厚生労働科学研究費補助金

化学物質リスク研究事業

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

令和元年度　総括・分担研究年度終了報告書

研究代表者　相場　節也

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目　　次

（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）

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（30210101）

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研究要旨

本課題においては，これまでに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，既にMITA を構成する試験法の一つである IL-2 Luc assay に関してvalidation試験を終了し，それに基づきvalidation report を作成しpeer review panelの評価を受けている。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1月に行われる海外からのliaison委員を交えたvalidation management team (VMT)会議にて予測性を除いた試験結果の評価がなされる施設内施設間再現生結果が承認された。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，in vitro免疫毒性試験法の現状とMITAの有用性に関してdetailed review paperを作成しOECDに提出する準備を始めた。

研究分担者氏名・所属研究機関名及び所属研究機関における職名
小島　肇･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･室長
中島　芳浩･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･研究グループ長
安野理恵･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･主任研究員
大森　崇･神戸大学医学部附属病院･臨床研究推進センター、生物統計学第二室長
木村　裕･東北大学病院･皮膚科･助教

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[1,2]。

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[3]。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)[4,5]。

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-β 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２）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。

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

B. 研究方法

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

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

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

（倫理面への配慮）

健常人からの採血に際しては、研究内容、採血における危険性、得られた検査結果により本人の人権が損なわれることのないこと、得られた検査結果は守秘され個人のプライバシーを侵害する可能性がないこと、研究に協力することに同意した後もいつでも自由に辞退できること、この研究によって生じる知的財産権は被験者には帰属しないことについて説明し、本人より同意書を取得している。

C. 研究結果

①IL-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[6-9]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。分類方法は添付資料１を参照。これによりIL-2 Luc assayの予測性が決定した（添付資料２）。それに基づきvalidation reportを作成し提出した（添付資料３抜粋）。

我々が提出したvalidation reportに対して，1st International peer review panel meeting on Multi-Immunotoxicity Test Assay (MITA))にて添付資料4のaction items（簡略版）が提案された。それに対して，添付資料5で対応した。さらに我々の回答に対して，2nd International peer review panel meeting on Multi-Immunotoxicity Test Assay (MITA) (Webex)で

は，添付資料6のaction itemsが提案され，それに対して添付資料7で対応した。
更に，3rd International peer review panel meeting on Multi-Immunotoxicity Test Assay (MITA) (Webex)では，添付資料8のaction itemsが提案され，それに対して添付資料8の赤字にて回答した。

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

IL-1 Luc assay Phase I試験を実施した。添付資料９に結果を示すが，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%（資料10），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.

③IL-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data setを作成した(添付資料11)

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12，添付資料13）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 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

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
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添付資料 14。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。修正後の draft を添付する（資料 15）

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

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[6-9]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にからも承認された。この評価法に基づくと，Phase I、IIをまとめたpredictivityは75%となった。以上の結果をもとにvalidation reportを提出し現在peer review panelからのコメントに対応している。

また，上記のようにIL-2 Luc assayのvalidation試験の予測性評価を通して本課題で作成した免疫毒性データベースの有用性が確認された。

IL-1 Luc assayに関しては，これまでに順調にPhase I ,Phase II試験を終了し，2020年1月に行われるVMT会議で良好な施設内，施設間再現性が評価され，現在予測性に関して検討中である。

最後に，IL-1 Luc assay，IL-2 Luc assayと免疫毒性評価法をOECDテストガイドライン化を進めるにあたり，detailed review paperを提出することにし既にOECDにSPSFを提出した。さらに，その中に含まれる項目と執筆担当者を決定した。さらに2020年1月において，draft案が提案され，それの修正を行った。修正後のdraftを添付する担当者が一同に介する会議を東京にて開催予定である。

一方，本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ベースの作成をNTPの協力を得て行った。25種類の化学物質の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、さらにそれらを添付資料11,12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。

IL-2 Luc assayのpredictivityに関しては、2019年2月27日から28日まで、東京にて開催予定のMITAのOECDガイドライン化に向けての国際評価会議にて検討する予定である。

E. 結論

本課題においては，これまで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的 validation 試験を行ってきた。2019 年度までに MITA を構成する試験法の一つである IL-2 Luc assay に関しては validation 試験を終了し，それに基づき validation report を作成し peer review panel の評価を受けている。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 1 月に行われる海外からの liaison 委員を交えた validation management team (VMT)会議にて施設内施設間再現生結果は承認された。また MITA の OECD テストガイドライン化にむけて，最終年度に提出する予定の in vitro 免疫毒性試験に関する detailed review paper の standard project submission form (SPSF)を提出した。また validation 試験において評価した化学物質，MITA の data set の中に含まれる化学物質に関して，既知の免疫毒性特性を文献的に収集し、本課題のもう一つのテーマである化学物質免疫毒性データベースの構築を進めた。

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F.研究発表

1．論文発表

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2．学会発表

1. 15th International Congress of Toxicology, Hawaii convention center, July 15, 2019. Immunotoxicological Profiling of Chemicals Using Novel In Vitro Assays. Setsuya Aiba

2. 木村裕他：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 年 11 月

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

添付資料１．化学物質免疫毒性評価基準 (Criteria to determine immunotoxicity of chemicals induced by directly targeting T cells) (IL-2 Luc assay validation report から抜粋)

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 (Table 1). Using these six findings, we defined TTCs by the 4 criteria according to the rationale for classifying immunotoxic chemicals reported by Luster et al (Luster et al., 1992) (Table 2). Namely, if chemicals satisfy one of 4 criteria, they are considered as TTCs. 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.

Table 1. The immunotoxicological data obtained from the literature

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

Table 2. The criteria to classify immunotoxic chemicals by affecting T cells.

Criteria	Definition
Criterion 1	If chemicals are demonstrated to decrease thymus weight, one finding among Finding 2 to Finding 5
Criterion 2	There are multiple reports of Finding 2 or Finding 3.
Criterion 3	There are reports of increased or decreased mRNA expression or protein production in two or more cytokines for Finding 2 or Finding 3.
Criterion 4	The presence of the NTP data including Finding 6.

添付資料 2．IL-2 Luc assay バリデーション試験最終結果（IL-2 Luc assay validation report から抜粋）

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
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
o-Benzyl-p-chorolophenol	120-32-1	P	P	P	1	No
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 carbamate	51-79-6	P	P	P	1	Yes
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

Within-laboratory reproducibilities (%)	**80 (4/5)**	**100 (5/5)**	**80 (4/5)**		
	Average 86.7 (13/15)				
Between-laboratory reproducibilities (%) (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)				

添付資料 3．IL-2 Luc assay バリデーションレポート （目次のみ抜粋）

Report on a Validation Study of the IL-2 Luc Assay for Evaluating the Potential Immunotoxic Effects of Chemicals on T-Cells

Validation Management Team

添付資料４．IL-2 Luc assay validation report に対する Peer review panel からのコメント (1)

201902

Action Items to peer reviewers for the validation report on the IL-2 Luc assay

Evaluation Criterion 1: A rationale for the test method should be available, including a description of the human health effect, a clear statement of scientific need, and regulatory application.

PRP Comment: Together with a new title, the rationale needs to be stated clearly to be T-cell targeting.

Evaluation Criterion 2: The toxicological mechanisms and the relationship between the test method endpoint(s) with the biological effect as well as the toxicity of interest should be addressed, describing limitations of the test method.

PRP Comment: Needs to focus on IL-2, including the limitations described in the meeting minutes. The introduction needs to focus solely on IL-2 and the IL-2 Luc Assay. Discussion about its part in MITA should be left until the discussion section.

Evaluation Criterion 3: A detailed test method protocol should be available

PRP Comment: The commercial availability of the #2H4 cell line needs to be described.

Evaluation Criterion 4: The within and between laboratory reproducibility of the test method should be demonstrated

PRP Comment: Acceptable

Evaluation Criterion 5: Demonstration of the test method's performance should be based on testing of representative, preferably coded reference chemicals

PRP Comment: We think only four or five negatives is not enough, so we suggest that some additional testing of negatives be performed.

Evaluation Criterion 6: Predictive capacity should be demonstrated using representative chemicals.

PRP Comment: Predictive capacity needs to be reassessed based on today's proposed definition of T-cell–targeting chemicals.

Evaluation Criterion 7: All data should adequately support the assessment of the validity of the test method for peer review.

PRP Comment: A clear definition of the 35% threshold and a clear explanation of Criteria 5 and how it was developed is needed. Should the table in Appendix 8 include the test judgment? Also, delete DTH, tumor, infection, and NK activity but specify T-cell proliferation in the table in Appendix 8.

Evaluation Criterion 8: All data from the validation study supporting the validity of a test method should be obtained in accordance with the principles of Good Laboratory Practice (GLP)

PRP Comment: The report needs to explain clearly and in detail what is meant by the phrase "in the spirit of GLP" and whether or not each laboratory performed their work in this spirit.

Evaluation Criterion 9: Applicability domain of the test method should be defined

PRP Comment: We recommend that the applicability domain be more clearly defined as noted in the PRP meeting minutes.

Evaluation Criterion 10: Proficiency chemicals should be set up in the proposed protocol

PRP Comment: None

Evaluation Criterion 11: Performance standards should be set up with the proposed protocol

PRP Comment: If performance standards are understood to be assay controls, then the use of three-fold stimulation of IL-2 Luc by PMA/IO and inhibition of stimulated IL-2 Luc by DEX and CYA are sufficient. We suggest that acceptance criteria for variability within test replicates be defined.

Evaluation Criterion 12: Advantages in terms of time, cost and animal welfare

PRP Comment: We suggest that the conclusion leave out mention of in vivo testing to confirm T-cell immunotoxicity and include discussion of the use of IL-2 Luc assay within MITA.

Evaluation Criterion 13: Completeness of all data and documents supporting the assessment of the validity of the test method.

PRP Comment: We suggest that data be redone to reassess predictive capacity based on today's proposed definition of T-cell–targeting chemicals. Also, a critical assessment of the 35% threshold in the context of the new definition of T-cell targeting is necessary.

Evaluation Criterion 14: Validation Study Management and Conduct

PRP Comment: None

Other considerations

PRP Comment: None

Conclusion

PRP Comment: We look forward to seeing a revised report based on our comments.

添付資料５． Peer review panel からのコメント (1)に対する対応

Dear the PRP:

Thank you for your kind and constructive comments and suggestions. We responded to each comment below and revised the VR taking the PRP comments into consideration. We used red fonts in the revised or newly added parts.

Evaluation Criterion 1: A rationale for the test method should be available, including a description of the human health effect, a clear statement of scientific need, and regulatory application.

PRP Comment: *Together with a new title, the rationale needs to be stated clearly to be T-cell targeting.*

The title was revised and changed to “Report on a Validation Study of the IL-2 Luc Assay for Evaluating the Potential Effect of Chemicals on T-Cells”.

The rationale to judge chemicals whether they were T-cell targeting or not was described in 10-3-1.

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

A well-functioning immune system is essential in maintaining the integrity of the organism. Therefore, immune dysregulation caused by chemicals, i.e., immunotoxic effects of chemicals, may make serious impacts on human health. It ranges from reduced resistance to infection and neoplasia to allergic and autoimmune conditions. 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. Chemicals can potentially affect the immune system by targeting either the innate immune system or the acquired immune system (Fig. 2 and Fig. 3). Therefore, *in vitro* test methods to detect immunotoxic effects of chemicals are needed to adequately assess their effects on both arms of immune system. However, it is impossible to predict the toxic effects of chemicals on the whole aspects of immune system by a single in vitro assay. Consequently, to accomplish the final goal of in vitro immunotoxicity tests that cover the whole aspects of immune system, it is indispensable to develop an integrated approach composed of multiple in vitro immunotoxic tests evaluating different aspects of immune responses. The MITA including the IL-2 Luc assay was developed to be components of the integrated approach.

Among various immune responses, one of pivotal responses is the development of antigen-specific effector T-helper subtypes, such as, Th1 cells, Th2 cells, Th17 cells, and regulatory T cells (Treg cells) that are associated with the clinical features and disease progression (reviewed by [1]). Therefore, the in vitro assay to clarify the effects

of chemicals on the development of these T-helper subtypes is one of the critical components of the integrated approach.

Now it is known that IL-2 exerts pleiotropic actions on CD4+ T cell differentiation via its modulation of cytokine receptor expression. It 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 [2]). Therefore, it is conceivable that chemicals, which affect IL-2 release by T cells, give significant impact on the development of Th cells.

When immunotoxic information of chemical is collected from the literature, however, most of the published data are not focusing on the effects of chemicals on the development of Th subsets. 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 collected the following 6 components in the literature.

#1. The decreased thymus weight

#2. The increased or decreased IL-2, IFN-g, or IL-4 mRNA expression or production by T cells in ex vivo.

#3. The increased or decreased IL-2, IFN-g, or IL-4 mRNA expression or production by T cells in vitro.

#4. The suppression of T cell proliferation

#5. The suppression of cytotoxic T cell response

#6. There is a clear statement in the NTP data that one of the immunotoxic mechanism of chemicals are attributed to its effect on T cells.

Then, we determined TTCs as chemicals that satisfied one of the following criteria

1) The combination of more than two components among #1 to #5 components
2) Multiple reports on #2 or #3
3) #2 or #3 on two or more cytokines
4) #5

Evaluation Criterion 2: The toxicological mechanisms and the relationship between the test method endpoint(s) with the biological effect as well as the toxicity of interest should be addressed, describing limitations of the test method.

PRP Comment: *Needs to focus on IL-2, including the limitations described in the meeting minutes. The introduction needs to focus solely on IL-2 and the IL-2 Luc Assay. Discussion about its part in MITA should be left until the discussion section.*

The limitation of this assay was described in the applicability domain (10-6).

10-6. Limitations and drawback, and applicability domain of the IL-2 Luc assay

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 [3]. 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 the conventional 28-day subacute toxicity test [4] or *in vitro* myelotoxicity tests [5]. Similar to other *in vitro* test methods, poor water soluble chemicals are not suitable for this assay.

The introduction was revised according to the PRP comment. The detailed discussion on the MITA was moved to the Discussion.

Evaluation Criterion 3: A detailed test method protocol should be available

PRP Comment: The commercial availability of the #2H4 cell line needs to be described.

2H4 cells will be obtained from the GPC laboratory, Tottori, Japan after this assay is accepted as the test guideline.

Evaluation Criterion 4: The within and between laboratory reproducibility of the test method should be demonstrated

PRP Comment:*Acceptable*

Evaluation Criterion 5: Demonstration of the test method's performance should be based on testing of representative, preferably coded reference chemicals

PRP Comment: *We think only four or five negatives is not enough, so we suggest that some additional testing of negatives be performed.*

We reconsidered the immunotoxic characteristics of chemicals evaluated in Phase I and II studies. Finally, these two studies contained 7 negative chemicals (Appendix 8).

Evaluation Criterion 6: Predictive capacity should be demonstrated using representative chemicals.

PRP Comment: *Predictive capacity needs to be reassessed based on today's proposed definition of T-cell–targeting chemicals.*

We admit that it is crucial to more clearly define the criteria to classify chemicals into T cell-targeting chemical (TTC) and non-T cell-targeting chemical (NTTC). So, we proposed the new criteria with the international expert members, Dr. Emanuela Corsini and Dr. Dori Germolec taking PRP's proposal into consideration. The following was the revised session of predictivity (Revised VR 9-1-3).

9-1-3. Predictivity

To determine the predictivity of the IL-2 Luc assay, it is crucial to understand the immunotoxic characteristics of 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 tried to classify chemicals into those that affect T cell function, i.e., T cell-targeting chemical (TTC) and those that do not directly affect T cell function, i.e., non-T cell-targeting chemicals (NTTC). In this assay, to define TTCs, we collected the following 6 components in the literature.

#1. The decreased thymus weight

#2. The increased or decreased IL-2, IFN-g, or IL-4 mRNA expression or production by T cells in ex vivo.

#3. The increased or decreased IL-2, IFN-g, or IL-4 mRNA expression or production by T cells in vitro.

#4. The suppression of T cell proliferation

#5. The suppression of cytotoxic T cell response

#6. There is a clear statement in the NTP data that one of the immunotoxic mechanism of chemicals are attributed to its effect on T cells.

Then, we defined TTCs as chemicals that satisfy one of the following criteria

1) The combination of more than two components among #1 to #5 components
2) Multiple reports on #2 or #3
3) #2 or #3 on two or more cytokines
4) #5

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 and their summarized data are shown in Appendix 8. The table in Appendix 8 is the combined data of the NTP data and the data collected by the VMT member. 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 augmentation or suppression, they were both considered as positive (P) and if not, they were judged as negative (N). Then we examined concordance between positive judgment and TTC.

Based on the new criterial for chemical classification, the predictivity of the Phase I and Phase II studies was summarized in 10-3-2.

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

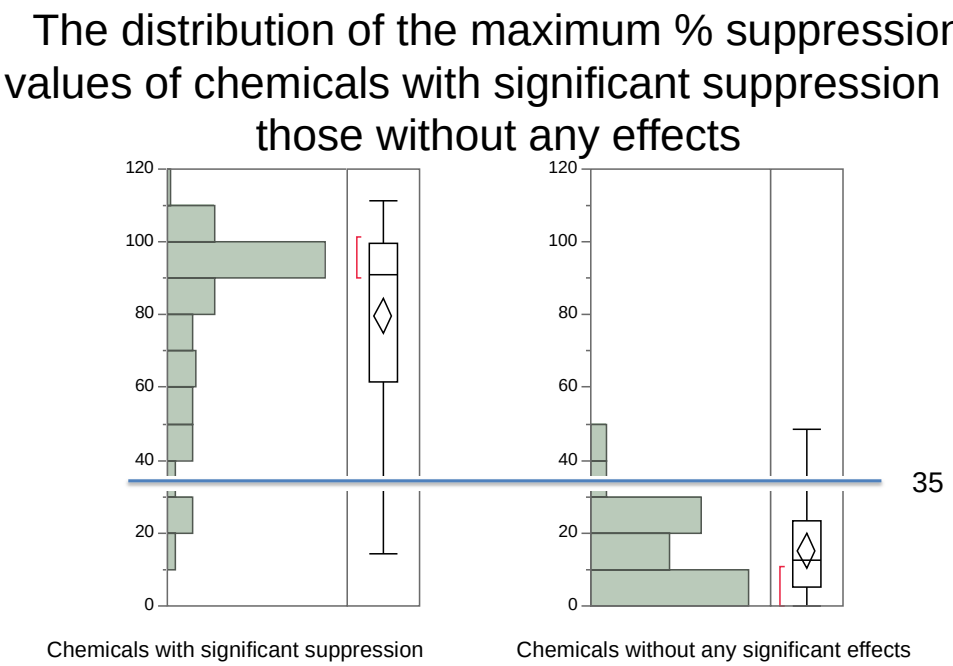
To classify 25 chemicals used in the Phase I and II studies, we used the chemical information kindly provided by the National Toxicology Program (NTP) and those collected by the VMT members. The immunotoxic characteristics of each chemical are shown in Appendix 7 and their summarized data are shown in Appendix 8. Based on the criteria, the 25 chemicals were classified into 14 TTCs, 9 NTTCs, and 2 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 80.0%, 80.0%, 73.3% and 77.7%, 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 78.2%, 78.2%, 73.9%, and 76.8%, respectively.

Evaluation Criterion 7: All data should adequately support the assessment of the validity of the test method for peer review.

PRP Comment: *A clear definition of the 35% threshold and a clear explanation of Criteria 5 and how it was developed is needed. Should the table in Appendix 8*

include the test judgment? Also, delete DTH, tumor, infection, and NK activity but specify T-cell proliferation in the table in Appendix 8.

To determine the optimum threshold, we first potted the maximum % suppression values of chemicals with statistically significant suppression or those without any effects. The comparison of these two graphs showed that the threshold 35 can divide chemicals with significant suppression and those without any effects with minimum false positive or negative results.



The values of the maximum % suppression were derived from the data set made by the lead laboratory in our recent publication in Arch Toxicol (see the attached file)

We revised Appendix 8. As suggested, we deleted test judgment, DTH, infection, tumor rejection, and NK activity, and specified T cell proliferation.

Evaluation Criterion 8: All data from the validation study supporting the validity of a test method should be obtained in accordance with the principles of Good Laboratory Practice (GLP)

PRP Comment: The report needs to explain clearly and in detail what is meant by the phrase "in the spirit of GLP" and whether or not each laboratory performed their work in this spirit.

Evaluation Criterion 9: Applicability domain of the test method should be defined

PRP Comment: We recommend that the applicability domain be more clearly defined as noted in the PRP meeting minutes.

We described the applicability domain more precisely, taking the PRP comments into consideration in 10-6.

10-6. Limitations and drawback, and applicability domain of the IL-2 Luc assay

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 [3]. 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 the conventional 28-day subacute toxicity test [4] or *in vitro* myelotoxicity tests [5]. Similar to other *in vitro* test methods, poor water soluble chemicals are not suitable for this assay.

Evaluation Criterion 10: Proficiency chemicals should be set up in the proposed protocol

PRP Comment:*None*

Evaluation Criterion 11: Performance standards should be set up with the proposed protocol

PRP Comment: *If performance standards are understood to be assay controls, then the use of three-fold stimulation of IL-2 Luc by PMA/IO and inhibition of stimulated IL-2 Luc by DEX and CYA are sufficient. We suggest that acceptance criteria for variability within test replicates be defined.*

Based on the PRP comments, we added the performance standard in the revised VR, Appendix 15.

Evaluation Criterion 12: Advantages in terms of time, cost and animal welfare

PRP Comment: *We suggest that the conclusion leave out mention of in vivo testing to confirm T-cell immunotoxicity and include discussion of the use of IL-2 Luc assay within MITA.*

In the revised VR, we deleted the description of requirement of in vivo testing. In addition, we described the potential of the IL-2 Luc assay (10-7).

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 [6, 7]. The downstream signaling after the stimulation by TCR/CD28 is shown in Fig. 17. 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-kB, 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-kB and AP1/2 pathways [8]. 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-kB pathway (reviewed by Zhang and Sun [9]. 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.

Evaluation Criterion 13: Completeness of all data and documents supporting the assessment of the validity of the test method.

PRP Comment: *We suggest that data be redone to reassess predictive capacity based on today's proposed definition of T-cell–targeting chemicals. Also, a critical assessment of the 35% threshold in the context of the new definition of T-cell targeting is necessary.*

In the revised validation report, we clearly defined the T cell-targeting chemicals. Based on the definition, we classified chemicals into T cell-targeting chemicals (TTCs) or non-T cell targeting chemicals (NTTCs). According to this classification, we recalculated the sensitivity, specificity, and accuracy of the Phase I and II studies.

Evaluation Criterion 14: Validation Study Management and Conduct

PRP Comment: None

Other considerations

PRP Comment: None

Conclusion

PRP Comment: We look forward to seeing a revised report based on our comments.

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添付資料 6．Peer review panel との teleconferance の議事録

Teleconference for IL-2 PRP

October 1, 2019

Peer Review Panel: Henk van Loveren, Haley Neff-LaFord, Barbara Kaplan, Fujio Kayama, Takao Ashikaga
VMT: Hajime Kojima
Observers: Steve Venti (meeting minutes)

Kojima:	In this meeting, we will discuss the revised validation report and the schedule going forward. I will explain the changes in the report, which are shown in red. One important point is Appendix 7. It has 290 pages and discusses the data available on immunotoxic effects of chemicals. Mainly, the figures for predictivity and the summary were revised. I heard Dr. Aiba is on-going to revise minorly. After the meeting, I ill share the newest Validation report.
Kaplan:	This summary is in line with what we discussed at the FTF meeting.
Kojima:	Does everyone accept this summary?
Everyone:	Yes.
Kojima:	Section 9-1-3 addresses predictivity and describes the effects of chemicals on T-cells. And there is a definition of T-cell targeting chemicals (TTCs).
Kaplan:	Criterion 3 says "#2 or #3 on two or more cytokines." Does that refer only to the three cytokines mentioned in #2 and #3? For example, is IL-17 excluded? This is not clear. If there is a report for other cytokines, would they be considered TTCs?
Kojima:	I can't answer at the moment, but I will ask Dr. Aiba.
Kaplan:	This is an improvement over the original report. Once we have some clarification on Criterion 3, I think that these criteria are acceptable.
van Loveren:	Although I think it would be good to extend this to other cytokines, not just the ones listed.
Kojima:	(Brief review of other changes in red. Please see revised Validation Study Report.) If you are happy with this report, then we can move on to reviewing the PRP Evaluation Criteria and creating the PRP report.
Kaplan:	Do we need to read this and provide comments? What do you need from the PRP to submit to the OECD?
Kojima:	If you feel that the Validation Study Report satisfies the 14 PRP Evaluation Criteria, then you can prepare a Peer Review Report of about 12 pages with a comment about each criterion. And then the Validation Study Report and the Peer Review Report will be reviewed by an OECD expert working group.
van Loveren:	Are there specific places we should comment on?
Kojima:	We revised the Validation Report based on the comments from the PRP.
Kaplan:	So we have already covered the critical issues. But if there is anything specific you want us to look at, please tell us now.
van Loveren:	Is there any issue we need to address now?
Kojima:	I will share these documents with you, and after we have your comments, Dr. Kayama will write the final PRP report.
Neff-LaFord:	Once you see the documents, it is pretty easy to follow what has been changed, so we should be able to follow it.
Kojima:	The deadline for comments if possible, would be by the end of October and then we can have another teleconference in early or mid-November. OK, I will send you meeting minutes, the newest validation Study Report, and the evaluation

添付資料７． Teleconferance のコメントに対する対応

October 4th, 2019

The response to the reviewers' comments:

Thank you for your kind consideration and important suggestions to the validation report. We revised the validation report according to the reviewers' comments. In addition, we corrected the values of the predictivity of this assay because there was one calculation error and we changed the classification of chemicals based on several references we found. The modified part was as follows. All the modified parts were written in red.

1. We modified the criteria to classify immunotoxic chemicals according to the reviewers' comments. (9-1-3. Predictivity in Page 61 and 10-3-1. Rationale to determine..... in Page 82)
2. We recalculated the predictivity. Consequently, the predictivity of the Phase II study, the combined Phase I and Phase II studies, and the data set was slightly changed. Briefly, the average predictivity of the Phase II was changed from 74.0% (40/54) to 70.2% (40/57). The average predictivity of the combined Phase I and Phase II studies was changed from 76.8% (53/69) to 75.0% (54/72). The predictivity of 60 chemicals was not changed. These changes were precisely described in Abstract, 9-4-3. Predictivity in the Phase II study, Table 22, 9-6-3. Predictivity in the Phases I and II studies, Table 23, 10-3-2. The predictivity of the Phase I and Phase II studies, 10-4. IL-2 Luc assay data set for 60 chemicals, and Table 24.
3. While revising the VR, we found a very crucial report by Luster et al, 1992. In their manuscript (Luster et al., 1992b), they proposed the rationale for immunotoxic classification. Namely, their proposal was that a positive was established on the basis that the test material either produced significant dose-response effect in the immune tests or significantly altered two or more test results at the highest dose of chemical tested. Furthermore, they classified chemicals based on their results of immune tests according to this rationale and found that there was a significant correlation between the judgment of immunotoxic chemicals and the host resistance (Luster et al., 1993). Therefore, we referred to their paper in 9-1-3. Predictivity and 10-3-1. Rationale to determine…..

4. We also added the comparison between the predictivity of the IL-2 Luc assay and that reported by Luster et al (Luster et al., 1992a; Luster et al., 1993; Luster et al., 1992b)and between the predictivity of the IL-2 Luc assay and that of the human whole blood cytokine release assay by Langezaal et al(Langezaal et al., 2002) in 10.4. IL-2 Luc assay data set for 60 chemicals.

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添付資料 8.

