厚生労働科学研究費補助金（化学リスク研究事業）多色発光細胞を用いたhigh-throughput免疫毒性評価試験法の開発

分担研究報告書

化学物質のMulti-ImmunoTox assayによる解析，精度管理

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研究要旨
多色発光タンパク質を用いたMulti-ImmunoTox assay（MITA）だけでは、皮膚感作性物質の多くがLPSで刺激したTHP-G8細胞のIL-8転写活性を抑制し、単球/樹状細胞に抑制的に作用する免疫抑制物質と皮膚感作性物質を区別できないことが明らかとなった。そこで従来法のMITAに、開発を進めていた皮膚感作性物質試験法であるIL-8 Luc assayを加えたmodified MITAを開発、バリデーションに進むためのプロトコールの最適化を行うための5物質のIL-8 Luc assayを実施した。また、免疫毒性試験系として、MITAのIL-2レポーター活性抑制評価系のバリデーション試験を行うための最初の段階である技術移転性を確認するために、5物質のIL-2レポーター活性抑制作用を調べた。

キーワード：免疫毒性、動物実験代替法、*in vitro*

A．研究目的

我々はこれまでに多色発光タンパク質による新たな *in vitro* 免疫毒性評価試験法、いわゆる Multi-ImmunoTox assay（MITA）を確立し各種毒性評価発光細胞を樹立した[1)]。現在、これらの細胞群を用いた化学物質の免疫毒性評価法の確立を目指している。そこで本研究では、化学物質の免疫毒性評価のための MITA 法の OECD ガイドライン化を視野に、ラボ間バリデーション試験の実施と MITA 法の精度管理に必要な周辺技術の開発を目的とした。

より具体的には、東北大学病院で樹立された Jurkat 細胞における INF-γ，IL-2，G3PDH プロモータ活性を測定する細胞株 2H4 及び THP-１細胞における IL-8 と G3PDH プロモータ活性を定量化できる細胞株 TGCHAC-A4、IL-1β と G3PDH プロモータ活性を定量化できる細胞株 THP-G8 をモデル細胞として施設内、施設間バリデーション試験を実施、ガイドライン化するための手法の最適化を目指す。本年度は、IL-8 Luc assay の結果を含む Modified MITA の data set を構築するために 5 物質に関して IL-8 Luc assay を実施した。また、免疫毒性の評価系として IL-2 レポーター活性抑制評価系のバリデーション試験の最初のステップとして、技術移転性確認のため 5 物質の試験を行った。

B．研究方法

B-1）IL-8 Luc assay

IL-8とG3PDHプロモータにそれぞれSLRおよびSLOルシフェラーゼ遺伝子を繋いだ発現ベクターをTHP-1細胞に導入した2色発光細胞株THP-G8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についてはIL-8 Luc assay protocol Ver. 020E 20150703に準ずる。

試験化学物質として2,4-Diaminotoluene, 2-Aminoanthracene, Dapsone, Dibutyl phthalate, Isoniazidの5物質を供試し、発光測定装置はアトー社製Pheliosを用いた。

B-1) IL2レポーター活性抑制物質評価のためのMITA assay

IL-2とIFN-γ、G3PDHプロモータにそれぞれSLG、SLOおよびSLRルシフェラーゼ遺伝子を繋いだ発現ベクターをJurkat細胞に導入した3色発光細胞株#2H4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についてはMulti-Immuno Tox Assay protocol 案 Ver.008.1E 20160202に準ずる。

試験化学物質として、2-Aminoanthracene, Citral, Chloroquine, Dexamethasone, Methyl mercuric(II) chlorideの5物質とを供試した。

（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.結果

C-1) IL-8 Luc assay

a) 試験開始当初、リードラボである東北大で出された発光値と比べ、約50～60%と低く、反応性の低さが懸念された。そのため、新たに東北大から分与された細胞株を用いて試験を実施したところ反応性の向上が確認され、試験に用いた細胞（当研究機関において凍結保存した株）自体の反応性が落ちている可能性が示唆された。そこで改めて、東北大からの分与株を用いて試験を実施し、その結果（図1）と各Criteriaにおける評価（図2）を示す。

b) 28年度より予定しているMITAバリデーション試験 phaseIの実施に向け、#2H4細胞株を用いた技術移転性の確認試験を実施した。各物質に対し3回繰り返し試験を行った結果を図3に示す。現在、リードラボである東北大にて、試験参加各施設の結果をもって評価、考察を進めている。

D. 考察

従来のMITAでは一部の感作性物質がIL-8レポーター活性抑制作用を示し、一方、デキサメサドンなどの免疫抑制剤との区別ができないため、IL-8プロモータ活性測定系を加えたmodified MITAを構築することは本年度の免疫毒性物質の評価においては重要である。そこで、modified MITAのdata setを構築するために、MITAのdata setから選択した5物質についてIL-8 Luc assayを実施した。その結果、criteriaによって、結果が異なる場合もあるが、概ね再現性良く免疫抑制物質と感作性物質との識別が可能となった。

さらに、今年度の技術移転性結果をもとに、次年度以降は免疫毒性試験のIL-2プロモータ活性評価系のバリデーション試験を行うため、プレバリデーション試験を5つの試験物質について行った。最終的には再現性の高いデータを得ることができたが、細胞を活性化する処理によるバラつきの問題や細胞の維持管理に関しての問題点が浮き彫りになった。来年度以降、これらバラつきの問題を解消し、試験プロトコールの最適化が重要な課題となった。今後、プロトコールの最適化と共に精度管理に関する研究を行う予定である。

E. 結論

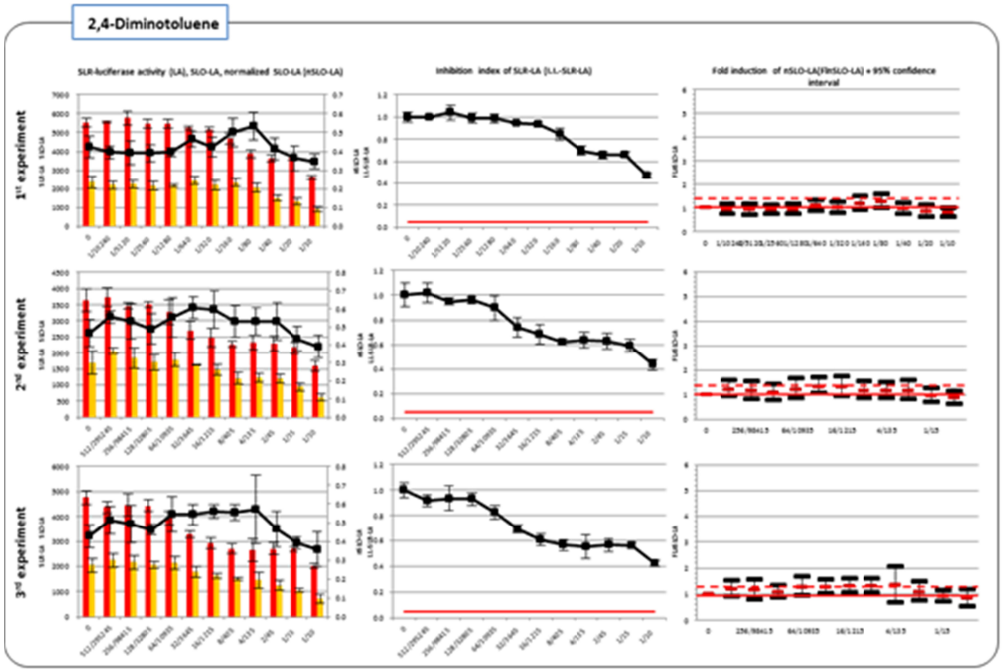
MITAのdata setの構築に協力し、AOP作成に貢献した。また、免疫毒性試験のIL-2プロモータ活性評価系のバリデーション試験に進むための技術移転性の確認実験を終了、プロトコールの最適化のための課題を見出した。

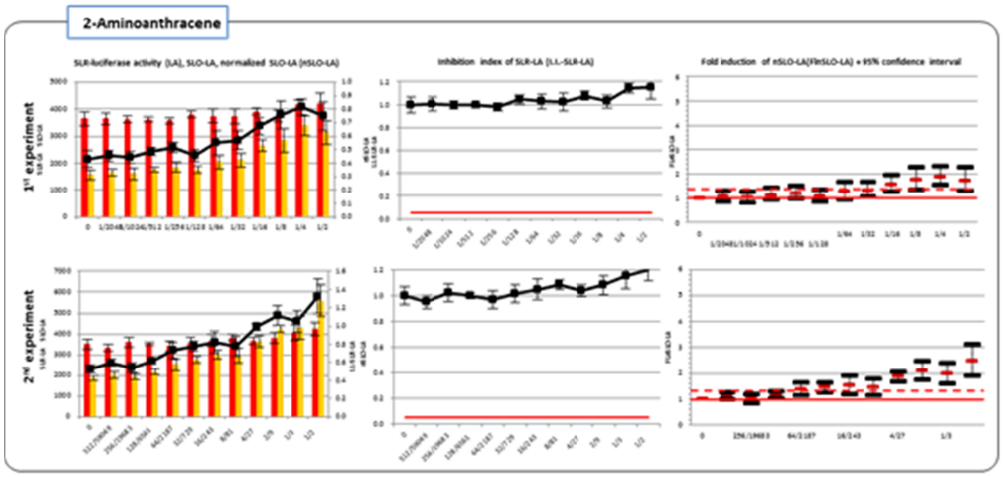
F. 参考文献

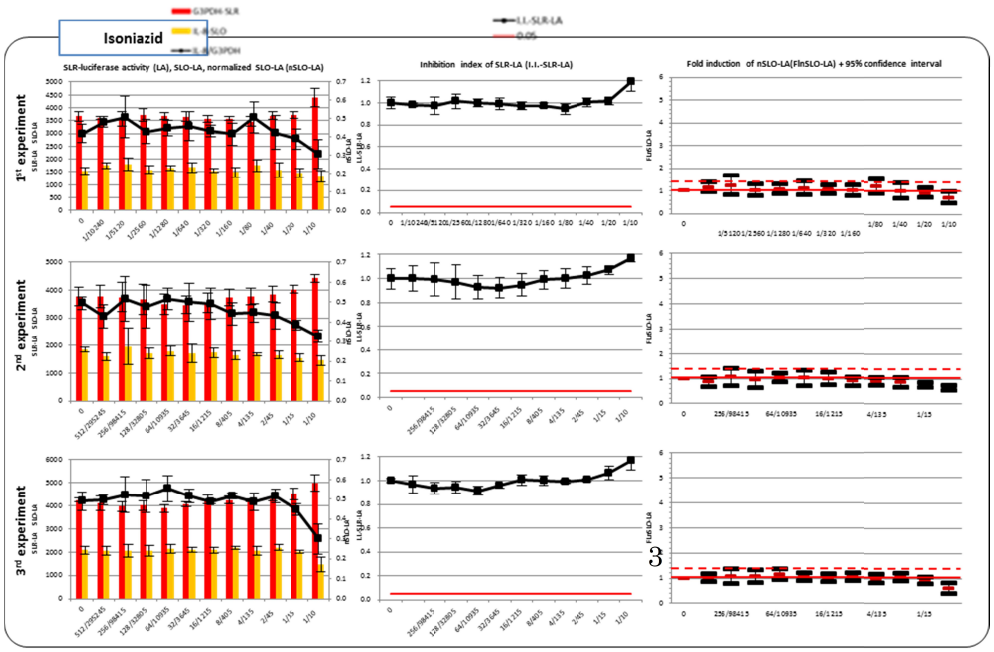
1) Takahashi T, Kimura Y, Saito R, Nakajima Y, Ohmiya Y, Yamasaki K, Aiba S: An in vitro test to screen skin sensitizers using a stable THP-1-derived IL-8 reporter cell line, THP-G8. Toxicol Sci., 124, 359-69, 2011

G. 研究発表

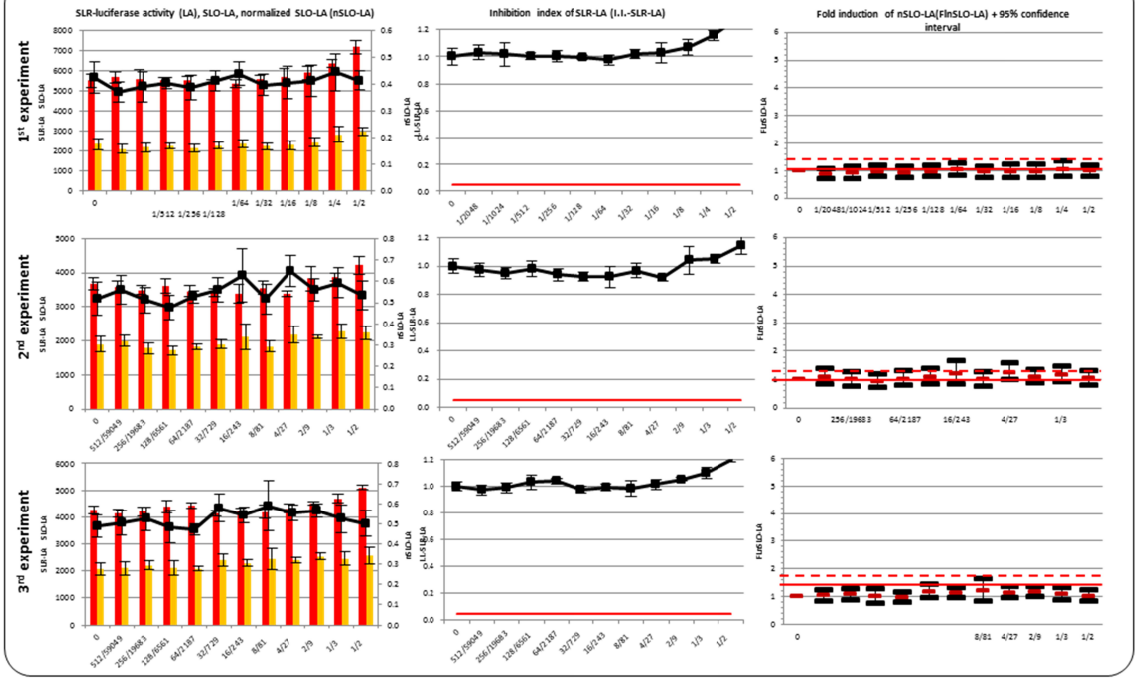
図１ Modified MITA による IL-8 Luc assay の結果







Dapson



Dibutyl phthalate

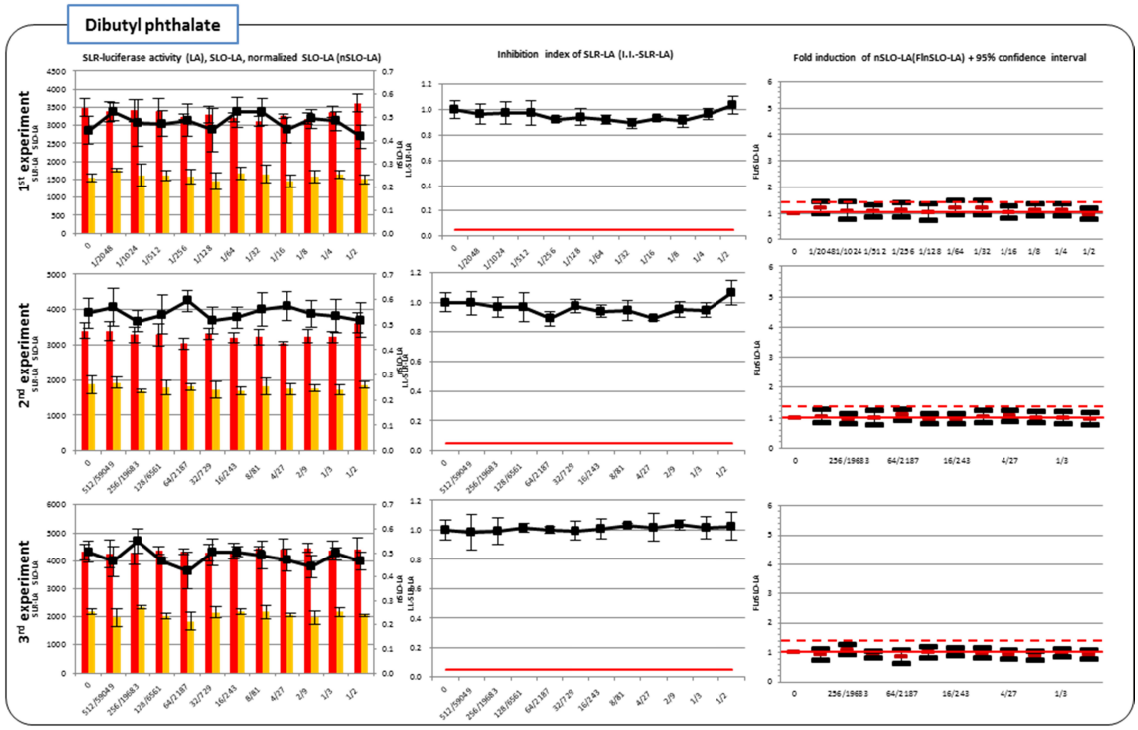


図 2　Modified MITA による IL-8 Luc assay の各 criteria における評価

	1st	2nd	3rd	4th	Judge
2,4-Diaminotoluene	N	N	N	-	Non-sensitizer

図 3　Jurkat 細胞由来株#2H4 における各試験化学物質に対する細胞応答性。

Crite

	1st	2nd	3rd	4th	Judge
Dibutyl phthalate	N	N	N	-	Non-sensitizer
Isoniazid	N	N	N	-	Non-sensitizer

