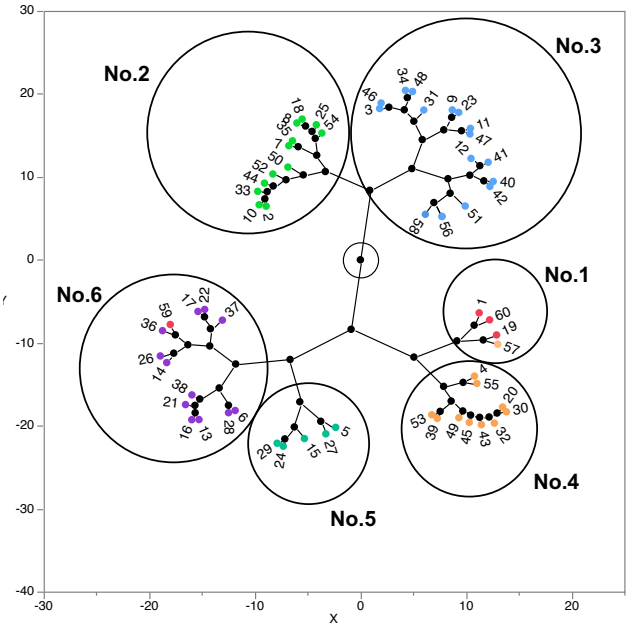


Fig. 17. The correlation between the LOEL for the 4 luciferase assays.

Next, we demonstrated that K-means clustering and hierarchical clustering of the 60 chemicals based on the LOEL for their effects on IL-2 and IL-8 promoter activities, and the judgment by the IL-8 Luc assay, resulted in the same 6-cluster solution: cluster 1 with preferential suppression of IL-8, cluster 2 with suppression of IL-2 and a positive IL-8 Luc assay result, cluster 3 with suppression of both IL-2 and IL-8, cluster 4 with no effects on IL-2 or IL-8 and a negative IL-8 Luc assay result, cluster 5 with suppression of both IL-2 and IL-8 and a negative IL-8 Luc assay result, and cluster 6 with preferential suppression of IL-2 (Kimura et al., 2018) (Figs. 18, 19 and 20). These data suggest that the mMITA is a promising novel high-throughput approach for detecting unrecognized immunological effects of chemicals and for profiling their

immunotoxic effects. The data obtained from these assays can be used by both industry and regulatory agencies to assess the immunotoxicity risks of chemicals. Toward this particular goal, the IL-2 Luc assay and the IL-8 or IL-1β Luc assay should be officially validated and a larger number of chemicals must be evaluated using the MITA to fully determine the potential and limits of this technique.



Cluster No	No	Chemicals
1	1	2,4-Diaminotoluene
1	19	Dimethyl sulfoxide
1	57	Nicotinamide
1	60	Warfarin
2	2	2-Aminoanthracene
2	7	Amphoterycin B
2	8	Benzethonium chloride
2	10	Cisplatin
2	18	Diethanolamin
2	25	Hydrogen peroxide
2	33	Mitomycin C
2	35	Nitrofurazone
2	44	Citral
2	50	Chloroquine
2	52	Digoxin
2	54	Minocycline
3	3	2-Mercaptobenzothiazole
3	9	Chlorpromazine
3	11	Cobalt chloride
3	12	Cyclophosphamide
3	23	Histamine
3	31	Mercuric chloride
3	34	Nickel sulfate
3	40	Sodium bromate
3	41	Sodium dodecyl sulfate
3	42	Triethanolamine
3	46	Chloroplatinic acid
3	47	Formaldehyde
3	48	Diesel exhaust particles
3	51	Colchicine
3	56	Mycophenolic acid
3	58	Rapamycin

Cluster No	No	Chemicals
4	4	Acetaminophen
4	20	Ethanol
4	30	Magnesium sulfate
4	32	Methanol
4	39	Ribavirin
4	43	Hexachlorobenzene
4	45	Trichloroethylene
4	49	Azathioprine
4	53	Methotrexate
4	55	Mizoribine
5	5	Actinomycin D
5	15	Dexamethasone
5	24	Hydrocortisone
5	27	Isophorone diisocyanate
5	29	Lithium carbonate
6	6	Aluminum chloride
6	13	Cyclosporine A
6	14	Dapsone
6	16	Dibenzopyrene
6	17	Dibutyl phthalate
6	21	FK 506
6	22	FR167653
6	26	Isoniazid
6	28	Lead(II) acetate
6	36	Pentamidine isethionate
6	37	p-Nitroaniline
6	38	Pyrimethamine
6	59	Sulfasalazine

Fig. 18. Hierarchical clustering of 60 chemicals by the mMITA

Hierarchical clustering of 60 chemicals was performed for these 3 immunotoxic parameters and visualized using JMP pro 13.1.0. Table is the list of chemicals that belong to each cluster.

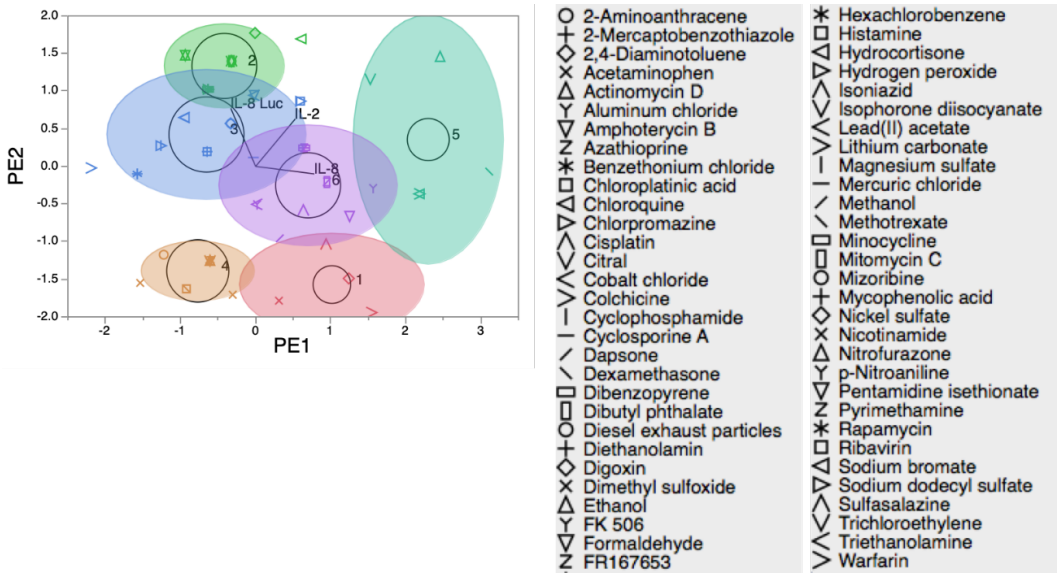


Fig. 19. K-means clustering analysis of chemicals by MITA

K-means clustering of 60chemicals was performed for these 3 immunotoxic parameters and visualized using JMP pro 13.1.0.

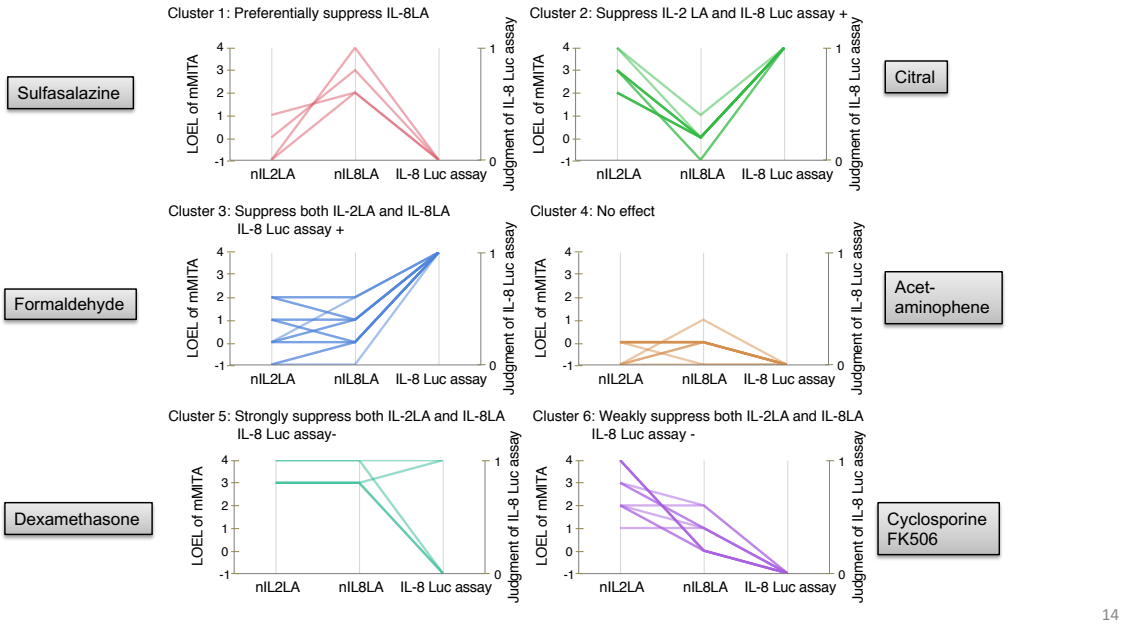


Fig. 20. Characteristics of each cluster and their representative chemicals

The scores for the LOEL of IL2LA, IL8LA and the IL8 Luc assay was plotted for each chemical belonging to different clusters.

10-9. The regulatory application of the IL-2 Luc assay.

The CAS REGISTRY^{SM} currently contains more than 130 million unique organic and inorganic chemical substances, such as alloys, coordination compounds, minerals, mixtures, polymers, and salts. Humans are exposed to many of these substances, which are present as environmental contaminants or used as food additives and drugs. Some of these compounds can target the immune system, resulting in adverse health effects such as the development of allergies, autoimmune disorders, increased susceptibility to infection and cancer, and other diseases. Accordingly, immunotoxicity, which is defined as the toxicological effects of xenobiotics on the function of the immune system, is a matter of serious concern to the public as well as regulatory agencies. To address these concerns, the World Health Organization published its Guidance for Immunotoxicity Risk Assessment for Chemicals (WHO). Currently, the assessment of chemical immunotoxicity relies mainly on animal models and assays that characterize immunosuppression and sensitization. However, animal studies have so many drawbacks, such as high cost, ethical concerns, and questionable relevance to risk assessment for humans, that they cannot screen immunotoxicity of more than 130 million chemicals. Therefore, it is an urgent matter to develop alternative testing methods and assessment strategies to reduce the use of laboratory animals and, if possible, replace animals used in scientific studies (Adler et al., 2011). So far, however, there is no OECD test guidelines to detect chemical immunotoxicity in vitro. Therefore, we would like to propose the IL-2 Luc assay, and the MITA in near future, as a screening toolbox of alternative test methods for immunotoxicity.

Finally, the VMT recommend that the proficiency chemicals (Appendix 15) to users and the performance standard chemicals (Appendix 16) to me-too validation study.

11. Conclusion

In this study, we conducted the validation study of the IL-2 Luc assay among the 4 luciferase assays that comprise the MITA. The results of both Phase I and Phase II studies satisfied the acceptance criteria for the validation study. Although the predictivity could not reach 80%, it may be acceptable when considering its applicability domain and limited target. So, we would like to propose the IL-2 Luc assay for the OECD test guideline of *in vitro* immunotoxicity test.

12. Acknowledgement

This validation study was supported by the Grants-in-Aid for the Ministry of Economy, Trade and Industry (METI), the Ministry of Health, Labor and Welfare (MHLW) and the Japanese Society for Alternatives to Animal Experiments (JSAAE). We gratefully acknowledge the voluntary works by the participated laboratories and the European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM), and the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM).

13. References

Adler, S., Basketter, D., Creton, S., et al. (2011), Alternative (non-animal) methods for cosmetics testing: current status and future prospects-2010. *Arch Toxicol* 85: 367-485, 10.1007/s00204-011-0693-2

Alegre, M.L., Frauwirth, K.A., Thompson, C.B. (2001), T-cell regulation by CD28 and CTLA-4. *Nat Rev Immunol* 1: 220-228, 10.1038/35105024

Allison, A.C. (2000), Immunosuppressive drugs: the first 50 years and a glance forward. *Immunopharmacol* 47: 63-83,

Corsini, E., Roggen, E. (2017), Overview of in vitro assessment of immunotoxicity. *DOI:10.1016/j.cotox.2017.06.016*

Elentner, A., Schmuth, M., Yannoutsos, N., et al. (2018), Epidermal Overexpression of Xenobiotic Receptor PXR Impairs the Epidermal Barrier and Triggers Th2 Immune Response. *J Invest Dermatol* 138: 109-120, 10.1016/j.jid.2017.07.846

Fulda, S., Gorman, A.M., Hori, O., et al. (2010), Cellular stress responses: cell survival and cell death. *Int J Cell Biol* 2010: 214074, 10.1155/2010/214074

Galbiati, V., Mitjans, M., Corsini, E. (2010), Present and future of in vitro immunotoxicology in drug development. *J Immunotoxicol* 7: 255-267, 10.3109/1547691X.2010.509848

Gallucci, S., Matzinger, P. (2001), Danger signals: SOS to the immune system. *Curr Opin Immunol.* 13: 114-119,

Gennari, A., Ban, M., Braun, A., et al. (2005), The Use of In Vitro Systems for Evaluating Immunotoxicity: The Report and Recommendations of an ECVAM Workshop. *J Immunotoxicol* 2: 61-83, 10.1080/15476910590965832

Hidaka, T., Ogawa, E., Kobayashi, E.H., et al. (2017), The aryl hydrocarbon receptor AhR links atopic dermatitis and air pollution via induction of the neurotrophic factor artemin. *Nat Immunol* 18: 64-73, 10.1038/ni.3614

Kaiko, G.E., Horvat, J.C., Beagley, K.W., et al. (2008), Immunological decision-making: how does the immune system decide to mount a helper T-cell response? *Immunology* 123: 326-338, 10.1111/j.1365-2567.2007.02719.x

Kimura, Y., Fujimura, C., Ito, Y., et al. (2014), Evaluation of the Multi-ImmunoTox Assay composed of 3 human cytokine reporter cells by examining immunological effects of drugs. *Toxicol In Vitro* 28: 759-768, 10.1016/j.tiv.2014.02.013

Kimura, Y., Fujimura, C., Ito, Y., et al. (2018), Profiling the immunotoxicity of chemicals based on in vitro evaluation by a combination of the Multi-ImmunoTox assay and the IL-8 Luc

assay. *Arch Toxicol* 92: 2043-2054, 10.1007/s00204-018-2199-7

Kimura, Y, Yasuno R, Watanabe M., et al. (2020), An international validation study of the IL-2 Luc assay for evaluating the potential immunnotoxic effects of chemicals on T cells and a proposal for reference data for immunotoxic chemicals. Toxicol in Vitro in press, 10.1016/j.tiv.2020.104832

Kultz, D. (2005), Molecular and evolutionary basis of the cellular stress response. *Ann Rev Physiol* 67: 225-257, 10.1146/annurev.physiol.67.040403.103635

Kumagai, N., Benedict, S.H., Mills, G.B., et al. (1987), Requirements for the simultaneous presence of phorbol esters and calcium ionophores in the expression of human T lymphocyte proliferation-related genes. *J Immunol* 139: 1393-1399,

Langezaal, I., Hoffmann, S., Hartung, T., et al. (2002), Evaluation and prevalidation of an immunotoxicity test based on human whole-blood cytokine release. *ATLA* 30: 581-595,

Lankveld, D.P., Van Loveren, H., Baken, K.A., et al. (2010), In vitro testing for direct immunotoxicity: state of the art. *Meth Mol Biol* 598: 401-423, 10.1007/978-1-60761-401-2_26

Liao, W., Lin, J.X., Wang, L., et al. (2011), Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. *Nat Immunol* 12: 551-559, 10.1038/ni.2030

Luster, M.I., Munsosn, A.E., Thomas, P.D., et al. (1988), Development of a testing battery to assess chemical-induced immunotoxicity: National Toxicology Program's guidelines for immunotoxicity evaluation in mice. *Fundam Appl Toxicol* 10: 2-19, 10.1016/0272-0590(88)90247-3

Luster, M.I., Portier, C., Pait, D.G., et al. (1992a), Risk assessment in immunotoxicology. I. Sensitivity and predictability of immune tests. *Fundam Appl Toxicol* 18: 200-210, 10.1016/0272-0590(92)90047-l

Luster, M.I., Pait, D.G., Portier, C., et al. (1992b), Qualitative and quantitative experimental models to aid in risk assessment for immunotoxicology. *Toxicol Lett* 64-65 Spec No: 71-8, 10.1016/0378-4274(92)90174-i

Luster, M.I., Portier, C., Pait, D.G., et al. (1993), Risk assessment in immunotoxicology. II. Relationships between immune and host resistance tests. *Fundam Appl Toxicol* 21: 71-82,

Michelini, E., Cevenini, L., Calabretta, M.M., et al., (2014). Exploiting in vitro and in vivo bioluminescence for the implementation of the three Rs principle (replacement, reduction, and refinement) in drug discovery. Anal Bioanal Chem 406, 5531-5539, 10.1007/s00216-

014-7925-2

Milisav, I., (2011), Cellular stress responses. Advance in regererative medicine ect. Sabine Wislet-Gendebien InTech, 978-953-307-732-1

Nakajima, Y., Ikeda, M., Kimura, T., et al. (2004), Bidirectional role of orphan nuclear receptor RORalpha in clock gene transcriptions demonstrated by a novel reporter assay system. *FEBS Lett* 565: 122-126, 10.1016/j.febslet.2004.03.083

Nakajima, Y., Kimura, T., Sugata, K., et al. (2005), Multicolor luciferase assay system: one-step monitoring of multiple gene expressions with a single substrate. *Biotechniques* 38: 891-894, 05386ST03 [pii]

Nakajima Y, Ohmiya Y., (2010), Bioluminescence assays: multicolor luciferase assay, secreted luciferase assay and imaging luciferase assay. *Expert Opin Drug Discov* 5:835-49, 10.1517/17460441.2010.506213

Newton, K., Dixit, V.M. (2012), Signaling in innate immunity and inflammation. *Cold Spring Harb Perspect Biol* 4, 10.1101/cshperspect.a006049

Niwa, K., Ichino, Y., Kumata, S., et al. (2010), Quantum yields and kinetics of the firefly bioluminescence reaction of beetle luciferases. *Photochem Photobiol* 86: 1046-1049, 10.1111/j.1751-1097.2010.00777.x

Noguchi, T., Ikeda, M., Ohmiya, Y., et al. (2008), Simultaneous monitoring of independent gene expression patterns in two types of cocultured fibroblasts with different color-emitting luciferases. *BMC Biotechnol* 8: 40, 1472-6750-8-40 [pii]10.1186/1472-6750-8-40

OECD (1998), OECD Principles on Good Laboratory Practice, OECD SERIES ON PRINCIPLES OF GOOD LABORATORY PRACTICE AND COMPLIANCE MONITORING, No 1, Available at: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/mc/chem(98)17&doclanguage=en

OECD (2017), OECD Test Gudeline for the Testing of Chemicals No.442E: In Vitro Skin Sensitisation assays addressing the key Event on activation of dendritic cells on the Adverse Outcome pathway for Skin Sensitisation. http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788.

Pessina, A., Albella, B., Bayo, M., et al. (2003), Application of the CFU-GM assay to predict acute drug-induced neutropenia: an international blind trial to validate a prediction model for the maximum tolerated dose (MTD) of myelosuppressive xenobiotics. *Toxicol Sci* 75: 355-367, 10.1093/toxsci/kfg188

Roda, A., Pasini, P., Mirasoli, M., et al. (2004), Biotechnological applications of bioluminescence and chemiluminescence. *Trends Biotechnol* 22: 295-303, 10.1016/j.tibtech.2004.03.011

Saito, R., Hirakawa, S., Ohara, H., et al. (2011), Nickel differentially regulates NFAT and NF-kappaB activation in T cell signaling. *Toxicol Appl Pharmacol* 254: 245-255, 10.1016/j.taap.2011.04.017

Sasaki, Y., Aiba, S. (2007), Dendritic cells and contact dermatitis. *Clinl Rev Allergy Immunol* 33: 27-34, 10.1007/s12016-007-0034-7 [doi]

Takahashi, T., Kimura, Y., Saito, R., et al. (2011), An in vitro test to screen skin sensitizers using a stable THP-1-derived IL-8 reporter cell line, THP-G8. *Toxicol Sci* 124: 359-369, 10.1093/toxsci/kfr237

Truneh, A., Albert, F., Golstein, P., et al. (1985), Calcium ionophore plus phorbol ester can substitute for antigen in the induction of cytolytic T lymphocytes from specifically primed precursors. *J Immunol* 135: 2262-2267,

Wagner, W., Walczak-Drzewiecka, A., Slusarczyk, A., et al. (2006), Fluorescent Cell Chip a new in vitro approach for immunotoxicity screening. *Toxicol letters* 162: 55-70, 10.1016/j.toxlet.2005.10.017

W.H.O., Meeting, I.P.o.C.S.I.S., (2012), Guidance for immunotoxicity risk assessment for chemicals. WHO press,

Zhang, H., Sun, S.C. (2015), NF-kappaB in inflammation and renal diseases. *Cell Biosci* 5: 63, 10.1186/s13578-015-0056-4

14. List of abbreviations

95% CI : the 95% confidence interval

AIST : National Institute of Advanced Industrial Science and Technology

AOP : Adverse outcome pathway

ARE: Antioxidant response element

CAS No. : Chemical Abstract Service Number

CMV : Cytomegalovirus

CSC : the Chemical Selection Committee

DMSO : Dimethyl sulphoxide

DPRA : the Direct Peptide Reactivity Assay

ECVAM : the European Centre for Validation of Alternative Methods

EDTA : Ethylenediaminetetraacetic acid

EGFR : Epidermal growth factor receptor

EGR-1 : Early growth response-1

EU : European Union

FBS : Fetal bovine serum

FN : False Negative Rate

GLP : Good laboratory Practice

GSH : Glutathione

HRI/FDSC : Hatano Research Institute, Food & Drug Safety Center

HSV : Herpes simplex viruses

ICCVAM : Interagency Coordinating Committee on the Validation of Alternative Methods

ID : Identification

IFN-γ : Interferon-γ

Inh-GAPLA : Inhibition index of GAPLA

IL-2 : Interleukin-2

IL-8 : Interleukin-8

JaCVAM : the Japanese Center for the Validation of Alternative Methods

Keap-1 : Kelch-like ECH-associated protein 1

KoCVAM : Korean Center for the Validation of Alternative Methods

LLNA : Local lymph node assay

LPS : Lipopolysaccharide

MIT : Minimum induction threshold

MITA : Multi-Immuno Tox Assay

mMUSST : modified myeloid U937 dendritic cell activation test

MoDCs : Monocyte-derived dendritic cells

MOVS: Management Office of Validation Study

mRNA : messenger ribonucleic acid

MSDS : Material safety data sheet

NICEATM : the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

NIHS : National Institute of Health Sciences

NPV : Negative predictive value

Nqo1 : NADPH-quinone oxidoreductase 1

Nrf2 : Nuclear factor (erythroid-derived 2)-like factor 2

nIL2LA : normalized IL2LA

nIFNLA : normalized IFNLA

OECD : the Organization for Economic Co-operation and Development

PCR : Polymerase chain reaction

PI : Propidium iodide

PMA/Io : Phorbol 12-myristate 13-acetate/Ionomycin

PN : False Positive Rate

PPV : Positive Predictive Value

QC : Quality Control

REACH : Registration, Evaluation, Authorization and Restriction of CHemicals

RFI : Relative fluorescence intensity

RT : Ring trial

SLG : Stable luciferase green

IL2LA : SLG luciferase activity

SLO : Stable luciferase orange

IFNLA : SLO luciferase activity

SLR : Stable luciferase red

GAPLA : SLR luciferase activity

SLS : Sodium lauryl sulfate

SLR : Stable luciferase red

SLR-LA : SLR luciferase activity

SV40 : Simian virus 40

TG : Test Guideline

TNF-α : Tumor necrosis factor-α

UN GHS : United Nations Globally Harmonized System of Classification and Labeling of Chemicals

VMT : Validation Management Team

15. Appendixes

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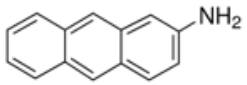
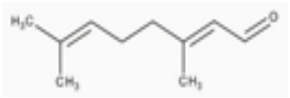
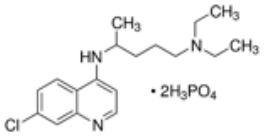
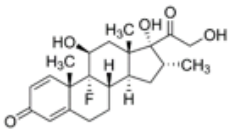
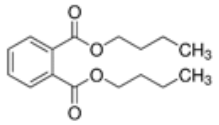
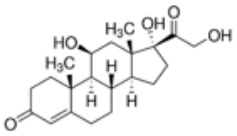
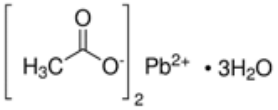
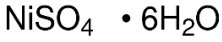
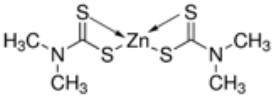
15. Appendixes

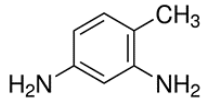
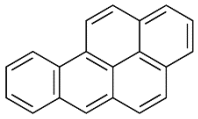
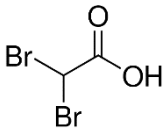
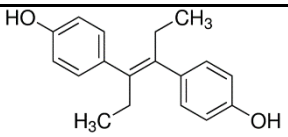
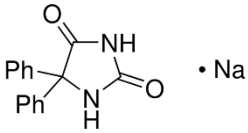
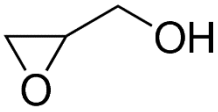
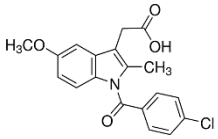
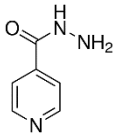
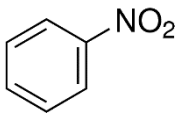
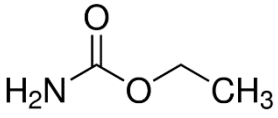
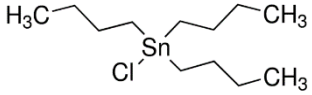
Appendix 1. Chemical structure of the test chemicals for Phase 0 study

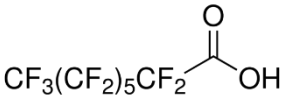
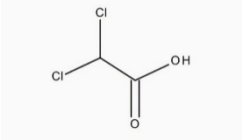
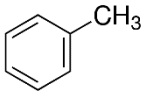
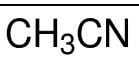
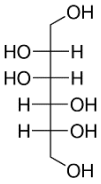
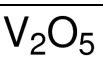
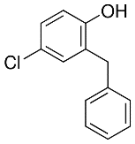
No.	Chemical name	CAS No.	Molecular weight	Chemical structure
0-1	2-Aminoanthracene	613-13-8	193.24	
0-2	Citral	5392-40-5	152.23	
0-3	Chloroquine diphosphate salt	50-63-5	515.86	
0-4	Dexamethasone	50-02-2	392.46	
0-5	Methylmercury(II) chloride	115-09-3	251.08	CH_3HgCl

Appendix 2. Chemical structure of the test chemicals for the Phase I study

No.	Chemical name	CAS No.	Molecular weight	Chemical structure
I-1	Dibutyl phthalate	84-74-2	278.34	
I-2	Hydrocortisone watersoluble	50-23-7	362.46	
I-3	Lead(II)acetate	6080-56-4	379.33	
I-4	Nickel sulfate hexahydrate	10101-97-0	262.85	
I-5	Dimethyldithiocarbamate	137-30-4	305.82	

Appendix 3. Chemical structure of the test chemicals for the Phase II study

No.	Chemical name	CAS No.	Molecular weight	Chemical structure
II-1	2,4-Diaminotoluene	95-80-7	122.17	
II-2	Benzo(a)pyrene	50-32-8	252.31	
II-3	Cadmium chloride	10108-64-2	183.32	$CdCl_2$
II-4	Dibromoacetic acid	631-64-1	217.84	
II-5	Diethylstilbestrol	56-53-1	268.35	
II-6	Diphenylhydantoin	630-93-3	274.25	
II-7	Ethylene dibromide	106-93-4	187.86	$BrCH_2CH_2Br$
II-8	Glycidol	556-52-5	74.08	
II-9	Indomethacin	53-86-1	357.79	
II-10	Isoniazid	54-85-3	137.14	
II-11	Nitrobenzene	98-95-3	123.11	
II-12	Urethane, Ethyl carbamate	51-79-6	89.09	
II-13	Tributyltin chloride	1461-22-9	325.51	

II-14	Perfluorooctanoic acid	335-67-1	414.07	
II-15	Dichloroacetic acid	79-43-6	128.94	
II-16	Toluene	108-88-3	92.14	
II-17	Acetonitrile	75-05-8	41.05	
II-18	Mannitol	69-65-8	182.17	
II-19	Vanadium pentoxide	1314-62-1	181.88	
II-20	o-Benzyl-p-chlorophenol	120-32-1	218.68	

Appendix 4. Protocol of the Multi-Immuno Tox Assay (ver. 011E)

Multi-Immuno Tox Assay protocol ver. 011E

May. 10th, 2018

Department of Dermatology, Tohoku University Graduate School of Medicine

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1. Introduction

This protocol describes how to maintain the cells, how to prepare the test chemicals, and how to measure the luciferase activity of 2H4 cells transfected with 3 luciferase genes, stable luciferase green (SLG), stable luciferase orange (SLO) and stable luciferase red (SLR), under the control of IL-2, IFNγ and G3PDH promoters, respectively, for the Multi-Immuno Tox Assay.

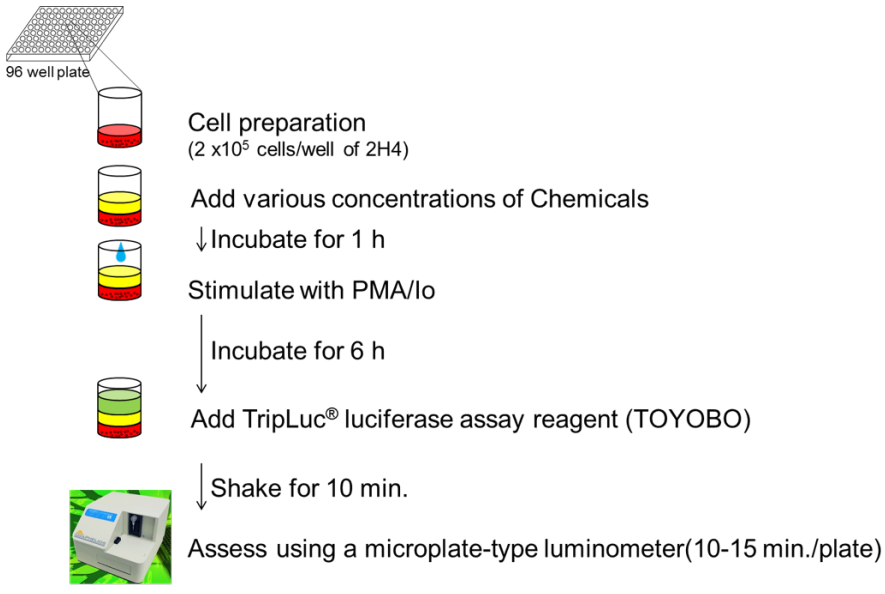
(Kimura Y. et al. Evaluation of the Multi-Immuno Tox Assay composed of 3 human cytokine reporter cells by examining immunological effects of drugs *Toxicol in Vitro*, 28, 759-768, 2014)

Figure 1

Assay design (2 chemicals per one plate)

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	cont (distilled water or DMSO)	PMA/Io only	A/2^9 μg/ml	A/2^8 μg/ml	A/2^7 μg/ml	A/2^6 μg/ml	A/2^5 μg/ml	A/2^4 μg/ml	A/2^3 μg/ml	A/2^2 μg/ml	A/2^1 μg/ml	A μg/ml
B												
C												
D			Chemical A (common ratio of 2、10 concentrations、n=4)									
E	cont (distilled water or DMSO)	PMA/Io only	B/2^9 μg/ml	B/2^8 μg/ml	B/2^7 μg/ml	B/2^6 μg/ml	B/2^5 μg/ml	B/2^4 μg/ml	B/2^3 μg/ml	B/2^2 μg/ml	B/2^1 μg/ml	B μg/ml
F												
G												
H			Chemical B (common ratio of 2、10 concentrations、n=4)									

PMA/Io or LPS



2. Materials

2-1 Cells

2H4 (IL2-SLG、IFNγ-SLO、G3PDH-SLR)

The human acute T lymphoblastic leukemia cell line Jurkat was obtained from the American Type Culture Collection (Manassas, VA, USA). A Jurkat-derived IL-2 and IFNγ reporter cell line, 2H4,

that harbors the SLG, SLO and SLR luciferase genes under the control of the IL-2, IFNγ and GAPDH promoters, respectively, was established by Tsuruga Institute of Biotechnology, TOYOBO Co. Ltd. (Saito R. et al. Nickel differentially regulates NFAT and NF-κB activation in T cell signaling *Toxicology and Applied Pharmacology,* 254, 245–255, 2011)

2-2 Reagents and equipment

2-2-1 For maintenance of the 2H4 cells

RPMI-1640 (GIBCO Cat#11875-093, 500 mL)

FBS (Biological Industries Cat#04-001-1E Lot: 715004)

Antibiotic-Antimycotic (GIBCO Cat#15240-062)

HygromycinB (CAS:31282-04-9, Invitrogen Cat#10687-010)

G418 (CAS:108321-42-2, Nacalai Tesque Cat#16513-84)

Puromycin (CAS:58-58-2, InvivoGen Cat#ant-pr-1)

2-2-2 For chemical exposure, stimulation and solvents

Ionomycin (CAS:56092-82-1, Sigma Cat#I0634)

Phorbol 12-myristate 13-acetate (PMA) (CAS:16561-29-8, Sigma Cat#P8139)

Ethanol (e.g., Wako Cat#057-00456)

Dimethyl sulfoxide (DMSO) (CAS:67-68-5, Sigma Cat#D5879)

Distilled water (GIBCO Cat#10977-015)

2-2-3 For measurement of the luciferase activity

Triplu[®] Luciferase assay reagent (TOYOBO Cat#MRA-301)

2-2-4 Expendable supplies

T-75 flask tissue culture treated (e.g., Corning Cat#353136)

96 well μ clear black plate (flat-bottom, for measurement of the luciferase activity, e.g. Greiner Bio-one Cat#655090)

96 well clear plate (round-bottom, for preparation of chemicals and stimulants)

96 well assay block, 2 mL (e.g., Costar Cat#3960)

Seal for 96 well plate (e.g., Perkin Elmer TopSeal-A PLUS Cat#6050185, EXCEL Scientific SealMate Cat#SM-KIT-SP)

Reservoir

Pipette

2-2-5 Equipment for measurement of luciferase activity

Measuring device: a microplate-type luminometer with a multi-color detection system that can accept two optical filter

e.g. Phelios AB-2350 (ATTO), ARVO (PerkinElmer), Tristar LB941 (Berthold)

Optical filter: 560 nm long-pass filter and 600 nm long-pass filter

Measuring time: set at 1～5 sec/well measuring time

2-2-6 Others

Pipetman

8 channel or 12 channel pipetman (optimized for 10~100 μL)

Plate shaker (for 96 well plate)

CO_2 incubator (37°C, 5% CO_2)

Water bath

Cell counter: hemocytometer, trypan blue

2-3 Culture medium

2-3-1 A medium: for maintenance of 2H4 cells (500 mL, stored at 2-8°C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	440 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	50 mL
Antibiotic-Antimycotic	GIBCO #15240-062	100×	1×	5 mL
Puromycin	InvivoGen # ant-pr-1	10 mg/mL	0.15 μg/mL	7.5 μL
G418	Nacalai Tesque #16513-84	50 mg/mL	300 μg/mL	3 mL
HygromycinB	Invitrogen #10687-010	50 mg/mL	200 μg/mL	2 mL

2-3-2 B medium: for luciferase assay (30 mL, stored at 2-8°C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	27 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	3 mL

2-3-3 C medium: for thawing 2H4 cells (30 mL, stored at 2-8°C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	26.7 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	3 mL
Antibiotic-Antimycotic	GIBCO #15240-062	100×	1×	0.3 mL

2-4 Preparation of the stimulant of 2H4

2-4-1 Phorbol 12-myristate 13-acetate (PMA)

Reagent	Company	Concentration of the stock solution	Final concentration
Phorbol 12-myristate 13-acetate (PMA)	Sigma #P8139	2 mM	25 nM
DMSO	Sigma #D5789		

Dissolve 1 mg PMA using DMSO 811 μL, dispend at 5 μL/tube and store at freezer at -30°C. Use these stocks within 6 months after dissolution.

2-4-2 Ionomycin

Reagent	Company	Concentration of the stock solution	Final concentration
Ionomycin	Sigma # I0634	2 mM	1 μM
Ethanol	Wako #057-00456		

Dissolve 1mg Ionomycin using ethanol 669.3 μL, dispend at 30 μL/tube and store at freezer at -30°C. Use these stocks within 6 months after dissolution.

3. Cell culture

3-1 Thawing of 2H4 cells

Pre-warm 9 mL of C medium in a 15 mL polypropylene conical tube in a 37°C water bath (for centrifugation) and 15 mL of C medium in a T-75 Flask at 37°C in a 5% CO_2 incubator (for culture).

Thaw frozen cells ($2x10^6$ cells / 0.5 mL of freezing medium) in a 37°C water bath, then add to a 15 mL polypropylene conical tube containing 9 mL of pre-warmed C medium. Centrifuge the tube at 120-350 x *g* at room temperature for 5 min, discard the supernatant, and resuspend in 15 mL of pre-warmed C medium in a T-75 Flask. Cells are incubated at 37°C, 5% CO_2.

3-2 Maintenance of 2H4 cells

Pre-warm the A medium in a T-75 Flask at 37°C in a 5% CO_2 incubator. The culture medium should be changed to the A medium 3 or 4 days after thawing. At that time, count the number of cells, centrifuge the tube at 120-350 x *g* at room temperature for 5 min, discard the supernatant, and resuspend in pre-warmed the A medium in a T-75 Flask. Cells are passaged at $3x10^5$/mL and incubated at 37°C, 5% CO_2.

The interval between subcultures should be 3~4 days. Cells can be used between one and six weeks after thawing.

4. Preparation of cells for assay

A cell passage should be done 2-4 days before the assay.

Use cells between 1 and 6 weeks after thawing.

Pre-warm the B medium in a 37°C water bath. Count the number of cells and collect the number of cells needed (2.0 x 10^7 cells for two chemicals are required, but to have some leeway, 3.0 x 10^7 cells for two chemicals should be prepared), centrifuge the tube at 120-350 x *g*, 5 min. Resuspend in pre-warmed the B medium at a cell density of 4×10^6/mL. Transfer the cell suspension to a reservoir, and add 50 µL of cell suspension to each well of a 96 well µclear black plate (flat bottom) using an 8 channel or 12 channel pipetman. (cf. Figure 2)

Figure 2

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
B	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
C	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
D	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
E	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
F	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
G	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
H	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL

5. Preparation of chemicals and cell treatment with chemicals

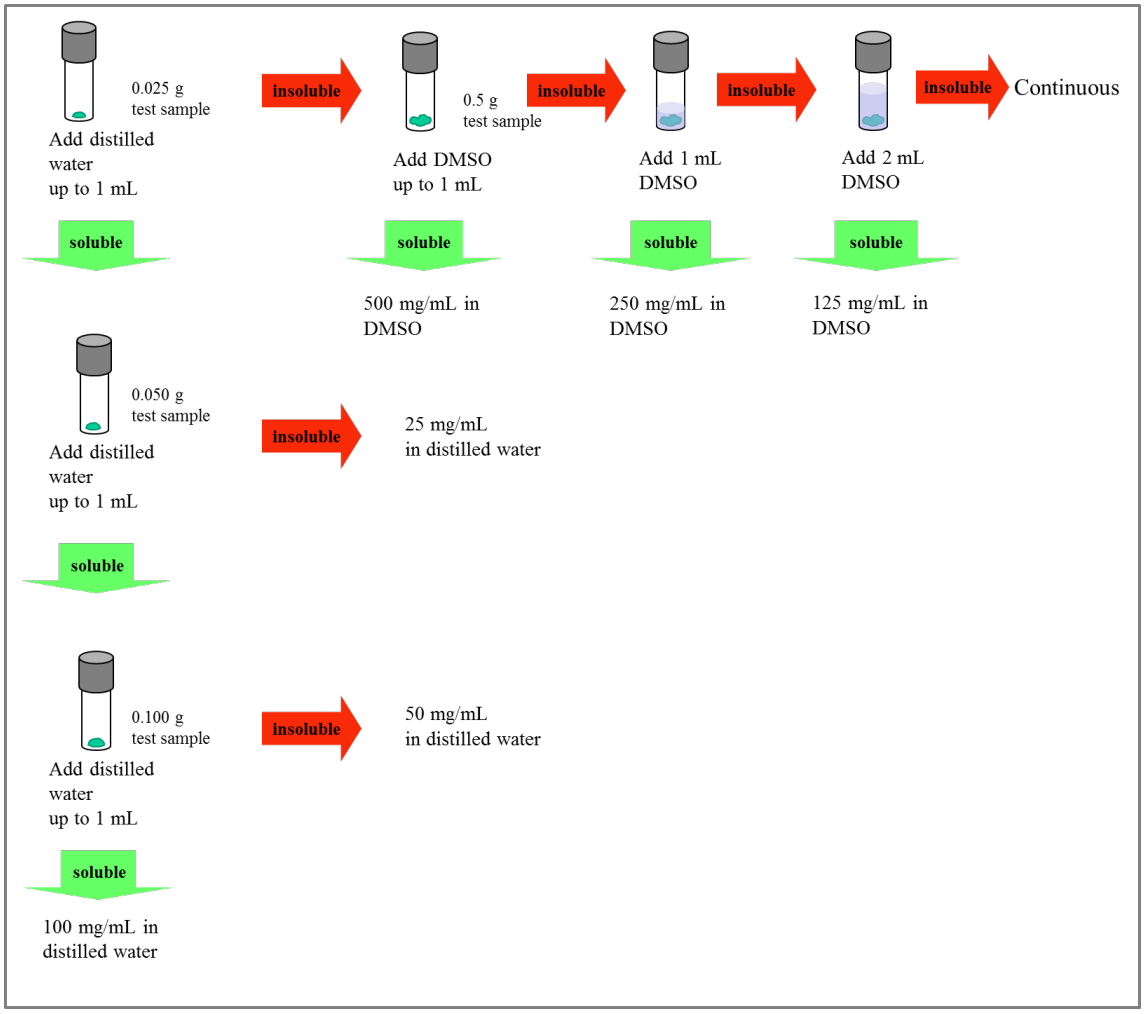
5-1 Dissolution by vehicle (cf. Figure 3)

Dissolve the chemical first in distilled water. Namely, weigh 0.025 g of the test chemical in a volumetric flask and add distilled water up to 1 mL. If the chemical is soluble at 25 mg/mL, weigh 0.050 g of the test chemical in a volumetric flask and add distilled water up to 1 mL. If the chemical is not soluble at 50 mg/mL, 25 mg/mL is the highest soluble concentration. If the chemical is soluble at 50 mg/mL, weigh 0.100 g of the test chemical in a volumetric flask and add distilled water up to 1 mL. If the chemical is not soluble at 100 mg/mL, 50 mg/mL is the highest soluble concentration. If the chemical is soluble at 100 mg/mL, 100 mg/mL is the highest soluble concentration.

If the chemical is not soluble in water, the chemical should be dissolved in DMSO at 500 mg/mL. Namely, weigh 0.5 g of the test chemical in volumetric flask and add DMSO up to 1 mL.
If the chemical is not soluble at 500 mg/mL, the highest soluble concentration should be determined by diluting the solution from 500 mg/mL at a common ratio of two (250 mg/mL → 125 mg/mL → continued if needed) with DMSO.

Sonication and vortex may be used if needed, and attempt to dissolve the chemical for at least 5 minutes. Being soluble should be confirmed by centrifugation at 15,000 rpm (≈20,000 x g) for 5 min and absence of precipitation. The chemical should be used within 4 hours after being dissolved in distilled water or DMSO.

Figure 3



In the first experiment (1st experiment), when the chemical is prepared in distilled water, conduct 10 serial dilutions at a common ratio of 2 from the highest concentration using distilled water. When the chemical is prepared as a DMSO solution, conduct 10 serial dilutions at a common ratio of 2 from the highest concentration using DMSO.

In the second to fourth experiment (2nd to 4th experiment), determine the minimum concentration at which I.I.-SLR-LA (mentioned later in **10**) became lower than 0.05 in the 1st experiment, use the concentration one step (2-times) higher than this determined concentration as the highest concentration of the chemical to examine, and conduct 10 serial dilutions at a common ratio of 2 from the highest concentration. If I.I.-SLR-LA did not become lower than 0.05 or became lower than 0.05 at the highest concentration in the 1st experiment, conduct 10 serial dilutions at a common ratio of 2 from the highest concentration in the 1st experiment.

For example, in Figure 3 below, the minimum concentration at which I.I.-SLR-LA became lower than 0.05 is 1.95 μg/ml. The highest concentration of the chemical to examine is the concentration one step (2-times) higher than 1.95 μg/ml, which is 3.91 μg/ml.

In Figure 4 below, I.I.-SLR-LA did not become lower than 0.05. In such a case, the highest concentration of the chemical to examine is the highest concentration in the 1st experiment, namely 125 μg/ml.

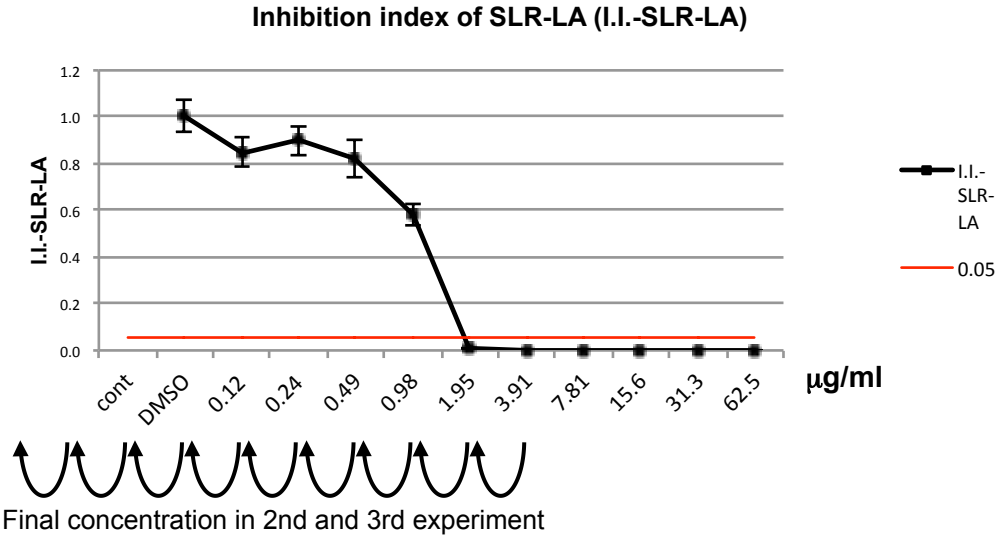


Figure 3.

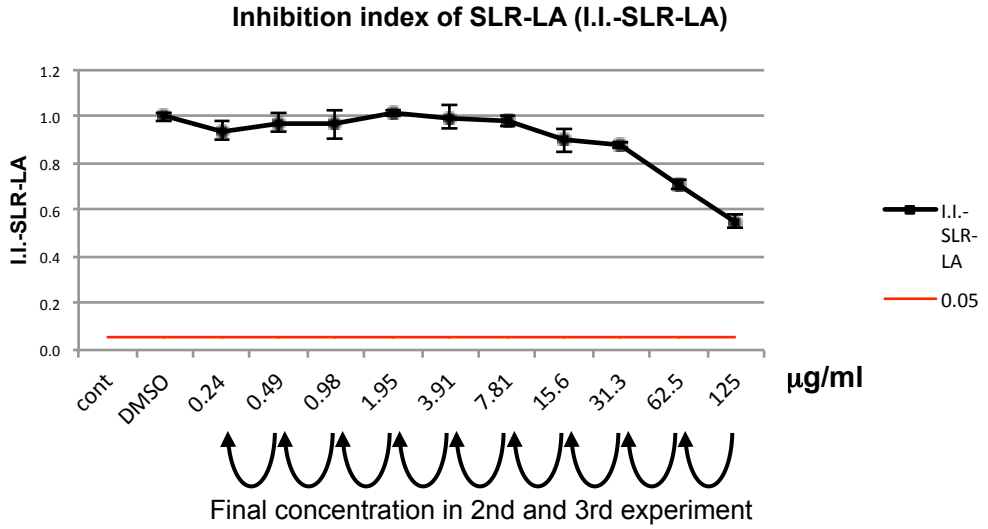


Figure 4

5-2 When the chemical is prepared in distilled water

If the chemical is prepared at a lower concentration, use the prepared concentration instead of the 100 mg/mL distilled water solution.

5-2-1 Arrangement of chemicals and vehicle

Add 100 µL of the 100 mg/mL distilled water solution of the chemical to well #A12, and 50 µL of the distilled water to wells #A1-#A11 of the 96 well clear plate (round bottom).

5-2-2 Serial dilution

Conduct 9 serial dilutions at a common ratio of 2 as indicated in Figure 4 from well #A11 to well #A3. Transfer 50 µL to the next (left) well. (cf. Figure 4)

Figure 4

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Chemical 100 mg/mL in distiled water 100 uL
B												
C												
D												
E												
F												
G												
H												

2-fold dilution : transfer 50 uL（pipetman、yellow tip）

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 50uL	Distilled water 50uL	Chemical 0.2 mg/mL in distilled water 100uL	Chemical 0.4 mg/mL in distilled water 50uL	Chemical 0.8 mg/mL in distilled water 50uL	Chemical 1.6 mg/mL in distilled water 50uL	Chemical 3.1 mg/mL in distilled water 50uL	Chemical 6.3 mg/mL in distilled water 50uL	Chemical 13 mg/mL in distilled water 50uL	Chemical 25 mg/mL in distilled water 50uL	Chemical 50 mg/mL in distilled water 50uL	Chemical 100 mg/mL in distilled water 50uL
B												
C												
D												
E												
F												
G												
H												

5-2-3　2 step dilution

Add 20 µL of the diluted chemical to 480 µL of the B medium prepared in the assay block. And add 50 µL to 2H4 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 1 hour (37°C, CO_2, 5%) (cf. Figure 5-7).

Figure 5

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 50uL	Distilled water 50uL	Chemical 0.2 mg/mL in distilled water 100uL	Chemical 0.4 mg/mL in distilled water 50uL	Chemical 0.8 mg/mL in distilled water 50uL	Chemical 1.6 mg/mL in distilled water 50uL	Chemical 3.1 mg/mL in distilled water 50uL	Chemical 6.3 mg/mL in distilled water 50uL	Chemical 13 mg/mL in distilled water 50uL	Chemical 25 mg/mL in distilled water 50uL	Chemical 50 mg/mL in distilled water 50uL	Chemical 100 mg/mL in distilled water 50uL
B												
C												
D												
E												
F												
G												
H												

20uL

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL
B												
C												
D												
E												
F												
G												
H												

Figure 6

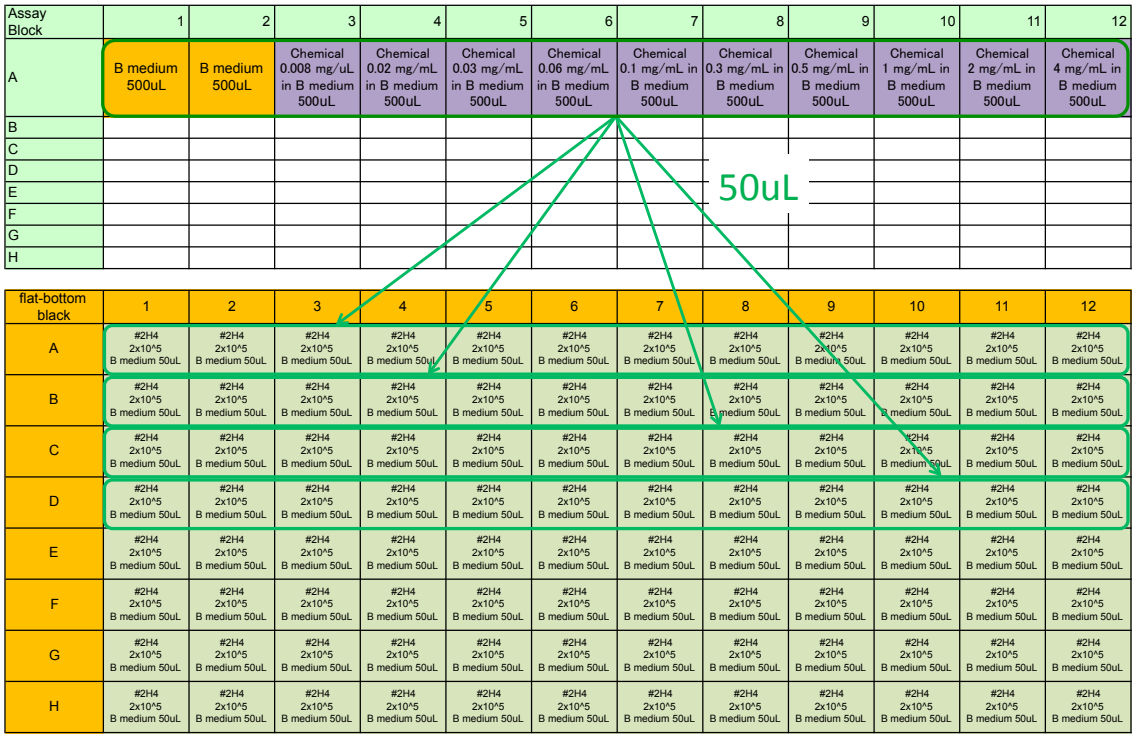


Figure 7 Final constituents of each well of the plate

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	Chemical 0 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.004 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.008 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.02 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.03 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.06 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.1 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.3 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.5 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 1 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 2 mg/mL #2H4 2x10^5 B medium 100uL
B	Chemical 0 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.004 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.008 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.02 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.03 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.06 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.1 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.3 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.5 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 1 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 2 mg/mL #2H4 2x10^5 B medium 100uL
C	Chemical 0 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.004 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.008 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.02 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.03 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.06 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.1 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.3 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.5 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 1 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 2 mg/mL #2H4 2x10^5 B medium 100uL
D	Chemical 0 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.004 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.008 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.02 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.03 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.06 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.1 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.3 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.5 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 1 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 2 mg/mL #2H4 2x10^5 B medium 100uL
E	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
F	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
G	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
H	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL

5-3 When the chemical is prepared as a DMSO solution

<u>If the chemical is prepared at a lower concentration, use the prepared concentration instead of 500 mg/mL DMSO solution.</u>

5-3-1 Arrangement of chemicals and vehicle

Add 100 μL of the 500 mg/mL DMSO solution of the chemical to well #A12, 50 μL of DMSO to wells #A1-#A11, and 90 μL of the B medium to wells #B1-#B12 of the 96 well clear plate (round bottom)

5-3-2 Serial dilution

Conduct 9 serial dilutions at a common ratio of 2 as indicated in Figure 8 from well #A11 to well #A3. Transfer 50 μL to the next (left) well. (cf. Figure 8)

Figure 8

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	Chemical 500 mg/mL in DMSO 100uL
B	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL
C												
D												
E												
F												
G												
H												

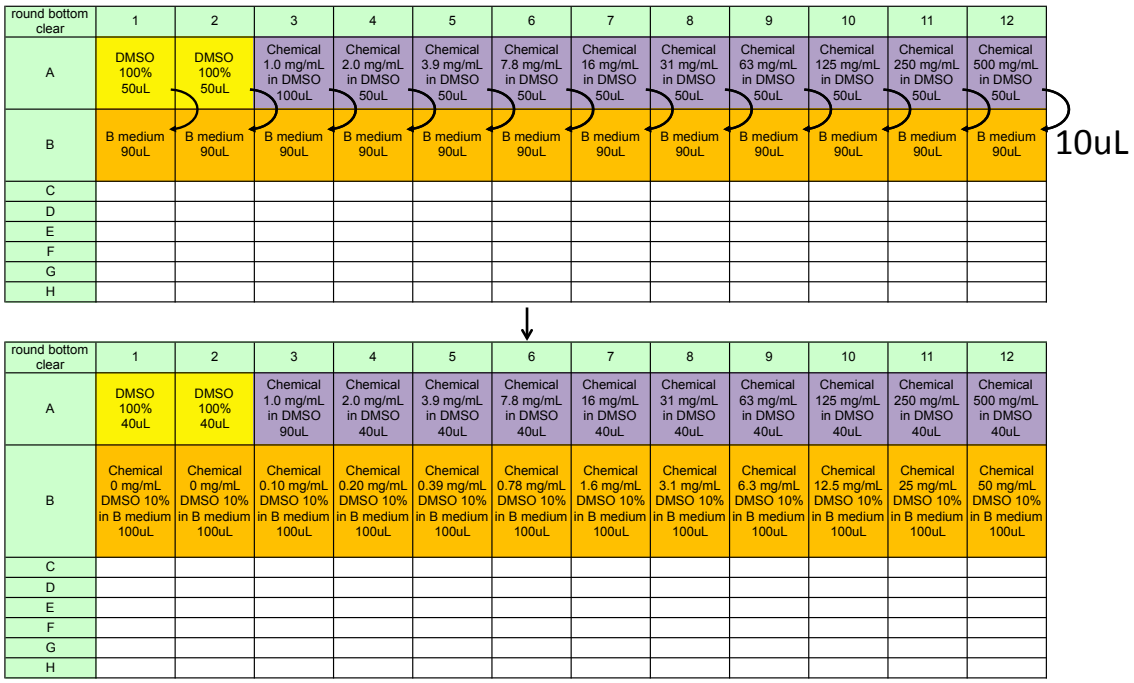
2-fold dilution : transfer 50 uL（pipetman、yellow tip）

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 50uL	DMSO 100% 50uL	Chemical 1.0 mg/mL in DMSO 100uL	Chemical 2.0 mg/mL in DMSO 50uL	Chemical 3.9 mg/mL in DMSO 50uL	Chemical 7.8 mg/mL in DMSO 50uL	Chemical 16 mg/mL in DMSO 50uL	Chemical 31 mg/mL in DMSO 50uL	Chemical 63 mg/mL in DMSO 50uL	Chemical 125 mg/mL in DMSO 50uL	Chemical 250 mg/mL in DMSO 50uL	Chemical 500 mg/mL in DMSO 50uL
B	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL
C												
D												
E												
F												
G												
H												

5-3-3 Dilution of DMSO solution with the B medium

Dilute 10 μL of the DMSO solution of the chemical in wells #A1-#A12 with 90 μL of the B medium using an 8-12 channel pipetman. (cf. Figure 9)

Figure 9



5-3-4　2 step dilution

Add 10 μL of the diluted chemical to 490 μL of the B medium prepared in the assay block. And add 50 μL to 2H4 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Manipulate the procedures from 5-3-3 to 5-3-4 as quickly as you can, and do not leave a long time at step after 5-3-3 or Figure 10. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 1 hour (37°C, CO_2, 5%) (cf. Figure 10-12).

Figure 10

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 40uL	DMSO 100% 40uL	Chemical 1.0 mg/mL in DMSO 90uL	Chemical 2.0 mg/mL in DMSO 40uL	Chemical 3.9 mg/mL in DMSO 40uL	Chemical 7.8 mg/mL in DMSO 40uL	Chemical 16 mg/mL in DMSO 40uL	Chemical 31 mg/mL in DMSO 40uL	Chemical 63 mg/mL in DMSO 40uL	Chemical 125 mg/mL in DMSO 40uL	Chemical 250 mg/mL in DMSO 40uL	Chemical 500 mg/mL in DMSO 40uL
B	Chemical 0 mg/mL DMSO 10% in B medium 100uL	Chemical 0 mg/mL DMSO 10% in B medium 100uL	Chemical 0.10 mg/mL DMSO 10% in B medium 100uL	Chemical 0.20 mg/mL DMSO 10% in B medium 100uL	Chemical 0.39 mg/mL DMSO 10% in B medium 100uL	Chemical 0.78 mg/mL DMSO 10% in B medium 100uL	Chemical 1.6 mg/mL DMSO 10% in B medium 100uL	Chemical 3.1 mg/mL DMSO 10% in B medium 100uL	Chemical 6.3 mg/mL DMSO 10% in B medium 100uL	Chemical 12.5 mg/mL DMSO 10% in B medium 100uL	Chemical 25 mg/mL DMSO 10% in B medium 100uL	Chemical 50 mg/mL DMSO 10% in B medium 100uL
C												
D												
E												
F												
G												
H												

10uL

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL
B												
C												
D												
E												
F												
G												
H												

Figure 11

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	Chemical 0ug/mL DMSO 0.2% in B medium 500uL	Chemical 0ug/mL DMSO 0.2% in B medium 500uL	Chemical 2.0ug/mL DMSO 0.2% in B medium 500uL	Chemical 3.9ug/mL DMSO 0.2% in B medium 500uL	Chemical 7.8ug/mL DMSO 0.2% in B medium 500uL	Chemical 15.6ug/mL DMSO 0.2% in B medium 500uL	Chemical 31.3ug/mL DMSO 0.2% in B medium 500uL	Chemical 62.5ug/mL DMSO 0.2% in B medium 500uL	Chemical 125ug/mL DMSO 0.2% in B medium 500uL	Chemical 250ug/mL DMSO 0.2% in B medium 500uL	Chemical 500ug/mL DMSO 0.2% in B medium 500uL	Chemical 1000ug/mL DMSO 0.2% in B medium 500uL
B												
C												
D												
E												
F												
G												
H												

50uL

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
B	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
C	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
D	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
E	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
F	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
G	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
H	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL

Figure 12 Final constituents of each well of the plate

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	Chemical 0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 1.0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 2.0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 3.9ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 7.8ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 16ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 31ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 63ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 125ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 250ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 500ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL
B	Chemical 0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 1.0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 2.0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 3.9ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 7.8ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 16ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 31ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 63ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 125ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 250ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 500ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL
C	Chemical 0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 1.0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 2.0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 3.9ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 7.8ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 16ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 31ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 63ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 125ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 250ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 500ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL
D	Chemical 0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 1.0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 2.0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 3.9ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 7.8ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 16ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 31ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 63ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 125ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 250ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 500ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL
E	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
F	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
G	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
H	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL

6. Preparation of the stimulant (PMA/ionomycin) and addition to 2H4

6-1 Material

・2 mM PMA stock

・2 mM Ionomycin stock

・B medium

・Ethanol

6-2 Preparation of 100 μM PMA

Dilute 2 mM PMA stock with the B medium as follows (20 times, final concentration is 100 μM).

2 mM PMA	B medium	Total	final concentration
5 μL	95 μL	100 μL	100 μM

6-3 Preparation of control and x10 PMA/ionomycin solution

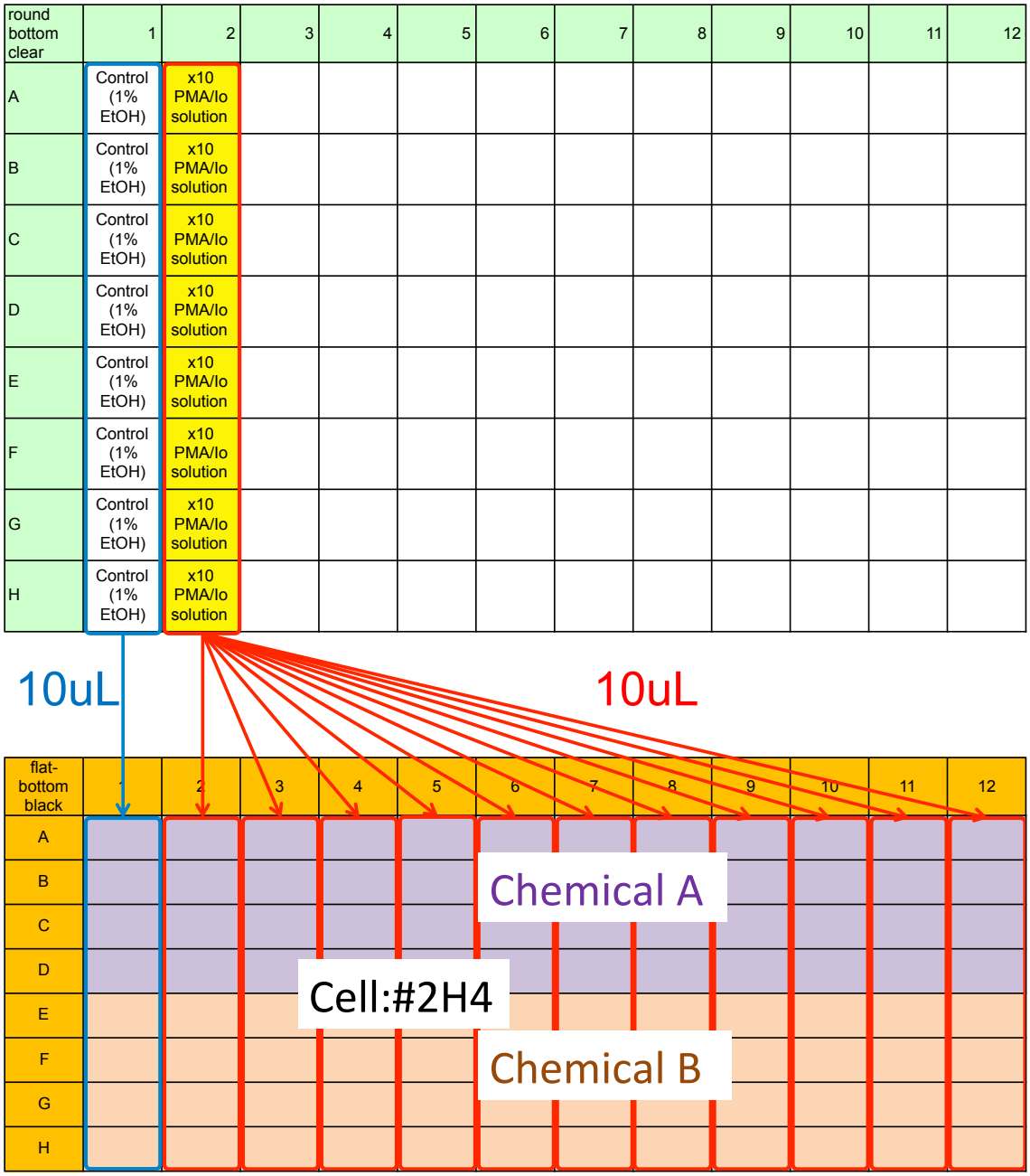
Dilute ethanol, 2 mM ionomycin and 100 μM PMA with the B medium to prepare control or x10 PMA/ionomycin solution. Add the control to well #A1-#H1 of the 96 well clear plate (round bottom), and add x10 PMA/ionomycin solution to wells #A2-#H2 of the 96 well clear plate (round bottom).

	B medium	2 mM Ionomycin	100 μM PMA	Ethanol	Total
Control	995 μL	-		5 μL	1000 μL
x10 PMA/ionomycin solution	2382 μL	12 μL	6 μL	-	2400 μL

6-4 Addition of PMA/ionomycin to 2H4

One hour after the addition of chemicals, add 10 μL of control or PMA/ionomycin solution to the cells (#A1-#H1 or #A2-#H12, respectively) using an 8 channel or 12 channel pipetman after pipetting 20 times. Make sure that the apex of the tip is dipped into the medium. Change tips every line you add. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 6 hour (37°C, CO_2, 5%). (cf. Figure 13)

Figure 13



7. Control

7-1 Preparing control chemical (dexamethasone, cyclosporine A)

7-1-1 Preparing dexamethasone stock

Reagent	Company	Concentration of the stock solution	Preparing concentration	Final concentration
Dexamethasone-water soluble	Sigma #D2915-100MG	2.5 mg/mL	2.5 mg/mL	50 μg/mL
Distilled water	GIBCO Cat#10977-015			

Dissolve 100 mg of Dexamethasone-water soluble with distilled water 40 mL, dispend at 50 μL/tube and store a freezer at -30°C.

7-1-2 Preparing cyclosporine A stock

Reagent	Company	Concentration of the stock solution	Preparing concentration	Final concentration
Cyclosporine A	Sigma #C1832-5MG	100 μg/mL	100 μg/mL	100 μg/mL
DMSO	Sigma #D5789			

Dissolve 5 mg of cyclosporine A with DMSO 50 mL, dispend at 50 μL/tube and store a freezer at -30°C.

7-2 Preparation of cells for assay

A cell passage should be done 2-4 days before the assay.

Use cells between 1 and 6 weeks after thawing.

Pre-warm the B medium in a 37°C water bath. Count the number of cells and collect the number of cells needed (5.0 x 10^6 cells are required, but to have some leeway, 7.5 x 10^6 cells should be prepared), centrifuge the tube at 120-350 x *g*, 5 min. Resuspend in pre-warmed the B medium at a cell density of 4×10^6/mL. Transfer the cell suspension to a reservoir, and add 50 µL of cell suspension to each well of a 96 well µclear black plate (flat bottom) using an 8 channel or 12 channel pipetman. (cf. Figure 14)

Figure 14

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
B	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
C	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
D	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
E												
F												
G												
H												

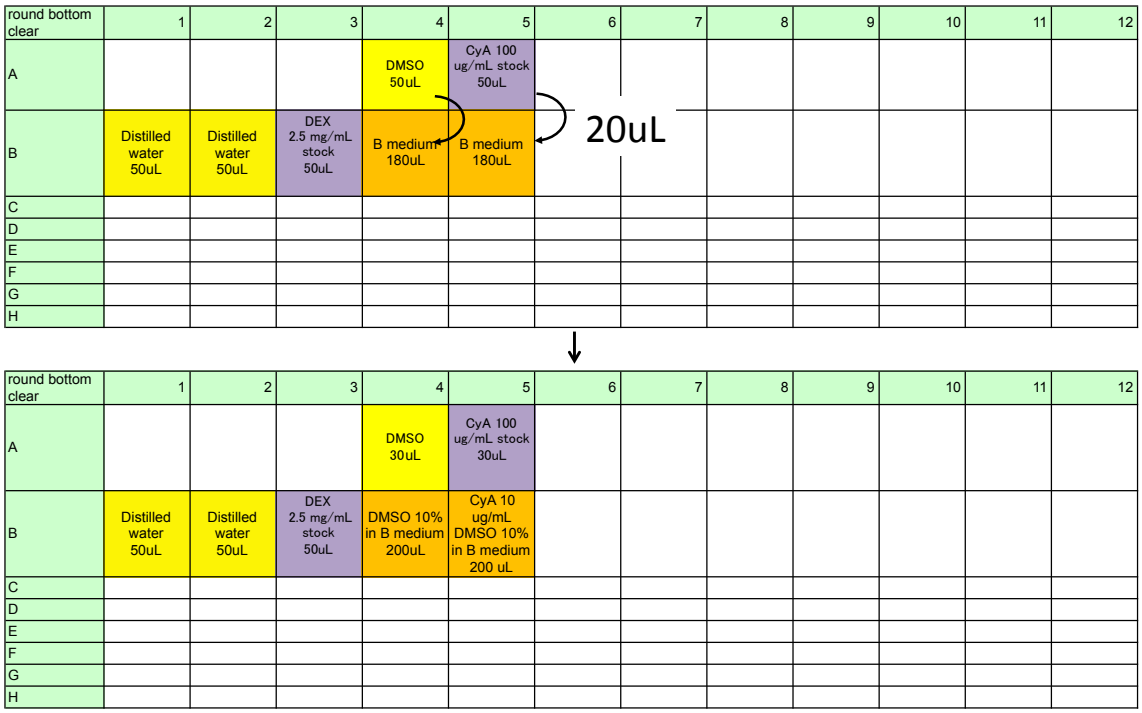
7-3 Arrangement of chemicals and vehicle

Add DMSO 50 µL to #A4, 100 µg/mL cyclosporine A stock 50 µl to #A5, distilled water 50 µl to #B1 and #B2, 2.5 mg/ml dexamethasone stock 50 µl to #B3 and the B medium 180 µl to #B4 and #B5 of the 96 well clear plate (round bottom). (cf. Figure 15)

7-4 Dilution with the B medium

Dilute DMSO in #A4 and cyclosporine A DMSO solution in #A5 by adding 20 µL to the B medium in #B4 and #B5, respectively. (cf. Figure 15)

Figure 15



7-5　　2 step dilution

Add 20 μL of the diluted chemical or vehicle to 480 μL (1-3 lanes) or 980 μL (4-5 lanes) of the B medium prepared in the assay block. And add 50 μL to 2H4 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Manipulate the procedures from 7-4 to 7-5 as quickly as you can, and do not leave a long time at step after 7-4 or Figure 16. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 1 hour (37°C, CO_2, 5%). (cf. Figure 16-18)

Figure 16

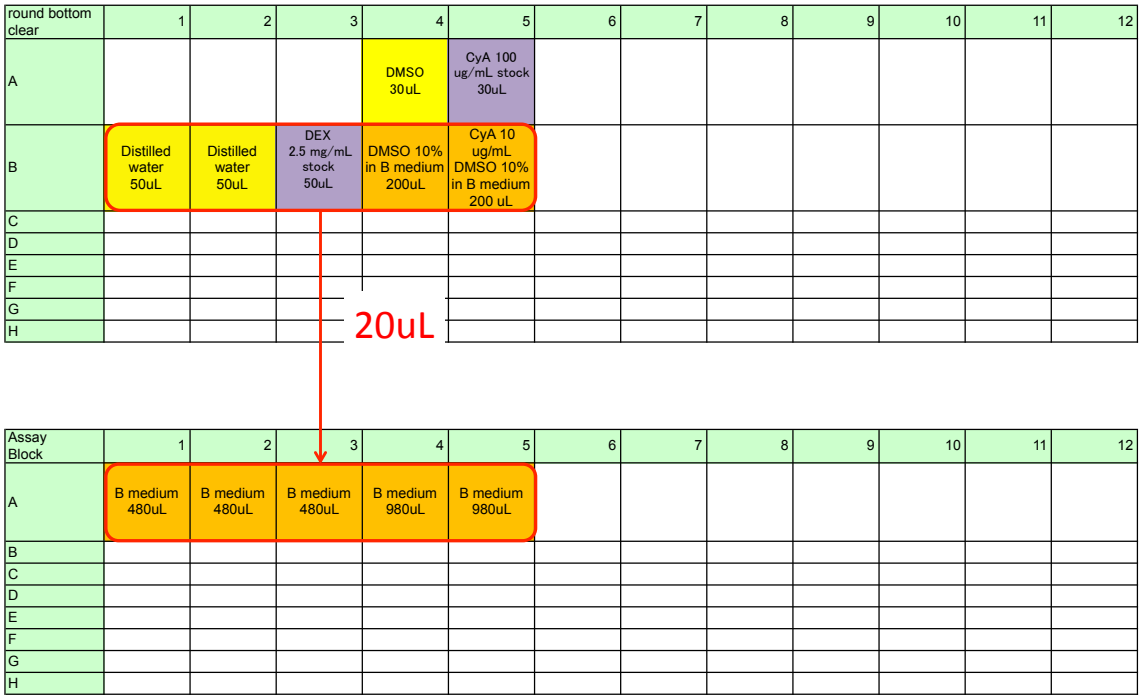


Figure 17

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 500uL	B medium 500uL	DEX 100 ug/mL B medium 500uL	DMSO 0.2% B medium 1000uL	CyA 200 ng/mL DMSO 0.2% B medium 1000uL							
B												
C												
D												
E												
F												
G												
H												

50ul

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
B	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
C	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
D	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
E												
F												
G												
H												

Figure 18 Final constituents of each well of the plate

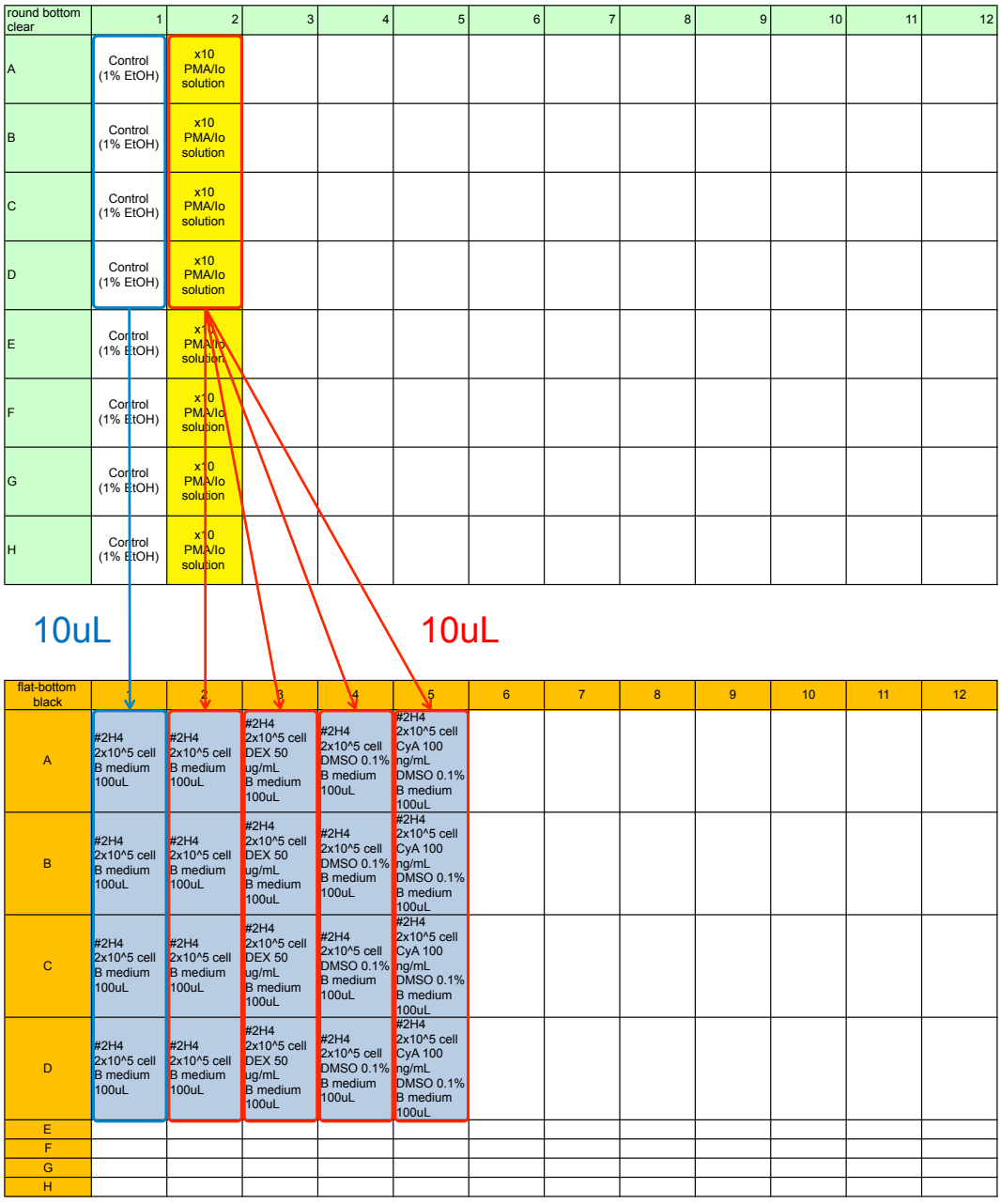
flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell DEX 50 ug/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 100 ng/mL DMSO 0.1% B medium 100uL							
B	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell DEX 50 ug/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 100 ng/mL DMSO 0.1% B medium 100uL							
C	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell DEX 50 ug/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 100 ng/mL DMSO 0.1% B medium 100uL							
D	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell DEX 50 ug/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 100 ng/mL DMSO 0.1% B medium 100uL							
E												
F												
G												
H												

7-6 Addition of PMA/ionomycin to 2H4

One hour after the addition of dexamethasone and cyclosporine A, add 10 µL of control or PMA/ionomycin solution prepared in §6-3 to the cells (#A1-#D1 or #A2-#D5, respectively) using an 8 channel or 12 channel pipetman after pipetting 20 times. Make sure that the apex of the tip is

dipped into the medium. Change tips every line you add. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 6 hour (37°C, CO_2, 5%). (cf. Figure 19)

Figure 19



8. Calculation of the transmittance factors

Color discrimination in multi-color reporter assays is generally achieved using detectors (luminometer and plate reader) equipped with optical filters, such as sharp-cut (long-pass) filters and band-pass filters. The transmittance factors of these filters for each bio-luminescence signal color must be calibrated prior to all experiments by following the protocols below.

8-1 Reagents

Single reference samples:

Lyophilized luciferase enzyme reagent for stable luciferase green (SLG)

Lyophilized luciferase enzyme reagent for stable luciferase orange (SLO)

Lyophilized luciferase enzyme reagent for stable luciferase red (SLR)

Assay reagent:

Tripluc® Luciferase assay reagent（TOYOBO Cat#MRA-301）

B medium: for luciferase assay (30 mL, stored at 2- 8°C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	27 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	3 mL

8-2 Preparation of luminescence reaction solution

Thaw Tripluc® Luciferase assay reagent (Tripluc) and keep it at room temperature either in a water bath or at ambient air temperature. Power on the luminometer 30 min before starting the measurement to allow the photomultiplier to stabilize.

Add 200 μL of 100 mM Tris-HCl (pH8.0) contains 10 % glycerol to each tube of lyophilized reference sample to dissolve the enzymes, divide into 10 μL aliquots in 1.5 mL disposable tubes and store in a freezer at -80°C. The stored frozen solution of the reference samples can be used for up to 6 months.

Add 1 mL of the B medium to each tube of frozen reference sample (10 μL sample per tube). Keep the reference samples on ice to prevent deactivation.

8-3 Bioluminescence measurement

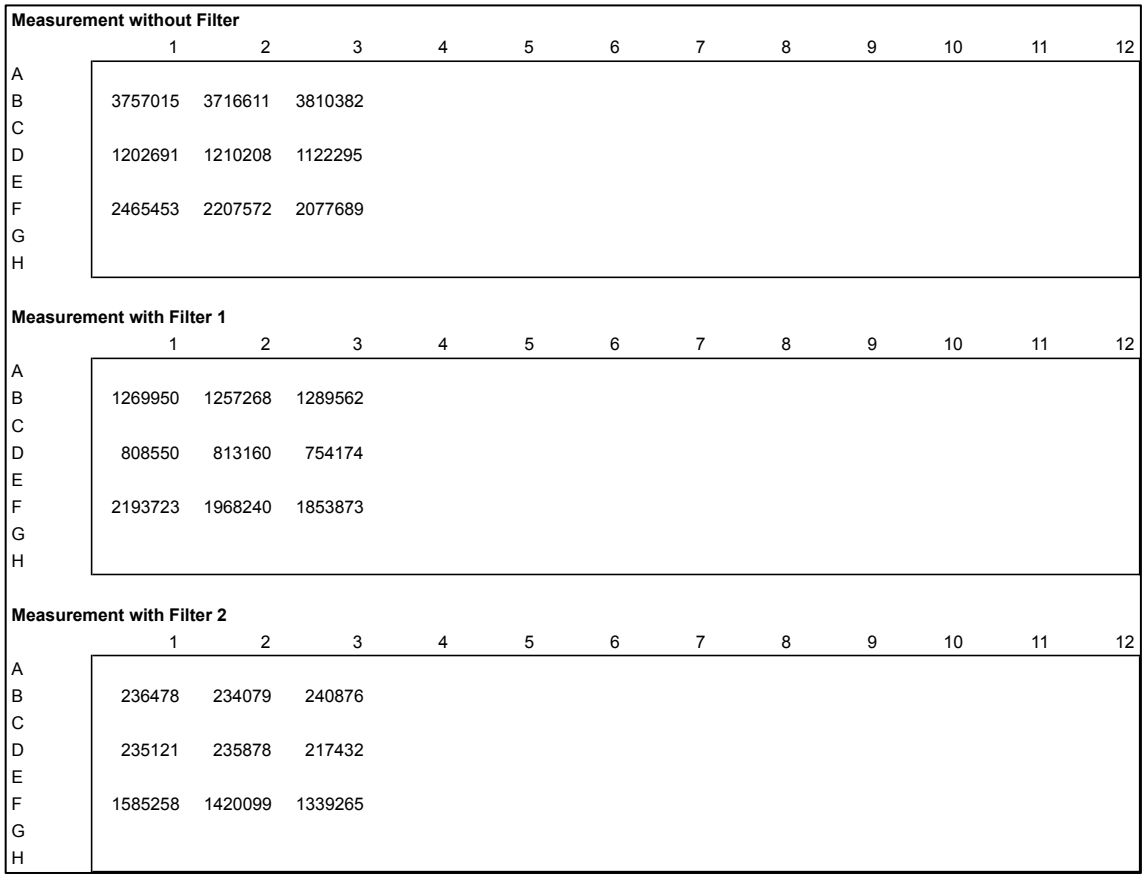
Transfer 100 μL of the diluted reference samples to a black 96 well plate (flat bottom) as shown below.

Figure 20

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	SLG 100 µL	SLG 100 µL	SLG 100 µL									
C												
D	SLO 100 µL	SLO 100 µL	SLO 100 µL									
E												
F	SLR 100 µL	SLR 100 µL	SLR 100 µL									
G												
H												

Transfer 100 µL of pre-warmed Tripluc to each well of the plate containing the reference sample using a pipetman. Shake the plate for 10 min at room temperature (about 25°C) using a plate shaker. Remove bubbles in the solutions in wells if they appear. Place the plate in the luminometer to measure the luciferase activity. Bioluminescence is measured for 3 sec each in the absence (F0) and presence (F1, F2) of the optical filters. An example of the raw output data is shown below.

Figure 22



Measurement without Filter

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	3757015	3716611	3810382									
C												
D	1202691	1210208	1122295									
E												
F	2465453	2207572	2077689									
G												
H												

Measurement with Filter 1

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	1269950	1257268	1289562									
C												
D	808550	813160	754174									
E												
F	2193723	1968240	1853873									
G												
H												

Measurement with Filter 2

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	236478	234079	240876									
C												
D	235121	235878	217432									
E												
F	1585258	1420099	1339265									
G												
H												

Six transmittance factors of the optical filters were calculated as follow:

Transmittance factor (κG_{R56})=$\frac{\#B1 \text{ of } F1+ \#B2 \text{ of } F1+ \#B3 \text{ of } F1}{\#B1 \text{ of } F0+ \#B2 \text{ of } F0+ \#B3 \text{ of } F0}$

Transmittance factor (κO_{R56})=$\frac{\#D1 \text{ of } F1+\#D2 \text{ of } F1+\#D3 \text{ of } F1}{\#D1 \text{ of } F0+\#D2 \text{ of } F0+\#D3 \text{ of } F0}$

Transmittance factor (κR_{R56})= $\frac{\#F1 \text{ of } F1+\#F2 \text{ of } F1+\#F3 \text{ of } F1}{\#F1 \text{ of } F0+\#F2 \text{ of } F0+\#F3 \text{ of } F0}$

Transmittance factor (κG_{R60})=$\frac{\#B1 \text{ of } F2+ \#B2 \text{ of } F2+ \#B3 \text{ of } F2}{\#B1 \text{ of } F0+ \#B2 \text{ of } F0+ \#B3 \text{ of } F0}$

Transmittance factor (κO_{R60})=$\frac{\#D1 \text{ of } F2+\#D2 \text{ of } F2+\#D3 \text{ of } F2}{\#D1 \text{ of } F0+\#D2 \text{ of } F0+\#D3 \text{ of } F0}$

Transmittance factor (κR_{R60})=$\frac{\#F1 \text{ of } F2+\#F2 \text{ of } F2+\#F3 \text{ of } F2}{\#F1 \text{ of } F0+\#F2 \text{ of } F0+\#F3 \text{ of } F0}$

In the case shown above,

Transmittance factors (κG_{R56})=$\frac{1269550+1257268+1289562}{3757015+3716611+3810382}$=0.338

Transmittance factors (κO_{R56})=$\frac{808550+813160+754174}{1202691+1210208+1122295}$=0.672

Transmittance factors (κR_{R56})=$\frac{2193723+1968240+1853873}{2465453+2207572+2077689}$=0.891

Transmittance factors (κG_{R60})= $\frac{236478+234079+240876}{3757015+3716611+3810382}$=0.06

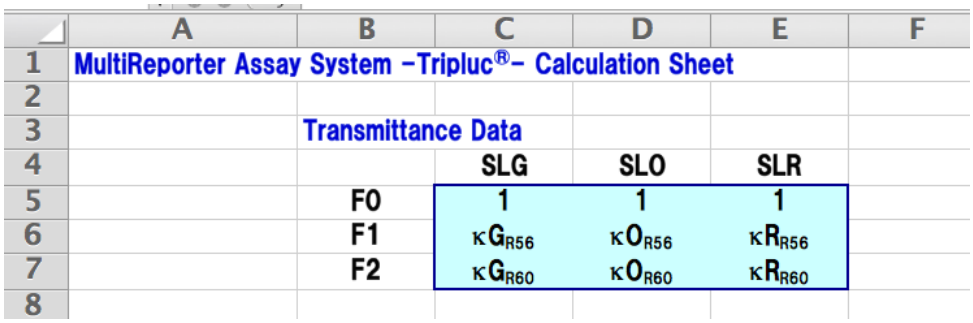
Transmittance factors (κO_{R60})=$\frac{235121+235878+217432}{1202691+1210208+1122295}$=0.195

Transmittance factors (κR_{R60})=$\frac{1585258+1420099+1339265}{2465453+2207572+2077689}$=0.644

Calculated transmittance factors are used for all the measurements executed using the same luminometer.

Input the transmittance factors to #C6-#E7 of the “Data Input” sheet of the Data sheet as follow.

Figure 23



	A	B	C	D	E	F
1	MultiReporter Assay System -Tripluc®- Calculation Sheet					
2						
3		Transmittance Data				
4			SLG	SLO	SLR	
5		F0	1	1	1	
6		F1	κG_{R56}	κO_{R56}	κR_{R56}	
7		F2	κG_{R60}	κO_{R60}	κR_{R60}	
8						

9. Measurement

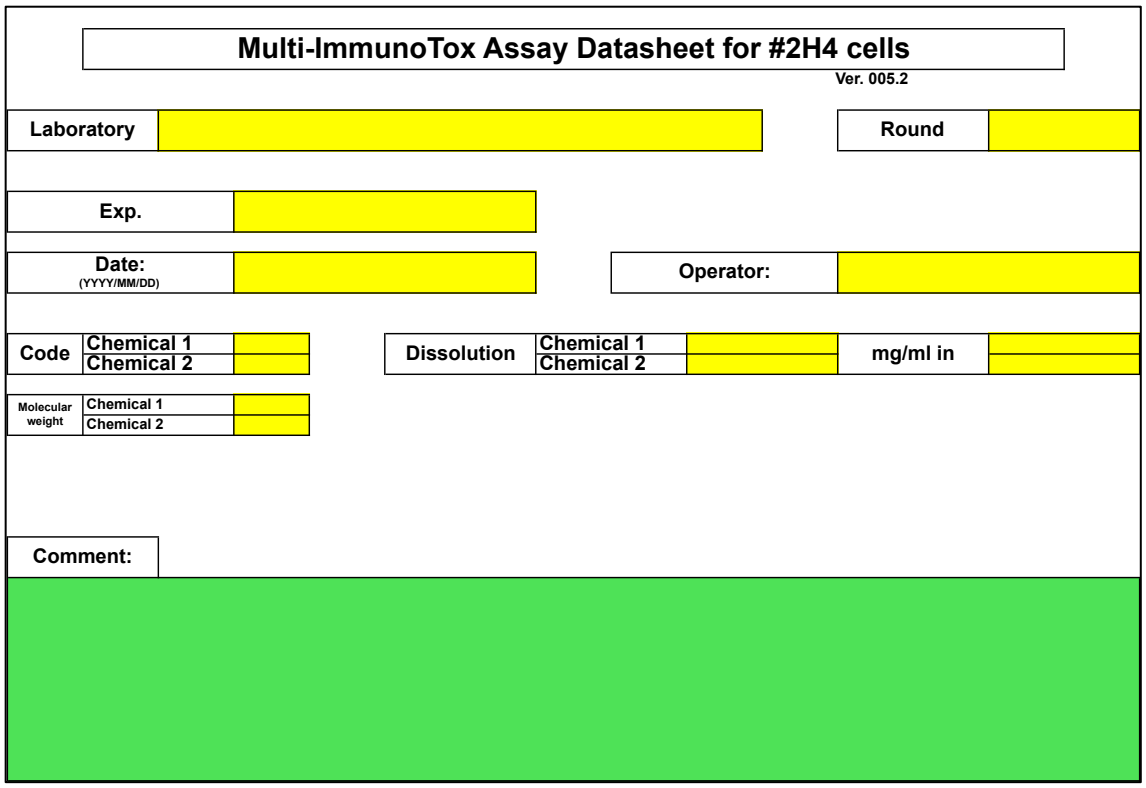
Please refer Appendix 1 for the principle of measurement of luciferase activity.

Thaw Tripluc® Luciferase assay reagent (Tripluc) and keep it at room temperature either in a water bath or at ambient air temperature. Power on the luminometer 30 min before starting the measurement to allow the photomultiplier to stabilize.

Transfer 100 µL of pre-warmed Tripluc from the reservoir to each well of the plate containing the reference sample using an 8 channel or 12 channel pipetman. Shake the plate for 10 min at room temperature (about 25°C) on a plate shaker. Remove bubbles in the solutions in the wells if they appear. Place the plate in the luminometer to measure the luciferase activity. Bioluminescence is measured for 3 sec each in the absence of (F0) and presence (F1, F2) of the optical filters.

1st. Add the information regarding the name of laboratory, the round of experiments if multiple experimental sets are performed, the experiment number, date, the operator, chemical codes, dissolved in distilled water or DMSO, the prepared concentration, molecular weight of the chemicals and comments if any to Face Sheet of the data sheet.

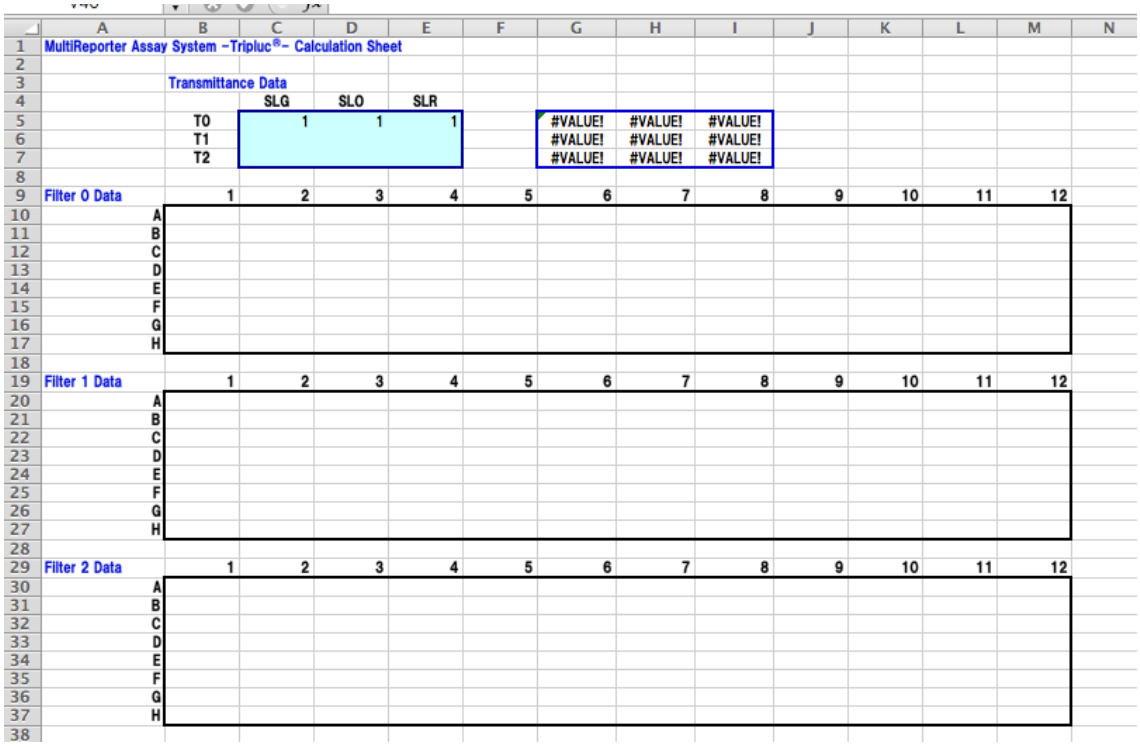
Figure 24 “Face Sheet” of the data sheet



2nd. Copy the results of the F0, F1 and F2 measurements (values are expressed as counts) and paste them into the appropriate area in the “Data Input” sheet of the data sheet shown below. In addition, input the transmittance factors calculated in "§5. Calculation of the transmittance factors" to #C6-

#E7 of the “Data Input” sheet.

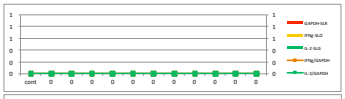
Figure 25 “Data Input” sheet of the data sheet

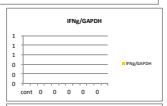


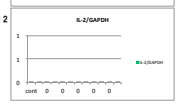
	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	MultiReporter Assay System –Tripluc®– Calculation Sheet													
2														
3		Transmittance Data												
4			SLG	SLO	SLR									
5		T0	1	1	1		#VALUE!	#VALUE!	#VALUE!					
6		T1					#VALUE!	#VALUE!	#VALUE!					
7		T2					#VALUE!	#VALUE!	#VALUE!					
8														
9	Filter 0 Data	1	2	3	4	5	6	7	8	9	10	11	12	
10	A													
11	B													
12	C													
13	D													
14	E													
15	F													
16	G													
17	H													
18														
19	Filter 1 Data	1	2	3	4	5	6	7	8	9	10	11	12	
20	A													
21	B													
22	C													
23	D													
24	E													
25	F													
26	G													
27	H													
28														
29	Filter 2 Data	1	2	3	4	5	6	7	8	9	10	11	12	
30	A													
31	B													
32	C													
33	D													
34	E													
35	F													
36	G													
37	H													
38														

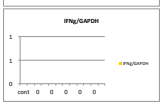
Next, the calculated results for the parameters of the Multi-Immuno Tox assay for each concentration, e.g., SLG-LA, SLO-LA, SLR-LA, nSLG-LA, nSLO-LA, the mean ± SD of SLG-LA, the mean ± SD of SLO-LA, the mean ± SD of SLR-LA %suppression and graphs will automatically appear on the “Result Format” sheet of the data sheet.

Figure 26 “Result Format” sheet of the data sheet









MultiReporter Assay System -Tripluc®- Calculation Sheet

SLG-LA

	1	2	3	4	5	6	7	8	9	10	11	12
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

SLO-LA

	1	2	3	4	5	6	7	8	9	10	11	12
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

SLR-LA

	1	2	3	4	5	6	7	8	9	10	11	12
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

nSLG-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
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nSLO-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
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	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
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Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
I.I.-SLR-LA		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
input "*"												
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Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
input "*"												
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	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

Chemical 1													
	cont	0	0	0	0	0	0	0	0	0	0	0	ug/ml
Chemical 2													
	cont	0	0	0	0	0	0	0	0	0	0	0	ug/ml

%Suppression (IL-2)		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
%Suppression (IFNg)		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

10. Data analysis

Definition of the parameters used in the Multi-Immuno Tox assay

SLG-luciferase activity (SLG-LA) : Luciferase activity of stable luciferase green
(Under the control of IL-2 promoter)

SLO-luciferase activity (SLO-LA) : Luciferase activity of stable luciferase orange
(Under the control of IFN-γ promoter)

SLR-luciferase activity (SLR-LA) : Luciferase activity of stable luciferase red
(Under the control of G3PDH promoter)

Normalized SLG-LA (nSLG-LA) : =(SLG-LA)/(SLR-LA)

Normalized SLO-LA (nSLO-LA) : =(SLO-LA)/(SLR-LA)

Inhibition index of SLR-LA (I.I.-SLR-LA) : The cytotoxic effect of chemicals
=(SLR-LA of 2H4 treated with chemicals)/(SLR-LA of untreated 2H4)

%suppression : The effect of chemicals on IL-2 or IFN-γ promoter
=(1-(nSLG-LA or nSLO-LA of 2H4 treated with chemicals)
/(nSLG-LA or nSLO-LA of non-treated 2H4)) x 100

11. Criteria

11-1 Acceptance criteria

The following acceptance criteria should be satisfied when using the Multi-Immuno Tox Assay method.

If Fold induction of nSLO-LA of PMA/Ionomycin wells without chemicals (=(nSLO-LA of 2H4 cells treated with PMA/Ionomycin) / (nSLO-LA of non-treated 2H4 cells)) demonstrate less than 3.0, the results obtained from the plate containing the control wells should be rejected.

11-2 Criterion

The experiments are repeated until two consistent positive (negative) results or two consistent “no effect results” are obtained. When two consistent results are obtained, the chemicals are judged as the obtained consistent results.

Identification of immunotoxicant is evaluated by the mean of %suppression and its 95% simultaneous confidence interval.

In each experiment, when the chemicals clear the following 3 criteria, they are judged as suppressive or stimulatory. Otherwise, they are judged as no effect chemicals.

1. The mean of %suppression is $\geq$ 35 (suppressive) or $\leq$ -35 (stimulatory) with statistical significance. The statistical significance is judged by its 95% confidence interval.

2. The result shows two or more consecutive statistically significant positive (negative) data or one statistically significant positive (negative) data with a trend in which at least 3 consecutive data increase (decrease) in a dose dependent manner. In the latter case, the trend can cross 0, as long as only one data point shows the opposite effect without statistical significance.

3. The results are judged using only data obtained in the concentration at which I.I.-SLR-LA is $\geq$ 0.05.

12. Update record

Ver. 0011.0E 2018.5.10

Change the criteria

Ver. 0010.0E 2018.1.15 distribution

Change the criteria

Ver. 009.1E 2017.5.8 distribution

Change the criteria

Ver. 009.0E 2017.4.7 distribution

Change the preparation of chemicals

Change the acceptance criteria

Change the criteria

Ver. 008.5E 2016.9.14 distribution

Change the criteria

Ver. 008.4E 2016.9.9 distribution

Change the criteria

Ver. 008.3E 2016.8.1 distribution

Correction of the preparation of PMA and ionomycin

Change the preparation of PMA and ionomycin

Change the preparation of controls

Addition of Acceptance criteria

Ver. 008.1E 2016.2.2 distribution

Changes after the VMT meeting

Ver. 008.0E 2016.1.19

Translation to English

Addition of appendix

Ver. 006.0J 2015.8.17

Change the preparation of chemicals (same method to the IL-8 Luc assay)

Delete the alteration in Ver. 005.0J

Ver. 005.0J 2015.1.9　distribution

Change to use SLR-LA of THP-G8 at the calculation of nSLG-LA of TGCHAC-A4

Ver. 004.1J 2014.12.10　distribution

Change the cellar concentration at cell passage

Modify figure 16, 17

Ver. 004.0J 2014.11.17　distribution

For the validation study at AIST, FDSC and Tohoku university (chemicals: Sodium Bromate ($NaBrO_3$), Nickel (II) sulfate ($NiSO_4$), Dibutyl phthalate (DP), 2-Mercaptobenzothiazole (2-MBT))

Change THP-G1b cells to TGCHAC-A4 cells

Change cell number of THP-G8 and TGCHAC-A4 5x10^4/well to 1x10^5/well

Change concentration of chemicals 11 steps to 10 steps

Change final concentration of LPS (THP-G8 : 25 ng/mL, TGCHAC-A4 : 1 ng/mL)

Change the way of addition of LPS (2 mL/well to 10 mL/well)

Change the criteria

Ver. 002.0J 2013.08.19 distribution

For the validation study at AIST and FDSC (chemicals: $CoCl_2$, $NiSO_4$, Isophorone diisocyanate, 2-Mercaptobenzothiazole)

Change the common ratio 3 to 2

Change the concentration of LPS 100 ng/mL to 25 ng/mL

Add description about the control (dexamethasone)

Delete the appendix about THP-G8 cell

Ver. 001.1J 2012. Nov. 13 distribution

Add the appendix about THP-G8 cell

Ver. 001J 2012. Nov. 09 distribution

Appendix 5 Principle of measurement of luciferase activity

MultiReporter Assay System -Tripluc- can be used with a microplate-type luminometer with a multi-color detection system, which can equip two optical filters (e.g. Phelios AB-2350 (ATTO), ARVO (PerkinElmer), Tristar LB941 (Berthold)). The optical filters used in measurement are a 560 nm long-pass filter and a 600 nm long-pass filter.

(1) Measurement of three-color luciferase with two optical filters.

This is an example using Phelios AB-2350 (ATTO). This luminometer equips a 560 nm long-pass filter (560 nm LP, Filter 1) and a 600 nm long pass filter (600 nm LP, Filter 2) for optical isolation.

First, using luciferase enzyme reagent of SLG (λmax = 550 nm), SLO (λmax = 580 nm) and SLR (λmax = 630 nm), measure i) the intensity of light without filter (all optical), ii) the intensity of 560 nm LP (Filter 1) transmitted light iii) the intensity of 600 nm LP (Filter 2) transmitted light, and calculate the coefficient factor listed below.

Coefficient factor		Abbreviation	Definition
SLG	Filter 1 transmittance factor	κG_{R56}	The intensity of 560 nm LP (Filter 1) transmitted SLG / the intensity of SLG without filter (all optical)
	Filter 2 transmittance factor	κG_{R60}	The intensity of 600 nm LP (Filter 2) transmitted SLG / the intensity of SLG without filter (all optical)
SLO	Filter 1 transmittance factor	κO_{R56}	The intensity of 560 nm LP (Filter 1) transmitted SLO / the intensity of SLO without filter (all optical)
	Filter 2 transmittance factor	κO_{R60}	The intensity of 600 nm LP (Filter 2) transmitted SLO / the intensity of SLO without filter (all optical)
SLR	Filter 1 transmittance factor	κR_{R56}	The intensity of 560 nm LP (Filter 1) transmitted SLR / the intensity of SLR without filter (all optical)
	Filter 2 transmittance factor	κR_{R60}	The intensity of 600 nm LP (Filter 2) transmitted SLR / the intensity of SLR without filter (all optical)

When the intensity of SLG, SLO and SLR in test sample are defined as G, O and R, respectively, i) the intensity of light without filter (all optical): F0, ii) the intensity of 560 nm LP (Filter 1) transmitted light and iii) the intensity of 600 nm LP (Filter 2) transmitted light are described as below.

$F0=G+O+R$

$F1=\kappa G_{R56} \times G + \kappa O_{R56} \times O + \kappa R_{R56} \times R$

$F2=\kappa G_{R60} \times G + \kappa O_{R60} \times O + \kappa R_{R60} \times R$

These formulas can be rephrased as follows

$$\begin{pmatrix} F0 \\ F1 \\ F2 \end{pmatrix} = \begin{pmatrix} 1 & 1 & 1 \\ \kappa G_{R56} & \kappa O_{R56} & \kappa R_{R56} \\ \kappa G_{R60} & \kappa O_{R60} & \kappa R_{R60} \end{pmatrix} \begin{pmatrix} G \\ O \\ R \end{pmatrix}$$

Then using calculated coefficient factors and measured F0, F1 and F2, you can calculate G, O and R-value as follows.

$$\begin{pmatrix} G \\ O \\ R \end{pmatrix} = \begin{pmatrix} 1 & 1 & 1 \\ \kappa G_{R56} & \kappa O_{R56} & \kappa R_{R56} \\ \kappa G_{R60} & \kappa O_{R60} & \kappa R_{R60} \end{pmatrix}^{-1} \begin{pmatrix} F0 \\ F1 \\ F2 \end{pmatrix}$$

This calculation can be performed using the functions "MININVERSE" and "MMULT" in Microsoft Excel. These calculations are integrated in the Data Sheet.

Appendix 6　Validation of reagents and equipment

5-1 Measurement of transmittance of optical filter for multicolor measurement

For color discriminations in the multi-color reporter assay, detectors (luminometer and plate reader) are usually equipped with optical filters, such as sharp-cut (long-pass) filters and band-pass filters. The transmittance factors of these filters for each bioluminescence signal color have to be calibrated prior to all experiments by following the protocols below.

5-1-1　Reagents

・Single reference samples:

Lyophilized luciferase enzyme reagent of SLG

Lyophilized luciferase enzyme reagent of SLO

Lyophilized luciferase enzyme reagent of SLR

・Assay reagent:

Tripluc® Luciferase assay reagent（TOYOBO Cat#MRA-301）

・B medium: for luciferase assay (30 mL, stored at 2 – 8°C)

Reagent	Company	Conc.	Final conc. in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	27 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	3 mL

5-1-2 Calibration

5-1-2-1 Preparation of luminescence reaction solution

Thaw Tripluc® Luciferase assay reagent (Tripluc) and keep it at room temperature by bathing in water or ambient air. Start the luminometer 30 min before starting the measurement for stabilization of the photomultiplier.

Add 200 μL of 100 mM Tris-HCl (pH8.0) contains 10% glycerol to each tube of the lyophilized reference samples to dissolve the enzymes, followed by separating them into 1.5 mL disposable tubes at 10 μL each and storing in a freezer at -80°C. The stored frozen solution of the reference samples can be used for one half year.

Add 1 mL of the B medium to each tube of the frozen reference sample (10 μL in a tube) and label them as SLG1/1, SLO1/1 and SLR1/1. Keep the reference samples on ice to prevent deactivation.

Prepare dilution series of the single reference samples of SLG, SLO and SLras follows. Dilute 0.3 mL of each 1/1 solution with 0.9 mL of the B medium to make SLG1/4, SLO1/4 and SLR1/4. In the same manner, prepare 1/16 and 1/64 solution of each. Keep diluted reference samples on ice.

5-1-2-2 Bioluminescence measurement

Transfer 100 μL of the diluted reference samples to a black 96 well plate (flat bottom) as shown below.

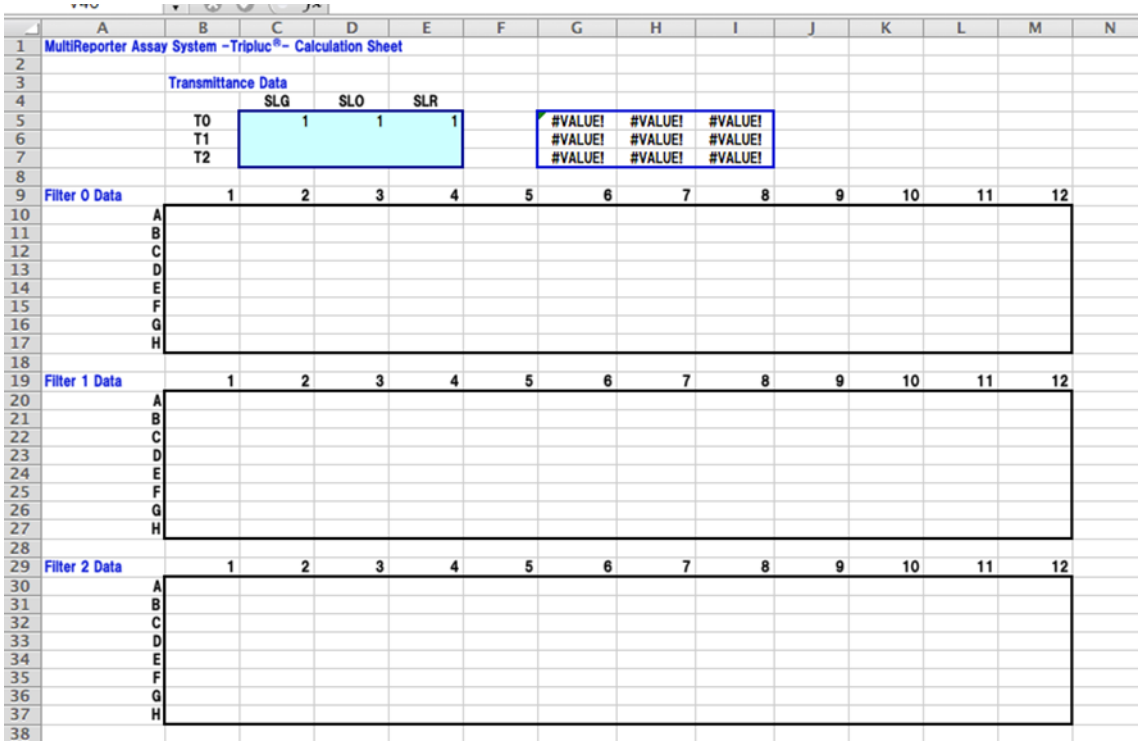
Figure 27

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	SLG 1/1	SLG 1/1	SLG 1/1	SLG 1/4	SLG 1/4	SLG 1/4	SLG 1/16	SLG 1/16	SLG 1/16	SLG 1/64	SLG 1/64	SLG 1/64
C												
D	SLO 1/1	SLO 1/1	SLO 1/1	SLO 1/4	SLO 1/4	SLO 1/4	SLO 1/16	SLO 1/16	SLO 1/16	SLO 1/64	SLO 1/64	SLO 1/64
E												
F	SLR 1/1	SLR 1/1	SLR 1/1	SLR 1/4	SLR 1/4	SLR 1/4	SLR 1/16	SLR 1/16	SLR 1/16	SLR 1/64	SLR 1/64	SLR 1/64
G												
H												

Transfer 100 μL of pre-warmed Tripluc to each well containing the reference samples of the plate using a pipetman. Shake the plate for 10 min at room temperature (about 25°C) with a plate shaker. Remove bubbles on the solutions in wells if they appear. Place the plate into the luminometer to measure the luciferase activity. Bioluminescence is measured for 3 sec each in the absence (F0) and presence (F1, F2) of the optical filters.

Copy the results of the F0, F1 and F2 measurement (values are expressed as counts) and paste it to the appropriate area in the “Data Input” sheet of the data sheet for data analyses shown below.

Figure 28



	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	MultiReporter Assay System –Triplue®– Calculation Sheet													
2														
3		Transmittance Data												
4			SLG	SLO	SLR									
5		T0	1	1	1		#VALUE!	#VALUE!	#VALUE!					
6		T1					#VALUE!	#VALUE!	#VALUE!					
7		T2					#VALUE!	#VALUE!	#VALUE!					
8														
9	Filter 0 Data	1	2	3	4	5	6	7	8	9	10	11	12	
10	A													
11	B													
12	C													
13	D													
14	E													
15	F													
16	G													
17	H													
18														
19	Filter 1 Data	1	2	3	4	5	6	7	8	9	10	11	12	
20	A													
21	B													
22	C													
23	D													
24	E													
25	F													
26	G													
27	H													
28														
29	Filter 2 Data	1	2	3	4	5	6	7	8	9	10	11	12	
30	A													
31	B													
32	C													
33	D													
34	E													
35	F													
36	G													
37	H													
38														

Record all the results for quality control.

5-2 Quality control of equipment

In order to confirm the detector stability as the quality control, the reference luciferase sample, optical property, the protocol described here should be performed at the beginning of the experiments every day.

5-2-1 Light source

LED Plate: Reference LED light source plates equipped with stabilized red, green, and blue LEDs are commercially available. For example,

TRIANT® (wSL-0001) by ATTO (Tokyo, Japan)

L12367 by Hamamatsu Photonics (Shizuoka, Japan)

5-2-2 Data collection (an example using TRIANT® by ATTO)

1) Start luminometer 30 min before starting the measurement for stabilization of the photomultiplier.
2) Start LED plate and select “PMT” mode.
3) Select three-color (BRG) mode and adjust light intensity to 1/10 (10E-1).
4) Place the LED plate into the luminometer. Light intensity is measured for 3 sec each in the absence (F0) and presence (F2) of the optical filter.
5) Blue, green, and red LEDs are located at the position of #F6, #E6, and #D6, respectively. Copy

the collected data of each position to the appropriate area on Sheet “LED” in the excel file of the data sheet.

6) Check the photo-detector performance by comparing with old data of the LED plate. For quality control purpose, every collected data should be recorded.

7) LED plate data typically fluctuates up to 1.5% (σ). Disagreement to the old data should be less than 3×σ (= 4.5%).

Appendix 7. Immunotoxicologic information of 25 chemicals used in the validation study

MITA Literature Reports

August 8, 2018

Table of Contents

2,4-Diaminotoluene (DAT) [CASRN 95-80-7] 1

5,5-Diphenylhydantoin (DPH) [CASRN 57-41-0] 3

Benzo(a)pyrene [B(a)P] [CASRN 50-32-8] 9

Cadmium Chloride [CASRN 10108-64-2] 14

Dibromoacetic Acid (DBAA) [CASRN 631-61-1] 17

Dibutyl phthalate (DBP) [CASRN 84-74-2] 21

Dichloroacetic Acid (DCAA) [CASRN 79-43-6] 25

Diethylstilbestrol (DES) [CASRN 56-53-1] 27

Ethylene Dibromide (EDB) [CASRN 106-93-4] 32

Glycidol [CASRN 556-52-5] 34

Hydrocortisone (HC) [CASRN 50-23-7] 37

Indomethacin [CASRN 53-86-1] 40

Isonicotinic Acid Hydrazide (IAH) [CASRN 54-85-3] 45

Lead (II) Acetate Trihydrate [CASRN 6080-56-4] 49

Mannitol [CASRN 69-65-8] 53

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Nitrobenzene [CASRN 98-95-3] 60

o-Benzyl-p-chlorophenol (BCP) [CASRN 120-32-1] 62

Perflouorooctanoic Acid (PFOA) [CASRN 335-67-1] 63

Toluene [CASRN 108-88-3] 68

Tributyltin Chloride (TBTC) [CASRN 1461-22-9] 71

Urethane [CASRN 51-79-6] 76

Vanadium Pentoxide [CASRN 1314-62-1] 80

Zinc Dimethyldithiocarbamate (ZDMDC) [CASRN 137-30-4] 84

2,4-Diaminotoluene (DAT) [CASRN 95-80-7]

Human Data

Data from epidemiology studies

No data were located.

In vitro data with cells or cell lines

No data were located.

Mode of action information

No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

Female B6C3F1 mice were orally dosed with 25, 50, or 100 mg/kg DAT for 14 days. Absolute and relative (to body weight and to brain weight) liver weights (LOAEL = 100 mg/kg) were increased compared to controls. No effect on absolute or relative spleen weights were reported. However, trend analyses indicated significant changes in relative spleen weight and spleen/brain ratio in treated mice. Leukocyte and lymphocyte numbers also were increased (LOAEL =100 mg/kg). The percentage of lymphocytes and polymorphonuclear leukocytes also were increased (LOAEL = 50 mg/kg). No changes in serum chemistry parameters (e.g., ALT levels) and bone marrow parameters (e.g., number of cells in the femur) were noted. The number of spleen cells, and percentage of T- and B-cells (LOAELs = 100 and 25 mg/kg, respectively) were altered in treated animals. While the number of spleen cells was decreased 18% at the highest dose tested, the percentage of T-cells and B-cells were increased 75% and 15%, respectively.

Peak IgM and IgG responses (in response to sheep erythrocytes) were observed on days 4 and 5 after immunization, respectively. DAT produced a dose-dependent decrease in IgM (46% at 100 mg/kg) and IgG (56% at 100 mg/kg) AFC responses based on total

spleen activity. DAT exposure also produced a dose-dependent increase in delayed hypersensitivity response to keyhole limpet hemocyanin (2.2-fold increase at 100 mg/kg). Serum CH50 and C3 levels were not significantly affected in mice treated with DAT. The activity of the reticuloendothelial system was increased in the liver (LOAEL = 100 mg/kg), decreased in the spleen (LOAEL = 50 mg/kg) and kidney (LOAEL = 100 mg/kg), and not affected in the lung or thymus of treated mice. Decreased host resistance (LOAEL = 100 mg/kg) to *Streptococcus pneumoniae* and *Listeria monocytogenes*. However host resistance to B16F10 fibrosarcoma and PYB6 melanoma were not affected (Burns et al. 1994).

In vitro data with cells or cell lines

Spleen cells from female B6C3F1 cells treated with 25, 50, or 100 mg/kg DAT for 14 days were evaluated for response to mitogens and DBA/2 spleen cells. DAT exposure did not affect cell

responses to T-cell mitogens PHA and ConA. An increase in responsiveness to LPS was reported in cells obtained from mice treated with 25 or 50 mg/kg, but not those treated with 100 mg/kg.

Spleen cellularity was decreased 20% and 15% at 50 and 100 mg/kg DAT. In response to DBA/2 cells, an enhanced response was observed in responder cells (LOAEL = 100 mg/kg) while no mixed lymphocyte response was noted (Burns et al. 1994).

Peritoneal exudate cells from female B6C3F1 cells treated with 25, 50, or 100 mg/kg DAT for 14 days were allowed to adhere to plastic and the percentage of cells phagocytizing fluorescent Covaspheres or chicken erythrocytes was measured. No significant change in the percentage of phagocytosis was noted at any of the doses (Burns et al. 1994).

Splenic NK cell activity was decreased in cells obtained from mice exposure to DAT for 14 days. A dose-dependent decrease was observed at all effector/target ratios tested (100/1, 50/1, and 25/1). The LOAEL was 50 mg/kg (Burns et al. 1994).

Spleen cell suspensions from female NMRI mice were evaluated to determine whether DAT could modulate luminol-dependent chemiluminescence of phagocytotic cells. Cells were treated with 0.01, 0.1, 1.0, 10, or 100 mg/L DAT. At concentrations greater than 1 mg/L, a dose- dependent decrease in response was observed. When compared to control levels, chemiluminescence was decreased 43%, 90%, and 100% at 1.0, 10, and 100 mg/kg, respectively (Thierfelder and Masihi 1995).

Mode of action information

Based on the combined effects, Burns and colleagues (1994) proposed that DAT affects differentiation and maturation of leukocytes.

References

Burns LA, Bradley SG, White KL, McCay JA, Fuchs BA, Stern M, et al. 1994. Immunotoxicity of 2,4-diaminotoluene in female B6C3F1 mice. Drug and chemical toxicology 17:401–36; doi:10.3109/01480549409017865.

Thierfelder W, Masihi KN. 1995. Effects of trinitrotoluene (TNT) metabolites on chemiluminescence response of phagocytic cells. International journal of immunopharmacology 17: 453–6.

5,5-Diphenylhydantoin (DPH) [CASRN 57-41-0]

Human Data

Data from epidemiology studies

In a study of 51 epileptic patients, 20 of whom had not received anticonvulsant treatment for at least two years and 31 of whom had received DPH at 300 mg/24 hours for at least 4 months, the DPH treated group had decreased serum levels of IgA (156 ± 65 mg/100 mL) and IgM (121 ± 43 mg/100 mL) as compared to untreated epileptics (IgA, 179 ± 70 mg/100 mL; IgM, 133 ± 50 mg/100 mL) or control subjects (n= 15; IgA, 223 ± 49 mg/100 mL; IgM, 163 ± 48 mg/100 mL). Serum IgG levels were not statistically significantly different among the groups. The authors concluded that DPH treatment suppresses the normal function of the humoral immune response and that epilepsy may be a contributing factor (Badawy et al. 1991).

Peripheral blood lymphocytes, serum immunoglobulins and complement (C3 and C4) protein concentrations were determined in 20 patients with idiopathic epilepsy who were receiving 200- 300 mg DPH treatment and 30 healthy controls. A significant decrease in T-suppressor cells (28%) and subsequently higher T-helper to T-suppresor lymphocyte ratio (36%) were observed in DPH treated patients. A significant increase in B-lymphocytes (39%) and in serum IgM levels (data in graph) was also observed in DPH treated patients as compared to controls. No significant changes in serum concentrations of IgG, IgA or complement proteins was observed (Basaran et al. 1989).

Peripheral blood lymphocyte subsets, serum immunoglobulins and complement (C3 and C4) protein concentrations were determined in 40 healthy subjects, 30 DPH treated patients (200-300 mg/day), 22 carbamazepine treated patients, and 38 untreated epilepsy patients. Subjects receiving drug therapy had been taking the drug for 3 months up to 20 years. The DPH treated group had decreased IgA (19% and 24%, respectively) and IgG (16% and 14%, respectively) as compared to both healthy subjects and untreated epileptic patients. Significantly lower T- suppressor lymphocyte counts (23% decrease) was observed when compared to healthy controls. Significantly higher T-helper to T-suppressor lymphocyte ratio was observed when compared to healthy subjects and untreated epileptic patients. No significant differences in C3 or C4 protein

levels were observed in DPH treated patients as compared to controls (Basaran et al. 1994).

Serum IgA values were determined in 191 patients taking DPH (dosage not provided). A reduction in serum IgA levels was observed in up to 20% of the patients. Cellular immune status was assessed in the 11% of patients with IgA values lower than two standard deviations below the mean and included: lymphocyte counts, lymphocyte population studies and responses to *in vitro* mitogen stimulation. No significant variations from control values were observed in any of the evaluated endpoints (Burks et al. 1989).

In vitro data with cells or cell lines

No data were located.

Mode of action information

DPH (20 μg/mL) induced IL-1 activity and potentiated LPS-induced IL-1 production in human PMBC and in U-937 cells, a stable monocytic cell line (Modeer et al. 1989).

DPH treatment can lead to a decrease of suppressor T cells and a reversible IgA deficiency in patients with epilepsy. Gingival overgrowth, which often develops in patients taking DPH, is hypothesized to be due to increased production of both IL-6 and IL-8, combined with elevations of basic fibroblast growth factor as observed *in vitro* using human gingival fibroblasts (Beghi and Shorvon 2011; Godhwani and Bahna 2016).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

Male Balb/C mice were given DPH at doses of 0, 25, 50 or 100 mg/kg via oral gavage for 7 days. DPH significantly increased cellularity in the spleen (LOAEL = 25 mg/kg), however, both the direct and indirect plaque-forming cells responses following intraperitoneal injection with sheep erythrocytes, were significantly depressed (LOAELs = 25 mg/kg). A significant decrease in the delayed type hypersensitivity in response to sheep erythrocytes was also observed (LOAEL = 25 mg/kg) (Andrade-Mena et al. 1994).

Pregnant Balb/C mice were treated with DPH at doses of 0, 20, 40, and 60 mg/kg via oral gavage on days 9 through 18 of gestation. A dose-related suppression of humoral immune function (measured as the antibody response to type III pneumococcal polysaccharide) was observed in male and female offspring at 25 days, but not at 15 weeks of age (NOAEL = 20 mg/kg). Female offspring of dams treated with 20 or 60 mg/kg DPH had greater antibody levels than controls. No difference was noted in female offspring of dams treated with 40 mg/kg DPH when compared to controls. Cell-mediated immune function (as measured by delayed-type hypersensitivity response to oxazolone) was not affected in offspring of treated dams. Immunosuppressive effects also were greater in offspring born with an open eye defect, also attributed to DPH treatment (Chapman and Roberts 1984).

Female B10.s, B10.d2 and DBA/2 mice were injected with 2 mg DPH and received a single injection of 10 μg TNP-OVA subcutaneously into the right hind footpad. Popliteal

lymph nodes (PLN) were isolated 7 days after injection. DPH increased the number of cells in all three strains (B10.s>B10.d2>DBA/2) (data in graph). IgG1 production to TNP-OVA was increased in all three mouse strains (in B10.d2 about 850-fold; and in B10.s and DBA/2 about 120-fold). DPH treatment did not facilitate immune complex deposition in any of the mouse strains, six days after challenge (Albers et al. 1999).

DPH (administered subcutaneously) produced a significant, dose-dependent response in the PLN assay at 0.5 mg (mean PLN index =1.60 ± 0.18) and 1.0 mg (mean PLN index = 2.79 ± 0.30) as compared to control (mean PLN index = 1.11 ± 0.24) in C3Hf mice. The maximal response occurred at 6-8 days post treatment and returned to normal after 3-4 weeks. The observed response was proposed to be T-lymphocyte dependent since only heterozygous C3H +/nu mice developed PLN enlargement whereas their congenitally athymic C3H nu/nu counterparts did not.

The PLN response to DPH was significantly amplified in thymectomized C57BL/10 mice (PLN index = 6.73 ± 0.83 vs. control PLN index = 2.93 ± 0.53). Proliferation of B lymphocytes was considered a major contributor to the PLN enlargement. A marked increase in IgM and IgG secreting cells was observed following inoculation of BALB/c mice with 1 mg DPH. A maximal increase was observed 10 days after treatment (Gleichmann et al. 1982).

Male C3H/HeN mice were given intraperitoneal injections of DPH (10 mg/mL, once per day) for 28 days and immunized with 100 μg KLH on day 14 and 21. Serum levels of anti-KLH IgG and IgE antibodies were determined on day 28. The KLH-specific IgE response was significantly increased compared to control (data in graph); the IgG response was not changed. Plasma ACTH and corticosterone were significantly higher in DPH-treated mice as compared to controls (data not provided) (Okada et al. 2001).

In vitro data with cells or cell lines

Splenocytes from DPH-treated mice (10 mg/mL for 28 days) immunized with KLH were cultured for 3 days with 50 or 100 μg/mL KLH. No effect on proliferation was noted in splenocytes from DPH-treated mice at either concentration of KLH. Comparatively, splenocytes from control mice immunized with KLH showed a potent proliferative response to stimulation with 50 or 100 μg/mL KLH. T cell function was also impaired in splenocytes from DPH-treated mice, in response to nonspecific mitogens (ConA and LPS) and in response to cross-linking of CD3. The accessory cell function (e.g. macrophages) was also impaired in spleen cells from DPH-treated mice. IL-4 production was significantly enhanced, while IFN-γ and IL-2 production, and NK cell activity were significantly reduced in spleen cells from DPH-treated mice (data in graphs or not provided). IL-1α production was decreased in spleen adherent cells from DPH-treated mice stimulated with *S. aureus*. No effect on IL-6 or IL-12 levels was reported (Okada et al. 2001).

The offspring of female C3H Orleans mice treated with 25 mg/kg diphenylhydantoin 2 times/day throughout gestation, exhibited a reduced thymic cortex and low mitotic activity in the lymphoid population. The reticuloepithelial tissue was enlarged. In the spleen, the white pulp was enlarged due to lymphocyte accumulation. The dams did not

exhibit any changes in the thymus or spleen following treatment (Kohler et al. 1987).

Mode of action information

Heat shock proteins were not induced in the PLNs in female BALB/c mice injected subcutaneously with 2 mg DPH (Albers et al. 1996).

Male ICR mice injected intraperitoneally with 60 mg diphenylhydantoin for 3, 8 and 30 days exhibited elevated levels of serum glucocorticoids and thymic atrophy throughout the experiment (Hirai and Ichikawa 1991).

References

Albers R, van der Pijl A, Bol M, Bleumink R, Seinen W, Pieters R. 1999. Distinct immunomodulation by autoimmunogenic xenobiotics in susceptible and resistant mice. Toxicology and applied pharmacology 160:156–62; doi:10.1006/taap.1999.8761.

Albers R, van der Pijl A, Bol M, Seinen W, Pieters R. 1996. Stress proteins (HSP) and chemical- induced autoimmunity. Toxicology and applied pharmacology 140: 70–6.

Andrade-Mena CE, Sardo-Olmedo JA, Ramirez-Lizardo EJ. 1994. Effects of phenytoin administration on murine immune function. Journal of neuroimmunology 50: 3–7.

Badawy AH, Shalaby SA, Abdel Aal SF. 1991. Hydantoin immunosuppression clinical study. Journal of the Egyptian Society of Parasitology 21: 257–62.

Basaran N, Hincal F, Kansu E, Ciger A. 1994. Humoral and cellular immune parameters in untreated and phenytoin-or carbamazepine-treated epileptic patients. International journal of immunopharmacology 16: 1071–7.

Basaran N, Kansu E, Hincal F. 1989. Serum immunoglobulins, complement levels and lymphocyte subpopulations in phenytoin-treated epileptic patients. Immunopharmacology and immunotoxicology 11:335–46; doi:10.3109/08923978909005374.

Beghi E, Shorvon S. 2011. Antiepileptic drugs and the immune system. Epilepsia 52 Suppl 3:40– 4; doi:10.1111/j.1528-1167.2011.03035.x.

Burks AW, Charlton R, Casey P, Poindexter A, Steele R. 1989. Immune function in patients treated with phenytoin. Journal of child neurology 4:25–9; doi:10.1177/088307388900400104.

Chapman JR, Roberts DW. 1984. Humoral immune dysfunction as a result of prenatal exposure to diphenylhydantoin: correlation with the occurrence of physical defects. Teratology 30:107–17; doi:10.1002/tera.1420300115.

Gleichmann H, Pals S, Radaszkiewicz T, Wasser M. 1982. T cell-dependent B cell lymphoproliferation and activation induced by the drug diphenylhydantoin. Advances in experimental medicine and biology 149: 617–22.

Godhwani N, Bahna SL. 2016. Antiepilepsy drugs and the immune system. Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology 117:634–640; doi:10.1016/j.anai.2016.09.443.

Hirai M, Ichikawa M. 1991. Changes in serum glucocorticoid levels and thymic atrophy induced by phenytoin administration in mice. Toxicology letters 56: 1–6.

Kohler C, Jeanvoine G, Pierrez J, Olive D, Gerard H. 1987. Modifications of the thymus and splenic thymic dependent zones after in utero exposure to phenytoin: qualitative

and quantitative analysis in C3H mice. Developmental pharmacology and therapeutics 10: 405–12.
Modeer T, Karsten J, Weintraub A, Gidlund M, Sundqvist KG. 1989. Phenytoin induces interleukin-1 production in vitro. Life sciences 44: 35–40.
Okada K, Sugiura T, Kuroda E, Tsuji S, Yamashita U. 2001. Phenytoin promotes Th2 type immune response in mice. Clinical and experimental immunology 124: 406–13.

Acetonitrile [CASRN 75-05-8]

Human Data

Data from epidemiology studies

No data were located.

In vitro data with cells or cell lines

No data were located.

Mode of action information

No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

In F344/N rats exposed to acetonitrile by inhalation for 13 weeks, gross and histophathologic changes were evaluated in males (800 and 1600 ppm) and females (1600 ppm) that died during the study. Changes reported included thymic atrophy and splenic lymphoid depletion. Decreased absolute and relative thymus weights also were reported in male and female rats (LOAEL =
800 ppm). In F344/N rats exposed to 100, 200, or 400 ppm acetonitrile for 2 years, no immune related effects were reported (National Toxicology Program 1996)

In B6C3F1 mice exposed to acetonitrile by inhalation for 13 weeks, lymphoid depletion and lymphocytolysis in the thymus, spleen and bone marrow was reported in animals that died. A lack of immune effects were reported in mice exposed to acetonitrile for 2 years (NOAEL = 200 ppm) (National Toxicology Program 1996).

Based on a 14-day inhalation study in B6C3F1 mice (doses not provided), acetonitrile was not identified as an immunotoxicant (Luster et al. 1992).

Male Wistar rats were subcutaneously injected with acetonitrile at a dose of 0.8 LD50 (dose not provided). Antibody titer to sheep erythrocytes was decreased by 43%. Additionally, the number of antibody producing cells against sheep erythrocytes and Vi-

Ag (no further information provided in article) were decreased by 52% and 27%, respectively. Thymus T-cell count, percentage of natural cytotoxicity (used as a surrogate for NK cell activity), and antibody- dependent cell cytotoxicity also were significantly decreased after acetonitrile exposure. The percentage decreases were calculated as 31%, 52%, and 41%, respectively (Zabrodskii et al.
2002).

In vitro data with cells or cell lines

No data were located.

Mode of action information

No data were located.

References

Luster MI, Portier C, Pait DG, White KL, Gennings C, Munson AE, et al. 1992. Risk assessment in immunotoxicology. I. Sensitivity and predictability of immune tests. Fundam Appl Toxicol 18: 200–210.

National Toxicology Program. 1996. Toxicology and carcinogenesis studies of acetonitrile (CAS NO. 75-05-8) in F44/N rats and B6C3F1 mice (inhalation studies).

Zabrodskii PF, Germanchuk VG, Kirichuk VF, Birbin VS, Chuev AN. 2002. Combined effects of toxicants with various mechanisms of action and mechanical trauma on the immune system. Bulletin of experimental biology and medicine 133: 594–6.

Benzo(a)pyrene [B(a)P] [CASRN 50-32-8]

Human Data

Data from epidemiology studies

No data were located

In vitro data with cells or cell lines

B(a)P (1 μM) and related metabolites significantly increased IgE-mediated histamine release from human basophils, but did not induce cell death. Additionally, a B(a)P metabolite significantly increased IgE-mediated IL-4 production in human basophils (Kepley et al. 2003). In primary human macrophages, 10 μM B(a)P increased expression of TNF-α and IL-1ß and produced no effect on IFNγ, IL-6, or IL-12 expression (Lecureur et al. 2005). Comparatively, B(a)P did not modulate IL-6 or IL-8 production in BEAS-2B cells at concentrations ranging from 0.1 to 10 μM (Chowdhury et al. 2017).

B(a)P inhibited anti-CD3 antibody stimulation of human lymphocyte proliferation (IC50 =

12.82 μM) (Carfi et al. 2007).

Six breast epithelial cell strains were incubated with 4μM B(a)P for 24 hours. Gene expression studies (using Hu-Gene 133A arrays) showed that signal log ratio (SLR) was altered by ≥1.5 for 5 immune-related genes in at least one of the tested cell strains. Four genes were upregulated, while one was down regulated. Up regulated genes were IL1B, MAL, HTLF, and SECTM1.

CXCL14 gene expression was down-regulated (John et al. 2009).

PBMCs were exposed to ConA and B(a)P and assessed after 3 days. B(a)P dose-dependently decreased DNA synthesis and cell viability in treated cells (LOAELs = 0.01 and 0.1 μM, respectively). The number of cells recovered during the same period also was decreased (LOAEL = 0.01 μM). B(a)P did not affect IL-2 activity or expression of CD25 on small cells or blasts at concentrations up to 1 μM. B(a)P decreased the percentage of blasts that were CD71+ by 13% at 1 μM. Cell cycle analysis indicated that B(a)P increased the percentage of cells in S- phase and decreased the percentage in G0/G1 phase (Mudzinski 1993).

Mode of action information

Calcium mobilization in human T-cells is a proposed mode of action for B(a)P (Krieger et al. 1994). Additionally, Ah receptor activation by B(a)P is proposed to inhibit differentiation of monocytes to macrophages and cell growth of B-cells which may contribute to immunotoxic effects (Allan and Sherr 2005, 2010; van Grevenynghe et al. 2003).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

Lactating C3H/HeJ dams were dosed with 0.25, 5.0, or 100 pmol/week B(a)P via oral gavage on PND 1, 8, and 15. Pups (5-weeks old) were treated with OVA via intratracheal instillation every

2 weeks for 6 weeks. B(a)P had no effect on the number of macrophages or lymphocytes in BAL from male or female offspring not treated with OVA. Additionally, no effect was noted on the number of macrophages or lymphocytes in B(a)P-treated offspring that were immunized with OVA (when compared with offspring only treated with OVA). IL-4, IL-5, IL-13, IL-33, and

IFN-γ levels in the BAL were not affected in offspring not treated with OVA. Increased IL-33 and IFN-γ levels were observed in OVA-sensitized female offspring lactationally exposed to 5.0 and 100 pmol/week B(a)P, respectively. Lactational exposure to 0.25 B(a)P increased the total number of mediastinal lymph node cells in males. Lactational 100 pmol/week B(a)P increased numbers of TCRβ+ and CD86+ cells compared with vehicle in non-sensitized male offspring. In non-sensitized female offspring, lactational exposure to 100 pmol/week B(a)P increased numbers of CD11c+ PDCA-1-, CD28+, TCRβ+CD28+, MHC Class II+, and MHC Class II+CD86+ cells.

In OVA-sensitized female offspring, a significant increase in CD11c+PDCA-1+ and CD11c+PDCA-1- cells was observed after exposure to 0.25 and 5.0 pmol/week, respectively (Yanagisawa et al. 2018).

Pregnant C3H/HeB mice were administered 150 mg/kg B(a)P via intraperitoneal injection on GD 11; immune effects were assessed at parturition and again one week after parturition. A significant reduction in newborn CD4+CD8+ (46%), CD4+CD8+Vγ2+ (60%), and CD4+CD8+Vβ2+ (53%) thymocytes were noted. Additionally, CD4+ splenocytes from 1-week- old offspring were significantly reduced (50%) (Rodriguez et al. 1999).

B6C3F1 mice were administered 0.4, 4.0, or 40 mg/kg B(a)P by intratracheal instillation for seven days and immunized with sheep erythrocytes after the last B(a)P exposure. Decreased formation of antigen-specific AFC (by 60%) was observed at 40 mg/kg B(a)P in LALN. When sheep erythrocytes were administered by intraperitoneal injection, an increase in antigen-specific AFC was observed at 40 mg/kg B(a)P in LALN. However, the levels of AFC in the spleen were decreased (Schnizlein et al. 1987).

B6C3F1 mice (3-6 months, 13-16 months, and 23-26 months) were administered 40 mg/kg B(a)P for 8 days by intraperitoneal injection. Mice also were immunized with sheep erythrocytes after day 4 of the B(a)P treatment. Spleens were removed and

splenocytes assessed for formation of AFCs. Decreased formation of AFCs was noted in splenocytes from all three age groups. In two sets of experiments, the observed decreases were 23%-43% in mice ages 3-6 months, 63%- 84% in mice ages 16-18 months, and 93% in mice ages 23-26 months (Lyte and Bick 1985).

B6C3F1 mice were administered 5, 20, or 40 mg/kg B(a)P for 14 days by subcutaneous injection. Spleens were removed and ConA-induced production of IL-2 and IL-3 were assessed. While splenocyte IL-2 production was decreased in a dose dependent manner, no effect on splenocyte IL-3 production was noted. As shown in other studies, B(a)P decreased responses to sheep erythrocytes (>95% inhibition). Addition of exogenous IL-2 to the treated splenocytes, reversed the B(a)P-induced inhibition of responses to sheep erythrocytes (Lyte et al. 1987; Lyte and Bick 1986).

Female B6C3F1 mice were administered 10 subcutaneous injections of B(a)P over a 14-day period at doses of 5, 20, or 40 μg/g. KLH-sensitization did not affect delayed hypersensitivity

responses at the tested doses. Additionally, B(a)P treatment did not induce rejection to DBA mice skin that was grafted onto mice. Proliferative responses to PHA were dose-dependently decreased (LOAEL = 20 μg/g B(a)P). Spontaneous and LPS-induced proliferative responses were increased at 5 μg/g B(a)P and significantly decreased at 40 μg/g B(a)P. MLC responses, and the percentage of spleen cells with T- and B-cell surface markers were not significantly affected at any of the tested doses. Additionally, NK cell activity against YAC- I target cells was not impacted in mice treated with 40 μg/g B(a)P (data not provided). Serum IgG levels were dose-dependently decreased in treated mice (18-24%). A reduction in the number of antibody plaque forming cells to sheep erythrocytes and LPS were noted (LOAELs = 20 and 5 μg/g B(a)P, respectively). B(a)P exposure decreased response to TNP-Ficoll without effects on TNP-LPS response. Host resistance studies showed that B(a)P had no effect on PYB6 tumor incidence or susceptibility to *L. monocytogenes* (Dean et al. 1983).

In vitro data with cells or cell lines

Rat and mouse spleen cells were treated with B(a)P for 24 hours and then LPS or PHA for 48 hours. Then, mitogen stimulation was assessed by 5-bromo-2'-deoxyuridine incorporation. B(a)P inhibited cellular proliferation for both species at similar concentrations (data in graphs). B(a)P also inhibited rat spleen proliferation that was stimulated by ConA (data provided in graph).

B(a)P inhibited anti-CD3 antibody stimulation of mouse lymphocyte proliferation (IC50 > 160 μM) (Carfi et al. 2007).

B(a)P decreased viability of mouse antigen presenting cells (APC) and increased expression of CD86 expression on APC (LOAELs = 0.1 μM). In murine splenocytes, B(a)P decreased cell viability and proliferation (LOAELs = 0.1 and 1.0 μM, respectively). B(a)P did not modulate the expression of T-cell receptors or CD19 at any of the tested concentrations in murine splenocytes (Chowdhury et al. 2017).

B(a)P decreased ConA induced cellular proliferation of mouse splenic T-cells in a dose-dependent manner (LOAEL = 0.1 μg/mL). Inhibition of IL-2, IL-4, and IFN-γ also was observed in ConA-stimulated splenic T-cells (LOAELs = 0.1, 0.2, and 0.1 μg/mL, respectively) (Guan et al. 2017).

B(a)P inhibited spleen cell response to sheep erythrocytes in a concentration dependent manner (LOAEL = 0.01 μM). B(a)P also inhibited one-way mixed lymphocyte response with a maximal inhibition of 19% (Urso et al. 1986). Similar response of murine spleen cell response to sheep erythrocytes was reported by Kawabata and White (1987) (LOAEL = 1 nM) after incubation for 5 days.

Splenocytes from B6C3F1 mice (3-6 months and 23-26 months) were exposed to 1, 10, or

50 μg/mL B(a)P and sheep erythrocytes for 4-5 days. After end of exposure period, the number of AFCs was determined. Dose-dependent decrease in the number of cells was observed in splenocytes from both age groups (data in graphs) (Lyte and Bick 1985).

B(a)P (in PVP-NaCl) dose-dependently increased LPS-induced IL-1 production by peritoneal exudate macrophages isolated from B6C3F1 mice; tested concentrations ranged from 25 to 800

μg/mL. A concurrent decrease in cell viabilities was noted at the same test concentrations. Comparatively, when B(a)P was dissolved in corn oil no effect on IL-1 production or cell viabilities was noted (Lyte and Bick 1986).

Mode of action information

Disruption of T-cell activities has been associated with B(a)P induced immunotoxic effects (Urso et al. 1986). Modulation of mouse splenic T-cell effects was associated with modulation of calcium levels; which was associated with suppression of the NF-□B and NFAT pathways (Guan et al. 2017).

In addition to T-cell effects, modulation of B-cell population or responses, or macrophage functions also have been implicated in B(a)P mode of action (Saxena et al. 2018; Urso et al. 1986). Hardin and colleagues (1992) proposed that B(a)P-induced suppression of B-cell lymphopoiesis was, partially, produced through induction of programmed cell death. Ah-receptor dependent- and/or independent-pathways could produce the observed effects.

References

Allan LL, Sherr DH. 2005. Constitutive activation and environmental chemical induction of the aryl hydrocarbon receptor/transcription factor in activated human B lymphocytes. Molecular pharmacology 67:1740–50; doi:10.1124/mol.104.009100.

Allan LL, Sherr DH. 2010. Disruption of human plasma cell differentiation by an environmental polycyclic aromatic hydrocarbon: a mechanistic immunotoxicological study. Environmental health : a global access science source 9:15; doi:10.1186/1476-069x-9-15.

Carfi M, Gennari A, Malerba I, Corsini E, Pallardy M, Pieters R, et al. 2007. In vitro tests to evaluate immunotoxicity: a preliminary study. Toxicology 229:11–22; doi:10.1016/j.tox.2006.09.003.

Chowdhury PH, Kitamura G, Honda A, Sawahara T, Hayashi T, Fukushima W, et al. 2017. Synergistic effect of carbon nuclei and polyaromatic hydrocarbons on respiratory and immune responses. Environmental toxicology 32:2172–2181; doi:10.1002/tox.22430.

Dean JH, Luster MI, Boorman GA, Lauer LD, Leubke RW, Lawson L. 1983. Selective immunosuppression resulting from exposure to the carcinogenic congener of

benzopyrene in B6C3F1 mice. Clinical and experimental immunology 52: 199–206.
Guan S, Huang Y, Feng Z, Xu L, Jin Y, Lu J. 2017. The toxic effects of benzo[a]pyrene on activated mouse T cells in vitro. Immunopharmacology and immunotoxicology 39:117–123; doi:10.1080/08923973.2017.1299173.
Hardin JA, Hinoshita F, Sherr DH. 1992. Mechanisms by which benzo[a]pyrene, an environmental carcinogen, suppresses B cell lymphopoiesis. Toxicology and applied pharmacology 117: 155–64.
John K, Keshava C, Richardson DL, Weston A, Nath J. 2009. Immune response signatures of benzo{alpha}pyrene exposure in normal human mammary epithelial cells in the absence or presence of chlorophyllin. Cancer genomics & proteomics 6: 1–11.

Kawabata TT, White KL Jr. 1987. Suppression of the vitro humoral immune response of mouse splenocytes by benzo(a)pyrene metabolites and inhibition of benzo(a)pyrene-induced immunosuppression by alpha-naphthoflavone. Cancer research 47: 2317–22.

Kepley CL, Lauer FT, Oliver JM, Burchiel SW. 2003. Environmental polycyclic aromatic hydrocarbons, benzo(a) pyrene (BaP) and BaP-quinones, enhance IgE-mediated histamine release and IL-4 production in human basophils. Clinical immunology (Orlando, Fla) 107: 10–9.

Krieger JA, Born JL, Burchiel SW. 1994. Persistence of calcium elevation in the HPB-ALL human T cell line correlates with immunosuppressive properties of polycyclic aromatic hydrocarbons. Toxicology and applied pharmacology 127:268–74; doi:10.1006/taap.1994.1161.

Lecureur V, Ferrec EL, N'Diaye M, Vee ML, Gardyn C, Gilot D, et al. 2005. ERK-dependent induction of TNFalpha expression by the environmental contaminant benzo(a)pyrene in primary human macrophages. FEBS letters 579:1904–10; doi:10.1016/j.febslet.2005.01.081.

Lyte M, Bick PH. 1985. Differential immunotoxic effects of the environmental chemical benzo[a]pyrene in young and aged mice. Mechanisms of ageing and development 30: 333–41.

Lyte M, Bick PH. 1986. Modulation of interleukin-1 production by macrophages following benzo(a)pyrene exposure. International journal of immunopharmacology 8: 377–81.

Lyte M, Blanton RH, Myers MJ, Bick PH. 1987. Effect of in vivo administration of the carcinogen benzo(a)pyrene on interleukin-2 and interleukin-3 production. International journal of immunopharmacology 9: 307–12.

Mudzinski SP. 1993. Effects of benzo[a]pyrene on concanavalin A-stimulated human peripheral blood mononuclear cells in vitro: inhibition of proliferation but no effect on parameters related to the G1 phase of the cell cycle. Toxicology and applied pharmacology 119:166–74; doi:10.1006/taap.1993.1057.

Rodriguez JW, Kirlin WG, Wirsiy YG, Matheravidathu S, Hodge TW, Urso P. 1999. Maternal exposure to benzo[a]pyrene alters development of T lymphocytes in offspring. Immunopharmacology and immunotoxicology 21:379–96;

doi:10.3109/08923979909052769.

Saxena N, Kaur AP, Chandra NC. 2018. Differential Response of B Cells to an Immunogen, a Mitogen and a Chemical Carcinogen in a Mouse Model System. Asian Pacific journal of cancer prevention : APJCP 19:81–90; doi:10.22034/apjcp.2018.19.1.81.

Schnizlein CT, Munson AE, Rhoades RA. 1987. Immunomodulation of local and systemic immunity after subchronic pulmonary exposure of mice to benzo(a)pyrene. International journal of immunopharmacology 9: 99–106.

Urso P, Gengozian N, Rossi RM, Johnson RA. 1986. Suppression of humoral and cell-mediated immune responses in vitro by benzo(a)pyrene. Journal of immunopharmacology 8: 223–41.

van Grevenynghe J, Rion S, Le Ferrec E, Le Vee M, Amiot L, Fauchet R, et al. 2003. Polycyclic aromatic hydrocarbons inhibit differentiation of human monocytes into macrophages. Journal of immunology (Baltimore, Md : 1950) 170: 2374–81.

Yanagisawa R, Koike E, Win-Shwe TT, Ichinose T, Takano H. 2018. Effects of lactational exposure to low-dose BaP on allergic and non-allergic immune responses in mice offspring. Journal of immunotoxicology 15:31–40; doi:10.1080/1547691x.2018.1442379.

Cadmium Chloride [CASRN 10108-64-2]

Human Data

Data from epidemiology studies

No data were located.

In vitro data with cells or cell lines

Cadmium chloride (10-100 μM) inhibited NK (against K562 cells) and antibody-dependent cellular (against P815 cells) cytotoxicity (ADCC) in peripheral blood lymphocytes in a concentration-dependent manner. The estimated 50% inhibition doses (ID50) for NK and ADCC activities were 50 and 100 μM, respectively. NK and ADCC activities were not significantly affected by changing the effector cell:target cell ratios. Cadmium chloride also inhibited cytotoxic activity against K562 or Daudi cells in activated IL-2 cells (data in graph). Time- course studies showed that a significant decrease in NK and ADCC activities was observed when added at 90 minutes after the start of the experiment (Cifone et al. 1990).

Viability of A549 cells was decreased (44.5% of control) after exposure to 75 μM cadmium chloride. At the same concentration, cadmium chloride increased select cytokine levels (e.g., IFN-γ, IL-3, IL-5, IL-10, IL-15, and IL-16). Comparatively, cadmium chloride decreased TGF- ß3 levels (Odewumi et al. 2016).

Mode of action information

In vitro studies suggest that in peripheral blood lymphocytes, cadmium chloride modulated phosphoinositide hydrolysis induced by a target molecule. This modulation is proposed to lead to inhibited NK activity (Cifone et al. 1990).

Proposed direct action of cadmium on immunocompetent cells stimulates production and release of cytokines, which may produce proinflammatory effects (Marth et al. 2000).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

Female BDF1 mice were provided drinking water containing 5, 10, or 50 μg/mL cadmium (as cadmium chloride) for 3 weeks. Antibody response to sheep erythrocytes was decreased in a dose-dependent manner. Splenic plaque-forming cell number decreased by 16% to 28%. A dose- dependent increase in LPS-induced proliferation also was observed (LOAEL = 10 μg/mL). In the absence of a mitogen, cadmium chloride also increased lymphocyte proliferation (LOAEL = 10 μg/mL). No effect was observed when ConA mitogen was used to stimulate proliferation (Blakley 1985).

Female CD1 mice were provided drinking water containing 5, 10, or 50 μg/mL cadmium (as cadmium chloride) for 3 weeks. *In vivo* T-lymphocyte independent (against DNP-Ficoll) and T-

lymphocyte and macrophage independent (against *E. coli*) responses were increased by cadmium exposure (Blakley and Tomar 1986).

Female BDF1 mice were provided drinking water containing 50 μg/mL cadmium (as cadmium chloride) for 3 weeks. Spleen cell suspensions from pooled spleens were separated by adherence techniques and antibody production against sheep erythrocytes was assessed. Suppressed antibody production (26%-34%) was noted in cultures that contained cadmium-exposed T- lymphocytes. Antibody production was similar to controls in cultures that contained cadmium- exposed macrophages (Blakley and Tomar 1986).

Male Sprague-Dawley rats were administered 0.7 or 6 mg/kg cadmium (as cadmium chloride) by oral gavage for 28 days. Splenocyte proliferation was significantly decreased (76% of control) in rats that were administered 6 mg/kg cadmium. Splenocyte IL-2 production also was increased after administration of 6 mg/kg cadmium, when production was normalized with cell number.

No effect was noted on splenocyte IFN-γ production (Wang et al. 2017).

Immunotoxic effects in offspring were noted after exposure to cadmium chloride *in utero* or through milk. In ICR mice administered 2.5 or 5.0 mg/kg cadmium chloride on GD 16, a significant increase in offspring spleen weight was reported (LOAEL = 2.5 mg/kg).

Unstimulated spleen lymphocyte proliferation was significantly increased at both tested doses (1.5- to 2-fold). Additionally, ConA, PHA, and LPS stimulation was increased in treated animals (LOAELs = 5.0, 5.0, and 2.5 mg/kg). No effect on delayed-type hypersensitivity to sheep erythrocytes was reported, but an increase in total Ig and IgM antibody titer was noted at 2.5 mg/kg (Soukupova et al. 1991). In offspring that were exposed to cadmium chloride through maternal milk (dams received 5 ppm or 10 ppb cadmium chloride in water until weaning) decreased spleen weights were observed in females, but not males (data in graphs). The effect was greater in lower dosed females. Effects on organ weight did not persist to adulthood. In adult and juvenile rats, effects on cytotoxic activity of splenic NK-cells was noted (data in graphs). Additionally, cadmium chloride inhibited ConA-induced thymocyte proliferation in both male and female adult rats (Pillet et al. 2005).

Female C57BL/6 mice were exposed (nose-only) to aerosolized cadmium chloride (60-minute exposure to 0.88 mg Cd/m^3) and examined 5-18 days later. Decreased splenic cell viability was observed (data in graph). Significant decreases of proliferative responses to LPS and PHA, and inhibition of IgM secretion in response to sheep erythrocytes were observed. Comparatively, oral chronic exposure (5, 100, or 300 ppm cadmium chloride in water for 12-16 weeks) suppressed IgM response to sheep erythrocytes, without effects on cell viability (Krzystyniak et al. 1987).

In vitro data with cells or cell lines

Splenocytes isolated from male Sprague-Dawley rats were treated with ConA for 24 hours, followed by incubation with 5, 10, or 20 μM cadmium chloride for 4 or 24 hours. After exposure for 4 hours, decreased IL-2 (LOAEL = 5 μM) and IFN-γ (LOAEL = 10 μM) production was observed in the absence of effects on cell proliferation. After exposure for 24 hours, decreased IL-2 (LOAEL = 5 μM) production and cell proliferation (LOAEL = 10 μM) were observed.

When cytokine production after 24-hour exposure was normalized based on cell number,

increased IFN-γ production (LOAEL = 10 μM) was noted. For IL-2 production, a significant decrease was noted at 5 μM and an increase was noted at 20 μM (Wang et al. 2017).

Mode of action information

In RAW264.7 cells, cadmium chloride upregulation of COX-2 and MIP-2 was associated with activation of the phosphatidylinositol 3-kinase/Akt signaling pathway (Huang et al. 2014).

Cadmium chloride also may induce overstimulation of nuclear factors of activated T-cells to activate Jurkat T cells (Colombo et al. 2004).

References

Blakley BR. 1985. The effect of cadmium chloride on the immune response in mice. Canadian journal of comparative medicine : Revue canadienne de medecine comparee 49: 104–8.

Blakley BR, Tomar RS. 1986. The effect of cadmium on antibody responses to antigens with different cellular requirements. International journal of immunopharmacology 8: 1009–15.

Cifone MG, Procopio A, Napolitano T, Alesse E, Santoni G, Santoni A. 1990. Cadmium inhibits spontaneous (NK), antibody-mediated (ADCC) and IL-2-stimulated cytotoxic functions of natural killer cells. Immunopharmacology 20: 73–80.

Colombo M, Hamelin C, Kouassi E, Fournier M, Bernier J. 2004. Differential effects of mercury, lead, and cadmium on IL-2 production by Jurkat T cells. Clinical immunology (Orlando, Fla) 111:311–22; doi:10.1016/j.clim.2004.02.005.

Huang YY, Xia MZ, Wang H, Liu XJ, Hu YF, Chen YH, et al. 2014. Cadmium selectively induces MIP-2 and COX-2 through PTEN-mediated Akt activation in RAW264.7 cells. Toxicological sciences : an official journal of the Society of Toxicology 138:310–21; doi:10.1093/toxsci/kfu013.

Krzystyniak K, Fournier M, Trottier B, Nadeau D, Chevalier G. 1987. Immunosuppression in mice after inhalation of cadmium aerosol. Toxicology letters 38: 1–12.

Marth E, Barth S, Jelovcan S. 2000. Influence of cadmium on the immune system.

Description of stimulating reactions. Central European journal of public health 8: 40–4.

Odewumi CO, Latinwo LM, Ruden ML, Badisa VL, Fils-Aime S, Badisa RB. 2016. Modulation of cytokines and chemokines expression by NAC in cadmium chloride treated human lung cells. Environmental toxicology 31:1612–1619; doi:10.1002/tox.22165.

Pillet S, Rooney AA, Bouquegneau JM, Cyr DG, Fournier M. 2005. Sex-specific effects of neonatal exposures to low levels of cadmium through maternal milk on development and immune functions of juvenile and adult rats. Toxicology 209:289–301; doi:10.1016/j.tox.2004.12.007.

Soukupova D, Dostal M, Piza J. 1991. Developmental toxicity of cadmium in mice. II. Immunotoxic effects. Functional and developmental morphology 1: 31–6.

Wang P, Wang J, Sun YJ, Yang L, Wu YJ. 2017. Cadmium and chlorpyrifos inhibit cellular immune response in spleen of rats. Environmental toxicology 32:1927–1936; doi:10.1002/tox.22415.

Dibromoacetic Acid (DBAA) [CASRN 631-61-1]

Human Data

Data from epidemiology studies

No studies were located.

In vitro data with cells or cell lines

In cultured PBMCs collected from healthy, non-smoking volunteers and cultured in DBAA for four hours, DBAA increased the percentage of necrotic human PBMC and decreased PBMC cell size (LOAEL = 5 mM). Increases in the percentage of apoptotic cells and PBMC granulation also was reported (LOAEL = 1 and 5mM, respectively). Caspase-8, -9, and -3 expression were upregulated at 1 and 5 mM. Increased transmembrane mitochondrial potential and levels of reactive oxygen species (ROS) also were noted with DBAA exposure (LOAEL = 1 and 0.1 mM) (Michalowicz et al. 2015).

Mode of action information

DBAA may increase ROS levels and transmembrane mitochondrial potentials (Michalowicz et al. 2015).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

In female F344/N rats exposed to DBAA for 3 months (0-2000 mg/L in drinking water), minimal to mild hematopoietic cell proliferation was noted at the highest dose. A similar effect was not observed in males. While no spleen effects were noted in B6C3F1 mice exposed to DBAA for 3 months (0-2000 mg/L in drinking water), thymus atrophy was reported in males and females (LOAEL = 1000 and 2000 mg/L, respectively) (National Toxicology Program 2007).

In male and female BALB/c mice orally gavaged with 5, 20, or 50 mg/kg DBAA for 28 days altered spleen and thymus weights, and splenic and thymic cellularity were reported. DBAA also inhibited B-cell proliferation (LOAEL = 20 mg/kg). DBAA

increased T-cell mitogenesis (value not provided) at 20 mg/kg. DBAA increased apoptosis in spleen and thymus in a dose-dependent manner (values not provided). Additionally, DBAA exposure altered the expression of apoptosis- related genes in spleens and thymus of treated mice. In the thymus, expression of Fas and TRAF2 were altered (2-2.5 fold). In spleens of treated mice, expression of Fas and TRAF2 were increased 5-fold while bcl-2 expression was decreased 1.5-fold. Increased protein expression of Fas and FasL also were observed in spleen and thymus of treated mice (LOAEL = 5 mg/kg) (Gao et al. 2008).

Table 1. Data from Gao et al. (2008)

Endpoint	0 mg/kgs	5 mg/kg	20 mg/kg	50 mg/kg
Male				
Spleen weight (mg)	80.5 ± 2.7	88.7 ± 3.7	91.2 ± 3.4**	94.0 ± 2.5***
Thymus weight (mg)	43.1 ± 3.4	37.6 ± 2.0	33.3 ± 2.8**	33.4 ± 2.3**
Relative spleen weight (mg/g)	3.68 ± 0.14	3.87 ± 0.22	4.18 ± 0.20*	4.23 ± 0.09*
Relative thymus weight (mg/g)	1.85 ± 0.14	1.65 ± 0.09	1.50 ± 0.13**	1.50 ± 0.11**
Splenic cellularity ($x10^7$)	9.00 ± 0.44	9.09 ± 0.28	7.63 ± 0.65	6.11 ± 0.38***
Thymic cellularity ($x10^7$)	8.88 ± 1.06	9.16 ± 0.28	7.39 ± 0.47	5.37 ± 0.82**
Female				
Spleen weight (mg)	78.9 ± 2.2	100.1 ± 7.7**	102.4 ± 5.0**	101.2 ± 4.8**
Thymus weight (mg)	46.9 ± 3.7	47.3 ± 3.9	35.8 ± 2.3*	29.5 ± 3.3***
Relative spleen weight (mg/g)	3.99 ± 0.18	5.33 ± 0.45**	5.29 ± 0.27**	5.22 ± 0.19**
Relative thymus weight (mg/g)	2.39 ± 0.17	2.41 ± 0.18	1.87 ± 0.11*	1.55 ± 0.17***
Splenic cellularity ($x10^7$)	8.60 ± 0.55	8.28 ± 1.19	6.14 ± 1.27	4.65 ± 0.43**
Thymic cellularity ($x10^7$)	7.97 ± 0.53	7.08 ± 0.74	5.42 ± 0.79*	4.28 ± 0.39***

Data are presented as mean ± SEM.

***p < 0.001, **p < 0.01, *p < 0.05, significance assessed by ANOVA when compared with control group (DBA 0 mg/kg).

Increased neuronal expression of immune factors was noted in Sprague-Dawley rats administered 20, 50, or 125 mg/kg DBAA via intragastric injection for 4-weeks. mRNA

expression of Iba-1, NK-□B, IL-6, IL-1ß, and TNF-α were increased in the pre-frontal cortex and hippocampus of treated rats (LOAEL = 50 mg/kg for all brain regions). Protein levels of Iba-1, NK-□B, IL-6, IL-1ß, and TNF-α also were significantly increased in the same brain regions.

Protein expression LOAEL in the pre-frontal cortex for Iba-1, NK-□B, IL-6, IL-1ß, and TNF-α was 50 mg/kg. Protein expression LOAEL In the hippocampus was 100 mg/kg for NK-□B and 50 mg/kg for other evaluated cytokines (Jiang et al. 2017).

Female B6C3F1 mice were given drinking water with 125, 500, or 1000 mg/L for 28 days. A significant decrease in thymus weight was noted at 500 and 1000 mg/L (19%). No effect on absolute or relative spleen weight, or relative thymus weight was reported. A non-dose response decrease (19%) in total spleen cell number and number of CD+CD- T-lymphocytes (13%) was observed at 500 and 125 mg/L, respectively. A significant decrease in absolute (38%) and percent (22%) NK1.1+CD3- cells was noted at 500 mg/L. Significant decreases in absolute and percent splenic macrophages also were observed (LOAEL = 500 and 1000 mg/L, respectively). No effects on absolute or percent Ig+, CD3+, CD4-CD+, or CD4+CD8+ markers were noted. No effects on AFC response or IgM antibody titers in response to exposure to sheep red blood cells were noted. Additionally, no impact on response to allogeneic spleen cell stimulation was noted. A significant decrease in cytotoxicity was only observed after splenocyte NK cell activity was augmented with poly-IC; the effect was only observed at 125 mg/L. Host resistance to *Streptococcus pneumoniae*, *Plasmodoium yoelii*, and B16F10 melanoma tumors was not affect by treatment (Smith et al. 2010).

In vitro data with cells or cell lines

DBAA decreased thymocyte (obtained from BALB/c mice) proliferation at exposure lengths of at least 6 hours. At 6 hours, a significant decrease in proliferation was only observed at 40 μM. Comparatively, at 12, 24, and 48 hour exposure periods a significant decrease in proliferation was observed at 5, 10, 20 and 40 μM. DBAA also decreased IL-2 and IL-4 secretion (LOAEL = 10 and 5 μM, respectively). DBAA also increased late and early apoptosis (LOAEL = 5 and

10 μM), without effects on the percentage of necrotic cells. DBAA induced an increase in the percentage of cells in the G0/G1 phase and decreased the percentage of cells in the S phase. Increased intracellular thymocyte calcium levels (LOAEL = 5μM) and thymocyte Fas and FasL protein levels were reported (LOAELs = 10 μM for both proteins). Additionally, bcl-2 protein level was significantly decreased at all tested concentrations (LOAEL = 5 μM) (Gao et al. 2016).

Peritoneal exudate cells, obtained from B6C3F1 mice treated with 125, 500, or 1000 mg/L DBAA for 28 days, were evaluated for their ability to suppress B16F10 melanoma tumor cell proliferation *in vitro.* Treatment did not affect the ability of macrophages obtained from treated animals to suppress proliferation (Smith et al. 2010).

In Cl.Ly1 + 2/-9 cells, non-adherent cloned T-cell line derived from spleen cells from C57BL/6TL+ mice, DBAA (1-40 μM) decreased cell viability after exposure for 24, 48, or 72 hours (LOAEL = 1 μM). An increase in the mean percentage of early, late and total apoptotic cells also was noted (LOAEL = 5 μM) (Zhou et al. 2018).

Mode of action information

Overall, studies suggest that DBAA produces immunotoxic effects through modulation of T-cell mediated cell immunity. T-cell apoptosis, through extrinsic and intrinsic pathways, are proposed to play a role in the mode of action. Apoptosis may occur through a variety of pathways including modulation of transmembrane potential, the Fas/FasL pathway, modulation of intracellular calcium, and cell cycle arrest (Gao et al. 2008; Gao et al. 2016).

References

Gao S, Wang Y, Zhang P, Dong Y, Li B. 2008. Subacute oral exposure to dibromoacetic

acid induced immunotoxicity and apoptosis in the spleen and thymus of the mice. Toxicological sciences : an official journal of the Society of Toxicology 105:331–41; doi:10.1093/toxsci/kfn139.

Gao SY, Zhou XR, Gong TT, Jia LM, Li BX. 2016. Dibromoacetic Acid Induces Thymocyte Apoptosis by Blocking Cell Cycle Progression, Increasing Intracellular Calcium, and the Fas/FasL Pathway in Vitro. Toxicologic pathology 44:88–97; doi:10.1177/0192623315612939.

Jiang W, Li B, Chen Y, Gao S. 2017. The toxic influence of dibromoacetic acid on the hippocampus and pre-frontal cortex of rat: involvement of neuroinflammation response and oxidative stress. Metabolic brain disease 32:2009–2019; doi:10.1007/s11011-017-0095-0.

Michalowicz J, Wroblewski W, Mokra K, Macczak A, Kwiatkowska M. 2015. Comparative study of the effect of chloro-, dichloro-, bromo-, and dibromoacetic acid on necrotic, apoptotic and morphological changes in human peripheral blood mononuclear cells (in vitro study).

Toxicology in vitro : an international journal published in association with BIBRA 29:1416–24; doi:10.1016/j.tiv.2015.05.021.

National Toxicology Program. 2007. Toxicology and carcinogenesis studies of dibromoacetic acid (Cas No. 631-64-1) in F344/N rats and B6C3F1 mice (drinking water studies). National Toxicology Program technical report series 1–320.

Smith MJ, Germolec DR, Luebke RW, Sheth CM, Auttachoat W, Guo TL, et al. 2010. Immunotoxicity of dibromoacetic acid administered via drinking water to female B(6)C(3)F(1) mice. Journal of immunotoxicology 7:333–43; doi:10.3109/1547691x.2010.519744.

Zhou XR, Jiang WB, Zhang YT, Gong TT, Gao SY. 2018. Dibromoacetic acid induced Cl.Ly1+2/-9 T-cell apoptosis and activation of MAPKs signaling cascades. Toxicology in vitro : an international journal published in association with BIBRA 47:156–164; doi:10.1016/j.tiv.2017.11.006.

Dibutyl phthalate (DBP) [CASRN 84-74-2]

Human Data

Data from epidemiology studies

No data were located.

In vitro data with cells or cell lines

DBP significantly decreased phagocytotic capacity of differentiated THP-1 cells at all tested concentrations (LOAEL = 0.001μM). DBP also increased TNF-α secretion (LOAEL = 0.1 μM). Comparatively, DBP had no effect on IL-1ß or IL-8 secretion from differentiated THP-1 cells (NOAEL = 0.001 μM) (Couleau et al. 2015).

High-density microarray studies were conducted using normal human mammary epithelial cell strains obtained from discarded tissues; cells were treated with 1 μM DBP for 10 hours. Gene expression of 29 genes were increased in all four isolated cell strains. Gene expression of 28 genes were decreased in all four isolated cell strains including genes involved in the immune response (TNF-α-induced protein 3; values not provided) (Gwinn et al. 2007).

DBP (tested at 0.1 and 100 μM) increased IL-6, CXCL8, and IL-10 secretion from monocytes/macrophages, isolated from blood of healthy individuals. The cells were, stimulated with *E. coli* lipopolysaccharide (LPS) for 1 hour. Comparatively, DBP did not affect IL-1ß and decreased TNF-α secretion from the cells. For all affected cytokines the LOAEL was 100 μM. For phytohemagglutinin-P (PHA-P) stimulated T cells, DBP decreased IL-2, IL-4, TNF-α and IFN-γ secretion (LOAEL for all cytokines = 100 μM). No effect on IL-6 or IL-10 secretion was observed in the PHA-P stimulated T cells treated with DBP. Metabolism studies indicated that DBP was metabolized to monobutyl phthalate *in vitro*. Additionally, secretion patterns of monobutyl phthalate was similar to those observed for DBP (Hansen et al. 2015).

DBP increased IL-1ß gene expression (as assessed by RT-PCR) in human corneal endothelial cell line B4G12 at all tested concentrations (LOAEL = 1 μM). IL-8 gene expression was increased at 1 and 10 μM (values not provided). IL-ß, IL-8, and IL-6 secretion from cells also was increased. IL-6 and IL-8 LOAEL values were 10 and 5 μM,

respectively. Significant IL-1ß secretion was only observed at 1 µM. [Note: The authors note that secretion for IL-1ß and IL-6 was low and quantification was approximate] (Kruger et al. 2012).

In THP-1 cells, DBP did not induce release of IL-18 (doses tested not provided) or IL-8 (NOAEL = 250 µM), or expression of CD86 (NOAEL = 250 µM). However, DBP did induce IL-8 mRNA expression at 500 µM after exposure for 3 hours (values not provided in paper) (Lourenco et al. 2015).

In HepG2 and L02 (normal human liver) cell lines, DBP (10 µM and 25 µM, respectively) significantly increased levels of mature caspase-1, IL-1ß, and nucleotide oligomerization domain (NOD) like receptor family, pyrin domain containing 3 (NLRP3) (values not provided). KN-62,

a P2X7 receptor inhibitor, attenuated DBP-induced effects on caspase-1, IL-1ß, and NLRP3 (Ni et al. 2016).

In primary human keratinocytes cultured on an amorphous pseudodermis, DBP increased TSLP (thymic stromal lymphopoietin) mRNA expression (Schuepbach-Mallepell et al. 2013).

Mode of action information

Studies suggest that innate and adaptive immune system is impacted by DBP exposure (Hansen et al. 2015). DBP is proposed to be metabolized to the monoester *in vitro*. This compound then is proposed to modulate cytokine secretion from both monocytes/macrophages and T cells.

The results from Couleau and colleagues (2015) suggest that some effects may occur through activation of the endocrine pathway. DBP also may regulate gene and protein expression of a variety of immune factors (e.g., cytokines) without impacting cell viability.

Immunomodulation by DBP also may occur through receptor-mediated effects on the inflammasome.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

DBP did not increase proliferative responses in lymph nodes of BALB/c mice at concentrations up to 20% (v/v in acetone; dermal route of exposure). Additionally, 10% DBP did not increase dendritic cell accumulation in draining lymph nodes (Dearman et al. 1996).

Wistar rats were fed a diet containing 0.5% or 5% DBP for 34-36 days. While no effect on absolute spleen weight was reported, a significant increase (1.8-fold) in relative spleen weight was reported at 5% DBP (Murakami et al. 1986).

Female BALB/cJ mice were subcutaneously exposed to ovalbumin (antigen) and 2-2000 µg/mL DBP. After the primary immunization, one or two booster shots were given to the mice. No effects on the IgG1 or IgE serum levels after either one or two booster shots were noted. A dose- dependent effect was observed on IgG1 serum levels;

maximum responses were observed at 200 μg/ml (value not provided). No effect was noted on IgE serum levels (data not provided) (Larsen et al. 2002).

Thymic stromal lymphopoietin (TSLP) mRNA expression was significantly increased in BALB/c mouse ears 24 hours after exposure to DBP (in acetone, 1:1) (values not provided). An increase in TSLP protein levels was also measured at 24 hours (values not provided) (Larson et al. 2010; Schuepbach-Mallepell et al. 2013). DBP-induced induction of TSLP was strain dependent (BALB/c was more sensitive than C57Bl/6 mice). DBP also produced effects on TSLP in IL-1 receptor or apoptosis-associated speck-like protein containing a caspase recruitment domain deficient mice (Schuepbach-Mallepell et al. 2013).

In vitro data with cells or cell lines

DBP was cytotoxic to murine peritoneal exudate macrophages (PEM) after exposure to 50 or 100 μM for 24 hours. Annexin V and PI double stained cells (markers of apoptosis) were significantly increased after treatment with 100 μM DBP for 24 hours. Additionally, using trypan blue exclusion, a significant decrease in viable cells was reported after DBP exposure (LOAEL = 50 μM). Using two-color flow cytometry, DBP was shown to decrease expression of CD80, CD36, and major histocompatibility-II molecules on F4/80+ macrophages at 1 and 10 μM. Cytokine expression (IL-1ß, IL-6, IL-12, and TNF-α) also were decreased at the same concentrations. Phagocytotic capacity of PEM to apoptotic thymocytes and *E. coli* was decreased after exposure to DBP when compared to controls (LOAEL = 1 μM). DBP exposure also decreased PEM immunogenicity to allogenic T cells (LOAEL = 1 μM) (Li et al. 2013).

DBP decreased cell viability of RAW 264.7 macrophages (LOAEL = 100 μM for 60 minutes) but did not increase cellular apoptosis (NOAEL = 1 mM for 60 minutes) (Naarala and Korpi 2009).

In RBL-2H3 mast cells sensitized with anti-dinitrophenyl monoclonal IgE, DBP potentiated ß- hexosaminidase activity, which was used as a measurement of degranulation (LOAEL = 50 μM for 10 minutes). DBP did not induce degranulation in the cells that were not sensitized (NOAEL
= 500 μM for 10 minutes) (Nakamura et al. 2002).

In PAM212 keratinocytes, 1% DBP increased relative expression of TSLP; maximal effect (values not provided) was observed at 36 hours post treatment (Larson et al. 2010). DBP-induced TSLP expression was associated with epidermal mouse skin and human abdominal skin transplanted on mice (Schuepbach-Mallepell et al. 2013).

Mode of action information

In vivo rodent studies suggest that DBP impacts the Th2 response. Inflammasome activation by DBP impacts TSLP expression and Th2 response.

In vitro studies suggest that while high doses of DBP induced macrophage apoptosis, moderate doses induced protein expression and production of cytokines. DBP also impacted the antigen- presenting capacity of macrophages.

References

Couleau N, Falla J, Beillerot A, Battaglia E, D'Innocenzo M, Plancon S, et al. 2015. Effects of Endocrine Disruptor Compounds, Alone or in Combination, on Human Macrophage-Like THP-1 Cell Response. PloS one 10:e0131428; doi:10.1371/journal.pone.0131428.

Dearman RJ, Cumberbatch M, Hilton J, Clowes HM, Fielding I, Heylings JR, et al. 1996. Influence of dibutyl phthalate on dermal sensitization to fluorescein isothiocyanate. Fundamental and applied toxicology : official journal of the Society of Toxicology 33: 24–30.

Gwinn MR, Whipkey DL, Tennant LB, Weston A. 2007. Gene expression profiling of di-n-butyl phthalate in normal human mammary epithelial cells. Journal of environmental pathology, toxicology and oncology : official organ of the International Society for Environmental Toxicology and Cancer 26: 51–61.

Hansen JF, Nielsen CH, Brorson MM, Frederiksen H, Hartoft-Nielsen ML, Rasmussen AK, et al. 2015. Influence of phthalates on in vitro innate and adaptive immune responses. PloS one 10:e0131168; doi:10.1371/journal.pone.0131168.

Kruger T, Cao Y, Kjaergaard SK, Knudsen LE, Bonefeld-Jorgensen EC. 2012. Effects of phthalates on the human corneal endothelial cell line B4G12. International journal of toxicology 31:364–71; doi:10.1177/1091581812449660.

Larsen ST, Lund RM, Nielsen GD, Thygesen P, Poulsen OM. 2002. Adjuvant effect of di-n- butyl-, di-n-octyl-, di-iso-nonyl- and di-iso-decyl phthalate in a subcutaneous injection model using BALB/c mice. Pharmacology & toxicology 91: 264–72.

Larson RP, Zimmerli SC, Comeau MR, Itano A, Omori M, Iseki M, et al. 2010. Dibutyl phthalate-induced thymic stromal lymphopoietin is required for Th2 contact hypersensitivity responses. Journal of immunology (Baltimore, Md : 1950) 184:2974–84; doi:10.4049/jimmunol.0803478.

Li L, Li HS, Song NN, Chen HM. 2013. The immunotoxicity of dibutyl phthalate on the macrophages in mice. Immunopharmacology and immunotoxicology 35:272–81; doi:10.3109/08923973.2013.768267.

Lourenco AC, Galbiati V, Corti D, Papale A, Martino-Andrade AJ, Corsini E. 2015. The plasticizer dibutyl phthalate (DBP) potentiates chemical allergen-induced THP-1 activation. Toxicology in vitro : an international journal published in association with BIBRA 29:2001–8; doi:10.1016/j.tiv.2015.08.011.

Murakami K, Nishiyama K, Higuti T. 1986. Toxicity of dibutyl phthalate and its metabolites in rats. Nihon eiseigaku zasshi Japanese journal of hygiene 41: 775–81.

Naarala J, Korpi A. 2009. Cell death and production of reactive oxygen species by murine macrophages after short term exposure to phthalates. Toxicology letters 188:157–60; doi:10.1016/j.toxlet.2009.04.001.

Nakamura R, Teshima R, Sawada J. 2002. Effect of dialkyl phthalates on the degranulation and Ca2+ response of RBL-2H3 mast cells. Immunology letters 80: 119–24.

Ni J, Zhang Z, Luo X, Xiao L, Wang N. 2016. Plasticizer DBP Activates NLRP3 Inflammasome through the P2X7 Receptor in HepG2 and L02 Cells. Journal of biochemical and molecular toxicology 30:178–85; doi:10.1002/jbt.21776.

Schuepbach-Mallepell S, Philippe V, Bruggen MC, Watanabe H, Roques S, Baldeschi C, et al. 2013. Antagonistic effect of the inflammasome on thymic stromal lymphopoietin expression in the skin. The Journal of allergy and clinical immunology 132:1348–57; doi:10.1016/j.jaci.2013.06.033.

Dichloroacetic Acid (DCAA) [CASRN 79-43-6]

Human Data

Data from epidemiology studies

No studies were identified.

In vitro data with cells or cell lines

A single study suggested that DCAA may produce immunosuppressive effects. Using a two-way mixed lymphocyte reaction, DCAA (LOAEL = 0.33 mM; lowest dose tested) increased IL-10 production and FOX P3 expression 11.4- and 4.5-fold, respectively (Eleftheriadis et al. 2013).

DCAA (3.0 mM and 0.5 mM, respectively) increased IL-2 production after incubation for 16 hours and expression of the T-cell activation marker CD25 in Jurkat cells. Comparatively, no effect on CD69 expression (0.5 and 3.0 mM) was noted. IL-2 and IFN-γ mRNA expression was significantly increased after DCAA treatment (3.0 and 0.5 mM, respectively) (Pan et al. 2015).

DCAA (N/LOAEL = 0.1/1.0 mM) induced statistically significant increases in necrosis in PBMC, as shown by a decrease in PBMC cell size combined with an increase in cellular granulation. Statistically significant increases in the percentage of apoptotic cells were observed at similar concentrations of DCAA (N/LOAEL = 1.0/2.0 mM) (Michalowicz et al. 2015).

Mode of action information

T-cell activation was one proposed mode of action for DCAA. Increased IL-10 production, combined with increased FOX P3 expression, is proposed to increase regulatory T-cell differentiation which may lead to increased IL-10 production. Additionally, DCAA increased expression of T-cell activation markers in Jurkat cells.

Apoptosis was proposed be associated with a variety of mechanisms including ROS generation, alterations in mitochondrial transmembrane potential, and activation of caspase activity.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

In a 90-day drinking water study (0, 50, 500 and 5000 ppm (w/v) DCAA) with male Sprague- Dawley rats, a significant increase in relative spleen weight was noted at 5000 ppm (0.25% vs. 0.21%). No consistent effects on T cell-dependent anti-keyhole limpet hemocyanin IgG antibody production (measured by ELISA), delayed hypersensitivity to bovine serum albumin, NK cell cytotoxicity, or production of peritoneal macrophage-derived PGE2 or spleen lymphocyte- derived IL-2 were detected at tested doses (data not shown in paper) (Exon et al. 1986; Mather et al. 1990).

In autoimmune-prone MRL +/+ female mice, 0.5 mg/mL DCAA (provided *ad libitum* in drinking water for 12 weeks) significantly increased serum IgG (32%) and IgM (30%) levels. DCAA significantly decreased IL-10 (34%) and KC chemokine (31%) in liver extracts from

MRL +/+ mice. Comparatively, a significant increase in serum IgG3 levels (27%) was observed in wild-type B6C3F1 after DCAA exposure. In liver extracts from treated B6C3F1 mice, DCAA significantly increased IL-4 (400%), IL-5 (33%), IL-6 (53%), IL-10 (25%), IL-12 (32%), KC

chemokine (18%), GM-CSF (42%), G-CSF (56%), and IFN-γ (45%) compared to controls. Compared to isolated MRL +/+ splenic lymphocytes from controls, DCAA decreased IL-4 and IL-10 secretion in MRL+/+ treated mice. DCAA decreased IL-4 and increased IFN-γ secretion from splenic lymphocytes from treated B6C3F1 mice when compared to controls (values not provided). DCAA also significantly decreased IL-4 and IL-2 secretion and significantly increased IL-5, IFN-γ, and GM-CSF secretion from B6C3F1 isolated splenic lymphocytes when compared to secretion from MRL +/+ isolated splenic lymphocytes from treated animals (values not provided) (Cai et al. 2007).

In vitro data with cells or cell lines

No studies were identified.

Mode of action information

DCAA-induced increase of p53 accumulation has been proposed to lead to increased formation of cells in G2-M phase (Staneviciute et al. 2016).

References

Cai P, Boor PJ, Khan MF, Kaphalia BS, Ansari GAS, Konig R. 2007. Immuno- and hepato- toxicity of dichloroacetic acid in MRL(+/+) and B(6)C(3)F(1) mice. J Immunotoxicol 4:107– 115; doi:10.1080/15476910701337225.

Eleftheriadis T, Pissas G, Karioti A, Antoniadi G, Antoniadis N, Liakopoulos V, et al. 2013. Dichloroacetate at therapeutic concentration alters glucose metabolism and induces regulatory T- cell differentiation in alloreactive human lymphocytes. J Basic Clin Physiol Pharmacol 24:271– 276; doi:10.1515/jbcpp-2013-0001.

Exon JH, Koller LD, Talcott, P.A., O'Reilly CA, Henningsen GM. 1986. Immunotoxicity Testing: An Economical Multiple-Assay Approach. Fundamental and Applied Toxicology 7: 387–397.

Mather GG, Exon JH, Koller LD. 1990. Subchronic 90 day toxicity of dichloroacetic and

trichloroacetic acid in rats. Toxicology 64: 71–80.
Michalowicz J, Wroblewski W, Mokra K, Macczak A, Kwiatkowska M. 2015. Comparative study of the effect of chloro-, dichloro-, bromo-, and dibromoacetic acid on necrotic, apoptotic and morphological changes in human peripheral blood mononuclear cells (in vitro study).
Toxicol In Vitro 29:1416–1424; doi:10.1016/j.tiv.2015.05.021.
Pan Y, Wei X, Hao W. 2015. Trichloroethylene and Its Oxidative Metabolites Enhance the Activated State and Th1 Cytokine Gene Expression in Jurkat Cells. Int J Environ Res Public Health 12:10575–10586; doi:10.3390/ijerph120910575.
Staneviciute J, Urboniene D, Valanciute A, Balnyte I, Vitkauskiene A, Grigaleviciene B, Stakisaitis D. 2016. The effect of dicloroacetate on male rat thymus and on thymocyte cell cycle. Int J Immunopathol Pharmacol 29:818-822.

Diethylstilbestrol (DES) [CASRN 56-53-1]

Human Data

Data from epidemiology studies

Male and female offspring of pregnant women given DES doses from the seventh week to 34th week of pregnancy, were interviewed about immune-related health problems. A total of 549 DES-exposed offspring and 487 placebo-exposed offspring participated in the study. Rates of allergy-related health problems (e.g., asthma, drug allergy, hives) were similar between DES- and placebo-offspring. Infection (e.g., shingles, flu) and autoimmune disease (e.g., diabetes, rheumatoid arthritis) also were similar between the two groups (Baird et al. 1996).

The frequency of any autoimmune disease in women exposed to DES *in utero* (n = 1711) was higher than the frequency observed in control women. The overall frequency was 28.6 per 1000 women compared to 16.3 per 1000 women. Hashimoto's thyroiditis was significantly more prevalent (relative prevalence = 5.4) in exposed women compared to controls (Noller et al.

1988).

Increased incidence of asthma, arthritis, and diabetes mellitus was reported in sons and daughters exposed to DES *in utero* when compared to unexposed individuals. Additionally, the number of respiratory tract conditions (e.g., colds) was increased in the exposed population vs. the unexposed population (Wingard and Turiel 1988).

In vitro data with cells or cell lines

Lymphocyte NK activity (assessed using chromium release from K562 cells) from 12 patients exposed to DES *in utero* was greater than observed from controls; however, effects were not significant. No effects on adherent cells were noted (Ford et al. 1983). Comparatively, DES dose-dependently inhibited lysis of K562 cells in PBMCs obtained from 12 patients. At the highest concentration tested (100 μM), an 82% reduction in activity was observed compared to control samples (Ablin et al. 1988b, 1988a)

Responses to 0.125 μg/mL PHA (as measured by uptake of radiolabeled thymidine) was significantly greater in peripheral blood monocytes from women exposed to DES *in*

utero compared to controls (88.6×10^3 vs. 44.0×10^3 cpm; $p < 0.002$). Maximal blastogenic response to PHA in lymphocytes from DES-exposed women was observed at 0.125 μg/mL while it was observed at 0.25-0.50 μg/mL in controls (Ways et al. 1987).

Mode of action information

DES inhibits the lytic activity of human NK cells (Kalland and Campbell 1984).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

C57BL/6 dams were orally administered 48 μg/kg DES from GD 14-16 and then sacrificed on GD 18. Fetal thymic weight and cellularity were significantly decreased (44% and 51%, respectively) in treated animals. Relative fetal thymic weight was decreased 28% when compared to controls. The percentage of thymocytes in the CD4-8- and CD4-8+ populations were increased 87% and 138%, respectively. Comparatively, CD4+8+ thymocyte population was decreased 12%. Increased apoptosis of CD4+8+, CD4-8+, and CD4-8- thymocytes also was observed (Besteman et al. 2005).

Male and female CD-1 mice were subcutaneously injected with 5, 15, or 30 μg/kg DES, four times on alternate days. Relative thymic weight (LOAEL = 30 μg/kg) was decreased, and absolute and relative splenic weights (LOAELs = 15 μg/kg) were increased in female mice. A similar effect in male mice was not observed. Relative expression of thymocyte populations (e.g., CD4-8-) were not affected in males or females. However, an increase in the number of total apoptotic and decrease in the number of live CD4+8+ and CD4+8- thymocytes was observed in male and female mice. Additionally, an increase in the number of total apoptotic and decrease in the number of live CD4-8- cells were observed in females. Increased proliferative response to ConA, LPS, or PMA was observed in splenic lymphocytes isolated from female mice treated with 5 μg/kg DES. At higher doses, a trend for decreased proliferation was observed in female splenic lymphocytes. Proliferative responses by splenic lymphocytes were only modulated in response to ConA at 15 μg/kg DES (Calemine et al. 2002).

Female mice (strain not provided) were administered (route not provided) 0.2, 2.0, or 8.0 mg/kg DES for 5 days. Antibody response to sheep erythrocytes and LPS were decreased 15% to 45% (LOAELs = 2.0 mg/kg). Delayed hypersensitivity response to keyhole lymphet hemocyanin was similar to controls when mice were exposed to DES before sensitization. However, when mice were exposed to DES after sensitization and before challenge a decrease in response was observed (LOAEL = 2 mg/kg). The percentage of splenic T lymphocytes was decreased 25% at the highest dose tested. No effect on the percentage of splenic B lymphocytes was observed.

Splenic lymphoproliferative response to PHA and ConA were decreased (>30%) at all

tested doses. Responses to *Staphylococcus* enterotoxin A were increased at 0.2 mg/kg and decreased at higher doses, while responses to LPS were increased at 0.2 and 2.0 mg/kg and decreased at 8.0 mg/kg. MLC responses also were decreased (LOAEL = 2 mg/kg). Suppressor cell activity was decreased after exposure to 8 mg/kg DES (Luster et al. 1980).

Differential effects on the immune system were observed in female NMRI mice depending on the time of DES exposure. Thymus weights were increased in 56-day-old mice that were subcutaneously injected with 5 µg from PND 1-5, 6-10, or 30-34 (1.2- to 1.4-fold).

Comparatively, thymus weight was decreased in mice subcutaneously injected with DES from PND 48-52 (29%). A dose-related effect on thymus weight was observed in mice treated from PND 1-5; no effects on absolute or relative spleen weight were noted. Differences in thymus weight also were noted depending on when the mice were killed after treatment. Four days after treatment, thymus weights were decreased in all test groups. However, 4 to 8 weeks after

treatment showed an increase in thymus weight in mice treated on PND 1-5 and weights similar to controls in other treatment groups. DES treatment on PND 1-5 also reduced the number of cells in S-phase in the thymus (Forsberg 1996).

C57BL/6 mice were treated with DES once *in utero* and/or once at 12-16 months of age via subcutaneous injection. Increased secretion of IFNγ was observed in splenic lymphocytes obtained from mice exposed to DES *in utero* and as adults. Increased IFNγ also was observed when splenocytes were stimulated with anti-CD3 antibodies. This increase was not observed in other treatment conditions (data in graphs). An increase in IFNγ production also was observed in T-cells from mice exposed to DES *in utero* and as adults (Karpuzoglu-Sahin et al. 2001).

In vitro data with cells or cell lines

DES stimulated IL-1 production from peritoneal exudate macrophages at concentrations ranging from 0.01 to 1 μM; the maximal response was observed at 0.1 μM. DES (0.1 μM) also significantly increased production of IL-6 (1.7-fold), IL-12 (9.5-fold) TNF-α (3.1-fold), and macrophage chemotactic protein 1 (7.2-fold), and surface expression of CD86 (1.6-fold). DES also increased proliferative responses (8.6-fold) and IL-2 production (5.6-fold) observed when macrophages were incubated with purified T cells. Anti-MHC-II, -CD-80, and -CD86 blocked effects produced by DES (Yamashita et al. 2005).

DES increased IgE levels in male BALB/c mouse splenocytes at concentrations greater than 1 μM. Comparatively, DES had no effect on IgM, IgG, or IgA levels at concentrations up to 1 mM (Han et al. 2002).

Mode of action information

DES-induced thymic atrophy was proposed to be due, in part, to estrogen-related thymocyte apoptosis (Besteman et al. 2005; Fenaux et al. 2004). Brown and colleagues suggested that DES exposure upregulates TNF family members, which leads to altered T-cell development. This alteration was suggested to lead to thymic atrophy (Brown et al. 2006). Direct effects on T lymphocytes also may occur (Luster et al. 1980).

In mice, DES exposure was associated with down-regulation of gene expression in the TCR complex, and the TCR and CD28 signaling pathways. Genes in the B-cell receptor

signaling pathway, antigen presentation, and dendritic cell pathways also was altered by DES exposure. It was proposed that DES dysregulation of T-cell development plays a role in thymus effects (Frawley et al. 2011). Alterations of microRNA expression also has been proposed as playing a role in the immunotoxic effects produced by DES (Singh et al. 2015).

Additional proposed modes of action on the immune system include effects on adherent suppressor cells, modulation of NK activity by interfering with bone marrow lymphoid precursors, and modulation of the mononuclear phagocyte system (Dean et al. 1986; Forsberg 1984; Kalland 1984; Luster et al. 1980).

References

Ablin RJ, Bartkus JM, Gonder MJ. 1988a. In vitro effects of diethylstilboestrol and the LHRH analogue leuprolide on natural killer cell activity. Immunopharmacology 15: 95–101.

Ablin RJ, Gonder MJ, Bartkus JM. 1988b. Leuprolide vs. diethylstilboestrol: effect on natural killer cells. Anticancer research 8: 73–6.

Baird DD, Wilcox AJ, Herbst AL. 1996. Self-reported allergy, infection, and autoimmune diseases among men and women exposed in utero to diethylstilbestrol. Journal of Clinical Epidemiology 49:263–266; doi:10.1016/0895-4356(95)00521-8.

Besteman EG, Zimmerman KL, Holladay SD. 2005. Diethylstilbestrol (DES)-induced fetal thymic atrophy in C57BL/6 mice: inhibited thymocyte differentiation and increased apoptotic cell death. International journal of toxicology 24:231–9; doi:10.1080/10915810591000703.

Brown N, Nagarkatti M, Nagarkatti PS. 2006. Induction of apoptosis in murine fetal thymocytes following perinatal exposure to diethylstilbestrol. International journal of toxicology 25:9–15; doi:10.1080/10915810500488353.

Calemine JB, Gogal RM Jr, Lengi A, Sponenberg P, Ahmed SA. 2002. Immunomodulation by diethylstilbestrol is dose and gender related: effects on thymocyte apoptosis and mitogen- induced proliferation. Toxicology 178: 101–18.

Dean JH, Lauer LD, Murray MJ, Luster MI, Neptun D, Adams DO. 1986. Functions of mononuclear phagocytes in mice exposed to diethylstilbestrol: a model of aberrant macrophage development. Cellular immunology 102: 315–22.

Fenaux JB, Gogal RM Jr, Ahmed SA. 2004. Diethylstilbestrol exposure during fetal development affects thymus: studies in fourteen-month-old mice. Journal of reproductive immunology 64:75–90; doi:10.1016/j.jri.2004.08.003.

Ford CD, Johnson GH, Smith WG. 1983. Natural killer cells in in utero diethylstilbesterol- exposed patients. Gynecologic oncology 16: 400–4.

Forsberg JG. 1984. Short-term and long-term effects of estrogen on lymphoid tissues and lymphoid cells with some remarks on the significance for carcinogenesis. Archives of toxicology 55: 79–90.

Forsberg JG. 1996. The different responses of the female mouse thymus to estrogen

after treatment of neonatal, prepubertal, and adult animals. Acta anatomica 157: 275–90.

Frawley R, White K Jr, Brown R, Musgrove D, Walker N, Germolec D. 2011. Gene expression alterations in immune system pathways in the thymus after exposure to immunosuppressive chemicals. Environmental health perspectives 119:371–6; doi:10.1289/ehp.1002358.

Han D, Denison MS, Tachibana H, Yamada K. 2002. Effects of estrogenic compounds on immunoglobulin production by mouse splenocytes. Biological & pharmaceutical bulletin 25: 1263–7.

Kalland T. 1984. Exposure of neonatal female mice to diethylstilbestrol persistently impairs NK activity through reduction of effector cells at the bone marrow level. Immunopharmacology 7: 127–34.

Kalland T, Campbell T. 1984. Effects of diethylstilbestrol on human natural killer cells in vitro. Immunopharmacology 8: 19–25.

Karpuzoglu-Sahin E, Hissong BD, Ansar Ahmed S. 2001. Interferon-gamma levels are upregulated by 17-beta-estradiol and diethylstilbestrol. Journal of reproductive immunology 52: 113–27.

Luster MI, Boorman GA, Dean JH, Luebke RW, Lawson LD. 1980. The effect of adult exposure to diethylstilbestrol in the mouse: alterations in immunological functions. Journal of the Reticuloendothelial Society 28: 561–9.

Noller KL, Blair PB, O'Brien PC, Melton LJ, Offord JR, Kaufman RH, et al. 1988. Increased occurrence of autoimmune disease among women exposed in utero to diethylstilbestrol. Fertil Steril 49: 1080–1082.

Singh NP, Abbas IK, Menard M, Singh UP, Zhang J, Nagarkatti P, et al. 2015. Exposure to diethylstilbestrol during pregnancy modulates microRNA expression profile in mothers and fetuses reflecting oncogenic and immunological changes. Molecular pharmacology 87:842–54; doi:10.1124/mol.114.096743.

Ways SC, Mortola JF, Zvaifler NJ, Weiss RJ, Yen SS. 1987. Alterations in immune responsiveness in women exposed to diethylstilbestrol in utero. Fertil Steril 48: 193–197.

Wingard DL, Turiel J. 1988. Long-term effects of exposure to diethylstilbestrol. West J Med 149: 551–554.

Yamashita U, Sugiura T, Yoshida Y, Kuroda E. 2005. Effect of endocrine disrupters on macrophage functions in vitro. Journal of UOEH 27: 1–10.

Ethylene Dibromide (EDB) [CASRN 106-93-4]

Human Data

Data from epidemiology studies

The prevalence of adult-onset asthma, in relation to lifetime pesticide use, were assessed using data from the Agricultural Health Study (19,704 male farmers). Adult-onset asthma was reported in 441 individuals; 127 classified as allergic and 314 classified as non-allergic. EDB exposure was positively associated with allergic asthma (OR: 2.07 [1.02-4.20]) (Hoppin et al. 2009).

In vitro data with cells or cell lines

No data were located.

Mode of action information

No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

Female B6C3F1 mice were intragastrically treated with 100, 125, 160, or 200 mg/kg EDB for 14 days. Relative thymus and spleen weights were decreased in a dose-related manner (LOAEL = 200 mg/kg). Comparatively, relative liver and kidney weights were increased at higher doses (LOAEL = 125 and 160 mg/kg, respectively). Significant increases in white blood cells (LOAEL
= 200 mg/kg) and neutrophils (LOAEL = 160 mg/kg) were noted. Host resistance to influenza A2, *Listeria monocytogenes*, and herpes simplex virus types 1 and 2 was not significantly affected by EDB exposure. The total number of resident peritoneal exudate cells were significantly increased in EDB-treated mice (LOAEL = 160 mg/kg). However, the percentage of cell types present in the exudates were similar to those observed in control exudates (macrophages: 53%; lymphocytes: 47%). Phagocytosis of radiolabeled chicken red blood cells was increased in peritoneal macrophages obtained

from EDB-treated mice (187% of control; LOAEL = 125 mg/kg). Splenic NK cell activity was evaluated in animals treated with 100, 125, or 160 mg/kg; a significant decrease in activity was observed at 160 mg/kg. The number of viable cells in the spleen decreased at 125, 160, and 200 mg/kg (not significant), while a significant increase in the number of anti-SE PFC/10^6 viable spleen cells was significantly increased at 160 mg/kg. Splenic lymphocyte responses to allogenic spleen cells, PHA and ConA, but not LPS, were significantly decreased at 125 and 160 mg/kg (Ratajczak et al. 1994).

Female B6C3F1 were intragastrically treated with 31.25, 62.5, or 125 mg/kg EDB for 5 days per week for 12 weeks. No effect on white blood cell numbers, or the percentage of neutrophils or lymphocytes were noted at the doses tested. Splenic lymphocyte responses to PHA and LPS were significantly decreased at the highest dose tested (data not provided) (Ratajczak et al.

1995).

Relative spleen weights were not significantly affected in male Sprague-Dawley rats inhalationally exposed to EDB 7 hours per day, 5 days per week, for 30 days. However, relative liver weights were increased at the highest dose tested (LOAEL = 455 ppm) (Igwe et al. 1986).

In vitro data with cells or cell lines

No data were located.

Mode of action information

No data were located.

References

Hoppin JA, Umbach DM, London SJ, Henneberger PK, Kullman GJ, Coble J, et al. 2009. Pesticide use and adult-onset asthma among male farmers in the Agricultural Health Study. The European respiratory journal 34:1296–303; doi:10.1183/09031936.00005509.

Igwe OJ, Que Hee SS, Wagner WD. 1986. Interaction between 1,2-dichloroethane and disulfiram. I. Toxicologic effects. Fundamental and applied toxicology : official journal of the Society of Toxicology 6: 733–46.

Ratajczak HV, Aranyi C, Bradof JN, Barbera P, Fugmann R, Fenters JD, et al. 1994. Ethylene dibromide: evidence of systemic and immunologic toxicity without impairment of in vivo host defenses. In vivo (Athens, Greece) 8: 879–84.

Ratajczak HV, Thomas PT, Gerhart J, Sothern RB. 1995. Immunotoxicologic effects of ethylene dibromide in the mouse and their modulation by the estrous cycle. In vivo (Athens, Greece) 9: 299–304.

Glycidol [CASRN 556-52-5]

Human Data

Data from epidemiology studies

No data were located.

In vitro data with cells or cell lines

No data were located.

Mode of action information

No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

Of 10 F344/N female rats that received 400 mg/kg glycidol for 13-weeks (via gavage), lymphoid necrosis of the thymus was observed in nine (Irwin et al. 1996; National Toxicology Program 1990). Enlarged spleen were observed in haploinsufficient $p16^{Ink4a}/p19^{Arf}$ male mice treated with 200 mg/kg glycidol for 40 weeks via gavage (National Toxicology Program 2007).

Increased splenic fibrosis incidence was reported in male and female F344/N rats gavaged with

37.5 and 75 mg/kg glycidol for 2 years. In males, splenic fibrosis incidences were 26% in controls, 68% in rats treated with 37.5 mg/kg, and 56% in rats treated with 75 mg/kg. In females, splenic fibrosis incidences were 6%, 29%, and 40% for control, 37.5 mg/kg rats and 75 mg/kg rats, respectively (National Toxicology Program 1990).

In female B6C3F1 mice administered glycidol via gastric intubation (25, 125, and 250 mg/kg) for 14 days, no effect on spleen or thymus weights, or leukocyte or lymphocytes counts were reported. To assess AFC response, treated mice were intravenously exposed to sheep erythrocytes on day 11 and spleen IgM AFC response was measured 4 days later. At the highest treatment dose, there was a 31% reduction in specific

activity. When expressed as total spleen activity, significant decreases were noted at 125 and 250 mg/kg (29% and 41%, respectively).

Splenic T-cell proliferation, in response to 10 μg/mL ConA was significantly decreased (16% and 26%, respectively) in splenocytes obtained from mice treated with 125 and 250 mg/kg glycidol. B-cell proliferation, in response to Il-4 or Il-4 and goat anti-mouse IgM F(ab')2, was only decreased in splenocytes obtained from mice treated with 125 mg/kg glycidol (13% and 16%, respectively). Comparatively, proliferation in response to goat anti-mouse IgM F(ab')2 was decreased in splenocytes from mice treated with 125 and 250 mg/kg glycidol (30-32%). While glycidol had no effect on lymphocyte blastogenesis (as assessed by splenocyte proliferative response) alone, in the presence of allogenic DBA/2 spleen cells a 25% decrease in response was noted at the middle dose only. NK cell activity in spleens was decreased at two ratios of effector:target ratios (100:1 and 50:1); the LOAELs at both ratios were 125 and 250 mg/kg, respectively. Using flow cytometry, the number and percent of B lymphocytes, T-lymphocytes,

$CD4^+CD8^-$, $CD4^-CD8^+$, and $CD4^-CD8^-$ cells from spleens isolated from treated mice were quantified. The total number of spleen cells, B lymphocytes, and $CD4^+CD8^-$ were significantly decreased at 250 mg/kg. The LOAEL also was 250 mg/kg when the percent values of B and T lymphocytes were assessed (Guo et al. 2000).

In vitro data with cells or cell lines

To further assess the effect of glycidol on the immune function, Guo and colleagues (2000) conducted a set of *ex vivo* assays. Glycidol inhibited cytotoxic T cell activity in spleens obtained from mice administered glycidol via gastric intubation (25, 125, and 250 mg/kg) for 14 days.

Splenocytes were sensitized with mitomycin C-exposed P815 mastocytoma cells, and co- cultured with labeled P815 cells at a variety of effector:target ratios. At an effector:target ratio of 25:1 and 0.75:1, glycidol inhibited CTL activity at a 25 mg/kg when compared to vehicle (53.8 vs. 31.5, and 8.8 vs. 2.1, respectively). At a ratio of 12.5:1, CTL activity was decreased significantly (39%) in spleens from mice treated with 125 mg/kg glycidol (Guo et al. 2000).

Resident macrophage activity (in the presence of macrophage stimulators) was assessed in mice treated with glycidol via gastric intubation (25, 125, and 250 mg/kg) for 14 days. Increased cytotoxicity was only observed after treatment with 25 mg/kg glycidol in the presence or absence of macrophage stimulators (1.7- to 2.5-fold increase) (Guo et al. 2000).

Host resistance to B16F10 melanoma cells, *Listeria monocytogenes* and *Streptococcus pneumoniae* were assessed in mice treated with glycidol via gastric intubation (25, 125, and 250 mg/kg) for 14 days. Glycidol increased pulmonary tumor formation in mice treated with B16F10 melanoma cells (LOAEL = 125 mg/kg). No effect on host resistance was noted at the three challenge levels of *Listera monocytogenes* (1, 2, or 4×10^4 CFU/mouse). At the challenge

level 5.52×10^7 CFU *Streptococcus phenumoniae*/mouse, increased host resistance was observed in the 250-mg/kg glycidol treated mice (Guo et al. 2000).

Mode of action information

Studies suggest that glycidol modulates B-cell function, and NK cell and macrophage activities (Guo et al. 2000).

References

Guo TL, McCay JA, Brown RD, Musgrove DL, Butterworth L, Munson AE, et al. 2000. Glycidol modulation of the immune responses in female B6C3F1 mice. Drug and chemical toxicology 23:433–57; doi:10.1081/dct-100100127.

Irwin RD, Eustis SL, Stefanski S, Haseman JK. 1996. Carcinogenicity of glycidol in F344 rats and B6C3F1 mice. Journal of applied toxicology : JAT 16:201–9; doi:10.1002/(sici)1099- 1263(199605)16:3<201::aid-jat333>3.0.co;2-0.

National Toxicology Program. 1990. NTP Toxicology and Carcinogenesis Studies of Glycidol (CAS No. 556-52-5) In F344/N Rats and B6C3F1 Mice (Gavage Studies). Natl Toxicol Program Tech Rep Ser 374: 1–229.

National Toxicology Program. 2007. Toxicology and carcinogenesis study of glycidol (CAS No. 556-52-5) in genetically modified haploinsufficient p16(Ink4a)/p19(Arf) mice (gavage study).
National Toxicology Program genetically modified model report 1–81.

Hydrocortisone (HC) [CASRN 50-23-7]

Human Data

Data from epidemiology studies

No data were located.

In vitro data with cells or cell lines

The effect of HC on IL-4-induced IgE production was measured in PBMCs isolated from healthy volunteers. HC induced an ~20 fold increase in IgE production at a LOEL of 1×10^{-7} M. HC did not have any effect on IgE production in the absence of IL-4 (data not shown) (Nüsslein et al.
1994).

Blood samples from healthy adults were pre-treated with 30 μg/dL HC (identified as cortisol); INF production was then stimulated with Newcastle disease virus. HC decreased IFN-α response by 50-60% (data in graph) (Reissland and Wandinger 1999).

Mode of action information

Keh and colleagues (2003) reported that in septic shock patients, HC attenuated inflammatory and anti-inflammatory responses without inducing immunosuppression.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

Swiss inbred mice were intraperitoneally injected with 0, 1.5, 5 or 15 mg/kg of HC. Forty-eight hours later, there was a significant decrease in thymus weight at 15 mg/kg (data in graph). To test the effect of HC on delayed hypersensitivity, mice were immunized with sheep erythrocytes in FCA, challenged in the footpad on day 5, and treated simultaneously with increasing amounts of HC. The mice received another injection of HC two hours before measuring 24-hour footpad swelling. 5 and 15 mg/kg HC suppressed footpad swelling (data in graph). Glucocorticoid- induced leukopenia and monocytopenia was evaluated in mice 2.5 hours after intravenous injection with

HC. The numbers of circulating nucleated and monocytic cells was maximally decreased at the lowest dose tested (1.5 mg/kg), with the number of both cell types increasing with increasing dose (data in graph). A plasma transfer study found that 2.5 hours after transfer, the plasma of HC treated mice raised the number of nucleated cells in saline treated acceptor mice by 46%. To evaluate feedback-inhibition, mice were injected (route not specified) with 5 mg/kg HC for four days and examined 7 or 11 days (data not shown) after the last injection. At day 7, HC had no effect on delayed hypersensitivity, serum corticosterone, or numbers of circulating nucleated and monocytic cells (data in graphs) (Van Dijk et al. 1979).

In a trio of studies by El Fouhil and colleagues (El Fouhil et al. 1993a, 1993b; El Fouhil and Turkall 1993), immunologically immature rats were treated subcutaneously with 400 mg/M^2/day HC, administered on alternate days from PND 7 to PND 19. At two days after the last treatment (PND 21), thymus and spleen weights were decreased (71 and 28%) compared to vehicle control,

but at PND 42 organ weights were increased (18 and 7%). Leucocytosis was increased in PND 21 and 42 rats (12 and 24%), with a decrease in IgM concentration in serum (45 and 15%). At PND 21 there was a 46% decrease in the percentage of lymphocytes, which resolved by PND 42 (El Fouhil and Turkall 1993). On PND 21, splenic white pulp was largely depleted of small lymphocytes. There were no distinct periartiolar lymphoid sheaths and no primary follicles. The number of T cells surrounding the central arteriole was decreased (data not shown). By PND 42, the pulp appeared normal (El Fouhil et al. 1993a). On PND 21, the outer cortex of mesenteric lymph nodes was found to be depleted of small lymphocytes and primary follicles, and neither cortical expansion nor capsular indentations were detected. There was a marked depletion of B lymphocytes, which were more or less discrete and did not aggregate to form follicles. There was no apparent change in T lymphocytes. On PND 42, the lymph nodes were comparable between HC treated and control rats (El Fouhil et al. 1993b).

HC (1.5 mg intraperitoneally administered) decreased formation of splenic anti-sheep erythrocyte (4×10^7 sheep erythrocytes) PFC in female BALB/c mice (data in graph). HC did not affect IgM-PFC or IgG-PFC response or serum antibody titers (data in graphs) (Jokay et al.
1980).

In vitro data with cells or cell lines

No data were located.

Mode of action information

No data were located.

References

El Fouhil AFI, Iskander FA, Turkall RM. 1993a. Effect of Alternate-Day Hydrocortisone Therapy on the Immunologically Immature Rat. II: Changes in T- and B-Cell Areas in Spleen. Toxicologic Pathology 21:383–390; doi:10.1177/019262339302100406.

El Fouhil AFI, Iskander FA, Turkall RM. 1993b. Effect of Alternate-Day Hydrocortisone Therapy on the Immunologically Immature Rat. III: Changes in T- and B-Cell Areas in

Lymph Nodes. Toxicologic Pathology 21:391–396; doi:10.1177/019262339302100407.
El Fouhil AFI, Turkall RM. 1993. Effect of Alternate-Day Hydrocortisone Therapy on the Immunologically Immature Rat. I: Effect on Blood Cell Count, Immunoglobulin Concentrations, and Body and Organ Weights. Toxicologic Pathology 21:377–382; doi:10.1177/019262339302100405.
Jokay I, Kelemenics K, Karczag E, Foldes I. 1980. Interactions of glucocorticoids and heparin on the humoral immune response of mice. Immunobiology 157:390–400; doi:10.1016/s0171- 2985(80)80008-8.
Keh D, Boehnke T, Weber-Cartens S, Schulz C, Ahlers O, Bercker S, et al. 2003. Immunologic and Hemodynamic Effects of "Low-Dose" Hydrocortisone in Septic Shock: A Double-Blind, Randomized, Placebo-controlled, Crossover Study. American Journal of Respiratory and Critical Care Medicine 167:512–520; doi:10.1164/rccm.200205-446OC.

Nüsslein HG, Weber G, Kalden JR. 1994. Synthetic glucocorticoids potentiate IgE synthesis.: Influence of steroid and nonsteroid hormones on human in vitro IgE secretion. Allergy 49:365– 370; doi:10.1111/j.1398-9995.1994.tb02283.x.

Reissland P, Wandinger KP. 1999. Increased Cortisol Levels in Human Umbilical Cord Blood Inhibit Interferon Alpha Production of Neonates. Immunobiology 200:227–233; doi:10.1016/S0171-2985(99)80072-2.

Van Dijk H, Bloksma N, Rademaker PM, Schouten WJ, Willers JM. 1979. Differential potencies of corticosterone and hydrocortisone in immune and immune-related processes in the mouse.

International Journal of Immunopharmacology 1:285–292; doi:10.1016/0192-0561(79)90004-3.

Indomethacin [CASRN 53-86-1]

Human Data

Data from epidemiology studies

No data were located.

In vitro data with cells or cell lines

Heparinized whole blood, from healthy adult volunteers, was incubated with 1 to 50 μM indomethacin for 1 hour prior to stimulation with LPS. A significant increase in IL-6 expression was only noted at 50 μM indomethacin (129.7%). Comparatively, a dose-dependent increase in TNF-α was observed, and at 50 μM the number of TNF-α positive cells had doubled (204.7%) (Hartel et al. 2004).

Human PBMC were treated with 1, 10 or 100 μM indomethacin. At all tested doses, indomethacin decreased LPS-induced PGE2 synthesis to near 0%; the calculated IC50 was

0.039 μM (data in graph). Indomethacin also decreased IgG and IgM production (data in graph) at all doses tested. Indomethacin up-regulated IL-2 production and down-regulated IL-6 production in treated PBMC (data not shown). Increased PHA-, anti-CD3, and IL-2-induced lymphocyte proliferation was reported after indomethacin exposure. NK activity (against K562 target cells) was increased at 1 (1.5-fold) and 10 (1.5-fold) μM. A significant effect on LAK cell activity was not observed at 50 μM. Co-incubation of PBMCs with IL-2 and indomethacin caused an increase in IFN-γ production by LAK cells at 1, 10 or 100 μM (data not shown) (Tanaka et al. 1998).

Indomethacin (5.6 μM) increased proliferation of PHA- and ConA-stimulated lymphocytes (in mononuclear cell cultures) (data in graph). The effect was only observed at suboptimal concentrations of PHA and ConA. The observed increased proliferation was lost at optimal and supraoptimal concentrations. Additional testing showed indomethacin increased PHA-stimulated lymphocyte proliferation in a dose-dependent manner (LOEL = 0.04 μM). Removal of adherent cells from the culture negated the stimulatory effect produced by indomethacin. Indomethacin did not affect cell viability, but increased incorporation of tritiated thymidine in a dose- dependent

manner (Jawad and Rogers 1984).

Mode of action information

No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

Female C57BL/6 mice were orally administered 5 mg/kg indomethacin for 4 days. Animals were then immunized with sheep erythrocytes and then serum hemagglutination and AFC titers were assessed 4 and 8 days, or 6 days later, respectively. Indomethacin decreased both titers by approximately 40% (data in graphs). Indomethacin also decreased ConA- and LPS-induced

stimulation of lymphocyte proliferation (data in graphs). Incubation of indomethacin (3 μM) with LPS-stimulated lymphocytes isolated from indomethacin-treated mice decreased proliferation (Barasoain et al. 1980).

Female B6C3F1 mice were subcutaneously injected with 1, 2, or 4 mg/kg indomethacin for 6 days. Studies were conducted on mice 3 days after final treatment. No effect on thymus weight was reported, while 4 mg/kg indomethacin caused a 38% increase in spleen weight. A

dose-dependent increase in splenic lymphocyte proliferation (10%-80%) was observed in non- stimulated cultures. Increased proliferation was observed in LPS-stimulated cultures from mice treated with 1 or 2 mg/kg indomethacin (13% and 22%, respectively), while a decrease was observed at the highest indomethacin dose. Comparatively, decreased proliferation was observed in PHA-, ConA-, or MLC-treated splenic cultures from indomethacin treated mice. Increased formation of PFC/10^6 splenocytes also were observed in treated mice (149% increase at 4 mg/kg). Indomethacin did not affect macrophage-induced inhibition of tumor cell growth (MBL- 2), but did increase phagocytosis of sheep erythrocytes. Host resistance to *Listeria* was increased in treated mice. No effect on delayed hypersensitivity was noted (Boorman et al. 1982).

Oral exposure to indomethacin (2.5, 5, or 10 mg/kg/day for 3 days) decreased formation of PFC in C57BL/6 mice after immunization to sheep erythrocytes. Studies showed a dose-dependent decrease in the number of PFC/10^6 spleen cells; decrease ranged from 43% to 97%. Similar inhibition was observed at 5 mg/kg/day indomethacin and various concentrations of sheep erythrocytes (2.5×10^8 and 5×10^8); decreases ranged from 47% to 68%. Indomethacin also inhibited antibody response to *P. aeruginosa* LPS; total response was decreased by 44% (Rojo et al. 1981).

Oral administration of 6 mg/kg/day indomethacin for 4 days produced a 32% decrease in total number of lymphocytes in Swiss male mice. No effect was noted at earlier time points (i.e., 2 or 3 days). An increase in the number of colonies/10^5 bone marrow cells (2.7- to 3.9-fold) also was noted in mice that were administered indomethacin for 4 days. Indomethacin also decreased PGE2 (25-43%) and PGF2α (41-56%) levels in bone marrow cells after 4 days of administration (Fontagne et al. 1980).

Male CBA mice were intraperitoneally injected with 0.7, 4, or 8 mg/kg indomethacin. Two to 24 hours after exposure, mice were euthanized and spleens removed. A dose-dependent increase in splenocyte proliferation was noted after 2 hours, with a 14.3-fold increase in proliferation at the highest dose tested. A time-dependent increase in proliferation was also noted when mice were treated with 4 mg/kg indomethacin, with a maximal fold change of 31.4-fold at 24 hours.

Distribution of T-cell phenotypes was not affected by indomethacin administration (Gonzalez- Cabello et al. 1987).

Kushima and colleagues (2007, 2009) evaluated effects of indomethacin in young Sprague- Dawley rats after *in utero* exposure. In 3-week old pups from dams treated with 0.25, 0.5, or

1.0 mg/kg indomethacin on GD 18-21, a significant increase (31%) in the number of spleen cells was observed in males from the highest dose group. Immunophenotyping of splenocytes showed a dose-dependent increase in the proportion of CD45RA+ cells in male pups. However, a similar

increase in peripheral blood lymphocytes was noted. No effect on serum IgM or IgG levels was reported in males or females. A significant decrease in anti-KLH IgG titers, but not IgM titers was reported in males from the highest dose group tested (Kushima et al. 2007). When doses of 0.5, 1.0, or 2.0 mg/kg indomethacin were used, a significant decrease in splenocyte IL-10 levels were reported in males; no effects on IL-6, IL-2, IL-4, TNF, or IFN-γ levels were noted in either sex (Kushima et al. 2009).

Indomethacin (1 or 2 mg/kg administered twice daily for 3 days to adjuvant induced arthritic Sprague-Dawley rats) reduced PHA-induced lymphocyte proliferation in a dose-dependent manner (data in graph). LPS-stimulated proliferation was also inhibited at both doses, however the response was partially recovered at the higher tested indomethacin dose (data in graph) (Seng et al. 1990).

Indomethacin increased the total number of cells, and number of T- and B-cells up to 14 days after birth, in newborn ddy mice intraperitoneally injected with 5 μg/g every 2 days from birth (data in graphs) (Shibuya et al. 1986).

In vitro data with cells or cell lines

Indomethacin (3 μM) inhibited proliferation of lymphocytes isolated from C57BL/6 mice (data in graph). Additionally, dose-dependent inhibition LPS-induced proliferation of isolated lymphocytes was noted (Barasoain et al. 1980).

Indomethacin dose-dependently increased male rat (strain not provided) ConA-induced lymphocyte proliferation after an 18-hour incubation (LOAEC = 1 μM). A time-course evaluation with 1 μM indomethacin showed that ConA-inducted lymphocyte proliferation was enhanced at incubation times up to 30 hours. Proliferation at exposure times ranging from 36 to 66 hours were not different from controls (Calder et al. 1991).

Indomethacin (50 nM to 50 μM) dose dependently increased LAK activity in BALB/c mouse splenocytes that were cocultured with recombinant IL-2. Increased lysis of JC tumor cells was observed, reaching a maximum response of 123.6 lytic units at 50 μM compared to 43.6 lytic units for IL-2 alone. Studies also showed that the increased response, compared to addition of IL-2 alone, was observed when culture conditions were maintained for up to 4 days. Addition of nylon wool to the culture, abrogated the

induction of LAK response observed in the presence of indomethacin (Chao et al. 1989). Increased time-dependent proliferation was observed in lymphocytes, from CBA mice, treated with 10 μg/mL indomethacin. After 6 and 24 hours, proliferation was increased 4.3- and 46.6- fold, respectively (Gonzalez-Cabello et al. 1987).

Indomethacin decreased IL-4 levels in ConA-stimulated splenocytes isolated from 3-week old male rats (LOAEL = 50 μM). No effect was noted in splenocytes from females. Decreased IL-6 splenocyte levels was observed in cells obtained from females and treated with 2.0 μM indomethacin. No effect on IL-2, IL-10, IFN-γ, and TNF-α were noted (data not shown or in graph) (Kushima et al. 2009).

Mode of action information

Indomethacin induced effects on prostaglandin synthesis was associated with several immune effects. Lala and Parhar (1988) suggested that indomethacin effects are associated with suppression of prostaglandin synthesis. Rojo and colleagues (1981) and Franceschi and colleagues (1988) proposed that indomethacin inhibition of prostaglandin synthesis leads to altered T-cell function, and NK- and antibody-dependent cytotoxicity.

Differential effects on T-cell and B-cell-induced lymphocyte proliferation were reported. A dose- dependent effect on T-cell function was reported, while an inverse effect on B-cell function was noted (Seng et al. 1990).

Indomethacin has been postulated to produce immune effects through inhibition of Th1, and to a lesser extent Th2, responses (Yamaki et al. 2003). Studies conducted by Jaramillo and colleagues (1992) supported this proposed mode of action.

References

Barasoain I, Rojo JM, Portolés A. 1980. “In vivo” effects of acetylsalicylic acid and two ether derived compounds on primary immune response and lymphoblastic transformation.

Immunopharmacology 2: 293–300.

Boorman GA, Luster MI, Dean JH, Luebke RW. 1982. Effect of indomethacin on the bone marrow and immune system of the mouse. Journal of clinical & laboratory immunology 7: 119– 26.

Calder PC, Bond JA, Newsholme EA. 1991. Effect of inhibitors of eicosanoid synthesis upon lymphocyte proliferation. Biochemical Society transactions 19: 88s.

Chao TY, Ohnishi H, Chu TM. 1989. Augmentation of murine lymphokine (rIL-2)-activated killer cell activity by indomethacin. Molecular biotherapy 1: 318–22.

Fontagne J, Adolphe M, Semichon M, Zizine L, Lechat P. 1980. Effect of in vitro treatment with indomethacin on mouse granulocyte-macrophage colony-forming cells in culture (CFUC).

Possible role of prostaglandins. Experimental hematology 8: 1157–64.

Franceschi D, Warnaka P, Kim B. 1988. Indomethacin inhibition of IL-2-induced splenocyte proliferation. Current surgery 45: 474–6.

Gonzalez-Cabello R, Perl A, Kalmar L, Gergely P. 1987. Short-term stimulation of lymphocyte proliferation by indomethacin in vitro and in vivo. Acta physiologica Hungarica 70: 25–30.

Hartel C, von Puttkamer J, Gallner F, Strunk T, Schultz C. 2004. Dose-dependent immunomodulatory effects of acetylsalicylic acid and indomethacin in human whole blood: potential role of cyclooxygenase-2 inhibition. Scandinavian journal of immunology 60:412–20; doi:10.1111/j.0300-9475.2004.01481.x.

Jaramillo A, Bhattacherjee P, Sonnenfeld G, Paterson CA. 1992. Modulation of immune responses by cyclo-oxygenase inhibitors during intraocular inflammation. Curr Eye Res 11: 571– 579.

Jawad AM, Rogers HJ. 1984. The effects of flurbiprofen and indomethacin on the mitogenic response of human peripheral mononuclear cells. Immunopharmacology 7: 59–67.

Kushima K, Oda K, Sakuma S, Furusawa S, Fujiwara M. 2007. Effect of prenatal administration of NSAIDs on the immune response in juvenile and adult rats. Toxicology 232:257–67; doi:10.1016/j.tox.2007.01.012.

Kushima K, Sakuma S, Furusawa S, Fujiwara M. 2009. Prenatal administration of indomethacin modulates Th2 cytokines in juvenile rats. Toxicology letters 185:32–7; doi:10.1016/j.toxlet.2008.11.013.

Lala PK, Parhar RS. 1988. Cure of B16F10 melanoma lung metastasis in mice by chronic indomethacin therapy combined with repeated rounds of interleukin 2: characteristics of killer cells generated in situ. Cancer research 48: 1072–9.

Rojo JM, Barasoain I, Portoles A. 1981. Further studies on the immunosuppressive effects of indomethacin. International journal of clinical pharmacology, therapy, and toxicology 19: 220–2.

Seng GF, Benensohn J, Bayer BM. 1990. Changes in T and B lymphocyte proliferative responses in adjuvant-arthritic rats: antagonism by indomethacin. European journal of pharmacology 178: 267–73.

Shibuya T, Izuchi K, Koga Y, Nomoto K, Shirakawa K. 1986. Prostaglandin modulation of the postnatal development of T and B lymphocytes in the spleens of newborn mice. Dev Comp Immunol 10: 419–428.

Tanaka K, Tanaka H, Kanemoto Y, Tsuboi I. 1998. The effects of nonsteroidal anti-inflammatory drugs on immune functions of human peripheral blood mononuclear cells. Immunopharmacology 40: 209–17.

Yamaki K, Uchida H, Harada Y, Yanagisawa R, Takano H, Hayashi H, et al. 2003. Effect of the nonsteroidal anti-inflammatory drug indomethacin on Th1 and Th2 immune responses in mice. Journal of pharmaceutical sciences 92:1723–9; doi:10.1002/jps.10380.

Isonicotinic Acid Hydrazide (IAH) [CASRN 54-85-3]

Human Data

Data from epidemiology studies

In 19 cases of INH-induced liver failure, antibodies were present in sera of 15 patients. Anti-INH antibodies were present in 8 patients. Additionally, anti-cytochrome P450 antibodies were identified in up to 14 patients. Antibodies were not detected in patients that were treated with INH but did not have significant liver injury (Metushi et al. 2014c). In eight INH-induced liver failure patients, the dominant serum immunoglobulin isotype of anti-INH antibodies was IgG. A low titer of IgM was observed in two patients, while IgA and IgE antibodies were not detected.

Phenotyping the IgG antibody indicated that the isotype was IgG3 (Metushi et al. 2014b).

