

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

国際バリデーシヨンの施行

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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）の一つであるIL-2レポーターアッセイを、経済協力開発機構（Organisation for Economic Co-operation and Development：OECD）の試験法ガイドラインとしての公定化するため、国際バリデーシヨン研究を施行した。本年度に実施されたバリデーシヨン研究phase Iの結果を受け、昨年度同様、国際的な専門家を招聘してバリデーシヨン結果に対して意見を求めた。その結果、phase Iバリデーシヨン研究が適切に実施されたことを確認できた。

キーワード：免疫毒性、バリデーシヨン、OECD

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A．研究目的

相場らにより、新たに開発された *in vitro* 免疫毒性評価試験法（Multi-ImmunoTox assay：MITA）の一つであるIL-2レポーターアッセイを、経済協力開発機構（Organisation for Economic Co-operation and Development：OECD）の試験法ガイドライン（Test Guideline：TG）としての公定化するため、国際バリデーシヨン研究を施行する。

B．研究方法

B-1. バリデーシヨン研究の支援

B-1-1. バリデーシヨン被験物質の送付

IL-2レポーターアッセイのバリデーシヨン研究Phase I（以下、Phase I と記す）にて、施設内および施設間再現性を求めるために用いた5物質を、コード化して各施設に送付した。

B-1-2. バリデーシヨン研究の被験物質選択

B-2-2.に示す第2回会議にて、より広範な物質を用いて施設間再現性を評価するためのIL-2レポーターアッセイのバリデーシヨン研究Phase II（以下、Phase IIと記す）の被験物質を選択した。

B-1-3. バリデーシヨン結果の記録確認

Phase I で用いられた各施設の記録用紙およびデータを回収し、バリデーシヨンが適切に実施さ

れたかを確認した。

## B-2. 国際的な専門家との意見交換

### B-2-1. IL-2レポーターアッセイのバリデーション研究Phase 0 (以下、Phase 0)後の電話会議

Phase 0 終了後の平成28年9月に電話会議を開催し、Phase I の開始について議論した。

### B-2-2. 第2回対面会議

本年度に実施されたMITAに関する国際バリデーション結果を検証するため、平成29年2月に国際バリデーション第2回実行委員会の会議を企画した。

## C. 結果

### C-1. バリデーション研究の支援

#### C-1-1. バリデーション被験物質の送付

Phase I で用いた5物質を、施設内再現性を求めるために、15物質(5物質×3セット)にコード化してリード施設を含む参加4施設に送付した。コード化して実施していることもあり、本報告書には物質名を記載していない。

実験の終了まで、被験物質に関するトラブルは生じなかった。

#### C-1-2. バリデーション研究の被験物質選択

C-2-2.に示す第2回対面会議にて、施設間再現性を確認するため、phase II の25物質を選定した。コード化して実施することもあり、本報告書には物質名を記載していない。*In vivo*結果が明確な物質を中心に選択した。

この25物質から、国内在庫、価格等を考慮して20物質に被験物質を絞った。

#### C-1-3. バリデーション結果の記録確認

Phase I 終了後に回収した記録用紙の一覧を表1に示した。施設によって一部不備はあったが、GLP ( Good Laboratory Procedure ) の精神に則り、適切に実験が実施され、その記録が残されていることを確認した。

なお、装置のバリデーションは各施設では行わ

ず、添付資料1に従うことになった。

## C-2. 国際的な専門家との意見交換

### C-2-1. Phase 0後の電話会議

Phase 0 終了後の平成28年9月に電話会議を開催し、結果を確認するとともに、Phase I の開始について議論した。

Phase 0 には表2に示す5物質が4施設で実施された。そのうち、産総研四国は経験の浅い施設として、本年度よりバリデーションに参加することになった。データ解析は複数の判別式で解析された。電話会議に使用されたAgendaおよび会議後の結論を添付資料2にまとめた。

Phase 0 の結果では、経験の浅い施設の初期の実験を除き、4施設の結果は同様の反応パターンを示した。実際には、nSLO-LAおよびnSLG-LAという生データを用いた場合、4施設で異なっていたが、%suppressionを計算した場合、4施設間に有意な差はなかった。これらの原因を探るため、細胞を集めるための遠心スピード、抗生物質の選択、T細胞を活性化するPMA ( phorbol myristate acetate ) /Ionomycinの作成法を検討したが、いずれも原因ではなかった。nSLO-LAはルミノメーターの特性によりばらつきを引き起こすので、受け入れ基準 (fold induction) としてnSLO-LA > 1.5を設定している。なお、nSLG-LAには受け入れ基準はない。nSLG-LAがルミノメーターに依存しているため、溶媒で処理された細胞と化学物質で処理された細胞のnSLG-LAによって求められる%suppressionのデータを解析に用いている。判別式の選択は現時点では保留となり、phase I 終了後に決定することになった。

今後、一部の計画を修正して、平成28年9月より、phase I に移行することで合意された。

### C-2-2. 第2回対面会議

国際バリデーション研究における第2回対面会議には、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. Emanuel Corsini (Milan Univ.)、Dr. Erwin L. Roggen (3Rs Management and Consulting ApS) および Dr. Dori Germolec

(NTP/NIEHS) を、国内からは、日本免疫毒性学会の推薦者である井上智彰博士(中外製薬)を外部専門家として招聘し、研究班の班員を含む表3に示すメンバーにて2日間掛けて、MITA バリデーション結果の確認、判別式の選択を含む試験法プロトコルの改訂などについて討論した。会議の議事次第を添付文書3 および議事概要を添付文書4に示す。