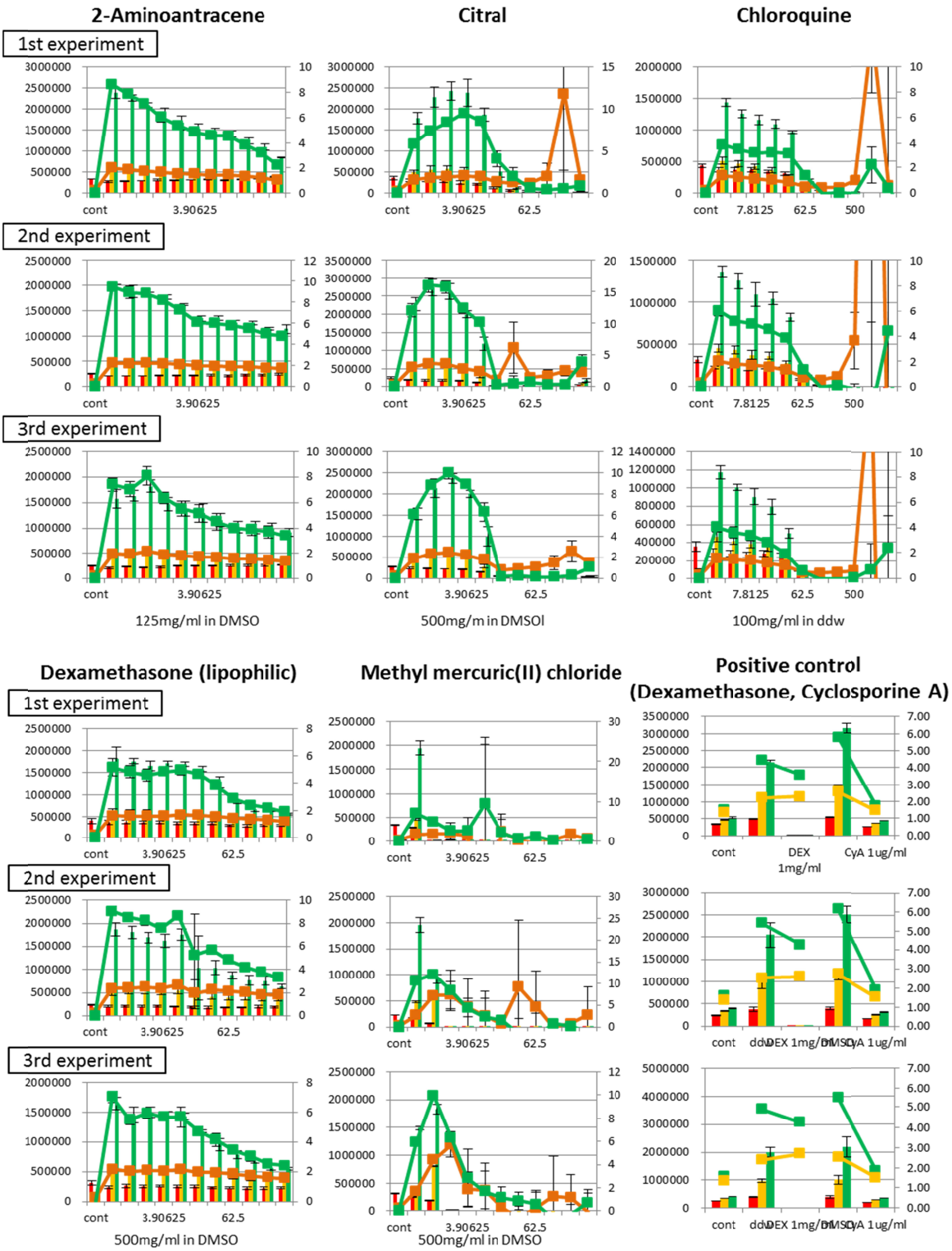
Criteria 2

	1st	2nd	3rd	4th	Judge
2,4-Diaminotoluene	N	P	P	-	sensitizer
2-Aminoanthracene	P	P	-	-	sensitizer
Dapson	N	N	N	-	Non-sensitizer
Dibutyl phthalate	N	N	N	-	Non-sensitizer
Isoniazid	N	N	N	-	Non-sensitizer

Criteria 3

	1st	2nd	3rd	4th	Judge
2,4-Diaminotoluene	N	N	N	-	Non-sensitizer
2-Aminoanthracene	P	P	-	-	sensitizer
Dapson	N	N	N	-	Non-sensitizer
Dibutyl phthalate	N	N	N	-	Non-sensitizer
Isoniazid	N	N	N	-	Non-sensitizer

N;Negative, P;Positive


2-Aminoantracene
Citral
Chloroquine
1st experiment
2nd experiment
3rd experiment
cont
3.90625
62.5
7.8125
500
125mg/ml in DMSO
500mg/m in DMSOl
100mg/ml in ddw
Dexamethasone (lipophilic)
Methyl mercuric(II) chloride
Positive control
(Dexamethasone, Cyclosporine A)
DEX
1mg/ml
CyA 1ug/ml
500mg/ml in DMSO
500mg/ml in DMSO

厚生労働科学研究費補助金（化学リスク研究事業）
免疫毒性評価試験法Multi-ImmunoTox assayの国際validationへ向けての検討
分担研究報告書

化学物質のMITAによる解析,validation

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研究要旨
Multi-ImmunoTox assay（MITA）では、皮膚感作性物質の多くがLPSで刺激したTHP-G8細胞のIL-8転写活性を抑制し、単球/樹状細胞に抑制的に作用する免疫抑制物質と皮膚感作性物質を区別できないことが明らかとなった。そこで従来法のMITAに、これまで我々が進めてきた皮膚感作性物質試験法であるIL-8 Luc assayを加えたmodified MITAを構築するために、5物質のIL-8 Luc assayを実施した。また、免疫毒性試験系として、MITAのIL-2レポーター活性抑制評価系のバリデーション試験を行うための最初の段階である技術移転性を確認するために、5物質のIL-2レポーター活性抑制作用を調べた。

キーワード：IL-8 Luc assay、IL-2 レポーター活性抑制、技術移転性

A．研究目的

免疫毒性評価試験法である Multi-ImmunoToxicity assay (MITA)の data set（60 化学物質）を構築した結果、MITA のプロトコールでハプテンを評価すると $CoCl_2$、$NiCl_2$、isophorone diisocyanate などの感作性物質が IL-8 レポーター活性抑制作用を示し、免疫抑制剤と区別できないことが明らかとなった。真の免疫抑制剤と感作性物質を区別して評価するために、MITA と感作性物質評価系との組み合わせが不可欠であることから、感作性物質の評価系である IL-8 Luc assay の結果を含む Modified MITA の data set を構築するために 5 物質に関して IL-8 Luc assay を実施した。また、免疫毒性の評価系として IL-2 レポーター活性抑制評価系のバリデーション試験の最初のステップとして、技術移転性確認のため 5 物質の試験を行った。

B. 研究方法

B-1)用いた細胞

IL-8 Luc assayには、IL-8およびG3PDHの各プロモーター領域にそれぞれ橙および赤色のルシフェラーゼ遺伝子を繋いだベクターをTHP-1細胞に導入した安定細胞株THP-G8を使用した。

IL-2レポーター活性抑制試験には、緑、橙、赤色の発光色の異なるルシフェラーゼ遺伝子をIL-2, IFN-γ, G3PDHの各プロモーター領域に繋いだベクター（それぞれ緑、橙、赤色）をJurkat細胞に導入した安定細胞株#2H4を使用した。

B-2) 使用した化学物質

IL-8 Luc assay には、isophorone diisocyanate、pentamidine isethionate salt、4-nitroaniline、magnesium sulfate heptahydrate、lithium carbonateの5物質を用いた。

IL-2レポーター活性抑制試験の技術移転性には、2-aminoanthracece、chloroquine、citral、dexamethasone、methylmercury(II) chlorideを用いた。

B-3) 実験方法

IL-8 Luc assay については、バリデーション試験で実施した方法に従った。すなわち、1×10⁶/mLに調整した THP-G8細胞の50 μLを96 wellプレートに播種し、X-VIVO™に溶解した原液または懸濁液を遠心した原液とそれを希釈した各濃度の化学物質溶液50 μLを添加し、16時間処理(37°C、 5%CO_2)した。処理終了後、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、各色ルシフェラーゼ活性をPherios（アトー社製）で測定し、色分離式により各プロモーター活性を算出した。

IL-2レポーター活性抑制試験は、MITAプロトコールに準じて行った。概要としては、#2H4細胞を96 wellプレートに播種し、各種濃度の化学物質を添加した。1時間後にPMA/ionomycin (#2H4細胞)による活性化処理を行い、6時間処理(37°C、 5%CO_2)後にTripluc luciferase assay reagentを用いて各色ルシフェラーゼ活性をPheriosで測定し、IL-2プロモーター活性を算出した。

C.結果

C-1) IL-8 Luc assay

実施した5物質の結果を図1に示した。化学物質処理群のG3PDHプロモーター活性（SLR-LA）を陰性対照のその活性で割った阻害指数（I.I.-SLR-LA）が0.05以上で、標準化した（G3PDHプロモーター活性で割った）IL-8プロモーター活性（SLO-LA）を化学物質処理群と陰性対照群とで比較したfold induction（FInSLO-LA）が1.4以上でかつ、その95%信頼限界の下限が1.0以上の場合を陽性とし、2回以上陽性結果が得られた場合を感作性有りと判定した。

試験した5物質中、pentamidine isethionate saltと4-nitroanilineは非感作性物質と判定され、残りの3物質は感作性物質と判定された。

C-2) IL-2レポーター活性抑制試験の技術移転性

5物質の実験を3回繰り返し、その結果を東北大へ送付した。バリデーション試験の試験実施施設である3施設（秦野研究所、産総研バイオメディカル研究部門、産総研健康工学研究部門）の結果を東北大で比較検討している。

D. 考察

MITA data setの解析の結果、MITAでは$CoCl_2$、$NiCl_2$、isophorone diisocyanateなどの感作性物質がIL-8 レポーター活性抑制作用を示し、Dex、hydrocortisoneあるいはFR167653 (p38 mitogen activated kinase (MAPK)阻害剤)などの免疫抑制剤との区別ができないことが明らかとなった。この問題点を解決するために、IL-8プロモーター活性測定系を加えたmodified MITAを構築することは免疫毒性物質の評価において重要であると考えられる。そこで、modified MITAのdata setを構築するために、MITAのdata setから選択した5物質についてIL-8 Luc assayを実施した。これにより免疫抑制物質と感作性物質との識別が可能となった。

また、今年度の技術移転性結果をもとに、次年度以降は免疫毒性試験のIL-2プロモーター活性評価系のバリデーション試験を継続する予定である。

E. 結論

MITAのdata set（60化学物質）を構築に協力し、AOP作成に貢献した。また、免疫毒性試験のIL-2プロモーター活性評価系のバリデーション試験の最初のステップである技術移転性の確認実験を終了した。

F. 参考文献

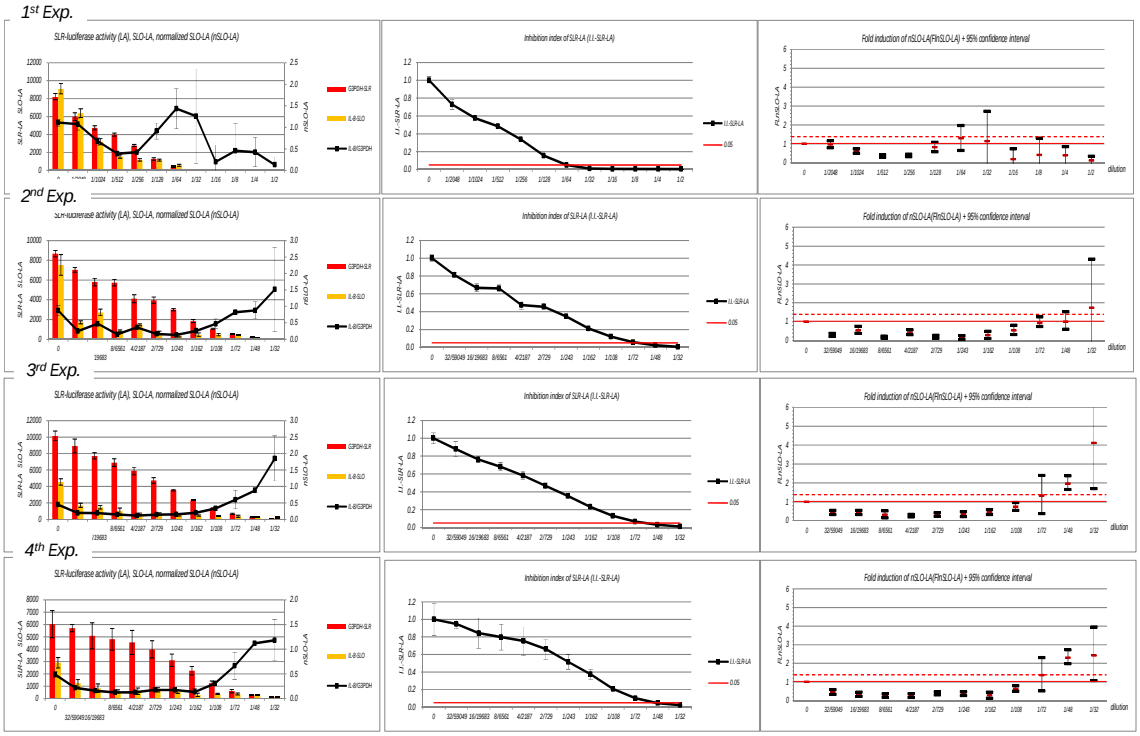
なし

G. 研究発表

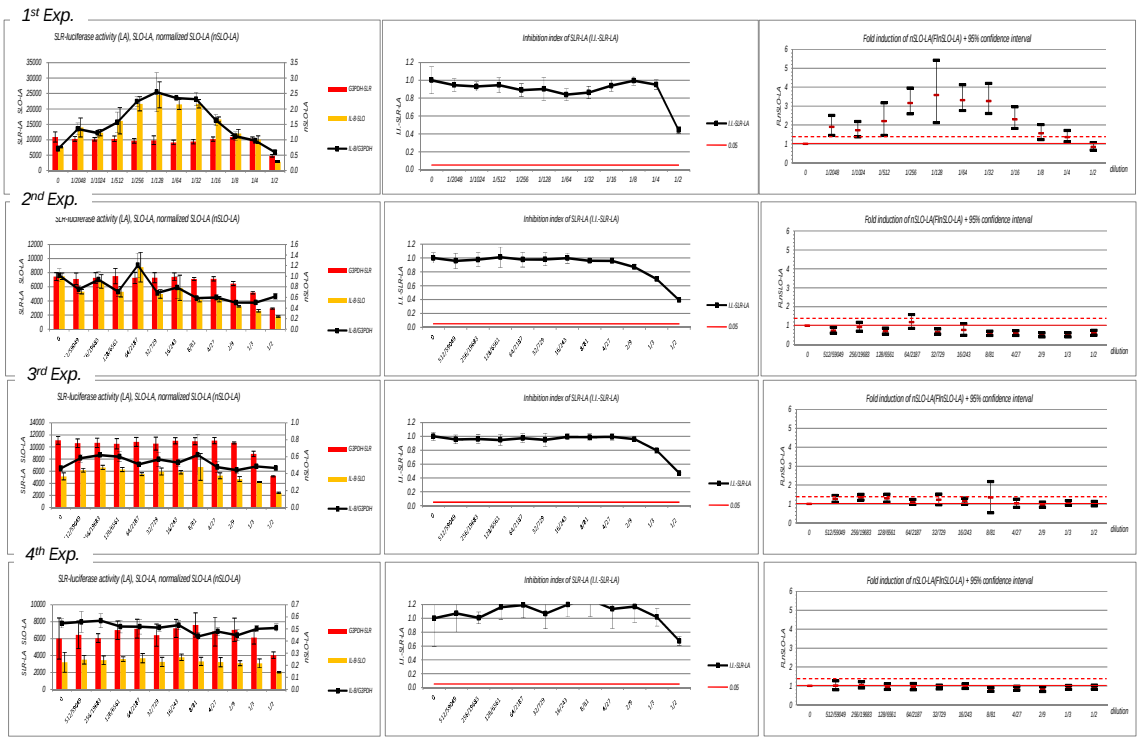
なし

図１　Modified MITA による data set 構築のための IL-8 Luc assay の結果

<Pentamidine isethionate salt>



< 4-Nitroaniline >



<Isophorone diisocyanate>

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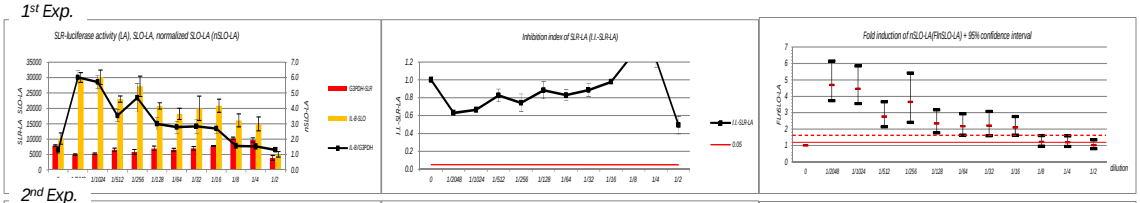
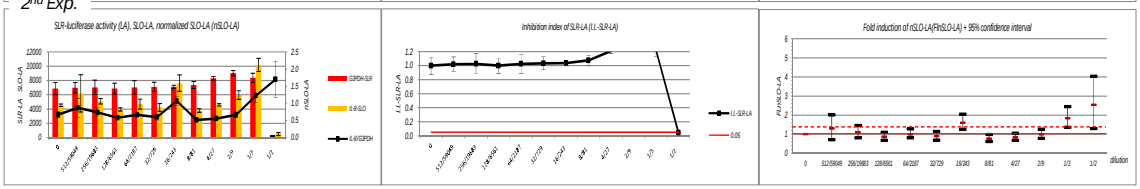
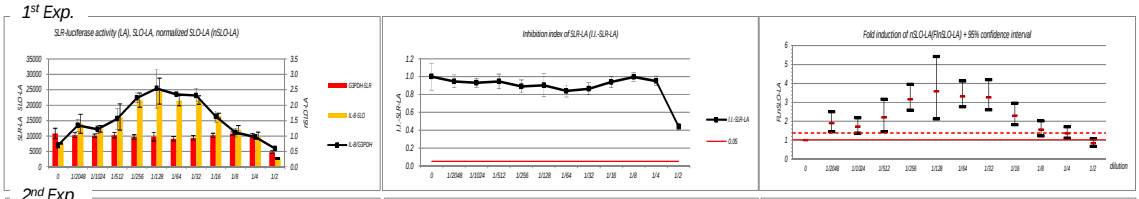


2nd Exp.

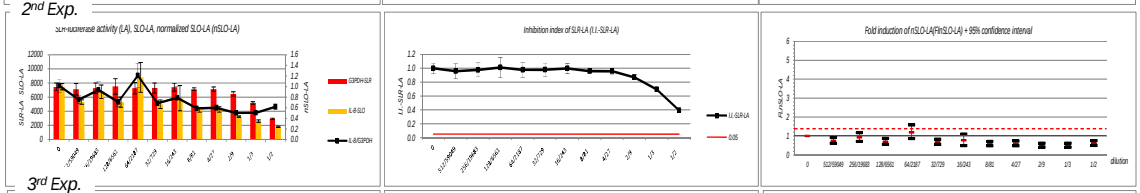


<Magnesium sulfate heptahydrate>

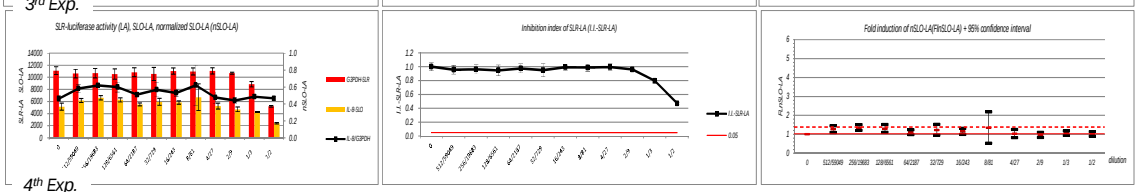
1st Exp.



