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

平成30年度　総括・分担研究報告書

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総括・分担研究年度終了報告書

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

（ 30210101 ）

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研究要旨

今年度は、1)化学物質の免疫毒性データ収集、2)MITA による免疫毒性 clustering の有用性検討、3)IL-2 Luc assay プロトコールならびにクライテリアの改訂、4)免疫毒性評価基準の検討ならびに validation 試験の predictivity 評価、5)Validation report 作成、6)IL-1 Luc assay プロトコールならびにクライテリアの設定、7)IL-1 Luc assay Phase 0、8)IL-1 Luc assay Phase 1 を行った。その結果、1)に関しては、IL-2 Luc assay の validation に用いた 25 化学物質に関して National Toxicology Program (NTP)の協力を仰ぎ，免疫毒性データベースを構築した。2)に関しては、IL-8 Luc assay と組み合わせた MITA により分類された 6 つの cluster の特性ならびに MITA の適応限界を明らかにした。3)4)に関しては、IL-2 Luc assay のこれまでの結果に基づき、クライテリアの詳細を決定し、それに基づき validation 結果を総括した。5)に関しては，さらにその結果をもとに validation report を作成し、2019 年 2 月 27 日､28 日に OECD テストガイドライン化を目指して peer review 会議を開催した。6)に関しては、IL-1 Luc assay validation 試験に向けてプロトコールとクライテリアを確立した。7) に関しては、Phase 0 試験により IL-1 Luc assay の技術移転性を確認した。8)に関しては、Phase I 試験を終了し，その結果を検討中である。

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Ａ．研究目的

研究背景 ：

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

我々は，平成18-22年NEDO「高機能簡易型有害性評価手法の開発」プロジェクトにおいて、産業総合研究所が開発した３色発光細胞の技術を応用し，Jurkat細胞におけるINF-γ、IL-2、G3PDHプロモーター活性、THP-１細胞におけるIL-8、IL-1β、G3PDHプロモーター活性をhigh throughputに評価できる長期細胞株を樹立し、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。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、我々はまず作用機序の明らかな種々の免疫抑制剤をMITAを用いて評価するなかで、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、そのプロトコールを用いた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]。

そこで平成27年度以降は、合計60化学物質からなるdata setを作成した[3]。また、MITAによる化学物質の免疫毒性を評価するなかで、MITAのみによる分類では、免疫抑制物質中に感作性物質が含まれてしまうことが明らかとなり皮膚感作性試験法IL-8 Luc assayとMITAを組み合わせたmodified MITAを構築し、IL-8 Luc assayの評価結果もdata setに追加した。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[4,5]。

計画全体の目的（図１）：

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

上記データベースに基づき，我々がこれまでに確立してきた多項目免疫毒性評価系 (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。

また、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)(国際 validation phase I、II が既に終了）と IL-1β転写活性抑制評価試験(IL-1 luciferase reporter assay; IL-1 Luc assay)の国際 validation study を行い、MITA の多項目免疫毒性評価系として OECD テストガイドライン化を目指す。

2018 年度

① 免疫毒性化学物質の in vivo 毒性データの構築

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

B. 研究方法

① 免疫毒性物質データの作成

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

② MITA による免疫毒性 clustering の有用性の検討

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

③ IL-2 Luc assayに関するvalidation 試験の最終評価ならびにOECD提出用validation report作成

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

④ IL-1 Luc assayに関するPhase 0ならびにPhase I validation試験

IL-8 転写活性抑制評価系の国際 validation study を行い、MITA を多項目免疫毒性評価系として OECD テストガイドライン化を目指す。

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

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

（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

① 化学物質の免疫毒性データの作成

IL-2 Luc assay の validation に用いた 25 化学物質に関して National Toxicology Program (NTP)の協力を仰ぎ, 免疫毒性データベースを構築した。個々の化学物質に関する毒性データは Appendix 1(IL-2 Luc assay validation report draft)に記載し, それらを総括したデータを表 1 にまとめた。表 1 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。

② MITAによる免疫毒性clusteringの有用性の検討

IL-8 Luc assay と組み合わせた MITA により化学物質が、大きく 6 つの cluster に分類できることが示された。その特性と代表的化学物質を表 2 にした。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。

③ IL-2 Luc assayプロトコールならびにクライテリアの改訂

国際validation委員会にて，昨年度に策定されたクライテリア5（図3）を記載した最終プロトコール(Multi-Immuno Tox Assay protocol Ver.011E) Appendix 1(IL-2 Luc assay validation report draft Appendix)を作成した。このクライテリアを用いバリデーション研究を再評価したところPhase Iでは施設間再現性、施設内再現性はそれぞれ80.0 % (4/5)、86.7 % (13/15)であり、Phase IIでは施設間再現性が80.0 % (16/20)と良好な結果が得られた（表３）。

④ 免疫毒性評価基準の検討ならびにvalidation 試験のpredictivity評価

免疫毒性分野では皮膚感作性試験におけるLLNAのようなゴールドスタンダードが存在せずpredictivity (accuracy)の算出ができない。Validation management team (VMT) のLiaison membersであるGermolec博士らによる化学物質の免疫機能に対する影響をまとめたレポートAppendix 1(IL-2 Luc assay validation report draft Appendix)をもとに化学物質がTリンパ球をターゲットとするか否かの分類を試みた(表１)。この分類に基づきPhase I, Phase IIを統合した際のpredictivityを66.7 % (16/24) と算出した（表３）。

⑤ 60化学物質のIL-2 Luc assayによるpredictivity評価

また，東北大学で検討した60化学物質の解析結果（表４）では，感度84%，特異度54%，精度78% であった。

⑥ MITAを用いた免疫毒性評価系国際化へ向けての国際バリデーション実行委員会

平成30年度:2018年10月4-6日、神戸にて第5回国際バリデーション実行委員会会議を行った。（参加者：小島肇、足利太可雄、S.Venti、相場節也、木村裕、大森崇、真下奈々、高木佑実、門、安野理恵、山影康次、渡辺美香、小林美和子、中島芳浩、Emanuela Corsini、Erwin L. Roggen、Dori Germolec、Tomoaki Inoue）その際に，lead laboratoryからcriteriaの変更が提案され承認された。

⑦ Validation report作成とpeer review 会議の開催

IL-2 Luc assayのOECDガイドライン化を目指しバリデーションレポート（Appendix 1.）を作成した。

⑧ IL-1 Luc assayプロトコールならびにクライテリアの設定

昨年度、MITAとIL-8 Luc assayの結果を用い免疫毒性物質を６つのカテゴリーに分類する方法を提案した[3]。 既にIL-8 Luc assayは、OECD test guideline (442E)に承認され、またIL-2転写活性抑制評価系は、上述のように国際バリデーションphase I、IIが完了している。MITAのもう一つの構成因子として、THP-1細胞をベースとしたIL-1βレポーター細胞であるTHP-G1b細胞を用いた国際バリデーション試験を開始した。

⑨ IL-1 Luc assay Phase 0

国際バリデーション実行委員会にて選定したDapson, Diethanolamine, p-Nitroanilineについて参加3施設、産総研つくば、食薬センター、産総研高松においてMulti-Immuno Tox Assay protocol for TGCHAC-A4 ver. 007E (Appendix 3)にのっとり各物質3回繰り返し1セットの試験を2セット行った（図４）。表5に示すように%suppressionの閾値を20%と設定した場合、産総研つくば、産総研高松においてはリードラボと同様の結果が得られた。食薬センターについてはLPSによるFInSLG-LAの数値が得られない、再現性が得られない等の問題が認められた。食薬センターを含めた際の施設間再現性は83.3% (5/6)であった。

食薬センターについてはその後LPSによるFInSLG-LAの数値が得られない原因を検討し、FCSの非動化の方法、細胞へのLPSへの添加方法を再確認しアッセイしたところリードラボと同様の結果を得られた。（図5)

⑩ IL-1 Luc assay Phase 1

国際バリデーション実行委員会にて選定した５化学物質をコード化し、参加3施設、東北大学、産総研つくば、産総研高松においてMulti-Immuno Tox Assay protocol for TGCHAC-A4 ver. 008Eにのっとり各物質3回繰り返し1セットの試験を３セット実施した(表６)。次年度，Phase 1試験の結果をValidation management teamにて評価し今後の対応を決定する。

E. 考察

これまで行ってきたMITAのvalidation試験の内、IL-2 Luc assayのPhase I、Phase II試験が終了した。これらの試験を通して、IL-2 Luc

assayの施設間、施設内再現性が十分にOECDガイドライン化に必要な基準を満たしていることが明らかになった。しかし、これらの試験の予測性を評価する際に大きな壁に突き当たった。

今回の試験では、Phase I、IIあわせて25種類の化学物質を用いたが、これまで研究者が行ってきた感作性試験法と異なり、化学物質の免疫毒性の有無を評価した確立したデータが存在しないことに気がついた。

そこで、本研究課題のもう一つのテーマである化学物質の免疫毒性データの集積をNTPの協力を得て行った。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、さらにそれらを表１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。

次に、IL-2 Luc assayは免疫毒性評価のなかでも、T細胞を標的にした免疫毒性を評価する試験であることから、ex vivo、in vitroのT細胞由来サイトカイン産生能に影響を与える物質ないしは各化学物質のmode of actionにT細胞への作用が明記されている化学物質をPhase I、II試験のpositive物質とした。

その結果、Phase I、IIをまとめたpredictivityは約67%となった。この値は、必ずしも十分な値ではないが、化学物質の免疫毒性の有無が必ずしも明確ではないこと、またT細胞を標的とした免疫毒性にもIL-2転写活性以外を標的とした作用が存在することは容易に想像できるので、免疫毒性評価の一歩としては満足すべき値なのかもしれない。

IL-2 Luc assayのpredictivityに関しては、2019年2月27日から28日まで、東京にて開催された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されて修正，さらなる検討の必要な個所が指摘された。次年度に対応予定である。

免疫毒性クラスター解析では、クラスター1,2,3,5,6が免疫毒性抑制に分類される。各clusterには臨床で使われている薬剤が含まれているため、それらの主作用ないし副作用から、そのclusterに含まれる一般化学物質の毒性が予測できる。Cluster 1に含まれるdiaminotolueneはwarfarinに類似した特性が予想され、比較的安全な化学物質である可能性が考えられる。Cluster 2に分類されるdiethanolaminはminocyclineと、cluster 3に分類されるcobalt chlorideはchlorpromazineと、Cluster 5に分類されるactinomycin Dはdexamethasoneと、Cluster 6に分類されるFR167653はcyclosporinやFK506と類似したin vivoでの免疫毒性が予想される。

E. 結論

１）25化学物質に関してNational Toxicology Program (NTP)の協力を仰ぎ、免疫毒性データベースを構築した。

２）IL-2 Luc assayに関するvalidationを終了し、validation reportをOECD テストガイドライン化に向けて，peer review 会議に提出した。2019年2月の東京における会議において外部委員により検討され修正点，改善点が示され現在対応中である。

３）IL-1 Luc assayに関する国際的validationを開始し、Phase 0，Phase I を終了し，validation management teamによる評価結果をもとに今後の対応を検討する予定である。

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F. 研究発表

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

なし

表１．化学物質の免疫毒性データまとめ

Chemicals	In vivo				Ex vivo		In vitro			T cell targeting	Expert opinion	Mode of action
	immune sytem organ weight	DTH	infection	tumor	cytokine production	NK activity	TDAR	cytokine production	cell proliferation			
Phase I study												
Dibutyl phthalate	**A (spleen)**							**S (IL-2, 4, IFN-g)(H) A (IL-1b)(H) x 3 S (IL-1b)**		**YES**	**YES**	This compound then is proposed to modulate cytokine secretion from both monocytes/macrophages and T cells.
Hydrocortisone	**S (thymus) x 2 S (spleen)**	**S**					**N**	**S (IFN-a)**		**YES**	**YES**	
Lead(II) acetate	**A(thymus)**	**S A**	**S**				**S N**	**S (IFN-g, IL-1b)(H) A (IL-4)(H)**	**S(H)**	**YES**	**YES**	
Nickel(II) sulfate	**N S (thymus)**	**S**		**S**		**S N**	**N**	**A (IL-4, IFN-g)(H) S (IL-2) S (IFN-g)**		**YES**	**YES**	
dimethyldithiocarbamate (DMDTC)		**A x 2**						**S (IL-1b)**	**N(H)**	**NO**	**NO**	
Phase II study												
2.4-diaminotoluene	**N (spleen) A (spleen)**	**A**	**S**	**N**		**S**	**S**	**-**	**-**	**NO**	**NO**	
Benzo(a)pyrene		**N**	**N**		**S(IL-2)**	**N**	**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**	**YES**	**YES**	Disruption of T-cell activities has been associated with B(a)P induced immunotoxic effects (Urso et al. 1986).
Cadmium Chloride	**A (spleen) S (spleen)**	**N**			**A (IL-2) N (IFN-γ)**	**N**	**S x 4**	**A (IFN-g)(H) S (IL-2, IFN-g) A (IFN-g) S (IL-2) A (IL-2)**	**S**	**YES**	**YES**	
Dibromoacetic acid (DBAA)	**A (spleen) S (thymus) x 2**		**N**	**N**		**S**	**N**	**S (IL-2, 4)**	**S**	**YES**	**No**	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)	**S (thymus) x 4 A (thymus) x 2 A (spleen)**	**N S**	**N(H) A(H)**		**A (IFN-g) x 3**	**A(H)**	**S**	**A (IL-1) A (IL-2)**		**YES**	**YES**	DES exposure was associated with down-regulation of gene expression in the TCR complex, and the TCR and CD28 signaling pathways.
Diphenylhydantoin		**S N**			**A (IL-4) S (IFN-γ, IL-2) S (IL-1α) N (IL-6, 12)**	**S**	**S A x 2**	**-**	**-**	**YES**	**YES**	DPH treatment can lead to a decrease of suppressor T cells
Ethylene Dibromide (EDB)	**S (thymus) S (spleen) N**		**N**			**S**	**A**	**-**	**-**	**NO**	**YES**	

Glycidol	**N**		**A**	**S**		**S**	**S**	**-**	**-**	**NO**	**YES**	Studies suggest that glycidol modulates B-cell function, and NK cell and macrophage
Indomethacin	**N** **A (spleen)**		**A**				**S x 3** **A x 1**	**A (IL-2)(H)** **A (IFN-g)(H)**	**A (H) x 4** **S** **A x 3**	**YES**	**YES**	indomethacin inhibition of prostaglandin synthesis leads to altered T-cell function,
Isonicotinic Acid Hydrazide (IAH)	**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**	**YES**	**YES**	
Nitrobenzene	**A (spleen) x 3** **A (thymus) x 2**		**N** **S**	**N**			**S** **N**	**-**	**-**	**YES**	**YES**	effects on T-cell function may play a role in increased susceptibility to L. monocytogenes (Burns et al. 1994).
Urethane, Ethyl carbamate	**S (thymus) x2** **S (spleen) x 2** **N** **A (thymus)** **A (spleen)**	**S**			**N (IL-2)**	**S x 4** **N x 3**	**S x 2** **N**	**N (IL-2, 4, IFN-g)(H)** **A (IFN-g)(H)** **S (IFN-g)(H)**	**N x 2**	**YES**	**NO**	
Tributyltin Chloride (TBTC)	**S (thymus) x4** **S (spleen) x 3**	**N** **S**	**S x 2**			**S**	**N** **S**	**A (INF-g)(H)** **N (IL-2, 4)(H)** **S (IFN-g)(H)**	**S (H)** **S x 3**	**YES**	**YES**	
Perflouorooctanoic Acid (PFOA)	**S (thymus) x2** **S (spleen) x 2**		**S(H)** **N(H)**		**N (IFN-g)**			**S (IL-4)(H)** **N (IL-2)(H)**	**A (H)** **S (H)** **N (H)**	**YES**	**YES**	Direct modulation of NF-kB has been implicated in modulation of cytokine production and secretion (Corsini et al. 2012).
Dichloroacetic Acid (DCAA)	**A(spleen)**	**N**			**N (IL-2)** **A (IFN-γ) x 3** **S (IL-4) x 2** **S (IL-2)**	**N**	**N**	**A (IL-2)(H)** **A (IL-2, IFN-g)**		**YES**	**YES**	T-cell activation was one proposed mode of action for DCAA.
Toluene									**A**	**NO**	**NO**	Toluene also enhanced NF-kB, STAT5, and NF-AT in thymus cells of C3H/HeN mice inhalationally (Liu et al. 2010).
Acetonitrile	**S(thymus)**					**S**	**S** **S**	**-**	**-**	**Undetemined**	**Undetemined**	Toluene modulation of IL-2 synthesis, after oral No data were located.
Mannnitol									**N (H)**	**NO**	**NO**	
Vanadium Pentoxide	**N** **A (spleen)**		**N x 2**					**S (IL-2, IFN-g)(H)**	**S (H)**	**YES**	**YES**	
o-Benzyl-p-chlorophenol (BCP)	**N**	**N** **A**		**N**			**N**	**-**	**-**	**NO**	**NO**	

表２．化学物質の免疫毒性に基づく分類

Cluster	IL-2転写活性	IL-8転写活性	IL-2 Luc assay	代表的な化学物質
1	N	S	N	Sulfasarzine
2	S	N	P	Citral
3	N	N	P	Formaldehyde Cyclophosphamide Mycophenolic acid Rapamycin
4	N	N	N	Acetaminophen Azathioprine Methotrexate Mizoribine
5	S	S	N	Dexamethosone
6	S	N	N	Cyclosporin, FK506

N: No effect, S: Suppression, P: Positive

表 3. IL-2 Luc assay Phase I, Phase II study まとめ

Chemical	CAS	Lab.A	Lab.B	Lab.C	concordance	T cell targeting
Phase I						
Dibutyl phthalate	84-74-2	SSS	SSS	SSS	1	Yes
Hydrocortisone	50-23-7	SNN	SSS	SSN	0	Yes
Lead(II) acetate	6080-56-4	SSS	SSS	SSS	1	Yes
Nickel(II) sulfate	10101-97-0	SSS	SSS	SSS	1	Yes
Zinc dimethyldithiocarbamate (DMDTC)	137-30-4	NNN	NNN	NNN	1	No
Phase II						
2,4-Diaminotoluene	95-80-7	N	N	N	1	No
Benzo(a)pyrene	50-32-8	S	S	S	1	Yes
Cadmium chloride	10108-64-2	N	N	N	1	Yes
Dibromoacetic acid	631-64-1	A/S	A	N	0	No
Diethylstilbestol	56-53-1	S	S	S	1	Yes
Diphenylhydantoin	630-93-3	N	N	N	1	Yes
Ethylene dibromide	106-93-4	N	N	N	1	Yes
Glycidol	556-52-5	A	A	A	1	Yes
Indomethacin	53-86-1	A	A	A	1	Yes
Isonicotinic Acid Hydrazide	54-85-3	S	N	S	0	Yes
Nitrobenzene	98-95-3	N	S	N	0	Yes
Urethane, Ethyl carbamate	51-79-6	A	A	A	1	No
Tributyltin chloride	1461-22-9	S	S	S	1	Yes
Perfluorooctanoic acid	335-67-1	A	A	A	1	Yes
Dichloracetic acid	79-43-6	A	S	S	0	Yes
Toluene	108-88-3	N	N	N	1	No
Acetonitril	75-05-8	N	N	N	1	undetermined
Mannitol	69-65-8	N	N	N	1	No

Vanadium pentoxide	1314-62-1	N	N	N	1	Yes
o-Benzyl-p-chorolophenol	120-32-1	S	S	S	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 (%)		64.7 (11/17)	70.6 (12/17)	70.6 (12/17)		
		Average 68.6 (35/51)				
Specificity (%)		57.1 (4/7)	57.1 (4/7)	71.4 (5/7)		
		Average 61.9 (13/21)				
Accuracy (%)		62.5 (15/24)	66.7 (16/24)	70.8 (17/24)		
		Average 66.7 (48/72)				

S : Immunosuppression, A : Immunoaugmentation, N : No effect,
A/S : Immunoaugmentation/suppression.
T cell targeting と一致する結果を黄色のハイライトで示した。

表4 IL-2 Luc assay data set

Chemical name	Judge	Ave.LOEL(35%)	Ave.LOEL(-35%)	Immunotoxicity in references
FK506	S	0.0002		S
Cyclosporine A	S	0.0041		S
Actinomycin D	S	0.0156		S
Digoxin	S	0.0686		N
Colchichine	S	0.2743		S
FR1667953	S	1.3021		S
Benzethonium chloride	S	1.6276		U
Mercuric chloride	S	1.9531		S
Chlorpromazine	S	1.9531		U
Amphoterycin B	S	2.6042		U
Dibutyl phthalate	S	2.6042		S
2-Aminoanthracene	S	5.8594		U
Formaldehyde	S	7.8125		S
Pyrimethamine	S	7.8125		S
Isophorone diisocyanate	S	15.6250		U
Cisplatin	S	16.9271		S
Cobalt chloride	S	16.9271		S
Chloroquine	S	17.8326		S
Minocycline	S	18.5185		S
Mitomycin C	S	20.0000		S
Hydrogen peroxide	S	23.4375		U
Citral	S	25.0000		U
Dexamethasone	S	41.1692		S
Pentamidine isethionate	S	52.0833		U
Lead(II)acetate	S	57.2917		S
Azathioprine	S	58.4778		S
Diesel exaust particle	S	62.5000		S
Sodium dodecyl sulfate	S	62.5000		N
Dapsone	S	72.9167		S
Nitrofurazone	S	83.3333		U
p-Nitroaniline	S	83.3333		U
Sulfasalazine	S	92.9444		S
Aluminium chloride	S	104.1667		S
Nickel sulfate	S	104.1667		S
Diethanolamine	S	250.0000		U
Chloroplatinic acid	S	250.0000		U
Sodium bromate	S	500.0000		S
Histamine	S	750.0000		S
Isoniazid	N			N
Triethanolamine	N			U
Magnesium sulfate	N			U
Hydrocortisone	N			S
Rapamycin	N			S
Mizoribine	N			S
Warfarin	N			N
2,4-Diaminotoluene	N			U
Cyclophosphamide	N			S
Dibenzopyrene	N			U
Ethanol	N			N
Hexachlorobenzene	N			S
Lithium carbonate	N			U
Methanol	N			N
Methotrexate	N			S
Dimethyl sulfoxide	N			N
Trichloroethylene	N			U
Mycophenolic acid	A		0.395061728	S
2-Mercaptobenzothiazole	A		16.11328125	N
Ribavirin	A		26.04166667	U
Nicotinamide	A		288.0658436	N
Acetaminophen	A		100	S

S: Suppression; N: No effects on T cells, U: Undetermined

U	Unclassified
S	Suppression, correctly judged
S	Suppression, misjudged
N	No effects, correctly judged
N	No effects, misjudged

表 5 IL-1 Luc assay Phase 0 結果

Chemical	Lab.A (Lead Lab)	Lab.B (AISTTS)	Lab.C (FDSC)	Lab.D (AISTTA)	concordance	Based on Majority
Dapson Set1	S:SSS	S:SSS	S:SSS	S:SSS	1	S
Dapson Set2	S:SSS	S:SSS	S:SSS	S:SSS	1	S
Diethanolamine Set1	S:SSS	S:SSS	?:S/A AS	S:SSS	0	S
Diethanolamine Set2	S:SSS	S:SSS	S:SS S/A	S:SSS	1	S
p-Nitroaniline Set1	S:SSS	S:SSS	S:SSS	S:SSS	1	S
p-Nitroaniline Set2	S:SSS	S:SSS	S:SSS	S:SSS	1	S

表6 IL-1 Luc assay Phase 1 結果

MITA　Phase1

2019.3.25現在

LabA Tohoku							LabB AIST tsukuba							LabC AIST shikoku						
setNo.	code No.	1回目	2回目	3回目	4回目	judge	setNo.	code No.	1回目	2回目	3回目	4回目	judge	setNo.	code No.	1回目	2回目	3回目	4回目	judge
Set1	MITA103	S	S			S	Set1	MITB402	S	S			S	Set1	MITC704	S	S	S		S
Set2	MITA203	S	S			S	Set2	MITB501	S	S			S	Set2	MITC803	S	S			S
Set3	MITA304	S	S			S	Set3	MITB605	S	S			S	Set3	MITC902	S	S			S
Set1	MITA101	A	N	A		A	Set1	MITB404	N	N			N	Set1	MITC701	N	A	A		A
Set2	MITA205	S	N	A	N	N	Set2	MITB505	N	N			N	Set2	MITC802	N	A	N		N
Set3	MITA305	S/A	N	N		N	Set3	MITB603	N	N			N	Set3	MITC905	S	A	A		A
Set1	MITA104	N	S	S		S	Set1	MITB403	N	N			N	Set1	MITC705	N	N			N
Set2	MITA202	S	S			S	Set2	MITB502	N	N			N	Set2	MITC805	S	S			S
Set3	MITA303	N	N			N	Set3	MITB601	N	S	N		N	Set3	MITC901	S	S			S
Set1	MITA105	S	S			S	Set1	MITB401	S	S			S	Set1	MITC702	S	S			S
Set2	MITA204	S	S			S	Set2	MITB503	N	S	S		S	Set2	MITC801	S	S			S
Set3	MITA301	S	S			S	Set3	MITB602	S	N	S		S	Set3	MITC904	S	S			S
Set1	MITA102	A	S	N	N	N	Set1	MITB405	N	N			N	Set1	MITC703	S	S			S
Set2	MITA201	N	N			N	Set2	MITB504	N	N			N	Set2	MITC804	N	N			N
Set3	MITA302	N	S	N		N	Set3	MITB604	N	N			N	Set3	MITC903	N	N			N

図 1.　研究計画

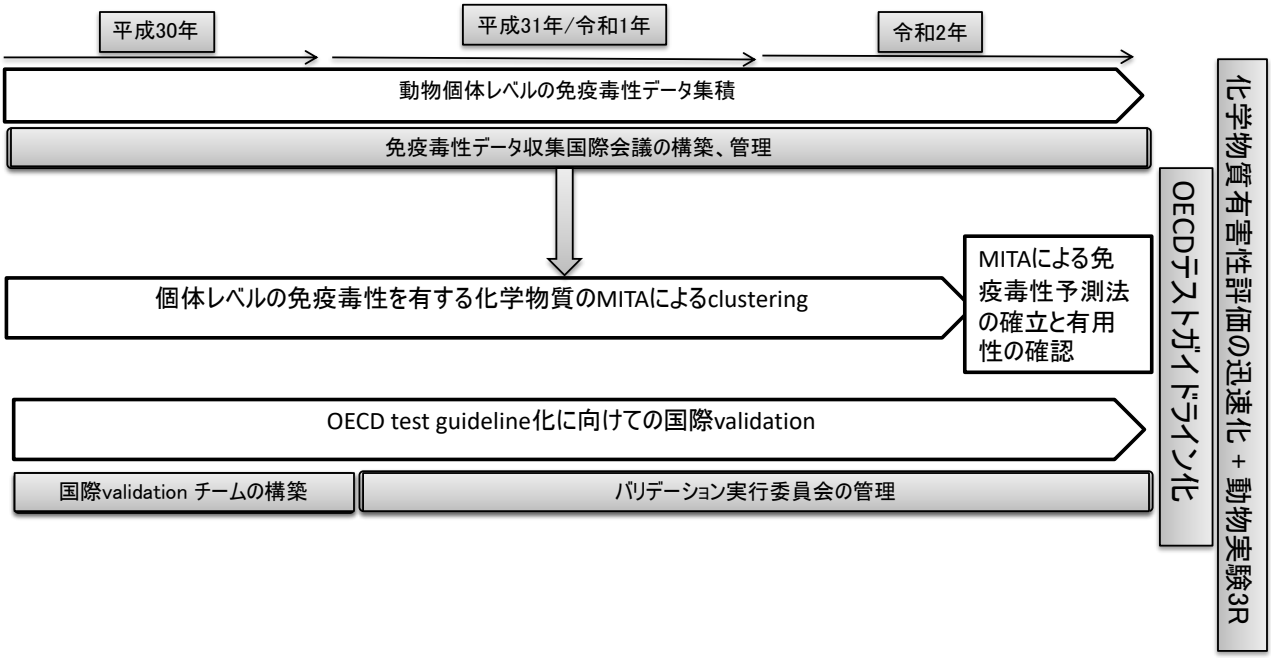


図 2．Multi-ImmunoTox assay (MITA)

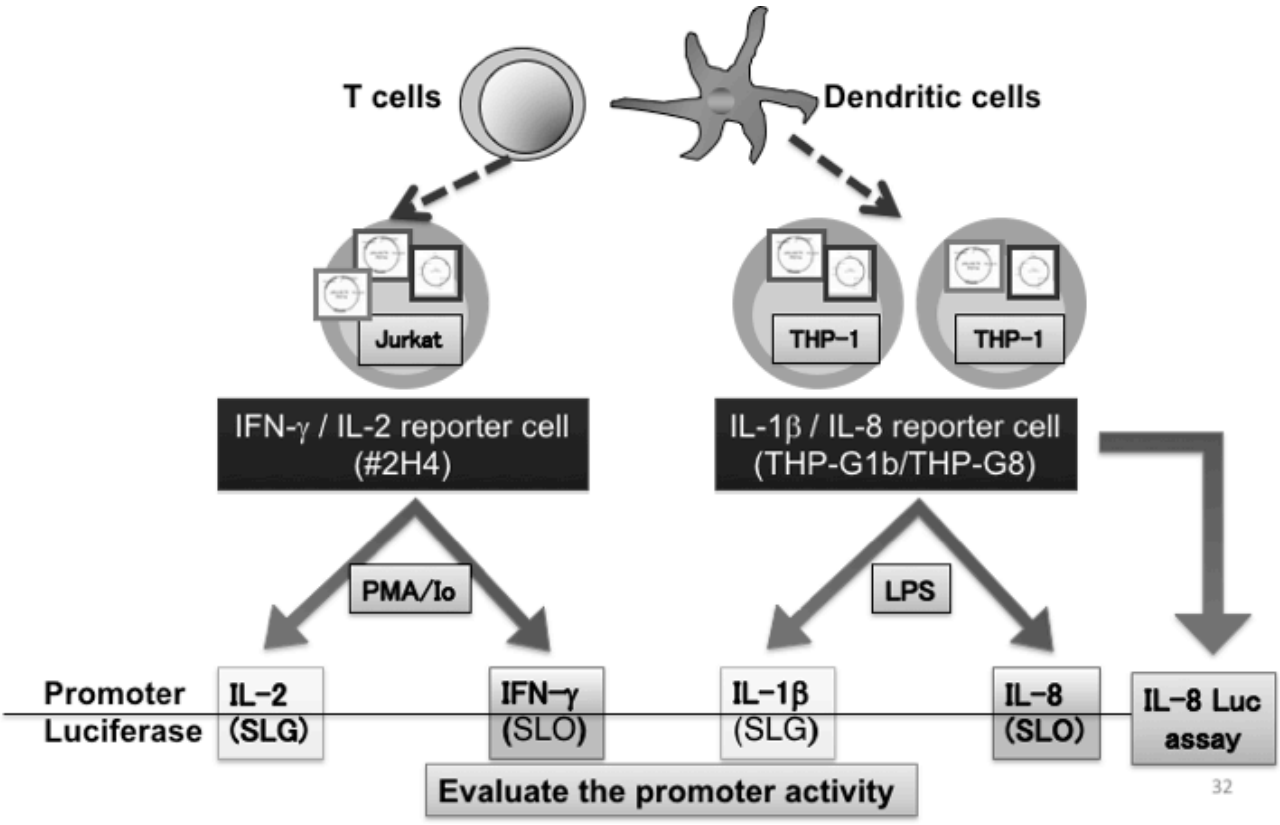


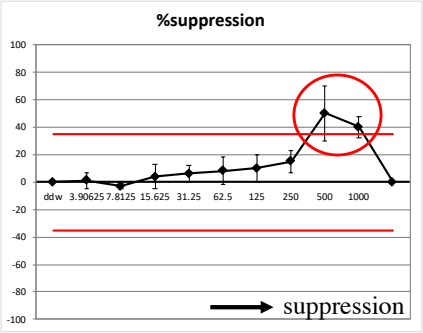
図 3. IL-2 Luc assay 判定基準

Criteria 5

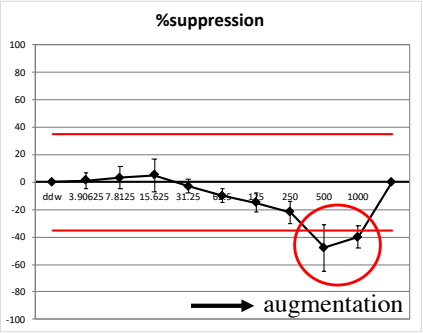
The experiments are repeated until two consistent positive (negative) results or two consistent “no effect results” are obtained. When two consistent results are obtained, the chemicals are judged as the obtained consistent results.
Identification of immunotoxicant is evaluated by the mean of %suppression and its 95% simultaneous confidence interval.

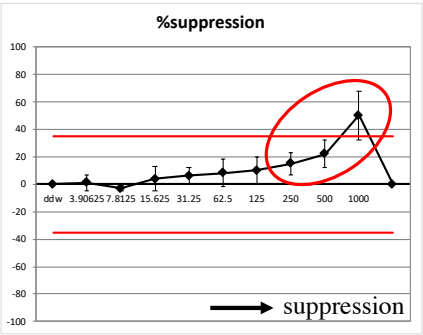
In each experiment, when the chemicals clear the following 3 criteria, they are judged as suppressive or stimulatory. Otherwise, they are judged as no effect chemicals.

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

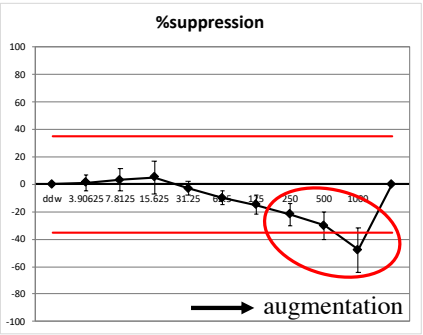


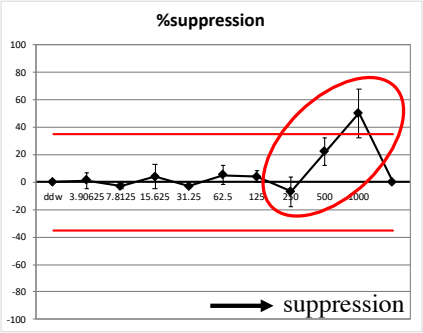
2. The result shows two or more consecutive statistically significant positive (negative) data,



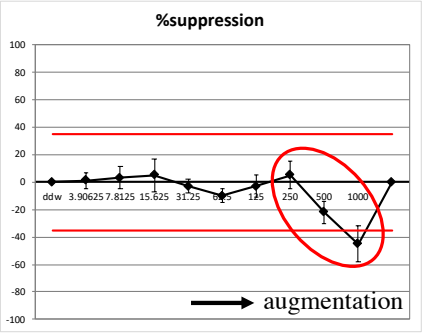


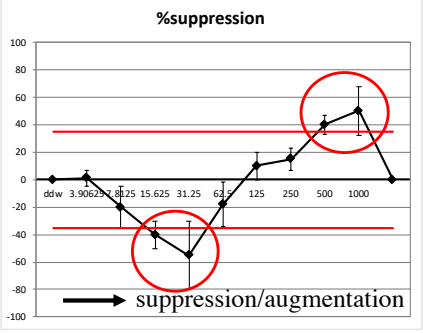
or one statistically significant positive (negative) data with a trend in which at least 3 consecutive data increase (decrease) in a dose dependent manner.





In the latter case, the trend can cross 0, as long as only one data point shows the opposite effect without statistical significance.





両方の条件を満たす場合は suppression/augmentation と判定する。

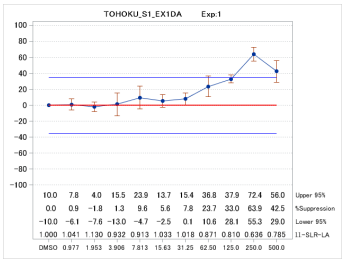
3. The results are judged using only data obtained in the concentration at which I.I.-SLR-LA is ≥ 0.05
（0.05未満の濃度は 0 と表示される。）

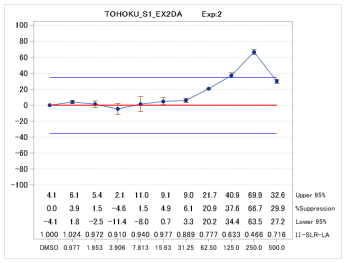
図 4　IL-1 Luc assay Phase 0 グラフ

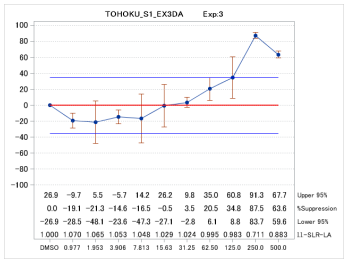
Lab.A(Lead Lab) Dapsone

Set1

S:SSS

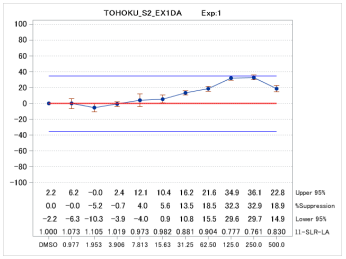


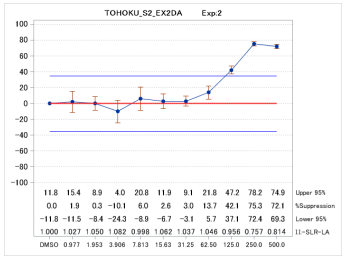


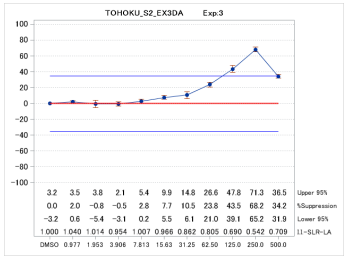


Set2

S:SSS



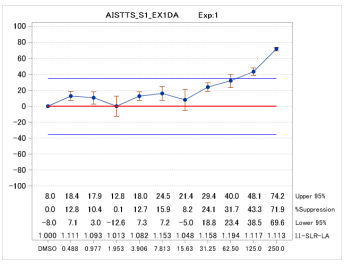


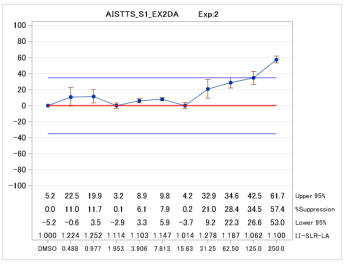


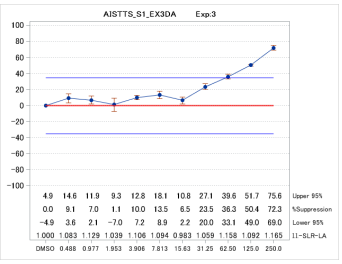
Lab.B(AISTTS) Dapsone

Set1

S:SSS

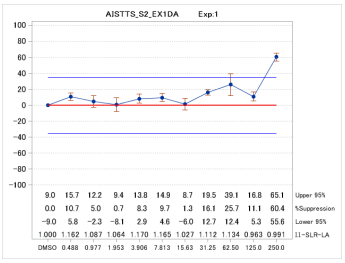


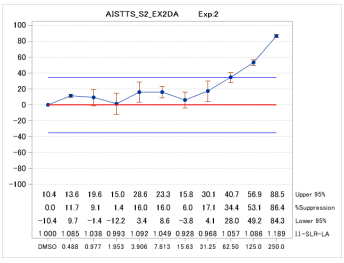


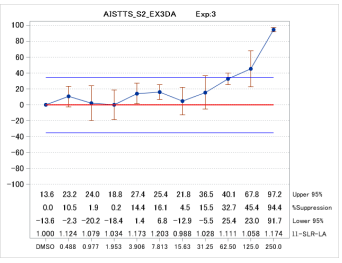


Set2

S:SSS







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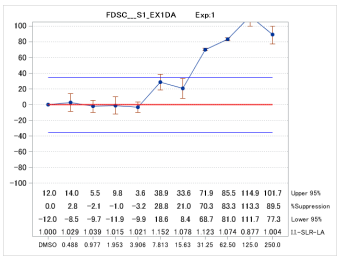
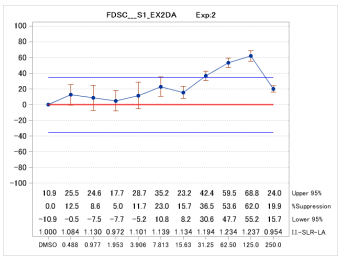
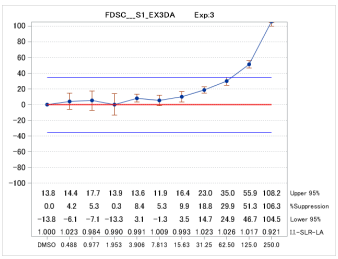
Lab.C(FDSC) Dapsone

Set1

S:SSS

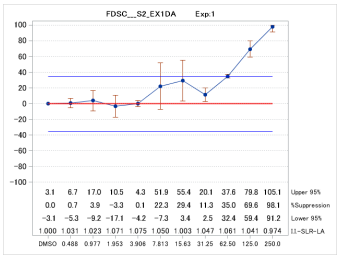


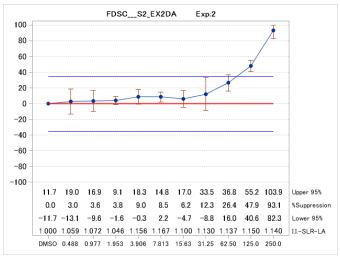


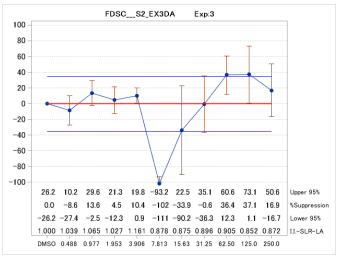


Set2

S:SSS



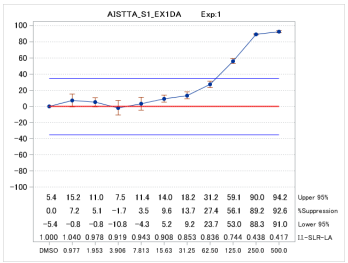


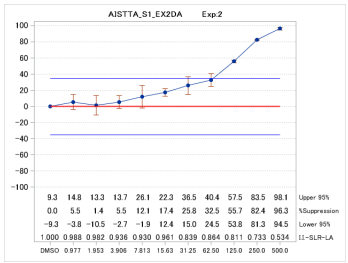


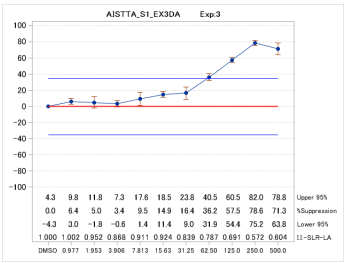
Lab.D(AIST-Shikoku) Dapsone

Set1

S:SSS

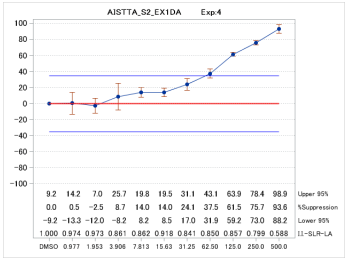


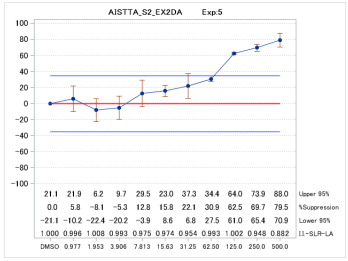


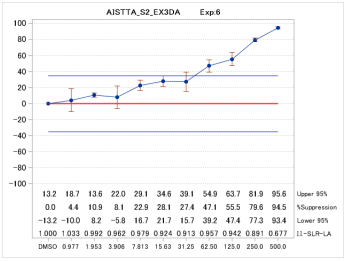


Set2

S:SSS







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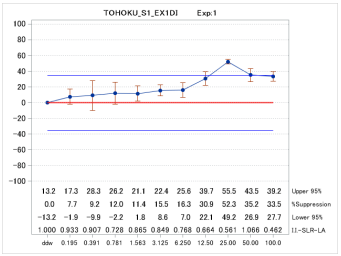
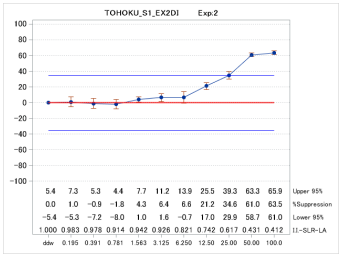
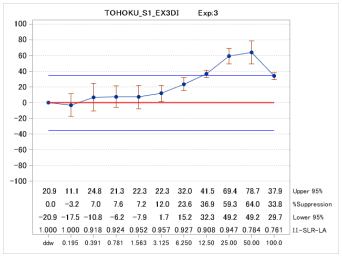
Lab.A(Lead Lab) Diethanolamine

Set1

S:SSS

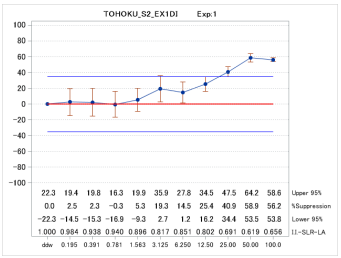


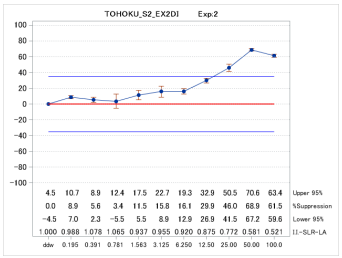


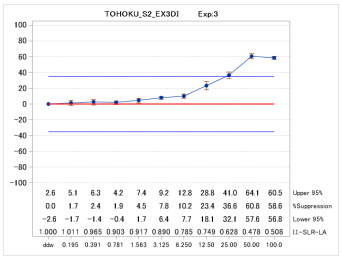


Set2

S:SSS



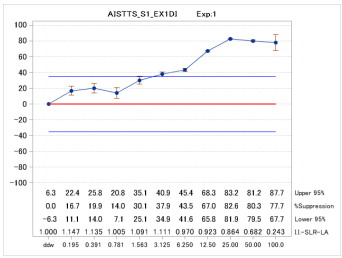


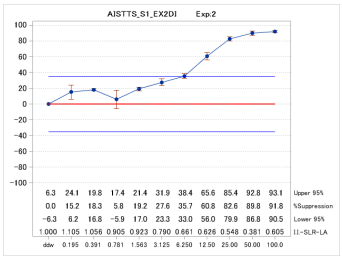


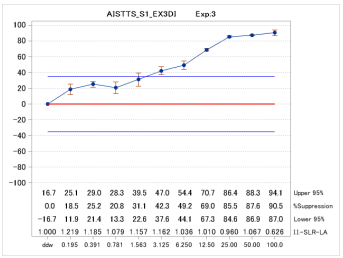
Lab.B(AISTST) Diethanolamine

Set1

S:SSS

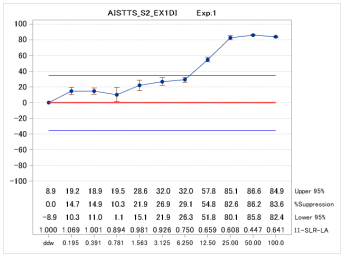


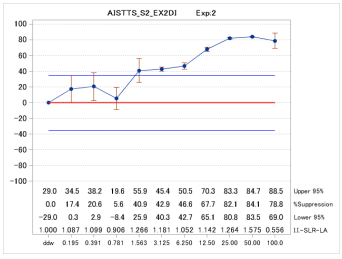


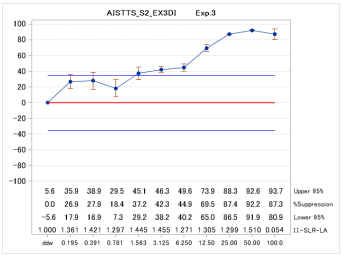


Set2

S:SSS







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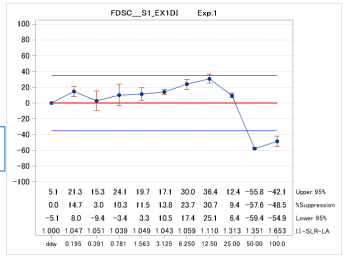
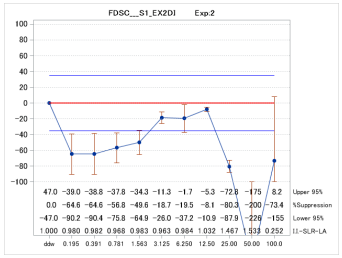
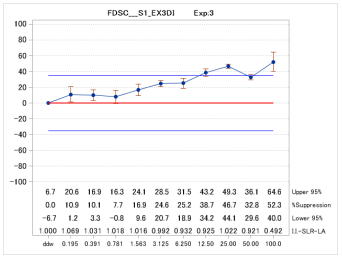
Lab.C(FDSC) Diethanolamine

Set1

?:S/A AS

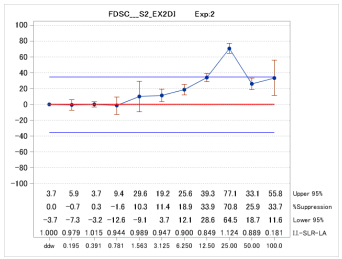


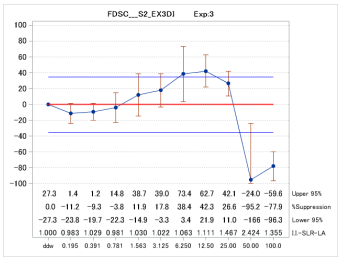




Set2

S:SS S/A

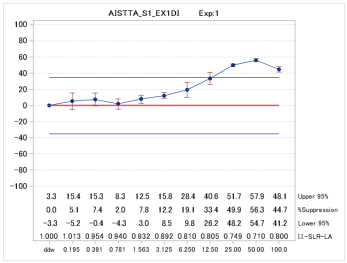


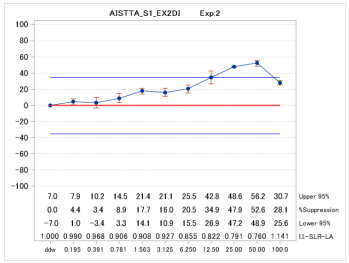


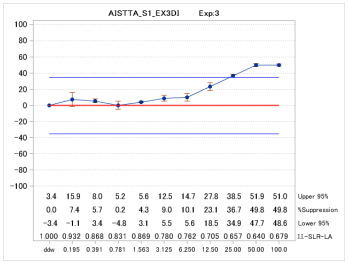
Lab.D(AIST-Shikoku) Diethanolamine

Set1

S:SSS

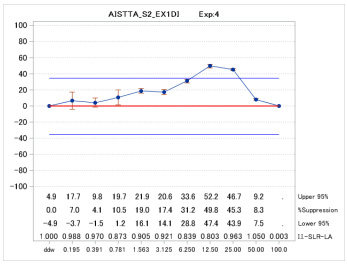


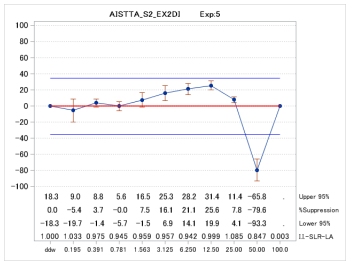


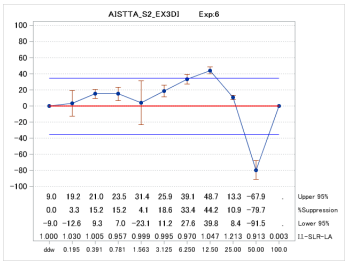


Set2

S:SSS







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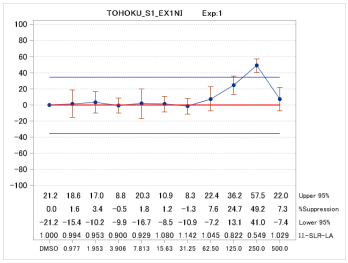
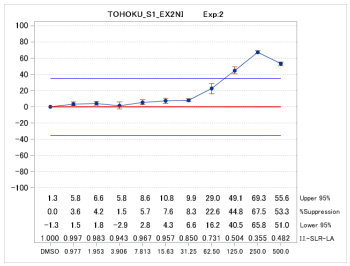
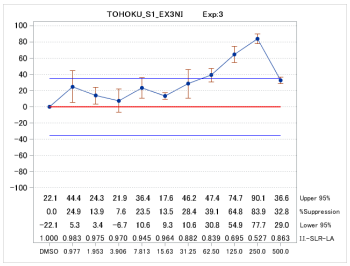
Lab.A(Lead Lab) p-Nitroaniline

Set1

S:SSS







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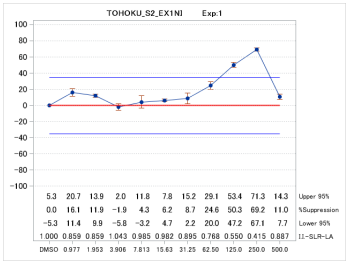
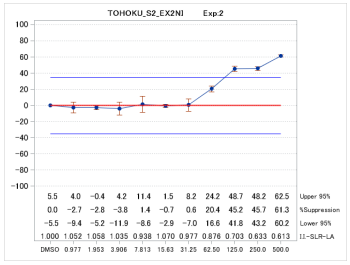
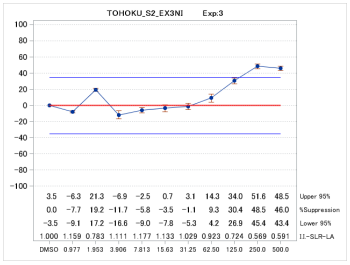


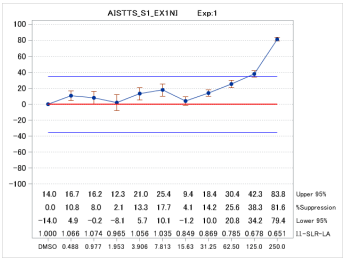


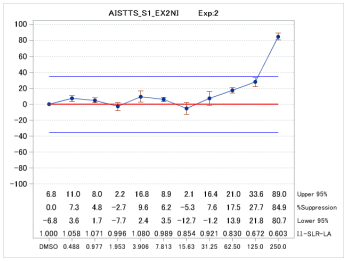


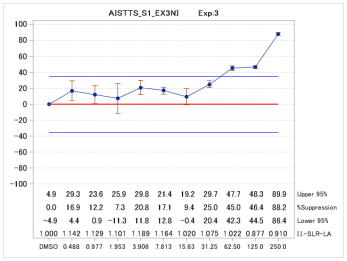
Lab.B(AISTTS) p-Nitroaniline

Set1

S:SSS







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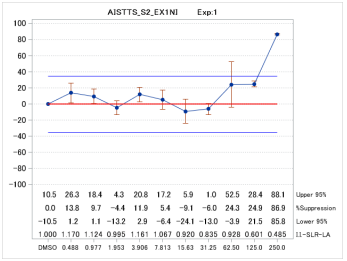

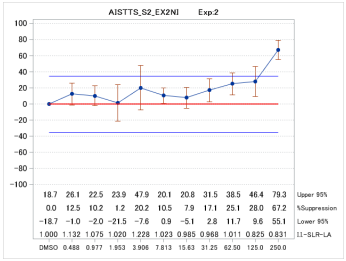

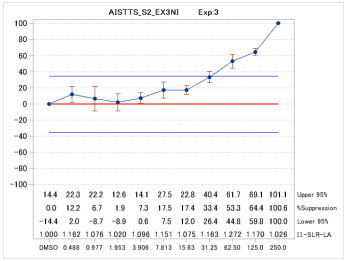

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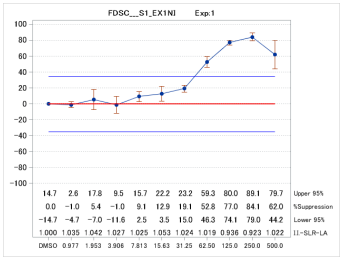
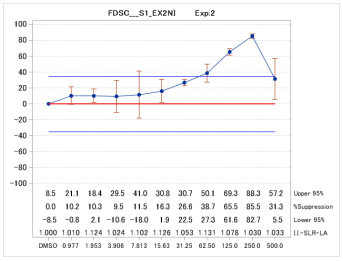
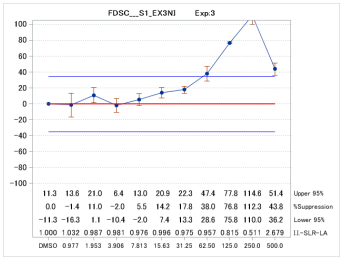
Lab.C(FDSC) p-Nitroaniline

Set1

S:SSS







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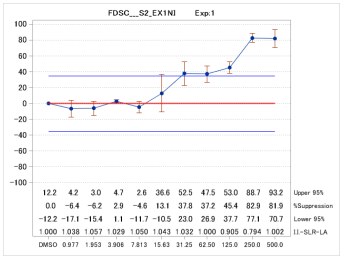

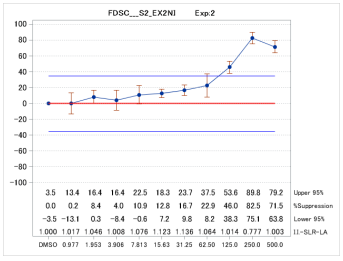

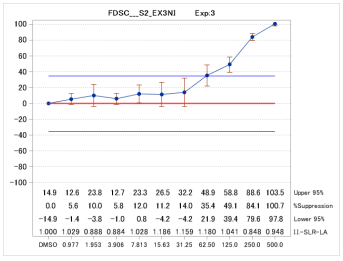

Lab.D(AIST-Shikoku) p-Nitroaniline

Set1

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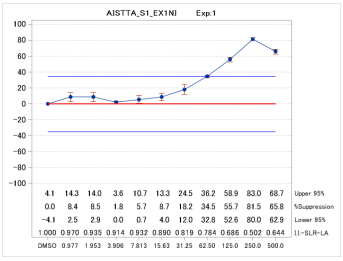
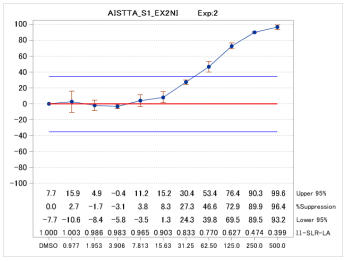
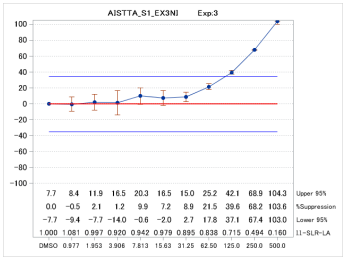
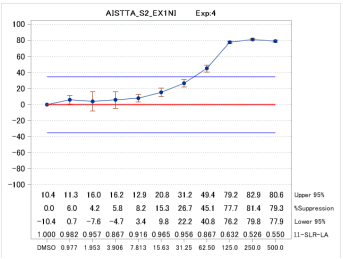


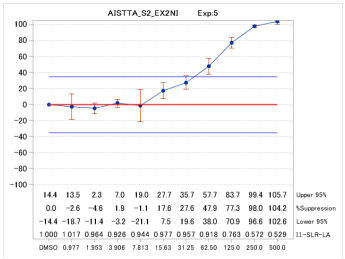




Set2

S:SSS





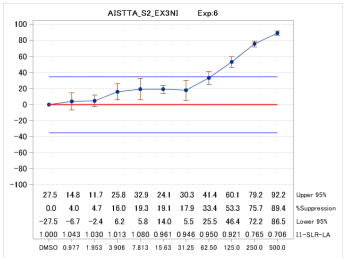
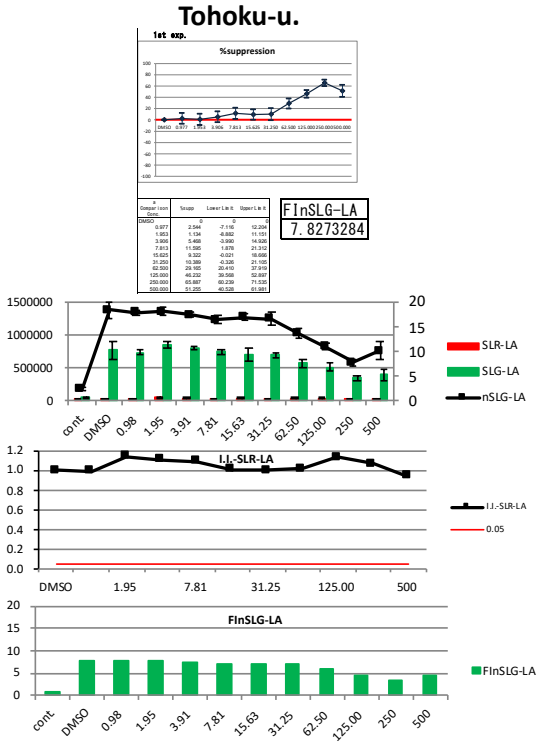


図 5 技術確認会結果

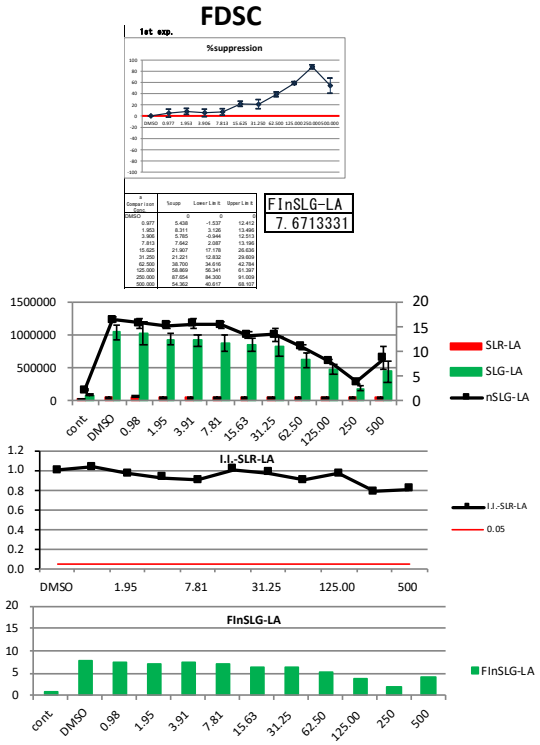
Dapsone

2018.12.19 FDSC&TU MITA技術確認会

Tohoku-u.



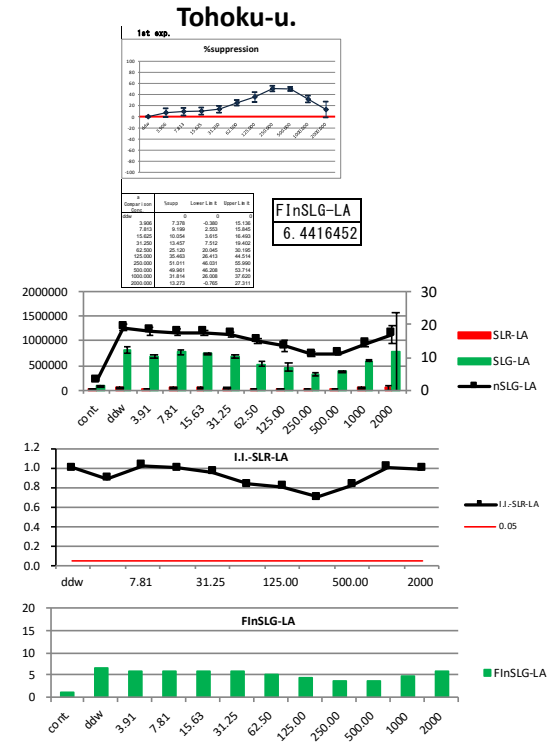
FDSC



2018.12.19 FDSC&TU MITA技術確認会

Diethanolamine

Tohoku-u.



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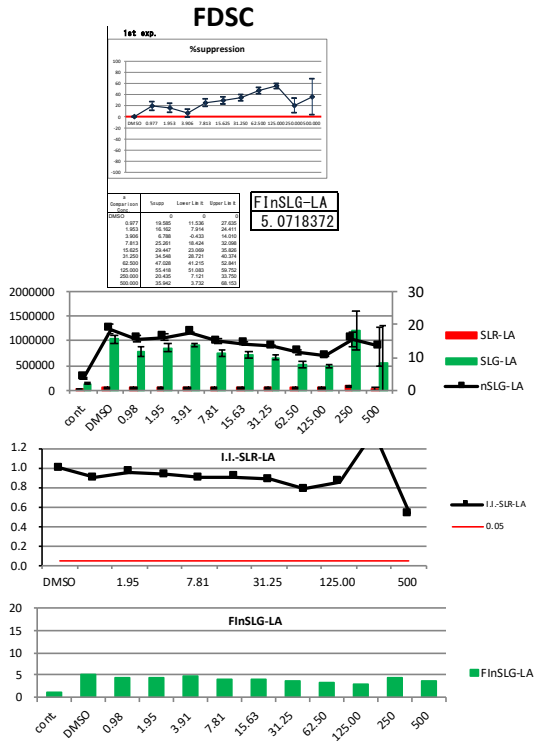

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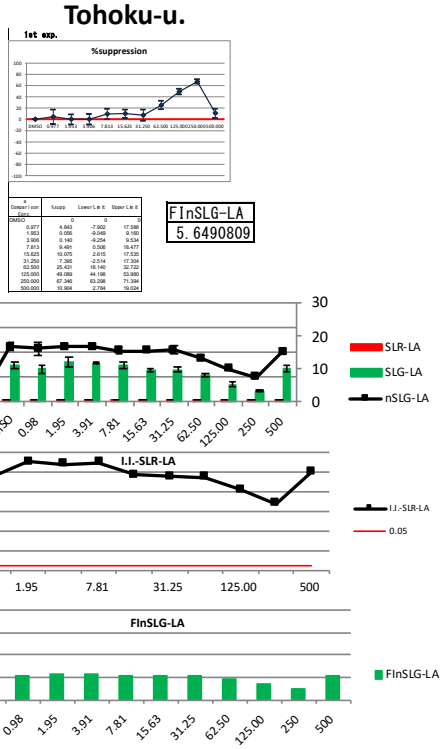
2018.12.19 FDSC&TU MITA技術確認会

p-Nitroaniline

Tohoku-u.



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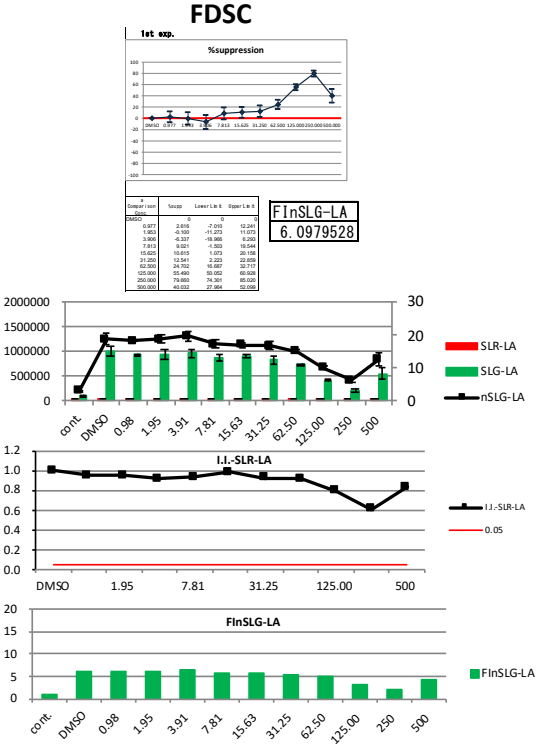

厚生労働科学研究費補助金（化学リスク研究事業）
化学物質の動物個体レベルの免疫毒性データ集積とそれに基づくMulti-ImmunoTox assay（MITA）による予測性試験法の確立と国際標準化
平成30年度分担研究報告書

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

分担研究者　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　安全性予測評価部　室長

研究要旨

*in vitro*免疫毒性評価試験法（Multi-ImmunoTox assay：MITA)である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）としての公定化するため、国際バリデーション研究を施行した。本年度は、IL-2 Lucアッセイのバリデーション報告書を作成するとともに、IL-1β Lucアッセイの施設内再現性を検証するため、バリデーション研究（phase I）を実施した。その結果、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。

研究協力者氏名・所属機関名及び所属機関における職名

相場節也　東北大学医学系研究科・医学部・皮膚科学分野教授
木村　裕　東北大学医学系研究科・医学部・皮膚科学分野助教
足利太可雄　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　安全性予測評価部主任研究員

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）である 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）としての公定化するため、国際バリデーション研究を施行する。

B．研究方法

B-1. バリデーション研究の支援

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)の最終結果の報告書を作成する。

B-1.2.　IL-1β Lucアッセイバリデーション計画の作成

IL-１βLucアッセイのバリデーション研究の計画をバリデーション実行委員会のメンバーに対面会議にて、提示した。

B-1.3.　IL-１βLucアッセイバリデーション被験物質の送付

IL-１βLucアッセイのバリデーション研究Phase I(以下、Phase I と記す)にて、施設内再現性を求めるために選ばれた5物質の3セット分をコード化し、合計15物質を各施設に送付した。

被験物質は、以下に示す対面会議にて、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-1-4. IL-１βLucアッセイバリデーション結果の記録確認

Phase I 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。

B-2. 国際的な専門家との意見交換

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、2018年10月4-6日、神戸にて対面会議を企画した。

C. 結果

C-1. バリデーション研究の支援

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

バリデーション実行員であるDr. Dori Germolec（NTP/NIEHS）の協力を得て、免疫毒性のヒト及び動物実験データを集積し、相場らに提供した。相場らがまとめたIL-2 Luc アッセイの最終結果の報告書を確認し、被験物質の配布、コード化及び質的確保に関する部分を加筆・修正した。

C-1.2. IL-1β Lucアッセイバリデーション計画の作成

対面会議にて、添付資料１に示すバリデーション計画が承認された。

C-1.3. IL-1β Lucアッセイバリデーション被験物質の送付

Phase I にて、施設内再現性を求めるために実行委員会で選ばれた5物質3セットの15物質を選定し、コード化してリード施設を含む参加4施設に送付した。表1に被験物質のコード番号を示す。

実験の終了まで、被験物質に関するトラブルは生じなかった。平成31年4月5日及び5月2日に開催された参加施設との電話会議にて、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であるとの見解で合意を得た。

C-1-4. IL-1β Lucアッセイバリデーション結果の記録確認

Phase I 終了後に回収した記録用紙の一覧を表2に示した。施設によって一部不備はあったが、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。

C-2. 国際的な専門家との意見交換

国際バリデーション研究における対面会議には、免疫毒性及びその試験法に関する専門家として、海外から Dr. Emanual Corsini (Milan Univ.)、Dr. Erwin L. Roggen（3Rs Management and Consulting ApS）及び Dr. Dori Germolec を、国内からは、日本免疫毒性学会の推薦者である井上智彰博士（中外製薬）を外部専門家として招聘し、研究班の班員を含む表 3 に示すメンバーにて 2 日間掛けて、IL-2 Luc アッセイバリデーション報告書内容の確認、IL-1β Luc アッセイのプロトコルの改訂、予備試験結果の確認及びバリデーション被験物質の選定などについて討論した。会議の議事概要を添付文書 2 に示す。

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を作成し、次の段階として、国際的なindependent peer review（第三者評価）に送ることができた。なお、この第三者評価は、別途研究班で実施されている。

一方、MITAのもう一つの試験法であるIL-１βLucアッセイの計画書を作成し、Phase Iとして施設内再現性の確認を目的としたバリデーション研究が施行された。次年度, 追加実験結果と合わせ、Phase 1試験の結果をバリデーション実行委員会にて評価し、今後の対応を決定する予定である。

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、バリデーション報告書を完成できた。IL-1β Lucアッセイについては、このバリデーションの施設内再現性を確保するため、被験物質5点をコード化し、配布、実験記録の回収及び確認を担当し、適切な実験が実施されていることを確認できた。ただし、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。

F. 添付文書

1) 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
2) Minutes of MITA at 5th meeting in October, 2018

表 1. Phase I 被験物質とコード番号

LabA Tohoku							LabB AIST tsukuba							LabC AIST shikoku					
setNo.	code No.	1 回目	2 回目	3 回目	4 回目	judge	setNo.	code No.	1 回目	2 回目	3 回目	4 回目	judge	setNo.	code No.	1 回目	2 回目	3 回目	4 回目	judge
Set1	MITA103	S	S			S	Set1	MITB402	S	S			S	Set1	MITC704	S	S	S		S
Set2	MITA203	S	S			S	Set2	MITB501	S	S			S	Set2	MITC803	S	S			S
Set3	MITA304	S	S			S	Set3	MITB605	S	S			S	Set3	MITC902	S	S			S
Set1	MITA101	A	N	A		A	Set1	MITB404	N	N			N	Set1	MITC701	N	A	A		A
Set2	MITA205	S	N	A	N	N	Set2	MITB505	N	N			N	Set2	MITC802	N	A	N		N
Set3	MITA305	S/A	N	N		N	Set3	MITB603	N	N			N	Set3	MITC905	S	A	A		A
Set1	MITA104	N	S	S		S	Set1	MITB403	N	N			N	Set1	MITC705	N	N			N
Set2	MITA202	S	S			S	Set2	MITB502	N	N			N	Set2	MITC805	S	S			S
Set3	MITA303	N	N			N	Set3	MITB601	N	S	N		N	Set3	MITC901	S	S			S
Set1	MITA105	S	S			S	Set1	MITB401	S	S			S	Set1	MITC702	S	S			S
Set2	MITA204	S	S			S	Set2	MITB503	N	S	S		S	Set2	MITC801	S	S			S
Set3	MITA301	S	S			S	Set3	MITB602	S	N	S		S	Set3	MITC904	S	S			S
Set1	MITA102	A	S	N	N	N	Set1	MITB405	N	N			N	Set1	MITC703	S	S			S
Set2	MITA201	N	N			N	Set2	MITB504	N	N			N	Set2	MITC804	N	N			N
Set3	MITA302	N	S	N		N	Set3	MITB604	N	N			N	Set3	MITC903	N	N			N

表2. Phase I　記録用紙の一覧表

MITA（P1）資料確認記録　　2019年3月19日

setNo.	参加施設名 / 資料名	東北大学（LabA）				AISTつくば（LabB）				AIST四国（LabC）			
		資料有無	ファイルNo.	●施設確認	備考	資料有無	ファイルNo.	●施設確認	備考	資料有無	ファイルNo.	●施設確認	備考
set1	①試薬管理（５物質）	○	35			○	19			○	1		
	②溶解性試験（５物質）	○	36			○	20			○	2		
	③細胞増殖記録	○	37			○	21		A-1~3,B-1,2	○	3		1,2
set1-1 実験記録	④実験担当者・試験物質コード	○	38			○	22			○	4		
	⑤細胞調整	○	38			○	22			○	4		
	⑥コントロールの調製と細胞への処理	○	38			○	22			○	4		
	⑦被試験試薬の調整（DW、DWSO）	○	38			○	22		5物質	○	4		5物質
set1-2 実験記録	④実験担当者・試験物質コード	○	39			○	23			○	5		
	⑤細胞調整	○	39			○	23			○	5		
	⑥コントロールの調製と細胞への処理	○	39			○	23			○	5		
	⑦被試験試薬の調整（DW、DWSO）	○	39			○	23		5物質	○	5		5物質
set1-3	④実験担当者・試験物質コード	○	40			-	-			○	6		
	⑤細胞調整	○	40			-	-			○	6		
	⑥コントロールの調製と細胞への処理	○	40			-	-			○	6		
	⑦被試験試薬の調整（DW、DWSO）	○	40			-	-			○	6		4物質
set1-4	④実験担当者・試験物質コード	○	41			-	-			-	-		
	⑤細胞調整	○	41			-	-			-	-		
	⑥コントロールの調製と細胞への処理	○	41			-	-			-	-		
	⑦被試験試薬の調整（DW、DWSO）	○	41			-	-			-	-		
set1-5	④実験担当者・試験物質コード	○	42			-	-			-	-		
	⑤細胞調整	○	42			-	-			-	-		
	⑥コントロールの調製と細胞への処理	○	42			-	-			-	-		
	⑦被試験試薬の調整（DW、DWSO）	○	42			-	-			-	-		
set1-6	④実験担当者・試験物質コード	○	43			-	-			-	-		
	⑤細胞調整	○	43			-	-			-	-		
	⑥コントロールの調製と細胞への処理	○	43			-	-			-	-		
	⑦被試験試薬の調整（DW、DWSO）	○	43			-	-			-	-		
set1-7	④実験担当者・試験物質コード	○	44			-	-			-	-		
	⑤細胞調整	○	44			-	-			-	-		
	⑥コントロールの調製と細胞への処理	○	44			-	-			-	-		
	⑦被試験試薬の調整（DW、DWSO）	○	44			-	-			-	-		
set1-8	④実験担当者・試験物質コード	○	45			-	-			-	-		
	⑤細胞調整	○	45			-	-			-	-		
	⑥コントロールの調製と細胞への処理	○	45			-	-			-	-		
	⑦被試験試薬の調整（DW、DWSO）	○	45			-	-			-	-		

MITA（P1）資料確認記録

2019年3月19日

setNo.	参加施設名 / 資料名	東北大学（LabA）				AISTつくば（LabB）				AIST四国（LabC）			
		資料有無	ファイルNo.	事務局確認	備考	資料有無	ファイルNo.	事務局確認	備考	資料有無	ファイルNo.	事務局確認	備考
set2	①試薬管理（５物質）	○	46			○	24			○	7		
	②溶解性試験（５物質）	○	47			○	24			○	8		
	③細胞培養記録	○	48				21			○	9		
set2-1	④実験担当者・試験物質コード	○	49			○	26			○	10		
	⑤細胞調整	○	49			○	26			○	10		
	⑥コントロールの調製と細胞への処理	○	49			○	26			○	10		
	⑦被試験試薬の調整（DW、DWSO）	○	49			○	26		5物質	○	10		5物質
set2-2	④実験担当者・試験物質コード	○	50			○	27			○	11		
	⑤細胞調整	○	50			○	27			○	11		
	⑥コントロールの調製と細胞への処理	○	50			○	27			○	11		
	⑦被試験試薬の調整（DW、DWSO）	○	50			○	27		5物質	○	11		5物質
set2-3	④実験担当者・試験物質コード	○	51			○	28			○	12		
	⑤細胞調整	○	51			○	28			○	12		
	⑥コントロールの調製と細胞への処理	○	51			○	28			○	12		
	⑦被試験試薬の調整（DW、DWSO）	○	51			○	28		1物質	○	12		2物質
set2-4	④実験担当者・試験物質コード	○	52			-	-			-	-		
	⑤細胞調整	○	52			-	-			-	-		
	⑥コントロールの調製と細胞への処理	○	52			-	-			-	-		
	⑦被試験試薬の調整（DW、DWSO）	○	52			-	-			-	-		
set2-5	④実験担当者・試験物質コード	○	53			-	-			-	-		
	⑤細胞調整	○	53			-	-			-	-		
	⑥コントロールの調製と細胞への処理	○	53			-	-			-	-		
	⑦被試験試薬の調整（DW、DWSO）	○	53			-	-			-	-		
set3	①試薬管理（５物質）	○	54			○	29			○	13		
	②溶解性試験（５物質）	○	55			○	30			○	14		
	③細胞培養記録	○	56			○	21			○	15		
set3-1	④実験担当者・試験物質コード	○	57			○	31			○	16		
	⑤細胞調整	○	57			○	31			○	16		
	⑥コントロールの調製と細胞への処理	○	57			○	31			○	16		
	⑦被試験試薬の調整（DW、DWSO）	○	57			○	31		5物質	○	16		
set3-2	④実験担当者・試験物質コード	○	58			○	32			○	17		
	⑤細胞調整	○	58			○	32			○	17		
	⑥コントロールの調製と細胞への処理	○	58			○	32		5物質	○	17		
	⑦被試験試薬の調整（DW、DWSO）	○	58			○	32			○	17		
set3-2（再試験）	④実験担当者・試験物質コード		-	-		○	33		set3-2の		-	-	
	⑤細胞調整		-	-		○	33		やり直し		-	-	
	⑥コントロールの調製と細胞への処理		-	-		○	33				-	-	
	⑦被試験試薬の調整（DW、DWSO）		-	-		○	33		5物質		-	-	
set3-3	④実験担当者・試験物質コード	○	59			○	34			○	18		
	⑤細胞調整	○	59			○	34			○	18		
	⑥コントロールの調製と細胞への処理	○	59			○	34			○	18		
	⑦被試験試薬の調整（DW、DWSO）	○	59			○	34		2物質	○	18		2物質

表3. 2018年度　MITA国際バリデーション対面会議の主な参加者リスト

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	YoshihiroNakajima	National Institute of Advanced Industrial Science and Technology	Japan
8	Rie Yasuno	National Institute of Advanced Industrial Science and Technology	Japan
9	Kohji Yamakage	Food and Drug Safety Center, Hatano Research Institute	Japan
10	Takashi Omori	Kobe University	Japan
11	Hajime Kojima	JaCVAM, National Institute of Health Sciences	Japan
12	Takao Ashikaga	JaCVAM, National Institute of Health Sciences	Japan
13	Steven Venti	Translator	Japan

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Yamakage K, Saito K, Nakajima Y, Fujimura C, Ohmiya Y, Omori T, Kojima H, Aiba S: The performance of an in vitro skin sensitisation test, IL-8 Luc assay (OECD442E), and the integrated approach with direct peptide reactive assay (DPRA), J Toxicol Sci. 2018; 43(12):741-749. doi: 10.2131/jts.43.741.

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4. Recent Activities for safety assessment, Kojima H, International Symposium on Cosmetic Regulation, 2018/7/13, **国内**.
5. **教育講演：ガイドライン化を目指した**in vitro**試験系導入の具体的な留意点**, **小島肇**, **第**45**回日本毒性学会学術年会**, 2018/7/20, **国内**.
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17. ヒト健康影響を予測するための非動物実験の開発動向, 小島 肇, Translational and Regulatory Science Symposium, 2019/2/7, 国内.
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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

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

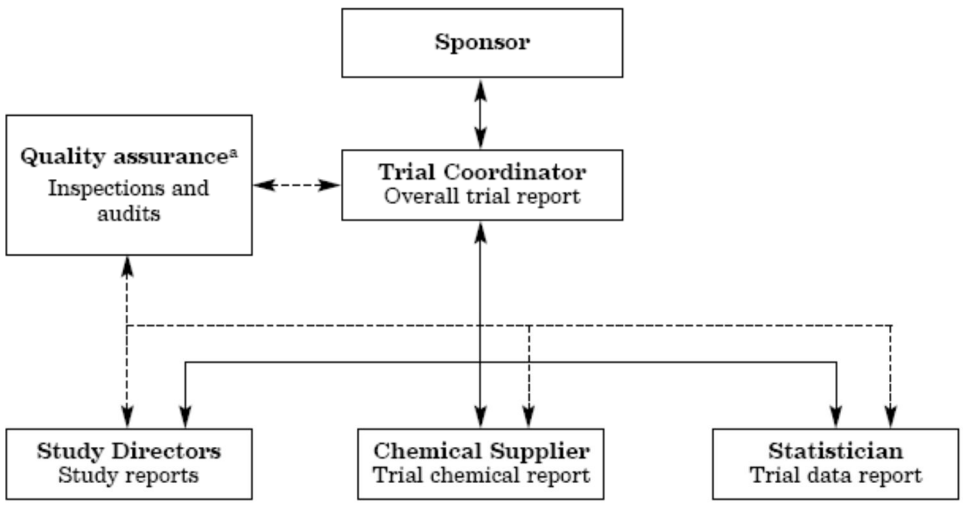
Test Facility 2: AIST, Tsukuba SD : Rie Yasuno

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

4th Meeting for the MITA Validation Study

October 4–5th, 2018
Kobe University, Kobe, Japan

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

	October 4
	Welcome address and housekeeping
Ashikaga:	We have postponed the discussion about reproducibility until tomorrow.
Aiba:	Thank you for being here today. This is an important meeting for our validation study. We have found that the problems we face are really very difficult yet also interesting, and I hope will be able to reach agreement at this meeting.
Kojima:	Today's discussion will focus on predictive capacity. And Dr. Aiba has a new proposal. Tomorrow we will discuss reproducibility, the study plan, and other issues.
	Report of draft validation report and discussion of predictive capacity
Aiba:	(PowerPoint presentation)
Corsini:	We need to look at all data that shows T-cell response to chemicals for which we have mechanistic information and be aware that some of those mechanisms might be affecting other things. And compare this with any other data for which we know the response to these chemicals.
Germolec:	Data from the IL2 assay seems to suggest that there are chemicals that are immunosuppressive but probably are not targeting T-cells. This also shows the power of the combined assays to enable predictions where individual assays give only equivocal results. Perhaps we can rethink the definition of immunotoxic and specify that this assay is predictive of chemicals that target T-cells, and it becomes a tool in the toolbox.
Inoue:	Cytokine release and cell proliferation are important for judging T-cell dependent mechanisms.
Aiba:	That will be added.
Corsini:	What are the criteria for the tentative designation of immunotoxic chemicals?
Aiba:	We look at different effects such as changes in the thymus weight, proliferation, serum immunoglobin.
Germolec:	I think that the observed endpoints are more important than the number of assays that give positive or negative results. So, for example, if we have relevant human epidemiology data, that is all would need to classify based on human health effects. It is good to have mechanistic and other data, but if you are doing risk assessment, all you need is evidence of an effect on a human population.
Inoue:	Very few chemicals show immuno-augmentation in in vivo studies. So even if IL2 shows augmentation, that might not be corroborated by in vivo studies.
Aiba:	We are going to classify 25 chemicals for potency and target?
Germolec:	For this assay, the first thing we should look at is whether it targets T-cells or not. That is its strength and where the focus should be. Then we can discuss other immune effects or potency and other things.
Aiba:	Chemical Cytokine production (in vivo, ex vivo) Mitogen response DTH TDAR T-cell immunotoxic Non T-cell immunotoxic None
Germolec:	If we have trouble finding chemicals with in vivo T-cell data, we might consider running a small set of chemicals on animals.
Kojima:	How many chemicals have you tested in-house?

Aiba:	60. Here is a list of chemicals we have published, and many of them are drugs, so the effects are well known. The criteria used here is slightly different, however.
Corsini:	So, you could apply the new criteria to this data.
Germolec:	Instead of saying "T-cell immunotoxic or non-immunotoxic," perhaps we should say "Targets T-cells: Yes or No."
Corsini:	But it is difficult to determine a direction, because there will be in vivo data that sometimes show changes in both directions.
Germolec:	So, we should focus just on whether it affects T-cells or not.
	October 5
	Discussion of Reproducibility
Omori:	We used criteria 5 shown on page 30 of section 7-2. Recalculation was done for Phase I and Phase II. For Phase I, WLR was 80%, 100%, and 80% for the three laboratories, and BLR was 80% for five chemicals. For Phase II, BLR was 80% for twenty chemicals.
Aiba:	We found a problem in the calculation sheet but applied a correction to get these figures.
Corsini:	A concordance of 80% is a reasonable level. Even if there was a mistake in the calculation sheet, once it was corrected, the figures are what we expected, so I think we can all agree that the method achieves reproducibility targets.
Kojima:	
	Conclusions
Corsini:	Yesterday we discussed how we cannot expect accurate predictions for chemicals that do not target T-cells, so we have 25 chemicals that we reviewed to determine whether or not they target T-cells.
Aiba:	We established criteria and reviewed the 25 chemicals yesterday, but there are some points we need to discuss.
Corsini:	If we have a chemical that we consider undetermined but there are in vivo or other test results that suggest it does target T-cells, should we include it?
Germolec:	There are some chemicals for which other tests find both suppression and augmentation, so I think that when we see the same thing in IL2 results, it is an indication of how sensitive this test is.
Roggen:	I wonder if we should go back to look at the ones that are undetermined because there was no relevant data.
Germolec:	I think we need to distinguish between cases where there is not enough data to determine whether or not it targets T-cells and cases where there is evidence that other types of cells are also targeted.
Corsini:	So, results for chemicals that are undetermined should not be included in the discussion of predictive capacity.
Aiba:	So, at the moment, our predictive capacity is 16/24 or 67%.
Corsini:	How does that compare with your earlier in-house data? Although you probably should apply the current criteria to make a relevant comparison.
Aiba:	I think that drugs that we know target T-cells can be predicted accurately, but I am not so sure that other chemicals with effects that are not so well known can be predicted.
Corsini:	We won't be able to make any conclusions using the in-house data until we do a further review of the literature and then apply criteria 5. And it would be helpful in determining an applicability domain to be able to provide a mechanistic explanation for chemicals that give IL2 results that aren't concordant with results from other test methods.
Corsini:	It is important that accuracy be greater than 50%, but an accuracy of 65 or 70% is fine for one tool in the toolbox
Roggen:	I wonder if it might not make the test more useful just to forget about suppression and augmentation and focus on saying yes or no for targeting the biological function of T-cells. Aspects that are concentration-dependent are not so useful, because concentrations used in vitro are not necessarily relevant in vivo.
Germolec:	Many of the in vitro tests currently being used are only looking for biological effects. They aren't concerned with whether the effect is up or down, but rather is there an effect at all.
	Plan for a peer review
Kojima:	Here is my plan for the peer review. (PowerPoint presentation)
Aiba:	I hope to finish the first draft validation report by the end of November.
Kojima:	We would like to fix the PRP members by the end of November, and early discussions will be by email and a teleconference if necessary. And a F2F meeting at the end of February 2019. And the have the report by the end of July.
Corsini:	I suggest Henk Van Loveren or Marc Pallardy for the EU expert.