以下に結果の概要を示す。

#### 1)細胞毒性

細胞毒性が II-SLR-LA 活性の低下と無関係といえる根拠について意見交換がなされた。

II-SLR-LA は細胞死の初期を示すとの仮説が東北大より示され、細胞死が弱い濃度で免疫抑制または増強を評価するとの見解で合意を得た。

#### 2)プロトコル改訂

##### 2-1) 溶解性

最高適用濃度で沈殿が見られた被験物質もあったが、低濃度で評価できている。改訂なし。

##### 2-2) 細胞密度

$3 \times 10^5$ /mL で播種され、培養4または5日目で評価することが最適である。

##### 2-3) nSLO-LA

Fold induction を 1.5 から 3 に変更する。

#### 3) 施設内再現性

Phase I の結果においても、どの判別式を用いても、施設内再現性は良好であった。

#### 4)判別式

複数の判別式を用い、それぞれの解析結果から、適切な評価法について議論された。その結果、以下の判別式をプロトコルに組み込むことで合意を得た。

判別式：Dunnet テストで検定された3回の独立した実験の%suppression を使用する。その際に

3濃度以上で濃度依存性が見られ、その中で1点でも有意差がある場合を陽性とする。

以上の議論の末、phase Iバリデーション研究が適切に実施されたことを確認できた。さらに、新判別式を含むプロトコルの改訂に合意が得られ、より広範な被験物質を用いて施設間再現性を確認するためのphase IIを実施することになった。

添付文書5に示すバリデーション計画案を、外部専門家の意見をもとに、一部改訂した。

#### D. 考察

*in vitro*免疫毒性評価試験法(MITA)の一つであるIL-2アッセイの技術移転性および再現性の確認を目的としたバリデーション研究が施行された。トレーニングにあたるphase 0を経て、phase Iの5物質で施設内再現性および施設間再現性が確認された。国際的な専門家との会議では、Phase I およびIIの解析に用いる判別式についても合意が取れ、プロトコルがほぼ固まった。Phase IIの円滑な実施により、より広範な被験物質を用いて施設間再現性の確保に務めたい。

#### E. 結論

相場らにより、新たに開発されたMITAの一つであるIL-2レポーターアッセイの公定化を目指すため、国際的なバリデーション研究が施行された。このバリデーションの客観性を確保するため、被験物質のコード化および配布、実験記録の回収および確認を担当し、適切な実験が実施されていることを確認できた。

#### F. 添付文書

- 1) Verification of luminometer
- 2) Teleconference for the MITA assay
- 3) Agenda : 2nd meeting for the MITA assay
- 4) Minutes of MITA at 2nd meeting,

5) Study plan for the validation trial on multicolor reporter assay using IL-2 Luc as a test evaluating the immunotoxic potential of chemicals

表 1 . 記録用紙確認リスト

		LabB (AIST-TSUKUBA)					LabC (FDSC)				LabD (AIST-TAKAMATSU)				LabA (TOHOKU univ.)		
		Code	try1	try2	exp1	exp2	exp3	Code	exp1	exp2	exp3	Code	exp1	exp2	exp3	exp4	
setA	1	MIB014A	×	×	○	○	○	MIC027A	○	○	○	MID036A	○	○	×	○	MIA003A
	2	MIB017A	×	×	○	○	○	MIC029A	○	○	○	MID038A	○	○	×	○	MIA005A
	3	MIB018A	×	×	○	○	○	MIC021A	○	○	○	MID310A	○	○	×	○	MIA007A
	4	MIB110A	×	×	○	○	○	MIC023A	○	○	○	MID037A	○	○	×	○	MIA009A
	5	MIB012A	×	×	○	○	○	MIC025A	○	○	○	MID034A	○	○	×	○	MIA001A
setB	1	MIB017B			○	○	○	MIC026B	○	○	○	MID033B	×	○	○	○	MIA004B
	2	MIB019B			○	○	○	MIC028B	○	○	○	MID035B	×	○	○	○	MIA007B
	3	MIB011B			○	○	○	MIC210B	○	○	○	MID037B	×	○	○	○	MIA008B
	4	MIB013B			○	○	○	MIC027B	○	○	○	MID039B	×	○	○	○	MIA010B
	5	MIB015B			○	○	○	MIC024B	○	○	○	MID031B	×	○	○	○	MIA002B
setC	1	MIB016C			○	○	○	MIC023C	○	○	○	MID034C	×	○	○	○	MIA007C
	2	MI0018C			○	○	○	MIC025C	○	○	○	MID037C	○	○	×	○	MIA009C
	3	MIB110C			○	○	○	MIC027C	○	○	○	MID038C	○	○	×	○	MIA001C
	4	MIB017C			○	○	○	MIC029C	○	○	○	MID310C	○	○	○		MIA003C
	5	MIB014C			○	○	○	MIC021C	○	○	○	MID032C	×	○	○	○	MIA005C

○	passed	×	failed		needless	
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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 <sub>3</sub> HgCl	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(-/-/-)