INH (1.25 μg/mL) did not stimulate PGE3 production in polymorphonuclear leukocytes or modulate PHA-stimulated mononuclear leukocytes transformations. No effect on PG2 production was observed at 5 μg/mL (Zeis 1987).

In vitro data with cells or cell lines

In a series of studies, Kucharz and colleagues studies the immunomodulatory effects of IAH. In 5 μg/mL PHA-stimulated T-cells, IAH increased cellular proliferation (16% to 27%) at concentrations ranging from 0.01 to 0.0001 mM. (Kucharz and Sierakowski 1990a). In PBMC stimulated with 5 ng/mL anti-CD3 antibody, IAH produced a biphasic response. At 1 and 10 mM IAH decreased (53.6% and 24.4%, respectively) cell proliferation. Increased cellular proliferation (18-47%) was observed at concentrations ranging from 0.0001 to 0.1 mM. A similar biphasic pattern was observed when 10 ng/mL anti-CD3 antibody was used. In T-cells stimulated with anti-CD3 antibody, PHA, or PHA with PMA, IAH also modulated proliferation in a biphasic manner (Kucharz and Sierakowski 1990a). In cells stimulated with 5 μg/mL PHA and 20 ng/mL PMA, 0.1 to 10 mM IAH decreased T-cell proliferation 17% to 46%. At 0.001 mM IAH, at significant increase (21%) in T-cell proliferation was observed (Kucharz and Sierakowski 1990d). In cells stimulated with IL-2, IAH decreased cell proliferation 0.1 and 1 mM (71% and

47%, respectively) and increased proliferation at 0.01 to 0.001 mM (8% to 12%, respectively (Kucharz 1995).

IAH also decreased T-cell IL-2 production at 0.1 and 1 mM (44.7% and 71.6%, respectively) and increased T-cell IL-2 production at 0.01 to 0.0001 mM (105% to 115%). No effect on IL-2 receptor expression in T-cells was observed (Kucharz and Sierakowski 1990b).

IAH decreased IL-1 production from human monocytes in a dose-dependent manner in the absence or presence of lipopolysaccharide (LOAEL = 0.001 mM) (Kucharz and Sierakowski 1992).

In the absence of PHA, IAH stimulated proliferation of Jurkat cells (LOAEL = 0.01 mM). In the presence of PHA (2 or 5 μg/mL), IAH stimulation was observed at higher concentrations (1 and 10 mM) while at lower concentrations no effect was observed (Kucharz and Sierakowski 1990c).

When PMA (20 ng/mL) or PMA (20 ng/mL) and PHA (5 μg/mL) were added to the media, increased Jurkat cellular proliferation was observed at 0.001 mM (32%) and 0.01 and 0.001 mM (8% and 18%, respectively) (Kucharz and Sierakowski 1990d).
INH (5 μg/mL) did not have any effect on the phagocytic activity or intracellular killing activity on polymorphonuclear leukocytes obtained from healthy volunteers (Okuyan et al. 2005).

Mode of action information

Metushi and colleagues proposed that INH produced an immune response that leads to liver injury (Metushi et al. 2014c, 2014b).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

Female *Nat1/2(-/-)* mice were treated with INH either by oral gavage (100 mg/kg/day) for up to 7 days or by feed (0.2%) for 35 days. In mice treated by gavage, significant decrease in M1 macrophages and increase in M2a and M2b macrophages in cervical lymph nodes was noted. No effect on the M2c macrophages was observed. Comparatively, no effect was noted in the macrophage phenotypes obtained from mice that were exposed by feed (Metushi et al. 2014a).
INH (0.1 to 1.0 mg/10 μL) did not alter the weight of popliteal lymph nodes from C57BL/10 mice 7 days after subcutaneous injection (Kammuller et al. 1989). A lack of effect on popliteal lymph nodes from Brown Norway rats also was observed when exposed to 5 mg/50 μL INH (Verdier et al. 1990).
Four female Cbl-b-/-, C57BL/6 background that lack an E3 ubiquitin ligase, were provided diets containing 0.2% w/w INH for 5 weeks. Blood was collected to assess serum cytokine levels.
Significant decreases in serum IL-12 and IL-1α was noted in female Cbl-b-/- mice (data provided in graph). No effects on IL-1ß, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-13, IL- 17A, eotaxin, GCSF, GMCSF, IFNγ, KC, MCP-1, MIP-1α, MIP-1ß, RANTES, and TNF-α were
observed (data provided in supplementary materials) (Metushi and Uetrecht 2014).

In vitro data with cells or cell lines

In HT-2 cells, stimulated with IL-2 (3 or 30 U/mL), increased proliferation was observed at 1 and 10 mM IAH at 30 U/mL and only at 1 mM at 3 U/mL. No effect on proliferation was observed in cells stimulated with 60 U/mL IL-2 (Kucharz and Sierakowski 1990c). Additionally, no effect on proliferation by IAH was observed in HT-2 cells stimulated with PMA (data not provided) (Kucharz and Sierakowski 1990d).

Mode of action information

No data were located.

References

Kammuller ME, Thomas C, De Bakker JM, Bloksma N, Seinen W. 1989. The popliteal lymph node assay in mice to screen for the immune disregulating potential of chemicals--a preliminary study. International journal of immunopharmacology 11: 293–300.

Kucharz EJ. 1995. Studies on immunomodulatory properties of isoniazid. Influence of isoniazid on responsiveness of peripheral blood mononuclear cells to interleukin-2. Central European journal of public health 3: 65–6.

Kucharz EJ, Sierakowski SJ. 1990a. Studies on immunomodulatory properties of isoniazid. I. Effect of isoniazid on mitogen- and anti-CD3 antibody-induced proliferation of human peripheral blood mononuclear cells and T cells. Journal of hygiene, epidemiology, microbiology, and immunology 34: 99–105.

Kucharz EJ, Sierakowski SJ. 1990b. Studies on immunomodulatory properties of isoniazid. II. Effect of isoniazid on interleukin 2 production and interleukin 2-receptor expression. Journal of hygiene, epidemiology, microbiology, and immunology 34: 207–11.

Kucharz EJ, Sierakowski SJ. 1990c. Studies on immunomodulatory properties of isoniazid. III. Effect of isoniazid on proliferation of interleukin-dependent and interleukin-independent cell line. Journal of hygiene, epidemiology, microbiology, and immunology 34: 305–8.

Kucharz EJ, Sierakowski SJ. 1990d. Studies on immunomodulatory properties of isoniazid. IV. Effect of isoniazid on T lymphocyte activation by phorbol ester tumor promoter. Journal of hygiene, epidemiology, microbiology, and immunology 34: 381–5.

Kucharz EJ, Sierakowski SJ. 1992. Studies on immunomodulatory properties of isoniazid: V. Influence of isoniazid on secretion of interleukin-1. Journal of hygiene, epidemiology, microbiology, and immunology 36: 119–22.

Metushi IG, Cai P, Vega L, Grant DM, Uetrecht J. 2014a. Paradoxical attenuation of autoimmune hepatitis by oral isoniazid in wild-type and N-acetyltransferase-deficient mice. Drug metabolism and disposition: the biological fate of chemicals 42:963–73; doi:10.1124/dmd.113.056622.

Metushi IG, Lee WM, Uetrecht J. 2014b. IgG3 is the dominant subtype of anti-isoniazid

antibodies in patients with isoniazid-induced liver failure. Chemical research in toxicology 27:738–40; doi:10.1021/tx500108u.

Metushi IG, Sanders C, Lee WM, Uetrecht J. 2014c. Detection of anti-isoniazid and anti- cytochrome P450 antibodies in patients with isoniazid-induced liver failure. Hepatology (Baltimore, Md) 59:1084–93; doi:10.1002/hep.26564.

Metushi IG, Uetrecht J. 2014. Isoniazid-induced liver injury and immune response in mice. Journal of immunotoxicology 11:383–92; doi:10.3109/1547691x.2013.860644.

Okuyan B, Izzettin FV, Sancar M, Ertas O, Cevikbas A, Gurer US. 2005. Effect of antituberculous drugs on human polymorphonuclear leukocyte functions in vitro. International immunopharmacology 5:1337–42; doi:10.1016/j.intimp.2005.03.002.

Verdier F, Virat M, Descotes J. 1990. Applicability of the popliteal lymph node assay in the Brown-Norway rat. Immunopharmacology and immunotoxicology 12:669–77; doi:10.3109/08923979009019683.

Zeis BM. 1987. Effects of anti-tuberculosis drugs on the production of prostaglandin E2 and on mononuclear leucocyte transformation. Chemotherapy 33:204–10; doi:10.1159/000238496.

Lead (II) Acetate Trihydrate [CASRN 6080-56-4]

Human Data

Data from epidemiology studies

No data were located.

In vitro data with cells or cell lines

Lead (lead acetate 5.0 mg - 1.5 ng/mL, or lead chloride 0.5 mg - 0.15 ng/mL for 24 hours) significantly reduced cell vitality and/or proliferation and affected secretion of proinflammatory, TH1 and TH2 cytokines in human peripheral mononuclear blood cells that were stimulated with either heat-killed *Salmonella enteritidis* (hk-SE) or monoclonal antibodies. At lower lead levels, expression of IFN-γ, IL-1ß and TNF-α were reduced. Monoclonal antibody induced IL-4, IL-6 and IL-10 and hk-SE induced IL-10 and IL-6 levels were increased in the presence of lower lead levels. The authors suggest that lower dose lead suppresses the TH1 cytokine and the proinflammatory cytokines while the increased IL-4 and/or IL-10 production can induce and maintain a TH2 immune response (Hemdan et al. 2005).

Thirty male lead-exposed (battery recycling industry) workers with a blood lead level > 10 µg/dL and 27 unexposed healthy volunteers without any history of occupational exposure to lead were selected for this study. The serum level of IgA was found to be significantly increased in the lead-exposed group as compared to controls. No differences were observed in serum IgG and IgM levels. Both the level of nitric oxide production after stimulation with zymosan-A and the neutrophil respiratory burst as measured by nitroblue tetrazolium reduction were comparable in neutrophils from lead-exposed and unexposed volunteers (Mishra et al. 2006).

Mode of action information

Lead acetate (1 µM) induced activation of NF-κB in primary human CD4+ T lymphocytes. This lead induced activation was blocked by antibodies for p65 and p50 subunits (indicating that the p65:p50 heterodimer (NF-κB) is involved), but not by cRel. Lead acetate (100 pM – 100 µM) did not activate NF-κB in 4 different T cell lines,

suggesting that these cell lines may not be a reliable system for studying transcriptional activation in human T cells (Pyatt et al. 1996).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

Lead acetate suppressed macrophage-dependent immune responses in cells taken from female BDF1 mice exposed to lead in drinking water at concentrations from 0 to 1000 ppm for 3 weeks. The T-cell dependent sheep erythrocyte primary immune response was suppressed by approximately 40-50% in all lead-exposed groups. In contrast, the *E. coli* lipopolysaccharide (T- cell and macrophage-independent) induced response, was not suppressed (Blakley and Archer 1981). Lead did not alter the ability of T-cell mitogens to induce interferon (Blakley et al. 1982).

Lead acetate effects were modulated by maternal protein intake. Fischer 344 rats were exposed to lead acetate (250 ppm) in the drinking water during breeding and pregnancy until parturition and were fed isocaloric diets (either 20% casein or 10% casein). Offspring exposed to lead and high maternal dietary protein had significantly elevated levels of both IL-4 and TNF-α (values not provided). Offspring exposed to lead and low maternal dietary protein had significantly reduced IL-4 levels compared to the lead control group (values not provided). No other changes were observed, and immune parameters measured in the dams were not affected by treatment (Chen et al. 2004).

In a study comparing immunotoxic effects of various lead salts, Balb/c mice were treated for five consecutive days between immunization and elicitation with intraperitoneal injections of 0.5 or 6 mg/kg of a lead salt. A statistically significant increase in delayed hypersensitivity (as measured by footpad swelling) was observed following administration of lead acetate (55.0% increase in footpad thickness as compared to controls; LOAEL = 6 mg/kg) (Descotes et al. 1984).

Exposure to lead acetate resulted in a decreased ability of mice to survive a sublethal dose of a virulent strain of *S. typhimurium*. C3H/HeN mice were exposed to lead acetate (5 or 10 mM) in the drinking water for up to 18 weeks. At week 16, mice were infected with *S. typhimurium*. 40% of the mice exposed to 5 mM lead acetate survived the infection with a median survival of 26 days. None of the mice treated with 10 mM lead acetate survived, with death occurring within three weeks of becoming infected. In contrast, 80% of control mice survived with a median survival of 60 days. The ability of splenocytes, cultured from the lead-treated and control mice showed a marked reduction in the production of IFN-γ (27% and 35% in mice treated with 5 and 10 mM lead acetate, respectively) and IL-12p40 (42-45% in mice treated with 5 and 10 mM lead acetate, respectively, as compared to induced control). Secretion of IL-4 by splenocytes from lead-treated mice was 3 to 3.6-fold higher than in control mice (Fernandez-Cabezudo et al.

2007).

Adult Sprague-Dawley females were treated with 500 ppm lead acetate via drinking water either early in gestation (days 3-9) or late in gestation (days 15-21). Offsping were assessed as adults. Significantly depressed DTH responses as well as increased IL-10

production, relative monocyte numbers and relative thymic weights were reported in female offspring exposed to lead during late gestation. Male offspring exposed during late gestation had significantly increased IL-12 production and decreased IL-10 production while the DTH response, relative monocyte numbers and thymic weights were unchanged compared to controls. The authors found that adherent splenocytes (likely macrophages) and T lymphocytes are the primary immune cells affected during fetal lead exposure and that gender may influence immunotoxicity due to lead exposure (Bunn et al. 2001).

Lead acetate increased IL-4 production in mice at 40 and 400 mg/L and decreased IFN-γ levels in mice at 400 mg/L. Adult Swiss mice were administered lead acetate in drinking water for 14 days. The authors concluded that low level lead exposure enhances a Th2 response while high lead levels can either stimulate Th2 immune activity or reduce Th1 activity, thus resulting in an imbalance between Th1 and Th2 activation (Iavicoli et al. 2004).

Lead acetate (100 or 1000 ppm in drinking water) did not alter the ability of splenocytes isolated from exposed male Alderly Park rats to mediate native and interferon activated natural cytotoxicity at 2,4,6 and 8 weeks following commencement of exposure. Splenic T-cell function of treated rats as determined by phytohaemagglutinin induced proliferation was comparable to control values (Kimber et al. 1986).

Lead acetate (10 mM in the drinking water for 8 weeks) did not suppress the primary direct humoral immune response to T-dependent antigen (sheep erythrocyte) and T-independent antigens (TNP-LPS, TNP-Ficoll) in several inbred (A, BALB/c, C57Bl/6, DBA/1, SJL, and NZW/NZB F1) and an outbred (CFW) strains of mice (Mudzinski et al. 1986).

Lead acetate (200 ppm either in the drinking water or given intraperitoneally for 4 weeks) decreased the number of lymphocyte cells and cellularity (i.e., number of cells per mg tissue) in the thymus, but no significant changes in either parameter were reported for the submaxillary lymph nodes. Proliferation of T cells stimulated by ConA and proliferation of B cells stimulated by LPS was increased by lead in the thymus by both routes of exposure. In the submaxillary lymph nodes, there was a decrease in the proliferation of T cells following treatment by either route (Teijon et al. 2010).

In vitro data with cells or cell lines

RAW 264.7 cells were treated with 100 ppm lead acetate for 24 hours in the presence or absence of LPS. Lead produced a statistically significant inhibition of the level of LPS-induced nitric oxide (data not provided). No effect on cytotoxicity was observed (Mishra et al. 2006).

Mode of action information

C3H/HeN mice were exposed to lead acetate (0, 5 or 10 mM in drinking water for periods of up to 18 weeks) and inoculated with a virulent strain of *S. typhimurium*. Sera were collected on days 15 and 38 post infection. The authors report that the IgG2a antibodies were elevated in control mice by day 38 post infection (0.09 ± 0.05 on day 15 vs. 0.30 ± 0.03 on day 38; an increase of 300% from day 15), but were only slightly increased in lead-exposed mice (0.11 ± 0.01 on day 15 vs. 0.16 ± 0.02 on day 38). IgG1 isotype

antibodies (an isotype induced by IL-4) were significantly elevated in lead exposed mice on day 38, as compared to control mice. The authors conclude that lead acetate induces a subtle but substantial shift toward a Th2-type immune response to infection with Salmonella organism (Fernandez-Cabezudo et al. 2007).

A single intraperitoneal exposure to lead acetate (12 mg/kg) in B6C3F1 mice produced changes in cell surface markers on discrete subpopulations of lymphoid cells from the spleen and bone marrow. The authors concluded that while the changes may not correlate with functional activity of the cells, they seemed to predict a shift to immature cell types, which correlated with the increase in progenitor cells observed (Burchiel et al. 1987).

References

Blakley BR, Archer DL. 1981. The effect of lead acetate on the immune response in mice. Toxicology and applied pharmacology 61: 18–26.

Blakley BR, Archer DL, Osborne L. 1982. The effect of lead on immune and viral interferon production. Canadian journal of comparative medicine : Revue canadienne de medecine comparee 46: 43–6.

Bunn TL, Parsons PJ, Kao E, Dietert RR. 2001. Exposure to lead during critical windows of embryonic development: differential immunotoxic outcome based on stage of exposure and gender. Toxicological sciences : an official journal of the Society of Toxicology 64: 57–66.

Burchiel SW, Hadley WM, Cameron CL, Fincher RH, Lim TW, Elias L, et al. 1987. Analysis of heavy metal immunotoxicity by multiparameter flow cytometry: correlation of flow cytometry and immune function data in B6CF1 mice. International journal of immunopharmacology 9: 597–610.

Chen S, Golemboski K, Piepenbrink M, Dietert R. 2004. Developmental immunotoxicity of lead in the rat: influence of maternal diet. Journal of toxicology and environmental health Part A 67:495–511; doi:10.1080/15287390490276520.

Descotes J, Evreux JC, Laschi-Locquerie A, Tachon P. 1984. Comparative effects of various lead salts on delayed hypersensitivity in mice. Journal of applied toxicology : JAT 4: 265–6.

Fernandez-Cabezudo MJ, Ali SA, Ullah A, Hasan MY, Kosanovic M, Fahim MA, et al. 2007. Pronounced susceptibility to infection by Salmonella enterica serovar Typhimurium in mice chronically exposed to lead correlates with a shift to Th2-type immune responses. Toxicology and applied pharmacology 218:215–26; doi:10.1016/j.taap.2006.11.018.

Hemdan NY, Emmrich F, Adham K, Wichmann G, Lehmann I, El-Massry A, et al. 2005. Dose- dependent modulation of the in vitro cytokine production of human immune competent cells by lead salts. Toxicological sciences : an official journal of the Society of Toxicology 86:75–83; doi:10.1093/toxsci/kfi177.

Iavicoli I, Marinaccio A, Castellino N, Carelli G. 2004. Altered cytokine production in mice exposed to lead acetate. International journal of immunopathology and pharmacology 17:97– 102; doi:10.1177/03946320040170s216.

Kimber I, Jackson JA, Stonard MD. 1986. Failure of inorganic lead exposure to impair natural killer (NK) cell and T lymphocyte function in rats. Toxicology letters 31: 211–8.

Mishra KP, Chauhan UK, Naik S. 2006. Effect of lead exposure on serum immunoglobulins and reactive nitrogen and oxygen intermediate. Human & experimental toxicology 25:661–5; doi:10.1177/0960327106070453.

Mudzinski SP, Rudofsky UH, Mitchell DG, Lawrence DA. 1986. Analysis of lead effects on in vivo antibody-mediated immunity in several mouse strains. Toxicology and applied pharmacology 83: 321–30.

Pyatt DW, Zheng JH, Stillman WS, Irons RD. 1996. Inorganic lead activates NF-kappa B in primary human CD4+ T lymphocytes. Biochemical and biophysical research communications 227:380–5; doi:10.1006/bbrc.1996.1516.

Teijon C, Blanco MD, Romero CS, Beneit JV, Villarino AL, Guerrero S, et al. 2010. Study of response of thymic and submaxillary lymph node lymphocytes to administration of lead by different routes. Biological trace element research 135:74–85; doi:10.1007/s12011-009-8495-6.

Mannitol [CASRN 69-65-8]

Human Data

Data from epidemiology studies

No data were located.

In vitro data with cells or cell lines

Increased urinary excretion of 9α,11ß-prostaglandin F2 and leukotriene-4 were reported in association with mannitol-induced bronchoconstriction in 14 asthmatic patients. Urinary excretion of 9α,11ß-prostaglandin F2 and leukotriene-4 increased from 61 to 92 and 19 to 31 ng

× mmol/creatinine, respectively (Brannan et al. 2006). A separate study reported that repeated challenge with mannitol induced refractoriness in asthma patients. The mannitol refractoriness was associated with maintained release of 9α,11ß-prostaglandin F2 and leukotriene-4 (Larsson et al. 2011).

Increased proportion of submucosal MCTC was observed in asthmatic individuals with airway hyperresponsiveness to mannitol compared to asthmatic individuals without responses to mannitol. The percentage MCTC increased from 18.7% to 40.3%, but the increase in the numbers of MCTC between the two groups was not significantly increased. Increased gene expression of thymic stromal lymphopoietin and carboxypeptidase AM also were reported (Sverrild et al.

2016).

Mannitol significantly increased 9α,11ß-prostaglandin F2, leukotriene-C4, and histamine release from cord blood-derived mast cells (LOAEL = 0.7 M for all endpoints). At the same tested mannitol concentrations (0.3-1.0 M), no concordant increase in lactate dehydrogenase release was observed suggesting cell viability was not affected. The ratio of 9α,11ß-prostaglandin F2 to leukotriene-C4 was 156-1 (Gulliksson et al. 2006).

Mannitol did not induce DNA damage in human leukocytes at concentrations from 1.25 to 10 mM (Frenzilli et al. 2000).

Mannitol (22 mmol/L) did not increase IL-6 or TNF-α secretion from monocytes treated

with glucose (11 mmol/glucose) for 24 hours. A similar lack of effect was observed when cells were incubated for 48 hours (Morohoshi et al. 1996).

At the highest concentration tested (100,000 μM), mannitol did not reduce cell viability in human LCLs or PBMCs. Mannitol (50,000 μM) did not modulate TNF-α, IL-6, IL-2, IL-4, IL- 10, or IFNγ release in LCLs pre-treated with phorbol 12-myristate 13-acetate/ionomycin stimulated cells (Markovic et al. 2015).

Mannitol did not inhibit growth of human granulocyte precursor cells at a concentration up to 5 mM (Holdener et al. 1983).

Mode of action information

Mannitol is shown to narrow the airway in asthmatic, but not healthy, test subjects (Brannan et al. 2001, 2003, 2000). Mannitol is proposed to increase osmolarity of airway surface liquid, leading to an increase in mediator release (e.g., histamine, prostaglandins, and leukotrienes) from inflammatory cells which induces bronchoconstriction (Brannan et al. 2006; Sverrild et al. 2016). One mediator that is proposed to be released is prostaglandin D2 from mast cells (Brannan et al. 2003, 2006).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

No data were located.

In vitro data with cells or cell lines

No data were located.

Mode of action information

No data were located.

References

Brannan JD, Anderson SD, Freed R, Leuppi JD, Koskela H, Chan H-K. 2000. Nedocromil Sodium Inhibits Responsiveness to Inhaled Mannitol in Asthmatic Subjects. American Journal of Respiratory and Critical Care Medicine 161:2096–2099; doi:10.1164/ajrccm.161.6.9908096.

Brannan JD, Anderson SD, Gomes K, King GG, Chan HK, Seale JP. 2001. Fexofenadine decreases sensitivity to and montelukast improves recovery from inhaled mannitol. Am J Respir Crit Care Med 163:1420–1425; doi:10.1164/ajrccm.163.6.2006019.

Brannan JD, Gulliksson M, Anderson SD, Chew N, Kumlin M. 2003. Evidence of mast cell activation and leukotriene release after mannitol inhalation. Eur Respir J 22: 491–496.

Brannan JD, Gulliksson M, Anderson SD, Chew N, Seale JP, Kumlin M. 2006. Inhibition of mast cell PGD2 release protects against mannitol-induced airway

narrowing. The European respiratory journal 27:944–50; doi:10.1183/09031936.06.00078205.

Frenzilli G, Bosco E, Barale R. 2000. Validation of single cell gel assay in human leukocytes with 18 reference compounds. Mutation research 468: 93–108.

Gulliksson M, Palmberg L, Nilsson G, Ahlstedt S, Kumlin M. 2006. Release of prostaglandin D2 and leukotriene C4 in response to hyperosmolar stimulation of mast cells. Allergy 61:1473–9; doi:10.1111/j.1398-9995.2006.01213.x.

Holdener EE, Park CH, Belt RJ, Stephens RL, Hoogstraten B. 1983. Effect of mannitol and plasma on the cytotoxicity of cisplatin. European journal of cancer & clinical oncology 19: 515– 8.

Larsson J, Perry CP, Anderson SD, Brannan JD, Dahlen SE, Dahlen B. 2011. The occurrence of refractoriness and mast cell mediator release following mannitol-induced bronchoconstriction.

Journal of applied physiology (Bethesda, Md : 1985) 110:1029–35; doi:10.1152/japplphysiol.00978.2010.

Markovic T, Gobec M, Gurwitz D, Mlinaric-Rascan I. 2015. Characterization of human lymphoblastoid cell lines as a novel in vitro test system to predict the immunotoxicity of xenobiotics. Toxicology letters 233:8–15; doi:10.1016/j.toxlet.2014.12.013.

Morohoshi M, Fujisawa K, Uchimura I, Numano F. 1996. Glucose-dependent interleukin 6 and tumor necrosis factor production by human peripheral blood monocytes in vitro. Diabetes 45: 954–9.

Sverrild A, Bergqvist A, Baines KJ, Porsbjerg C, Andersson CK, Thomsen SF, et al. 2016. Airway responsiveness to mannitol in asthma is associated with chymase-positive mast cells and eosinophilic airway inflammation. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology 46:288–97; doi:10.1111/cea.12609.

Nickel (II) Sulfate Hexahydrate ($NiSO_4$) [CASRN 10101-97-0]

Human Data

Data from epidemiology studies

No data were located.

In vitro data with cells or cell lines

In PBMC from five nickel allergic individuals 0.1 mM $NiSO_4$ increased IL-4 and IFN-γ production. The peak effect was lower than when PBMC were incubated with PHA (data shown in graph) (Thomas et al. 2003).

$NiSO_4$ (85 μg/mL) significantly upregulated expression of CD40, CD83, CD86, and CD54 markers on THP-1 cells. $NiSO_4$ also significantly increased production of TNF-α and IL-8 in a dose-dependent manner. IL-6 production was significantly increased after exposure to

170 μg/mL (Miyazawa et al. 2007). Ade and colleagues noted that $NiSO_4$ induced CD83, CD86, HLA-DR, and CD40 in a dose dependent manner in dendritic cells (Ade et al. 2007).

Mode of action information

$NiSO_4$ was shown to alter dendritic cell phenotypes by activation of MAPKs and NF-□B. Additionally, $NiSO_4$ induced IL-8, IL-6, and IL-12 p40 production (Ade et al. 2007; Antonios et al. 2009). Activation of the MAPK pathway may lead to upregulation of the Cys-Cys chemokine receptor, CCR7, which allows dendritic cells to migrate to the draining lymph nodes (Boisleve et al. 2004).

$NiSO_4$ has a similar capacity to stimulate polyclonal CD+4 in Ni-allergic and -nonallergic individuals. Differences in clonal expansion or presence of Ni-binding motifs in MHC class II complexes could be involved in the development of allergic contact dermatitis (Lisby et al.

1999).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

Male Wistar rats were intratracheally instilled with 1, 2, 4, or 8 μmole $NiSO_4$ per rat. The rats were euthanized up to 7 days after treatment. Treatment decreased the percentage of lymphocytes in pulmonary lymphoid cells (~55% to ~40%). NK activity in lymphoid lung cells was dependent on concentration and effector:target cell ratio. NK activity was decreased 1 day after treatment of 4 and 8 μmole $NiSO_4$ at the effector to target cell ratio of 6:1. Two days after treatment suppression of NK activity was significant at doses ≥2 μmole $NiSO_4$ and at the effector to target cell ratio of 6:1. After 7 days, a significant decrease was only observed at 8 μmole.

$NiSO_4$ did not significantly modulate alveolar macrophage cytotoxic activity towards 3T12 target cells. Decreased levels of TNF-α was reported at all time points, while increased IFN-γ level was only noted after exposure to 8 μmole/rat on day 2 (data in graph) (Goutet et al. 2000).

Female B6C3F1 mice were exposed to $NiSO_4$ aerosol for 6 hours per day, 5 days per week for 65 days. The actual exposure concentrations tested were 0.027, 0.11, or 0.45 mg Ni/m3. No change in thymic weight was reported. A significant increase in the number of nucleated cell numbers from lung-associated lymph nodes (LALN) and lavage fluid, after mice were immunized with sheep red blood cells, was noted at the highest dose tested (1.72- and 3.86-fold, respectively). Nonsignificant increase in the total antibody-forming cells (AFC)/(LALN) and nonsignificant decrease in AFC/spleen, after immunization with sheep red blood cells, were also noted after $NiSO_4$ exposure. $NiSO_4$ had no effect on mixed lymphocyte response of spleen cells after exposure to mitomycin C-treated spleen cells from DBA/2 mice. No effect in mitogen- stimulation assays also were noted by $NiSO_4$ exposure. $NiSO_4$ modulated pulmonary alveolar macrophage function, as measured by phagocytosis of opsonized erythrocytes; activity was significantly increased at 0.11 mg Ni/m3 (data not provided). Comparatively, $NiSO_4$ had no effect on peritoneal macrophage phagocytosis activity at any tested dose. The highest dose of $NiSO_4$ was associated with a significant two-fold increase in the number of B16F10 tumor nodules in the lungs of treated animals. However, incorporation of radiolabeled uridine was not considered biologically significant. $NiSO_4$ did not affect splenic NK cell cytolytic activity (Haley et al. 1990).

Histopathological lesions in lungs, liver, thymus, kidneys, spleen, and lymph nodes were noted in male F344 rats intramuscularly injected with 125 μmole/kg $NiSO_4$ over 26 days. Thymus glands from rats treated with the highest dose were much smaller than controls.

Corticomedullary junction was not distinct and extensive degeneration and depletion of lymphocytes in the thymic cortex were noted. Additional tissues from these rats were evaluated further. In the lungs, large alveolar macrophages and polymorphonuclear leukocytes were noted in alveolar spaces and exudate. In the spleen and lymph nodes, lymphocytes were focally depleted in the white and red pulp (Knight et al. 1991).

Male Sprague-Dawley rats were exposed to 0.02, 0.05, and 0.1% $NiSO_4$ in drinking water for 13 weeks. Effects on splenic lymphocyte and thymocyte subpopulations were evaluated. In splenic lymphocytes, increases in the total number of T-cells (LOAEL = 0.05%) and CD8+ T-cells (LOAEL = 0.02%) were reported. For CD4+ T-cells, the number

of cells increased at 0.05% $NiSO_4$ and then decreased at 0.1% dose. An increase in the total number of B cells was noted at 0.05% $NiSO_4$. Subchronic exposure to 0.02% $NiSO_4$ also increased the percentage and absolute number of thymocyte CD8+ cells. Exposure to 0.05% $NiSO_4$ increased the total number of thymocyte cells, the percentage and absolute number of CD8+ cells, and absolute numbers of both CD4+ and B-cell populations. Exposure to 0.1% $NiSO_4$ decreased the total number of thymocytes, the percentage and absolute number of CD4+ T cells, and absolute numbers of CD8+ T cells and of B cells (Obone et al. 1999).

Male C3H/He mice were provided 0.01, 0.05, 0.1, 0.25, 0.5, or 1% $NiSO_4$ for 7 or 10 weeks. Mice were then sensitized with $NiSO_4$ for 7 days and the footpad thickness was measured. The mice were then challenged with 0.4% $NiSO_4$ and footpad swelling was measured 24 hours later. After 7 weeks of oral exposure, footpad swelling was not reduced at any of the tested doses.

However, after 10 weeks of exposure swelling was decreased (LOAEL = 0.1%) (Ishii et al. 1993).

Lymph nodes from C3H/He mice sensitized to $NiSO_4$ were incubated with various monoclonal antibodies and then injected into naïve mice. After challenging with $NiSO_4$, footpad swelling was measured. Cells treated with CD4-, Thy1.2-, or Ig-specific antibodies showed reduced swelling while cells treated with CD8 antibodies induced footpad swelling (Ishii et al. 1993).

Macrophage and PMN chemotactic activities in bronchoalveolar fluid were increased at 2 days after intratracheal instillation of 50 μg Ni per male Wistar rat. Activity then decreased until end of the experiment (14 days). Comparatively, LTB4 were maximally decreased at day 1 and then increased to control levels by day 14 (Hirano et al. 1994).

In vitro data with cells or cell lines

Spleen cells from C57BL/6 and Rag-1 deficient mice were stimulated with varying concentrations of $NiSO_4$ (concentrations not provided). Using the ELISPOT assay, IL-2, IL-4 and IFN-γ secreting cells were identified in splenic cells from C57BL/6 mice. The number of IFN-γ cells were greater than the IL-2 and IL-4 cells. At higher concentrations (≥400 μM), the numbers of IL-2 and IL-2 secreting cells decreased while those secreting IFN-γ remained high. The number of IFN-γ cells did not increase due to previous immunization of $NiSO_4$. In splenic cells from Rag-1 deficient mice, $NiSO_4$ also contained IFN-γ secreting cells. However, at higher concentrations the cell levels decreased (in comparison to wild-type). Addition of NK1.1 antibodies produced a partial depletion in the cells. Further studies showed that addition of NKG2D antibodies reduced the number of IFN-γ secreting cells in wild-type and RAG-1 deficient mice (Kim et al. 2009).

Mode of action information

No data were located.

References

Ade N, Antonios D, Kerdine-Romer S, Boisleve F, Rousset F, Pallardy M. 2007. NF-kappaB plays a major role in the maturation of human dendritic cells induced by

NiSO(4) but not by DNCB. Toxicological sciences : an official journal of the Society of Toxicology 99:488–501; doi:10.1093/toxsci/kfm178.

Antonios D, Ade N, Kerdine-Romer S, Assaf-Vandecasteele H, Larange A, Azouri H, et al. 2009. Metallic haptens induce differential phenotype of human dendritic cells through activation of mitogen-activated protein kinase and NF-kappaB pathways. Toxicology in vitro : an international journal published in association with BIBRA 23:227–34; doi:10.1016/j.tiv.2008.11.009.

Boisleve F, Kerdine-Romer S, Rougier-Larzat N, Pallardy M. 2004. Nickel and DNCB induce CCR7 expression on human dendritic cells through different signalling pathways: role of TNF- alpha and MAPK. The Journal of investigative dermatology 123:494–502; doi:10.1111/j.0022- 202X.2004.23229.x.

Goutet M, Ban M, Binet S. 2000. Effects of nickel sulfate on pulmonary natural immunity in Wistar rats. Toxicology 145: 15–26.

Haley PJ, Shopp GM, Benson JM, Cheng YS, Bice DE, Luster MI, et al. 1990. The immunotoxicity of three nickel compounds following 13-week inhalation exposure in the mouse. Fundamental and applied toxicology : official journal of the Society of Toxicology 15: 476–87.

Hirano S, Asami T, Kodama N, Suzuki KT. 1994. Correlation between inflammatory cellular responses and chemotactic activity in bronchoalveolar lavage fluid following intratracheal instillation of nickel sulfate in rats. Archives of toxicology 68: 444–9.

Ishii N, Moriguchi N, Nakajima H, Tanaka S, Amemiya F. 1993. Nickel sulfate-specific suppressor T cells induced by nickel sulfate in drinking water. Journal of dermatological science 6: 159–64.

Kim JY, Huh K, Lee KY, Yang JM, Kim TJ. 2009. Nickel induces secretion of IFN-gamma by splenic natural killer cells. Experimental & molecular medicine 41:288–95; doi:10.3858/emm.2009.41.4.032.

Knight JA, Plowman MR, Hopfer SM, Sunderman FW Jr. 1991. Pathological reactions in lung, liver, thymus, and spleen of rats after subacute parenteral administration of nickel sulfate. Annals of clinical and laboratory science 21: 275–83.

Lisby S, Hansen LH, Menn T, Baadsgaard O. 1999. Nickel-induced proliferation of both memory and naive T cells in patch test-negative individuals. Clinical and experimental immunology 117: 217–22.

Miyazawa M, Ito Y, Yoshida Y, Sakaguchi H, Suzuki H. 2007. Phenotypic alterations and cytokine production in THP-1 cells in response to allergens. Toxicology in vitro : an international journal published in association with BIBRA 21:428–37; doi:10.1016/j.tiv.2006.10.005.

Obone E, Chakrabarti SK, Bai C, Malick MA, Lamontagne L, Subramanian KS. 1999. Toxicity and bioaccumulation of nickel sulfate in Sprague-Dawley rats following 13 weeks of subchronic exposure. Journal of toxicology and environmental health Part A 57: 379–401.

Thomas P, Barnstorf S, Summer B, Willmann G, Przybilla B. 2003. Immuno-allergological properties of aluminium oxide (Al2O3) ceramics and nickel sulfate in

humans. Biomaterials 24: 959–66.

Nitrobenzene [CASRN 98-95-3]

Human Data

Data from epidemiology studies

No data were located.

In vitro data with cells or cell lines

No data were located.

Mode of action information

No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

Female B6C3F1 mice were exposed to 30, 100, or 300 mg/kg nitrobenzene for 14 days via gastric intubation. Hepatomegaly and splenomegaly were observed in mice that received 100 and 300 mg/kg nitrogenzene. Mild congestion in the red pulp areas of the spleen was noted in mice that received 100 mg/kg, while the spleen was dark red in those that received 300 mg/kg.

Absolute and relative spleen weight were significantly increased (LOAEL = 100 mg/kg). Comparatively, absolute and relative thymus weights were increased only at 100 mg/kg. The number of bone marrow cells increased in a dose-dependent manner (LOAEL = 30 mg/kg). At the highest dose tested the increase was 60% above controls. DNA synthesis and the number of CFU-GM per femur also were increased (LOAELs = 30 mg/kg). In response to sheep erythrocytes, a significant increase in spleen weight (62%) and spleen cell number (29%) was observed at 300 mg/kg, when animals were sensitized four days after nitrobenzene exposure.

Comparatively, a decrease in IgM AFCs were decreased (LOAEL = 100 mg/kg). When responses to sheep erythrocytes were observed (sensitization occurred 5 days after nitrobenzene exposure), spleen weight and cells were increased at 100 and 300 mg/kg.

However, no effects on IgG AFC were noted. When 20 days lapsed between nitrobenzene exposure and sensitization to sheep erythrocytes, no effects were reported. No effect on delayed hypersensitivity was reported at any of the tested doses. Splenic proliferation responses induced by PHA and ConA were suppressed by exposure to nitrobenzene (LOAEL = 100 mg/kg). No effect on LPS-induced proliferation were reported. Responses to DBA/2 mice spleen cells also were decreased (LOAEL
= 100 mg/kg). Using radiolabeled sheep erythrocytes, the phagocytic index was shown to be increased in a dose-dependent manner. The phagocytic activity of peritoneal cells also was increased in a dose-dependent manner (LOAEL = 300 mg/kg). The ability of spleen cells to lyse radioactivity from YAC-1 target cells also was evaluated. Nitrobenzene exposure produced a decrease in lysis capacity at 100 and 300 mg/kg at effector:target ratios of 100:1 and 30:1.

Nitrobenzene did not affect host resistance to *Streptococcus pneumonia, Plasmodium berghei,* herpes simplex 2, or B16F10 melanoma. Comparatively, host resistance to *Listeria monocytogenes* was decreased. A challenge of 6 x 10^3 *L. monocytogenes* per mouse killed 13%

of control animals and 57% of animals treated with 300 mg/kg nitrobenzene. A challenge with

1.2 x 10^4 *L. monocytogenes* increased animal death from 19% in controls to 100% at 100 mg/kg nitrobenzene and 86% at 300 mg/kg nitrobenzene (Burns et al. 1994).

In vitro data with cells or cell lines

No data were located.

Mode of action information

Two proposed targets of nitrobenzene are: (1) erythrocytes and (2) precursors to erythrocytes and other cells (e,g., granulocytes). The site of action is proposed to be the bone marrow.

Additionally, effects on T-cell function may play a role in increased susceptibility to *L. monocytogenes* (Burns et al. 1994).

References

Burns LA, Bradley SG, White KL Jr, McCay JA, Fuchs BA, Stern M, et al. 1994. Immunotoxicity of nitrobenzene in female B6C3F1 mice. Drug and chemical toxicology 17:271– 315; doi:10.3109/01480549409017862.

o-Benzyl-p-chlorophenol (BCP) [CASRN 120-32-1]

Human Data

Data from epidemiology studies

No data were located.

In vitro data with cells or cell lines

No data were located.

Mode of action information

No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

B6C3F1 mice were orally administered 100, 300, or 500 mg/kg BCP for 14 days. No effect on spleen or thymus weight were reported. No effect on delayed hypersensitivity response (to keyhold limpet hemocyanin), antibody response to sheep erythrocytes, serum IgM, IgA, or IgG levels, or splenic lymphocyte proliferation were noted. Absolute and relative liver weights were increased at the highest dose group. Additionally, BCP-treated mice did not develop tumors after challenge with PYB6 tumor cells (vs. controls which had a 15% tumor incidence) (Birnbaum et al. 1986).

BCP produced contact hypersensitivity in female B6C3F1 mice (Stern et al. 1991).

In vitro data with cells or cell lines

No data were located.

Mode of action information

No data were located.

References

Birnbaum LS, Deskin R, Grumbein SL, Kurtz P, Fowler KL, Peters AC. 1986.

Prechronic toxicity of o-benzyl-p-chlorophenol in rats and mice. Fundamental and applied toxicology : official journal of the Society of Toxicology 7: 615–25.
Stern ML, Brown TA, Brown RD, Munson AE. 1991. Contact hypersensitivity response to o- benzyl-p-chlorophenol in mice. Drug and chemical toxicology 14:231–42; doi:10.3109/01480549109002186.

Perflouorooctanoic Acid (PFOA) [CASRN 335-67-1]

Human Data

Data from epidemiology studies

Several studies have suggested that prenatal PFOA exposure is linked to immunosuppressive and immunotoxicant effects observed in offspring. Granum and colleagues (2013) reported that maternal PFOA blood levels, collected at birth, were positively associated with decreased rubella antibody-levels (β = -0.40) and an increased number of common cold episodes in children from 0-3 and 2-3 years old. Cord blood IgE levels also were suppressed in female infants with high maternal PFOA levels. However, no effects on number of 18 month-old infants with allergies (e.g., food allergy, eczema) or infections (e.g., otitis media, pneumonia, skin infections, chicken pox) were noted (Okada et al. 2012). Okada and colleagues noted that while the correlation between fetal PFOA levels and the evaluated endpoints were not available, the results suggest that PFOA produced immunosuppressive effects after prenatal exposure.

A positive association between serum PFOA in adults and development of ulcerative colitis also was reported. However, a positive association with other autoimmune diseases, such as Type 1 diabetes, lupus, multiple sclerosis, Chron's disease, and rheumatoid arthritis, was not observed (Steenland et al. 2013).

Chang and colleagues (2016) conducted a systematic review to summarize and evaluate epidemiological literature on PFOA and perfluorooctanesulfonate (PFOS) with relation to evaluated immune endpoints. Endpoints evaluated included immune biomarker levels (e.g., IgE levels, white blood cell count, and C-reactive protein), immune gene expression patterns, atopic or allergic disorders (e.g., asthma, eczema, and food allergy), infectious disease (e.g., common cold), vaccine response, and autoimmune and inflammatory conditions (e.g., ulcerative colitis, rheumatoid arthritis, and osteoarthritis). The authors stated that the totality of the data limited development of a conclusion on the causal relationship between PFOA and/or PFOS exposure and evaluated endpoints due to inconsistent results and confounding factors.

In vitro data with cells or cell lines

Studies with human cells or human-derived cell lines indicate that PFOA modulates cell activation and cytokine production. In human PBMC, PFOA significantly increased the percentage of viable cells at concentrations <125 μg/mL. At higher concentrations (250 and
500 μg/mL), a significant decrease in cell viability was reported (values not reported). No effects on T-cell proliferation (NOAEL = 1 μg/mL) or, TNF-α or IL-6 release (NOAEL = 1 μg/mL) were noted. PFOA also increased monocyte differentiation in HL-60 cells (LOAEL = 100 μg/mL) (Brieger et al. 2011). Comparatively, PFOA decreased TNF-α, IL-4, and IL-10 (LOAEL
= 1 μg/mL, 10 and 10 pg/mL, respectively) in peripheral leukocytes. PFOA also decreased TNF- α (LOAEL = 10 μg/mL) production in THP-1 cells (value not reported). PFOA did not affect IL- 2 production in Jurkat cells (value not reported) (NOAEL = 0.005 μg/mL) (Corsini et al. 2011, 2012; Midgett et al. 2015).

Mode of action information

Direct modulation of NF-□B has been implicated in modulation of cytokine production and secretion (Corsini et al. 2012). PFOA interaction with the PPARα receptor also was implicated in immunomodulatory effects in human cells. Receptor interaction was associated with reduced p65 phosphorylation and NF-□B-mediated transcription (Corsini et al. 2011). The extent the role of PPARα receptor activation plays in human effects is unclear given the low level of human receptor expression (Corsini et al. 2014).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

Animal studies suggest that PFOA exposure can affect innate and adaptive immune functions *in vivo*. Dietary exposure to PFOA (0.02% w/w) for 7 days significantly decreased spleen and thymus weight, and splenocyte and thymocyte levels in wild-type C57Bl/6 mice. Spleen weight and splenocyte numbers were not affected in PPARα-null mice (Yang et al. 2002).

Table 1. Data from Yang et al. (2002)

Group and Treatment	Body weight (g)	Spleen weight (g)	Splenocyte number (x 10^6)	Thymus weight (g)	Thymocyte number (x 10^6)
Wild-type mice					
None	24.5 ± 1.58	0.082 ± 0.006	68.8 ± 16.8	0.061 ± 0.014	81.0 ± 28.2
PFOA	21.0 ± 0.74**	0.050 ± 0.001***	15.3 ± 5.84***	0.013 ± 0.001***	12.8 ±7.98***
PPARα-null mice					
None	23.6 ± 2.9	0.064 ± 0.021	84.0 ± 19.3	0.054 ± 0.006	88.3 ± 7.04

PFOA	23.5 ± 1.0†	0.054 ± 0.015	73.8 ± 26.2†††	0.033 ± 0.005***†††	54.0 ± 12.7**†††

All values are means ± SEM for four animals in each group. **P<0.01, ***P<0.001 compared to the corresponding control.

†P<0.05, †††P<0.001 compared to the corresponding wild-type group.

Dose response studies in C57Bl/6N mice reported that PFOA decreased absolute and relative spleen weights (LOAEL = 7.5 and 15 mg/kg/day, respectively) and absolute and relative thymus weights (LOAEL =15 mg/kg/day for both endpoints). Organ weight effects were generally reversed by 15 days after exposure was terminated (DeWitt et al. 2008). No effect on organ weights was reported in PPARα knockout mice treated with 7.5 or 30 mg/kg/day PFOA for 15 days (DeWitt et al. 2016).

PFOA exposure in drinking water was associated with reduced IgM antibody titers in C57Bl/6J and C57Bl/6N mice (DeWitt et al. 2008, 2016). Removal of the adrenal glands in C57Bl/6N mice did not reverse reductions in IgM antibody titer levels, suggesting that the observed suppression was not in response to corticosterone production (DeWitt et al. 2009).

Modulation of the complement system was observed in C57Bl/6 mice administered PFOA- treated diets. In mice provided diets containing PFOA for 10 days, activity of the classical and alternative pathways of the complement system was decreased (N/LOAEL = 0.01%/0.02%, respectively). Serum C3 levels also was decreased by PFOA (N/LOAEL = 0.01%/0.02%, respectively). Results showed that PFOA-induced hepatotoxicity was associated with activation of the complement system (Botelho et al. 2015).

Dietary PFOA (0.02% w/w) for 10 days significantly decreased total white blood cell count (72%) and number of macrophages in the bone marrow (12.2%) (Qazi et al. 2009). Exposure of mice to 0.002% PFOA for 10 days modulated levels of intrahepatic immune cells. The total number of all leukocytes (CD45+) was increased 2-fold in treated mice. Additionally, changes in cell numbers other cell types also were noted (e.g., granulocytes and myeloid suppressor cells). Hepatic levels of TNF-α (33%), IFN-γ (37%), and IL-4 (31%) were decreased in treated mice; IL-6 levels were not affected (Qazi et al. 2010).
Hu and colleagues reported effects in offspring of dams exposed to PFOA. Dams were gavaged with 0.02, 0.2, or 2 mg/kg PFOA from before pregnancy to PND 21. Splenic CD4+CD25+Foxp3+ T cells was decreased by 22% in exposed offspring (LOAEL = 2 mg/kg) (Hu et al. 2012).

In vitro data with cells or cell lines

Reduced lymphocyte proliferation was observed in cells isolated from C57Bl/6 mice treated with diets containing 0.02% PFOA for 7 days. No effect was observed in lymphocytes isolated from PPARα-null mice also provided diets containing 0.02% PFOA (values not provided) (Yang et al. 2002). Increased *ex vivo* production of TNF-α in cells isolated from peritoneal cavity (2.2-fold) and bone marrow (1.7-fold), and IL-6 in cells isolated from peritoneal cavity (2.6-fold) was observed in mice treated with 0.02% dietary PFOA for 10 days. Comparatively, TNF-α production was decreased (0.8-fold) in cells isolated from spleen of treated animals (Qazi et al.
2009). IgM or IFN-γ production levels were not modulated in intrahepatic immune cells isolated from male C57Bl/6 mice provided diets with 0.002% (w/w) PFOA for 10 days (Qazi et al. 2010).
Ex vivo co-cultures of splenic CD4+CD25+ and CD4+CD25- T cells offspring gestationally and lactationally exposed to PFOA were assessed for effects on IL-10 production. Results showed IL-10 produced was significantly decreased at all doses 61%-75% in cells obtained from male offspring (LOAEL = 0.02 mg/kg). *Ex vivo* measurement of autoreactivity antibodies in female mice gestationally and lactationally exposed to 0.02 and 2 mg/kg PFOA showed an decrease (26%) in anti-ssDNA (Hu et al. 2012).

Mode of action information

PFOA suppresses T-cell-dependent and T-cell-independent antibody responses (DeWitt et al. 2012). The role of PPARα in PFOA-induced immunosuppression may be strain dependent (Corsini et al. 2014). PFOA-induced effects on humoral immunity may occur through effects on B-cell/plasma cell function (DeWitt et al. 2016). Direct effects on immune cells also are a proposed mode of action of PFOA (Corsini et al. 2014).

The lack of impact of removal of the adrenal gland on PFOA-induced inhibition of IgM antibody titer levels suggests that the observed effects are not dependent on elevated corticosterone levels in mice (DeWitt et al. 2009).

Effects on lymphoid organ weights and measures of immune function (i.e., thymus and spleen) indicate that they are differentially sensitive to PFOA effect. The biological basis for this difference is not known (DeWitt et al. 2016).

References

Botelho SC, Saghafian M, Pavlova S, Hassan M, DePierre JW, Abedi-Valugerdi M. 2015. Complement activation is involved in the hepatic injury caused by high-dose exposure of mice to perfluorooctanoic acid. Chemosphere 129:225–231; doi:10.1016/j.chemosphere.2014.06.093.

Brieger A, Bienefeld N, Hasan R, Goerlich R, Haase H. 2011. Impact of perfluorooctanesulfonate and perfluorooctanoic acid on human peripheral leukocytes. Toxicol Vitro Int J Publ Assoc BIBRA 25:960–968; doi:10.1016/j.tiv.2011.03.005.

Chang ET, Adami H-O, Boffetta P, Wedner HJ, Mandel JS. 2016. A critical review of perfluorooctanoate and perfluorooctanesulfonate exposure and immunological health conditions in humans. Crit Rev Toxicol 46:279–331; doi:10.3109/10408444.2015.1122573.

Corsini E, Avogadro A, Galbiati V, dell'Agli M, Marinovich M, Galli CL, et al. 2011. In vitro evaluation of the immunotoxic potential of perfluorinated compounds (PFCs). Toxicol Appl Pharmacol 250:108–116; doi:10.1016/j.taap.2010.11.004.

Corsini E, Luebke RW, Germolec DR, DeWitt JC. 2014. Perfluorinated compounds: emerging POPs with potential immunotoxicity. Toxicol Lett 230:263–270; doi:10.1016/j.toxlet.2014.01.038.

Corsini E, Sangiovanni E, Avogadro A, Galbiati V, Viviani B, Marinovich M, et al. 2012. In vitro characterization of the immunotoxic potential of several perfluorinated compounds (PFCs). Toxicol Appl Pharmacol 258:248–255; doi:10.1016/j.taap.2011.11.004.

DeWitt JC, Copeland CB, Luebke RW. 2009. Suppression of humoral immunity by perfluorooctanoic acid is independent of elevated serum corticosterone concentration in mice. Toxicol Sci Off J Soc Toxicol 109:106–112; doi:10.1093/toxsci/kfp040.

DeWitt JC, Copeland CB, Strynar MJ, Luebke RW. 2008. Perfluorooctanoic acid-induced immunomodulation in adult C57BL/6J or C57BL/6N female mice. Environ Health Perspect 116:644–650; doi:10.1289/ehp.10896.

DeWitt JC, Peden-Adams MM, Keller JM, Germolec DR. 2012. Immunotoxicity of perfluorinated compounds: recent developments. Toxicol Pathol 40:300–311; doi:10.1177/0192623311428473.

DeWitt JC, Williams WC, Creech NJ, Luebke RW. 2016. Suppression of antigen-specific antibody responses in mice exposed to perfluorooctanoic acid: Role of PPARα and T- and B-cell targeting. J Immunotoxicol 13:38–45; doi:10.3109/1547691X.2014.996682.

Granum B, Haug LS, Namork E, Stølevik SB, Thomsen C, Aaberge IS, et al. 2013. Pre-natal exposure to perfluoroalkyl substances may be associated with altered vaccine antibody levels and immune-related health outcomes in early childhood. J Immunotoxicol 10:373–379; doi:10.3109/1547691X.2012.755580.

Hu Q, Franklin JN, Bryan I, Morris E, Wood A, DeWitt JC. 2012. Does developmental exposure to perflurooctanoic acid (PFOA) induce immunopathologies commonly observed in neurodevelopmental disorders? Neurotoxicology 33:1491–1498; doi:10.1016/j.neuro.2012.10.016.

Midgett K, Peden-Adams MM, Gilkeson GS, Kamen DL. 2015. In vitro evaluation of the effects of perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) on IL-2 production in human T-cells. J Appl Toxicol JAT 35:459–465; doi:10.1002/jat.3037.

Okada E, Sasaki S, Saijo Y, Washino N, Miyashita C, Kobayashi S, et al. 2012. Prenatal exposure to perfluorinated chemicals and relationship with allergies and infectious diseases in infants. Environ Res 112:118–125; doi:10.1016/j.envres.2011.10.003.

Qazi MR, Abedi MR, Nelson BD, DePierre JW, Abedi-Valugerdi M. 2010. Dietary exposure to perfluorooctanoate or perfluorooctane sulfonate induces hypertrophy in centrilobular hepatocytes and alters the hepatic immune status in mice. Int Immunopharmacol 10:1420–1427; doi:10.1016/j.intimp.2010.08.009.

Qazi MR, Bogdanska J, Butenhoff JL, Nelson BD, DePierre JW, Abedi-Valugerdi M. 2009. High-dose, short-term exposure of mice to perfluorooctanesulfonate (PFOS) or perfluorooctanoate (PFOA) affects the number of circulating neutrophils differently, but enhances the inflammatory responses of macrophages to lipopolysaccharide (LPS) in a similar fashion. Toxicology 262:207–214; doi:10.1016/j.tox.2009.06.010.

Steenland K, Zhao L, Winquist A, Parks C. 2013. Ulcerative colitis and perfluorooctanoic acid (PFOA) in a highly exposed population of community residents and workers in the mid-Ohio valley. Environ Health Perspect 121:900–905; doi:10.1289/ehp.1206449.

Yang Q, Xie Y, Alexson SEH, Nelson BD, DePierre JW. 2002. Involvement of the peroxisome proliferator-activated receptor alpha in the immunomodulation caused by peroxisome proliferators in mice. Biochem Pharmacol 63: 1893–1900.

Toluene [CASRN 108-88-3]

Human Data

Data from epidemiology studies

No difference was noted in lymphocyte counts between individuals with or without toluene exposure (Akbas et al. 2004).

In vitro data with cells or cell lines

No data were located.

Mode of action information

No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

Male C3H mice were exposed to 9 ppm toluene (nose-only inhalation exposure) for 30 minutes on study days 0, 1, 2, 7, 14, 21, and 28. Mice also were immunized with ovalbumin. Toluene exposure significantly increased total cell (3-fold) and macrophage (3.1-fold) count in BAL 24 hours after final exposure. No effect on lymphocyte count was noted. BDNF level in BAL was increased in toluene-exposed mice that were immunized with ovalbumin (data in figure). Splenic ratio of CD4 and CD8 cells in control and toluene-exposed mice were not significantly different;
3.95 and 4.14, respectively. Treatment with ani-CD4 antibody decreased the ratios to 0.65 and 0.49, respectively. Toluene exposure significantly increased plasma levels of nerve growth factor (data in figure), but did not increase plasma BDNF levels (data not provided) (Fujimaki et al. 2009).
Male C57BL/10 and B10.BR/Sg mice were inhalationally exposed to 0, 5, and 50 ppm toluene for 6 hours per day, 5 days per week for 6 weeks. Subgroups of control and treated mice were administered ovalbumin prior to exposure. Toluene exposure did not impact ConA- or LPS- induced proliferation of spleen cells from C57BL/10 mice. While

no effect of ConA was noted in B10.BR/Sg (not treated with ovalbumin) mice spleen cells, 50 ppm toluene significantly increased the LPS-induced proliferation of spleen cells. Comparatively, 50 ppm toluene significantly decreased spleen cell proliferation in B10.BR/Sg mice treated with ovalbumin (data in graphs). Toluene did not alter expression of CD3, CD19, and CD11b (data not provided).

Forkhead box P3 (Foxp3) transcription was significantly increased in spleen cells from B10.BR/Sg mice exposed to 5 ppm toluene and ovalbumin, when compared to controls and those not treated with ovalbumin. No effect on GATA3 or T-bet expression was noted (Fujimaki et al. 2010).

Pregnant C3H/HeN mice were exposed to 50 ppm toluene via inhalation on GD 14-18. Additionally, male offspring of unexposed dams were exposed to 50 ppm toluene on PND 2-6 or 8-12. The following table summarizes the effects observed in male offspring on PND 21.

Table 1. Summary of effects in male offspring

Origin	Biomarker	GD 14-18	PND 2-6	PND 8-12
Plasma	IgG2a	No effect	Decrease	Increase
	IgG1	Decrease	Decrease	Decrease
Spleen	CD4+ lymphocyte subset	No effect	Decrease	Decrease
	CD8+ lymphocyte subset	No effect	No effect	Decrease
	T-bet mRNA	No effect	Decrease	Decrease
	Foxp3 mRNA	No effect	Decrease	Decrease
	GATA3 mRNA	No effect	No effect	No effect

On PND 42, IgG2a levels were decreased in mice exposed to 50 ppm toluene on PND 8-12. No effect on IgG1 was noted. CD19+ B-lymphocytes and CD4+ T-lymphocytes were significantly decreased, while CD3+ T-lymphocytes were increased at PND 42 after exposure on PND 8-12. Additionally, T-bet expression was significantly decreased, while no effects on GATA3 or Foxp3 mRNA expression were reported (Win-Shwe et al. 2012a). Pregnant C3H/HeN mice were exposed to 5 or 50 ppm toluene via inhalation on GD 14-18. Additionally, male offspring of unexposed dams were exposed to 5 or 50 ppm toluene on PND 2-6 or 8-12. In the hippocampus of PND 21 male offspring, TNF-α and NF-κB mRNA were significantly increased in mice exposed to 50 ppm on PND 2-6 when compared to controls (data in graphs). TNF-α, CCL3, and NF-κB were increased in mice exposed to 5 ppm on PND 8-12 (data in graphs) (Win-Shwe et al. 2012b).

In vitro data with cells or cell lines

Toluene (500 μM) exposure significantly increased ConA- (1.8-fold) and LPS- (2.1-fold) induced proliferation of spleen cells from female C57BL/6 mice. However, at the same concentration toluene did not modulate NK activity or suppress CTL formation (Grayson and Gill 1986).

Mode of action information

Low-level (5 ppm) inhalational exposure to toluene activates the STAT6, STAT5, and Foxp3 signaling pathway to enhance Th2-related and T_{reg}-related responses in B10.BR/Sg mice treated with ovalbumin (Fujimaki et al. 2010). Toluene also enhanced NF-□B, STAT5, and NF-AT in thymus cells of C3H/HeN mice inhalationally exposed to toluene (Liu et al. 2010).

Toluene modulation of IL-2 synthesis, after oral exposure, may play a role in observed immunotoxic effects (Hsieh et al. 1989).

References

Akbas E, Derici E, Soylemez F, Kanik A, Polat F. 2004. An investigation of effects of toluene and cigarette smoking on some blood parameters and lymphocyte life span. Cell biology and toxicology 20: 33–40.

Fujimaki H, Win-Shwe TT, Yamamoto S, Kunugita N, Yoshida Y, Arashidani K. 2010. Different sensitivity in expression of transcription factor mRNAs in congenic mice following

exposure to low-level toluene. Inhalation toxicology 22:903–9; doi:10.3109/08958378.2010.494256.

Fujimaki H, Win-Shwe TT, Yamamoto S, Nakajima D, Goto S. 2009. Role of CD4(+) T cells in the modulation of neurotrophin production in mice exposed to low-level toluene. Immunopharmacology and immunotoxicology 31:146–9; doi:10.1080/08923970802504762.

Grayson MH, Gill SS. 1986. Effect of in vitro exposure to styrene, styrene oxide, and other structurally related compounds on murine cell-mediated immunity. Immunopharmacology 11: 165–73.

Hsieh GC, Sharma RP, Parker RD. 1989. Immunotoxicological evaluation of toluene exposure via drinking water in mice. Environmental research 49: 93–103.

Liu J, Yoshida Y, Kunugita N, Noguchi J, Sugiura T, Ding N, et al. 2010. Thymocytes are activated by toluene inhalation through the transcription factors NF-kappaB, STAT5 and NF-AT. Journal of applied toxicology : JAT 30:656–60; doi:10.1002/jat.1536.

Win-Shwe TT, Kunugita N, Nakajima D, Yoshida Y, Fujimaki H. 2012a. Developmental stage- specific changes in immunological biomarkers in male C3H/HeN mice after early life toluene exposure. Toxicology letters 208:133–41; doi:10.1016/j.toxlet.2011.10.015.

Win-Shwe TT, Kunugita N, Yoshida Y, Nakajima D, Tsukahara S, Fujimaki H. 2012b. Differential mRNA expression of neuroimmune markers in the hippocampus of infant mice following toluene exposure during brain developmental period. Journal of applied toxicology : JAT 32:126–34; doi:10.1002/jat.1643.

Tributyltin Chloride (TBTC) [CASRN 1461-22-9]

Human Data

Data from epidemiology studies

No data were located.

In vitro data with cells or cell lines

TBTC dose-dependently decreased the percentage of colony forming unit-granulocyte macrophage (CFU-GM) colonies at concentrations ranging from 0.001 to 3.3 μM (data not provided) (Carfi et al. 2007).

Cytokine release from human lymphocytes was assessed in whole blood obtained from three donors. IFN-γ was evaluated in blood from two donors after PHA stimulation for 72 hours. Comparatively, TNF-α was evaluated in blood from three donors after LPS stimulation for 72 hours. Overall, IFN-γ and TNF-α was modulated (i.e., either increase or decrease release) in all tested samples (Carfi et al. 2007).

Long-term cultures of human bone marrow cells were incubated with 0.001 μM TBTC in the presence or absence of a cytokine mixture for 7 or 14 days. A significant decrease in the percentage of CD19+CD22+ cells, in the absence of effects on the total lymphocyte population or percentage of T-cell subsets was reported after 7 and 14 days. Addition of cytokine mixture had no effect on TBTC effects. TBTC also induced cell death in CD19+ lymphocytes, in the absence of PPAR-γ receptor expression (Carfi et al. 2010).

The IC50s for cell viability in human LCLs or PBMCs were 0.25 and 0.33 μM, respectively. TBTC (0.1 μM) did not modulate TNF-α, IL-2, IL-4, or IL-10 release in LCLs pre-treated with phorbol 12-myristate 13-acetate/ionomycin stimulated cells. Comparatively, TBTC significantly decreased IL-6 and IFN-γ release (Markovic et al. 2015).

Mode of action information

In vitro toxicogenomic studies in Jurkat cells (human lymphoblastic T-cell line) showed that TBTC activated cellular stress response and retinoic-acid mediated response genes (Shao et al. 2013).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

In a 2-week study, male Wistar rats were provided diets containing 15, 50, or 150 ppm TBTC. A dose-related decrease in relative and absolute spleen and thymus weights were reported (LOAEL
= 50 and 15 ppm, respectively). Concurrent to the change in thymus weight, a decrease in thymic cell counts also was observed (LOAEL = 50 ppm). However, no signs of increased lymphocyte destruction in the spleen was observed. A dose-related increase in relative liver weight was reported (LOAEL = 50 ppm). Decreased thymus weight also was observed in rats fed 100 ppm

TBTC for 4-weeks (43% of control weight); no effects on spleen or liver weight were noted (Snoeij et al. 1985).

In utero and lactational exposure effects of TBTC (0.025, 0.25, or 2.5 mg/kg/day) were evaluated in Sprague-Dawley rats. Dams were orally dosed with TBTC from GD 8 until weaning. After weaning, pups were orally exposed to the same dose as the dam until sacrifice (up to PND 90). In males, a significant decrease in spleen weight was only observed in pups treated with 0.25 mg/kg/day on PND 30. A significant decrease in thymus weight also was noted on PND 30 (LOAEL = 2.5 mg/kg/day) (Cooke et al. 2004). Serum IgM levels were increased in 30- and 60-day old female offspring, while IgA, IgM, IgG, and IgG2a levels were increased in 90-day old male rats (Tables 1 and 2) (Tryphonas et al. 2004).

Table 1. Serum IgM levels in 30- and 60-day old females†.

	30-day old females				60-day old females			
	Control	0.025 mg/kg/day	0.25 mg/kg/day	2.5 mg/kg/day	Control	0.025 mg/kg/day	0.25 mg/kg/day	2.5 mg/kg/day
IgM	51.6 ± 8.8	41.2 ± 6.8	39.6 ± 5.8	66.5 ± 9.9	34.0 ± 3.2	63.8 ± 5.8	68.1 ± 16.4	73.0 ± 15.0

†Values provided as pg Ig/mL serum × 10^4 (standard error of the mean ± standard error).

Table 2. Serum immunoglobulin levels in 90-day old males†

	Control	0.025 mg/kg/day	0.25 mg/kg/day	2.5 mg/kg/day	Pearson product moment correlation
IgA	32.0 ± 8.8	13.9 ± 3.3	9.7 ± 3.4*	11.9 ± 1.6	>0.05
IgM	46.2 ± 8.9	65.1 ± 4.9	69.6 ± 5.8	232.5 ± 90.1*	0.00168
IgG	96.8 ± 9.6	184.2 ± 86.7	194.6 ±	314.1 ±	0.0134

			25.7*	57.5*	
IgG1	41.5 ± 8.6	77.2 ± 28.6	85.2 ± 18.6	58.1 ± 15.9	>0.05
IgG2a	53.1 ± 5.7	59.1 ± 6.3	50.8 ± 4.6	31.3 ± 4.4*	0.00041
IgG2b	34.1 ± 3.3	39.1 ± 5.6	39.3 ± 3.5	31.6 ± 4.2	>0.05
IgG2c	13.6 ± 1.7	20.6 ± 3.6	41.0 ± 19.4	20.9 ± 2.2	>0.05

†Values provided as pg Ig/mL serum × 10^4 (standard error of the mean ± standard error).

* Significantly different from control.

The number and percentage of NK cells was increased in 30-day female and male offspring (LOAEL = 2.5 mg/kg/day). A dose-dependent increase in the number and percentage of NK cells also was noted in 90-day male rats. In 60-day female offspring an increase in the percentage of CD4+8+ T lymphocytes (LOAEL = 0.25 mg/kg/day). No anti-sheep erythrocyte IgM response or lymphoproliferative activity of splenocytes in response to mitogen stimulation was noted in 60- day old female rats or 90-day old male rats (data not provided). Delayed-type hypersensitivity to oxazolone was increased in 90-day old male rats at 0.025 and 0.25 mg/kg/day and decreased at

2.50 mg/kg/day. Mean colony forming *L. monocytogenes* bacteria was non-linearly increased at 48 hours post-infection and statistically significant in pairwise comparisons (0.25 mg/kg/day) in 60-day old females. In 90-day old males, a non-linear dose–response trend 3 days after infection was reported. No effects in serum levels of IL-2, TNF-α, IFN-γ, and IL-1ß were reported in males or females. A non-linear dose-response increase in NK activity in 60-day females was reported (Tryphonas et al. 2004).

Lactational exposure in mice to TBTC also impaired innate immunodefenses in offspring. C57BL/6 pregnant mice were given drinking water with 15 or 50 µg/mL TBTC from parturition to weaning. Clearance of *Escherichia coli* K-12 from the peritoneal cavity and spleen of offspring treated with 15 µg/mL TBTC was significantly decreased (Kimura et al. 2005).

ICR mice were orally dosed with 0.5, 4, or 20 mg/kg TBTC for 28 days. Relative spleen and thymus weights were significantly decreased at the highest dose tested (46% and 59% decrease, respectively). TBTC also decreased the number of plaque forming cells in response to exposure to sheep red blood cells (LOAEL = 4 mg/kg). TBTC also suppressed delayed-type hypersensitivity response to sheep red blood cells when assessed 24 and 48 hours after injection (LOAEL = 4 mg/kg). TBTC suppressed T-lymphocyte proliferation in a dose dependent manner (LOAEL = 20 mg/kg). Increased percentage of early- and late-stage thymocyte apoptosis, and expression of Fas protein expression in proteins also were noted (LOAEL = 4 mg/kg) (Chen et al. 2011).

Esophageal tubing of male C3H/Hen mice with 10 or 100 ppm TBTC for 1 week was associated with decreased NK activity. NK activities were inhibited 36% to 46% at effector:target (YAC-1 cells) ratios of 25:1 and 50:1, respectively. A significant decrease in the percentage of large granular lymphocytes (~60%) also was noted 1 week after end of treatment (Ghoneum et al.
1990).

In vitro data with cells or cell lines

Neutrophils and macrophages from mice lactationally exposed to TBTC (15 or 50 µg/mL) were isolated from peritoneal exudates. Bacterial binding to isolated neutrophils from offspring treated with 50 µg/mL TBTC was significantly decreased (data not provided). Comparatively, bacterial binding was increased in macrophages isolated from offspring treated with 50 µg/mL TBTC. Decreased phagocytosis (LOAEL = 15 µg/mL) and killing activities (15 µg/mL) only were observed in neutrophils. No effect on IL-1ß, IL-6, or TNF-α production was noted from macrophages or neutrophils. MCP-1 production was significantly increased in neutrophils isolated from offspring treated with 50 µg/mL TBTC (Kimura et al. 2005).

Rat and mouse spleen cells were treated with TBTC for 24 hours and then LPS or PHA for 48 hours. Then, mitogen stimulation was assessed by 5-bromo-2'-deoxyuridine incorporation.

TBTC inhibited cellular proliferation for both species; the inhibitory response was more potent in mice cells vs. rat cells (IC50 with LPS: 0.0025 vs. 0.007 μM, IC50 with PHA: 0.002 vs.

0.007 μM). TBTC also inhibited rat spleen proliferation that was stimulated by ConA (no data provided). TBTC also inhibited anti-CD3 antibody stimulation of mouse lymphocyte proliferation (IC50 > 0.1 μM) (Carfi et al. 2007).

NK activity was dose-dependently inhibited in splenic lymphocytes incubated with 0.01 to

1 ppm TBTC. The LOAEL values at effector:target ratios of 25:1 and 50:1 were 0.05 and 0.01 ppm, respectively. Decreased viability of splenic lymphocytes also was reported after exposure to TBTC (LOAEL = 0.1 ppm) (Ghoneum et al. 1990).

Mode of action information

In vivo effects of TBTC on the thymus of orally treated rats are proposed to be due to the metabolite dibutyltin chloride (Snoeij et al. 1988).

The role of apoptosis is not clear. In one study the authors indicated that apoptosis does not appear to be involved in inhibition of immature thymocyte proliferation, which may lead to thymus atrophy (Gennari et al. 1997). In a separate study, the authors proposed oxidative stress plays a role in TBTC-caspase-dependent apoptosis in murine thymocytes (Sharma and Kumar 2014).

In vitro studies suggest that TBTC promotes Th2 polarization via depletion of glutathione in antigen-presenting cells, which leads to modulation of IL-10 and IL-12 production (Kato et al. 2006).

References

Carfi M, Bowe G, Pieters R, Gribaldo L. 2010. Selective inhibition of B lymphocytes in TBTC- treated human bone marrow long-term culture. Toxicology 276:33–40; doi:10.1016/j.tox.2010.06.012.

Carfi M, Gennari A, Malerba I, Corsini E, Pallardy M, Pieters R, et al. 2007. In vitro tests to evaluate immunotoxicity: a preliminary study. Toxicology 229:11–22; doi:10.1016/j.tox.2006.09.003.

Chen Q, Zhang Z, Zhang R, Niu Y, Bian X, Zhang Q. 2011. Tributyltin chloride-induced immunotoxicity and thymocyte apoptosis are related to abnormal Fas expression. International journal of hygiene and environmental health 214:145–50; doi:10.1016/j.ijheh.2011.01.008.

Cooke GM, Tryphonas H, Pulido O, Caldwell D, Bondy GS, Forsyth D. 2004. Oral (gavage), in utero and postnatal exposure of Sprague-Dawley rats to low doses of tributyltin chloride. Part 1: Toxicology, histopathology and clinical chemistry. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association 42: 211–20.

Gennari A, Potters M, Seinen W, Pieters R. 1997. Organotin-induced apoptosis as observed in vitro is not relevant for induction of thymus atrophy at antiproliferative doses. Toxicol Appl Pharmacol 147:259–266; doi:10.1006/taap.1997.8265.

Ghoneum M, Hussein AE, Gill G, Alfred LJ. 1990. Suppression of murine natural killer

cell activity by tributyltin: in vivo and in vitro assessment. Environmental research 52: 178–86.

Kato T, Tada-Oikawa S, Takahashi K, Saito K, Wang L, Nishio A, et al. 2006. Endocrine disruptors that deplete glutathione levels in APC promote Th2 polarization in mice leading to the exacerbation of airway inflammation. European journal of immunology 36:1199–209; doi:10.1002/eji.200535140.

Kimura K, Kobayashi K, Naito H, Suzuki Y, Sugita-Konishi Y. 2005. Effect of lactational exposure to tributyltin chloride on innate immunodefenses in the F1 generation in mice.

Bioscience, biotechnology, and biochemistry 69:1104–10; doi:10.1271/bbb.69.1104.

Markovic T, Gobec M, Gurwitz D, Mlinaric-Rascan I. 2015. Characterization of human lymphoblastoid cell lines as a novel in vitro test system to predict the immunotoxicity of xenobiotics. Toxicology letters 233:8–15; doi:10.1016/j.toxlet.2014.12.013.

Shao J, Katika MR, Schmeits PC, Hendriksen PJ, van Loveren H, Peijnenburg AA, et al. 2013. Toxicogenomics-based identification of mechanisms for direct immunotoxicity. Toxicological sciences : an official journal of the Society of Toxicology 135:328–46; doi:10.1093/toxsci/kft151.

Sharma N, Kumar A. 2014. Mechanism of immunotoxicological effects of tributyltin chloride on murine thymocytes. Cell biology and toxicology 30:101–12; doi:10.1007/s10565-014-9272-7.

Snoeij NJ, Penninks AH, Seinen W. 1988. Dibutyltin and tributyltin compounds induce thymus atrophy in rats due to a selective action on thymic lymphoblasts. Int J Immunopharmacol 10: 891–899.

Snoeij NJ, van Iersel AA, Penninks AH, Seinen W. 1985. Toxicity of triorganotin compounds: comparative in vivo studies with a series of trialkyltin compounds and triphenyltin chloride in male rats. Toxicol Appl Pharmacol 81: 274–286.

Tryphonas H, Cooke G, Caldwell D, Bondy G, Parenteau M, Hayward S, et al. 2004. Oral (gavage), in utero and post-natal exposure of Sprague-Dawley rats to low doses of tributyltin chloride. Part II: effects on the immune system. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association 42: 221–35.

Urethane [CASRN 51-79-6]

Human Data

Data from epidemiology studies

No data were located.

In vitro data with cells or cell lines

The IC50s for cell viability in human LCLs or PBMCs were 82,329 and 140,768 μM, respectively. Urethane (5000 μM) did not modulate TNF-α, IFN-γ, IL-2, IL-4, IL-6, or IL-10 release in LCLs pre-treated with phorbol 12-myristate 13-acetate/ionomycin stimulated cells (Markovic et al. 2015).

Urethane dose-dependently decreased the percentage of CFU-GM colonies at concentrations greater than 1000 μM (data provided in graph) (Carfi et al. 2007).

Cytokine release from human lymphocytes was assessed in whole blood obtained from four donors. IFN-γ was evaluated in blood from three donors after PHA stimulation for 72 hours. Comparatively, TNF-α was evaluated in blood from four donors after LPS stimulation for 72 hours. IFN-γ was modulated (i.e., either increase or decrease release) in a single tested sample. TNF-α was not modulated any of the tested samples (Carfi et al. 2007).

Mode of action information

No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

Inbred A/J mice were administered urethane (1 mg/g) via intraperitoneal injection. A biphasic response on splenic NK cell activity was noted. At one day after the injection activity was decreased 60%, activity then increased (decreased 35%), and then remained decreased until 14 days after exposure (decreased 98%). Spleen size was initially reduced, but then increased to control levels. Mitogen response (against YAC-

1 or RL♂1 target cells) was initially depressed after urethane exposure and then returned to control levels (Gorelik and Herberman 1981a).

Inbred A/J mice (5-24 days old) were administered urethane (0.5 mg/g or 1 mg/g) up to 24 days old. In all tested groups, splenic NK activity was inhibited without effects on cellularity on spleens. Decreased NK activity remained until at least 8-10 weeks of age (Gorelik and Herberman 1981a).

Inbred A/J, CBA/J, and C57BL/6 mice were administered urethane (1 mg/g) via intraperitoneal injection. One day after injection, cytotoxicity (against YAC-1 target cells) of A/J and CBA/] spleen cells was significantly decreased (63% and 25%, respectively). Activity was similar to control levels at day 4. Activity then decreased in splenic cells from A/J mice (58%), while a

similar effect in cells from CBA/J mice was not observed. No effect on activity was observed in C57BL/6 mice (Gorelik and Herberman 1981b).

Female B6C3F1 mice were administered 1, 2, or 4 mg/g urethane over a 14-day period via intraperitoneal injection. Decreased spleen weight (decreased 47%) and thymic atrophy (decreased 40%) were observed at 4 mg/g. Splenic lymphoproliferative response to ConA was decreased at 4 mg/g (42%). Responses to PHA and spleen cells from DBA mice were similar to controls. Delayed hypersensitivity responses also were not affected by exposure to urethane.

Serum immunoglobulin levels and antibody responses to sheep erythrocytes and LPS were decreased in mice administered 4 mg/kg (decreased 61% and 46%, respectively). Macrophage cytostasis of MBL-2 target cells was decreased (LOAEL = 1 mg/g). However, phagocytosis and bactericidal activity against *S. aureus* was not affected. Pluripotent stem cells proliferation was inhibited at all doses. Urethane decreased NK activity against all YAC-1 target to cell ratios at all doses (Luster et al. 1982).

C57BL/6J dams were subcutaneously injected with 0.05 or 0.1 mg/g urethane on GD 7-17. Offspring were evaluated 8 weeks after parturition. Increased relative spleen weight was reported for the litter at 0.05 mg/g urethane. When evaluated based on sex, only an increase in relative thymus weight was observed at 0.05 and 0.1 mg/g. Decreased white blood cell count was also observed (LOAEL = 0.05 mg/g). No effect on lymphoproliferative responses or NK cell activity was noted. However, a decrease in the levels of plaque forming cells in response to sheep erythrocytes was noted (LOAEL = 0.1 mg/g) (Luebke et al. 1986).

C57BL/6J offspring were subcutaneously injected with 0.2 mg/g urethane on PND 5-14. No effects on organ weight or lymphoproliferative responses were noted. NK cell activity was decreased at an effector:target (YAC-1) ratio of 50:1. Splenic cellularity was increased in female offspring and decreased in male offspring (Luebke et al. 1986).

Female C57BL/6J mice were subcutaneously injected with 1, 2, or 4 mg/g urethane. Significant reduction in absolute (LOAEL = 1 mg/g) and relative (data not provided) spleen weights were observed. Additionally, absolute thymus weight was decreased (LOAEL = 4 mg/g). Dose- dependent reduction in leukocyte number was noted, but differential counts of white blood cells were not altered. Lymphoproliferative responses,

induced by ConA, PHA, and LPS, were suppressed by urethane (LOAELs = 1, 1, and 4 mg/g, respectively). were noted.

Lymphoproliferative responses to allogenic cells (mitomycin C treated CBA/J mouse spleen cels) were not affected by urethane exposure. NK cell activity was not affected at any effector:target (YAC-1) ratio. Splenic cellularity of mice treated with urethane and sheep erythrocytes was decreased (LOAEL = 2 mg/g) without effects on PFC/spleen or PFC/splenocytes. Decreased DTH index (to keyhole limpet hemocyanin) was decreased in urethane treated mice (LOAEL = 4 mg/g) (Luebke et al. 1987).

mRNA expression of interleukins and TNF-α were evaluated in spleens of male Wistar rats exposed to 1500 mg/kg urethane. Increased expression of IL-6 was noted, while decreased expression of IL-1ß and TNF-α were reported. No effects on IL-2 expression were observed (Bette et al. 2004).

Urethane (10%) did not deplete ear epidermis Ia-positive LCs after male BALB/c mice were treated with topical application. Similarly, urethane did not alter the density of ß-glucuronidase- positive LC in C57BL mouse tails topically treated for 1 or 3 weeks (Halliday et al. 1988).

Urethane administration to pregnant ICR mice (1.5 mg/g subcutaneous injection on GD 10) produced a transient decrease in dam thymocyte cell count. At 3 days after treatment, a significant decrease in cell count was noted. By 5 days after treatment, the cell count had recovered to control levels (data in graph). A similar phenomenon was noted with thymocyte phenotypes; decrease in CD4+8+ thymocytes (88%) at day 3 after treatment was recovered by day 5. Transient changes in dam splenocyte cell count and splenocyte phenotype CD4+8-, CD4- 8+, and CD4-8- also were reported. Gene expression analyses identified changes in spleen gene expression due to urethane exposure with or without immune stimulation (FCA). Increased expression of TGFß3 was observed in the presence or absence of immune stimulation one day after treatment. IGF-I, IGF-II and IL-2 were also differentially expressed (Sharova et al. 2002).

In vitro data with cells or cell lines

Rat and mouse spleen cells were treated with urethane for 24 hours and then LPS or PHA for 48 hours. Then, mitogen stimulation was assessed by 5-bromo-2'-deoxyuridine incorporation.

Urethane did not inhibit cellular proliferation in either species (data not provided). Urethane also did not modulate rat spleen proliferation that was stimulated by ConA or inhibit anti-CD3 antibody stimulation of mouse lymphocyte proliferation (data not provided) (Carfi et al. 2007).

Mode of action information

In vitro and *in vivo* studies suggest that urethane metabolism by cytochrome P450 is needed to produce the observed immunomodulatory effects (Cha et al. 2000). Macrophage effects are based on urethane effects on the inductive phase of immune responses (Foris et al. 1983).

References

Bette M, Schlimme S, Mutters R, Menendez S, Hoffmann S, Schulz S. 2004. Influence of different anaesthetics on pro-inflammatory cytokine expression in rat spleen. Laboratory animals 38:272–9; doi:10.1258/002367704323133655.

Carfi M, Gennari A, Malerba I, Corsini E, Pallardy M, Pieters R, et al. 2007. In vitro tests to evaluate immunotoxicity: a preliminary study. Toxicology 229:11–22; doi:10.1016/j.tox.2006.09.003.

Cha SW, Gu HK, Lee KP, Lee MH, Han SS, Jeong TC. 2000. Immunotoxicity of ethyl carbamate in female BALB/c mice: role of esterase and cytochrome P450. Toxicology letters 115: 173–81.

Foris G, Bojan F, Medgyesi GA, Szilagyi T. 1983. Effects of urethan on lymphokine-producing activity of lymphocytes and on some functions of peritoneal macrophages in rats. Agents and actions 13: 63–8.

Gorelik E, Herberman RB. 1981a. Inhibition of the activity of mouse natural killer cells by urethan. Journal of the National Cancer Institute 66: 543–8.

Gorelik E, Herberman RB. 1981b. Susceptibility of various strains of mice to urethan-induced lung tumors and depressed natural killer cell activity. Journal of the National Cancer Institute 67: 1317–22.

Halliday GM, Odling KA, Ruby JC, Muller HK. 1988. Suppressor cell activation and enhanced skin allograft survival after tumor promotor but not initiator induced depletion of cutaneous Langerhans cells. The Journal of investigative dermatology 90: 293–7.

Luebke RW, Riddle MM, Rogers RR, Rowe DG, Garner RJ, Smialowicz RJ. 1986. Immune function in adult C57BL/6J mice following exposure to urethan pre- or postnatally. Journal of immunopharmacology 8: 243–57.

Luebke RW, Rogers RR, Riddle MM, Rowe DG, Smialowicz RJ. 1987. Alteration of immune function in mice following carcinogen exposure. Immunopharmacology 13: 1–9.

Luster MI, Dean JH, Boorman GA, Dieter MP, Hayes HT. 1982. Immune functions in methyl and ethyl carbamate treated mice. Clinical and experimental immunology 50: 223–30.

Markovic T, Gobec M, Gurwitz D, Mlinaric-Rascan I. 2015. Characterization of human lymphoblastoid cell lines as a novel in vitro test system to predict the immunotoxicity of xenobiotics. Toxicology letters 233:8–15; doi:10.1016/j.toxlet.2014.12.013.

Sharova LV, Gogal RM Jr, Sharov AA, Chrisman MV, Holladay SD. 2002. Immune stimulation in urethane-exposed pregnant mice increases expression level of spleen leukocyte genes for TGFbeta3 GM-CSF and other cytokines that may play a role in reduced chemical-induced birth defects. International immunopharmacology 2: 1477–89.

Human Data

Data from epidemiology studies

No data were located.

In vitro data with cells or cell lines

Vanadium pentoxide (25 – 400 μM) inhibited cell proliferation and induced cell apoptosis in a dose and time-related manner in the IL-2-independent human NK cell line, NK-92MI. Cell proliferation was maximally inhibited (78%) at 400 μM vanadium pentoxide, and the percentage of cells undergoing apoptosis increased at 12 and 24 hours of exposure (51.2 and 64.7%, respectively) as the concentration of vanadium pentoxide increased. IL-2, IL-10 and IFNγ secretion were all inhibited by vanadium pentoxide after 24 hours at the highest concentration tested. IL-2 secretion also was inhibited after 12 hours. Expression of CD25 significantly increased above background starting at 50 μM, reaching a maximal migration inhibitory factor (MIF) of 47.4% at 400 μM. A similar pattern was observed for IL-15Rα, with a maximal MIF of 55.2% at 400 μM. Fas expression began to increase at 100 μM and reached a maximal MIF of 48.9% at 400 μM, while FasL peaked at 200 μM (62.1%). Jak3 phosphorylation was increased at 12 and 24 hours after treatment with 200 and 400 μM vanadium pentoxide (data in figure), and intracellular staining showed a strong presence of pJak3 in the internal cell membranes after treatment. (Gallardo-Vera et al. 2016).

Mode of action information

Vanadium in the +2, +3, and +4 (but not the +5) valence states interacted with human FMLP- activated neutrophils and statistically significantly increased the formation of hydroxyl radicals, with additional augmentation observed in the presence of sodium azide (values not provided) (Fickl et al. 2006).

Vanadium pentoxide induced toxic effects on the IL-2-independent human NK cell line, NK- 92MI, through dysregulation of signaling pathways mediated by IL-2 via increased

PTEN and decreased SHP1 expression (Gallardo-Vera et al. 2018).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

Male F344 rats were exposed to vanadium pentoxide (100 μg V/m^3) via inhalation, 5 hours per day for 5 days. The animals were infected with Listeria following the 5-day exposure and the bacterial burden assessed at 24, 48 and 72 hours, post-infection. Vanadium pentoxide did not have any significant effect on Listeria burdens at any of the timepoints observed (Cohen et al. 2007).

Male CD-1 mice were exposed to vanadium pentoxide (0 or an average of 1436 μg/m^3) via inhalation, 1 hour per day, 2 times per week over 12 weeks. An increase in the number (3.8±0.12 vs. 2.1±0.12 μm per field) and the size (36±0.52 vs. 25±0.35 μm) of megakaryocytes in the

spleen was observed in vanadium pentoxide exposed mice, as compared to controls. These same types of changes were also observed in the bone marrow (values not provided). No statistical difference was observed in spleen weight between treated and control mice (Fortoul et al. 2008). When male and female CD-1 mice were exposed to vanadium pentoxide (0 or 1.4 mg/m^3) using the same protocol as in Fortoul et al., 2008, a sex difference was observed in the expression of Ki-67, a specific proliferation marker for lymphocytes. The percentage of Ki-67 immunopositive lymphocytes increased in male mice (38.86, 41.75 and 41.91%) after 4, 8 and 12 weeks of exposure, respectively, with both cytoplasmic and nuclear expression of Ki-67 observed. In female mice, the percentage of proliferating lymphocytes increased only after the first week of exposure (34.87%) and the signal was observed only in the nucleus. Subsequent exposures did not produce significant changes in the percentage of proliferating cells in females. The authors concluded there is a role for sex hormones in potential protection against vanadium immunoxicity (Rodriguez-Lara et al. 2016).

Male CD-1 mice were exposed to vanadium pentoxide (0 or an average of 1.4 mg/m^3) via inhalation, 1 hour per day, 2 times per week over 12 weeks. Spleen weight of vanadium exposed animals peaked at 9 weeks (546 ± 45 vs. 274 ± 27 mg in controls) and progressively decreased afterwards (321 ± 39 mg at 12 weeks vs. 298 ± 35 mg in controls). The spleens of vanadium exposed animals had histological changes that included increased numbers of lymphocytes and megakaryocytes as compared to controls. The number of $CD19^+$ cells was also increased within the hyperplastic germinal node (values not provided) and the mean hepatitis B surface antigen levels in immunized control mice was greater than in the exposed hosts (OD=0.39 ± 0.03 vs. 0.11 ± 0.05). The authors concluded that vanadium pentoxide induces functional changes in the spleen which appear to result in effects on the humoral immune response (Pinon-Zarate et al. 2008).

Male CD-1 mice were exposed to vanadium pentoxide (0 or an average of 1.4 mg/m^3) via inhalation, 1 hour per day, 2 times per week over 4 weeks. The expression of CD11c in the thymic medulla was decreased in vanadium pentoxide exposed mice, as compared to controls (values not provided), based on immunohistochemistry. Flow cytometry also

demonstrated a decrease in $CD11c^+$ and $MHC\text{-}II^+$ cells in vanadium pentoxide exposed mice, as compared to controls (values not provided). The decrease was both, in terms of number and in mean fluorescence intensity values (Ustarroz-Cano et al. 2012).

Male F344/N rats and female B6C3F1 mice were exposed to 0, 4,8, or 16 mg/m^3 vanadium pentoxide, via inhalation, 6 hours per day, 5 days per week for 16 days. Pulmonary inflammation was assessed via analysis of BAL fluid. Significant alterations in the percentage of recoverable macrophages and neutrophils (NOAEL 4 mg/m^3), and increased lung protein and lysozyme in male rats (LOAEL 4 mg/m^3) were observed. In female mice, an increase in lymphocytes, protein and lysozymes was observed (LOAEL 4 mg/m^3). No effects were observed on systemic immunity as evidenced by a normal response to *Klebsiella pneumoniae* (National Toxicology Program 2002).

The induction of pulmonary inflammation was examined in three different strains of mice [A/J (sensitive strain for pulmonary inflammation and carcinogenesis), BALB/c (intermediate

sensitivity), and C57Bl/6J (resistant)]. Mice were aspirated with vanadium pentoxide (4 mg/kg) or phosphate-buffered saline, four times per week, with BALF collected at 6 hours, and 1, 3, 6 and 21 days. In A/J mice, vanadium pentoxide increased BALF levels of total cells (95.7%) inflammatory markers (PMNs, macrophages and lymphocytes, 74.6, 99.5, and 623.8%, respectively). Levels of inflammatory chemokines (keratinocyte-derived chemokine, macrophage inflammatory protein-2 and monocyte chemoattractant protein 1), transcription factor activity (NFκB and c-Fos) and signaling pathway activation (MAPK) were increased with highest levels observed in A/J mice followed by BALB/c and then C57BL/6J mice (data in graphs). All results returned to baseline 21 days post exposure (Rondini et al. 2010).

In vitro data with cells or cell lines

No data were located.

Mode of action information

Rondini and colleagues (2010) reported that vanadium pentoxide impacts pulmonary levels of inflammatory markers, induction of chemokines, and modulation of transcription factors Alterations in macrophage mediated functions have been associated with vanadium exposure (Cohen et al. 1996).

The ability of several vanadium compounds to increase mRNA levels of cytokines in BALF was investigated in female CD rats. Rats received 42 or 420 μg of vanadium pentoxide or phosphate- buffered saline by intratracheal instillation. BALF was collected at times ranging from 1 hour to 10 days. Influx of neutrophils was significantly increased 24 hours after exposure to vanadium pentoxide and peaked 24-48 hours post exposure (data in graph). Macrophage inflammatory protein-2 mRNA expression levels were significantly elevated in vanadium pentoxide treated rats at 1 to 48-hour timepoints, as compared to controls (Pierce et al. 1996).

References

Cohen MD, McManus TP, Yang Z, Qu Q, Schlesinger RB, Zelikoff JT. 1996. Vanadium affects macrophage interferon-gamma-binding and -inducible responses. Toxicology and applied pharmacology 138:110–20; doi:10.1006/taap.1996.0104.

Cohen MD, Sisco M, Prophete C, Chen LC, Zelikoff JT, Ghio AJ, et al. 2007. Pulmonary immunotoxic potentials of metals are governed by select physicochemical properties: vanadium agents. Journal of immunotoxicology 4:49–60; doi:10.1080/15476910601119350.

Fickl H, Theron AJ, Grimmer H, Oommen J, Ramafi GJ, Steel HC, et al. 2006. Vanadium promotes hydroxyl radical formation by activated human neutrophils. Free radical biology & medicine 40:146–55; doi:10.1016/j.freeradbiomed.2005.09.019.

Fortoul TI, Pinon-Zarate G, Diaz-Bech ME, Gonzalez-Villalva A, Mussali-Galante P, Rodriguez-Lara V, et al. 2008. Spleen and bone marrow megakaryocytes as targets for inhaled vanadium. Histology and histopathology 23:1321–6; doi:10.14670/hh-23.1321.

Gallardo-Vera F, Diaz D, Tapia-Rodriguez M, Fortoul van der Goes T, Masso F, Rendon-Huerta E, et al. 2016. Vanadium pentoxide prevents NK-92MI cell proliferation and IFNgamma

secretion through sustained JAK3 phosphorylation. Journal of immunotoxicology 13:27–37; doi:10.3109/1547691x.2014.996681.

Gallardo-Vera F, Tapia-Rodriguez M, Diaz D, Fortoul van der Goes T, Montano LF, Rendon- Huerta EP. 2018. Vanadium pentoxide increased PTEN and decreased SHP1 expression in NK- 92MI cells, affecting PI3K-AKT-mTOR and Ras-MAPK pathways. Journal of immunotoxicology 15:1–11; doi:10.1080/1547691x.2017.1404662.

National Toxicology Program. 2002. NTP toxicology and carcinogensis studies of vanadium pentoxide (CAS No. 1314-62-1) in F344/N rats and B6C3F1 mice (inhalation). National Toxicology Program technical report series 1–343.

Pierce LM, Alessandrini F, Godleski JJ, Paulauskis JD. 1996. Vanadium-induced chemokine mRNA expression and pulmonary inflammation. Toxicology and applied pharmacology 138:1– 11; doi:10.1006/taap.1996.9999.

Pinon-Zarate G, Rodriguez-Lara V, Rojas-Lemus M, Martinez-Pedraza M, Gonzalez-Villalva A, Mussali-Galante P, et al. 2008. Vanadium pentoxide inhalation provokes germinal center hyperplasia and suppressed humoral immune responses. Journal of immunotoxicology 5:115–22; doi:10.1080/15476910802085749.

Rodriguez-Lara V, Muniz-Rivera Cambas A, Gonzalez Villalva A, Fortoul TI. 2016. Sex-based differences in lymphocyte proliferation in the spleen after vanadium inhalation. Journal of immunotoxicology 13:498–508; doi:10.3109/1547691x.2015.1134731.

Rondini EA, Walters DM, Bauer AK. 2010. Vanadium pentoxide induces pulmonary inflammation and tumor promotion in a strain-dependent manner. Particle and fibre toxicology 7:9; doi:10.1186/1743-8977-7-9.

Ustarroz-Cano M, Garcia-Pelaez I, Pinon-Zarate G, Herrera-Enriquez M, Soldevila G, Fortoul TI. 2012. CD11c decrease in mouse thymic dendritic cells after vanadium inhalation. Journal of immunotoxicology 9:374–80; doi:10.3109/1547691x.2012.673181.

Zinc Dimethyldithiocarbamate (ZDMDC) [CASRN 137-30-4]

Human Data

Data from epidemiology studies

No data were located.

In vitro data with cells or cell lines

ZDMDC induced cytotoxicity in purified NK cells from healthy donors. Exposure to 2.5 μM ZDMDC for 24 hours produced a 99% decrease in lytic function (against K562 target cells) and at 1 μM for 6 days produced a 96% decrease. When a preparation containing T- and NK-cells were exposed to 2.5 μM for 24 hours a 41% decrease in function was observed. Comparatively, a 6-day exposure to 1 μM ziram did not inhibit lytic function. (Whalen et al. 2003). Wilson and colleagues showed that concentrations as low as 125 nM decreased cytotoxic function of purified NK cells (Wilson et al. 2004).

ZDMDC significantly inhibited NK-92MI activity (against K562 target cells) in a dose- and concentration-dependent manner (LOAEL = 0.125 μM at 2 hours incubation). A similar dose- and concentration-dependent inhibition of NK activity was observed with human lymphokine activated killer cells (LOAEL = 0.125 μM at 2 hours incubation) (Li et al. 2012a).

Purified, human NK cells were exposed to ZDMDC (0.5-5 μM) for 1 hour. Then the cells were incubated for 24 or 48 hours, or 6 days in ZDMDC-free media. A decrease in NK activity was observed at 2.5 and 5 μM. The loss of activity lasted up to 6 days after exposure (Taylor et al. 2005).

ZDMDC (5 μg/mL) decreased LPS-induced TNF-α production in THP-1 cells (data in graph). ZDMDC (5 μg/mL) also blocked LPS-induced degradation of I□B (data in Western blot) (Corsini et al. 2006).

ZDMDC induced apoptosis and necrosis in U937, NK-92MI, NK-92CI, Jurkat, and human T cells. Of U937 cells treated with 2 μM ZDMDC, 49.3% were apoptotic and 18.5% were necrotic (Li et al. 2011). In Jurkat cells treated with 0.5 μM ZDMDC, 52.5% were apoptotic and 7.9% were late apoptotic/necrotic (Li et al. 2012c). In NK-92MI cells treated with 0.5 μM ZDMDC, 47.4% were apoptotic and 12.2% were late

apoptotic/necrotic (Li et al. 2012b). In NK-92CI cells treated with 0.5 μM ZDMDC, 28.7% were apoptotic and 38.5% were necrotic (Li et al. 2014).

Increased apoptosis and late apoptosis/necrosis also was observed in a time- and dose-dependent manner in isolated primary T-cells (data in graph) (Li et al. 2012c).

At concentrations ranging from 0.1 to 10 μg/mL, ZDMDC was not cytotoxic to lymphocyte cultures obtained from peripheral blood from healthy volunteers (Zenzen et al. 2001).

Mode of action information

Effects in U937, NK-92MI, and Jurkat cells were dose- and time-dependent. Increased DNA fragmentation, level of active caspase-3, and level of cytochrome c release from U937 and Jurkat

cells also were noted after ZDMDC exposure (Li et al. 2011, 2012c, 2012b, 2015). Increased levels of caspase-7, -8, and -9 also were detected in NK-92MI and Jurkat cells (Li et al. 2012c, 2012b).

ZDMDC-induced inhibition of NK and LAK activity was mediated, in part, by decreases in intracellular levels of Gr3/K, granulysin, perforin, granzyme (Gr) A, and GrB (Li et al. 2012a). Decreased levels of GrB was associated with activation of p38 while activation of p44/42 was associated with decreased levels of perforin (Taylor and Whalen 2011).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

The EC3 in the local lymph node assay was between 1.0% and 5.0% in female BALB/c mice (De Jong et al. 2002). ZDMDC also was identified as a skin sensitizer in the guinea pig maximization test (TS5 = 0.01%) (van Och et al. 2001).

In vitro data with cells or cell lines

ZDMDC inhibited murine (C57BL/6J) cytotoxic T lymphocyte activity in a dose- and concentration-dependent manner (LOAEL = 0.125 μM) (Li et al. 2012a).

ZDMDC (10 μM) decreased expression of pro-caspase-1 and NLRP3 in J774A.1 cells. Studies in RAW264.7 cells showed that 10 μM ZDMDC increased pro-caspase-1 degradation and not protein cleavage. ZDMDC also decreased LPS-induced production of IL-18 and IL-1ß in bone marrow macrophages. Inhibition of LPS-induced IL-1ß production occurred in a dose-dependent manner in J774A.1 (LOAEL = 5 μM) (Muroi and Tanamoto 2015).

J774A.1 cells were infected with *S. typhimurium* TA98 and then treated with ZDMDC. ZDMDC (1-10 μM) increased the number of infected bacteria in a concentration-dependent manner (LOAEL = 5 μM) (Muroi and Tanamoto 2015).

Mode of action information

ZDMDC increased intracellular level of zinc in rat thymic lymphocytes, which may be associated with induction of apoptosis (Kanemoto-Kataoka et al. 2015).

References

Corsini E, Viviani B, Birindelli S, Gilardi F, Torri A, Codeca I, et al. 2006. Molecular mechanisms underlying mancozeb-induced inhibition of TNF-alpha production. Toxicology and applied pharmacology 212:89–98; doi:10.1016/j.taap.2005.07.002.

De Jong WH, Tentij M, Spiekstra SW, Vandebriel RJ, Van Loveren H. 2002. Determination of the sensitising activity of the rubber contact sensitisers TMTD, ZDMC, MBT and DEA in a modified local lymph node assay and the effect of sodium dodecyl sulfate pretreatment on local lymph node responses. Toxicology 176: 123–34.

Kanemoto-Kataoka Y, Oyama TM, Ishibashi H, Oyama Y. 2015. Dithiocarbamate fungicides increase intracellular Zn(2+) levels by increasing influx of Zn(2+) in rat thymic lymphocytes. Chemico-biological interactions 237:80–6; doi:10.1016/j.cbi.2015.05.014.

Li Q, Kobayashi M, Kawada T. 2014. Carbamate pesticide-induced apoptosis and necrosis in human natural killer cells. Journal of biological regulators and homeostatic agents 28: 23–32.

Li Q, Kobayashi M, Kawada T. 2015. Carbamate pesticide-induced apoptosis in human T lymphocytes. International journal of environmental research and public health 12:3633–45; doi:10.3390/ijerph120403633.

Li Q, Kobayashi M, Kawada T. 2012a. Effect of ziram on natural killer, lymphokine-activated killer, and cytotoxic T lymphocyte activity. Archives of toxicology 86:475–81; doi:10.1007/s00204-011-0771-5.

Li Q, Kobayashi M, Kawada T. 2012b. Mechanism of ziram-induced apoptosis in human natural killer cells. International journal of immunopathology and pharmacology 25:883–91; doi:10.1177/039463201202500406.

Li Q, Kobayashi M, Kawada T. 2012c. Mechanism of ziram-induced apoptosis in human T lymphocytes. Archives of toxicology 86:615–23; doi:10.1007/s00204-011-0791-1.

Li Q, Kobayashi M, Kawada T. 2011. Ziram induces apoptosis and necrosis in human immune cells. Archives of toxicology 85:355–61; doi:10.1007/s00204-010-0586-9.

Muroi M, Tanamoto K. 2015. Zinc- and oxidative property-dependent degradation of pro- caspase-1 and NLRP3 by ziram in mouse macrophages. Toxicology letters 235:199–205; doi:10.1016/j.toxlet.2015.04.012.

Taylor TR, Tucker T, Whalen MM. 2005. Persistent inhibition of human natural killer cell function by ziram and pentachlorophenol. Environmental toxicology 20:418–24; doi:10.1002/tox.20127.

Taylor TR, Whalen MM. 2011. Ziram activates mitogen-activated protein kinases and decreases cytolytic protein levels in human natural killer cells. Toxicology mechanisms and methods 21:577–84; doi:10.3109/15376516.2011.578170.

van Och FM, Vandebriel RJ, Prinsen MK, De Jong WH, Slob W, van Loveren H. 2001. Comparison of dose-responses of contact allergens using the guinea

pig maximization test and the local lymph node assay. Toxicology 167: 207–15.

Whalen MM, Loganathan BG, Yamashita N, Saito T. 2003. Immunomodulation of human natural killer cell cytotoxic function by triazine and carbamate pesticides. Chemico-biological interactions 145: 311–9.

Wilson S, Dzon L, Reed A, Pruitt M, Whalen MM. 2004. Effects of in vitro exposure to low levels of organotin and carbamate pesticides on human natural killer cell cytotoxic function. Environmental toxicology 19:554–63; doi:10.1002/tox.20061.

Zenzen V, Fauth E, Zankl H, Janzowski C, Eisenbrand G. 2001. Mutagenic and cytotoxic effectiveness of zinc dimethyl and zinc diisononyldithiocarbamate in human lymphocyte cultures. Mutation research 497: 89–99.

Appendix 8. The summary of immunotoxicological data of 25 chemicals.

The table is attached as an independent Excel file (Appendix Table 1).

References of immunotoxicological information of the chemicals used in Phase I and II studies.

Chen, S., Golemboski, K., Piepenbrink, M., et al., 2004. Developmental immunotoxicity of lead in the rat: influence of maternal diet. J Toxicol Environ Health A 67, 495-511.

Chikanza, L.C., Panayi, G.S., 1993. The effects of hydrocortisone on in vitro lymphocyte proliferation and interleukin-2 and -4 production in corticosteroid sensitive and resistant subjects. Eur J Clin Invest 23, 845-850.

Demenesku, J., Mirkov, I., Ninkov, M., et al., 2014. Acute cadmium administration to rats exerts both immunosuppressive and proinflammatory effects in spleen. Toxicology 326, 96-108.

Fernandez-Cabezudo, M.J., Ali, S.A., Ullah, A., et al., 2007. Pronounced susceptibility to infection by Salmonella enterica serovar Typhimurium in mice chronically exposed to lead correlates with a shift to Th2-type immune responses. Toxicol Appl Pharmacol 218, 215-226.

Goodwin, J.S., Atluru, D., Sierakowski, S., et al., 1986. Mechanism of action of glucocorticosteroids. Inhibition of T cell proliferation and interleukin 2 production by hydrocortisone is reversed by leukotriene B4. J Clin Invest 77, 1244-1250.

Goutet, M., Ban, M., Binet, S., 2000. Effects of nickel sulfate on pulmonary natural immunity in Wistar rats. Toxicology 145, 15-26.

Hansen, J.F., Nielsen, C.H., Brorson, M.M., et al., 2015. Influence of phthalates on in vitro innate and adaptive immune responses. PLoS One 10, e0131168.

Hemdan, N.Y., Emmrich, F., Adham, K., et al., 2005. Dose-dependent modulation of the in vitro cytokine production of human immune competent cells by lead salts. Toxicol Sci 86, 75-83.

Iavicoli, I., Marinaccio, A., Castellino, N., et al., 2004. Altered cytokine

production in mice exposed to lead acetate. Int J Immunopathol Pharmacol 17, 97-102.

Kim, J.Y., Huh, K., Lee, K.Y., et al., 2009. Nickel induces secretion of IFN-gamma by splenic natural killer cells. Exp Mol Med 41, 288-295.

Kooijman, R., Devos, S., Hooghe-Peters, E., 2010. Inhibition of in vitro cytokine production by human peripheral blood mononuclear cells treated with xenobiotics: implications for the prediction of general toxicity and immunotoxicity. Toxicol In Vitro 24, 1782-1789.

Metushi, I.G., Uetrecht, J., 2014. Isoniazid-induced liver injury and immune response in mice. J Immunotoxicol 11, 383-392.

Pathak, N., Khandelwal, S., 2008. Comparative efficacy of piperine, curcumin and picroliv against Cd immunotoxicity in mice. Biometals 21, 649-661.

Ringerike, T., Ulleras, E., Volker, R., et al., 2005. Detection of immunotoxicity using T-cell based cytokine reporter cell lines ("Cell Chip"). Toxicology 206, 257-272.

Thomas, P., Barnstorf, S., Summer, B., et al., 2003. Immuno-allergological properties of aluminium oxide (Al2O3) ceramics and nickel sulfate in humans. Biomaterials 24, 959-966.

Tsuboi, I., Tanaka, H., Nakao, M., et al., 1995. Nonsteroidal anti-inflammatory drugs differentially regulate cytokine production in human lymphocytes: up-regulation of TNF, IFN-gamma and IL-2, in contrast to down-regulation of IL-6 production. Cytokine 7, 372-379.

Wagner, W., Walczak-Drzewiecka, A., Slusarczyk, A., et al., 2006. Fluorescent Cell Chip a new in vitro approach for immunotoxicity screening. Toxicol Lett 162, 55-70.

Wang, P., Wang, J., Sun, Y.J., et al., 2017. Cadmium and chlorpyrifos inhibit cellular immune response in spleen of rats. Environ Toxicol 32, 1927-1936.

Appendix 9. The summary of immunotoxicological data of 60 chemicals.

The table is attached as an independent Excel file (Appendix 9).

References for toxicological information of 60 chemicals

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1993a. NTP Toxicology and Carcinogenesis Studies of Acetaminophen (CAS No. 103-90-2) in F344 Rats and B6C3F1 Mice (Feed Studies). Natl Toxicol Program Tech Rep Ser 394, 1-274.

1993b. NTP Toxicology and Carcinogenesis Studies of p-Nitroaniline (CAS No. 100-01-6) in B6C3F1 Mice (Gavage Studies). Natl Toxicol Program Tech Rep Ser 418, 1-203.

1995. NTP Toxicology and Carcinogenesis Studies of Benzethonium Chloride (CAS No. 121-54-0) in F344/N Rats and B6C3F1 Mice (Dermal Studies). Natl Toxicol Program Tech Rep Ser 438, 1-220.

1996. NTP Toxicology and Carcinogenesis Studies of Nickel Sulfate Hexahydrate (CAS No. 10101-97-0) in F344 Rats and B6C3F1 Mice (Inhalation Studies). Natl Toxicol Program Tech Rep Ser 454, 1-380.

1997. NTP Toxicology and Carcinogenesis Studies of Salicylazosulfapyridine (CAS No. 599-79-1) in F344/N Rats and B6C3F1 Mice (Gavage Studies). Natl Toxicol Program Tech Rep Ser 457, 1-327.

2003. NTP toxicology and carcinogenesiss studies of citral (microencapsulated) (CAS No. 5392-40-5) in F344/N rats and B6C3F1 mice (feed studies). Natl Toxicol Program Tech Rep Ser, 1-268.

Almousa, L.A., Salter, A.M., Langley-Evans, S.C., 2018. Magnesium deficiency heightens lipopolysaccharide-induced inflammation and enhances monocyte adhesion in human umbilical vein endothelial cells. Magnes Res 31, 39-48.

Auli, M., Domenech, A., Andres, A., et al., 2012. Multiparametric immunotoxicity screening in mice during early drug development. Toxicol Lett 214, 200-208.

Beschorner, W.E., Namnoum, J.D., Hess, A.D., et al., 1987. Cyclosporin A and the thymus. Immunopathology. Am J Pathol 126, 487-496.

Bessler, H., Straussberg, R., Gurary, N., et al., 1996. Effect of dexamethasone on IL-2 and IL-3 production by mononuclear cells in neonates and adults. Arch Dis Child Fetal Neonatal Ed 75, F197-201.

Blanke, T.J., Little, J.R., Shirley, S.F., et al., 1977. Augmentation of murine immune responses by amphotericin B. Cell Immunol 33, 180-190.

Bruserud, O., Lundin, K., 1987. The effect of drugs used in anticoagulation therapy on T lymphocyte activation in vitro. II. Warfarin inhibits T lymphocyte activation. J Clin Lab Immunol 23, 169-173.

Bunn, T.L., Parsons, P.J., Kao, E., et al., 2001. Exposure to lead during critical windows of embryonic development: differential immunotoxic outcome based on stage of exposure and gender. Toxicol Sci 64, 57-66.

Bygbjerg, I.C., Svenson, M., Theander, T.G., et al., 1987. Effect of antimalarial drugs on stimulation and interleukin 2 production of human lymphocytes. Int J Immunopharmacol 9, 513-519.

Caren, L.D., Oven, H.M., Mandel, A.D., 1985. Dimethyl sulfoxide: lack of suppression of the humoral immune response in mice. Toxicol Lett 26, 193-197.

Cesario, T.C., Slater, L.M., Kaplan, H.S., et al., 1984. Effect of antineoplastic agents on gamma-interferon production in human peripheral blood mononuclear cells. Cancer Res 44, 4962-4966.

Chetty, K.N., Subba Rao, D.S., Drummond, L., et al., 1979. Cobalt induced changes in immune response and adenosine triphosphatase activities in rats. J Environ Sci Health B 14, 525-544.

Chikanza, L.C., Panayi, G.S., 1993. The effects of hydrocortisone on in vitro lymphocyte proliferation and interleukin-2 and -4 production in corticosteroid sensitive and resistant subjects. Eur J Clin Invest 23, 845-850.

de Abreu Costa, L., Henrique Fernandes Ottoni, M., Dos Santos, M.G., et al., 2017. Dimethyl Sulfoxide (DMSO) Decreases Cell Proliferation and TNF-alpha, IFN-gamma, and IL-2 Cytokines Production in Cultures of Peripheral Blood Lymphocytes. Molecules 22.

De Waal, E.J., Timmerman, H.H., Dortant, P.M., et al., 1995. Investigation of a screening battery for immunotoxicity of pharmaceuticals within a 28-day oral toxicity study using azathioprine and cyclosporin A as model compounds. Regul

Toxicol Pharmacol 21, 327-338.

Dieter, M.P., Luster, M.I., Boorman, G.A., et al., 1983. Immunological and biochemical responses in mice treated with mercuric chloride. Toxicol Appl Pharmacol 68, 218-228.

Dupont, E., Huygen, K., Schandene, L., et al., 1985. Influence of in vivo immunosuppressive drugs on production of lymphokines. Transplantation 39, 143-147.

Dupuis, G., Martel, J., Bastin, B., et al., 1993. Microtubules are not an essential component of phytohemagglutinin-dependent signal transduction in Jurkat T lymphocytes. Cell Immunol 146, 38-51.

el Fouhil, A.F., Iskander, F.A., Turkall, R.M., 1993a. Effect of alternate-day hydrocortisone therapy on the immunologically immature rat. II: Changes in T- and B-cell areas in spleen. Toxicol Pathol 21, 383-390.

el Fouhil, A.F., Iskander, F.A., Turkall, R.M., 1993b. Effect of alternate-day hydrocortisone therapy on the immunologically immature rat. III: Changes in T- and B-cell areas in lymph nodes. Toxicol Pathol 21, 391-396.

el Fouhil, A.F., Turkall, R.M., 1993. Effect of alternate-day hydrocortisone therapy on the immunologically immature rat. I: Effect on blood cell count, immunoglobulin concentrations, and body and organ weights. Toxicol Pathol 21, 377-382.

Exon, J.H., Koller, L.D., Talcott, P.A., et al., 1986. Immunotoxicity testing: an economical multiple-assay approach. Fundam Appl Toxicol 7, 387-397.

Ezendam, J., Hassing, I., Bleumink, R., et al., 2004. Hexachlorobenzene-induced Immunopathology in Brown Norway rats is partly mediated by T cells. Toxicol Sci 78, 88-95.

Freed, B.M., Lempert, N., Lawrence, D.A., 1989. The inhibitory effects of N-ethylmaleimide, colchicine and cytochalasins on human T-cell functions. Int J Immunopharmacol 11, 459-465.

Freed, B.M., Rapoport, R., Lempert, N., 1987. Inhibition of early events in the human T-lymphocyte response to mitogens and alloantigens by hydrogen peroxide. Arch Surg 122, 99-104.

Fujiwara, M., Mitsui, K., Yamamoto, I., 1990. Inhibition of proliferative responses

and interleukin 2 productions by salazosulfapyridine and its metabolites. Jpn J Pharmacol 54, 121-131.

Gabryel, B., Labuzek, K., Malecki, A., et al., 2004. Immunophilin ligands decrease release of pro-inflammatory cytokines (IL-1beta, TNF-alpha and IL-2 in rat astrocyte cultures exposed to simulated ischemia in vitro. Pol J Pharmacol 56, 129-136.

Garly, M.L., Trautner, S.L., Marx, C., et al., 2008. Thymus size at 6 months of age and subsequent child mortality. J Pediatr 153, 683-688, 688.e681-683.

Gentile, D.A., Henry, J., Katz, A.J., et al., 1997. Inhibition of peripheral blood mononuclear cell proliferation by cardiac glycosides. Ann Allergy Asthma Immunol 78, 466-472.

Ghare, S., Patil, M., Hote, P., et al., 2011. Ethanol inhibits lipid raft-mediated TCR signaling and IL-2 expression: potential mechanism of alcohol-induced immune suppression. Alcohol Clin Exp Res 35, 1435-1444.

Goodwin, J.S., Atluru, D., Sierakowski, S., et al., 1986. Mechanism of action of glucocorticosteroids. Inhibition of T cell proliferation and interleukin 2 production by hydrocortisone is reversed by leukotriene B4. J Clin Invest 77, 1244-1250.

Haley, P.J., Shopp, G.M., Benson, J.M., et al., 1990. The immunotoxicity of three nickel compounds following 13-week inhalation exposure in the mouse. Fundam Appl Toxicol 15, 476-487.

Hanke, J.H., Nichols, L.N., Coon, M.E., 1992. FK506 and rapamycin selectively enhance degradation of IL-2 and GM-CSF mRNA. Lymphokine Cytokine Res 11, 221-231.

Hansen, J.F., Nielsen, C.H., Brorson, M.M., et al., 2015. Influence of phthalates on in vitro innate and adaptive immune responses. PLoS One 10, e0131168.

Hattori, A., Kunz, H.W., Gill, T.J., 3rd, et al., 1987. Thymic and lymphoid changes and serum immunoglobulin abnormalities in mice receiving cyclosporine. Am J Pathol 128, 111-120.

He, Y.W., Deftos, M.L., Ojala, E.W., et al., 1998. RORgamma t, a novel isoform of an orphan receptor, negatively regulates Fas ligand expression and IL-2 production in T cells. Immunity 9, 797-806.

Henderson, D.J., Naya, I., Bundick, R.V., et al., 1991. Comparison of the effects of

FK-506, cyclosporin A and rapamycin on IL-2 production. Immunology 73, 316-321.

Himmerich, H., Schonherr, J., Fulda, S., et al., 2011. Impact of antipsychotics on cytokine production in-vitro. J Psychiatr Res 45, 1358-1365.

Hu, H., Abedi-Valugerdi, M., Moller, G., 1997. Pretreatment of lymphocytes with mercury in vitro induces a response in T cells from genetically determined low-responders and a shift of the interleukin profile. Immunology 90, 198-204.

Huchet, R., Grandjon, D., 1988. Histamine-induced regulation of IL-2 synthesis in man: characterization of two pathways of inhibition. Ann Inst Pasteur Immunol 139, 485-499.

Iatropoulos, M.J., Hobson, W., Knauf, V., et al., 1976. Morphological effects of hexachlorobenzene toxicity in female rhesus monkeys. Toxicol Appl Pharmacol 37, 433-444.

Kanariou, M., Huby, R., Ladyman, H., et al., 1989. Immunosuppression with cyclosporin A alters the thymic microenvironment. Clin Exp Immunol 78, 263-270.

Karas, K., Salkowska, A., Sobalska-Kwapis, M., et al., 2018. Digoxin, an Overlooked Agonist of RORgamma/RORgammaT. Front Pharmacol 9, 1460.

Khan, M.M., Melmon, K.L., Fathman, C.G., et al., 1985. The effects of autacoids on cloned murine lymphoid cells: modulation of IL 2 secretion and the activity of natural suppressor cells. J Immunol 134, 4100-4106.

Kim, J.H., Park, J.S., 2002. Potentiation of the immunotoxicity of ethanol by acetaminophen in mice. Int Immunopharmacol 2, 15-24.

Kim, J.Y., Huh, K., Lee, K.Y., et al., 2009. Nickel induces secretion of IFN-gamma by splenic natural killer cells. Exp Mol Med 41, 288-295.

Kim, S.K., Kwon, D.A., Lee, H.S., et al., 2019. Preventive Effect of the Herbal Preparation, HemoHIM, on Cisplatin-Induced Immune Suppression. Evid Based Complement Alternat Med 2019, 3494806.

Kloppenburg, M., Verweij, C.L., Miltenburg, A.M., et al., 1995. The influence of tetracyclines on T cell activation. Clin Exp Immunol 102, 635-641.

Knight, J.A., Plowman, M.R., Hopfer, S.M., et al., 1991. Pathological reactions in lung, liver, thymus, and spleen of rats after subacute parenteral administration of

nickel sulfate. Ann Clin Lab Sci 21, 275-283.
Kouchi, Y., Maeda, Y., Ohuchida, A., et al., 1996. Immunotoxic effect of low dose cisplatin in mice. J Toxicol Sci 21, 227-233.
Kucharz, E.J., Sierakowski, S.J., 1990. Studies on immunomodulatory properties of isoniazid. II. Effect of isoniazid on interleukin 2 production and interleukin 2-receptor expression. J Hyg Epidemiol Microbiol Immunol 34, 207-211.
Labuzek, K., Kowalski, J., Gabryel, B., et al., 2005. Chlorpromazine and loxapine reduce interleukin-1beta and interleukin-2 release by rat mixed glial and microglial cell cultures. Eur Neuropsychopharmacol 15, 23-30.
Landewe, R.B., Miltenburg, A.M., Verdonk, M.J., et al., 1995. Chloroquine inhibits T cell proliferation by interfering with IL-2 production and responsiveness. Clin Exp Immunol 102, 144-151.
Lee, J., Lim, K.T., 2012. SJSZ glycoprotein (38 kDa) modulates expression of IL-2, IL-12, and IFN-gamma in cyclophosphamide-induced Balb/c. Inflamm Res 61, 1319-1328.
Lehmann, D.M., Williams, W.C., 2018. Development and utilization of a unique in vitro antigen presentation co-culture model for detection of immunomodulating substances. Toxicol In Vitro 53, 20-28.
Lemster, B., Woo, J., Strednak, J., et al., 1992. Cytokine gene expression in murine lymphocytes activated in the presence of FK 506, bredinin, mycophenolic acid, or brequinar sodium. Transplant Proc 24, 2845-2846.
Loose, L.D., Silkworth, J.B., Pittman, K.A., et al., 1978. Impaired host resistance to endotoxin and malaria in polychlorinated biphenyl- and hexachlorobenzene-treated mice. Infect Immun 20, 30-35.
Lu, Z., Liu, F., Chen, L., et al., 2015. Effect of Chronic Administration of Low Dose Rapamycin on Development and Immunity in Young Rats. PLoS One 10, e0135256.
Maeda, M., Ishii, H., Tanaka, S., et al., 2010. Suppressive efficacies of antimicrobial agents against human peripheral-blood mononuclear cells stimulated with T cell mitogen and bacterial superantigen. Arzneimittelforschung 60, 760-768.
Meredith, C., Scott, M.P., 1994. Altered gene expression in immunotoxicology screening in vitro: Comparison with ex vivo analysis. Toxicol In Vitro 8, 751-753.

Miller, L.C., Kaplan, M.M., 1992. Serum interleukin-2 and tumor necrosis factor-alpha in primary biliary cirrhosis: decrease by colchicine and relationship to HLA-DR4. Am J Gastroenterol 87, 465-470.

Miller, T.E., Golemboski, K.A., Ha, R.S., et al., 1998. Developmental exposure to lead causes persistent immunotoxicity in Fischer 344 rats. Toxicol Sci 42, 129-135.

Munson, A.E., Sanders, V.M., Douglas, K.A., et al., 1982. In vivo assessment of immunotoxicity. Environ Health Perspect 43, 41-52.

Nalesnik, M.A., Todo, S., Murase, N., et al., 1987. Toxicology of FK-506 in the Lewis rat. Transplant Proc 19, 89-92.

Northoff, H., Carter, C., Oppenheim, J.J., 1980. Inhibition of concanavalin A-induced human lymphocyte mitogenic factor (Interleukin-2) production by suppressor T lymphocytes. J Immunol 125, 1823-1828.

Palacios, R., Sugawara, I., 1982. Hydrocortisone abrogates proliferation of T cells in autologous mixed lymphocyte reaction by rendering the interleukin-2 Producer T cells unresponsive to interleukin-1 and unable to synthesize the T-cell growth factor. Scand J Immunol 15, 25-31.

Pally, C., Tanner, M., Rizvi, H., et al., 2001. Tolerability profile of sodium mycophenolate (ERL080) and mycophenolate mofetil with and without cyclosporine (Neoral) in the rat. Toxicology 157, 207-215.

Parenti, D.M., Simon, G.L., Scheib, R.G., et al., 1988. Effect of lithium carbonate in HIV-infected patients with immune dysfunction. J Acquir Immune Defic Syndr 1, 119-124.

Parthasarathy, N.J., Kumar, R.S., Devi, R.S., 2005. Effect of methanol intoxication on rat neutrophil functions. J Immunotoxicol 2, 115-121.

Peterson, K.P., Van Hirtum, M., Peterson, C.M., 1997. Dapsone decreases the cumulative incidence of diabetes in non-obese diabetic female mice. Proc Soc Exp Biol Med 215, 264-268.

Poluektova, L.Y., Huggler, G.K., Patterson, E.B., et al., 1999. Involvement of protein kinase A in histamine-mediated inhibition of IL-2 mRNA expression in mouse splenocytes. Immunopharmacology 41, 77-87.

Quemeneur, L., Flacher, M., Gerland, L.M., et al., 2002. Mycophenolic acid inhibits

IL-2-dependent T cell proliferation, but not IL-2-dependent survival and sensitization to apoptosis. J Immunol 169, 2747-2755.

Ress, N.B., Hailey, J.R., Maronpot, R.R., et al., 2003. Toxicology and carcinogenesis studies of microencapsulated citral in rats and mice. Toxicol Sci 71, 198-206.

Riesbeck, K., 1999. Cisplatin at clinically relevant concentrations enhances interleukin-2 synthesis by human primary blood lymphocytes. Anticancer Drugs 10, 219-227.

Ringerike, T., Ulleras, E., Volker, R., et al., 2005. Detection of immunotoxicity using T-cell based cytokine reporter cell lines ("Cell Chip"). Toxicology 206, 257-272.

Roche, Y., Fay, M., Gougerot-Pocidalo, M.A., 1988. Enhancement of interleukin 2 production by quinolone-treated human mononuclear leukocytes. Int J Immunopharmacol 10, 161-167.

Saito, R., Hirakawa, S., Ohara, H., et al., 2011. Nickel differentially regulates NFAT and NF-kappaB activation in T cell signaling. Toxicol Appl Pharmacol 254, 245-255.

Salazar, V., Castillo, C., Ariznavarreta, C., et al., 2004. Effect of oral intake of dibutyl phthalate on reproductive parameters of Long Evans rats and pre-pubertal development of their offspring. Toxicology 205, 131-137.

Santarelli, L., Bracci, M., Mocchegiani, E., 2006. In vitro and in vivo effects of mercuric chloride on thymic endocrine activity, NK and NKT cell cytotoxicity, cytokine profiles (IL-2, IFN-gamma, IL-6): role of the nitric oxide-L-arginine pathway. Int Immunopharmacol 6, 376-389.

Schleuning, M.J., Duggan, A., Reem, G.H., 1989. Inhibition by chlorpromazine of lymphokine-specific mRNA expression in human thymocytes. Eur J Immunol 19, 1491-1495.

Sfikakis, P.P., Souliotis, V.L., Katsilambros, N., et al., 1996. Downregulation of interleukin-2 and apha-chain interleukin-2 receptor biosynthesis by cisplatin in human peripheral lymphocytes. Clin Immunol Immunopathol 79, 43-49.

She, Y., Wang, N., Chen, C., et al., 2012. Effects of aluminum on immune functions of cultured splenic T and B lymphocytes in rats. Biol Trace Elem Res 147, 246-250.

Sheikhi, A., Jaberi, Y., Esmaeilzadeh, A., et al., 2007. The effect of cardiovascular

drugs on pro-inflammatory cytokine secretion and natural killer activity of peripheral blood mononuclear cells of patients with chronic heart failure in vitro. Pak J Biol Sci 10, 1580-1587.

Silvestrini, B., Lisciani, R., Barcellona, P.S., 1967. Anti-granuloma and thymolytic activity of certain drugs. Eur J Pharmacol 1, 240-246.

Song, Y., Han, S., Kim, H., et al., 2006. Effects of mizoribine on MHC-restricted exogenous antigen presentation in dendritic cells. Arch Pharm Res 29, 1147-1153.

Sookoian, S., Castano, G., Flichman, D., et al., 2004. Effects of ribavirin on cytokine production of recall antigens and phytohemaglutinin-stimulated peripheral blood mononuclear cells. (Inhibitory effects of ribavirin on cytokine production). Ann Hepatol 3, 104-107.

Sugiyama, K., Ueda, H., Ichio, Y., et al., 1995. Improvement of cisplatin toxicity and lethality by juzen-taiho-to in mice. Biol Pharm Bull 18, 53-58.

Synzynys, B.I., Sharetskii, A.N., Kharlamova, O.V., 2004. [Immunotoxicity of aluminum chloride]. Gig Sanit, 70-72.

Sztein, M.B., Simon, G.L., Parenti, D.M., et al., 1987. In vitro effects of thymosin and lithium on lymphoproliferative responses of normal donors and HIV seropositive male homosexuals with AIDS-related complex. Clin Immunol Immunopathol 44, 51-62.

Takai, K., Jojima, K., Sakatoku, J., et al., 1990. Effects of FK506 on rat thymus: time-course analysis by immunoperoxidase technique and flow cytofluorometry. Clin Exp Immunol 82, 445-449.

Tam, R.C., Pai, B., Bard, J., et al., 1999. Ribavirin polarizes human T cell responses towards a Type 1 cytokine profile. J Hepatol 30, 376-382.

Tsukue, N., Toda, N., Tsubone, H., et al., 2001. Diesel exhaust (DE) affects the regulation of testicular function in male Fischer 344 rats. J Toxicol Environ Health A 63, 115-126.

Turka, L.A., Dayton, J., Sinclair, G., et al., 1991. Guanine ribonucleotide depletion inhibits T cell activation. Mechanism of action of the immunosuppressive drug mizoribine. J Clin Invest 87, 940-948.

Van Dijk, H., Bloksma, N., Rademaker, P.M., et al., 1979. Differential potencies of corticosterone and hydrocortisone in immune and immune-related processes in the

mouse. Int J Immunopharmacol 1, 285-292.

Van Wauwe, J., Aerts, F., Van Genechten, H., et al., 1996. The inhibitory effect of pentamidine on the production of chemotactic cytokines by in vitro stimulated human blood cells. Inflamm Res 45, 357-363.

Vandebriel, R.J., Meredith, C., Scott, M.P., et al., 1998. Effects of in vivo exposure to bis(tri-n-butyltin)oxide, hexachlorobenzene, and benzo(a)pyrene on cytokine (receptor) mRNA levels in cultured rat splenocytes and on IL-2 receptor protein levels. Toxicol Appl Pharmacol 148, 126-136.

Vargova, M., Wagnerova, J., Liskova, A., et al., 1993. Subacute immunotoxicity study of formaldehyde in male rats. Drug Chem Toxicol 16, 255-275.

Vos, J.G., van Logten, M.J., Kreeftenberg, J.G., et al., 1979. Hexachlorobenzene-induced stimulation of the humoral immune response in rats. Ann N Y Acad Sci 320, 535-550.

Vos, J.G., Van Loveren, H., 1994. Developments of immunotoxicology methods in the rat and applications to the study of environmental pollutants. Toxicol In Vitro 8, 951-956.

Wagner, W., Sachrajda, I., Pulaski, L., et al., 2011. Application of cellular biosensors for analysis of bioactivity associated with airborne particulate matter. Toxicol In Vitro 25, 1132-1142.

Wagner, W., Walczak-Drzewiecka, A., Slusarczyk, A., et al., 2006. Fluorescent Cell Chip a new in vitro approach for immunotoxicity screening. Toxicol Lett 162, 55-70.

Wang, Y., Walker, C., Stadler, B.M., et al., 1984. Transcription and translation dependent induction of interleukin 2 (IL-2) and IL-2 receptors. Immunol Lett 8, 227-231.

Wilson, R., Fraser, W.D., McKillop, J.H., et al., 1989. The "in vitro" effects of lithium on the immune system. Autoimmunity 4, 109-114.

Yamamoto, N., Sakai, F., Yamazaki, H., et al., 1996. Effect of FR167653, a cytokine suppressive agent, on endotoxin-induced disseminated intravascular coagulation. Eur J Pharmacol 314, 137-142.

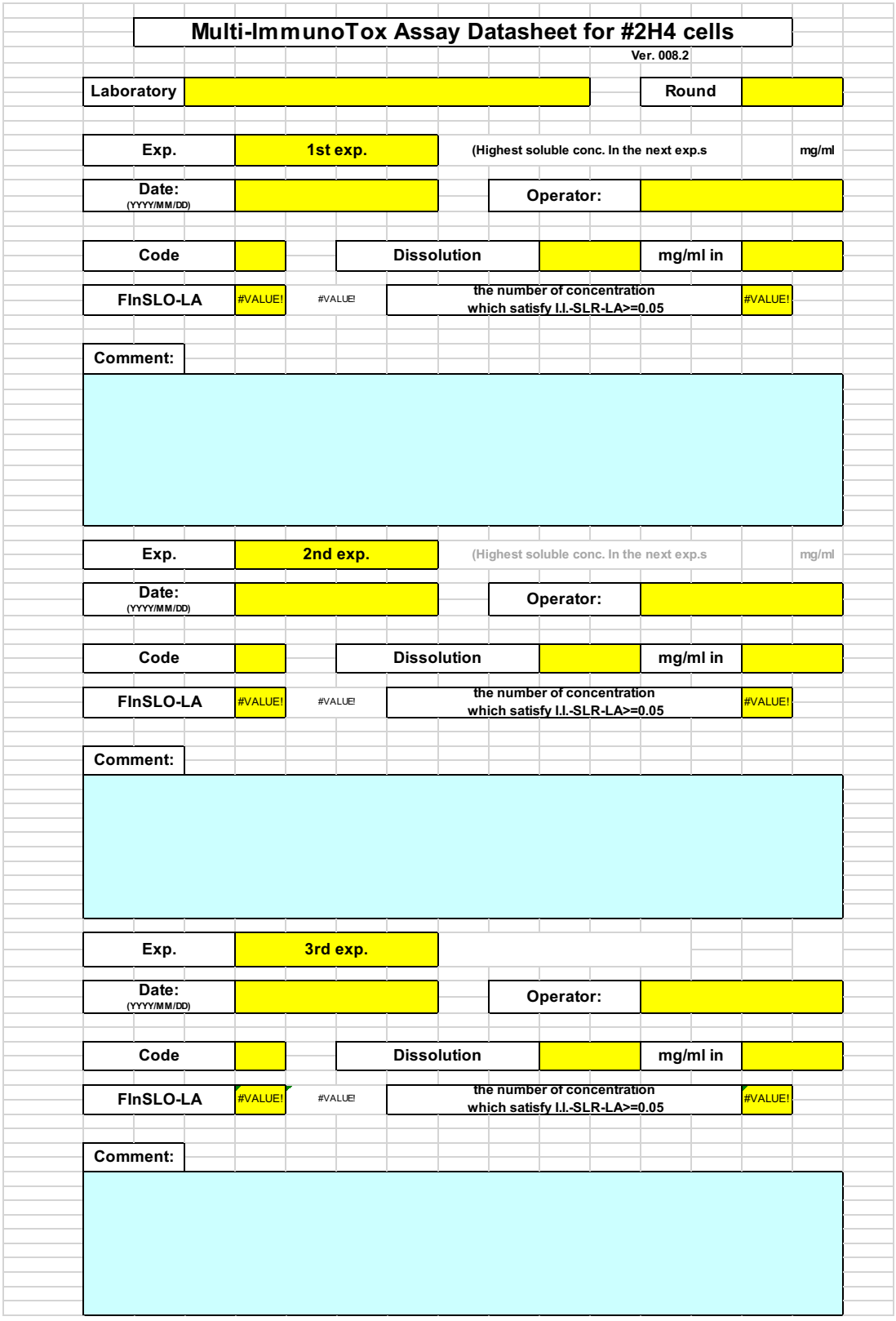
Yoshimura, N., Matsui, S., Hamashima, T., et al., 1989. Effect of a new immunosuppressive agent, FK506, on human lymphocyte responses in vitro. II.

Inhibition of the production of IL-2 and gamma-IFN, but not B cell-stimulating factor 2. Transplantation 47, 356-359.

Zhang, W.Z., Yong, L., Jia, X.D., et al., 2013. Combined subchronic toxicity of bisphenol A and dibutyl phthalate on male rats. Biomed Environ Sci 26, 63-69.

Appendix 10. The Multi-Immuno Tox Assay Data sheet

face sheet



Multi-ImmunoTox Assay Datasheet for #2H4 cells					
Ver. 008.2					
Laboratory				Round	
Exp.	1st exp.	(Highest soluble conc. In the next exp.s			mg/ml
Date: (YYYY/MM/DD)		Operator:			
Code		Dissolution		mg/ml in	
FInSLO-LA	#VALUE!	#VALUE!	the number of concentration which satisfy I.I.-SLR-LA>=0.05		#VALUE!
Comment:					
Exp.	2nd exp.	(Highest soluble conc. In the next exp.s			mg/ml
Date: (YYYY/MM/DD)		Operator:			
Code		Dissolution		mg/ml in	
FInSLO-LA	#VALUE!	#VALUE!	the number of concentration which satisfy I.I.-SLR-LA>=0.05		#VALUE!
Comment:					
Exp.	3rd exp.				
Date: (YYYY/MM/DD)		Operator:			
Code		Dissolution		mg/ml in	
FInSLO-LA	#VALUE!	#VALUE!	the number of concentration which satisfy I.I.-SLR-LA>=0.05		#VALUE!
Comment:					

Exp.	4th exp.				
Date: (YYYY/MM/DD)		Operator:			
Code		Dissolution		mg/ml in	
FInSLO-LA	#VALUE!	#VALUE!	the number of concentration which satisfy I.I.-SLR-LA>=0.05	#VALUE!	

Comment:

Exp.	5th exp.				
Date: (YYYY/MM/DD)		Operator:			
Code		Dissolution		mg/ml in	
FInSLO-LA	#VALUE!	#VALUE!	the number of concentration which satisfy I.I.-SLR-LA>=0.05	#VALUE!	

Comment:

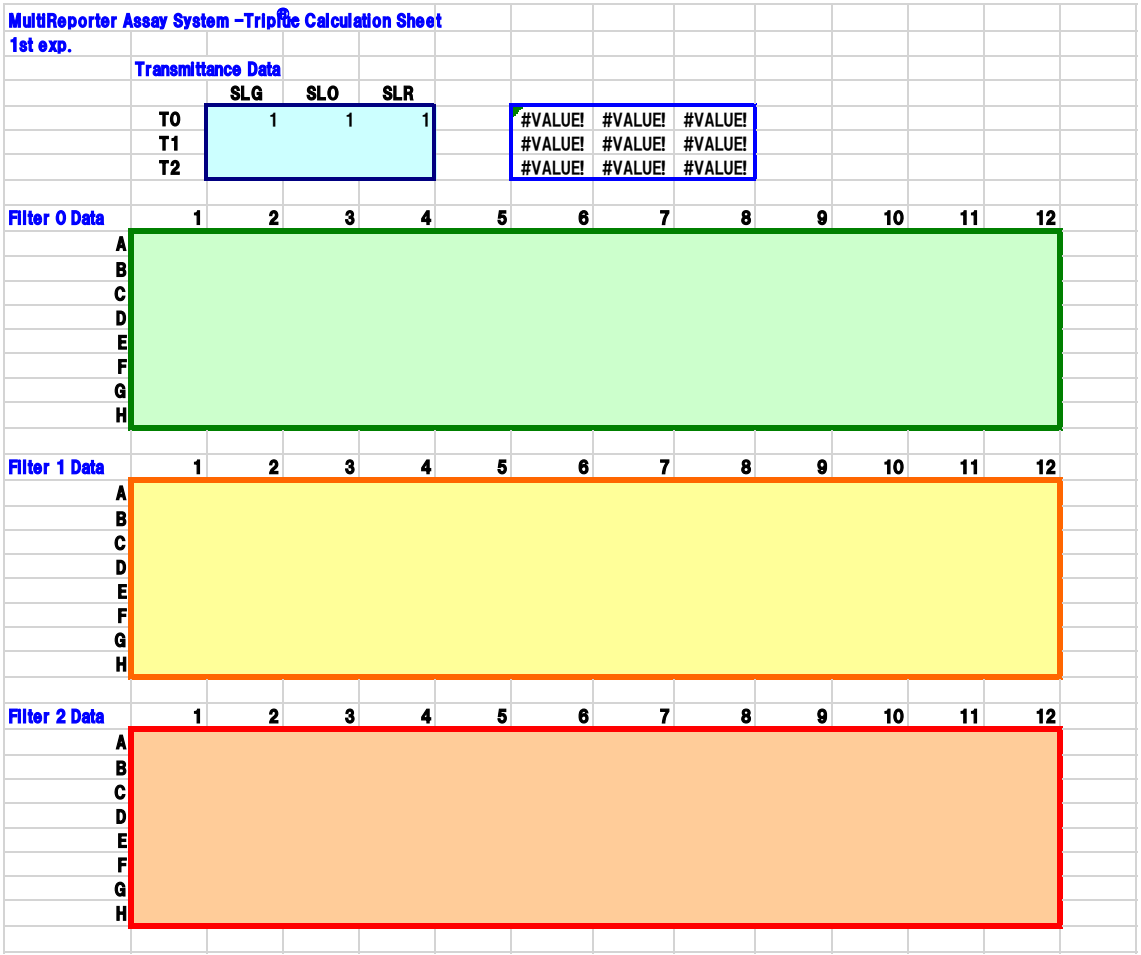
Exp.	6th exp.				
Date: (YYYY/MM/DD)		Operator:			
Code		Dissolution		mg/ml in	
FInSLO-LA	#VALUE!	#VALUE!	the number of concentration which satisfy I.I.-SLR-LA>=0.05	#VALUE!	

Comment:

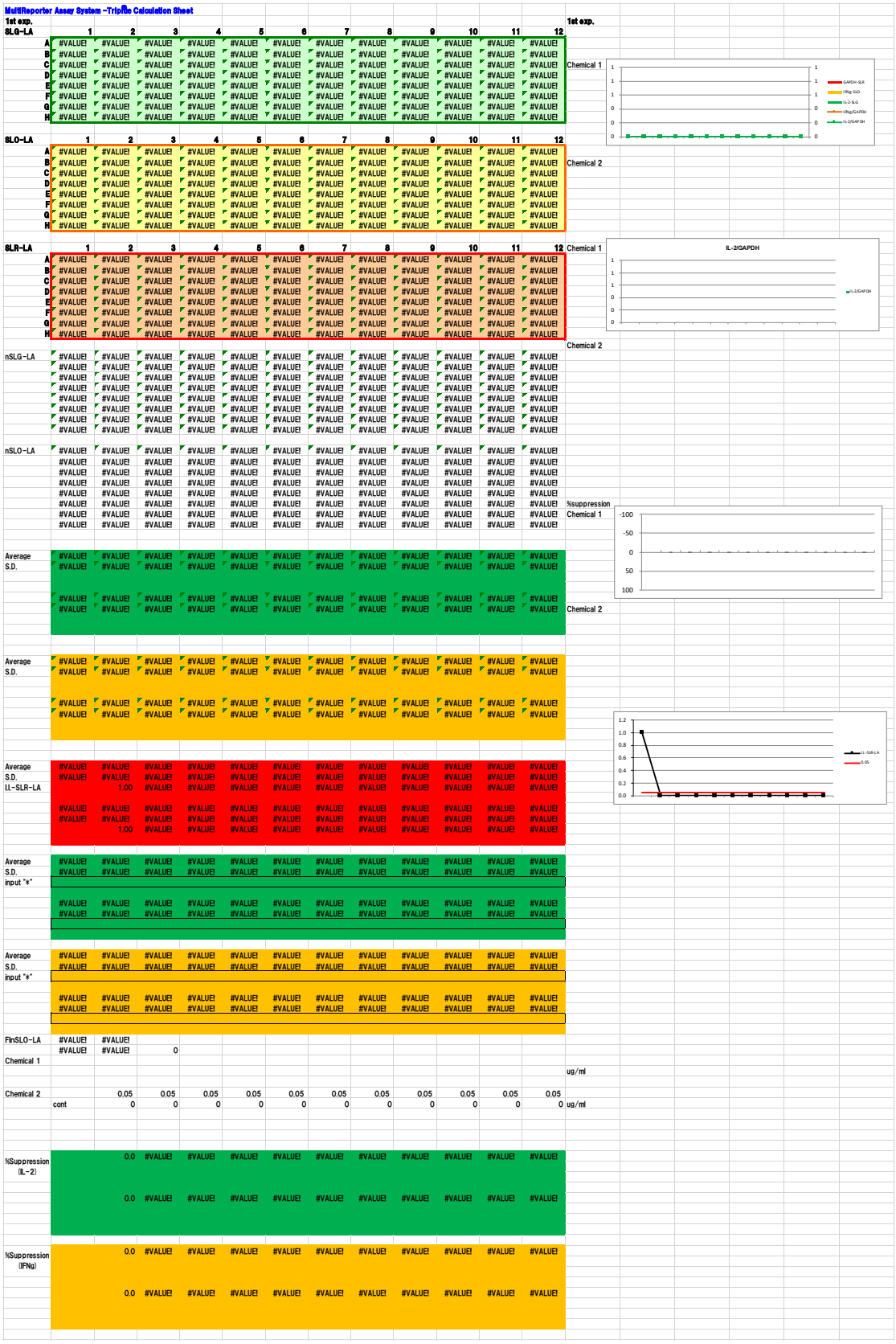
																									ug/mL
1st																									
2nd																									
3rd																									
4th																									
5th																									
6th																									

1st Exp.	
2nd Exp.	
3rd Exp.	
4th Exp.	
5th Exp.	
6th Exp.	

data input sheet

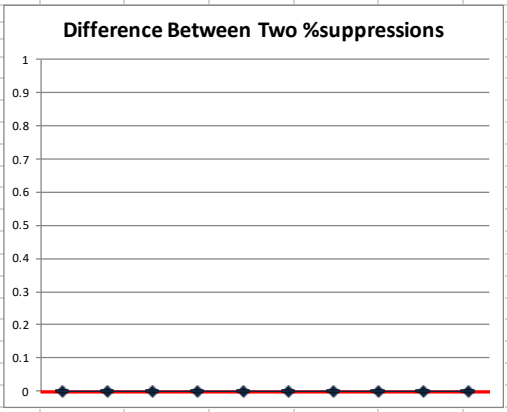


result format sheet



graph sheet

Results



a Comparison Conc.	Difference	Lower Limit	Upper Limit
	0.000	0.000	0.000
	0.000	0.000	0.000
	0.000	0.000	0.000
	0.000	0.000	0.000
	0.000	0.000	0.000
	0.000	0.000	0.000
	0.000	0.000	0.000
	0.000	0.000	0.000
	0.000	0.000	0.000
	0.000	0.000	0.000

Final Judgement
no effect

Appendix 11. The summary of the study by the independent biostatistician

1. Results

The concentration-response plot for each experiment is shown in appendix A. We strongly suggest to see the graphs to understand the result of each judgement of experiment.

1.1 Basic results

The judgment of each experiment by chemical is shown in Table 1 for the phase 1 study and Table 2 for the phase II study. Symbols in column “exp.” means “S” for suppression, “A” for augmentation and “N” for no effect. The column “Judge” lists the final judgment of the assay in each laboratory. The column “Chem. Code” is chemical code.

Table 1. Judgment for 3 independet experiments in Phase I study

(a)

Lab A	exp.			
Chem. Code	1	2	3	Judge
P101_R1	S	S	S	S
P101_R2	S	S	S	S
P101_R3	S	S	S	S
P102_R1	S	A	S	S
P102_R2	N	N	N	N
P102_R3	N	N	N	N
P103_R1	S	S	S	S
P103_R2	S	S	S	S
P103_R3	S	S	S	S
P104_R1	S	S	S	S
P104_R2	S	S	S	S
P104_R3	S	S	S	S
P105_R1	A	N	N	N
P105_R2	N	N	N	N
P105_R3	N	S	N	N

(b)

Lab B	exp.			
Chem. Code	1	2	3	Judge
P101_R1	S	S	S	S
P101_R2	S	S	S	S
P101_R3	S	S	S	S
P102_R1	S	S	S	S
P102_R2	S	S	S	S
P102_R3	S	S	N	S
P103_R1	S	S	S	S
P103_R2	S	S	S	S
P103_R3	S	S	S	S
P104_R1	S	S	S	S
P104_R2	S	S	S	S
P104_R3	S	S	S	S
P105_R1	N	N	N	N
P105_R2	N	S	N	N
P105_R3	N	N	N	N

(c)

Lab C	exp.			
Chem. Code	1	2	3	Judge
P101_R1	S	S	S	S
P101_R2	S	S	S	S
P101_R3	S	S	S	S
P102_R1	N	S	S	S
P102_R2	S	S	N	S
P102_R3	N	N	N	N
P103_R1	S	S	S	S
P103_R2	S	S	S	S
P103_R3	S	S	S	S
P104_R1	S	S	S	S
P104_R2	S	S	S	S
P104_R3	S	S	S	S
P105_R1	N	N	A	N
P105_R2	N	N	N	N
P105_R3	N	N	N	N

S :Suppression, A :Augmentation, N :No effect,
A/S :Augmentation/Suppression

Table 2. Judgment for 3 independent experiments in Phase II study.

(a)

Lab A	exp.				
Chem. Code	1	2	3	4	Judge
P201	N	N	N		N
P202	S	S	S		S
P203	N	N	N		N
P204	A/S	A/S	A/S		A/S
P205	S	S	S		S
P206	N	N	N		N
P207	N	N	N		N
P208	A	A	A		A
P209	A	A	A		A
P210	S	S	S		S
P211	N	N	N		N
P212	A	A	A		A
P213	S	N	S		S
P214	A	A	A		A
P215	A	A	N		A
P216	N	N	N		N
P217	N	N	N		N
P218	S	A	N	N	N
P219	N	A	N		N
P220	S	S	S		S

(b)

Lab B	exp.				
Chem. Code	1	2	3	4	Judge
P201	N	N	N		N
P202	A	S	S		S
P203	N	S	N		N
P204	N	A	A		A
P205	S	S	S		S
P206	N	N	N		N
P207	N	N	N		N
P208	S	A	A		A
P209	A	A	A		A
P210	A	N	N		N
P211	S	S	S		S
P212	A	A	A		A
P213	S	S	S		S
P214	A	A	A		A
P215	S	S	S		S
P216	N	N	N		N
P217	N	N	N		N
P218	N	N	N		N
P219	N	N	N		N
P220	S	S	S		S

(c)

Lab C	exp.				
Chem. Code	1	2	3	4	Judge
P201	N	N	N		N
P202	N	S	S		S
P203	N	A	N		N
P204	N	N	A		N
P205	S	S	S		S
P206	N	N	N		N
P207	N	N	N		N
P208	A	N	A		A
P209	A	A	A		A
P210	S	S	S		S
P211	N	N	N		N
P212	A	A	A		A
P213	S	S	S		S
P214	A	A	A		A
P215	S	S	N		S
P216	N	N	N		N
P217	A	N	N		N
P218	N	N	N		N
P219	A	N	N		N
P220	S	S	S		S

S : Immunosuppression, A : Immunoaugmentation, N : No effect, A/S : Immunoaugmentation/suppression

1.2 Within-laboratory reproducibility

Table 3 shows the final judgment of each assay by chemical and the concordance based on the results in the phase I study. “R” means round.

Table 3. Judgment for independent 3 rounds and concordanc

(a)

Lab A

Chem. Code	R1	R2	R3	Concordance
P101	S	S	S	1
P102	S	N	N	0
P103	S	S	S	1
P104	S	S	S	1
P105	N	N	N	1

(b)

Lab B

Chem. Code	R1	R2	R3	Concordance
P101	S	S	S	1
P102	S	S	S	1
P103	S	S	S	1
P104	S	S	S	1
P105	N	N	N	1

(c)

Lab C

Chem. Code	R1	R2	R3	Concordance
P101	S	S	S	1
P102	S	S	N	0
P103	S	S	S	1
P104	S	S	S	1
P105	N	N	N	1

Table 4 shows the concordance rate of the within-laboratory reproducibility which is estimated by data of Table 3.

Table 4. Withing laboratory concordance

Statistics	Lab A	Lab B	Lab C	Average
Within-laboratory concodance rate	80% (4/5)	100% (5/5)	80% (4/5)	86.7%

1.3 Between-laboratory reproducibility

Table 5 shows the final judgment of the assay for each laboratory and the concordance in the phase II study.

Table 5. Final judgment of the assay for each laboratory and concordance

Chem. Code	Lab A	Lab B	Lab C	Concordance
P201	N	N	N	1
P202	S	S	S	1
P203	N	N	N	1
P204	A/S	A	N	0
P205	S	S	S	1
P206	N	N	N	1
P207	N	N	N	1
P208	A	A	A	1
P209	A	A	A	1
P210	S	N	S	0
P211	N	S	N	0
P212	A	A	A	1
P213	S	S	S	1
P214	A	A	A	1
P215	A	S	S	0
P216	N	N	N	1
P217	N	N	N	1
P218	N	N	N	1
P219	N	N	N	1
P220	S	S	S	1

Table 6 shows the concordance rate of the between-laboratory reproducibility which is estimated by data of Table 5.

Table 6. Between laboratory reproducibility in Phase II study

Statistics	
Between-laboratory concodance rate	80% (16/20)

Table 7 is the result from Table 3 and Table 6. The final judgment in Table 3 was summarized with based on the majority.

Statistics	
Between-laboratory concodance rate	80% (20/25)

Appendix 17

The concentration-response plots for each experiment in the phase I study is contained.

Appendix 18

The concentration-response plot for each experiment in the phase II study is contained.

Appendix 12. Study plan

Study plan
for the validation trial on multicolor reporter assay using IL-2 Luc (IL-2 Luc assay) as a test evaluating the immunotoxic potential of chemicals

Version 1.4 February, 2017

Conducted by:

IL-2 Luc assay Validation Management Team

INDEX

Background

Objective of the trial

3. Validation Management Team

4. Protocol

5. Chemical

6. Records and archiving

7. Study timeline

1. Background

The multicolor reporter assay using IL-2 Luc in Jurkat cells (IL-2 assay) is important for evaluating the immunotoxic potential of chemicals. This assay forms part of the Multi-ImmunoTox assay (MITA) and has the advantages of technical simplicity and a short test period, and the accuracy of the test result is based on the mechanism underlying immunotoxicity.

The aim of this trial is to (pre)validate the IL-2 Luc assay method to assess its transferability and inter-laboratory variability so that this test can be used to screen for immunotoxic chemicals. The IL-2 Luc assay for the validation trial was undertaken i) in accordance with the principles and criteria documented in the OECD No. 34 Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment [OECD, 2005], ii) according to the Modular Approach to validation [Hartung et al., 2004], iii) according to the concept discussed in the validation trials with the participation of GLP Test Facilities [Cooper-Hannan et al., 1999] where the whole concept of validation trials is described in the context of GLP, and iv) in line with the ISO procedure JRC.I.03.GP.01v.01 (http://ihcpnet.jrc.it/quality-safety/quality-documents/unit-03-ivm/doc/JRC.I.03.GP.01v.01.pdf).

The studies comprising a validation trial should ideally be performed in accordance with GLP [OECD, 1998-2007; FDA, 1999; EPA, 1998a&b; JSQA, 2010; SCC, 2010]. As a minimum, use of standard operating procedures (SOP) and adequate data recording, reporting and record keeping are essential.

A general conceptual framework [Hartung et al., 2004; OECD, 2005] will be used for documenting the entire study to assess the validation status of the test method. This is called a “modular approach” to validation. In this approach, the information needed to support the validity of the method is organized into modules that provide the following information:

Module 1: Test Definition

Module 2: Within-laboratory repeatability and reproducibility

Module 3: Between-laboratory transferability

Module 4: Between-laboratory reproducibility

Module 5: Predictive capacity

Module 6: Applicability domain

Module 7: Performance standards

The modular approach as introduced by Hartung et al. allows the use of datasets from

various data sources and studies. This advantage is used in the following proposal to assess the scientific validity of the IL-2 Luc assay. This IL-2 Luc assay for the validation trial has been performed under GLP principles.

2. Objective of the trial

The validation trial will assess the reliability (reproducibility within and between laboratories) and relevance (predictive capacity) of the IL-2 Luc assay with a challenging set of test substances (test items) for which high quality *in vitro* and *in vivo* data are available.

3. Validation Management Team (VMT)

The VMT encompasses collective expertise with the test, in the underlying science, and the scientific design, management and evaluation of a validation trial.

The VMT, which plays a central role overseeing the conduct of the validation trial, includes:

Table 1. Members for IL-2 Luc assay Validation Management Team

Name	Role and expertise	Affiliation
Trial Coordinator Hajime Kojima	VMT trial coordinator, Chemical supplier and Management of quality control	JaCVAM, NIHS, Japan (JaCVAM representative)
Lead Lab Yutaka Kimura* Setsuya Aiba*	*Developer of this assay Test method, expertise underlying science	Tohoku Univ., Japan
Takashi Omori	Data analysis, biostatistics dossier	Kobe Univ., Japan
International expert members		
EU liaison Emanuela Corcini	Test system expertise, validation expertise, immunotoxicity expertise	Milan Univ., Italy
EU liaison Erwin L. Roggen	Test system expertise, validation expertise, immunotoxicity expertise	3Rs Management and Consulting ApS, Denmark
ICCVAM liaison	Immunotoxicity expertise	NTP/NIEHS, USA

Dori Germolec		
JSIT liaison Tomoaki Inoue	Immunotoxicity expertise	Chugai Pharmaceutical Co., Ltd.

Participating Test Facilities

The laboratories participating in the trial are defined as follow:

Test Facility 1: Hatano Res. Inst., FDSC. Study Director (SD): Kohji Yamakage

Test Facility 2: AIST, Tsukuba SD: Yoshihiro Ohmiya

Test Facility 3: AIST, Takamatsu SD: Yoshihiro Nakajima

Information relevant for Modules 1, 2, 3 performed by all laboratories. Data obtained by these laboratories have demonstrated that the IL-2 Luc assay is transferable and reproducible between experienced laboratories. All laboratories participating in this validation trial will act as unexperienced laboratories to assess between-laboratory transferability, reliability, and relevance of the IL-2 Luc assay method under non-GLP conditions (GLP principle).

Trial management structure

1) Chemical management group

The members of the chemical management group are elected by recommendation of the IL-2 Luc assay VMT. The members prepare a tentative list of test chemicals and work with the VMT to make a final decision on the test chemicals to be used in the validation trial. The coded test chemicals listed in Table 6 and 7 are distributed by the JaCVAM.

2) Data analysis group

The members of the data analysis group are elected by recommendation of the IL-2 Luc assay VMT and check and analyze the data obtained in this validation trial from a third-party standpoint. The members also take charge of statistical processing in this validation trial.

3) Quality assurance group

The members of the record management group are elected by recommendation of the IL-2 Luc assay VMT. The members prepare the protocol, the test chemical preparation record forms, blank data sheets, etc., and distribute them to the research laboratories participating in this validation trial. The members also collect completed forms and data sheets after completion of the experiments, and point out omissions or flaws in recording, if any, and request corrections of such errors.

4) Lead laboratory

The lead laboratory representing the test method is responsible for providing the test method protocol and the necessary data recording or calculation templates. The Trial Coordinator must ensure that such data recording or calculation templates have been validated before distribution to the test facilities involved in the validation trial. The lead laboratory is also responsible for providing, if necessary, new versions of the protocols during the entire validation trial. The lead laboratory and the other participating test facilities might be contacted by the VMT regarding technical issues.

Sponsor

The validation trial for assessing the validity of IL-2 Luc assay will be financed by the Ministry of Health, Labour and Welfare (MHLW), Japan.

The lead laboratory will support the IL-2 Luc assay validation trial by assuring that reliability is assessed. At the same time, preliminary results of the test method can be evaluated. For this purpose, Lead laboratory will support:

- financial aspects related to the coordination of a validation trial (e.g. organization of VMT meetings where also the involved test facilities can be invited for technical clarifications to the VMT, the publication of the validation trial results)
- test, reference and control item purchase, coding and distribution to the test facility
- availability of the test systems to the participating laboratories by supporting the lead laboratory with the logistics for delivering the test system to the facility
- independent analysis of data and statistical support (biostatistician) based on the study reports generated
- other costs incurred by the participating laboratories

Trial coordination

Dr. Hajime Kojima was appointed as the Trial Coordinator with well-defined roles and responsibilities to coordinate the trial and to establishment of a VMT by supporting of JaCVAM.

The name and location of the Trial Coordinator should be identified in each individual study plan. For the IL-2 Luc assay validation trial, the Trial Coordinator has direct access to the test item coding.

The Trial Coordinator's responsibilities include:

a) Establishment of/support to lead laboratory, including meeting organization
b) Trial communication and coordination with test facilities
c) Recording of document and data flow between test facilities
d) Assessing and documenting the impact of any amendments and/or deviations from the trial plan and study plans on the quality and integrity of the validation trial
e) Ensuring that the individual study reports are forwarded, in a timely manner, for data and statistical analysis
f) Preparing the trial plan and report, which can be based on the study reports from the lead laboratories and other test facilities involved in the validation trial, and should reflect the overall trial
g) Approval with date and signature of all protocols, Study Plans and Study Reports
h) The communication of the results of the trial into the public domain
The Trial Coordinator's responsibilities include:
a) Establishment/support of the lead laboratory, including meeting organization
b) Trial communication and coordination with the test facilities
c) Recording of documents and data flow between the test facilities
d) Assessing and documenting the impact of any amendments and/or deviations from the trial plan and study plans on the quality and integrity of the validation trial
e) Ensuring that the individual study reports are forwarded, in a timely manner, for data and statistical analysis
f) Preparing the trial plan and report, which can be based on the study reports from the lead laboratory and other test facilities involved in the validation trial, and should reflect the overall trial
g) Approval with date and signature of all protocols, study plans and study reports
h) Communication of the results of the trial to the public domain
The role of the Trial Coordinator (as the formal representative of the VMT and the single contact point with the SDs) is of fundamental importance. The Trial Coordinator is the single critical point of trial control and must ensure clear lines of communication between the involved test facilities in the trial. The communication line of the Trial Coordinator is with the SDs of the different test facilities. The SDs are the single point of contact with the Trial Coordinator (unless otherwise communicated by the participating test facilities) to assure a transparent and recorded documentation flow during the trial. The Trial Coordinator should also ensure that appropriate arrangements have been made for the supply of the test systems, and test, control and reference items, which meet the requirements of the trial, and that there are appropriate

test method protocols (dated signature by the Trial Coordinator and the lead laboratory) and, if appropriate, validated data recording, data analysis, and data reporting sheets for the test method.
It is the responsibility of the Trial Coordinator to approve the study plans sent for approval by the test facilities, and any amendments to the study plan, by dated signature.

Training
The lead laboratory will be responsible for issuing a training agenda to the Trial Coordinator for further distribution to all test facilities, giving details of what training aspects will be covered during the training of the other SDs and study personnel at the lead laboratory. Furthermore, after the training during the Phase 0 study, the lead laboratory will issue to the Trial Coordinator a training report and indicate if critical observations are made by the other test facilities regarding the IL-2 Luc assay protocols. In case any critical observations are made, a new version of the IL-2 Luc assay protocols might need be issued to the other test facilities before initiating the between-laboratory transferability test.

[Module 2] Within-laboratory reproducibility
The within-laboratory reproducibility of all test facilities has been done by an independent biostatistical analysis using 5 coded chemicals under the VMT. The concordance should be equal to or greater than 80% as a tentative acceptance criterion for the Phase I study.

3.7 [Module 3] Between-laboratory transferability
This between-laboratory transferability (Module 3) study is performed in order to assess the successful transfer of the assay to a test facility unexperienced with that particular test method but having knowledge of similar test systems and endpoint detection methods.
Transfer of the IL-2 Luc assay to all test facilities in the Phase 0 study using 5 coded five chemicals was achieved. A few concentrations of each test item were tested in triplicate in 3 independent runs according to the IL-2 Luc assay protocol describing the details of the experimental design.
The 5 test items selected for the Phase I study are coded as A, B, C, D, and E. The facilities will prepare a study according to internal GLP principles. This plan will be submitted to the Trial Coordinator and lead laboratory for approval.

The results of the between-laboratory transferability study will be reviewed before progressing with module 4 in the between-laboratory reproducibility study. If the transferability data do not meet the test acceptance criteria, the Trial Coordinator representing the VMT will try to identify the problems and make corrections where needed. At the end of testing, the test facilities will submit a QC certified copy of the entire study dossier to the Trial Coordinator (study plan adhering to GLP principles, raw data, records and data analysis, study report adhering to GLP principles).

3.8 [Module 4] Between-laboratory reproducibility

Twenty-five coded test items have been selected to confirm the between-laboratory reproducibilities in the Phase I and II studies. Several concentrations of each test item will be tested in triplicate according to the IL-2 Luc assay method protocol describing the details of the experimental design.

At the end of testing, the test facilities will submit a QC certified copy of the entire study dossier to the Trial Coordinator (study plan adhering to GLP principles, raw data, records and data analysis, study report adhering to GLP principles). The concordance for between-laboratory reproducibility should be equal or greater than 80% to meet the acceptance criteria.

[Module 5] Predictive capacity

The necessity for further chemical analysis will be subject to a VMT decision once the data for between-laboratory reproducibility has been assessed. Depending on the statistical analysis, lean design for validation as well as automatisation of the test leading to an increased dataset will be considered.

Protocol

In this validation trial, the protocols ver. 0.08E, Phase I and 0.1E, Phase II will be used. These protocols will be drafted by the lead laboratory and will be finalized by the VMT. The criteria to identify immunotoxicants by the MITA are provisionally fixed in protocol ver. 0.08E prior to the Phase I study. There are 2 temporary criteria to identify imunotoxicants. The VMT adopted these criteria after the Phase I validation study.

A measurement of bioluminescence intensity induced by chemical treatment will be measured by a luminometer (Phelios: ATTO, Cat #:AB-2350) calibrated using stabilized SLG, SLO and SLR enzymes in this validation trial.

Chemicals

5.1 Chemical Selection

Test chemicals have been selected from a chemical repository based on published papers on *in vivo* immunotoxicity.

The applied selection criteria were:

information on mode/site of action

coverage of range of relevant chemical classes and product classes

quality and quantity of reference data (*in vivo* and *in vitro*)

high quality data derived from animals and (if available) also humans

knowledge of interspecies variations (for example: variability with regard to the uptake of chemicals, metabolism, etc.)

coverage of range of toxic effects/potencies

chemicals that do not need metabolic activation

appropriate negative and positive controls

physical and chemical properties (feasibility of use in the experimental set-up as defined by the CAS No.)

single chemical entities or formulations of known high purity

availability

cost

In the first phase of the selection procedure, the chemical management group identified and collected several existing lists of potential chemical sensitizers in order to establish a primary database. These chemicals had originally been compiled by international experts for various purposes, such as reference compounds for validation studies. An extensive literature research was performed by the chemical management group, insuring that the preselected chemicals fulfilled the selection criteria described above. Emphasis was laid on the fact that different potencies (strong, weak and no activity) have been chosen. In addition, it was decided that at least 20% of the total substances to be tested should be negative in order to increase the statistical power of the data analysis.

In the first phase of the IL-2 Luc assay validation trial using data generated at the test facilities, 5 chemicals will be tested 3 times for each test chemical for between-laboratory reproducibility and to confirm transferability. After discussion of the Phase I results, detailed test planning for Phase II will be established. Currently, it is planned that 20 chemicals will be tested in the Phase II trial to establish predictive capacity (Table 2).

Table 2. Outline of test planning at each study in the validation trial.

Study	Chemicals	Test Number	Information obtained
Phase I	5 non- coded	1	Between-lab transferability
Phase I	5 coded	3	Within & between-lab reproducibility
Phase II	20 coded	1	Between-lab reproducibility & predictability

5.2 Chemical Acquisition, Coding and Distribution

The within-laboratory reproducibility (Module 2) and between-laboratory transferability (Module 3) in all test facilities have been assessed with coded chemicals. This IL-2 Luc validation trial plan describes generation of the missing data sets under coded test item. If the results obtained are not highly similar to the previously obtained sets, the VMT must assess if coded chemicals need to be tested in all the test facilities.

Coding will be supervised by the Trial Coordinator, in collaboration with the chemical repository responsible for coding and distribution of the test, reference and control items for the validation trial.

5.3 Handling

Each test facility shall receive through the Trial Coordinator essential information about the test chemicals (physical state, weight or volume of sample, specific density for liquid test chemicals, and storage instructions). Moreover, the SD should receive safety information concerning hazards identification and exposure controls/personal protection.

Records and archiving

At the end of the trial, the IL-2 Luc assay validation trial report is prepared by the Trial Coordinator or the VMT personnel who appointed by the Trial Coordinator. The trial report summarizes the trial goals, procedures, results and conclusions of the validation trial. This represents the whole validation trial, including archiving and, as such, will cover several study reports, as well as reports for test item supply, data management and statistics. The Trial Coordinator oversees the preparation of the trial report. The Trial Coordinator will be representing the VMT discussions responsible for preparation of the scientific conclusions. Signatories to the trial report include the Trial Coordinator, the statistician, and the SDs of the involved test facilities. Although the SDs may not be involved with the preparation of the trial report, their signatures confirm that the trial report is an accurate reflection of the management and study events. The trial report should contain a statement, signed by the Trial Coordinator, commenting on the

accuracy and completeness of the trial report and identifying any significant issues which could have affected the integrity of the trial, including matters of GLP compliance. A QC statement will be included in the trial report, in order to identify what QC monitoring was done and to confirm whether or not the trial report is an accurate reflection of the validation trial data.

Study timeline

An approximate schedule for IL-2 Luc assay validation trial is shown in Table 3. The duration of this validation trial is around 20 months, from May 2016 to December 2017.

Table 3. Schedule of IL-2 Luc assay validation trial

Month	Activity
January 2016	Establish the VMT
	Selection of participating research laboratories
	Deliberation, decision and read-through of draft study plan
	Deliberation and decision of protocol
	Preparation of a tentative list of test chemicals
	Distribution of test chemicals, standard chemicals and positive control chemicals
February,2016	Technical transfer using five known chemicals (non-coded) Start of technical transfer **to know between laboratory transferability**
	Data collection of technical transfer (**Phase 0 study**)
Phase I study	
September 2016	Coding and distribution of five coded test chemicals
September, 2016	Start of Phase I study
December, 2016	End of Phase I study
February, 2017	**2nd VMT Meeting** / Phase I results and planning of Phase II study
Phase II study to know between- and within-laboratory reproducibility	
April, 2017	Coding and distribution of coded test chemicals and positive chemicals
May, 2017	Start of Phase II study using 20 coded test chemicals
August,2017	End of Phase II study
November-December, 2017	**3rd VMT Meeting** /reviewing of Phase II study results
2018	Completed validation report

Abbreviations

CAS: Chemical Abstracts Service
GLP: Good Laboratory Practice
HRI: Hatano Research Institute
FDSC: Food and Drug Safety Center
JaCVAM: Japanese Centre for the Validation of Alternative Methods
NIHS: National Institute of Health Sciences
OECD: Organization for Economic Co-operation and Development
QC: Quality Control
TG: Test Guideline
VMT: Validation Management Team

Appendix 13. MITA QC confirmation table

MITA(P1) confirmation tabale

	LabB (AIST, Tsukuba)			LabC (FDSC)			LabD (AIST, Takamatsu)		
setA-1 (run 1)	date	2016.9.12	(document 4 - 5)						
	Cell culture records	○							
	Weighting records	○							
	Test records	○							
	Datasheet	×							
	Graph	○							
setA-1 (run 2)	date	2016.10.4	(document 4 - 6)						
	Cell culture records	○							
	Weighting records	○							
	Test records	○							
	Datasheet	×							
	Graph	○							
setA	Weighting records	○		Weighting records	○		Weighting records	○	
	Cell culture records	○		Cell culture records	×		Cell culture records	○	wake up 2016.8.26~~ Last culture 2016.9.29
setA-1	date	2016.10.26		date	2016.10.4.		date	2016.9.9	
	Cell culture records	○		Cell culture records	○		Cell culture records	○	
	Weighting records	○		Weighting records	○		Weighting records	○	
	Test records	○		Test records	○		Test records	○	
	Datasheet	○		Datasheet	○		Datasheet	○	
	Graph	○		Graph			Graph	○	
setA-2	date	2016.11.1		date	2016.10.17		date	2016.9.12	
	Cell culture records	○		Cell culture records	○		Cell culture records	○	
	Weighting records	○		Weighting records	○		Weighting records	×	
	Test records	○		Test records	○		Test records	○	
	Datasheet	○		Datasheet	○		Datasheet	○	
	Graph	○		Graph			Graph	○	
setA-3	date	2016.11.4		date	2016.10.21		date	2016.9.15	(document 7 - 8)
	Cell culture records	○		Cell culture records	○		Cell culture records	○	
	Weighting records	○		Weighting records	○		Weighting records	×	
	Test records	○		Test records	○		Test records	○	
	Datasheet	○		Datasheet	○		Datasheet	○	
	Graph	○		Graph			Graph	○	
setA-4	date			date			date	2016.9.20	(3rd re trial)
	Cell culture records			Cell culture records			Cell culture records	○	
	Weighting records			Weighting records			Weighting records	×	
	Test records			Test records			Test records	○	
	Datasheet			Datasheet			Datasheet	○	
	Graph			Graph			Graph	○	
setB	Weighting records	○		Weighting records	○		Weighting records	○	
	Cell culture records	○		Cell culture records	×		Cell culture records	○ Newly starting cell culture on the way	continue from SetA + (wake up from 20160923 cell culture till 20161014)
setB-1	date	2016.11.8		date	2016.10.27		date	2016.9.23	(document 7 - 8)
	Cell culture records	○		Cell culture records	○		Cell culture records	○	
	Weighting records	○		Weighting records	○		Weighting records	○	
	Test records	○		Test records	○		Test records	○	
	Datasheet	○		Datasheet	○		Datasheet	○	
	Graph	○		Graph	○		Graph	○	
setB-2	date	2016.11.12		date	2016.10.28		date	2016.9.26	(1st re-trial)
	Cell culture records	○		Cell culture records	○		Cell culture records	○	
	Weighting records	○		Weighting records	○		Weighting records	×	
	Test records	○		Test records	○		Test records	○	
	Datasheet	○		Datasheet	○		Datasheet	○	
	Graph	○		Graph	○		Graph	○	
setB-3	date	2016.11.16		date	2016.10.31		date	2016.9.29	(2nd trial)
	Cell culture records	○		Cell culture records	○		Cell culture records	○	
	Weighting records	○		Weighting records	○		Weighting records	×	
	Test records	○		Test records	○		Test records	○	
	Datasheet	○		Datasheet	○		Datasheet	○	
	Graph	○		Graph	○		Graph	○	
setB-4	date			date			date	2016.10.3	(3rd trial)
	Cell culture records			Cell culture records			Cell culture records	○	
	Weighting records			Weighting records			Weighting records	×	
	Test records			Test records			Test records	○	
	Datasheet			Datasheet			Datasheet	○	
	Graph			Graph			Graph	○	

1

MITA(P1) confirmation tabale

	LabB (AIST, Tsukuba)			LabC (FDSC)			LabD (AIST, Takamatsu)		
setC	Weighting records	○		Weighting records	○		Weighting records	○	
	Cell culture records	○		Cell culture records	×		Cell culture records	○	
setC-1	date	2016.11.10		date	2016.11.14		date	2016.10.6	(document 7 - 8)
	Cell culture records	○		Cell culture records	○		Cell culture records	○	
	Weighting records	○		Weighting records	○		Weighting records	○	
	Test records	○		Test records	○		Test records	○	
	Datasheet			Datasheet	○		Datasheet	○	
	Graph	○		Graph	○		Graph	○	
setC-2	date	2016.11.14		date	2016.11.25		date	2016.10.14	(document 7 - 8)
	Cell culture records	○		Cell culture records	○		Cell culture records	○	
	Weighting records	○		Weighting records	○		Weighting records	×	
	Test records	○		Test records	○		Test records	○	
	Datasheet			Datasheet	○		Datasheet	○	
	Graph	○		Graph	○		Graph	○	
setC-3	date	2016.11.18		date	2016.12.09		date	2016.10.17	(document 7 - 8)
	Cell culture records	○		Cell culture records	○		Cell culture records	○	
	Weighting records	○		Weighting records	○		Weighting records	×	
	Test records	○		Test records	○		Test records	○	
	Datasheet			Datasheet	○		Datasheet	○	
	Graph	○		Graph	○		Graph	○	
setC-4	date			date			date	2016.10.20	(document 7 - 8)
	Cell culture records			Cell culture records			Cell culture records	○	
	Weighting records			Weighting records			Weighting records	×	
	Test records			Test records			Test records	○	
	Datasheet			Datasheet			Datasheet	○	
	Graph			Graph			Graph	○	
	SDS back	○	20170322受領	SDS back	○		SDS back	○	
	calibration records			calibration records			calibration records		

2

MITA(P2) Confirmation table

	項目	LabB (AIST, Tsukuba)	LabC (FDSC)	LabD (AIST, Shikoku)
	Weighing records		○	○
	Cell culture records	○ 2017.05.02 3sets 2017.05.19 2017.06.12	○ 2017.05.29 3sets 2017.07.03 2017.07.31	○ 2017.05.08 3sets 2017.06.06 2017.07.03
	Solubility check records	○ per each samples	○ per each tests	○ per each samples
1	Test date	2017.5.19.	2017.06.30	2017.05.22
	Test samples No.（repeat No.)	1-5(1)	6,4,6,7(1)	2,7,8,12(1)
	Others records	○	○	○
	Datasheets	○	○	○
2	Test date	2017.5.31	2017.07.06	2017.05.23
	Test samples No.	1,3-5(2),2(re1)	4,6,7(2)	14,16,17,19,20,01(1)
	Others records	○	○	○
	Datasheets	○	○	○
3	Test date	2017.6.8	2017.07.07	2017.05.29
	Test samples No.	1,3-5(3),2(2)	4,6,7(3)	3,4,10,11(1)
	Others records	○	○	○
	Datasheets	○	○	○
4	Test date	2017.6.12	2017.07.13	2017.05.30
	Test samples No.	2(3).5(re3)	1,3,5,8(1)	5,6,9,13,15,18(1)
	Others records	○	○	○
	Datasheets	○	○	○
5	Test date	2017.6.5	2017.07.14	2017.06.12
	Test samples No.	6-10(1)	1,3,5,8(2),9,10(1)	5,6,9,13,15,18(re1)
	Others records	○	○	○
	Datasheets	○	○	○
6	Test date	2017.6.6.	2017.07.18	2017.06.19
	Test samples No.	6(re1),7-10(2)	1,3,5,8(3),9,10(2)	3,4,10,11(re1)
	Others records	○	○	○
	Datasheets	○	○	○
7	Test date	2017.6.9.	2017.07.21	2017.06.20
	Test samples No.	6(2),7-10(3)	9,10(3),11-14(1)	2,7,8,12(2)
	Others records	○	○	○
	Datasheets	○	○	○
8	Test date	2017.6.14	2017.07.24	2017.06.26
	Test samples No.	11-15(1)	11.12.14(2),13(re1).15,16(1)	14,16,17,19,20,01(re1)
	Others records	○	○	○
	Datasheets	○	○	○
9	Test date	2017.6.21	2017.07.27	2017.06.27
	Test samples No.	11-15(2)	11,12,14(3),13(2)	5,6,10,11,3,4（2）
	Others records	○	○	○
	Datasheets	○	○	○
10	Test date	2017.6.22	2017.07.28	2017.07.03
	Test samples No.	11-13.15(3)14(re2),	2(re1),13(3),15,16(2),18,20(1)	16,17,19,20,15,18（2）
	Others records	○	○	○
	Datasheets	○	○	○
11	Test date	2017.6.29	2017.08.03	2017.07.04
	Test samples No.	6,14(3)	15,16(3),17,19(1),18,20(2)	1,9,13,14（2）
	Others records	○	○	○
	Datasheets	○	○	○
12	Test date	2017.6.28	2017.08.04	2017.07.10
	Test samples No.	16-20(1)	1,3(4),2(3).19(2),18,20(3)	17,19,7,3（3）
	Others records	○	○	○
	Datasheets	○	○	○
13	Test date	2017.7.7	2017.08.07	2017.07.11
	Test samples No.	16-20(2)	2(4),17(2),19(3)	2,8,12,16,14,20（3）
	Others records	○	○	○
	Datasheets	○	○	○
14	Test date	2017.7.11	2017.08.08	2017.07.18
	Test samples No.	16-20(3)	5,8,19(4),17(3)	1,4,5,6,10,11（3）
	Others records	○	○	○
	Datasheets	○	○	○
15	Test date		2017.08.14	2017.07.24
	Test samples No.		13(4)	9,13,15,18(3),3,19(4)
	Others records		○	○
	Datasheets		○	○
16	Test date			2017.07.25
	Test samples No.			10,13,14,9,4(4)
	Others records			○
	Datasheets			○

Appendix 14. MITA coded chemical list

【Phase I coded list for the MITA validation study in Sep 2016】

No.	Chemical	CASRN	MW	Supplier	Catalog No.	Content	Physical characteristics	Lot	Storage	Purity	LabA	LabB	LabC	LabD
											TOHOKU univ.	AIST-TSUKUBA	FDSC	AIST-SHIKOKU
1	Dibutyl phthalate	84-74-2	278.34	Wako	021-06936	500mL	Liquid	TLN0112	RT	98.0+% (Capillary GC)	MIA003A	MIB014A	MIC027A	MID036A
											MIA004B	MIB017B	MIC026B	MID033B
											MIA007C	MIB016C	MIC023C	MID034C
2	Hydrocortisone (for Cell Culture)	50-23-7	362.46	Wako	080-10194	50g	Solid	SAH3714	RT	97% (HPLC)	MIA005A	MIB017A	MIC029A	MID038A
											MIA007B	MIB019B	MIC028B	MID035B
											MIA009C	MIB018C	MIC025C	MID037C
3	Lead(II) acetate trihydrate (Deleterious substances)	6080-56-4	379.33	Sigma-Aldrich	316512-100G	100g	Solid	09901TS	RT	99.999% trace metals basis	MIA007A	MIB018A	MIC021A	MID310A
											MIA008B	MIB011B	MIC210B	MID037B
											MIA001C	MIB110C	MIC027C	MID038C
4	Zinc dimethyldithiocarbamate (DMDTC)	137-30-4	305.82	Kanto Chemical	48028-31	25g x2	Solid	403N2204	RT	>95.0% (T)	MIA009A	MIB110A	MIC023A	MID037A
											MIA010B	MIB013B	MIC027B	MID039B
											MIA003C	MIB017C	MIC029C	MID310C
5	Nickel (II) sulfate hexahydrate	10101-97-0	262.85	Wako	146-01171	100g	Solid	LKQ2263	RT	99.0-102.0% (as NiSO4 - 6H2O) (Titration)	MIA001A	MIB012A	MIC025A	MID034A
											MIA002B	MIB015B	MIC024B	MID031B
											MIA005C	MIB014C	MIC021C	MID032C

MITA(Phase2) coded chemicals

	Chemical	Cas.no.	LabA	LabB	LabC	LabD	Note	State	Storage	Supplier	Lot
			TOHOKU unv.	AIST-TSUKUBA	FDSC	AIST-SHIKOKU					
1	2,4-Diaminotoluene	95-80-7	MIA401	MIB515	MIC618	MID702	Deleterious	S	RT	Wako	CDF0347
2	Benzo(a)pyrene	50-32-8	MIA413	MIB516	MIC601	MID703		S	RT	TCI	M8DFD
3	Cadmium chloride	10108-64-2	MIA403	MIB502	MIC602	MID714	Deleterious	S	RT	Wako	DEE3332
4	Dibromoacetic acid	631-64-1	MIA406	MIB518	MIC610	MID720		S	RT	ALDRICH	BCBR5175V
5	Diethylstilbestol	56-53-1	MIA420	MIB509	MIC611	MID711		S	RT	SIGMA	BCBR9766V
6	Diphenylhydantoin	630-93-3	MIA412	MIB510	MIC615	MID704		S	RT	SIGMA	SLBB3874
7	Ethylene dibromide	106-93-4	MIA407	MIB507	MIC605	MID705	Deleterious	L	RT	Wako	KWG5479
8	Glycidol	556-52-5	MIA408	MIB505	MIC607	MID712		L	2-8℃	ALDRICH	MKBX5752V
9	Indomethacin	53-86-1	MIA409	MIB508	MIC609	MID715		S	RT	SIGMA	122K0718
10	Isonicotinic Acid Hydrazide (Isoniazid)	54-85-3	MIA411	MIB517	MIC612	MID707		S	RT	Fluka	SLBF8371V
11	Nitrobenzene	98-95-3	MIA402	MIB519	MIC603	MID701	Deleterious	L	RT	Sigma-Aldrich	SHBG5577V
12	Urethane, Ethyl carbamate	51-79-6	MIA415	MIB520	MIC604	MID719		S	RT	Sigma-Aldrich	WXBC3505V
13	Tributyltin chloride	1461-22-9	MIA404	MIB506	MIC613	MID713	Deleterious	L	RT	TCI	2442A-IQ
14	Perfluorooctanoic acid	335-67-1	MIA414	MIB514	MIC614	MID718		S	RT	TCI	O3U70
15	Dichloracetic acid	79-43-6	MIA416	MIB511	MIC606	MID716	Deleterious	L	RT	Sigma-Aldrich	SHBH3492V
16	Toluene	108-88-3	MIA417	MIB512	MIC616	MID706	Deleterious	L	RT	Sigma-Aldrich	J5136
17	Acetonitril	75-05-8	MIA405	MIB501	MIC617	MID708	Deleterious	L	RT	Wako	KWH4805
18	Mannitol	69-65-8	MIA418	MIB503	MIC619	MID717		S	RT	Wako	LKP4362
19	Vanadium pentoxide	1314-62-1	MIA419	MIB504	MIC608	MID709	Deleterious	S	RT	Wako	SAE6958
20	o-Benzyl-p-chorolophenol	120-32-1	MIA410	MIB513	MIC620	MID710		S	RT	Wako	KPQ0988

positive
negative

Appendix 15. The list of proficiency chemicals

The list of proficiency chemicals

No.	Chemical name	CAS No.	T cell targeting	Physical state	Phase
1	Dibutyl phthalate	84-74-2	Yes	Liquid	I
2	Lead(II) acetate trihydrate	6080-56-4	Yes	Solid	I
3	Nickel（II) sulfate hexahydrate	10101-97-0	Yes	Solid	I
4	Benzo(a)pyrene	50-32-8	Yes	Solid	II
5	Diethylstilbestol	56-53-1	Yes	Solid	II
6	Urethane, Ethyl carbamate	51-79-6	Yes	Solid	II
7	Tributyltin chloride	1461-22-9	Yes	Liquid	II
8	2.4-diaminotoluene	95-80-7	NO	Solid	II
9	Acetonitril	75-05-8	NO	Liquid	II
10	Vanadium pentoxide	1314-62-1	NO	Solid	II

Prior to routine use of the test method described in this Annex to Test Guideline 442E, laboratories should demonstrate technical proficiency, using the 10 Proficiency Substances listed in Appendix 15 in compliance with the Good in vitro Method Practices (1). Moreover, test method users should maintain a historical database of data generated with the reactivity checks (see paragraph 15) and with the positive and solvent/vehicle controls (see paragraphs 21-24), and use these data to confirm the reproducibility of the test method in their laboratory is maintained over time.

1. OECD (2017), Draft Guidance document: Good *In Vitro €i0Method Practices (GIVIMP) for the Development and Implementation of In Vitro €i0Methods for Regulatory Use in Human Safety Assessment. Organisation for Economic Cooperation and Development, Paris. Available at: [http://www.oecd.org/env/ehs/testing/OECD%20Draft%20GIVIMP_v05%20-%20clean.pdf].*

Appendix 16. The list of performance standard chemicals

No.	Chemical name	CAS No.	T cell targeting	Physical state	Phase
1	Dexamethasone	50-02-2	Yes	Solid	positive control
2	Cyclosporine	59865-13-3	Yes	Solid	-
3	Indomethacin	53-86-1	Yes	Solid	II
4	Perfluorooctanoic acid	335-67-1	Yes	Solid	II
5	Zinc dimethyldithiocarbamate (DMDTC)	137-30-4	No	Solid	I
6	Mannitol	69-65-8	No	Solid	II

Performance standards (PS) (15) are shown to facilitate the validation of modified in vitro IL-2 luciferase test methods similar to the IL-2 Luc assay and allow for timely amendment of this Test Guideline for their inclusion. Mutual Acceptance of Data (MAD) will only be guaranteed for test methods validated according to the PS, if these test methods have been reviewed and included in this Test Guideline by the OECD.

IL2 Graph P1

2019.02.24

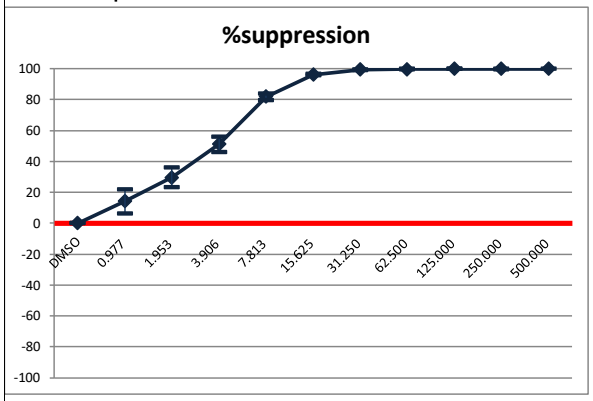
Takashi Omori

Lead Lab.

Chem.1

S:SSS

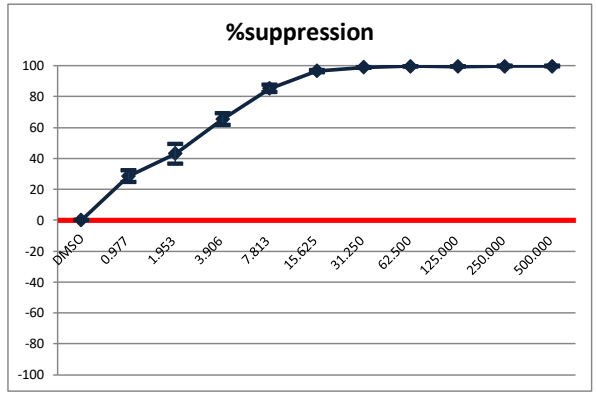
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	14.226	6.496	21.957
1.953	29.723	23.166	36.280
3.906	51.119	46.123	56.116
7.813	81.861	79.524	84.197
15.625	96.223	95.765	96.681
31.250	99.509	99.340	99.678
62.500	99.821	99.751	99.892
125.000	99.904	99.848	99.960
250.000	99.893	99.788	99.998
500.000	99.995	99.954	100.036

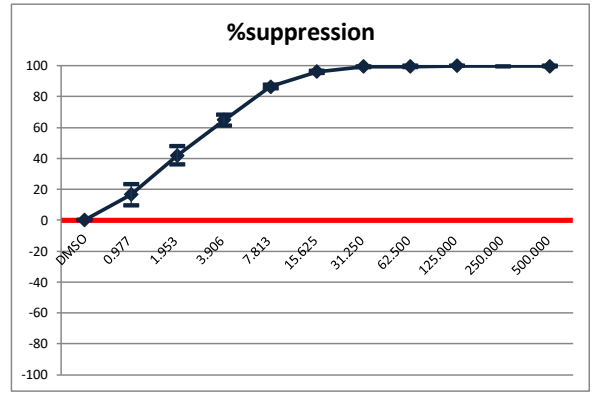
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	28.414	24.636	32.191
1.953	43.176	36.764	49.588
3.906	65.479	61.507	69.451
7.813	85.358	83.095	87.622
15.625	96.594	96.008	97.179
31.250	99.026	98.808	99.244
62.500	99.636	99.496	99.777
125.000	99.564	99.435	99.694
250.000	99.747	99.547	99.946
500.000	99.707	99.569	99.844

3rd Exp.



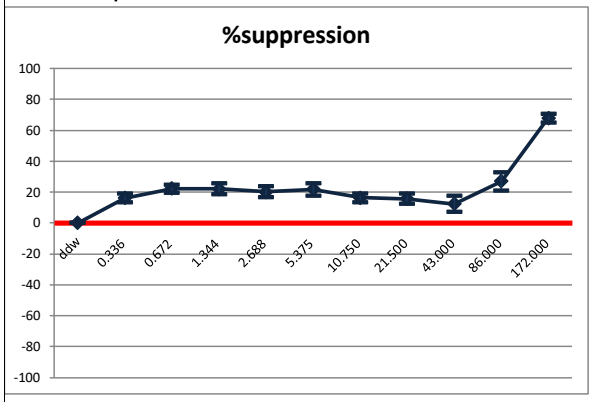
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	16.638	9.844	23.433
1.953	41.956	36.076	47.835
3.906	64.883	61.176	68.590
7.813	86.548	85.247	87.849
15.625	96.112	95.504	96.721
31.250	99.411	99.306	99.516
62.500	99.592	99.241	99.942
125.000	99.990	99.859	100.121
250.000	100.068	99.802	100.334
500.000	99.827	99.667	99.986

Chem.2

N:SNN

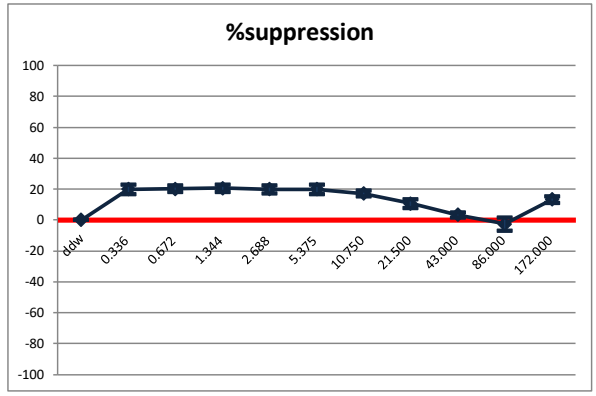
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.336	16.248	13.314	19.183
0.672	22.330	19.755	24.905
1.344	22.201	18.628	25.774
2.688	20.189	16.636	23.742
5.375	21.810	17.820	25.801
10.750	16.303	13.426	19.179
21.500	15.693	12.465	18.921
43.000	12.445	7.173	17.717
86.000	26.978	20.988	32.968
172.000	68.016	65.152	70.879

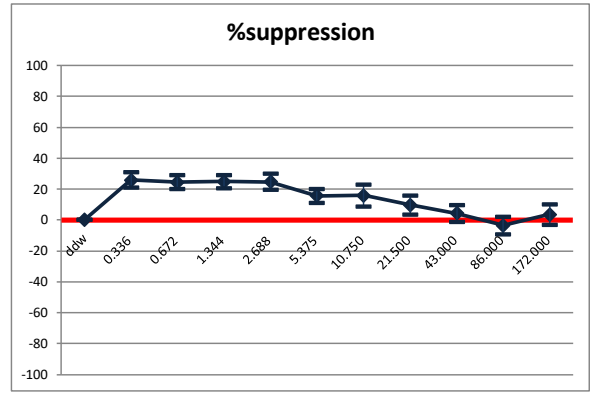
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.336	19.980	16.979	22.981
0.672	20.284	18.298	22.271
1.344	20.566	18.297	22.836
2.688	20.057	17.465	22.649
5.375	19.925	16.976	22.874
10.750	17.105	15.259	18.952
21.500	10.678	7.865	13.491
43.000	3.370	1.633	5.106
86.000	-2.602	-7.005	1.801
172.000	13.239	11.245	15.233

3rd Exp.



3rd Exp.

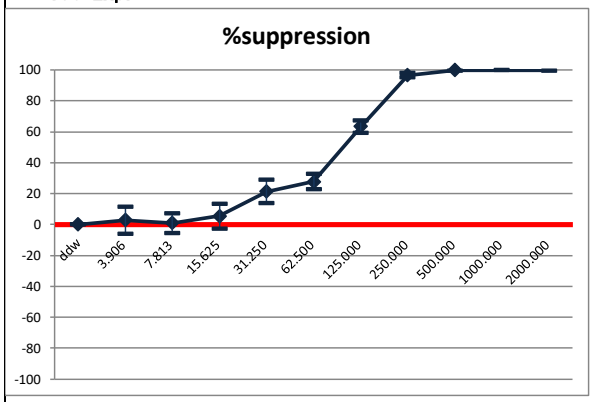
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.336	25.978	20.976	30.981
0.672	24.540	19.863	29.216
1.344	24.815	20.434	29.195
2.688	24.645	19.421	29.868
5.375	15.609	10.939	20.279
10.750	15.942	8.818	23.066
21.500	9.738	3.508	15.969
43.000	4.252	-1.169	9.672
86.000	-3.498	-9.306	2.311
172.000	3.680	-2.988	10.347

Lead Lab.

Chem.3

S:SSS

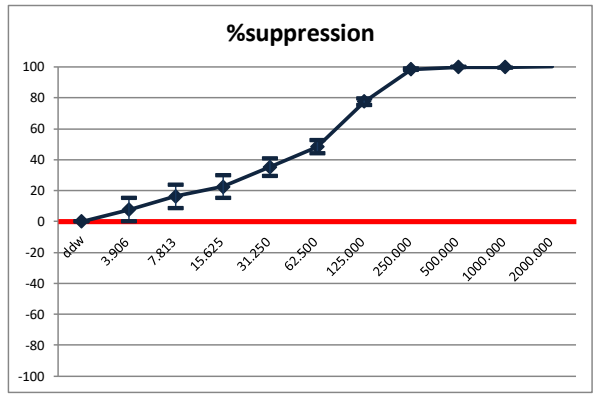
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	2.885	-5.803	11.573
7.813	1.021	-5.490	7.532
15.625	5.570	-2.507	13.646
31.250	21.468	13.822	29.113
62.500	27.763	22.811	32.715
125.000	63.414	59.357	67.471
250.000	96.733	95.422	98.045
500.000	99.908	99.812	100.004
1000.000	100.002	99.885	100.118
2000.000	100.033	99.816	100.251

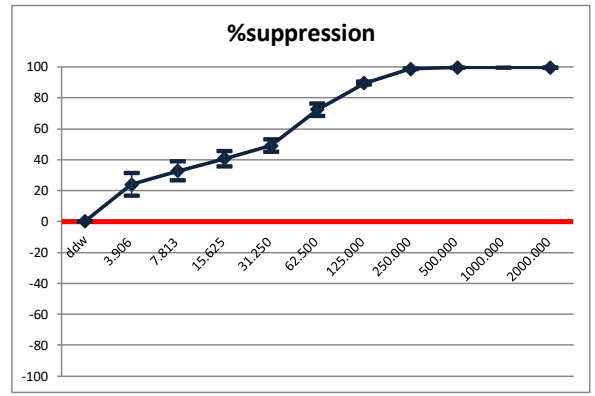
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	7.728	-0.011	15.468
7.813	16.337	8.694	23.980
15.625	22.559	15.190	29.929
31.250	35.234	29.749	40.718
62.500	48.476	44.120	52.833
125.000	77.669	75.604	79.734
250.000	98.494	98.080	98.908
500.000	99.915	99.842	99.989
1000.000	99.870	99.711	100.030
2000.000	100.360	100.311	100.409

3rd Exp.



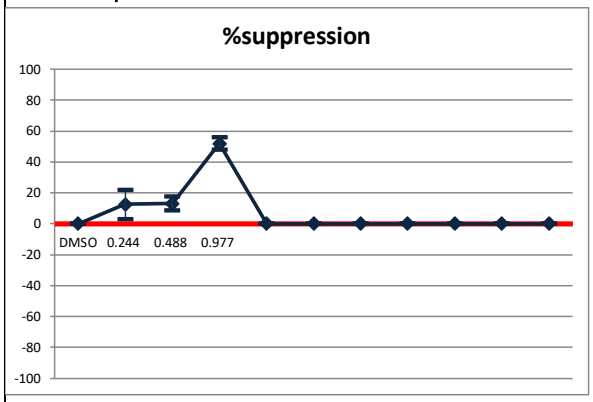
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	24.034	16.790	31.278
7.813	32.777	26.659	38.896
15.625	40.806	35.812	45.799
31.250	49.016	44.970	53.061
62.500	72.526	68.439	76.613
125.000	89.575	88.729	90.420
250.000	98.869	98.721	99.017
500.000	99.784	99.440	100.128
1000.000	100.009	99.779	100.240
2000.000	99.710	99.587	99.833

Chem.4

N:SNN

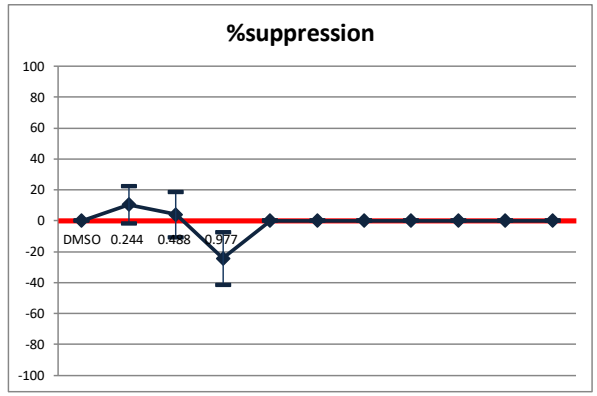
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	12.644	3.249	22.040
0.488	13.196	8.730	17.663
0.977	51.870	47.893	55.847

2nd Exp.

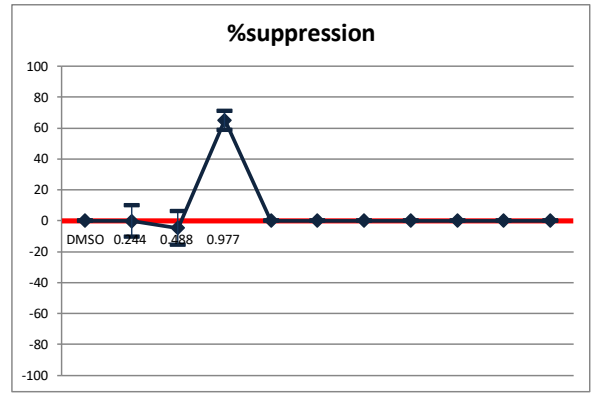


2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	10.430	-1.679	22.539
0.488	4.000	-10.718	18.718
0.977	-24.350	-41.246	-7.454

382

3rd Exp.



3rd Exp.

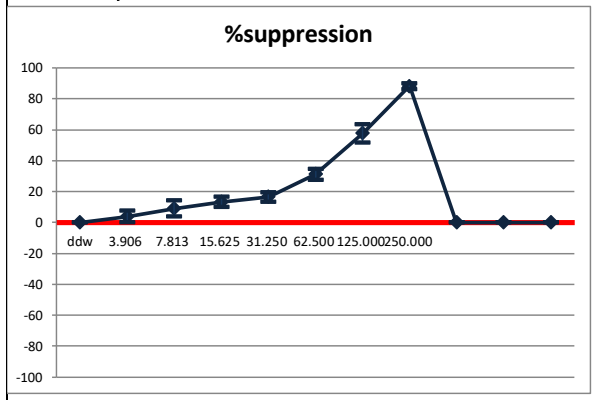
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	-0.143	-10.192	9.906
0.488	-4.596	-15.321	6.129
0.977	65.117	59.116	71.118

Lead Lab.

Chem.5

S:SSS

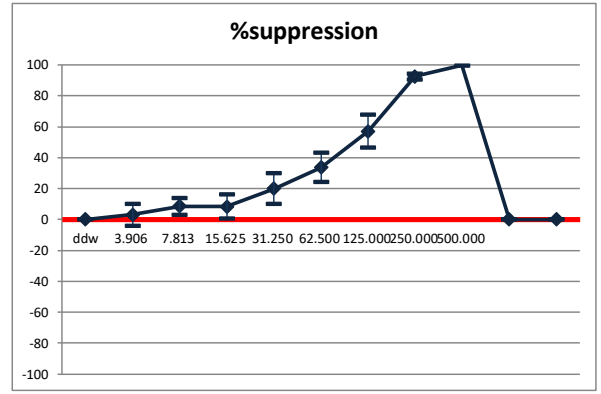
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	3.901	0.241	7.562
7.813	9.123	4.053	14.193
15.625	13.438	10.165	16.712
31.250	16.684	13.541	19.828
62.500	31.287	27.652	34.921
125.000	57.733	52.013	63.452
250.000	88.330	86.340	90.319

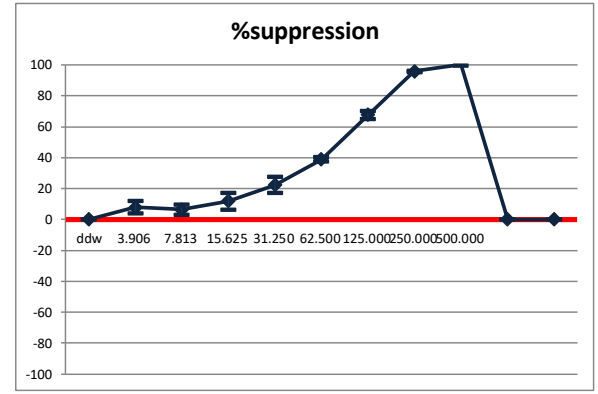
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	3.159	-3.869	10.187
7.813	8.670	3.240	14.100
15.625	8.354	0.465	16.242
31.250	19.973	9.928	30.019
62.500	33.772	24.139	43.406
125.000	57.167	46.591	67.744
250.000	92.520	90.706	94.334
500.000	100.026	99.439	100.614

3rd Exp.



3rd Exp.

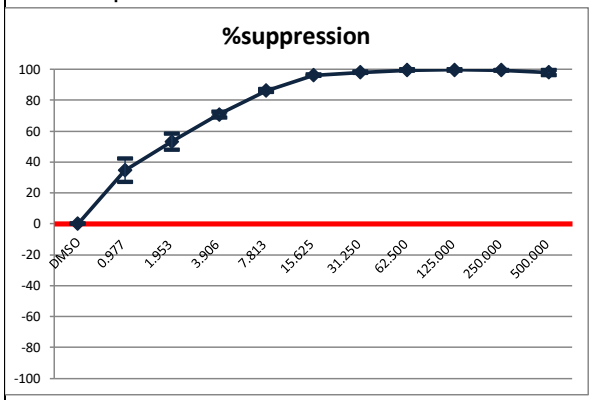
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	7.936	3.796	12.076
7.813	6.408	3.161	9.654
15.625	11.972	6.524	17.421
31.250	22.343	17.259	27.427
62.500	38.994	37.483	40.504
125.000	67.688	65.026	70.351
250.000	95.886	95.552	96.220
500.000	100.316	99.694	100.937

Lab A chem.1

round1

S:SSS

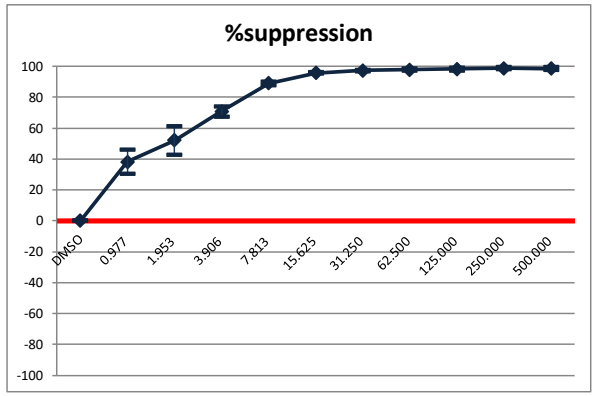
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	34.721	27.171	42.272
1.953	53.241	47.931	58.550
3.906	70.784	68.790	72.778
7.813	86.198	85.207	87.189
15.625	96.139	95.729	96.549
31.250	98.149	97.843	98.454
62.500	99.534	99.168	99.901
125.000	99.609	99.294	99.924
250.000	99.537	99.291	99.782
500.000	97.948	96.518	99.377

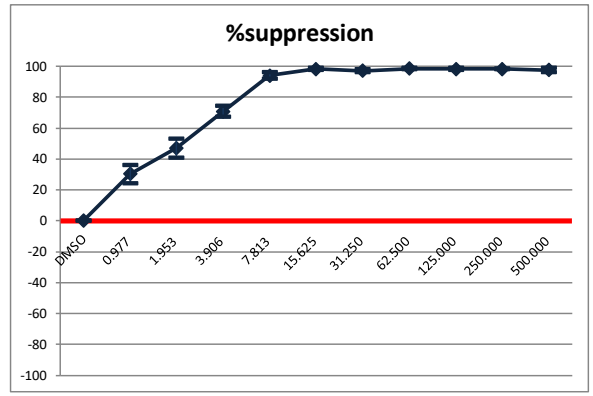
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	38.255	30.623	45.888
1.953	52.040	42.711	61.369
3.906	70.749	67.321	74.177
7.813	89.001	87.853	90.148
15.625	95.766	95.220	96.313
31.250	97.274	96.769	97.779
62.500	97.877	97.036	98.718
125.000	98.211	97.271	99.151
250.000	98.835	98.022	99.647
500.000	98.636	97.883	99.389

3rd Exp.



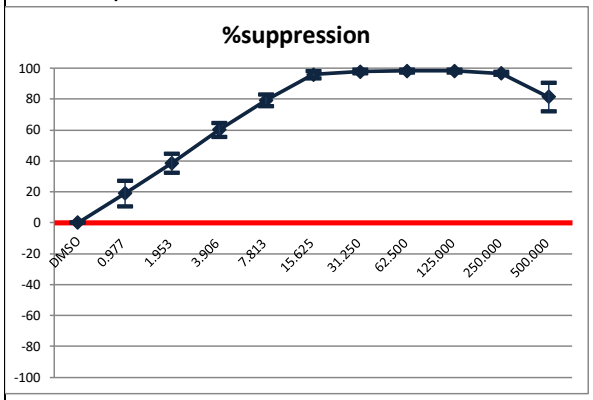
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	30.382	24.396	36.369
1.953	47.148	40.945	53.352
3.906	70.956	67.400	74.511
7.813	94.153	91.924	96.381
15.625	98.355	97.480	99.231
31.250	97.164	96.139	98.188
62.500	98.646	98.137	99.155
125.000	98.353	97.631	99.075
250.000	98.383	98.082	98.684
500.000	97.583	96.257	98.909

round2

S:SSS

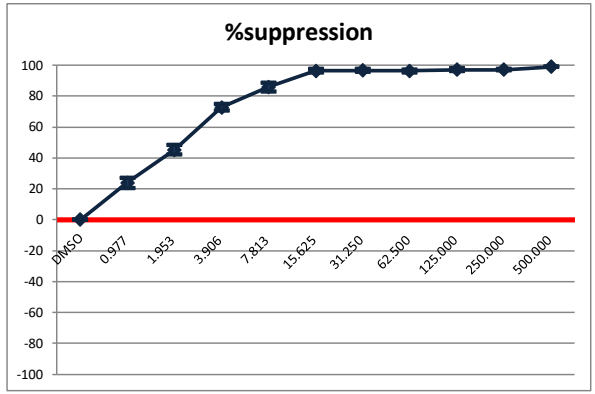
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	18.898	10.495	27.301
1.953	38.592	32.507	44.678
3.906	60.086	55.623	64.550
7.813	79.289	75.424	83.153
15.625	95.848	93.418	98.279
31.250	97.923	96.953	98.892
62.500	98.186	97.119	99.253
125.000	98.258	97.340	99.175
250.000	96.643	95.581	97.706
500.000	81.535	72.228	90.842

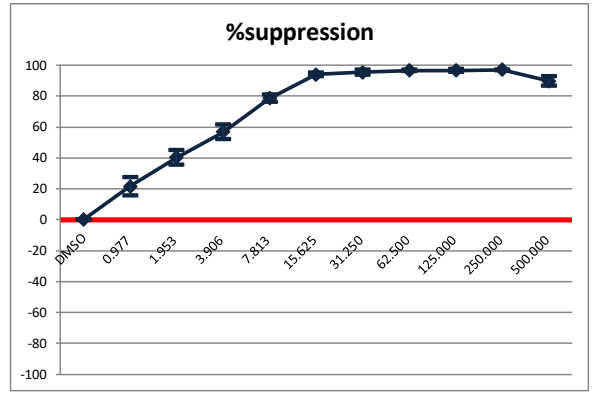
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	23.938	20.646	27.230
1.953	45.339	42.434	48.244
3.906	72.795	70.518	75.071
7.813	85.893	83.192	88.594
15.625	96.410	95.218	97.601
31.250	96.733	95.910	97.556
62.500	96.462	95.538	97.385
125.000	97.192	96.310	98.075
250.000	97.084	96.643	97.525
500.000	99.108	98.916	99.299

3rd Exp.



3rd Exp.

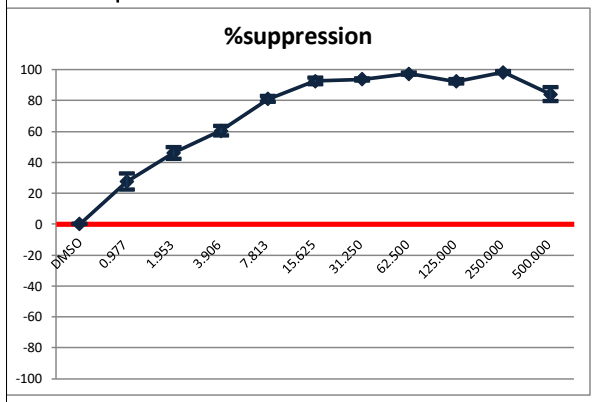
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	21.664	15.691	27.637
1.953	40.316	35.650	44.982
3.906	56.889	52.192	61.585
7.813	78.759	76.503	81.014
15.625	94.144	93.162	95.127
31.250	95.575	93.964	97.186
62.500	96.575	96.072	97.078
125.000	96.670	95.738	97.601
250.000	97.031	96.698	97.363
500.000	89.782	86.629	92.936

Lab A chem.1

round3

S:SSS

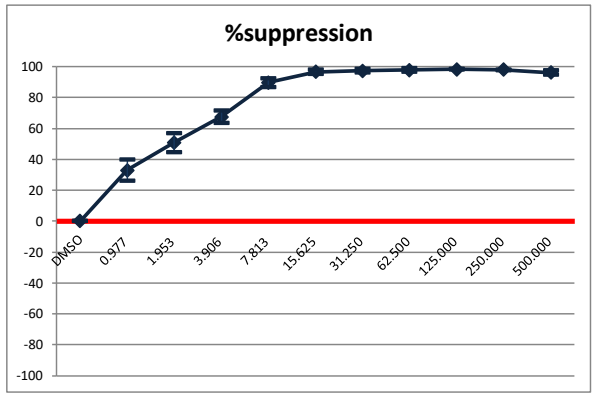
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	27.645	22.391	32.898
1.953	46.125	42.277	49.973
3.906	60.531	57.534	63.529
7.813	81.017	79.218	82.816
15.625	92.588	90.506	94.670
31.250	93.784	93.160	94.409
62.500	97.430	96.581	98.280
125.000	92.486	91.061	93.912
250.000	98.295	97.575	99.015
500.000	84.207	79.914	88.500

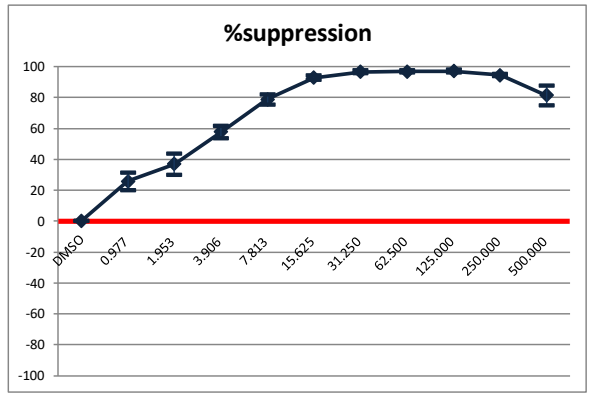
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	33.055	26.364	39.746
1.953	51.005	44.914	57.096
3.906	67.564	63.465	71.663
7.813	89.666	86.631	92.700
15.625	96.615	95.209	98.021
31.250	97.466	96.496	98.437
62.500	97.934	96.880	98.988
125.000	98.298	97.989	98.607
250.000	98.106	97.801	98.410
500.000	96.133	94.673	97.593

3rd Exp.



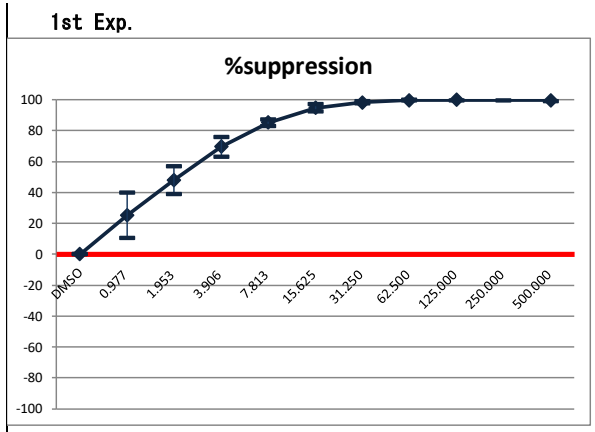
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	25.797	19.943	31.650
1.953	36.972	30.192	43.751
3.906	57.820	53.735	61.904
7.813	78.970	75.663	82.277
15.625	92.859	91.511	94.207
31.250	96.648	95.750	97.547
62.500	96.829	96.125	97.533
125.000	97.115	96.094	98.135
250.000	94.559	93.697	95.420
500.000	81.450	74.923	87.977

Lab B chem.1

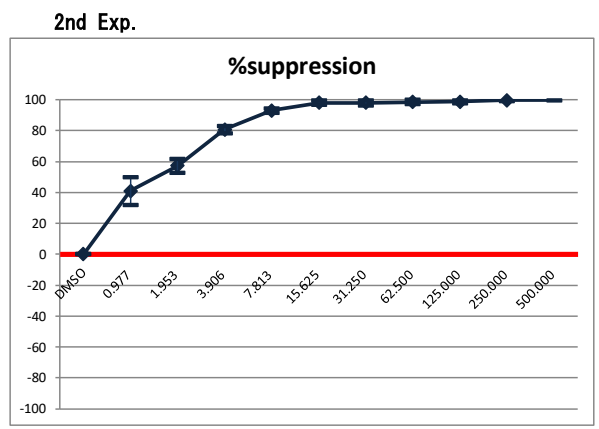
round1

S:SSS



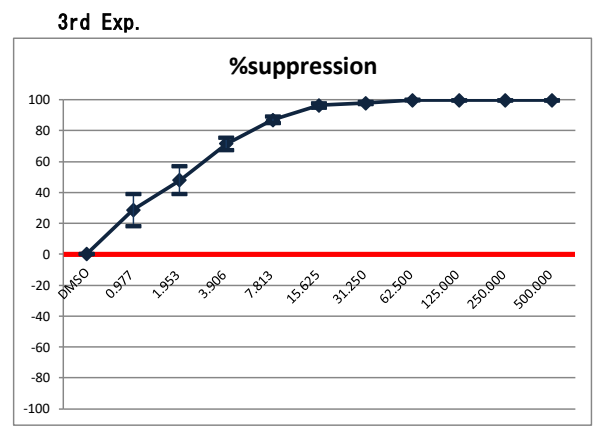
1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	25.105	10.379	39.832
1.953	48.085	39.073	57.097
3.906	69.647	63.157	76.137
7.813	85.168	82.912	87.423
15.625	94.850	92.631	97.068
31.250	98.389	97.598	99.181
62.500	99.705	99.487	99.924
125.000	99.872	99.664	100.079
250.000	100.055	99.556	100.554
500.000	99.796	99.076	100.516



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	40.932	32.044	49.819
1.953	57.257	52.897	61.617
3.906	80.723	78.399	83.048
7.813	92.977	91.738	94.215
15.625	98.082	96.535	99.630
31.250	97.947	96.292	99.603
62.500	98.620	97.388	99.852
125.000	98.850	97.884	99.816
250.000	99.709	99.066	100.352
500.000	100.047	99.751	100.344

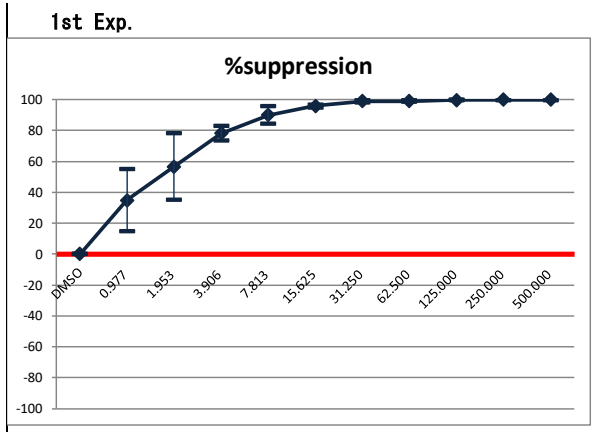


3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	28.684	18.244	39.123
1.953	47.983	39.050	56.916
3.906	71.438	67.329	75.547
7.813	87.029	84.780	89.278
15.625	96.393	95.031	97.754
31.250	97.843	97.256	98.430
62.500	99.690	99.457	99.922
125.000	99.720	99.622	99.818
250.000	99.777	99.738	99.816
500.000	99.626	99.494	99.758

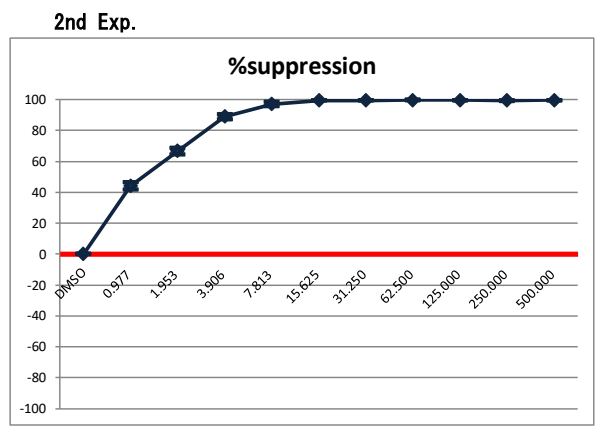
round2

S:SSS



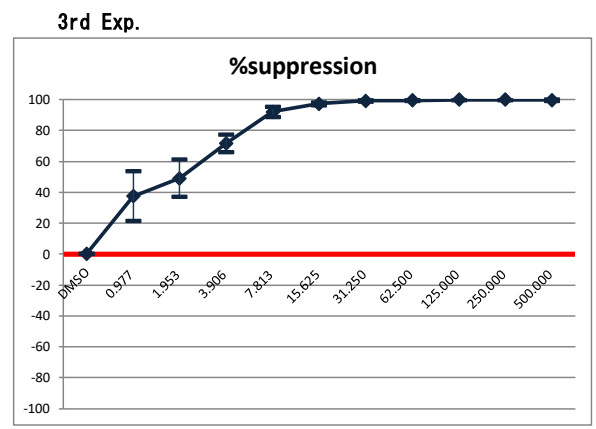
1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	34.922	14.778	55.066
1.953	56.757	35.410	78.105
3.906	78.264	73.608	82.919
7.813	90.051	84.504	95.599
15.625	95.848	94.931	96.765
31.250	98.948	98.312	99.584
62.500	98.945	98.463	99.427
125.000	99.763	99.530	99.995
250.000	99.887	99.384	100.389
500.000	99.939	99.636	100.242



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	44.048	41.678	46.417
1.953	66.834	64.626	69.041
3.906	88.960	87.160	90.761
7.813	97.151	95.756	98.547
15.625	99.578	99.372	99.784
31.250	99.594	99.399	99.789
62.500	99.707	99.560	99.854
125.000	99.633	99.471	99.795
250.000	99.543	99.360	99.726
500.000	99.648	99.476	99.819



3rd Exp.

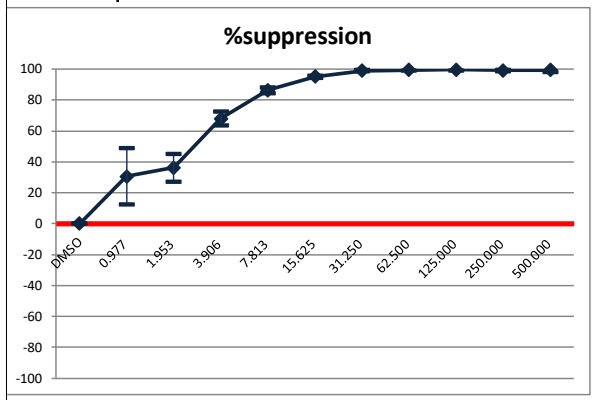
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	37.576	21.496	53.657
1.953	49.128	37.062	61.193
3.906	71.679	65.894	77.464
7.813	92.176	88.923	95.429
15.625	97.418	96.436	98.400
31.250	99.241	98.777	99.706
62.500	99.370	99.004	99.736
125.000	99.881	99.689	100.073
250.000	99.916	99.693	100.138
500.000	99.619	99.312	99.926

Lab B chem.1

round3

S:SSS

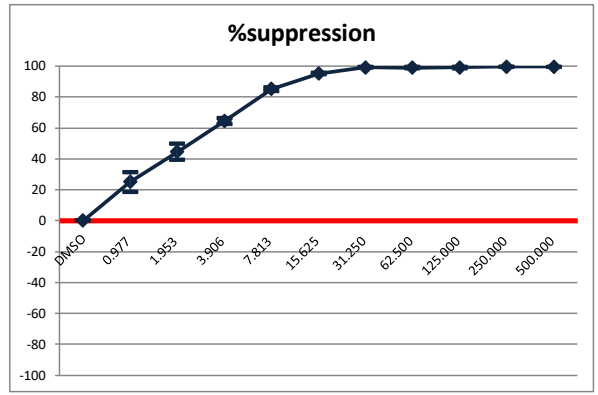
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	30.705	12.682	48.727
1.953	36.219	27.325	45.112
3.906	68.072	63.655	72.490
7.813	86.553	84.667	88.439
15.625	95.166	94.293	96.040
31.250	99.061	98.456	99.666
62.500	99.571	99.009	100.133
125.000	99.642	99.233	100.051
250.000	99.231	98.724	99.738
500.000	99.554	98.397	100.712

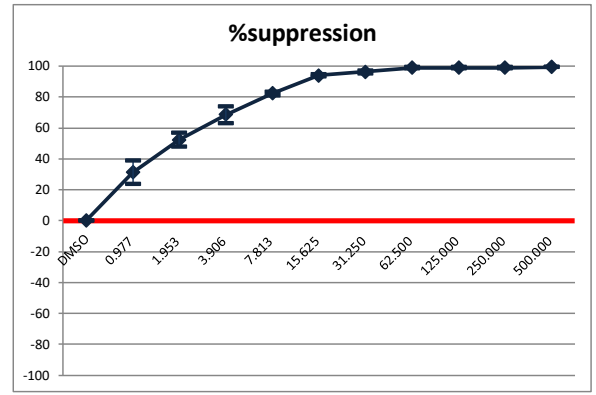
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	25.078	18.616	31.540
1.953	44.566	39.294	49.837
3.906	64.561	62.828	66.294
7.813	85.167	84.079	86.255
15.625	95.188	94.757	95.618
31.250	99.254	99.091	99.418
62.500	98.989	98.584	99.393
125.000	99.268	98.811	99.724
250.000	99.635	99.449	99.821
500.000	99.765	99.526	100.003

3rd Exp.



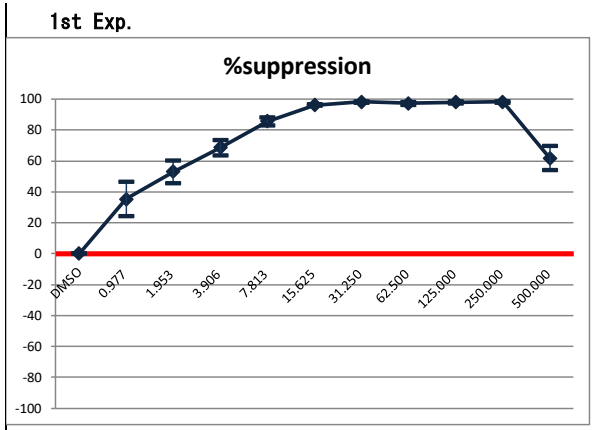
3rd Exp.