Germolec:	Mike Luster, Madeline Fort, or Haley LaNef Ford for the US expert. And for the Korean expert, Sanghyun Kim from Korea.
Kojima:	I would like to propose that Japan submit a SPSF, and if it is approved, then I will coordinate a DRP with the VMT and the PRP and would like to discuss the TOC next July. The target is the WNT meeting in April 2020.
	Validation plan for the IL-1β assay
	Introduction and Protocol
Aiba:	Please look at page 5 of the file Multi-Immuno Tox Assay protocol for-TGCHAC-A4 ver. 007E 20180712. This shows the protocol for the assay. And on page 36, the criteria are described. Acceptance criteria will be determined based on Phase 0 results.
Inoue:	What about the passages of the cells before the assay?
Aiba:	Cell cultures requirements are described on page 10.
Corsini:	Do the cell lines and reagents need to be purchased from a specific supplier? Do you provide a catalog number? In the future, will these materials be available from several sources?
Aiba:	In the future, yes, although we tried several different LPS and settled on this one.
Roggen:	It would be good to describe the different LPSs in terms of stimulation potency and show what kind of differences can be expected.
Corsini:	We should keep in mind the potential to replace bovine calf serum with human serum.
Aiba:	The prediction criteria are 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.
Corsini:	So, this is the same as Criteria 5.
Roggen:	Again, it should be focused on yes or no.
Germolec:	The criteria need to define suppression and augmentation, but then the data analysis can be based on yes or no.
Aiba:	We used different plates which show different backgrounds of DMSO. Also, we thought about what kind of counts should be expected. Three of four laboratories achieved similar stimulation with LPS. So, we think that a 5-fold or greater induction of FinSLG-LA should be included in the acceptance criteria.
Omori:	Do you have any ideas how to increase the fold induction at the lab that was low?
Aiba:	The lab that was low needs to increase its fold induction the same level as the other labs before starting Phase I.
Corsini:	Did all the participating laboratories have materials from the same manufacturing lots?
Aiba:	Yes.
Corsini:	Were the readers all calibrated to the same standards, as well?
Aiba:	Yes.
Germolec:	Are there any other markers of proliferation that could be used to understand whether or not they are all getting the same levels of activation? What will you do in the next phase so to improve this situation for that lab?
Aiba:	We will investigate the situation to find out why they had lower fold induction.
Roggen:	When you saw there was a problem, did you send them new cells to use?
Aiba:	No, we just did the one test. But we do understand that this is an issue that we have to correct before we proceed.
	Results of the Pre-Validation Study
Omori:	Criteria 5 is from IL2 and these graphs have reference lines at ±35%. There are a number of tests, however, in which changing the cutoff value from 35% to 20% would change the results to suppression. The last sheet summarizes the results using 35%, 20%, and 25% cutoff values.
Inoue:	Please explain the why the judgement is suppression rather than augmentation on page 9.
Omori:	If the confidence interval had extended below the zero line then it could be considered a trend, but it did not.
Germolec:	Even though the response was not as high as some of the data from the other labs, the lab that did not perform as well showed a similar trend as the results from the other labs.
Roggen:	There are two things here. The ratio is important to help people using a different machine to define a window, but then an absolute value can be used to make the judgment.
Germolec:	In terms of stimulation, you need a cutoff for DMSO. And it looks like 5 is too low but 10 is two high. So, we

	need to determine what fold induction is sufficient to ensure optimal LPS response.
Corsini:	The readers are the same and the materials are the same, so there needs to be an explanation of why one lab had significantly lower figures.
Aiba:	Unfortunately, we changed the plates before the Phase 0 study. So, it is difficult to determine what was different based on in-house data.
Corsini:	Perhaps viability is one explanation for why there was variation in the response.
Inoue:	Perhaps the time since the passage affected the response.
Aiba:	This is stipulated in the protocol.
Roggen:	One shows high response with large variation, but another shows low response but small variation. It's possible that different techniques cause some of these variations. There are many variables, even the age of the equipment could be a factor.
Aiba:	We agree that we have to devise criteria to ensure a sufficient fold induction and find a reason and a countermeasure for this problem. Maybe we need to redo some tests.
Yamakage:	Well, it will take some time to do more tests, but first we need to resolve our problem.
Germolec:	Stimulation by LPS should be independent of the plate being used.
Aiba:	Yes, we will examine our data.
Roggen:	If there is no historical data, then you will need to do some additional testing to acquire data you can use.
Germolec:	Looking at the dates, it appears that the response was dropping in the later experiments, and since FDSC performed their tests last, perhaps their low counts were related to that.
	Study Plan for the Validation Study
Kojima:	This is the same study plan as for IL2 and calls for 25 test chemicals: 5 in Phase I and 20 in Phase II. Is this workable for the participating laboratories?
Yamakage:	It might be difficult.
Kojima:	We need to start the Phase I testing by January. The timeline calls for starting in November, but we have to find a solution to the Phase 0 problem, so I think the start date will be January. Do we need a teleconference in December before starting Phase I?
Roggen:	We need to see a list of what has been done to solve the problem.
Germolec:	And I think email might be better, because December is a busy time of year to try to arrange a teleconference.
Aiba:	I think the problem is clear and we should be able to find a solution.
	Comments from the Participating Laboratories
Yamakage:	We don't have any clear ideas for solving the problem, so I will need some time to check.
Roggen:	It takes time to identify problems in reproducibility. Sometimes it is something as simple as differences in pipette technique, so there are many things to consider and it is difficult to plan beforehand. You have to stay openminded and compare one thing after the other.
Germolec:	But it also needs to be noted that if the protocol is that sensitive to the techniques used, then its transferability to other laboratories will be in question.
Corsini:	In a situation where only one out of four labs is getting different results, then you have to ask why.

（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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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 I 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、本年度はバリデーション試験に先立ち、Phase 0 として既知の 3 物質について試験を実施し、技術移転性を確認した。続いて 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 0では3物質(Dapsone，Diethanolamine，p-Nitroaniline)を用いた。

Phase Iでは1セット5種類のコード化した被験物質3セットを用いた。

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、被験物質調製及び添加方法、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.008.1E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 0 では 3 物質 (Dapsone，Diethanolamine，p-Nitroaniline)を 1 セットとし、それぞれ 3 回ずつ 2 セットの試験を行った。続いて行った Phase I バリデーション試験では、1 セット 5 種類のコード化した試験化学物質 3 セットを用いて 1 セットにつき 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3つの基準を満たす場合をSuppressionまたはAugmentation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20%以上(Suppression)か-20%以下（Augmentation）でかつ、同時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か、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)。

（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

Phase 0ではコード化を行わない3物質に対して各3回ずつの実験を2セット行った。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）の結果を比較検討した。その結果、良好な施設間再現性が得られ、技術移転性を確認できたことから、Phase I試験を実施することとした。

Phase Iでは施設内再現性の確認を行う目的で、コード化された5物質を1セットとする群が3セット配布された。1物質につき2回もしくは3回の実験を実施した。1セットの実験がすべて終了した後に次のセットの実験を行うことで、セット毎の実験の独立性を担保した。図1にSet 1、図2にSet 2、図3にSet 3の結果を示す。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1に示した。

D．考察

Phase I studyでは施設内再現性および施設間再現性を確認するために、コード化した5物質を1セットとし、3セットの合計15物質について実験を行った。

当施設では、5物質のうち2物質については3セットとも一致した判定が得られた。一方、3物質は3セット中2セットについては判定が一致したが、1セットのみ異なる判定となった。

概ね再現性は良好といってよいが、プロトコールを改善すればガイドライン化可能なレベルにまで正確性や施設間および施設内再現性を向上できるかもしれない。

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。まずPhase 0として3物質の試験を行い、技術移転性について確認した。次に5種類のコード化した被験物質を用いた1セット2回以上の試験を繰り返すPhase Iバリデーション試験を実施した。また、再現性や精度を高めるための実験操作等の改善点を抽出し、Phase Iバリデーション試験における諸条件の改善のための情報およびサポートデータを提供した。

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

該当なし

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

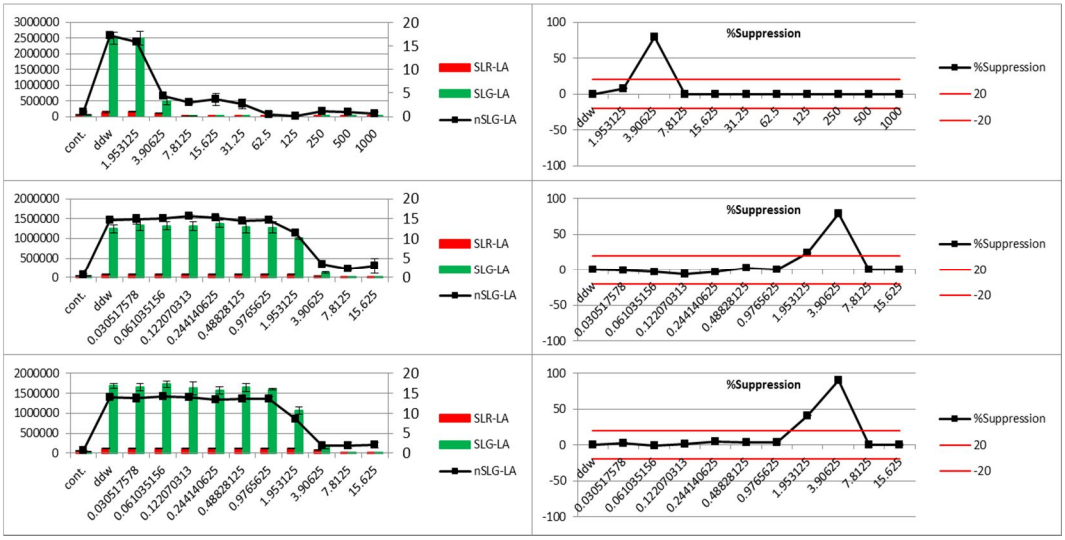
該当なし

3.その他

該当なし

図１　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(Set 1).

<MITC701>



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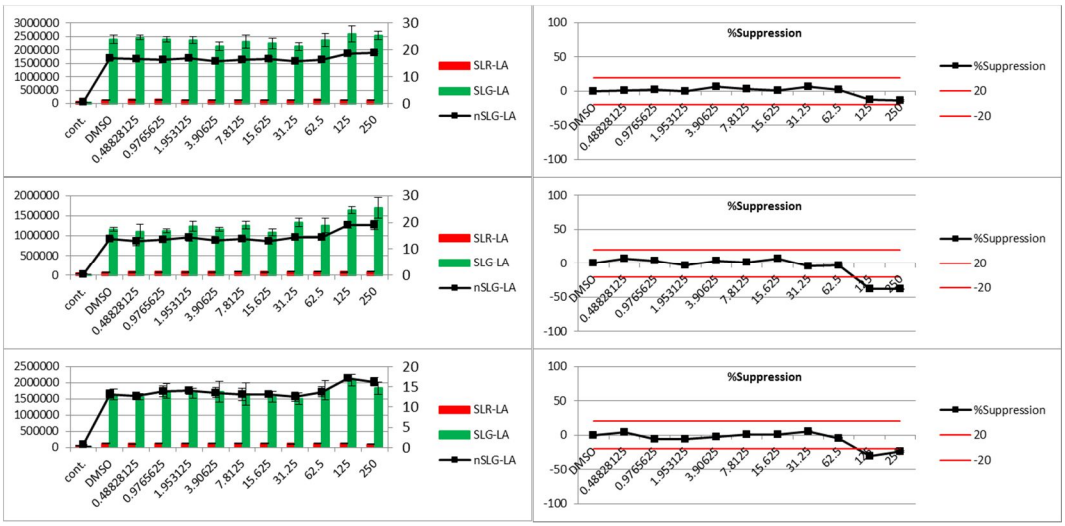

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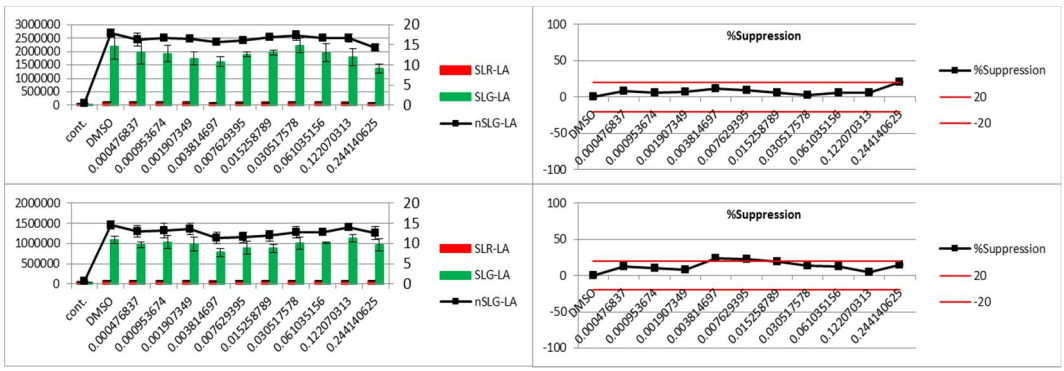

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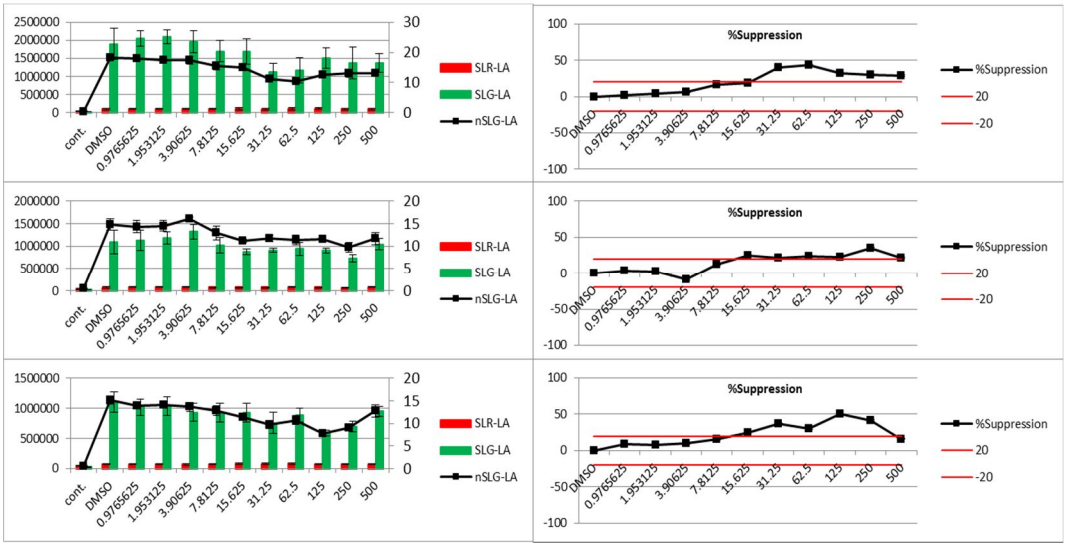

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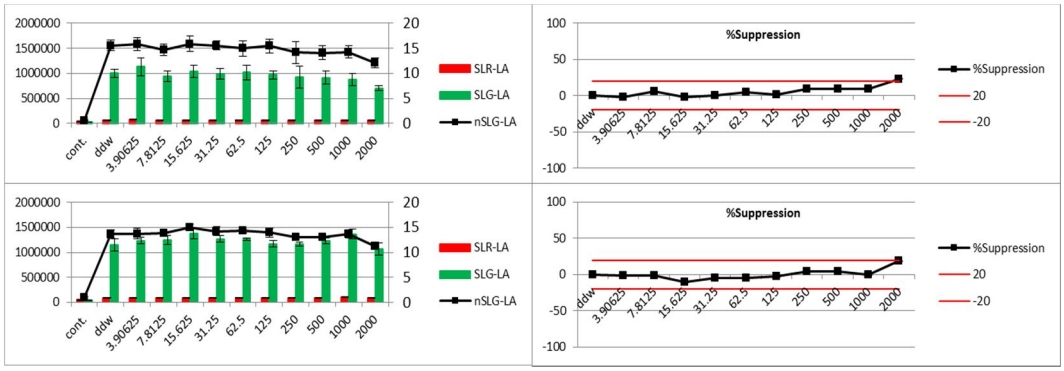
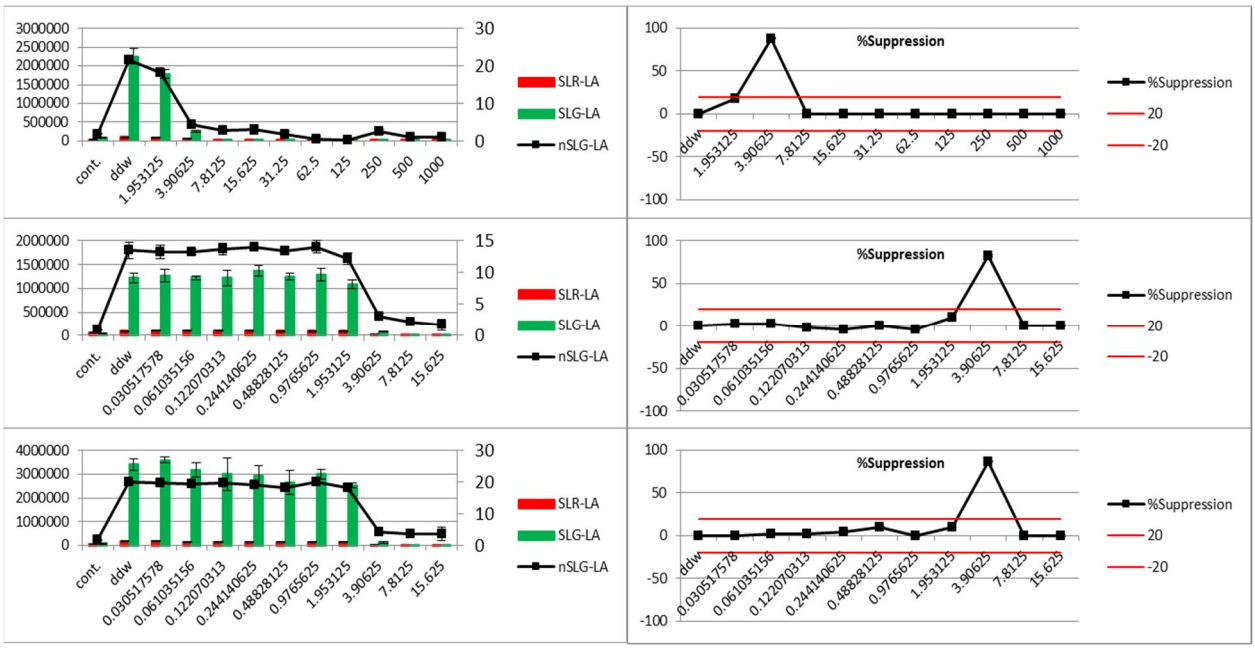


図 2　THP-G1b(TGCHAC-A4)細胞株における各試験化学物質に対する細胞応答性(Set 2).

<MITC801>



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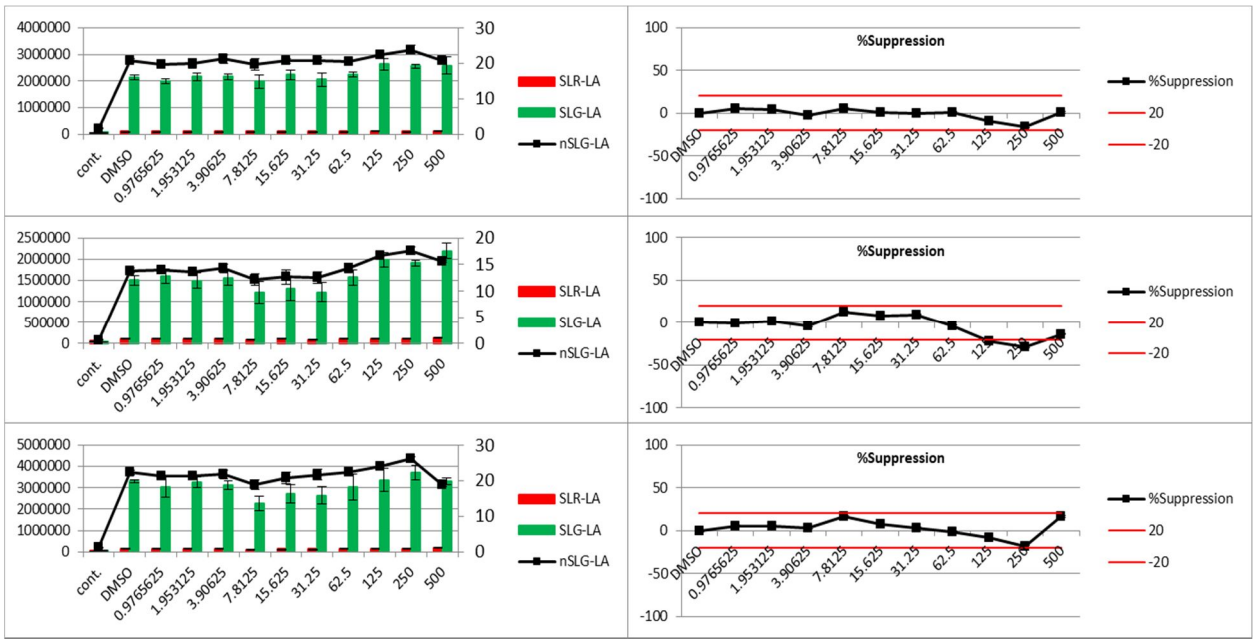

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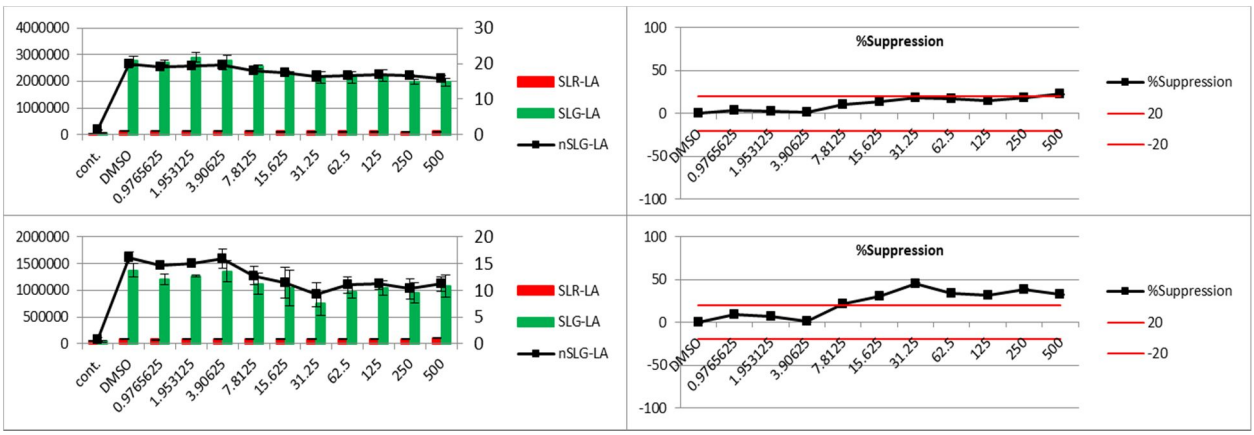

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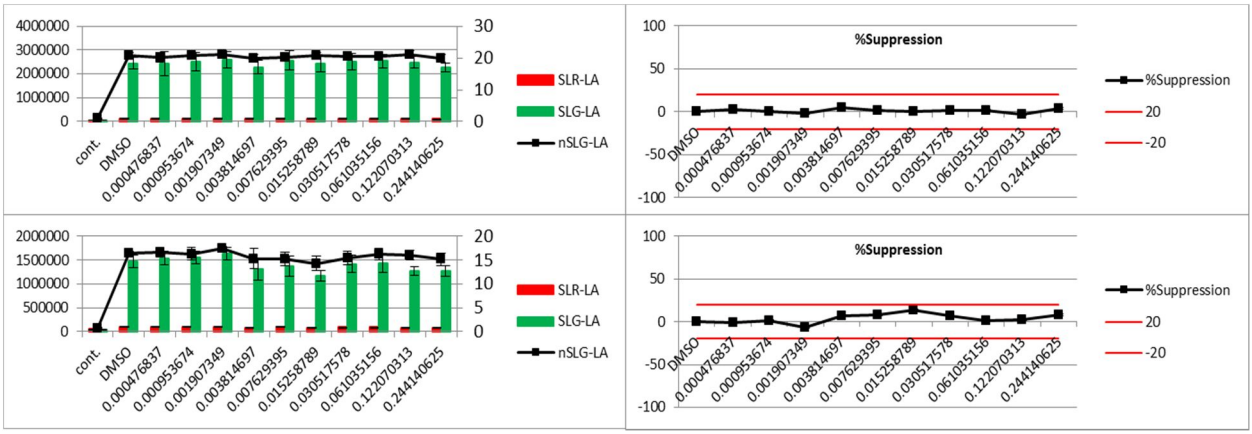

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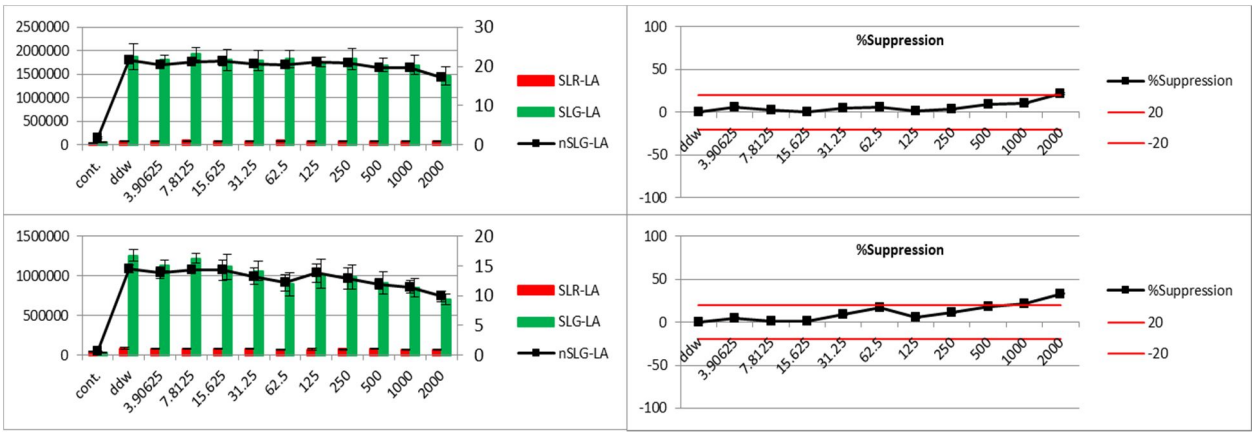
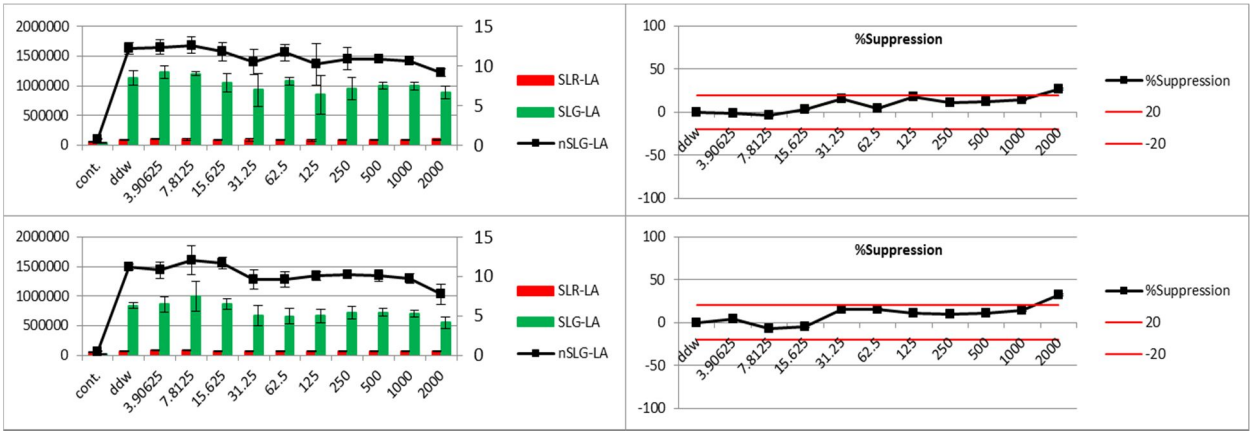


図3　THP-G1b(TGCHAC-A4)細胞株における各試験化学物質に対する細胞応答性(Set 3)．

<MITC901>



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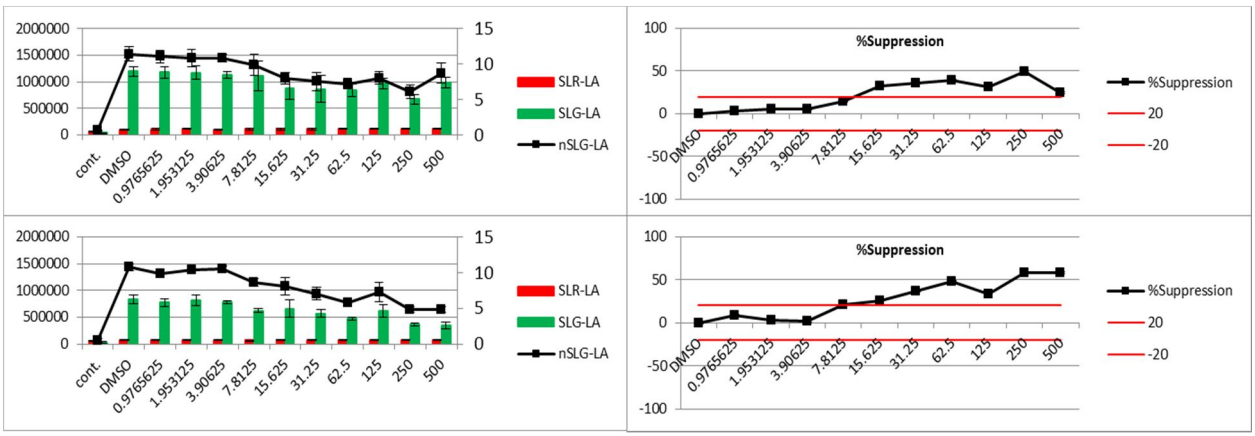

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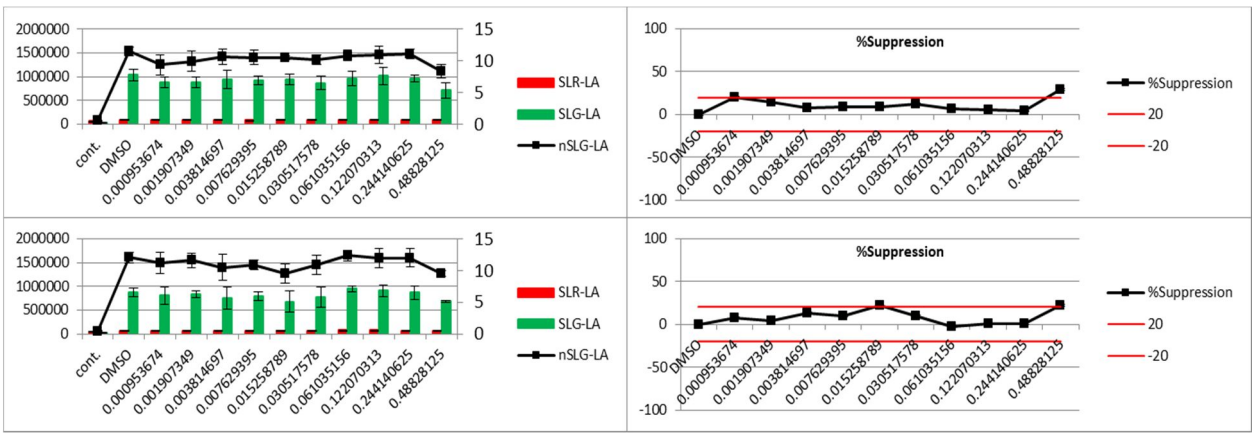

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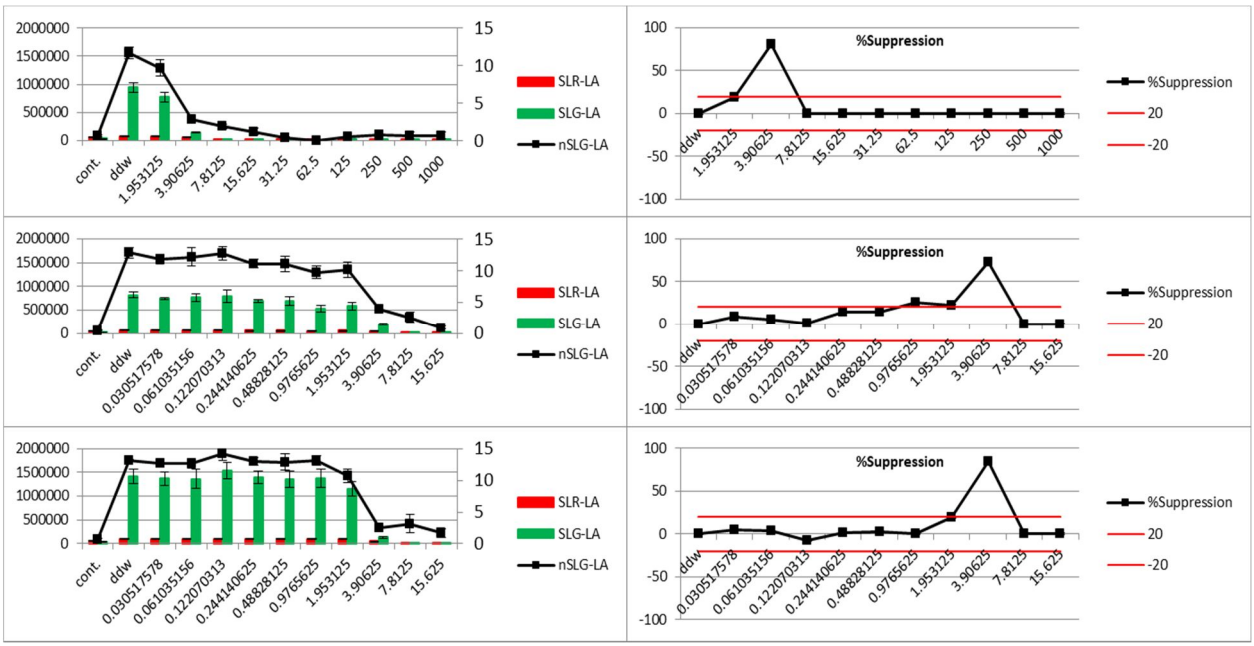

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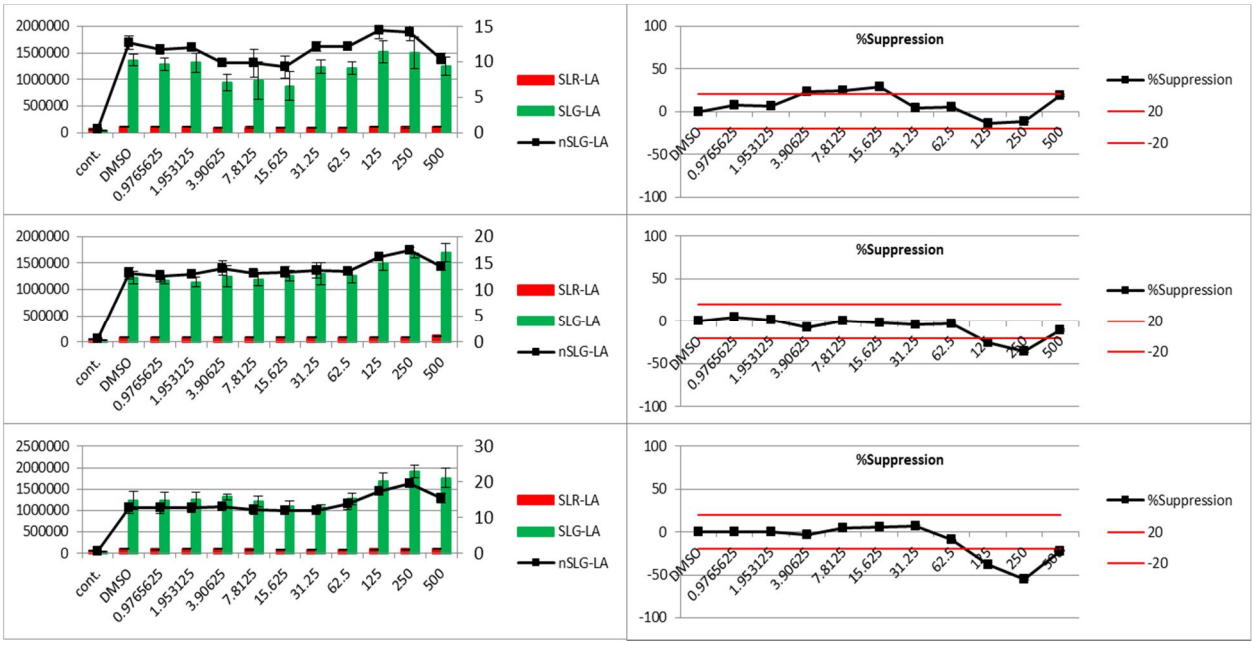

表１　各被験物質に対する評価.

Chemicals	Code No.	Test			Judge
		1st	2nd	3rd	
1	MITC701	N	A	A	A
	MITC802	N	A	N	N
	MITC905	S	A	A	A
2	MITC702	R	S	S	S
	MITC801	R	S	S	S
	MITC904	R	S	S	S
3	MITC703	S	S		S
	MITC804	N	N		N
	MITC903	N	N		N
4	MITC704	S	S	S	S
	MITC803	S	S		S
	MITC902	S	S		S
5	MITC705	N	N		N
	MITC805	S	S		S
	MITC901	S	S		S

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

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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

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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、まず 3 化学物質を用いて Phase0 試験を実施し、技術移転性を確認した。つづいて、コード化された 5 物質において Phase1 試験（施設内再現性確認試験）を実施した。

A．研究目的

東北大学らによって開発された 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、バリデーションの技術移転性の確認（Phase0）および Phase1 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.007E(Phase0)および Ver.008E(Phase1)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。

Phase0 試験;試験には 3 化学物質(Dapsone, Diethanolamine, p-Nitroaniline)を供試し、各物質 3 回×2 セットの試験を行った。

Phase1 試験；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 5 種類のコード化した被験物質を供試した。各物質 1 セットにつき 2 回の同一結果を得られるまで試験を繰り返し被験物質を判定、これを 3 セット実施した。

（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

Phase0 試験；被験 3 物質の試験結果を図 1 に示す。これらの結果を、リードラボの東北大学及びバリデーション実施施設である食薬センター、産総研高松と比較検証したところ、良好な施設内、施設間再現性が確認されたことから、つづいて Phase1 試験を実施した。

Phase1 試験；

コード化された 5 種類の化学物質（5 物質×3 セット分の 15 被験試薬）の試験結果および判定結果を図 2 に示す。Multi-Immuno Tox Assay protocol 案 Ver.008 E の判定基準に準じ、%suppression の±20%を基準として被験試薬の効果を判定した。各試薬の判定を得るまで、それぞれ 2～4 回の実験を繰り返した。3 セット目（MIB601~605）の 2nd experience では、LPS による誘導の基準となる FInSLG-LA=>5%を満たさなかったので、判定対象外とした。

D．考察

Phase1 試験では、コード化された 15 被験試薬（5 物質×3）の試験を実施した。5 物質全てにおいて、3 セットで同一の判定結果となり、良好な施設内再現性を確認した。一方、各セットにおける 1st~4th experience を確認すると、3 つの被験試薬（MIB503、MIB601、MIB602）において、N:No effect と S；suppression の両判定があった。MIB601 に関しては、「N、S、N」で最終的に No effect 判定であったが、2nd exp.の suppression 判定も+20%ラインを僅かに上回る微妙なものであった。MIB503、602 においては、被験試薬の毒性が強く、細胞の I.I.-SLR-LA が 0.5 を下回る直前の非常に狭い濃度範囲において明らかな IL1-β の転写活性抑制反応が出ている。しかし、反応が計測できる濃度が 1 点のみであったため判断基準となる「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か、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」に合致せず、No effect の判定となっている。これらの結果から、プロトコルの改善や判定基準の再検証を進めることにより、より正確性、再現性を向上した試験法の確立が見込まれる

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（Phase0 および Phase1）を実施した。Phase0 試験において技術移転性を確認し、Phase1 試験において 5 物質×3 セットからなる試験を実施した。

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、小林 眞弓、大森 崇、足利 太可雄、小島 肇、相場 節也：Multi-ImmunoTox Assay (MITA) バリデーション研究の結果 日本動物実験代替法学会 第 31 回大会（熊本）2018 年 11 月

該当なし

H．知的財産権の出願・登録状況
（予定を含む。）

1. **特許取得**

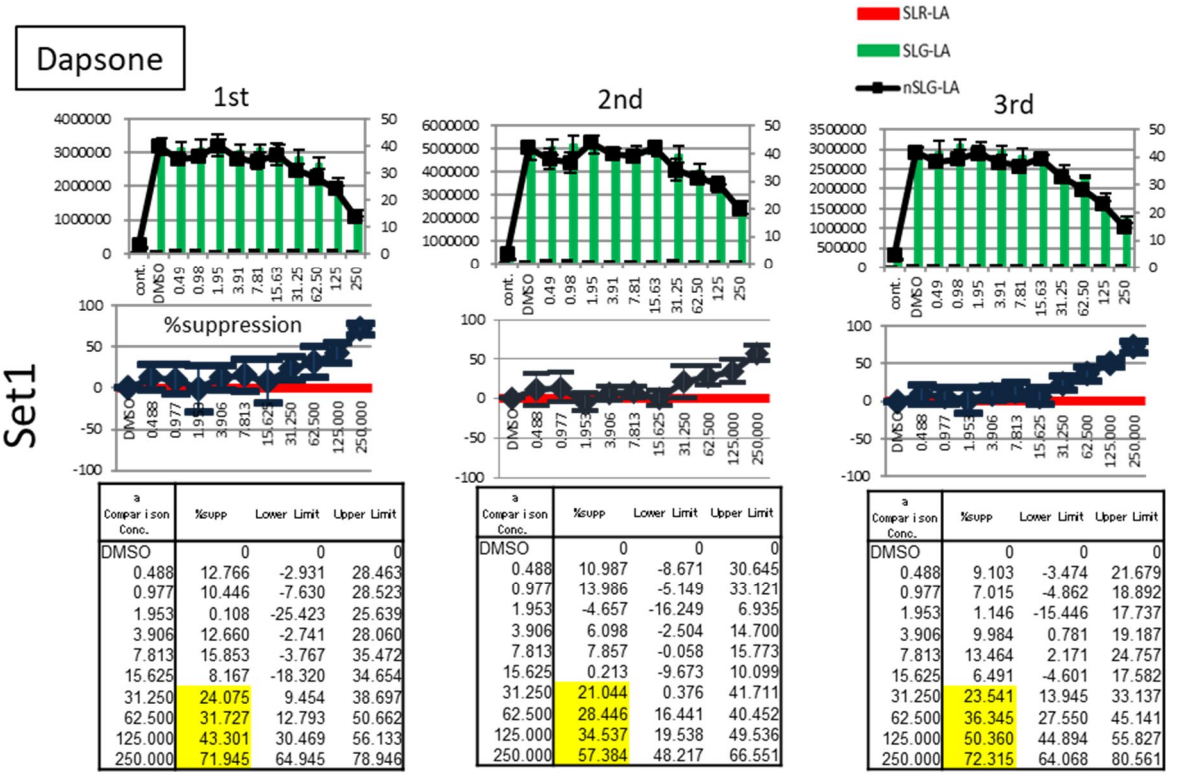
該当なし

2. **実用新案登録**

3.**その他**

該当なし

図1　Phase0試験；被験物質に対する細胞応答性

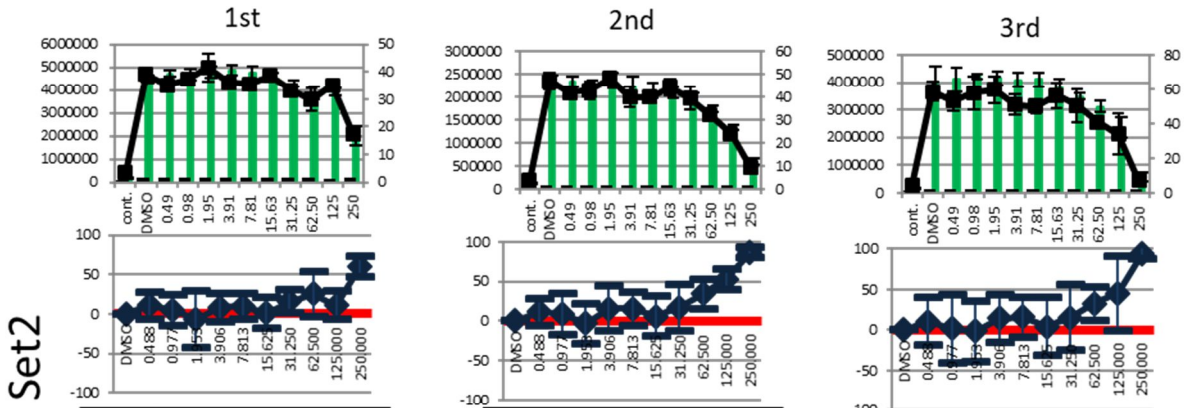


Set1

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.488	12.766	-2.931	28.463
0.977	10.446	-7.630	28.523
1.953	0.108	-25.423	25.639
3.906	12.660	-2.741	28.060
7.813	15.853	-3.767	35.472
15.625	8.167	-18.320	34.654
31.250	24.075	9.454	38.697
62.500	31.727	12.793	50.662
125.000	43.301	30.469	56.133
250.000	71.945	64.945	78.946

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.488	10.987	-8.671	30.645
0.977	13.986	-5.149	33.121
1.953	-4.657	-16.249	6.935
3.906	6.098	-2.504	14.700
7.813	7.857	-0.058	15.773
15.625	0.213	-9.673	10.099
31.250	21.044	0.376	41.711
62.500	28.446	16.441	40.452
125.000	34.537	19.538	49.536
250.000	57.384	48.217	66.551

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.488	9.103	-3.474	21.679
0.977	7.015	-4.862	18.892
1.953	1.146	-15.446	17.737
3.906	9.984	0.781	19.187
7.813	13.464	2.171	24.757
15.625	6.491	-4.601	17.582
31.250	23.541	13.945	33.137
62.500	36.345	27.550	45.141
125.000	50.360	44.894	55.827
250.000	72.315	64.068	80.561



Set2

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.488	10.720	-5.974	27.414
0.977	4.985	-14.657	24.626
1.953	-7.325	-43.204	28.553
3.906	8.350	-9.084	25.783
7.813	9.725	-7.260	26.711
15.625	1.339	-18.649	21.328
31.250	16.142	1.409	30.875
62.500	25.733	-2.795	54.262
125.000	11.055	-6.486	28.596
250.000	60.359	47.619	73.099

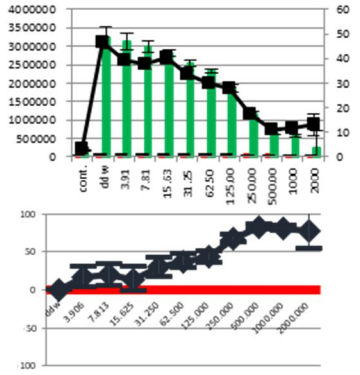
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.488	11.656	-5.241	28.552
0.977	9.118	-16.735	34.971
1.953	-2.957	-28.047	22.133
3.906	16.002	-12.839	44.843
7.813	15.950	-5.167	37.068
15.625	5.952	-19.075	30.978
31.250	17.104	-12.436	46.644
62.500	34.380	16.127	52.633
125.000	53.053	40.181	65.926
250.000	86.386	80.023	92.749

a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.488	10.456	-18.804	39.716
0.977	1.885	-39.988	43.757
1.953	-1.684	-38.380	35.011
3.906	14.372	-14.964	43.709
7.813	16.110	-8.525	40.745
15.625	4.478	-30.939	39.895
31.250	15.493	-25.389	56.375
62.500	32.734	12.273	53.194
125.000	45.404	-0.573	91.381
250.000	94.412	87.654	101.170

Diethanolamine

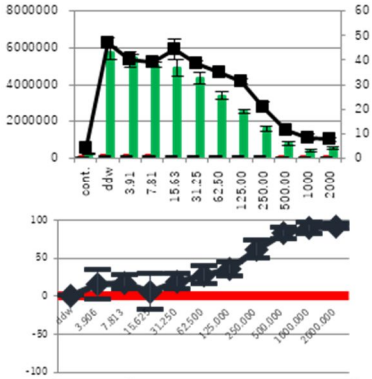
Set1

1st



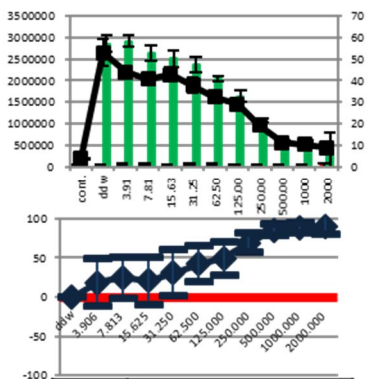
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	16.742	2.625	30.859
7.813	19.913	5.486	34.341
15.625	13.951	-1.942	29.844
31.250	30.121	17.365	42.878
62.500	37.856	28.414	47.298
125.000	43.514	35.839	51.189
250.000	67.038	61.992	72.085
500.000	82.566	79.532	85.600
1000.000	80.310	76.854	83.766
2000.000	77.715	53.745	101.685

2nd



a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	15.172	-4.209	34.552
7.813	18.301	8.661	27.941
15.625	5.784	-17.706	29.273
31.250	19.166	9.130	29.203
62.500	27.619	15.752	39.487
125.000	35.686	26.430	44.942
250.000	60.780	48.771	72.789
500.000	82.616	75.430	89.802
1000.000	89.817	82.264	97.370
2000.000	91.768	88.124	95.412

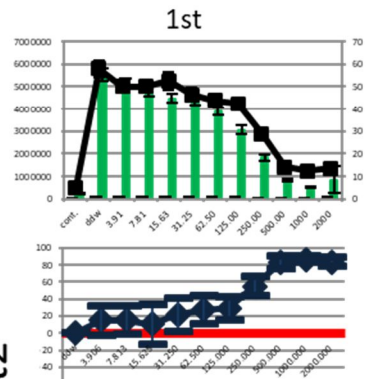
3rd



a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	18.503	-11.460	48.466
7.813	25.188	-0.750	51.125
15.625	20.828	-9.481	51.138
31.250	31.076	1.579	60.572
62.500	42.312	19.905	64.718
125.000	49.220	27.869	70.571
250.000	68.960	56.470	81.450
500.000	85.493	78.273	92.713
1000.000	87.601	81.134	94.068
2000.000	90.509	79.604	101.414

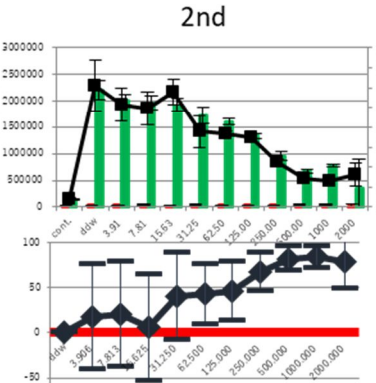
Set2

1st



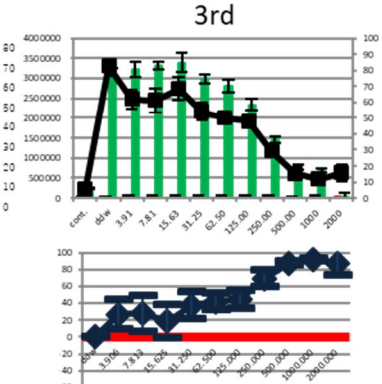
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	14.738	-2.275	31.751
7.813	14.942	-1.529	31.412
15.625	10.340	-13.239	33.919
31.250	21.864	2.572	41.157
62.500	26.921	10.414	43.429
125.000	29.143	15.428	42.858
250.000	54.824	43.826	65.822
500.000	82.590	74.934	90.247
1000.000	86.157	82.457	89.856
2000.000	83.646	78.581	88.710

2nd



a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	17.399	-40.836	75.633
7.813	20.550	-37.480	78.580
15.625	5.631	-53.501	64.763
31.250	40.875	-7.613	89.364
62.500	42.873	9.079	76.668
125.000	46.592	13.904	79.280
250.000	67.695	46.137	89.253
500.000	82.055	68.539	95.571
1000.000	84.096	71.922	96.271
2000.000	78.745	49.404	108.086

3rd

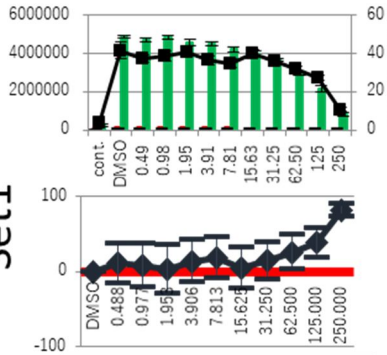


a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	26.922	9.815	44.030
7.813	27.890	7.342	48.438
15.625	18.377	-1.737	38.491
31.250	37.156	21.379	52.933
62.500	42.265	32.923	51.607
125.000	44.883	34.551	55.215
250.000	69.451	59.762	79.139
500.000	87.404	84.899	89.910
1000.000	92.219	90.730	93.708
2000.000	87.267	73.464	101.069

p-Nitroanilline

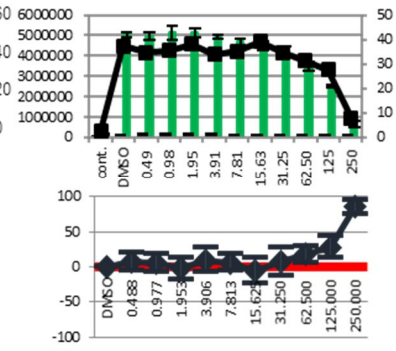
Set1

1st



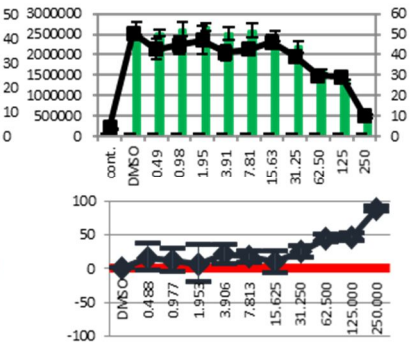
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.488	10.798	-15.855	37.451
0.977	8.031	-21.359	37.421
1.953	2.083	-30.450	34.615
3.906	13.350	-14.554	41.253
7.813	17.742	-9.448	44.932
15.625	4.067	-23.438	31.572
31.250	14.195	-10.364	38.755
62.500	25.576	2.851	48.301
125.000	38.277	18.807	57.747
250.000	81.564	72.706	90.422

2nd



a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.488	7.340	-5.954	20.634
0.977	4.849	-8.144	17.843
1.953	-2.737	-18.049	12.575
3.906	9.624	-7.854	27.102
7.813	6.202	-6.336	18.739
15.625	-5.289	-23.473	12.896
31.250	7.560	-12.343	27.463
62.500	17.469	5.141	29.797
125.000	27.721	12.654	42.789
250.000	84.881	74.302	95.460

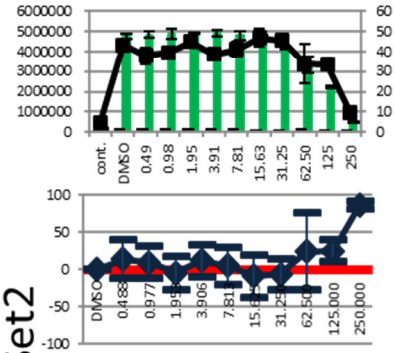
3rd



a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.488	16.876	-2.486	36.237
0.977	12.247	-5.318	29.812
1.953	7.277	-19.981	34.535
3.906	20.804	6.125	35.482
7.813	17.109	8.538	25.680
15.625	9.398	-6.038	24.833
31.250	25.026	16.157	33.895
62.500	45.040	38.984	51.097
125.000	46.390	41.348	51.432
250.000	88.165	84.598	91.733

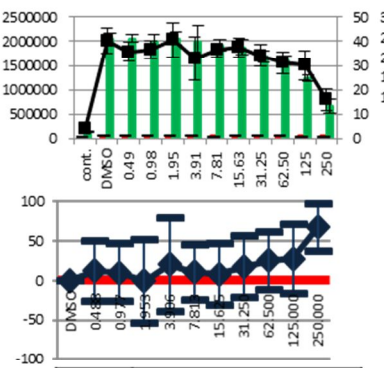
Set2

1st



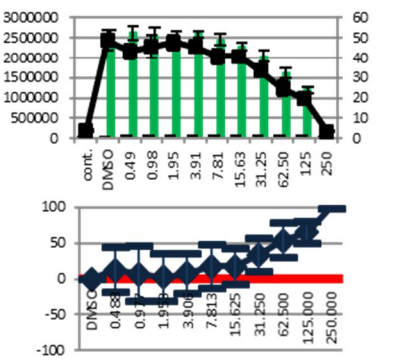
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.488	13.767	-12.206	39.739
0.977	9.723	-11.417	30.862
1.953	-4.432	-26.698	17.834
3.906	11.853	-9.525	33.232
7.813	5.409	-19.753	30.571
15.625	-9.124	-38.533	20.285
31.250	-6.026	-26.872	14.819
62.500	24.313	-27.870	76.497
125.000	24.920	10.461	39.380
250.000	86.931	82.447	91.415

2nd



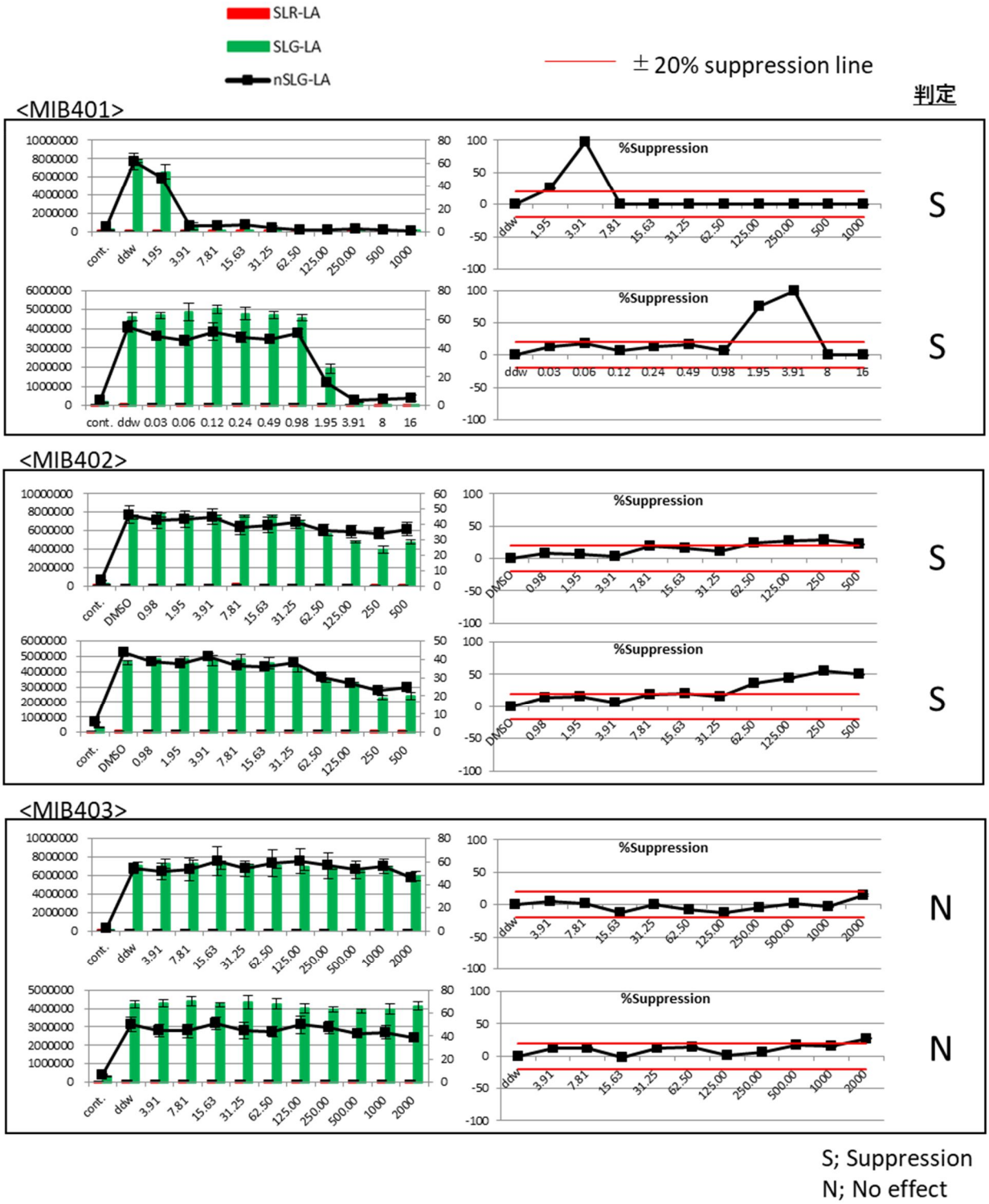
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.488	12.528	-25.834	50.890
0.977	10.230	-26.989	47.448
1.953	-0.662	-53.725	52.401
3.906	20.183	-39.331	79.698
7.813	10.514	-23.950	44.978
15.625	7.859	-30.587	46.305
31.250	17.150	-21.603	55.903
62.500	25.085	-11.367	61.538
125.000	28.034	-15.855	71.923
250.000	67.192	36.436	97.947

3rd



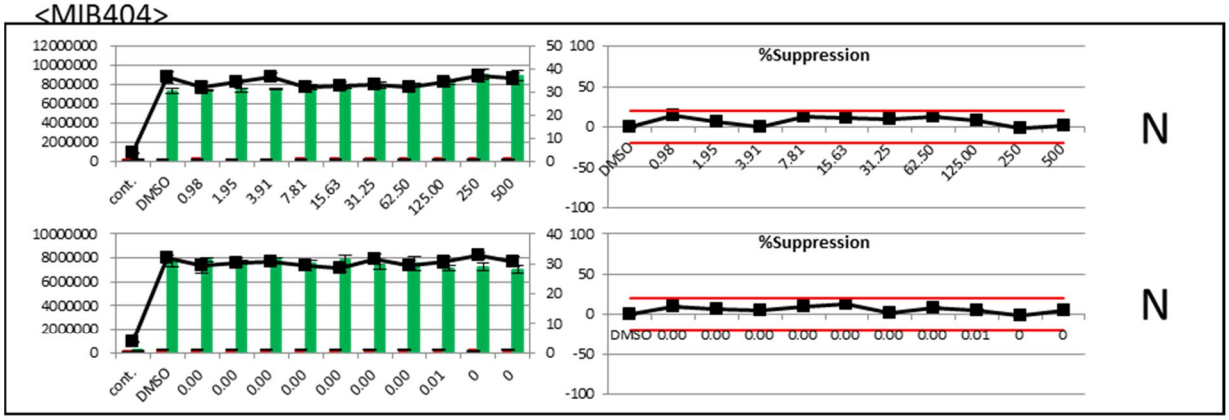
a Comparison Conc.	%supp	Lower Limit	Upper Limit
DMSO	0	0	0
0.488	12.161	-18.753	43.074
0.977	6.737	-31.988	45.461
1.953	1.867	-31.206	34.940
3.906	7.334	-20.703	35.371
7.813	17.517	-12.523	47.557
15.625	17.362	-7.538	42.263
31.250	33.411	9.612	57.210
62.500	53.268	29.353	77.183
125.000	64.419	48.774	80.064
250.000	100.591	98.208	102.974

図2　Phase1試験；被験物質に対する細胞応答性



S; Suppression
N; No effect

<MIB404>



N

N

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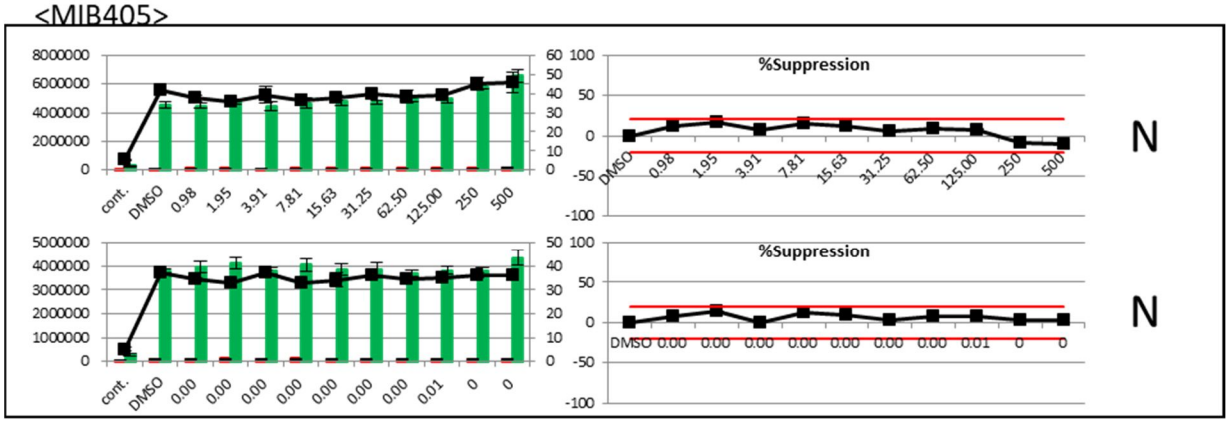

N

N

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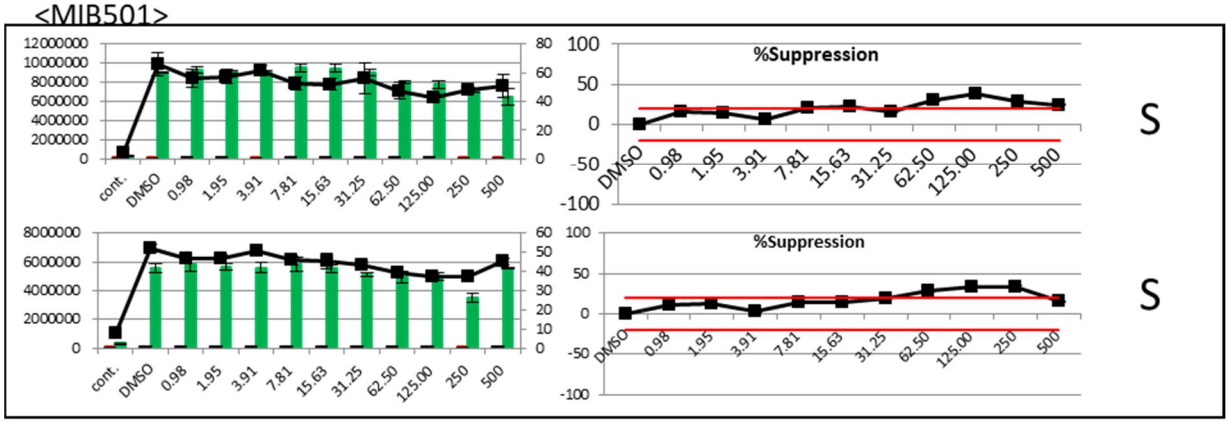
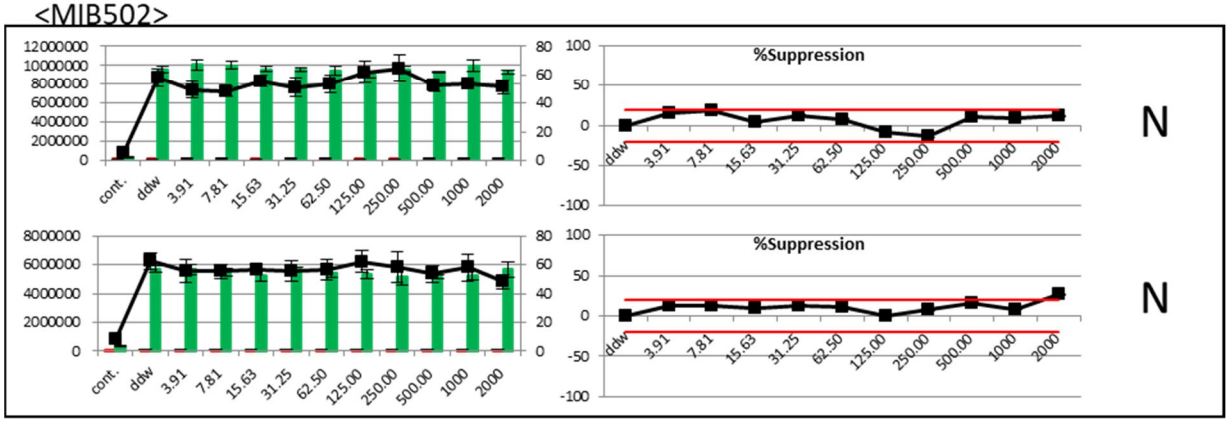


S

S

<MIB502>



N

N

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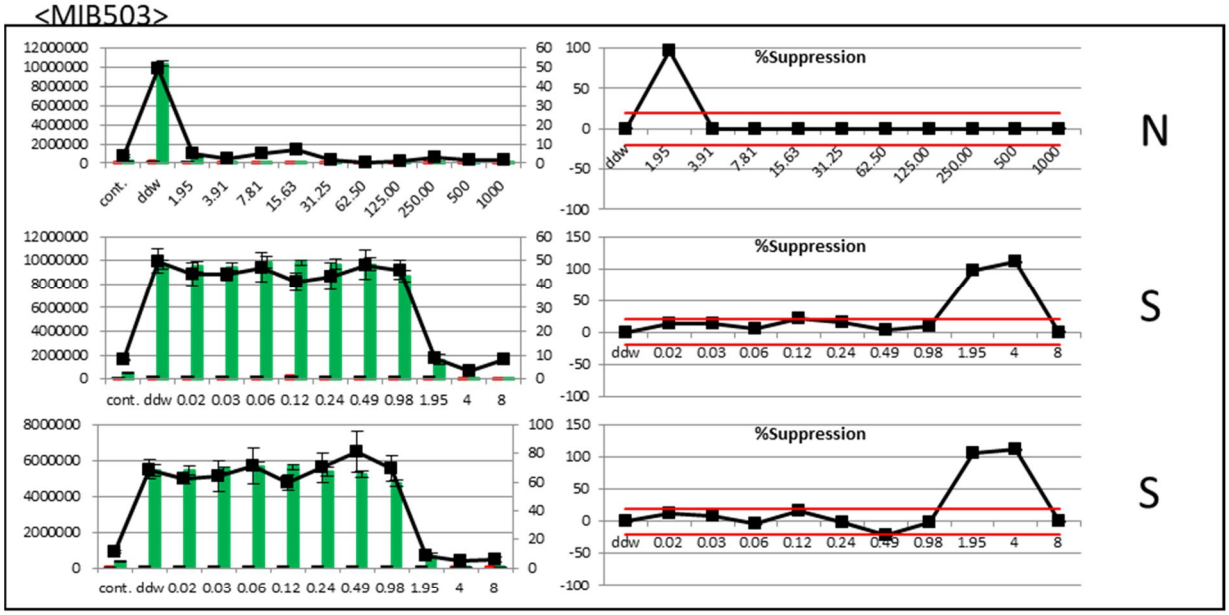

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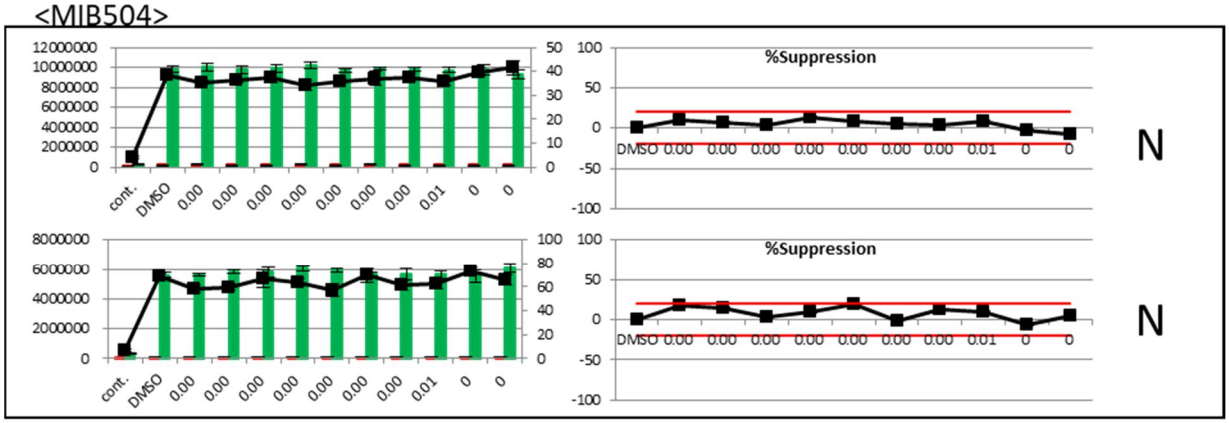

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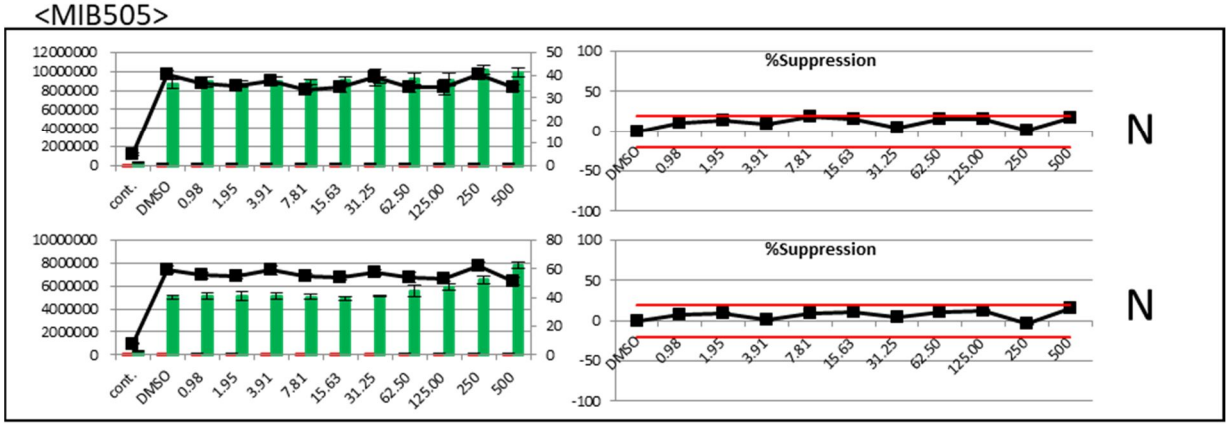

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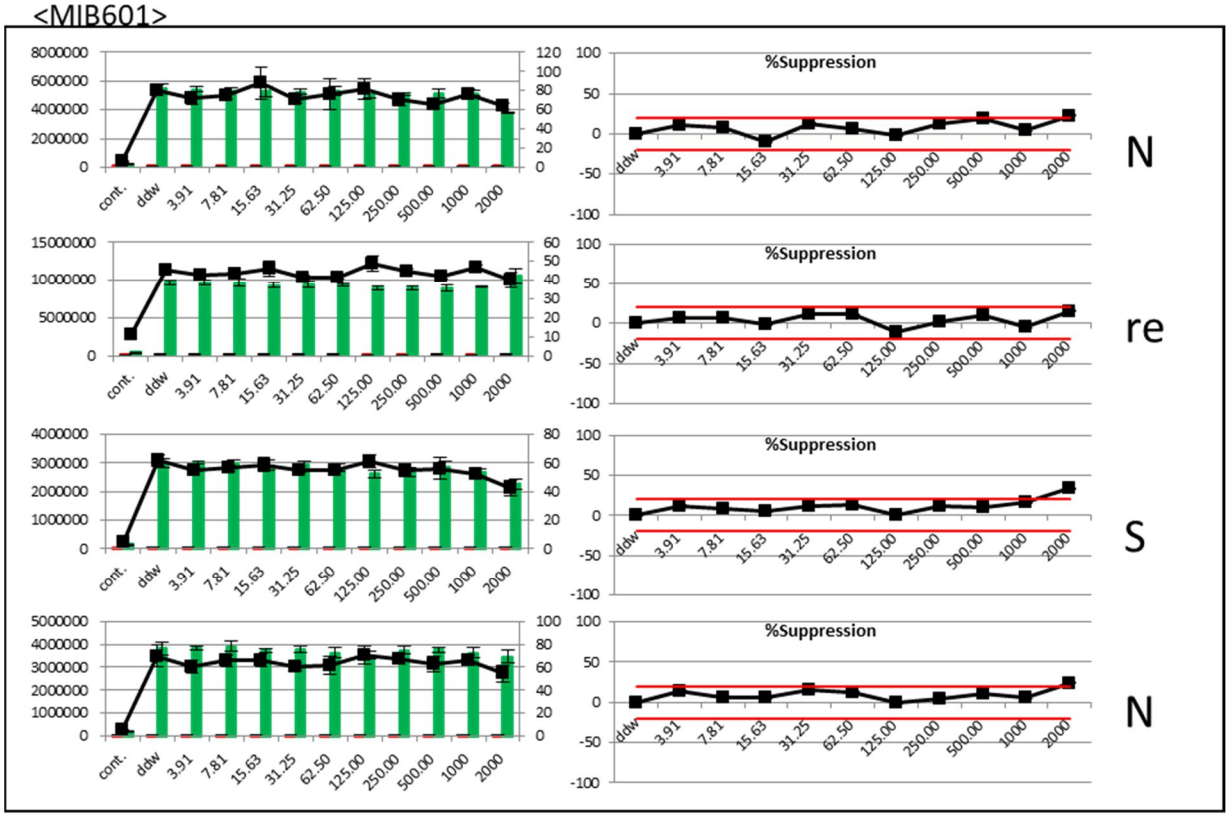

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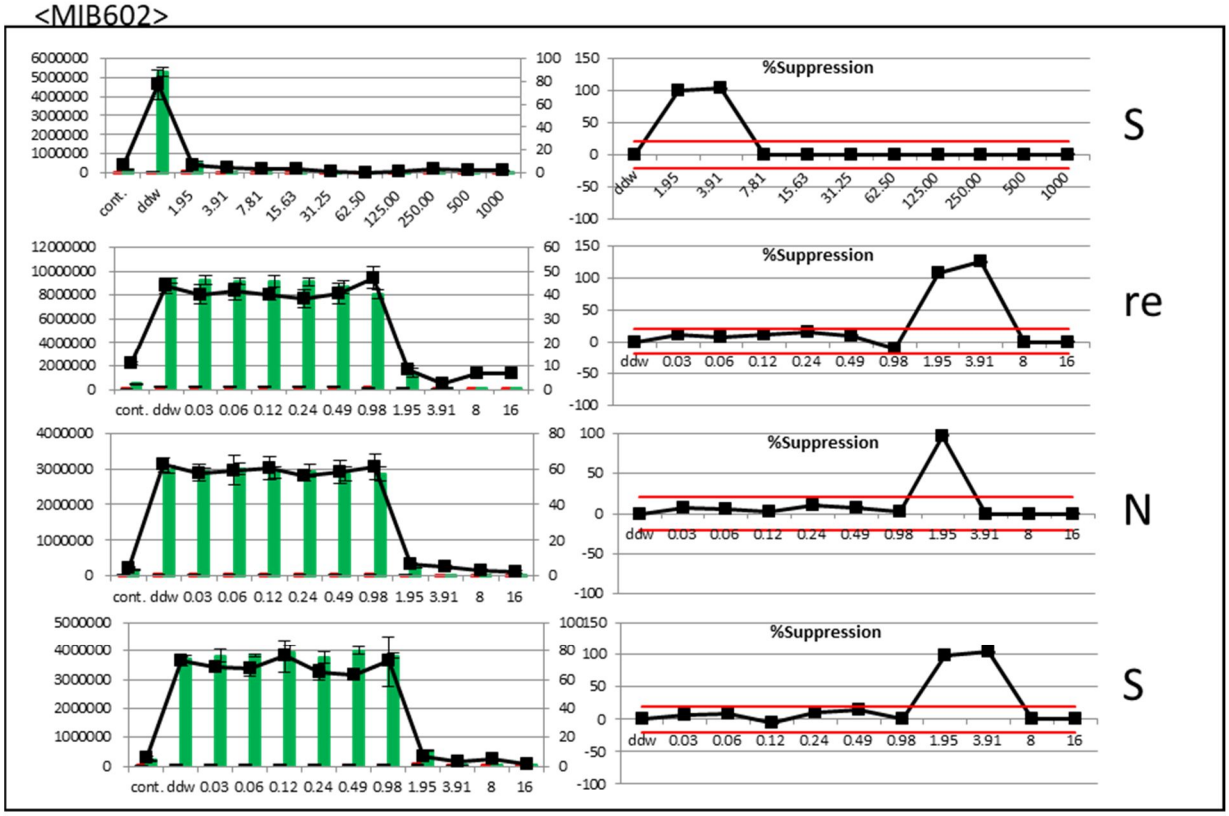
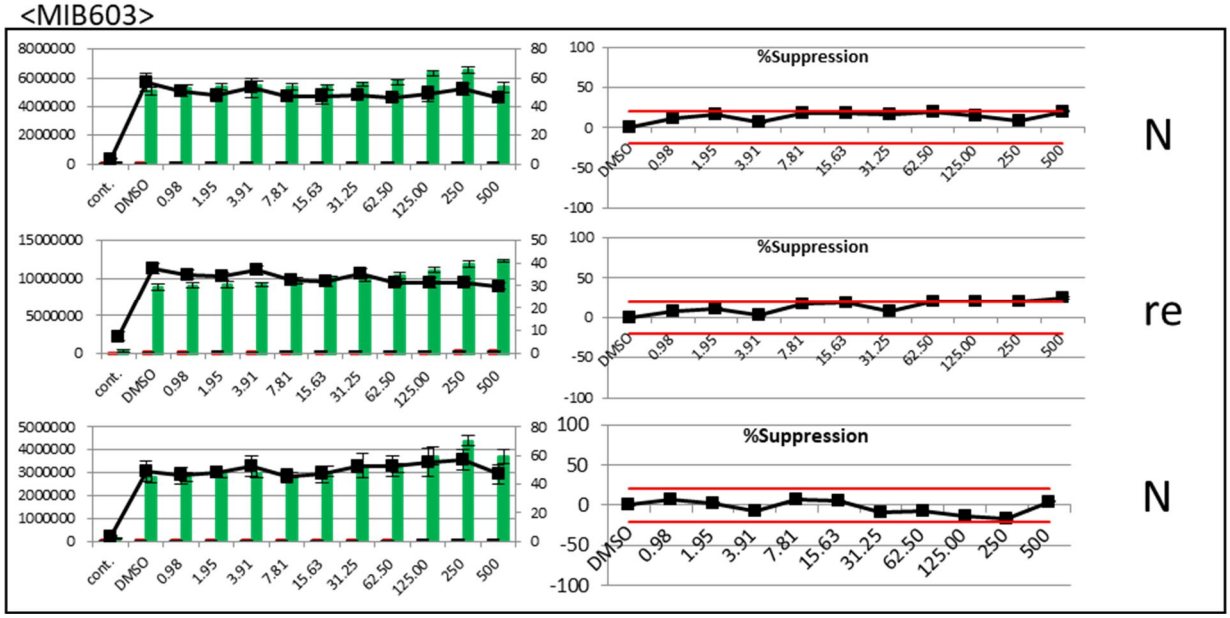


Re: rejected

（FInSLG-LAが<5のため、判定基準を満たしていない）

<MIB603>



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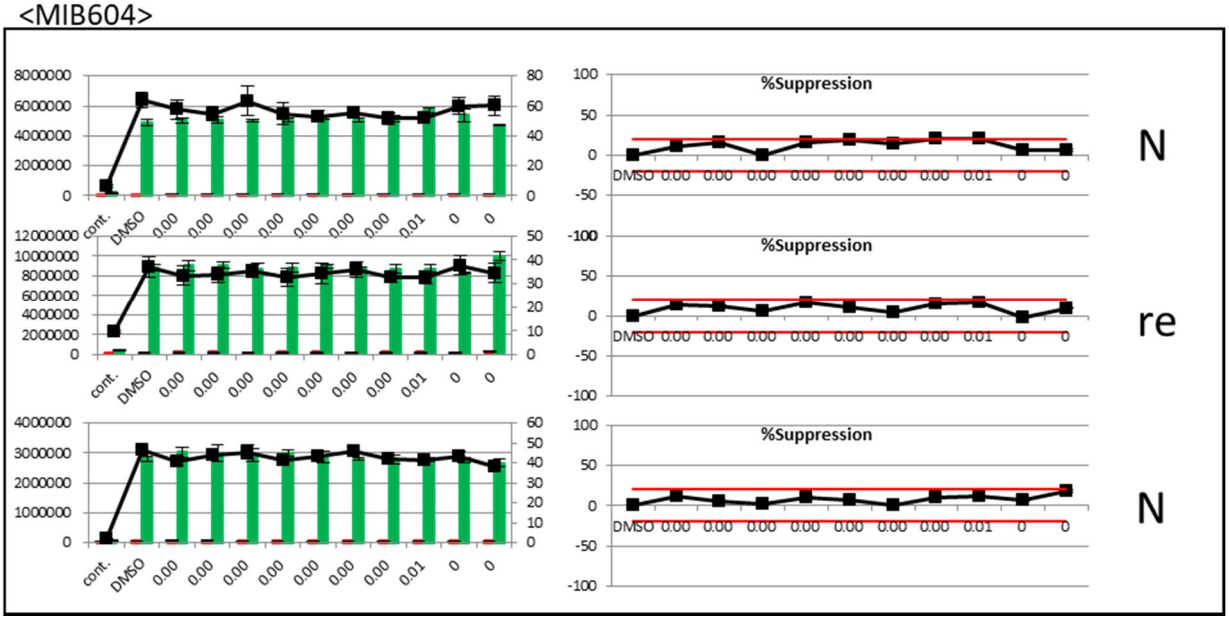

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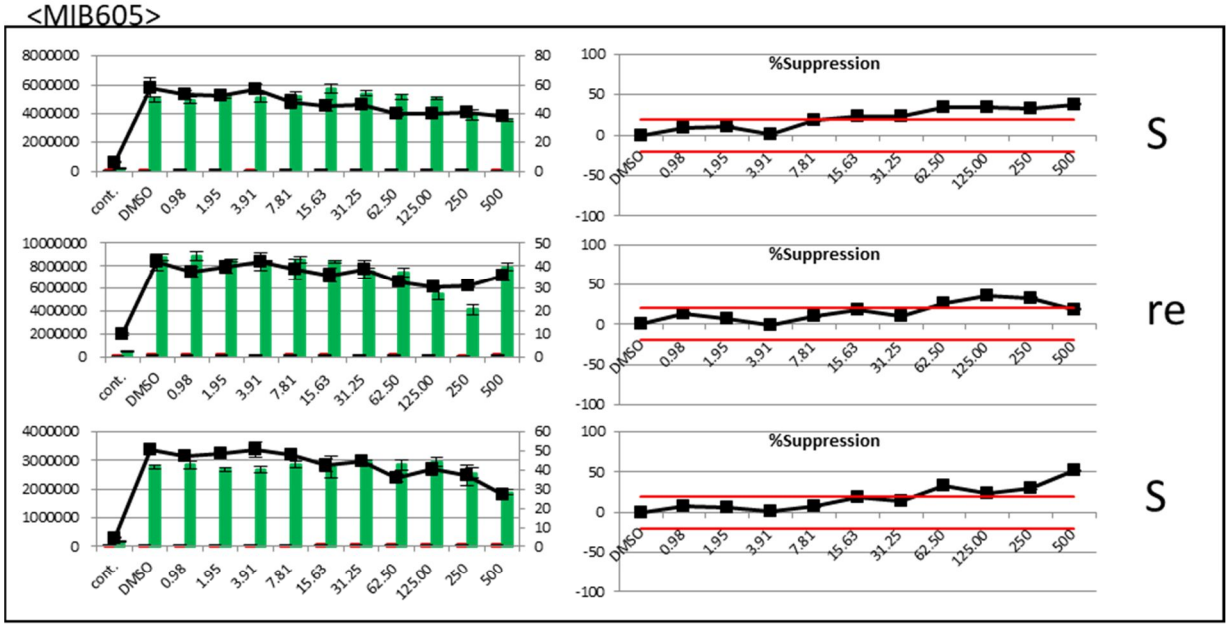

Phase1試験判定結果

MIB	1st	2nd	3rd	4th	判定
401	S	S	-	-	S
503	N	S	S	-	S
602	S	re	N	S	S
402	S	S	-	-	S
501	S	S	-	-	S
605	S	re	S	-	S
403	N	N	-	-	N
502	N	N	-	-	N
601	N	re	S	N	N
404	N	N	-	-	N
505	N	N	-	-	N
603	N	re	N	-	N
405	N	N	-	-	N
504	N	N	-	-	N
604	N	re	N	-	N

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

MITAの国際標準化へ向けてのvalidation試験

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研究要旨

Multi-ImmunoTox assay（MITA）は、T細胞および単球のサイトカイン転写調節に及ぼす化学物質の影響をレポーター遺伝子の発光を利用して評価する免疫毒性評価試験法である。

MITAのOECDガイドライン化を目指し、T細胞におけるIL-2 転写活性抑制を指標とする免疫毒性物質評価系の国際バリデーション試験が終了したことから、今年度から、単球におけるIL-1β転写活性抑制を指標とする免疫毒性物質評価系の国際バリデーション試験を開始した。