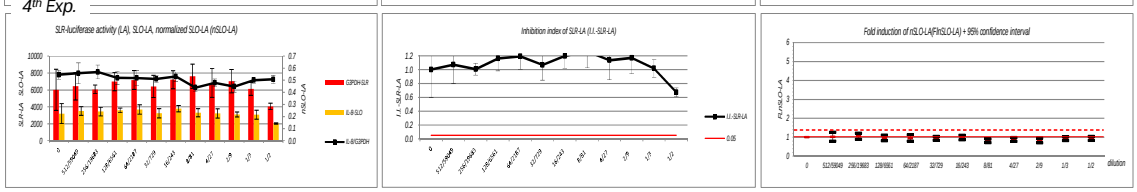
2nd Exp.



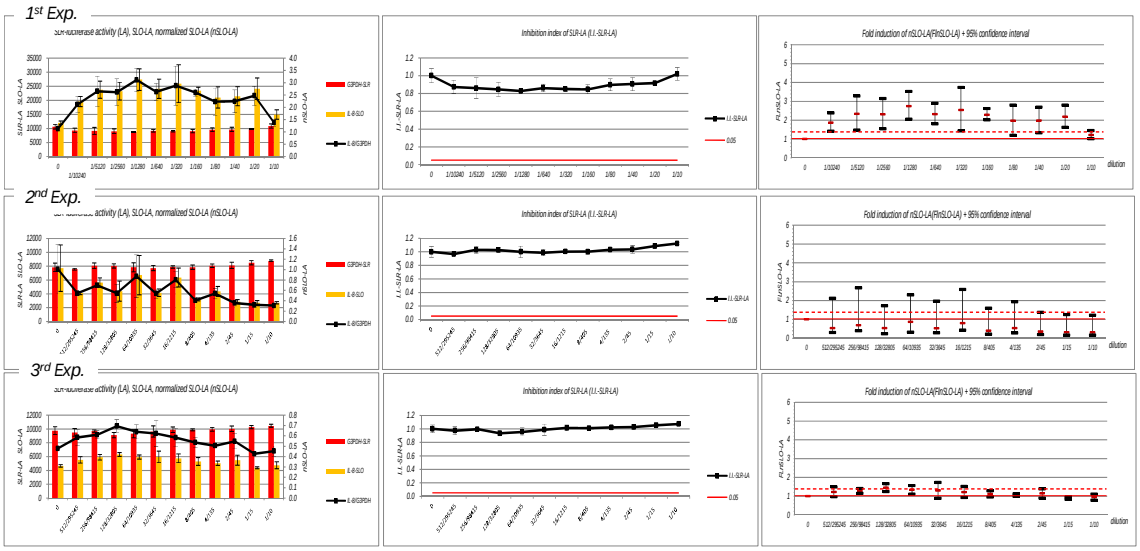
3rd Exp.



4th Exp.



<Lithium carbonate>



厚生労働科学研究費補助金（化学リスク研究事業）
免疫毒性評価試験法Multi-ImmunoTox assayの国際validationへ向けての検討
分担研究報告書

国際バリデーションの施行

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研究要旨

新たな*in vitro*免疫毒性評価試験法（Multi-ImmunoTox assay：MITA)のOECD（Organisation for Economic Co-operation and Development）試験法ガイドラインとしての公定化を目指し、国際バリデーションに向けた活動を行った。国際的な専門家を招聘して本試験法に対して意見を求めた結果、平成28年度以降、MITAに関する国際バリデーションを開始することになった。

キーワード：免疫毒性、動物実験代替法、バリデーション

A．研究目的

新たに開発された *in vitro* 免疫毒性評価試験法（Multi-ImmunoTox assay：MITA）の OECD（Organisation for Economic Co-operation and Development）における試験法ガイドライン（Test Guideline：TG）を目指し、国際バリデーションの開始を模索する。

B．研究方法

B-1. 国際的な専門家との意見交換

平成28年度以降、MITAに関する国際バリデーションを開始し、国際的なTGへの道程を明確にすることを予定している。その第一歩として、平成28年１月国際バリデーションのキックオフ会議を企画した。

B-2.バリデーション研究の被験物質選択

キックオフ会議において、Phase0として、トレーニングに用いる被験物質およびphase I として施設内再現性を評価するための被験物質の選択を行った。

B-3.IL-8 Lucアッセイの公定化

平成27年10月、OECDの皮膚感作性試験専門家会議に参加し、日本で開発されたin vitro皮膚感作性試験のうち、IL-8 LucアッセイのTGとしての意義、必要性について意見交換した。

C．結果

C-1.　国際的な専門家との意見交換

国際バリデーションのキックオフ会議には、免疫毒性およ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）および Dr. Dori Germolec（NTP/NIEHS：電話でのみ参加）を、国内からは、景山茂樹博士（富士フィルム）および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１に示すメンバーにて 2 日間掛けて、MITA の科学的意義、試験法プロトコルの妥当性などについて討論した。会議の議事次第を添付文書 1 に示す。

会議に先立ち、研究代表者の相場は、当初の計画を一部変更する以下の提案を示した。

１）細胞の選択と測定指標の妥当性

平成 28 年度からの 2 年間は IL-2 レポーター細胞の 2H4 を用いてバリデーション研究を実施する。この細胞は今年度に作成した 60 種類の化学物質からなるデータセットにおいても鋭敏に IL-2 転写活性抑制物質を検出することができている。したがって今年度に作成した IL-2 転写抑制を指標としたＴ細胞サブセット分化異常の AOP に則った評価も可能である。この IL-2 の結果とすでにバリデーション研究が終了している IL-8　Luc アッセイの組み合わせにより、免疫毒性を評価することとした。

２）一つ一つの試験法を独立してバリデーション研究を行うべきであるが、そのスケジュール

これから行うバリデーション研究においては予算の関係もあり、上記 IL-2 転写活性抑制に関する試験法に関してのみ施行する。

３）アンタゴニスト試験系に対する懸念（これまでの経験ではアンタゴニストのプロトコルがよく練られていないと頓挫する可能性が高い）

Lipopolysaccharide (LPS)に対するアンタゴニストを用いる評価系は、現時点では再現性が不十分である。現在、種々検討しているところである。

４）試験法データを組み合わせた判別式の確立

今後、作成する予定である。

この案をもとに表１に示すメンバーにて議論を重ねた。その議事録を添付文書2に示す。主な論点を以下に示す。

・免疫毒性は多岐に渡り、IL-2やIL-8にエンドポイントを特定することが妥当か。
・IL-2は免疫毒性に重要であり、事実的なエンドポイントである。作用機構の点から有用である。
・細胞毒性は免疫抑制の作用機構の一つであり、IL-2を細胞毒性の代用として測定すべきである。
・IL-2が関与したAOP(Adverse Outcome Pathway)の開発も必要である。
・評価には他の試験結果との組み合わせが必要である。
・バリデーション研究の開始は早すぎる。バリデーション研究を行う理由が見当たらない。
・偽陰性の原因を明確にすべきほうが重要である。
・今すべきことは、ヒトや動物の免疫毒性データを整備することである。
・MITAはスクリーニングツールとして有用であり、真の陰性結果を見つけられることを確かめるためにバリデーション研究は必要である。
・IL-2レポーター細胞のもとであるJarkat細胞は他の細胞よりは再現性が得られやすい。
・バリデーション研究を実施するなら、再現性の確認のために実施すべきである。80%の施設内再現性が必要である。
・被験物質の選択が重要である。
・数年前に作成した免疫毒性物質リストと比較して、相場らの選択物質は主な免疫毒性物質を網羅している。
・過去の経験から、陰性物質を見つけることが難しい。
・まず明らかな陽性、陰性物質を含む5物質で結果の一致性を確認すべきである。
・プロトコルに細胞の管理や最大適用濃度を明記することが重要である。

以上の議論の末、再現性を確認するための国際バリデーション研究の実施に合意が得られた。

早速、添付文書3に示すバリデーション計画案を示し、外部専門家の意見をもとに、平成28年度以降に実施するバリデーション計画を検討した。まずphase0としてトレーニングを実施すること、次に

phase1として施設内再現性を確認する計画に合意を得た。

C-2.バリデーション研究の被験物質選択

まずはphase 0 として、3施設のトレーニングを行う5物質を選定した。表2にそのリストを示す。

施設内再現性を確認するphase I の5物質も選定したが、コード化して実施することもあり、本報告書には記載していない。

C-3.IL-8 Luc アッセイの公定化

OECD における専門家会議において、日本からOECD に提案している皮膚感作性試験代替法 IL-8 Luc アッセイが専門家会議で議論された。議事概要を添付文書 4 に示す。

日本から開発者の相場らが本会議に参加し、概要説明を行うとともに、内容の詳細について議論した。まだ第三者評価が進行中であるが、TG 化が期待される IL-8 Luc アッセイの国際的な理解が深まった。

D. 考察

免疫毒性は多岐に渡り、作用機構がわかっているものは少ない。そのような状況下でIL-2やIL-8に特化した試験法の開発には懐疑的な意見が外部専門家からあった。

とはいえ、IL-2が免疫毒性の重要なエンドポイントであることは間違いなく、この試験法の開発を中断するほどの大きな理由は見当たらない。そこで、再現性の確認を目的としたバリデーション研究を行うことで外部専門家の合意が得られた。その際の被験物質の選択も重要であり、phase 0を経て、phase Iの5物質で施設内再現性を確認した後、施設間再現性を確認するためのPhase IIの被験物質選択が重要との見解で一致した。

E. 結論

新たな*in vitro*免疫毒性評価試験法（MITA）のOECDにおける公定化の道筋を明確にするため、国際的な専門家を招聘して意見を求めた。

その結果、平成28年度以降、MITAに関する国際バリデーション研究を開始することになった。

F. 添付文書

1) Agenda：Kick-off meeting for the MITA assay
2) Minutes of MITA Kick-off meeting, Jan. 27 & 28, 2016
3) 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
4) Draft Summary Record: Expert Group Meeting on Skin Sensitisation, 14-15 October 2015, Paris, France

表１． MITA**第一回国際バリデーション会議　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	Shigeki Kageyama	Fujifilm Corporation	Japan
4	Tomoaki Inoue	Chugai Pharmaceutical Co., Ltd.	Japan
5	Setsuya Aiba	Tohoku University Graduate School of Medicine	Japan
6	Yutaka Kimura	Tohoku University Graduate School of Medicine	Japan
7	Yoshihiro Ohmiya	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	Shihori Tanabe	National Institute of Health Sciences	Japan
12	Hajime Kojima	JaCVAM, National Institute of Health Sciences	Japan
13	Steven Venti	Translator	Japan

表２． Phase 0 **トレーニング用物質**

Chemical	CAS No.	MW	Physical state	MITA IL-2 result
2-Aminoanthracene	613-13-8	193.24	Solid	S(-/-/-)
CH_3HgCl	115-09-3	251.08	Solid	+-
Chloroquine diphosphate salt	50-63-5	515.86	Solid	S(-/-/-)
Citral	5392-40-5	152.23	Liquid	S(+-/+-/+-*)
Dexamethasone	50-02-2	392.46	Solid	S(-/-/-)

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27）Furukawa M, Sakakibara T, Ito K, Sasaki S, Koshita M, Okumura S, Kawamura K, Matsuura M, Kojima H: Histopathological Findings on the Cornea in the Bovine Corneal Opacity and Permeability Test (BCOP Test) for Alternative to Eye Irritation Test, 55th annual meeting of the Society of Toxicology (2016.3) (New Orleans, U.S.A.)

28）Narita K, Vo P.T, Nakagawa F, Kojima H, Itagakai H: Reducing False Negatives of Chemicals in the in vitro Skin Sensitization Test, 55th annual meeting of the Society of Toxicology (2016.3) (New Orleans, U.S.A.)

29）Tsukumo H, Matsunari N, Sugiyama M, Toyoda A, Kojima H, Itagakai H: Development of an in vitro test for Allergenic Potency of Proteins, 55th annual meeting of the Society of Toxicology (2016.3) (New Orleans, U.S.A.)

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

2. 実用新案登録
なし

3.その他
なし

添付文書 1

Agenda
Kick-off meeting for the MITA assay

Date ： January 27, 2016, 13:00 - 17:00
January 28, 2016, 9:00 - 13:00
Venue：New Mitoya, Sendai, Japan (http://www.mitoya-group.co.jp/access/)
Participants: Corsini, E., Rogen, E., Kageyama, S.
Aiba, S., Kimura, Y., Yamakage, K., Ohmiya, Y., Yasuno, R., Omori, T., Kojima, H., Tanabe, S.

January 27
Introduction (13:00-13:10)
1. Welcome address and house keeping (Kojima, H.)

Chair: Ohmiya, Y.

Presentations (13:10-17:00)
2. Development of AOP on immunosuppression in EGMST, OECD (Kojima, H.)
3. Outline of the MITA assay (Aiba, S.)
4. Research on the immunotoxicity (E. Corsini)
5. *Applying Toxicogenomics for In Vitro Assessment of Immunotoxicants.(E.L Roggen)*

Coffee Break

Chair: Yamakage, K.

6. Research on the immunotoxicity (Kageyama, S.)
7. Research on the immunotoxicity (Inoue T.)
8. Research on the immunotoxicity (S. Tanabe)
9. Development of reporter gene assay (Ohmiya, Y.)

January 28
Proposal of validation study (9:00-11:00)

Chair: Omori, T.

10. Outline of the IL-2 Luc assay (Aiba, S.)
11. Protocol (Kimura, Y.)
12. Results of preliminary test by three laboratories (Kimura, Y.)
13. Study plan (Kojima, H.)

Coffee Break

Chair: Dr. Kojima, H.

14. Discussion and suggestion (11:30-12:30)

Closing session (12:30-13:00)
15. Wrap-up on discussion
16. Future plan
17. Any other business
18. Closing remark (Aiba, S.)

MITA Kick-off meeting **添付資料２**

Jan. 27, 2016

Kojima	Opening remarks and review of the agenda, followed by self-introductions Review of new and pending OECD Test Guidelines Increased emphasis on identifying AOP, because of the benefits of using AOP as a framework for development of IATA for skin sensitization testing.
Corsini	Do we need to discuss which aspects immunosuppressive or immunomodulatory aspects are to be tested?
Roggen	Need to keep it simple but also ensure that all essential elements are included.
Aiba	Presentation on Multi-Immuno Tox (MITA) assay (See presentation.)
Corsini	Presentation on Research in Immunotoxicity (See presentation.)
Roggen	Presentation on Applying Toxicogenomics for In Vitro Assessment of Immunotoxicants (See presentation.)
Kageyama	Presentation on Research in Immunotoxicity (See presentation.)
Tanabe	Presentation on Research in Immunotoxicity (See presentation.)

MITA Kick-off meeting, Day Two

Jan. 28, 2016

Kojima	Agenda will change a little. Dr. Aiba will summarize the proposed protocol and then we will discuss. If everyone agrees, we will discuss whether to pursue this approach or not.
Aiba	(Summary of proposed protocol)
Omori	I would like to discuss whether this is a viable test or not. MITA appears to be valuable as an immunotoxicological assay. If you agree, I suggest we discuss a validation study. We have completed a validation for IL=8, and we should validate IL-2.
Roggen	Although not a general test for immunotoxicity, it could be useful for some specific things, which we need to identify.
Aiba	I think this modified MITA could be useful to regulators. It should help identify the characteristics of immunotoxicants.
Germolec	Very hard to hear what is being said.
Inoue	With regard to differentiating T-helper cells, IL-2 is related but so is IL-10 and others, so perhaps the results will differ from in vivo testing.
Aiba	Yes, this is not a complete method. But it could be useful in screening chemicals.
Corsini	I agree that as a screening tool it could be useful. Negative results will require additional testing. But immunotoxicity will require weight of evidence, so combined with other tests it will be useful.
Ohmori	These comments seen to suggest that a validation study is needed. Does anyone disagree?
Aiba	Is it possible apply to OECD for a guideline?
Kojima	Immunosuppression is an important factor in safety assessment of chemicals.
Tanabe	What will the endpoint be?
Aiba	We will need to combine with other cell lines, so we would like to validate for 2H4 cell line alone not just MITA.
Roggen	From ECVAM point of view, I am afraid that reproducibility needs to be improved. And from this data, I'm not sure I understand the limits in terms of applicability domain. Also, it seems there were some false negatives, so it seems there is something we don't yet understand.
Aiba	There are so many different chemicals but there is limited information about their toxicity. Most chemicals lack information about immunotoxicity.
Roggen	Yes, we need tests to identify immunotoxins that are not sensitizers, which is

why it is too early for a validation study. Still need more information about reproducibility and how it will be used for screening, etc.

Corsini Perhaps you can comment more about table on page 24.

Aiba The data shows that IL-2 gives consistent results but is still difficult to get results for THP cells.

Corsini If I understand, Jurkat gives good between-lab reproducibility, but the other cell lines are more difficult. Do you have figures for the 60 chemicals for accuracy, sensitivity, and specificity?

Aiba Looking at page 23, there are chemicals that don't give clear results. For example, acetaminophen. Some of these chemicals have known characteristics, but for many, it is not so clear.

Corsini It is difficult to find a compound that is always negative. So the chemical selection will be crucial and not so easy for this validation.

Aiba There are many chemicals that do not have clear information about immunosuppression.

Germolec We are working to find some true negatives, but it has been a struggle. It is difficult to find a chemical that is active but doesn't have an effect on something. So I wonder if we can find a true negative.

Aiba We thought that we had a good negative control, but when we increased the doses, we found it was an immunosuppressant.

Most of the chemicals that affect the pathways can be detected by IL-2 and with just these two tests in the modified MITA, we can detect the characteristics of most chemicals.

Kageyama Compared with other assays, for example, incubation time is longer and concentrations are higher, so I think that this is important. On page 23 these problems occur when time is short or concentration is low.

Roggen With regard to the Fluorescent cell chip method, which uses mouse cells that would be extensively used. We are focusing more and more on human cells now than 10 years ago. It is difficult to find negative compounds, but cytotoxicity is one mechanism of immunosuppression, so perhaps IL-2 upregulation should be measured in the absence of cytotoxicity.

Aiba We do check to see if viability of our cell line contains luciferase activity stimulated by GADPH promoters.

Inoue On page 23, not just sensitivity or cytotoxicity, there are some substances here that give opposite results between Fluorescent cell chip method and MITA.

Ohmori Is the purpose of the validation study to establish the test method? Perhaps we should discuss whether or not there is a need for this test method.

Aiba If we decide that the method is not sufficiently established, we will have to give up this study. I think I have done almost everything necessary to establish the method and categorize chemicals with it. But the situation surrounding MITA is still immature. Perhaps there is no clear need to screen immunotoxicity and there is not clear target. Pharmaceutical makers have a clear need for skin sensitizer test methods but I don't know if there is a need for screening immunotoxicity.

It is probably just a small step to where a need will be established, but we are not there yet.