表3 . MITA第2回国際バリデーション対面会議 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	Tomoaki Inoue	Chugai Pharmaceutical Co., Ltd.	Japan
4	Setsuya Aiba	Tohoku University Graduate School of Medicine	Japan
5	Yutaka Kimura	Tohoku University Graduate School of Medicine	Japan
6	Nakajima	National Institute of Advanced Industrial Science and Technology	Japan
7	Rie Yasuno	National Institute of Advanced Industrial Science and Technology	Japan
8	Kohji Yamakage	Food and Drug Safety Center, Hatano Research Institute	Japan
9	Takashi Omori	Kobe University	Japan
10	Hajime Kojima	JaCVAM, National Institute of Health Sciences	Japan
11	Steven Venti	Translator	Japan

## G. 研究発表

### G-1. 論文発表

- 1) 小島 肇: 日本で開発または評価された OECD テストガイドライン, 生物化学的測定研究会年報, 20 (2016)
- 2) Yamamoto N, Kato Y, Sato A, Hiramatsu N, Yamashita H, Ohkuma M, Miyachi E, Horiguchi M, Hirano K, Kojima H: Establishment of a new immortalized human corneal epithelial cell line (iHCE-NY1) for use in evaluating eye irritancy by in vitro test methods, *In Vitro Cell.Dev.Biol.-Animal*.2016; Aug;52(7):742-8
- 3) Yamaguchi H, Kojima H, Takezawa T: Predictive performance of the Vitrigel-eye irritancy test method using 118 chemicals, *J Appl Toxicol*. 2016 Aug;36(8):1025-37.
- 4) 小島 肇: 皮膚毒性評価に関する最近の話題, 評価方法, 第 17 回日本毒学会生涯教育講習会テキスト, 89-108 (2016)
- 5) Uchino T, Kuroda Y, Ishida S, Yamashita K, Miyazaki H, Oshikata A, Shimizu K, Kojima H, Takezawa T, Akiyama T, Ikarashi Y: Increase of  $\beta$ 2-integrin on adhesion of THP-1 cells to collagen vitrigel membrane, *Biosci Biotechnol Biochem*. 2016; Jul 4:1-6
- 6) Marx U, Andersson TB, Bahinski A, Beilmann M, Beken S, Cassee FR, Cirit M, Daneshian, Fitzpatrick S, Frey O, Gaertner C, Giese C, Griffith L, Hartung T, Heringa MB, Hoeng J, Jong WH, Kojima H, Kuehnl J, Leist M, Luch A, Maschmeyer I, Sakharov D, Sips AJAM, Steger-Hartmann T, Tagle DA, Tonevitsky A, Tralau T, Tsyb S, Stolpe A, Vandebriel R, Vulto P, Wang J, Wiest J, Rodenburg M, Roth A: Biology-inspired microphysiological system approaches to solve the prediction dilemma of substance testing. *ALTEX*. 2016; 33(3):272-321
- 7) Barroso J, Ahn IY, Caldeira C, Carmichael PL, Casey W, Coecke S, Curren R, Desprez B, Eskes C, Griesinger C, Guo J, Hill E, Roi AJ, Kojima H, Li J, Lim CH, Moura W, Nishikawa A, Park H, Peng S, Presgrave O, Singer T, Sohn SJ, Westmoreland C, Whelan M, Yang X, Yang Y, Zuang V.: International Harmonization and Cooperation in the Validation of Alternative Methods, *Advance in Experimental Medicine and Biology. Validation of Alternative Methods for Toxicity Testing*, Springer, 2016, pp.793-803
- 8) Kojima H., Safety Assessment of Cosmetic Ingredients, *COSMETIC SCIENCE AND TECHNOLOGY: THEORETICAL PRINCIPLES AND APPLICATIONS*, Elsevier 2017; 343-386
- 9) 小島 肇: 医薬品に係わる新添加物の安全性評価, 月刊ファームステージ, 16(6), 1 (2016)
- 10) 小島 肇: 皮膚細胞を用いた最新の in vitro 皮膚安全性評価研究, 月刊コスメティックステージ, 12, 1-4 (2016)
- 11) 小島 肇, 西川秋佳: 日本動物実験代替法評価センター (JaCVAM) 平成 27 年度報告書. *AATEX-JaCVAM*, 5(1), 45-56 (2016)

### G-2. 学会発表

- 1) Kojima H: View and suggestion about how to promote progress and cooperation in Asia, 2016 上海化粧品科学フォーラム (2016.4) (Shanghai, China)
- 2) 小島 肇: 国際機関で承認されている in vitro 試験法, 日本組織培養学会 第 89 回大会 (2016.5) (大阪)
- 3) 山本直樹, 平松範子, 加藤義直, 佐藤淳, 中田 悟, 松井優子, 真野陽介,