フェーズ0として、技術移転性を確認するために、3試験施設がプロトコル(ver. 007E）に従って3物質の実験を行った。その結果、Dapsoneと*p*-Nitroanilineは3試験施設の結果が一致したが、Diethanolamineの我々の結果は他の2試験施設と一致しなかった。不一致の原因を調査した結果、化学物質処理1時間後に実施するIL-1β転写活性剤（LPS）添加後の攪拌の有無が秦野研究所とリードラボ（東北大学）との違いであることが判明した。その他の操作上の差異を確認するために、秦野研究所と東北大学のそれぞれの担当者が秦野研究所において同時に試験を実施した。その結果、結果に影響するような操作上の差異は認められず、フェーズ0 で使用した 3 物質の結果もほぼ一致した。また、LPS添加後の攪拌を実施してDiethanolamineの3回の繰り返し実験を行なった結果、すべて一致した結果が得られ、他の2試験施設の結果とも一致することを確認した。さらに、その影響を検討した結果、LPS添加後に攪拌しない場合には結果が安定せず、攪拌することによって安定した結果が得られることが明らかとなった。

以上の結果より、フェーズ0における結果の不一致は、プロトコルの記述に起因して発生したLPS添加後の撹拌操作の差異が主要因であり、それを改善することにより、良好な施設内再現性および施設間再現性が得られると考えられる。

キーワード：レポータアッセイ、IL-1βプロモーター活性、バリデーション試験、技術移転性、LPS

A．研究目的

MITAの開発者である相場らは、IL-2転写調節障害をkey eventとするT細胞分化異常誘導に関するAOPを作成した。それに基づき、MITAを構成するIL-2転写活性抑制を指標としたT細胞分化異常誘導化学物質評価系のOECDテストガイドライン化を目指し、国際バリデーション試験を実施し、昨年度終了した。

また、相場らは、60種類の化学物質をMITAの複数項目に関して効果発現最低濃度（Lowest observed effect level；LOEL）を基にクラスター分類することにより、化学物質が6種類のクラスターに分類できることを明らかにした。

このクラスター分類は、既にOECDテストガイドライン（442E）に承認されている皮膚感作性試験（IL-8 Luc assay）に加え、MITAを構成するIL-2転写活性抑制評価系とIL-1β転写活性抑制評価系の結果に基づいて行われる。そこで、MITAを構成するIL-1β転写活性抑制評価系をOECDテストガイドライン化するために、その国際バリデーション試験が開始された。

今年度は、フェーズ0として、IL-1β転写活性抑制作用が知られている3物質を用いて、3試験施設による技術移転性を確認した。

B．研究方法

B-1)使用した細胞

IL-1β転写活性抑制試験には、IL-1βおよびG3PDHの各プロモーター領域にそれぞれ緑色ルシフェラーゼ遺伝子（SLG）および赤色ルシフェラーゼ遺伝子（SLR）を繋ぎ、それらの遺伝子を挿入したヒト人工染色体（HAC）ベクターをヒト急性単球性白血病由来のTHP-1細胞に導入した安定細胞株THP-G1b（TGCHAC-A4）を使用した。

細胞の解凍および継代にはC培地、即ち、非働化した仔ウシ胎児血清（FSC、ロット番号：715004、Biological Industries）を10 vol%、および抗生物質-抗真菌剤混合溶液（GIBCO）を1倍となるように添加したRPMI-1640（GIBCO）を使用した。

試験には、非働化FCSを10 vol%添加したRPMI-1640、即ち、B培地を使用した。

なお、FCSの非働化は56℃で30分保温して行い、そのまま室温に放置したのち、冷蔵保存した。

B-2）使用した試薬

技術移転性を確認するために、Dapsone、Diethanolamine、*p*-Nitroanilineの3物質が国立医薬品食品衛生研究所または東北大学から送付され、それらを試験に使用した。

また、陽性対照物質として東北大学から送付されたDexamethasone（DEX、富士フイルム和光純薬株式会社）の100 mg/mL溶液（凍結保存）を使用した。

溶媒として、蒸留水またはジメチルスルホキシド（DMSO）を使用した。

IL-1βの活性化剤として、東北大学から送付された大腸菌K12株由来のLipopolysaccharide（LPS、InvivoGen）の1 mg/mL溶液（凍結保存）を使用した。

発光試薬としては、Tripluc® Luciferase assay reagent（東洋紡株式会社）を使用した。

B-3）研究方法

IL-1β転写活性抑制試験は、リードラボ（東北大学）の指示により、解凍時の培養液をA培地（PuromycinおよびBlasticidin S HClを添加）からC培地に変更したこと、発光測定装置のフィルターをF1 からF2 に変更したこと、実験回数を1セット3回の2セットとしたこと以外は、MITAプロトコル（Ver.007E）に従って行った。概要は以下の通りである。

2×10^6細胞/mLのTHP-G1b（TGCHAC-A4）細胞懸濁液を調製し、その50 µLを96 wellプレートの各ウェルに播種（1×10^5細胞/ウェル）した。また、溶解性検討結果から、プロトコルに従い、DapsoneではDMSO、Diethanolamineでは蒸留水、*p*-NitroanilineではDMSOを使用し、それぞれ250 mg/mL、100 mg/mLおよび500 mg/mLの原液を調製した。さらに、原液を溶媒で段階希釈（公比2）して10濃度の化学物質調製液を調製した。なお、溶解性は、15,000 rpmで5分間遠心し、沈澱の有無を確認することにより判定した。

調製した化学物質調製液をB培地に添加（蒸留水の場合は25倍希釈、DMSOの場合は10倍希釈後さらに5倍希釈）して2倍濃度の処理液を調製し、その50 µLを細胞懸濁液（50 µL）が入っている各ウェルに添加し、

プレートシェーカーで約30秒振盪した（溶媒の最終濃度：蒸留水は2 vol%、DMSOは0.1 vol%）。処理開始1時間後に1 μg/mLのLPSをウェルあたり10 μL添加し、約30秒プレートシェーカーで振盪することにより活性化処理を行った。6時間培養（37℃、5%CO_2）後にTripluc® Luciferase assay reagentを各ウェルあたり100 μL添加し、10分間プレートシェーカーで振盪後、発光測定装置（AB-2350、フェリオス、アトー社製）でフィルターのない発光量（F0）とF2フィルターをセットしたときの発光量をそれぞれ3秒間測定した。その測定値をデータシート「Data sheet for MITA THP-G1b (TGCHAC-A4) Ver. 008 20181203」に入力して、LPSを添加しない細胞に対するLPSを添加した細胞の標準化IL-1β転写活性の増加率（FInSRG-LA）および化学物質処理した細胞における標準化IL-1β転写活性の抑制率（%suppression）を求めた。

C．研究結果

C-1)フェーズ0

各物質について、実験日または継代後の日数を変えて、実験を繰り返し、3実験を1セットとし、2セットの実験（計6回の実験）を行った。

Dapsoneのセット1では、すべての実験で15.6 μg/mLから濃度依存的に発光量の減少、即ち、IL-1βの転写活性抑制が認められた（図1）。セット2では、実験1および2では明らかに濃度依存的にIL-1βの転写活性抑制が認められた。一方、実験3ではバラツキが大きく、高濃度でIL-1βの転写活性抑制が認められたが、濃度依存性は明確ではなかった（図2）。

Diethanolamineのセット1では、弱い転写活性抑制と高濃度での転写活性促進（実験1)、低濃度と高濃度での転写活性促進(実験2)、濃度依存的な転写活性抑制（実験3）が認められ、再現性のある結果が得られなかった（図3）。セット2では、62.5 μg/mLないし125 μg/mLから濃度依存的に転写活性抑制が認められ、1000 μg/mLないし2000 μg/mLで転写活性抑制作用の低下または転写活性促進作用を示すような類似の反応性を示した（図4）

p-Nitroanilineのセット1の実験では、いずれも15.6 μg/mLから濃度依存的に転写活性抑制が認められ、500 μg/mLでその作用が低下した（図5）。セット2の実験においても同様の結果が得られた（図6）。

フェーズ0の3試験施設の結果と比較した結果（表1）、Dapsoneと*p*-Nitroanilineは3試験施設ともに転写活性抑制作用を示し、一致した結果が得られた。一方、Diethanolamineについては、2試験施設では転写活性抑制作用を示し、我々の結果で得られたような明らかな転写活性促進は認められなかった。

C-2) LPS添加方法の影響

この試験系は化学物質で処理し、1時間経過後にIL-1βの転写活性を誘導するためにLPSを添加する。プロトコルでは、「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.」と記載されており、我々は、「LPS溶液を20回ピペッティングしたのち、10 μLのLPS溶液をサンプリングし、それを培養液にチップを確実に浸けた状態で添加する」と解釈した。FInSRG-LAは、蒸留水を10 μL添加したウェルの標準化発光量に対して、LPSを10 μL添加したウェルの標準化発光量の比で求められることから、LPS添加の影響は大きいと考えられた。そこで、化学物質の代わりにDMSOを添加して1時間培養したのち、我々のLPS添加方法、即ち、10 μLを静かに添加する「添加のみ群」、添加してチップをウェル内で回転して撹拌する「回転群」およびウェル内でピペッティングにより撹拌する「ピペッティング群」を設定してLPS添加6時間後の発光量を測定した。なお、LPS添加後のプレートシェーカーによる撹拌は実施しなかった。その結果、撹拌した群のFInSLG-LAは「添加のみ群」よりも高い値、即ち、IL-1βの転写活性促進を示す結果が得られた（図7）。また、この傾向にFCSの差（秦野研究所または東北大学で非働化した同一ロットのFCS、ならびに、MITA用ではなく、かつ、非働化していないFCS）は認められなかった（図7）。

リードラボにLPS添加方法に関するプロ

トコルの記載について確認したところ、プロトコルの記載は、「チップを培地につけた状態でLPSを 10 µL添加し、その後 20 回ピペッティングする」であることが明らかとなった。そこで、秦野研究所で非働化したFCSを用いてLPSの添加方法の差による影響について更に検討した。8 連のピペットで細胞を播種し、必要なすべてのウェルにDMSOを添加した。1 時間培養後、DMSOコントロール（2 列目）に相当する上段 4 ウェルはLPS添加のみ実施（ピッティング無し）し、その下段 4 ウェルはLPS添加後 20 回ピペッティングし、3 列目以降は 8 連ピペットでLPS添加操作を行った（図 8）。その結果を表 2 にまとめた。すべてDMSO処理群であるが、LPS添加のみ（ピペッティング無し）をコントロールにした場合、ピペッティング無しの条件であっても、転写活性抑制結果になることや、20 回ピペッティングでは転写活性促進の結果になることもあり、その傾向は、LPS添加後にプレートを撹拌しない場合にはさらに強く表れた（図 8）。また、20 回ピペッティングをコントロールとした場合には、同じ 20 回ピペッティングしたウェルでは%suppressionに差は認められなかったが、ピペッティング無しまたは 1 回の場合には、転写活性抑制の結果が得られ、プレートを撹拌しない場合にはその傾向は強く表れた（図 8）。

LPSの添加方法以外の操作上の差異を確認するために、秦野研究所においてリードラボの担当者が秦野研究所の担当者の実験操作を観察した。観察は、LPS添加後に攪拌する方法に変更してフェーズ 0 の 3 物質の実験について実施した。それと同時にリードラボの担当者もフェーズ 0 の 3 物質の実験を実施した。その結果、操作上の差異は認められず、3 物質の結果はいずれもほぼ同じ反応が得られた（図 9）。そこで、改善したLPS添加法でDiethanolamineの再試験（3 実験、1 セット）を実施した。その結果、すべての実験において 31.3 µg/mLから濃度依存的に転写活性抑制が強くなり、1000 µg/mLおよび 2000 µg/mLでその作用が低下する反応を示し、再現性が得られた（図 10）。

C-3) 血清の影響

DEX（25、50、100 µg/mL）を用いてFCSの影響を検討した。その結果、継代時の細胞密度をプロトコルに記載されている範囲、即ち、2×10^5 細胞/mLと 5×10^5 細胞/mLとし、2 種類のFCS（秦野研究所で非働化したFCSと東北大学で非働化したFCS）で培養し、継代 3 日後の細胞を用いて、1 枚のプレートでDEXの反応性を比較した（図 11）。その結果、2×10^5 細胞/mLで継代した場合、東北大学で非働化したFCSの方がやや転写活性抑制が強く、5×10^5 細胞/mLの場合も東北大学のFCSで転写活性抑制作用がやや高い傾向があるが、ほぼ同じ反応性を示した（図 11）。

C-4) 継代時細胞密度と培養日数の影響

秦野研究所で非働化したFCSを用いて継代日数の異なる細胞を 1 枚のプレートに播種し、DEXの反応性を比較した（図 12）。DEXは、継代 3 日後に播種して実験した場合には 40～60%の転写活性抑制作用を示した。一方、継代 4 日後の場合、2×10^5 細胞/mLで継代した細胞は継代 3 日後の細胞と同様の反応性を示したが、5×10^5 細胞/mLで継代した細胞では 40%未満の転写活性抑制を示した（図 12）。再現性を確認した結果、5×10^5 細胞/mLで継代し、継代 4 日後の細胞で実験した場合、DEXの反応性低下が再現された（図 13）。

C-5) 細胞増殖率

今回の試験に使用したFCSは、指定された同一ロットのFCSを各施設で非働化して使用したが、非働化方法の詳細はプロトコルに記載されていない。そこで、秦野研究所で使用している非働化FCSと東北大学で非働化したFCS、MITA用ではなく、かつロットの異なる非働化していないFCSで培養した細胞の増殖率を求めた。その結果、2×10^5 細胞/mLで継代した場合、倍加時間はいずれのFCSもほぼ 34 時間であり、ロット差、非働化による差は認められなかった。また、5×10^5 細胞/mLで継代した場合、播種後 4 日目では対数増殖期が終了し定常期にあると考えられた（図 14）

D．考察

フェーズ 0 では、1 物質について 6 回（3 回/セット×2 セット）の実験を行なったが、*p*-Nitroanilineでは濃度依存性および最終判定ともに再現性のある結果が得られ、他の 2 試験施設の最終判定とも一致し、施設

間再現性が得られた。Dapsoneについては、高濃度での反応性に差が認められたが、5回の実験の判定は一致した。残り1回の実験結果は、転写活性促進を示す濃度が存在し、バラツキも大きかったが、判定は一致しており、他の2試験施設の最終判定とも一致した。Diethanolamineでは、転写活性抑制のみを示した実験が2回、転写活性抑制と転写活性促進の両方の反応を示した実験が3回、転写活性促進のみを示した実験が1回となり、再現性が得られなかった。他の2試験施設では単発的に転写活性促進を示す濃度が存在するものの、判定はすべて転写活性抑制であり、我々の結果のみ施設間再現性なしとなった。

Diethanolamineの結果が、他の試験施設と明らかに異なることから、その原因を特定するために検討実験を行なった。

フェーズ0における我々の結果では、コントロールのFInSRG-LA（LPSを添加しない細胞に対するLPS添加による標準化IL-1β転写活性の増加率）が他の施設と比較して低いことから、これがバラツキの原因の一つとして議論された。我々は、「チップを培養液につけた状態で（攪拌せずに）LPSを添加する」とプロトコル（Ver.007）を解釈していたが、LPSの添加方法が重要であると考えたことから、LPS添加後の攪拌の影響を検討した。その結果、LPS添加後に攪拌することによって、転写活性促進様の結果が得られることが明らかとなり、フェーズ0のDiethanolamineで認められた転写活性促進結果の原因となった可能性が示唆された。さらに、%suppressionの溶媒対照となるLPS添加コントロールのLPS添加後の撹拌条件が転写活性に影響することが明らかとなった。即ち、化学物質の代わりにDMSOを使用した場合、すべての処理群が理論的には同じ転写活性、即ち、%suppressionに差の無い結果となることが期待される。しかしながら、LPS添加後に攪拌しないコントロールの場合、攪拌条件にかかわらず結果が一致せず、安定した結果が得られなかった。一方、20回攪拌したコントロールの場合では、同じ条件、即ち20回攪拌した群では期待通り%suppressionに差は認められないが、攪拌しない群では結果が一致しなかった。LPS添加後にプレートを撹拌しない場合には、これらの傾向が明確となった。特に、20回攪拌したコントロールの場合では、20回攪拌しない群ではすべて明らかな転写活性抑制を示す結果となった。この結果は、LPS添加後のプレートシェーカーによる撹拌のみでは、LPSを十分に作用させることができないことを示唆している。また、プロトコルでは、LPS添加後のプレートシェーカーによる攪拌条件が細かく規定されていない。これらのことから、我々がフェーズ0で実施したLPS添加後にピペッティングによる撹拌をしない条件下では、LPS添加後のプレートの攪拌状態が結果のバラツキに影響した可能性も考えられる。

この試験系で安定した結果を得るために、LPS添加後の撹拌が重要であることを支持する結果として、LPS添加後に攪拌して実施したリードラボとの比較実験の結果に差はなく、その後に実施したDiethanolamineの結果もフェーズ0で認められた転写活性促進を示す結果は得られず、他の2試験施設と同じく、転写活性抑制のみを示す結果が再現良く得られたことがあげられる。

フェーズ0の結果の不一致の原因として、FCSの非働化の差が影響した可能性も考えられたことから、FCSによる影響をLPS添加法の比較実験（図7）および継代細胞密度と培養日数の影響の検討実験（図11）によって検証した。LPS添加比較実験ではFCSの違による明確な差は認められず、継代細胞密度と培養日数の検討実験では東北大学のFCSで良好な反応を示す傾向が認められたが、明らかな差とは考えられなかった。

また、継代後の培養日数がLPSによる転写活性化に影響している可能性についても検討した。継代細胞密度をプロトコル記載の最低とし、プロトコル記載の最大日数である4日間培養してDEX処理による反応性を調べた結果、転写活性抑制作用は40～60%の範囲内の値を示した。一方、継代細胞密度をプロトコル記載の最大である5×10^5細胞/mLとし、最大日数培養してDEX処理した場合、転写活性抑制作用は40%未満となり、再現性も得られた。細胞増殖率の結果は、5×10^5細胞/mLで継代した場合、培養4日後では細胞密度が2×10^6細胞/mLに達し、細胞増殖が止まる定常状態であることを示したことから、この状態では、IL-1βの転写活性が低下するとともに化学物質に対する反応性も低下する可能性が考えられた。

Ｅ．結論

MITAの技術移転性を評価するためにフェーズ 0 として既知の 3 物質について実験したが、1 物質（Diethanolamine）で再現性が得られず、他の 2 試験施設の結果とも一致しなかった。そこで、その原因を検討した結果、MITAプロトコル（Ver.007）に記載されているLPSの添加法の解釈が他の試験施設と異なっていたことが原因であり、改善した方法でリードラボと同時に技術確認実験を実施したところ、同等の結果が得られたほか、追加で実施したDiethanolamineの 3 回繰り返し実験では、良好な再現性が得られ、また、他の 2 試験施設と同様の結果であった。以上のことから、プロトコルのLPS添加法の記載を修正することによって、MITAは良好な施設内再現性および施設間再現性が得られると考えられる。

Ｇ． 研究発表

１．論文発表

なし

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、小林 眞弓、大森 崇、足利　太可雄、小島 肇、相場 節也：Multi-ImmunoTox Assay (MITA) バリデーション研究の結果　日本動物実験代替法学会　第 31 回大会(熊本)2018 年 11 月

表 1　フェーズ 0 における 3 物質（3 実験/セット、2 セット）の結果

IL-1β Phase0 Result(35%)

Chemical	Lab.A (Lead Lab)	Lab.B (AISTTS)	Lab.C (FDSC)	Lab.D (AISTTA)	2018/10/5 concordance	Based on Majority
Dapson Set1	S:SSS	S:SSS	S:SSS	S:SSS	1	S
Dapson Set2	S:NSS	S:SSS	S:SSS	S:SSS	1	S
Diethanolamine Set1	S:SSS	S:SSS	?:NAS	S:SSS	0	S
Diethanolamine Set2	S:SSS	S:SSS	S:SSS	S:SNS	1	S
p-Nitroaniline Set1	S:SSS	S:SSS	S:SSS	S:SSS	1	S
p-Nitroaniline Set2	S:SSS	S:SSS	S:SSS	S:SSS	1	S
					Between 83.33%	

IL-1β Phase0 Result(20%)

Chemical	Lab.A (Lead Lab)	Lab.B (AISTTS)	Lab.C (FDSC)	Lab.D (AISTTA)	2018/10/5 concordance	Based on Majority
Dapson Set1	S:SSS	S:SSS	S:SSS	S:SSS	1	S
Dapson Set2	S:SSS	S:SSS	S:SSS	S:SSS	1	S
Diethanolamine Set1	S:SSS	S:SSS	S:SAS	S:SSS	1	S
Diethanolamine Set2	S:SSS	S:SSS	S:SSS	S:SSS	1	S
p-Nitroaniline Set1	S:SSS	S:SSS	S:SSS	S:SSS	1	S
p-Nitroaniline Set2	S:SSS	S:SSS	S:SSS	S:SSS	1	S
					Between 100%	

IL-1β Phase0 Result(25%)

Chemical	Lab.A (Lead Lab)	Lab.B (AISTTS)	Lab.C (FDSC)	Lab.D (AISTTA)	2018/10/5 concordance	Based on Majority
Dapson Set1	S:SSS	S:SSS	S:SSS	S:SSS	1	S
Dapson Set2	S:SSS	S:SSS	S:SSS	S:SSS	1	S
Diethanolamine Set1	S:SSS	S:SSS	S:SAS	S:SSS	1	S
Diethanolamine Set2	S:SSS	S:SSS	S:SSS	S:SSS	1	S
p-Nitroaniline Set1	S:SSS	S:SSS	S:SSS	S:SSS	1	S
p-Nitroaniline Set2	S:SSS	S:SSS	S:SSS	S:SSS	1	S
					Between 100%	

S

: Suppression, N: No response, A: Augmentation

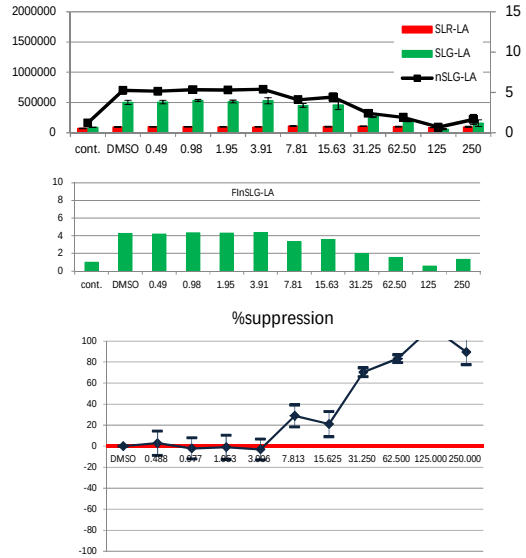
表 2　LPS 添加後のピペッティングの有無による IL-1β 転写活性への影響のまとめ

Plate No.	Pipetting in Control (Row 2)	Shaking of a plate	Fold Increase (P : Significance, N : Not significance) Control (Row 2)	No. of times of pipetting 0 (Row 3)	0 (Row 6)	1 (Row 4)	20 (Row 5)
1	No	Yes	7	7 (N)	7 (N)	7 (N)	8 (P)
3	No	Yes	13	11 (P)	11 (P)	13 (N)	13 (N)
2	No	No	8	8 (N)	6 (P)	7 (N)	11 (P)
1	Yes	Yes	8	9 (N)	7 (N)	8 (N)	8 (N)
3	Yes	Yes	10	9 (P)	8 (P)	9 (P)	9 (N)
2	Yes	No	11	7 (P)	6 (P)	7 (P)	10 (N)

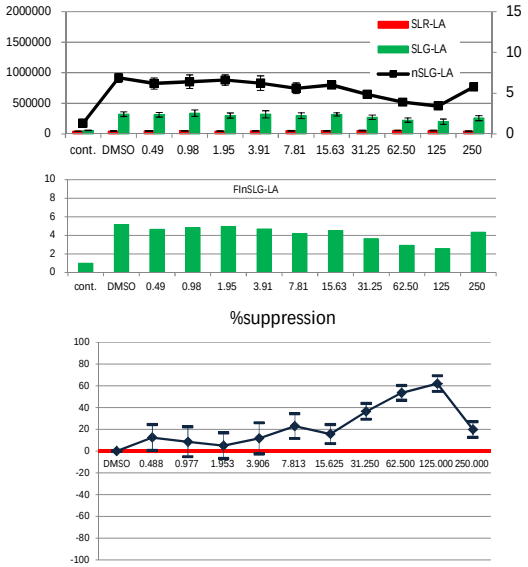
コントロールのピペッティングは 20 回実施した。

Set 1

(1st Experiment)



(2nd Experiment)



(3rd Experiment)

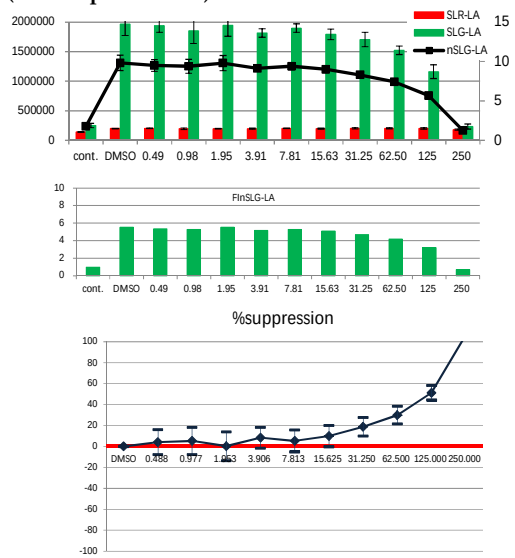
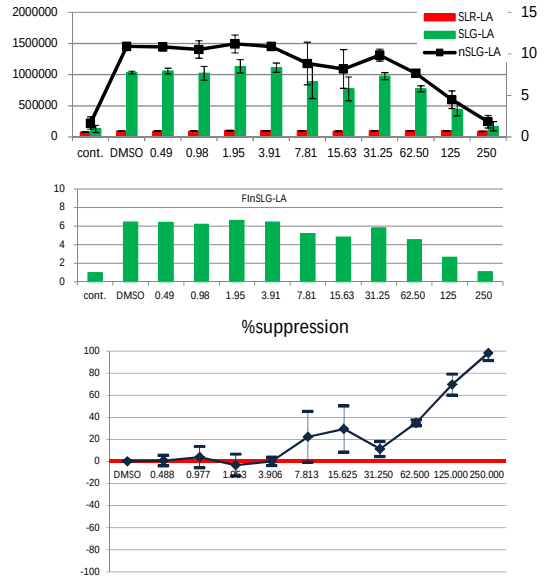


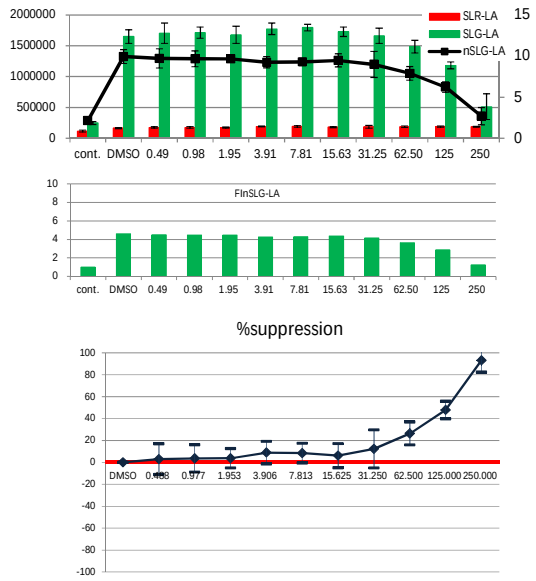
図１　Dapsone の IL-1β転写活性抑制試験の結果（Phase 0，Set 1）

Set 2

(1st Experiment)



(2nd Experiment)



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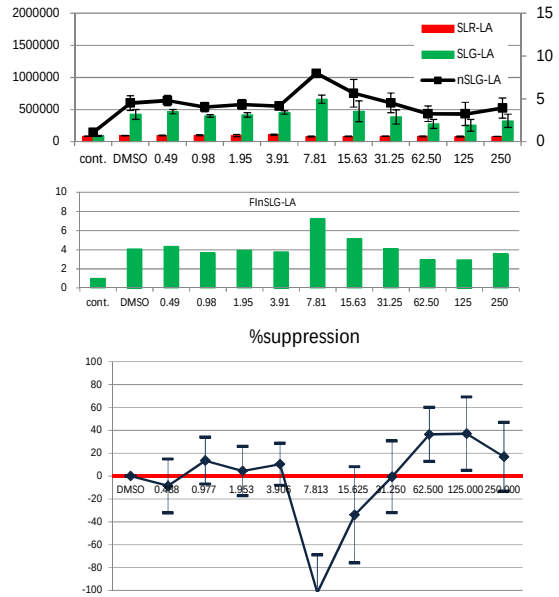
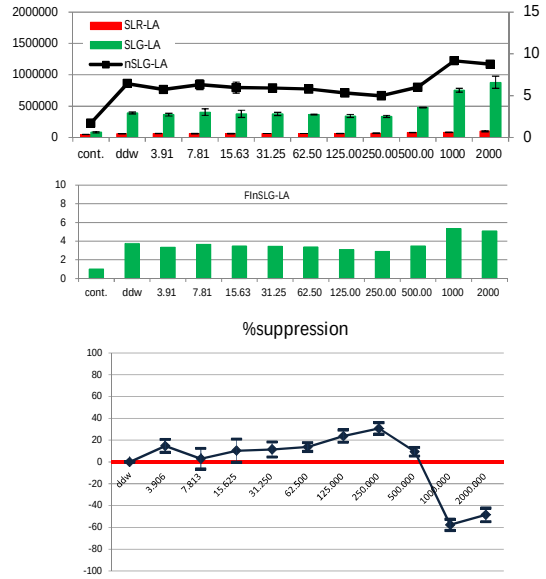


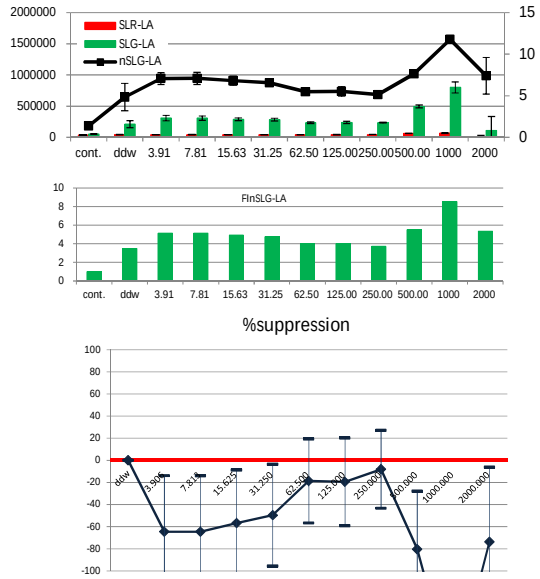
図２　Dapsone の IL-1β転写活性抑制試験の結果（Phase 0，Set 2）

Set 1

(1st Experiment)



(2nd Experiment)



(3rd Experiment)

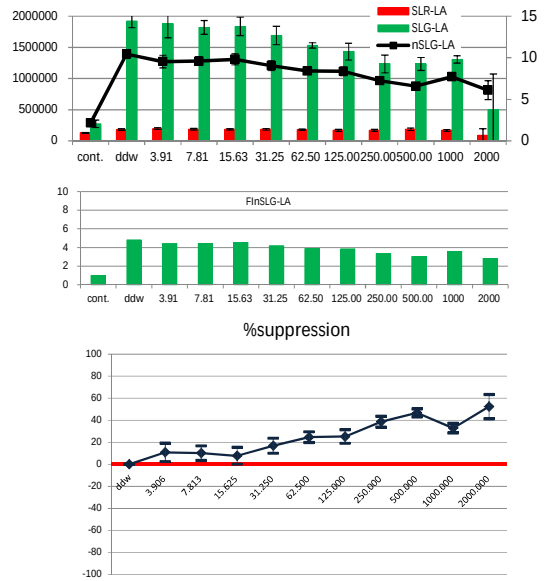
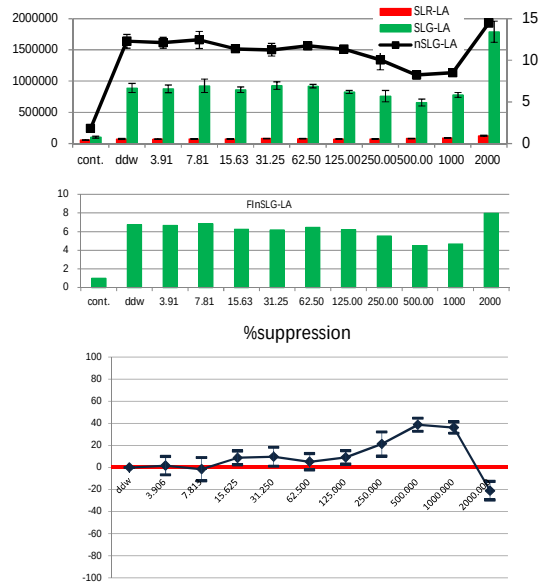


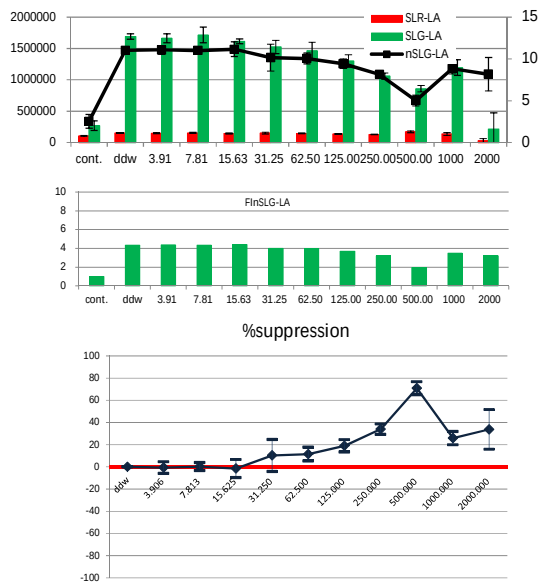
図３　Diethanolamine の IL-1β転写活性抑制試験の結果（Phase 0, Set 1）

Set 2

(1st Experiment)



(2nd Experiment)



(3rd Experiment)

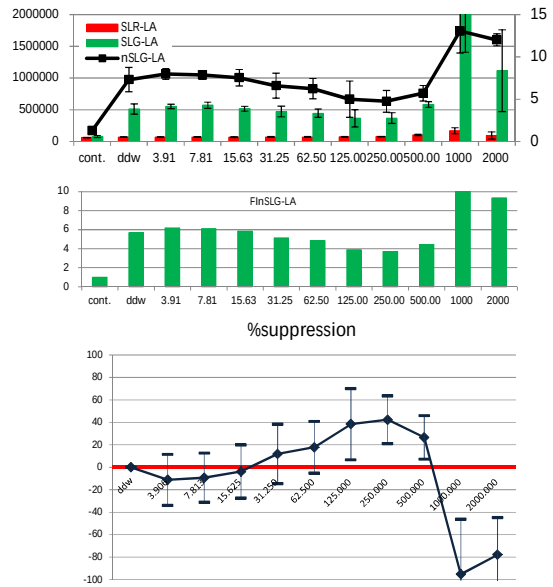
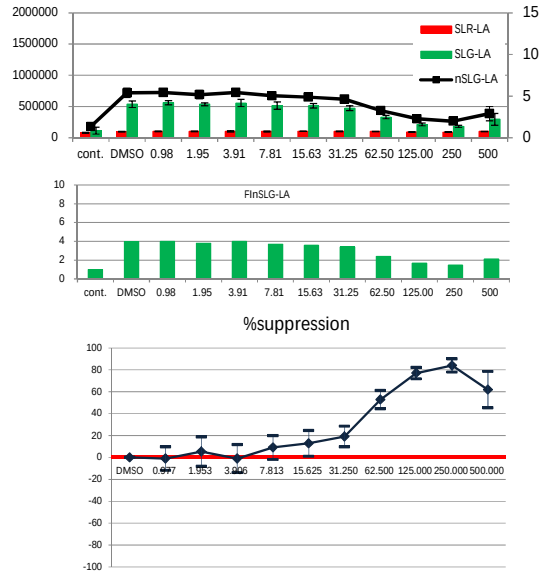


図４　Diethanolamine の IL-1β転写活性抑制試験の結果（Phase 0, Set 2）

Set 1

(1st Experiment)



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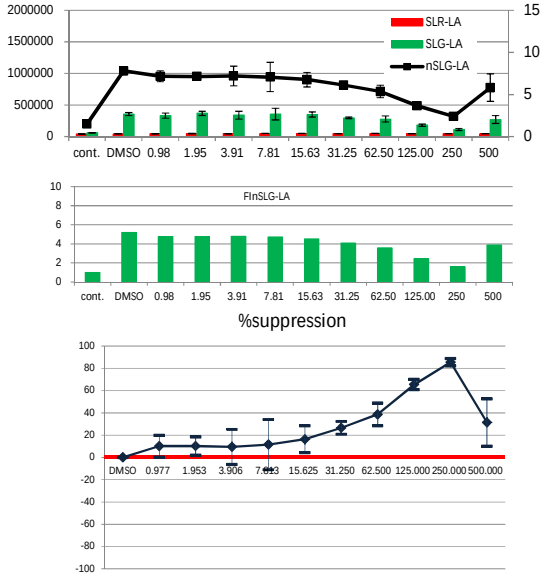


(3rd Experiment)

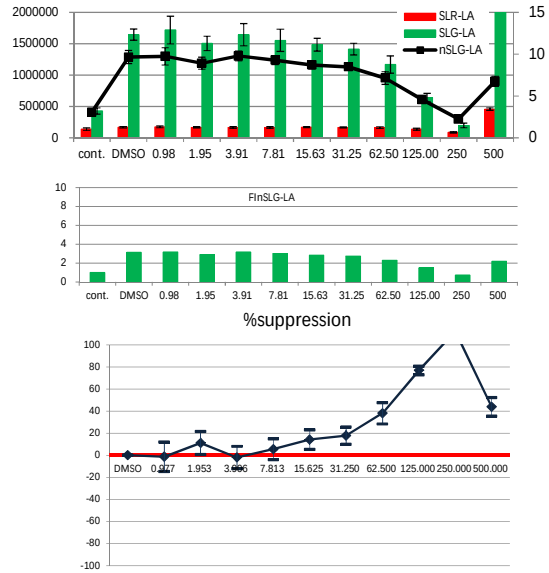
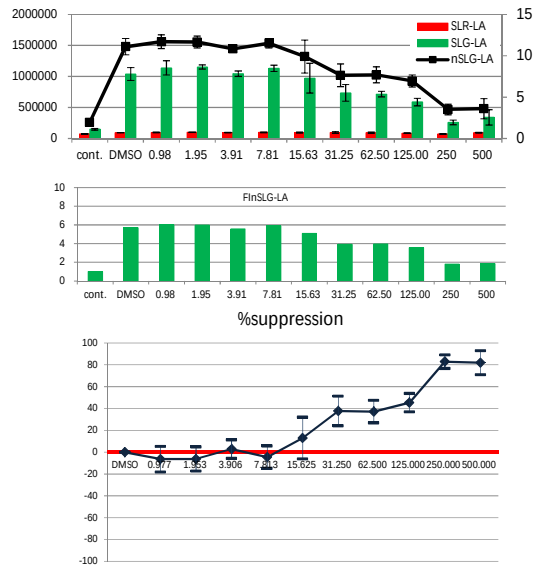


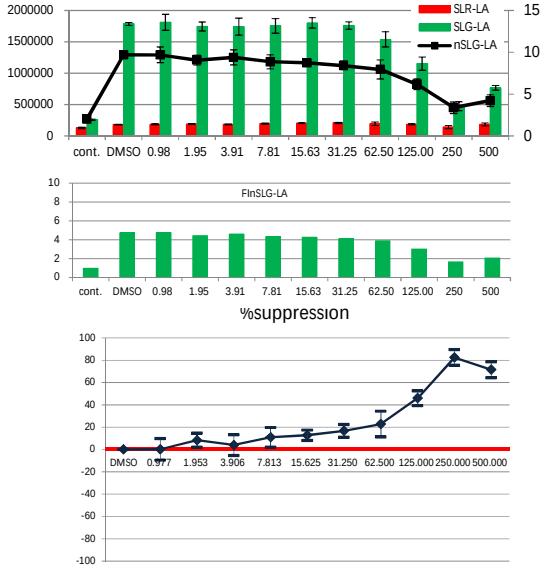
図５　*p*-Nitroaniline の IL-1β転写活性抑制試験の結果（Phase 0，Set 1）

Set 2

(1st Experiment)



(2nd Experiment)



(3rd Experiment)

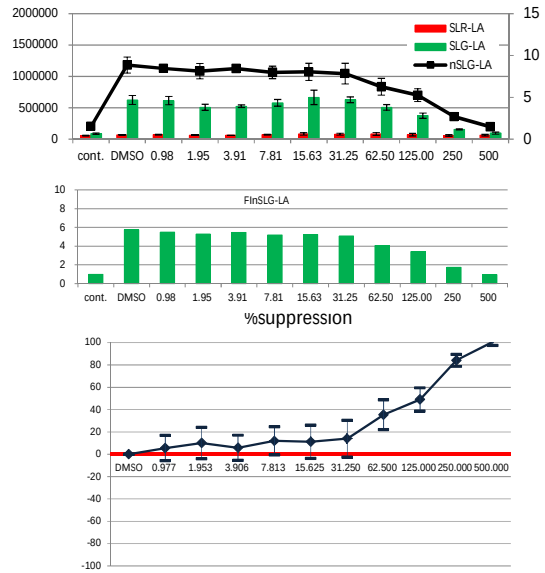
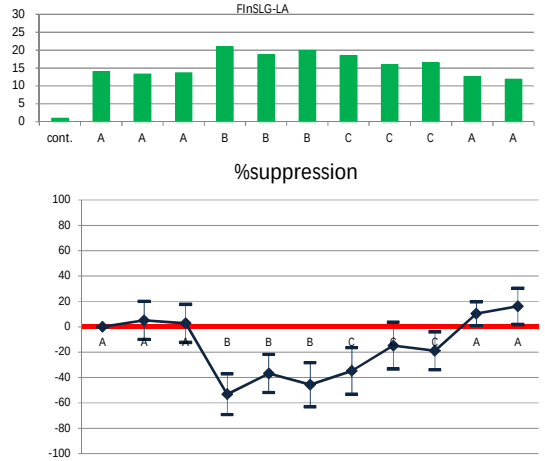
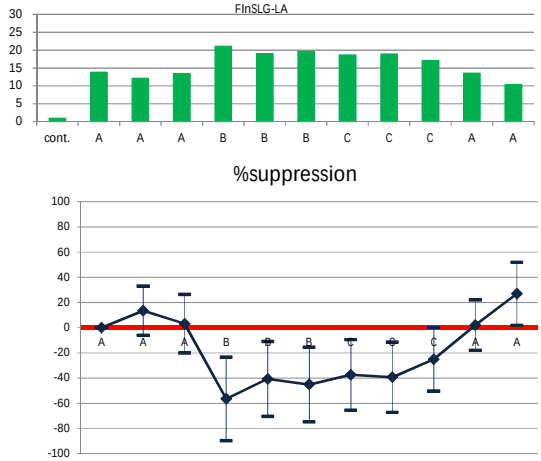


図 6　*p*-Nitroaniline の IL-1β転写活性抑制試験結果（Phase 0, Set 2）

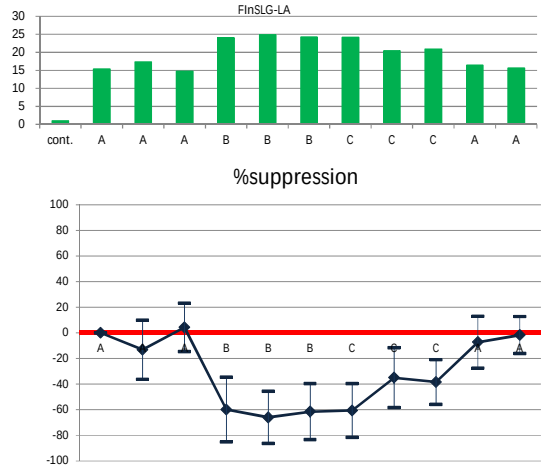
Heat-inactivated FBS (for MITA)



Heat-inactivated FBS from Tohiku Univ.



Non-inactivated FBS (not for MITA)



A: LPS was added to each well without mixing according to the protocol.
B: LPS was added to each well and it was mixed by circulation of a tip in each well.
C: LPS was added to each well and it was mixed by pipetting.

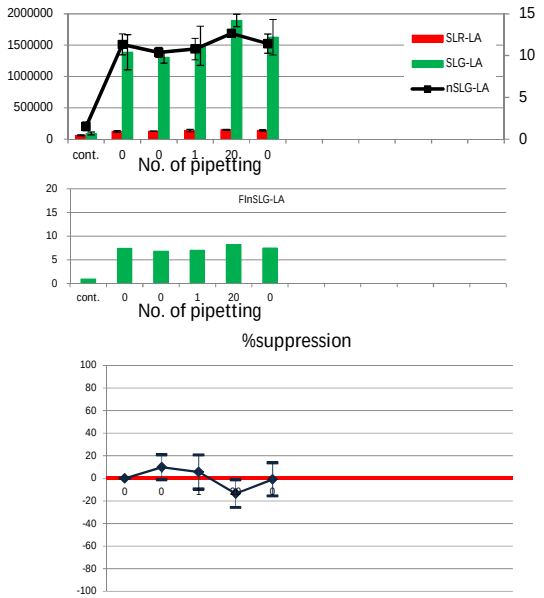
図 7　LPS 添加後の撹拌が IL-1β転写活性に及ぼす影響

Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Control (without LPS)	LPS, no pipetting	LPS, no pipetting	LPS, 1 time pipetting	LPS, 20 times pipetting	LPS, no pipetting						
B												
C												
D												
E		LPS, 20 times pipetting										
F												
G												
H												

Plate 1 (Shaking)

Row 2：No pipetting



Row 2：20 times pipetting

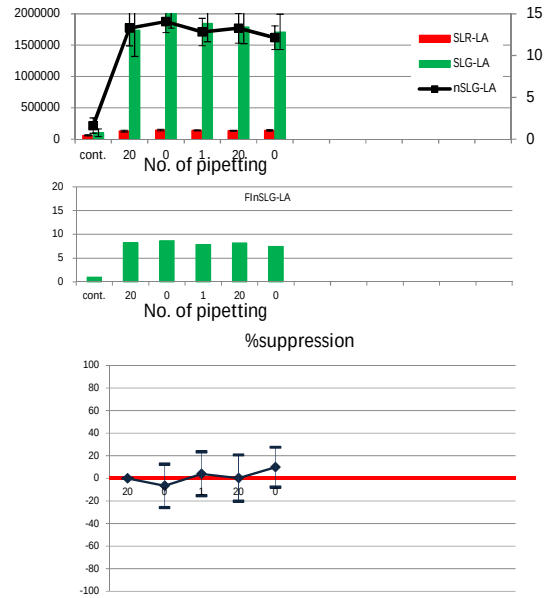
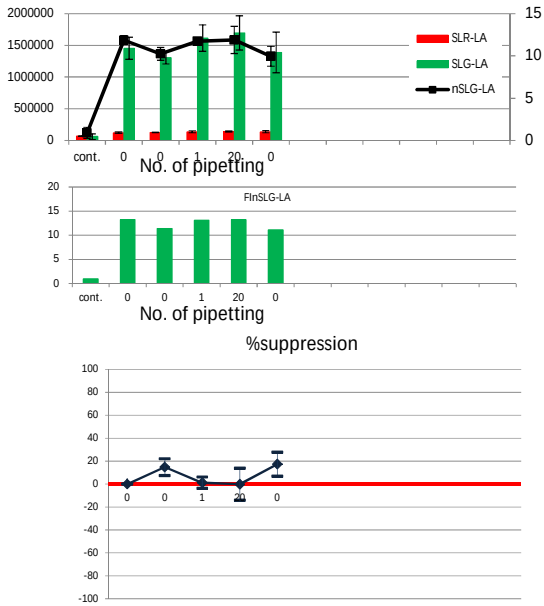


Plate 2 (Shaking, Reproducibility on the same day)

Row 2：No pipetting



Row 2：20 times pipetting

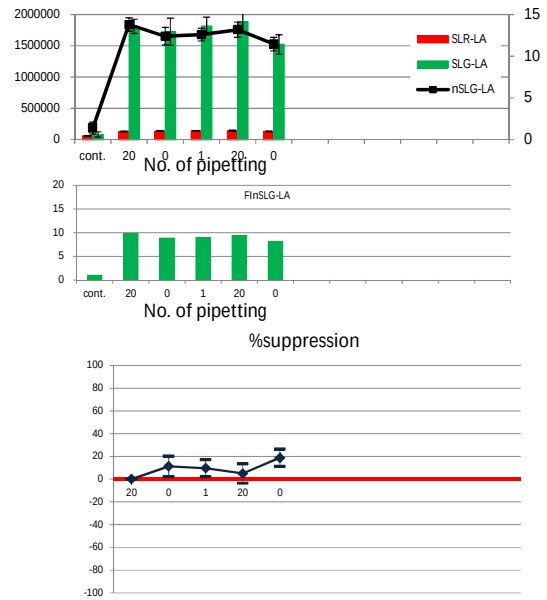
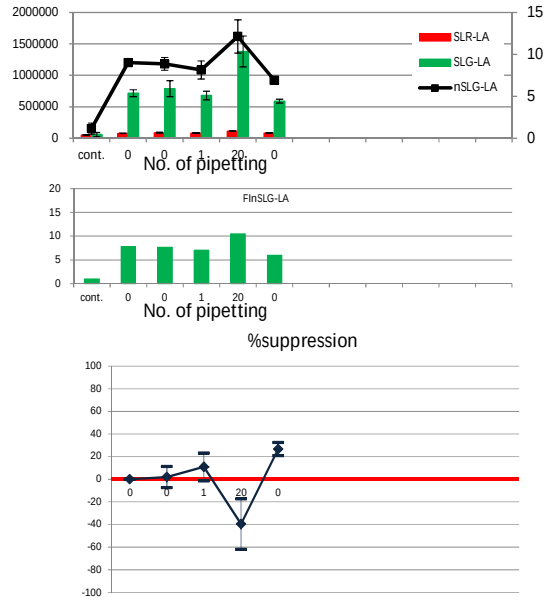


図 8　LPS 添加後のピペッティングの有無による IL-1β転写活性への影響

Plate 3 (No shaking)

Row 2：No pipetting



Row 2：20 times pipetting

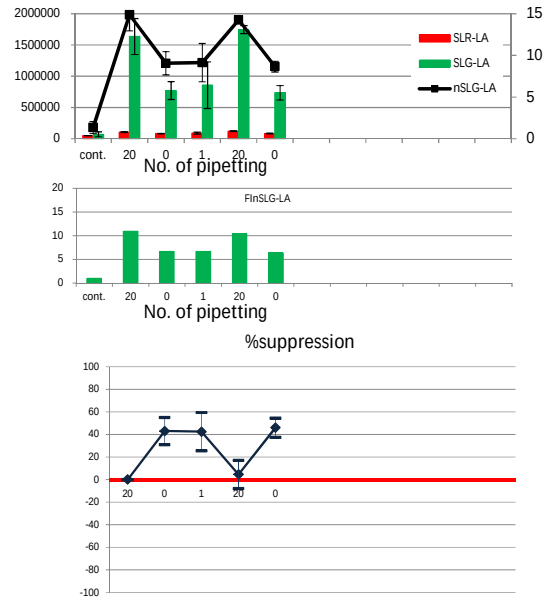
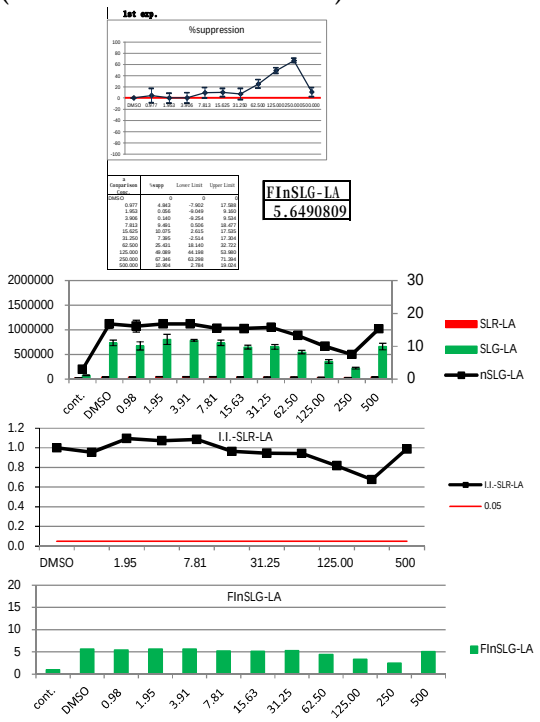


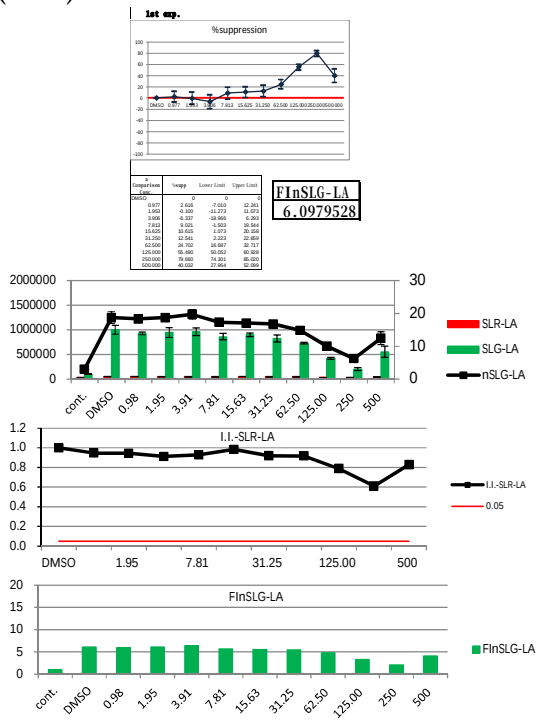
図 8　LPS 添加後のピペッティングの有無による IL-1β転写活性への影響（続き）

Dapsone

(Lead Lab.: Tohoku Univ.)

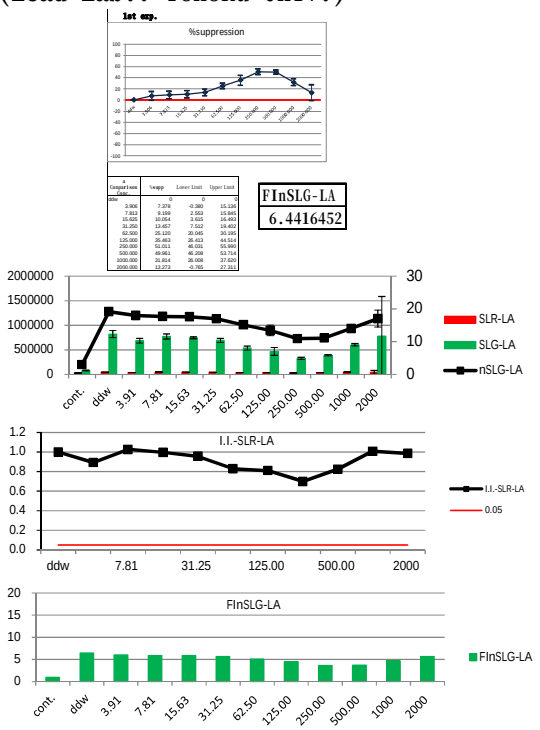


(FDSC)



Diethanolamine

(Lead Lab.: Tohoku Univ.)



(FDSC)

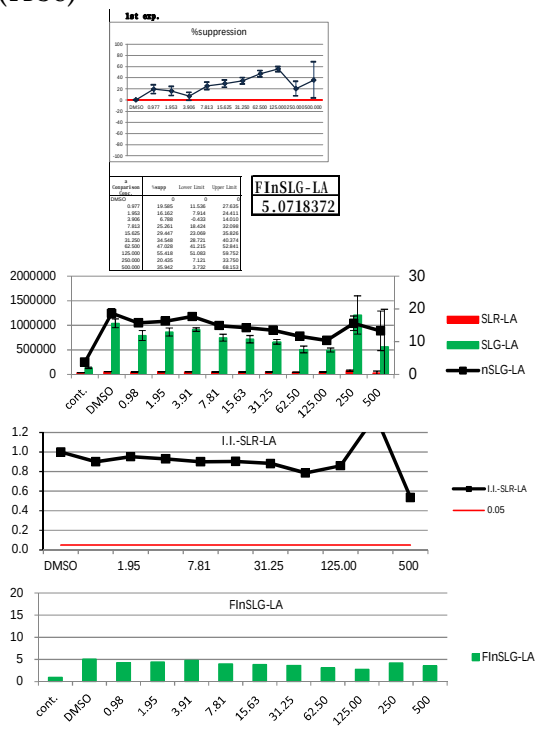
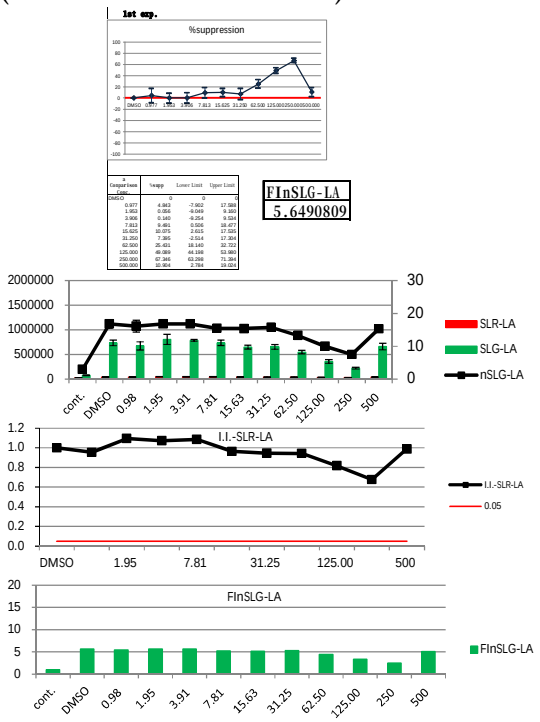


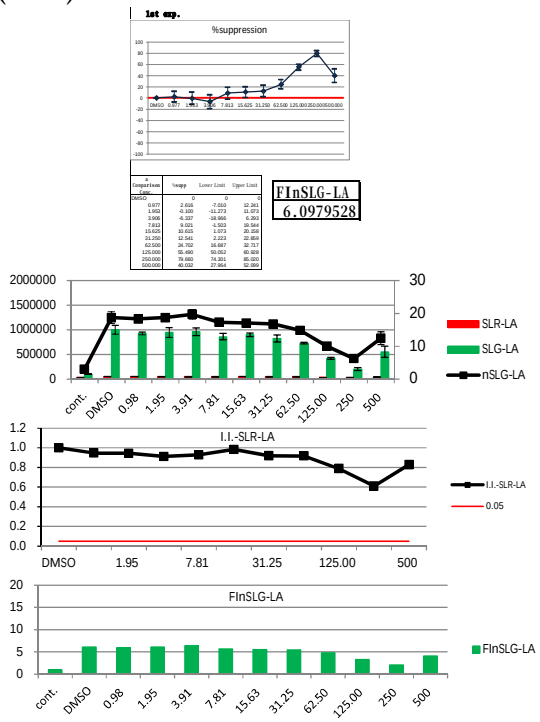
図９　リードラボ（東北大学）による秦野研究所の技術確認試験結果

p-Nitroaniline

(Lead Lab.: Tohoku Univ.)

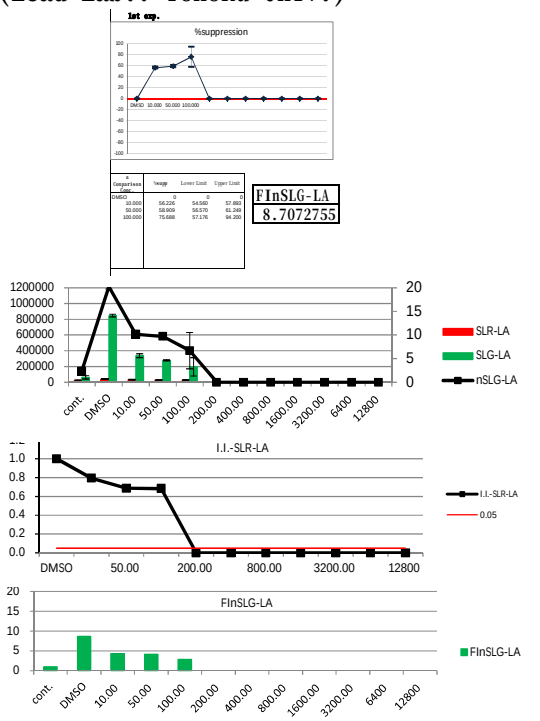


(FDSC)



Dexamethasone (Positive control)

(Lead Lab.: Tohoku Univ.)



(FDSC)

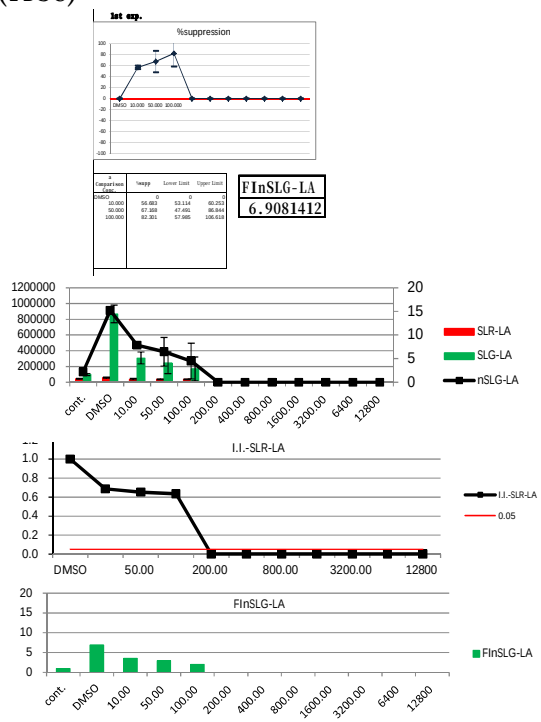
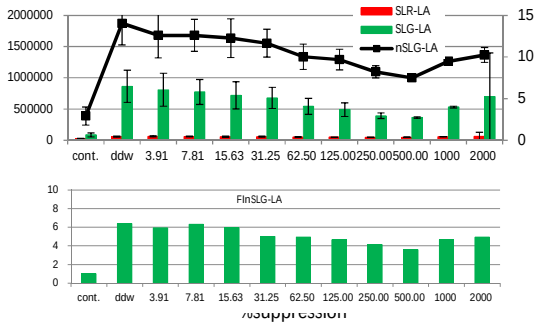
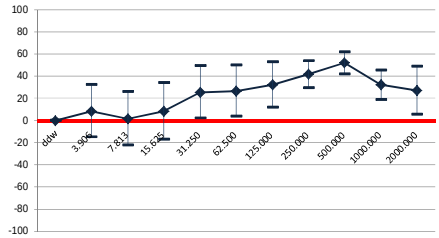


図９　リードラボ（東北大学）による秦野研究所の技術確認試験結果（続き）

Retest

(1st Experiment)

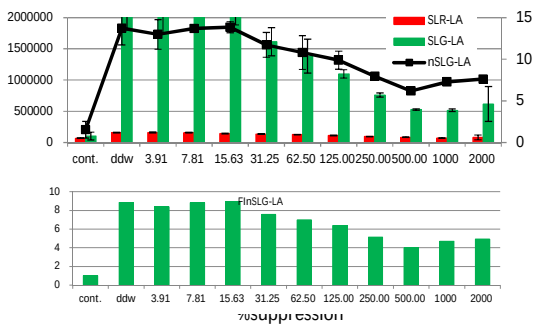


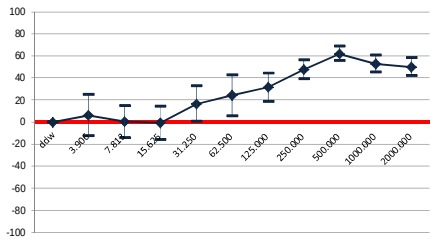


a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	8.537	-15.206	32.279
7.813	1.781	-22.371	25.932
15.625	8.165	-17.412	33.742
31.250	25.596	2.163	49.029
62.500	26.670	3.767	49.573
125.000	32.189	11.559	52.819
250.000	41.640	29.322	53.958
500.000	51.853	41.808	61.897
1000.000	32.038	18.752	45.324
2000.000	27.105	5.553	48.658

FInSLG-LA
6.3935882

(2nd Experiment)

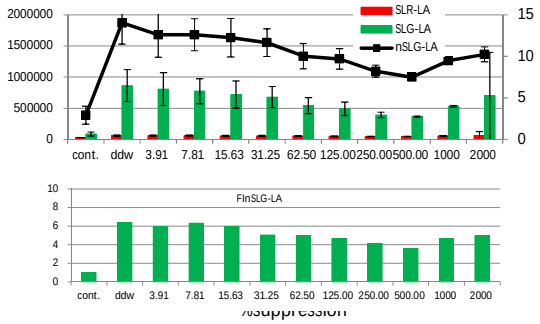


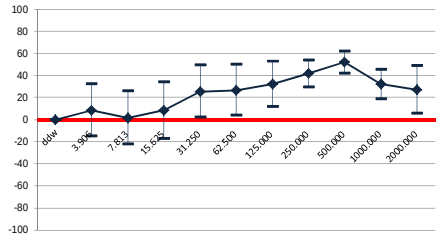


a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	6.011	-12.539	24.560
7.813	0.220	-14.311	14.751
15.625	-1.008	-16.246	14.230
31.250	16.425	0.192	32.657
62.500	24.022	5.707	42.337
125.000	31.406	18.484	44.327
250.000	47.310	38.850	55.771
500.000	61.836	55.284	68.388
1000.000	52.787	45.050	60.524
2000.000	50.069	41.692	58.446

FInSLG-LA
8.8610082

(3rd Experiment)





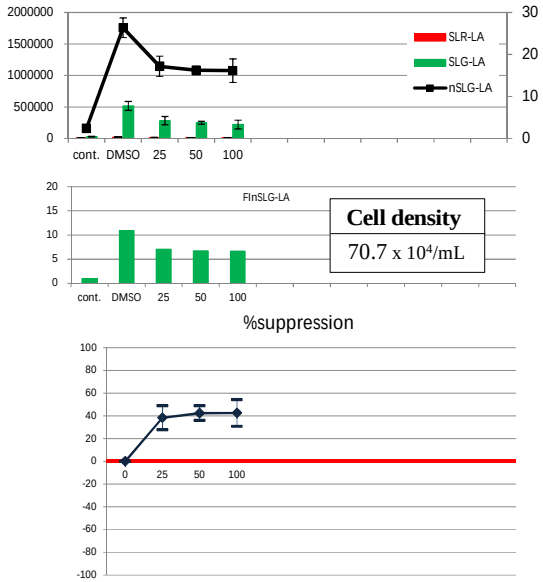
a Comparison Conc.	%supp	Lower Limit	Upper Limit
ddw	0	0	0
3.906	8.537	-15.206	32.279
7.813	1.781	-22.371	25.932
15.625	8.165	-17.412	33.742
31.250	25.596	2.163	49.029
62.500	26.670	3.767	49.573
125.000	32.189	11.559	52.819
250.000	41.640	29.322	53.958
500.000	51.853	41.808	61.897
1000.000	32.038	18.752	45.324
2000.000	27.105	5.553	48.658

FInSLG-LA
6.3935882

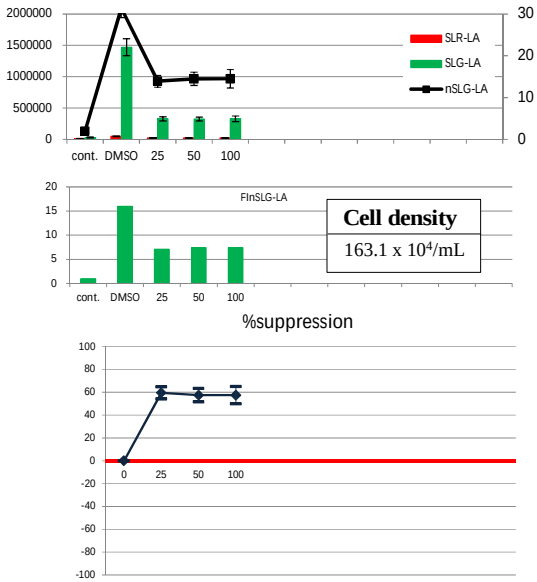
図 10　Diethanolamine の再実験結果

FDSC serum (Heat inactivated)

・Subculture at 2×10^5 cells/mL

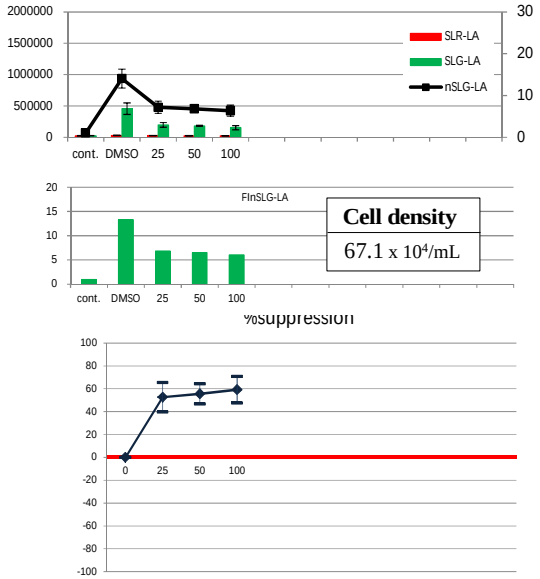


・Subculture at 5×10^5 cells/mL



Tohoku Univ. serum (Heat inactivated)

・Subculture at 2×10^5 cells/mL



・Subculture at 5×10^5 cells/mL

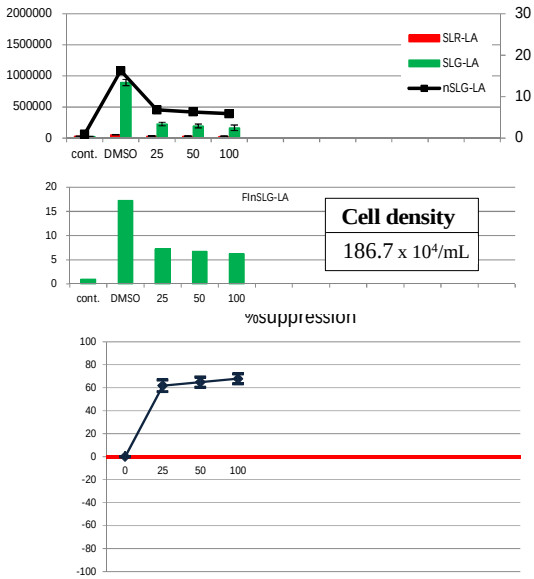
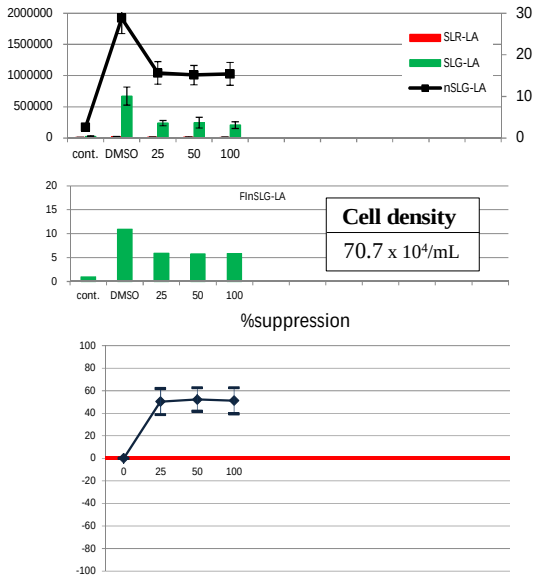


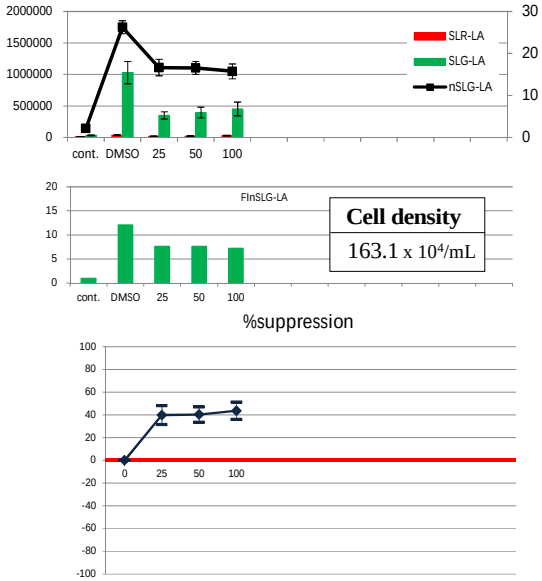
図 11　異なる FCS で継代後 3 日間培養した場合の IL-1β転写活性

3 Days after subculture

・Subculture at 2×10^5 cells/mL

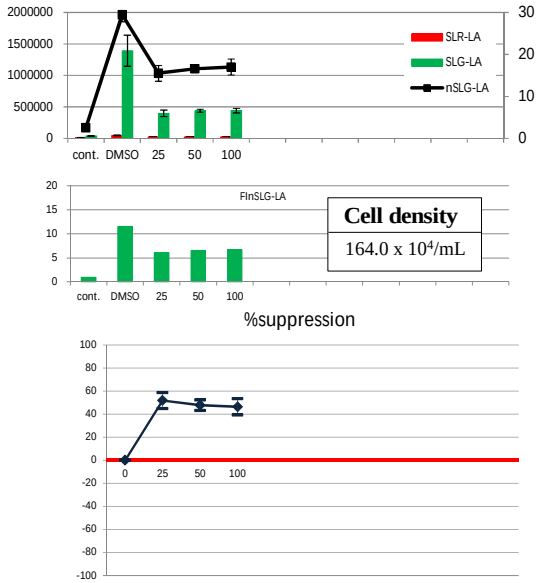


・Subculture at 5×10^5 cells/mL



4 Days after subculture

・Subculture at 2×10^5 cells/mL



・Subculture at 5×10^5 cells/mL

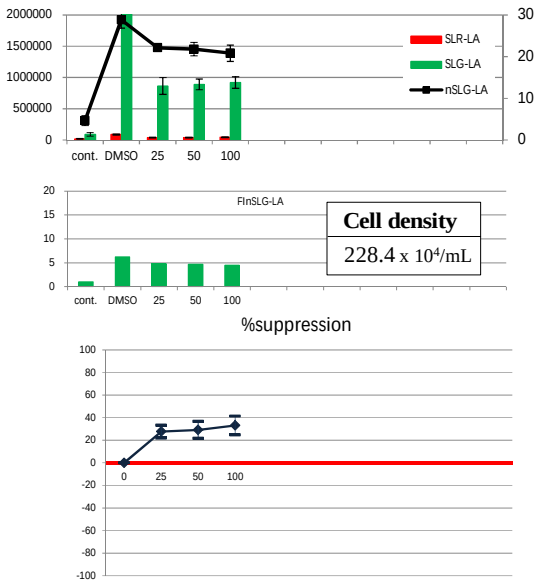
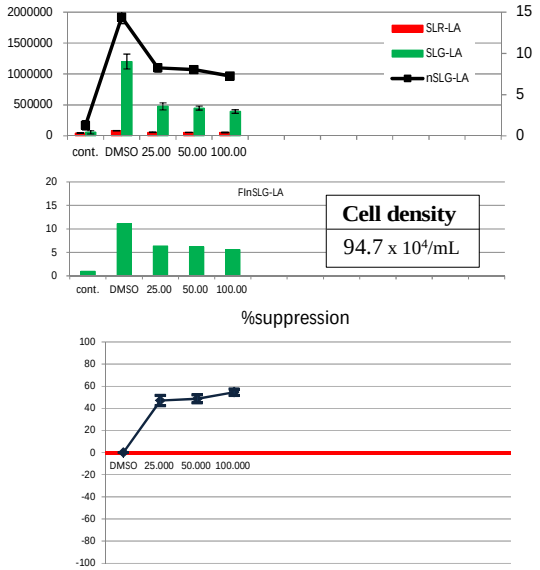


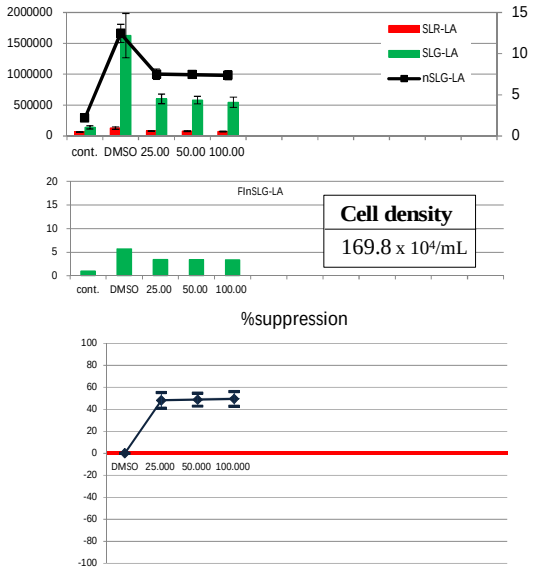
図 12　IL-1β転写活性に対する継代時の細胞密度と継代後の培養日数の影響

3 Days after subculture (Retest on the different day)

・Subculture at 20×10^4 cells/mL

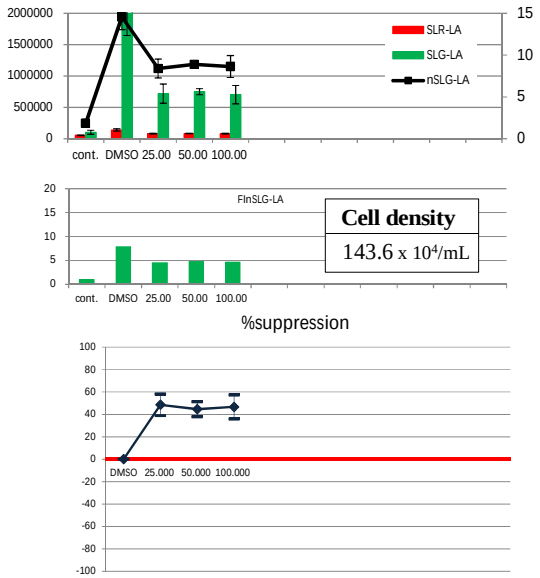


・Subculture at 50×10^4 cells/mL



4 Days after subculture (Retest on the different day)

・Subculture at 20×10^4 cells/mL



・Subculture at 50×10^4 cells/mL

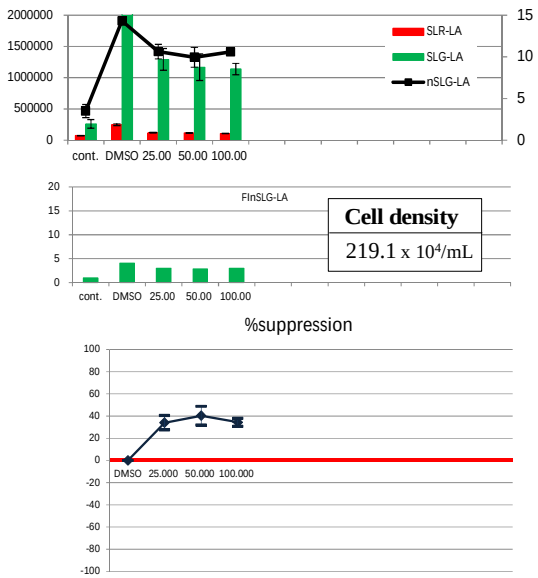


図 13　IL-1β転写活性に対する継代時の細胞密度と継代後の培養日数の影響（再現性）

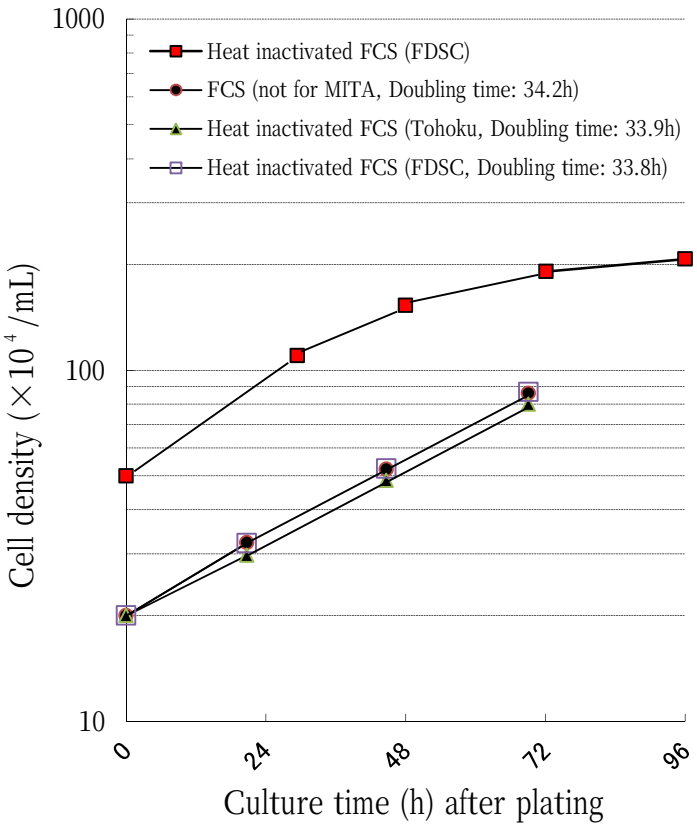


図 14　THP-G1b（TGCHAC-A4）細胞の増殖曲線

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 30 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* 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。

[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-2 Luc アッセイ及び 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、immumoaugmentation、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、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
という意見を出し合い判定方法が決定されてきた。

IL-2 Luc アッセイ及び 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。

本報告では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

<u>IL-2 Luc アッセイ及び IL-1β Luc アッセイのデータの特徴</u>

IL-2 Luc アッセイ及び 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。

<u>各実験から得られる測定値と指標について</u>

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。

<u>IL-2 Luc アッセイ及び IL-1β Luc アッセイの実験ごとの判定</u>

アッセイの最終的な判定は各実験の判定である「Suppression」「Augmentation」「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。

本研究の検討

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

判定アルゴリズム

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 $Cut^{(S)}$、カットオフ値の下限（Augmentation に関係する）を $Cut^{(A)}$ とする。

Step 1

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。

Step 2-1

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 $Lower_i > 0$

Step 2-2

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

$Upper_i^{(-1)} < 0$ かつ $Upper_i < 0$

Step 3-1

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

$Upper_i^{(-2)} > 0$

Step 3-2

以下の条件を満たした場合に新しい変数 $Trend\text{-}A_i$ 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

$\mathrm{Lower}_i^{(-2)} < 0$

Step 4-1

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 4-2

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

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

かつ $\%\mathrm{suppresion}_i^{(-1)} < \mathrm{Cut}^{(A)}\}$

または

$\{\mathrm{Trend\text{-}A}_i = 1$ かつ $\mathrm{Upper}_i < 0$

かつ $\%\mathrm{suppresion}_i < \mathrm{Cut}^{(A)}\}$

Step 5

いずれかの濃度で IndConc-$S_i = 1$ の場合に Suppression と判断する。

いずれかの濃度で IndConc-$A_i = 1$ の場合に Augmentation と判断する。

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 アッセイは、現在バリデーション研究（Phase I）を実施している。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 から 4 に示す。なお、カットオフ値は上限が 20、下限を 20 とした。図中 S が Suppression、A が Augmentation，N が No effect を示す。

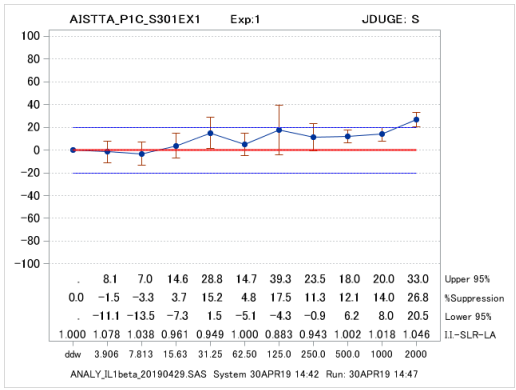


図 1　Suppression と判定される例

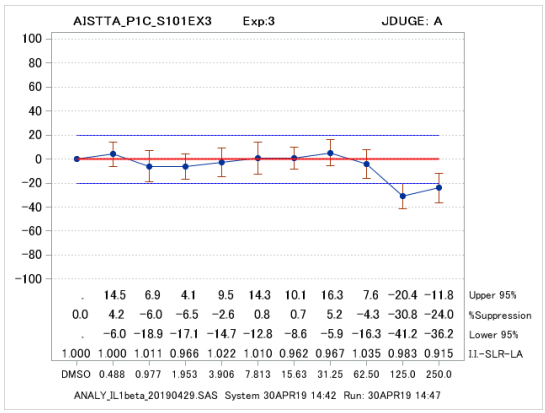


図 2　Augmentation と判定される例

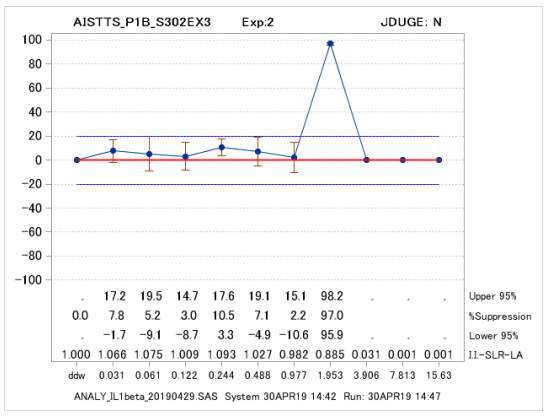


図 3　No effect と判定される例

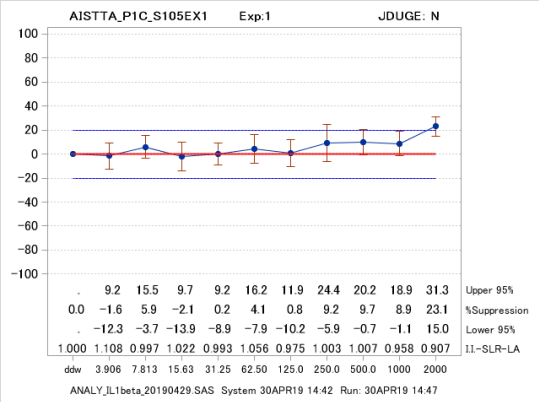


図 4　No effect と判定される例

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 アッセイは現在バリデーションが実施されている。これまで、これらの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きた。判定アルゴリズムの開発における重要な点は、このアルゴリズムをデータシートに含めることができることである。これまでデータシートには、%suppression の濃度反応曲線のグラフが出力されるようにはなっていたので、判定の基準を照らし合わせることで実験を行った者も判定することができたが、自分たちが基準に照らし合わせた判定が適切であったかどうか不安だという声も上がっていた。また、バリデーション研究において、各被験物質について実施されたアッセイの最終的な判定を下す際も、複数人で確認を行うことをしていた。

今後、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

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

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

今年度は、分担者としてIL-1β転写活性抑制評価試験(IL-1 luciferase reporter assay; IL-1 Luc assay)のクライテリアの設定ならびにプロトコールの作成を行った。昨年度までに国際バリデーションが終了したIL-2 転写活性抑制評価試験(IL-2 Luciferase reporter assay; IL-2 Luc assay)のプロトコールを参照とし、使用するIL-1βレポーター細胞であるTHP-G1b細胞の培養条件設定（選択抗生剤を使用しない等)、刺激するLPSの選定および濃度設定（E. coli K12, 100 ng/mL)、使用する96ウェルプレートの選定を行った（black & whiteプレートを使用)。過去の施設間試験で使用した化学物質のデータを参考としPhase 0試験（2018年8月～9月）用の３つの化学物質を選定した。当教室で作成した60化学物質でのデータベースの結果およびPhase 0試験での各施設のデータをもとに｜%suppression｜>=20%を陽性とするクライテリアを設定しPhase Ⅰ用のプロトコールを作成した。神戸大学の協力を得てバリデーション試験において使用するデータシート(Appendix 1)を作成、その他記録用紙(Appendix 2)を作成しPhase Ⅰ試験（2018年12月～2019年3月）を行った。Phase Ⅰの中途で、化学物質の毒性が強い場合判定できないという問題が生じたため、その対応を盛り込んだプロトコールに改訂した。(Multi-Immuno Tox Assay protocol for THP-G1b ver.008.1E:最新プロトコール、総括Appendix 2)

A．研究目的

IL-1 Luc assayの国際バリデーションの実施に向け、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
I． アッセイ方法の検討
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THP-1細胞をベースとしたIL-1βレポーター細胞であるTHP-G1b(TGCHAC-A4)細胞を樹立した。本研究でIL-1β転写活性抑制評価試験(IL-1 luciferase reporter assay; IL-1 Luc assay)に使用するにあたり以下の条件設定を行った。

① LPS、96ウェルプレートの選定
従来のLPS(E.coli 026:B6)ではSLR-LAに比べSLG-LAの値が極端に高くなり結果が不安定になるという問題があったため刺激するLPSについて再検討を行った。入手可能なLPS４種について反応性を測定したところE. coli K12でE.coli 026:B6の３分の１の反応性を示すことが示された。また従来96ウェルプレートについてはblackプレートを使用していたがウェルの内側を白色、ウェル間を含めた他の部分が黒色のblack & whiteプレートの使用を検討したところシグナルの増強が認められ安定した結果が得られた。
以上の結果からLPSはE. coli K12へ、96ウェルプレートはblack & whiteプレートへ変更した。

② 培養条件の検討
THP-G1b(TGCHAC-A4)細胞の培養の際に選択抗生剤としてブラストサイジンを使用していたがブラストサイジンにより細胞のLPSへの反応性が低下する可能性が考えられたためブラストサイジンが入っていない培地を使用することとした。

II．クライテリアの設定
① 当教室で作成した60化学物質でのデータベースの結果およびPhase 0試験での各施設のデータをもとに｜%suppression｜>=20%を陽性とするクライテリアを設定しPhase I用のプロトコールを作成した。
② Phase Iの中途で、化学物質の毒性が強い場合に有効なデータが得られず判定ができないという問題が生じたため、有効なデータが６濃度未満でかつ陰性と判定されるときそのアッセイは棄却されるクライテリアに変更した。

以上I、IIを反映したプロトコールを作成した。(Multi-Immuno Tox Assay protocol for THP-G1b ver.008.1E:最新プロトコール、総括Appendix 2)

III.データシートの作成
95%信頼区間の算出法について神戸大学の協力を得てPhase I用のデータシートを作成した。(Appendix 1)

IV．Phase I用の記録用紙を作成した。(Appendix 2)

D．考察
培養条件、LPS、96ウェルプレート、クライテリアの変更によりPhase 0における技術移転性が確認された。

E．結論
IL-2 Luc assayのプロトコールを参照とし、THP-G1b(TGCHAC-A4)細胞の培養条件、LPS、96ウェルプレート、クライテリアを変更しプロトコールを作成した。

F．健康危険情報
なし

G．研究発表
1．論文発表
1) Kimura, Y., Fujimura, C., Ito, Y., Takahashi, T., Terui, H., Aiba, S., 2018. Profiling the immunotoxicity of chemicals based on in vitro evaluation by a combination of the Multi-ImmunoTox

assay and the IL-8 Luc assay. Arch Toxicol 92, 2043-2054.
2) Kimura, Y., Watanabe, M., Suzuki, N., Iwaki, T., Yamakage, K., Saito, K.,Nakajima, Y., Fujimura, C., Ohmiya, Y., Omori, T., Kojima, H., Aiba, S., 2018. The performance of an in vitro skin sensitisation test, IL-8 Luc assay (OECD442E), and the integrated approach with direct peptide reactive assay (DPRA). J Toxicol Sci 43, 741-749.