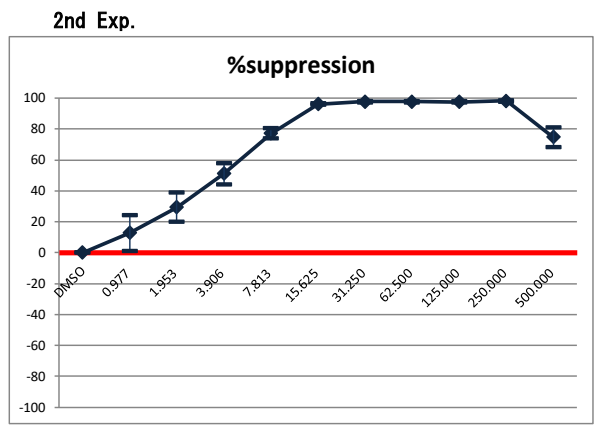
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	31.264	23.643	38.885
1.953	52.400	47.979	56.820
3.906	68.655	63.164	74.145
7.813	82.351	81.077	83.624
15.625	93.935	93.239	94.631
31.250	96.289	95.236	97.342
62.500	99.113	98.628	99.599
125.000	99.102	98.794	99.409
250.000	99.059	98.470	99.648
500.000	99.536	99.381	99.690

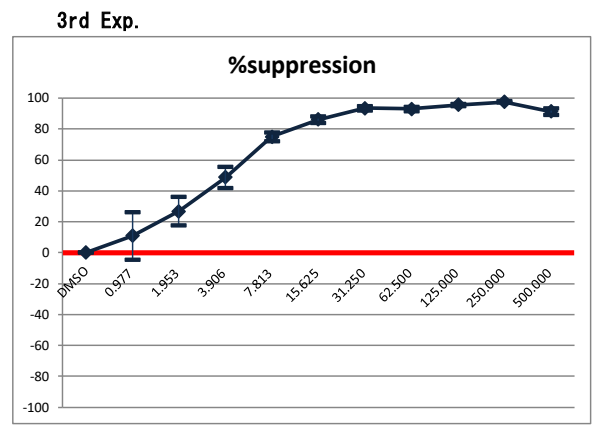
Lab C chem.1

round1

S:SSS







1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	35.459	24.559	46.358
1.953	52.990	45.644	60.336
3.906	68.746	63.730	73.761
7.813	85.762	83.093	88.430
15.625	96.243	95.636	96.850
31.250	98.219	97.854	98.584
62.500	97.277	96.418	98.137
125.000	98.008	97.367	98.650
250.000	98.263	97.716	98.810
500.000	61.883	54.014	69.752

2nd Exp.

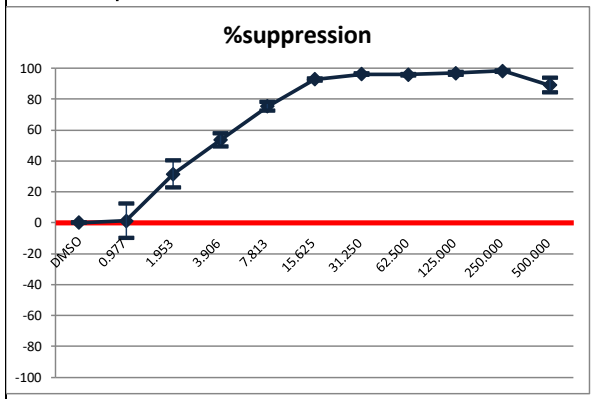
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	12.839	1.367	24.311
1.953	29.519	20.192	38.846
3.906	51.221	44.294	58.148
7.813	77.253	74.060	80.447
15.625	96.175	95.585	96.766
31.250	97.840	97.391	98.289
62.500	97.734	97.313	98.154
125.000	97.608	97.200	98.016
250.000	98.320	97.759	98.880
500.000	74.783	68.587	80.979

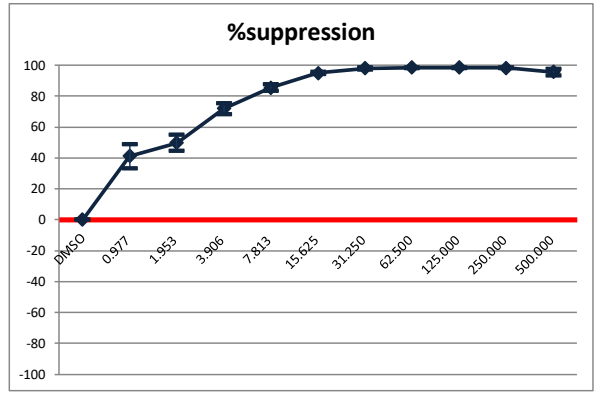
3rd Exp.

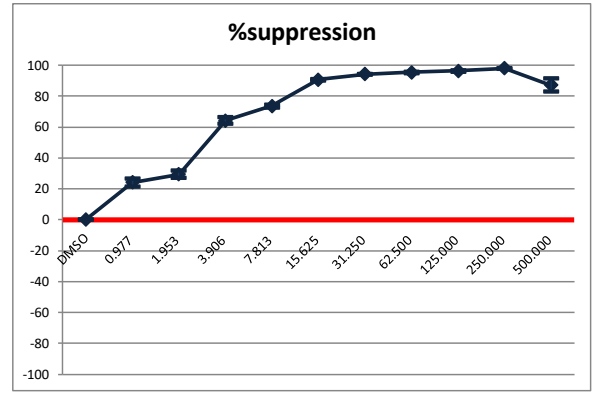
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	10.946	-4.361	26.252
1.953	26.938	17.930	35.945
3.906	48.729	41.726	55.732
7.813	74.994	72.100	77.887
15.625	86.159	84.160	88.159
31.250	93.466	92.250	94.681
62.500	93.158	91.788	94.528
125.000	95.693	95.044	96.343
250.000	97.598	97.219	97.977
500.000	91.328	89.147	93.508

round2

S:SSS







1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	1.383	-9.868	12.633
1.953	31.561	22.817	40.305
3.906	53.616	49.239	57.993
7.813	75.394	72.667	78.121
15.625	92.787	92.108	93.466
31.250	96.230	95.704	96.755
62.500	95.876	95.271	96.480
125.000	96.789	95.758	97.821
250.000	98.384	97.933	98.836
500.000	89.163	84.375	93.951

2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	41.152	33.520	48.783
1.953	49.685	44.486	54.883
3.906	71.934	68.545	75.322
7.813	85.621	83.616	87.625
15.625	95.056	94.507	95.605
31.250	97.966	97.470	98.462
62.500	98.528	98.208	98.848
125.000	98.467	98.108	98.827
250.000	98.349	97.953	98.744
500.000	95.579	93.590	97.567

3rd Exp.

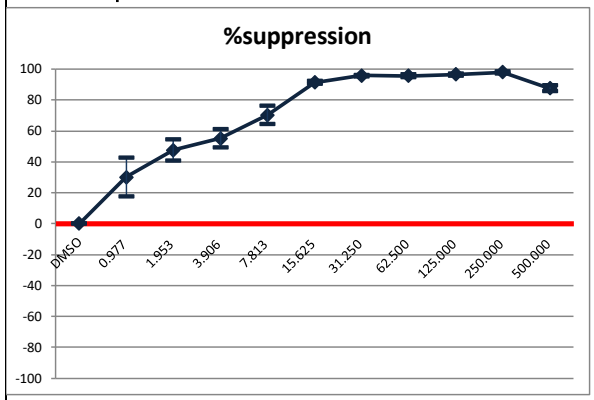
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	24.148	21.378	26.917
1.953	29.517	26.974	32.060
3.906	64.125	61.983	66.266
7.813	73.671	72.683	74.660
15.625	90.786	90.287	91.285
31.250	94.231	93.839	94.623
62.500	95.514	95.053	95.975
125.000	96.479	96.018	96.941
250.000	98.041	97.920	98.163
500.000	87.222	83.001	91.443

Lab C chem.1

round3

S:SSS

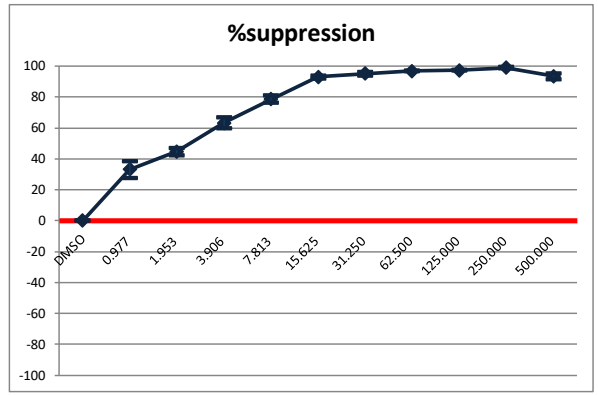
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	30.256	17.494	43.018
1.953	47.757	40.849	54.665
3.906	55.176	49.212	61.140
7.813	70.489	64.412	76.567
15.625	91.473	90.678	92.267
31.250	95.825	95.300	96.350
62.500	95.691	94.763	96.619
125.000	96.593	95.985	97.200
250.000	97.972	97.357	98.587
500.000	87.753	85.885	89.621

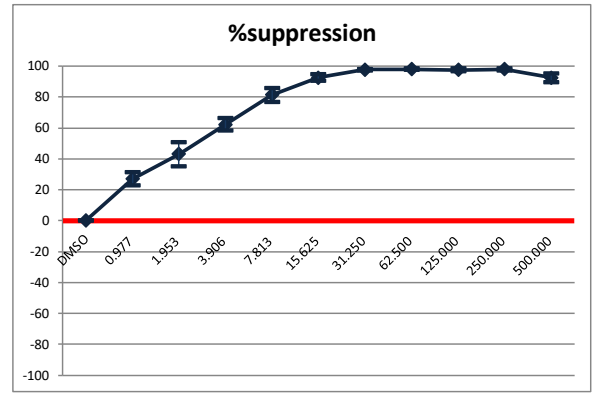
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	33.115	27.656	38.573
1.953	44.712	42.238	47.186
3.906	63.277	59.767	66.786
7.813	78.697	76.489	80.904
15.625	93.038	91.946	94.131
31.250	95.181	94.085	96.276
62.500	96.899	96.700	97.097
125.000	97.371	97.220	97.522
250.000	98.974	98.543	99.405
500.000	93.519	91.781	95.257

3rd Exp.



3rd Exp.

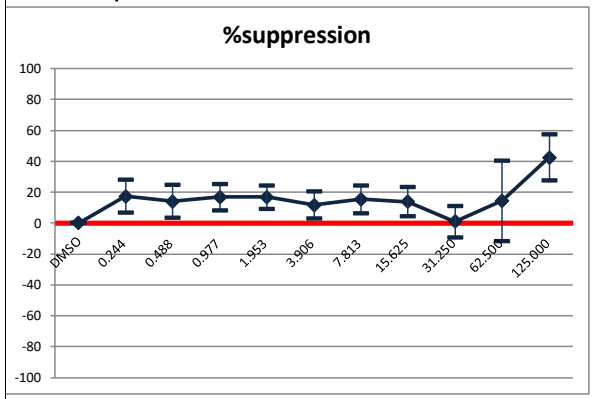
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	27.145	22.948	31.342
1.953	43.115	35.165	51.065
3.906	62.424	58.566	66.282
7.813	81.495	76.958	86.032
15.625	92.589	90.528	94.650
31.250	97.818	97.301	98.335
62.500	98.058	97.511	98.604
125.000	97.615	96.673	98.557
250.000	98.020	97.458	98.582
500.000	92.518	89.508	95.527

Lab A chem.2

round1

S:SAS

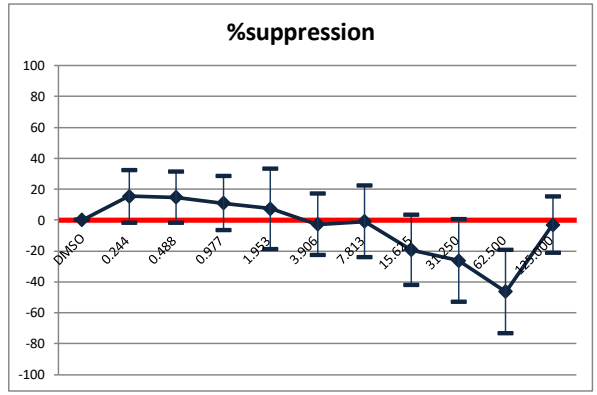
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	17.394	6.591	28.198
0.488	14.120	3.422	24.819
0.977	16.792	8.347	25.237
1.953	16.822	9.346	24.298
3.906	11.775	2.989	20.561
7.813	15.395	6.257	24.532
15.625	13.749	4.326	23.172
31.250	0.951	-9.193	11.095
62.500	14.456	-11.680	40.592
125.000	42.523	27.758	57.288

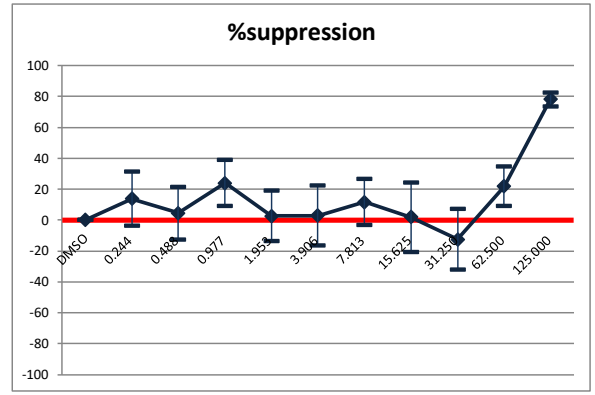
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	15.440	-1.687	32.567
0.488	14.757	-1.699	31.213
0.977	10.885	-6.596	28.365
1.953	7.317	-18.538	33.173
3.906	-2.744	-22.521	17.032
7.813	-0.823	-24.008	22.361
15.625	-19.428	-42.142	3.287
31.250	-26.222	-53.049	0.605
62.500	-46.300	-73.250	-19.350
125.000	-2.961	-21.195	15.273

3rd Exp.



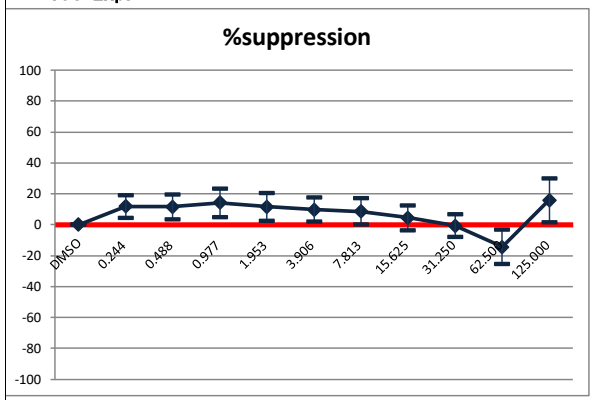
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	13.926	-3.382	31.234
0.488	4.492	-12.692	21.676
0.977	24.074	9.331	38.817
1.953	2.730	-13.445	18.904
3.906	2.951	-16.531	22.433
7.813	11.641	-3.190	26.472
15.625	2.000	-20.519	24.519
31.250	-12.355	-31.908	7.197
62.500	21.958	9.270	34.646
125.000	78.172	73.730	82.615

round2

N:NNN

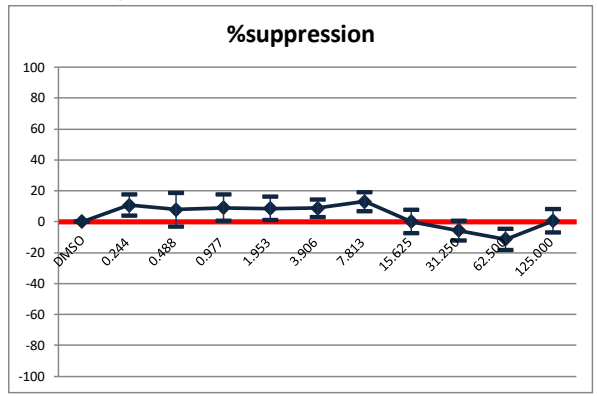
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	11.897	4.635	19.160
0.488	11.658	3.701	19.615
0.977	14.170	4.968	23.372
1.953	11.744	2.742	20.745
3.906	9.891	2.038	17.744
7.813	8.505	-0.016	17.026
15.625	4.644	-3.446	12.733
31.250	-0.511	-7.626	6.604
62.500	-14.272	-25.195	-3.348
125.000	15.852	1.536	30.169

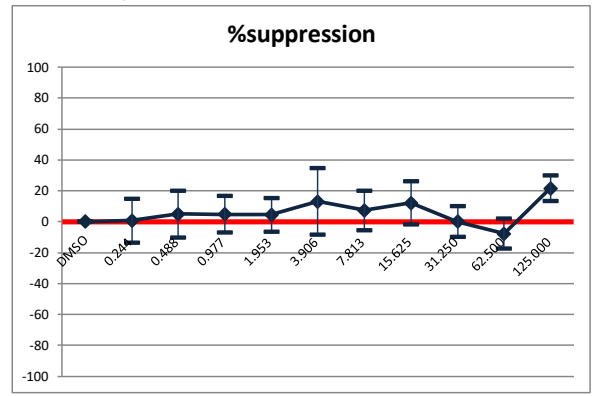
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	10.808	3.992	17.624
0.488	7.854	-3.015	18.723
0.977	9.070	0.560	17.580
1.953	8.597	1.105	16.090
3.906	8.785	3.077	14.494
7.813	13.105	7.037	19.174
15.625	0.175	-7.535	7.686
31.250	-5.494	-11.896	0.907
62.500	-11.341	-18.333	-4.349
125.000	0.654	-7.098	8.406

3rd Exp.



3rd Exp.

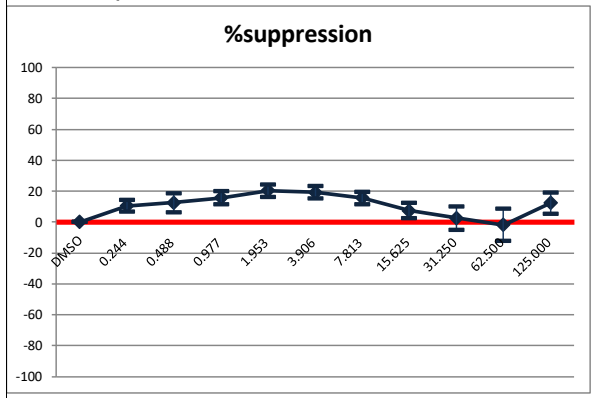
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	0.696	-13.611	15.003
0.488	4.990	-10.213	20.192
0.977	4.838	-6.903	16.579
1.953	4.491	-6.473	15.455
3.906	13.094	-8.480	34.669
7.813	7.356	-5.573	20.284
15.625	12.184	-1.907	26.275
31.250	0.119	-9.932	10.171
62.500	-7.635	-17.324	2.054
125.000	21.663	13.271	30.055

Lab A chem.2

round3

N:NNN

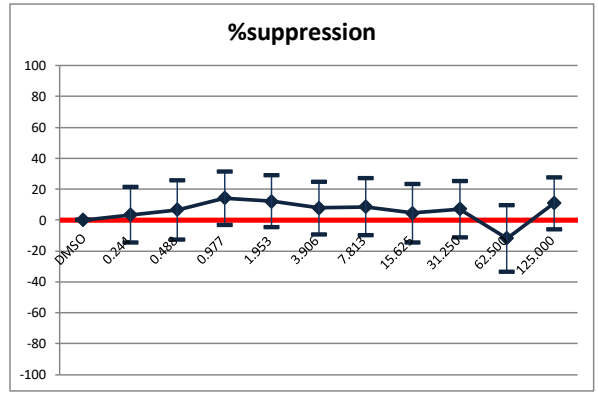
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	10.582	6.634	14.529
0.488	12.612	6.431	18.793
0.977	15.689	11.441	19.936
1.953	20.382	16.352	24.413
3.906	19.298	15.413	23.184
7.813	15.700	11.593	19.808
15.625	7.445	2.520	12.371
31.250	2.584	-5.123	10.290
62.500	-1.786	-12.248	8.676
125.000	12.301	5.598	19.004

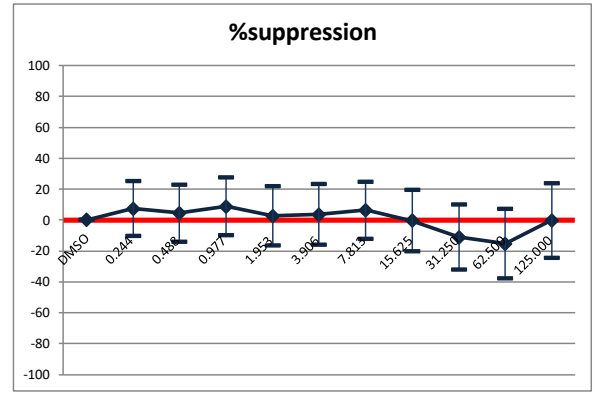
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	3.429	-14.480	21.338
0.488	6.660	-12.541	25.860
0.977	14.270	-3.064	31.604
1.953	12.204	-4.610	29.019
3.906	7.806	-9.306	24.917
7.813	8.651	-9.707	27.009
15.625	4.624	-14.279	23.526
31.250	7.111	-11.050	25.272
62.500	-11.800	-33.441	9.842
125.000	11.043	-5.780	27.866

3rd Exp.



3rd Exp.

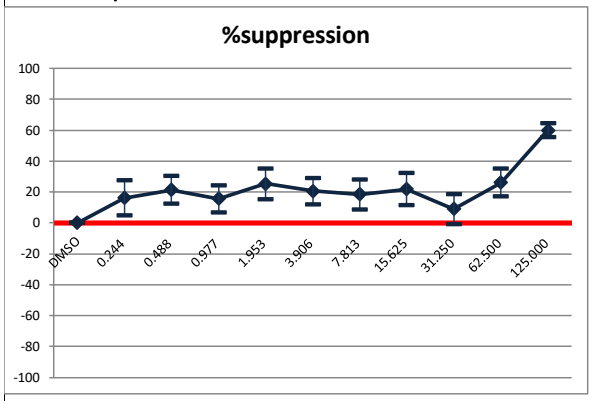
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	7.394	-10.436	25.223
0.488	4.485	-13.804	22.774
0.977	8.824	-9.963	27.611
1.953	2.781	-16.345	21.907
3.906	3.663	-15.856	23.182
7.813	6.391	-12.169	24.951
15.625	-0.288	-20.212	19.635
31.250	-11.091	-32.209	10.027
62.500	-15.200	-37.732	7.332
125.000	-0.121	-24.226	23.984

Lab B chem.2

round1

S:SSS

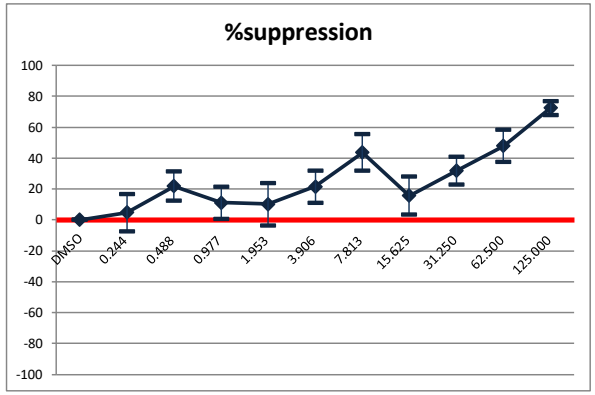
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	16.237	4.953	27.521
0.488	21.482	12.555	30.410
0.977	15.626	6.867	24.385
1.953	25.390	15.433	35.347
3.906	20.576	12.213	28.940
7.813	18.528	8.938	28.118
15.625	21.891	11.523	32.260
31.250	9.052	-0.551	18.654
62.500	26.232	17.425	35.038
125.000	59.954	55.456	64.453

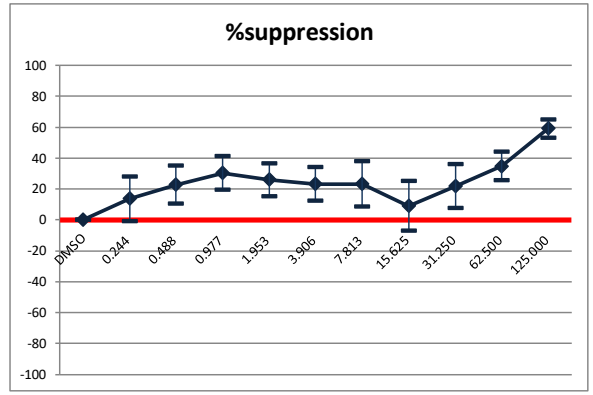
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	4.829	-7.239	16.898
0.488	21.912	12.546	31.279
0.977	11.111	0.534	21.687
1.953	10.184	-3.477	23.846
3.906	21.520	11.067	31.973
7.813	43.744	31.740	55.749
15.625	15.751	3.483	28.020
31.250	31.872	22.823	40.922
62.500	47.945	37.428	58.462
125.000	72.385	67.825	76.946

3rd Exp.



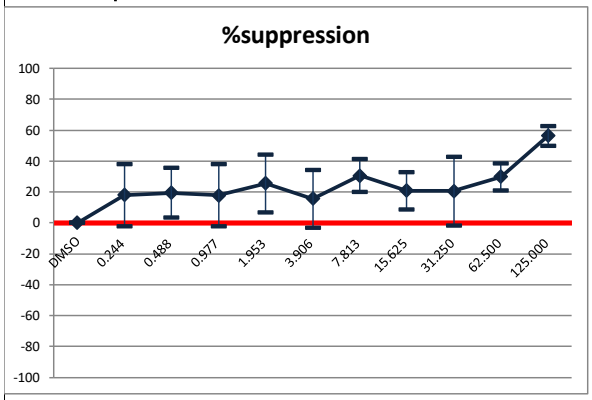
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	13.836	-0.592	28.263
0.488	22.856	10.702	35.009
0.977	30.398	19.519	41.277
1.953	26.050	15.448	36.653
3.906	23.344	12.427	34.260
7.813	23.354	8.627	38.082
15.625	9.115	-6.908	25.137
31.250	21.925	7.822	36.028
62.500	34.895	25.609	44.181
125.000	59.203	53.157	65.249

round2

S:SSS

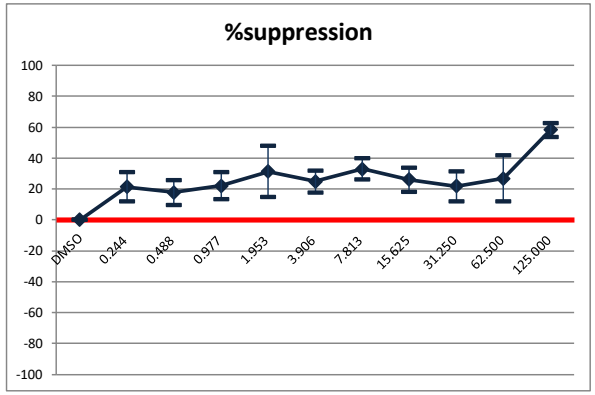
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	17.976	-2.104	38.055
0.488	19.569	3.452	35.686
0.977	17.893	-2.324	38.110
1.953	25.594	6.776	44.411
3.906	15.602	-3.038	34.242
7.813	30.628	19.998	41.258
15.625	20.843	8.712	32.973
31.250	20.704	-1.485	42.892
62.500	29.984	21.207	38.761
125.000	56.303	50.050	62.557

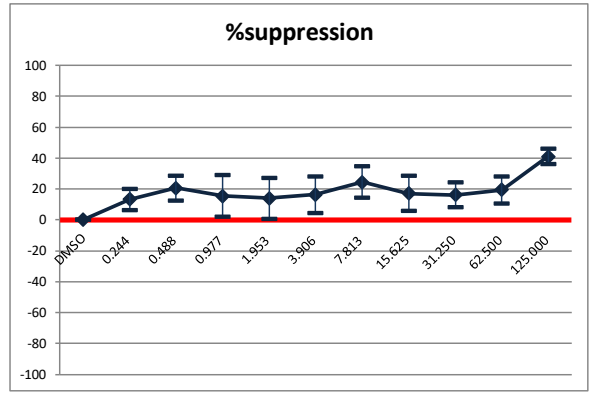
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	21.472	12.122	30.822
0.488	17.807	9.897	25.717
0.977	22.055	13.282	30.827
1.953	31.246	14.680	47.812
3.906	24.851	17.753	31.950
7.813	32.994	26.184	39.803
15.625	26.036	18.032	34.039
31.250	21.760	12.132	31.387
62.500	26.788	11.880	41.695
125.000	58.215	53.754	62.676

3rd Exp.



3rd Exp.

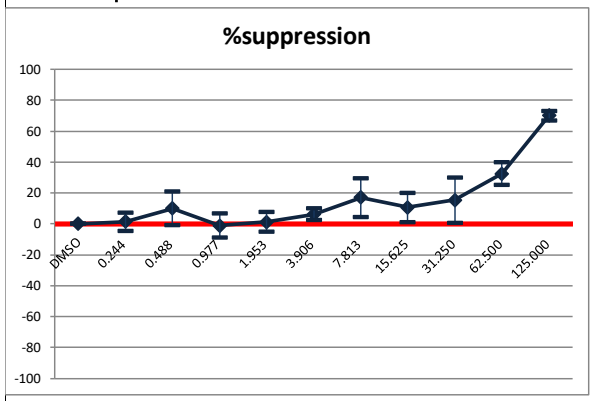
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	13.251	6.429	20.074
0.488	20.591	12.650	28.533
0.977	15.438	1.916	28.960
1.953	14.047	0.824	27.270
3.906	16.464	4.631	28.296
7.813	24.430	14.258	34.601
15.625	17.197	5.696	28.699
31.250	16.223	8.174	24.272
62.500	19.388	10.488	28.287
125.000	41.138	36.331	45.945

Lab B chem.2

round3

S:SSN

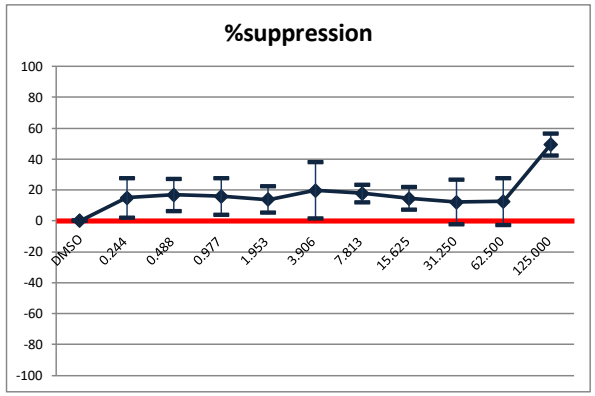
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	1.459	-4.458	7.377
0.488	10.008	-0.795	20.811
0.977	-1.013	-8.684	6.659
1.953	1.361	-5.024	7.746
3.906	6.321	2.726	9.916
7.813	17.043	4.314	29.772
15.625	10.669	1.117	20.220
31.250	15.367	0.797	29.938
62.500	32.522	25.147	39.897
125.000	70.091	66.892	73.291

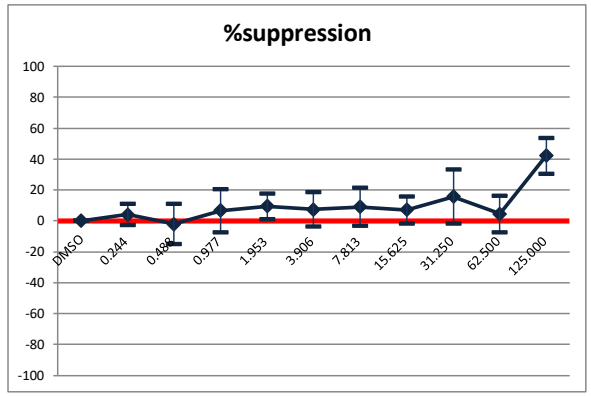
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	14.966	2.286	27.645
0.488	16.856	6.310	27.401
0.977	15.858	4.205	27.511
1.953	13.877	5.512	22.242
3.906	19.822	1.761	37.882
7.813	17.740	12.016	23.464
15.625	14.628	7.171	22.086
31.250	12.256	-1.999	26.511
62.500	12.538	-2.589	27.665
125.000	49.404	42.403	56.404

3rd Exp.



3rd Exp.

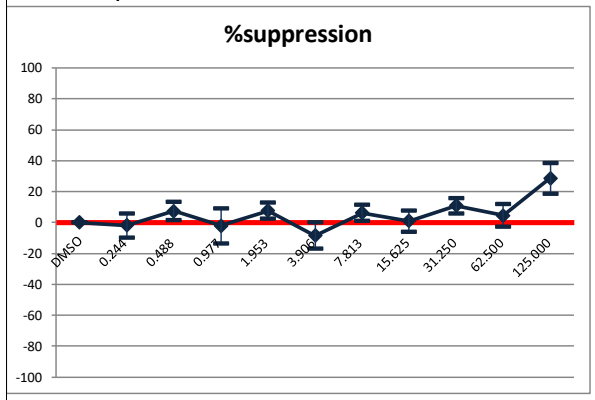
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	4.034	-2.861	10.929
0.488	-2.085	-15.035	10.865
0.977	6.602	-7.447	20.651
1.953	9.432	1.011	17.854
3.906	7.376	-3.759	18.511
7.813	9.126	-3.022	21.274
15.625	7.174	-1.492	15.840
31.250	15.797	-1.514	33.108
62.500	4.604	-7.251	16.460
125.000	42.251	30.674	53.828

Lab C chem.2

round1

S:NSS

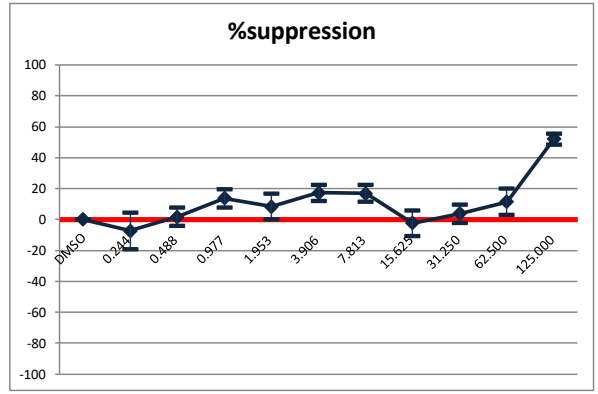
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	-1.900	-9.868	6.068
0.488	7.386	1.489	13.282
0.977	-2.102	-13.539	9.335
1.953	7.739	2.549	12.930
3.906	-8.330	-16.967	0.307
7.813	6.260	0.963	11.557
15.625	0.931	-6.020	7.883
31.250	10.916	5.847	15.985
62.500	4.552	-2.761	11.866
125.000	28.710	18.819	38.600

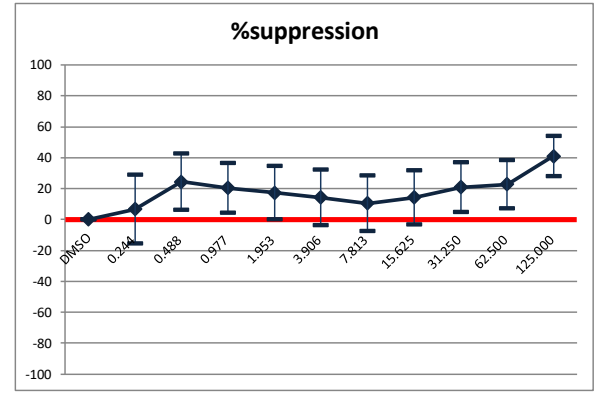
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	-7.331	-19.106	4.443
0.488	1.722	-4.250	7.693
0.977	13.712	7.715	19.708
1.953	8.440	0.288	16.592
3.906	17.277	11.908	22.646
7.813	16.947	11.475	22.419
15.625	-2.197	-10.464	6.071
31.250	3.842	-1.933	9.618
62.500	11.434	2.884	19.984
125.000	52.105	48.399	55.811

3rd Exp.



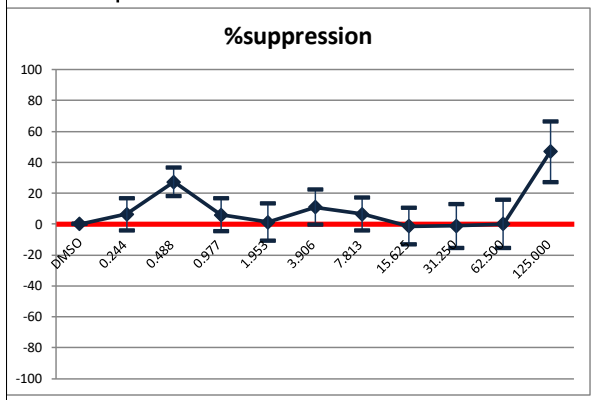
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	6.813	-15.302	28.928
0.488	24.481	6.203	42.760
0.977	20.506	4.415	36.598
1.953	17.299	0.071	34.528
3.906	14.275	-3.635	32.184
7.813	10.424	-7.604	28.451
15.625	14.280	-3.276	31.836
31.250	20.884	4.843	36.925
62.500	22.832	7.184	38.480
125.000	41.002	27.954	54.050

round2

S:SSN

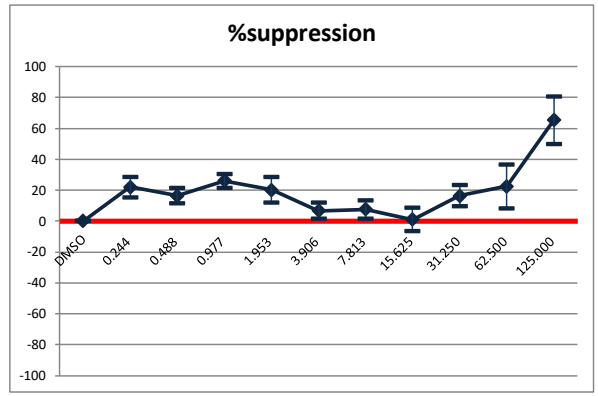
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	6.525	-3.942	16.991
0.488	27.412	17.955	36.868
0.977	6.008	-4.540	16.556
1.953	1.334	-10.571	13.240
3.906	11.047	-0.469	22.562
7.813	6.560	-4.230	17.349
15.625	-1.258	-12.947	10.431
31.250	-1.067	-15.230	13.096
62.500	0.176	-15.450	15.801
125.000	46.904	27.229	66.579

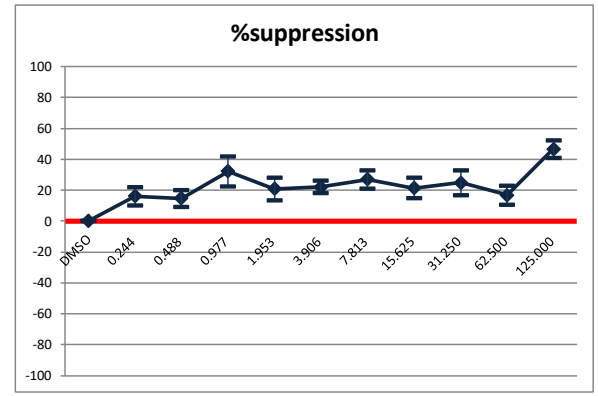
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	22.104	15.460	28.749
0.488	16.452	11.625	21.278
0.977	26.066	21.648	30.484
1.953	20.256	12.025	28.486
3.906	6.655	1.503	11.807
7.813	7.726	1.837	13.616
15.625	1.073	-6.616	8.762
31.250	16.358	9.529	23.187
62.500	22.546	8.336	36.756
125.000	65.306	50.123	80.489

3rd Exp.



3rd Exp.

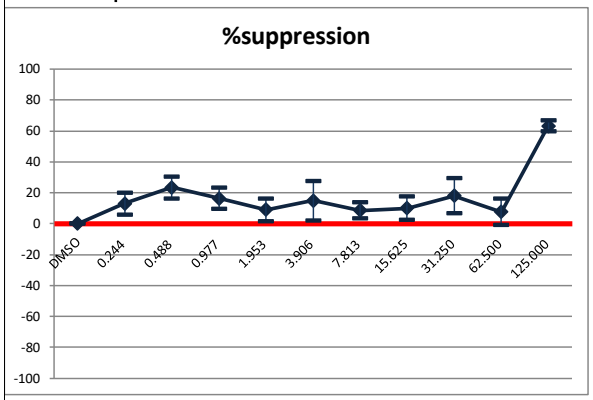
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	16.158	10.304	22.011
0.488	14.672	9.358	19.986
0.977	32.221	22.377	42.065
1.953	20.795	13.546	28.045
3.906	22.116	18.195	26.037
7.813	26.975	21.083	32.867
15.625	21.459	14.904	28.013
31.250	24.954	16.977	32.931
62.500	16.936	10.764	23.107
125.000	46.762	41.022	52.503

Lab C chem.2

round3

N:NNN

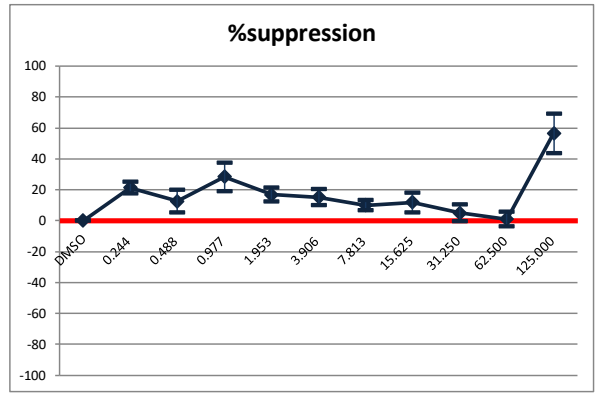
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	13.011	5.885	20.138
0.488	23.394	16.230	30.557
0.977	16.484	9.610	23.359
1.953	9.054	1.745	16.364
3.906	15.047	2.313	27.780
7.813	8.667	3.493	13.841
15.625	10.116	2.704	17.529
31.250	18.089	6.828	29.349
62.500	7.695	-0.762	16.151
125.000	63.220	59.698	66.741

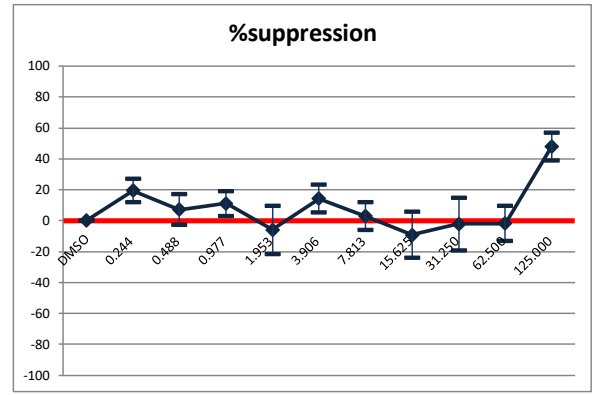
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	21.442	17.522	25.361
0.488	12.674	5.454	19.894
0.977	28.512	19.359	37.666
1.953	16.965	12.384	21.545
3.906	15.279	9.974	20.583
7.813	10.049	6.867	13.231
15.625	11.982	5.638	18.325
31.250	5.083	-0.230	10.395
62.500	1.114	-3.518	5.746
125.000	56.529	43.733	69.324

3rd Exp.



3rd Exp.

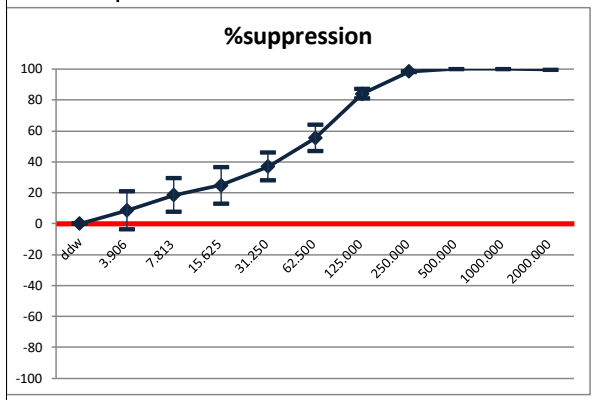
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	19.417	11.846	26.989
0.488	7.154	-2.753	17.061
0.977	11.187	3.181	19.192
1.953	-5.878	-21.442	9.685
3.906	14.368	5.485	23.252
7.813	3.001	-6.156	12.158
15.625	-9.245	-24.171	5.681
31.250	-2.094	-19.181	14.993
62.500	-1.818	-13.159	9.523
125.000	47.891	38.904	56.878

Lab A chem.3

round1

S:SSS

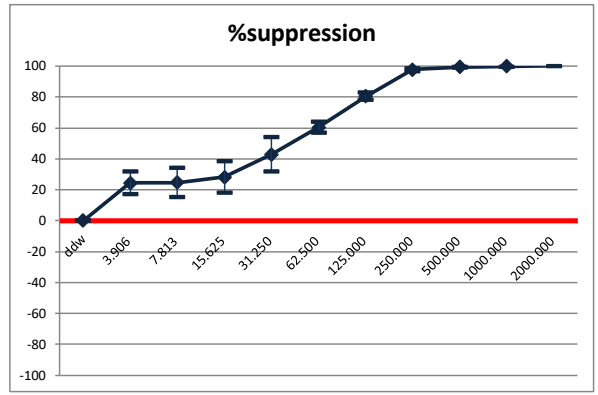
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	8.668	-3.714	21.050
7.813	18.525	7.653	29.398
15.625	24.949	13.188	36.710
31.250	36.927	27.972	45.882
62.500	55.540	46.974	64.106
125.000	84.197	81.304	87.091
250.000	98.557	98.259	98.856
500.000	100.186	99.866	100.507
1000.000	100.166	99.890	100.442
2000.000	100.035	99.570	100.500

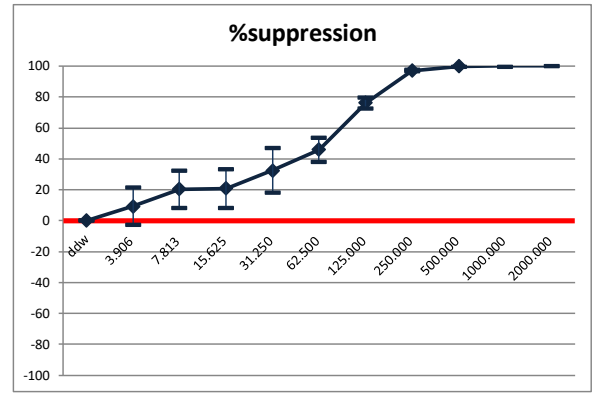
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	24.525	17.277	31.774
7.813	24.713	15.326	34.100
15.625	28.317	18.289	38.344
31.250	42.979	31.743	54.215
62.500	60.401	56.779	64.023
125.000	80.616	78.329	82.903
250.000	97.816	96.949	98.683
500.000	99.472	99.194	99.750
1000.000	99.968	99.793	100.143
2000.000	100.273	99.898	100.648

3rd Exp.



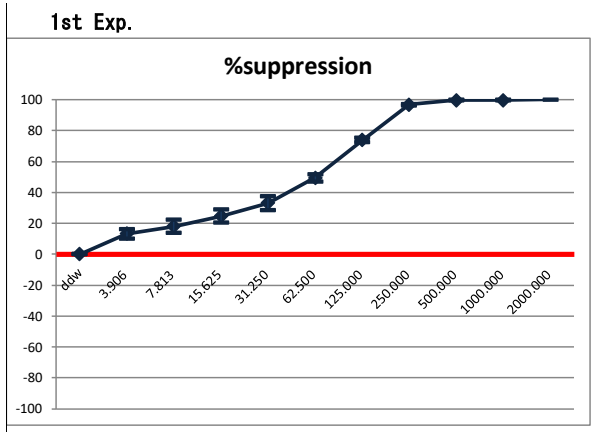
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	9.343	-2.867	21.553
7.813	20.418	8.275	32.560
15.625	20.881	8.421	33.341
31.250	32.478	18.102	46.853
62.500	45.881	38.268	53.494
125.000	76.226	72.565	79.887
250.000	97.095	96.544	97.647
500.000	99.917	99.728	100.106
1000.000	100.076	99.824	100.327
2000.000	100.151	99.893	100.409

round2

S:SSS

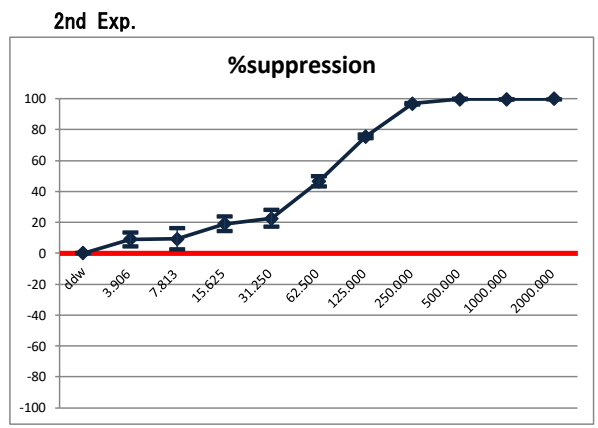
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	13.290	10.074	16.506
7.813	18.094	13.850	22.339
15.625	24.803	20.574	29.032
31.250	33.206	28.705	37.708
62.500	49.441	47.284	51.598
125.000	74.007	72.484	75.530
250.000	96.767	96.143	97.391
500.000	99.791	99.708	99.873
1000.000	99.829	99.779	99.878
2000.000	100.093	99.926	100.259

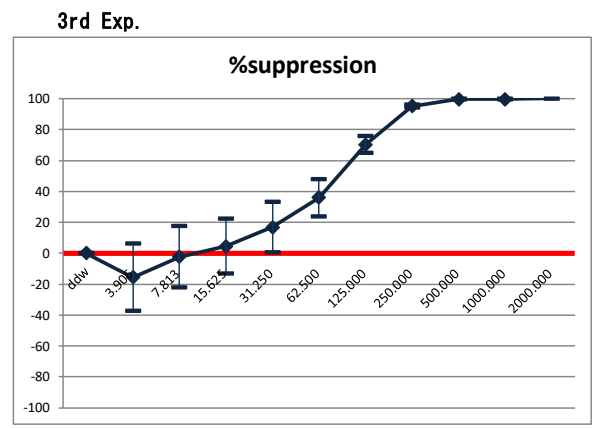
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	9.024	4.465	13.584
7.813	9.291	2.360	16.222
15.625	19.099	14.380	23.819
31.250	22.665	17.063	28.268
62.500	46.446	43.066	49.825
125.000	75.671	74.468	76.874
250.000	96.781	96.470	97.092
500.000	99.765	99.605	99.924
1000.000	99.618	99.540	99.696
2000.000	99.981	99.766	100.196

3rd Exp.



3rd Exp.

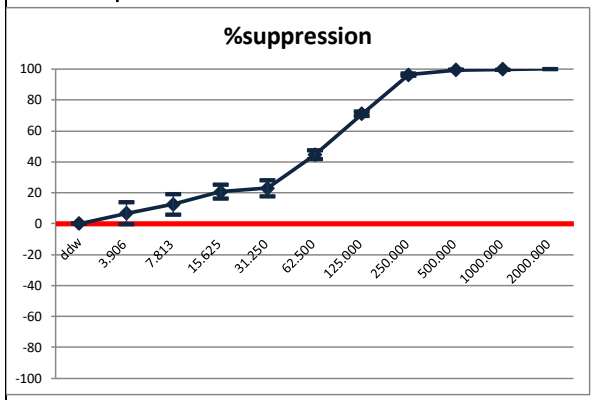
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-15.476	-37.379	6.427
7.813	-2.358	-22.276	17.559
15.625	4.534	-13.248	22.316
31.250	16.883	0.647	33.119
62.500	36.091	24.045	48.137
125.000	70.366	64.850	75.881
250.000	95.256	94.285	96.227
500.000	99.757	99.564	99.950
1000.000	99.717	99.462	99.971
2000.000	100.141	99.970	100.313

Lab A chem.3

round3

S:SSS

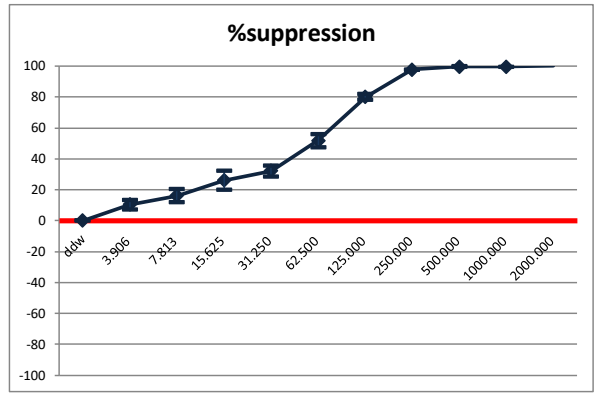
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	6.812	-0.423	14.047
7.813	12.582	5.897	19.266
15.625	20.727	16.119	25.334
31.250	22.956	17.689	28.223
62.500	44.654	41.647	47.661
125.000	71.167	69.566	72.767
250.000	96.374	95.581	97.168
500.000	99.442	99.393	99.491
1000.000	99.877	99.660	100.094
2000.000	100.095	99.929	100.261

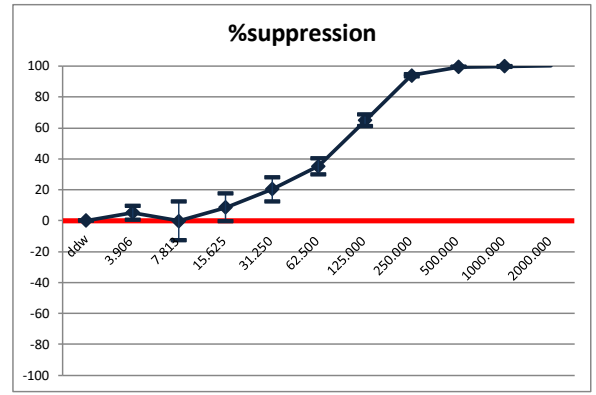
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	10.471	7.503	13.439
7.813	16.205	11.834	20.577
15.625	26.180	20.073	32.287
31.250	32.274	28.825	35.723
62.500	51.929	47.678	56.179
125.000	80.165	78.094	82.236
250.000	97.712	97.536	97.888
500.000	99.704	99.416	99.993
1000.000	99.779	99.512	100.045
2000.000	100.165	100.004	100.327

3rd Exp.



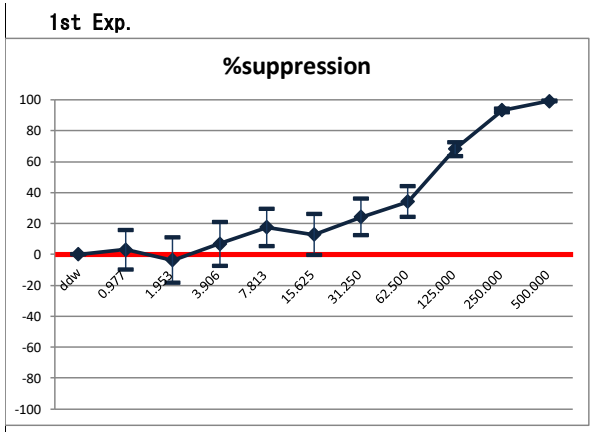
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	5.179	0.609	9.749
7.813	-0.054	-12.677	12.568
15.625	8.624	-0.472	17.720
31.250	20.335	12.489	28.182
62.500	35.359	30.153	40.566
125.000	65.010	61.198	68.823
250.000	94.035	93.385	94.685
500.000	99.555	99.386	99.724
1000.000	99.878	99.805	99.951
2000.000	100.214	100.046	100.382

Lab B chem.3

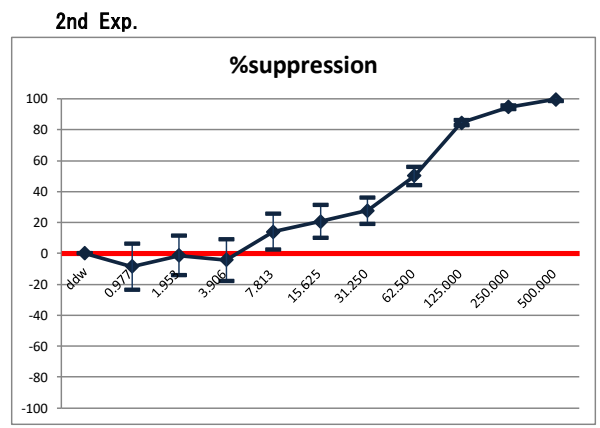
round1

S:SSS



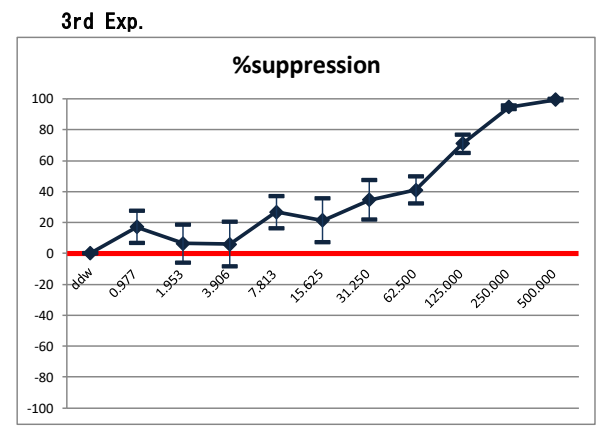
1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.977	3.110	-9.671	15.891
1.953	-3.737	-18.355	10.881
3.906	6.962	-7.234	21.158
7.813	17.487	5.617	29.358
15.625	12.953	-0.186	26.092
31.250	24.254	12.308	36.200
62.500	34.127	24.157	44.097
125.000	68.190	63.804	72.577
250.000	93.244	92.192	94.295
500.000	99.350	99.058	99.642



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.977	-8.522	-23.445	6.401
1.953	-1.266	-14.155	11.624
3.906	-4.219	-17.788	9.350
7.813	14.039	2.404	25.674
15.625	20.786	10.081	31.491
31.250	27.674	18.939	36.410
62.500	50.251	44.313	56.188
125.000	84.599	83.032	86.165
250.000	94.676	93.420	95.932
500.000	99.810	98.526	101.095

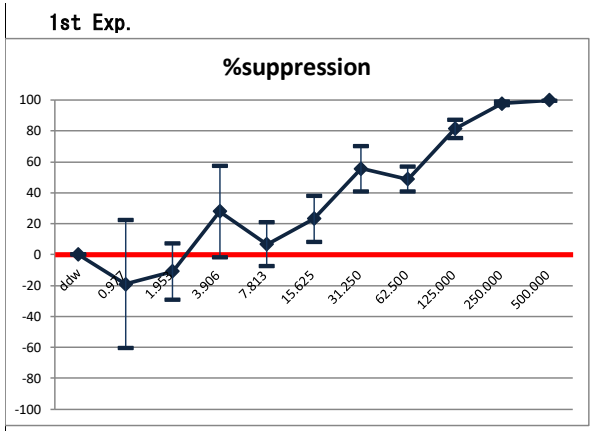


3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.977	17.177	6.688	27.666
1.953	6.525	-5.725	18.774
3.906	6.043	-8.306	20.392
7.813	26.746	16.265	37.227
15.625	21.431	7.074	35.787
31.250	34.673	21.977	47.369
62.500	41.132	32.355	49.909
125.000	71.054	65.135	76.974
250.000	94.671	93.456	95.886
500.000	99.421	98.933	99.908

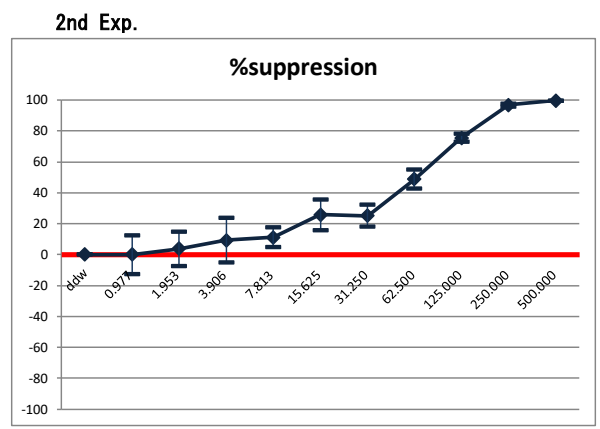
round2

S:SSS



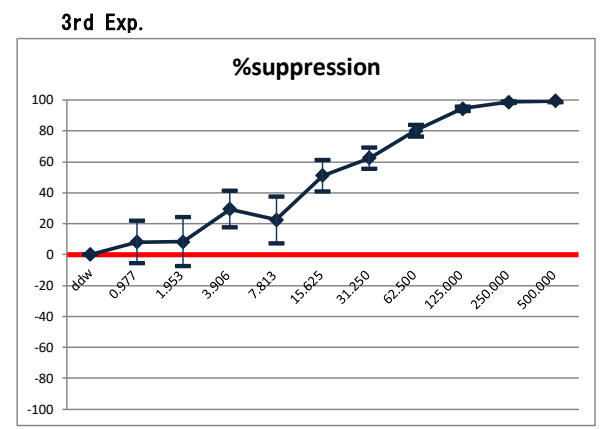
1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.977	-18.993	-60.319	22.333
1.953	-10.837	-28.970	7.297
3.906	28.042	-1.540	57.624
7.813	6.794	-7.450	21.037
15.625	23.266	8.453	38.080
31.250	55.733	41.095	70.370
62.500	48.903	40.787	57.019
125.000	81.540	75.620	87.459
250.000	97.758	96.577	98.938
500.000	99.952	99.766	100.138



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.977	0.024	-12.477	12.526
1.953	3.881	-7.183	14.945
3.906	9.264	-5.206	23.733
7.813	11.306	4.779	17.834
15.625	25.808	16.038	35.579
31.250	25.237	18.285	32.189
62.500	48.752	42.607	54.896
125.000	75.680	73.013	78.347
250.000	96.809	96.048	97.570
500.000	99.727	99.683	99.771



3rd Exp.

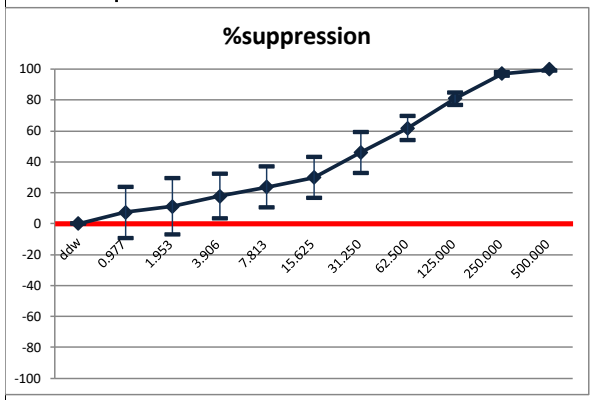
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.977	8.229	-5.585	22.043
1.953	8.480	-7.506	24.467
3.906	29.507	17.528	41.486
7.813	22.680	7.531	37.828
15.625	51.295	41.104	61.485
31.250	62.529	55.577	69.481
62.500	80.223	76.341	84.104
125.000	94.398	92.999	95.798
250.000	98.827	98.368	99.285
500.000	99.445	98.878	100.012

Lab B chem.3

round3

S:SSS

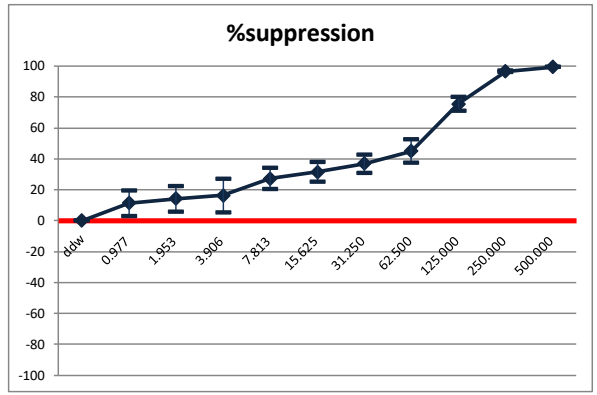
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.977	7.332	-9.383	24.046
1.953	11.180	-7.034	29.394
3.906	17.923	3.452	32.394
7.813	23.703	10.384	37.022
15.625	29.977	16.793	43.162
31.250	46.205	33.028	59.382
62.500	61.806	53.963	69.648
125.000	80.994	76.887	85.101
250.000	97.002	95.917	98.087
500.000	99.888	99.167	100.610

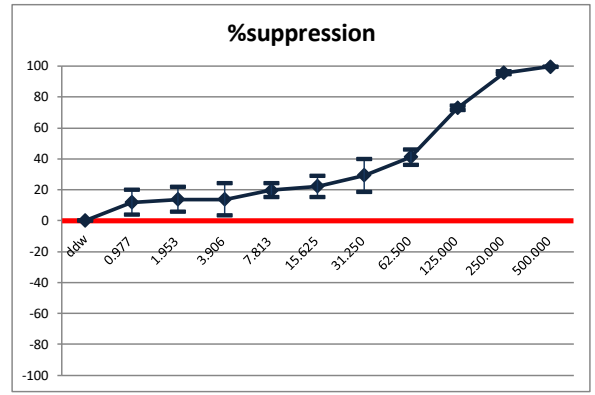
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.977	11.438	3.101	19.774
1.953	14.199	5.740	22.659
3.906	16.373	5.466	27.280
7.813	27.338	20.470	34.207
15.625	31.656	25.319	37.993
31.250	37.080	31.148	43.013
62.500	45.112	37.556	52.667
125.000	75.587	71.179	79.995
250.000	96.730	96.094	97.365
500.000	99.564	99.432	99.695

3rd Exp.



3rd Exp.

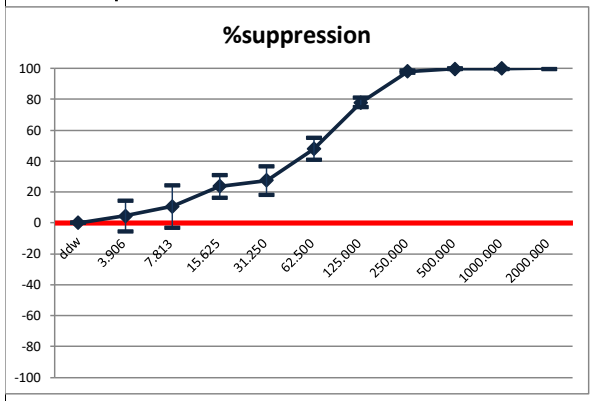
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.977	11.910	3.760	20.061
1.953	13.919	5.718	22.119
3.906	13.899	3.342	24.456
7.813	19.828	15.235	24.420
15.625	22.359	15.556	29.162
31.250	29.420	18.767	40.074
62.500	41.166	36.039	46.292
125.000	73.030	71.512	74.549
250.000	95.771	94.988	96.554
500.000	99.666	99.578	99.754

Lab C chem.3

round1

S:SSS

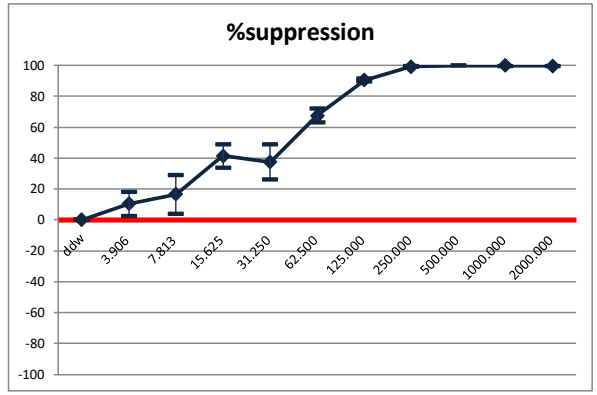
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	4.511	-5.545	14.566
7.813	10.640	-2.971	24.251
15.625	23.726	16.417	31.034
31.250	27.455	18.412	36.497
62.500	47.885	40.797	54.973
125.000	78.004	74.878	81.130
250.000	97.949	97.427	98.470
500.000	99.719	99.514	99.923
1000.000	99.958	99.779	100.137
2000.000	100.094	99.708	100.481

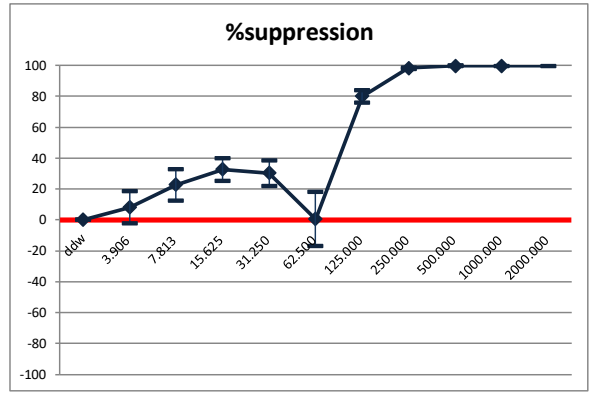
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	10.491	2.754	18.228
7.813	16.543	3.874	29.213
15.625	41.487	34.024	48.949
31.250	37.465	26.200	48.730
62.500	67.635	62.984	72.286
125.000	90.526	89.711	91.340
250.000	99.344	99.045	99.643
500.000	100.011	99.874	100.149
1000.000	99.939	99.697	100.181
2000.000	99.775	99.422	100.128

3rd Exp.



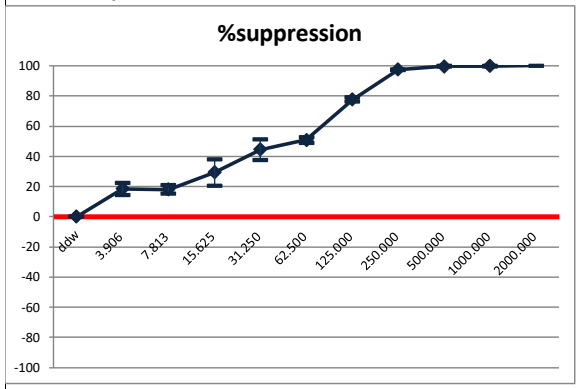
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	8.215	-2.107	18.537
7.813	22.725	12.715	32.735
15.625	32.791	25.496	40.085
31.250	30.384	22.027	38.741
62.500	0.623	-16.785	18.031
125.000	80.020	76.147	83.892
250.000	98.238	97.925	98.550
500.000	99.687	99.408	99.966
1000.000	99.764	99.508	100.021
2000.000	100.003	99.771	100.235

round2

S:SSS

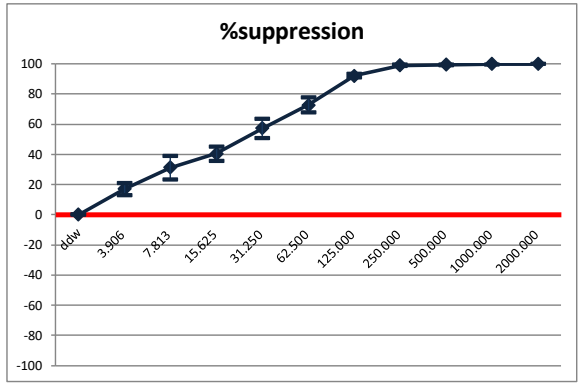
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	18.472	14.404	22.541
7.813	18.030	15.247	20.813
15.625	29.446	20.718	38.173
31.250	44.486	37.589	51.383
62.500	50.885	49.012	52.758
125.000	77.825	76.308	79.343
250.000	97.581	97.457	97.705
500.000	99.739	99.505	99.974
1000.000	99.844	99.728	99.961
2000.000	100.081	99.929	100.232

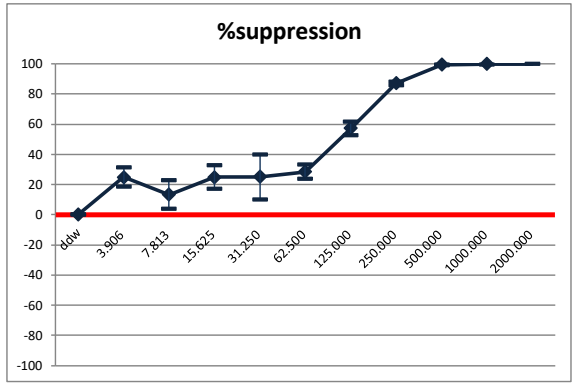
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	17.113	13.140	21.086
7.813	31.396	23.583	39.209
15.625	40.536	35.853	45.219
31.250	57.411	51.070	63.752
62.500	72.700	67.765	77.635
125.000	92.211	90.985	93.436
250.000	99.064	98.708	99.419
500.000	99.486	99.259	99.713
1000.000	99.870	99.450	100.290
2000.000	99.929	99.837	100.022

3rd Exp.



3rd Exp.

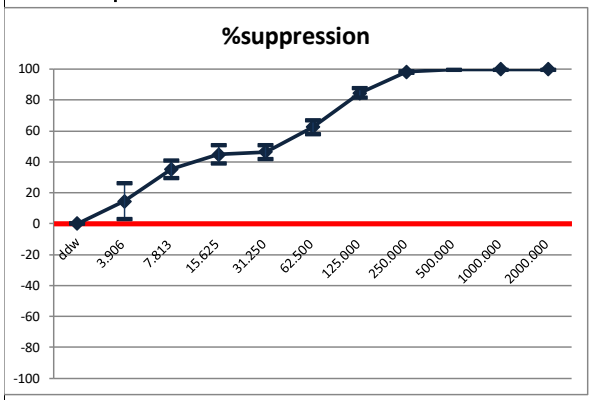
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	24.973	18.426	31.520
7.813	13.251	3.818	22.684
15.625	24.996	17.298	32.695
31.250	25.156	10.182	40.130
62.500	28.555	23.811	33.298
125.000	57.247	52.555	61.939
250.000	87.072	85.829	88.315
500.000	99.376	99.183	99.568
1000.000	99.943	99.834	100.052
2000.000	100.052	99.875	100.229

Lab C chem.3

round3

S:SSS

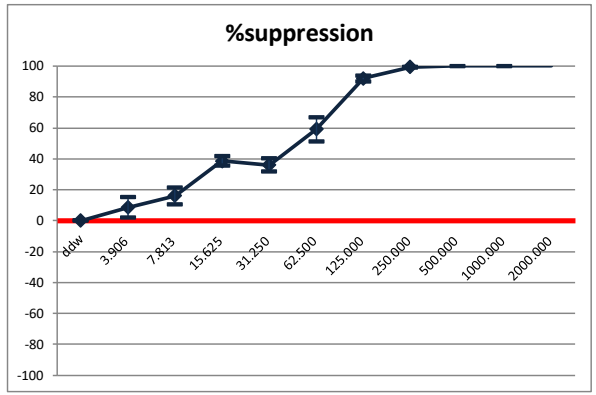
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	14.574	2.979	26.169
7.813	35.312	29.589	41.036
15.625	44.874	39.099	50.649
31.250	46.400	41.804	50.997
62.500	62.477	58.181	66.774
125.000	84.688	81.444	87.932
250.000	98.363	97.933	98.792
500.000	100.010	99.782	100.239
1000.000	99.920	99.835	100.006
2000.000	99.939	99.729	100.149

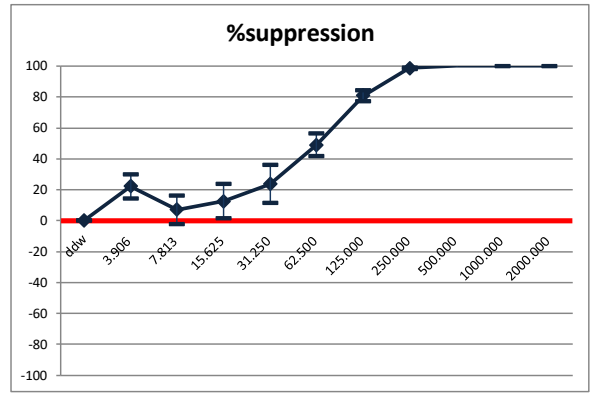
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	8.649	1.882	15.415
7.813	16.262	10.809	21.716
15.625	38.675	35.705	41.645
31.250	36.145	31.819	40.472
62.500	59.272	51.490	67.055
125.000	92.039	90.085	93.994
250.000	99.521	99.210	99.833
500.000	100.108	99.889	100.327
1000.000	100.079	99.922	100.235
2000.000	100.293	100.120	100.466

3rd Exp.



3rd Exp.

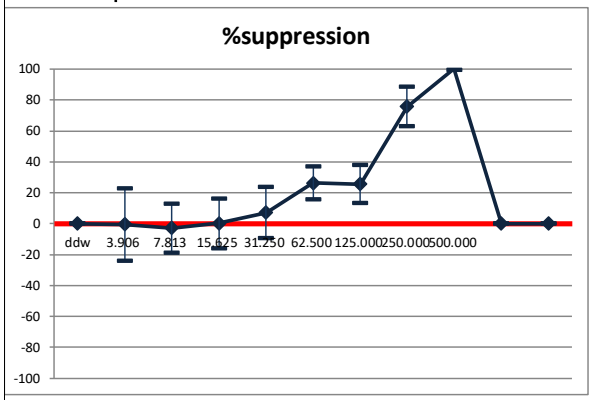
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	22.307	14.396	30.217
7.813	7.068	-2.326	16.462
15.625	12.719	1.570	23.867
31.250	23.750	11.559	35.940
62.500	49.183	41.678	56.688
125.000	80.961	77.405	84.517
250.000	98.679	98.346	99.012
500.000	100.154	100.029	100.279
1000.000	100.109	99.980	100.239
2000.000	100.179	99.934	100.425

Lab A chem.4

round1

S:SSS

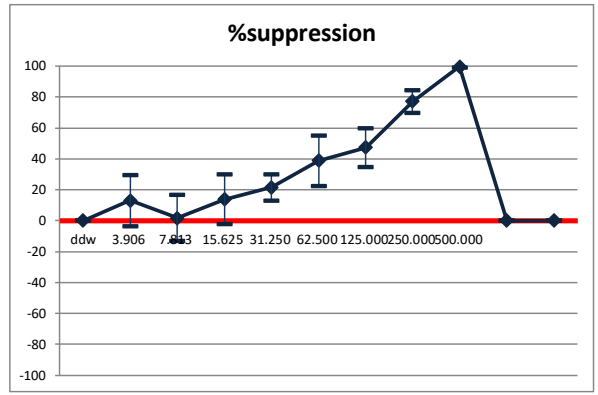
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-0.450	-23.945	23.045
7.813	-2.855	-18.694	12.983
15.625	0.218	-15.674	16.111
31.250	7.176	-9.285	23.638
62.500	26.432	15.780	37.084
125.000	25.594	13.263	37.925
250.000	75.789	62.942	88.636
500.000	100.103	99.460	100.745

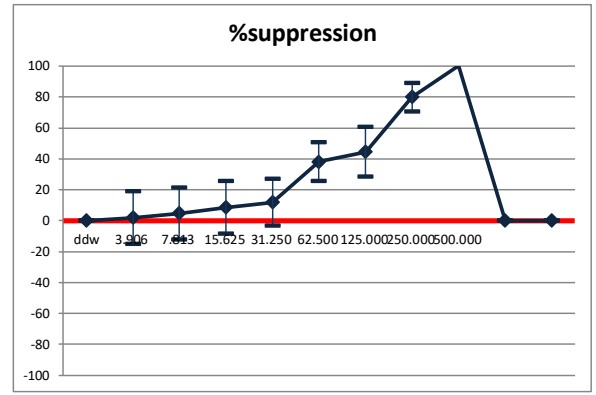
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	12.981	-3.697	29.660
7.813	1.747	-13.143	16.637
15.625	13.839	-2.197	29.875
31.250	21.529	13.122	29.936
62.500	38.841	22.559	55.122
125.000	47.339	34.771	59.907
250.000	77.207	69.933	84.480
500.000	99.604	99.154	100.055

3rd Exp.



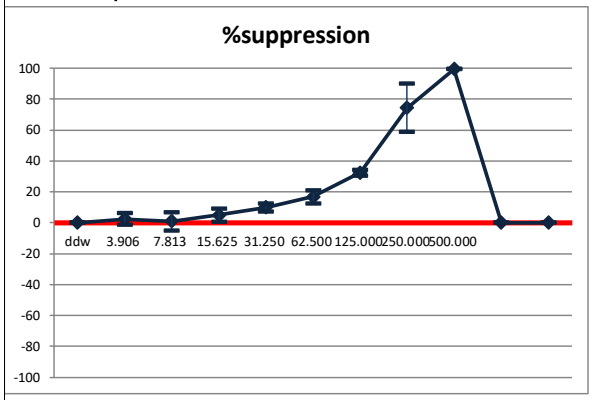
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	2.091	-14.969	19.150
7.813	4.739	-12.001	21.479
15.625	8.705	-8.337	25.748
31.250	12.022	-3.254	27.299
62.500	38.197	25.555	50.840
125.000	44.629	28.608	60.651
250.000	80.029	70.896	89.163
500.000	100.269	100.050	100.488

round2

S:SSS

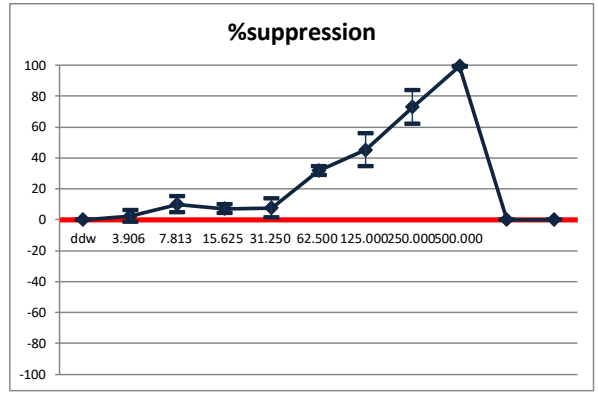
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	2.454	-1.315	6.224
7.813	1.018	-4.982	7.017
15.625	5.018	0.847	9.189
31.250	9.913	7.325	12.501
62.500	16.855	12.456	21.253
125.000	32.471	30.598	34.345
250.000	74.509	59.046	89.972
500.000	99.517	99.385	99.649

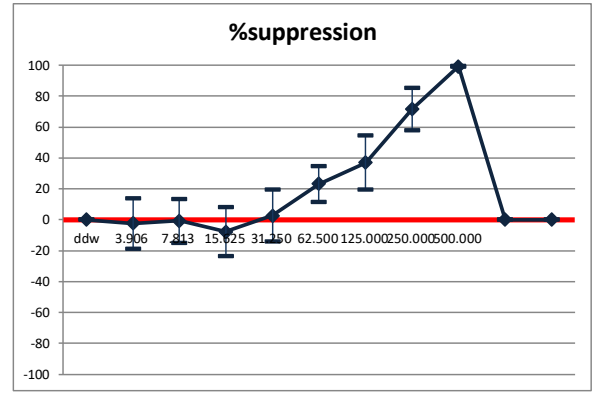
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	2.531	-1.206	6.268
7.813	10.103	5.077	15.129
15.625	7.280	4.419	10.141
31.250	7.662	1.467	13.858
62.500	31.862	29.049	34.675
125.000	45.299	34.714	55.885
250.000	72.945	61.975	83.916
500.000	99.532	99.274	99.788

3rd Exp.



3rd Exp.

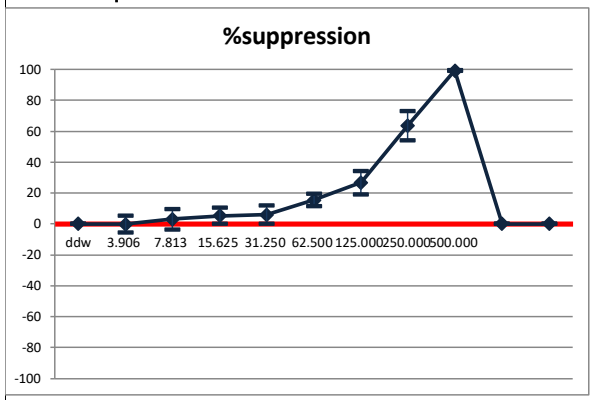
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-2.367	-18.739	14.005
7.813	-0.633	-14.763	13.497
15.625	-7.732	-23.669	8.205
31.250	2.717	-14.095	19.528
62.500	23.169	11.752	34.585
125.000	37.114	19.585	54.642
250.000	71.554	57.884	85.224
500.000	99.316	99.111	99.520

Lab A chem.4

round3

S:SSS

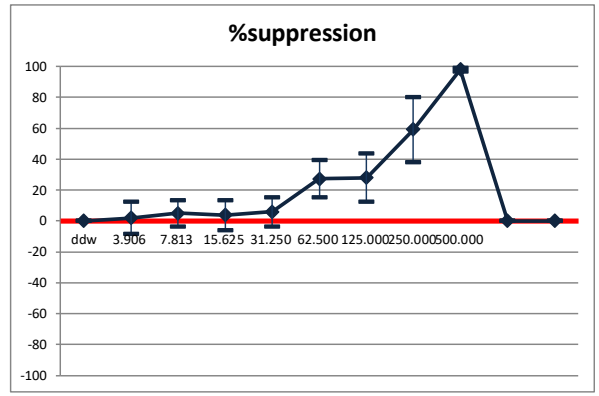
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-0.192	-5.622	5.239
7.813	3.065	-3.577	9.707
15.625	5.367	0.024	10.709
31.250	6.109	0.249	11.969
62.500	15.551	11.454	19.649
125.000	26.712	18.936	34.487
250.000	63.634	54.382	72.887
500.000	99.216	99.036	99.395

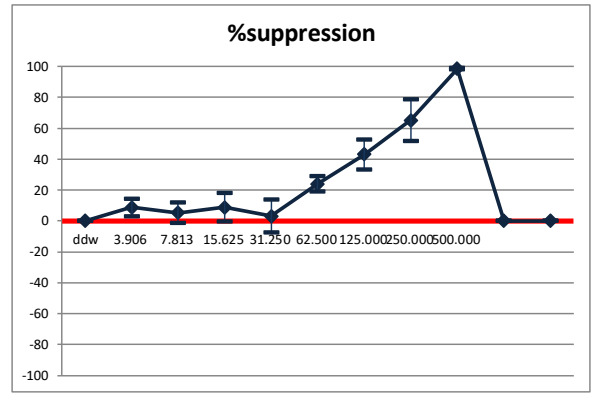
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	1.990	-8.487	12.468
7.813	4.934	-3.459	13.326
15.625	3.890	-5.823	13.603
31.250	5.930	-3.581	15.441
62.500	27.383	15.513	39.254
125.000	27.938	12.332	43.543
250.000	59.270	38.215	80.326
500.000	98.004	96.836	99.173

3rd Exp.



3rd Exp.

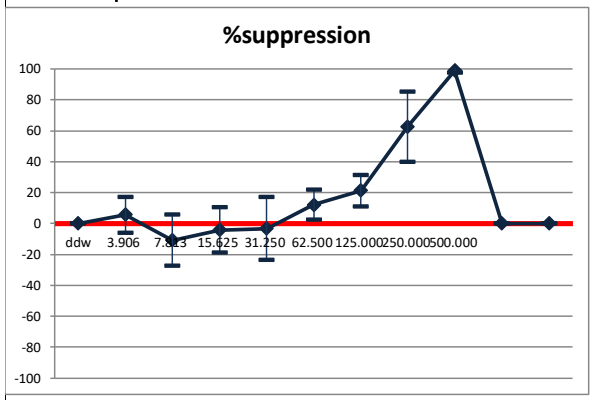
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	8.816	3.024	14.608
7.813	5.329	-1.405	12.063
15.625	8.882	-0.479	18.243
31.250	3.270	-7.534	14.074
62.500	23.895	18.935	28.856
125.000	43.110	33.487	52.733
250.000	65.240	51.590	78.890
500.000	98.527	97.989	99.065

Lab B chem.4

round1

S:SSS

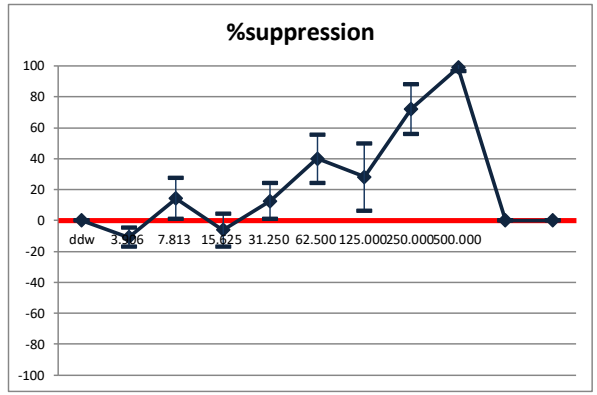
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	5.646	-5.956	17.249
7.813	-10.812	-27.488	5.864
15.625	-4.064	-18.914	10.786
31.250	-3.290	-23.623	17.043
62.500	12.229	2.509	21.948
125.000	21.351	11.095	31.606
250.000	62.695	40.073	85.317
500.000	99.111	97.667	100.556

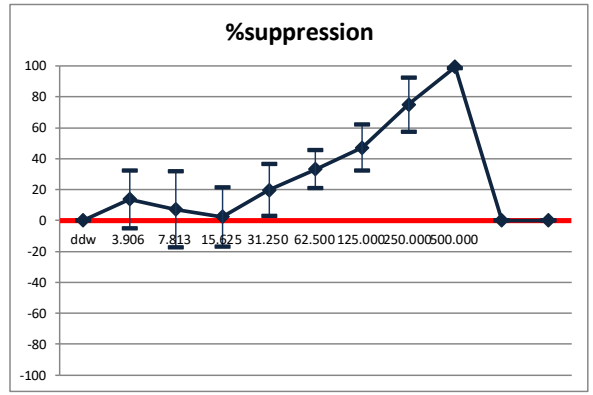
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-10.698	-16.987	-4.410
7.813	14.354	1.136	27.571
15.625	-6.047	-16.690	4.597
31.250	12.686	1.068	24.303
62.500	40.115	24.524	55.706
125.000	28.281	6.569	49.992
250.000	72.198	56.175	88.221
500.000	99.057	96.932	101.183

3rd Exp.



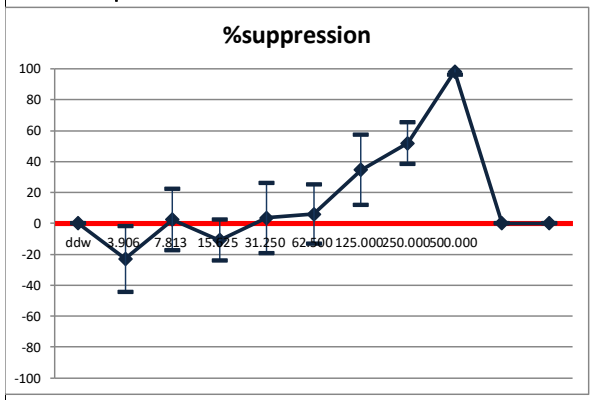
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	13.778	-5.003	32.560
7.813	7.245	-17.529	32.020
15.625	2.414	-16.729	21.557
31.250	19.805	3.028	36.582
62.500	33.246	20.924	45.569
125.000	47.252	32.291	62.213
250.000	75.080	57.651	92.509
500.000	99.415	98.643	100.188

round2

S:SSS

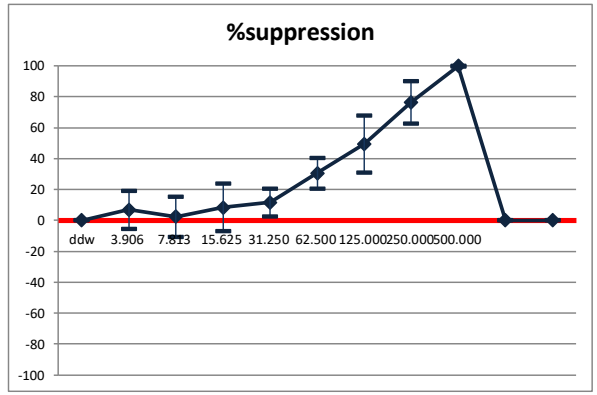
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-22.847	-44.230	-1.463
7.813	2.378	-17.515	22.272
15.625	-10.706	-24.073	2.662
31.250	3.529	-19.213	26.271
62.500	6.017	-13.026	25.061
125.000	34.752	12.187	57.316
250.000	51.924	38.385	65.462
500.000	98.331	96.464	100.199

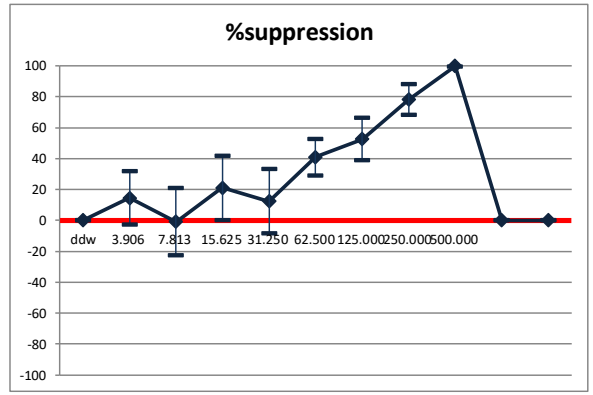
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	6.865	-5.425	19.155
7.813	2.436	-10.471	15.343
15.625	8.436	-7.132	24.005
31.250	11.567	2.551	20.583
62.500	30.656	20.751	40.561
125.000	49.551	30.987	68.116
250.000	76.526	62.786	90.265
500.000	99.850	99.720	99.979

3rd Exp.



3rd Exp.

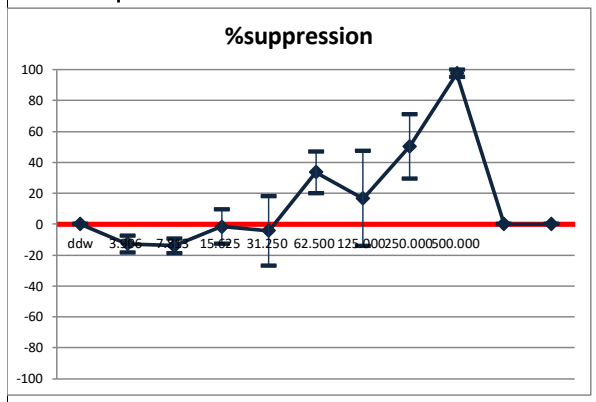
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	14.623	-2.519	31.765
7.813	-0.823	-22.589	20.942
15.625	21.154	0.346	41.962
31.250	12.447	-8.447	33.341
62.500	41.039	29.117	52.962
125.000	52.633	38.801	66.466
250.000	78.134	68.157	88.111
500.000	99.857	99.580	100.135

Lab B chem.4

round3

S:SSS

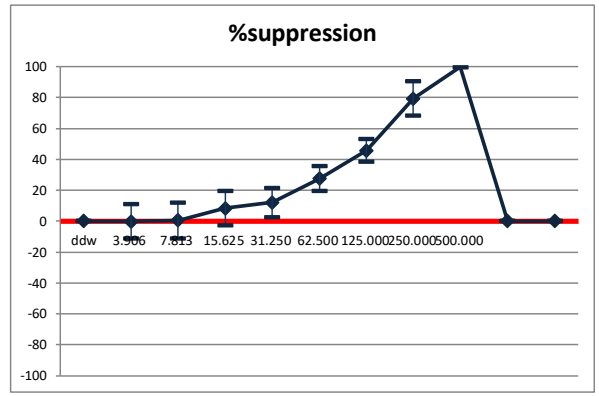
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-12.768	-18.029	-7.508
7.813	-13.941	-18.554	-9.329
15.625	-1.488	-12.739	9.762
31.250	-4.184	-26.623	18.256
62.500	33.591	19.967	47.215
125.000	16.626	-14.094	47.346
250.000	50.420	29.671	71.169
500.000	97.571	95.267	99.876

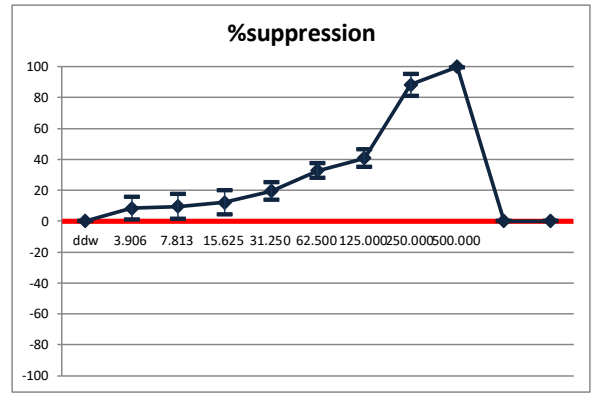
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-0.116	-11.165	10.934
7.813	0.497	-11.086	12.079
15.625	8.452	-2.524	19.429
31.250	12.080	2.577	21.583
62.500	27.608	19.419	35.798
125.000	45.763	38.349	53.177
250.000	79.448	68.154	90.741
500.000	100.029	99.738	100.321

3rd Exp.



3rd Exp.

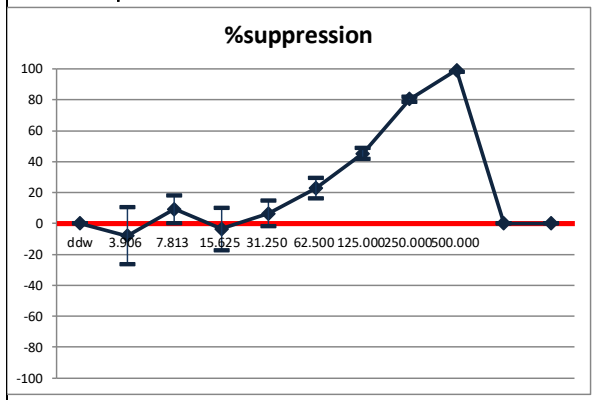
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	8.343	1.029	15.656
7.813	9.598	1.428	17.768
15.625	12.061	4.272	19.851
31.250	19.558	13.970	25.146
62.500	32.816	27.953	37.680
125.000	40.797	35.093	46.501
250.000	88.246	81.094	95.398
500.000	99.967	99.606	100.329

Lab C chem.4

round1

S:SSS

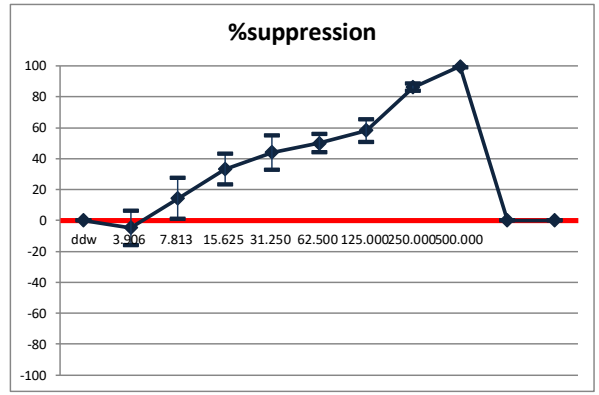
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-7.959	-26.483	10.564
7.813	9.394	0.400	18.387
15.625	-3.500	-17.186	10.187
31.250	6.428	-1.890	14.747
62.500	22.820	16.236	29.404
125.000	45.343	41.679	49.008
250.000	80.491	78.900	82.082
500.000	99.246	98.279	100.212

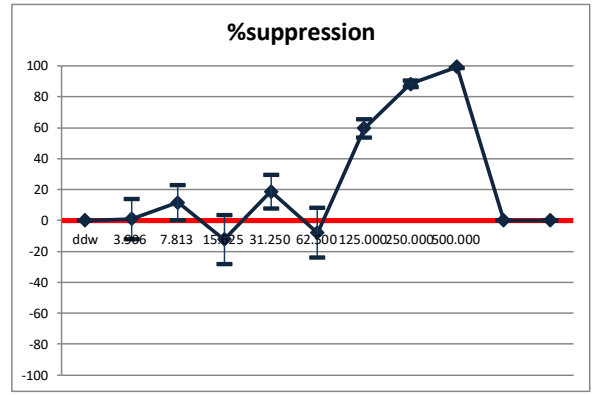
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-4.706	-15.951	6.538
7.813	14.392	0.934	27.849
15.625	33.295	23.506	43.084
31.250	44.042	32.756	55.328
62.500	50.010	44.034	55.986
125.000	58.354	50.952	65.756
250.000	86.269	84.025	88.513
500.000	99.730	99.104	100.356

3rd Exp.



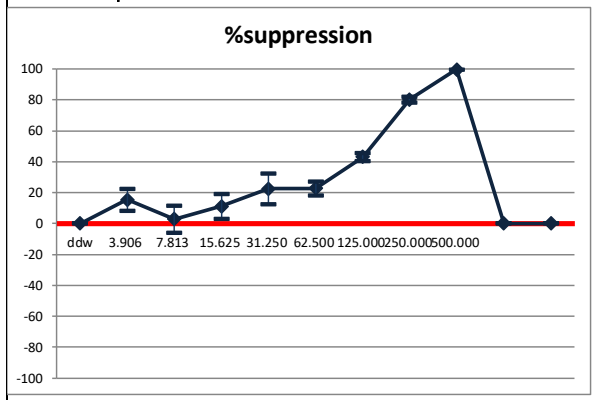
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	0.996	-12.060	14.051
7.813	11.685	0.339	23.032
15.625	-12.221	-28.167	3.726
31.250	18.857	7.965	29.749
62.500	-8.036	-24.132	8.060
125.000	59.723	53.834	65.611
250.000	88.378	86.182	90.574
500.000	99.597	98.645	100.549

round2

S:SSS

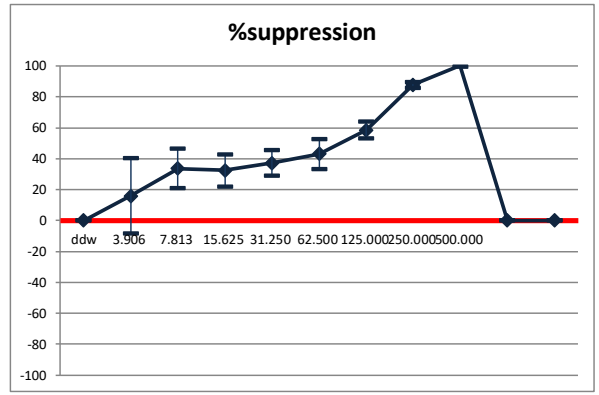
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	15.452	8.249	22.655
7.813	2.967	-5.754	11.688
15.625	11.160	3.068	19.253
31.250	22.486	12.627	32.344
62.500	22.754	18.311	27.197
125.000	43.086	40.386	45.785
250.000	80.344	78.456	82.232
500.000	99.736	99.395	100.076

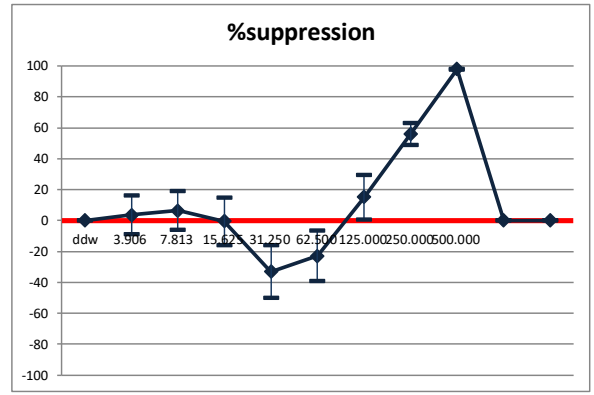
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	15.954	-8.444	40.353
7.813	33.629	20.907	46.350
15.625	32.403	21.938	42.867
31.250	37.234	28.837	45.631
62.500	43.130	33.342	52.918
125.000	58.500	53.064	63.936
250.000	87.824	86.094	89.554
500.000	100.456	99.718	101.193

3rd Exp.



3rd Exp.

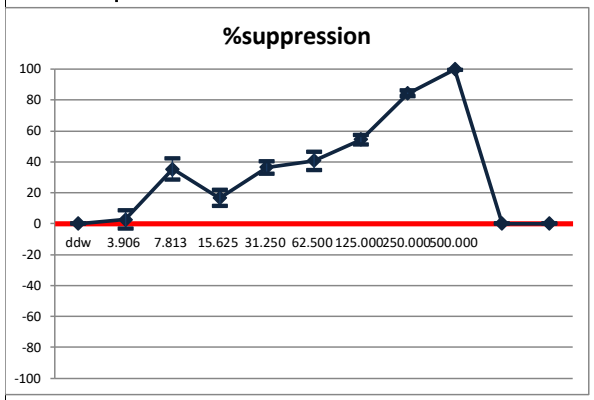
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	3.704	-8.777	16.184
7.813	6.472	-6.061	19.005
15.625	-0.478	-15.909	14.952
31.250	-33.054	-50.166	-15.941
62.500	-22.906	-39.175	-6.638
125.000	15.200	0.726	29.675
250.000	55.874	48.825	62.923
500.000	98.088	97.769	98.408

Lab C chem.4

round3

S:SSS

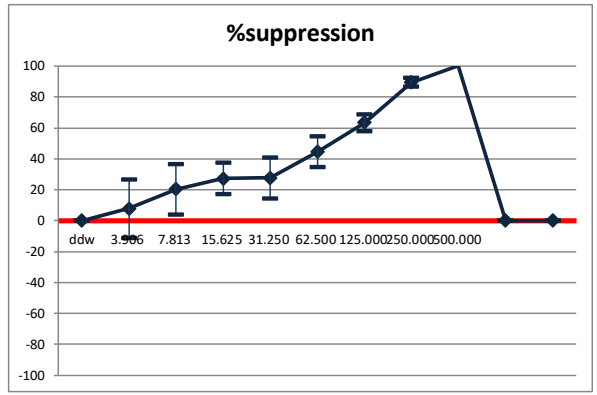
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	2.781	-3.077	8.639
7.813	35.449	28.552	42.346
15.625	16.709	11.665	21.753
31.250	36.471	32.316	40.626
62.500	40.807	34.983	46.630
125.000	54.407	51.157	57.657
250.000	84.376	82.498	86.254
500.000	99.945	99.715	100.175

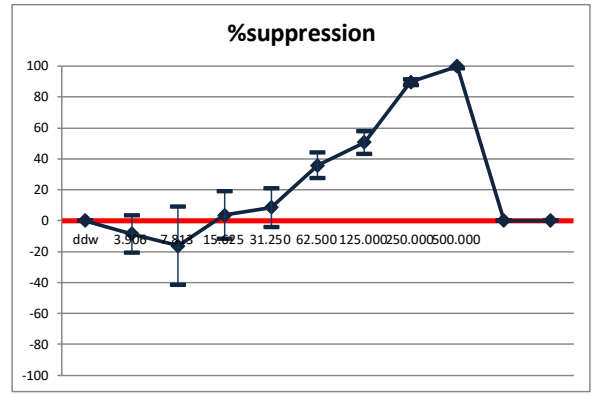
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	7.886	-10.949	26.721
7.813	20.429	4.073	36.784
15.625	27.388	17.342	37.433
31.250	27.670	14.625	40.716
62.500	44.667	34.719	54.615
125.000	63.481	58.021	68.940
250.000	89.649	86.632	92.667
500.000	100.130	100.006	100.253

3rd Exp.



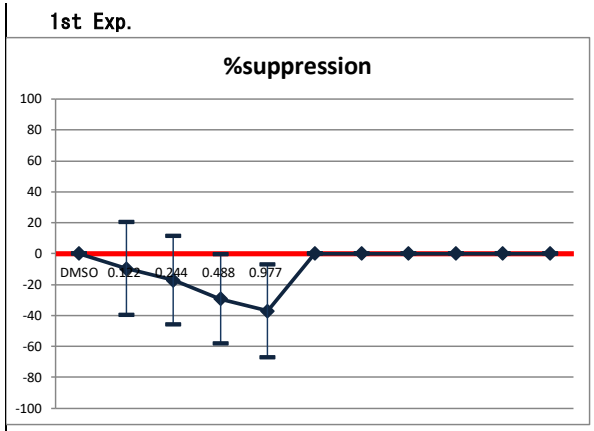
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-8.551	-20.674	3.573
7.813	-16.157	-41.469	9.155
15.625	3.564	-11.786	18.913
31.250	8.584	-3.990	21.159
62.500	35.799	27.440	44.158
125.000	50.724	43.503	57.945
250.000	89.781	87.848	91.715
500.000	99.840	98.696	100.984

Lab A chem.5

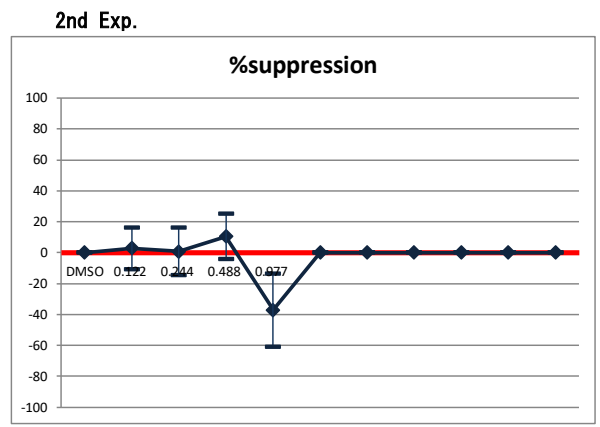
round1

N:ANN



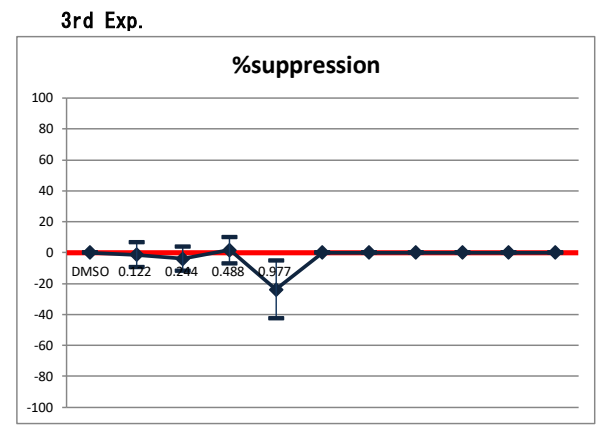
1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	-9.690	-39.716	20.335
0.244	-17.294	-45.941	11.354
0.488	-29.209	-58.114	-0.305
0.977	-37.020	-67.153	-6.888



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	2.820	-10.464	16.104
0.244	0.873	-14.589	16.335
0.488	10.537	-4.191	25.265
0.977	-37.090	-60.883	-13.298

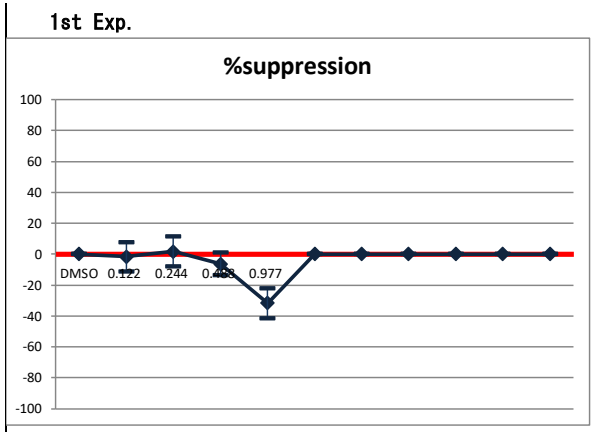


3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	-1.294	-9.232	6.644
0.244	-3.904	-11.571	3.762
0.488	1.759	-6.667	10.184
0.977	-23.708	-42.339	-5.077

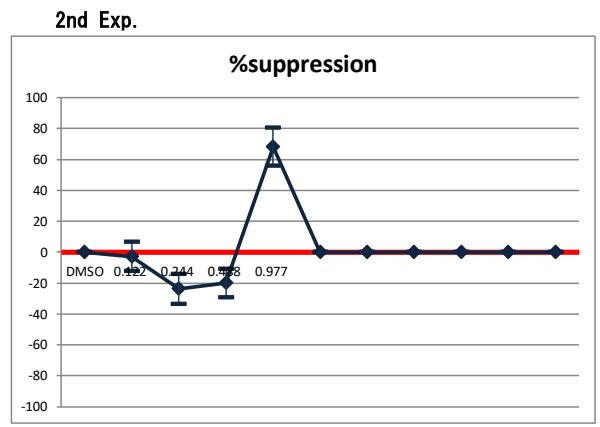
round2

N:NNN



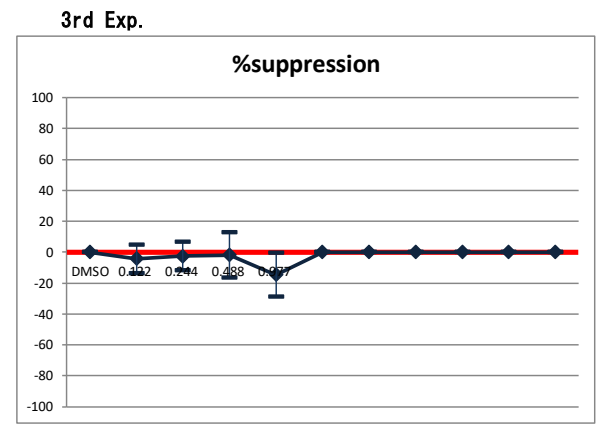
1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	-1.602	-11.025	7.821
0.244	1.747	-8.071	11.565
0.488	-6.325	-13.675	1.025
0.977	-31.703	-41.372	-22.033



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	-2.668	-12.023	6.687
0.244	-23.637	-33.416	-13.858
0.488	-19.852	-29.097	-10.607
0.977	68.256	55.899	80.612



3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	-4.278	-13.634	5.078
0.244	-2.409	-11.595	6.777
0.488	-1.753	-16.320	12.814
0.977	-14.642	-28.789	-0.495

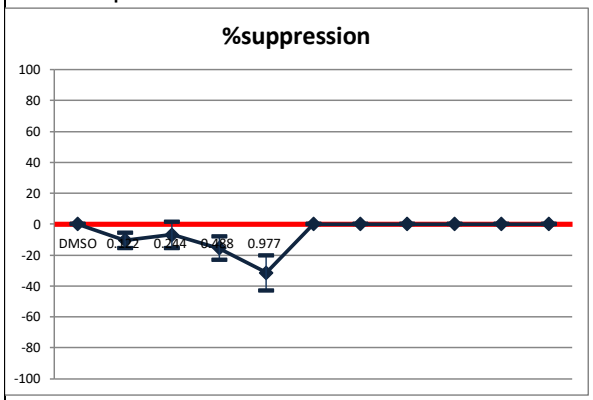
408

Lab A chem.5

round3

N:NSN

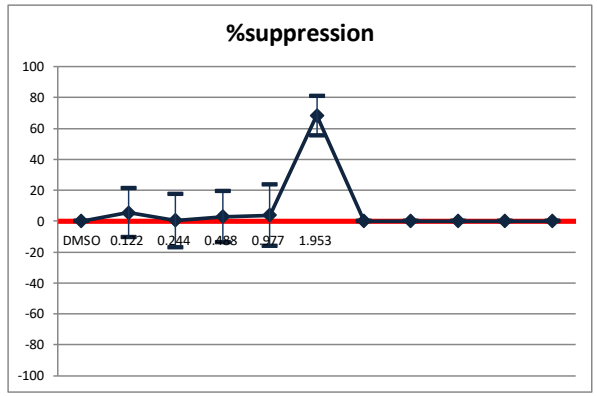
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	-10.415	-15.410	-5.421
0.244	-6.713	-15.233	1.808
0.488	-15.462	-22.921	-8.003
0.977	-31.451	-42.955	-19.947

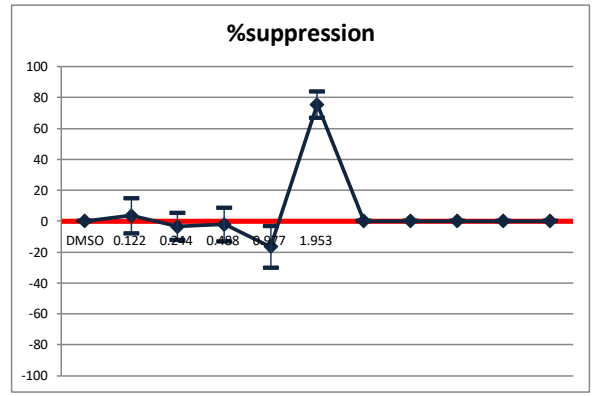
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	5.567	-10.221	21.355
0.244	0.541	-16.756	17.839
0.488	2.905	-13.601	19.411
0.977	3.847	-16.102	23.795
1.953	68.488	55.674	81.301

3rd Exp.



3rd Exp.

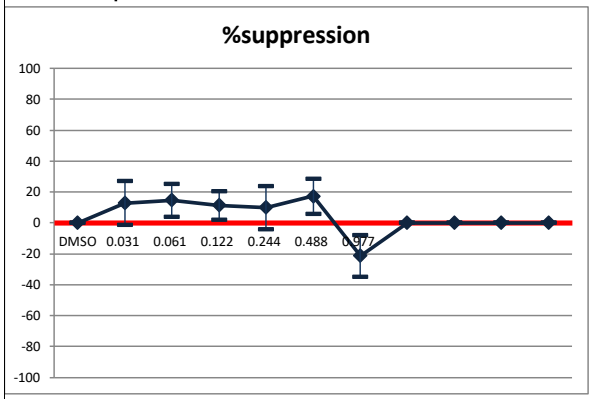
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	3.601	-7.661	14.863
0.244	-3.396	-12.074	5.283
0.488	-2.083	-13.101	8.936
0.977	-16.526	-30.056	-2.995
1.953	75.610	67.152	84.068

Lab B chem.5

round1

N:NNN

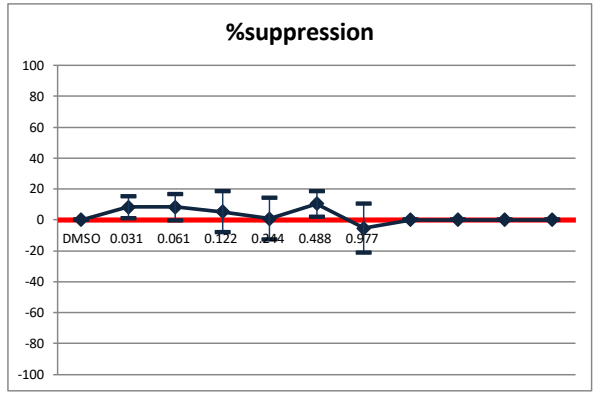
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.031	12.945	-1.084	26.974
0.061	14.715	4.048	25.383
0.122	11.450	2.204	20.696
0.244	9.922	-3.832	23.676
0.488	17.405	6.004	28.806
0.977	-21.277	-34.861	-7.693

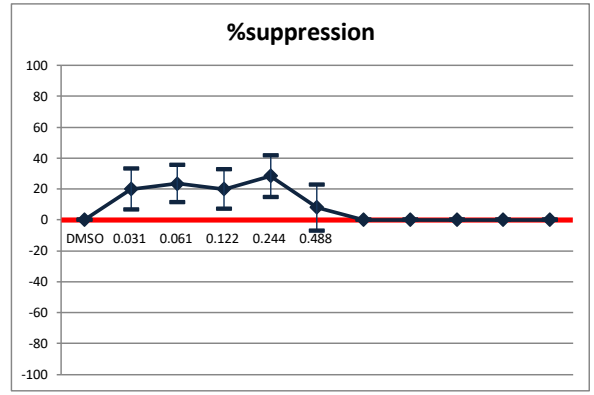
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.031	8.252	1.227	15.276
0.061	8.370	-0.094	16.834
0.122	5.367	-7.785	18.519
0.244	0.740	-12.805	14.286
0.488	10.496	2.184	18.808
0.977	-5.395	-21.337	10.546

3rd Exp.



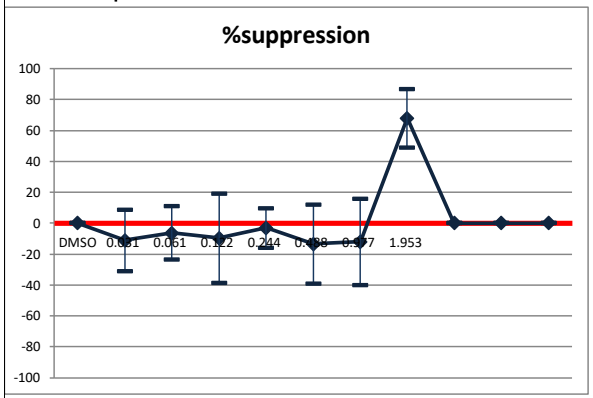
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.031	20.019	6.877	33.161
0.061	23.536	11.589	35.483
0.122	19.971	7.273	32.669
0.244	28.397	14.808	41.987
0.488	8.058	-6.820	22.936

round2

N:NSN

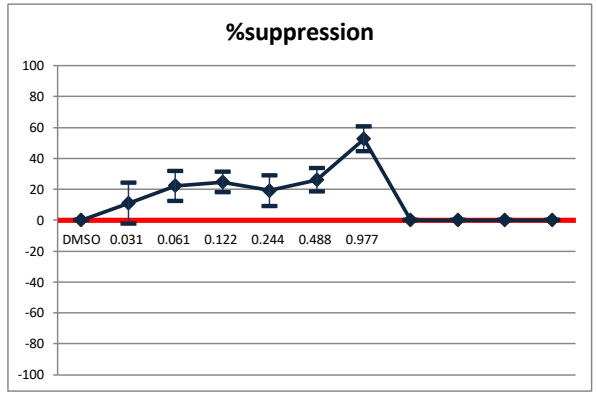
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.031	-10.969	-30.857	8.919
0.061	-6.227	-23.428	10.974
0.122	-9.615	-38.459	19.230
0.244	-2.964	-15.735	9.806
0.488	-13.438	-39.040	12.163
0.977	-12.075	-40.071	15.921
1.953	67.907	49.187	86.627

2nd Exp.

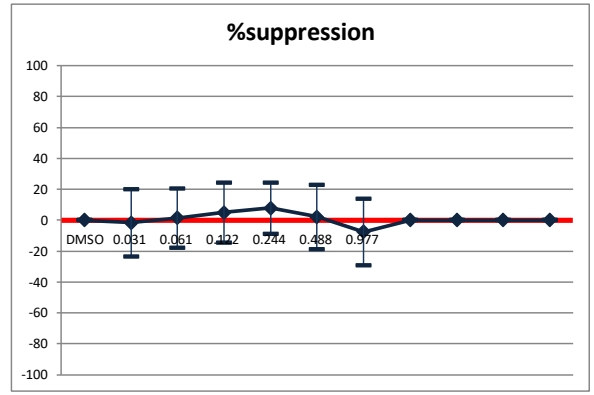


2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.031	11.027	-2.389	24.442
0.061	22.211	12.328	32.094
0.122	24.776	18.247	31.305
0.244	19.224	9.223	29.224
0.488	26.158	18.688	33.628
0.977	52.723	44.771	60.675

410

3rd Exp.



3rd Exp.

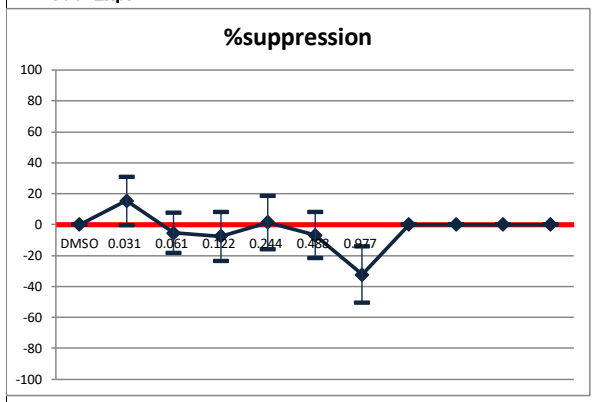
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.031	-1.686	-23.375	20.003
0.061	1.388	-17.986	20.762
0.122	4.936	-14.505	24.378
0.244	7.855	-8.628	24.338
0.488	2.104	-18.534	22.742
0.977	-7.567	-28.915	13.782

Lab B chem.5

round3

N:NNN

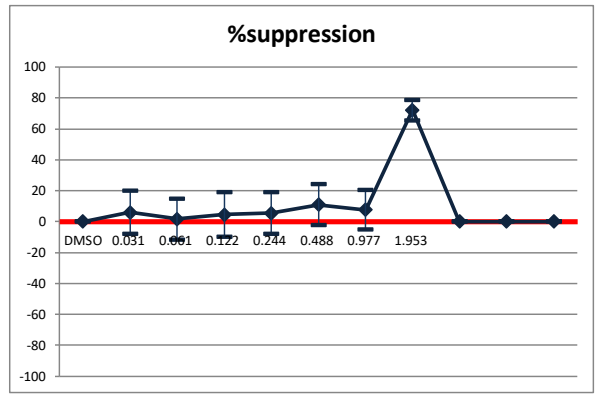
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.031	15.371	-0.206	30.948
0.061	-5.299	-18.213	7.615
0.122	-7.529	-23.457	8.399
0.244	1.393	-15.693	18.478
0.488	-6.813	-21.662	8.036
0.977	-32.237	-50.408	-14.066

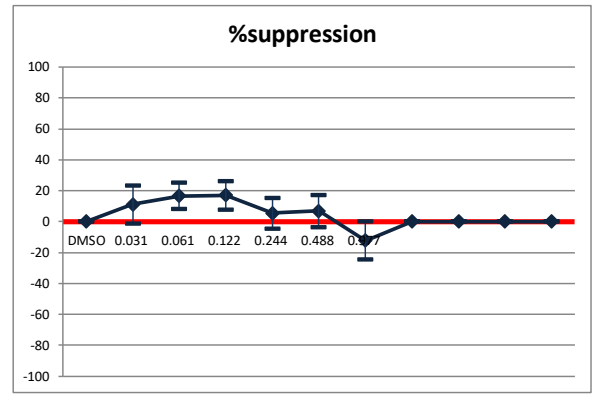
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.031	6.107	-7.795	20.009
0.061	1.672	-11.717	15.061
0.122	4.695	-9.749	19.138
0.244	5.565	-7.983	19.112
0.488	11.043	-2.120	24.207
0.977	7.672	-5.043	20.387
1.953	72.147	65.675	78.620

3rd Exp.



3rd Exp.

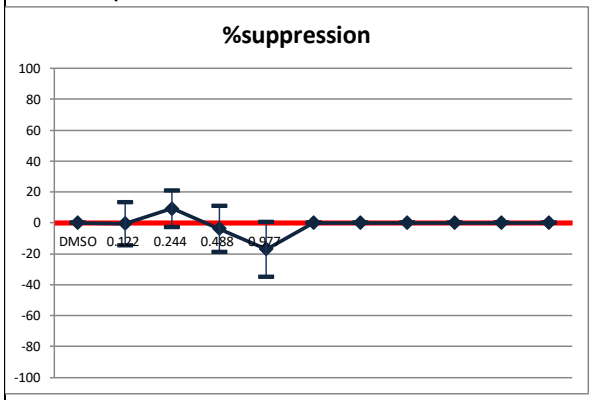
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.031	11.220	-1.046	23.486
0.061	16.628	8.056	25.200
0.122	17.028	7.657	26.399
0.244	5.584	-4.327	15.494
0.488	6.905	-3.492	17.302
0.977	-12.229	-24.572	0.115

Lab C chem.5

round1

N:NNA

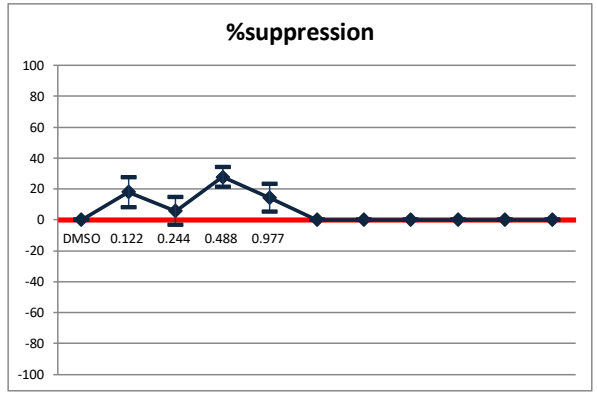
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	-0.356	-14.252	13.540
0.244	9.249	-2.611	21.109
0.488	-3.802	-18.560	10.957
0.977	-17.010	-34.875	0.855

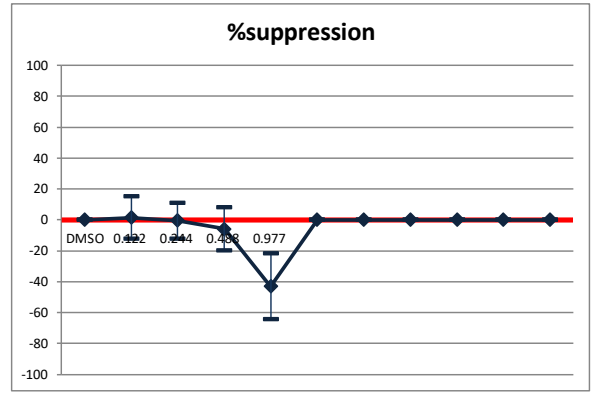
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	18.051	8.249	27.853
0.244	5.741	-3.339	14.821
0.488	27.752	21.267	34.238
0.977	14.342	5.435	23.250

3rd Exp.



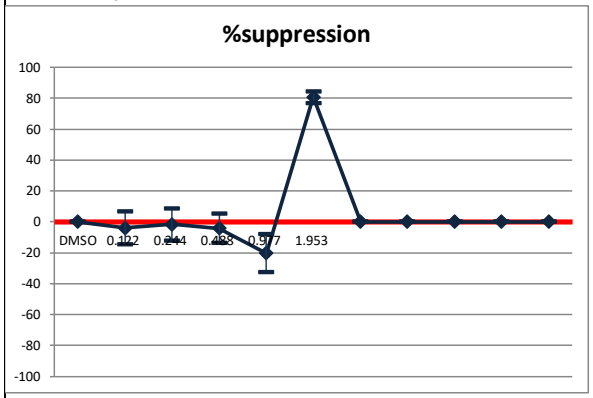
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	1.596	-12.052	15.243
0.244	-0.373	-11.954	11.208
0.488	-5.794	-19.869	8.281
0.977	-42.890	-64.072	-21.708

round2

N:NNN

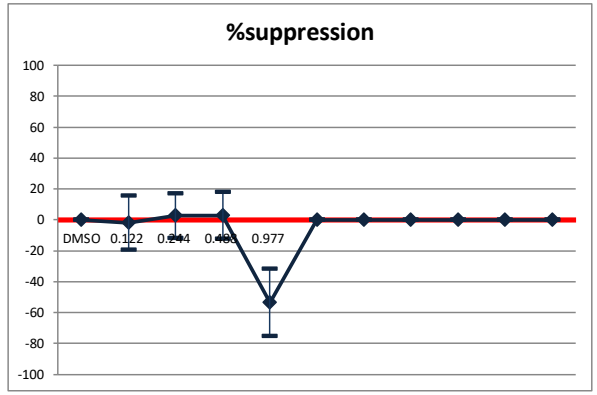
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	-3.833	-14.422	6.757
0.244	-1.618	-11.878	8.641
0.488	-4.105	-13.712	5.501
0.977	-20.258	-32.471	-8.045
1.953	80.874	77.103	84.646

2nd Exp.

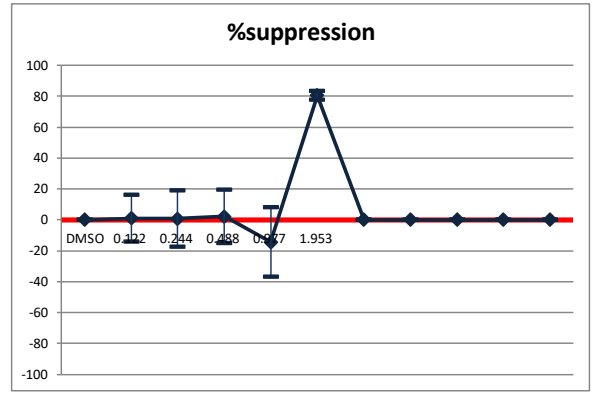


2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	-1.727	-19.139	15.685
0.244	2.916	-11.579	17.410
0.488	3.014	-12.226	18.254
0.977	-53.426	-75.108	-31.743

412

3rd Exp.



3rd Exp.

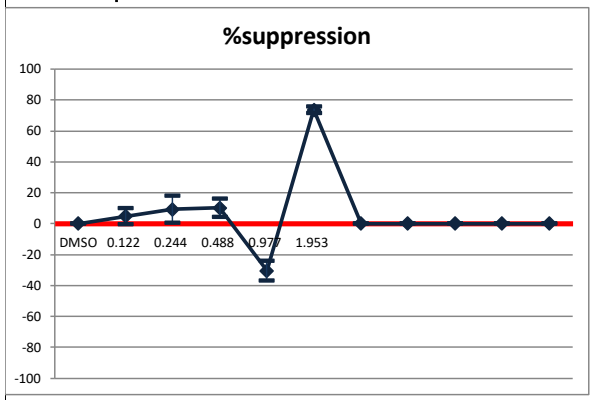
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	0.976	-14.140	16.092
0.244	0.842	-17.461	19.145
0.488	2.325	-14.722	19.373
0.977	-14.421	-36.887	8.044
1.953	80.631	77.711	83.551

Lab C chem.5

round3

N:NNN

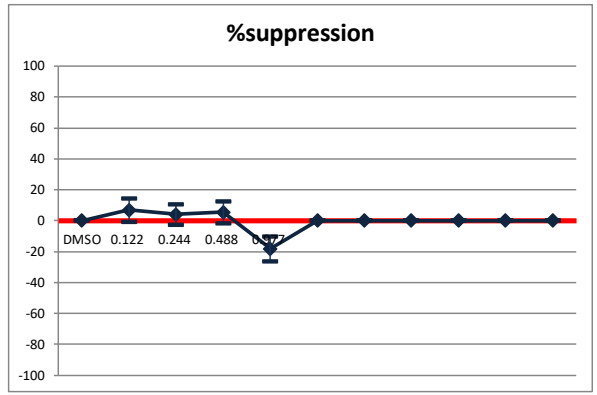
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	4.856	-0.371	10.082
0.244	9.406	0.705	18.107
0.488	10.284	4.269	16.299
0.977	-30.405	-36.691	-24.120
1.953	73.751	71.738	75.764

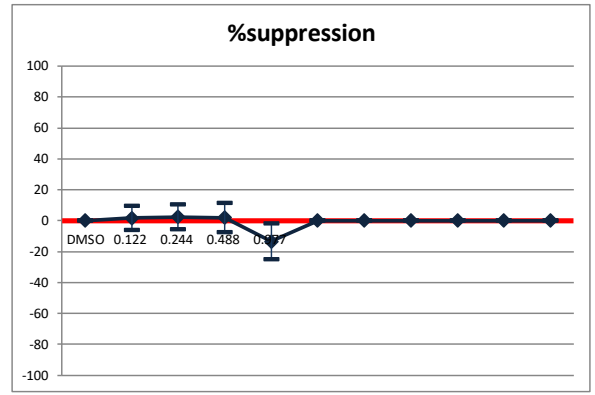
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	6.907	-0.690	14.504
0.244	4.025	-2.437	10.487
0.488	5.416	-1.699	12.532
0.977	-18.158	-26.216	-10.099

3rd Exp.



3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	1.818	-6.109	9.745
0.244	2.483	-5.446	10.411
0.488	2.042	-7.268	11.352
0.977	-13.346	-24.864	-1.829

Data analysis report for the IL-2 Luc assay validation study Appendix B

IL2 Graph PII

2019.02.24

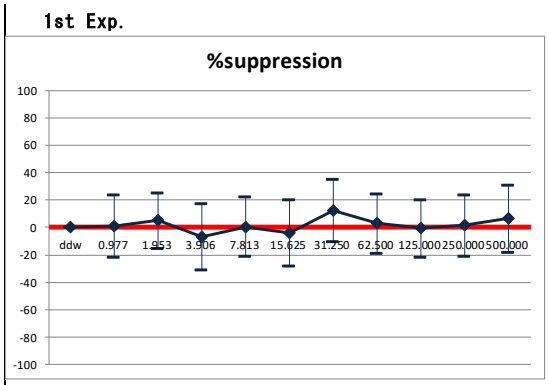
Takashi Omori

Chemical.1

Lead Lab.

N:NNN

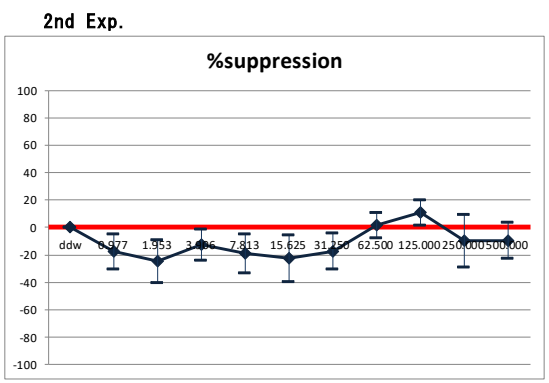
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.977	0.759	-21.891	23.408
1.953	4.960	-15.258	25.178
3.906	-6.523	-30.700	17.653
7.813	0.447	-21.203	22.096
15.625	-4.003	-28.040	20.035
31.250	12.716	-9.991	35.424
62.500	2.868	-19.038	24.775
125.000	-0.645	-21.427	20.137
250.000	1.466	-21.050	23.981
500.000	6.628	-17.822	31.077

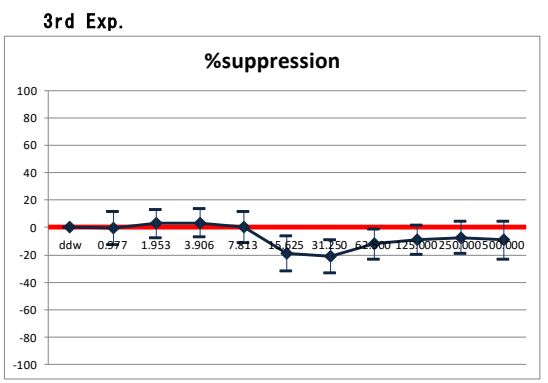
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.977	-17.305	-30.130	-4.480
1.953	-24.317	-39.874	-8.759
3.906	-12.595	-23.981	-1.209
7.813	-18.980	-33.038	-4.922
15.625	-22.465	-39.474	-5.456
31.250	-17.412	-30.551	-4.273
62.500	1.894	-7.215	11.003
125.000	10.851	1.386	20.317
250.000	-9.571	-28.776	9.634
500.000	-9.401	-22.392	3.591

3rd Exp.



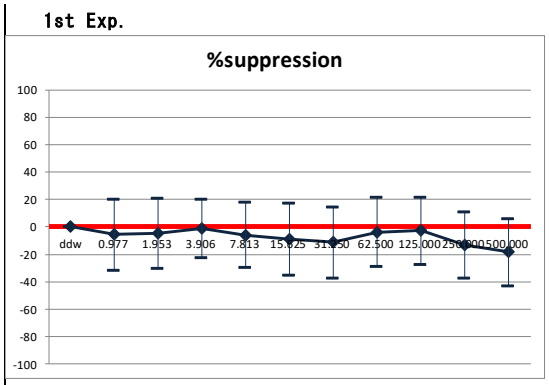
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.977	-0.469	-12.613	11.675
1.953	3.039	-7.230	13.307
3.906	3.245	-7.073	13.562
7.813	0.035	-11.346	11.415
15.625	-19.016	-31.874	-6.158
31.250	-21.140	-33.287	-8.994
62.500	-11.955	-23.105	-0.806
125.000	-8.795	-19.273	1.682
250.000	-7.247	-18.836	4.342
500.000	-9.200	-22.827	4.428

Lab A

N:NNN

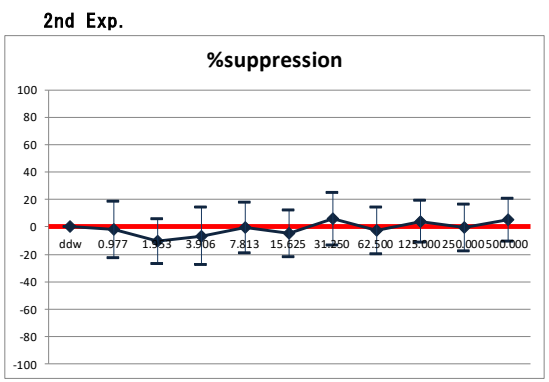
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.977	-5.649	-31.517	20.218
1.953	-4.700	-30.489	21.089
3.906	-1.099	-22.557	20.359
7.813	-5.979	-29.815	17.857
15.625	-8.785	-35.028	17.458
31.250	-11.124	-37.105	14.857
62.500	-3.661	-28.614	21.292
125.000	-2.863	-27.200	21.474
250.000	-13.230	-37.485	11.025
500.000	-18.446	-43.164	6.272

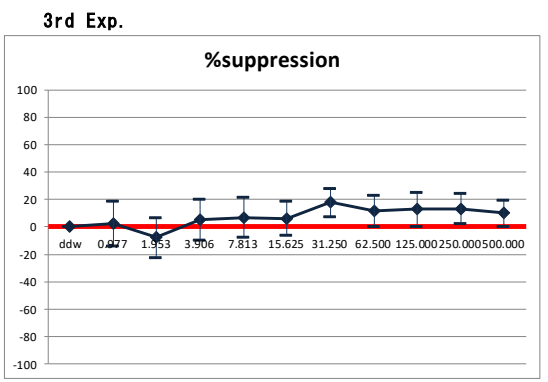
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.977	-1.862	-22.680	18.957
1.953	-10.436	-26.573	5.702
3.906	-6.459	-27.689	14.771
7.813	-0.473	-19.188	18.242
15.625	-4.864	-21.928	12.201
31.250	6.202	-13.037	25.440
62.500	-2.548	-19.917	14.821
125.000	4.092	-11.200	19.384
250.000	-0.445	-17.441	16.551
500.000	5.315	-9.990	20.619

3rd Exp.



3rd Exp.

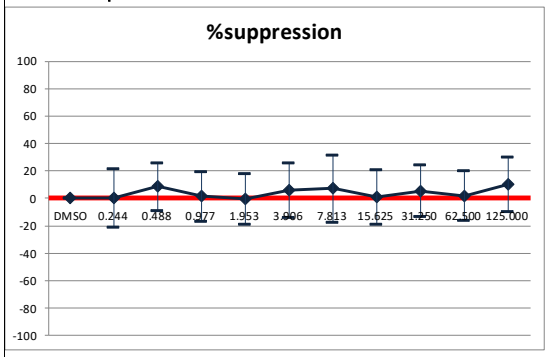
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.977	2.481	-13.584	18.546
1.953	-7.826	-22.227	6.575
3.906	5.211	-9.670	20.092
7.813	6.860	-7.675	21.396
15.625	6.101	-6.281	18.483
31.250	17.780	7.391	28.168
62.500	11.809	0.324	23.294
125.000	12.813	0.187	25.438
250.000	13.288	2.471	24.104
500.000	9.919	0.463	19.376

Chemical.1

Lab B

N:NNN

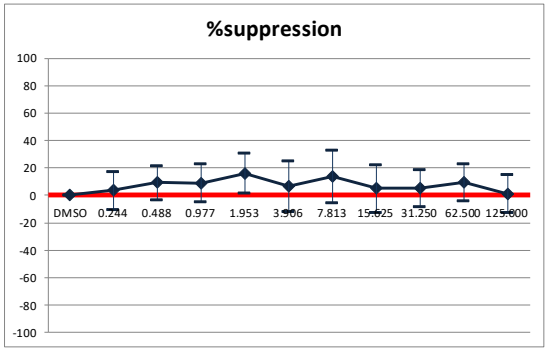
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	0.180	-21.076	21.436
0.488	8.666	-8.635	25.967
0.977	1.457	-16.627	19.541
1.953	-0.467	-19.120	18.186
3.906	6.041	-13.749	25.830
7.813	7.275	-17.228	31.777
15.625	0.846	-19.192	20.884
31.250	5.481	-13.275	24.237
62.500	2.019	-16.194	20.232
125.000	10.221	-9.419	29.860

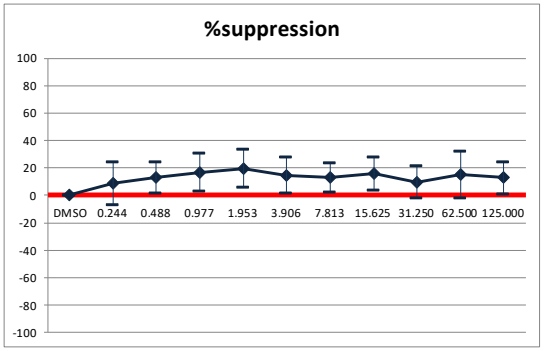
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	3.572	-10.048	17.191
0.488	9.206	-2.973	21.384
0.977	8.987	-4.959	22.932
1.953	16.212	1.831	30.592
3.906	6.653	-11.749	25.055
7.813	13.780	-5.120	32.679
15.625	5.019	-12.346	22.384
31.250	5.128	-8.228	18.484
62.500	9.411	-3.864	22.686
125.000	1.176	-12.708	15.059

3rd Exp.



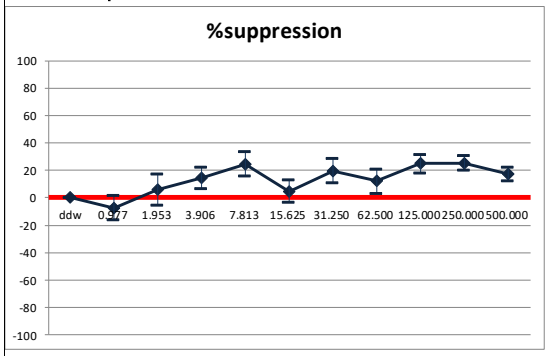
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	8.741	-6.654	24.137
0.488	13.001	1.725	24.276
0.977	16.752	2.855	30.649
1.953	19.773	5.811	33.736
3.906	14.761	1.744	27.778
7.813	13.192	2.472	23.913
15.625	16.082	3.911	28.254
31.250	9.829	-2.139	21.797
62.500	15.258	-1.776	32.291
125.000	12.744	1.207	24.282

Lab C

N:NNN

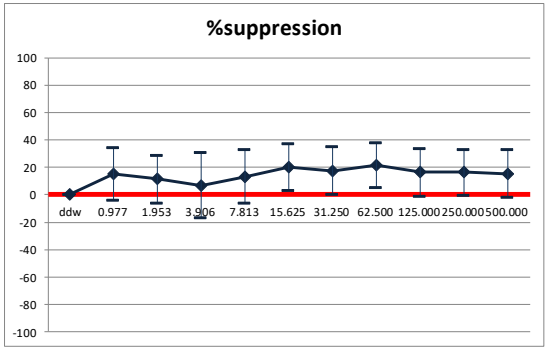
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.977	-7.291	-15.977	1.395
1.953	6.241	-5.211	17.693
3.906	14.368	6.654	22.082
7.813	24.567	15.734	33.400
15.625	4.841	-3.326	13.008
31.250	19.833	10.749	28.916
62.500	12.077	3.312	20.842
125.000	24.859	18.149	31.569
250.000	25.443	20.220	30.665
500.000	17.521	12.563	22.480

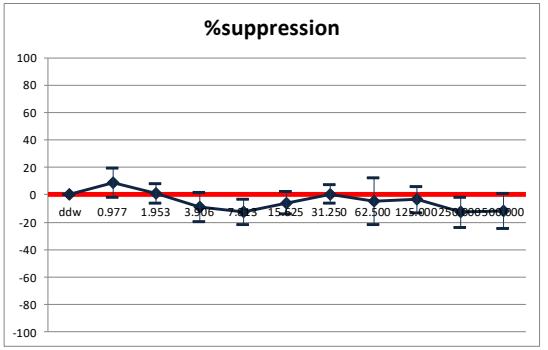
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.977	15.184	-4.162	34.530
1.953	11.371	-6.299	29.040
3.906	7.016	-16.491	30.523
7.813	13.332	-6.019	32.682
15.625	20.186	2.867	37.506
31.250	17.538	0.242	34.834
62.500	21.499	5.098	37.899
125.000	16.359	-1.168	33.886
250.000	16.395	-0.418	33.209
500.000	15.454	-1.744	32.652

3rd Exp.



3rd Exp.

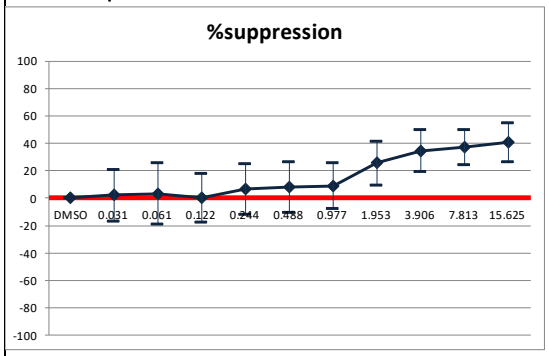
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.977	8.952	-1.716	19.620
1.953	0.928	-6.235	8.091
3.906	-8.778	-19.584	2.029
7.813	-12.295	-21.643	-2.947
15.625	-5.854	-14.226	2.518
31.250	0.377	-6.414	7.168
62.500	-4.615	-21.616	12.386
125.000	-3.365	-12.892	6.163
250.000	-12.774	-24.065	-1.483
500.000	-11.687	-24.219	0.845

Chemical.2

Lead Lab.

S:SNS

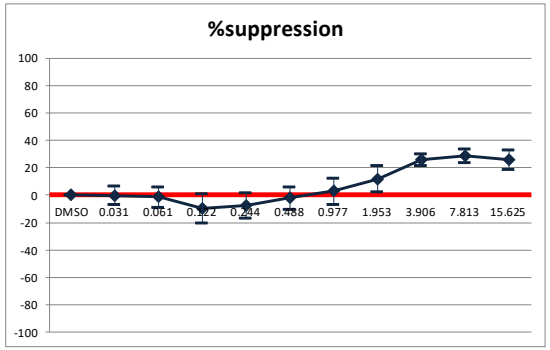
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.031	2.176	-16.687	21.039
0.061	3.499	-19.096	26.093
0.122	0.290	-17.557	18.138
0.244	6.953	-11.450	25.357
0.488	8.317	-10.070	26.705
0.977	9.107	-7.342	25.555
1.953	25.644	9.845	41.443
3.906	34.610	19.306	49.914
7.813	36.976	24.119	49.833
15.625	40.734	26.388	55.080

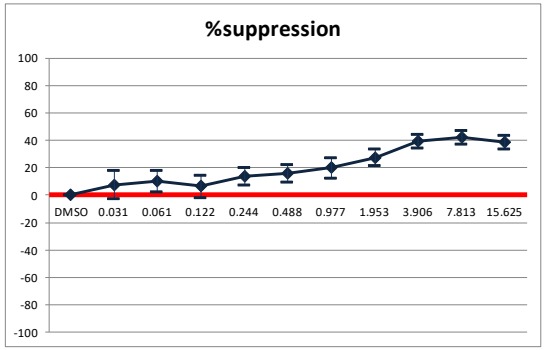
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.031	-0.194	-6.971	6.583
0.061	-1.424	-8.606	5.759
0.122	-9.714	-20.125	0.697
0.244	-7.390	-16.816	2.037
0.488	-2.009	-10.041	6.023
0.977	2.907	-6.541	12.356
1.953	11.974	2.609	21.339
3.906	25.922	21.743	30.101
7.813	28.777	24.076	33.478
15.625	25.779	18.423	33.134

3rd Exp.



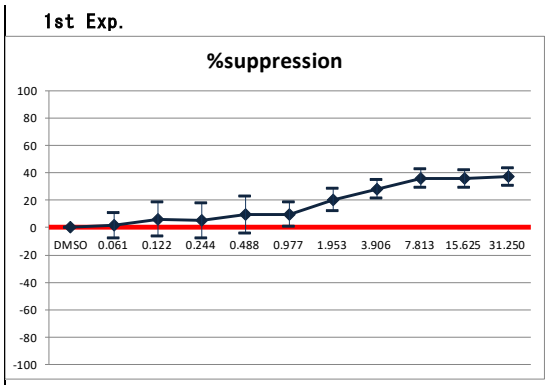
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.031	7.719	-2.512	17.949
0.061	10.424	2.585	18.263
0.122	6.415	-1.828	14.657
0.244	13.935	7.354	20.515
0.488	15.970	9.494	22.446
0.977	19.870	12.583	27.156
1.953	27.536	21.394	33.677
3.906	39.105	34.057	44.152
7.813	41.880	36.914	46.847
15.625	38.858	34.020	43.697

Lab A

S:SSS

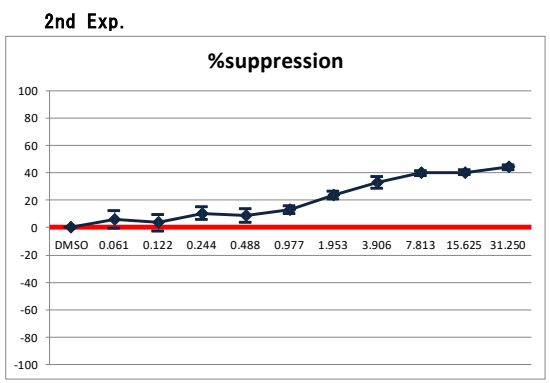
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.061	2.049	-7.220	11.317
0.122	6.196	-6.296	18.687
0.244	5.275	-7.512	18.062
0.488	9.468	-3.766	22.702
0.977	9.873	0.770	18.975
1.953	20.312	12.140	28.484
3.906	28.275	21.794	34.756
7.813	36.128	29.372	42.884
15.625	35.663	29.452	41.874
31.250	37.230	30.581	43.879

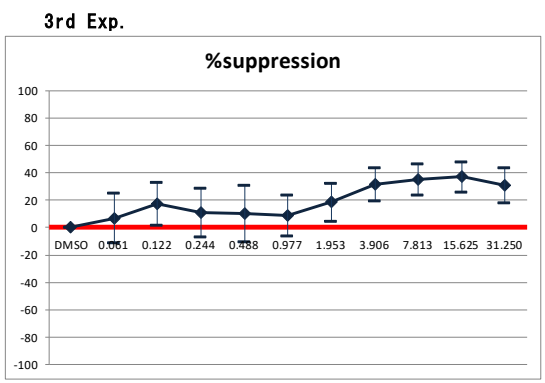
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.061	5.753	-0.746	12.252
0.122	3.646	-2.564	9.856
0.244	10.603	6.192	15.014
0.488	8.833	3.945	13.722
0.977	13.090	10.048	16.133
1.953	23.826	20.989	26.663
3.906	33.064	29.026	37.103
7.813	39.899	38.049	41.748
15.625	40.431	38.358	42.503
31.250	44.058	42.316	45.800

3rd Exp.



3rd Exp.

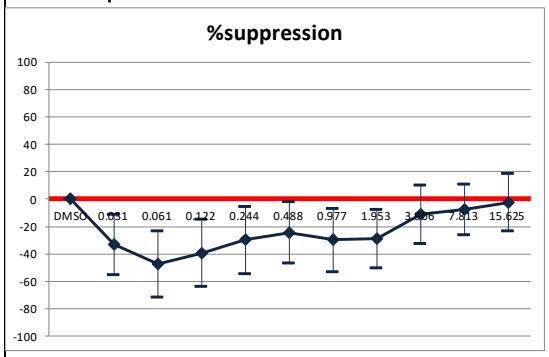
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.061	6.851	-11.287	24.988
0.122	17.248	1.441	33.056
0.244	11.050	-6.442	28.543
0.488	10.058	-10.389	30.505
0.977	8.876	-5.945	23.696
1.953	18.453	4.700	32.207
3.906	31.448	19.269	43.628
7.813	34.960	23.442	46.477
15.625	36.921	25.796	48.047
31.250	30.988	18.010	43.966

Chemical.2

Lab B

S:ASS

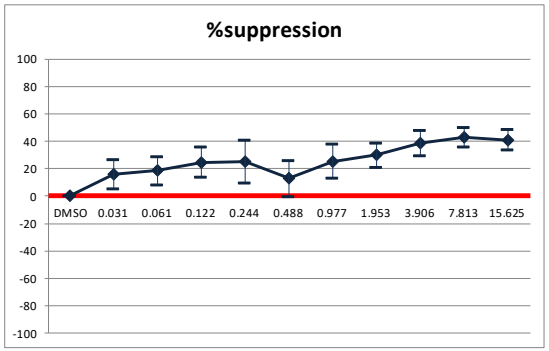
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.031	-32.822	-54.831	-10.812
0.061	-47.466	-71.673	-23.258
0.122	-39.276	-63.616	-14.936
0.244	-29.590	-54.133	-5.048
0.488	-24.319	-46.849	-1.788
0.977	-29.744	-52.719	-6.770
1.953	-28.997	-50.380	-7.613
3.906	-10.800	-32.104	10.504
7.813	-7.519	-26.140	11.103
15.625	-2.368	-23.303	18.567

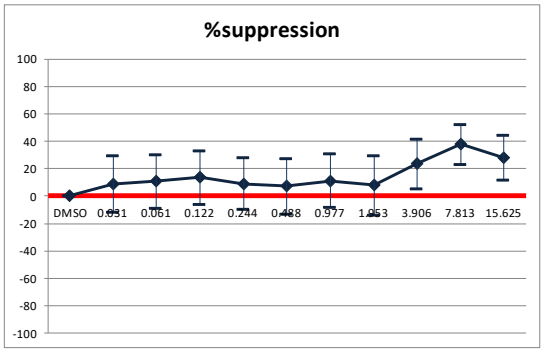
3rd Exp.



3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.031	15.791	5.160	26.422
0.061	18.476	8.167	28.785
0.122	24.737	13.864	35.610
0.244	25.023	9.541	40.505
0.488	12.760	-0.379	25.899
0.977	25.432	12.888	37.976
1.953	29.798	20.840	38.755
3.906	38.826	29.535	48.118
7.813	43.095	36.050	50.140
15.625	41.116	33.620	48.612

4th Exp.



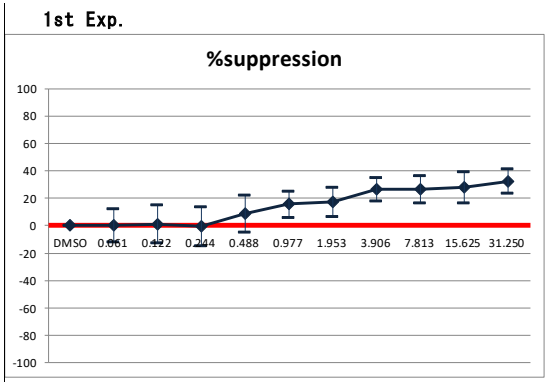
4th Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.031	8.866	-12.002	29.734
0.061	10.748	-8.875	30.371
0.122	13.754	-5.738	33.246
0.244	8.895	-9.935	27.724
0.488	7.176	-13.133	27.484
0.977	11.266	-8.182	30.713
1.953	7.780	-13.813	29.373
3.906	23.539	5.583	41.494
7.813	37.710	22.985	52.434
15.625	27.968	11.793	44.143

Lab C

S:NSS

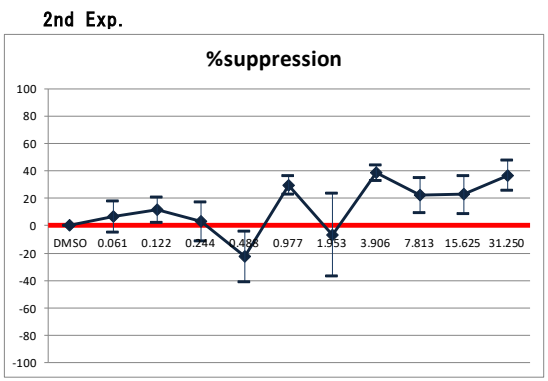
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.061	0.328	-11.575	12.230
0.122	1.378	-12.190	14.947
0.244	-0.458	-14.484	13.569
0.488	8.841	-4.402	22.083
0.977	15.586	5.680	25.492
1.953	17.599	7.004	28.194
3.906	26.646	18.156	35.137
7.813	26.295	16.325	36.266
15.625	28.020	16.836	39.204
31.250	32.425	23.693	41.156

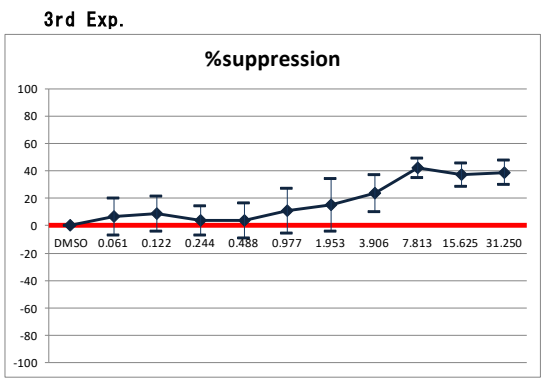
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.061	6.572	-4.804	17.949
0.122	11.632	2.266	20.997
0.244	2.843	-11.384	17.070
0.488	-22.238	-40.539	-3.936
0.977	29.646	23.027	36.265
1.953	-6.726	-36.862	23.410
3.906	38.477	32.966	43.988
7.813	22.488	9.829	35.147
15.625	22.784	8.966	36.603
31.250	36.695	25.603	47.787

3rd Exp.



3rd Exp.

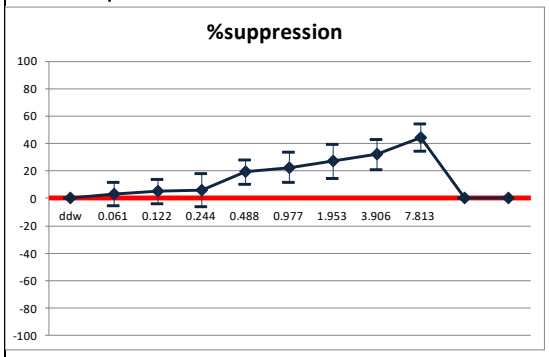
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.061	6.875	-6.692	20.442
0.122	8.540	-4.242	21.323
0.244	3.866	-7.043	14.774
0.488	4.036	-8.732	16.803
0.977	10.768	-5.591	27.127
1.953	15.365	-3.726	34.456
3.906	23.743	9.993	37.493
7.813	41.962	34.911	49.014
15.625	37.194	28.464	45.924
31.250	38.873	29.874	47.873

Chemical.3

Lead Lab.

N:SNN

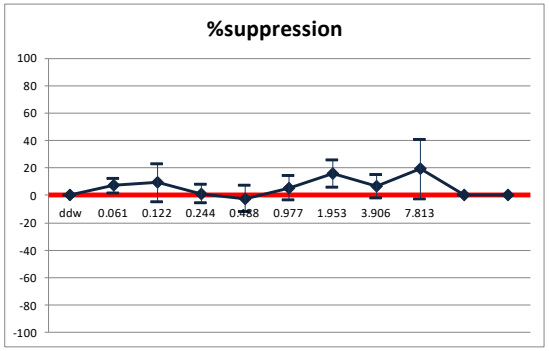
3rd Exp.



3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.061	3.378	-5.140	11.896
0.122	5.074	-3.797	13.946
0.244	5.997	-6.162	18.155
0.488	19.376	10.403	28.350
0.977	22.595	11.778	33.412
1.953	27.025	14.424	39.627
3.906	31.938	20.866	43.011
7.813	44.316	34.513	54.119

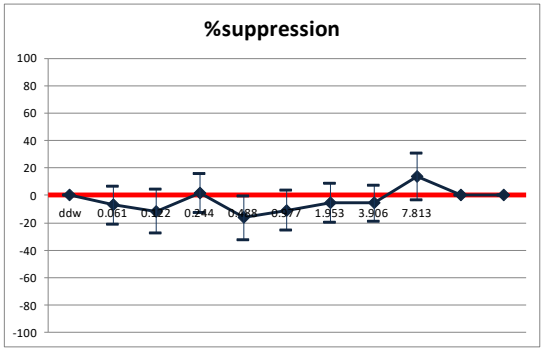
4th Exp.



4th Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.061	7.090	1.745	12.435
0.122	9.344	-4.602	23.290
0.244	1.341	-5.349	8.030
0.488	-2.186	-11.922	7.551
0.977	5.446	-3.352	14.243
1.953	15.845	6.146	25.544
3.906	6.625	-1.695	14.945
7.813	19.413	-2.225	41.051

6th Exp.



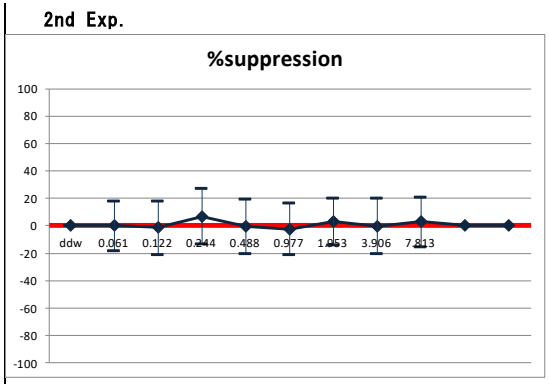
6th Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.061	-6.995	-21.049	7.059
0.122	-11.412	-27.359	4.534
0.244	1.862	-12.320	16.043
0.488	-16.335	-32.034	-0.636
0.977	-10.910	-25.502	3.682
1.953	-5.631	-19.814	8.551
3.906	-5.534	-18.629	7.561
7.813	13.759	-3.345	30.864

Lab A

N:NNN

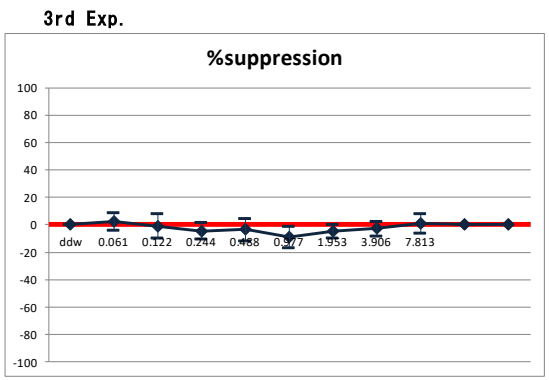
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.061	0.123	-17.788	18.034
0.122	-1.393	-20.851	18.066
0.244	6.983	-12.985	26.951
0.488	-0.639	-20.506	19.228
0.977	-2.181	-21.082	16.720
1.953	3.154	-13.825	20.133
3.906	-0.157	-20.226	19.912
7.813	2.924	-15.093	20.942

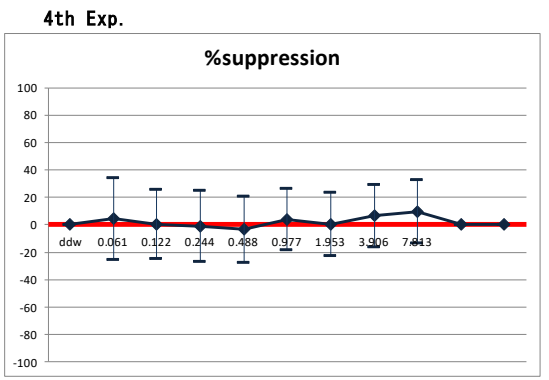
3rd Exp.



3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.061	2.679	-3.661	9.019
0.122	-0.971	-9.856	7.914
0.244	-4.319	-10.248	1.609
0.488	-3.335	-11.503	4.833
0.977	-8.689	-16.560	-0.817
1.953	-4.746	-9.820	0.327
3.906	-2.822	-7.876	2.232
7.813	1.096	-6.178	8.370

4th Exp.



4th Exp.

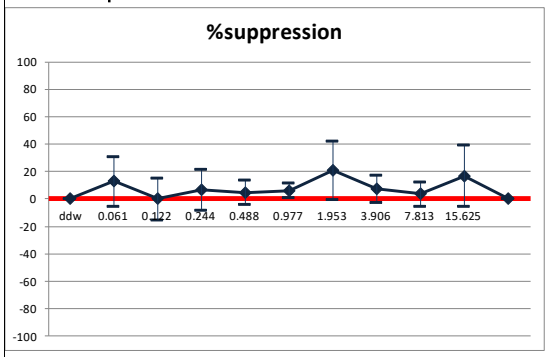
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.061	4.611	-25.508	34.730
0.122	0.587	-24.587	25.761
0.244	-1.028	-27.001	24.945
0.488	-3.251	-27.612	21.110
0.977	4.094	-18.165	26.354
1.953	0.574	-22.387	23.534
3.906	6.478	-16.120	29.077
7.813	9.855	-12.944	32.653

Chemical.3

Lab B

N:NSN

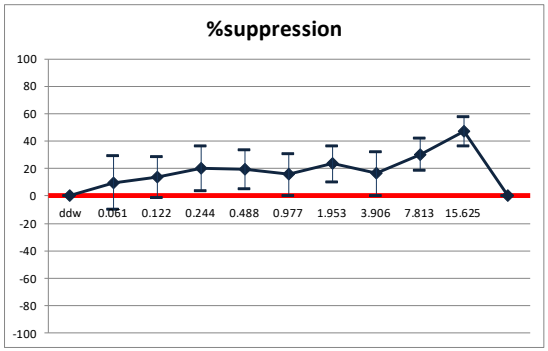
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.061	12.918	-5.099	30.935
0.122	-0.030	-15.518	15.457
0.244	6.808	-8.026	21.643
0.488	4.641	-4.177	13.458
0.977	6.227	0.927	11.527
1.953	20.931	-0.241	42.104
3.906	7.450	-2.654	17.554
7.813	3.582	-5.551	12.715
15.625	16.896	-5.373	39.164

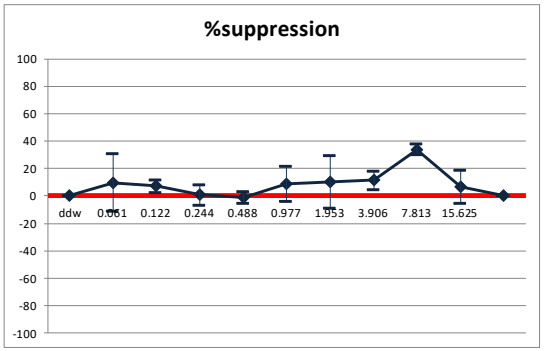
3rd Exp.



3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.061	9.758	-9.612	29.129
0.122	13.695	-1.434	28.824
0.244	20.373	4.082	36.663
0.488	19.551	5.116	33.986
0.977	15.776	0.537	31.014
1.953	23.483	10.414	36.551
3.906	16.403	0.453	32.354
7.813	30.418	18.689	42.148
15.625	47.487	36.819	58.155

4th Exp.



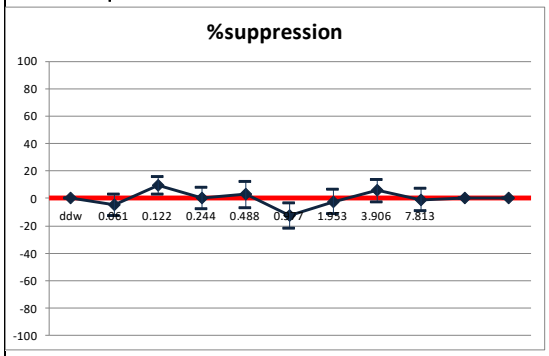
4th Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.061	9.695	-11.120	30.509
0.122	7.180	2.600	11.761
0.244	0.740	-6.491	7.972
0.488	-1.101	-5.350	3.147
0.977	9.102	-3.705	21.909
1.953	10.447	-8.761	29.654
3.906	11.630	4.842	18.418
7.813	34.032	30.259	37.806
15.625	6.881	-5.012	18.774

Lab C

N:NAN

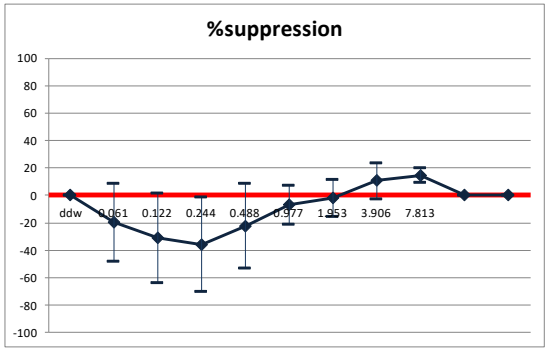
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.061	-4.575	-12.171	3.021
0.122	9.810	3.356	16.265
0.244	0.219	-7.611	8.049
0.488	2.804	-6.648	12.257
0.977	-12.298	-21.419	-3.178
1.953	-2.333	-11.156	6.490
3.906	5.854	-2.402	14.110
7.813	-0.773	-9.239	7.694

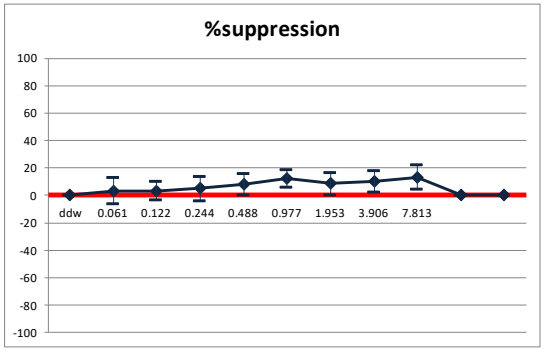
3rd Exp.



3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.061	-19.548	-47.632	8.537
0.122	-30.865	-63.417	1.686
0.244	-35.585	-69.756	-1.414
0.488	-22.381	-53.252	8.490
0.977	-6.700	-20.892	7.492
1.953	-1.832	-15.216	11.552
3.906	10.716	-2.336	23.768
7.813	14.873	9.839	19.906

4th Exp.



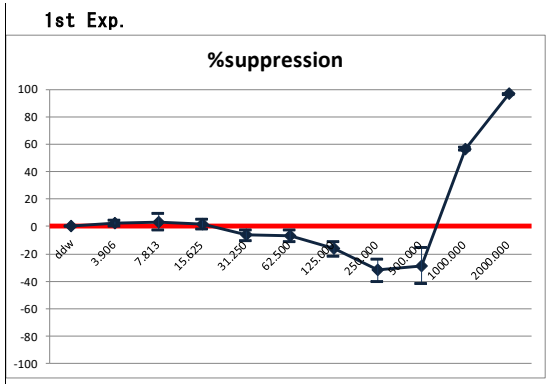
4th Exp.

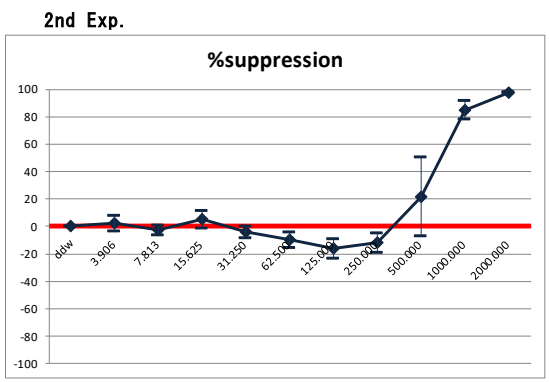
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
0.061	3.474	-5.833	12.780
0.122	3.339	-3.475	10.154
0.244	4.978	-3.897	13.853
0.488	8.094	0.253	15.936
0.977	12.250	5.793	18.707
1.953	8.519	0.333	16.704
3.906	10.525	2.727	18.323
7.813	13.302	4.411	22.192

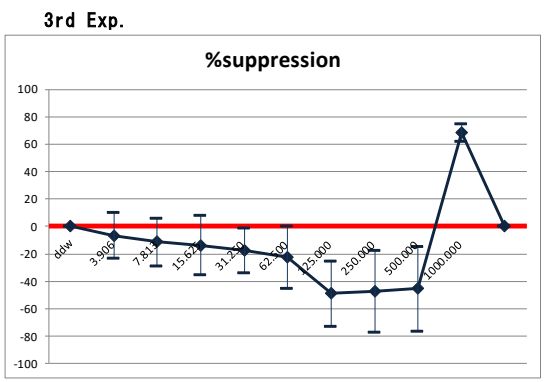
Chemical.4

Lead Lab.

S:SSA







1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	2.394	0.344	4.445
7.813	3.369	-2.762	9.500
15.625	1.516	-1.993	5.026
31.250	-6.401	-10.377	-2.424
62.500	-7.016	-11.213	-2.818
125.000	-16.202	-21.357	-11.047
250.000	-31.840	-39.946	-23.735
500.000	-28.617	-41.749	-15.485
1000.000	56.537	55.560	57.515
2000.000	96.800	96.451	97.150

2nd Exp.

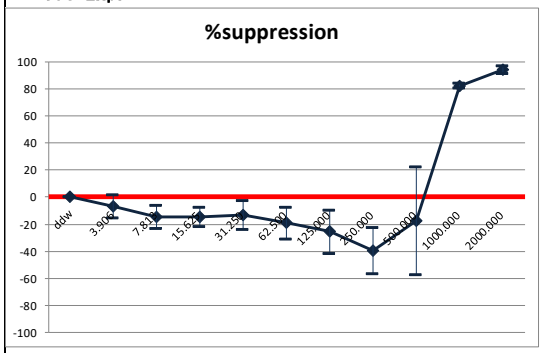
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	2.524	-3.101	8.149
7.813	-2.312	-5.934	1.311
15.625	5.293	-0.797	11.382
31.250	-3.955	-8.461	0.550
62.500	-9.723	-15.356	-4.091
125.000	-15.848	-23.054	-8.642
250.000	-11.676	-18.895	-4.457
500.000	21.926	-6.997	50.849
1000.000	85.141	78.393	91.888
2000.000	97.904	97.331	98.477

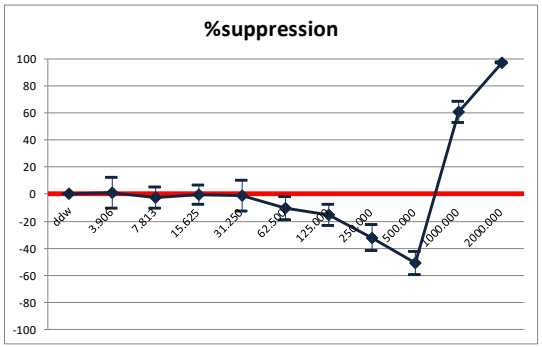
3rd Exp.

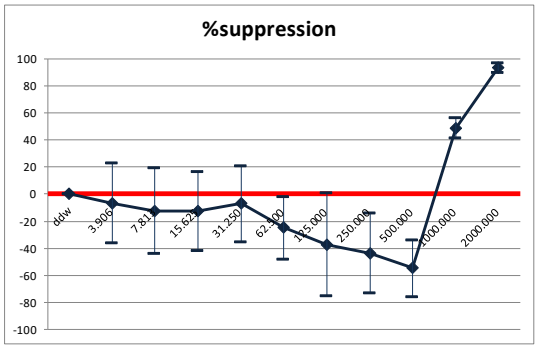
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-6.551	-23.043	9.942
7.813	-11.184	-28.677	6.309
15.625	-13.665	-35.113	7.784
31.250	-17.628	-33.926	-1.329
62.500	-22.156	-44.852	0.541
125.000	-48.850	-72.750	-24.950
250.000	-47.524	-77.388	-17.660
500.000	-45.373	-76.432	-14.314
1000.000	68.509	62.099	74.918

Lab A

A/S:A/S A/S A/S







1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-6.651	-15.175	1.873
7.813	-14.605	-22.910	-6.300
15.625	-14.425	-21.676	-7.173
31.250	-13.145	-23.563	-2.726
62.500	-19.146	-30.768	-7.525
125.000	-25.514	-41.362	-9.665
250.000	-39.240	-56.298	-22.183
500.000	-17.485	-57.087	22.118
1000.000	82.071	80.199	83.942
2000.000	93.817	91.071	96.564

2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	1.352	-10.018	12.721
7.813	-2.551	-10.581	5.480
15.625	-0.335	-7.480	6.810
31.250	-1.106	-12.119	9.906
62.500	-10.457	-18.786	-2.127
125.000	-15.563	-23.378	-7.748
250.000	-32.134	-41.806	-22.462
500.000	-50.751	-59.257	-42.245
1000.000	60.436	52.733	68.139
2000.000	97.183	96.845	97.521

3rd Exp.

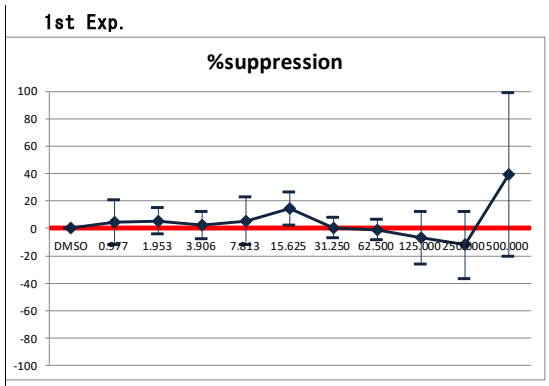
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-6.639	-35.992	22.714
7.813	-12.237	-43.777	19.304
15.625	-12.254	-41.309	16.800
31.250	-7.094	-35.205	21.017
62.500	-24.788	-47.681	-1.894
125.000	-37.010	-75.118	1.097
250.000	-43.447	-72.866	-14.029
500.000	-54.706	-75.906	-33.507
1000.000	48.813	41.220	56.405
2000.000	93.187	89.614	96.761

Chemical.4

Lab B

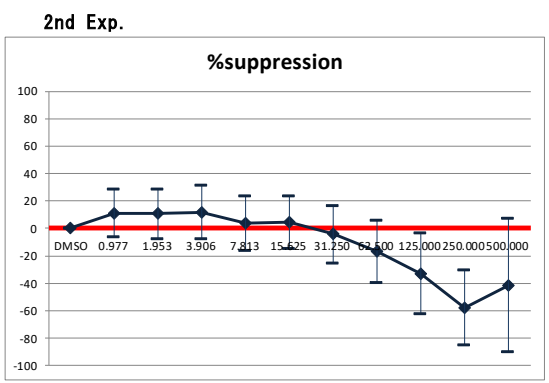
A:NAA

1st Exp.



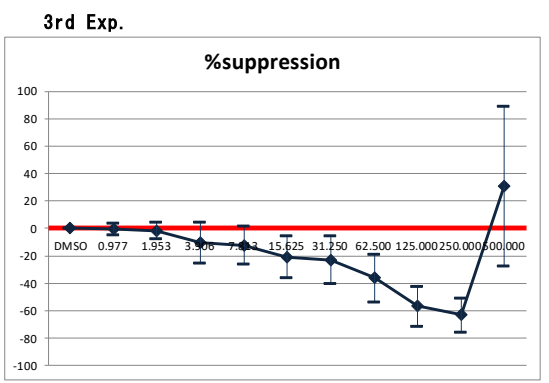
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	4.482	-11.811	20.776
1.953	5.506	-4.134	15.145
3.906	2.545	-7.390	12.480
7.813	5.639	-11.658	22.936
15.625	14.256	2.130	26.382
31.250	0.563	-6.750	7.877
62.500	-0.754	-8.225	6.717
125.000	-6.624	-25.927	12.679
250.000	-11.865	-36.461	12.731
500.000	39.428	-20.186	99.042

2nd Exp.



a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	11.299	-5.835	28.433
1.953	10.736	-7.496	28.968
3.906	11.943	-7.715	31.602
7.813	3.788	-16.208	23.784
15.625	4.492	-14.424	23.407
31.250	-4.135	-24.938	16.668
62.500	-16.613	-39.308	6.081
125.000	-32.870	-62.209	-3.531
250.000	-57.685	-84.903	-30.467
500.000	-41.447	-89.983	7.090

3rd Exp.

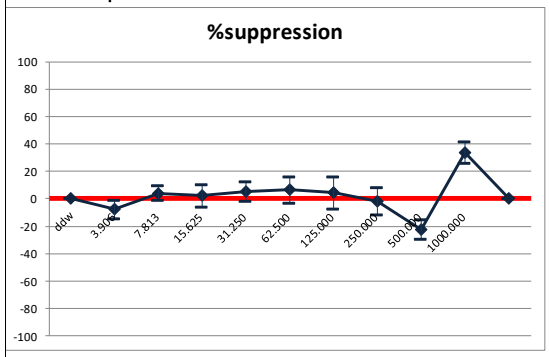


a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-0.612	-4.930	3.705
1.953	-1.592	-7.496	4.313
3.906	-9.996	-24.899	4.907
7.813	-12.392	-26.291	1.507
15.625	-20.927	-36.216	-5.638
31.250	-23.084	-40.504	-5.663
62.500	-36.012	-53.456	-18.568
125.000	-56.801	-71.519	-42.083
250.000	-62.994	-75.361	-50.628
500.000	30.754	-27.524	89.031

Lab C

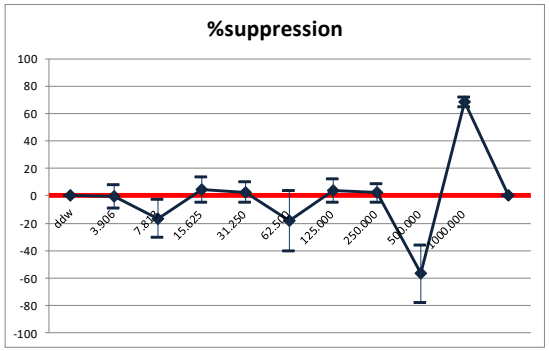
N:NNA

1st Exp.



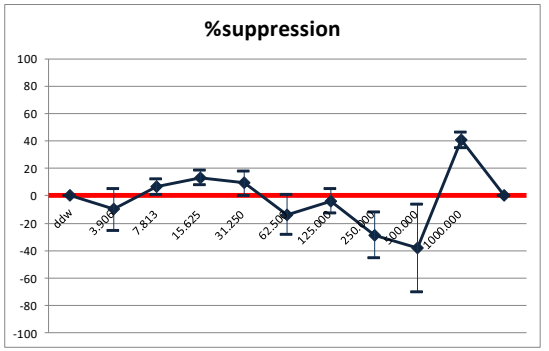
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-7.810	-14.689	-0.932
7.813	4.090	-1.168	9.348
15.625	2.174	-6.218	10.567
31.250	5.126	-1.799	12.051
62.500	6.602	-2.945	16.149
125.000	4.365	-7.291	16.021
250.000	-1.986	-11.989	8.016
500.000	-22.561	-29.701	-15.421
1000.000	33.653	25.562	41.745

2nd Exp.



a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-0.195	-8.683	8.293
7.813	-16.391	-30.094	-2.688
15.625	4.842	-4.469	14.154
31.250	2.797	-4.622	10.216
62.500	-18.247	-40.416	3.923
125.000	3.556	-5.004	12.117
250.000	2.161	-4.515	8.837
500.000	-56.803	-77.704	-35.902
1000.000	68.458	65.012	71.904

3rd Exp.



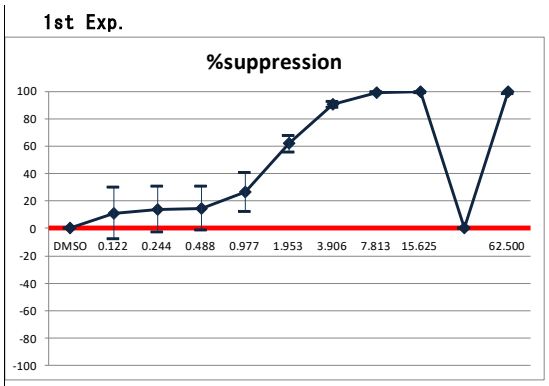
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-9.717	-24.908	5.474
7.813	6.984	1.250	12.718
15.625	13.177	7.897	18.457
31.250	9.291	0.160	18.422
62.500	-13.653	-28.137	0.832
125.000	-3.845	-12.794	5.103
250.000	-28.510	-45.322	-11.698
500.000	-38.227	-70.196	-6.259
1000.000	40.759	34.952	46.566

Chemical.5

Lead Lab.

S:SSS

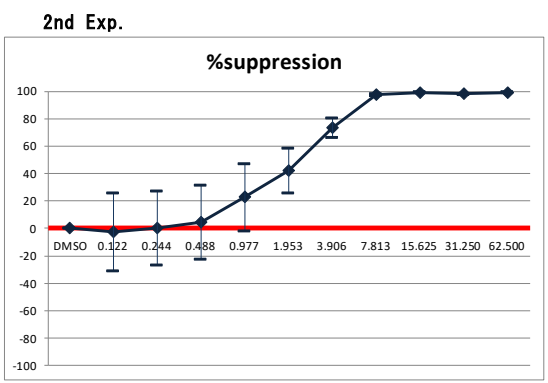
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	11.150	-7.667	29.966
0.244	14.121	-2.775	31.016
0.488	14.809	-1.019	30.636
0.977	26.533	12.336	40.730
1.953	61.893	55.870	67.916
3.906	90.615	88.496	92.735
7.813	99.279	99.031	99.528
15.625	99.422	99.017	99.827
62.500	99.380	98.629	100.132

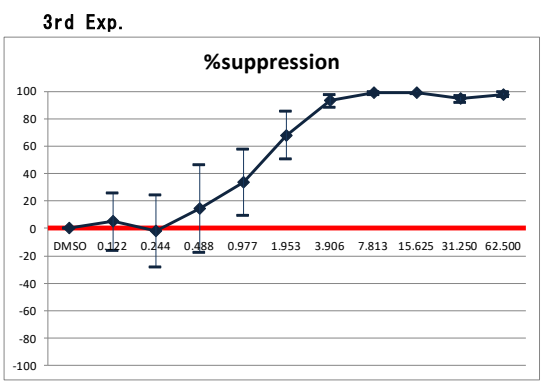
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	-2.385	-30.717	25.947
0.244	0.134	-26.752	27.020
0.488	4.375	-22.721	31.470
0.977	22.701	-1.697	47.099
1.953	42.246	26.188	58.303
3.906	73.246	66.031	80.460
7.813	97.737	96.821	98.653
15.625	99.225	98.970	99.480
31.250	98.044	97.569	98.520
62.500	99.346	99.060	99.631

3rd Exp.



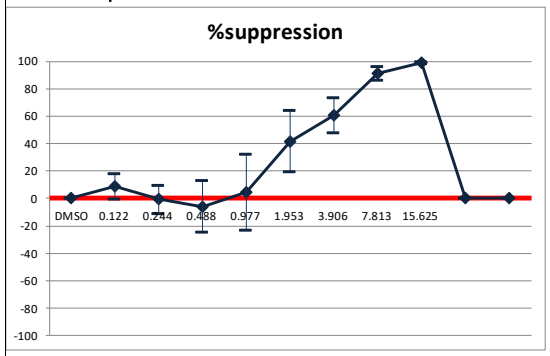
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	5.038	-15.987	26.063
0.244	-1.802	-28.135	24.531
0.488	14.453	-17.464	46.369
0.977	33.710	9.773	57.647
1.953	68.117	50.854	85.379
3.906	93.175	88.469	97.880
7.813	98.674	97.620	99.727
15.625	99.031	97.989	100.074
31.250	94.401	91.965	96.837
62.500	97.808	96.027	99.590

Lab A

S:SSS

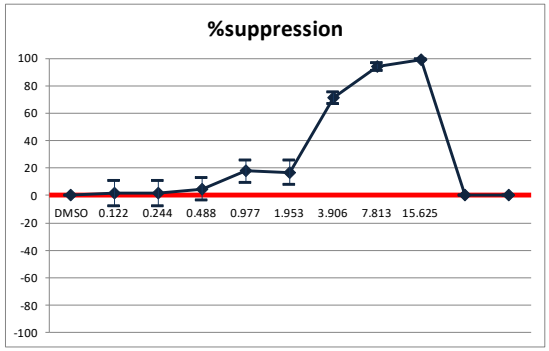
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	8.716	-0.579	18.011
0.244	-0.730	-11.296	9.835
0.488	-5.852	-24.595	12.891
0.977	4.807	-22.896	32.511
1.953	41.630	19.143	64.118
3.906	60.724	47.878	73.570
7.813	91.397	86.525	96.269
15.625	98.771	98.157	99.386

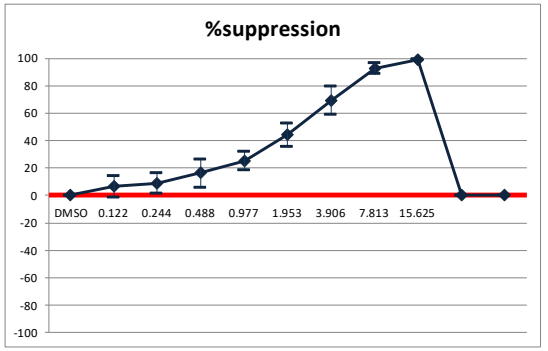
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	1.705	-7.544	10.954
0.244	1.805	-7.631	11.241
0.488	4.725	-3.529	12.979
0.977	17.762	9.424	26.099
1.953	16.803	7.870	25.735
3.906	71.488	67.358	75.618
7.813	94.010	91.181	96.839
15.625	99.284	98.994	99.574

3rd Exp.



3rd Exp.

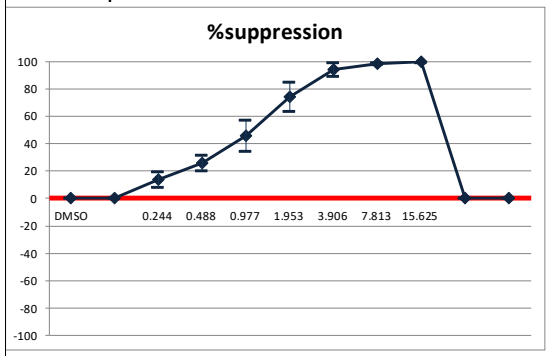
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	6.843	-0.841	14.527
0.244	8.914	1.420	16.407
0.488	16.523	6.341	26.704
0.977	25.511	18.772	32.249
1.953	44.463	36.098	52.828
3.906	69.464	59.255	79.673
7.813	92.930	88.875	96.984
15.625	99.328	98.930	99.726

Chemical.5

Lab B

S:SSS

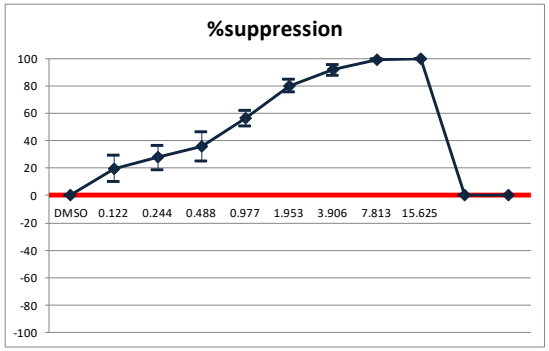
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	13.709	7.995	19.422
0.488	25.936	20.218	31.654
0.977	45.696	34.539	56.853
1.953	74.090	63.498	84.683
3.906	93.995	89.045	98.945
7.813	98.174	97.409	98.939
15.625	99.599	99.180	100.018

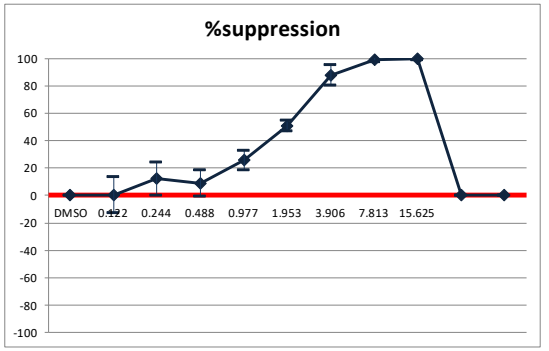
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	19.662	10.197	29.128
0.244	27.687	18.925	36.449
0.488	35.940	25.067	46.813
0.977	56.527	50.678	62.377
1.953	80.185	75.389	84.982
3.906	91.656	87.828	95.483
7.813	98.804	97.964	99.644
15.625	99.735	99.539	99.930

3rd Exp.



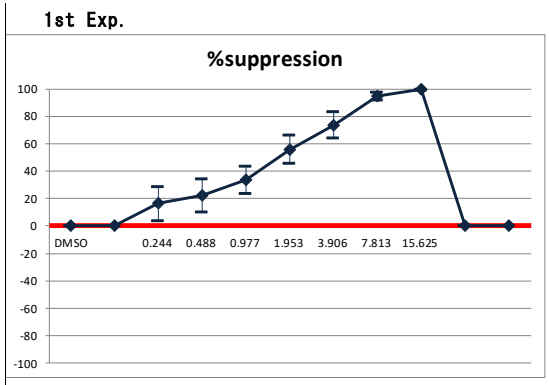
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	0.608	-12.608	13.825
0.244	12.402	0.593	24.212
0.488	9.026	-0.376	18.429
0.977	25.881	19.123	32.640
1.953	51.064	46.988	55.141
3.906	87.995	80.420	95.571
7.813	98.823	97.345	100.300
15.625	99.807	99.204	100.411

Lab C

S:SSS

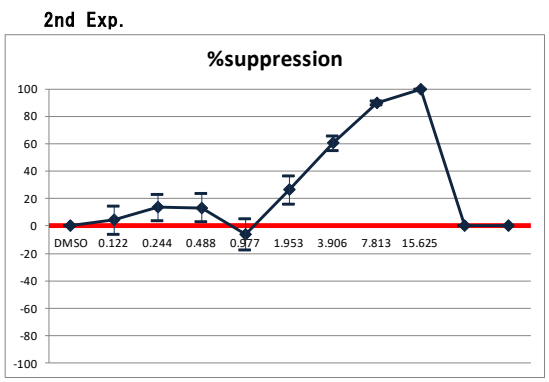
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	16.549	4.054	29.044
0.488	22.383	10.601	34.165
0.977	33.551	23.559	43.543
1.953	55.888	45.738	66.039
3.906	73.591	64.107	83.074
7.813	94.860	91.955	97.764
15.625	99.599	99.354	99.844

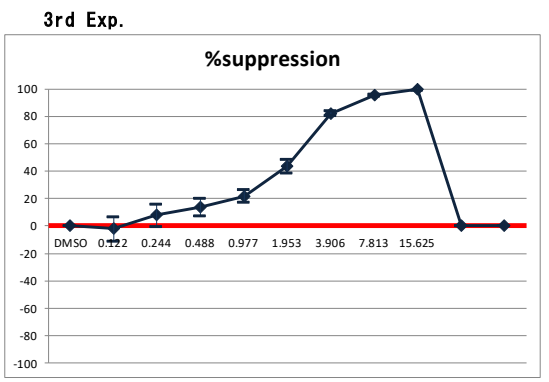
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	4.423	-5.859	14.705
0.244	13.475	4.015	22.936
0.488	13.259	2.893	23.625
0.977	-6.115	-17.331	5.101
1.953	26.348	16.109	36.588
3.906	60.450	55.159	65.740
7.813	89.916	88.406	91.426
15.625	99.697	99.462	99.933

3rd Exp.



3rd Exp.

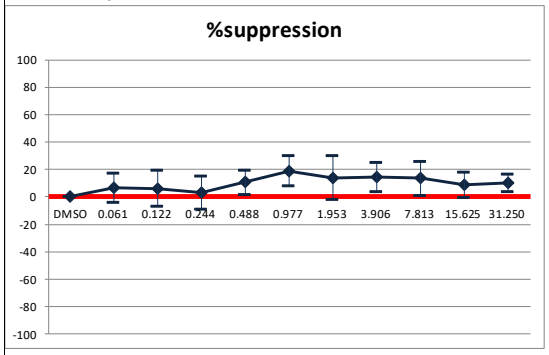
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	-2.073	-10.759	6.612
0.244	7.954	-0.177	16.085
0.488	13.997	7.523	20.472
0.977	21.890	17.035	26.746
1.953	43.727	38.961	48.492
3.906	82.162	80.325	83.999
7.813	95.553	94.817	96.288
15.625	99.792	99.265	100.319

Chemical.6

Lead Lab.

N:NNN

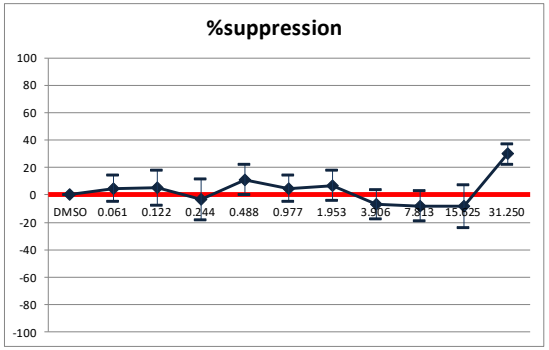
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.061	6.517	-4.149	17.183
0.122	6.225	-7.086	19.537
0.244	3.149	-9.251	15.548
0.488	10.704	1.616	19.792
0.977	19.072	7.810	30.334
1.953	14.126	-2.045	30.298
3.906	14.570	4.171	24.969
7.813	13.673	1.267	26.079
15.625	9.093	-0.219	18.406
31.250	10.086	3.532	16.640

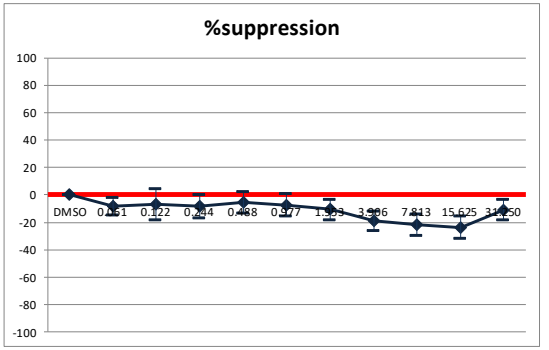
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.061	4.783	-4.601	14.166
0.122	5.281	-7.580	18.142
0.244	-3.324	-18.043	11.394
0.488	11.232	0.210	22.254
0.977	4.806	-4.904	14.515
1.953	6.835	-4.053	17.723
3.906	-6.786	-17.273	3.701
7.813	-7.938	-19.020	3.144
15.625	-8.440	-24.154	7.274
31.250	29.935	22.386	37.485

3rd Exp.



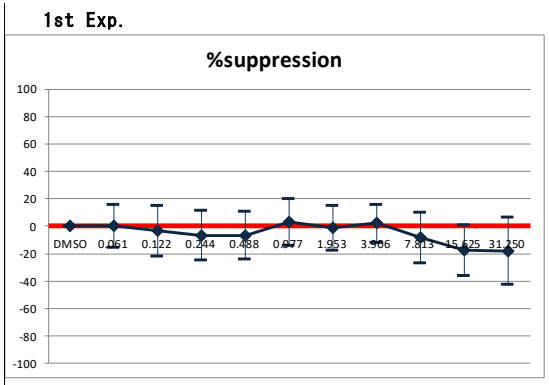
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.061	-8.151	-14.623	-1.679
0.122	-6.850	-18.080	4.380
0.244	-8.306	-16.721	0.109
0.488	-5.264	-13.136	2.609
0.977	-7.433	-15.616	0.749
1.953	-10.571	-18.077	-3.066
3.906	-18.821	-25.699	-11.943
7.813	-21.763	-29.657	-13.868
15.625	-23.733	-31.880	-15.586
31.250	-10.823	-18.434	-3.213

Lab A

N:NNN

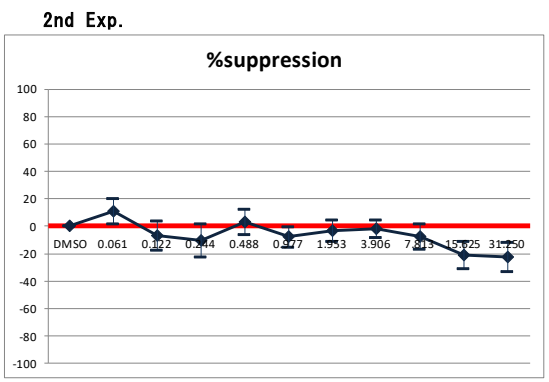
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.061	0.091	-15.471	15.652
0.122	-3.089	-21.722	15.544
0.244	-6.429	-24.262	11.404
0.488	-6.502	-24.122	11.118
0.977	3.343	-13.756	20.443
1.953	-1.052	-17.256	15.153
3.906	2.186	-11.411	15.784
7.813	-8.323	-26.821	10.174
15.625	-17.379	-35.698	0.940
31.250	-17.886	-42.236	6.465

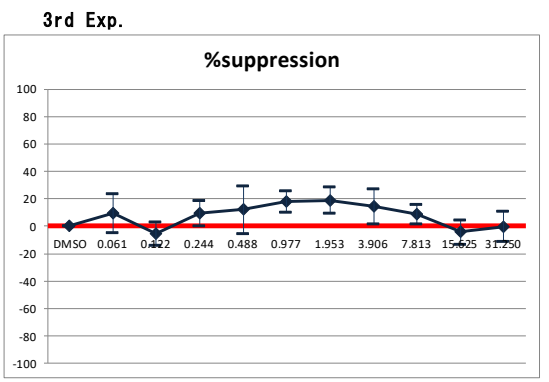
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.061	11.081	1.833	20.329
0.122	-6.665	-17.233	3.902
0.244	-10.174	-22.077	1.728
0.488	3.356	-5.858	12.571
0.977	-7.718	-15.377	-0.059
1.953	-3.058	-11.015	4.899
3.906	-1.705	-8.252	4.841
7.813	-7.270	-16.471	1.932
15.625	-21.093	-30.945	-11.240
31.250	-22.587	-33.234	-11.940

3rd Exp.



3rd Exp.

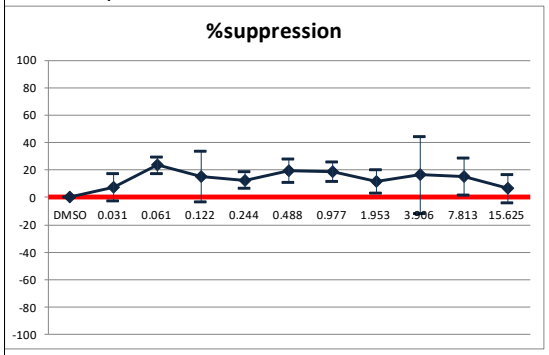
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.061	9.419	-4.917	23.755
0.122	-5.329	-13.777	3.119
0.244	9.554	0.082	19.025
0.488	12.054	-5.281	29.389
0.977	18.013	10.290	25.736
1.953	19.130	9.573	28.686
3.906	14.600	1.694	27.506
7.813	8.983	1.754	16.212
15.625	-4.150	-13.151	4.850
31.250	-0.067	-10.904	10.770

Chemical.6

Lab B

N:NNN

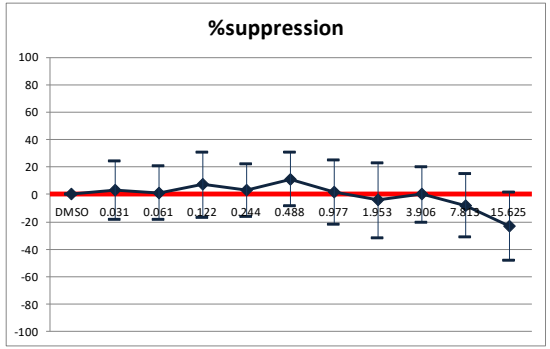
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.031	7.242	-2.849	17.333
0.061	23.665	17.610	29.721
0.122	15.165	-3.054	33.385
0.244	12.692	6.443	18.941
0.488	19.489	11.046	27.933
0.977	18.930	11.985	25.874
1.953	11.857	3.260	20.454
3.906	16.440	-11.644	44.525
7.813	14.979	1.437	28.522
15.625	6.442	-3.907	16.791

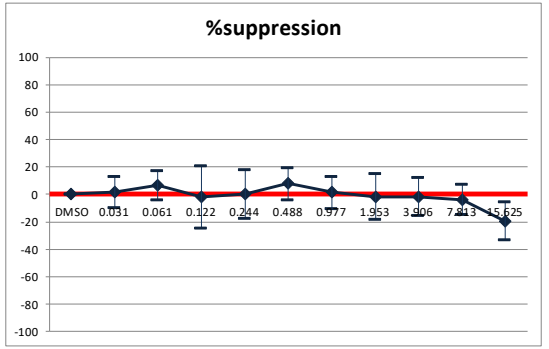
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.031	3.240	-17.845	24.326
0.061	1.136	-18.356	20.629
0.122	7.102	-16.627	30.831
0.244	3.189	-16.208	22.586
0.488	11.314	-7.921	30.548
0.977	1.640	-21.984	25.264
1.953	-4.286	-31.489	22.918
3.906	-0.012	-20.504	20.480
7.813	-7.911	-30.897	15.075
15.625	-23.273	-47.972	1.426

3rd Exp.



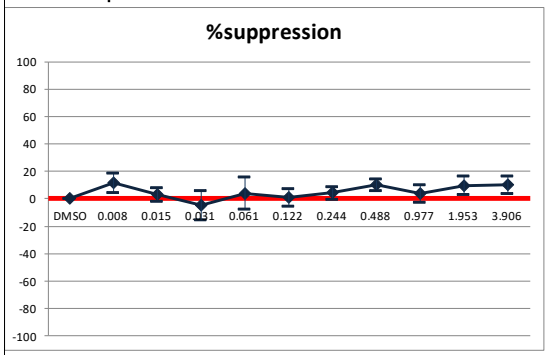
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.031	1.945	-9.445	13.335
0.061	6.695	-3.649	17.040
0.122	-1.915	-24.884	21.053
0.244	0.071	-17.717	17.859
0.488	7.973	-3.799	19.745
0.977	1.548	-10.113	13.210
1.953	-1.752	-18.434	14.930
3.906	-1.466	-15.240	12.307
7.813	-3.764	-14.842	7.314
15.625	-19.292	-33.159	-5.425

Lab C

N:NNN

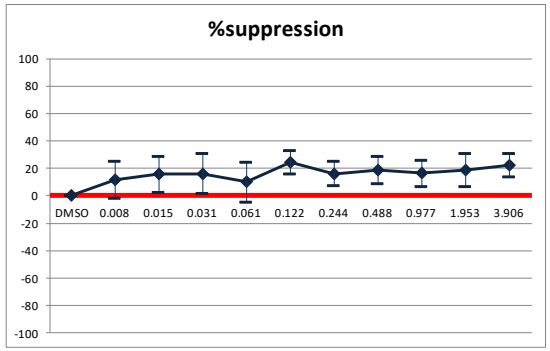
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.008	11.495	4.395	18.596
0.015	3.125	-1.856	8.105
0.031	-4.690	-15.158	5.779
0.061	4.173	-7.328	15.674
0.122	1.109	-5.139	7.358
0.244	4.408	-0.361	9.178
0.488	10.430	6.114	14.747
0.977	3.778	-2.484	10.040
1.953	9.817	2.888	16.746
3.906	10.062	3.748	16.375

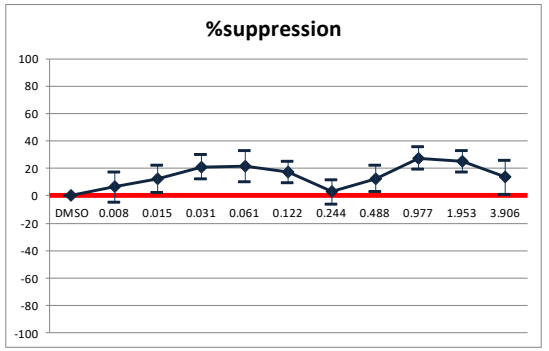
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.008	11.879	-1.634	25.392
0.015	15.745	2.707	28.784
0.031	16.044	1.445	30.642
0.061	9.960	-4.606	24.526
0.122	24.475	16.101	32.848
0.244	16.193	7.083	25.302
0.488	18.802	8.824	28.779
0.977	16.301	6.855	25.746
1.953	18.652	6.725	30.579
3.906	22.554	14.162	30.946

4th Exp.



4th Exp.

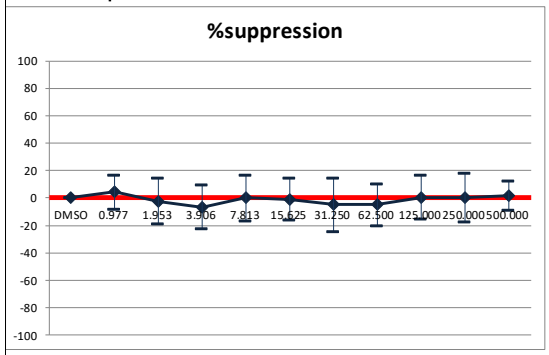
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.008	6.382	-4.306	17.070
0.015	12.206	2.436	21.976
0.031	21.178	12.385	29.971
0.061	21.515	10.336	32.693
0.122	17.218	9.295	25.141
0.244	3.059	-5.874	11.991
0.488	12.613	3.194	22.031
0.977	27.356	19.195	35.518
1.953	25.228	17.603	32.853
3.906	13.585	1.378	25.793

Chemical.7

Lead Lab.

N:NNN

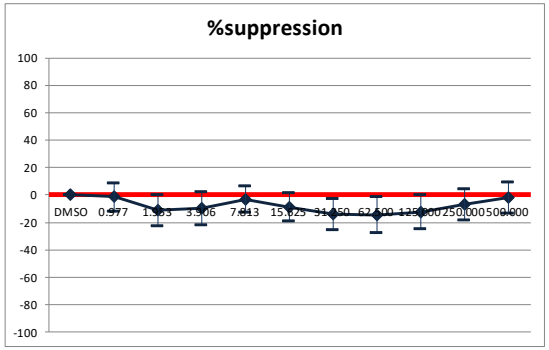
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	4.280	-8.358	16.918
1.953	-2.207	-18.588	14.173
3.906	-6.540	-22.415	9.335
7.813	0.132	-16.437	16.700
15.625	-0.748	-15.865	14.369
31.250	-4.813	-24.488	14.861
62.500	-4.945	-20.495	10.605
125.000	0.562	-15.405	16.528
250.000	0.220	-17.690	18.131
500.000	1.619	-8.959	12.198

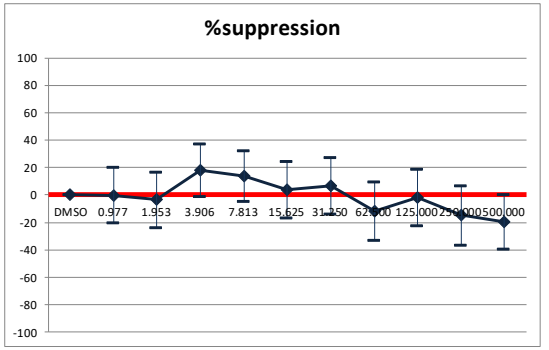
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-1.370	-11.548	8.808
1.953	-11.359	-22.687	-0.032
3.906	-9.562	-21.856	2.731
7.813	-2.972	-12.446	6.503
15.625	-8.803	-19.128	1.522
31.250	-14.002	-25.296	-2.709
62.500	-14.511	-27.697	-1.325
125.000	-12.268	-24.506	-0.029
250.000	-6.686	-18.008	4.636
500.000	-1.944	-13.128	9.240

3rd Exp.



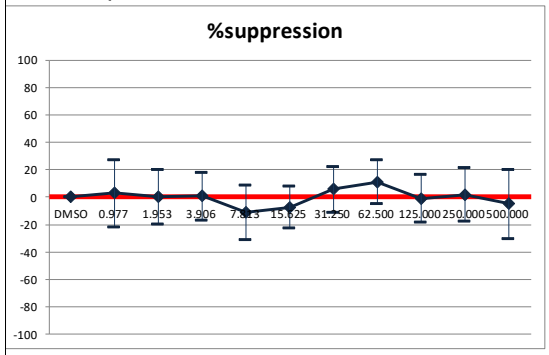
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-0.254	-20.496	19.988
1.953	-3.437	-23.530	16.656
3.906	18.345	-0.829	37.520
7.813	13.876	-4.592	32.344
15.625	3.871	-16.453	24.194
31.250	6.636	-14.070	27.342
62.500	-11.877	-33.334	9.581
125.000	-1.974	-22.699	18.752
250.000	-14.729	-36.398	6.940
500.000	-19.447	-39.418	0.525

Lab A

N:NNN

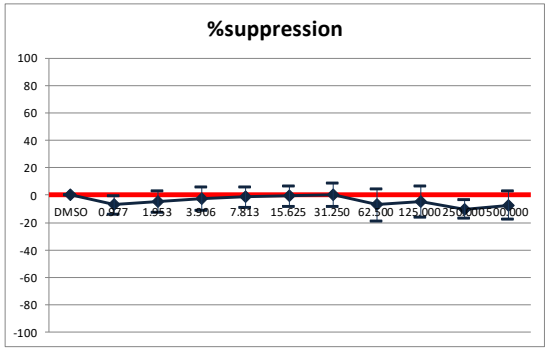
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	2.839	-21.654	27.332
1.953	0.649	-19.218	20.516
3.906	0.846	-16.560	18.252
7.813	-11.236	-31.094	8.622
15.625	-7.176	-22.555	8.203
31.250	5.711	-10.789	22.211
62.500	11.200	-4.600	27.000
125.000	-1.001	-18.325	16.323
250.000	2.057	-17.617	21.731
500.000	-4.876	-30.045	20.293

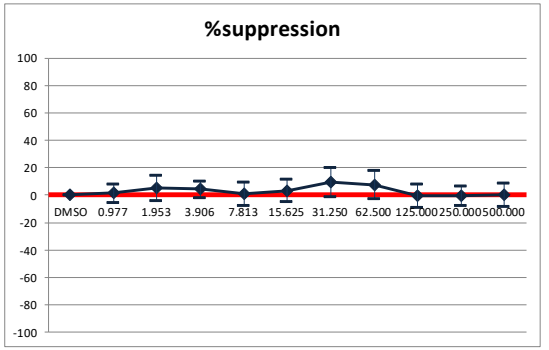
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-7.079	-13.710	-0.448
1.953	-4.534	-12.200	3.132
3.906	-2.530	-10.729	5.669
7.813	-1.270	-8.889	6.350
15.625	-0.604	-8.162	6.954
31.250	0.384	-8.357	9.124
62.500	-6.972	-18.795	4.850
125.000	-4.768	-15.925	6.389
250.000	-10.007	-16.985	-3.029
500.000	-7.177	-17.511	3.156

3rd Exp.



3rd Exp.

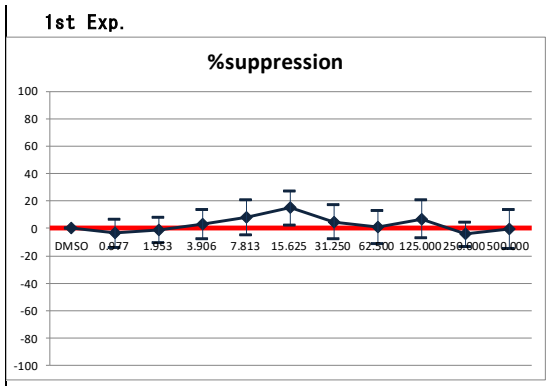
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	1.471	-5.209	8.152
1.953	5.199	-4.258	14.656
3.906	4.388	-1.748	10.524
7.813	1.195	-7.302	9.692
15.625	3.336	-4.951	11.622
31.250	9.460	-1.131	20.050
62.500	7.769	-2.238	17.776
125.000	-0.240	-8.559	8.078
250.000	-0.382	-7.467	6.702
500.000	0.252	-8.496	9.000

Chemical.7

Lab B

N:NNN

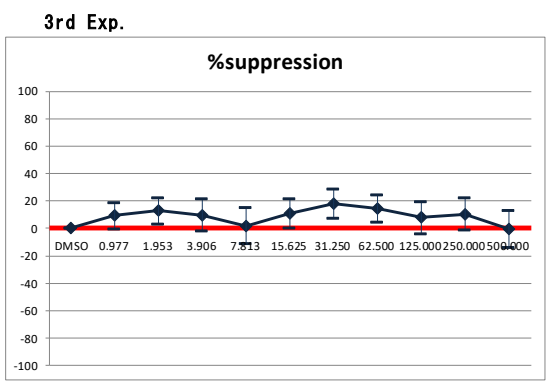
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-3.431	-13.677	6.814
1.953	-1.044	-10.023	7.934
3.906	3.337	-7.353	14.027
7.813	8.121	-4.822	21.063
15.625	15.006	2.767	27.246
31.250	4.730	-7.612	17.073
62.500	1.056	-11.263	13.375
125.000	7.036	-6.513	20.584
250.000	-3.971	-12.819	4.877
500.000	-0.137	-14.310	14.035

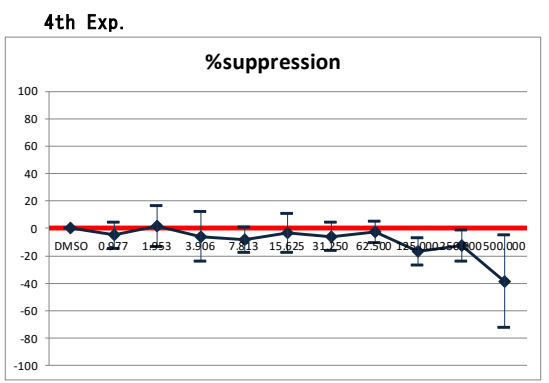
3rd Exp.



3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	9.319	-0.287	18.924
1.953	12.822	3.234	22.410
3.906	9.901	-1.505	21.308
7.813	1.991	-11.020	15.003
15.625	10.906	0.323	21.489
31.250	18.048	7.511	28.584
62.500	14.584	4.581	24.588
125.000	7.862	-3.658	19.382
250.000	10.564	-1.384	22.513
500.000	-0.258	-13.548	13.031

4th Exp.



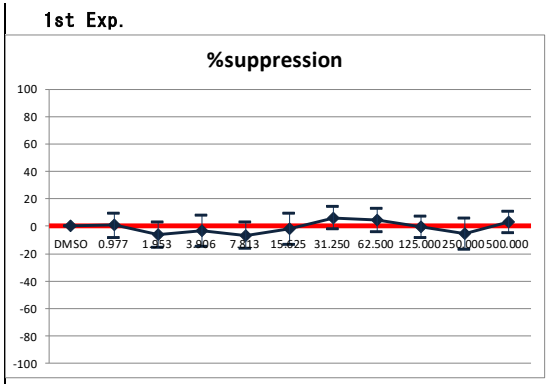
4th Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-4.896	-14.274	4.482
1.953	1.410	-13.525	16.345
3.906	-5.770	-23.599	12.059
7.813	-8.170	-17.423	1.083
15.625	-3.205	-17.431	11.022
31.250	-5.848	-16.224	4.528
62.500	-2.384	-10.388	5.619
125.000	-16.721	-26.471	-6.971
250.000	-12.525	-24.004	-1.046
500.000	-38.661	-72.341	-4.981

Lab C

N:NNN

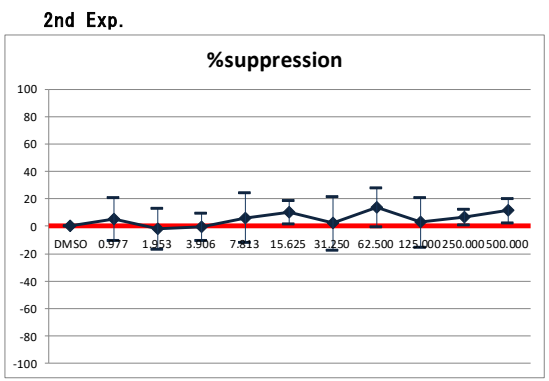
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	0.722	-8.313	9.756
1.953	-6.139	-15.419	3.142
3.906	-3.123	-14.309	8.063
7.813	-6.534	-16.305	3.238
15.625	-2.070	-13.487	9.347
31.250	6.220	-1.794	14.235
62.500	4.467	-3.815	12.750
125.000	-0.351	-8.173	7.471
250.000	-5.623	-17.076	5.831
500.000	2.932	-4.871	10.735

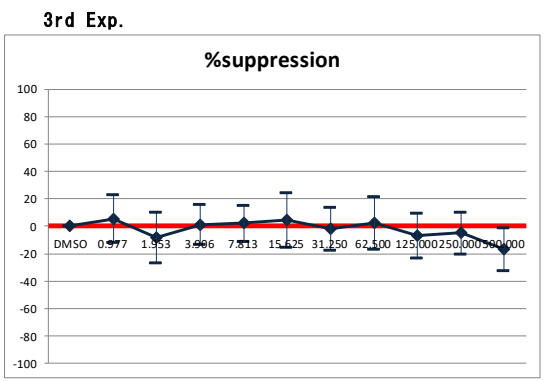
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	5.276	-10.616	21.168
1.953	-1.758	-16.728	13.212
3.906	-0.448	-10.264	9.368
7.813	6.341	-11.477	24.159
15.625	10.094	1.752	18.437
31.250	2.231	-17.130	21.592
62.500	13.904	-0.189	27.997
125.000	2.850	-15.331	21.032
250.000	6.619	0.775	12.464
500.000	11.383	2.759	20.007

3rd Exp.



3rd Exp.

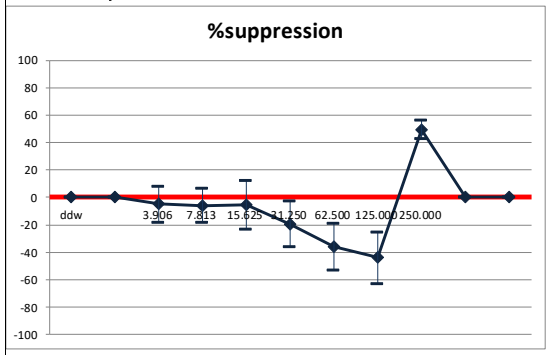
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	5.604	-11.636	22.844
1.953	-8.148	-26.396	10.099
3.906	1.335	-13.106	15.776
7.813	2.096	-10.749	14.941
15.625	4.648	-15.020	24.316
31.250	-1.885	-17.364	13.594
62.500	2.512	-16.525	21.550
125.000	-6.797	-22.866	9.272
250.000	-4.770	-20.044	10.503
500.000	-16.727	-32.000	-1.455

Chemical.8

Lead Lab.

A:A A/S A

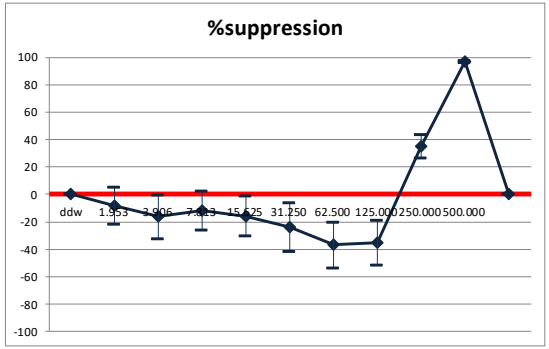
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-4.713	-17.878	8.451
7.813	-5.828	-18.228	6.571
15.625	-5.495	-23.192	12.201
31.250	-19.233	-35.776	-2.689
62.500	-36.169	-53.169	-19.169
125.000	-43.920	-62.780	-25.061
250.000	49.531	42.853	56.209

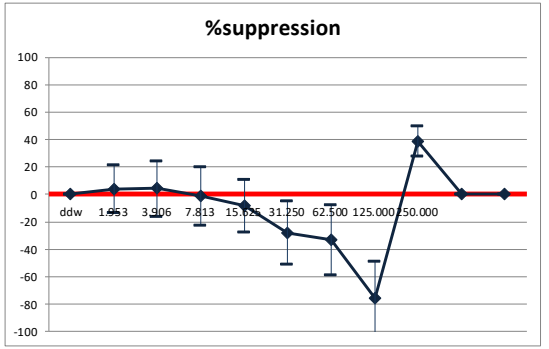
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
1.953	-8.181	-21.880	5.519
3.906	-16.127	-32.119	-0.135
7.813	-11.894	-26.277	2.489
15.625	-16.003	-30.553	-1.454
31.250	-24.068	-41.897	-6.239
62.500	-36.833	-53.694	-19.973
125.000	-35.017	-51.536	-18.498
250.000	35.101	26.404	43.798
500.000	96.938	96.042	97.835

3rd Exp.



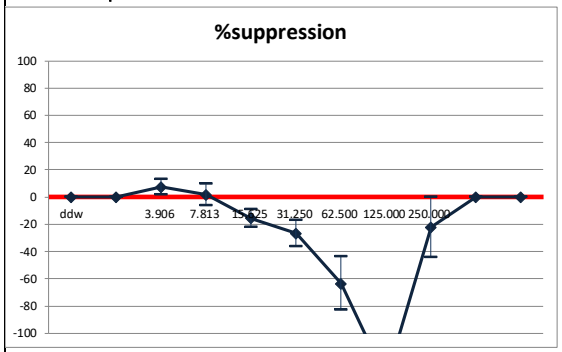
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
1.953	4.116	-13.054	21.287
3.906	4.274	-16.016	24.565
7.813	-1.188	-22.540	20.164
15.625	-8.448	-27.684	10.788
31.250	-27.811	-50.738	-4.883
62.500	-33.183	-58.609	-7.756
125.000	-75.721	-102.474	-48.967
250.000	38.934	28.057	49.811

Lab A

A:AAA

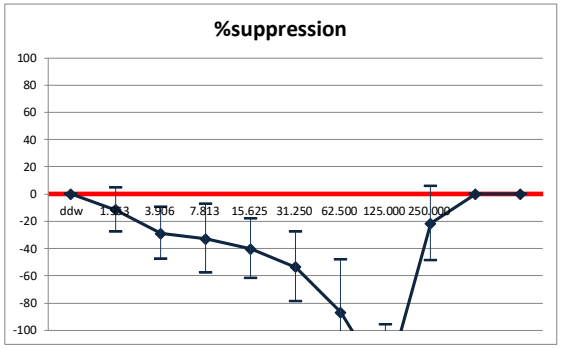
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	7.244	1.804	12.683
7.813	1.530	-6.419	9.480
15.625	-15.728	-22.444	-9.012
31.250	-26.832	-36.686	-16.979
62.500	-63.564	-82.956	-44.171
125.000	-133.259	-159.265	-107.254
250.000	-22.160	-44.368	0.048

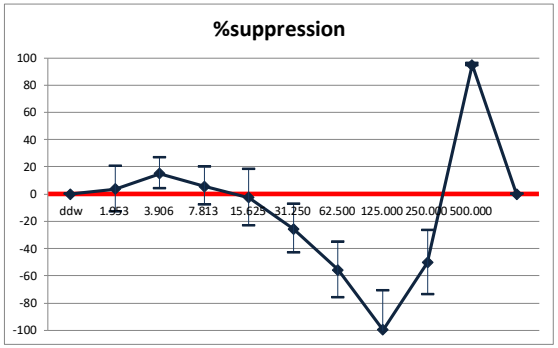
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
1.953	-11.811	-27.957	4.335
3.906	-28.946	-48.050	-9.842
7.813	-33.023	-58.336	-7.711
15.625	-40.348	-62.253	-18.442
31.250	-53.669	-79.185	-28.154
62.500	-86.805	-125.327	-48.283
125.000	-140.610	-184.854	-96.367
250.000	-21.735	-49.118	5.648

3rd Exp.



3rd Exp.