- 原 和宏, 増藺夕紀子, 中村政志, 小島 肇: ヒト不死化角膜上皮細胞を用いた三次元角膜モデルの有用性, 日本組織培養学会 第 89 回大会 (2016.5) (大阪)
- 4) 小島 肇: 医薬品に係わる新添加剤の安全性評価における諸課題, 第 43 回日本毒性学会学術年会 (2016.6) (名古屋)
- 5) 小島 肇: 経済産業省プロジェクト「石油精製物質等の新たな化学物質規制に必要な国際先導的有害性試験法の開発: Arch-Tox」の計画概要, 第 43 回日本毒性学会学術年会 (2016.6) (名古屋)
- 6) 伊藤浩太, 榊原隆史, 古川正敏, 奥村宗平, 越田 美, 川村公太郎, 松浦正男, 小島 肇: 牛摘出角膜を用いた混濁度及び透過性試験 (BCOP 法: 眼刺激性代替法試験) における角膜病理学的検査により弱刺激性物質の評価, 第 43 回日本毒性学会学術年会 (2016.6) (名古屋)
- 7) Kojima H: Japanese activities for alternative to animal testing around the world, 6th Workshop & Training of Alternative Methods (2016.6) (Guangzhou, China)
- 8) 小島 肇: 皮膚毒性評価に関する最近の話題, 評価方法, 第 17 回日本毒性学会生涯教育講習会テキスト (2016.7) (名古屋)
- 9) 小島 肇: 代替法試験の基礎から最新知見まで, マツモト交商 安全性試験セミナー (2016.7) (東京)
- 10) 小島 肇: 動物実験代替法の国内外の動向, 皮膚基礎研究クラスターフォーラム第 11 回教育セミナー (2016.7) (東京)
- 11) Kojima H: Strategy on the OECD TG in Japan, 13th Annual meeting of Korean Society for Alternatives to Animal Experiments (2016.8) (Seoul, Korea)
- 12) Kojima H: The current status of non-animal test methods and prospects for Asian cooperation, 17th Annual Congress of European Society for Alternative to Animal Testing (2016.8) (Linz, Austria)
- 13) 小島 肇: AOP の考え方, OECD による AOP プロジェクトの目的, 経緯と最終的なゴール, 第 23 回日本免疫毒性学会学術年会 (2016.9) (北九州, 福岡)
- 14) Kojima H: International validation study on Hand1-Luc Embryonic stem cell test (Hand1-Luc EST): A reporter gene assay using engineered mouse ES cells evaluate embryotoxicity in vitro, 5th Annual meeting of American Society for Cellular and Computational Toxicology (2016.9) (North Carolina, USA)
- 15) 伊藤浩太, 榊原隆史, 古川正敏, 奥村宗平, 越田 美, 河村公太郎, 松浦正男, 小島 肇: 牛摘出角膜を用いた混濁度及び透過性試験法 (BCOP 法: 眼刺激性代替法試験) における角膜の病理組織学的検査による弱刺激性物質の評価, 日本動物実験代替法学会第 29 回大会 (2016.11) (福岡)
- 16) 小島 肇: JaCVAM における 3Rs 原則と動物実験代替法, 日本動物実験代替法学会第 29 回大会 (2016.11) (福岡)
- 17) 萩原沙織, 篠田伸介, 仲原 聡, 小島 肇, 大森 崇, 遠藤麻衣, 佐竹真悠子, 池田英史, 西浦英樹, 笠原利彦, 山本祐介, 加藤雅一, 菅原 桂: 培養角膜



- 上 皮 モ デ ル LabCyte CORNEA-MODEL24 眼刺激性試験の多施設バリデーション研究, 日本動物実験代替法学会第 29 回大会( 2016.11 )( 福岡 )
- 18) 加藤義直, 山本直樹, 佐藤 淳, 中田悟, 小島 肇: 不死化ヒト角膜細胞株 (iHCE-NY) を用いて作製した三次元角膜再構築モデルの眼刺激性試験代替法 ~再構築ヒト角膜様上皮 (RhCE) 試験法用性能標準の 30 物質(TG492PS) に対する回復性を取り入れた予測性~, 日本動物実験代替法学会第 29 回大会 ( 2016.11 )( 福岡 )
- 19) 藤田正晴, 笠原利彦, 山本裕介, 渡辺真一, 菅原経継, 若林晃次, 田原 宥, 堀江宣行, 藤本恵一, 高橋寛明, 黒川嘉彦, 小野 敦, 小島 肇: Cys および Lys 誘導体を用いた皮膚感作性試験代替法 (ADRA 法) のバリデーション研究のための技術移転結果報告, 日本動物実験代替法学会第 29 回大会( 2016.11 ) ( 福岡 )
- 20) 松成夏美, 九十九英恵, 謝 丹, 岡 朱音, 小島 肇, 板垣 宏: タンパク質のアレルギー性を評価する in vitro 試験法の開発, 日本動物実験代替法学会第 29 回大会 ( 2016.11 )( 福岡 )
- 21) 内野 正, 宮崎 洋, 山 邦彦, 竹澤俊明, 小島 肇, 秋山卓美, 五十嵐良明: 改良型コラーゲンビトリゲル膜チャンバーでの THP-1 細胞の細胞接着性及びサイトカイン産生量, 日本動物実験代替法学会第 29 回大会( 2016.11 )( 福岡 )
- 22) VO P.T.H, Narita K, Nakagawa F, Kojima H, Itagaki H: Reducing false negative results in an in vitro skin sensitization test: The human cell line activation test, 日本動物実験代替法学会第 29 回大会 ( 2016.11 )( 福岡 )
- 23) Kojima H: Guidance on use of alternative methods for testing in the safety assessment of cosmetics and quasi-drug, Asian Congress on Alternatives and Animal Use in the Life Sciences (Asian Congress) 2016, (2016.11) (Karatsu, Saga)
- 24) 小島 肇: 医薬品食品領域での動物愛護管理法の現在と未来, NPO 法人動物実験関係者連絡協議会 第 5 回シンポジウム 「動物愛護管理法」の過去・現在・未来 ( 2016.12 )( 東京 )
- 25) Furukawa, M., Sakakibara, T., Kouta I., Kawamura, K., Matsuura, M., Kojima, H.: Special stain for detection of corneal histopathological changes in BCOP (Bovine Corneal Opacity and Permeability) assay, 56th Annual meeting of Society of Toxicology, March 12-16, Baltimore USA
- 26) 小島 肇: 日本における動物実験代替法研究の胎動, シンポジウム「日本における動物実験代替法の新たな技術展開」, 第 90 回日本薬理学会年会 ( 2017.3 )( 長崎 )
3. 知的所有権の取得状況
- G - 1) 特許取得  
特になし
- G - 2) 実用新案登録  
特になし
- G - 3) その他  
特になし