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、小林 眞弓、大森 崇、足利 太可雄、小島 肇、相場 節也：**Multi-ImmunoTox Assay (MITA) **：バリデーション研究の結果 日本動物実験代替法学会 第31回大会（熊本）2018年11月**

H．知的財産権の出願・登録状況
（予定を含む。）
なし

Appendix 1：Data sheet for MITA THP-G1b (TGCHAC-A4)　Ver. 008 20181203

① フェイスシート

Multi-ImmunoTox Assay Datasheet for THP-G1b (TGCHAC-A4) cells

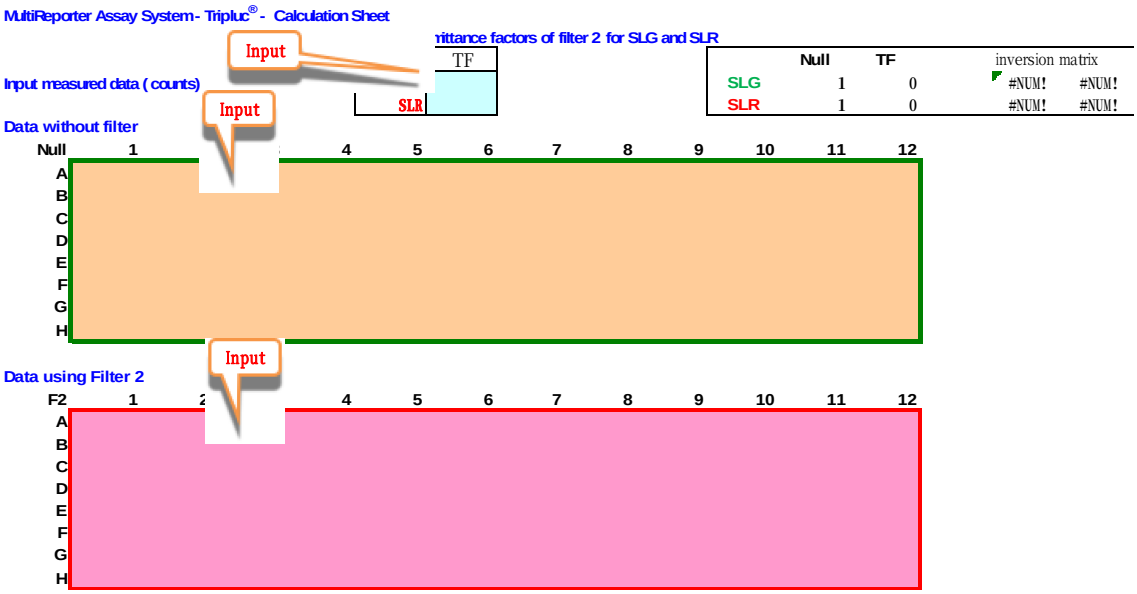
Ver. 008

Laboratory		Round	
Exp.	1st exp.	(Highest soluble conc. In the next exp.s	mg/ml)
Date: (YYYY/MM/DD)		Operator:	
Code		Dissolution	mg/ml in
FInSLG-LA	#NUM!	#NUM!	

Comment:

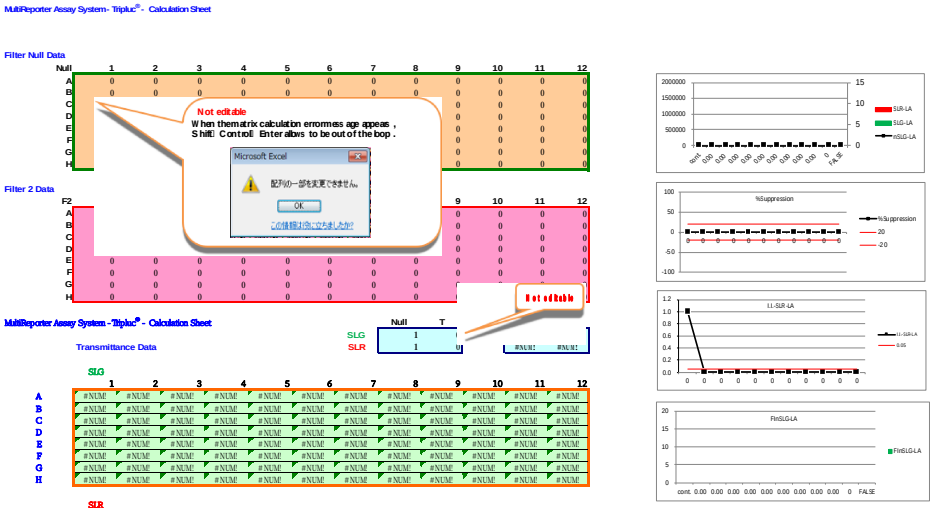
Appendix 1：Data sheet for MITA THP-G1b (TGCHAC-A4)　Ver. 008 20181203

② data 入力シート



Appendix 1：Data sheet for MITA THP-G1b (TGCHAC-A4)　Ver. 008 20181203

③ ResultFormat シート

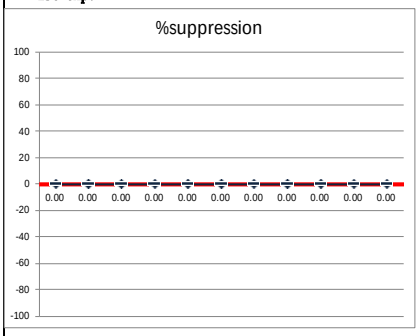


Appendix 1：Data sheet for MITA THP-G1b (TGCHAC-A4)　Ver. 008 20181203

④ %suppression グラフシート

Results

1st exp.



a Comparison Conc.	%supp	Lower Limit	Upper Limit
0	0	0	0
#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!

Appendix 2：MITA 記録用紙 Ver. 005J 20181203

① 試薬管理シート

実験名　MITA バリデーション研究

被試験試薬コード

被試験試薬管理

受領日　　年　月　日　　受領者氏名

保管場所　　温度（℃）　　℃

備考

受領量（容器込）　　g

月 日	使用量(g)	残存量(g)	実験担当者名	備考	Exp. No.	溶解性検討
H. / /						
/						
/						
/						
/						
/						
/						
/						
/						
/						
/						
/						

Appendix 2：MITA 記録用紙 Ver. 005J 20181203
② 試験者シート

実験名　MITA バリデーション研究

実験日

施設名

実験責任者名

実験担当者名

実験担当者名

実験担当者名

実験担当者名

試験物質コード

	回目
	回目
	回目
	回目
	回目
	回目
	回目
	回目
	回目
	回目

Appendix 2：MITA 記録用紙 Ver. 005J 20181203

③ 細胞継代シート

3-1 THP-G1b (TGCHAC-A4) 培養方法

3-1-1 細胞蘇生(P1)

□ あらかじめ、THP-G1b (TGCHAC-A4)用A培地15 mLを37° C恒温槽で温めておく(培養用)。

□ 凍結細胞を37° C恒温槽で融解し、THP-G1b (TGCHAC-A4)用A培地9 mLを入れておいた15 mLの遠沈管に加える（細胞液0.5 mL+C培地 9 mL=計9.5 mL)

□ 遠心して細胞を集める(120-350 x *g*, 5分程度)。

□ 上清を吸引除去し、先に温めておいたTHP-G1b (TGCHAC-A4)用A培地15 mLに細胞を懸濁してT-75 Flaskで培養を開始する(37℃, 5%CO_2)。

□上記より一部細胞浮遊液を採取し、培養開始時の細胞生存率を計測する。

(計算)

生細胞数：

死細胞数：

実施日：　　　年　月　日、実施者：

3-1-2 通常の継代培養(P2以降)

P-

□ あらかじめ、THP-G1b (TGCHAC-A4)用A培地必要量を37° C恒温槽で温めておく。

□ 細胞数を計測する。

継代細胞濃度は2~5 × 10^5/mL、継代間隔は3~4日程度で行う。

(　　＋　　)/　　×　　=　　× 10^4/mLーA液

□ 必要細胞量を取り、遠心して細胞を集める(120-350 x g, 5分程度)。上清を吸引除去し、先に温めておいたA培地15mLに2~5 × 10^5/mLで細胞を懸濁してT-75 Flaskで培養する。

※ 継代濃度が同一であれば、容量の変更や維持本数変更は構わない。

A液の採取量：　　　mL

実施日：　　　年　月　日、実施者：

P-

□ あらかじめ、THP-G1b (TGCHAC-A4)用A培地必要量を37° C恒温槽で温めておく。

□ 細胞数を計測する。

継代細胞濃度は2~5 × 10^5/mL、継代間隔は3~4日程度で行う。

(　　＋　　)/　　×　　=　　× 10^4/mLーA液

□ 必要細胞量を取り、遠心して細胞を集める(120-350 x g, 5分程度)。上清を吸引除去し、先に温めておいたA培地15mLに2~5 × 10^5/mLで細胞を懸濁してT-75 Flaskで培養する。

※ 継代濃度が同一であれば、容量の変更や維持本数変更は構わない。

A液の採取量：　　　mL

実施日：　　　年　月　日、実施者：

P-

□ あらかじめ、THP-G1b (TGCHAC-A4)用A培地必要量を37° C恒温槽で温めておく。

□ 細胞数を計測する。

継代細胞濃度は2~5 × 10^5/mL、継代間隔は3~4日程度で行う。

(　　＋　　)/　　×　　=　　× 10^4/mLーA液

□ 必要細胞量を取り、遠心して細胞を集める(120-350 x g, 5分程度)。上清を吸引除去し、先に温めておいたA培地15mLに2~5 × 10^5/mLで細胞を懸濁してT-75 Flaskで培養する。

※ 継代濃度が同一であれば、容量の変更や維持本数変更は構わない。

A液の採取量：　　　mL

実施日：　　　年　月　日、実施者：

P-

□ あらかじめ、THP-G1b (TGCHAC-A4)用A培地必要量を37° C恒温槽で温めておく。

□ 細胞数を計測する。

継代細胞濃度は2~5 × 10^5/mL、継代間隔は3~4日程度で行う。

(　　＋　　)/　　×　　=　　× 10^4/mLーA液

□ 必要細胞量を取り、遠心して細胞を集める(120-350 x g, 5分程度)。上清を吸引除去し、先に温めておいたA培地15mLに2~5 × 10^5/mLで細胞を懸濁してT-75 Flaskで培養する。

※ 継代濃度が同一であれば、容量の変更や維持本数変更は構わない。

A液の採取量：　　　mL

実施日：　　　年　月　日、実施者：

Appendix 2：MITA 記録用紙 Ver. 005J 20181203
④ 細胞調製シート

実験名 MITA バリデーション研究

実験日 _____

施設名 _____

細胞調製

室温 ____℃

予定プレート数 ____枚 x $10x10^6$ cells/枚 x1.5=____cells（必要細胞数）

細胞調製（試験物質用）

細胞蘇生年月日 ____年____月____日

前回継代年月日 ____年____月____日

前回継代時
細胞濃度・培養液量 ____cells/mL X ____mL

実験当日細胞濃度 ____cells/mL －①

遠心した細胞数 ____cells[*1] ①を____mLを採取

再懸濁した培地量 ____mL（[*1]の細胞数÷($2x10^6$)）

それぞれのプレートに50 ㎕ /wellで分注 □ （ : ）

細胞調製（コントロール(dexamethasone)用）

上で調製した細胞を別のプレートの#A1-#D5に50 ㎕/wellで分注 □ （ : ）

Appendix 2：MITA 記録用紙 Ver. 005J 20181203
⑤ 被試験試薬の調製①シート

実験名　MITA バリデーション研究

実験日　＿＿＿＿＿＿＿＿＿＿＿＿

施設名　＿＿＿＿＿＿＿＿＿＿＿＿

被試験試薬コード　＿＿＿＿　＿＿＿＿回目

被試験試薬の調製①（溶媒への溶解）

25mg/mL水溶液で	□完全に溶解せず	□完全に溶解 （□遠心でペレットを生じない）	
		↓	
50mg/mL水溶液で		□完全に溶解せず □完全に溶解 （□遠心でペレットを生じない）	→25mg/mL水溶液を調製
		↓	
100mg/mL水溶液で		□完全に溶解せず □完全に溶解 （□遠心でペレットを生じない）	→50mg/mL水溶液を調製 →100mg/mL水溶液として調製を継続
500mg/mL DMSO溶液で	□完全に溶解 （□遠心でペレットを生じない） □完全に溶解せず	→500mg/mL DMSO溶液として調製を継続	
	↓		
250mg/mL DMSO溶液で	□完全に溶解 （□遠心でペレットを生じない） □完全に溶解せず	→250mg/mL DMSO溶液として調製を継続	
	↓		
125mg/mL DMSO溶液で	□完全に溶解 （□遠心でペレットを生じない） □完全に溶解せず	→125mg/mL DMSO溶液として調製を継続	
	↓		
62.5mg/mL DMSO溶液で	□完全に溶解 （□遠心でペレットを生じない） □完全に溶解せず	→62.5mg/mL DMSO溶液として調製を継続	
	↓		
31.25mg/mL DMSO溶液で	□完全に溶解 （□遠心でペレットを生じない） □完全に溶解せず	→31.25mg/mL DMSO溶液として調製を継続	
	↓		

Appendix 2：MITA 記録用紙 Ver. 005J 20181203

⑥ 被試験試薬の調製②(ddw)シート

実験名 MITA バリデーション研究

実験日 _____

施設名 _____

被試験試薬コード _____ 1 2 3 4 5 回目

Distilled water溶液に調製された場合

1st experimentまたは2nd experimentより決定された調整濃度
(最終濃度が、I.I.-SLR-LA≦0.05となる最も低い濃度の1段階高い(2倍の)濃度になるように設定、
その50倍の濃度のDistilled water溶液を調整する。データシートのface sheetに算出されます) _____mg/mL

試験液の調製と細胞への処理

被試験試薬_____mgをDistilled waterに溶解し_____mLとする。 →_____mg/mL
さらにDistilled waterで_____倍希釈する→_____mg/ml

調製時間
(:)

96 well clear plate(丸底)に下図のようにDistilled water、被試験試薬Distilled water溶液を分注する。

丸底・透明	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	被試験試薬 水溶液 100uL
B												
C												
D												
E												
F												
G												
H												

□

well#A11から#A3までDistilled waterで公比2で段階希釈を9段階おこなう。

添加時間

アッセイブロックにB培地480 μLを分注し、上図の希釈液を20 μL添加して25倍希釈し、これを50 μL/wellずつ細胞に添加する。 □ (:)

プレートをシールし、プレートシェーカーを使用し、撹拌して混合する。 □

細胞をインキュベーターへ入れ、1時間反応させる。 □

賦活剤(LPS)の調製と細胞への処理

1 mg/mL LPSストックをDistilled waterで200倍希釈し5 μg/mL溶液を作製する。(1 mg/mL LPSストック 5 μL + Distilled water 995 μL) □

5 μg/mL LPS溶液をDistilled waterで5倍希釈し1000 ng/mL LPS溶液を作製する。(5 μg/mL LPS溶液 250 μL + Distilled water 1000 μL) □

添加時間

Distilled waterを#C1-#F1、1000 ng/mL LPS溶液を#C2-#F12に10 μLずつ分注する。 □ (:)

プレートをシールし、プレートシェーカーを使用し、撹拌して混合する。

細胞をインキュベーターへ入れ、6時間反応させる。

測定(被試験物質)

Tripluc® Luciferase assay reagentを溶解させる。 □

光電子増倍管を安定させるため、ルミノメータは測定開始30 分前には電源を入れる。 □

添加時間

リザーバーにTriplucを移し、8チャンネルもしくは12チャンネルピペットマンを使用して、
反応終了後のアッセイプレートに100 μL/wellずつ分注する。 □ (:)

Tripluc添加後、プレートシェーカーを使用して室温(23-27 ℃)で10分間(30分間まで可)撹拌し、
細胞を溶解させる。 □ 撹拌中温度

(℃)

ルミノメータでLuciferase活性を測定する。(測定中温度が26-31℃であることを確認する。)
フィルタ無し、フィルタ有りで各々3 秒/well測定する(アトー社製Phelosの場合はF0、F2を使用)。 □ 測定時間

(:)

測定中温度
(℃)

Appendix 2：MITA 記録用紙 Ver. 005J 20181203

⑦ 被試験試薬の調製③(DMSO)シート

実験名 MITA バリデーション研究

実験日 _____

施設名 _____

被試験試薬コード _____ 1 2 3 4 5 回目

DMSO溶液に調製された場合

1st experimentまたは2nd experimentより決定された調整濃度
(最終濃度が、LL-SLR-LA≦0.05となる最も低い濃度の1段階高い(2倍の)濃度になるように設定、
その1000倍の濃度のDMSO溶液を調整する。データシートのface sheetに算出されます) _____mg/mL

試験液の調製と細胞への処理

被試験試薬_____mgをDMSOに溶解し_____mLとする。 → _____mg/mL 調製時間
さらにDMSOで_____倍希釈する→_____mg/ml (:)

96 well clear plate(丸底)に下図のようにDMSO、B培地、被試験試薬DMSO溶液を分注する。

丸底・透明	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 50uL	DMSO 50uL	DMSO 50uL	DMSO 50uL	DMSO 50uL	DMSO 50uL	DMSO 50uL	DMSO 50uL	DMSO 50uL	DMSO 50uL	DMSO 50uL	被試験試薬 DMSO溶液 100uL
B	B培地 90uL	B培地 90uL	B培地 90uL	B培地 90uL	B培地 90uL	B培地 90uL	B培地 90uL	B培地 90uL	B培地 90uL	B培地 90uL	B培地 90uL	B培地 90uL
C												
D												
E												
F												
G												
H												

☐

well#A11から#A3までDMSOで公比2で段階希釈を9段階おこなう。 ☐

段階希釈した被試験試薬DMSO溶液 10 μLを8チャンネルもしくは12チャンネルピペットマンを使用して下のB培地90 μLにうつし10倍に希釈する。 ☐

希釈した段階での沈殿の有無、性状

#B1	#B2	#B3	#B4	#B5	#B6	#B7	#B8	#B9	#B10	#B11	#B12
有□無□	有□無□	有□無□	有□無□	有□無□	有□無□	有□無□	有□無□	有□無□	有□無□	有□無□	有□無□

沈殿の性状 (例:粉状、泥状、膜状、ミセル様)

アッセイブロックにB培地490 μLを分注し、上図の希釈液を10 μL添加して50倍希釈し、これを50 μL/wellずつ細胞に添加する。 ☐ 添加時間 (:)

プレートをシールし、プレートシェーカーを使用し、撹拌して混合する。 ☐

細胞をインキュベーターへ入れ、1時間反応させる。 ☐

賦活剤(LPS)の調製と細胞への処理

1 mg/mL LPSストックをDistilled waterで200倍希釈し5 μg/mL溶液を作製する。(1 mg/mL LPSストック 5 μL + Distilled water 995 μL) ☐

5 μg/mL LPS溶液をDistilled waterで5倍希釈し1000 ng/mL LPS溶液を作製する。(5 μg/mL LPS溶液 250 μL + Distilled water 1000 μL) ☐

Distilled waterを#C1-#F1、1000 ng/mL LPS溶液を#C2-#F12に10 μLずつ分注する。 ☐ 添加時間 (:)

プレートをシールし、プレートシェーカーを使用し、撹拌して混合する。

細胞をインキュベーターへ入れ、6時間反応させる。

測定(被試験物質)

Tripluc® Luciferase assay reagentを溶解させる。 ☐

光電子増倍管を安定させるため、ルミノメータは測定開始30 分前には電源を入れる。 ☐

リザーバーにTriplucを移し、8チャンネルもしくは12チャンネルピペットマンを使用して、
反応終了後のアッセイプレートに100 μL/wellずつ分注する。 ☐ 添加時間 (:)

Tripluc添加後、プレートシェーカーを使用して室温(23-27 ℃)で10分間(30分間まで可)撹拌し、
細胞を溶解させる。 ☐ 撹拌中温度 (℃)

ルミノメータでLuciferase活性を測定する。(測定中温度が26-31℃であることを確認する。)
フィルタ無し、フィルタ有りで各々3 秒/well測定する(アトー社製Pheliosの場合はF0、F2を使用)。 ☐ 測定時間 (:)
測定中温度 (℃)

Appendix 2：MITA 記録用紙 Ver. 005J 20181203
⑧ 被試験試薬の調製④(コントロール)シート

実験名 MITA バリデーション研究

実験日 _____

施設名 _____

被試験試薬コード _____ _____回目

コントロールの調製と細胞への処理

dexamethasone (DEX)の調製
96 well clear plate(丸底)に下図のようにDMSO 50 μL (#A1, #A2)、DEX 10 mg/mL DMSO溶液 50 μL (#A3)、DEX 50 mg/mL DMSO溶液 50 mL (#A4)、DEX 100 mg/mL DMSO溶液 50 mL (#A5)、B培地 90 μl (#B1-5)を分注する。 ☐ 添加時間 (:)

丸底・透明	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 50 uL	DMSO 50 uL	DEX 10 mg/mL DMSO溶液 50 uL	DEX 50 mg/mL DMSO溶液 50 uL	DEX 100 mg/mL DMSO溶液 50 uL							
B	B培地 90 uL	B培地 90 uL	B培地 90 uL	B培地 90 uL	B培地 90 uL							
C												
D												
E												
F												
G												
H												

#A1-2のDMSOと#A3-5のDEX DMSO溶液 10 μLを下のB培地90 μLにうつし10倍に希釈する。 ☐

アッセイブロックの#A1-5にB培地490 μLを分注し、上図の希釈液を10 μL添加して混合し、50 μL/wellずつ細胞に添加する。 ☐ 添加時間 (:)

プレートをシールし、プレートシェーカーを使用し、撹拌して混合する。 ☐

細胞をインキュベーターへ入れ、1時間反応させる。 ☐

賦活剤(LPS)の調製と細胞への処理

1 mg/mL LPSストックをDistilled waterで200倍希釈し5 μg/mL溶液を作製する。(1 mg/mL LPSストック 5 μL + Distilled water 995 μL) ☐

5 μg/mL LPS溶液をDistilled waterで5倍希釈し1000 ng/mL LPS溶液を作製する。(5 μg/mL LPS溶液 250 μL + Distilled water 1000 μL) ☐

Distilled waterを#A1-#D1、1000 ng/mL LPS溶液を#A2-#D5に10 μLずつ分注する。 ☐ 添加時間 (:)

プレートをシールし、プレートシェーカーを使用し、撹拌して混合する。

細胞をインキュベーターへ入れ、6時間反応させる。

測定(被試験物質)

Tripluc® Luciferase assay reagentを溶解させる。 ☐

光電子増倍管を安定させるため、ルミノメータは測定開始30 分前には電源を入れる。 ☐

リザーバーにTriplucを移し、8チャンネルもしくは12チャンネルピペットマンを使用して、
反応終了後のアッセイプレートに100 μL/wellずつ分注する。 ☐ 添加時間 (:)

Tripluc添加後、プレートシェーカーを使用して室温(23-27 ℃)で10分間(30分間まで可)撹拌し、
細胞を溶解させる。 ☐ 撹拌中温度 (℃)

ルミノメータでLuciferase活性を測定する。(測定中温度が26-31℃であることを確認する。)
フィルタ無し、フィルタ有りで各々3 秒/well測定する(アトー社製Pheliosの場合はF0、F2を使用)。 ☐ 測定時間 (:)
測定中温度 (℃)

Appendix 1. IL-2 Luc assay validation report draft

January, 2019

Report on the international validation study of the IL-2 Luc assay for evaluating the immunotoxic potential of chemicals

Validation Management Team*****

1. Summary

Tohoku University developed a high-throughput screening system to evaluate chemical immunotoxicity. We first established 3 stable lines of reporter cells transfected with luciferase genes under control of the IL-2, IFN-γ, IL-8, and IL-1β promoters: 2H4 derived from Jurkat cells containing stable luciferase green (SLG) regulated by the IL-2 promoter, stable luciferase orange (SLO) regulated by the IFN-γ promoter, and stable luciferase red (SLR) regulated by the G3PDH promoter; THP-G8 cells derived from THP-1 cells containing SLO regulated by the IL-8 promoter and SLR regulated by the G3PDH promoter; and THP-G1b cells derived from THP-1 cells containing SLG regulated by the IL-1β promoter and SLR regulated by the G3PDH promoter. We selected these 4 cytokines because IL-2 and IFN-γ are primarily produced by T cells (a type of adaptive immune cell), whereas IL-8 and IL-1β are primarily produced by monocytes and dendritic cells (types of innate immune cells).

Using these 3 cell lines, we established the Multi-ImmunoTox assay (MITA) in which the effects of chemicals on the IL-2 and IFN-γ luciferase activity of 2H4 cells are evaluated in the presence of the stimulants phorbol 12-myristate 13-acetate (PMA) and ionomycin (Io). The effects of chemicals on the IL-1β and IL-8 luciferase activity of THP-G1b and THP-G8 cells, respectively, were examined in the presence of the stimulant lipopolysaccharide (LPS).

To date, we have demonstrated the following:

1) The luciferase activities of the 3 MITA cell lines correspond with mRNA expression in the mother cell lines or in human whole blood cells when stimulated with PMA/Io or LPS in the presence of the 3 representative immunosuppressive drugs dexamethasone (Dex), cyclosporine A (CyA), and tacrolimus (Tac).
2) The MITA indicates that Dex significantly suppresses IL-2, IL-1β, and IL-8 reporter activities, while CyA and Tac suppress IL-2 and IFN-γ reporter activities but have no effect on IL-1β and IL-8 reporter activities. On the other hand, the MITA cannot detect the immunosuppressive effects of an alkylating agent (cyclophosphamide), inhibitors of de novo purine synthesis (azathioprine (AZ), mycophenolic acid (MPA) and mizoribine (MZR)), and an inhibitor of pyrimidine and purine synthesis (methotrexate (MT)).

3) Since sensitization is a major form of toxicity that must be investigated in the immunotoxicity screening of chemicals, we combined the MITA with an *in vitro* sensitization test, the IL-8 Luc assay, recently approved as an OECD test guideline for *in vitro* skin sensitization testing (OECD TG442E) (modified MITA; mMITA). The lead laboratory established a data set of 60 chemicals evaluated by the mMITA. Using this data set, we demonstrated a significant correlation between Lowest Observed Effect Levels (LOELs) or the effect on IL-2 luciferase activity and for that on IFN luciferase activity, and between LOELs for the effect on IL-1β luciferase activity and for that on IL-8 luciferase activity. These results indicated that evaluation of the effects of chemicals on IL-2 luciferase activity and on IL-8 luciferase activity can provide immunotoxicological information almost equivalent to evaluations conducted using IL-2, IFN-γ, IL-1β, and IL-8 luciferase activities. In addition, K-means clustering and hierarchical clustering of the 60 chemicals using the mMITA resulted in the same 6-cluster solution: cluster 1 with preferential suppression of IL-8, cluster 2 with suppression of IL-2 and a positive IL-8 Luc assay result, cluster 3 with suppression of both IL-2 and IL-8, cluster 4 with no effects on IL-2 or IL-8 and a negative IL-8 Luc assay result, cluster 5 with suppression of both IL-2 and IL-8 and a negative IL-8 Luc assay result, and cluster 6 with preferential suppression of IL-8. These data suggest that the mMITA is a promising novel high-throughput approach for detecting unrecognized immunological effects of chemicals and for profiling their immunotoxic effects.

Our final long term goal is to officially validate the MITA for within- and between- laboratory reproducibility and predictivity. Since the IL-8 Luc assay has already been accepted as an OECD test guideline (442E), the purpose of the current effort was to conduct the validation of the IL-2 luciferase reporter assay (IL-2 Luc assay).

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

In the Phase I study, a total of 5 coded chemicals (4 T cell targeting and 1 non-T cell targeting) were evaluated by 3 experimental sets based on MITA protocol Ver. 008.5E. The average within-laboratory reproducibility was 86.7% (13/15). The between-laboratory reproducibility was 80.0% (4/5). The average predictivity was 93.3% (14/15).

In the Phase II study, between-laboratory reproducibility and predictivity using a total of 20 coded chemicals (13 T cell targeting, 6 non-T cell targeting, and 1 undetermined) were evaluated by 1 experiment set based on IL-2 Luc assay protocol Ver. 009.1E. The between-laboratory reproducibility was 80% (16/20) and the average predictivity was 59.6% (34/57)

In the combined results of the Phase I and II studies, the average within-laboratory reproducibility was 86.7% (13/15). The between laboratory reproducibility was 80% (20/25). The average predictivity was 66.7% (48/72).

Although the within- and between-laboratory reproducibilities could satisfy the acceptance criteria for the validation study, the predictivity was not satisfactory. We considered several possible reasons for this unsatisfactory predictivity.

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

Our long term goal is to complete the official validation of the IL-2 Luc assay and the IL-1β Luc assay to provide a novel *in vitro* immunotoxicity screening test by combining the MITA with the OECD test guideline 442E for the IL-8 Luc assay (mMITA).

2. Background

2-1. What is immunotoxicity?

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

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

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

primary cells may decrease reproducibility and makes the assay unsuitable for high-throughput approaches

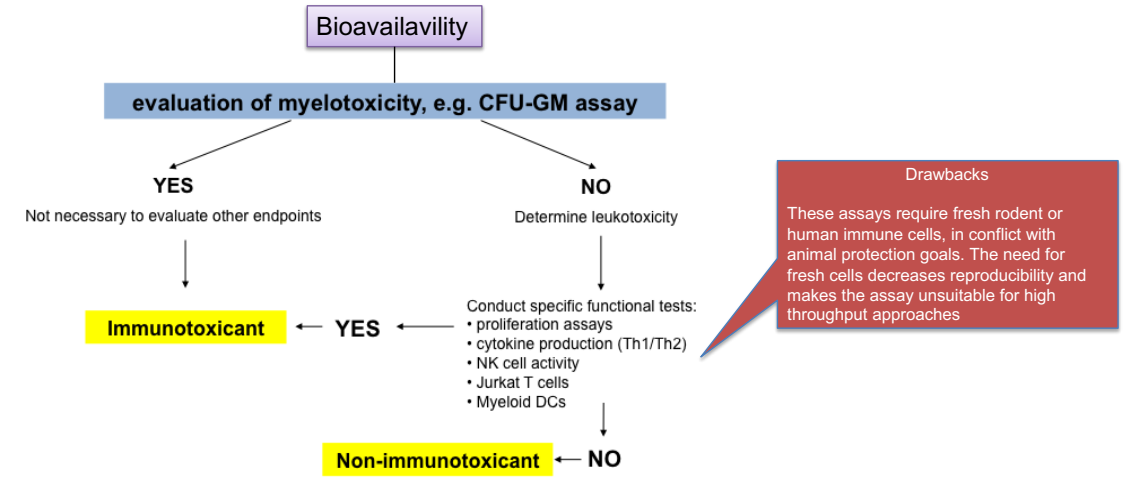


Fig. 1. Decision tree approach for *in vitro* assessment of chemical-induced immunosuppression.

2-3. *In vitro* immunotoxicity tests in principle should evaluate effects on both innate and acquired immunity

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

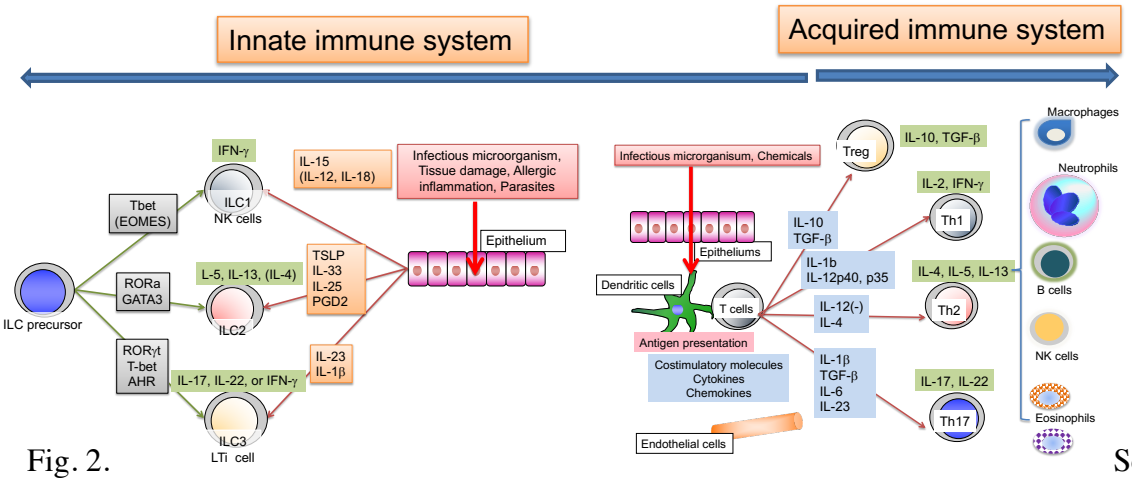


Fig. 2. Schematic representation of the innate immune system and acquired immune system.

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

intracellular signaling pathways also play roles in innate and adaptive immune responses.

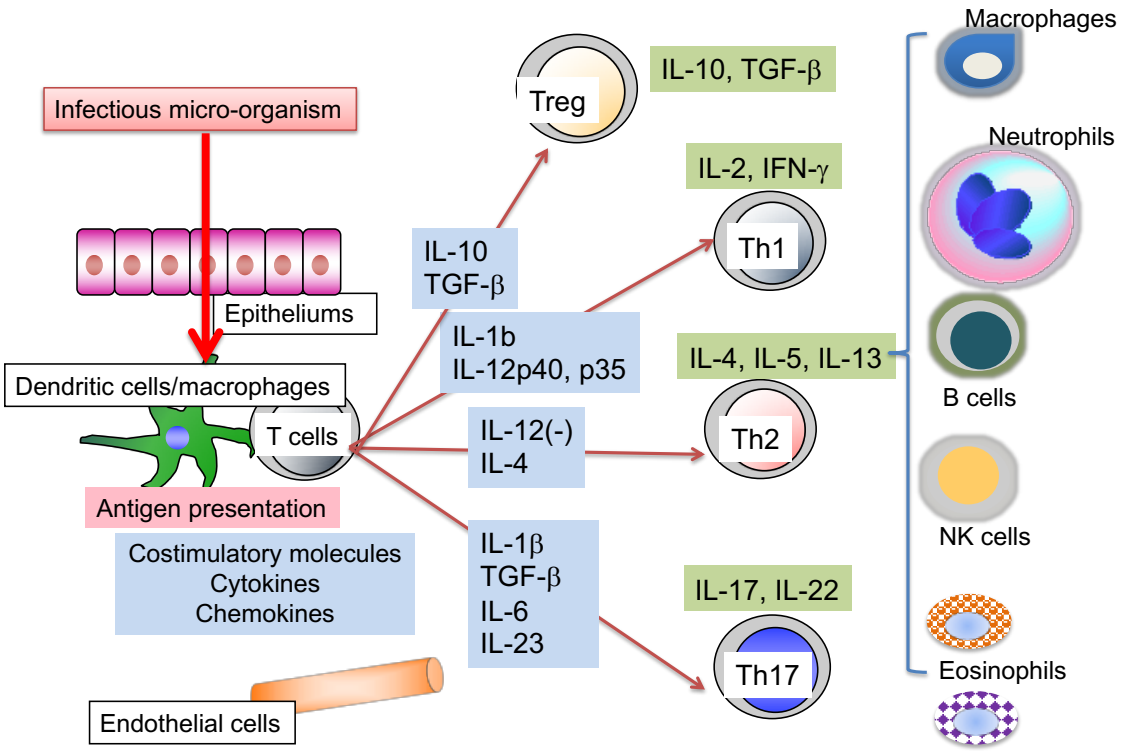
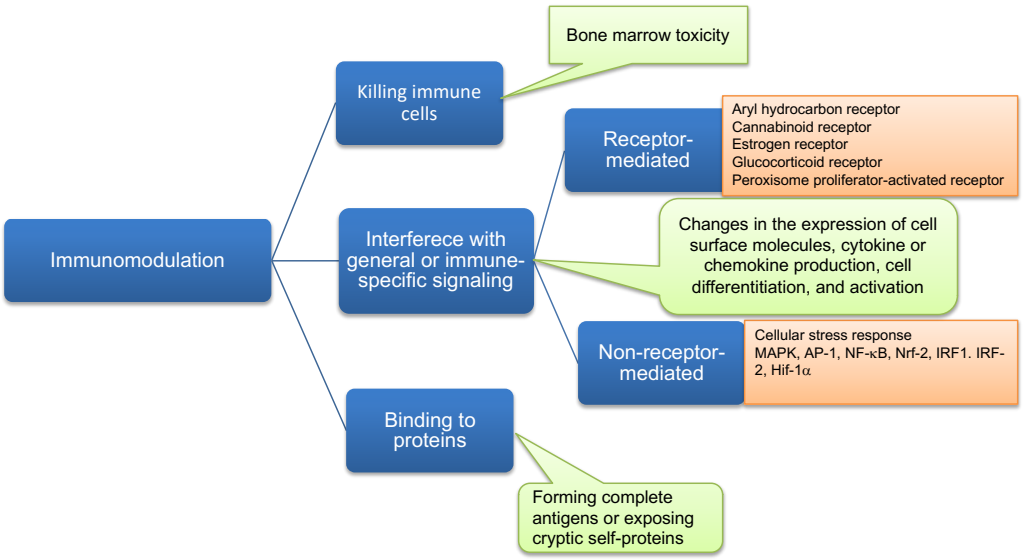


Fig. 3. Dendritic cells link the innate immune response to the acquired immune response.

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

2-4. Mechanism for the induction of immunotoxicity by chemicals

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



Corsini and Roggen. Overview of in vitro assessment of immunotoxicity DOI: 10.1016/j.cotox.2017.06.016.

4

Fig. 4. Main mechanisms of immunotoxicity

Chemicals can interfere with immune-related cell signaling through receptor-mediated pathways using xenobiotic receptors such as the aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane X receptor (PXR), (Hidaka et al., 2017, Elentner et al., 2018) or through non-receptor-mediated ways. In contrast, it is easy to understand the mechanism underlying non-receptor-mediated immunotoxicity by considering the cellular stress response (Kultz, 2005, Fulda et al., 2010). In essence, as long as the stress stimulus does not cross a certain threshold, a cell can cope and survive by mounting an appropriate protective response. Conversely, the failure to activate or maintain a protective response (e.g., when the stressor is too strong) results in activation of stress signaling cascades that eventually activate cell death pathways. Depending on the type of stress and its severity, a cell's response can be manifold. However, most cellular protective responses induced by chemicals can be classified into one of several categories, such as heat shock, unfolded protein, DNA damage, and oxidative stress responses, in addition to the response to danger signals (Gallucci & Matzinger, 2001). These responses are independent of the chemical species (Fig. 5). In addition, these cellular stress responses can affect immune function because they share the same cellular signaling pathways (such as those mediated by MAP kinase, NF-kB, and mTOR) used by the immune response (Milisav, 2011). Indeed, although sensitizers (which are chemicals that induce allergic contact hypersensitivity) include numerous compounds with different molecular structures, it has become clear that their ability to sensitize is based simply

on their reactivity to mainly cysteine residues, which induces a response to oxidative stress (Sasaki & Aiba, 2007). Therefore, although it is assumed that there may be many chemicals with the potential to produce immunotoxicity, only a limited number of assay systems may be required to detect their effects.

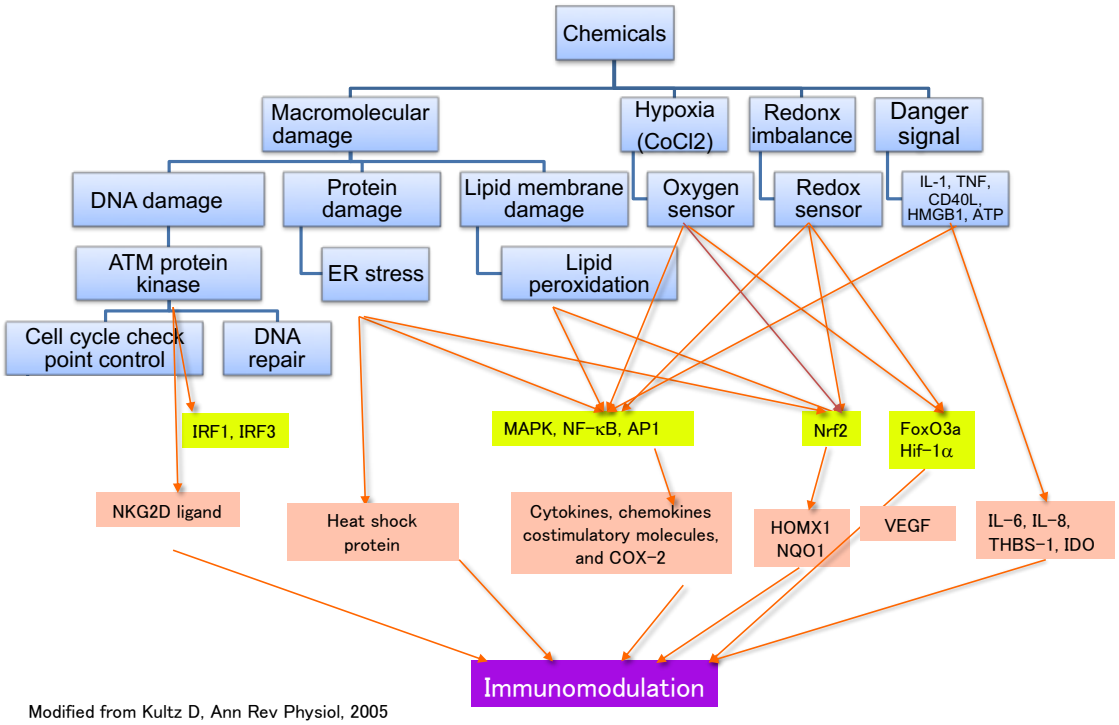


Fig. 5. Cellular stress response and danger signals.

2-5. Multi-ImmunoTox assay (MITA)

Our group developed a high-throughput screening system to evaluate chemical immunotoxicity. We first established 3 stable reporter cell lines transfected with luciferase genes under control of the IL-2, IFN-γ, IL-8, and IL-1β promoters: 2H4 derived from Jurkat cells containing stable luciferase green (SLG) regulated by the IL-2 promoter, stable luciferase orange (SLO) regulated by the IFN-γ promoter, and stable luciferase red (SLR) regulated by the G3PDH promoter (Saito et al., 2011); THP-G8 cells derived from THP-1 cells containing SLO regulated by the IL-8 promoter and SLR regulated by G3PDH promoter (Takahashi et al., 2011); and THP-G1b cells derived from THP-1 cells containing SLG regulated by the IL-1β promoter and SLR by the G3PDH promoter (Kimura et al., 2014). These 4 cytokines were selected because IL-2 and IFN-γ are primarily produced by T cells (adaptive immune cells), whereas IL-8 and IL-1β are primarily

produced by monocytes and dendritic cells (innate immune cells). Using these 3 cell lines, we established the Multi-ImmunoTox assay (MITA). This assay identifies the effects of chemicals on the IL-2 and IFN-γ luciferase activity in 2H4 cells in the presence of the stimulants phorbol 12-myristate 13-acetate (PMA) and ionomycin (Io), and on the IL-1β and IL-8 luciferase activities in THP-G1b and THP-G8 cells, respectively, in the presence of the stimulant lipopolysaccharide (LPS) (Fig. 6).

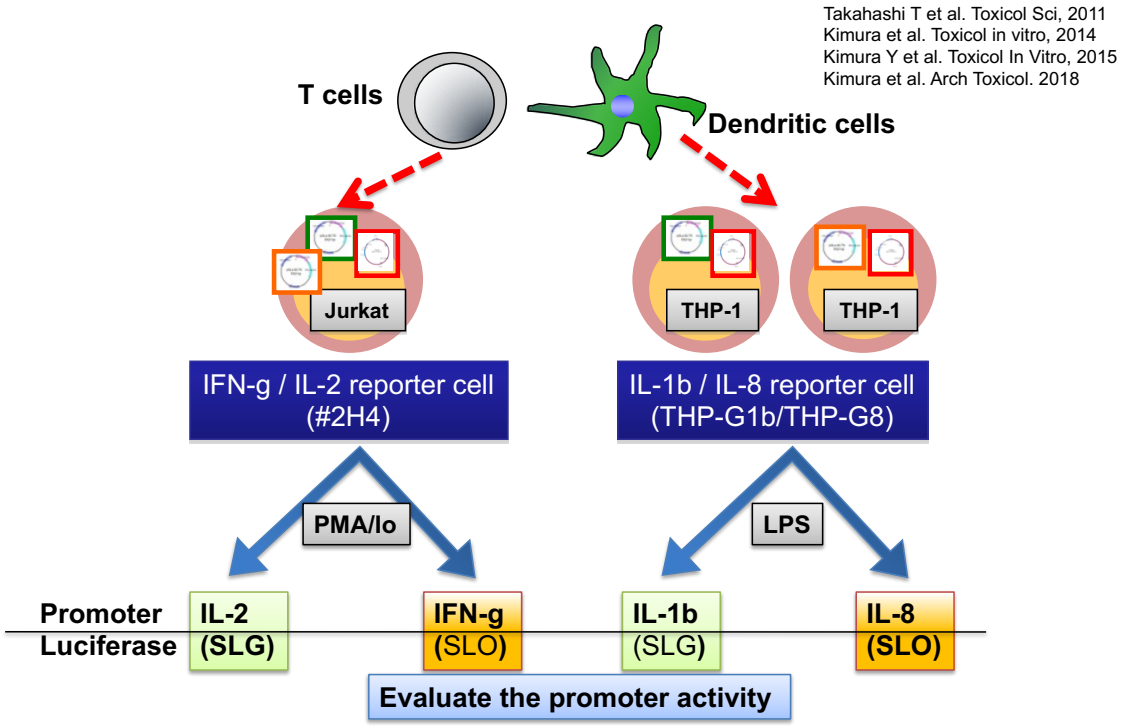


Fig. 6. The Muti-ImmunoTox assay (MITA)

2-6. The luciferase activities of the three MITA cell lines correspond with mRNA expression in the wild type cell lines or in human whole blood cells when stimulated with PMA/Io or LPS in the presence of 3 representative immunosuppressive drugs

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

2-7. The MITA can evaluate the immunotoxicity profiles of well-known immunosuppressive drugs

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

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

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

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

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

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

2-8. Evaluation of the immunotoxicity of 60 chemicals by the modified MITA (mMITA)

Regulatory authorities worldwide require testing for allergic contact dermatitis (ACD) and appropriate hazard labeling to minimize exposures. Thus, we combined the MITA with an *in vitro* sensitization test, the IL-8 Luc assay, recently approved as an OECD test guideline for *in vitro* skin sensitization testing (OECD TG442E) (OECD, 2017). We designated this combined assay 'modified MITA' (mMITA). We established a data set of 60 chemicals by referring to the publication by Wagner et al. (Wagner et al., 2006) in which they examined 46 chemicals characterized to different degrees for their immunotoxic and immunomodulatory properties using the Fluorescent Cell Chip (FCP) assay. In addition, we also evaluated the chemicals listed in the case studies in the Guidance for Immunotoxicity Risk Assessment for Chemicals published by WHO ((WHO)/ & Meeting, 2012). Since there were several overlaps between the chemicals we examined in our previous publication and those examined by the FCP, our final data set comprised 60 chemicals evaluated by the mMITA (Kimura et al., 2018) (Table 2). Table 2 lists the chemicals that affected the normalized IL-2 luciferase activity in increasing order of their Lowest Observed Effect Level (LOEL), the results of the MITA evaluation (suppression (S), augmentation (A) or no effect (N)), the LOEL for each parameter of each chemical, and the results of the IL-8 Luc assay evaluation (positive (P) and negative (N)).

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

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

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

0.00 of the LOEL menas less than 0.001.

Using this data set, we first demonstrated a significant correlation between LOELs for effects on the IL-2 luciferase assay and those on the IFN luciferase assay, and between LOELs for effects on the IL-1β luciferase assay and those on the IL-8 luciferase assay (Kimura et al., 2018) (Fig. 7). These results indicated that evaluations of the effects of chemicals on the IL-2 and IL-8 luciferase assays can provide immunotoxicological information equivalent to the evaluation of these chemicals using the IL-2, IFN-γ, IL-1β, and IL-8 luciferase assays.

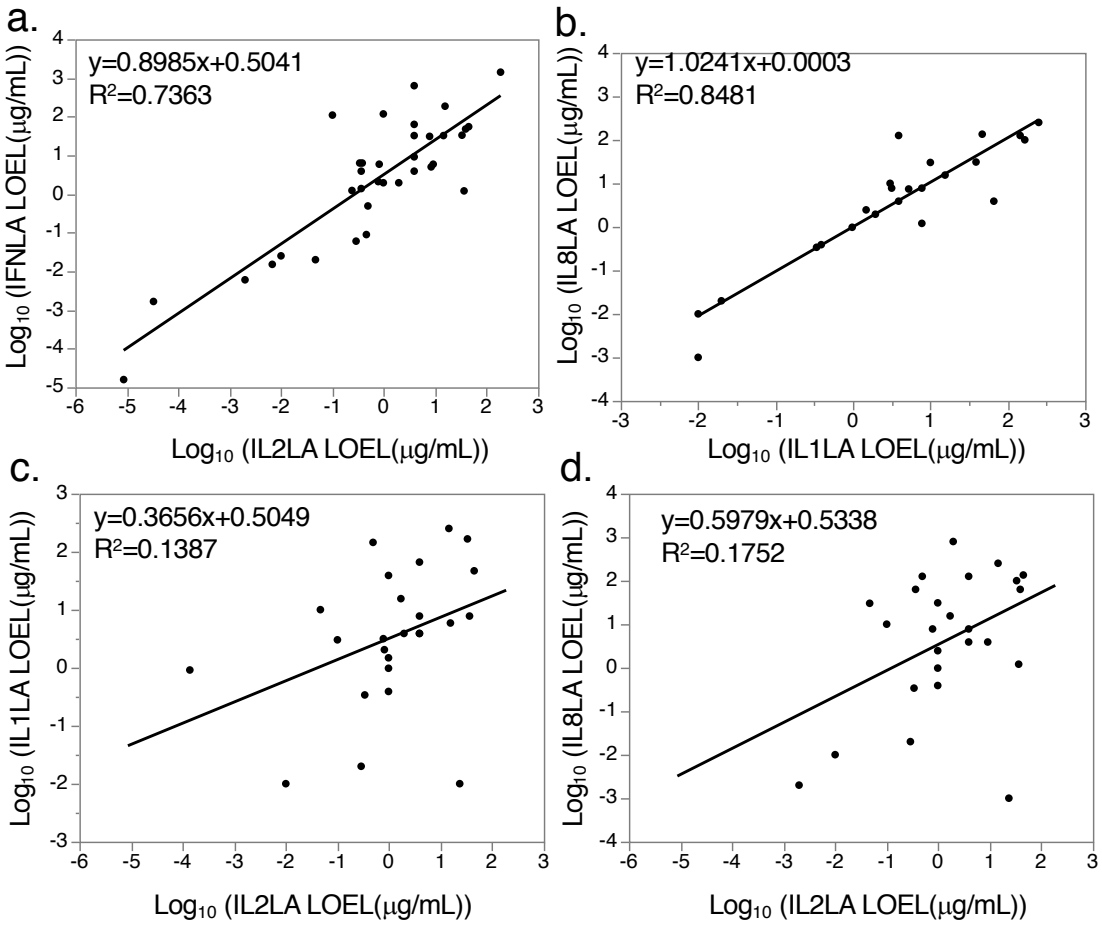


Fig. 7. The correlation between the LOEL for the 4 luciferase assays.

Next, we demonstrated that K-means clustering and hierarchical clustering of the 60 chemicals based on the LOEL for their effects on IL-2 and IL-8 promoter activities, and the judgment by the IL-8 Luc assay, resulted in the same 6-cluster solution: cluster 1 with preferential suppression of IL-8, cluster 2 with suppression of IL-2 and a positive IL-8 Luc assay result, cluster 3 with suppression of both IL-2 and IL-8, cluster 4 with no effects on IL-2 or IL-8 and a negative IL-8 Luc assay result, cluster 5 with suppression of both IL-2 and IL-8 and a negative IL-8 Luc assay result, and cluster 6 with preferential suppression of IL-8 (Kimura et al., 2018) (Fig. 8 and Fig. 9). These data suggest that the mMITA is a promising novel high-throughput approach for detecting unrecognized immunological effects of chemicals and for profiling their immunotoxic effects.

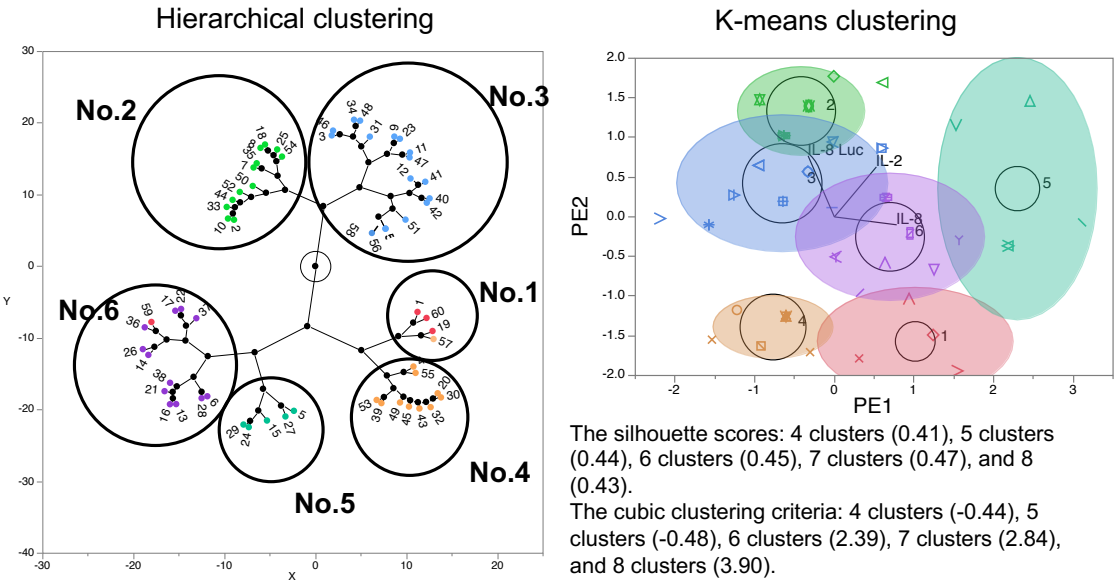


Fig. 8. Clustering of 60 chemicals by the mMITA

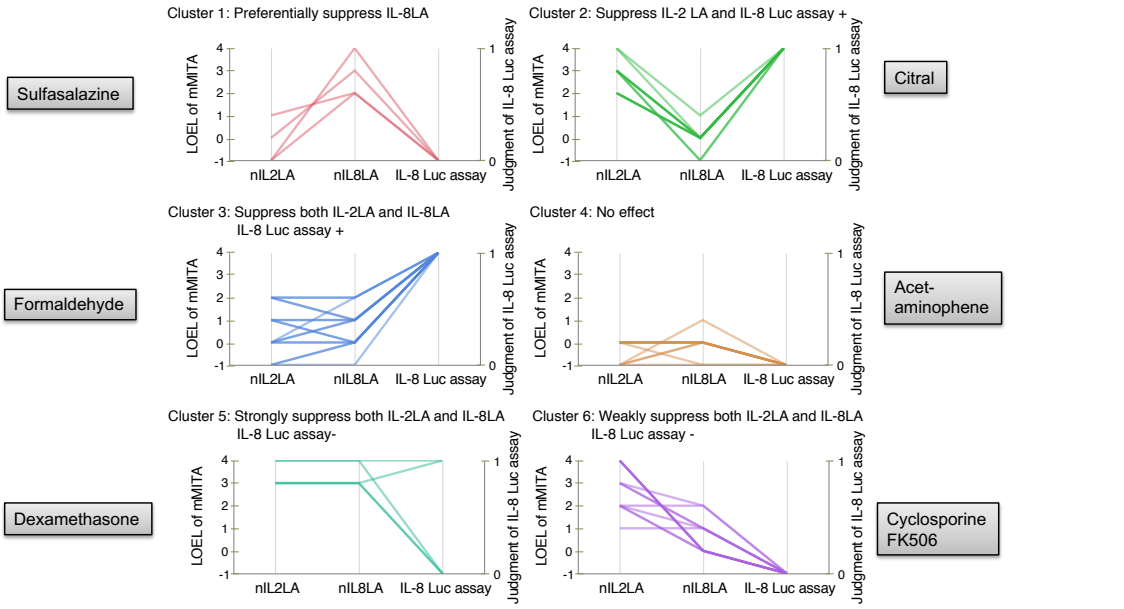


Fig. 9. Characteristics of each cluster and their representative chemicals

2-9. The process of validation of the mMITA

Our final goal is to officially validate the mMITA for within- and between- laboratory reproducibility and predictivity. Since the IL-8 Luc assay had already been accepted as OECD test

guideline 442E, in the current study we conducted the validation study for the IL-2 luciferase assay and the IL-1β luciferase assay using a tiered approach. The first step was the conduct of a validation study of the IL-2 luciferase reporter assay (IL-2 Luc assay).

2-10. The Adverse Outcome Pathway (AOP) of chemicals that affect IL-2 transcription

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

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

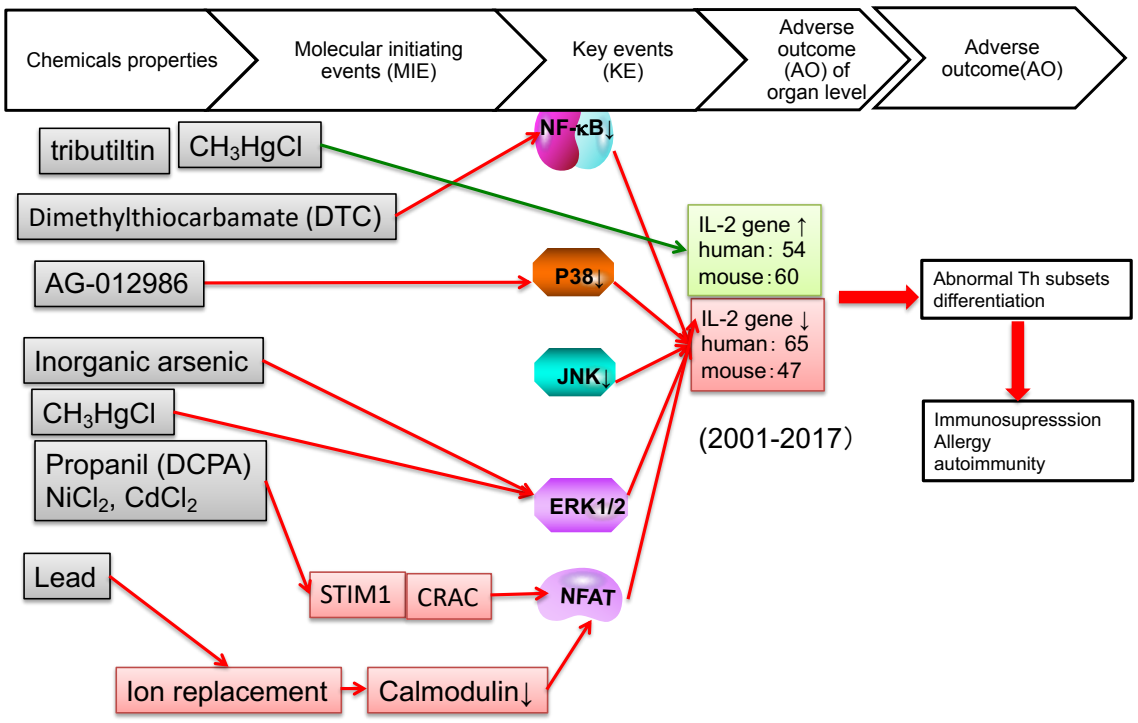
IL-2 mRNA is transcribed after T cell receptor stimulation. Therefore, chemicals that affect any pathway leading to IL-2 transcription after T cell activation can induce dysregulation of IL-2 mRNA and protein expression by T cells. In antigen presentation, T cells are stimulated by T cell receptor (TCR) with co-receptor CD4 or CD8 and CD28. The TCR with CD4 or CD8 recognizes the major histocompatibility complex (MHC)–peptide complex, which results in activation of the SRC kinase Lck and subsequent phosphorylation of immunoglobulin family tyrosine (Y)-based activation motifs (ITAMs) in the CD3 complex (Y-p). This leads to recruitment and phosphorylation of ζ-chain-associated protein (ZAP70), which phosphorylates adaptor proteins, resulting in activation of phospholipase Cγ1 (PLCγ1) and the guanine triphosphatase RAC. PLCγ, in turn, promotes Ca^{2+} mobilization and RAS activation. The combination of these upstream events leads, by complex signaling cascades, to activation of the mitogen-activated protein (MAP)

kinases: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, as well as phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB/Akt). Together, these signals promote different events, including the activation of transcription factors, which result in gene expression and, presumably, T-cell function. On the other hand, CD28 might associate, in its unphosphorylated state, with the serine/threonine phosphatase protein phosphatase 2A (PP2A). Upon T-cell stimulation, CD28 undergoes phosphorylation on its intracellular tyrosine residues (Y), presumably resulting in dissociation from PP2A and recruitment of phosphatidylinositol 3-kinase (PI3K) and growth-factor-receptor-bound protein 2 (GRB2). Activation of PI3K, which induces phosphorylation of phosphatidylinositol (PI) into phosphatidylinositol 3-phosphate (PIP3), might promote activation of protein kinase B (PKB/Akt), followed by activation of nuclear factor-κB (NF-κB), resulting in BCL-XL upregulation that favors T-cell survival. Akt activation might also promote interleukin-2 (IL-2) production. PI3K is negatively regulated by phosphatase and tensin homologue (PTEN). The carboxy-terminal proline (P)-rich region might promote IL-2 production and proliferation, perhaps by recruiting and activating Lck (reviewed by Alegre et al., 2001).

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

Based on recent advances in immunology, we tentatively propose the following AOP for immunosuppression focusing on IL-2 transcription. Figure 10 shows the AOP with representative chemicals that affect IL-2 transcription. From 2001 to 2007, 54 chemicals were reported to augment IL-2 gene or protein expression in human and 60 chemicals had this effect in mice, while 65 chemicals in human and 47 chemicals in mice were reported to decrease IL-2 gene or protein expression, as determined by a PubMed search.

Fig. 10. AOP for dysregulation of Th subset differentiation triggered by disrupted IL-2 transcription.



3. Objective of the study

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

The specific objectives of the study were to establish:

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

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

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

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

4. Test Method and modification

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

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

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

Based on previous reports (Saito et al., 2011, Takahashi et al., 2011), 2H4 cells (2×10^5 cells/50 μl/well) in 96-well black plates (Greiner Bio-One GmbH, Frickenhausen, Germany) were pretreated with different concentrations of individual chemicals for 1 h. The 2H4 cells were then stimulated with 25 nM PMA and 1 μM ionomycin (PMA/Io) for 6 h. Three luciferase activities (SLG luciferase activity (SLG-LA), SLO luciferase activity (SLO-LA), and SLR luciferase activity (SLR-LA)) were simultaneously determined using a microplate-type luminometer with a multi-color detection system (Phelios; Atto Co., Tokyo, Japan) and Tripluc luciferase assay reagent (TOYOBO Co., Ltd., Osaka, Japan) according to the manufacturers' instructions. Use of the 2H4 cell line enabled measurement of SLO-LA driven by the IL-2 promoter (IL2LA), SLG-LA driven by the INF-γ promoter (IFNLA), and SLR-LA driven by G3PDH (GAPLA) in 2H4 cells. We accounted for the variation in cell number and cell viability after chemical treatment by normalizing the data for IL2LA (nIL2LA) or IFNLA (nIFNLA) by dividing IL2LA or IFNLA,

respectively, with GAPLA in the 2H4 cells. In addition, we calculated % suppression, % augmentation, and Inh-GAPLA as follows:

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

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

Inh-GAPLA = GAPLA of 2H4 cells treated with chemicals/GAPLA of untreated cells. Definitions of these terms are provided in Table 3.

Table 3. Definition of the parameters in the IL-2 Luc assay.

Abbreviation	Definition
SLG-LA (SLG-luciferase activity)	Luciferase activity of stable luciferase green (Under the control of IL-2 promoter)
SLO-LA (SLO-luciferase activity)	Luciferase activity of stable luciferase orange (Under the control of IFN-γ promoter)
SLR-LA(SLR-luciferase activity)	Luciferase activity of stable luciferase red (Under the control of G3PDH promoter)
Normalized SLG-LA (nSLG-LA)	SLG-LA/SLR-LA
Normalized SLO-LA (nSLO-LA)	SLO-LA/SLR-LA
Inhibition index of SLR-LA (I.I.-SLR-LA) (Cytotoxic effect of chemicals)	SLR-LA of #2H4 treated with chemicals/SLR-LA of untreated #2H4
% suppression (Effect of chemicals on IL-2 promoter)	(1-(nSLG-LA of #2H4 treated with chemicals) /(nSLG-LA of non-treated #2H4)) x 100

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

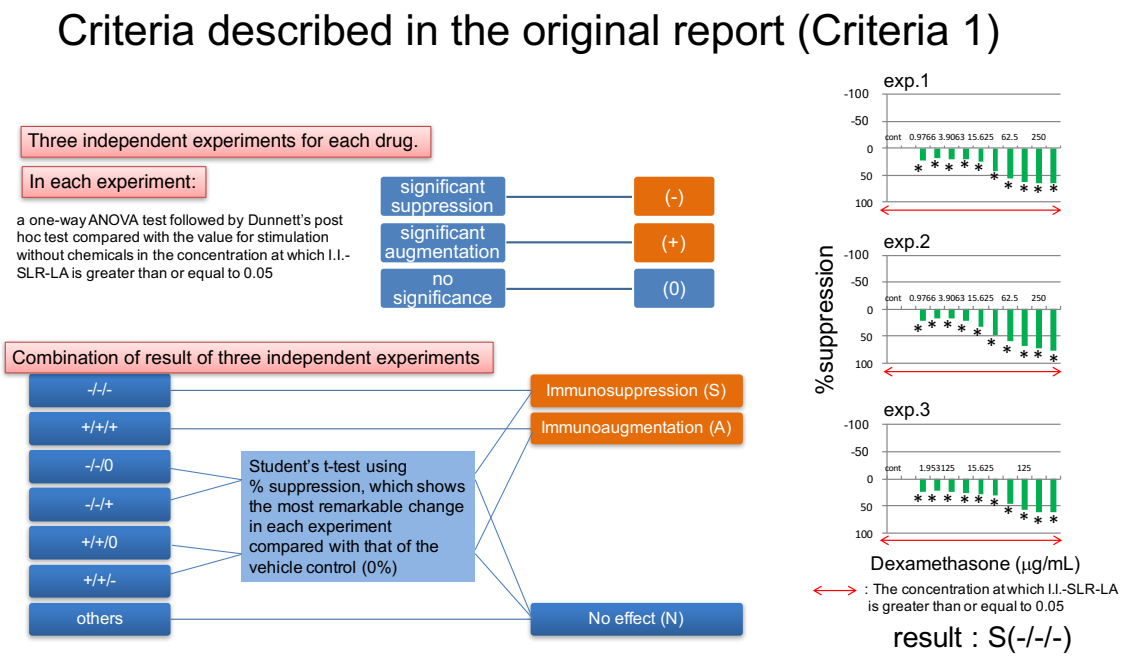
During the validation study, we modified the criteria to determine the effects of chemicals on T cells to determine the criteria for the MITA.

4-3-1. Criteria used in our first publication describing the MITA.

We used the following Criteria 1 in our first publication describing the MITA.

Three independent experiments were conducted for each chemical. For each experiment, a one-way ANOVA test followed by Dunnett's post hoc test was used to evaluate statistical significance. If chemicals showed statistically significant immunosuppression or immunostimulation in 3 experiments, they were judged as immunosuppressive or immunostimulatory drugs, respectively. If chemicals showed statistically significant immunosuppression or immunostimulation in only 2 independent experiments, they were judged as potential immunosuppressive or immunostimulatory drugs, respectively. If not, they were judged as ineffective. Then, for potential immunosuppressive

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



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

4-4. Bioluminescence system

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

concomitant quenching of firefly luciferase luminescence. Finally, firefly luciferase activity is normalized by Renilla luciferase activity as the promoter activity (Michelini et al., 2014; Nakajima and Ohmiya, 2010; Roda et al., 2004).

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

In the IL-2 Luc assay, the triple-color assay system consisted of a green-emitting luciferase (SLG; λmax = 550 nm) (? et al., ?) for the gene expression of the IL-2 promoter, an orange-emitting luciferase (SLO; λmax = 580 nm) (Viviani et al., 2001) for the gene expression of the IFN-γ promoter, and a red-emitting luciferase (SLR; λmax = 630 nm) (Viviani et al., 1999) for the gene expression of the internal control promoter, GAPDH. The three luciferases emit different colors upon reacting with firefly D-luciferin and their luminescence is measured simultaneously in a one-step reaction by dividing the emission from the assay mixture using an optical filter (Nakajima et al., 2005). First, the total relative light units (F0) are measured in the absence of the filters. Then, the F1 and F2 values that passed through the R56 filter (>560-nm long-pass filters) or the R60 filter (>600-nm long-pass filters), respectively, is measured. The three luciferase activities are calculated using the simultaneous equation shown below by substituting the F0, F1 and F2 values. In this equation, G, O and R are the activities of the green-, orange- and red-emitting luciferases, respectively, κG_{R56}, κO_{R56} and κR_{R56} are the transmission coefficients of the green-, orange- and red-emitting luciferases of the R56 filter, respectively, κG_{R60}, κO_{R60} and κR_{R60} are the transmission coefficients of the green-, orange- and red-emitting luciferases of the R60 filter, respectively.

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

Luminescence activity is measured using a filtered 96-well microplate luminometer (for example, Phelios (ATTO, Tokyo, Japan), Tristan 941 (Berthold, Bad Wildbad, Germany), and the ARVO series (PerkinElmer, Waltham, MA). It is necessary to calibrate the luminometer in each

experiment to ensure reproducibility (Niwa et al., 2010). Recombinant green-, orange- and red-emitting luciferases are available for this calibration.

5. Validation Management Structure

5-1. Validation Management Team (VMT)

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

Participating Test Facilities	Test Facility 1: Hatano Res. Inst., FDSC, Study Director (SD): Kohji Yamakage Test Facility 2: AIST, Tsukuba, SD: Rie Yasuno Test Facility 3: AIST, Takamatsu, SD: Yoshihiro Nakajima

5-2. Management office

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

5-3. Meetings held

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

13/9/2016 (Skype-meeting)
Meeting by Skype
Subjects: Result of the phase 0 study and proposal of the revised protocol
VMT members: Corsini, E., Roggen, E., Germolec, D., Inoue, T., Aiba, S., Kimura, Y., Omori, T., Kojima, H.

4-5/2/2017 (Nayamachi community hall, Kyoto, Japan)
2nd International VMT Meeting
Subjects: Validation results, discussion and suggestion
VMT members: Corsini, E., Roggen, E., Germolec, D., Inoue, T., Aiba, S., Kimura, Y., Yamakage, K., Watanabe, M., Kobayashi, M.,

Yasuno, R., Nakajima, Y., Omori, T., Mori, A., Kobayashi, M., Kojima, H., Venti, S.

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

18-19/11/2017 (Umeda Center Building, Osaka, Japan)

3rd International VMT Meeting

Subjects: Validation results, discussion and suggestion

VMT members: Corsini, E., Roggen, E., Germolec, D., Inoue, T., Aiba, S., Kimura, Y., Yamakage, K., Watanabe, M., Kobayashi, M., Yasuno, R., Nakajima, Y., Omori, T., Mori, A., Kobayashi, M., Kojima, H., Venti, S.

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

29/3/2018 (Skype-meeting)

Meeting by Skype

Subjects: Proposal of the revised protocol

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

10/4/2018 (telephone-meeting)

Meeting by telephone

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

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

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

4th meeting for the MITA Validation study

Subjects: Validation report for the IL-2 assay

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

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

6. Study Design

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

The validation study (Phase I and Phase II trials) was conducted by 3 laboratories, based on the study design and schedule shown in Tables 8 and 9 and using the test chemicals shown in Tables 10 and 11. The methods were described above in section 4: 'Test Method 4.1 IL-2 Luc assay', and the precise protocol is described below in section 8: 'Protocol 8.2 Protocol for the IL-2 Luc assay'.

Table 4. The number of chemicals analyzed in the validation study

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

7. Test Chemicals

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

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

Table 5. Breakdown of the IL-2 Luc assay validation study

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

7-1. Basic rule for chemical selection

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

7-1-1. The applied selection criteria

- information on mode/site of action
- coverage of a range of relevant chemical classes and product classes
- quality and quantity of reference data (*in vivo* and *in vitro*)
- high-quality data derived from animal and (if available) human studies
- information on interspecies variations (for example: variability with regard to the uptake of chemicals, metabolism, etc.)
- coverage of a range of toxic effects/potencies
- chemicals that do not require metabolic activation
- appropriate negative and positive controls
- physical and chemical properties (feasibility of use in the experimental set-up as implicated by the CAS No.)
- single chemical entities or formulations of known high purity
- availability
- cost

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

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

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

7-1-3. Handling

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

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

7-2. Pre-validation study

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

7-3. Validation study -Phase I trial

Within- and between-laboratory reproducibility of this assay was checked using 5 coded chemicals in 3 test facilities, as shown in Table 6 (Appendix 2). These chemicals were selected by CSC based on the in-house data set of the lead laboratory. The chemicals were coded by JaCVAM as shown in Table 10 and distributed to the test facilities.

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

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

7-4. Validation study -Phase II trial

Twenty test chemicals were selected by CSC for between-laboratory reproducibility as shown in Table 7 (Appendix 3). The chemicals were coded by JaCVAM as shown in Table 7 and distributed to the test facilities.

Table 7. Chemical code list on the phase II validation trial for IL-2 Luc assay

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

7-5. Acceptance criteria

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

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

8. Protocols

8-1. Overview of the IL-2 Luc assay

An overview of the IL-2 Luc assay is shown in Fig. 9. In addition, the final protocol of the present test (version 023E) is provided as attached Appendix 4 and the procedures are described in detail below.

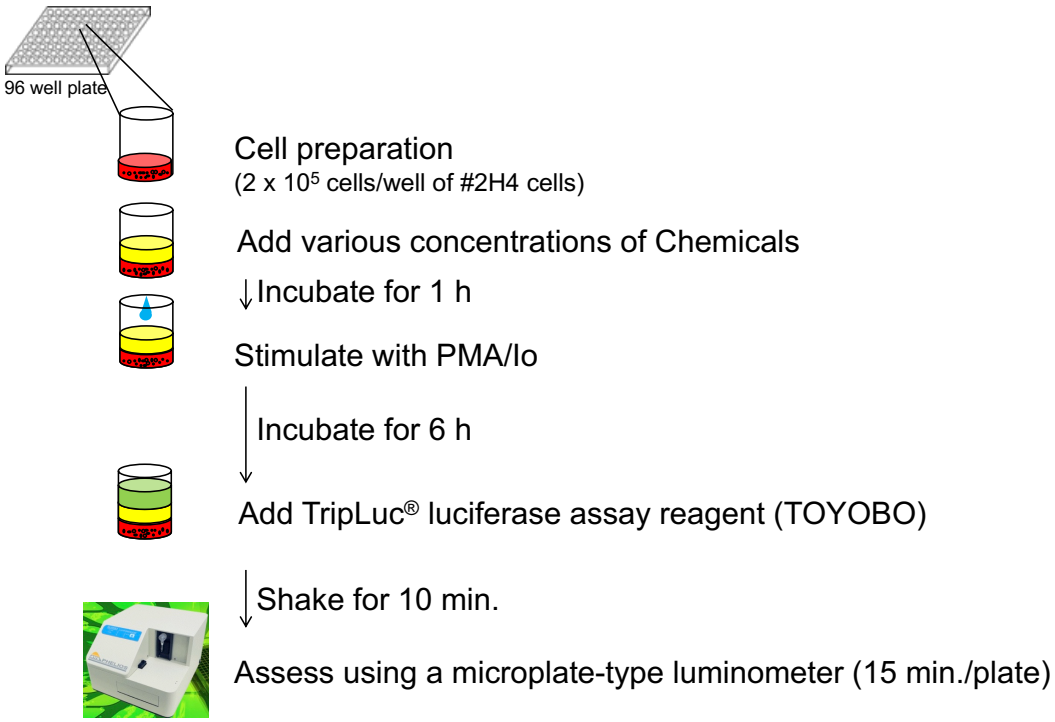


Fig. 11. Overview of the IL-2 Luc assay

8-1 Cells

- 2H4 (IL2-SLG、IFNγ-SLO、G3PDH-SLR)

  The human acute T lymphoblastic leukemia cell line Jurkat was obtained from the ATCC. A Jurkat-derived IL-2 and IFN-γ reporter cell line, 2H4, that harbors the SLG, SLO and SLR luciferase genes under the control of the IL-2, IFN-γ and GAPDH promoters, respectively, was established by Tsuruga Institute of Biotechnology, TOYOBO Co. Ltd., Fukui, Japan. (Saito R. et al. Nickel differentially regulates NFAT and NF-κB activation in T cell signaling *Toxicology and Applied Pharmacology,* 254, 245–255, 2011)

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

8-2-1. Reagents and equipment

The following reagents and equipment were used.

For maintenance of 2H4 cells

- RPMI-1640 (GIBCO Cat#11875-093, 500 mL)
- FBS (Biological Industries Cat#04-001-1E Lot: 715004)
- Antibiotic-Antimycotic (GIBCO Cat#15240-062)
- HygromycinB (CAS:31282-04-9, Invitrogen Cat#10687-010)
- G418 (CAS:108321-42-2, Nacalai Tesque Cat#16513-84)
- Puromycin (CAS:58-58-2, InvivoGen Cat#ant-pr-1)

For chemical exposure, stimulation and solvents

- Ionomycin (CAS:56092-82-1, Sigma Cat#I0634)
- Phorbol 12-myristate 13-acetate (PMA) (CAS:16561-29-8, Sigma Cat#P8139)
- Ethanol (e.g., Wako Cat#057-00456)
- Dimethyl sulfoxide (DMSO) (CAS:67-68-5, Sigma Cat#D5879)
- Distilled water (GIBCO Cat#10977-015)

For measurement of luciferase activity

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

8-2-2. Culture medium

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

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

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

Table 9. B medium: for luciferase assay (30 mL, stored at 2-8°C)

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

Table 10. C medium: for thawing 2H4 cells (30 mL, stored at 2-8°C)

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

8-2-3. Cell line

The Jurkat human acute T lymphoblastic leukemia cell line (ATCC, Manassas, VA, USA), was cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) with Antibiotic-Antimycotic (Invitrogen, Carlsbad, CA, USA) and 10% Hyclone™ fetal calf serum (Thermo Fisher Scientific, Wilmington, NC, USA) (Jurkat growth medium) at 37 °C with 5% CO_2. The luciferase reporter assay system was constructed using three different luciferases, SLG, SLO and SLR, that emit green, orange, and red light, respectively, with a single substrate. In brief, we constructed three luciferase vectors, pSLG-test/Hygr, pSLO-test/Neor, and pSLR-test/Purr, by ligating the BamHI/SacI site of resistant gene vectors containing one of the three resistant genes, hygromycin (SLG), neomycin (SLO) or puromycin (SLR), the SV40 promoter, and HSVtk polyA into the luciferase gene vectors, pSLG-test, pSLO-test and pSLRtest (Toyobo, Osaka, Japan), respectively.

The activities of the luciferases can be measured simultaneously and quantitatively using optical filters. This system can rapidly and easily monitor multiple gene expression (Nakajima et al., 2005; Noguchi et al., 2008). Promoter cloning was carried out as follows. The IL-2 promoter construct containing nt −3006 to +286, the IFN-γ promoter construct containing nt −4971 to +111, and the G3PDH promoter construct containing nt −1373 to +128 from transcription initiation sites that were identified using DBTSS (http://dbtss.hgc.jp/), were amplified from genomic DNA by PCR using KOD-Plus- ver. 2 (Toyobo) for the IL-2 promoter or KOD-Plus- (Toyobo) for the IFN-γ and G3PDH promoters and specific primers. The IL-2 promoter, IFN-γ promoter, or G3PDH promoter was ligated into pSLG-test/Hygr, pSLOtest/Neor or pSLR-test/Purr vectors that had been digested with MluI and XhoI, MluI and SalI, or MluI and EcoRI, respectively. Before transfection, we confirmed the sequence of the 5' and 3' regions of each promoter using a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). IL-2, IFN-γ and G3PDH reporter plasmids (1 μg) were transfected into Jurkat T cells ($5x10^5$ cells) using SuperFect (Qiagen, Valencia, CA, USA). After transfection, cells were cultured in Jurkat growth medium containing 200 μg/ml hygromycin (Invitrogen), 300 μg/ml G418 (Nacalai tesque, Kyoto, Japan) and 0.15 μg/ml puromycin (InvivoGen, San Diego, CA, USA) for selection. After repeated limiting dilution, we established a stable cell line (2H4 cells).

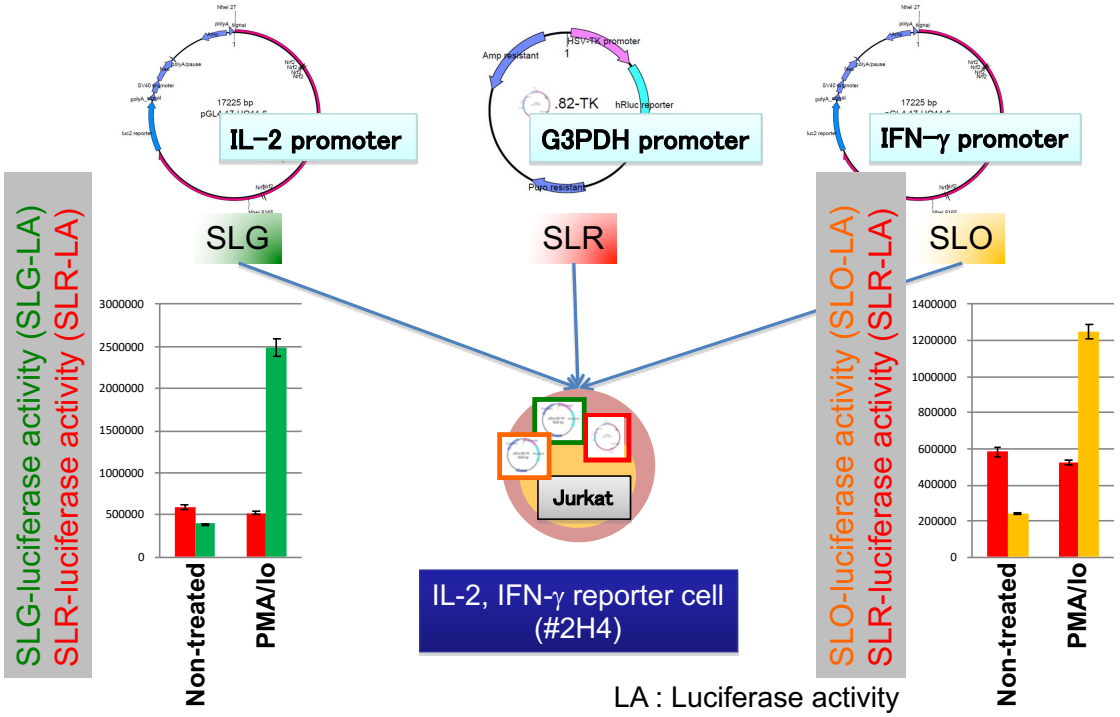


Fig. 12. IL-2 reporter cell, 2H4

8-2-4. Thawing of 2H4 cells

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

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

8-2-4. Maintenance of 2H4 cells

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

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

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

8-2-5. Preparation of cells for assay

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

Use cells between 1 and 6 weeks after thawing.

Pre-warm the B medium in a 37°C water bath. Count the number of cells and collect the number of cells needed (2.0×10^7 cells for two chemicals are required, but to have some leeway, 3.0×10^7 cells for two chemicals should be prepared), centrifuge the tube at 120-350 x *g*, 5 min. Resuspend in pre-warmed the B medium at a cell density of 4×10^6/mL. Transfer the cell suspension to a reservoir (Thermo Scientific), and add 50 μL of cell suspension to each well of a 96 well μclear black plate (flat bottom) using an 8 channel or 12 channel pipetman (Gison, Inc, Middleton, WI, USA). (cf. Figure 13)

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

Fig. 13. Components in each well of 96-well plates after cell preparation.

8-2-6. Preparation of chemicals and cell treatment with chemicals

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

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

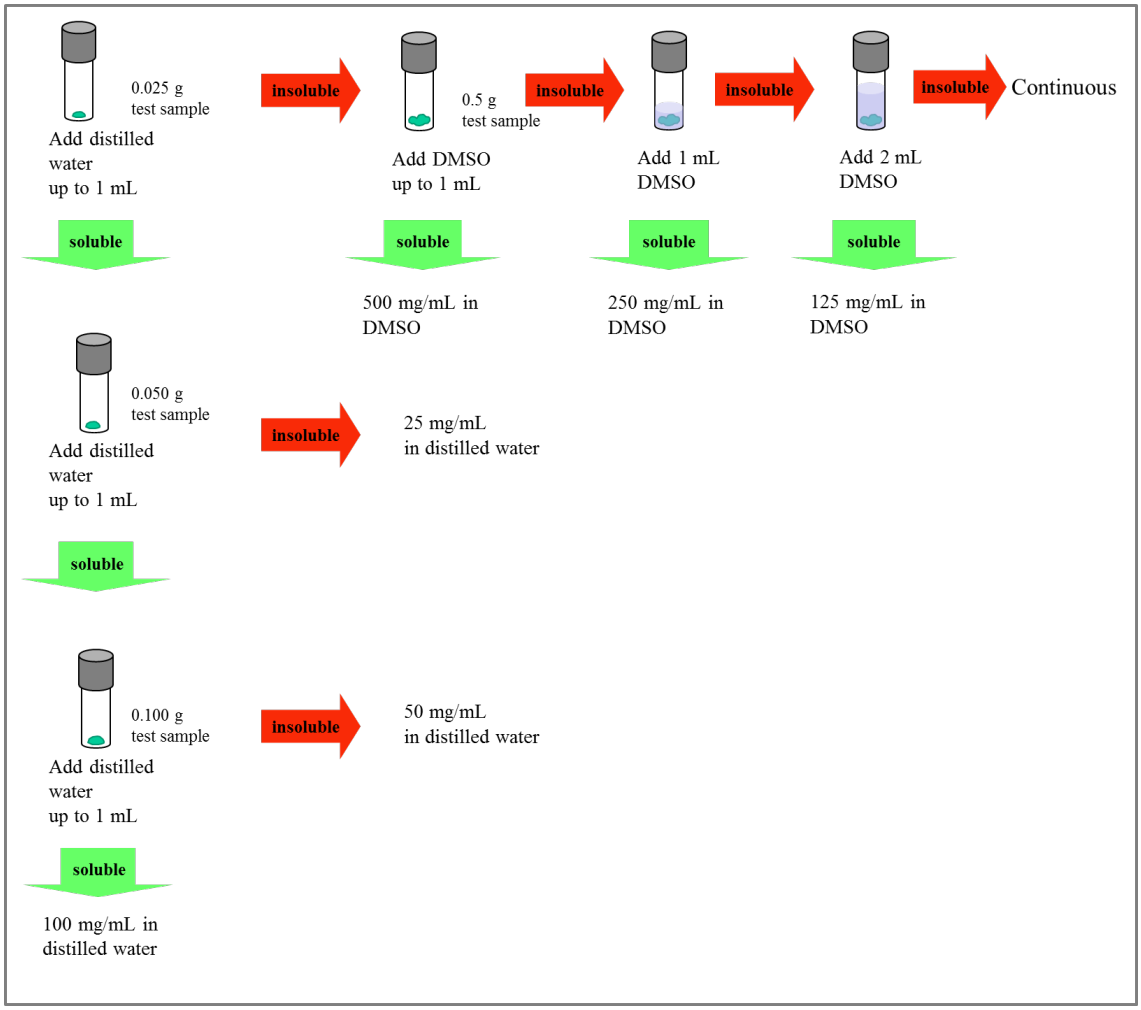


Fig. 14. Dissolution by vehicle

8-2-7. Dilution of chemicals

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

8-2-8. Measurements

After incubation with the chemical and PMA/Io for 6 h at 37°C in a 5% CO_2 incubator, 100 µL of pre-warmed Tripluc is added to each well in the plate containing reference samples using a pipetman and the plate is shaken for 10 min at room temperature (about 25°C) using a plate shaker. Surface bubbles are removed if present and bioluminescence in each well is measured using

a microplate-type luminometer with a multi-color detection system (Phelios; Atto Co., Tokyo, Japan) for 3 sec each in the absence (F0) and presence (F1, F2) of the optical filter. The F0, F1 and F2 data (values are expressed as counts) are processed using an Excel-based data sheet (Appendix 12). SLG-LA, SLO-LA and SLR-LA are calculated for each well based on the algorithm to calculate SLG-LA, SLO-LA and SLR-LA from the raw luminescence data reported previously (Nakajima et al., 2005; Noguchi et al., 2008). In addition to being used to calculate SLG-LA, SLO-LA and SLR-LA, this data sheet can automatically generate final graphs showing the correlation between %suppression and the concentration of chemicals, and between II-SLR-LA and the concentration of the chemical.