Teleconference for IL-2 PRP

November 11, 2019

Peer Review Panel: Henk van Loveren, Barbara Kaplan, Haley Neff-LaFord, Fujio Kayama, Takao Ashikaga, Lin Shi, Xingchao Geng
VMT: Hajime Kojima, Setsuya Aiba, Takuya Kimura
Observers: Steve Venti (meeting minutes)

Kojima:	In this meeting, we will discuss the revised validation report prior to discus items. We revised the report based on your comments. After the previous we received it in accordance with the comments from Barbara, and you hav comments that have not been reflected yet, so I think we need to discuss th
Kaplan:	I think these revisions are fine as long as things are separated into a table o intelligible.
Aiba:	I don't know who made this table, but it presents what I wanted to say, so I this if the PRP agrees.
Kojima:	Dr. Aiba will calculate predictive capacity based on this table, so the most i that the PRP finds this table acceptable.
Kayama:	I think these criteria are easier to understand as presented in the table.
van Loveren:	I am still concerned that the introduction is confusing to a naïve reader. We understand that MITA is the context, *not* the aim, of this study. But the intr clear statement at the start of the introduction that the aim of this validatio not MITA in general. Mentioning MITA in the introduction is fine, but you at the start of the introduction. The introduction must begin with the aim of IL-2. According to the reviewer's suggestion, I changed the abstract and began it this study.
Kaplan:	The first time I read this introduction, I thought that you were validating th later I realized that is not the case. The goal is to validate the IL-2 assay. I a Haley that the goal of the validation needs to be stated clearly at the start o Even just one sentence is enough. Just clearly state that the goal is to valida As described in the response to Dr. van Loveren's comment, I changed the it with the purpose of this study.
Neff-LaFord:	Yes, just more section 3 up higher. As suggested by the reviewer, we moved the objective of the study to secti
van Loveren:	We need to say "proposed AOP" because this AOP has not yet been accept As suggested, we added "proposed " in 3-9. The proposed Adverse Outcom of chemicals that affect IL-2 transcription.
Neff-LaFord:	The expression "IL-2 LA" appears to mean the same thing as "IL-2 Luc As intended to mean something different, then this needs to be spelled out mo According to the reviewer's comment, we modified Table 3. Definition of t

Aiba:	Yes, I will clarify that.
van Loveren:	On page seven in introduction, I have suggested a revision, but perhaps the the applicability range that I deleted needs to be added back.
Kaplan:	I think that in context, the meaning of “applicability domain” is clear enou the word “however” should be removed for clarity. As suggested by several reviewers, we deleted “however”.
van Loveren:	The applicability domain is discussed in the preceding paragraph, so mayb Haley’s suggestion as is.
Kojima:	In section 9-5, I will inform you the detailed records collected in the princi
Neff-LaFord:	I don’t understand what “almost comparable” means in section 10-3-1. We changed “comparable” to “similar to”, which is now in section 10-7.
Kaplan:	Given the emphasis on comparing IL-2 results with the results of other test section needs to be expressed more clearly. I think this information is impo it should be described more clearly. In the revised VL, we tried to describe more clearly the following sections,
Ashikaga:	I couldn’t find any description about regulatory application in the report. We added a new section describing the regulatory application (10-9)
Aiba:	Do I need to respond to each of these comments one by one?
Ashikaga:	Why is SFO-luciferase activity measured in this assay? We made a comment for the reason to ignore SLO-luciferase activity or IF
Aiba:	It is automatically measured but it is not necessary for this assay.
Kaplan:	This is related to what we were talking about before. This report contains a that is only incidentally related to IL-2, which confuses the reader.
Ashikaga:	I could not find a list of proficiency chemicals. Shouldn’t the developer sub
Aiba:	Yes. Appendix 14 and 15 have a list of proficiency chemicals.
Kojima:	Are there any other comments?
Xingchao:	I agree with the comments and I think the report is improved.
Lin:	(inaudible)
Aiba:	(inaudible)

van Loveren:	The applicability domain does not seem to be defined anywhere. Where is t applicability domain? All the information is there, but there is no single cle could rename 10-6 and start with a simple explanation of the applicability d According to the reviewers' suggestion, we changed the name of 10-6 to th domain and the limitation of the IL-2 Luc assay and added a simple explan applicability domain.
Kaplan:	This is a good point. We have defined a T-cell target, so we need to say that applicability domain. We have answered to Dr. van Loveren's comment.
Aiba:	OK, I will provide a clear definition of what the applicability domain is.
Kojima:	I will share the minutes of this meeting, and then Dr. Aiba and the VMT wi validation report to share with the PRP. Perhaps you can then submit your d Kayama within one month and to be created the PRP report by Dr. Kayama
Kayama:	The most important comment today is Henk's last comment.
Aiba:	I'd like to ask Dr. Kayama to summarize the PRP comments, because I alre original comments. I would like to know what I should respond to.
Kayama:	Will the PRP report be incorporated into the validation report or separately
Kojima:	Separately attached.

添付資料 9. IL-1 Luc assay Phase I validation 試験結果

The results of the Phase I study

Line25 judge		25									
LabA Tohoku				LabB AIST tsukuba				LabC AIST shikoku			
setNo.	code No.			setNo.	code No.			setNo.	code No.		
Set1	MITA103	MITA103	S	Set1	MITB402	MITB402	S	Set1	MITC704	MITC704	S
Set2	MITA203	MITA203	S	Set2	MITB501	MITB501	S	Set2	MITC803	MITC803	S
Set3	MITA304	MITA304	S	Set3	MITB605	MITB605	S	Set3	MITC902	MITC902	S
Set1	MITA101	MITA101	N	Set1	MITB404	MITB404	N	Set1	MITC701	MITC701	N
Set2	MITA205	MITA205	N	Set2	MITB505	MITB505	N	Set2	MITC802	MITC802	N
Set3	MITA305	MITA305	N	Set3	MITB603	MITB603	N	Set3	MITC905	MITC905	N
Set1	MITA104	MITA104	N	Set1	MITB403	MITB403	N	Set1	MITC705	MITC705	N
Set2	MITA202	MITA202	N	Set2	MITB502	MITB502	N	Set2	MITC805	MITC805	N
Set3	MITA303	MITA303	N	Set3	MITB601	MITB601	N	Set3	MITC901	MITC901	N
Set1	MITA105	MITA105	S	Set1	MITB401	MITB401	S	Set1	MITC702	MITC702	S
Set2	MITA204	MITA204	S	Set2	MITB503	MITB503	S	Set2	MITC801	MITC801	S
Set3	MITA301	MITA301	S	Set3	MITB602	MITB602	S	Set3	MITC904	MITC904	S
Set1	MITA102	MITA102	N	Set1	MITB405	MITB405	N	Set1	MITC703	MITC703	N
Set2	MITA201	MITA201	N	Set2	MITB504	MITB504	N	Set2	MITC804	MITC804	N
Set3	MITA302	MITA302	N	Set3	MITB604	MITB604	N	Set3	MITC903	MITC903	N

Within laboratory reproducibility: Lab A: 100% (5/5), Lab B: 100% (5/5), Lab C 100% (5/5)
Between laboratory reproducibility: 100% (5/5)

添付資料 10.　IL-1 Luc assay Phase II validation 試験結果

	LabA Tohoku		LabB Tsukuba		LabC AIST Shikoku		Between-labolatory concodance or disconcodance
Chem No.	Code No.	Judge	Code No.	Judge	Code No.	Judge	
2	MTA117	S	MIB221	S	MTC305	S	concodance
3	MTA105	N	MIB220	N	MTC301	N	concodance
4	MTA120	N	MIB203	N	MTC318	S	disconcodance
5	MTA115	N	MIB211	N	MTC307	S	disconcodance
6	MTA111	N	MIB224	N	MTC302	N	concodance
7	MTA112	N	MIB208	N	MTC312	N	concodance
8	MTA125	S	MIB214	S	MTC303	S	concodance
11	MTA110	N	MIB218	N	MTC322	N	concodance
12	MTA124	S	MIB217	S	MTC313	S	concodance
13	MTA102	N	MIB206	N	MTC317	N	concodance
14	MTA121	N	MIB205	N	MTC324	N	concodance
15	MTA116	N	MIB223	N	MTC309	N	concodance
16	MTA118	N	MIB202	S	MTC316	N	disconcodance
17	MTA108	S	MIB204	S	MTC315	S	concodance
20	MTA113	S	MIB219	S	MTC323	S	concodance
22	MTA107	S	MIB222	S	MTC314	S	concodance
23	MTA119	N	MIB201	N	MTC306	S	disconcodance
25	MTA104	N	MIB210	N	MTC311	N	concodance
26	MTA114	S	MIB216	S	MTC304	S	concodance
27	MTA127	N	MIB227	N	MTC327	N	concodance
	Between-labolatory concodance rate						80%（16/20）

添付資料 11. IL-1 Luc assay, IL-2 Luc assay, IL-8 Luc assay data set

Chemicals	IL-2		IL-1β		IL-8 Luc
	Judge	LOEL (ug/mL)	Judge	LOEL (ug/mL)	Judge
FK506	S	0.00	N		N
Cyclosporine A	S	0.00	N		N
Actinomycin D	S	0.02	S	0.13	P
Digoxin	S	0.07	S	0.59	P
Colchicine	S	0.27	N		P
FR167653	S	1.30	S	0.49	N
Benzethonium chloride	S	1.63	N		P
Mercuric chloride	S	1.95	S	1.95	P
Chlorpromazine	S	1.95	S	3.91	P
Dibutyl phthalate	S	2.60	S	15.63	N
Amphoterycin B	S	2.60	S	1.17	P
2-Aminoanthracene	S	5.86	S	11.72	P
Isophorone diisocyanate	S	7.81	S	3.91	P
Formaldehyde	S	7.81	N		P
Pyrimethamine	S	7.81	N		P
Cobalt chloride	S	16.93	S	46.88	P
Cisplatin	S	16.93	S	46.88	P
Chloroquine	S	17.83	S	39.06	P
Minocycline	S	18.52	S	62.50	P
Mitomycin C	S	20.00	N		P
Hydrogen peroxide	S	23.44	S	375.00	P
Citral	S	25.00	S	4.88	P
Dexamethasone	S	41.67	S	0.98	N
Pentamidine isethionate	S	52.08	S	64.45	P
Lead(II) acetate	S	57.29	N		N
Azathioprine	S	58.48	S	41.55	N
Diesel exhaust particles	S	62.50	S	39.06	P
Sodium dodecyl sulfate	S	62.50	S	62.50	P
Dapsone	S	72.92	S	125.00	N
p-Nitroaniline	S	83.33	S	125.00	N
Nitrofurazone	S	83.33	N		P
Sulfasalazine	S	92.94	S	44.81	N
Nickel sulfate	S	104.17	S	375.00	P
Aluminum chloride	S	104.17	N		N
Chloroplatinicacid	S	250.00	S	23.44	P
Diethanolamin	S	250.00	S	333.33	P
Sodium bromate	S	500.00	S	500.00	P
Histamine	S	750.00	N		P
Isoniazid	S	1000.00	N		N
Triethanolamine	S	1333.33	S	1000.00	P
Magnesium sulfate	S	2000.00	N		N
Warfarin	N		N		N
Hydrocortisone	N		N		N
Lithium carbonate	N		N		P
2,4-Diaminotoluene	N		N		N
Dibenzopyrene	N		N		N
Cyclophosphamide	N		N		P
Ethanol	N		N		N
Methanol	N		N		N
Hexachlorobenzene	N		N		N
Trichloroethylene	N		N		N
Methotrexate	N		N		P
Rapamycin	N		N		N
Mizoribine	N		N		N
Mycophenolicacid	A	0.40	S	72.00	P
2-Mercaptobenzothiazole	A	16.11	S	93.75	P
Ribavirin	A	26.04	S	750.00	N
Acetaminophen	A	100.00	N		N
Nicotinamide	A	288.07	N		N
Dimethyl sulfoxide	A	2000.00	N		N

AFC antibody forming cell, *CSM* cell surface marer, *NK* natural killer cell activity, *LOEL* lowest observed effect level

添付資料 12．IL-2 Luc assay Phase I，Phase II 化学物質の免疫毒性データーベース

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 IL-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

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。

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添付資料 13. IL-2 data set 化学物質の免疫毒性データベース

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
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

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-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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#: 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.

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				

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-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						

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	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

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-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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#: 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
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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#: 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**
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		

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#: 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**	-	-	

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#: 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

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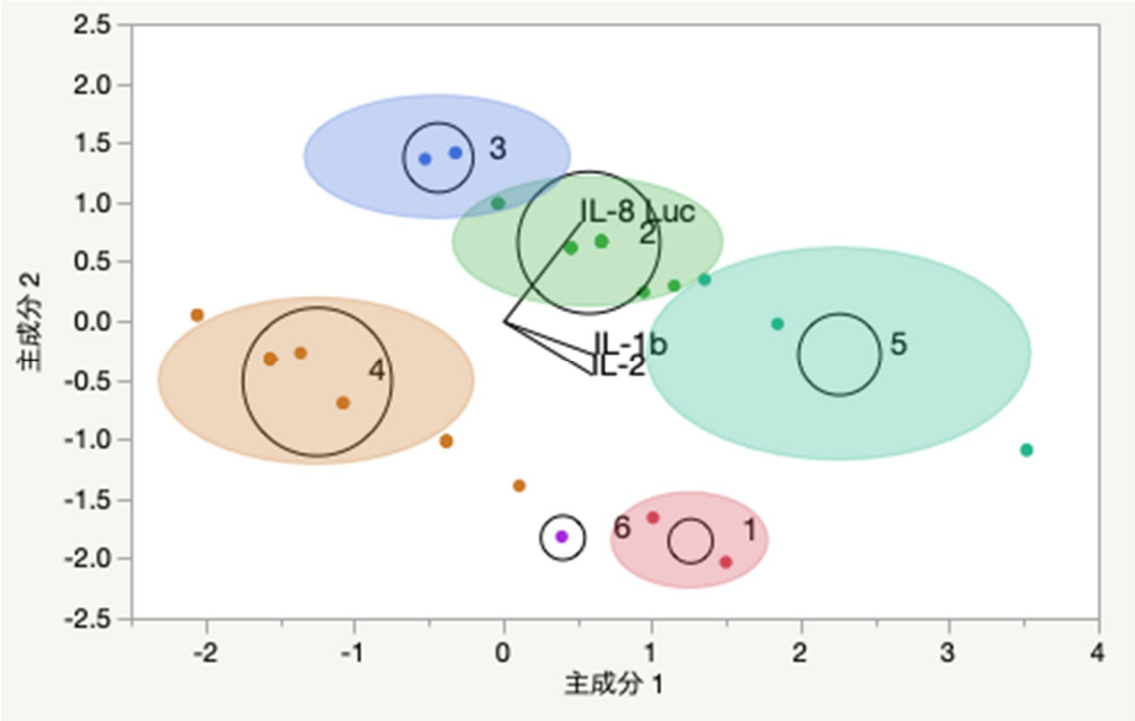
添付資料 14．MITA による化学物質の免疫毒性プロフィル



添付資料 15. Detailed review paper content.

Potential title:
"*In vitro* immunotoxicity testing"
Draft TABLE OF CONTENTS
Ver.2.1

厚生労働科学研究費補助金（化学リスク研究事業）

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

令和元年度分担研究報告書
免疫毒性データの集積、国際標準化へ向けてのvalidation試験の計画、国際会議の企画、進行

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研究要旨

*in vitro*免疫毒性評価試験法（Multi-ImmunoTox assay：MITA)に含まれるIL-2 Luc アッセイ及びIL-1β Lucアッセイを、経済協力開発機構（Organisation for Economic Co-operation and Development：OECD）の試験法ガイドライン（Test Guideline：TG）として公定化するため、国際バリデーション研究を施行した。本年度、IL-2 Luc アッセイに関しては、国際的な第三者評価委員会の指摘を受け、バリデーション報告書を改訂した。IL-1β Lucアッセイについては、施設間再現性及び予測性を検証するため、バリデーション研究（phase II）を実施した。その結果、いずれの施設も施設間再現性の目標値である80%を達成でき、実験の終了を確認できた。

研究協力者氏名・所属機関名及び所属機関における職名

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A．研究目的

相場らにより、新たに開発された *in vitro* 免疫毒性評価試験法（Multi-ImmunoTox assay：MITA）に含まれるIL-2 Luc アッセイ及びIL-1β Luc アッセイを、経済協力開発機構（Organisation for Economic Co-operation and Development：OECD）の試験法ガイドライン（Test Guideline：TG）として公定化するため、国際バリデーション研究を施行する。

B．研究方法

B-1. 国際的な専門家との意見交換

B-1-1.　IL-2 Luc アッセイのバリデーション研究の報告書作成

免疫毒性データを集積し、IL-2 Luc assay(国際バリデーション研究 phase I、IIが既に終了)の最終結果を反映した報告書を完成した。

B-1-2.　バリデーション実行委員会会議

本年度に実施されたMITAに関する国際バリデーション結果を検証するため、対面会議及び電話会議を企画した。

B-2. バリデーション研究の実験支援

B-2-1.　IL-1β Lucアッセイバリデーション被験物

質の送付

IL-1β Luc アッセイのバリデーション研究Phase II (以下、Phase II と記す)にて、施設内再現性を求めるために選ばれた20物質をコード化し、各施設に送付した。

被験物質は、対面会議にて、より広範な物質を用いて施設内再現性を評価するために選択された。

B-2-2. IL-1β Lucアッセイバリデーション結果の記録確認

Phase II で用いられた各施設の記録用紙及びデータを回収し、バリデーション研究が適切に実施されたかを確認した。

C. 結果

C-1. 国際的な専門家との意見交換

C-1-1. IL-2 Lucアッセイのバリデーション研究の報告書作成

国際的な independent peer review panel（第三者評価委員会）の指摘を受け、バリデーション実行委員会（表 1 参照）の協力のもと、相場らが中心となってバリデーション報告書を改訂した。なお、この第三者評価委員会の運営は、別途研究班で実施されている。

C-1-2. バリデーション実行委員会会議

昨年度の検討では、一施設が施設内再現性の目標値である80%を達成できず、プロトコルの見直し及び再試験の追加が必要となった。

そこで、令和元年6月26日にバリデーション実行委員会の電話会議を開催し、委員に結果を説明した後、データ採用及び陽性基準の変更を提案した。その結果、以下に示す下線部の変更がなされた基準案に概ね合意を得た（添付資料1）。これにより、追加試験の実施は延期となった。

11. Criteria

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)) demonstrate less than going to be decided after Phase 0 study, the results obtained from the plate containing the control wells should be rejected.

□11-2 Positive 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 > going to be decided after Phase 0 study (suppressive) or < -going to be decided after Phase 0 study (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 > 0.05

令和元年7月15日にハワイで開催されたバリデーション実行委員会の対面会議にて、変更基準をもとに改訂された結果が確認された。これをもとに、表2に示すphase Iの結果をもって、いずれの施設も施設内再現性の目標値である80%を達成でき、

追加試験の必要もないという見解で一致し、phase Iの終了が合意された。さらに、phase IIのために用いる被験物質についても表3に示すように、合意がなされた。以上のバリデーション実行委員会の結論をへて、令和元年8月～のphase IIの開始で合意した（添付資料2）。

令和元年12月までに終了した実験を受け、令和2年1月30日及び31日に川崎市にてバリデーション実行委員会の対面会議を開催した。その結果、いずれの施設も施設間再現性の目標値である80%を達成でき、phase IIの終了に合意がなされた。(添付資料3)。解析結果については、大森らの報告書を参照されたい。

C-2. バリデーション研究の実験支援

C-2-1. IL-1β Lucアッセイバリデーション被験物質の送付

試験計画（添付資料4）に示すように、phase II バリデーションとして施設間再現性を求めるために実行委員会で選ばれた20物質を、コード化してリード施設を含む参加3施設に送付した。表3に被験物質のコード番号を示す。

実験の終了まで、被験物質による誤使用などによる健康障害などのトラブルは生じなかった。

C-2-2. IL-1β Lucアッセイバリデーション結果の記録確認

Phase II 終了後に回収した記録用紙の一覧を添付資料5に示した。施設によって一部記載の不備があったが、GLP（Good Laboratory Procedure）の精神に則り、適切に実験が実施され、その記録が残されていることを確認した。その結果が、QC報告書にまとめられた（添付資料6)。

D. 考察

MITAの一つであるIL-2 Lucアッセイのバリデーション報告書は、第三者評価委員会の意見に従い、修正された。これにより、評価報告書はまもなく、完成すると推察している。

一方、MITAのもう一つの試験法であるIL-１βLucアッセイのバリデーション研究の実験は無事終了した。昨年度のphaseIの検討では、一施設が目標値である80%を達成できず、プロトコルの見直し及び再試験の追加が必要となったが、相場らにデータ採用基準の変更提案が受け入れられ、追加実験なく、phase IIに移行することができ、無事実験終了となった。

来年度は、バリデーション報告書が作成され、第三者評価委員会に移行される。

いずれの方法も将来的には、OECDにてTGと採択されることを目指しており、来年度にはいずれの方法もOECDに提案できる段階となると考えている。

E. 結論

相場らにより、新たに開発されたMITAであるIL-2 Luc アッセイ及びIL-1β Lucアッセイの公定化を目指すため、国際的なバリデーション研究を施行した。IL-2 Lucアッセイについては、第三者評価委員会の指摘を受け、バリデーション報告書を改訂した。

MITAのもう一つの試験法であるIL-１βLucアッセイのバリデーション研究においては、いずれの施設も施設内及び施設間再現性の目標値である80%を達成でき、実験の終了を確認できた。

F. 添付文書

1) Minutes of MITA, June 26th, 2019
2) Minutes of MITA, July 15th, 2019
3) Minutes of MITA, January 30th & 31th, 2020
4) 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
5) Confirmation table for phase II
6) QC report for IL-1β Luc assay validation study

表１． 2019年度　MITA国際バリデーション実行委員会及び参加施設の主なリスト

No.	Name	Affiliation	Country
1	Emanuela Corsini	Universit.AN` degli Studi di Milano	Italy
2	Erwin L. Roggen	3Rs Management and Consulting ApS	Denmark
3	Dori Germolec	NIH/NIEHS	USA
4	Tomoaki Inoue	Chugai Pharmaceutical Co., Ltd.	Japan
5	Setsuya Aiba	Tohoku University Graduate School of Medicine	Japan
6	Yutaka Kimura	Tohoku University Graduate School of Medicine	Japan
7	Yoshihiro Nakajima	National Institute of Advanced Industrial Science and Technology (AIST), Shikoku	Japan
8	Rie Yasuno	AIST, Tsukuba	Japan
9	Takashi Omori	Kobe University	Japan
10	Nana Mashimo	Kobe University	Japan
11	K. Okayama	Kobe University	Japan
12	Hajime Kojima	JaCVAM, National Institute of Health Sciences	Japan

表２． Phase I **結果（最終合意）**

LabA Tohoku			LabB AIST Tsukuba			LabC AIST Shikoku		
setNo.	code No.		setNo.	code No.		setNo.	code No.	
Set1	MITA103	S	Set1	MITB402	S	Set1	MITC704	S
Set2	MITA203	S	Set2	MITB501	S	Set2	MITC803	S
Set3	MITA304	S	Set3	MITB605	S	Set3	MITC902	S
Set1	MITA101	N	Set1	MITB404	N	Set1	MITC701	N
Set2	MITA205	N	Set2	MITB505	N	Set2	MITC802	N
Set3	MITA305	N	Set3	MITB603	N	Set3	MITC905	N
Set1	MITA104	N	Set1	MITB403	N	Set1	MITC705	N
Set2	MITA202	N	Set2	MITB502	N	Set2	MITC805	N
Set3	MITA303	N	Set3	MITB601	N	Set3	MITC901	N
Set1	MITA105	S	Set1	MITB401	S	Set1	MITC702	S
Set2	MITA204	S	Set2	MITB503	S	Set2	MITC801	S
Set3	MITA301	S	Set3	MITB602	S	Set3	MITC904	S
Set1	MITA102	N	Set1	MITB405	N	Set1	MITC703	N
Set2	MITA201	N	Set2	MITB504	N	Set2	MITC804	N
Set3	MITA302	N	Set3	MITB604	N	Set3	MITC903	N

Within-laboratory concodance rate: 100% (5/5) in all laboratory and between-labolatory concodance rate: 100% (5/5)

表３．Phase II で用いた被験物質とコード表

MITA　Phase2　　2019/6/25

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-KQRC	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

G. **研究発表**

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H. **知的所有権の取得状況**

H－1）**特許取得**

特になし

H－2）**実用新案登録**

特になし

H－3）**その他**

特になし

Draft minutes

Conference call for the MITA assay validation study

Date ： June 26th, 2019

Validation Management Team: Corsini, E., Roggen, E., Germolec, D., Inoue, T., Aiba, S., Kimura, Y., Omori, T., Kojima, H.

1. Welcome address and approve draft agenda
   Kojima welcomed to join this meeting and the VMT members approved the agenda.
2. Results of phase I
   Omori introduced results of phase I. For within-laboratory reproducibility, the ratio of AIST tukuba was 100%(5/5) and one of Tohoku Univ. was 80% (4/5). Unfortunately, one of AIST, Shikoku was 60% (3/5) as shown in Table and the results of this laboratory had not met the success criteria of within-laboratory reproducibility (80%) in the study plan..
3. Proposal of the revised positive criteria
   To dissolve this concern, Aiba suggested three proposals. They are 1) Change the acceptance criteria, 2)Judgement and 3)The threshold and the positive results at 2000 μg/mL. Based on these proposals and the data re-analyzed by Kimura, all the members discussed and considered the current data may meet the success criteria if the changed acceptance criteria uses. The all agreed to change the acceptance criteria (see the attached new SOP).
4. Re-analysis of phase I
   Omori promised to perform re-analysis of phase I data and provide the results by e-mail after this meeting.
5. Future plan
   Kojima talked about the future plan as a prerequisite the success criteria of within-laboratory reproducibility meet using re-analysis data of phase I. In this case, he declares the completed phase I and coordinate the next F2F meeting on July 15th in Hawaii for chemical selection phase II.

   He mentioned to request the VMT members support the revision of IL-2 Luc validation study and develop the Detailed Review Paper on in vitro immunotoxicity in the next meeting.

MITA F2F meeting draft minutes in Hawaii

Date: July 15, 12:00-14:45

Venue: Syokudo & StarBucks in Ala Moana Hotel

Participants: Emanuela Corsini, Dori Germolec, Setsuya Aiba, Hajime Kojima

1. Development of DRP

   Kojima reported the OECD WNT approved to develop a detailed review paper (DRP) on in vitro immunotoxicity and proposed to support this project to the MITA validation management team (VMT). Based on the previous Corsini & Rogen's papers, he considers to develop the DRP. All the participants agreed and Kojima asked them to select additional experts for the project. Corsini and Germolec recommend him following candidate experts. He will contact them soon and he will coordinate the F2F meeting in January or February, 2020. Before the F2F meeting, he will make a draft ToC with Corsini and Dori and share to all.

   -Laura Gribaldo (JRC)

   -Henk van Loveren (Maastricht Univ.)

   - Barbara Kaplan (Mississippi State Univ.)

2. IL-1βassay validation study

   Kojima welcomed you agreed to complete the experiment of phase I. According to the revised criteria, the within-laboratory reproducibility of this assay is perfect at three laboratories. However, do not forget Corsini's previous comments.

   1. Regarding the maximum concentration, it is always possible write in the SOP that final concentrations higher than 1 or 1.5 mg/ml should not be tested to avoid false positive response.

   2. It is important that in the next phase, classification criteria remains as there are now. It is not correct to continue changing the criteria to fit the results, to me, this indicates the non-optimization of the method.

3. Reply to peer review panel

   To reply to the comments from peer review panel for the IL-2 assay validation report, Aiba's comments as their reply confirmed. Especially, we discussed the positive criteria of test chemicals for predictive capacity.

   As the positive criteria, we fixed the thymus weight reduction plus T cell proliferation, T cell mediated function, cytokine induction or DTH response by in vivo, in vitro and ex vivo data. The data depending on only one paper is not accepted.

   Based on this discussion, Aiba will revise the validation report and share it to all. After checking it, Kojima will share the final one to the peer review panel.

Date: July 15, 14:55-15:15

Venue: StarBucks in Ala Moana Hotel

Participants: Emanuela Corsini, Dori Germolec, Hajime Kojima

Chemical selection for phase II

Based on the candidate chemicals recommended by Corsini and Germolec, we discussed 20 test chemicals for phase II. We agreed 17 test chemicals in accordance with their suggestion and discussed more three chemicals. Kojima requested one more negative test chemical including total more than 6 negatives considering balance of test chemicals. The following three chemicals was selected.

5th Meeting for the MITA Validation Study

January 30 & 31, 2020

VMT: E. Corsini, D. Germolec, T. Inoue, S. Aiba, Y. Kimura, T. Omori, N. Mashimo, K. Okayama, H. Kojima, T. Ashikaga, S. Venti

Participating Labs: R. Yasuno, Y. Nakajima

	January 30
Kojima:	Today is the 5th meeting of the MITA VMT. (See agenda.)
Omori:	(See presentation.) Although we judge each chemical to be either positive or negative, I think it is important to emphasize the dose response information shown on the graphs.
Aiba:	It is interesting to see chemicals like No. 13 show borderline results.
Germolec:	There are some graphs that are visually similar but have different results. And this is a little bit strange.
Omori:	The results are determined by an algorithm, which does not "look" at the graph.
Aiba:	But the between-lab reproducibility is 80%. We have to wonder if the criteria are entirely correct, but we cannot change them now.
Kojima:	For the validation study, we have to classify each chemical as positive or negative, but in the future, we need to consider the needs of users who are classifying new compounds.
Corsini:	Without knowing what the chemicals are, we cannot consider any biological factors. For now, we must just base our classifications on the numbers. But there will always be some false positives or false negatives when the results are close to the threshold value.

Germolec:	But the fact that the between-lab reproducibility is very good is a very positive thing, irrespective of these borderline results.
Inoue:	What about the I.I.-SLR-LA valve of ≥ 0.05?
Aiba:	We have several ways to determine cell viability. This assay measures the transcription of housekeeping genes. And the cells maintain their membrane integrity. So, this number is analogous to 80% cell viability.
Kojima:	The data sheets are available on the JaCVAM website. (See username and password shown separately.) If you agree that the data is acceptable, we can open the code sheet.
All:	We achieved our goals for within- and between laboratory reproducibility, and we agree that the data is acceptable.
Aiba:	The Luster data is difficult to use for reference data. So, we looked through the literature, and compiled this list. I would like to add more but I am not sure what is available. But there are many chemicals that we do not have data for. So, this is a problem we must solve.
Corsini:	Without reference data, we cannot define predictive capacity, but what we can try to do is use the clustering to see how many in vivo positives we missed. It will never be a standalone test, so we should use the cluster data to see how it correlates. And there are some chemicals that have discordant results.
Germolec:	There are some chemicals we might have data for, but it would difficult to create a list of chemicals we think are positive in vivo. But this test is not going to be standalone, it will be just one tool in the toolbox.
Corsini:	We should stay withing the MITA, because there are also differences in human response for macrophage and monocytes for IL-1 A or B. This study shows good reproducibility, so this assay should be evaluated only within the context of MITA. Because this assay is testing only a very small part of the immune system at large. What is important is to evaluate the predictive

	capacity of MITA as a whole, not just this individual test. Of course, we will need to explain this.
Germolec:	We have looked at predictive capacity of the IL-2, so how does that improve if we add the IL-1 assay to that?
Aiba:	Unfortunately, the IL-2 assay covers most of the results from the IL-1 assay.
Inoue:	Perhaps changing the main components will reveal the third axis more clearly.
Corsini:	Perhaps at the end of the validation we can see how they fit with the cluster data and if IL-1 adds to predictive capacity.
Germolec:	Possibly, the addition of the IL-1 assay will not increase predictivity. It is possible that the IL-1 will not be needed for all permutations of the modified MITA.
	(Lunch break)
Germolec:	I will provide Dr. Aiba a list of chemicals that can be used for reference. So, it seems that we have a way forward to say something about predictive capacity. But even if we cannot assess the predictive capacity because of a lack of in vivo data, that does not imply a limitation of the assay.
Kojima:	The OECD is now promoting the development of AOPs
Germolec:	I think in terms of immunotoxicity, we are going to have to show how multiple AOPs intersect.
Aiba:	The immune system is highly redundant, so there are many cytokines that need to be described. But that does not mean that each of these cytokines presents a specific effect.
Corsini:	There are a lot of chemicals that have been tested with the whole blood assay, so it might be interesting to compare with those results.
Germolec:	Do we need to go over what our follow up activities are for predictive capacity?