F. 添付文書

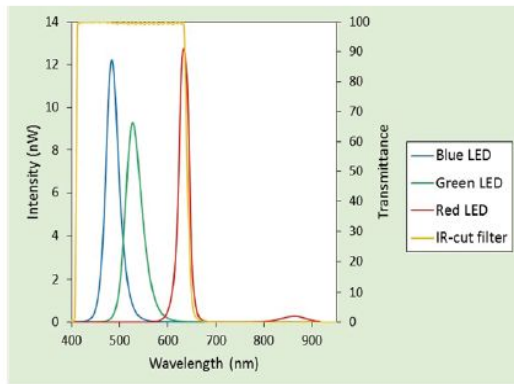
1) Verification of luminometer

## Reference light source (LED plate)

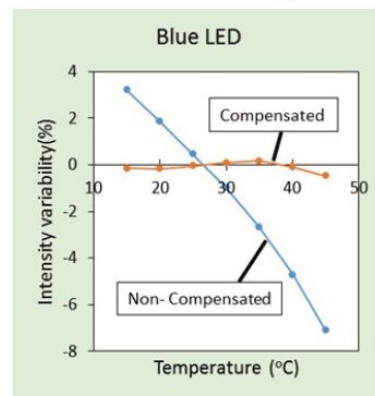


ATTO Co.

### RGB LED Spectra

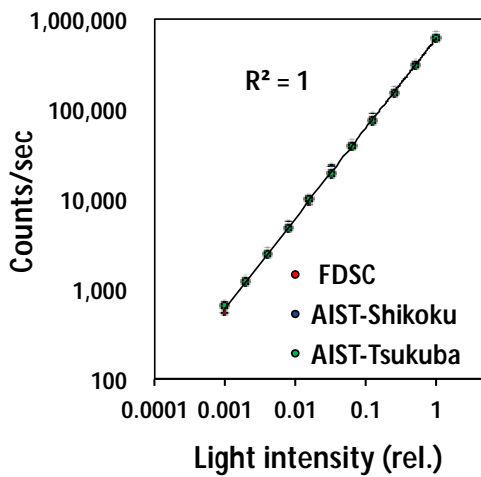


Light intensity is compensated for temperature change.

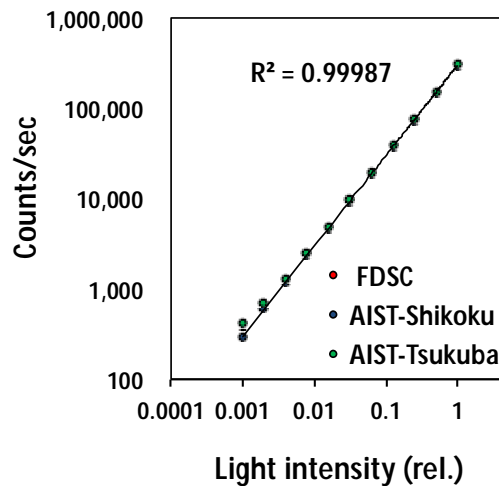


## Linear dynamic range and detection sensitivity of luminometers in the test laboratories.

Green ( $\lambda_{max} = 524 \text{ nm}$ )



Red ( $\lambda_{max} = 624 \text{ nm}$ )



F. 添付文書

2) Teleconference for the MITA assay

## **Teleconference for the MITA assay Agenda**

- Date : September 13, 2016, 16:30 - 18:00 (Italy) , 10:30-12:00 (East, USA), 23:30-25:00 (Japan)
- Corsini, E., Rogen, E., Germolec, D., Inoue ,T., Aiba, S., Kimura, Y., Omori, T., Kojima, H.

1. Welcome address (Hajime)
2. Results of the phase 0 study (Dr. Aiba)
3. Proposal of the revised protocol (Dr. Aiba, Dr. Kimura and Dr. Omori)
4. Study plan of the phase I study (Hajime)
5. Other information (next F2F meeting)

F. 添付文書

3) Agenda : 2nd meeting for the MITA assay

Draft agenda

2<sup>nd</sup> meeting for the MITA assay

Date : February 3, 2017, 14:00 - 17:00

February 4, 2017, 10:00 - 17:00

Venue : Nayamachi community hall ([http://nayamachi.or.jp/community\\_hall/](http://nayamachi.or.jp/community_hall/))

Participants: Corsini, E., Roggen, E., Germolec, D, Inoue, T

Aiba, S., Kimura, Y., Yamakage, K., Watanabe, M., Kobayashi Mayumi., Yasuno, R.,

Omori, T., Nakajima, Y., Kojima, H., Mori, A., Kobayashi Miwako, Venti, S.

February 3

Introduction (14:00-17:00)

Chair: Nakajima, Y.

1. Welcome address and house keeping (Kojima, H.)
2. Outline and protocol of the MITA assay (Aiba, S.)
3. Study plan for the MITA assay validation study (Kojima, H.)
4. Results of the MITA assay (Omori, T.)
5. QC check (Kojima, H.)
6. Comments from the participated laboratories

February 4

Chair: Aiba, S.

Discussion and suggestion (10:00-13:30)

**Working Lunch**

Chair: Omori, T..