8-2-9. Luminometer apparatus

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

8-2-10. Positive control

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

8-2-11. Calculation and definition of parameters for the IL-2 Luc assay

In the IL-2 Luc assay, the lead laboratory defined nSLG-LA to represent IL-2 promoter activity by the SLG luciferase activity (SLG-LA) normalized by SLR luciferase activity (SLR-LA). The suppression index of SLR-LA (I.I.-SLR-LA) was obtained by dividing SLR-LA of 2H4 treated with chemicals with SLR-LA of non-treated 2H4. %suppression reflects the effect of chemicals on IL-2 promoter. (Table 19).

Table 19. Abbreviations used in the 2H4 luciferase assay protocol

Parameter	Definition
SLG-luciferase activity (SLG-LA)	Luciferase activity of stable luciferase green (Under the control of IL-2 promoter)
SLO-luciferase activity (SLO-LA)	Luciferase activity of stable luciferase orange (Under the control of IFN-γ promoter)
SLR-luciferase activity (SLR-LA)	Luciferase activity of stable luciferase red (Under the control of G3PDH promoter)
Normalized SLG-LA (nSLG-LA)	=(SLG-LA)/(SLR-LA)
Normalized SLO-LA (nSLO-LA)	=(SLO-LA)/(SLR-LA)
Inhibition index of SLR-LA (I.I.-SLR-LA)	=(SLR-LA of #2H4 treated with chemicals)/(SLR-LA of untreated #2H4) (The cytotoxic effect of chemicals)
%suppression	=(1-(nSLG-LA of #2H4 treated with chemicals) /(nSLG-LA of non-treated #2H4)) x 100 (The effect of chemicals on IL-2 promoter)

8-2-11 Acceptance criteria

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

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

8-2-12 Criteria

The experiments are repeated until 2 consistent positive (or negative) results or 2 consistent "no effect results" are obtained. When 2 consistent results are obtained, the chemicals are judged as indicated by the obtained consistent results.

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

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

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

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

8-3. Data collection

8-3-1. Operating procedure

The operating procedure for Phase I is described in protocol version 008.5E, and that for Phase II is described in protocol version 009.1E. These protocols differ slightly regarding the decision rules for identifying immunotoxicants but are the same regarding the operating procedure.

The result of each experiment is judged “Positive” or “Negative”, and the final judgment for “Immunosuppression”, “Immunoaugmentation” or “No effect” of a test chemical is based on the results of 2-4 experiments. This rule was decided after discussion of the Phase II study at the VMT meeting, and the details are described in protocol ver. 0.11E.

8-3-2. Chemicals

The main aim of the Phase I study was to evaluate the within- and between-laboratory reliability of the assay. Three sets of 5 chemicals were distributed to 3 laboratories. A different code for each set of chemicals was used for each laboratory. The chemicals are re-coded in the present document. Each set of chemicals is indicated by a suffix, providing code names such as MIB012A, MIB014A, MIB017A, and so on.

The main aim of the Phase II study was to evaluate predictively and thus only 1 set of 20 chemicals was used in this phase. The Table 20 shows the chemical codes used throughout this document.

Table 20. The chemical codes

Phase		Chemical		
		Lab A	Lab B	Lab C
I	set A	MIB012A MIB014A MIB017A MIB018A MIB110A	MIC021A MIC023A MIC025A MIC027A MIC029A	MID034A MID036A MID037A MID038A MID310A
	set B	MIB011B MIB013B MIB015B MIB017B MIB019B	MIC024B MIC026B MIC027B MIC028B MIC210B	MID031B MID033B MID035B MID037B MID039B
	set C	MIB014C MIB016C MIB017C MIB018C MIB110C	MIC021C MIC023C MIC025C MIC027C MIC029C	MID032C MID034C MID037C MID038C MID310C
II		MIB501 MIB502 MIB503 MIB504 MIB505 MIB506 MIB507 MIB508 MIB509 MIB510 MIB511 MIB512 MIB513 MIB514 MIB515 MIB516 MIB517 MIB518 MIB519 MIB520	MIC601 MIC602 MIC603 MIC604 MIC605 MIC606 MIC607 MIC608 MIC609 MIC610 MIC611 MIC612 MIC613 MIC614 MIC615 MIC616 MIC617 MIC618 MIC619 MIC620	MID701 MID702 MID703 MID704 MID705 MID706 MID707 MID708 MID709 MID710 MID711 MID712 MID713 MID714 MID715 MID716 MID717 MID718 MID719 MID720

8-3-3. Data handling

The Excel data sheet developed for this study was distributed to the laboratories. The data management team received data files from the 3 laboratories. Since the Excel data sheet is able to display a concentration-response plot for %suppression with its 95% confidence interval, we were able to judge “Suppression”, “Stimulation” or “Negative” for each experiment by seeing the plot.

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

The j-th repetition (j = 1 to 4) of the i-th concentration (j = 0 to 11) is measured for SLG-LA and SLR-LA respectively. The normalized SLG-LA is referred as nSLG-LA, and is defined as $nSLG\text{-}LA_{ij} = SLG\text{-}LA_{ij} / SLR\text{-}LA_{ij}$. This is the basic unit of measurement in this assay.

8-3-4-1. %suppression

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

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

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

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

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

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

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

where mean_i is the mean of nSLG-LA at the i-th concentration, mean_0 is the mean of nSLG-LA at 0 concentration, sd_i is the standard deviation of nSLG-LA at the i-th concentration and sd_0 is the standard deviation of nSLG-LA at 0 concentration.

$z_{0.975}$ is 97.5 percentile of the standard normal distribution.

8-3-4-2. I.I.-SLR-LA

The I.I.-SLR-LA is a ratio of the averaged SLR-LA for the repetition of the i-th concentration compared with it of the 0 concentration, and this is written by

$$\text{I.I.-SLR-LA}_i = \left\{(1/4)\times\sum_j \text{SLL - LA}_{ij}\right\} / \left\{(1/4)\times\sum_j \text{SLL - LA}_{0j}\right\}.$$

Since the SLR-LA is the denominator of the nSLG-LA, the extremely smaller value of this is considered to cause the large variation of the nSLG-LA. Therefore, the i-th %suppression value with extremely smaller value of the I.I.- SLR-LA might be considered to be poor precision.

8-3-4-3. Judgment for “Suppression”, “Stimulation” or “Negative” in each experiment

In each experiment, when the following 3 criteria are satisfied, they are judged as “suppression” or “stimulation”. Otherwise, they are judged as no effect chemicals.

1. %suppression is $\geq$ 35 (suppressive) or ≤ -35 (stimulatory) at any dose and statistically significant.

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

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

For 1, 2, the statistically significant is judged by the lower limit of 95%

confidence interval of %suppression is over 0 or the upper limit of it is under 0.

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

In this assay, “Suppression” or “Stimulation” is defined as in case that the 2 same judgments were found in a set of experiments; “No effect” is defined as in case that the 3 “Negative” judgments were found in a set of experiments.

8-3-5. Reliability

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

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

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

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

8-3-5-2 Between-laboratory reproducibility

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

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

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8-3-6. Predictivity

8-3-6-1. Definition of concordance, sensitivity and specificity

In the evaluation of predictivity, we did not distinguish suppression and stimulation, because both of these indicate modulation of immune function. Then, we dealt as “Positive” in case of “suppression” or “stimulation”, and “Negative” in case of “No effect” for each chemical judgement.

The concordance, sensitivity and specificity were estimated as the indexes of predictivity. These indexes were estimated using the frequency results obtained from the 2 by 2 contingency table for T cell targeting. The definitions of these indexes are summarized in Table 21 below. This calculation was based on the results decided by a majority for the between-laboratory results for each chemical.

Table 21. Definition of the concordance, sensitivity and specificity

Sensitivity = $100 \times a / (a+c)$
Specificity = $100 \times d / (b+d)$
Accuracy = $100 \times (a+d) / N$

8-4. Quality assurance

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

9. Results

We conducted Phase I and II studies in this validation. The assay procedure and criteria used to judge immunotoxicants in the validation studies are summarized in Fig. 15.

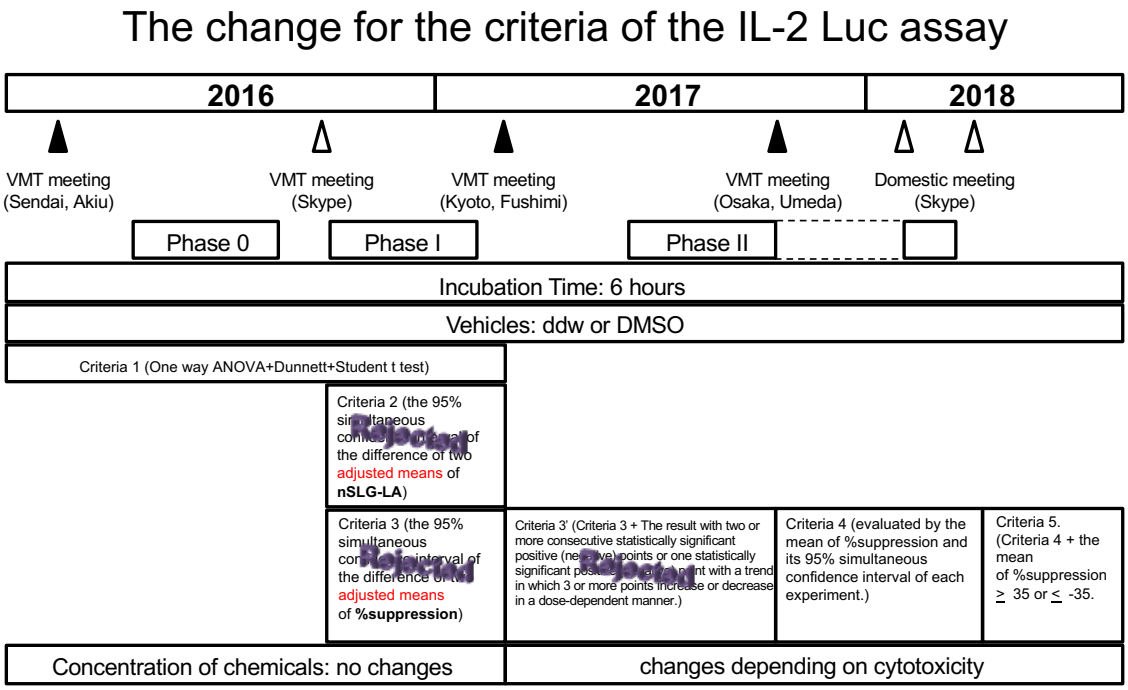


Fig. 15. The modification of the protocols of the IL-2 Luc assay.

9-1. The final criteria

9-1-1. Acceptance criteria

The following acceptance criteria should be satisfied when using the MITA method.

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

9-1-2. Criteria

The experiments are repeated until 2 consistent positive (or negative) results or 2 consistent "no effect results" are obtained. When 2 consistent results are obtained, the chemicals are judged as indicated by the obtained consistent results.

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

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

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

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

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

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

- We changed the speed of centrifugation of the cells, and the preparation method for the selection antibiotics and PMA/Io.
- We set nSLO-LA >3 as an acceptance criterion.
- Because nSLG-LA is dependent on the properties of the specific luminometer used, we expressed the results of the data by %suppression, which is determined by dividing nSLG-LA of the chemically treated cells by nSLGLA of the vehicle-treated cells.
- Volatile chemicals were to be sealed.

- We determined the criteria to judge chemicals from a statistical standpoint (Criteria 2).

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

9-3-1. Test conditions

A total of 5 coded chemicals (4 T cell targeting and 1 non-T cell targeting) were evaluated by 3 experimental sets in the Phase I study based on the MITA protocol Ver. 008.5E.

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

Chemicals that satisfied criteria 5 were judged as positive. Chemicals that provided 2 positive results were judged as immunotoxicants.

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

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

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

Between-Lab reproducibility (Based on Majority)

80.0% (4/5)

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

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

Table 21. Results of the Phase I study

Chemical	CAS	Set	Lab. A	Lab. B	Lab. C	concordance	T cell targeting

Dibutyl phthalate	84-74-2	1st round	S	S	S	1	Yes
		2nd round	S	S	S		
		3rd round	S	S	S		
Hydrocortisone	50-23-7	1st round	S	S	S	0	Yes
		2nd round	N	S	S		
		3rd round	N	S	N		
Lead(II) acetate	6080-56-4	1st round	S	S	S	1	Yes
		2nd round	S	S	S		
		3rd round	S	S	S		
Nickel(II) sulfate	10101-97-0	1st round	S	S	S	1	Yes
		2nd round	S	S	S		
		3rd round	S	S	S		
Zinc dimethyldithiocarbamate (DMDTC)	137-30-4	1st round	N	N	N	1	No
		2nd round	N	N	N		
		3rd round	N	N	N		
Within-laboratory reproducibilities (%)			80.0 (4/5)	100 (5/5)	80.0 (4/5)		
			Average 86.7 (13/15)				
Between-laboratory reproducibilities (%) (Based on Majority)						80 (4/5)	
Sensitivity (%) (Based on Majority)			75.0 (3/4)	100 (4/4)	100 (4/4)		
			Average 91.7 (11/12)				
Specificity (%) (Based on Majority)			100 (1/1)	100 (1/1)	100 (1/1)		
			100 (3/3)				
Accuracy (%) (Based on Majority)			80.0 (4/5)	100 (5/5)	100 (5/5)		
			Average				

93.3 (14/15)

S : Immunosuppression, A : Immunoaugmentation, N : No effect, A/S : Immunoaugmentation/suppression

9-2-5. Contingency tables for the Phase I study

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

Sensitivity : 83.3% (10/12)

Specificity : 100% (3/3)

Accuracy : 86.7% (13/15)

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

Sensitivity : 100% (12/12)

Specificity : 100% (3/3)

Accuracy : 100% (15/15)

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

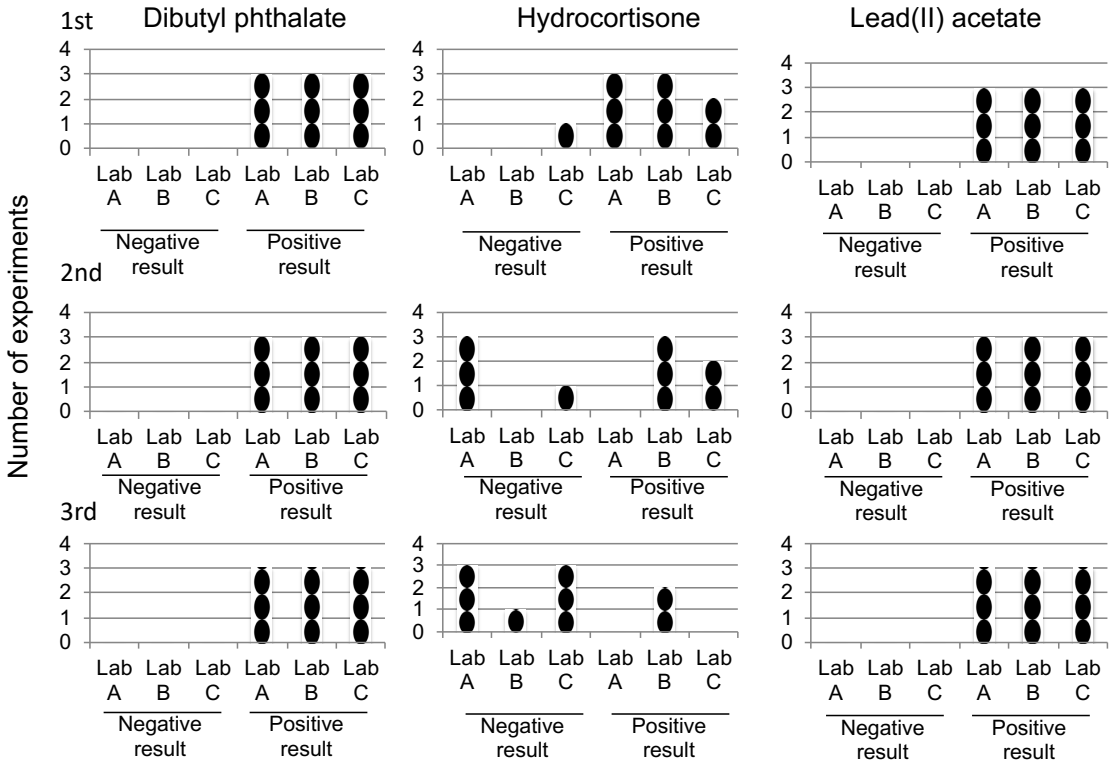
Sensitivity : 91.7% (11/12)

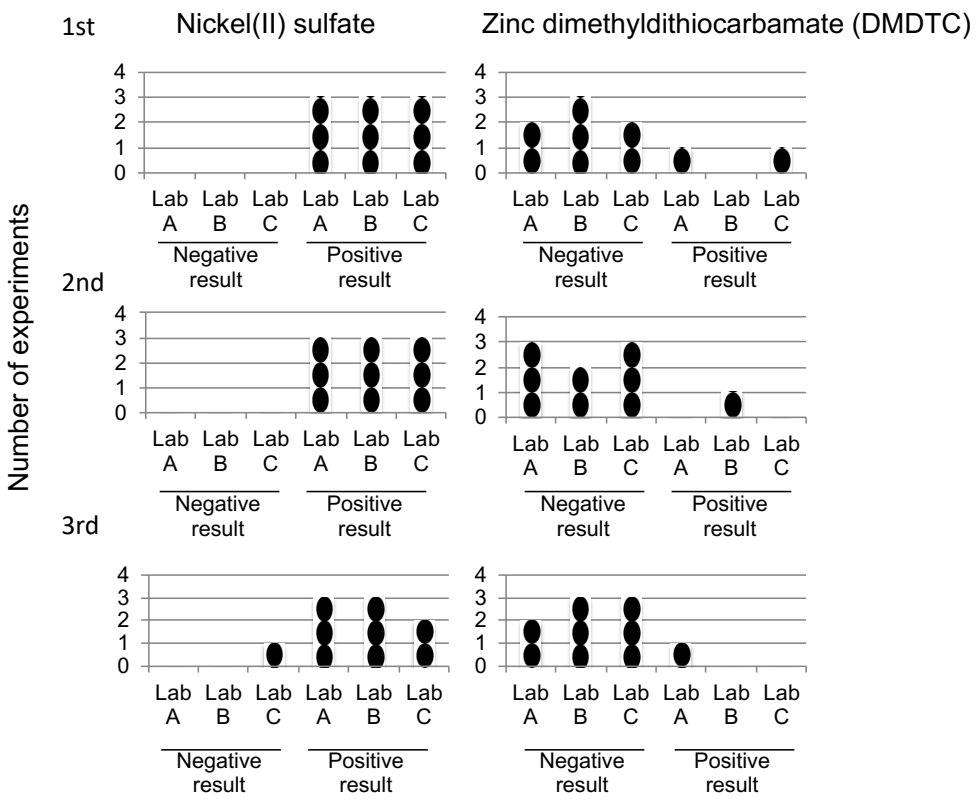
Specificity : 100% (3/3)

Accuracy : 93.3% (14/15)

A graphical presentation of between- and within-laboratory variation in Phase I study is shown in Fig. 15.

Within-laboratory reproducibility





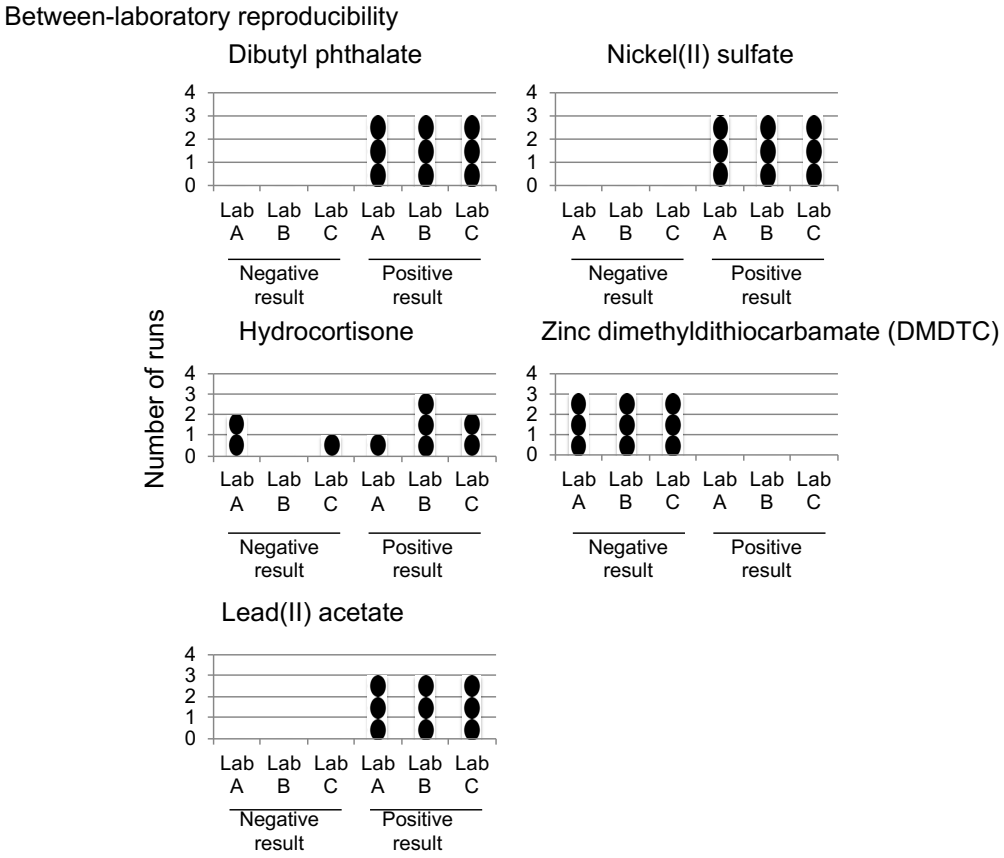


Fig. 15. Between- and within- laboratory variation assessments in Phase I study

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

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

9-4-1. Test conditions

The Phase II study for between-laboratory reproducibility and predictivity was conducted using a total of 20 coded chemicals (13 T cell targeting, 6 non-T cell targeting and 1 undetermined) and evaluated by 1 experiment set based on the IL-2 Luc assay protocol Ver. 009.1E.

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

Between-Lab reproducibility 80% (16/20)

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

Accuracy of Lab A	57.9% (11/19)
Accuracy of Lab B	57.9% (11/19)
Accuracy of Lab C	63.2% (12/19)
Average	59.6% (34/57)

Table 22. Results of the Phase II study

Chemical	CAS	Lab.A	Lab.B	Lab.C	concordance	T cell targeting
2,4-Diaminotoluene	95-80-7	N	N	N	1	No
Benzo(a)pyrene	50-32-8	S	S	S	1	Yes
Cadmium chloride	10108-64-2	N	N	N	1	Yes
Dibromoacetic acid	631-64-1	A/S	A	N	0	No
Diethylstilbestol	56-53-1	S	S	S	1	Yes
Diphenylhydantoin	630-93-3	N	N	N	1	Yes
Ethylene dibromide	106-93-4	N	N	N	1	Yes
Glycidol	556-52-5	A	A	A	1	Yes
Indomethacin	53-86-1	A	A	A	1	Yes
Isonicotinic Acid Hydrazide	54-85-3	S	N	S	0	Yes
Nitrobenzene	98-95-3	N	S	N	0	Yes
Urethane, Ethyl carbamate	51-79-6	A	A	A	1	No
Tributyltin chloride	1461-22-9	S	S	S	1	Yes
Perfluorooctanoic acid	335-67-1	A	A	A	1	Yes
Dichloracetic acid	79-43-6	A	S	S	0	Yes
Toluene	108-88-3	N	N	N	1	No
Acetonitril	75-05-8	N	N	N	1	undetermined
Mannitol	69-65-8	N	N	N	1	No

Vanadium pentoxide	1314-62-1	N	N	N	1	Yes
o-Benzyl-p-chorolophenol	120-32-1	S	S	S	1	No
Between-laboratory reproducibilities (%)					80 (16/20)	
Sensitivity (%)		61.5 (8/13)	61.5 (8/13)	61.5 (8/13)		
Specificity (%)		50.0 (3/6)	50.0 (3/6)	66.7 (4/6)		
Accuracy (%)		57.9 (11/19)	57.9 (11/19)	63.2 (12/19)		

S : Immunosuppression, A : Immunoaugmentation, N : No effect, A/S : Immunoaugmentation/suppression

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

Lab A		IL-2 Luc assay		Total
		+	-	
T cell targeting	+	8	5	13
	-	3	3	6
Total		11	8	19

Sensitivity : 61.5% (8/13)

Specificity : 50.0% (3/6)

Accuracy : 57.9% (11/19)

Lab B		IL-2 Luc assay		Total
		+	-	
T cell targeting	+	8	5	13
	-	3	3	6
Total		11	8	19

Sensitivity : 61.5% (8/13)

Specificity : 50.0% (3/6)

Accuracy : 57.9% (11/19)

Lab C	IL-2 Luc assay	Total

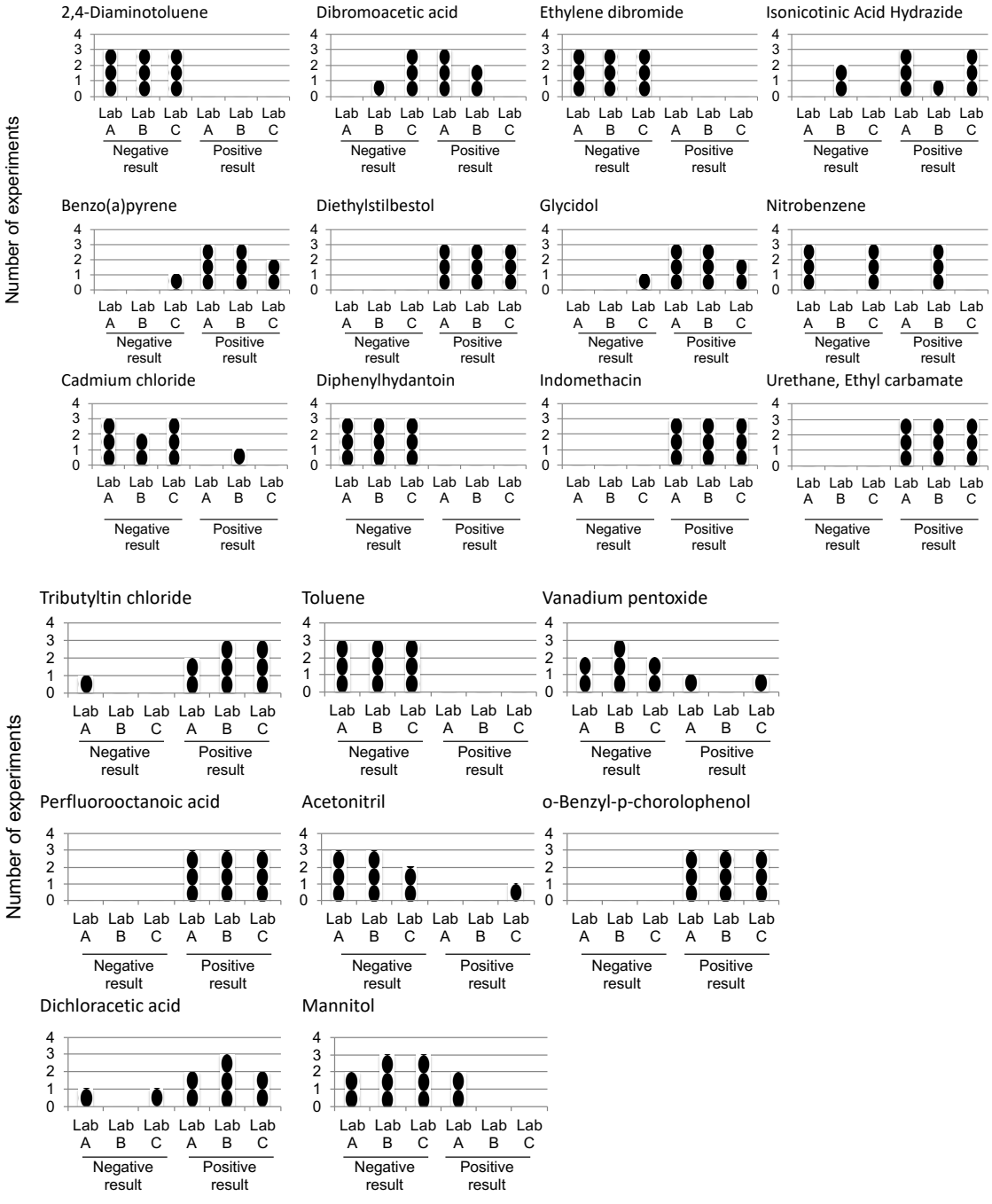
		+	-	
T cell targeting	+	8	5	13
	-	2	4	6
Total		11	4	19

Sensitivity : 61.5% (8/13)

Specificity : 66.7% (4/6)

Accuracy : 63.2% (12/19)

The graphical presentation of between- and within-laboratory variation in Phase II study is shown in Fig. 16.



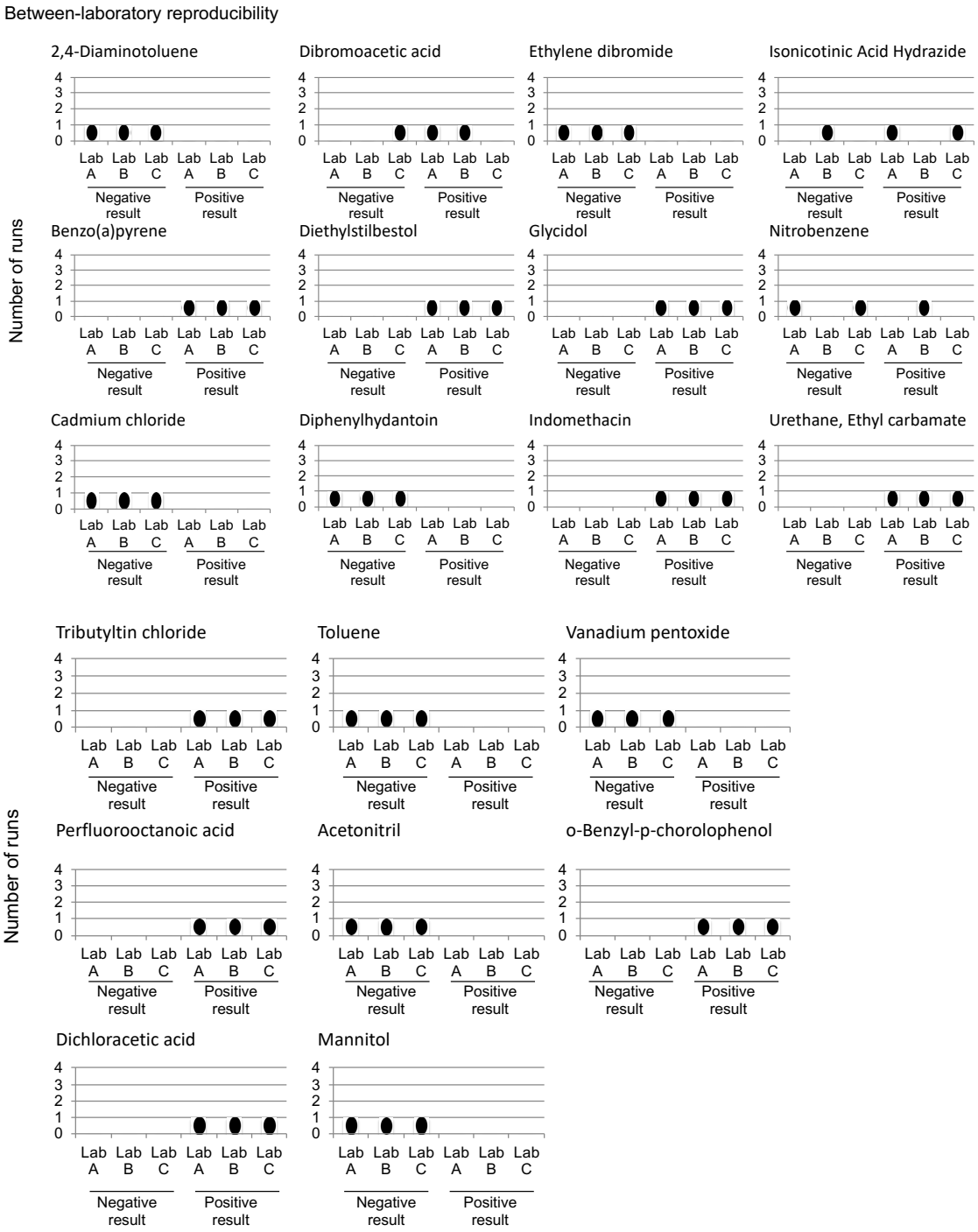


Fig. 16. Between variation assessments in Phase II study

The Phase II study examined between-laboratory reproducibilities using a total of 20 coded chemicals (13 T cell targeting, 6 non-T cell targeting and 1 undetermined) evaluated by 1 experiment sets based on Multi-Immuno Tox Assay protocol Ver. 009.1E. Closed circles represent the judgments for individual experiments in within-laboratory reproducibility or represent the judgments in individual experimental sets for between-laboratory reproducibility.

9-5. Quality assurance

All the records (data sheets and record sheets) from the participating laboratories were checked by Kobe univ. and JaCVAM, As a result, all record sheets on the maintenance of measuring instruments, the culture of cell line and the preparation and application of test chemicals were completed. JaCVAM considered these records had no effect on quality of data in the validation study.

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

9-6-1. Test conditions

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

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

Between-Lab reproducibility	80% (20/25)
Within-Lab reproducibility	Lab. A 80.0% (4/5)
	Lab. B 100% (5/5)
	Lab. C 80.0% (4/5)
	Average 86.7% (13/15)

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

Accuracy of Lab. A	62.5% (15/24)
Accuracy of Lab. B	66.7% (16/24)
Accuracy of Lab. C	70.8% (17/24)
Average	66.7% (48/72)

Table 23. Combined results of the Phase I and II studies

Chemical	CAS	Lab.A	Lab.B	Lab.C	concordance	T cell targeting
		Phase I				
Dibutyl phthalate	84-74-2	SSS	SSS	SSS	1	Yes

Hydrocortisone	50-23-7	SNN	SSS	SSN	0	Yes
Lead(II) acetate	6080-56-4	SSS	SSS	SSS	1	Yes
Nickel(II) sulfate	10101-97-0	SSS	SSS	SSS	1	Yes
Zinc dimethyldithiocarbamate (DMDTC)	137-30-4	NNN	NNN	NNN	1	No
		Phase II				
2,4-Diaminotoluene	95-80-7	N	N	N	1	No
Benzo(a)pyrene	50-32-8	S	S	S	1	Yes
Cadmium chloride	10108-64-2	N	N	N	1	Yes
Dibromoacetic acid	631-64-1	A/S	A	N	0	No
Diethylstilbestol	56-53-1	S	S	S	1	Yes
Diphenylhydantoin	630-93-3	N	N	N	1	Yes
Ethylene dibromide	106-93-4	N	N	N	1	Yes
Glycidol	556-52-5	A	A	A	1	Yes
Indomethacin	53-86-1	A	A	A	1	Yes
Isonicotinic Acid Hydrazide	54-85-3	S	N	S	0	Yes
Nitrobenzene	98-95-3	N	S	N	0	Yes
Urethane, Ethyl carbamate	51-79-6	A	A	A	1	No
Tributyltin chloride	1461-22-9	S	S	S	1	Yes
Perfluorooctanoic acid	335-67-1	A	A	A	1	Yes
Dichloracetic acid	79-43-6	A	S	S	0	Yes
Toluene	108-88-3	N	N	N	1	No
Acetonitril	75-05-8	N	N	N	1	undetermined
Mannitol	69-65-8	N	N	N	1	No

Vanadium pentoxide	1314-62-1	N	N	N	1	Yes
o-Benzyl-p-chorolophenol	120-32-1	S	S	S	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 (%)		64.7 (11/17)	70.6 (12/17)	70.6 (12/17)		
			Average 68.6 (35/51)			
Specificity (%)		57.1 (4/7)	57.1 (4/7)	71.4 (5/7)		
			Average 61.9 (13/21)			
Accuracy (%)		62.5 (15/24)	66.7 (16/24)	70.8 (17/24)		
			Average 66.7 (48/72)			

9-8-4. Contingency tables for the Phase II study

Lab A		IL-2 Luc assay		Total
		+	-	
T cell targeting	+	11	6	17
	-	3	4	7
Total		15	9	24

Sensitivity : 64.7% (11/17)

Specificity : 57.1% (4/7)

Accuracy : 62.5% (15/24)

Lab B		IL-2 Luc assay		Total
		+	-	
T cell targeting	+	12	5	17
	-	3	4	7
Total		15	9	24

Sensitivity : 70.6% (12/17)

Specificity : 57.1% (4/7)

Accuracy : 66.7% (16/24)

Lab C		IL-2 Luc assay		Total
		+	-	
T cell targeting	+	12	5	17
	-	2	5	7
Total		14	10	24

Sensitivity : 70.6% (12/17)

Specificity : 71.4% (5/7)

Accuracy : 70.8% (17/24)

10. Discussion

10-1. Reliability

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

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

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

10-2. Between- and within-laboratory reproducibility

We examined within-laboratory reproducibility in the Phase I study. Lab A, Lab B, and Lab C demonstrated 80%, 100%, and 80% reproducibility, respectively. On the other hand, Lab A, Lab B, and Lab C demonstrated 80% between-laboratory reproducibility in the combined data of the Phase I and Phase II studies. These results satisfied the acceptance criteria for the validation study with a within-laboratory reproducibility of at least 80% and a between-laboratory reproducibility of at least 80%.

10-3. Predictivity

To determine the predictivity of this assay, it is necessary to classify chemicals used in the validation study as immunotoxic or non-immunotoxic. In addition, since the IL-2 Luc assay focused on T cell response, we further classified immunotoxic chemicals into those that affect T cell function and those that do not. We therefore collected immunotoxicological information regarding the 25 test chemicals with the support of National Toxicology Program (NTP). The information for each chemical is provided in Appendix 5. The summarized data are shown in Appendix 6. The information for each chemical is composed of *in vivo*, *ex vivo*, and *in vitro* data. The *in vivo* data present the change in weight of immune system organs such as spleen and thymus, delayed hypersensitivity response, and the susceptibility to infection and resistance to transplanted tumors. The *ex vivo* data contain the effects of chemicals on cytokine production, T cell-dependent antibody response *in vitro*, as well as cytotoxic T cell response, mixed lymphocyte reaction, T cell mitogen-induced proliferation using cells from animals treated with the chemicals *in vivo*. The *in vitro* data provide the effects of the chemicals on cytokine production or on T cell proliferation after mitogen stimulation *in vitro*.

From these data we extracted the effects of the chemicals on the change of thymus weight in vivo, and T cell function ex vivo and in vitro. We also used their reported mode of action on T cell function as criteria to determine the immunotoxic effects of the chemicals on T cells. Based on these criteria, the 25 chemicals were classified into 16 immunotoxic chemicals, 8 non-immunotoxic chemicals, and 1 chemical with undetermined effects. 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 64.7%, 70.6%, 70.6%, and 68.6%, respectively. The specificities of the assays as conducted by Lab A, Lab B, Lab C, and their average are 57.1%, 57.1%, 71.4%, and 61.9%, respectively. The accuracies of the assays as conducted by Lab A, Lab B, Lab C, and their average are 62.5%, 66.7%, 70.8%, and 66.7%, respectively.

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

Based on the IL-2 Luc assay protocol (version 011E) and the Criteria 5, the lead laboratory reevaluated the data of 60 chemicals reported previously (Table 24). Similar to the classification by the criteria used in our published paper (Kimura et al., 2018), TAC, CyA, and Dex significantly suppressed IL-2 luciferase activity (IL-2 LA), although average LOEL of TAC and CyA was significantly lower that of DEX. The off-label immunosuppressive drugs, chloroquine, minocycline, and dapsone significantly suppressed IL-2 LA. Anti-cancer drugs, actinomycin D and cisplatin also significantly suppressed IL-2LA. In addition, azathioprine and colchicine were

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

If we calculated the predictivity of this assay based on the reported effects of the chemicals on the change of thymus weight in vivo, and T cell function ex vivo and in vitro that were obtained from the literature (Viora et al., 1996, Haley et al., 1990, Guo et al., 2001, Ulrich et al., 2004, Kobayashi et al., 2006, Wagner et al., 2006, Li et al., 2013, Kimura et al., 2014, Kimura et al., 2018), the sensitivity, specificity and accuracy (predictivity) are 84%, 56%, and 78%, respectively. In this calculation, we considered whether chemicals are targeting T cells or not irrespective of suppression or augmentation.

Table 24. Data set of the IL-2 Luc assay based on Criteria 5.

Chemical name	Judge	Ave.LOEL(35%)	Ave.LOEL(-35%)	Immunotoxicity in references
FK506	S	0.0002		S
Cyclosporine A	S	0.0041		S
Actinomycin D	S	0.0156		S
Digoxin	S	0.0686		N
Colchichine	S	0.2743		S
FR1667953	S	1.3021		S
Benzethonium chloride	S	1.6276		U
Mercuric chloride	S	1.9531		S
Chlorpromazine	S	1.9531		U
Amphoterycin B	S	2.6042		U
Dibutyl phthalate	S	2.6042		S
2-Aminoanthracene	S	5.8594		U
Formaldehyde	S	7.8125		S
Pyrimethamine	S	7.8125		S
Isophorone diisocyanate	S	15.6250		U
Cisplatin	S	16.9271		S
Cobalt chloride	S	16.9271		S
Chloroquine	S	17.8326		S
Minocycline	S	18.5185		S
Mitomycin C	S	20.0000		S
Hydrogen peroxide	S	23.4375		U
Citral	S	25.0000		U
Dexamethasone	S	41.1692		S
Pentamidine isethionate	S	52.0833		U
Lead(II)acetate	S	57.2917		S
Azathioprine	S	58.4778		S
Diesel exaust particle	S	62.5000		S
Sodium dodecyl sulfate	S	62.5000		N
Dapsone	S	72.9167		S
Nitrofurazone	S	83.3333		U
p-Nitroaniline	S	83.3333		U
Sulfasalazine	S	92.9444		S
Aluminium chloride	S	104.1667		S
Nickel sulfate	S	104.1667		S
Diethanolamine	S	250.0000		U
Chloroplatinic acid	S	250.0000		U
Sodium bromate	S	500.0000		S
Histamine	S	750.0000		S
Isoniazid	N			N
Triethanolamine	N			U
Magnesium sulfate	N			U
Hydrocortisone	N			S
Rapamycin	N			S
Mizoribine	N			S
Warfarin	N			N
2,4-Diaminotoluene	N			U
Cyclophosphamide	N			S
Dibenzopyrene	N			U
Ethanol	N			N
Hexachlorobenzene	N			S
Lithium carbonate	N			U
Methanol	N			N
Methotrexate	N			S
Dimethyl sulfoxide	N			N
Trichloroethylene	N			U
Mycophenolic acid	A		0.395061728	S
2-Mercaptobenzothiazole	A		16.11328125	N
Ribavirin	A		26.04166667	U
Nicotinamide	A		288.0658436	N
Acetaminophen	A		100	S

S: Suppression; N: No effects on T cells, U: Undetermined

U	Unclassified
S	Suppression, correctly judged
S	Suppression, misjudged
N	No effects, correctly judged
N	No effects, misjudged

Luster et al (Luster et al., 1993) proposed a screening battery using a 'tier' approach for detecting potential immunotoxic compounds in mice. In their study, they conducted the following immune test for approximately 30 to 50 chemicals, such as IgM plaque forming cell (PFC) response to sheep red blood cells, NK cell activity against YAC-I tumor cells, mixed leucocyte response (MLR) to allogeneic leucocytes, cytotoxic T-lymphocyte (CTL) response to P815 tumor

cells, T-cell mitogenic response to concanavalin A, B-Cell mitogenic response to lipopolysaccharide, delayed hypersensitivity response to keyhole limpet hemocyanin, surface marker expression including SIg, Thyl.2, CD4 and CD8, peripheral leucocyte counts, spleen cellularity (nucleated cells), thymus:body weight ratio, and spleen : body weight ratio. The conclusions derived from their study were as follows: (1) A good correlation exists between changes in the immune tests and altered host resistance, in that there were no instances where host resistance was altered without affecting one or more immune test(s). However, in some instances immune changes occurred without corresponding changes in host resistance. (2) No single immune test could be identified that was fully predictive for altered host resistance, although most assays were relatively good indicators (i.e. > 70%). (3) The ability to resist infectious agent challenge is dependent on the degrees of immunosuppression and the quantity of infectious agent administered. (4) Logistic and standard regression modelling using one extensive chemical data set from the immunosuppressive agent, cyclophosphamide, indicated that most immune function-host resistance relationships followed linear rather than linear~luadratic (threshold-like) models.

In their test, they obtained cells or organs from animals. Therefore, these tests are classified into ex vivo study. Using even these ex vivo studies, the top three predictivity values were 82% by delayed hypersensitivity, 76% by thymus/BW ration, and 74% by spleen/BW.

Considering these predictivity values by ex vivo tests and the limited target of the IL-2 Luc assay that is a high-throughput *in vitro* test, the performance of the IL-2 Luc assay might be acceptable.

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

Although the within- and between-laboratory reproducibilities satisfied the acceptance criteria for the validation study, the predictivity results were not satisfactory. We considered at least 2 reasons for the poor predictivity of the assay.

1) We collected as much immunotoxicological information on the chemicals as possible and determined whether or not the chemicals exhibited T-cell dependent immunotoxicity or not based on information in the peer reviewed literature. The criteria used for classification were the effects of the chemicals on the production of cytokines predominantly produced by T cells, *in vitro* or *ex vivo*, and their reported mode of action on T cell function. However, the information available was very limited for most chemicals and very little data had been reproduced by different laboratories. Therefore, the reliability of the criteria is sometimes uncertain.
2) It is reasonable to consider that the IL-2 Luc assay does not cover every aspect of the effects of the chemicals on T cell function. Using PMA + ionomycin de facto limits effects to chemicals

targeting calcium and PKC-mediated T cell activation. Other assays targeting T cell functions may be mandatory.

10-6. Limitations and drawback, and applicability domain of the modified IL-8 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 (Kimura et al., 2014). Thus, these chemicals in addition to chemicals that need metabolic activation need to 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 (Investigators, 1998) or *in vitro* myelotoxicity tests (Pessina et al., 2003).

10-7. Potential of the MITA

The MITA can evaluate the effects of chemicals on IL-2, IFN-γ, IL-1β, and IL-8 promoter activities. The induction of these cytokines is mediated by a wide range of signaling pathways, including the MAP kinase, NF-kB, and calcium/calmodulin pathways. It is also well known that the induction of different immune-related molecules such as cytokines or chemokines commonly use the same signaling pathways. Therefore, although MITA evaluate only the effects of chemicals on the transcription of 4 cytokines, it may be able to cover the effects of chemicals on much wider range of immune response.

Furthermore, the combination of the MITA with the IL-8 Luc assay can evaluate the effects of chemicals on T cells and macrophages, and on the sensitizing potential of chemicals. The data obtained from these assays can be used by both industry and regulatory agencies to assess the immunotoxicity risks of chemicals. At the least, the IL-2 Luc assay and the IL-8 or IL-1β Luc assay should be officially validated and a larger number of chemicals must be evaluated using the MITA to fully determine the potential and limits of this technique.

11. Conclusion

Using 3 luciferase reporter cell lines, we established the MITA in which the effects of chemicals on the IL-2 luciferase activity of 2H4 cells are evaluated in the presence of PMA/Io. In

addition, the effect of chemicals on the IL-1β and IL-8 luciferase activity of THP-G1b and THP-G8 cells, respectively, were examined in the presence of the stimulant LPS. Our final goal is to officially validate the MITA for within- and between-laboratory reproducibility and predictivity. In this validation study, we first conducted a validation study of the IL-2 luciferase reporter assay (IL-2 Luc assay). After confirming the transferability of the IL-2 Luc assay among different laboratories, we conducted Phase I and II studies. These studies produced satisfactory within- and between-laboratory reproducibilities. However, the predictivity was below 80%, possibly due to inadequate immunotoxicological data for several chemicals used in the validation, leading to misjudgment, and possibly due to the IL-2 Luc assay covering limited aspects of the effects of chemicals on T cell function.

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14. List of abbreviations.

95% CI : the 95% confidence interval

AIST : National Institute of Advanced Industrial Science and Technology

AOP : Adverse outcome pathway

ARE: Antioxidant response element

CAS No. : Chemical Abstract Service Number

CMV : Cytomegalovirus

CSC : the Chemical Selection Committee

DMSO : Dimethyl sulphoxide

DPRA : the Direct Peptide Reactivity Assay

ECVAM : the European Centre for Validation of Alternative Methods

EDTA : Ethylenediaminetetraacetic acid

EGFR : Epidermal growth factor receptor

EGR-1 : Early growth response-1

EU : European Union

FBS : Fetal bovine serum

FN : False Negative Rate

GLP : Good laboratory Practice

GSH : Glutathione

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

HSV : Herpes simplex viruses

ICCVAM : Interagency Coordinating Committee on the Validation of Alternative Methods

ID : Identification

IFN-γ : Interferon-γ

I.I.-SLR-LA : Inhibition index of SLR-LA

IL-2 : Interleukin-2

IL-8 : Interleukin-8

JaCVAM : the Japanese Center for the Validation of Alternative Methods

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

KoCVAM : Korean Center for the Validation of Alternative Methods

LLNA : Local lymph node assay

LPS : Lipopolysaccharide

MIT : Minimum induction threshold

MITA : Multi-Immuno Tox Assay

mMUSST : modified myeloid U937 dendritic cell activation test

MoDCs : Monocyte-derived dendritic cells

MOVS: Management Office of Validation Study

mRNA : messenger ribonucleic acid

MSDS : Material safety data sheet

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

NIHS : National Institute of Health Sciences

NPV : Negative predictive value

Nqo1 : NADPH-quinone oxidoreductase 1

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

nSLG-LA : normalized SLG luciferase activity

nSLO-LA : normalized SLO luciferase activity

OECD : the Organization for Economic Co-operation and Development

PCR : Polymerase chain reaction

PI : Propidium iodide

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

PN : False Positive Rate

PPV : Positive Predictive Value

QC : Quality Control

REACH : Registration, Evaluation, Authorization and Restriction of CHemicals

RFI : Relative fluorescence intensity

RT : Ring trial

SLG : Stable luciferase green

SLG-LA : SLG luciferase activity

SLO : Stable luciferase orange

SLO-LA : SLO luciferase activity

SLR : Stable luciferase red

SLR-LA : SLR luciferase activity

SLS : Sodium lauryl sulfate

SLR : Stable luciferase red

SLR-LA : SLR luciferase activity

SV40 : Simian virus 40

TG : Test Guideline

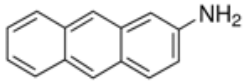
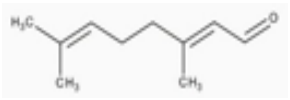
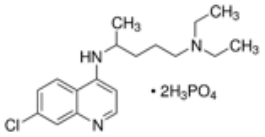
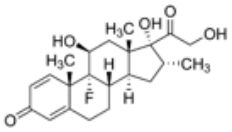
TNF-α : Tumor necrosis factor-α

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

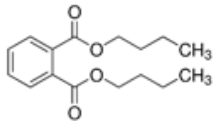
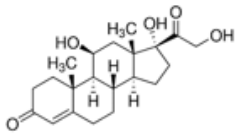
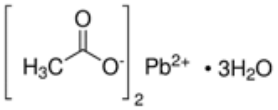
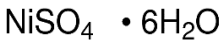
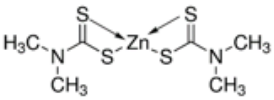
VMT : Validation Management Team

15. Appendixes

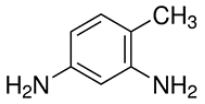
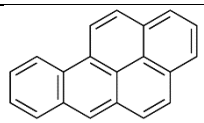
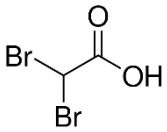
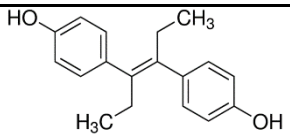
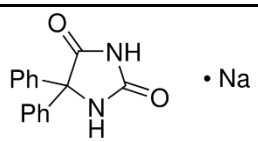
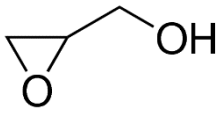
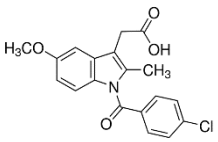
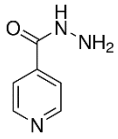
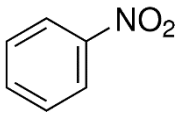
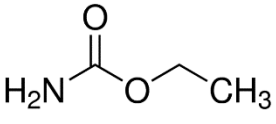
Appendix 1. Chemical structure of the test chemicals for Phase 0 study

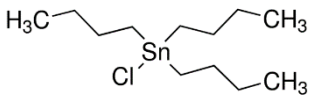
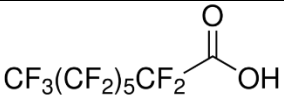
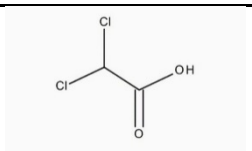
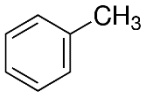
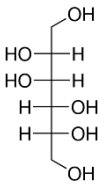
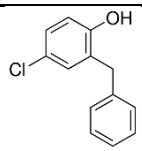
No.	Chemical name	CAS No.	Molecular weight	Chemical structure
0-1	2-Aminoanthracene	613-13-8	193.24	
0-2	Citral	5392-40-5	152.23	
0-3	Chloroquine diphosphate salt	50-63-5	515.86	
0-4	Dexamethasone	50-02-2	392.46	
0-5	Methylmercury(II) chloride	115-09-3	251.08	CH_3HgCl

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

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

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

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

II-13	Tributyltin chloride	1461-22-9	325.51	
II-14	Perfluorooctanoic acid	335-67-1	414.07	
II-15	Dichloroacetic acid	79-43-6	128.94	
II-16	Toluene	108-88-3	92.14	
II-17	Acetonitrile	75-05-8	41.05	CH_3CN
II-18	Mannitol	69-65-8	182.17	
II-19	Vanadium pentoxide	1314-62-1	181.88	V_2O_5
II-20	o-Benzyl-p-chlorophenol	120-32-1	218.68	

Multi-Immuno Tox Assay protocol ver. 011E

May. 10th, 2018

Department of Dermatology, Tohoku University Graduate School of Medicine
Yutaka Kimura, M.D., Ph.D.
Setsuya Aiba, M.D., Ph.D.

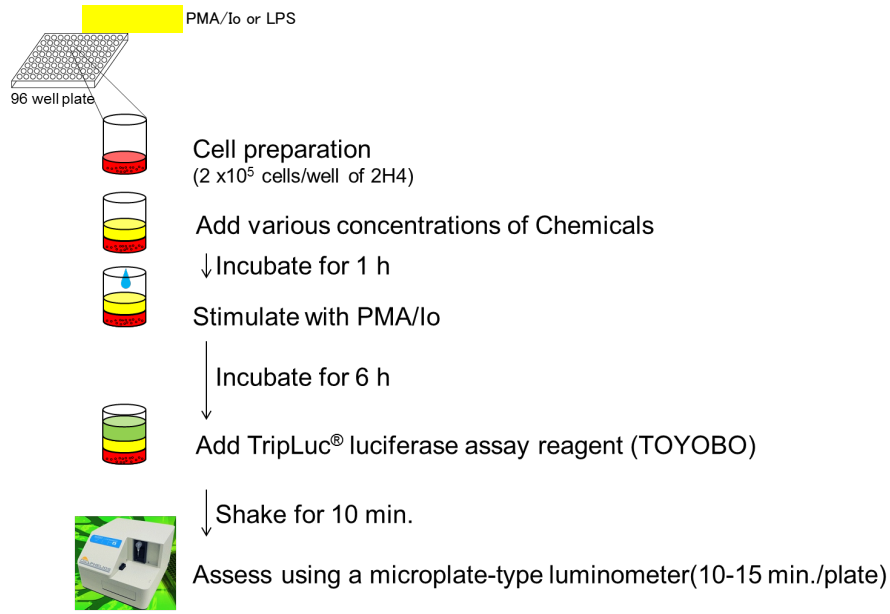
1. Introduction

This protocol describes how to maintain the cells, how to prepare the test chemicals, and how to measure the luciferase activity of 2H4 cells transfected with 3 luciferase genes, stable luciferase green (SLG), stable luciferase orange (SLO) and stable luciferase red (SLR), under the control of IL-2, IFNγ and G3PDH promoters, respectively, for the Multi-Immuno Tox Assay.
(Kimura Y. et al. Evaluation of the Multi-Immuno Tox Assay composed of 3 human cytokine reporter cells by examining immunological effects of drugs *Toxicol in Vitro*, 28, 759-768, 2014)

Figure 1

Assay design (2 chemicals per one plate)

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



2. Materials

2-1 Cells

2H4 (IL2-SLG、IFNγ-SLO、G3PDH-SLR)
The human acute T lymphoblastic leukemia cell line Jurkat was obtained from the American Type Culture Collection (Manassas, VA, USA). A Jurkat-derived IL-2 and IFNγ reporter cell line, 2H4, that harbors the SLG, SLO and SLR luciferase genes under the control of the IL-2, IFNγ and GAPDH promoters, respectively, was established by Tsuruga Institute of Biotechnology, TOYOBO Co. Ltd.
(Saito R. et al. Nickel differentially regulates NFAT and NF-κB activation in T cell signaling *Toxicology and Applied Pharmacology*, 254, 245–255, 2011)

2-2 Reagents and equipment

2-2-1 For maintenance of the 2H4 cells
RPMI-1640 (GIBCO Cat#11875-093, 500 mL)
FBS (Biological Industries Cat#04-001-1E Lot: 715004)
Antibiotic-Antimycotic (GIBCO Cat#15240-062)
HygromycinB (CAS:31282-04-9, Invitrogen Cat#10687-010)
G418 (CAS:108321-42-2, Nacalai Tesque Cat#16513-84)
Puromycin (CAS:58-58-2, InvivoGen Cat#ant-pr-1)

2-2-2 For chemical exposure, stimulation and solvents
Ionomycin (CAS:56092-82-1, Sigma Cat#I0634)
Phorbol 12-myristate 13-acetate (PMA) (CAS:16561-29-8, Sigma Cat#P8139)
Ethanol (e.g., Wako Cat#057-00456)
Dimethyl sulfoxide (DMSO) (CAS:67-68-5, Sigma Cat#D5879)
Distilled water (GIBCO Cat#10977-015)

2-2-3 For measurement of the luciferase activity
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 μ clear black plate (flat-bottom, for measurement of the luciferase activity, e.g. Greiner Bio-one Cat#655090)
96 well clear plate (round-bottom, for preparation of chemicals and stimulants)
96 well assay block, 2 mL (e.g., Costar Cat#3960)
Seal for 96 well plate (e.g., Perkin Elmer TopSeal-A PLUS Cat#6050185, EXCEL Scientific SealMate Cat#SM-KIT-SP)
Reservoir
Pipette

2-2-5 Equipment for measurement of luciferase activity
Measuring device: a microplate-type luminometer with a multi-color detection system that can accept two optical filter
e.g. Phelios AB-2350 (ATTO), ARVO (PerkinElmer), Tristar LB941 (Berthold)
Optical filter: 560 nm long-pass filter and 600 nm long-pass filter
Measuring time: set at 1〜5 sec/well measuring time

2-2-6 Others
Pipetman
8 channel or 12 channel pipetman (optimized for 10~100 μL)
Plate shaker (for 96 well plate)
CO_2 incubator (37°C, 5% CO_2)
Water bath
Cell counter: hemocytometer, trypan blue

2-3 Culture medium

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

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

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

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

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

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

2-4 Preparation of the stimulant of 2H4

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

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

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

2-4-2 Ionomycin

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

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

3. Cell culture

3-1 Thawing of 2H4 cells

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

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

3-2 Maintenance of 2H4 cells

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

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

4. Preparation of cells for assay

A cell passage should be done 2-4 days before the assay.
Use cells between 1 and 6 weeks after thawing.
Pre-warm the B medium in a 37°C water bath. Count the number of cells and collect the number of cells needed (2.0 x 10^7 cells for two chemicals are required, but to have some leeway, 3.0 x 10^7 cells for two chemicals should be prepared), centrifuge the tube at 120-350 x *g*, 5 min. Resuspend in pre-warmed the B medium at a cell density of 4×10^6/mL. Transfer the cell suspension to a reservoir, and add 50 µL of cell suspension to each well of a 96 well µclear black plate (flat bottom) using an 8 channel or 12 channel pipetman. (cf. Figure 2)

Figure 2

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

5. Preparation of chemicals and cell treatment with chemicals

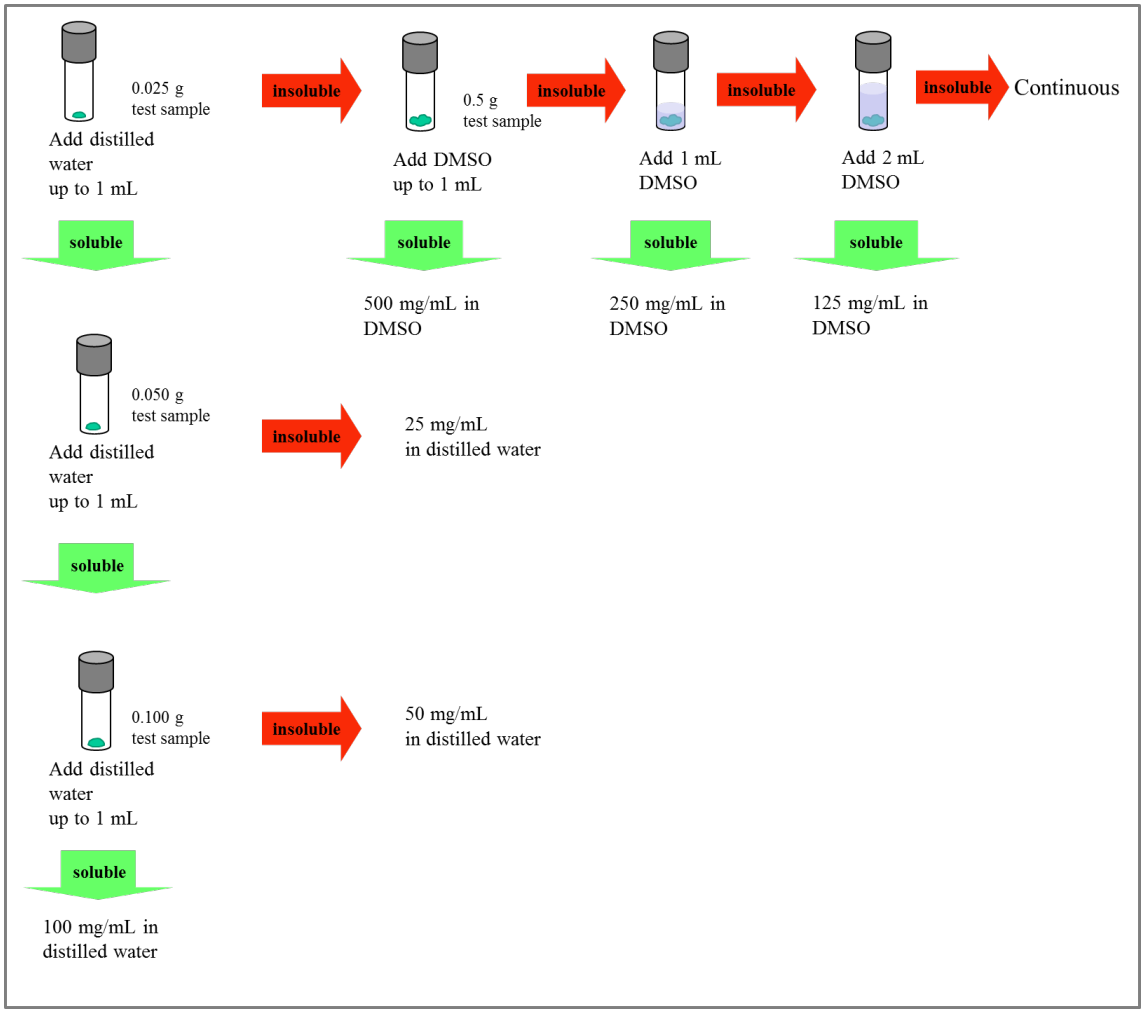
5-1 Dissolution by vehicle (cf. Figure 3)

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

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

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

Figure 3



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

In the second to fourth experiment (2nd to 4th experiment), determine the minimum concentration at which I.I.-SLR-LA (mentioned later in **10**) became lower than 0.05 in the 1st experiment, use the concentration one step (2-times) higher than this determined concentration as the highest concentration of the chemical to examine, and conduct 10 serial dilutions at a common ratio of 2 from the highest concentration. If I.I.-SLR-LA did not become lower than 0.05 or became lower than 0.05 at the highest concentration in the 1st experiment, conduct 10 serial dilutions at a common ratio of 2 from the highest concentration in the 1st experiment.
For example, in Figure 3 below, the minimum concentration at which I.I.-SLR-LA became lower than 0.05 is 1.95 μg/ml. The highest concentration of the chemical to examine is the concentration one step (2-times) higher than 1.95 μg/ml, which is 3.91 μg/ml.
In Figure 4 below, I.I.-SLR-LA did not become lower than 0.05. In such a case, the highest concentration of the chemical to examine is the highest concentration in the 1st experiment, namely 125 μg/ml.

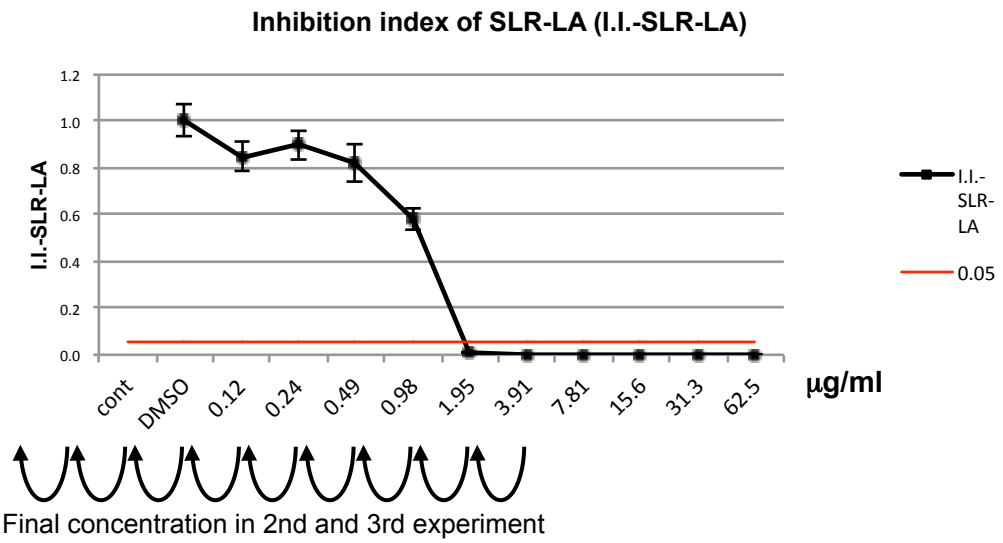


Figure 3.

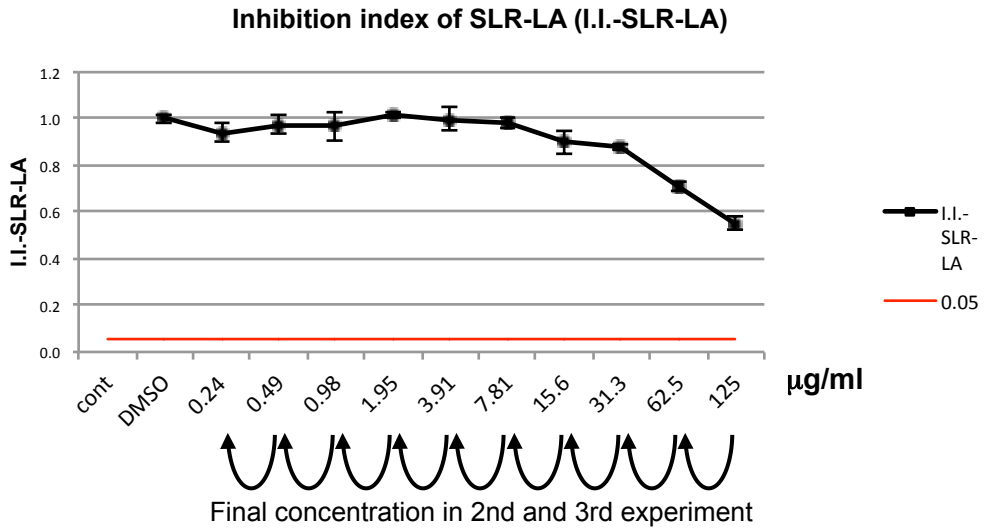


Figure 4

5-2 When the chemical is prepared in distilled water

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

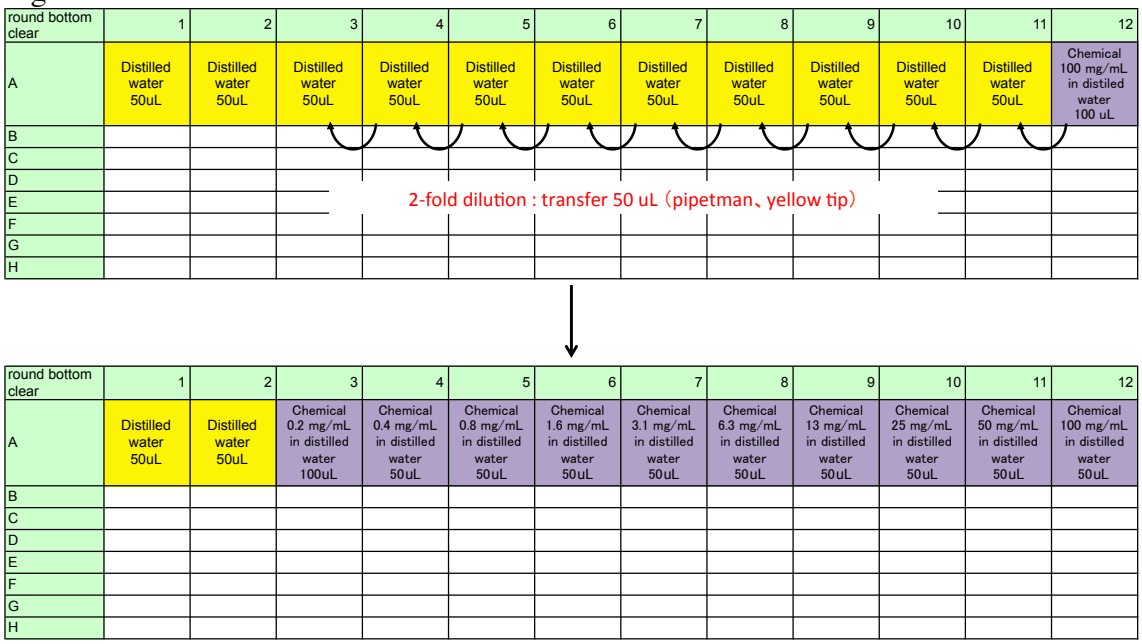
5-2-1 Arrangement of chemicals and vehicle

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

5-2-2 Serial dilution

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

Figure 4



5-2-3　2 step dilution

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

Figure 5

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

20uL

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

Figure 6

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 500uL	B medium 500uL	Chemical 0.008 mg/uL in B medium 500uL	Chemical 0.02 mg/mL in B medium 500uL	Chemical 0.03 mg/mL in B medium 500uL	Chemical 0.06 mg/mL in B medium 500uL	Chemical 0.1 mg/mL in B medium 500uL	Chemical 0.3 mg/mL in B medium 500uL	Chemical 0.5 mg/mL in B medium 500uL	Chemical 1 mg/mL in B medium 500uL	Chemical 2 mg/mL in B medium 500uL	Chemical 4 mg/mL in B medium 500uL
B												
C												
D												
E												
F												
G												
H												

50uL

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

Figure 7 Final constituents of each well of the plate

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

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

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

5-3-1 Arrangement of chemicals and vehicle

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

5-3-2 Serial dilution

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

Figure 8

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

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

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

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

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

Figure 9

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

Figure 10

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

10uL

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

Figure 11

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

50uL

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

Figure 12 Final constituents of each well of the plate

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

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

6-1 Material

・2 mM PMA stock
・2 mM Ionomycin stock
・B medium
・Ethanol

6-2 Preparation of 100 μM PMA

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

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

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

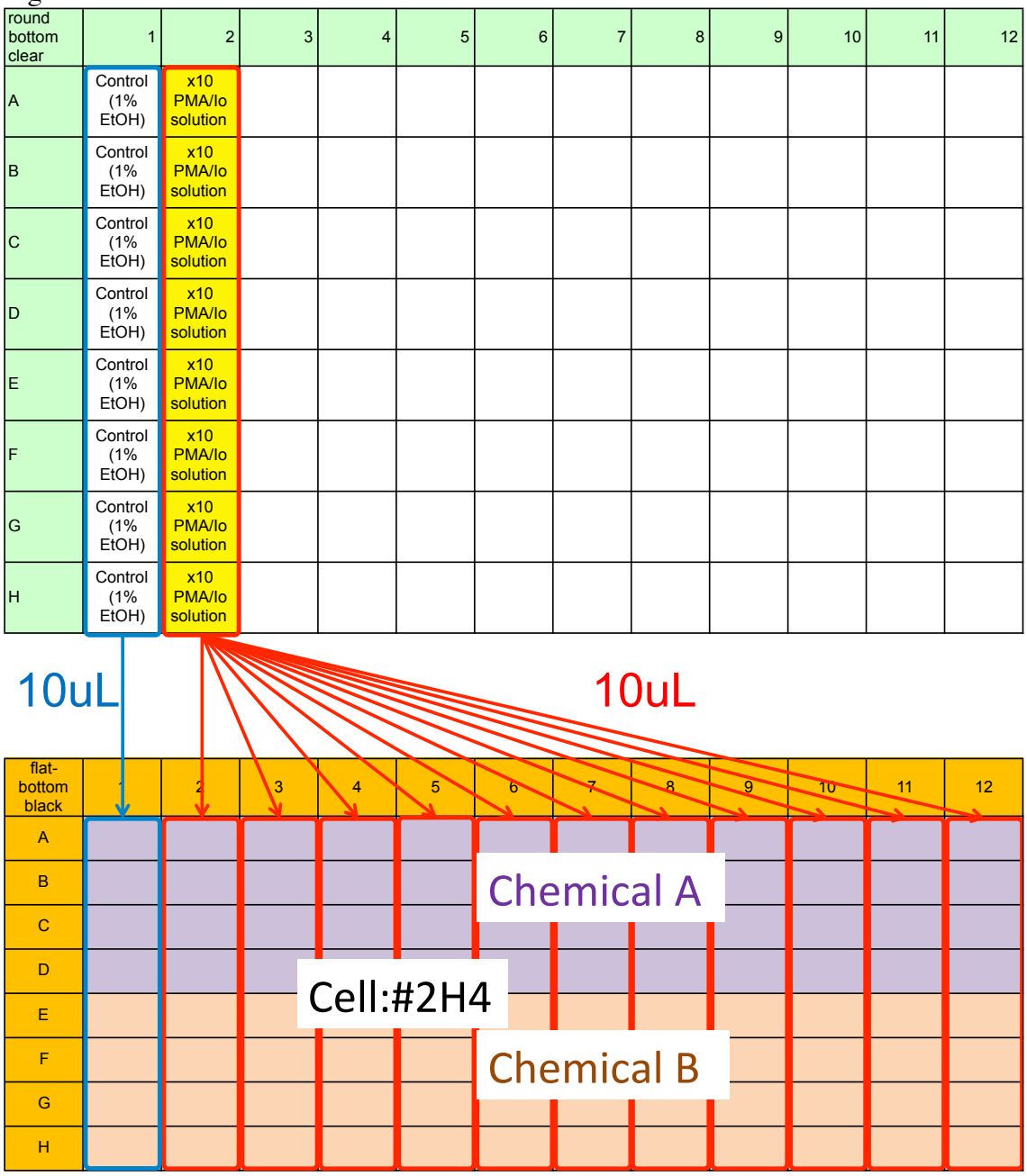
Dilute ethanol, 2 mM ionomycin and 100 μM PMA with the B medium to prepare control or x10 PMA/ionomycin solution. Add the control to well #A1-#H1 of the 96 well clear plate (round bottom), and add x10 PMA/ionomycin solution to wells #A2-#H2 of the 96 well clear plate (round bottom).

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

6-4 Addition of PMA/ionomycin to 2H4

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

Figure 13



7. Control

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

7-1-1 Preparing dexamethasone stock

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

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

7-1-2 Preparing cyclosporine A stock

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

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

7-2 Preparation of cells for assay

A cell passage should be done 2-4 days before the assay.
Use cells between 1 and 6 weeks after thawing.
Pre-warm the B medium in a 37°C water bath. Count the number of cells and collect the number of cells needed (5.0 x 10^6 cells are required, but to have some leeway, 7.5 x 10^6 cells should be prepared), centrifuge the tube at 120-350 x *g*, 5 min. Resuspend in pre-warmed the B medium at a cell density of 4×10^6/mL. Transfer the cell suspension to a reservoir, and add 50 µL of cell suspension to each well of a 96 well µclear black plate (flat bottom) using an 8 channel or 12 channel pipetman. (cf. Figure 14)

Figure 14

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

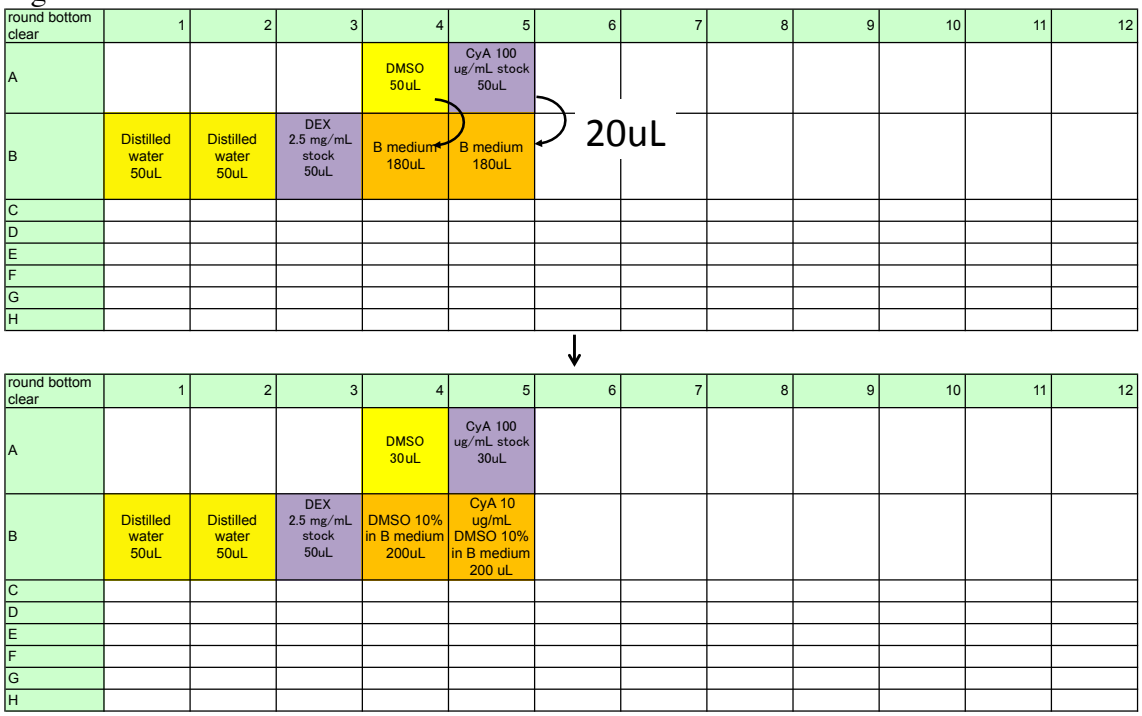
7-3 Arrangement of chemicals and vehicle

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

7-4 Dilution with the B medium

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

Figure 15



7-5 2 step dilution

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

Figure 16

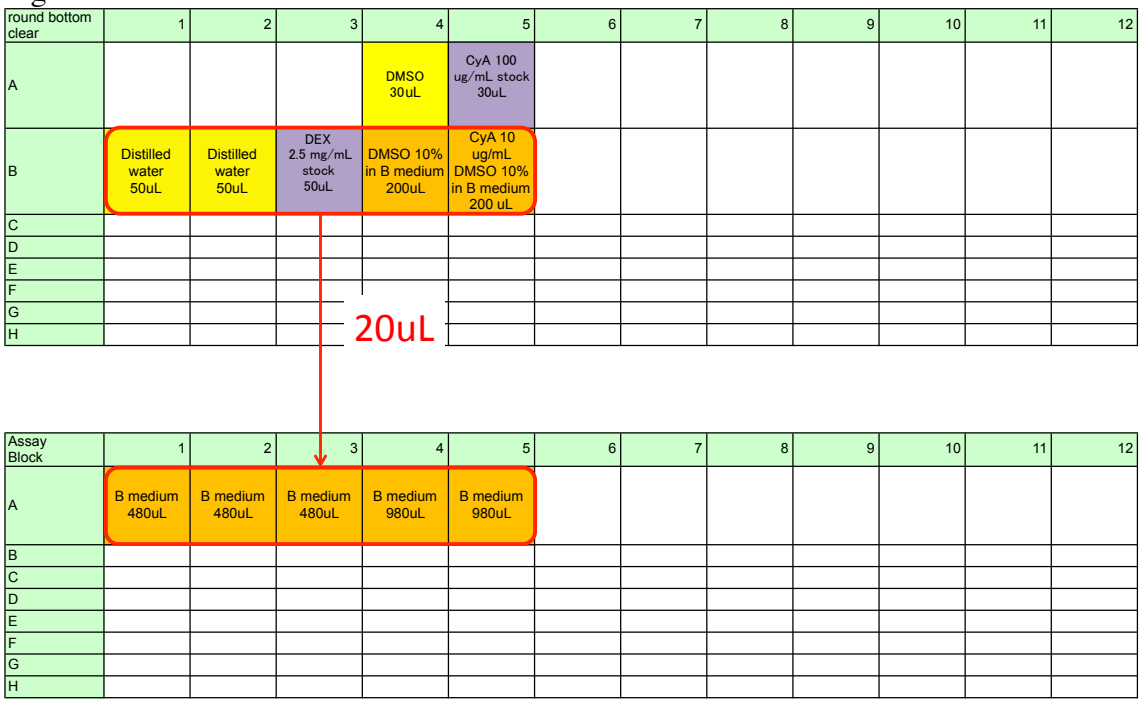


Figure 17

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

50ul

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

Figure 18 Final constituents of each well of the plate

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

7-6 Addition of PMA/ionomycin to 2H4

One hour after the addition of dexamethasone and cyclosporine A, add 10 µL of control or PMA/ionomycin solution prepared in §6-3 to the cells (#A1-#D1 or #A2-#D5, respectively) using an 8 channel or 12 channel pipetman after pipetting 20 times. Make sure that the apex of the tip is dipped into the medium. Change tips every line you add. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 6 hour (37°C, CO_2, 5%). (cf. Figure 19)

Figure 19

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Control (1% EtOH)	x10 PMA/Io solution										
B	Control (1% EtOH)	x10 PMA/Io solution										
C	Control (1% EtOH)	x10 PMA/Io solution										
D	Control (1% EtOH)	x10 PMA/Io solution										
E	Control (1% EtOH)	x10 PMA/Io solution										
F	Control (1% EtOH)	x10 PMA/Io solution										
G	Control (1% EtOH)	x10 PMA/Io solution										
H	Control (1% EtOH)	x10 PMA/Io solution										

10uL

10uL

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

8. Calculation of the transmittance factors

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

8-1 Reagents

Single reference samples:
Lyophilized luciferase enzyme reagent for stable luciferase green (SLG)
Lyophilized luciferase enzyme reagent for stable luciferase orange (SLO)
Lyophilized luciferase enzyme reagent for stable luciferase red (SLR)
Assay reagent:
Tripluc® Luciferase assay reagent（TOYOBO Cat#MRA-301）
B medium: for luciferase assay (30 mL, stored at 2- 8°C)

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

8-2 Preparation of luminescence reaction solution

Thaw Tripluc® Luciferase assay reagent (Tripluc) and keep it at room temperature either in a water bath or at ambient air temperature. Power on the luminometer 30 min before starting the measurement to allow the photomultiplier to stabilize.
Add 200 µL of 100 mM Tris-HCl (pH8.0) contains 10 % glycerol to each tube of lyophilized reference sample to dissolve the enzymes, divide into 10 µL aliquots in 1.5 mL disposable tubes and store in a freezer at -80°C. The stored frozen solution of the reference samples can be used for up to 6 months.
Add 1 mL of the B medium to each tube of frozen reference sample (10 µL sample per tube). Keep the reference samples on ice to prevent deactivation.

8-3 Bioluminescence measurement

Transfer 100 µL of the diluted reference samples to a black 96 well plate (flat bottom) as shown below.
Figure 20

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

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

Figure 22

Measurement without Filter

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

Measurement with Filter 1

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

Measurement with Filter 2

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

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

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

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

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

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

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

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

In the case shown above,

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

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

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

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

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

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

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

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

Figure 23

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

9. Measurement

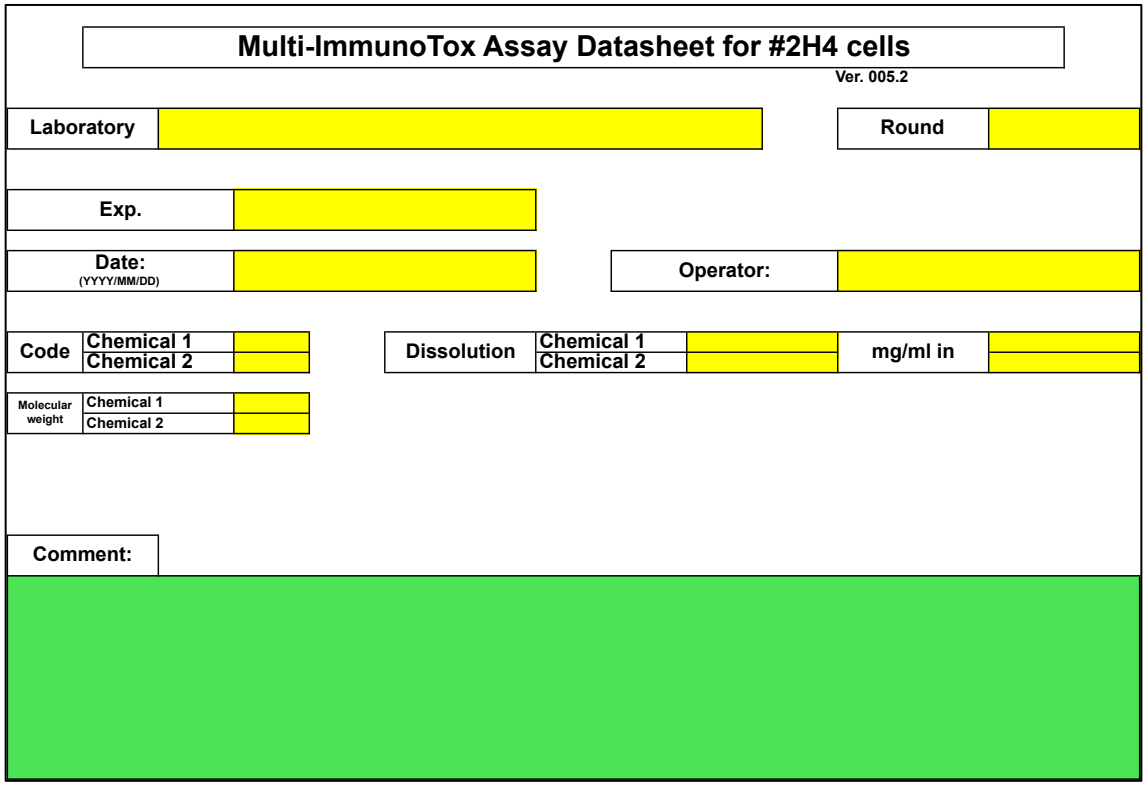
Please refer Appendix 1 for the principle of measurement of luciferase activity.