Inoue:	Does the IL-1 data ignores augmentation, but the IL-8 Luc assay includes augmentation? So maybe the vector will be different?
Aiba:	Yes, that is correct.
Kojima:	If we have not proposed it to the OECD, we will need to complete the validation study and peer review report next year.
Inoue:	In the table with information on monocytes, is this data from human monocytes? It would be good to know which is human and which is animal monocytes.
Aiba:	Yes, it shows whether it is human or animal.
Corsini:	Maybe we could add a column to show positive or negative results for NTP to this table.
Aiba:	We will have to decide that ourselves from the data we got from Dori.
Germolec:	I'm not sure we have data for all the chemicals that Dr. Aiba has.

	January 31
Kojima:	Today I would like to discuss the validation report and related issues.
Corsini:	Item 4-3 should not be T-cells. This is a monocyte cell line.
Aiba:	(Review of who will revise each section of the ToC.)
Germolec:	As we discussed yesterday, we need to be careful with the predictivity. We might want to remove parts of section 10 on predictivity for now but discuss in section 10-7 about potential of the assay within MITA. This is a strong assay, but we don't have an appropriate data set with which to discuss predictivity as a standalone test.
Omori:	Also delete section 8-3-6 from my section.

Corsini:	Also introduce this issue in section 2 about the object of the study. The majority of chemicals are well-known immunotoxicants for which in vivo data is available, so we have lots of data that is relevant to judging whether or not MITA is a suitable model for predicting immunotoxicity.
Aiba:	Once I revise the discussion, I will share with the VMT for comments.
Omori:	I can discuss clustering but after that it is not possible to discuss predictivity without a data set for comparison.
Aiba:	We might be able to identify and characterize several different clusters. I will make IL-2 assay the first tier, and then IL-1 or IL-8 as the second tier. And if we have a positive result in the first tier, then we accept the positive result.
Corsini:	We need to be clear about what we consider immunotoxicity and what we consider skin sensitization when comparing in vitro with in vivo results.
Germolec:	The IL-8 with give you a yes or no for skin sensitization, but you are still going to need another tier for immunosuppression. So, it might be interesting to look at the predictivity of different combinations of the IL-1, IL-2, and IL-8. You might find that you only need one or two rather than all three tests.
Corsini:	The IL-8 Luc is similar to in vivo testing in that it challenges the immune system.
Germolec:	I will try to identify some chemicals that target monocytes and macrophages from the Tox21 data.
Corsini:	An added value of MITA is that it gives some additional information about whether the chemical acts on acquired immunity or innate immunity.
Aiba:	The IL-8 Luc in the presence of LPS could add some predictivity.
Kojima:	My concern is the use of different solvents. DMSO and EXVIVO will result in different predictive capacities.

Corsini:	Maybe we can note that the performance is very good and that we are responding to the need to remove serum from the system by using EXVIVO culture medium.
Kojima:	But I think the solvent will affect the clustering analysis.
Aiba:	In this context, I don't think the use of EXVIVO really matters. I wonder if I need define an applicability domain when trying different combinations.
Corsini:	When you study predicative capacity, you will need to consider an applicability domain.
Germolec:	You can explain what you think the applicability domain should be in the discussion.
Corsini:	In places where we have a discrepancy, the applicability domain provides additional information that helps us understand why. After you determine your tiered approach, maybe we can have a conference call to discuss the results. We are more concerned about missing a positive than about overpredicting a negative as positive.
Aiba:	Dori, when can you get us the information about chemicals?
Germolec:	I would say end of March. But I also need to see what action items I am responsible for before I can promise. One thing is that the calls—positive or negative—I can get by the end of March, but it will probably take until the end of summer for the chemicals on which you need more detailed information.
Aiba:	I think there will be 19 chemicals for which I would like detailed information.
Germolec:	So please send me that list.
Kojima:	When do we think we can finalize the validation report?

Aiba:	By the end of September?
Kojima:	So, we will need to have conference calls in October or November.
Omori:	Will you do more testing?
Aiba:	We have to retest some chemicals so they can be judged on new criteria. And there are some that have never been tested using IL-8 in the presence of LPS. So, I might have to request some additional testing from the participating laboratories.
	Action Items ✓ Dr. Germolec will send Dr. Aiba a file will all Tox21 chemicals that were tested for immunotoxicity by the end of February. ✓ Dr. Aiba will send Dr. Germolec a list of 19 chemicals for which a literature search and detailed information is requested. Dr. Germolc will respond by the end of July. ✓ Dr. Kojima will coordinate a conference call for May or June. ✓ Dr. Aiba will revise the validation report by the end of September. ✓ Dr. Kojima will organize a final F2F meeting to be held in November.

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

INDEX

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

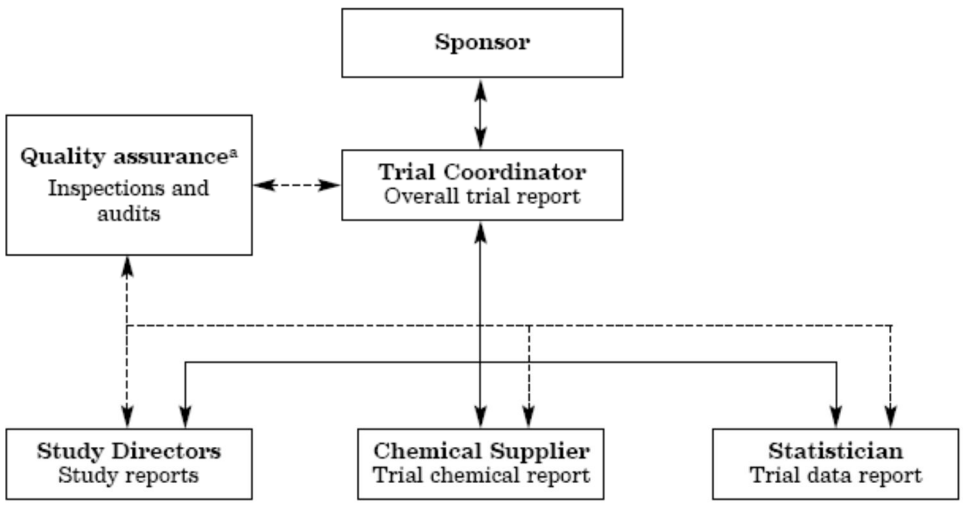
Test Facility 2: AIST, Tsukuba SD : Rie Yasuno

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 II (P2)2017　Confirmation Table ①

		LabB AIST, Tsukuba	LabC FDSC	LabD AIST, Takamatsu
Set	Reagent Records	IL II 2017-B01	IL II 2017-C01	IL II 2017-D01
	Solubility Test		IL II 2017-C02	IL II 2017-D02
	Cell Culture Records	IL II 2017-B03	IL II 2017-C03	IL II 2017-D03
	date	2017.5.19	2017.6.9	2017.5.22
	Test records	IL II 2017-B04	IL II 2017-C04	IL II 2017-D04
	Datasheet	IL II 2017-B20	IL II 2017-C20	IL II 2017-D20
	date	2017.5.31	2017.6.30	2017.5.23
	Test records	IL II 2017-B05	IL II 2017-C05	IL II 2017-D05
	Datasheet	IL II 2017-B21	IL II 2017-C21	IL II 2017-D21
	date	2017.6.5	2017.7.6	2017.5.29
	Test records	IL II 2017-B06	IL II 2017-C06	IL II 2017-D06
	Datasheet	IL II 2017-B22	IL II 2017-C22	IL II 2017-D22
	date	2017.6.6	2017.7.7	2017.5.30
	Test records	IL II 2017-B07	IL II 2017-C07	IL II 2017-D07
	Datasheet	IL II 2017-B23	IL II 2017-C23	IL II 2017-D23
	date	2017.6.8	2017.7.10	2017.6.12
	Test records	IL II 2017-B08	IL II 2017-C08	IL II 2017-D08
	Datasheet	IL II 2017-B24	IL II 2017-C24	IL II 2017-D24
	date	2017.6.9	2017.7.13	2017.6.19
	Test records	IL II 2017-B09	IL II 2017-C09	IL II 2017-D09
	Datasheet	IL II 2017-B25	IL II 2017-C25	IL II 2017-D25
	date	2017.6.12	2017.7.14	2017.6.20
	Test records	IL II 2017-B10	IL II 2017-C10	IL II 2017-D10
	Datasheet	IL II 2017-B26	IL II 2017-C26	IL II 2017-D26
	date	2017.6.14	2017.7.18	2017.6.26
	Test records	IL II 2017-B11	IL II 2017-C11	IL II 2017-D11
	Datasheet	IL II 2017-B27	IL II 2017-C27	IL II 2017-D27
	date	2017.6.21	2017.7.21	2017.6.27
	Test records	IL II 2017-B12	IL II 2017-C12	IL II 2017-D12
	Datasheet	IL II 2017-B28	IL II 2017-C28	IL II 2017-D28
	date	2017.6.22	2017.7.24	2017.7.3
	Test records	IL II 2017-B13	IL II 2017-C13	IL II 2017-D13
	Datasheet	IL II 2017-B29	IL II 2017-C29	IL II 2017-D29
	date	2017.6.28	2017.7.27	2017.7.4
	Test records	IL II 2017-B14	IL II 2017-C14	IL II 2017-D14
	Datasheet	IL II 2017-B30	IL II 2017-C30	IL II 2017-D30
	date	2017.6.29	2017.7.28	2017.7.10
	Test records	IL II 2017-B15	IL II 2017-C15	IL II 2017-D15
	Datasheet	IL II 2017-B31	IL II 2017-C31	IL II 2017-D31
	date	2017.7.7	2017.8.3	2017.7.11
	Test records	IL II 2017-B16	IL II 2017-C16	IL II 2017-D16
	Datasheet	IL II 2017-B32	IL II 2017-C32	IL II 2017-D32
	date	2017.7.11	2017.8.4	2017.7.18
	Test records	IL II 2017-B17	IL II 2017-C17	IL II 2017-D17
	Datasheet	IL II 2017-B33	IL II 2017-C33	IL II 2017-D33
	date		2017.8.7	2017.7.24
	Test records		IL II 2017-C18	IL II 2017-D18
	Datasheet		IL II 2017-C34	IL II 2017-D34
	date		2017.8.8	2017.7.25
	Test records		IL II 2017-C19	IL II 2017-D19
	Datasheet		IL II 2017-C35	IL II 2017-D35
	date		2017.8.14	
	Test records		IL II 2017-C20	
	Datasheet		IL II 2017-C36	

IL II (P2)2017　Confirmation Table ②

	LabB AIST, Tsukuba		LabC FDSC		LabD AIST, Takamatsu	
datasheet	501	IL II 2017-B40	601	IL II 2017-C40	701	IL II 2017-D40
	502	IL II 2017-B41	602	IL II 2017-C41	702	IL II 2017-D41
	503	IL II 2017-B42	603	IL II 2017-C42	703	IL II 2017-D42
	504	IL II 2017-B43	604	IL II 2017-C43	704	IL II 2017-D43
	505	IL II 2017-B44	605	IL II 2017-C44	705	IL II 2017-D44
	506	IL II 2017-B45	606	IL II 2017-C45	706	IL II 2017-D45
	507	IL II 2017-B46	607	IL II 2017-C46	707	IL II 2017-D46
	508	IL II 2017-B47	608	IL II 2017-C47	708	IL II 2017-D47
	509	IL II 2017-B48	609	IL II 2017-C48	709	IL II 2017-D48
	510	IL II 2017-B49	610	IL II 2017-C49	710	IL II 2017-D49
	511	IL II 2017-B50	611	IL II 2017-C50	711	IL II 2017-D50
	512	IL II 2017-B51	612	IL II 2017-C51	712	IL II 2017-D51
	513	IL II 2017-B52	613	IL II 2017-C52	713	IL II 2017-D52
	514	IL II 2017-B53	614	IL II 2017-C53	714	IL II 2017-D53
	515	IL II 2017-B54	615	IL II 2017-C54	715	IL II 2017-D54
	516	IL II 2017-B55	616	IL II 2017-C55	716	IL II 2017-D55
	517	IL II 2017-B56	617	IL II 2017-C56	717	IL II 2017-D56
	518	IL II 2017-B57	618	IL II 2017-C57	718	IL II 2017-D57
	519	IL II 2017-B58	619	IL II 2017-C58	719	IL II 2017-D58
	520	IL II 2017-B59	620	IL II 2017-C59	720	IL II 2017-D59

IL II (P2)2017　Table of Contents

Chemical name	CAS No.	(LabA Tohoku)	LabB AIST, Tsukuba	LabC FDSC	LabD AIST, Takamatsu
2,4-diaminotoluene	95-80-7	MIA401	MIB515	MIC618	MID702
Benzo(a)pyrene	50-32-8	MIA413	MIB516	MIC601	MID703
Cadmium chloride	10108-64-2	MIA403	MIB502	MIC602	MID714
Dibromoacetic acid	631-64-1	MIA406	MIB518	MIC610	MID720
Diethylstilbestol	56-53-1	MIA420	MIB509	MIC611	MID711
Diphenylhydantoin	630-93-3	MIA412	MIB510	MIC615	MID704
Ethylene dibromide	106-93-4	MIA407	MIB507	MIC605	MID705
Glycidol	556-52-5	MIA408	MIB505	MIC607	MID712
Indomethacin	53-86-1	MIA409	MIB508	MIC609	MID715
Isonicotinic Acid Hydrazide (Isoniazid)	54-85-3	MIA411	MIB517	MIC612	MID707
Nitrobenzene	98-95-3	MIA402	MIB519	MIC603	MID701
Urethane, Ethyl carbamate	51-79-6	MIA415	MIB520	MIC604	MID719
Tributyltin chloride	1461-22-9	MIA404	MIB506	MIC613	MID713
Perfluorooctanoic acid	335-67-1	MIA414	MIB514	MIC614	MID718
Dichloracetic acid	79-43-6	MIA416	MIB511	MIC606	MID716
Toluene	108-88-3	MIA417	MIB512	MIC616	MID706
acetonitril	75-05-8	MIA405	MIB501	MIC617	MID708
mannitol	69-65-8	MIA418	MIB503	MIC619	MID717
Vanadium pentoxide	1314-62-1	MIA419	MIB504	MIC608	MID709
o-benzyl-p-chorolophenol	120-32-1	MIA410	MIB513	MIC620	MID710

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

厚生労働科学研究費補助金（化学物質リスク研究事業）
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
（ H30-化学-一般-001 ）

分担研究報告書
免疫毒性評価試験法 Multi-ImmunoToxicity assay の国際 validation へ向けての検討

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産業技術総合研究所　健康工学研究部門

研究要旨

IL-1β プロモーター活性を緑色発光ルシフェラーゼおよびプロモーター活性を補正するための内部標準プロモーターG3PDH 活性を赤色ルシフェラーゼでモニターするヒト単球由来 THP-1 細胞（TGCHAC-A4 細胞）を用いた化学物質免疫毒性評価系 Multi-ImmunoToxicity assay (MITA)の Phase II バリデーション試験を実施した。

A．研究目的

環境中に存在する何万という化学物質のなかには、免疫系を標的として健康被害を及ぼすものが多数存在する。したがって、免疫毒性は、消費者、生産者はもとより公衆衛生行政にとっても重要な課題となっている。当該研究では、免疫毒性に影響を及ぼす化学物質を簡便に評価するための発光レポーターを利用した*in vitro*免疫毒性評価試験法（Multi-ImmunoToxicity assay）を構築、本試験法のガイドライン化を目指し、昨年度までにPhase 0として既知の3物質について試験を実施して技術移転性を確認した後、Phase Iバリデーション試験として、1組5種類のコード化した試験化学物質3組を供試して施設内再現性および施設間再現性について検討してきた。本年度は引き続きPhase IIバリデーション試験として、コード化した試験化学物質20種類を供試し、施設間再現性等についてさらに検討した。

B．研究方法

B-1) 使用した細胞

IL-1βとG3PDHプロモーターにそれぞれSLG、SLRルシフェラーゼ遺伝子をつないで人工染色体発現ベクターにノックインし、ヒト単球由来細胞株THP-1に導入した2色発光細胞株THP-G1b(TGCHAC-A4)を用いて試験を行った。

B-2) 使用した化学物質

試験化学物質としてPhase IIでは1セット20種類のコード化した被験物質1セットを用いた。物質名とCAS番号およびコード番号の対応を表１に示す。

B-3) 実験方法

化学物質の免疫毒性試験法における細胞培養方法、被験物質調整及び添加方法、及びルシフェラーゼアッセイの方法については Multi-Immuno Tox Assay protocol for THP-G1b (TGCHAC-A4) Ver. 009Eに準ずる。発光測定装置はアトー社製フェリオス(AB-2350)を用いた。

Phase IIバリデーション試験では、1セット20種類のコード化した試験化学物質1セットを用いて1被験物質につき2回以上、判定が決定できるまで試験を行った。判定基準は以下の通りである。

以下の４つの基準を満たす場合をSuppressionとし、それ以外をNo effectとする。2回一致した結果が得られたとき、その結果を当該物質の評価として扱う。

- SLR-LAの阻害指標（I.I.-SLR-LA）が0.05以上の濃度のみを判定に使用する。I.I.-SLR-LAが0.05以上の濃度が6点より少ない場合は、以下の条件を満たす場合のみ判定を採用し、他は続いて濃度を下げた試験を行う。
- %suppressionの平均値が25%以上でかつ、同時に95%信頼区間を用いた判定で濃度0と有意差が認められる場合に有意（統計学的有意）とする。
- 統計学的有意となる連続した2つ以上の濃度が得られる。もしくは統計学的有意

となる濃度は1つであるが、すくなくとも連続した3濃度で濃度依存性を示す（この場合、統計学的有意を示さなければ、0を挟んでもよい）。
- 被験物質濃度2000 μg/mLの結果は除外する。

（倫理面への配慮）
倫理的な問題が生じる実験を実施しておらず、配慮すべき問題はない。

C．研究結果
Phase IIでは施設間再現性および試験の正確性を検討する目的で、コード化された20物質を1セットとする群が1セット配布された。1物質につき判定を決定できるまで、2回から4回の実験を実施した。結果を図1に示す。提案された判定基準に基づいて各物質を評価した結果を表2に示した。

D．考察
Phase II studyでは施設間再現性等の確認のために、コード化した20物質1セットについて実験を行った。
当施設では、20物質のうち10物質がSuppression、残り10物質がNo effectと判定された。バリデーション試験の試験実施施設である3施設（産総研健康工学研究部門、産総研バイオメディカル研究部門、東北大医学部皮膚科）の結果を比較したところ、20物質中16物質で判定が一致しており(16/20)、施設間再現性は80%であった。
また、昨年度行ったPhase I studyの結果を今回と同じ判定基準を用いて改めて再判定した。Phase I studyは、コード化された5物質を1組とする群が3組配布され、1組毎に実験を行ったものである。その結果、施設内再現性は100% (5/5)であり、施設間再現性もまた100% (5/5)となった。
以上の結果、Phase IおよびIIを通して新しい判定基準を適用することにより、施設間ならびに施設内再現性が80%以上に向上した。
Phase II studyにおいて判定が一致しなかった4物質には、全濃度で10～20%程度のSuppressionが見られるものが2物質含まれている。このような物質の場合は、偶発的に1点の濃度で25%を超えていて濃度依存性があるように見えることがあるが、試験1回目と2回目ではピークとなる濃度が異なる等濃度依存性に違いが見られる。このような擬陽性の可能性がある物質データの取り扱いには注意が必要かもしれない。

E．結論
IL-1β転写誘導抑制を指標とした免疫毒性評価試験法のOECDテストガイドライン化を目的として、試験実施施設としてバリデーション試験に参加した。Phase I studyの結果を元に判定基準の見直しを行い、新たに提案された基準を用いて判定を行うこととした。Phase II studyではコード化した20物質について試験を実施した。得られた結果を比較して施設間再現性を検討したところ、80%という良好な結果が得られた。また、同判定基準を適用してPhase I studyの結果を再判定したところ、施設内再現性、施設間再現性ともに100%となり、特に再現性に関して非常に良好な系を構築できた。

F．健康危険情報
該当なし

G．研究発表
1. 論文発表
該当なし

2. 学会発表
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、足利太可雄、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、第32回日本動物実験代替法学会

H．知的財産権の出願・登録状況
（予定を含む。）
1. 特許取得
該当なし

2. 実用新案登録
該当なし

3.その他
該当なし

表１．試験化学物質名と CAS **番号およびコード番号との対応.**

化学物質名	CAS No.	コード番号
Cadmium Chloride	10108-64-2	MTC305
5,5-Diphenylhydantoin Sodium salt	630-93-3	MTC301
Indomethacin	53-86-1	MTC318
Pentachlorophenol	87-86-5	MTC307
Urethane	51-79-6	MTC302
Tributyltin Chloride	1461-22-9	MTC312
Perfluorooctanoic Acid	335-67-1	MTC303
Hydroquinone	123-31-9	MTC322
Bis(4-aminophenyl) Sulfone	80-08-0	MTC313
Ethanol	64-17-5	MTC317
5-Nitro-2-furaldehyde Semicarbazone	59-87-0	MTC324
Trichloroethylene	79-01-6	MTC309
Zinc Dimethyldithiocarbamate	137-30-4	MTC316
Citral	5392-40-5	MTC315
t- Butylhydroquinone	1948-33-0	MTC323
Bisphenol A	80-05-7	MTC314
2,6-Di-tert-butyl-4-methylphenol	128-37-0	MTC306
Nonylphenol	84852-15-3	MTC311
Sodium Chloride	7758-19-2	MTC304
D(-)-Mannitol	69-65-8	MTC327

図 1　THP-G1b(TGCHAC-A4)細胞株における各試験化学物質に対する細胞応答性.

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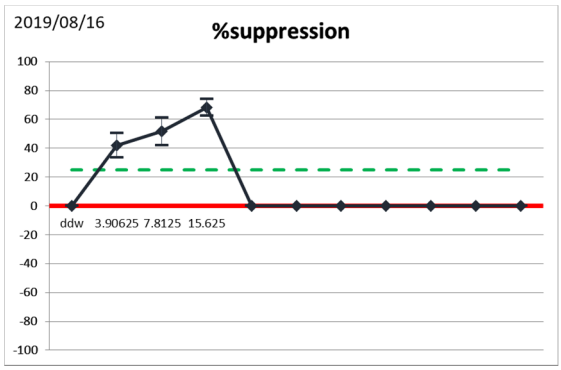

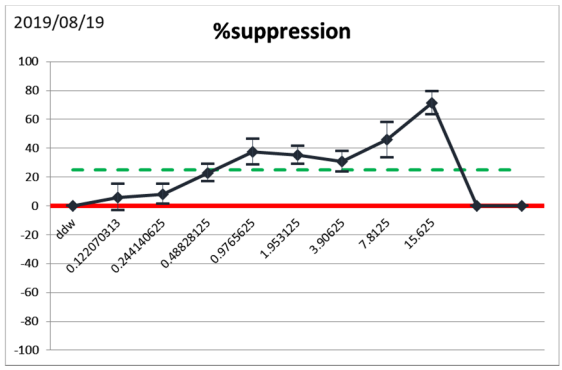

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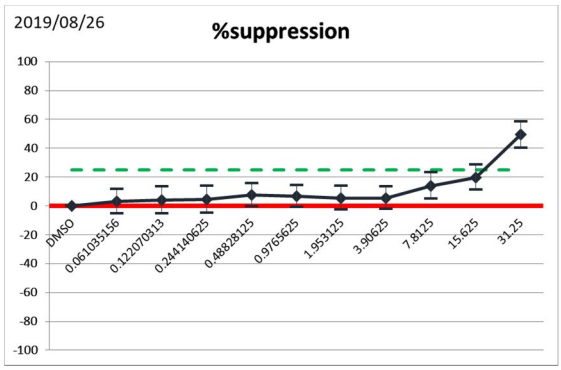

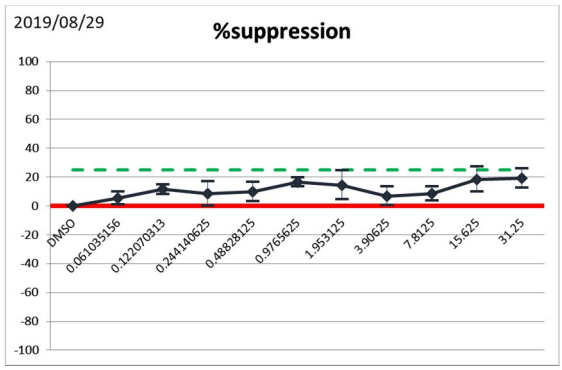

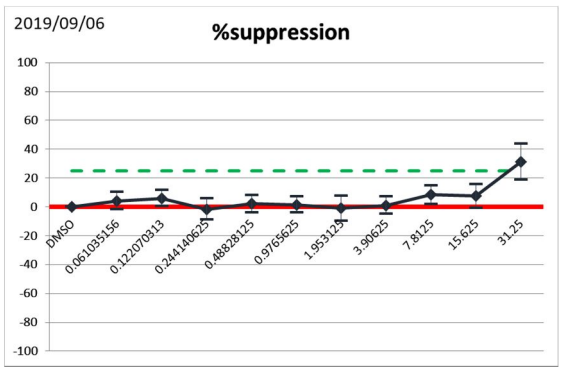

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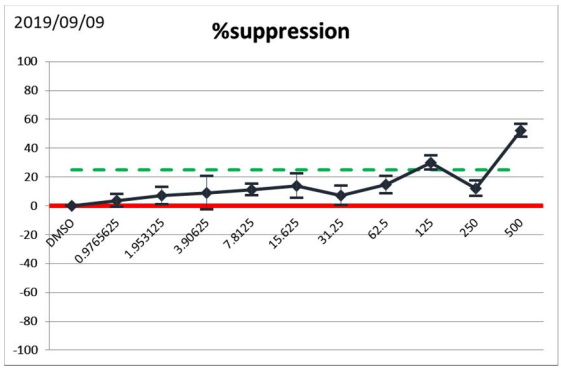

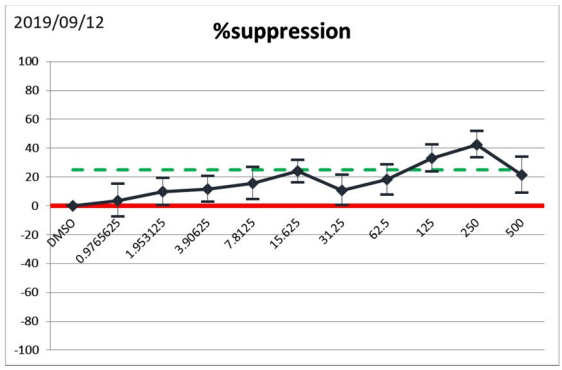

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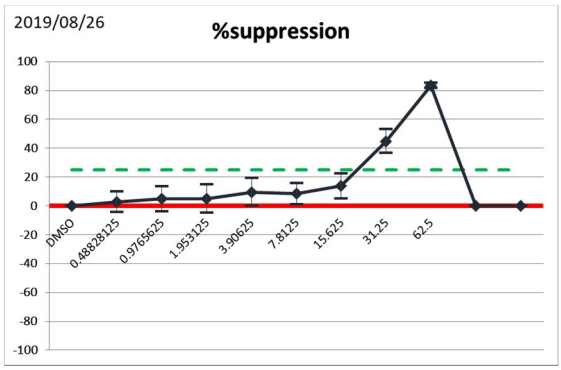

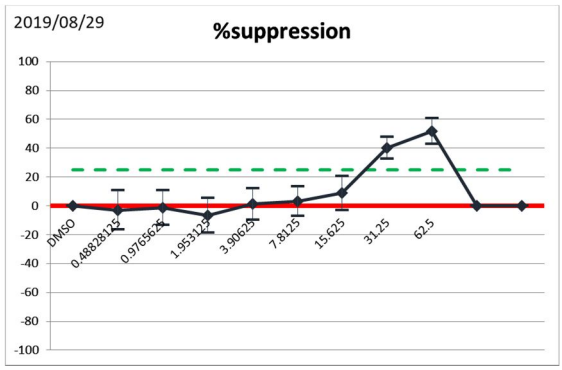

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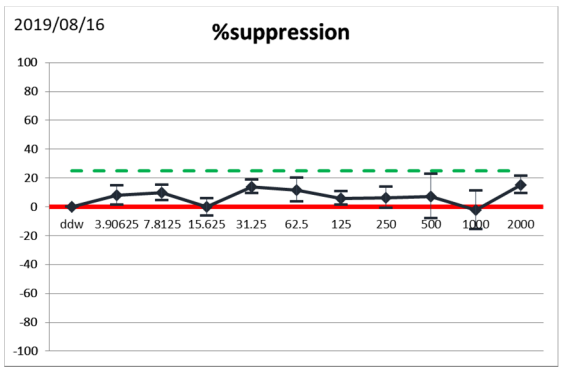