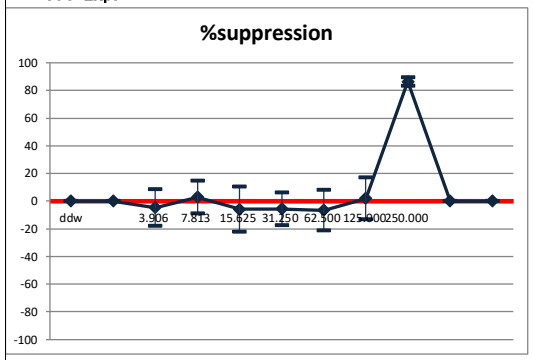
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
1.953	3.689	-13.131	20.510
3.906	14.970	3.577	26.362
7.813	5.782	-8.126	19.691
15.625	-2.709	-23.185	17.768
31.250	-25.550	-43.381	-7.720
62.500	-55.783	-76.350	-35.215
125.000	-99.649	-128.010	-71.288
250.000	-50.252	-73.849	-26.656
500.000	94.796	93.983	95.609

Chemical.8

Lab B

A:SAA

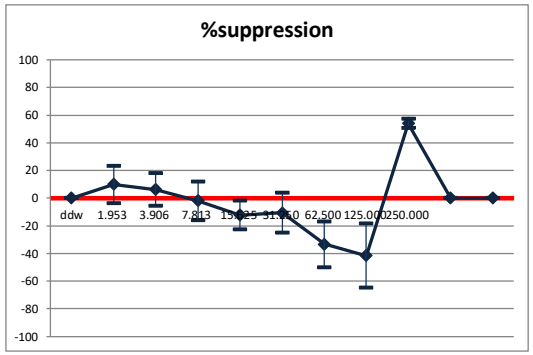
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-4.675	-17.900	8.550
7.813	3.006	-8.932	14.945
15.625	-5.800	-22.270	10.669
31.250	-5.514	-17.457	6.430
62.500	-6.521	-21.055	8.013
125.000	1.902	-13.273	17.077
250.000	86.457	83.476	89.437

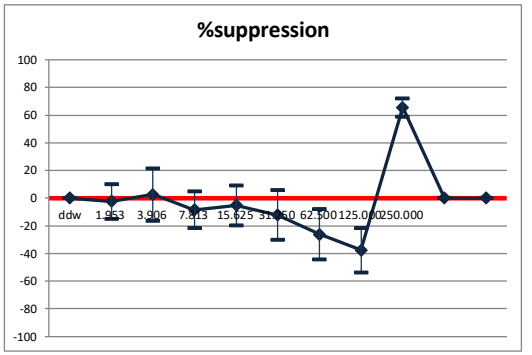
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
1.953	9.927	-3.705	23.558
3.906	6.331	-5.444	18.107
7.813	-1.921	-16.021	12.180
15.625	-12.231	-22.571	-1.892
31.250	-10.453	-24.973	4.068
62.500	-33.392	-50.050	-16.735
125.000	-41.674	-64.873	-18.475
250.000	54.098	50.831	57.364

3rd Exp.



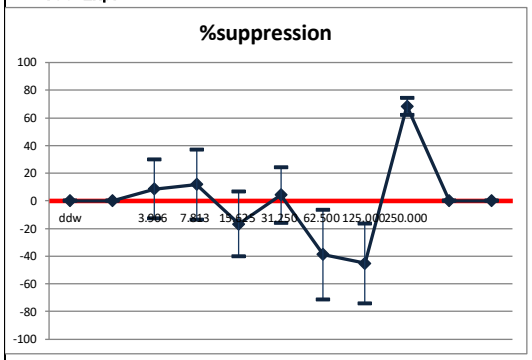
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
1.953	-2.264	-14.800	10.272
3.906	2.568	-16.199	21.336
7.813	-8.372	-21.487	4.743
15.625	-5.232	-19.833	9.368
31.250	-12.118	-30.236	6.001
62.500	-26.141	-44.330	-7.952
125.000	-37.560	-53.625	-21.495
250.000	65.531	58.840	72.221

Lab C

A:ANA

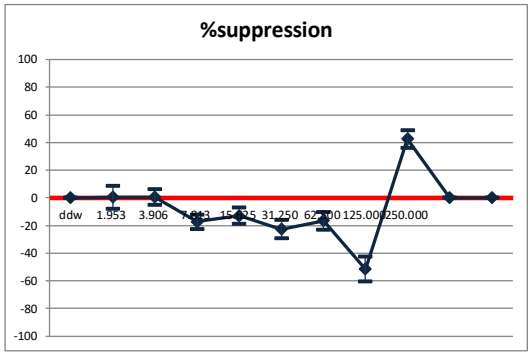
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	8.596	-12.786	29.978
7.813	11.844	-13.598	37.287
15.625	-16.726	-40.243	6.792
31.250	4.268	-15.966	24.502
62.500	-38.846	-71.193	-6.498
125.000	-45.188	-74.162	-16.214
250.000	68.475	62.203	74.746

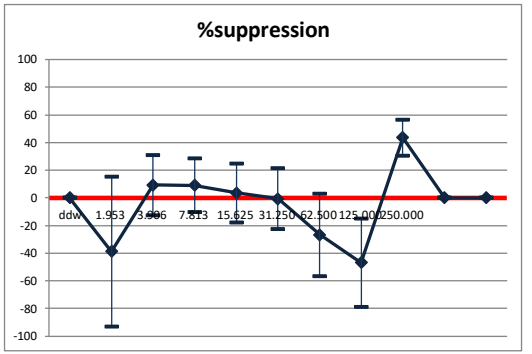
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
1.953	0.455	-7.935	8.845
3.906	0.593	-5.045	6.231
7.813	-17.205	-22.533	-11.877
15.625	-13.003	-18.911	-7.094
31.250	-22.527	-29.111	-15.943
62.500	-16.536	-22.836	-10.236
125.000	-51.582	-60.587	-42.576
250.000	42.591	36.234	48.948

3rd Exp.



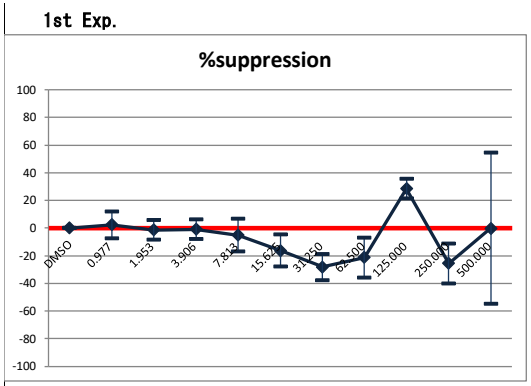
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
1.953	-38.715	-92.946	15.516
3.906	9.197	-12.582	30.976
7.813	9.191	-10.315	28.696
15.625	3.525	-17.911	24.960
31.250	-0.546	-22.584	21.493
62.500	-26.719	-56.522	3.084
125.000	-46.886	-78.693	-15.080
250.000	43.553	30.632	56.474

Chemical.9

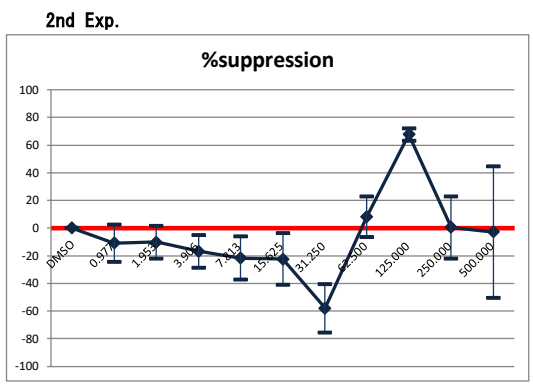
Lead Lab.

A/S:N A/S A/S



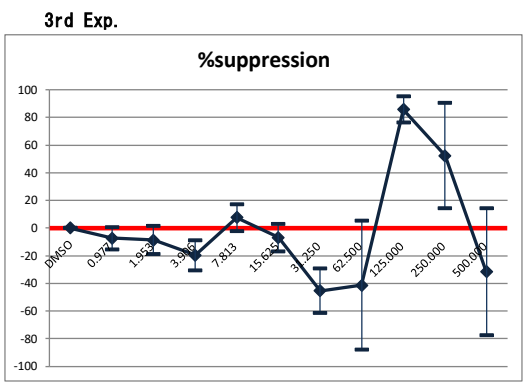
1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	2.444	-7.142	12.029
1.953	-1.333	-8.513	5.847
3.906	-0.799	-7.754	6.155
7.813	-5.124	-16.890	6.642
15.625	-16.188	-27.740	-4.635
31.250	-28.124	-37.662	-18.586
62.500	-21.286	-35.644	-6.928
125.000	28.577	21.589	35.564
250.000	-25.555	-39.934	-11.176
500.000	-0.214	-54.832	54.404



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-10.922	-24.327	2.483
1.953	-10.156	-21.930	1.617
3.906	-16.838	-28.630	-5.046
7.813	-21.666	-37.285	-6.048
15.625	-22.313	-41.030	-3.596
31.250	-57.900	-75.446	-40.354
62.500	8.261	-6.563	23.084
125.000	67.767	63.374	72.161
250.000	0.439	-22.032	22.909
500.000	-2.771	-50.313	44.771

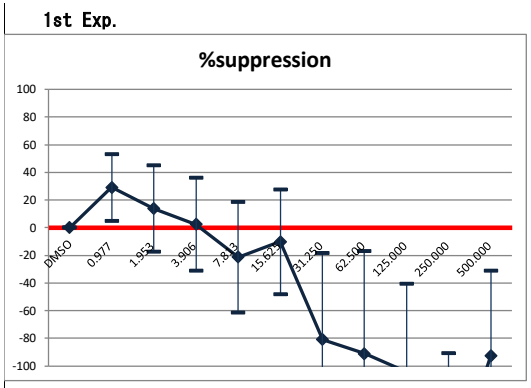


3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-7.215	-15.192	0.762
1.953	-8.587	-18.832	1.659
3.906	-19.643	-30.729	-8.558
7.813	7.645	-1.974	17.265
15.625	-6.793	-16.713	3.127
31.250	-45.245	-61.527	-28.963
62.500	-41.233	-87.917	5.450
125.000	85.857	76.373	95.341
250.000	52.414	14.433	90.394
500.000	-31.606	-77.590	14.377

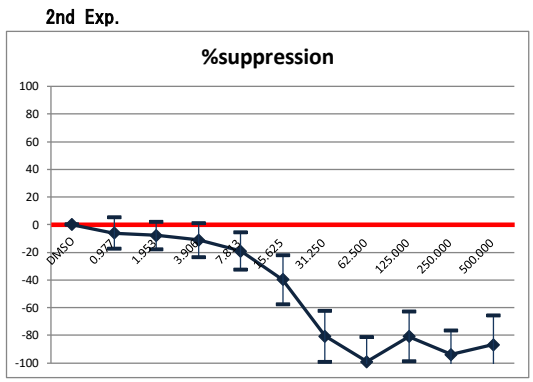
Lab A

A:AAA



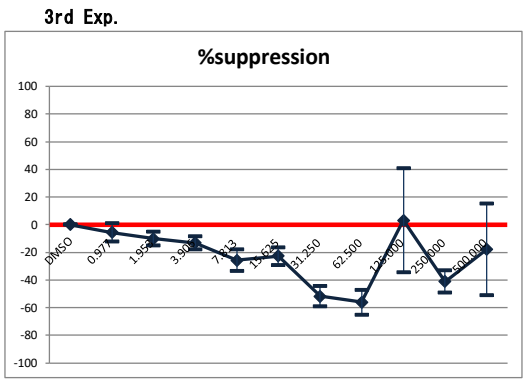
1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	29.114	4.869	53.358
1.953	13.864	-17.514	45.242
3.906	2.494	-31.224	36.212
7.813	-21.241	-61.266	18.784
15.625	-10.179	-48.223	27.864
31.250	-80.810	-143.242	-18.378
62.500	-90.935	-165.204	-16.666
125.000	-104.125	-167.969	-40.280
250.000	-185.321	-279.878	-90.764
500.000	-92.698	-154.588	-30.809



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-6.110	-17.394	5.174
1.953	-7.684	-17.630	2.262
3.906	-11.116	-23.541	1.309
7.813	-19.161	-32.669	-5.652
15.625	-39.756	-57.513	-22.000
31.250	-80.547	-98.979	-62.114
62.500	-99.159	-117.281	-81.037
125.000	-80.883	-98.914	-62.851
250.000	-93.864	-111.402	-76.326
500.000	-86.799	-107.919	-65.679



3rd Exp.

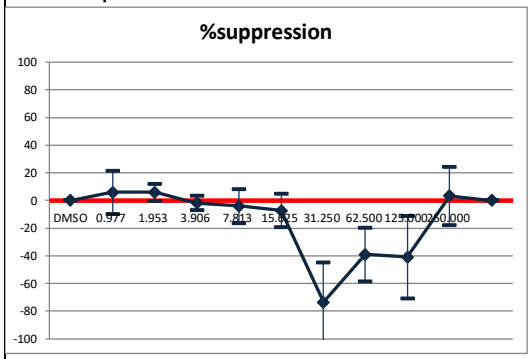
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-5.572	-12.070	0.927
1.953	-10.053	-14.896	-5.209
3.906	-13.060	-17.825	-8.296
7.813	-25.608	-33.305	-17.911
15.625	-22.670	-29.139	-16.202
31.250	-51.699	-59.117	-44.281
62.500	-56.104	-64.975	-47.234
125.000	3.114	-34.446	40.674
250.000	-40.988	-49.020	-32.956
500.000	-17.857	-51.131	15.417

Chemical.9

Lab B

A:AAA

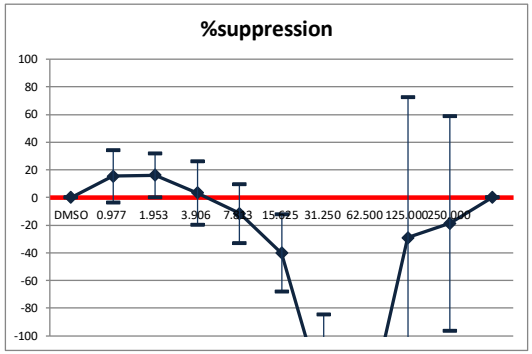
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	5.973	-9.610	21.557
1.953	5.987	-0.120	12.094
3.906	-1.748	-6.872	3.377
7.813	-3.891	-16.171	8.389
15.625	-7.143	-19.179	4.893
31.250	-73.464	-102.392	-44.536
62.500	-38.948	-58.280	-19.616
125.000	-40.960	-70.933	-10.986
250.000	3.395	-17.671	24.460

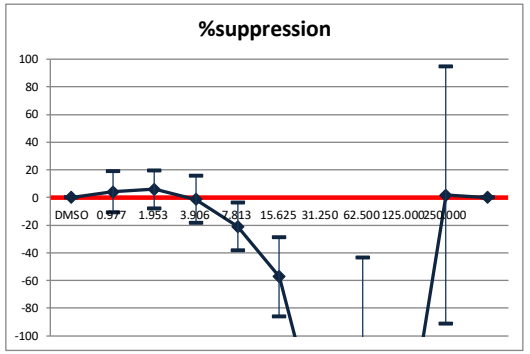
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	15.373	-3.420	34.167
1.953	16.076	0.007	32.145
3.906	3.278	-19.592	26.148
7.813	-11.618	-32.900	9.664
15.625	-40.128	-67.922	-12.334
31.250	-146.261	-207.903	-84.618
62.500	-162.603	-217.870	-107.337
125.000	-29.118	-130.867	72.631
250.000	-18.624	-96.246	58.998

3rd Exp.



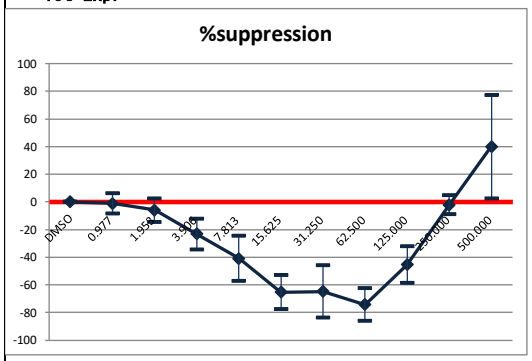
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	4.209	-10.769	19.188
1.953	6.043	-7.735	19.822
3.906	-1.391	-18.429	15.647
7.813	-20.885	-38.047	-3.723
15.625	-57.256	-85.785	-28.727
31.250	-173.722	-237.918	-109.527
62.500	-152.868	-262.606	-43.129
125.000	-186.417	-267.514	-105.320
250.000	1.715	-91.370	94.799

Lab C

A:AAA

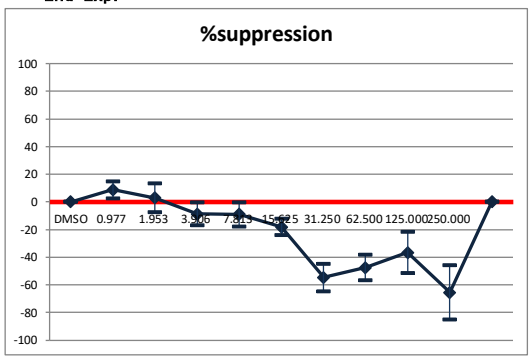
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-1.023	-8.408	6.362
1.953	-5.846	-14.492	2.800
3.906	-23.185	-34.243	-12.127
7.813	-40.887	-57.244	-24.530
15.625	-65.262	-77.617	-52.907
31.250	-64.823	-83.806	-45.841
62.500	-74.214	-86.019	-62.409
125.000	-45.352	-58.699	-32.005
250.000	-1.972	-8.648	4.704
500.000	40.091	2.703	77.479

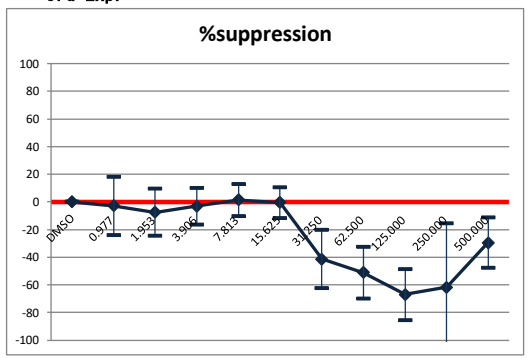
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	8.742	2.596	14.887
1.953	3.008	-7.349	13.366
3.906	-8.582	-16.921	-0.243
7.813	-8.842	-17.605	-0.080
15.625	-18.081	-23.921	-12.242
31.250	-54.640	-64.581	-44.699
62.500	-47.378	-56.450	-38.307
125.000	-36.592	-51.527	-21.656
250.000	-65.505	-85.162	-45.849

3rd Exp.



3rd Exp.

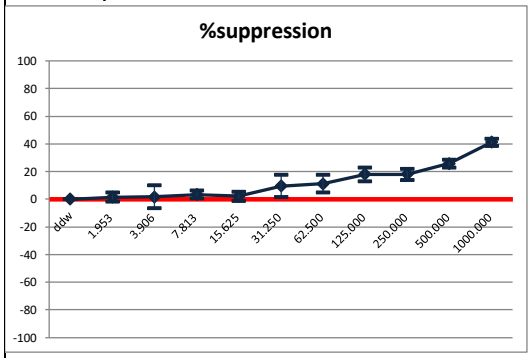
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-2.839	-23.777	18.100
1.953	-7.375	-24.238	9.487
3.906	-3.068	-16.282	10.147
7.813	1.579	-10.037	13.196
15.625	-0.482	-11.747	10.783
31.250	-41.243	-62.503	-19.984
62.500	-51.094	-69.904	-32.283
125.000	-66.830	-85.267	-48.392
250.000	-61.803	-108.121	-15.486
500.000	-29.448	-47.688	-11.208

Chemical.10

Lead Lab.

S:SSN

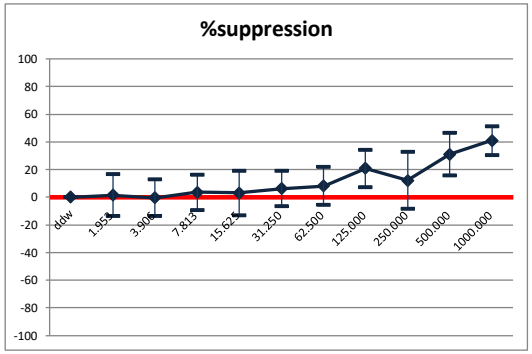
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
1.953	1.553	-1.600	4.706
3.906	1.781	-6.347	9.909
7.813	3.465	0.537	6.394
15.625	2.191	-1.181	5.563
31.250	9.479	1.459	17.498
62.500	11.294	4.894	17.693
125.000	18.112	13.215	23.009
250.000	17.988	13.909	22.067
500.000	25.876	23.036	28.717
1000.000	41.306	38.704	43.908

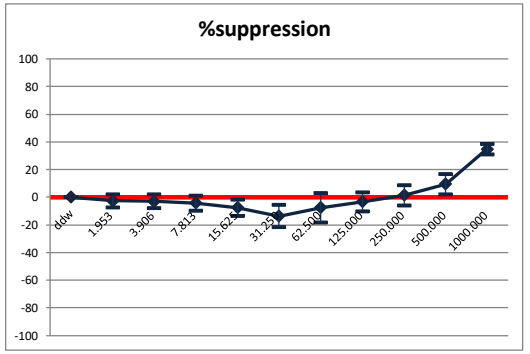
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
1.953	1.469	-13.622	16.559
3.906	-0.336	-13.611	12.939
7.813	3.674	-9.050	16.398
15.625	3.116	-12.907	19.139
31.250	6.302	-6.354	18.958
62.500	8.058	-5.635	21.751
125.000	20.904	7.472	34.335
250.000	12.207	-8.342	32.755
500.000	31.119	15.819	46.419
1000.000	40.966	30.561	51.370

3rd Exp.



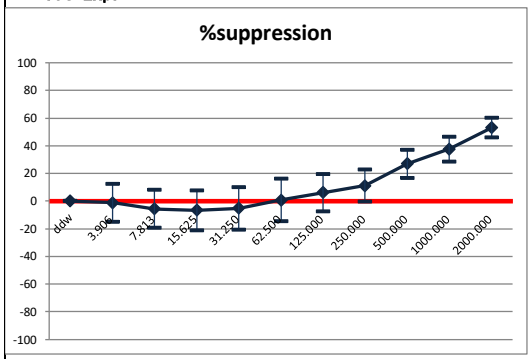
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
1.953	-2.601	-7.306	2.104
3.906	-2.959	-7.829	1.911
7.813	-4.295	-9.770	1.181
15.625	-7.458	-13.449	-1.467
31.250	-13.609	-21.742	-5.475
62.500	-7.421	-18.110	3.269
125.000	-3.260	-10.241	3.721
250.000	1.483	-5.729	8.695
500.000	9.537	2.295	16.778
1000.000	34.755	30.757	38.753

Lab A

S:SSS

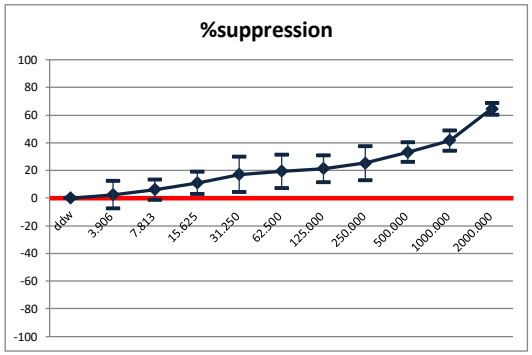
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-1.155	-14.760	12.449
7.813	-5.649	-19.423	8.126
15.625	-6.459	-20.888	7.970
31.250	-5.171	-20.434	10.093
62.500	0.777	-14.575	16.129
125.000	6.255	-7.283	19.793
250.000	11.290	-0.479	23.059
500.000	27.027	16.760	37.294
1000.000	37.648	28.557	46.738
2000.000	53.208	46.001	60.415

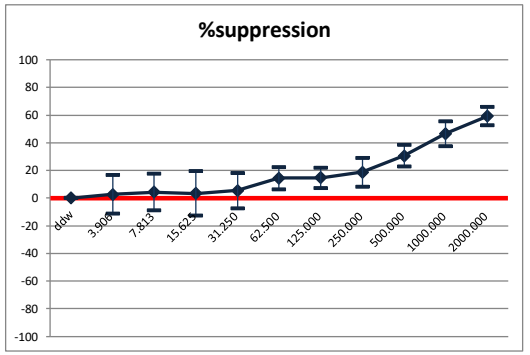
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	2.399	-7.606	12.404
7.813	6.307	-1.015	13.629
15.625	11.035	2.826	19.245
31.250	17.065	4.341	29.788
62.500	19.507	7.404	31.610
125.000	21.298	11.697	30.899
250.000	25.331	12.840	37.822
500.000	33.219	26.111	40.327
1000.000	41.760	34.366	49.153
2000.000	64.589	60.347	68.830

3rd Exp.



3rd Exp.

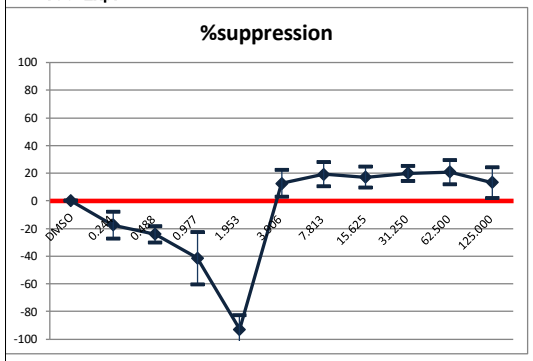
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	2.635	-11.264	16.534
7.813	4.415	-8.696	17.527
15.625	3.498	-12.708	19.704
31.250	5.418	-7.537	18.372
62.500	14.472	6.290	22.654
125.000	14.666	7.451	21.882
250.000	18.660	8.132	29.189
500.000	30.709	22.879	38.539
1000.000	46.648	37.629	55.667
2000.000	59.312	52.682	65.943

Chemical.10

Lab B

N:ANN

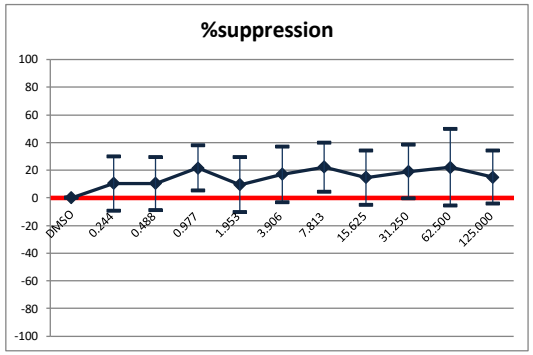
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	-17.405	-27.148	-7.663
0.488	-24.139	-30.192	-18.086
0.977	-41.358	-60.207	-22.508
1.953	-92.817	-103.106	-82.528
3.906	12.586	2.865	22.307
7.813	19.357	10.511	28.203
15.625	17.227	9.744	24.711
31.250	19.912	14.535	25.289
62.500	20.841	12.256	29.426
125.000	13.279	2.221	24.337

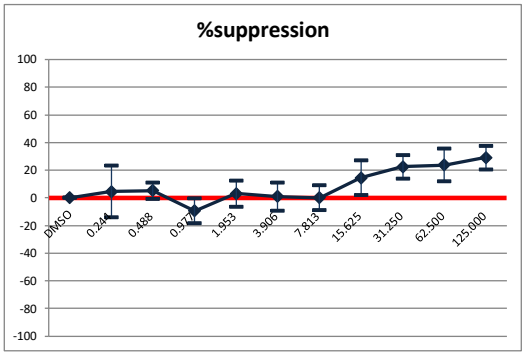
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	10.400	-9.437	30.237
0.488	10.529	-8.696	29.753
0.977	21.694	5.485	37.904
1.953	9.644	-10.308	29.597
3.906	17.012	-2.977	37.002
7.813	22.233	4.302	40.165
15.625	14.653	-5.010	34.316
31.250	19.050	-0.210	38.311
62.500	22.187	-5.335	49.709
125.000	15.098	-4.174	34.371

3rd Exp.



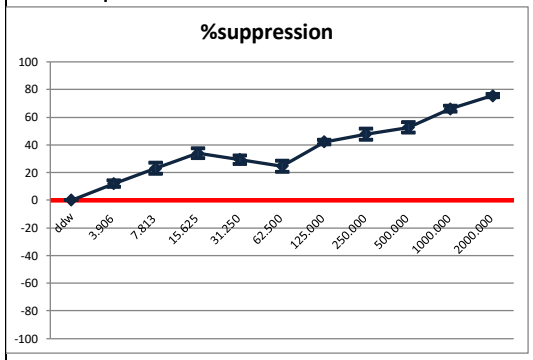
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	4.679	-14.134	23.492
0.488	5.318	-0.654	11.289
0.977	-9.349	-18.349	-0.348
1.953	3.124	-6.221	12.469
3.906	1.094	-9.035	11.223
7.813	0.157	-9.024	9.337
15.625	14.586	2.142	27.030
31.250	22.478	13.863	31.092
62.500	23.748	11.960	35.535
125.000	29.229	20.712	37.745

Lab C

S:SSS

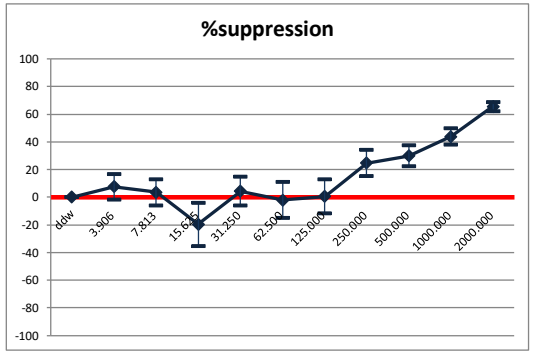
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	11.912	9.589	14.235
7.813	22.939	18.897	26.980
15.625	34.022	30.673	37.371
31.250	29.331	26.359	32.303
62.500	24.602	20.769	28.435
125.000	42.298	40.638	43.957
250.000	47.771	43.892	51.650
500.000	52.545	48.772	56.319
1000.000	66.137	63.978	68.296
2000.000	75.652	74.532	76.773

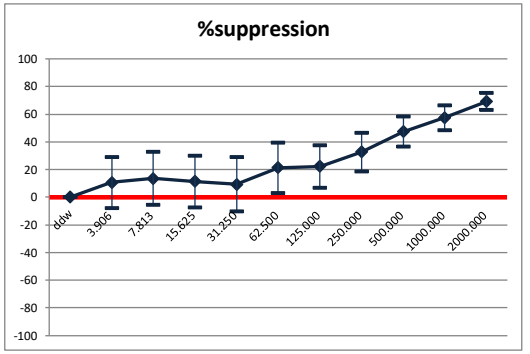
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	7.557	-1.796	16.910
7.813	3.688	-5.767	13.143
15.625	-19.676	-35.299	-4.053
31.250	4.433	-6.004	14.870
62.500	-2.036	-15.087	11.015
125.000	0.604	-11.575	12.782
250.000	24.807	15.243	34.372
500.000	29.897	22.269	37.526
1000.000	43.970	37.940	50.001
2000.000	65.547	62.230	68.864

3rd Exp.



3rd Exp.

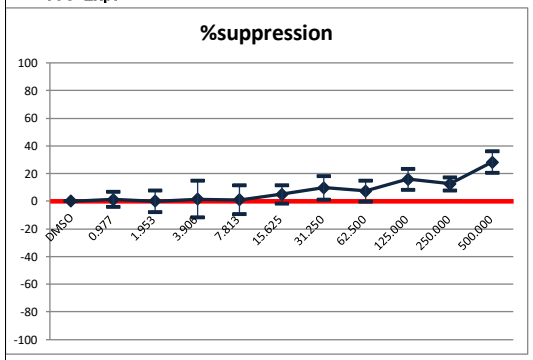
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	10.711	-7.805	29.226
7.813	13.688	-5.454	32.831
15.625	11.383	-7.172	29.938
31.250	9.403	-10.252	29.058
62.500	21.462	3.209	39.714
125.000	22.305	6.885	37.725
250.000	32.709	18.682	46.736
500.000	47.513	36.563	58.463
1000.000	57.512	48.682	66.342
2000.000	69.289	63.025	75.554

Chemical.11

Lead Lab.

N:NNN

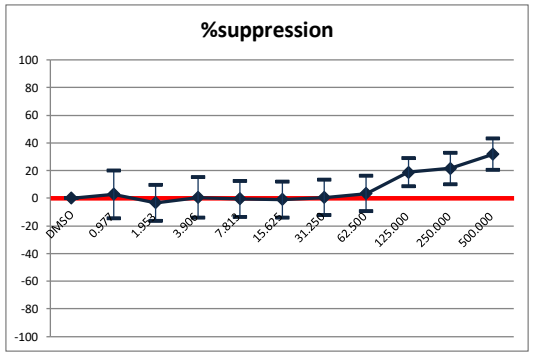
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	1.305	-4.084	6.693
1.953	0.047	-7.716	7.810
3.906	1.552	-11.839	14.943
7.813	1.040	-9.311	11.391
15.625	5.155	-1.473	11.782
31.250	9.716	1.178	18.254
62.500	7.474	-0.093	15.042
125.000	15.832	8.178	23.486
250.000	12.635	7.980	17.290
500.000	28.333	20.367	36.300

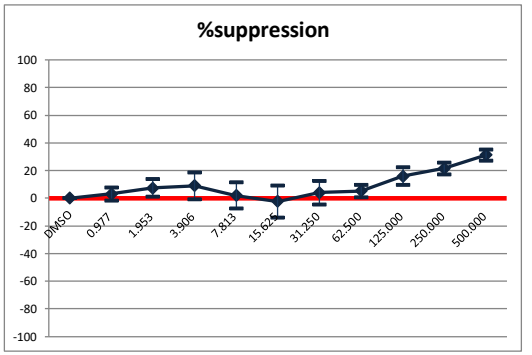
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	2.807	-14.421	20.036
1.953	-3.190	-16.180	9.800
3.906	0.583	-13.998	15.163
7.813	-0.436	-13.319	12.447
15.625	-0.972	-13.777	11.833
31.250	0.537	-12.293	13.367
62.500	3.467	-9.351	16.284
125.000	18.686	8.506	28.866
250.000	21.584	10.156	33.013
500.000	31.913	20.756	43.070

3rd Exp.



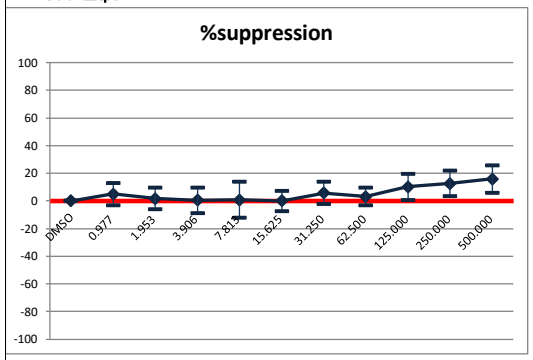
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	3.058	-1.706	7.823
1.953	7.371	0.945	13.797
3.906	9.104	-0.520	18.728
7.813	2.043	-7.581	11.668
15.625	-2.273	-13.969	9.424
31.250	4.147	-4.381	12.675
62.500	5.261	0.816	9.707
125.000	15.881	9.485	22.277
250.000	21.515	17.245	25.784
500.000	31.378	27.342	35.415

Lab A

N:NNN

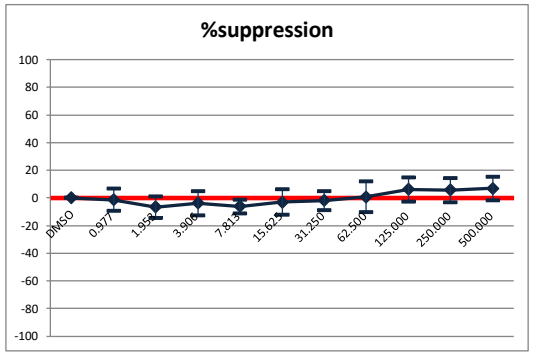
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	5.005	-3.126	13.137
1.953	1.705	-6.164	9.574
3.906	0.509	-8.617	9.634
7.813	0.844	-12.193	13.880
15.625	0.084	-7.289	7.458
31.250	5.801	-2.122	13.723
62.500	3.092	-3.281	9.464
125.000	10.184	0.668	19.701
250.000	12.607	3.359	21.855
500.000	15.824	5.652	25.996

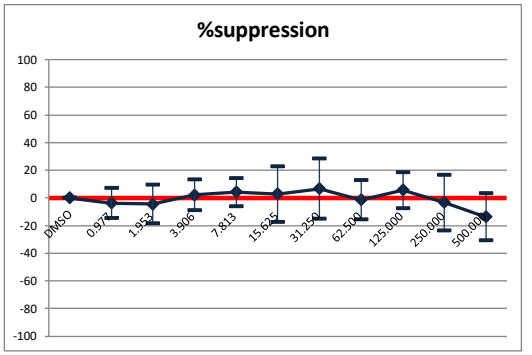
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-1.300	-9.381	6.782
1.953	-6.549	-14.478	1.379
3.906	-3.806	-12.555	4.943
7.813	-6.127	-10.938	-1.316
15.625	-2.924	-12.309	6.462
31.250	-1.912	-8.834	5.009
62.500	0.773	-10.367	11.913
125.000	6.345	-2.406	15.097
250.000	5.759	-3.050	14.568
500.000	6.981	-1.594	15.555

3rd Exp.



3rd Exp.

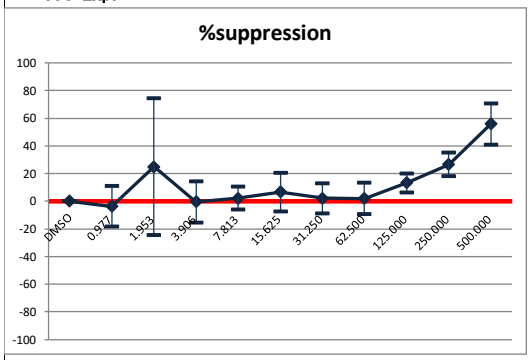
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-3.706	-14.493	7.080
1.953	-4.407	-18.301	9.488
3.906	2.292	-8.632	13.217
7.813	4.255	-5.897	14.408
15.625	2.957	-17.140	23.054
31.250	6.794	-14.902	28.490
62.500	-1.316	-15.425	12.792
125.000	5.827	-7.155	18.809
250.000	-3.297	-23.375	16.781
500.000	-13.647	-30.644	3.351

Chemical.11

Lab B

S:SSS

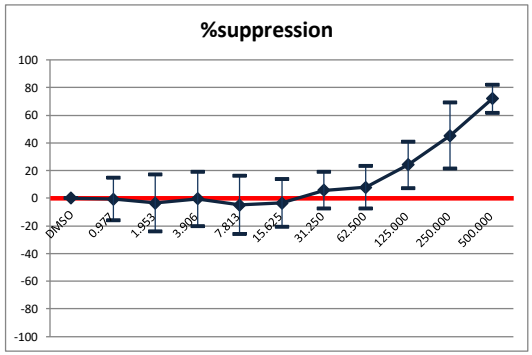
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-3.665	-18.235	10.904
1.953	25.035	-24.397	74.467
3.906	-0.455	-15.201	14.291
7.813	2.285	-6.114	10.684
15.625	6.748	-7.160	20.656
31.250	2.288	-8.583	13.158
62.500	2.018	-9.352	13.389
125.000	13.267	6.409	20.126
250.000	26.560	17.966	35.154
500.000	55.829	40.699	70.960

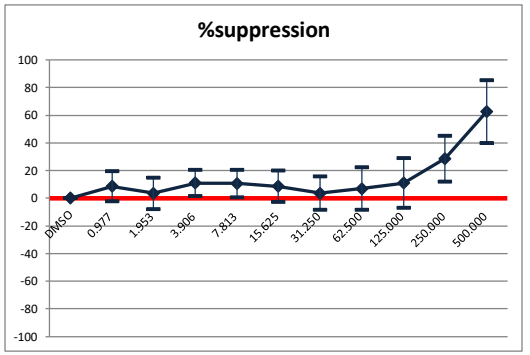
3rd Exp.



3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-0.603	-15.856	14.651
1.953	-3.399	-23.910	17.111
3.906	-0.422	-20.074	19.229
7.813	-4.830	-25.929	16.270
15.625	-3.505	-20.813	13.803
31.250	5.745	-7.477	18.967
62.500	7.978	-7.318	23.275
125.000	24.212	7.410	41.015
250.000	45.369	21.610	69.129
500.000	71.987	61.863	82.110

4th Exp.



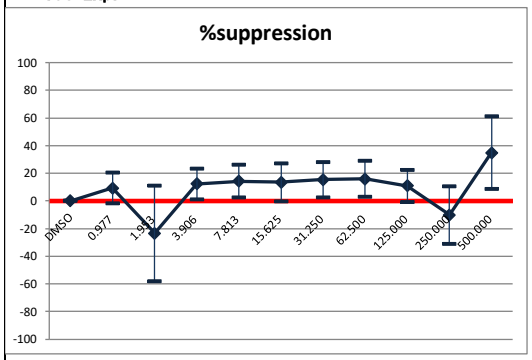
4th Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	8.621	-2.360	19.602
1.953	3.549	-7.694	14.792
3.906	11.051	1.649	20.452
7.813	10.705	0.897	20.514
15.625	8.685	-2.698	20.068
31.250	3.647	-8.555	15.849
62.500	6.973	-8.397	22.343
125.000	10.938	-7.030	28.906
250.000	28.673	12.011	45.335
500.000	62.642	39.873	85.412

Lab C

N:NNN

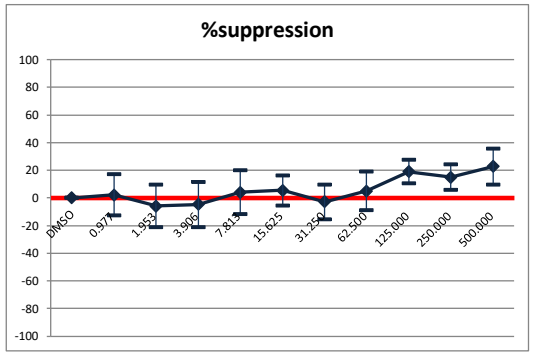
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	9.371	-1.841	20.582
1.953	-23.461	-58.229	11.306
3.906	12.292	1.079	23.506
7.813	14.226	2.423	26.028
15.625	13.494	-0.299	27.287
31.250	15.355	2.359	28.351
62.500	15.944	2.970	28.918
125.000	10.904	-0.751	22.559
250.000	-10.103	-30.975	10.769
500.000	34.818	8.603	61.033

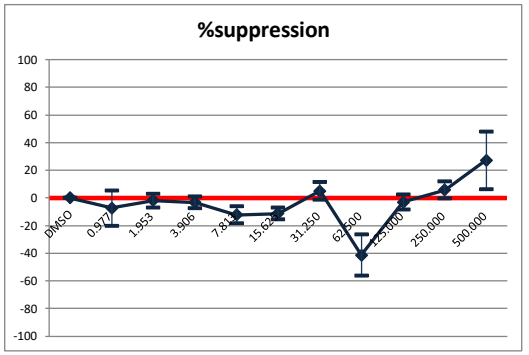
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	2.233	-12.720	17.185
1.953	-5.737	-21.162	9.688
3.906	-4.604	-20.895	11.688
7.813	4.145	-11.572	19.863
15.625	5.491	-5.505	16.487
31.250	-2.875	-15.512	9.762
62.500	5.053	-9.006	19.112
125.000	19.088	10.381	27.794
250.000	15.101	6.075	24.128
500.000	22.727	9.848	35.605

3rd Exp.



3rd Exp.

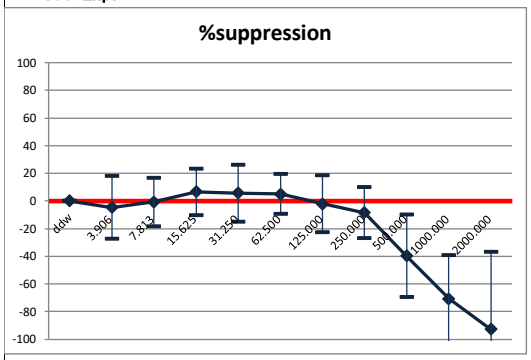
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-7.353	-20.101	5.395
1.953	-1.926	-6.994	3.142
3.906	-3.143	-7.324	1.037
7.813	-12.290	-18.450	-6.129
15.625	-11.260	-15.563	-6.956
31.250	5.152	-1.044	11.349
62.500	-41.342	-56.328	-26.357
125.000	-2.981	-8.364	2.403
250.000	5.854	-0.177	11.884
500.000	27.249	6.332	48.166

Chemical.12

Lead Lab.

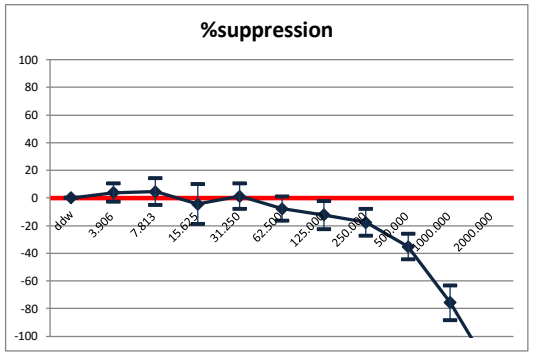
A:AAA

1st Exp.



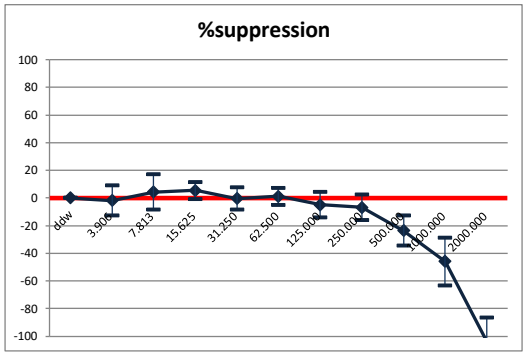
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-4.641	-27.344	18.063
7.813	-0.661	-18.264	16.941
15.625	6.805	-10.005	23.616
31.250	5.726	-14.839	26.292
62.500	5.035	-9.337	19.406
125.000	-2.026	-22.757	18.704
250.000	-8.362	-26.847	10.123
500.000	-39.635	-69.496	-9.774
1000.000	-70.687	-102.453	-38.922
2000.000	-92.679	-148.679	-36.679

2nd Exp.



a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	3.892	-2.812	10.597
7.813	4.567	-5.069	14.203
15.625	-4.389	-18.776	9.998
31.250	1.335	-8.020	10.689
62.500	-7.635	-16.519	1.249
125.000	-12.256	-22.349	-2.163
250.000	-17.591	-27.178	-8.005
500.000	-35.067	-44.336	-25.798
1000.000	-75.663	-88.228	-63.097
2000.000	-126.213	-146.875	-105.551

3rd Exp.

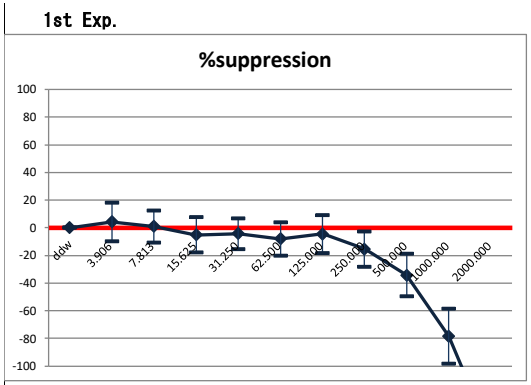


a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-1.700	-12.653	9.254
7.813	4.349	-8.437	17.135
15.625	5.498	-0.605	11.602
31.250	-0.288	-8.270	7.693
62.500	1.188	-5.144	7.519
125.000	-4.788	-13.936	4.360
250.000	-6.682	-15.699	2.334
500.000	-23.567	-34.344	-12.790
1000.000	-45.922	-63.019	-28.826
2000.000	-103.102	-119.770	-86.434

Lab A

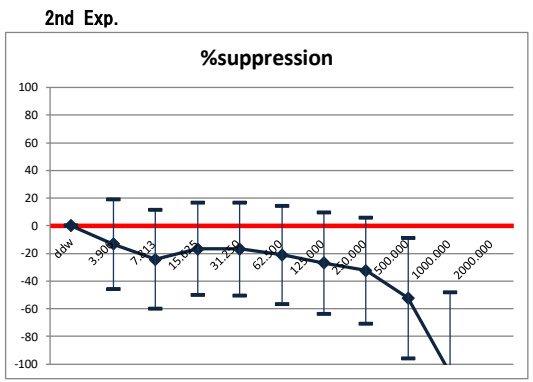
A:AAA

1st Exp.



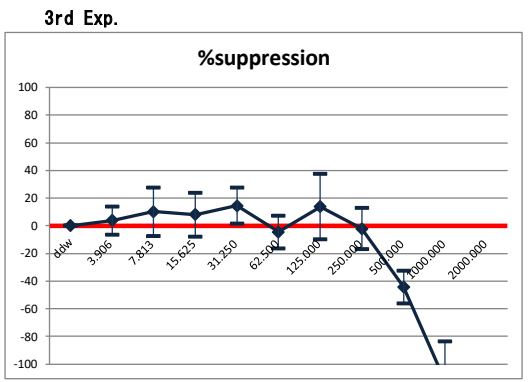
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	4.247	-9.906	18.399
7.813	0.930	-10.673	12.534
15.625	-5.053	-17.873	7.768
31.250	-4.280	-15.537	6.976
62.500	-7.912	-19.935	4.112
125.000	-4.444	-18.226	9.339
250.000	-15.290	-28.157	-2.422
500.000	-34.148	-49.370	-18.926
1000.000	-78.478	-98.421	-58.535
2000.000	-150.443	-182.012	-118.873

2nd Exp.



a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-13.198	-45.535	19.138
7.813	-24.326	-59.977	11.324
15.625	-16.626	-50.104	16.852
31.250	-16.756	-50.313	16.802
62.500	-21.084	-56.736	14.569
125.000	-26.836	-63.475	9.802
250.000	-32.399	-70.790	5.992
500.000	-52.248	-95.852	-8.644
1000.000	-107.058	-166.151	-47.964
2000.000	-189.106	-271.730	-106.483

3rd Exp.



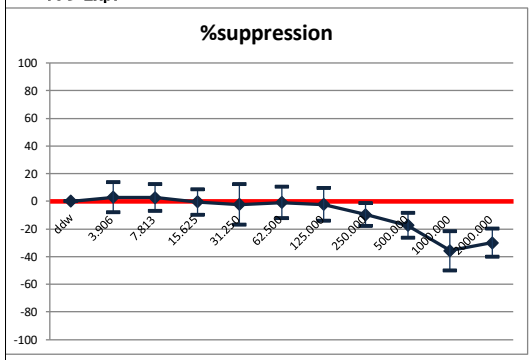
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	3.836	-6.365	14.038
7.813	10.237	-7.414	27.888
15.625	8.146	-7.774	24.065
31.250	14.502	1.391	27.614
62.500	-4.357	-16.154	7.440
125.000	13.904	-9.697	37.504
250.000	-1.983	-17.013	13.048
500.000	-44.254	-56.036	-32.471
1000.000	-112.658	-141.620	-83.696
2000.000	-256.468	-293.966	-218.970

Chemical.12

Lab B

A:AAA

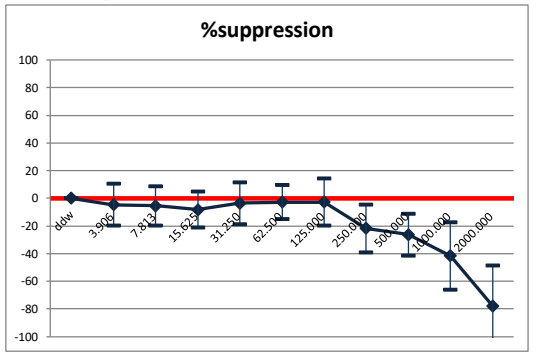
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	2.999	-7.961	13.958
7.813	2.779	-7.086	12.645
15.625	-0.347	-9.611	8.918
31.250	-2.193	-16.871	12.486
62.500	-0.944	-12.324	10.437
125.000	-2.195	-13.852	9.461
250.000	-9.524	-17.983	-1.066
500.000	-17.372	-26.530	-8.214
1000.000	-35.743	-49.970	-21.516
2000.000	-29.918	-39.964	-19.872

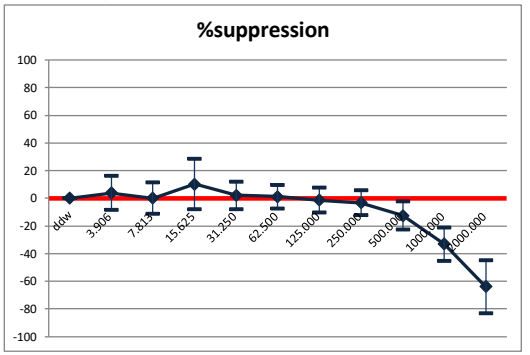
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-4.561	-19.568	10.447
7.813	-5.457	-19.824	8.910
15.625	-8.235	-21.192	4.722
31.250	-3.523	-18.667	11.621
62.500	-2.788	-15.070	9.493
125.000	-2.665	-19.881	14.552
250.000	-21.711	-39.096	-4.325
500.000	-26.274	-41.571	-10.976
1000.000	-41.567	-65.845	-17.289
2000.000	-77.752	-106.739	-48.765

3rd Exp.



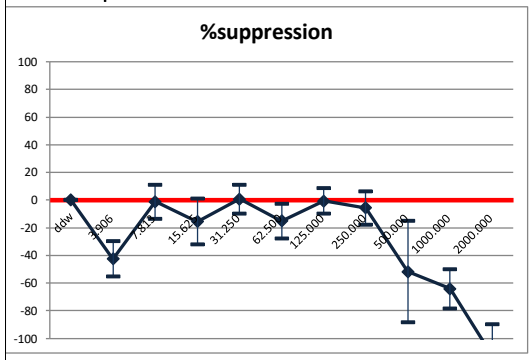
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	3.922	-8.365	16.209
7.813	-0.005	-11.347	11.336
15.625	10.368	-8.018	28.753
31.250	2.191	-7.656	12.038
62.500	1.237	-7.299	9.773
125.000	-1.223	-10.438	7.992
250.000	-3.209	-12.308	5.889
500.000	-12.348	-22.564	-2.133
1000.000	-33.092	-45.084	-21.101
2000.000	-63.915	-83.291	-44.538

Lab C

A:AAA

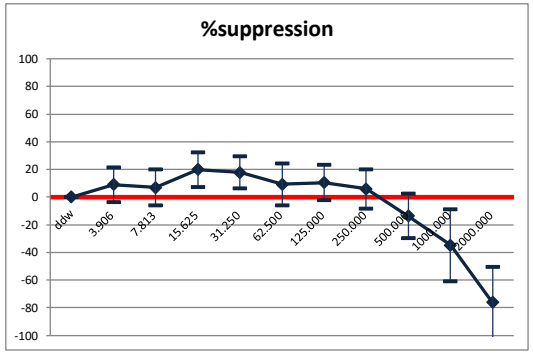
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-42.455	-55.294	-29.617
7.813	-1.112	-13.326	11.102
15.625	-15.454	-32.124	1.217
31.250	0.851	-9.621	11.322
62.500	-15.139	-27.610	-2.669
125.000	-0.612	-9.784	8.559
250.000	-5.705	-17.755	6.344
500.000	-51.671	-88.445	-14.897
1000.000	-64.147	-78.419	-49.876
2000.000	-112.453	-135.078	-89.828

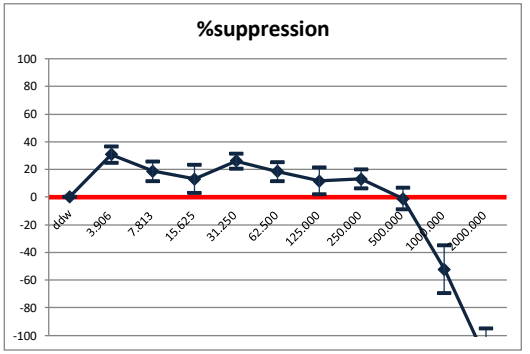
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	9.061	-3.370	21.493
7.813	7.014	-5.901	19.929
15.625	20.017	7.412	32.622
31.250	17.945	6.480	29.411
62.500	9.369	-5.816	24.554
125.000	10.606	-2.108	23.321
250.000	5.890	-8.245	20.025
500.000	-13.549	-29.552	2.455
1000.000	-34.647	-60.716	-8.578
2000.000	-76.116	-101.681	-50.551

4th Exp.



4th Exp.

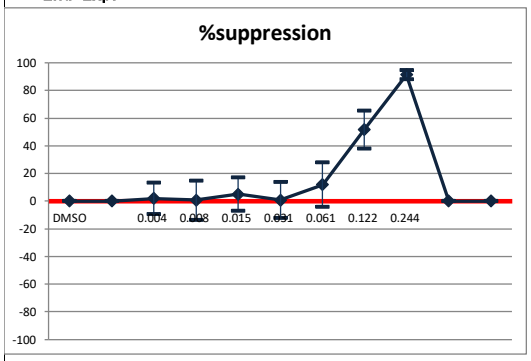
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	30.875	25.047	36.704
7.813	18.760	11.656	25.864
15.625	13.037	2.812	23.262
31.250	26.006	20.773	31.238
62.500	18.565	11.748	25.381
125.000	11.725	1.887	21.564
250.000	13.122	6.267	19.978
500.000	-1.044	-9.020	6.932
1000.000	-52.234	-69.509	-34.959
2000.000	-116.503	-138.147	-94.860

Chemical.13

Lead Lab.

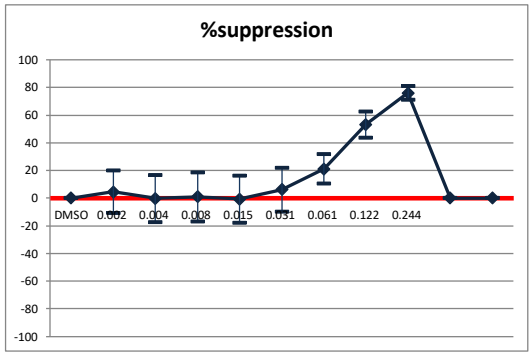
S:SSS

2nd Exp.



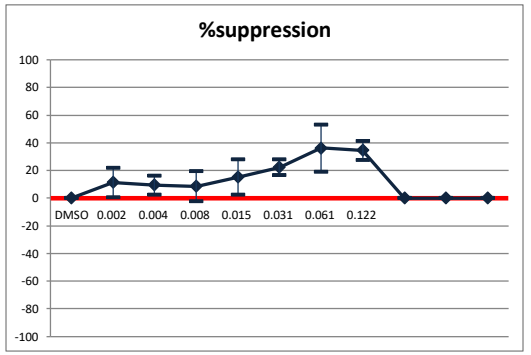
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.004	2.014	-9.440	13.467
0.008	0.726	-13.423	14.875
0.015	5.006	-7.059	17.070
0.031	0.717	-12.259	13.693
0.061	11.940	-4.219	28.099
0.122	51.801	37.891	65.711
0.244	91.431	88.029	94.833

3rd Exp.



a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.002	4.593	-10.824	20.009
0.004	-0.171	-17.158	16.815
0.008	1.020	-16.704	18.743
0.015	-0.578	-17.596	16.440
0.031	6.122	-9.875	22.120
0.061	21.095	10.471	31.720
0.122	53.239	43.738	62.740
0.244	76.102	71.107	81.097

4th Exp.

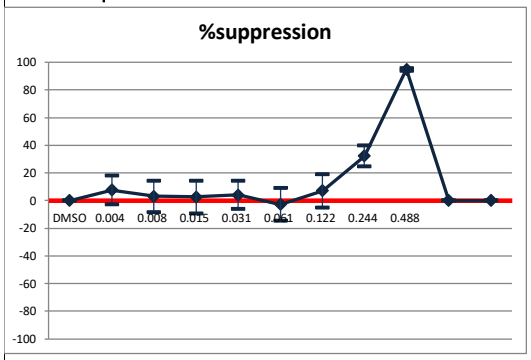


a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.002	11.401	0.648	22.153
0.004	9.432	2.596	16.267
0.008	8.703	-2.144	19.551
0.015	15.243	2.362	28.125
0.031	22.398	16.561	28.235
0.061	36.309	19.228	53.391
0.122	34.611	27.752	41.470

Lab A

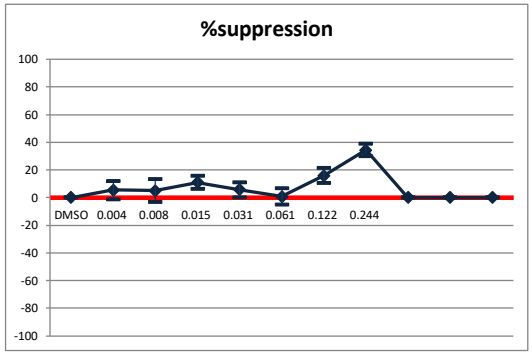
S:SNS

2nd Exp.



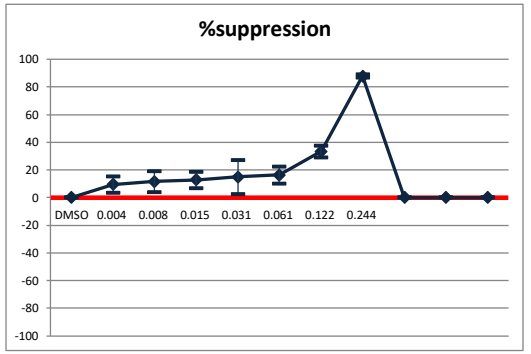
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.004	7.559	-2.865	17.984
0.008	3.057	-8.183	14.297
0.015	2.696	-9.175	14.567
0.031	4.139	-5.897	14.174
0.061	-2.650	-14.593	9.293
0.122	7.175	-4.977	19.328
0.244	32.330	24.906	39.754
0.488	94.992	94.085	95.899

3rd Exp.



a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.004	5.564	-1.107	12.235
0.008	5.125	-3.225	13.474
0.015	11.031	6.417	15.644
0.031	5.777	0.406	11.149
0.061	0.880	-5.064	6.823
0.122	16.044	10.432	21.656
0.244	34.469	30.129	38.808

4th Exp.



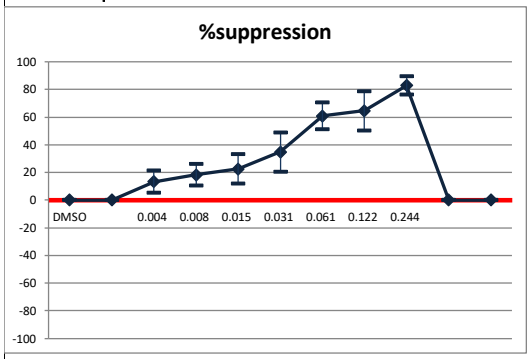
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.004	9.436	3.355	15.516
0.008	11.650	4.217	19.084
0.015	12.805	7.055	18.555
0.031	14.914	2.781	27.047
0.061	16.309	10.231	22.388
0.122	33.337	29.273	37.400
0.244	87.945	86.625	89.266

Chemical.13

Lab B

S:SSS

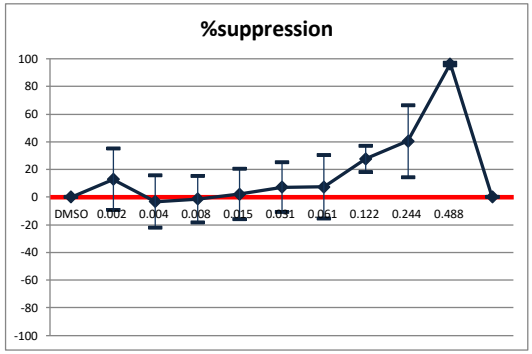
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.004	13.370	5.435	21.306
0.008	18.324	10.418	26.231
0.015	22.640	11.834	33.445
0.031	34.982	20.779	49.186
0.061	60.987	51.095	70.879
0.122	64.658	50.553	78.762
0.244	82.848	76.185	89.511

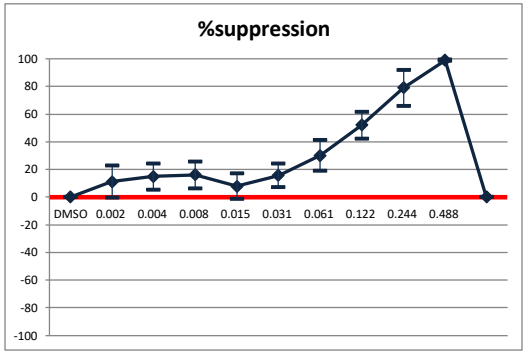
3rd Exp.



3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.002	12.924	-9.449	35.298
0.004	-3.161	-21.920	15.598
0.008	-1.417	-18.258	15.424
0.015	2.279	-16.076	20.634
0.031	7.203	-10.754	25.161
0.061	7.425	-15.502	30.352
0.122	27.812	18.400	37.225
0.244	40.467	14.348	66.586
0.488	96.294	95.345	97.242

5th Exp.



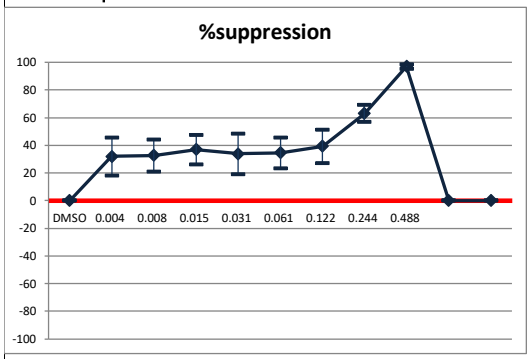
5th Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.002	11.123	-0.500	22.746
0.004	14.904	5.365	24.444
0.008	16.165	6.508	25.821
0.015	7.918	-1.239	17.076
0.031	15.699	7.187	24.211
0.061	30.245	19.313	41.178
0.122	52.078	42.388	61.768
0.244	79.062	66.164	91.961
0.488	99.068	98.499	99.637

Lab C

S:SSS

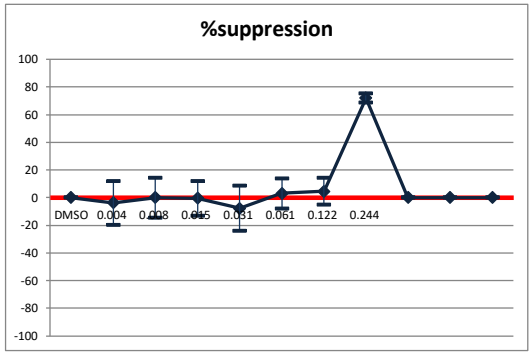
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.004	31.931	18.038	45.824
0.008	32.821	21.188	44.454
0.015	36.991	26.257	47.725
0.031	33.810	19.282	48.338
0.061	34.537	23.584	45.490
0.122	39.253	27.280	51.227
0.244	62.951	56.827	69.075
0.488	97.038	95.384	98.692

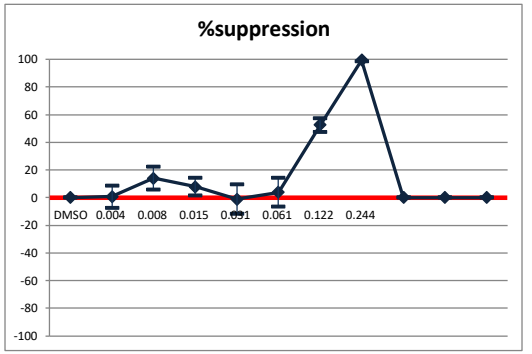
3rd Exp.



3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.004	-3.664	-19.571	12.243
0.008	0.036	-14.476	14.548
0.015	-0.472	-13.012	12.069
0.031	-7.624	-24.049	8.801
0.061	3.172	-7.694	14.037
0.122	4.684	-5.237	14.606
0.244	72.011	68.599	75.423

4th Exp.



4th Exp.

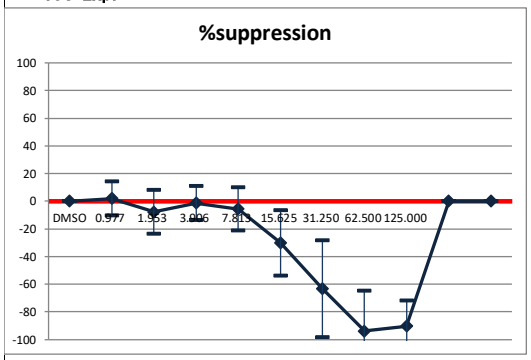
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.004	0.775	-7.205	8.755
0.008	14.107	5.976	22.238
0.015	7.937	1.677	14.196
0.031	-1.114	-11.837	9.609
0.061	3.950	-6.476	14.376
0.122	52.589	47.495	57.682
0.244	99.431	98.563	100.299

Chemical.14

Lead Lab.

A:AAN

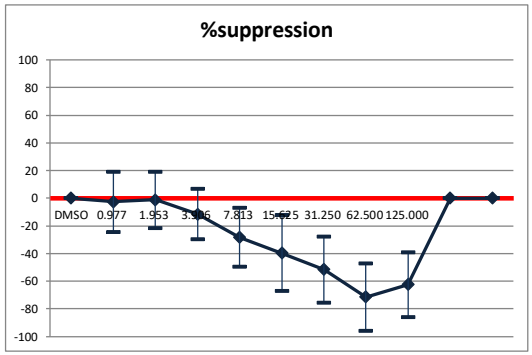
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	1.986	-10.288	14.259
1.953	-7.766	-23.669	8.136
3.906	-1.368	-13.762	11.026
7.813	-5.620	-21.190	9.950
15.625	-30.091	-53.544	-6.638
31.250	-63.413	-98.470	-28.357
62.500	-93.778	-123.086	-64.470
125.000	-90.424	-109.154	-71.693

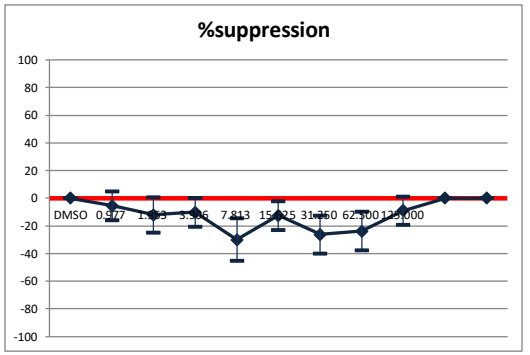
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-2.638	-24.293	19.018
1.953	-1.179	-21.508	19.150
3.906	-11.436	-29.665	6.793
7.813	-28.287	-49.670	-6.903
15.625	-39.566	-67.048	-12.084
31.250	-51.468	-75.363	-27.574
62.500	-71.495	-95.833	-47.157
125.000	-62.384	-85.908	-38.859

3rd Exp.



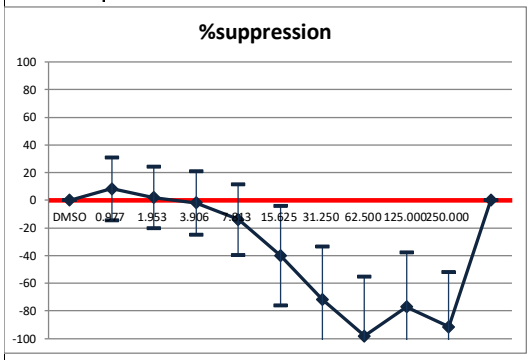
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-5.417	-15.928	5.093
1.953	-12.100	-24.795	0.594
3.906	-10.062	-20.549	0.425
7.813	-29.939	-45.361	-14.517
15.625	-12.556	-23.076	-2.036
31.250	-26.184	-39.809	-12.558
62.500	-23.741	-37.817	-9.664
125.000	-9.024	-19.199	1.151

Lab A

A:AAA

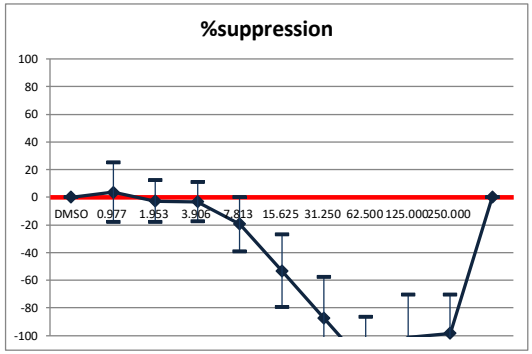
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	8.250	-14.447	30.947
1.953	2.039	-20.369	24.447
3.906	-1.882	-24.859	21.094
7.813	-13.894	-39.541	11.753
15.625	-39.924	-75.984	-3.865
31.250	-71.976	-110.629	-33.323
62.500	-98.055	-140.893	-55.218
125.000	-76.975	-116.311	-37.638
250.000	-91.427	-130.873	-51.981

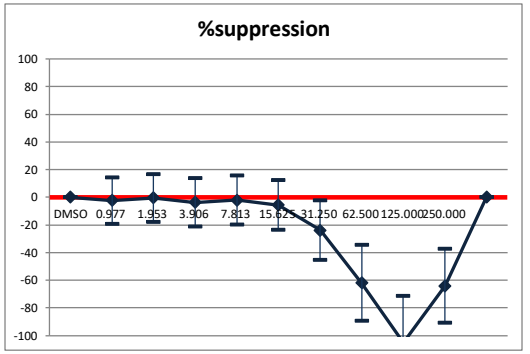
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	3.700	-17.718	25.117
1.953	-2.767	-17.814	12.279
3.906	-3.143	-17.473	11.186
7.813	-19.418	-39.166	0.329
15.625	-53.069	-79.269	-26.869
31.250	-87.557	-117.375	-57.738
62.500	-121.886	-157.345	-86.427
125.000	-101.411	-132.725	-70.097
250.000	-98.370	-126.633	-70.107

3rd Exp.



3rd Exp.

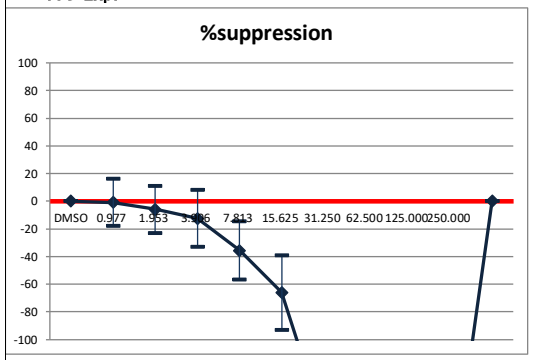
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-2.199	-19.012	14.613
1.953	-0.453	-17.551	16.644
3.906	-3.746	-21.231	13.738
7.813	-2.055	-19.800	15.690
15.625	-5.582	-23.570	12.405
31.250	-23.882	-45.402	-2.363
62.500	-61.876	-89.272	-34.480
125.000	-105.051	-138.972	-71.130
250.000	-63.946	-90.784	-37.108

Chemical.14

Lab B

A:AAA

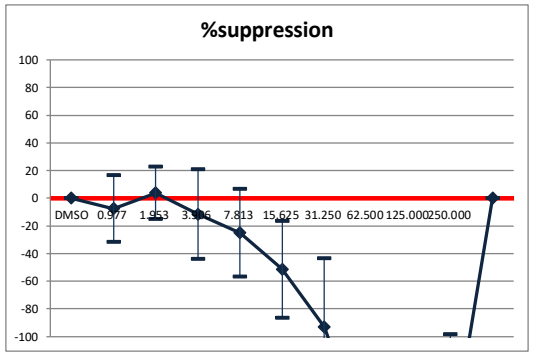
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-0.817	-17.770	16.135
1.953	-5.820	-22.759	11.119
3.906	-12.497	-33.041	8.047
7.813	-35.616	-56.572	-14.660
15.625	-66.063	-92.850	-39.276
31.250	-161.516	-203.347	-119.684
62.500	-230.228	-296.184	-164.273
125.000	-206.648	-275.708	-137.587
250.000	-215.975	-328.111	-103.839

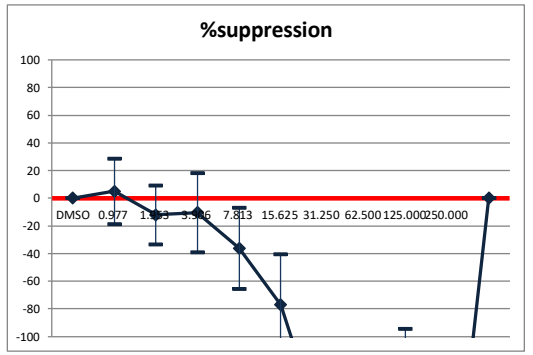
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-7.416	-31.678	16.847
1.953	3.914	-14.946	22.774
3.906	-11.484	-43.959	20.991
7.813	-24.993	-56.728	6.743
15.625	-51.363	-86.588	-16.138
31.250	-93.239	-143.318	-43.161
62.500	-179.785	-238.396	-121.173
125.000	-164.571	-218.243	-110.898
250.000	-182.874	-267.333	-98.414

3rd Exp.



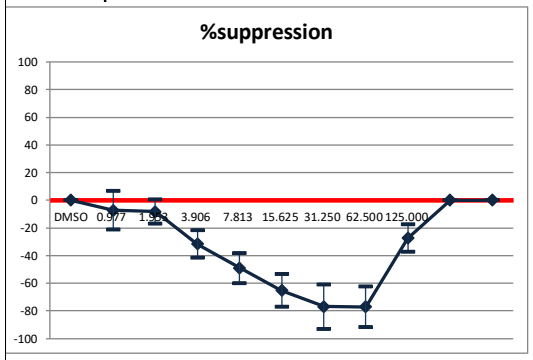
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	4.936	-18.883	28.755
1.953	-12.070	-33.237	9.096
3.906	-10.392	-38.866	18.082
7.813	-36.232	-65.439	-7.024
15.625	-77.143	-113.988	-40.298
31.250	-170.681	-225.401	-115.961
62.500	-199.869	-273.430	-126.308
125.000	-172.637	-250.802	-94.473
250.000	-266.374	-352.495	-180.254

Lab C

A:AAA

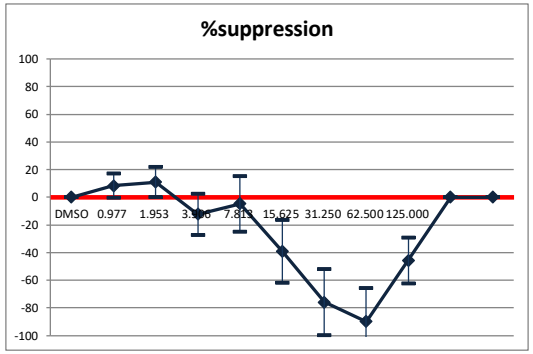
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-7.168	-21.177	6.841
1.953	-8.203	-16.955	0.548
3.906	-31.608	-41.524	-21.692
7.813	-48.929	-59.945	-37.913
15.625	-65.220	-76.944	-53.495
31.250	-76.874	-92.848	-60.899
62.500	-77.111	-91.806	-62.416
125.000	-27.292	-37.416	-17.168

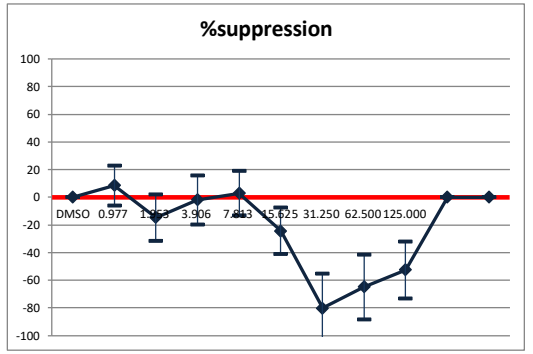
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	8.476	-0.096	17.047
1.953	11.080	0.031	22.129
3.906	-12.222	-27.155	2.711
7.813	-4.723	-24.756	15.310
15.625	-38.997	-61.725	-16.268
31.250	-75.830	-99.565	-52.095
62.500	-89.772	-113.860	-65.684
125.000	-45.658	-62.092	-29.225

3rd Exp.



3rd Exp.

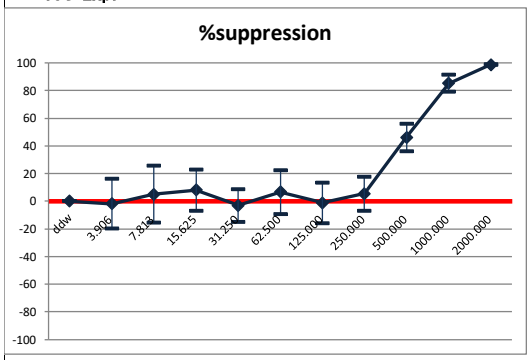
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	8.551	-5.792	22.894
1.953	-14.692	-31.461	2.077
3.906	-1.857	-19.725	16.010
7.813	2.827	-13.263	18.916
15.625	-24.214	-41.008	-7.419
31.250	-80.164	-105.325	-55.003
62.500	-64.751	-88.221	-41.281
125.000	-52.474	-72.943	-32.004

Chemical.15

Lead Lab.

S:SSS

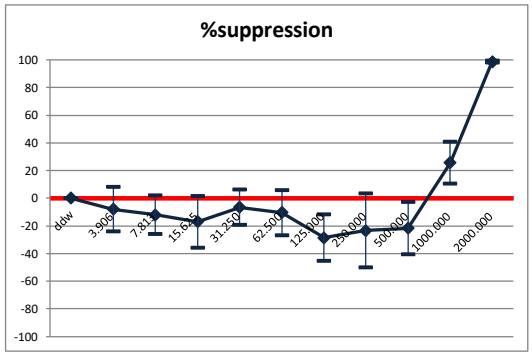
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-1.761	-19.879	16.357
7.813	5.060	-15.417	25.537
15.625	8.016	-6.791	22.824
31.250	-3.099	-15.029	8.831
62.500	6.698	-9.097	22.494
125.000	-1.058	-15.714	13.598
250.000	5.453	-6.762	17.669
500.000	46.205	36.291	56.119
1000.000	85.272	79.070	91.474
2000.000	98.854	98.489	99.219

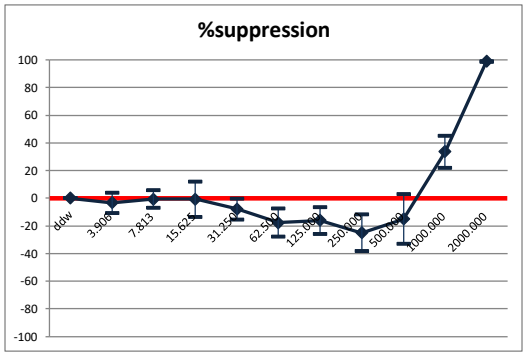
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-7.897	-24.003	8.209
7.813	-11.872	-25.891	2.148
15.625	-17.040	-35.543	1.462
31.250	-6.470	-19.329	6.390
62.500	-10.252	-26.601	6.096
125.000	-28.625	-45.391	-11.860
250.000	-23.410	-50.131	3.310
500.000	-21.702	-40.605	-2.800
1000.000	25.569	10.424	40.715
2000.000	98.755	97.968	99.542

3rd Exp.



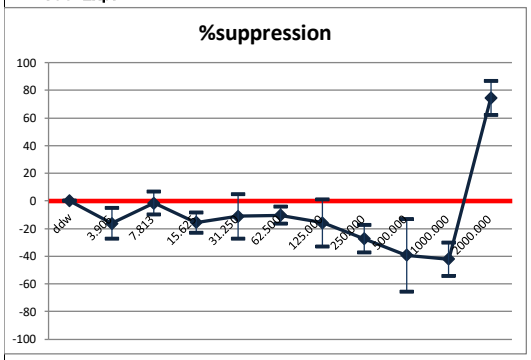
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-3.281	-10.749	4.187
7.813	-0.599	-7.133	5.936
15.625	-0.680	-13.419	12.060
31.250	-7.744	-15.277	-0.211
62.500	-17.594	-27.724	-7.465
125.000	-16.085	-25.893	-6.278
250.000	-24.966	-38.333	-11.600
500.000	-15.041	-33.042	2.960
1000.000	33.598	22.133	45.064
2000.000	99.016	98.688	99.344

Lab A

A:AAN

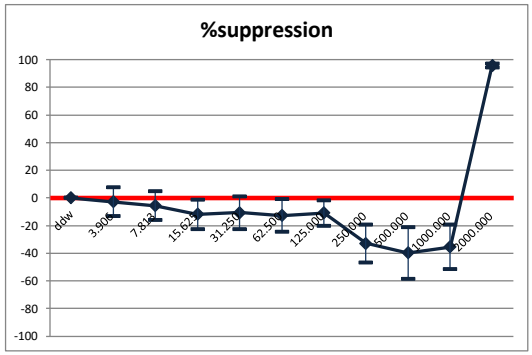
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-16.167	-27.294	-5.039
7.813	-1.503	-9.764	6.757
15.625	-15.650	-23.070	-8.230
31.250	-11.079	-27.019	4.861
62.500	-10.264	-16.248	-4.281
125.000	-15.777	-32.738	1.184
250.000	-27.300	-37.158	-17.441
500.000	-39.130	-65.431	-12.829
1000.000	-42.149	-54.371	-29.928
2000.000	74.377	62.107	86.648

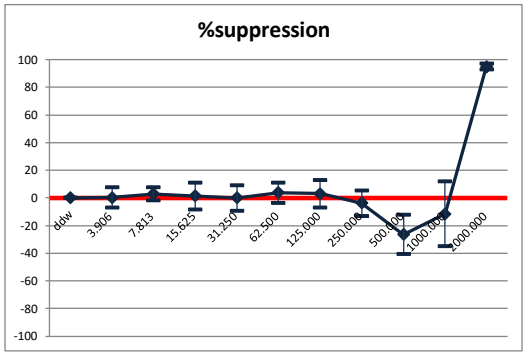
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-2.749	-13.158	7.660
7.813	-5.551	-15.972	4.869
15.625	-11.693	-22.300	-1.086
31.250	-10.687	-22.431	1.057
62.500	-12.688	-24.599	-0.777
125.000	-10.865	-19.956	-1.773
250.000	-32.845	-46.703	-18.987
500.000	-39.597	-58.289	-20.906
1000.000	-35.322	-51.425	-19.218
2000.000	95.877	94.457	97.298

3rd Exp.



3rd Exp.

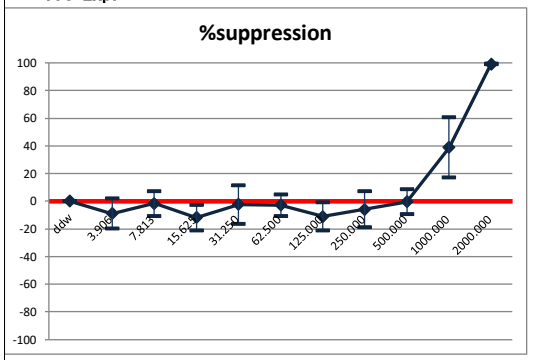
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	0.404	-7.020	7.827
7.813	2.991	-1.701	7.683
15.625	1.401	-8.225	11.027
31.250	0.014	-9.146	9.174
62.500	3.876	-3.462	11.213
125.000	3.186	-6.699	13.071
250.000	-3.651	-12.901	5.599
500.000	-26.476	-40.683	-12.269
1000.000	-11.520	-35.036	11.995
2000.000	95.022	92.756	97.287

Chemical.15

Lab B

S:SSS

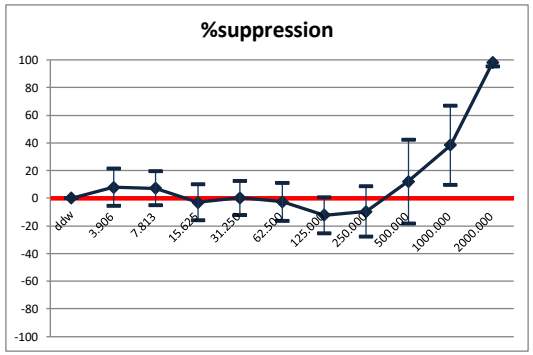
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-8.809	-19.497	1.878
7.813	-1.668	-10.676	7.341
15.625	-11.757	-21.081	-2.432
31.250	-2.393	-16.510	11.725
62.500	-2.873	-10.663	4.916
125.000	-10.947	-21.039	-0.854
250.000	-5.727	-18.570	7.115
500.000	-0.388	-9.453	8.677
1000.000	38.977	17.038	60.916
2000.000	99.354	99.138	99.570

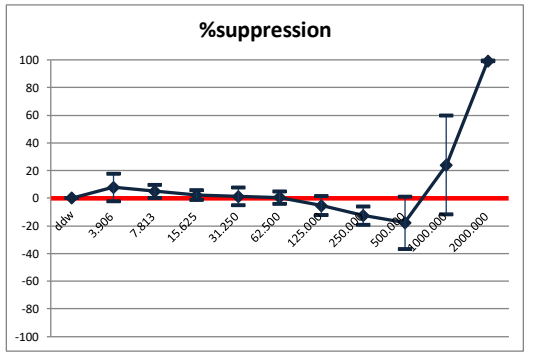
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	7.865	-5.692	21.421
7.813	7.150	-5.093	19.393
15.625	-3.096	-16.101	9.910
31.250	0.287	-11.910	12.484
62.500	-2.592	-16.303	11.119
125.000	-12.284	-25.312	0.743
250.000	-9.620	-27.863	8.624
500.000	12.200	-18.069	42.468
1000.000	38.496	9.842	67.150
2000.000	98.084	95.210	100.958

3rd Exp.



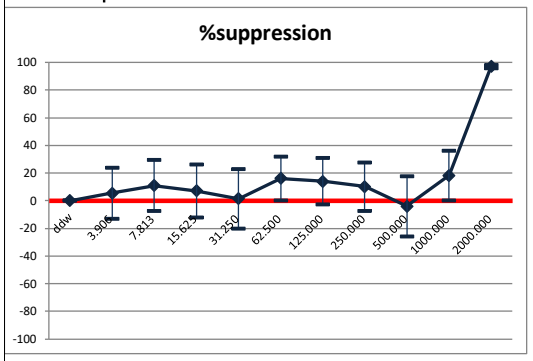
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	7.803	-2.231	17.838
7.813	5.052	0.237	9.866
15.625	2.311	-1.428	6.050
31.250	1.364	-5.013	7.742
62.500	0.453	-3.997	4.903
125.000	-5.366	-12.229	1.497
250.000	-12.501	-19.115	-5.887
500.000	-17.787	-36.857	1.284
1000.000	24.049	-11.718	59.816
2000.000	99.260	98.900	99.620

Lab C

S:SSN

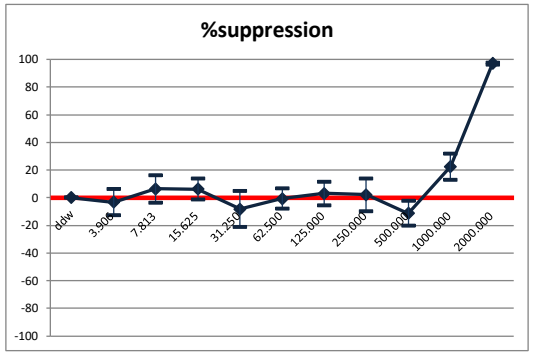
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	5.608	-12.878	24.094
7.813	10.958	-7.462	29.379
15.625	7.130	-12.186	26.445
31.250	1.385	-19.923	22.693
62.500	16.080	0.163	31.998
125.000	14.100	-2.629	30.828
250.000	10.143	-7.451	27.737
500.000	-4.025	-25.997	17.947
1000.000	18.219	0.070	36.368
2000.000	97.026	95.950	98.103

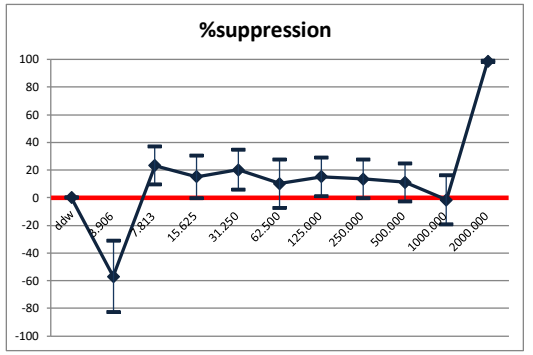
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-3.149	-12.757	6.458
7.813	6.507	-3.516	16.529
15.625	6.329	-1.252	13.910
31.250	-8.237	-21.254	4.779
62.500	-0.669	-7.951	6.612
125.000	3.109	-5.551	11.769
250.000	2.099	-9.952	14.150
500.000	-11.232	-20.204	-2.259
1000.000	22.549	13.000	32.098
2000.000	97.094	96.458	97.731

3rd Exp.



3rd Exp.

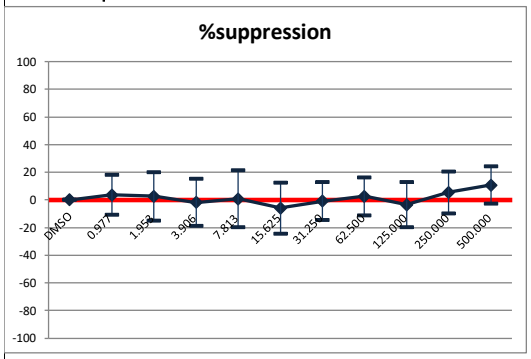
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-56.932	-82.726	-31.138
7.813	23.365	9.532	37.198
15.625	15.168	-0.159	30.495
31.250	20.269	5.700	34.839
62.500	10.141	-7.190	27.472
125.000	15.236	1.246	29.226
250.000	13.605	-0.507	27.718
500.000	11.114	-2.531	24.759
1000.000	-1.640	-19.408	16.128
2000.000	98.675	98.327	99.023

Chemical.16

Lead Lab.

N:NNN

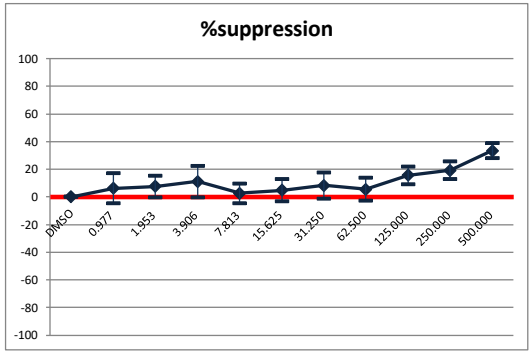
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	3.592	-10.808	17.992
1.953	2.654	-14.773	20.081
3.906	-1.809	-18.875	15.257
7.813	0.813	-19.767	21.393
15.625	-5.831	-24.387	12.726
31.250	-0.793	-14.610	13.024
62.500	2.733	-11.056	16.522
125.000	-3.441	-19.911	13.028
250.000	5.462	-9.756	20.680
500.000	10.764	-2.680	24.208

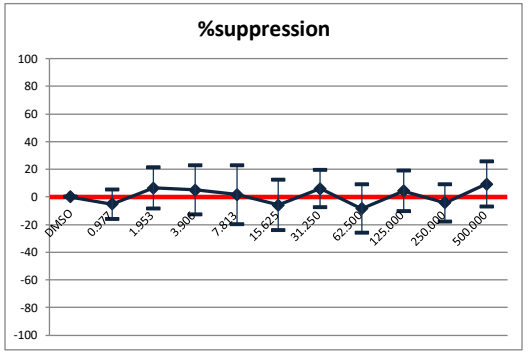
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	6.315	-4.566	17.196
1.953	7.542	-0.404	15.488
3.906	11.181	-0.230	22.592
7.813	2.725	-4.405	9.855
15.625	4.896	-3.118	12.909
31.250	8.433	-1.041	17.908
62.500	5.565	-2.710	13.839
125.000	15.654	9.188	22.120
250.000	19.344	12.784	25.904
500.000	33.515	27.938	39.091

3rd Exp.



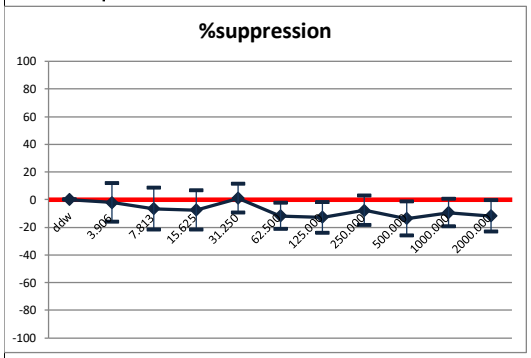
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-5.153	-15.767	5.462
1.953	6.531	-8.452	21.513
3.906	5.146	-12.753	23.044
7.813	1.640	-19.786	23.066
15.625	-5.827	-23.950	12.295
31.250	6.103	-7.292	19.497
62.500	-8.365	-25.857	9.128
125.000	4.419	-10.124	18.962
250.000	-4.091	-17.580	9.398
500.000	9.354	-6.852	25.561

Lab A

N:NNN

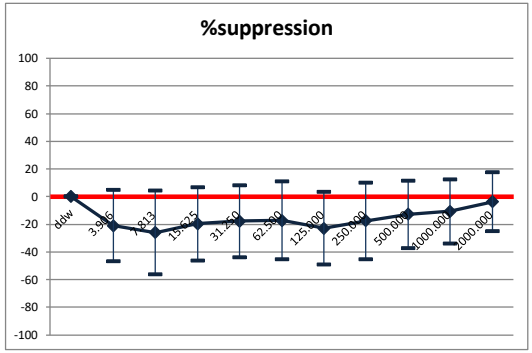
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-2.009	-15.904	11.887
7.813	-6.476	-21.458	8.507
15.625	-7.380	-21.415	6.654
31.250	1.089	-9.176	11.354
62.500	-11.788	-21.236	-2.340
125.000	-12.777	-23.820	-1.735
250.000	-7.709	-18.429	3.011
500.000	-13.541	-25.764	-1.319
1000.000	-9.440	-19.383	0.503
2000.000	-11.814	-23.200	-0.428

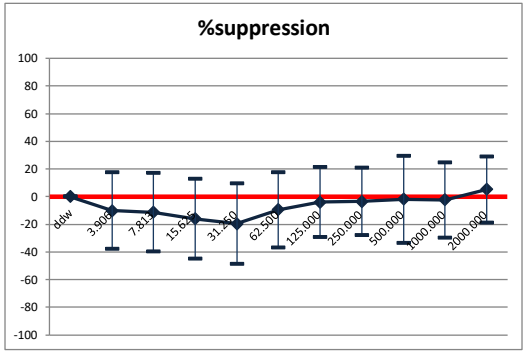
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-20.904	-46.760	4.952
7.813	-25.922	-56.330	4.486
15.625	-19.583	-46.181	7.016
31.250	-17.784	-43.659	8.090
62.500	-17.131	-45.176	10.914
125.000	-22.808	-48.977	3.361
250.000	-17.550	-45.070	9.970
500.000	-12.752	-37.220	11.716
1000.000	-10.615	-33.923	12.692
2000.000	-3.724	-25.111	17.663

3rd Exp.



3rd Exp.

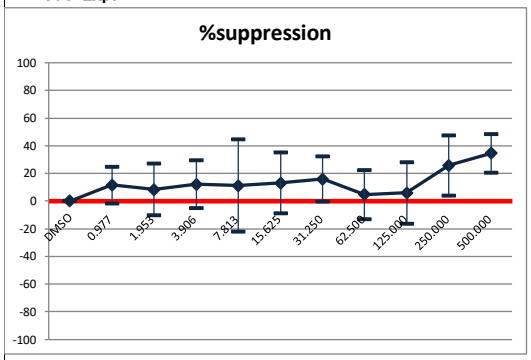
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-10.060	-37.710	17.590
7.813	-11.230	-39.664	17.203
15.625	-15.932	-44.908	13.045
31.250	-19.478	-48.621	9.665
62.500	-9.419	-36.757	17.919
125.000	-3.986	-29.252	21.281
250.000	-3.375	-27.826	21.076
500.000	-1.921	-33.315	29.473
1000.000	-2.292	-29.508	24.925
2000.000	5.172	-18.927	29.272

Chemical.16

Lab B

N:NNN

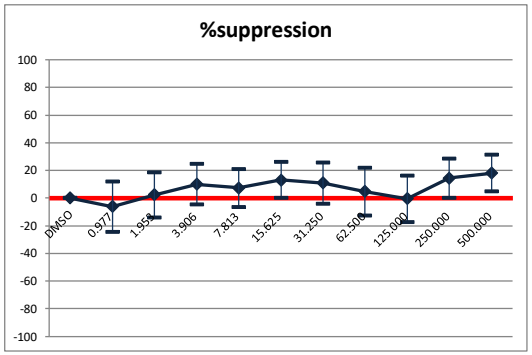
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	11.669	-1.646	24.983
1.953	8.371	-10.358	27.100
3.906	12.144	-5.178	29.466
7.813	11.157	-22.231	44.546
15.625	13.097	-8.993	35.186
31.250	15.840	-0.491	32.172
62.500	4.772	-12.930	22.474
125.000	5.911	-16.171	27.994
250.000	25.903	4.098	47.708
500.000	34.543	20.553	48.533

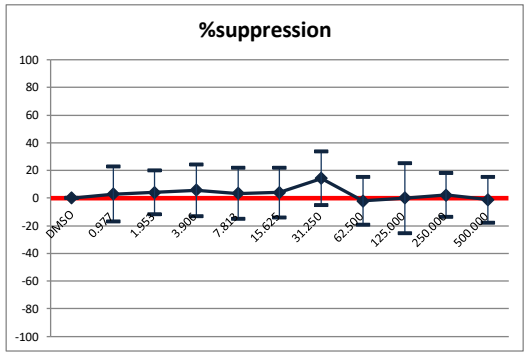
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-6.073	-24.290	12.143
1.953	2.330	-13.881	18.541
3.906	9.949	-4.720	24.618
7.813	7.341	-6.416	21.099
15.625	13.151	0.255	26.048
31.250	10.914	-3.909	25.737
62.500	4.786	-12.558	22.130
125.000	-0.471	-17.316	16.375
250.000	14.447	0.269	28.626
500.000	18.064	4.834	31.295

3rd Exp.



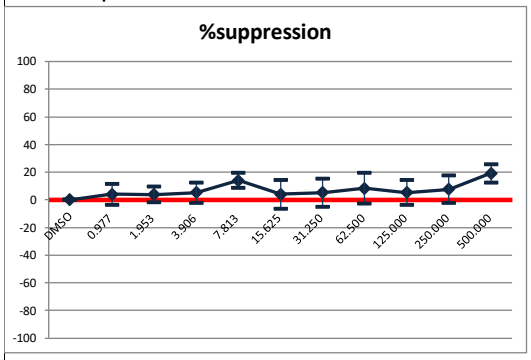
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	2.904	-16.930	22.738
1.953	4.103	-11.726	19.933
3.906	5.822	-12.857	24.502
7.813	3.377	-15.034	21.787
15.625	3.989	-13.894	21.871
31.250	14.322	-5.060	33.703
62.500	-2.022	-19.351	15.307
125.000	0.040	-25.289	25.369
250.000	2.328	-13.350	18.006
500.000	-1.134	-17.734	15.466

Lab C

N:NNN

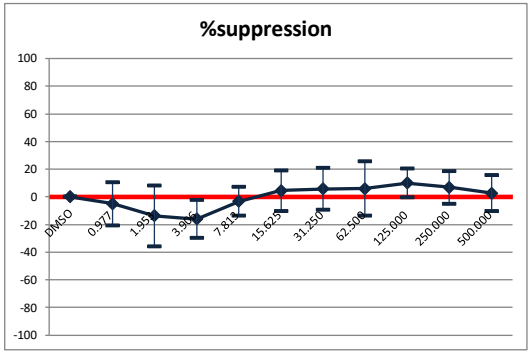
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	4.040	-3.365	11.446
1.953	3.970	-1.669	9.609
3.906	5.273	-2.092	12.638
7.813	14.058	8.690	19.426
15.625	3.995	-6.610	14.600
31.250	5.326	-4.841	15.493
62.500	8.386	-2.751	19.523
125.000	5.278	-3.701	14.257
250.000	7.573	-2.362	17.509
500.000	19.164	12.524	25.803

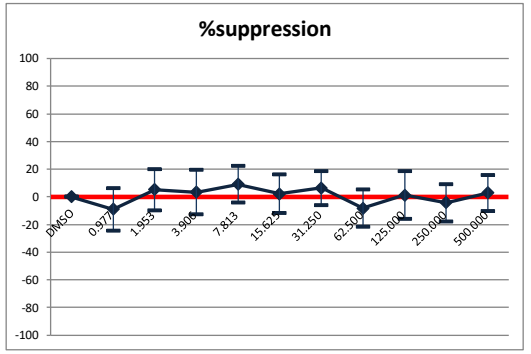
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-4.984	-20.593	10.625
1.953	-13.578	-35.583	8.427
3.906	-16.102	-29.853	-2.351
7.813	-3.292	-13.650	7.067
15.625	4.477	-10.227	19.180
31.250	5.760	-9.305	20.825
62.500	5.968	-13.742	25.677
125.000	10.091	-0.480	20.661
250.000	6.965	-4.954	18.885
500.000	2.629	-10.440	15.698

3rd Exp.



3rd Exp.

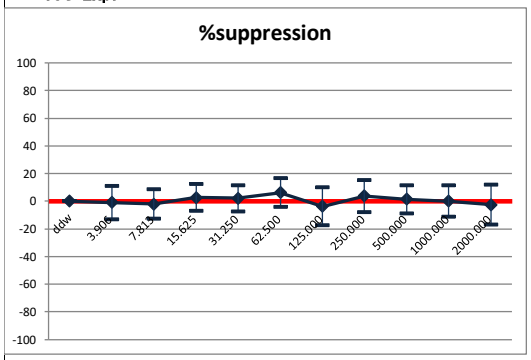
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	-8.953	-24.279	6.373
1.953	5.185	-9.783	20.153
3.906	3.463	-12.582	19.509
7.813	9.161	-4.046	22.367
15.625	2.329	-11.866	16.524
31.250	6.531	-5.775	18.837
62.500	-8.205	-21.780	5.369
125.000	1.331	-15.950	18.611
250.000	-4.198	-17.747	9.351
500.000	2.862	-10.063	15.786

Chemical.17

Lead Lab.

N:NNN

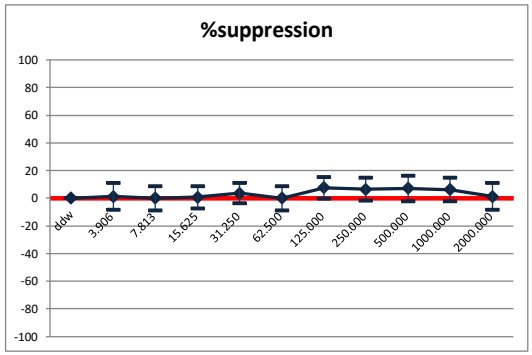
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-0.809	-12.819	11.202
7.813	-1.992	-12.762	8.777
15.625	2.773	-7.006	12.552
31.250	2.170	-7.432	11.771
62.500	6.168	-4.227	16.564
125.000	-3.663	-17.420	10.095
250.000	3.889	-7.796	15.574
500.000	1.597	-8.571	11.764
1000.000	0.197	-11.058	11.453
2000.000	-2.417	-16.956	12.122

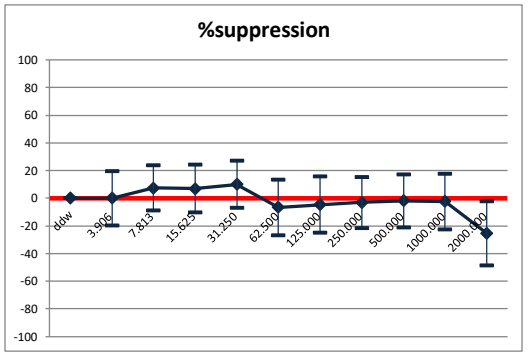
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	1.353	-8.244	10.950
7.813	0.044	-8.582	8.671
15.625	0.786	-7.378	8.950
31.250	3.724	-3.807	11.256
62.500	0.079	-8.704	8.861
125.000	7.568	-0.291	15.427
250.000	6.569	-1.535	14.673
500.000	7.235	-1.958	16.428
1000.000	6.167	-2.315	14.648
2000.000	1.359	-8.278	10.995

3rd Exp.



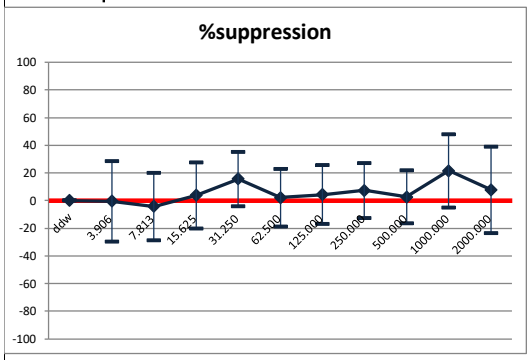
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-0.037	-19.680	19.606
7.813	7.488	-8.995	23.970
15.625	7.041	-10.430	24.512
31.250	10.010	-7.011	27.031
62.500	-6.659	-26.595	13.278
125.000	-4.581	-25.072	15.909
250.000	-3.056	-21.442	15.330
500.000	-1.839	-20.909	17.230
1000.000	-2.356	-22.397	17.686
2000.000	-25.320	-48.649	-1.991

Lab A

N:NNN

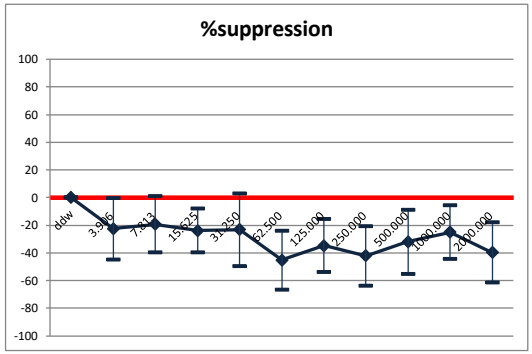
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-0.458	-29.409	28.493
7.813	-4.087	-28.456	20.282
15.625	3.753	-20.216	27.722
31.250	15.606	-3.883	35.094
62.500	2.121	-18.801	23.042
125.000	4.399	-16.939	25.737
250.000	7.424	-12.500	27.349
500.000	2.730	-16.389	21.850
1000.000	21.580	-4.962	48.121
2000.000	7.850	-23.412	39.112

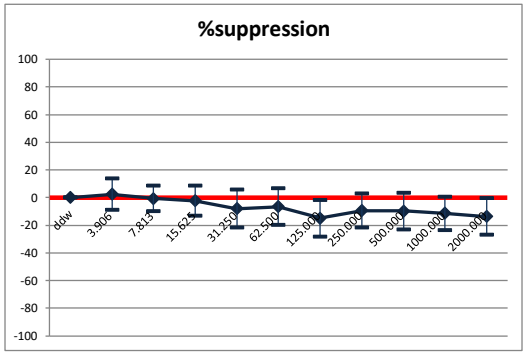
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-22.394	-44.751	-0.038
7.813	-19.300	-39.726	1.126
15.625	-23.749	-39.625	-7.872
31.250	-23.166	-49.558	3.226
62.500	-45.188	-66.627	-23.749
125.000	-34.665	-53.971	-15.359
250.000	-42.100	-63.584	-20.616
500.000	-31.869	-55.028	-8.710
1000.000	-24.967	-44.504	-5.429
2000.000	-39.594	-61.262	-17.925

3rd Exp.



3rd Exp.

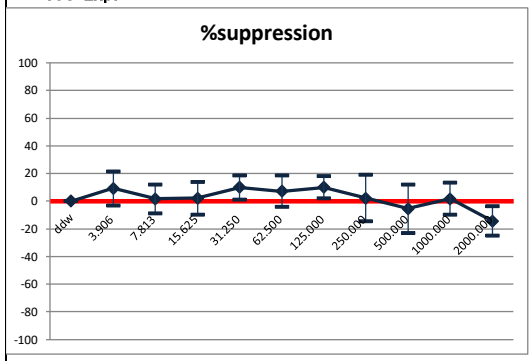
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	2.464	-8.939	13.867
7.813	-0.527	-9.967	8.913
15.625	-2.169	-13.119	8.781
31.250	-7.939	-21.788	5.910
62.500	-6.505	-19.872	6.863
125.000	-14.931	-28.063	-1.798
250.000	-9.407	-21.667	2.854
500.000	-9.734	-22.882	3.414
1000.000	-11.386	-23.532	0.760
2000.000	-13.532	-26.604	-0.460

Chemical.17

Lab B

N:NNN

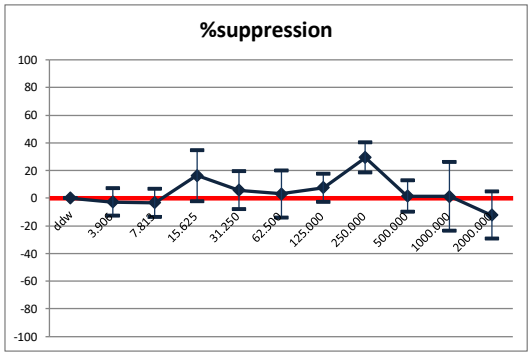
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	9.235	-2.900	21.370
7.813	1.675	-8.728	12.078
15.625	2.170	-9.789	14.129
31.250	9.964	1.347	18.581
62.500	7.276	-4.252	18.804
125.000	10.022	1.888	18.155
250.000	2.193	-14.572	18.957
500.000	-5.313	-22.794	12.167
1000.000	1.834	-9.956	13.623
2000.000	-14.284	-25.081	-3.487

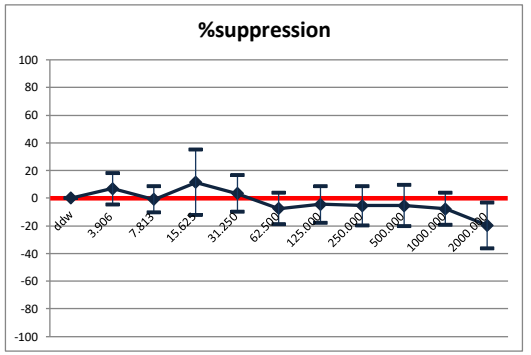
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-2.702	-12.640	7.236
7.813	-3.208	-13.428	7.012
15.625	16.367	-1.989	34.723
31.250	5.867	-7.923	19.657
62.500	3.124	-14.017	20.265
125.000	7.616	-2.404	17.637
250.000	29.492	18.627	40.357
500.000	1.537	-9.722	12.797
1000.000	1.324	-23.592	26.241
2000.000	-12.153	-29.247	4.941

3rd Exp.



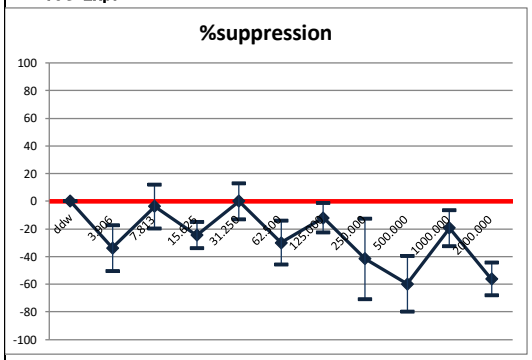
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	6.870	-4.552	18.292
7.813	-0.889	-10.386	8.607
15.625	11.479	-12.060	35.017
31.250	3.487	-9.613	16.588
62.500	-7.520	-18.958	3.918
125.000	-4.518	-17.717	8.682
250.000	-5.460	-19.592	8.672
500.000	-5.260	-20.266	9.746
1000.000	-7.750	-19.275	3.776
2000.000	-19.546	-36.099	-2.993

Lab C

N:ANN

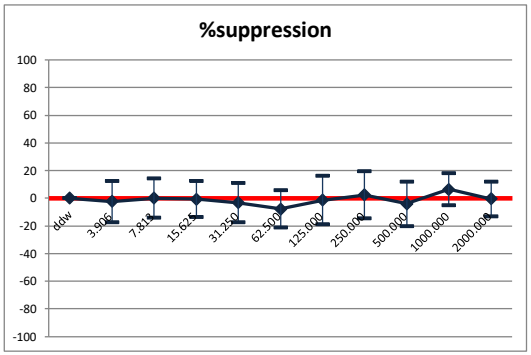
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-33.802	-50.469	-17.136
7.813	-3.814	-19.693	12.065
15.625	-24.447	-33.829	-15.065
31.250	0.065	-13.040	13.171
62.500	-29.859	-45.639	-14.078
125.000	-11.985	-22.517	-1.453
250.000	-41.603	-70.701	-12.505
500.000	-59.757	-79.734	-39.781
1000.000	-19.430	-32.418	-6.441
2000.000	-56.165	-67.932	-44.398

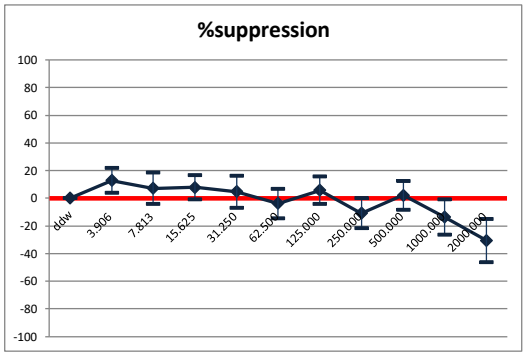
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-2.342	-17.411	12.727
7.813	0.169	-14.169	14.506
15.625	-0.646	-13.699	12.406
31.250	-3.186	-17.276	10.903
62.500	-7.721	-21.135	5.693
125.000	-1.286	-18.942	16.370
250.000	2.467	-14.619	19.552
500.000	-4.052	-20.088	11.984
1000.000	6.434	-5.197	18.066
2000.000	-0.440	-13.020	12.141

3rd Exp.

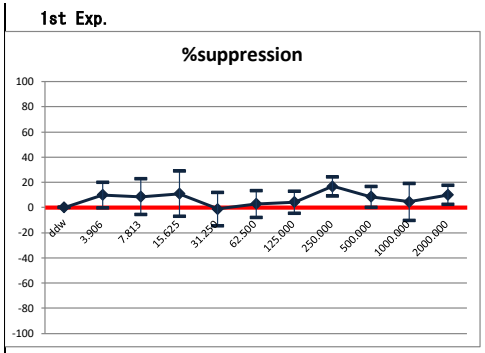


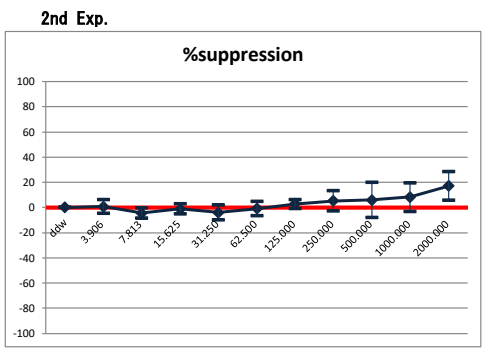
3rd Exp.

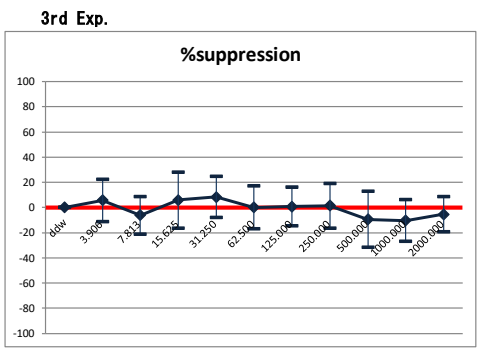
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	12.816	3.838	21.795
7.813	7.254	-4.056	18.563
15.625	7.999	-0.974	16.973
31.250	4.711	-6.903	16.325
62.500	-3.807	-14.603	6.989
125.000	5.802	-4.024	15.627
250.000	-10.854	-21.678	-0.030
500.000	2.166	-8.395	12.727
1000.000	-13.639	-26.341	-0.936
2000.000	-30.528	-46.059	-14.997

Lead Lab.

N:NNN







1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	9.961	-0.354	20.275
7.813	8.677	-5.514	22.868
15.625	10.943	-6.996	28.881
31.250	-1.130	-14.268	12.008
62.500	2.844	-7.643	13.332
125.000	4.384	-4.394	13.163
250.000	16.888	9.212	24.564
500.000	8.616	0.334	16.898
1000.000	4.650	-10.031	19.331
2000.000	9.973	2.341	17.606

2nd Exp.

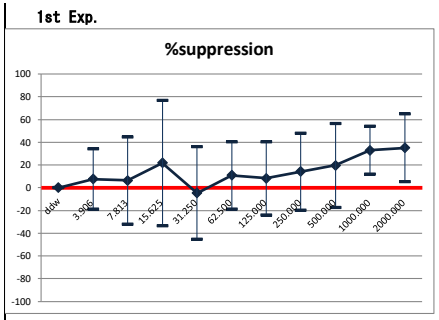
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	0.904	-4.327	6.136
7.813	-4.343	-8.552	-0.134
15.625	-1.002	-5.173	3.169
31.250	-3.842	-9.873	2.189
62.500	-0.839	-6.526	4.849
125.000	2.834	-0.769	6.437
250.000	5.357	-2.558	13.272
500.000	6.017	-7.877	19.911
1000.000	8.311	-2.893	19.515
2000.000	17.232	5.992	28.471

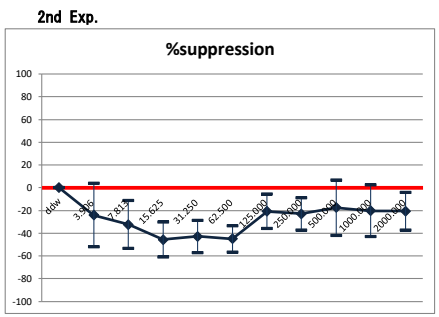
3rd Exp.

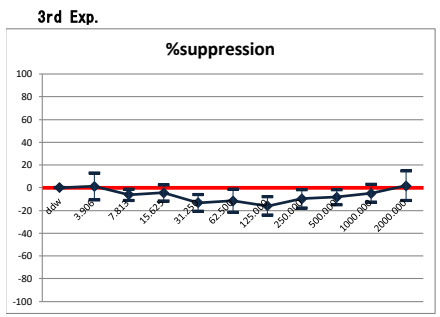
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	5.654	-10.979	22.288
7.813	-6.002	-20.939	8.935
15.625	5.965	-16.367	28.297
31.250	8.455	-7.900	24.810
62.500	0.108	-16.940	17.157
125.000	0.770	-14.648	16.187
250.000	1.495	-16.216	19.206
500.000	-9.339	-31.444	12.766
1000.000	-10.259	-26.776	6.259
2000.000	-5.255	-19.102	8.592

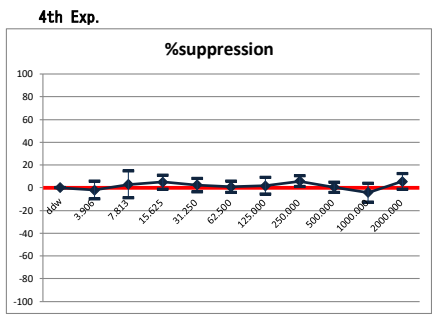
Lab A

N:SANN









1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	7.759	-18.633	34.151
7.813	6.362	-31.987	44.712
15.625	21.847	-33.376	77.070
31.250	-4.615	-45.452	36.222
62.500	10.885	-18.765	40.535
125.000	8.253	-23.934	40.439
250.000	14.218	-19.683	48.120
500.000	19.683	-17.377	56.742
1000.000	33.016	12.010	54.022
2000.000	35.210	5.558	64.862

2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-24.049	-51.936	3.839
7.813	-32.322	-53.497	-11.148
15.625	-45.630	-61.017	-30.243
31.250	-42.868	-56.939	-28.796
62.500	-44.828	-56.482	-33.173
125.000	-20.730	-35.847	-5.612
250.000	-22.908	-37.212	-8.604
500.000	-17.532	-41.982	6.918
1000.000	-20.199	-42.999	2.602
2000.000	-20.573	-37.115	-4.030

3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	1.233	-10.504	12.971
7.813	-6.089	-11.091	-1.088
15.625	-4.508	-11.599	2.583
31.250	-13.259	-20.666	-5.853
62.500	-11.543	-21.732	-1.354
125.000	-15.938	-24.173	-7.702
250.000	-9.728	-17.801	-1.655
500.000	-8.279	-15.078	-1.480
1000.000	-4.948	-12.746	2.850
2000.000	1.788	-11.121	14.697

4th Exp.

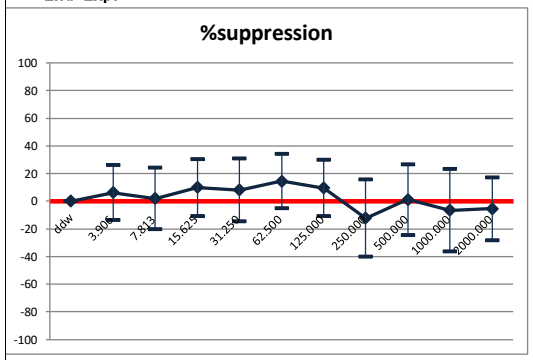
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-1.978	-9.890	5.934
7.813	3.029	-8.694	14.753
15.625	5.057	-1.082	11.196
31.250	2.404	-3.412	8.220
62.500	0.872	-4.022	5.765
125.000	1.825	-5.323	8.973
250.000	5.836	0.960	10.712
500.000	0.488	-4.154	5.131
1000.000	-4.296	-12.620	4.029
2000.000	5.603	-1.098	12.304

Chemical.18

Lab B

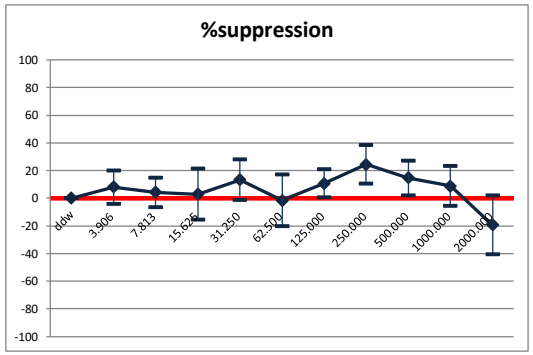
N:NNN

2nd Exp.



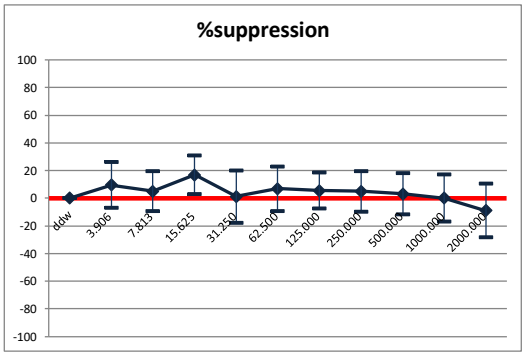
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	6.165	-13.679	26.010
7.813	1.950	-20.287	24.187
15.625	9.955	-10.579	30.489
31.250	8.156	-14.419	30.731
62.500	14.596	-5.149	34.342
125.000	9.564	-10.781	29.910
250.000	-12.198	-40.100	15.704
500.000	1.185	-24.321	26.691
1000.000	-6.478	-36.183	23.228
2000.000	-5.441	-28.302	17.419

3rd Exp.



a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	8.072	-3.853	19.998
7.813	4.435	-6.232	15.103
15.625	3.023	-15.487	21.534
31.250	13.275	-1.367	27.917
62.500	-1.502	-20.290	17.286
125.000	10.646	0.468	20.824
250.000	24.490	10.528	38.451
500.000	14.725	2.277	27.173
1000.000	8.829	-5.519	23.177
2000.000	-19.233	-40.445	1.979

4th Exp.

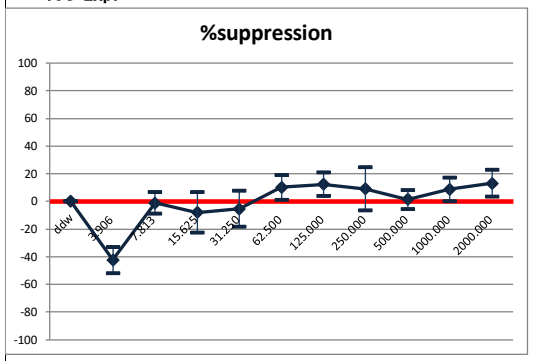


a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	9.630	-6.871	26.132
7.813	5.167	-9.443	19.777
15.625	16.994	2.925	31.062
31.250	1.151	-17.569	19.872
62.500	6.849	-9.298	22.996
125.000	5.561	-7.445	18.567
250.000	5.045	-9.572	19.663
500.000	3.266	-11.770	18.301
1000.000	0.032	-17.053	17.118
2000.000	-8.904	-28.422	10.614

Lab C

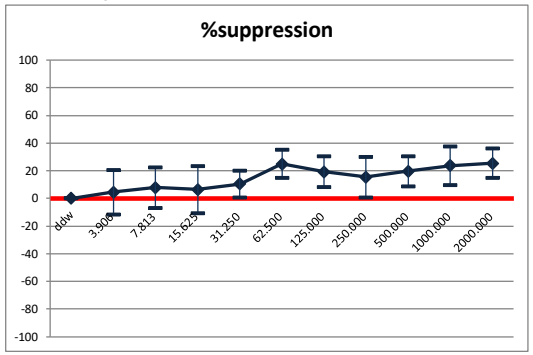
N:NNN

1st Exp.



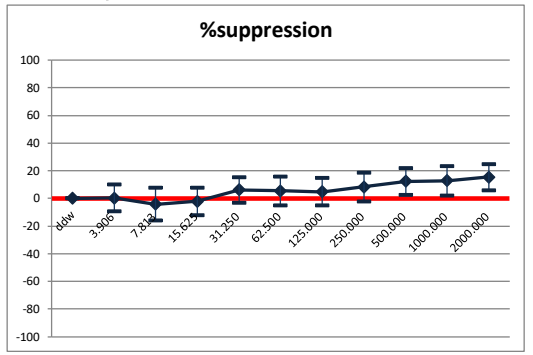
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	-42.250	-51.799	-32.701
7.813	-1.016	-8.898	6.867
15.625	-7.849	-22.421	6.724
31.250	-5.323	-18.378	7.733
62.500	10.152	1.020	19.284
125.000	12.482	4.042	20.922
250.000	9.130	-6.405	24.665
500.000	1.550	-5.354	8.454
1000.000	8.730	0.238	17.221
2000.000	13.153	3.356	22.951

2nd Exp.



a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	4.487	-11.451	20.425
7.813	7.862	-6.919	22.644
15.625	6.521	-10.479	23.521
31.250	10.432	0.822	20.042
62.500	24.966	14.683	35.249
125.000	19.360	8.035	30.685
250.000	15.499	0.870	30.128
500.000	19.710	8.808	30.612
1000.000	23.660	9.537	37.782
2000.000	25.474	15.001	35.948

3rd Exp.



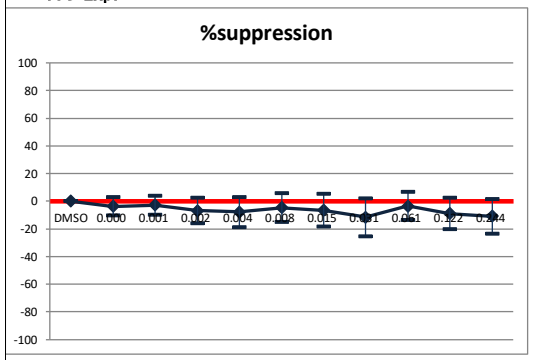
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	0.328	-9.460	10.116
7.813	-4.151	-16.025	7.722
15.625	-2.027	-11.945	7.890
31.250	6.299	-2.896	15.493
62.500	5.530	-4.894	15.954
125.000	4.768	-5.234	14.770
250.000	8.316	-1.945	18.577
500.000	12.477	2.768	22.185
1000.000	12.751	2.317	23.185
2000.000	15.406	6.007	24.805

Chemical.19

Lead Lab.

N:NNN

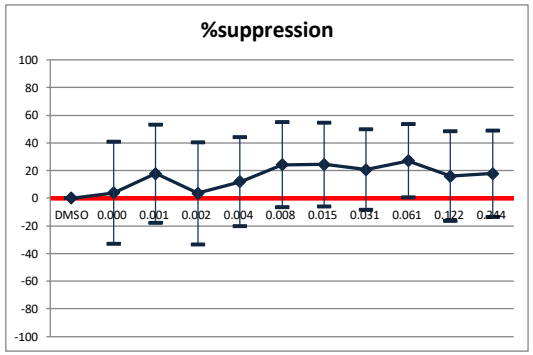
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.000	-3.616	-10.292	3.061
0.001	-2.843	-9.832	4.145
0.002	-6.886	-16.122	2.350
0.004	-7.806	-18.516	2.904
0.008	-4.664	-15.070	5.742
0.015	-6.470	-18.190	5.249
0.031	-11.594	-25.174	1.986
0.061	-3.443	-13.750	6.864
0.122	-8.831	-20.146	2.484
0.244	-10.773	-23.309	1.764

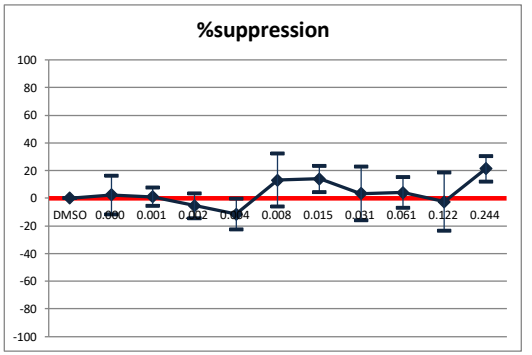
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.000	3.929	-33.085	40.942
0.001	17.729	-17.552	53.009
0.002	3.601	-33.428	40.631
0.004	12.001	-20.330	44.332
0.008	24.250	-6.652	55.151
0.015	24.529	-5.812	54.870
0.031	20.648	-8.405	49.702
0.061	27.064	0.583	53.545
0.122	16.038	-16.541	48.618
0.244	17.800	-13.526	49.127

3rd Exp.



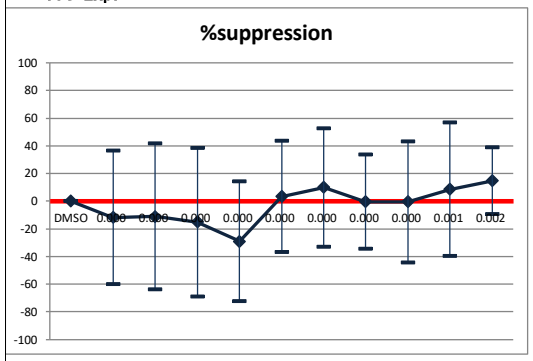
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.000	2.430	-11.447	16.307
0.001	1.009	-5.602	7.620
0.002	-5.357	-14.302	3.588
0.004	-11.428	-22.519	-0.337
0.008	13.065	-6.182	32.313
0.015	13.970	4.431	23.509
0.031	3.457	-15.898	22.813
0.061	4.193	-7.056	15.442
0.122	-2.458	-23.475	18.559
0.244	21.367	12.209	30.524

Lab A

N:NAN

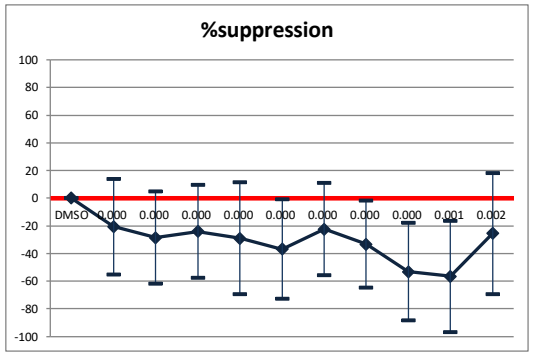
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.000	-11.828	-60.090	36.435
0.000	-11.091	-63.830	41.648
0.000	-15.176	-68.946	38.595
0.000	-29.024	-72.388	14.339
0.000	3.390	-36.956	43.735
0.000	9.936	-32.797	52.669
0.000	-0.331	-34.390	33.729
0.000	-0.399	-44.293	43.494
0.001	8.708	-39.371	56.786
0.002	14.780	-9.331	38.890

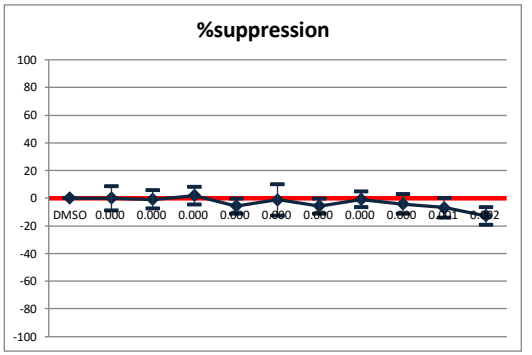
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.000	-20.594	-54.953	13.765
0.000	-28.541	-61.955	4.873
0.000	-24.076	-57.727	9.576
0.000	-29.004	-69.353	11.345
0.000	-36.769	-72.590	-0.947
0.000	-22.426	-55.801	10.949
0.000	-33.252	-64.581	-1.924
0.000	-53.123	-88.496	-17.750
0.001	-56.545	-96.720	-16.370
0.002	-25.424	-69.270	18.423

3rd Exp.



3rd Exp.

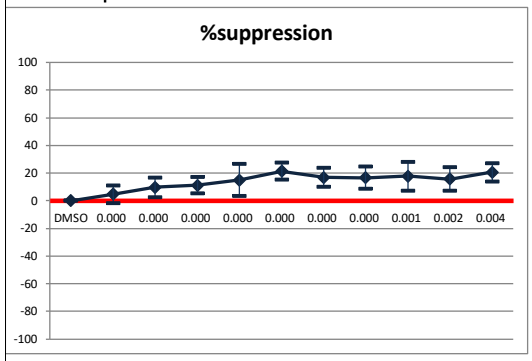
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.000	0.073	-8.630	8.775
0.000	-0.847	-7.489	5.795
0.000	1.915	-4.527	8.356
0.000	-5.512	-10.960	-0.063
0.000	-1.127	-12.483	10.229
0.000	-5.574	-10.951	-0.197
0.000	-0.761	-6.554	5.032
0.000	-4.135	-11.237	2.968
0.001	-6.842	-13.992	0.308
0.002	-12.787	-19.324	-6.249

Chemical.19

Lab B

N:NNN

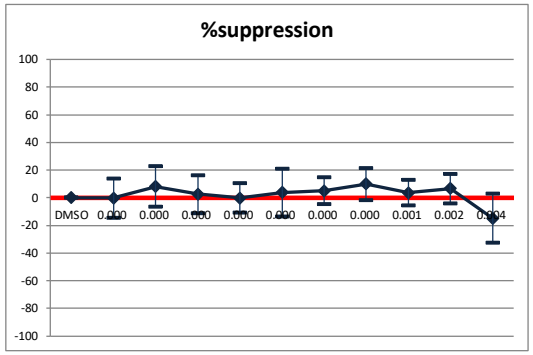
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.000	4.730	-1.639	11.098
0.000	9.816	2.735	16.897
0.000	11.287	5.508	17.065
0.000	15.015	3.522	26.509
0.000	21.359	15.135	27.583
0.000	16.948	10.246	23.649
0.000	16.649	8.688	24.610
0.001	17.783	7.288	28.277
0.002	15.809	7.468	24.151
0.004	20.702	13.992	27.411

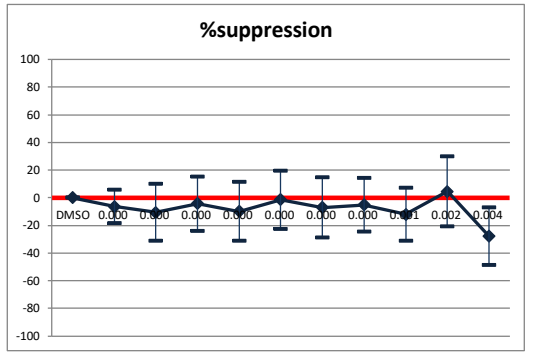
3rd Exp.



3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.000	-0.181	-14.335	13.973
0.000	8.095	-6.652	22.841
0.000	2.670	-11.080	16.419
0.000	-0.063	-10.626	10.501
0.000	3.758	-13.442	20.957
0.000	5.051	-4.565	14.667
0.000	9.971	-1.503	21.445
0.001	3.692	-5.611	12.996
0.002	6.711	-3.825	17.248
0.004	-14.901	-32.691	2.890

4th Exp.



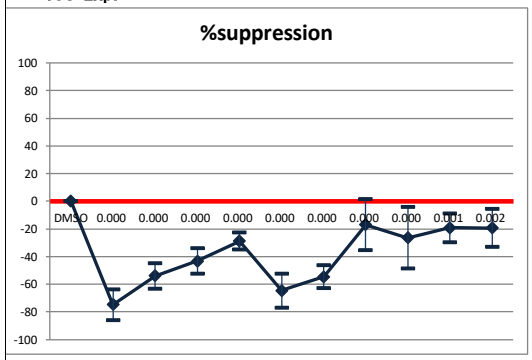
4th Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.000	-6.364	-18.418	5.690
0.000	-10.514	-31.040	10.012
0.000	-4.177	-23.790	15.436
0.000	-9.786	-30.924	11.352
0.000	-1.449	-22.471	19.573
0.000	-6.970	-28.758	14.818
0.000	-5.019	-24.513	14.476
0.001	-11.890	-31.230	7.450
0.002	4.666	-20.722	30.053
0.004	-27.686	-48.330	-7.043

Lab C

N:ANN

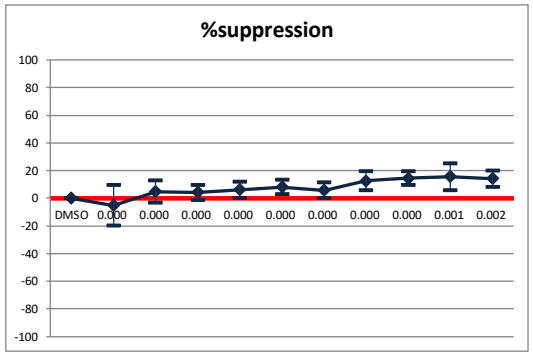
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.000	-74.720	-85.932	-63.507
0.000	-53.984	-63.077	-44.892
0.000	-43.112	-52.401	-33.822
0.000	-28.857	-35.048	-22.666
0.000	-64.546	-76.963	-52.129
0.000	-54.591	-62.900	-46.283
0.000	-16.922	-35.418	1.574
0.000	-26.312	-48.775	-3.849
0.001	-19.135	-29.428	-8.841
0.002	-19.237	-32.867	-5.608

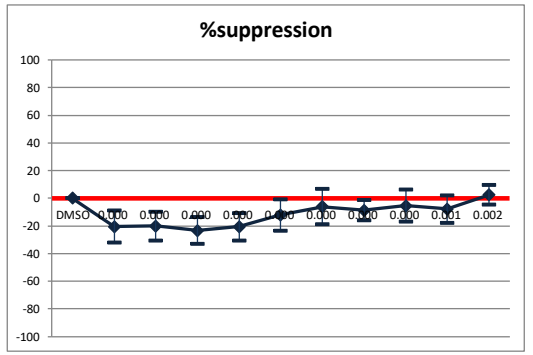
3rd Exp.



3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.000	-5.125	-19.688	9.438
0.000	4.849	-3.082	12.779
0.000	4.305	-1.060	9.670
0.000	6.121	0.386	11.857
0.000	8.125	2.899	13.352
0.000	5.765	0.041	11.488
0.000	12.667	5.694	19.640
0.000	14.563	9.518	19.608
0.001	15.692	6.090	25.295
0.002	14.243	8.260	20.225

4th Exp.



4th Exp.

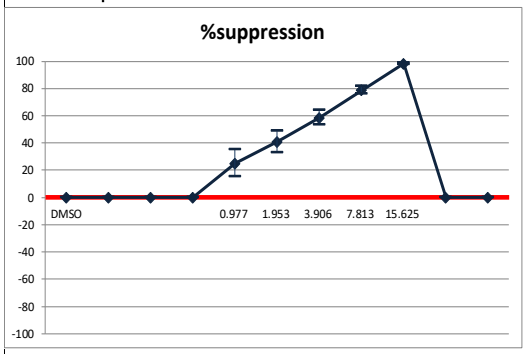
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.000	-20.526	-32.081	-8.971
0.000	-20.130	-30.358	-9.902
0.000	-23.430	-33.140	-13.720
0.000	-20.614	-30.587	-10.641
0.000	-12.015	-23.421	-0.609
0.000	-6.104	-18.940	6.731
0.000	-8.618	-15.919	-1.317
0.000	-5.326	-17.068	6.416
0.001	-7.754	-17.678	2.170
0.002	2.543	-4.491	9.577

Chemical.20

Lead Lab.

S:SSS

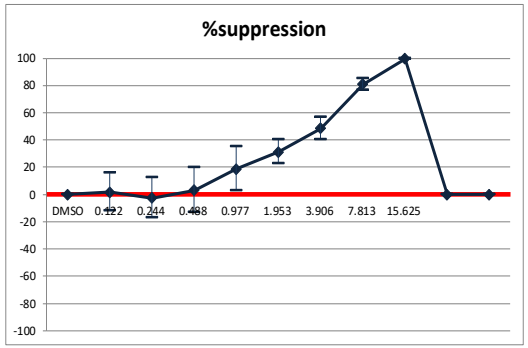
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	25.042	15.192	34.892
1.953	40.767	32.957	48.576
3.906	58.491	52.949	64.033
7.813	78.670	75.760	81.581
15.625	98.129	97.624	98.635

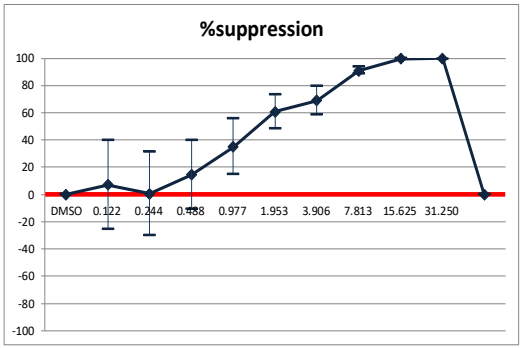
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	1.705	-12.388	15.798
0.244	-2.609	-17.385	12.167
0.488	3.173	-13.084	19.430
0.977	18.648	2.382	34.915
1.953	31.340	22.501	40.179
3.906	48.386	40.235	56.538
7.813	80.909	76.712	85.106
15.625	99.585	99.306	99.863

3rd Exp.



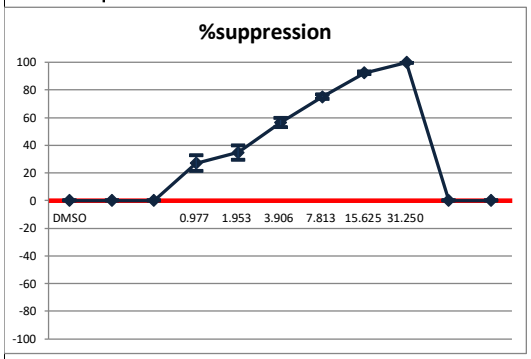
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	6.943	-25.889	39.775
0.244	0.559	-30.222	31.341
0.488	14.368	-10.724	39.461
0.977	35.036	14.713	55.360
1.953	60.598	48.277	72.918
3.906	68.843	58.262	79.424
7.813	90.872	88.334	93.410
15.625	99.592	99.359	99.825
31.250	99.805	99.285	100.326

Lab A

S:SSS

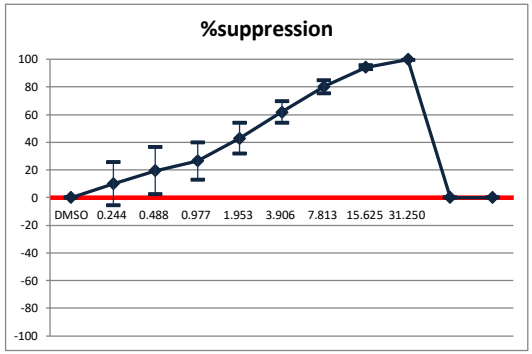
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.977	27.054	21.403	32.704
1.953	34.773	29.365	40.182
3.906	56.318	52.979	59.657
7.813	75.183	73.337	77.029
15.625	92.338	91.437	93.239
31.250	99.852	99.460	100.243

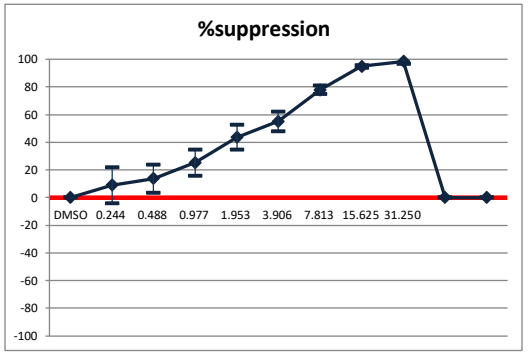
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	10.006	-5.641	25.653
0.488	19.584	2.382	36.787
0.977	26.526	13.019	40.033
1.953	43.032	31.952	54.113
3.906	61.864	54.000	69.728
7.813	80.235	75.657	84.814
15.625	94.242	92.863	95.621
31.250	99.966	99.811	100.120

3rd Exp.



3rd Exp.

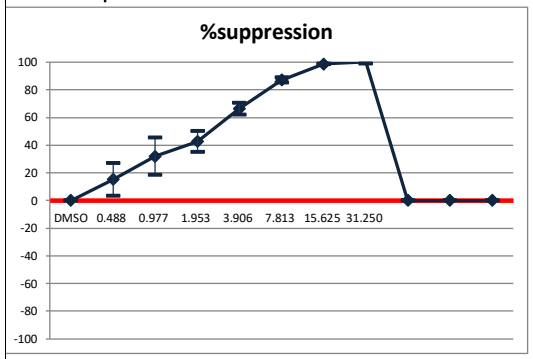
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.244	9.016	-3.948	21.979
0.488	13.737	3.701	23.773
0.977	25.344	15.851	34.836
1.953	43.641	34.636	52.646
3.906	55.206	48.053	62.359
7.813	78.054	75.023	81.085
15.625	95.011	94.125	95.898
31.250	98.462	96.851	100.072

Chemical.20

Lab B

S:SSS

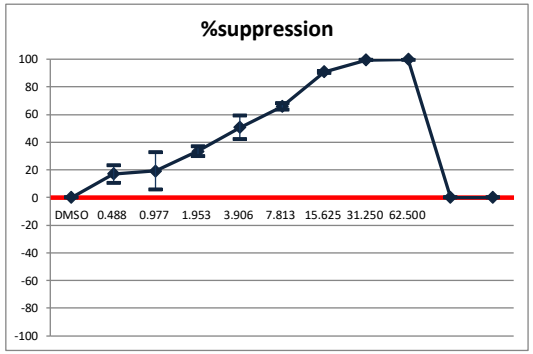
1st Exp.



1st Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.488	15.311	3.499	27.124
0.977	31.988	18.441	45.536
1.953	42.752	35.147	50.356
3.906	66.510	62.193	70.828
7.813	87.281	85.558	89.003
15.625	98.884	98.488	99.280
31.250	100.084	99.157	101.010

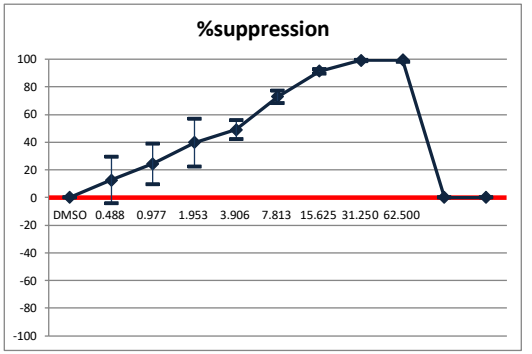
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.488	17.067	10.604	23.530
0.977	19.289	5.830	32.749
1.953	33.504	30.005	37.003
3.906	50.762	42.211	59.312
7.813	65.811	63.394	68.227
15.625	90.948	90.187	91.710
31.250	99.511	99.394	99.627
62.500	99.943	99.684	100.203

3rd Exp.



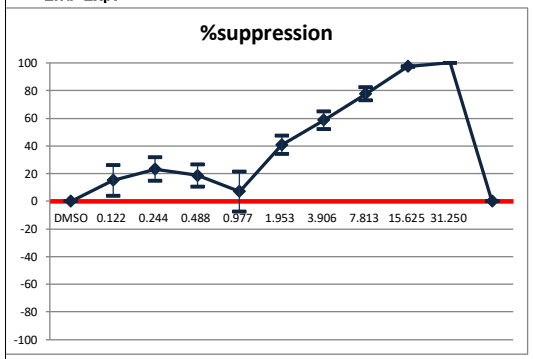
3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.488	12.683	-4.034	29.400
0.977	24.417	9.720	39.114
1.953	39.881	22.578	57.183
3.906	49.098	42.358	55.838
7.813	72.990	68.578	77.402
15.625	91.380	89.608	93.152
31.250	99.312	98.886	99.738
62.500	99.474	98.022	100.927

Lab C

S:SSS

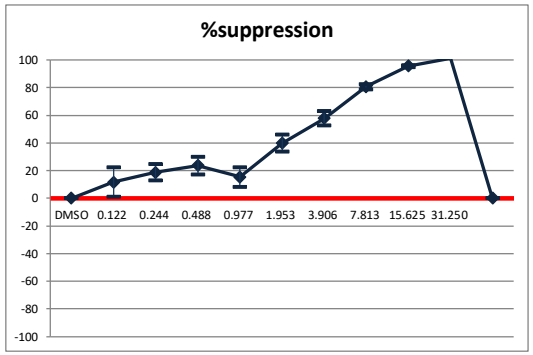
2nd Exp.



2nd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	15.162	4.084	26.240
0.244	23.336	14.837	31.835
0.488	18.681	10.526	26.836
0.977	7.222	-7.177	21.622
1.953	40.686	34.075	47.297
3.906	58.669	52.112	65.226
7.813	77.794	73.182	82.407
15.625	97.514	97.186	97.843
31.250	100.085	99.977	100.194

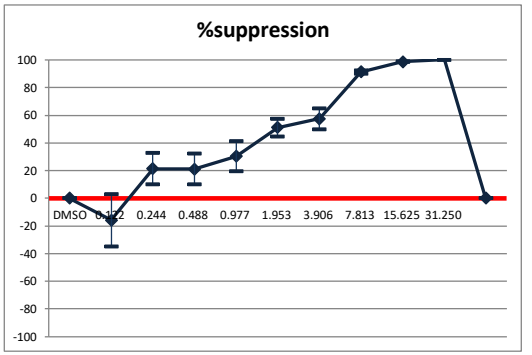
3rd Exp.



3rd Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	11.789	1.093	22.485
0.244	18.884	13.011	24.758
0.488	23.821	17.428	30.215
0.977	15.509	8.399	22.619
1.953	39.799	33.659	45.938
3.906	57.864	52.531	63.197
7.813	80.698	78.604	82.791
15.625	95.797	95.073	96.520
31.250	101.041	100.221	101.860

4th Exp.



4th Exp.

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.122	-15.730	-34.703	3.242
0.244	21.434	10.133	32.736
0.488	21.177	10.084	32.270
0.977	30.429	19.494	41.364
1.953	51.242	44.819	57.666
3.906	57.691	50.118	65.264
7.813	91.417	90.273	92.561
15.625	98.819	98.624	99.015
31.250	100.159	99.961	100.357

添付資料１．Appendix Table 1
化学物質の免疫毒性データーベース（Phase I, 5 chemicals and Phase II, 20 chemicals）

Chemical name	Immunotoxicity classification		NTP data					
			In vivo	*Ex vivo*		*In vitro*		Mode of action
	Classification	Rationale	immune sytem organ weight	cytokine production	TDAR	cytokine production	T cell proliferation	
Phase I study								
Dibutyl phthalate	TTC	3), 4)	**A (spleen)**			**S (IL-2, 4, IFN-g)(H) A (IL-1b)(H) x 3 S (IL-1b)**		This compound then is proposed to modulate cytokine secretion from both monocytes/macrophages and T cells.
Hydrocortisone	TTC	1)	**S (thymus) x 2 S (spleen)**		**N**	**S (IFN-a)**		
Lead(II) acetate	TTC	1)	**A(thymus)**		**S N**	**S (IFN-g, IL-1b)(H) A (IL-4)(H)**	**S(H)**	
Nickel(II) sulfate	TTC	1)	**N S (thymus)**		**N**	**A (IL-4, IFN-g)(H) S (IL-2) S (IFN-g)**		
dimethyldithiocarbamate (DMDTC)	NTTC					**S (IL-1b)**	**N(H)**	
Phase II study								
2.4-diaminotoluene	NTTC		**N (spleen) A (spleen)**		**S**	-	-	
Benzo(a)pyrene	TTC	2), 3)		**S(IL-2)**	**S x 5 A**	**A (IL-4)(H) N (IFNγ)(H) N (IL-2)(H) S (IL-2, 4, IFN-g)**	**S (H) x 2 S x 6**	Disruption of T-cell activities has been associated with B(a)P induced immunotoxic effects (Urso et al. 1986).
Cadmium Chloride	TTC	2), 3)	**A (spleen) S (spleen)**	**A (IL-2) N (IFN-γ)**	**S x 4**	**A (IFN-g)(H) S (IL-2, IFN-g) A (IFN-g) S (IL-2) A (IL-2)**	**S**	
Dibromoacetic acid (DBAA)	TTC	1), 4)	**A (spleen) S (thymus) x 2**		**N**	**S (IL-2, 4)**	**S**	Overall, studies suggest that DBAA produces immunotoxic effects through modulation of T-cell mediated cell immunity. T-cell apoptosis, through extrinsic and intrinsic pathways, are proposed to play a role in the mode of action.
Diethylstilbestrol (DES)	TTC	1), 2), 4)	**S (thymus) x 4 A (thymus) x 2 A (spleen)**	**A (IFN-g) x 3**	**S**	**A (IL-1) A (IL-2)**		DES exposure was associated with down-regulation of gene expression in the TCR complex, and the TCR and CD28 signaling pathways.
Diphenylhydantoin	TTC	2), 3), 4)		**A (IL-4) S (IFN-γ, IL-2) S (IL-1α) N (IL-6, 12)**	**S A x 2**	-	-	DPH treatment can lead to a decrease of suppressor T cells
Ethylene Dibromide (EDB)	TTC	1)	**S (thymus) S (spleen) N**		**A**	-	**S**	
Glycidol	NTTC		**N**		**S**	-	-	Studies suggest that glycidol modulates B-cell function, and NK cell and macrophage activities.111 and decreased cytotoxic T cell activity
Indomethacin	TTC	3), 4)	**N A (spleen)**		**S x 3 A x 1**	**A (IL-2)(H) A (IFN-g)(H)**	**A (H) x 4 S A x 3**	indomethacin inhibition of prostaglandin synthesis leads to altered T-cell function,
Isonicotinic Acid Hydrazide (IAH)	TTC	2)	**N x 2**			**S (IL-2)(H) A (IL-2)(H) S (IL-1)(H)**	**S (H) x 3 A (H) x 6 A N**	
Nitrobenzene	Undetermined		**A (spleen) x 3 A (thymus) x 2**		**S N**	-		effects on T-cell function may play a role in increased susceptibility to L. monocytogenes (Burns et al. 1994).
Urethane, Ethyl carbamate	TTC	1)	**S (thymus) x2 S (spleen) x 2 N A (thymus) A (spleen)**	**N (IL-2)**	**S x 2 N**	**N (IL-2, 4, IFN-g)(H) A (IFN-g)(H) S (IFN-g)(H)**	**N x 2**	
Tributyltin Chloride (TBTC)	TTC	1)	**S (thymus) x4 S (spleen) x 3**		**N S**	**A (INF-g)(H) N (IL-2, 4)(H) S (IFN-g)(H)**	**S (H) S x 3**	
Perflouorooctanoic Acid (PFOA)	TTC	1)	**S (thymus) x2 S (spleen) x 2**	**N (IFN-g)**		**S (IL-4)(H) N (IL-2)(H)**	**A (H) S (H) N (H)**	Direct modulation of NF-kB has been implicated in modulation of cytokine production and secretion (Corsini et al. 2012).
Dichloroacetic Acid (DCAA)	TTC	2), 3)	**A(spleen)**	**N (IL-2) A (IFN-γ) x 3 S (IL-4) x 2 S (IL-2)**	**N**	**A (IL-2)(H) A (IL-2, IFN-g)**		T-cell activation was one proposed mode of action for DCAA.
Toluene	NTTC		**N**		**N**		**N**	
Acetonitrile	NTTC		**S(thymus)**		**S S**	-	-	
Mannnitol	NTTC						**N (H)**	
Vanadium Pentoxide	NTTC		**N A (spleen)**			**N**	**N**	
o-Benzyl-p-chlorophenol (BCP)	NTTC		**N**		**N**	-	-	

S: Suppression, A: Augumentation, N: No effect, (H) humana study,
#: The criterion number used to define immunotoxicity

Appendix 8 Table. The summary of immunotoxicological data of 25 chemicals (continue)

The data collected by the VMT												
Chemical name	*In vitro* effect on IL-2				*In vitro* effect on IFN-γ				*In vitro* effect on IIL-4			
	Effect	Animal	*in vitro (method)*	References	Effect	Animal	*in vitro (method)*	References	Effect	Animal	*in vitro (method)*	References
Phase I study												
Dibutyl phthalate					S	human	T cells (in vitro)	Hansen et al. 2015	S	human	T cells (in vitro)	Hansen et al. 2015 (0.0278~27.8 ug/mL)
Hydrocortisone	S S	human human	lymphocyte (in vitro) PBL (in vitro)	Chikanza and Panayi 1993 Goodwin et al. 1986								
Lead(II) acetate					S no effect S	mice mice human	splenocyte (ex vivo) cell line (EL-4) PBMC	Fernandez-Cabezudo et al. 2007 Wagner et al. 2006 Hemdan et al. 2005	A no effect A A	mice mice human rat	splenocyte (ex vivo) cell line (EL-4) PBMC (in vitro) ?	Fernandez-Cabezudo et al. 2007 Wagner et al. 2006 Hemdan et al. 2005 Chen et al. 2004
Nickel(II) sulfate					A A (NiCl2) A A	mice mice human rat	spleen cell (in vitro) cell line (EL-4) PBMC (in vitro) lymphoid lung cell (ex vivo)	Kim et al. 2009 Wagner et al. 2006 Thomas et al. 2003 Goutet et al. 2000	A, S A (NiCl2) A	mice mice human	spleen cell (in vitro) cell line (EL-4) PBMC (in vitro)	Kim et al. 2009 Wagner et al. 2006 Thomas et al. 2003
dimethyldithiocarbamate (DMDTC)												
Phase II study												
2.4-diaminotoluene												
Benzo(a)pyrene												
Cadmium Chloride					N (ex vivo), A (in vitro) S S (IC50=7.05E-05 M) S	rat rat human mice	splenocyte (ex vivo, in vitro) spleen cell (ex vivo) PBMC (in vitro) thymocyte, splenocyte (in vitro)	Wang et al. 2017 Demenesku et al. 2014 Kooijman et al. 2010 Pathak and Khandelwal 2008	no effect	rat	spleen cell (ex vivo)	Demenesku et al. 2014
Dibromoacetic acid (DBAA)												
Diethylstilbestrol (DES)												
Diphenylhydantoin												
Ethylene Dibromide (EDB)												
Glycidol												
Indomethacin												
Isonicotinic Acid Hydrazide (IAH)	A	human	PBMC (in vitro), cell line (Jurkat)	Tsuboi et al. 1995								
Nitrobenzene												
Urethane, Ethyl carbamate												
Tributyltin Chloride (TBTC)					no effect (TBTO)	mice	cell line (EL-4)	Ringerike et al. 2005				
Perflouorooctanoic Acid (PFOA)												
Dichloroacetic Acid (DCAA)												
Toluene												
Acetonitrile												
Mannnitol												
Vanadium Pentoxide												
o-Benzyl-p-chlorophenol (BCP)												

S: Suppression, A: Augumentation, N: No effect, (H) humana study,
#: The criterion number used to define immunotoxicity

引用文献の記されていないデータは NTP の好意により作成して頂いた免疫毒性データベースに基づいている（昨年度の成果報告書に記載）。引用文献が書かれている文献は以下の通りである。

Chen, S., Golemboski, K., Piepenbrink, M., et al., 2004. Developmental immunotoxicity of lead in the rat: influence of maternal diet. J Toxicol Environ Health A 67, 495-511.

Chikanza, L.C., Panayi, G.S., 1993. The effects of hydrocortisone on in vitro lymphocyte proliferation and interleukin-2 and -4 production in corticosteroid sensitive and resistant subjects. Eur J Clin Invest 23, 845-850.

Demenesku, J., Mirkov, I., Ninkov, M., et al., 2014. Acute cadmium administration to rats exerts both immunosuppressive and proinflammatory effects in spleen. Toxicology 326, 96-108.

Fernandez-Cabezudo, M.J., Ali, S.A., Ullah, A., et al., 2007. Pronounced susceptibility to infection by Salmonella enterica serovar Typhimurium in mice chronically exposed to lead correlates with a shift to Th2-type immune responses. Toxicol Appl Pharmacol 218, 215-226.

Goodwin, J.S., Atluru, D., Sierakowski, S., et al., 1986. Mechanism of action of glucocorticosteroids. Inhibition of T cell proliferation and interleukin 2 production by hydrocortisone is reversed by leukotriene B4. J Clin Invest 77, 1244-1250.

Goutet, M., Ban, M., Binet, S., 2000. Effects of nickel sulfate on pulmonary natural immunity in Wistar rats. Toxicology 145, 15-26.

Hansen, J.F., Nielsen, C.H., Brorson, M.M., et al., 2015. Influence of phthalates on in vitro innate and adaptive immune responses. PLoS One 10, e0131168.

Hemdan, N.Y., Emmrich, F., Adham, K., et al., 2005. Dose-dependent modulation of the in vitro cytokine production of human immune competent cells by lead salts. Toxicol Sci 86, 75-83.

Iavicoli, I., Marinaccio, A., Castellino, N., et al., 2004. Altered cytokine production in mice exposed to lead acetate. Int J Immunopathol Pharmacol 17, 97-102.

Kim, J.Y., Huh, K., Lee, K.Y., et al., 2009. Nickel induces secretion of IFN-gamma by splenic natural killer cells. Exp Mol Med 41, 288-295.

Kooijman, R., Devos, S., Hooghe-Peters, E., 2010. Inhibition of in vitro cytokine production by human peripheral blood mononuclear cells treated with

xenobiotics: implications for the prediction of general toxicity and immunotoxicity. Toxicol In Vitro 24, 1782-1789.

Metushi, I.G., Uetrecht, J., 2014. Isoniazid-induced liver injury and immune response in mice. J Immunotoxicol 11, 383-392.

Pathak, N., Khandelwal, S., 2008. Comparative efficacy of piperine, curcumin and picroliv against Cd immunotoxicity in mice. Biometals 21, 649-661.

Ringerike, T., Ulleras, E., Volker, R., et al., 2005. Detection of immunotoxicity using T-cell based cytokine reporter cell lines ("Cell Chip"). Toxicology 206, 257-272.

Thomas, P., Barnstorf, S., Summer, B., et al., 2003. Immuno-allergological properties of aluminium oxide (Al2O3) ceramics and nickel sulfate in humans. Biomaterials 24, 959-966.

Tsuboi, I., Tanaka, H., Nakao, M., et al., 1995. Nonsteroidal anti-inflammatory drugs differentially regulate cytokine production in human lymphocytes: up-regulation of TNF, IFN-gamma and IL-2, in contrast to down-regulation of IL-6 production. Cytokine 7, 372-379.

Wagner, W., Walczak-Drzewiecka, A., Slusarczyk, A., et al., 2006. Fluorescent Cell Chip a new in vitro approach for immunotoxicity screening. Toxicol Lett 162, 55-70.

Wang, P., Wang, J., Sun, Y.J., et al., 2017. Cadmium and chlorpyrifos inhibit cellular immune response in spleen of rats. Environ Toxicol 32, 1927-1936.

添付資料１．Appendix Table 2
化学物質の免疫毒性データーベース（Data set 60 chemicals）

Chemical name	Immunotoxicity classification		Thymus weight			Ex Vivo effect on IL-2			
	Classification	Rationale[#]	Weight	Animal	Reference	Effect	Animal	*ex vivo (method)*	Reference
FK506	TTC	1,3	decrease decrease	rat rat	Nalesnik et al. 1987 Takai et al. 1990				
Cyclosporine A	TTC	1,3	decrease no effect decrease decrease	mice mice rat mice	Auli et al. 2012 Kanariou et al. 1989 Beschorner et al. 1987 Hattori et al. 1987				
Actinomycin D	TTC	3							
Digoxin	TTC	2, 3							
Colchicine	TTC	2,3				A	human	PBMC (ex vivo)	Freed et al. 1989
FR167653	Undetermined	2, 3							
Benzethonium chloride	Undetermined	1	decrease	rat, mice	National Toxicology Program 1995				
Mercuric chloride	TTC	1,3	decrease	mice	Dieter et al. 1983				
Chlorpromazine	TTC	1,3	decrease decrease	mice rat	Auli et al. 2012 Silvestrini et al. 1967				
Amphotericin B	Undetermined	1	decrease	mice	Blanke et al. 1977				
Dibutyl phthalate	TTC	3	no effect no effect	rat rat	Zhang et al. 2013 Salazar et al. 2004				
2-Aminoanthracene	Undetermined								
Formaldehyde	TTC	2,3	no effect	rat	Vargova et al. 1993				
Pyrimethamine	Undetermined								
Isophorone diisocyanate	Undetermined								
Cisplatin	TTC	1,2,3	decrease decrease	mice mice	Kouchi et al. 1996 Sugiyama et al. 1995	S	mice	Spleen cell (ex vivo)	Kim et al. 2019
Cobalt chloride	TTC	1, 3	decrease	rat	Chetty et al. 1979				
Chloroquine	TTC	1,3	decrease	human	Garly et al. 2008				
Minocycline	TTC	3							
Mitomycin C	Undetermined								
Hydrogen peroxide	TTC	3							

S: Suppression, A: Augumentation, N: No effect, (H) humana study,

#: The criterion number used to define immunotoxicity

Appendix 9 Table . The summary of immunotoxicological data of 60 chemicals in the IL-2 Luc assay data set.(continue)

Chemical name	In vitro effect on IL-2				In vitro effeon on IFN-γ			
	Effect	**Animal**	***in vitro (method)***	**Reference**	**Effect**	**Animal**	***in vitro (method)***	**Reference**
FK506	S S S S	mice rat human human	cell line (EL-4) primary astrocyte cell (in vitro) cell line (Jurkat, Hut-78) PBMC	Wagner et al. 2006 Gabryel et al. 2004 Henderson et al. 1991 Yoshimura et al. 1989	S	mice	cell line (EL-4)	Wagner et al. 2006
Cyclosporine A	S S S S	mice mice rat human	cell line (3A9 Tcell hybridoma) cell line (EL-4) primary astrocyte cell (in vitro) cell line (Jurkat, Hut-78)	Lehmann and Williams 2018 Ringerike et al. 2005 Gabryel et al. 2004 Henderson et al. 1991	IC50=5.00E-08 M S S	human mice mice	PBMC (in vitro) cell line (EL-4) cell line (EL-4)	Kooijman et al. 2010 Wagner et al. 2006 Ringerike et al. 2005
Actinomycin D	S S	mice human	cell line (EL-4) PBMC (in vitro)	Wagner et al. 2006 Wang et al. 1984	no effect	mice	cell line (EL-4)	Wagner et al. 2006
Digoxin	S no effect S	human human human	cell line (HepG2), Th17 cell, thymocytes PBMC (in vitro) PBMC (in vitro)	Karas et al. 2018, He et al. 1998 Sheikhi et al. 2007 Gentile et al. 1997	S (ex vivo), no effect (in vitro) S (IC50=4.31E-07 M)	mice human	spleen cell (ex vivo, in vitro) PBMC (in vitro)	Hinshaw et al. 2016 Kooijman et al. 2010
Colchicine	A	human	cell line (Jurkat)	Dupuis et al. 1993	N (IC50>5.00E-04 M(=200 ug/mL)) S (in vitro) A S	human mice human human	PBMC (in vitro) spleen cell (in vitro) PBMC (in vitro)	Kooijman et al. 2010 Sosroseno 2009 Tzortzaki et al. 2007 Altindag et al. 1997
FR167653	no effect no effect	mice human	cell line (EL-4) lymphocyte (in vitro)	Wagner et al. 2006 Yamamoto et al. 1996	no effect S no effect	mice mice human	cell line (EL-4) spleen cell (ex vivo) lymphocyte (in vitro)	Wagner et al. 2006 Ando et al. 2004 Yamamoto et al. 1996
Benzethonium chloride	no effect	mice	cell line (EL-4)	Wagner et al. 2006	no effect	mice	cell line (EL-4)	Wagner et al. 2006
Mercuric chloride	S no effect A	mice mice mice	plasma (in vivo) cell line (EL-4) spleen cell	Santarelli et al. 2006 Wagner et al. 2006 Hu et al. 1997	S (IC50=3.06E-06 M) A S	human mice mice	PBMC (in vitro) cell line (EL-4) cell line (EL-4)	Kooijman et al. 2010 Wagner et al. 2006 Ringerike et al. 2005
Chlorpromazine	A S S	human rat human	whole blood (in vitro) mixed glial and microglial cell cultures (in vitro) thymocytes (in vitro)	Himmerich et al. 2011 Labuzek et al. 2005 Schleuning et al. 1989	S S	human mice	thymocytes (in vitro) Spleen cell (in vitro)	Schleuning et al. 1989 Johnson et al. 1985
Amphotericin B								
Dibutyl phthalate	S	human	T cell (in vitro)	Hansen et al. 2015	S	human	T cells (in vitro)	Hansen et al. 2015
2-Aminoanthracene	A	mice	cell line (EL-4)	Wagner et al. 2006	A	mice	cell line (EL-4)	Wagner et al. 2006
Formaldehyde					S (mRNA and protein) A	human mice	T cell (in vitro) spleen cell (ex vivo)	Sasaki et al. 2009 Fujimaki et al. 2004
Pyrimethamine	A no effect (<LOEL)	mice human	cell line (EL-4) lymphocyte (in vitro)	Wagner et al. 2006 Bygbjerg et al. 1987	no effect	mice	cell line (EL-4)	Wagner et al. 2006
Isophorone diisocyanate					no effect	mice	Lymph node (ex vivo)	Selgrade et al. 2006
Cisplatin	no effect (<LOEL) A S	mice human human	cell line (EL-4) PBL (in vitro) PBL (in vitro)	Wagner et al. 2006 Riesbeck 1999 Sfikakis et al. 1996	S A	mice mice	Spleen cell (ex vivo) cell line (EL-4)	Kim et al. 2019 Wagner et al. 2006
Cobalt chloride	S	mice	cell line (EL-4)	Wagner et al. 2006	A	mice	cell line (EL-4)	Wagner et al. 2006
Chloroquine	S	human	Synovial T cell clones	Landewe et al. 1995	A S	mice human	? (ex vivo) T cell clone	Rosa et al. 1999 Landewe et al. 1992
Minocycline	S S	human human	PBMC (in vitro) T cell clones (in vitro)	Maeda et al. 2010 Kloppenburg et al. 1995	no effect S	mice human	splenocyte (ex vivo) T cell clones (in vitro)	Chen et al. 2010 Kloppenburg et al. 1995
Mitomycin C	no effect (<LOEL) S	mice human	cell line (EL-4) mononuclear leukocyte (in vitro)	Wagner et al. 2006 Roche et al. 1988	no effect	mice	cell line (EL-4)	Wagner et al. 2006
Hydrogen peroxide	A S	mice human	cell line (EL-4) PBMC (in vitro)	Wagner et al. 2006 Freed et al. 1987	A	mice	cell line (EL-4)	Wagner et al. 2006

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#: The criterion number used to define immunotoxicity

Appendix 9 Table . The summary of immunotoxicological data of 60 chemicals in the IL-2 Luc assay data set.(continue)

Chemical name	In vitro effect on IL-4			
	Effect	Animal	*in vitro (method)*	Reference
FK506	S	mice	cell line (EL-4)	Wagner et al. 2006
Cyclosporine A	S S S	mice mice human	cell line (EL-4) cell line (EL-4) cell line (D10.G4.1)	Wagner et al. 2006 Ringerike et al. 2005 Schmidt et al. 1994
Actinomycin D	A	mice	cell line (EL-4)	Wagner et al. 2006
Digoxin				
Colchicine	A (in vitro)	mice	spleen cell (in vitro)	Sosroseno 2009
FR167653	S no effect	mice mice	cell line (EL-4) spleen cell (ex vivo)	Wagner et al. 2006 Ando et al. 2004
Benzethonium chloride	A	mice	cell line (EL-4)	Wagner et al. 2006
Mercuric chloride	A	mice	cell line (EL-4)	Wagner et al. 2006
Chlorpromazine	S A	mice human	splenic lymphocyte (in vitro) whole blood (in vitro)	Pei et al. 2014 Himmerich et al. 2011
Amphotericin B				
Dibutyl phthalate	S	human	T cells (in vitro)	Hansen et al. 2015
2-Aminoanthracene	A	mice	cell line (EL-4)	Wagner et al. 2006
Formaldehyde	no effect	human	T cell (in vitro)	Sasaki et al. 2009
Pyrimethamine	A	mice	cell line (EL-4)	Wagner et al. 2006
Isophorone diisocyanate				
Cisplatin	A no effect	mice mice	Spleen cell (ex vivo) cell line (EL-4)	Kim et al. 2019 Wagner et al. 2006
Cobalt chloride	A	mice	cell line (EL-4)	Wagner et al. 2006
Chloroquine	no effect	mice	? (ex vivo)	Rosa et al. 1999
Minocycline	no effect	mice	splenocyte (ex vivo)	Chen et al. 2010
Mitomycin C	no effect	mice	cell line (EL-4)	Wagner et al. 2006
Hydrogen peroxide	A	mice	cell line (EL-4)	Wagner et al. 2006

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Appendix 9 Table . The summary of immunotoxicological data of 60 chemicals in the IL-2 Luc assay data set.

Chemical name	Immunotoxicity classification		Thymus weight			Ex Vivo effect on IL-2			
	Classification	Rationale#	Weight	Animal	Reference	Effect	Animal	*ex vivo (method)*	Reference
Citral	Undetermined	1	decrease decrease	rat rat, mice	Ress et al. 2003 National Toxicology Program 2003				
Dexamethasone	TTC	1,3	decrease decrease decrease	mice mice rat	Auli et al. 2012 Munson et al. 1982 Exon et al. 1986				
Pentamidine isethionate	TTC	3							
Lead(II)acetate	TTC	1, 3	increase	rat	Bunn et al. 2001	no effect no effect	rat rat	spleen cell (ex vivo) spleen cell (ex vivo)	Bunn et al. 2001 Miller et al. 1998
Azathioprine	TTC	1,2, 3	decrease decrease	rat rat	De Waal et al. 1995 Vos and Van Loveren 1994	S S	mice, rat human	lymphocyte, thymocyte (in vitro, ex vivo) PBMC (ex vivo)	Meredith and Scott 1994 Dupont et al. 1985
Diesel exhaust particle	TTC	1, 3	decrease	rat	Tsukue et al. 2001				
Sodium dodecyl sulfate	TTC	3							
Dapsone	TTC	3	No Effect	mice	https://ntp.niehs.nih.gov/testing/types/imm/abstracts/imm90015/index.html				
Nitrofurazone	NTTC		No Effect	mice	https://ntp.niehs.nih.gov/testing/types/imm/abstracts/imm90011/index.html				
p-Nitroaniline	TTC	1,3	increase, decrease	mice	National Toxicology Program 1993b				
Sulfasalazine	TTC	1,3	decrease	rat	National Toxicology Program 1997				
Aluminium chloride	TTC	1,3	diminished thymic cellularity	mice	Synzynys et al. 2004				
Nickel sulfate	TTC	1, 3	no effect decrease decrease	mice rat rat, mice	Knight et al. 1991 Haley et al. 1990 National Toxicology Program 1996				
Hydrocortisone	TTC	1,3	decrease decrease (PND 21), increase (PND 42)	mice rat	Van Dijk et al. 1979 El Fouhil et al. 1993a, El Fouhil et al.1993b, El Fouhil and Turkall 1993				
Diethanolamine	Undetermined	1	decrease	mice	https://ntp.niehs.nih.gov/testing/types/imm/abstracts/imm20004/imm20004.html				
Chloroplatinic acid	Undetermined				X				
Sodium bromate	Undetermined	1	No Effect	mice	https://ntp.niehs.nih.gov/testing/types/imm/abstracts/imm98004/index.html				
S: Suppression, A: Augumentation, N: No effect, (H) humana study,									
#: The criterion number used to define immunotoxicity									

Appendix 9 Table . The summary of immunotoxicological data of 60 chemicals in the IL-2 Luc assay data set.(continue)

Chemical name	In vitro effect on IL-2				In vitro effeon on IFN-γ			
	Effect	Animal	*in vitro (method)*	Reference	Effect	Animal	*in vitro (method)*	Reference
Citral								X
Dexamethasone	S no effect S	mice mice human	cell line (3A9 Tcell hybridoma) cell line (EL-4) CBMC, PBMC (in vitro)	Lehmann and Williams 2018 Wagner et al. 2006 Bessler et al. 1996	S S S S no effect	human human mice mice mice	PBL (in vitro) T cell (in vitro) T cell clone (in vitro) splenocyte (ex vivo) cell line (EL-4)	Arya et al. 1984 Reen and Yeh 1984 Kelso and Munck 1984 Kunicka et al. 1993 Wagner et al. 2006
Pentamidine isethionate	S no effect no effect (<LOEL)	mice mice human	cell line (EL-4) cell line (EL-4) whole blood (in vitro)	Ringerike et al. 2005 Wagner et al. 2006 Van Wauwe et al. 1996	A S	mice mice	cell line (EL-4) cell line (EL-4)	Wagner et al. 2006 Ringerike et al. 2005
Lead(II)acetate	S	mice	cell line (EL-4)	Wagner et al. 2006	S no effect S	mice mice human	splenocyte (ex vivo) cell line (EL-4) PBMC	Fernandez-Cabezudo et al. 2007 Wagner et al. 2006 Hemdan et al. 2005
Azathioprine	S S	mice mice, rat	cell line (3A9 Tcell hybridoma) lymphocyte, thymocyte (in vitro, ex vivo)	Lehmann and Williams 2018 Meredith et al. 1994	S S	human human	PBMC (ex vivo) PBMC (ex vivo)	Weimar et al. 1995 Dupont et al. 1985
Diesel exhaust particle	A	mice	cell line (EL-4)	Wagner et al. 2011	S	human	T cell (in vitro)	Sasaki et al. 2009
Sodium dodecyl sulfate	S	mice	cell line (EL-4)	Ringerike et al. 2005	S (IC50=1.61E-04 M)	human mice	PBMC (in vitro) cell line (EL-4)	Kooijman et al. 2010 Ringerike et al. 2005
Dapsone	S, A S	mice mice	cell line (EL-4) splenocyte (in vitro)	Wagner et al. 2006 Peterson et al. 1997	S, A	mice	cell line (EL-4)	Wagner et al. 2006
Nitrofurazone	A	mice	cell line (EL-4)	Wagner et al. 2006	no effect	mice	cell line (EL-4)	Wagner et al. 2006
p-Nitroaniline	A	mice	cell line (EL-4)	Wagner et al. 2006	A	mice	cell line (EL-4)	Wagner et al. 2006
Sulfasalazine	S	mice	splenocyte (in vitro)	Fujiwara et al. 1990	S A	human rat	BAL cell (in vitro) CNS (in vivo)	Dobis et al. 2010 Correale et al. 1991
Aluminium chloride	S	rat	lymphocyte (in vitro)	She et al. 2012				
Nickel sulfate	S ($NiCl_2$) A A ($NiCl_2$)	human mice mice	Cell line (Jurkat) spleen cell (in vitro) cell line (EL-4)	Saito et al. 2011 Kim et al. 2009 Wagner et al. 2006	A A (NiCl2) A A	mice mice human rat	spleen cell (in vitro) cell line (EL-4) PBMC (in vitro) lymphoid lung cell (ex vivo)	Kim et al. 2009 Wagner et al. 2006 Thomas et al. 2003 Goutet et al. 2000
Hydrocortisone	S S S S	human human human human	lymphocyte (in vitro) PBL (in vitro) lymphocyte (in vitro) PBMC (in vitro)	Chikanza and Panayi 1993 Goodwin et al. 1986 Palacios and Sugawara 1982 Northoff et al. 1980				
Diethanolamine				X				
Chloroplatinic acid				X				
Sodium bromate				X				

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#: The criterion number used to define immunotoxicity

Appendix 9 Table . The summary of immunotoxicological data of 60 chemicals in the IL-2 Luc assay data set.(continue)

Chemical name	In vitro effect on IL-4					
	Effect	Animal	*in vitro (method)*	Reference		
Citral				X		
Dexamethasone	A S S	mice human mice	cell line (EL-4) cell line (D10.G4.1) splenocyte (ex vivo)	Wagner et al. 2006 Schmidt et al. 1994 Kunicka et al. 1993		
Pentamidine isethionate	A S	mice mice	cell line (EL-4) cell line (EL-4)	Wagner et al. 2006 Ringerike et al. 2005		
Lead(II)acetate	A no effect A A	mice mice human rat	splenocyte (ex vivo) cell line (EL-4) PBMC (in vitro) ?	Fernandez-Cabezudo et al. 2007 Wagner et al. 2006 Hemdan et al. 2005 Chen et al. 2004		
Azathioprine						
Diesel exhaust particle	no effect	human	T cell (in vitro)	Sasaki et al. 2009		
Sodium dodecyl sulfate						
Dapsone	S	mice	cell line (EL-4)	Wagner et al. 2006		
Nitrofurazone	no effect	mice	cell line (EL-4)	Wagner et al. 2006		
p-Nitroaniline	A	mice	cell line (EL-4)	Wagner et al. 2006		
Sulfasalazine	S	mice	mesangial cell (in vitro)	Tsai et al. 2000		
Aluminium chloride						
Nickel sulfate	A, S A (NiCl2) A	mice mice human	spleen cell (in vitro) cell line (EL-4) PBMC (in vitro)	Kim et al. 2009 Wagner et al. 2006 Thomas et al. 2003		
Hydrocortisone						
Diethanolamine						
Chloroplatinic acid						
Sodium bromate						

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#: The criterion number used to define immunotoxicity

Appendix 9 Table . The summary of immunotoxicological data of 60 chemicals in the IL-2 Luc assay data set.(continue)

Chemical name	Immunotoxicity classification		Thymus weight			Ex Vivo effect on IL-2			
	Classification	Rationale#	Weight	Animal	Reference	Effect	Animal	*ex vivo* (method)	Reference
Histamine	TTC	3							
Isoniazid	NTTC	1	No Effect	mice	https://ntp.niehs.nih.gov/testing/types/imm/abstracts/imm96002/index.html				
Triethanolamine	Undetermined								
Magnesium sulfate	Undetermined								
Rapamycin	TTC	1, 3	decrease	rat	Lu et al. 2015				
Mizoribine	Undetermined								
Warfarin	TTC	3							
2,4-Diaminotoluene	NTTC	1	No Effect	mice	https://ntp.niehs.nih.gov/testing/types/imm/abstracts/imm87034/index.html				
Cyclophosphamide	TTC	1	decrease decrease decrease	mice mice rat	Auli et al. 2012 https://ntp.niehs.nih.gov/testing/types/imm/abstracts/imm90015/index.html Exon et al. 1986	S	mice	splenocyte (ex vivo)	Tabi et al. 1988
Dibenzopyrene	Undetermined	3							
Ethanol	TTC	1, 3	decrease	mice	Kim and Park 2002				
Hexachlorobenzene	Undetermined	1,2	no effect decrease cortical atrophy	rat mice monkey	Vos et al. 1979 Loose et al. 1978 Iatropoulos et al. 1976	A A	rat rat	spleen cell (ex vivo) spleen cell (ex vivo)	Ezendam et al. 2004 Vandebriel et al. 1998
Lithium carbonate	TTC	1,3	decrease	mice	https://ntp.niehs.nih.gov/testing/types/imm/abstracts/imm85001/index.html				
Methanol	NTTC	1	decrease	rat	Parthasarathy et al. 2005				
Methotrexate	TTC	3							
Dimethyl sulfoxide	NTTC	1,3	no effect	mice	Caren et al. 1985				

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#: The criterion number used to define immunotoxicity

Appendix 9 Table . The summary of immunotoxicological data of 60 chemicals in the IL-2 Luc assay data set.(continue)

Chemical name	In vitro effect on IL-2				In vitro effeon on IFN-γ			
	Effect	**Animal**	***in vitro (method)***	**Reference**	**Effect**	**Animal**	***in vitro (method)***	**Reference**
Histamine	S S A, S	mice human mice	splenocyte (in vitro) PBMC (in vitro) spleen cell (in vitro)	Poluektova et al. 1999 Huchet and Grandjon 1988 Khan et al. 1985	no effect	mice	serum (in vivo)	Metushi and Uetrecht 2014
Isoniazid	S (13.7, 137.1 ug/mL), A (0.0137~1.37 ug/mL)	human	T cell (in vitro)	Kucharz and Sierakowski 1990				
Triethanolamine				X				
Magnesium sulfate								
Rapamycin	A, S A (0.0009ug/mL), S (0.457ug/mL) S S	mice rat human human	cell line (EL-4) primary astrocyte cell (in vitro) T cell (in vitro) cell line (Jurkat, Hut-78)	Ringerike et al. 2005 Gabryel et al. 2004 Hanke et al. 1992 Henderson et al. 1991	no effect	mice	cell line (EL-4)	Ringerike et al. 2005
Mizoribine	S (>LOEL) no effect	mice human	T cells (in vitro) peripheral blood T cells (in vitro)	Song et al. 2006 Turka et al. 1991				
Warfarin	S	human	T cell (in vitro)	Bruserud and Lundin 1	S (IC50=3.16E-04 M)	human	PBMC (in vitro)	Kooijman et al. 2010
2,4-Diaminotoluene				X X				X
Cyclophosphamide	no effect (needs metabolization)	mice	cell line (3A9 Tcell hybridoma)	Lehmann and Williams 2018				
Dibenzopyrene	A	mice	cell line (EL-4)	Wagner et al. 2006	A	mice	cell line (EL-4)	Wagner et al. 2006
Ethanol	S	human	cell line (Jurkat), primary CD4+ T lymphocytes (in vitro)	Ghare et al. 2011	N (IC50>1.00E-03 M)	human	PBMC (in vitro)	Kooijman et al. 2010
Hexachlorobenzene					N (IC50>1.00E-05 M)	human	PBMC (in vitro)	Kooijman et al. 2010
Lithium carbonate	A A A	human human human	PBMC (in vitro) PBMC (in vitro) PBMC (in vitro)	Wilson et al. 1989 Parenti et al. 1988 Sztein et al. 1987	N (IC50>1.00E-03 M)	human	PBMC (in vitro)	Kooijman et al. 2010
Methanol	no effect	mice	cell line (EL-4)	Wagner et al. 2006	N (IC50>1.00E-03 M) no effect	human mice	PBMC (in vitro) cell line (EL-4)	Kooijman et al. 2010 Wagner et al. 2006
Methotrexate	S A	mice human	cell line (3A9 Tcell hybridoma) PBMC (in vitro)	Lehmann and Williams 2018 Cesario et al. 1984				
Dimethyl sulfoxide	S, A no effect (1 %), S (2.5, 5, 10 %)	mice human	cell line (EL-4) PBMC (in vitro)	Wagner et al. 2006 de Abreu Costa et al. 2017	no effect	mice	cell line (EL-4)	Wagner et al. 2006

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#: The criterion number used to define immunotoxicity

Appendix 9 Table . The summary of immunotoxicological data of 60 chemicals in the IL-2 Luc assay data set.(continue)

Chemical name	In vitro effect on IL-4					
	Effect	Animal	*in vitro (method)*	Reference		
Histamine						
Isoniazid						
Triethanolamine						
Magnesium sulfate						
Rapamycin	S	mice	cell line (EL-4)	Ringerike et al. 2005		
Mizoribine						
Warfarin						
2,4-Diaminotoluene				X		
Cyclophosphamide						
Dibenzopyrene	A	mice	cell line (EL-4)	Wagner et al. 2006		
Ethanol						
Hexachlorobenzene						
Lithium carbonate						
Methanol	A	mice	cell line (EL-4)	Wagner et al. 2006		
Methotrexate	no effect	human	cell line (D10.G4.1)	Schmidt et al. 1994		
Dimethyl sulfoxide	A	mice	cell line (EL-4)	Wagner et al. 2006		

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Appendix 9 Table . The summary of immunotoxicological data of 60 chemicals in the IL-2 Luc assay data set.(continue)

Chemical name	Immunotoxicity classification		Thymus weight			Ex Vivo effect on IL-2			
	Classification	Rationale#	Weight	Animal	Reference	Effect	Animal	*ex vivo (method)*	Reference
Trichloroethylene	NTTC	1	No Effect	mice, rat	https://ntp.niehs.nih.gov/testing/types/imm/abstracts/imm20006/imm20006.html https://ntp.niehs.nih.gov/testing/types/imm/abstracts/imm96007/imm96007.html				
Mycophenolic acid	Undetermined	1, 3	decrease	rat	Pally et al. 2001				
2-Mercaptobenzothiazole	Undetermined								
Ribavirin	TTC	1, 3	decrease	mice	https://ntp.niehs.nih.gov/testing/types/imm/abstracts/imm90010/index.html				
Nicotinamide	Undetermined								
Acetaminophen	Undetermined		no effect decrease (rat), no effect (mice)	mice rat, mice	Kim and Park 2002 National Toxicology Program 1993a				

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Appendix 9 Table . The summary of immunotoxicological data of 60 chemicals in the IL-2 Luc assay data set.(continue)

Chemical name	In vitro effect on IL-2				In vitro effeon on IFN-γ			
	Effect	**Animal**	***in vitro (method)***	**Reference**	**Effect**	**Animal**	***in vitro (method)***	**Reference**
Trichloroethylene								
Mycophenolic acid	no effect no effect	human mice	PBL (in vitro) spleen cell (in vitro)	Quemeneur et al. 2002 Lemster et al. 1992				
2-Mercaptobenzothiazole								
Ribavirin	A A	human human	PBMC (in vitro) T cells (in vitro)	Sookoian et al. 2004 Tam et al. 1999				
Nicotinamide								
Acetaminophen	A	mice	cell line (EL-4)	Wagner et al. 2006	A N (C50>5.00E-04 M)	mice human	cell line (EL-4) PBMC (in vitro)	Wagner et al. 2006 Kooijman et al. 2010

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#: The criterion number used to define immunotoxicity

Appendix 9 Table . The summary of immunotoxicological data of 60 chemicals in the IL-2 Luc assay data set.(continue)

Chemical name	In vitro effect on IL-4					
	Effect	Animal	*in vitro (method)*	Reference		
Trichloroethylene						
Mycophenolic acid						
2-Mercaptobenzothiazole						
Ribavirin						
Nicotinamide						
Acetaminophen	A	mice	cell line (EL-4)	Wagner et al. 2006		
S: Suppression, A: Augumentation, N: No effect, (H) humana study,						
#: The criterion number used to define immunotoxicity						

Chemical name	Immunotoxicity classification		NTP data					
			In vivo	*Ex vivo*		*In vitro*		
	Classification	Rationale	immune sytem organ weight	cytokine production	TDAR	cytokine production	T cell proliferation	Mode of action
Phase I study								
Dibutyl phthalate	TTC	3), 4)	**A (spleen)**			**S (IL-2, 4, IFN-g)(H) A (IL-1b)(H) x 3 S (IL-1b)**		This compound then is proposed to modulate cytokine secretion from both monocytes/macrophages and T cells.
Hydrocortisone	TTC	1)	**S (thymus) x 2 S (spleen)**		**N**	**S (IFN-a)**		
Lead(II) acetate	TTC	1)	**A(thymus)**		**S N**	**S (IFN-g, IL-1b)(H) A (IL-4)(H)**	**S(H)**	
Nickel(II) sulfate	TTC	1)	**N S (thymus)**		**N**	**A (IL-4, IFN-g)(H) S (IL-2) S (IFN-g)**		
dimethyldithiocarbamate (DMDTC)	NTTC					**S (IL-1b)**	**N(H)**	
Phase II study								
2.4-diaminotoluene	NTTC		**N (spleen) A (spleen)**		**S**	-	-	
Benzo(a)pyrene	TTC	2), 3)		**S(IL-2)**	**S x 5 A**	**A (IL-4)(H) N (IFNγ)(H) N (IL-2)(H) S (IL-2, 4, IFN-g)**	**S (H) x 2 S x 6**	Disruption of T-cell activities has been associated with B(a)P induced immunotoxic effects (Urso et al. 1986).
Cadmium Chloride	TTC	2), 3)	**A (spleen) S (spleen)**	**A (IL-2) N (IFN-γ)**	**S x 4**	**A (IFN-g)(H) S (IL-2, IFN-g) A (IFN-g) S (IL-2) A (IL-2)**	**S**	
Dibromoacetic acid (DBAA)	TTC	1), 4)	**A (spleen) S (thymus) x 2**		**N**	**S (IL-2, 4)**	**S**	Overall, studies suggest that DBAA produces immunotoxic effects through modulation of T-cell mediated cell immunity. T-cell apoptosis, through extrinsic and intrinsic pathways, are proposed to play a role in the mode of action.
Diethylstilbestrol (DES)	TTC	1), 2), 4)	**S (thymus) x 4 A (thymus) x 2 A (spleen)**	**A (IFN-g) x 3**	**S**	**A (IL-1) A (IL-2)**		DES exposure was associated with down-regulation of gene expression in the TCR complex, and the TCR and CD28 signaling pathways.
Diphenylhydantoin	TTC	2), 3), 4)		**A (IL-4) S (IFN-γ, IL-2) S (IL-1α) N (IL-6, 12)**	**S A x 2**	-	-	DPH treatment can lead to a decrease of suppressor T cells
Ethylene Dibromide (EDB)	TTC	1)	**S (thymus) S (spleen) N**		**A**	-	**S**	
Glycidol	NTTC		**N**		**S**	-	-	Studies suggest that glycidol modulates B-cell function, and NK cell and macrophage activities.111 and decreased cytotoxic T cell activity
Indomethacin	TTC	3), 4)	**N A (spleen)**		**S x 3 A x 1**	**A (IL-2)(H) A (IFN-g)(H)**	**A (H) x 4 S A x 3**	indomethacin inhibition of prostaglandin synthesis leads to altered T-cell function,
Isonicotinic Acid Hydrazide (IAH)	TTC	2)	**N x 2**			**S (IL-2)(H) A (IL-2)(H) S (IL-1)(H)**	**S (H) x 3 A (H) x 6 A N**	
Nitrobenzene	Undetermined		**A (spleen) x 3 A (thymus) x 2**		**S N**	-		effects on T-cell function may play a role in increased susceptibility to L. monocytogenes (Burns et al. 1994).
Urethane, Ethyl carbamate	TTC	1)	**S (thymus) x2 S (spleen) x 2 N A (thymus) A (spleen)**	**N (IL-2)**	**S x 2 N**	**N (IL-2, 4, IFN-g)(H) A (IFN-g)(H) S (IFN-g)(H)**	**N x 2**	
Tributyltin Chloride (TBTC)	TTC	1)	**S (thymus) x4 S (spleen) x 3**		**N S**	**A (INF-g)(H) N (IL-2, 4)(H) S (IFN-g)(H)**	**S (H) S x 3**	
Perflouorooctanoic Acid (PFOA)	TTC	1)	**S (thymus) x2 S (spleen) x 2**	**N (IFN-g)**		**S (IL-4)(H) N (IL-2)(H)**	**A (H) S (H) N (H)**	Direct modulation of NF-kB has been implicated in modulation of cytokine production and secretion (Corsini et al. 2012).
Dichloroacetic Acid (DCAA)	TTC	2), 3)	**A(spleen)**	**N (IL-2) A (IFN-γ) x 3 S (IL-4) x 2 S (IL-2)**	**N**	**A (IL-2)(H) A (IL-2, IFN-g)**		T-cell activation was one proposed mode of action for DCAA.
Toluene	NTTC		**N**		**N**		**N**	
Acetonitrile	NTTC		**S(thymus)**		**S S**	-	-	
Mannnitol	NTTC						**N (H)**	
Vanadium Pentoxide	NTTC		**N A (spleen)**			**N**	**N**	
o-Benzyl-p-chlorophenol (BCP)	NTTC		**N**		**N**	-	-	

S: Suppression, A: Augumentation, N: No effect, (H) humana study,
#: The criterion number used to define immunotoxicity

Appendix 8 Table. The summary of immunotoxicological data of 25 chemicals (continue)

The data collected by the VMT												
Chemical name	*In vitro* effect on IL-2				*In vitro* effect on IFN-γ				*In vitro* effect on IIL-4			
	Effect	Animal	*in vitro (method)*	References	Effect	Animal	*in vitro (method)*	References	Effect	Animal	*in vitro (method)*	References
Phase I study												
Dibutyl phthalate					S	human	T cells (in vitro)	Hansen et al. 2015	S	human	T cells (in vitro)	Hansen et al. 2015 (0.0278~27.8 ug/mL)
Hydrocortisone	S S	human human	lymphocyte (in vitro) PBL (in vitro)	Chikanza and Panayi 1993 Goodwin et al. 1986								
Lead(II) acetate					S no effect S	mice mice human	splenocyte (ex vivo) cell line (EL-4) PBMC	Fernandez-Cabezudo et al. 2007 Wagner et al. 2006 Hemdan et al. 2005	A no effect A A	mice mice human rat	splenocyte (ex vivo) cell line (EL-4) PBMC (in vitro) ?	Fernandez-Cabezudo et al. 2007 Wagner et al. 2006 Hemdan et al. 2005 Chen et al. 2004
Nickel(II) sulfate					A A (NiCl2) A A	mice mice human rat	spleen cell (in vitro) cell line (EL-4) PBMC (in vitro) lymphoid lung cell (ex vivo)	Kim et al. 2009 Wagner et al. 2006 Thomas et al. 2003 Goutet et al. 2000	A, S A (NiCl2) A	mice mice human	spleen cell (in vitro) cell line (EL-4) PBMC (in vitro)	Kim et al. 2009 Wagner et al. 2006 Thomas et al. 2003
dimethyldithiocarbamate (DMDTC)												
Phase II study												
2.4-diaminotoluene												
Benzo(a)pyrene												
Cadmium Chloride					N (ex vivo), A (in vitro) S S (IC50=7.05E-05 M) S	rat rat human mice	splenocyte (ex vivo, in vitro) spleen cell (ex vivo) PBMC (in vitro) thymocyte, splenocyte (in vitro)	Wang et al. 2017 Demenesku et al. 2014 Kooijiman et al. 2010 Pathak and Khandelwal 2008	no effect	rat	spleen cell (ex vivo)	Demenesku et al. 2014
Dibromoacetic acid (DBAA)												
Diethylstilbestrol (DES)												
Diphenylhydantoin												
Ethylene Dibromide (EDB)												
Glycidol												
Indomethacin												
Isonicotinic Acid Hydrazide (IAH)	A	human	PBMC (in vitro), cell line (Jurkat)	Tsuboi et al. 1995								
Nitrobenzene												
Urethane, Ethyl carbamate												
Tributyltin Chloride (TBTC)					no effect (TBTO)	mice	cell line (EL-4)	Ringerike et al. 2005				
Perflouorooctanoic Acid (PFOA)												
Dichloroacetic Acid (DCAA)												
Toluene												
Acetonitrile												
Mannnitol												
Vanadium Pentoxide												
o-Benzyl-p-chlorophenol (BCP)												

S: Suppression, A: Augumentation, N: No effect, (H) humana study,
#: The criterion number used to define immunotoxicity

文献

1993a. NTP Toxicology and Carcinogenesis Studies of Acetaminophen (CAS No. 103-90-2) in F344 Rats and B6C3F1 Mice (Feed Studies). Natl Toxicol Program Tech Rep Ser 394, 1-274.

1993b. NTP Toxicology and Carcinogenesis Studies of p-Nitroaniline (CAS No. 100-01-6) in B6C3F1 Mice (Gavage Studies). Natl Toxicol Program Tech Rep Ser 418, 1-203.

1995. NTP Toxicology and Carcinogenesis Studies of Benzethonium Chloride (CAS No. 121-54-0) in F344/N Rats and B6C3F1 Mice (Dermal Studies). Natl Toxicol Program Tech Rep Ser 438, 1-220.

1996. NTP Toxicology and Carcinogenesis Studies of Nickel Sulfate Hexahydrate (CAS No. 10101-97-0) in F344 Rats and B6C3F1 Mice (Inhalation Studies). Natl Toxicol Program Tech Rep Ser 454, 1-380.

1997. NTP Toxicology and Carcinogenesis Studies of Salicylazosulfapyridine (CAS No. 599-79-1) in F344/N Rats and B6C3F1 Mice (Gavage Studies). Natl Toxicol Program Tech Rep Ser 457, 1-327.

2003. NTP toxicology and carcinogenesiss studies of citral (microencapsulated) (CAS No. 5392-40-5) in F344/N rats and B6C3F1 mice (feed studies). Natl Toxicol Program Tech Rep Ser, 1-268.

Almousa, L.A., Salter, A.M., Langley-Evans, S.C., 2018. Magnesium deficiency heightens lipopolysaccharide-induced inflammation and enhances monocyte adhesion in human umbilical vein endothelial cells. Magnes Res 31, 39-48.

Auli, M., Domenech, A., Andres, A., et al., 2012. Multiparametric immunotoxicity screening in mice during early drug development. Toxicol Lett 214, 200-208.

Beschorner, W.E., Namnoum, J.D., Hess, A.D., et al., 1987. Cyclosporin A and the thymus. Immunopathology. Am J Pathol 126, 487-496.

Bessler, H., Straussberg, R., Gurary, N., et al., 1996. Effect of dexamethasone on IL-2 and IL-3 production by mononuclear cells in neonates and adults. Arch Dis Child Fetal Neonatal Ed 75, F197-201.

Blanke, T.J., Little, J.R., Shirley, S.F., et al., 1977. Augmentation of murine immune responses by amphotericin B. Cell Immunol 33, 180-190.

Bruserud, O., Lundin, K., 1987. The effect of drugs used in anticoagulation therapy on T lymphocyte activation in vitro. II. Warfarin inhibits T lymphocyte activation. J Clin Lab Immunol 23, 169-173.

Bunn, T.L., Parsons, P.J., Kao, E., et al., 2001. Exposure to lead during critical windows of embryonic development: differential immunotoxic outcome based on stage of exposure and gender. Toxicol Sci 64, 57-66.

Bygbjerg, I.C., Svenson, M., Theander, T.G., et al., 1987. Effect of antimalarial drugs on stimulation and interleukin 2 production of human lymphocytes. Int J Immunopharmacol 9, 513-519.

Caren, L.D., Oven, H.M., Mandel, A.D., 1985. Dimethyl sulfoxide: lack of suppression of the humoral immune response in mice. Toxicol Lett 26, 193-197.

Cesario, T.C., Slater, L.M., Kaplan, H.S., et al., 1984. Effect of antineoplastic agents on gamma-interferon production in human peripheral blood mononuclear cells. Cancer Res 44, 4962-4966.

Chetty, K.N., Subba Rao, D.S., Drummond, L., et al., 1979. Cobalt induced changes in immune response and adenosine triphosphatase activities in rats. J Environ Sci Health B 14, 525-544.

Chikanza, L.C., Panayi, G.S., 1993. The effects of hydrocortisone on in vitro lymphocyte proliferation and interleukin-2 and -4 production in corticosteroid sensitive and resistant subjects. Eur J Clin Invest 23, 845-850.

de Abreu Costa, L., Henrique Fernandes Ottoni, M., Dos Santos, M.G., et al., 2017. Dimethyl Sulfoxide (DMSO) Decreases Cell Proliferation and TNF-alpha, IFN-gamma, and IL-2 Cytokines Production in Cultures of Peripheral Blood Lymphocytes. Molecules 22.

De Waal, E.J., Timmerman, H.H., Dortant, P.M., et al., 1995. Investigation of a screening battery for immunotoxicity of pharmaceuticals within a 28-day oral toxicity study using azathioprine and cyclosporin A as model compounds. Regul Toxicol Pharmacol 21, 327-338.

Dieter, M.P., Luster, M.I., Boorman, G.A., et al., 1983. Immunological and biochemical responses in mice treated with mercuric chloride. Toxicol Appl Pharmacol 68, 218-228.

Dupont, E., Huygen, K., Schandene, L., et al., 1985. Influence of in vivo immunosuppressive drugs on production of lymphokines. Transplantation 39, 143-147.

Dupuis, G., Martel, J., Bastin, B., et al., 1993. Microtubules are not an essential component of phytohemagglutinin-dependent signal transduction in Jurkat T lymphocytes. Cell Immunol 146, 38-51.

el Fouhil, A.F., Iskander, F.A., Turkall, R.M., 1993a. Effect of alternate-day hydrocortisone therapy on the immunologically immature rat. II: Changes in T- and B-cell areas in spleen. Toxicol Pathol 21, 383-390.

el Fouhil, A.F., Iskander, F.A., Turkall, R.M., 1993b. Effect of alternate-day hydrocortisone therapy on the immunologically immature rat. III: Changes in T- and B-cell areas in lymph nodes. Toxicol Pathol 21, 391-396.

el Fouhil, A.F., Turkall, R.M., 1993. Effect of alternate-day hydrocortisone therapy on the immunologically immature rat. I: Effect on blood cell count, immunoglobulin concentrations, and body and organ weights. Toxicol Pathol 21, 377-382.

Exon, J.H., Koller, L.D., Talcott, P.A., et al., 1986. Immunotoxicity testing: an economical multiple-assay approach. Fundam Appl Toxicol 7, 387-397.

Ezendam, J., Hassing, I., Bleumink, R., et al., 2004. Hexachlorobenzene-induced Immunopathology in Brown Norway rats is partly mediated by T cells. Toxicol Sci 78, 88-95.

Freed, B.M., Lempert, N., Lawrence, D.A., 1989. The inhibitory effects of N-ethylmaleimide, colchicine and cytochalasins on human T-cell functions. Int J Immunopharmacol 11, 459-465.

Freed, B.M., Rapoport, R., Lempert, N., 1987. Inhibition of early events in the human T-lymphocyte response to mitogens and alloantigens by hydrogen peroxide. Arch Surg 122, 99-104.

Fujiwara, M., Mitsui, K., Yamamoto, I., 1990. Inhibition of proliferative responses and interleukin 2 productions by salazosulfapyridine and its metabolites. Jpn J Pharmacol 54, 121-131.

Gabryel, B., Labuzek, K., Malecki, A., et al., 2004. Immunophilin ligands decrease release of pro-inflammatory cytokines (IL-1beta, TNF-alpha and IL-2 in rat

astrocyte cultures exposed to simulated ischemia in vitro. Pol J Pharmacol 56, 129-136.

Garly, M.L., Trautner, S.L., Marx, C., et al., 2008. Thymus size at 6 months of age and subsequent child mortality. J Pediatr 153, 683-688, 688.e681-683.

Gentile, D.A., Henry, J., Katz, A.J., et al., 1997. Inhibition of peripheral blood mononuclear cell proliferation by cardiac glycosides. Ann Allergy Asthma Immunol 78, 466-472.

Ghare, S., Patil, M., Hote, P., et al., 2011. Ethanol inhibits lipid raft-mediated TCR signaling and IL-2 expression: potential mechanism of alcohol-induced immune suppression. Alcohol Clin Exp Res 35, 1435-1444.

Goodwin, J.S., Atluru, D., Sierakowski, S., et al., 1986. Mechanism of action of glucocorticosteroids. Inhibition of T cell proliferation and interleukin 2 production by hydrocortisone is reversed by leukotriene B4. J Clin Invest 77, 1244-1250.

Haley, P.J., Shopp, G.M., Benson, J.M., et al., 1990. The immunotoxicity of three nickel compounds following 13-week inhalation exposure in the mouse. Fundam Appl Toxicol 15, 476-487.

Hanke, J.H., Nichols, L.N., Coon, M.E., 1992. FK506 and rapamycin selectively enhance degradation of IL-2 and GM-CSF mRNA. Lymphokine Cytokine Res 11, 221-231.

Hansen, J.F., Nielsen, C.H., Brorson, M.M., et al., 2015. Influence of phthalates on in vitro innate and adaptive immune responses. PLoS One 10, e0131168.

Hattori, A., Kunz, H.W., Gill, T.J., 3rd, et al., 1987. Thymic and lymphoid changes and serum immunoglobulin abnormalities in mice receiving cyclosporine. Am J Pathol 128, 111-120.

He, Y.W., Deftos, M.L., Ojala, E.W., et al., 1998. RORgamma t, a novel isoform of an orphan receptor, negatively regulates Fas ligand expression and IL-2 production in T cells. Immunity 9, 797-806.

Henderson, D.J., Naya, I., Bundick, R.V., et al., 1991. Comparison of the effects of FK-506, cyclosporin A and rapamycin on IL-2 production. Immunology 73, 316-321.

Himmerich, H., Schonherr, J., Fulda, S., et al., 2011. Impact of antipsychotics on cytokine production in-vitro. J Psychiatr Res 45, 1358-1365.

Hu, H., Abedi-Valugerdi, M., Moller, G., 1997. Pretreatment of lymphocytes with mercury in vitro induces a response in T cells from genetically determined low-responders and a shift of the interleukin profile. Immunology 90, 198-204.

Huchet, R., Grandjon, D., 1988. Histamine-induced regulation of IL-2 synthesis in man: characterization of two pathways of inhibition. Ann Inst Pasteur Immunol 139, 485-499.

Iatropoulos, M.J., Hobson, W., Knauf, V., et al., 1976. Morphological effects of hexachlorobenzene toxicity in female rhesus monkeys. Toxicol Appl Pharmacol 37, 433-444.

Kanariou, M., Huby, R., Ladyman, H., et al., 1989. Immunosuppression with cyclosporin A alters the thymic microenvironment. Clin Exp Immunol 78, 263-270.

Karas, K., Salkowska, A., Sobalska-Kwapis, M., et al., 2018. Digoxin, an Overlooked Agonist of RORgamma/RORgammaT. Front Pharmacol 9, 1460.

Khan, M.M., Melmon, K.L., Fathman, C.G., et al., 1985. The effects of autacoids on cloned murine lymphoid cells: modulation of IL 2 secretion and the activity of natural suppressor cells. J Immunol 134, 4100-4106.

Kim, J.H., Park, J.S., 2002. Potentiation of the immunotoxicity of ethanol by acetaminophen in mice. Int Immunopharmacol 2, 15-24.

Kim, J.Y., Huh, K., Lee, K.Y., et al., 2009. Nickel induces secretion of IFN-gamma by splenic natural killer cells. Exp Mol Med 41, 288-295.

Kim, S.K., Kwon, D.A., Lee, H.S., et al., 2019. Preventive Effect of the Herbal Preparation, HemoHIM, on Cisplatin-Induced Immune Suppression. Evid Based Complement Alternat Med 2019, 3494806.

Kloppenburg, M., Verweij, C.L., Miltenburg, A.M., et al., 1995. The influence of tetracyclines on T cell activation. Clin Exp Immunol 102, 635-641.

Knight, J.A., Plowman, M.R., Hopfer, S.M., et al., 1991. Pathological reactions in lung, liver, thymus, and spleen of rats after subacute parenteral administration of nickel sulfate. Ann Clin Lab Sci 21, 275-283.

Kouchi, Y., Maeda, Y., Ohuchida, A., et al., 1996. Immunotoxic effect of low dose cisplatin in mice. J Toxicol Sci 21, 227-233.

Kucharz, E.J., Sierakowski, S.J., 1990. Studies on immunomodulatory properties of isoniazid. II. Effect of isoniazid on interleukin 2 production and interleukin 2-receptor expression. J Hyg Epidemiol Microbiol Immunol 34, 207-211.

Labuzek, K., Kowalski, J., Gabryel, B., et al., 2005. Chlorpromazine and loxapine reduce interleukin-1beta and interleukin-2 release by rat mixed glial and microglial cell cultures. Eur Neuropsychopharmacol 15, 23-30.

Landewe, R.B., Miltenburg, A.M., Verdonk, M.J., et al., 1995. Chloroquine inhibits T cell proliferation by interfering with IL-2 production and responsiveness. Clin Exp Immunol 102, 144-151.

Lee, J., Lim, K.T., 2012. SJSZ glycoprotein (38 kDa) modulates expression of IL-2, IL-12, and IFN-gamma in cyclophosphamide-induced Balb/c. Inflamm Res 61, 1319-1328.

Lehmann, D.M., Williams, W.C., 2018. Development and utilization of a unique in vitro antigen presentation co-culture model for detection of immunomodulating substances. Toxicol In Vitro 53, 20-28.

Lemster, B., Woo, J., Strednak, J., et al., 1992. Cytokine gene expression in murine lymphocytes activated in the presence of FK 506, bredinin, mycophenolic acid, or brequinar sodium. Transplant Proc 24, 2845-2846.

Loose, L.D., Silkworth, J.B., Pittman, K.A., et al., 1978. Impaired host resistance to endotoxin and malaria in polychlorinated biphenyl- and hexachlorobenzene-treated mice. Infect Immun 20, 30-35.

Lu, Z., Liu, F., Chen, L., et al., 2015. Effect of Chronic Administration of Low Dose Rapamycin on Development and Immunity in Young Rats. PLoS One 10, e0135256.

Maeda, M., Ishii, H., Tanaka, S., et al., 2010. Suppressive efficacies of antimicrobial agents against human peripheral-blood mononuclear cells stimulated with T cell mitogen and bacterial superantigen. Arzneimittelforschung 60, 760-768.

Meredith, C., Scott, M.P., 1994. Altered gene expression in immunotoxicology screening in vitro: Comparison with ex vivo analysis. Toxicol In Vitro 8, 751-753.

Miller, L.C., Kaplan, M.M., 1992. Serum interleukin-2 and tumor necrosis factor-alpha in primary biliary cirrhosis: decrease by colchicine and relationship to HLA-DR4. Am J Gastroenterol 87, 465-470.

Miller, T.E., Golemboski, K.A., Ha, R.S., et al., 1998. Developmental exposure to lead causes persistent immunotoxicity in Fischer 344 rats. Toxicol Sci 42, 129-135.

Munson, A.E., Sanders, V.M., Douglas, K.A., et al., 1982. In vivo assessment of immunotoxicity. Environ Health Perspect 43, 41-52.

Nalesnik, M.A., Todo, S., Murase, N., et al., 1987. Toxicology of FK-506 in the Lewis rat. Transplant Proc 19, 89-92.

Northoff, H., Carter, C., Oppenheim, J.J., 1980. Inhibition of concanavalin A-induced human lymphocyte mitogenic factor (Interleukin-2) production by suppressor T lymphocytes. J Immunol 125, 1823-1828.

Palacios, R., Sugawara, I., 1982. Hydrocortisone abrogates proliferation of T cells in autologous mixed lymphocyte reaction by rendering the interleukin-2 Producer T cells unresponsive to interleukin-1 and unable to synthesize the T-cell growth factor. Scand J Immunol 15, 25-31.

Pally, C., Tanner, M., Rizvi, H., et al., 2001. Tolerability profile of sodium mycophenolate (ERL080) and mycophenolate mofetil with and without cyclosporine (Neoral) in the rat. Toxicology 157, 207-215.

Parenti, D.M., Simon, G.L., Scheib, R.G., et al., 1988. Effect of lithium carbonate in HIV-infected patients with immune dysfunction. J Acquir Immune Defic Syndr 1, 119-124.

Parthasarathy, N.J., Kumar, R.S., Devi, R.S., 2005. Effect of methanol intoxication on rat neutrophil functions. J Immunotoxicol 2, 115-121.

Peterson, K.P., Van Hirtum, M., Peterson, C.M., 1997. Dapsone decreases the cumulative incidence of diabetes in non-obese diabetic female mice. Proc Soc Exp Biol Med 215, 264-268.

Poluektova, L.Y., Huggler, G.K., Patterson, E.B., et al., 1999. Involvement of protein kinase A in histamine-mediated inhibition of IL-2 mRNA expression in mouse splenocytes. Immunopharmacology 41, 77-87.

Quemeneur, L., Flacher, M., Gerland, L.M., et al., 2002. Mycophenolic acid inhibits IL-2-dependent T cell proliferation, but not IL-2-dependent survival and sensitization to apoptosis. J Immunol 169, 2747-2755.

Ress, N.B., Hailey, J.R., Maronpot, R.R., et al., 2003. Toxicology and carcinogenesis studies of microencapsulated citral in rats and mice. Toxicol Sci 71, 198-206.

Riesbeck, K., 1999. Cisplatin at clinically relevant concentrations enhances interleukin-2 synthesis by human primary blood lymphocytes. Anticancer Drugs 10, 219-227.

Ringerike, T., Ulleras, E., Volker, R., et al., 2005. Detection of immunotoxicity using T-cell based cytokine reporter cell lines ("Cell Chip"). Toxicology 206, 257-272.

Roche, Y., Fay, M., Gougerot-Pocidalo, M.A., 1988. Enhancement of interleukin 2 production by quinolone-treated human mononuclear leukocytes. Int J Immunopharmacol 10, 161-167.

Saito, R., Hirakawa, S., Ohara, H., et al., 2011. Nickel differentially regulates NFAT and NF-kappaB activation in T cell signaling. Toxicol Appl Pharmacol 254, 245-255.

Salazar, V., Castillo, C., Ariznavarreta, C., et al., 2004. Effect of oral intake of dibutyl phthalate on reproductive parameters of Long Evans rats and pre-pubertal development of their offspring. Toxicology 205, 131-137.

Santarelli, L., Bracci, M., Mocchegiani, E., 2006. In vitro and in vivo effects of mercuric chloride on thymic endocrine activity, NK and NKT cell cytotoxicity, cytokine profiles (IL-2, IFN-gamma, IL-6): role of the nitric oxide-L-arginine pathway. Int Immunopharmacol 6, 376-389.

Schleuning, M.J., Duggan, A., Reem, G.H., 1989. Inhibition by chlorpromazine of lymphokine-specific mRNA expression in human thymocytes. Eur J Immunol 19, 1491-1495.

Sfikakis, P.P., Souliotis, V.L., Katsilambros, N., et al., 1996. Downregulation of interleukin-2 and apha-chain interleukin-2 receptor biosynthesis by cisplatin in human peripheral lymphocytes. Clin Immunol Immunopathol 79, 43-49.

She, Y., Wang, N., Chen, C., et al., 2012. Effects of aluminum on immune functions of cultured splenic T and B lymphocytes in rats. Biol Trace Elem Res 147, 246-250.

Sheikhi, A., Jaberi, Y., Esmaeilzadeh, A., et al., 2007. The effect of cardiovascular drugs on pro-inflammatory cytokine secretion and natural killer activity of

peripheral blood mononuclear cells of patients with chronic heart failure in vitro. Pak J Biol Sci 10, 1580-1587.

Silvestrini, B., Lisciani, R., Barcellona, P.S., 1967. Anti-granuloma and thymolytic activity of certain drugs. Eur J Pharmacol 1, 240-246.

Song, Y., Han, S., Kim, H., et al., 2006. Effects of mizoribine on MHC-restricted exogenous antigen presentation in dendritic cells. Arch Pharm Res 29, 1147-1153.

Sookoian, S., Castano, G., Flichman, D., et al., 2004. Effects of ribavirin on cytokine production of recall antigens and phytohemaglutinin-stimulated peripheral blood mononuclear cells. (Inhibitory effects of ribavirin on cytokine production). Ann Hepatol 3, 104-107.

Sugiyama, K., Ueda, H., Ichio, Y., et al., 1995. Improvement of cisplatin toxicity and lethality by juzen-taiho-to in mice. Biol Pharm Bull 18, 53-58.

Synzynys, B.I., Sharetskii, A.N., Kharlamova, O.V., 2004. [Immunotoxicity of aluminum chloride]. Gig Sanit, 70-72.

Sztein, M.B., Simon, G.L., Parenti, D.M., et al., 1987. In vitro effects of thymosin and lithium on lymphoproliferative responses of normal donors and HIV seropositive male homosexuals with AIDS-related complex. Clin Immunol Immunopathol 44, 51-62.

Takai, K., Jojima, K., Sakatoku, J., et al., 1990. Effects of FK506 on rat thymus: time-course analysis by immunoperoxidase technique and flow cytofluorometry. Clin Exp Immunol 82, 445-449.

Tam, R.C., Pai, B., Bard, J., et al., 1999. Ribavirin polarizes human T cell responses towards a Type 1 cytokine profile. J Hepatol 30, 376-382.

Tsukue, N., Toda, N., Tsubone, H., et al., 2001. Diesel exhaust (DE) affects the regulation of testicular function in male Fischer 344 rats. J Toxicol Environ Health A 63, 115-126.

Turka, L.A., Dayton, J., Sinclair, G., et al., 1991. Guanine ribonucleotide depletion inhibits T cell activation. Mechanism of action of the immunosuppressive drug mizoribine. J Clin Invest 87, 940-948.

Van Dijk, H., Bloksma, N., Rademaker, P.M., et al., 1979. Differential potencies of corticosterone and hydrocortisone in immune and immune-related processes in the mouse. Int J Immunopharmacol 1, 285-292.

Van Wauwe, J., Aerts, F., Van Genechten, H., et al., 1996. The inhibitory effect of pentamidine on the production of chemotactic cytokines by in vitro stimulated human blood cells. Inflamm Res 45, 357-363.

Vandebriel, R.J., Meredith, C., Scott, M.P., et al., 1998. Effects of in vivo exposure to bis(tri-n-butyltin)oxide, hexachlorobenzene, and benzo(a)pyrene on cytokine (receptor) mRNA levels in cultured rat splenocytes and on IL-2 receptor protein levels. Toxicol Appl Pharmacol 148, 126-136.

Vargova, M., Wagnerova, J., Liskova, A., et al., 1993. Subacute immunotoxicity study of formaldehyde in male rats. Drug Chem Toxicol 16, 255-275.

Vos, J.G., van Logten, M.J., Kreeftenberg, J.G., et al., 1979. Hexachlorobenzene-induced stimulation of the humoral immune response in rats. Ann N Y Acad Sci 320, 535-550.

Vos, J.G., Van Loveren, H., 1994. Developments of immunotoxicology methods in the rat and applications to the study of environmental pollutants. Toxicol In Vitro 8, 951-956.

Wagner, W., Sachrajda, I., Pulaski, L., et al., 2011. Application of cellular biosensors for analysis of bioactivity associated with airborne particulate matter. Toxicol In Vitro 25, 1132-1142.

Wagner, W., Walczak-Drzewiecka, A., Slusarczyk, A., et al., 2006. Fluorescent Cell Chip a new in vitro approach for immunotoxicity screening. Toxicol Lett 162, 55-70.

Wang, Y., Walker, C., Stadler, B.M., et al., 1984. Transcription and translation dependent induction of interleukin 2 (IL-2) and IL-2 receptors. Immunol Lett 8, 227-231.

Wilson, R., Fraser, W.D., McKillop, J.H., et al., 1989. The "in vitro" effects of lithium on the immune system. Autoimmunity 4, 109-114.

Yamamoto, N., Sakai, F., Yamazaki, H., et al., 1996. Effect of FR167653, a cytokine suppressive agent, on endotoxin-induced disseminated intravascular coagulation. Eur J Pharmacol 314, 137-142.

Yoshimura, N., Matsui, S., Hamashima, T., et al., 1989. Effect of a new immunosuppressive agent, FK506, on human lymphocyte responses in vitro. II. Inhibition of the production of IL-2 and gamma-IFN, but not B cell-stimulating factor 2. Transplantation 47, 356-359.

Zhang, W.Z., Yong, L., Jia, X.D., et al., 2013. Combined subchronic toxicity of bisphenol A and dibutyl phthalate on male rats. Biomed Environ Sci 26, 63-69.

OECD TEST GUIDELINES PROGRAMME

Standard Project Submission Form

If you require further information please contact the OECD Secretariat
Return completed forms to:
Anne Gourmelon (anne.gourmelon@oecd.org)
and
Anna Rourke (anna.rourke@oecd.org)

PROJECT TITLE

Test guideline for identifying the T cell-mediated immunotoxic potential of chemicals using the IL-2 Luc assay

SUBMITTED BY (Country / European Commission / Secretariat)

Japan

DATE OF SUBMISSION TO THE SECRETARIAT

November 11th, 2019

DETAILS OF LEAD COUNTRY/CONSORTIUM

Country /Organisation:	Japan
Agency/ministry/Other:	Japanese Center for the Validation of Alternative Methods / National Institute of Health Sciences / Ministry of Health, Labour Welfare (MHLW) / Ministry of Economy, Trade and Industry (METI)
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PROJECT OUTCOMES

☒ New Test Guideline	☐ Guidance document
☐ Revised Test Guideline	☐ Detailed Review Paper
☐ Deletion of an existing Test Guideline	☐ Other, please specify below

MAIN OBJECTIVE OF THE PROPOSAL (max. 150 words)

We propose an *in vitro* immunotoxicity test using a stable luciferase reporter cell line that contain IL-2 promoter-driven luciferase. This assay designated as the IL-2 Luc assay is aimed to be a component of Integrated Approaches to Testing and Assessment (IATA*)* to detect immunotoxic potential of chemicals *in vitro.*

PROPOSED WORK PLAN and RESOURCE NEEDS:

1. Draft workplan for development of the proposal, including any need to establish Ad Hoc Expert Group and mode of meetings (face-to-face, teleconference; electronic discussion group). Indicate key milestones, including first and subsequent drafts of documents and timing of meetings.

Dr. Setuya Aiba et al (Tohoku University, Japan) developed the IL-2 Luc assay as a new *in vitro* immunotoxicity testing in addition to the IL-8 Luc assay; OECD Test Guideline (TG) 442Ef for *in vitro* skin sensitisation assay. The IL-2 Luc assay is a cell-based *in vitro* assay (not involving the use of animals) designed to be used in combination with other *in vitro* tests as part of a battery or ITS (will have to be defined) to detect the immunotoxic potential of chemicals *in vitro*. They have also been involved in the development of an adverse outcome pathway (AOP) contains IL-2 suppression as Key Event in the extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST) (AOP154).

The Japanese Center for the Validation of Alternative Methods (JaCVAM) recently coordinated a validation study to assess the reliability (transferability, within- and between-laboratory reproducibility) and preliminary predictive capacity of the IL-2 Luc assay. The validation report has been submitted to the JaCVAM International peer review panel. The IL-2 Luc assay has undergone independent peer review during 2019 and the peer review finished in June, 2020. Japan has submitted the validation report and peer review report to the OECD secretariat as attachments of the SPSF.

Prior to the development of a Test Guideline, Japan has developed Detailed Review Paper (DRP) for in vitro immunotoxicity testing. Therefore, Japan wish to discuss a draft TG on IL-2 Luc assay as well as DRP in the expert meeting and WNT. In the near future, the DRP and TG on in vitro immunotoxicity will be approved as the OECD documents.

2. Will additional information, including generation or collection of data, be required? If yes, please describe the anticipated process and timelines.

No additional information is submitted.

3. Indicate the estimated overall resource need (time/money) for member country / consortium and Secretariat

Resources for drafting the document will be provided by the National Institute of Health Sciences (NIHS)/JaCVAM. This effort will be coordinated in collaboration with the other International Cooperation on Alternative Test Methods (ICATM) partners (EURL ECVAM, NTP Interagency Centre for the Evaluation of Alternative Toxicological Methods (NICEATM)/US Coordinating Committee on the Validation of Alternative Methods (ICCVAM), Korean Centre for the Validation of Alternative methods (KoCVAM), Brazilian Centre for the Validation of Alternative methods (BraCVAM) and Health Canada).

4. Is this proposal intended to replace an existing Test Guideline or lead to the deletion of an existing Test Guideline?