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

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

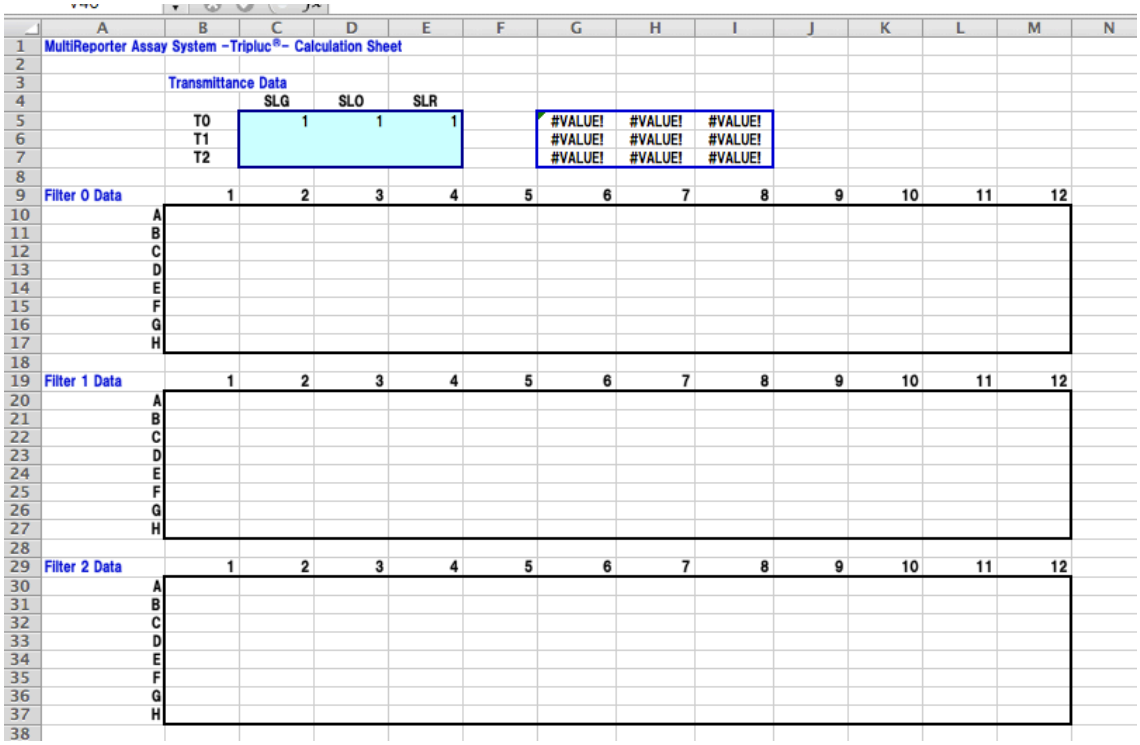
1st. Add the information regarding the name of laboratory, the round of experiments if multiple experimental sets are performed, the experiment number, date, the operator, chemical codes, dissolved in distilled water or DMSO, the prepared concentration, molecular weight of the chemicals and comments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

Figure 24 “Face Sheet” of the data sheet



2nd. Copy the results of the F0, F1 and F2 measurements (values are expressed as counts) and paste them into the appropriate area in the “Data Input” sheet of the data sheet shown below. In addition, input the transmittance factors calculated in "§5. Calculation of the transmittance factors" to #C6-#E7 of the “Data Input” sheet.

Figure 25 “Data Input” sheet of the data sheet



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

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

Figure 26 "Result Format" sheet of the data sheet

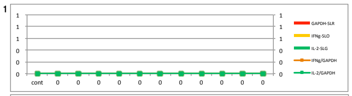
MultiReporter Assay System -Tripluc®- Calculation Sheet

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

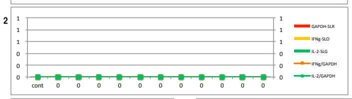
SLO-LA	1	2	3	4	5	6	7	8	9	10	11	12
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!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

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

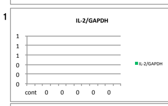
Chemical 1

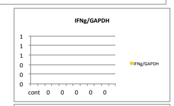


Chemical 2

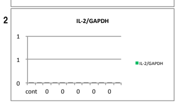


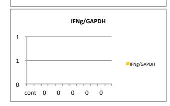
Chemical 1





Chemical 2





nSLG-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
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nSLO-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
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Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

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

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

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

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

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

10. Data analysis

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

SLG-luciferase activity (SLG-LA): Luciferase activity of stable luciferase green (Under the control of IL-2 promoter)
SLO-luciferase activity (SLO-LA): Luciferase activity of stable luciferase orange (Under the control of IFN-γ promoter)
SLR-luciferase activity (SLR-LA): Luciferase activity of stable luciferase red (Under the control of G3PDH promoter)
Normalized SLG-LA (nSLG-LA): =(SLG-LA)/(SLR-LA)
Normalized SLO-LA (nSLO-LA): =(SLO-LA)/(SLR-LA)
Inhibition index of SLR-LA (I.I.-SLR-LA): The cytotoxic effect of chemicals
=(SLR-LA of 2H4 treated with chemicals)/(SLR-LA of untreated 2H4)
%suppression: The effect of chemicals on IL-2 or IFN-γ promoter
=(1-(nSLG-LA or nSLO-LA of 2H4 treated with chemicals)
/(nSLG-LA or nSLO-LA of non-treated 2H4)) x 100

11. Criteria

11-1 Acceptance criteria

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

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

11-2 Criterion

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

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

In each experiment, when the chemicals clear the following 3 criteria, they are judged as suppressive or stimulatory. Otherwise, they are judged as no effect chemicals.
1. The mean of %suppression is $\geq$ 35 (suppressive) or $\leq$ -35 (stimulatory) with statistical significance. The statistical significance is judged by its 95% confidence interval.
2. The result shows two or more consecutive statistically significant positive (negative) data or one statistically significant positive (negative) data with a trend in which at least 3 consecutive data increase (decrease) in a dose dependent manner. In the latter case, the trend can cross 0, as long as only one data point shows the opposite effect without statistical significance.
3. The results are judged using only data obtained in the concentration at which I.I.-SLR-LA is $\geq$ 0.05

12. Update record

Ver. 0011.0E 2018.5.10
Change the criteria

Ver. 0010.0E 2018.1.15 distribution
Change the criteria

Ver. 009.1E 2017.5.8 distribution
Change the criteria

Ver. 009.0E 2017.4.7 distribution
Change the preparation of chemicals
Change the acceptance criteria
Change the criteria

Ver. 008.5E 2016.9.14 distribution
Change the criteria

Ver. 008.4E 2016.9.9 distribution
Change the criteria

Ver. 008.3E 2016.8.1 distribution
Correction of the preparation of PMA and ionomycin
Change the preparation of PMA and ionomycin
Change the preparation of controls
Addition of Acceptance criteria

Ver. 008.1E 2016.2.2 distribution
Changes after the VMT meeting

Ver. 008.0E 2016.1.19
Translation to English
Addition of appendix

Ver. 006.0J 2015.8.17
Change the preparation of chemicals (same method to the IL-8 Luc assay)
Delete the alteration in Ver. 005.0J

Ver. 005.0J 2015.1.9 distribution
Change to use SLR-LA of THP-G8 at the calculation of nSLG-LA of TGCHAC-A4

Ver. 004.1J 2014.12.10 distribution
Change the cellar concentration at cell passage
Modify figure 16, 17

Ver. 004.0J 2014.11.17 distribution
For the validation study at AIST, FDSC and Tohoku university (chemicals: Sodium Bromate ($NaBrO_3$), Nickel (II) sulfate ($NiSO_4$), Dibutyl phthalate (DP), 2-Mercaptobenzothiazole (2-MBT))
Change THP-G1b cells to TGCHAC-A4 cells
Change cell number of THP-G8 and TGCHAC-A4 5x10^4/well to 1x10^5/well
Change concentration of chemicals 11 steps to 10 steps

Change final concentration of LPS (THP-G8 : 25 ng/mL, TGCHAC-A4 : 1 ng/mL)
Change the way of addition of LPS (2 mL/well to 10 mL/well)
Change the criteria

Ver. 002.0J 2013.08.19 distribution
For the validation study at AIST and FDSC (chemicals: $CoCl_2$, $NiSO_4$, Isophorone diisocyanate, 2-Mercaptobenzothiazole)
Change the common ratio 3 to 2
Change the concentration of LPS 100 ng/mL to 25 ng/mL
Add description about the control (dexamethasone)
Delete the appendix about THP-G8 cell

Ver. 001.1J 2012. Nov. 13 distribution
Add the appendix about THP-G8 cell

Ver. 001J 2012. Nov. 09 distribution

Appendix 1 Principle of measurement of luciferase activity

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

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

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

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

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

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

transmitted light and iii) the intensity of 600 nm LP (Filter 2) transmitted light are described as below.

$$F0=G+O+R$$

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

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

These formulas can be rephrased as follows

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

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

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

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

Appendix 2　　Validation of reagents and equipment

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

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

5-1-1　Reagents

・Single reference samples:

Lyophilized luciferase enzyme reagent of SLG

Lyophilized luciferase enzyme reagent of SLO

Lyophilized luciferase enzyme reagent of SLR

・Assay reagent:

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

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

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

5-1-2 Calibration

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

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

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

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

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

5-1-2-2 Bioluminescence measurement

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

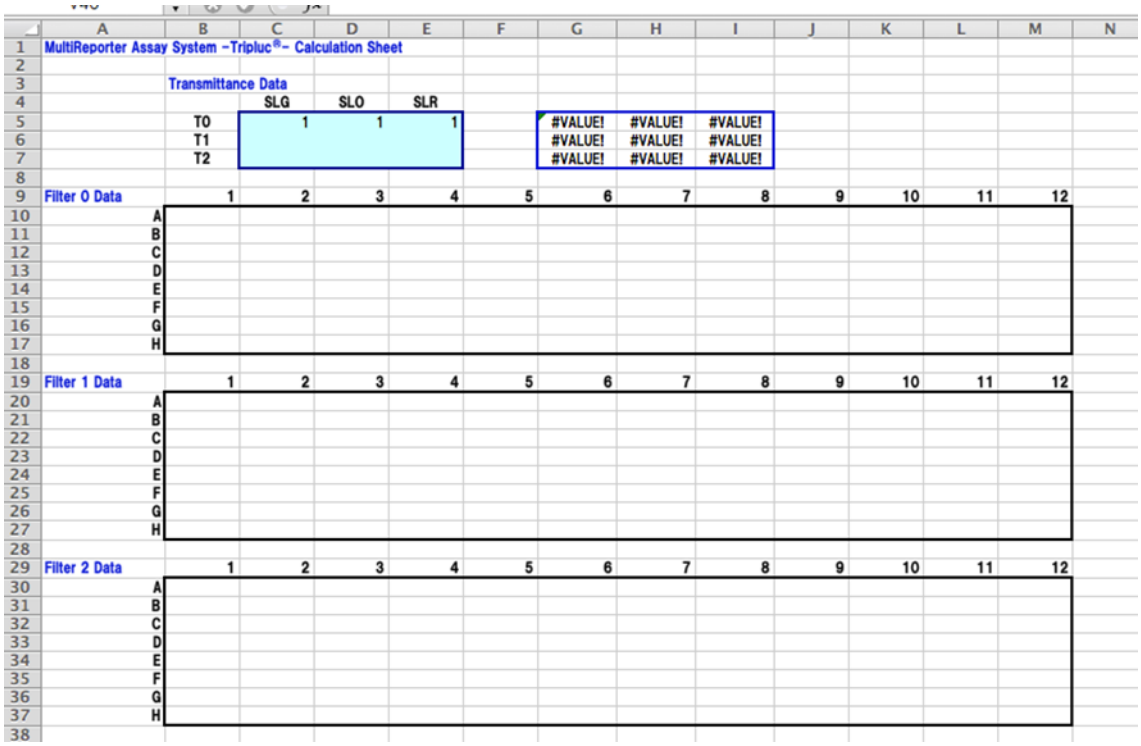
Figure 27

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

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

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

Figure 28



MultiReporter Assay System –Tripluc®– Calculation Sheet

Transmittance Data

	SLG	SLO	SLR			
T0	1	1	1	#VALUE!	#VALUE!	#VALUE!
T1				#VALUE!	#VALUE!	#VALUE!
T2				#VALUE!	#VALUE!	#VALUE!

Filter 0 Data	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

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

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

Record all the results for quality control.

5-2 Quality control of equipment

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

5-2-1 Light source

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

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

L12367 by Hamamatsu Photonics (Shizuoka, Japan)

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

1) Start luminometer 30 min before starting the measurement for stabilization of the photomultiplier.

2) Start LED plate and select “PMT” mode.

3) Select three-color (BRG) mode and adjust light intensity to 1/10 (10E-1).

4) Place the LED plate into the luminometer. Light intensity is measured for 3 sec each in the absence (F0) and presence (F2) of the optical filter.
5) Blue, green, and red LEDs are located at the position of #F6, #E6, and #D6, respectively. Copy the collected data of each position to the appropriate area on Sheet "LED" in the excel file of the data sheet.
6) Check the photo-detector performance by comparing with old data of the LED plate. For quality control purpose, every collected data should be recorded.
7) LED plate data typically fluctuates up to 1.5% (σ). Disagreement to the old data should be less than $3\times\sigma$ (= 4.5%).

Appendix 5. Immunotoxicological information of 25 chemicals used in the validation study

Table of Contents

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

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
No data were located.

Mode of action information
No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Female B6C3F1 mice were orally dosed with 25, 50, or 100 mg/kg DAT for 14 days. Absolute and relative (to body weight and to brain weight) liver weights (LOAEL = 100 mg/kg) were increased compared to controls. No effect on absolute or relative spleen weights were reported. However, trend analyses indicated significant changes in relative spleen weight and spleen/brain ratio in treated mice. Leukocyte and lymphocyte numbers also were increased (LOAEL =
100 mg/kg). The percentage of lymphocytes and polymorphonuclear leukocytes also were increased (LOAEL = 50 mg/kg). No changes in serum chemistry parameters (e.g., ALT levels) and bone marrow parameters (e.g., number of cells in the femur) were noted. The number of spleen cells, and percentage of T- and B-cells (LOAELs = 100 and 25 mg/kg, respectively) were altered in treated animals. While the number of spleen cells was decreased 18% at the highest dose tested, the percentage of T-cells and B-cells were increased 75% and 15%, respectively.
Peak IgM and IgG responses (in response to sheep erythrocytes) were observed on days 4 and 5 after immunization, respectively. DAT produced a dose-dependent decrease in IgM (46% at 100 mg/kg) and IgG (56% at 100 mg/kg) AFC responses based on total spleen activity. DAT exposure also produced a dose-dependent increase in delayed hypersensitivity response to keyhole limpet hemocyanin (2.2-fold increase at 100 mg/kg). Serum CH50 and C3 levels were not significantly affected in mice treated with DAT. The activity of the reticuloendothelial system was increased in the liver (LOAEL = 100 mg/kg), decreased in the spleen (LOAEL = 50 mg/kg) and kidney (LOAEL = 100 mg/kg), and not affected in the lung or thymus of treated mice. Decreased host resistance (LOAEL = 100 mg/kg) to *Streptococcus pneumoniae* and *Listeria monocytogenes*. However host resistance to B16F10 fibrosarcoma and PYB6 melanoma were not affected (Burns et al. 1994).

In vitro data with cells or cell lines

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

responses to T-cell mitogens PHA and ConA. An increase in responsiveness to LPS was reported in cells obtained from mice treated with 25 or 50 mg/kg, but not those treated with 100 mg/kg.
Spleen cellularity was decreased 20% and 15% at 50 and 100 mg/kg DAT. In response to DBA/2 cells, an enhanced response was observed in responder cells (LOAEL = 100 mg/kg) while no mixed lymphocyte response was noted (Burns et al. 1994).
Peritoneal exudate cells from female B6C3F1 cells treated with 25, 50, or 100 mg/kg DAT for 14 days were allowed to adhere to plastic and the percentage of cells phagocytizing fluorescent Covaspheres or chicken erythrocytes was measured. No significant change in the percentage of phagocytosis was noted at any of the doses (Burns et al. 1994).
Splenic NK cell activity was decreased in cells obtained from mice exposure to DAT for 14 days. A dose-dependent decrease was observed at all effector/target ratios tested (100/1, 50/1, and 25/1). The LOAEL was 50 mg/kg (Burns et al. 1994).
Spleen cell suspensions from female NMRI mice were evaluated to determine whether DAT could modulate luminol-dependent chemiluminescence of phagocytotic cells. Cells were treated with 0.01, 0.1, 1.0, 10, or 100 mg/L DAT. At concentrations greater than 1 mg/L, a dose-dependent decrease in response was observed. When compared to control levels, chemiluminescence was decreased 43%, 90%, and 100% at 1.0, 10, and 100 mg/kg, respectively (Thierfelder and Masihi 1995).

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

References
Burns LA, Bradley SG, White KL, McCay JA, Fuchs BA, Stern M, et al. 1994. Immunotoxicity of 2,4-diaminotoluene in female B6C3F1 mice. Drug and chemical toxicology 17:401-36; doi:10.3109/01480549409017865.
Thierfelder W, Masihi KN. 1995. Effects of trinitrotoluene (TNT) metabolites on chemiluminescence response of phagocytic cells. International journal of immunopharmacology 17: 453-6.

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

Human Data

Data from epidemiology studies
In a study of 51 epileptic patients, 20 of whom had not received anticonvulsant treatment for at least two years and 31 of whom had received DPH at 300 mg/24 hours for at least 4 months, the DPH treated group had decreased serum levels of IgA (156 ± 65 mg/100 mL) and IgM (121 ± 43 mg/100 mL) as compared to untreated epileptics (IgA, 179 ± 70 mg/100 mL; IgM, 133 ± 50 mg/100 mL) or control subjects (n= 15; IgA, 223 ± 49 mg/100 mL; IgM, 163 ± 48 mg/100 mL). Serum IgG levels were not statistically significantly different among the groups. The authors concluded that DPH treatment suppresses the normal function of the humoral immune response and that epilepsy may be a contributing factor (Badawy et al. 1991).
Peripheral blood lymphocytes, serum immunoglobulins and complement (C3 and C4) protein concentrations were determined in 20 patients with idiopathic epilepsy who were receiving 200- 300 mg DPH treatment and 30 healthy controls. A significant decrease in T-suppressor cells (28%) and subsequently higher T-helper to T-suppresor lymphocyte ratio (36%) were observed in DPH treated patients. A significant increase in B-lymphocytes (39%) and in serum IgM levels (data in graph) was also observed in DPH treated patients as compared to controls. No significant changes in serum concentrations of IgG, IgA or complement proteins was observed (Basaran et al. 1989).
Peripheral blood lymphocyte subsets, serum immunoglobulins and complement (C3 and C4) protein concentrations were determined in 40 healthy subjects, 30 DPH treated patients (200-300 mg/day), 22 carbamazepine treated patients, and 38 untreated epilepsy patients. Subjects receiving drug therapy had been taking the drug for 3 months up to 20 years. The DPH treated group had decreased IgA (19% and 24%, respectively) and IgG (16% and 14%, respectively) as compared to both healthy subjects and untreated epileptic patients. Significantly lower T-suppressor lymphocyte counts (23% decrease) was observed when compared to healthy controls. Significantly higher T-helper to T-suppressor lymphocyte ratio was observed when compared to healthy subjects and untreated epileptic patients. No significant differences in C3 or C4 protein levels were observed in DPH treated patients as compared to controls (Basaran et al. 1994).
Serum IgA values were determined in 191 patients taking DPH (dosage not provided). A reduction in serum IgA levels was observed in up to 20% of the patients. Cellular immune status was assessed in the 11% of patients with IgA values lower than two standard deviations below the mean and included: lymphocyte counts, lymphocyte population studies and responses to *in vitro* mitogen stimulation. No significant variations from control values were observed in any of the evaluated endpoints (Burks et al. 1989).

In vitro data with cells or cell lines
No data were located.

Mode of action information
DPH (20 µg/mL) induced IL-1 activity and potentiated LPS-induced IL-1 production in human PMBC and in U-937 cells, a stable monocytic cell line (Modeer et al. 1989).
DPH treatment can lead to a decrease of suppressor T cells and a reversible IgA deficiency in patients with epilepsy. Gingival overgrowth, which often develops in patients taking DPH, is hypothesized to be due to increased production of both IL-6 and IL-8, combined with elevations of basic fibroblast growth factor as observed *in vitro* using human gingival fibroblasts (Beghi and Shorvon 2011; Godhwani and Bahna 2016).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Male Balb/C mice were given DPH at doses of 0, 25, 50 or 100 mg/kg via oral gavage for 7 days. DPH significantly increased cellularity in the spleen (LOAEL = 25 mg/kg), however, both the direct and indirect plaque-forming cells responses following intraperitoneal injection with sheep erythrocytes, were significantly depressed (LOAELs = 25 mg/kg). A significant decrease in the delayed type hypersensitivity in response to sheep erythrocytes was also observed (LOAEL = 25 mg/kg) (Andrade-Mena et al. 1994).
Pregnant Balb/C mice were treated with DPH at doses of 0, 20, 40, and 60 mg/kg via oral gavage on days 9 through 18 of gestation. A dose-related suppression of humoral immune function (measured as the antibody response to type III pneumococcal polysaccharide) was observed in male and female offspring at 25 days, but not at 15 weeks of age (NOAEL = 20 mg/kg). Female offspring of dams treated with 20 or 60 mg/kg DPH had greater antibody levels than controls. No difference was noted in female offspring of dams treated with 40 mg/kg DPH when compared to controls. Cell-mediated immune function (as measured by delayed-type hypersensitivity response to oxazolone) was not affected in offspring of treated dams. Immunosuppressive effects also were greater in offspring born with an open eye defect, also attributed to DPH treatment (Chapman and Roberts 1984).
Female B10.s, B10.d2 and DBA/2 mice were injected with 2 mg DPH and received a single injection of 10 µg TNP-OVA subcutaneously into the right hind footpad. Popliteal lymph nodes (PLN) were isolated 7 days after injection. DPH increased the number of cells in all three strains (B10.s>B10.d2>DBA/2) (data in graph). IgG1 production to TNP-OVA was increased in all three mouse strains (in B10.d2 about 850-fold; and in B10.s and DBA/2 about 120-fold). DPH treatment did not facilitate immune complex deposition in any of the mouse strains, six days after challenge (Albers et al. 1999).
DPH (administered subcutaneously) produced a significant, dose-dependent response in the PLN assay at 0.5 mg (mean PLN index =1.60 ± 0.18) and 1.0 mg (mean PLN index = 2.79 ± 0.30) as compared to control (mean PLN index = 1.11 ± 0.24) in C3Hf mice. The maximal response occurred at 6-8 days post treatment and returned to normal after 3-4 weeks. The observed response was proposed to be T-lymphocyte dependent since only heterozygous C3H +/nu mice developed PLN enlargement whereas their congenitally athymic C3H nu/nu counterparts did not.

The PLN response to DPH was significantly amplified in thymectomized C57BL/10 mice (PLN index = 6.73 ± 0.83 vs. control PLN index = 2.93 ± 0.53). Proliferation of B lymphocytes was considered a major contributor to the PLN enlargement. A marked increase in IgM and IgG secreting cells was observed following inoculation of BALB/c mice with 1 mg DPH. A maximal increase was observed 10 days after treatment (Gleichmann et al. 1982).
Male C3H/HeN mice were given intraperitoneal injections of DPH (10 mg/mL, once per day) for 28 days and immunized with 100 µg KLH on day 14 and 21. Serum levels of anti-KLH IgG and IgE antibodies were determined on day 28. The KLH-specific IgE response was significantly increased compared to control (data in graph); the IgG response was not changed. Plasma ACTH and corticosterone were significantly higher in DPH-treated mice as compared to controls (data not provided) (Okada et al. 2001).

In vitro data with cells or cell lines
Splenocytes from DPH-treated mice (10 mg/mL for 28 days) immunized with KLH were cultured for 3 days with 50 or 100 µg/mL KLH. No effect on proliferation was noted in splenocytes from DPH-treated mice at either concentration of KLH. Comparatively, splenocytes from control mice immunized with KLH showed a potent proliferative response to stimulation with 50 or 100 µg/mL KLH. T cell function was also impaired in splenocytes from DPH-treated mice, in response to nonspecific mitogens (ConA and LPS) and in response to cross-linking of CD3. The accessory cell function (e.g. macrophages) was also impaired in spleen cells from DPH-treated mice. IL-4 production was significantly enhanced, while IFN-γ and IL-2 production, and NK cell activity were significantly reduced in spleen cells from DPH-treated mice (data in graphs or not provided). IL-1α production was decreased in spleen adherent cells from DPH-treated mice stimulated with *S. aureus*. No effect on IL-6 or IL-12 levels was reported (Okada et al. 2001).
The offspring of female C3H Orleans mice treated with 25 mg/kg diphenylhydantoin 2 times/day throughout gestation, exhibited a reduced thymic cortex and low mitotic activity in the lymphoid population. The reticuloepithelial tissue was enlarged. In the spleen, the white pulp was enlarged due to lymphocyte accumulation. The dams did not exhibit any changes in the thymus or spleen following treatment (Kohler et al. 1987).

Mode of action information
Heat shock proteins were not induced in the PLNs in female BALB/c mice injected subcutaneously with 2 mg DPH (Albers et al. 1996).
Male ICR mice injected intraperitoneally with 60 mg diphenylhydantoin for 3, 8 and 30 days exhibited elevated levels of serum glucocorticoids and thymic atrophy throughout the experiment (Hirai and Ichikawa 1991).

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Modeer T, Karsten J, Weintraub A, Gidlund M, Sundqvist KG. 1989. Phenytoin induces interleukin-1 production in vitro. Life sciences 44: 35–40.
Okada K, Sugiura T, Kuroda E, Tsuji S, Yamashita U. 2001. Phenytoin promotes Th2 type immune response in mice. Clinical and experimental immunology 124: 406–13.

Acetonitrile [CASRN 75-05-8]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
No data were located.

Mode of action information
No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
In F344/N rats exposed to acetonitrile by inhalation for 13 weeks, gross and histophathologic changes were evaluated in males (800 and 1600 ppm) and females (1600 ppm) that died during the study. Changes reported included thymic atrophy and splenic lymphoid depletion. Decreased absolute and relative thymus weights also were reported in male and female rats (LOAEL =
800 ppm). In F344/N rats exposed to 100, 200, or 400 ppm acetonitrile for 2 years, no immune related effects were reported (National Toxicology Program 1996)
In B6C3F1 mice exposed to acetonitrile by inhalation for 13 weeks, lymphoid depletion and lymphocytolysis in the thymus, spleen and bone marrow was reported in animals that died. A lack of immune effects were reported in mice exposed to acetonitrile for 2 years (NOAEL = 200 ppm) (National Toxicology Program 1996).
Based on a 14-day inhalation study in B6C3F1 mice (doses not provided), acetonitrile was not identified as an immunotoxicant (Luster et al. 1992).
Male Wistar rats were subcutaneously injected with acetonitrile at a dose of 0.8 LD50 (dose not provided). Antibody titer to sheep erythrocytes was decreased by 43%. Additionally, the number of antibody producing cells against sheep erythrocytes and Vi-Ag (no further information provided in article) were decreased by 52% and 27%, respectively. Thymus T-cell count, percentage of natural cytotoxicity (used as a surrogate for NK cell activity), and antibody- dependent cell cytotoxicity also were significantly decreased after acetonitrile exposure. The percentage decreases were calculated as 31%, 52%, and 41%, respectively (Zabrodskii et al.
2002).

In vitro data with cells or cell lines
No data were located.

Mode of action information
No data were located.

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

Human Data

Data from epidemiology studies
No data were located

In vitro data with cells or cell lines
B(a)P (1 μM) and related metabolites significantly increased IgE-mediated histamine release from human basophils, but did not induce cell death. Additionally, a B(a)P metabolite significantly increased IgE-mediated IL-4 production in human basophils (Kepley et al. 2003). In primary human macrophages, 10 μM B(a)P increased expression of TNF-α and IL-1β and produced no effect on IFNγ, IL-6, or IL-12 expression (Lecureur et al. 2005). Comparatively, B(a)P did not modulate IL-6 or IL-8 production in BEAS-2B cells at concentrations ranging from 0.1 to 10 μM (Chowdhury et al. 2017).
B(a)P inhibited anti-CD3 antibody stimulation of human lymphocyte proliferation (IC50 = 12.82 μM) (Carfi et al. 2007).
Six breast epithelial cell strains were incubated with 4μM B(a)P for 24 hours. Gene expression studies (using Hu-Gene 133A arrays) showed that signal log ratio (SLR) was altered by ≥1.5 for 5 immune-related genes in at least one of the tested cell strains. Four genes were upregulated, while one was down regulated. Up regulated genes were IL1B, MAL, HTLF, and SECTM1. CXCL14 gene expression was down-regulated (John et al. 2009).
PBMCs were exposed to ConA and B(a)P and assessed after 3 days. B(a)P dose-dependently decreased DNA synthesis and cell viability in treated cells (LOAELs = 0.01 and 0.1 μM, respectively). The number of cells recovered during the same period also was decreased (LOAEL = 0.01 μM). B(a)P did not affect IL-2 activity or expression of CD25 on small cells or blasts at concentrations up to 1 μM. B(a)P decreased the percentage of blasts that were CD71+ by 13% at 1 μM. Cell cycle analysis indicated that B(a)P increased the percentage of cells in S- phase and decreased the percentage in G0/G1 phase (Mudzinski 1993).

Mode of action information
Calcium mobilization in human T-cells is a proposed mode of action for B(a)P (Krieger et al. 1994). Additionally, Ah receptor activation by B(a)P is proposed to inhibit differentiation of monocytes to macrophages and cell growth of B-cells which may contribute to immunotoxic effects (Allan and Sherr 2005, 2010; van Grevenynghe et al. 2003).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Lactating C3H/HeJ dams were dosed with 0.25, 5.0, or 100 pmol/week B(a)P via oral gavage on PND 1, 8, and 15. Pups (5-weeks old) were treated with OVA via intratracheal instillation every

2 weeks for 6 weeks. B(a)P had no effect on the number of macrophages or lymphocytes in BAL from male or female offspring not treated with OVA. Additionally, no effect was noted on the number of macrophages or lymphocytes in B(a)P-treated offspring that were immunized with OVA (when compared with offspring only treated with OVA). IL-4, IL-5, IL-13, IL-33, and IFN-γ levels in the BAL were not affected in offspring not treated with OVA. Increased IL-33 and IFN-γ levels were observed in OVA-sensitized female offspring lactationally exposed to 5.0 and 100 pmol/week B(a)P, respectively. Lactational exposure to 0.25 B(a)P increased the total number of mediastinal lymph node cells in males. Lactational 100 pmol/week B(a)P increased numbers of TCRβ+ and CD86+ cells compared with vehicle in non-sensitized male offspring. In non-sensitized female offspring, lactational exposure to 100 pmol/week B(a)P increased numbers of CD11c+ PDCA-1-, CD28+, TCRβ+CD28+, MHC Class II+, and MHC Class II+CD86+ cells.
In OVA-sensitized female offspring, a significant increase in CD11c+PDCA-1+ and CD11c+PDCA-1- cells was observed after exposure to 0.25 and 5.0 pmol/week, respectively (Yanagisawa et al. 2018).
Pregnant C3H/HeB mice were administered 150 mg/kg B(a)P via intraperitoneal injection on GD 11; immune effects were assessed at parturition and again one week after parturition. A significant reduction in newborn CD4+CD8+ (46%), CD4+CD8+Vγ2+ (60%), and CD4+CD8+Vβ2+ (53%) thymocytes were noted. Additionally, CD4+ splenocytes from 1-week-old offspring were significantly reduced (50%) (Rodriguez et al. 1999).
B6C3F1 mice were administered 0.4, 4.0, or 40 mg/kg B(a)P by intratracheal instillation for seven days and immunized with sheep erythrocytes after the last B(a)P exposure. Decreased formation of antigen-specific AFC (by 60%) was observed at 40 mg/kg B(a)P in LALN. When sheep erythrocytes were administered by intraperitoneal injection, an increase in antigen-specific AFC was observed at 40 mg/kg B(a)P in LALN. However, the levels of AFC in the spleen were decreased (Schnizlein et al. 1987).
B6C3F1 mice (3-6 months, 13-16 months, and 23-26 months) were administered 40 mg/kg B(a)P for 8 days by intraperitoneal injection. Mice also were immunized with sheep erythrocytes after day 4 of the B(a)P treatment. Spleens were removed and splenocytes assessed for formation of AFCs. Decreased formation of AFCs was noted in splenocytes from all three age groups. In two sets of experiments, the observed decreases were 23%-43% in mice ages 3-6 months, 63%- 84% in mice ages 16-18 months, and 93% in mice ages 23-26 months (Lyte and Bick 1985).
B6C3F1 mice were administered 5, 20, or 40 mg/kg B(a)P for 14 days by subcutaneous injection. Spleens were removed and ConA-induced production of IL-2 and IL-3 were assessed. While splenocyte IL-2 production was decreased in a dose dependent manner, no effect on splenocyte IL-3 production was noted. As shown in other studies, B(a)P decreased responses to sheep erythrocytes (>95% inhibition). Addition of exogenous IL-2 to the treated splenocytes, reversed the B(a)P-induced inhibition of responses to sheep erythrocytes (Lyte et al. 1987; Lyte and Bick 1986).
Female B6C3F1 mice were administered 10 subcutaneous injections of B(a)P over a 14-day period at doses of 5, 20, or 40 μg/g. KLH-sensitization did not affect delayed hypersensitivity

responses at the tested doses. Additionally, B(a)P treatment did not induce rejection to DBA mice skin that was grafted onto mice. Proliferative responses to PHA were dose-dependently decreased (LOAEL = 20 µg/g B(a)P). Spontaneous and LPS-induced proliferative responses were increased at 5 µg/g B(a)P and significantly decreased at 40 µg/g B(a)P. MLC responses, and the percentage of spleen cells with T- and B-cell surface markers were not significantly affected at any of the tested doses. Additionally, NK cell activity against YAC- I target cells was not impacted in mice treated with 40 µg/g B(a)P (data not provided). Serum IgG levels were dose-dependently decreased in treated mice (18-24%). A reduction in the number of antibody plaque forming cells to sheep erythrocytes and LPS were noted (LOAELs = 20 and 5 µg/g B(a)P, respectively). B(a)P exposure decreased response to TNP-Ficoll without effects on TNP-LPS response. Host resistance studies showed that B(a)P had no effect on PYB6 tumor incidence or susceptibility to *L. monocytogenes* (Dean et al. 1983).

In vitro data with cells or cell lines
Rat and mouse spleen cells were treated with B(a)P for 24 hours and then LPS or PHA for 48 hours. Then, mitogen stimulation was assessed by 5-bromo-2'-deoxyuridine incorporation.
B(a)P inhibited cellular proliferation for both species at similar concentrations (data in graphs).
B(a)P also inhibited rat spleen proliferation that was stimulated by ConA (data provided in graph).
B(a)P inhibited anti-CD3 antibody stimulation of mouse lymphocyte proliferation (IC50 > 160 µM) (Carfi et al. 2007).
B(a)P decreased viability of mouse antigen presenting cells (APC) and increased expression of CD86 expression on APC (LOAELs = 0.1 µM). In murine splenocytes, B(a)P decreased cell viability and proliferation (LOAELs = 0.1 and 1.0 µM, respectively). B(a)P did not modulate the expression of T-cell receptors or CD19 at any of the tested concentrations in murine splenocytes (Chowdhury et al. 2017).
B(a)P decreased ConA induced cellular proliferation of mouse splenic T-cells in a dose-dependent manner (LOAEL = 0.1 µg/mL). Inhibition of IL-2, IL-4, and IFN-γ also was observed in ConA-stimulated splenic T-cells (LOAELs = 0.1, 0.2, and 0.1 µg/mL, respectively) (Guan et al. 2017).
B(a)P inhibited spleen cell response to sheep erythrocytes in a concentration dependent manner (LOAEL = 0.01 µM). B(a)P also inhibited one-way mixed lymphocyte response with a maximal inhibition of 19% (Urso et al. 1986). Similar response of murine spleen cell response to sheep erythrocytes was reported by Kawabata and White (1987) (LOAEL = 1 nM) after incubation for 5 days.
Splenocytes from B6C3F1 mice (3-6 months and 23-26 months) were exposed to 1, 10, or 50 µg/mL B(a)P and sheep erythrocytes for 4-5 days. After end of exposure period, the number of AFCs was determined. Dose-dependent decrease in the number of cells was observed in splenocytes from both age groups (data in graphs) (Lyte and Bick 1985).
B(a)P (in PVP-NaCl) dose-dependently increased LPS-induced IL-1 production by peritoneal exudate macrophages isolated from B6C3F1 mice; tested concentrations ranged from 25 to 800

μg/mL. A concurrent decrease in cell viabilities was noted at the same test concentrations. Comparatively, when B(a)P was dissolved in corn oil no effect on IL-1 production or cell viabilities was noted (Lyte and Bick 1986).

Mode of action information

Disruption of T-cell activities has been associated with B(a)P induced immunotoxic effects (Urso et al. 1986). Modulation of mouse splenic T-cell effects was associated with modulation of calcium levels; which was associated with suppression of the NF-□B and NFAT pathways (Guan et al. 2017).

In addition to T-cell effects, modulation of B-cell population or responses, or macrophage functions also have been implicated in B(a)P mode of action (Saxena et al. 2018; Urso et al. 1986). Hardin and colleagues (1992) proposed that B(a)P-induced suppression of B-cell lymphopoiesis was, partially, produced through induction of programmed cell death. Ah-receptor dependent- and/or independent-pathways could produce the observed effects.

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Cadmium Chloride [CASRN 10108-64-2]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
Cadmium chloride (10–100 µM) inhibited NK (against K562 cells) and antibody-dependent cellular (against P815 cells) cytotoxicity (ADCC) in peripheral blood lymphocytes in a concentration-dependent manner. The estimated 50% inhibition doses (ID50) for NK and ADCC activities were 50 and 100 µM, respectively. NK and ADCC activities were not significantly affected by changing the effector cell:target cell ratios. Cadmium chloride also inhibited cytotoxic activity against K562 or Daudi cells in activated IL-2 cells (data in graph). Time-course studies showed that a significant decrease in NK and ADCC activities was observed when added at 90 minutes after the start of the experiment (Cifone et al. 1990).
Viability of A549 cells was decreased (44.5% of control) after exposure to 75 µM cadmium chloride. At the same concentration, cadmium chloride increased select cytokine levels (e.g., IFN-γ, IL-3, IL-5, IL-10, IL-15, and IL-16). Comparatively, cadmium chloride decreased TGF-β3 levels (Odewumi et al. 2016).

Mode of action information
In vitro studies suggest that in peripheral blood lymphocytes, cadmium chloride modulated phosphoinositide hydrolysis induced by a target molecule. This modulation is proposed to lead to inhibited NK activity (Cifone et al. 1990).
Proposed direct action of cadmium on immunocompetent cells stimulates production and release of cytokines, which may produce proinflammatory effects (Marth et al. 2000).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Female BDF1 mice were provided drinking water containing 5, 10, or 50 µg/mL cadmium (as cadmium chloride) for 3 weeks. Antibody response to sheep erythrocytes was decreased in a dose-dependent manner. Splenic plaque-forming cell number decreased by 16% to 28%. A dose-dependent increase in LPS-induced proliferation also was observed (LOAEL = 10 µg/mL). In the absence of a mitogen, cadmium chloride also increased lymphocyte proliferation (LOAEL = 10 µg/mL). No effect was observed when ConA mitogen was used to stimulate proliferation (Blakley 1985).
Female CD1 mice were provided drinking water containing 5, 10, or 50 µg/mL cadmium (as cadmium chloride) for 3 weeks. *In vivo* T-lymphocyte independent (against DNP-Ficoll) and T-

lymphocyte and macrophage independent (against *E. coli*) responses were increased by cadmium exposure (Blakley and Tomar 1986).
Female BDF1 mice were provided drinking water containing 50 µg/mL cadmium (as cadmium chloride) for 3 weeks. Spleen cell suspensions from pooled spleens were separated by adherence techniques and antibody production against sheep erythrocytes was assessed. Suppressed antibody production (26%–34%) was noted in cultures that contained cadmium–exposed T– lymphocytes. Antibody production was similar to controls in cultures that contained cadmium– exposed macrophages (Blakley and Tomar 1986).
Male Sprague–Dawley rats were administered 0.7 or 6 mg/kg cadmium (as cadmium chloride) by oral gavage for 28 days. Splenocyte proliferation was significantly decreased (76% of control) in rats that were administered 6 mg/kg cadmium. Splenocyte IL–2 production also was increased after administration of 6 mg/kg cadmium, when production was normalized with cell number.
No effect was noted on splenocyte IFN–γ production (Wang et al. 2017).
Immunotoxic effects in offspring were noted after exposure to cadmium chloride *in utero* or through milk. In ICR mice administered 2.5 or 5.0 mg/kg cadmium chloride on GD 16, a significant increase in offspring spleen weight was reported (LOAEL = 2.5 mg/kg). Unstimulated spleen lymphocyte proliferation was significantly increased at both tested doses (1.5– to 2–fold). Additionally, ConA, PHA, and LPS stimulation was increased in treated animals (LOAELs = 5.0, 5.0, and 2.5 mg/kg). No effect on delayed–type hypersensitivity to sheep erythrocytes was reported, but an increase in total Ig and IgM antibody titer was noted at 2.5 mg/kg (Soukupova et al. 1991). In offspring that were exposed to cadmium chloride through maternal milk (dams received 5 ppm or 10 ppb cadmium chloride in water until weaning) decreased spleen weights were observed in females, but not males (data in graphs). The effect was greater in lower dosed females. Effects on organ weight did not persist to adulthood. In adult and juvenile rats, effects on cytotoxic activity of splenic NK–cells was noted (data in graphs).
Additionally, cadmium chloride inhibited ConA–induced thymocyte proliferation in both male and female adult rats (Pillet et al. 2005).
Female C57BL/6 mice were exposed (nose–only) to aerosolized cadmium chloride (60–minute exposure to 0.88 mg Cd/m^3) and examined 5–18 days later. Decreased splenic cell viability was observed (data in graph). Significant decreases of proliferative responses to LPS and PHA, and inhibition of IgM secretion in response to sheep erythrocytes were observed. Comparatively, oral chronic exposure (5, 100, or 300 ppm cadmium chloride in water for 12–16 weeks) suppressed IgM response to sheep erythrocytes, without effects on cell viability (Krzystyniak et al. 1987).

In vitro data with cells or cell lines
Splenocytes isolated from male Sprague–Dawley rats were treated with ConA for 24 hours, followed by incubation with 5, 10, or 20 µM cadmium chloride for 4 or 24 hours. After exposure for 4 hours, decreased IL–2 (LOAEL = 5 µM) and IFN–γ (LOAEL = 10 µM) production was observed in the absence of effects on cell proliferation. After exposure for 24 hours, decreased IL–2 (LOAEL = 5 µM) production and cell proliferation (LOAEL = 10 µM) were observed.

When cytokine production after 24-hour exposure was normalized based on cell number,

increased IFN-γ production (LOAEL = 10 μM) was noted. For IL-2 production, a significant decrease was noted at 5 μM and an increase was noted at 20 μM (Wang et al. 2017).

Mode of action information

In RAW264.7 cells, cadmium chloride upregulation of COX-2 and MIP-2 was associated with activation of the phosphatidylinositol 3-kinase/Akt signaling pathway (Huang et al. 2014). Cadmium chloride also may induce overstimulation of nuclear factors of activated T-cells to activate Jurkat T cells (Colombo et al. 2004).

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

Human Data

Data from epidemiology studies
No studies were located.

In vitro data with cells or cell lines
In cultured PBMCs collected from healthy, non-smoking volunteers and cultured in DBAA for four hours, DBAA increased the percentage of necrotic human PBMC and decreased PBMC cell size (LOAEL = 5 mM). Increases in the percentage of apoptotic cells and PBMC granulation also was reported (LOAEL = 1 and 5mM, respectively). Caspase-8, -9, and -3 expression were upregulated at 1 and 5 mM. Increased transmembrane mitochondrial potential and levels of reactive oxygen species (ROS) also were noted with DBAA exposure (LOAEL = 1 and 0.1 mM) (Michalowicz et al. 2015).

Mode of action information
DBAA may increase ROS levels and transmembrane mitochondrial potentials (Michalowicz et al. 2015).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
In female F344/N rats exposed to DBAA for 3 months (0-2000 mg/L in drinking water), minimal to mild hematopoietic cell proliferation was noted at the highest dose. A similar effect was not observed in males. While no spleen effects were noted in B6C3F1 mice exposed to DBAA for 3 months (0-2000 mg/L in drinking water), thymus atrophy was reported in males and females (LOAEL = 1000 and 2000 mg/L, respectively) (National Toxicology Program 2007).
In male and female BALB/c mice orally gavaged with 5, 20, or 50 mg/kg DBAA for 28 days altered spleen and thymus weights, and splenic and thymic cellularity were reported. DBAA also inhibited B-cell proliferation (LOAEL = 20 mg/kg). DBAA increased T-cell mitogenesis (value not provided) at 20 mg/kg. DBAA increased apoptosis in spleen and thymus in a dose-dependent manner (values not provided). Additionally, DBAA exposure altered the expression of apoptosis- related genes in spleens and thymus of treated mice. In the thymus, expression of Fas and TRAF2 were altered (2-2.5 fold). In spleens of treated mice, expression of Fas and TRAF2 were increased 5-fold while bcl-2 expression was decreased 1.5-fold. Increased protein expression of Fas and FasL also were observed in spleen and thymus of treated mice (LOAEL = 5 mg/kg) (Gao et al. 2008).

Table 1. Data from Gao et al. (2008)

Endpoint	0 mg/kgs	5 mg/kg	20 mg/kg	50 mg/kg
Male				
Spleen weight (mg)	80.5 ± 2.7	88.7 ± 3.7	91.2 ± 3.4**	94.0 ± 2.5***
Thymus weight (mg)	43.1 ± 3.4	37.6 ± 2.0	33.3 ± 2.8**	33.4 ± 2.3**
Relative spleen weight (mg/g)	3.68 ± 0.14	3.87 ± 0.22	4.18 ± 0.20*	4.23 ± 0.09*
Relative thymus weight (mg/g)	1.85 ± 0.14	1.65 ± 0.09	1.50 ± 0.13**	1.50 ± 0.11**
Splenic cellularity ($x10^7$)	9.00 ± 0.44	9.09 ± 0.28	7.63 ± 0.65	6.11 ± 0.38***
Thymic cellularity ($x10^7$)	8.88 ± 1.06	9.16 ± 0.28	7.39 ± 0.47	5.37 ± 0.82**
Female				
Spleen weight (mg)	78.9 ± 2.2	100.1 ± 7.7**	102.4 ± 5.0**	101.2 ± 4.8**
Thymus weight (mg)	46.9 ± 3.7	47.3 ± 3.9	35.8 ± 2.3*	29.5 ± 3.3***
Relative spleen weight (mg/g)	3.99 ± 0.18	5.33 ± 0.45**	5.29 ± 0.27**	5.22 ± 0.19**
Relative thymus weight (mg/g)	2.39 ± 0.17	2.41 ± 0.18	1.87 ± 0.11*	1.55 ± 0.17***
Splenic cellularity ($x10^7$)	8.60 ± 0.55	8.28 ± 1.19	6.14 ± 1.27	4.65 ± 0.43**
Thymic cellularity ($x10^7$)	7.97 ± 0.53	7.08 ± 0.74	5.42 ± 0.79*	4.28 ± 0.39***

Data are presented as mean ± SEM.

***p < 0.001, **p < 0.01, *p < 0.05, significance assessed by ANOVA when compared with control group (DBA 0 mg/kg).

Increased neuronal expression of immune factors was noted in Sprague-Dawley rats administered 20, 50, or 125 mg/kg DBAA via intragastric injection for 4-weeks. mRNA expression of Iba-1, NK-□B, IL-6, IL-1β, and TNF-α were increased in the pre-frontal cortex and hippocampus of treated rats (LOAEL = 50 mg/kg for all brain regions). Protein levels of Iba-1, NK-□B, IL-6, IL-1β, and TNF-α also were significantly increased in the same brain regions. Protein expression LOAEL in the pre-frontal cortex for Iba-1, NK-□B, IL-6, IL-1β, and TNF-α was 50 mg/kg. Protein expression LOAEL In the hippocampus was 100 mg/kg for NK-□B and 50 mg/kg for other evaluated cytokines (Jiang et al. 2017).

Female B6C3F1 mice were given drinking water with 125, 500, or 1000 mg/L for 28 days. A significant decrease in thymus weight was noted at 500 and 1000 mg/L (19%). No effect on absolute or relative spleen weight, or relative thymus weight was reported. A non-dose response decrease (19%) in total spleen cell number and number of CD+CD- T-lymphocytes

(13%) was observed at 500 and 125 mg/L, respectively. A significant decrease in absolute (38%) and percent (22%) NK1.1+CD3- cells was noted at 500 mg/L. Significant decreases in absolute and percent splenic macrophages also were observed (LOAEL = 500 and 1000 mg/L, respectively). No effects on absolute or percent Ig+, CD3+, CD4-CD+, or CD4+CD8+ markers were noted. No effects on AFC response or IgM antibody titers in response to exposure to sheep red blood cells were noted. Additionally, no impact on response to allogeneic spleen cell stimulation was noted. A significant decrease in cytotoxicity was only observed after splenocyte NK cell activity was augmented with poly-IC; the effect was only observed at 125 mg/L. Host resistance to *Streptococcus pneumoniae*, *Plasmodoium yoelii*, and B16F10 melanoma tumors was not affect by treatment (Smith et al. 2010).

In vitro data with cells or cell lines

DBAA decreased thymocyte (obtained from BALB/c mice) proliferation at exposure lengths of at least 6 hours. At 6 hours, a significant decrease in proliferation was only observed at 40 μM. Comparatively, at 12, 24, and 48 hour exposure periods a significant decrease in proliferation was observed at 5, 10, 20 and 40 μM. DBAA also decreased IL-2 and IL-4 secretion (LOAEL = 10 and 5 μM, respectively). DBAA also increased late and early apoptosis (LOAEL = 5 and 10 μM), without effects on the percentage of necrotic cells. DBAA induced an increase in the percentage of cells in the G0/G1 phase and decreased the percentage of cells in the S phase. Increased intracellular thymocyte calcium levels (LOAEL = 5μM) and thymocyte Fas and FasL protein levels were reported (LOAELs = 10 μM for both proteins). Additionally, bcl-2 protein level was significantly decreased at all tested concentrations (LOAEL = 5 μM) (Gao et al. 2016).

Peritoneal exudate cells, obtained from B6C3F1 mice treated with 125, 500, or 1000 mg/L DBAA for 28 days, were evaluated for their ability to suppress B16F10 melanoma tumor cell proliferation *in vitro.* Treatment did not affect the ability of macrophages obtained from treated animals to suppress proliferation (Smith et al. 2010).

In Cl.Ly1 + 2/-9 cells, non-adherent cloned T-cell line derived from spleen cells from C57BL/6TL+ mice, DBAA (1-40 μM) decreased cell viability after exposure for 24, 48, or 72 hours (LOAEL = 1 μM). An increase in the mean percentage of early, late and total apoptotic cells also was noted (LOAEL = 5 μM) (Zhou et al. 2018).

Mode of action information

Overall, studies suggest that DBAA produces immunotoxic effects through modulation of T-cell mediated cell immunity. T-cell apoptosis, through extrinsic and intrinsic pathways, are proposed to play a role in the mode of action. Apoptosis may occur through a variety of pathways including modulation of transmembrane potential, the Fas/FasL pathway, modulation of intracellular calcium, and cell cycle arrest (Gao et al. 2008; Gao et al. 2016).

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

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
DBP significantly decreased phagocytotic capacity of differentiated THP-1 cells at all tested concentrations (LOAEL = 0.001μM). DBP also increased TNF-α secretion (LOAEL = 0.1 μM). Comparatively, DBP had no effect on IL-1β or IL-8 secretion from differentiated THP-1 cells (NOAEL = 0.001 μM) (Couleau et al. 2015).
High-density microarray studies were conducted using normal human mammary epithelial cell strains obtained from discarded tissues; cells were treated with 1 μM DBP for 10 hours. Gene expression of 29 genes were increased in all four isolated cell strains. Gene expression of 28 genes were decreased in all four isolated cell strains including genes involved in the immune response (TNF-α-induced protein 3; values not provided) (Gwinn et al. 2007).
DBP (tested at 0.1 and 100 μM) increased IL-6, CXCL8, and IL-10 secretion from monocytes/macrophages, isolated from blood of healthy individuals. The cells were, stimulated with *E. coli* lipopolysaccharide (LPS) for 1 hour. Comparatively, DBP did not affect IL-1β and decreased TNF-α secretion from the cells. For all affected cytokines the LOAEL was 100 μM. For phytohemagglutinin-P (PHA-P) stimulated T cells, DBP decreased IL-2, IL-4, TNF-α and IFN-γ secretion (LOAEL for all cytokines = 100 μM). No effect on IL-6 or IL-10 secretion was observed in the PHA-P stimulated T cells treated with DBP. Metabolism studies indicated that DBP was metabolized to monobutyl phthalate *in vitro*. Additionally, secretion patterns of monobutyl phthalate was similar to those observed for DBP (Hansen et al. 2015).
DBP increased IL-1β gene expression (as assessed by RT-PCR) in human corneal endothelial cell line B4G12 at all tested concentrations (LOAEL = 1 μM). IL-8 gene expression was increased at 1 and 10 μM (values not provided). IL-β, IL-8, and IL-6 secretion from cells also was increased. IL-6 and IL-8 LOAEL values were 10 and 5 μM, respectively. Significant IL-1β secretion was only observed at 1 μM. [Note: The authors note that secretion for IL-1β and IL-6 was low and quantification was approximate] (Kruger et al. 2012).
In THP-1 cells, DBP did not induce release of IL-18 (doses tested not provided) or IL-8 (NOAEL = 250 μM), or expression of CD86 (NOAEL = 250 μM). However, DBP did induce IL-8 mRNA expression at 500 μM after exposure for 3 hours (values not provided in paper) (Lourenco et al. 2015).
In HepG2 and L02 (normal human liver) cell lines, DBP (10 μM and 25 μM, respectively) significantly increased levels of mature caspase-1, IL-1β, and nucleotide oligomerization domain (NOD) like receptor family, pyrin domain containing 3 (NLRP3) (values not provided). KN-62,

a P2X7 receptor inhibitor, attenuated DBP-induced effects on caspase-1, IL-1β, and NLRP3 (Ni et al. 2016).
In primary human keratinocytes cultured on an amorphous pseudodermis, DBP increased TSLP (thymic stromal lymphopoietin) mRNA expression (Schuepbach-Mallepell et al. 2013).

Mode of action information
Studies suggest that innate and adaptive immune system is impacted by DBP exposure (Hansen et al. 2015). DBP is proposed to be metabolized to the monoester *in vitro*. This compound then is proposed to modulate cytokine secretion from both monocytes/macrophages and T cells.
The results from Couleau and colleagues (2015) suggest that some effects may occur through activation of the endocrine pathway. DBP also may regulate gene and protein expression of a variety of immune factors (e.g., cytokines) without impacting cell viability.
Immunomodulation by DBP also may occur through receptor-mediated effects on the inflammasome.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
DBP did not increase proliferative responses in lymph nodes of BALB/c mice at concentrations up to 20% (v/v in acetone; dermal route of exposure). Additionally, 10% DBP did not increase dendritic cell accumulation in draining lymph nodes (Dearman et al. 1996).
Wistar rats were fed a diet containing 0.5% or 5% DBP for 34-36 days. While no effect on absolute spleen weight was reported, a significant increase (1.8-fold) in relative spleen weight was reported at 5% DBP (Murakami et al. 1986).
Female BALB/cJ mice were subcutaneously exposed to ovalbumin (antigen) and 2-2000 µg/mL DBP. After the primary immunization, one or two booster shots were given to the mice. No effects on the IgG1 or IgE serum levels after either one or two booster shots were noted. A dose- dependent effect was observed on IgG1 serum levels; maximum responses were observed at 200 µg/ml (value not provided). No effect was noted on IgE serum levels (data not provided) (Larsen et al. 2002).
Thymic stromal lymphopoietin (TSLP) mRNA expression was significantly increased in BALB/c mouse ears 24 hours after exposure to DBP (in acetone, 1:1) (values not provided). An increase in TSLP protein levels was also measured at 24 hours (values not provided) (Larson et al. 2010; Schuepbach-Mallepell et al. 2013). DBP-induced induction of TSLP was strain dependent (BALB/c was more sensitive than C57Bl/6 mice). DBP also produced effects on TSLP in IL-1 receptor or apoptosis-associated speck-like protein containing a caspase recruitment domain deficient mice (Schuepbach-Mallepell et al. 2013).

In vitro data with cells or cell lines

DBP was cytotoxic to murine peritoneal exudate macrophages (PEM) after exposure to 50 or 100 μM for 24 hours. Annexin V and PI double stained cells (markers of apoptosis) were significantly increased after treatment with 100 μM DBP for 24 hours. Additionally, using trypan blue exclusion, a significant decrease in viable cells was reported after DBP exposure (LOAEL = 50 μM). Using two-color flow cytometry, DBP was shown to decrease expression of CD80, CD36, and major histocompatibility-II molecules on F4/80+ macrophages at 1 and 10 μM. Cytokine expression (IL-1β, IL-6, IL-12, and TNF-α) also were decreased at the same concentrations. Phagocytotic capacity of PEM to apoptotic thymocytes and *E. coli* was decreased after exposure to DBP when compared to controls (LOAEL = 1 μM). DBP exposure also decreased PEM immunogenicity to allogenic T cells (LOAEL = 1 μM) (Li et al. 2013).

DBP decreased cell viability of RAW 264.7 macrophages (LOAEL = 100 μM for 60 minutes) but did not increase cellular apoptosis (NOAEL = 1 mM for 60 minutes) (Naarala and Korpi 2009).

In RBL-2H3 mast cells sensitized with anti-dinitrophenyl monoclonal IgE, DBP potentiated β-hexosaminidase activity, which was used as a measurement of degranulation (LOAEL = 50 μM for 10 minutes). DBP did not induce degranulation in the cells that were not sensitized (NOAEL = 500 μM for 10 minutes) (Nakamura et al. 2002).

In PAM212 keratinocytes, 1% DBP increased relative expression of TSLP; maximal effect (values not provided) was observed at 36 hours post treatment (Larson et al. 2010). DBP-induced TSLP expression was associated with epidermal mouse skin and human abdominal skin transplanted on mice (Schuepbach-Mallepell et al. 2013).

Mode of action information

In vivo rodent studies suggest that DBP impacts the Th2 response. Inflammasome activation by DBP impacts TSLP expression and Th2 response.

In vitro studies suggest that while high doses of DBP induced macrophage apoptosis, moderate doses induced protein expression and production of cytokines. DBP also impacted the antigen-presenting capacity of macrophages.

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

Human Data

Data from epidemiology studies
No studies were identified.

In vitro data with cells or cell lines
A single study suggested that DCAA may produce immunosuppressive effects. Using a two-way mixed lymphocyte reaction, DCAA (LOAEL = 0.33 mM; lowest dose tested) increased IL-10 production and FOX P3 expression 11.4- and 4.5-fold, respectively (Eleftheriadis et al. 2013).
DCAA (3.0 mM and 0.5 mM, respectively) increased IL-2 production after incubation for 16 hours and expression of the T-cell activation marker CD25 in Jurkat cells. Comparatively, no effect on CD69 expression (0.5 and 3.0 mM) was noted. IL-2 and IFN-γ mRNA expression was significantly increased after DCAA treatment (3.0 and 0.5 mM, respectively) (Pan et al. 2015).
DCAA (N/LOAEL = 0.1/1.0 mM) induced statistically significant increases in necrosis in PBMC, as shown by a decrease in PBMC cell size combined with an increase in cellular granulation. Statistically significant increases in the percentage of apoptotic cells were observed at similar concentrations of DCAA (N/LOAEL = 1.0/2.0 mM) (Michalowicz et al. 2015).

Mode of action information
T-cell activation was one proposed mode of action for DCAA. Increased IL-10 production, combined with increased FOX P3 expression, is proposed to increase regulatory T-cell differentiation which may lead to increased IL-10 production. Additionally, DCAA increased expression of T-cell activation markers in Jurkat cells.
Apoptosis was proposed be associated with a variety of mechanisms including ROS generation, alterations in mitochondrial transmembrane potential, and activation of caspase activity.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
In a 90-day drinking water study (0, 50, 500 and 5000 ppm (w/v) DCAA) with male Sprague-Dawley rats, a significant increase in relative spleen weight was noted at 5000 ppm (0.25% vs. 0.21%). No consistent effects on T cell-dependent anti-keyhole limpet hemocyanin IgG antibody production (measured by ELISA), delayed hypersensitivity to bovine serum albumin, NK cell cytotoxicity, or production of peritoneal macrophage-derived PGE_2 or spleen lymphocyte-derived IL-2 were detected at tested doses (data not shown in paper) (Exon et al. 1986; Mather et al. 1990).
In autoimmune-prone MRL +/+ female mice, 0.5 mg/mL DCAA (provided *ad libitum* in drinking water for 12 weeks) significantly increased serum IgG (32%) and IgM (30%) levels. DCAA significantly decreased IL-10 (34%) and KC chemokine (31%) in liver extracts from

MRL +/+ mice. Comparatively, a significant increase in serum IgG3 levels (27%) was observed in wild-type B6C3F1 after DCAA exposure. In liver extracts from treated B6C3F1 mice, DCAA significantly increased IL-4 (400%), IL-5 (33%), IL-6 (53%), IL-10 (25%), IL-12 (32%), KC chemokine (18%), GM-CSF (42%), G-CSF (56%), and IFN-γ (45%) compared to controls. Compared to isolated MRL +/+ splenic lymphocytes from controls, DCAA decreased IL-4 and IL-10 secretion in MRL+/+ treated mice. DCAA decreased IL-4 and increased IFN-γ secretion from splenic lymphocytes from treated B6C3F1 mice when compared to controls (values not provided). DCAA also significantly decreased IL-4 and IL-2 secretion and significantly increased IL-5, IFN-γ, and GM-CSF secretion from B6C3F1 isolated splenic lymphocytes when compared to secretion from MRL +/+ isolated splenic lymphocytes from treated animals (values not provided) (Cai et al. 2007).

In vitro data with cells or cell lines
No studies were identified.

Mode of action information
DCAA-induced increase of p53 accumulation has been proposed to lead to increased formation of cells in G2-M phase (Staneviciute et al. 2016).

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

Human Data

Data from epidemiology studies

Male and female offspring of pregnant women given DES doses from the seventh week to 34th week of pregnancy, were interviewed about immune-related health problems. A total of 549 DES-exposed offspring and 487 placebo-exposed offspring participated in the study. Rates of allergy-related health problems (e.g., asthma, drug allergy, hives) were similar between DES- and placebo-offspring. Infection (e.g., shingles, flu) and autoimmune disease (e.g., diabetes, rheumatoid arthritis) also were similar between the two groups (Baird et al. 1996).
The frequency of any autoimmune disease in women exposed to DES *in utero* (n = 1711) was higher than the frequency observed in control women. The overall frequency was 28.6 per 1000 women compared to 16.3 per 1000 women. Hashimoto's thyroiditis was significantly more prevalent (relative prevalence = 5.4) in exposed women compared to controls (Noller et al. 1988).
Increased incidence of asthma, arthritis, and diabetes mellitus was reported in sons and daughters exposed to DES *in utero* when compared to unexposed individuals. Additionally, the number of respiratory tract conditions (e.g., colds) was increased in the exposed population vs. the unexposed population (Wingard and Turiel 1988).

In vitro data with cells or cell lines
Lymphocyte NK activity (assessed using chromium release from K562 cells) from 12 patients exposed to DES *in utero* was greater than observed from controls; however, effects were not significant. No effects on adherent cells were noted (Ford et al. 1983). Comparatively, DES dose-dependently inhibited lysis of K562 cells in PBMCs obtained from 12 patients. At the highest concentration tested (100 µM), an 82% reduction in activity was observed compared to control samples (Ablin et al. 1988b, 1988a)
Responses to 0.125 µg/mL PHA (as measured by uptake of radiolabeled thymidine) was significantly greater in peripheral blood monocytes from women exposed to DES *in utero* compared to controls (88.6×10^3 vs. 44.0×10^3 cpm; $p < 0.002$). Maximal blastogenic response to PHA in lymphocytes from DES-exposed women was observed at 0.125 µg/mL while it was observed at 0.25-0.50 µg/mL in controls (Ways et al. 1987).

Mode of action information
DES inhibits the lytic activity of human NK cells (Kalland and Campbell 1984).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
C57BL/6 dams were orally administered 48 µg/kg DES from GD 14–16 and then sacrificed on GD 18. Fetal thymic weight and cellularity were significantly decreased (44% and 51%, respectively) in treated animals. Relative fetal thymic weight was decreased 28% when compared to controls. The percentage of thymocytes in the CD4–8– and CD4–8+ populations were increased 87% and 138%, respectively. Comparatively, CD4+8+ thymocyte population was decreased 12%. Increased apoptosis of CD4+8+, CD4–8+, and CD4–8– thymocytes also was observed (Besteman et al. 2005).
Male and female CD–1 mice were subcutaneously injected with 5, 15, or 30 µg/kg DES, four times on alternate days. Relative thymic weight (LOAEL = 30 µg/kg) was decreased, and absolute and relative splenic weights (LOAELs = 15 µg/kg) were increased in female mice. A similar effect in male mice was not observed. Relative expression of thymocyte populations (e.g., CD4–8–) were not affected in males or females. However, an increase in the number of total apoptotic and decrease in the number of live CD4+8+ and CD4+8– thymocytes was observed in male and female mice. Additionally, an increase in the number of total apoptotic and decrease in the number of live CD4–8– cells were observed in females. Increased proliferative response to ConA, LPS, or PMA was observed in splenic lymphocytes isolated from female mice treated with 5 µg/kg DES. At higher doses, a trend for decreased proliferation was observed in female splenic lymphocytes. Proliferative responses by splenic lymphocytes were only modulated in response to ConA at 15 µg/kg DES (Calemine et al. 2002).
Female mice (strain not provided) were administered (route not provided) 0.2, 2.0, or 8.0 mg/kg DES for 5 days. Antibody response to sheep erythrocytes and LPS were decreased 15% to 45% (LOAELs = 2.0 mg/kg). Delayed hypersensitivity response to keyhole lymphet hemocyanin was similar to controls when mice were exposed to DES before sensitization. However, when mice were exposed to DES after sensitization and before challenge a decrease in response was observed (LOAEL = 2 mg/kg). The percentage of splenic T lymphocytes was decreased 25% at the highest dose tested. No effect on the percentage of splenic B lymphocytes was observed. Splenic lymphoproliferative response to PHA and ConA were decreased (>30%) at all tested doses. Responses to *Staphylococcus* enterotoxin A were increased at 0.2 mg/kg and decreased at higher doses, while responses to LPS were increased at 0.2 and 2.0 mg/kg and decreased at 8.0 mg/kg. MLC responses also were decreased (LOAEL = 2 mg/kg). Suppressor cell activity was decreased after exposure to 8 mg/kg DES (Luster et al. 1980).
Differential effects on the immune system were observed in female NMRI mice depending on the time of DES exposure. Thymus weights were increased in 56–day–old mice that were subcutaneously injected with 5 µg from PND 1–5, 6–10, or 30–34 (1.2– to 1.4–fold). Comparatively, thymus weight was decreased in mice subcutaneously injected with DES from PND 48–52 (29%). A dose–related effect on thymus weight was observed in mice treated from PND 1–5; no effects on absolute or relative spleen weight were noted. Differences in thymus weight also were noted depending on when the mice were killed after treatment. Four days after treatment, thymus weights were decreased in all test groups. However, 4 to 8 weeks after

treatment showed an increase in thymus weight in mice treated on PND 1–5 and weights similar to controls in other treatment groups. DES treatment on PND 1–5 also reduced the number of cells in S–phase in the thymus (Forsberg 1996).
C57BL/6 mice were treated with DES once *in utero* and/or once at 12–16 months of age via subcutaneous injection. Increased secretion of IFN γ was observed in splenic lymphocytes obtained from mice exposed to DES *in utero* and as adults. Increased IFN γ also was observed when splenocytes were stimulated with anti–CD3 antibodies. This increase was not observed in other treatment conditions (data in graphs). An increase in IFN γ production also was observed in T–cells from mice exposed to DES *in utero* and as adults (Karpuzoglu–Sahin et al. 2001).

In vitro data with cells or cell lines
DES stimulated IL–1 production from peritoneal exudate macrophages at concentrations ranging from 0.01 to 1 µM; the maximal response was observed at 0.1 µM. DES (0.1 µM) also significantly increased production of IL–6 (1.7–fold), IL–12 (9.5–fold) TNF–α (3.1–fold), and macrophage chemotactic protein 1 (7.2–fold), and surface expression of CD86 (1.6–fold). DES also increased proliferative responses (8.6–fold) and IL–2 production (5.6–fold) observed when macrophages were incubated with purified T cells. Anti–MHC–II, –CD–80, and –CD86 blocked effects produced by DES (Yamashita et al. 2005).
DES increased IgE levels in male BALB/c mouse splenocytes at concentrations greater than 1 µM. Comparatively, DES had no effect on IgM, IgG, or IgA levels at concentrations up to 1 mM (Han et al. 2002).

Mode of action information
DES–induced thymic atrophy was proposed to be due, in part, to estrogen–related thymocyte apoptosis (Besteman et al. 2005; Fenaux et al. 2004). Brown and colleagues suggested that DES exposure upregulates TNF family members, which leads to altered T–cell development. This alteration was suggested to lead to thymic atrophy (Brown et al. 2006). Direct effects on T lymphocytes also may occur (Luster et al. 1980).
In mice, DES exposure was associated with down–regulation of gene expression in the TCR complex, and the TCR and CD28 signaling pathways. Genes in the B–cell receptor signaling pathway, antigen presentation, and dendritic cell pathways also was altered by DES exposure. It was proposed that DES dysregulation of T–cell development plays a role in thymus effects (Frawley et al. 2011). Alterations of microRNA expression also has been proposed as playing a role in the immunotoxic effects produced by DES (Singh et al. 2015).
Additional proposed modes of action on the immune system include effects on adherent suppressor cells, modulation of NK activity by interfering with bone marrow lymphoid precursors, and modulation of the mononuclear phagocyte system (Dean et al. 1986; Forsberg 1984; Kalland 1984; Luster et al. 1980).

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

Human Data

Data from epidemiology studies
The prevalence of adult-onset asthma, in relation to lifetime pesticide use, were assessed using data from the Agricultural Health Study (19,704 male farmers). Adult-onset asthma was reported in 441 individuals; 127 classified as allergic and 314 classified as non-allergic. EDB exposure was positively associated with allergic asthma (OR: 2.07 [1.02-4.20]) (Hoppin et al. 2009).

In vitro data with cells or cell lines
No data were located.

Mode of action information
No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Female B6C3F1 mice were intragastrically treated with 100, 125, 160, or 200 mg/kg EDB for 14 days. Relative thymus and spleen weights were decreased in a dose-related manner (LOAEL = 200 mg/kg). Comparatively, relative liver and kidney weights were increased at higher doses (LOAEL = 125 and 160 mg/kg, respectively). Significant increases in white blood cells (LOAEL = 200 mg/kg) and neutrophils (LOAEL = 160 mg/kg) were noted. Host resistance to influenza A2, *Listeria monocytogenes*, and herpes simplex virus types 1 and 2 was not significantly affected by EDB exposure. The total number of resident peritoneal exudate cells were significantly increased in EDB-treated mice (LOAEL = 160 mg/kg). However, the percentage of cell types present in the exudates were similar to those observed in control exudates (macrophages: 53%; lymphocytes: 47%). Phagocytosis of radiolabeled chicken red blood cells was increased in peritoneal macrophages obtained from EDB-treated mice (187% of control; LOAEL = 125 mg/kg). Splenic NK cell activity was evaluated in animals treated with 100, 125, or 160 mg/kg; a significant decrease in activity was observed at 160 mg/kg. The number of viable cells in the spleen decreased at 125, 160, and 200 mg/kg (not significant), while a significant increase in the number of anti-SE PFC/10^6 viable spleen cells was significantly increased at 160 mg/kg. Splenic lymphocyte responses to allogenic spleen cells, PHA and ConA, but not LPS, were significantly decreased at 125 and 160 mg/kg (Ratajczak et al. 1994). Female B6C3F1 were intragastrically treated with 31.25, 62.5, or 125 mg/kg EDB for 5 days per week for 12 weeks. No effect on white blood cell numbers, or the percentage of neutrophils or lymphocytes were noted at the doses tested. Splenic lymphocyte responses to PHA and LPS were significantly decreased at the highest dose tested (data not provided) (Ratajczak et al. 1995).

Relative spleen weights were not significantly affected in male Sprague-Dawley rats inhalationally exposed to EDB 7 hours per day, 5 days per week, for 30 days. However, relative liver weights were increased at the highest dose tested (LOAEL = 455 ppm) (Igwe et al. 1986).

In vitro data with cells or cell lines
No data were located.

Mode of action information
No data were located.

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Glycidol [CASRN 556-52-5]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
No data were located.

Mode of action information
No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Of 10 F344/N female rats that received 400 mg/kg glycidol for 13-weeks (via gavage), lymphoid necrosis of the thymus was observed in nine (Irwin et al. 1996; National Toxicology Program 1990). Enlarged spleen were observed in haploinsufficient $p16^{Ink4a}/p19^{Arf}$ male mice treated with 200 mg/kg glycidol for 40 weeks via gavage (National Toxicology Program 2007). Increased splenic fibrosis incidence was reported in male and female F344/N rats gavaged with 37.5 and 75 mg/kg glycidol for 2 years. In males, splenic fibrosis incidences were 26% in controls, 68% in rats treated with 37.5 mg/kg, and 56% in rats treated with 75 mg/kg. In females, splenic fibrosis incidences were 6%, 29%, and 40% for control, 37.5 mg/kg rats and 75 mg/kg rats, respectively (National Toxicology Program 1990).
In female B6C3F1 mice administered glycidol via gastric intubation (25, 125, and 250 mg/kg) for 14 days, no effect on spleen or thymus weights, or leukocyte or lymphocytes counts were reported. To assess AFC response, treated mice were intravenously exposed to sheep erythrocytes on day 11 and spleen IgM AFC response was measured 4 days later. At the highest treatment dose, there was a 31% reduction in specific activity. When expressed as total spleen activity, significant decreases were noted at 125 and 250 mg/kg (29% and 41%, respectively).
Splenic T-cell proliferation, in response to 10 μg/mL ConA was significantly decreased (16% and 26%, respectively) in splenocytes obtained from mice treated with 125 and 250 mg/kg glycidol. B-cell proliferation, in response to Il-4 or Il-4 and goat anti-mouse IgM F(ab')2, was only decreased in splenocytes obtained from mice treated with 125 mg/kg glycidol (13% and 16%, respectively). Comparatively, proliferation in response to goat anti-mouse IgM F(ab')2 was decreased in splenocytes from mice treated with 125 and 250 mg/kg glycidol (30-32%). While glycidol had no effect on lymphocyte blastogenesis (as assessed by splenocyte proliferative response) alone, in the presence of allogenic DBA/2 spleen cells a 25% decrease in response was noted at the middle dose only. NK cell activity in spleens was decreased at two ratios of effector:target ratios (100:1 and 50:1); the LOAELs at both ratios were 125 and 250 mg/kg, respectively. Using flow cytometry, the number and percent of B lymphocytes, T-lymphocytes,

$CD4^+CD8^-$, $CD4^-CD8^+$, and $CD4^-CD8^-$ cells from spleens isolated from treated mice were quantified. The total number of spleen cells, B lymphocytes, and $CD4^+CD8^-$ were significantly decreased at 250 mg/kg. The LOAEL also was 250 mg/kg when the percent values of B and T lymphocytes were assessed (Guo et al. 2000).

In vitro data with cells or cell lines

To further assess the effect of glycidol on the immune function, Guo and colleagues (2000) conducted a set of *ex vivo* assays. Glycidol inhibited cytotoxic T cell activity in spleens obtained from mice administered glycidol via gastric intubation (25, 125, and 250 mg/kg) for 14 days.

Splenocytes were sensitized with mitomycin C–exposed P815 mastocytoma cells, and co-cultured with labeled P815 cells at a variety of effector:target ratios. At an effector:target ratio of 25:1 and 0.75:1, glycidol inhibited CTL activity at a 25 mg/kg when compared to vehicle (53.8 vs. 31.5, and 8.8 vs. 2.1, respectively). At a ratio of 12.5:1, CTL activity was decreased significantly (39%) in spleens from mice treated with 125 mg/kg glycidol (Guo et al. 2000).

Resident macrophage activity (in the presence of macrophage stimulators) was assessed in mice treated with glycidol via gastric intubation (25, 125, and 250 mg/kg) for 14 days. Increased cytotoxicity was only observed after treatment with 25 mg/kg glycidol in the presence or absence of macrophage stimulators (1.7- to 2.5-fold increase) (Guo et al. 2000).

Host resistance to B16F10 melanoma cells, *Listeria monocytogenes* and *Streptococcus pneumoniae* were assessed in mice treated with glycidol via gastric intubation (25, 125, and 250 mg/kg) for 14 days. Glycidol increased pulmonary tumor formation in mice treated with B16F10 melanoma cells (LOAEL = 125 mg/kg). No effect on host resistance was noted at the three challenge levels of *Listera monocytogenes* (1, 2, or 4×10^4 CFU/mouse). At the challenge level 5.52×10^7 CFU *Streptococcus phenumoniae*/mouse, increased host resistance was observed in the 250-mg/kg glycidol treated mice (Guo et al. 2000).

Mode of action information

Studies suggest that glycidol modulates B-cell function, and NK cell and macrophage activities (Guo et al. 2000).

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

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
The effect of HC on IL-4-induced IgE production was measured in PBMCs isolated from healthy volunteers. HC induced an ~20 fold increase in IgE production at a LOEL of 1×10^{-7} M. HC did not have any effect on IgE production in the absence of IL-4 (data not shown) (Nüsslein et al.
1994).
Blood samples from healthy adults were pre-treated with 30 µg/dL HC (identified as cortisol); INF production was then stimulated with Newcastle disease virus. HC decreased IFN-α response by 50-60% (data in graph) (Reissland and Wandinger 1999).

Mode of action information
Keh and colleagues (2003) reported that in septic shock patients, HC attenuated inflammatory and anti-inflammatory responses without inducing immunosuppression.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Swiss inbred mice were intraperitoneally injected with 0, 1.5, 5 or 15 mg/kg of HC. Forty-eight hours later, there was a significant decrease in thymus weight at 15 mg/kg (data in graph). To test the effect of HC on delayed hypersensitivity, mice were immunized with sheep erythrocytes in FCA, challenged in the footpad on day 5, and treated simultaneously with increasing amounts of HC. The mice received another injection of HC two hours before measuring 24-hour footpad swelling. 5 and 15 mg/kg HC suppressed footpad swelling (data in graph). Glucocorticoid- induced leukopenia and monocytopenia was evaluated in mice 2.5 hours after intravenous injection with HC. The numbers of circulating nucleated and monocytic cells was maximally decreased at the lowest dose tested (1.5 mg/kg), with the number of both cell types increasing with increasing dose (data in graph). A plasma transfer study found that 2.5 hours after transfer, the plasma of HC treated mice raised the number of nucleated cells in saline treated acceptor mice by 46%. To evaluate feedback-inhibition, mice were injected (route not specified) with 5 mg/kg HC for four days and examined 7 or 11 days (data not shown) after the last injection. At day 7, HC had no effect on delayed hypersensitivity, serum corticosterone, or numbers of circulating nucleated and monocytic cells (data in graphs) (Van Dijk et al. 1979).
In a trio of studies by El Fouhil and colleagues (El Fouhil et al. 1993a, 1993b; El Fouhil and Turkall 1993), immunologically immature rats were treated subcutaneously with 400 mg/M^2/day HC, administered on alternate days from PND 7 to PND 19. At two days after the last

treatment (PND 21), thymus and spleen weights were decreased (71 and 28%) compared to vehicle control,

but at PND 42 organ weights were increased (18 and 7%). Leucocytosis was increased in PND 21 and 42 rats (12 and 24%), with a decrease in IgM concentration in serum (45 and 15%). At PND 21 there was a 46% decrease in the percentage of lymphocytes, which resolved by PND 42 (El Fouhil and Turkall 1993). On PND 21, splenic white pulp was largely depleted of small lymphocytes. There were no distinct periartiolar lymphoid sheaths and no primary follicles. The number of T cells surrounding the central arteriole was decreased (data not shown). By PND 42, the pulp appeared normal (El Fouhil et al. 1993a). On PND 21, the outer cortex of mesenteric lymph nodes was found to be depleted of small lymphocytes and primary follicles, and neither cortical expansion nor capsular indentations were detected. There was a marked depletion of B lymphocytes, which were more or less discrete and did not aggregate to form follicles. There was no apparent change in T lymphocytes. On PND 42, the lymph nodes were comparable between HC treated and control rats (El Fouhil et al. 1993b).
HC (1.5 mg intraperitoneally administered) decreased formation of splenic anti-sheep erythrocyte (4×10^7 sheep erythrocytes) PFC in female BALB/c mice (data in graph). HC did not affect IgM-PFC or IgG-PFC response or serum antibody titers (data in graphs) (Jokay et al.
1980).

In vitro data with cells or cell lines
No data were located.

Mode of action information
No data were located.

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Indomethacin [CASRN 53-86-1]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
Heparinized whole blood, from healthy adult volunteers, was incubated with 1 to 50 µM indomethacin for 1 hour prior to stimulation with LPS. A significant increase in IL-6 expression was only noted at 50 µM indomethacin (129.7%). Comparatively, a dose-dependent increase in TNF-α was observed, and at 50 µM the number of TNF-α positive cells had doubled (204.7%) (Hartel et al. 2004).
Human PBMC were treated with 1, 10 or 100 µM indomethacin. At all tested doses, indomethacin decreased LPS-induced PGE2 synthesis to near 0%; the calculated IC50 was 0.039 µM (data in graph). Indomethacin also decreased IgG and IgM production (data in graph) at all doses tested. Indomethacin up-regulated IL-2 production and down-regulated IL-6 production in treated PBMC (data not shown). Increased PHA-, anti-CD3, and IL-2-induced lymphocyte proliferation was reported after indomethacin exposure. NK activity (against K562 target cells) was increased at 1 (1.5-fold) and 10 (1.5-fold) µM. A significant effect on LAK cell activity was not observed at 50 µM. Co-incubation of PBMCs with IL-2 and indomethacin caused an increase in IFN-γ production by LAK cells at 1, 10 or 100 µM (data not shown) (Tanaka et al. 1998).
Indomethacin (5.6 µM) increased proliferation of PHA- and ConA-stimulated lymphocytes (in mononuclear cell cultures) (data in graph). The effect was only observed at suboptimal concentrations of PHA and ConA. The observed increased proliferation was lost at optimal and supraoptimal concentrations. Additional testing showed indomethacin increased PHA-stimulated lymphocyte proliferation in a dose-dependent manner (LOEL = 0.04 µM). Removal of adherent cells from the culture negated the stimulatory effect produced by indomethacin. Indomethacin did not affect cell viability, but increased incorporation of tritiated thymidine in a dose- dependent manner (Jawad and Rogers 1984).

Mode of action information
No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Female C57BL/6 mice were orally administered 5 mg/kg indomethacin for 4 days. Animals were then immunized with sheep erythrocytes and then serum hemagglutination and AFC titers were assessed 4 and 8 days, or 6 days later, respectively. Indomethacin decreased both titers by approximately 40% (data in graphs). Indomethacin also decreased ConA- and LPS-induced

stimulation of lymphocyte proliferation (data in graphs). Incubation of indomethacin (3 μM) with LPS-stimulated lymphocytes isolated from indomethacin-treated mice decreased proliferation (Barasoain et al. 1980).

Female B6C3F1 mice were subcutaneously injected with 1, 2, or 4 mg/kg indomethacin for 6 days. Studies were conducted on mice 3 days after final treatment. No effect on thymus weight was reported, while 4 mg/kg indomethacin caused a 38% increase in spleen weight. A dose-dependent increase in splenic lymphocyte proliferation (10%-80%) was observed in non-stimulated cultures. Increased proliferation was observed in LPS-stimulated cultures from mice treated with 1 or 2 mg/kg indomethacin (13% and 22%, respectively), while a decrease was observed at the highest indomethacin dose. Comparatively, decreased proliferation was observed in PHA-, ConA-, or MLC-treated splenic cultures from indomethacin treated mice. Increased formation of PFC/10^6 splenocytes also were observed in treated mice (149% increase at 4 mg/kg). Indomethacin did not affect macrophage-induced inhibition of tumor cell growth (MBL- 2), but did increase phagocytosis of sheep erythrocytes. Host resistance to *Listeria* was increased in treated mice. No effect on delayed hypersensitivity was noted (Boorman et al. 1982).

Oral exposure to indomethacin (2.5, 5, or 10 mg/kg/day for 3 days) decreased formation of PFC in C57BL/6 mice after immunization to sheep erythrocytes. Studies showed a dose-dependent decrease in the number of PFC/10^6 spleen cells; decrease ranged from 43% to 97%. Similar inhibition was observed at 5 mg/kg/day indomethacin and various concentrations of sheep erythrocytes (2.5×10^8 and 5×10^8); decreases ranged from 47% to 68%. Indomethacin also inhibited antibody response to *P. aeruginosa* LPS; total response was decreased by 44% (Rojo et al. 1981).

Oral administration of 6 mg/kg/day indomethacin for 4 days produced a 32% decrease in total number of lymphocytes in Swiss male mice. No effect was noted at earlier time points (i.e., 2 or 3 days). An increase in the number of colonies/10^5 bone marrow cells (2.7- to 3.9-fold) also was noted in mice that were administered indomethacin for 4 days. Indomethacin also decreased PGE2 (25-43%) and PGF2α (41-56%) levels in bone marrow cells after 4 days of administration (Fontagne et al. 1980).

Male CBA mice were intraperitoneally injected with 0.7, 4, or 8 mg/kg indomethacin. Two to 24 hours after exposure, mice were euthanized and spleens removed. A dose-dependent increase in splenocyte proliferation was noted after 2 hours, with a 14.3-fold increase in proliferation at the highest dose tested. A time-dependent increase in proliferation was also noted when mice were treated with 4 mg/kg indomethacin, with a maximal fold change of 31.4-fold at 24 hours. Distribution of T-cell phenotypes was not affected by indomethacin administration (Gonzalez-Cabello et al. 1987).

Kushima and colleagues (2007, 2009) evaluated effects of indomethacin in young Sprague-Dawley rats after *in utero* exposure. In 3-week old pups from dams treated with 0.25, 0.5, or 1.0 mg/kg indomethacin on GD 18-21, a significant increase (31%) in the number of spleen cells was observed in males from the highest dose group. Immunophenotyping of splenocytes showed a dose-dependent increase in the proportion of CD45RA+ cells in male pups. However, a similar

increase in peripheral blood lymphocytes was noted. No effect on serum IgM or IgG levels was reported in males or females. A significant decrease in anti-KLH IgG titers, but not IgM titers was reported in males from the highest dose group tested (Kushima et al. 2007). When doses of 0.5, 1.0, or 2.0 mg/kg indomethacin were used, a significant decrease in splenocyte IL-10 levels were reported in males; no effects on IL-6, IL-2, IL-4, TNF, or IFN-γ levels were noted in either sex (Kushima et al. 2009).
Indomethacin (1 or 2 mg/kg administered twice daily for 3 days to adjuvant induced arthritic Sprague-Dawley rats) reduced PHA-induced lymphocyte proliferation in a dose-dependent manner (data in graph). LPS-stimulated proliferation was also inhibited at both doses, however the response was partially recovered at the higher tested indomethacin dose (data in graph) (Seng et al. 1990).
Indomethacin increased the total number of cells, and number of T- and B-cells up to 14 days after birth, in newborn ddy mice intraperitoneally injected with 5 μg/g every 2 days from birth (data in graphs) (Shibuya et al. 1986).