Discussion and suggestion (13:30-16:00)

7. Revised protocol (If need)
8. Study plan for the next phase

Chair: Dr. Kojima, H.

Closing remark (16:00-17:00)

9. Wrap-up on discussion (Venti, S.)
10. Any other business
11. Closing remark (Aiba, S.)

Draft agenda

2<sup>nd</sup> meeting for the MITA assay

Date : February 5, 2017, 10:00 - 12:00

Venue : Nayamachi community hall ([http://nayamachi.or.jp/community\\_hall/](http://nayamachi.or.jp/community_hall/))

Participants: Corsini, E., Rogen, E., Germolec, D, Inoue, T

Aiba, S., Kimura, Y., Omori, T., Mori, A., Kobayashi, M., Kojima, H., Venti, S.

Chemical selection meeting

1. Confirmation of study plan
2. Code open for phase I
3. Chemical selection for phase II
4. Closing remark

## F. 添付文書

4) Minutes of MITA at 2nd meeting,

2<sup>nd</sup> MITA Validation Study meeting

Date : February 4, 2017, 10:00 - 17:00

Venue : Nayamachi community hall ([http://nayamachi.or.jp/community\\_hall/](http://nayamachi.or.jp/community_hall/))

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

Kojima: Opening remarks and self-introductions, followed by review of documentation

Aiba: Presentation

Omori: Lower left result of criteria 2 should be immunoaugmentation not immunostimulation

Roggen: Were these groups 1 to 6 defined by yourself?

Aiba: It's not based in vivo effects but rather on in vitro LOEL effect levels in the IL-2 assay.

Corsini: When a chemical shows both an increase and a decrease, do you also look at cytotoxicity?

Roggen: And what is an acceptable cell viability?

Aiba: When looking at concentrations that show 80% viability, there was very low II-SLR-LA activity. Chemicals with high PI-exclusion often have a low II-SLR-LA.

Nakajima: We recently published a paper correlating SLR and viability

Roggen: Did you separate the dead cells from the living cells?

Aiba: Most test chemicals exhibit some decrease in cell viability. The II-SLR-LA indicates cells that will die sooner.

Roggen: When you separate the living and dead cells, you might see a decrease in the population but no decrease on the cellular level. In other words, the decrease in the population was due to the cells dying.

Corsini: But you can't expect all the cells to be activated.

Aiba: This is a very common way to normalize. We use IL-8 promoter activity normalized by GAPDH promoter activity to overcome the concern. We have examined the correlation many times. It is difficult to understand the meaning, but we are sure the correlation exists. The assumption is the II-SLR-LA is the early stage of cell death.

Roggen: My concern is discerning between the effects of cytotoxicity and suppression.

Germolec: So how do you use these classes when you see an increase and decrease? If you just use the low level, you might be misclassifying.

Aiba: We accept the most significant effect.

Corsini: So, you are assuming that the lower concentrations are the most significant?

Nakajima: Let's look at the study plan next

Kojima: We have new criteria that is not included in the study plan yet. And after the teleconference, Shihori Tanabe left the VMT. So, we will update the study plan. We coded five chemicals for distribution in Phase 1. Each replicate was coded separately, so 15 samples were sent to each laboratory.

Omori: What is the difference between "try" and "exp" at Lab B?

Lab B: It should be Exp. 1 to 5.

Omori: Were the Labs told they were testing 15 chemicals or three replicates of five chemicals?

Lab B: Three replicates of five chemicals.

Omori: They were aware they were testing WLR with three replicates of five chemicals.

We now have three different criteria, which are difficult to understand. Criteria 1 is based on three independent test results. If all three results are concordant, we can make a call. If there are non-concordant results, we calculate % suppression using the Student t-test.

Roggen: What does "+ and -" mean?

Omori: A single test result showed both significant augmentation and significant suppression.

I wanted to simplify Criteria 1, so we used an adjusted mean for nSLG-LA from three independent test results for Criteria 2. Which we explained during the teleconference in September. But it was suggested that it would be better to use % suppression. So, Criteria 3 uses an adjusted mean for % suppression from three independent tests.

Perhaps we need to discuss which Criteria we should use, but before we do, I would like to look at the dose-response curves.

Germolec: Some immunosuppressants are very consistent but maybe chemicals that do not have a clear usage are not so consistent.

Omori: Chemical 3 shows good WLR at all three Labs. Chemical 4 does too. For Chemical 2, WLR is good. Chemical 5 has good WLR but maybe BLR is not as good.

The WLR appears to be good at all labs.

Roggen: Does the problem with Chemical 5 have to do with solubility?

Aiba: It shows precipitation in higher concentrations, but the significant effects are at

lower concentrations.

Roggen: Would it be useful to address solubility in the protocol?

Yamakage: Solubility is judged visually.

Germolec: Results at the two AIST labs appears consistent but some variability between those two and FDSC.

Kojima: We want to finalize the experiment data. We checked the record sheets from each Lab and we will make them available on the JaCVAM website.

Roggen: If cells are more responsive from Day 4, perhaps that should be noted in the protocol.

Germolec: Instead of optimizing to a particular day, would it be better to optimize to PMA or some other indicator of cell response?

Inoue: What does the protocol say?

Yamakage: At least two days. Page 11 of the protocol says "cell passage ... two to four days before the assay." Maybe this should say "four days before the assay."