No expectation to replace or delete any existing TG. The IL-2 Luc assay may also be a partial method designed to be used within a test battery or ITS for assessing the immunotoxic potential of chemicals.

ESSENTIAL INFORMATION

In this section, please provide the information required by the Working Group of National Coordinators of the Test Guidelines Programme to assess the suitability of the project for the workplan of the Test Guidelines Programme

1. What is the existing or expected regulatory need/data requirement that will be met by the proposed outcome of the project? Please provide details below or as an attachment.

This assay is expected to meet some regulatory needs (e.g., ICH S8 and WHO/IPCS guidance on immunotoxicity). Japanese researchers have been involved recently in developing six AOPs for immunotoxicity in the EAGMST, in which two AOPs contain Key Event of IL-2 suppression. Japan is coordinating to develop a DRP on in vitro immunotoxicity, which includes a tiered approach for a testing strategy on immunotoxicity, with International experts who cooperated IL-2 Luc assay. Considering these advances, JaCVAM will continues to support the development of IL-2 Luc assay.

or as attachment No.__

2. How will the work contribute to further international harmonisation of hazard and risk assessment? Please provide details below or as an attachment.

With the REACH legislation (Regulation 1907/2006/EC), the EU also promotes alternative methods for safety testing. In addition, REACH article 25 states that animal testing must be used as a last resort, which encourages the exploitation of useful alternative methods to the absolute maximum.

The ICCVAM, EURL ECVAM and KoCVAM were liaisons to the Validation Management Group. In addition, JaCVAM was represented on the Validation Management Group. The peer review conducted in line with the Memorandum of Cooperation signed by ICATM aiming at reaching harmonised recommendations on the usefulness and limitations of alternative test methods and their potential role in an ITS for regulatory testing purposes in member countries.

or as attachment No.__

3. How will the proposed project address issues and /or endpoints which are of major human health or environmental concerns? If there are existing Test Guidelines or projects in the work plan of the Test Guidelines Programme covering the same endpoint, please refer to these and

describe the added value and usability of the proposed new test method. Please provide details below or as an attachment.

The IL-2 Luc assay is one of the components constituting the Multi-ImmunoTox assay that we have recently developed to detect immunotoxicity of chemicals [1] [2] [3]. The IL-2 Luc assay uses a stable reporter cell line, 2H4 cell, which is derived from Jurkat cells containing stable luciferase green (SLG) regulated by the IL-2 promoter, stable luciferase orange (SLO) regulated by the IFN-γ promoter, and stable luciferase red (SLR) regulated by the G3PDH promoter. Using this cell line, the IL-2 Luc assay identifies the effects of chemicals on the IL-2 promoter activity in 2H4 cells in the presence of the stimulants phorbol 12-myristate 13-acetate (PMA) and ionomycin (Io).

Immune dysregulation may have serious impacts on human health, ranging from reduced resistance to infection and neoplasia to allergic and autoimmune conditions. Pivotal immune elements of these diseases are the development of antigen-specific effector T-helper type (Th) cells, Th1 cells, Th2 cells, Th17 cells, and regulatory T cells (Treg cells) that are associated with clinical features and disease progression. Consequently, identifying the immunotoxicity of chemicals requires clarifying their effects on the development of these T cells (reviewed by [4]). IL-2 exerts pleiotropic actions on CD4+ T cell differentiation via its modulation of cytokine receptor expression. IL-2 promotes Th1 differentiation by inducing IL-12Rb2 (and IL-12Rb1), promotes Th2 differentiation by inducing IL-4Ra, inhibits Th17 differentiation by inhibiting gp130 (and IL-6Ra), and drives Treg differentiation by inducing IL-2Ra. IL-2 also potently represses IL-7Ra, which decreases survival signals that normally promote cell survival and memory cell development [5]. It is therefore assumed that chemicals that affect IL-2 release by T cells could significantly impact immune function. Needless to say, the evaluation of the effects of chemicals on the IL-2 transcription cannot cover the immunotoxicity of all the chemicals and should be used as IAT. However, the transcription of IL-2 by T cells after stimulation with PMA/Io involves the pathways leading the activation of mitogen activated protein kinases (MAPs), mammalian target of rapamycin (mTOR), nuclear factor-κB (NF-κB), and nuclear factor of activated T-cells (NF-AT) (reviewed by [6]). Since these pathways play a crucial role in intracellular signaling utilized by a variety of immune responses other than IL-2 transcription, the IL-2 Luc assay has the potential to cover the immunotoxicity of substantial numbers of chemicals.

In addition, AOP 154 (Inhibition of calcineurin activity leading to impaired T-cell dependent antibody response) indicates the crucial role of optimal IL-2 production in maintaining normal immune response.

References

[1] Saito R, Hirakawa S, Ohara H, Yasuda M, Yamazaki T, Nishii S, et al.: Nickel differentially regulates NFAT and NF-kappaB activation in T cell signaling. Toxicology and applied pharmacology 254: 245-255, 2011.
[2] Takahashi T, Kimura Y, Saito R, Nakajima Y, Ohmiya Y, Yamasaki K, et al.: An in vitro test to screen skin sensitizers using a stable THP-1-derived IL-8 reporter cell line, THP-G8. Toxicological sciences : an official journal of the Society of Toxicology 124: 359-369, 2011.
[3] Kimura Y, Fujimura C, Ito Y, Takahashi T, Aiba S: Evaluation of the Multi-ImmunoTox Assay composed of 3 human cytokine reporter cells by examining immunological effects of drugs. Toxicol In Vitro 28: 759-768, 2014.
[4] Kaiko GE, Horvat JC, Beagley KW, Hansbro PM: Immunological decision-making: how does the immune system decide to mount a helper T-cell response? Immunology 123: 326-338, 2008.
[5] Liao W, Lin JX, Wang L, Li P, Leonard WJ: Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. Nat Immunol 12: 551-559, 2011.
[6] Alegre ML, Frauwirth KA, Thompson CB: T-cell regulation by CD28 and CTLA-4. Nat Rev Immunol 1: 220-228, 2001.

or as attachment No._ _

4. Will the project have general support from OECD member countries or is the outcome relevant for just one or a few member countries / stakeholders? Provide details of the countries and the rationale for this view below.

☒ Many countries ☐ A few countries ☐ Only for the submitting country

Given the global interest in accurately labelling products for immunotoxic potential without using animals, it is anticipated that the proposed Test Guideline and the RP will have general support from OECD member countries if determined to be scientifically justified by the peer review process.

5. If the Test Guideline is not intended for general use, indicate if the Test Guideline would be intended for:

☐ Specific (limited) applications such as pesticide usage, or

☐ for specific classes of chemicals (e.g. surfactants) rather than for chemicals in general.

6. If the expected outcome of this proposal is a Test Guideline or a Guidance Document, provide information on the intended use, applicability and limitations of the test method.

Since the 2H4 cell line used in the IL-2 Luc assay is derived from Jurkat cells, it is conceivable that this cell line is more resistant to the cytotoxic effects of chemicals than bone marrow cells. Indeed, our study demonstrated that the IL-2 Luc assay cannot evaluate the immunotoxic effects of some immunosuppressive drugs which act by inhibiting DNA synthesis leading to myelotoxicity [1]. Thus, these chemicals in addition to chemicals that need metabolic activation should be outside the applicability domain. To overcome this drawback at present, the IL-2 Luc assay must be combined with assays capable of detecting myelotoxicity, such as *in vitro* myelotoxicity tests [2]. Similar to other *in vitro* test methods, poor insoluble chemicals are not suitable for this assay.

[1] Kimura Y, Fujimura C, Ito Y, Takahashi T, Aiba S: Evaluation of the Multi-ImmunoTox Assay composed of 3 human cytokine reporter cells by examining immunological effects of drugs. Toxicol In Vitro 28: 759-768, 2014.

[2] OECD: OECD Test Gudeline for the Testing of Chemicals No.442E: In Vitro Skin Sensitisation assays addressing the key Event on activation of dendritic cells on the Adverse Outcome pathway for Skin Sensitisation. http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788. 2017.

7. Provide supporting information on the validation status (i.e. relevance and reliability) of the method. Principles for validation of test methods for OECD Test Guidelines are described in Guidance Document 34.

Provide justification and rationale for the test, including data.

If there are no or limited data available to support the reliability and relevance of the proposed test, indicate if validation work is included in the project.

If there is no need for validation, provide a detailed justification.

Experimental data was generated with the IL-2 Luc assay in a JaCVAM-coordinated validation study designed to allow for an assessment of the reliability and preliminary predictive capacity. The experiment part of IL-2 Luc assay validation study completed in Oct. 2018. For the within-laboratory reproducibility, 5 coded chemicals were tested by 3 laboratories and the average within-laboratory reproducibility was 86.7% (13/15). On the other hand, for the inter-laboratory reproducibility, a total of 25 coded chemicals were evaluated and the concordance rate was 80.0% (22/25).

To determine the predictivity of the IL-2 Luc assay, classification chemicals into those that affect T cell function (T cell-targeting chemicals, TTC) and those that do not directly affect T cell function (non-T cell-targeting chemicals, NTTC) was needed. According to the classification, accuracy is 75% (18/24), specificity is 75% (6/8) and sensitivity is 75% (12/16). The PRP concluded the predictive capacity of the test is not sufficient to detect all immunotoxic chemicals if used as a stand-alone test.

Based on the results, the IL-2 Luc assay may be also a partial method designed to be used within a test battery or ITS for assessing the immunotoxic potential of chemicals and therefore it is expected to contribute to the evaluation depending on member countries' regulatory requirements. The peer review is not foreseen to assess the test method's relevance as a stand-alone method.

8. Describe if the test method includes components, equipment or other scientific procedures that are covered (or pending) by Intellectual Property Rights (IPR) (e.g., patents, patent applications, industrial designs and trademarks). Information should be provided on the overall availability of the IPR-protected components including whether they are commercially available or require a Material Transfer Agreement (MTA) or other licensing agreements. In addition, a description of the IPR-covered component/test system should be disclosed. Note that the OECD has developed Guiding Principles on good practices for the availability/distribution of protected elements in OECD Test Guidelines. The test method developer will be requested to fill in and sign the FRAND Terms Licensing Declaration Form annexed to the Guiding Principles.

8.1 Nature of protected elements (e.g. reagent identity, cell line identity, specific process, etc.):

None

8.2 Form of protection (e.g. trademark, patent, etc.):

None

8.3 For users to access protected elements, please tick the relevant box(es):

☒ MTA required ☐ License requirement ☐ No agreement required

If a license or other agreement is foreseen, please note that terms and conditions should comply with FRAND and a signed declaration needs to be submitted if the project gets onto the work plan. See Annex 2 of the OECD Guiding Principles on Good Licensing Practices for Protected Elements in OECD TGs (2019).

8.4 Are you providing the agreement document(s) referred to in 8.3 with the SPSF:

☐ Yes ☒ No If no, what's the reason?

The Material Transfer Agreement (MTA) in line with the conditions of the OECD template is currently under preparation. It will be prepared by the time when this assay is accepted as the OECD TG.

8.5 How and where can users get access to protected elements?

The Standard Operating Procedure (SOP) for the IL-2 Luc assay and 2H4 cells become available when this assay is accepted as the OECD TG. Laboratories that want to perform the test would obtain the 2H4 cell line from GPC Lab. Co. Ltd., Tottori, Japan, upon signing a MTA in line with the conditions of the OECD template. 2H4 cells will be maintained and quality-checked at regular intervals in GPC.

8.6 Has any search for existing patent(s) possibly associated with this test method been performed (e.g. through patent search or Freedom-To-Operate search). If yes, what was the outcome?

Yes, we have performed. But so far, IL-2 Luc assay does not conflict the other patents.

8.7 Does the test method include any Confidential Business Information? No

If yes, which ones?

<u>IMPORTANT NOTE:</u> Should the OECD and Expert Group working on the Test Guideline development discover that the information provided under Item 3 on IP elements be erroneous or be evolving in the course of the project, the project itself might be re-considered, suspended or cancelled.

9 Have Performance Standards been developed? ☒ Yes ☐ No ☐ N/A

ADDITIONAL INFORMATION

In this section please provide further information to allow the Working Group of National Coordinators of the Test Guidelines Programme to assess the suitability of the project for the workplan of the Test Guidelines Programme

1. If the expected outcome of the project proposal is a Test Guideline and is based on existing, regional or international documents such as guidelines, protocols or guidance material, please provide that information here or as an attachment.

We have been exposed to an enomous number of chemicals as environmental contaminants, food additives, and drugs. Some of them can target the immune system resulting in immune dysregulation, which can have serious adverse health consequences, ranging from reduced resistance to infection and neoplasia to allergic and autoimmune conditions. Accordingly, the potential for immunotoxicity, which is defined as the toxicological effects of xenobiotics on the function of the immune system, has raised serious concerns from the public as well as regulatory agencies. On such a ground, World Health Organization published the Guidance for Immunotoxicity Risk Assessment for Chemicals ((WHO)/ & Meeting, 2012). Furthermore, in the OECD TG 443, assessment of potential developmental immunotoxicity was described as a part of reproductive and developmental effects. The ICH guideline (ICH HARMONISED TRIPARTITE GUIDELINE IMMUNOTOXICITY STUDIES FOR HUMAN PHARMACEUTICALS S8) mentions on nonclinical testing approaches to identify compounds which have the potential to be immunotoxic. Currently, the assessment of chemical immunotoxicity relies mainly on animal models and assays that characterize immunosuppression and sensitization. However, animal studies have many drawbacks, such as high cost, ethical concerns, and questionable relevance to risk assessment for humans.

or as attachment No.__

2. If Animal Welfare considerations are addressed in the project proposal, provide details below or as an attachment. Explain if the project is aimed at refining, reducing and/or replacing the use of animals.

If the project is not specifically developed for animal welfare purposes, indicate if the animal welfare considerations have been a component of the project proposal.

Indicate if animal welfare considerations are irrelevant to the project, for example for physico-chemical properties.

The IL-2 Luc assay is an *in vitro* method not involving the use of animals. Its proposed role is to be a part of a screening approach in combination with other *in vitro* tests, with the purpose of reducing the number of animals used, or when regulatory requirements allows also to replace existing *in vivo* tests for immunotoxicity hazard assessment.

or as attachment No.__

3. Provide information on expected or possible resource savings in member countries as a result of this project.

The Test Guideline will contribute to the reduction of animal usage and provide a screening test that is cost efficient to perform.

4. If the expected outcome of the proposed project is a Guidance Document or Detailed Review Paper, will it be directly linked to the development of a particular Test Guideline or a series of Test Guidelines?

☐ Yes, it is the initial step in the development of a new or revision of existing Guidelines.

☐ Yes, additional guidance is needed for the most appropriate selection of the Guidelines on the subject.

☐ No, the guidance is on issues related to testing or the development of Test Guidelines in general.

There are _9_ attachments added to this form.

1. Multi-Immuno Tox Assay proto col Ver.011.1E 20200514
2. Kimura et al., Toxicology in Vitro 28 (2014) 759–768
3. Kimura et al., Archives of Toxicology, https://doi.org/10.1007/s00204-018-2199-7
4. IL-2 Luc assay validation report
5. IL-2 Luc assay peer review report
6. Kimura et al., Toxicology in Vitro 66 (2020) 1-8
7. FRAND Terms Licensing Declaration Forms (Prof. Aiba)
8. Material Transfer Agreement (for Jurkat cells)
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Report on a Validation Study of the IL-1 Luc Assay for Evaluating the Potential Immunotoxic Effects of Chemicals on Monocyte/Dendritic Cells

Validation Management Team

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1. Summary

The IL-1 Luciferase reporter assay (IL-1 Luc assay) was developed as one of three luciferase reporter assays in the Multi-ImmunoTox Assay (MITA), a high-throughput screening system that our group developed to evaluate chemical immunotoxicity. Although our final long-term goal is to officially validate the MITA for within- and between-laboratory reproducibility and predictivity, in this study we conducted a validation of the IL-1 Luc assay as a second step following the IL-2 Luc assay.

In the MITA, we used three stable lines of reporter cells transfected with luciferase genes under control of the IL-2, IFN-γ, IL-8, and IL-1β promoters: 2H4 cells derived from Jurkat cells containing stable luciferase green (SLG) regulated by the IL-2 promoter, stable luciferase orange (SLO) regulated by the IFN-γ promoter, and stable luciferase red (SLR) regulated by the G3PDH promoter; THP-G8 cells derived from THP-1 cells containing SLO regulated by the IL-8 promoter, SLR regulated by the G3PDH promoter; THP-G1b cells derived from THP-1 cells containing SLG regulated by the IL-1β promoter, and SLR regulated by the G3PDH promoter. We selected these four cytokines because IL-2 and IFN-γ are primarily produced by T cells (a type of adaptive immune cells), whereas IL-8 and IL-1β are primarily produced by monocytes and dendritic cells (types of innate immune cells).

Using these three cell lines, the MITA can evaluate the effects of chemicals on the IL-2 and IFN-γ luciferase activity of 2H4 cells stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Io), and those on the IL-1β and IL-8 luciferase activity of THP-G1b and THP-G8 cells, stimulated by lipopolysaccharide (LPS).

In the validation study of the IL-1 Luc assay, the preliminary test trial (Phase 0) was performed by the participating laboratories following explicit explanations of the Multi-ImmunoTox Assay protocol for THP-G1b ver. 007E by the lead laboratory, Tohoku University. Three laboratories participated in the Phase 0 study of the IL-1 Luc assay using the three open labeled chemicals dapson, diethanolamine and p-nitroaniline, and conducted one set (three experiments) for each chemical. The response patterns for the

three chemicals were similar among the three laboratories. Based on these results, the VMT judged that technical and protocol transfer of the IL-1 Luc assay is acceptable.

In the Phase I study, a total of five coded chemicals were evaluated by three experimental sets based on the Multi-ImmunoTox Assay protocol for THP-G8 ver. 008E made by the lead laboratory, Tohoku University. The within-laboratory reproducibility was 100.0% (15/15). The between-laboratory reproducibility was 100.0% (5/5). The predictivity was 40.0% (6/15).

The Phase II study for between-laboratory reproducibility and predictivity was conducted using a total of 20 coded chemicals (15 immunotoxic chemicals and 5 non-immunotoxic chemicals) and evaluated by one experiment set based on the Multi-ImmunoTox Assay protocol for THP-G1b ver. 009E. The between-laboratory reproducibility was 80% (16/20) and the predictivity was 46.7% (28/60).

In the combined results of the Phase I and II studies, the within-laboratory reproducibility was 100.0% (15/15). The between laboratory reproducibility was 84.0% (21/25). The predictivity was 45.3% (34/75).

Although the within- and between-laboratory reproducibilities could satisfy the acceptance criteria for the validation study, the predictivity was below 80%. As suggested by non-animal tests for skin sensitization, there is a wide consensus that due to the complex mechanisms involved in the human immune system, there is no single non-animal alternative assay that covers the effects of chemicals on the entire immune response. Instead, it is necessary to develop integrated approaches for testing and assessment (IATA) using multiple assays for different aspects of the immune system. Therefore, this low predictability of the IL-1 Luc assay does not necessarily indicate its limited usefulness as an immunotoxicity test.

We have completed an official validation study of the IL-2 Luc assay, in which the immunotoxicity of chemicals was evaluated by their effects on IL-2 promoter-driven luciferase activity of 2H4 cells. To clarify the characteristics of the IL-1 Luc assay, the lead laboratory examined data for 60 chemicals previously evaluated by the IL-2 Luc assay and two new chemicals added for this study for evaluation by the IL-1 Luc assay.

Although the performance of the IL-1 Luc assay alone was 52.1% for sensitivity, 35.7% for specificity, and 46.7% for predictivity, the performance of the IL-2 Luc assay improved from 77.8% to 83.3% for sensitivity and from 82.5% to 83.3% for predictability when combined with the IL-1 Luc assay. These data indicate the potential usefulness of the IL-1 Luc assay as a component of the IATA for immunotoxicity.

Moreover, by comparing the signaling pathway to induce IL-1β mRNA expression after LPS stimulation with the signaling pathway to induce IL-2 mRNA expression after PMA/Io, it became clear that the signaling molecules TLR4, Mal, TRAM, Myd88, IRAK4 and IRAK1/2 are specific to IL-1β mRNA expression after LPS stimulation. Indeed, the activity of the TLR4 inhibitor TAK-242 and the IRAK4 inhibitor PF06650833 was only detected by the IL-1 Luc assay, and not by the IL-2 Luc assay. The IL-1 Luc assay is thus a promising tool for detecting the effects of chemicals on these signaling molecules. IRAK4 is a key signaling node for transducing the responses of the interleukin-1 (IL-1) receptor family (IL-1, IL-18 and IL-33 receptors) and TLRs (except for TLR3), and has recently attracted widespread attention as a therapeutic target for inflammation and tumor diseases.

These data suggest that although the performance of the IL-1 Luc assay is not satisfactory as a stand alone method when used to examine the chemicals used for the validation study and the dataset, it is a promising approach for detecting the immunotoxicity of chemicals towards a certain aspect of the immune response or as a component of the IATA.

2. Objective of study

The objective of the present validation study was to determine the usefulness and limitations of the IL-1 Luc assay as a Multi-ImmunoTox Assay (MITA): specifically, as a non-animal screening method to detect and assess the immunotoxicity of chemicals. The specific objectives of the study were to establish:

1) “Transferability”, i.e., the extent to which a laboratory can adapt and easily implement the IL-1 Luc assay;

2) “Between- or inter-laboratory reproducibility”, i.e., the extent to which results agree among different laboratories;

3) "Within- or intra-laboratory reproducibility", i.e., the extent to which results agree in the same laboratory; and

3) “Predictivity”, i.e., the extent to which the *in vitro* results agree with the known immunological profiles of the chemicals.

3. Background

3-1. What is immunotoxicity?

A well-functioning immune system is essential for maintaining the integrity of an organism. Immune dysregulation can have serious adverse health consequences, ranging from reduced resistance to infection and neoplasia to allergic and autoimmune conditions. Environmental contaminants, food additives, and drugs can target the immune system, resulting in immune dysregulation. Accordingly, the potential for immunotoxicity, which is defined as the toxicological effects of xenobiotics on the function of the immune system, has raised serious concerns from the public as well as regulatory agencies. Currently, the assessment of chemical immunotoxicity relies mainly on animal models and assays that characterize immunosuppression and sensitization. However, animal studies have many drawbacks, such as high cost, ethical concerns, and questionable relevance to risk assessment for humans. Furthermore, there is global recognition of the need for alternative testing methods and assessment strategies to reduce the use of laboratory animals and, if possible, replace animals used in scientific studies (Adler et al. 2011).

3-2. *In vitro* immunotoxicity tests should evaluate effects on both innate and acquired immunity

The immune system comprises innate and adaptive immunity (Fig. 1). Both arms of the immune response function differently and are driven by different populations of cells. In innate immunity, pathogens are recognized through various pattern recognition molecules, such as C-type lectin receptors, toll-like receptors, nod-like receptors, and retinoic acid–inducible gene-I (RIG-I)-like receptors. In addition, a variety of different cells are involved in this type of response, including neutrophils and other types of granulocytes, macrophages, natural killer (NK) cells, innate lymphoid cells, and mast cells. Adaptive immune responses involve specific antigen receptors encoded by rearranged genes, and T cells and B cells play critical roles in these responses.

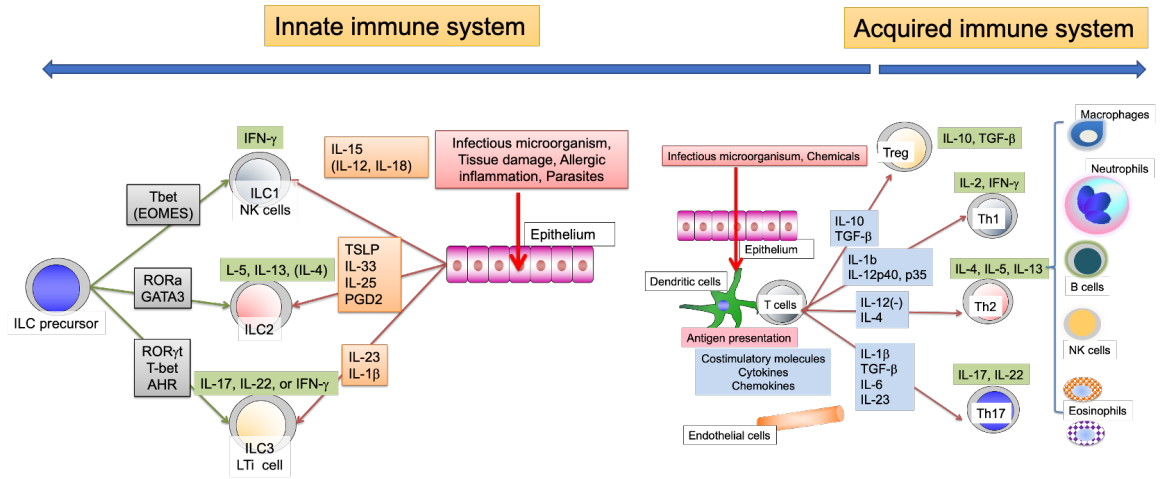

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Fig. 1. Schematic representation of the innate immune system and acquired immune system.

Macrophages and dendritic cells (DCs), which act as antigen-presenting cells (APCs), link the innate and adaptive immune responses because they can present antigens to T lymphocytes in the context of major histocompatibility complex (MHC) class I or II molecules and stimulate their proliferation and effector functions after being stimulated via pathogen recognition receptors (Fig. 2). To induce optimal immune responses to various pathogens and minimize autoreactivity, innate and adaptive

immune cells produce a vast array of cytokines, chemokines, and chemical mediators and present the molecules required for direct cell-cell interaction on their surface. A variety of intracellular signaling pathways also play roles in innate and adaptive immune responses.

Theoretically, chemicals can affect the immune system by targeting either the innate immune system or the acquired immune system (Fig. 1). Therefore, novel *in vitro* test methods are needed to adequately assess the immunotoxic effects of chemicals on both arms of the immune system.

3-3. The current status of *in vitro* approaches to detect immunotoxicants

The workshop hosted by the European Centre for the Validation of Alternative Methods (ECVAM) in 2003 focused on state-of-the-art *in vitro* systems for evaluating immunotoxicity (Galbiati et al. 2010; Gennari et al. 2005; Lankveld et al. 2010). A tiered approach was proposed. Since useful information can be obtained from regular 28-day general toxicity tests, pre-screening for direct immunotoxicity would begin with the evaluation of myelotoxicity in the proposed tiered approach (Corsini and Roggen 2017). Compounds that are capable of damaging or destroying bone marrow will most likely have immunotoxic effects. If compounds are not potentially myelotoxic, they are tested for leukotoxicity. Compounds are then tested for immunotoxicity using various approaches such as the human whole-blood cytokine release assay (HWBCRA), lymphocyte proliferation assay, mixed lymphocyte reaction, NK cell assay, T cell–dependent antibody response, dendritic cell maturation assay, and fluorescent cell chip (FCP) assay. Among these assays, the HWBCRA has undergone formal pre-validation, although other techniques are being examined or have been examined in a rigorous pre-validation effort by the ECVAM and other groups (Fig. 2). However, these assays require fresh rodent or human immune cells, in conflict with animal protection goals. The need for primary cells may decrease reproducibility and makes the assay unsuitable for high-throughput approaches.

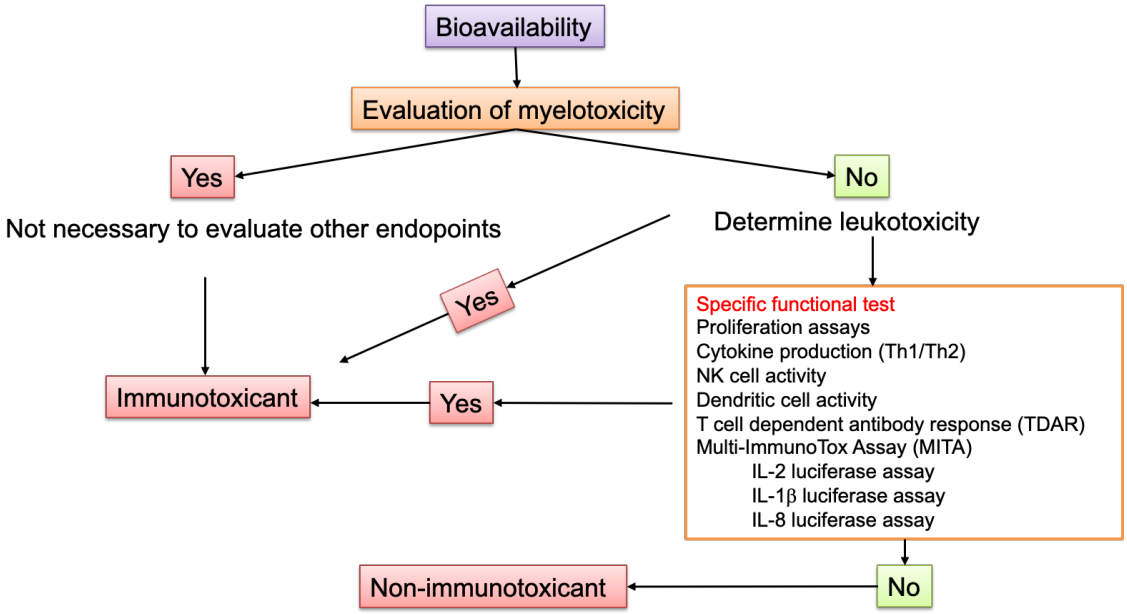

Fig. 2. Decision tree approach for *in vitro* assessment of chemical-induced immunosuppression (modified from Corsini and Roggen. Overview of *in vitro* assessment of immunotoxicity DOI: 10.1016/j.cotox.2017.06.016)

3-4. Further consideration of *in vitro* immunotoxicity tests

Although the above decision tree approach can classify chemicals into those with immunotoxic effects and those without, it seems to be oversimplified. Recent advances in immunosuppressive or immunomodulatory drugs have shown that drugs that affect the immune system can be classified into at least two categories (Isaacs and Burmester 2020; Nelson and Ballow 2003). One comprises immunosuppressants that globally impair the host immune response, typically in a dose-dependent fashion, and are characterized by a low therapeutic index (narrow window between the therapeutic and toxic range) and significant intra- and inter-individual pharmacokinetic variability. The second comprises immunomodulators that act more selectively by targeting only specific portions of the immune system and therefore pose a lower risk of complications related to immune dysfunction, as well as having a wider therapeutic index, a greater safety margin, more predictable pharmacokinetic properties, and less inter-individual variability. The major classes of immunosuppressants are mostly used in transplantation and are classified into glucocorticoids, calcineurin inhibitors such as cyclosporine and tacrolimus, antiproliferative/antimetabolic agents such as azathioprine, mycophnolate

mefetil, sirolimus and everolimus, and biologics such as belatecept, alemtuzumab, muromonab-CD3, daclizumab and basiliximab. On the other hand, the immunomodulators comprise various monoclonal antibodies, such as antibodies against IL-1, IL-4, IL-6, IL-12, IL-17, IL-23 and IL-31.

Taking the classification of the drugs into account, it is reasonable that *in vitro* immunotoxicity tests are also classified as assays to detect chemicals that give global immunosuppression, designated as global immunotoxicity tests, or as assays to detect chemicals that affect a part of the immune response, designated as specific immunotoxicity tests (Fig. 3). The former typically includes myelotoxicity tests, lymphocyte toxicity tests, T cell-dependent antibody response (TDAR), the human whole blood cell cytokine release assay and the IL-2 Luc assay, while the latter includes assays to examine the effects of chemicals on NK cell activity, DC activity, or cytokine or chemokine production.

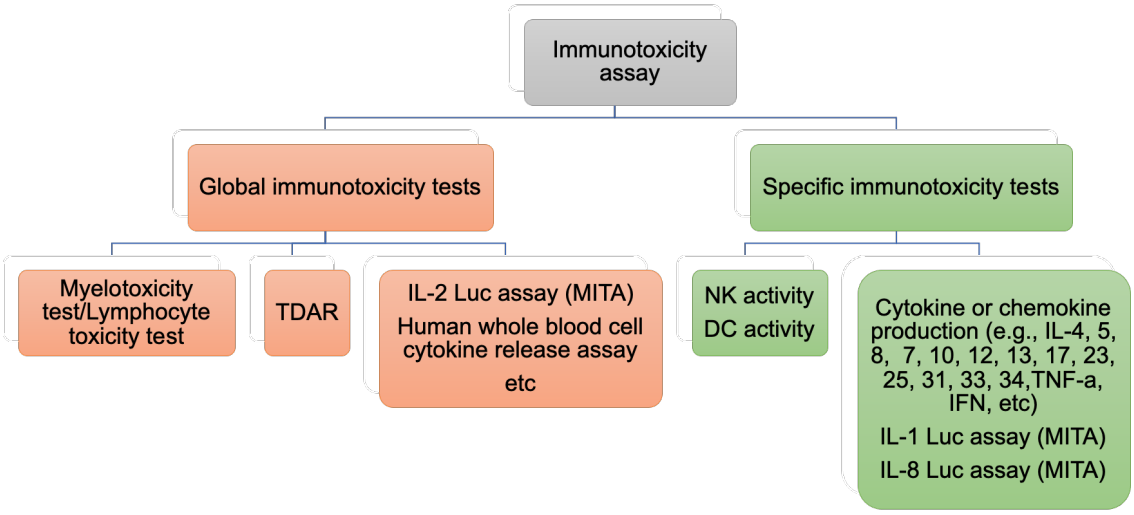

Fig. 3. Classification of immunotoxicity tests in vitro

3-5. Predictivity of *in vitro* immunotoxicity tests

A crucial step in developing an *in vitro* immunotoxicity test involves determining its predictivity. Determining the predictability of tests requires reference data that list the chemicals used to make positive or negative decisions based on results obtained by

gold standard analysis or data obtained from the literature. The reference data for the global immunotoxicity tests and specific immunotoxicity tests should be different.

As suggested by non-animal tests for skin sensitization, determining the predictivity of global immunotoxicity tests cannot be dependent on a single non-animal alternative assay; rather, it is necessary to develop integrated approaches to testing and assessment (IATA) using combinations of assays representing different KEs of the AOP. Moreover, although skin sensitization can be explained by a single AOP, immunotoxicity may be represented by multiple AOPs. Therefore, the predictivity of the *in vitro* test depends on the percentage of chemicals that affect the relevant AOP among the total examined chemicals, making it difficult to determine the goal of predictivity of *in vitro* immunotoxicity tests for global immunotoxicity in the validation study.

3-6. Multi-ImmunoTox Assay (MITA)

Our group developed a high-throughput screening system to evaluate chemical immunotoxicity. We first established three stable reporter cell lines transfected with luciferase genes under control of the IL-2, IFN-γ, IL-8, and IL-1β promoters: 2H4 cells derived from Jurkat cells containing stable luciferase green (SLG) regulated by the IL-2 promoter, stable luciferase orange (SLO) regulated by the IFN-γ promoter, and stable luciferase red (SLR) regulated by the G3PDH promoter (Saito et al. 2011): THP-G8 cells derived from THP-1 cells containing SLO regulated by the IL-8 promoter, SLR regulated by the G3PDH promoter (Takahashi et al. 2011), THP-G1b cells derived from THP-1 cells containing SLG regulated by the IL-1β promoter, and SLR regulated by the G3PDH promoter (Kimura et al. 2014). These four cytokines were selected because IL-2 and IFN-γ are primarily produced by T cells (adaptive immune cells), whereas IL-8 and IL-1β are primarily produced by monocytes and dendritic cells (innate immune cells). Using these three cell lines, we established the Multi-ImmunoTox Assay (MITA). This assay identifies the effects of chemicals on IL-2 luciferase activity (IL2LA) and IFN-γ luciferase activity (IFNLA) in 2H4 cells in the presence of the stimulants phorbol 12-myristate 13-acetate (PMA) and ionomycin (Io), and on IL-1

luciferase activity (IL1LA) and IL-8 luciferase activity (IL8LA) in THP-G1b and THP-G8 cells, respectively, in the presence of the stimulant lipopolysaccharide (LPS) (Fig. 4).

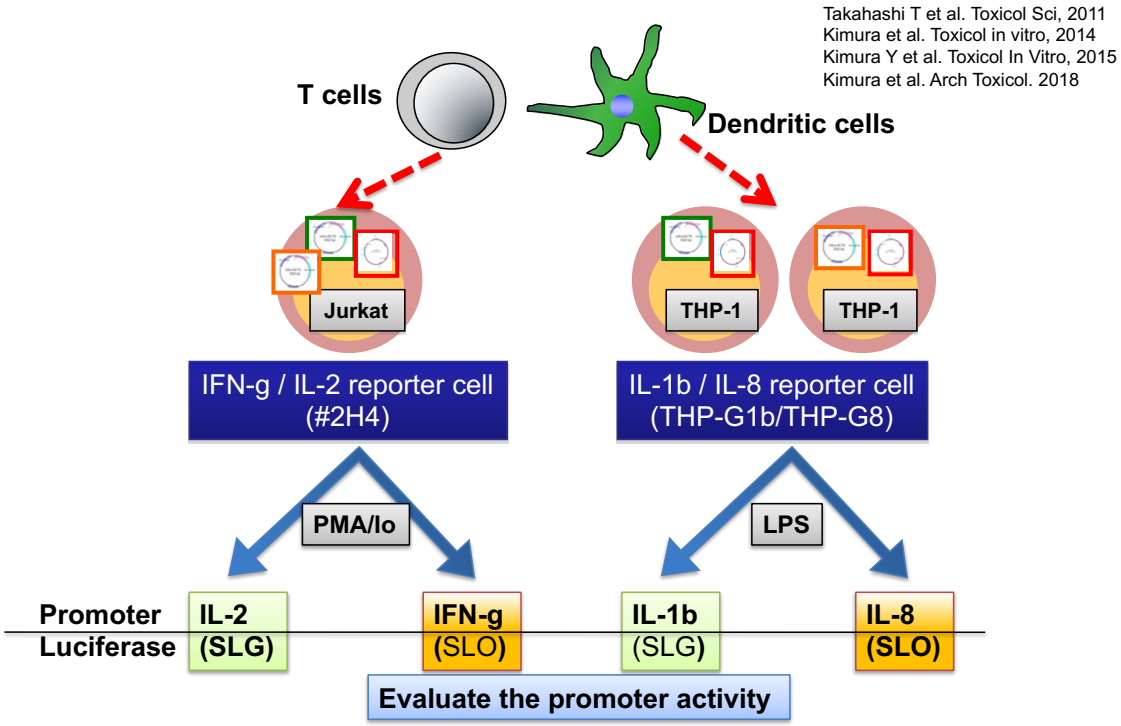

Fig. 4 The Multi-ImmunoTox Assay (MITA)

After establishing the MITA, we first compared the effects of dexamethasone (Dex), cyclosporine (CyA) and tacrolimus (Tac) on the mRNA expression of the three MITA cell lines, and on wild type cell lines or in human whole-blood cells stimulated with PMA/Io or LPS. The results confirmed that the MITA correctly reflects changes in mRNA expression in the mother cell lines and whole-blood cells (Kimura et al. 2014).

3-7. MITA evaluation of immunotoxicity profiles of well-known immunosuppressive drugs

We next evaluated the performance of the MITA by examining immunosuppressive or immunomodulatory drugs with well-known clinical effects on the human immune system (Kimura et al. 2014). The results obtained with

immunosuppressive drugs classified by their principal mechanism of action are shown in Table 1. Drug classifications are based on a review by Allison (Allison 2000).

The MITA demonstrated that Dex significantly suppressed IL-2, IL-1β and IL-8 reporter activity, while CyA and Tac suppressed IL-2 and IFN-γ reporter activity but had no effect on IL-1β and IL-8 reporter activity. However, the MITA could not detect the immunosuppressive effects of the alkylating agent cyclophosphamide, of the inhibitors of *de novo* purine synthesis azathioprine (AZ), mycophenolic acid (MPA) and mizoribine (MZR), or of the inhibitor of pyrimidine and purine synthesis, methotrexate (MT). These data suggest that the MITA correctly evaluates the effects of chemicals on cytokine expression but cannot detect immunotoxicity associated with the inhibition of DNA synthesis and cell division. This drawback has also been reported for other assays, such as the HWBCRA (Langezaal et al. 2002) and the FCP assay (Wagner et al. 2006). On the other hand, the MITA has the advantage that it can discriminate the effects of chemicals on T cells from those on macrophages/dendritic cells.

Table 1. The MITA can detect immunosuppressive effects of representative immunosuppressive drugs

Principal mechanism of action	Drugs	The effects of transcriptional activity			
		IL-2	IFN-γ	IL-1β	IL-8
Immunosuppressing drugs					
Regulation of gene expression	Dexamethasone (Dex)	S	N	S	S
Kinase and phosphatase inhibitors	Cyclosporin A (CyA)	S	S	N	N
	Tacrolimus (Tac)	S	S	N	N
	Rapamycin (RPM)	A	N	N	N
Alkylation	Cyclophosphamide (CP)	N	N	N	N
Inhibition of de novo purine synthesis	Azathioprine (AZ)	N	N	N	N
	Mycophenolic acid (MPA)	A	A	N	N
	Mizoribine (MZR)	N	N	A	A
Inhibition of pyrimidine and purine synthesis	Methotrexate (MTX)	N	A	N	N
Off-label immunosuppressing drugs					
	Sulfasalazine (SASP)	S	S	S	S
	Colchicine	S	N	A	N
	Chloroquine (CQ)	S	N	N	N
	Minocycline (MC)	S	S	N	N
	Nicotinamide (NA)	S	N	S	S
Non-immunomodulatory drugs					
	Acetaminophen (AA)	N	N	N	N
	Digoxin	S	S	N	N
	Warfarin	N	N	S	S

Kimura et al. Toxicol in Vitro 28: 759-769, 2014

*S and A indicate drugs that showed statistically significant suppression or augmentation in triplicate experiments for each parameter, while N indicates drugs that did not show significant effects.

3-8. Process of validation of MITA and purpose of current validation study

Our final goal is to officially validate the MITA for within- and between-laboratory reproducibility and predictivity. As the initial step, we completed a validation study on the IL-2 Luc assay that evaluated the effects of chemicals on IL2LA in the presence of PMA/Io (Kimura et al. 2020), and the validation report is now under peer review by the OECD. In the current study, we conducted a validation study for the IL-1

Luc assay as the second step. The IL-1 Luc assay is an *in vitro* immunotoxicity test that examines the effects of chemicals on IL1LA in the presence of LPS. Thus, the IL-1 Luc assay is likely to detect chemicals that affect the signaling cascade from TLR4 to the transcription of IL-1β mRNA.

3-9. Significance of assay for detecting effects of chemicals on IL-1β mRNA expression by monocytes

3-9-1. Regulation of IL-1β production

Molecules such as nuclear or mitochondrial DNA, adenosine triphosphate (ATP), uridine triphosphate (UTP), uric acid and high mobility group box 1 (HMGB1) are classified as damage-associated molecular patterns (DAMPs). DAMPs are secreted or produced upon cellular injury or death and induce sterile inflammation. On the other hand, bacterial products like LPS, peptidoglycans, lipoprotein flagellins, and bacterial RNA and DNA are well-characterized pathogen-associated molecular patterns (PAMPs). These DAMPs and PAMPs, with a few exceptions, bind to pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs). Proinflammatory mediators such as DAMPs, PAMPs and various inflammatory cytokines or mediators, including IL-1β itself, activate innate immune mechanisms in the host, leading to IL-1β production (Handa et al. 2016; Newton and Dixit 2012; Yang et al. 2017).

Transcriptional and post-transcriptional level regulation by the RNA-binding protein pro-IL-1β requires proteolytic cleavage by active caspase-1 as the effector component of stimulation-induced multi-protein inflammasomes to acquire functional activity. These different layers of regulation allow fine tuning of IL-1β production under different pathophysiological conditions (Bent et al. 2018).

3-9-2 Role of IL-1β in pathophysiological conditions

The crucial role of IL-1 has been demonstrated by several experiments using IL-1α- or IL-1β-deficient, both IL-1α- and IL-1β-deficient, or IL-1 receptor 1- (IL-1R1-)

deficient mice. IL-1β is essential for antigen-specific T cell activation and the induction of delayed-type hypersensitivity (Nambu et al. 2006). IL-1α/β deficiency impairs T cell-dependent antibody production through the induction of CD40 ligands and OX40 on T cells (Nakae et al. 2001). IL-1 α/β-deficient or IL-1R-deficient mice have a decreased ability to control infection by *Pseudomonas aeruginosa* (Horino et al. 2009), *Listeria monocytogenes* (Labow et al. 1997), *Leishmania major* (Satoskar et al. 1998), and *Mycobacterium tuberculosis* (Juffermans et al. 2000).

The more precise role of IL-1 in inflammation or immune response has been reviewed (Bent et al. 2018). Briefly, IL-1β is a potent stimulator of antigen-presenting cells (APCs), and can induce maturation of Langerhans cells in the epidermis. IL-1β can also promote the differentiation of monocytes to conventional DCs. IL-1β generated by activated APC induces type 1 immune responses. However, IL-1β also induces IL-4 receptor expression on CD4+ T cells, which is necessary for the maintenance of Th2 cells. In combination with IL-6 and IL-23, IL-1β favors the differentiation of CD4+ T cells towards Th17. On the other hand, IL-1β counteracts TGF-β-induced Foxp3 expression in CD4+ T cells, thereby inhibiting the differentiation of regulatory T cells (Treg). Moreover, IL-1β induces alternative splicing of Foxp3 in Treg, resulting in a functional switch towards Th17. IL-1β not only promotes Th17 polarization via IL1R signaling, but also supports APC-induced Th17 production by CD4+ memory T cells.

IL-1β also supports the proliferation of activated B cells and their differentiation into plasma cells. IL-1β in combination with IL-2 promotes the expansion of NK cells, as well as of CD4+ CD8+ T cells. IL-1β induced in the course of acute inflammation promotes the upregulation of adhesion receptors on immune and endothelial cells as a prerequisite for the infiltration of leukocytes to sites of infection.

In addition to the beneficial role of IL-1β for clearing infections, this cytokine contributes to the severity of several inflammatory diseases and mediates autoinflammatory disorders, such as cryopyrin-associated periodic syndrome, neonatal-onset multisystem inflammatory disease, and familial Mediterranean fever.

3-9-3. Signaling cascade leading to IL-1β mRNA expression after LPS stimulation

The IL-1 Luc assay involves stimulation by LPS, with the binding of LPS to TLR4 and co-receptor MD2 triggering interactions between the cytoplasmic TIR domain of TLR4 and TIR-containing adaptor proteins (Mal, MyD88, and TRAM). MyD88 binds IRAK4, utilizing the kinase activity of IRAK4 to bind the kinases IRAK1 and IRAK2 sequentially. The MyD88–IRAK complex also engages the ubiquitin ligase TRAF6 to generate polyubiquitin chains that activate the IKK complex for NF-κB - and ERK-dependent gene transcription. Ubiquitin ligases cIAP1 and cIAP2 recruited to the TLR4 signaling complex regulate translocation of a subset of signaling components to the cytoplasm, where TAK1 activation initiates a MAPK cascade to activate p38a and JNK, thereby stimulating gene expression. TLR4 activated at the plasma membrane is endocytosed but can signal within the endosomal compartment via the adaptors TRAM and TRIF. The kinase and ubiquitin ligase combination of RIP1 and Peli1 interacts with TRIF to signal NF-κB activation, whereas TBK1 and TRAF3 stimulate IRF3-dependent transcription. These signaling cascades activate nuclear factor-κB (NF-κB), activator protein-1 (AP-1), cAMP responsive element binding protein (CREB)/activating transcription factor (ATF), CCAAT-enhancer-binding protein β *(*c/EBP β) and interferon regulatory factor 3 (IRF3). These transcription factors induce the expression of various inflammatory cytokines, such as IL-1β, TNFα, IL-6, and several chemokines (reviewed by Newton and Dixit (Newton and Dixit 2012)).

On the other hand, the activation of NLRs promotes assembly of inflammasome multiprotein complexes, consisting of NLR family CARD domain-containing proteins, NLRPs, adaptor proteins such as the apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), and serine protease caspase 1(Casp1), as well as activing NF-κB (Newton and Dixit 2012). Casp1 is cleaved and activated, further inducing interleukin-1β and IL-18 maturation, which contributes to inflammation.

3-9-4. Suppression of IL-1β production by chemicals

Decreased IL-1 production by macrophages can be induced by suppressed IL-1β

mRNA induction or suppressed maturation of pro-IL-1β, leading to decreased IL-1β secretion. Dexamethasone is a representative drug that significantly suppresses IL-1β production by monocytes (Finch-Arietta and Cochran 1991). As mentioned earlier, the binding of LPS to TLR4 activates the transcription factors NF-kB, CREB/ATF, c/EBPb, AP1 and IRF3. Therefore, chemicals that affect the signaling pathway leading to the activation of these transcription factors are likely to suppress IL-1β production, and chemicals that affect NF-κB signaling have been investigated thoroughly. Numerous compounds have been reported to inhibit NF-κB signaling via several different mechanisms, as reviewed by (Fuchs 2010). A list of representative chemicals and their mechanisms for inhibition of NF-κB is shown in Table 2. Dimethyl fumarate inhibits the activation of NF-κB, resulting in a loss of proinflammatory cytokine production, distorted maturation and function of antigen-presenting cells, and immune deviation of T helper cells (Th) from the type 1 (Th1) and type 17 (Th17) profiles to a type 2 (Th2) phenotype (McGuire et al. 2016; Peng et al. 2012). Several studies have shown intriguing pharmacologic effects associated with curcumin, which inhibits NF-κB expression by regulating the NF-κB/IκB pathway and down-regulating the expression of pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, IL-8, and tumor necrosis factor (TNF)-α (Wang et al. 2018). Iguratimod, a methanesulfonanilide novel disease-modifying antirheumatic drug, inhibits the nuclear transcription factor NF-κB but not its inhibitor, IκBα (Mucke 2012). Epigaloocatechin gallate (EGCG) has been reported to inhibit NF-κB activation by inhibiting p65 phosphorylation (Wheeler et al. 2004).

Although the binding of LPS to TLR4 activates several transcription factors that can be stimulated by various means other than TLR4 stimulation, there are also signaling molecules that are specific to TLR4 signaling, such as TLR4, Mal, TRAM, Myd88, IRAK4 and IRAK1/2 (Vallabhapurapu and Karin 2009). Several chemicals target some of these molecules, including the TLR4 inhibitor TAK-242 (Matsunaga et al. 2011) and various IRAK4 inhibitors (Lee et al. 2017). IRAK4 has recently attracted widespread attention as a therapeutic target for inflammation and tumor diseases.

Minocycline is orally adsorbed, as are the two prodrugs pralnacasan (VX-740) and belnacasan (VX-765), which are converted into the active compounds VRT-018858 and VRT-043198, respectively (Fenini et al. 2017). All three compounds suppress IL-1 signaling by inhibiting caspase-1 activation. Caspase-1 is an essential enzyme for the maturation of pro-IL-1β and the secretion of mature IL-1β (Vincent and Mohr 2007). It was recently reported that cinnamicaldehyde suppresses serum IL-1β levels in endotoxin poisoned mice (Xu et al. 2017), suggesting that both chemicals and drugs can suppress IL-1 signaling through their inhibitory effects on IL-1β production.

Table 2. List of compounds that inhibit NF-κB signaling (modified from Fuchs 2010)

Target and Function	Compound
AKT/NF-κB inhibitor	AT514 (a cyclic depsipeptide), Xanthohumol
Antiinflammatory and prostaglandin synthase inhibition	Etodolac (SDX-101)
Caspase activation, poly (ADP-ribose) polymerase cleavage and apoptosis	SDX-308 (CEP-180802)
IκB kinase inhibitor	BMS-345541, MLN120B (b-carboline derivative), PS-1145 (b-carboline derivative)
IκB kinase inhibitor, inhibitor of NF-κB expression on both, the protein and mRNA level	Celastrol
IKKβ inhibitor, inhibitor of NF-κB nuclear translocation and induction of apoptosis	AS602868 (anilino-pyrimidine derivative)
IKKα inhibitor	Flavopiridol
Inhibition of RelA binding to DNA	LC-1 (dimethylaminoparthenolide, DMAPT)
Inhibitor of both canonical and non-canonical NF-κB activating pathways at the level of nuclear translocation	DHMEQ
Inhibitor of NF-κB activation, induces G1/S arrest and induces apoptosis	Curcumin
Inhibitor of NF-κB binding to DNA	Epicatechin
Inhibitor of p50 binding to DNA	Kamebakaurin
Mitochondrial dysfunction and apoptosis	Bay117082
NF-κB nuclear translocation inhibitor	SN50 (cell-permeable inhibitor peptide)
Proteasome inhibition, stabilization of IκB	Bortezomib, Carfilzomib (PR-171), CEP-18770
Proteasome inhibition, stabilization of IκB, mitochondrial dysfunction and apoptosis	MG132 (peptidyl aldehyde of tri-leucine), Salinosporamide A (NPI-0052 or ML858)
Reduction of IκBα mRNA levels and decrease in phosphorylated IκBα	4-hydroxy-2-nonenal
ROS generation,caspase activation and apoptosis	Triptolide
Upregulation of A20, downregulation of IKKa and inhibition of p65 nuclear translocation	Berbamine

4. Test method and modification

4-1. IL-1 reporter cell line THP-G1b

A THP-1-derived IL-1β reporter cell line, THP-G1b, harbors the SLG and SLR luciferase genes under the control of the IL-1β and G3PDH promoters, respectively, and was established by the Department of Dermatology, Tohoku University School of Medicine, and GPC Laboratory Co. Ltd. (Kimura et al. 2018). To establish this line, we used THP-1 cells containing stable luciferase red (SLR) regulated by the G3PDH promoter. The IL-1β reporter cassette containing stable luciferase green (SLG) under the IL-1β promoter was transfected into a gene-loading site of a human artificial chromosome (HAC) vector in CHO cells. We transfected the HAC vector into the THP-1-derived cell line containing SLR regulated by the G3PDH promoter from CHO cells using a microcell-mediated chromosome transfer technique to obtain the THP-G1b cell line (Kimura et al. 2018).

4-2. Chemical treatment of THP-G1b cells and measurement of luciferase activity

Based on a previous report (Kimura et al. 2018), THP-G1b cells (1×10^5 cells/50 μL/well) in a 96-well black-frame and white-well plate were pretreated with different concentrations of individual chemicals for 1 h. Next, THP-G1b cells were stimulated with 100 ng/mL of LPS for 6 h. Two luciferase activities (SLG luciferase activity (SLG-LA) and SLR luciferase activity (SLR-LA)) were simultaneously determined using a microplate-type luminometer with a multi-color detection system (e.g., Phelios AB-2350 (ATTO), ARVO (PerkinElmer), Tristar LB941 (Berthold)) and Tripluc Luciferase Assay Reagent (TOYOBO Co., Ltd., Osaka, Japan) according to the manufacturers' instructions. Using these cell lines, we obtained SLG-LA driven by the IL-1β promoter (IL1LA) and SLR-LA driven by G3PDH promoter (GAPLA) in THP-G1b cells. We accounted for the variation in cell number and cell viability after chemical treatment by normalizing the data for IL1LA (nIL1LA) by dividing IL1LA with GAPLA in the THP-G1b cells. In addition, we calculated suppression (%), augmentation (%) and Inh-GAPLA as follows:
Suppression (%) = (1 − (nIL1LA of THP-G1b cells treated with drugs/nIL1LA of non-treated THP-G1b cells)) × 100

Augmentation (%) = ((nIL1LA of THP-G1b cells treated with drugs/nIL1LA of non-treated THP-G1b cells) − 1) × 100
Inh-GAPLA = GAPLA of THP-G1b cells treated with chemicals/GAPLA of untreated THP-G1b cells
Definitions of these terms are provided in Table 3.

Table 3. Definition of the parameters in the IL-1 Luc assay.

Abbreviations	Definition
IL-1 Luc assay	IL-1 luciferase assay
GAPLA	SLR luciferase activity reflecting GAPDH promoter activity
IL1LA	SLG luciferase activity reflecting IL-1 promoter activity of THP-G1b cells
nIL1LA	IL1LA/GAPLA of THP-G1b cells
Suppression (%)	(1 − (nIL1LA of THP-G1b cells treated with drugs/nIL1LA of non-treated THP-G1b cells)) x 100
Augmentation (%)	((nIL1LA of THP-G1b cells treated with drugs/nIL1LA of non-treated THP-G1b cells) − 1) x 100
CV05	The lowest concentration of the chemical at which Inh-GAPLA becomes < 0.05.
Inh-GAPLA	GAPLA of THP-G1b cells treated with chemicals/GAPLA of untreated THP-G1b cells.

4-3. Criteria to determine effects of chemicals on monocyte/dendritic cells

During the validation study, we modified the criteria to determine the effects of chemicals on monocyte/dendritic cells. Considering the criteria used in the IL-1 Luc assay described in Multi-ImmunoTox Assay protocol Ver.011E, we set the acceptance

criteria and criteria for the Phase I study of the IL-1 Luc assay as follows (Multi-ImmunoTox Assay protocol for THP-G1b ver. 008E):

Acceptance criteria
If the fold induction of nIL1LA in LPS wells without chemicals (= (nIL1LA of THP-G1b cells treated with LPS)/(nIL1LA of non-treated THP-G1b cells)) is less than 5, the results obtained from the plate containing the control wells should be rejected.

Criteria
The experiments are repeated until two consistent positive (negative) results or two consistent "no effect results" are obtained. When two consistent results are obtained, the chemicals are judged as the obtained consistent results.
An immunotoxicant is identified by the mean of Suppression (%) and its 95% confidence interval.

In each experiment, when a chemical clears the following three criteria, it is judged as suppressive or stimulatory. Otherwise, it is judged as a 'no effect' chemical.
1. The mean of Suppression (%) is $\geq$ 20 (suppressive) or $\leq$ -20 (stimulatory) with statistical significance. The statistical significance is judged by its 95% confidence interval.
2. The result shows two or more consecutive statistically significant positive (negative) data points or one statistically significant positive (negative) data point with a trend in which at least three consecutive data points increase (decrease) in a dose-dependent manner. In the latter case, the trend can cross 0, as long as only one data point shows the opposite effect without statistical significance.
3. The results are judged using only data obtained at the concentration at which Inh-GAPLA is $\geq$ 0.05.

After the Phase 1 study, the criteria were changed to improve the performance of the assay. The following are the final acceptance criteria and the criteria for judgment accepted by the internal expert members (Multi-ImmunoTox Assay protocol for THP-G1b ver. 009E).

Acceptance criteria

- If fold induction for nIL1LA in LPS wells without chemicals (= (nIL1LA of THP-G1b cells treated with LPS)/(nIL1LA of non-treated THP-G1b cells)) is less than 3, the results obtained from the plate containing the control wells should be rejected.
- If the number of concentrations which satisfy Inh-GAPLA $\geq$ 0.05 is less than 6, the experiment is accepted only when viable wells satisfy the following positive criteria. In this case, the following experiments should be conducted using the concentration described in Section 5-1 of Multi-Immuno Tox Assay protocol for THP-G1b ver. 009E (Appendix 6).

Criteria

The experiments are repeated until two consistent positive results or two consistent "non-suppression" results are obtained. When two consistent results are obtained, the chemical is judged as per the obtained consistent results.

An immunotoxicant is identified by the mean of Suppression (%) and its 95% confidence interval.

In each experiment, when a chemical clears the following four criteria, it is judged as being a suppressant. Otherwise, it is judged as a non-suppressant.

1. Suppression (%) is $\geq$ 25 with statistical significance. The statistical significance is judged when the 95% confidence interval does not include 0.
2. The result shows two or more consecutive statistically significant positive data points or one statistically significant positive data point with a trend in which at least three consecutive data points increase in a dose-dependent manner. In the latter case, the trend can cross 0, as long as only one data point shows the opposite effect without statistical significance.
3. The results are judged using only data obtained at the concentration at which Inh-GAPLA is $\geq$ 0.05
4. The results at 2000 μg/mL are excluded.

4-4. Bioluminescence system

In a typical dual-reporter assay, firefly luciferase from *Photinus pyralis* (FLuc) is used as the experimental reporter and *Renilla* luciferase is used as the internal control reporter. This internal control reporter connects to a constitutively expressed promoter, such as a virus promoter (e.g., the herpes simplex virus thymidine kinase promoter, simian virus 40 promoter) or a housekeeping gene promoter (e.g., G3PDH, β-actin). This assay system has been commercialized as the Dual-Luciferase Reporter Assay System by Promega Corporation. In this system, both luciferase activities are measured sequentially from single extracts on the basis of their bioluminescent substrate specificity. Firefly luciferase activity is measured first by adding firefly D-luciferin, then *Renilla* luciferase activity is measured by adding coelenterazine (another name for *Renilla* luciferin), with concomitant quenching of firefly luciferase luminescence. Finally, firefly luciferase activity is normalized by *Renilla* luciferase activity as the promoter activity (Michelini et al. 2014; Nakajima and Ohmiya 2010; Roda et al. 2004).

An alternative chemical test using a cell-based assay requires the analysis of a large number of samples. It is preferable to use an improved assay system whereby gene expression can be monitored simultaneously. In the MITA, therefore, three kinds of beetle luciferases that emit either green, orange or red light with a single bioluminescent substrate, D-luciferin, are used. Multiple promoter activities are conventionally evaluated in a one-step reaction by combined use of a commercially available bioluminescent reagent (Tripluc Luciferase Assay Reagent, TOYOBO) and a microplate luminometer equipped optical filters (Nakajima et al. 2005, 2010).

In the IL-2 Luc assay, the triple-color assay system consists of a green-emitting luciferase (SLG; λmax = 550 nm) (Ohmiya et al. 2000; Nakajima et al. 2005) for monitoring IL-2 promoter activity, an orange-emitting luciferase (SLO; λmax = 580 nm) (Viviani et al. 2001; Nakajima et al. 2005) for monitoring IFN-γ promoter activity, and a red-emitting luciferase (SLR; λmax = 630 nm) (Viviani et al. 1999; Nakajima et al. 2005) for monitoring internal control promoter (G3PDH) activity. The three luciferases emit different colors upon reacting with firefly D-luciferin and their

luminescence is measured simultaneously in a one-step reaction by dividing the emission from the assay mixture, measured using optical filters (Nakajima et al. 2005). First, the total relative light units (F0) are measured in the absence of the filters. Then, the F1 and F2 values of light that passed through the R56 filter (>560-nm long-pass filter) or the R60 filter (>600-nm long-pass filter), respectively, are measured. The three luciferase activities are calculated using the simultaneous equation shown below by substituting the F0, F1 and F2 values. In this equation, G, O and R are the activities of the green-, orange- and red-emitting luciferases, respectively, κG_{R56}, κO_{R56} and κR_{R56} are the transmission coefficients for the green-, orange- and red-emitting luciferases of the R56 filter, respectively, and κG_{R60}, κO_{R60} and κR_{R60} are the transmission coefficients for the green-, orange- and red-emitting luciferases of the R60 filter, respectively. The transmission coefficients are simply estimated using purified recombinant luciferase enzymes (Niwa et al. 2010).

$$\begin{pmatrix} F0 \\ F1 \\ F2 \end{pmatrix} = \begin{pmatrix} 1 & 1 & 1 \\ \kappa G_{O56} & \kappa O_{O56} & \kappa R_{O56} \\ \kappa G_{R60} & \kappa O_{R60} & \kappa R_{R60} \end{pmatrix} \begin{pmatrix} G \\ O \\ R \end{pmatrix}$$

Luminescence activity is measured using a filtered 96-well microplate luminometer (for example, Phelios (ATTO, Tokyo, Japan), Tristan 941 (Berthold, Bad Wildbad, Germany), and the ARVO series (PerkinElmer, Waltham, MA).

5. Validation Management Structure

5-1. Validation Management Team (VMT)

Trial Coordinator:	Hajime Kojima (Japanese Center for the Validation of Alternative Methods (JaCVAM), National Institute of Health Sciences (NIHS), Kawasaki, Japan), VMT trial coordinator, Chemical supplier and Management of quality control
Lead laboratory:	Setsuya Aiba (Tohoku University, Miyagi, Japan), Developer of this assay, Test method, expertise underlying science Yutaka Kimura (Tohoku University, Miyagi, Japan)
International expert members	
EU liaison:	Emanuela Corsini (Milan Univ., Italy), Test system expertise, validation expertise, immunotoxicity expertise Erwin L. Roggen (3Rs Management and Consulting ApS, Denmark), Test system expertise, validation expertise, immunotoxicity expertise
ICCVAM liaison:	Dori Germolec (NTP/NIEHS, USA), Immunotoxicity expertise
JSIT liaison:	Tomoaki Inoue (Chugai Pharmaceutical Co., Ltd.), Immunotoxicity expertise
Data management team:	Takashi Omori (Kobe University, Kobe, Japan), Data analysis, biostatistics dossier
Chemical Selection Committee （CSC）	Setsuya Aiba (Tohoku University) Yutaka Kimura (Tohoku University Hajime Kojima (JaCVAM) Emanuela Corsini (Milan Univ) Erwin L. Roggen (3Rs Management and

	Consulting ApS) Dori Germolec (NTP/NIEHS) Tomoaki Inoue (Chugai Pharmaceutical Co., Ltd.)
Participating Test Facilities	Test Facility 1: Hatano Res. Inst., FDSC only for Phase 0 study, Study Director (SD): Kohji Yamakage Test Facility 2: AIST, Tsukuba, SD: Rie Yasuno Test Facility 3: AIST, Takamatsu, SD: Yoshihiro Nakajima Test Facility 4: Tohoku univ. (Phase I, II), SD: Chizu Fujimura

5-2. Management office

Hajime Kojima (JaCVAM)
3-25-26 Tonomachi Kawasaki, Kawasaki, 210-9501
TEL: +81-44-270-6600
h-kojima@nihs.go.jp

5-3. Meetings held

14/5/2018 (Web meeting)
Subjects: Preparation of IL-1β Luc assay procedure
VMT members: Yasuno, R., Yamakage, K., Watanabe, M., Kobayashi, M., Nakajima, Y., Iwaki, T., Aiba, S., Kimura, Y., Fujimura, C., Omori, T., Mashimo, N., Kojima, H.

28/6/2018 (Web meeting)
Subjects: Preparation of IL-1β Luc assay procedure
VMT members: Yasuno, R., Yamakage, K., Watanabe, M., Kobayashi, M., Nakajima, Y., Iwaki, T., Aiba, S., Kimura, Y., Fujimura, C., Omori, T., Kojima, H.

4-6/10/2018 (Kobe Univ., Kobe, Japan)

4th meeting for the MITA Validation study

Subjects: Result of Pre-Validation study of IL-1β Luc assay and validation plan for the Phase I study.

VMT members: Corsini, E., Roggen, E., Germolec, D., Inoue, T. Aiba, S., Kimura, Y., Yamakage, K., Watanabe, M., Yasuno, R., Nakajima, Y., Omori, T., Takagi, Y., Mashimo, N., Kado, Y., Kojima, H., Venti, S.

Participating laboratories: Tohoku University (Phase I, II), AIST(Tsukuba), FDSC (Phase 0), AIST(Takamatsu)

7/2/2019 (Web meeting)

Subjects: Result of Phase I study of IL-1β Luc assay and Proposal of the revised positive criteria

VMT members: Yasuno, R., Nakajima, Y., Aiba, S., Kimura, Y., Omori, T., Kojima, H.

5/4/2019 (Web meeting)

Subjects: Result of Phase I study of IL-1β Luc assay and Proposal of the revised positive criteria

VMT members: Aiba, S., Kimura, Y., Omori, T., Takagi, Y., Kojima, H.

26/6/2019 (Web meeting)

Subjects: Result of Phase I study of IL-1β Luc assay and Proposal of the revised positive criteria

VMT members: Corsini, E., Roggen, E., Germolec, D., Inoue, T., Aiba, S., Kimura, Y., Omori, T., Kojima, H.

30-31/1/2020 (National Institute of Health Sciences, Richmond Hotel Premier Musashikosugi, Kawasaki, Japan)

5th meeting for the MITA Validation study

Subjects: Validation results for the IL-1β Luc assay**,** Validation report for the IL-1 Luc assay

VMT members: Corsini, E., Germolec, D., Inoue, T. Aiba, S., Kimura, Y., M., Yasuno, R., Nakajima, Y., Omori, T., Mashimo, N., Okayama, K., Kojima, H., Venti, S.

Participating laboratories: Tohoku University, AIST(Tsukuba), AIST(Takamatsu)

6. Study Design (Appendix 1)

The aim of this phase is to (pre)validate the IL-1 Luc assay method to assess transferability and inter-laboratory variability so that this test can be used to screen for immunotoxic chemicals.

The validation study (Phase I and Phase II trials) was conducted by three laboratories, based on the study design and schedule shown in Tables 4 and 5 and using the test chemicals shown in Tables 6 and 7. The methods were described above in Section 4 and the precise protocol is described later in Section 8.

Table 4. Number of chemicals analyzed in validation study

Studies	Within-Laboratory	Between-laboratories	Predictivity
I	5	5	5
II		20	20
Total	5	25	25

7. Test Chemicals

The selection process for the test chemicals for the IL-1β Luc assay validation study is described below.

In addition, the chemical categories or physical state and chemical properties (e.g., solid, liquid, etc.) are included in the tables of these test chemicals in order to investigate the applicable domain.

Table 5. Breakdown of the IL-1 Luc assay validation study

Study	Number of test compounds	Number of repetitions	Information obtained	Experiment date
Phase 0	5 non-coded	1	Between-lab transferability	July, 2018
Phase I	5 coded	3	Within & between-lab reproducibility	November, 2018
Phase II	20 coded	1	Between-lab reproducibility & predictability	July, 2019

7-1. Basic rules for chemical selection

The selection of test chemicals by the CSC in the VMT was based on published papers on *in vivo* immunotoxicity tests and validation studies for *in vitro* alternative assays on immunotoxicity test methods.

7-1-1. Applied selection criteria

- information on mode/site of action
- coverage of a range of relevant chemical classes and product classes
- quality and quantity of reference data (*in vivo* and *in vitro*)
- high-quality data derived from animal and (if available) human studies
- information on interspecies variations (for example: variability with regard to the uptake of chemicals, metabolism, etc.)
- coverage of a range of toxic effects/potencies
- chemicals that do not require metabolic activation
- appropriate negative and positive controls
- physical and chemical properties (feasibility of use in the experimental set-up as implied by the CAS No.)
- single chemical entities or formulations of known high purity
- availability
- cost

In the first phase of the selection procedure, the CSC identified and collected several existing lists of potential chemical immunotoxicants, such as NTP IMMUNOTOX, and an EPA candidate list. An extensive literature search was performed by the CSC in order to ensure that all the pre-selected chemicals fulfilled the selection criteria described above. In addition, it was decided that at least 20% of the total chemicals to be tested should provide negative results (i.e., not immunotoxic) in order to increase the statistical power of the data analysis.

7-1-2. Chemical acquisition, coding and distribution

Laboratory transferability, and within- and between-laboratory reproducibility and predictivity, in all test facilities were assessed using coded chemicals. Coding was supervised by JaCVAM (Appendixes 2-1 and 2-2), in collaboration with the CSC. The CSC was responsible for coding and distributing the test chemicals, references, and controls for the validation study.

7-1-3. Handling

The chemical master at each test facility received complete information considered essential regarding the test chemicals (physical state, weight or volume of sample, specific density for liquid test chemicals, and storage instructions) by JaCVAM. Moreover, the test facility chemical master stored each chemical under conditions in accordance with the storage instructions and received sealed safety information such as the Material Safety Data Sheet (MSDS) describing hazard identification and exposure control/personal protection for each chemical. The test chemicals were delivered directly to the study director and the study director was not shown the MSDSs. The study director was to refer to the MSDSs only in the event of an accident. If the study director referred to the MSDS, he/she was not to reveal the content of the MSDS to the test facility technicians.

No accidents occurred during the course of the validation study, and all test facilities will return the MSDSs for the test chemicals to JaCVAM in a sealed envelope upon completion of the validation study. All test chemicals will be disposed of in compliance with the rules and regulations of the test facilities upon completion of the validation study.

7-2. Pre-validation study

Transferability of this assay was checked using five non-coded chemicals (2-aminoanthracene, citral, chloroquine diphosphate salt, dexamethasone and methylmercury(II) chloride) (Appendix 3) in four test facilities, including the lead laboratory. These chemicals were selected by the CSC.

7-3. Validation study - Phase I trial

Within- and between-laboratory reproducibility of this assay was checked using five coded chemicals in three test facilities, as shown in Table 6 (Appendix 4). These chemicals were selected by the CSC based on the in-house dataset of the lead

laboratory. The chemicals were coded by JaCVAM as shown in Table 6 and distributed to the test facilities.

Table 6. Chemical code list on the phase I validation trial for IL-1 Luc assay

No.	Chemical	CASRN	MW	Supplier	Catalog No.	Content	Physical characteristics	Lot	Storage	Purity	LabA	LabB	LabC	LabD
											TOHOKU unv.	AIST-TSUKUBA	FDSC	AIST-SHIKOKU
1	Dibutyl phthalate	84-74-2	278.34	Wako	021-06936	500mL	Liquid	TLN0112	RT	98.0+% (Capillary GC)	MIA003A	MIB014A	MIC027A	MID036A
											MIA004B	MIB017B	MIC026B	MID033B
											MIA007C	MIB016C	MIC023C	MID034C
2	Hydrocortisone (for Cell Culture)	50-23-7	362.46	Wako	080-10194	50g	Solid	SAH3714	RT	97% (HPLC)	MIA005A	MIB017A	MIC029A	MID038A
											MIA007B	MIB019B	MIC028B	MID035B
											MIA009C	MIB018C	MIC025C	MID037C
3	Lead(II) acetate trihydrate (Deleterious substances)	6080-56-4	379.33	Sigma-Aldrich	316512-100G	100g	Solid	09901TS	RT	99.999% trace metals basis	MIA007A	MIB018A	MIC021A	MID310A
											MIA008B	MIB011B	MIC210B	MID037B
											MIA001C	MIB110C	MIC027C	MID038C
4	Zinc dimethyldithiocarbamate (DMDTC)	137-30-4	305.82	Kanto Chemical	48028-31	25g x2	Solid	403N2204	RT	>95.0% (T)	MIA009A	MIB110A	MIC023A	MID037A
											MIA010B	MIB013B	MIC027B	MID039B
											MIA003C	MIB017C	MIC029C	MID310C
5	Nickel (II) sulfate hexahydrate	10101-97-0	262.85	Wako	146-01171	100g	Solid	LKQ2263	RT	99.0-102.0% (as NiSO4・6H2O) (Titration)	MIA001A	MIB012A	MIC025A	MID034A
											MIA002B	MIB015B	MIC024B	MID031B
											MIA005C	MIB014C	MIC021C	MID032C

7-4. Validation study -Phase II trial

Twenty test chemicals were selected by CSC for between-laboratory reproducibility as shown in Table 7 (Appendix 5). The chemicals were coded by JaCVAM as shown in Table 7 and distributed to the test facilities.

Table 7. Chemical code list on the phase II validation trial for IL-1 Luc assay

No.	Chemical name	CAS No.	LabA Tohoku	LabB Tsukuba	LabC Shikoku	Remark	Storage	Physicality	Supplier	Lot	Product code
1	Cadmium chloride	10108-64-2	MTA117	MTB221	MTC305	D	R	Solid	Wako	PEE3332	032-00122
2	5,5-Diphenylhydantoin sodium salt	630-93-3	MTA105	MTB220	MTC301		R	Solid	SIGMA-ALDRICH	BCBV6645	D4505
3	Indomethacin	53-86-1	MTA120	MTB203	MTC318		R	Solid	SIGMA-ALDRICH	122K0718	17378
4	Pentachlorophenol	87-86-5	MTA115	MTB211	MTC307		R	Solid	TCI	AK01-KGRC	P0033
5	Urethane	51-79-6	MTA111	MTB224	MTC302		R	Solid	SIGMA-ALDRICH	WXBC3505V	U2500
6	Tributyltin chloride	1461-22-9	MTA112	MTB208	MTC312	D	R	Liquid	SIGMA-ALDRICH	STBH8190	T50202
7	Perfluorooctanois acid	335-67-1	MTA125	MTB214	MTC303		R	Solid	TCI	ODJ8C-DL	P0764
8	Hydroquinone	123-31-9	MTA110	MTB218	MTC322		R	Solid	Wako	CDH5977	085-01212
9	4-Aminophenyl sulfone	80-08-0	MTA124	MTB217	MTC313		R	Solid	SIGMA-ALDRICH	MKBG7137V	A74807-100G
10	Ethanol	64-17-5	MTA102	MTB206	MTC317		R	Liquid	Wako	KWJ3722	053-06531
11	5-Nitro-2-furaldehyde semicarbazone	59-87-0	MTA121	MTB205	MTC324		R	Solid	SIGMA-ALDRICH	BCBG1878V	73340-100G
12	Trichloroethylene	79-01-6	MTA116	MTB223	MTC309		R	Liquid	Wako	KPF6884	209-18565
13	Zinc dimethyldithiocarbamate	137-30-4	MTA118	MTB202	MTC316		R	Solid	Cica	403N2204	48028-31
14	Citral	5392-40-5	MTA108	MTB204	MTC315		R	Liquid	Wako	TSK3117	032-05982
15	t-Buthlhydroquinone	1948-33-0	MTA113	MTB219	MTC323		R	Solid	Wako	CDH6008	027-07212
16	Bisphenol A	80-05-7	MTA107	MTB222	MTC314		R	Solid	SIGMA-ALDRICH	MKCD7508	239658
17	2,6-Di-tert-butyl-4-methylphenol	128-37-0	MTA119	MTB201	MTC306		R	Solid	SIGMA-ALDRICH	BCCB4438	B1378
18	Nonylphenol	84852-15-3	MTA104	MTB210	MTC311	H	R	Liquid	SIGMA-ALDRICH	MKCG3412	290858
19	Sodium chlorite	7758-19-2	MTA114	MTB216	MTC304	D	R	Solid	SIGMA-ALDRICH	BCBV1836	244155
20	D(-)-Mannitol	69-65-8	MTA127	MTB227	MTC327		R	Solid	Wako	LKP4365	139-00842

D=Deleterious Substance
H=Dangerous Substance

R=Room Temperature

7-5. Acceptance criteria

The within-laboratory reproducibility for all the test facilities was determined by independent biostatistical analysis using five coded chemicals, under supervision by the VMT. The proportion of concordance should be greater than or equal to 80% to be accepted as tentative acceptance criteria for the Phase I study.

Twenty-five coded test items were selected to confirm the between-laboratory reproducibility in the Phase I and II studies. At the end of the testing, the test facilities submitted a QC certified copy of the whole study dossier to the trial coordinator (study plan in accordance with the principles of GLP, raw data, records and data analysis, study report in accordance with the principles of GLP). The proportion of concordance of between-laboratory reproducibility should be greater than or equal to 80% to be accepted as acceptance criteria.

8. Protocols

8-1. Overview of IL-1 Luc assay

An overview of the IL-1β Luc assay is shown in Fig. 5. In addition, the final protocol for the present test (version 009E) is provided as Appendix 6, 7, and 8 and the procedures are described in detail below.

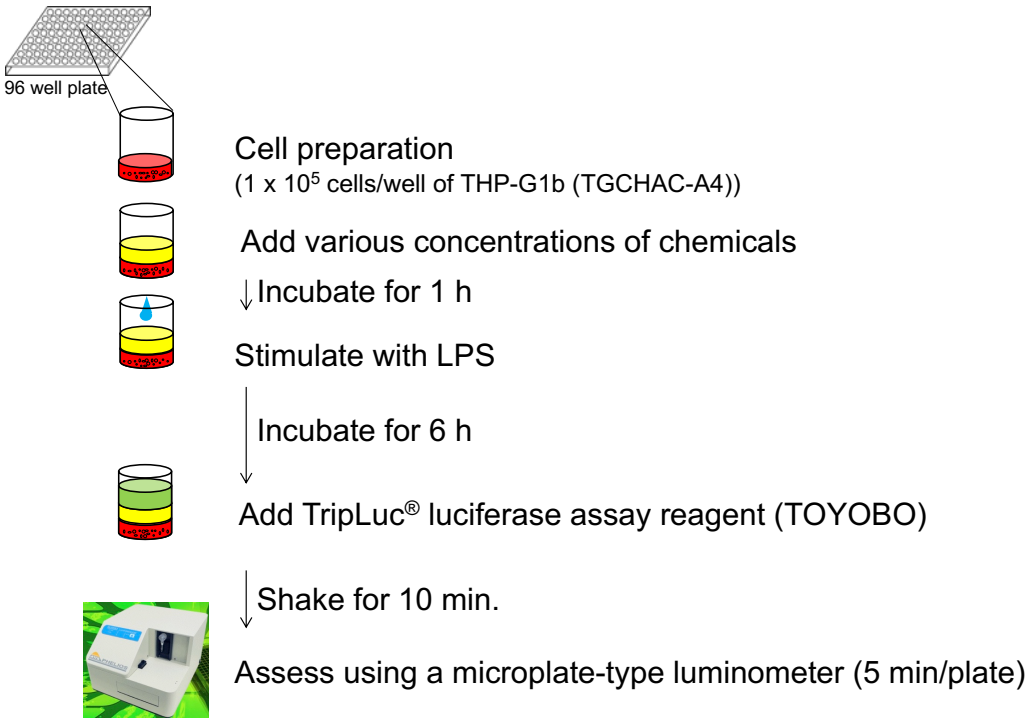

Fig. 5. Overview of the IL-1 Luc assay

8-1 Cells

・/ THP-G1b (TGCHAC-A4) (IL1β-SLG, G3PDH-SLR)

A THP-1-derived IL-1β reporter cell line, THP-G1b (TGCHAC-A4), harbors the SLG and SLR luciferase genes under the control of the IL-1β and G3PDH promoters, respectively, and was established by the Department of Dermatology, Tohoku University School of Medicine, and GPC laboratory Co. Ltd. (Kimura et al. 2018). To establish the THP-G1b cell line, we used THP-1 cells containing stable luciferase red (SLR) regulated by the G3PDH promoter. Then, an IL-1β reporter cassette containing stable luciferase green (SLG) under the IL-1β promoter was transfected into a gene-loading site of a human artificial chromosome (HAC) vector in CHO cells. We transfected the HAC vector into the THP-1-derived cell line containing SLR regulated by the G3PDH promoter from CHO cells using a microcell-mediated chromosome transfer technique to obtain the THP-G1b cell line (Kimura et al. 2018).

8-2. Protocol for IL-1 Luc assay

8-2-1. Reagents and equipment

The following reagents and equipment were used.

For maintenance of the THP-G1b (TGCHAC-A4) cells

- RPMI-1640 (GIBCO Cat#11875-093, 500 mL)
- FBS (Biological Industries Cat#04-001-1E Lot: 715004)
- 100× concentrated antibiotic and antimycotic (10,000 U/mL of penicillin G, 10,000 μg/mL of streptomycin and 25 μg/mL of amphotericin B in 0.85% saline) (e.g., GIBCO Cat#15240-062)

For chemical exposure, stimulation, positive control and solvents

- Lipopolysaccharide (LPS) from *Escherichia coli* K12 (Invivogen Cat#tlrl-eklps, Lot#: LEK-39-01)
- Dexamethasone (CAS:50-02-2, Fujifilm Wako Pure Chemical Cat#041-18861)
- Dimethyl sulfoxide (DMSO) (CAS:67-68-5, Sigma Cat#D5879)
- Distilled water (GIBCO Cat#10977-015)

For measurement of luciferase activity

- Tripluc® Luciferase Assay Reagent (TOYOBO Cat#MRA-301)

Expendable supplies

- T-75 flask, tissue culture treated (e.g., Corning Cat#353136)
- 96-well black-flame and white-well plate (flat-bottom, for measurement of luciferase activity, e.g., PerkinElmer B&W Isoplate-96 TC Cat#6005060)
- 96-well clear plate (round-bottom, for preparation of chemicals and stimulants)
- 96-well assay block, 2 mL (e.g., Costar Cat#3960)
- Seal for 96-well plate (e.g., Perkin Elmer TopSeal-A PLUS Cat#6050185, EXCEL Scientific SealMate Cat#SM-KIT-SP)
- Reservoir
- Pipette

Equipment for measurement of luciferase activity

- Measuring device: a microplate-type luminometer with a multi-color detection system that can accept an optical filter
  e.g., Phelios AB-2350 (ATTO), ARVO (PerkinElmer), Tristar LB941 (Berthold)
- Optical filter: a 600 nm long-pass filter or a 600-700 nm band-pass filter

Others

- Pipetman
- 8 channel or 12 channel pipetman (optimized for 10-100 μL)
- Plate shaker (for 96-well plate)
- CO_2 incubator (37°C, 5% CO_2)
- Water bath
- Cell counter: hemocytometer, trypan blue

8-2-2. Culture media

Various culture media were used, depending on the purpose of the cell culture.

Table 8. A medium: for maintenance of THP-G1b cells (500 mL, stored at 2-8°C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO Cat #11875-093	-	-	445 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	50 mL
Antibiotic-Antimycotic	e.g., GIBCO Cat #15240-062	100×	1×	5 mL

Table 9. B medium: for luciferase assay (30 mL, stored at 2-8°C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO Cat #11875-093	-	-	27 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10%	3 mL

8-2-3. Preparation of stimulant for THP-G1b cells

Table 10. Lipopolysaccharide (LPS) from *Escherichia coli* K12

Reagent	Company	Concentration of stock solution	Final concentration
Lipopolysaccharide (LPS) from *Escherichia coli* K12	Invivogen Cat#tlrl-eklps	1 mg/mL	100 ng/mL
Distilled water	GIBCO Cat#10977-015		

Dissolve 5 mg LPS using 5 mL distilled water, dispense at 5 μL/tube and store in freezer at -30°C. Use these stocks within 6 months of dissolution.

8-2-4. Thawing of THP-G1b cells

Pre-warm 9 mL of A medium in a 15 mL polypropylene conical tube in a 37°C water bath (for centrifugation) and 15 mL of A medium in a T-75 flask at 37°C in a 5% CO_2 incubator (for culture).

Thaw frozen cells (2×10^6 cells/0.5 mL of freezing medium) in a 37°C water bath, then add to a 15 mL polypropylene conical tube containing 9 mL of pre-warmed A

medium. Centrifuge the tube at 120-350 × *g* at room temperature for 5 min, discard the supernatant, and resuspend the cells in 15 mL of pre-warmed A medium in a T-75 flask. Cells are incubated at 37°C, 5% CO_2.

8-2-4. Maintenance of THP-G1b cells

Three or 4 days after thawing, pre-warm A medium in a T-75 flask at 37°C in a 5% CO_2 incubator. Count the number of cells, centrifuge the tube at 120-350 × *g* at room temperature for 5 min, discard the supernatant, and resuspend the cells in the pre-warmed A medium in a T-75 flask. Cells are passaged at 2-5 × 10^5/mL, depending on the condition of the cells, and incubated at 37°C, 5% CO_2.

The interval between subcultures should be 3-4 days. Cells can be used between 1 and 6 weeks after thawing.

The lead laboratory has examined how long THP-G1b cells can be cultured without losing their reactivity to LPS. THP-G1b cells maintained their response to LPS for up to 16 weeks or 33 passages.

8-2-5. Preparation of cells for assay

Cells should be passaged 2-4 days before the assay.

Use cells between 1 and 6 weeks after thawing.

Pre-warm the B medium in a 37°C water bath. Count the number of cells and collect the number of cells needed (5.0 × 10^6 cells are required, but to have some leeway, 7.5 × 10^6 cells should be prepared), centrifuge the tube at 120-350 x *g*, 5 min. Resuspend in the pre-warmed B medium at a cell density of 2 × 10^6/mL. Transfer the cell suspension to a reservoir (Thermo Scientific), and add 50 μL of cell suspension to each well of a 96-well black-frame and white-well plate (flat bottom) using an 8 channel or 12 channel pipetman (Gison, Inc., Middleton, WI, USA) (cf. Fig. 6).

flat-bottom B&W	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL
D	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL
E	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL
F	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL
G												
H												

Fig. 6. Components in each well of 96-well plates after cell preparation.

8-2-6. Preparation of chemicals and cell treatment with chemicals

Water soluble chemicals were dissolved in distilled water at a concentration of 25 mg/mL. If the chemicals were soluble at 25 mg/mL, then 50 mg/mL solutions were prepared if their solubility was sufficient. If they were not soluble at 50 mg/mL, then 25 mg/mL was judged to be the highest soluble concentration. If the chemicals were soluble at 50 mg/mL, then 100 mg/mL solutions were prepared if their solubility was sufficient. If they were not soluble at 100 mg/mL, then 50 mg/mL was judged to be the highest soluble concentration. If they were soluble at 100 mg/mL, then 100 mg/mL was judged to be the highest soluble concentration.

Chemicals not soluble in water were dissolved in DMSO at 500 mg/mL. If they were not soluble at 500 mg/mL, the highest soluble concentration was determined by diluting the suspension from 500 mg/mL by a factor of 2 with DMSO. Sonication and vortex mixing were used if needed and the attempt to dissolve the chemicals was continued for at least 5 minutes. The dissolution of the chemicals was confirmed by the absence of precipitation after centrifugation at 15,000 rpm for 5 minutes. Dissolved chemicals were used within 4 h after being dissolved in distilled water or DMSO (Fig. 7).

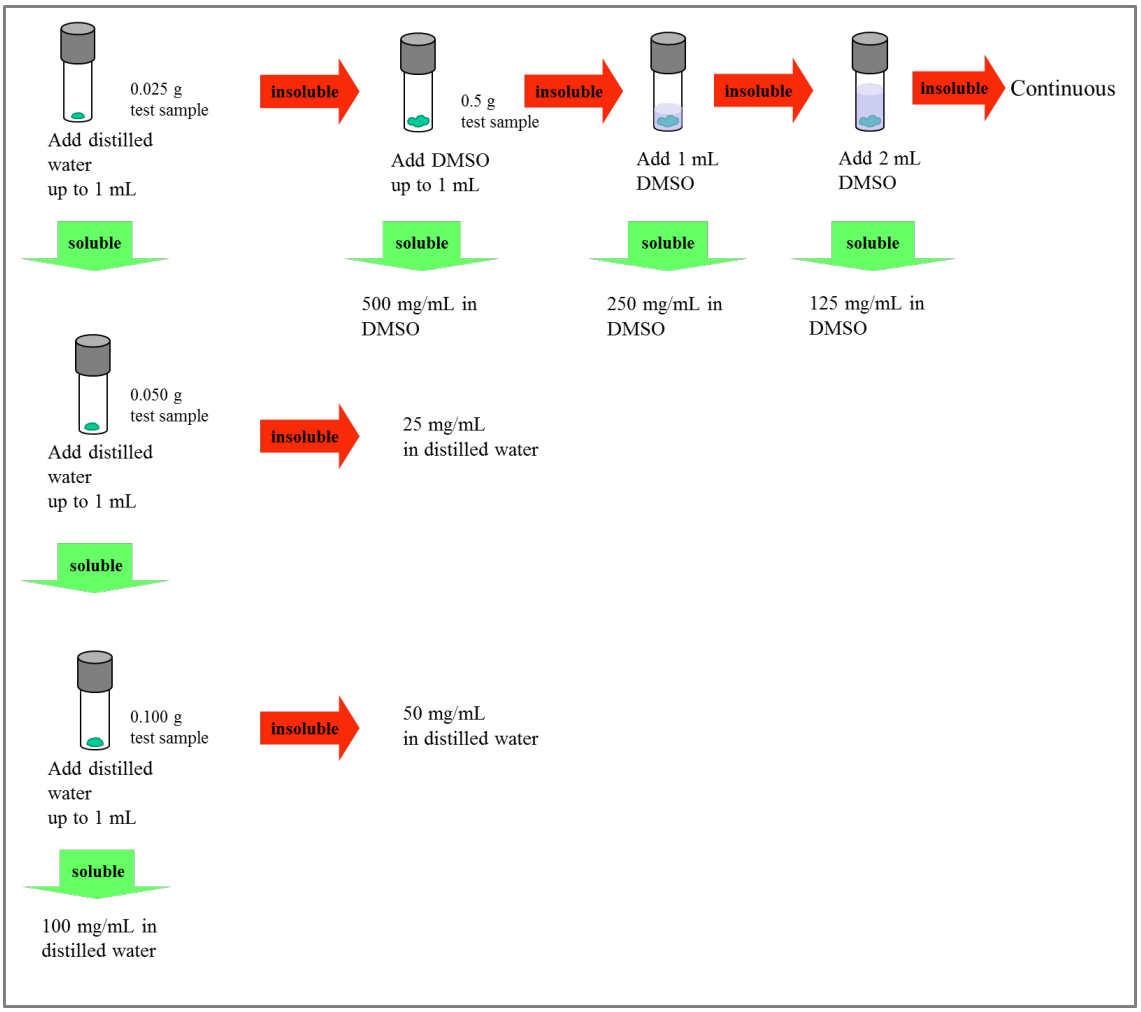

Fig. 7. Dissolution by solvent

8-2-7. Dilution of chemicals

For water soluble chemicals, 11 serial dilutions were conducted using distilled water, diluting by a factor of 2. For water insoluble chemicals, 11 serial dilutions were conducted using DMSO as the solvent, diluting by a factor of 2. The diluted chemicals were added to THP-G1b cells in a 96-well plate. After 1 h incubation at 37°C in a 5% CO_2 incubator, THP-G1b cells were added to 10 μL of LPS solution and incubated again at 37°C in a 5% CO_2 incubator for 6 h.

8-2-8. Measurements

After incubation with the chemical and LPS for 6 h at 37°C in a 5% CO_2 incubator, 100 μL of pre-warmed Tripluc Luciferase Assay Reagent was added to each

well in the plate containing reference samples using a pipetman and the plate was shaken for 10 min at room temperature (about 25°C) using a plate shaker. Surface bubbles were removed if present and bioluminescence from each well was measured using a microplate-type luminometer with a multi-color detection system (Phelios; Atto Co., Tokyo, Japan) for 3 s each in the absence (F0) and presence (F1) of the optical filter. The F0 and F1 data (values are expressed as counts) were processed using an Excel-based data sheet (Appendix 9). IL1LA and GAPLA were calculated for each well based on the algorithm to calculate IL1LA and GAPLA from the raw luminescence data reported previously (Nakajima et al. 2005; Noguchi et al. 2008). In addition to being used to calculate IL1LA and GAPLA, this data sheet can automatically generate final graphs showing the correlation between %suppression and the concentration of the chemical, and between Inh-GAPLA and the concentration of the chemical.

8-2-9. Luminometer apparatus

Multi-color detection systems such as microplate luminometers are available (e.g., Phelios (ATTO, Tokyo, Japan), Tristan 941 (Berthold, Bad Wildbad, Germany), and the ARVO series (PerkinElmer, Waltham, MA)). The luminometer detectors must have high sensitivity (especially for the red region) and low background noise, and are usually equipped with optical filters such as sharp-cut (long-pass) filters or band-pass filters. The transmission coefficients for these filters against each luciferase must be estimated prior to initiating the experiments because the coefficients are dependent on the luminometer due to lot-to-lot variations in detectors.

8-2-10. Positive control

In each experimental set, dexamethasone was used as a positive control.

8-2-11. Calculation and definition of parameters for IL-1 Luc assay

In the IL-1 Luc assay, nIL1LA was defined to represent IL-1β promoter-driven SLG luciferase activity (IL1LA) normalized by the SLR luciferase activity (GAPLA).

The inhibition of GAPLA (Inh-GAPLA) was determined by dividing the GAPLA for THP-G1b treated with chemicals with the GAPLA for non-treated THP-G1b. Percent suppression reflects the effect of chemicals on the IL-1β promoter (Table 11).

Abbreviation	Definition
IL-1 Luc assay	IL-1 luciferase assay
GAPLA	SLR luciferase activity reflecting GAPDH promoter activity
IL1LA	SLG luciferase activity reflecting IL-1 promoter activity of THP-G1b cells
nIL1LA	IL1LA/GAPLA of THP-G1b cells
Suppression (%)	(1 – (nIL1LA of THP-G1b cells treated with drugs/nIL1LA of non-treated THP-G1b cells)) × 100
Augmentation (%)	((nIL1LA of THP-G1b cells treated with drugs/nIL1LA of non-treated THP-G1b cells) – 1) × 100
CV05	The lowest concentration of the chemical at which Inh-GAPLA becomes < 0.05.
Inh-GAPLA	GAPLA for THP-G1b cells treated with chemicals/GAPLA for untreated THP-G1b cells.

Table 11. Abbreviations used in the IL-1 Luc assay protocol

8-2-11 Acceptance criteria (for Phase I study, Multi-ImmunoTox Assay protocol for THP-G1b ver. 008E)

The following acceptance criteria should be satisfied when using the IL-1 Luc assay method.

- If the fold induction of nIL1LA in LPS wells without chemicals (= (nIL1LA for THP-G1b cells treated with LPS)/(nIL1LA for non-treated THP-G1b cells)) is less than 5, the results obtained from the plate containing the control wells should be rejected.

8-2-12 Criteria (for Phase I study, Multi-ImmunoTox Assay protocol for THP-G1b

ver. 008E)

The experiments were repeated until two consistent positive (negative) results or two consistent “no effect results” were obtained. When two consistent results were obtained, the chemicals were judged as the obtained consistent results.

An immunotoxicant was identified by the mean of Suppression (%) and its 95% confidence interval.

In each experiment, when a chemical cleared the following three criteria, it was judged as suppressive or stimulatory. Otherwise, they were judged as ‘no effect’ chemicals.

1. The mean of Suppression (%) is $\geq$ 20 (suppressive) or $\leq$ -20 (stimulatory) with statistical significance. The statistical significance is judged by its 95% confidence interval.
2. The result shows two or more consecutive statistically significant positive (negative) data points or one statistically significant positive (negative) data point, with a trend in which at least three consecutive data points increase (decrease) in a dose-dependent manner. In the latter case, the trend can cross 0, as long as only one data point shows the opposite effect without statistical significance.
3. The results are judged using only data obtained at the concentration at which Inh-GAPLA is $\geq$ 0.05

8-3. Data collection

8-3-1. Operating procedure

Details of the operating procedure for this assay are described in protocol version 008E. Versions of the protocols were updated during the validation studies, but the descriptions of the operating procedures described in these protocols were the same for the two validation studies.

8-3-2. Chemicals

For the Phase I study, in which the main aim was to evaluate intra- and inter-laboratory reliability, a total of 15 coded chemicals were tested in three rounds of five chemicals distributed to three laboratories. Different codes were used in the rounds and thus the technician in each laboratory could not identify the chemicals. For the Phase II study, in which the main aim was to evaluate inter-laboratory reliability, 20 coded chemicals were distributed.

In this document, the chemicals were re-coded. The round is indicated by a suffix such as P101_R1 for the first chemical of the first round of the Phase I study: P1 means Phase I; 01 means the first chemical; _R1 means first round.

Table 12. Chemical codes used throughout this document.

Phase	Chemical code	Lab A	Lab B	Lab C
I	P101_R1, P101_R2, P101_R3, P102_R1, P102_R2, P102_R3, P103_R1, P103_R2, P103_R3, P104_R1, P104_R2, P104_R3, P105_R1, P105_R2, P105_R3	3 rounds	3 rounds	3 rounds
II	P201, P202, P203, P204, P205, P206, P207, P208, P209, P210, P211, P212, P213, P214, P215, P216, P217, P218, P219, P220	1 round	1 round	1 round

8-3-3. Data handling

The Excel data sheet developed for this study was distributed to the laboratories. We received data files from the three laboratories.

From JaCVAM, we received files listing the chemical codes for the five distributed chemicals in the Phase I study, and 20 chemicals in the Phase II study.

For data analysis, these files were combined and datasets were constructed. SAS ver. 9.4 and Microsoft Excel were used for the data analyses described in this report.

Since Excel data sheets can display a concentration-response plot for Suppression (%) with its 95% confidence interval, we could judge “Suppression” or “Negative” visually for each experiment from the plot (Appendix 10 and 11).

8-3-4. Index from each experiment and decision criteria for judgment

The j-th repetition (j = 1 to 4) of the i-th concentration (j = 0 to 11) was measured for IL1LA and GAPLA. The normalized IL1LA is referred as nIL1LA and is defined as $nIL1LA_{ij} = IL1LA_{ij}/GAPLA_{ij}$.

This is the basic unit of measurement in this assay.

8-3-4-1. Suppression (%)

Suppression (%) is an index for the averaged nIL1LA for the repetition using the i-th concentration compared with at 0 concentration and is the primary measure in this assay. Suppression (%) is described by the following formula

$$\text{Suppression}_{\text{i}}(\%) = \left\{1 - \frac{\left(\frac{1}{4}\right)\sum_{\text{i}} \text{nIL1LA}_{\text{ij}}}{\left(\frac{1}{4}\right)nIL1\text{LA}_{0\text{j}}}\right\} \times 100 \qquad (1)$$

The acceptance criteria for a tested chemical was set at 25. This value was based on the results of discussion at the VMT meeting because there are few historical data to help the lead laboratory decide the value for the assay. Before the Phase I study, the data management team prepared three cut-off values (20, 25, and 35) for the acceptance criteria. After the Phase I study, the team presented the results for each cut-off value at the VMT meeting. Based on performance, the VMT decided on 25 as the value and the criteria in the protocol were changed before the Phase II study.

The primary outcome measure, Suppression (%), is basically the ratio of two arithmetic means of nIL1LA, as shown in equation. The 95% confidence interval (95% CI) for Suppression (%) for the i-th concentration can be estimated.

The lower limit of the 95% CI above 0 is interpreted as the nIL1LA of the i-th concentration being statistically significantly greater than at 0 concentration.

There are several ways to construct the 95% CI. We used the method known as the Delta method. This 95% confidence interval is obtained from the following formula

$$\text{Suppression (\%)} \pm 100 \times \left\{ z_{0.975} \times \sqrt{\frac{\text{sd}_\text{i}^2}{\text{mean}_0^2} + \frac{\text{mean}_\text{i}^2 \times \text{sd}_0^2}{\text{mean}_0^4}} \right\},$$

where mean_i is the mean of nIL1LA at the i-th concentration, mean_0 is the mean of nIL1LA at 0 concentration, sd_i is the standard deviation of nIL1LA at the i-th concentration, and sd_0 is the standard deviation of nIL1LA at 0 concentration. $z_{0.975}$ is the 97.5 percentile of the standard normal distribution.

8-3-4-2. Inh-GAPLA

Inh-GAPLA is a ratio of the averaged GAPLA for the repetition of the i-th concentration compared with 0 concentration, and is written as

$$\text{Inh} - \text{GAPLA}_\text{i} = \left(\frac{1}{4}\right) \sum_\text{i} \text{GAPLA}_\text{ij} / \left(\frac{1}{4}\right) \sum_\text{i} \text{GAPLA}_\text{0j}.$$

Since GAPLA is the denominator of the nIL1LA, an extremely small GAPLA value causes a large variation in nIL1LA. Therefore, the i-th Suppression (%) value with an extremely small value of Inh-GAPLA might result in poor precision.

8-3-4-3. Judgment for “Suppression” or “Negative” in each experiment

In each experiment, when a chemical clears the following three criteria, it is judged as being suppressive or stimulatory. Otherwise, it is judged as a ‘no effect’ chemical.

1. The mean of Suppression (%) is ≥ 25 (suppressive) with statistical significance. The statistical significance is judged by its 95% confidence interval.
2. The result shows two or more consecutive statistically significant positive data points or one statistically significant positive data point, with a trend in which at least three

consecutive data points increase in a dose-dependent manner. In the latter case, the trend can cross 0, as long as only one data point shows the opposite effect without statistical significance.

3. The results are judged using only data obtained at the concentration at which Inh-GAPLA is $\geq$ 0.05.

8-3-4-4. Final judgment for "Suppression" or "No effect" using this assay

In this assay, "Suppression" or "No effect" is defined as the case in which there are two identical judgments in a set of experiments.

8-3-5. Reliability

8-3-5-1. Within-laboratory reproducibility for five common chemicals

Within-laboratory reproducibility was determined by whether or not tables of three sets for the final judgment for each chemical by each laboratory were concordant. The concordance rate was then calculated as the proportion of concordance for each laboratory.

The concordance rate for within-laboratory reproducibility was based on the results of three sets.

To summarize, the concordance rate for within-laboratory reproducibility from the three laboratories was used to calculate the averaged concordance rate.

8-3-5-2 Between-laboratory reproducibility

Between-laboratory reproducibility was determined using the results from the final judgment from the three laboratories for 20 chemicals, this is, five chemicals in the Phase I study and 20 chemicals in the Phase II study. These judgements were tabulated, **and** then the concordance rate was calculated as a proportion of the concordance in each laboratory.

To summarize, the concordance rate for between-laboratory reproducibility from the three laboratories was used to calculate the averaged concordance rate.

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8-3-6. Predictivity

8-3-6-1. Definition of concordance, sensitivity and specificity

In the evaluation of predictivity, we did not distinguish between suppression and stimulation because both indicate modulation of immune function. Rather, we used "Positive (P)" in the case of "suppression" or "stimulation", and "No effect (N)" in the case of no significant effects for each chemical judgement.

The concordance, sensitivity and specificity were estimated as the indices of predictivity. These indices were estimated using the frequency results obtained from the 2 × 2 contingency table for T cell targeting. The definitions of these indices are summarized in Table 13 below. This calculation was based on the results decided by the majority for the between-laboratory results for each chemical.

Table 13. Definition of concordance, sensitivity and specificity

Judgment from IL-1 Luc assay	Chemical category		Total
	Positive	Negative	
Positive	a	b	a+b
Negative	c	d	c+d
Total	a+c	b+d	N

Sensitivity = 100 x a/(a+c)

Specificity = 100 x d/(b+d)

Accuracy = 100 x (a+d)/N

8-3-5. Reliability

8-3-5-1. Within-laboratory reproducibility for five common chemicals

Within-laboratory reproducibility was determined by whether or not tables of three sets for the final judgment for each chemical by each laboratory were concordant. The concordance rate was then calculated as a proportion of the concordance for each laboratory.

The concordance rate for within-laboratory reproducibility was based on the results of three sets.

To summarize, the concordance rate for within-laboratory reproducibility for three laboratories was used to calculate the averaged concordance rate.

8-3-5-2 Between-laboratory reproducibility

Between-laboratory reproducibility was determined using the results from the final judgment from the three laboratories for 25 chemicals, this is, five chemicals in the Phase I study and 20 chemicals in the Phase II study. These judgements were tabulated, and then the concordance rate was calculated as a proportion of the concordance for each laboratory.

To summarize, the concordance rate for between-laboratory reproducibility for the three laboratories was used to calculate the averaged concordance rate.

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8-3-6. Predictivity

8-3-6-1. Definition of concordance, sensitivity and specificity

In the evaluation of predictivity, we did not distinguish between suppression and stimulation because both indicate modulation of immune function. Rather, we used “Positive (P)” in the case of “suppression” or “stimulation”, and “No effect (N)” in the case of no significant effects for each chemical judgement.

The concordance, sensitivity and specificity were estimated as the indices of predictivity. These indices were estimated using the frequency results obtained from the 2×2 contingency table for T cell targeting. The definitions of these indices are summarized in Table 13. This calculation was based on the results decided by the majority for the between-laboratory results for each chemical.

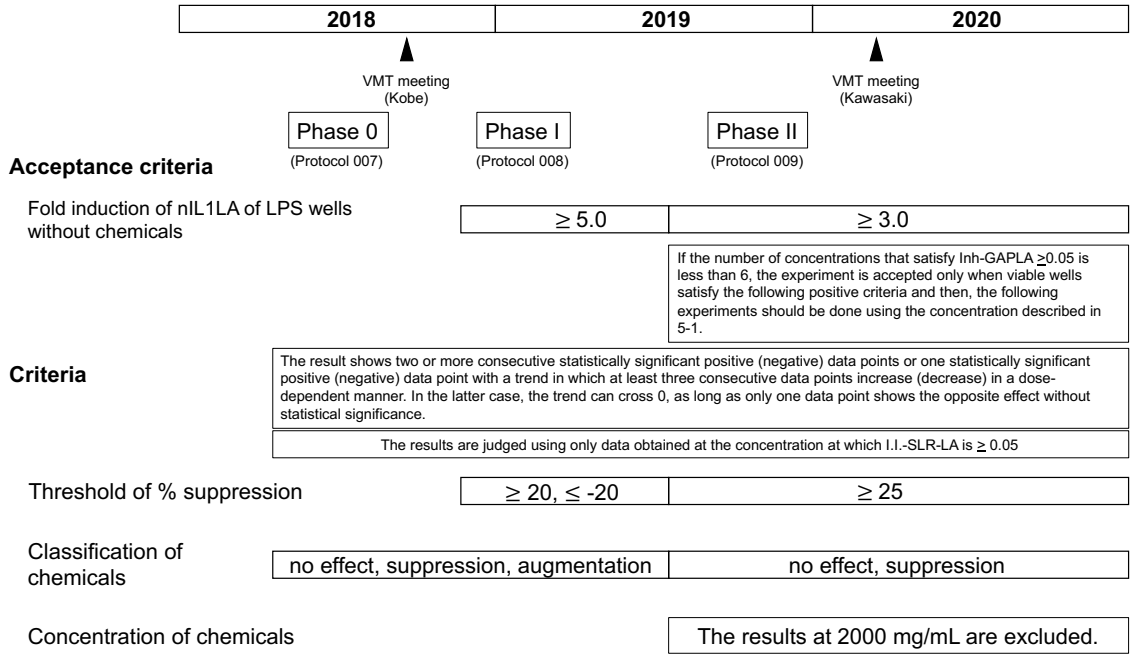
8-4. Quality assurance

Assays and quality assurance were carried out in the spirit of GLP, although not all the participating laboratories routinely worked under GLP certification. The participating laboratories conducted the experiments in accordance with the protocol provided by the VMT. All raw data and data analysis sheets were pre-checked for quality by each laboratory and were then reviewed by the VMT quality assurance team. The results accurately reflected the raw data.

9. Results

We conducted Phase I and II studies in this validation. The assay procedure and criteria used to judge immunotoxicants in the validation studies are summarized in Fig. 8.

Fig. 8. Modification of the protocols of the IL-1 Luc assay.



9-1. Final criteria for Phase I study

9-1-1. Acceptance criteria

The following acceptance criteria should be satisfied when using the MITA method.

Each time an experiment was conducted, a control experiment examining nIL1LA for THP-G1b cells treated with LPS, and nIL1LA for non-treated THP-G1b cells, was conducted. Then, the fold induction for nIL1LA for LPS wells without chemicals (= (nIL1LA for THP-G8 cells treated with LPS)/(nIL1LA for non-treated THP-G1b cells)) was calculated. If the fold induction was less than 5.0, the results obtained from these experiments was rejected.

9-1-2. Criteria

The experiments were repeated until two consistent positive (negative) results or two consistent "no effect results" were obtained. When two consistent results were obtained, the chemicals were judged as the obtained consistent results.

An immunotoxicant was identified by the mean of Suppression (%) and its 95% confidence interval.

In each experiment, when a chemical clears the following three criteria, it is judged as suppressive or stimulatory. Otherwise, it is judged as a 'no effect' chemical.

1. The mean Suppression (%) is $\geq$ 20 (suppressive) or $\leq$ -20 (stimulatory) with statistical significance. The statistical significance is judged by its 95% confidence interval.
2. The results show two or more consecutive statistically significant positive (negative) data points or one statistically significant positive (negative) data point with a trend in which at least three consecutive data points increase (decrease) in a dose-dependent manner. In the latter case, the trend can cross 0, as long as only one data point shows the opposite effect without statistical significance.
3. The results are judged using only data obtained at the concentration at which Inh-GAPLA is $\geq$ 0.05

9-1-3. Predictivity

As already described, there are at least two targets of *in vitro* immunotoxicity tests: global immunosuppression, and limited immune response. In this validation report we

therefore first examined the predictivity of the IL-1 Luc assay as an immunotoxicity test that targets global immunosuppression (global immunotoxicity test), since IL-1β has pleiotropic effects, as described in section 3-9-2. Although reference data that aid positive or negative judgments are essential in determining the predictability of tests, no such reference data are currently available to determine the predictability of the IL-1Luc assay. Thus, we tried to generate reference data for the chemicals used in this validation study of the IL-1 Luc assay. We referred to the rationale for immunotoxic classification proposed by Luster et al. where they presented a screening battery using a 'tier' approach to detect potential immunotoxic compounds in mice (Luster 1998). According to their rationale, a positive reference chemical would either produce a significant dose-response effect in the immune test or significantly alter two or more immune test results at the highest dose of the chemical tested. They classified chemicals based on the results obtained in 12 immune tests according to this rationale and found a significant correlation between the judgment of immunotoxic chemicals and host resistance (Luster et al. 1993). Therefore, using this rationale, we classified chemicals as described in our previous publication. (Kimura et al. 2020).

We first surveyed the literature (Appendix 12 and 13), collected the following eight endpoints regarding each chemical used in the study (Table 14), generated reference data for their immunotoxic profiles, and identified chemicals that satisfy at least one of the following three criteria for immunotoxic chemicals (Table 15). The summarized immunotoxicity information and the classifications of the chemicals are shown in Appendix 14 and 15.

Table 14. Immunotoxicological data obtained from literature.

Endpoint	**Information**
Endpoint 1	Decreased antibody response
Endpoint 2	Suppressed T cell proliferation upon stimulation with innate immune cells, such as dendritic cells or macrophages

Endpoint 3	Decreased LPS response *in vivo*, *ex vivo*, or *in vitro*
Endpoint 4	Suppressed DHR
Endpoint 5	Suppressed host resistance
Endpoint 6	The NTP data or Tox 21 data indicate immunotoxicity of chemicals
Endpoint 7	Increased or decreased mRNA expression or protein production of IL-1β, TNF-α, IL-6, or other proinflammatory cytokines by innate immune cells *ex vivo.*
Endpoint 8	Increased or decreased mRNA expression or protein production of IL-1β, TNF-α, IL-6, or other proinflammatory cytokines by innate immune cells *in vitro.*

Table 15. Criteria to classify immunotoxic chemicals

Criterion	Definition
Criterion 1	Satisfy one of Endpoints 1 to 6
Criterion 2	Increased or decreased mRNA expression or protein production in one or more cytokines in Endpoints 7 or 8 in multiple reports
Criterion 3	Increased or decreased mRNA expression or protein production in two or more cytokines in Endpoints 7 or 8

The predictivity of the IL-1 Luc assay was determined by comparing the results of the IL-1 Luc assay (positive or no effect) with the classification of the chemicals (immunotoxic or not).

9-2. Phase 0 study (for technical transfer)

The preliminary test trial (Phase 0) was performed by the participating laboratories following explicit explanations of the Multi-ImmunTox Assay protocol for THP-G1b ver. 007E by the lead laboratory, Tohoku University. Three laboratories participated in the Phase 0 study of the IL-1 Luc assay using the three open-labeled chemicals dapson, diethanolamine and p-nitroaniline, and conducted one set (three experiments) for each chemical. When the threshold of Suppression (%) was the highest, the response patterns for the three chemicals were similar among the three laboratories. Based on these results, the VMT judged that technical and protocol transfer of the IL-1 Luc assay is acceptable.

9-3. Phase I study (for within- and between-laboratory reproducibility)

9-3-1. Test conditions

A total of five coded chemicals were evaluated by three experimental sets in the Phase I study based on the Multi-ImmunoTox Assay protocol for THP-G8 ver. 008E.

In each experimental set, three or more experiments were conducted for each chemical.

Chemicals that satisfied the criteria were judged as positive. Chemicals that provided two positive results were judged as immunotoxicants.

9-3-2. Within-laboratory variation assessments in Phase I study

Lab A	60.0% (3/5)
Lab B	100.0% (5/5)
Lab C	40.0% (2/5)
Average	66.7% (10/15)

9-3-3. Between-laboratory variation assessments in Phase I study

Between-Lab reproducibility (based on majority)

60.0% (3/5)

9-3-4. Predictivity in Phase I study (based on majority)

Accuracy of Lab A	60.0% (3/5)
Accuracy of Lab B	40.0% (2/5)
Accuracy of Lab C	80.0% (4/5)
Average	60.0% (9/15)

Table 16. Results of Phase I study

Chemical	CAS	Set	Lab. A	Lab. B	Lab. C	Concordance	Immunotoxicity	Rationale
Dibutyl phthalate	84-74-2	1st	S	S	S	1	Yes	1, 2, 3
		2nd	S	S	S			
		3rd	S	S	S			
		1st	A	N	A	0	Yes	1

Acetaminophen	103-90-2	2nd 3rd	N N	N N	N A			
Isonicotinic Acid Hydrazide (Isoniazid)	54-85-3	1st 2nd 3rd	S S N	N N N	N S S	0	Yes	1
Sulem Mercury(II) Chloride	7487-94-7	1st 2nd 3rd	S S S	S S S	S S S	1	Yes	1, 2, 3
Hexachlorobenzene	118-74-1	1st 2nd 3rd	N N N	N N N	S N N	1	Yes	1
Within-laboratory reproducibility (%)			60.0 (3/5)	100.0 (5/5)	40.0 (2/5)			
				Average 66.7 (10/15)				
Between-laboratory reproducibility (%) (based on majority)						60.0 (3/5)		
Sensitivity (%) (based on majority)			60.0 (3/5)	40.0 (2/5)	80.0 (4/5)			
				Average 60.0 (9/15)				
Specificity (%) (based on majority)			N.D.	N.D.	N.D.			
				N.D.				
Accuracy (%) (based on majority)			60.0 (3/5)	40.0 (2/5)	80.0 (4/5)			
				Average 60.0 (9/15)				

S: Immunosuppression, A: Immunoaugmentation, N: No effect, A/S: Immunoaugmentation/suppression, N.D.: Not determined

9-3-5. Contingency tables for Phase I study

Lab A		Immunotoxicity		Total
		+	-	
IL-1 Luc assay	+	3	0	3
	-	2	0	2
Total		5	0	5

Sensitivity: 60.0% (3/5)

Specificity: not determined

Accuracy: 86.7% (13/15)

Lab B		Immunotoxicity		Total
		+	-	
IL-1 Luc assay	+	2	0	2
	-	3	0	3
Total		5	0	5

Sensitivity: 40.0% (2/5)

Specificity: not determined

Accuracy: 40.0% (2/5)

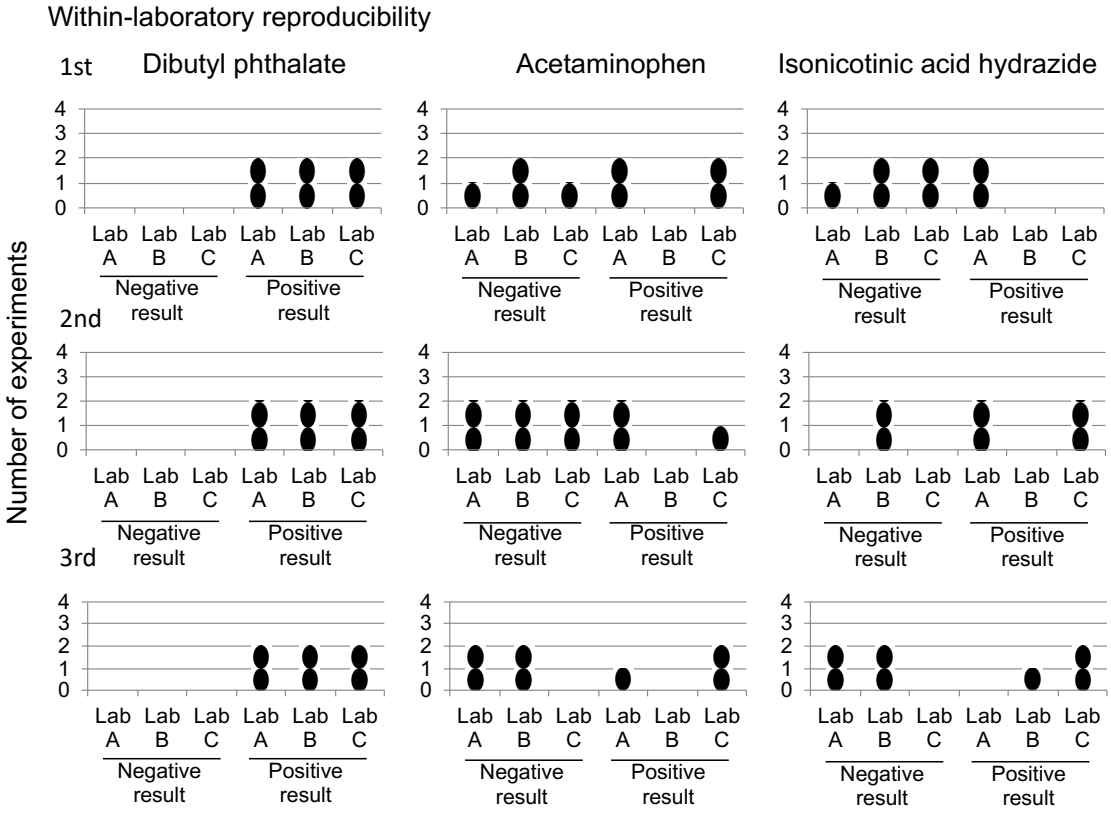
Lab C		Immunotoxicity		Total
		+	-	
IL-1 Luc assay	+	4	0	4
	-	1	0	1
Total		5	0	5

Sensitivity: 80.0% (4/5)

Specificity: not determined

Accuracy: 80.0% (4/5)

A graphical presentation of between- and within-laboratory variation in the Phase I study is shown in Fig. 9.



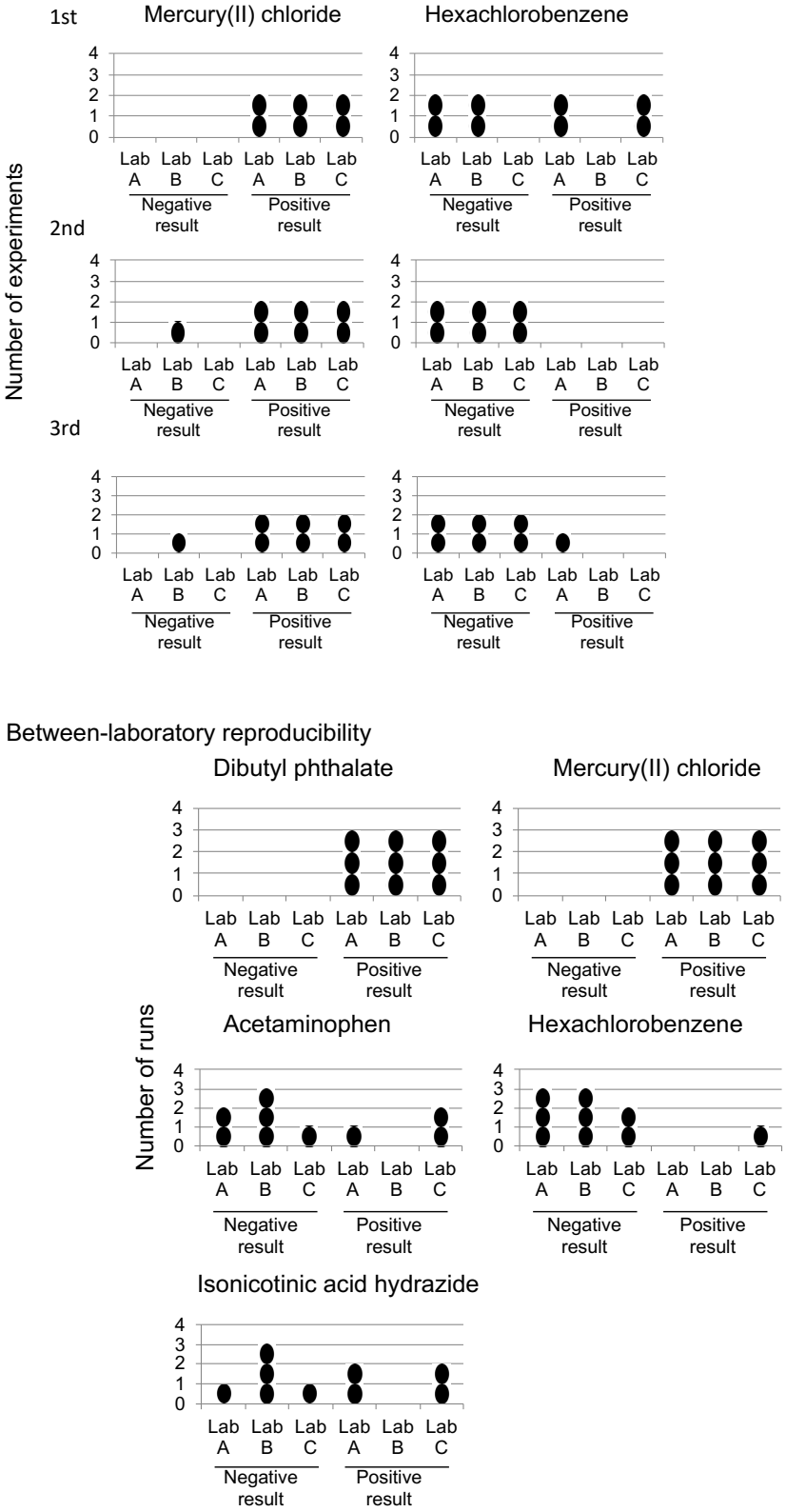


Fig. 9. Between- and within-laboratory variation assessments in Phase I study

The Phase I study examined within-and between-laboratory reproducibilities using a total of five coded chemicals (five immunotoxic chemicals and no non-immunotoxic chemicals) evaluated by three experimental sets based on the MITA protocol for THP-G1b ver. 008E. Closed circles represent the judgments for individual experiments for within-laboratory reproducibility or the judgments in individual experimental sets for between-laboratory reproducibility.

After the Phase I study, we amended the protocol as follows:

- We changed the fold induction for nIL1LA of LPS wells without chemicals to >5 in the acceptance criterion to the fold induction for nIL1LA of LPS wells without chemicals >3.
- For assaying highly cytotoxic chemicals, a new acceptance criterion was added: “If the number of concentrations that satisfy Inh-GAPLA >0.05 is less than 6, the experiment is accepted only when viable wells satisfy the following positive criteria and then, the following experiments should be conducted using in Section 5-1 of Multi-Immuno Tox Assay protocol for THP-G1b ver. 009E (Appendix 6).
- In this assay, since LPS is very potent, a minute variation in the amount of added LPS results in a large variation in nSLG-LA. Therefore, when chemicals have no effect or some stimulatory effect on nSLG-LA, a small variation in LPS concentration causes a significant variation in nSLG-LA. Indeed, there is much more variation in the data showing augmentation than in the data showing suppression. We therefore proposed that the judgment of IL-1 LA is suppression or non-suppression, with the latter including both no effect and augmentation. Indeed, even though we classify chemicals into the categories, suppression, augmentation or no effect, judging a chemical effect as augmentation will cause significant problems when we evaluate the predictivity of this chemical because it is difficult to find chemicals in the literature that further stimulate IL-1 production by monocytes stimulated with LPS.
- The lead laboratory found that some chemicals that should be judged as no effect gave results with Suppression (%) greater than 20. In particular, at 2000 μg/mL,

significant numbers of chemicals showed more than 20% suppression, likely due to high osmotic pressure caused by such a high concentration of dissolved chemical. It was proposed that the threshold should be 25% suppression and that the results at 2000 μg/mL should be excluded.

The following are the final acceptance criteria and criteria for judgment accepted by the internal expert members (Multi-ImmunoTox Assay protocol for THP-G1b ver. 009E).

Acceptance criteria

- If the fold induction of nIL1LA of LPS wells without chemicals (= (nIL1LA of THP-G1b cells treated with LPS)/(nIL1LA of non-treated THP-G1b cells)) is less than 3, the results obtained from the plate containing the control wells should be rejected.
- If the number of concentrations which satisfy Inh-GAPLA >=0.05 is less than 6, the experiment is accepted only when viable wells satisfy the following positive criteria and then, the following experiments should be conducted using the concentration described in Section 5-1 of Multi-Immuno Tox Assay protocol for THP-G1b ver. 009E (Appendix 6).

Criteria

The experiments are repeated until two consistent positive results or two consistent "non-suppression" results are obtained. When two consistent results are obtained, the chemicals are judged as the obtained consistent results.

An immunotoxicant is identified by the mean of Suppression (%) and its 95% simultaneous confidence interval.

In each experiment, when a chemical clears the following four criteria, it is judged as suppression. Otherwise, it is judged as non-suppression.

1. Suppression (%) is $\geq$ 25 with statistical significance. Statistical significance is judged when the 95% confidence interval does not include 0.
2. The result shows two or more consecutive statistically significant positive data points or one statistically significant positive data point with a trend in which at least three consecutive data points increase in a dose-dependent manner. In the latter case, the

trend can cross 0, as long as only one data point shows the opposite effect without statistical significance.
3. The results are judged using only data obtained at the concentration at which Inh-GAPLA is $\geq$ 0.05
4. The results at 2000 μg/mL are excluded.

9-3-6. Within-laboratory variation assessments in Phase I study when new criteria are applied.

Lab A	100.0% (5/5)
Lab B	100.0% (5/5)
Lab C	100.0% (5/5)
Average	100.0% (15/15)

9-3-7. Between-laboratory variation assessments in Phase I study when new criteria are applied.

Between-Lab reproducibility (based on majority)

100.0% (5/5)

9-3-8. Predictivity in Phase I study (based on majority) when new criteria are applied.

Accuracy of Lab A	40.0% (2/5)
Accuracy of Lab B	40.0% (2/5)
Accuracy of Lab C	40.0% (2/5)
Average	40.0% (6/15)

Table 17. Results of Phase I study

Chemical	CAS	Set	Lab. A	Lab. B	Lab. C	Concordance	Immunotoxicity	Rationale
Dibutyl phthalate	84-74-2	1st	P	P	P	1	Yes	1, 2, 3
		2nd	P	P	P			
		3rd	P	P	P			
Acetaminophen	103-90-2	1st	N	N	N	1	Yes	1
		2nd	N	N	N			
		3rd	N	N	N			
Isonicotinic acid hydrazide (Isoniazid)	54-85-3	1st	N	N	N	1	Yes	1
		2nd	N	N	N			
		3rd	N	N	N			
Mercury(II) chloride	7487-94-7	1st	P	P	P	1	Yes	1, 2, 3
		2nd	P	P	P			
		3rd	P	P	P			
Hexachlorobenzene	118-74-1	1st	N	N	N	1	Yes	1
		2nd	N	N	N			
		3rd	N	N	N			
Within-laboratory reproducibility (%)			100 (5/5)	100 (5/5)	100 (5/5)			
				Average 100 (15/15)				
Between-laboratory reproducibility (%) (based on majority)						100 (5/5)		
Sensitivity (%) (based on majority)			40.0 (2/5)	40.0 (2/5)	40.0 (2/5)			
				Average 40.0 (6/15)				
Specificity (%) (based on majority)			N.D.	N.D.	N.D.			
				N.D.				

Accuracy (%) (based on majority)	40.0 (2/5)	40.0 (2/5)	40.0 (2/5)
	Average 40.0 (6/15)		

P: Positive, N: No effect, N.D: Not determined

9-3-9. Contingency tables the Phase I study when new criteria are applied.

Lab A		Immunotoxicity		Total
		+	-	
IL-1 Luc assay	+	6	0	6
	-	9	0	9
Total		15	0	15

Sensitivity: 40.0% (6/15)

Specificity: not determined

Accuracy: 40.0% (6/15)

Lab B		Immunotoxicity		Total
		+	-	
IL-1 Luc assay	+	6	0	6
	-	9	0	9
Total		15	0	15

Sensitivity: 40.0% (6/15)

Specificity: not determined

Accuracy : 40.0% (6/15)

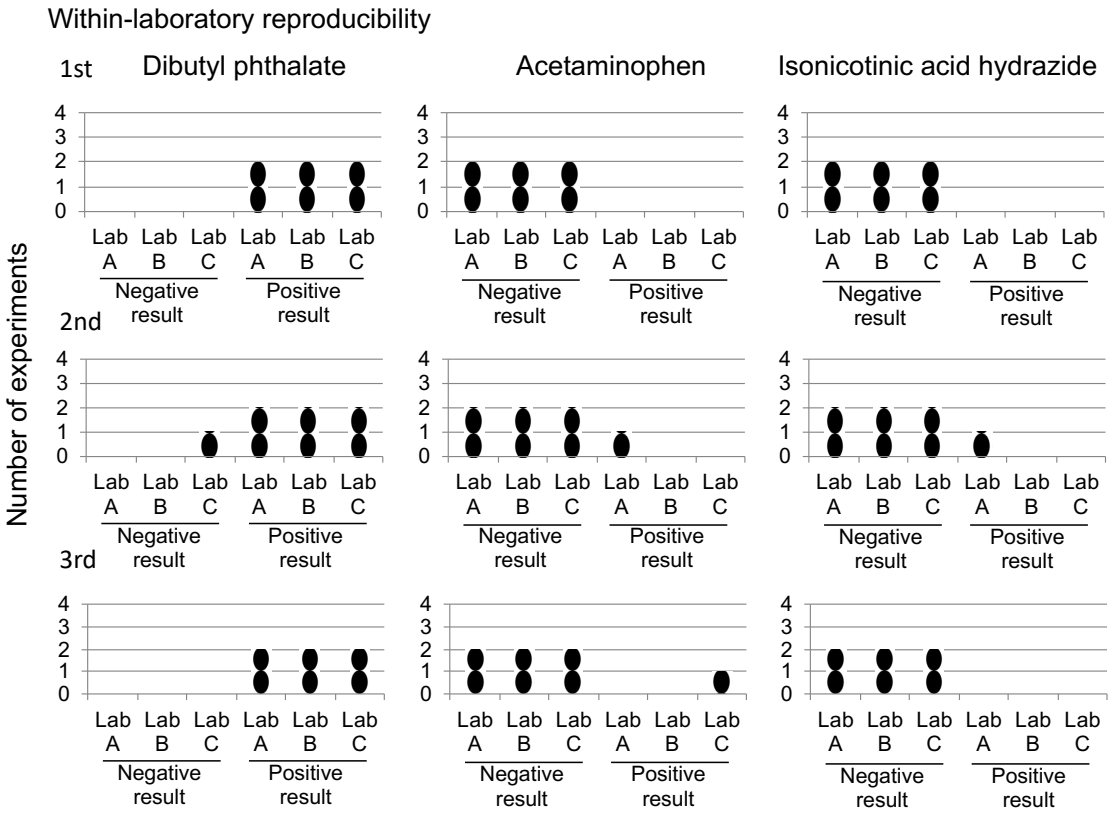
Lab C		Immunotoxicity		Total
		+	-	
IL-1 Luc assay	+	6	0	6
	-	9	0	9
Total		15	0	15

Sensitivity: 40.0% (6/15)

Specificity: not determined

Accuracy: 40.0% (6/15)

A graphical presentation of between- and within-laboratory variation in the Phase I study is shown in Fig. 10.



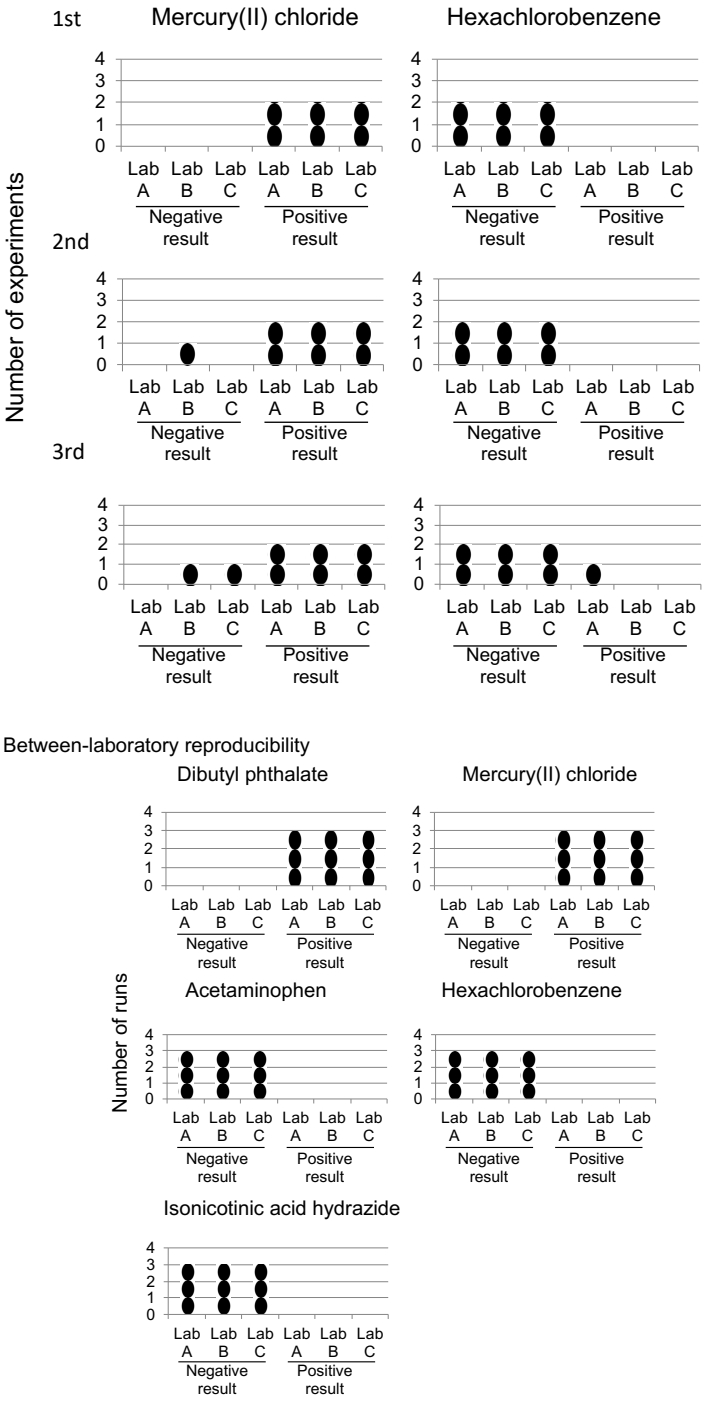


Fig. 10. Between- and within-laboratory variation assessments in Phase I study

The Phase I study examined within-and between-laboratory reproducibilities using a total of five coded chemicals (five immunotoxic chemicals and no non-immunotoxic chemicals) evaluated by three experimental sets based on the MITA protocol for THP-G1b ver. 008E. Closed circles represent the judgments for individual experiments for

within-laboratory reproducibility or the judgments in individual experimental sets for between-laboratory reproducibility.

9-4. Phase II study (for between-laboratory reproducibility and predictivity)

9-4-1. Test conditions

The Phase II study for between-laboratory reproducibility and predictivity was conducted using a total of 20 coded chemicals (15 immunotoxic chemicals and five non-immunotoxic chemicals) and evaluated by one experiment set based on the Multi-ImmunoTox Assay protocol for THP-G1b ver. 009E.

9-4-2. Between-laboratory variation assessments in Phase II study

Between-Lab reproducibility 80% (16/20)

9-4-3. Predictivity in Phase II study

Accuracy of Lab A 40.0% (8/20)

Accuracy of Lab B 45.0% (9/20)

Accuracy of Lab C 55.5% (11/20)

Average 46.7% (28/60)

Table 18. Results of Phase II study

Chemical	CAS	Lab. A	Lab. B	Lab. C	Based on majority	Concordance	Immunotoxicity	Rationale
Cadmium chloride	10108-64-2	P	P	P	P	1	Yes	1, 2, 3
5,5-Diphenylhydantoin sodium salt	630-93-3	N	N	N	N	1	Yes	1, 3
Indomethacin	53-86-1	N	N	P	N	0	Yes	1, 2, 3

Pentachlorophenol	87-86-5	N	N	P	N	0	Yes	1, 3
Urethane	51-79-6	N	N	N	N	1	Yes	1, 3
Tributyltin chloride	1461-22-9	N	N	N	N	1	Yes	1
Perfluorooctanois acid	335-67-1	P	P	P	P	1	Yes	1, 2, 3
Hydroquinone	123-31-9	N	N	N	N	1	Yes	2, 3
4-Aminophenyl sulfone	80-08-0	P	P	P	P	1	Yes	1, 2, 3
Ethanol	64-17-5	N	N	N	N	1	Yes	3
5-Nitro-2-furaldehydesemicarbazone	59-87-0	N	N	N	N	1	No	
Trichloroethylene	79-01-6	N	N	N	N	1	No	
Zinc dimethyldithiocarbamate	137-30-4	N	P	N	N	0	Yes	1, 3
Citral	5392-40-5	P	P	P	P	1	Yes	3
t-Buthlhydroquinone	1948-33-0	P	P	P	P	1	No	
Bisphenol A	80-05-7	P	P	P	P	1	Yes	1, 2, 3
2,6-Di-tert-butyl-4-methylphenol	128-37-0	N	N	P	N	0	Yes	1
Nonylphenol	84852-15-3	N	N	N	N	1	Yes	3
Sodium chlorite	7758-19-2	P	P	P	P	1	No	
D(-)-Mannitol	69-65-8	N	N	N	N	1	No	

Between-laboratory reproducibility (%)					80 (16/ 20)		
Sensitivity (%)	33.3 (5/1 5)	40.0 (6/1 5)	53.3 (8/1 5)	33.3 (5/1 5)			
Specificity (%)	60.0 (3/5)	60.0 (3/5)	60.0 (3/5)	60.0 (3/5)			
Accuracy (%)	40.0 (8/2 0)	45.0 (9/2 0)	55.0 (11/ 20)	40.0 (8/2 0)			

P: Positive, N: No effect

9-4-4. Contingency tables for Phase II study

Lab A		Immunotoxicity		Total
		+	-	
IL-1 Luc assay	+	5	2	7
	-	10	3	13
Total		15	5	20

Sensitivity	33.3 (5/15)
Specificity	60.0 (3/5)
Accuracy	40.0 (8/20)

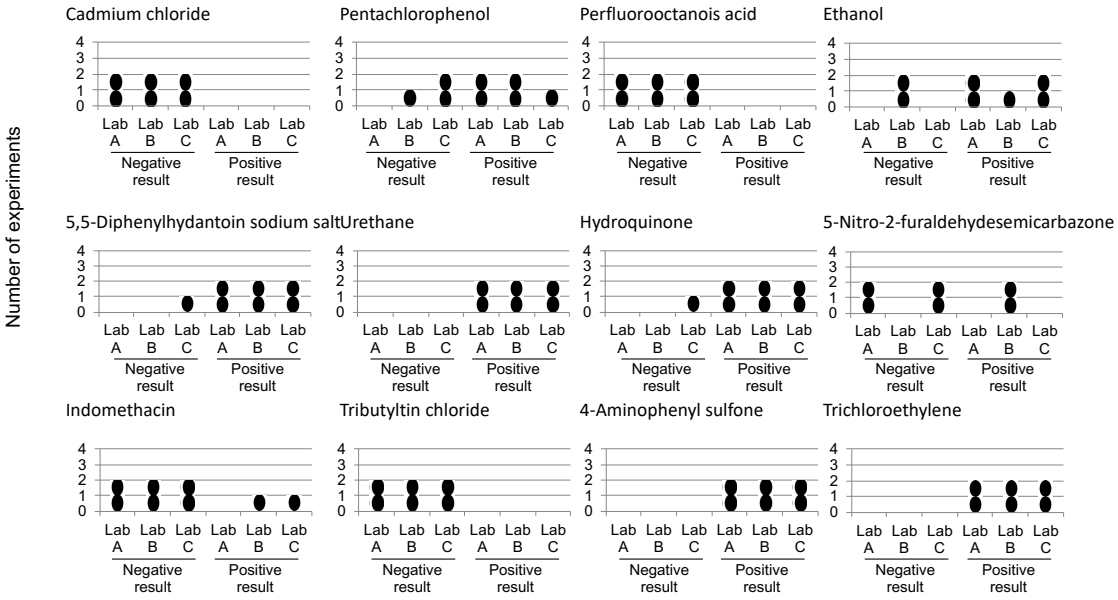
Lab B		Immunotoxicity		Total
		+	-	
IL-1 Luc assay	+	6	2	8
	-	9	3	12
Total		15	5	20

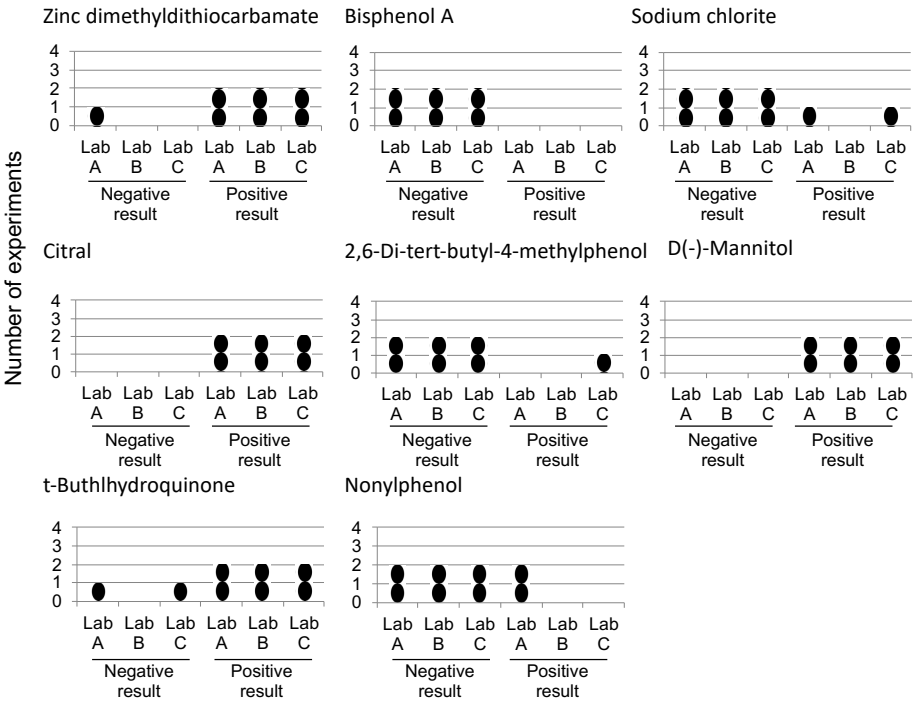
Sensitivity	40.0 (6/15)
Specificity	60.0 (3/5)
Accuracy	45.0 (9/20)

Lab C		Immunotoxicity		Total
		+	-	
IL-1 Luc assay	+	8	2	10
	-	7	3	10
Total		15	5	20

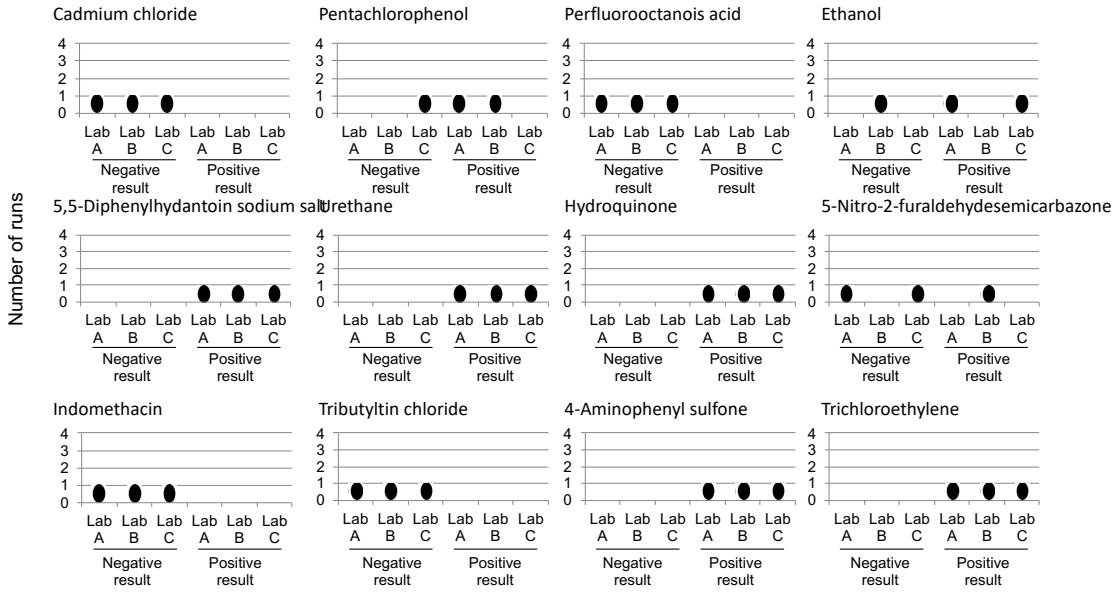
Sensitivity	53.3 (8/15)
Specificity	60.0 (3/5)
Accuracy	55.0 (11/20)

A graphical presentation of between- and within-laboratory variation in the Phase II study is shown in Fig. 11.





Between-laboratory reproducibility



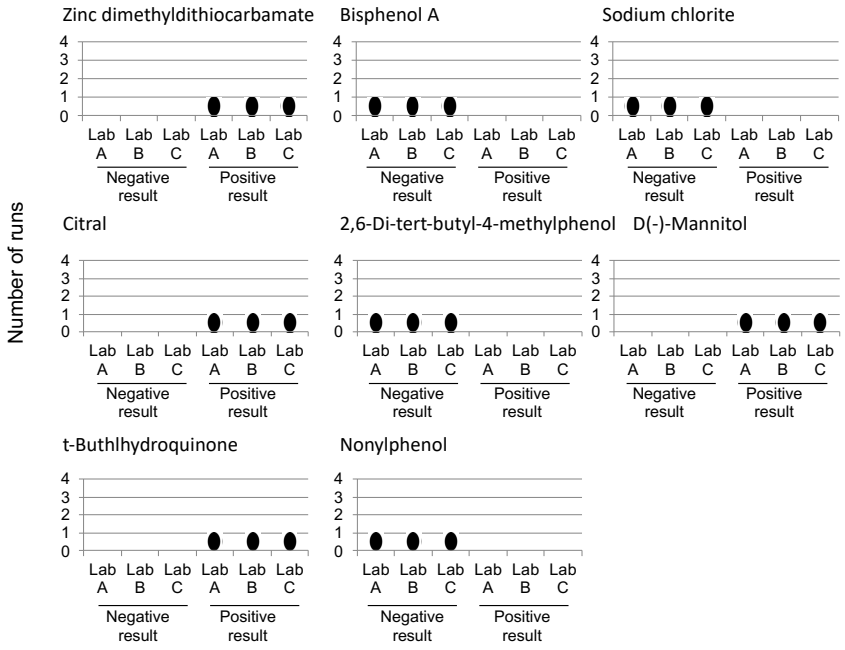


Fig. 11. Between-laboratory variation assessments in Phase II study

The Phase II study examined between-laboratory reproducibility using a total of 20 coded chemicals (15 immunotoxic chemicals and five non-immunotoxic chemicals) evaluated by one experiment set based on Multi-ImmunoTox Assay protocol for THP-G1b ver. 009E. Closed circles represent the judgments for individual experiments for within-laboratory reproducibility or represent the judgments in individual experimental sets for between-laboratory reproducibility.

9-5. Quality assurance

No accidents occurred during the course of the validation study, and all test facilities returned the MSDSs for the test chemicals to JaCVAM in sealed envelopes upon completion of the validation study. All test chemicals were disposed of in compliance with the rules and regulations of the test facilities upon completion of the validation study.

All the records (data sheets and record sheets) from the participating laboratories were checked by Dr. Takashi Omori, Kobe University, and JaCVAM (See Appendix 17-1 and 17-2). The record sheets include “Reagent records, Solubility test, Cell culture records, Test records and Data sheets”. They are more than 300 pages long in total and

are available at the JaCVAM website (http://www.jacvam.jp/validation08-login.html). Tests performed as part of a validation study were carried out in accordance with the principles of GLP (OECD 1998) and necessarily included, but were not limited to, the use of protocol and adequate recording of data as well as suitable reporting of results and archival record keeping.

Cell culture, preparation and application of the test chemicals, and generation of the data sheets were completed and the results accurately reflected the raw data. Unfortunately, the record sheets for maintenance of the measuring instruments were not collected prior to the validation study. JaCVAM thus had concerns regarding the quality of the data in the validation study. However, JaCVAM checked carefully all the results and judged all data to be within the acceptable range.

The reliability of the measuring instruments was checked by an independent organization before the validation study. JaCVAM recommended to the validation management team that the formal validation study be conducted with GLP laboratories.

9-6. Combined results of Phase I and II studies (for between- and within-laboratory reproducibility and predictive capacity)

9-6-1. Test conditions

The within- and between-laboratory reproducibilities, and the predictivity of the IL-1 Luc assay, were evaluated using all the results from Phases I and II.

9-6-2. Within- and between-laboratory variation assessments from Phase I and II studies.

Between-Lab reproducibility 84.0% (21/25)

Within-Lab reproducibility

Lab. A 100.0% (5/5)

Lab. B 100% (5/5)

Lab. C 100.0% (5/5)

Average 100.0% (15/15)

9-6-3. Predictivity in Phases I and II studies

Accuracy of Lab. A 40.0% (10/25)

Accuracy of Lab. B 44.0% (11/25)

Accuracy of Lab. C 52.0% (13/25)

Average 45.3% (34/75)

Table 19. Combined results of Phase I and II studies

Chemical	CAS	Lab.A	Lab.B	Lab.C	Concordance	Immunotoxicity
Phase I						
Dibutyl phthalate	84-74-2	PPP	PPP	PPP	1	Yes
Acetaminophen	103-90-2	NNN	NNN	NNN	1	Yes
Isonicotinic acid hydrazide (Isoniazid)	54-85-3	NNN	NNN	NNN	1	Yes
Sulem Mercury(II) chloride	7487-94-7	PPP	PPP	PPP	1	Yes
Hexachlorobenzene	118-74-1	NNN	NNN	NNN	1	Yes
Phase II						
Cadmium chloride	10108-64-2	P	P	P	1	Yes
5,5-Diphenylhydantoin sodium salt	630-93-3	N	N	N	1	Yes
Indomethacin	53-86-1	N	N	P	0	Yes
Pentachlorophenol	87-86-5	N	N	P	0	Yes
Urethane	51-79-6	N	N	N	1	Yes
Tributyltin chloride	1461-22-9	N	N	N	1	Yes

Perfluorooctanoic acid	335-67-1	P	P	P	1	Yes
Hydroquinone	123-31-9	N	N	N	1	Yes
4-Aminophenyl sulfone	80-08-0	P	P	P	1	Yes
Ethanol	64-17-5	N	N	N	1	Yes
5-Nitro-2-furaldehydesemicarbazone	59-87-0	N	N	N	1	No
Trichloroethylene	79-01-6	N	N	N	1	No
Zinc dimethyldithiocarbamate	137-30-4	N	P	N	0	Yes
Citral	5392-40-5	P	P	P	1	Yes
t-Buthlhydroquinone	1948-33-0	P	P	P	1	No
Bisphenol A	80-05-7	P	P	P	1	Yes
2,6-Di-tert-butyl-4-methylphenol	128-37-0	N	N	P	0	Yes
Nonylphenol	84852-15-3	N	N	N	1	Yes
Sodium chlorite	7758-19-2	P	P	P	1	No
D(-)-Mannitol	69-65-8	N	N	N	1	No
Within-laboratory reproducibilities (%)		100.0 (5/5)	100.0 (5/5)	100.0 (5/5)		
		Average 100.0 (15/15)				
Between-laboratory reproducibility (%) (Based on majority for Phase I)					84.0 (21/25)	

Sensitivity (%)	35.0 (7/20)	40.0 (8/20)	50.0 (10/20)		
	Average 41.7 (25/60)				
Specificity (%)	60.0 (3/5)	60.0 (3/5)	60.0 (3/5)		
	Average 75.0 (9/15)				
Accuracy (%)	40.0 (10/25)	44.0 (11/25)	52.0 (13/25)		
	Average 45.3 (34/75)				

P: Positive, N: No effect

9-6-4. Contingency tables for Phase I and II studies

Lab A		Immunotoxicity		Total
		+	-	
IL-1 Luc assay	+	7	2	9
	-	13	3	16
Total		20	5	25

Sensitivity: 35.0% (7/20)

Specificity: 60.0% (3/5)

Accuracy: 40.0% (10/25)

Lab B		Immunotoxicity		Total
		+	-	
IL-1 Luc assay	+	8	2	10
	-	12	3	15
Total		20	5	25

Sensitivity: 40.0% (8/20)

Specificity: 60.0% (3/5)

Accuracy: 44.0% (11/25)

Lab C		Immunotoxicity		Total
		+	-	
IL-1 Luc assay	+	10	2	12
	-	10	3	13
Total		20	5	25

Sensitivity: 50.0% (10/20)

Specificity: 60.0% (3/5)

Accuracy: 52.0% (13/25)

10. Discussion

10-1. Reliability

The IL-1 Luc assay is based on the modulation of LPS-induced luciferase activity in the IL-1β reporter cell line, THP-G1b. Therefore, it is important that THP-G1b cells retain their ability to induce luciferase activity after LPS stimulation even after passage for a sufficient number of times to perform the assay in the long term. We confirmed that a frozen stock of THP-G1b cells can be cultured without losing luciferase activity for at least 16 weeks or 33 passages.

Culturing of THP-G1b cells is relatively simple and does not require the use of trypsin or EDTA because THP-G1b cells do not adhere to the culture dishes. First, chemicals at graded concentrations are added to the wells of a 96-well culture plate. Then, cells adjusted to the optimum concentration are seeded into each well. After 6 h incubation, 100 μL of pre-warmed Tripluc Luciferase Assay Reagent is added to each of the 96 wells. The subsequent process is completely automated, except for calculating the results using the predesigned Excel spreadsheet. Therefore, the IL-1 Luc assay is a test method that can significantly reduce human error.

Moreover, the IL-1 Luc assay does not require the determination of cell viability after chemical treatment. THP-G1b cells can present IL-1β promoter activity as well as promoter activity of GAPDH, a well-known housekeeping gene; therefore, information regarding the effects of the chemical on both IL-1β promoter activity and cell viability is obtained in each experiment. Furthermore, a single experiment takes only 8 h, including the time required for chemical preparation and cell plating, indicating that the IL-1 Luc assay is a true high-throughput method.

10-2. Between- and within-laboratory reproducibility

We examined within-laboratory reproducibility in the Phase I study. Lab A, Lab B, and Lab C demonstrated 100%, 100%, and 100% reproducibility, respectively. On the other hand, the between-laboratory reproducibility result for Lab A, Lab B, and Lab C

was 84.0% for the combined data of the Phase I and Phase II studies. These results satisfied the acceptance criteria for the validation study of a within-laboratory reproducibility of at least 80% and a between-laboratory reproducibility of at least 80%.

10-3. Predictivity

10-3-1. Predictivity of Phase I and Phase II studies

As described in the Background section, the immunotoxicity test can target global immunotoxicity or a specific immune response. Since IL-1β has pleiotropic functions in immune response, it could potentially be used as a global immunotoxicity test. Therefore, we first examined the predictivity of the IL-1 Luc assay in detecting immunosuppressants. According to the procedure used to determine the predictivity of the IL-2 Luc assay (Kimura et al. 2020), we first constructed reference data for making positive or negative judgments by collecting literature information on the eight endpoints for each chemical and identifying chemicals that satisfied one of the three criteria for immunotoxic chemicals. Based on these reference data, the IL-1 Luc assay performance was 41.7% for mean sensitivity, 60.0% for mean specificity, and 45.3% for mean prediction for the combined data from the Phase 1 and Phase 2 studies. These data suggest that the IL-1 Luc assay alone cannot detect chemicals with global immunotoxicity and must be used in combination with other global immunotoxicity tests.

10-3-2. IL-1 Luc assay data set for 63 chemicals

To clarify the characteristics of the IL-1 Luc assay, the lead laboratory evaluated the data for 60 chemicals previously evaluated by the IL-2 Luc assay (Kimura et al. 2020) and three new chemicals added in this study, and evaluated by the IL-1 Luc assay. To determine the performance of the IL-1 Luc assay for these 63 chemicals, we referred to reference data generated by collecting literature information on the eight endpoints for each chemical (Appendixes 12 and 13) and identifying chemicals that satisfied at least one of the three criteria as immunotoxic chemicals. The summarized

immunotoxicity information, together with the classification of each chemical, are shown in Appendixes 14 and 15. The judgment of these chemicals by the IL-1 Luc assay is shown in Table 20, and the judgment by the IL-2 Luc assay is also shown for comparison. The performance of the IL-1 Luc assay was 53.1% for sensitivity, 35.7% for specificity, and 49.2% for predictivity (Table 21). Again, these data suggest that the IL-1 Luc assay alone is not sufficient to detect chemicals with global immunotoxicity. On the other hand, the performance of the IL-2 Luc assay was 69.4% for sensitivity, 21.4% for specificity, and 58.7% for predictivity, when the same reference data used for the IL-1 Luc assay was applied. Although the performance of the IL-2 Luc assay is better than that of the IL-1 Luc assay, it is also insufficient to be used as an immunotoxicity test alone. Next, we examined the performance of the combination of the IL-1 Luc assay and the IL-2 Luc assay. It improved the performance of the IL-2 Luc assay from 69.4% to 73.5% for sensitivity and from 58.7% to 61.9% for predictability (Table 22 and 23). Even after combining these two assays, its performance is insufficient to cover the immunotoxicity of whole chemicals. Probably, the performance of the assay will be improved by the IATA combining various immunotoxicity tests that target different aspects of our immune responses step by step. The combination of the IL-1 Luc assay and the IL-2 Luc assay can be the first step of this IATA approach.

Next, we compared the judgment for 63 chemicals by the IL-1 Luc assay with that by the IL-2 Luc assay and found that 31 of 33 chemicals that were judged as exhibiting suppression by the IL-1 Luc assay were also judged as exhibiting either suppression or augmentation by the IL-2 Luc assay. In contrast, 31 of 47 chemicals that were judged as exhibiting either suppression or augmentation were judged as exhibiting suppression by the IL-1 Luc assay.

This significant overlap of suppression judgment is likely due to the signaling pathway of TLR4 stimulation to induce IL-1β mRNA being at least partially shared by the signaling pathway for PMA/Io stimulation to induce IL-2 mRNA.

As described in section 3-2-2, LPS stimulation leads to the activation of several transcription factors, including NF-κB, CREB/ATF, c/EBPb, AP1 and IRF3 (reviewed

by Newton et al. (Newton and Dixit 2012)). Although the relative contributions of these factors to IL-1β transcription are currently unclear, the IL-1 Luc assay is considered a test method for finding chemicals that primarily block the NF-κB, AP1 and p38a MAPK signaling cascades after TLR4 receptor stimulation.

On the other hand, the simultaneous stimulation of PMA and calcium ionophore or ionomycin is known to mimic the stimulation by T cell receptor (TCR) and CD28 (Kumagai et al. 1987; Truneh et al. 1985; (Gholijani et al. 2015). In addition, the signaling pathways leading to IL-2 transcription after PMA/Io stimulation overlap significantly with the signaling pathways leading to IL-2 transcription after T cell receptor and CD28 stimulation. In fact, both stimulations ultimately activate NF-AT, c-Fos, c-Jun and NF-κB (Gholijani et al. 2015; Lee et al. 1994).

These data suggest that TLR4 stimulation and PMA/CI stimulation share at least a part of the signaling pathway leading to the activation of NF-κB. Indeed, TLR4, TCR/CD28 and PMA stimulation is known to activate the canonical pathway of NF-κB (Meininger and Krappmann 2016; Vallabhapurapu and Karin 2009; Wang et al. 2011). Therefore, it is quite reasonable that the two assays share the same judgments for a significant number of chemicals (Fig. 12).

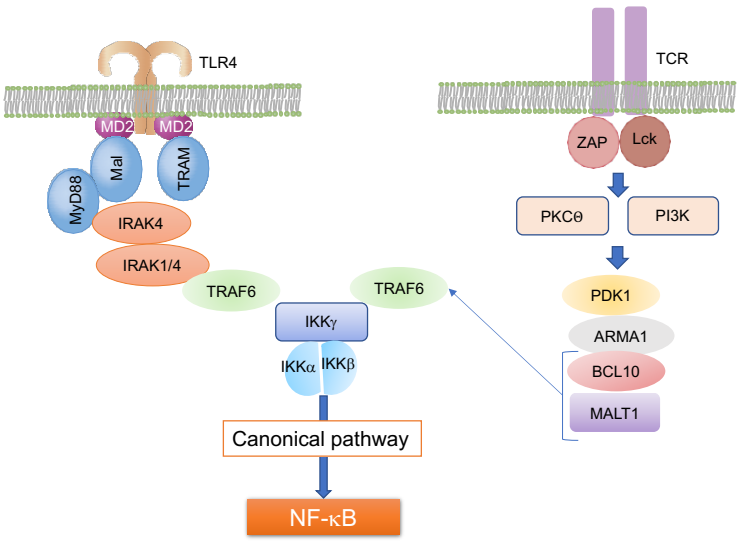


Fig. 12. The signaling cascade of TLR4 stimulation and TCR stimulation leading to NF-κB

Table 20. Judgments for 63 chemicals evaluated by IL-1 Luc and IL-2 Luc assays

Chemical	IL-2		IL-1β		
	Judgment	LOEL (μg/mL)	Judgment	LOEL (μg/mL)	Immunotoxicity judgment
PF06650833	S	0.451	P	0.00176	Positive
TAK-242	N		P	0.014	Positive
Actinomycin D	S	0.02	P	0.13	Positive
FR167653	S	1.30	P	0.49	Positive
Digoxin	S	0.07	P	0.59	Negative
Dexamethasone	S	41.67	P	0.98	Positive
Amphoterycin B	S	2.60	P	1.17	Positive
Mercuric chloride	S	1.95	P	1.95	Positive
VIPER	N		P	2.71	Positive
Chlorpromazine	S	1.95	P	3.91	Negative
Isophorone diisocyanate	S	7.81	P	3.91	Positive
Citral	S	25.00	P	4.88	Positive
2-Aminoanthracene	S	5.86	P	11.72	Negative
Dibutyl phthalate	S	2.60	P	15.63	Positive
Chloroplatinic acid	S	250.00	P	23.44	Positive
Chloroquine	S	17.83	P	39.06	Positive
Diesel exhaust particles	S	62.50	P	39.06	Positive
Azathioprine	S	58.48	P	41.55	Positive
Sulfasalazine	S	92.94	P	44.81	Negative
Cobalt chloride	S	16.93	P	46.88	Negative
Cisplatin	S	16.93	P	46.88	Positive
Minocycline	S	18.52	P	62.50	Positive
Sodium dodecyl sulfate	S	62.50	P	62.50	Positive
Pentamidine isethionate	S	52.08	P	64.45	Positive
Mycophenolicacid	A	0.40	P	72.00	Positive
2-Mercaptobenzothiazole	A	16.11	P	93.75	Positive
Dapsone	S	72.92	P	125.00	Positive
p-Nitroaniline	S	83.33	P	125.00	Negative
Diethanolamin	S	250.00	P	333.33	Positive
Hydrogen peroxide	S	23.44	P	375.00	Negative
Nickel sulfate	S	104.17	P	375.00	Positive
Sodium bromate	S	500.00	P	500.00	Negative
Ribavirin	A	26.04	P	750.00	Positive
Triethanolamine	S	1333.33	P	1000.00	Negative
FK506	S	0.0002	N		Positive
Cyclosporine A	S	0.0041	N		Positive
Colchicine	S	0.27	N		Negative
Benzethonium chloride	S	1.63	N		Negative
Formaldehyde	S	7.81	N		Positive
Pyrimethamine	S	7.81	N		Positive
Mitomycin C	S	20.00	N		Positive
Lead(II) acetate	S	57.29	N		Positive
Nitrofurazone	S	83.33	N		Negative
Aluminum chloride	S	104.17	N		Positive
Histamine	S	750.00	N		Positive
Isoniazid	S	1000.00	N		Positive
Magnesium sulfate	S	2000.00	N		Positive
Warfarin	N		N		Positive
Hydrocortisone	N		N		Positive
Lithium carbonate	N		N		Positive
2,4-Diaminotoluene	N		N		Positive
Dibenzopyrene	N		N		Negative
Cyclophosphamide	N		N		Positive
Ethanol	N		N		Positive
Methanol	N		N		Positive
Hexachlorobenzene	N		N		Positive
Trichloroethylene	N		N		Negative
Methotrexate	N		N		Positive
Rapamycin	N		N		Positive
Mizoribine	N		N		Positive
Acetaminophen	A	100.00	N		Positive
Nicotinamide	A	288.07	N		Positive
Dimethyl sulfoxide	A	2000.00	N		Positive
LOEL: lowest observed effect level					

Table 21. The performance of the IL-1 Luc assay, the IL-2 Luc assay and the combination

IL-1 Luc assay	Immunosuppression		
	Positive	Negative	Total
Positive	26	9	35
Negative	23	5	28
Total	49	14	63

Sensitivity	0.531
Specificity	0.357
Predictivity	0.492

IL-2 Luc assay	Immunosuppression		
	Positive	Negative	Total
Positive	34	11	45
Negative	15	3	18
Total	49	14	63

Sensitivity	0.694
Specificity	0.214
Predictivity	0.587

IL-1 Luc assay + IL-2 Luc assay	Immunosuppression		
	Positive	Negative	Total
Positive	36	11	47
Negative	13	3	16
Total	49	14	63

Sensitivity	0.735
Specificity	0.214
Predictivity	0.619

10-4. IL-1 Luc assay as novel assay for evaluating effects of chemicals on TLR4 receptor and IRAK4.

Although TLR4 and PMA/Io stimulate the canonical pathway of NF-κB, their upstream signaling to activate the canonical pathway differ. TRAF6 used to activate the canonical pathway by TLR4 stimulation is likely involved in the activation of the canonical pathway by T cell receptor activation (Sun et al. 2004; Vallabhapurapu and Karin 2009), and thus upstream signaling to stimulate TRF6 can be different between TLR4 stimulation and PMA/Io stimulation (Fig. 12).

Indeed, the signaling cascade leading to TRAF6 activation after TLR4 stimulation comprises at least TLR4, Mal, TRAM, Myd88, IRAK4 and IRAK1/2, which are not shared by TCR signaling. IRAK4 is a key signaling node for transducing the responses of the interleukin-1 (IL-1) receptor family (IL-1, IL-18, and IL-33 receptors), and TLRs (except for TLR3) have recently attracted widespread attention as therapeutic targets for inflammation and tumor diseases (Chaudhary et al. 2015). We therefore examined the effects of TAK-242 and VIPER, inhibitors of TLR4 (Matsunaga et al. 2011) (Lysakova-Devine et al. 2010) and of PF06650833, an inhibitor of IRAK4 (Lee et al. 2017) using the IL-1 Luc assay and the IL-2 Luc assay. As expected, both inhibitors significantly suppressed IL1LA, suggesting the utility of the IL-1 Luc assay in identifying chemicals that block TLR4 signaling upstream of TRAF6 activation. Although PF06650833 significantly suppressed IL2LA in the IL-2 Luc assay, the LOWEL to suppress IL2LA in the IL-2 Luc assay was 400 times higher than that to suppress IL1LA in the IL-1 Luc assay. Consistently with the finding, the role of IRAK4 in T-cell receptor signaling to activate NF-κB has been reported (Suzuki and Saito 2006).

Currently there is no high-throughput approach to detect the effects of chemicals or drugs that target TLR4, Mal, TRAM, Myd88, IRAK4 or IRAK1/2. In addition, some of these molecules are also used in the signaling cascade after the stimulation of other TLRs and IL-1R (Suzuki and Saito 2006). The IL-1 Luc assay is a promising tool for detecting the immunotoxicity of chemicals that target these molecules.

10-5. Factors responsible for false negative results in IL-1 Luc assay

Although the within- and between-laboratory reproducibility results satisfied the acceptance criteria for the validation study, the predictivity was far less than 80%, likely because the IL-1 Luc assay does not cover every aspect of the effects of the chemicals on the global immune response. In addition, like other *in vitro* tests, the IL-1 Luc assay does not have metabolic activity and cannot evaluate water insoluble chemicals.

10-6. Applicability domain and limitations of IL-1 Luc assay

The IL-1 Luc assay is not intended to evaluate global immunotoxicity alone. This assay can evaluate the effects of chemicals that affect the signaling pathway to activate NF-κB, AP1 and p38a MAPK in innate immune cells, especially after TLR4 receptor stimulation. Chemicals that potentially induce immunosuppression by other signaling pathways are not suitable for the IL-1 Luc assay. In addition, chemicals that require metabolic activation or are water insoluble fall outside the applicability domain.

10-7. Regulatory application of the IL-1 Luc assay

The CAS REGISTRY^{SM} currently contains more than 130 million unique organic and inorganic chemical substances, such as alloys, coordination compounds, minerals, mixtures, polymers, and salts. Humans are exposed to many of these substances, which are present as environmental contaminants or used as food additives and drugs. Some of these compounds can target the immune system, resulting in adverse health effects such as the development of allergies, autoimmune disorders, increased susceptibility to infection and cancer, and other diseases. Accordingly, immunotoxicity, which is defined as the toxicological effects of xenobiotics on the function of the immune system, is a matter of serious concern to the public as well as regulatory agencies. To address these concerns, the World Health Organization published its Guidance for Immunotoxicity Risk Assessment for Chemicals (World Health Organization (WHO). Currently, the assessment of chemical immunotoxicity relies mainly on animal models and assays that

characterize immunosuppression and sensitization. However, animal studies have so many drawbacks, such as high cost, ethical concerns, and questionable relevance to risk assessment for humans, that they cannot be used to screen the immunotoxicity of more than 130 million chemicals. Therefore, there is an urgent need to develop alternative testing methods and assessment strategies to reduce the use of laboratory animals and, if possible, replace animals used in scientific studies (Adler et al. 2011). To date, however, there are no OECD test guidelines to detect chemical immunotoxicity *in vitro*. We would therefore like to propose the IL-1 Luc assay, and the MITA in the near future, as a screening toolbox of alternative test methods for immunotoxicity. Finally, the VMT recommend that the proficiency chemicals (Appendix 18) to users and the performance standard chemicals (Appendix 19) to me-too validation study.

11. Conclusion

Using three luciferase reporter cell lines, we established the MITA, in which the effects of chemicals on IL-2 and IFN-γ promoter activity of 2H4 cells and those on IL-1β and IL-8 promoter activity of THP-G1b and THP-G8 cells can be evaluated. Here, we conducted a validation study of the IL-1 Luc assay among the four luciferase assays that comprise the MITA. The results of both Phase I and Phase II studies satisfied the acceptance criteria for the validation study. Although a predictivity of 80% was not attained, it may nonetheless be acceptable when considering the applicability domain , limited targets of the IL-1 Luc assay, and future use as one of the components of the IATA. We would therefore like to propose the IL-1 Luc assay as the OECD test guideline for *in vitro* immunotoxicity tests.

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13. References

Adler S, Basketter D, Creton S, et al. (2011) Alternative (non-animal) methods for cosmetics testing: current status and future prospects-2010. Arch Toxicol 85(5):367-485 10.1007/s00204-011-0693-2

Allison AC (2000) Immunosuppressive drugs: the first 50 years and a glance forward. Immunopharmacology 47(2-3):63-83

Bent R, Moll L, Grabbe S, Bros M (2018) Interleukin-1 Beta-A Friend or Foe in Malignancies? Int J Mol Sci 19(8) 10.3390/ijms19082155

Chaudhary D, Robinson S, Romero DL (2015) Recent advances in the discovery of small molecule inhibitors of interleukin-1 receptor-associated kinase 4 (IRAK4) as a therapeutic target for inflammation and oncology disorders. J Med Chem 58(1):96-110 10.1021/jm5016044

Corsini E, Roggen E (2017) Overview of in vitro assessment of immunotoxicity. DOI:101016/jcotox201706016

Fenini G, Contassot E, French LE (2017) Potential of IL-1, IL-18 and Inflammasome Inhibition for the Treatment of Inflammatory Skin Diseases. Frontiers in pharmacology 8:278 10.3389/fphar.2017.00278

Finch-Arietta MB, Cochran FR (1991) Cytokine production in whole blood ex vivo. Agents Actions 34(1-2):49-52 10.1007/BF01993235

Fuchs O (2010) Transcription factor NF-kappaB inhibitors as single therapeutic agents or in combination with classical chemotherapeutic agents for the treatment of hematologic malignancies. Curr Mol Pharmacol 3(3):98-122 10.2174/1874467211003030098

Galbiati V, Mitjans M, Corsini E (2010) Present and future of in vitro immunotoxicology in drug development. Journal of immunotoxicology 7(4):255-67 10.3109/1547691X.2010.509848

Gennari A, Ban M, Braun A, et al. (2005) The Use of In Vitro Systems for Evaluating Immunotoxicity: The Report and Recommendations of an ECVAM Workshop. Journal of immunotoxicology 2(2):61-83 10.1080/15476910590965832

Gholijani N, Gharagozloo M, Kalantar F, Ramezani A, Amirghofran Z (2015) Modulation of Cytokine Production and Transcription Factors Activities in Human Jurkat T Cells by Thymol and Carvacrol. Adv Pharm Bull 5(Suppl 1):653-60 10.15171/apb.2015.089

Handa P, Vemulakonda A, Kowdley KV, Uribe M, Mendez-Sanchez N (2016) Mitochondrial

DNA from hepatocytes as a ligand for TLR9: Drivers of nonalcoholic steatohepatitis? World J Gastroenterol 22(31):6965-71 10.3748/wjg.v22.i31.6965

Horino T, Matsumoto T, Ishikawa H, et al. (2009) Interleukin-1 deficiency in combination with macrophage depletion increases susceptibility to Pseudomonas aeruginosa bacteremia. Microbiol Immunol 53(9):502-11 10.1111/j.1348-0421.2009.00143.x

Isaacs JD, Burmester GR (2020) Smart battles: immunosuppression versus immunomodulation in the inflammatory RMDs. Ann Rheum Dis 79(8):991-993 10.1136/annrheumdis-2020-218019

Juffermans NP, Florquin S, Camoglio L, et al. (2000) Interleukin-1 signaling is essential for host defense during murine pulmonary tuberculosis. J Infect Dis 182(3):902-8 10.1086/315771

Kimura Y, Fujimura C, Ito Y, Takahashi T, Aiba S (2014) Evaluation of the Multi-ImmunoTox Assay composed of 3 human cytokine reporter cells by examining immunological effects of drugs. Toxicol In Vitro 28(5):759-68 10.1016/j.tiv.2014.02.013

Kimura Y, Fujimura C, Ito Y, Takahashi T, Terui H, Aiba S (2018) Profiling the immunotoxicity of chemicals based on in vitro evaluation by a combination of the Multi-ImmunoTox assay and the IL-8 Luc assay. Arch Toxicol 92(6):2043-2054 10.1007/s00204-018-2199-7

Kimura Y, Yasuno R, Watanabe M, et al. (2020) An international validation study of the IL-2 Luc assay for evaluating the potential immunotoxic effects of chemicals on T cells and a proposal for reference data for immunotoxic chemicals. Toxicol In Vitro 66:104832 10.1016/j.tiv.2020.104832

Labow M, Shuster D, Zetterstrom M, et al. (1997) Absence of IL-1 signaling and reduced inflammatory response in IL-1 type I receptor-deficient mice. J Immunol 159(5):2452-61

Langezaal I, Hoffmann S, Hartung T, Coecke S (2002) Evaluation and prevalidation of an immunotoxicity test based on human whole-blood cytokine release. Alternatives to laboratory animals : ATLA 30(6):581-95

Lankveld DP, Van Loveren H, Baken KA, Vandebriel RJ (2010) In vitro testing for direct immunotoxicity: state of the art. Methods in molecular biology 598:401-23 10.1007/978-1-60761-401-2_26

Lee KL, Ambler CM, Anderson DR, et al. (2017) Discovery of Clinical Candidate 1-{[(2S,3S,4S)-3-Ethyl-4-fluoro-5-oxopyrrolidin-2-yl]methoxy}-7-methoxyisoquinoli ne-

6-carboxamide (PF-06650833), a Potent, Selective Inhibitor of Interleukin-1 Receptor Associated Kinase 4 (IRAK4), by Fragment-Based Drug Design. J Med Chem 60(13):5521-5542 10.1021/acs.jmedchem.7b00231

Luster AD (1998) Chemokines--chemotactic cytokines that mediate inflammation. New England Journal of Medicine 338(7):436-45

Luster MI, Portier C, Pait DG, et al. (1993) Risk assessment in immunotoxicology. II. Relationships between immune and host resistance tests. Fundam Appl Toxicol 21(1):71-82

Lysakova-Devine T, Keogh B, Harrington B, et al. (2010) Viral inhibitory peptide of TLR4, a peptide derived from vaccinia protein A46, specifically inhibits TLR4 by directly targeting MyD88 adaptor-like and TRIF-related adaptor molecule. J Immunol 185(7):4261-71 10.4049/jimmunol.1002013

Matsunaga N, Tsuchimori N, Matsumoto T, Ii M (2011) TAK-242 (resatorvid), a small-molecule inhibitor of Toll-like receptor (TLR) 4 signaling, binds selectively to TLR4 and interferes with interactions between TLR4 and its adaptor molecules. Mol Pharmacol 79(1):34-41 10.1124/mol.110.068064

McGuire VA, Ruiz-Zorrilla Diez T, Emmerich CH, et al. (2016) Dimethyl fumarate blocks pro-inflammatory cytokine production via inhibition of TLR induced M1 and K63 ubiquitin chain formation. Sci Rep 6:31159 10.1038/srep31159

Meininger I, Krappmann D (2016) Lymphocyte signaling and activation by the CARMA1-BCL10-MALT1 signalosome. Biol Chem 397(12):1315-1333 10.1515/hsz-2016-0216

Michelini E, Cevenini L, Calabretta MM, Calabria D, Roda A (2014) Exploiting in vitro and in vivo bioluminescence for the implementation of the three Rs principle (replacement, reduction, and refinement) in drug discovery. Anal Bioanal Chem 406(23):5531-9 10.1007/s00216-014-7925-2

Mucke HA (2012) Iguratimod: a new disease-modifying antirheumatic drug. Drugs Today (Barc) 48(9):577-86 10.1358/dot.2012.48.9.1855758

Nakae S, Asano M, Horai R, Sakaguchi N, Iwakura Y (2001) IL-1 enhances T cell-dependent antibody production through induction of CD40 ligand and OX40 on T cells. J Immunol 167(1):90-7 10.4049/jimmunol.167.1.90

Nakajima Y, Ohmiya Y (2010) Bioluminescence assays: multicolor luciferase assay, secreted luciferase assay and imaging luciferase assay. Expert Opin Drug Discov 5(9):835-49 10.1517/17460441.2010.506213

Nambu A, Nakae S, Iwakura Y (2006) IL-1beta, but not IL-1alpha, is required for antigen-specific T cell activation and the induction of local inflammation in the delayed-type hypersensitivity responses. International Immunology 18(5):701-12 dxl007 [pii]
10.1093/intimm/dxl007 [doi]

Nelson RP, Jr., Ballow M (2003) 26. Immunomodulation and immunotherapy: drugs, cytokines, cytokine receptors, and antibodies. J Allergy Clin Immunol 111(2 Suppl):S720-43 10.1067/mai.2003.146

Newton K, Dixit VM (2012) Signaling in innate immunity and inflammation. Cold Spring Harb Perspect Biol 4(3) 10.1101/cshperspect.a006049

Peng H, Guerau-de-Arellano M, Mehta VB, et al. (2012) Dimethyl fumarate inhibits dendritic cell maturation via nuclear factor kappaB (NF-kappaB) and extracellular signal-regulated kinase 1 and 2 (ERK1/2) and mitogen stress-activated kinase 1 (MSK1) signaling. The Journal of biological chemistry 287(33):28017-26 10.1074/jbc.M112.383380

Roda A, Pasini P, Mirasoli M, Michelini E, Guardigli M (2004) Biotechnological applications of bioluminescence and chemiluminescence. Trends Biotechnol 22(6):295-303 10.1016/j.tibtech.2004.03.011

Saito R, Hirakawa S, Ohara H, et al. (2011) Nickel differentially regulates NFAT and NF-kappaB activation in T cell signaling. Toxicology and applied pharmacology 254(3):245-55 10.1016/j.taap.2011.04.017

Satoskar AR, Okano M, Connaughton S, Raisanen-Sokolwski A, David JR, Labow M (1998) Enhanced Th2-like responses in IL-1 type 1 receptor-deficient mice. Eur J Immunol 28(7):2066-74 10.1002/(SICI)1521-4141(199807)28:07<2066::AID-IMMU2066>3.0.CO;2-X

Sun L, Deng L, Ea CK, Xia ZP, Chen ZJ (2004) The TRAF6 ubiquitin ligase and TAK1 kinase mediate IKK activation by BCL10 and MALT1 in T lymphocytes. Mol Cell 14(3):289-301 10.1016/s1097-2765(04)00236-9

Suzuki N, Saito T (2006) IRAK-4--a shared NF-kappaB activator in innate and acquired immunity. Trends Immunol 27(12):566-72 10.1016/j.it.2006.10.003

Takahashi T, Kimura Y, Saito R, et al. (2011) An in vitro test to screen skin sensitizers using a stable THP-1-derived IL-8 reporter cell line, THP-G8. Toxicological sciences : an official journal of the Society of Toxicology 124(2):359-69 10.1093/toxsci/kfr237

Vallabhapurapu S, Karin M (2009) Regulation and function of NF-kappaB transcription factors

in the immune system. Annu Rev Immunol 27:693-733 10.1146/annurev.immunol.021908.132641

Vincent JA, Mohr S (2007) Inhibition of caspase-1/interleukin-1beta signaling prevents degeneration of retinal capillaries in diabetes and galactosemia. Diabetes 56(1):224-30 10.2337/db06-0427

Wagner W, Walczak-Drzewiecka A, Slusarczyk A, Biecek P, Rychlewski L, Dastych J (2006) Fluorescent Cell Chip a new in vitro approach for immunotoxicity screening. Toxicology letters 162(1):55-70 10.1016/j.toxlet.2005.10.017

Wang Y, Huang Z, Wang L, et al. (2011) The anti-malarial artemisinin inhibits pro-inflammatory cytokines via the NF-kappaB canonical signaling pathway in PMA-induced THP-1 monocytes. Int J Mol Med 27(2):233-41 10.3892/ijmm.2010.580

Wang Y, Tang Q, Duan P, Yang L (2018) Curcumin as a therapeutic agent for blocking NF-kappaB activation in ulcerative colitis. Immunopharmacol Immunotoxicol 40(6):476-482 10.1080/08923973.2018.1469145

Wheeler DS, Catravas JD, Odoms K, Denenberg A, Malhotra V, Wong HR (2004) Epigallocatechin-3-gallate, a green tea-derived polyphenol, inhibits IL-1 beta-dependent proinflammatory signal transduction in cultured respiratory epithelial cells. J Nutr 134(5):1039-44 10.1093/jn/134.5.1039

Xu F, Wang F, Wen T, Sang W, Wang D, Zeng N (2017) Inhibition of NLRP3 inflammasome: a new protective mechanism of cinnamaldehyde in endotoxin poisoning of mice. Immunopharmacol Immunotoxicol 39(5):296-304 10.1080/08923973.2017.1355377

Yang, Han Z, Oppenheim JJ (2017) Alarmins and immunity. Immunol Rev 280(1):41-56 10.1111/imr.12577

14. List of abbreviations

95% CI : the 95% confidence interval

AIST : National Institute of Advanced Industrial Science and Technology

AOP : Adverse outcome pathway

ARE: Antioxidant response element

CAS No. : Chemical Abstract Service Number

CMV : Cytomegalovirus

CSC : the Chemical Selection Committee

DMSO : Dimethyl sulphoxide

DPRA : the Direct Peptide Reactivity Assay

ECVAM : the European Centre for Validation of Alternative Methods

EDTA : Ethylenediaminetetraacetic acid

EGFR : Epidermal growth factor receptor

EGR-1 : Early growth response-1

EU : European Union

FBS : Fetal bovine serum

FN : False Negative Rate

GLP : Good laboratory Practice

GSH : Glutathione

HRI/FDSC : Hatano Research Institute, Food & Drug Safety Center

HSV : Herpes simplex viruses

ICCVAM : Interagency Coordinating Committee on the Validation of Alternative Methods

ID : Identification

IFN-γ : Interferon-γ

I.I.-SLR-LA : Inhibition index of SLR-LA

IL-2 : Interleukin-2

IL-8 : Interleukin-8

JaCVAM : the Japanese Center for the Validation of Alternative Methods

Keap-1 : Kelch-like ECH-associated protein 1

KoCVAM : Korean Center for the Validation of Alternative Methods

LLNA : Local lymph node assay

LPS : Lipopolysaccharide

MIT : Minimum induction threshold

MITA : Multi-Immuno Tox Assay

MoDCs : Monocyte-derived dendritic cells

MOVS: Management Office of Validation Study

mRNA : messenger ribonucleic acid

MSDS : Material safety data sheet

NICEATM : the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

NIHS : National Institute of Health Sciences

NPV : Negative predictive value

Nqo1 : NADPH-quinone oxidoreductase 1

Nrf2 : Nuclear factor (erythroid-derived 2)-like factor 2

nSLG-LA : normalized SLG luciferase activity

nSLO-LA : normalized SLO luciferase activity

OECD : the Organization for Economic Co-operation and Development

PCR : Polymerase chain reaction

PI : Propidium iodide

PMA/Io : Phorbol 12-myristate 13-acetate/Ionomycin

PN : False Positive Rate

PPV : Positive Predictive Value

QC : Quality Control

REACH : Registration, Evaluation, Authorization and Restriction of CHemicals

RFI : Relative fluorescence intensity

RT : Ring trial

SLG : Stable luciferase green

SLG-LA : SLG luciferase activity

SLO : Stable luciferase orange

SLO-LA : SLO luciferase activity

SLR : Stable luciferase red

SLR-LA : SLR luciferase activity

SLS : Sodium lauryl sulfate

SLR : Stable luciferase red

SLR-LA : SLR luciferase activity

SV40 : Simian virus 40

TG : Test Guideline

TNF-α : Tumor necrosis factor-α

UN GHS : the United Nations Globally Harmonized System of Classification and Labeling of Chemicals

VMT : Validation Management Team

Appendix 01 IL-1b Study plan Ver1.0

Version 1.0 September, 2018

Study plan for the validation trial on multicolor reporter assay using THP-G1b (TGCHAC-A4) (IL-1β Luc assay) as a test evaluating the immunotoxic potential of chemicals

Conducted by:

IL-1β Luc assay Validation Management Team

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1. Background

The use of multicolor reporter assay using THP-G1b (TGCHAC-A4), IL-1β Luc assay is an important for evaluating the immunotoxic potential of chemicals as a part of Multi-ImmunoTox assay (MITA), because of its technical simplicity, short-term test period and accuracy of test result based on a mechanism of immunotoxicity.

The aim of this trial is to (pre)validate the IL-1β Luc assay method to assess transferability and inter-laboratory variability, in order to incorporate this test for screening the immunotoxic chemicals. The IL-1β Luc assay for the validation trial will be undertaken i) in accordance with the principles and criteria documented in the OECD No. 34 Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment [OECD, 2005], ii) according to the Modular Approach to validation [Hartung et al., 2004] ,iii) according to the concept discussed on the validation trials with participation of GLP Test Facilities [Cooper-Hannan et al., 1999] where the whole concept of the validation trials is described in the context of GLP, iv) and in line with the ISO procedure JRC.I.03.GP.01v.01 (http://ihcpnet.jrc.it/quality-safety/quality-documents/unit-03-ivm/doc/JRC.I.03.GP.01v.01.pdf).

The studies part of a validation trial should ideally be performed in accordance with GLP [OECD, 1998-2007; FDA, 1999; EPA, 1998a&b; JSQA, 2010; SCC, 2010]. As a minimum, but not necessary limited, use of standard operating procedures (SOP), adequate data recording, reporting and record keeping are essential.

A general conceptional framework [Hartung et al., 2004; OECD, 2005] will be used

for documenting all the study to assess the validation status of a test method, called "modular approach" to validation. In this approach, the information needed to support the validity of the method is organized into modules that provide the following information:

Module 1: Test Definition

Module 2: Within-laboratory repeatability and reproducibility

Module 3: Between-laboratory transferability

Module 4: Between-laboratory reproducibility

Module 5: Predictive capacity

Module 6: Applicability domain

Module 7: Performance standards

The Modular approach as introduced by Hartung et al., allows using datasets from various data sources and studies. This advantage is used in the following proposal to assess the scientific validity of the IL-1β Luc assay. The IL-1β Luc assay for the validation trial has performed under the GLP principle.

2. Objective of the trial

The validation trial will assess the reliability (reproducibility within and between laboratories) and relevance (predictive capacity) of the IL-1β Luc assay with a challenging set of test substances (test items) for which high quality *in vitro and in vivo* data are available.

3. Validation Management Team (VMT)

The VMT encompasses collective expertise with the test, in the underlying science

and the scientific design, management and evaluation of a validation trial.

The VMT, which plays a central role overseeing the conduct of the validation trial, includes:

Table 1. Members for IL-1β Luc assay Validation Management Team

Name	Role and expertise	Affiliation
Trial Coordinator Hajime Kojima	VMT trial coordinator , Management of quality control	JaCVAM, NIHS, Japan (JaCVAM representative)
Lead Lab Yutaka Kimura* Setsuya Aiba*	*Developer of this assay Test method, expertise underlying science	Tohoku Univ., Japan
Takao Ashikaga	Chemical supplier	JaCVAM, NIHS, Japan (JaCVAM representative)
Takashi Omori	Data analysis, biostatistics dossier	Kobe Univ., Japan
International expert members		
EU liaison Emanuela Corcini	Test system expertise, validation expertise, immunotoxicity expertise	Milan Univ., Italy
EU liaison Erwin L. Roggen,	Test system expertise, validation expertise, immunotoxicity expertise	3Rs Management and Consulting ApS, Denmark
ICCVAM liaison Dori Germolec	Immunotoxicity expertise	NTP/NIEHS, USA
JSIT liason Tomoaki Inoue	Immunotoxicity expertise	Chugai Pharmaceutical Co., Ltd.

3.1 Participating Test Facilities

The laboratories participating in the trial are defined as follow:

Test Facility 1: Hatano Res. Inst., FDSC. Study Director (SD) : Kohji Yamakage

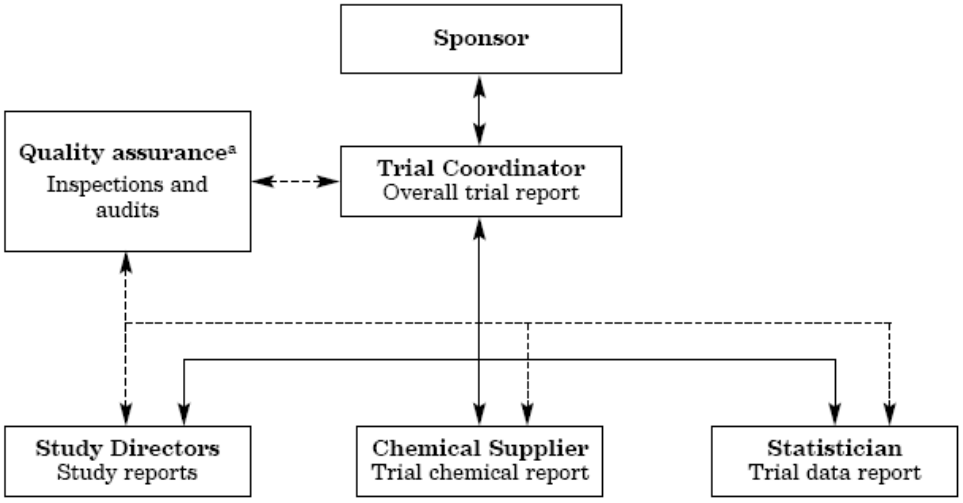
Test Facility 2: AIST, Tsukuba SD : Rie Yasuno

Test Facility 3: AIST, Takamatsu SD : Yoshihiro Nakajima

Information relevant for Modules 1, 2, 3 performed by all laboratories. Data obtained by these laboratories have demonstrated that the IL-1B Luc assay is transferable and reproducible between experienced laboratories. The all facility will be the laboratory participating in this validation trial acting as unexperienced laboratory to assess between laboratory transferability, reliability and relevance of the IL-1B Luc assay method under non-GLP conditions (GLP principle).

3.1 Trial management structure

The management structure of the validation trial is shown in **Figure 1**



[a]Several Quality Assurance units might be involved in a multi-study trial.
Dashed lines indicate assurance staff involvement.

Figure 1: Management Structure of the IL-1β **Luc assay validation trial**

1) Chemical management group

The members of chemical management group are elected by recommendation of the IL-1β Luc assay VMT. They prepare a tentative list of test chemicals and works with the VMT to make a final decision on the test chemicals to be used in the validation trial. The coded test chemicals listed are distributed by JaCVAM.

2) Data analysis group

The members of data analysis group are elected by recommendation of the IL-1β Luc assay VMT, and check and analyze the data obtained in this validation trial from a third-party standpoint. They also take charge of statistical processing in this validation trial.

3) Quality assurance group

The members of record management group are elected by recommendation of the

IL-1β Luc assay VMT. They prepare protocol, test chemical preparation record forms, blank data sheets, etc. and distributes them to the research laboratories participating in this validation trial. They also collect filled out forms and data sheets after completion of experiments, pointing out omissions or flaws in recording, if any, and requesting correction of such errors.

4) Lead laboratory

The lead laboratory representing the test method is responsible for providing the test method protocol and the eventually necessary data recording or calculation templates. The Trial Coordinator has to ensure that such data recording or calculation templates have been validated before distribution to the test facilities involved in the validation trial. The lead laboratory is also responsible for providing, if necessary, new versions of the protocols during the entire validation trial. The lead lab and the other participating test facilities might be contacted by the VMT for technical issues.

3.2 Sponsor

The validation trial for assessing the validity of IL-1β Luc assay will be financed by the Ministry of Health, Labour and Welfare (MHLW), Japan.

The lead laboratory will support the IL-1β Luc assay validation trial by assuring that reliability is assessed. At the same time, preliminary results of the test method can be evaluated. For this purpose, Lead laboratory will support:

- the financial aspects related to the coordination of a validation trial (e.g. organization of VMT meetings where also the involved test facilities can be invited for technical clarifications to the VMT, the publication of the validation

trial results)

- the test, reference and control item purchase, coding and distribution to the test facility
- the availability of the test systems to the participating laboratories by supporting the Lead laboratory with the logistics for delivering the test system to the facility
- the independent data analysis and statistical support (biostatistician) based on the study reports generated
- the other costs for participating laboratories

3.3 Trial coordination

Dr. Hajime Kojima was appointed as the Trial Coordinator with well-defined roles and responsibilities to coordinate the trial and to establishment of a VMT by supporting of JaCVAM.

The name and location of the Trial Coordinator should be identified in each individual study plan. For the IL-1β Luc assay validation trial, the Trial Coordinator has direct access to the test item coding.

The Trial Coordinator's responsibilities include:

a) Establishment of/support to lead laboratory, including meeting organization

b) Trial communication and coordination with test facilities

c) Recording of document and data flow between test facilities

d) Assessing and documenting the impact of any amendments and/or deviations from the trial plan and study plans on the quality and integrity of the validation trial

e) Ensuring that the individual study reports are forwarded, in a timely manner, for data and statistical analysis

f) Preparing the trial plan and report, which can be based on the study reports from the lead laboratories and other test facilities involved in the validation trial, and should reflect the overall trial

g) Approval with date and signature of all protocols, Study Plans and Study Reports

h) The communication of the results of the trial into the public domain

The role of Trial Coordinator (as the formal representative of the VMT and the single contact point with the SDs) is of fundamental importance. The Trial Coordinator is the single critical point of trial control and must ensure clear lines of communication between the involved test facilities in the trial. The communication line of the Trial Coordinator is with the SDs of the different test facilities. The SDs are the single point of contact with the Trial coordinator (unless otherwise communicated by the participating Test Facilities) to assure a transparent and recorded documentation flow during the trial. The Trial Coordinator should also ensure that appropriate arrangements have been made for the supply of the test systems, and test, control and reference items, which meet the requirements of the trial, and that there are appropriate test method protocols (dated signature by the trial coordinator and the Lead Laboratories) and, if appropriate, validated data recording, data analysis, data reporting sheets for the test method.

It is the responsibility of the Trial Coordinator to approve the study plans send for approval by the test facilities, and any amendments to the study plan, by dated signature.

3.4 Training

The lead laboratory will be responsible for issuing a training agenda to the Trial Coordinator for further distribution to the all test facility giving details what training aspects will be covered during the training of the other SDs and Study Personnel at the lead laboratory. Furthermore, after the training, the lead laboratory will issue to the Trial Coordinator a training report and indicating if critical observations are made by the other test facilities regarding the IL-1β Luc assay protocols. In case any critical observations are made a new version of the IL-1β Luc assay protocols might necessary be issued to the other test facilities before initiating the between-laboratory transferability.

3.5 [Module 3] Between-laboratory transferability

This between-laboratory transferability (Module 3, identical to ICCVAM proficiency testing phase) is performed in order to assess the successful transfer of the assay to a test facility unexperienced with that particular test method but having knowledge of similar test systems and endpoint detection methods.

For the transfer of IL-1β Luc assay to the all test facility, the Phase 0 study using non-coded three chemicals was performed. A few concentrations of each test item will be tested in triplicate in 2 independent runs according to the IL-1β Luc assay protocol describing the details of the experimental design. The results of the between-laboratory transferability will be reviewed before progressing with module 4 on the between laboratory reproducibility. If the transferability data do not meet test acceptance criteria, the Trial Coordinator representing the VMT will try to identify the problems

and make corrections where needed.

3.6 [Module 2] Within-laboratory reproducibility

The within-laboratory reproducibility of the all test facility has been done by an independent biostatistical analysis, under the VMT. The proportion of concordance should be equal or more than 80% as tentative acceptance criteria for phase I validation.

The five test items selected for the phase I study are coded as follows: A, B, C, D, and E. The all facility will prepare a study according to internal GLP principle. This plan will be submitted to the Trial Coordinator and lead laboratory for approval.

At the end of the testing, the test facilities will submit a QC certified copy of whole study dossier to the Trial Coordinator (study plan in GLP principle, raw data, records and data analysis, study report in GLP principle).

3.7 [Module 4] Between-laboratory reproducibility

Ten coded test items have been selected to confirm the between-laboratory reproducibility in the phase I study. A few concentrations of each test item will be tested in triplicate according to the IL-1β Luc assay method protocol describing the details of the experimental design.

At the end of the testing, the test facilities will submit a QC certified copy of whole study dossier to the trial coordinator (study plan in GLP principle, raw data, records and data analysis, study report in GLP principle). The proportion of concordance between-laboratory reproducibility should be equal or more than 80% as acceptance criteria,

3.8 [Module 5] Predictive capacity

The necessity for further chemical analysis will be subject to a VMT decision once the data of the between laboratory reproducibility has been assessed. Depending on the statistical analysis the lean design for validation as well as the automatisation of the test leading to an increased dataset will be considered.

4. Protocol

In this validation trial, the protocol (ver. 1E) will be used (attached Document #2). This protocol will make up a draft by the lead laboratory and be finalized by VMT.

A measurement of bioluminescence intensity induced with chemical treatment will be measured by luminometer (Phelios: ATTO, Cat #:AB-2350) calibrated using stabilized SLG, SLO and SLR enzymes in this validation trial.

5. Chemicals

5.1 Chemicals Selection

Test chemicals have been selected by chemical repository based on published papers on in vivo immunotoxicity

The applied selection criteria were:

- information on mode/site of action
- coverage of range of relevant chemical classes and product classes quality and quantity of reference data (*in vivo* and *in vitro*)
- high quality data derived from animals and (if available) also humans
- knowledge on interspecies variations (for example: variability with regard to the uptake of chemicals, metabolism, etc.)
- coverage of range of toxic effects/potencies
- chemicals that do not need metabolic activation

- appropriate negative and positive controls
- physical and chemical properties (feasibility of use in the experimental set-up as defined by the CAS No.)
- single chemical entities or formulations of known high purity
- availability
- costs

In the first phase of the selection procedure, the Chemical Selection Committee identified and collected several existing lists of potential chemical sensitizing in order to establish a primary database. These chemicals had originally been compiled by international experts for various purposes e.g. as reference compounds for validation studies. An extensive literature research was performed by the Chemical Selection Committee in order to insure that the preselected chemical fulfilled the selection criteria described above.

Emphasis was laid on the fact that different potencies (strong, weak and no activity) have been chosen. In addition, it was decided that at least 20% of the total substances to be tested should be negative in order to increase the statistical power of the data analysis.

In the first phase IL-1β Luc assay validation trial with data generation at the test facilities, five chemicals will be tested three times in each test chemical for between-laboratory reproducibility and to confirm transferability. After discussion of Phase I results, detailed test planning of the Phase II will be determined. At this moment, twenty chemicals will be planned in the phase II trial for predictive capacity (Table 2).

Table 2. Outline of test planning at each study in the validation trial.

Study	Chemicals	Test Number	Information obtained
Phase 0	3 non- coded	2	Between-lab transferability
Phase I	5 coded	3	Within & between-lab reproducibility
Phase II (planning)	20 coded	1	Between-lab reproducibility & predictability

(Planning of Phase II will be determined after discussion of the results of Phase I)

5.2 Chemicals Acquisition, Coding and Distribution

The assessment of within-laboratory reproducibility (Module 2), between laboratory transferability (Module 3) in the all test facilities have been performed with coded chemicals. This IL-1β Luc validation trial plan describes the generation of the missing data sets under coded test item. If the results obtained are not very similar to the previous obtained sets, the VMT has to assess if coded chemicals need to be tested in the all test facilities.

The coding will be supervised by the Trial Coordinator, in collaboration with the chemical repository responsible of coding and distribution of test, reference and control items for the validation trial.

5.3 Handling

Each test facility shall receive through the Trial Coordinator essential information about the test chemicals (physical state, weight or volume of sample, specific density for liquid test chemicals, and storage instructions). Moreover, the SD should receive

the safety information concerning the hazards identification and exposure controls/personal protection.

6. Records and archiving

At the end of the trial, the IL-1β Luc assay validation trial report is prepared by the Trial Coordinator or the VMT personnel who appointed by the Trial Coordinator. The trial report summarizes the trial goals, procedures, results and conclusions of the validation trial. This represents the whole validation trial, including archiving and, as such, will cover several study reports, as well as reports for test item supply, data management and statistics. The Trial Coordinator oversees the preparation of the trial report. The Trial Coordinator will be representing the VMT discussions responsible for preparation of the scientific conclusions. Signatories to the trial report include the Trial Coordinator, the statistician, and the SDs of the involved test facilities. Although the SDs may not be involved with the preparation of the trial report, their signatures confirm that the trial report is an accurate reflection of the management and study events. The trial report should contain a statement, signed by the Trial Coordinator, commenting on the accuracy and completeness of the trial report and identifying any significant issues which could have affected the integrity of the trial, including matters of GLP compliance. A QC statement will be included in the trial report, in order to identify what QC monitoring was done and to confirm whether or not the trial report is an accurate reflection of the validation trial data.

7. Study timeline

An approximate schedule for IL-1β Luc assay validation trial is shown in Table 3.

Duration of this validation trial is around twenty -month from August 2018 to 2020.

Table 3. Schedule of IL-1β Luc assay validation trial

Month	Activity
August, 2018	Establish the VMT
	Selection of participating research laboratories
	Deliberation, decision and read-through of draft study plan
	Deliberation and decision of protocol
	Preparation of a tentative list of test chemicals
	Distribution of test chemicals, standard chemicals and positive control chemicals
October,2018	Technical transfer using five known chemicals (non-coded) Start of technical transfer **to know between laboratory transferability**
	Data collection of technical transfer (**Phase 0 study**)
Phase I study	
October, 2018	Coding and distribution of five coded test chemicals
November, 2018	Start of Phase I study
March, 2019	End of Phase I study
May, 2019	**2nd VMT Meeting** / Phase I results and planning of Phase II study
Phase II study to know between- and within-laboratory reproducibility	
2019	Coding and distribution of coded test chemicals and positive chemicals
2019	Start of Phase II study using 20 coded test chemicals
2019	End of Phase II study
2020	**3rd VMT Meeting** /reviewing of Phase II study results
2020	Completed validation report

Abbreviations

CAS: Chemical Abstracts Service
GLP: Good Laboratory Practice
HRI: Hatano Research Institute
FDSC: Food and Drug Safety Center
JaCVAM: Japanese Centre for the Validation of Alternative Methods
NIHS: National Institute of Health Sciences
OECD: Organization for Economic Co-operation and Development
QC: Quality Control
TG: Test Guideline
VMT: Validation Management Team

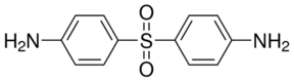
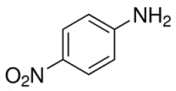
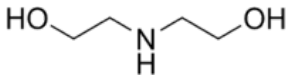
IL-1β (P1)2018 List of Coded Chemicals

Chemical name	CAS No.	set	LabA Tohoku	LabB Tsukuba	LabC Takamatsu
Dibutyl Phthalate	84-74-2	1	MITA103	MITB402	MITC704
		2	MITA203	MITB501	MITC803
		3	MITA304	MITB605	MITC902
Acetaminophen	103-90-2	1	MITA101	MITB404	MITC701
		2	MITA205	MITB505	MITC802
		3	MITA305	MITB603	MITC905
Isonicotinic Acid Hydrazide (Isoniazid)	54-85-3	1	MITA104	MITB403	MITC705
		2	MITA202	MITB502	MITC805
		3	MITA303	MITB601	MITC901
Sulem Mercury(II) Chloride	7487-94-7	1	MITA105	MITB401	MITC702
		2	MITA204	MITB503	MITC801
		3	MITA301	MITB602	MITC904
Hexachlorobenzene	118-74-1	1	MITA102	MITB405	MITC703
		2	MITA201	MITB504	MITC804
		3	MITA302	MITB604	MITC903

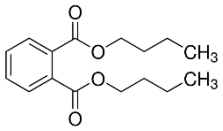
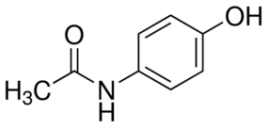
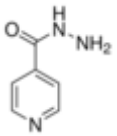
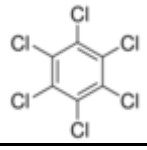
IL-1β (P2)2019 List of Coded Chemicals

Chemical name	CAS No.	LabA Tohoku	LabB Tsukuba	LabC Takamatsu
Cadmium chloride	10108-64-2	MTA117	MTB221	MTC305
5,5-Diphenylhydantoin　sodium salt	630-93-3	MTA105	MTB220	MTC301
Indomethacin	53-86-1	MTA120	MTB203	MTC318
Pentachlorophenol	87-86-5	MTA115	MTB211	MTC307
Urethane	51-79-6	MTA111	MTB224	MTC302
Tributyltin chloride	1461-22-9	MTA112	MTB208	MTC312
Perfluorooctanois acid	335-67-1	MTA125	MTB214	MTC303
Hydroquinone	123-31-9	MTA110	MTB218	MTC322
4-Aminophenyl sulfone	80-08-0	MTA124	MTB217	MTC313
Ethanol	64-17-5	MTA102	MTB206	MTC317
5-Nitro-2-furaldehyde semicarbazone	59-87-0	MTA121	MTB205	MTC324
Trichloroethylene	79-01-6	MTA116	MTB223	MTC309
Zinc dimethyldithiocarbamate	137-30-4	MTA118	MTB202	MTC316
Citral	5392-40-5	MTA108	MTB204	MTC315
t-Buthlhydroquinone	1948-33-0	MTA113	MTB219	MTC323
Bisphenol A	80-05-7	MTA107	MTB222	MTC314
2,6-Di-tert-butyl-4-methylphenol	128-37-0	MTA119	MTB201	MTC306
Nonylphenol	84852-15-3	MTA104	MTB210	MTC311
Sodium chlorite	7758-19-2	MTA114	MTB216	MTC304
D(-)-Mannitol	69-65-8	MTA127	MTB227	MTC327

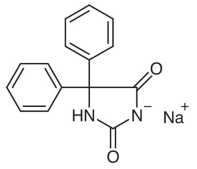
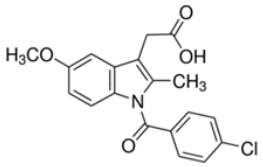
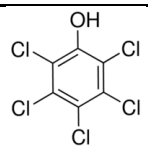
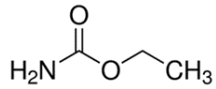
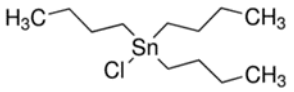
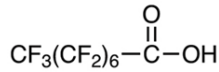
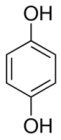
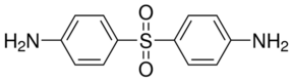
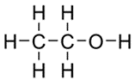
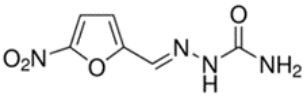
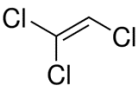
Appendix 03 Chemical structure of the test chemicals for the Phase 0 study

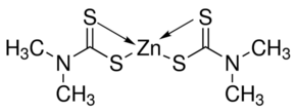
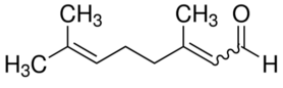
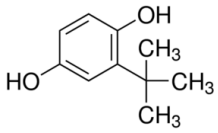
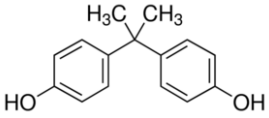
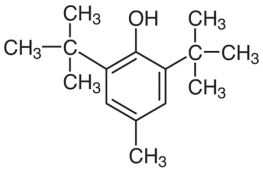
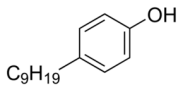
No.	Chemical name	CAS No.	Molecular weight	Chemical structure
0-1	4-Aminophenyl sulfone(Dapsone)	80-08-0	248.30	
0-2	4-Nitroaniline	100-01-6	138.12	
0-3	Diethanolamine	111-42-2	105.14	

Appendix 04 Chemical structure of the test chemicals for the Phase I study

No.	Chemical name	CAS No.	Molecular weight	Chemical structure
I-1	Dibutyl phthalate	84-74-2	278.34	
I-2	Acetaminophene	103-90-2	151.16	
I-3	Isonicotinic acid hydrazide(Isoniazid)	54-85-3	137.14	
I-4	Mercury(II) chloride	7487-94-7	271.50	$HgCl_2$
I-5	Hexachlorobenzene	118-74-1	284.78	

Appendix 05 Chemical structure of the test chemicals for the Phase II study

No.	Chemical name	CAS No.	Molecular weight	Chemical structure
II-1	Cadmium chloride	10108-64-2	183.32	$CdCl_2$
II-2	5,5-Diphenylhydantoin sodium salt	630-93-3	274.25	
II-3	Indomethacin	53-86-1	357.79	
II-4	Pentachlorophenol	87-86-5	266.34	
II-5	Urethane	51-79-6	89.09	
II-6	Tributyltin chloride	1461-22-9	325.51	
II-7	Perfluorooctanoic acid	335-67-1	414.07	
II-8	Hydroquinone	123-31-9	110.11	
II-9	4-Aminophenyl sulfone(Dapsone)	80-08-0	248.30	
II-10	Ethanol	64-17-5	46.07	
II-11	5-Nitro-2-furaldehyde semicarbazone (Nitrofurazone)	59-87-0	198.14	
II-12	Trichloroethylene	79-01-6	131.39	

II-13	Zinc dimethyldithiocarbamate (Ziram)	137-30-4	305.82	
II-14	Citral	5392-40-5	152.23	
II-15	tert-Butylhydroquinone	1948-33-0	166.22	
II-16	Bisphenol A	80-05-7	228.29	
II-17	2,6-Di-tert-butyl-4-methylphenol	128-37-0	220.35	
II-18	Nonylphenol	84852-15-3	220.35	
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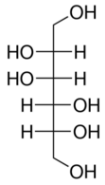
II-20	D(-)Mannitol	69-65-8	182.17	

Appendix 06

Multi-Immuno Tox Assay protocol for THP-G1b (TGCHAC-A4) ver. 009E

July 1st, 2019

Department of Dermatology, Tohoku University Graduate School of Medicine
Yutaka Kimura, M.D., Ph.D.
Setsuya Aiba, M.D., Ph.D.

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Appendix 1 Principle of measurement of luciferase activity....

Appendix 2 Validation of reagents and equipment....

1. Introduction

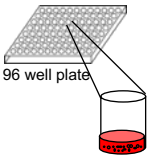
This protocol describes how to maintain the cells, how to prepare the test chemicals, and how to measure the luciferase activity of THP-G1b (TGCHAC-A4), THP-1 cells transfected with 2 luciferase genes, stable luciferase orange (SLG) on the human artificial chromosome (HAC) vector and stable luciferase red (SLR), under the control of IL-1β and G3PDH promoters, respectively, for the Multi-Immuno Tox Assay.
(Kimura Y. et al. Evaluation of the Multi-Immuno Tox Assay composed of 3 human cytokine reporter cells by examining immunological effects of drugs *Toxicol in Vitro*, 28, 759-768, 2014)

Figure 1

Assay design

flat-bottom B&W	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	cont (distilled water or DMSO)	LPS only	$A/2^9$ μg/mL	$A/2^8$ μg/mL	$A/2^7$ μg/mL	$A/2^6$ μg/mL	$A/2^5$ μg/mL	$A/2^4$ μg/mL	$A/2^3$ μg/mL	$A/2^2$ μg/mL	$A/2^1$ μg/mL	A μg/mL
D			Chemical (common ratio of 2, 10 concentrations, n=4)									
E												
F												
G												
H												

LPS



Cell preparation
(1 x 10^5 cells/well of THP-G1b (TGCHAC-A4))

Add various concentrations of Chemicals

↓Incubate for 1 h

Stimulate with LPS

↓Incubate for 6 h

Add TripLuc® luciferase assay reagent (TOYOBO)

↓Shake for 10 min.

Assess using a microplate-type luminometer (5 min./plate)

2. Materials

2-1 Cells

・ THP-G1b (TGCHAC-A4) (IL1β-SLG、G3PDH-SLR)

The human macrophage-like cell line THP-1 was obtained from the American Type Culture Collection (Manassas, VA, USA). A THP-1-derived IL-1β reporter cell line, THP-G1b (TGCHAC-A4), that harbors the SLG and SLR luciferase genes under the control of the IL-1β and G3PDH promoters, respectively, was established by the Dept. of Dermatology, Tohoku University School of Medicine and GPC laboratory Co. Ltd.
(Kimura Y. et al. Profiling the immunotoxicity of chemicals based on in vitro evaluation by a combination of the Multi-ImmunoTox assay and the IL-8 Luc assay. Archives of Toxicology, 92, 2043-2054, 2018)

2-2 Reagents and equipment

2-2-1 For maintenance of the THP-G1b (TGCHAC-A4) cells

・ RPMI-1640 (GIBCO Cat#11875-093, 500 mL)
・ FBS (Biological Industries Cat#04-001-1E Lot: 715004)
・ 100 X concentrated antibiotic and antimycotic (10,000 U/mL of penicillin G, 10,000 μg/mL of streptomycin and 25 μg/mL of amphotericin B in 0.85 % saline) (e.g., GIBCO Cat#15240-062)

2-2-2 For chemical exposure, stimulation, positive control and solvents

・ Lipopolysaccharide (LPS) from Escherichia coli K12 (Invivogen Cat#tlrl-eklps, Lot#: LEK-39-01)
・ Dexamethasone (CAS:50-02-2, Fujifilm Wako Pure Chemical Cat#041-18861)
・ Dimethyl sulfoxide (DMSO) (CAS:67-68-5, Sigma Cat#D5879)
・ Distilled water (GIBCO Cat#10977-015)

2-2-3 For measurement of the luciferase activity

・ Tripluc® Luciferase assay reagent (TOYOBO Cat#MRA-301)

2-2-4 Expendable supplies

・ T-75 flask tissue culture treated (e.g., Corning Cat#353136)
・ 96 well black-flame and white-well plate (flat-bottom, for measurement of the luciferase activity, e.g. PerkinElmer B&W Isoplate-96 TC Cat#6005060)
・ 96 well clear plate (round-bottom, for preparation of chemicals and stimulants)
・ 96 well assay block, 2 mL (e.g., Costar Cat#3960)

- Seal for 96 well plate (e.g., Perkin Elmer TopSeal-A PLUS Cat#6050185, EXCEL Scientific SealMate Cat#SM-KIT-SP)
- Reservoir
- Pipette

2-2-5 Equipment for measurement of luciferase activity

- Measuring device: a microplate-type luminometer with a multi-color detection system that can accept an optical filter
  e.g. Phelios AB-2350 (ATTO), ARVO (PerkinElmer), Tristar LB941 (Berthold)
- Optical filter: 600 nm long-pass filter, 600～700 nm band-pass filter
- Measuring time: set at 1～5 sec/well measuring time

2-2-6 Others

- Pipetman
- 8 channel or 12 channel pipetman (optimized for 10~100 μL)
- Plate shaker (for 96 well plate)
- CO_2 incubator (37°C, 5% CO_2)
- Water bath
- Cell counter: hemocytometer, trypan blue

2-3 Culture medium

2-3-1A medium: for maintenance of THP-G1b (TGCHAC-A4) cells (500 mL, stored at 2-8°C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO Cat #11875-093	-	-	445 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	50 mL
Antibiotic-Antimycotic	e.g., GIBCO Cat #15240-062	100×	1×	5 mL

2-3-2 B medium: for luciferase assay (30 mL, stored at 2-8°C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO Cat #11875-093	-	-	27 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	3 mL

2-4 Preparation of the stimulant of THP-G1b (TGCHAC-A4) cells

2-4-1 Lipopolysaccharide (LPS) from Escherichia coli K12

Reagent	Company	Concentration of the stock solution	Final concentration
Lipopolysaccharide (LPS) from Escherichia coli K12	Invivogen Cat#tlrl-eklps	1 mg/mL	100 ng/mL
Distilled water	GIBCO Cat#10977-015		

Dissolve 5 mg LPS using distilled water 5 mL, dispend at 5 μL/tube and store at freezer at -30°C. Use these stocks within 6 months after dissolution.

3. Cell culture

3-1 Thawing of THP-G1b (TGCHAC-A4) cells

Pre-warm 9 mL of A medium in a 15 mL polypropylene conical tube in a 37°C water bath (for centrifugation) and 15 mL of A medium in a T-75 Flask at 37°C in a 5% CO_2 incubator (for culture).

Thaw frozen cells ($2x10^6$ cells / 0.5 mL of freezing medium) in a 37°C water bath, then add to a 15 mL polypropylene conical tube containing 9 mL of pre-warmed A medium. Centrifuge the tube at 120-350 x *g* at room temperature for 5 min, discard the supernatant, and resuspend in 15 mL of pre-warmed A medium in a T-75 Flask. Cells are incubated at 37°C, 5% CO_2.

3-2 Maintenance of THP-G1b (TGCHAC-A4) cells

3 or 4 days after thawing, pre-warm the A medium in a T-75 Flask at 37°C in a 5% CO_2 incubator. Count the number of cells, centrifuge the tube at 120-350 x *g* at room temperature for 5 min, discard the supernatant, and resuspend in the pre-warmed A medium in a T-75 Flask. Cells are passaged at $2\text{-}5x10^5$/mL, depending on the condition of the cells and incubated at 37°C, 5% CO_2.

The interval between subcultures should be 3~4 days. Cells can be used between one and six weeks after thawing.

4. Preparation of cells for assay

A cell passage should be done 2-4 days before the assay.

Use cells between 1 and 6 weeks after thawing.

Pre-warm the B medium in a 37°C water bath. Count the number of cells and collect the number of cells needed (5.0 x 10^6 cells are required, but to have some leeway, 7.5 x 10^6 cells should be prepared), centrifuge the tube at 120-350 x *g*, 5 min. Resuspend in pre-warmed the B medium at a cell density of 2×10^6/mL. Transfer the cell suspension to a reservoir, and add 50 µL of cell suspension to each well of a 96 well black-flame and white-well plate (flat bottom) using an 8 channel or 12 channel pipetman. (cf. Figure 2)

Figure 2

flat-bottom B&W	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL
D	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL
E	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL
F	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL
G												
H												

5. Preparation of chemicals and cell treatment with chemicals

5-1 Dissolution by vehicle (cf. Figure 3)

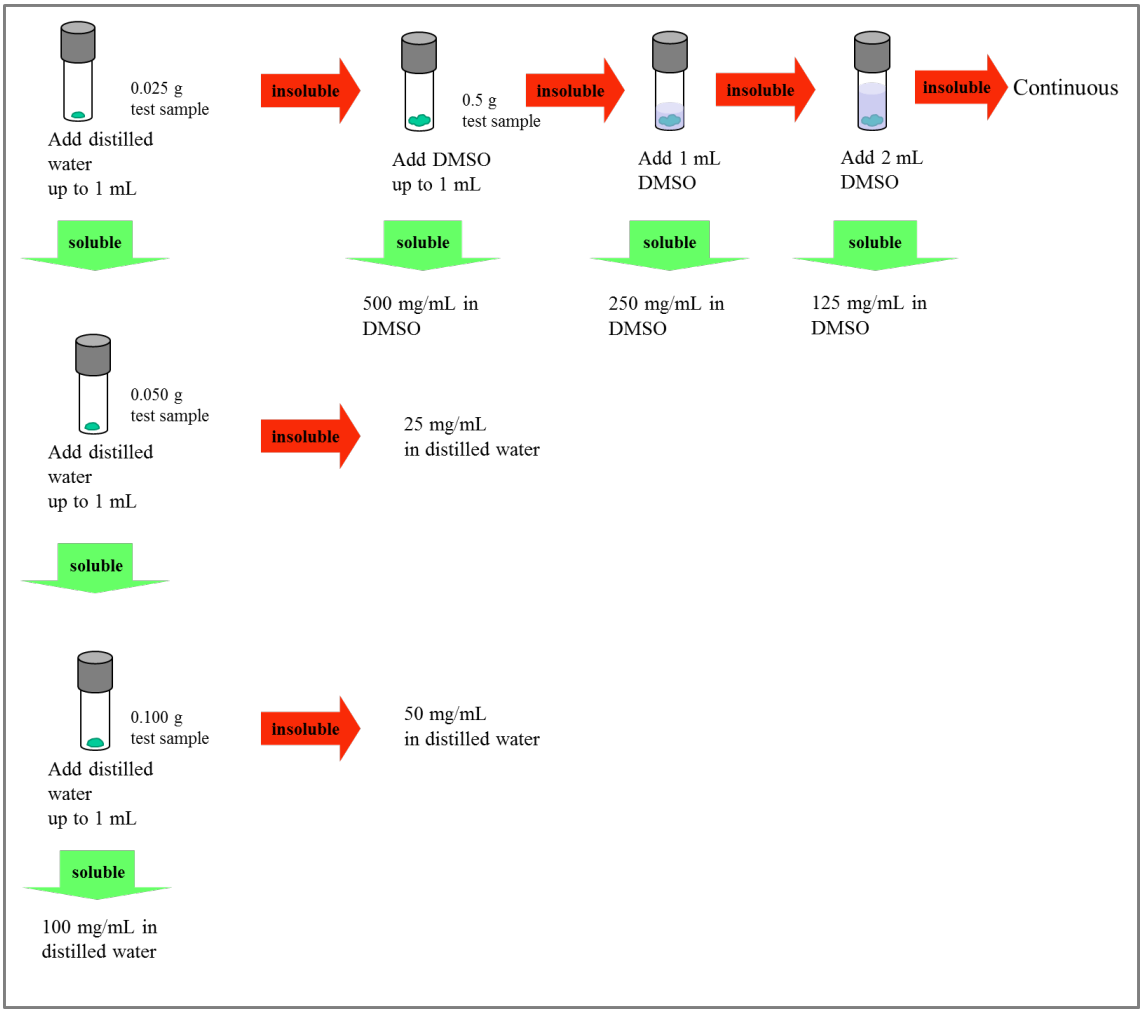
Dissolve the chemical first in distilled water. Namely, weigh 0.025 g of the test chemical in a volumetric flask and add distilled water up to 1 mL. If the chemical is soluble at 25 mg/mL, weigh 0.050 g of the test chemical in a volumetric flask and add distilled water up to 1 mL. If the chemical is not soluble at 50 mg/mL, 25 mg/mL is the highest soluble concentration. If the chemical is soluble at 50 mg/mL, weigh 0.100 g of the test chemical in a volumetric flask and add distilled water up to 1 mL. If the chemical is not soluble at 100 mg/mL, 50 mg/mL is the highest soluble concentration. If the chemical is soluble at 100 mg/mL, 100 mg/mL is the highest soluble concentration.

If the chemical is not soluble in water, the chemical should be dissolved in DMSO at 500 mg/mL. Namely, weigh 0.5 g of the test chemical in volumetric flask and add DMSO up to 1 mL.

If the chemical is not soluble at 500 mg/mL, the highest soluble concentration should be determined by diluting the solution from 500 mg/mL at a common ratio of two (250 mg/mL → 125 mg/mL → continued if needed) with DMSO.

Sonication and vortex may be used if needed, and attempt to dissolve the chemical for at least 5 minutes. Being soluble should be confirmed by centrifugation at 15,000 rpm (≈20,000 x g) for 5 min and absence of precipitation. The chemical should be used within 4 hours after being dissolved in distilled water or DMSO.

Figure 3



In the first experiment (1st experiment), when the chemical is prepared in distilled water, conduct 10 serial dilutions at a common ratio of 2 from the highest concentration using distilled water. When the chemical is prepared as a DMSO solution, conduct 10 serial dilutions at a common ratio of 2 from the highest concentration using DMSO.

In the second to fifth experiment (2nd to 5th experiment), determine the minimum concentration at which I.I.-SLR-LA (mentioned later in **10**) became lower than 0.05 in the 1st experiment, use the concentration one step (2-times) higher than this determined concentration as the highest concentration of the chemical to examine, and conduct 10 serial dilutions at a common ratio of 2 from the highest concentration. If I.I.-SLR-LA did not become lower than 0.05 or became lower than 0.05 at the highest concentration in the 1st experiment, conduct 10 serial dilutions at a common ratio of 2 from the highest concentration in the 1st experiment.

For example, in Figure 3 below, the minimum concentration at which I.I.-SLR-LA became lower than 0.05 is 1.95 μg/ml. The highest concentration of the chemical to examine is the concentration one step (2-times) higher than 1.95 μg/ml, which is 3.91 μg/ml.

In Figure 4 below, I.I.-SLR-LA did not become lower than 0.05. In such a case, the highest concentration of the chemical to examine is the highest concentration in the 1st experiment, namely 125 μg/ml.