In vitro data with cells or cell lines
Indomethacin (3 μM) inhibited proliferation of lymphocytes isolated from C57BL/6 mice (data in graph). Additionally, dose-dependent inhibition LPS-induced proliferation of isolated lymphocytes was noted (Barasoain et al. 1980).
Indomethacin dose-dependently increased male rat (strain not provided) ConA-induced lymphocyte proliferation after an 18-hour incubation (LOAEC = 1 μM). A time-course evaluation with 1 μM indomethacin showed that ConA-inducted lymphocyte proliferation was enhanced at incubation times up to 30 hours. Proliferation at exposure times ranging from 36 to 66 hours were not different from controls (Calder et al. 1991).
Indomethacin (50 nM to 50 μM) dose dependently increased LAK activity in BALB/c mouse splenocytes that were cocultured with recombinant IL-2. Increased lysis of JC tumor cells was observed, reaching a maximum response of 123.6 lytic units at 50 μM compared to 43.6 lytic units for IL-2 alone. Studies also showed that the increased response, compared to addition of IL-2 alone, was observed when culture conditions were maintained for up to 4 days. Addition of nylon wool to the culture, abrogated the induction of LAK response observed in the presence of indomethacin (Chao et al. 1989).
Increased time-dependent proliferation was observed in lymphocytes, from CBA mice, treated with 10 μg/mL indomethacin. After 6 and 24 hours, proliferation was increased 4.3- and 46.6-fold, respectively (Gonzalez-Cabello et al. 1987).
Indomethacin decreased IL-4 levels in ConA-stimulated splenocytes isolated from 3-week old male rats (LOAEL = 50 μM). No effect was noted in splenocytes from females. Decreased IL-6 splenocyte levels was observed in cells obtained from females and treated with 2.0 μM indomethacin. No effect on IL-2, IL-10, IFN-γ, and TNF-α were noted (data not shown or in graph) (Kushima et al. 2009).

Mode of action information

Indomethacin induced effects on prostaglandin synthesis was associated with several immune effects. Lala and Parhar (1988) suggested that indomethacin effects are associated with suppression of prostaglandin synthesis. Rojo and colleagues (1981) and Franceschi and colleagues (1988) proposed that indomethacin inhibition of prostaglandin synthesis leads to altered T-cell function, and NK- and antibody-dependent cytotoxicity.

Differential effects on T-cell and B-cell-induced lymphocyte proliferation were reported. A dose- dependent effect on T-cell function was reported, while an inverse effect on B-cell function was noted (Seng et al. 1990).

Indomethacin has been postulated to produce immune effects through inhibition of Th1, and to a lesser extent Th2, responses (Yamaki et al. 2003). Studies conducted by Jaramillo and colleagues (1992) supported this proposed mode of action.

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

Human Data

Data from epidemiology studies
In 19 cases of INH-induced liver failure, antibodies were present in sera of 15 patients. Anti-INH antibodies were present in 8 patients. Additionally, anti-cytochrome P450 antibodies were identified in up to 14 patients. Antibodies were not detected in patients that were treated with INH but did not have significant liver injury (Metushi et al. 2014c). In eight INH-induced liver failure patients, the dominant serum immunoglobulin isotype of anti-INH antibodies was IgG. A low titer of IgM was observed in two patients, while IgA and IgE antibodies were not detected. Phenotyping the IgG antibody indicated that the isotype was IgG3 (Metushi et al. 2014b).
INH (1.25 µg/mL) did not stimulate PGE3 production in polymorphonuclear leukocytes or modulate PHA-stimulated mononuclear leukocytes transformations. No effect on PG2 production was observed at 5 µg/mL (Zeis 1987).

In vitro data with cells or cell lines
In a series of studies, Kucharz and colleagues studies the immunomodulatory effects of IAH. In 5 µg/mL PHA-stimulated T-cells, IAH increased cellular proliferation (16% to 27%) at concentrations ranging from 0.01 to 0.0001 mM. (Kucharz and Sierakowski 1990a). In PBMC stimulated with 5 ng/mL anti-CD3 antibody, IAH produced a biphasic response. At 1 and 10 mM IAH decreased (53.6% and 24.4%, respectively) cell proliferation. Increased cellular proliferation (18-47%) was observed at concentrations ranging from 0.0001 to 0.1 mM. A similar biphasic pattern was observed when 10 ng/mL anti-CD3 antibody was used. In T-cells stimulated with anti-CD3 antibody, PHA, or PHA with PMA, IAH also modulated proliferation in a biphasic manner (Kucharz and Sierakowski 1990a). In cells stimulated with 5 µg/mL PHA and 20 ng/mL PMA, 0.1 to 10 mM IAH decreased T-cell proliferation 17% to 46%. At 0.001 mM IAH, at significant increase (21%) in T-cell proliferation was observed (Kucharz and Sierakowski 1990d). In cells stimulated with IL-2, IAH decreased cell proliferation 0.1 and 1 mM (71% and 47%, respectively) and increased proliferation at 0.01 to 0.001 mM (8% to 12%, respectively (Kucharz 1995).
IAH also decreased T-cell IL-2 production at 0.1 and 1 mM (44.7% and 71.6%, respectively) and increased T-cell IL-2 production at 0.01 to 0.0001 mM (105% to 115%). No effect on IL-2 receptor expression in T-cells was observed (Kucharz and Sierakowski 1990b).
IAH decreased IL-1 production from human monocytes in a dose-dependent manner in the absence or presence of lipopolysaccharide (LOAEL = 0.001 mM) (Kucharz and Sierakowski 1992).
In the absence of PHA, IAH stimulated proliferation of Jurkat cells (LOAEL = 0.01 mM). In the presence of PHA (2 or 5 µg/mL), IAH stimulation was observed at higher concentrations (1 and 10 mM) while at lower concentrations no effect was observed (Kucharz and Sierakowski 1990c).

When PMA (20 ng/mL) or PMA (20 ng/mL) and PHA (5 µg/mL) were added to the media, increased Jurkat cellular proliferation was observed at 0.001 mM (32%) and 0.01 and 0.001 mM (8% and 18%, respectively) (Kucharz and Sierakowski 1990d).
INH (5 µg/mL) did not have any effect on the phagocytic activity or intracellular killing activity on polymorphonuclear leukocytes obtained from healthy volunteers (Okuyan et al. 2005).

Mode of action information
Metushi and colleagues proposed that INH produced an immune response that leads to liver injury (Metushi et al. 2014c, 2014b).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Female *Nat1/2(-/-)* mice were treated with INH either by oral gavage (100 mg/kg/day) for up to 7 days or by feed (0.2%) for 35 days. In mice treated by gavage, significant decrease in M1 macrophages and increase in M2a and M2b macrophages in cervical lymph nodes was noted. No effect on the M2c macrophages was observed. Comparatively, no effect was noted in the macrophage phenotypes obtained from mice that were exposed by feed (Metushi et al. 2014a).
INH (0.1 to 1.0 mg/10 µL) did not alter the weight of popliteal lymph nodes from C57BL/10 mice 7 days after subcutaneous injection (Kammuller et al. 1989). A lack of effect on popliteal lymph nodes from Brown Norway rats also was observed when exposed to 5 mg/50 µL INH (Verdier et al. 1990).
Four female Cbl-b-/-, C57BL/6 background that lack an E3 ubiquitin ligase, were provided diets containing 0.2% w/w INH for 5 weeks. Blood was collected to assess serum cytokine levels.
Significant decreases in serum IL-12 and IL-1α was noted in female Cbl-b-/- mice (data provided in graph). No effects on IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-13, IL- 17A, eotaxin, GCSF, GMCSF, IFNγ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α were
observed (data provided in supplementary materials) (Metushi and Uetrecht 2014).

In vitro data with cells or cell lines
In HT-2 cells, stimulated with IL-2 (3 or 30 U/mL), increased proliferation was observed at 1 and 10 mM IAH at 30 U/mL and only at 1 mM at 3 U/mL. No effect on proliferation was observed in cells stimulated with 60 U/mL IL-2 (Kucharz and Sierakowski 1990c). Additionally, no effect on proliferation by IAH was observed in HT-2 cells stimulated with PMA (data not provided) (Kucharz and Sierakowski 1990d).

Mode of action information
No data were located.

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

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
Lead (lead acetate 5.0 mg – 1.5 ng/mL, or lead chloride 0.5 mg – 0.15 ng/mL for 24 hours) significantly reduced cell vitality and/or proliferation and affected secretion of proinflammatory, TH1 and TH2 cytokines in human peripheral mononuclear blood cells that were stimulated with either heat-killed *Salmonella enteritidis* (hk-SE) or monoclonal antibodies. At lower lead levels, expression of IFN-γ, IL-1β and TNF-α were reduced. Monoclonal antibody induced IL-4, IL-6 and IL-10 and hk-SE induced IL-10 and IL-6 levels were increased in the presence of lower lead levels. The authors suggest that lower dose lead suppresses the TH1 cytokine and the proinflammatory cytokines while the increased IL-4 and/or IL-10 production can induce and maintain a TH2 immune response (Hemdan et al. 2005).
Thirty male lead-exposed (battery recycling industry) workers with a blood lead level > 10 µg/dL and 27 unexposed healthy volunteers without any history of occupational exposure to lead were selected for this study. The serum level of IgA was found to be significantly increased in the lead-exposed group as compared to controls. No differences were observed in serum IgG and IgM levels. Both the level of nitric oxide production after stimulation with zymosan-A and the neutrophil respiratory burst as measured by nitroblue tetrazolium reduction were comparable in neutrophils from lead-exposed and unexposed volunteers (Mishra et al. 2006).

Mode of action information
Lead acetate (1 µM) induced activation of NF-κB in primary human CD4+ T lymphocytes. This lead induced activation was blocked by antibodies for p65 and p50 subunits (indicating that the p65:p50 heterodimer (NF-κB) is involved), but not by cRel. Lead acetate (100 pM – 100 µM) did not activate NF-κB in 4 different T cell lines, suggesting that these cell lines may not be a reliable system for studying transcriptional activation in human T cells (Pyatt et al. 1996).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Lead acetate suppressed macrophage-dependent immune responses in cells taken from female BDF1 mice exposed to lead in drinking water at concentrations from 0 to 1000 ppm for 3 weeks. The T-cell dependent sheep erythrocyte primary immune response was suppressed by approximately 40-50% in all lead-exposed groups. In contrast, the *E. coli* lipopolysaccharide (T-cell and macrophage-independent) induced response, was not suppressed (Blakley and Archer 1981). Lead did not alter the ability of T-cell mitogens to induce interferon (Blakley et al. 1982).

Lead acetate effects were modulated by maternal protein intake. Fischer 344 rats were exposed to lead acetate (250 ppm) in the drinking water during breeding and pregnancy until parturition and were fed isocaloric diets (either 20% casein or 10% casein). Offspring exposed to lead and high maternal dietary protein had significantly elevated levels of both IL-4 and TNF-α (values not provided). Offspring exposed to lead and low maternal dietary protein had significantly reduced IL-4 levels compared to the lead control group (values not provided). No other changes were observed, and immune parameters measured in the dams were not affected by treatment (Chen et al. 2004).

In a study comparing immunotoxic effects of various lead salts, Balb/c mice were treated for five consecutive days between immunization and elicitation with intraperitoneal injections of 0.5 or 6 mg/kg of a lead salt. A statistically significant increase in delayed hypersensitivity (as measured by footpad swelling) was observed following administration of lead acetate (55.0% increase in footpad thickness as compared to controls; LOAEL = 6 mg/kg) (Descotes et al. 1984).

Exposure to lead acetate resulted in a decreased ability of mice to survive a sublethal dose of a virulent strain of *S. typhimurium*. C3H/HeN mice were exposed to lead acetate (5 or 10 mM) in the drinking water for up to 18 weeks. At week 16, mice were infected with *S. typhimurium*. 40% of the mice exposed to 5 mM lead acetate survived the infection with a median survival of 26 days. None of the mice treated with 10 mM lead acetate survived, with death occurring within three weeks of becoming infected. In contrast, 80% of control mice survived with a median survival of 60 days. The ability of splenocytes, cultured from the lead-treated and control mice showed a marked reduction in the production of IFN-γ (27% and 35% in mice treated with 5 and 10 mM lead acetate, respectively) and IL-12p40 (42-45% in mice treated with 5 and 10 mM lead acetate, respectively, as compared to induced control). Secretion of IL-4 by splenocytes from lead-treated mice was 3 to 3.6-fold higher than in control mice (Fernandez-Cabezudo et al.
2007).

Adult Sprague-Dawley females were treated with 500 ppm lead acetate via drinking water either early in gestation (days 3-9) or late in gestation (days 15-21). Offsping were assessed as adults. Significantly depressed DTH responses as well as increased IL-10 production, relative monocyte numbers and relative thymic weights were reported in female offspring exposed to lead during late gestation. Male offspring exposed during late gestation had significantly increased IL-12 production and decreased IL-10 production while the DTH response, relative monocyte numbers and thymic weights were unchanged compared to controls. The authors found that adherent splenocytes (likely macrophages) and T lymphocytes are the primary immune cells affected during fetal lead exposure and that gender may influence immunotoxicity due to lead exposure (Bunn et al. 2001).

Lead acetate increased IL-4 production in mice at 40 and 400 mg/L and decreased IFN-γ levels in mice at 400 mg/L. Adult Swiss mice were administered lead acetate in drinking water for 14 days. The authors concluded that low level lead exposure enhances a Th2 response while high lead levels can either stimulate Th2 immune activity or reduce Th1 activity, thus resulting in an imbalance between Th1 and Th2 activation (Iavicoli et al. 2004).

Lead acetate (100 or 1000 ppm in drinking water) did not alter the ability of splenocytes isolated from exposed male Alderly Park rats to mediate native and interferon activated natural cytotoxicity at 2,4,6 and 8 weeks following commencement of exposure. Splenic T-cell function of treated rats as determined by phytohaemagglutinin induced proliferation was comparable to control values (Kimber et al. 1986).
Lead acetate (10 mM in the drinking water for 8 weeks) did not suppress the primary direct humoral immune response to T-dependent antigen (sheep erythrocyte) and T-independent antigens (TNP-LPS, TNP-Ficoll) in several inbred (A, BALB/c, C57Bl/6, DBA/1, SJL, and NZW/NZB F1) and an outbred (CFW) strains of mice (Mudzinski et al. 1986).
Lead acetate (200 ppm either in the drinking water or given intraperitoneally for 4 weeks) decreased the number of lymphocyte cells and cellularity (i.e., number of cells per mg tissue) in the thymus, but no significant changes in either parameter were reported for the submaxillary lymph nodes. Proliferation of T cells stimulated by ConA and proliferation of B cells stimulated by LPS was increased by lead in the thymus by both routes of exposure. In the submaxillary lymph nodes, there was a decrease in the proliferation of T cells following treatment by either route (Teijon et al. 2010).

In vitro data with cells or cell lines
RAW 264.7 cells were treated with 100 ppm lead acetate for 24 hours in the presence or absence of LPS. Lead produced a statistically significant inhibition of the level of LPS-induced nitric oxide (data not provided). No effect on cytotoxicity was observed (Mishra et al. 2006).

Mode of action information
C3H/HeN mice were exposed to lead acetate (0, 5 or 10 mM in drinking water for periods of up to 18 weeks) and inoculated with a virulent strain of *S. typhimurium*. Sera were collected on days 15 and 38 post infection. The authors report that the IgG2a antibodies were elevated in control mice by day 38 post infection (0.09 ± 0.05 on day 15 vs. 0.30 ± 0.03 on day 38; an increase of 300% from day 15), but were only slightly increased in lead-exposed mice (0.11 ± 0.01 on day 15 vs. 0.16 ± 0.02 on day 38). IgG1 isotype antibodies (an isotype induced by IL-4) were significantly elevated in lead exposed mice on day 38, as compared to control mice. The authors conclude that lead acetate induces a subtle but substantial shift toward a Th2-type immune response to infection with Salmonella organism (Fernandez-Cabezudo et al. 2007).
A single intraperitoneal exposure to lead acetate (12 mg/kg) in B6C3F1 mice produced changes in cell surface markers on discrete subpopulations of lymphoid cells from the spleen and bone marrow. The authors concluded that while the changes may not correlate with functional activity of the cells, they seemed to predict a shift to immature cell types, which correlated with the increase in progenitor cells observed (Burchiel et al. 1987).

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Mannitol [CASRN 69-65-8]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
Increased urinary excretion of 9α,11β-prostaglandin F2 and leukotriene-4 were reported in association with mannitol-induced bronchoconstriction in 14 asthmatic patients. Urinary excretion of 9α,11β-prostaglandin F2 and leukotriene-4 increased from 61 to 92 and 19 to 31 ng
× mmol/creatinine, respectively (Brannan et al. 2006). A separate study reported that repeated challenge with mannitol induced refractoriness in asthma patients. The mannitol refractoriness was associated with maintained release of 9α,11β-prostaglandin F2 and leukotriene-4 (Larsson et al. 2011).
Increased proportion of submucosal MCTC was observed in asthmatic individuals with airway hyperresponsiveness to mannitol compared to asthmatic individuals without responses to mannitol. The percentage MCTC increased from 18.7% to 40.3%, but the increase in the numbers of MCTC between the two groups was not significantly increased. Increased gene expression of thymic stromal lymphopoietin and carboxypeptidase AM also were reported (Sverrild et al.
2016).
Mannitol significantly increased 9α,11β-prostaglandin F2, leukotriene-C4, and histamine release from cord blood-derived mast cells (LOAEL = 0.7 M for all endpoints). At the same tested mannitol concentrations (0.3-1.0 M), no concordant increase in lactate dehydrogenase release was observed suggesting cell viability was not affected. The ratio of 9α,11β-prostaglandin F2 to leukotriene-C4 was 156-1 (Gulliksson et al. 2006).
Mannitol did not induce DNA damage in human leukocytes at concentrations from 1.25 to 10 mM (Frenzilli et al. 2000).
Mannitol (22 mmol/L) did not increase IL-6 or TNF-α secretion from monocytes treated with glucose (11 mmol/glucose) for 24 hours. A similar lack of effect was observed when cells were incubated for 48 hours (Morohoshi et al. 1996).
At the highest concentration tested (100,000 μM), mannitol did not reduce cell viability in human LCLs or PBMCs. Mannitol (50,000 μM) did not modulate TNF-α, IL-6, IL-2, IL-4, IL-10, or IFNγ release in LCLs pre-treated with phorbol 12-myristate 13-acetate/ionomycin stimulated cells (Markovic et al. 2015).
Mannitol did not inhibit growth of human granulocyte precursor cells at a concentration up to 5 mM (Holdener et al. 1983).

Mode of action information
Mannitol is shown to narrow the airway in asthmatic, but not healthy, test subjects (Brannan et al. 2001, 2003, 2000). Mannitol is proposed to increase osmolarity of airway surface liquid, leading to an increase in mediator release (e.g., histamine, prostaglandins, and leukotrienes) from inflammatory cells which induces bronchoconstriction (Brannan et al. 2006; Sverrild et al. 2016). One mediator that is proposed to be released is prostaglandin D2 from mast cells (Brannan et al. 2003, 2006).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
No data were located.

In vitro data with cells or cell lines
No data were located.

Mode of action information
No data were located.

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Nickel (II) Sulfate Hexahydrate ($NiSO_4$) [CASRN 10101-97-0]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
In PBMC from five nickel allergic individuals 0.1 mM $NiSO_4$ increased IL-4 and IFN-γ production. The peak effect was lower than when PBMC were incubated with PHA (data shown in graph) (Thomas et al. 2003).
$NiSO_4$ (85 µg/mL) significantly upregulated expression of CD40, CD83, CD86, and CD54 markers on THP-1 cells. $NiSO_4$ also significantly increased production of TNF-α and IL-8 in a dose-dependent manner. IL-6 production was significantly increased after exposure to 170 µg/mL (Miyazawa et al. 2007). Ade and colleagues noted that $NiSO_4$ induced CD83, CD86, HLA-DR, and CD40 in a dose dependent manner in dendritic cells (Ade et al. 2007).

Mode of action information
$NiSO_4$ was shown to alter dendritic cell phenotypes by activation of MAPKs and NF-□B. Additionally, $NiSO_4$ induced IL-8, IL-6, and IL-12 p40 production (Ade et al. 2007; Antonios et al. 2009). Activation of the MAPK pathway may lead to upregulation of the Cys-Cys chemokine receptor, CCR7, which allows dendritic cells to migrate to the draining lymph nodes (Boisleve et al. 2004).
$NiSO_4$ has a similar capacity to stimulate polyclonal CD+4 in Ni-allergic and -nonallergic individuals. Differences in clonal expansion or presence of Ni-binding motifs in MHC class II complexes could be involved in the development of allergic contact dermatitis (Lisby et al. 1999).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Male Wistar rats were intratracheally instilled with 1, 2, 4, or 8 µmole $NiSO_4$ per rat. The rats were euthanized up to 7 days after treatment. Treatment decreased the percentage of lymphocytes in pulmonary lymphoid cells (~55% to ~40%). NK activity in lymphoid lung cells was dependent on concentration and effector:target cell ratio. NK activity was decreased 1 day after treatment of 4 and 8 µmole $NiSO_4$ at the effector to target cell ratio of 6:1. Two days after treatment suppression of NK activity was significant at doses ≥2 µmole $NiSO_4$ and at the effector to target cell ratio of 6:1. After 7 days, a significant decrease was only observed at 8 µmole.
$NiSO_4$ did not significantly modulate alveolar macrophage cytotoxic activity towards 3T12 target cells. Decreased levels of TNF-α was reported at all time points, while increased IFN-

γ level was only noted after exposure to 8 μmole/rat on day 2 (data in graph) (Goutet et al. 2000).

Female B6C3F1 mice were exposed to $NiSO_4$ aerosol for 6 hours per day, 5 days per week for 65 days. The actual exposure concentrations tested were 0.027, 0.11, or 0.45 mg Ni/m3. No change in thymic weight was reported. A significant increase in the number of nucleated cell numbers from lung-associated lymph nodes (LALN) and lavage fluid, after mice were immunized with sheep red blood cells, was noted at the highest dose tested (1.72- and 3.86-fold, respectively). Nonsignificant increase in the total antibody-forming cells (AFC)/(LALN) and nonsignificant decrease in AFC/spleen, after immunization with sheep red blood cells, were also noted after $NiSO_4$ exposure. $NiSO_4$ had no effect on mixed lymphocyte response of spleen cells after exposure to mitomycin C-treated spleen cells from DBA/2 mice. No effect in mitogen- stimulation assays also were noted by $NiSO_4$ exposure. $NiSO_4$ modulated pulmonary alveolar macrophage function, as measured by phagocytosis of opsonized erythrocytes; activity was significantly increased at 0.11 mg Ni/m3 (data not provided). Comparatively, $NiSO_4$ had no effect on peritoneal macrophage phagocytosis activity at any tested dose. The highest dose of $NiSO_4$ was associated with a significant two-fold increase in the number of B16F10 tumor nodules in the lungs of treated animals. However, incorporation of radiolabeled uridine was not considered biologically significant. $NiSO_4$ did not affect splenic NK cell cytolytic activity (Haley et al. 1990).

Histopathological lesions in lungs, liver, thymus, kidneys, spleen, and lymph nodes were noted in male F344 rats intramuscularly injected with 125 μmole/kg $NiSO_4$ over 26 days. Thymus glands from rats treated with the highest dose were much smaller than controls.

Corticomedullary junction was not distinct and extensive degeneration and depletion of lymphocytes in the thymic cortex were noted. Additional tissues from these rats were evaluated further. In the lungs, large alveolar macrophages and polymorphonuclear leukocytes were noted in alveolar spaces and exudate. In the spleen and lymph nodes, lymphocytes were focally depleted in the white and red pulp (Knight et al. 1991).

Male Sprague-Dawley rats were exposed to 0.02, 0.05, and 0.1% $NiSO_4$ in drinking water for 13 weeks. Effects on splenic lymphocyte and thymocyte subpopulations were evaluated. In splenic lymphocytes, increases in the total number of T-cells (LOAEL = 0.05%) and CD8+ T-cells (LOAEL = 0.02%) were reported. For CD4+ T-cells, the number of cells increased at 0.05% $NiSO_4$ and then decreased at 0.1% dose. An increase in the total number of B cells was noted at 0.05% $NiSO_4$. Subchronic exposure to 0.02% $NiSO_4$ also increased the percentage and absolute number of thymocyte CD8+ cells. Exposure to 0.05% $NiSO_4$ increased the total number of thymocyte cells, the percentage and absolute number of CD8+ cells, and absolute numbers of both CD4+ and B-cell populations. Exposure to 0.1% $NiSO_4$ decreased the total number of thymocytes, the percentage and absolute number of CD4+ T cells, and absolute numbers of CD8+ T cells and of B cells (Obone et al. 1999).

Male C3H/He mice were provided 0.01, 0.05, 0.1, 0.25, 0.5, or 1% $NiSO_4$ for 7 or 10 weeks. Mice were then sensitized with $NiSO_4$ for 7 days and the footpad thickness was measured. The mice were then challenged with 0.4% $NiSO_4$ and footpad swelling was measured 24 hours later. After 7 weeks of oral exposure, footpad swelling was not reduced at any of the tested doses.

However, after 10 weeks of exposure swelling was decreased (LOAEL = 0.1%) (Ishii et al. 1993). Lymph nodes from C3H/He mice sensitized to $NiSO_4$ were incubated with various monoclonal antibodies and then injected into naïve mice. After challenging with $NiSO_4$, footpad swelling was measured. Cells treated with CD4-, Thy1.2-, or Ig-specific antibodies showed reduced swelling while cells treated with CD8 antibodies induced footpad swelling (Ishii et al. 1993). Macrophage and PMN chemotactic activities in bronchoalveolar fluid were increased at 2 days after intratracheal instillation of 50 μg Ni per male Wistar rat. Activity then decreased until end of the experiment (14 days). Comparatively, LTB4 were maximally decreased at day 1 and then increased to control levels by day 14 (Hirano et al. 1994).

In vitro data with cells or cell lines

Spleen cells from C57BL/6 and Rag-1 deficient mice were stimulated with varying concentrations of $NiSO_4$ (concentrations not provided). Using the ELISPOT assay, IL-2, IL-4 and IFN-γ secreting cells were identified in splenic cells from C57BL/6 mice. The number of IFN-γ cells were greater than the IL-2 and IL-4 cells. At higher concentrations (≥400 μM), the numbers of IL-2 and IL-2 secreting cells decreased while those secreting IFN-γ remained high. The number of IFN-γ cells did not increase due to previous immunization of $NiSO_4$. In splenic cells from Rag-1 deficient mice, $NiSO_4$ also contained IFN-γ secreting cells. However, at higher concentrations the cell levels decreased (in comparison to wild-type). Addition of NK1.1 antibodies produced a partial depletion in the cells. Further studies showed that addition of NKG2D antibodies reduced the number of IFN-γ secreting cells in wild-type and RAG-1 deficient mice (Kim et al. 2009).

Mode of action information

No data were located.

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Nitrobenzene [CASRN 98-95-3]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
No data were located.

Mode of action information
No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Female B6C3F1 mice were exposed to 30, 100, or 300 mg/kg nitrobenzene for 14 days via gastric intubation. Hepatomegaly and splenomegaly were observed in mice that received 100 and 300 mg/kg nitrogenzene. Mild congestion in the red pulp areas of the spleen was noted in mice that received 100 mg/kg, while the spleen was dark red in those that received 300 mg/kg. Absolute and relative spleen weight were significantly increased (LOAEL = 100 mg/kg). Comparatively, absolute and relative thymus weights were increased only at 100 mg/kg. The number of bone marrow cells increased in a dose-dependent manner (LOAEL = 30 mg/kg). At the highest dose tested the increase was 60% above controls. DNA synthesis and the number of CFU-GM per femur also were increased (LOAELs = 30 mg/kg). In response to sheep erythrocytes, a significant increase in spleen weight (62%) and spleen cell number (29%) was observed at 300 mg/kg, when animals were sensitized four days after nitrobenzene exposure. Comparatively, a decrease in IgM AFCs were decreased (LOAEL = 100 mg/kg). When responses to sheep erythrocytes were observed (sensitization occurred 5 days after nitrobenzene exposure), spleen weight and cells were increased at 100 and 300 mg/kg. However, no effects on IgG AFC were noted. When 20 days lapsed between nitrobenzene exposure and sensitization to sheep erythrocytes, no effects were reported. No effect on delayed hypersensitivity was reported at any of the tested doses. Splenic proliferation responses induced by PHA and ConA were suppressed by exposure to nitrobenzene (LOAEL = 100 mg/kg). No effect on LPS-induced proliferation were reported. Responses to DBA/2 mice spleen cells also were decreased (LOAEL
= 100 mg/kg). Using radiolabeled sheep erythrocytes, the phagocytic index was shown to be increased in a dose-dependent manner. The phagocytic activity of peritoneal cells also was increased in a dose-dependent manner (LOAEL = 300 mg/kg). The ability of spleen cells to lyse radioactivity from YAC-1 target cells also was evaluated. Nitrobenzene exposure produced a decrease in lysis capacity at 100 and 300 mg/kg at effector:target ratios of 100:1 and 30:1.

Nitrobenzene did not affect host resistance to *Streptococcus pneumonia, Plasmodium berghei,* herpes simplex 2, or B16F10 melanoma. Comparatively, host resistance to *Listeria monocytogenes* was decreased. A challenge of 6 x 10^3 *L. monocytogenes* per mouse killed 13%

of control animals and 57% of animals treated with 300 mg/kg nitrobenzene. A challenge with 1.2×10^4 *L. monocytogenes* increased animal death from 19% in controls to 100% at 100 mg/kg nitrobenzene and 86% at 300 mg/kg nitrobenzene (Burns et al. 1994).

In vitro data with cells or cell lines
No data were located.

Mode of action information
Two proposed targets of nitrobenzene are: (1) erythrocytes and (2) precursors to erythrocytes and other cells (e,g., granulocytes). The site of action is proposed to be the bone marrow. Additionally, effects on T-cell function may play a role in increased susceptibility to *L. monocytogenes* (Burns et al. 1994).

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

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
No data were located.

Mode of action information
No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
B6C3F1 mice were orally administered 100, 300, or 500 mg/kg BCP for 14 days. No effect on spleen or thymus weight were reported. No effect on delayed hypersensitivity response (to keyhold limpet hemocyanin), antibody response to sheep erythrocytes, serum IgM, IgA, or IgG levels, or splenic lymphocyte proliferation were noted. Absolute and relative liver weights were increased at the highest dose group. Additionally, BCP-treated mice did not develop tumors after challenge with PYB6 tumor cells (vs. controls which had a 15% tumor incidence) (Birnbaum et al. 1986).
BCP produced contact hypersensitivity in female B6C3F1 mice (Stern et al. 1991).

In vitro data with cells or cell lines
No data were located.

Mode of action information
No data were located.

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

Human Data

Data from epidemiology studies
Several studies have suggested that prenatal PFOA exposure is linked to immunosuppressive and immunotoxicant effects observed in offspring. Granum and colleagues (2013) reported that maternal PFOA blood levels, collected at birth, were positively associated with decreased rubella antibody-levels (β = -0.40) and an increased number of common cold episodes in children from 0-3 and 2-3 years old. Cord blood IgE levels also were suppressed in female infants with high maternal PFOA levels. However, no effects on number of 18 month-old infants with allergies (e.g., food allergy, eczema) or infections (e.g., otitis media, pneumonia, skin infections, chicken pox) were noted (Okada et al. 2012). Okada and colleagues noted that while the correlation between fetal PFOA levels and the evaluated endpoints were not available, the results suggest that PFOA produced immunosuppressive effects after prenatal exposure.
A positive association between serum PFOA in adults and development of ulcerative colitis also was reported. However, a positive association with other autoimmune diseases, such as Type 1 diabetes, lupus, multiple sclerosis, Chron's disease, and rheumatoid arthritis, was not observed (Steenland et al. 2013).
Chang and colleagues (2016) conducted a systematic review to summarize and evaluate epidemiological literature on PFOA and perfluorooctanesulfonate (PFOS) with relation to evaluated immune endpoints. Endpoints evaluated included immune biomarker levels (e.g., IgE levels, white blood cell count, and C-reactive protein), immune gene expression patterns, atopic or allergic disorders (e.g., asthma, eczema, and food allergy), infectious disease (e.g., common cold), vaccine response, and autoimmune and inflammatory conditions (e.g., ulcerative colitis, rheumatoid arthritis, and osteoarthritis). The authors stated that the totality of the data limited development of a conclusion on the causal relationship between PFOA and/or PFOS exposure and evaluated endpoints due to inconsistent results and confounding factors.

In vitro data with cells or cell lines
Studies with human cells or human-derived cell lines indicate that PFOA modulates cell activation and cytokine production. In human PBMC, PFOA significantly increased the percentage of viable cells at concentrations <125 µg/mL. At higher concentrations (250 and 500 µg/mL), a significant decrease in cell viability was reported (values not reported). No effects on T-cell proliferation (NOAEL = 1 µg/mL) or, TNF-α or IL-6 release (NOAEL = 1 µg/mL) were noted. PFOA also increased monocyte differentiation in HL-60 cells (LOAEL = 100 µg/mL) (Brieger et al. 2011). Comparatively, PFOA decreased TNF-α, IL-4, and IL-10 (LOAEL
= 1 µg/mL, 10 and 10 pg/mL, respectively) in peripheral leukocytes. PFOA also decreased TNF- α (LOAEL = 10 µg/mL) production in THP-1 cells (value not reported). PFOA did not affect IL- 2 production in Jurkat cells (value not reported) (NOAEL = 0.005 µg/mL) (Corsini et al. 2011, 2012; Midgett et al. 2015).

Mode of action information

Direct modulation of NF-□B has been implicated in modulation of cytokine production and secretion (Corsini et al. 2012). PFOA interaction with the PPARα receptor also was implicated in immunomodulatory effects in human cells. Receptor interaction was associated with reduced p65 phosphorylation and NF-□B-mediated transcription (Corsini et al. 2011). The extent the role of PPARα receptor activation plays in human effects is unclear given the low level of human receptor expression (Corsini et al. 2014).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

Animal studies suggest that PFOA exposure can affect innate and adaptive immune functions *in vivo*. Dietary exposure to PFOA (0.02% w/w) for 7 days significantly decreased spleen and thymus weight, and splenocyte and thymocyte levels in wild-type C57Bl/6 mice. Spleen weight and splenocyte numbers were not affected in PPARα-null mice (Yang et al. 2002).

Table 1. Data from Yang et al. (2002)

Group and Treatment	Body weight (g)	Spleen weight (g)	Splenocyte number (x 10^6)	Thymus weight (g)	Thymocyte number (x 10^6)
Wild-type mice					
None	24.5 ± 1.58	0.082 ± 0.006	68.8 ± 16.8	0.061 ± 0.014	81.0 ± 28.2
PFOA	21.0 ± 0.74**	0.050 ± 0.001***	15.3 ± 5.84***	0.013 ± 0.001***	12.8 ±7.98***
PPARα-null mice					
None	23.6 ± 2.9	0.064 ± 0.021	84.0 ± 19.3	0.054 ± 0.006	88.3 ± 7.04
PFOA	23.5 ± 1.0†	0.054 ± 0.015	73.8 ± 26.2†††	0.033 ± 0.005***†††	54.0 ± 12.7**†††

All values are means ± SEM for four animals in each group. **P<0.01, ***P<0.001 compared to the corresponding control.

†P<0.05, †††P<0.001 compared to the corresponding wild-type group.

Dose response studies in C57Bl/6N mice reported that PFOA decreased absolute and relative spleen weights (LOAEL = 7.5 and 15 mg/kg/day, respectively) and absolute and relative thymus weights (LOAEL =15 mg/kg/day for both endpoints). Organ weight effects were generally reversed by 15 days after exposure was terminated (DeWitt et al. 2008). No effect on organ weights was reported in PPARα knockout mice treated with 7.5 or 30 mg/kg/day PFOA for 15 days (DeWitt et al. 2016).

PFOA exposure in drinking water was associated with reduced IgM antibody titers in C57Bl/6J and C57Bl/6N mice (DeWitt et al. 2008, 2016). Removal of the adrenal glands in C57Bl/6N mice did not reverse reductions in IgM antibody titer levels, suggesting that the observed suppression was not in response to corticosterone production (DeWitt et al. 2009). Modulation of the complement system was observed in C57Bl/6 mice administered PFOA-treated diets. In mice provided diets containing PFOA for 10 days, activity of the classical and alternative pathways of the complement system was decreased (N/LOAEL = 0.01%/0.02%, respectively). Serum C3 levels also was decreased by PFOA (N/LOAEL = 0.01%/0.02%, respectively). Results showed that PFOA-induced hepatotoxicity was associated with activation of the complement system (Botelho et al. 2015).

Dietary PFOA (0.02% w/w) for 10 days significantly decreased total white blood cell count (72%) and number of macrophages in the bone marrow (12.2%) (Qazi et al. 2009). Exposure of mice to 0.002% PFOA for 10 days modulated levels of intrahepatic immune cells. The total number of all leukocytes (CD45+) was increased 2-fold in treated mice. Additionally, changes in cell numbers other cell types also were noted (e.g., granulocytes and myeloid suppressor cells). Hepatic levels of TNF-α (33%), IFN-γ (37%), and IL-4 (31%) were decreased in treated mice; IL-6 levels were not affected (Qazi et al. 2010).
Hu and colleagues reported effects in offspring of dams exposed to PFOA. Dams were gavaged with 0.02, 0.2, or 2 mg/kg PFOA from before pregnancy to PND 21. Splenic CD4+CD25+Foxp3+ T cells was decreased by 22% in exposed offspring (LOAEL = 2 mg/kg) (Hu et al. 2012).

In vitro data with cells or cell lines
Reduced lymphocyte proliferation was observed in cells isolated from C57Bl/6 mice treated with diets containing 0.02% PFOA for 7 days. No effect was observed in lymphocytes isolated from PPARα-null mice also provided diets containing 0.02% PFOA (values not provided) (Yang et al. 2002). Increased *ex vivo* production of TNF-α in cells isolated from peritoneal cavity (2.2-fold) and bone marrow (1.7-fold), and IL-6 in cells isolated from peritoneal cavity (2.6-fold) was observed in mice treated with 0.02% dietary PFOA for 10 days. Comparatively, TNF-α production was decreased (0.8-fold) in cells isolated from spleen of treated animals (Qazi et al. 2009). IgM or IFN-γ production levels were not modulated in intrahepatic immune cells isolated from male C57Bl/6 mice provided diets with 0.002% (w/w) PFOA for 10 days (Qazi et al. 2010).
Ex vivo co-cultures of splenic CD4+CD25+ and CD4+CD25- T cells offspring gestationally and lactationally exposed to PFOA were assessed for effects on IL-10 production. Results showed IL-10 produced was significantly decreased at all doses 61%-75% in cells obtained from male offspring (LOAEL = 0.02 mg/kg). *Ex vivo* measurement of autoreactivity antibodies in female mice gestationally and lactationally exposed to 0.02 and 2 mg/kg PFOA showed an decrease (26%) in anti-ssDNA (Hu et al. 2012).

Mode of action information
PFOA suppresses T-cell-dependent and T-cell-independent antibody responses (DeWitt et al. 2012). The role of PPARα in PFOA-induced immunosuppression may be strain dependent (Corsini et al. 2014). PFOA-induced effects on humoral immunity may occur through effects on B-cell/plasma cell function (DeWitt et al. 2016). Direct effects on immune cells also are a proposed mode of action of PFOA (Corsini et al. 2014).
The lack of impact of removal of the adrenal gland on PFOA-induced inhibition of IgM antibody titer levels suggests that the observed effects are not dependent on elevated corticosterone levels in mice (DeWitt et al. 2009).
Effects on lymphoid organ weights and measures of immune function (i.e., thymus and spleen) indicate that they are differentially sensitive to PFOA effect. The biological basis for this difference is not known (DeWitt et al. 2016).

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Human Data

Data from epidemiology studies
No difference was noted in lymphocyte counts between individuals with or without toluene exposure (Akbas et al. 2004).

In vitro data with cells or cell lines
No data were located.

Mode of action information
No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Male C3H mice were exposed to 9 ppm toluene (nose-only inhalation exposure) for 30 minutes on study days 0, 1, 2, 7, 14, 21, and 28. Mice also were immunized with ovalbumin. Toluene exposure significantly increased total cell (3-fold) and macrophage (3.1-fold) count in BAL 24 hours after final exposure. No effect on lymphocyte count was noted. BDNF level in BAL was increased in toluene-exposed mice that were immunized with ovalbumin (data in figure). Splenic ratio of CD4 and CD8 cells in control and toluene-exposed mice were not significantly different;
3.95 and 4.14, respectively. Treatment with ani-CD4 antibody decreased the ratios to 0.65 and 0.49, respectively. Toluene exposure significantly increased plasma levels of nerve growth factor (data in figure), but did not increase plasma BDNF levels (data not provided) (Fujimaki et al. 2009).
Male C57BL/10 and B10.BR/Sg mice were inhalationally exposed to 0, 5, and 50 ppm toluene for 6 hours per day, 5 days per week for 6 weeks. Subgroups of control and treated mice were administered ovalbumin prior to exposure. Toluene exposure did not impact ConA- or LPS-induced proliferation of spleen cells from C57BL/10 mice. While no effect of ConA was noted in B10.BR/Sg (not treated with ovalbumin) mice spleen cells, 50 ppm toluene significantly increased the LPS-induced proliferation of spleen cells. Comparatively, 50 ppm toluene significantly decreased spleen cell proliferation in B10.BR/Sg mice treated with ovalbumin (data in graphs). Toluene did not alter expression of CD3, CD19, and CD11b (data not provided). Forkhead box P3 (Foxp3) transcription was significantly increased in spleen cells from B10.BR/Sg mice exposed to 5 ppm toluene and ovalbumin, when compared to controls and those not treated with ovalbumin. No effect on GATA3 or T-bet expression was noted (Fujimaki et al. 2010).
Pregnant C3H/HeN mice were exposed to 50 ppm toluene via inhalation on GD 14-18. Additionally, male offspring of unexposed dams were exposed to 50 ppm toluene on PND 2-6 or 8-12. The following table summarizes the effects observed in male offspring on PND 21.

Table 1. Summary of effects in male offspring

Origin	Biomarker	GD 14–18	PND 2–6	PND 8–12
Plasma	IgG2a	No effect	Decrease	Increase
	IgG1	Decrease	Decrease	Decrease
Spleen	CD4+ lymphocyte subset	No effect	Decrease	Decrease
	CD8+ lymphocyte subset	No effect	No effect	Decrease
	T-bet mRNA	No effect	Decrease	Decrease
	Foxp3 mRNA	No effect	Decrease	Decrease
	GATA3 mRNA	No effect	No effect	No effect

On PND 42, IgG2a levels were decreased in mice exposed to 50 ppm toluene on PND 8–12. No effect on IgG1 was noted. CD19+ B-lymphocytes and CD4+ T-lymphocytes were significantly decreased, while CD3+ T-lymphocytes were increased at PND 42 after exposure on PND 8–12. Additionally, T-bet expression was significantly decreased, while no effects on GATA3 or Foxp3 mRNA expression were reported (Win-Shwe et al. 2012a).
Pregnant C3H/HeN mice were exposed to 5 or 50 ppm toluene via inhalation on GD 14–18. Additionally, male offspring of unexposed dams were exposed to 5 or 50 ppm toluene on PND 2–6 or 8–12. In the hippocampus of PND 21 male offspring, TNF-α and NF-κB mRNA were significantly increased in mice exposed to 50 ppm on PND 2–6 when compared to controls (data in graphs). TNF-α, CCL3, and NF-κB were increased in mice exposed to 5 ppm on PND 8–12 (data in graphs) (Win-Shwe et al. 2012b).

In vitro data with cells or cell lines
Toluene (500 μM) exposure significantly increased ConA- (1.8-fold) and LPS- (2.1-fold) induced proliferation of spleen cells from female C57BL/6 mice. However, at the same concentration toluene did not modulate NK activity or suppress CTL formation (Grayson and Gill 1986).

Mode of action information
Low-level (5 ppm) inhalational exposure to toluene activates the STAT6, STAT5, and Foxp3 signaling pathway to enhance Th2-related and T_{reg}-related responses in B10.BR/Sg mice treated with ovalbumin (Fujimaki et al. 2010). Toluene also enhanced NF-□B, STAT5, and NF-AT in thymus cells of C3H/HeN mice inhalationally exposed to toluene (Liu et al. 2010). Toluene modulation of IL-2 synthesis, after oral exposure, may play a role in observed immunotoxic effects (Hsieh et al. 1989).

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

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
TBTC dose-dependently decreased the percentage of colony forming unit-granulocyte macrophage (CFU-GM) colonies at concentrations ranging from 0.001 to 3.3 µM (data not provided) (Carfi et al. 2007).
Cytokine release from human lymphocytes was assessed in whole blood obtained from three donors. IFN-γ was evaluated in blood from two donors after PHA stimulation for 72 hours. Comparatively, TNF-α was evaluated in blood from three donors after LPS stimulation for 72 hours. Overall, IFN-γ and TNF-α was modulated (i.e., either increase or decrease release) in all tested samples (Carfi et al. 2007).
Long-term cultures of human bone marrow cells were incubated with 0.001 µM TBTC in the presence or absence of a cytokine mixture for 7 or 14 days. A significant decrease in the percentage of CD19+CD22+ cells, in the absence of effects on the total lymphocyte population or percentage of T-cell subsets was reported after 7 and 14 days. Addition of cytokine mixture had no effect on TBTC effects. TBTC also induced cell death in CD19+ lymphocytes, in the absence of PPAR-γ receptor expression (Carfi et al. 2010).
The IC50s for cell viability in human LCLs or PBMCs were 0.25 and 0.33 µM, respectively. TBTC (0.1 µM) did not modulate TNF-α, IL-2, IL-4, or IL-10 release in LCLs pre-treated with phorbol 12-myristate 13-acetate/ionomycin stimulated cells. Comparatively, TBTC significantly decreased IL-6 and IFN-γ release (Markovic et al. 2015).

Mode of action information
In vitro toxicogenomic studies in Jurkat cells (human lymphoblastic T-cell line) showed that TBTC activated cellular stress response and retinoic-acid mediated response genes (Shao et al. 2013).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
In a 2-week study, male Wistar rats were provided diets containing 15, 50, or 150 ppm TBTC. A dose-related decrease in relative and absolute spleen and thymus weights were reported (LOAEL
= 50 and 15 ppm, respectively). Concurrent to the change in thymus weight, a decrease in thymic cell counts also was observed (LOAEL = 50 ppm). However, no signs of increased lymphocyte destruction in the spleen was observed. A dose-related increase in relative liver weight was reported (LOAEL = 50 ppm). Decreased thymus weight also was observed in rats fed 100 ppm

TBTC for 4-weeks (43% of control weight); no effects on spleen or liver weight were noted (Snoeij et al. 1985).

In utero and lactational exposure effects of TBTC (0.025, 0.25, or 2.5 mg/kg/day) were evaluated in Sprague-Dawley rats. Dams were orally dosed with TBTC from GD 8 until weaning. After weaning, pups were orally exposed to the same dose as the dam until sacrifice (up to PND 90). In males, a significant decrease in spleen weight was only observed in pups treated with 0.25 mg/kg/day on PND 30. A significant decrease in thymus weight also was noted on PND 30 (LOAEL = 2.5 mg/kg/day) (Cooke et al. 2004). Serum IgM levels were increased in 30- and 60-day old female offspring, while IgA, IgM, IgG, and IgG2a levels were increased in 90-day old male rats (Tables 1 and 2) (Tryphonas et al. 2004).

Table 1. Serum IgM levels in 30- and 60-day old females†.

	30-day old females				60-day old females			
	Control	0.025 mg/kg/day	0.25 mg/kg/day	2.5 mg/kg/day	Control	0.025 mg/kg/day	0.25 mg/kg/day	2.5 mg/kg/day
IgM	51.6 ± 8.8	41.2 ± 6.8	39.6 ± 5.8	66.5 ± 9.9	34.0 ± 3.2	63.8 ± 5.8	68.1 ± 16.4	73.0 ± 15.0

†Values provided as pg Ig/mL serum × 10^4 (standard error of the mean ± standard error).

Table 2. Serum immunoglobulin levels in 90-day old males†

	Control	0.025 mg/kg/day	0.25 mg/kg/day	2.5 mg/kg/day	Pearson product moment correlation
IgA	32.0 ± 8.8	13.9 ± 3.3	9.7 ± 3.4*	11.9 ± 1.6	>0.05
IgM	46.2 ± 8.9	65.1 ± 4.9	69.6 ± 5.8	232.5 ± 90.1*	0.00168
IgG	96.8 ± 9.6	184.2 ± 86.7	194.6 ± 25.7*	314.1 ± 57.5*	0.0134
IgG1	41.5 ± 8.6	77.2 ± 28.6	85.2 ± 18.6	58.1 ± 15.9	>0.05
IgG2a	53.1 ± 5.7	59.1 ± 6.3	50.8 ± 4.6	31.3 ± 4.4*	0.00041
IgG2b	34.1 ± 3.3	39.1 ± 5.6	39.3 ± 3.5	31.6 ± 4.2	>0.05
IgG2c	13.6 ± 1.7	20.6 ± 3.6	41.0 ± 19.4	20.9 ± 2.2	>0.05

†Values provided as pg Ig/mL serum × 10^4 (standard error of the mean ± standard error).
* Significantly different from control.

The number and percentage of NK cells was increased in 30-day female and male offspring (LOAEL = 2.5 mg/kg/day). A dose-dependent increase in the number and percentage of NK cells also was noted in 90-day male rats. In 60-day female offspring an increase in the percentage of CD4+8+ T lymphocytes (LOAEL = 0.25 mg/kg/day). No anti-sheep erythrocyte IgM response or lymphoproliferative activity of splenocytes in response to mitogen stimulation

was noted in 60- day old female rats or 90-day old male rats (data not provided). Delayed-type hypersensitivity to oxazolone was increased in 90-day old male rats at 0.025 and 0.25 mg/kg/day and decreased at
2.50 mg/kg/day. Mean colony forming *L. monocytogenes* bacteria was non-linearly increased at 48 hours post-infection and statistically significant in pairwise comparisons (0.25 mg/kg/day) in 60-day old females. In 90-day old males, a non-linear dose-response trend 3 days after infection was reported. No effects in serum levels of IL-2, TNF-α, IFN-γ, and IL-1β were reported in males or females. A non-linear dose-response increase in NK activity in 60-day females was reported (Tryphonas et al. 2004).

Lactational exposure in mice to TBTC also impaired innate immunodefenses in offspring. C57BL/6 pregnant mice were given drinking water with 15 or 50 µg/mL TBTC from parturition to weaning. Clearance of *Escherichia coli* K-12 from the peritoneal cavity and spleen of offspring treated with 15 µg/mL TBTC was significantly decreased (Kimura et al. 2005).
ICR mice were orally dosed with 0.5, 4, or 20 mg/kg TBTC for 28 days. Relative spleen and thymus weights were significantly decreased at the highest dose tested (46% and 59% decrease, respectively). TBTC also decreased the number of plaque forming cells in response to exposure to sheep red blood cells (LOAEL = 4 mg/kg). TBTC also suppressed delayed-type hypersensitivity response to sheep red blood cells when assessed 24 and 48 hours after injection (LOAEL = 4 mg/kg). TBTC suppressed T-lymphocyte proliferation in a dose dependent manner (LOAEL = 20 mg/kg). Increased percentage of early- and late-stage thymocyte apoptosis, and expression of Fas protein expression in proteins also were noted (LOAEL = 4 mg/kg) (Chen et al. 2011).
Esophageal tubing of male C3H/Hen mice with 10 or 100 ppm TBTC for 1 week was associated with decreased NK activity. NK activities were inhibited 36% to 46% at effector:target (YAC-1 cells) ratios of 25:1 and 50:1, respectively. A significant decrease in the percentage of large granular lymphocytes (~60%) also was noted 1 week after end of treatment (Ghoneum et al. 1990).

In vitro data with cells or cell lines
Neutrophils and macrophages from mice lactationally exposed to TBTC (15 or 50 µg/mL) were isolated from peritoneal exudates. Bacterial binding to isolated neutrophils from offspring treated with 50 µg/mL TBTC was significantly decreased (data not provided). Comparatively, bacterial binding was increased in macrophages isolated from offspring treated with 50 µg/mL TBTC. Decreased phagocytosis (LOAEL = 15 µg/mL) and killing activities (15 µg/mL) only were observed in neutrophils. No effect on IL-1β, IL-6, or TNF-α production was noted from macrophages or neutrophils. MCP-1 production was significantly increased in neutrophils isolated from offspring treated with 50 µg/mL TBTC (Kimura et al. 2005).
Rat and mouse spleen cells were treated with TBTC for 24 hours and then LPS or PHA for 48 hours. Then, mitogen stimulation was assessed by 5-bromo-2'-deoxyuridine incorporation. TBTC inhibited cellular proliferation for both species; the inhibitory response was more potent in mice cells vs. rat cells (IC50 with LPS: 0.0025 vs. 0.007 µM, IC50 with PHA: 0.002 vs. 0.007 µM). TBTC also inhibited rat spleen proliferation that was stimulated by ConA (no data provided). TBTC also inhibited anti-CD3 antibody stimulation of mouse lymphocyte proliferation (IC50 > 0.1 µM) (Carfi et al. 2007).
NK activity was dose-dependently inhibited in splenic lymphocytes incubated with 0.01 to 1 ppm TBTC. The LOAEL values at effector:target ratios of 25:1 and 50:1 were 0.05 and 0.01 ppm, respectively. Decreased viability of splenic lymphocytes also was reported after exposure to TBTC (LOAEL = 0.1 ppm) (Ghoneum et al. 1990).

Mode of action information

In vivo effects of TBTC on the thymus of orally treated rats are proposed to be due to the metabolite dibutyltin chloride (Snoeij et al. 1988).

The role of apoptosis is not clear. In one study the authors indicated that apoptosis does not appear to be involved in inhibition of immature thymocyte proliferation, which may lead to thymus atrophy (Gennari et al. 1997). In a separate study, the authors proposed oxidative stress plays a role in TBTC-caspase-dependent apoptosis in murine thymocytes (Sharma and Kumar 2014).

In vitro studies suggest that TBTC promotes Th2 polarization via depletion of glutathione in antigen-presenting cells, which leads to modulation of IL-10 and IL-12 production (Kato et al. 2006).

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Urethane [CASRN 51-79-6]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
The IC50s for cell viability in human LCLs or PBMCs were 82,329 and 140,768 μM, respectively. Urethane (5000 μM) did not modulate TNF-α, IFN-γ, IL-2, IL-4, IL-6, or IL-10 release in LCLs pre-treated with phorbol 12-myristate 13-acetate/ionomycin stimulated cells (Markovic et al. 2015).
Urethane dose-dependently decreased the percentage of CFU-GM colonies at concentrations greater than 1000 μM (data provided in graph) (Carfi et al. 2007).
Cytokine release from human lymphocytes was assessed in whole blood obtained from four donors. IFN-γ was evaluated in blood from three donors after PHA stimulation for 72 hours. Comparatively, TNF-α was evaluated in blood from four donors after LPS stimulation for 72 hours. IFN-γ was modulated (i.e., either increase or decrease release) in a single tested sample. TNF-α was not modulated any of the tested samples (Carfi et al. 2007).

Mode of action information
No data were located.

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
Inbred A/J mice were administered urethane (1 mg/g) via intraperitoneal injection. A biphasic response on splenic NK cell activity was noted. At one day after the injection activity was decreased 60%, activity then increased (decreased 35%), and then remained decreased until 14 days after exposure (decreased 98%). Spleen size was initially reduced, but then increased to control levels. Mitogen response (against YAC-1 or RL♂1 target cells) was initially depressed after urethane exposure and then returned to control levels (Gorelik and Herberman 1981a).
Inbred A/J mice (5-24 days old) were administered urethane (0.5 mg/g or 1 mg/g) up to 24 days old. In all tested groups, splenic NK activity was inhibited without effects on cellularity on spleens. Decreased NK activity remained until at least 8-10 weeks of age (Gorelik and Herberman 1981a).
Inbred A/J, CBA/J, and C57BL/6 mice were administered urethane (1 mg/g) via intraperitoneal injection. One day after injection, cytotoxicity (against YAC-1 target cells) of A/J and CBA/] spleen cells was significantly decreased (63% and 25%, respectively). Activity was similar to control levels at day 4. Activity then decreased in splenic cells from A/J mice (58%), while a

similar effect in cells from CBA/J mice was not observed. No effect on activity was observed in C57BL/6 mice (Gorelik and Herberman 1981b).
Female B6C3F1 mice were administered 1, 2, or 4 mg/g urethane over a 14-day period via intraperitoneal injection. Decreased spleen weight (decreased 47%) and thymic atrophy (decreased 40%) were observed at 4 mg/g. Splenic lymphoproliferative response to ConA was decreased at 4 mg/g (42%). Responses to PHA and spleen cells from DBA mice were similar to controls. Delayed hypersensitivity responses also were not affected by exposure to urethane. Serum immunoglobulin levels and antibody responses to sheep erythrocytes and LPS were decreased in mice administered 4 mg/kg (decreased 61% and 46%, respectively). Macrophage cytostasis of MBL-2 target cells was decreased (LOAEL = 1 mg/g). However, phagocytosis and bactericidal activity against *S. aureus* was not affected. Pluripotent stem cells proliferation was inhibited at all doses. Urethane decreased NK activity against all YAC-1 target to cell ratios at all doses (Luster et al. 1982).
C57BL/6J dams were subcutaneously injected with 0.05 or 0.1 mg/g urethane on GD 7-17. Offspring were evaluated 8 weeks after parturition. Increased relative spleen weight was reported for the litter at 0.05 mg/g urethane. When evaluated based on sex, only an increase in relative thymus weight was observed at 0.05 and 0.1 mg/g. Decreased white blood cell count was also observed (LOAEL = 0.05 mg/g). No effect on lymphoproliferative responses or NK cell activity was noted. However, a decrease in the levels of plaque forming cells in response to sheep erythrocytes was noted (LOAEL = 0.1 mg/g) (Luebke et al. 1986).
C57BL/6J offspring were subcutaneously injected with 0.2 mg/g urethane on PND 5-14. No effects on organ weight or lymphoproliferative responses were noted. NK cell activity was decreased at an effector:target (YAC-1) ratio of 50:1. Splenic cellularity was increased in female offspring and decreased in male offspring (Luebke et al. 1986).
Female C57BL/6J mice were subcutaneously injected with 1, 2, or 4 mg/g urethane. Significant reduction in absolute (LOAEL = 1 mg/g) and relative (data not provided) spleen weights were observed. Additionally, absolute thymus weight was decreased (LOAEL = 4 mg/g). Dose-dependent reduction in leukocyte number was noted, but differential counts of white blood cells were not altered. Lymphoproliferative responses, induced by ConA, PHA, and LPS, were suppressed by urethane (LOAELs = 1, 1, and 4 mg/g, respectively). were noted. Lymphoproliferative responses to allogenic cells (mitomycin C treated CBA/J mouse spleen cels) were not affected by urethane exposure. NK cell activity was not affected at any effector:target (YAC-1) ratio. Splenic cellularity of mice treated with urethane and sheep erythrocytes was decreased (LOAEL = 2 mg/g) without effects on PFC/spleen or PFC/splenocytes. Decreased DTH index (to keyhole limpet hemocyanin) was decreased in urethane treated mice (LOAEL = 4 mg/g) (Luebke et al. 1987).
mRNA expression of interleukins and TNF-α were evaluated in spleens of male Wistar rats exposed to 1500 mg/kg urethane. Increased expression of IL-6 was noted, while decreased expression of IL-1β and TNF-α were reported. No effects on IL-2 expression were observed (Bette et al. 2004).

Urethane (10%) did not deplete ear epidermis Ia-positive LCs after male BALB/c mice were treated with topical application. Similarly, urethane did not alter the density of β-glucuronidase- positive LC in C57BL mouse tails topically treated for 1 or 3 weeks (Halliday et al. 1988).
Urethane administration to pregnant ICR mice (1.5 mg/g subcutaneous injection on GD 10) produced a transient decrease in dam thymocyte cell count. At 3 days after treatment, a significant decrease in cell count was noted. By 5 days after treatment, the cell count had recovered to control levels (data in graph). A similar phenomenon was noted with thymocyte phenotypes; decrease in CD4+8+ thymocytes (88%) at day 3 after treatment was recovered by day 5. Transient changes in dam splenocyte cell count and splenocyte phenotype CD4+8-, CD4- 8+, and CD4-8- also were reported. Gene expression analyses identified changes in spleen gene expression due to urethane exposure with or without immune stimulation (FCA). Increased expression of TGFβ3 was observed in the presence or absence of immune stimulation one day after treatment. IGF-I, IGF-II and IL-2 were also differentially expressed (Sharova et al. 2002).

In vitro data with cells or cell lines
Rat and mouse spleen cells were treated with urethane for 24 hours and then LPS or PHA for 48 hours. Then, mitogen stimulation was assessed by 5-bromo-2'-deoxyuridine incorporation. Urethane did not inhibit cellular proliferation in either species (data not provided). Urethane also did not modulate rat spleen proliferation that was stimulated by ConA or inhibit anti-CD3 antibody stimulation of mouse lymphocyte proliferation (data not provided) (Carfi et al. 2007).

Mode of action information
In vitro and *in vivo* studies suggest that urethane metabolism by cytochrome P450 is needed to produce the observed immunomodulatory effects (Cha et al. 2000). Macrophage effects are based on urethane effects on the inductive phase of immune responses (Foris et al. 1983).

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Vanadium Pentoxide [CASRN 1314-62-1]

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
Vanadium pentoxide (25 – 400 μM) inhibited cell proliferation and induced cell apoptosis in a dose and time-related manner in the IL-2-independent human NK cell line, NK-92MI. Cell proliferation was maximally inhibited (78%) at 400 μM vanadium pentoxide, and the percentage of cells undergoing apoptosis increased at 12 and 24 hours of exposure (51.2 and 64.7%, respectively) as the concentration of vanadium pentoxide increased. IL-2, IL-10 and IFN γ secretion were all inhibited by vanadium pentoxide after 24 hours at the highest concentration tested. IL-2 secretion also was inhibited after 12 hours. Expression of CD25 significantly increased above background starting at 50 μM, reaching a maximal migration inhibitory factor (MIF) of 47.4% at 400 μM. A similar pattern was observed for IL-15R α, with a maximal MIF of 55.2% at 400 μM. Fas expression began to increase at 100 μM and reached a maximal MIF of 48.9% at 400 μM, while FasL peaked at 200 μM (62.1%). Jak3 phosphorylation was increased at 12 and 24 hours after treatment with 200 and 400 μM vanadium pentoxide (data in figure), and intracellular staining showed a strong presence of pJak3 in the internal cell membranes after treatment. (Gallardo-Vera et al. 2016).

Mode of action information
Vanadium in the +2, +3, and +4 (but not the +5) valence states interacted with human FMLP-activated neutrophils and statistically significantly increased the formation of hydroxyl radicals, with additional augmentation observed in the presence of sodium azide (values not provided) (Fickl et al. 2006).
Vanadium pentoxide induced toxic effects on the IL-2-independent human NK cell line, NK-92MI, through dysregulation of signaling pathways mediated by IL-2 via increased PTEN and decreased SHP1 expression (Gallardo-Vera et al. 2018).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies

Male F344 rats were exposed to vanadium pentoxide (100 μg V/m^3) via inhalation, 5 hours per day for 5 days. The animals were infected with Listeria following the 5-day exposure and the bacterial burden assessed at 24, 48 and 72 hours, post-infection. Vanadium pentoxide did not have any significant effect on Listeria burdens at any of the timepoints observed (Cohen et al. 2007).

Male CD-1 mice were exposed to vanadium pentoxide (0 or an average of 1436 μg/m^3) via inhalation, 1 hour per day, 2 times per week over 12 weeks. An increase in the number

(3.8±0.12 vs. 2.1±0.12 μm per field) and the size (36±0.52 vs. 25±0.35 μm) of megakaryocytes in the

spleen was observed in vanadium pentoxide exposed mice, as compared to controls. These same types of changes were also observed in the bone marrow (values not provided). No statistical difference was observed in spleen weight between treated and control mice (Fortoul et al. 2008). When male and female CD-1 mice were exposed to vanadium pentoxide (0 or 1.4 mg/m^3) using the same protocol as in Fortoul et al., 2008, a sex difference was observed in the expression of Ki-67, a specific proliferation marker for lymphocytes. The percentage of Ki-67 immunopositive lymphocytes increased in male mice (38.86, 41.75 and 41.91%) after 4, 8 and 12 weeks of exposure, respectively, with both cytoplasmic and nuclear expression of Ki-67 observed. In female mice, the percentage of proliferating lymphocytes increased only after the first week of exposure (34.87%) and the signal was observed only in the nucleus. Subsequent exposures did not produce significant changes in the percentage of proliferating cells in females. The authors concluded there is a role for sex hormones in potential protection against vanadium immunoxicity (Rodriguez-Lara et al. 2016).

Male CD-1 mice were exposed to vanadium pentoxide (0 or an average of 1.4 mg/m^3) via inhalation, 1 hour per day, 2 times per week over 12 weeks. Spleen weight of vanadium exposed animals peaked at 9 weeks (546 ± 45 vs. 274 ± 27 mg in controls) and progressively decreased afterwards (321 ± 39 mg at 12 weeks vs. 298 ± 35 mg in controls). The spleens of vanadium exposed animals had histological changes that included increased numbers of lymphocytes and megakaryocytes as compared to controls. The number of $CD19^+$ cells was also increased within the hyperplastic germinal node (values not provided) and the mean hepatitis B surface antigen levels in immunized control mice was greater than in the exposed hosts (OD=0.39 ± 0.03 vs.
0.11 ± 0.05). The authors concluded that vanadium pentoxide induces functional changes in the spleen which appear to result in effects on the humoral immune response (Pinon-Zarate et al. 2008).

Male CD-1 mice were exposed to vanadium pentoxide (0 or an average of 1.4 mg/m^3) via inhalation, 1 hour per day, 2 times per week over 4 weeks. The expression of CD11c in the thymic medulla was decreased in vanadium pentoxide exposed mice, as compared to controls (values not provided), based on immunohistochemistry. Flow cytometry also demonstrated a decrease in $CD11c^+$ and $MHC\text{-}II^+$ cells in vanadium pentoxide exposed mice, as compared to controls (values not provided). The decrease was both, in terms of number and in mean fluorescence intensity values (Ustarroz-Cano et al. 2012).
Male F344/N rats and female B6C3F1 mice were exposed to 0, 4,8, or 16 mg/m^3 vanadium pentoxide, via inhalation, 6 hours per day, 5 days per week for 16 days. Pulmonary inflammation was assessed via analysis of BAL fluid. Significant alterations in the percentage of recoverable macrophages and neutrophils (NOAEL 4 mg/m^3), and increased lung protein and lysozyme in male rats (LOAEL 4 mg/m^3) were observed. In female mice, an increase in lymphocytes, protein and lysozymes was observed (LOAEL 4 mg/m^3). No effects were observed on systemic immunity as evidenced by a normal response to *Klebsiella pneumoniae* (National Toxicology Program 2002).

The induction of pulmonary inflammation was examined in three different strains of mice [A/J (sensitive strain for pulmonary inflammation and carcinogenesis), BALB/c (intermediate

sensitivity), and C57Bl/6J (resistant)]. Mice were aspirated with vanadium pentoxide (4 mg/kg) or phosphate-buffered saline, four times per week, with BALF collected at 6 hours, and 1, 3, 6 and 21 days. In A/J mice, vanadium pentoxide increased BALF levels of total cells (95.7%) inflammatory markers (PMNs, macrophages and lymphocytes, 74.6, 99.5, and 623.8%, respectively). Levels of inflammatory chemokines (keratinocyte-derived chemokine, macrophage inflammatory protein-2 and monocyte chemoattractant protein 1), transcription factor activity (NF κB and c-Fos) and signaling pathway activation (MAPK) were increased with highest levels observed in A/J mice followed by BALB/c and then C57BL/6J mice (data in graphs). All results returned to baseline 21 days post exposure (Rondini et al. 2010).

In vitro data with cells or cell lines
No data were located.

Mode of action information
Rondini and colleagues (2010) reported that vanadium pentoxide impacts pulmonary levels of inflammatory markers, induction of chemokines, and modulation of transcription factors
Alterations in macrophage mediated functions have been associated with vanadium exposure (Cohen et al. 1996).
The ability of several vanadium compounds to increase mRNA levels of cytokines in BALF was investigated in female CD rats. Rats received 42 or 420 µg of vanadium pentoxide or phosphate- buffered saline by intratracheal instillation. BALF was collected at times ranging from 1 hour to 10 days. Influx of neutrophils was significantly increased 24 hours after exposure to vanadium pentoxide and peaked 24-48 hours post exposure (data in graph). Macrophage inflammatory protein-2 mRNA expression levels were significantly elevated in vanadium pentoxide treated rats at 1 to 48-hour timepoints, as compared to controls (Pierce et al. 1996).

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

Human Data

Data from epidemiology studies
No data were located.

In vitro data with cells or cell lines
ZDMDC induced cytotoxicity in purified NK cells from healthy donors. Exposure to 2.5 μM ZDMDC for 24 hours produced a 99% decrease in lytic function (against K562 target cells) and at 1 μM for 6 days produced a 96% decrease. When a preparation containing T- and NK-cells were exposed to 2.5 μM for 24 hours a 41% decrease in function was observed. Comparatively, a 6-day exposure to 1 μM ziram did not inhibit lytic function. (Whalen et al. 2003). Wilson and colleagues showed that concentrations as low as 125 nM decreased cytotoxic function of purified NK cells (Wilson et al. 2004).
ZDMDC significantly inhibited NK-92MI activity (against K562 target cells) in a dose- and concentration-dependent manner (LOAEL = 0.125 μM at 2 hours incubation). A similar dose- and concentration-dependent inhibition of NK activity was observed with human lymphokine activated killer cells (LOAEL = 0.125 μM at 2 hours incubation) (Li et al. 2012a).
Purified, human NK cells were exposed to ZDMDC (0.5-5 μM) for 1 hour. Then the cells were incubated for 24 or 48 hours, or 6 days in ZDMDC-free media. A decrease in NK activity was observed at 2.5 and 5 μM. The loss of activity lasted up to 6 days after exposure (Taylor et al. 2005).
ZDMDC (5 μg/mL) decreased LPS-induced TNF-α production in THP-1 cells (data in graph). ZDMDC (5 μg/mL) also blocked LPS-induced degradation of I□B (data in Western blot) (Corsini et al. 2006).
ZDMDC induced apoptosis and necrosis in U937, NK-92MI, NK-92CI, Jurkat, and human T cells. Of U937 cells treated with 2 μM ZDMDC, 49.3% were apoptotic and 18.5% were necrotic (Li et al. 2011). In Jurkat cells treated with 0.5 μM ZDMDC, 52.5% were apoptotic and 7.9% were late apoptotic/necrotic (Li et al. 2012c). In NK-92MI cells treated with 0.5 μM ZDMDC, 47.4% were apoptotic and 12.2% were late apoptotic/necrotic (Li et al. 2012b). In NK-92CI cells treated with 0.5 μM ZDMDC, 28.7% were apoptotic and 38.5% were necrotic (Li et al. 2014). Increased apoptosis and late apoptosis/necrosis also was observed in a time- and dose-dependent manner in isolated primary T-cells (data in graph) (Li et al. 2012c).
At concentrations ranging from 0.1 to 10 μg/mL, ZDMDC was not cytotoxic to lymphocyte cultures obtained from peripheral blood from healthy volunteers (Zenzen et al. 2001).

Mode of action information
Effects in U937, NK-92MI, and Jurkat cells were dose- and time-dependent. Increased DNA fragmentation, level of active caspase-3, and level of cytochrome c release from U937 and Jurkat

cells also were noted after ZDMDC exposure (Li et al. 2011, 2012c, 2012b, 2015). Increased levels of caspase-7, -8, and -9 also were detected in NK-92MI and Jurkat cells (Li et al. 2012c, 2012b).
ZDMDC-induced inhibition of NK and LAK activity was mediated, in part, by decreases in intracellular levels of Gr3/K, granulysin, perforin, granzyme (Gr) A, and GrB (Li et al. 2012a). Decreased levels of GrB was associated with activation of p38 while activation of p44/42 was associated with decreased levels of perforin (Taylor and Whalen 2011).

Rodent Data

Data from in vivo immunotoxicology or toxicology studies
The EC3 in the local lymph node assay was between 1.0% and 5.0% in female BALB/c mice (De Jong et al. 2002). ZDMDC also was identified as a skin sensitizer in the guinea pig maximization test (TS_5 = 0.01%) (van Och et al. 2001).

In vitro data with cells or cell lines
ZDMDC inhibited murine (C57BL/6J) cytotoxic T lymphocyte activity in a dose- and concentration-dependent manner (LOAEL = 0.125 μM) (Li et al. 2012a).
ZDMDC (10 μM) decreased expression of pro-caspase-1 and NLRP3 in J774A.1 cells. Studies in RAW264.7 cells showed that 10 μM ZDMDC increased pro-caspase-1 degradation and not protein cleavage. ZDMDC also decreased LPS-induced production of IL-18 and IL-1β in bone marrow macrophages. Inhibition of LPS-induced IL-1β production occurred in a dose-dependent manner in J774A.1 (LOAEL = 5 μM) (Muroi and Tanamoto 2015).
J774A.1 cells were infected with *S. typhimurium* TA98 and then treated with ZDMDC. ZDMDC (1-10 μM) increased the number of infected bacteria in a concentration-dependent manner (LOAEL = 5 μM) (Muroi and Tanamoto 2015).

Mode of action information
ZDMDC increased intracellular level of zinc in rat thymic lymphocytes, which may be associated with induction of apoptosis (Kanemoto-Kataoka et al. 2015).

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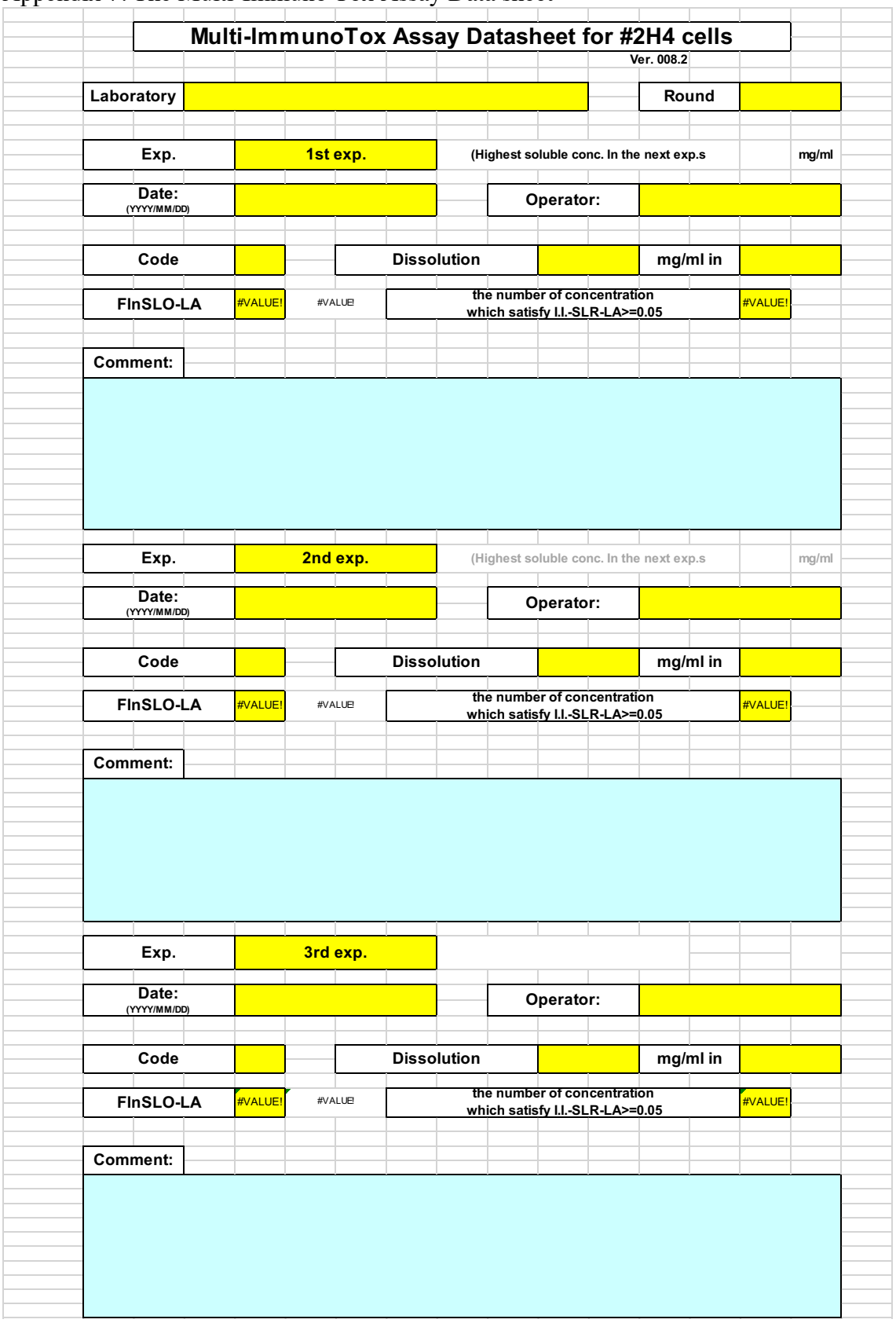
Appendix 6. The summary of immunotoxicological data of 25 chemicals.

Chemicals	In vivo				Ex vivo			In vitro		T cell targeting	Mode of action
	immune sytem organ weight	DTH	infection	tumor	cytokine production	NK activity	TDAR	cytokine production	cell proliferation		
Phase I study											
Dibutyl phthalate	A (spleen)							S (IL-2, 4, IFN-g)(H) A (IL-1b)(H) x 3 S (IL-1b)		YES	This compound then is proposed to modulate cytokine secretion from both monocytes/macrophages and T cells.
Hydrocortisone	S (thymus) x 2 S (spleen)	S					N	S (IFN-a)		YES	
Lead(II) acetate	A(thymus)	S A	S				S N	S (IFN-g, IL-1b)(H) A (IL-4)(H)	S(H)	YES	
Nickel(II) sulfate	N S (thymus)	S		S		S N	N	A (IL-4, IFN-g)(H) S (IL-2) S (IFN-g)		YES	
dimethyldithiocarbamate (DMDTC)		A x 2						S (IL-1b)	N(H)	NO	
Phase II study											
2.4-diaminotoluene	N (spleen) A (spleen)	A	S	N		S	S	-	-	NO	
Benzo(a)pyrene		N	N		S(IL-2)	N	S x 5 A	A (IL-4)(H) N (IFNγ)(H) N (IL-2)(H)	S (H) x 2 S x 6	YES	Disruption of T-cell activities has been associated with B(a)P induced immunotoxic effects (Urso et al. 1986).
Cadmium Chloride	A (spleen) S (spleen)	N			A (IL-2) N (IFN-γ)	N	S x 4	S (IL-2, 4, IFN-g) A (IFN-g)(H) S (IL-2, IFN-g) A (IFN-g) S (IL-2) A (IL-2)	S	YES	
Dibromoacetic acid (DBAA)	A (spleen) S (thymus) x 2		N	N		S	N	S (IL-2, 4)	S	YES	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)	**S (thymus) x 4 A (thymus) x 2 A (spleen)**	**N S**	**N(H) A(H)**		**A (IFN-g) x 3**	**A(H)**	**S**	**A (IL-1) A (IL-2)**		**YES**	DES exposure was associated with down-regulation of gene expression in the TCR complex, and the TCR and CD28 signaling pathways.
Diphenylhydantoin		**S N**			**A (IL-4) S (IFN-γ, IL-2) S (IL-1α) N (IL-6, 12)**	**S**	**S A x 2**	-	-	**YES**	DPH treatment can lead to a decrease of suppressor T cells
Ethylene Dibromide (EDB)	**S (thymus) S (spleen) N**		**N**			**S**	**A**	-	-	**NO**	
Glycidol	**N**		**A**	**S**		**S**	**S**	-	-	**NO**	
Indomethacin	**N A (spleen)**		**A**				**S x 3 A x 1**	**A (IL-2)(H) A (IFN-g)(H)**	**A (H) x 4 S A x 3**	**YES**	indomethacin inhibition of prostaglandin synthesis leads to altered T-cell function,
Isonicotinic Acid Hydrazide (IAH)	**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**	**YES**	
Nitrobenzene	**A (spleen) x 3 A (thymus) x 2**		**N S**	**N**			**S N**	-	-	**NO**	effects on T-cell function may play a role in increased susceptibility to L. monocytogenes (Burns et al. 1994).
Urethane, Ethyl carbamate	**S (thymus) x2 S (spleen) x 2 N A (thymus) A (spleen)**	**S**			**N (IL-2)**	**S x 4 N x 3**	**S x 2 N**	**N (IL-2, 4, IFN-g)(H) A (IFN-g)(H) S (IFN-g)(H)**	**N x 2**	**YES**	
Tributyltin Chloride (TBTC)	**S (thymus) x4 S (spleen) x 3**	**N S**	**S x 2**			**S**	**N S**	**A (INF-g)(H) N (IL-2, 4)(H) S (IFN-g)(H)**	**S (H) S x 3**	**YES**	
Perflouorooctanoic Acid (PFOA)	**S (thymus) x2 S (spleen) x 2**		**S(H) N(H)**		**N (IFN-g)**			**S (IL-4)(H) N (IL-2)(H)**	**A (H) S (H) N (H)**	**YES**	Direct modulation of NF-kB has been implicated in modulation of cytokine production and secretion (Corsini et al. 2012).
Dichloroacetic Acid (DCAA)	**A(spleen)**	**N**			**N (IL-2) A (IFN-γ) x 3 S (IL-4) x 2 S (IL-2)**	**N**	**N**	**A (IL-2)(H) A (IL-2, IFN-g)**		**YES**	T-cell activation was one proposed mode of action for DCAA.
Toluene									**A**	**NO**	Toluene also enhanced NF-kB, STAT5, and NF-AT in thymus cells of C3H/HeN mice inhalationally (Liu et al. 2010). Toluene modulation of IL-2 synthesis, after oral exposure, may play a role in observed immunotoxic effects (Hsieh et al. 1989).
Acetonitrile	**S(thymus)**					**S**	**S S**	-	-	**Undetemined**	No data were located.
Mannnitol									**N (H)**	**NO**	
Vanadium Pentoxide	**N A (spleen)**		**N x 2**					**S (IL-2, IFN-g)(H)**	**S (H)**	**YES**	
o-Benzyl-p-chlorophenol (BCP)	**N**	**N A**		**N**			**N**	-	-	**NO**	

Appendix 7. The Multi-Immuno Tox Assay Data sheet



Multi-ImmunoTox Assay Datasheet for #2H4 cells

Ver. 008.2

Laboratory		Round	

Exp.	1st exp.	(Highest soluble conc. In the next exp.s	mg/ml
Date: (YYYY/MM/DD)		Operator:	
Code		Dissolution	mg/ml in
FInSLO-LA	#VALUE!	#VALUE!	the number of concentration which satisfy I.I.-SLR-LA>=0.05

Comment:

Exp.	2nd exp.	(Highest soluble conc. In the next exp.s	mg/ml
Date: (YYYY/MM/DD)		Operator:	
Code		Dissolution	mg/ml in
FInSLO-LA	#VALUE!	#VALUE!	the number of concentration which satisfy I.I.-SLR-LA>=0.05

Comment:

Exp.	3rd exp.		
Date: (YYYY/MM/DD)		Operator:	
Code		Dissolution	mg/ml in
FInSLO-LA	#VALUE!	#VALUE!	the number of concentration which satisfy I.I.-SLR-LA>=0.05

Comment:

Exp.	4th exp.		
Date: (YYYY/MM/DD)		Operator:	
Code		Dissolution	mg/ml in
FInSLO-LA	#VALUE!	#VALUE!	the number of concentration which satisfy I.I.-SLR-LA>=0.05

Comment:

Exp.	5th exp.		
Date: (YYYY/MM/DD)		Operator:	
Code		Dissolution	mg/ml in
FInSLO-LA	#VALUE!	#VALUE!	the number of concentration which satisfy I.I.-SLR-LA>=0.05

Comment:

Exp.	6th exp.		
Date: (YYYY/MM/DD)		Operator:	
Code		Dissolution	mg/ml in
FInSLO-LA	#VALUE!	#VALUE!	the number of concentration which satisfy I.I.-SLR-LA>=0.05

Comment:

																								ug/mL
1st																								
2nd																								
3rd																								
4th																								
5th																								
6th																								

1st Exp.	
2nd Exp.	
3rd Exp.	
4th Exp.	
5th Exp.	
6th Exp.	

MultiReporter Assay System -Tripluc® - Calculation Sheet

1st exp.

Transmittance Data

	SLG	SLO	SLR			
T0				#VALUE!	#VALUE!	#VALUE!
T1				#VALUE!	#VALUE!	#VALUE!
T2				#VALUE!	#VALUE!	#VALUE!

Filter 0 Data

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

Filter 1 Data

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

Filter 2 Data

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

2nd exp.

Transmittance Data

	SLG	SLO	SLR			
T0				#VALUE!	#VALUE!	#VALUE!
T1				#VALUE!	#VALUE!	#VALUE!
T2				#VALUE!	#VALUE!	#VALUE!

Filter 0 D

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

Filter 1 D

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

Filter 2 D

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

3rd exp.

Transmittance Data

	SLG	SLO	SLR									
T0					#VALUE!	#VALUE!	#VALUE!					
T1					#VALUE!	#VALUE!	#VALUE!					
T2					#VALUE!	#VALUE!	#VALUE!					

Filter 0 D	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

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

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

4th exp.

Transmittance Data

	SLG	SLO	SLR									
T0					#VALUE!	#VALUE!	#VALUE!					
T1					#VALUE!	#VALUE!	#VALUE!					
T2					#VALUE!	#VALUE!	#VALUE!					

Filter 0 D	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

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

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

5th exp.

Transmittance Data

	SLG	SLO	SLR			
T0				#VALUE!	#VALUE!	#VALUE!
T1				#VALUE!	#VALUE!	#VALUE!
T2				#VALUE!	#VALUE!	#VALUE!

Filter 0 D

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

Filter 1 D

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

Filter 2 D

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

6th exp.

Transmittance Data

	SLG	SLO	SLR			
T0				#VALUE!	#VALUE!	#VALUE!
T1				#VALUE!	#VALUE!	#VALUE!
T2				#VALUE!	#VALUE!	#VALUE!

Filter 0 D

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

Filter 1 D

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

Filter 2 D

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

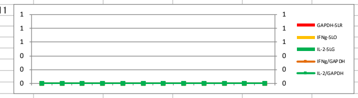
MultiReporter Assay System -Tripluc® - Calculation Sheet

1st exp.

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

1st exp.