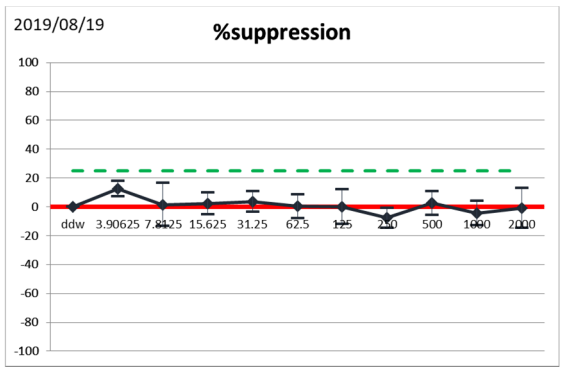

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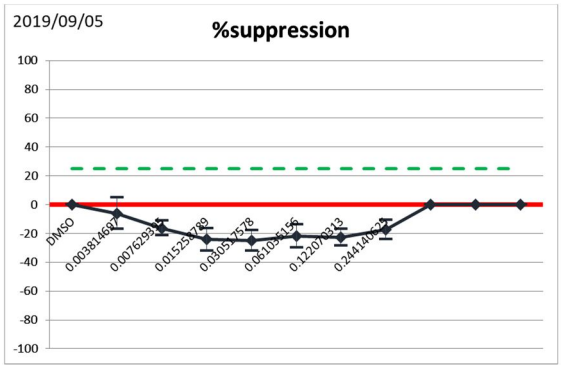

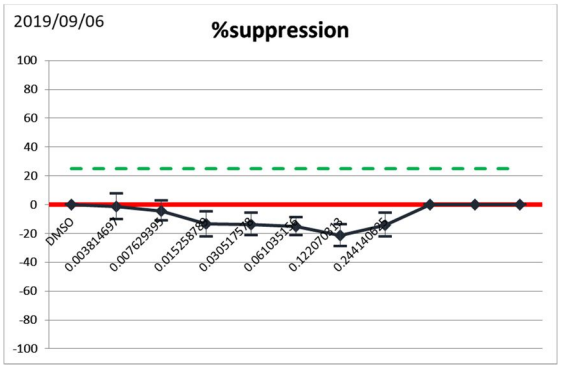

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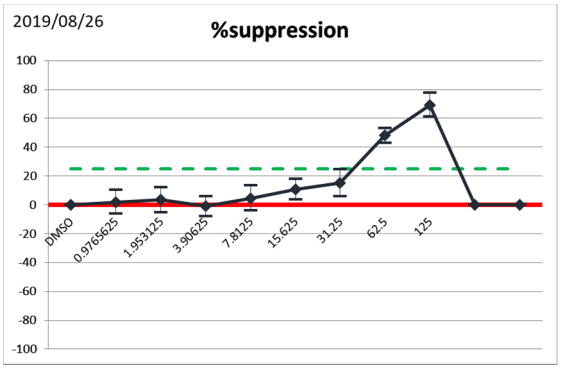

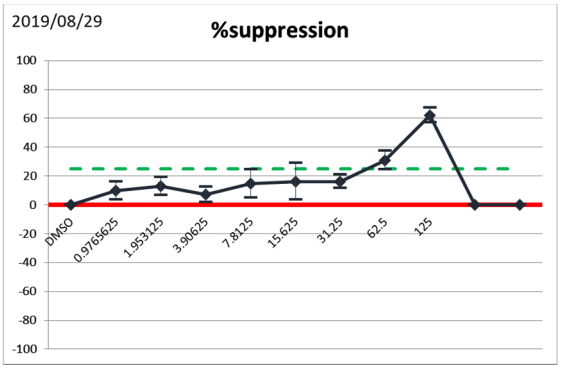

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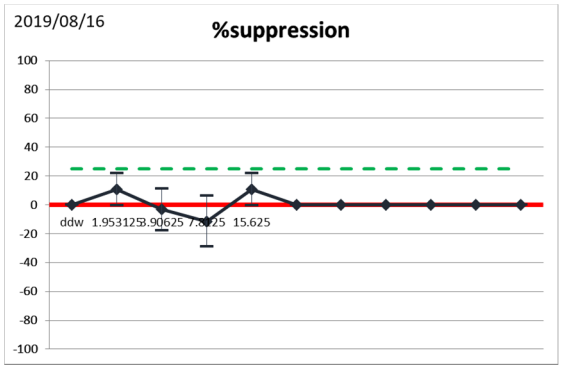

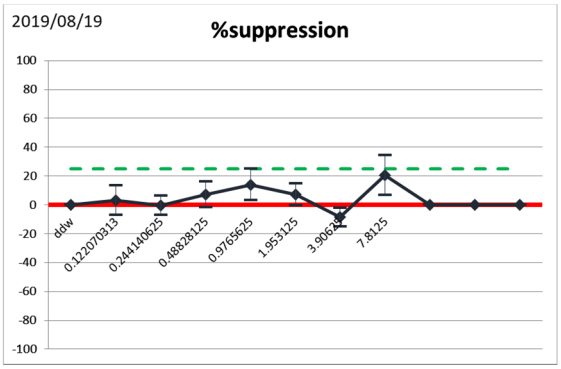

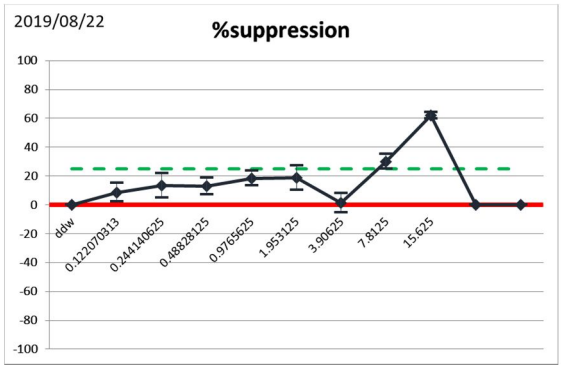

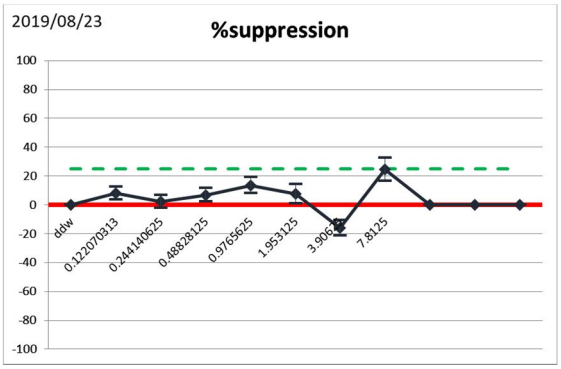

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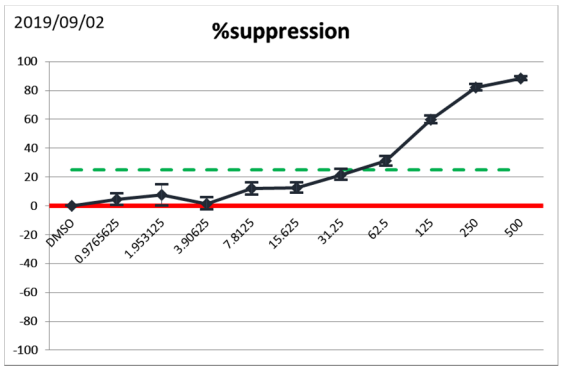

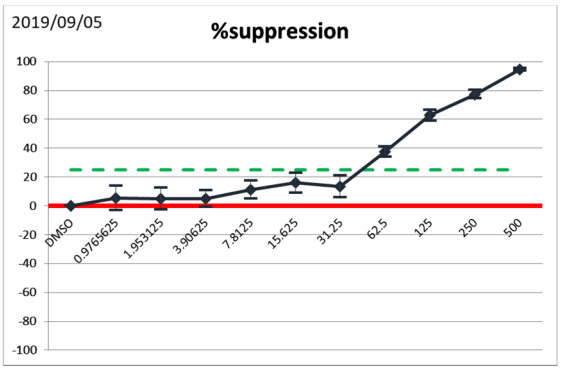

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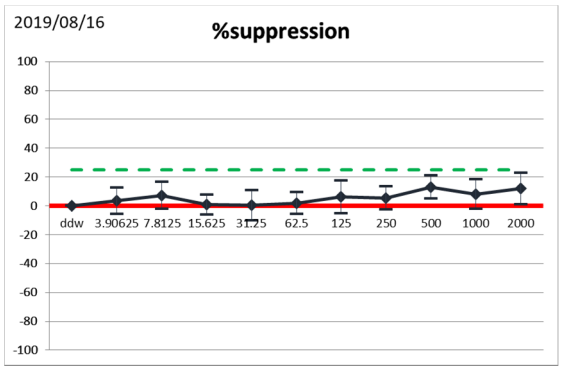

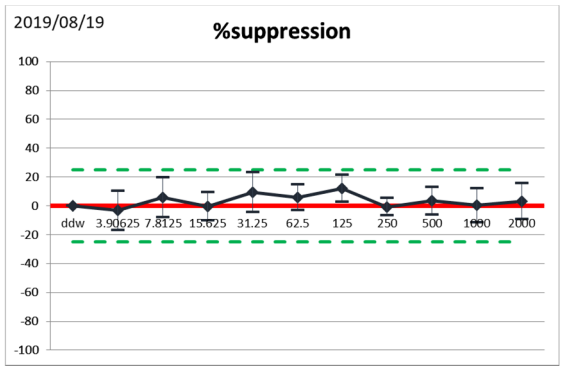

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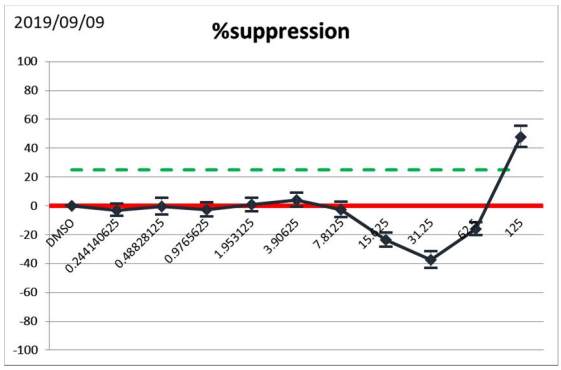

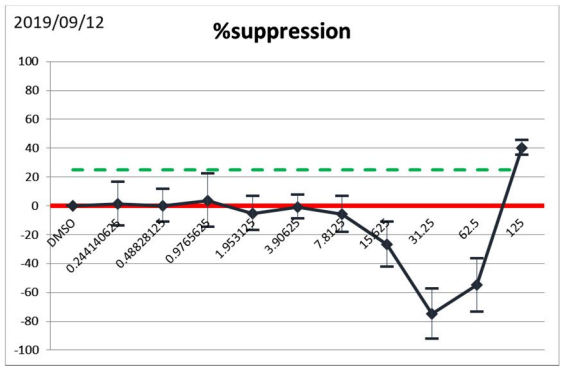

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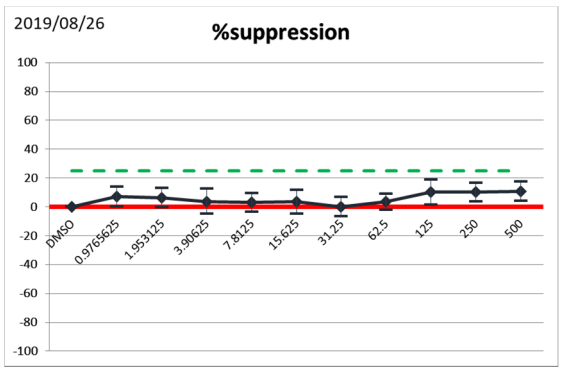

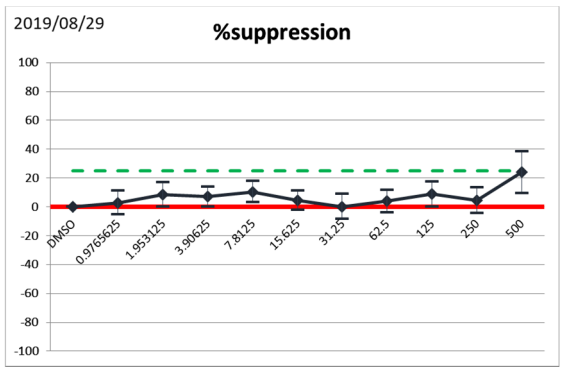

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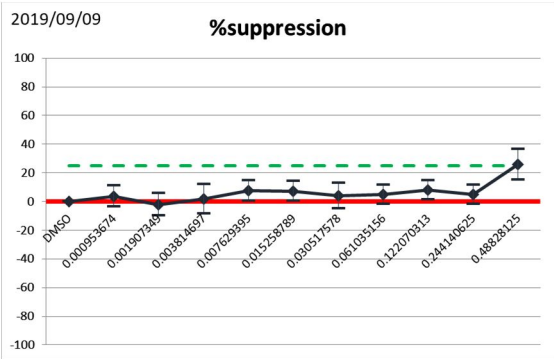

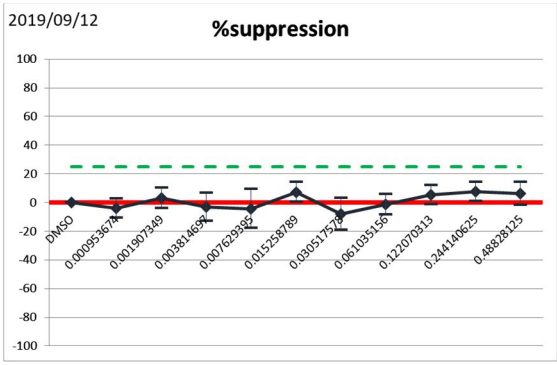

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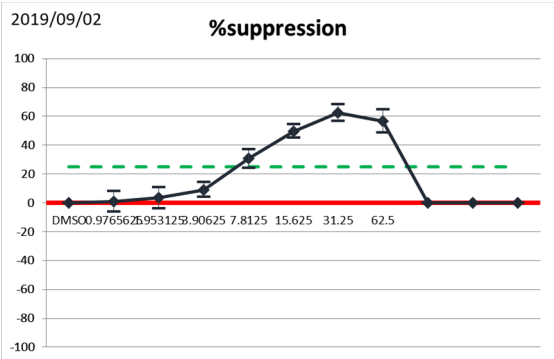

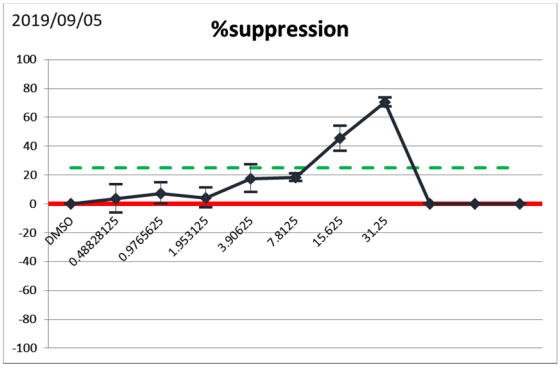

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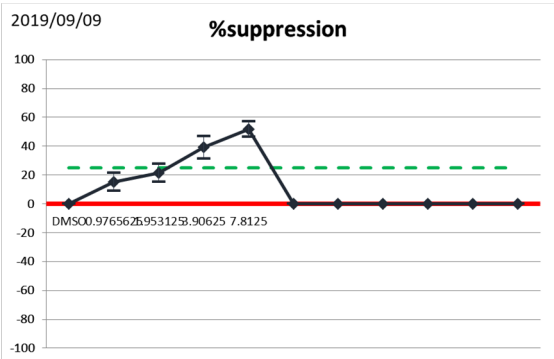

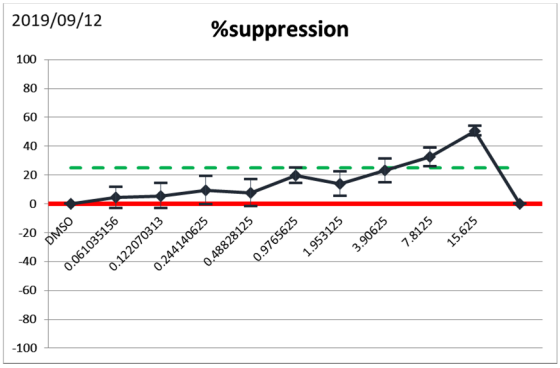

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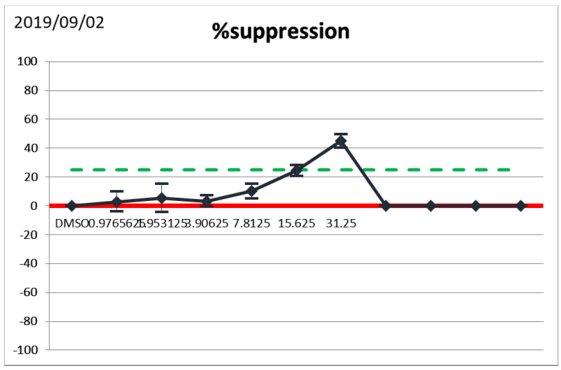

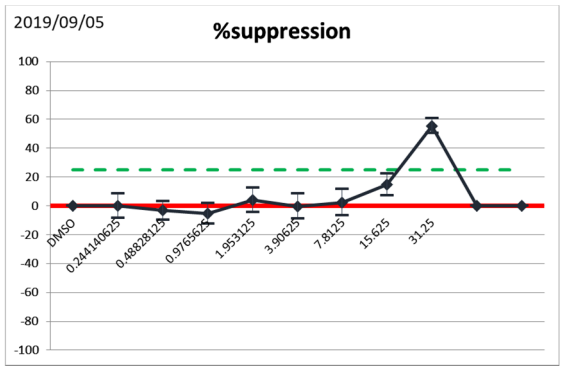

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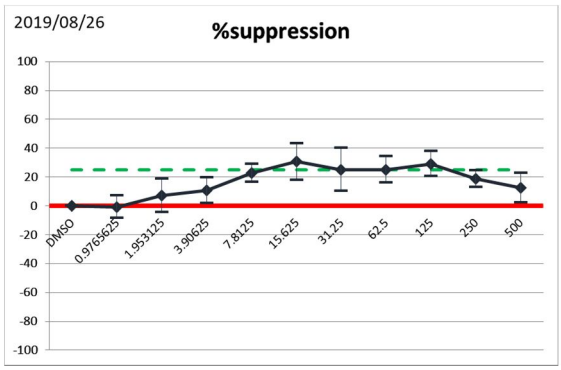

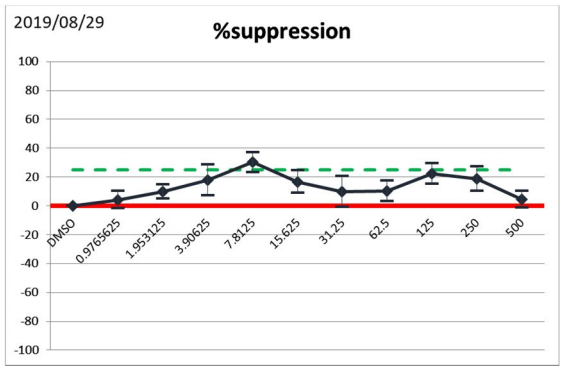

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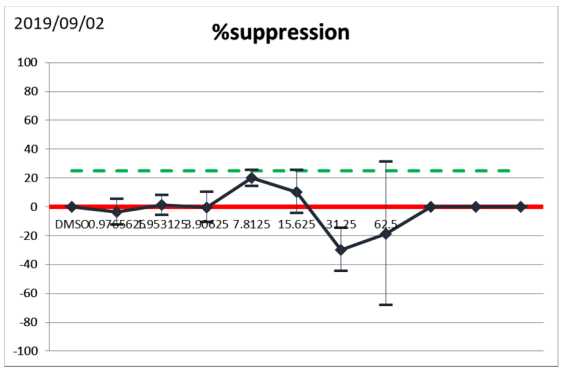

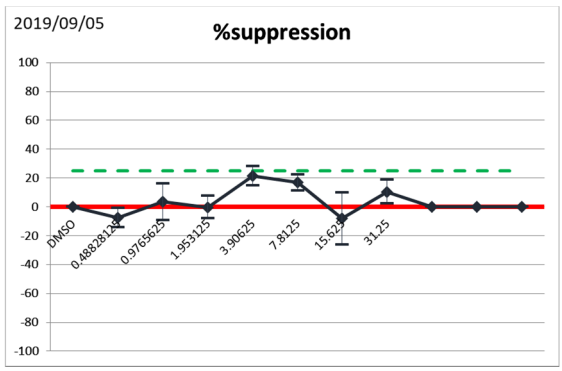

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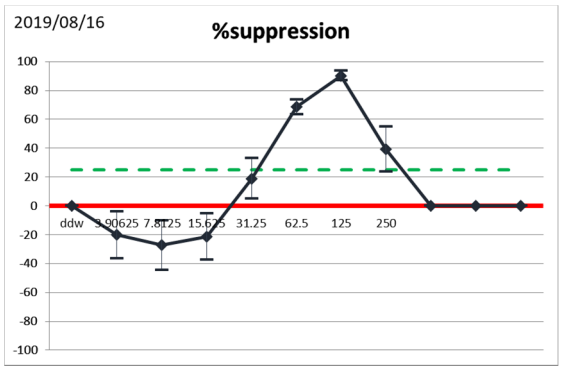

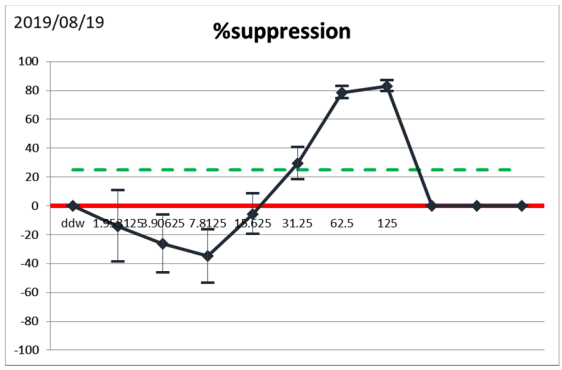

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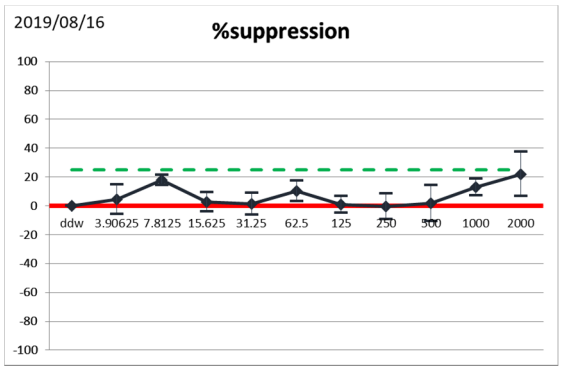

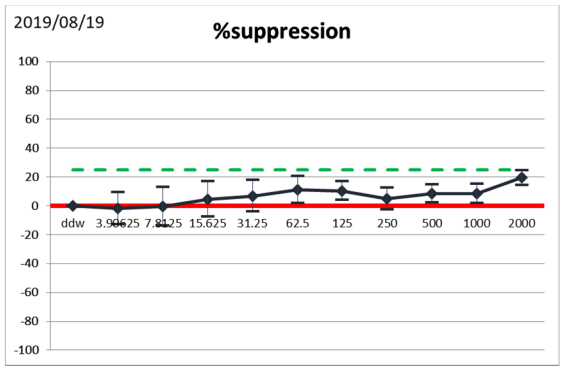

表２．試験化学物質の評価

コード番号	試験回数				判定
	1	2	3	4	
MTC305	S	S			S
MTC301	S	N	N		N
MTC318	S	S			S
MTC307	S	S			S
MTC302	N	N			N
MTC312	R	N	N		N
MTC303	S	S			S
MTC322	R	N	S	N	N
MTC313	S	S			S
MTC317	N	N			N
MTC324	N	N			N
MTC309	N	N			N
MTC316	N	N			N
MTC315	S	S			S
MTC323	S	S			S
MTC314	S	S			S
MTC306	S	S			S
MTC311	N	N			N
MTC304	S	S			S
MTC327	N	N			N

S; Suppression, N; No Effect, R; Reject.

（具体的かつ詳細に記入すること）

厚生労働科学研究費補助金（化学物質リスク研究事業）
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
（ H30-化学-一般-001 ）

分担研究報告書
免疫毒性評価試験法（Multi-ImmunoTox assay）国際標準化へ向けた評価法の検討

研究分担者　安野　理恵
産業技術総合研究所　細胞分子工学研究部門

研究要旨

THP-1 細胞における IL-1β転写活性抑制を指標とした化学物質免疫毒性評価系のバリデーション試験を実施した。今年度は、昨年度実施した Phase1 試験（施設内再現性）に引き続き、コード化された 20 化学物質を用いて Phase2 試験を実施し、施設間再現性を確認した。

A．研究目的

東北大学を中心に開発された Multi-ImmunoTox assay（MITA）は、多色発光タンパク質による *in vitro* 免疫毒性評価試験法で、各種の毒性評価発光細胞によって構成される。本研究では、化学物質の免疫毒性評価のための MITA 試験法確立と OECD ガイドライン化を目指してバリデーション試験を実施する。MITA の構成要素の一つである TGCHAC-4A（THP-G1β）細胞は、THP-1 細胞において IL-1β転写活性を定量化する。本年度は TGCHAC-4A 細胞を用いた試験法の確立を目指し、バリデーションの Phase2 試験の実施を目的とする。

B．研究方法

IL-1β と内部標準としての G3PDH プロモーターに SLG および SLR ルシフェラーゼ遺伝子をそれぞれ繋いだ人工染色体発現ベクターを THP-1 細胞に導入した 2 色発光細胞株 TGCHAC-4A（THP-G1β）を用いて試験を行った。化学物質の免疫毒性試験法における細胞培養方法、被験物質調整及び添加方法、及びルシフェラーゼアッセイの方法,試験結果の判定基準等については Multi-Immuno Tox Assay protocol 案 Ver.009E に準ずる。発光の計測には、多検体発光測定装置 Phelios（ATTO 社）を用いた。Phase2 試験には、国際バリデーション実行委員会にて選定された 20 種類のコード化した被験物質を供試した。各物質において 2 回の同一結果を得られるまで試験を繰り返して被験物質を判定した。

（倫理面への配慮）

倫理的な問題が生じる実験を実施しておらず、特に配慮すべき問題はない。

C．研究結果

コード化された 20 種類の化学物質の試験結果（図 1）および判定結果(表 1)を示す。得られた試験結果のうち、Multi-Immuno

Tox Assay protocol 案 Ver.009E の Acceptance criterion をクリアしたものに関して、判定基準に準じ“suppression”または“non-suppression”の判定を行った。各被験試薬に対して、同一結果が 2 回得られた時点で最終判定とした。Acceptance criterion をクリアしなかった結果に関しては判定不可(表 1)とした。

D．考察

昨年度実施した Phase1(施設内再現性)に引き続き、Phase2(施設間再現性)試験をコード化された 20 被験試薬を対して実施した。得られた判定結果を、他のバリデーション実施機関の結果を比較したところ、20 被験物質中 16 物質で結果が一致し（80%一致)、良好な施設間再現性が確認された。結果が一致しなかった物質に関しても各機関の%suppression カーブを比較すると、ほぼ同様の傾向が見られた。例えば MIB202 に関して、他の 2 機関が Non-suppression に対し、我々の結果では Suppression 判定であったが、東北大学の試験結果を比較すると、被験物質の濃度 1.953mg/ml においてのみ%suppression 25%を超える positive を示し、それより低濃度域では Non-suppression という結果が一致している。際どいところで「positive を含む 3 点の連続的上昇」有無の判断が分かれ、Criteria に準じた判定では異なる結果となったものの、実際の試験結果はほぼ同一であった。一方、産総研四国では被験物質の希釈率が高く、1.953mg/ml 未満の濃度で判定を行っており、同じ希釈率で判断した場合、同様の試験結果が得られる可能性もある。Phase2 試験では、参画施設間の再現性は非常によく一致しており、不一致だった物質に関しては判断基準の境界のもの、または希釈濃度の違いによるものがほとんどであった。

E．結論

免疫毒性評価試験法（Multi-ImmunoTox assay）の国際標準化を目指し、TGCHAC-4A 細胞を用いた THP-G1β転写発現抑制を指標とした評価試験のバリデーション試験（Phase2）を実施した。バリデーション参加の 3 機関の結果を検討した結果、80%の物質で同一の結果が得られ、施設間再現性が確認された。

F．健康危険情報

該当なし

G．研究発表

1．論文発表

1) Yutaka Kimura, Rie Yasuno, Mika Watanabe, Miwako Kobayashi, Tomoko Iwaki, Chizu Fujimura, Yoshihiro Ohmiya, Kohji Yamakage, Yoshihiro Nakajima, Mayumi Kobayashi, Nana Mashimo, Yumi Takagi, Takashi Omori, Emanuela Corsini, Dori Germolec, Tomoaki Inoue, Erwin L. Rogen, Hajime Kojima, Setsuya Aiba: An internationalvalidationstudyoftheIL-2LucassayforevaluatingthepotentialimmunotoxiceffectsofchemicalsonTcellsandaproposalforreferencedataforimmunotoxicchemicals. Toxicology in Vitro 66(2020)

2．学会発表

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)

H．知的財産権の出願・登録状況

（予定を含む。）

1．特許取得：該当なし

2．実用新案登録：該当なし

3.その他：該当なし

図1　Phase2試験；各被験試薬の解析結果

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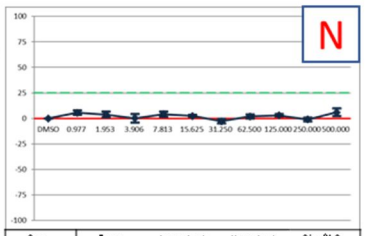
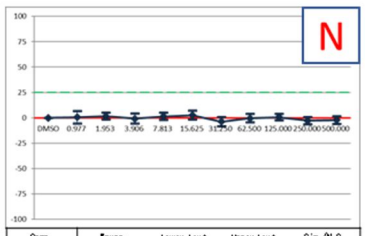
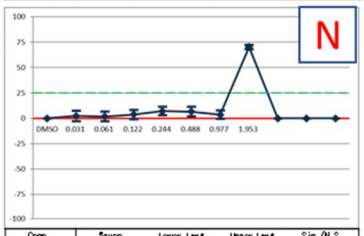


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.977	5.566	3.534	7.597	Sig.
1.953	3.816	1.130	6.503	Sig.
3.906	0.119	-4.093	4.331	N.S.
7.813	4.034	1.395	6.672	Sig.
15.625	2.335	1.402	3.269	Sig.
31.250	-2.758	-4.848	-0.669	Sig.
62.500	1.916	-0.003	3.834	N.S.
125.000	3.019	1.801	4.236	Sig.
250.000	-1.101	-2.905	0.704	N.S.
500.000	5.994	2.292	9.697	Sig.