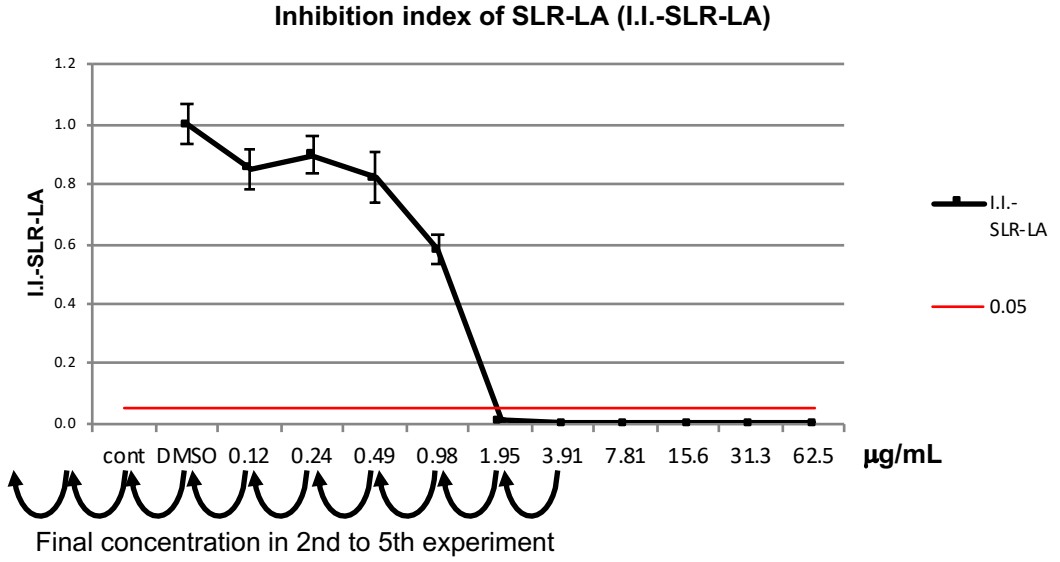


Figure 3.

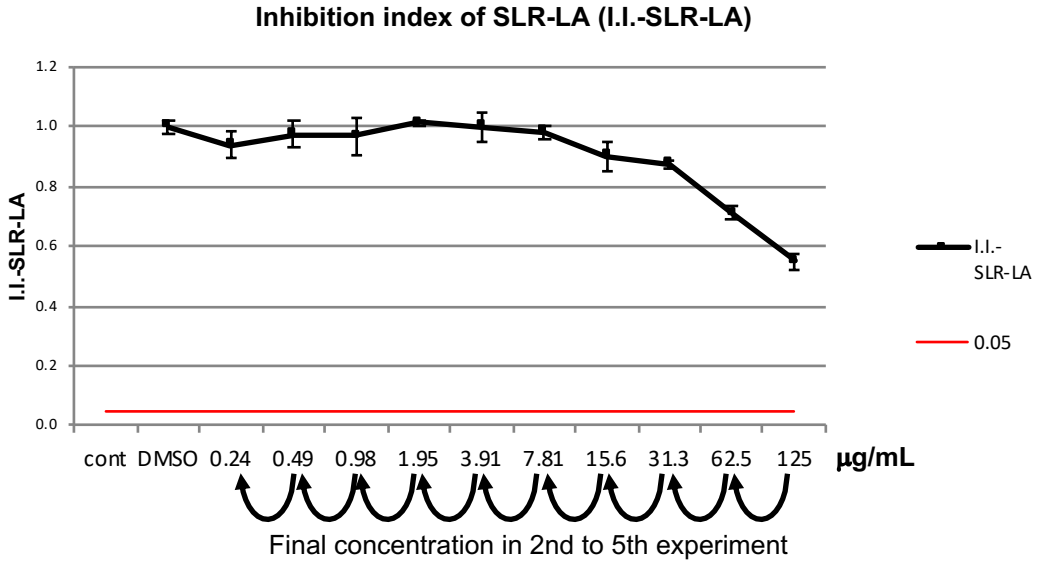


Figure 4

5-2 When the chemical is prepared in distilled water

If the chemical is prepared at a lower concentration, use the prepared concentration instead of the 100 mg/mL distilled water solution.

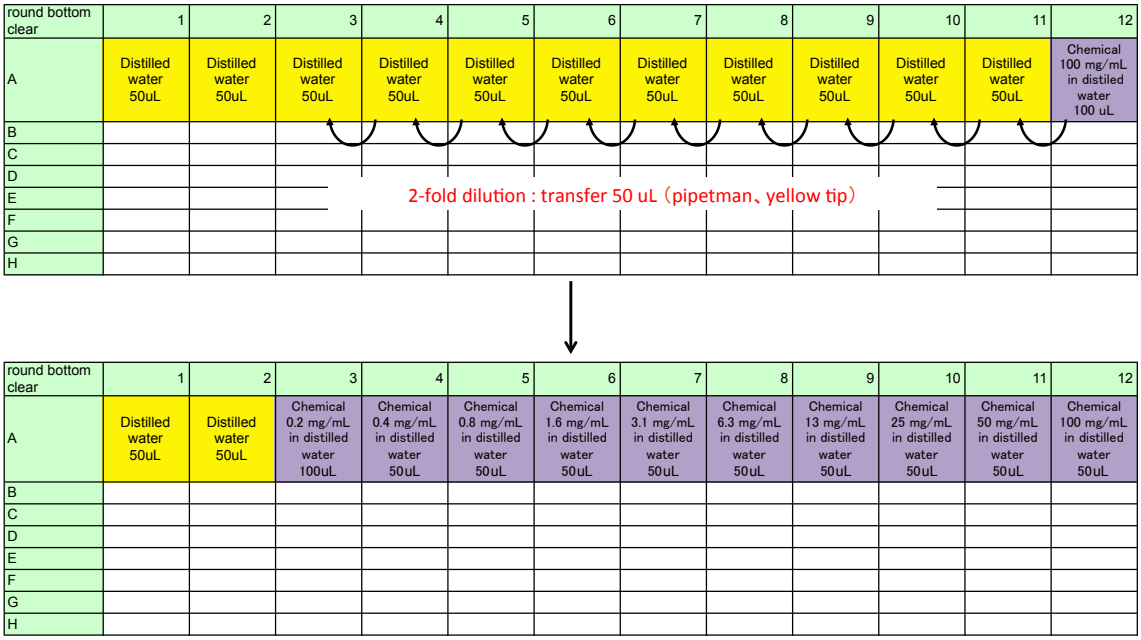
5-2-1 Arrangement of chemicals and vehicle

Add 100 µL of the 100 mg/mL distilled water solution of the chemical to well #A12, and 50 µL of the distilled water to wells #A1-#A11 of the 96 well clear plate (round bottom).

5-2-2 Serial dilution

Conduct 9 serial dilutions at a common ratio of 2 as indicated in Figure 4 from well #A11 to well #A3. Transfer 50 µL to the next (left) well. (cf. Figure 4)

Figure 4



5-2-3　2 step dilution

Add 20 μL of the diluted chemical to 480 μL of the B medium prepared in the assay block. And add 50 μL to THP-G8 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 1 hour (37°C, CO_2, 5%) (cf. Figure 5-7).

Figure 5

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 50uL	Distilled water 50uL	Chemical 0.2 mg/mL in distilled water 100uL	Chemical 0.4 mg/mL in distilled water 50uL	Chemical 0.8 mg/mL in distilled water 50uL	Chemical 1.6 mg/mL in distilled water 50uL	Chemical 3.1 mg/mL in distilled water 50uL	Chemical 6.3 mg/mL in distilled water 50uL	Chemical 13 mg/mL in distilled water 50uL	Chemical 25 mg/mL in distilled water 50uL	Chemical 50 mg/mL in distilled water 50uL	Chemical 100 mg/mL in distilled water 50uL
B												
C												
D												
E												
F												
G												
H												

20uL

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL
B												
C												
D												
E												
F												
G												
H												

Figure 6

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 500uL	B medium 500uL	Chemical 0.008 mg/uL in B medium 500uL	Chemical 0.02 mg/mL in B medium 500uL	Chemical 0.03 mg/mL in B medium 500uL	Chemical 0.06 mg/mL in B medium 500uL	Chemical 0.1 mg/mL in B medium 500uL	Chemical 0.3 mg/mL in B medium 500uL	Chemical 0.5 mg/mL in B medium 500uL	Chemical 1 mg/mL in B medium 500uL	Chemical 2 mg/mL in B medium 500uL	Chemical 4 mg/mL in B medium 500uL
B												
C												
D												
E												
F												
G												
H												

50uL

flat-bottom B&W	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL
D	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL
E	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL
F	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL
G												
H												

Figure 7 Final constituents of each well of the plate

flat-bottom B&W	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	Chemical 0 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.004 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.008 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.02 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.03 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.06 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.1 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.3 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.5 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 1 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 2 mg/mL THP-G1b 1x10^5 B medium 100uL
D	Chemical 0 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.004 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.008 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.02 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.03 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.06 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.1 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.3 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.5 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 1 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 2 mg/mL THP-G1b 1x10^5 B medium 100uL
E	Chemical 0 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.004 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.008 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.02 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.03 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.06 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.1 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.3 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.5 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 1 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 2 mg/mL THP-G1b 1x10^5 B medium 100uL
F	Chemical 0 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.004 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.008 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.02 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.03 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.06 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.1 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.3 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.5 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 1 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 2 mg/mL THP-G1b 1x10^5 B medium 100uL
G												
H												

5-3 When the chemical is prepared as a DMSO solution

If the chemical is prepared at a lower concentration, use the prepared concentration instead of 500 mg/mL DMSO solution.

5-3-1 Arrangement of chemicals and vehicle

Add 100 µL of the 500 mg/mL DMSO solution of the chemical to well #A12, 50 µL of DMSO to wells #A1-#A11, and 90 µL of the B medium to wells #B1-#B12 of the 96 well clear plate (round bottom)

5-3-2 Serial dilution

Conduct 9 serial dilutions at a common ratio of 2 as indicated in Figure 8 from well #A11 to well #A3. Transfer 50 µL to the next (left) well. (cf. Figure 8)

Figure 8

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	Chemical 500 mg/mL in DMSO 100uL
B	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL
C												
D												
E												
F												
G												
H												

2-fold dilution : transfer 50 uL (pipetman、yellow tip)

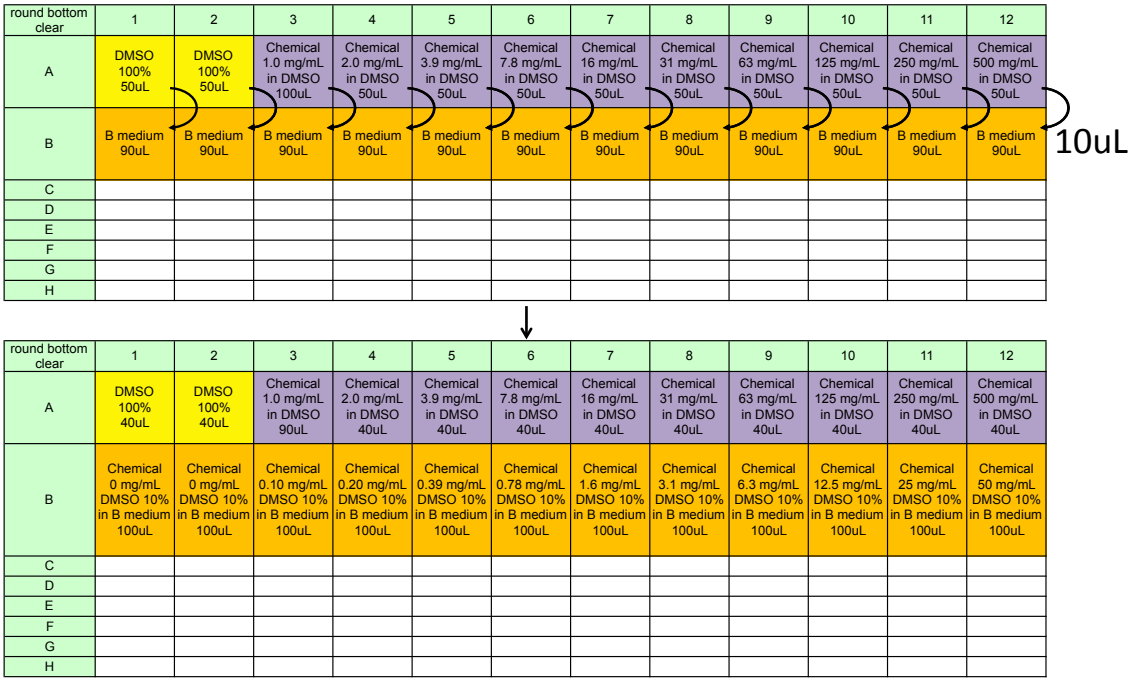
↓

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 50uL	DMSO 100% 50uL	Chemical 1.0 mg/mL in DMSO 100uL	Chemical 2.0 mg/mL in DMSO 50uL	Chemical 3.9 mg/mL in DMSO 50uL	Chemical 7.8 mg/mL in DMSO 50uL	Chemical 16 mg/mL in DMSO 50uL	Chemical 31 mg/mL in DMSO 50uL	Chemical 63 mg/mL in DMSO 50uL	Chemical 125 mg/mL in DMSO 50uL	Chemical 250 mg/mL in DMSO 50uL	Chemical 500 mg/mL in DMSO 50uL
B	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL
C												
D												
E												
F												
G												
H												

5-3-3 Dilution of DMSO solution with the B medium

Dilute 10 μL of the DMSO solution of the chemical in wells #A1-#A12 with 90 μL of the B medium using an 8-12 channel pipetman. (cf. Figure 9)

Figure 9



5-3-4　2 step dilution

Add 10 µL of the diluted chemical to 490 µL of the B medium prepared in the assay block. And add 50 µL to THP-G8 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Manipulate the procedures from 5-3-3 to 5-3-4 as quickly as you can, and do not leave a long time at step after 5-3-3 or Figure 10. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 1 hour (37°C, CO_2, 5%) (cf. Figure 10-12).

Figure 10

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 40uL	DMSO 100% 40uL	Chemical 1.0 mg/mL in DMSO 90uL	Chemical 2.0 mg/mL in DMSO 40uL	Chemical 3.9 mg/mL in DMSO 40uL	Chemical 7.8 mg/mL in DMSO 40uL	Chemical 16 mg/mL in DMSO 40uL	Chemical 31 mg/mL in DMSO 40uL	Chemical 63 mg/mL in DMSO 40uL	Chemical 125 mg/mL in DMSO 40uL	Chemical 250 mg/mL in DMSO 40uL	Chemical 500 mg/mL in DMSO 40uL
B	Chemical 0 mg/mL DMSO 10% in B medium 100uL	Chemical 0 mg/mL DMSO 10% in B medium 100uL	Chemical 0.10 mg/mL DMSO 10% in B medium 100uL	Chemical 0.20 mg/mL DMSO 10% in B medium 100uL	Chemical 0.39 mg/mL DMSO 10% in B medium 100uL	Chemical 0.78 mg/mL DMSO 10% in B medium 100uL	Chemical 1.6 mg/mL DMSO 10% in B medium 100uL	Chemical 3.1 mg/mL DMSO 10% in B medium 100uL	Chemical 6.3 mg/mL DMSO 10% in B medium 100uL	Chemical 12.5 mg/mL DMSO 10% in B medium 100uL	Chemical 25 mg/mL DMSO 10% in B medium 100uL	Chemical 50 mg/mL DMSO 10% in B medium 100uL
C												
D												
E												
F												
G							10uL					
H												

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL
B												
C												
D												
E												
F												
G												
H												

Figure 11

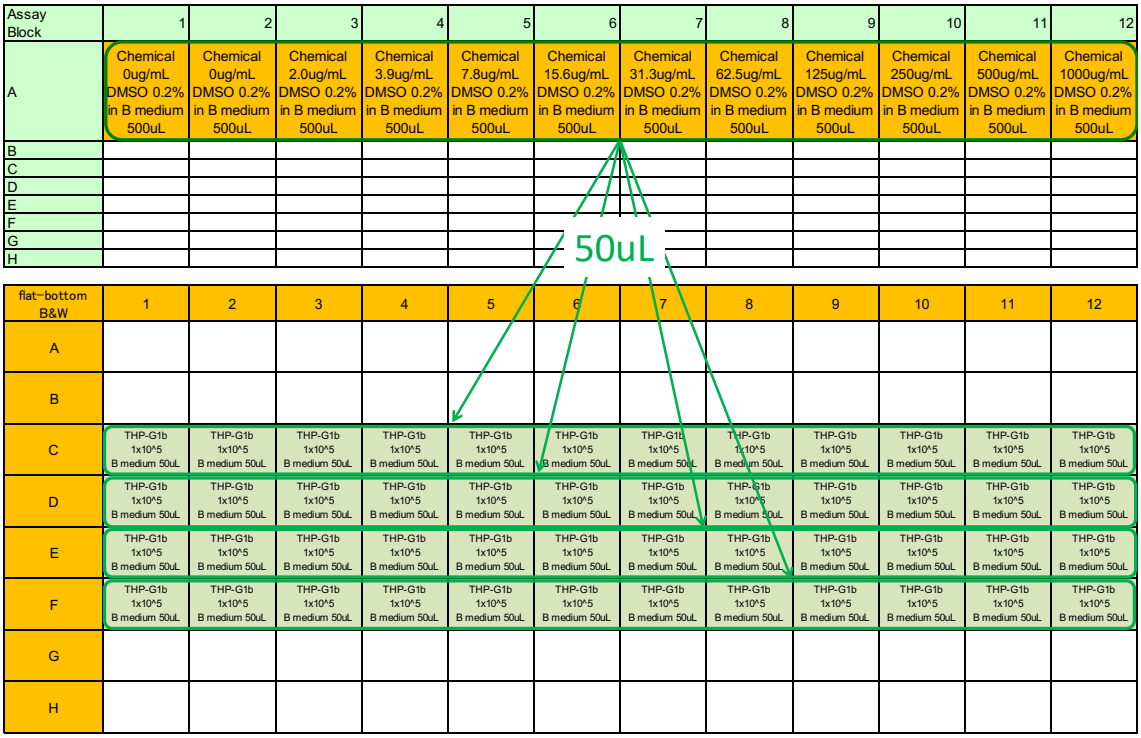


Figure 12 Final constituents of each well of the plate

flat-bottom B&W	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	Chemical 0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 1.0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 2.0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 3.9ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 7.8ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 16ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 31ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 63ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 125ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 250ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 500ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL
D	Chemical 0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 1.0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 2.0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 3.9ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 7.8ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 16ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 31ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 63ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 125ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 250ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 500ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL
E	Chemical 0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 1.0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 2.0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 3.9ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 7.8ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 16ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 31ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 63ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 125ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 250ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 500ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL
F	Chemical 0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 1.0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 2.0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 3.9ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 7.8ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 16ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 31ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 63ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 125ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 250ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 500ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL
G												
H												

6. Preparation of the stimulant (Lipopolysaccharide (LPS)) and addition to THP-G1b (TGCHAC-A4)

6-1 Material

・ 1 mg/mL LPS stock

6-2 Preparation of 1000 ng/mL LPS solution

Dilute 1 mg/mL LPS stock with distilled water as follows (1000 times, final concentration is 1000 ng/mL). Add distilled water as control to well #A1-#D1 of the 96 well clear plate (round bottom), and add 1000 ng/mL LPS solution to wells #A2-#D2 of the 96 well clear plate (round bottom).

1st step

1 mg/mL LPS	distilled water	Total	final concentration
5 µL	995 µL	1000 µL	5 µg/mL

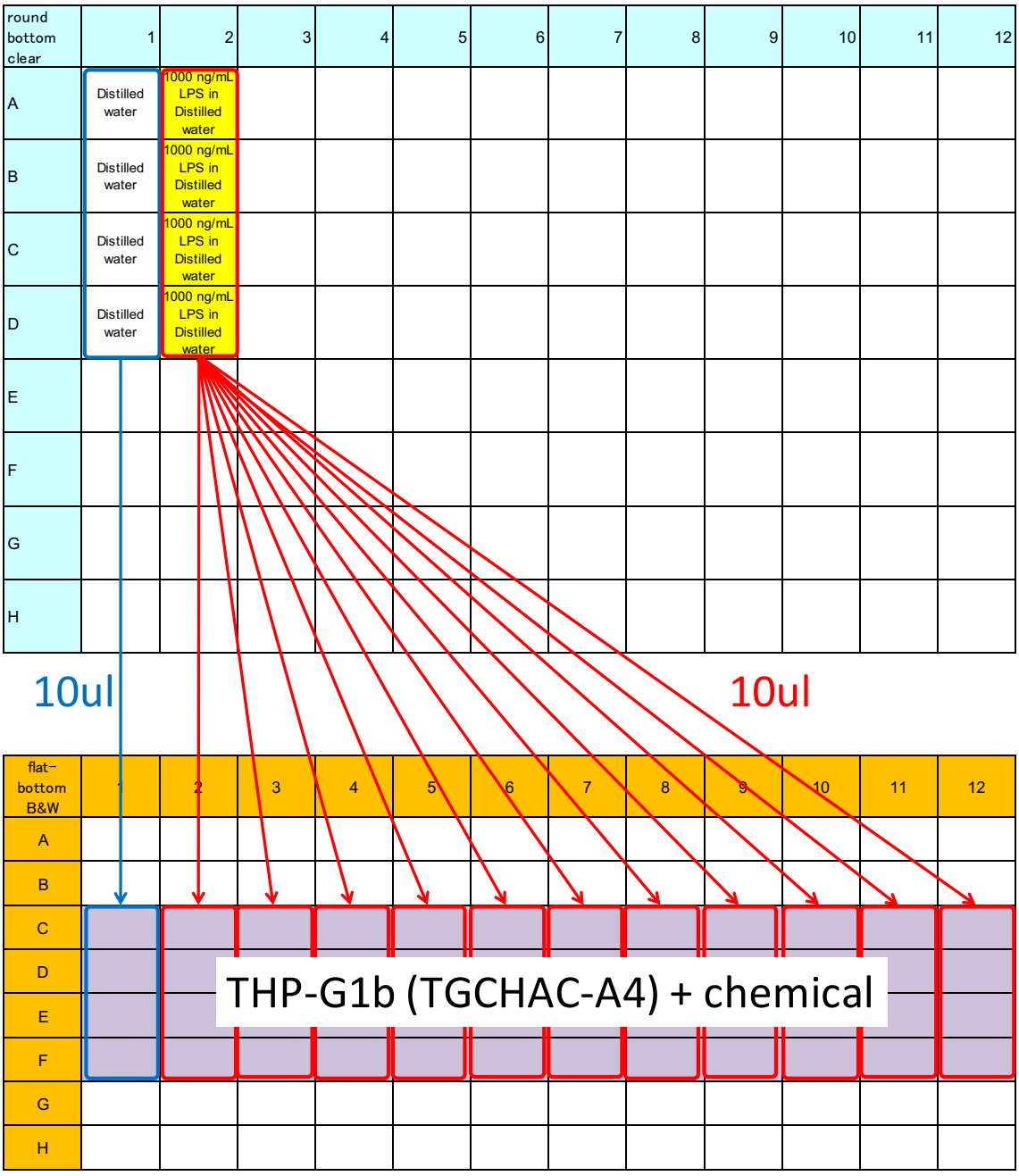
2nd step

5 µg/mL LPS	distilled water	Total	final concentration
250 µL	1000 µL	1250 µL	1000 ng/mL

6-3 Addition of LPS to THP-G1b (TGCHAC-A4)

One hour after the addition of chemicals, add 10 µL of control or 1000 ng/mL LPS solution to the cells (#C1-#F1 or #C2-#F12, respectively) using an 8 channel or 12 channel pipetman after pipetting 20 times. Make sure that the apex of the tip is dipped into the medium. Change tips every line you add. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 6 hours (37°C, CO_2, 5%). (cf. Figure 13)

Figure 13



7. Positive control

7-1 Preparing control chemical (dexamethasone)

7-1-1 Preparing dexamethasone stock

Reagent	Company	Concentration of the stock solution	Preparing concentration	Final concentration
Dexamethasone	Fujifilm Wako Pure Chemical Cat#041-18861	100 mg/mL	10, 50, 100 mg/mL	10, 50, 100 μg/mL
Dimethyl sulfoxide (DMSO)	Sigma Cat#D5879			

Dissolve 1 g of Dexamethasone with DMSO 10 mL, dispend at 100 μL/tube and store at freezer at -30°C.

7-2 Preparation of cells for assay

A cell passage should be done 2-4 days before the assay.

Use cells between 1 and 6 weeks after thawing.

Pre-warm the B medium in a 37°C water bath. Count the number of cells and collect the number of cells needed (2.0 x 10^6 cells are required, but to have some leeway, 3 x 10^6 cells should be prepared), centrifuge the tube at 120-350 x *g*, 5 min. Resuspend in pre-warmed the B medium at a cell density of 2×10^6/mL. Transfer the cell suspension to a reservoir, and add 50 µL of cell suspension to each well of a 96 well black-flame and white-well plate (flat bottom) using an 8 channel or 12 channel pipetman. (cf. Figure 14)

Figure 14

flat-bottom B&W	1	2	3	4	5	6	7	8	9	10	11	12
A	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL							
B	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL							
C	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL							
D	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL							
E												
F												
G												
H												

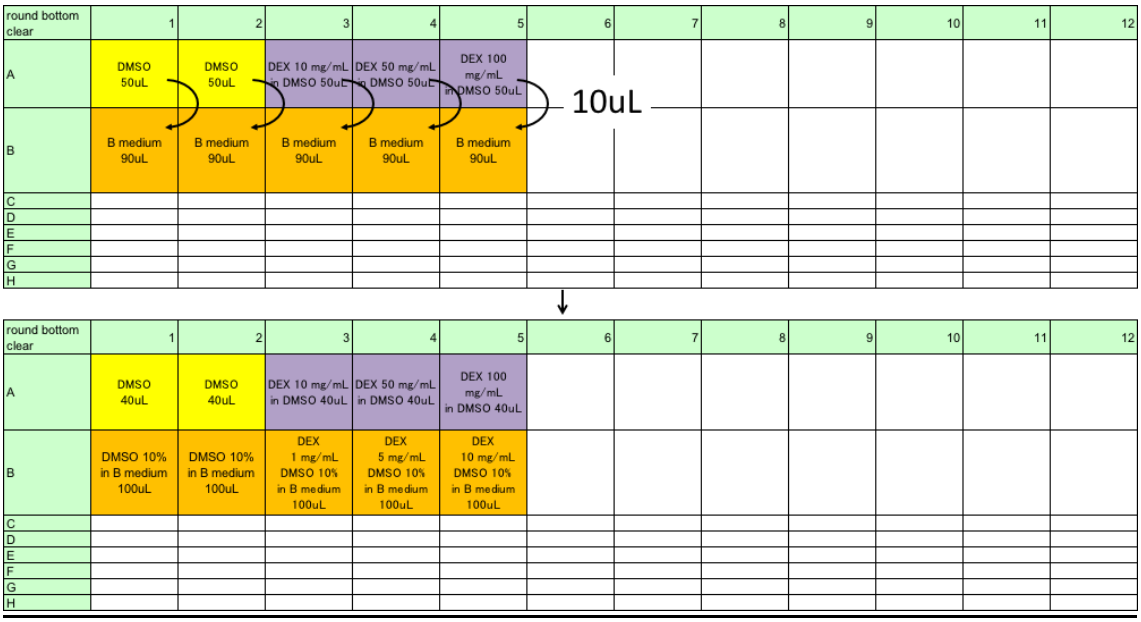
7-3 Arrangement of chemicals and vehicle

Add DMSO 50 µL to #A1-2, 10 mg/mL dexamethasone 50 µl to #A3, 50 mg/mL dexamethasone 50 µl to #A4, 100 mg/mL dexamethasone 50 µl to #A5 and B medium 90 µl to #B1-5 of the 96 well clear plate (round bottom). (cf. Figure 15)

7-4 Dilution with the B medium

Dilute DMSO in #A1-2 and dexamethasone DMSO solution in #A3-5 by adding 10 µL to the B medium in #B1-5. (cf. Figure 15)

Figure 15



7-5　　2 step dilution

Add 10 µL of the diluted DMSO or dexamethasone to 490 µL of the B medium prepared in the assay block. And add 50 µL to THP-G1b (TGCHAC-A4) in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Manipulate the procedures from 7-4 to 7-5 as quickly as you can, and do not leave a long time at step after 7-4 or Figure 16. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 1 hour (37°C, CO_2, 5%). (cf. Figure 16-18)

Figure 16

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 40uL	DMSO 40uL	DEX 10 mg/mL in DMSO 40uL	DEX 50 mg/mL in DMSO 40uL	DEX 100 mg/mL in DMSO 40uL							
B	DMSO 10% in B medium 100uL	DMSO 10% in B medium 100uL	DEX 1 mg/mL DMSO 10% in B medium 100uL	DEX 5 mg/mL DMSO 10% in B medium 100uL	DEX 10 mg/mL DMSO 10% in B medium 100uL							
C												
D												
E												
F												
G												
H												

10uL

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL							
B												
C												
D												
E												
F												
G												
H												

Figure 17

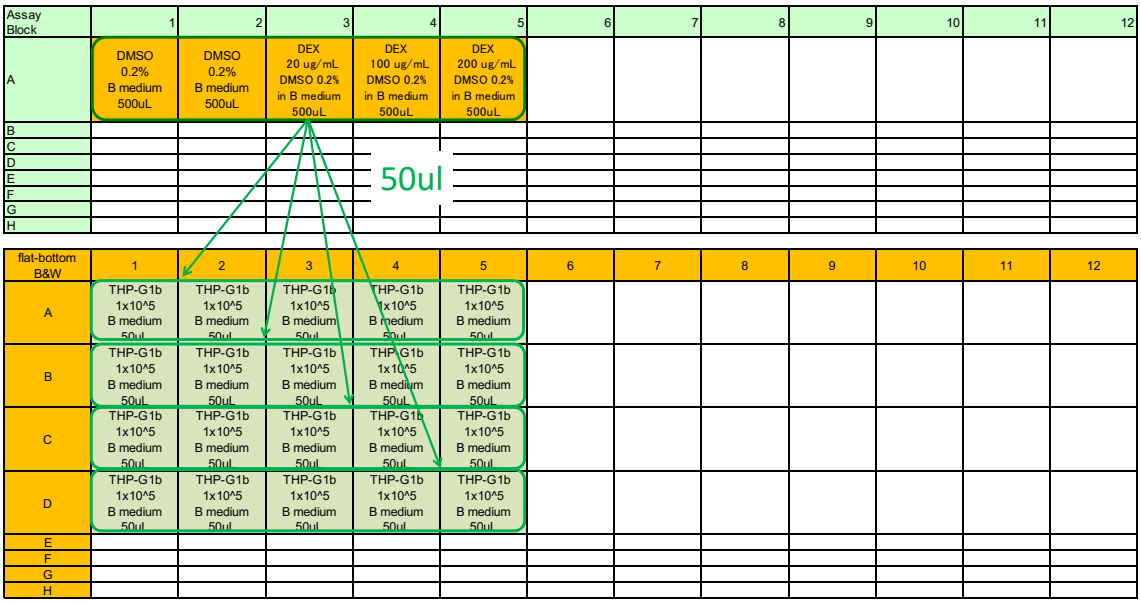


Figure 18 Final constituents of each well of the plate

flat-bottom B&W	1	2	3	4	5	6	7	8	9	10	11	12
A	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 10 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 50 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 100 ug/mL DMSO 0.1% B medium 100uL							
B	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 10 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 50 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 100 ug/mL DMSO 0.1% B medium 100uL							
C	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 10 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 50 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 100 ug/mL DMSO 0.1% B medium 100uL							
D	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 10 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 50 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 100 ug/mL DMSO 0.1% B medium 100uL							
E												
F												
G												
H												

7-6 Addition of LPS to THP-G1b (TGCHAC-A4)

One hour after the addition of dexamethasone, add 10 µL of distilled water or 1000 ng/mL LPS solution prepared in §6 to the cells (#A1-#D1 or #A2-#D5, respectively) using an 8 channel or 12 channel pipetman after pipetting 20 times. Make sure that the apex of the tip is dipped into the medium. Change tips every line you add. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 6 hours (37°C, CO_2, 5%). (cf. Figure 19)

Figure 19

8. Calculation of the transmittance factors

Color discrimination in multi-color reporter assays is generally achieved using detectors (luminometer and plate reader) equipped with optical filters, such as sharp-cut (long-pass) filters and band-pass filters. The transmittance factors of these filters for each bio-luminescence signal color must be calibrated prior to all experiments by following the protocols below.

8-1 Reagents

- Single reference samples:
  Lyophilized luciferase enzyme reagent for stable luciferase green (SLG)
  Lyophilized luciferase enzyme reagent for stable luciferase red (SLR)
- Assay reagent:
  Tripluc® Luciferase assay reagent（TOYOBO Cat#MRA-301）
- B medium: for luciferase assay (30 mL, stored at 2 – 8°C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	27 mL
FBS	Biological Industries Cat#04-001-1E Lot:715004	-	10 %	3 mL

8-2 Preparation of luminescence reaction solution

Thaw Tripluc® Luciferase assay reagent (Tripluc) and keep it at room temperature either in a water bath or at ambient air temperature. Power on the luminometer 30 min before starting the measurement to allow the photomultiplier to stabilize.

Add 200 μL of 100 mM Tris-HCl (pH8.0) contains 10 % glycerol to each tube of lyophilized reference sample to dissolve the enzymes, divide into 10 μL aliquots in 1.5 mL disposable tubes and store in a freezer at -80°C. The stored frozen solution of the reference samples can be used for up to 6 months.

Add 1 mL of the B medium to each tube of frozen reference sample (10 μL sample per tube). Keep the reference samples on ice to prevent deactivation.

8-3 Bioluminescence measurement

Transfer 100 µL of the diluted reference samples to a 96 well black-flame and white-well plate (flat bottom) as shown below (the SLG reference sample to #B1, #B2, #B3, the SLR reference sample to #D1, #D2, #D3).

Figure 20.

flat-bottom B&W	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	SLG 100 µL	SLG 100 µL	SLG 100 µL									
C												
D	SLR 100 µL	SLR 100 µL	SLR 100 µL									
E												
F												
G												
H												

Transfer 100 µL of pre-warmed Tripluc to each well of the plate containing the reference sample using a pipetman. Shake the plate for 10 min at room temperature (about 25°C) using a plate shaker. Remove bubbles in the solutions in wells if they appear. Place the plate in the luminometer to measure the luciferase activity. Bioluminescence is measured for 3 sec each in the absence (F0) and presence (F1) of the optical filter. An example of the raw output data is shown below.

Figure 21. An example of the raw output data

Measurement without Filter

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	3757015	3716611	3810382									
C												
D	2465453	2207572	2077689									
E												
F												
G												
H												

Measurement with Filter

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	236478	234079	240876									
C												
D	1585258	1420099	1339265									
E												
F												
G												
H												

Two transmittance factors of the optical filter were calculated as follow:

$$\text{Transmittance factor } (\kappa G_{R60}) = \frac{\#B1 \text{ of } F1 + \#B2 \text{ of } F1 + \#B3 \text{ of } F1}{\#B1 \text{ of } F0 + \#B2 \text{ of } F0 + \#B3 \text{ of } F0}$$

$$\text{Transmittance factor } (\kappa R_{R60}) = \frac{\#D1 \text{ of } F2 + \#D2 \text{ of } F2 + \#D3 \text{ of } F2}{\#D1 \text{ of } F0 + \#D2 \text{ of } F0 + \#D3 \text{ of } F0}$$

In the case shown above,

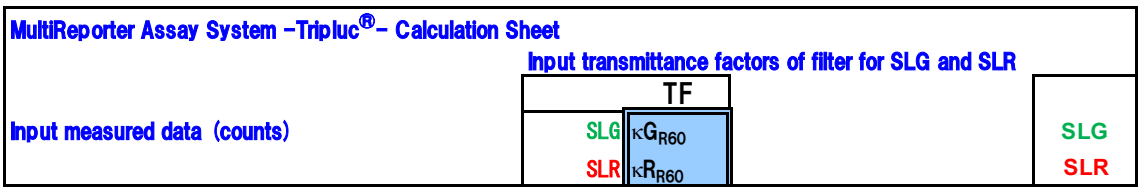
$$\text{Transmittance factors } (\kappa G_{R60}) = \frac{236478+234079+240876}{3757015+3716611+3810382} = 0.063$$

$$\text{Transmittance factors } (\kappa R_{R60}) = \frac{1585258+1420099+1339265}{2465453+2207572+2077689} = 0.644$$

Calculated transmittance factors are used for all the measurements executed using the same luminometer.

Input the transmittance factors to #G4-5 of the “Data Input” sheet of the Data sheet as follow.

Figure 22



9. Measurement

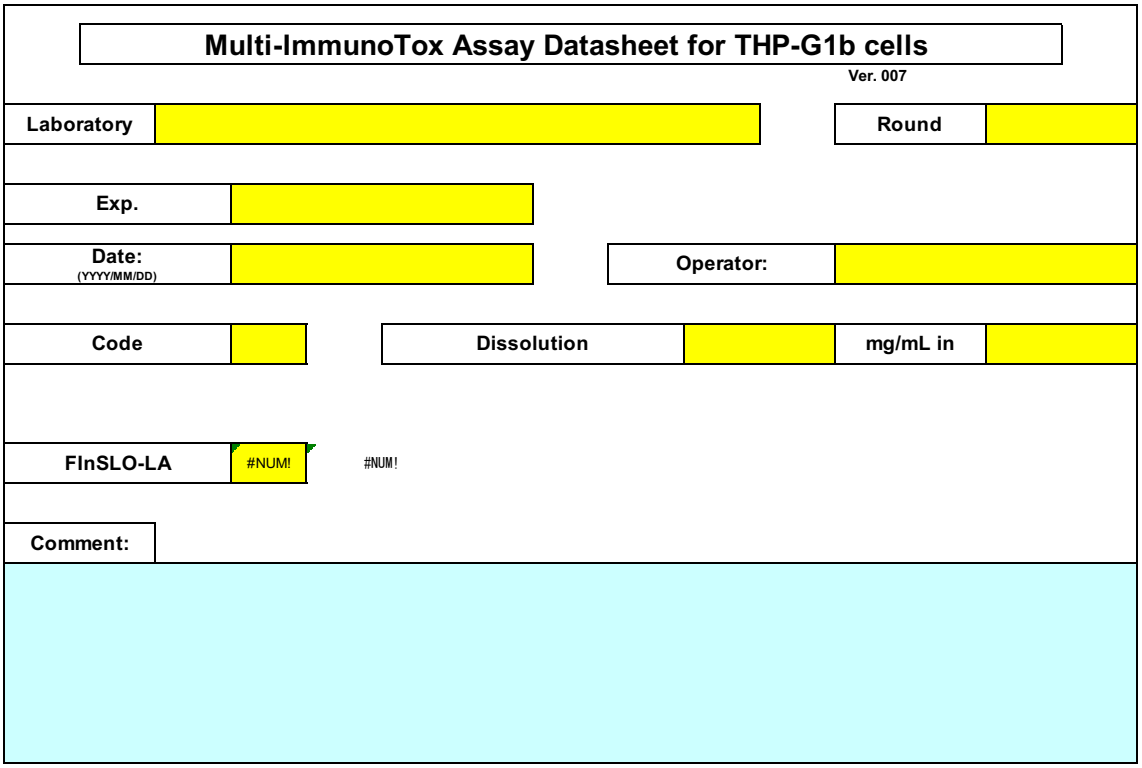
Please refer Appendix 1 for the principle of measurement of luciferase activity.

Thaw Tripluc® Luciferase assay reagent (Tripluc) and keep it at room temperature either in a water bath or at ambient air temperature. Power on the luminometer 30 min before starting the measurement to allow the photomultiplier to stabilize.

Transfer 100 μL of pre-warmed Tripluc from the reservoir to each well of the plate containing the reference sample using an 8 channel or 12 channel pipetman. Shake the plate for 10 min at room temperature (about 25°C) using a plate shaker. Remove bubbles in the solutions in the wells if they appear. Place the plate in the luminometer to measure the luciferase activity. Bioluminescence is measured for 3 sec each in the absence (F0) and presence (F1) of the optical filter. In case alternative settings are used, e.g., depending on the model of luminometer used, these settings should be justified.

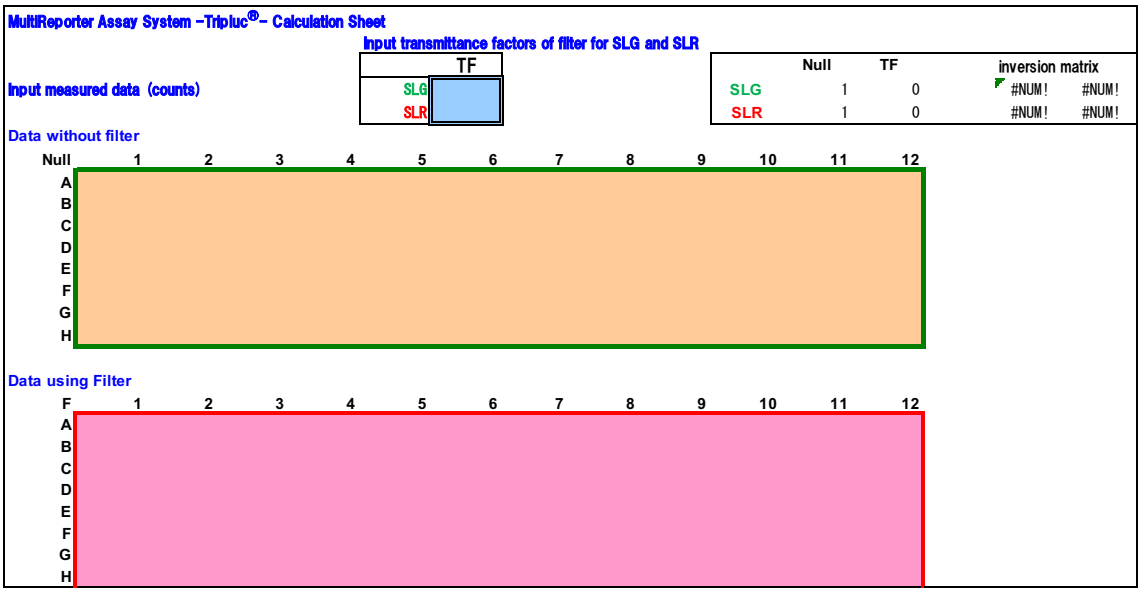
1st. Add the information regarding the name of laboratory, the round of experiments if multiple experimental sets are performed, the experiment number, date, the operator, chemical codes, dissolved in distilled water or DMSO, the prepared concentration and comments if any to Face Sheet of the data sheet.

Figure 23 “Face Sheet” of the data sheet



2nd. Copy the results of the F0 and F1 measurements (values are expressed as counts) and paste them into the appropriate area in the “Data Input” sheet of the data sheet shown below (Figure 28). In addition, input the transmittance factors calculated in chapter 5. Calculation of the transmittance factors to TF of the “Data Input” sheet (Figure 24).

Figure 24 “Data Input” sheet of the data sheet



Next, the calculated results for the parameters of the Multi-Immuno Tox assay for each concentration, e.g., SLG-LA, SLR-LA, nSLG-LA, the mean ± SD of SLG-LA, the mean ± SD of SLR-LA, %suppression and graphs will automatically appear on the “Result Format” sheet of the data sheet.

Figure 25 “Result Format” sheet of the data sheet

10. Data analysis

Definition of the parameters used in the Multi-Immuno Tox assay

- SLG-luciferase activity (SLG-LA): Luciferase activity of stable luciferase orange (Under the control of IL-1β promoter)
- SLR-luciferase activity (SLR-LA): Luciferase activity of stable luciferase red (Under the control of G3PDH promoter)
- Normalized SLG-LA (nSLG-LA): =(SLG-LA)/(SLR-LA)
- Inhibition index of SLR-LA (I.I.-SLR-LA): The cytotoxic effect of chemicals
  =(SLR-LA of THP-G1b treated with chemicals)/(SLR-LA of untreated THP-G1b)
- %suppression: The effect of chemicals on IL-8 promoter
  =(1-(nSLG-LA of THP-G1b treated with chemicals)
  /(nSLG-LA of non-treated THP-G1b)) x 100

11. Criteria

11-1 Acceptance criteria

The following acceptance criteria should be satisfied when using the Multi-Immuno Tox Assay method.

- If Fold induction of nSLG-LA of LPS wells without chemicals (=(nSLG-LA of THP-G1b cells treated with LPS) / (nSLG-LA of non-treated THP-G1b cells)) demonstrate less than 3, the results obtained from the plate containing the control wells should be rejected.
- If the number of concentrations which satisfy I.I.-SLR-LA>=0.05 is less than 6, the experiment, is accepted only when viable wells satisfy the following positive criteria and then, the following experiments should be done using the concentration described in **5-1**.

11-2 Criterion

The experiments are repeated until two consistent positive results or two consistent “non-suppression” are obtained. When two consistent results are obtained, the chemicals are judged as the obtained consistent results.

Identification of immunotoxicant is evaluated by the mean of %suppression and its 95% simultaneous confidence interval.

In each experiment, when the chemicals clear the following 4 criteria, they are judged as suppression. Otherwise, they are judged as non-suppression.
1. The %suppression is ≥ 25 with statistical significance. The statistical significance is judged when the 95% confidence interval does not include 0.
2. The result shows two or more consecutive statistically significant positive data or one statistically significant positive data with a trend in which at least 3 consecutive data increase in a dose dependent manner. In the latter case, the trend can cross 0, as long as only one data point shows the opposite effect without statistical significance.
3. The results are judged using only data obtained in the concentration at which I.I.-SLR-LA is ≥ 0.05
4. The results at 2000 μg/ml is excluded.

The acceptance criteria and the criterion for judgment can be described in the following with the same meaning and possibly more understandable way.

11-1 Acceptance criteria
The following acceptance criteria should be satisfied when using the Multi-ImmunoTox Assay method.

- If Fold induction of nSLG-LA of LPS wells without chemicals (=(nSLG-LA of THP-G1b cells treated with LPS) / (nSLG-LA of non-treated THP-G1b cells)) as a control demonstrates less than 3, the results obtained from the plate containing the control wells should be rejected.
- If the number of concentrations which satisfies I.I.-SLR-LA>=0.05 is less than 6, the experiment is accepted only when viable wells satisfy the following positive criteria and then, the following experiments should be conducted using the concentration described in **5-1**.
- When chemicals are dissolved at 100 mg/ml in water, the data of the wells treated with chemicals at the highest concentration, i.e. 2 mg/ml, was omitted.
-

11-2 Criterion
The experiments are repeated until two consistent suppression or two consistent non-suppression are obtained. When two consistent results are obtained, the chemicals are judged as the obtained consistent results.

Identification of immunotoxicant is evaluated by the %suppression and its 95% confidence interval.

In each experiment, when the chemicals clear the following 3 criteria, they are judged as suppression Otherwise, they are judged as non-suppression.

1. The result shows two or more consecutive statistically significant positive data with $\geq$ 25 of the % suppression or one statistically significant data with $\geq$ 25 of the % suppression and a trend in which at least 3 consecutive data increase in a dose dependent manner. In the latter case, the trend can cross 0, as long as only one data point shows negative data without statistical significance. The statistical significance is judged when the 95% confidence interval does not include 0.
2. The results are judged using only data obtained in the concentration at which I.I.-SLR-LA is $\geq$ 0.05

12. Update record

Ver. 009E for THP-G1b (TGCHAC-A4) 2019.7.1
Change the Acceptance criteria
Change the criteria

Ver. 008.1E for THP-G1b (TGCHAC-A4) 2019.2.7
Change the Acceptance criteria
Change the criteria

Ver. 008E for THP-G1b (TGCHAC-A4) 2018.12.3
Addition of thresholds to the criteria.
Change the composition of the culture medium
Change the prepareation of the dexamethasone solution

Ver. 007E for THP-G1b (TGCHAC-A4) 2018.7.12

Ver. 0011.0E 2018.5.10
Change the criteria

Ver. 0010.0E 2018.1.15 distribution
Change the criteria

Ver. 009.1E 2017.5.8 distribution
Change the criteria

Ver. 009.0E 2017.4.7 distribution
Change the preparation of chemicals
Change the acceptance criteria
Change the criteria

Ver. 008.5E 2016.9.14 distribution
Change the criteria

Ver. 008.4E 2016.9.9 distribution
Change the criteria

Ver. 008.3E 2016.8.1 distribution
Correction of the preparation of PMA and ionomycin
Change the preparation of PMA and ionomycin
Change the preparation of controls
Addition of Acceptance criteria

Ver. 008.1E 2016.2.2 distribution
Changes after the VMT meeting

Ver. 008.0E 2016.1.19
Translation to English
Addition of appendix

Ver. 006.0J 2015.8.17
Change the preparation of chemicals (same method to the IL-8 Luc assay)
Delete the alteration in Ver. 005.0J

Ver. 005.0J 2015.1.9 distribution
Change to use SLR-LA of THP-G8 at the calculation of nSLG-LA of TGCHAC-A4

Ver. 004.1J 2014.12.10 distribution
Change the cellar concentration at cell passage
Modify figure 16, 17

Ver. 004.0J 2014.11.17 distribution
For the validation study at AIST, FDSC and Tohoku university (chemicals: Sodium Bromate ($NaBrO_3$), Nickel (II) sulfate ($NiSO_4$), Dibutyl phthalate (DP), 2-Mercaptobenzothiazole (2-MBT))
Change THP-G1b cells to TGCHAC-A4 cells
Change cell number of THP-G8 and TGCHAC-A4 5x10^4/well to 1x10^5/well
Change concentration of chemicals 11 steps to 10 steps
Change final concentration of LPS (THP-G8 : 25 ng/mL, TGCHAC-A4 : 1 ng/mL)
Change the way of addition of LPS (2 mL/well to 10 mL/well)
Change the criteria

Ver. 002.0J 2013.08.19 distribution

For the validation study at AIST and FDSC (chemicals: $CoCl_2$, $NiSO_4$, Isophorone diisocyanate, 2-Mercaptobenzothiazole）
Change the common ratio 3 to 2
Change the concentration of LPS 100 ng/mL to 25 ng/mL
Add description about the control (dexamethasone)
Delete the appendix about THP-G8 cell

Ver. 001.1J 2012. Nov. 13 distribution
Add the appendix about THP-G8 cell

Ver. 001J 2012. Nov. 09 distribution

Appendix 7 Principle of measurement of luciferase activity

MultiReporter Assay System -Tripluc- can be used with a microplate-type luminometer with a multi-color detection system, which can equip an optical filter. (e.g. Phelios AB-2350 (ATTO), ARVO (PerkinElmer), Tristar LB941 (Berthold)) The optical filter used in measurement is 600 ～ 620 nm long or short pass filter, or 600～700 nm band pass filter.

(1) Measurement of two-color luciferases with an optical filter.

This is an example using Phelios AB-2350 (ATTO). This luminometer equips a 600 nm long pass filter (600 nm LP (Filter 1); R60 HOYA Co.), for splitting SLG and SLR luminescence.

To determine transmission coefficients of the 600 nm LP, first, using purified SLG and SLR luciferase enzymes, measure i) the intensity of SLG and SLR bioluminescence intensity without filter (F0), ii) the SLG and SLR bioluminescence intensity that passed through 600 nm LP (Filter 1), and calculate the transmission coefficients of 600 nm LP for SLG and SLR listed below.

Transmission coefficients		Abbreviation	Definition
SLG	Filter 1 Transmission coefficients	κG_{R60}	The filter's transmission coefficient for the SLG
SLR	Filter 1 Transmission coefficients	κR_{R60}	The filter's transmission coefficient for the SLR

When the intensity of SLG and SLR in test sample are defined as G and R, respectively, i) the intensity of light without filter (all optical): F0 and ii) the intensity of 600 nm LP (Filter 1) transmitted light are described as below.

$F0=G+R$

$F1=\kappa G_{R60} \times G + \kappa R_{R60} \times R$

These formulas can be rephrased as follows

$$\begin{pmatrix} F0 \\ F1 \end{pmatrix} = \begin{pmatrix} 1 & 1 \\ \kappa G_{R60} & \kappa R_{R60} \end{pmatrix} \begin{pmatrix} G \\ R \end{pmatrix}$$

Then using calculated coefficient factors (κG_{R60} and κR_{R60}) and measured F0 and F1, you can calculate G and R-value as follows.

$$\begin{pmatrix} G \\ R \end{pmatrix} = \begin{pmatrix} 1 & 1 \\ \kappa G_{R60} & \kappa R_{R60} \end{pmatrix}^{-1} \begin{pmatrix} F0 \\ F1 \end{pmatrix}$$

This calculation can be performed using the functions "MININVERSE" and "MMULT" in Microsoft Excel. These calculations are integrated in the Data sheet for MITA THP-G1b.

Appendix 8 Validation of reagents and equipment

1 Measurement of transmittance of optical filter for multicolor measurement

For color discriminations in the multi-color reporter assay, detectors (luminometer and plate reader) are usually equipped with optical filters, such as sharp-cut (long-pass) filters and band-pass filters. The transmittance factors of these filters for each bioluminescence signal color have to be calibrated prior to all experiments by following the protocols below.

1-1 Reagents

・Single reference samples:

Lyophilized luciferase enzyme reagent of SLG

Lyophilized luciferase enzyme reagent of SLR

・Assay reagent:

Tripluc® Luciferase assay reagent（TOYOBO Cat#MRA-301）

・B medium: for luciferase assay (30 mL, stored at 2−8°C)

Reagent	Company		Final conc. in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	27 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	3 mL

1-2 Calibration

1-2-1 Preparation of luminescence reaction solution

Thaw Tripluc® Luciferase assay reagent (Tripluc) and keep it at room temperature by bathing in water or ambient air. Start the luminometer 30 min before starting the measurement for stabilization of the photomultiplier.

Add 200 μL of 100 mM Tris-HCl (pH8.0) contains 10% glycerol to each tube of the lyophilized reference samples to dissolve the enzymes, followed by separating them into 1.5 mL disposable tubes at 10 μL each and storing in a freezer at -80°C. The stored frozen solution of the reference samples can be used for one half year.

Add 1 mL of B medium to each tube of the frozen reference sample (10 μL in a tube) and label them as SLG1/1, SLR1/1, and SLG/SLR1/1. Keep the reference samples on ice to prevent deactivation.

Prepare dilution series of the single reference samples of SLG and SLR as follows. Dilute 0.3 mL of each 1/1 solution with 0.9 mL of B medium to make SLG1/4 and SLR1/4. In the same manner, prepare 1/16 and 1/64 solution of each. Keep diluted reference samples on ice.

1-2-2 Bioluminescence measurement

Transfer 100 μL of the diluted reference samples to a 96-well flat-bottom black plate as shown below.

Figure 26.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	SLG 1/1	SLG 1/1	SLG 1/1	SLG 1/4	SLG 1/4	SLG 1/4	SLG 1/16	SLG 1/16	SLG 1/16	SLG 1/64	SLG 1/64	SLG 1/64
C												
D	SLR 1/1	SLR 1/1	SLR 1/1	SLR 1/4	SLR 1/4	SLR 1/4	SLR 1/16	SLR 1/16	SLR 1/16	SLR 1/64	SLR 1/64	SLR 1/64
E												
F												
G												
H												

Transfer 100 μL of pre-warmed Tripluc to each well containing the reference samples of the plate using a pipetman. Shake the plate for 10 min at room temperature (about 20°C) with a plate shaker. Remove bubbles on the solutions in wells if they appear. Place the plate into the luminometer of the under-test to measure the luciferase activity. Bioluminescence is measured for 3 sec each in the absence (F0) and presence (F1) of the optical filter. The example of the raw output data was shown below.

Figure 32.

Measurement without Filter

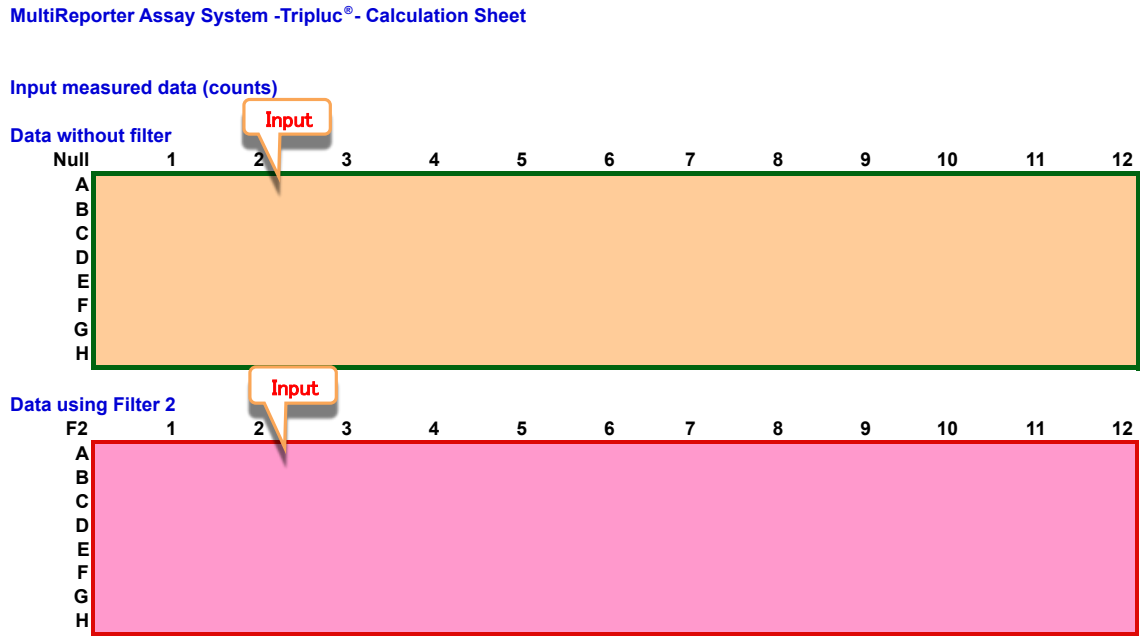
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	9689	9691	9677	2402	2388	2412	704	689	721	177	189	182
C												
D	8588	8444	8462	2281	2128	2239	609	578	690	150	132	129
E												
F												
G												
H												

Measurement with Filter

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	2022	1945	2067	502	496	510	143	149	153	37	49	45
C												
D	5722	5756	5721	1523	1459	1589	413	397	468	102	108	97
E												
F												
G												
H												

Copy the results of the F0 and F1 measurement (values are expressed as counts) and paste it to the appropriate area in the “Data Input” sheet of the data sheet for data analyses shown below.

Figure 33.



Record all the results for quality control.

2 Quality control of equipment

In order to confirm the detector stability as the quality control, the reference luciferase sample, optical property, the protocol described hereafter should be performed at the beginning of the experiments every day using reference LED light source plates equipped

with stabilized LEDs. LED plate data typically fluctuates up to 1.5% (σ). Disagreement to the old data should be less than 3×σ (= 4.5%). Here describes an example using a commercially available reference light source, TRIANT® (wSL-0001) by ATTO (Tokyo, Japan).

Start LED plate and select “PMT” mode.

Select three-color (BRG) mode and adjust light intensity to 1/10 (10E-1).

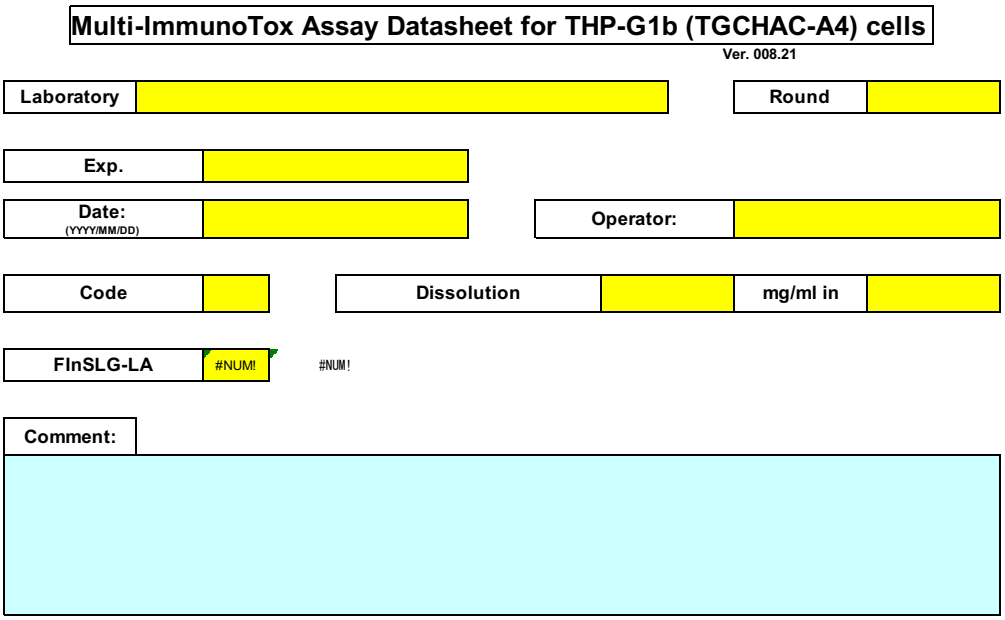
Place the LED plate into the luminometer. Light intensity is measured for 3 sec each in the absence (F0) and presence (F2) of the optical filter.

Read and record the data at the position of #F6, #E6, and #D6 that are LEDs of blue, green, and red LEDs, respectively.

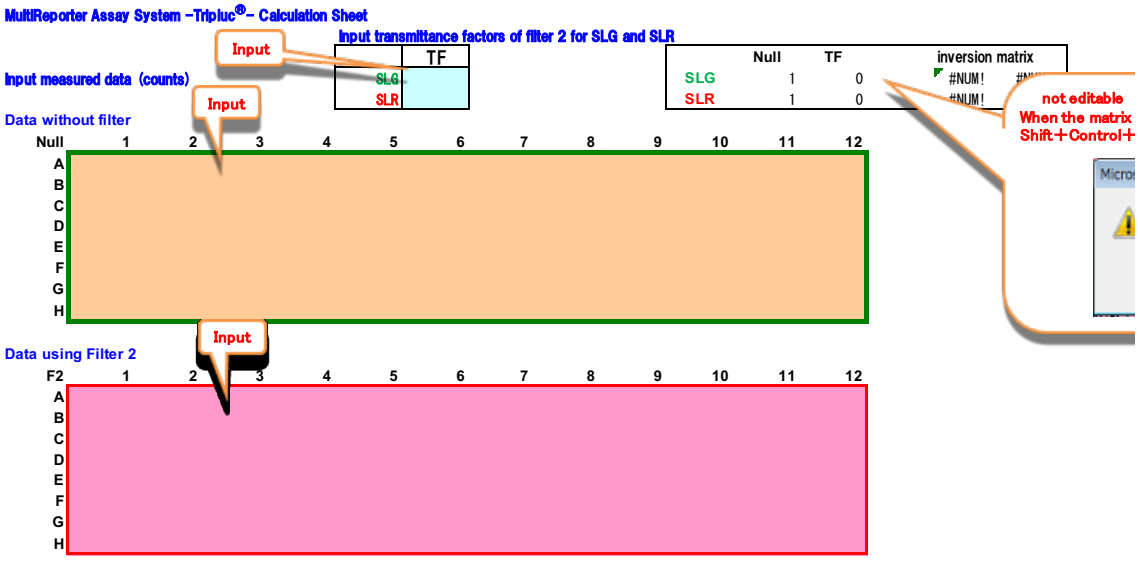
LED plate data typically fluctuates up to 1.5% (σ). Disagreement to the old data should be less than 3×σ (= 4.5%).

Appendix 10 Multi-ImmunoTox Assay Datasheet for THP-G1b (TGCHAC-A4) cells ver.008.21E

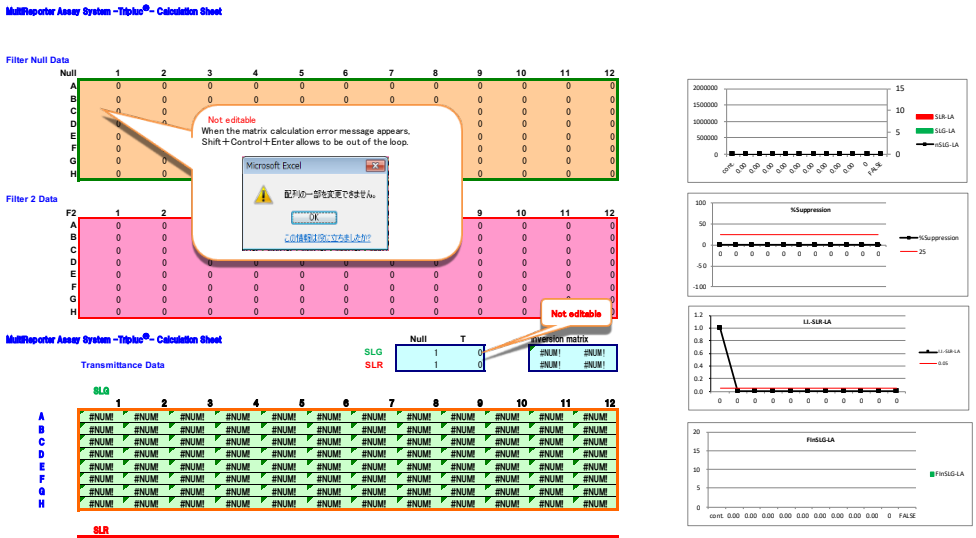
1. Face sheet

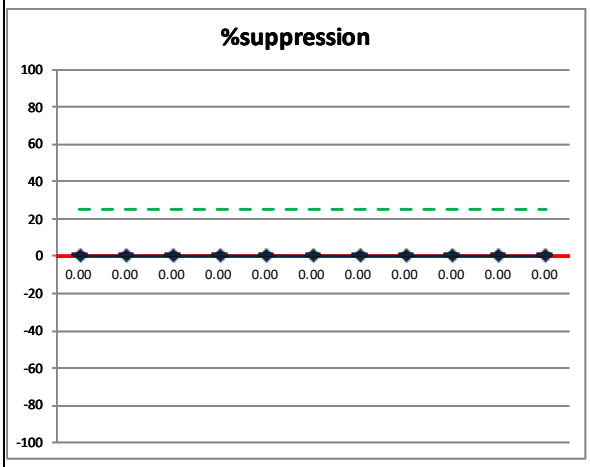


2. Data input sheet



3. Result format sheet





4. Graph sheet

Results

0 LINE 入力 25

a Comparison Conc	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
0	0	0	0		
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

IL1-β Graph P1(Line25)

2019.06.26

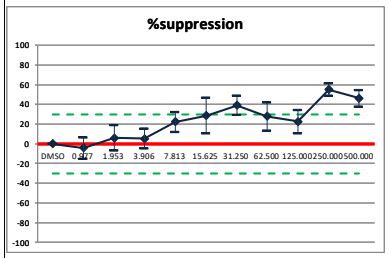
Takashi Omori

Chem.1 Set1 (MITA103,MITB402,MITC704)

LabA Tohoku

Exp.1

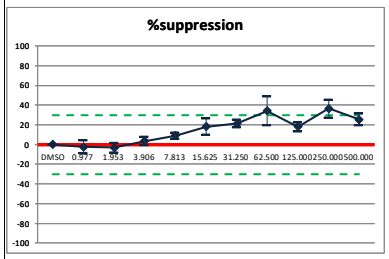
1st exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	-4.142	-15.015	6.731	N.S.	
1.953	5.862	-6.956	18.680	N.S.	
3.906	5.321	-4.490	15.133	N.S.	
7.813	22.124	11.916	32.332	Sig.	
15.625	28.596	10.390	46.802	Sig.	
31.250	39.008	29.157	48.859	Sig.	Over
62.500	27.738	13.647	41.828	Sig.	
125.000	22.511	10.702	34.320	Sig.	
250.000	55.038	48.566	61.510	Sig.	Over
500.000	46.124	38.065	54.183	Sig.	Over

Exp.2

2nd exp. LINE 入力 30

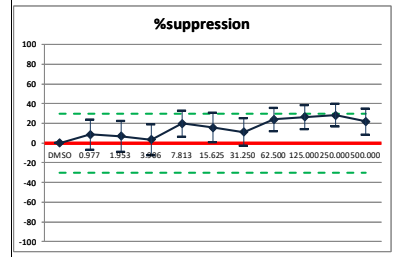


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	-2.312	-8.731	4.107	N.S.	
1.953	-3.100	-8.021	1.822	N.S.	
3.906	3.369	-0.711	7.450	N.S.	
7.813	9.007	5.850	12.164	Sig.	
15.625	18.232	10.112	26.351	Sig.	
31.250	21.128	17.293	24.963	Sig.	
62.500	34.240	19.773	48.707	Sig.	Over
125.000	18.201	13.691	22.711	Sig.	
250.000	36.614	27.580	45.647	Sig.	Over
500.000	25.460	19.739	31.180	Sig.	

Exp.3

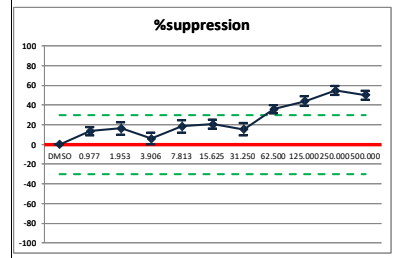
LabB AIST tsukuba

1st exp. LINE 入力 30



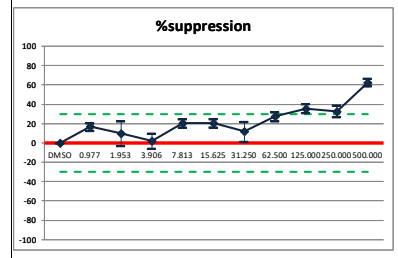
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	8.544	-6.710	23.798	N.S.	
1.953	6.767	-8.827	22.361	N.S.	
3.906	3.410	-12.260	19.081	N.S.	
7.813	19.723	6.434	33.012	Sig.	
15.625	15.669	0.766	30.573	Sig.	
31.250	11.248	-2.921	25.416	N.S.	
62.500	23.973	12.009	35.937	Sig.	
125.000	26.487	14.228	38.746	Sig.	
250.000	28.178	16.801	39.556	Sig.	
500.000	21.907	8.568	35.246	Sig.	

2nd exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	13.484	9.292	17.676	Sig.	
1.953	16.157	9.700	22.614	Sig.	
3.906	5.875	0.107	11.643	Sig.	
7.813	18.390	12.057	24.723	Sig.	
15.625	20.441	16.001	24.881	Sig.	
31.250	15.293	9.080	21.507	Sig.	
62.500	35.708	31.634	39.782	Sig.	Over
125.000	43.736	38.831	48.641	Sig.	Over
250.000	54.735	49.992	59.479	Sig.	Over
500.000	49.972	45.419	54.524	Sig.	Over

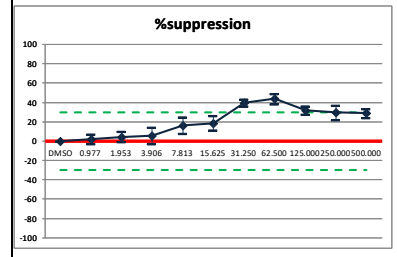
3rd exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	16.794	13.033	20.554	Sig.	
1.953	9.847	-3.054	22.747	N.S.	
3.906	2.131	-5.625	9.887	N.S.	
7.813	20.594	16.081	25.108	Sig.	
15.625	20.413	16.066	24.759	Sig.	
31.250	12.010	1.694	22.326	Sig.	
62.500	27.535	22.990	32.080	Sig.	
125.000	35.723	31.030	40.417	Sig.	Over
250.000	32.742	26.661	38.823	Sig.	Over
500.000	62.769	58.919	66.618	Sig.	Over

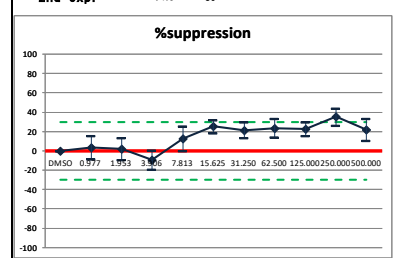
LabC AIST shikoku

1st exp. LINE 入力 30



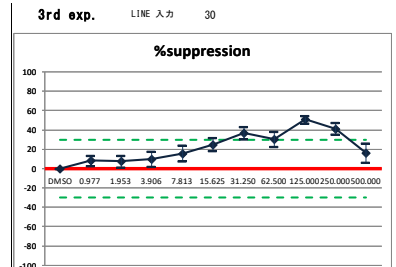
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	2.076	-3.024	7.176	N.S.	
1.953	4.533	-1.060	10.125	N.S.	
3.906	5.712	-2.816	14.241	N.S.	
7.813	16.165	7.638	24.692	Sig.	
15.625	18.731	11.362	26.100	Sig.	
31.250	39.762	36.206	43.319	Sig.	Over
62.500	43.772	38.445	49.098	Sig.	Over
125.000	31.782	27.736	35.829	Sig.	Over
250.000	29.455	22.072	36.839	Sig.	
500.000	28.999	24.419	33.579	Sig.	

2nd exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	3.200	-8.833	15.234	N.S.	
1.953	1.927	-9.423	13.277	N.S.	
3.906	-9.132	-18.945	0.681	N.S.	
7.813	12.584	-0.146	25.315	N.S.	
15.625	25.198	18.163	32.233	Sig.	
31.250	21.362	13.208	29.517	Sig.	
62.500	23.739	14.033	33.444	Sig.	
125.000	22.713	15.322	30.104	Sig.	
250.000	35.299	26.404	44.193	Sig.	Over
500.000	21.653	10.290	33.017	Sig.	

3rd exp. LINE 入力 30



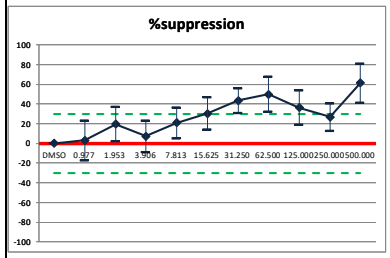
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	8.359	3.096	13.621	Sig.	
1.953	7.478	1.512	13.445	Sig.	
3.906	9.850	1.764	17.936	Sig.	
7.813	15.797	7.722	23.873	Sig.	
15.625	25.131	18.465	31.797	Sig.	
31.250	36.995	30.739	43.251	Sig.	Over
62.500	30.350	22.667	38.034	Sig.	Over
125.000	50.875	47.074	54.676	Sig.	Over
250.000	41.334	35.377	47.292	Sig.	Over
500.000	16.190	6.302	26.079	Sig.	

Chem.1 Set2 (MITA203,MITB501,MITC803)

LabA Tohoku

Exp.1

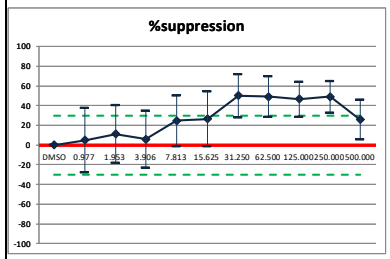
1st exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	3.025	-17.085	23.135	N.S.	
1.953	19.504	1.899	37.109	Sig.	
3.906	7.109	-9.051	23.269	N.S.	
7.813	20.754	5.230	36.278	Sig.	
15.625	30.288	14.087	46.490	Sig.	Over
31.250	43.396	30.904	55.887	Sig.	Over
62.500	49.782	31.879	67.686	Sig.	Over
125.000	36.145	18.833	53.457	Sig.	Over
250.000	26.860	12.917	40.804	Sig.	
500.000	61.391	41.533	81.249	Sig.	Over

Exp.2

2nd exp. LINE 入力 30

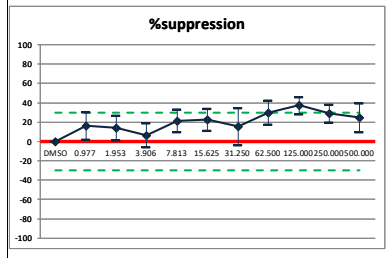


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	4.964	-27.928	37.855	N.S.	
1.953	11.256	-18.010	40.521	N.S.	
3.906	5.923	-23.034	34.881	N.S.	
7.813	24.622	-1.101	50.346	N.S.	
15.625	26.548	-1.050	54.146	N.S.	
31.250	50.035	28.002	72.069	Sig.	Over
62.500	49.072	28.349	69.794	Sig.	Over
125.000	46.657	28.858	64.456	Sig.	Over
250.000	49.153	33.161	65.146	Sig.	Over
500.000	25.904	5.592	46.215	Sig.	

Exp.3

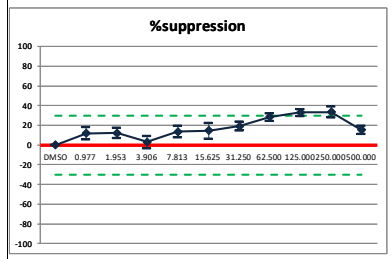
LabB AIST tsukuba

1st exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	16.133	2.056	30.211	Sig.	
1.953	14.074	1.313	26.835	Sig.	
3.906	6.561	-6.035	19.156	N.S.	
7.813	21.465	9.780	33.150	Sig.	
15.625	22.802	11.496	34.108	Sig.	
31.250	15.714	-3.331	34.759	N.S.	
62.500	30.052	17.619	42.485	Sig.	Over
125.000	37.475	28.633	46.318	Sig.	Over
250.000	28.938	19.613	38.263	Sig.	
500.000	24.631	9.868	39.395	Sig.	

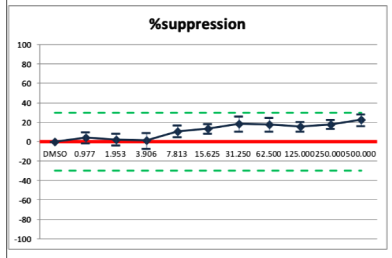
2nd exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	11.756	5.450	18.062	Sig.	
1.953	12.194	6.775	17.612	Sig.	
3.906	3.063	-3.029	9.156	N.S.	
7.813	13.629	7.731	19.527	Sig.	
15.625	14.538	6.406	22.671	Sig.	
31.250	19.238	15.007	23.469	Sig.	
62.500	28.351	24.503	32.200	Sig.	
125.000	32.917	29.172	36.662	Sig.	Over
250.000	33.457	28.061	38.853	Sig.	Over
500.000	15.251	11.087	19.415	Sig.	

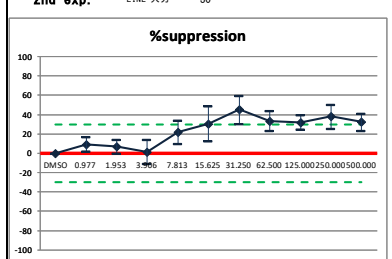
LabC AIST shikoku

1st exp. LINE 入力 30



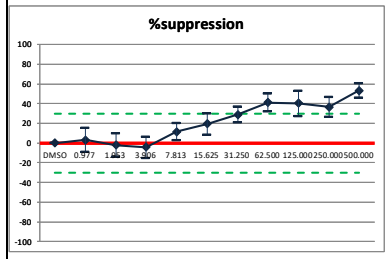
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	4.130	-1.529	9.790	N.S.	
1.953	2.371	-3.795	8.538	N.S.	
3.906	1.239	-6.982	9.459	N.S.	
7.813	10.959	5.268	16.651	Sig.	
15.625	13.655	8.797	18.514	Sig.	
31.250	18.427	10.658	26.196	Sig.	
62.500	17.729	10.765	24.692	Sig.	
125.000	15.536	10.757	20.316	Sig.	
250.000	17.964	13.453	22.474	Sig.	
500.000	22.333	16.207	28.459	Sig.	

2nd exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	9.547	2.228	16.866	Sig.	
1.953	7.057	0.105	14.008	Sig.	
3.906	1.651	-10.983	14.284	N.S.	
7.813	21.827	9.948	33.707	Sig.	
15.625	30.838	12.421	49.256	Sig.	Over
31.250	45.145	30.717	59.573	Sig.	Over
62.500	33.444	23.118	43.771	Sig.	Over
125.000	32.213	24.489	39.936	Sig.	Over
250.000	38.033	25.869	50.197	Sig.	Over
500.000	32.331	23.315	41.346	Sig.	Over

3rd exp. LINE 入力 30



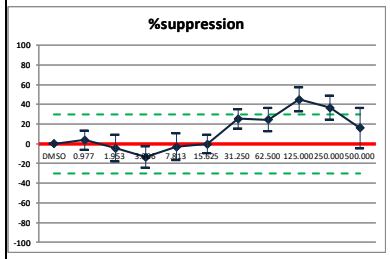
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	3.198	-8.993	15.389	N.S.	
1.953	-2.049	-13.639	9.541	N.S.	
3.906	-4.177	-15.040	6.687	N.S.	
7.813	11.548	2.813	20.282	Sig.	
15.625	19.369	8.491	30.246	Sig.	
31.250	28.882	20.782	36.983	Sig.	
62.500	41.090	31.941	50.239	Sig.	Over
125.000	40.333	27.392	53.275	Sig.	Over
250.000	36.679	26.511	46.847	Sig.	Over
500.000	53.319	46.173	60.465	Sig.	Over

Chem.1 Set3 (MITA304,MITB605,MITC902)

LabA Tohoku

Exp.1

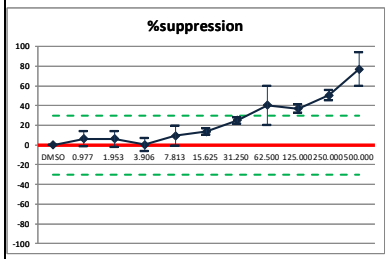
1st exp. LINE 入力 30



a Comparison Conc.	%supp	LowerLimit	UpperLimit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	3.684	-5.830	13.198	N.S.	
1.953	-4.589	-18.207	9.028	N.S.	
3.906	-13.708	-24.561	-2.854	Sig.	
7.813	-3.058	-16.456	10.339	N.S.	
15.625	-0.281	-9.664	9.101	N.S.	
31.250	25.284	15.586	34.982	Sig.	
62.500	24.415	12.542	36.287	Sig.	
125.000	44.932	32.751	57.114	Sig.	Over
250.000	36.615	24.265	48.966	Sig.	Over
500.000	15.965	-4.721	36.652	N.S.	

Exp.2

2nd exp. LINE 入力 30



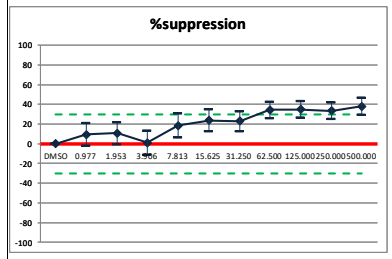
a Comparison Conc.	%supp	LowerLimit	UpperLimit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	6.321	-1.268	13.909	N.S.	
1.953	6.213	-1.811	14.237	N.S.	
3.906	0.370	-6.371	7.110	N.S.	
7.813	9.294	-0.725	19.313	N.S.	
15.625	13.649	10.266	17.033	Sig.	
31.250	25.140	22.023	28.257	Sig.	
62.500	40.218	20.658	59.778	Sig.	Over
125.000	36.910	32.823	40.996	Sig.	Over
250.000	50.563	45.109	56.016	Sig.	Over
500.000	77.029	59.971	94.087	Sig.	Over

Exp.3

LabB AIST tsukuba

Exp.1

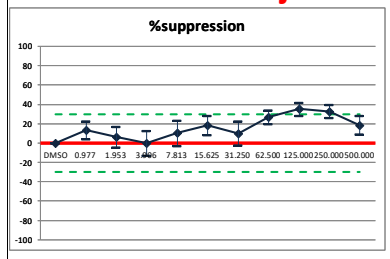
1st exp. LINE 入力 30



a Comparison Conc.	%supp	LowerLimit	UpperLimit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	9.529	-2.219	21.277	N.S.	
1.953	10.814	-0.433	22.061	N.S.	
3.906	1.117	-11.118	13.352	N.S.	
7.813	18.496	6.110	30.881	Sig.	
15.625	23.805	12.342	35.268	Sig.	
31.250	22.813	12.553	33.073	Sig.	
62.500	34.309	25.652	42.966	Sig.	Over
125.000	34.920	26.370	43.470	Sig.	Over
250.000	33.342	24.949	41.734	Sig.	Over
500.000	38.007	29.307	46.706	Sig.	Over

Exp.2

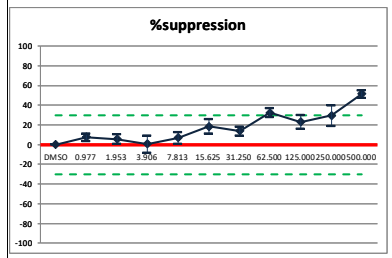
2nd exp. LINE 入力 30 **reject**



a Comparison Conc.	%supp	LowerLimit	UpperLimit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	13.578	4.177	22.980	Sig.	
1.953	6.493	-4.009	16.995	N.S.	
3.906	-0.201	-12.870	12.468	N.S.	
7.813	10.306	-3.051	23.663	N.S.	
15.625	18.281	8.143	28.418	Sig.	
31.250	10.179	-2.221	22.579	N.S.	
62.500	26.844	19.982	33.706	Sig.	
125.000	35.188	28.561	41.815	Sig.	Over
250.000	32.879	26.315	39.443	Sig.	Over
500.000	18.725	9.339	28.111	Sig.	

Exp.3

2nd exp. LINE 入力 30

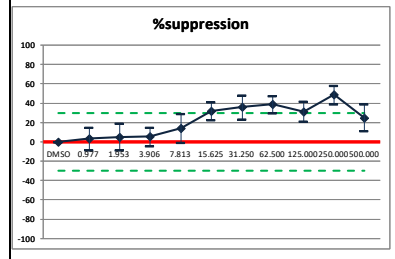


a Comparison Conc.	%supp	LowerLimit	UpperLimit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	7.577	3.616	11.539	Sig.	
1.953	5.596	0.546	10.645	Sig.	
3.906	0.650	-8.034	9.334	N.S.	
7.813	6.897	1.068	12.725	Sig.	
15.625	18.336	11.115	25.558	Sig.	
31.250	13.729	9.396	18.063	Sig.	
62.500	32.383	27.786	36.980	Sig.	Over
125.000	22.903	16.022	29.784	Sig.	
250.000	29.684	19.196	40.173	Sig.	
500.000	51.393	47.322	55.464	Sig.	Over

LabC AIST shikoku

Exp.1

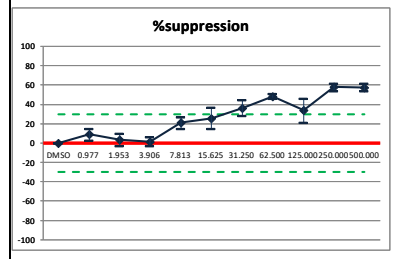
1st exp. LINE 入力 30



a Comparison Conc.	%supp	LowerLimit	UpperLimit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	3.350	-8.436	15.136	N.S.	
1.953	5.251	-8.634	19.135	N.S.	
3.906	5.455	-4.144	15.054	N.S.	
7.813	13.943	-0.985	28.870	N.S.	
15.625	32.021	22.809	41.232	Sig.	Over
31.250	35.958	23.419	48.497	Sig.	Over
62.500	38.762	30.012	47.511	Sig.	Over
125.000	31.437	21.307	41.568	Sig.	Over
250.000	48.875	39.347	58.402	Sig.	Over
500.000	24.976	11.275	38.677	Sig.	

Exp.2

2nd exp. LINE 入力 30



a Comparison Conc.	%supp	LowerLimit	UpperLimit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	8.861	2.861	14.860	Sig.	
1.953	3.434	-2.762	9.630	N.S.	
3.906	1.688	-3.153	6.529	N.S.	
7.813	21.011	15.163	26.858	Sig.	
15.625	25.767	14.534	37.000	Sig.	
31.250	36.313	28.041	44.585	Sig.	Over
62.500	48.544	46.206	50.883	Sig.	Over
125.000	33.848	21.357	46.340	Sig.	Over
250.000	57.879	53.690	62.067	Sig.	Over
500.000	57.666	53.797	61.535	Sig.	Over

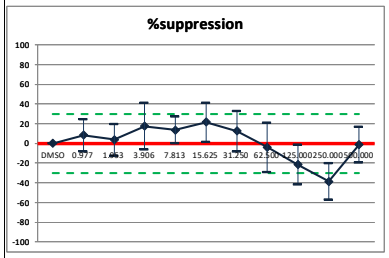
Exp.3

Chem.2 Set1 (MITA101,MITB404,MITC701)

LabA Tohoku

Exp.1

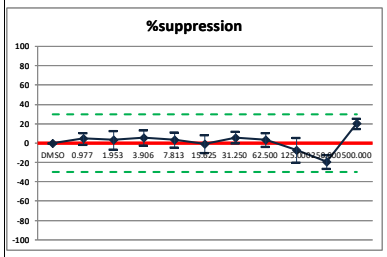
1st exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	8.243	-7.979	24.466	N.S.	
1.953	3.927	-12.057	19.912	N.S.	
3.906	17.536	-5.908	40.979	N.S.	
7.813	13.698	0.412	26.984	Sig.	
15.625	21.436	1.332	41.540	Sig.	
31.250	12.363	-8.406	33.132	N.S.	
62.500	-4.036	-29.188	21.116	N.S.	
125.000	-21.515	-41.473	-1.557	Sig.	
250.000	-38.529	-56.701	-20.356	Sig.	Over
500.000	-1.370	-19.675	16.935	N.S.	

Exp.2

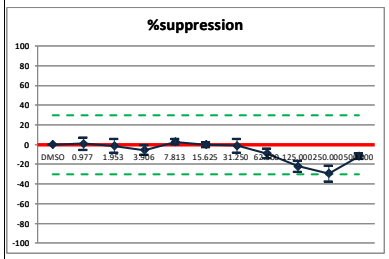
2nd exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	4.744	-1.290	10.779	N.S.	
1.953	3.182	-6.188	12.552	N.S.	
3.906	5.642	-1.975	13.259	N.S.	
7.813	3.567	-4.074	11.207	N.S.	
15.625	-0.828	-10.092	8.435	N.S.	
31.250	5.826	-0.233	11.884	N.S.	
62.500	3.672	-3.570	10.915	N.S.	
125.000	-7.042	-19.871	5.788	N.S.	
250.000	-19.364	-26.443	-12.286	Sig.	
500.000	20.240	14.974	25.506	Sig.	

Exp.3

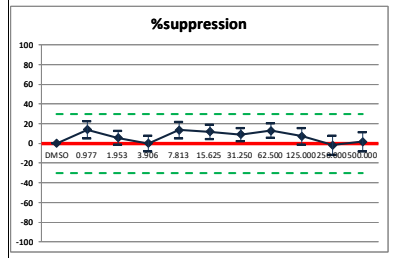
3rd exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	0.960	-5.377	7.296	N.S.	
1.953	-1.424	-8.492	5.644	N.S.	
3.906	-5.725	-10.596	-0.854	Sig.	
7.813	2.909	-0.139	5.958	N.S.	
15.625	0.131	-2.305	2.566	N.S.	
31.250	-1.101	-8.036	5.834	N.S.	
62.500	-9.021	-14.017	-4.026	Sig.	
125.000	-22.097	-27.662	-16.531	Sig.	
250.000	-29.361	-37.414	-21.309	Sig.	
500.000	-11.748	-14.793	-8.703	Sig.	

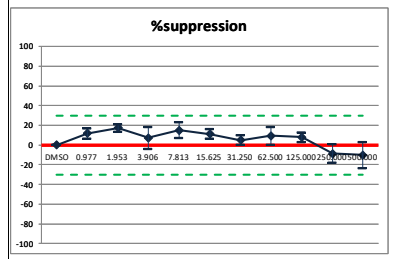
LabB AIST tsukuba

1st exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	13.846	4.975	22.717	Sig.	
1.953	5.517	-1.316	12.350	N.S.	
3.906	-0.180	-8.497	8.136	N.S.	
7.813	13.370	4.759	21.981	Sig.	
15.625	11.740	4.434	19.045	Sig.	
31.250	8.931	2.078	15.785	Sig.	
62.500	12.902	5.740	20.064	Sig.	
125.000	7.107	-1.381	15.596	N.S.	
250.000	-1.704	-11.552	8.144	N.S.	
500.000	1.649	-7.915	11.213	N.S.	

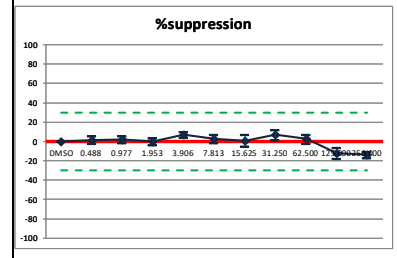
2nd exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	11.640	6.454	16.825	Sig.	
1.953	17.292	13.284	21.300	Sig.	
3.906	7.133	-3.723	17.990	N.S.	
7.813	14.947	7.121	22.772	Sig.	
15.625	11.252	6.075	16.429	Sig.	
31.250	5.001	-0.163	10.165	N.S.	
62.500	9.286	0.310	18.263	Sig.	
125.000	7.853	2.953	12.754	Sig.	
250.000	-8.868	-18.316	0.580	N.S.	
500.000	-10.171	-23.456	3.113	N.S.	

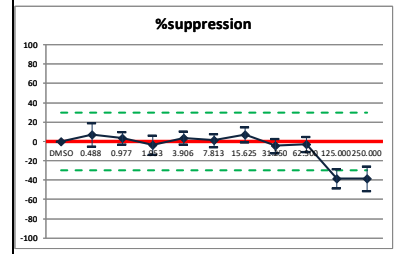
LabC AIST shikoku

1st exp. LINE 入力 30



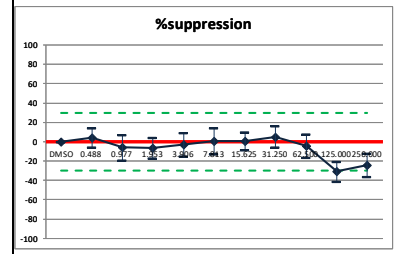
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.488	1.574	-2.303	5.452	N.S.	
0.977	2.162	-1.502	5.826	N.S.	
1.953	-0.125	-3.876	3.626	N.S.	
3.906	6.985	4.089	9.880	Sig.	
7.813	2.869	-1.111	6.850	N.S.	
15.625	0.868	-5.200	6.936	N.S.	
31.250	6.985	1.599	12.371	Sig.	
62.500	2.473	-2.446	7.392	N.S.	
125.000	-12.055	-17.563	-6.547	Sig.	
250.000	-13.809	-17.166	-10.452	Sig.	

2nd exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.488	7.150	-4.725	19.024	N.S.	
0.977	3.310	-3.180	9.800	N.S.	
1.953	-3.474	-13.400	6.453	N.S.	
3.906	3.749	-2.986	10.485	N.S.	
7.813	1.123	-5.518	7.765	N.S.	
15.625	7.176	-0.820	15.172	N.S.	
31.250	-4.450	-11.925	3.024	N.S.	
62.500	-2.992	-10.770	4.786	N.S.	
125.000	-38.421	-48.059	-28.783	Sig.	Over
250.000	-38.428	-51.385	-25.470	Sig.	Over

3rd exp. LINE 入力 30

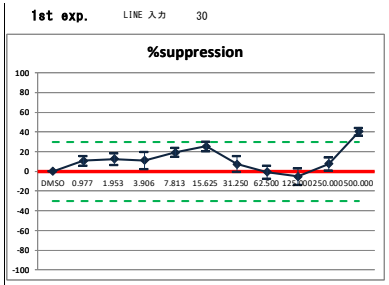


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.488	4.246	-5.988	14.480	N.S.	
0.977	-5.967	-18.878	6.944	N.S.	
1.953	-6.479	-17.109	4.150	N.S.	
3.906	-2.595	-14.691	9.501	N.S.	
7.813	0.771	-12.751	14.293	N.S.	
15.625	0.727	-8.609	10.063	N.S.	
31.250	5.209	-5.885	16.303	N.S.	
62.500	-4.334	-16.282	7.614	N.S.	
125.000	-30.811	-41.226	-20.396	Sig.	Over
250.000	-23.980	-36.174	-11.785	Sig.	

Chem.2 Set2 (MITA205,MITB505,MITC802)

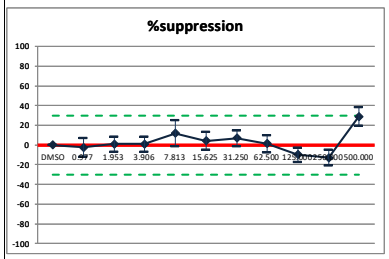
LabA Tohoku

Exp.1



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	10.585	5.393	15.776	Sig.	
1.953	12.396	6.276	18.517	Sig.	
3.906	11.042	2.131	19.953	Sig.	
7.813	19.148	14.609	23.687	Sig.	
15.625	25.437	20.538	30.336	Sig.	
31.250	7.212	-0.701	15.124	N.S.	
62.500	-0.772	-7.256	5.712	N.S.	
125.000	-5.310	-13.619	3.000	N.S.	
250.000	7.571	1.079	14.062	Sig.	
500.000	40.297	36.650	43.944	Sig.	Over

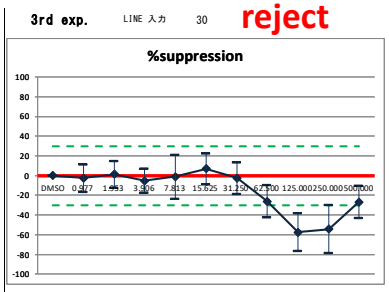
Exp.2



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	-2.370	-12.024	7.284	N.S.	
1.953	0.936	-6.807	8.680	N.S.	
3.906	0.883	-6.677	8.442	N.S.	
7.813	11.883	-1.286	25.052	N.S.	
15.625	4.124	-4.816	13.064	N.S.	
31.250	6.764	-1.333	14.861	N.S.	
62.500	1.281	-7.273	9.834	N.S.	
125.000	-9.930	-17.524	-2.335	Sig.	
250.000	-12.813	-20.729	-4.897	Sig.	
500.000	28.938	19.674	38.201	Sig.	

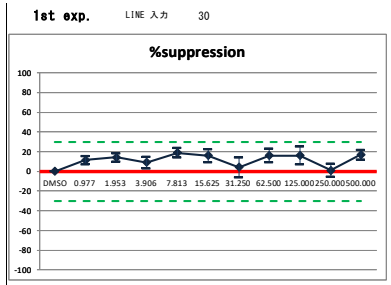
Exp.3

reject

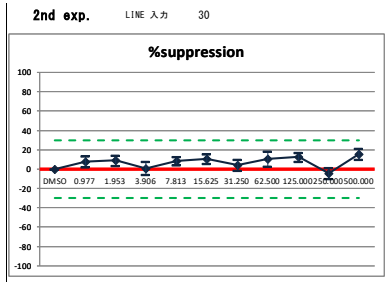


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	-2.601	-16.242	11.041	N.S.	
1.953	1.230	-12.177	14.638	N.S.	
3.906	-5.102	-16.970	6.767	N.S.	
7.813	-1.135	-23.229	20.959	N.S.	
15.625	6.801	-8.795	22.397	N.S.	
31.250	-2.513	-18.452	13.426	N.S.	
62.500	-26.081	-42.502	-9.660	Sig.	
125.000	-57.387	-76.513	-38.262	Sig.	Over
250.000	-54.191	-78.251	-30.131	Sig.	Over
500.000	-26.850	-43.343	-10.357	Sig.	

LabB AIST tsukuba

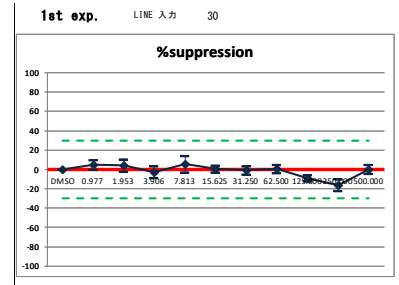


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	11.425	7.387	15.464	Sig.	
1.953	14.084	9.917	18.251	Sig.	
3.906	9.017	3.090	14.944	Sig.	
7.813	18.779	14.043	23.515	Sig.	
15.625	16.051	9.499	22.602	Sig.	
31.250	4.077	-5.825	13.979	N.S.	
62.500	16.153	9.311	22.995	Sig.	
125.000	15.972	7.073	24.872	Sig.	
250.000	0.913	-5.673	7.498	N.S.	
500.000	16.876	11.988	21.764	Sig.	

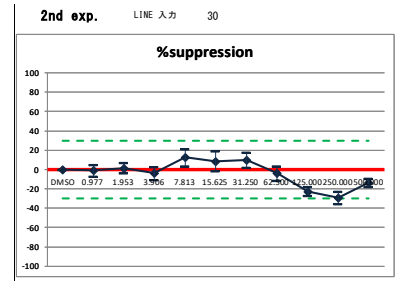


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	7.948	2.260	13.637	Sig.	
1.953	8.876	3.807	13.944	Sig.	
3.906	0.915	-5.933	7.762	N.S.	
7.813	8.319	3.970	12.667	Sig.	
15.625	10.414	5.442	15.387	Sig.	
31.250	4.266	-1.372	9.904	N.S.	
62.500	10.606	3.120	18.092	Sig.	
125.000	12.474	8.103	16.844	Sig.	
250.000	-4.385	-10.299	1.528	N.S.	
500.000	15.435	9.857	21.012	Sig.	

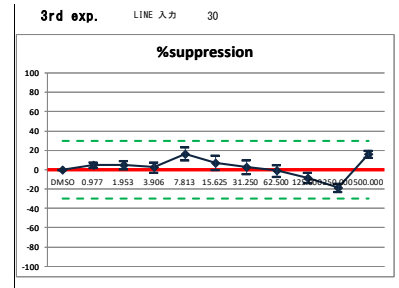
LabC AIST shikoku



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	4.856	-0.128	9.841	N.S.	
1.953	3.911	-2.444	10.266	N.S.	
3.906	-2.580	-8.890	3.731	N.S.	
7.813	5.549	-2.760	13.859	N.S.	
15.625	0.462	-3.049	3.973	N.S.	
31.250	-0.484	-4.707	3.739	N.S.	
62.500	0.737	-3.340	4.813	N.S.	
125.000	-8.949	-12.056	-5.841	Sig.	
250.000	-16.063	-22.275	-9.851	Sig.	
500.000	0.316	-4.034	4.666	N.S.	



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	-0.876	-6.796	5.045	N.S.	
1.953	1.708	-3.453	6.870	N.S.	
3.906	-3.699	-10.555	3.158	N.S.	
7.813	12.481	3.769	21.193	Sig.	
15.625	8.819	-1.577	19.215	N.S.	
31.250	9.917	1.841	17.994	Sig.	
62.500	-3.766	-11.117	3.584	N.S.	
125.000	-22.416	-26.795	-18.036	Sig.	
250.000	-29.198	-35.764	-22.633	Sig.	
500.000	-13.662	-17.709	-9.615	Sig.	



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	4.841	1.831	7.851	Sig.	
1.953	4.930	0.964	8.896	Sig.	
3.906	2.574	-2.890	8.038	N.S.	
7.813	16.452	9.800	23.105	Sig.	
15.625	7.260	-0.003	14.523	N.S.	
31.250	2.861	-4.424	10.146	N.S.	
62.500	-1.027	-6.887	4.833	N.S.	
125.000	-8.310	-13.858	-2.761	Sig.	
250.000	-18.499	-22.594	-14.404	Sig.	
500.000	16.215	12.728	19.703	Sig.	

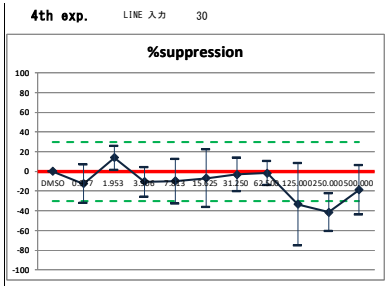
Chem.2 Set2 (MITA205,505,802)

LabA Tohoku

LabB AIST tsukuba

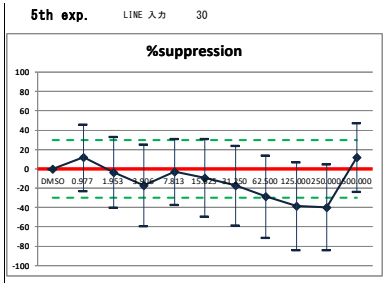
LabC AIST shikoku

Exp.4



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	-12.595	-31.989	6.799	N.S.	
1.953	13.745	1.438	26.053	Sig.	
3.906	-10.582	-25.589	4.426	N.S.	
7.813	-9.850	-32.650	12.949	N.S.	
15.625	-6.962	-36.315	22.392	N.S.	
31.250	-3.129	-20.058	13.799	N.S.	
62.500	-1.778	-13.965	10.410	N.S.	
125.000	-33.573	-75.331	8.186	N.S.	Over
250.000	-41.491	-60.528	-22.454	Sig.	Over
500.000	-18.825	-43.874	6.225	N.S.	

Exp.5



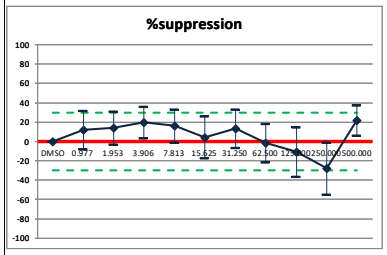
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	11.692	-22.703	46.087	N.S.	
1.953	-3.245	-39.485	32.994	N.S.	
3.906	-16.868	-59.052	25.315	N.S.	
7.813	-3.161	-37.219	30.896	N.S.	
15.625	-9.070	-49.086	30.947	N.S.	
31.250	-16.995	-58.277	24.287	N.S.	
62.500	-28.580	-71.100	13.940	N.S.	
125.000	-38.670	-84.137	6.797	N.S.	Over
250.000	-39.624	-84.162	4.914	N.S.	Over
500.000	12.083	-23.657	47.822	N.S.	

Chem.2 Set3 (MITA305,MITB603,MITC905)

LabA Tohoku

Exp.1

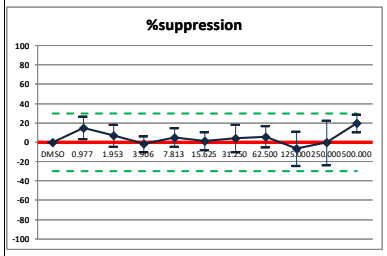
1st exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	12.134	-7.880	32.149	N.S.	
1.953	13.836	-3.226	30.897	N.S.	
3.906	20.032	3.830	36.235	Sig.	
7.813	16.456	-0.616	33.529	N.S.	
15.625	4.397	-17.320	26.113	N.S.	
31.250	13.530	-6.294	33.354	N.S.	
62.500	-1.559	-21.431	18.313	N.S.	
125.000	-10.474	-36.082	15.135	N.S.	
250.000	-27.408	-54.410	-0.405	Sig.	
500.000	22.043	6.484	37.603	Sig.	

Exp.2

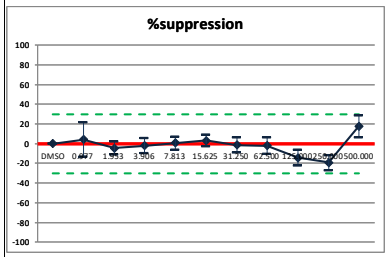
2nd exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	15.059	3.364	26.754	Sig.	
1.953	6.895	-4.385	18.176	N.S.	
3.906	-1.625	-9.795	6.545	N.S.	
7.813	5.155	-4.238	14.549	N.S.	
15.625	1.399	-7.825	10.623	N.S.	
31.250	4.290	-9.692	18.272	N.S.	
62.500	5.913	-5.244	17.070	N.S.	
125.000	-6.631	-24.425	11.163	N.S.	
250.000	-0.114	-23.233	23.006	N.S.	
500.000	19.813	10.695	28.932	Sig.	

Exp.3

3rd exp. LINE 入力 30

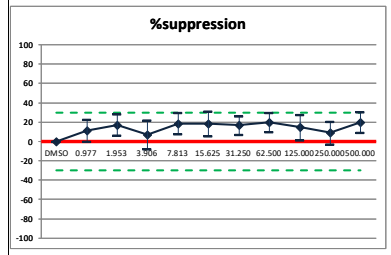


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	4.051	-13.321	21.423	N.S.	
1.953	-4.464	-11.004	2.077	N.S.	
3.906	-1.965	-9.344	5.415	N.S.	
7.813	0.487	-6.131	7.105	N.S.	
15.625	3.255	-2.988	9.498	N.S.	
31.250	-1.272	-8.729	6.185	N.S.	
62.500	-2.019	-10.524	6.486	N.S.	
125.000	-14.209	-21.987	-6.431	Sig.	
250.000	-19.279	-26.937	-11.621	Sig.	
500.000	17.853	6.699	29.008	Sig.	

LabB AIST tsukuba

Exp.1

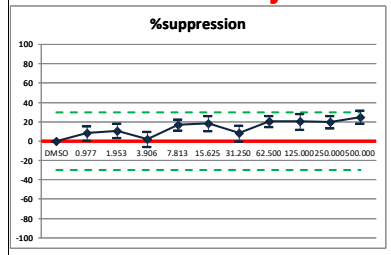
1st exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	11.533	0.252	22.814	Sig.	
1.953	17.274	6.339	28.209	Sig.	
3.906	7.143	-7.950	22.236	N.S.	
7.813	18.761	7.921	29.602	Sig.	
15.625	18.475	5.597	31.353	Sig.	
31.250	16.981	7.407	26.555	Sig.	
62.500	19.901	9.960	29.842	Sig.	
125.000	14.538	1.492	27.584	Sig.	
250.000	8.853	-2.779	20.485	N.S.	
500.000	20.128	9.515	30.740	Sig.	

Exp.2

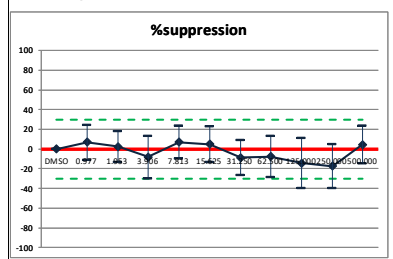
2nd exp. LINE 入力 30 **reject**



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	8.399	0.914	15.885	Sig.	
1.953	10.862	3.439	18.286	Sig.	
3.906	2.278	-5.611	10.167	N.S.	
7.813	16.783	11.166	22.400	Sig.	
15.625	18.589	10.743	26.435	Sig.	
31.250	8.221	-0.023	16.466	N.S.	
62.500	20.547	14.721	26.373	Sig.	
125.000	20.400	12.188	28.613	Sig.	
250.000	19.782	13.266	26.299	Sig.	
500.000	25.144	18.542	31.747	Sig.	

Exp.3

2nd exp. LINE 入力 30

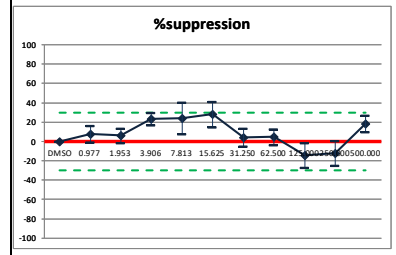


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	6.884	-10.679	24.447	N.S.	
1.953	2.560	-13.074	18.194	N.S.	
3.906	-7.989	-29.554	13.575	N.S.	
7.813	7.054	-9.596	23.705	N.S.	
15.625	4.938	-13.137	23.013	N.S.	
31.250	-8.639	-26.638	9.361	N.S.	
62.500	-7.535	-28.583	13.513	N.S.	
125.000	-14.183	-39.768	11.401	N.S.	
250.000	-17.282	-39.563	4.999	N.S.	
500.000	4.390	-14.793	23.574	N.S.	

LabC AIST shikoku

Exp.1

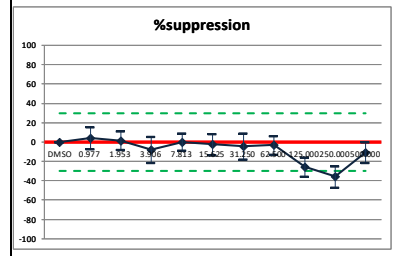
1st exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	7.881	-0.527	16.289	N.S.	
1.953	6.079	-1.262	13.420	N.S.	
3.906	23.357	16.778	29.936	Sig.	
7.813	23.944	7.469	40.419	Sig.	
15.625	28.265	15.079	41.450	Sig.	
31.250	4.310	-4.675	13.295	N.S.	
62.500	4.650	-3.433	12.734	N.S.	
125.000	-14.214	-26.770	-1.659	Sig.	
250.000	-12.243	-25.211	0.724	N.S.	
500.000	18.592	10.024	27.159	Sig.	

Exp.2

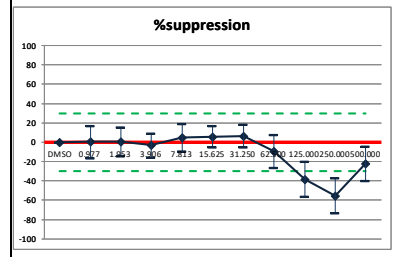
2nd exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	4.359	-6.806	15.523	N.S.	
1.953	1.442	-8.135	11.019	N.S.	
3.906	-7.734	-21.061	5.593	N.S.	
7.813	0.204	-8.573	8.981	N.S.	
15.625	-2.253	-13.328	8.822	N.S.	
31.250	-4.478	-17.967	9.012	N.S.	
62.500	-2.978	-12.559	6.604	N.S.	
125.000	-25.470	-35.620	-15.320	Sig.	
250.000	-35.570	-46.599	-24.542	Sig.	Over
500.000	-10.706	-21.339	-0.073	Sig.	

Exp.3

3rd exp. LINE 入力 30

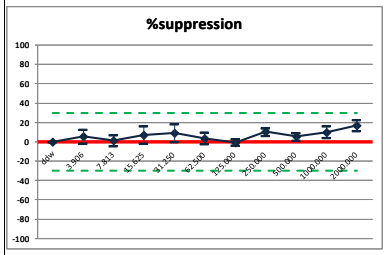


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.977	0.481	-16.109	17.071	N.S.	
1.953	0.589	-14.077	15.255	N.S.	
3.906	-2.952	-15.408	9.504	N.S.	
7.813	4.950	-9.498	19.399	N.S.	
15.625	5.781	-5.249	16.810	N.S.	
31.250	6.640	-5.157	18.437	N.S.	
62.500	-9.344	-26.415	7.728	N.S.	
125.000	-38.027	-55.815	-20.240	Sig.	Over
250.000	-55.272	-73.365	-37.178	Sig.	Over
500.000	-21.925	-39.483	-4.367	Sig.	

LabA Tohoku

Exp.1

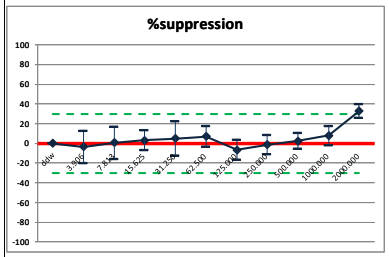
1st exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	5.656	-1.530	12.843	N.S.	
7.813	1.426	-4.101	6.953	N.S.	
15.625	7.313	-1.350	15.975	N.S.	
31.250	9.137	-0.175	18.449	N.S.	
62.500	3.650	-2.426	9.726	N.S.	
125.000	-0.524	-3.761	2.712	N.S.	
250.000	10.307	6.347	14.267	Sig.	
500.000	5.423	1.638	9.208	Sig.	
1000.000	10.167	4.042	16.291	Sig.	
2000.000	17.274	11.649	22.900	Sig.	

Exp.2

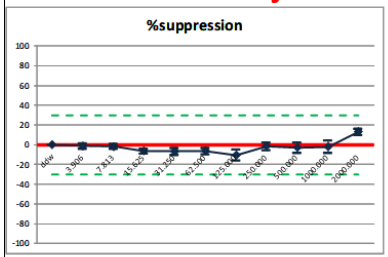
2nd exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	-3.566	-19.860	12.728	N.S.	
7.813	0.749	-15.639	17.136	N.S.	
15.625	3.131	-6.790	13.053	N.S.	
31.250	4.808	-12.675	22.290	N.S.	
62.500	7.012	-3.284	17.309	N.S.	
125.000	-6.555	-16.745	3.634	N.S.	
250.000	-1.350	-11.088	8.388	N.S.	
500.000	2.454	-5.443	10.351	N.S.	
1000.000	7.926	-1.936	17.787	N.S.	
2000.000	32.856	26.000	39.712	Sig.	Over

Exp.3

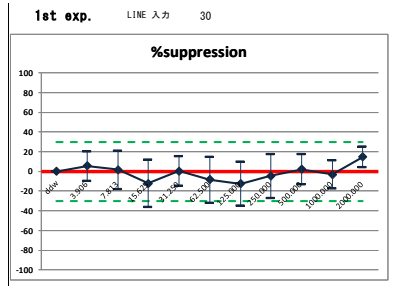
3rd exp. LINE 入力 30 **reject**



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	-1.055	-3.735	1.625	N.S.	
7.813	-1.665	-4.347	1.018	N.S.	
15.625	-5.271	-8.694	-3.848	Sig.	
31.250	-6.804	-10.025	-3.583	Sig.	
62.500	-5.401	-9.614	-3.187	Sig.	
125.000	-10.414	-15.767	-5.061	Sig.	
250.000	-1.396	-5.178	2.386	N.S.	
500.000	-2.779	-7.893	2.335	N.S.	
1000.000	-1.960	-8.497	4.577	N.S.	
2000.000	13.049	9.760	16.337	Sig.	

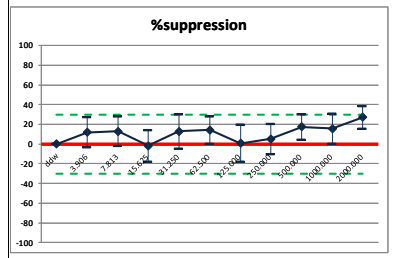
LabB AIST tsukuba

1st exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	5.398	-9.565	20.362	N.S.	
7.813	1.599	-18.051	21.248	N.S.	
15.625	-12.054	-36.080	11.972	N.S.	
31.250	0.430	-14.777	15.637	N.S.	
62.500	-8.422	-31.699	14.856	N.S.	
125.000	-12.442	-34.456	9.572	N.S.	
250.000	-4.668	-27.125	17.789	N.S.	
500.000	2.132	-13.419	17.683	N.S.	
1000.000	-3.137	-17.335	11.061	N.S.	
2000.000	14.664	4.220	25.109	Sig.	

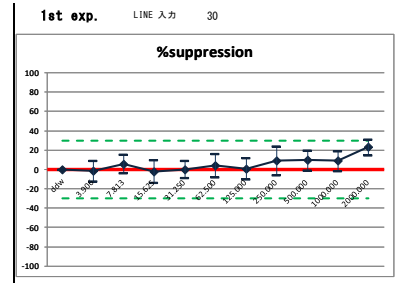
2nd exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	11.927	-3.134	26.987	N.S.	
7.813	12.893	-2.258	28.045	N.S.	
15.625	-1.905	-17.853	14.043	N.S.	
31.250	12.778	-4.632	30.188	N.S.	
62.500	14.167	0.069	28.265	Sig.	
125.000	0.608	-18.284	19.499	N.S.	
250.000	5.345	-9.965	20.655	N.S.	
500.000	17.280	4.406	30.154	Sig.	
1000.000	15.525	0.479	30.570	Sig.	
2000.000	27.040	15.734	38.346	Sig.	

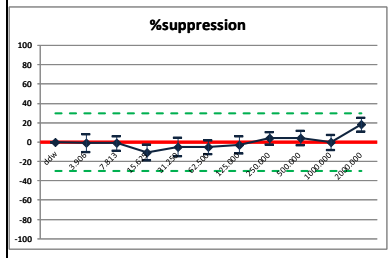
LabC AIST shikoku

1st exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	-1.567	-12.329	9.196	N.S.	
7.813	5.907	-3.670	15.484	N.S.	
15.625	-2.084	-13.877	9.709	N.S.	
31.250	0.187	-8.856	9.229	N.S.	
62.500	4.144	-7.899	16.186	N.S.	
125.000	0.836	-10.215	11.887	N.S.	
250.000	9.242	-5.939	24.424	N.S.	
500.000	9.746	-0.701	20.194	N.S.	
1000.000	8.859	-1.139	18.857	N.S.	
2000.000	23.124	14.958	31.290	Sig.	

2nd exp. LINE 入力 30

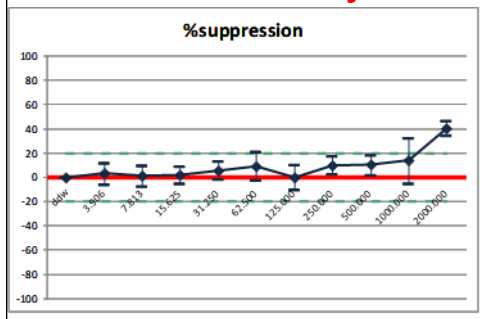


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	-0.783	-10.295	8.730	N.S.	
7.813	-1.057	-8.419	6.305	N.S.	
15.625	-10.389	-18.565	-2.213	Sig.	
31.250	-4.700	-14.089	4.688	N.S.	
62.500	-5.069	-12.010	1.872	N.S.	
125.000	-2.745	-11.751	6.261	N.S.	
250.000	4.278	-2.004	10.560	N.S.	
500.000	4.231	-3.217	11.680	N.S.	
1000.000	0.155	-7.706	8.017	N.S.	
2000.000	18.432	11.045	25.819	Sig.	

LabA Tohoku

Exp.4

4th exp. LINE 入力 20 **reject**

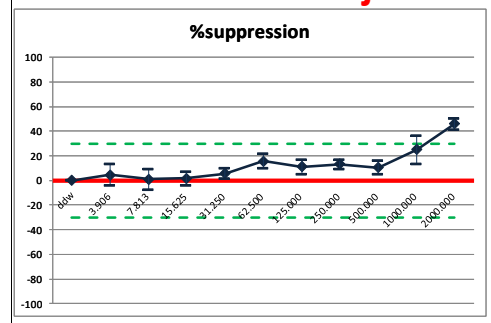


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	3.357	-5.607	12.321	N.S.	
7.813	1.428	-6.893	9.749	N.S.	
15.625	2.106	-4.780	8.993	N.S.	
31.250	5.896	-1.781	13.572	N.S.	
62.500	9.524	-2.321	21.369	N.S.	
125.000	0.165	-10.187	10.517	N.S.	
250.000	10.122	2.537	17.707	Sig.	
500.000	10.446	2.219	18.673	Sig.	
1000.000	13.884	-5.176	32.944	N.S.	
2000.000	40.684	34.671	46.698	Sig.	Over

LabA Tohoku

Exp.6

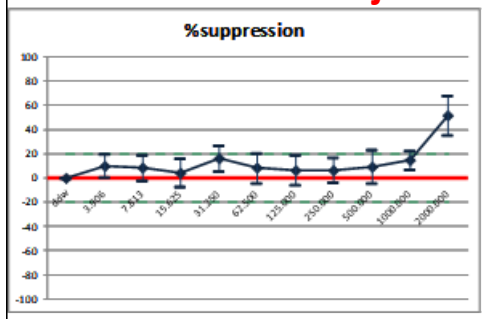
6th exp. LINE 入力 30 **reject**



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	4.515	-4.145	13.174	N.S.	
7.813	0.852	-7.671	9.376	N.S.	
15.625	1.868	-3.705	7.441	N.S.	
31.250	5.685	1.473	9.897	Sig.	
62.500	15.726	9.856	21.595	Sig.	
125.000	11.124	5.198	17.050	Sig.	
250.000	13.149	9.155	17.143	Sig.	
500.000	10.554	5.216	15.892	Sig.	
1000.000	24.975	13.272	36.678	Sig.	
2000.000	45.876	41.191	50.561	Sig.	Over

Exp.5

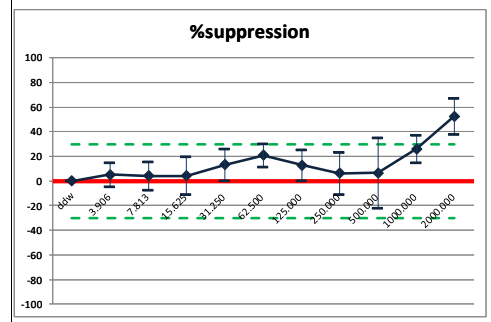
5th exp. LINE 入力 20 **reject**



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	10.052	0.337	19.767	Sig.	
7.813	8.495	-2.099	19.089	N.S.	
15.625	4.449	-7.160	16.059	N.S.	
31.250	16.403	5.868	26.937	Sig.	
62.500	8.151	-4.600	20.903	N.S.	
125.000	6.500	-5.956	18.955	N.S.	
250.000	6.544	-3.686	16.773	N.S.	
500.000	9.296	-4.457	23.050	N.S.	
1000.000	14.650	6.943	22.357	Sig.	
2000.000	51.878	35.291	68.464	Sig.	Over

Exp.7

6th exp. LINE 入力 30

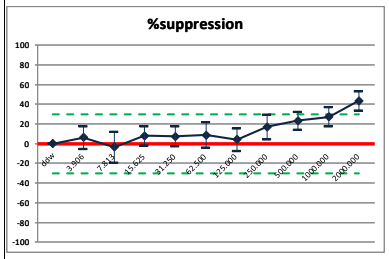


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	5.219	-4.464	14.902	N.S.	
7.813	4.022	-7.670	15.713	N.S.	
15.625	4.278	-11.246	19.803	N.S.	
31.250	13.056	0.453	25.660	Sig.	
62.500	20.695	11.271	30.119	Sig.	
125.000	12.775	0.175	25.374	Sig.	
250.000	6.292	-10.732	23.316	N.S.	
500.000	6.495	-22.181	35.171	N.S.	
1000.000	26.058	14.810	37.305	Sig.	
2000.000	52.500	37.981	67.018	Sig.	Over

LabA Tohoku

Exp.1

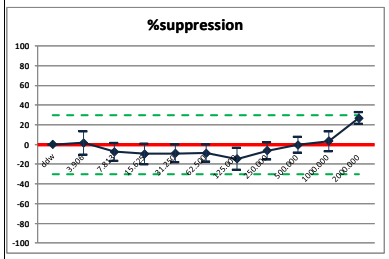
1st exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	6.126	-5.261	17.512	N.S.	
7.813	-3.511	-19.243	12.220	N.S.	
15.625	8.036	-1.723	17.795	N.S.	
31.250	7.393	-2.849	17.634	N.S.	
62.500	8.687	-4.161	21.536	N.S.	
125.000	3.982	-7.589	15.553	N.S.	
250.000	17.148	4.659	29.637	Sig.	
500.000	23.159	14.070	32.248	Sig.	
1000.000	27.201	17.391	37.011	Sig.	
2000.000	43.387	33.599	53.174	Sig.	Over

Exp.2

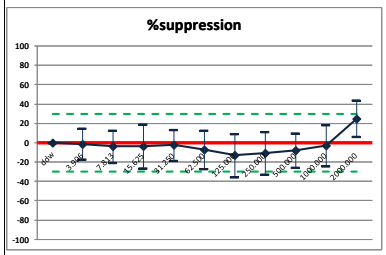
2nd exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	1.707	-10.114	13.528	N.S.	
7.813	-7.310	-16.240	1.619	N.S.	
15.625	-9.601	-20.119	0.917	N.S.	
31.250	-9.069	-18.229	0.092	N.S.	
62.500	-8.752	-17.518	0.014	N.S.	
125.000	-14.530	-25.881	-3.179	Sig.	
250.000	-6.301	-14.938	2.337	N.S.	
500.000	-0.341	-8.436	7.754	N.S.	
1000.000	3.460	-6.665	13.584	N.S.	
2000.000	26.781	20.996	32.567	Sig.	

Exp.3

3rd exp. LINE 入力 30

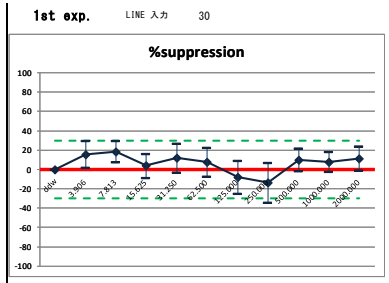


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	-1.426	-17.422	14.569	N.S.	
7.813	-3.900	-20.690	12.889	N.S.	
15.625	-3.364	-26.162	19.435	N.S.	
31.250	-2.477	-18.543	13.589	N.S.	
62.500	-7.093	-26.917	12.732	N.S.	
125.000	-12.959	-35.438	9.520	N.S.	
250.000	-10.914	-33.026	11.197	N.S.	
500.000	-7.649	-25.543	10.245	N.S.	
1000.000	-2.847	-24.027	18.332	N.S.	
2000.000	25.164	6.064	44.264	Sig.	

LabB AIST tsukuba

Exp.1

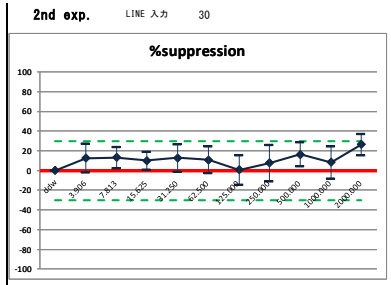
1st exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	15.934	2.439	29.430	Sig.	
7.813	18.725	7.975	29.474	Sig.	
15.625	3.950	-8.454	16.354	N.S.	
31.250	12.273	-2.680	27.227	N.S.	
62.500	7.790	-7.285	22.865	N.S.	
125.000	-7.965	-24.982	9.052	N.S.	
250.000	-13.278	-33.867	7.310	N.S.	
500.000	10.246	-1.519	22.010	N.S.	
1000.000	8.054	-2.147	18.254	N.S.	
2000.000	11.640	-0.940	24.221	N.S.	

Exp.2

2nd exp. LINE 入力 30

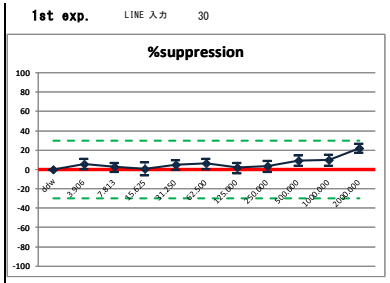


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	12.659	-1.807	27.126	N.S.	
7.813	13.139	2.397	23.880	Sig.	
15.625	10.051	1.145	18.957	Sig.	
31.250	12.698	-1.450	26.846	N.S.	
62.500	10.882	-2.457	24.220	N.S.	
125.000	0.528	-14.230	15.286	N.S.	
250.000	7.706	-10.744	26.156	N.S.	
500.000	16.443	4.326	28.560	Sig.	
1000.000	8.230	-8.296	24.757	N.S.	
2000.000	26.303	15.784	36.821	Sig.	

LabC AIST shikoku

Exp.1

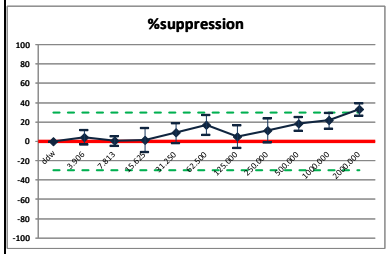
1st exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	5.860	0.530	11.190	Sig.	
7.813	2.450	-1.864	6.764	N.S.	
15.625	0.937	-5.754	7.627	N.S.	
31.250	4.804	-0.112	9.720	N.S.	
62.500	6.232	0.809	11.656	Sig.	
125.000	1.889	-3.535	7.313	N.S.	
250.000	3.794	-1.833	9.421	N.S.	
500.000	9.516	4.357	14.675	Sig.	
1000.000	10.163	4.560	15.766	Sig.	
2000.000	22.196	17.632	26.760	Sig.	

Exp.2

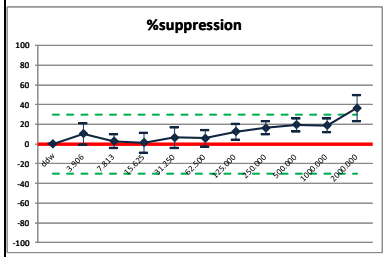
2nd exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	4.570	-3.074	12.214	N.S.	
7.813	0.786	-4.239	5.811	N.S.	
15.625	1.734	-10.853	14.320	N.S.	
31.250	8.929	-1.238	19.097	N.S.	
62.500	17.087	6.857	27.317	Sig.	
125.000	5.204	-6.241	16.649	N.S.	
250.000	11.675	-0.568	23.917	N.S.	
500.000	18.469	11.364	25.575	Sig.	
1000.000	21.798	13.626	29.971	Sig.	
2000.000	33.264	26.690	39.839	Sig.	Over

Exp.3

3rd exp. LINE 入力 30



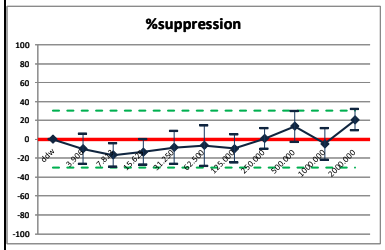
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	10.466	-0.270	21.203	N.S.	
7.813	2.639	-4.296	9.574	N.S.	
15.625	1.237	-8.923	11.397	N.S.	
31.250	6.500	-3.963	16.963	N.S.	
62.500	5.717	-2.712	14.147	N.S.	
125.000	12.328	4.027	20.629	Sig.	
250.000	16.384	9.764	23.004	Sig.	
500.000	19.519	13.002	26.037	Sig.	
1000.000	18.650	11.729	25.570	Sig.	
2000.000	36.581	23.419	49.743	Sig.	Over

Chem.3 Set3 (MITA303,MITB601,MITC901)

LabA Tohoku

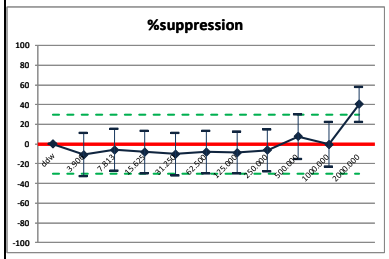
Exp.1

1st exp. LINE 入力 30



a Comparison Conc.	%supp	LowerLimit	UpperLimit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	-9.975	-25.797	5.848	N.S.	
7.813	-16.563	-28.751	-4.375	Sig.	
15.625	-13.473	-27.032	0.085	N.S.	
31.250	-8.635	-26.247	8.977	N.S.	
62.500	-6.715	-28.315	14.884	N.S.	
125.000	-9.755	-24.317	4.807	N.S.	
250.000	0.789	-9.873	11.451	N.S.	
500.000	13.658	-2.561	29.876	N.S.	
1000.000	-4.851	-21.504	11.802	N.S.	
2000.000	20.515	9.163	31.868	Sig.	

2nd exp. LINE 入力 30

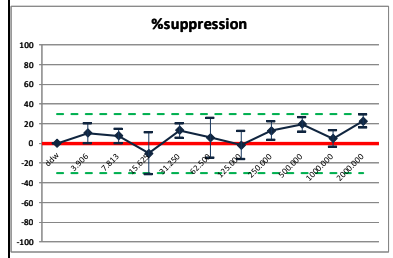


a Comparison Conc.	%supp	LowerLimit	UpperLimit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	-10.730	-32.771	11.311	N.S.	
7.813	-5.791	-26.955	15.373	N.S.	
15.625	-7.980	-29.593	13.633	N.S.	
31.250	-10.292	-31.684	11.100	N.S.	
62.500	-8.179	-29.750	13.391	N.S.	
125.000	-8.630	-29.735	12.475	N.S.	
250.000	-6.377	-27.767	15.013	N.S.	
500.000	7.463	-15.484	30.409	N.S.	
1000.000	-0.237	-22.565	22.090	N.S.	
2000.000	40.409	22.748	58.070	Sig.	Over

Exp.2

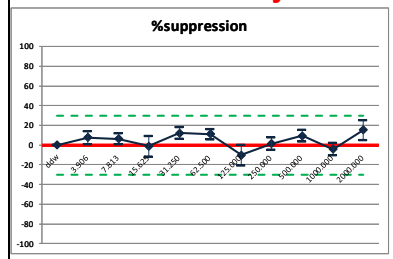
LabB AIST tsukuba

1st exp. LINE 入力 30



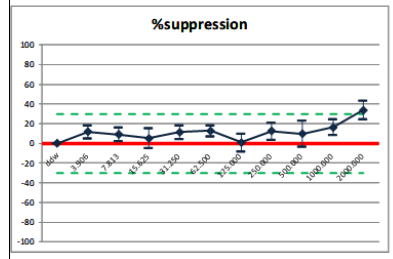
a Comparison Conc.	%supp	LowerLimit	UpperLimit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	10.443	0.387	20.498	Sig.	
7.813	7.467	0.048	14.885	Sig.	
15.625	-10.245	-31.438	10.949	N.S.	
31.250	13.259	5.921	20.598	Sig.	
62.500	5.964	-14.300	26.228	N.S.	
125.000	-1.775	-15.962	12.413	N.S.	
250.000	12.803	3.334	22.271	Sig.	
500.000	19.492	12.220	26.765	Sig.	
1000.000	4.846	-3.487	13.179	N.S.	
2000.000	22.568	16.015	29.122	Sig.	

2nd exp. LINE 入力 30 **reject**



a Comparison Conc.	%supp	LowerLimit	UpperLimit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	7.513	1.090	13.936	Sig.	
7.813	6.191	0.746	11.635	Sig.	
15.625	-1.120	-11.391	9.152	N.S.	
31.250	12.198	6.277	18.119	Sig.	
62.500	10.937	5.389	16.486	Sig.	
125.000	-10.248	-20.565	0.069	N.S.	
250.000	1.234	-5.039	7.506	N.S.	
500.000	9.517	3.685	15.349	Sig.	
1000.000	-4.204	-10.316	1.907	N.S.	
2000.000	15.167	5.072	25.262	Sig.	

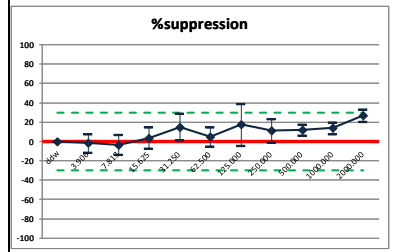
2nd exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	11.726	4.902	18.551	Sig.	
7.813	9.118	2.352	15.883	Sig.	
15.625	5.321	-4.790	15.433	N.S.	
31.250	11.404	4.412	18.397	Sig.	
62.500	12.698	7.101	18.296	Sig.	
125.000	0.951	-8.298	10.200	N.S.	
250.000	12.556	3.759	21.353	Sig.	
500.000	9.826	-3.596	23.247	N.S.	
1000.000	16.290	8.283	24.297	Sig.	
2000.000	33.830	24.304	43.357	Sig.	Over

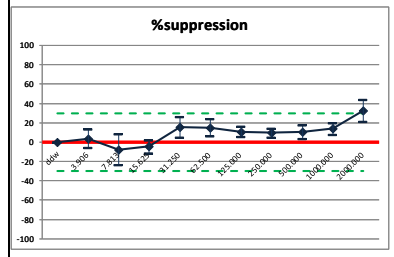
LabC AIST shikoku

1st exp. LINE 入力 30



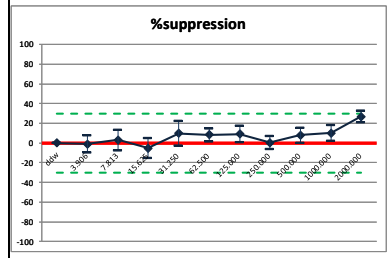
a Comparison Conc.	%supp	LowerLimit	UpperLimit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	-1.499	-11.068	8.071	N.S.	
7.813	-3.280	-13.545	6.985	N.S.	
15.625	3.650	-7.293	14.593	N.S.	
31.250	15.156	1.465	28.846	Sig.	
62.500	4.798	-5.059	14.656	N.S.	
125.000	17.499	-4.282	39.281	N.S.	
250.000	11.298	-0.913	23.510	N.S.	
500.000	12.105	6.242	17.968	Sig.	
1000.000	13.998	8.044	19.951	Sig.	
2000.000	26.758	20.494	33.021	Sig.	

2nd exp. LINE 入力 30



a Comparison Conc.	%supp	LowerLimit	UpperLimit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	3.743	-5.645	13.131	N.S.	
7.813	-7.643	-23.744	8.457	N.S.	
15.625	-4.521	-11.425	2.383	N.S.	
31.250	15.400	4.588	26.213	Sig.	
62.500	15.014	6.271	23.758	Sig.	
125.000	10.822	5.322	16.321	Sig.	
250.000	9.596	5.035	14.157	Sig.	
500.000	10.507	3.535	17.478	Sig.	
1000.000	13.828	7.629	20.028	Sig.	
2000.000	32.588	20.913	44.263	Sig.	Over

3rd exp. LINE 入力 30



a Comparison Conc.	%supp	LowerLimit	UpperLimit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	-0.964	-9.432	7.504	N.S.	
7.813	3.093	-7.299	13.486	N.S.	
15.625	-5.121	-15.219	4.978	N.S.	
31.250	9.751	-2.992	22.494	N.S.	
62.500	8.392	1.811	14.973	Sig.	
125.000	9.157	1.108	17.206	Sig.	
250.000	0.240	-6.469	6.949	N.S.	
500.000	7.874	0.372	15.377	Sig.	
1000.000	10.111	1.945	18.276	Sig.	
2000.000	26.762	20.898	32.626	Sig.	

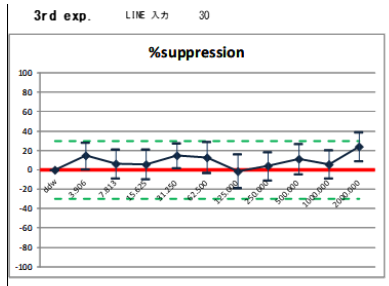
Exp.3

LabA Tohoku

LabB AIST tsukuba

LabC AIST shikoku

Exp.4

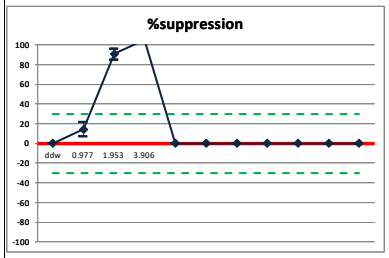


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
3.906	14.531	0.401	28.660	Sig.	
7.813	6.177	-8.594	20.947	N.S.	
15.625	5.985	-9.136	21.106	N.S.	
31.250	15.014	2.104	27.924	Sig.	
62.500	12.954	-3.130	29.039	N.S.	
125.000	-1.123	-18.724	16.477	N.S.	
250.000	3.903	-10.634	18.439	N.S.	
500.000	11.337	-4.548	27.223	N.S.	
1000.000	5.927	-8.666	20.519	N.S.	
2000.000	24.000	9.263	38.737	Sig.	

LabA Tohoku

Exp.1

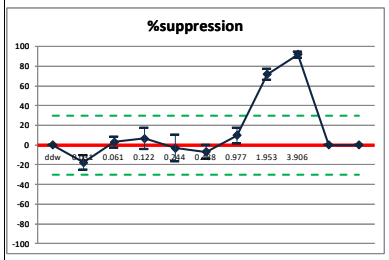
1st exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
0.977	14.272	6.953	21.590	Sig.	
1.953	90.859	85.373	96.344	Sig.	Over
3.906	105.156	103.899	106.413	Sig.	Over
					Over
					Over
					Over
					Over
					Over
					Over
					Over

Exp.2

2nd exp. LINE 入力 30

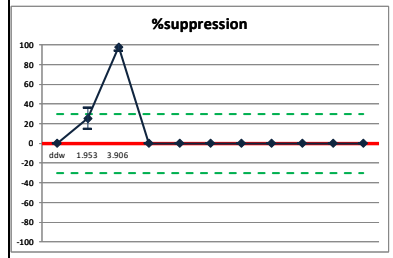


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
0.031	-17.914	-25.191	-10.636	Sig.	
0.061	3.064	-2.376	8.505	N.S.	
0.122	6.713	-3.779	17.204	N.S.	
0.244	-3.019	-16.907	10.869	N.S.	
0.488	-6.838	-13.662	-0.015	Sig.	
0.977	9.583	1.579	17.587	Sig.	
1.953	71.669	66.012	77.327	Sig.	Over
3.906	91.732	88.342	95.121	Sig.	Over
					Over
					Over

Exp.3

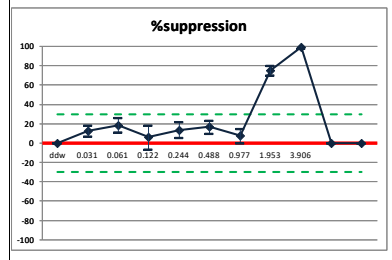
LabB AIST tsukuba

1st exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
1.953	25.529	14.527	36.530	Sig.	
3.906	97.958	93.808	102.109	Sig.	Over
					Over
					Over
					Over
					Over
					Over
					Over
					Over
					Over

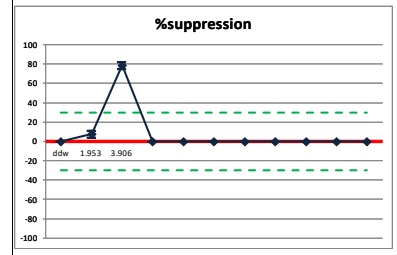
2nd exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
0.031	12.822	6.986	18.657	Sig.	
0.061	18.468	11.003	25.933	Sig.	
0.122	6.221	-6.118	18.560	N.S.	
0.244	13.642	5.407	21.877	Sig.	
0.488	16.896	10.096	23.696	Sig.	
0.977	7.661	0.136	15.186	Sig.	
1.953	75.133	70.200	80.067	Sig.	Over
3.906	99.058	97.690	100.425	Sig.	Over
					Over
					Over

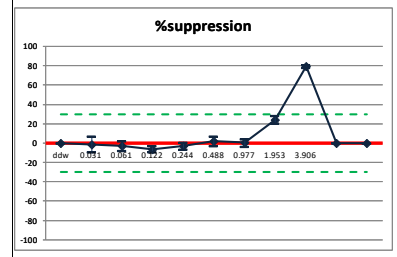
LabC AIST shikoku

1st exp. LINE 入力 30



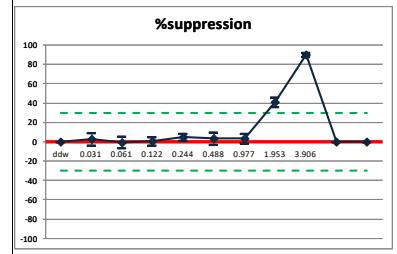
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
1.953	7.966	4.445	11.487	Sig.	
3.906	78.832	75.358	82.305	Sig.	Over
					Over
					Over
					Over
					Over
					Over
					Over
					Over
					Over

2nd exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
0.031	-1.123	-9.288	7.042	N.S.	
0.061	-2.719	-7.576	2.139	N.S.	
0.122	-6.181	-9.453	-2.909	Sig.	
0.244	-3.038	-6.611	0.536	N.S.	
0.488	2.230	-2.820	7.279	N.S.	
0.977	0.360	-3.778	4.497	N.S.	
1.953	24.386	20.688	28.085	Sig.	
3.906	79.483	78.202	80.763	Sig.	Over
					Over
					Over

3rd exp. LINE 入力 30



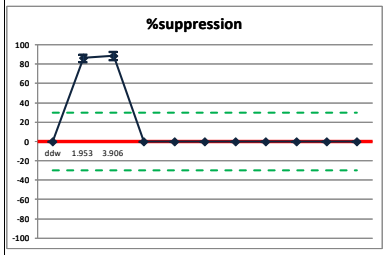
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
0.031	2.671	-3.932	9.274	N.S.	
0.061	-0.580	-6.769	5.609	N.S.	
0.122	0.834	-3.532	5.201	N.S.	
0.244	4.902	1.322	8.482	Sig.	
0.488	3.432	-3.135	9.999	N.S.	
0.977	3.337	-1.549	8.223	N.S.	
1.953	41.156	36.251	46.060	Sig.	Over
3.906	90.322	88.206	92.438	Sig.	Over
					Over
					Over

Chem.4 Set2 (MITA204,MITB503,MITC801)

LabA Tohoku

Exp.1

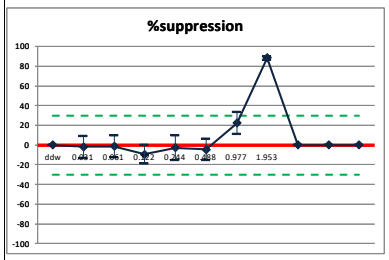
1st exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
1.953	86.232	82.549	89.915	Sig.	Over
3.906	88.848	84.400	93.296	Sig.	Over

Exp.2

2nd exp. LINE 入力 30

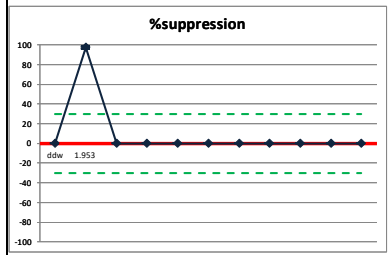


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
0.031	-1.951	-13.259	9.358	N.S.	
0.061	-1.371	-12.296	9.553	N.S.	
0.122	-9.356	-18.859	0.147	N.S.	
0.244	-2.723	-15.017	9.570	N.S.	
0.488	-4.487	-15.257	6.282	N.S.	
0.977	22.394	11.115	33.673	Sig.	
1.953	88.330	86.337	90.322	Sig.	Over

Exp.3

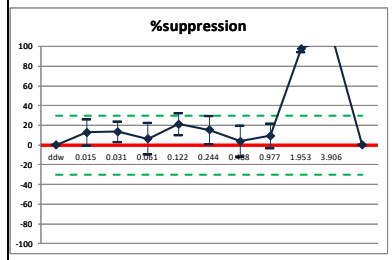
LabB AIST tsukuba

1st exp. LINE 入力 30



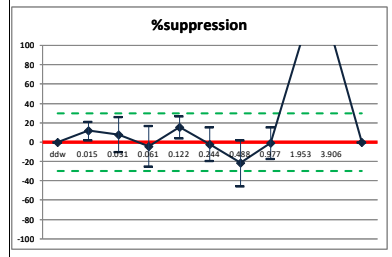
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
1.953	97.514	96.441	98.586	Sig.	Over

2nd exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
0.015	12.764	-0.407	25.934	N.S.	
0.031	13.483	2.927	24.040	Sig.	
0.061	6.288	-9.557	22.134	N.S.	
0.122	21.104	9.888	32.321	Sig.	
0.244	15.203	1.092	29.314	Sig.	
0.488	3.861	-11.746	19.467	N.S.	
0.977	9.216	-3.180	21.612	N.S.	
1.953	97.762	93.878	101.647	Sig.	Over
3.906	110.846	109.940	111.752	Sig.	Over

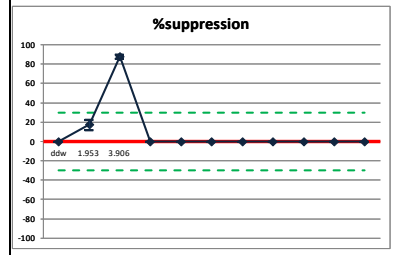
3rd exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
0.015	11.894	2.219	21.569	Sig.	
0.031	8.105	-9.861	26.071	N.S.	
0.061	-4.133	-24.990	16.723	N.S.	
0.122	15.368	3.888	26.848	Sig.	
0.244	-1.975	-19.470	15.521	N.S.	
0.488	-21.674	-45.375	2.026	N.S.	
0.977	-0.811	-16.908	15.285	N.S.	
1.953	105.048	103.181	106.915	Sig.	Over
3.906	111.023	110.141	111.906	Sig.	Over

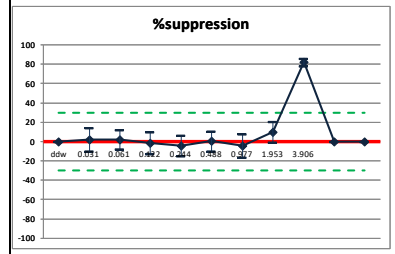
LabC AIST shikoku

1st exp. LINE 入力 30



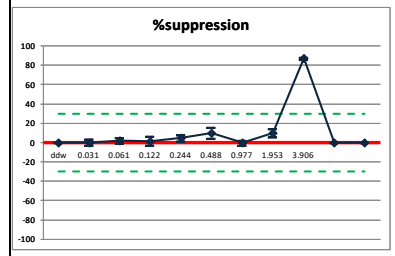
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
1.953	17.398	12.144	22.653	Sig.	
3.906	88.091	86.033	90.150	Sig.	Over

2nd exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
0.031	2.013	-10.271	14.296	N.S.	
0.061	2.186	-7.700	12.072	N.S.	
0.122	-1.496	-13.163	10.171	N.S.	
0.244	-4.260	-14.858	6.337	N.S.	
0.488	0.380	-9.750	10.510	N.S.	
0.977	-4.302	-16.572	7.969	N.S.	
1.953	10.075	-0.595	20.745	N.S.	
3.906	82.057	78.389	85.725	Sig.	Over

3rd exp. LINE 入力 30

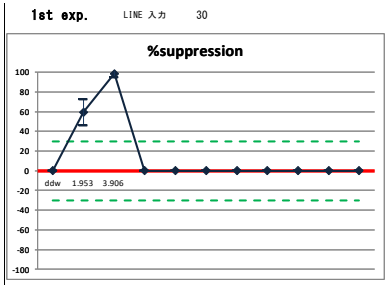


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
0.031	0.220	-2.808	3.248	N.S.	
0.061	1.997	-0.605	4.598	N.S.	
0.122	1.619	-3.118	6.356	N.S.	
0.244	4.578	1.624	7.532	Sig.	
0.488	9.912	4.167	15.657	Sig.	
0.977	-0.264	-2.713	2.186	N.S.	
1.953	9.941	5.956	13.927	Sig.	
3.906	86.981	85.903	88.059	Sig.	Over

Chem.4 Set3 (MITA301,MITB602,MITC904)

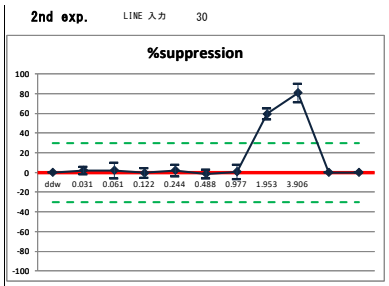
LabA Tohoku

Exp.1



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
1.953	59.460	46.305	72.616	Sig.	Over
3.906	98.494	94.859	102.130	Sig.	Over

Exp.2

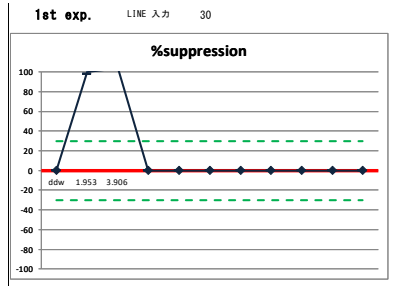


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
0.031	1.916	-2.161	5.992	N.S.	
0.061	2.030	-5.875	9.935	N.S.	
0.122	-0.422	-5.423	4.580	N.S.	
0.244	2.101	-3.842	8.045	N.S.	
0.488	-1.521	-5.991	2.949	N.S.	
0.977	0.803	-6.516	8.122	N.S.	
1.953	59.484	53.872	65.095	Sig.	Over
3.906	80.766	71.329	90.204	Sig.	Over

Exp.3

LabB AIST tsukuba

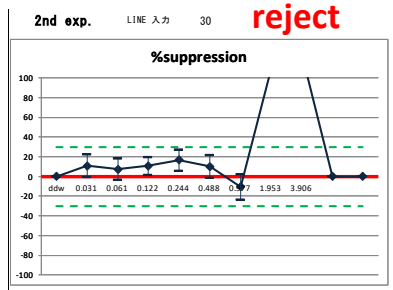
Exp.1



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
1.953	100.151	98.665	101.637	Sig.	Over
3.906	103.346	102.332	104.359	Sig.	Over

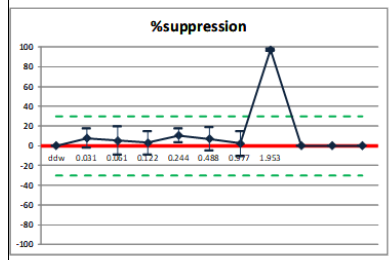
Exp.2

reject



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
0.031	10.914	-0.524	22.351	N.S.	
0.061	7.335	-3.459	18.129	N.S.	
0.122	10.770	1.801	19.740	Sig.	
0.244	16.549	5.974	27.124	Sig.	
0.488	10.089	-1.599	21.778	N.S.	
0.977	-10.442	-23.348	2.464	N.S.	
1.953	108.015	103.794	112.236	Sig.	Over
3.906	125.115	124.470	125.760	Sig.	Over

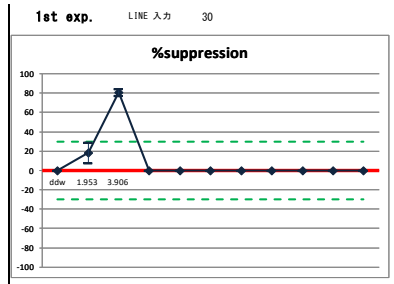
Exp.3



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
0.031	7.779	-1.676	17.234	N.S.	
0.061	5.226	-9.054	19.506	N.S.	
0.122	2.999	-8.681	14.679	N.S.	
0.244	10.459	3.333	17.584	Sig.	
0.488	7.061	-4.949	19.050	N.S.	
0.977	2.228	-10.612	15.068	N.S.	
1.953	97.039	95.906	98.171	Sig.	Over

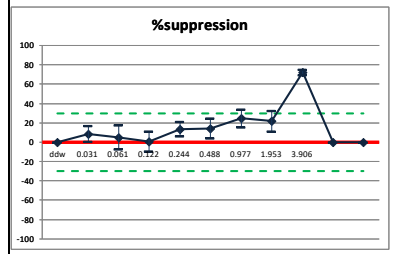
LabC AIST shikoku

Exp.1



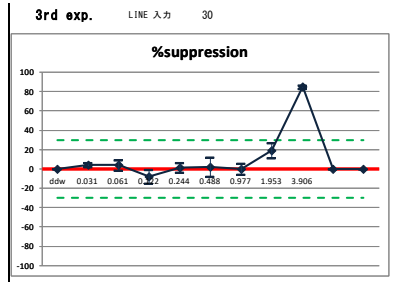
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
1.953	18.465	8.017	28.913	Sig.	
3.906	80.825	77.437	84.213	Sig.	Over

Exp.2



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
0.031	8.539	0.373	16.705	Sig.	
0.061	5.275	-7.287	17.837	N.S.	
0.122	0.868	-9.496	11.232	N.S.	
0.244	13.626	6.270	20.981	Sig.	
0.488	14.445	3.900	24.991	Sig.	
0.977	24.886	15.811	33.961	Sig.	
1.953	21.941	11.537	32.346	Sig.	
3.906	72.146	69.350	74.942	Sig.	Over

Exp.3



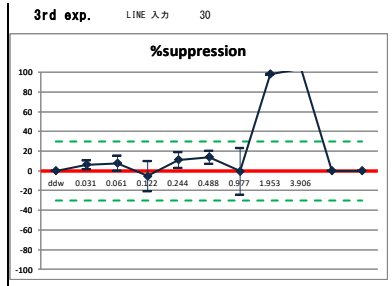
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
ddw	0	0	0		
0.031	4.342	2.055	6.629	Sig.	
0.061	3.968	-1.349	9.285	N.S.	
0.122	-7.777	-14.999	-0.554	Sig.	
0.244	1.364	-3.342	6.071	N.S.	
0.488	2.180	-7.789	12.148	N.S.	
0.977	-0.077	-5.589	5.434	N.S.	
1.953	19.093	11.103	27.084	Sig.	
3.906	84.823	83.293	86.353	Sig.	Over

LabA Tohoku

LabB AIST tsukuba

LabC AIST shikoku

Exp.4



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig. /N.S.	%supp Over Line
ddw	0	0	0		
0.031	6.155	1.407	10.902	Sig.	
0.061	7.655	0.157	15.153	Sig.	
0.122	-5.409	-20.784	9.966	N.S.	
0.244	10.992	3.161	18.824	Sig.	
0.488	13.758	7.282	20.234	Sig.	
0.977	-0.353	-24.027	23.321	N.S.	
1.953	98.041	97.652	98.430	Sig.	Over
3.906	103.525	103.051	104.000	Sig.	Over
					Over
					Over

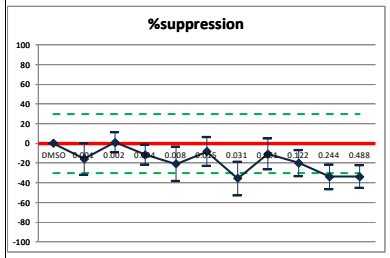
LabA Tohoku

LabB AIST tsukuba

LabC AIST shikoku

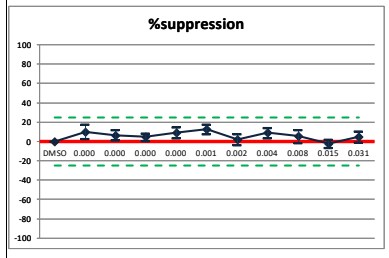
Exp.1

1st exp. LINE 入力 30



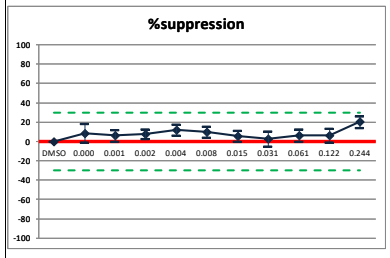
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.001	-15.761	-31.944	0.422	N.S.	
0.002	1.145	-8.952	11.243	N.S.	
0.004	-11.391	-21.356	-1.426	Sig.	
0.008	-20.930	-38.248	-3.613	Sig.	
0.015	-8.357	-22.850	6.135	N.S.	
0.031	-35.626	-52.462	-18.791	Sig.	Over
0.061	-10.764	-26.276	4.747	N.S.	
0.122	-19.714	-32.946	-6.482	Sig.	
0.244	-33.976	-46.779	-21.173	Sig.	Over
0.488	-33.901	-45.328	-22.474	Sig.	Over

1st exp. LINE 入力 25



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.000	10.086	2.584	17.588	Sig.	
0.000	6.435	1.146	11.725	Sig.	
0.000	4.636	0.473	8.800	Sig.	
0.000	9.273	3.657	14.889	Sig.	
0.001	12.791	8.053	17.528	Sig.	
0.002	1.978	-3.604	7.560	N.S.	
0.004	8.862	3.417	14.307	Sig.	
0.008	5.307	-1.309	11.923	N.S.	
0.015	-2.101	-6.567	2.364	N.S.	
0.031	5.046	-0.872	10.963	N.S.	

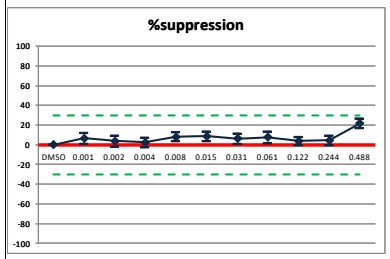
1st exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.000	8.607	-1.076	18.289	N.S.	
0.001	6.242	0.189	12.294	Sig.	
0.002	7.600	2.671	12.529	Sig.	
0.004	11.849	6.138	17.560	Sig.	
0.008	9.892	4.051	15.733	Sig.	
0.015	5.587	0.093	11.082	Sig.	
0.031	2.954	-4.939	10.846	N.S.	
0.061	6.433	0.002	12.864	Sig.	
0.122	6.451	-0.896	13.798	N.S.	
0.244	20.449	14.333	26.564	Sig.	

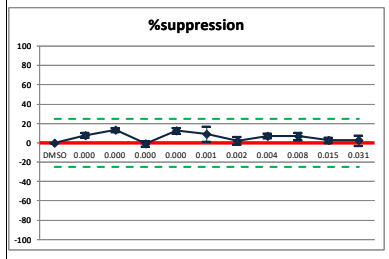
Exp.2

2nd exp. LINE 入力 30



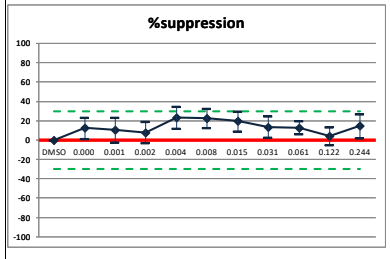
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.001	6.452	1.125	11.780	Sig.	
0.002	3.879	-1.618	9.375	N.S.	
0.004	2.321	-2.672	7.314	N.S.	
0.008	8.059	3.351	12.767	Sig.	
0.015	8.530	3.560	13.499	Sig.	
0.031	6.074	0.860	11.288	Sig.	
0.061	7.596	1.517	13.675	Sig.	
0.122	3.646	-0.725	8.018	N.S.	
0.244	4.588	-0.334	9.511	N.S.	
0.488	21.419	16.553	26.285	Sig.	

2nd exp. LINE 入力 25



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.000	8.104	5.451	10.758	Sig.	
0.000	13.433	11.361	15.506	Sig.	
0.000	-0.676	-3.594	2.242	N.S.	
0.000	12.580	9.572	15.588	Sig.	
0.001	8.983	1.141	16.824	Sig.	
0.002	2.430	-1.685	6.545	N.S.	
0.004	7.350	4.734	9.965	Sig.	
0.008	6.965	3.009	10.921	Sig.	
0.015	2.773	-0.131	5.676	N.S.	
0.031	2.668	-2.788	8.125	N.S.	

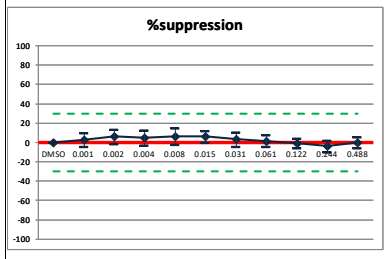
2nd exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.000	12.548	1.619	23.477	Sig.	
0.001	10.663	-2.362	23.689	N.S.	
0.002	7.964	-3.154	19.083	N.S.	
0.004	23.372	12.180	34.565	Sig.	
0.008	22.633	12.797	32.470	Sig.	
0.015	19.589	9.214	29.964	Sig.	
0.031	13.690	2.816	24.565	Sig.	
0.061	13.003	6.380	19.627	Sig.	
0.122	4.223	-4.768	13.214	N.S.	
0.244	14.776	2.330	27.221	Sig.	

Exp.3

3rd exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.001	2.741	-4.144	9.625	N.S.	
0.002	6.087	-1.508	13.682	N.S.	
0.004	4.749	-3.199	12.697	N.S.	
0.008	6.162	-2.250	14.573	N.S.	
0.015	6.067	0.113	12.021	Sig.	
0.031	3.184	-4.034	10.402	N.S.	
0.061	1.587	-4.307	7.482	N.S.	
0.122	-0.614	-5.745	4.517	N.S.	
0.244	-3.645	-9.655	2.364	N.S.	
0.488	0.083	-5.440	5.606	N.S.	

Chem.5 Set1 (MITA102,MITB405,MITC703)

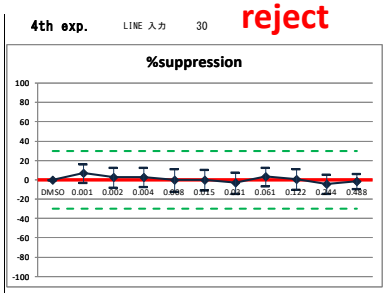
LabA Tohoku

LabB AIST tsukuba

LabC AIST shikoku

Exp.4

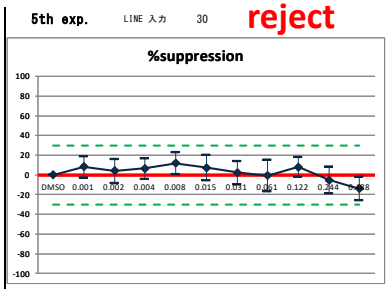
reject



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.001	6.782	-2.861	16.425	N.S.	
0.002	2.690	-7.647	13.027	N.S.	
0.004	2.745	-6.946	12.435	N.S.	
0.008	-0.280	-12.222	11.662	N.S.	
0.015	0.206	-10.546	10.959	N.S.	
0.031	-3.193	-14.205	7.818	N.S.	
0.061	3.235	-6.501	12.971	N.S.	
0.122	0.626	-10.012	11.263	N.S.	
0.244	-4.319	-14.268	5.630	N.S.	
0.488	-1.608	-9.525	6.309	N.S.	

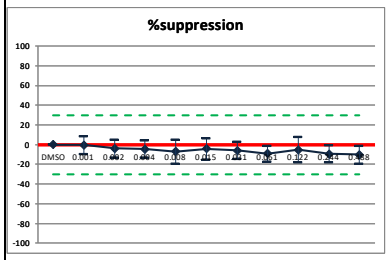
Exp.5

reject



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.001	8.419	-2.328	19.167	N.S.	
0.002	4.001	-8.310	16.313	N.S.	
0.004	6.695	-3.698	17.088	N.S.	
0.008	11.922	1.023	22.821	Sig.	
0.015	7.314	-5.735	20.363	N.S.	
0.031	2.405	-9.525	14.335	N.S.	
0.061	-0.604	-16.671	15.464	N.S.	
0.122	7.976	-2.052	18.003	N.S.	
0.244	-5.145	-18.740	8.451	N.S.	
0.488	-13.896	-25.927	-1.866	Sig.	

Exp.6

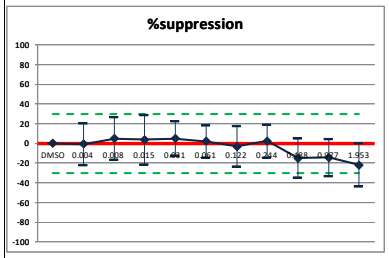


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.001	-0.281	-9.340	8.779	N.S.	
0.002	-3.976	-13.006	5.054	N.S.	
0.004	-4.444	-13.130	4.242	N.S.	
0.008	-7.082	-19.330	5.167	N.S.	
0.015	-4.142	-14.983	6.700	N.S.	
0.031	-5.789	-14.473	2.896	N.S.	
0.061	-9.126	-16.940	-1.311	Sig.	
0.122	-5.183	-18.308	7.943	N.S.	
0.244	-9.402	-17.988	-0.817	Sig.	
0.488	-10.225	-19.454	-0.997	Sig.	

LabA Tohoku

Exp.1

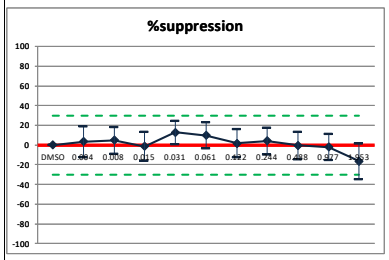
1st exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.004	-0.672	-22.403	20.659	N.S.	
0.008	4.945	-16.548	26.438	N.S.	
0.015	3.656	-21.234	28.547	N.S.	
0.031	5.001	-12.516	22.519	N.S.	
0.061	2.006	-14.281	18.292	N.S.	
0.122	-3.054	-23.450	17.343	N.S.	
0.244	2.235	-14.282	18.753	N.S.	
0.488	-14.860	-34.604	4.884	N.S.	
0.977	-14.411	-33.418	4.597	N.S.	
1.953	-22.102	-44.046	-0.158	Sig.	

Exp.2

2nd exp. LINE 入力 30

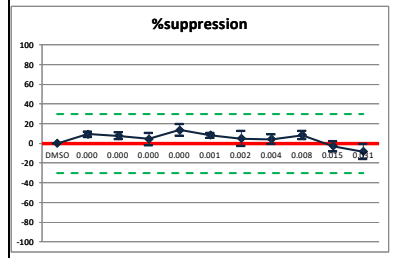


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.004	3.550	-12.079	19.179	N.S.	
0.008	4.711	-9.090	18.512	N.S.	
0.015	-1.316	-15.716	13.085	N.S.	
0.031	12.916	1.027	24.804	Sig.	
0.061	9.835	-3.456	23.127	N.S.	
0.122	1.663	-12.635	15.961	N.S.	
0.244	3.975	-9.300	17.249	N.S.	
0.488	-0.514	-14.499	13.471	N.S.	
0.977	-2.033	-15.369	11.303	N.S.	
1.953	-16.429	-34.525	1.667	N.S.	

Exp.3

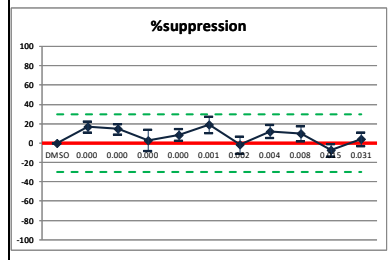
LabB AIST tsukuba

1st exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.000	9.397	6.646	12.148	Sig.	
0.000	7.632	4.312	10.952	Sig.	
0.000	4.378	-1.607	10.363	N.S.	
0.000	13.688	7.936	19.439	Sig.	
0.001	8.372	5.820	10.925	Sig.	
0.002	4.911	-2.585	12.406	N.S.	
0.004	4.261	-0.570	9.091	N.S.	
0.008	8.418	4.369	12.467	Sig.	
0.015	-2.869	-8.144	2.405	N.S.	
0.031	-8.246	-16.047	-0.445	Sig.	

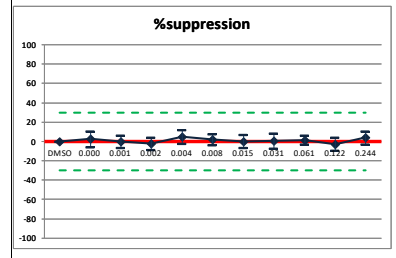
2nd exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.000	16.905	11.317	22.492	Sig.	
0.000	14.772	9.476	20.067	Sig.	
0.000	3.113	-8.057	14.283	N.S.	
0.000	8.829	2.835	14.822	Sig.	
0.001	19.153	10.898	27.407	Sig.	
0.002	-1.605	-10.443	7.234	N.S.	
0.004	12.298	5.768	18.828	Sig.	
0.008	9.960	2.302	17.619	Sig.	
0.015	-7.248	-13.560	-0.937	Sig.	
0.031	4.091	-2.980	11.163	N.S.	

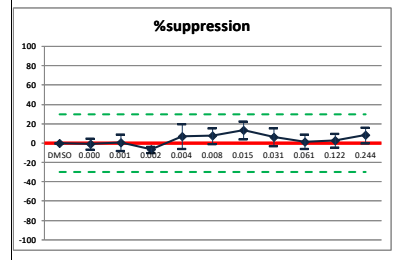
LabC AIST shikoku

1st exp. LINE 入力 30



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.000	2.488	-5.775	10.751	N.S.	
0.001	0.024	-6.215	6.264	N.S.	
0.002	-2.134	-8.784	4.516	N.S.	
0.004	5.025	-2.055	12.106	N.S.	
0.008	2.085	-3.505	7.675	N.S.	
0.015	0.185	-6.742	7.112	N.S.	
0.031	0.979	-6.850	8.807	N.S.	
0.061	1.558	-2.928	6.045	N.S.	
0.122	-2.591	-9.112	3.930	N.S.	
0.244	4.152	-2.626	10.931	N.S.	

2nd exp. LINE 入力 30

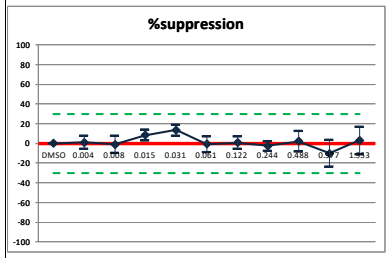


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.000	-0.713	-6.103	4.678	N.S.	
0.001	0.838	-7.665	9.341	N.S.	
0.002	-6.548	-9.763	-3.333	Sig.	
0.004	7.175	-5.395	19.746	N.S.	
0.008	7.644	-0.575	15.863	N.S.	
0.015	13.669	4.409	22.928	Sig.	
0.031	6.454	-2.712	15.620	N.S.	
0.061	1.440	-5.967	8.848	N.S.	
0.122	2.799	-3.969	9.566	N.S.	
0.244	8.183	0.119	16.247	Sig.	

LabA Tohoku

Exp.1

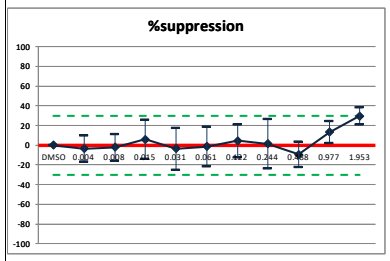
1st exp. LINE 入力 30



a Comparison Conc.	%supp	LowerLimit	UpperLimit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.004	0.970	-5.713	7.652	N.S.	
0.008	-1.119	-9.758	7.521	N.S.	
0.015	8.387	3.028	13.747	Sig.	
0.031	13.561	7.966	19.156	Sig.	
0.061	-0.852	-8.788	7.084	N.S.	
0.122	0.827	-5.655	7.308	N.S.	
0.244	-2.610	-7.319	2.098	N.S.	
0.488	2.221	-8.262	12.703	N.S.	
0.977	-10.112	-23.778	3.554	N.S.	
1.953	3.089	-10.947	17.125	N.S.	

Exp.2

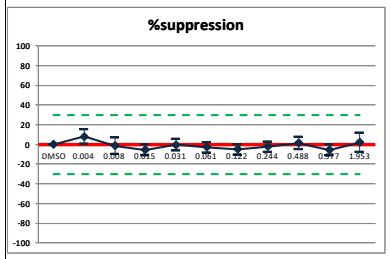
2nd exp. LINE 入力 30



a Comparison Conc.	%supp	LowerLimit	UpperLimit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.004	-3.625	-16.848	9.598	N.S.	
0.008	-1.968	-15.537	11.600	N.S.	
0.015	5.998	-13.603	25.600	N.S.	
0.031	-3.426	-24.700	17.848	N.S.	
0.061	-1.313	-21.630	19.004	N.S.	
0.122	4.374	-12.126	20.874	N.S.	
0.244	1.293	-23.803	26.389	N.S.	
0.488	-9.279	-22.239	3.680	N.S.	
0.977	13.331	1.918	24.745	Sig.	
1.953	29.618	21.110	38.126	Sig.	

Exp.3

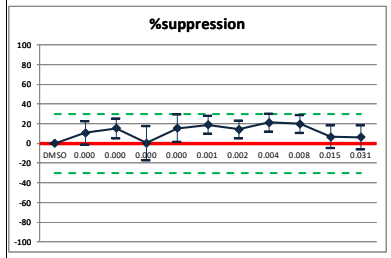
3rd exp. LINE 入力 30



a Comparison Conc.	%supp	LowerLimit	UpperLimit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.004	8.047	0.741	15.354	Sig.	
0.008	-1.369	-9.949	7.210	N.S.	
0.015	-5.519	-11.247	0.209	N.S.	
0.031	-0.259	-5.953	5.434	N.S.	
0.061	-2.824	-8.002	2.354	N.S.	
0.122	-4.957	-10.184	0.271	N.S.	
0.244	-2.136	-7.176	2.905	N.S.	
0.488	1.510	-4.723	7.744	N.S.	
0.977	-5.537	-11.028	-0.046	Sig.	
1.953	2.001	-7.854	11.857	N.S.	

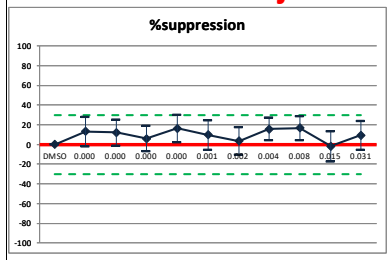
LabB AIST tsukuba

1st exp. LINE 入力 30



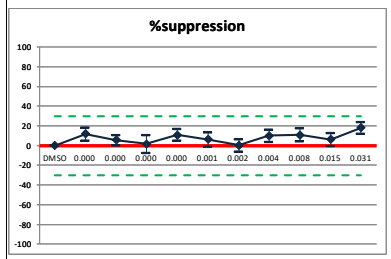
a Comparison Conc.	%supp	LowerLimit	UpperLimit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.000	10.712	-1.186	22.610	N.S.	
0.000	15.307	5.279	25.334	Sig.	
0.000	0.423	-17.000	17.845	N.S.	
0.000	15.274	1.484	29.065	Sig.	
0.001	18.676	9.590	27.763	Sig.	
0.002	14.076	4.821	23.331	Sig.	
0.004	21.146	12.004	30.289	Sig.	
0.008	19.780	10.921	28.640	Sig.	
0.015	6.616	-4.874	18.105	N.S.	
0.031	6.141	-6.287	18.570	N.S.	

2nd exp. LINE 入力 30 **reject**



a Comparison Conc.	%supp	LowerLimit	UpperLimit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.000	13.155	-1.752	28.062	N.S.	
0.000	12.128	-1.049	25.304	N.S.	
0.000	5.972	-6.971	18.915	N.S.	
0.000	16.453	2.513	30.393	Sig.	
0.001	9.689	-5.339	24.717	N.S.	
0.002	3.580	-10.358	17.518	N.S.	
0.004	15.588	4.168	27.009	Sig.	
0.008	16.518	4.296	28.739	Sig.	
0.015	-1.904	-17.310	13.502	N.S.	
0.031	9.228	-5.668	24.124	N.S.	

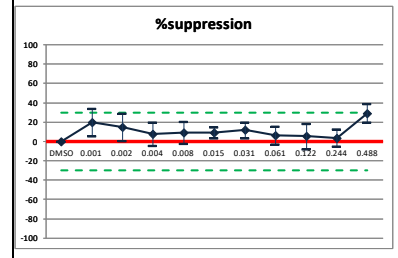
2nd exp. LINE 入力 30



a Comparison Conc.	%supp	LowerLimit	UpperLimit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.000	11.800	5.216	18.384	Sig.	
0.000	5.607	0.417	10.798	Sig.	
0.000	1.703	-7.304	10.709	N.S.	
0.001	10.652	4.777	16.526	Sig.	
0.002	6.156	-1.040	13.353	N.S.	
0.004	0.144	-6.273	6.560	N.S.	
0.008	9.904	3.367	16.442	Sig.	
0.015	10.908	4.325	17.491	Sig.	
0.031	6.062	-0.787	12.912	N.S.	
0.061	18.067	12.242	23.892	Sig.	

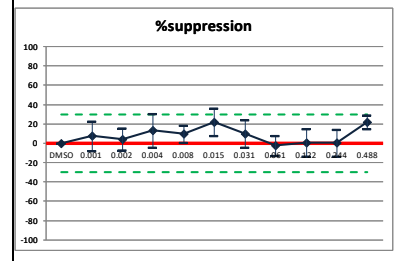
LabC AIST shikoku

1st exp. LINE 入力 30



a Comparison Conc.	%supp	LowerLimit	UpperLimit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.001	19.795	5.851	33.739	Sig.	
0.002	14.606	0.320	28.891	Sig.	
0.004	7.799	-4.196	19.793	N.S.	
0.008	8.927	-2.353	20.208	N.S.	
0.015	9.112	3.188	15.037	Sig.	
0.031	11.781	3.545	20.016	Sig.	
0.061	6.258	-2.939	15.455	N.S.	
0.122	5.352	-7.676	18.381	N.S.	
0.244	3.792	-4.999	12.583	N.S.	
0.488	29.290	19.515	39.066	Sig.	

2nd exp. LINE 入力 30



a Comparison Conc.	%supp	LowerLimit	UpperLimit	Sig./N.S.	%supp Over Line
DMSO	0	0	0		
0.001	7.490	-7.926	22.907	N.S.	
0.002	4.062	-7.110	15.233	N.S.	
0.004	13.346	-4.064	30.756	N.S.	
0.008	9.614	0.770	18.457	Sig.	
0.015	21.807	7.709	35.906	Sig.	
0.031	9.848	-4.271	23.966	N.S.	
0.061	-2.507	-12.921	7.907	N.S.	
0.122	0.818	-13.300	14.937	N.S.	
0.244	0.469	-13.215	14.154	N.S.	
0.488	22.045	15.084	29.007	Sig.	

Phase2 Line25 results

Takashi Omori

2019.11.29

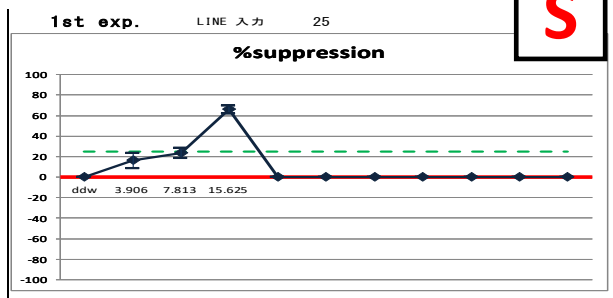
Chem.2

LabA Tohoku MTA117

Judge **S**

Exp.1

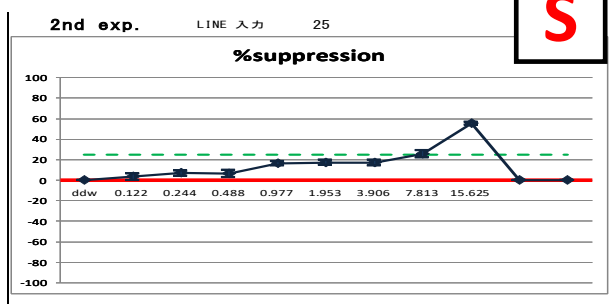
S



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	16.270	8.703	23.837	Sig.	
7.813	23.408	18.234	28.583	Sig.	
15.625	65.825	62.007	69.643	Sig.	

Exp.2

S



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
0.122	3.492	0.231	6.753	Sig.	
0.244	6.840	4.325	9.355	Sig.	
0.488	6.435	2.844	10.025	Sig.	
0.977	16.433	14.138	18.729	Sig.	
1.953	17.406	15.012	19.799	Sig.	
3.906	17.146	14.128	20.164	Sig.	
7.813	25.683	22.413	28.954	Sig.	
15.625	55.470	53.750	57.190	Sig.	

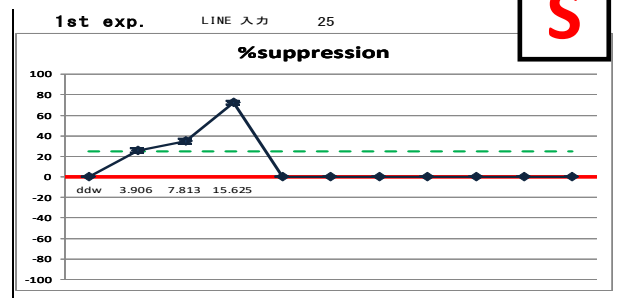
Exp.3

LabB Tsukuba MTB221

Judge **S**

Exp.1

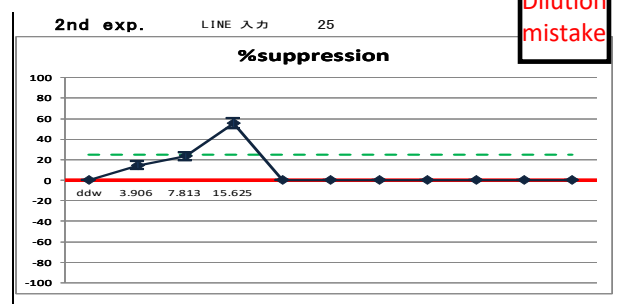
S



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	25.771	23.836	27.707	Sig.	
7.813	34.722	32.216	37.228	Sig.	
15.625	72.308	70.637	73.980	Sig.	

Exp.2

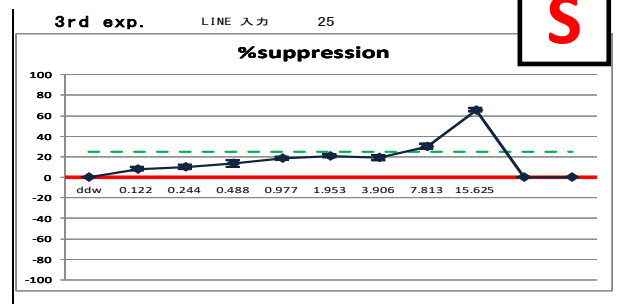
Dilution mistake



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	14.568	10.773	18.363	Sig.	
7.813	23.199	19.290	27.107	Sig.	
15.625	55.398	50.594	60.202	Sig.	

Exp.3

S



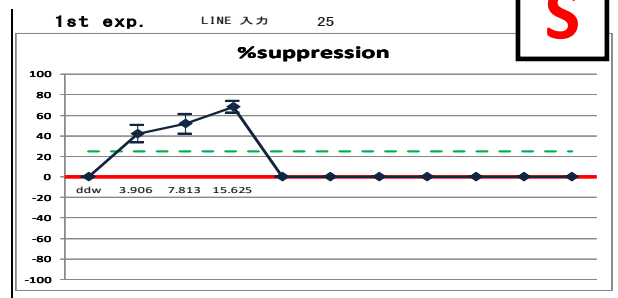
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
0.122	8.156	6.624	9.687	Sig.	
0.244	10.026	8.245	11.807	Sig.	
0.488	13.372	10.336	16.409	Sig.	
0.977	18.606	17.132	20.081	Sig.	
1.953	20.674	19.206	22.143	Sig.	
3.906	19.080	16.553	21.607	Sig.	
7.813	30.193	27.987	32.400	Sig.	
15.625	65.719	63.855	67.583	Sig.	

LabC Shikoku MTC305

Judge **S**

Exp.1

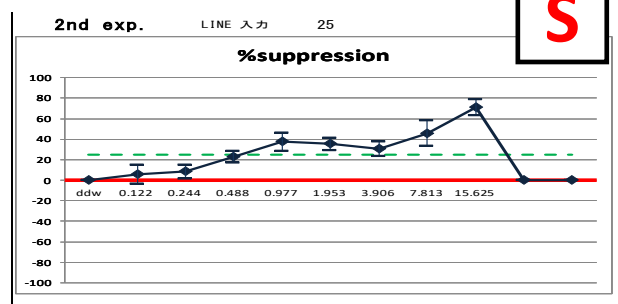
S



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	41.856	33.366	50.347	Sig.	
7.813	51.689	42.128	61.249	Sig.	
15.625	68.259	62.450	74.068	Sig.	

Exp.2

S

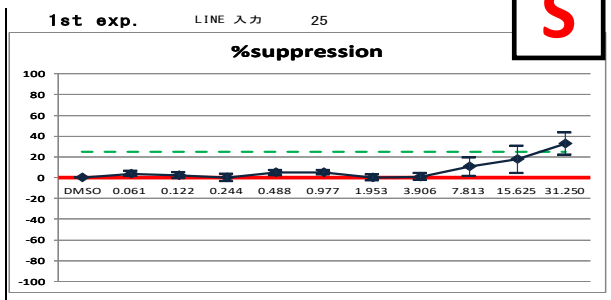


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
0.122	5.914	-3.235	15.064	N.S.	
0.244	8.256	1.227	15.285	Sig.	
0.488	22.869	16.916	28.822	Sig.	
0.977	37.458	28.726	46.190	Sig.	
1.953	35.261	28.907	41.615	Sig.	
3.906	30.579	23.458	37.700	Sig.	
7.813	45.799	33.440	58.159	Sig.	
15.625	71.264	63.403	79.125	Sig.	

Exp.3

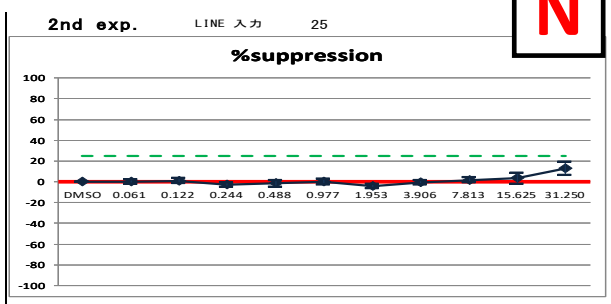
Chem.3	LabA Tohoku MTA105	LabB Tsukuba MTB220	LabC Shikoku MTC301
Judge	N	N	N
Exp.1	S	N	S
Exp.2	N	N	N
Exp.3	N		N

LabA Tohoku MTA105 — Exp.1 — S



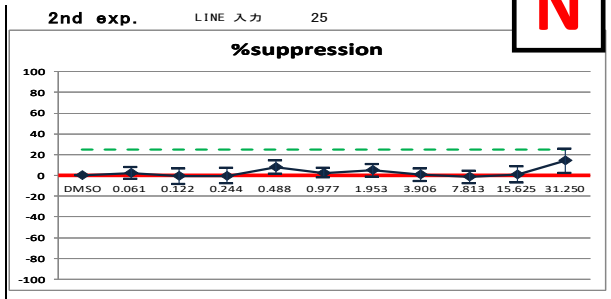
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.061	3.915	1.447	6.383	Sig.	
0.122	2.237	-0.688	5.162	N.S.	
0.244	-0.167	-3.752	3.418	N.S.	
0.488	4.842	2.490	7.194	Sig.	
0.977	5.344	3.220	7.468	Sig.	
1.953	0.074	-3.028	3.175	N.S.	
3.906	0.974	-2.200	4.149	N.S.	
7.813	10.458	1.762	19.154	Sig.	
15.625	17.624	4.513	30.735	Sig.	
31.250	32.451	21.812	43.090	Sig.	

LabA Tohoku MTA105 — Exp.2 — N



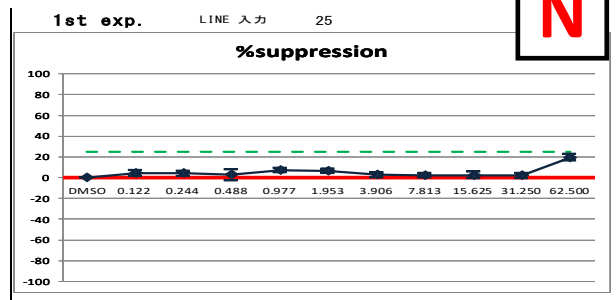
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.061	0.190	-2.061	2.441	N.S.	
0.122	0.992	-1.607	3.590	N.S.	
0.244	-2.469	-4.569	-0.370	Sig.	
0.488	-1.516	-4.882	1.850	N.S.	
0.977	0.367	-2.439	3.173	N.S.	
1.953	-4.192	-6.531	-1.853	Sig.	
3.906	-0.830	-2.843	1.184	N.S.	
7.813	1.678	-0.682	4.039	N.S.	
15.625	3.299	-1.995	8.593	N.S.	
31.250	12.921	6.358	19.484	Sig.	

LabA Tohoku MTA105 — Exp.3 — N



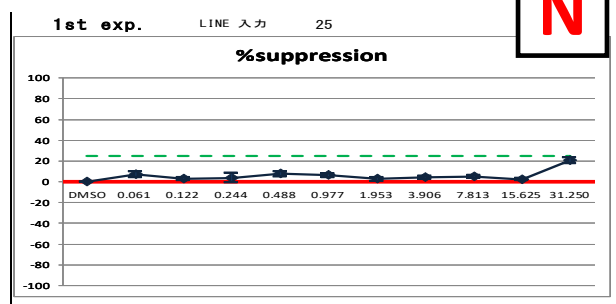
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.061	2.194	-3.230	7.617	N.S.	
0.122	-0.855	-8.202	6.492	N.S.	
0.244	-0.463	-7.865	6.938	N.S.	
0.488	8.124	1.688	14.560	Sig.	
0.977	2.529	-2.288	7.346	N.S.	
1.953	4.709	-1.135	10.554	N.S.	
3.906	0.692	-5.405	6.789	N.S.	
7.813	-1.678	-7.817	4.461	N.S.	
15.625	0.870	-6.763	8.503	N.S.	
31.250	13.928	1.880	25.975	Sig.	

LabB Tsukuba MTB220 — Exp.1 — N



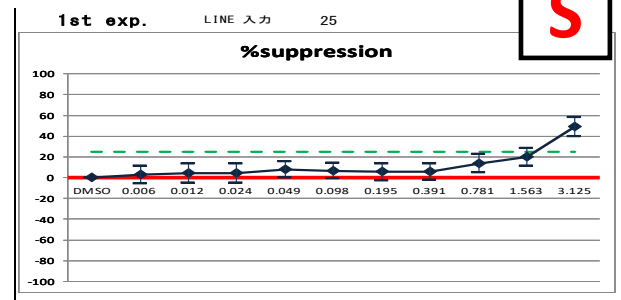
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.122	4.385	1.781	6.989	Sig.	
0.244	4.106	1.830	6.383	Sig.	
0.488	2.593	-2.787	7.973	N.S.	
0.977	7.281	5.218	9.345	Sig.	
1.953	6.477	4.642	8.313	Sig.	
3.906	2.725	0.340	5.110	Sig.	
7.813	1.865	-0.274	4.004	N.S.	
15.625	2.546	-0.946	6.037	N.S.	
31.250	1.961	-0.772	4.694	N.S.	
62.500	19.543	16.521	22.565	Sig.	

LabB Tsukuba MTB220 — Exp.2 — N



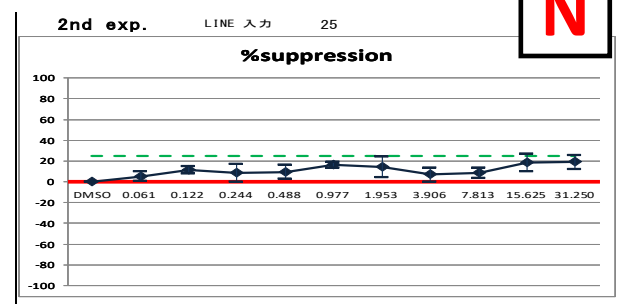
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.061	6.988	4.170	9.806	Sig.	
0.122	3.232	1.825	4.638	Sig.	
0.244	3.828	-0.739	8.396	N.S.	
0.488	7.705	5.336	10.075	Sig.	
0.977	6.283	4.551	8.015	Sig.	
1.953	2.706	0.994	4.419	Sig.	
3.906	4.238	2.582	5.894	Sig.	
7.813	5.301	3.969	6.633	Sig.	
15.625	2.490	1.243	3.738	Sig.	
31.250	20.970	18.164	23.777	Sig.	

LabC Shikoku MTC301 — Exp.1 — S



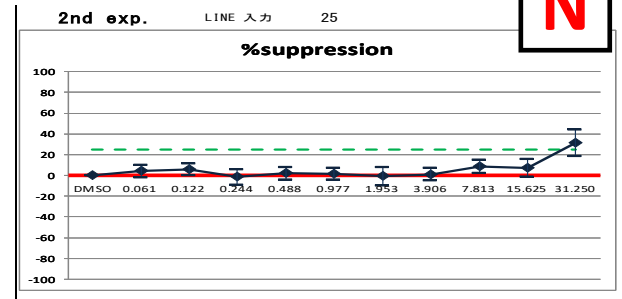
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.006	3.236	-5.291	11.762	N.S.	
0.012	4.055	-5.141	13.250	N.S.	
0.024	4.398	-5.103	13.900	N.S.	
0.049	7.729	-0.246	15.703	N.S.	
0.098	6.691	-0.677	14.059	N.S.	
0.195	5.415	-2.818	13.648	N.S.	
0.391	5.545	-2.152	13.242	N.S.	
0.781	13.890	4.767	23.013	Sig.	
1.563	19.845	11.262	28.427	Sig.	
3.125	49.320	40.108	58.532	Sig.	

LabC Shikoku MTC301 — Exp.2 — N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.061	5.328	0.839	9.816	Sig.	
0.122	11.381	8.059	14.703	Sig.	
0.244	8.582	0.157	17.006	Sig.	
0.488	9.665	2.910	16.419	Sig.	
0.977	16.500	13.469	19.531	Sig.	
1.953	14.429	4.350	24.508	Sig.	
3.906	6.907	0.294	13.520	Sig.	
7.813	8.654	3.745	13.563	Sig.	
15.625	18.436	9.745	27.127	Sig.	
31.250	19.090	12.433	25.746	Sig.	

LabC Shikoku MTC301 — Exp.3 — N

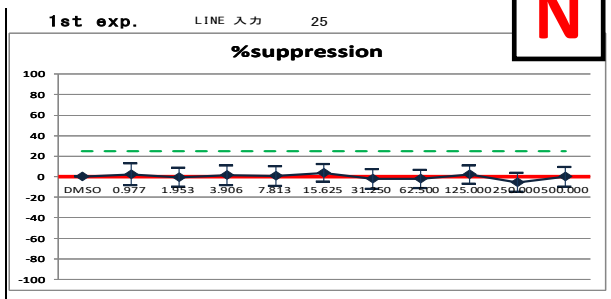


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.061	4.218	-1.882	10.318	N.S.	
0.122	5.936	0.389	11.483	Sig.	
0.244	-1.600	-8.857	5.658	N.S.	
0.488	2.040	-4.099	8.180	N.S.	
0.977	1.424	-4.196	7.044	N.S.	
1.953	-0.979	-9.571	7.613	N.S.	
3.906	0.986	-5.040	7.012	N.S.	
7.813	8.406	1.981	14.832	Sig.	
15.625	7.389	-1.024	15.802	N.S.	
31.250	31.207	18.647	43.767	Sig.	

Chem.4

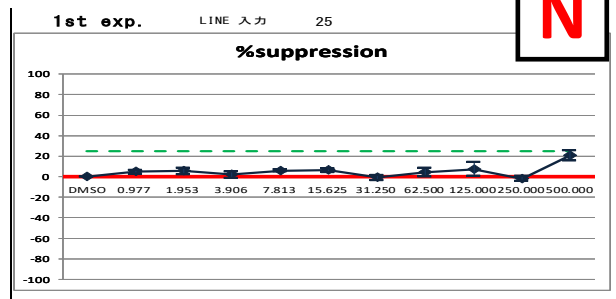
	LabA Tohoku MTA120	LabB Tsukuba MTB203	LabC Shikoku MTC318
Judge	**N**	**N**	**S**

Exp.1



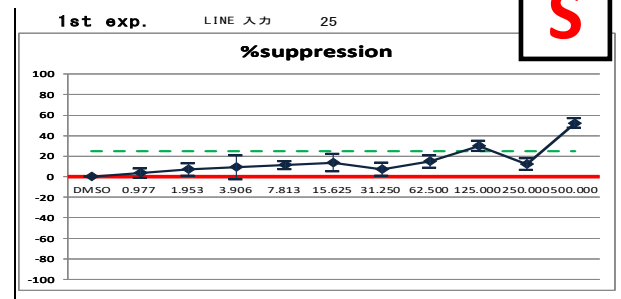
N

a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	2.414	-8.262	13.090	N.S.	
1.953	-0.656	-9.645	8.333	N.S.	
3.906	1.482	-8.108	11.073	N.S.	
7.813	0.498	-9.144	10.139	N.S.	
15.625	3.352	-5.234	11.939	N.S.	
31.250	-2.105	-11.663	7.452	N.S.	
62.500	-2.194	-11.153	6.764	N.S.	
125.000	2.176	-6.701	11.053	N.S.	
250.000	-5.385	-14.749	3.980	N.S.	
500.000	-0.139	-9.808	9.530	N.S.	



N

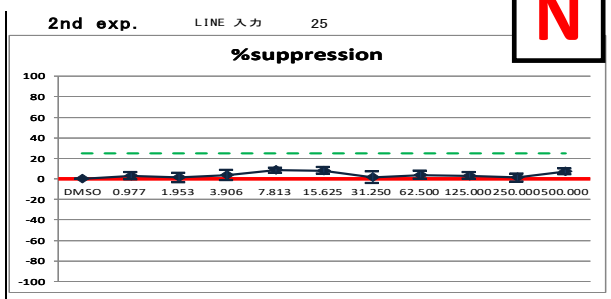
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	4.976	3.255	6.698	Sig.	
1.953	5.629	2.432	8.826	Sig.	
3.906	1.966	-1.442	5.373	N.S.	
7.813	6.096	5.088	7.104	Sig.	
15.625	6.224	4.437	8.010	Sig.	
31.250	-0.976	-3.394	1.442	N.S.	
62.500	4.222	0.135	8.308	Sig.	
125.000	7.461	0.735	14.188	Sig.	
250.000	-1.812	-4.459	0.836	N.S.	
500.000	20.453	15.420	25.487	Sig.	



S

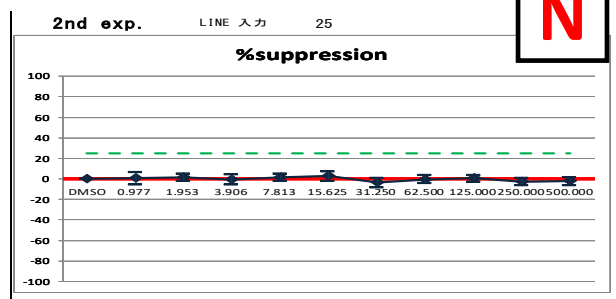
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	3.427	-1.039	7.892	N.S.	
1.953	7.028	1.076	12.980	Sig.	
3.906	9.056	-2.476	20.588	N.S.	
7.813	11.144	7.010	15.278	Sig.	
15.625	13.834	5.368	22.300	Sig.	
31.250	7.123	0.548	13.698	Sig.	
62.500	14.673	8.645	20.702	Sig.	
125.000	29.878	24.826	34.930	Sig.	
250.000	12.112	6.743	17.482	Sig.	
500.000	52.234	47.764	56.705	Sig.	

Exp.2



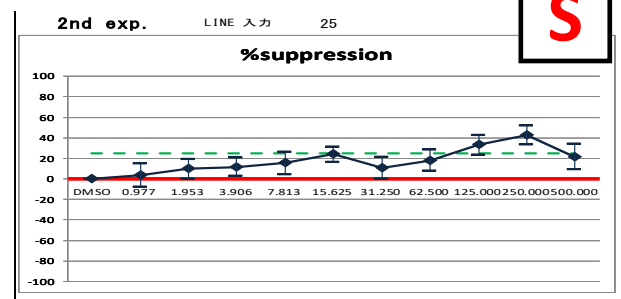
N

a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	2.959	-0.677	6.594	N.S.	
1.953	1.300	-3.166	5.765	N.S.	
3.906	3.421	-1.579	8.420	N.S.	
7.813	8.271	5.463	11.079	Sig.	
15.625	8.231	4.749	11.714	Sig.	
31.250	1.467	-4.076	7.009	N.S.	
62.500	3.873	0.142	7.605	Sig.	
125.000	3.249	-0.191	6.690	N.S.	
250.000	1.272	-2.817	5.360	N.S.	
500.000	6.974	4.007	9.941	Sig.	



N

a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	0.607	-5.500	6.714	N.S.	
1.953	1.592	-1.856	5.039	N.S.	
3.906	-0.767	-5.711	4.177	N.S.	
7.813	1.420	-2.216	5.057	N.S.	
15.625	2.597	-1.764	6.957	N.S.	
31.250	-3.773	-8.291	0.744	N.S.	
62.500	-0.348	-4.415	3.718	N.S.	
125.000	0.480	-2.692	3.652	N.S.	
250.000	-2.846	-6.537	0.844	N.S.	
500.000	-2.297	-6.111	1.516	N.S.	



S

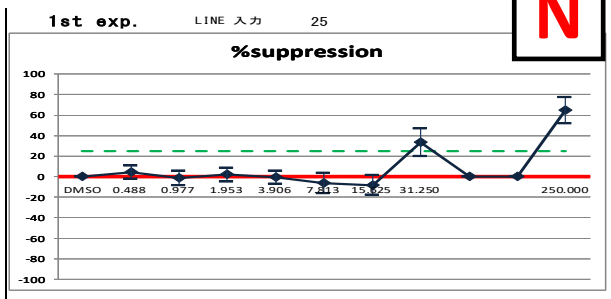
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	3.747	-7.768	15.263	N.S.	
1.953	9.723	0.334	19.112	Sig.	
3.906	11.664	2.789	20.539	Sig.	
7.813	15.609	4.530	26.688	Sig.	
15.625	23.887	16.103	31.671	Sig.	
31.250	10.842	0.427	21.256	Sig.	
62.500	18.107	7.770	28.444	Sig.	
125.000	33.194	23.852	42.536	Sig.	
250.000	42.516	33.357	51.675	Sig.	
500.000	21.478	8.967	33.990	Sig.	

Exp.3

Chem.5	LabA Tohoku MTA115	LabB Tsukuba MTB211	LabC Shikoku MTC307
Judge	**N**	**N**	**S**

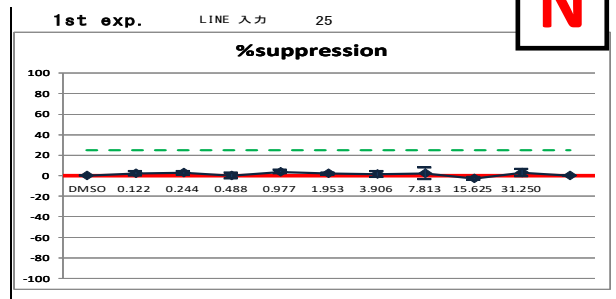
Exp.1

N



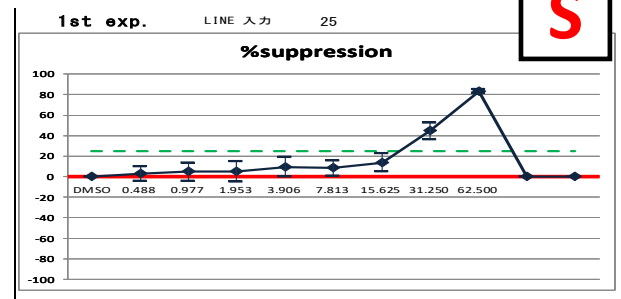
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.488	4.121	-2.176	10.418	N.S.	
0.977	-1.480	-8.525	5.565	N.S.	
1.953	1.886	-4.626	8.398	N.S.	
3.906	-0.643	-7.359	6.074	N.S.	
7.813	-6.165	-15.909	3.579	N.S.	
15.625	-8.181	-17.798	1.436	N.S.	
31.250	33.473	19.732	47.213	Sig.	
250.000	64.652	51.719	77.585	Sig.	

N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.122	2.192	-0.248	4.631	N.S.	
0.244	2.625	1.057	4.194	Sig.	
0.488	0.116	-2.726	2.959	N.S.	
0.977	3.806	2.181	5.432	Sig.	
1.953	1.926	0.820	3.031	Sig.	
3.906	1.559	-1.254	4.371	N.S.	
7.813	2.091	-3.542	7.724	N.S.	
15.625	-2.605	-4.021	-1.188	Sig.	
31.250	2.814	-0.629	6.257	N.S.	

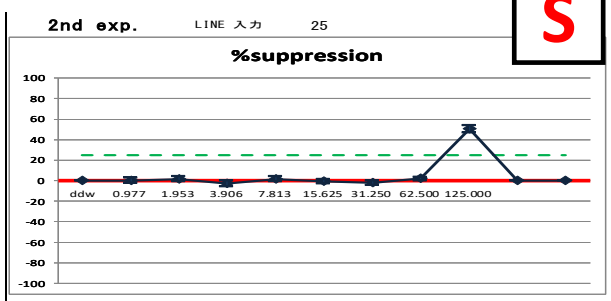
S



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.488	2.595	-4.503	9.693	N.S.	
0.977	4.699	-3.848	13.245	N.S.	
1.953	4.992	-4.797	14.781	N.S.	
3.906	9.463	-0.053	18.979	N.S.	
7.813	8.322	0.851	15.794	Sig.	
15.625	13.645	4.836	22.455	Sig.	
31.250	44.677	36.398	52.956	Sig.	
62.500	83.314	81.626	85.002	Sig.	

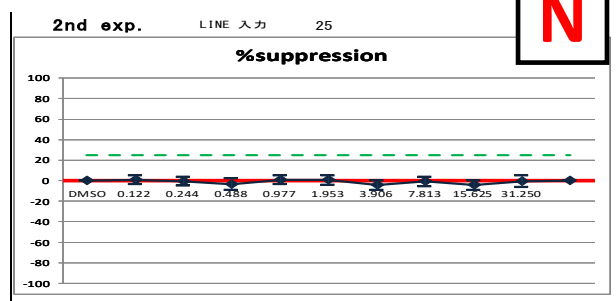
Exp.2

S



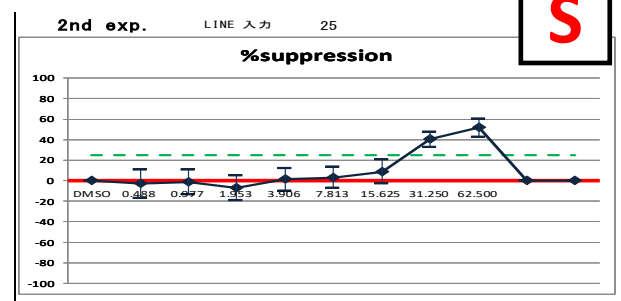
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
0.977	0.220	-2.137	2.576	N.S.	
1.953	1.630	-0.816	4.077	N.S.	
3.906	-2.979	-5.766	-0.192	Sig.	
7.813	1.843	-0.645	4.330	N.S.	
15.625	-0.622	-2.643	1.398	N.S.	
31.250	-2.188	-4.474	0.098	N.S.	
62.500	2.141	0.353	3.929	Sig.	
125.000	50.454	46.657	54.251	Sig.	

N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.122	0.799	-3.375	4.972	N.S.	
0.244	-0.379	-4.549	3.790	N.S.	
0.488	-3.472	-8.927	1.983	N.S.	
0.977	1.084	-3.167	5.335	N.S.	
1.953	0.527	-3.833	4.887	N.S.	
3.906	-4.429	-9.263	0.406	N.S.	
7.813	-0.976	-5.302	3.350	N.S.	
15.625	-4.437	-9.152	0.277	N.S.	
31.250	-0.770	-6.433	4.892	N.S.	

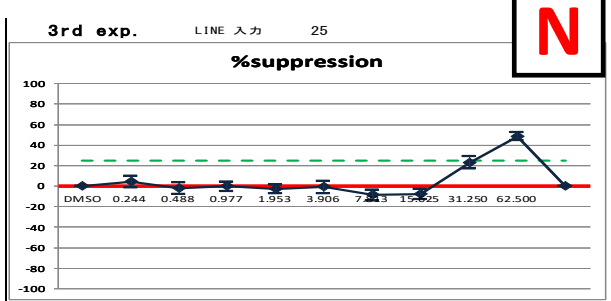
S



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.488	-2.902	-16.650	10.846	N.S.	
0.977	-1.345	-13.548	10.857	N.S.	
1.953	-6.755	-18.765	5.256	N.S.	
3.906	1.259	-9.649	12.167	N.S.	
7.813	3.212	-7.013	13.436	N.S.	
15.625	8.857	-3.013	20.728	N.S.	
31.250	40.218	32.696	47.740	Sig.	
62.500	51.835	42.972	60.699	Sig.	

Exp.3

N

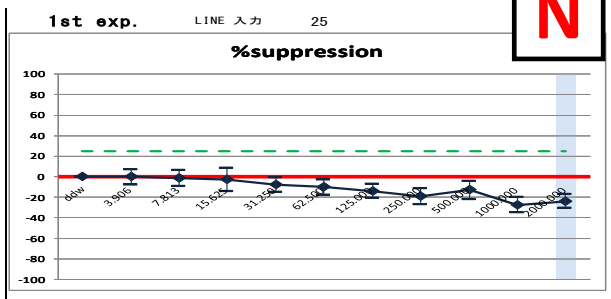


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.244	4.438	-1.485	10.361	N.S.	
0.488	-1.987	-7.815	3.841	N.S.	
0.977	-0.020	-4.713	4.673	N.S.	
1.953	-2.884	-6.992	1.223	N.S.	
3.906	-0.808	-6.681	5.064	N.S.	
7.813	-8.729	-14.155	-3.303	Sig.	
15.625	-7.878	-12.908	-2.847	Sig.	
31.250	23.106	17.240	28.973	Sig.	
62.500	48.607	44.622	52.592	Sig.	

Chem.6	LabA Tohoku MTA111	LabB Tsukuba MTB224	LabC Shikoku MTC302
Judge	N	N	N

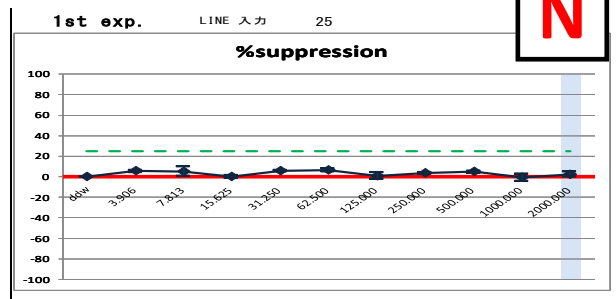
Exp.1

N



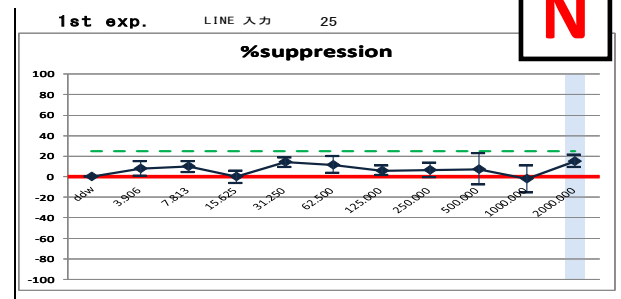
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	-0.015	-7.501	7.470	N.S.	
7.813	-1.344	-9.447	6.759	N.S.	
15.625	-2.699	-13.838	8.439	N.S.	
31.250	-7.784	-14.958	-0.610	Sig.	
62.500	-10.135	-17.470	-2.799	Sig.	
125.000	-13.873	-20.559	-7.187	Sig.	
250.000	-19.085	-27.070	-11.100	Sig.	
500.000	-12.907	-21.893	-3.921	Sig.	
1000.000	-27.305	-34.547	-20.063	Sig.	
2000.000	-23.832	-30.779	-16.885	Sig.	eliminate

N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	5.497	4.335	6.658	Sig.	
7.813	5.244	0.662	9.825	Sig.	
15.625	0.116	-1.030	1.262	N.S.	
31.250	5.817	5.218	6.416	Sig.	
62.500	6.656	5.396	7.915	Sig.	
125.000	0.939	-2.180	4.058	N.S.	
250.000	3.619	2.669	4.569	Sig.	
500.000	4.744	3.607	5.881	Sig.	
1000.000	-0.612	-4.146	2.923	N.S.	
2000.000	2.289	-0.223	4.802	N.S.	eliminate

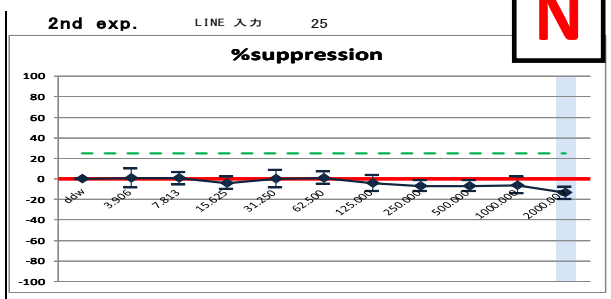
N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	8.040	1.144	14.935	Sig.	
7.813	9.799	4.378	15.221	Sig.	
15.625	-0.072	-6.009	5.864	N.S.	
31.250	13.935	9.328	18.541	Sig.	
62.500	11.786	3.722	19.849	Sig.	
125.000	5.898	1.195	10.600	Sig.	
250.000	6.456	-0.719	13.631	N.S.	
500.000	7.308	-7.908	22.524	N.S.	
1000.000	-2.260	-15.507	10.987	N.S.	
2000.000	15.324	9.309	21.339	Sig.	eliminate

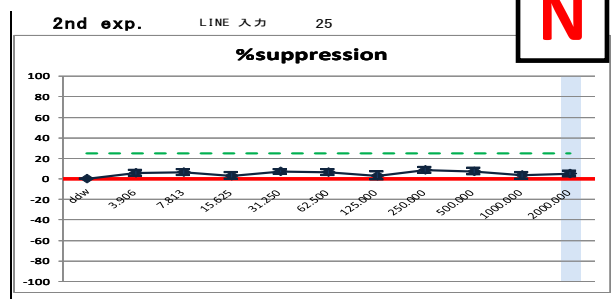
Exp.2

N



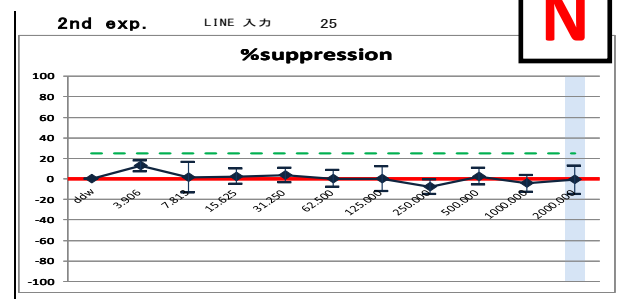
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	0.500	-8.701	9.700	N.S.	
7.813	0.525	-5.605	6.655	N.S.	
15.625	-4.063	-10.022	1.896	N.S.	
31.250	0.367	-8.204	8.939	N.S.	
62.500	0.956	-5.237	7.149	N.S.	
125.000	-4.152	-11.659	3.355	N.S.	
250.000	-6.889	-12.282	-1.496	Sig.	
500.000	-6.740	-12.272	-1.209	Sig.	
1000.000	-6.132	-14.271	2.007	N.S.	
2000.000	-13.679	-19.755	-7.603	Sig.	eliminate

N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	5.551	2.661	8.440	Sig.	
7.813	6.616	3.789	9.444	Sig.	
15.625	3.152	0.096	6.208	Sig.	
31.250	7.161	4.698	9.624	Sig.	
62.500	6.261	3.352	9.170	Sig.	
125.000	3.148	-0.890	7.186	N.S.	
250.000	8.798	6.049	11.548	Sig.	
500.000	7.524	4.114	10.934	Sig.	
1000.000	3.468	0.342	6.593	Sig.	
2000.000	5.064	2.149	7.978	Sig.	eliminate

N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	12.632	7.365	17.899	Sig.	
7.813	1.570	-13.473	16.612	N.S.	
15.625	2.305	-5.238	9.848	N.S.	
31.250	3.687	-3.419	10.794	N.S.	
62.500	0.316	-7.963	8.596	N.S.	
125.000	0.049	-12.089	12.188	N.S.	
250.000	-7.751	-14.680	-0.822	Sig.	
500.000	2.475	-5.665	10.616	N.S.	
1000.000	-4.387	-12.738	3.963	N.S.	
2000.000	-0.876	-14.529	12.777	N.S.	eliminate

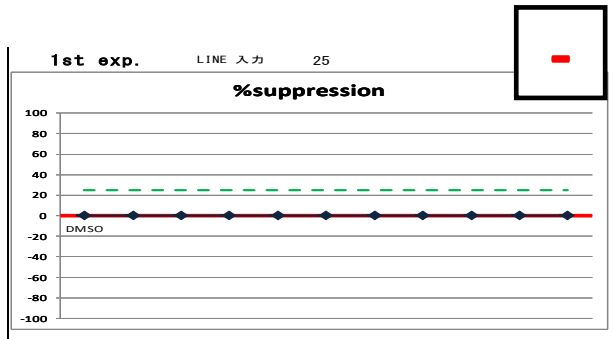
Exp.3

Chem.7

LabA Tohoku MTA112

Judge **N**

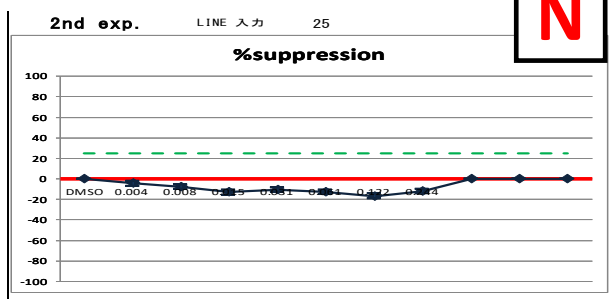
Exp.1



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		

N

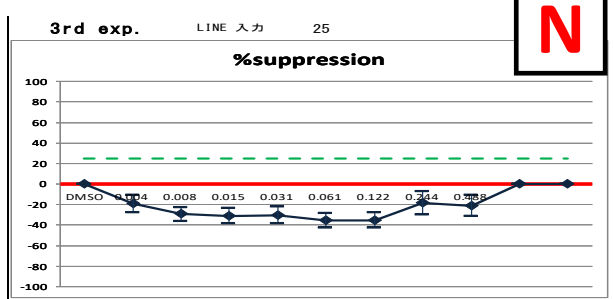
Exp.2



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.004	-4.370	-6.996	-1.744	Sig.	
0.008	-7.779	-9.993	-5.565	Sig.	
0.015	-12.993	-15.634	-10.351	Sig.	
0.031	-10.496	-12.640	-8.353	Sig.	
0.061	-12.617	-14.884	-10.350	Sig.	
0.122	-16.756	-19.436	-14.075	Sig.	
0.244	-11.963	-14.109	-9.817	Sig.	

N

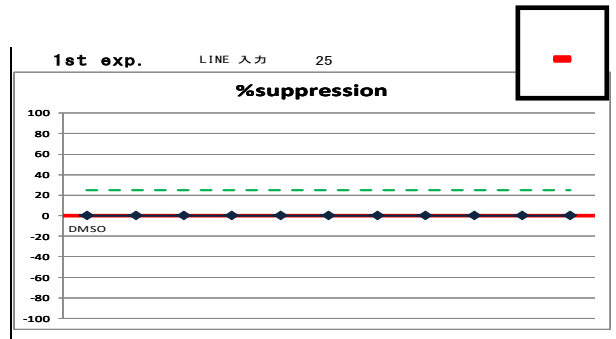
Exp.3



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.004	-19.198	-27.788	-10.609	Sig.	
0.008	-29.166	-35.866	-22.467	Sig.	
0.015	-30.830	-38.569	-23.091	Sig.	
0.031	-30.150	-38.481	-21.820	Sig.	
0.061	-35.570	-42.569	-28.572	Sig.	
0.122	-35.150	-42.860	-27.441	Sig.	
0.244	-18.160	-29.468	-6.851	Sig.	
0.488	-20.990	-31.404	-10.575	Sig.	

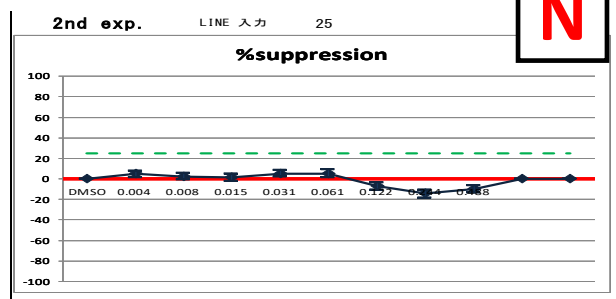
LabB Tsukuba MTB208

Judge **N**



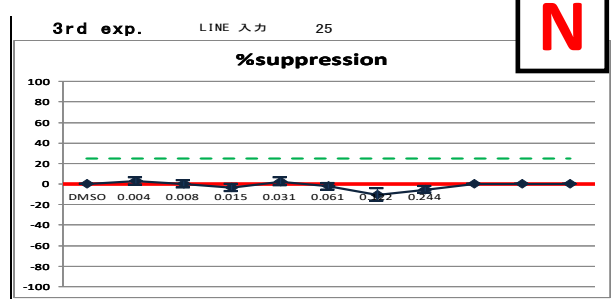
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		

N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.004	4.807	1.779	7.834	Sig.	
0.008	2.540	-0.449	5.529	N.S.	
0.015	1.773	-1.717	5.263	N.S.	
0.031	5.335	2.229	8.441	Sig.	
0.061	5.231	1.487	8.975	Sig.	
0.122	-7.084	-10.575	-3.592	Sig.	
0.244	-14.446	-18.077	-10.815	Sig.	
0.488	-10.112	-13.656	-6.568	Sig.	

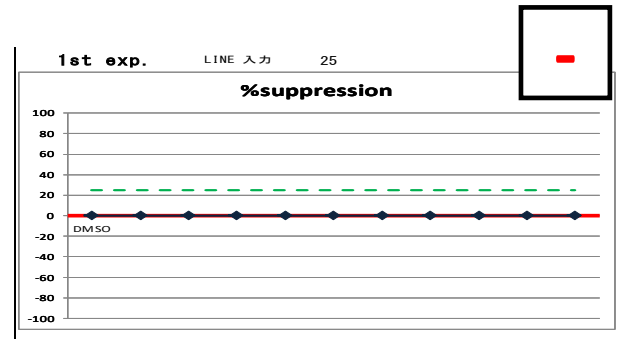
N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.004	3.036	-0.463	6.536	N.S.	
0.008	0.105	-3.291	3.501	N.S.	
0.015	-3.293	-6.676	0.090	N.S.	
0.031	2.537	-1.392	6.466	N.S.	
0.061	-2.106	-5.343	1.130	N.S.	
0.122	-10.291	-16.081	-4.502	Sig.	
0.244	-5.251	-8.504	-1.999	Sig.	

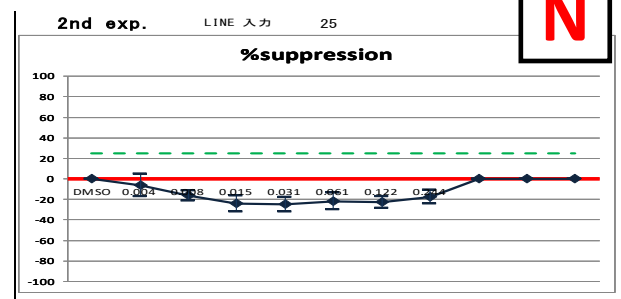
LabC Shikoku MTC312

Judge **N**



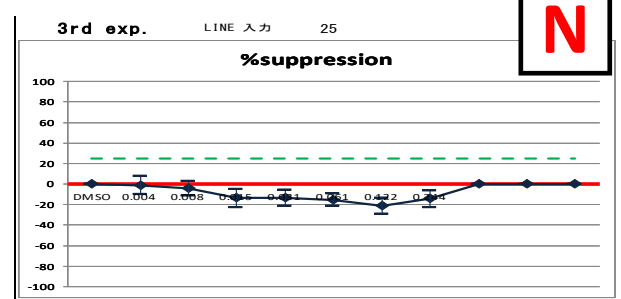
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		

N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.004	-6.016	-16.903	4.870	N.S.	
0.008	-16.422	-21.534	-11.311	Sig.	
0.015	-24.137	-31.870	-16.405	Sig.	
0.031	-24.963	-32.206	-17.721	Sig.	
0.061	-21.832	-29.976	-13.688	Sig.	
0.122	-22.746	-28.648	-16.844	Sig.	
0.244	-17.334	-24.074	-10.595	Sig.	

N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.004	-1.317	-10.192	7.559	N.S.	
0.008	-4.228	-11.116	2.660	N.S.	
0.015	-13.541	-22.379	-4.702	Sig.	
0.031	-13.683	-21.422	-5.943	Sig.	
0.061	-15.215	-21.564	-8.866	Sig.	
0.122	-21.328	-28.931	-13.725	Sig.	
0.244	-14.194	-22.411	-5.977	Sig.	