Chemical1

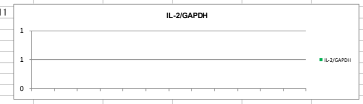


SLO-LA		1	2	3	4	5	6	7	8	9	10	11	12
	A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!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

Chemical2

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

Chemical1

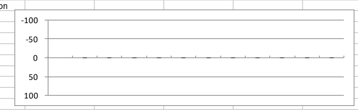


Chemical2

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

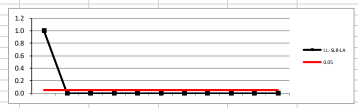
%suppression

Chemical1



Chemical2

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



Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
II-SLR-LA		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
input"*"												
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
input"*"												
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
FInSLO-LA	#VALUE!	#VALUE!										
	#VALUE!	#VALUE!	0									
Chemical1												
													ug/ml
Chemical2		0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
	cont	0	0	0	0	0	0	0	0	0	0	0	ug/ml
%Suppression (IL-2)		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
%Suppression (IFNg)		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
濃度の数(n)		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

MultiReporter Assay System -Tripluc® - Calculation Sheet

2nd exp.													2nd exp.
SLG-LA	1	2	3	4	5	6	7	8	9	10	11	12	
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical1
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
SLO-LA	1	2	3	4	5	6	7	8	9	10	11	12	
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical2
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
SLR-LA	1	2	3	4	5	6	7	8	9	10	11	12	Chemical1
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
													Chemical2
nSLG-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
nSLO-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	%suppression
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical1
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical2
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
II-SLR-LA		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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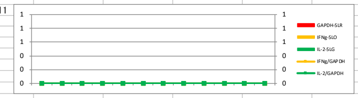
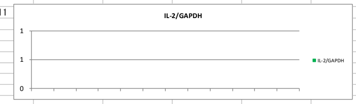
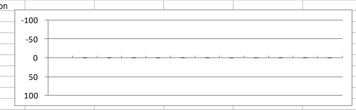
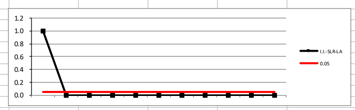
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
input"*"													
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
input"*"													
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
FInSLO-LA	#VALUE!	#VALUE!											
	#VALUE!	#VALUE!	0										
Chemical1													
													ug/ml
Chemical2													
	cont	0	0	0	0	0	0	0	0	0	0	0	ug/ml
%Suppression (IL-2)		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
%Suppression (IFNg)		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
濃度の数(n)		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	









MultiReporter Assay System -Tripluc® - Calculation Sheet

3rd exp.													3rd exp.
SLG-LA	1	2	3	4	5	6	7	8	9	10	11	12	
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical1
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
SLO-LA	1	2	3	4	5	6	7	8	9	10	11	12	
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical2
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
SLR-LA	1	2	3	4	5	6	7	8	9	10	11	12	Chemical1
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
													Chemical2
nSLG-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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nSLO-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	%suppression
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical1
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical2
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
II-SLR-LA		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
input"*"													
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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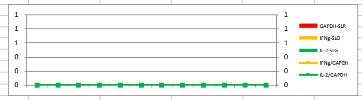
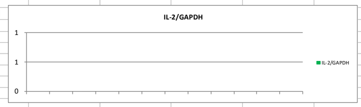
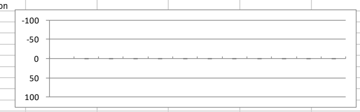
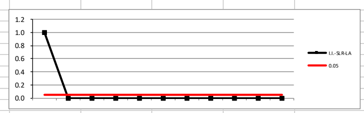
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
input"*"													
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
FInSLO-LA	#VALUE!	#VALUE!											
	#VALUE!	#VALUE!	0										
Chemical1													
													ug/ml
Chemical2													
	cont	0	0	0	0	0	0	0	0	0	0	0	ug/ml
%Suppression (IL-2)		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
%Suppression (IFNg)		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
濃度の数(n)		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	









MultiReporter Assay System -Tripluc® - Calculation Sheet

	1	2	3	4	5	6	7	8	9	10	11	12	
4th exp.													4th exp.
SLG-LA	1	2	3	4	5	6	7	8	9	10	11	12	
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical1
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
SLO-LA	1	2	3	4	5	6	7	8	9	10	11	12	
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical2
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
SLR-LA	1	2	3	4	5	6	7	8	9	10	11	12	Chemical1
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
													Chemical2
nSLG-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
nSLO-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	%suppression
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical1
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical2
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
II-SLR-LA		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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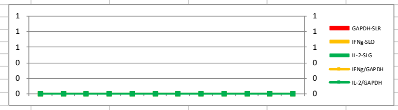
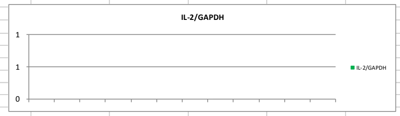
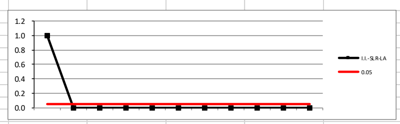
		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
input"*"													
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
input"*"													
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
FInSLO-LA	#VALUE!	#VALUE!											
	#VALUE!	#VALUE!	0										
Chemical1													
													ug/ml
Chemical2													
	cont	0	0	0	0	0	0	0	0	0	0	0	ug/ml
%Suppression (IL-2)		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
%Suppression (IFNg)		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
濃度の数(n)		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	







MultiReporter Assay System -Tripluc® - Calculation Sheet

	1	2	3	4	5	6	7	8	9	10	11	12	
5th exp.													5th exp.
SLG-LA	1	2	3	4	5	6	7	8	9	10	11	12	
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical1
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
SLO-LA	1	2	3	4	5	6	7	8	9	10	11	12	
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical2
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
SLR-LA	1	2	3	4	5	6	7	8	9	10	11	12	Chemical1
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
D	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
E	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
F	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
G	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
H	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
													Chemical2
nSLG-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#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!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
nSLO-LA	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	%suppression
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical1
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	Chemical2
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
II-SLR-LA		1.00	#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!	
		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
input"*"													
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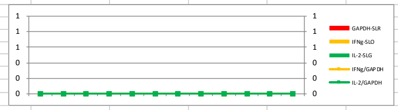
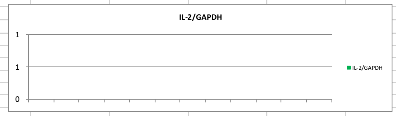
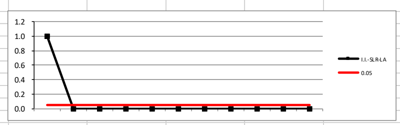
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
input"*"													
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
FInSLO-LA	#VALUE!	#VALUE!											
	#VALUE!	#VALUE!	0										
Chemical1													
													ug/ml
Chemical2													
	cont	0	0	0	0	0	0	0	0	0	0	0	ug/ml







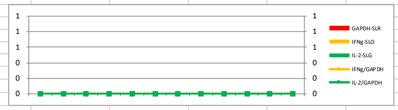
MultiReporter Assay System -Tripluc® - Calculation Sheet

6th exp.

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

6th exp.

Chemical1

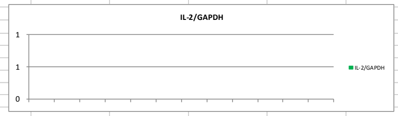


Chemical2

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

Chemical1

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



Chemical2

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

%suppression

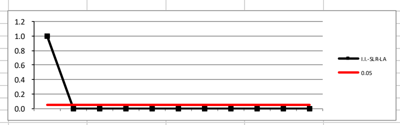
Chemical1

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

Chemical2

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

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

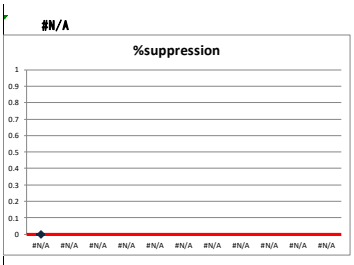


Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
input"*"												
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!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

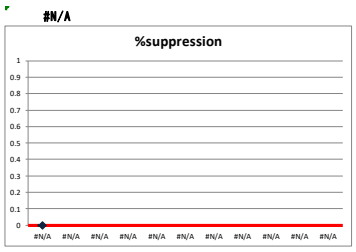
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
input"*"												
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!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

FInSLO-LA	#VALUE!	#VALUE!											
	#VALUE!	#VALUE!	0										
Chemical1													
													ug/ml
Chemical2													
	cont	0	0	0	0	0	0	0	0	0	0	0	ug/ml

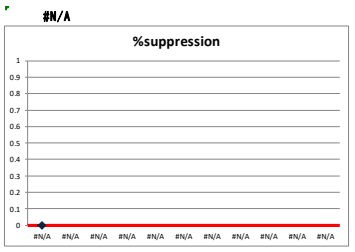
#N/A



#N/A



#N/A



#N/A

a Comparison Conc.	%supp	Lower Limit	Upper Limit
#N/A	0	0	0
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A

#N/A

a Comparison Conc.	%supp	Lower Limit	Upper Limit
#N/A	0	0	0
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A

#N/A

a Comparison Conc.	%supp	Lower Limit	Upper Limit
#N/A	0	0	0
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A

1st exp. Chemical 1

conc.	%supp	average	sd^2	ave/Dave	I.I.SLR	dummy
	0	#VALUE!	#VALUE!	1	1	1
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

cont. #VALUE!

2nd exp. Chemical 1

conc.	%supp	average	sd^2	ave/Dave	I.I.SLR	dummy
	0	#VALUE!	#VALUE!	1	1	1
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

#VALUE!

3rd exp. Chemical 1

conc.	%supp	average	sd^2	ave/Dave	I.I.SLR	dummy
	0	#VALUE!	#VALUE!	1	1	1
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

#VALUE!

Accept Exp.

#N/A
#N/A
#N/A

%suppression

	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A

dummy

#N/A	#N/A	#N/A
#N/A	#N/A	#N/A
#N/A	#N/A	#N/A
#N/A	#N/A	#N/A
#N/A	#N/A	#N/A
#N/A	#N/A	#N/A
#N/A	#N/A	#N/A
#N/A	#N/A	#N/A
#N/A	#N/A	#N/A
#N/A	#N/A	#N/A
#N/A	#N/A	#N/A
#N/A	#N/A	#N/A

Accept %suppression

	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A

#N/A

a Comparison Conc.	1-(ave/Dave)	Lower Limit	Upper Limit	ACCEPT Dummy*n
#N/A	0	0	0	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A

ACCEPTV（併合分散） #N/A ACCEPTdunnet 棄却域 #N/A

average

	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A

Accept average

	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A

#N/A

a Comparison Conc.	1-(ave/Dave)	Lower Limit	Upper Limit	ACCEPT Dummy*n
#N/A	0	0	0	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A

ACCEPTV（併合分散） #N/A ACCEPTdunnet 棄却域 #N/A

sd^2

	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A

Accept sd^2

	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A

#N/A

a Comparison Conc.	1-(ave/Dave)	Lower Limit	Upper Limit	ACCEPT Dummy*n
#N/A	0	0	0	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A	#N/A

ACCEPTV（併合分散） #N/A ACCEPTdunnet 棄却域 #N/A

ave/Dave

	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A

ave/Dave

	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A
#N/A	#N/A	#N/A	#N/A

	conc.	%supp	average	sd^2	ave/Dave	I.I.SLR	dummy
			4th exp.	Chemical 1			
	conc.	%supp	average	sd^2	ave/Dave	I.I.SLR	dummy
		0	#VALUE!	#VALUE!	1	1	1
		#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
			#VALUE!				

conc.	%supp	average	sd^2	ave/Dave	I.I.SLR	dummy
		5th exp.	Chemical 1			
conc.	%supp	average	sd^2	ave/Dave	I.I.SLR	dummy
	0	#VALUE!	#VALUE!	1	1	1
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		#VALUE!				

conc.	%supp	average	sd^2	ave/Dave	I.I.SLR	dummy
		6th exp.	Chemical 1			
conc.	%supp	average	sd^2	ave/Dave	I.I.SLR	dummy
	0	#VALUE!	#VALUE!	1	1	1
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		#VALUE!				

EXP	Dummycount	V 併合分散)	dunnetDF(全例数ー対象群を含めた群の数)	dunnet比較数(対照群を含めた群の数ー1)	dunnet棄却域
1st exp.	1	#VALUE!	#VALUE!	0	FALSE
2nd exp.	1	#VALUE!	#VALUE!	0	FALSE
3rd exp.	1	#VALUE!	#VALUE!	0	FALSE
4th exp.	1	#VALUE!	#VALUE!	0	FALSE
5th exp.	1	#VALUE!	#VALUE!	0	FALSE
6th exp.	1	#VALUE!	#VALUE!	0	FALSE

1st exp.

conc.	n	SS	Dummy*df	Dummy*n
	0		-1	0
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!

2nd exp.

conc.	n	SS	Dummy*df	Dummy*n
	0		-1	0
	0		#VALUE!	#VALUE!
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3rd exp.

conc.	n	SS	Dummy*df	Dummy*n
	0		-1	0
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
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4th exp.

conc.	n	SS	Dummy*df	Dummy*n
	0		-1	0
	0		#VALUE!	#VALUE!
	0		#VALUE!	#VALUE!
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5th exp.

conc.	n	SS	Dummy*df	Dummy*n
	0		-1	0
	0		#VALUE!	#VALUE!
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6th exp.

conc.	n	SS	Dummy*df	Dummy*n
	0		-1	0
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3	3.18245	3.86634	4.26254	4.5382	4.74793	4.91629	5.05639	5.17604	5.28024	5.37237
4	2.77631	3.31035	3.61766	3.83152	3.99447	4.12549	4.23469	4.32808	4.4095	4.48158
5	2.57058	3.03015	3.29305	3.47589	3.61512	3.72711	3.8205	3.90042	3.97015	4.0319
6	2.44692	2.86275	3.09929	3.26336	3.38838	3.48878	3.57273	3.64448	3.70709	3.76256
7	2.36464	2.75171	2.97085	3.12264	3.23813	3.33099	3.40834	3.47465	3.53251	3.58376
8	2.30603	2.67282	2.87967	3.02271	3.13145	3.21885	3.29171	3.35406	3.40847	3.45667
9	2.26218	2.61395	2.81166	2.94817	3.05188	3.13519	3.20463	3.26403	3.31587	3.36178
10	2.22817	2.56837	2.75902	2.8905	2.9903	3.07044	3.13722	3.19433	3.24416	3.2883
11	2.20102	2.53204	2.71709	2.84456	2.94126	3.01887	3.08352	3.1388	3.18703	3.22974
12	2.17885	2.50241	2.68291	2.80712	2.90128	2.97683	3.03975	3.09354	3.14045	3.182
13	2.16041	2.4778	2.65452	2.77602	2.86809	2.94193	3.0034	3.05595	3.10177	3.14234
14	2.14483	2.45702	2.63057	2.74979	2.84009	2.91248	2.97273	3.02423	3.06913	3.10889
15	2.13149	2.43926	2.6101	2.72737	2.81615	2.88731	2.94652	2.99712	3.04124	3.08029
16	2.11994	2.42389	2.59239	2.70799	2.79546	2.86555	2.92387	2.97369	3.01712	3.05556
17	2.10985	2.41047	2.57694	2.69107	2.7774	2.84656	2.90408	2.95323	2.99606	3.03397
18	2.10096	2.39865	2.56332	2.67617	2.7615	2.82983	2.88667	2.93521	2.97752	3.01496
19	2.09306	2.38815	2.55115	2.66285	2.74738	2.81499	2.87121	2.91923	2.96107	2.99809
20	2.086	2.37878	2.54035	2.65103	2.73468	2.80164	2.85731	2.90486	2.94628	2.98303
21	2.07965	2.37035	2.53064	2.64041	2.72334	2.78972	2.8449	2.89202	2.93307	2.96939
22	2.07391	2.36264	2.52188	2.63081	2.7131	2.77895	2.83369	2.88042	2.92113	2.95715
23	2.06869	2.35572	2.51391	2.6221	2.7038	2.76918	2.82351	2.86989	2.91029	2.94604
24	2.06393	2.34941	2.50665	2.61415	2.69532	2.76026	2.81422	2.86029	2.90041	2.9359
25	2.05957	2.34363	2.5	2.60688	2.68756	2.7521	2.80572	2.8515	2.89136	2.92662
26	2.05556	2.33831	2.49389	2.60019	2.68042	2.7446	2.79791	2.84342	2.88304	2.91809
27	2.05186	2.33341	2.48825	2.59402	2.67384	2.73768	2.79071	2.83596	2.87537	2.91023
28	2.04843	2.32888	2.48304	2.58832	2.66776	2.73128	2.78404	2.82907	2.86828	2.90296
29	2.04526	2.32467	2.4782	2.58302	2.66211	2.72534	2.77786	2.82267	2.8617	2.89621
30	2.0423	2.32075	2.47369	2.5781	2.65685	2.71981	2.77211	2.81673	2.85557	2.88993
31	2.03954	2.3171	2.46949	2.57351	2.65195	2.71466	2.76674	2.81118	2.84986	2.88407
32	2.03696	2.31368	2.46557	2.56921	2.64737	2.70985	2.76173	2.80599	2.84452	2.8786
33	2.03454	2.31048	2.46189	2.56519	2.64308	2.70533	2.75703	2.80113	2.83952	2.87347
34	2.03227	2.30748	2.45844	2.56141	2.63905	2.7011	2.75262	2.79657	2.83483	2.86866

1st exp.	nSLG-LA		**2nd exp.**	nSLG-LA		**3rd exp.**	nSLG-LA		**4th exp.**	nSLG-LA		**5th exp.**	nSLG-LA		**6th exp.**	nSLG-LA	
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Appendix 8 Study plan

Study plan

for the validation trial on multicolor reporter assay using IL-2 Luc (IL-2 Luc assay) as a test evaluating the immunotoxic potential of chemicals

Version 1.4 February, 2017

Conducted by:

IL-2 Luc assay Validation Management Team

INDEX

1. Background

The multicolor reporter assay using IL-2 Luc in Jurkat cells (IL-2 assay) is important for evaluating the immunotoxic potential of chemicals. This assay forms part of the Multi-ImmunoTox assay (MITA) and has the advantages of technical simplicity and a short test period, and the accuracy of the test result is based on the mechanism underlying immunotoxicity.

The aim of this trial is to (pre)validate the IL-2 Luc assay method to assess its transferability and inter-laboratory variability so that this test can be used to screen for immunotoxic chemicals. The IL-2 Luc assay for the validation trial was undertaken i) in accordance with the principles and criteria documented in the OECD No. 34 Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment [OECD, 2005], ii) according to the Modular Approach to validation [Hartung et al., 2004], iii) according to the concept discussed in the validation trials with the participation of GLP Test Facilities [Cooper-Hannan et al., 1999] where the whole concept of validation trials is described in the context of GLP, and iv) in line with the ISO procedure JRC.I.03.GP.01v.01 (http://ihcpnet.jrc.it/quality-safety/quality-documents/unit-03-ivm/doc/JRC.I.03.GP.01v.01.pdf).

The studies comprising a validation trial should ideally be performed in accordance with GLP [OECD, 1998-2007; FDA, 1999; EPA, 1998a&b; JSQA, 2010; SCC, 2010]. As a minimum, use of standard operating procedures (SOP) and adequate data recording, reporting and record keeping are essential.

A general conceptual framework [Hartung et al., 2004; OECD, 2005] will be used for documenting the entire study to assess the validation status of the test method. This is called a "modular approach" to validation. In this approach, the information needed to support the validity of the method is organized into modules that provide the following information:

Module 1: Test Definition
Module 2: Within-laboratory repeatability and reproducibility
Module 3: Between-laboratory transferability
Module 4: Between-laboratory reproducibility
Module 5: Predictive capacity
Module 6: Applicability domain
Module 7: Performance standards

The modular approach as introduced by Hartung et al. allows the use of datasets from various data sources and studies. This advantage is used in the following proposal to assess the scientific validity of the IL-2 Luc assay. This IL-2 Luc assay for the validation trial has been performed under GLP principles.

2. Objective of the trial

The validation trial will assess the reliability (reproducibility within and between laboratories) and relevance (predictive capacity) of the IL-2 Luc assay with a challenging set of test substances (test items) for which high quality *in vitro* and *in vivo* data are available.

3. Validation Management Team (VMT)

The VMT encompasses collective expertise with the test, in the underlying science, and the scientific design, management and evaluation of a validation trial.

The VMT, which plays a central role overseeing the conduct of the validation trial, includes:

Table 1. Members for IL-2 Luc assay Validation Management Team

Name	Role and expertise	Affiliation
Trial Coordinator Hajime Kojima	VMT trial coordinator, Chemical supplier and Management of quality control	JaCVAM, NIHS, Japan (JaCVAM representative)
Lead Lab Yutaka Kimura* Setsuya Aiba*	*Developer of this assay Test method, expertise underlying science	Tohoku Univ., Japan
Takashi Omori	Data analysis, biostatistics dossier	Kobe Univ., Japan
International expert members		
EU liaison Emanuela Corcini	Test system expertise, validation expertise, immunotoxicity expertise	Milan Univ., Italy
EU liaison Erwin L. Roggen	Test system expertise, validation expertise, immunotoxicity expertise	3Rs Management and Consulting ApS, Denmark
ICCVAM liaison Dori Germolec	Immunotoxicity expertise	NTP/NIEHS, USA
JSIT liaison Tomoaki Inoue	Immunotoxicity expertise	Chugai Pharmaceutical Co., Ltd.

Participating Test Facilities

The laboratories participating in the trial are defined as follow:

Test Facility 1: Hatano Res. Inst., FDSC. Study Director (SD): Kohji Yamakage

Test Facility 2: AIST, Tsukuba SD: Yoshihiro Ohmiya

Test Facility 3: AIST, Takamatsu SD: Yoshihiro Nakajima

Information relevant for Modules 1, 2, 3 performed by all laboratories. Data obtained by these laboratories have demonstrated that the IL-2 Luc assay is transferable and reproducible between experienced laboratories. All laboratories participating in this validation trial will act as unexperienced laboratories to assess between-laboratory transferability, reliability, and relevance of the IL-2 Luc assay method under non-GLP conditions (GLP principle).

Trial management structure

1) Chemical management group

The members of the chemical management group are elected by recommendation of the IL-2 Luc assay VMT. The members prepare a tentative list of test chemicals and work with the VMT to make a final decision on the test chemicals to be used in the validation trial. The coded test chemicals listed in Table 6 and 7 are distributed by the JaCVAM.

2) Data analysis group

The members of the data analysis group are elected by recommendation of the IL-2 Luc assay VMT and check and analyze the data obtained in this validation trial from a third-party standpoint. The members also take charge of statistical processing in this validation trial.

3) Quality assurance group

The members of the record management group are elected by recommendation of the IL-2 Luc assay VMT. The members prepare the protocol, the test chemical preparation record forms, blank data sheets, etc., and distribute them to the research laboratories participating in this validation trial. The members also collect completed forms and data sheets after completion of the experiments, and point out omissions or flaws in recording, if any, and request corrections of such errors.

4) Lead laboratory

The lead laboratory representing the test method is responsible for providing the test method protocol and the necessary data recording or calculation templates. The Trial Coordinator must ensure that such data recording or calculation templates have been validated before distribution to the test facilities involved in the validation trial. The lead laboratory is also responsible for providing, if necessary, new versions of the protocols during the entire validation trial. The lead laboratory and the other participating test facilities might be contacted by the VMT regarding technical issues.

Sponsor

The validation trial for assessing the validity of IL-2 Luc assay will be financed by the Ministry of Health, Labour and Welfare (MHLW), Japan.

The lead laboratory will support the IL-2 Luc assay validation trial by assuring that reliability is assessed. At the same time, preliminary results of the test method can be evaluated. For this purpose, Lead laboratory will support:

- financial aspects related to the coordination of a validation trial (e.g. organization of VMT meetings where also the involved test facilities can be invited for technical clarifications to the VMT, the publication of the validation trial results)
- test, reference and control item purchase, coding and distribution to the test facility
- availability of the test systems to the participating laboratories by supporting the lead laboratory with the logistics for delivering the test system to the facility
- independent analysis of data and statistical support (biostatistician) based on the study reports generated
- other costs incurred by the participating laboratories

Trial coordination

Dr. Hajime Kojima was appointed as the Trial Coordinator with well-defined roles and responsibilities to coordinate the trial and to establishment of a VMT by supporting of JaCVAM. The name and location of the Trial Coordinator should be identified in each individual study plan. For the IL-2 Luc assay validation trial, the Trial Coordinator has direct access to the test item coding.

The Trial Coordinator's responsibilities include:
a) Establishment of/support to lead laboratory, including meeting organization
b) Trial communication and coordination with test facilities
c) Recording of document and data flow between test facilities
d) Assessing and documenting the impact of any amendments and/or deviations from the trial plan and study plans on the quality and integrity of the validation trial
e) Ensuring that the individual study reports are forwarded, in a timely manner, for data and statistical analysis
f) Preparing the trial plan and report, which can be based on the study reports from the lead laboratories and other test facilities involved in the validation trial, and should reflect the overall trial
g) Approval with date and signature of all protocols, Study Plans and Study Reports
h) The communication of the results of the trial into the public domain
The Trial Coordinator's responsibilities include:
a) Establishment/support of the lead laboratory, including meeting organization
b) Trial communication and coordination with the test facilities
c) Recording of documents and data flow between the test facilities
d) Assessing and documenting the impact of any amendments and/or deviations from the trial plan and study plans on the quality and integrity of the validation trial
e) Ensuring that the individual study reports are forwarded, in a timely manner, for data and statistical analysis
f) Preparing the trial plan and report, which can be based on the study reports from the lead laboratory and other test facilities involved in the validation trial, and should reflect the overall trial
g) Approval with date and signature of all protocols, study plans and study reports
h) Communication of the results of the trial to the public domain
The role of the Trial Coordinator (as the formal representative of the VMT and the single contact point with the SDs) is of fundamental importance. The Trial Coordinator is the single critical point of trial control and must ensure clear lines of communication between the involved test facilities in the trial. The communication line of the Trial Coordinator is with the SDs of the different test facilities. The SDs are the single point of contact with the Trial Coordinator (unless otherwise communicated by the participating test facilities) to assure a transparent and recorded documentation flow during the trial. The Trial Coordinator should also ensure that appropriate arrangements have been made for the supply of the test systems, and test, control and reference items, which meet the requirements of the trial, and that there are appropriate test method protocols (dated signature by the Trial Coordinator and the lead laboratory) and, if appropriate, validated data recording, data analysis, and data reporting sheets for the test method.
It is the responsibility of the Trial Coordinator to approve the study plans sent for approval by the test facilities, and any amendments to the study plan, by dated signature.

Training

The lead laboratory will be responsible for issuing a training agenda to the Trial Coordinator for further distribution to all test facilities, giving details of what training aspects will be covered during the training of the other SDs and study personnel at the lead laboratory. Furthermore, after the training during the Phase 0 study, the lead laboratory will issue to the Trial Coordinator a training report and indicate if critical observations are made by the other test facilities regarding the IL-2 Luc assay protocols. In case any critical observations are made, a new version of the IL-2 Luc assay protocols might need be issued to the other test facilities before initiating the between-laboratory transferability test.

[Module 2] Within-laboratory reproducibility

The within-laboratory reproducibility of all test facilities has been done by an independent biostatistical analysis using 5 coded chemicals under the VMT. The concordance should be equal to or greater than 80% as a tentative acceptance criterion for the Phase I study.

3.7 [Module 3] Between-laboratory transferability

This between-laboratory transferability (Module 3) study is performed in order to assess the successful transfer of the assay to a test facility unexperienced with that particular test method but having knowledge of similar test systems and endpoint detection methods.

Transfer of the IL-2 Luc assay to all test facilities in the Phase 0 study using 5 coded five chemicals was achieved. A few concentrations of each test item were tested in triplicate in 3 independent runs according to the IL-2 Luc assay protocol describing the details of the experimental design.

The 5 test items selected for the Phase I study are coded as A, B, C, D, and E. The facilities will prepare a study according to internal GLP principles. This plan will be submitted to the Trial Coordinator and lead laboratory for approval.

The results of the between-laboratory transferability study will be reviewed before progressing with module 4 in the between-laboratory reproducibility study. If the transferability data do not meet the test acceptance criteria, the Trial Coordinator representing the VMT will try to identify the problems and make corrections where needed. At the end of testing, the test facilities will submit a QC certified copy of the entire study dossier to the Trial Coordinator (study plan adhering to GLP principles, raw data, records and data analysis, study report adhering to GLP principles).

3.8 [Module 4] Between-laboratory reproducibility

Twenty-five coded test items have been selected to confirm the between-laboratory reproducibilities in the Phase I and II studies. Several concentrations of each test item will be tested in triplicate according to the IL-2 Luc assay method protocol describing the details of the experimental design.

At the end of testing, the test facilities will submit a QC certified copy of the entire study dossier to the Trial Coordinator (study plan adhering to GLP principles, raw data, records and data analysis, study report adhering to GLP principles). The concordance for between-laboratory reproducibility should be equal or greater than 80% to meet the acceptance criteria.

[Module 5] Predictive capacity

The necessity for further chemical analysis will be subject to a VMT decision once the data for between-laboratory reproducibility has been assessed. Depending on the statistical analysis, lean design for validation as well as automatisation of the test leading to an increased dataset will be considered.

Protocol
In this validation trial, the protocols ver. 0.08E, Phase I and 0.1E, Phase II will be used. These protocols will be drafted by the lead laboratory and will be finalized by the VMT. The criteria to identify immunotoxicants by the MITA are provisionally fixed in protocol ver. 0.08E prior to the Phase I study. There are 2 temporary criteria to identify imunotoxicants. The VMT adopted these criteria after the Phase I validation study.
A measurement of bioluminescence intensity induced by chemical treatment will be measured by a luminometer (Phelios: ATTO, Cat #:AB-2350) calibrated using stabilized SLG, SLO and SLR enzymes in this validation trial.

Chemicals
5.1 Chemical Selection
Test chemicals have been selected from a chemical repository based on published papers on *in vivo* immunotoxicity.
The applied selection criteria were:
information on mode/site of action
coverage of range of relevant chemical classes and product classes
quality and quantity of reference data (*in vivo* and *in vitro*)
high quality data derived from animals and (if available) also humans
knowledge of interspecies variations (for example: variability with regard to the uptake of chemicals, metabolism, etc.)
coverage of range of toxic effects/potencies
chemicals that do not need metabolic activation
appropriate negative and positive controls
physical and chemical properties (feasibility of use in the experimental set-up as defined by the CAS No.)
single chemical entities or formulations of known high purity
availability
cost

In the first phase of the selection procedure, the chemical management group identified and collected several existing lists of potential chemical sensitizers in order to establish a primary database. These chemicals had originally been compiled by international experts for various purposes, such as reference compounds for validation studies. An extensive literature research was performed by the chemical management group, insuring that the preselected chemicals fulfilled the selection criteria described above.

Emphasis was laid on the fact that different potencies (strong, weak and no activity) have been chosen. In addition, it was decided that at least 20% of the total substances to be tested should be negative in order to increase the statistical power of the data analysis.

In the first phase of the IL-2 Luc assay validation trial using data generated at the test facilities, 5 chemicals will be tested 3 times for each test chemical for between-laboratory reproducibility and to confirm transferability. After discussion of the Phase I results, detailed test planning for Phase II will be established. Currently, it is planned that 20 chemicals will be tested in the Phase II trial to establish predictive capacity (Table 2).

Table 2. Outline of test planning at each study in the validation trial.

Study	Chemicals	Test Number	Information obtained
Phase I	5 non- coded	1	Between-lab transferability
Phase I	5 coded	3	Within & between-lab reproducibility
Phase II	20 coded	1	Between-lab reproducibility & predictability

5.2 Chemical Acquisition, Coding and Distribution

The within-laboratory reproducibility (Module 2) and between-laboratory transferability (Module 3) in all test facilities have been assessed with coded chemicals. This IL-2 Luc validation trial plan describes generation of the missing data sets under coded test item. If the results obtained are not highly similar to the previously obtained sets, the VMT must assess if coded chemicals need to be tested in all the test facilities.

Coding will be supervised by the Trial Coordinator, in collaboration with the chemical repository responsible for coding and distribution of the test, reference and control items for the validation trial.

5.3 Handling

Each test facility shall receive through the Trial Coordinator essential information about the test chemicals (physical state, weight or volume of sample, specific density for liquid test chemicals, and storage instructions). Moreover, the SD should receive safety information concerning hazards identification and exposure controls/personal protection.

Records and archiving

At the end of the trial, the IL-2 Luc assay validation trial report is prepared by the Trial Coordinator or the VMT personnel who appointed by the Trial Coordinator. The trial report summarizes the trial goals, procedures, results and conclusions of the validation trial. This represents the whole validation trial, including archiving and, as such, will cover several study reports, as well as reports for test item supply, data management and statistics. The Trial Coordinator oversees the preparation of the trial report. The Trial Coordinator will be representing the VMT discussions responsible for preparation of the scientific conclusions. Signatories to the trial report include the Trial Coordinator, the statistician, and the SDs of the involved test facilities. Although the SDs may not be involved with the preparation of the trial report, their signatures confirm that the trial report is an accurate reflection of the management and study events. The trial report should contain a statement, signed by the Trial Coordinator,

commenting on the accuracy and completeness of the trial report and identifying any significant issues which could have affected the integrity of the trial, including matters of GLP compliance. A QC statement will be included in the trial report, in order to identify what QC monitoring was done and to confirm whether or not the trial report is an accurate reflection of the validation trial data.

Study timeline

An approximate schedule for IL-2 Luc assay validation trial is shown in Table 3. The duration of this validation trial is around 20 months, from May 2016 to December 2017.

Table 3. Schedule of IL-2 Luc assay validation trial

Month	Activity
January 2016	Establish the VMT
	Selection of participating research laboratories
	Deliberation, decision and read-through of draft study plan
	Deliberation and decision of protocol
	Preparation of a tentative list of test chemicals
	Distribution of test chemicals, standard chemicals and positive control chemicals
February,2016	Technical transfer using five known chemicals (non-coded) Start of technical transfer **to know between laboratory transferability**
	Data collection of technical transfer (**Phase 0 study**)
Phase I study	
September 2016	Coding and distribution of five coded test chemicals
September, 2016	Start of Phase I study
December, 2016	End of Phase I study
February, 2017	**2nd VMT Meeting** / Phase I results and planning of Phase II study
Phase II study to know between- and within-laboratory reproducibility	
April, 2017	Coding and distribution of coded test chemicals and positive chemicals
May, 2017	Start of Phase II study using 20 coded test chemicals
August,2017	End of Phase II study
November-December, 2017	**3rd VMT Meeting** /reviewing of Phase II study results
2018	Completed validation report

Abbreviations

CAS: Chemical Abstracts Service
GLP: Good Laboratory Practice
HRI: Hatano Research Institute
FDSC: Food and Drug Safety Center
JaCVAM: Japanese Centre for the Validation of Alternative Methods
NIHS: National Institute of Health Sciences
OECD: Organization for Economic Co-operation and Development
QC: Quality Control
TG: Test Guideline
VMT: Validation Management Team

Appendix 9. The list of proficiency chemicals

Undetermined yet

Appendix 10. MITA QC confirmation table

MITA(P1) confirmation tabale

	LabB (AIST, Tsukuba)			LabC (FDSC)			LabD (AIST, Takamatsu)		
setA-1 (run 1)	date	2016.9.12	(document 4 - 5)						
	Cell culture records	○							
	Weighting records	○							
	Test records	○							
	Datasheet	×							
	Graph	○							
setA-1 (run 2)	date	2016.10.4	(document 4 - 6)						
	Cell culture records	○							
	Weighting records	○							
	Test records	○							
	Datasheet	×							
	Graph	○							
setA	Weighting records	○		Weighting records	○		Weighting records	○	
	Cell culture records	○		Cell culture records	×		Cell culture records	○	wake up 2016.8.26~ Last culture 2016.9.29
setA-1	date	2016.10.26		date	2016.10.4.		date	2016.9.9	
	Cell culture records	○		Cell culture records	○		Cell culture records	○	
	Weighting records	○		Weighting records	○		Weighting records	○	
	Test records	○		Test records	○		Test records	○	
	Datasheet	○		Datasheet	○		Datasheet	○	
	Graph	○		Graph			Graph	○	
setA-2	date	2016.11.1		date	2016.10.17		date	2016.9.12	
	Cell culture records	○		Cell culture records	○		Cell culture records	○	
	Weighting records	○		Weighting records	○		Weighting records	×	
	Test records	○		Test records	○		Test records	○	
	Datasheet	○		Datasheet	○		Datasheet	○	
	Graph	○		Graph			Graph	○	
setA-3	date	2016.11.4		date	2016.10.21		date	2016.9.15	(document 7 - 8)
	Cell culture records	○		Cell culture records	○		Cell culture records	○	
	Weighting records	○		Weighting records	○		Weighting records	×	
	Test records	○		Test records	○		Test records	○	
	Datasheet	○		Datasheet	○		Datasheet	○	
	Graph	○		Graph			Graph	○	
setA-4	date			date			date	2016.9.20	(3rd re trial)
	Cell culture records			Cell culture records			Cell culture records	○	
	Weighting records			Weighting records			Weighting records	×	
	Test records			Test records			Test records	○	
	Datasheet			Datasheet			Datasheet	○	
	Graph			Graph			Graph	○	
setB	Weighting records	○		Weighting records	○		Weighting records	○	
	Cell culture records	○		Cell culture records	×		Cell culture records	○ Newly starting cell culture on the way	continue from SetA + (wake up from 20160923 cell culture till 20161014)
setB-1	date	2016.11.8		date	2016.10.27		date	2016.9.23	(document 7 - 8)
	Cell culture records	○		Cell culture records	○		Cell culture records	○	
	Weighting records	○		Weighting records	○		Weighting records	○	
	Test records	○		Test records	○		Test records	○	
	Datasheet	○		Datasheet	○		Datasheet	○	
	Graph	○		Graph	○		Graph	○	
setB-2	date	2016.11.12		date	2016.10.28		date	2016.9.26	(1st re-trial)
	Cell culture records	○		Cell culture records	○		Cell culture records	○	
	Weighting records	○		Weighting records	○		Weighting records	×	
	Test records	○		Test records	○		Test records	○	
	Datasheet	○		Datasheet	○		Datasheet	○	
	Graph	○		Graph	○		Graph	○	
setB-3	date	2016.11.16		date	2016.10.31		date	2016.9.29	(2nd trial)
	Cell culture records	○		Cell culture records	○		Cell culture records	○	
	Weighting records	○		Weighting records	○		Weighting records	×	
	Test records	○		Test records	○		Test records	○	
	Datasheet	○		Datasheet	○		Datasheet	○	
	Graph	○		Graph	○		Graph	○	
setB-4	date			date			date	2016.10.3	(3rd trial)
	Cell culture records			Cell culture records			Cell culture records	○	
	Weighting records			Weighting records			Weighting records	×	
	Test records			Test records			Test records	○	
	Datasheet			Datasheet			Datasheet	○	
	Graph			Graph			Graph	○	

1

MITA(P1) confirmation tabale

	LabB (AIST, Tsukuba)			LabC (FDSC)			LabD (AIST, Takamatsu)		
setC	Weighting records	○		Weighting records	○		Weighting records	○	
	Cell culture records	○		Cell culture records	×		Cell culture records	○	
setC-1	date	2016.11.10		date	2016.11.14		date	2016.10.6	(document 7 - 8)
	Cell culture records	○		Cell culture records	○		Cell culture records	○	
	Weighting records	○		Weighting records	○		Weighting records	○	
	Test records	○		Test records	○		Test records	○	
	Datasheet			Datasheet	○		Datasheet	○	
	Graph	○		Graph	○		Graph	○	
setC-2	date	2016.11.14		date	2016.11.25		date	2016.10.14	(document 7 - 8)
	Cell culture records	○		Cell culture records	○		Cell culture records	○	
	Weighting records	○		Weighting records	○		Weighting records	×	
	Test records	○		Test records	○		Test records	○	
	Datasheet			Datasheet	○		Datasheet	○	
	Graph	○		Graph	○		Graph	○	
setC-3	date	2016.11.18		date	2016.12.09		date	2016.10.17	(document 7 - 8)
	Cell culture records	○		Cell culture records	○		Cell culture records	○	
	Weighting records	○		Weighting records	○		Weighting records	×	
	Test records	○		Test records	○		Test records	○	
	Datasheet			Datasheet	○		Datasheet	○	
	Graph	○		Graph	○		Graph	○	
setC-4	date			date			date	2016.10.20	(document 7 - 8)
	Cell culture records			Cell culture records			Cell culture records	○	
	Weighting records			Weighting records			Weighting records	×	
	Test records			Test records			Test records	○	
	Datasheet			Datasheet			Datasheet	○	
	Graph			Graph			Graph	○	
	SDS back	○	20170322受領	SDS back	○		SDS back	○	
	calibration records			calibration records			calibration records		

MITA(P2) Confirmation table

	項目	LabB (AIST, Tsukuba)	LabC (FDSC)	LabD (AIST, Shikoku)
	Weighing records		○	○
	Cell culture records	○ 2017.05.02 3sets 2017.05.19 2017.06.12	○ 2017.05.29 3sets 2017.07.03 2017.07.31	○ 2017.05.08 3sets 2017.06.06 2017.07.03
	Solubility check records	○ per each samples	○ per each tests	○ per each samples
1	Test date	2017.5.19.	2017.06.30	2017.05.22
	Test samples No.（repeat No.）	1-5(1)	6,4,6,7(1)	2,7,8,12(1)
	Others records	○	○	○
	Datasheets	○	○	○
2	Test date	2017.5.31	2017.07.06	2017.05.23
	Test samples No.	1,3-5(2),2(re1)	4,6,7(2)	14,16,17,19,20,01(1)
	Others records	○	○	○
	Datasheets	○	○	○
3	Test date	2017.6.8	2017.07.07	2017.05.29
	Test samples No.	1,3-5(3),2(2)	4,6,7(3)	3,4,10,11(1)
	Others records	○	○	○
	Datasheets	○	○	○
4	Test date	2017.6.12	2017.07.13	2017.05.30
	Test samples No.	2(3).5(re3)	1,3,5,8(1)	5,6,9,13,15,18(1)
	Others records	○	○	○
	Datasheets	○	○	○
5	Test date	2017.6.5	2017.07.14	2017.06.12
	Test samples No.	6-10(1)	1,3,5,8(2),9,10(1)	5,6,9,13,15,18(re1)
	Others records	○	○	○
	Datasheets	○	○	○
6	Test date	2017.6.6.	2017.07.18	2017.06.19
	Test samples No.	6(re1),7-10(2)	1,3,5,8(3),9,10(2)	3,4,10,11(re1)
	Others records	○	○	○
	Datasheets	○	○	○
7	Test date	2017.6.9.	2017.07.21	2017.06.20
	Test samples No.	6(2),7-10(3)	9,10(3),11-14(1)	2,7,8,12(2)
	Others records	○	○	○
	Datasheets	○	○	○
8	Test date	2017.6.14	2017.07.24	2017.06.26
	Test samples No.	11-15(1)	11.12.14(2),13(re1).15,16(1)	14,16,17,19,20,01(re1)
	Others records	○	○	○
	Datasheets	○	○	○
9	Test date	2017.6.21	2017.07.27	2017.06.27
	Test samples No.	11-15(2)	11,12,14(3),13(2)	5,6,10,11,3,4（2）
	Others records	○	○	○
	Datasheets	○	○	○
10	Test date	2017.6.22	2017.07.28	2017.07.03
	Test samples No.	11-13.15(3)14(re2),	2(re1),13(3),15,16(2),18,20(1)	16,17,19,20,15,18（2）
	Others records	○	○	○
	Datasheets	○	○	○
11	Test date	2017.6.29	2017.08.03	2017.07.04
	Test samples No.	6,14(3)	15,16(3),17,19(1),18,20(2)	1,9,13,14（2）
	Others records	○	○	○
	Datasheets	○	○	○
12	Test date	2017.6.28	2017.08.04	2017.07.10
	Test samples No.	16-20(1)	1,3(4),2(3).19(2),18,20(3)	17,19,7,3（3）
	Others records	○	○	○
	Datasheets	○	○	○
13	Test date	2017.7.7	2017.08.07	2017.07.11
	Test samples No.	16-20(2)	2(4),17(2),19(3)	2,8,12,16,14,20（3）
	Others records	○	○	○
	Datasheets	○	○	○
14	Test date	2017.7.11	2017.08.08	2017.07.18
	Test samples No.	16-20(3)	5,8,19(4),17(3)	1,4,5,6,10,11（3）
	Others records	○	○	○
	Datasheets	○	○	○
15	Test date		2017.08.14	2017.07.24
	Test samples No.		13(4)	9,13,15,18(3),3,19(4)
	Others records		○	○
	Datasheets		○	○
16	Test date			2017.07.25
	Test samples No.			10,13,14,9,4(4)
	Others records			○
	Datasheets			○

【Phase I coded list for the MITA validation study in Sep 2016】

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

MITA(Phase2) coded chemicals

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

positive
negative

Appendix 2.

Multi-Immuno Tox Assay protocol for THP-G1b (TGCHAC-A4) ver. 008.1E Februaly 7th, 2019

Department of Dermatology, Tohoku University Graduate School of Medicine
Yutaka Kimura, M.D., Ph.D.
Setsuya Aiba, M.D., Ph.D.

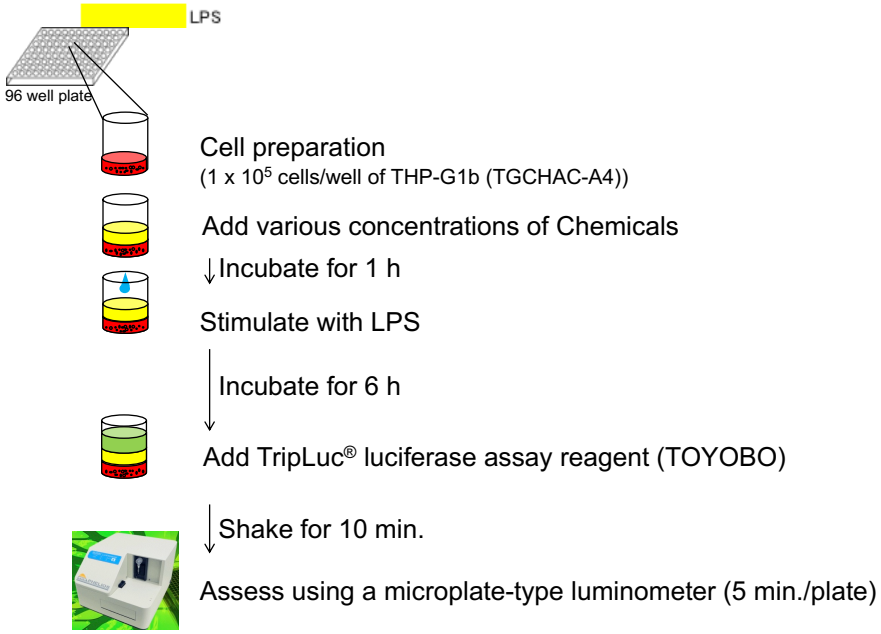
1. Introduction

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

2-3-1 A medium: for maintenance of THP-G1b (TGCHAC-A4) cells (500 mL, stored at 2-8°C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO Cat #11875-093	-	-	445 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	50 mL
Antibiotic-Antimycotic	e.g., GIBCO Cat #15240-062	100×	1×	5 mL

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

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

2-4 Preparation of the stimulant of THP-G1b (TGCHAC-A4) cells

2-4-1 Lipopolysaccharide (LPS) from Escherichia coli K12

Reagent	Company	Concentration of the stock solution	Final concentration
Lipopolysaccharide (LPS) from Escherichia coli K12	Invivogen Cat#tlrl-eklps	1 mg/mL	100 ng/mL
Distilled water	GIBCO Cat#10977-015		

Dissolve 5 mg LPS using distilled water 5 mL, dispend at 5 μL/tube and store at freezer at -30°C. Use these stocks within 6 months after dissolution.

3. Cell culture

3-1 Thawing of THP-G1b (TGCHAC-A4) cells

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

3-2 Maintenance of THP-G1b (TGCHAC-A4) cells

3 or 4 days after thawing, pre-warm the A medium in a T-75 Flask at 37°C in a 5% CO_2 incubator. Count the number of cells, centrifuge the tube at 120-350 x *g* at room temperature for 5 min, discard the supernatant, and resuspend in the pre-warmed A medium in a T-75 Flask. Cells are passaged at $2\text{-}5x10^5$/mL, depending on the condition of the cells and incubated at 37°C, 5% CO_2.

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

4. Preparation of cells for assay

A cell passage should be done 2-4 days before the assay.
Use cells between 1 and 6 weeks after thawing.
Pre-warm the B medium in a 37°C water bath. Count the number of cells and collect the number of cells needed (5.0 x 10^6 cells are required, but to have some leeway, 7.5 x 10^6 cells should be prepared), centrifuge the tube at 120-350 x *g*, 5 min. Resuspend in pre-warmed the B medium at a cell density of 2×10^6/mL. Transfer the cell suspension to a reservoir, and add 50 µL of cell suspension to each well of a 96 well black-flame and white-well plate (flat bottom) using an 8 channel or 12 channel pipetman. (cf. Figure 2)

Figure 2

flat-bottom B&W	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL
D	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL
E	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL
F	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL
G												
H												

5. Preparation of chemicals and cell treatment with chemicals

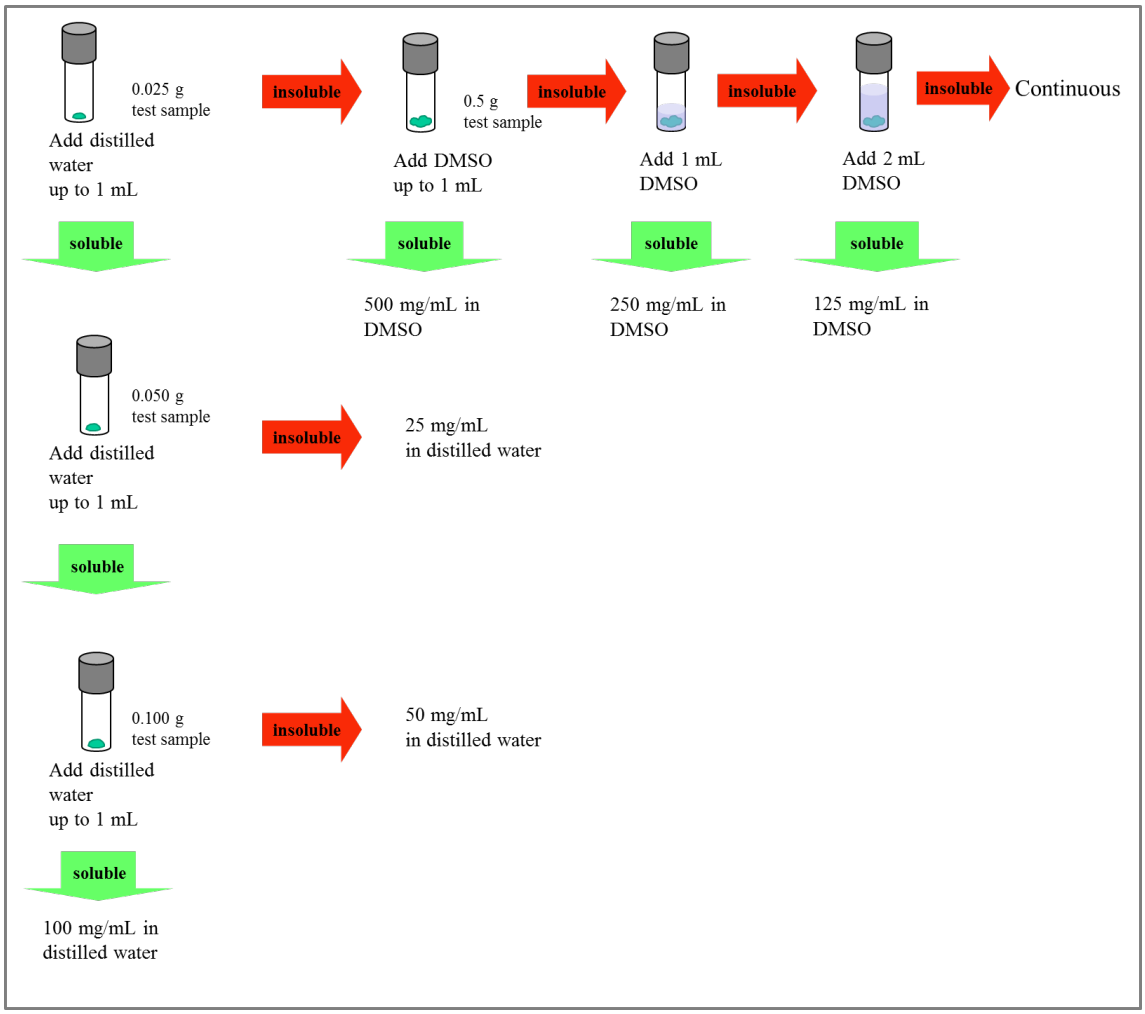
5-1 Dissolution by vehicle (cf. Figure 3)

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

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

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

Figure 3



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

In the second to fifth experiment (2nd to 5th experiment), determine the minimum concentration at which I.I.-SLR-LA (mentioned later in **10**) became lower than 0.05 in the 1st experiment, use the concentration one step (2-times) higher than this determined concentration as the highest concentration of the chemical to examine, and conduct 10 serial dilutions at a common ratio of 2 from the highest concentration. If I.I.-SLR-LA did not become lower than 0.05 or became lower than 0.05 at the highest concentration in the 1st experiment, conduct 10 serial dilutions at a common ratio of 2 from the highest concentration in the 1st experiment.

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

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

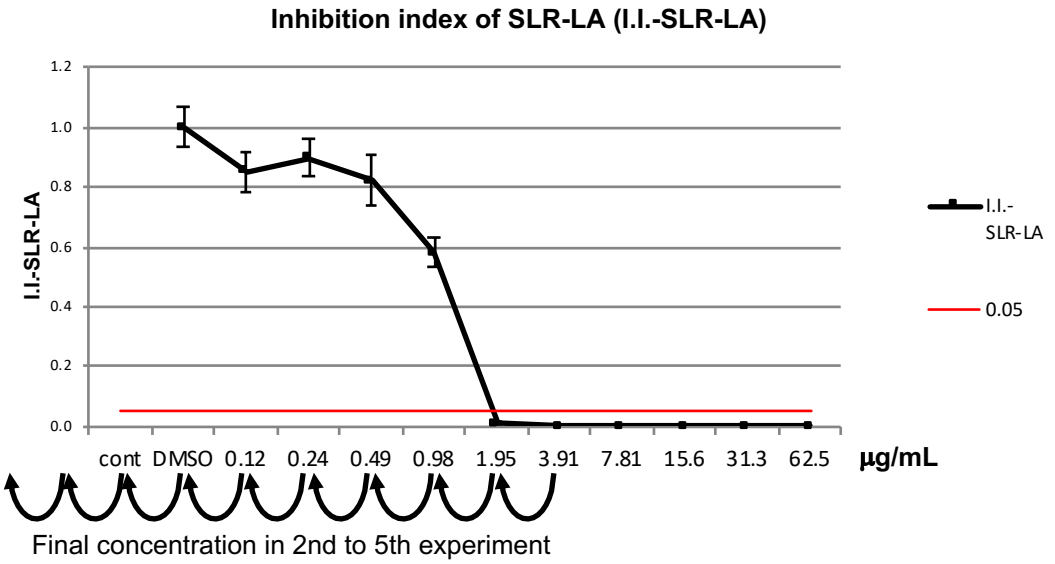


Figure 3.

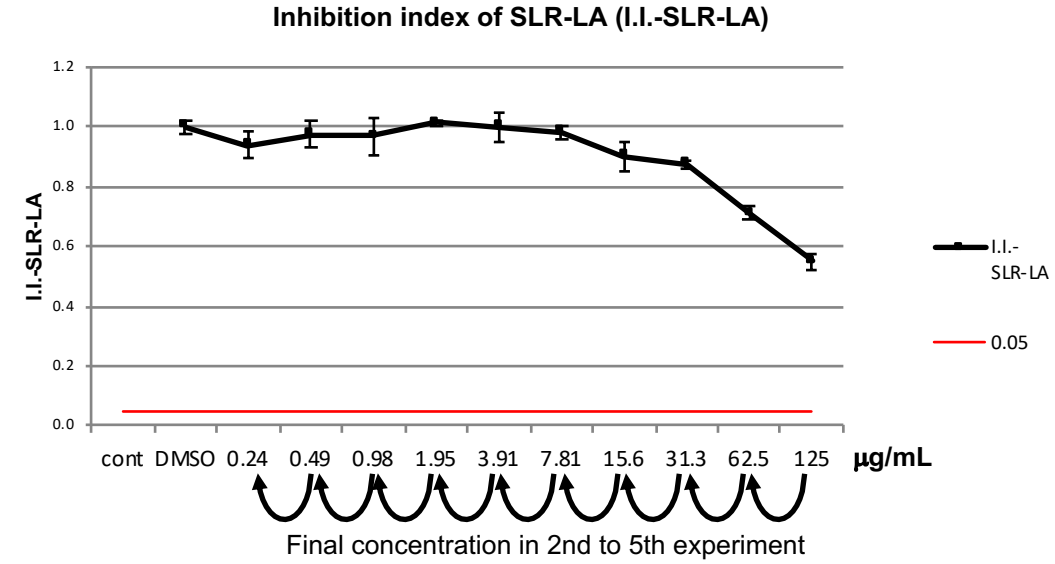


Figure 4

5-2 When the chemical is prepared in distilled water

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

5-2-1 Arrangement of chemicals and vehicle

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

5-2-2 Serial dilution

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

Figure 4

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Chemical 100 mg/mL in distiled water 100 uL
B												
C												
D												
E			2-fold dilution : transfer 50 uL（pipetman、yellow tip）									
F												
G												
H												

↓

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

5-2-3 2 step dilution

Add 20 µL of the diluted chemical to 480 µL of the B medium prepared in the assay block. And add 50 µL to THP-G8 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 1 hour (37°C, CO_2, 5%) (cf. Figure 5-7).

Figure 5

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

20uL

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

Figure 6

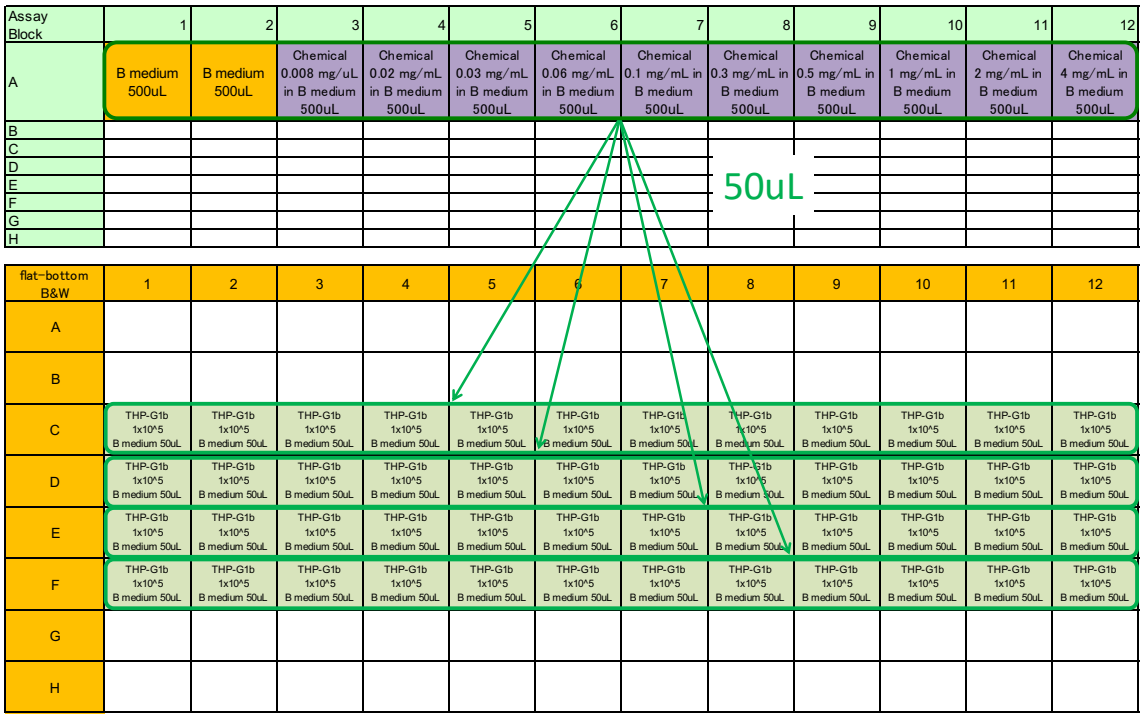


Figure 7 Final constituents of each well of the plate

flat-bottom B&W	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	Chemical 0 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.004 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.008 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.02 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.03 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.06 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.1 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.3 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.5 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 1 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 2 mg/mL THP-G1b 1x10^5 B medium 100uL
D	Chemical 0 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.004 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.008 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.02 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.03 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.06 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.1 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.3 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.5 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 1 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 2 mg/mL THP-G1b 1x10^5 B medium 100uL
E	Chemical 0 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.004 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.008 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.02 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.03 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.06 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.1 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.3 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.5 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 1 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 2 mg/mL THP-G1b 1x10^5 B medium 100uL
F	Chemical 0 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.004 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.008 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.02 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.03 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.06 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.1 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.3 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.5 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 1 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 2 mg/mL THP-G1b 1x10^5 B medium 100uL
G												
H												

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

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

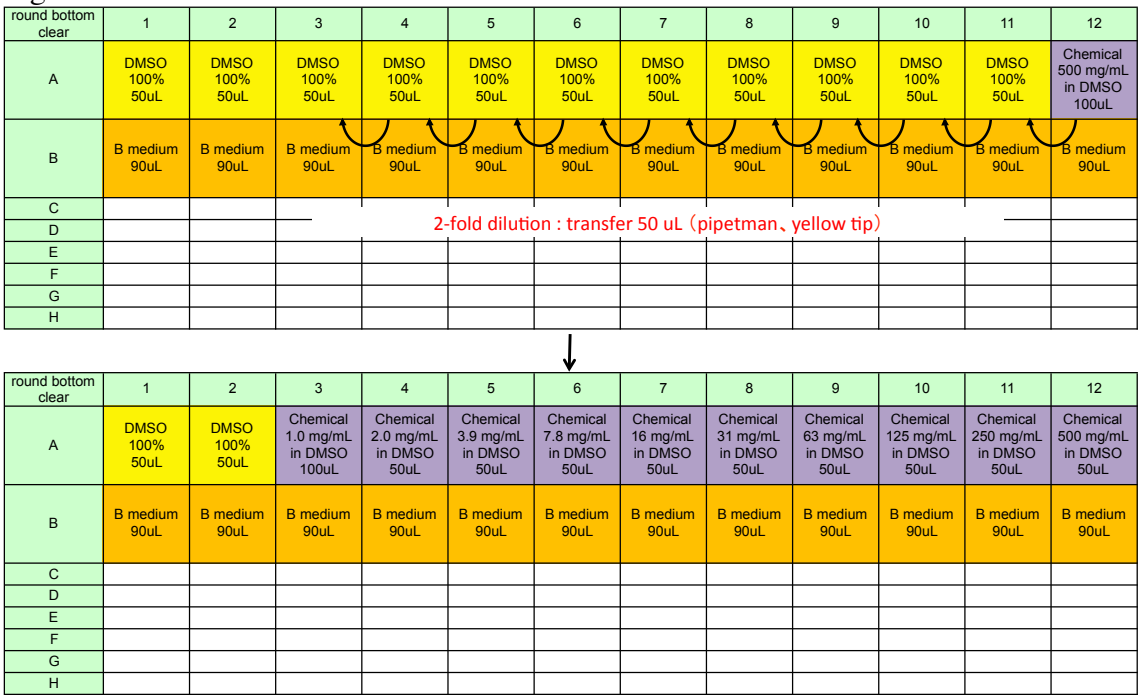
5-3-1 Arrangement of chemicals and vehicle

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

5-3-2 Serial dilution

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

Figure 8



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

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

Figure 9

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 10-12).

Figure 10

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

10uL

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

Figure 11

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

50uL

flat-bottom 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 12 Final constituents of each well of the plate

flat-bottom B&W	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	Chemical 0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 1.0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 2.0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 3.9ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 7.8ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 16ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 31ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 63ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 125ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 250ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 500ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL
D	Chemical 0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 1.0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 2.0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 3.9ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 7.8ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 16ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 31ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 63ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 125ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 250ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 500ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL
E	Chemical 0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 1.0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 2.0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 3.9ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 7.8ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 16ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 31ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 63ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 125ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 250ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 500ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL
F	Chemical 0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 1.0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 2.0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 3.9ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 7.8ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 16ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 31ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 63ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 125ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 250ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 500ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL
G												
H												

6. Preparation of the stimulant (Lipopolysaccharide (LPS)) and addition to THP-G1b (TGCHAC-A4)

6-1 Material

・ 1 mg/mL LPS stock

6-2 Preparation of 1000 ng/mL LPS solution

Dilute 1 mg/mL LPS stock with distilled water as follows (1000 times, final concentration is 1000 ng/mL). Add distilled water as control to well #A1-#D1 of the 96 well clear plate (round bottom), and add 1000 ng/mL LPS solution to wells #A2-#D2 of the 96 well clear plate (round bottom).

1st step

1 mg/mL LPS	distilled water	Total	final concentration
5 μL	995 μL	1000 μL	5 μg/mL

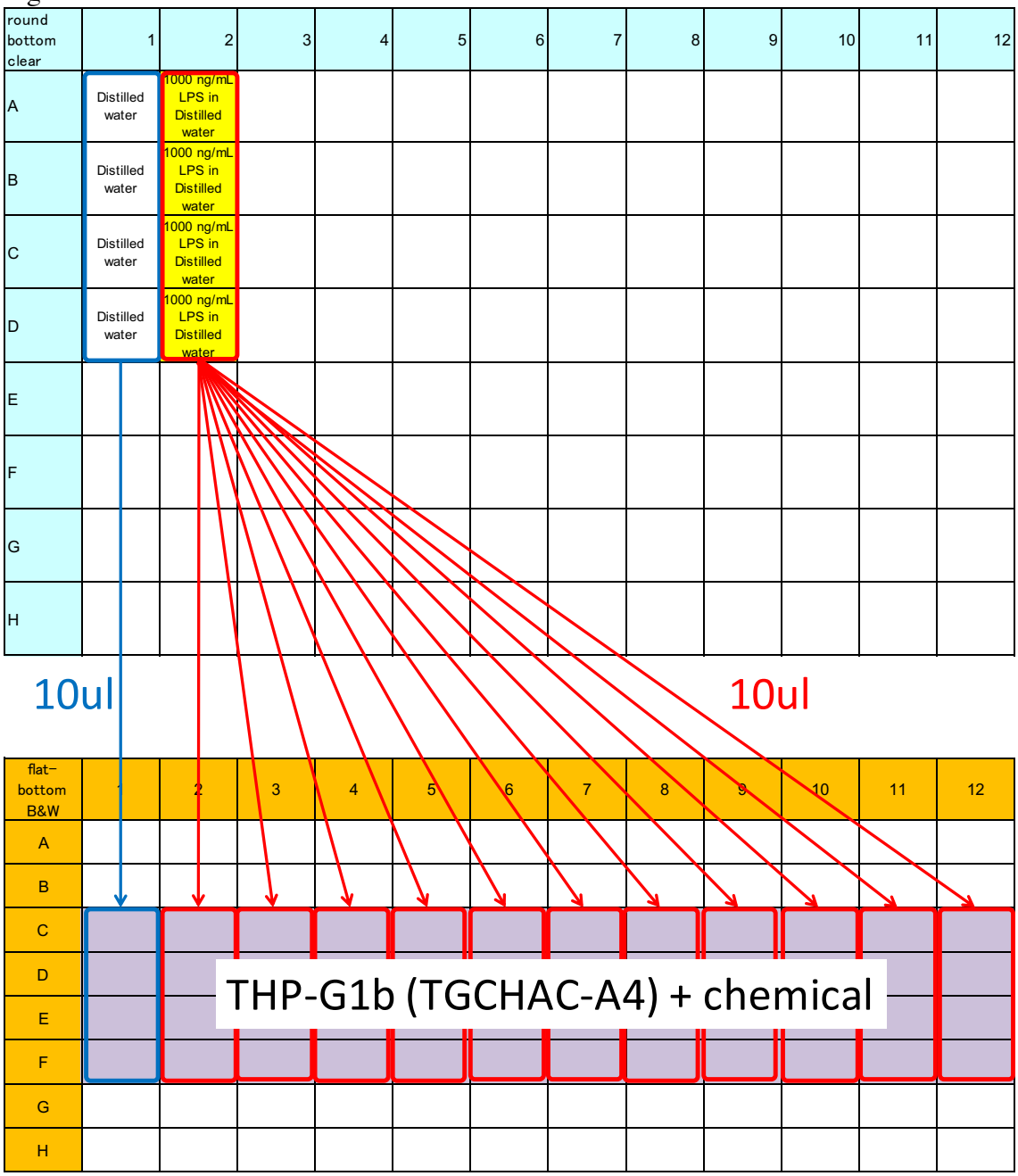
2nd step

5 μg/mL LPS	distilled water	Total	final concentration
250 μL	1000 μL	1250 μL	1000 ng/mL

6-3 Addition of LPS to THP-G1b (TGCHAC-A4)

One hour after the addition of chemicals, add 10 µL of control or 1000 ng/mL LPS solution to the cells (#C1-#F1 or #C2-#F12, respectively) using an 8 channel or 12 channel pipetman after pipetting 20 times. Make sure that the apex of the tip is dipped into the medium. Change tips every line you add. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 6 hours (37°C, CO_2, 5%). (cf. Figure 13)

Figure 13



7. Positive control

7-1 Preparing control chemical (dexamethasone)

7-1-1 Preparing dexamethasone stock

Reagent	Company	Concentration of the stock solution	Preparing concentration	Final concentration
Dexamethasone	Fujifilm Wako Pure Chemical Cat#041-18861	100 mg/mL	10, 50, 100 mg/mL	10, 50, 100 μg/mL
Dimethyl sulfoxide (DMSO)	Sigma Cat#D5879			

Dissolve 1 g of Dexamethasone with DMSO 10 mL, dispend at 100 μL/tube and store at freezer at -30°C.

7-2 Preparation of cells for assay

A cell passage should be done 2-4 days before the assay.
Use cells between 1 and 6 weeks after thawing.
Pre-warm the B medium in a 37°C water bath. Count the number of cells and collect the number of cells needed (2.0 x 10^6 cells are required, but to have some leeway, 3 x 10^6 cells should be prepared), centrifuge the tube at 120-350 x *g*, 5 min. Resuspend in pre-warmed the B medium at a cell density of 2×10^6/mL. Transfer the cell suspension to a reservoir, and add 50 µL of cell suspension to each well of a 96 well black-flame and white-well plate (flat bottom) using an 8 channel or 12 channel pipetman. (cf. Figure 14)

Figure 14

flat-bottom B&W	1	2	3	4	5	6	7	8	9	10	11	12
A	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL							
B	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL							
C	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL							
D	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL							
E												
F												
G												
H												

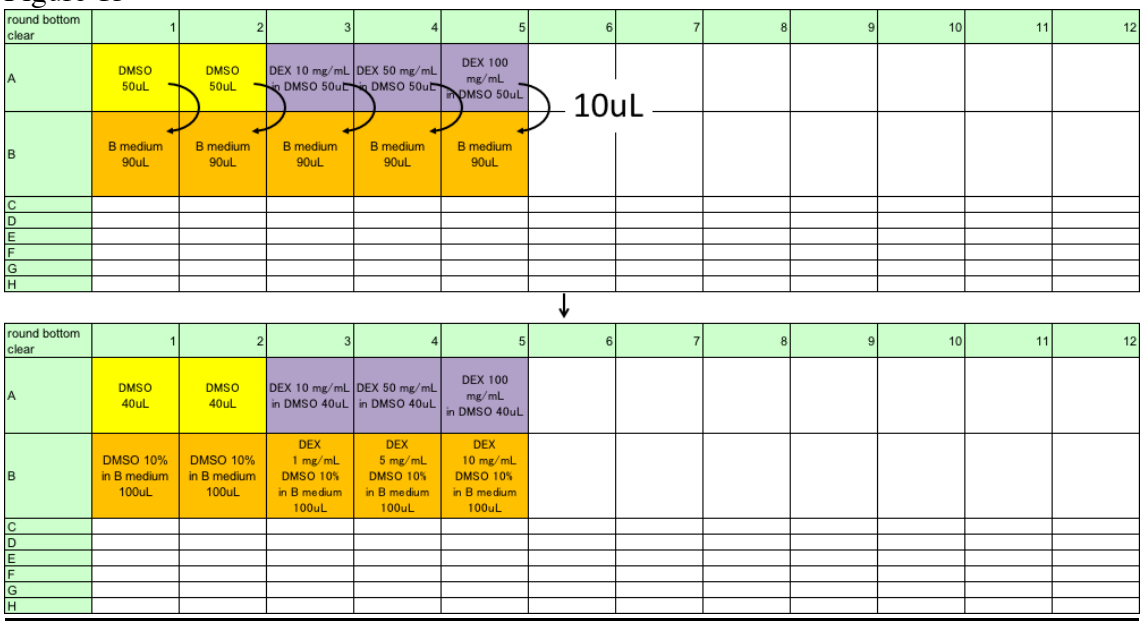
7-3 Arrangement of chemicals and vehicle

Add DMSO 50 µL to #A1-2, 10 mg/mL dexamethasone 50 µl to #A3, 50 mg/mL dexamethasone 50 µl to #A4, 100 mg/mL dexamethasone 50 µl to #A5 and B medium 90 µl to #B1-5 of the 96 well clear plate (round bottom). (cf. Figure 15)

7-4 Dilution with the B medium

Dilute DMSO in #A1-2 and dexamethasone DMSO solution in #A3-5 by adding 10 µL to the B medium in #B1-5. (cf. Figure 15)

Figure 15



7-5 2 step dilution

Add 10 µL of the diluted DMSO or dexamethasone to 490 µL of the B medium prepared in the assay block. And add 50 µL to THP-G1b (TGCHAC-A4) in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Manipulate the procedures from 7-4 to 7-5 as quickly as you can, and do not leave a long time at step after 7-4 or Figure 16. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 1 hour (37°C, CO_2, 5%). (cf. Figure 16-18)

Figure 16

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 40uL	DMSO 40uL	DEX 10 mg/mL in DMSO 40uL	DEX 50 mg/mL in DMSO 40uL	DEX 100 mg/mL in DMSO 40uL							
B	DMSO 10% in B medium 100uL	DMSO 10% in B medium 100uL	DEX 1 mg/mL DMSO 10% in B medium 100uL	DEX 5 mg/mL DMSO 10% in B medium 100uL	DEX 10 mg/mL DMSO 10% in B medium 100uL							
C												
D												
E												
F												
G												
H												

10uL

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

Figure 17

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 0.2% B medium 500uL	DMSO 0.2% B medium 500uL	DEX 20 ug/mL DMSO 0.2% in B medium 500uL	DEX 100 ug/mL DMSO 0.2% in B medium 500uL	DEX 200 ug/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	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												

Figure 18 Final constituents of each well of the plate

flat-bottom B&W	1	2	3	4	5	6	7	8	9	10	11	12
A	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 10 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 50 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 100 ug/mL DMSO 0.1% B medium 100uL							
B	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 10 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 50 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 100 ug/mL DMSO 0.1% B medium 100uL							
C	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 10 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 50 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 100 ug/mL DMSO 0.1% B medium 100uL							
D	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 10 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 50 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 100 ug/mL DMSO 0.1% B medium 100uL							
E												
F												
G												
H												

7-6 Addition of LPS to THP-G1b (TGCHAC-A4)

One hour after the addition of dexamethasone, add 10 µL of distilled water or 1000 ng/mL LPS solution prepared in §6 to the cells (#A1-#D1 or #A2-#D5, respectively) using an 8 channel or 12 channel pipetman after pipetting 20 times. Make sure that the apex of the tip is dipped into the medium. Change tips every line you add. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 6 hours (37°C, CO_2, 5%). (cf. Figure 19)

Figure 19

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 20.

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

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

Figure 21. An example of the raw output data

Measurement without Filter

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

Measurement with Filter

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

Two transmittance factors of the optical filter were calculated as follow:

$$\text{Transmittance factor } (\kappa G_{R60}) = \frac{\#B1 \text{ of } F1 + \#B2 \text{ of } F1 + \#B3 \text{ of } F1}{\#B1 \text{ of } F0 + \#B2 \text{ of } F0 + \#B3 \text{ of } F0}$$

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

In the case shown above,

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

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

Calculated transmittance factors are used for all the measurements executed using the same luminometer.
Input the transmittance factors to #G4-5 of the “Data Input” sheet of the Data sheet as follow.

Figure 22

MultiReporter Assay System –Tripluc®– Calculation Sheet

Input transmittance factors of filter for SLG and SLR

	TF	
Input measured data (counts)	SLG κG_{R60}	SLG
	SLR κR_{R60}	SLR

9. Measurement

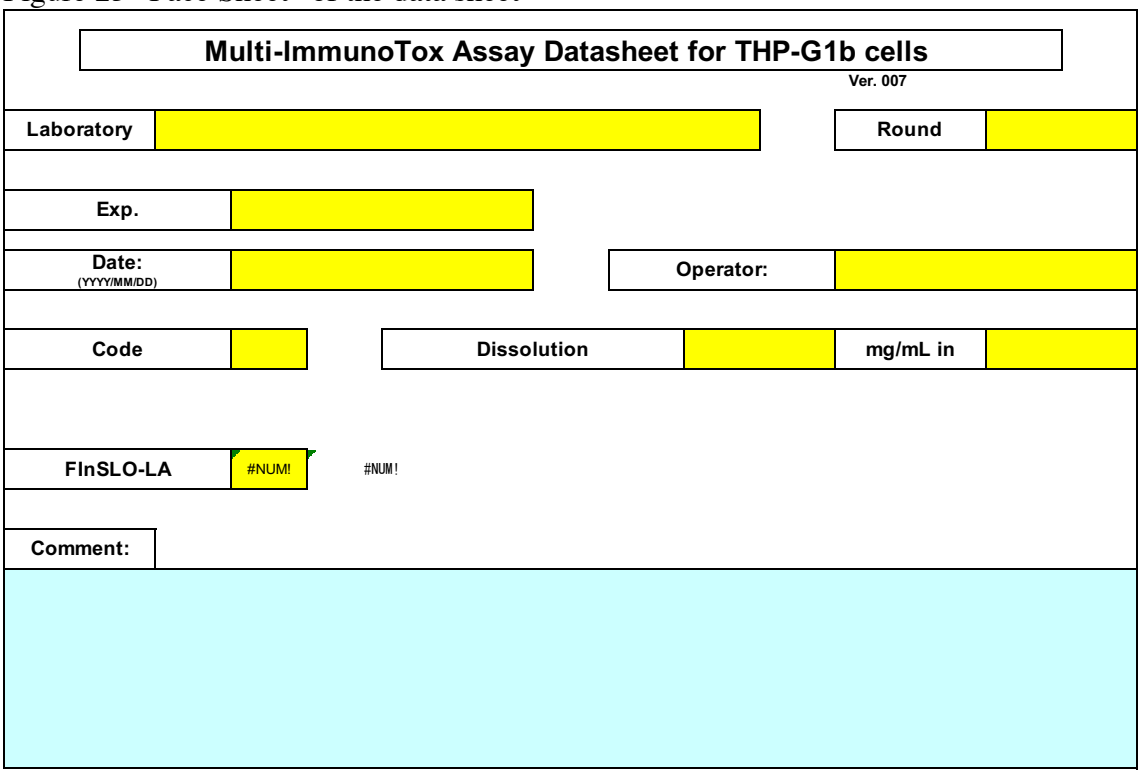
Please refer Appendix 1 for the principle of measurement of luciferase activity.

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

Transfer 100 μL of pre-warmed Tripluc from the reservoir to each well of the plate containing the reference sample using an 8 channel or 12 channel pipetman. Shake the plate for 10 min at room temperature (about 25°C) using a plate shaker. Remove bubbles in the solutions in the wells if they appear. Place the plate in the luminometer to measure the luciferase activity. Bioluminescence is measured for 3 sec each in the absence (F0) and presence (F1) of the optical filter. In case alternative settings are used, e.g., depending on the model of luminometer used, these settings should be justified.

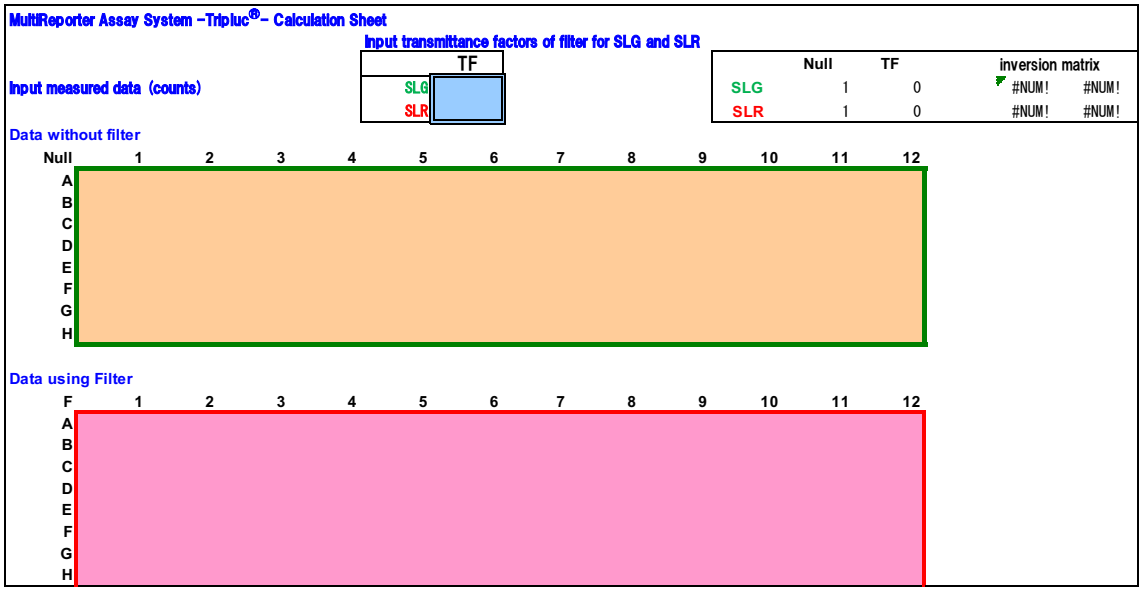
1st. Add the information regarding the name of laboratory, the round of experiments if multiple experimental sets are performed, the experiment number, date, the operator, chemical codes, dissolved in distilled water or DMSO, the prepared concentration and comments if any to Face Sheet of the data sheet.

Figure 23 “Face Sheet” of the data sheet



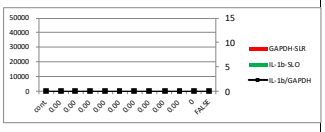
2nd. Copy the results of the F0 and F1 measurements (values are expressed as counts) and paste them into the appropriate area in the “Data Input” sheet of the data sheet shown below (Figure 28). In addition, input the transmittance factors calculated in chapter 5. Calculation of the transmittance factors to TF of the “Data Input” sheet (Figure 24).

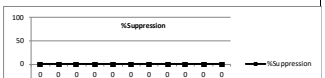
Figure 24 “Data Input” sheet of the data sheet

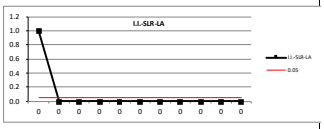


Next, the calculated results for the parameters of the Multi-Immuno Tox assay for each concentration, e.g., SLG-LA, SLR-LA, nSLG-LA, the mean ± SD of SLG-LA, the mean ± SD of SLR-LA, %suppression and graphs will automatically appear on the "Result Format" sheet of the data sheet.

Figure 25 "Result Format" sheet of the data sheet







MultiReporter Assay System -Tripluc®- Calculation Sheet

Filter Null Data

Null	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	0	0	0	0	0	0	0	0	0
B	0	0	0	0	0	0	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0	0
D	0	0	0	0	0	0	0	0	0	0	0	0
E	0	0	0	0	0	0	0	0	0	0	0	0
F	0	0	0	0	0	0	0	0	0	0	0	0
G	0	0	0	0	0	0	0	0	0	0	0	0
H	0	0	0	0	0	0	0	0	0	0	0	0

Filter 2 Data

F2	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	0	0	0	0	0	0	0	0	0
B	0	0	0	0	0	0	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0	0
D	0	0	0	0	0	0	0	0	0	0	0	0
E	0	0	0	0	0	0	0	0	0	0	0	0
F	0	0	0	0	0	0	0	0	0	0	0	0
G	0	0	0	0	0	0	0	0	0	0	0	0
H	0	0	0	0	0	0	0	0	0	0	0	0

MultiReporter Assay System -Tripluc®- Calculation Sheet

Transmittance Data

	Null	T
SLG	1	0
SLR	1	0

inversion matrix

#NUM!	#NUM!
#NUM!	#NUM!

SLG

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

SLR

A	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
B	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
C	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
D	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
E	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
F	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
G	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
H	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!

SLG mod

A	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
B	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
C	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
D	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
E	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
F	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
G	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
H	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!

SLR mod

A	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
B	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
C	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
D	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
E	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
F	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
G	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
H	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!

nSLG-LA

#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!

Chemical concentration	cont.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	FALSE	μg/ml
SLG-LA Average	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	
S.D.	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	
SLR-LA Average	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	
S.D.	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	
nSLG-LA Average	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	
S.D.	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	
I.I-SLR-LA	#NUM!	1.000	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	
%suppression (IL-1β)		0.000	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	
FInSLO-LA	#NUM!	#NUM!											

10. Data analysis

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

- SLG-luciferase activity (SLG-LA): Luciferase activity of stable luciferase orange (Under the control of IL-1β promoter)
- SLR-luciferase activity (SLR-LA): Luciferase activity of stable luciferase red (Under the control of G3PDH promoter)
- Normalized SLG-LA (nSLG-LA): =(SLG-LA)/(SLR-LA)
- Inhibition index of SLR-LA (I.I.-SLR-LA): The cytotoxic effect of chemicals
  =(SLR-LA of THP-G1b treated with chemicals)/(SLR-LA of untreated THP-G1b)
- %suppression: The effect of chemicals on IL-8 promoter
  =(1-(nSLG-LA of THP-G1b treated with chemicals)
  /(nSLG-LA of non-treated THP-G1b)) x 100

11. Criteria

11-1 Acceptance criteria

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

- If Fold induction of nSLG-LA of LPS wells without chemicals (=(nSLG-LA of THP-G1b cells treated with LPS) / (nSLG-LA of non-treated THP-G1b cells)) demonstrate less than 5, 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, only if viable wells satisfy the following positive criteria is accepted 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 (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 ≥ 20 (suppressive) or ≤ -20 (stimulatory) 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 (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

12. Update record

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 26.

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

Transfer 100 µL of pre-warmed Tripluc to each well containing the reference samples of the plate using a pipetman. Shake the plate for 10 min at room temperature (about 20°C) with a plate shaker. Remove bubbles on the solutions in wells if they appear. Place the plate into the luminometer of the under-test to measure the luciferase activity. Bioluminescence is measured for 3 sec each in the absence (F0) and presence (F1) of the optical filter. The example of the raw output data was shown below.

Figure 32.

Measurement without Filter

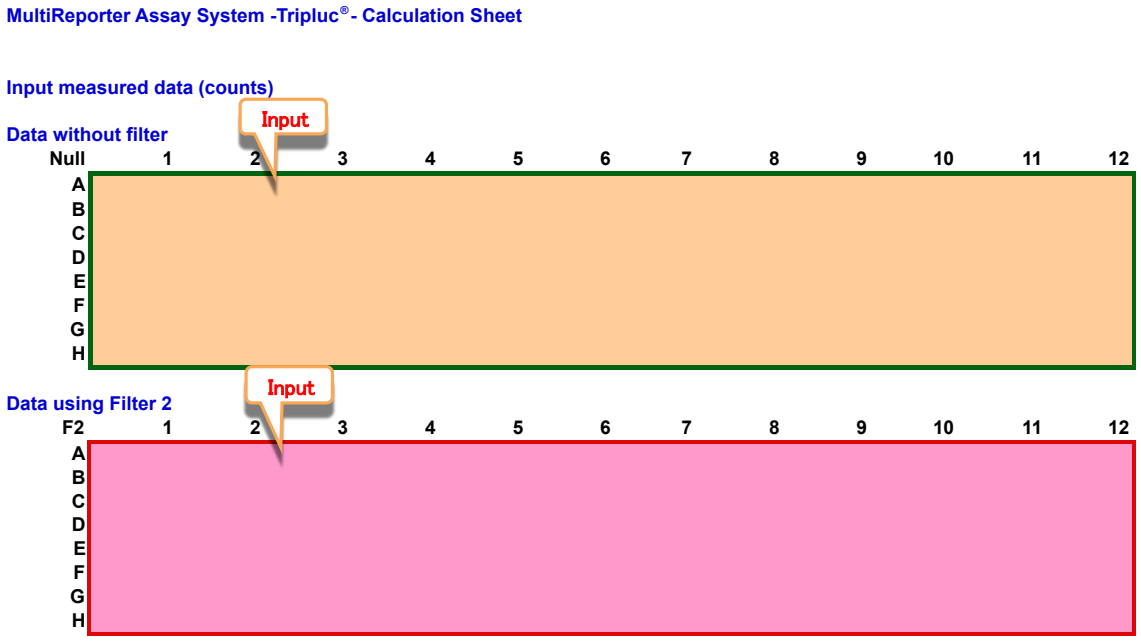
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	9689	9691	9677	2402	2388	2412	704	689	721	177	189	182
C												
D	8588	8444	8462	2281	2128	2239	609	578	690	150	132	129
E												
F												
G												
H												

Measurement with Filter

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	2022	1945	2067	502	496	510	143	149	153	37	49	45
C												
D	5722	5756	5721	1523	1459	1589	413	397	468	102	108	97
E												
F												
G												
H												

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

Figure 33.



Record all the results for quality control.

2 Quality control of equipment

In order to confirm the detector stability as the quality control, the reference luciferase sample, optical property, the protocol described hereafter should be performed at the beginning of the experiments every day using reference LED light source plates equipped with stabilized LEDs. LED plate data typically fluctuates up to 1.5% (σ). Disagreement to the old data should be less than 3×σ (= 4.5%). Here describes an example using a commercially available reference light source, TRIANT® (wSL-0001) by ATTO (Tokyo, Japan).

Start LED plate and select “PMT” mode.

Select three-color (BRG) mode and adjust light intensity to 1/10 (10E-1).

Place the LED plate into the luminometer. Light intensity is measured for 3 sec each in the absence (F0) and presence (F2) of the optical filter.

Read and record the data at the position of #F6, #E6, and #D6 that are LEDs of blue, green, and red LEDs, respectively.

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

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
小島　肇	皮膚・粘膜毒性		トキシコロジー第３版	朝倉書店			279-286
小島　肇	動物実験代替法		トキシコロジー第３版	朝倉書店			320-325
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平成３１年２月２１日

国立医薬品食品衛生研究所長 殿

機関名　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３０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。

平成31年3月28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

次の職員の平成３０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）　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）

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）

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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。

平成３１年３月１４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 中鉢 良治 印

次の職員の平成 30 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）健康工学研究部門 細胞光シグナル研究グループ

（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

	該当性の有無		左記で該当がある場合のみ記入（※1）		
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研究倫理教育の受講状況	受講 ■ 未受講 □

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・分担研究者の所属する機関の長も作成すること。

平成３１年3月１４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　　中鉢 良治　印

次の職員の平成 30 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。

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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

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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

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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。

2019年　3月　13日

国立医薬品食品衛生研究所長 殿

機関名　一般財団法人食品薬品安全センター

所属研究機関長　職　名　代表理事

氏　名　小島　幸一　　印

次の職員の平成30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。

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3．研究者名　（所属部局・職名）　主任研究員

（氏名・フリガナ）　山影　康次（ヤマカゲ　コウジ）

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）		
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遺伝子治療等臨床研究に関する指針	□	☑	□		□
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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：　　）	□	☑	□		□

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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 □

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（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。

令和元年　5月　8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

次の職員の平成30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）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。

平成31年 2 月 18 日

国立医薬品食品衛生研究所長 殿

機関名　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 30 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。

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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。

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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。