Aiba: The lead lab is studying this issue and we will revise the protocol. We had different cell densities and the cell functionality appears to be best on Day 4 or Day 5 when seeded at a cell density of  $3 \times 10^5$ .

Germolec: Perhaps you can normalize cell density. The question might be that, since there will be slight differences in doubling times even at the same lab, how to quantify cell density.

Aiba: We looked at data for SLO-LA and SLG-LA. We think we might have change the criteria to exclude data if fold induction of nSLO-LA is less than 3. I would like to discuss this issue.

Nakajima: Another QC issue involved the luminometer at each laboratory. LED plate is easy to check. These figures show that the luminometers used at each lab are equivalent.

Roggen: When transferring this test to another lab, can their luminometer be calibrated?

Nakajima: Yes.

Aiba: We need to be able to exclude certain results, so we want to revise the acceptance criteria on page 36 of the protocol. We want to change the value for fold induction from 1.5 to 3.

Omori: We now have three proposed criteria, which we should discuss.

Corsini: Looking at the dose response curves, the error bar for each data point has to be either completely above or completely below the threshold line to be considered significant. Classification of Chemical 2 needs to be revised.

Omori: Using Criteria 2 gives immunosuppression but Criteria 3 gives both immunosuppression and immunoaugmentation.

Roggen: I don't trust classification based on a single point. I think maybe classification should be based on two successive significant values. Or you could do a trend analysis.

Aiba: Looking at pages 2 and 7, we see the response varies between laboratories.



Roggen: Is there any in vivo data for Chemical 2?  
Kojima: It is positive.  
Roggen: Chemical 5?  
Kojima: It is negative.

Germolec: Criteria 1 seems to provide the best transparency and is very consistent, with only one non-concordant result out of 15. And if Chemical 5 is negative, then Criteria 1 is the most acceptable from a biological perspective.

Omori: I think we can agree to use Criteria 1 for the study.

Kojima: We will revise the protocol to use Criteria 1. For Phase 2, we will use 20 chemicals to evaluate BLR. Is that acceptable? Also, when will Phase 2 begin?

Aiba: The end of April or beginning of May.

Kojima: How long will it take to test 20 chemicals?

Roggen: One way to check solubility would be to centrifuge the test chemical solution once it appears fully dissolved. Also, add instructions in the protocol about what to do if precipitation is found after centrifuging.

Inoue: Precipitation after mixing in final concentration with culture medium is also important.

Aiba: We will revise the protocol to include checking the concentration of the stock solution, using a centrifuge to check solubility in DMSO, and making notes of any changes in appearance that are observed in the well.

Kojima: It will take three months for the participating labs to do Phase 2 testing, so we can expect testing in May to July and then data will be available for analysis around August.

Omori: Please give us two months for analysis, so August and September. The purpose of Phase 2 is to verify BLR and predictive capacity with 25 chemicals.

Germolec: Is there are minimum number of concentrations needed to make a judgment?  
Aiba: We need at least four valid data points of different concentrations.

Roggen: You could test for maximum concentration at which you have 90% viability and then select concentrations below that. You have to do three runs, so you could use the first run for dose finding using a broad range of concentrations, which can be narrowed in the second and third runs as necessary. It is not necessary to change the dilution ratio, just slide the window to where there is immuno-modulatory response.

Nakajima: I am worried about some chemicals that might show immunosuppression but only in a very narrow range of concentrations.

Aiba: There might be some cases where we get 4 valid data points during the first run but then get only 3 valid data points in a subsequent run. But I think that is a very rare case.

Since we have adopted Criteria 1, we have to discuss what to do when a chemical shows two distinct tendencies.

Corsini: Citral shows two tendencies, so you can't call it negative.

Roggen: The question is, is it ever immunosuppressive? And you have to say yes.

Germolec: I think you need to call it "A/S," because it shows both.

Aiba: So, what procedure should we use to make a call in a case like Citral?

Inoue: You do a statistical analysis for both % suppression and % augmentation.

Aiba: We have to revise Criteria 1 to include this situation.

Omori: It is possible that Chemical 5 will no longer be No Effect if we revise Criteria 1 this way. I would like to check how to implement this revision to the data sheet, after which we might need a teleconference to review it.

Dori: If you bear in mind that you need successive data points to make a call, then Chemical 5 won't change because there is only one valid data point.

Roggen: Dori's suggestion is good. If it shows both, call it "modulation." But it will make the assay stronger if you could show a way to discriminate between augmentation and suppression.

## 2<sup>nd</sup> MITA Validation Study meeting—Day Two

Date : February 5, 2017, 10:00 - 12:00

Venue : Nayamachi community hall ([http://nayamachi.or.jp/community\\_hall/](http://nayamachi.or.jp/community_hall/))

Participants: Corsini, E., Roggen, E., Germolec, D, Inoue, T, Aiba, S., Kimura, Y., Omori, T., Mori, A., Kobayashi, M., Kojima, H., Venti, S.

Kojima: Today we would like to select chemicals for Phase 2 and then discuss the Acceptance Criteria.

Germolec: We know there is "standardized" in vivo data for the NTP chemicals.

Kojima: I will check the cost of the twenty-five proposed chemicals this week. And then the VMT will finalize a list of 20 chemicals for Phase 2 by the end of this month.

Omori: We have modified Criteria 1 and Criteria 3 to require either

1) two consecutive statistically significant points

or

2) one statistically significant point as well as a trend in which 3 or more

points above the red line are increasing or 3 or more points below the red line are decreasing.