Roggen I understand your reasoning, but I also wish that work on immunotoxicity will continue. Unfortunately, I do not see a rationale for proceeding with a validation now. Perhaps we need more human and in vivo data about immunotoxicity.

Corsini I see your point, and I think immunotoxicity should be given much more importance. But we are not studying it enough. I agree that we should find some rationale for this study to proceed.

Germolec It is a constantly moving thing. We have a list of 25 compounds. Mostly environmental chemicals. We are still working on making a larger list, so let

me update you all about the status of that list.

Roggen You said the biggest challenge is finding negative substances.

Germolec It is difficult to run through all the assays and find a substance that is truly negative across all parameters. It is often based on cytotoxicity. We were looking at very wide dose-response curves to try to find cytotoxicity and then refine that immunological responses. It has been hard to find immunotoxicity in the absence of cytotoxicity.

Roggen Do you know the Jerka test using 25 genes to identify immunotoxicants? Maybe you need to look at the gene level to achieve that.

Aiba Can we reach a conclusion? The situation is difficult, I know. But I would like to submit an AOP for airway hyper sensitivity or contaminated water. But it is difficult to select chemicals. I would be happy to hear ideas. There is little info on chemicals, key events, and outcome. What chemicals might be useful?

Corsini There are probably close to 100 compounds that are immunotoxic or non-immunotoxic. There was work in the 90s listing non-immunotoxic chemicals. How many do we need?

Germolec There is crossover between the list we are making and the MITA list.

Roggen And to validate, you will need chemicals that affect IL-2 expression.

Corsini Or chemical that affect T-cell expression. You have tested 60 chemicals. How did you select them?

Ohmori One purpose of validation study is to validate reliability. How about that? Most of this discussion is about relevance.

Roggen I think this is a pragmatic approach, since we know IL-2 is important. But to validate that for regulatory use, we have to identify the chemicals that follow that mechanism, and we might not have enough to do that. From a mechanistic point of view, this is an important approach. Reliability is a technical aspect that has to be done but can be improved.

Aiba How can we examine chemicals for immunotoxicity? Is there a method that can be applied to the compounds? It would require a high concentration. How do people examine the effects of pesticides? Skin sensitizers can be tested in a high concentration, but working with low concentrations makes it hard to discern immunotoxicity.

Corsini Pesticides are immunotoxic in animal experiments. So you compare in vivo and in vitro data. Most pesticides today are not immunotoxic, but some old ones are.

Kageyama On page 23, discrepancies are important. By evaluating cytotoxicity, you can examine the effect on T-cells. So combining IL-2 and cytotoxicity is important. So page 23 shows a mixture of two assays.

Roggen All different aspects are important. Here we have two assays that can be combined eventually with other assays.

Ohmori I think this test method is not yet established. We should discuss whether or not this method is valuable enough to establish.

Aiba I think the method is established. We can still modify the assay. We have to optimize certain aspects, but the issue here is that we cannot find a good positive control. But we have no information about a chemical that is clearly immunotoxic.

Roggen I think we agree the tests are important and address specific mechanistic events that are related to immunosuppression. So the test is valuable. The next step is a project to improve reproducibility. Then establish a list of reliable positive and negative controls. It won't be easy but you can do it. And after you have that, you can think about a validation. Chemical selection is crucial to validation planning, and will take time. These are the three steps needed to proceed with this project.

Corsini I agree with Erwin.

Kojima I also agree. It is also necessary to establish AOP.

Roggen AOPs are never finished, so do that in parallel with other activities.
Aiba It is a chicken and egg issue. The modified MITA can provide some insight into the characteristics of chemicals. This method will be useful to people who want to identify the mechanism of immunotoxicity.
Ohmori So, the conclusion is to continue the project. So what is the next step? Should we discuss the plan?
Corsini It is important to come up with a good list of chemicals that we are confident of in terms of immunotoxicity.
Germolec Yes we are willing to share what we have and what we will find in the future.
Corsini We started a few years ago with making a list.
Germolec Yes, there have been many stops and starts. But we are willing to share.

Break

Aiba (Explanation of protocol)
Corsini PMA has a shelf life. Is there is shelf life to the stock solution? From my own experience, PMA stops working after 6 months, so please indicate a shelf life.
Will you include a positive and negative control?
Aiba See page 23.
Corsini What is the cost of this cell line?
Aiba Will be determined in the future.
Roggen I have a comment about the cells. Cell lines lose functionality, especially in transfected parts. It is important to define the No. of permissible passages.
Aiba We are now confirming that. But I think more than 30 times. This line is already 5 years old but we are still using it.
Roggen I understand but you need to define that in the procedure.
Kageyama What about interference from FCS?
Aiba We have used this line for more than 5 years, and have changed a couple of times. I don't think it is a factor.
Inoue On pg 23, DMSO could affect the performance of the cells.
Aiba Page 27 shows DMSO control
Kojima Regarding the criteria, the student t-test is perhaps not the best method. Could you change it to a different method?
Aiba This criteria was accepted for my paper, but if we need to change it for validation, we can do so.
Roggen From the reviewer's point of view, dose dependent response is important. It would be better to say "and" instead of "or" on pg 36.
Kageyama Why don't you use beta mercaptoethanol for the T-cell culture?
Ohmiya Perhaps use a different name rather than 2H4.
Tanabe How many chemicals per plate?
Aiba Can test two chemicals on each plate.
Omori Please send other questions by mail.

Kojima (Presents draft study plan)
Lead lab is responsible for training. Is there a plan for training?
Aiba Yes, this is similar to earlier assays and we do not anticipate any problem.
Roggen For within-lab reproducibility, you have 80% acceptance criteria, but I think a rationale for that target is necessary. I always have this question. This figure is very strict.
Why is there no target for between-lab transferability?
Kojima We expect 100% transferability.
As to the 80% target, we have five chemicals. So we allow one chemical to be out of concordance.
Roggen Maybe better to say "4 of 5" instead of "80%."
Kojima Do you think we need more than 5 chemicals?

Roggen	5 is enough, depending on how clear cut the results are.
Corsini	Is five chemicals enough for within-lab reproducibility with blind chemicals?
Roggen	Five is a common number for within-lab reproducibility.
Kojima	The Phase II will add 20 chemicals, other than the five used for the within-lab reproducibility.
Roggen	The more IL-2 immunotoxicants that you have, the better.
Kojima	(review of schedule) End of Phase 1 study is at the end of this 2016. In the Table 3 it is says Phase 2, but that is a typo. It should be Phase 1.
Aiba	You need 100% concordance?
Kojima	For Phase 0 Study, yes.
Aiba	What happens if there is a non-concordance?
Kojima	Then solve the technical problem.
Roggen	For the five chemicals, the number of positive or negative is not that important. Concordance of results is what is important. (discussion of chemicals)
Aiba	I propose these chemicals for Phase 0: 2-aminoanthracere (DMSO), CH_3HgCl (DMSO), chloroquine (ddw), citral (DMSO), and dexamethansone (water insoluble, DMSO)
Kojima	Is DMSO for positive control?
Aiba	I don't think so.
Roggen	We are looking for five chemicals that are clearly positive? But to challenge the labs, you will want to use chemicals with differing levels of effect.
Aiba	Many of these chemicals are not so toxic, so we can increase the concentration. We have to think about the effect of the chemicals on blood.
Roggen	Is the maximum concentration the concentration that gives the highest toxicity? That is one way of defining it.
Corsini	Max. concentration is 1 mg/mM for Keratinsens and HCLAT.
Roggen	Here we are limited to drugs. We want this test to work for everything. But we need to provide information that will enable people to find the correct concentration for any chemical. There should be some that have only a weak effect, either positive or negative. To see if they can clearly identify it. And then others that are clearly positive or negative.
Aiba	Finding two negatives is a problem.
Corsini	Can we select substances that haven't been tested yet but are known immunotoxicants and affect T-cells.
Roggen	It's better to select chemicals for Phase 1 that have been tested already. For the real validation, you can expand (in Phase 2).
Corsini	So better to select chemicals listed on pages 32 and 33.
Aiba	For Phase 2, I propose: lead acetate ++ (ddw), hydrocortisone + (ddw), dibutyl phthalate +, DMDTC - (DMSO), and nickel sulfate + (ddw)
Roggen	It is good to have one clear positive and one clear negative and then the others that are not so clear. Even if you have to repeat this Phase a couple of times, it is time well spent.
Omori	After checking the properties of these chemicals, Dr. Aiba will discuss the final selection by email. Time to close the meeting now.
Aiba	Thanks for participating. There is still a lot of information that has to be collected and many decisions to make. Thank you for your support.

添付資料３

Version 1,1 January, 2016

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

IL-2 Luc assay Validation Management Team

INDEX

1. Background

The use of multicolor reporter assay using IL-2 Luc, Jurkat cell (IL-2 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-2 Luc assay method to assess transferability and inter-laboratory variability, in order to incorporate this test for screening the immunotoxic chemicals. The IL-2 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-2 Luc assay. This IL-2 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-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	JaCVAM, NIHS, Japan (JaCVAM representative)
Lead Lab Yutaka Kimura* Setsuya Aiba*	*Developer of this assay Test method, expertise underlying science	Tohoku Univ., Japan
Shihori Tanabe	Management of quality control	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
Japan liaison Shigeki Kageyama	Immunotoxicity expertise	Fujifilm Corporation, Japan
JSIT liason Tomoaki Inoue	Immunotoxicity expertise	Chugai Pharmaceutical Co., Ltd.

3.1Participating Test Facilities

The laboratories participating in the trial are defined as follow:

Test Facility 1: Hatano Res. Inst., FDSC. Study Director (SD) : Kohji Yamakage

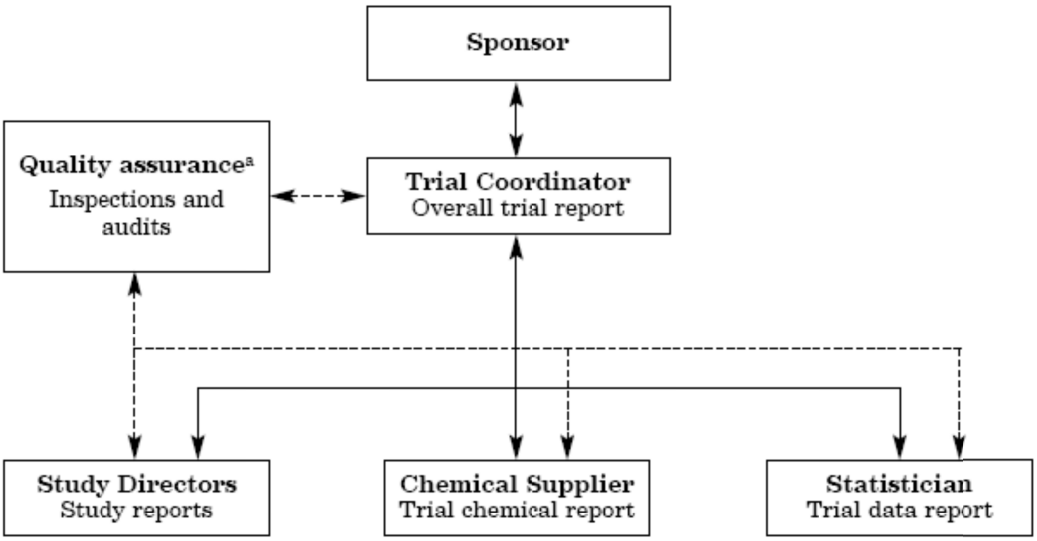
Test Facility 2: AIST, Tsukuba SD : Yoshihiro Ohmiya

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. 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-2 Luc assay method under non-GLP conditions (GLP principle).

3.2 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-2 Luc assay validation trial

1) Chemical management group

The members of chemical management group are elected by recommendation of the IL-2 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-2 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-2 Luc assay VMT. They prepares protocol, test chemical preparation record forms, blank data sheets, etc. and distributes them to the research laboratories participating in this validation trial. They also collects 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.

3.3Sponsor

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:

- 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

3.4Trial 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 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.

3.5Training

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-2 Luc assay protocols. In case any critical observations are made a new version of the IL-2 Luc assay protocols might necessary be issued to the other test facilities before initiating the between-laboratory transferability.

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.

3.7[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-2 Luc assay to the all test facility, the Phase 0 study using non-coded five chemicals was performed. A few concentrations of each test item will be tested in triplicate in 3 independent runs according to the IL-2 Luc assay protocol describing the details of the experimental design.

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.

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. 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).

3.8 [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-2 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,

3.9[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-2 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 (planning)	0	5 non- coded	1	Between-lab transferability
Phase (planning)	I	5 coded	3	Within & between-lab reproducibility
Phase (planning)	II	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-2 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-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.

7. Study timeline

An approximate schedule for IL-2 Luc assay validation trial is shown in Table 3.

Duration of this validation trial is around twenty -month from May 2016 to Nov 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	
May 2016	Coding and distribution of five coded test chemicals
June, 2016	Start of Phase I study
September, 2016	End of Phase I study
January, 2017	**2nd VMT Meeting** / Phase I results and planning of Phase II study
Phase II study to know between- and within-laboratory reproducibility	
2017	Coding and distribution of coded test chemicals and positive chemicals
2017	Start of Phase II study using 20 coded test chemicals
2017	End of Phase II study
January, 2018	**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

ENV/JM/TG/M(2015)6

Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development

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ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

Test Guidelines Programme

Draft Summary Record: Expert Group Meeting on Skin Sensitisation

14-15 October 2015 Paris,
France

Contact(s):
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Draft Summary Record: Expert Group Meeting on Skin Sensitisation

14-15 October 2015

Paris, France

1. Opening of the meeting

1. The meeting was chaired by the OECD Secretariat. Participants from Denmark, France, Japan, Korea, Netherlands, Spain, Switzerland, United Kingdom, European Commission (European Chemicals Agency and Joint Research Center), BIAC and ICAPO attended the meeting. The list of participants is available in Annex 1. The Chair introduced the draft agenda, indicating that the main objective was to address comments received from the WNT in July 2015 on the draft Test Guidelines for human Cell Line Activation test (h-CLAT). The second objective was to discuss the status and issues related to other assays on skin sensitisation, either on-going projects included in the workplan or potential new projects.

Part 1: human Cell Line Activation test (h-CLAT)

2. Discussion of the main issues raised by the WNT comments

2. Joao Barroso (EC) presented the main issues with h-CLAT, raised by WNT comments, mainly h-CLAT reproducibility, applicability and limitations. This was followed by a presentation from Roman Liška (EC) of the re- analysis of the within and between laboratory reproducibility of the h-CLAT. He explained how new approaches were used to assess the within and between laboratory reproducibility of the assay in the validation study, i.e. the permutation of runs' predictions that takes into account all possible sequences of events and the bootstrap probabilistic approach.

3. It was clarified that these new approaches can be used since the runs conducted in the test are fully independent: the runs are conducted on different days and if done on the same day, all preparations have to be re-done. How they happen in time is thus random and the runs are interchangeable. The group agreed on the methodology for calculation of the WLR. Based on the new approaches, the value of the WLR in the validation study was found to be between 82.4 and 84.8% (while the WLR in the h-CLAT validation report was of 80.0% and the target WLR was of 85%). Despite the fact that the level of WLR is slightly below the target, it was agreed that the level of WLR is transparently reflected in the TG, which specifies that "The level of reproducibility in predictions that can be expected from the test method is in the order of 80% within and between laboratories". It was noted that the 'Report on re-analysis of the within and between laboratory reproducibility of the h-CLAT' would be made available together with the validation report (after approval process) in the Series on Testing and Assessment on the OECD public website.

4. To better control certain parameters that could be a source of variability and thus to reduce the variability of the assay, some revisions to the draft Test Guideline had been proposed before the meeting. The optimisation work conducted throughout the development of the test method to minimise sources of variability were presented by Masaki Miyazawa and Takao Ashikaga (BIAC). It was in particular proposed to better control cell density (relevant changes brought to paragraph 19 of the draft TG) and the exposure time (paragraphs 24, 25 and 29 revised). In addition, as the reactivity check using controls is regularly performed, the group also agreed to emphasise in the draft TG, on the need to use the data from the reactivity check as a mean of verification of laboratory proficiency. For this purpose, a new paragraph was added in the section on laboratory proficiency and the test report was also revised accordingly.

5. It was agreed that since the result of a test is based on 2 concordant runs, when the first two runs are concordant, it is not necessary to run a third one. The group agreed on the wording of the draft TG that says: "each test chemical is tested in at least two independent runs to derive a single prediction". "If however, the first two runs are not concordant for at least one of the markers (CD54 or CD86), a third run is needed and the final prediction will be

based on the majority result of the three individual runs (i.e., 2 out of 3)" (paragraphs 29 and 33). A figure of the prediction model used in the h-CLAT test method (figure 1 of draft TG) was considered to be a very useful addition to the draft TG.

6. The group also discussed the number of replicates that should be used in the TG and it was agreed that because a prediction is obtained from at least two independent runs, one replicate could be enough. Paragraph 29 was revised to provide this rationale.

7. Regarding the limitation of the assay and description of the applicability domain, it was recognised that the assay presents limitations for the detection of three types of chemicals, which were described in the TG: (i) pro/pre- haptens, (ii) substances of low water solubility and (iii) strong fluorescent chemicals. The group agreed with the proposed text (paragraph 12), after further harmonised with the wording used in the Keratinosens TG. It was acknowledged that the detection of pro/pre-haptens is an area where progress needs to be made (an upcoming workshop dedicated to discussing this topic was mentioned), but the current state of knowledge doesn't allow to provide more details in the TG.

3. Other issues

8. A few other topics were discussed such as the use of the word 'solvent' vs 'vehicle' in the TG. It was agreed to replace 'solvent' by 'solvent/vehicle' throughout the text of the TG to acknowledge the fact that some test chemicals can be dispersed (and 'solvent' would not be appropriate in this case).

9. There was some discussion and request for clarification about the sentence in paragraph 11 relative to the testing of mixture: "However, before use of this Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed when there is a regulatory requirement for the testing of the mixture." This sentence was developed and agreed by the WNT at the meeting in April 2014. It was clarified that it should be understood in a context of Classification and Labelling, to express that when a mixture is tested and the result is negative, the substances that are part of the mixture can't be declassified on the basis of the results obtained from the mixture.

10. It was suggested to provide the historical relative fluorescence activity (RFI) value and the CV75 value (75% of cell viability) obtained for each proficiency substance included in Table 1 of Annex 2 of the TG. Having the ranges for these values, derived from the 4 laboratories which participated in the validation study, was considered particularly useful to facilitate comparison of data in case other antibodies and cytotoxicity markers are used. Japan/ EURL ECVAM will go back to the data obtained from the laboratories involved in the validation study and investigate the relevance of providing this information. If relevant, these data will be included in the revised version of the draft TG.