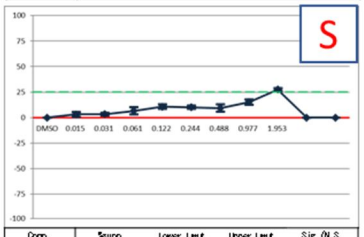


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.977	0.607	-5.500	6.714	N.S.
1.953	1.592	-1.856	5.039	N.S.
3.906	-0.767	-5.711	4.177	N.S.
7.813	1.420	-2.216	5.057	N.S.
15.625	2.597	-1.764	6.957	N.S.
31.250	-3.773	-8.291	0.744	N.S.
62.500	-0.348	-4.415	3.718	N.S.
125.000	0.480	-2.692	3.652	N.S.
250.000	-2.846	-6.537	0.844	N.S.
500.000	-2.297	-6.111	1.516	N.S.

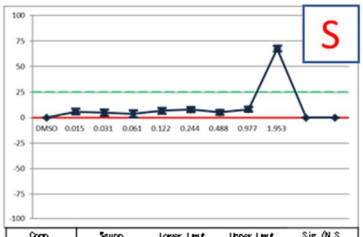
202



Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
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.

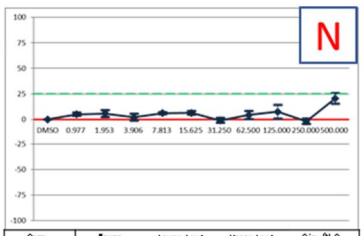


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.015	3.175	0.851	5.499	Sig.
0.031	3.233	1.771	4.695	Sig.
0.061	6.563	2.991	10.136	Sig.
0.122	10.704	8.638	12.770	Sig.
0.244	10.079	8.543	11.616	Sig.
0.488	9.369	5.914	12.824	Sig.
0.977	15.209	12.529	17.889	Sig.
1.953	27.478	26.029	28.926	Sig.

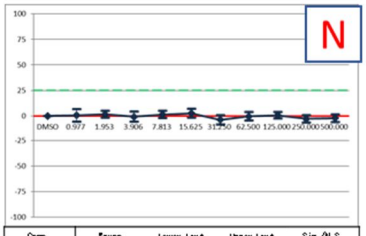


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.015	5.648	2.873	8.422	Sig.
0.031	4.895	1.981	7.810	Sig.
0.061	3.665	0.405	6.924	Sig.
0.122	6.648	3.839	9.457	Sig.
0.244	7.885	5.455	10.316	Sig.
0.488	5.190	2.825	7.554	Sig.
0.977	8.156	5.802	10.511	Sig.
1.953	67.597	64.817	70.377	Sig.

203

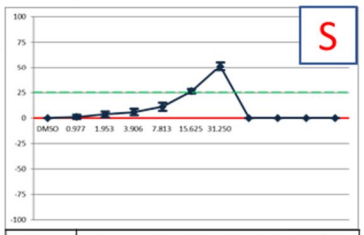


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.977	4.976	3.255	6.698	Sig.
1.953	5.629	2.432	8.826	Sig.
3.906	1.966	-1.442	5.373	N.S.
7.813	6.096	5.088	7.104	Sig.
15.625	6.224	4.437	8.010	Sig.
31.250	-0.976	-3.394	1.442	N.S.
62.500	4.222	0.135	8.308	Sig.
125.000	7.461	0.735	14.188	Sig.
250.000	-1.812	-4.459	0.836	N.S.
500.000	20.453	15.420	25.487	Sig.

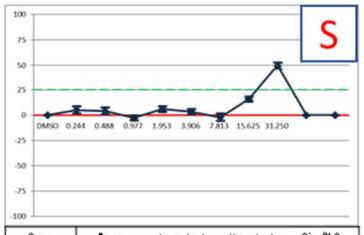


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.977	0.607	-5.500	6.714	N.S.
1.953	1.592	-1.856	5.039	N.S.
3.906	-0.767	-5.711	4.177	N.S.
7.813	1.420	-2.216	5.057	N.S.
15.625	2.597	-1.764	6.957	N.S.
31.250	-3.773	-8.291	0.744	N.S.
62.500	-0.348	-4.415	3.718	N.S.
125.000	0.480	-2.692	3.652	N.S.
250.000	-2.846	-6.537	0.844	N.S.
500.000	-2.297	-6.111	1.516	N.S.

204

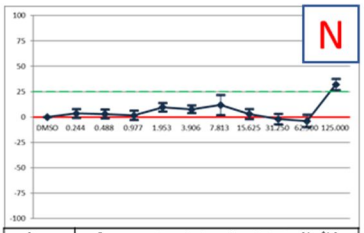


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.977	1.005	-0.920	2.929	N.S.
1.953	3.429	0.834	6.024	Sig.
3.906	5.693	2.591	8.795	Sig.
7.813	10.995	6.942	15.047	Sig.
15.625	26.250	24.081	28.418	Sig.
31.250	51.250	47.211	55.289	Sig.

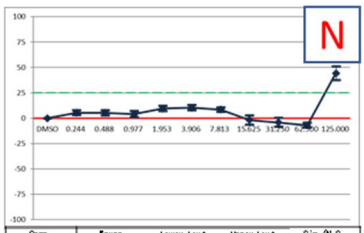


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.244	4.884	1.374	8.394	Sig.
0.488	3.985	0.732	7.238	Sig.
0.977	-2.874	-5.225	-0.522	Sig.
1.953	5.844	3.163	8.525	Sig.
3.906	3.101	0.523	5.679	Sig.
7.813	-2.189	-5.664	1.286	N.S.
15.625	15.795	13.418	18.173	Sig.
31.250	49.182	45.821	52.542	Sig.

205

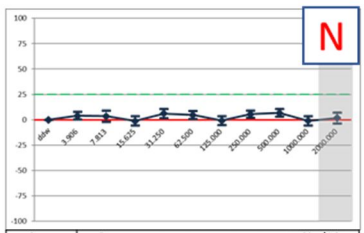


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.244	3.429	-1.078	7.936	N.S.
0.488	3.014	-1.457	7.485	N.S.
0.977	1.602	-3.047	6.251	N.S.
1.953	9.704	5.564	13.844	Sig.
3.906	7.519	3.781	11.257	Sig.
7.813	11.902	1.988	21.816	Sig.
15.625	2.842	-2.152	7.837	N.S.
31.250	-2.084	-7.092	2.924	N.S.
62.500	-3.970	-10.198	2.257	N.S.
125.000	31.978	26.377	37.580	Sig.

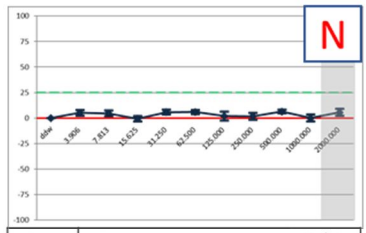


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.244	5.382	3.005	7.718	Sig.
0.488	5.271	2.700	7.843	Sig.
0.977	4.000	1.110	6.890	Sig.
1.953	9.555	6.835	12.274	Sig.
3.906	10.361	7.780	12.941	Sig.
7.813	8.400	6.241	10.558	Sig.
15.625	-1.874	-6.393	2.645	N.S.
31.250	-4.255	-8.626	0.116	N.S.
62.500	-6.973	-9.327	-4.619	Sig.
125.000	44.336	37.651	51.020	Sig.

206

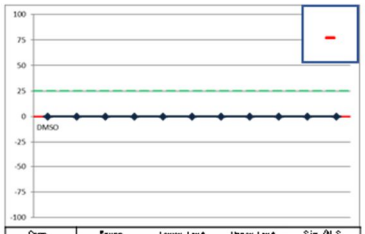


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
ddw	0	0	0	
3.906	4.010	0.361	7.659	Sig.
7.813	3.513	-2.037	9.063	N.S.
15.625	-1.250	-5.788	3.288	N.S.
31.250	6.021	1.462	10.581	Sig.
62.500	4.620	0.810	8.431	Sig.
125.000	-0.804	-5.229	3.621	N.S.
250.000	5.456	1.943	8.969	Sig.
500.000	6.743	2.948	10.537	Sig.
1000.000	-1.002	-5.509	3.505	N.S.
2000.000	1.646	-3.730	7.022	N.S.

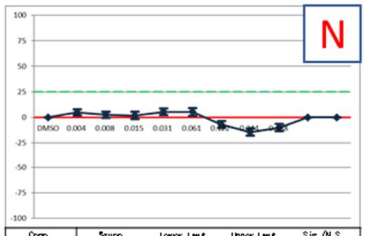


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
ddw	0	0	0	
3.906	5.062	2.391	7.734	Sig.
7.813	4.584	1.828	7.341	Sig.
15.625	-0.599	-3.229	2.032	N.S.
31.250	5.765	3.498	8.032	Sig.
62.500	5.913	3.995	7.831	Sig.
125.000	1.894	-2.545	6.333	N.S.
250.000	1.686	-1.789	5.161	N.S.
500.000	6.246	4.435	8.057	Sig.
1000.000	0.002	-3.353	3.357	N.S.
2000.000	5.581	2.048	9.114	Sig.

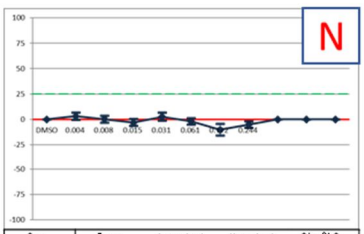
208



Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	

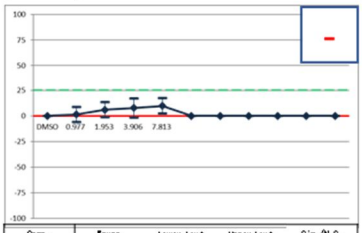


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.004	4.807	1.779	7.834	Sig.
0.008	2.540	-0.449	5.529	N.S.
0.015	1.773	-1.717	5.263	N.S.
0.031	5.335	2.229	8.441	Sig.
0.061	5.231	1.487	8.975	Sig.
0.122	-7.094	-10.575	-3.592	Sig.
0.244	-14.446	-18.077	-10.815	Sig.
0.488	-10.112	-13.656	-6.568	Sig.

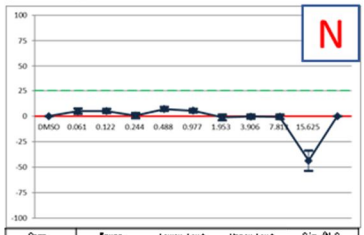


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.004	3.036	-0.463	6.536	N.S.
0.008	0.105	-3.291	3.501	N.S.
0.015	-3.293	-6.676	0.090	N.S.
0.031	2.537	-1.392	6.466	N.S.
0.061	-2.106	-5.343	1.130	N.S.
0.122	-10.291	-16.081	-4.502	Sig.
0.244	-5.251	-8.504	-1.999	Sig.

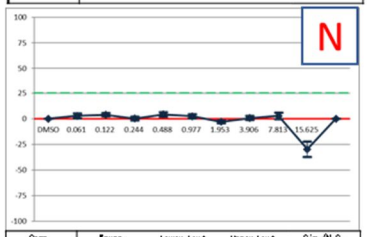
210



Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
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.



Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.061	4.779	2.121	7.436	Sig.
0.122	4.849	2.889	6.809	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.



Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
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.

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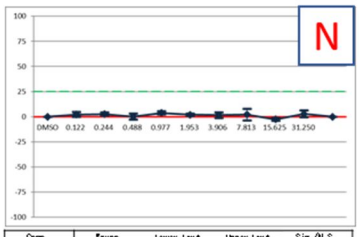
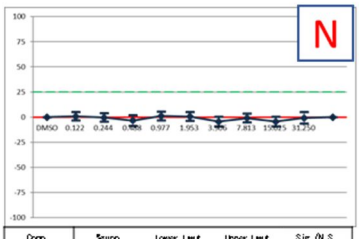
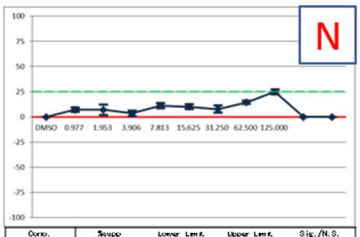


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.122	2.192	-0.248	4.631	N.S.
0.244	2.625	1.057	4.194	Sig.
0.488	0.116	-2.726	2.959	N.S.
0.977	3.806	2.181	5.432	Sig.
1.953	1.926	0.820	3.031	Sig.
3.906	1.559	-1.254	4.371	N.S.
7.813	2.091	-3.542	7.724	N.S.
15.625	-2.605	-4.021	-1.188	Sig.
31.250	2.814	-0.629	6.257	N.S.

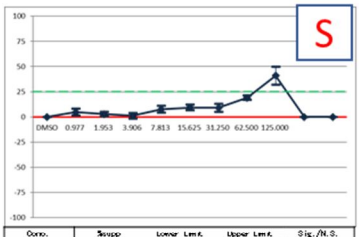


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.122	0.799	-3.375	4.972	N.S.
0.244	-0.379	-4.549	3.790	N.S.
0.488	-3.472	-8.927	1.983	N.S.
0.977	1.084	-3.167	5.335	N.S.
1.953	0.527	-3.833	4.887	N.S.
3.906	-4.429	-9.263	0.406	N.S.
7.813	-0.976	-5.302	3.350	N.S.
15.625	-4.437	-9.152	0.277	N.S.
31.250	-0.770	-6.433	4.892	N.S.

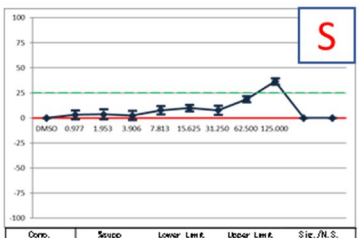
214



Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.977	7.164	5.099	9.230	Sig.
1.953	7.024	2.012	12.037	Sig.
3.906	3.489	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.



Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
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	5.178	13.059	Sig.
62.500	18.959	16.712	21.205	Sig.
125.000	40.944	31.895	49.994	Sig.



Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
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.

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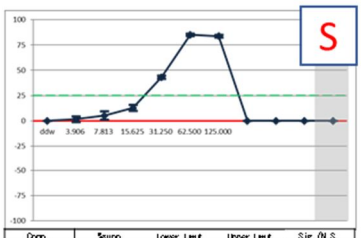
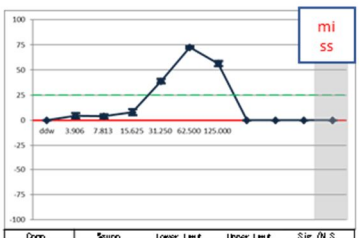
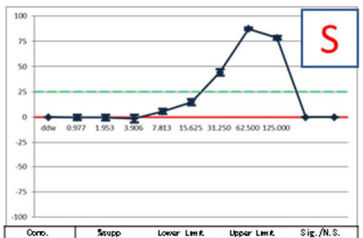


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
ddw	0	0	0	
3.906	1.489	-1.203	4.181	N.S.
7.813	5.174	1.139	9.208	Sig.
15.625	12.570	9.604	15.536	Sig.
31.250	43.068	41.355	44.781	Sig.
62.500	85.077	84.073	86.081	Sig.
125.000	83.759	82.255	85.263	Sig.



Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
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.



Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
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.

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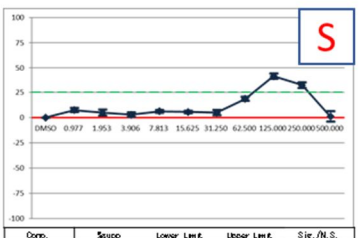
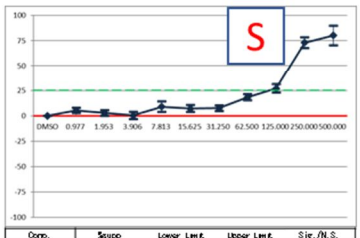
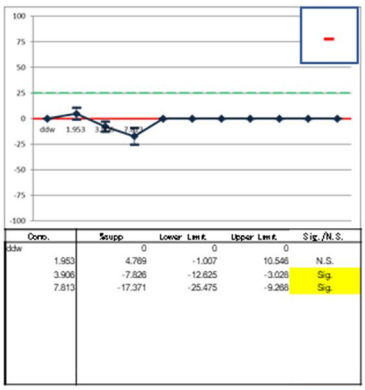


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.977	7.336	5.411	9.260	Sig.
1.953	4.802	1.835	7.769	Sig.
3.906	2.798	0.489	5.127	Sig.
7.813	6.013	4.534	7.492	Sig.
15.625	5.506	4.285	6.727	Sig.
31.250	4.792	2.382	7.202	Sig.
62.500	18.391	16.506	20.276	Sig.
125.000	40.774	38.135	43.413	Sig.
250.000	31.968	29.125	34.851	Sig.
500.000	0.976	-4.098	6.049	N.S.

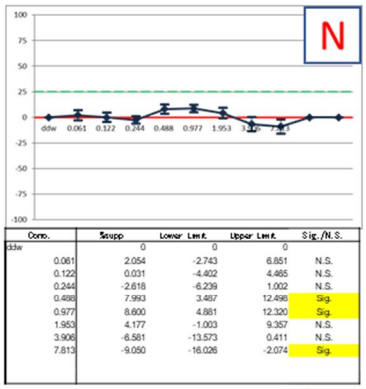


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.977	5.096	2.547	7.645	Sig.
1.953	2.662	-0.016	5.340	N.S.
3.906	0.273	-3.374	3.920	N.S.
7.813	8.974	3.865	14.083	Sig.
15.625	7.017	3.643	10.392	Sig.
31.250	7.408	4.813	10.004	Sig.
62.500	18.228	15.251	21.205	Sig.
125.000	27.006	22.768	31.245	Sig.
250.000	73.060	67.695	78.425	Sig.
500.000	80.152	70.545	89.759	Sig.

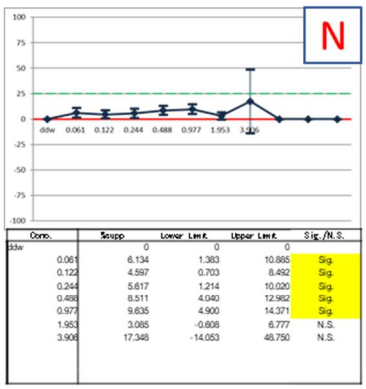
218



Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
ddw	0	0	0	
1.953	4.789	-1.007	10.586	N.S.
3.906	-7.826	-12.625	-3.028	Sig.
7.813	-17.371	-25.475	-9.268	Sig.

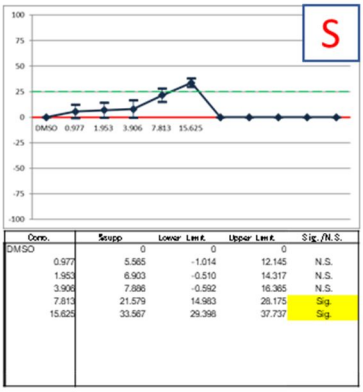


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
ddw	0	0	0	
0.061	2.054	-2.743	6.851	N.S.
0.122	0.031	-4.402	4.465	N.S.
0.244	-2.618	-6.239	1.002	N.S.
0.488	7.993	3.487	12.498	Sig.
0.977	8.600	4.881	12.320	Sig.
1.953	4.177	-1.003	9.357	N.S.
3.906	-6.581	-13.573	0.411	N.S.
7.813	-9.050	-16.026	-2.074	Sig.

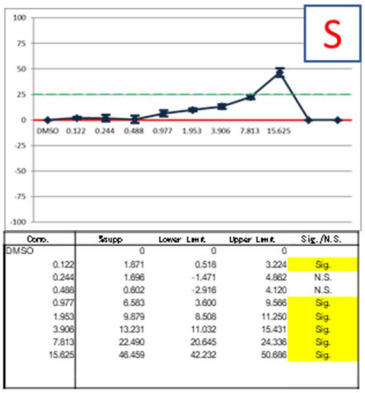


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
ddw	0	0	0	
0.061	6.134	1.383	10.885	Sig.
0.122	4.597	0.703	8.492	Sig.
0.244	5.617	1.214	10.020	Sig.
0.488	8.511	4.040	12.982	Sig.
0.977	9.635	4.900	14.371	Sig.
1.953	3.085	-0.808	6.777	N.S.
3.906	17.348	-14.053	48.750	N.S.

219

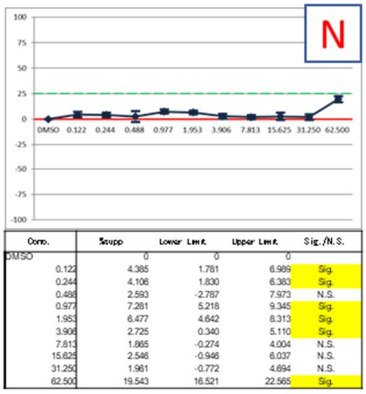


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.977	5.565	-1.014	12.145	N.S.
1.953	6.903	-0.510	14.317	N.S.
3.906	7.886	-0.592	16.365	N.S.
7.813	21.579	14.983	28.175	Sig.
15.625	33.567	29.398	37.737	Sig.

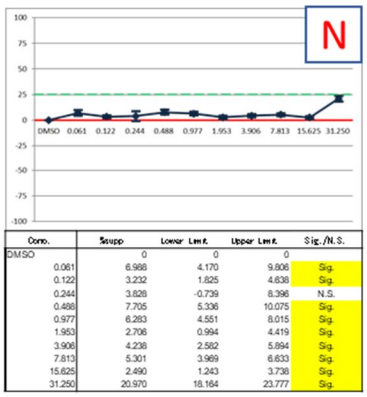


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.122	1.871	0.518	3.224	Sig.
0.244	1.696	-1.471	4.862	N.S.
0.488	0.602	-2.916	4.120	N.S.
0.977	6.583	3.600	9.566	Sig.
1.953	9.879	8.508	11.250	Sig.
3.906	13.231	11.032	15.431	Sig.
7.813	22.490	20.645	24.336	Sig.
15.625	46.459	42.232	50.686	Sig.

220

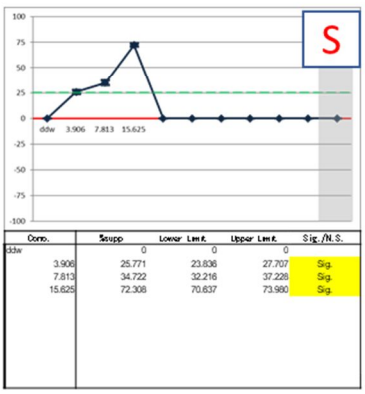


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.122	4.385	1.781	6.989	Sig.
0.244	4.106	1.830	6.383	Sig.
0.488	2.593	-2.787	7.973	N.S.
0.977	7.281	5.218	9.345	Sig.
1.953	6.477	4.642	8.313	Sig.
3.906	2.725	0.340	5.110	Sig.
7.813	1.865	-0.274	4.004	N.S.
15.625	2.546	-0.946	6.037	N.S.
31.250	1.961	-0.772	4.694	N.S.
62.500	19.543	16.521	22.565	Sig.

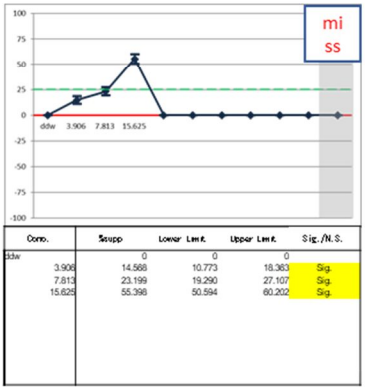


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.061	6.988	4.170	9.806	Sig.
0.122	3.232	1.825	4.638	Sig.
0.244	3.828	-0.739	8.396	N.S.
0.488	7.705	5.336	10.075	Sig.
0.977	6.283	4.551	8.015	Sig.
1.953	2.706	0.994	4.419	Sig.
3.906	4.238	2.582	5.894	Sig.
7.813	5.301	3.989	6.633	Sig.
15.625	2.490	1.243	3.738	Sig.
31.250	20.970	18.164	23.777	Sig.

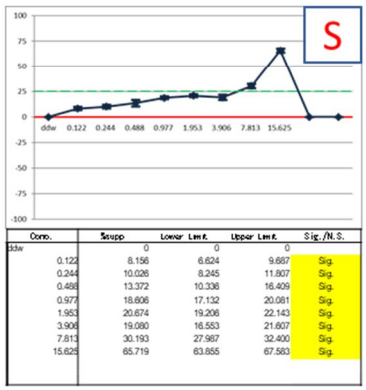
221



Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
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.

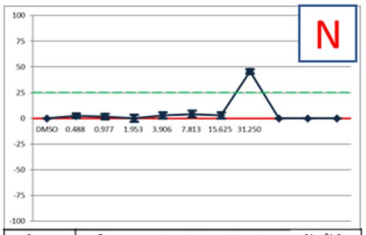


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
ddw	0	0	0	
3.906	14.568	10.773	18.363	Sig.
7.813	23.199	19.290	27.107	Sig.
15.625	55.398	50.594	60.202	Sig.

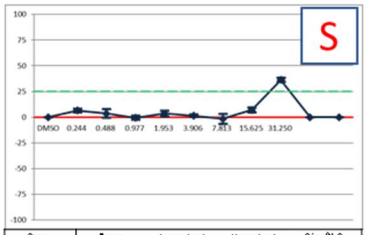


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
ddw	0	0	0	
0.122	8.156	6.624	9.687	Sig.
0.244	10.026	8.245	11.807	Sig.
0.488	13.372	10.336	16.409	Sig.
0.977	18.606	17.132	20.081	Sig.
1.953	20.674	19.206	22.143	Sig.
3.906	19.080	16.553	21.607	Sig.
7.813	30.193	27.987	32.400	Sig.
15.625	65.719	63.855	67.583	Sig.

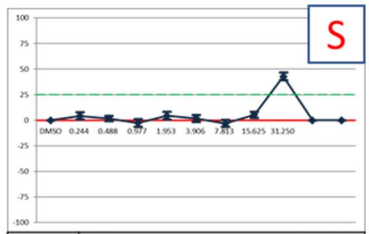
222



Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
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.

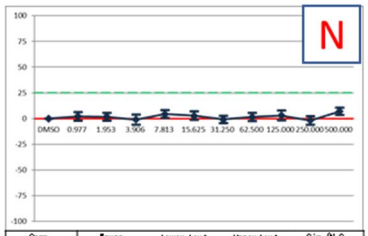


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.244	6.495	4.707	8.283	Sig.
0.488	3.597	-0.703	7.898	N.S.
0.977	-0.650	-2.589	1.289	N.S.
1.953	3.434	0.490	6.378	Sig.
3.906	1.421	0.134	2.708	Sig.
7.813	-1.645	-6.311	3.021	N.S.
15.625	7.043	4.652	9.433	Sig.
31.250	36.319	34.495	38.144	Sig.

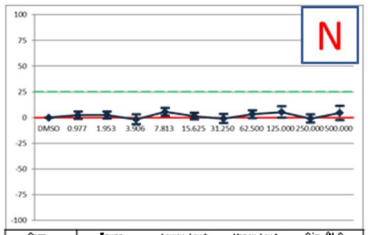


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.244	4.133	0.564	7.702	Sig.
0.488	1.684	-0.804	4.172	N.S.
0.977	-2.691	-6.327	0.946	N.S.
1.953	4.301	0.487	8.115	Sig.
3.906	1.789	-1.626	5.163	N.S.
7.813	-3.170	-6.619	0.278	N.S.
15.625	5.309	2.261	8.358	Sig.
31.250	42.854	39.141	46.567	Sig.

223

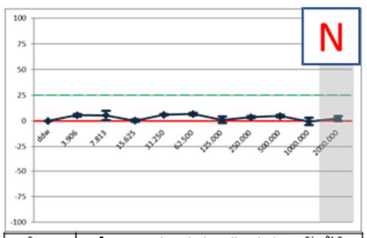


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.977	2.128	-2.119	6.376	N.S.
1.953	1.709	-1.939	5.356	N.S.
3.906	-1.097	-6.020	3.825	N.S.
7.813	4.355	0.593	8.117	Sig.
15.625	2.744	-1.331	6.819	N.S.
31.250	-0.898	-4.596	2.799	N.S.
62.500	1.524	-2.410	5.457	N.S.
125.000	2.959	-1.896	7.814	N.S.
250.000	-1.906	-6.138	2.327	N.S.
500.000	6.811	3.245	10.378	Sig.

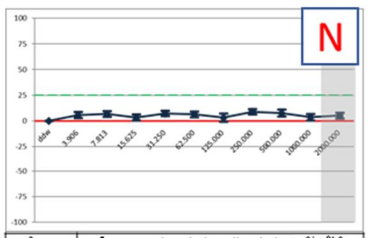


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
DMSO	0	0	0	
0.977	2.375	-1.195	5.945	N.S.
1.953	2.479	-0.752	5.709	N.S.
3.906	-1.739	-6.559	3.081	N.S.
7.813	5.515	1.700	9.329	Sig.
15.625	1.324	-1.875	4.524	N.S.
31.250	-0.975	-5.299	3.349	N.S.
62.500	3.300	-0.456	7.057	N.S.
125.000	5.329	-0.146	10.803	N.S.
250.000	-0.964	-4.783	2.855	N.S.
500.000	4.518	-2.515	11.550	N.S.