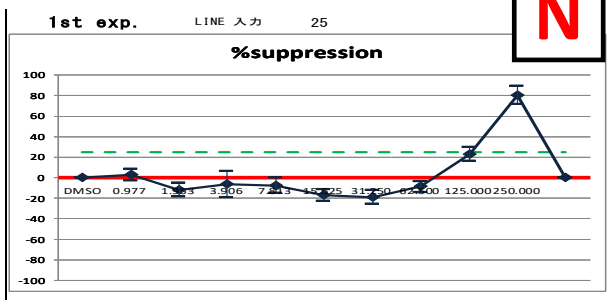
Chem.8

LabA Tohoku MTA125

Judge: **S**

Exp.1

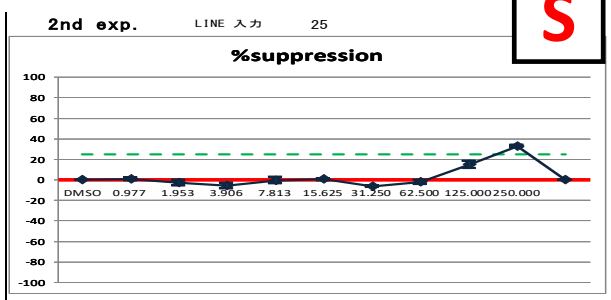
N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	3.007	-2.490	8.504	N.S.	
1.953	-11.909	-18.656	-5.162	Sig.	
3.906	-6.279	-19.131	6.573	N.S.	
7.813	-7.444	-14.983	0.095	N.S.	
15.625	-16.832	-22.495	-11.168	Sig.	
31.250	-18.920	-25.616	-12.224	Sig.	
62.500	-8.756	-13.998	-3.515	Sig.	
125.000	22.907	16.125	29.688	Sig.	
250.000	80.568	71.767	89.370	Sig.	

Exp.2

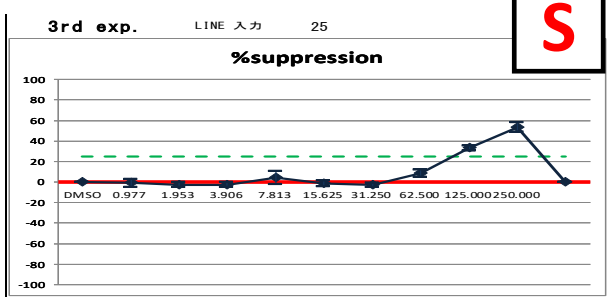
S



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	0.857	-0.546	2.260	N.S.	
1.953	-3.080	-5.921	-0.239	Sig.	
3.906	-5.365	-8.226	-2.503	Sig.	
7.813	-0.327	-3.759	3.106	N.S.	
15.625	0.543	-0.481	1.567	N.S.	
31.250	-6.159	-6.837	-5.481	Sig.	
62.500	-1.974	-4.160	0.213	N.S.	
125.000	14.994	11.780	18.207	Sig.	
250.000	33.063	32.313	33.813	Sig.	

Exp.3

S



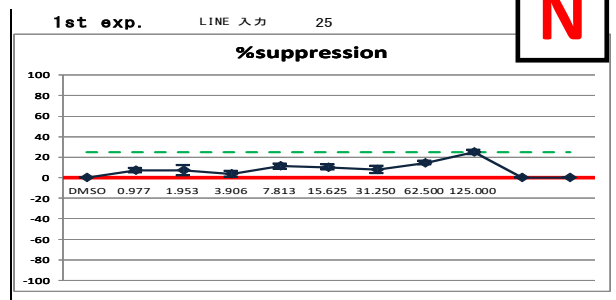
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	-0.947	-5.077	3.182	N.S.	
1.953	-2.436	-5.172	0.299	N.S.	
3.906	-2.428	-5.053	0.198	N.S.	
7.813	4.354	-2.265	10.973	N.S.	
15.625	-1.355	-4.392	1.681	N.S.	
31.250	-2.754	-5.145	-0.363	Sig.	
62.500	8.773	5.200	12.346	Sig.	
125.000	33.195	30.658	35.732	Sig.	
250.000	53.431	48.807	58.055	Sig.	

LabB Tsukuba MTB214

Judge: **S**

Exp.1

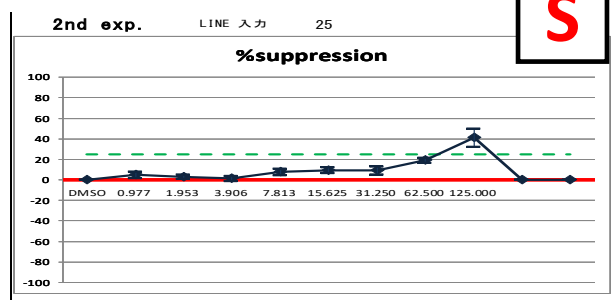
N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	7.164	5.099	9.230	Sig.	
1.953	7.024	2.012	12.037	Sig.	
3.906	3.469	0.590	6.348	Sig.	
7.813	11.279	8.733	13.825	Sig.	
15.625	10.161	7.765	12.556	Sig.	
31.250	7.655	4.094	11.217	Sig.	
62.500	14.505	12.910	16.099	Sig.	
125.000	24.939	22.503	27.374	Sig.	

Exp.2

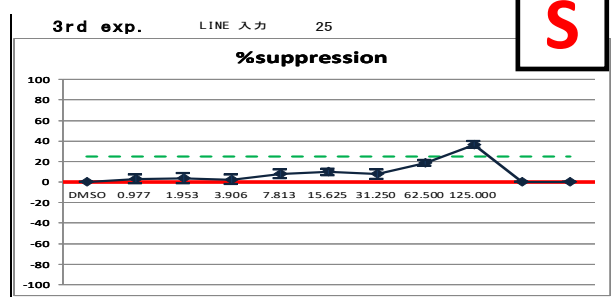
S



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	4.787	1.507	8.068	Sig.	
1.953	2.776	0.521	5.031	Sig.	
3.906	1.170	-1.614	3.954	N.S.	
7.813	7.579	4.134	11.024	Sig.	
15.625	9.277	6.549	12.005	Sig.	
31.250	9.118	5.176	13.059	Sig.	
62.500	18.959	16.712	21.205	Sig.	
125.000	40.944	31.895	49.994	Sig.	

Exp.3

S



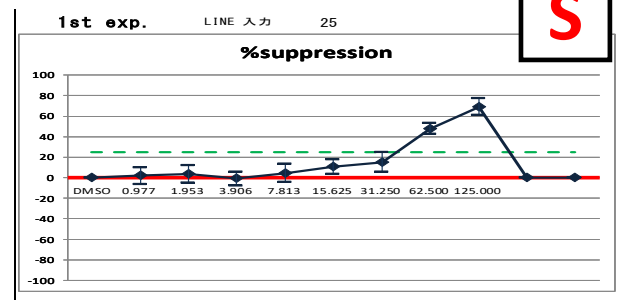
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	3.044	-1.233	7.322	N.S.	
1.953	3.624	-1.266	8.513	N.S.	
3.906	2.387	-2.052	6.827	N.S.	
7.813	7.669	3.420	11.919	Sig.	
15.625	9.809	6.708	12.909	Sig.	
31.250	7.611	3.106	12.116	Sig.	
62.500	18.637	15.738	21.537	Sig.	
125.000	36.396	33.268	39.525	Sig.	

LabC Shikoku MTC303

Judge: **S**

Exp.1

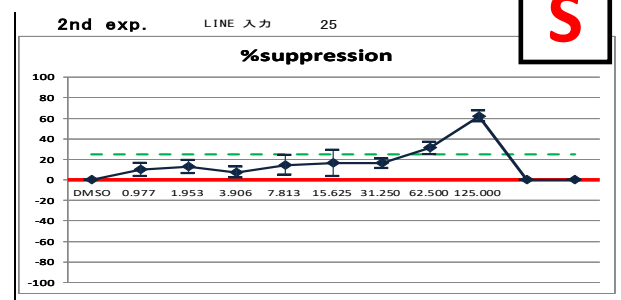
S



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	1.907	-6.366	10.179	N.S.	
1.953	3.457	-5.149	12.064	N.S.	
3.906	-0.963	-7.931	6.006	N.S.	
7.813	4.633	-4.007	13.273	N.S.	
15.625	10.632	3.639	17.625	Sig.	
31.250	15.281	5.920	24.641	Sig.	
62.500	47.982	42.779	53.184	Sig.	
125.000	69.288	61.125	77.451	Sig.	

Exp.2

S



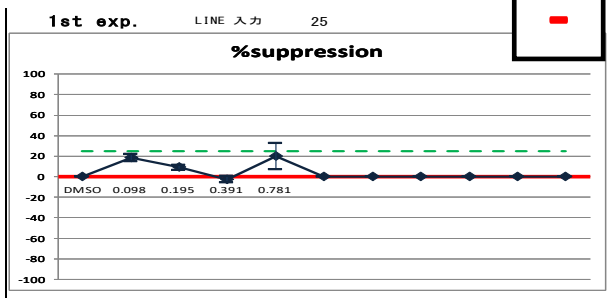
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	9.944	3.727	16.161	Sig.	
1.953	12.775	6.493	19.058	Sig.	
3.906	7.197	1.881	12.513	Sig.	
7.813	14.599	4.763	24.435	Sig.	
15.625	16.213	3.533	28.893	Sig.	
31.250	16.206	11.572	20.840	Sig.	
62.500	30.984	24.636	37.331	Sig.	
125.000	62.103	56.999	67.207	Sig.	

Chem.11

LabA Tohoku MTA110

Judge **N**

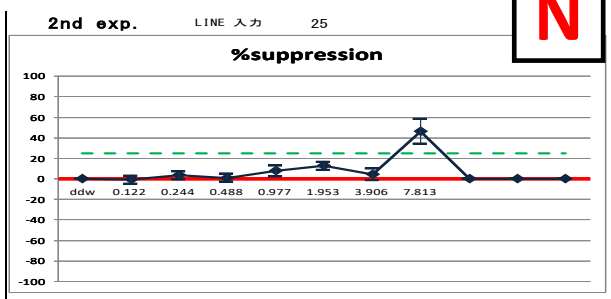
Exp.1



Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.098	18.468	15.184	21.752	Sig.	
0.195	9.006	6.349	11.663	Sig.	
0.391	-2.581	-5.809	0.646	N.S.	
0.781	19.744	6.901	32.587	Sig.	

Exp.2

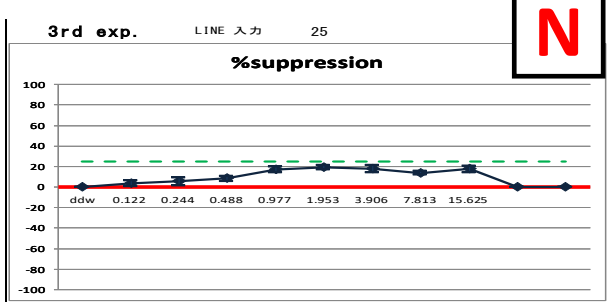
N



Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
0.122	-0.867	-4.931	3.198	N.S.	
0.244	3.447	-0.543	7.437	N.S.	
0.488	1.103	-2.614	4.820	N.S.	
0.977	7.578	2.487	12.669	Sig.	
1.953	12.699	8.834	16.564	Sig.	
3.906	4.150	-1.531	9.831	N.S.	
7.813	46.141	34.147	58.136	Sig.	

Exp.3

N

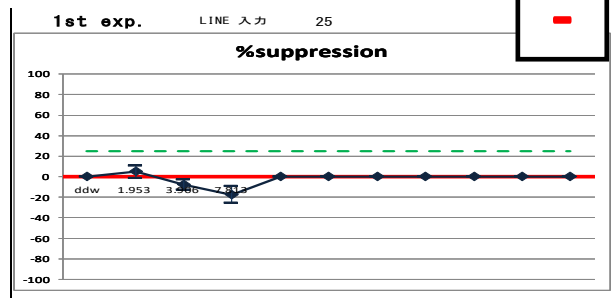


Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
0.122	3.518	0.564	6.473	Sig.	
0.244	5.588	1.594	9.583	Sig.	
0.488	8.347	5.682	11.013	Sig.	
0.977	16.967	14.236	19.698	Sig.	
1.953	19.285	16.959	21.612	Sig.	
3.906	17.996	14.593	21.398	Sig.	
7.813	13.764	11.954	15.574	Sig.	
15.625	17.590	14.589	20.591	Sig.	

LabB Tsukuba MTB218

Judge **N**

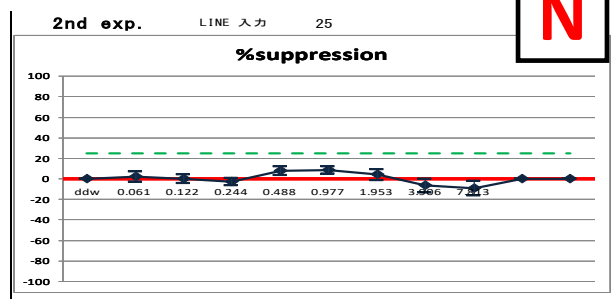
Exp.1



Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
1.953	4.769	-1.007	10.546	N.S.	
3.906	-7.826	-12.625	-3.028	Sig.	
7.813	-17.371	-25.475	-9.268	Sig.	

Exp.2

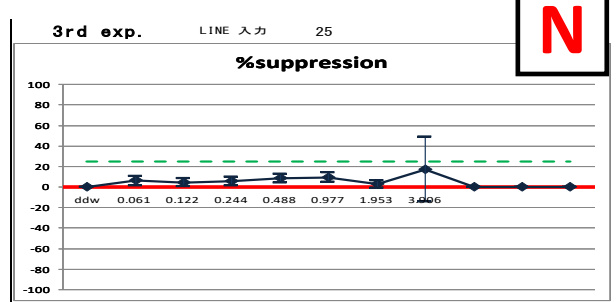
N



Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
0.061	2.054	-2.743	6.851	N.S.	
0.122	0.031	-4.402	4.465	N.S.	
0.244	-2.618	-6.239	1.002	N.S.	
0.488	7.993	3.487	12.498	Sig.	
0.977	8.600	4.881	12.320	Sig.	
1.953	4.177	-1.003	9.357	N.S.	
3.906	-6.581	-13.573	0.411	N.S.	
7.813	-9.050	-16.026	-2.074	Sig.	

Exp.3

N

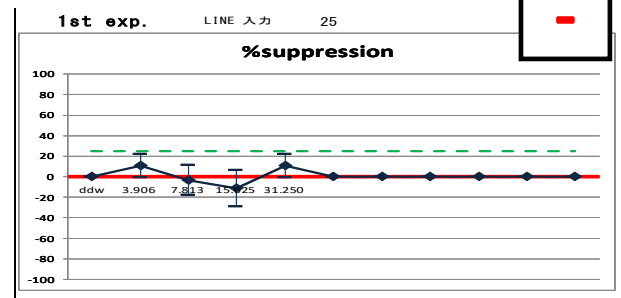


Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
0.061	6.134	1.383	10.885	Sig.	
0.122	4.597	0.703	8.492	Sig.	
0.244	5.617	1.214	10.020	Sig.	
0.488	8.511	4.040	12.982	Sig.	
0.977	9.635	4.900	14.371	Sig.	
1.953	3.085	-0.608	6.777	N.S.	
3.906	17.348	-14.053	48.750	N.S.	

LabC Shikoku MTC322

Judge **N**

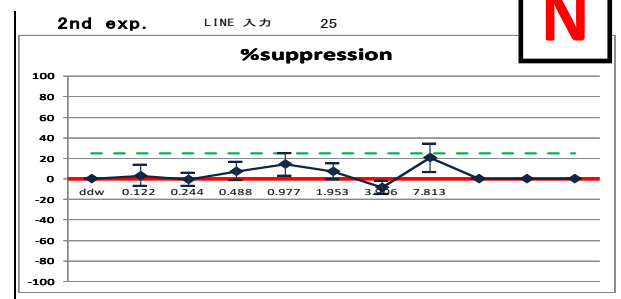
Exp.1



Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	10.722	-0.336	21.781	N.S.	
7.813	-3.286	-17.839	11.267	N.S.	
15.625	-11.436	-29.121	6.249	N.S.	
31.250	10.595	-0.610	21.800	N.S.	

Exp.2

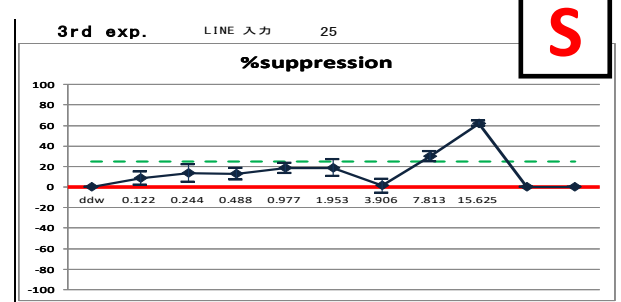
N



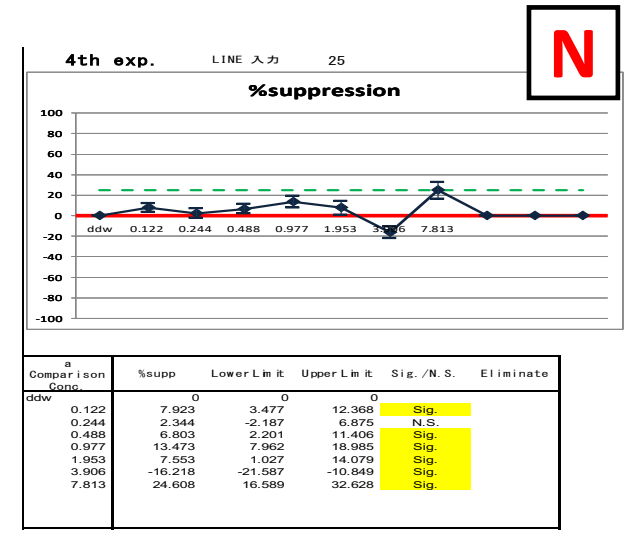
Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
0.122	3.132	-7.052	13.316	N.S.	
0.244	-0.642	-7.331	6.046	N.S.	
0.488	7.292	-1.656	16.240	N.S.	
0.977	14.051	3.182	24.919	Sig.	
1.953	7.075	-0.524	14.673	N.S.	
3.906	-8.598	-15.154	-2.042	Sig.	
7.813	20.562	6.824	34.299	Sig.	

Exp.3

S



Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
0.122	8.593	2.150	15.035	Sig.	
0.244	13.570	5.066	22.074	Sig.	
0.488	13.106	7.321	18.891	Sig.	
0.977	18.501	13.590	23.412	Sig.	
1.953	18.779	10.380	27.177	Sig.	
3.906	1.392	-5.263	8.046	N.S.	
7.813	30.042	24.883	35.202	Sig.	
15.625	61.941	59.523	64.359	Sig.	

Chem.11	LabA Tohoku MTA110	LabB Tsukuba MTB218	LabC Shikoku MTC322
Judge			N
Exp.4			
Exp.5			
Exp.6			

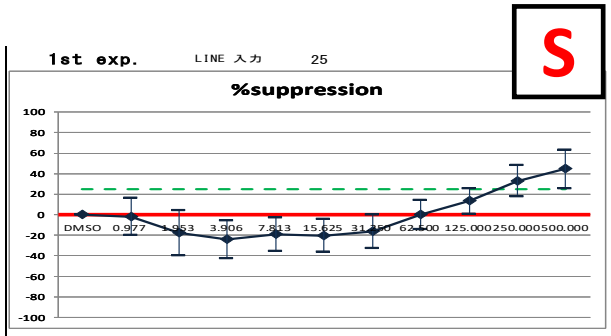
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
0.122	7.923	3.477	12.368	Sig.	
0.244	2.344	-2.187	6.875	N.S.	
0.488	6.803	2.201	11.406	Sig.	
0.977	13.473	7.962	18.985	Sig.	
1.953	7.553	1.027	14.079	Sig.	
3.906	-16.218	-21.587	-10.849	Sig.	
7.813	24.608	16.589	32.628	Sig.	

Chem.12

LabA Tohoku MTA124

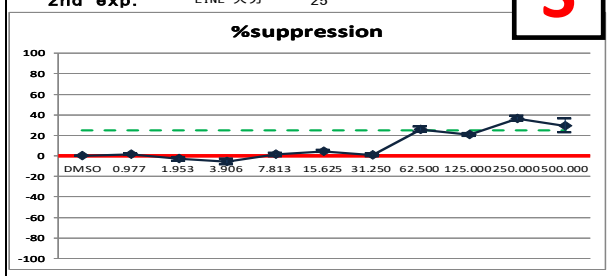
Judge **S**

Exp.1 **S**



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	-1.846	-20.122	16.430	N.S.	
1.953	-17.876	-39.780	4.028	N.S.	
3.906	-24.110	-42.690	-5.530	Sig.	
7.813	-18.905	-35.112	-2.698	Sig.	
15.625	-20.378	-36.242	-4.515	Sig.	
31.250	-16.145	-32.327	0.038	N.S.	
62.500	0.038	-13.968	14.045	N.S.	
125.000	13.241	0.894	25.588	Sig.	
250.000	33.087	17.919	48.255	Sig.	
500.000	44.505	25.840	63.170	Sig.	

Exp.2 **S**

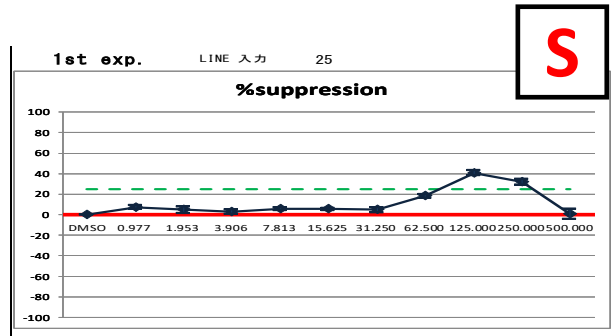


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	1.417	0.478	2.357	Sig.	
1.953	-3.102	-4.644	-1.559	Sig.	
3.906	-5.811	-8.759	-2.864	Sig.	
7.813	1.350	-0.377	3.077	N.S.	
15.625	4.688	3.570	5.806	Sig.	
31.250	0.913	-0.412	2.238	N.S.	
62.500	25.956	23.456	28.457	Sig.	
125.000	20.750	19.252	22.248	Sig.	
250.000	36.495	34.019	38.971	Sig.	
500.000	29.498	22.650	36.345	Sig.	

Exp.3

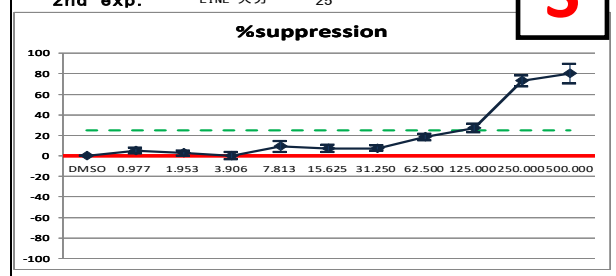
LabB Tsukuba MTB217

S



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	7.336	5.411	9.260	Sig.	
1.953	4.802	1.835	7.769	Sig.	
3.906	2.798	0.469	5.127	Sig.	
7.813	6.013	4.534	7.492	Sig.	
15.625	5.506	4.285	6.727	Sig.	
31.250	4.792	2.382	7.202	Sig.	
62.500	18.391	16.506	20.276	Sig.	
125.000	40.774	38.135	43.413	Sig.	
250.000	31.988	29.125	34.851	Sig.	
500.000	0.976	-4.098	6.049	N.S.	

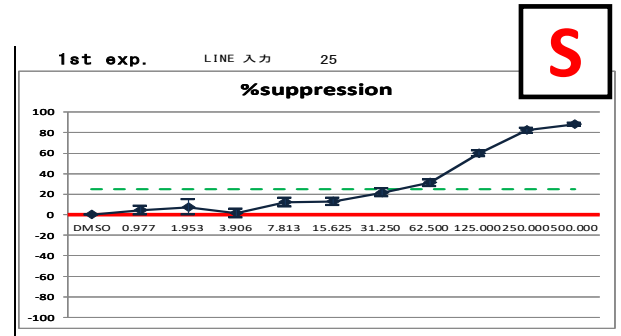
S



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	5.096	2.547	7.645	Sig.	
1.953	2.662	-0.016	5.340	N.S.	
3.906	0.273	-3.374	3.920	N.S.	
7.813	8.974	3.865	14.083	Sig.	
15.625	7.017	3.643	10.392	Sig.	
31.250	7.408	4.813	10.004	Sig.	
62.500	18.228	15.251	21.205	Sig.	
125.000	27.006	22.768	31.245	Sig.	
250.000	73.060	67.695	78.425	Sig.	
500.000	80.152	70.545	89.759	Sig.	

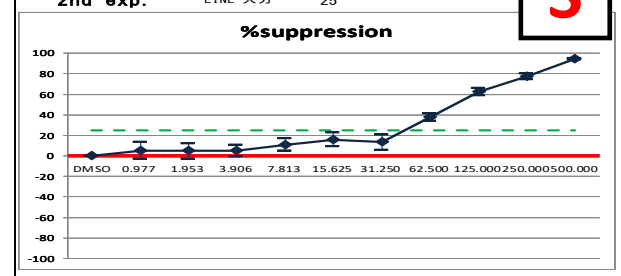
LabC Shikoku MTC313

S



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	4.301	0.336	8.267	Sig.	
1.953	7.409	-0.051	14.869	N.S.	
3.906	1.493	-2.817	5.802	N.S.	
7.813	11.872	7.658	16.086	Sig.	
15.625	12.606	9.033	16.178	Sig.	
31.250	21.623	17.819	25.426	Sig.	
62.500	31.131	27.853	34.408	Sig.	
125.000	59.754	57.231	62.277	Sig.	
250.000	82.136	79.938	84.333	Sig.	
500.000	88.313	87.049	89.576	Sig.	

S



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	5.391	-2.975	13.756	N.S.	
1.953	4.836	-2.742	12.415	N.S.	
3.906	4.978	-0.702	10.658	N.S.	
7.813	11.033	4.863	17.203	Sig.	
15.625	15.906	8.983	22.829	Sig.	
31.250	13.230	5.705	20.755	Sig.	
62.500	37.488	34.000	40.977	Sig.	
125.000	62.672	58.986	66.359	Sig.	
250.000	77.303	74.588	80.017	Sig.	
500.000	94.457	93.535	95.380	Sig.	

Chem.13

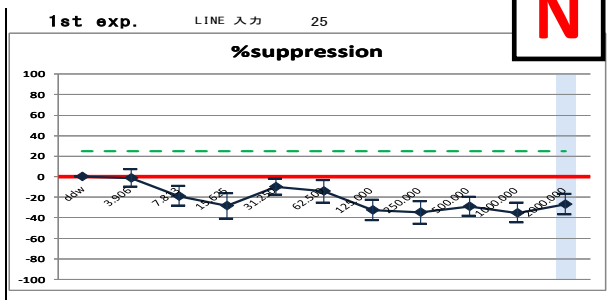
LabA Tohoku MTA102

LabB Tsukuba MTB206

LabC Shikoku MTC317

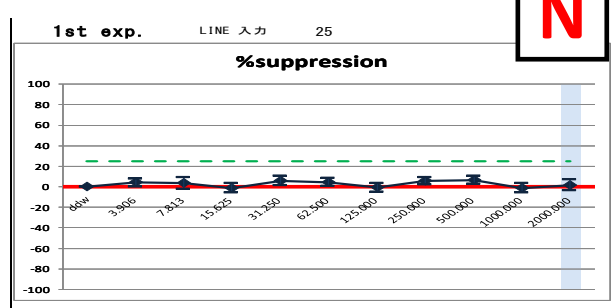
Judge **N** **N** **N**

Exp.1



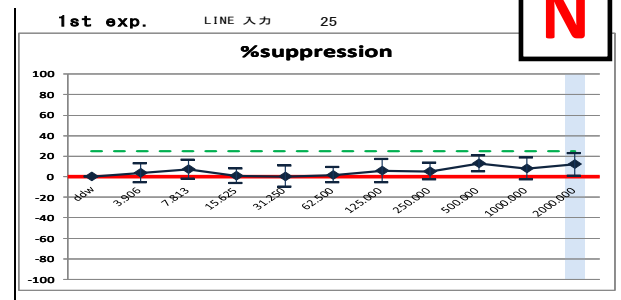
N

a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	-1.272	-9.519	6.975	N.S.	
7.813	-18.746	-28.567	-8.926	Sig.	
15.625	-28.657	-40.920	-16.394	Sig.	
31.250	-9.673	-17.524	-1.823	Sig.	
62.500	-14.402	-25.143	-3.660	Sig.	
125.000	-32.487	-42.608	-22.366	Sig.	
250.000	-34.885	-45.784	-23.986	Sig.	
500.000	-29.028	-38.352	-19.703	Sig.	
1000.000	-35.095	-44.983	-25.207	Sig.	
2000.000	-26.894	-36.861	-16.927	Sig.	elimina



N

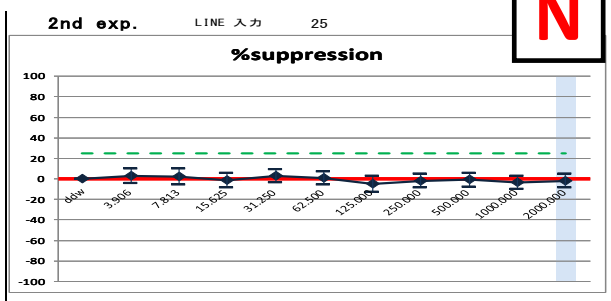
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	4.010	0.361	7.659	Sig.	
7.813	3.513	-2.037	9.063	N.S.	
15.625	-1.250	-5.788	3.288	N.S.	
31.250	6.021	1.462	10.581	Sig.	
62.500	4.620	0.810	8.431	Sig.	
125.000	-0.804	-5.229	3.621	N.S.	
250.000	5.456	1.943	8.969	Sig.	
500.000	6.743	2.948	10.537	Sig.	
1000.000	-1.002	-5.509	3.505	N.S.	
2000.000	1.646	-3.730	7.022	N.S.	elimina



N

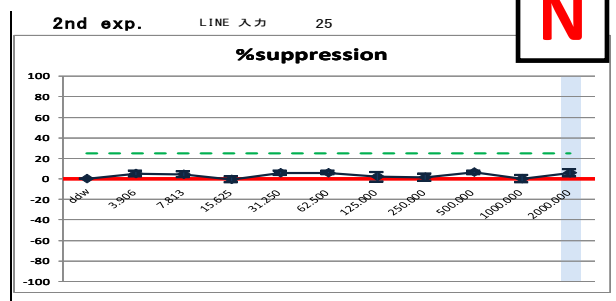
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	3.474	-5.681	12.630	N.S.	
7.813	7.064	-2.350	16.478	N.S.	
15.625	0.823	-6.126	7.771	N.S.	
31.250	0.308	-10.032	10.647	N.S.	
62.500	1.855	-5.760	9.469	N.S.	
125.000	6.030	-5.278	17.337	N.S.	
250.000	5.317	-2.776	13.411	N.S.	
500.000	12.853	4.787	20.918	Sig.	
1000.000	7.995	-2.422	18.413	N.S.	
2000.000	12.004	1.120	22.889	Sig.	elimina

Exp.2



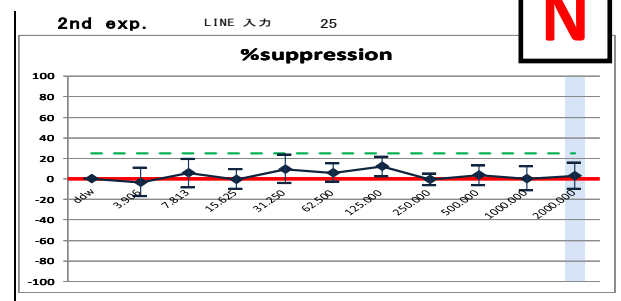
N

a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	2.744	-4.502	9.991	N.S.	
7.813	2.330	-5.458	10.118	N.S.	
15.625	-1.322	-8.469	5.824	N.S.	
31.250	2.952	-3.234	9.138	N.S.	
62.500	0.754	-5.726	7.234	N.S.	
125.000	-4.650	-12.396	3.096	N.S.	
250.000	-1.861	-8.428	4.706	N.S.	
500.000	-0.972	-7.430	5.486	N.S.	
1000.000	-3.328	-9.793	3.137	N.S.	
2000.000	-1.902	-8.665	4.861	N.S.	eliminate



N

a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	5.062	2.391	7.734	Sig.	
7.813	4.584	1.828	7.341	Sig.	
15.625	-0.599	-3.229	2.032	N.S.	
31.250	5.765	3.498	8.032	Sig.	
62.500	5.913	3.995	7.831	Sig.	
125.000	1.894	-2.545	6.333	N.S.	
250.000	1.686	-1.789	5.161	N.S.	
500.000	6.246	4.435	8.057	Sig.	
1000.000	0.002	-3.353	3.357	N.S.	
2000.000	5.581	2.048	9.114	Sig.	eliminate



N

a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	-3.272	-17.003	10.460	N.S.	
7.813	5.706	-8.193	19.606	N.S.	
15.625	-0.332	-10.048	9.384	N.S.	
31.250	9.480	-4.394	23.353	N.S.	
62.500	5.872	-2.964	14.708	N.S.	
125.000	11.928	2.555	21.300	Sig.	
250.000	-0.717	-6.659	5.226	N.S.	
500.000	3.380	-6.128	12.888	N.S.	
1000.000	0.314	-11.633	12.262	N.S.	
2000.000	3.011	-9.551	15.573	N.S.	eliminate

Exp.3

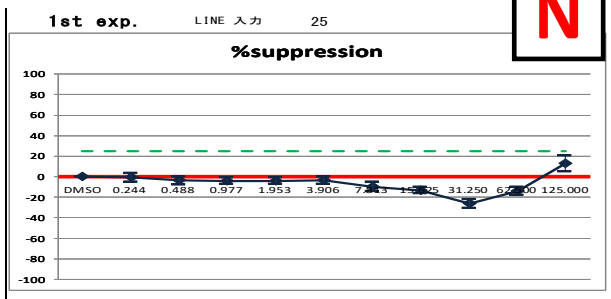
Chem.14

LabA Tohoku MTA121

Judge **N**

Exp.1

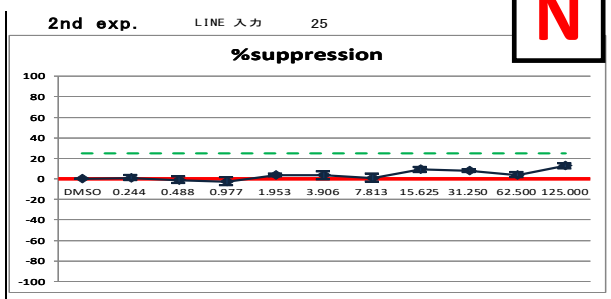
N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.244	-0.758	-4.971	3.455	N.S.	
0.488	-3.716	-7.589	0.158	N.S.	
0.977	-4.023	-7.309	-0.737	Sig.	
1.953	-4.033	-7.102	-0.964	Sig.	
3.906	-3.318	-6.922	0.286	N.S.	
7.813	-9.643	-14.425	-4.862	Sig.	
15.625	-13.165	-16.377	-9.953	Sig.	
31.250	-25.912	-30.164	-21.660	Sig.	
62.500	-13.856	-17.537	-10.176	Sig.	
125.000	13.030	5.260	20.800	Sig.	

Exp.2

N



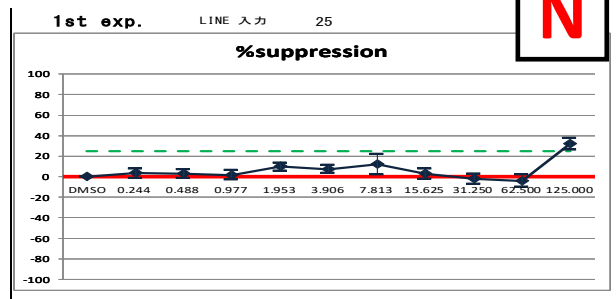
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.244	1.070	-1.426	3.566	N.S.	
0.488	-1.068	-4.079	1.942	N.S.	
0.977	-2.621	-6.403	1.160	N.S.	
1.953	3.623	1.993	5.252	Sig.	
3.906	3.435	-0.284	7.154	N.S.	
7.813	1.003	-2.815	4.820	N.S.	
15.625	8.965	6.378	11.553	Sig.	
31.250	7.898	6.600	9.197	Sig.	
62.500	3.891	1.645	6.137	Sig.	
125.000	12.560	9.878	15.242	Sig.	

LabB Tsukuba MTB205

Judge **N**

Exp.1

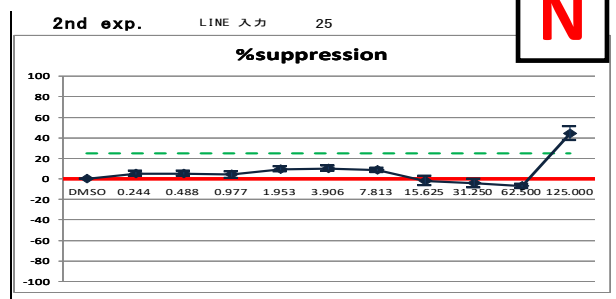
N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.244	3.429	-1.078	7.936	N.S.	
0.488	3.014	-1.457	7.485	N.S.	
0.977	1.602	-3.047	6.251	N.S.	
1.953	9.704	5.564	13.844	Sig.	
3.906	7.519	3.781	11.257	Sig.	
7.813	11.902	1.988	21.816	Sig.	
15.625	2.842	-2.152	7.837	N.S.	
31.250	-2.084	-7.092	2.924	N.S.	
62.500	-3.970	-10.198	2.257	N.S.	
125.000	31.978	26.377	37.580	Sig.	

Exp.2

N



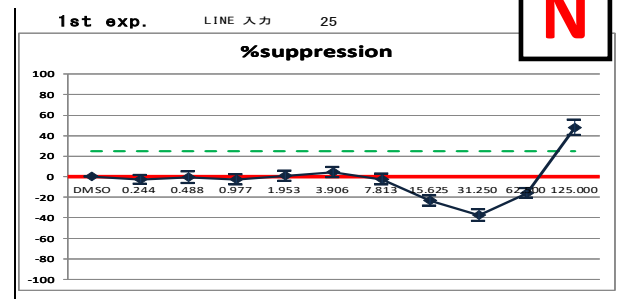
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.244	5.362	3.005	7.718	Sig.	
0.488	5.271	2.700	7.843	Sig.	
0.977	4.000	1.110	6.890	Sig.	
1.953	9.555	6.835	12.274	Sig.	
3.906	10.361	7.780	12.941	Sig.	
7.813	8.400	6.241	10.558	Sig.	
15.625	-1.874	-6.393	2.645	N.S.	
31.250	-4.255	-8.626	0.116	N.S.	
62.500	-6.973	-9.327	-4.619	Sig.	
125.000	44.336	37.651	51.020	Sig.	

LabC Shikoku MTC324

Judge **N**

Exp.1

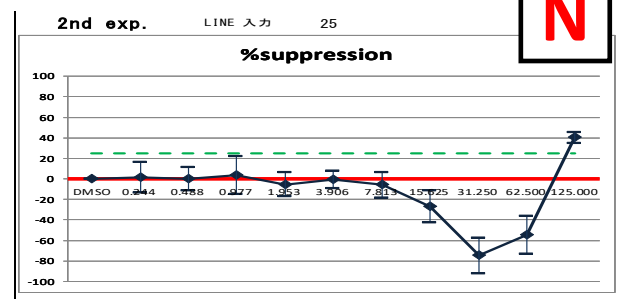
N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.244	-2.952	-7.052	1.148	N.S.	
0.488	-0.554	-6.249	5.140	N.S.	
0.977	-2.800	-7.757	2.157	N.S.	
1.953	0.736	-4.089	5.561	N.S.	
3.906	4.065	-0.832	8.961	N.S.	
7.813	-2.555	-7.845	2.736	N.S.	
15.625	-23.529	-28.564	-18.494	Sig.	
31.250	-37.397	-43.256	-31.538	Sig.	
62.500	-16.075	-20.533	-11.618	Sig.	
125.000	47.890	40.470	55.311	Sig.	

Exp.2

N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.244	1.436	-13.716	16.587	N.S.	
0.488	0.096	-11.287	11.480	N.S.	
0.977	3.800	-14.788	22.389	N.S.	
1.953	-5.298	-17.146	6.550	N.S.	
3.906	-0.755	-9.103	7.593	N.S.	
7.813	-5.842	-18.359	6.675	N.S.	
15.625	-26.705	-42.352	-11.058	Sig.	
31.250	-74.806	-92.341	-57.272	Sig.	
62.500	-54.891	-73.359	-36.423	Sig.	
125.000	40.314	35.196	45.431	Sig.	

Exp.3

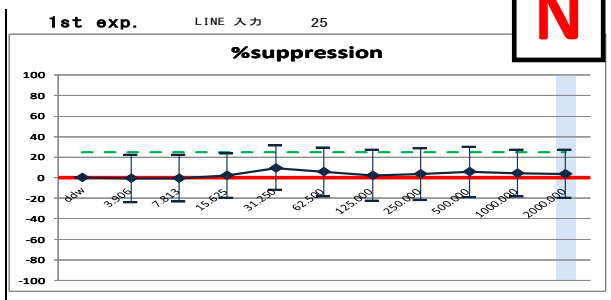
Chem.15

LabA Tohoku MTA116

Judge **N**

Exp.1

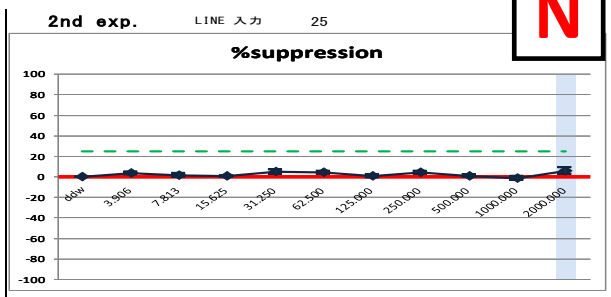
N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	-0.787	-23.873	22.299	N.S.	
7.813	-0.785	-23.349	21.779	N.S.	
15.625	1.970	-19.483	23.422	N.S.	
31.250	9.408	-12.284	31.100	N.S.	
62.500	5.575	-18.306	29.456	N.S.	
125.000	2.523	-22.347	27.392	N.S.	
250.000	3.362	-21.768	28.492	N.S.	
500.000	5.466	-18.759	29.691	N.S.	
1000.000	4.404	-18.326	27.134	N.S.	
2000.000	3.615	-20.117	27.346	N.S.	elimina

Exp.2

N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	3.500	2.239	4.760	Sig.	
7.813	1.752	-0.056	3.560	N.S.	
15.625	0.721	-0.076	1.517	N.S.	
31.250	4.800	2.725	6.875	Sig.	
62.500	4.280	2.986	5.574	Sig.	
125.000	0.718	-0.704	2.140	N.S.	
250.000	4.342	2.749	5.935	Sig.	
500.000	0.941	-0.133	2.015	N.S.	
1000.000	-1.104	-2.968	0.760	N.S.	
2000.000	5.814	2.653	8.975	Sig.	eliminate

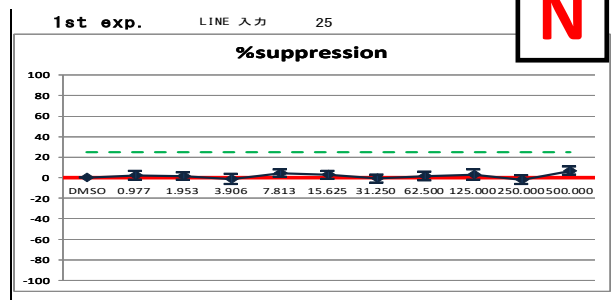
Exp.3

LabB Tsukuba MTB223

Judge **N**

Exp.1

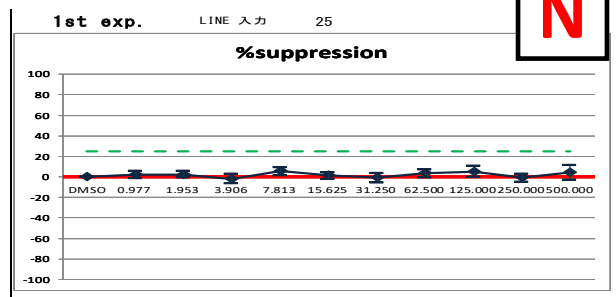
N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	2.128	-2.119	6.376	N.S.	
1.953	1.709	-1.939	5.356	N.S.	
3.906	-1.097	-6.020	3.825	N.S.	
7.813	4.355	0.593	8.117	Sig.	
15.625	2.744	-1.331	6.819	N.S.	
31.250	-0.898	-4.596	2.799	N.S.	
62.500	1.524	-2.410	5.457	N.S.	
125.000	2.959	-1.896	7.814	N.S.	
250.000	-1.906	-6.138	2.327	N.S.	
500.000	6.811	3.245	10.378	Sig.	

Exp.2

N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	2.375	-1.195	5.945	N.S.	
1.953	2.479	-0.752	5.709	N.S.	
3.906	-1.739	-6.559	3.081	N.S.	
7.813	5.515	1.700	9.329	Sig.	
15.625	1.324	-1.875	4.524	N.S.	
31.250	-0.975	-5.299	3.349	N.S.	
62.500	3.300	-0.456	7.057	N.S.	
125.000	5.329	-0.146	10.803	N.S.	
250.000	-0.964	-4.783	2.855	N.S.	
500.000	4.518	-2.515	11.550	N.S.	

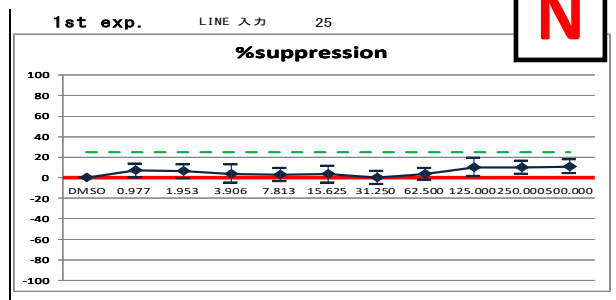
Exp.3

LabC Shikoku MTC309

Judge **N**

Exp.1

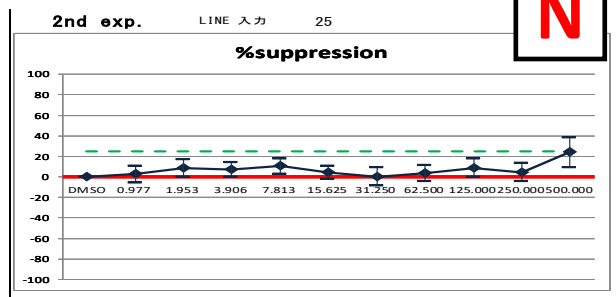
N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	6.989	0.073	13.906	Sig.	
1.953	6.322	-0.368	13.013	N.S.	
3.906	3.752	-5.069	12.573	N.S.	
7.813	2.990	-3.564	9.544	N.S.	
15.625	3.435	-4.759	11.629	N.S.	
31.250	0.165	-6.496	6.825	N.S.	
62.500	3.460	-2.227	9.146	N.S.	
125.000	10.114	1.280	18.948	Sig.	
250.000	10.148	3.647	16.649	Sig.	
500.000	10.796	4.076	17.517	Sig.	

Exp.2

N

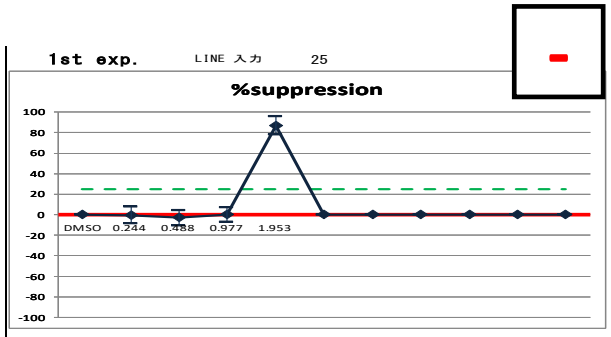


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	2.845	-5.344	11.035	N.S.	
1.953	8.418	-0.064	16.900	N.S.	
3.906	7.078	0.219	13.936	Sig.	
7.813	10.430	3.012	17.848	Sig.	
15.625	4.406	-2.164	10.977	N.S.	
31.250	0.211	-8.535	8.957	N.S.	
62.500	3.837	-4.025	11.698	N.S.	
125.000	8.848	0.190	17.506	Sig.	
250.000	4.515	-4.407	13.437	N.S.	
500.000	23.941	9.465	38.416	Sig.	

Chem.16

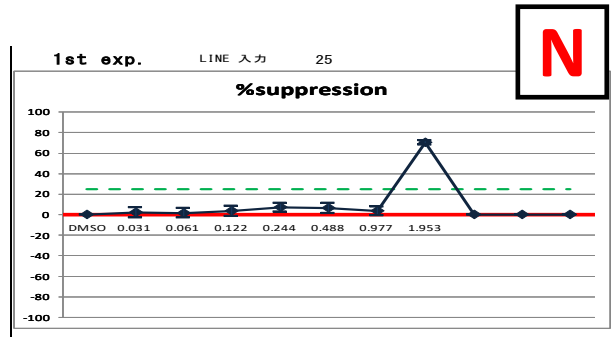
	LabA Tohoku MTA118	LabB Tsukuba MTB202	LabC Shikoku MTC316
Judge	N	S	N

Exp.1



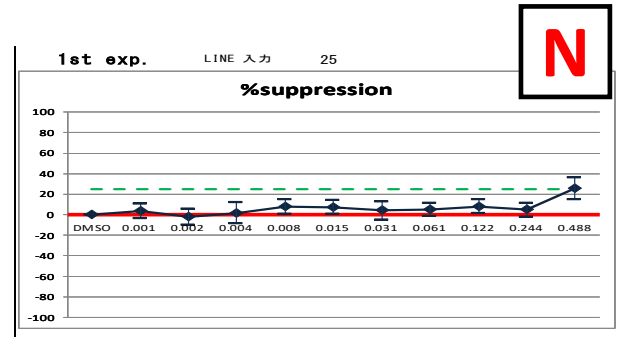
-

Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.244	-0.404	-8.578	7.770	N.S.	
0.488	-3.053	-10.247	4.141	N.S.	
0.977	0.004	-7.268	7.276	N.S.	
1.953	86.977	77.872	96.081	Sig.	



N

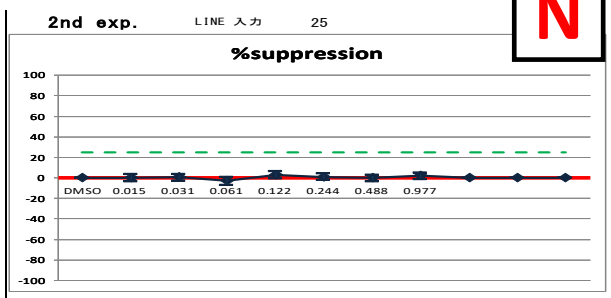
Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.031	2.334	-2.797	7.464	N.S.	
0.061	1.814	-2.858	6.486	N.S.	
0.122	3.647	-1.011	8.306	N.S.	
0.244	7.173	2.901	11.444	Sig.	
0.488	6.499	1.779	11.219	Sig.	
0.977	3.494	-0.674	7.662	N.S.	
1.953	70.239	68.096	72.383	Sig.	



N

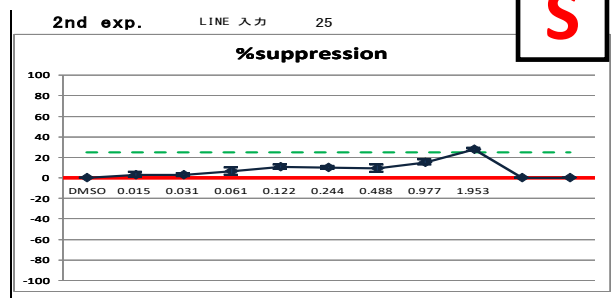
Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.001	3.740	-3.502	10.982	N.S.	
0.002	-2.025	-10.004	5.955	N.S.	
0.004	1.789	-8.495	12.073	N.S.	
0.008	7.735	0.580	14.889	Sig.	
0.015	7.280	0.479	14.082	Sig.	
0.031	4.026	-4.983	13.035	N.S.	
0.061	5.034	-1.604	11.673	N.S.	
0.122	7.999	1.342	14.655	Sig.	
0.244	4.797	-1.962	11.555	N.S.	
0.488	25.762	14.982	36.542	Sig.	

Exp.2



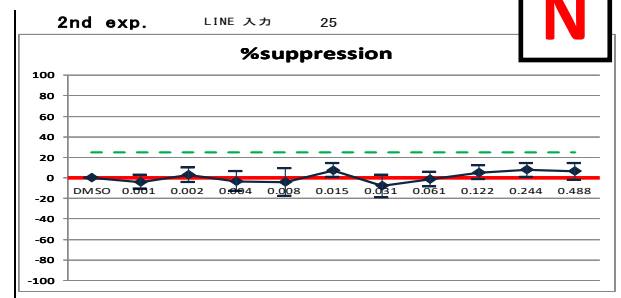
N

Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.015	0.152	-3.318	3.622	N.S.	
0.031	0.632	-2.551	3.816	N.S.	
0.061	-3.092	-6.900	0.715	N.S.	
0.122	2.732	-0.843	6.307	N.S.	
0.244	1.021	-2.115	4.156	N.S.	
0.488	-0.198	-3.252	2.856	N.S.	
0.977	1.958	-1.115	5.031	N.S.	



S

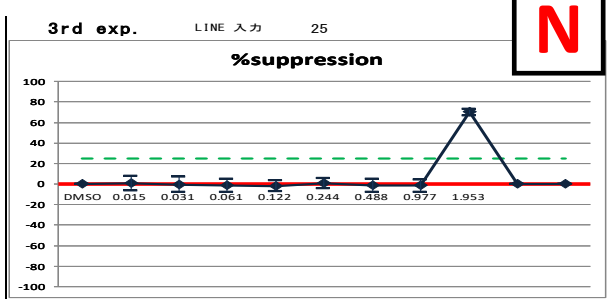
Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.015	3.175	0.851	5.499	Sig.	
0.031	3.233	1.771	4.695	Sig.	
0.061	6.563	2.991	10.136	Sig.	
0.122	10.704	8.638	12.770	Sig.	
0.244	10.079	8.543	11.616	Sig.	
0.488	9.369	5.914	12.824	Sig.	
0.977	15.209	12.529	17.889	Sig.	
1.953	27.478	26.029	28.926	Sig.	



N

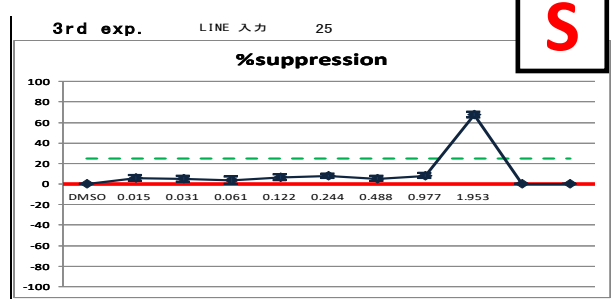
Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.001	-3.873	-10.470	2.724	N.S.	
0.002	3.171	-4.000	10.341	N.S.	
0.004	-3.185	-13.042	6.671	N.S.	
0.008	-4.396	-17.994	9.202	N.S.	
0.015	7.350	0.645	14.054	Sig.	
0.031	-7.977	-19.101	3.146	N.S.	
0.061	-1.330	-8.591	5.931	N.S.	
0.122	5.302	-1.499	12.102	N.S.	
0.244	7.638	1.055	14.222	Sig.	
0.488	6.324	-1.731	14.380	N.S.	

Exp.3



N

Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.015	0.884	-6.155	7.922	N.S.	
0.031	-0.327	-7.982	7.329	N.S.	
0.061	-1.245	-7.570	5.079	N.S.	
0.122	-1.764	-7.056	3.528	N.S.	
0.244	0.594	-4.522	5.711	N.S.	
0.488	-1.140	-7.432	5.151	N.S.	
0.977	-1.526	-7.652	4.599	N.S.	
1.953	70.031	66.939	73.123	Sig.	



S

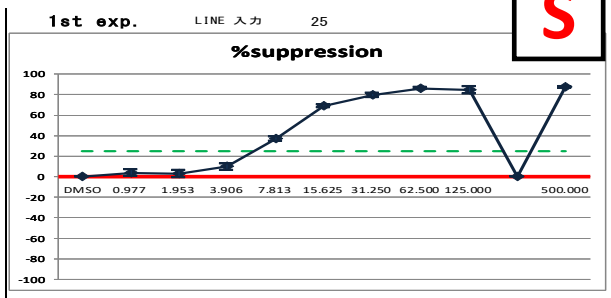
Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.015	5.648	2.873	8.422	Sig.	
0.031	4.895	1.981	7.810	Sig.	
0.061	3.665	0.405	6.924	Sig.	
0.122	6.648	3.839	9.457	Sig.	
0.244	7.885	5.455	10.316	Sig.	
0.488	5.190	2.825	7.554	Sig.	
0.977	8.156	5.802	10.511	Sig.	
1.953	67.597	64.817	70.377	Sig.	

Chem.17

LabA Tohoku MTA108

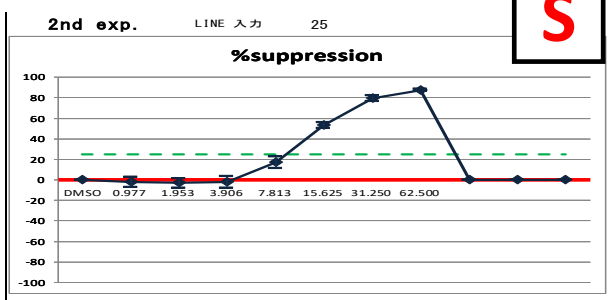
Judge **S**

Exp.1 **S**



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	3.888	0.758	7.018	Sig.	
1.953	2.938	-0.754	6.631	N.S.	
3.906	9.760	6.710	12.810	Sig.	
7.813	37.041	35.170	38.912	Sig.	
15.625	68.865	67.228	70.502	Sig.	
31.250	79.561	77.176	81.946	Sig.	
62.500	86.271	85.232	87.310	Sig.	
125.000	84.442	80.699	88.184	Sig.	
500.000	87.306	86.482	88.131	Sig.	

Exp.2 **S**



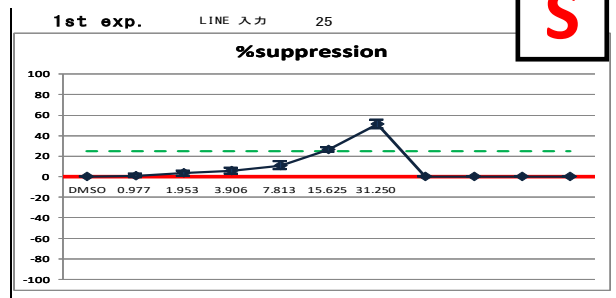
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	-2.132	-6.955	2.692	N.S.	
1.953	-2.857	-7.510	1.796	N.S.	
3.906	-1.943	-7.655	3.769	N.S.	
7.813	16.842	11.156	22.527	Sig.	
15.625	53.296	50.683	55.909	Sig.	
31.250	79.697	76.970	82.423	Sig.	
62.500	87.579	86.548	88.611	Sig.	

Exp.3

LabB Tsukuba MTB204

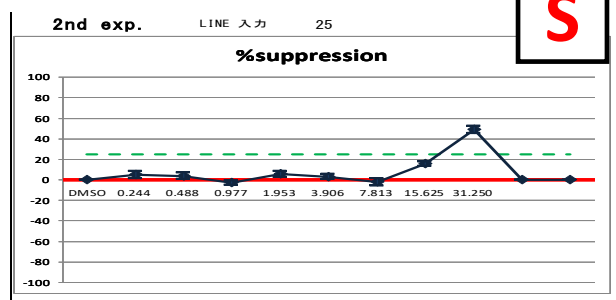
S

S



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	1.005	-0.920	2.929	N.S.	
1.953	3.429	0.834	6.024	Sig.	
3.906	5.693	2.591	8.795	Sig.	
7.813	10.995	6.942	15.047	Sig.	
15.625	26.250	24.081	28.418	Sig.	
31.250	51.250	47.211	55.289	Sig.	

S

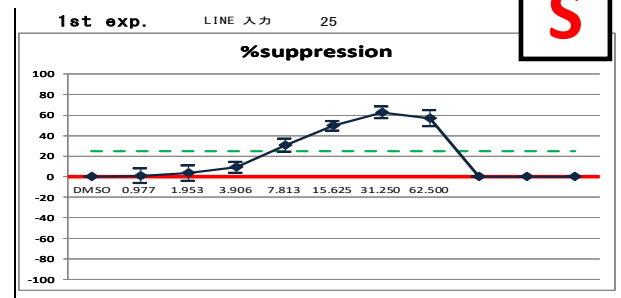


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.244	4.884	1.374	8.394	Sig.	
0.488	3.985	0.732	7.238	Sig.	
0.977	-2.874	-5.225	-0.522	Sig.	
1.953	5.844	3.163	8.525	Sig.	
3.906	3.101	0.523	5.679	Sig.	
7.813	-2.189	-5.664	1.286	N.S.	
15.625	15.795	13.418	18.173	Sig.	
31.250	49.182	45.821	52.542	Sig.	

LabC Shikoku MTC315

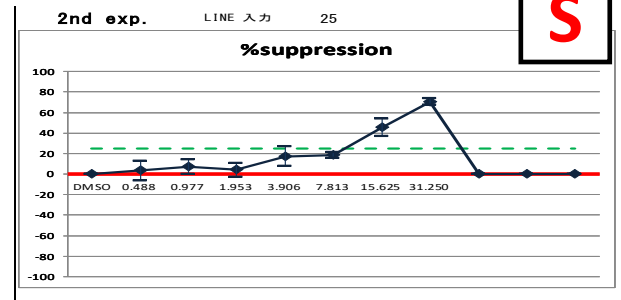
S

S



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	0.973	-6.194	8.141	N.S.	
1.953	3.372	-4.188	10.932	N.S.	
3.906	9.066	3.907	14.225	Sig.	
7.813	30.618	24.103	37.134	Sig.	
15.625	49.580	44.884	54.276	Sig.	
31.250	62.480	56.695	68.265	Sig.	
62.500	56.611	48.754	64.468	Sig.	

S



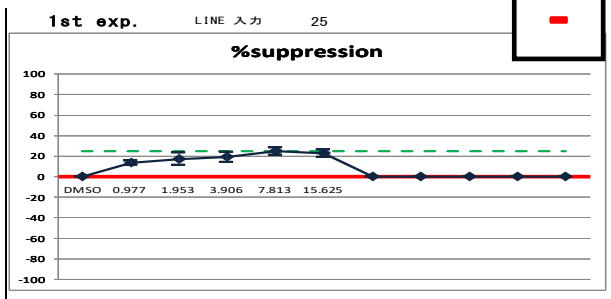
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.488	3.388	-6.437	13.213	N.S.	
0.977	7.199	-0.139	14.536	N.S.	
1.953	4.239	-2.596	11.075	N.S.	
3.906	17.428	7.836	27.019	Sig.	
7.813	18.357	15.667	21.046	Sig.	
15.625	45.414	36.683	54.145	Sig.	
31.250	70.468	67.184	73.751	Sig.	

Chem.20

LabA Tohoku MTA113

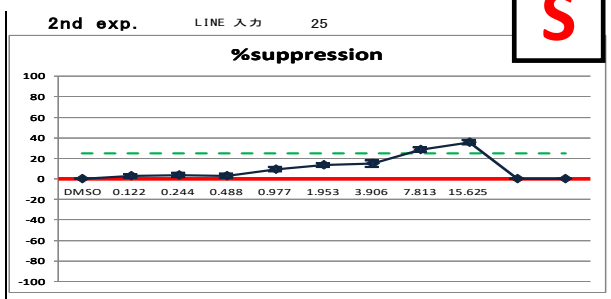
Judge **S**

Exp.1 **-**



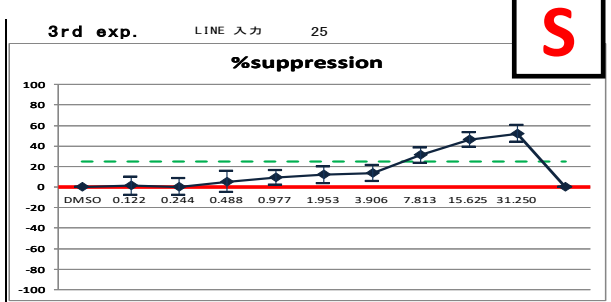
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	13.382	11.224	15.540	Sig.	
1.953	17.346	11.118	23.574	Sig.	
3.906	19.143	14.075	24.212	Sig.	
7.813	24.853	21.090	28.615	Sig.	
15.625	23.090	19.531	26.649	Sig.	

Exp.2 **S**



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.122	2.654	0.937	4.371	Sig.	
0.244	3.610	1.782	5.438	Sig.	
0.488	3.124	0.960	5.288	Sig.	
0.977	9.352	7.474	11.230	Sig.	
1.953	13.335	11.643	15.028	Sig.	
3.906	14.857	11.619	18.095	Sig.	
7.813	28.666	26.607	30.725	Sig.	
15.625	35.771	33.593	37.949	Sig.	

Exp.3 **S**

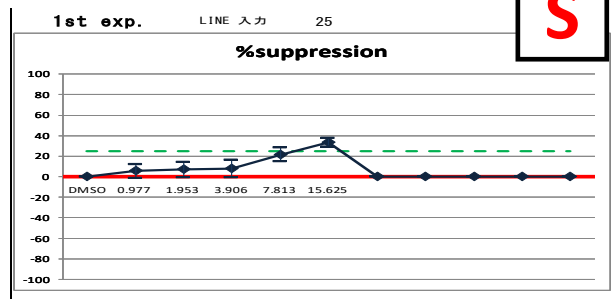


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.122	1.212	-7.856	10.280	N.S.	
0.244	0.423	-7.830	8.675	N.S.	
0.488	5.337	-5.108	15.782	N.S.	
0.977	9.471	2.260	16.681	Sig.	
1.953	11.867	3.427	20.308	Sig.	
3.906	13.642	6.052	21.231	Sig.	
7.813	30.997	23.666	38.328	Sig.	
15.625	46.320	39.111	53.529	Sig.	
31.250	52.125	43.786	60.464	Sig.	

LabB Tsukuba MTB219

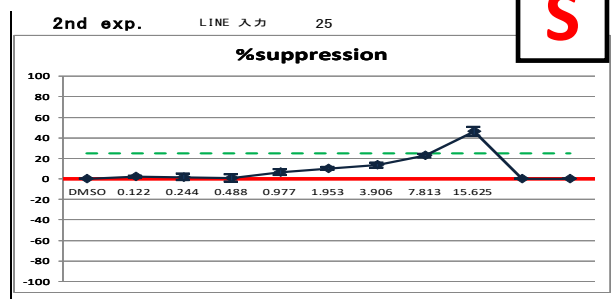
Judge **S**

Exp.1 **S**



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	5.565	-1.014	12.145	N.S.	
1.953	6.903	-0.510	14.317	N.S.	
3.906	7.886	-0.592	16.365	N.S.	
7.813	21.579	14.983	28.175	Sig.	
15.625	33.567	29.398	37.737	Sig.	

Exp.2 **S**

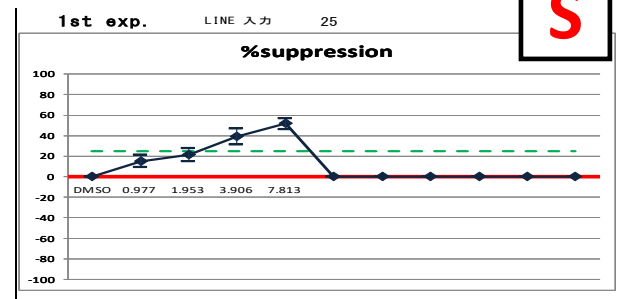


a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.122	1.871	0.518	3.224	Sig.	
0.244	1.696	-1.471	4.862	N.S.	
0.488	0.602	-2.916	4.120	N.S.	
0.977	6.583	3.600	9.566	Sig.	
1.953	9.879	8.508	11.250	Sig.	
3.906	13.231	11.032	15.431	Sig.	
7.813	22.490	20.645	24.336	Sig.	
15.625	46.459	42.232	50.686	Sig.	

LabC Shikoku MTC323

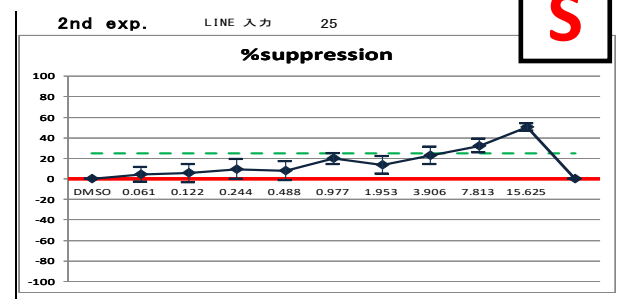
Judge **S**

Exp.1 **S**



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	15.196	8.977	21.415	Sig.	
1.953	21.304	15.139	27.468	Sig.	
3.906	39.064	31.216	46.912	Sig.	
7.813	51.705	46.397	57.013	Sig.	

Exp.2 **S**



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.061	4.343	-3.057	11.743	N.S.	
0.122	5.540	-3.322	14.403	N.S.	
0.244	9.496	-0.273	19.266	N.S.	
0.488	7.749	-1.633	17.130	N.S.	
0.977	19.618	14.087	25.150	Sig.	
1.953	13.785	5.296	22.274	Sig.	
3.906	22.972	14.535	31.408	Sig.	
7.813	32.380	25.845	38.914	Sig.	
15.625	50.484	47.118	53.849	Sig.	

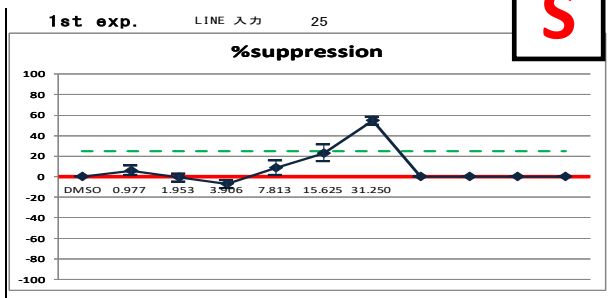
Chem.22

LabA Tohoku MTA107

Judge **S**

Exp.1

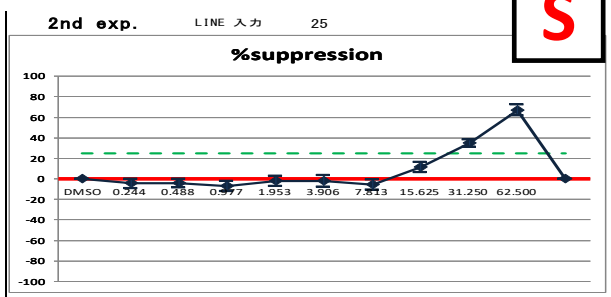
S



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	6.066	1.164	10.967	Sig.	
1.953	-0.833	-4.884	3.217	N.S.	
3.906	-7.128	-10.995	-3.261	Sig.	
7.813	8.579	1.167	15.992	Sig.	
15.625	23.017	14.714	31.319	Sig.	
31.250	54.460	50.372	58.548	Sig.	

Exp.2

S



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.244	-4.427	-8.907	0.053	N.S.	
0.488	-4.102	-8.566	0.362	N.S.	
0.977	-7.165	-12.008	-2.321	Sig.	
1.953	-2.288	-7.284	2.707	N.S.	
3.906	-2.017	-7.659	3.624	N.S.	
7.813	-5.560	-10.237	-0.884	Sig.	
15.625	11.225	6.332	16.118	Sig.	
31.250	34.958	31.437	38.479	Sig.	
62.500	67.029	61.790	72.267	Sig.	

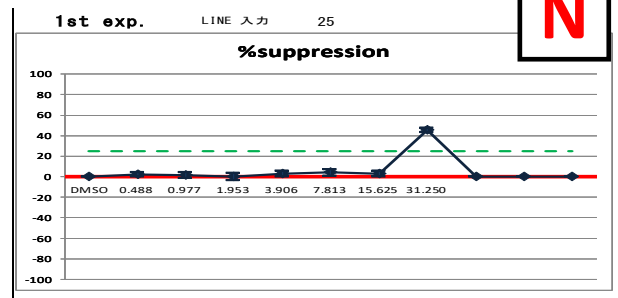
Exp.3

LabB Tsukuba MTB222

Judge **S**

Exp.1

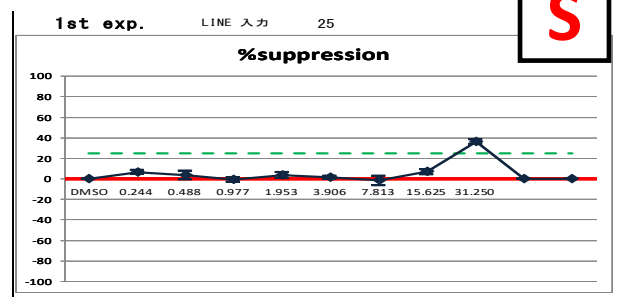
N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.488	2.394	0.227	4.561	Sig.	
0.977	1.693	-0.995	4.381	N.S.	
1.953	-0.026	-3.329	3.278	N.S.	
3.906	2.888	-0.155	5.931	N.S.	
7.813	4.132	0.973	7.292	Sig.	
15.625	2.964	0.011	5.917	Sig.	
31.250	45.590	43.425	47.754	Sig.	

Exp.2

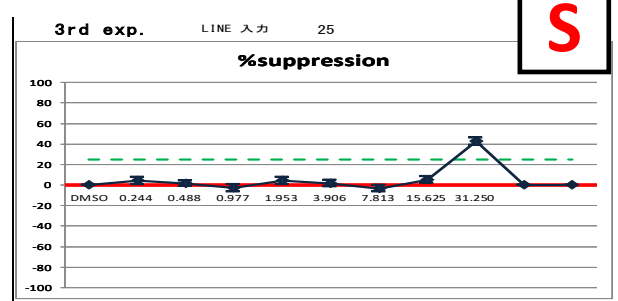
S



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.244	6.495	4.707	8.283	Sig.	
0.488	3.597	-0.703	7.898	N.S.	
0.977	-0.650	-2.589	1.289	N.S.	
1.953	3.434	0.490	6.378	Sig.	
3.906	1.421	0.134	2.708	Sig.	
7.813	-1.645	-6.311	3.021	N.S.	
15.625	7.043	4.652	9.433	Sig.	
31.250	36.319	34.495	38.144	Sig.	

Exp.3

S



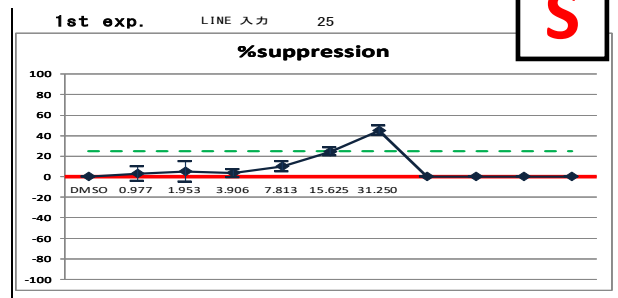
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.244	4.133	0.564	7.702	Sig.	
0.488	1.684	-0.804	4.172	N.S.	
0.977	-2.691	-6.327	0.946	N.S.	
1.953	4.301	0.487	8.115	Sig.	
3.906	1.769	-1.626	5.163	N.S.	
7.813	3.170	-6.619	0.278	N.S.	
15.625	5.309	2.261	8.358	Sig.	
31.250	42.854	39.141	46.567	Sig.	

LabC Shikoku MTC314

Judge **S**

Exp.1

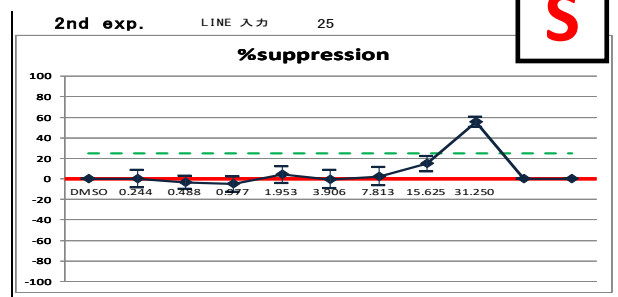
S



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	2.804	-4.118	9.727	N.S.	
1.953	5.191	-4.663	15.044	N.S.	
3.906	3.340	-0.524	7.204	N.S.	
7.813	10.059	4.946	15.172	Sig.	
15.625	24.373	20.439	28.308	Sig.	
31.250	44.890	40.246	49.533	Sig.	

Exp.2

S



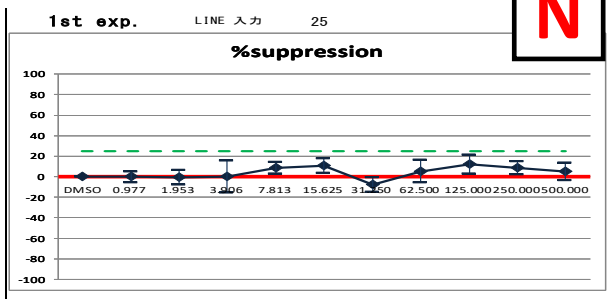
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.244	-0.077	-8.481	8.327	N.S.	
0.488	-3.228	-9.707	3.250	N.S.	
0.977	-5.207	-12.394	1.980	N.S.	
1.953	4.113	-4.237	12.462	N.S.	
3.906	-0.277	-8.990	8.437	N.S.	
7.813	2.437	-6.569	11.443	N.S.	
15.625	14.788	7.366	22.211	Sig.	
31.250	55.482	50.474	60.490	Sig.	

Exp.3

Chem.23

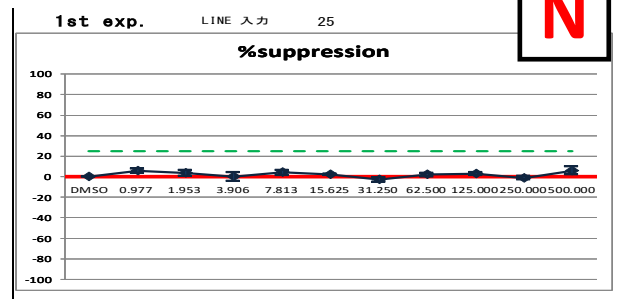
	LabA Tohoku MTA119	LabB Tsukuba MTB201	LabC Shikoku MTC306
Judge	N	N	S

Exp.1



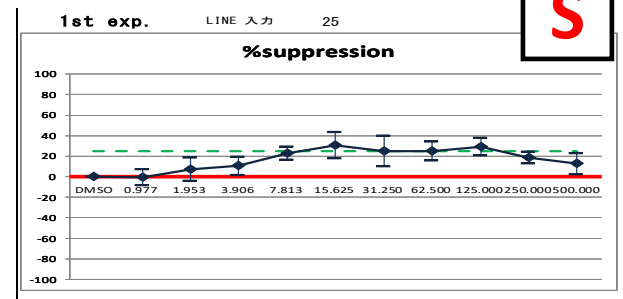
N

a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	-0.142	-5.247	4.964	N.S.	
1.953	-0.678	-7.625	6.269	N.S.	
3.906	0.045	-15.833	15.923	N.S.	
7.813	8.547	2.947	14.147	Sig.	
15.625	10.647	3.643	17.651	Sig.	
31.250	-7.468	-14.547	-0.388	Sig.	
62.500	5.402	-5.282	16.086	N.S.	
125.000	12.143	3.194	21.093	Sig.	
250.000	8.898	2.487	15.310	Sig.	
500.000	4.779	-3.775	13.334	N.S.	



N

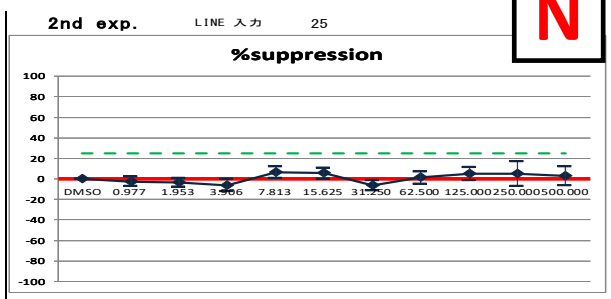
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	5.566	3.534	7.597	Sig.	
1.953	3.816	1.130	6.503	Sig.	
3.906	0.119	-4.093	4.331	N.S.	
7.813	4.034	1.395	6.672	Sig.	
15.625	2.335	1.402	3.269	Sig.	
31.250	-2.758	-4.848	-0.669	Sig.	
62.500	1.916	-0.003	3.834	N.S.	
125.000	3.019	1.801	4.236	Sig.	
250.000	-1.101	-2.905	0.704	N.S.	
500.000	5.994	2.292	9.697	Sig.	



S

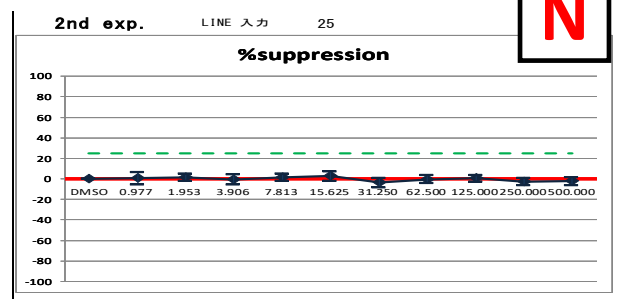
a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	-0.854	-8.675	6.967	N.S.	
1.953	7.108	-4.479	18.694	N.S.	
3.906	10.604	1.805	19.403	Sig.	
7.813	22.838	16.723	28.953	Sig.	
15.625	30.614	17.910	43.319	Sig.	
31.250	25.138	10.196	40.079	Sig.	
62.500	25.105	16.016	34.194	Sig.	
125.000	29.160	20.435	37.886	Sig.	
250.000	18.789	13.131	24.446	Sig.	
500.000	12.553	2.433	22.674	Sig.	

Exp.2



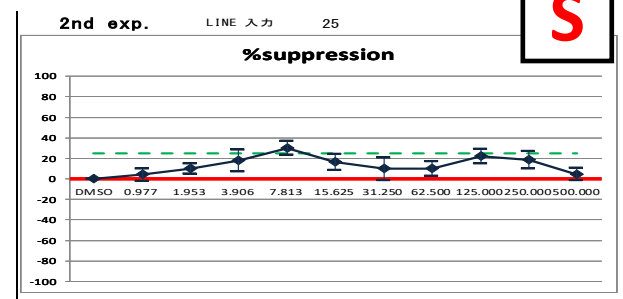
N

a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	-2.452	-6.797	1.894	N.S.	
1.953	-3.662	-7.881	0.557	N.S.	
3.906	-5.994	-11.907	-0.082	Sig.	
7.813	6.659	0.884	12.433	Sig.	
15.625	5.563	0.393	10.734	Sig.	
31.250	-6.150	-10.950	-1.351	Sig.	
62.500	1.236	-4.642	7.114	N.S.	
125.000	5.101	-1.263	11.466	N.S.	
250.000	5.026	-7.059	17.111	N.S.	
500.000	3.247	-5.970	12.463	N.S.	



N

a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	0.607	-5.500	6.714	N.S.	
1.953	1.592	-1.856	5.039	N.S.	
3.906	-0.767	-5.711	4.177	N.S.	
7.813	1.420	-2.216	5.057	N.S.	
15.625	2.597	-1.764	6.957	N.S.	
31.250	-3.773	-8.291	0.744	N.S.	
62.500	-0.348	-4.415	3.718	N.S.	
125.000	0.480	-2.692	3.652	N.S.	
250.000	-2.846	-6.537	0.844	N.S.	
500.000	-2.297	-6.111	1.516	N.S.	



S

a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	4.097	-1.935	10.129	N.S.	
1.953	9.836	4.817	14.854	Sig.	
3.906	17.898	7.286	28.511	Sig.	
7.813	30.145	23.378	36.912	Sig.	
15.625	16.700	8.898	24.501	Sig.	
31.250	9.789	-0.994	20.572	N.S.	
62.500	10.093	2.973	17.213	Sig.	
125.000	22.347	15.263	29.431	Sig.	
250.000	18.655	10.237	27.074	Sig.	
500.000	4.656	-1.121	10.434	N.S.	

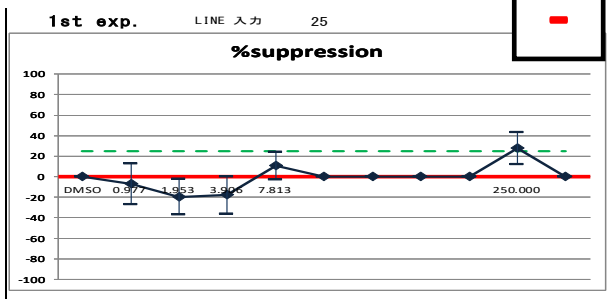
Exp.3

Chem.25

LabA Tohoku MTA104

Judge **N**

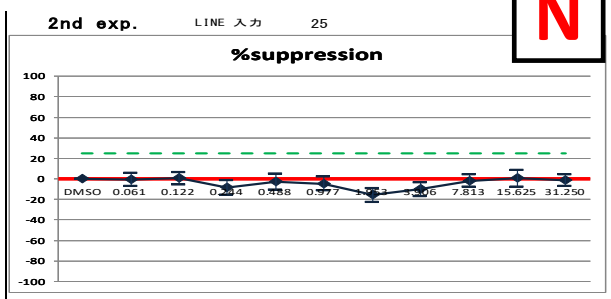
Exp.1



-

a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	-7.137	-26.945	12.672	N.S.	
1.953	-19.526	-36.801	-2.252	Sig.	
3.906	-17.815	-35.808	0.178	N.S.	
7.813	10.896	-2.703	24.495	N.S.	
250.000	27.705	11.912	43.498	Sig.	

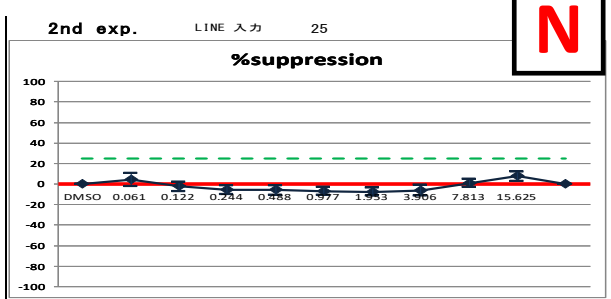
Exp.2



N

a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.061	-0.509	-6.736	5.717	N.S.	
0.122	0.552	-5.310	6.414	N.S.	
0.244	-8.416	-15.557	-1.275	Sig.	
0.488	-2.775	-10.577	5.026	N.S.	
0.977	-4.561	-11.136	2.014	N.S.	
1.953	-15.800	-22.454	-9.145	Sig.	
3.906	-10.054	-16.667	-3.442	Sig.	
7.813	-1.731	-7.973	4.511	N.S.	
15.625	0.505	-7.942	8.953	N.S.	
31.250	-1.428	-7.169	4.314	N.S.	

Exp.3



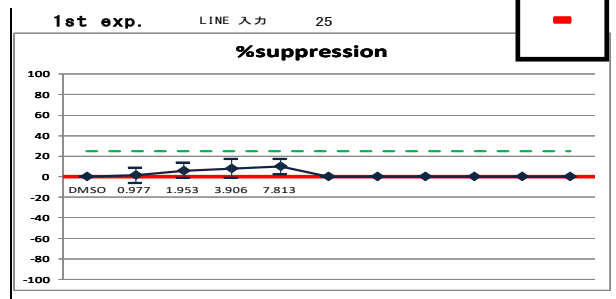
N

a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.061	4.280	-1.902	10.461	N.S.	
0.122	-2.188	-6.900	2.523	N.S.	
0.244	-5.363	-9.548	-1.179	Sig.	
0.488	-5.842	-10.539	-1.145	Sig.	
0.977	-6.842	-10.839	-2.845	Sig.	
1.953	-7.486	-11.364	-3.609	Sig.	
3.906	-6.039	-11.415	-0.664	Sig.	
7.813	0.956	-3.058	4.970	N.S.	
15.625	7.679	3.261	12.097	Sig.	

LabB Tsukuba MTB210

Judge **N**

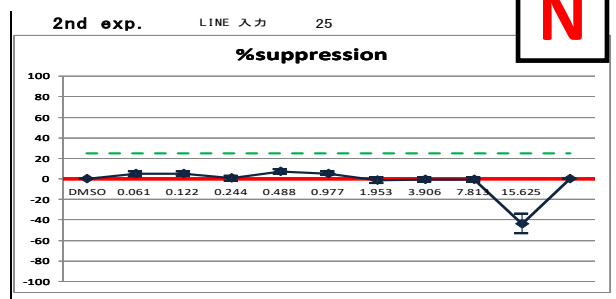
Exp.1



-

a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	1.267	-6.239	8.774	N.S.	
1.953	5.980	-1.524	13.483	N.S.	
3.906	7.605	-1.565	16.775	N.S.	
7.813	9.691	2.072	17.311	Sig.	

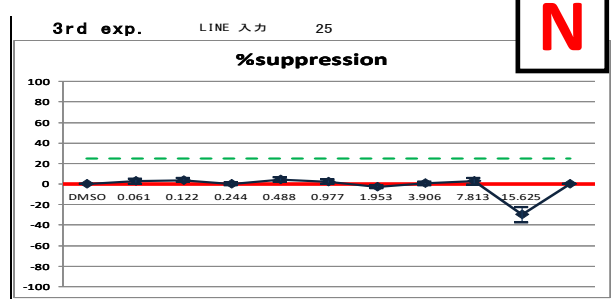
Exp.2



N

a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.061	4.779	2.121	7.436	Sig.	
0.122	4.849	2.869	6.829	Sig.	
0.244	0.532	-2.162	3.225	N.S.	
0.488	6.940	4.911	8.969	Sig.	
0.977	5.249	3.457	7.040	Sig.	
1.953	-1.315	-4.105	1.476	N.S.	
3.906	-0.451	-2.512	1.610	N.S.	
7.813	-0.641	-2.799	1.518	N.S.	
15.625	-43.684	-53.484	-33.884	Sig.	

Exp.3



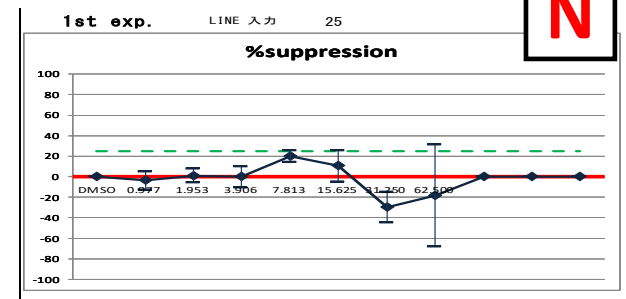
N

a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.061	2.811	0.410	5.212	Sig.	
0.122	3.817	2.163	5.472	Sig.	
0.244	0.109	-1.531	1.749	N.S.	
0.488	4.029	1.857	6.201	Sig.	
0.977	2.254	0.423	4.085	Sig.	
1.953	2.927	-4.330	-1.525	Sig.	
3.906	0.527	-1.448	2.503	N.S.	
7.813	2.738	-0.362	5.839	N.S.	
15.625	-29.823	-37.271	-22.374	Sig.	

LabC Shikoku MTC311

Judge **N**

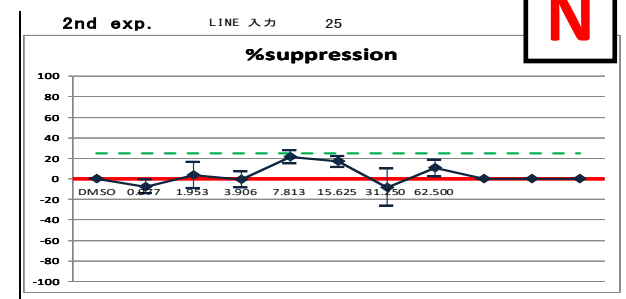
Exp.1



N

a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	-3.696	-12.690	5.298	N.S.	
1.953	1.136	-5.814	8.086	N.S.	
3.906	-0.244	-10.710	10.221	N.S.	
7.813	19.915	14.496	25.334	Sig.	
15.625	10.424	-4.560	25.407	N.S.	
31.250	-29.776	-44.635	-14.916	Sig.	
62.500	-18.670	-68.335	30.996	N.S.	

Exp.2



N

a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
DMSO	0	0	0		
0.977	-7.492	-14.186	-0.797	Sig.	
1.953	3.443	-9.328	16.214	N.S.	
3.906	-0.280	-8.096	7.536	N.S.	
7.813	21.436	14.918	27.954	Sig.	
15.625	16.792	11.206	22.377	Sig.	
31.250	-8.145	-26.320	10.031	N.S.	
62.500	10.468	2.175	18.760	Sig.	

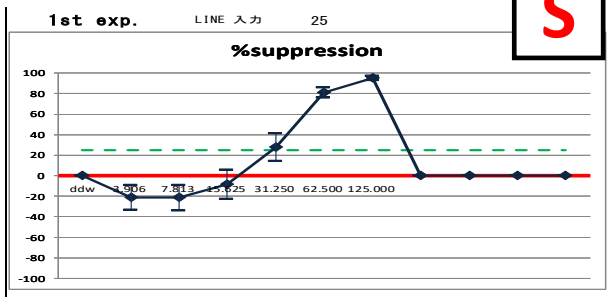
Chem.26

LabA Tohoku MTA114

Judge **S**

Exp.1

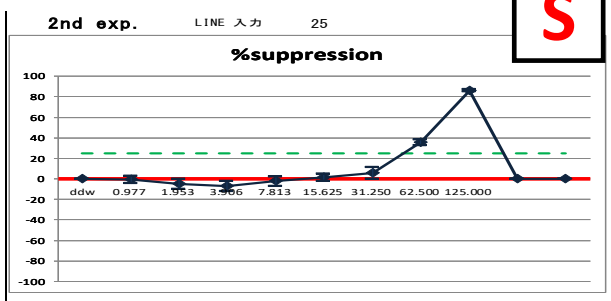
S



Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	-21.353	-33.531	-9.176	Sig.	
7.813	-21.512	-34.133	-8.891	Sig.	
15.625	-8.578	-22.969	5.813	N.S.	
31.250	27.861	14.488	41.234	Sig.	
62.500	81.011	76.148	85.873	Sig.	
125.000	95.247	93.287	97.206	Sig.	

Exp.2

S



Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
0.977	-0.757	-4.428	2.915	N.S.	
1.953	-4.674	-9.564	0.217	N.S.	
3.906	-6.795	-11.767	-1.822	Sig.	
7.813	-2.339	-6.738	2.060	N.S.	
15.625	1.481	-2.150	5.113	N.S.	
31.250	5.902	0.102	11.701	Sig.	
62.500	35.681	32.614	38.748	Sig.	
125.000	86.212	84.996	87.429	Sig.	

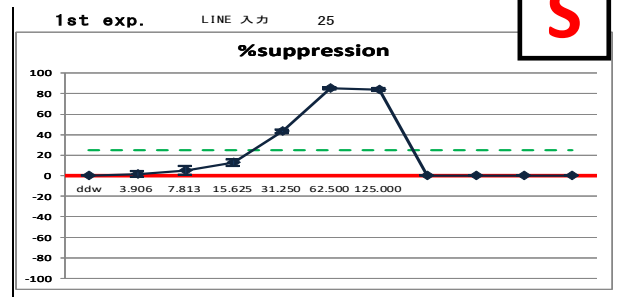
Exp.3

LabB Tsukuba MTB216

Judge **S**

Exp.1

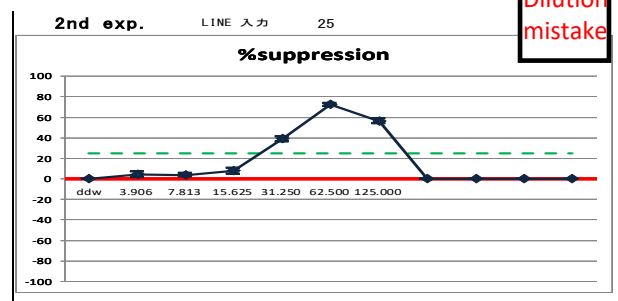
S



Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	1.489	-1.203	4.181	N.S.	
7.813	5.174	1.139	9.208	Sig.	
15.625	12.570	9.604	15.536	Sig.	
31.250	43.068	41.355	44.781	Sig.	
62.500	85.077	84.073	86.081	Sig.	
125.000	83.759	82.255	85.263	Sig.	

Exp.2

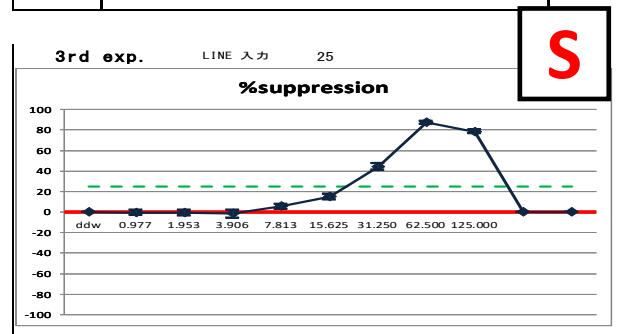
Dilution mistake



Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	4.416	1.752	7.079	Sig.	
7.813	3.900	1.869	5.932	Sig.	
15.625	7.921	4.931	10.911	Sig.	
31.250	38.861	36.549	41.172	Sig.	
62.500	72.458	71.338	73.578	Sig.	
125.000	56.484	53.862	59.106	Sig.	

Exp.3

S



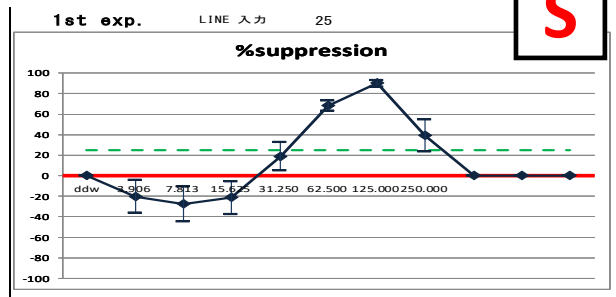
Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
0.977	-0.396	-2.983	2.190	N.S.	
1.953	-0.467	-3.204	2.269	N.S.	
3.906	-1.510	-5.340	2.319	N.S.	
7.813	5.597	3.096	8.099	Sig.	
15.625	14.886	11.886	17.886	Sig.	
31.250	44.254	40.988	47.520	Sig.	
62.500	87.372	86.106	88.637	Sig.	
125.000	78.426	76.722	80.130	Sig.	

LabC Shikoku MTC304

Judge **S**

Exp.1

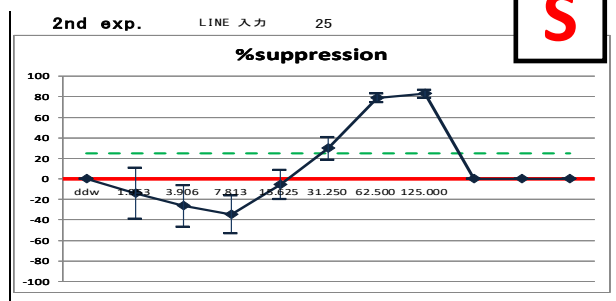
S



Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	-20.196	-36.356	-4.037	Sig.	
7.813	-27.360	-44.442	-10.278	Sig.	
15.625	-21.410	-37.380	-5.439	Sig.	
31.250	18.835	4.825	32.845	Sig.	
62.500	68.451	63.354	73.547	Sig.	
125.000	90.124	86.823	93.424	Sig.	
250.000	39.379	23.752	55.006	Sig.	

Exp.2

S



Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
1.953	-14.055	-38.774	10.665	N.S.	
3.906	-26.352	-46.426	-6.278	Sig.	
7.813	-34.900	-53.301	-16.499	Sig.	
15.625	-5.606	-19.709	8.497	N.S.	
31.250	29.546	18.405	40.688	Sig.	
62.500	78.644	74.478	82.810	Sig.	
125.000	83.094	79.226	86.963	Sig.	

Exp.3

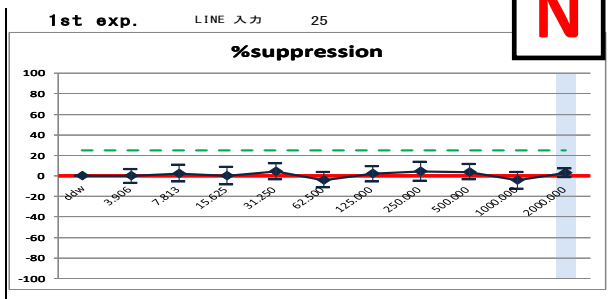
Chem.27

LabA Tohoku MTA127

Judge **N**

Exp.1

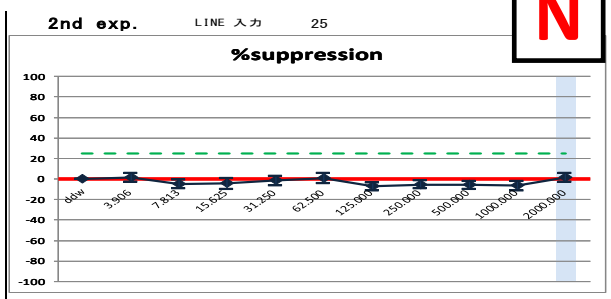
N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	-0.139	-6.813	6.535	N.S.	
7.813	2.395	-5.842	10.632	N.S.	
15.625	0.136	-8.583	8.855	N.S.	
31.250	4.388	-3.147	11.924	N.S.	
62.500	-3.833	-11.542	3.875	N.S.	
125.000	1.969	-5.470	9.409	N.S.	
250.000	4.069	-5.104	13.241	N.S.	
500.000	3.893	-3.498	11.285	N.S.	
1000.000	-4.404	-12.461	3.654	N.S.	
2000.000	2.808	-1.540	7.156	N.S.	eliminate

Exp.2

N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	1.280	-2.970	5.530	N.S.	
7.813	-5.098	-9.223	-0.974	Sig.	
15.625	-4.282	-9.508	0.943	N.S.	
31.250	-1.592	-6.050	2.867	N.S.	
62.500	0.530	-4.347	5.407	N.S.	
125.000	-7.232	-11.215	-3.249	Sig.	
250.000	-5.447	-9.206	-1.687	Sig.	
500.000	-5.898	-9.887	-1.910	Sig.	
1000.000	-6.500	-10.929	-2.071	Sig.	
2000.000	1.613	-2.442	5.669	N.S.	eliminate

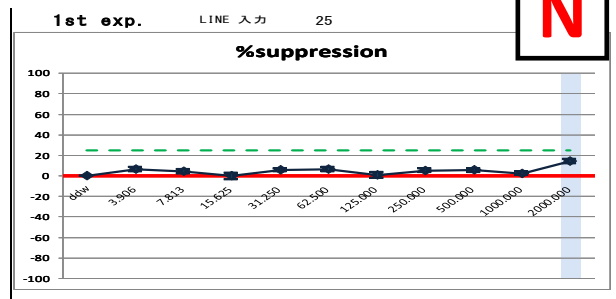
Exp.3

LabB Tsukuba MTB227

Judge **N**

Exp.1

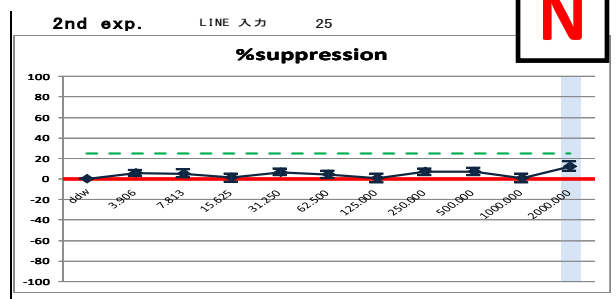
N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	6.352	4.275	8.429	Sig.	
7.813	4.418	2.140	6.697	Sig.	
15.625	-0.244	-3.509	3.021	N.S.	
31.250	5.729	4.153	7.306	Sig.	
62.500	6.393	4.406	8.379	Sig.	
125.000	0.833	-1.716	3.382	N.S.	
250.000	5.271	3.568	6.974	Sig.	
500.000	5.717	3.982	7.452	Sig.	
1000.000	2.491	0.455	4.527	Sig.	
2000.000	14.376	12.266	16.485	Sig.	eliminate

Exp.2

N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	5.810	2.955	8.666	Sig.	
7.813	5.398	1.783	9.013	Sig.	
15.625	1.256	-2.492	5.004	N.S.	
31.250	6.726	3.557	9.894	Sig.	
62.500	4.053	0.478	7.628	Sig.	
125.000	0.957	-3.317	5.231	N.S.	
250.000	6.918	3.588	10.247	Sig.	
500.000	7.102	3.785	10.418	Sig.	
1000.000	1.013	-3.283	5.308	N.S.	
2000.000	12.301	7.672	16.930	Sig.	eliminate

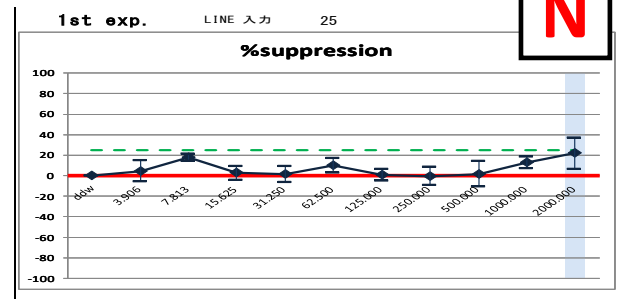
Exp.3

LabC Shikoku MTC327

Judge **N**

Exp.1

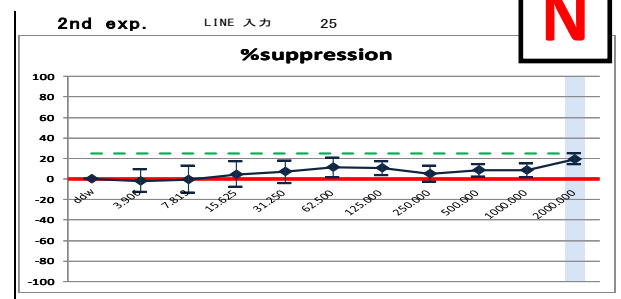
N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	4.411	-5.874	14.696	N.S.	
7.813	17.838	14.247	21.428	Sig.	
15.625	2.871	-3.825	9.567	N.S.	
31.250	1.370	-6.353	9.093	N.S.	
62.500	10.166	3.010	17.322	Sig.	
125.000	0.878	-4.889	6.644	N.S.	
250.000	-0.285	-9.152	8.583	N.S.	
500.000	1.709	-10.721	14.139	N.S.	
1000.000	13.031	7.318	18.744	Sig.	
2000.000	22.001	6.737	37.264	Sig.	eliminate

Exp.2

N



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
ddw	0	0	0		
3.906	-1.887	-13.000	9.226	N.S.	
7.813	-0.357	-13.584	12.870	N.S.	
15.625	4.641	-7.758	17.040	N.S.	
31.250	6.885	-4.202	17.971	N.S.	
62.500	11.117	1.617	20.617	Sig.	
125.000	10.447	3.834	17.059	Sig.	
250.000	5.082	-2.491	12.655	N.S.	
500.000	8.518	2.455	14.581	Sig.	
1000.000	8.466	1.818	15.114	Sig.	
2000.000	19.452	14.248	24.655	Sig.	eliminate

Exp.3

Appendix 12 Refference 25 chemicals

Abe, M., Shimizu, A., Yokoyama, Y., Takeuchi, Y., Ishikawa, O., 2008. A possible inhibitory action of diaminodiphenyl sulfone on tumour necrosis factor-alpha production from activated mononuclear cells on cutaneous lupus erythematosus. Clin Exp Dermatol 33, 759-763.

Andrade-Mena, C.E., Sardo-Olmedo, J.A., Ramirez-Lizardo, E.J., 1994. Effects of phenytoin administration on murine immune function. J Neuroimmunol 50, 3-7.

Babu, U.S., Wiesenfeld, P.L., Jenkins, M.Y., 1999. Effect of dietary rosemary extract on cell-mediated immunity of young rats. Plant Foods Hum Nutr 53, 169-174.

Bachiega, T.F., Sforcin, J.M., 2011. Lemongrass and citral effect on cytokines production by murine macrophages. J Ethnopharmacol 137, 909-913.

Banerjee, N., Wang, H., Wang, G., Khan, M.F., 2020. Enhancing the Nrf2 antioxidant signaling provides protection against trichloroethene-mediated inflammation and autoimmune response. Toxicol Sci.

Barasoain, I., Rojo, J.M., Portoles, A., 1980. "In vivo" effects of acetylsalicylic acid and two ether derived compounds on primary immune response and lymphoblastic transformation. Immunopharmacology 2, 293-300.

Bette, M., Schlimme, S., Mutters, R., Menendez, S., Hoffmann, S., Schulz, S., 2004. Influence of different anaesthetics on pro-inflammatory cytokine expression in rat spleen. Lab Anim 38, 272-279.

Bilrha, H., Roy, R., Moreau, B., Belles-Isles, M., Dewailly, E., Ayotte, P., 2003. In vitro activation of cord blood mononuclear cells and cytokine production in a remote coastal population exposed to organochlorines and methyl mercury. Environ Health Perspect 111, 1952-1957.

Blakley, B.R., 1985. The effect of cadmium chloride on the immune response in mice. Can J Comp Med 49, 104-108.

Blakley, B.R., Tomar, R.S., 1986. The effect of cadmium on antibody responses to antigens with different cellular requirements. Int J Immunopharmacol 8, 1009-1015.

Blakley, B.R., Yole, M.J., Brousseau, P., Boermans, H., Fournier, M., 1998. Effect of pentachlorophenol on immune function. Toxicology 125, 141-148.

Boorman, G.A., Luster, M.I., Dean, J.H., Luebke, R.W., 1982. Effect of indomethacin on the bone marrow and immune system of the mouse. J Clin Lab Immunol 7, 119-126.

Brieger, A., Bienefeld, N., Hasan, R., Goerlich, R., Haase, H., 2011. Impact of perfluorooctanesulfonate and perfluorooctanoic acid on human peripheral leukocytes. Toxicol In Vitro 25, 960-968.

Carfi, M., Gennari, A., Malerba, I., Corsini, E., Pallardy, M., Pieters, R., Van Loveren, H., Vohr, H.W., Hartung, T., Gribaldo, L., 2007. In vitro tests to evaluate immunotoxicity: a preliminary

study. Toxicology 229, 11-22.
Chang, D.M., Baptiste, P., Schur, P.H., 1990. The effect of antirheumatic drugs on interleukin 1 (IL-1) activity and IL-1 and IL-1 inhibitor production by human monocytes. J Rheumatol 17, 1148-1157.
Chang, M.P., Norman, D.C., 1991. Immunotoxicity of alcohol in young and old mice. II. Impaired T cell proliferation and T cell-dependent antibody responses of young and old mice fed ethanol-containing liquid diet. Mech Ageing Dev 57, 175-186.
Chapman, J.R., Roberts, D.W., 1984. Humoral immune dysfunction as a result of prenatal exposure to diphenylhydantoin: correlation with the occurrence of physical defects. Teratology 30, 107-117.
Chaudhri, G., Clark, I.A., 1989. Reactive oxygen species facilitate the in vitro and in vivo lipopolysaccharide-induced release of tumor necrosis factor. J Immunol 143, 1290-1294.
Chen, H.M., Lee, Y.H., Chen, R.J., Chiu, H.W., Wang, B.J., Wang, Y.J., 2013. The immunotoxic effects of dual exposure to PCP and TCDD. Chem Biol Interact 206, 166-174.
Chen, Q., Zhang, Z., Zhang, R., Niu, Y., Bian, X., Zhang, Q., 2011. Tributyltin chloride-induced immunotoxicity and thymocyte apoptosis are related to abnormal Fas expression. Int J Hyg Environ Health 214, 145-150.
Corsini, E., Avogadro, A., Galbiati, V., dell'Agli, M., Marinovich, M., Galli, C.L., Germolec, D.R., 2011. In vitro evaluation of the immunotoxic potential of perfluorinated compounds (PFCs). Toxicol Appl Pharmacol 250, 108-116.
Corsini, E., Sangiovanni, E., Avogadro, A., Galbiati, V., Viviani, B., Marinovich, M., Galli, C.L., Dell'Agli, M., Germolec, D.R., 2012. In vitro characterization of the immunotoxic potential of several perfluorinated compounds (PFCs). Toxicol Appl Pharmacol 258, 248-255.
Corsini, E., Viviani, B., Birindelli, S., Gilardi, F., Torri, A., Codeca, I., Lucchi, L., Bartesaghi, S., Galli, C.L., Marinovich, M., Colosio, C., 2006. Molecular mechanisms underlying mancozeb-induced inhibition of TNF-alpha production. Toxicol Appl Pharmacol 212, 89-98.
Couleau, N., Falla, J., Beillerot, A., Battaglia, E., D'Innocenzo, M., Plancon, S., Laval-Gilly, P., Bennasroune, A., 2015. Effects of Endocrine Disruptor Compounds, Alone or in Combination, on Human Macrophage-Like THP-1 Cell Response. PLoS One 10, e0131428.
Del Bufalo, A., Bernad, J., Dardenne, C., Verda, D., Meunier, J.R., Rousset, F., Martinozzi-Teissier, S., Pipy, B., 2011. Contact sensitizers modulate the arachidonic acid metabolism of PMA-differentiated U-937 monocytic cells activated by LPS. Toxicol Appl Pharmacol 256, 35-43.
Devos, S., Van Den Heuvel, R., Hooghe, R., Hooghe-Peters, E.L., 2004. Limited effect of selected organic pollutants on cytokine production by peripheral blood leukocytes. Eur Cytokine Netw 15, 145-151.
Dewitt, J.C., Copeland, C.B., Strynar, M.J., Luebke, R.W., 2008. Perfluorooctanoic acid-induced

immunomodulation in adult C57BL/6J or C57BL/6N female mice. Environ Health Perspect 116, 644-650.
Dieter, M.P., Luster, M.I., Boorman, G.A., Jameson, C.W., Dean, J.H., Cox, J.W., 1983. Immunological and biochemical responses in mice treated with mercuric chloride. Toxicol Appl Pharmacol 68, 218-228.
DiMartino, M.J., Johnson, W.J., Votta, B., Hanna, N., 1987. Effect of antiarthritic drugs on the enhanced interleukin-1 (IL-1) production by macrophages from adjuvant-induced arthritic (AA) rats. Agents Actions 21, 348-350.
Eugui, E.M., DeLustro, B., Rouhafza, S., Ilnicka, M., Lee, S.W., Wilhelm, R., Allison, A.C., 1994. Some antioxidants inhibit, in a co-ordinate fashion, the production of tumor necrosis factor-alpha, IL-beta, and IL-6 by human peripheral blood mononuclear cells. Int Immunol 6, 409-422.
Gardner, R.M., Nyland, J.F., Evans, S.L., Wang, S.B., Doyle, K.M., Crainiceanu, C.M., Silbergeld, E.K., 2009. Mercury induces an unopposed inflammatory response in human peripheral blood mononuclear cells in vitro. Environ Health Perspect 117, 1932-1938.
Gaworski, C.L., Vollmuth, T.A., Dozier, M.M., Heck, J.D., Dunn, L.T., Ratajczak, H.V., Thomas, P.T., 1994. An immunotoxicity assessment of food flavouring ingredients. Food Chem Toxicol 32, 409-415.
Hansen, J.F., Nielsen, C.H., Brorson, M.M., Frederiksen, H., Hartoft-Nielsen, M.L., Rasmussen, A.K., Bendtzen, K., Feldt-Rasmussen, U., 2015. Influence of phthalates on in vitro innate and adaptive immune responses. PLoS One 10, e0131168.
Hartel, C., von Puttkamer, J., Gallner, F., Strunk, T., Schultz, C., 2004. Dose-dependent immunomodulatory effects of acetylsalicylic acid and indomethacin in human whole blood: potential role of cyclooxygenase-2 inhibition. Scand J Immunol 60, 412-420.
Hemdan, N.Y., Emmrich, F., Sack, U., Wichmann, G., Lehmann, J., Adham, K., Lehmann, I., 2006. The in vitro immune modulation by cadmium depends on the way of cell activation. Toxicology 222, 37-45.
Hemdan, N.Y., Lehmann, I., Wichmann, G., Lehmann, J., Emmrich, F., Sack, U., 2007. Immunomodulation by mercuric chloride in vitro: application of different cell activation pathways. Clin Exp Immunol 148, 325-337.
Hu, C., Meng, X., Huang, C., Shen, C., Li, J., 2017. Frontline Science: ATF3 is responsible for the inhibition of TNF-alpha release and the impaired migration of acute ethanol-exposed monocytes and macrophages. J Leukoc Biol 101, 633-642.
Jin, Y., Liu, L., Zhang, S., He, R., Wu, Y., Chen, G., Fu, Z., 2016. Cadmium exposure to murine macrophages decreases their inflammatory responses and increases their oxidative stress. Chemosphere 144, 168-175.
Kerkvliet, N.I., Baecher-Steppan, L., Claycomb, A.T., Craig, A.M., Sheggeby, G.G., 1982.

Immunotoxicity of technical pentachlorophenol (PCP-T): depressed humoral immune responses to T-dependent and T-independent antigen stimulation in PCP-T exposed mice. Fundam Appl Toxicol 2, 90-99.

Kerkvliet, N.I., Brauner, J.A., Baecher-Steppan, L., 1985. Effects of dietary technical pentachlorophenol exposure on T cell, macrophage and natural killer cell activity in C57Bl/6 mice. Int J Immunopharmacol 7, 239-247.

Kim, H.M., Han, S.B., Chang, W.I., Hyun, B.H., Oh, G.T., Ahn, C.J., Cha, Y.N., 1996. Selective suppression of in vitro T-dependent humoral immunity by synthetic food additive antioxidants. J Toxicol Sci 21, 41-45.

Kimura, K., Kobayashi, K., Naito, H., Suzuki, Y., Sugita-Konishi, Y., 2005. Effect of lactational exposure to tributyltin chloride on innate immunodefenses in the F1 generation in mice. Biosci Biotechnol Biochem 69, 1104-1110.

Krzystyniak, K., Fournier, M., Trottier, B., Nadeau, D., Chevalier, G., 1987. Immunosuppression in mice after inhalation of cadmium aerosol. Toxicol Lett 38, 1-12.

Kucharz, E.J., Sierakowski, S.J., 1992. Studies on immunomodulatory properties of isoniazid: V. Influence of isoniazid on secretion of interleukin-1. J Hyg Epidemiol Microbiol Immunol 36, 119-122.

Kushima, K., Sakuma, S., Furusawa, S., Fujiwara, M., 2009. Prenatal administration of indomethacin modulates Th2 cytokines in juvenile rats. Toxicol Lett 185, 32-37.

Larsen, S.T., Lund, R.M., Nielsen, G.D., Thygesen, P., Poulsen, O.M., 2002. Adjuvant effect of di-n-butyl-, di-n-octyl-, di-iso-nonyl- and di-iso-decyl phthalate in a subcutaneous injection model using BALB/c mice. Pharmacol Toxicol 91, 264-272.

Lee, J.Y., Kim, J.Y., Lee, Y.G., Shin, W.C., Chun, T., Rhee, M.H., Cho, J.Y., 2007. Hydroquinone, a reactive metabolite of benzene, reduces macrophage-mediated immune responses. Mol Cells 23, 198-206.

Li, L., Li, H.S., Song, N.N., Chen, H.M., 2013. The immunotoxicity of dibutyl phthalate on the macrophages in mice. Immunopharmacol Immunotoxicol 35, 272-281.

Lombardi, P., Fournier, M., Bernier, J., Mansour, S., Neveu, P., Krzystyniak, K., 1991. Evaluation of the immunomodulatory potential of diethyl dithiocarbamate derivatives. Int J Immunopharmacol 13, 1073-1084.

Lourenco, A.C., Galbiati, V., Corti, D., Papale, A., Martino-Andrade, A.J., Corsini, E., 2015. The plasticizer dibutyl phthalate (DBP) potentiates chemical allergen-induced THP-1 activation. Toxicol In Vitro 29, 2001-2008.

Luebke, R.W., Riddle, M.M., Rogers, R.R., Rowe, D.G., Garner, R.J., Smialowicz, R.J., 1986. Immune function in adult C57BL/6J mice following exposure to urethan pre- or postnatally. J Immunopharmacol 8, 243-257.

Luebke, R.W., Rogers, R.R., Riddle, M.M., Rowe, D.G., Smialowicz, R.J., 1987. Alteration of immune function in mice following carcinogen exposure. Immunopharmacology 13, 1-9.
Luster, M.I., Dean, J.H., Boorman, G.A., Dieter, M.P., Hayes, H.T., 1982. Immune functions in methyl and ethyl carbamate treated mice. Clin Exp Immunol 50, 223-230.
Ma, Q., Kinneer, K., 2002. Chemoprotection by phenolic antioxidants. Inhibition of tumor necrosis factor alpha induction in macrophages. J Biol Chem 277, 2477-2484.
Marshall, M., Moore, P.K., 2004. Effect of nitric oxide releasing paracetamol and flurbiprofen on cytokine production in human blood. Eur J Pharmacol 483, 317-322.
Marth, E., Barth, S., Jelovcan, S., 2000. Influence of cadmium on the immune system. Description of stimulating reactions. Cent Eur J Public Health 8, 40-44.
Martin, T.J., Whalen, M.M., 2017. Exposures to the environmental toxicants pentachlorophenol (PCP) and dichlorodiphenyltrichloroethane (DDT) modify secretion of interleukin 1-beta (IL-1beta) from human immune cells. Arch Toxicol 91, 1795-1808.
Modeer, T., Karsten, J., Weintraub, A., Gidlund, M., Sundqvist, K.G., 1989. Phenytoin induces interleukin-1 production in vitro. Life Sci 44, 35-40.
Morohoshi, M., Fujisawa, K., Uchimura, I., Numano, F., 1996. Glucose-dependent interleukin 6 and tumor necrosis factor production by human peripheral blood monocytes in vitro. Diabetes 45, 954-959.
Nastevska, C., Gerber, E., Horbach, M., Rohrdanz, E., Kahl, R., 1999. Impairment of TNF-alpha expression and secretion in primary rat liver cell cultures by acetaminophen treatment. Toxicology 133, 85-92.
Okada, K., Sugiura, T., Kuroda, E., Tsuji, S., Yamashita, U., 2001. Phenytoin promotes Th2 type immune response in mice. Clin Exp Immunol 124, 406-413.
Qazi, M.R., Bogdanska, J., Butenhoff, J.L., Nelson, B.D., DePierre, J.W., Abedi-Valugerdi, M., 2009. High-dose, short-term exposure of mice to perfluorooctanesulfonate (PFOS) or perfluorooctanoate (PFOA) affects the number of circulating neutrophils differently, but enhances the inflammatory responses of macrophages to lipopolysaccharide (LPS) in a similar fashion. Toxicology 262, 207-214.
Rojo, J.M., Barasoain, I., Portoles, A., 1981. Further studies on the immunosuppressive effects of indomethacin. Int J Clin Pharmacol Ther Toxicol 19, 220-222.
Rordorf-Adam, C., Lazdins, J., Woods-Cook, K., Alteri, E., Henn, R., Geiger, T., Feige, U., Towbin, H., Erard, F., 1989. An assay for the detection of interleukin-1 synthesis inhibitors: effects of antirheumatic drugs. Drugs Exp Clin Res 15, 355-362.
Sakazaki, H., Ueno, H., Nakamuro, K., 2002. Estrogen receptor alpha in mouse splenic lymphocytes: possible involvement in immunity. Toxicol Lett 133, 221-229.
Seng, G.F., Benensohn, J., Bayer, B.M., 1990. Changes in T and B lymphocyte proliferative

responses in adjuvant-arthritic rats: antagonism by indomethacin. Eur J Pharmacol 178, 267-273.
Serra, R., Al-Saidi, A.G., Angelov, N., Nares, S., 2010. Suppression of LPS-induced matrix-metalloproteinase responses in macrophages exposed to phenytoin and its metabolite, 5-(p-hydroxyphenyl-), 5-phenylhydantoin. J Inflamm (Lond) 7, 48.
Son, H.Y., Lee, S., Tak, E.N., Cho, H.S., Shin, H.I., Kim, S.H., Yang, J.H., 2009. Perfluorooctanoic acid alters T lymphocyte phenotypes and cytokine expression in mice. Environ Toxicol 24, 580-588.
Soukupova, D., Dostal, M., Piza, J., 1991. Developmental toxicity of cadmium in mice. II. Immunotoxic effects. Funct Dev Morphol 1, 31-36.
Tanaka, K., Tanaka, H., Kanemoto, Y., Tsuboi, I., 1998. The effects of nonsteroidal anti-inflammatory drugs on immune functions of human peripheral blood mononuclear cells. Immunopharmacology 40, 209-217.
Teixeira, D., Marques, C., Pestana, D., Faria, A., Norberto, S., Calhau, C., Monteiro, R., 2016. Effects of xenoestrogens in human M1 and M2 macrophage migration, cytokine release, and estrogen-related signaling pathways. Environ Toxicol 31, 1496-1509.
Theocharis, S.E., Souliotis, V.L., Panayiotidis, P.G., 1994. Suppression of interleukin-1 beta and tumour necrosis factor-alpha biosynthesis by cadmium in in vitro activated human peripheral blood mononuclear cells. Arch Toxicol 69, 132-136.
Thomas, D.J., Reasor, M.J., Wierda, D., 1989. Macrophage regulation of myelopoiesis is altered by exposure to the benzene metabolite hydroquinone. Toxicol Appl Pharmacol 97, 440-453.
Tonk, E.C., Verhoef, A., Gremmer, E.R., van Loveren, H., Piersma, A.H., 2013. Developmental immunotoxicity in male rats after juvenile exposure to ethanol. Toxicology 309, 91-99.
Tryphonas, H., Cooke, G., Caldwell, D., Bondy, G., Parenteau, M., Hayward, S., Pulido, O., 2004. Oral (gavage), in utero and post-natal exposure of Sprague-Dawley rats to low doses of tributyltin chloride. Part II: effects on the immune system. Food Chem Toxicol 42, 221-235.
Urbaschek, R., Mannel, D.N., Urbanczik, R., 1991. Isoniazid protects mice against endotoxin lethality without influencing tumor necrosis factor synthesis and release. Antimicrob Agents Chemother 35, 1666-1668.
Utsunomiya, I., Nagai, S., Oh-ishi, S., 1994. Differential effects of indomethacin and dexamethasone on cytokine production in carrageenin-induced rat pleurisy. Eur J Pharmacol 252, 213-218.
Vos, J.G., van Logten, M.J., Kreeftenberg, J.G., Kruizinga, W., 1979. Hexachlorobenzene-induced stimulation of the humoral immune response in rats. Ann N Y Acad Sci 320, 535-550.
Yamashita, U., Kuroda, E., Yoshida, Y., Sugiura, T., 2003a. Effect of endocrine disrupters on immune responses in vivo. J uoeh 25, 365-374.
Yamashita, U., Sugiura, T., Yoshida, Y., Kuroda, E., 2003b. Effect of endocrine disrupters on

thymocytes in vitro. J uoeh 25, 161-170.
Yamashita, U., Sugiura, T., Yoshida, Y., Kuroda, E., 2005. Effect of endocrine disrupters on macrophage functions in vitro. J uoeh 27, 1-10.
Yang, M., Qiu, W., Chen, B., Chen, J., Liu, S., Wu, M., Wang, K.J., 2015. The in vitro immune modulatory effect of bisphenol A on fish macrophages via estrogen receptor alpha and nuclear factor-kappaB signaling. Environ Sci Technol 49, 1888-1895.
Zdolsek, J.M., Soder, O., Hultman, P., 1994. Mercury induces in vivo and in vitro secretion of interleukin-1 in mice. Immunopharmacology 28, 201-208.
Zidell, R.H., Hatoum, N.S., Thomas, P.T., 1988. Fetal alcohol effects: evidence of developmental impairment in the absence of immunotoxicity. Fundam Appl Toxicol 10, 189-198.

Appendix 13 Refference 60 chemicals

Abe, M., Shimizu, A., Yokoyama, Y., Takeuchi, Y., Ishikawa, O., 2008. A possible inhibitory action of diaminodiphenyl sulfone on tumour necrosis factor-alpha production from activated mononuclear cells on cutaneous lupus erythematosus. Clin Exp Dermatol 33, 759-763.

Ade, N., Antonios, D., Kerdine-Romer, S., Boisleve, F., Rousset, F., Pallardy, M., 2007. NF-kappaB plays a major role in the maturation of human dendritic cells induced by NiSO(4) but not by DNCB. Toxicol Sci 99, 488-501.

Akiyoshi, T., Arinaga, S., Tsuji, H., 1987. Augmentation of the generation of cell-mediated cytotoxicity in culture by mitomycin C. Cancer Immunol Immunother 24, 259-262.

Arkusz, J., Stepnik, M., Lewińska, D., Stańczyk, M., Palus, J., Dziubałtowska, E., 2007. Interleukin-1beta expression in murine J774A.1 macrophages exposed to platinum compounds: the role of p38 and ERK 1/2 mitogen-activated protein kinases. Toxicol In Vitro 21, 371-379.

Bachiega, T.F., Sforcin, J.M., 2011. Lemongrass and citral effect on cytokines production by murine macrophages. J Ethnopharmacol 137, 909-913.

Banerjee, N., Wang, H., Wang, G., Khan, M.F., 2020. Enhancing the Nrf2 antioxidant signaling provides protection against trichloroethene-mediated inflammation and autoimmune response. Toxicol Sci.

Berkarda, B., Marrack, P., Kappler, J.W., Bakemeier, R.F., 1978. Effects of warfarin administration on the immune response of mice. Arzneimittelforschung 28, 1407-1410.

Bilrha, H., Roy, R., Moreau, B., Belles-Isles, M., Dewailly, E., Ayotte, P., 2003. In vitro activation of cord blood mononuclear cells and cytokine production in a remote coastal population exposed to organochlorines and methyl mercury. Environ Health Perspect 111, 1952-1957.

Blakley, B.R., Archer, D.L., 1981. The effect of lead acetate on the immune response in mice. Toxicol Appl Pharmacol 61, 18-26.

Brown, D.M., Donaldson, K., Stone, V., 2004. Effects of PM10 in human peripheral blood monocytes and J774 macrophages. Respir Res 5, 29.

Bunn, T.L., Parsons, P.J., Kao, E., Dietert, R.R., 2001. Exposure to lead during critical windows of embryonic development: differential immunotoxic outcome based on stage of exposure and gender. Toxicol Sci 64, 57-66.

Burns, L.A., Bradley, S.G., White, K.L., McCay, J.A., Fuchs, B.A., Stern, M., Brown, R.D., Musgrove, D.L., Holsapple, M.P., Luster, M.I., et al., 1994. Immunotoxicity of 2,4-diaminotoluene in female B6C3F1 mice. Drug Chem Toxicol 17, 401-436.

Carfi, M., Gennari, A., Malerba, I., Corsini, E., Pallardy, M., Pieters, R., Van Loveren, H., Vohr, H.W., Hartung, T., Gribaldo, L., 2007. In vitro tests to evaluate immunotoxicity: a preliminary study. Toxicology 229, 11-22.

Chang, D.M., Baptiste, P., Schur, P.H., 1990. The effect of antirheumatic drugs on interleukin 1 (IL-1) activity and IL-1 and IL-1 inhibitor production by human monocytes. J Rheumatol 17, 1148-1157.
Chang, M.P., Norman, D.C., 1991. Immunotoxicity of alcohol in young and old mice. II. Impaired T cell proliferation and T cell-dependent antibody responses of young and old mice fed ethanol-containing liquid diet. Mech Ageing Dev 57, 175-186.
Chen, H., Luo, H., Daloze, P., Xu, D., Shan, X., St-Louis, G., Wu, J., 1993. Long-term in vivo effects of rapamycin on humoral and cellular immune responses in the rat. Immunobiology 188, 303-315.
Chen, S., Golemboski, K., Piepenbrink, M., Dietert, R., 2004. Developmental immunotoxicity of lead in the rat: influence of maternal diet. J Toxicol Environ Health A 67, 495-511.
Cohen, S., Benacerraf, B., McCluskey, R.T., Ovary, Z., 1967. Effect of anticoagulants on delayed hypersensitivity reactions. J Immunol 98, 351-358.
Couleau, N., Falla, J., Beillerot, A., Battaglia, E., D'Innocenzo, M., Plancon, S., Laval-Gilly, P., Bennasroune, A., 2015. Effects of Endocrine Disruptor Compounds, Alone or in Combination, on Human Macrophage-Like THP-1 Cell Response. PLoS One 10, e0131428.
Czuprynski, C.J., Henson, P.M., Campbell, P.A., 1984. Effect of dimethyl sulfoxide on the in vitro and in vivo bactericidal activity of human and mouse neutrophils and mononuclear phagocytes. Inflammation 8, 181-191.
Darisipudi, M.N., Allam, R., Rupanagudi, K.V., Anders, H.J., 2011. Polyene macrolide antifungal drugs trigger interleukin-1beta secretion by activating the NLRP3 inflammasome. PLoS One 6, e19588.
Descotes, J., Evreux, J.C., Laschi-Locquerie, A., Tachon, P., 1984. Comparative effects of various lead salts on delayed hypersensitivity in mice. J Appl Toxicol 4, 265-266.
Devos, S., Van Den Heuvel, R., Hooghe, R., Hooghe-Peters, E.L., 2004. Limited effect of selected organic pollutants on cytokine production by peripheral blood leukocytes. Eur Cytokine Netw 15, 145-151.
Dieter, M.P., Luster, M.I., Boorman, G.A., Jameson, C.W., Dean, J.H., Cox, J.W., 1983. Immunological and biochemical responses in mice treated with mercuric chloride. Toxicol Appl Pharmacol 68, 218-228.
DiMartino, M.J., Johnson, W.J., Votta, B., Hanna, N., 1987. Effect of antiarthritic drugs on the enhanced interleukin-1 (IL-1) production by macrophages from adjuvant-induced arthritic (AA) rats. Agents Actions 21, 348-350.
Dohlsten, M., Kalland, T., Sjögren, H.O., Carlsson, R., 1988. Histamine inhibits interleukin 1 production by lipopolysaccharide-stimulated human peripheral blood monocytes. Scand J Immunol 27, 527-532.

Edwards, R.L., Rickles, F.R., 1978. Delayed hypersensitivity in man: effects of systemic anticoagulation. Science 200, 541-543.
Enose-Akahata, Y., Matsuura, E., Tanaka, Y., Oh, U., Jacobson, S., 2012. Minocycline modulates antigen-specific CTL activity through inactivation of mononuclear phagocytes in patients with HTLV-I associated neurologic disease. Retrovirology 9, 16.
Eugui, E.M., Almquist, S.J., Muller, C.D., Allison, A.C., 1991. Lymphocyte-selective cytostatic and immunosuppressive effects of mycophenolic acid in vitro: role of deoxyguanosine nucleotide depletion. Scand J Immunol 33, 161-173.
Freund, Y.R., Riccio, E.S., Phillips, S.J., Dousman, L., MacGregor, J.T., 1998. Pyrimethamine impairs host resistance to infection with Listeria monocytogenes in BALB/c mice. Toxicol Sci 42, 91-98.
Fukuzawa, M., Satoh, J., Muto, G., Muto, Y., Nishimura, S., Miyaguchi, S., Qiang, X.L., Toyota, T., 1997. Inhibitory effect of nicotinamide on in vitro and in vivo production of tumor necrosis factor-alpha. Immunol Lett 59, 7-11.
Futamura, Y., Matsumoto, K., 1995. Characteristics of peripheral blood monocytes and bone marrow macrophages from rats treated with mitomycin C, 5-fluorouracil or phenylhydrazine. J Toxicol Sci 20, 1-7.
Gardner, R.M., Nyland, J.F., Evans, S.L., Wang, S.B., Doyle, K.M., Crainiceanu, C.M., Silbergeld, E.K., 2009. Mercury induces an unopposed inflammatory response in human peripheral blood mononuclear cells in vitro. Environ Health Perspect 117, 1932-1938.
Gaworski, C.L., Vollmuth, T.A., Dozier, M.M., Heck, J.D., Dunn, L.T., Ratajczak, H.V., Thomas, P.T., 1994. An immunotoxicity assessment of food flavouring ingredients. Food Chem Toxicol 32, 409-415.
Gray, B.N., Walker, C., 1979. Augmentation of lymphocyte surface immunogenicity following treatment with dimethyl-sulphoxide. Int Arch Allergy Appl Immunol 60, 390-397.
Gupta, P., Sodhi, A., 1987. Increased release of interleukin-1 from mouse peritoneal macrophages in vitro after cisplatin treatment. Int J Immunopharmacol 9, 385-388.
Haley, P.J., Shopp, G.M., Benson, J.M., Cheng, Y.S., Bice, D.E., Luster, M.I., Dunnick, J.K., Hobbs, C.H., 1990. The immunotoxicity of three nickel compounds following 13-week inhalation exposure in the mouse. Fundam Appl Toxicol 15, 476-487.
Hansen, J.F., Nielsen, C.H., Brorson, M.M., Frederiksen, H., Hartoft-Nielsen, M.L., Rasmussen, A.K., Bendtzen, K., Feldt-Rasmussen, U., 2015. Influence of phthalates on in vitro innate and adaptive immune responses. PLoS One 10, e0131168.
Hemdan, N.Y., Emmrich, F., Adham, K., Wichmann, G., Lehmann, I., El-Massry, A., Ghoneim, H., Lehmann, J., Sack, U., 2005. Dose-dependent modulation of the in vitro cytokine production of human immune competent cells by lead salts. Toxicol Sci 86, 75-83.

Hemdan, N.Y., Lehmann, I., Wichmann, G., Lehmann, J., Emmrich, F., Sack, U., 2007. Immunomodulation by mercuric chloride in vitro: application of different cell activation pathways. Clin Exp Immunol 148, 325-337.
Henderson, D.J., Naya, I., Bundick, R.V., Smith, G.M., Schmidt, J.A., 1991. Comparison of the effects of FK-506, cyclosporin A and rapamycin on IL-2 production. Immunology 73, 316-321.
Himmerich, H., Schonherr, J., Fulda, S., Sheldrick, A.J., Bauer, K., Sack, U., 2011. Impact of antipsychotics on cytokine production in-vitro. J Psychiatr Res 45, 1358-1365.
Hu, C., Meng, X., Huang, C., Shen, C., Li, J., 2017. Frontline Science: ATF3 is responsible for the inhibition of TNF-alpha release and the impaired migration of acute ethanol-exposed monocytes and macrophages. J Leukoc Biol 101, 633-642.
Ingham, E., 1990. Modulation of the proliferative response of murine thymocytes stimulated by IL-1, and enhancement of IL-1 beta secretion from mononuclear phagocytes by tetracyclines. J Antimicrob Chemother 26, 61-70.
Jahnová, E., Ferencík, M., Nyulassy, S., Devínsky, F., Lacko, I., 1993. Amphiphilic detergents inhibit production of IgG and IgM by human peripheral blood mononuclear cells. Immunol Lett 39, 71-75.
Johnson, W.J., DiMartino, M.J., Meunier, P.C., Muirhead, K.A., Hanna, N., 1988. Methotrexate inhibits macrophage activation as well as vascular and cellular inflammatory events in rat adjuvant induced arthritis. J Rheumatol 15, 745-749.
Jokay, I., Kelemenics, K., Karczag, E., Foldes, I., 1980. Interactions of glucocorticoids and heparin on the humoral immune response of mice. Immunobiology 157, 390-400.
Jonsson, C.A., Carlsten, H., 2002. Mycophenolic acid inhibits inosine 5'-monophosphate dehydrogenase and suppresses production of pro-inflammatory cytokines, nitric oxide, and LDH in macrophages. Cell Immunol 216, 93-101.
Kater, A.P., Peppelenbosch, M.P., Brandjes, D.P., Lumbantobing, M., 2002. Dichotomal effect of the coumadin derivative warfarin on inflammatory signal transduction. Clin Diagn Lab Immunol 9, 1396-1397.
Kawai, R., Ito, S., Aida, T., Hattori, H., Kimura, T., Furukawa, T., Mori, K., Sanbuissho, A., Kawada, T., 2013. Evaluation of primary and secondary responses to a T-cell-dependent antigen, keyhole limpet hemocyanin, in rats. J Immunotoxicol 10, 40-48.
Kay, J.E., Kromwel, L., Doe, S.E., Denyer, M., 1991. Inhibition of T and B lymphocyte proliferation by rapamycin. Immunology 72, 544-549.
Kimball, P.M., Kerman, R.H., Kahan, B.D., 1991. Production of synergistic but nonidentical mechanisms of immunosuppression by rapamycin and cyclosporine. Transplantation 51, 486-490.
Kloppenburg, M., Brinkman, B.M., de Rooij-Dijk, H.H., Miltenburg, A.M., Daha, M.R., Breedveld, F.C., Dijkmans, B.A., Verweij, C., 1996. The tetracycline derivative minocycline differentially

affects cytokine production by monocytes and T lymphocytes. Antimicrob Agents Chemother 40, 934-940.
Kucharz, E.J., Sierakowski, S.J., 1992. Studies on immunomodulatory properties of isoniazid: V. Influence of isoniazid on secretion of interleukin-1. J Hyg Epidemiol Microbiol Immunol 36, 119-122.
Kurohara, M., Yasuda, H., Moriyama, H., Nakayama, M., Sakata, M., Yamada, K., Kotani, R., Hara, K., Yokono, K., Nagata, M., 2008. Low-dose warfarin functions as an immunomodulator to prevent cyclophosphamide-induced NOD diabetes. Kobe J Med Sci 54, E1-13.
Langezaal, I., Hoffmann, S., Hartung, T., Coecke, S., 2002. Evaluation and prevalidation of an immunotoxicity test based on human whole-blood cytokine release. Altern Lab Anim 30, 581-595.
Larsen, S.T., Lund, R.M., Nielsen, G.D., Thygesen, P., Poulsen, O.M., 2002. Adjuvant effect of di-n-butyl-, di-n-octyl-, di-iso-nonyl- and di-iso-decyl phthalate in a subcutaneous injection model using BALB/c mice. Pharmacol Toxicol 91, 264-272.
Lee, S.W., Tsou, A.P., Chan, H., Thomas, J., Petrie, K., Eugui, E.M., Allison, A.C., 1988. Glucocorticoids selectively inhibit the transcription of the interleukin 1 beta gene and decrease the stability of interleukin 1 beta mRNA. Proc Natl Acad Sci U S A 85, 1204-1208.
Li, L., Li, H.S., Song, N.N., Chen, H.M., 2013. The immunotoxicity of dibutyl phthalate on the macrophages in mice. Immunopharmacol Immunotoxicol 35, 272-281.
Lin, C.Y., Tsai, P.S., Hung, Y.C., Huang, C.J., 2010. L-type calcium channels are involved in mediating the anti-inflammatory effects of magnesium sulphate. Br J Anaesth 104, 44-51.
Lourenco, A.C., Galbiati, V., Corti, D., Papale, A., Martino-Andrade, A.J., Corsini, E., 2015. The plasticizer dibutyl phthalate (DBP) potentiates chemical allergen-induced THP-1 activation. Toxicol In Vitro 29, 2001-2008.
Luebke, R.W., Rogers, R.R., Riddle, M.M., Rowe, D.G., Smialowicz, R.J., 1987. Alteration of immune function in mice following carcinogen exposure. Immunopharmacology 13, 1-9.
Manosroi, J., Manosroi, A., Vithayasai, V., 1987. Effect of histamine and antihistamines on interleukin-1 production by human monocytes. Microbiol Immunol 31, 1217-1230.
Marshall, M., Moore, P.K., 2004. Effect of nitric oxide releasing paracetamol and flurbiprofen on cytokine production in human blood. Eur J Pharmacol 483, 317-322.
Meredith, C., Scott, M.P., 1994. Altered gene expression in immunotoxicology screening in vitro: Comparison with ex vivo analysis. Toxicol In Vitro 8, 751-753.
Mitjans, M., Viviani, B., Lucchi, L., Galli, C.L., Marinovich, M., Corsini, E., 2008. Role of p38 MAPK in the selective release of IL-8 induced by chemical allergen in naive THp-1 cells. Toxicol In Vitro 22, 386-395.
Miyazawa, M., Ito, Y., Yoshida, Y., Sakaguchi, H., Suzuki, H., 2007. Phenotypic alterations and cytokine production in THP-1 cells in response to allergens. Toxicol In Vitro 21, 428-437.

Mudzinski, S.P., Rudofsky, U.H., Mitchell, D.G., Lawrence, D.A., 1986. Analysis of lead effects on in vivo antibody-mediated immunity in several mouse strains. Toxicol Appl Pharmacol 83, 321-330.

Nastevska, C., Gerber, E., Horbach, M., Rohrdanz, E., Kahl, R., 1999. Impairment of TNF-alpha expression and secretion in primary rat liver cell cultures by acetaminophen treatment. Toxicology 133, 85-92.

Nelson, D.S., 1965. The effects of anticoagulants and other drugs on cellular and cutaneous reactions to antigen in guinea-pigs with delayed-type hypersensitivity. Immunology 9, 219-234.

Neuhaus, V., Danov, O., Konzok, S., Obernolte, H., Dehmel, S., Braubach, P., Jonigk, D., Fieguth, H.G., Zardo, P., Warnecke, G., Martin, C., Braun, A., Sewald, K., 2018. Assessment of the Cytotoxic and Immunomodulatory Effects of Substances in Human Precision-cut Lung Slices. J Vis Exp.

Nowacki, W., Malpuech-Brugère, C., Rock, E., Rayssiguier, Y., 2009. High-magnesium concentration and cytokine production in human whole blood model. Magnes Res 22, 93-96.

Ohsaki, Y., Shirakawa, H., Miura, A., Giriwono, P.E., Sato, S., Ohashi, A., Iribe, M., Goto, T., Komai, M., 2010. Vitamin K suppresses the lipopolysaccharide-induced expression of inflammatory cytokines in cultured macrophage-like cells via the inhibition of the activation of nuclear factor κB through the repression of IKK α/β phosphorylation. J Nutr Biochem 21, 1120-1126.

Okamoto, M., Sasano, M., Goto, M., Nishioka, K., Aotsuka, S., Nakamura, K., Yokohari, R., 1991. Suppressive effect of anti-rheumatic drugs on interleukin-1 beta release from human peripheral blood monocytes. Int J Immunopharmacol 13, 39-43.

Pai, K., Shrivastava, A., Kumar, R., Khetarpal, S., Sarmah, B., Gupta, P., Sodhi, A., 1997. Activation of P388D1 macrophage cell line by chemotherapeutic drugs. Life Sci 60, 1239-1248.

Pang, T., Wang, J., Benicky, J., Saavedra, J.M., 2012. Minocycline ameliorates LPS-induced inflammation in human monocytes by novel mechanisms including LOX-1, Nur77 and LITAF inhibition. Biochim Biophys Acta 1820, 503-510.

Parthasarathy, N.J., Srikumar, R., Manikandan, S., Narayanan, G.S., Devi, R.S., 2007. Effect of methanol intoxication on specific immune functions of albino rats. Cell Biol Toxicol 23, 177-187.

Pellat-Deceunynck, C., Wietzerbin, J., Drapier, J.C., 1994. Nicotinamide inhibits nitric oxide synthase mRNA induction in activated macrophages. Biochem J 297 (Pt 1), 53-58.

Rogers, P.D., Jenkins, J.K., Chapman, S.W., Ndebele, K., Chapman, B.A., Cleary, J.D., 1998. Amphotericin B activation of human genes encoding for cytokines. J Infect Dis 178, 1726-1733.

Rogers, P.D., Stiles, J.K., Chapman, S.W., Cleary, J.D., 2000. Amphotericin B induces expression of genes encoding chemokines and cell adhesion molecules in the human monocytic cell line THP-1. J Infect Dis 182, 1280-1283.

Rordorf-Adam, C., Lazdins, J., Woods-Cook, K., Alteri, E., Henn, R., Geiger, T., Feige, U., Towbin, H., Erard, F., 1989. An assay for the detection of interleukin-1 synthesis inhibitors: effects of antirheumatic drugs. Drugs Exp Clin Res 15, 355-362.

Santos, B.C., Starobinas, N., Barbuto, J.A., Russo, M., Schor, N., 2003. Absence of peripheral blood mononuclear cells priming in hemodialysis patients. Braz J Med Biol Res 36, 219-225.

Sheikhi, A., Jaberi, Y., Esmaeilzadeh, A., Khani, M., Moosaeefard, M., Shafaqatian, M., 2007. The effect of cardiovascular drugs on pro-inflammatory cytokine secretion and natural killer activity of peripheral blood mononuclear cells of patients with chronic heart failure in vitro. Pak J Biol Sci 10, 1580-1587.

Shirota, H., Goto, M., Hashida, R., Yamatsu, I., Katayama, K., 1989. Inhibitory effects of E-5110 on interleukin-1 generation from human monocytes. Agents Actions 27, 322-324.

Snyder, D.S., Unanue, E.R., 1982. Corticosteroids inhibit murine macrophage Ia expression and interleukin 1 production. J Immunol 129, 1803-1805.

Stosic-Grujicic, S., Simic, M.M., 1982. Modulation of interleukin 1 production by activated macrophages: in vitro action of hydrocortisone, colchicine, and cytochalasin B. Cell Immunol 69, 235-247.

Tai, K., Iwasaki, H., Ikegaya, S., Ueda, T., 2013. Minocycline modulates cytokine and chemokine production in lipopolysaccharide-stimulated THP-1 monocytic cells by inhibiting IkappaB kinase alpha/beta phosphorylation. Transl Res 161, 99-109.

Takahashi, H.K., Iwagaki, H., Mori, S., Yoshino, T., Tanaka, N., Nishibori, M., 2004. Histamine inhibits lipopolysaccharide-induced interleukin (IL)-18 production in human monocytes. Clin Immunol 112, 30-34.

Teixeira, D., Marques, C., Pestana, D., Faria, A., Norberto, S., Calhau, C., Monteiro, R., 2016. Effects of xenoestrogens in human M1 and M2 macrophage migration, cytokine release, and estrogen-related signaling pathways. Environ Toxicol 31, 1496-1509.

Thomson, A.W., Woo, J., Yao, G.Z., Todo, S., Starzl, T.E., Zeevi, A., 1993. Effects of combined administration of FK 506 and the purine biosynthesis inhibitors mizoribine or mycophenolic acid on lymphocyte DNA synthesis and T cell activation molecule expression in human mixed lymphocyte cultures. Transpl Immunol 1, 146-150.

Thong, Y.H., Ferrante, A., 1980. Immunopotentiation by pyrimethamine in the mouse. Clin Exp Immunol 39, 190-194.

Tonk, E.C., de Groot, D.M., Wolterbeek, A.P., Penninks, A.H., Waalkens-Berendsen, I.D., Piersma, A.H., van Loveren, H., 2013. Developmental immunotoxicity of ethanol in an extended one-generation reproductive toxicity study. Arch Toxicol 87, 323-335.

Urbaschek, R., Mannel, D.N., Urbanczik, R., 1991. Isoniazid protects mice against endotoxin lethality without influencing tumor necrosis factor synthesis and release. Antimicrob Agents

Chemother 35, 1666-1668.
Utsunomiya, I., Nagai, S., Oh-ishi, S., 1994. Differential effects of indomethacin and dexamethasone on cytokine production in carrageenin-induced rat pleurisy. Eur J Pharmacol 252, 213-218.
Van Dijk, H., Bloksma, N., Rademaker, P.M., Schouten, W.J., Willers, J.M., 1979. Differential potencies of corticosterone and hydrocortisone in immune and immune-related processes in the mouse. Int J Immunopharmacol 1, 285-292.
Van Wauwe, J., Aerts, F., Van Genechten, H., Blockx, H., Deleersnijder, W., Walter, H., 1996. The inhibitory effect of pentamidine on the production of chemotactic cytokines by in vitro stimulated human blood cells. Inflamm Res 45, 357-363.
Vos, J.G., van Logten, M.J., Kreeftenberg, J.G., Kruizinga, W., 1979. Hexachlorobenzene-induced stimulation of the humoral immune response in rats. Ann N Y Acad Sci 320, 535-550.
Wang, J.Y., Wicklund, B.H., Gustilo, R.B., Tsukayama, D.T., 1996. Titanium, chromium and cobalt ions modulate the release of bone-associated cytokines by human monocytes/macrophages in vitro. Biomaterials 17, 2233-2240.
Woo, J., Stephen, M., Thomson, A.W., 1988. Spleen lymphocyte populations and expression of activation markers in rats treated with the potent new immunosuppressive agent FK-506. Immunology 65, 153-155.
Xu, F., Ji, Q., Zhang, J., Huang, W., Cao, Z., Li, Y., 2018. AlCl3 inhibits LPS-induced NLRP3 inflammasome activation and IL-1beta production through suppressing NF-kappaB signaling pathway in murine peritoneal macrophages. Chemosphere 209, 972-980.
Yamamoto, N., Sakai, F., Yamazaki, H., Nakahara, K., Okuhara, M., 1996. Effect of FR167653, a cytokine suppressive agent, on endotoxin-induced disseminated intravascular coagulation. Eur J Pharmacol 314, 137-142.
Yang, H.M., Barger, M.W., Castranova, V., Ma, J.K., Yang, J.J., Ma, J.Y., 1999. Effects of diesel exhaust particles (DEP), carbon black, and silica on macrophage responses to lipopolysaccharide: evidence of DEP suppression of macrophage activity. J Toxicol Environ Health A 58, 261-278.
Zdolsek, J.M., Soder, O., Hultman, P., 1994. Mercury induces in vivo and in vitro secretion of interleukin-1 in mice. Immunopharmacology 28, 201-208.
Zhang, Y., Liu, X., McHale, C., Li, R., Zhang, L., Wu, Y., Ye, X., Yang, X., Ding, S., 2013. Bone marrow injury induced via oxidative stress in mice by inhalation exposure to formaldehyde. PLoS One 8, e74974.
Zidell, R.H., Hatoum, N.S., Thomas, P.T., 1988. Fetal alcohol effects: evidence of developmental impairment in the absence of immunotoxicity. Fundam Appl Toxicol 10, 189-198.

Appendix 14 Table 1. The summary of immunotoxicological data of 25 chemicals

			Endpoint 1	Endpoint 2	Endpoint 3	Endpoint 4	Endpoint 5	Endpoint 6	Endpoint 7	
	Chemical name	cas no.	1. Decreased antibody response	2. Suppressed T cell proliferation in the stimulation with innate immune cells, such as dendritic cells or macrophages	3. Decreased LPS response in vivo, ex vivo, or in vitro	4. Suppressed DHR	5. Suppressed host resistance	6. The NTP data or Tox 21 data indicates immunotoxicity of chemicals	7. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.	
									IL-1	TNF-a
Phase I	Dibutyl phthalate	84-74-2	A mice Larsen et al. 2002							
	Acetaminophen	103-90-2	S mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm91019/index.html	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm91019/index.html	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm91019/index.html					
	Isonicotinic Acid Hydrazide (Isoniazid)	54-85-3	no effect (spleen IgM AFC) S (serum titers) mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm96002/index.html1	S mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm96002/index.html0			negative	positive		
	Sulem Mercury(II) Chloride	7487-94-7	S mice Dieter et al. 1983	S mice Dieter et al. 1983	A mice Dieter et al. 1983					
	Hexachlorobenzene	118-74-1	A rat Vos et al. 1979		A rat Vos et al. 1979	no effect rat Vos et al. 1979				S (PHA) human cord blood mononuclear cells Bilrha et al. 2003

Appendix 14 Table 1. The summary of immunotoxicological data of 25 chemicals(continue)

		Endpoint 7(continue)		Endpoint 8				Criterion 1	Criterion 2	Criterion 3	
	Chemical name	7(continue). Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.		8. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells in vitro.				Satisfy one of Endpoints 1 to 6	Increased or decreased mRNA expression or protein production in one or more cytokines in Endpoints 7 or 8 in multiple reports	Increased or decreased mRNA expression or protein production in two or more cytokines in Endpoints 3, 7 or 8	Result from refernece data
		IL-6	other	IL-1	TNF-a	IL-6	other				
Phase I	Dibutyl phthalate			S F4/80+ macrophages (chemical only) mice Li et al. 2013 no effect cell line (THP-1)(PMA) human Couleau et al. 2015 no effect monocytes/macrophages (LPS) human Hansen et al. 2015 no effect (M1) A (M2) human PBMC-derived macrophage (LPS or IL-4) Teixeira et al. 2016	S F4/80+ macrophages (chemical only) mice Li et al. 2013 A cell line (THP-1)(PMA) human Couleau et al. 2015 S monocytes/macrophages (LPS) human Hansen et al. 2015	S F4/80+ macrophages mice (chemical only) Li et al. 2013 A monocytes/macrophages (LPS) human Hansen et al. 2015 S human PBMC-derived macrophage (LPS or IL-4) Teixeira et al. 2016	no effect (IL-8) cell line (THP-1)(PMA) human Couleau et al. 2015 A (IL-8) monocytes/macrophages (LPS) human Hansen et al. 2015 no effect (IL-8) (protein) A (mRNA) cell line (THP-1) (chemical only) human Lourenco et al. 2015	satisfy	satisfy	satisfy	Positive
	Acetaminophen			S human monocyte (LPS) Chang et al. 1990 no effect (LPS) human whole blood Marshall & Moore 2004	no effect (chemical only) rat Kupffer cells Nastevska et al. 1999 no effect (LPS) human whole blood Marshall & Moore 2004			satisfy	not satisfy	not satisfy	Positive
	Isonicotinic Acid Hydrazide (Isoniazid)			S human monocyte (LPS) Kucharz and Sierakowski 1992	no effect (S. aureus) human PBMC Urbaschek et al. 1991			satisfy	not satisfy	not satisfy	Positive
	Sulem Mercury(II) Chloride			A mice peritoneal macrophage (chemical only) Zdolsek et al. 1994 A (LPS) human PBMC Gardner et al. 2009	S (heat-killed *Salmonella enterica*) human PBMC Hemdan et al. 2007 A (LPS) human PBMC Gardner et al. 2009	S (heat-killed *Salmonella enterica*) human PBMC Hemdan et al. 2007		satisfy	satisfy	satisfy	Positive
	Hexachlorobenzene				no effect (PHA, *Dermatophagoides pteronyssinus* extract, PMA) human PBMC Devos et al. 2004	no effect (PHA, *Dermatophagoides pteronyssinus* extract, PMA) human PBMC Devos et al. 2004		satisfy	not satisfy	not satisfy	Positive

Appendix 14 Table 1. The summary of immunotoxicological data of 25 chemicals(continue)

			Endpoint 1	Endpoint 2	Endpoint 3	Endpoint 4	Endpoint 5	Endpoint 6	Endpoint 7	
	Chemical name	cas no.	1. Decreased antibody response	2. Suppressed T cell proliferation in the stimulation with innate immune cells, such as dendritic cells or macrophages	3. Decreased LPS response in vivo, ex vivo, or in vitro	4. Suppressed DHR	5. Suppressed host resistance	6. The NTP data or Tox 21 data indicates immunotoxicity of chemicals	7. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.	
									IL-1	TNF-a
Phase II	Cadmium chloride	10108-64-2	S mice Blakley 1985 S mice Blakley and Tomar 1986 S mice Krzystyniak et al. 1987 A mice Soukupova et al. 1991		A mice Blakley 1985 S mice Krzystyniak et al. 1987 A mice Soukupova et al. 1991 1	S mice Soukupova et al. 1991 0	negative	positive		
	5,5-Diphenylhydantoin sodium salt	630-93-3	S mice Andrade-Mena et al. 1994 no effect mice Okada et al. 2001 0		S mice splenocytes Okada et al. 2001 0	S mice Andrade-Mena et al. 1994 no effect mice (offspring of dams treated with DPH) Chapman and Roberts 1984	positive	positive	S mice spleen adherent cells (Staphylococcus aureus Cowan I) Okada et al. 2001	
	Indomethacin	53-86-1	S mice Barasoain et al. 1980 S mice Rojo et al. 1981 S rat Kushima et al. 2009	1	S mice Barasoain et al. 1980 A (low dose) S (high dose) mice Boorman et al. 1982 S rat Seng et al. 1990	no effect mice Boorman et al. 1982 0	positive	positive	no effect (splenic cells) S (peritoneal cells) rat splenic cells peritoneal exudate (LPS) DiMartino et al. 1987	no effect rat splenocyte (ConA or LPS) Kushima et al. 2009
	Pentachlorophenol	87-86-5	1 S mice Kerkvliet et al. 1982 S (per 10^6 cells) N (per spleen) rat Blakley et al. 1998 S (7Days) no effect (14Days) mice Chen et al. 2013	0 S mice Kerkvliet et al. 1985 S (7Days) no effect (14Days) mice Chen et al. 2013	0 no effect mice Kerkvliet et al. 1985 A rat Blakley et al. 1998	0	negative	positive		

Appendix 14 Table 1. The summary of immunotoxicological data of 25 chemicals(continue)

		Endpoint 7(continue)		Endpoint 8				Criterion 1	Criterion 2	Criterion 3	
	Chemical name	7. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo. (continue)		8. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells in vitro.				Satisfy one of Endpoints 1 to 6	Increased or decreased mRNA expression or protein production in one or more cytokines in Endpoints 7 or 8 in multiple reports	Increased or decreased mRNA expression or protein production in two or more cytokines in Endpoints 3, 7 or 8	Result from refernece data
		IL-6	other	IL-1	TNF-a	IL-6	other				
Phase II	Cadmium chloride			S human PBMC (PHA) Theocharis et al. 1994 A (low), no effect (high) human PBMC (chemical only) Marth et al. 2000 S human PBMC (activated by heat-killed Salmonella Enteritidis) Hemdan et al. 2006 S (LPS) mice cell line (RAW264.7) Jin et al. 2016	S human PBMC (PHA) Theocharis et al. 1994 S human PBMC (activated by heat-killed Salmonella Enteritidis) Hemdan et al. 2006 S (LPS) mice cell line (RAW264.7) Jin et al. 2016	S (LPS) mice cell line (RAW264.7) Jin et al. 2016		satisfy	satisfy	satisfy	Positive
	5,5-Diphenylhydantoin sodium salt	no effect mice spleen adherent cells (Staphylococcus aureus Cowan I) Okada et al. 2001		A human PBMC, cell line (U937)(LPS) Modeer et al. 1989	S (LPS) human PBMC Serra et al. 2010	No effect (LPS) human PBMC Serra et al. 2010		satisfy	not satisfy	satisfy	Positive
	Indomethacin	no effect rat splenocyte (ConA or LPS) Kushima et al. 2009		A human monocyte (LPS) Rordorf-Adam et al.1989 S human monocyte (LPS) Chang et al. 1990 A rat pleural exudate (carrageenin) Utsunomiya et al. 1994	A rat pleural exudate (carrageenin) Utsunomiya et al. 1994 A human whole blood (LPS) Hartel et al. 2004 no effect rat splenocyte (ConA or LPS) Kushima et al. 2009	S rat pleural exudate (carrageenin) Utsunomiya et al. 1994 S human PBMC (LPS) Tanaka et al. 1998 A human whole blood (LPS) Hartel et al. 2004 S rat splenocyte (ConA or LPS) Kushima et al. 2009		satisfy	satisfy	satisfy	Positive
	Pentachlorophenol			A human MD-PBMCS, PBMCs Martin and Whalen 2017	S no effect (PHA, *Dermatophagoides pteronyssinus* extract, PMA) human PBMC Devos et al. 2004	no effect (PHA, *Dermatophagoides pteronyssinus* extract, PMA) human PBMC Devos et al. 2004		satisfy	not satisfy	satisfy	Positive

Appendix 14 Table 1. The summary of immunotoxicological data of 25 chemicals(continue)

			Endpoint 1	Endpoint 2	Endpoint 3	Endpoint 4	Endpoint 5	Endpoint 6	Endpoint 7	
	Chemical name	cas no.	1. Decreased antibody response	2. Suppressed T cell proliferation in the stimulation with innate immune cells, such as dendritic cells or macrophages	3. Decreased LPS response in vivo, ex vivo, or in vitro	4. Suppressed DHR	5. Suppressed host resistance	6. The NTP data or Tox 21 data indicates immunotoxicity of chemicals	7. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.	
									IL-1	TNF-a
Phase II	Urethane	51-79-6	S mice Luster et al. 1982 S mice Luebke et al. 1986 no effect mice Luebke et al. 1987 1	no effect mice Luebke et al. 1987 0	S mice Luebke et al. 1987 no effect rat, mice spleen cells Carfi et al. 2007	no effect mice Luster et al. 1982 S mice Luebke et al. 1987 0		positive	S rat spleen (chemical only) Bette et al. 2004	S rat spleen (chemical only) Bette et al. 2004
	Tributyltin chloride	1461-22-9	no effect rat Tryphonas et al. 2004 S mice Chen et al. 2011		S rat, mice spleen cells Carfi et al. 2007	A (low and middle doses) S (high dose) rat Tryphonas et al. 2004 S mice Chen et al. 2011			no effect mice macrophages (infected with E. coli) Kimura et al. 2005	no effect mice macrophages (infected with E. coli) Kimura et al. 2005
	Perfluorooctanois acid	335-67-1	S mice DeWitt et al. 2008						A mice spleen (chemical only) Son et al. 2009	A mice spleen (chemical only) Son et al. 2009 A (peritoneal cavity, bone marrow) S (spleen) mice (LPS) Qazi et al. 2009
	Hydroquinone	123-31-9								
	4-Aminophenyl sulfone	80-08-0	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm90015/index.html 0	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm90015/index.html 0	A mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm90015/index.html 1			negative		

Appendix 14 Table 1. The summary of immunotoxicological data of 25 chemicals(continue)

		Endpoint 7(continue)		Endpoint 8				Criterion 1	Criterion 2	Criterion 3	
	Chemical name	7(continue). Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.		8. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells in vitro.				Satisfy one of Endpoints 1 to 6	Increased or decreased mRNA expression or protein production in one or more cytokines in Endpoints 7 or 8 in multiple reports	Increased or decreased mRNA expression or protein production in two or more cytokines in Endpoints 3, 7 or 8	Result from refernece data
		IL-6	other	IL-1	TNF-a	IL-6	other				
Phase II	Urethane	A rat spleen (chemical only) Bette et al. 2004			no effect human whole blood (LPS) Carfi et al. 2007			satisfy	not satisfy	satisfy	Positive
	Tributyltin chloride	no effect mice macrophages (infected with E. coli) Kimura et al. 2005			S or A human whole blood (LPS) Carfi et al. 2007			satisfy	not satisfy	not satisfy	Positive
	Perfluorooctanois acid	A mice spleen (chemical only) Son et al. 2009 A (peritoneal cavity) mice (LPS) Qazi et al. 2009			no effect human PBMC (LPS) Brieger et al. 2011 S human PBMC, cell line (THP-1) (LPS) Corsini et al. 2011, 2012	no effect human PBMC (LPS) Brieger et al. 2011 no effect human PBMC (LPS) Corsini et al. 2011, 2012	no effect (IL-8) (PBMC) S (THP-1) human PBMC, cell line (THP-1) (LPS) Corsini et al. 2011, 2012	satisfy	satisfy	satisfy	Positive
	Hydroquinone			S mice macrophages (LPS) Thomas et al. 1989 S (LPS) mice cell line (RAW264.7) Lee et al. 2007 no effect (LPS) human cell line (U-937) Del Bufalo et al. 2011	S (LPS) mice cell line (RAW264.7) Ma and Kinneer 2002 S (LPS) mice cell line (RAW264.7) Lee et al. 2007 no effect (LPS) human cell line (U-937) Del Bufalo et al. 2011	S (LPS) mice cell line (RAW264.7) Lee et al. 2007		not satisfy	satisfy	satisfy	Positive
	4-Aminophenyl sulfone			no effect human PBMC (LPS) Abe et al. 2008 S human Macrophages (?) Interview form of dapsone	S human PBMC (LPS) Abe et al. 2008 S human Macrophages (?) Interview form of dapsone	no effect human PBMC (LPS) Abe et al. 2008 S human Macrophages (?) Interview form of dapsone	S (IL-8) human PBMC (LPS) Abe et al. 2008	satisfy	satisfy	satisfy	Positive

Appendix 14 Table 1. The summary of immunotoxicological data of 25 chemicals(continue)

			Endpoint 1	Endpoint 2	Endpoint 3	Endpoint 4	Endpoint 5	Endpoint 6	Endpoint 7	
	Chemical name	cas no.	1. Decreased antibody response	2. Suppressed T cell proliferation in the stimulation with innate immune cells, such as dendritic cells or macrophages	3. Decreased LPS response in vivo, ex vivo, or in vitro	4. Suppressed DHR	5. Suppressed host resistance	6. The NTP data or Tox 21 data indicates immunotoxicity of chemicals	7. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.	
									IL-1	TNF-a
Phase II	Ethanol	64-17-5	no effect mice Zidell et al. 1988 S mice Chang and Norman 1991 A rat Tonk et al. 2013		no effect mice Zidell et al. 1988 S (PND 21) A (PND 70) rat Tonk et al. 2013	no effect mice Zidell et al. 1988 S rat Tonk et al. 2013				A rat splenocytes Tonk et al. 2013
	5-Nitro-2-furaldehydesemicarbazone	59-87-0	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm90011/index.html 0	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm90011/index.html 0	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm90011/index.html 0		negative	negative		
	Trichloroethylene	79-01-6	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm20006/imm20006.html 0					negative		
	Zinc dimethyldithiocarbamate	137-30-4		A mice Lombardi et al. 1991						
	Citral	5392-40-5	no effect mice Gaworski et al. 1994							

Appendix 14 Table 1. The summary of immunotoxicological data of 25 chemicals(continue)

		Endpoint 7(continue)		Endpoint 8				Criterion 1	Criterion 2	Criterion 3	
	Chemical name	7(continue). Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.		8. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells in vitro.				Satisfy one of Endpoints 1 to 6	Increased or decreased mRNA expression or protein production in one or more cytokines in Endpoints 7 or 8 in multiple reports	Increased or decreased mRNA expression or protein production in two or more cytokines in Endpoints 3, 7 or 8	Result from refernece data
		IL-6	other	IL-1	TNF-a	IL-6	other				
Phase II	Ethanol			A (mRNA)(LPS) S (protein)(LPS) mice cell line (RAW246.7) Hu et al. 2017	S (LPS) mice cell line (RAW246.7) Hu et al. 2017	A (mRNA)(LPS) S (protein)(LPS) mice cell line (RAW246.7) Hu et al. 2017		not satisfy	not satisfy	satisfy	Positive
	5-Nitro-2-furaldehydesemicarbazone							not satisfy	not satisfy	not satisfy	negative
	Trichloroethylene				A (LPS) mice Kupffer cell line Banerjee et al. 2020 (dichloroacetyle chloride)			not satisfy	not satisfy	not satisfy	negative
	Zinc dimethyldithiocarbamate			S mice bone marrow macrophages macrophage-like cell line J774A.1 (LPS) Muroi and Tanamoto 2015	S human cell line (THP-1) (LPS) Corsini et al. 2006			satisfy	not satisfy	satisfy	Positive
	Citral			S mice peritoneal macrophages (LPS) Bachiega et al. 2011		S mice peritoneal macrophages (LPS) Bachiega et al. 2011		not satisfy	not satisfy	satisfy	Positive

Appendix 14 Table 1. The summary of immunotoxicological data of 25 chemicals(continue)

			Endpoint 1	Endpoint 2	Endpoint 3	Endpoint 4	Endpoint 5	Endpoint 6	Endpoint 7	
	Chemical name	cas no.	1. Decreased antibody response	2. Suppressed T cell proliferation in the stimulation with innate immune cells, such as dendritic cells or macrophages	3. Decreased LPS response in vivo, ex vivo, or in vitro	4. Suppressed DHR	5. Suppressed host resistance	6. The NTP data or Tox 21 data indicates immunotoxicity of chemicals	7. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.	
									IL-1	TNF-a
Phase II	t-Butylhydroquinone	1948-33-0	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm87036/index.html 0	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm87036/index.html 0	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm87036/index.html 0	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm87036/index.html 0	negative	negative		
	Bisphenol A	80-05-7	A mice Yoshino et al. 2003a A mice Yamashita et al. 2003b	A mice Yamashita et al. 2003a	S mice Sakazaki et al. 2002 no effect mice Yamashita et al. 2003b					
	2,6-Di-tert-butyl-4-methylphenol	128-37-0	S mice Kim et al. 1996		no effect rat Babu et al. 1998					
	Nonylphenol	84852-15-3	no effect rat https://ntp.niehs.nih.gov/publications/abstracts/imm/mg96003/index.html							
	Sodium chlorite	7758-19-2	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm98005/index.html 0	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm98005/index.html 0	0			negative		
	D(-)-Mannitol	69-65-8								

Appendix 14 Table 1. The summary of immunotoxicological data of 25 chemicals(continue)

		Endpoint 7(continue)		Endpoint 8				Criterion 1	Criterion 2	Criterion 3	
	Chemical name	7(continue). Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.		8. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells in vitro.				Satisfy one of Endpoints 1 to 6	Increased or decreased mRNA expression or protein production in one or more cytokines in Endpoints 7 or 8 in multiple reports	Increased or decreased mRNA expression or protein production in two or more cytokines in Endpoints 3, 7 or 8	Result from refernece data
		IL-6	other	IL-1	TNF-a	IL-6	other				
Phase II	t-Butylhydroquinone				S (LPS) mice cell line (RAW264.7) Ma et al. 2002 no effect (LPS) mice Kupffer cell line Banerjee et al. 2020			not satisfy	not satisfy	not satisfy	negative
	Bisphenol A			A mice peritoneal exudate macrophages (thioglycolate-induced) Yamashita et al. 2005 A human cell line (THP-1)(PMA) Couleau et al. 2015 A carp primary macrophages (chemical only) Yang et al. 2015 no effect human PBMC-derived macrophage (LPS or IL-4) Teixeira et al.	A mice peritoneal exudate macrophages (thioglycolate-induced) Yamashita et al. 2005	A mice peritoneal exudate macrophages (thioglycolate-induced) Yamashita et al. 2005 S human PBMC-derived macrophage (LPS or IL-4) Teixeira et al. 2016	A (IL-8) human cell line (THP-1)(PMA) Couleau et al. 2015	satisfy	satisfy	satisfy	Positive
	2,6-Di-tert-butyl-4-methylphenol			no effect human PBMC (LPS) Eugui et al. 1994	S (LPS) mice PEC Chaudhri et al. 1989 no effect human PBMC (LPS) Eugui et al. 1994	no effect human PBMC (LPS) Eugui et al. 1994		satisfy	not satisfy	not satisfy	Positive
	Nonylphenol			A mice peritoneal exudate macrophages (thioglycolate-induced) Yamashita et al. 2005	A mice peritoneal exudate macrophages (thioglycolate-induced) Yamashita et al. 2005	A mice peritoneal exudate macrophages (thioglycolate-induced) Yamashita et al. 2005		not satisfy	not satisfy	satisfy	Positive
	Sodium chlorite							not satisfy	not satisfy	not satisfy	negative
	D(-)-Mannitol			no effect (LPS) human PBMC Eugui et al. 1994	no effect human monocytes Morohoshi et al. 1996	no effect human monocytes Morohoshi et al. 1996		not satisfy	not satisfy	not satisfy	negative

Blue character: Data from Tox21

Red character: Data for cytokine expression or production after LPS stimulation.

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Appendix 15 Table 2. The summary of immunotoxicological data of 63 chemicals

			Endpoint 1	Endpoint 2	Endpoint 3	Endpoint 4	Endpoint 5	Endpoint 6	Endpoint 7	
	Chemical Name	cas no.	1. Decreased antibody response	2. Suppressed T cell proliferation in the stimulation with innate immune cells, such as dendritic cells or macrophages	3. Decreased LPS response in vivo, ex vivo, or in vitro	4. Suppressed DHR	5. Suppressed host resistance	6. The NTP data or Tox 21 data indicates immunotoxicity of chemicals	7. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.	
									IL-1	TNF-a
Data set	2-Aminoanthracene	613-13-8								
	2-Mercaptobenzothiazole	149-30-4				1		positive		
	2,4-Diaminotoluene	95-80-7	S mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm87034/index.html S mice Burns et al. 1994 0	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm87034/index.html no effect mice Burns et al. 1994 0	A mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm87034/index.html A mice Burns et al. 1994 1	A mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm87034/index.html A mice Burns et al. 1994 1	positive	positive		
	Acetaminophen	103-90-2	S mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm91019/index.html	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm91019/index.html	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm91019/index.html					
	Actinomycin D	50-76-0								
	Aluminum chloride	7784-13-6								
	Amphoterycin B	1397-89-3								
	Azathioprine	446-86-6	1	0	0			positive		
	Benzethonium chloride	121-54-0				0		negative test for contact hypersensitivity		

Appendix 15 Table 2. The summary of immunotoxicological data of 63 chemicals (continue)

		Endpoint 7(continue)		Endpoint 8				Criterion 1	Criterion 2	Criterion 3	
	Chemical Name	7. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.(continue)		8. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells in vitro.				Satisfy one of Endpoints 1 to 6	Increased or decreased mRNA expression or protein production in one or more cytokines in Endpoints 7 or 8 in multiple reports	Increased or decreased mRNA expression or protein production in two or more cytokines in Endpoints 3, 7 or 8	Result from refernece data
		IL-6	other	IL-1	TNF-a	IL-6	other				
Data set	2-Aminoanthracene							not satisfy	not satisfy	not satisfy	negative
	2-Mercaptobenzothiazole							satisfy	not satisfy	not satisfy	Positive
	2,4-Diaminotoluene							satisfy	not satisfy	not satisfy	Positive
	Acetaminophen			S human monocyte (LPS) Chang et al. 1990 no effect (LPS) human whole blood Marshall & Moore 2004	no effect (chemical only) rat Kupffer cells Nastevska et al. 1999 no effect (LPS) human whole blood Marshall & Moore 2004			satisfy	not satisfy	not satisfy	Positive
	Actinomycin D			S LPS human cell line (U937) Lee SW et al. 1988 S Amphotericyin B human cell line (THP-1) Rogers et al. 1998	S chemical only human PBMC Santos et al. 2003			not satisfy	satisfy	satisfy	Positive
	Aluminum chloride			S mice peritoneal macrophase Xu et al. 2018	S mice peritoneal macrophase Xu et al. 2018	S mice peritoneal macrophase Xu et al. 2018		not satisfy	not satisfy	satisfy	Positive
	Amphoterycin B			A chemical only human cell line (THP-1) Rogers et al. 1998 A mice dendritic cells Darisipudi et al. 2011	A chemical only human cell line (THP-1) Rogers et al. 1998		A (IL-8, MCP-1, MIP-1b) chemical only human cell line (THP-1) Rogers et al. 2000	not satisfy	satisfy	satisfy	Positive
	Azathioprine			S mice macrophage Meredith et al. 1994	S mice macrophage Meredith et al. 1994	S mice macrophage Meredith et al. 1994		satisfy	not satisfy	satisfy	Positive
	Benzethonium chloride							not satisfy	not satisfy	not satisfy	negative

Appendix 15 Table 2. The summary of immunotoxicological data of 63 chemicals (continue)

	Chemical Name	cas no.	Endpoint 1 1. Decreased antibody response	Endpoint 2 2. Suppressed T cell proliferation in the stimulation with innate immune cells, such as dendritic cells or macrophages	Endpoint 3 3. Decreased LPS response in vivo, ex vivo, or in vitro	Endpoint 4 4. Suppressed DHR	Endpoint 5 5. Suppressed host resistance	Endpoint 6 6. The NTP data or Tox 21 data indicates immunotoxicity of chemicals	Endpoint 7 7. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.	
									IL-1	TNF-a
	Chloroplatinic acid	18497-13-7								
	Chloroquine	50-63-5							no effect (splenic cells) no effect (peritoneal exudate) rat splenic cells peritoneal exudate DiMartino et al. 1987	
	Chlorpromazine	69-09-0								
	Cisplatin	15663-27-1								
	Citral	5392-40-5	no effect mice Gaworski et al. 1994							
	Cobalt chloride	7791-13-1			no effect human monocyte/macrophage, cell line (U937) Wang et al. 1996					

Appendix 15 Table 2. The summary of immunotoxicological data of 63 chemicals(continue)

		Endpoint 7(continue)		Endpoint 8				Criterion 1	Criterion 2	Criterion 3	
	Chemical Name	7. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.(continue)		8. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells in vitro.				Satisfy one of Endpoints 1 to 6	Increased or decreased mRNA expression or protein production in one or more cytokines in Endpoints 7 or 8 in multiple reports	Increased or decreased mRNA expression or protein production in two or more cytokines in Endpoints 3, 7 or 8	Result from refernece data
		IL-6	other	IL-1	TNF-a	IL-6	other				
	Chloroplatinic acid			A (Ammonium hexachloroplatinate) mice cell line (J774A.1 macrophage) Arkusz et al. 2007	A (Ammonium hexachloroplatinate) human lung (PCLS) Neuhaus et al. 2018		A (IL-8, Ammonium hexachloroplatinate) human cell line (THP-1) Mitjans et al. 2008 A (IL-1a, Ammonium hexachloroplatinate) human lung (PCLS) Neuhaus et al. 2018	not satisfy	satisfy	satisfy	Positive
	Chloroquine			S human monocyte (LPS) Rordorf-Adam et al.1989 S human whole blood Langezaal et al. 2002				not satisfy	satisfy	not satisfy	Positive
	Chlorpromazine			no effect human whole blood Himmerich et al. 2011	A human whole blood Himmerich et al. 2011	no effect human whole blood Himmerich et al. 2011		not satisfy	not satisfy	not satisfy	negative
	Cisplatin			S mice peritoneal macrophage Gupta et al. 1987 A (Ammonium hexachloroplatinate) mice cell line (J774A.1 macrophage) Arkusz et al. 2007				not satisfy	satisfy	not satisfy	Positive
	Citral			S mice peritoneal macrophages (LPS) Bachiega et al. 2011		S mice peritoneal macrophages (LPS) Bachiega et al. 2011		not satisfy	not satisfy	satisfy	Positive
	Cobalt chloride			no effect human monocyte/macrophage, cell line (U937) (LPS) Wang et al. 1996	A human monocyte/macrophage, cell line (U937) (LPS) Wang et al. 1996	no effect human monocyte/macrophage, cell line (U937) (LPS) Wang et al. 1996		not satisfy	not satisfy	not satisfy	negative

Appendix 15 Table 2. The summary of immunotoxicological data of 63 chemicals (continue)

			Endpoint 1	Endpoint 2	Endpoint 3	Endpoint 4	Endpoint 5	Endpoint 6	Endpoint 7	
	Chemical Name	cas no.	1. Decreased antibody response	2. Suppressed T cell proliferation in the stimulation with innate immune cells, such as dendritic cells or macrophages	3. Decreased LPS response in vivo, ex vivo, or in vitro	4. Suppressed DHR	5. Suppressed host resistance	6. The NTP data or Tox 21 data indicates immunotoxicity of chemicals	7. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.	
									IL-1	TNF-a
	Colchicine	64-86-8								
	Cyclophosphamide	6055-19-2	S mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm90015/index.html S mice Luebke et al. 1987 S rat Kawai et al. 2013 1	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm90015/index.html no effect mice Luebke et al. 1987 0	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm90015/index.html S mice Luebke et al. 1987 0	S mice Luebke et al. 1987	positive	positive		
	Cyclosporine A	59865-13-3	1		S rat, mice spleen cells Carfi et al. 2007			positive		
	4-Aminophenyl sulfone, Dapson	80-08-0	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm90015/index.html 0	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm90015/index.html 0	A mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm90015/index.html 1			negative		
	Dexamethasone	50-02-2								
	Dibenzopyrene, dibenzo[a,l]pyrene	191-30-0								

Appendix 15 Table 2. The summary of immunotoxicological data of 63 chemicals (continue)

		Endpoint 7(continue)		Endpoint 8				Criterion 1	Criterion 2	Criterion 3	
	Chemical Name	7. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.(continue)		8. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells in vitro.				Satisfy one of Endpoints 1 to 6	Increased or decreased mRNA expression or protein production in one or more cytokines in Endpoints 7 or 8 in multiple reports	Increased or decreased mRNA expression or protein production in two or more cytokines in Endpoints 3, 7 or 8	Result from refernece data
		IL-6	other	IL-1	TNF-a	IL-6	other				
	Colchicine			A rat peritoneal macrophage s Stosic- Grujicic et al. 1982 no effect human monocyte Chang et al. 1990				not satisfy	not satisfy	not satisfy	negative
	Cyclophosphamide			A (chemical only) mice cell line (macrophag e, P388D1) Pai et al. 1997	A (chemical only) mice cell line (macrophag e, P388D1) Pai et al. 1997			satisfy	not satisfy	satisfy	Positive
	Cyclosporine A			no effect human monocyte (LPS) Rordorf- Adam et al.1989	no effect human whole blood Carfi et al. 2007			satisfy	not satisfy	not satisfy	Positive
	4-Aminophenyl sulfone, Dapson			no effect human PBMC (LPS) Abe et al. 2008 S human Macrophage s (?) Interview form of dapsone	S human PBMC (LPS) Abe et al. 2008 S human Macrophage s (?) Interview form of dapsone	no effect human PBMC (LPS) Abe et al. 2008 S human Macrophage s (?) Interview form of dapsone	S (IL-8) human PBMC (LPS) Abe et al. 2008	satisfy	satisfy	satisfy	Positive
	Dexamethasone			S human cell line (U- 937) Lee et al. 1988 S human monocyte (LPS) Rordorf- Adam et al.1989 S human monocyte Chang et al. 1990 S rat pleural exudate (carrageenin) Utsunomiya et al. 1994	S rat pleural exudate (carrageenin) Utsunomiya et al. 1994	S rat pleural exudate (carrageenin) Utsunomiya et al. 1994		not satisfy	satisfy	satisfy	Positive
	Dibenzopyrene, dibenzo[a,l]pyrene							not satisfy	not satisfy	not satisfy	negative

Appendix 15 Table 2. The summary of immunotoxicological data of 63 chemicals (continue)

			Endpoint 1	Endpoint 2	Endpoint 3	Endpoint 4	Endpoint 5	Endpoint 6	Endpoint 7	
	Chemical Name	cas no.	1. Decreased antibody response	2. Suppressed T cell proliferation in the stimulation with innate immune cells, such as dendritic cells or macrophages	3. Decreased LPS response in vivo, ex vivo, or in vitro	4. Suppressed DHR	5. Suppressed host resistance	6. The NTP data or Tox 21 data indicates immunotoxicity of chemicals	7. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.	
									IL-1	TNF-a
	Dibutyl phthalate	84-74-2	A mice Larsen et al. 2002							
	Diesel exhaust particles									
	Diethanolamin	111-42-2	S mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm20004/imm20004.html no effect rat https://ntp.niehs.nih.gov/publications/abstracts/imm/imm20303/imm20303.html 1	A rat https://ntp.niehs.nih.gov/publications/abstracts/imm/imm20303/imm20303.html 0	no effect rat https://ntp.niehs.nih.gov/publications/abstracts/imm/imm98011/index.html 0		positive	positive		
	Digoxin	20830-75-5								
	Dimethyl sulfoxide	67-68-5		A rat Gray & Walker 1979			no effect mice Czuprynski et al. 1984			

Appendix 15 Table 2. The summary of immunotoxicological data of 63 chemicals (continue)

		Endpoint 7(continue)		Endpoint 8				Criterion 1	Criterion 2	Criterion 3	
	Chemical Name	7. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.(continue)		8. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells in vitro.				Satisfy one of Endpoints 1 to 6	Increased or decreased mRNA expression or protein production in one or more cytokines in Endpoints 7 or 8 in multiple reports	Increased or decreased mRNA expression or protein production in two or more cytokines in Endpoints 3, 7 or 8	Result from refernece data
		IL-6	other	IL-1	TNF-a	IL-6	other				
	Dibutyl phthalate			S F4/80+ macrophages (chemical only) mice Li et al. 2013 no effect cell line (THP-1)(PMA) human Couleau et al. 2015 no effect monocytes/macrophages (LPS) human Hansen et al. 2015 no effect (M1) A (M2) human PBMC-	S F4/80+ macrophages (chemical only) mice Li et al. 2013 A cell line (THP-1) (PMA) human Couleau et al. 2015 S monocytes/macrophages (LPS) human Hansen et al. 2015	S F4/80+ macrophages mice (chemical only) Li et al. 2013 A monocytes/macrophages (LPS) human Hansen et al. 2015 S human PBMC-derived macrophage (LPS or IL-4) Teixeira et al. 2016	no effect (IL-8) cell line (THP-1)(PMA) human Couleau et al. 2015 A (IL-8) monocytes/macrophages (LPS) human Hansen et al. 2015 no effect (IL-8) (protein) A (mRNA) cell line (THP-1) (chemical only) human Lourenco et al. 2015	satisfy	satisfy	satisfy	Positive
	Diesel exhaust particles			S rat alveolar macrophage Yang et al. 1999 A human monocytes Brown et al. 2004	S rat alveolar macrophage Yang et al. 1999 A human, mice monocytes, macrophage cell line (J774) Brown et al. 2004			not satisfy	satisfy	satisfy	Positive
	Diethanolamin							satisfy	not satisfy	not satisfy	Positive
	Digoxin			A human whole blood Langezaal et al. 2002 no effect human PBMC Sheikhi et al. 2007	no effect human PBMC Sheikhi et al. 2007	no effect human PBMC Sheikhi et al. 2007		not satisfy	not satisfy	not satisfy	negative
	Dimethyl sulfoxide							satisfy	not satisfy	not satisfy	Positive

Appendix 15 Table 2. The summary of immunotoxicological data of 63 chemicals (continue)

		Endpoint 7(continue)		Endpoint 8				Criterion 1	Criterion 2	Criterion 3	
	Chemical Name	7. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.(continue)		8. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells in vitro.				Satisfy one of Endpoints 1 to 6	Increased or decreased mRNA expression or protein production in one or more cytokines in Endpoints 7 or 8 in multiple reports	Increased or decreased mRNA expression or protein production in two or more cytokines in Endpoints 3, 7 or 8	Result from refernece data
		IL-6	other	IL-1	TNF-a	IL-6	other				
	Ethanol			A (mRNA)(LPS) S (protein)(LPS) mice cell line (RAW246.7) Hu et al. 2017	S (LPS) mice cell line (RAW246.7) Hu et al. 2017	A (mRNA)(LPS) S (protein)(LPS) mice cell line (RAW246.7) Hu et al. 2017		not satisfy	not satisfy	satisfy	Positive
	FK506							satisfy	not satisfy	not satisfy	Positive
	Formaldehyde			A mice bone marrow Zhang et al. 2013	A human cell line (THP-1) Miyazawa et al. 2007 A mice bone marrow Zhang et al. 2013			not satisfy	satisfy	satisfy	Positive
	FR167653			S human monocyte Yamamoto et al. 1996	S human monocyte Yamamoto et al. 1996	no effect human monocyte Yamamoto et al. 1996		not satisfy	not satisfy	satisfy	Positive
	Hexachlorobenzene				no effect (PHA, *Dermatophagoides pteronyssinus* extract, PMA) human PBMC Devos et al. 2004	no effect (PHA, *Dermatophagoides pteronyssinus* extract, PMA) human PBMC Devos et al. 2004		satisfy	not satisfy	not satisfy	Positive
	Histamine			S human adherent human monocyte (LPS) Manosroi et al. 1987 S human PBMC (LPS) Dohlsten et al. 1988			IL-18 S human PBMC (LPS) Takahashi et al. 2004	not satisfy	satisfy	satisfy	Positive
	Hydrocortisone			S rat peritoneal macrophages Stosic-Grujicic et al. 1982 S mice peritoneal exudate cells Snyder and Unanue 1982 S human cell line (U-937) Lee et al. 1988 S human monocytes Shirota et al. 1989				satisfy	satisfy	not satisfy	Positive

Appendix 15 Table 2. The summary of immunotoxicological data of 63 chemicals (continue)

	Chemical Name	cas no.	Endpoint 1 1. Decreased antibody response	Endpoint 2 2. Suppressed T cell proliferation in the stimulation with innate immune cells, such as dendritic cells or macrophages	Endpoint 3 3. Decreased LPS response in vivo, ex vivo, or in vitro	Endpoint 4 4. Suppressed DHR	Endpoint 5 5. Suppressed host resistance	Endpoint 6 6. The NTP data or Tox 21 data indicates immunotoxicity of chemicals	Endpoint 7 7. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.	
									IL-1	TNF-a
	Hydrogen peroxide	7722-84-1								
	Isonicotinic Acid Hydrazide (Isoniazid)	54-85-3	no effect (spleen IgM AFC) S (serum titers) mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm96002/index.html 1	S mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm96002/index.html 0			negative	positive		
	Isophorone diisocyanate	4098-71-9				1		positive		
	Lead(II) acetate	6080-56-4	S mice Blakley and Archer 1981 no effect mice Mudzinski et al. 1986		S mice Blakley and Archer 1981	A mice Descotes et al. 1984 S rat Bunn et al. 2001 S rat Chen et al. 2004				A rat spleen Chen et al. 2004
	Lithium carbonate	554-13-2	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm85001/index.html 0	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm85001/index.html 0	A mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm85001/index.html 1	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm85001/index.html 0	negative	positive		
	Magnesium sulfate	10034-99-8								
	Sulem Mercury(II) Chloride	7487-94-7	S mice Dieter et al. 1983	S mice Dieter et al. 1983	A mice Dieter et al. 1983					
	Methanol	67-56-1	S rat Parthasarathy et al. 2007			S rat Parthasarathy et al. 2007				

Appendix 15 Table 2. The summary of immunotoxicological data of 63 chemicals (continue)

	Chemical Name	cas no.	Endpoint 1 1. Decreased antibody response	Endpoint 2 2. Suppressed T cell proliferation in the stimulation with innate immune cells, such as dendritic cells or macrophages	Endpoint 3 3. Decreased LPS response in vivo, ex vivo, or in vitro	Endpoint 4 4. Suppressed DHR	Endpoint 5 5. Suppressed host resistance	Endpoint 6 6. The NTP data or Tox 21 data indicates immunotoxicity of chemicals	Endpoint 7 7. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.	
									IL-1	TNF-a
	Ethanol	64-17-5	no effect mice Zidell et al. 1988 S mice Chang and Norman 1991 A rat Tonk et al. 2013		no effect mice Zidell et al. 1988 S (PND 21) A (PND 70) rat Tonk et al. 2013	no effect mice Zidell et al. 1988 S rat Tonk et al. 2013				A rat splenocytes Tonk et al. 2013
	FK506	109581-93-3	S rat Woo et al. 1988							
	Formaldehyde	50-00-0	0			0	negative	negative		
	FR167653	158876-65-4								
	Hexachlorobenzene	118-74-1	A rat Vos et al. 1979		A rat Vos et al. 1979	no effect rat Vos et al. 1979				S (PHA) human cord blood mononuclear cells Bilrha et al. 2003
	Histamine	51-45-6								
	Hydrocortisone	50-23-7	S mice Jokay et al. 1980			S mice Van Dijk et al. 1979				

Appendix 15 Table 2. The summary of immunotoxicological data of 63 chemicals (continue)

		Endpoint 7(continue)		Endpoint 8				Criterion 1	Criterion 2	Criterion 3	
	Chemical Name	7. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.(continue)		8. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells in vitro.				Satisfy one of Endpoints 1 to 6	Increased or decreased mRNA expression or protein production in one or more cytokines in Endpoints 7 or 8 in multiple reports	Increased or decreased mRNA expression or protein production in two or more cytokines in Endpoints 3, 7 or 8	Result from refernece data
		IL-6	other	IL-1	TNF-a	IL-6	other				
	Hydrogen peroxide							not satisfy	not satisfy	not satisfy	negative
	Isonicotinic Acid Hydrazide (Isoniazid)			S human monocyte (LPS) Kucharz and Sierakowski 1992	no effect (S. aureus) human PBMC Urbaschek et al. 1991			satisfy	not satisfy	not satisfy	Positive
	Isophorone diisocyanate							satisfy	not satisfy	not satisfy	Positive
	Lead(II) acetate			S human PBMC (activated by heat-killed Salmonella Enteritidis) Hemdan et al. 2005	A rat Chen et al. 2004 S human PBMC (activated by heat-killed Salmonella Enteritidis) Hemdan et al. 2005	A human PBMC (activated by heat-killed Salmonella Enteritidis) Hemdan et al. 2005		satisfy	satisfy	satisfy	Positive
	Lithium carbonate							satisfy	not satisfy	not satisfy	Positive
	Magnesium sulfate			S mice cell line (RAW264.7) (LPS) Lin et al. 2010	no effect (LPS) S (chemical only) human whole blood Nowacki et al. 2009 S mice cell line (RAW264.7) (LPS) Lin et al. 2010	no effect (LPS) S (chemical only) human whole blood Nowacki et al. 2009 S mice cell line (RAW264.7) (LPS) Lin et al. 2010	IL-8 no effect (LPS) S (chemical only) human whole blood Nowacki et al. 2009	not satisfy	satisfy	satisfy	Positive
	Sulem Mercury(II) Chloride			A mice peritoneal macrophage (chemical only) Zdolsek et al. 1994 A (LPS) human PBMC Gardner et al. 2009	S (heat-killed *Salmonella enterica*) human PBMC Hemdan et al. 2007 A (LPS) human PBMC Gardner et al. 2009	S (heat-killed *Salmonella enterica*) human PBMC Hemdan et al. 2007		satisfy	satisfy	satisfy	Positive
	Methanol							satisfy	not satisfy	not satisfy	Positive

Appendix 15 Table 2. The summary of immunotoxicological data of 63 chemicals (continue)

			Endpoint 1	Endpoint 2	Endpoint 3	Endpoint 4	Endpoint 5	Endpoint 6	Endpoint 7	
	Chemical Name	cas no.	1. Decreased antibody response	2. Suppressed T cell proliferation in the stimulation with innate immune cells, such as dendritic cells or macrophages	3. Decreased LPS response in vivo, ex vivo, or in vitro	4. Suppressed DHR	5. Suppressed host resistance	6. The NTP data or Tox 21 data indicates immunotoxicity of chemicals	7. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.	
									IL-1	TNF-a
	Methotrexate	13307-73-1							S (splenic cells) no effect (peritoneal cells) rat splenic cells peritoneal exudate DiMartino et al. 1987 S rat splenic macrophages Johnson et al. 1988	
	Minocycline	13614-98-7								
	Mitomycin C	50-07-7							A rat bone marrow Futamura et al. 1995	
	Mizoribine	50924-49-7		S human Thomson et al. 1993						
	Mycophenolic acid	24280-93-1		S Eugui et al. 1991 S human Thomson et al. 1993						
	Nickel sulfate	10101-97-0	no effect mice Haley et al. 1990 0	no effect mice Haley et al. 1990	no effect mice Haley et al. 1990 1		negative	positive		

Appendix 15 Table 2. The summary of immunotoxicological data of 63 chemicals (continue)

		Endpoint 7(continue)		Endpoint 8				Criterion 1	Criterion 2	Criterion 3	
	Chemical Name	7. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.(continue)		8. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells in vitro.				Satisfy one of Endpoints 1 to 6	Increased or decreased mRNA expression or protein production in one or more cytokines in Endpoints 7 or 8 in multiple reports	Increased or decreased mRNA expression or protein production in two or more cytokines in Endpoints 3, 7 or 8	Result from refernece data
		IL-6	other	IL-1	TNF-a	IL-6	other				
	Methotrexate			no effect human monocyte (LPS) Rordorf-Adam et al. 1989 no effect human monocyte Chang et al. 1990				not satisfy	satisfy	not satisfy	Positive
	Minocycline			A (LPS) human PBMC Ingham et al. 1990 S (LPS) human monocyte Pang et al. 2012 S (chemical only) human PBMC Enose-Akahata et al. 2012	A (LPS) human whole blood, PBMC, monocytes Kloppenburg et al. 1996 S (LPS) human monocyte Pang et al. 2012 S (chemical only) human CD14+ cells Enose-Akahata et al. 2012 S (LPS) human cell line (THP-1) Tai et al. 2013	A (LPS) human whole blood, PBMC, monocytes Kloppenburg et al. 1996 S (LPS) human monocyte Pang et al. 2012 S (LPS) human cell line (THP-1) Tai et al. 2013	S (LPS) human cell line (THP-1) Tai et al. 2013	not satisfy	satisfy	satisfy	Positive
	Mitomycin C			A (antigen) human PBMC Akiyoshi et al. 1987 A (chemical only) mice cell line (macrophage, P388D1) Pai et al. 1997	A (chemical only) mice cell line (macrophage, P388D1) Pai et al. 1997			not satisfy	satisfy	satisfy	Positive
	Mizoribine			A human whole blood Langezaal et al. 2002				satisfy	not satisfy	not satisfy	Positive
	Mycophenolic acid			no effect human PBMC (LPS) Eugui et al. 1991 A mice cell line (IC-21) (LPS) Jonsson et al. 2002	S mice cell line (IC-21) (LPS) Jonsson et al. 2002			satisfy	not satisfy	satisfy	Positive
	Nickel sulfate				A human cell line (THP-1) Miyazawa et al. 2007	A human cell line (THP-1) Miyazawa et al. 2007 A human CD34-DC Ade et al. 2007	A human cell line (THP-1) Miyazawa et al. 2007 A human CD34-DC Ade et al. 2007	satisfy	satisfy	satisfy	Positive

Appendix 15 Table 2. The summary of immunotoxicological data of 63 chemicals (continue)

	Chemical Name	cas no.	Endpoint 1 1. Decreased antibody response	Endpoint 2 2. Suppressed T cell proliferation in the stimulation with innate immune cells, such as dendritic cells or macrophages	Endpoint 3 3. Decreased LPS response in vivo, ex vivo, or in vitro	Endpoint 4 4. Suppressed DHR	Endpoint 5 5. Suppressed host resistance	Endpoint 6 6. The NTP data or Tox 21 data indicates immunotoxicity of chemicals	Endpoint 7 7. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.	
									IL-1	TNF-a
	Nicotinamide	98-92-0								
	Nitrofurazone	59-87-0	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm90011/index.html 0	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm90011/index.html 0	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm90011/index.html 0		negative	negative		
	p -Nitroaniline	100-01-6								
	Pentamidine isethionate	140-64-7	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm88036/index.html 0	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm88036/index.html 0	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm88036/index.html 0			negative		
	Pyrimethamine	58-14-0	A mice Thong & Ferrante 1980 S(IgM), no effect(IgG) mice Freund et al. 1998 1			A mice Thong & Ferrante 1980	S mice Freund et al. 1998	positive		
	Rapamycin	53123-88-9	S rat Chen et al. 1993	S mice Henderson et al. 1991 S human Kimball et al. 1991	S mice spleen cells Kay et al. 1991					
	Ribavirin	36791-045	S mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm90010/index.html 1	no change mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm90010/index.html 0	no change mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm90010/index.html 0			positive		
	Sodium bromate	7789-38-0	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm98004/index.html 0	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm98004/index.html 0	1			negative		

Appendix 15 Table 2. The summary of immunotoxicological data of 63 chemicals (continue)

		Endpoint 7(continue)		Endpoint 8				Criterion 1	Criterion 2	Criterion 3	
	Chemical Name	7. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.(continue)		8. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells in vitro.				Satisfy one of Endpoints 1 to 6	Increased or decreased mRNA expression or protein production in one or more cytokines in Endpoints 7 or 8 in multiple reports	Increased or decreased mRNA expression or protein production in two or more cytokines in Endpoints 3, 7 or 8	Result from refernece data
		IL-6	other	IL-1	TNF-a	IL-6	other				
	Nicotinamide			no effect human PBMC (LPS) Fukuzawa et al. 1997	S mice cell line (RAW 264.7)(LPS+IFN-g) Pellat-Deceunynck et al. 1994 S human PBMC (LPS) Fukuzawa et al. 1997	S human PBMC (LPS) Fukuzawa et al. 1997		not satisfy	satisfy	satisfy	Positive
	Nitrofurazone							not satisfy	not satisfy	not satisfy	negative
	p -Nitroaniline							not satisfy	not satisfy	not satisfy	negative
	Pentamidine isethionate			no effect human whole blood (PHA) Van Wauwe et al. 1996	no effect human whole blood (PHA) Van Wauwe et al. 1996	S human whole blood (PHA) Van Wauwe et al. 1996	S (IL-8) human whole blood (LPS, PHA) Van Wauwe et al. 1996	not satisfy	not satisfy	satisfy	Positive
	Pyrimethamine							satisfy	not satisfy	not satisfy	Positive
	Rapamycin							satisfy	not satisfy	not satisfy	Positive
	Ribavirin							satisfy	not satisfy	not satisfy	Positive
	Sodium bromate							not satisfy	not satisfy	not satisfy	negative

Appendix 15 Table 2. The summary of immunotoxicological data of 63 chemicals (continue)

			Endpoint 1	Endpoint 2	Endpoint 3	Endpoint 4	Endpoint 5	Endpoint 6	Endpoint 7	
	Chemical Name	cas no.	1. Decreased antibody response	2. Suppressed T cell proliferation in the stimulation with innate immune cells, such as dendritic cells or macrophages	3. Decreased LPS response in vivo, ex vivo, or in vitro	4. Suppressed DHR	5. Suppressed host resistance	6. The NTP data or Tox 21 data indicates immunotoxicity of chemicals	7. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.	
									IL-1	TNF-a
	Sodium dodecyl sulfate	151-21-3	S human Jahnova et al. 1994							
	Sulfasalazine	599-79-1								
	Trichloroethylene	79-01-6	no effect mice https://ntp.niehs.nih.gov/publications/abstracts/imm/imm20006/imm20006.html 0					negative		
	Triethanolamine	102-71-6				0		negative		
	Warfarin	81-81-2	No effect mice Berkarda et al. 1978			S guinea-pig Nelson 1965 S guinea-pig Cohen et al. 1967 A mice Berkarda et al. 1978 S human Edwards and Rickles 1978				
	PF06650833	1817626-54-2								S rat serum (LPS) Lee et al. 2017
	TAK-242	243984-11-4								
	VIPER									

Appendix 15 Table 2. The summary of immunotoxicological data of 63 chemicals (continue)

		Endpoint 7(continue)		Endpoint 8				Criterion 1	Criterion 2	Criterion 3	
	Chemical Name	7. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells ex vivo.(continue)		8. Increased or decreased mRNA expression or protein production of IL-1b, TNF-a, IL-6 or other proinflammatory cytokines by innate immune cells in vitro.				Satisfy one of Endpoints 1 to 6	Increased or decreased mRNA expression or protein production in one or more cytokines in Endpoints 7 or 8 in multiple reports	Increased or decreased mRNA expression or protein production in two or more cytokines in Endpoints 3, 7 or 8	Result from refernece data
		IL-6	other	IL-1	TNF-a	IL-6	other				
	Sodium dodecyl sulfate							satisfy	not satisfy	not satisfy	Positive
	Sulfasalazine			S human monocyte Okamoto et al. 1991				not satisfy	not satisfy	not satisfy	negative
	Trichloroethylene				A (LPS) mice Kupffer cell line Banerjee et al. 2020 (dichloroacetyle chloride)			not satisfy	not satisfy	not satisfy	negative
	Triethanolamine							not satisfy	not satisfy	not satisfy	negative
	Warfarin					S mice clone 4/4 macrophages (LPS) Kater et al. 2002 S mice splenocyte (LPS) Kurohara et al. 2008 no effect human cell line (THP-1) (LPS) Ohsaki et al. 2010		satisfy	satisfy	not satisfy	Positive
	PF06650833				S human PBMC (R848) Lee et al. 2017	S human Whole blood (R848) Lee et al. 2017		not satisfy	not satisfy	satisfy	Positive
	TAK-242				S mice peritoneal macrophages (LPS and IFN-g) Matsunaga et al. 2011	S mice peritoneal macrophages (LPS and IFN-g) Matsunaga et al. 2011		not satisfy	not satisfy	satisfy	Positive
	VIPER		S (IL-12p40) mice serum (LPS) Lysakova-Devine et al. 2010		S mice RAW264.7 cells, iBMDMs (LPS) Lysakova-Devine et al. 2010	S mice iBMDMs (LPS) Lysakova-Devine et al. 2010		not satisfy	not satisfy	satisfy	Positive

Blue character: Data from Tox21

Red character: Data for cytokine expression or production after LPS stimulation.

Quality assurance report for IL-1β validation study

Hajime Kojima and Asako Ueda

JaCVAM, NIHS

2020.3.31

１． Chemical distribution

1-1. Chemical Acquisition, Coding and Distribution

The assessment of laboratory transferability, and within- and between-laboratory reproducibility and predictivity, in all test facilities were performed with the coded chemicals. The coding was supervised by JaCVAM (See Appendix 1). JaCVAM was responsible for coding and distributing the test chemicals for the validation study.

1-2. Handling

The chemical master at each test facility received complete information considered essential regarding the test chemicals (physical state, weight or volume of sample, specific density for liquid test chemicals, and storage instructions) by JaCVAM. Moreover, the test facility chemical master stored each chemical at conditions in accordance with the storage instructions and received sealed safety information such as the Material Safety Data Sheet (MSDS) describing the hazards identification and exposure controls/personal protection for each chemical (See Appendix 2.1 and 2.2). The test chemicals were delivered directly to the study director and the study director was not shown the MSDSs. The study director was to refer to the MSDSs only in the event of an accident. If the study director referred to the MSDS, he/she was not to reveal the content of the MSDS to the test facility technicians.

No accidents occurred during the course of the validation study, and all test facilities returned the MSDSs for the test chemicals to JaCVAM in their sealed envelope upon completion of the validation study. All test chemicals were disposed of in compliance with the rules and regulations of the test facilities upon completion of the validation study.

２． Quality assurance

All the records (data sheets and record sheets) from the participating laboratories were checked by Dr. Takashi Omori, Kobe univ. and JaCVAM (See Appendix 3). The record sheets mean “Reagent records, solubility test, Cell culture records, Test records and data sheets”. They are total more than 300 pages and available at JaCVAM website (http:// http://www.jacvam.jp/validation08-login.html). Testings performed as part of a validation study were carried out in accordance with the principles of GLP (OECD, 1998) and necessarily include, without being limited to, the use of protocol and adequate recording of data as well as suitable reporting of results and archival record keeping.

The culture of the cells, the preparation and application of test chemicals and data sheets were completed and the results accurately reflect the raw data. Unfortunately, the record sheets on the maintenance of measuring instruments had not collected before the

validation study. JaCVAM considered these records had concerns on quality of data in the validation study. However, JaCVAM checked carefully all the results and judged all data within acceptable ranges.

At least, the reliability of measuring instruments would be checked by an independent organization before the validation study. JaCVAM recommend the validation management team the formal validation study participated with GLP laboratories will be done.

Reference

OECD (1998), OECD Principles on Good Laboratory Practice, OECD SERIES ON PRINCIPLES OF GOOD LABORATORY PRACTICE AND COMPLIANCE MONITORING, No 1, Available at: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/mc/chem(98)17&doclanguage=en

Appendix 17-1

IL-1β (P1)2018 Check List

		LabA Tohoku	LabB AIST, Tsukuba	LabC AIST, Takamatsu
set1	Reagent Records	IL-1 2018-A01	IL-1 2018-B01	IL-1 2018-C01
	Solubility Test	IL-1 2018-A02	IL-1 2018-B02	IL-1 2018-C02
	Cell Culture Records	IL-1 2018-A03	IL-1 2018-B03	IL-1 2018-C03
	Date	2018.12.7	2019.1.23	2019.1.22
	Test Records	IL-1 2018-A04	IL-1 2018-B04	IL-1 2018-C04
	Datasheets	IL-1 2018-A41	IL-1 2018-B41	IL-1 2018-C41
	Date	2018.12.10	2019.1.29	2019.1.24
	Test Records	IL-1 2018-A05	IL-1 2018-B05	IL-1 2018-C05
	Datasheets	IL-1 2018-A42	IL-1 2018-B42	IL-1 2018-C42
	Date	2018.12.14		2019.1.28
	Test Records	IL-1 2018-A06		IL-1 2018-C06
	Datasheets	IL-1 2018-A43		IL-1 2018-C43
	Date	2018.12.17		
	Test Records	IL-1 2018-A07		
	Datasheets	IL-1 2018-A44		
	Date	2018.12.21		
	Test Records	IL-1 2018-A08		
	Datasheets	IL-1 2018-A45		
	Date	2018.12.26		
	Test Records	IL-1 2018-A09		
	Datasheets	IL-1 2018-A46		
	Date	2018.12.28		
	Test Records	IL-1 2018-A10		
	Datasheets	IL-1 2018-A47		
	Date	2019.1.11		
	Test Records	IL-1 2018-A11		
	Datasheets	IL-1 2018-A48		
	Date	2019.1.28		
	Test Records	IL-1 2018-A12		
	Datasheets	IL-1 2018-A49		
set1 retrial	Date		2019.5.13	
	Test Records		IL-1 2018-B10	
	Datasheets		IL-1 2018-B43	

		LabA Tohoku	LabB AIST, Tsukuba	LabC AIST, Takamatsu
set2	Reagent Records	IL-1 2018-A21	IL-1 2018-B21	IL-1 2018-C21
	Solubility Test	IL-1 2018-A22	IL-1 2018-B22	IL-1 2018-C22
	Cell Culture Records	IL-1 2018-A23	IL-1 2018-B03	IL-1 2018-C23
	Date	2019.2.18	2019.1.30	2019.1.31
	Test Records	IL-1 2018-A24	IL-1 2018-B24	IL-1 2018-C24
	Datasheets	IL-1 2018-A51	IL-1 2018-B51	IL-1 2018-C51
	Date	2019.2.20	2019.2.8	2019.2.4
	Test Records	IL-1 2018-A25	IL-1 2018-B25	IL-1 2018-C25
	Datasheets	IL-1 2018-A52	IL-1 2018-B52	IL-1 2018-C52
	Date	2019.2.25	2019.2.12	2019.2.12
	Test Records	IL-1 2018-A26	IL-1 2018-B26	IL-1 2018-C26
	Datasheets	IL-1 2018-A53	IL-1 2018-B53	IL-1 2018-C53
	Date	2019.2.27		
	Test Records	IL-1 2018-A27		
	Datasheets	IL-1 2018-A54		
	Date	2019.2.28		
	Test Records	IL-1 2018-A28		
	Datasheets	IL-1 2018-A55		
	Date	2019.3.1		
	Test Records	IL-1 2018-A29		
	Datasheets	IL-1 2018-A56		
	Date	2019.3.4		
	Test Records	IL-1 2018-A30		
	Datasheets	IL-1 2018-A57		
set2 retrial	Cell Culture Records	IL-1 2018-A73		IL-1 2018-C73
	Date	2019.5.9		2019.5.27
	Test Records	IL-1 2018-A70		IL-1 2018-C70
	Datasheets	IL-1 2018-A71		IL-1 2018-C71

		LabA Tohoku	LabB AIST, Tsukuba	LabC AIST, Takamatsu
set3	Reagent Records	IL-1 2018-A31	IL-1 2018-B31	IL-1 2018-C31
	Solubility Test	IL-1 2018-A32	IL-1 2018-B32	IL-1 2018-C32
	Cell Culture Records	IL-1 2018-A33	IL-1 2018-B03	IL-1 2018-C33
	Date	2019.3.11	2019.2.14	2019.2.18
	Test Records	IL-1 2018-A34	IL-1 2018-B34	IL-1 2018-C34
	Datasheets	IL-1 2018-A61	IL-1 2018-B61	IL-1 2018-C61
	Date	2019.3.13	2019.2.18	2019.2.21
	Test Records	IL-1 2018-A35	IL-1 2018-B35	IL-1 2018-C35
	Datasheets	IL-1 2018-A62	IL-1 2018-B62	IL-1 2018-C62
	Date	2019.3.14	2019.3.3	2019.2.25
	Test Records	IL-1 2018-A36	IL-1 2018-B36	IL-1 2018-C36
	Datasheets	IL-1 2018-A63	IL-1 2018-B63	IL-1 2018-C63
	Date	2019.3.15		
	Test Records	IL-1 2018-A37		
	Datasheets	IL-1 2018-A64		
	Date	2019.3.18		
	Test Records	IL-1 2018-A38		
	Datasheets	IL-1 2018-A65		
set3 retrial	Cell Culture Records		IL-1 2018-B03	IL-1 2018-C73
	Date		2019.2.28	2019.5.31
	Test Records		IL-1 2018-B70	IL-1 2018-C80
	Datasheets		IL-1 2018-B71	-
	Date			2019.6.3
	Test Records			IL-1 2018-C90
	Datasheets			IL-1 2018-C91

Appendix 17-2

IL-1β (P2)2019 Check List

	LabA Tohoku	LabB AIST, Tsukuba	LabC AIST, Takamatsu
Reagent Records	IL-1 2019-A01	IL-1 2019-B01	IL-1 2019-C01
Solubility Test	IL-1 2019-A02	IL-1 2019-B02	IL-1 2019-C02
Cell Culture Records	IL-1 2019-A03	IL-1 2019-B03	IL-1 2019-C03
Date	2019.9.2	2019.8.19	2019.8.16
Test Records	IL-1 2019-A04	IL-1 2019-B04	IL-1 2019-C04
Datasheets	IL-1 2019-A20	IL-1 2019-B20	IL-1 2019-C20
Date	2019.9.4	2019.8.20	2019.8.19
Test Records	IL-1 2019-A05	IL-1 2019-B05	IL-1 2019-C05
Datasheets	IL-1 2019-A21	IL-1 2019-B21	IL-1 2019-C21
Date	2019.9.6	2019.8.22	2019.8.22
Test Records	IL-1 2019-A06	IL-1 2019-B06	IL-1 2019-C06
Datasheets	IL-1 2019-A22	IL-1 2019-B22	IL-1 2019-C22
Date	2019.9.9	2019.8.23	2019.8.23
Test Records	IL-1 2019-A07	IL-1 2019-B07	IL-1 2019-C07
Datasheets	IL-1 2019-A23	IL-1 2019-B23	IL-1 2019-C23
Date	2019.9.12	2019.8.26	2019.8.26
Test Records	IL-1 2019-A08	IL-1 2019-B08	IL-1 2019-C08
Datasheets	IL-1 2019-A24	IL-1 2019-B24	IL-1 2019-C24
Date	2019.9.13	2019.8.27	2019.8.29
Test Records	IL-1 2019-A09	IL-1 2019-B09	IL-1 2019-C09
Datasheets	IL-1 2019-A25	IL-1 2019-B25	IL-1 2019-C25
Date	2019.10.3	2019.8.29	2019.9.2
Test Records	IL-1 2019-A10	IL-1 2019-B10	IL-1 2019-C10
Datasheets	IL-1 2019-A26	IL-1 2019-B26	IL-1 2019-C26
Date	2019.10.4	2019.9.2	2019.9.5
Test Records	IL-1 2019-A11	IL-1 2019-B11	IL-1 2019-C11
Datasheets	IL-1 2019-A27	IL-1 2019-B27	IL-1 2019-C27
Date		2019.9.6	2019.9.6
Test Records		IL-1 2019-B12	IL-1 2019-C12
Datasheets		IL-1 2019-B28	IL-1 2019-C28
Date		2019.9.9	2019.9.9
Test Records		IL-1 2019-B13	IL-1 2019-C13
Datasheets		IL-1 2019-B29	IL-1 2019-C29
Date			2019.9.12
Test Records			IL-1 2019-C14
Datasheets			IL-1 2019-C30

Appendix 18. The list of proficiency chemicals

The list of proficiency chemicals

No.	Chemical name	CAS No.	Immunotoxicity	Physical state	Phase
1	Dexamethasone	50-02-2	Yes	Solid	positive control
2	Dibutyl phthalate	84-74-2	Yes	Liquid	I
3	Perfluorooctanois acid	335-67-1	Yes	Solid	II
4	Citral	5392-40-5	Yes	Liquid	II
5	Trichloroethylene	79-01-6	No	Liquid	II
6	Mannitol	69-65-8	No	Solid	II

Prior to routine use of the test method described in this Annex to Test Guideline 442E, laboratories should demonstrate technical proficiency, using the 6 Proficiency Substances listed in Appendix 15 in compliance with the Good in vitro Method Practices (1). Moreover, test method users should maintain a historical database of data generated with the reactivity checks (see paragraph 15) and with the positive and solvent/vehicle controls (see paragraphs 21-24), and use these data to confirm the reproducibility of the test method in their laboratory is maintained over time.

1. OECD (2017), Draft Guidance document: Good *In Vitro Method Practices (GIVIMP) for the Development and Implementation of In Vitro €i0Methods for Regulatory Use in Human Safety Assessment. Organisation for Economic Cooperation and Development, Paris. Available at: [http://www.oecd.org/env/ehs/testing/OECD%20Draft%20GIVIMP_v05%20-%20clean.pdf].*

Appendix 19. The list of performance standard chemicals

No.	Chemical name	CAS No.	Immunotoxicity	Physical state	Phase	Validation results
1	Dexamethasone	50-02-2	Positive	Solid	positive control	P
2	Dibutyl phthalate	84-74-2	Positive	Liquid	I	P
3	Sulem Mercury(II) Chloride	7487-94-7	Positive	Solid	I	P
4	Perfluorooctanois acid	335-67-1	Positive	Solid	II	P
5	Citral	5392-40-5	Positive	Liquid	II	P
6	Acetaminophen	103-90-2	Positive	Solid	I	N
7	Isonicotinic Acid Hydrazide (Isoniazid)	54-85-3	Positive	Solid	I	N
8	5,5-Diphenylhydantoin sodium salt	630-93-3	Positive	Solid	II	N
9	Tributyltin chloride	1461-22-9	Positive	Liquid	II	N
10	Ethanol	64-17-5	Positive	Liquid	II	N
11	Nonylphenol	84852-15-3	Positive	Liquid	II	N
12	t-Butylhydroquinone	1948-33-0	negative	Solid	II	P
13	Sodium chlorite	7758-19-2	negative	Solid	II	P
14	5-Nitro-2-furaldehydesemicarbazone	59-87-0	negative	Solid	II	N
15	Trichloroethylene	79-01-6	negative	Liquid	II	N
16	D(-)-Mannitol	69-65-8	negative	Solid	II	N

Performance standards (PS) are shown to facilitate the validation of modified *in vitro* IL-2 luciferase test methods similar to the IL-2 Luc assay and allow for timely amendment of this Test Guideline for their inclusion. Mutual Acceptance of Data (MAD) will only be guaranteed for test methods validated according to the PS, if these test methods have been reviewed and included in this Test Guideline by the OECD.

添付資料４．Data set63 化学物質の IL-2 Luc assay, IL-1 Luc assay, IL-8 Luc assay による評価結果

Chemical	IL-2 Luc assay		IL-1 Luc assay			IL-8 Luc
	Judgment	LOEL (μg/mL)	Judgment	LOEL (μg/mL)	Immunotoxicity judgment	Judge
FK506	S	0.0002	N		Positive	N
Cyclosporine A	S	0.0041	N		Positive	N
Actinomycin D	S	0.02	P	0.13	Positive	P
Digoxin	S	0.07	P	0.59	Negative	P
Colchicine	S	0.27	N		Negative	P
PF06650833	S	0.451	P	0.00176	Positive	P
FR167653	S	1.30	P	0.49	Positive	
Benzethonium chloride	S	1.63	N		Negative	P
Mercuric chloride	S	1.95	P	1.95	Positive	P
Chlorpromazine	S	1.95	P	3.91	Negative	P
Amphoterycin B	S	2.60	P	1.17	Positive	P
Dibutyl phthalate	S	2.60	P	15.63	Positive	N
2-Aminoanthracene	S	5.86	P	11.72	Negative	P
Isophorone diisocyanate	S	7.81	P	3.91	Positive	P
Formaldehyde	S	7.81	N		Positive	P
Pyrimethamine	S	7.81	N		Positive	P
Cobalt chloride	S	16.93	P	46.88	Negative	P
Cisplatin	S	16.93	P	46.88	Positive	P
Chloroquine	S	17.83	P	39.06	Positive	P
Minocycline	S	18.52	P	62.50	Positive	P
Mitomycin C	S	20.00	N		Positive	P
Hydrogen peroxide	S	23.44	P	375.00	Negative	P
Citral	S	25.00	P	4.88	Positive	P
Dexamethasone	S	41.67	P	0.98	Positive	N
Pentamidine isethionate	S	52.08	P	64.45	Positive	P
Lead(II) acetate	S	57.29	N		Positive	N
Azathioprine	S	58.48	P	41.55	Positive	N
Diesel exhaust particles	S	62.50	P	39.06	Positive	P
Sodium dodecyl sulfate	S	62.50	P	62.50	Positive	P
Dapsone	S	72.92	P	125.00	Positive	N
p-Nitroaniline	S	83.33	P	125.00	Negative	N
Nitrofurazone	S	83.33	N		Negative	P
Sulfasalazine	S	92.94	P	44.81	Negative	N
Nickel sulfate	S	104.17	P	375.00	Positive	P
Aluminum chloride	S	104.17	N		Positive	N
Chloroplatinic acid	S	250.00	P	23.44	Positive	P
Diethanolamin	S	250.00	P	333.33	Positive	P
Sodium bromate	S	500.00	P	500.00	Negative	P
Histamine	S	750.00	N		Positive	P
Isoniazid	S	1000.00	N		Positive	N
Triethanolamine	S	1333.33	P	1000.00	Negative	P

Magnesium sulfate	S	2000.00	N		Positive	N
Warfarin	N		N		Positive	N
Hydrocortisone	N		N		Positive	N
Lithium carbonate	N		N		Positive	P
2,4-Diaminotoluene	N		N		Positive	N
Dibenzopyrene	N		N		Negative	N
Cyclophosphamide	N		N		Positive	P
Ethanol	N		N		Positive	N
Methanol	N		N		Positive	N
Hexachlorobenzene	N		N		Positive	N
Trichloroethylene	N		N		Negative	N
Methotrexate	N		N		Positive	P
Rapamycin	N		N		Positive	N
Mizoribine	N		N		Positive	N
TAK-242	N		P	0.014	Positive	
VIPER	N		P	2.71	Positive	
Mycophenolicacid	A	0.40	P	72.00	Positive	P
2-Mercaptobenzothiazole	A	16.11	P	93.75	Positive	P
Ribavirin	A	26.04	P	750.00	Positive	N
Acetaminophen	A	100.00	N		Positive	N
Nicotinamide	A	288.07	N		Positive	N
Dimethyl sulfoxide	A	2000.00	N		Positive	N
LOEL: lowest observed effect level						

添付資料５

IL-2 Luc leukocyte toxicity test (IL-2 Luc LTT)

protocol ver. 001.3

September 16th, 2020

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1. Introduction

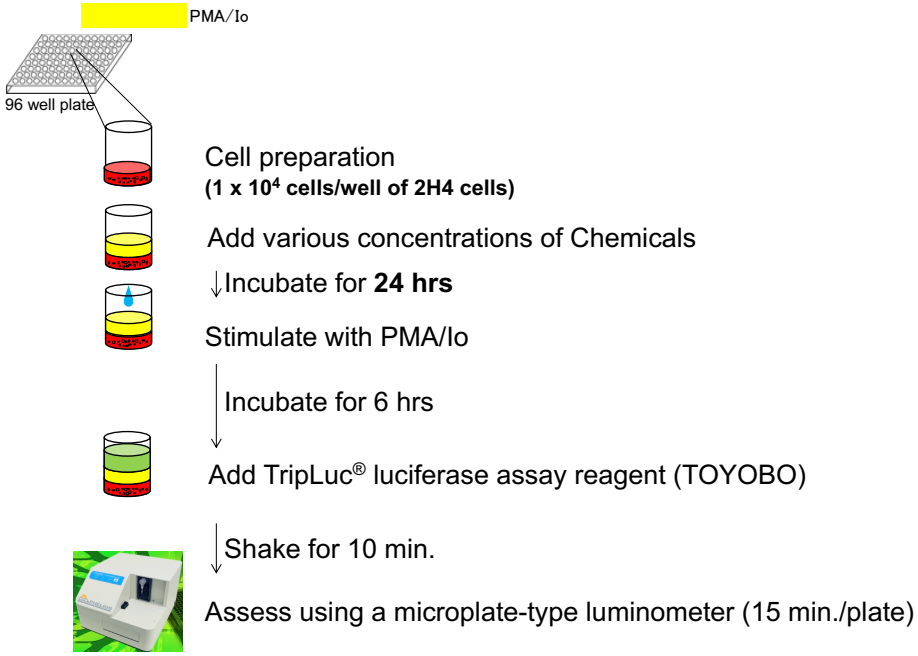
The IL-2 Luc leukocyte toxicity test (IL-2 Luc LTT) is aimed to detect immunosuppressive chemicals the mechanism of which is mostly due to suppression of cell proliferation. The IL-2 Luc LTT protocol is similar to that of the IL-2 Luc assay established previously, except for the duration of chemistry and cell seeding concentration.

(Kimura Y. et al. Evaluation of the Multi-Immuno Tox Assay composed of 3 human cytokine reporter cells by examining immunological effects of drugs *Toxicol in Vitro*, 28, 759-768, 2014)

Figure 1

Assay design (2 chemicals per one plate)

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	cont (distilled water or DMSO)	PMA/Io only	A/2^9 µg/ml	A/2^8 µg/ml	A/2^7 µg/ml	A/2^6 µg/ml	A/2^5 µg/ml	A/2^4 µg/ml	A/2^3 µg/ml	A/2^2 µg/ml	A/2^1 µg/ml	A µg/ml
B			Chemical A (common ratio of 2、10 concentrations、n=4)									
C												
D												
E	cont (distilled water or DMSO)	PMA/Io only	B/2^9 µg/ml	B/2^8 µg/ml	B/2^7 µg/ml	B/2^6 µg/ml	B/2^5 µg/ml	B/2^4 µg/ml	B/2^3 µg/ml	B/2^2 µg/ml	B/2^1 µg/ml	B µg/ml
F			Chemical B (common ratio of 2、10 concentrations、n=4)									
G												
H												



2. Materials

2-1 Cells

・/ 2H4 (IL2-SLG, IFNγ-SLO, G3PDH-SLR)

A Jurkat-derived IL-2 and IFNγ reporter cell line, 2H4, that harbors the SLG, SLO and SLR luciferase genes under the control of the IL-2, IFNγ and GAPDH promoters, respectively, was established by Tsuruga Institute of Biotechnology, TOYOBO Co. Ltd.