11. In terms of next steps, the Secretariat indicated that Japan/EURL ECVAM will now prepare the next version of the draft TG, based on the outcome of the meeting, and update the responses to comments received from the previous WNT commenting round, as appropriate. The revised draft TG will be circulated to the WNT for written comments before the end of the year. It is expected that the draft TG is then submitted for approval at the next meeting of the WNT, in April 2016.

Part 2: Other assays on the OECD Test Guideline workplan

4. Myeloid U937 Skin Sensitization Test (U-SENS) for identifying skin sensitization potential of chemicals

12. Nathalie Alépée (France) presented the history of the U-SENS™ skin sensitisation test, the outcome of the validation study and the statistical analysis that has been conducted. Like the h-CLAT, this assay addresses the 3rd key event in the skin sensitisation pathway, i.e. activation of dendritic cells. Following pre-validation of the assay, the validation phase started in 2013 and the validation test report was submitted to EURL ECVAM in July 2014. Following EURL ECVAM initial review, responses from France and the revised validation report are expected to be available in November 2015. In view of its subsequent peer review by ESAC, a new ESAC group for skin sensitisation is being established.

13. It was noted that the BLR and WLR values (respectively 84.2% and 91.7%) were above the h-CLAT values. It was suggested that this could come from a few differences between the 2 assays, such as the difference in cell line or the fact that this assay only looks at the expression of one type of cell surface markers (CD86). Some participants considered that the prediction model in case of inconclusive result is complex and might be difficult to use.

14. The possibility to develop a Performance-Based Test Guideline (PBTG) including h-CLAT and the U-SENS test methods was mentioned. It was noted however, that for the time being, considering the respective stages of development of the 2 draft TGs, the h-CLAT would be developed as a stand-alone TG and when the U-SENS is ready, the TG would be adapted and turned into a PBTG that would include both test methods. At that time, Performance Standards (PS) would also need to be developed. It was clarified that PS are a document that accompany a PBTGs and are intended to be a guide for the developers of new or modified test methods, similar to the validated reference methods. They communicate the basis by which new test methods can be determined to have sufficient accuracy and reliability for a specific testing purpose.

5. IL-8 Luc assay: An In Vitro Method for Identifying the Skin Sensitisation Potential of Chemicals

15. Setsuya Aiba (Japan) presented the validation report of the IL-8 Luc assay. This assay is based on IL-8 production, which is also a marker of dendritic cell activation in allergic contact dermatitis. The assay was optimised over the various phases of the validation, leading to significant improvement of the performance of the system.

16. Its peer review started in March 2015 and the final peer review meeting was expected to be held in Japan, on 23/24 October. Initial comments from the peer review panel (PRP) were presented by the chair of the PRP, David Basketter (UK). The validation report was well received by the panel. Regarding the BLR and WLR, the PRP concluded that the "data on BLR, with a sufficient number of test chemicals, exceeded the success criterion". However, although the "average WLR met the success criterion of 80%", "the data on WLR was more limited than comparable validation studies, particularly in respect to the number of chemicals tested". "Consequently, the PRP recommends additional assessment of within laboratory reproducibility with more and different test chemicals using the final protocol and prediction model". It is expected that the PRP report is available early 2016.

Part 3: New projects, not yet on the OECD workplan

6. LLNA: BrdU-FCM

17. Korea plans to submit a Standard Project Submission Form (SPSF) in November for inclusion in the TG workplan of a new project for the development of a TG on non-radioactive Mouse Local Lymph Node Assay using Flow-Cytometry Method (LLNA: BrdU-FCM). The method and outcome of the validation study were presented by Ilyoung Ahn (Korea). The method is a modified method of the LLNA: BrdU-ELISA (TG 442B) which enables to reduce the number of animals tested in pre-screen tests, compared with the existing LLNA test methods. The validation study has been conducted to evaluate its reliability and relevance based on the performance standards available in Annex I of TG 429 and Guidance Document 34. The validation study is almost finished and is planned to be completed in December.

18. The presentation was well received and the pre-screen step of the protocol considered an interesting development. However, as this assay is an in vivo assay it was uncertain if the WNT would consider it as a priority for inclusion in the workplan. As an alternative option, it was suggested that the project could consist in an update TG 442B to include also the BrdU-FCM method, rather than to create another LLNA TG.

7. SENS-IS assay

19. France plans to submit an SPSF in November for inclusion in the TG workplan of a new project for the development of the SENS-IS™ assay. The method and outcome of the validation study were

presented by Hervé Groux (France). The assay is a new approach for the identification of skin sensitisers where genes specifically modulated in sensitised skins allow the detection of sensitisers in a reconstructed human skin model. The results of the validation study, conducted in 3 laboratories were very promising and although it is a patented method the group showed interest and supported further development.

Part 4: Guidance documents for reporting IATA and IATA for skin sensitisation

8. Task Force on Hazard Assessment (TFHA) and skin sensitisation activities

20. Joop De Knecht (Secretariat) updated the Expert Group on the activities of the Task Force for Hazard Assessment (TFHA) related to Integrated Approaches to Testing and Assessment (IATA) and their application. He presented the scope of 2 draft documents currently under development and discussion by the TFHA: a Guidance Document on the reporting of IATA and a Guidance Document on the reporting of structured approaches to data integration and individual information sources used within IATA for skin sensitisation. The objective of developing these documents is to provide a consistent approach to the documentation of IATA, which is a preliminary step towards harmonisation. It was noted that a lot of strategies are currently proposed and questioned if the objective is to tend towards a final one with time. It was indicated that the objective is to provide tools for assessment but today it is hardly possible to say if one strategy is better than the other.

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分担研究報告書

データシートでの毒性判定結果提示のための平均値の比の95％信頼区間の計算の検討

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研究要旨

[*背景と目的*]　化学物質免疫毒性評価系として modified MITA が構築されつつある。modified MITA のバリデーション研究を行うにあたり、プロトコルに適したデータシートを作成する必要がある。試験施設の実験者がデータシートにデータを入力した段階で判定結果を知る必要が望ましい。毒性判定結果を平均値の比の 95%信頼区間を用いる場合、Excel の関数である t.inv 関数ではうまく結果を返すことができないことがわかっている。本研究では、t.inv 関数を用いずに山内の近似式として知られる近似式によって適切に平均値の比の 95%信頼区間を得ることができるかどうかを検討した。

[*方法*]　これまでに実施された計 4168 の実データを用い、山内の近似式による 97.5%点と比の 95%信頼区間の下限の値を統計解析ソフト R で算出したこれらの値と比較することを行った。

[*結果*]　検討に用いた実データの小数自由度が 3~6 の範囲であった。この範囲においてパーセント点も 95%区間の下限も R と山内の近似のどちらもほぼ同様な値を取っていることがわかった。

[*結論*]　Excel によるデータシートで平均値の比の 95%信頼区間を算出する際には、Excel の t 分布のパーセント点を計算する関数である t.inv 関数を用いるのではなく、山内の近似式である(1)式によってパーセント点を計算し、平均値の比の 95%信頼区間を得ればよい。

A. *研究目的*

Multi-ImmunoToxicity assay (MITA)は化学物質免疫毒性評価系として開発された試験法である。現在この試験法の改良がなされ IL-8 Luc assay を加えた modified MITA が構築されている。今後国内外から免疫毒性の専門家を招き、MITA の科学的意義、作成した adverse outcome pathway ならびに試験法プロトコルの妥当性などについて議論する予定である。本研究班では、modified MITA を用いた IL-2 転写活性抑制を指標とした T 細胞の分化異常誘導化学物質評価系と、IL-8 転写活性増強を指標とした気道刺激性物質評価系による試験法ガイドラインをめざしており、その目的のために多施設のバリデーション研究を計画する必要がある。

多施設バリデーション研究では、施設内/施設間再現性の評価ならびに関連性の評価が必要となる。その際、各試験実施施設に被験物質が送付され、これらの物質を用いて提案されたプロトコルに基づき実施された試験の結果が試験法のプロトコルに沿って作成されたデータシートに入力される。最終的な施設内/施設間再現性や関連性の評価は、

入力されたデータは解析施設に集められ、作成されたデータセットの下で行われる。

ところで提案される試験法は複数の繰り返し実験の結果で判定されることが多い。modified MITA においてもこの試験法を構成する一つである IL-8 Luc assay は複数の実験結果によって毒性の判定が行われる。IL-8 Luc assay は 4 回の実験結果の中で 2 回の陽性結果が得られれば毒性ありと判定することとなっている。このことはすべての被験物質について 4 回の実験を行うことをしなくても最低 2 回の実験結果で毒性を判定できる場合があることを意味する。必要最小限の実験でバリデーション研究を行うことができるならばコスト削減や期間短縮が期待できる。しかし、そのためには試験実施施設における実験実施者が、各実験の実験データをデータシートに入力した段階で実験結果が陽性であるのか陰性であるのかを知る必要がある。つまり、入力したデータシートは実験結果が提示されるように設計する必要がある。

データシートは多施設で試験を行うどのような施設でも簡便に扱うことができ、後の試験法の普及を考えると特別なソフトウエアを必要としないことが望ましいであろう。我々は Microsoft 社の Excel が広く普及している現状を考慮し、Excel を用いたデータシートを作成してきた。modified MITA の場合もその予定である。

毒性試験法はある被験物質について複数の濃度とその反応の関係で毒性を評価することが多い。ある 1 回の実験において、被験物質がの濃度が 0 である反応の測定値の平均値と特定の濃度の反応の測定値の平均値の比の大きさで陽性/陰性の判断を行うことが多く、modified MITA の場合も例外ではない。2 つの平均値の比の値に基づく判定では、陽性か陰性かの判定を行うための基準値の大きさは試験系が開発される過程で決められることが多い。一方で、比を算出する際に用いた個々の測定値のばらつきを考慮して、統計的な差によって判定することも可能であり、これは 95%信頼区間を用いることで行うことができる。

平均値の比の 95%信頼区間には、t 分布の 97.5%点が必要となる。[1] t 分布の 97.5%値は自由度と呼ばれるパラメータの関数であるため、自由度を与える必要がある。平均値の比の場合、比を構成するそれぞれの平均値を得るために用いたデータで得られるそれぞれの分散が等しいと仮定を置く場合には、自由度は整数となるが、そのような仮定ができない場合は、小数自由度を求めて使うことなる。[2] 毒性試験の場合、被験物質の濃度が 0 の測定値のばらつきは、特定での測定値のばらつきより小さくなることが観察されることが多いため、分散が等しいという仮定を置くことは避けることが望ましいであろう。つまり、小数自由度を用いた t 分布の 97.5%点が必要となる。ところが、Excel に組み込まれている t 分のパーセント点を計算する関数である t.inv 関数は、小数自由度を適切に反映しておらず、小数の値を入力しても整数に切り上げた自由度でのパーセント点を返すものになっている。先に記述したよう、データシートは Excel を用いることが望ましいとなると、小数自由度に対応した平均値の比の 95%信頼区間をシート上で計算できる必要がある。通常の統計ソフトに導入されているパーセント点の計算方法は公開されていないため知ることができない。一方、t 分布のパーセント点の近似式として山内の近似式として知られる方法がある。[3] 山内の近似式は Excel に組み込むことができる程度に簡便な近似式である。

そこで、本研究ではすでに研究結果が得られている modified MITA を構成する一つの試験法である IL-8 Luc assay のデータを用いて、山内の近似式により算出される 97.5%点と 95%信頼区間の下限について、統計解析ソフトである R で計算され

る結果の比較を行い、山内の近似式のデータシートへの適用可能性を検討することを目的とした。

B. *研究方法*

B.1. *検討に用いたデータ*

これまでに IL-8 Luc assay は、バリデーション研究を通して得られた 379 実験分データを用いた。この試験は 0 濃度と 11 の濃度の試験からなるため、計 4168 のパーセントが得られることになる。

B.2. *山内の近似式*

自由度 ν の t 分布の 97.5%点を $t_{0.975}(\nu)$ とすると、山内の近似式は $t_{0.975}(\nu)$ を

$$t_{0.975}(\nu) \approx u + \frac{y_1(u)}{\nu} + \frac{y_2(u)}{\nu^2} + \cdots + \frac{y_5(u)}{\nu^5} \qquad (1)$$

で近似する。ただし $y_1(u) = (u^3 + u)/4$,

$y_2(u) = (5u^5 + 16u^3 + 3u)/96$,

$y_3(u) = (3u^7 + 19u^5 + 17u^3 - 15u)/384$,

$y_4(u) = (79u^9 + 776u^7 + 1482u^5 - 1920u^3 - 945u)/92160$,

$y_5(u) = (27u^{11} + 339u^9 + 930u^7 - 1782u^5 - 756u^3 + 17955u)/368640$,

$u = 1.96$ である。[3)]

B.3. *パーセント点と 95%信頼区間の下限の比較*

1468 の比のデータについて、上記の式で得られる山内の近似式により算出される 97.5%点の値と 95%信頼区間の下限の値を、統計解析ソフトである R で計算されるそれぞれの値と比較した。

C. *研究結果*

C.1. *小数自由度の要約*

検討に用いた 1468 の比のデータについての要約統計量を表 1 に示す。

表 1　小数自由度の要約統計量

サイズ(n)	平均値	標準偏差	最小値	中央値	最大値
4169	4.49	1.12	3.00	4.58	6.00

自由度の範囲は 3～6 の範囲にあることがわかる。

C.2. *97.5%点の比較結果*

検討に用いた 1468 の比のデータについて R により得られる 97.5%を横軸に、山内の近似式により得られる 97.5%を縦軸にとった散布図を図 1 に示す。

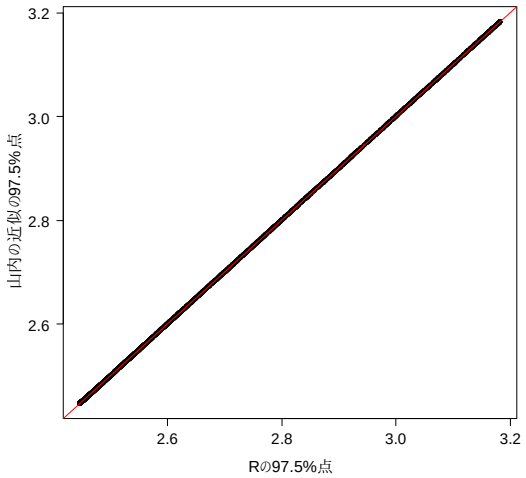

図 1　R と山内の近似の 97.5%点

C.3. *95%信頼区間の下限の比較結果*

検討に用いた 1468 の比のデータについて R により得られる 95%信頼区間の下限を横軸に、山内の近似式により得られる 95%信頼区間の下限を縦軸にとった散布図を図 2 に示す。図において点線で示した参照線は、それぞれ 1 の値のところであり、これは 95%信頼区間の下限を用いたときの統計的有意差の基準となる値である。

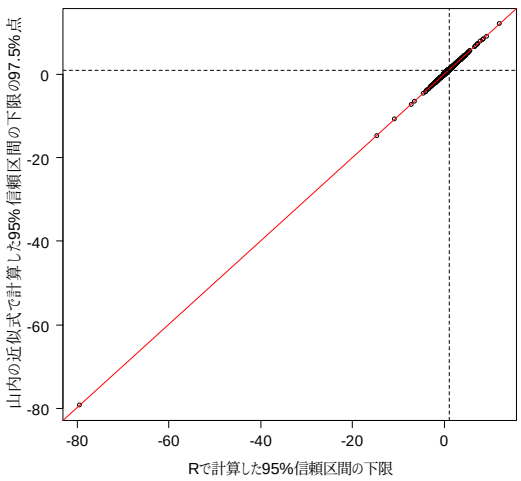

図 2　R と山内の近似の 95%信頼区間の下限

D. *考察*

表 1 では 1468 の比のデータの自由度の中央値は 4.58 である。この値を用いて Excel2010 の t.inv 関数で、97.5%点を計算するとエラーもなく 2.776445 という値が返される。しかし、この値は自由度を 4 として t.inv 関数で計算しても同じ値である 2.776445 が返されてしまう。一方、R のパーセント点を計算する関数 qt を用いた場合、自由度が 4.58 の場合には 2.643129 が、自由度が 4 の場合には 2.776445 が返され、小数自由度に対して適切な値を返していることがわかる。

図 1 よりパーセント点は R と山内の近似のどちらもほぼ同様な値を取っていることがわかる。また、図 2 より 95%区間の下限も R と山内の近似のどちらもほぼ同様な値を取っていることがわかる。よって、山内の近似式は統計解析ソフト R を用いて解析するのと同様の結果を得ることができることがわかった。

よって、Excel でデータシートを構築する場合には、Excel の t 分布のパーセント点を計算関数である t.inv 関数を用いるのではなく、山内の近似式である(1)式によって計算し、比の 95%信頼区間を得ればよい。この結果を今後 modified MITA のバリデーション研究で行う際に作成するデータシートを反映させることにする。

Excel は広く普及しているソフトウエアである。R はフリーのソフトウエアであるという利点があるものの、実験実施者にとって統計ソフトは馴染みがあるソフトウエアではないため、R での解析を実験実施者に求めるべきではないであろう。また、Excel の t.inv 関数の問題は Excel のバージョンが更新されることで問題は解消されるかもしれない。しかしながら、どのような実験施設も常に最新の Excel を有し、利用しているとは考えにくい。

E. *結論*

以上より、Excel によるデータシートで平均値の比の 95%信頼区間を算出する際には、Excel の t 分布のパーセント点を計算する関数である t.inv 関数を用いるのではなく、山内の近似式である(1)式によってパーセント点を計算し、平均値の比の 95%信頼区間を得ればよい。

謝辞：本検討を進めるにあたりデータの整理を協力してくれた同志社大学文化情報学部の丸谷あおいさんに感謝いたします。

F. *健康危険情報*

なし。

G. *研究発表*

なし。

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（化学リスク研究事業）
免疫毒性評価試験法Multi-ImmunoTox assayの国際validationへ向けての検討
分担研究報告書

化学物質のMITAによる解析、validation、プロトコール作成

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研究要旨
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」にて開発した新たな*in vitro*免疫毒性評価試験法（Multi-ImmunoTox assay：MITA）について現時点で得られたdata setおよび相場によるIL-2転写活性抑制を中心とした免疫毒性AOPをもとに国際バリデーション用の試験法プロトコール、データシート、記録用紙を作成した。施設間試験の実施者に対し試験法の説明会を実施し技術移転を図った。国際バリデーションに先立ち技術移転性を確認するため5物質でのトレーニングを行い、プロトコールの問題点を改良しPhase I試験に臨む予定である。

キーワード：試験法プロトコール、技術移転性、バリデーション

A．研究目的

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」にて開発した新たな *in vitro* 免疫毒性評価試験法（Multi-ImmunoTox assay：MITA）のOECD（Organisation for Economic Co-operation and Development）における試験法ガイドライン（Test Guideline：TG）化を目的とし、試験法プロトコールを作成し国際バリデーションの準備を行う。