224

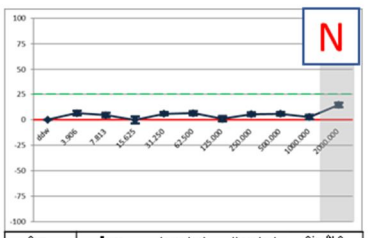


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
ddw	0	0	0	
3.906	5.497	4.335	6.658	Sig.
7.813	5.244	0.662	9.825	Sig.
15.625	0.116	-1.030	1.262	N.S.
31.250	5.817	5.218	6.416	Sig.
62.500	6.656	5.396	7.915	Sig.
125.000	0.939	-2.180	4.058	N.S.
250.000	3.619	2.689	4.569	Sig.
500.000	4.744	3.607	5.881	Sig.
1000.000	-0.612	-4.146	2.923	N.S.
2000.000	2.289	-0.223	4.802	N.S.

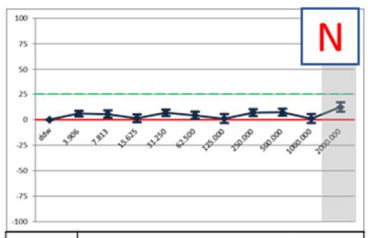


Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
ddw	0	0	0	
3.906	5.551	2.661	8.440	Sig.
7.813	6.616	3.789	9.444	Sig.
15.625	3.152	0.096	6.208	Sig.
31.250	7.161	4.698	9.624	Sig.
62.500	6.261	3.352	9.170	Sig.
125.000	3.148	-0.890	7.186	N.S.
250.000	8.798	6.049	11.548	Sig.
500.000	7.524	4.114	10.934	Sig.
1000.000	3.468	0.342	6.593	Sig.
2000.000	5.064	2.149	7.978	Sig.

227



Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
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.



Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.
ddw	0	0	0	
3.906	5.810	2.955	8.666	Sig.
7.813	5.398	1.783	9.013	Sig.
15.625	1.258	-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.

表 1　Phase2 試験；評価結果

MIB	1st	2nd	3rd	**Judge**
201	N	N	-	**N**
202	N	S	S	**S**
203	N	N	-	**N**
204	S	S	-	**S**
205	N	N	-	**N**
206	N	N	-	**N**
208	- 判断不可	N	N	**N**
210	- 判断不可	N	N	**N**
211	N	N	-	**N**
214	N	S	S	**S**
216	S	miss 希釈を間違えた	S	**S**
217	S	S	-	**S**
218	- 判断不可	N	N	**N**
219	S	S	-	**S**
220	N	N	-	**N**
221	S	miss 希釈を間違えた	S	**S**
222	N	S	S	**S**
223	N	N	-	**N**
224	N	N	-	**N**
227	N	N	-	**N**

S; Suppression

N; Non-suppression

厚生労働科学研究費補助金（化学リスク研究事業）
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
平成 31 年度分担研究報告書
分担研究報告書

分担研究報告書
免疫毒性評価試験法Multi-ImmunoToxicity assayの判定アルゴリズムの検討

研究分担者　大森崇
神戸大学医学研究科　生物統計学分野

研究要旨

[背景と目的] Multi-Immuno Tox Assay(以下、MITA)は、化学物質がヒトの免疫系に与える影響を評価することを目標とする *in vitro* で試験法である。現在、バリデーション研究が実施されている IL-2 Luc アッセイ及び IL-1β Luc アッセイの免疫毒性判定の方法は、過去の研究に基づき経験的に設定されているが、判定方法は複雑である。将来、これらのアッセイが広く利用されるようにするために自動的に判定できるアルゴリズムが提示されることが望ましい。IL-1β Luc アッセイのバリデーション研究が進むにつれ、IL-1β Luc アッセイの判定方法が新たに設定されたため、新たな判定アルゴリズムを作成することを目的とする。

[方法] バリデーション研究を通して提案された判定方法について、アルゴリズムを作成し、統計解析ソフトにアルゴリズムを実装し適用した。

[結果] 2 つ前からの濃度における反応の指標とその 95%信頼区間、カットオフ値を用いて 5 つのステップからなる判定アルゴリズムを開発した。バリデーション研究で実施された実際のデータにアルゴリズムを適用し、判定結果が含まれる図を描き判定を確認した。

[結論] これまで濃度反応関係の図を参照しながら基準に照らし合わせていた判定について、判定ルールを実装化可能なアルゴリズムを開発できた。このアルゴリズムをデータシート上で実装することが今後の課題である。

A. 研究目的

Multi-Immuno Tox Assay(以下、MITA)は、化学物質が免疫系に与える影響を簡便に評価するための光レポーターを利用した *in vitro* 免疫毒性評価試験法である。現在、IL-2 Luc アッセイ及び IL-1β Luc アッセイを、経済協力開発機構（Organisation for Economic Co-operation and Development：OECD）の試験法ガイドライン（Test Guideline：TG）としての公定化を目指し、バリデーション研究が施行されている。

IL-1β Luc アッセイでは、特定の化学物質の発現を評価は独立した 3 回の実験から得られる測定値を用いて行われ、最終的には immunosurpression、no effect のいずれかの判定がされる。バリデーション研究を行う中で、このアッセイに関する実験方法だけでなく、判定方法についても検討が行われてきた。これまでに、

・特定のある一濃度で発現が生じることがあるため、濃度別の統計的有意性のみで判定を行うと高感度になりすぎる

・濃度依存性をある程度考慮したい
・他の試験法の判定で行われてきたように 3 回の実験の判定は独立に行うことが望ましい
・特定の濃度以上は判定を行わない
という意見を出し合い判定方法が決定された。

IL-1β Luc アッセイでは、測定値から各濃度別に計算される%suppression という指標についてその点推定値と 95%信頼区間とともに、カットオフ値と呼ばれる参照線を引いた図から読み取り判定を行う。このような判定は、濃度に対するその反応の図を必ず確認することになるという利点があるが、基準が複雑で誤りを犯しやすいという欠点も有する。

昨年度、判定アルゴリズムを報告したが、IL-1β Luc アッセイのバリデーション研究が進行するに伴い、IL-1β Luc アッセイに対する判定方法は先行してバリデーション研究が実施された IL-2 Luc アッセイとは別の判定方法を採用することが必要となった。本報告では IL-1β Luc アッセイに対する判定方法の説明とその判定を自動的に行うアルゴリズムを開発することを目的として行った検討について記載する。

B. 研究方法

IL-1β Luc アッセイのデータの特徴

IL-1β Luc アッセイでは、独立に実施された 3 回の実験が行われる。個々の実験は濃度 0(DMSO)群と各濃度群との対比較により、判定を行う。1 回の実験には、96 穴プレートが用いられ、溶媒を用いた濃度 0 と濃度 1～濃度 10 までの計 11 段階の濃度群の測定値が得られる。個々の濃度群では 4 回の繰り返しがある。これらの測定値から、濃度ごとに、濃度 0 に対する被験物質の抑制度合を示す%suppression という 1 つの要約指標を計算できる。測定値に繰り返しがあるため、誤差的な変動を考慮して 95%信頼区間を構成できる。

各実験から得られる測定値と指標について

1 回の実験において、96 穴プレートの各セルから SLG-LA（SLG ルシフェラーゼ活性)、SLO-LA（SLO ルシフェラーゼ活性)、SLR-LA（SLR ルシフェラーゼ活性）の 3 種類の発光に関する測定値が得られる。化学物質の評価において、第 i 番目の濃度（i=0,1,2,…,10）の第 j 番目の繰り返しの測定値をそれぞれ $SLG\text{-}LA_{ij}$ 、$SLO\text{-}LA_{ij}$、$SLR\text{-}LA_{ij}$ とする。

これらの測定値を用いて、判定のための指標が得られる。

$I.I.SLR\text{-}LA_i=(\overline{SLR\text{-}LA_i})\,/(\overline{SLR\text{-}LA_0})$、

$nSLG\text{-}LA_{ij}=SLG\text{-}LA_{ij}\,/\,SLR\text{-}LA_{ij}$、

$\%suppression_i$

$$=\left\{1-\left(\overline{nSLG-LA_i}\right)/\left(\overline{nSLG-LA_0}\right)\right\}\times 100$$、

ただし、

$$\overline{SLG-LA_i}=\sum_{j=1}^{n_i} SLG-LA_{ij}\,/\,n_i$$、

$$\overline{SLR-LA_i}=\sum_{j=1}^{n_i} SLR-LA_{ij}\,/\,n_i$$

であり、n_i は第 i 濃度の繰り返し数であり実質 4 である。

$\%suppression_i$ は、第 i 濃度での免疫毒性の程度を%換算した値である。この指標は基本的には平均値の比であるため、デルタ法を用いてその 95%信頼区間を構成することができる。

$I.I.SLR\text{-}LA_i$ は細胞の状態が正常かどうかを判断するための指標である。

IL-1β Luc アッセイの実験ごとの判定

アッセイの最終的な判定は各実験の判定である「Suppression」か「No effect」に基づき行われる。各実験の免疫毒性ありの判定は以下の 3 つの基準をすべて満たした場合からなる。

1. ある濃度の%suppression の値がカットオフ値上限以上でかつ 95%信頼区間の下限が 0 よりも大きい、もしくは ある濃度の%suppression がカットオフ値下限以下で 95%信頼区間の上限が 0 よりも小さい。(1)

2. (1)に対して

隣り合ういずれかの 2 濃度で 95%信頼区間の下限が0より大きくそれぞれの%suppressionの値がカットオフの上限より大きくなるか、95%信頼区間の上限が0より小さくでそれぞれの%suppressionの値がカットオフの上限より小さくとなる。(2-1)

または、ある 1 濃度で 95%信頼区間の下限が 0 より大きくなる場合にその濃度を含めた続く 3 濃度の%suppression の値が増加傾向を示すか、95%信頼区間の上限が 0 より小さくなる場合にその濃度を含めた続く3濃度の%suppressionの値が減少傾向を示す。ただし、この場合、%suppression の値が 0 をまたいでよいのは 1 濃度のみであり、0 をまたいだ濃度の 95%信頼区間の上限が 0 以下にならないもしくは下限が 0 以上にならない。(2-2)

3. I.I.-SLR-LA が 0.05 以上となる濃度が判定では有効となる濃度である。

<u>本研究の検討</u>

本研究では上記の判定アルゴリズムを導出して、統計ソフト SAS に実装した。

C．研究結果

<u>判定アルゴリズム</u>

基準(3)に対して有効な濃度の測定値を用いることにする。

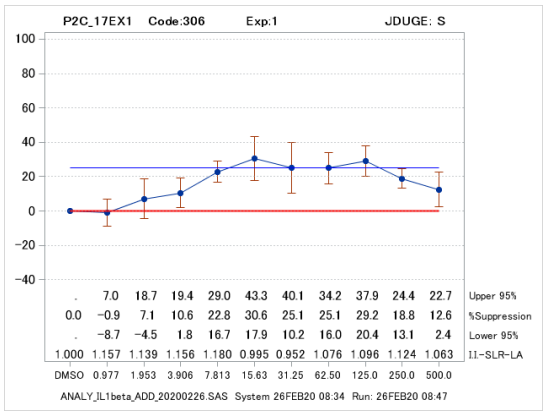
基準（2-2）では、続く 3 濃度の%suppression の値の大小関係が必要となるため、同時に 3 濃度を比べる必要がある。第 i 濃度の%suppression である $\%suppression_i$ に対して、1 つ前の濃度の値を $\%suppression_i^{(-1)}$、2 つ前の濃度の値を $\%suppression_i^{(-2)}$ とする。

基準（2.1）では、隣り合う 2 つの%suppression の 95%信頼区間の比較を行うことになる。$\%suppresion_i$ の 95%信頼区間の下限を $Lower_i$、上限を $Upper_i$ とし、これらの 95%信頼区間に対して、一つ前の濃度の下限を $Lower_i^{(-1)}$、上限を $Upper_i^{(-1)}$、2 つ前の濃度の下限を $Lower_i^{(-2)}$、上限を $Upper_i^{(-2)}$ とする。

また、カットオフ値の上限（Suppression に関係する）を $Cut^{(S)}$ とする。

<u>Step 1</u>

濃度が 2000 を超えるデータを除外する。



全ての濃度の $I.I.SLR\text{-}LA_i < 0.05$ となる $\%suppresion_i$ を欠測とする。

<u>Step 2</u>

以下の条件を満たした場合に新しい変数 $TwoSig\text{-}S_i$ を 1、そうでない場合は 0 とする。

$Lower_i^{(-1)} > 0$ かつ $Lower_i > 0$

<u>Step 3</u>

以下の条件を満たした場合に新しい変数 $Trend\text{-}S_i$ を 1、そうでない場合は 0 とする。

$\%suppresion_i^{(-1)} > 0$ かつ $\%suppresion_i > 0$

かつ

$\%suppresion_i^{(-2)} < \%suppresion_i^{(-1)}$

かつ

$\%suppresion_i^{(-1)} < \%suppresion_i$

かつ

$\mathrm{Upper}_i^{(-2)} > 0$

Step 4

以下の条件を満たした場合に新しい変数 IndConc-S_i を 1、そうでない場合は 0 とする。

$\{\mathrm{TwoSig\text{-}S}_i = 1$ かつ $\%\mathrm{suppresion}_i > \mathrm{Cut}^{(S)}$

かつ $\%\mathrm{suppresion}_i^{(-1)} > \mathrm{Cut}^{(S)}\}$

または

$\{\mathrm{Trend\text{-}S}_i = 1$ かつ $\mathrm{Lower}_i > 0$

かつ $\%\mathrm{suppresion}_i > \mathrm{Cut}^{(S)}\}$

Step 5

いずれかの濃度で IndConc-$S_i = 1$ の場合に

それ以外の場合には No effect とする。

判定アルゴリズムの実装

IL-1β Luc アッセイのバリデーション研究では、最終的に、カットオフ値は 25 とし、検討を行った。このバリデーション研究で行われたいくつかの実験データについて濃度と%suppression とその 95%信頼区間の図とともに上記の判定アルゴリズムによる結果を出力できるように作成した図を図 1 と図 2 に示す。図中の S が Suppression、N が No effect を示しており、図 1 が Suppression の例、図 2 が No effect の例である。

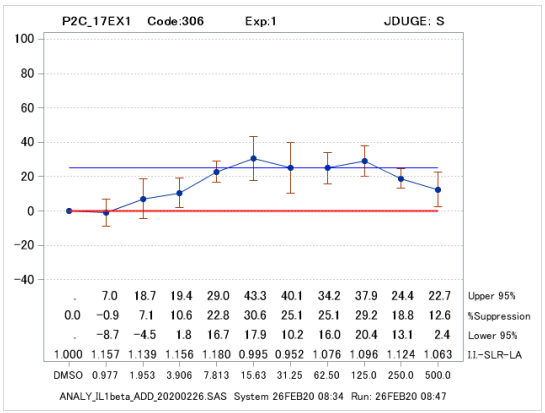


図 1a　Suppression と判定される例

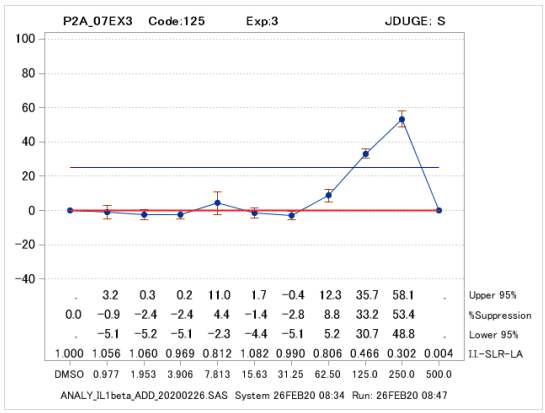


図 1b　Suppression と判定される例

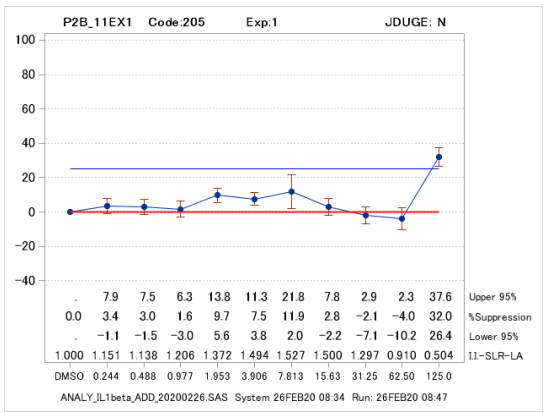


図 2b　No effect と判定される例

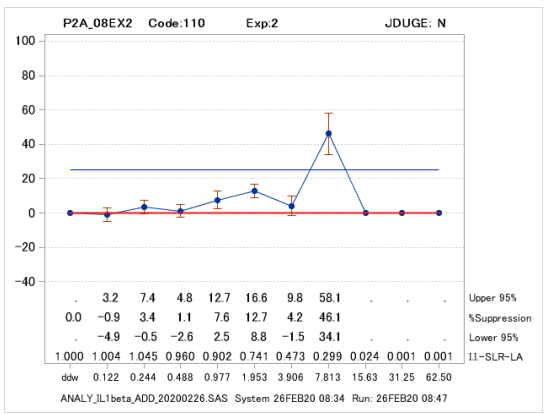


図 2b　No effect と判定される例

D. 考察

MITA の IL-2 Luc アッセイはすでにバリデーションが終了しており、IL-1β Luc アッセイの施設内お

よび施設間再現のバリデーション研究が終了したところである。これまで、アッセイの免疫毒性の判定は%suppression の濃度反応曲線のグラフから読み取ることで行われている。図 1 と図 2 に示す 4 つのグラフから瞬時に Suppression であるか No effect であるかを判断することは難しい。現在は、図を描くことで複数の目でチェックを行いながらバリデーション研究を行っているが、判定結果を誤る可能性が否定できない。

これらのアッセイが広く利用するようになる際には、本研究で検討したアルゴリズムをデータシートに組み込むことで、これまで費やされてきた多くの負担を減らすことができるであろう。

F. 健康危険情報

なし。

G. 研究発表

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. doi: 10.1016/j.tiv.2020.104832.

H. 知的財産権の出願・登録状況

なし。

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厚生労働科学研究費補助金（化学物質リスク研究事業）
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
（ H30-化学-一般-001 ）

分担研究報告書
IL-1 Luc assayクライテリアの設定ならびにプロトコールの作成

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研究要旨

平成 30 年度に実施された IL-1 Luc assay validation 試験 Phase I 試験後に validation management team (VMT)会議にて改変された IL-1 Luc assay 用クライテリアに沿って Phase II 試験用プロトコールを作成した。（Multi-Immuno Tox Assay protocol for TGCHAC-A4 ver.009E）そのプロトコールに則り令和元年 7 月より IL-1 Luc assay validation 試験 Phase II が実施された。Phase II 試験に先立ち３３候補被検物質の判定を行い VMT の chemical selection team にデータを提出し、そのデータをもとに Phase II における２０被検物質が決定された。また、Phase II 試験で使用するデータシートを作成した。Phase II 試験は１２月に終了し、その後令和２年１月に開催された VMT 会議にて施設内および施設間再現性の結果が承認され IL-1 Luc assay validation 試験が終了されたことを受け IL-1 Luc assay validation report の作成を開始した。

昨年度まで作成してきた６０化学物質による免疫毒性化学物質のデータベース作成を拡充し、９７化学物質からなるデータベースを作成した。

A．研究目的

IL-1 Luc assayの国際バリデーション試験 Phase II実施に向け、その際に使用するクライテリアの設定、プロコトールを作成することを目的とした。

B．研究方法

以下の方法によりIL-1βプロモーター活性の測定を行った。ヒト急性単球性白血病由来細胞株THP-1にIL-1βプロモーターに制御されたSLGルシフェラーゼ遺伝子（緑色に発色）、GAPDHプロモーターに制御されたSLRルシフェラーゼ遺伝子（赤色に発色）を導入したTHP-G1b(TGCHAC-A4)細胞を1ウェル当たり1×10^5個、96-well プレートに播種し化学物質を加え、37℃、5%CO_2下で1時間培養した。つづいてLipopolysaccharide (LPS)で刺激し37℃、5%CO_2下で6時間培養した。その後、細胞溶解剤とルシフェラーゼ反応の基質であるルシフェリンの混合剤であるTripluc luciferase assay reagent (TOYOBO)を混合し、室温で10分振盪させたのちマルチプレート対応型ルミノメーターにてルシフェラーゼ活性を測定した。SLG、SLRルシフェラーゼは共通の基質の存在により同時に発光するが、光学的フィルターにより分離し、各ルシフェラーゼの発光量(SLG-luciferase activity(SLG-LA)、SLR-luciferase activity(SLR-LA))を検出した。また細胞数の違いや各種刺激後の生存率の違いを勘案しSLG-LAをSLR-LAで除することにより normalized SLG-luciferase activity(nSLG-LA)を算出した。さらに以下の式により化学物質によるIL-1βプロモーター活性の抑制率%suppressionを計算した。

% suppression = (1-化学物質存在下でのnSLG-LA/化学物質非存在下でのnSLG-LA) X 100。

（倫理面への配慮）

本研究では主に細胞株を使用しており倫理面の問題はないと判断した。

C．研究結果

1． Phase II試験用プロトコールを作成

平成３０年度にIL-1 Luc assay validation試験 Phase I試験をMulti-Immuno Tox Assay protocol for TGCHAC-A4 ver.008Eに則り実施した。試験終了後、参加施設のデータを検討しPhase II試験に向け下記のようにアクセプタンスクライテリア、クライテリアを変更した。

アクセプタンスクライテリア

- LPS添加時のnIL1LAの誘導の許容下限が5.0であったのを3.0に変更した。
- Inh-GAPLAが0.05以上となる濃度が６未満かつ結果が陰性の場合はそのアッセイを棄却しその後のアッセイは濃度を低くして行うよう変更した。

クライテリア

- %suppressionの閾値を20%から25％に変更した。
- 結果をno effect, suppression, augmentationと３者に分類していたのを、augmentationをno effectに含めてno effect, suppressionの２者への分類に変更した。
- 2000 mg/mLの濃度における結果を除外した。

以上の結果を反映させたプロトコール、Multi-Immuno Tox Assay protocol for TGCHAC-A4 ver.009Eを作成した。(添付資料１)

2． Phase II試験３３候補被検物質の判定

Phase I終了後にVMTのchemical selection teamにより選定されたPhase II試験３３候補被検物質についてIL-1 Luc assayの判定結果、入手方法、IL-1発現への影響についての論文報告をまとめVMTに提出した。(添付資料２) このデータをもとにPhase IIにおける２０被検物質が決定された。

3． Phase II試験で使用するデータシート、記録用紙の作成

神戸大学の協力を得てPhase II試験用のデータシートを作成し、参加施設に配布した。(添付資料３)

4． 免疫毒性化学物質のデータベース作成

昨年度まで作成してきた６０化学物質による免疫毒性化学物質のデータベース作成を拡充し、９７化学物質からなるデータベースを作成した。(添付資料４)

D．考察

Phase I試験後に変更したクライテリアを適応したPhase II試験試験では施設内および施設間再現性共に良好な試験結果が得られた。予測性については今後論文を収集し検討する予定である。

E．結論

令和元年度に行われたIL-1 Luc assay validation試験 Phase II試験への準備として プロトコール、データシート、記録用紙を作成しPhase II試験が実施された。施設内および施設間再現性共に結果は良好でIL-1 Luc assay validation試験は終了しIL-1 Luc assay validation reportの作成を開始した。

F．健康危険情報

なし

G．研究発表

1． 論文発表

1) 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 immunotoxicchemicals. Toxicol In Vitro 2020 in press.

2． 学会発表

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（予定を含む。）

H．知的財産権の出願・登録状況　　**なし**

Multi-Immuno Tox Assay protocol for THP-G1b (TGCHAC-A4) ver. 009E

July 1st, 2019

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1. Introduction

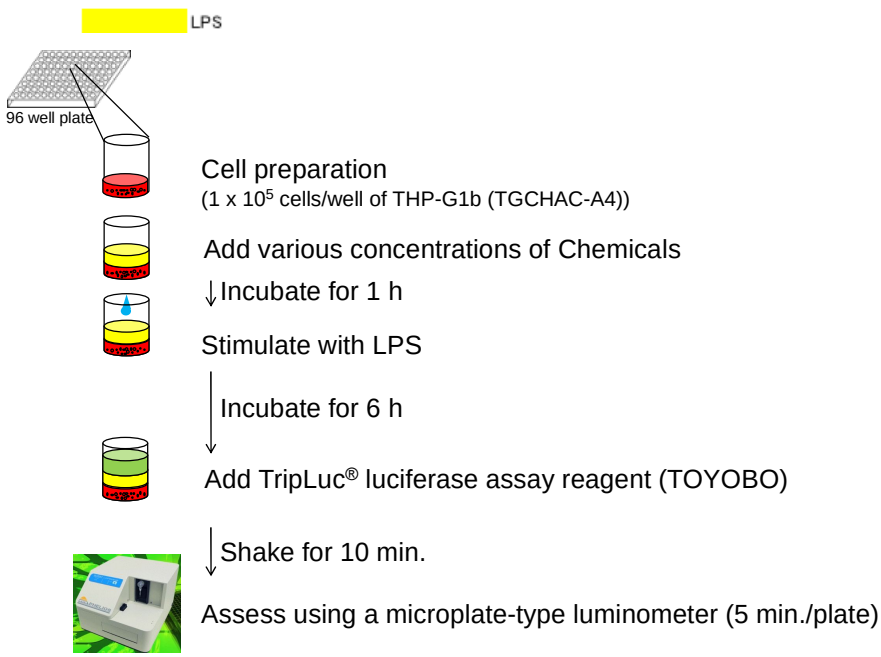
This protocol describes how to maintain the cells, how to prepare the test chemicals, and how to measure the luciferase activity of THP-G1b (TGCHAC-A4), THP-1 cells transfected with 2 luciferase genes, stable luciferase orange (SLG) on the human artificial chromosome (HAC) vector and stable luciferase 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												



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

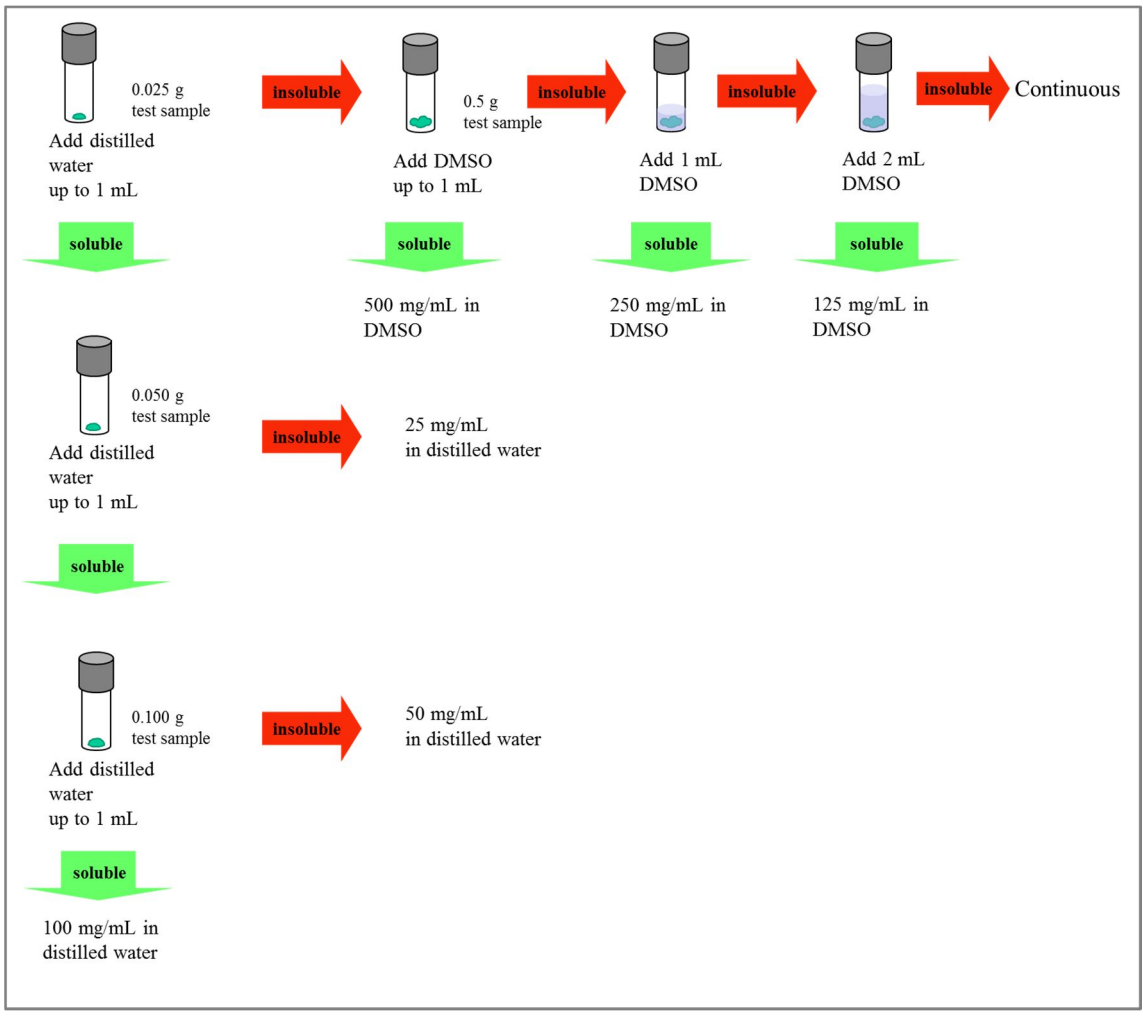
5-1 Dissolution by vehicle (cf. Figure 3)

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 4 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 5 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 4

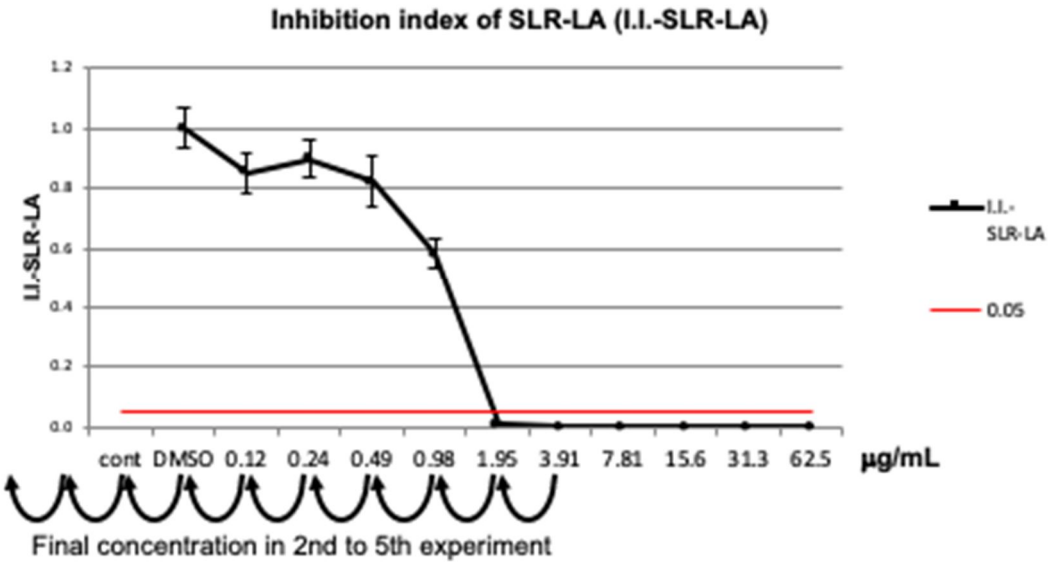
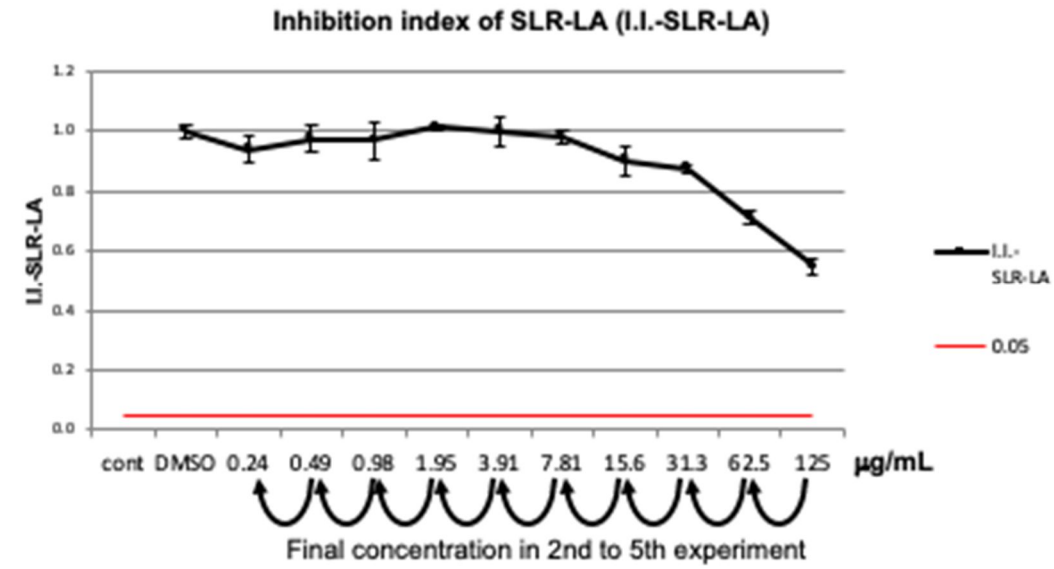


Figure 5



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.