(Saito R. et al. Nickel differentially regulates NFAT and NF-κB activation in T cell signaling *Toxicology and Applied Pharmacology*, 254, 245–255, 2011)

2-2 Reagents and equipment

2-2-1 For maintenance of the 2H4 cells

・/ RPMI-1640 (GIBCO Cat#11875-093, 500 mL)
・/ FBS (Biological Industries Cat#04-001-1A Lot: 1524129)
・/ Antibiotic-Antimycotic (GIBCO Cat#15240-062)
・/ HygromycinB (CAS:31282-04-9, Invitrogen Cat#10687-010)
・/ G418 (CAS:108321-42-2, WAKO Cat#071-06431)
・/ Puromycin (CAS:58-58-2, InvivoGen Cat#ant-pr-1)

2-2-2 For chemical exposure, stimulation and solvents

・/ Ionomycin (CAS:56092-82-1, Sigma Cat#I0634)
・/ Phorbol 12-myristate 13-acetate (PMA) (CAS:16561-29-8, Sigma Cat#P8139)
・/ Ethanol (e.g., Wako Cat#057-00456)
・/ Dimethyl sulfoxide (DMSO) (CAS:67-68-5, Sigma Cat#D5879)
・/ Distilled water (GIBCO Cat#10977-015)

2-2-3 For measurement of the luciferase activity

・/ Tripluc® Luciferase assay reagent (TOYOBO Cat#MRA-301)

2-2-4 Expendable supplies

・/ T-75 flask tissue culture treated (e.g., Corning Cat#353136)
・/ 96 well μclear black plate (flat-bottom, for measurement of the luciferase activity, e.g. Greiner Bio-one Cat#655090)
・/ 96 well clear plate (round-bottom, for preparation of chemicals and stimulants)
・/ 96 well assay block, 2 mL (e.g., Costar Cat#3960)
・/ Seal for 96 well plate (e.g., Perkin Elmer TopSeal-A PLUS Cat#6050185, EXCEL

Scientific SealMate Cat#SM-KIT-SP)

・/ Reservoir

・/ Pipette

2-2-5 Equipment for measurement of luciferase activity

・/ Measuring device: a microplate-type luminometer with a multi-color detection system that can accept two optical filter
e.g. Phelios AB-2350 (ATTO), ARVO (PerkinElmer), Tristar LB941 (Berthold)

・/ Optical filter: 560 nm long-pass filter and 600 nm long-pass filter

・/ Measuring time: set at 1〜5 sec/well measuring time

2-2-6 Others

・/ Pipetman

・/ 8 channel or 12 channel pipetman (optimized for 10~100 μL)

・/ Plate shaker (for 96 well plate)

・/ CO_2 incubator (37°C, 5% CO_2)

・/ Water bath

・/ Cell counter: hemocytometer, trypan blue

2-3 Culture medium

2-3-1 A medium: for maintenance of 2H4 cells (500 mL, stored at 2-8°C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	440 mL
FBS	Biological Industries Cat#04-001-1A Lot: 1524129	-	10 %	50 mL
Antibiotic-Antimycotic	GIBCO #15240-062	100×	1×	5 mL
Puromycin	InvivoGen # ant-pr-1	10 mg/mL	0.15 μg/mL	7.5 μL
G418	WAKO Cat#071-06431	50 mg/mL	300 μg/mL	3 mL
HygromycinB	Invitrogen #10687-010	50 mg/mL	200 μg/mL	2 mL

2-3-2 B medium: for luciferase assay (30 mL, stored at 2-8°C)/

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	27 mL
FBS	Biological Industries Cat#04-001-1A Lot: 1524129	-	10 %	3 mL

2-3-3 C medium: for thawing 2H4 cells (30 mL, stored at 2-8°C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	26.7 mL
FBS	Biological Industries Cat#04-001-1A Lot: 1524129	-	10 %	3 mL
Antibiotic-Antimycotic	GIBCO #15240-062	100×	1×	0.3 mL

2-4 Preparation of the stimulant of 2H4

2-4-1 Phorbol 12-myristate 13-acetate (PMA)

Reagent	Company	Concentration of the stock solution	Final concentration
Phorbol 12-myristate 13-acetate (PMA)	Sigma #P8139	2 mM	25 nM
DMSO	Sigma #D5789		

Dissolve 1 mg PMA using DMSO 811 μL, dispend at 5 μL/tube and store at freezer at -30°C. Use these stocks within 6 months after dissolution.

2-4-2 Ionomycin

Reagent	Company	Concentration of the stock solution	Final concentration
Ionomycin	Sigma # I0634	2 mM	1 μM
Ethanol	Wako #057-00456		

Dissolve 1mg Ionomycin using ethanol 669.3 μL, dispend at 15 μL/tube and store at freezer at -30°C. Use these stocks within 6 months after dissolution.

3. Cell culture

3-1 Thawing of 2H4 cells

Pre-warm 9 mL of C medium in a 15 mL polypropylene conical tube in a 37°C water bath (for centrifugation) and 15 mL of C medium in a T-75 Flask at 37°C in a 5% CO_2 incubator (for culture).

Thaw frozen cells ($5x10^6$ cells / 0.5 mL of freezing medium) in a 37°C water bath, then add to a 15 mL polypropylene conical tube containing 9 mL of pre-warmed C medium. Centrifuge the tube at 120-350 x *g* at room temperature for 5 min, discard the supernatant, and resuspend in 15 mL of pre-warmed C medium in a T-75 Flask. Cells are incubated at 37°C, 5% CO_2.

3-2 Maintenance of 2H4 cells

Pre-warm the A medium in a T-75 Flask at 37°C in a 5% CO_2 incubator. The culture medium should be changed to the A medium 3 or 4 days after thawing. At that time, count the number of cells, centrifuge the tube at 120-350 x *g* at room temperature for 5 min, discard the supernatant, and resuspend in pre-warmed the A medium in a T-75 Flask. Cells are passaged at $1\sim3x10^5$/mL and incubated at 37°C, 5% CO_2.

The interval between subcultures should be 3~4 days. Cells can be used between one and six weeks after thawing.

4. Preparation of cells for assay

A cell passage should be done 2-4 days before the assay.

Use cells between 1 and 6 weeks after thawing.

Pre-warm the B medium in a 37°C water bath. Count the number of cells and collect the number of cells needed (1.0 x 10^6 cells for two chemicals are required, but to have some leeway, 1.5 x 10^6 for two chemicals should be prepared), centrifuge the tube at 120-350 x *g*, 5 min. Resuspend in pre-warmed the B medium at a cell density of 2 x 10^5/mL. Transfer the cell suspension to a reservoir, and add 50 µL of cell suspension to each well of a 96 well µclear black plate (flat bottom) using an 8 channel or 12 channel pipetman. (cf. Figure 2)

Figure 2

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
B	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
C	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
D	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
E	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
F	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
G	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
H	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL

5. Preparation of chemicals and cell treatment with chemicals

5-1 Dissolution by vehicle

Dissolve the chemical first in distilled water. Weigh 10 mg of the test chemical in a volumetric flask and add distilled water up to 1 mL. If the chemical is soluble at 10 mg/mL, use 10 mg/ml solution for the stock solution.

If the chemical is not soluble at 10 mg/ml in water, the chemical should be dissolved in DMSO at 200 mg/mL. For example, weigh 200 mg of the test chemical in volumetric flask and add DMSO up to 1 mL. (cf. Figure 3). If the chemical does not dissolve in DMSO at 200 mg/ml, use the highest concentration possible after diluting with DMSO at a dilution factor of 2.

For expensive chemicals, prepare the highest concentration possible instead of 10 mg/mL distilled water. If the chemical is not soluble, prepare the highest concentration possible in DMSO.

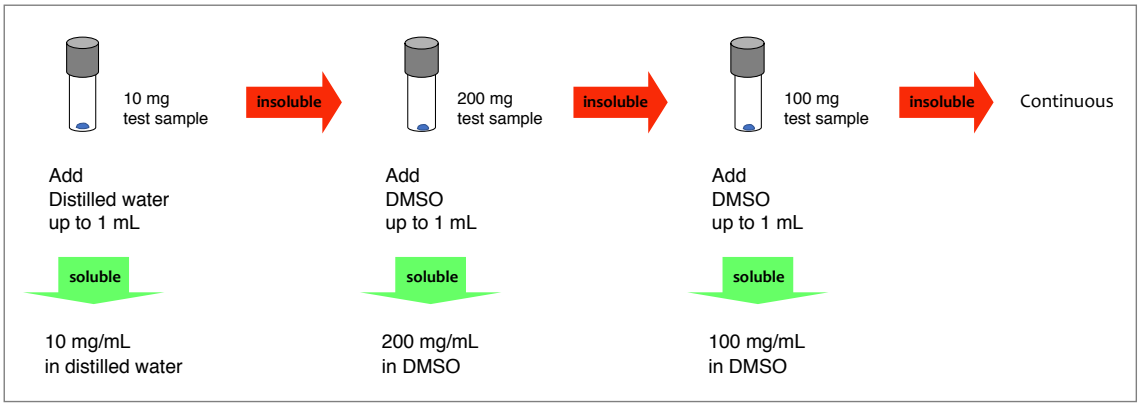
Sonication and vortex may be used if needed, and attempt to dissolve the chemical for at least 5 minutes. Being soluble should be confirmed by centrifugation at 15,000 rpm (≈20,000 x g) for 5 min and confirm the absence of precipitation. The chemical should be used within 4 hours after being dissolved in distilled water or DMSO.

In the first experiment (1st experiment), when the chemical is prepared in distilled water, conduct 10 serial dilutions at a dilution factor of 2 from the highest concentration using distilled water. When the chemical is prepared as a DMSO solution, conduct 10 serial dilutions at a dilution factor of 2 from the highest concentration using DMSO.

In the second and third experiment (2nd and 3rd experiment), determine the minimum concentration at which Inh-GAPLA (mentioned later in **10**) becomes lower than 0.05 in the 1st experiment, use the concentration one step (2-times) higher than this determined concentration as the highest concentration of the chemical to examine, and conduct 10 serial dilutions at a dilution factor of 2 from the highest concentration. (cf. Figure 4) If Inh-GAPLA did not become lower than 0.05 or became lower than 0.05 at the highest concentration in the 1st experiment, conduct 10 serial dilutions at a dilution factor of 2 from the highest concentration in the 1st experiment. (cf. Figure 5)

In addition, if the chemical gives Inh-GAPLA < 0.7 at the lowest concentration and does not give significant reduction of Inh-GAPLA at the higher concentrations, use the concentration two step (4-times) higher than the lowest concentration in the first experiment as the highest concentration of the chemical to examine (cf. Figure 6).

Figure 3



For example, in Figure 4 below, the minimum concentration at which Inh-GAPLA becomes lower than 0.05 is 6.25 μg/ml. The highest concentration of the chemical to examine is the concentration one step (2-times) higher than 6.25 μg/ml, which is 12.5 μg/ml.

In Figure 5 below, Inh-GAPLA does not become lower than 0.05. In such a case, the highest concentration of the chemical to examine is the highest concentration in the 1st experiment, namely 25 μg/ml.

In Figure 6 below, since the chemical gives Inh-GAPLA < 0.7 at the lowest concentration and does not give significant reduction of Inh-GAPLA at the higher concentrations, use the concentration two step (4-times) higher than the lowest concentration (0.39 μg/ml) in the first experiment as the highest concentration of the chemical to examine, which is 1.56 μg/ml.

Figure 4

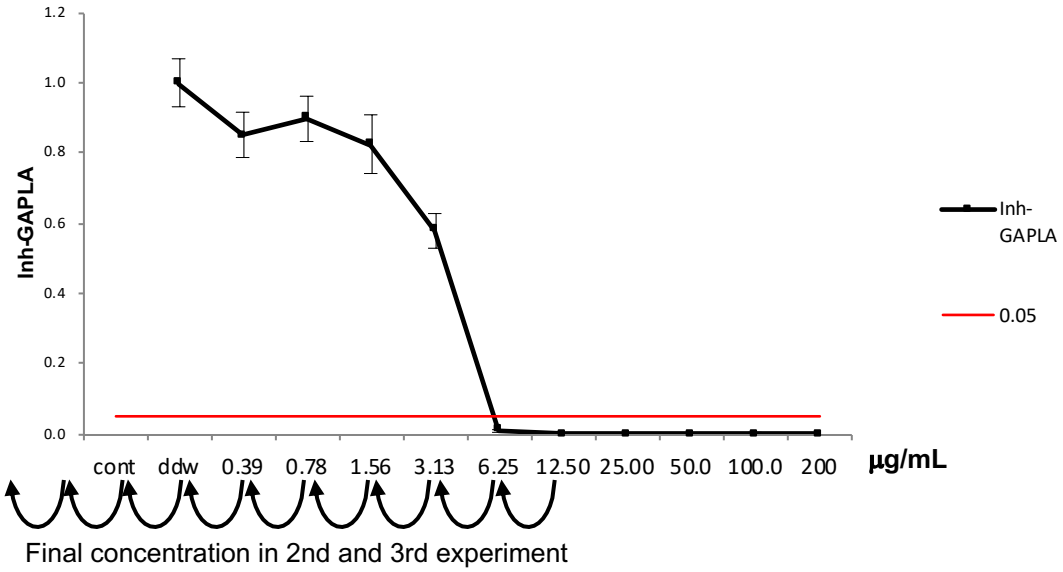


Figure 5

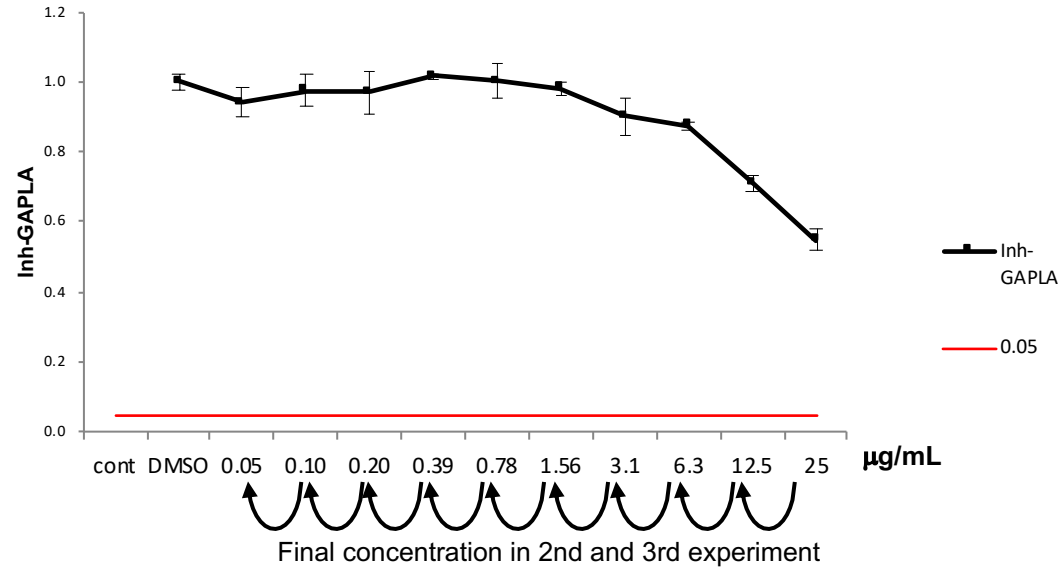
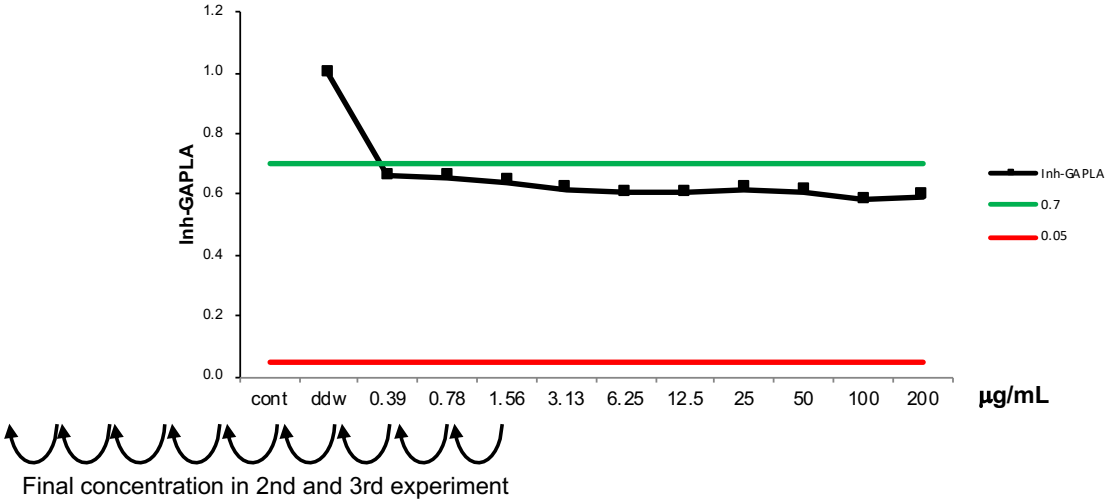


Figure 6



Final concentration in 2nd and 3rd experiment

5-2 When the chemical is prepared in distilled water

If the chemical is prepared at a lower concentration, use the prepared concentration instead of the 10 mg/mL distilled water solution.

5-2-1 Arrangement of chemicals and vehicle

Add 100 μL of the 10 mg/mL distilled water solution of the chemical to well #A12, and 50 μL of the distilled water to wells #A1-#A11 of the 96 well clear plate (round bottom).

5-2-2 Serial dilution

Conduct 9 serial dilutions at a common ratio of 2 as indicated in Figure 4 from well #A11 to well #A3. Transfer 50 μL to the next (left) well. (cf. Figure 7)

Figure 7

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Chemical 10 mg/mL in distilled water 100uL
B												
C												
D												
E												
F												
G												
H												

2-fold dilution : transfer 50 μL (pipetman, yellow tip)

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 50uL	Distilled water 50uL	Chemical 0.02 mg/mL in distilled water 100uL	Chemical 0.04 mg/mL in distilled water 50uL	Chemical 0.08 mg/mL in distilled water 50uL	Chemical 0.16 mg/mL in distilled water 50uL	Chemical 0.31 mg/mL in distilled water 50uL	Chemical 0.63 mg/mL in distilled water 50uL	Chemical 1.3 mg/mL in distilled water 50uL	Chemical 2.5 mg/mL in distilled water 50uL	Chemical 5 mg/mL in distilled water 50uL	Chemical 10 mg/mL in distilled water 50uL
B												
C												
D												
E												
F												
G												
H												

5-2-3 2 step dilution

Add 20 μL of the diluted chemical to 480 μL of the B medium prepared in the assay block. And add 50 μL to 2H4 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 24 hours (37°C, CO_2, 5%) (cf. Figure 8-10).

Figure 8

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 50uL	Distilled water 50uL	Chemical 0.02 mg/mL in distilled water 100uL	Chemical 0.04 mg/mL in distilled water 50uL	Chemical 0.08 mg/mL in distilled water 50uL	Chemical 0.16 mg/mL in distilled water 50uL	Chemical 0.31 mg/mL in distilled water 50uL	Chemical 0.63 mg/mL in distilled water 50uL	Chemical 1.3 mg/mL in distilled water 50uL	Chemical 2.5 mg/mL in distilled water 50uL	Chemical 5 mg/mL in distilled water 50uL	Chemical 10 mg/mL in distilled water 50uL
B												
C												
D												
E												
F												
G												
H												

20μL

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL
B												
C												
D												
E												
F												
G												
H												

Figure 9

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 500uL	B medium 500uL	Chemical 0.8 ug/mL in B medium 500uL	Chemical 1.6 ug/mL in B medium 500uL	Chemical 3.1 ug/mL in B medium 500uL	Chemical 6.3 ug/mL in B medium 500uL	Chemical 12.5 ug/mL in B medium 500uL	Chemical 25 ug/mL in B medium 500uL	Chemical 50 ug/mL in B medium 500uL	Chemical 100 ug/mL in B medium 500uL	Chemical 200 ug/mL in B medium 500uL	Chemical 400 ug/mL in B medium 500uL
B												
C												
D												
E												
F												
G												
H												

50µL

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
B	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
C	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
D	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
E	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
F	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
G	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
H	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL

Figure 10 Final constituents of each well of the plate

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	Chemical 0 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 0 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 0.4 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 0.8 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 1.6 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 3.1 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 6.3 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 12.5 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 25 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 50 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 100 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 200 ug/mL 2H4 1x10^4 B medium 100uL
B	Chemical 0 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 0 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 0.4 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 0.8 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 1.6 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 3.1 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 6.3 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 12.5 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 25 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 50 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 100 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 200 ug/mL 2H4 1x10^4 B medium 100uL
C	Chemical 0 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 0 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 0.4 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 0.8 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 1.6 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 3.1 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 6.3 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 12.5 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 25 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 50 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 100 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 200 ug/mL 2H4 1x10^4 B medium 100uL
D	Chemical 0 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 0 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 0.4 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 0.8 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 1.6 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 3.1 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 6.3 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 12.5 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 25 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 50 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 100 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 200 ug/mL 2H4 1x10^4 B medium 100uL
E	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
F	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
G	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
H	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL

5-3 When the chemical is prepared as a DMSO solution

If the chemical is prepared at a lower concentration, use the prepared concentration instead of 200 mg/mL DMSO solution.

5-3-1 Arrangement of chemicals and vehicle

Add 100 μL of the 200 mg/mL DMSO solution of the chemical to well #A12, 50 μL of DMSO to wells #A1-#A11, and 90 μL of the B medium to wells #B1-#B12 of the 96 well clear plate (round bottom)

5-3-2 Serial dilution// / / / /

Conduct 9 serial dilutions at a common ratio of 2 as indicated in Figure 8 from well #A11 to well #A3. Transfer 50 μL to the next (left) well. (cf. Figure 11)

Figure 11

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	Chemical 200 mg/mL in DMSO 100uL
B	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL
C												
D												
E												
F												
G												
H												

2-fold dilution : transfer 50 μL (pipetman, yellow tip)

↓

round botto m clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 50uL	DMSO 100% 50uL	Chemical 0.39 mg/mL in DMSO 100uL	Chemical 0.78 mg/mL in DMSO 50uL	Chemical 1.6 mg/mL in DMSO 50uL	Chemical 3.1 mg/mL in DMSO 50uL	Chemical 6.3 mg/mL in DMSO 50uL	Chemical 12.5 mg/mL in DMSO 50uL	Chemical 25 mg/mL in DMSO 50uL	Chemical 50 mg/mL in DMSO 50uL	Chemical 100 mg/mL in DMSO 50uL	Chemical 200 mg/mL in DMSO 50uL
B	B mediu m 90uL	B mediu m 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL
C												
D												
E												
F												
G												
H												

5-3-3 Dilution of DMSO solution with the B medium

Dilute 10 μL of the DMSO solution of the chemical in wells #A1-#A12 with 90 μL of the B medium using an 8-12 channel pipetman. (cf. Figure 12)

Figure 12

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 50uL	DMSO 100% 50uL	Chemical 0.39 mg/mL in DMSO 100uL	Chemical 0.78 mg/mL in DMSO 50uL	Chemical 1.6 mg/mL in DMSO 50uL	Chemical 3.1 mg/mL in DMSO 50uL	Chemical 6.3 mg/mL in DMSO 50uL	Chemical 12.5 mg/mL in DMSO 50uL	Chemical 25 mg/mL in DMSO 50uL	Chemical 50 mg/mL in DMSO 50uL	Chemical 100 mg/mL in DMSO 50uL	Chemical 200 mg/mL in DMSO 50uL
B	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL
C												
D												
E												
F												
G												
H												

10μL

↓

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 40uL	DMSO 100% 40uL	Chemical 0.39 mg/mL in DMSO 90uL	Chemical 0.78 mg/mL in DMSO 40uL	Chemical 1.6 mg/mL in DMSO 40uL	Chemical 3.1 mg/mL in DMSO 40uL	Chemical 6.3 mg/mL in DMSO 40uL	Chemical 12.5 mg/mL in DMSO 40uL	Chemical 25 mg/mL in DMSO 40uL	Chemical 50 mg/mL in DMSO 40uL	Chemical 100 mg/mL in DMSO 40uL	Chemical 200 mg/mL in DMSO 40uL
B	Chemical 0 mg/mL DMSO 10% in B medium 100uL	Chemical 0 mg/mL DMSO 10% in B medium 100uL	Chemical 0.039 mg/mL DMSO 10% in B medium 100uL	Chemical 0.078 mg/mL DMSO 10% in B medium 100uL	Chemical 0.16 mg/mL DMSO 10% in B medium 100uL	Chemical 0.31 mg/mL DMSO 10% in B medium 100uL	Chemical 0.63 mg/mL DMSO 10% in B medium 100uL	Chemical 1.25 mg/mL DMSO 10% in B medium 100uL	Chemical 2.5 mg/mL DMSO 10% in B medium 100uL	Chemical 50 mg/mL DMSO 10% in B medium 100uL	Chemical 10 mg/mL DMSO 10% in B medium 100uL	Chemical 20 mg/mL DMSO 10% in B medium 100uL
C												
D												
E												
F												
G												
H												

5-3-4　2 step dilution

Add 10 μL of the diluted chemical to 490 μL of the B medium prepared in the assay block. And add 50 μL to 2H4 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Manipulate the procedures from 5-3-3 to 5-3-4 as quickly as you can, and do not leave a long time at step after 5-3-3 or Figure 11. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 24 hours (37°C, CO_2, 5%) (cf. Figure 13-15).

Figure 13

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 40uL	DMSO 100% 40uL	Chemical 0.39 mg/mL in DMSO 90uL	Chemical 0.78 mg/mL in DMSO 40uL	Chemical 1.6 mg/mL in DMSO 40uL	Chemical 3.1 mg/mL in DMSO 40uL	Chemical 6.3 mg/mL in DMSO 40uL	Chemical 12.5 mg/mL in DMSO 40uL	Chemical 25 mg/mL in DMSO 40uL	Chemical 50 mg/mL in DMSO 40uL	Chemical 100 mg/mL in DMSO 40uL	Chemical 200 mg/mL in DMSO 40uL
B	Chemical 0 mg/mL DMSO 10% in B medium 100uL	Chemical 0 mg/mL DMSO 10% in B medium 100uL	Chemical 0.039 mg/mL DMSO 10% in B medium 100uL	Chemical 0.078 mg/mL DMSO 10% in B medium 100uL	Chemical 0.16 mg/mL DMSO 10% in B medium 100uL	Chemical 0.31 mg/mL DMSO 10% in B medium 100uL	Chemical 0.63 mg/mL DMSO 10% in B medium 100uL	Chemical 1.25 mg/mL DMSO 10% in B medium 100uL	Chemical 2.5 mg/mL DMSO 10% in B medium 100uL	Chemical 50 mg/mL DMSO 10% in B medium 100uL	Chemical 10 mg/mL DMSO 10% in B medium 100uL	Chemical 20 mg/mL DMSO 10% in B medium 100uL
C												
D												
E												
F												
G												
H												

10μL

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL
B												
C												
D												
E												
F												
G												
H												

Figure 14

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	Chemical 0ug/mL DMSO 0.2% in B medium 500uL	Chemical 0ug/mL DMSO 0.2% in B medium 500uL	Chemical 0.78ug/mL DMSO 0.2% in B medium 500uL	Chemical 1.6ug/mL DMSO 0.2% in B medium 500uL	Chemical 3.1ug/mL DMSO 0.2% in B medium 500uL	Chemical 6.3ug/mL DMSO 0.2% in B medium 500uL	Chemical 12.5ug/mL DMSO 0.2% in B medium 500uL	Chemical 25ug/mL DMSO 0.2% in B medium 500uL	Chemical 50ug/mL DMSO 0.2% in B medium 500uL	Chemical 100ug/mL DMSO 0.2% in B medium 500uL	Chemical 200ug/mL DMSO 0.2% in B medium 500uL	Chemical 400ug/mL DMSO 0.2% in B medium 500uL
B												
C												
D												
E												
F												
G												
H												

50µL

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
B	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
C	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
D	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
E	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
F	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
G	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
H	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL

Figure 15 Final constituents of each well of the plate

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	Chemical 0ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 0ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 0.39ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 0.78ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 1.6ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 3.1ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 6.3ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 12.5ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 25ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 50ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 100ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 200ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL
B	Chemical 0ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 0ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 0.39ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 0.78ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 1.6ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 3.1ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 6.3ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 12.5ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 25ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 50ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 100ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 200ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL
C	Chemical 0ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 0ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 0.39ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 0.78ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 1.6ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 3.1ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 6.3ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 12.5ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 25ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 50ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 100ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 200ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL
D	Chemical 0ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 0ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 0.39ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 0.78ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 1.6ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 3.1ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 6.3ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 12.5ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 25ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 50ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 100ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 200ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL
E	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
F	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
G	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
H	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL

6. Preparation of the stimulant (PMA/ionomycin) and addition to 2H4

6-1 Material

・2 mM PMA stock

・2 mM Ionomycin stock

・B medium

・Ethanol

6-2 Preparation of 100 μM PMA

Dilute 2 mM PMA stock with the B medium as follows (20 times, final concentration is 100 μM).

2 mM PMA	B medium	Total	final concentration
5 μL	95 μL	100 μL	100 μM

6-3 Preparation of control and x10 PMA/ionomycin solution

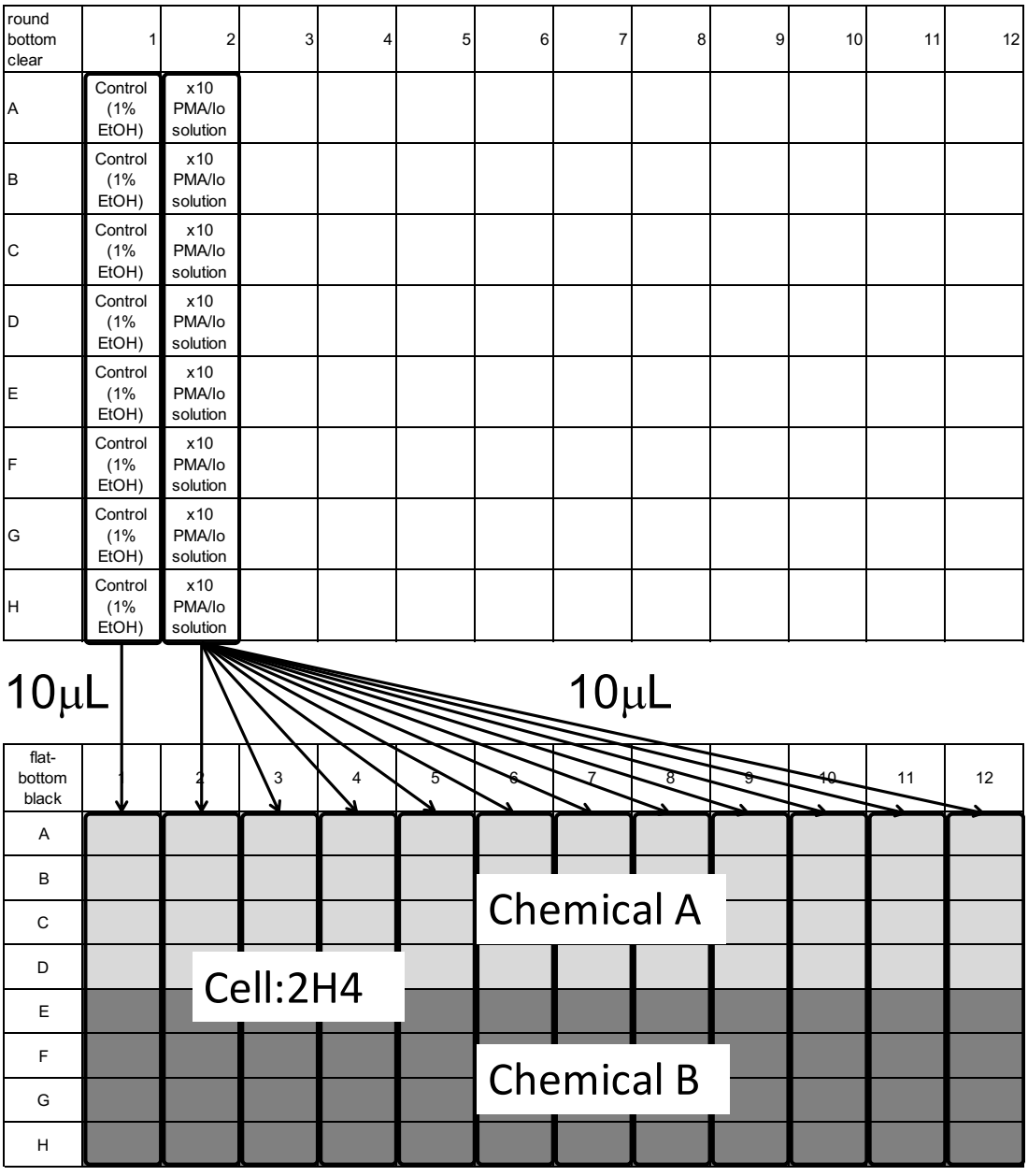
Dilute ethanol, 2 mM ionomycin and 100 μM PMA with the B medium to prepare control or x10 PMA/ionomycin solution. Add the control to well #A1-#H1 of the 96 well clear plate (round bottom), and add x10 PMA/ionomycin solution to wells #A2-#H2 of the 96 well clear plate (round bottom).

	B medium	2 mM Ionomycin	100 μM PMA	Ethanol	Total
Control	995 μL	-		5 μL	1000 μL
x10 PMA/ionomycin solution	2382 μL	12 μL	6 μL	-	2400 μL

6-4 Addition of PMA/ionomycin to 2H4

Twenty-four hours after the addition of chemicals, add 10 μL of control or PMA/ionomycin solution to the cells (#A1-#H1 or #A2-#H12, respectively) using an 8 channel or 12 channel pipetman after pipetting 20 times. Make sure that the apex of the tip is dipped into the medium. Change tips every line you add. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 6 hours (37°C, CO_2, 5%). (cf. Figure 16)

Figure 16



7. Control

7-1 Preparing control chemical (bleomycin sulfate, dexamethasone)

7-1-1 Preparing bleomycin sulfate stock

Reagent	Company	Concentration of the stock solution	Preparing concentration	Final concentration
bleomycin sulfate	TOKYO CHEMICAL INDUSTRY B3972	10 mg/mL	10 mg/mL	0.4~200 μg/mL
Distilled water	GIBCO Cat#10977-015			

Dissolve 10 mg of bleomycin sulfate with distilled water 1 mL, dispend at 100 μL/tube and store a freezer at -30°C.

7-1-2 Preparing dexamethasone stock

Reagent	Company	Concentration of the stock solution	Preparing concentration	Final concentration
dexamethasone	Wako 041-18861	500 mg/mL	500 mg/mL	1.0~500 μg/mL
DMSO	Sigma #D5789			

Weigh 1 g of dexamethasone in volumetric flask and add DMSO up to 2 mL, dispend at 100 μL/tube and store a freezer at -30°C.

7-2 Preparation of cells for assay

A cell passage should be done 2-4 days before the assay.

Use cells between 1 and 6 weeks after thawing.

Pre-warm the B medium in a 37°C water bath. Count the number of cells and collect the number of cells needed (1.0 x 10^6 for two chemicals are required, but to have some leeway, 1.5 x 10^6 for two controls should be prepared), centrifuge the tube at 120-350 x *g*, 5 min. Resuspend in pre-warmed the B medium at a cell density of 2 x 10^5/mL. Transfer the cell suspension to a reservoir, and add 50 µL of cell suspension to each well of a 96 well µclear black plate (flat bottom) using an 8 channel or 12 channel pipetman. (cf. Figure 17)

Figure 17

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
B	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
C	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
D	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
E	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
F	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
G	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
H	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL

7-3 Arrangement of chemicals and vehicle

Add 100 μL of the 10 mg/mL distilled water solution of bleomycin sulfate to well #A12, and 50 μL of the distilled water to wells #A1-#A11 of the 96 well clear plate (round bottom).

Add 100 μL of the 500 mg/mL DMSO solution of dexamethasone to well #E12, 50 μL of DMSO to wells #E1-#E11, and 90 μL of the B medium to wells #F1-#F12 of the 96 well clear plate (round bottom)

7-4 Serial dilution

Conduct 9 serial dilutions at a common ratio of 2 as indicated in Figure 17 from well #A11 to well #A3 and #E11 to well #E3. Transfer 50 μL to the next (left) well. (cf. Figure 18)

Figure 18

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	bleomycin sulfate 10 mg/mL in distilled water 100uL
B												
C				2-fold dilution : transfer 50 μL (pipetman, yellow tip)								
D												
E	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	DMSO 100% 50uL	dexa methasone 500 mg/mL in DMSO 100uL
F	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL
G				2-fold dilution : transfer 50 μL (pipetman, yellow tip)								
H												

↓

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 50uL	Distilled water 50uL	bleomycin sulfate 0.02 mg/mL in distilled water 100uL	bleomycin sulfate 0.04 mg/mL in distilled water 50uL	bleomycin sulfate 0.08 mg/mL in distilled water 50uL	bleomycin sulfate 0.16 mg/mL in distilled water 50uL	bleomycin sulfate 0.31 mg/mL in distilled water 50uL	bleomycin sulfate 0.63 mg/mL in distilled water 50uL	bleomycin sulfate 1.3 mg/mL in distilled water 50uL	bleomycin sulfate 2.5 mg/mL in distilled water 50uL	bleomycin sulfate 5 mg/mL in distilled water 50uL	bleomycin sulfate 10 mg/mL in distilled water 50uL
B												
C												
D												
E	DMSO 100% 50uL	DMSO 100% 50uL	dexa methasone 1.0 mg/mL in DMSO 100uL	dexa methasone 2.0 mg/mL in DMSO 50uL	dexa methasone 3.9 mg/mL in DMSO 50uL	dexa methasone 7.8 mg/mL in DMSO 50uL	dexa methasone 16 mg/mL in DMSO 50uL	dexa methasone 31 mg/mL in DMSO 50uL	dexa methasone 63 mg/mL in DMSO 50uL	dexa methasone 125 mg/mL in DMSO 50uL	dexa methasone 250 mg/mL in DMSO 50uL	dexa methasone 500 mg/mL in DMSO 50uL
F	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL
G												
H												

7-5 2 step dilution

Add 20 μL of the diluted bleomycin sulfate to 480 μL of the B medium prepared in the assay block. And add 50 μL to 2H4 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. (cf. Figure 19-20)

Figure 19

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 50uL	Distilled water 50uL	bleomycin sulfate 0.02 mg/mL in distilled water 100uL	bleomycin sulfate 0.04 mg/mL in distilled water 50uL	bleomycin sulfate 0.08 mg/mL in distilled water 50uL	bleomycin sulfate 0.16 mg/mL in distilled water 50uL	bleomycin sulfate 0.31 mg/mL in distilled water 50uL	bleomycin sulfate 0.63 mg/mL in distilled water 50uL	bleomycin sulfate 1.3 mg/mL in distilled water 50uL	bleomycin sulfate 2.5 mg/mL in distilled water 50uL	bleomycin sulfate 5 mg/mL in distilled water 50uL	bleomycin sulfate 10 mg/mL in distilled water 50uL
B												
C												
D												
E	DMSO 100% 50uL	DMSO 100% 50uL	dexamethasone 1.0 mg/mL in DMSO 100uL	dexamethasone 2.0 mg/mL in DMSO 50uL	dexamethasone 3.9 mg/mL in DMSO 50uL	dexamethasone 7.8 mg/mL in DMSO 50uL	dexamethasone 16 mg/mL in DMSO 50uL	dexamethasone 31 mg/mL in DMSO 50uL	dexamethasone 63 mg/mL in DMSO 50uL	dexamethasone 125 mg/mL in DMSO 50uL	dexamethasone 250 mg/mL in DMSO 50uL	dexamethasone 500 mg/mL in DMSO 50uL
F	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL
G												
H												

20μL

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL	B medium 480uL
B												
C												
D												
E	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL
F												
G												
H												

Figure 20

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 500uL	B medium 500uL	bleomycin sulfate 0.8 ug/mL in B medium 500uL	bleomycin sulfate 1.6 ug/mL in B medium 500uL	bleomycin sulfate 3.1 ug/mL in B medium 500uL	bleomycin sulfate 6.3 ug/mL in B medium 500uL	bleomycin sulfate 12.5 ug/mL in B medium 500uL	bleomycin sulfate 25 ug/mL in B medium 500uL	bleomycin sulfate 50 ug/mL in B medium 500uL	bleomycin sulfate 100 ug/mL in B medium 500uL	bleomycin sulfate 200 ug/mL in B medium 500uL	bleomycin sulfate 400 ug/mL in B medium 500uL
B												
C												
D												
E	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL
F												
G												
H												

50µL

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
B	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
C	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
D	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
E	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
F	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
G	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
H	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL

7-6 Dilution of DMSO solution with the B medium

Dilute 10 μL of the DMSO solution of dexamethasone in wells #E1-#E12 with 90 μL of the B medium using an 8-12 channel pipetman. (cf. Figure 21)

Figure 21

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 30uL	Distilled water 30uL	bleomycin sulfate 0.02 mg/mL in distilled water 80uL	bleomycin sulfate 0.04 mg/mL in distilled water 30uL	bleomycin sulfate 0.08 mg/mL in distilled water 30uL	bleomycin sulfate 0.16 mg/mL in distilled water 30uL	bleomycin sulfate 0.31 mg/mL in distilled water 30uL	bleomycin sulfate 0.63 mg/mL in distilled water 30uL	bleomycin sulfate 1.3 mg/mL in distilled water 30uL	bleomycin sulfate 2.5 mg/mL in distilled water 30uL	bleomycin sulfate 5 mg/mL in distilled water 30uL	bleomycin sulfate 10 mg/mL in distilled water 30uL
B												
C												
D												
E	DMSO 100% 50uL	DMSO 100% 50uL	dexamethasone 1.0 mg/mL in DMSO 100uL	dexamethasone 2.0 mg/mL in DMSO 50uL	dexamethasone 3.9 mg/mL in DMSO 50uL	dexamethasone 7.8 mg/mL in DMSO 50uL	dexamethasone 16 mg/mL in DMSO 50uL	dexamethasone 31 mg/mL in DMSO 50uL	dexamethasone 63 mg/mL in DMSO 50uL	dexamethasone 125 mg/mL in DMSO 50uL	dexamethasone 250 mg/mL in DMSO 50uL	dexamethasone 500 mg/mL in DMSO 50uL
F	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL
G												
H												

10μL

↓

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 30uL	Distilled water 30uL	bleomycin sulfate 0.02 mg/mL in distilled water 80uL	bleomycin sulfate 0.04 mg/mL in distilled water 30uL	bleomycin sulfate 0.08 mg/mL in distilled water 30uL	bleomycin sulfate 0.16 mg/mL in distilled water 30uL	bleomycin sulfate 0.31 mg/mL in distilled water 30uL	bleomycin sulfate 0.63 mg/mL in distilled water 30uL	bleomycin sulfate 1.3 mg/mL in distilled water 30uL	bleomycin sulfate 2.5 mg/mL in distilled water 30uL	bleomycin sulfate 5 mg/mL in distilled water 30uL	bleomycin sulfate 10 mg/mL in distilled water 30uL
B												
C												
D												
E	DMSO 100% 40uL	DMSO 100% 40uL	dexamethasone 1.0 mg/mL in DMSO 90uL	dexamethasone 2.0 mg/mL in DMSO 40uL	dexamethasone 3.9 mg/mL in DMSO 40uL	dexamethasone 7.8 mg/mL in DMSO 40uL	dexamethasone 16 mg/mL in DMSO 40uL	dexamethasone 31 mg/mL in DMSO 40uL	dexamethasone 63 mg/mL in DMSO 40uL	dexamethasone 125 mg/mL in DMSO 40uL	dexamethasone 250 mg/mL in DMSO 40uL	dexamethasone 500 mg/mL in DMSO 40uL
F	dexamethasone 0 mg/mL DMSO 10% in B medium 100uL	dexamethasone 0 mg/mL DMSO 10% in B medium 100uL	dexamethasone 0.10 mg/mL DMSO 10% in B medium 100uL	dexamethasone 0.20 mg/mL DMSO 10% in B medium 100uL	dexamethasone 0.39 mg/mL DMSO 10% in B medium 100uL	dexamethasone 0.78 mg/mL DMSO 10% in B medium 100uL	dexamethasone 1.6 mg/mL DMSO 10% in B medium 100uL	dexamethasone 3.1 mg/mL DMSO 10% in B medium 100uL	dexamethasone 6.3 mg/mL DMSO 10% in B medium 100uL	dexamethasone 12.5 mg/mL DMSO 10% in B medium 100uL	dexamethasone 25 mg/mL DMSO 10% in B medium 100uL	dexamethasone 50 mg/mL DMSO 10% in B medium 100uL
G												
H												

7-7 2 step dilution

Add 10 μL of the diluted dexamethasone to 490 μL of the B medium prepared in the assay block. And add 50 μL to 2H4 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Manipulate the procedures from 7-6 to 7-7 as quickly as you can, and do not leave a long time at step after 7-6 or Figure 20. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 24 hours (37°C, CO_2, 5%). (cf. Figure 22-24).

Figure 22

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 30uL	Distilled water 30uL	bleomycin sulfate 0.02 mg/mL in distilled water 80uL	bleomycin sulfate 0.04 mg/mL in distilled water 30uL	bleomycin sulfate 0.08 mg/mL in distilled water 30uL	bleomycin sulfate 0.16 mg/mL in distilled water 30uL	bleomycin sulfate 0.31 mg/mL in distilled water 30uL	bleomycin sulfate 0.63 mg/mL in distilled water 30uL	bleomycin sulfate 1.3 mg/mL in distilled water 30uL	bleomycin sulfate 2.5 mg/mL in distilled water 30uL	bleomycin sulfate 5 mg/mL in distilled water 30uL	bleomycin sulfate 10 mg/mL in distilled water 30uL
B												
C												
D												
E	DMSO 100% 40uL	DMSO 100% 40uL	dexamethasone 1.0 mg/mL in DMSO 90uL	dexamethasone 2.0 mg/mL in DMSO 40uL	dexamethasone 3.9 mg/mL in DMSO 40uL	dexamethasone 7.8 mg/mL in DMSO 40uL	dexamethasone 16 mg/mL in DMSO 40uL	dexamethasone 31 mg/mL in DMSO 40uL	dexamethasone 63 mg/mL in DMSO 40uL	dexamethasone 125 mg/mL in DMSO 40uL	dexamethasone 250 mg/mL in DMSO 40uL	dexamethasone 500 mg/mL in DMSO 40uL
F	dexamethasone 0 mg/mL DMSO 10% in B medium 100uL	dexamethasone 0 mg/mL DMSO 10% in B medium 100uL	dexamethasone 0.10 mg/mL DMSO 10% in B medium 100uL	dexamethasone 0.20 mg/mL DMSO 10% in B medium 100uL	dexamethasone 0.39 mg/mL DMSO 10% in B medium 100uL	dexamethasone 0.78 mg/mL DMSO 10% in B medium 100uL	dexamethasone 1.6 mg/mL DMSO 10% in B medium 100uL	dexamethasone 3.1 mg/mL DMSO 10% in B medium 100uL	dexamethasone 6.3 mg/mL DMSO 10% in B medium 100uL	dexamethasone 12.5 mg/mL DMSO 10% in B medium 100uL	dexamethasone 25 mg/mL DMSO 10% in B medium 100uL	dexamethasone 50 mg/mL DMSO 10% in B medium 100uL
G												
H												

10μL

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 300uL	B medium 300uL	bleomycin sulfate 0.8 ug/mL in B medium 300uL	bleomycin sulfate 1.6 ug/mL in B medium 300uL	bleomycin sulfate 3.1 ug/mL in B medium 300uL	bleomycin sulfate 6.3 ug/mL in B medium 300uL	bleomycin sulfate 12.5 ug/mL in B medium 300uL	bleomycin sulfate 25 ug/mL in B medium 300uL	bleomycin sulfate 50 ug/mL in B medium 300uL	bleomycin sulfate 100 ug/mL in B medium 300uL	bleomycin sulfate 200 ug/mL in B medium 300uL	bleomycin sulfate 400 ug/mL in B medium 300uL
B												
C												
D												
E	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL	B medium 490uL
F												
G												
H												

Figure 23

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 300uL	B medium 300uL	bleomycin sulfate 0.8 ug/mL in B medium 300uL	bleomycin sulfate 1.6 ug/mL in B medium 300uL	bleomycin sulfate 3.1 ug/mL in B medium 300uL	bleomycin sulfate 6.3 ug/mL in B medium 300uL	bleomycin sulfate 12.5 ug/mL in B medium 300uL	bleomycin sulfate 25 ug/mL in B medium 300uL	bleomycin sulfate 50 ug/mL in B medium 300uL	bleomycin sulfate 100 ug/mL in B medium 300uL	bleomycin sulfate 200 ug/mL in B medium 300uL	bleomycin sulfate 400 ug/mL in B medium 300uL
B												
C												
D												
E	dexamethasone 0ug/mL DMSO 0.2% in B medium 500uL	dexamethasone 0ug/mL DMSO 0.2% in B medium 500uL	dexamethasone 2.0ug/mL DMSO 0.2% in B medium 500uL	dexamethasone 3.9ug/mL DMSO 0.2% in B medium 500uL	dexamethasone 7.8ug/mL DMSO 0.2% in B medium 500uL	dexamethasone 15.6ug/mL DMSO 0.2% in B medium 500uL	dexamethasone 31.3ug/mL DMSO 0.2% in B medium 500uL	dexamethasone 62.5ug/mL DMSO 0.2% in B medium 500uL	dexamethasone 125ug/mL DMSO 0.2% in B medium 500uL	dexamethasone 250ug/mL DMSO 0.2% in B medium 500uL	dexamethasone 500ug/mL DMSO 0.2% in B medium 500uL	dexamethasone 1000ug/mL DMSO 0.2% in B medium 500uL
F												
G												
H												

50µL

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.4 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.8 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 1.6 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 3.1 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 6.3 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 12.5 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 25 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 50 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 100 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 200 ug/mL 2H4 1x10^4 B medium 100uL
B	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.4 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.8 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 1.6 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 3.1 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 6.3 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 12.5 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 25 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 50 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 100 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 200 ug/mL 2H4 1x10^4 B medium 100uL
C	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.4 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.8 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 1.6 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 3.1 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 6.3 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 12.5 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 25 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 50 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 100 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 200 ug/mL 2H4 1x10^4 B medium 100uL
D	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.4 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.8 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 1.6 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 3.1 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 6.3 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 12.5 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 25 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 50 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 100 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 200 ug/mL 2H4 1x10^4 B medium 100uL
E	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
F	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
G	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
H	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL

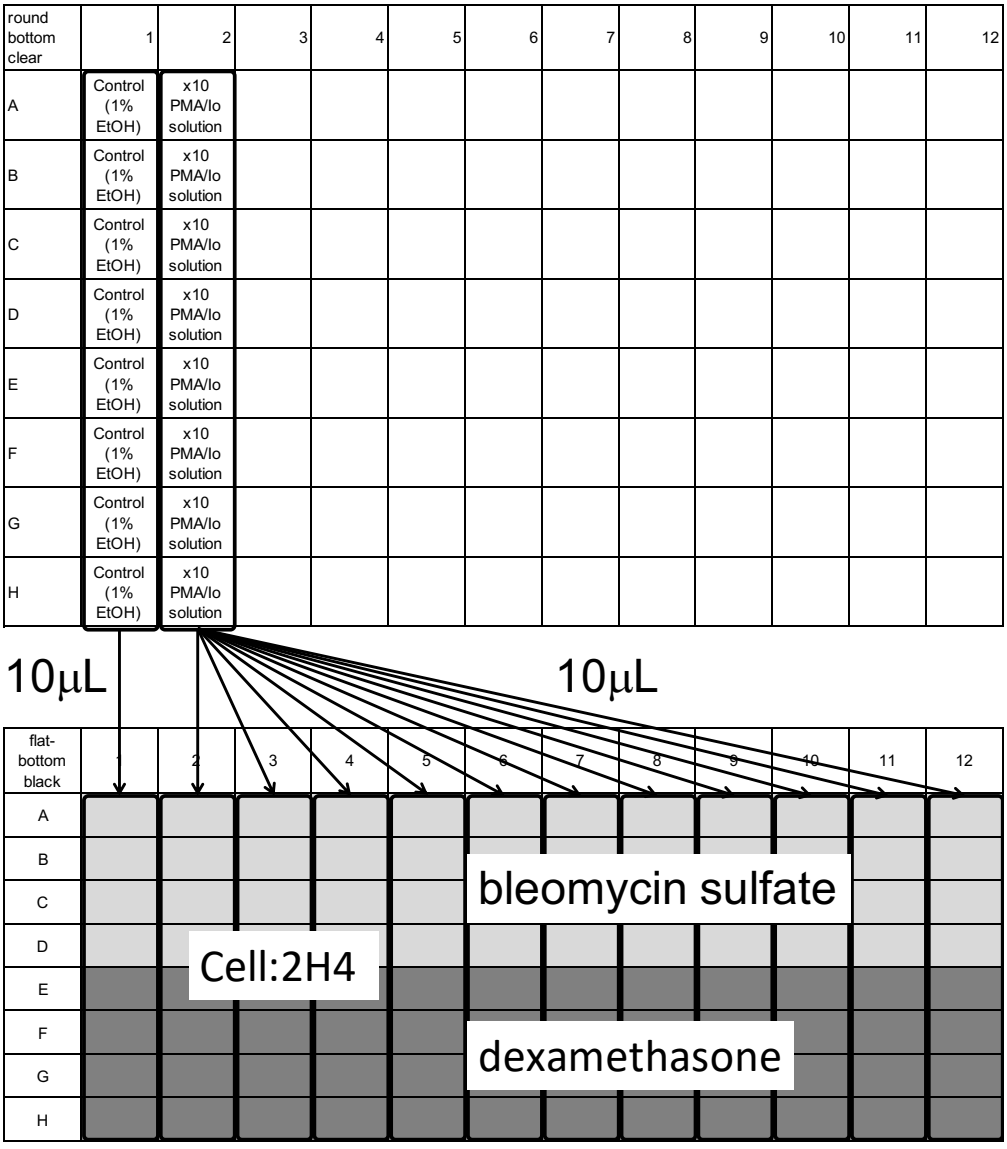
Figure 24 Final constituents of each well of the plate

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.4 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.8 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 1.6 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 3.1 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 6.3 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 12.5 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 25 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 50 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 100 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 200 ug/mL 2H4 1x10^4 B medium 100uL
B	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.4 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.8 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 1.6 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 3.1 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 6.3 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 12.5 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 25 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 50 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 100 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 200 ug/mL 2H4 1x10^4 B medium 100uL
C	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.4 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.8 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 1.6 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 3.1 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 6.3 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 12.5 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 25 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 50 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 100 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 200 ug/mL 2H4 1x10^4 B medium 100uL
D	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.4 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.8 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 1.6 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 3.1 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 6.3 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 12.5 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 25 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 50 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 100 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 200 ug/mL 2H4 1x10^4 B medium 100uL
E	dexamethasone 0ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 0ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 1.0ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 2.0ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 3.9ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 7.8ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 15.6ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 31.3ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 62.5ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 125ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 250ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 500ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL
F	dexamethasone 0ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 0ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 1.0ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 2.0ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 3.9ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 7.8ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 15.6ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 31.3ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 62.5ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 125ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 250ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 500ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL
G	dexamethasone 0ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 0ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 1.0ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 2.0ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 3.9ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 7.8ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 15.6ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 31.3ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 62.5ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 125ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 250ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 500ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL
H	dexamethasone 0ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 0ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 1.0ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 2.0ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 3.9ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 7.8ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 15.6ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 31.3ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 62.5ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 125ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 250ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 500ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL

7-8 Addition of PMA/ionomycin to 2H4

Twenty-four hours after the addition of bleomycin sulfate and dexamethasone, add 10 μL of control or PMA/ionomycin solution prepared in §6-3 to the cells (#A1-#H1 or #A2-#H12, respectively) using an 8 channel or 12 channel pipetman after pipetting 20 times. Make sure that the apex of the tip is dipped into the medium. Change tips every line you add. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 6 hours (37°C, CO_2, 5%). (cf. Figure 25)

Figure 25



8. Calculation of the transmittance factors

Color discrimination in multi-color reporter assays is generally achieved using detectors (luminometer and plate reader) equipped with optical filters, such as sharp-cut (long-pass) filters and band-pass filters. The transmittance factors of these filters for each bio-luminescence signal color must be calibrated prior to all experiments by following the protocols below.

8-1 Reagents

- Single reference samples:

Lyophilized luciferase enzyme reagent for stable luciferase green (SLG)
Lyophilized luciferase enzyme reagent for stable luciferase orange (SLO)
Lyophilized luciferase enzyme reagent for stable luciferase red (SLR)

- Assay reagent:

Tripluc® Luciferase assay reagent（TOYOBO Cat#MRA-301）

- B medium: for luciferase assay (30 mL, stored at 2- 8°C)

Reagent	Company	Conc.	Final conc. in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	27 mL
FBS	Biological Industries Cat#04-001-1A Lot: 1524129	-	10 %	3 mL

8-2 Preparation of luminescence reaction solution

Thaw Tripluc® Luciferase assay reagent (Tripluc) and keep it at room temperature either in a water bath or at ambient air temperature. Power on the luminometer 30 min before starting the measurement to allow the photomultiplier to stabilize.

Add 200 μL of 100 mM Tris-HCl (pH8.0) contains 10 % glycerol to each tube of lyophilized reference sample to dissolve the enzymes, divide into 10 μL aliquots in 1.5 mL disposable tubes and store in a freezer at -80°C. The stored frozen solution of the reference samples can be used for up to 6 months.

Add 1 mL of the B medium to each tube of frozen reference sample (10 μL sample per tube). Keep the reference samples on ice to prevent deactivation.

8-3 Bioluminescence measurement

Transfer 100 µL of the diluted reference samples to a black 96 well plate (flat bottom) as shown below.

Figure 26

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	SLG 100 µL	SLG 100 µL	SLG 100 µL									
C												
D	SLO 100 µL	SLO 100 µL	SLO 100 µL									
E												
F	SLR 100 µL	SLR 100 µL	SLR 100 µL									
G												
H												

Transfer 100 µL of pre-warmed Tripluc to each well of the plate containing the reference sample using a pipetman. Shake the plate for 10 min at room temperature (about 25°C) using a plate shaker. Remove bubbles in the solutions in wells if they appear. Place the plate in the luminometer to measure the luciferase activity. Bioluminescence is measured for 3 sec each in the absence (F0) and presence (F1, F2) of the optical filters. An example of the raw output data is shown below.

Figure 27

Measurement without Filter

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	3757015	3716611	3810382									
C												
D	1202691	1210208	1122295									
E												
F	2465453	2207572	2077689									
G												
H												

Measurement with Filter 1

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	1269950	1257268	1289562									
C												
D	808550	813160	754174									
E												
F	2193723	1968240	1853873									
G												
H												

Measurement with Filter 2

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	236478	234079	240876									
C												
D	235121	235878	217432									
E												
F	1585258	1420099	1339265									
G												
H												

Six transmittance factors of the optical filters were calculated as follow:

$$\text{Transmittance factor } (\kappa G_{R56}) = \frac{\#B1 \text{ of } F1 + \#B2 \text{ of } F1 + \#B3 \text{ of } F1}{\#B1 \text{ of } F0 + \#B2 \text{ of } F0 + \#B3 \text{ of } F0}$$

$$\text{Transmittance factor } (\kappa O_{R56}) = \frac{\#D1 \text{ of } F1 + \#D2 \text{ of } F1 + \#D3 \text{ of } F1}{\#D1 \text{ of } F0 + \#D2 \text{ of } F0 + \#D3 \text{ of } F0}$$

$$\text{Transmittance factor } (\kappa R_{R56}) = \frac{\#F1 \text{ of } F1 + \#F2 \text{ of } F1 + \#F3 \text{ of } F1}{\#F1 \text{ of } F0 + \#F2 \text{ of } F0 + \#F3 \text{ of } F0}$$

$$\text{Transmittance factor } (\kappa G_{R60}) = \frac{\#B1 \text{ of } F2 + \#B2 \text{ of } F2 + \#B3 \text{ of } F2}{\#B1 \text{ of } F0 + \#B2 \text{ of } F0 + \#B3 \text{ of } F0}$$

$$\text{Transmittance factor } (\kappa O_{R60}) = \frac{\#D1 \text{ of } F2 + \#D2 \text{ of } F2 + \#D3 \text{ of } F2}{\#D1 \text{ of } F0 + \#D2 \text{ of } F0 + \#D3 \text{ of } F0}$$

$$\text{Transmittance factor } (\kappa R_{R60}) = \frac{\#F1 \text{ of } F2 + \#F2 \text{ of } F2 + \#F3 \text{ of } F2}{\#F1 \text{ of } F0 + \#F2 \text{ of } F0 + \#F3 \text{ of } F0}$$

In the case shown above,

$$\text{Transmittance factors } (\kappa G_{R56}) = \frac{1269550 + 1257268 + 1289562}{3757015 + 3716611 + 3810382} = 0.338$$

Transmittance factors (κO_{R56})=$\frac{808550+813160+754174}{1202691+1210208+1122295}$=0.672

Transmittance factors (κR_{R56})=$\frac{2193723+1968240+1853873}{2465453+2207572+2077689}$=0.891

Transmittance factors (κG_{R60})= $\frac{236478+234079+240876}{3757015+3716611+3810382}$=0.06

Transmittance factors (κO_{R60})=$\frac{235121+235878+217432}{1202691+1210208+1122295}$=0.195

Transmittance factors (κR_{R60})=$\frac{1585258+1420099+1339265}{2465453+2207572+2077689}$=0.644

Calculated transmittance factors are used for all the measurements executed using the same luminometer.

Input the transmittance factors to #C6-#E7 of the “Data Input” sheet of the Data sheet as follow.

Figure 28

	A	B	C	D	E	F
1	MultiReporter Assay System –Tripluc®– Calculation Sheet					
2						
3		Transmittance Data				
4			SLG	SLO	SLR	
5		F0	1	1	1	
6		F1	κG_{R56}	κO_{R56}	κR_{R56}	
7		F2	κG_{R60}	κO_{R60}	κR_{R60}	
8						

9. Measurement

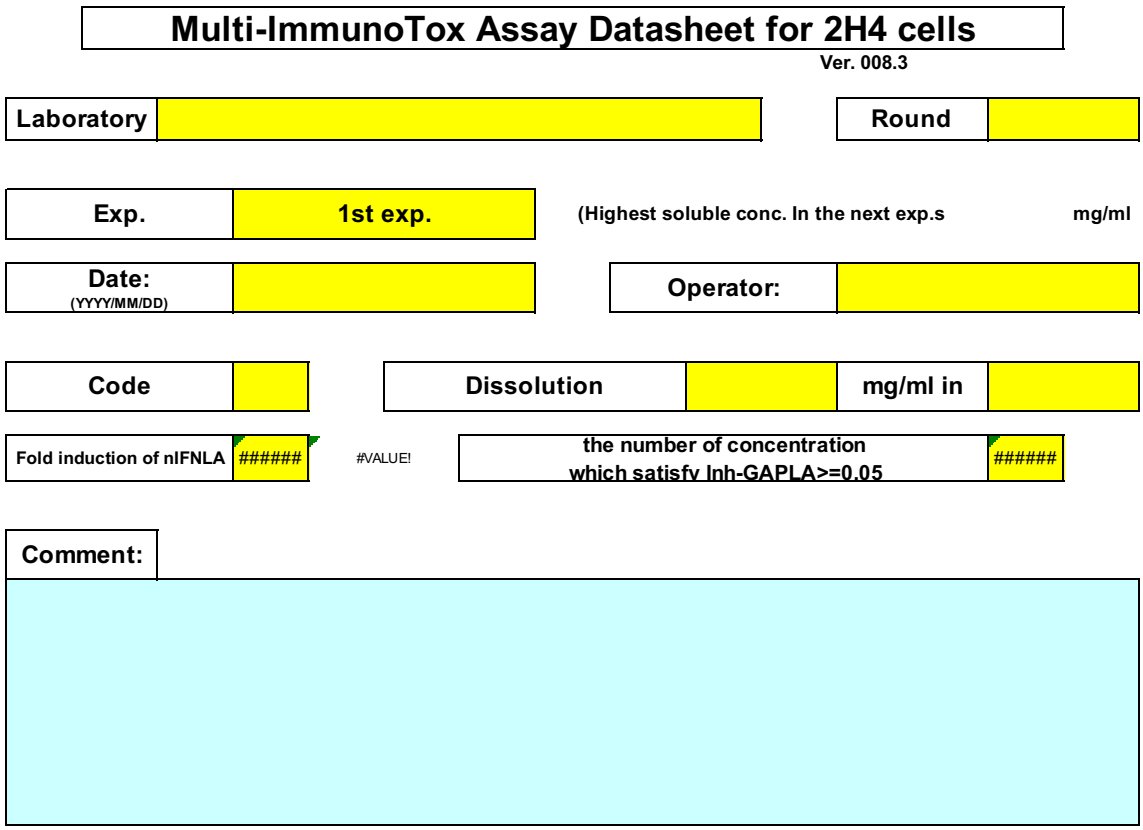
Please refer Appendix 1 for the principle of measurement of luciferase activity.

Thaw Tripluc® Luciferase assay reagent (Tripluc) and keep it at room temperature either in a water bath or at ambient air temperature. Power on the luminometer 30 min before starting the measurement to allow the photomultiplier to stabilize.

Transfer 100 μL of pre-warmed Tripluc from the reservoir to each well of the plate containing the reference sample using an 8 channel or 12 channel pipetman. Shake the plate for 10 min at room temperature (about 25°C) on a plate shaker. Remove bubbles in the solutions in the wells if they appear. Place the plate in the luminometer to measure the luciferase activity. Bioluminescence is measured for 3 sec each in the absence of (F0) and presence (F1, F2) of the optical filters.

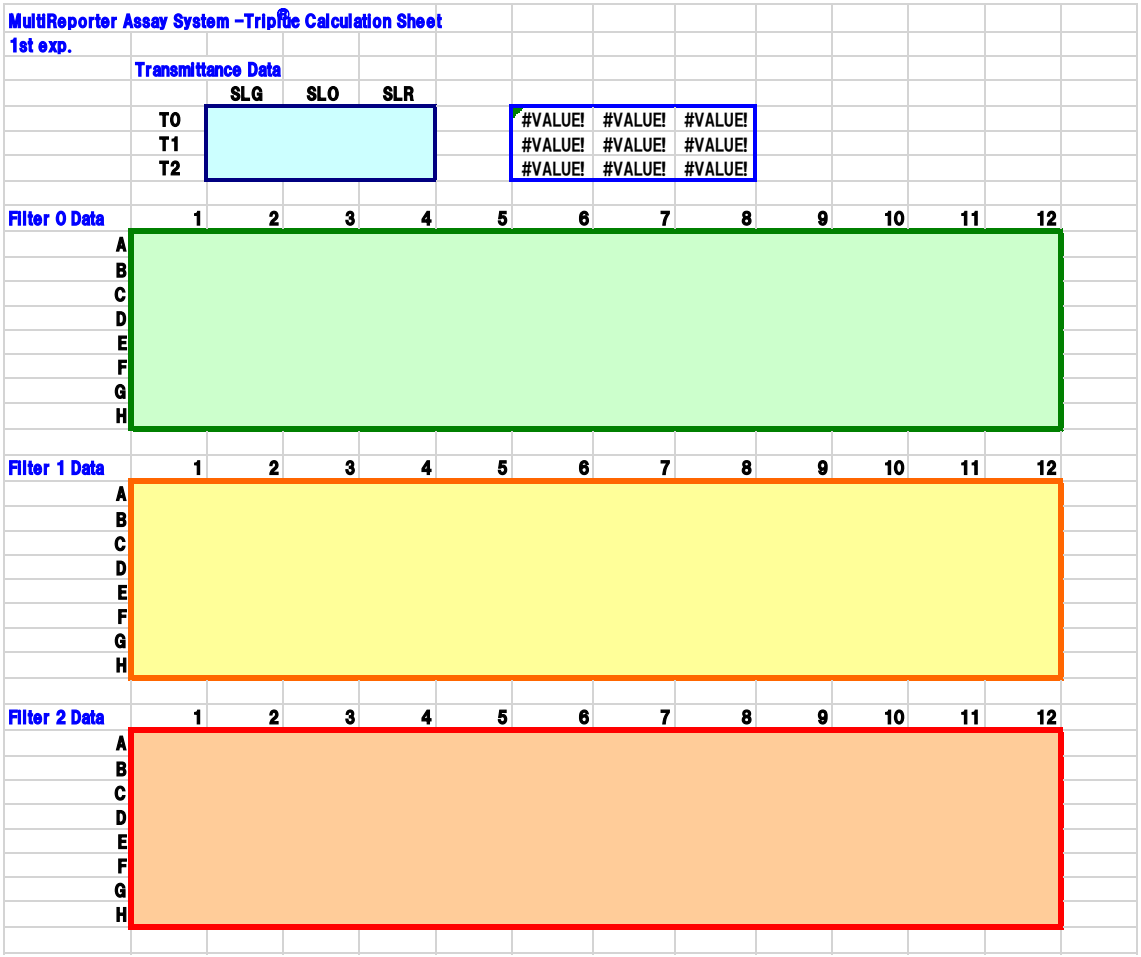
1st. Add the information regarding the name of laboratory, the round of experiments if multiple experimental sets are performed, the experiment number, date, the operator, chemical codes, dissolved in distilled water or DMSO, the prepared concentration, molecular weight of the chemicals and comments if any to Face Sheet of the data sheet.

Figure 29 “Face Sheet” of the data sheet



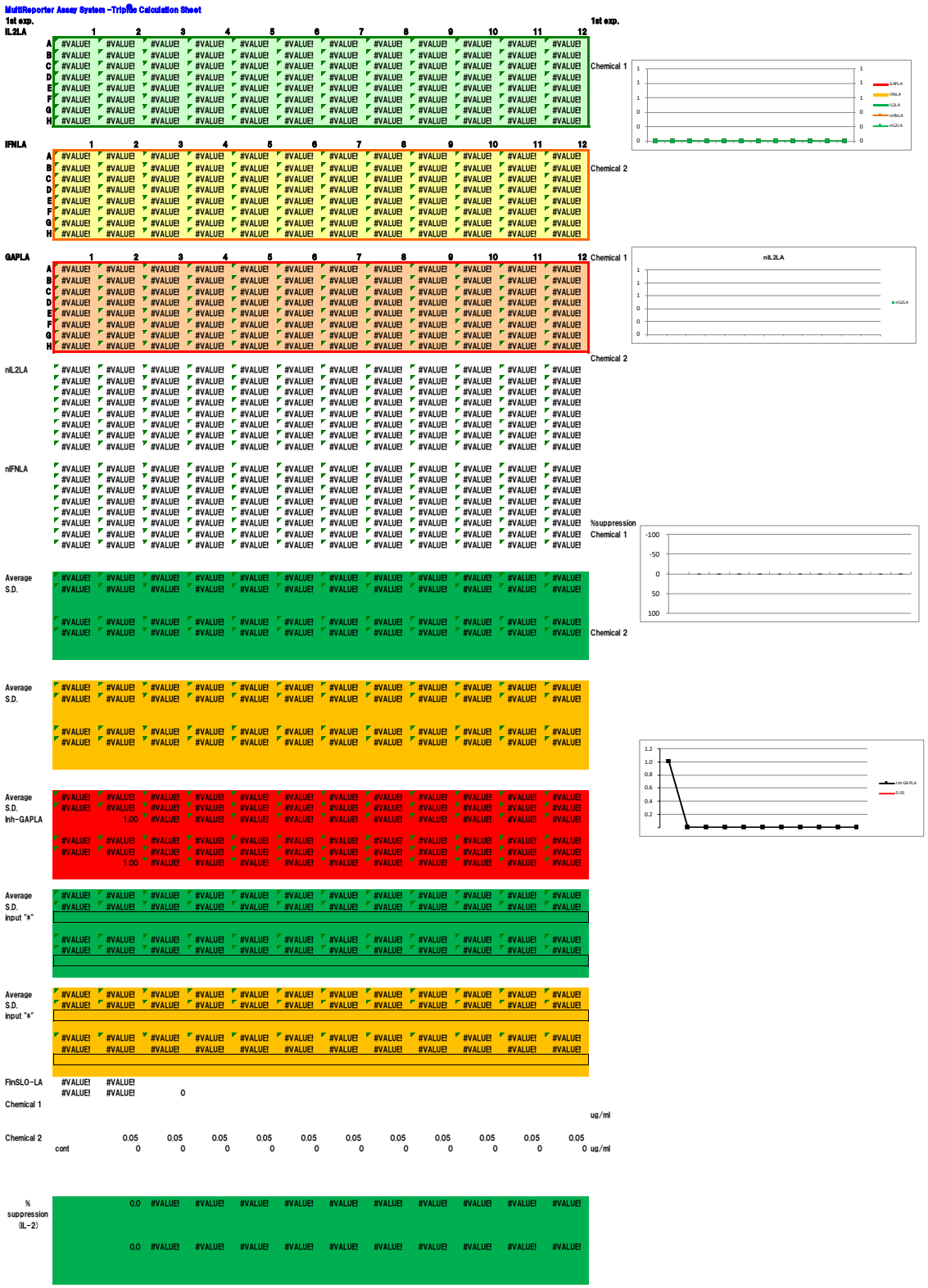
2nd. Copy the results of the F0, F1 and F2 measurements (values are expressed as counts) and paste them into the appropriate area in the “Data Input” sheet of the data sheet shown below. In addition, input the transmittance factors calculated in "§5. Calculation of the transmittance factors" to #C6-#E7 of the “Data Input” sheet.

Figure 30 “Data Input” sheet of the data sheet



Next, the calculated results for the parameters of the Multi-Immuno Tox assay for each concentration, e.g., IL2LA, IFNLA, GAPLA, nIL2LA, nIFNLA, the mean ± SD of IL2LA, the mean ± SD of IFNLA, the mean ± SD of GAPLA, % suppression and graphs will automatically appear on the “Result Format” sheet of the data sheet.

Figure 31 “Result Format” sheet of the data sheet



10. Data analysis

In the IL-2 Luc LTT, chemicals are first determined to be suppressive, stimulatory, or no effect based on the values of %suppression, which is defined as (nIL2LA of 2H4 cells treated with chemicals / nIL2LA of non-treated 2H4 cells) x 100. Then, considering the values of Inh-GAPLA, which is defined as GAPLA of 2H4 cells treated with chemicals / GAPLA of untreated cells, chemicals classified as suppressive, stimulatory, or no effect were further classified into leukocyte toxic, suppressive, stimulatory or no effect.

Definition of the parameters used in IL-2 Luc leukocyte toxicity test (IL-2 Luc LTT).

Abbreviations	Definition
GAPLA	SLR luciferase activity reflecting GAPDH promoter activity
IL2LA	SLG luciferase activity reflecting IL-2 promoter activity of 2H4 cells
IFNLA	SLO luciferase activity reflecting IFN-g promoter activity of 2H4 cells
nIL2LA	IL2LA/GAPLA of 2H4 cells
nIFNLA	IFNLA/GALA of 2H4 cells
% suppression	(nIL2LA of 2H4 cells treated with chemicals/ nIL2LA of non-treated 2H4 cells) x 100
CV05	The lowest concentration of the chemical at which Inh-GAPLA becomes < 0.05
Inh-GAPLA	GAPLA of 2H4 cells treated with chemicals /GAPLA of untreated cells
Min Inh-GAPLA	The minimum value of Inh-GAPLA of each experiment

11. Criteria

11-1 Acceptance criteria

The following acceptance criteria should be met when using the MITA method. In each time of the experiments, a control experiment examining nIFNLA of 2H4 cells treated with PMA/Io and nIFNLA of non-treated 2H4 cells must be conducted. Then, the fold induction of nIFNLA of 2H4 cells treated with PMA/Ionomycin to nIFNLA of non-treated 2H4 cells is calculated. If the fold induction is less than 3.0, the results obtained from these experiments should be rejected.

11-2 Criterion to determine leukocyte toxic or non-leukocyte toxic in the IL-2 Luc LTT

The experiments are repeated until 2 consistent leukocyte toxic results, indeterminate results, or non-leukocyte toxic results are obtained. When 2 consistent results are obtained, the chemicals are judged as indicated by the obtained consistent results.

In each experiment, if chemicals meet the following criteria described below and give Min Inh-GAPLA < 0.7, they are judged as leukocyte toxic. Otherwise, they are judged as provisional non-leukocyte toxic.

The criteria for stimulatory:

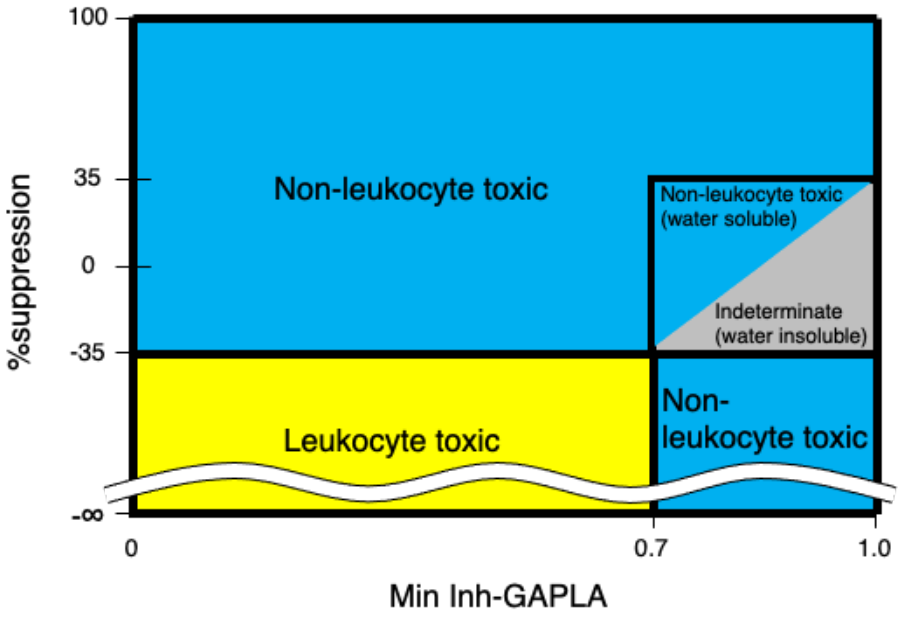
1./ The mean of % suppression is ≤-35 (stimulatory) with statistical significance. The statistical significance is judged by its 95% confidence interval.

2./ The result shows 2 or more consecutive statistically significant stimulatory data points or 1 statistically significant stimulatory data point with a trend in which at least 3 consecutive data points decrease in a dose-dependent manner. In the latter case, the trend can cross 0, as long as only 1 data point shows the opposite effect without statistical significance.

3./ The results are judged using only data obtained at the concentration at which Inh-GAPLA is ≥0.05.

Of chemicals that are not judged as provisional non-leukocyte toxic, if the chemicals that are insoluble at 10 mg/mL in distilled water do not give statistically significant suppressive or stimulatory data points and give Min Inh-GAPLA ≥ 0.7, they are judged as indeterminate because they may not be dissolved in the vehicle at the concentration sufficient to show the effects. Otherwise, they are judged as non-leukocyte toxic.

Figure 32


100
35
0
-35
-∞
%suppression
Non-leukocyte toxic
Non-leukocyte toxic
(water soluble)
Indeterminate
(water insoluble)
Leukocyte toxic
Non-
leukocyte toxic
0
0.7
1.0
Min Inh-GAPLA

12. Update record

Ver. 001.3 2020. September. 16th distribution
change cell culture
change the preparation of chemical
change the criterion

Ver. 001.2 2020. August. 7 distribution
change the preparation of chemical
change the criterion

Ver. 001.1 2020. July. 20 distribution
delete the description about D0-IL-2 Luc assay
change the criterion

Ver. 001 2020. June. 19 distribution

Appendix 1 Principle of measurement of luciferase activity

MultiReporter Assay System -Tripluc- can be used with a microplate-type luminometer with a multi-color detection system, which can equip two optical filters (e.g. Phelios AB-2350 (ATTO), ARVO (PerkinElmer), Tristar LB941 (Berthold)). The optical filters used in measurement are a 560 nm long-pass filter and a 600 nm long-pass filter.

(1) Measurement of three-color luciferase with two optical filters.

This is an example using Phelios AB-2350 (ATTO). This luminometer equips a 560 nm long-pass filter (560 nm LP, Filter 1) and a 600 nm long pass filter (600 nm LP, Filter 2) for optical isolation.

First, using luciferase enzyme reagent of SLG (λmax = 550 nm), SLO (λmax = 580 nm) and SLR (λmax = 630 nm), measure i) the intensity of light without filter (all optical), ii) the intensity of 560 nm LP (Filter 1) transmitted light iii) the intensity of 600 nm LP (Filter 2) transmitted light, and calculate the coefficient factor listed below.

Coefficient factor		Abbreviation	Definition
SLG	Filter 1 transmittance factor	κG_{R56}	The intensity of 560 nm LP (Filter 1) transmitted SLG / the intensity of SLG without filter (all optical)
	Filter 2 transmittance factor	κG_{R60}	The intensity of 600 nm LP (Filter 2) transmitted SLG / the intensity of SLG without filter (all optical)
SLO	Filter 1 transmittance factor	κO_{R56}	The intensity of 560 nm LP (Filter 1) transmitted SLO / the intensity of SLO without filter (all optical)
	Filter 2 transmittance factor	κO_{R60}	The intensity of 600 nm LP (Filter 2) transmitted SLO / the intensity of SLO without filter (all optical)
SLR	Filter 1 transmittance factor	κR_{R56}	The intensity of 560 nm LP (Filter 1) transmitted SLR / the

			intensity of SLR without filter (all optical)
	Filter 2 transmittance factor	κR_{R60}	The intensity of 600 nm LP (Filter 2) transmitted SLR / the intensity of SLR without filter (all optical)

When the intensity of SLG, SLO and SLR in test sample are defined as G, O and R, respectively, i) the intensity of light without filter (all optical): F0, ii) the intensity of 560 nm LP (Filter 1) transmitted light and iii) the intensity of 600 nm LP (Filter 2) transmitted light are described as below.

$F0=G+O+R$

$F1=\kappa G_{R56} \times G + \kappa O_{R56} \times O + \kappa R_{R56} \times R$

$F2=\kappa G_{R60} \times G + \kappa O_{R60} \times O + \kappa R_{R60} \times R$

These formulas can be rephrased as follows

$$\begin{pmatrix} F0 \\ F1 \\ F2 \end{pmatrix} = \begin{pmatrix} 1 & 1 & 1 \\ \kappa G_{R56} & \kappa O_{R56} & \kappa R_{R56} \\ \kappa G_{R60} & \kappa O_{R60} & \kappa R_{R60} \end{pmatrix} \begin{pmatrix} G \\ O \\ R \end{pmatrix}$$

Then using calculated coefficient factors and measured F0, F1 and F2, you can calculate G, O and R-value as follows.

$$\begin{pmatrix} G \\ O \\ R \end{pmatrix} = \begin{pmatrix} 1 & 1 & 1 \\ \kappa G_{R56} & \kappa O_{R56} & \kappa R_{R56} \\ \kappa G_{R60} & \kappa O_{R60} & \kappa R_{R60} \end{pmatrix}^{-1} \begin{pmatrix} F0 \\ F1 \\ F2 \end{pmatrix}$$

This calculation can be performed using the functions "MININVERSE" and "MMULT" in Microsoft Excel. These calculations are integrated in the Data Sheet.

Appendix 2　Validation of reagents and equipment

5-1 Measurement of transmittance of optical filter for multicolor measurement

For color discriminations in the multi-color reporter assay, detectors (luminometer and plate reader) are usually equipped with optical filters, such as sharp-cut (long-pass) filters and band-pass filters. The transmittance factors of these filters for each bioluminescence signal color have to be calibrated prior to all experiments by following the protocols below.

5-1-1 Reagents

・Single reference samples:

Lyophilized luciferase enzyme reagent of SLG

Lyophilized luciferase enzyme reagent of SLO

Lyophilized luciferase enzyme reagent of SLR

・Assay reagent:

Tripluc® Luciferase assay reagent（TOYOBO Cat#MRA-301）

・B medium: for luciferase assay (30 mL, stored at 2 – 8°C)

Reagent	Company	Conc.	Final conc. in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	27 mL
FBS	Biological Industries Cat#04-001-1A Lot: 1524129	-	10 %	3 mL

5-1-2 Calibration

5-1-2-1 Preparation of luminescence reaction solution

Thaw Tripluc® Luciferase assay reagent (Tripluc) and keep it at room temperature by bathing in water or ambient air. Start the luminometer 30 min before starting the measurement for stabilization of the photomultiplier.

Add 200 μL of 100 mM Tris-HCl (pH8.0) contains 10% glycerol to each tube of the lyophilized reference samples to dissolve the enzymes, followed by separating them into 1.5 mL disposable tubes at 10 μL each and storing in a freezer at -80°C. The stored frozen solution of the reference samples can be used for one half year.

Add 1 mL of the B medium to each tube of the frozen reference sample (10 μL in a tube) and label them as SLG1/1, SLO1/1 and SLR1/1. Keep the reference samples on ice to prevent deactivation.

Prepare dilution series of the single reference samples of SLG, SLO and SLR as follows. Dilute 0.3 mL of each 1/1 solution with 0.9 mL of the B medium to make SLG1/4, SLO1/4 and SLR1/4. In the same manner, prepare 1/16 and 1/64 solution of each. Keep diluted reference samples on ice.

5-1-2-2 Bioluminescence measurement

Transfer 100 µL of the diluted reference samples to a black 96 well plate (flat bottom) as shown below.

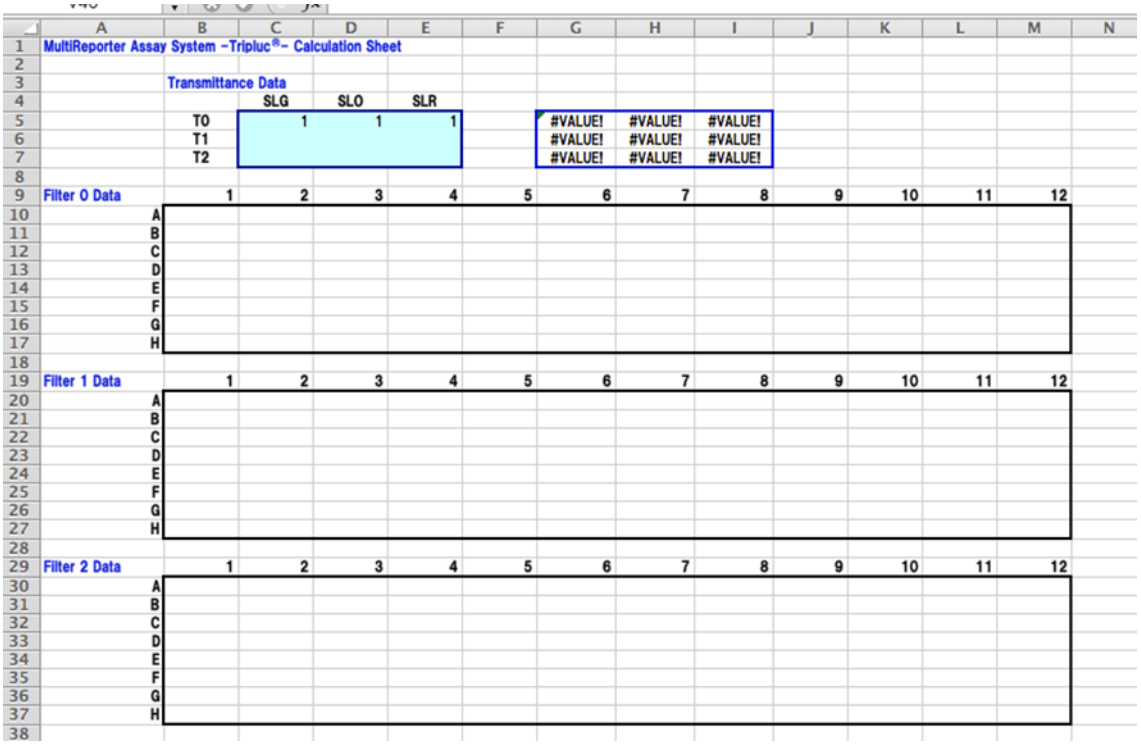
Figure 33

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	SLG 1/1	SLG 1/1	SLG 1/1	SLG 1/4	SLG 1/4	SLG 1/4	SLG 1/16	SLG 1/16	SLG 1/16	SLG 1/64	SLG 1/64	SLG 1/64
C												
D	SLO 1/1	SLO 1/1	SLO 1/1	SLO 1/4	SLO 1/4	SLO 1/4	SLO 1/16	SLO 1/16	SLO 1/16	SLO 1/64	SLO 1/64	SLO 1/64
E												
F	SLR 1/1	SLR 1/1	SLR 1/1	SLR 1/4	SLR 1/4	SLR 1/4	SLR 1/16	SLR 1/16	SLR 1/16	SLR 1/64	SLR 1/64	SLR 1/64
G												
H												

Transfer 100 µL of pre-warmed Tripluc to each well containing the reference samples of the plate using a pipetman. Shake the plate for 10 min at room temperature (about 25°C) with a plate shaker. Remove bubbles on the solutions in wells if they appear. Place the plate into the luminometer to measure the luciferase activity. Bioluminescence is measured for 3 sec each in the absence (F0) and presence (F1, F2) of the optical filters.

Copy the results of the F0, F1 and F2 measurement (values are expressed as counts) and paste it to the appropriate area in the “Data Input” sheet of the data sheet for data analyses shown below.

Figure 34



	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	MultiReporter Assay System –Tripluc®– Calculation Sheet													
2														
3		Transmittance Data												
4			SLG	SLO	SLR									
5		T0	1	1	1		#VALUE!	#VALUE!	#VALUE!					
6		T1					#VALUE!	#VALUE!	#VALUE!					
7		T2					#VALUE!	#VALUE!	#VALUE!					
8														
9	Filter 0 Data		1	2	3	4	5	6	7	8	9	10	11	12
10		A												
11		B												
12		C												
13		D												
14		E												
15		F												
16		G												
17		H												
18														
19	Filter 1 Data		1	2	3	4	5	6	7	8	9	10	11	12
20		A												
21		B												
22		C												
23		D												
24		E												
25		F												
26		G												
27		H												
28														
29	Filter 2 Data		1	2	3	4	5	6	7	8	9	10	11	12
30		A												
31		B												
32		C												
33		D												
34		E												
35		F												
36		G												
37		H												
38														

Record all the results for quality control.

5-2 Quality control of equipment

In order to confirm the detector stability as the quality control, the reference luciferase sample, optical property, the protocol described here should be performed at the beginning of the experiments every day.

5-2-1 Light source

LED Plate: Reference LED light source plates equipped with stabilized red, green, and blue LEDs are commercially available. For example,

TRIANT® (wSL-0001) by ATTO (Tokyo, Japan)

L12367 by Hamamatsu Photonics (Shizuoka, Japan)

5-2-2 Data collection (an example using TRIANT® by ATTO)

1) Start luminometer 30 min before starting the measurement for stabilization of the photomultiplier.
2) Start LED plate and select “PMT” mode.
3) Select three-color (BRG) mode and adjust light intensity to 1/10 (10E-1).

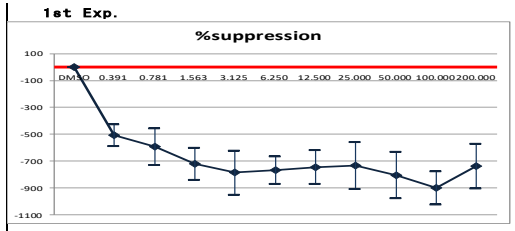
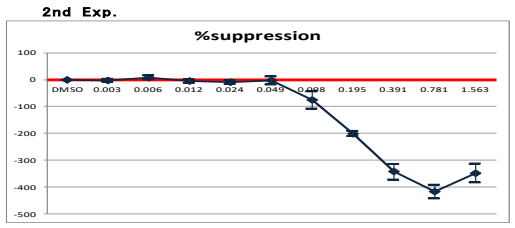
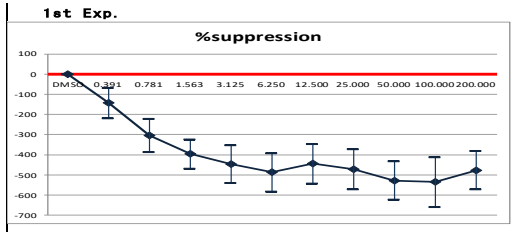
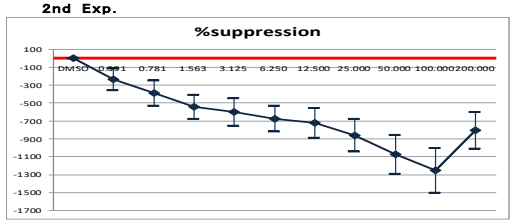
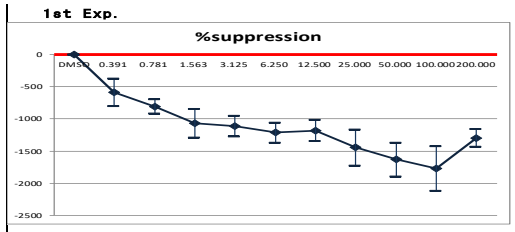
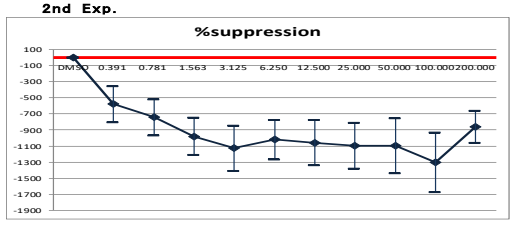
4) Place the LED plate into the luminometer. Light intensity is measured for 3 sec each in the absence (F0) and presence (F2) of the optical filter.
5) Blue, green, and red LEDs are located at the position of #F6, #E6, and #D6, respectively. Copy the collected data of each position to the appropriate area on Sheet "LED" in the excel file of the data sheet.
6) Check the photo-detector performance by comparing with old data of the LED plate. For quality control purpose, every collected data should be recorded.
7) LED plate data typically fluctuates up to 1.5% (σ). Disagreement to the old data should be less than 3×σ (= 4.5%).

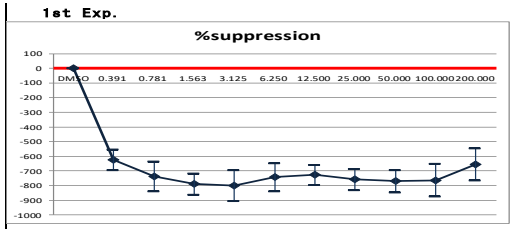
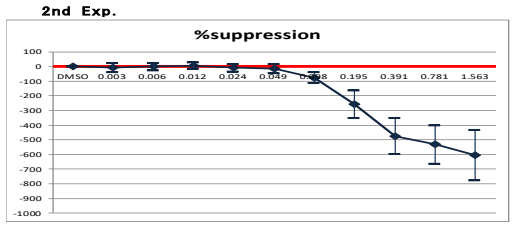
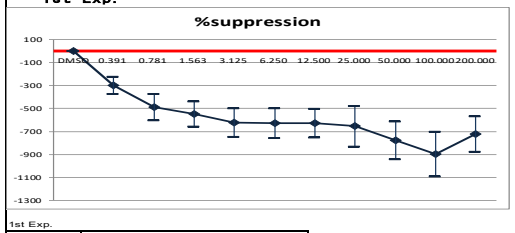
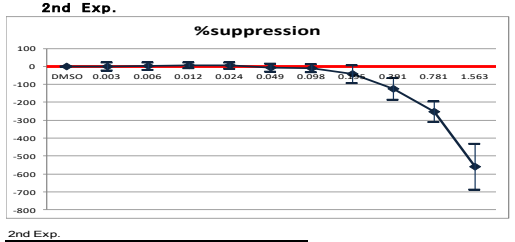
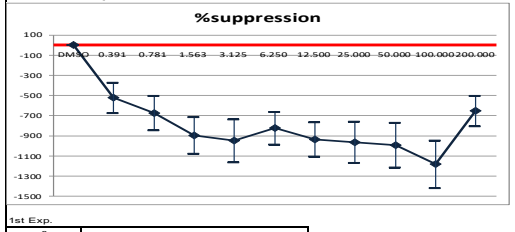
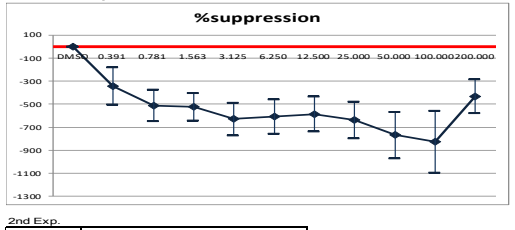
MITA IL-2 LUC LTT Phase1 results

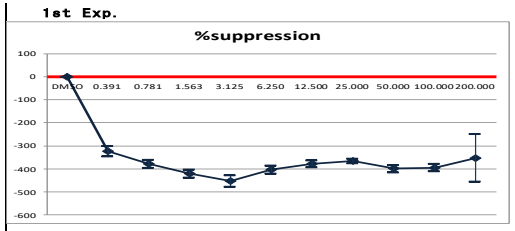
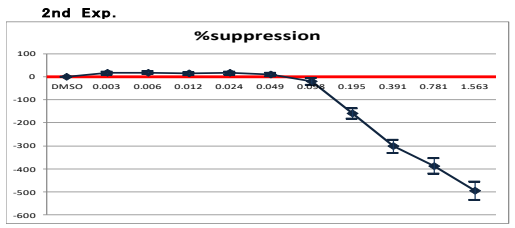
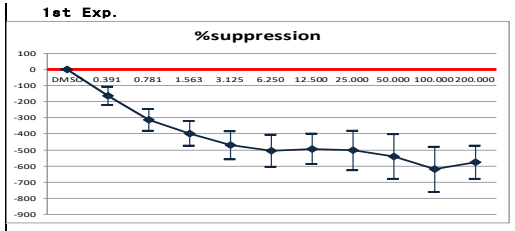
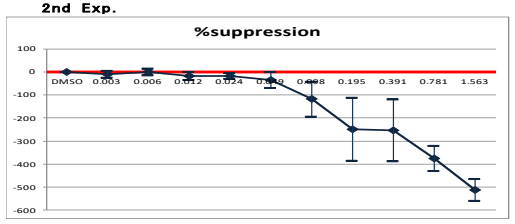
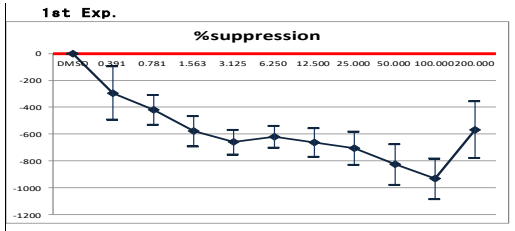
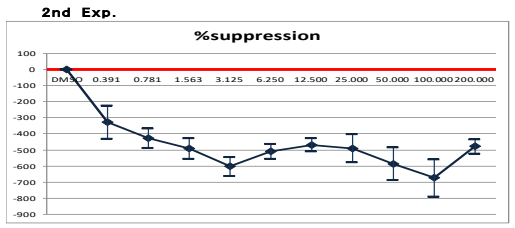
7th January 2021

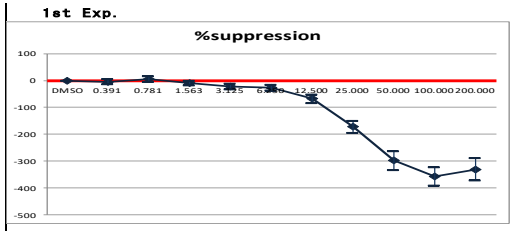
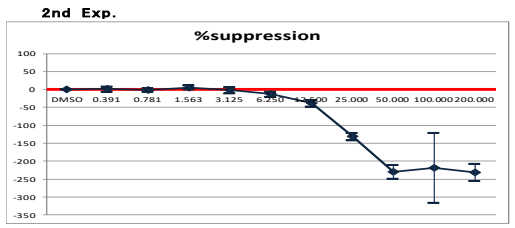
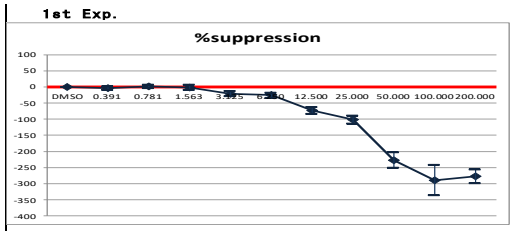
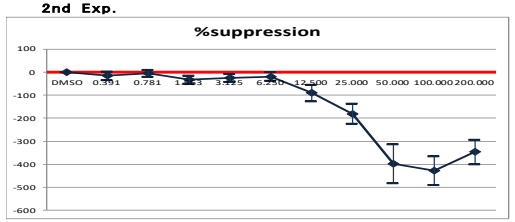
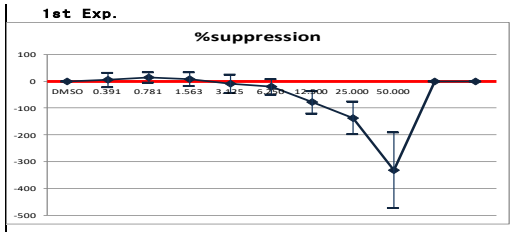
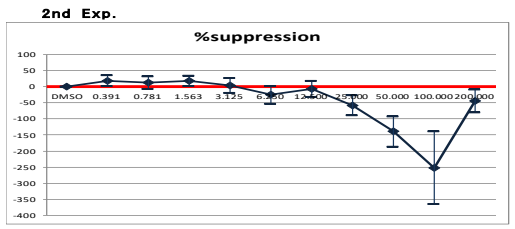
Chem No.	Set No.	LabA Tohoku		LabB tsukuba		LabC takamatsu	
		code No.	Judge	code No.	Judge	code No.	Judge
1	Set1	MLA102	Toxic	MLB402	Toxic	MLC705	Toxic
	Set2	MLA204	Toxic	MLB504	Toxic	MLC802	Toxic
	Set3	MLA303	Toxic	MLB602	Toxic	MLC903	Toxic
2	Set1	MLA101	Toxic	MLB404	Toxic	MLC701	Toxic
	Set2	MLA202	Toxic	MLB503	Toxic	MLC804	Toxic
	Set3	MLA304	Toxic	MLB604	Toxic	MLC905	Toxic
3	Set1	MLA104	Non	MLB401	Non	MLC702	Non
	Set2	MLA205	Non	MLB505	Non	MLC805	Non
	Set3	MLA305	Non	MLB603	Non	MLC902	Non
4	Set1	MLA105	Toxic	MLB405	Toxic	MLC704	Toxic
	Set2	MLA203	Toxic	MLB502	Toxic	MLC803	Toxic
	Set3	MLA301	Toxic	MLB601	Toxic	MLC904	Toxic
5	Set1	MLA103	Non	MLB403	Non	MLC703	Non
	Set2	MLA201	Non	MLB501	Non	MLC801	Non
	Set3	MLA302	Non	MLB605	Non	MLC901	Non
		Within-laboratory concordance rate	**100% (5/5)**	Within-laboratory concordance rate	**100% (5/5)**	Within-laboratory concordance rate	**100% (5/5)**
				Between-labolatory concordance rate		**100% (5/5)**	

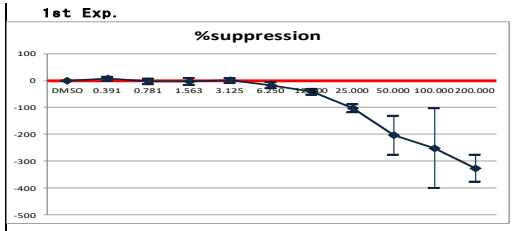
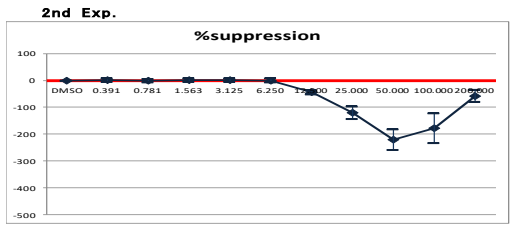
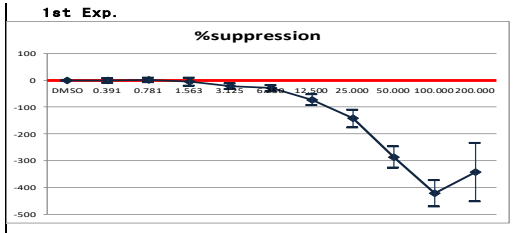
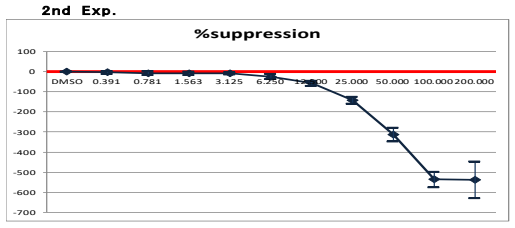
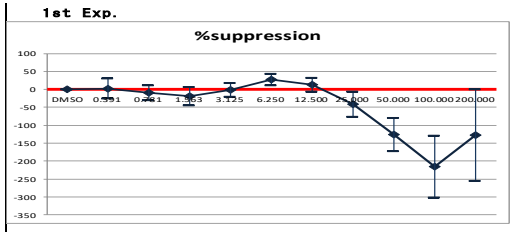
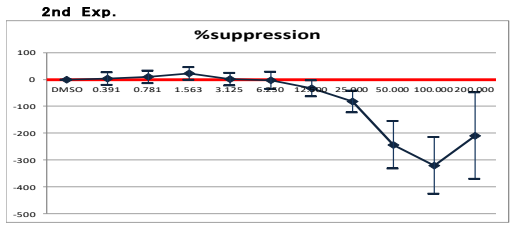
826

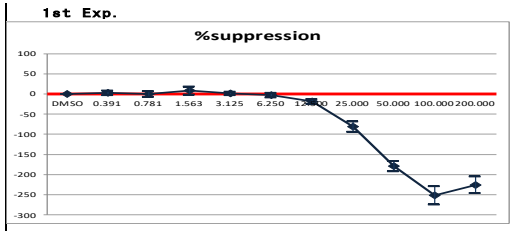
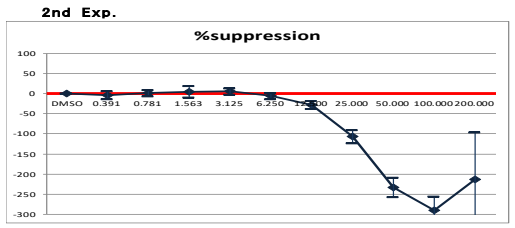
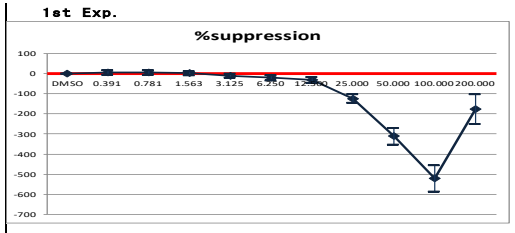
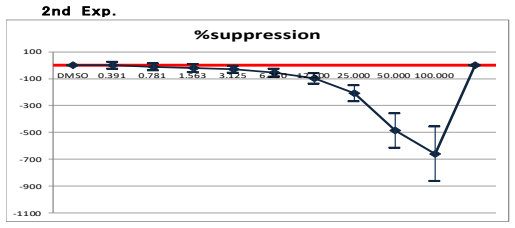
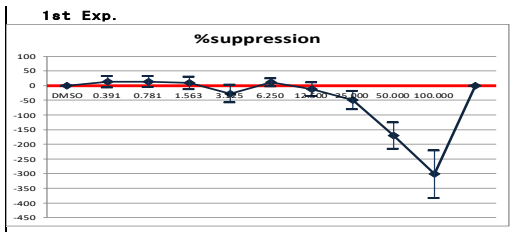
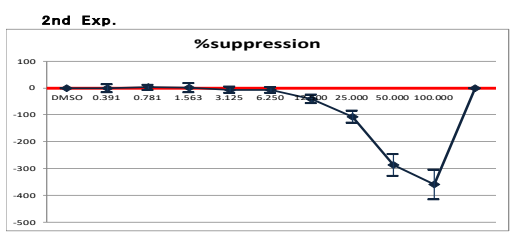
Chem1 Set1	Exp.1	Exp.2	Exp.3	Judge
MLA102	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: -504.544 / -585.480 / -423.608 0.781: -591.113 / -728.158 / -454.067 1.563: -720.460 / -839.623 / -601.297 3.125: -784.183 / -948.221 / -620.145 6.250: -766.605 / -870.628 / -662.583 12.500: -745.116 / -870.794 / -619.437 25.000: -731.140 / -905.396 / -556.884 50.000: -803.000 / -975.742 / -630.258 100.000: -898.780 / -1022.527 / -775.033 200.000: -736.797 / -902.913 / -570.681	Toxic	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.003: -2.297 / -8.134 / 3.540 0.006: 7.970 / 0.811 / 15.128 0.012: -4.493 / -11.164 / 2.179 0.024: -9.564 / -16.486 / -2.642 0.049: -3.186 / -17.861 / 11.490 0.098: -75.238 / -107.801 / -42.674 0.195: -200.150 / -210.227 / -190.072 0.391: -342.771 / -371.461 / -314.082 0.781: -416.355 / -441.908 / -390.803 1.563: -347.892 / -382.905 / -312.878	Toxic		Toxic
MLB402	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: -143.485 / -218.708 / -68.262 0.781: -303.767 / -386.018 / -221.516 1.563: -395.478 / -467.913 / -323.042 3.125: -446.037 / -540.354 / -351.719 6.250: -487.417 / -581.698 / -393.136 12.500: -444.854 / -543.221 / -346.487 25.000: -473.028 / -572.187 / -373.868 50.000: -527.491 / -623.309 / -431.674 100.000: -535.643 / -658.824 / -412.462 200.000: -476.627 / -571.262 / -381.993	Toxic	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: -234.533 / -358.578 / -110.489 0.781: -386.366 / -527.281 / -245.452 1.563: -540.989 / -675.938 / -406.040 3.125: -598.530 / -752.014 / -445.046 6.250: -673.529 / -818.793 / -528.265 12.500: -721.017 / -884.963 / -557.071 25.000: -858.843 / -1041.393 / -676.292 50.000: -1071.789 / -1287.657 / -855.921 100.000: -1252.762 / -1503.193 / -1002.331 200.000: -802.689 / -1009.493 / -595.884	Toxic		Toxic
MLC705	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: -591.316 / -803.959 / -378.673 0.781: -808.281 / -921.637 / -694.925 1.563: -1065.762 / -1286.225 / -845.299 3.125: -1113.441 / -1276.727 / -950.155 6.250: -1212.643 / -1370.052 / -1055.234 12.500: -1180.816 / -1345.185 / -1016.447 25.000: -1445.069 / -1721.367 / -1168.770 50.000: -1629.354 / -1891.461 / -1367.247 100.000: -1772.712 / -2118.018 / -1427.406 200.000: -1296.563 / -1435.947 / -1157.178	Toxic	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: -579.554 / -801.137 / -357.971 0.781: -743.351 / -966.961 / -519.741 1.563: -981.119 / -1211.213 / -751.026 3.125: -1126.850 / -1406.542 / -847.159 6.250: -1019.168 / -1265.596 / -772.740 12.500: -1058.563 / -1339.534 / -777.593 25.000: -1096.568 / -1378.982 / -814.155 50.000: -1095.029 / -1433.480 / -756.577 100.000: -1301.985 / -1673.895 / -930.076 200.000: -860.912 / -1059.885 / -661.939	Toxic		Toxic

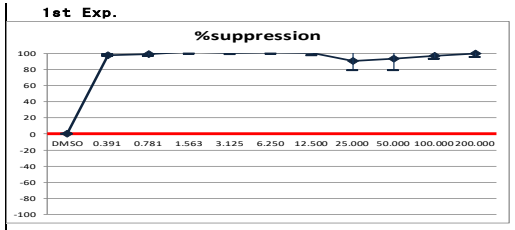
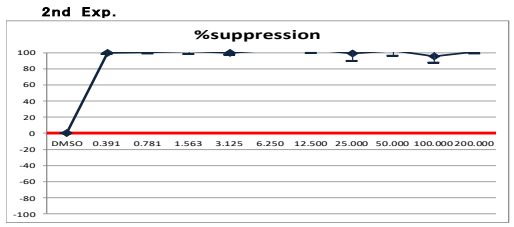
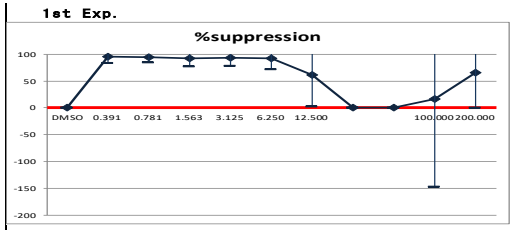
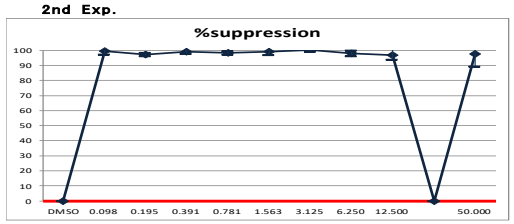
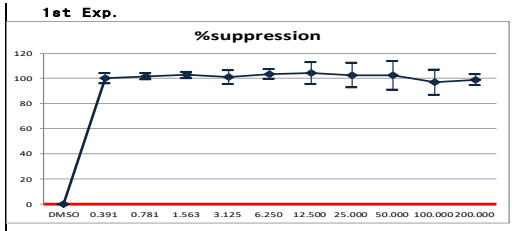
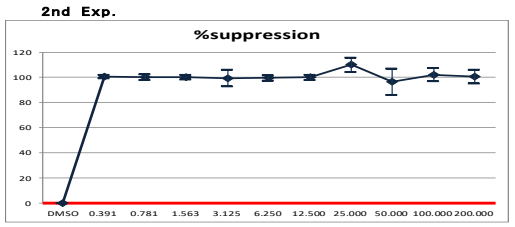
Chem1 Set2	Exp.1	Exp.2	Exp.3	Judge
MLA204	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: -623.506 / -695.151 / -551.861 0.781: -737.912 / -840.429 / -635.395 1.563: -789.896 / -862.592 / -717.201 3.125: -800.343 / -906.255 / -694.431 6.250: -741.763 / -837.183 / -646.343 12.500: -726.667 / -795.058 / -658.276 25.000: -756.824 / -829.106 / -684.541 50.000: -770.353 / -847.140 / -693.566 100.000: -762.841 / -874.084 / -651.598 200.000: -654.583 / -763.587 / -545.579	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.003: -7.840 / -38.246 / 22.566 0.006: -0.163 / -25.269 / 24.944 0.012: 6.557 / -16.231 / 29.346 0.024: -8.645 / -35.332 / 18.042 0.049: -13.784 / -44.284 / 16.716 0.098: -75.970 / -113.700 / -38.239 0.195: -256.519 / -350.359 / -162.679 0.391: -473.907 / -595.900 / -351.915 0.781: -531.285 / -662.109 / -400.461 1.563: -603.253 / -774.862 / -431.645		Toxic
MLB504	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: -298.634 / -371.990 / -225.278 0.781: -488.681 / -601.318 / -376.045 1.563: -548.199 / -659.789 / -436.610 3.125: -623.251 / -748.980 / -497.523 6.250: -629.340 / -758.524 / -500.155 12.500: -627.700 / -751.697 / -503.703 25.000: -654.198 / -831.860 / -476.536 50.000: -777.855 / -942.292 / -613.417 100.000: -895.817 / -1088.670 / -702.964 200.000: -722.130 / -878.564 / -565.696	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.003: -0.446 / -22.381 / 21.488 0.006: 1.319 / -19.818 / 22.455 0.012: 5.695 / -9.784 / 21.175 0.024: 5.501 / -12.389 / 23.392 0.049: -5.812 / -27.765 / 16.142 0.098: -8.574 / -31.038 / 13.890 0.195: -42.525 / -92.571 / 7.520 0.391: -124.909 / -185.958 / -63.860 0.781: -252.018 / -309.978 / -194.058 1.563: -560.218 / -688.032 / -432.404		Toxic
MLC802	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: -522.128 / -673.243 / -371.013 0.781: -673.500 / -843.731 / -503.270 1.563: -895.308 / -1078.038 / -712.579 3.125: -948.511 / -1161.858 / -735.164 6.250: -823.912 / -984.740 / -663.084 12.500: -934.966 / -1105.655 / -764.276 25.000: -964.407 / -1167.281 / -761.534 50.000: -992.312 / -1213.511 / -771.112 100.000: -1182.001 / -1416.979 / -947.022 200.000: -654.759 / -806.645 / -502.873	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: -342.542 / -505.477 / -179.607 0.781: -511.308 / -647.983 / -374.633 1.563: -523.595 / -644.638 / -402.552 3.125: -628.640 / -769.461 / -487.818 6.250: -608.948 / -758.592 / -459.304 12.500: -585.457 / -736.701 / -434.212 25.000: -637.471 / -797.894 / -477.048 50.000: -769.213 / -970.472 / -568.954 100.000: -825.864 / -1095.220 / -556.507 200.000: -432.490 / -578.426 / -286.554		Toxic

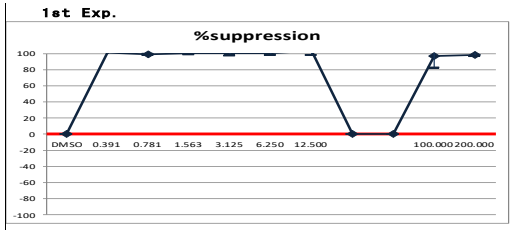
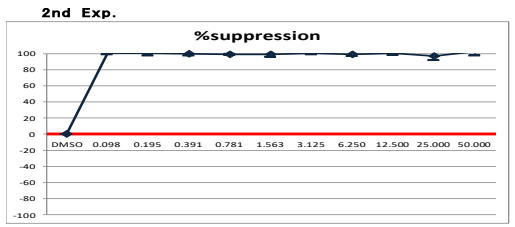
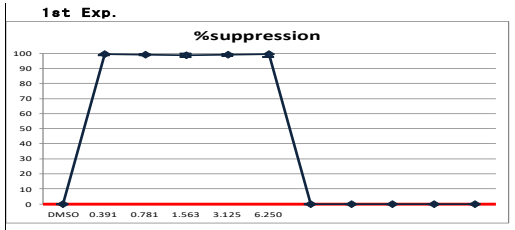
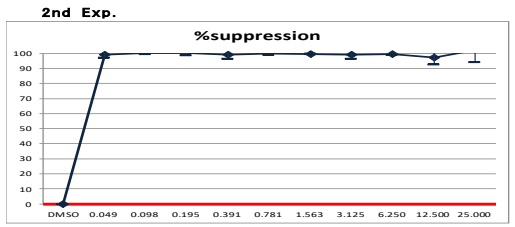
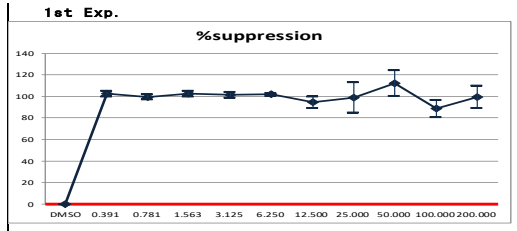
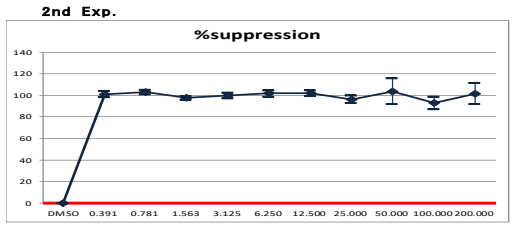
Chem1 Set3	Exp.1	Exp.2	Exp.3	Judge
MLA303	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: -323.360 / -344.995 / -301.725 0.781: -377.830 / -395.007 / -360.653 1.563: -420.617 / -437.234 / -404.000 3.125: -452.138 / -476.501 / -427.776 6.250: -402.076 / -419.704 / -384.448 12.500: -377.485 / -392.779 / -362.191 25.000: -366.039 / -376.277 / -355.801 50.000: -399.152 / -414.565 / -383.740 100.000: -394.287 / -410.403 / -378.171 200.000: -352.362 / -455.489 / -249.235	Toxic	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.003: 15.942 / 9.853 / 22.031 0.006: 17.895 / 12.280 / 23.510 0.012: 14.048 / 7.891 / 20.206 0.024: 16.228 / 10.032 / 22.425 0.049: 9.429 / 2.145 / 16.712 0.098: -21.296 / -36.330 / -6.261 0.195: -160.053 / -182.413 / -137.692 0.391: -302.144 / -329.661 / -274.627 0.781: -387.166 / -420.274 / -354.057 1.563: -494.931 / -535.406 / -454.456	Toxic		Toxic
MLB602	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: -165.288 / -221.771 / -108.805 0.781: -314.058 / -381.689 / -246.427 1.563: -398.379 / -474.694 / -322.064 3.125: -470.529 / -556.746 / -384.312 6.250: -505.865 / -605.804 / -405.926 12.500: -492.792 / -587.311 / -398.272 25.000: -503.115 / -625.276 / -380.954 50.000: -540.130 / -679.784 / -400.475 100.000: -620.052 / -760.093 / -480.011 200.000: -577.080 / -679.823 / -474.338	Toxic	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.003: -10.645 / -25.681 / 4.390 0.006: 0.025 / -14.629 / 14.679 0.012: -18.048 / -35.807 / -0.289 0.024: -18.071 / -29.735 / -6.407 0.049: -34.311 / -68.998 / 0.377 0.098: -118.010 / -193.296 / -42.723 0.195: -248.607 / -384.548 / -112.666 0.391: -254.635 / -389.081 / -120.189 0.781: -375.431 / -429.079 / -321.784 1.563: -513.203 / -560.145 / -466.261	Toxic		Toxic
MLC903	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: -293.575 / -491.156 / -95.994 0.781: -418.695 / -530.528 / -306.862 1.563: -578.598 / -693.066 / -464.131 3.125: -659.385 / -750.326 / -568.444 6.250: -620.895 / -702.452 / -539.339 12.500: -660.576 / -766.556 / -554.597 25.000: -704.482 / -829.001 / -579.963 50.000: -825.795 / -976.117 / -675.473 100.000: -931.385 / -1083.212 / -779.559 200.000: -567.330 / -777.591 / -357.068	Toxic	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: -325.988 / -429.198 / -222.778 0.781: -427.516 / -488.198 / -366.835 1.563: -491.856 / -556.201 / -427.510 3.125: -601.623 / -660.458 / -542.788 6.250: -508.298 / -554.826 / -461.770 12.500: -468.171 / -510.101 / -426.241 25.000: -490.211 / -577.646 / -402.775 50.000: -585.974 / -687.307 / -484.641 100.000: -672.665 / -787.758 / -557.573 200.000: -476.758 / -521.345 / -432.171	Toxic		Toxic

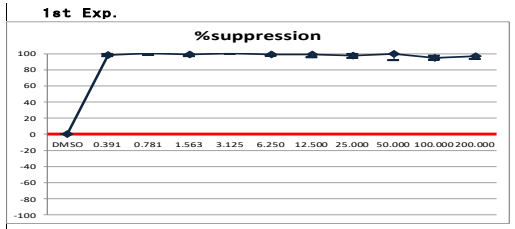
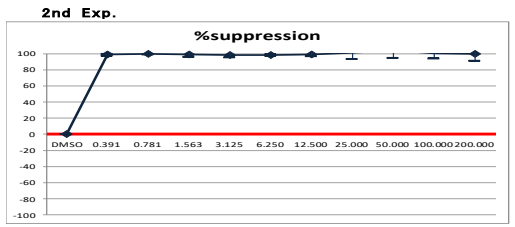
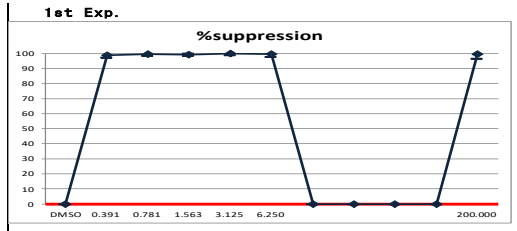
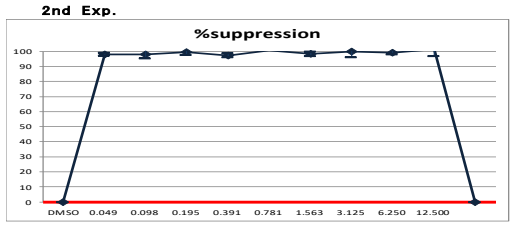
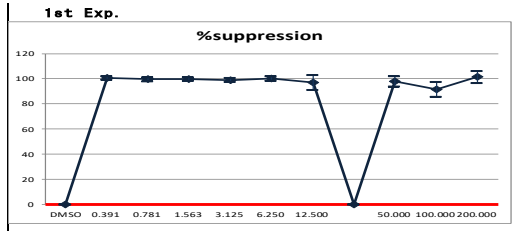
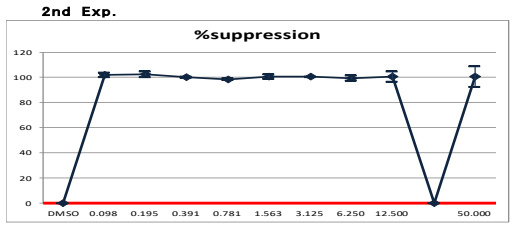
Chem2 Set1	Exp.1	Exp.2	Exp.3	Judge
MLA101	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: -4.600 / -14.578 / 5.378 0.781: 5.683 / -5.092 / 16.457 1.563: -9.271 / -17.919 / -0.623 3.125: -21.990 / -32.170 / -11.811 6.250: -26.432 / -36.326 / -16.538 12.500: -67.913 / -82.978 / -52.848 25.000: -172.355 / -195.655 / -149.054 50.000: -297.567 / -333.029 / -262.105 100.000: -356.735 / -391.585 / -321.884 200.000: -330.277 / -371.070 / -289.484 Toxic	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: 1.495 / -6.075 / 9.065 0.781: -1.706 / -6.150 / 2.738 1.563: 5.568 / 0.057 / 11.079 3.125: -1.965 / -10.378 / 6.447 6.250: -12.539 / -19.785 / -5.292 12.500: -38.430 / -46.859 / -30.002 25.000: -131.442 / -142.393 / -120.492 50.000: -229.784 / -248.303 / -211.265 100.000: -218.558 / -316.405 / -120.710 200.000: -230.645 / -254.561 / -206.729 Toxic		Toxic
MLB404	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: -3.519 / -9.102 / 2.063 0.781: 1.896 / -3.590 / 7.381 1.563: -1.208 / -9.058 / 6.642 3.125: -20.590 / -29.384 / -11.797 6.250: -25.326 / -33.353 / -17.298 12.500: -72.224 / -83.020 / -61.428 25.000: -101.504 / -114.369 / -88.639 50.000: -226.466 / -250.718 / -202.214 100.000: -288.808 / -335.980 / -241.635 200.000: -276.347 / -297.583 / -255.111 Toxic	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: -15.604 / -34.472 / 3.264 0.781: -6.045 / -21.390 / 9.299 1.563: -33.496 / -50.761 / -16.231 3.125: -24.874 / -42.105 / -7.643 6.250: -19.542 / -37.870 / -1.213 12.500: -90.358 / -125.959 / -54.757 25.000: -181.368 / -224.626 / -138.111 50.000: -397.568 / -482.278 / -312.858 100.000: -427.506 / -488.856 / -366.155 200.000: -346.018 / -399.546 / -292.491 Toxic		Toxic
MLC701	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: 4.539 / -21.236 / 30.315 0.781: 14.265 / -4.868 / 33.398 1.563: 6.884 / -18.968 / 32.735 3.125: -9.435 / -43.663 / 24.794 6.250: -21.020 / -49.666 / 7.625 12.500: -78.399 / -119.919 / -36.879 25.000: -136.234 / -195.959 / -76.509 50.000: -330.524 / -471.415 / -189.633 Toxic	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: 17.817 / 0.872 / 34.761 0.781: 12.688 / -6.408 / 31.784 1.563: 17.566 / 1.376 / 33.755 3.125: 2.904 / -19.909 / 25.718 6.250: -25.666 / -53.090 / 1.757 12.500: -7.385 / -32.171 / 17.401 25.000: -58.180 / -89.019 / -27.341 50.000: -139.159 / -186.397 / -91.922 100.000: -251.283 / -363.980 / -138.586 200.000: -44.083 / -79.376 / -8.789 Toxic		Toxic

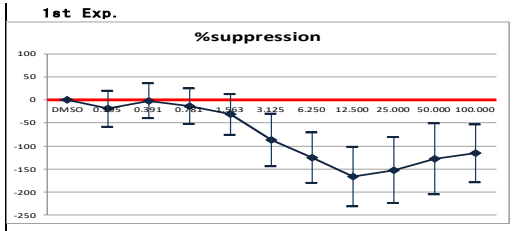
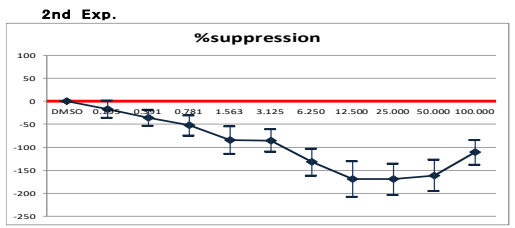
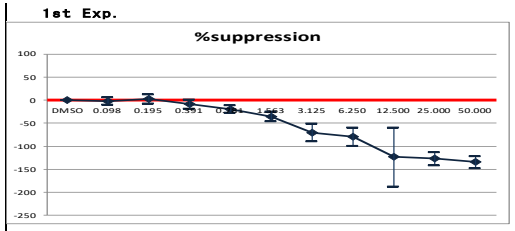
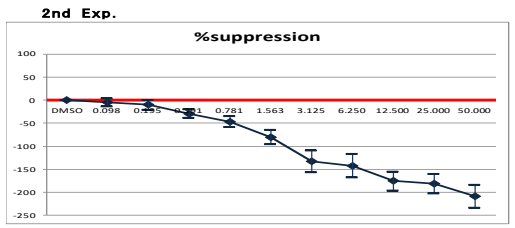
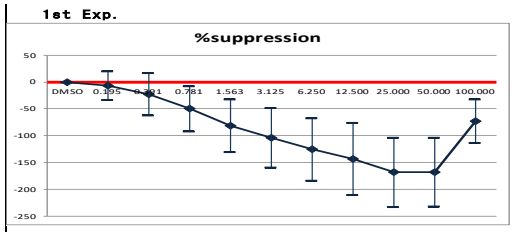
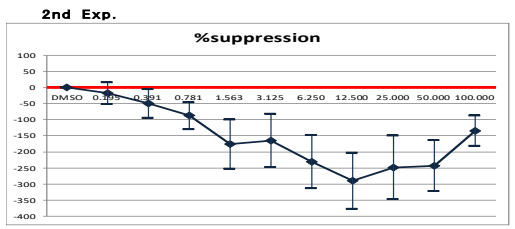
Chem2 Set2	Exp.1	Exp.2	Exp.3	Judge
MLA202	 1st Exp. a Comparison Conc. | %supp | Lower Limit | Upper Limit DMSO 0 0 0 0.391 6.588 -0.618 13.793 0.781 -3.011 -14.038 8.017 1.563 -3.266 -15.728 9.196 3.125 1.043 -8.466 10.552 6.250 -17.424 -29.319 -5.530 12.500 -41.614 -52.985 -30.243 25.000 -103.023 -117.289 -88.757 50.000 -202.840 -274.737 -130.944 100.000 -251.842 -400.160 -103.523 200.000 -326.193 -375.742 -276.645	Toxic	 2nd Exp. a Comparison Conc. | %supp | Lower Limit | Upper Limit DMSO 0 0 0 0.391 1.858 -4.611 8.326 0.781 -1.185 -6.823 4.452 1.563 0.444 -6.107 6.995 3.125 1.407 -4.225 7.038 6.250 -0.080 -7.825 7.665 12.500 -44.537 -51.620 -37.455 25.000 -119.795 -143.094 -96.495 50.000 -221.131 -259.715 -182.547 100.000 -178.392 -233.958 -122.826 200.000 -57.954 -80.106 -35.802	Toxic		Toxic
MLB503	 1st Exp. a Comparison Conc. | %supp | Lower Limit | Upper Limit DMSO 0 0 0 0.391 -1.915 -10.441 6.611 0.781 0.923 -7.813 9.658 1.563 -4.442 -19.408 10.523 3.125 -21.671 -32.707 -10.636 6.250 -29.089 -40.796 -17.381 12.500 -72.343 -93.264 -51.421 25.000 -142.282 -176.043 -108.521 50.000 -285.812 -325.129 -246.495 100.000 -420.621 -469.117 -372.126 200.000 -341.438 -450.272 -232.605	Toxic	 2nd Exp. a Comparison Conc. | %supp | Lower Limit | Upper Limit DMSO 0 0 0 0.391 -3.992 -11.260 3.276 0.781 -8.214 -17.894 1.465 1.563 -9.526 -18.319 -0.732 3.125 -8.988 -14.317 -3.660 6.250 -24.802 -38.111 -11.493 12.500 -58.514 -71.450 -45.578 25.000 -143.704 -160.744 -126.664 50.000 -313.481 -348.045 -278.917 100.000 -535.360 -573.561 -497.159 200.000 -537.138 -628.049 -446.227	Toxic		Toxic
MLC804	 1st Exp. a Comparison Conc. | %supp | Lower Limit | Upper Limit DMSO 0 0 0 0.391 2.456 -25.565 30.477 0.781 -9.656 -30.380 11.069 1.563 -18.666 -44.267 6.936 3.125 -1.605 -20.399 17.189 6.250 27.256 11.617 42.894 12.500 12.386 -6.791 31.564 25.000 -41.452 -76.396 -6.509 50.000 -126.287 -172.908 -79.667 100.000 -215.631 -301.969 -129.292 200.000 -127.454 -255.923 1.014	Toxic	 2nd Exp. a Comparison Conc. | %supp | Lower Limit | Upper Limit DMSO 0 0 0 0.391 3.284 -21.151 27.719 0.781 9.280 -13.587 32.148 1.563 22.500 0.045 44.955 3.125 1.042 -21.909 23.994 6.250 -2.734 -34.692 29.225 12.500 -32.515 -62.328 -2.702 25.000 -81.286 -121.610 -40.963 50.000 -242.732 -331.388 -154.076 100.000 -320.004 -425.635 -214.372 200.000 -208.666 -370.003 -47.328	Toxic		Toxic

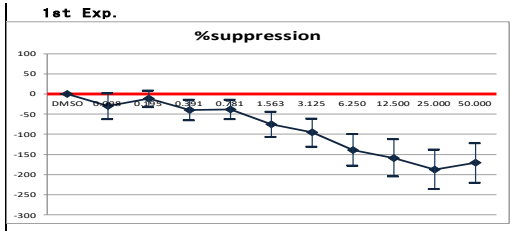
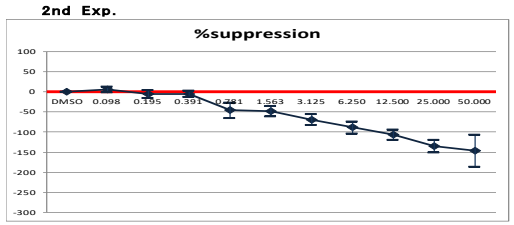
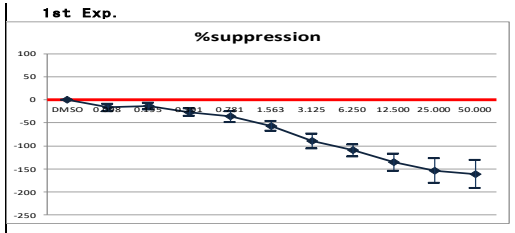
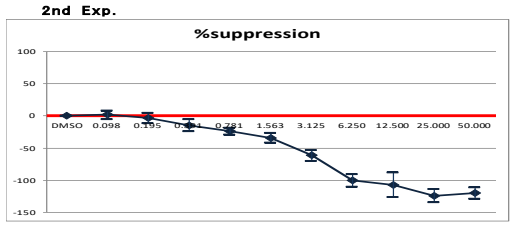
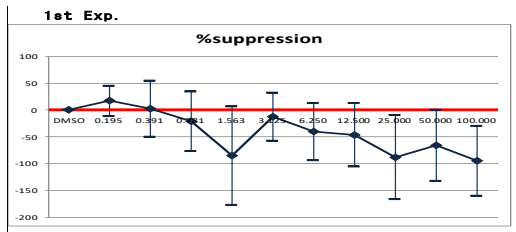
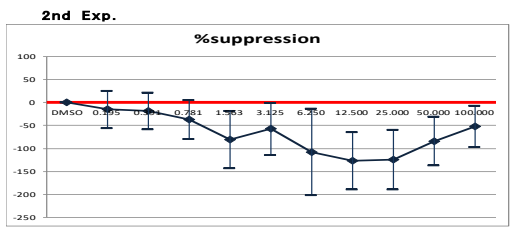
Chem2 Set3	Exp.1	Exp.2	Exp.3	Judge
MLA304	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: 2.731 / -3.004 / 8.466 0.781: 0.155 / -6.943 / 7.253 1.563: 8.236 / -1.647 / 18.119 3.125: 1.641 / -2.897 / 6.179 6.250: -2.896 / -8.688 / 2.896 12.500: -18.421 / -24.028 / -12.815 25.000: -81.443 / -94.251 / -68.634 50.000: -178.483 / -191.382 / -165.584 100.000: -251.640 / -274.500 / -228.779 200.000: -225.261 / -245.866 / -204.657	Toxic	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: -4.352 / -14.456 / 5.752 0.781: 0.767 / -7.527 / 9.060 1.563: 3.910 / -10.057 / 17.876 3.125: 5.087 / -2.455 / 12.629 6.250: -6.017 / -14.151 / 2.118 12.500: -28.537 / -38.204 / -18.869 25.000: -106.807 / -123.003 / -90.610 50.000: -233.009 / -256.743 / -209.274 100.000: -289.766 / -324.556 / -254.976 200.000: -213.245 / -330.321 / -96.168	Toxic		Toxic
MLB604	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: 4.570 / -6.685 / 15.826 0.781: 5.975 / -4.925 / 16.874 1.563: 1.129 / -9.379 / 11.637 3.125: -10.661 / -21.090 / -0.232 6.250: -21.710 / -33.616 / -9.805 12.500: -31.681 / -46.487 / -16.876 25.000: -124.811 / -146.323 / -103.298 50.000: -310.980 / -352.729 / -269.232 100.000: -519.512 / -584.712 / -454.313 200.000: -176.761 / -249.475 / -104.048	Toxic	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: 0.597 / -24.570 / 25.765 0.781: -12.108 / -38.332 / 14.116 1.563: -21.158 / -49.331 / 7.014 3.125: -29.705 / -54.021 / -5.389 6.250: -56.198 / -85.046 / -27.349 12.500: -96.934 / -136.173 / -57.695 25.000: -208.161 / -267.369 / -148.953 50.000: -484.822 / -611.327 / -358.317 100.000: -657.853 / -860.882 / -454.824	Toxic		Toxic
MLC905	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: 13.519 / -5.481 / 32.520 0.781: 14.009 / -3.997 / 32.014 1.563: 8.955 / -12.193 / 30.103 3.125: -26.939 / -57.166 / 3.288 6.250: 12.068 / -1.386 / 25.523 12.500: -11.564 / -35.466 / 12.338 25.000: -49.130 / -80.149 / -18.111 50.000: -170.066 / -214.209 / -125.923 100.000: -301.316 / -382.546 / -220.085	Toxic	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: -1.510 / -16.813 / 13.792 0.781: 2.870 / -6.574 / 12.314 1.563: 1.420 / -14.864 / 17.703 3.125: -6.447 / -18.626 / 5.732 6.250: -7.389 / -17.518 / 2.739 12.500: -40.474 / -56.809 / -22.138 25.000: -106.732 / -128.713 / -84.752 50.000: -286.123 / -327.334 / -244.911 100.000: -358.619 / -413.444 / -303.793	Toxic		Toxic

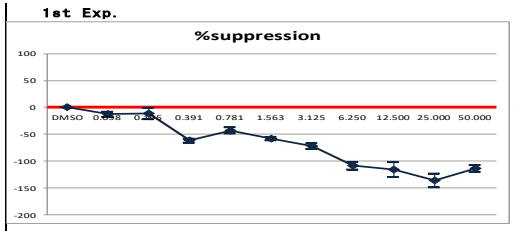
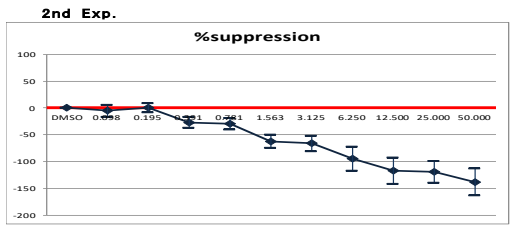
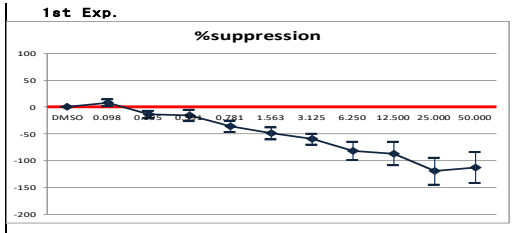
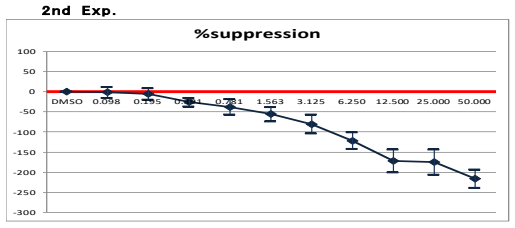
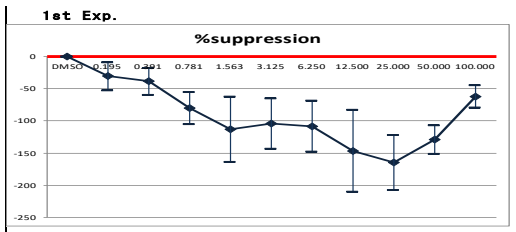
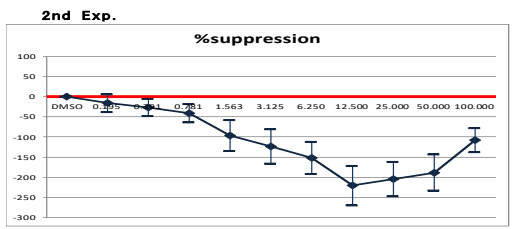
Chem3 Set1	Exp.1	Exp.2	Exp.3	Judge
MLA104	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: 97.721 / 96.525 / 98.918 0.781: 98.949 / 96.912 / 100.987 1.563: 101.984 / 99.452 / 104.516 3.125: 100.462 / 99.126 / 101.797 6.250: 101.430 / 99.590 / 103.270 12.500: 100.448 / 97.316 / 103.580 25.000: 90.599 / 79.308 / 101.890 50.000: 93.249 / 79.373 / 107.125 100.000: 96.905 / 93.439 / 100.371 200.000: 99.564 / 95.717 / 103.412	Non	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: 99.788 / 98.507 / 101.070 0.781: 100.279 / 98.739 / 101.818 1.563: 101.583 / 97.998 / 105.168 3.125: 99.950 / 97.382 / 102.519 6.250: 103.443 / 102.097 / 104.789 12.500: 104.233 / 99.621 / 108.846 25.000: 98.818 / 89.842 / 107.795 50.000: 102.211 / 96.234 / 108.188 100.000: 95.731 / 87.793 / 103.669 200.000: 100.815 / 98.961 / 102.669	Non		Non
MLB401	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: 95.008 / 83.378 / 106.637 0.781: 94.262 / 85.057 / 103.467 1.563: 91.865 / 77.140 / 106.590 3.125: 92.983 / 78.434 / 107.531 6.250: 92.625 / 71.867 / 113.383 12.500: 61.612 / 2.789 / 120.436 100.000: 16.935 / -145.921 / 179.791 200.000: 65.161 / 0.817 / 129.505	Non	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.098: 99.605 / 97.171 / 102.038 0.195: 97.144 / 96.132 / 98.155 0.391: 99.243 / 97.414 / 101.073 0.781: 98.385 / 97.223 / 99.546 1.563: 99.163 / 96.700 / 101.625 3.125: 100.184 / 99.137 / 101.231 6.250: 97.881 / 96.054 / 99.708 12.500: 96.971 / 93.804 / 100.138 50.000: 97.389 / 89.369 / 105.409	Non		Non
MLC702	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: 100.219 / 96.188 / 104.249 0.781: 101.601 / 99.068 / 104.134 1.563: 102.767 / 100.119 / 105.415 3.125: 101.311 / 95.831 / 106.791 6.250: 103.510 / 99.609 / 107.411 12.500: 104.410 / 95.792 / 113.028 25.000: 102.690 / 93.049 / 112.331 50.000: 102.526 / 91.005 / 114.047 100.000: 97.090 / 87.085 / 107.095 200.000: 99.042 / 94.796 / 103.287	Non	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: 100.492 / 99.166 / 101.817 0.781: 100.154 / 98.043 / 102.265 1.563: 100.132 / 98.223 / 102.040 3.125: 99.314 / 92.708 / 105.920 6.250: 99.549 / 97.684 / 101.415 12.500: 100.008 / 97.903 / 102.114 25.000: 110.119 / 104.388 / 115.849 50.000: 96.635 / 86.164 / 107.107 100.000: 102.179 / 96.796 / 107.562 200.000: 100.863 / 95.364 / 106.362	Non		Non

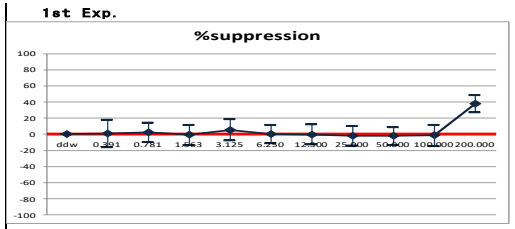
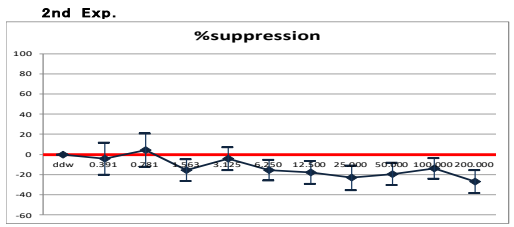
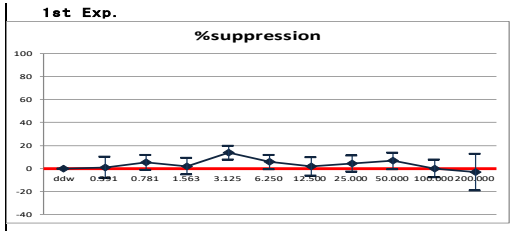
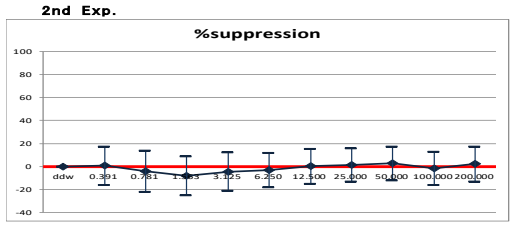
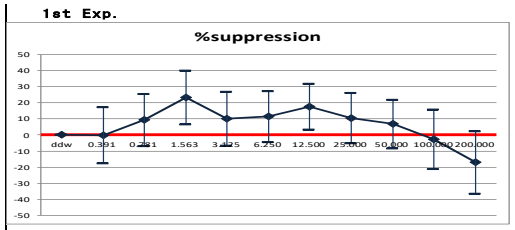
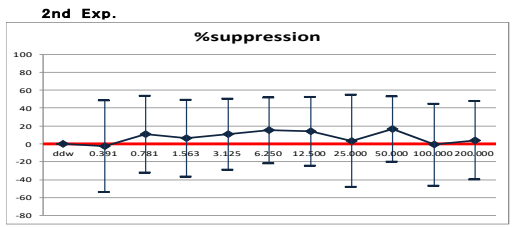
Chem3 Set2	Exp.1	Exp.2	Exp.3	Judge
MLA205	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: 101.300 / 100.473 / 102.126 0.781: 98.832 / 97.969 / 99.695 1.563: 100.378 / 99.145 / 101.611 3.125: 100.762 / 97.914 / 103.611 6.250: 100.598 / 98.255 / 102.941 12.500: 103.057 / 98.342 / 107.772 100.000: 97.068 / 82.918 / 111.217 200.000: 98.512 / 96.629 / 100.395	Non	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.098: 100.187 / 99.032 / 101.342 0.195: 100.238 / 97.588 / 102.887 0.391: 99.852 / 97.481 / 102.224 0.781: 98.867 / 98.174 / 99.559 1.563: 98.876 / 96.276 / 101.476 3.125: 100.559 / 99.137 / 101.981 6.250: 99.192 / 96.619 / 101.765 12.500: 100.272 / 97.946 / 102.598 25.000: 97.190 / 91.674 / 102.707 50.000: 102.878 / 97.828 / 107.929	Non		Non
MLB505	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: 99.541 / 98.619 / 100.464 0.781: 99.120 / 98.836 / 99.404 1.563: 98.661 / 97.509 / 99.813 3.125: 98.995 / 98.452 / 99.539 6.250: 99.557 / 97.735 / 101.378	Non	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.049: 99.163 / 97.277 / 101.050 0.098: 100.373 / 99.447 / 101.299 0.195: 100.133 / 98.835 / 101.432 0.391: 99.029 / 96.468 / 101.590 0.781: 100.000 / 99.250 / 100.751 1.563: 99.651 / 99.344 / 99.957 3.125: 99.227 / 96.580 / 101.875 6.250: 99.567 / 98.996 / 100.139 12.500: 97.087 / 92.811 / 101.362 25.000: 101.952 / 94.188 / 109.716	Non		Non
MLC805	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: 102.568 / 100.021 / 105.115 0.781: 99.466 / 97.155 / 101.777 1.563: 102.644 / 99.844 / 105.444 3.125: 101.554 / 98.803 / 104.304 6.250: 101.795 / 100.602 / 102.989 12.500: 94.361 / 89.029 / 99.693 25.000: 98.997 / 84.792 / 113.203 50.000: 112.304 / 100.316 / 124.292 100.000: 88.661 / 80.516 / 96.806 200.000: 99.531 / 89.218 / 109.843	Non	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: 100.980 / 98.027 / 103.933 0.781: 103.048 / 101.078 / 105.017 1.563: 97.579 / 96.065 / 99.094 3.125: 99.992 / 97.337 / 102.646 6.250: 101.843 / 98.844 / 104.842 12.500: 101.890 / 99.197 / 104.584 25.000: 96.289 / 92.728 / 99.849 50.000: 103.805 / 91.727 / 115.882 100.000: 92.798 / 86.959 / 98.636 200.000: 101.719 / 91.719 / 111.720	Non		Non

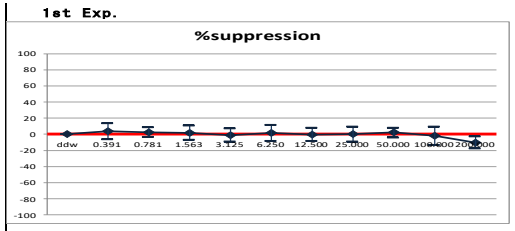
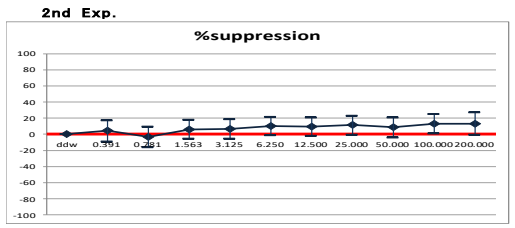
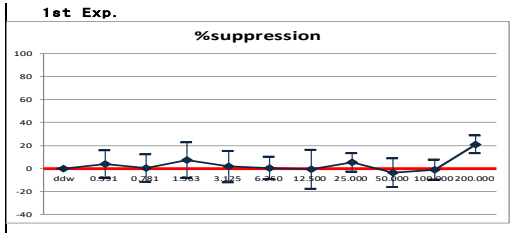
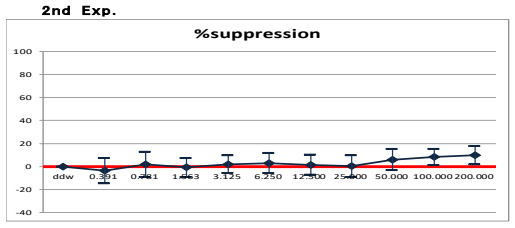
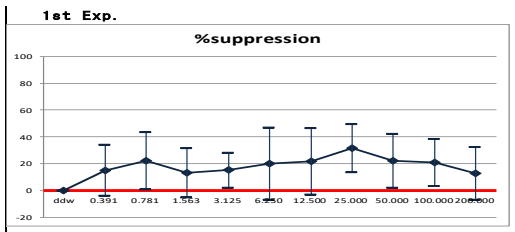
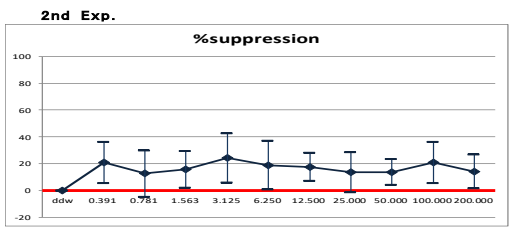
Chem3 Set3	Exp.1	Exp.2	Exp.3	Judge
MLA305	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: 98.489 / 97.210 / 99.769 0.781: 100.178 / 98.186 / 102.171 1.563: 99.179 / 97.070 / 101.288 3.125: 100.535 / 99.748 / 101.322 6.250: 98.888 / 97.591 / 100.185 12.500: 99.049 / 95.447 / 102.650 25.000: 97.273 / 95.051 / 99.494 50.000: 99.462 / 91.834 / 107.091 100.000: 94.833 / 92.409 / 97.258 200.000: 96.930 / 93.551 / 100.309	Non	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: 98.806 / 97.924 / 99.688 0.781: 99.635 / 98.902 / 100.369 1.563: 99.109 / 95.889 / 102.329 3.125: 98.335 / 95.196 / 101.473 6.250: 98.196 / 97.402 / 98.989 12.500: 98.794 / 96.662 / 100.927 25.000: 100.799 / 93.134 / 108.464 50.000: 104.173 / 94.704 / 113.643 100.000: 100.658 / 94.081 / 107.235 200.000: 99.397 / 90.866 / 107.929	Non		Non
MLB603	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: 98.845 / 97.322 / 100.367 0.781: 99.354 / 98.481 / 100.228 1.563: 98.928 / 98.181 / 99.676 3.125: 99.666 / 98.794 / 100.539 6.250: 99.398 / 97.752 / 101.045 200.000: 99.582 / 96.268 / 102.895	Non	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.049: 98.135 / 97.242 / 99.028 0.098: 97.874 / 95.259 / 100.489 0.195: 99.287 / 97.740 / 100.833 0.391: 97.350 / 95.861 / 98.838 0.781: 100.573 / 100.221 / 100.925 1.563: 98.312 / 96.905 / 99.719 3.125: 99.859 / 96.142 / 103.575 6.250: 99.077 / 97.904 / 100.251 12.500: 101.347 / 96.945 / 105.748	Non		Non
MLC902	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.391: 100.794 / 99.392 / 102.196 0.781: 99.641 / 97.950 / 101.333 1.563: 99.830 / 98.314 / 101.345 3.125: 98.919 / 97.340 / 100.498 6.250: 100.138 / 98.238 / 102.038 12.500: 97.024 / 90.895 / 103.154 50.000: 97.840 / 93.832 / 101.848 100.000: 91.560 / 85.474 / 97.646 200.000: 101.445 / 96.655 / 106.236	Non	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.098: 102.006 / 100.213 / 103.800 0.195: 102.445 / 100.049 / 104.842 0.391: 100.214 / 99.612 / 100.817 0.781: 98.573 / 98.039 / 99.106 1.563: 100.660 / 98.930 / 102.389 3.125: 100.438 / 100.113 / 100.763 6.250: 99.427 / 97.485 / 101.370 12.500: 100.593 / 96.371 / 104.815 50.000: 100.748 / 92.504 / 108.993	Non		Non

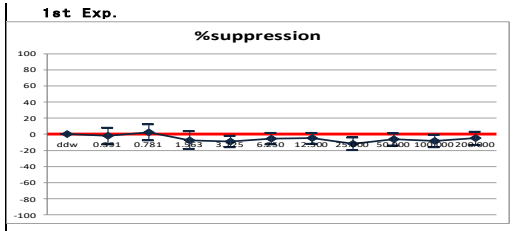
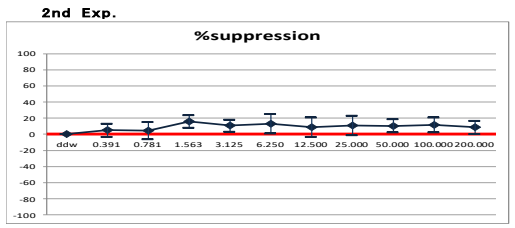
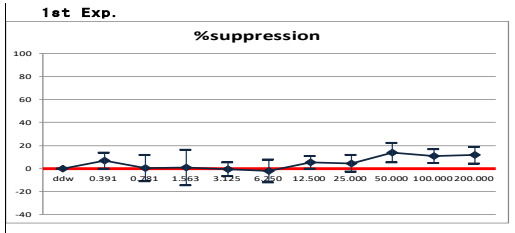
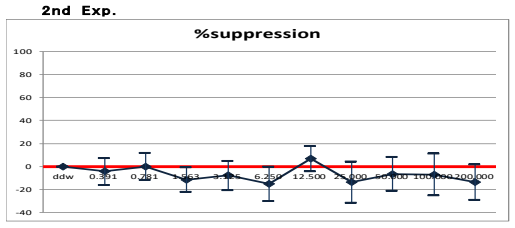
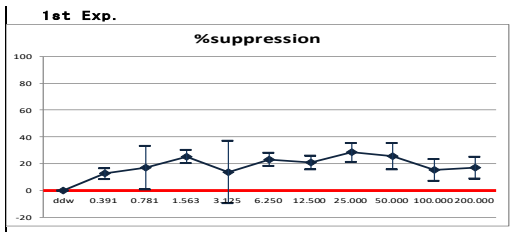
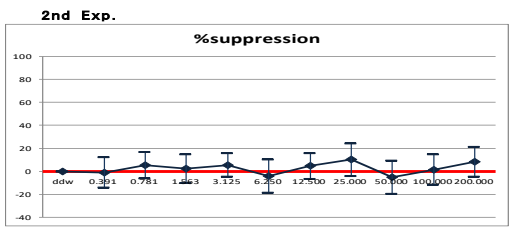
Chem4 Set1	Exp.1	Exp.2	Exp.3	Judge
MLA105	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.195: -18.863 / -57.691 / 19.965 0.391: -1.773 / -39.425 / 35.879 0.781: -13.653 / -51.857 / 24.551 1.563: -31.253 / -75.526 / 13.019 3.125: -86.641 / -144.162 / -29.121 6.250: -125.376 / -180.568 / -70.185 12.500: -166.387 / -231.599 / -101.176 25.000: -152.332 / -223.593 / -81.070 50.000: -128.182 / -204.924 / -51.440 100.000: -116.022 / -179.340 / -52.705	Toxic	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.195: -17.363 / -35.491 / 0.765 0.391: -36.003 / -53.151 / -18.855 0.781: -52.578 / -74.282 / -30.875 1.563: -83.898 / -113.751 / -54.046 3.125: -85.040 / -109.225 / -60.855 6.250: -132.020 / -161.441 / -102.599 12.500: -168.791 / -206.977 / -130.606 25.000: -169.143 / -203.178 / -135.109 50.000: -160.979 / -194.977 / -126.982 100.000: -110.880 / -137.482 / -84.278	Toxic		Toxic
MLB405	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.098: -1.861 / -9.644 / 5.922 0.195: 2.556 / -6.838 / 11.949 0.391: -8.984 / -18.925 / 0.957 0.781: -19.517 / -27.848 / -11.186 1.563: -35.379 / -45.760 / -24.997 3.125: -70.583 / -89.750 / -51.417 6.250: -79.567 / -99.024 / -60.110 12.500: -123.071 / -186.966 / -59.175 25.000: -126.804 / -141.031 / -112.577 50.000: -134.360 / -147.590 / -121.130	Toxic	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.098: -4.360 / -12.945 / 4.226 0.195: -10.279 / -20.851 / 0.294 0.391: -29.269 / -38.996 / -19.542 0.781: -46.534 / -58.322 / -34.747 1.563: -80.232 / -95.680 / -64.784 3.125: -132.810 / -155.946 / -109.674 6.250: -142.601 / -168.005 / -117.197 12.500: -175.297 / -195.842 / -154.752 25.000: -181.267 / -202.198 / -160.337 50.000: -208.497 / -233.330 / -183.663	Toxic		Toxic
MLC704	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.195: -6.924 / -33.848 / 20.000 0.391: -22.677 / -62.065 / 16.710 0.781: -49.849 / -92.244 / -7.454 1.563: -81.353 / -130.797 / -31.909 3.125: -103.434 / -158.987 / -47.881 6.250: -125.252 / -183.458 / -67.047 12.500: -142.943 / -209.919 / -75.967 25.000: -168.117 / -232.791 / -103.443 50.000: -167.892 / -231.893 / -103.892 100.000: -72.845 / -113.460 / -32.231	Toxic	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.195: -17.571 / -51.702 / 16.560 0.391: -49.561 / -93.925 / -5.196 0.781: -87.690 / -129.697 / -45.682 1.563: -175.887 / -252.120 / -99.655 3.125: -164.812 / -247.394 / -82.231 6.250: -230.584 / -312.984 / -148.183 12.500: -288.958 / -375.774 / -202.142 25.000: -248.386 / -346.994 / -149.778 50.000: -243.103 / -321.923 / -164.282 100.000: -134.258 / -181.801 / -86.716	Toxic		Toxic

Chem4 Set2	Exp.1	Exp.2	Exp.3	Judge
MLA203	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.098: -30.454 / -62.175 / 1.268 0.195: -12.015 / -33.292 / 9.261 0.391: -39.889 / -65.121 / -14.657 0.781: -37.826 / -62.019 / -13.633 1.563: -75.236 / -106.660 / -43.811 3.125: -95.587 / -130.447 / -60.727 6.250: -139.029 / -178.057 / -100.001 12.500: -158.704 / -205.000 / -112.407 25.000: -186.927 / -236.294 / -137.560 50.000: -170.590 / -219.546 / -121.635	Toxic	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.098: 5.631 / -1.249 / 12.510 0.195: -5.619 / -15.089 / 3.851 0.391: -5.409 / -13.290 / 2.472 0.781: -46.078 / -65.483 / -26.674 1.563: -48.008 / -60.438 / -35.579 3.125: -68.927 / -82.611 / -55.244 6.250: -88.398 / -103.360 / -73.436 12.500: -106.578 / -119.904 / -93.253 25.000: -135.084 / -150.821 / -119.348 50.000: -146.088 / -185.309 / -106.866	Toxic		Toxic
MLB502	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.098: -16.193 / -24.348 / -8.039 0.195: -13.231 / -20.449 / -6.012 0.391: -26.611 / -34.427 / -18.795 0.781: -36.163 / -48.131 / -24.194 1.563: -56.617 / -67.389 / -45.845 3.125: -89.377 / -105.024 / -73.730 6.250: -109.690 / -123.045 / -96.334 12.500: -135.363 / -153.965 / -116.762 25.000: -153.478 / -179.893 / -127.063 50.000: -161.182 / -191.645 / -130.719	Toxic	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.098: 1.945 / -4.687 / 8.578 0.195: -3.251 / -11.147 / 4.645 0.391: -14.404 / -24.147 / -4.660 0.781: -23.583 / -29.068 / -18.099 1.563: -34.234 / -41.682 / -26.786 3.125: -61.216 / -69.468 / -52.964 6.250: -100.406 / -110.220 / -90.593 12.500: -106.798 / -125.932 / -87.664 25.000: -123.588 / -133.488 / -113.687 50.000: -119.394 / -128.480 / -110.307	Toxic		Toxic
MLC803	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.195: 17.205 / -11.020 / 45.429 0.391: 2.543 / -49.333 / 54.419 0.781: -20.852 / -76.446 / 34.741 1.563: -84.602 / -175.916 / 6.713 3.125: -12.704 / -57.494 / 32.087 6.250: -39.725 / -92.679 / 13.228 12.500: -45.876 / -104.765 / 13.013 25.000: -87.467 / -166.030 / -8.904 50.000: -65.536 / -131.786 / 0.714 100.000: -94.359 / -159.245 / -29.473	Toxic	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.195: -15.119 / -55.737 / 25.499 0.391: -18.779 / -58.547 / 20.990 0.781: -37.221 / -78.939 / 4.497 1.563: -80.823 / -142.811 / -18.834 3.125: -57.350 / -113.657 / -1.042 6.250: -107.433 / -201.008 / -13.857 12.500: -126.472 / -188.937 / -64.007 25.000: -124.501 / -189.095 / -59.908 50.000: -83.976 / -136.645 / -31.308 100.000: -51.927 / -96.513 / -7.341	Toxic		Toxic

Chem4 Set3	Exp.1	Exp.2	Exp.3	Judge
MLA301	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.098: -12.551 / -17.439 / -7.663 0.195: -11.462 / -22.202 / -0.722 0.391: -61.396 / -65.247 / -57.546 0.781: -42.799 / -48.356 / -37.241 1.563: -58.155 / -61.428 / -54.881 3.125: -71.859 / -77.189 / -66.529 6.250: -108.578 / -115.180 / -101.975 12.500: -115.984 / -129.744 / -102.223 25.000: -135.613 / -148.237 / -122.989 50.000: -113.805 / -120.258 / -107.352	Toxic	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.098: -4.868 / -16.062 / 6.326 0.195: 0.496 / -7.708 / 8.699 0.391: -26.675 / -36.662 / -16.689 0.781: -29.476 / -40.468 / -18.484 1.563: -62.004 / -74.207 / -49.800 3.125: -65.608 / -80.046 / -51.170 6.250: -94.651 / -117.095 / -72.208 12.500: -116.866 / -141.173 / -92.558 25.000: -118.735 / -138.758 / -98.712 50.000: -137.650 / -162.743 / -112.558	Toxic		Toxic
MLB601	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.098: 8.006 / 1.268 / 14.744 0.195: -13.682 / -20.503 / -6.861 0.391: -15.723 / -26.600 / -4.847 0.781: -35.927 / -46.797 / -25.057 1.563: -48.682 / -59.918 / -37.447 3.125: -59.346 / -69.657 / -49.035 6.250: -81.848 / -99.006 / -64.690 12.500: -86.360 / -108.025 / -64.695 25.000: -118.046 / -143.786 / -94.105 50.000: -112.561 / -141.595 / -83.528	Toxic	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.098: -1.816 / -15.586 / 11.954 0.195: -5.583 / -19.357 / 8.190 0.391: -26.120 / -37.196 / -15.044 0.781: -37.917 / -57.286 / -18.549 1.563: -56.043 / -73.457 / -38.629 3.125: -80.429 / -104.136 / -56.722 6.250: -121.469 / -141.557 / -101.381 12.500: -171.996 / -200.531 / -143.462 25.000: -174.880 / -205.740 / -144.020 50.000: -215.802 / -238.961 / -192.643	Toxic		Toxic
MLC904	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.195: -30.859 / -52.405 / -9.314 0.391: -38.797 / -59.628 / -17.965 0.781: -80.158 / -105.374 / -54.943 1.563: -113.298 / -163.695 / -62.901 3.125: -104.260 / -143.032 / -65.487 6.250: -108.252 / -147.595 / -68.910 12.500: -146.608 / -210.151 / -83.065 25.000: -164.137 / -206.703 / -121.570 50.000: -129.182 / -151.110 / -107.254 100.000: -62.227 / -79.752 / -44.703	Toxic	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit DMSO: 0 / 0 / 0 0.195: -16.030 / -38.027 / 5.967 0.391: -27.282 / -48.668 / -5.896 0.781: -41.366 / -64.401 / -18.330 1.563: -96.447 / -134.337 / -58.557 3.125: -123.673 / -165.940 / -81.407 6.250: -151.635 / -191.423 / -111.848 12.500: -220.521 / -269.739 / -171.303 25.000: -204.351 / -247.239 / -161.463 50.000: -188.498 / -233.041 / -143.956 100.000: -108.154 / -137.569 / -78.740	Toxic		Toxic

Chem5 Set1	Exp.1	Exp.2	Exp.3	Judge
MLA103	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit ddw: 0 / 0 / 0 0.391: 1.186 / -16.040 / 18.412 0.781: 2.458 / -9.460 / 14.375 1.563: -0.656 / -12.981 / 11.668 3.125: 5.636 / -7.449 / 18.720 6.250: 0.594 / -10.782 / 11.971 12.500: -0.142 / -12.496 / 12.211 25.000: -1.968 / -14.176 / 10.240 50.000: -1.978 / -13.087 / 9.131 100.000: -1.364 / -14.461 / 11.734 200.000: 37.827 / 27.165 / 48.489	Non	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit ddw: 0 / 0 / 0 0.391: -4.033 / -19.994 / 11.927 0.781: 4.232 / -12.245 / 20.709 1.563: -15.407 / -26.144 / -4.670 3.125: -4.210 / -15.777 / 7.358 6.250: -15.508 / -25.884 / -5.131 12.500: -18.043 / -29.364 / -6.723 25.000: -23.138 / -35.320 / -10.957 50.000: -19.331 / -30.554 / -8.107 100.000: -13.937 / -24.073 / -3.802 200.000: -26.726 / -38.201 / -15.250	Non		Non
MLB403	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit ddw: 0 / 0 / 0 0.391: 1.045 / -8.053 / 10.142 0.781: 5.398 / -1.110 / 11.906 1.563: 2.132 / -4.905 / 9.168 3.125: 13.969 / 7.923 / 20.014 6.250: 5.662 / -0.644 / 11.968 12.500: 1.873 / -6.069 / 9.815 25.000: 4.356 / -2.621 / 11.333 50.000: 6.748 / -0.352 / 13.848 100.000: 0.134 / -7.494 / 7.762 200.000: -3.140 / -19.196 / 12.916	Non	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit ddw: 0 / 0 / 0 0.391: 0.697 / -16.106 / 17.499 0.781: -4.029 / -21.718 / 13.661 1.563: -8.238 / -25.150 / 8.674 3.125: -4.446 / -21.053 / 12.160 6.250: -3.258 / -18.141 / 11.626 12.500: 0.214 / -14.920 / 15.349 25.000: 1.463 / -12.980 / 15.906 50.000: 2.741 / -11.776 / 17.259 100.000: -1.586 / -15.782 / 12.609 200.000: 2.173 / -13.004 / 17.350	Non		Non
MLC703	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit ddw: 0 / 0 / 0 0.391: -0.226 / -17.523 / 17.070 0.781: 9.360 / -6.794 / 25.514 1.563: 23.123 / 6.483 / 39.763 3.125: 10.216 / -6.520 / 26.952 6.250: 11.461 / -4.282 / 27.203 12.500: 17.475 / 3.338 / 31.613 25.000: 10.423 / -5.267 / 26.114 50.000: 6.756 / -8.195 / 21.707 100.000: -2.752 / -21.148 / 15.643 200.000: -16.955 / -36.324 / 2.415	Non	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit ddw: 0 / 0 / 0 0.391: -2.444 / -53.563 / 48.675 0.781: 10.699 / -32.088 / 53.485 1.563: 6.681 / -36.173 / 49.535 3.125: 11.000 / -28.572 / 50.571 6.250: 15.115 / -21.613 / 51.844 12.500: 14.084 / -24.176 / 52.344 25.000: 3.339 / -48.108 / 54.786 50.000: 16.664 / -19.908 / 53.235 100.000: -0.829 / -46.342 / 44.684 200.000: 4.164 / -39.682 / 48.011	Non		Non

Chem5 Set2	Exp.1	Exp.2	Exp.3	Judge
MLA201	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit ddw: 0 / 0 / 0 0.391: 3.959 / -6.089 / 14.006 0.781: 2.722 / -3.420 / 8.864 1.563: 2.054 / -6.979 / 11.086 3.125: -0.771 / -8.837 / 7.295 6.250: 1.973 / -7.976 / 11.923 12.500: -0.323 / -8.446 / 7.801 25.000: 0.351 / -9.154 / 9.857 50.000: 2.275 / -3.678 / 8.228 100.000: -1.865 / -12.987 / 9.256 200.000: -10.116 / -17.667 / -2.565	Non	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit ddw: 0 / 0 / 0 0.391: 4.307 / -8.996 / 17.611 0.781: -3.378 / -16.042 / 9.287 1.563: 6.182 / -5.585 / 17.949 3.125: 6.942 / -5.195 / 19.079 6.250: 10.081 / -1.154 / 21.317 12.500: 9.573 / -1.590 / 20.736 25.000: 11.625 / -0.099 / 23.350 50.000: 8.668 / -3.619 / 20.955 100.000: 12.938 / 0.830 / 25.045 200.000: 13.362 / -0.379 / 27.102	Non		Non
MLB501	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit ddw: 0 / 0 / 0 0.391: 3.848 / -8.193 / 15.889 0.781: 0.267 / -11.708 / 12.242 1.563: 7.213 / -8.162 / 22.589 3.125: 1.724 / -12.003 / 15.451 6.250: 0.485 / -9.147 / 10.118 12.500: -0.496 / -17.523 / 16.532 25.000: 5.297 / -2.652 / 13.247 50.000: -3.801 / -16.247 / 8.645 100.000: -0.867 / -9.709 / 7.974 200.000: 20.944 / 13.245 / 28.642	Non	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit ddw: 0 / 0 / 0 0.391: -3.394 / -14.361 / 7.572 0.781: 1.917 / -9.070 / 12.904 1.563: -0.699 / -8.806 / 7.407 3.125: 1.925 / -5.787 / 9.637 6.250: 3.088 / -5.505 / 11.680 12.500: 1.510 / -7.293 / 10.313 25.000: 0.546 / -8.903 / 9.995 50.000: 6.058 / -3.160 / 15.275 100.000: 8.453 / 1.432 / 15.474 200.000: 9.826 / 1.981 / 17.672	Non		Non
MLC801	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit ddw: 0 / 0 / 0 0.391: 15.251 / -3.894 / 34.395 0.781: 22.328 / 1.138 / 43.518 1.563: 13.448 / -4.811 / 31.708 3.125: 15.283 / 2.205 / 28.361 6.250: 20.173 / -6.789 / 47.136 12.500: 21.956 / -2.694 / 46.606 25.000: 31.588 / 13.753 / 49.423 50.000: 22.478 / 2.439 / 42.516 100.000: 21.118 / 3.603 / 38.633 200.000: 12.936 / -6.675 / 32.547	Non	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit ddw: 0 / 0 / 0 0.391: 20.971 / 5.603 / 36.338 0.781: 12.830 / -4.397 / 30.057 1.563: 15.909 / 2.396 / 29.423 3.125: 24.458 / 6.298 / 42.618 6.250: 19.031 / 0.999 / 37.063 12.500: 17.748 / 7.340 / 28.155 25.000: 13.817 / -0.978 / 28.612 50.000: 13.810 / 4.194 / 23.427 100.000: 21.134 / 5.802 / 36.467 200.000: 14.354 / 1.783 / 26.925	Non		Non

Chem5 Set3	Exp.1	Exp.2	Exp.3	Judge
MLA302	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit ddw: 0 / 0 / 0 0.391: -2.007 / -12.218 / 8.204 0.781: 2.191 / -7.681 / 12.063 1.563: -7.160 / -17.882 / 3.563 3.125: -8.744 / -15.748 / -1.739 6.250: -5.229 / -12.384 / 1.925 12.500: -4.810 / -11.523 / 1.903 25.000: -11.944 / -19.737 / -4.151 50.000: -6.411 / -13.853 / 1.031 100.000: -8.145 / -16.164 / -0.126 200.000: -4.991 / -13.076 / 3.094	Non	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit ddw: 0 / 0 / 0 0.391: 5.051 / -2.892 / 12.994 0.781: 4.692 / -5.880 / 15.265 1.563: 16.198 / 8.444 / 23.952 3.125: 10.621 / 3.079 / 18.164 6.250: 13.165 / 1.225 / 25.105 12.500: 8.850 / -3.158 / 20.858 25.000: 10.912 / -0.934 / 22.758 50.000: 10.502 / 2.395 / 18.609 100.000: 11.666 / 2.397 / 20.935 200.000: 8.528 / 0.452 / 16.603	Non		Non
MLB605	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit ddw: 0 / 0 / 0 0.391: 6.984 / -0.025 / 13.993 0.781: 0.462 / -10.868 / 11.792 1.563: 0.884 / -14.742 / 16.510 3.125: -0.623 / -6.498 / 5.253 6.250: -2.137 / -12.051 / 7.777 12.500: 5.449 / -0.068 / 10.967 25.000: 4.541 / -2.705 / 11.787 50.000: 13.896 / 5.347 / 22.445 100.000: 10.883 / 4.862 / 16.903 200.000: 11.699 / 4.459 / 18.938	Non	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit ddw: 0 / 0 / 0 0.391: -4.187 / -15.888 / 7.514 0.781: 0.101 / -11.594 / 11.796 1.563: -11.301 / -22.174 / -0.429 3.125: -7.735 / -20.335 / 4.865 6.250: -14.934 / -29.844 / -0.023 12.500: 6.926 / -3.898 / 17.751 25.000: -13.466 / -31.418 / 4.486 50.000: -6.453 / -21.054 / 8.148 100.000: -6.836 / -24.807 / 11.136 200.000: -13.434 / -28.847 / 1.978	Non		Non
MLC901	 1st Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit ddw: 0 / 0 / 0 0.391: 12.739 / 8.538 / 16.939 0.781: 17.278 / 1.186 / 33.369 1.563: 25.364 / 20.496 / 30.232 3.125: 13.924 / -9.356 / 37.204 6.250: 23.291 / 18.460 / 28.122 12.500: 20.883 / 15.809 / 25.958 25.000: 28.586 / 21.605 / 35.567 50.000: 25.734 / 15.941 / 35.526 100.000: 15.421 / 7.224 / 23.618 200.000: 17.145 / 9.155 / 25.134	Non	 2nd Exp. a Comparison Conc. / %supp / Lower Limit / Upper Limit ddw: 0 / 0 / 0 0.391: -0.916 / -13.952 / 12.121 0.781: 5.539 / -5.848 / 16.926 1.563: 2.584 / -9.791 / 14.958 3.125: 5.592 / -4.762 / 15.946 6.250: -3.913 / -18.363 / 10.538 12.500: 4.725 / -6.504 / 15.953 25.000: 10.143 / -4.120 / 24.407 50.000: -4.963 / -19.495 / 9.570 100.000: 1.476 / -11.757 / 14.709 200.000: 8.285 / -4.785 / 21.356	Non		Non

OECD TEST GUIDELINES PROGRAMME

Standard Project Submission Form

If you require further information please contact the OECD Secretariat
Return completed forms to:
Anne Gourmelon (anne.gourmelon@oecd.org)
and Anna Rourke (anna.rourke@oecd.org)

PROJECT TITLE

The modification of the prediction model of the IL-8 Lu assay (OECD TG442E) to improve its performance

SUBMITTED BY (Country / European Commission / Secretariat)

Japan

DATE OF SUBMISSION TO THE SECRETARIAT

November 11th, 2020

DETAILS OF LEAD COUNTRY/CONSORTIUM

Country /Organisation:	Japan
Agency/ministry/Other:	National Institute of Health Sciences
Mail Address:	3-25-26 Tonomachi, Kawasaki-ku, Kawasaki 210-9501, Japan
Phone/fax:	+81-44-270-6597
Email:	h-kojima@nihs.go.jp

PROJECT OUTCOMES

☐ New Test Guideline	☐ Guidance document
☒ Revised Test Guideline	☐ Detailed Review Paper
☐ Deletion of an existing Test Guideline	☐ Other, please specify below

MAIN OBJECTIVE OF THE PROPOSAL (max. 150 words)

The proposed project aims to improve the performance of the IL8 Luc test method included in TG 442E by modifying the prediction model of the IL-8 Luc assay. The revised prediction model uses an indicator of cytotoxicity, based on glyceraldehyde 3-phosphate dehydrogenase luciferase activity (GAPLA). Based on this revised prediction model, inconclusive chemicals are judged negative if they show evidence of cytotoxicity. This modification reduces the number of inconclusive chemicals and increases the specificity of the assay.

PROPOSED WORK PLAN and RESOURCE NEEDS:

1. Draft workplan for development of the proposal, including any need to establish Ad Hoc Expert Group and mode of meetings (face-to-face, teleconference; electronic discussion group).

Indicate key milestones, including first and subsequent drafts of documents and timing of meetings.

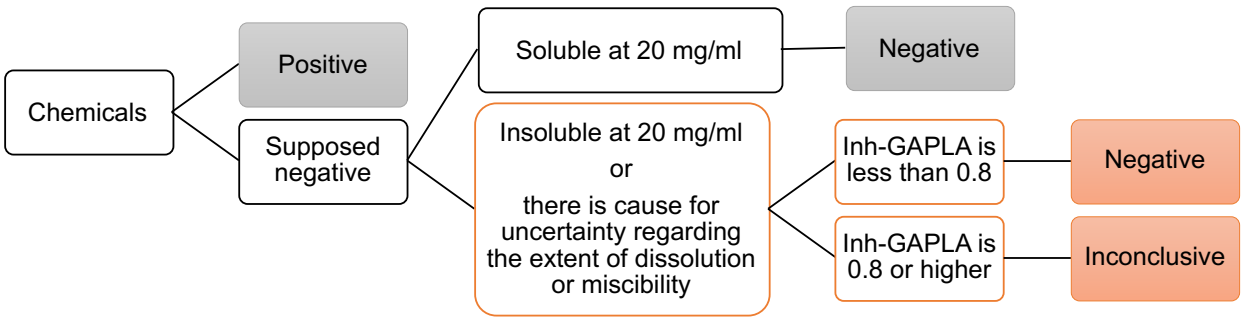
Tohoku University developed the IL-8 Luc assay as the *in vitro* test addressing the third key event of the skin sensitisation Adverse Outcome Pathway (AOP),. This assay detects the expression of IL-8 in the human monocytic leukaemia cell line THP-1. The IL-8 Luc assay has been adopted as Appendix III of Test Guideline (TG) 442E by the Organisation for Economic Co-operation and Development (OECD). According to TG 442E, the IL-8 Luc assay can be used for supporting the discrimination between skin sensitisers and non-sensitisers. In this project, it is proposed to modify the prediction model of the IL-8 Luc assay in order to improve the performance of the assay. The prediction model would focus on the evaluation cytotoxicity based on glyceraldehyde 3-phosphate dehydrogenase luciferase activity (GAPLA).

We propose to modify the prediction model. The modified part is written red.

Prediction model

32. Test chemicals that provide two positive results from among the 1st, 2nd, 3rd. and 4th runs are identified as positives whereas those that give three negative results from among the 1st, 2nd, 3rd and 4th runs are identified as supposed negative (Table 2). Among supposed negative chemicals, if chemicals are dissolved at 20 mg/ml in X-VOVO™ 15, they are judged as negative. If chemicals are not dissolved at 20 mg/ml in X-VOVO™ 15, or there is cause for uncertainty regarding the extent of dissolution or miscibility, chemicals that give Inh-less than 0.8 of GAPLA are judged as negative, while those that give 0.8 or higher of Inh-GAPLA should not be considered (Figure 1).

Figure 1. Prediction model for final judgment



This proposal with the revised text of TG 442E for the IL-8 Luc assay was already discussed at the tele-conference of OECD expert group for skin sensitisation (EG) on 30th October 2020. There was no particular comment on the proposed revisions which make the modified prediction model. If TG 442E can be revised upon approval of the SPSF by the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) in 2021 and the revised TG442E will be submitted by Japan 2nd Q in 2021. This draft will be discussed by EG and WNT and Japan wishes to be accepted this revised TG 442E in 2022.

2. Will additional information, including generation or collection of data, be required? If yes, please describe the anticipated process and timelines.

Japan proposes additional information and requires no further data. False negative judgment due to poor chemical solubility is a problem with *in vitro* skin sensitisation tests. Water-insoluble chemicals are typically dissolved in DMSO in most sensitisation tests but precipitate when diluted with medium beyond their solubility in water. Such tests lack procedures to rule out false negative judgments due to poor solubility. The IL-8 Luc assay (OECD442E) is unique in that if chemicals do not dissolve at 20 mg/mL in medium and have no effect on IL-8 luciferase activity (IL8LA), they are excluded from judgment by classifying them as inconclusive. The purpose of the present study is to reduce the number of inconclusive chemicals and improve assay performance.

The IL-8 Luc assay can simultaneously examine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) luciferase activity (GAPLA) and IL8LA. GAPDH mRNA is ubiquitously expressed at moderately abundant levels. It is frequently used as an endogenous control for quantitative real time polymerase chain reaction in some experimental systems, because its expression is constant at different times and after experimental manipulation (Edwards and Denhardt, 1985; Mori et al., 2008; Winer et al., 1999). Although there are several reports suggesting that its use as an internal standard is inappropriate in some cases (Oliveira et al., 1999; Thellin et al., 1999), in general within-tissue variation of GAPDH mRNA expression levels is small whereas between-tissue variation can be substantial, depending on tissue type (Barber et al., 2005). To improve the performance of the IL-8 Luc assay, we thought that GAPLA can be a marker of cytotoxicity of chemicals and thus it can be a marker indicating that chemicals are dissolved in medium because chemicals cannot induce cytotoxicity if they are not dissolved in medium. To demonstrate that GAPLA is a marker of cytotoxicity of chemicals, we examined the correlation between the reduction of GAPLA (defined as Inh-GAPLA) and the reduction of propidium iodide (PI)-excluding cells after chemical treatment for representative three sensitizers and three non-sensitizers.

Thus, THP-G8 cells were treated with various concentrations of the three sensitizers DNCB, 4-NBB and diethylmaleate, and the three non-sensitizers SLS, limonene and streptomycin sulfate. The cells were examined for Inh-GAPLA after 16 h of chemical treatment, and the number of PI-excluding cells was determined after 16 h, 24 h, and 48 h of chemical treatment. All the chemicals were dissolved and diluted according to the protocol for the IL-8 Luc assay, then THP-G8 cells were stimulated using the concentration of each chemical at which the values of Inh-GAPLA distributed from 0.02 to 1.0. These concentrations of chemicals were determined based on in-house data.

When these cells were cultured during 48 h, THP-G8 cells that gave less than 0.8 of Inh-GAPLA reduced PI excluding cells dose-dependently and chemicals providing an Inh-GAPLA value below 0.8 always reduced the ratio of PI-excluding cells to less than 0.6. On the other hand, the chemicals providing more than 0.8 of Inh-GAPLA maintained more than 0.8 of PI excluding cells (Fig. 1). Only THP-G8 cells treated with DNCB that gave 0.908 of Inf-GAPLA reduced PI reduction to 0.6 after 48 h of culture.

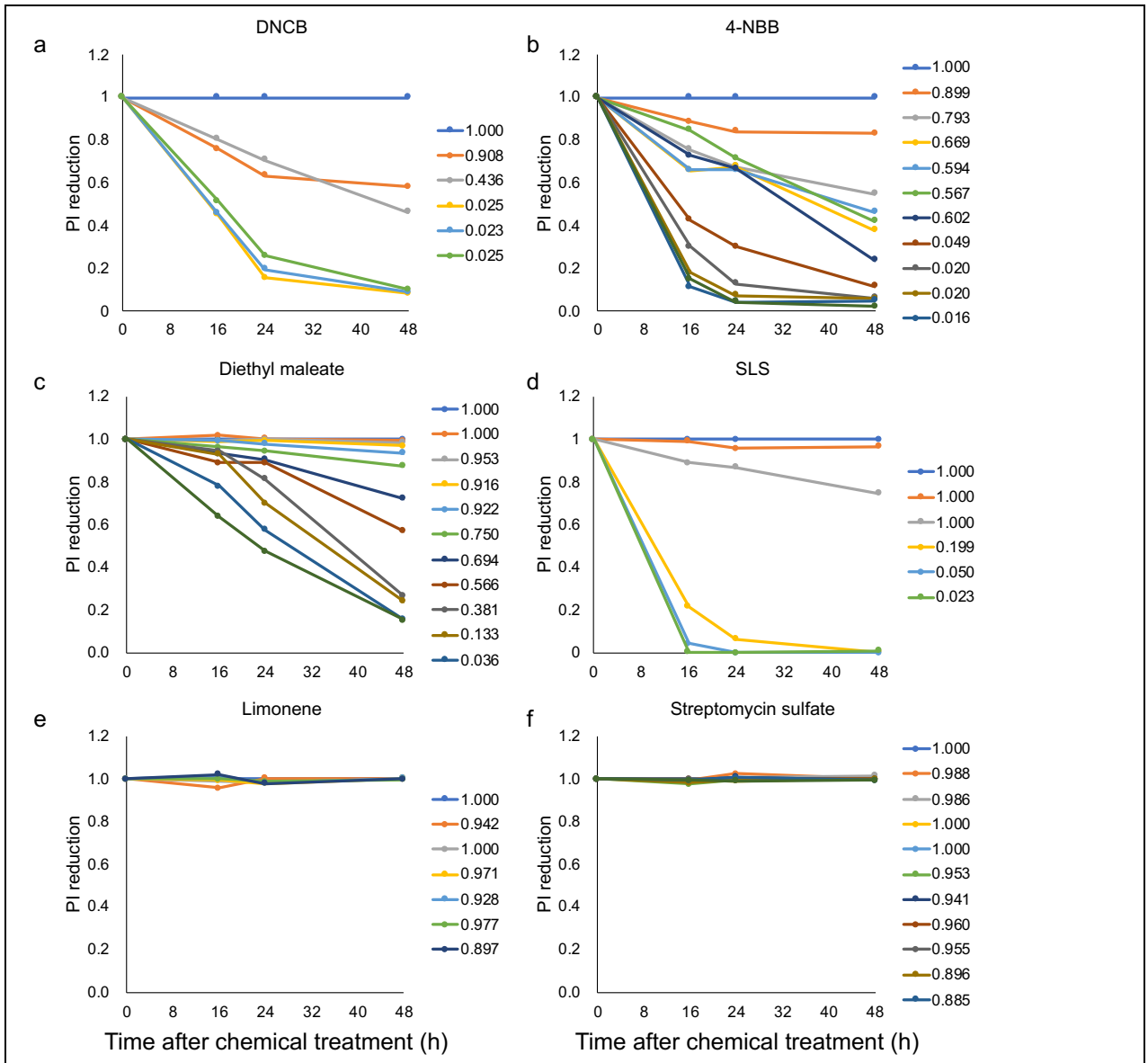


Fig. 1. PI reduction in THP-G8 cells treated with chemicals decreases as the level of Inh-GAPLA decreases and the culture period increases.
THP-G8 cells treated with various concentrations of the three sensitizers DNCB, 4-NBB and diethylmaleate, and the three non-sensitizers SLS, limonene and streptomycin sulfate, were examined for Inh-GAPLA after 16 h and for the number of PI-excluding cells after 16 h, 24 h, and 48 h of chemical treatment. THP-G8 cells were stimulated using concentrations of chemicals at which the values of Inh-GAPLA distributed from 0.02 to 1.0. The reduction in PI of THP-G8 cells treated with each concentration of chemical is shown at 0 h, 16 h, 24 h, and 48 h. Each line is labeled with an Inh-GAPLA value for THP-G8 cells treated with the concentration of chemical at which PI reduction was measured.

When Inh-GAPLA and PI reduction values for all chemicals were summarized and examined for correlation, these two parameters gave a significant correlation at 16 h, 24 h, and 48 h (Fig. 2). Furthermore, the correlation strengthened with increased culture period, with correlation coefficients after 16 h, 24 h, and 48 h of 0.775, 0.863, and 0.935, respectively (Kimura et al., 2020).

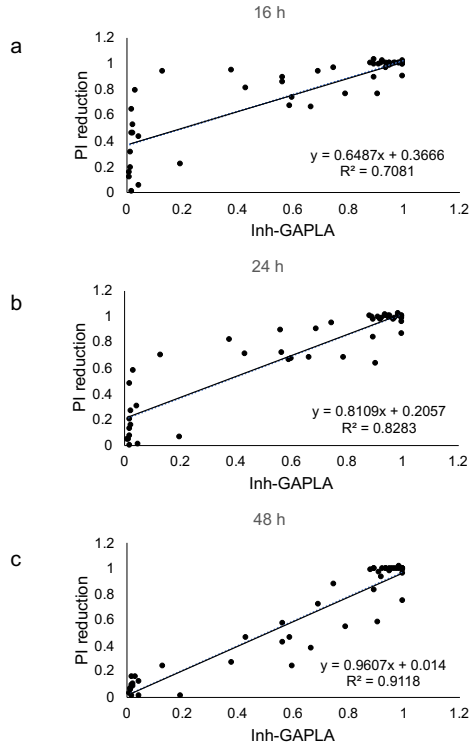


Fig. 2. There is a significant correlation between Inh-GAPLA and PI reduction values with strengthened correlation, depending on the culture period
Inh-GAPLA and PI reduction values for all chemicals were examined for correlation at three time points (16 h, 24 h, and 48 h) of chemical treatment.

These data suggest that Inh-GAPLA is a good marker for cell viability, with a strong correlation with PI-excluding cells, and that Inh-GAPLA below 0.8 indicates cytotoxicity of the test chemical, which in turn suggests that the chemical dissolved in X-VOVO^{TM} 15.

References

Barber, R.D., Harmer, D.W., Coleman, R.A., et al., 2005. GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. Physiol Genomics 21, 389-395.

Edwards, D.R., Denhardt, D.T., 1985. A study of mitochondrial and nuclear transcription with cloned cDNA probes. Changes in the relative abundance of mitochondrial transcripts after stimulation of quiescent mouse fibroblasts. Exp Cell Res 157, 127-143.

Mori, R., Wang, Q., Danenberg, K.D., et al., 2008. Both beta-actin and GAPDH are useful reference genes for normalization of quantitative RT-PCR in human FFPE tissue samples of prostate cancer. Prostate 68, 1555-1560.

Oliveira, J.G., Prados, R.Z., Guedes, A.C., et al., 1999. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase is inappropriate as internal control in comparative studies between skin tissue and cultured skin fibroblasts using Northern blot analysis. Arch Dermatol Res 291, 659-661.

Thellin, O., Zorzi, W., Lakaye, B., et al., 1999. Housekeeping genes as internal standards: use and limits. J Biotechnol 75, 291-295.

Winer, J., Jung, C.K., Shackel, I., et al., 1999. Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. Anal Biochem 270, 41-49.
Kimura, Y., Fujimura C., Aiba, S. 2020. The modified IL-8 Luc assay can significantly improve the false negative judgment of lipophilic sensitisers with LogKow>3.5. Arch Toxicol, in press.

3. Indicate the estimated overall resource need (time/money) for member country / consortium and Secretariat

No financial resources were needed from any stakeholder.

Tohoku University in collaboration with the NIHS will provided the revised text in 2021. Co-operation of the OECD Secretariat is requested for distributing revised versions of the prediction model in TG 442E for the IL-8 Luc assay that need to be addressed. We do not foresee any additional resources that are needed beyond this.

4. Is this proposal intended to replace an existing Test Guideline or lead to the deletion of an existing Test Guideline?

Yes, the current prediction model in TG 442E for the IL-8 Luc assay would be revised, as described previously.

ESSENTIAL INFORMATION

In this section, please provide the information required by the Working Group of National Coordinators of the Test Guidelines Programme to assess the suitability of the project for the workplan of the Test Guidelines Programme

1. What is the existing or expected regulatory need/data requirement that will be met by the proposed outcome of the project? Please provide details below or as an attachment.

Of the 143 chemicals examined by the original IL-8 Luc assay (TG442E), 23 were classified as inconclusive. On the other hand, by applying the modified criteria, 10 of the 23 inconclusive chemicals were classified as non-sensitizers. Consequently, the performance of the modified IL-8 Luc assay was 93.9% for sensitivity, 68.0% for specificity, and 88.6% for accuracy, while that of the original IL-8 Luc assay was 95.8% for sensitivity, 53.0% for specificity, and 89.4% for accuracy. In addition, the number of chemicals classified as inconclusive is 13 by the modified IL-8 Luc assay and 23 by the original IL-8 Luc assay (Table 1). We also demonstrated LLNA potency category, solubility in 20 mg/ml of X-VIVO^{TM} 15, the average of maximum values of induction of IL8LA (Ind-IL8LA) and the average of minimum values of inhibition of GAPLA (Inh-GAPLA) in repeated experiments, the judgments by the original IL-8 Luc assay and by the modified IL-8 Luc assay, and LogK_{ow} of the 143 chemicals (the Supplementary Table 1 of the attached document 3). The terms of Ind-IL8LA and Inh-GAPLA were defined as follows.
Ind-IL8LA = IL8LA of THP-G8 cells treated with chemicals/IL8LA of untreated cells
Inh-GAPLA = GAPLA of THP G8 cells treated with chemicals/GAPLA of untreated cells.

Table 1. The comparison of the performance of the original IL-8 Luc assay and modified IL-8 Luc assay

	IL-8 Luc assay (OECD TG442E)	moIL-8 Luc assay
Total chemicals	143	143
Haptens	107	107
Non-haptens	36	36
Correct Positive	92	92
False Negative	4	6
Correct negative	9	17
False positive	8	8
Inconclusive	22	12
Out of applicability domain	8	8
Accuracy	89.3%	88.5%
Sensitivity	95.8%	93.8%
Specificity	52.9%	68.0%
Positive predictive value	91.9%	91.9%
Negaive predictive value	69.2%	73.9%

or as attachment No._3_

2. How will the work contribute to further international harmonisation of hazard and risk assessment? Please provide details below or as an attachment.

The non-animal skin sensitization tests like the IL-8 Luc assay is used in combination with other sources of information in the context of an IATA. OECD draft test guideline for defined approach is published and under public comments. Modifying the prediction model in this assay will contribute to OECD draft test guidelines for defined approach.

or as attachment No.__

3. How will the proposed project address issues and /or endpoints which are of major human health or environmental concerns? If there are existing Test Guidelines or projects in the work plan of the Test Guidelines Programme covering the same endpoint, please refer to these and describe the added value and usability of the proposed new test method. Please provide details below or as an attachment.

The proposal is to modify the prediction model in TG 442E for the IL-8 Luc assay. The IL-8 Luc assay is used in combination with other sources of information in the context of an IATA. Modifying the prediction model will contribute to attainment of better compatibility of animal welfare and prediction of chemical safety in the skin sensitization field.

or as attachment No.__

4. Will the project have general support from OECD member countries or is the outcome relevant for just one or a few member countries / stakeholders? Provide details of the countries and the rationale for this view below.

☒ Many countries ☐ A few countries ☐ Only for the submitting country

The outcome was generated by Tohoku University. The outcome is relevant to all OECD member countries.

5. If the Test Guideline is not intended for general use, indicate if the Test Guideline would be intended for:

☐ Specific (limited) applications such as pesticide usage, or

☐ for specific classes of chemicals (e.g. surfactants) rather than for chemicals in general.

6. If the expected outcome of this proposal is a Test Guideline or a Guidance Document, provide information on the intended use, applicability and limitations of the test method.

Due to the proposed revision, the modified IL-8 Luc assay aims to improve its performance and expand the applicability domain. It is useful revision for end users.

7. Provide supporting information on the validation status (i.e. relevance and reliability) of the method. Principles for validation of test methods for OECD Test Guidelines are described in Guidance Document 34.

Provide justification and rationale for the test, including data.

If there are no or limited data available to support the reliability and relevance of the proposed test, indicate if validation work is included in the project.

If there is no need for validation, provide a detailed justification.

TG 442E for the IL-8 Luc assay has already been adopted. The proposed modified IL-8 Luc assay slightly differs from the earlier version of TG 442E described previously. However, it is foreseen that further validation is not required because the modified points are minor and supported by experimental work (manuscript submitted).

8. Describe if the test method includes components, equipment or other scientific procedures that are covered (or pending) by Intellectual Property Rights (IPR) (e.g., patents, patent applications, industrial designs and trademarks). Information should be provided on the overall availability of the IPR-protected components including whether they are commercially available or require a Material Transfer Agreement (MTA) or other licensing agreements. In addition, a description of the IPR-covered component/test system should be disclosed. Note that the OECD has developed Guiding Principles on good practices for the availability/distribution of protected elements in OECD Test Guidelines. The test method developer will be requested to fill in and sign the FRAND Terms Licensing Declaration Form annexed to the Guiding Principles.

8.1 Nature of protected elements (e.g. reagent identity, cell line identity, specific process, etc.):

None

8.2 Form of protection (e.g. trademark, patent, etc.):

None

8.3 For users to access protected elements, please tick the relevant box(es):

☐ MTA required ☐ License requirement ☒ No agreement required

If a license or other agreement is foreseen, please note that terms and conditions should comply with FRAND and a signed declaration needs to be submitted if the project gets onto the work plan. See Annex 2 of the OECD Guiding Principles on Good Licensing Practices for Protected Elements in OECD TGs (2019).

8.4 Are you providing the agreement document(s) referred to in 8.3 with the SPSF:

☐ Yes ☒ No If no, what's the reason?

Not applicable.

8.5 How and where can users get access to protected elements?

The Standard Operating Procedure (SOP) for the IL-8 Luc assay and THP-G8 cells become available when this assay is accepted as the OECD TG. Laboratories that want to perform the test would obtain the THP-G8 cell line from GPC Lab. Co. Ltd., Tottori, Japan, upon signing a Material Transfer Agreement (MTA) in line with the conditions of the OECD template. THP-G8 cells will be maintained and quality-checked at regular intervals in GPC.

8.6 Has any search for existing patent(s) possibly associated with this test method been performed (e.g. through patent search or Freedom-To-Operate search). If yes, what was the outcome?

Not applicable

8.7 Does the test method include any Confidential Business Information? ☐ Yes ☒ No ☐ N/A

If yes, which ones?

<u>IMPORTANT NOTE:</u> Should the OECD and Expert Group working on the Test Guideline development discover that the information provided under Item 3 on IP elements be erroneous or be evolving in the course of the project, the project itself might be re-considered, suspended or cancelled.

9 Have Performance Standards been developed? ☐ Yes ☐ No ☒ N/A

ADDITIONAL INFORMATION

In this section please provide further information to allow the Working Group of National Coordinators of the Test Guidelines Programme to assess the suitability of the project for the workplan of the Test Guidelines Programme

1. If the expected outcome of the project proposal is a Test Guideline and is based on existing, regional or international documents such as guidelines, protocols or guidance material, please provide that information here or as an attachment.

It is expected that the reliability and usability of the modified TG 442E for the IL-8 Luc assay have been already verified because the revised part is only minor point for the prediction model from the earlier version of TG 442E, as described previously.

or as attachment No.__

2. If Animal Welfare considerations are addressed in the project proposal, provide details below or as an attachment. Explain if the project is aimed at refining, reducing and/or replacing the use of animals.

If the project is not specifically developed for animal welfare purposes, indicate if the animal welfare considerations have been a component of the project proposal.

Indicate if animal welfare considerations are irrelevant to the project, for example for physico-chemical properties.

The OECD has already adopted the TG 442E for the IL-8 Luc assay. Modifying the prediction model would improve the performance of this assay by reducing the number of inconclusive chemicals and increasing their specificity.

or as attachment No.__

3. Provide information on expected or possible resource savings in member countries as a result of this project.

As mentioned in additional information 2, modifying the prediction model would lead to a decrease in the use of animals for identifying skin sensitization potential. Generally, in vitro does not take times comparing with in vivo sensitization tests. We believe these mean resource savings in member countries.

4. If the expected outcome of the proposed project is a Guidance Document or Detailed Review Paper, will it be directly linked to the development of a particular Test Guideline or a series of Test Guidelines?

☐ Yes, it is the initial step in the development of a new or revision of existing Guidelines.

☐ Yes, additional guidance is needed for the most appropriate selection of the Guidelines on the subject.

☐ No, the guidance is on issues related to testing or the development of Test Guidelines in general.

There are __2_ attachments added to this form.

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1. Arch Tox: The modified IL-8 Luc assay can significantly improve the false negative judgment of lipophilic sensitisers with LogKow >3.5
2. Arch Toxicol Supplement Table 1

国立医薬品食品衛生研究所
小島　肇　様

試験番号：GP-JKE-202005
報告書作成日：2021年2月26日

試験報告書

ご依頼の試験を実施し、下記の結果を得ましたのでご報告いたします。

【試験標題】　デュアルレポーターシステム搭載HACベクター導入THP-1細胞の構築

【試験期間】　2020年7月29日～2021年2月 25日

株式会社ジーピージー研究所
〒683-0826　鳥取県米子市西町86番地
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TEL 0859-21-7232

責任者	担当者	担当者
辻	澤西	泉

試験報告書

【試験標題】

デュアルレポーターシステム搭載HACベクター導入THP-1細胞の構築

【試験内容】

GAPDHSLR:IL8SLO遺伝子をHACベクターに搭載し、
これを保持するTHP-1細胞を作製する。

【使用機器・試薬等】

細胞培養関連

- CHO-H8細胞
- THP-1IDAC細胞
- Ham's F12, 富士フィルム和光純薬, cat#087-08335
- RPMI medium, Gibco, cat#11875093
- FBS, Sigma, cat#172012-500ML, lot#14L369
- Penicillin/Streptomycin, 富士フィルム和光純薬, cat#168-23191
- G418(50 mg/mL), ナカライテスク, cat#084-07681
- Blasticidin S (10 mg/mL), cat#A1113903
- Cytochalasin B, 富士フィルム和光純薬, cat#034-17554
- Cell Banker, TAKARA, cat#CB011
- その他汎用実験試薬、消耗品

【試験期間】

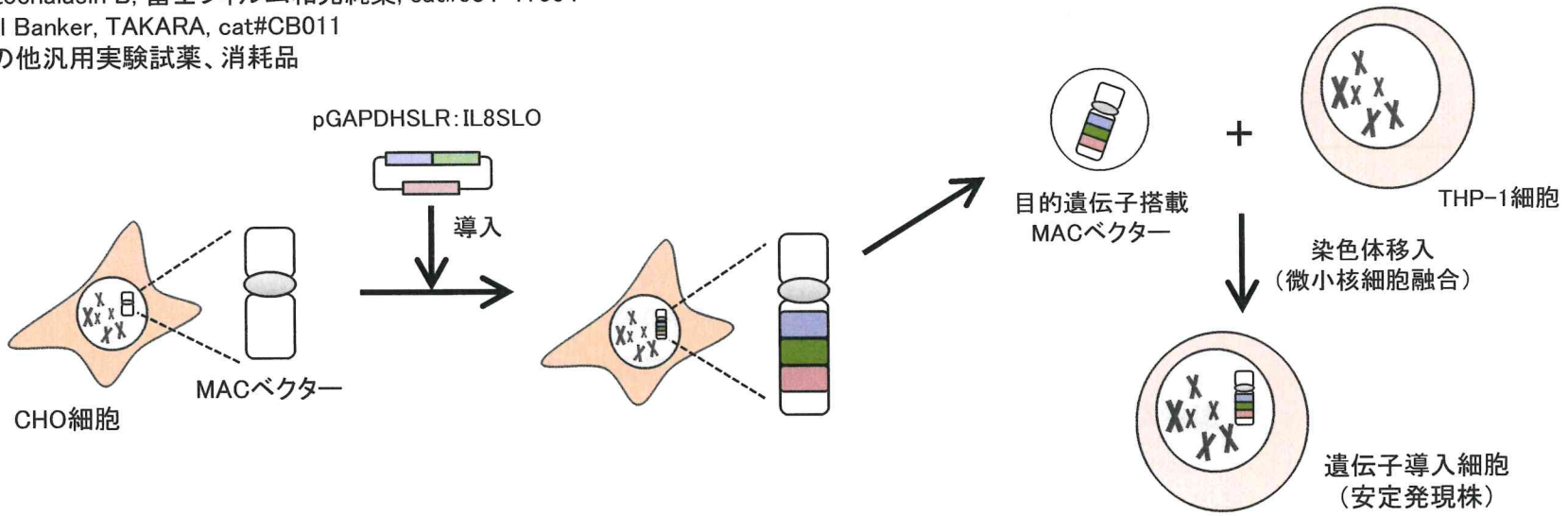
2020年7月29日～2021年2月26日

【試験概要】

デュアルレポータシステム用に構築されたプラスミドをCHO細胞内に保持されたHACベクターに搭載したのち、微小核細胞融合法を用いてTHP-1IDAC細胞に移入する。

試薬・機器関連

- KOD FX Neo, TOYOBO, cat#KFX-201
- Cell Lysis Solution, QIAGEN, cat#158906
- Protein Precipitation Solution, QIAGEN, cat#158910
- FHERIOS, ATTO
- Kronos H, ATTO
- Thermal Cycler Dice Touch, TaKaRa Bio



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【試験方法】

1. HACベクターへのGAPDHSLR:IL8SLO(GAPDHR-IL8O) 遺伝子搭載

1-1 HACベクター保持CHO細胞を培養する。
1-2 プラスミド(CMV-LoxP:GAPDHR:IL8O:をHACベクターに搭載する。
1-3 G418で薬剤選抜を行い、耐性クローンを獲得する。
1-4 耐性クローンは、PCRにより染色体上へのGAPDHR-IL8O遺伝子移入を確認する。

2. GAPDHR-IL8O遺伝子搭載HACベクター保持細胞の機能評価

2-1 取得したGAPDHR-IL8O保持細胞候補クローンについて、TNFα及びIL-1応答性評価および核型解析を実施する。
2-2 TNFαまたはIL1 を添加した培地で2日間の培養を行い、発光量の変動をクロノスHT(ATTO)を用いて測定する。
2-3 各クローンの核型標本を作製し、HACベクターを保持していることを確認する。
2-4 TNF-αおよびIL-1応答性が良く、HACベクターが独立して保持されていることが確認されるクローンを選択する。

3. GAPDR-IL8O遺子搭伝載HACベクタ-のTHP-1細胞への移入

3-1 選択した細胞が十分に増えたタイミングでコルセミド添加培地に置換し、微小核を形成させる。
3-2 微小核を形成した細胞をサイトカラシン含有培地で満たし、遠心(8000rpm, 60min, 34℃)して、微小核を回収する。
3-3 ポリエチレングリコール(PEG)を用いて、THP1-IDAC細胞と微小核を融合し、HACベクターを移入する。
3-4 Blasticidin S で薬剤選抜を行い、耐性クローンを獲得する。
3-5 耐性クローンは、PCRにより染色体移入を確認する。

【報告書一覧】

- ページ1～3　表紙、試験概要他
- ページ4～5　HACベクターへのGAPDHR -IL8SLO遺伝子搭載試験結果
- ページ6～8　GAPDHR-IL8O遺伝子搭載HACベクター保持細胞の評価結果
- ページ9　GAPDHR-IL8O遺子搭伝載HACベクタ-のTHP-1細胞への移入確認
- ページ9　総括

試験報告書

【1:HACベクターへのGAPDHR-IL8O遺伝子搭載】

- Cre-loXPシステムを用いてHACベクターへのプラスミド(pGAPDHSLR:IL8SLO)の搭載を実施した。
- Ripofectamin 2000を用いてCMV-CreとpGAPDHSLR:IL8SLOを共導入した。
- G418 (500ug/mL)で薬剤選抜を実施し、60個のクローンを得た.。その内、先行して増殖したクローンについてゲノム抽出とPCRおよびLucアッセイを実施し、発光量が高く、遺伝子の導入が確認できた5クローン(R4, R6, W3, W7, W9)を選択した。

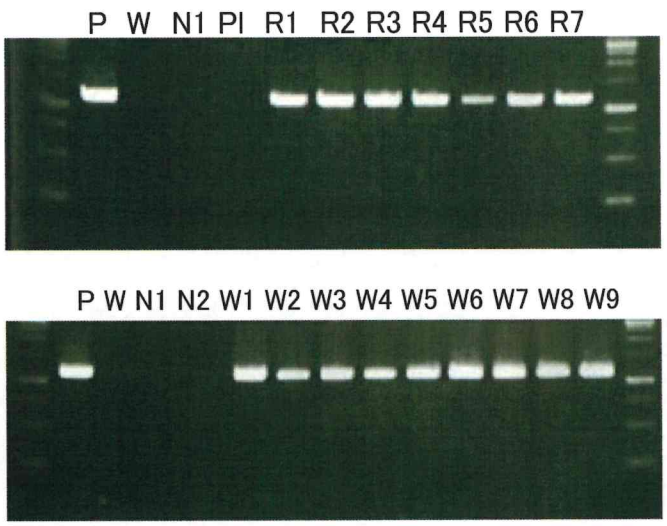
Luc アッセイ
20mM D-luciferin添加
全光(F0) 3秒測定

クローン名	発光値
R1	49
R2	30
R3	575
R4	2571
R5	65
R6	1284
R7	35
R8	252
R9	130
R10	240
R11	125
R13	124
R14	238
R15	1467
R16	36
R17	45
R18	154
R19	49
R20	40
R21	164
R22	138
R23	2575
R24	738

クローン名	発光値
w1	58
w2	44
w3	1947
w4	36
w5	63
w6	99
w7	1626
w8	68
w9	3087
w10	909
w11	1979
w12	3129
w13	1636
w14	640
w15	214
w16	17
w17	27
w18	367
w19	719
W20	53
W21	397
W22	2575
W23	21478

PCR結果

増幅領域 : HACベクター--プラスミド組換え部分(318bp)



・KODFXneo使用
94℃ 2min
98℃ 10 sec] 35 cycles
68℃ 1min

P: ポジティブコントロール
他遺伝子搭載HAC保持
CHO細胞
W: H_2O
N1: ネガティブコントロール
HAC保持CHO細胞(WT)
Pl: 導入プラスミド
pGAPDHSLR:IL8SLO

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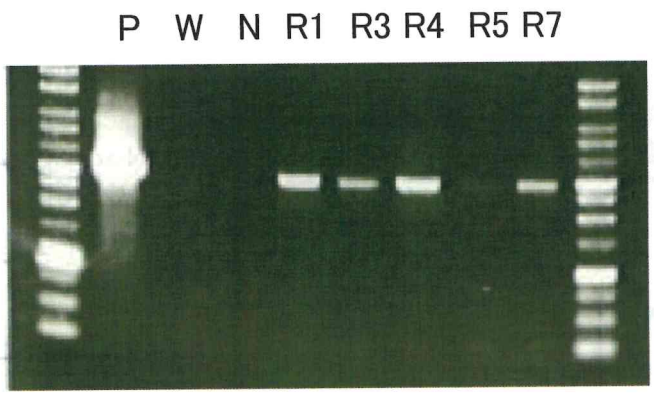
試験報告書

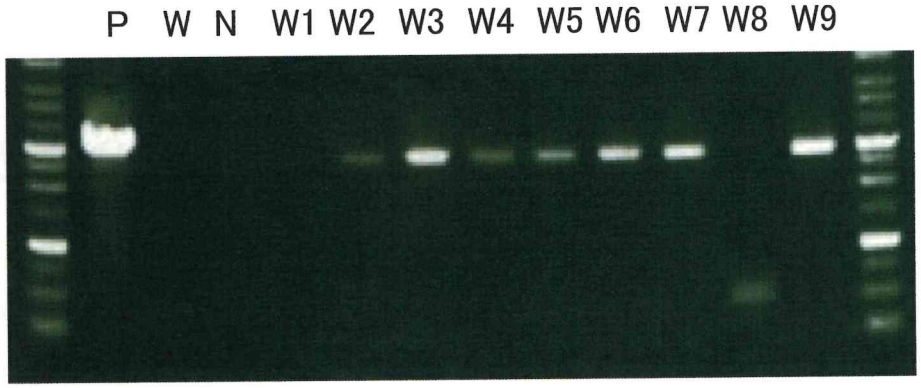
PCR結果

PI: 導入プラスミドpGAPDHSLR:IL8SLO
W: H_2O
N: ネガティブコントロール
HAC保持CHO(WT)

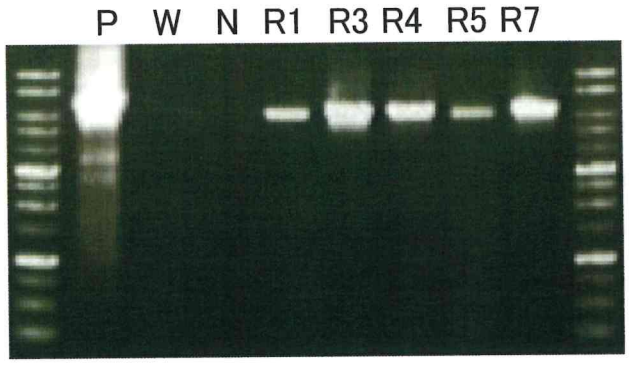
・KODFXneo使用
94℃ 2min
98℃ 10 sec ┐
68℃ 2min ┘ 35 cycles

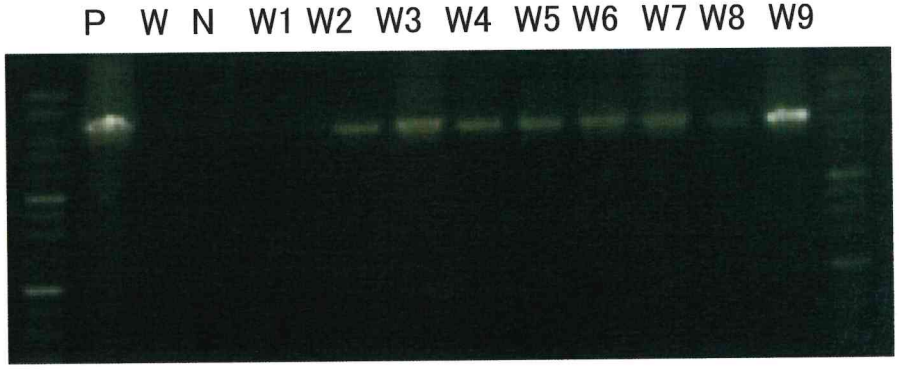
増幅領域: GAPDHプロモーター-SLR部分(3.0kb)





増幅領域: IL-8プロモーター-SLO部分(6.2kb)





W8を除く、クローンにおいてバンドが確認できた。

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試験報告書

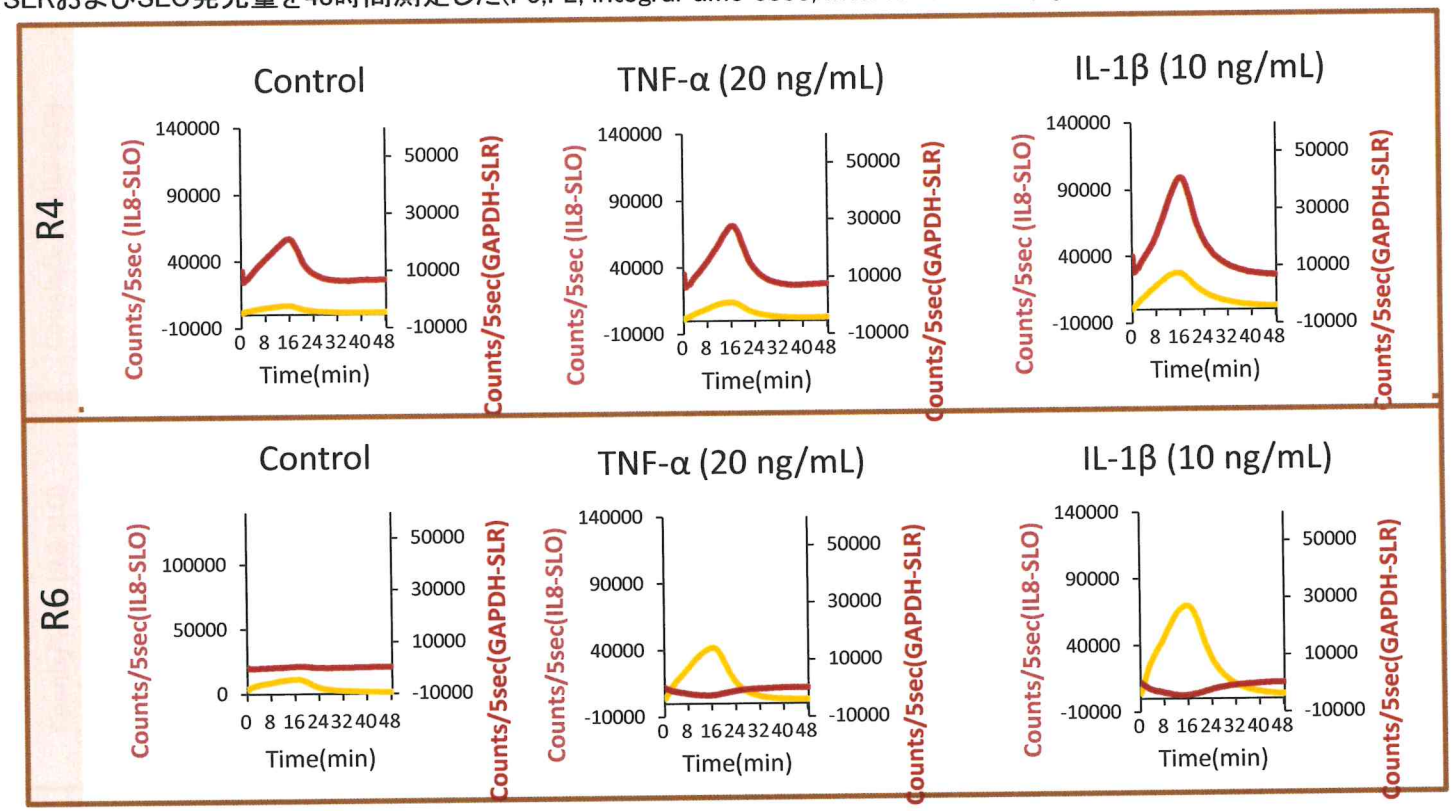
【2：GAPDHR-IL8O遺伝子搭載HACベクター保持細胞の機能評価TNFα・IL1応答性評価試験】

・PCRにて目的遺伝子の導入が確認でき、事前に実施したLucアッセイで高い蛍光値を示したHACベクター導入CHO細胞クローンについて下記条件で96well プレートに播種し、培地にTNFαおよびIL-1添加した際の発光量の変化を測定した。薬剤反応性と蛍光値および核型解析結果を考慮し、THP-1への微小核細胞誘導試験に用いるクローン（W3）を選択した。

TNF-α・IL-1β応答性評価

試験条件

・5X103cells/wellで播種し、48時間後に各薬剤（TNF-α：20 ng/mL, IL-1β：:: 100 μM10ng/mL, D-luciferin）を添加した培地に交換した。
・Kronos HTにてSLRおよびSLO発光量を48時間測定した(F0,F2, integral time 5sec, interval time min) 。



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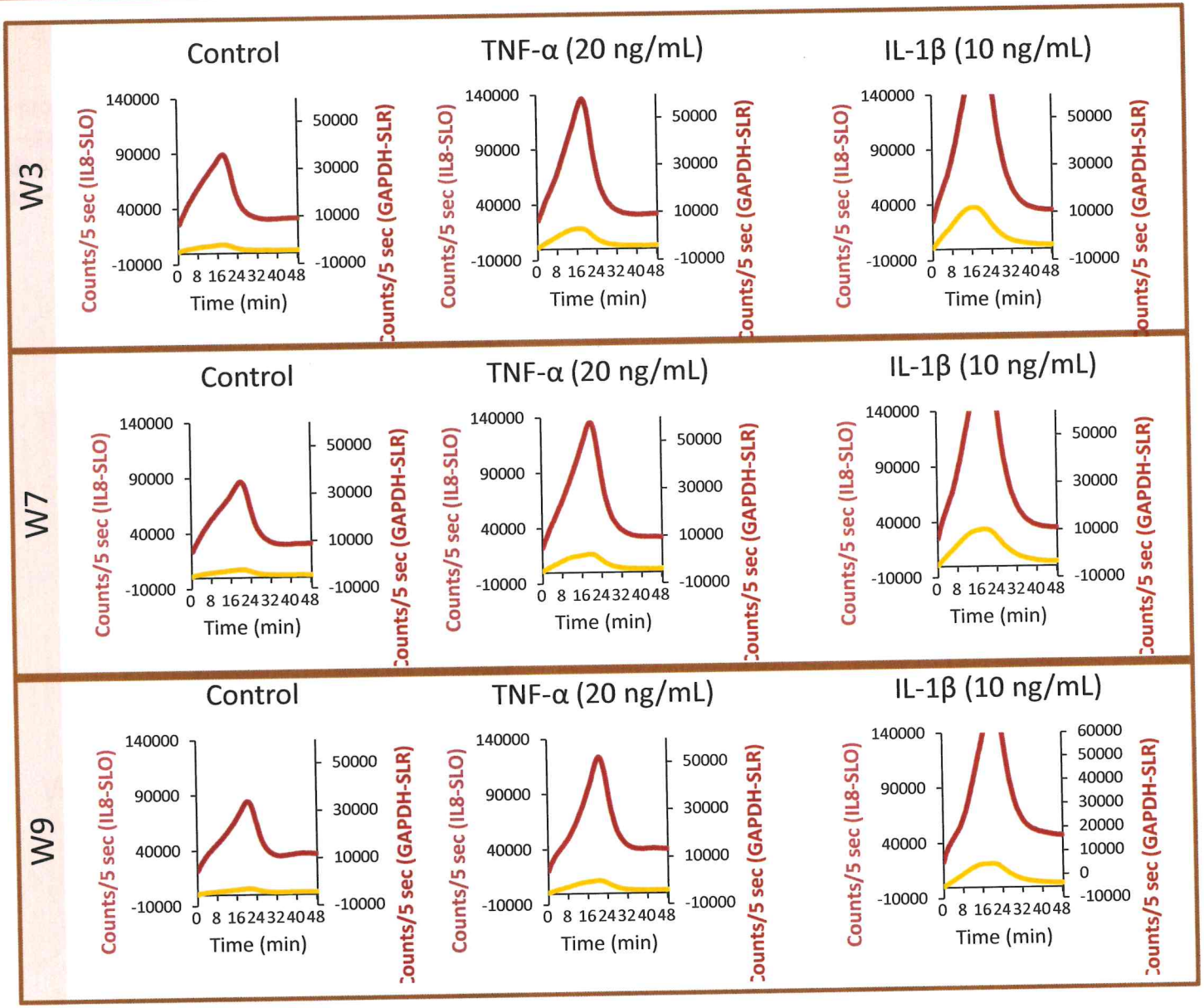
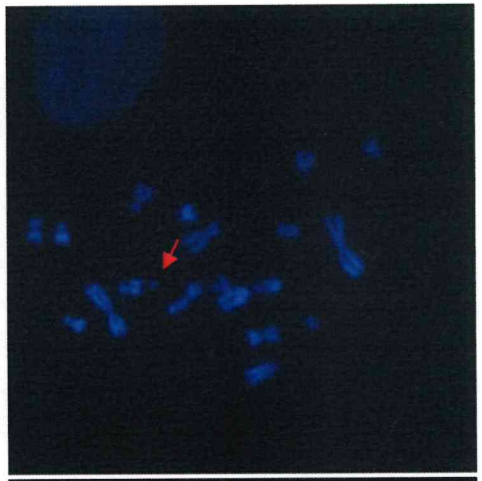


SLR・SLOの値が高W3, W7,W9を候補クローンとした。

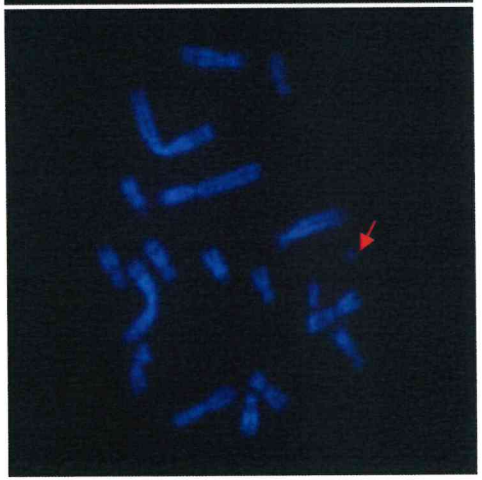
核型解析

・HACベクター保持CHO細胞 5クローン（R4, R6, W3, W7, W9)について、核型標本（DAPI染色）を作製し、宿主染色体本数及びHACベクター保持率を調べた。
・遺伝子が搭載されたHACベクターを確実にTHP細胞に移入するため、HACベクターが1個保持されているものとしてW3およびW9を候補クローンとして選択した。

W3



W9

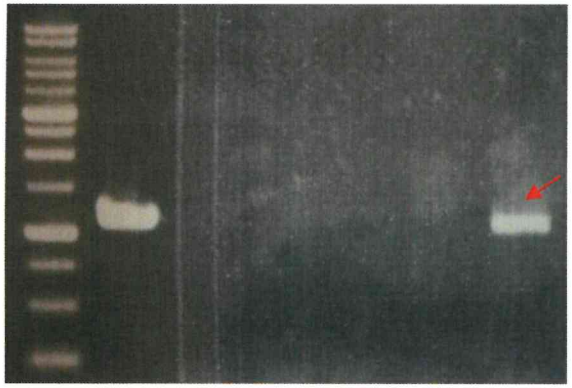


	染色体本数（本）	HACベクター数（個）	metaphase数（個）	metaphase 20個中の割合
R4	13	1	1	5%
	17	2	1	5%
	18	1	2	10%
	23	1	1	5%
	30	1	3	15%
	30	2	4	20%
	32	1	1	5%
	32	3	1	5%
	34	1	1	5%
	34	2	6	30%
R6	17	2	8	40%
	18	1	10	50%
	19	1	2	10%
W3	18	1	20	100%
W7	18	1	15	75%
	19	1	3	15%
	35	0	1	5%
	37	1	1	5%
W 9	18	1	19	95%
	4 n	1	1	5%

試験報告書

【3: GAPDHR-IL8O遺子搭伝載HACベクタ-のTHP-1細胞への移入移入】

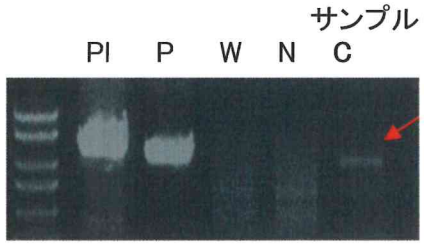
・HACベクター保持CHO細胞 W3からの微小核細胞融合試験を実施した。
・ PEGを用いた染色体移入（細胞融合）後、細胞集団をBlasticidin S (6ug/mL)にて培養。細胞増殖後、ゲノム（バルク）を回収し、PCRを実施したところ、HACベクターの存在を確認できた。



増幅領域：HACベクター--プラスミド組換え部分(318bp)

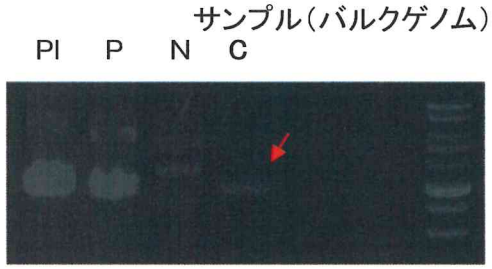
KODFXneo使用
94℃ 2min
98℃ 10 sec
68℃ 1min
35 cycles

P: ポジティブコントロール
HAC保持CHO細胞 W3
W: H_2O
N: ネガティブコントロール
THP-1細胞（WT）
Pl: 導入プラスミド
（pGAPDHSLR:IL8SLO）



増幅領域: IL-8プロモーター--SLO(6.2kb)

KODFXneo使用
94℃ 2min
98℃ 10 sec
68℃ 1min
35 cycles



増幅領域: GAPDHプロモーター--SLR(3.0kb)

総括

CHO細胞内においてHACベクターにGAPDHSLR:IL8SLO遺伝子を搭載することができた。さらにこのHACベクターを微小核細胞融合法によりTHP-1 IDAC細胞に移入させることに成功した。

今後は、クローニング作業を実施し、導入遺伝子の動作確認（TNF α・IL1応答性評価試験）、THP-1IDAC細胞内におけるHACの保持状態確認（核型解析・FISH解析）及び遺伝子保持確認（PCR解析）へと順次検証を進めていくこととなる。