B．研究方法

以下の方法によりIL-2およびIFN-γプロモーター活性の測定を行った。ヒトTリンパ芽球性白血病由来細胞株JurkatにIL-2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）、IFN-γプロモーターに制御されたSLOルシフェラーゼ遺伝子（橙色に発色）、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#2H4細胞を1ウェル当たり2×10^5個、黒色の96-well プレート(Greiner bio-one)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25nM PMAと1μM Ioの混合物(PMA/Io)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、SLO、 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、2枚の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)、SLO-luciferase activity (SLO-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、SLO-LAをSLR-LAで除することによりそれぞれnormalized SLG-luciferase activity(nSLG-LA), normalized SLO-luciferase activity(nSLO-LA)を算出した。さらに以下の式に%suppression抑制率を計算した。

% suppression = (1-薬物存在下でのnSLG-LAまたはnSLO-LA/薬物非存在下でのnSLG-LAまたはnSLO-LA) X 100

各実験において得られた結果は、一元配置分散分析を行い、その後Dunnett検定により有意な抑制効果，増強効果があるか否かを検討した。しかし、この実験を3回繰り返し検討すると、3回の実験結果が必ずしも一致していない薬剤が存在した。そこで、一致が見られなかった薬剤に関しては、3回の繰り返し実験の結果のなかから%suppressionの絶対値（免疫抑制物質に関しては正の値、増強物質に関しては負の値となる）が最も大きい値を選びStudent's t-testを行い、そこで統計的有意差の得られた場合、その結果を薬剤の最終的判定結果とした[1]。

C．結果

C-1. 試験法プロトコール、データシート、記録用紙の作成

現時点で得られたdata setおよび相場によるIL-2転写活性抑制を中心とした免疫毒性AOPを参考とし、IL-2，IFN-γレポーター細胞である#2H4細胞を用いた試験法プロトコール、Multi-Immuno Tox Assay protocol ver. 008.1Eを作成し、国際バリデーションに向け英訳した（添付文書１）。データ入力、結果表示用にエクセルファイルをベースとしたdata sheet、Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 006を作成した（添付文書２）。さらに参加施設用の記録用紙を作成し各施設に配布した（添付文書３）。

C-2. 試験法の説明会を実施

初めて MITA を行う参加施設の実施者を対象とし当研究室にて説明会を 2015 年 8 月と 2016 年 2 月の計２回開催した。当研究室において参加者の手技により PMA/Io に対する#2H4 細胞の反応および陽性コントロール化学物質による抑制が認められることを確認した（図１）。

C-3. Phase 0 試験の実施

MITA の国際バリデーション Phase I 試験に先立ち技術移転性を確認するため Phase 0 試験用に以下の化学物質を参加 3 施設に送付した。(2-Aminoanthracene, CH_3HgCl, Chloroquine diphosphate salt, Citral, Dexamethasone) 現在、これらの化学物質を３回ずつアッセイする Phase 0 試験を施行中である。

D. 考察

現時点でのクライテリアでは、低濃度で亢進し、高濃度で抑制が見られる化学物質については最終的な結果がばらつくことが予測される。今後Phase 0の結果を参照としPhase Iへ向けたクライテリアの改変を検討する。

E．結論

国際バリデーション用の試験法プロトコール、データシート、記録用紙を作成した。施設間試験の実施者に対し試験法の説明会を実施し技術移転を図った。国際バリデーションに先立ち技術移転性を確認するため5物質でのトレーニングを行い、プロトコールの問題点を改良しPhase I試験に臨む予定である。

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1. Kimura, Y., Fujimura, C., Ito, Y., Takahashi,

T., Aiba, S., 2014. Evaluation of the Multi-ImmunoTox Assay composed of 3 human cytokine reporter cells by examining immunological effects of drugs. Toxicol In Vitro 28, 759-768.

F. **添付文書**

1) Multi-Immuno Tox Assay protocol ver. 008.1E

2) Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 006

3）Multi-ImmunoTox Assay **記録用紙** Ver. 001

G. 研究発表

1. 論文発表

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２．学会発表

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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

2. 実用新案登録

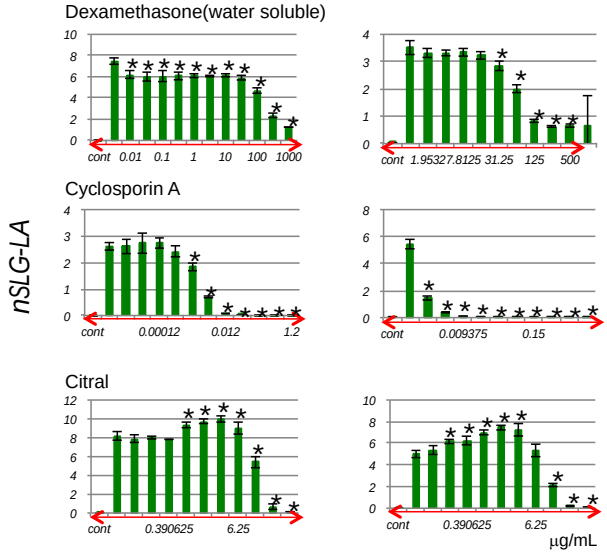
なし

3.その他

なし

図１　説明会データ

東北大学データ　　説明会参加者データ



Multi-Immuno Tox Assay protocol ver. 008.1E
Feb. 2, 2016

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

1. Introduction

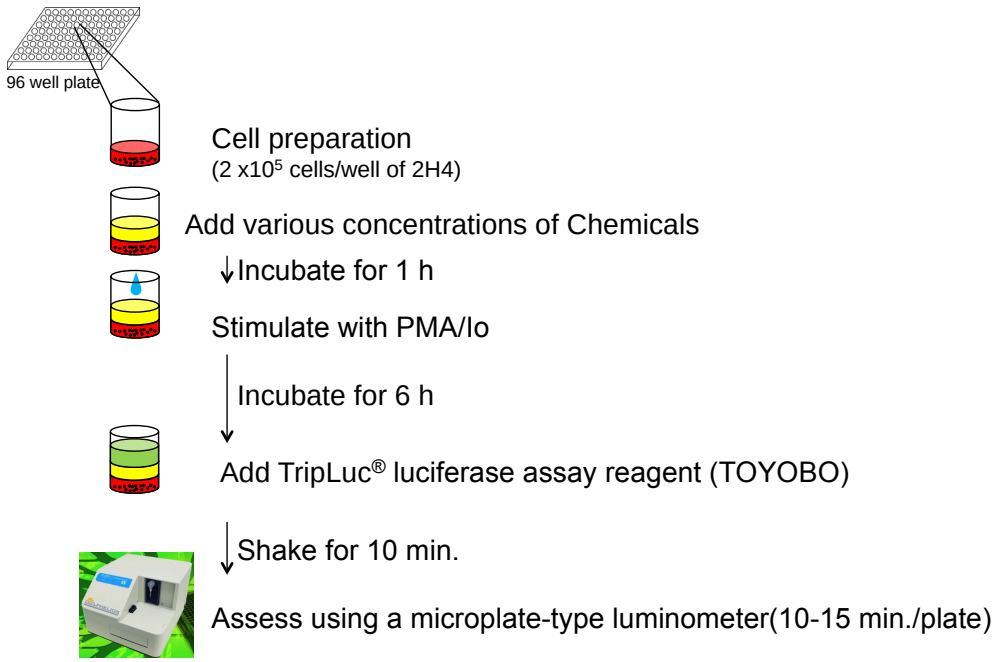
This protocol describes how to maintain the cells, how to prepare the test chemicals, and how to measure the luciferase activity of #2H4 cells transfected with 3 luciferase genes, stable luciferase green (SLG), stable luciferase orange (SLO) and stable luciferase red (SLR), under the control of IL-2,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

Figure 1

Assay design (2 chemicals per one plate)

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

PMA/Io or LPS



2. Materials

2-1 Cells

- #2H4 (IL2-SLG、IFNγ-SLO、G3PDH-SLR)

  The human acute T lymphoblastic leukemia cell line Jurkat was obtained from the American Type Culture Collection (Manassas, VA, USA). A Jurkat-derived IL-2 and IFNγ reporter cell line, #2H4, that harbors the SLG, SLO and SLR luciferase genes under the control of the IL-2, IFNγ and GAPDH promoters, respectively, was established by Tsuruga Institute of Biotechnology, TOYOBO Co. Ltd.

  (Saito R. et al. Nickel differentially regulates NFAT and NF-κB activation in T cell signaling *Toxicology and Applied Pharmacology,* 254, 245–255, 2011)

2-2 Reagents and equipment

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

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

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

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

2-2-3 For measurement of the luciferase activity

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

2-2-4 Expendable supplies

- T-75 flask tissue culture treated (e.g., Corning Cat#353136)
- 96 well μclear black plate (flat-bottom, for measurement of the luciferase activity, e.g. Greiner Bio-one Cat#655090)
- 96 well clear plate (round-bottom, for preparation of chemicals and stimulants)
- 96 well assay block, 2 mL (e.g., Costar Cat#3960)
- 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	1 mM	25 nM
DMSO	Sigma #D5789		

Dissolve 1 mg PMA using DMSO 1338.5 μL, dispend at 10 μL/tube and store at freezer at -30°C. Use these stocks within 6 month after dissolution.

2-4-2 Ionomycin

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

Dissolve 1mg Ionomycin using ethanol 1621 μL, dispend at 30 μL/tube and store at freezer at -30°C. Use these stocks within 6 month 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 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 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 chemical are required, but to have some leeway, 3.0 x 10^7 cells for two chemical should be prepared), centrifuge the tube at 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

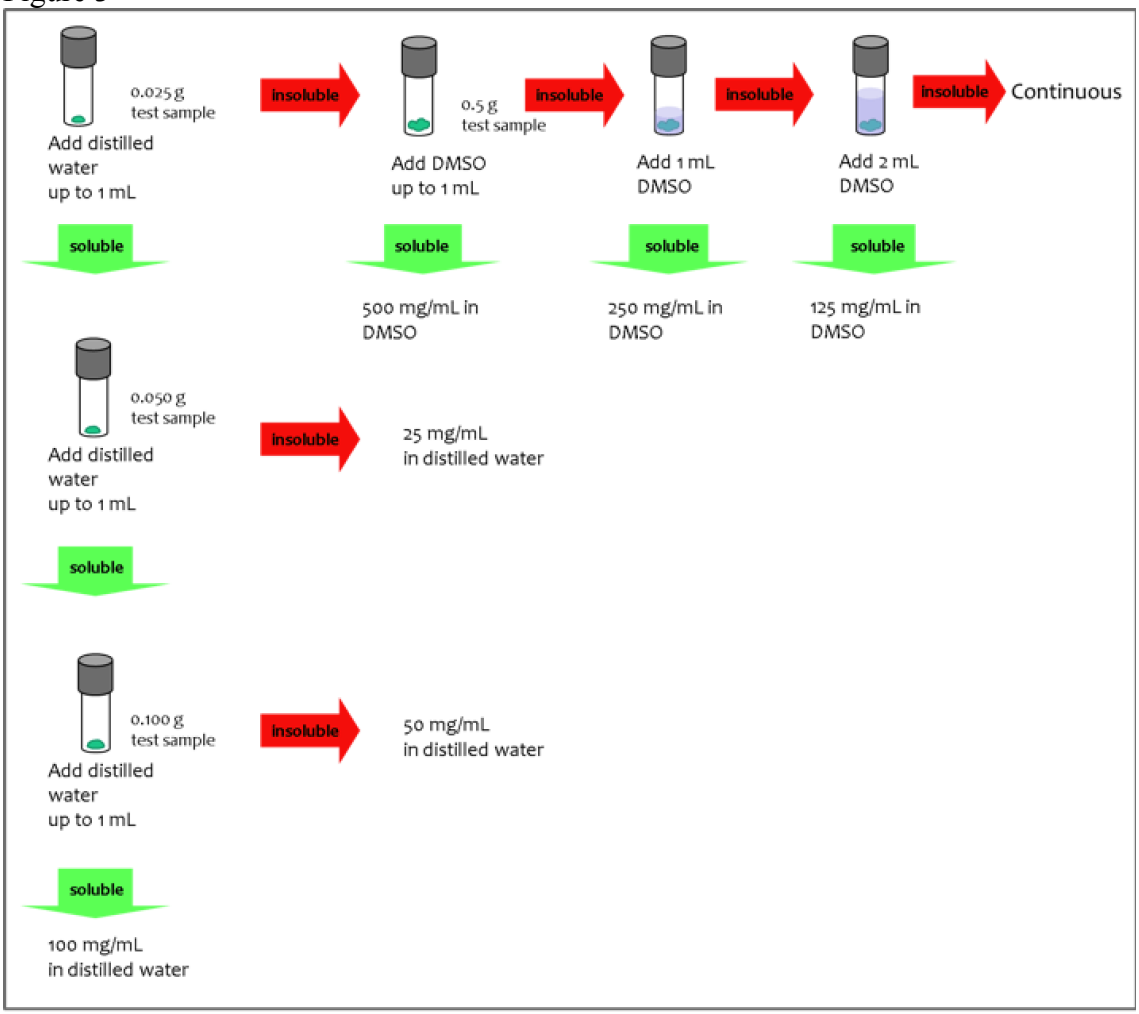
5. Preparation of chemicals and cell treatment with chemicals

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. The chemical should be used within 4 hours after being dissolved in distilled water or DMSO.

Figure 3


0.025 g
test sample
Add distilled
water
up to 1 mL
insoluble
0.5 g
test sample
Add DMSO
up to 1 mL
insoluble
Add 1 mL
DMSO
insoluble
Add 2 mL
DMSO
insoluble
Continuous
soluble
500 mg/mL in
DMSO
soluble
250 mg/mL in
DMSO
soluble
125 mg/mL in
DMSO
soluble
0.050 g
test sample
Add distilled
water
up to 1 mL
insoluble
25 mg/mL
in distilled water
soluble
0.100 g
test sample
Add distilled
water
up to 1 mL
insoluble
50 mg/mL
in distilled water
soluble
100 mg/mL
in distilled water

5-2 When the chemical is prepared as 25, 50 or 100 mg/mL in distilled water

If the chemical is prepared at 25 or 50 mg/mL in distilled water, use the prepared concentration instead of the 100 mg/mL distilled water solution.

5-2-1 Arrangement of chemicals and vehicle

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

5-2-2 Serial dilution

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

Figure 4

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

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

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

5-2-3 2 step dilution

Add 20 µL of the diluted chemical to 480 µL of the B medium prepared in the assay block. And add 50 µL to #2H4 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Shake the plate with a plateshaker, and incubate in a CO_2 incubator for 1 hour (37°C, 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 500 mg/mL 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. Shake the plate with a plateshaker and incubate in a CO_2 incubator for 1 hour (37°C, 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

- 1 mM PMA stock
- 1 mM Ionomycin stock
- B medium
- Ethanol

6-2 Preparation of 100 μM PMA

Dilute 1mM PMA stock with the B medium as follows (10 times, final concentration is 100 μM).

1 mM PMA	B medium	Total	final concentration
10 μL	90 μL	100 μL	100 μM

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

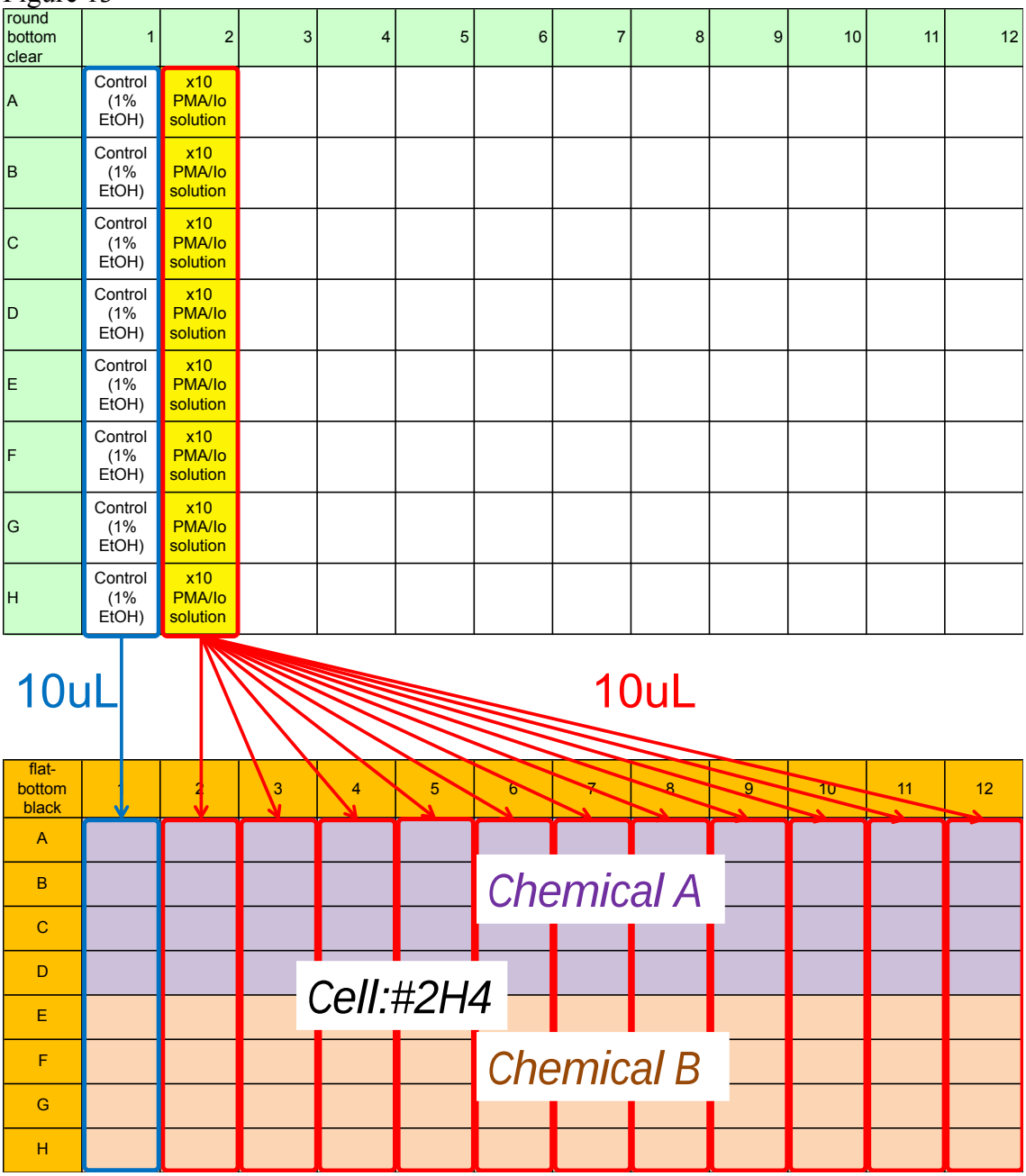
Dilute ethanol, 1mM ionomycin and 100 μM PMA with the B medium to prepare control or x10 PMA/ionomycin solution. Add the control to well #A1-#H1 of the 96 well clear plate (round bottom), and add x10 PMA/ionomycin solution to wells #A2-#H2 of the 96 well clear plate (round bottom).