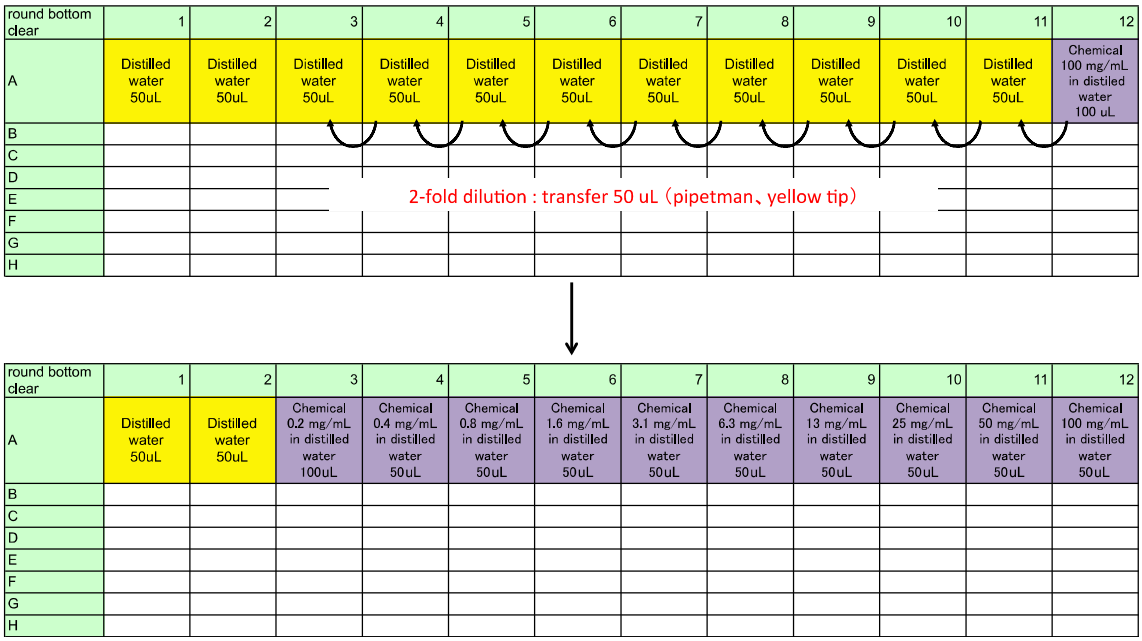
5-2-1 Arrangement of chemicals and vehicle

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 6)

Figure 6



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 7-9).

Figure 7

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 8

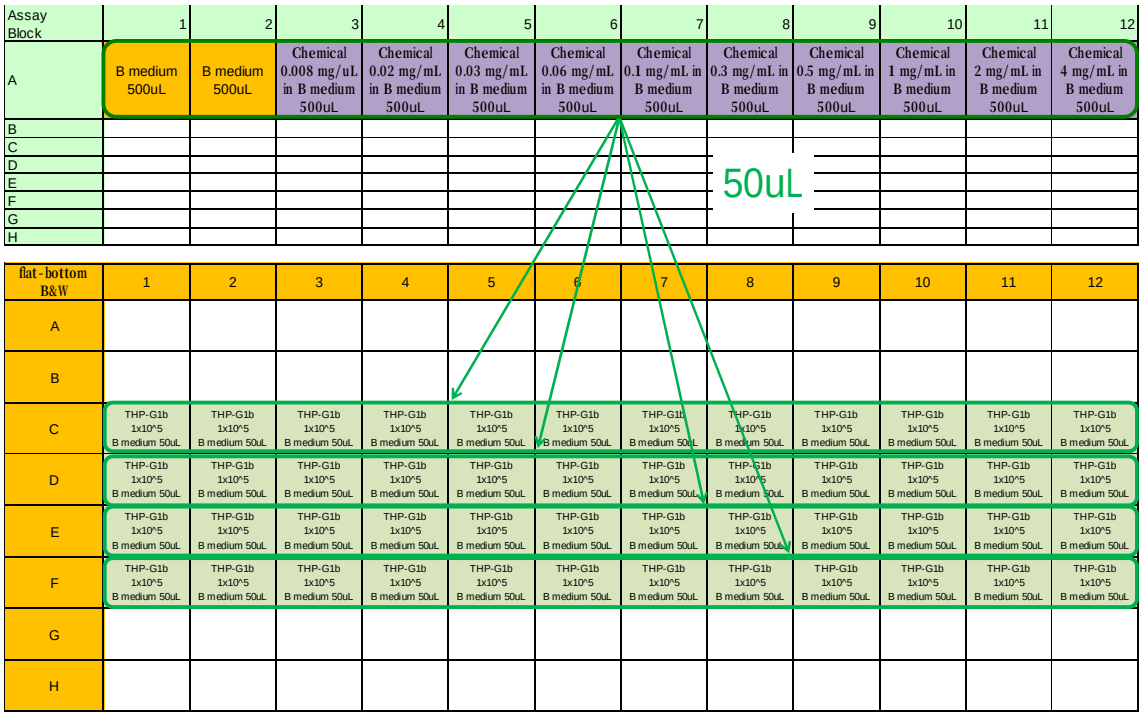


Figure 9 Final constituents of each well of the plate

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 10)

Figure 10

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 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	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												

10uL

↓

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												

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 12-14).

Figure 12

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 13

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 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 14 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

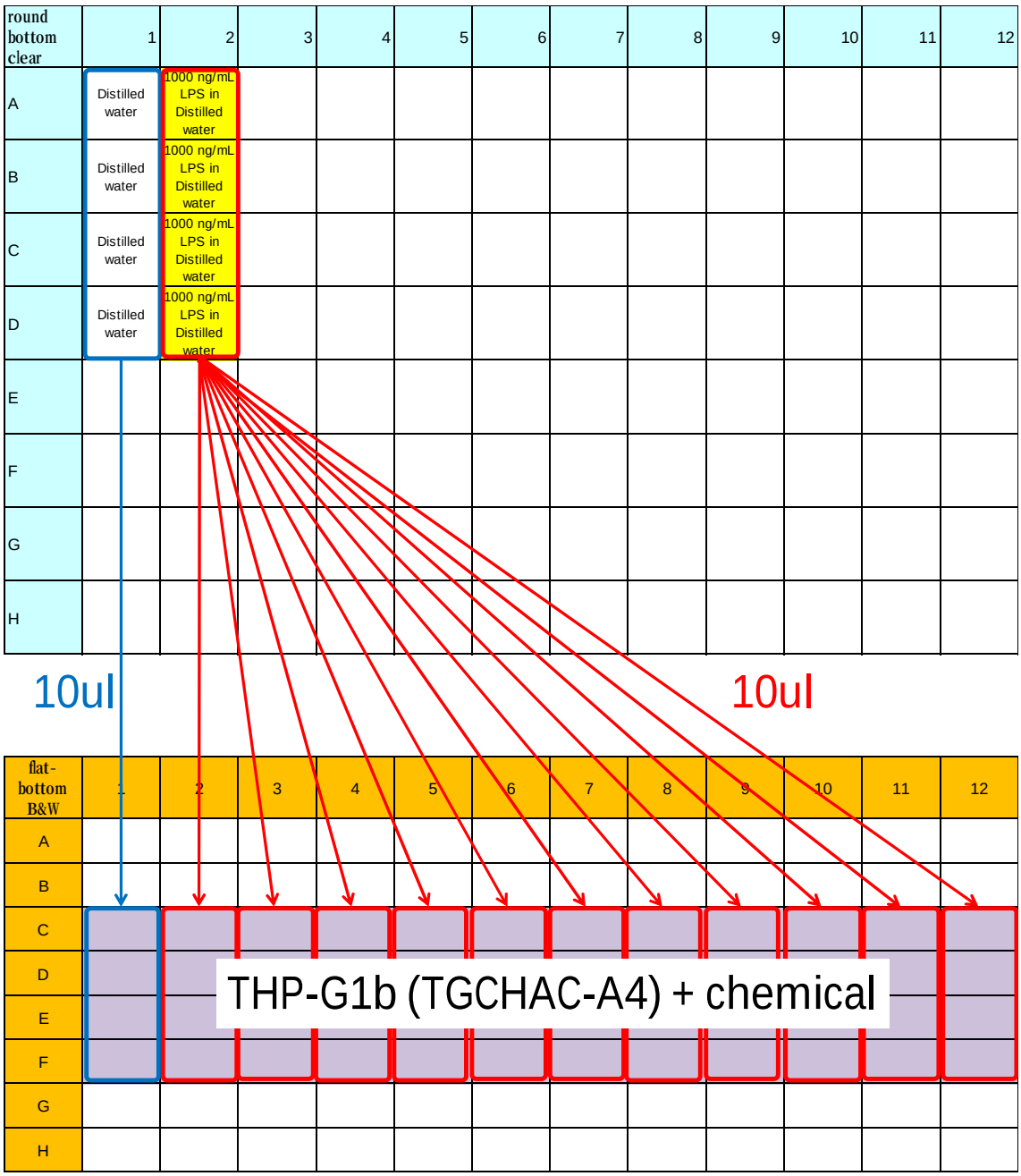
2nd step

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 15)

Figure 15



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 16)

Figure 16

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												

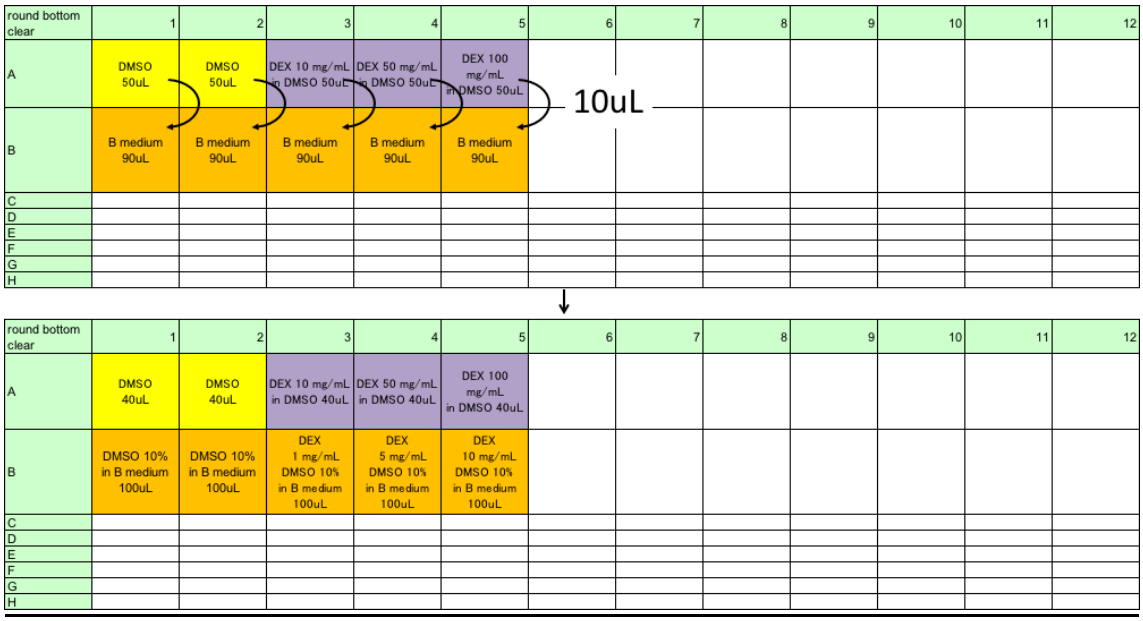
7-3 Arrangement of chemicals and vehicle

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 17)

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 17)

Figure 17



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 18-20)

Figure 18

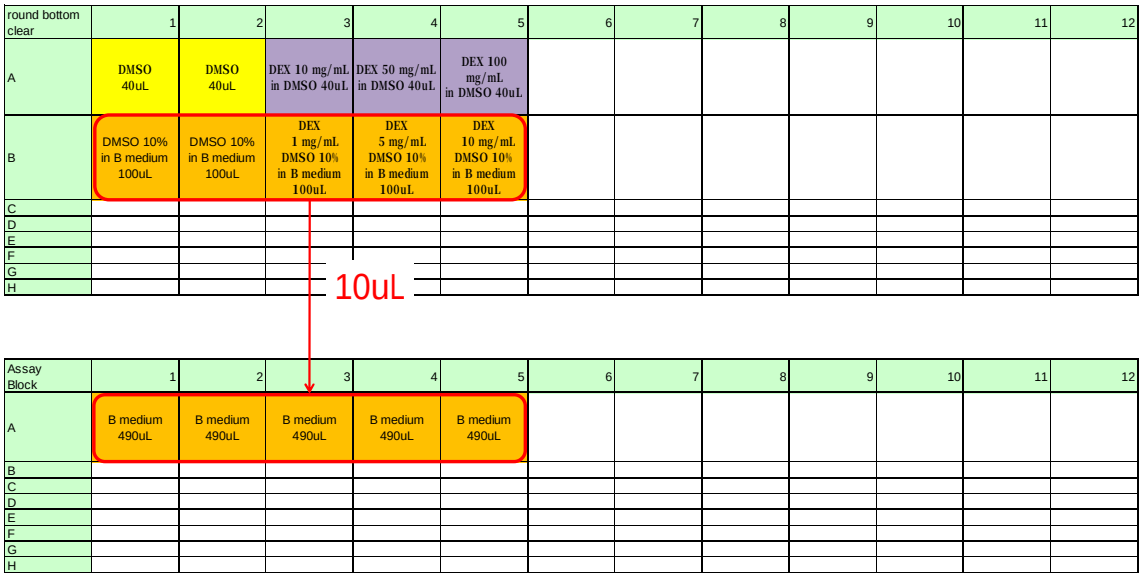


Figure 19

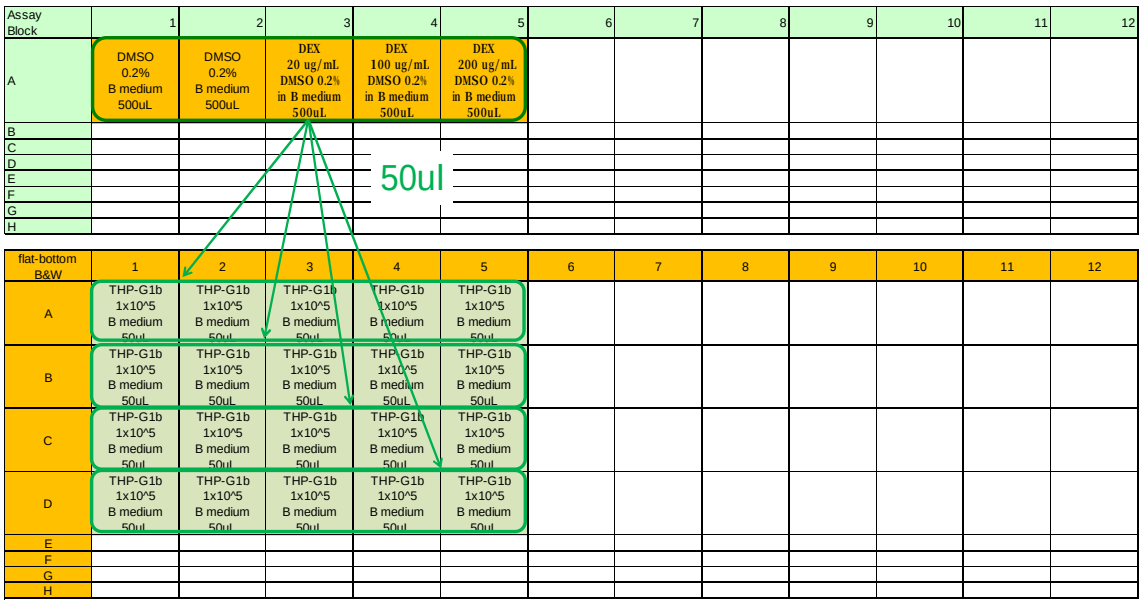


Figure 20 Final constituents of each well of the plate

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 21)

Figure 21

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water	1000 ng/mL LPS in Distilled water										
B	Distilled water	1000 ng/mL LPS in Distilled water										
C	Distilled water	1000 ng/mL LPS in Distilled water										
D	Distilled water	1000 ng/mL LPS in Distilled water										
E												
F												
G												
H												

10uL

10uL

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												

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 22.

flat-bottom B&W	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	SLG 100 ∝L	SLG 100 ∝L	SLG 100 ∝L									
C												
D	SLR 100 ∝L	SLR 100 ∝L	SLR 100 ∝L									
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 23. 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,

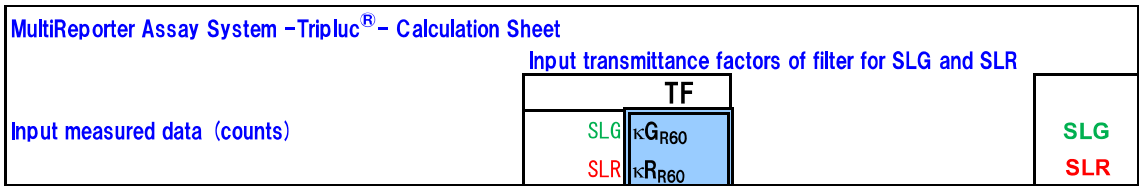
$$\text{Transmittance factors } (\kappa G_{R60}) = \frac{236478+234079+240876}{3757015+3716611+3810382} = 0.063$$

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

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 24



9. Measurement

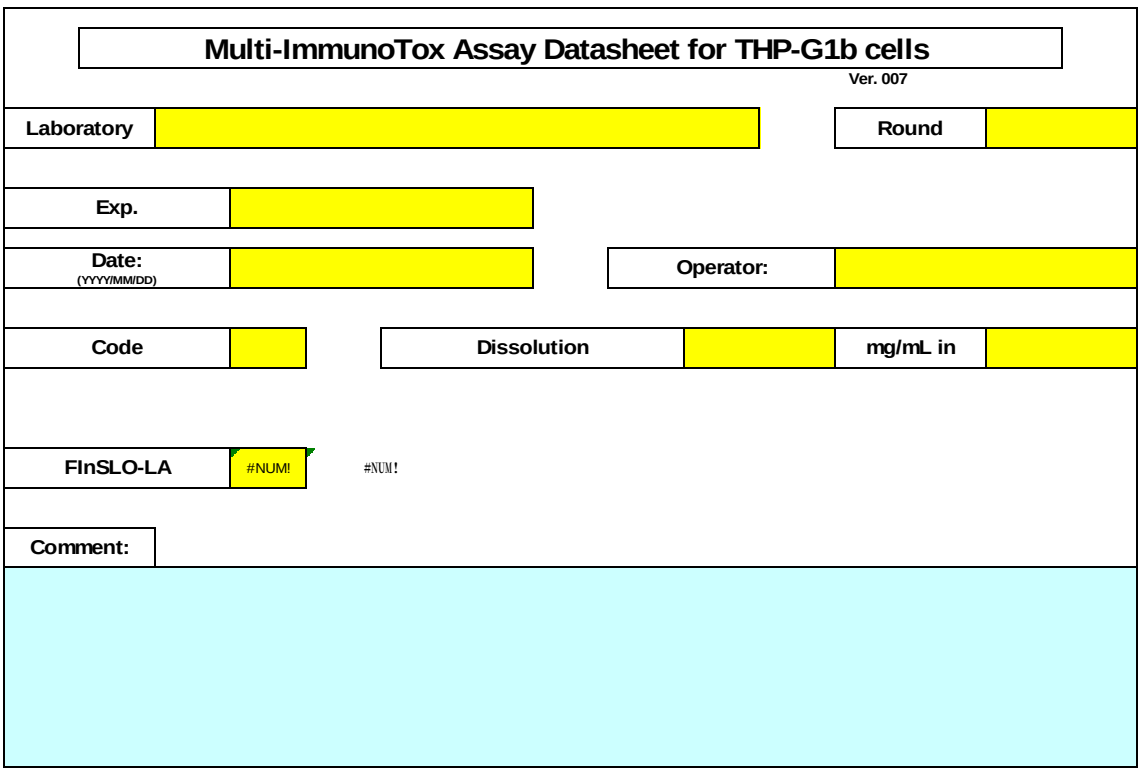
Please refer Appendix 1 for the principle of measurement of luciferase activity.

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.

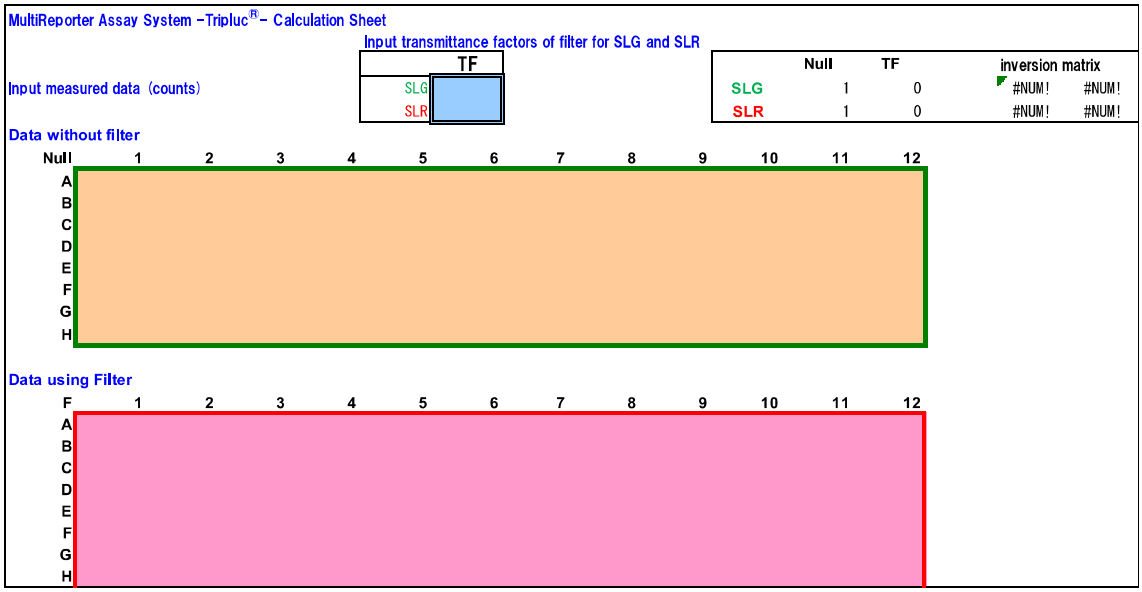
Figure 25 "Face Sheet" of the data sheet



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 26).

Figure 26 “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 27 “Result Format” sheet of the data sheet

10. Data analysis

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

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 $\geq$ 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 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 $\geq$ 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 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 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 2 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 28.

	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 29.

Measurement without Filter

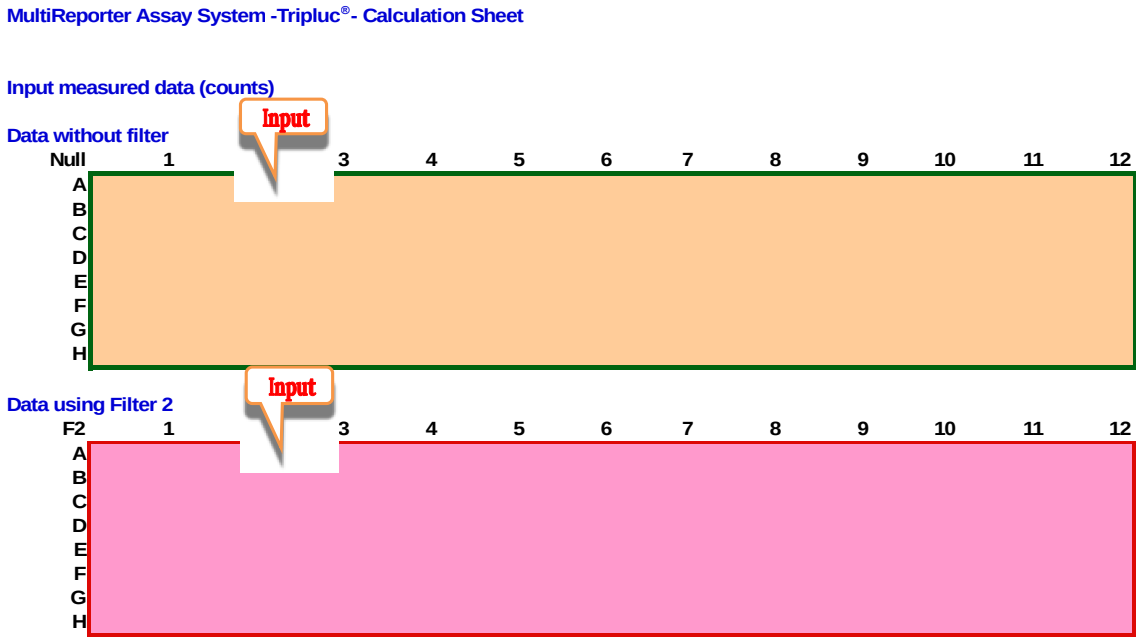
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	9689	9691	9677	2402	2388	2412	704	689	721	177	189	182
C												
D	8588	8444	8462	2281	2128	2239	609	578	690	150	132	129
E												
F												
G												
H												

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 30.



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.