	B medium	1 mM Ionomycin	100 μM PMA	Ethanol	Total
Control	990 μL	-		10 μL	1000 μL
x10 PMA/ionomycin solution	2370 μL	24 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. Shake the plate with a plateshaker and incubate in a CO_2 incubator for 6 hour (37°C, 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	50 mg/mL	50 mg/mL	1 mg/mL
Distilled water	GIBCO Cat#10977-015			

Dissolve 100 mg of Dexamethasone-water soluble with distilled water 2000 μL, 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	12 mg/mL	1 mg/mL	1 μg/mL
DMSO	Sigma #D5789			

Dissolve 5 mg of cyclosporine A with DMSO 416 μL, dispend at 10 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 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、12 mg/mL cyclosporine A stock 10 µl + DMSO 110 µL to #A5, distilled water 50 µl to #B1 and #B2, 50 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

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A				DMSO 50uL	CyA 12 mg/mL stock 10uL + DMSO 110uL							
B	Distilled water 50uL	Distilled water 50uL	DEX 50 mg/mL stock 50uL	B medium 180uL	B medium 180uL							
C												
D												
E												
F												
G												
H												

20uL

↓

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A				DMSO 30uL	CyA 1 mg/mL in DMSO 100uL							
B	Distilled water 50uL	Distilled water 50uL	DEX 50 mg/mL stock 50uL	DMSO 10% in B medium 200uL	CyA 100ug/mL DMSO 10% in B medium 200 uL							
C												
D												
E												
F												
G												
H												

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. Shake the plate with a plateshaker and incubate in a CO_2 incubator for 1 hour (37°C, 5%). (cf. Figure 16-18)

Figure 16

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A				DMSO 30uL	CyA 1 mg/mL in DMSO 100uL							
B	Distilled water 50uL	Distilled water 50uL	DEX 50 mg/mL stock 50uL	DMSO 10% in B medium 200uL	CyA 100ug/mL DMSO 10% in B medium 200 uL							
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 980uL	B medium 980uL							
B												
C												
D												
E												
F												
G												
H												

Figure 17

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 500uL	B medium 500uL	DEX 2mg/mL B medium 500uL	DMSO 0.2% B medium 1000uL	CyA 2ug/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 1mg/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 1ug/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 1mg/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 1ug/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 1mg/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 1ug/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 1mg/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 1ug/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. Shake the plate with a plateshaker and incubate in a CO_2 incubator for 6 hour (37°C, 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 1mg/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 1ug/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 1mg/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 1ug/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 1mg/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 1ug/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 1mg/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 1ug/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	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

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 **添付**

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

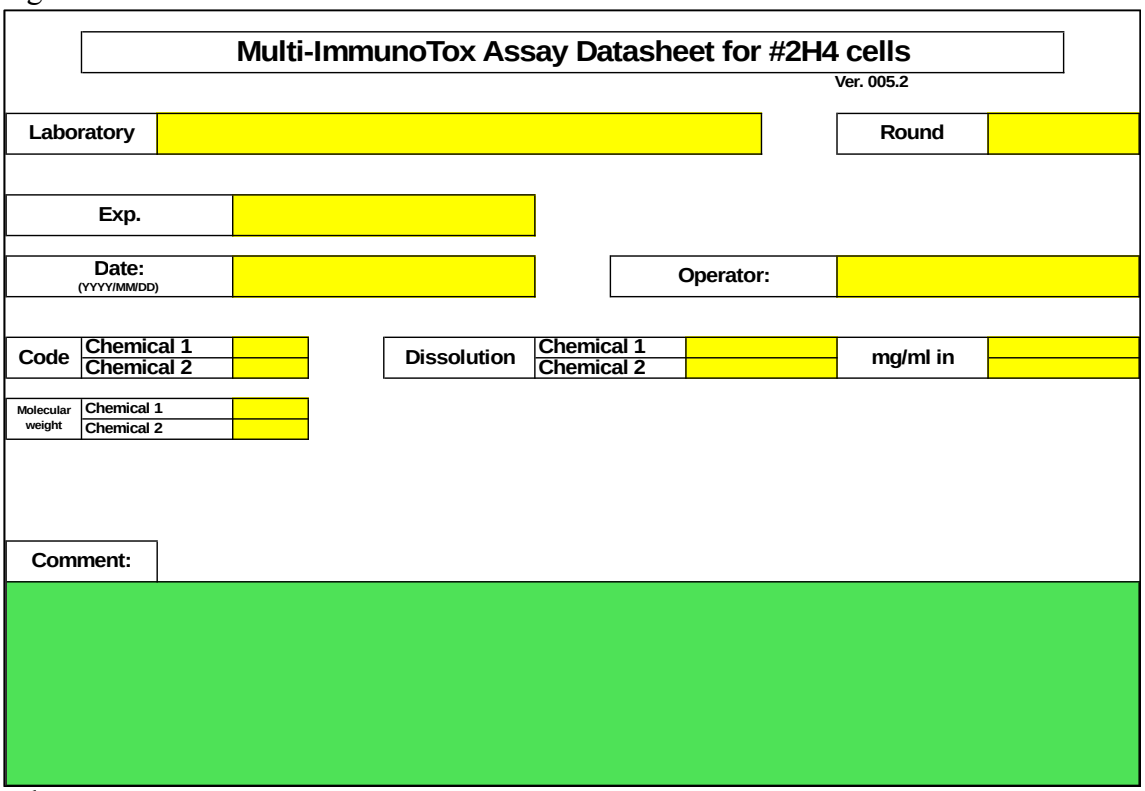
Please reffer Appendix 1 for the principal of measurement of luciferase activity.

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.

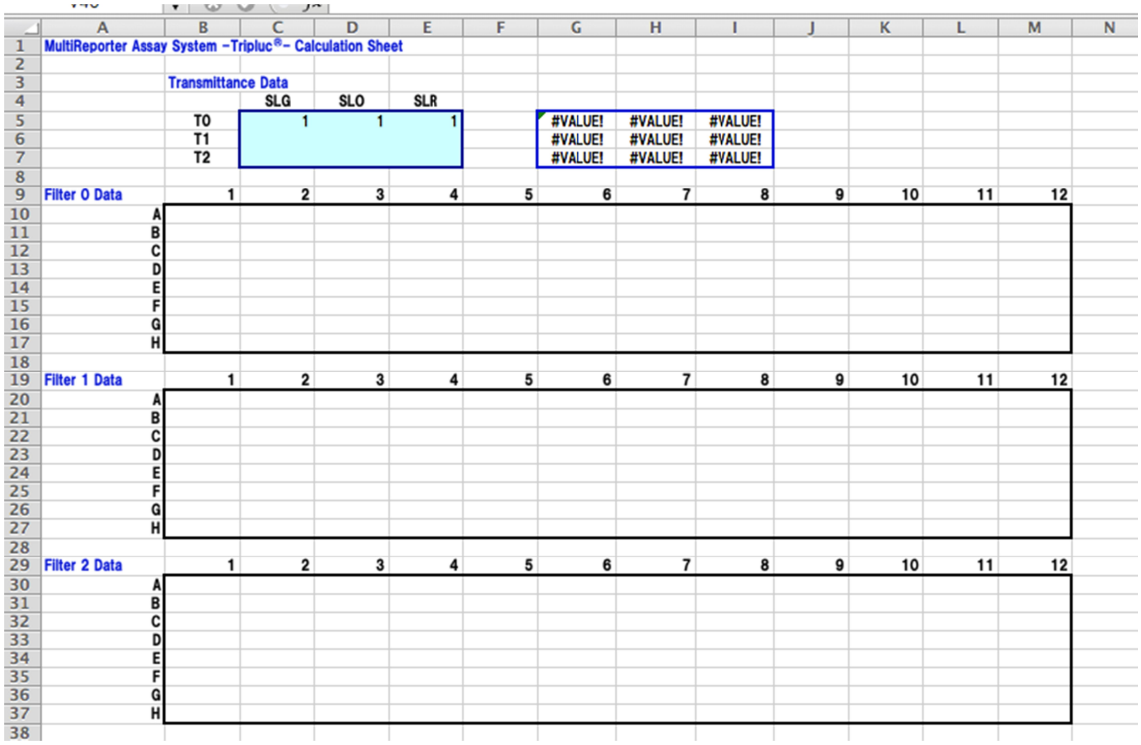
1st. Add the information regarding the name of laboratory, the round of experiments if multiple experimental sets are performed, the experiment number, date, the operator, chemical codes, dissolved in distilled water or DMSO, the prepared concentration, molecular weight of the chemicals and comments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

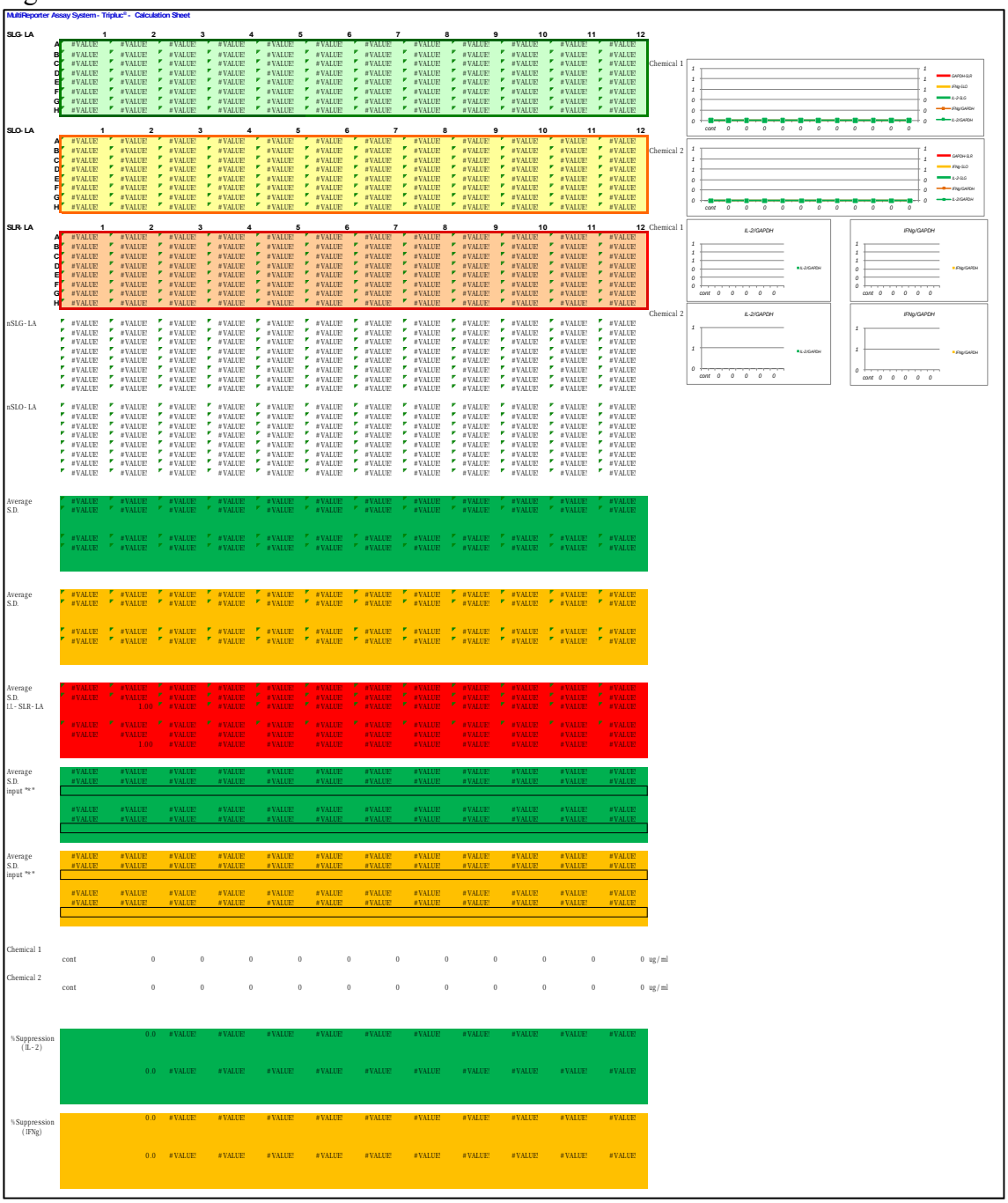
Figure 25 “Data Input” sheet of the data sheet



	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



10. Data analysis

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

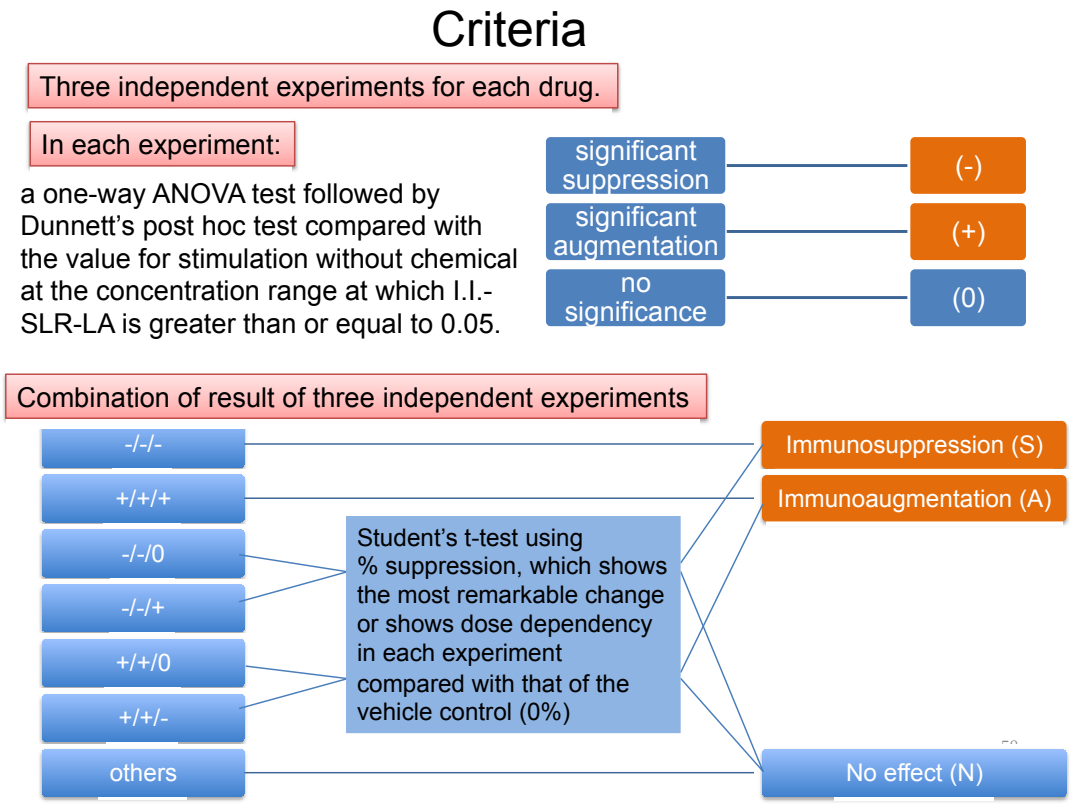
- SLG-luciferase activity (SLG-LA)：Luciferase activity of stable luciferase green (Under the control of IL-2 promoter)
- SLO-luciferase activity (SLO-LA)：Luciferase activity of stable luciferase orange (Under the control of IFN-□ promoter)
- SLR-luciferase activity (SLR-LA)：Luciferase activity of stable 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

Conduct three independent experiments for each chemical.
Determined in each experiment whether the chemicals induce statistically significant suppression or augmentation or no significant effects by a one-way ANOVA test followed by Dunnett's post hoc test at the concentration at which I.I.-SLR-LA is greater than or equal to 0.05.

If chemicals showed statistically significant immunosuppression or immunostimulation in three experiments, they were judged as immunosuppressive or immunostimulatory chemicals, respectively. If chemicals showed statistically significant immunosuppression or immunostimulation in only two independent experiments, they were judged as potential immunosuppressive or immunostimulatory drugs, respectively. If not, they were judged as "no effect". Then, for potential immunosuppressive or immunostimulatory chemicals, we selected their percent suppression or percent augmentation (negative percent suppression) in three experiments that showed the most remarkable change and that showed dose dependency and statistically compared suppression or augmentation of chemicals with that of vehicle control in three independent experiments by the Student's t-test. Only when chemicals demonstrated statistical significance, they were judged as immunosuppressive or immunostimulatory, respectively.

Figure 27


Criteria
Three independent experiments for each drug.
In each experiment:
a one-way ANOVA test followed by Dunnett's post hoc test compared with the value for stimulation without chemical at the concentration range at which I.I.-SLR-LA is greater than or equal to 0.05.
significant suppression
significant augmentation
no significance
(-)
(+)
(0)
Combination of result of three independent experiments
-/-/-
+/+/+
-/-/0
-/-/+
+/+/0
+/+/-
others
Student's t-test using % suppression, which shows the most remarkable change or shows dose dependency in each experiment compared with that of the vehicle control (0%)
Immunosuppression (S)
Immunoaugmentation (A)
No effect (N)

11. Update record

Ver. 008.1J 2016.2.2 distribution
Changes after the VMT meeting

Ver. 008.0J 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 □L□□□□□ to 10 □L□□□□□)
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　Principal 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.

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

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

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

5-1-2-2 Bioluminescence measurement

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

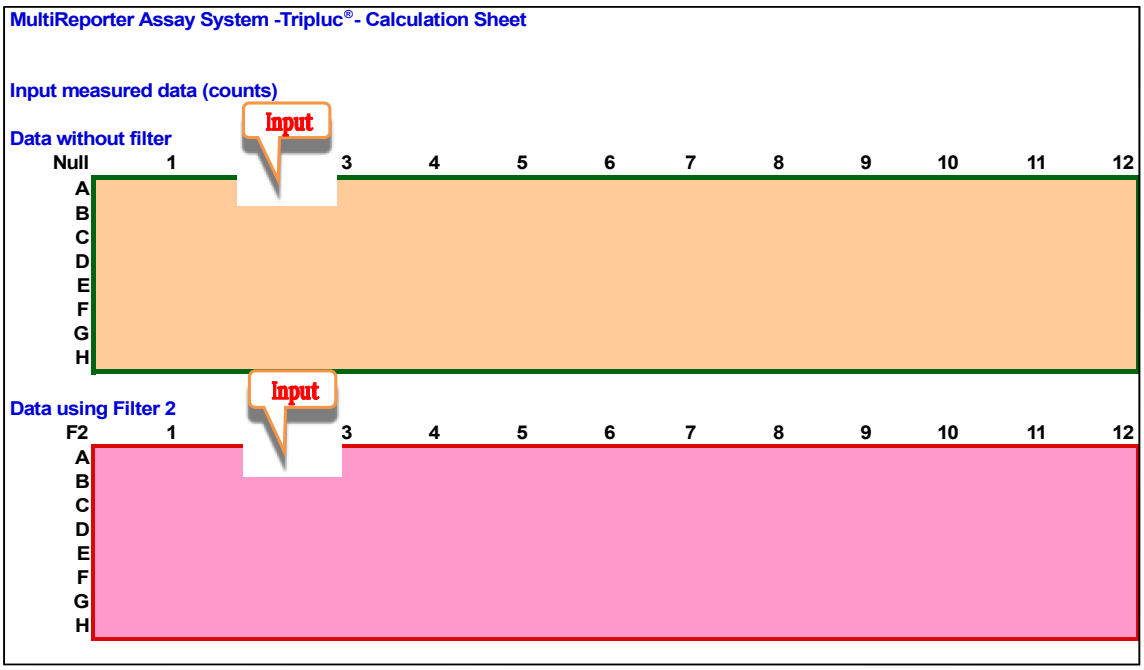
Figure 28

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 29



Record all the results for quality control.

5-2 Quality control of equipment

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

5-2-1 Light source

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

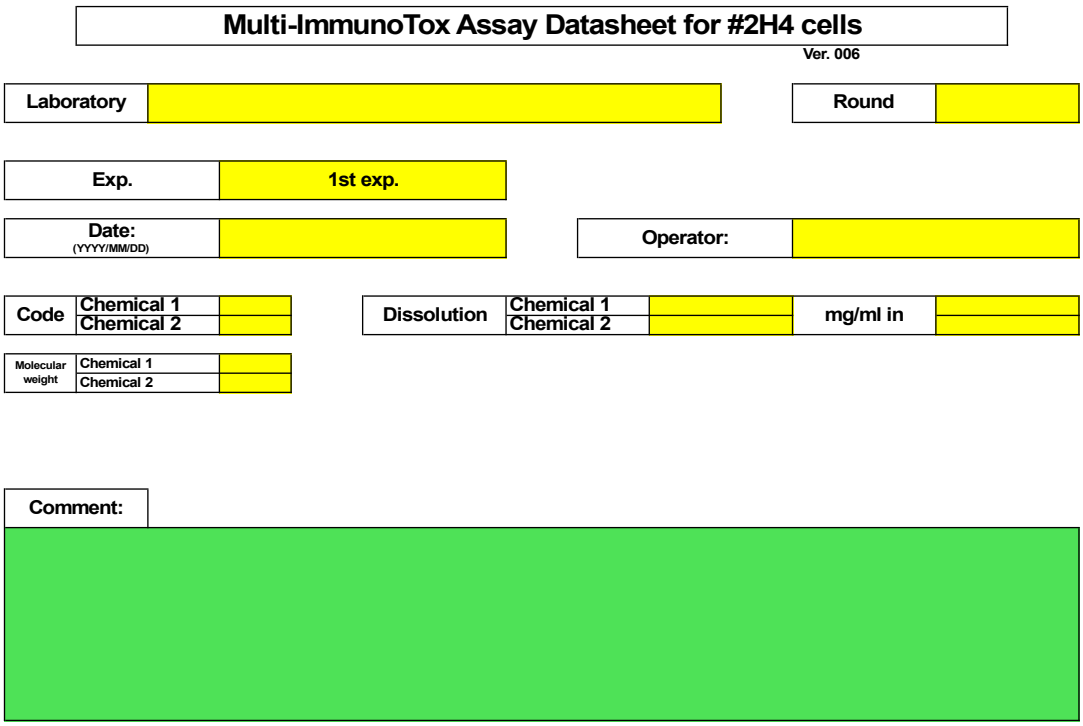
TRIANT® (wSL-0001) by ATTO (Tokyo, Japan)

L12367 by Hamamatsu Photonics (Shizuoka, Japan)

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

1) Start luminometer 30 min before starting the measurement for stabilization of the photomultiplier.
2) Start LED plate and select “PMT” mode.
3) Select three-color (BRG) mode and adjust light intensity to 1/10 (10E-1).
4) Place the LED plate into the luminometer. Light intensity is measured for 3 sec each in the absence (F0) and presence (F2) of the optical filter.
5) Blue, green, and red LEDs are located at the position of #F6, #E6, and #D6, respectively. Copy the collected data of each position to the appropriate area on Sheet “LED” in the excel file of the data sheet.
6) Check the photo-detector performance by comparing with old data of the LED plate. For quality control purpose, every collected data should be recorded.
7) LED plate data typically fluctuates up to 1.5% (σ). Disagreement to the old data should be less than 3×σ (= 4.5%).

Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 006, Face sheet



Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 006, Data imput sheet

MultiReporter Assay System -Tripluc®- Calculation Sheet

Transmittance Data

	SLG	SLO	SLR
T1	1	1	1
T2			
T3			

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

Filter 1 Data

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

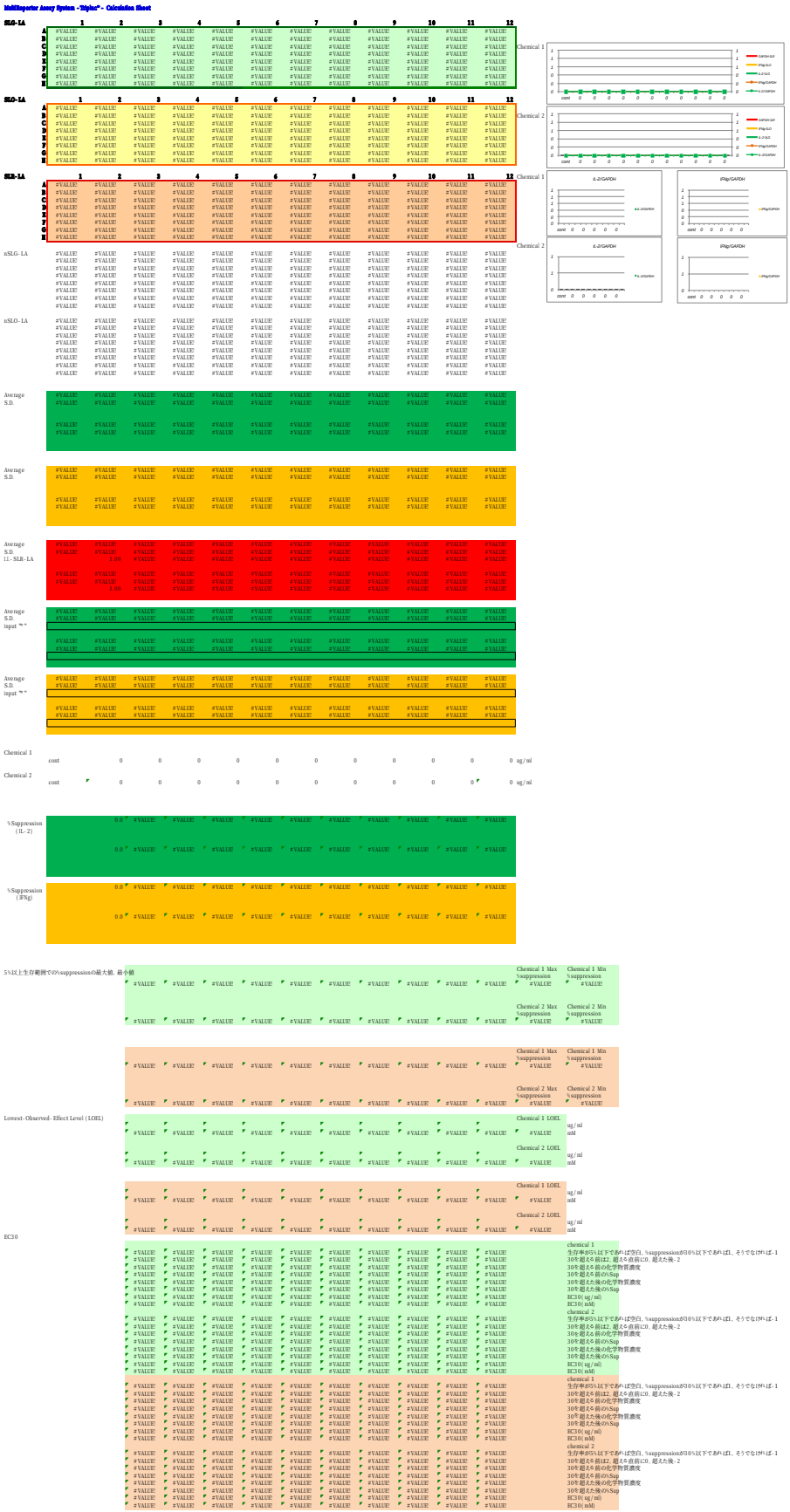
Filter 2 Data

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

Filter 3 Data

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

Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 006, Result format sheet



Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 006, Control 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!
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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!
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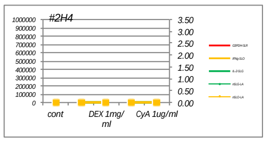

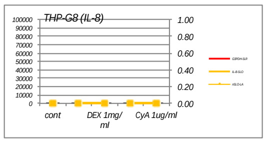

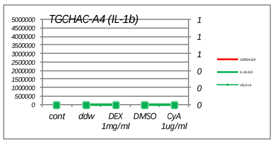

SLR-LA	1	2	3	4	5	6	7	8	9	10	11	12
A	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
B	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
C	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#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!
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nSLG-LA												
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nSLO-LA												
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	cont	ddw	DEX 1mg/ml	DMSO	CyA 1ug/ml						
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!						
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!						
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!		#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
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Average	#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!

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

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

Average	#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!

IL-2 抑制率(%)	0	0	#VALUE!	0	#VALUE!						
IL-1b 抑制率(%)	0	0	#VALUE!	0	#VALUE!						

IFN-g 抑制率(%)	0	0	#VALUE!	0	#VALUE!						
IL-8 抑制率(%)							0	0	#VALUE!	0	#VALUE!

添付文書２

Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 006, Update record

2016年2月2日 Ver.006
phase 0用

2015年11月17日 Ver.005.2
FaceSheetを追加
EC30、Lowest-Observed-Effect Level (LOEL)、Max %suppression, Min %suppressionが算出されるように改変

2014年11月26日 Ver.005.1
コントロール用のシートを修正

2014年11月17日 Ver.005
Multi-Immuno Tox Assayバリデーションプロトコール20141117 Ver.004J案の変更に合わせ、コントロール等のプレート配置を変更
%suppressionのグラフを削除

2013/11/6 Ver.004
統計処理用のシートを追加
Result Format2シートの化学物質の濃度表示を修正（公比2になるように）

未配布 Ver.003.1
グラフの大きさを縮小（パワーポイントにコピーペーストしやすくするため）

2013年9月19日 Ver.003
コントロール用のシートを追加（dexamethasone, cyclosporin A）

2012年11月13日 Ver.002.1
%Suppressionのグラフを追加

2012年8月31日 Ver.002
抑制率の計算方法を変更（バックグランドの値を引き算する方法）

2012年8月28日 Ver.001

添付文書３

Multi-ImmunoTox Assay **記録用紙** Ver. 001, **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. / /						
/						
/						
/						
/						
/						
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/						
/						
/						
/						
/						

添付文書３

Multi-ImmunoTox Assay **記録用紙** Ver. 001, **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

Multi-ImmunoTox Assay **記録用紙** Ver. 001, **細胞継代シート**

3-1 #2H4培養方法

3-1-1 細胞蘇生(P1)

□ あらかじめ、#2H4用C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、#2H4用C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 (350 x *g*, 5分程度)。
□ 上清を吸引除去し、先に温めておいた#2H4用C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 選択抗生剤での培養開始(P2)

□ あらかじめ、#2H4用A培地必要量を37° C恒温槽で温めておく。
□ 細胞蘇生して3日～4日後に、選択抗生剤を入れた培養(#2H4用A培地)を開始する。
フラスコ中の細胞塊を滅菌ピペットでピペッティングしてほぐし、細胞数を計測する。
(+)/ × = ×10^4/mL－A液

□ 必要細胞量を取り、遠心して細胞を集める (350 x *g*, 5分程度)。上清を吸引除去し、先に温めておいた
#2H4用A培地15mLに3×10^5/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:

3-1-3 通常の継代培養(P3以降)

P-
□ あらかじめ、#2H4用A培地必要量を37° C恒温槽で温めておく。
□ フラスコ中の細胞塊を滅菌ピペットでピペッティングしてほぐし、細胞数を計測する。
継代細胞濃度は3×10^5/mL、継代間隔は3~4日程度で行う。
(+)/ × = ×10^4/mL－A液
□ 必要細胞量を取り、遠心して細胞を集める (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3×10^5/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、#2H4用A培地必要量を37° C恒温槽で温めておく。
□ フラスコ中の細胞塊を滅菌ピペットでピペッティングしてほぐし、細胞数を計測する。
継代細胞濃度は3×10^5/mL、継代間隔は3~4日程度で行う。
(+)/ × = ×10^4/mL－A液
□ 必要細胞量を取り、遠心して細胞を集める (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3×10^5/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、#2H4用A培地必要量を37° C恒温槽で温めておく。
□ フラスコ中の細胞塊を滅菌ピペットでピペッティングしてほぐし、細胞数を計測する。
継代細胞濃度は2×10^5/mL、継代間隔は3~4日程度で行う。
(+)/ × = ×10^4/mL－A液
□ 必要細胞量を取り、遠心して細胞を集める (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3×10^5/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、#2H4用A培地必要量を37° C恒温槽で温めておく。
□ フラスコ中の細胞塊を滅菌ピペットでピペッティングしてほぐし、細胞数を計測する。
継代細胞濃度は2×10^5/mL、継代間隔は3~4日程度で行う。
(+)/ × = ×10^4/mL－A液
□ 必要細胞量を取り、遠心して細胞を集める (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3×10^5/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:

添付文書３

Multi-ImmunoTox Assay **記録用紙** Ver. 001, **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　2.0x10^{7} 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÷(4x10^{6})）

それぞれのプレートに50 μL /wellで分注　□　（　：　）

細胞調製（コントロール(dexamethasone, cyclosporine A)用）

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

添付文書３

Multi-ImmunoTox Assay **記録用紙** Ver. 001, **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	
		↓	
50mg/mL水溶液で		□完全に溶解せず	→25mg/mL水溶液を調製
		□完全に溶解	
		↓	
100mg/mL水溶液で		□完全に溶解せず	→50mg/mL水溶液を調製
		□完全に溶解	→100mg/mL水溶液として調製を継続
500mg/mL DMSO溶液で	□完全に溶解	→500mg/mL DMSO溶液として調製を継続	
	□完全に溶解せず		
	↓		
250mg/mL DMSO溶液で	□完全に溶解	→250mg/mL DMSO溶液として調製を継続	
	□完全に溶解せず		
	↓		
125mg/mL DMSO溶液で	□完全に溶解	→125mg/mL DMSO溶液として調製を継続	
	□完全に溶解せず		
	↓		
62.5mg/mL DMSO溶液で	□完全に溶解	→62.5mg/mL DMSO溶液として調製を継続	
	□完全に溶解せず		
	↓		
31.25mg/mL DMSO溶液で	□完全に溶解	→31.25mg/mL DMSO溶液として調製を継続	
	□完全に溶解せず		
	↓		

Multi-ImmunoTox Assay 記録用紙 Ver. 001, 被試験試薬の調製②(DW)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

水溶液に調製された場合

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　　調製時間 (　:　)

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

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(PMA/ionomycin)の調製と細胞への処理

1mM PMAストックをB培地で10倍希釈し100 µM溶液を作製する。(1mM PMA 10 µL + B培地 90 µL) □

下図のようにコントロール溶液と x10 PMA/ionomycin溶液を作製する。 □

	B medium	1 mM Ionomycin	100 µM PMA	Ethanol	Total
Control	990 µL	-		10 µL	1000 µL
x10 PMA/ ionomycin solution	2370 µL	24 µL	6 µL	-	2400 µL

添加時間

コントロール溶液を#A1-#H1、x10 PMA/ionomycin溶液を#A2-#H12に10 µLずつ分注する。 □ (　:　)

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Pheliosの場合はF0、F1、F2を使用)。 □ 測定時間 (　:　)

測定中温度 (　　℃)

Multi-ImmunoTox Assay 記録用紙 Ver. 001, 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 _____ _____回目

DMSO溶液に調製された場合

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 (　:　)

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10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(PMA/ionomycin)の調製と細胞への処理

1mM PMAストックをB培地で10倍希釈し100 μM溶液を作製する。(1mM PMA 10 μL + B培地 90 μL) ☐

下図のようにコントロール溶液と x10 PMA/ionomycin溶液を作製する。 ☐

	B medium	1 mM Ionomycin	100 μM PMA	Ethanol	Total
Control	990 μL	-		10 μL	1000 μL
x10 PMA/ ionomycin solution	2370 μL	24 μL	6 μL	-	2400 μL

コントロール溶液を#A1–#H1、x10 PMA/ionomycin溶液を#A2–#H12に10 μLずつ分注する。 ☐　添加時間 (　:　)

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、F1、F2を使用)。 ☐　測定時間 (　:　)
測定中温度 (　　℃)

Multi-ImmunoTox Assay 記録用紙 Ver. 001, 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, cyclosporine Aの調製

96 well clear plate（丸底）に下図のようにDMSO 50 μL (#A4)、12 mg/mL cyclosporine A stock 10 μL + DMSO 110 μL (#A5)、Distilled water 50 μL (#B1、 #B2)、50 mg/mL dexamethasone stock 50 μL (#B3)、B培地 180 μl (#B4、 #B5)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 50uL	CyA 12 mg/mL ストック 10uL + DMSO 110uL							
B	Distilled water 50uL	Distilled water 50uL	DEX 60 mg/mL ストック 50 uL	B培地 180uL	B培地 180uL							
C												
D												
E												
F												
G												
H												

#A4のDMSOと#A5のcyclosporine A DMSO溶液 20μLを下のB培地180 μLにうつし10倍に希釈する。 ☐

アッセイブロックの#A1–#A3にB培地480 μL、#A1–#A3にB培地980 μLを分注し、上図の希釈液を20 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(PMA/ionomycin)の調製と細胞への処理

1mM PMAストックをB培地で10倍希釈し100 μM溶液を作製する。（1mM PMA 10 μL + B培地 90 μL） ☐

下図のようにコントロール溶液と x10 PMA/ionomycin溶液を作製する。 ☐

	B medium	1 mM Ionomycin	100 μM PMA	Ethanol	Total
Control	990 μL	-		10 μL	1000 μL
x10 PMA/ ionomycin solution	2370 μL	24 μL	6 μL	-	2400 μL

コントロール溶液を#A1–#D1、x10 PMA/ionomycin溶液を#A2–#D5に10 μLずつ分注する。 ☐ 添加時間 （ ： ）

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（コントロール）

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、F1、F2を使用）。 ☐ 測定時間 （ ： ）
測定中温度 （ ℃）

Multi-ImmunoTox Assay **記録用紙** Ver. 001, **更新履歴シート**

Ver. 001J　2016年02月02日配布