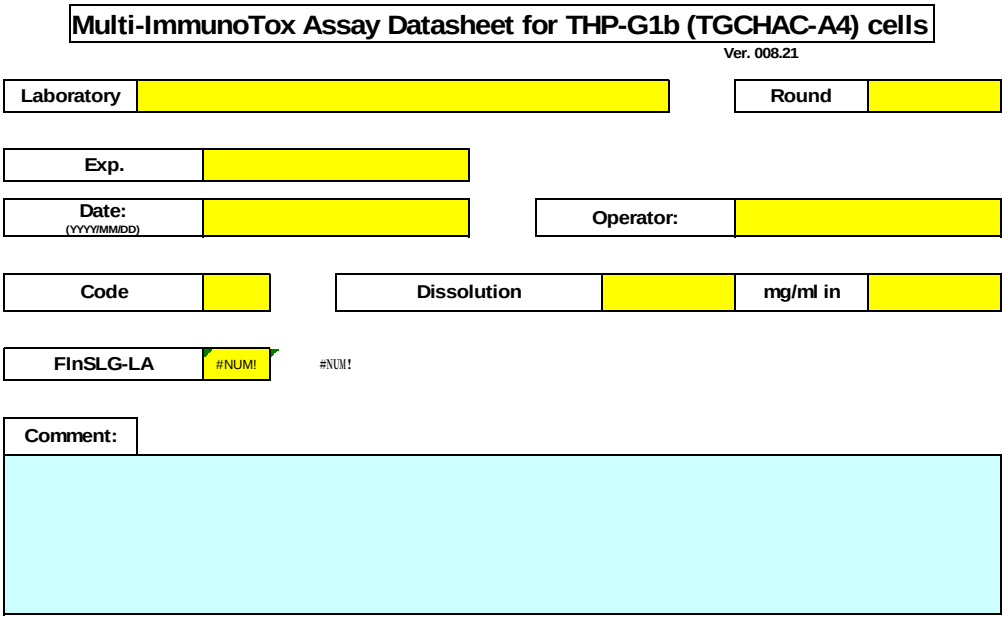
LED plate data typically fluctuates up to 1.5% (σ). Disagreement to the old data should be less than 3×σ (= 4.5%).

添付資料２ ： Phase II 試験３３候補被検物質の判定ならびに入手経路、論文報告

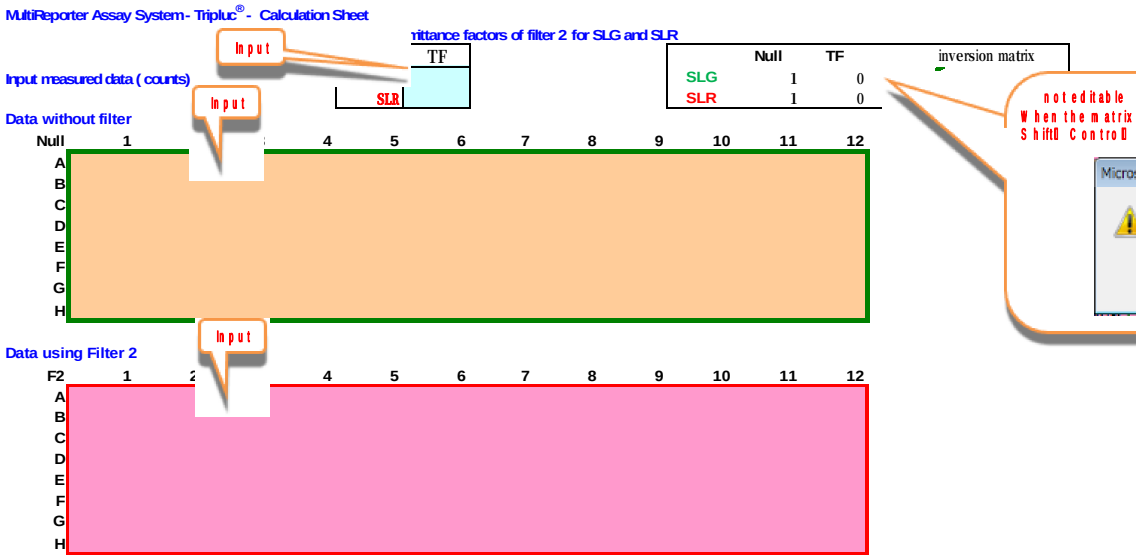
No.	Chemical	Cas.no.	Physical state	store	conc. (mg/ml)	vehicle	THP-G1b (HACA4)	Supplier	Ref. no.	Volume	Price	The effects of chemicals on IL-1 mRNA expression or IL-1 production in the literature			
1	2,4-Diaminotoluene	95-80-7	solid	r.t.	25	ddw	A	sigma	101915	50g	¥ 5,300	X			
2	Cadmium chloride	10108-64-2	solid	暗	100	ddw	S	aldrich	655198	5g	¥ 6,100	Odewumi et al. 2016 Li et al. 2016 Hemdan et al. 2006 Marth et al. 2000	human mice human human	cell line (A549, adenocarcinomic human alveolar basal epithelial cells) testis PBMC PBMC	A A S A (low), no effect (high)
3	Diethylstilbestol	56-53-1	solid	r.t.	62.5	DMSO	S	sigma	D4628	1g	¥ 20,300	Yamashita et al. 2005		peritoneal exudate macrophages	A
4	5,5-Diphenylhydantoin sodium salt	630-93-3	solid	r.t.	31.25	DMSO	S	sigma	D4505	25g	¥ 11,700	Okada et al. 2001 Modeer et al. 1989	mice human	spleen adherent cells PBMC, cell line (U937)	S A
5	Indomethacin	53-86-1	solid	r.t.	500	DMSO	S	sigma	I7378	5g	¥ 12,500	Muñoz-Miralles et al. 2018 Utsunomiya et al. 1994 Chang et al. 1990 Rordorf-Adam et al.1989	mice rat human human	intestine pleural exudate monocyte monocyte	A A S A
6	Isonicotinic Acid Hydrazide (Isoniazid)	54-85-3	solid	r.t.	50	ddw	N	fulka	I3377	5g	¥ 5,000	Metushi and Uetrecht 2014 Kucharz and Sierakowski 1992	mice human	serum (in vivo) monocyte	S (il-1a), no effect (IL-1b) S
7	Pentachlorophenol	87-86-5	solid	暗	125	DMSO	S	aldrich	P2604	5g	¥ 5,300	Martin and Whalen 2017 Chen et al. 2005	human crucian carp	immune cell macrophage	A S
8	Urethane, Ethyl carbamate	51-79-6	solid	r.t.	100	ddw	N	sigma	U2500	100g	¥ 9,100	Balharry et al. 2008 Bette et al. 2004	human rat	in vitro model of human upper respiratory tract epithelium (EpiAirway-100) spleen (in vivo)	A/S S
9	Tributyltin chloride	1461-22-9	liquid	暗	500	DMSO	A	aldrich	T50202	5g	¥ 3,400	Kimura et al. 2005 Tryphonas et al. 2004 Corsini et al. 1996	mice rat mice	macrophages, neutrophils (ex vivo) serum (in vivo) ear, keratinocyte cell line (HEL30)	no effect no effect A
10	Perfluorooctanoic acid	335-67-1	solid	r.t.	500	DMSO	S	TCI	P0764	25g	¥ 17,000	Son et al. 2009	mice	spleen, thymus	A (spleen), no effect (thymus)
11	Dichloracetic acid	79-43-6	liquid	4c暗	100	ddw	S	sigma	D54702	100ml	¥ 4,600	X			
12	Toluene	108-88-3	liquid	暗	500	DMSO	N	sigma	244511	100ml	¥ 4,500	X			
13	Hydroquinone(1,4-Dihydroquinone)	123-31-9	solid	r.t.	50	ddw	N	sigma	H9003	100g	¥ 4,400	Heluany et al. 2018 Thomas et al. 1989	rat mice	synoviocytes (ex vivo) macrophages (in vitro)	A S
14	4,4'-Sulfonyldianiline (Dapsone)	80-08-0	solid	r.t.	500	DMSO	SSS	sigma	A74807	100g	¥ 4,400	Abe et al. 2008 Interview form of dapsone	human human	PBMC (in vitro) Macrophages (in vitrio)	no effect S
15	Ethanol	64-17-5	liquid	r.t.	100	ddw	N	WAKO	057-00456	500ml	¥ 2,160	Tornier et al. 2006	human	reconstituted epidermis	No effect
16	Nitrofurazone	59-87-0	solid	r.t.	125	DMSO	N	aldrich	73340	100g	¥ 6,800	X			
17	Pentamidine isethionate	140-64-7	solid	-20	100	ddw	S	sigma	P0547	1g	¥ 31,900	Van Wauwe et al. 1996	human	whole blood	no effect
18	Trichloroethylene	79-01-6	liquid	r.t.	500	DMSO	S	WAKO	206-18575	500ml	¥ 1,400	Jia et al. 2012	human	serum, cell line (HaCaT)	A
19	Ziram(DMDTC)	137-30-4	solid	r.t.	125	DMSO	S	aldrich	329711	1kg	¥ 12,744	Muroi and Tanamoto 2015	mice	macrophage-like cell line J774A.1	S
20	Acetaminophen	103-90-2	solid	r.t.	500	DMSO	N	sigma	A7085	100g	¥ 9,800	Williams et al. 2010 Dambach et al. 2002 Blazka et al. 1995 Chang et al. 1990	mice mice mice human	plasma, liver liver serum (in vivo) monocyte	A A A S (1mg/ml)
21	Citral	5392-40-5	liquid	r.t.	500	DMSO	S	aldrich	C83007	5ml	¥ 4,100	Bachiega et al. 2011	mice	Peritoneal macrophages	S
22	Dibutyl phthalate	84-74-2	liquid	r.t.	500	DMSO	S	aldrich	524980	25ml	¥ 1,800	Ni et al. 2016 Couleau et al. 2015 Hansen et al. 2015 Li et al. 2013 Kruger et al. 2012	human human human mice human	HepG2 and L02 (normal human liver) cell lines cell line (THP-1) monocytes/macrophages F4/80+ macrophages corneal endothelial cell line B4G12	A no effect no effect S A
23	Hexachlorobenzene	118-74-1	solid	r.t.	3.9	DMSO	S	TCI	H0053	25 g	¥ 3,400	Fu et al. 2016	Rat	cell line (pheochromocytoma, PC12)	A
24	Lead(II) acetate	6080-56-4	solid	暗	100	ddw	N	aldrich	316512	5g	¥ 4,700	Hemdan et al. 2005	human	PBMC	S
25	Mercuric chloride	7487-94-7	solid	暗	50	ddw	S	WAKO	138-01152	25g	¥ 3,300	Zdolsek et al. 1994	mice	peritoneal macrophage	A
26	Triethanolamine	102-71-6	liquid	r.t.	100	ddw	S	sigma	90279	100ml	¥ 4,900	Tornier et al. 2006 Muller-Decker et al. 1998 Muller-Decker et al. 1994	human human human	reconstituted epidermis suction blister fluid cell line (keratinocyte)	A, no effect no effect A
27	t-Butylhydroquinone	1948-33-0	solid	r.t.	250	DMSO	S	aldrich	112941	5g	¥ 4,900	Hao et al. 2018 Bai et al. 2017 Sukumari-Ramesh and Alleyne 2016	rat rat mice	bone marrow mast cells paraventricular nucleus brain	A S S
28	5,5-Diphenylhydantoin	57-41-0	solid	r.t.	500	DMSO	S	sigma	D4007	100g	¥ 11,700	Okada et al. 2001 Modeer et al. 1989	mice human	spleen adherent cells PBMC, cell line (U937)	S A
29	Bisphenol A	80-05-7	solid	r.t.	500	DMSO	S	aldrich	239658	50g	¥ 10,400	Teixeira et al. 2016 Couleau et al. 2015 Yang et al. 2015	human human carp	PBMC-derived Macrophage cell line (differentiated THP-1) macrophage primary macrophages	no effect A A
30	Butylated hydroxytoluene	128-37-0	solid	r.t.	500	DMSO	S	aldrich	B1378	100g	¥ 2,900	Eugui et al. 1994	human	PBMC	no effect
31	D-Penicillamine	52-67-5	solid	4c	100	ddw	N	sigma	P4875	5g	¥ 21,900	Chang et al. 1990 Rordorf-Adam et al. 1989 DiMartino et al. 1987 Brisset et al. 1986	human human rat rabbit	monocyte monocyte peritoneal exudate mononuclear cells	S no effect S S
32	Nonylphenol	84852-15-3	liquid	r.t.	100	ddw	N	aldrich	290858	25ml	¥ 2,000	Gu et al. 2018 Yu et al. 2016	rat rat	hippocampus liver	A A
33	Sodium chlorite	7758-19-2	solid	暗	100	ddw	A/S	sigma	244155	100g	¥ 6,800	X			

添付資料３：Multi-ImmunoTox Assay Datasheet for THP-G1b (TGCHAC-A4) cells ver.008.21E

①フェイスシート



②データ入力シート



③計算結果シート

MultiReporter Assay System- Triplus® - Calculation Sheet

Filter Null Data

Filter 2 Data

MultiReporter Assay System - Triplus® - Calculation Sheet

Transmittance Data

SLG

SLR

SLG mod

SLR mod

nSLG-LA

Chemical concentration

SLG-LA Average

S.D.

SLR-LA Average

S.D.

nSLG-LA Average

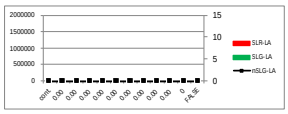
S.D.

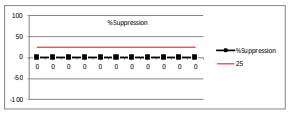
濃度の数(n)

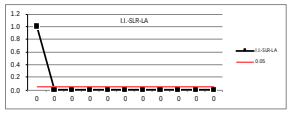
Il-SLR-LA

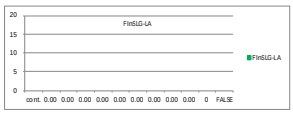
%suppression (IL-1 β)

FlnSLG-LA





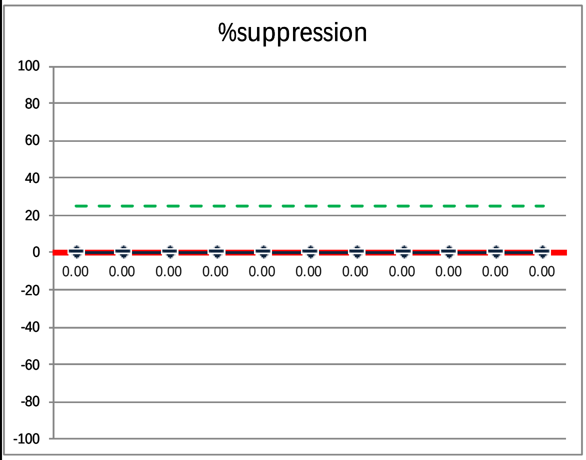




④グラフシート

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!
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#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
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#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
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#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

添付資料４ ： MITAデータベース（97化学物質）

No.	Chemicals	CAS No.	IL-2		IL-1β		IL-8 Luc
			Judge	LOEL (ug/mL)	Judge	LOEL (ug/mL)	Judge
1	2-Aminoanthracene	613-13-8	S	5.86	S	11.72	P
2	2-Mercaptobenzothiazole	149-30-4	A	16.11	S	93.75	P
3	2,4-Diaminotoluene	95-80-7	N		N		N
4	Acetaminophen	103-90-2	A	100.00	N		N
5	Actinomycin D	50-76-0	S	0.02	S	0.13	P
6	Aluminum chloride	7784-13-6	S	104.17	N		N
7	Amphoterycin B	1397-89-3	S	2.60	S	1.17	P
8	Azathioprine	446-86-6	S	58.48	S	41.55	N
9	Benzethonium chloride	121-54-0	S	1.63	N		P
10	Chloroplatinic acid	18497-13-7	S	250.00	S	23.44	P
11	Chloroquine	50-63-5	S	17.83	S	39.06	P
12	Chlorpromazine	69-09-0	S	1.95	S	3.91	P
13	Cisplatin	15663-27-1	S	16.93	S	46.88	P
14	Citral	5392-40-5	S	25.00	S	4.88	P
15	Cobalt chloride	7791-13-1	S	16.93	S	46.88	P
16	Colchicine	64-86-8	S	0.27	N		P
17	Cyclophosphamide	6055-19-2	N		N		P
18	Cyclosporine A	59865-13-3	S	0.00	N		N
19	4-Aminophenyl sulfone, Dapsone	80-08-0	S	72.92	S	125.00	N
20	Dexamethasone	50-02-2	S	41.67	S	0.98	N
21	Dibenzopyrene	191-30-0	N		N		N
22	Dibutyl phthalate	84-74-2	S	2.60	S	15.63	N
23	Diesel exhaust particles		S	62.50	S	39.06	P
24	Diethanolamin	111-42-2	S	250.00	S	333.33	P
25	Digoxin	20830-75-5	S	0.07	S	0.59	P
26	Dimethyl sulfoxide	67-68-5	A	2000.00	N		N
27	Ethanol	64-17-5	N		N		N
28	FK506	109581-93-3	S	0.00	N		N
29	Formaldehyde	50-00-0	S	7.81	N		P
30	FR167653	158876-65-4	S	1.30	S	0.49	N
31	Hexachlorobenzene	118-74-1	N		N		N
32	Histamine	51-45-6	S	750.00	N		P
33	Hydrocortisone	50-23-7	N		S	0.24	N
34	Hydrogen peroxide	7722-84-1	S	23.44	S	375.00	P
35	Isoniazid	54-85-3	S	1000.00	N		N
36	Isophorone diisocyanate	4098-71-9	S	7.81	S	3.91	P
37	Lead(II) acetate	6080-56-4	S	57.29	N		N
38	Lithium carbonate	554-13-2	N		N		P
39	Magnesium sulfate	10034-99-8	S	2000.00	N		N
40	Mercuric chloride	7487-94-7	S	1.95	S	1.95	P
41	Methanol	67-56-1	N		N		N
42	Methotrexate	13307-73-1	N		N		P
43	Minocycline	13614-98-7	S	18.52	S	62.50	P
44	Mitomycin C	50-07-7	S	20.00	N		P
45	Mizoribine	50924-49-7	N		N		N
46	Mycophenolic acid	24280-93-1	A	0.40	S	72.00	P
47	Nickel sulfate	10101-97-0	S	104.17	S	375.00	P
48	Nicotinamide	98-92-0	A	288.07	N		N
49	Nitrofurazone	59-87-0	S	83.33	N		P
50	*p* -Nitroaniline	100-01-6	S	83.33	S	125.00	N
51	Pentamidine isethionate	140-64-7	S	52.08	S	64.45	P
52	Pyrimethamine	58-14-0	S	7.81	N		P
53	Rapamycin	53123-88-9	N		N		N
54	Ribavirin	36791-045	A	26.04	S	750.00	N
55	Sodium bromate	7789-38-0	S	500.00	S	500.00	P
56	Sodium dodecyl sulfate	151-21-3	S	62.50	S	62.50	P
57	Sulfasalazine	599-79-1	S	92.94	S	44.81	N
58	Trichloroethylene	79-01-6	N		N		N
59	Triethanolamine	102-71-6	S	1333.33	S	1000.00	P
60	Warfarin	81-81-2	N		N		N
61	4-Chloro-o-phenylenediamine	95-83-0	S	0.98	S	0.98	P
62	4,4-Thiobis(6-t-butyl-m-cresol)	96-69-5	S	3.91	S	3.91	P
63	Acetonitrile	75-05-8	N		N		N
64	Benzo(a)pyrene	50-32-8	S	5.86	N		N
65	Cadmium chloride	10108-64-2	N		S	15.63	P
66	Diethylstilbestrol	56-53-1	S	1.95	N		P
67	Dimethylbenz(a)-anthracene	57-97-6	S	6.41	S	25.63	N
68	Dimethylvinyl chloride	513-37-1	N		N		N
69	Diphenylhydantoin	630-93-3	N		N		P
70	Urethane, Ethyl carbamate	51-79-6	A	666.67	N		N
71	Ethylene dibromide	106-93-4	N		N		P
72	Indomethacin	53-86-1	A	31.25	N		P
73	m-Nitrotoluene	99-08-1	N		N		N
74	Methyl carbamate	598-55-0	A	750.00	N		N
75	n-Nitrosodimethylamine	62-75-9	A	1500.00	N		N
76	Nitrobenzene	98-95-3	N		N		N
77	o-Benzyl-p-chlorophenyl	120-32-1	S	2.93	S	2.93	P
78	o-Phenylphenol	90-43-7	S	7.81	S	46.88	P
79	Oxymetholone	434-07-1	S	7.81	S	0.98	N
80	p-Nitrotoluene	99-99-0	N		N		N
81	Pentachlorophenol	87-86-5	S	31.25	S	23.44	P
82	Phorbol myristate acetate (TPA)	16561-29-8	S		S	0.04	P
83	t-Butylhydroquinone	1948-33-0	N		S	4.88	P
84	Toluene	108-88-3	N		N		N
85	Vanadium pentoxide	1314-62-1	N		N		P
86	Zinc dimethyldithiocarbamate	137-30-4	N		N		P
87	Dibromoacetic acid	631-64-1	A	156.25	N		P
88	Glycidol	556-52-5	A	82.59	S	500	P
89	Tributyltin chloride	1461-22-9	S	0.19	N		P
90	Perfluorooctanoic acid	335-67-1	A	19.97	S	125.00	P
91	Dichloracetic acid	79-43-6	S	1285.71	N		P
92	Mannitol	69-65-8	N		N		N
93	Hydroquinone	123-31-9	S	3.91	N		P
94	Bisphenol A	80-05-7	S	7.81	S	31.25	P
95	2,6-Di-tert-butyl-4-methylpheno	128-37-0	S	7.81	N		N
96	Nonylphenol	84852-15-3	N		N		P
97	Sodium chlorite	7758-19-2	S	2.93	S	41.67	P

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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小島　肇	**化粧品の安全性評価における国内外の動向**	**フレグランスジャーナル**	9	17-22	2019

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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.	in press		2020

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	in press		2020

令和２年　３月　２７日

国立医薬品食品衛生研究所長 殿

機関名　国立大学法人東北大学

所属研究機関長　職　名　　総長

氏　名　大野　英男　印

次の職員の令和元年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

１．研究事業名　　化学物質リスク研究事業

２．研究課題名　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

３．研究者名　（所属部局・職名）　大学院医学系研究科・教授

（氏名・フリガナ）　相場　節也　（アイバ　セツヤ）

４．倫理審査の状況

	該当性の有無	左記で該当がある場合のみ記入（※1）		
	有　無	審査済み	審査した機関	未審査（※2）
ヒトゲノム・遺伝子解析研究に関する倫理指針	□　■	□		□
遺伝子治療等臨床研究に関する指針	□　■	□		□
人を対象とする医学系研究に関する倫理指針（※3）	□　■	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□　■	□		□
その他、該当する倫理指針があれば記入すること（指針の名称：　　）	□　■	□		□

（※1）当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

（※2）未審査に場合は、その理由を記載すること。
（※3）廃止前の「疫学研究に関する倫理指針」や「臨床研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ■　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ■　無 □（無の場合はその理由：　　）
当研究機関におけるＣＯＩ委員会設置の有無	有 ■　無 □（無の場合は委託先機関：　　）
当研究に係るＣＯＩについての報告・審査の有無	有 ■　無 □（無の場合はその理由：　　）
当研究に係るＣＯＩについての指導・管理の有無	有 ■　無 □（有の場合はその内容：研究実施の際の留意点を示した　　）

（留意事項）　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和２年３月27日

国立医薬品食品衛生研究所長　殿

機関名　国立医薬品食品衛生研究所

所属研究機関長　職　名　所長

氏　名　奥田晴宏　印

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３．研究者名　（所属部局・職名）　安全性予測評価部　第二室　室長

（氏名・フリガナ）　小島　肇（コジマ　ハジメ）

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当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ☑（有の場合はその内容：　　）

（留意事項）　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和２年４月８日

国立医薬品食品衛生研究所長 殿

機関名　国立研究開発法人 産業技術総合研究所

所属研究機関長　職　名　理事長

氏　名　石村　和彦　印

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３．研究者名　（所属部局・職名）　健康工学研究部門　研究グループ長

（氏名・フリガナ）　　中島芳浩・ナカジマヨシヒロ

４．倫理審査の状況

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遺伝子治療等臨床研究に関する指針	□　■	□		□
人を対象とする医学系研究に関する倫理指針（※3）	□　■	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□　■	□		□
その他、該当する倫理指針があれば記入すること（指針の名称：　　）	□　■	□		□

（※1）当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

（※2）未審査に場合は、その理由を記載すること。
（※3）廃止前の「疫学研究に関する倫理指針」や「臨床研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ■　　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ■　無 □（無の場合はその理由：　　）
当研究機関におけるＣＯＩ委員会設置の有無	有 ■　無 □（無の場合は委託先機関：　　）
当研究に係るＣＯＩについての報告・審査の有無	有 ■　無 □（無の場合はその理由：　　）
当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ■（有の場合はその内容：　　）

（留意事項）　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和２年４月８日

国立医薬品食品衛生研究所長 殿

機関名　国立研究開発法人 産業技術総合研究所

所属研究機関長　職　名　理事長

氏　名　石村　和彦　印

次の職員の平成　　年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

１．研究事業名　化学物質リスク研究事業

２．研究課題名　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（H30-化学-一般-001）

３．研究者名　（所属部局・職名）　バイオメディカル研究部門　主任研究員

（氏名・フリガナ）　安野理恵・ヤスノリエ

４．倫理審査の状況

	該当性の有無		左記で該当がある場合のみ記入（※1）		
	有	無	審査済み	審査した機関	未審査（※2）
ヒトゲノム・遺伝子解析研究に関する倫理指針	□	■	□		□
遺伝子治療等臨床研究に関する指針	□	■	□		□
人を対象とする医学系研究に関する倫理指針（※3）	□	■	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□	■	□		□
その他、該当する倫理指針があれば記入すること（指針の名称：　　）	□	■	□		□

（※1）当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

（※2）未審査に場合は、その理由を記載すること。
（※3）廃止前の「疫学研究に関する倫理指針」や「臨床研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ■　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ■　無 □（無の場合はその理由：　　）
当研究機関におけるＣＯＩ委員会設置の有無	有 ■　無 □（無の場合は委託先機関：　　）
当研究に係るＣＯＩについての報告・審査の有無	有 ■　無 □（無の場合はその理由：　　）
当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ■（有の場合はその内容：　　）

（留意事項）　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和　2年　4月　30日

国立医薬品食品衛生研究所長　殿

機関名　国立大学法人神戸大学

所属研究機関長　職　名　学長

氏　名　武田　廣　　印

次の職員の令和元年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

1．研究事業名　化学物質リスク研究事業

2．研究課題名　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

3．研究者名　（所属部局・職名）医学部附属病院・特命教授

（氏名・フリガナ）大森　崇・オオモリ　タカシ

4．倫理審査の状況

	該当性の有無	左記で該当がある場合のみ記入（※1）		
	有　無	審査済み	審査した機関	未審査（※2）
ヒトゲノム・遺伝子解析研究に関する倫理指針	□　■	□		□
遺伝子治療等臨床研究に関する指針	□　■	□		□
人を対象とする医学系研究に関する倫理指針（※3）	□　■	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□　■	□		□
その他、該当する倫理指針があれば記入すること（指針の名称：　　）	□　■	□		□

（※1）当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

（※2）未審査に場合は、その理由を記載すること。

（※3）廃止前の「疫学研究に関する倫理指針」や「臨床研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

5．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ■　未受講 □

6．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ■　無 □（無の場合はその理由：　　）
当研究機関におけるＣＯＩ委員会設置の有無	有 ■　無 □（無の場合は委託先機関：　　）
当研究に係るＣＯＩについての報告・審査の有無	有 ■　無 □（無の場合はその理由：　　）
当研究に係るＣＯＩについての指導・管理の有無	有 □　無 ■（有の場合はその内容：　　）

（留意事項）　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。

令和２年　３月　１８日

国立医薬品食品衛生研究所長 殿

機関名　国立大学法人　東北大学

所属研究機関長　職　名　　総長

氏　名　大野　英男　印

次の職員の平成31年度厚生労働科学研究費の調査研究における、倫理審査状況及び利益相反等の管理については以下のとおりです。

１．研究事業名　　化学物質リスク研究事業

２．研究課題名　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

３．研究者名　（所属部局・職名）　東北大学病院・助教

（氏名・フリガナ）　　木村　裕　　（キムラ　ユタカ）

４．倫理審査の状況

	該当性の有無	左記で該当がある場合のみ記入（※1）		
	有　無	審査済み	審査した機関	未審査（※2）
ヒトゲノム・遺伝子解析研究に関する倫理指針	□　■	□		□
遺伝子治療等臨床研究に関する指針	□　■	□		□
人を対象とする医学系研究に関する倫理指針（※3）	□　■	□		□
厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針	□　■	□		□
その他、該当する倫理指針があれば記入すること（指針の名称：　　）	□　■	□		□

（※1）当該研究者が当該研究を実施するに当たり遵守すべき倫理指針に関する倫理委員会の審査が済んでいる場合は、「審査済み」にチェックし一部若しくは全部の審査が完了していない場合は、「未審査」にチェックすること。

その他（特記事項）

（※2）未審査に場合は、その理由を記載すること。
（※3）廃止前の「疫学研究に関する倫理指針」や「臨床研究に関する倫理指針」に準拠する場合は、当該項目に記入すること。

５．厚生労働分野の研究活動における不正行為への対応について

研究倫理教育の受講状況	受講 ■　未受講 □

６．利益相反の管理

当研究機関におけるＣＯＩの管理に関する規定の策定	有 ■　無 □（無の場合はその理由：　　）
当研究機関におけるＣＯＩ委員会設置の有無	有 ■　無 □（無の場合は委託先機関：　　）
当研究に係るＣＯＩについての報告・審査の有無	有 ■　無 □（無の場合はその理由：　　）
当研究に係るＣＯＩについての指導・管理の有無	有 ■　無 □（有の場合はその内容：研究実施の際の留意事項を示した　）

（留意事項）　・該当する□にチェックを入れること。
・分担研究者の所属する機関の長も作成すること。