Roggen: The call is based on two out of three runs, in which case nothing changes. Chemical 2 at Tsukuba is the only non-concordant call.

Inoue: The old Criteria 3 was too sensitive, but the new Criteria 3' is good.

Germolec: Criteria 1 is less relevant to Phase 2 because you won't be looking at multiple rounds of each chemical.

Aiba: Criteria 3 has an advantage in that data is normalized and is not affected by the different luminometers.

Germolec: I worry a little about normalizing data, but as long as you show the data from each independent run together with the final normalized data, you will have all the information you need.

Omori: We will show individual dose-response curves and normalized curves. The final call for each chemical will be based on the normalized curve, per Criteria 3' but we will also show statistical analysis for all data.

Roggen: I recommend that you present this process step by step to ESAC, so that they can see the details of how it works for themselves.

F. 添付文書

- 5) Study plan for the validation trial on multicolor reporter assay using IL-2 Luc as a test evaluating the immunotoxic potential of chemicals

**Study plan  
for the validation trial on multicolor reporter assay using IL-2 Luc (IL-2 Luc  
assay) as a test evaluating the immunotoxic potential of chemicals**

Version 1.4 February, 2017

Conducted by:

IL-2 Luc assay Validation Management Team

## **INDEX**

1. Background
2. Objective of the trial
3. Validation Management Team
4. Protocol
5. Chemical
6. Records and archiving
7. Study timeline

## 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
<b>Trial Coordinator</b> Hajime Kojima	VMT trial coordinator, Chemical supplier and Management of quality control	JaCVAM, NIHS, Japan (JaCVAM representative)
<b>Lead Lab</b> Yutaka Kimura* Setsuya Aiba*	*Developer of this assay Test method, expertise underlying science	Tohoku Univ., Japan
<del>Shihori Tanabe</del>	<del>Chemical supplier</del>	<del>JaCVAM, NIHS, Japan (JaCVAM representative)</del>
Takashi Omori	Data analysis, biostatistics dossier	Kobe Univ., Japan
International expert members		
<b>EU liaison</b> Emanuela Corcini	Test system expertise, validation expertise, immunotoxicity expertise	Milan Univ., Italy
<b>EU liaison</b> Erwin L. Roggen	Test system expertise, validation expertise, immunotoxicity expertise	3Rs Management and Consulting ApS, Denmark
<b>ICCVAM liaison</b> Dori Germolec	Immunotoxicity expertise	NTP/NIEHS, USA
<b>JSIT liaison</b> Tomoaki Inoue	Immunotoxicity expertise	Chugai Pharmaceutical Co., Ltd.

#### 3.1 Participating Test Facilities

The laboratories participating in the trial are defined as follow:

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

Test Facility 2: AIST, Tsukuba SD: 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

#### 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 prepare protocol, test chemical preparation record forms, blank data sheets, etc. and distributes them to the research laboratories participating in this validation trial. They also collect filled out forms and data sheets after completion of experiments, pointing out omissions or flaws in recording, if any, and requesting correction of such errors.

#### 4) Lead laboratory

The lead laboratory representing the test method is responsible for providing the test method protocol and the eventually necessary data recording or calculation templates. The Trial Coordinator has to ensure that such data recording or calculation templates have been validated before distribution to the test facilities involved in the validation trial. The lead laboratory is also responsible for providing, if necessary, new versions of the protocols during the entire validation trial. The lead lab and the other participating test facilities might be contacted by the VMT for technical issues.

### 3.3 Sponsor

The validation trial for assessing the validity of IL-2 Luc assay will be financed by the Ministry of Health, Labour and Welfare (MHLW), Japan.

The lead laboratory will support the IL-2 Luc assay validation trial by assuring that reliability is assessed. At the same time, preliminary results of the test method can be evaluated. For this purpose, Lead laboratory will support:

- 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

### 3.4 Trial coordination

Dr. Hajime Kojima was appointed as the Trial Coordinator with well-defined roles and responsibilities to coordinate the trial and to establishment of a VMT by supporting of JaCVAM.

The name and location of the Trial Coordinator should be identified in each individual study plan. For the IL-2 Luc assay validation trial, the Trial Coordinator has direct access to the test item coding.

The Trial Coordinator's responsibilities include:

- a) Establishment of/support to lead laboratory, including meeting organization
- b) Trial communication and coordination with test facilities
- c) Recording of document and data flow between test facilities
- d) Assessing and documenting the impact of any amendments and/or deviations from the trial plan and study plans on the quality and integrity of the validation trial
- e) Ensuring that the individual study reports are forwarded, in a timely manner, for data and statistical analysis
- f) Preparing the trial plan and report, which can be based on the study reports from the lead laboratories and other test facilities involved in the validation trial, and should reflect the overall trial
- g) Approval with date and signature of all protocols, Study Plans and Study Reports
- h) The communication of the results of the trial into the public domain

The 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.

### 3.5 Training

The lead laboratory will be responsible for issuing a training agenda to the Trial Coordinator for further distribution to the all test facility giving details what training aspects will be covered during the training of the other SDs and Study Personnel at the lead laboratory. Furthermore, after the training as Phase 0 study, 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 using coded five chemicals, under the VMT. The proportion of concordance should be equal or more than 80% as tentative acceptance criteria for phase I study.

### 3.7 [Module 3] Between-laboratory transferability

This between-laboratory transferability (Module 3) 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 -coded five chemicals were 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

Twenty-five coded test items have been selected to confirm the between-laboratory reproducibility in the phase I and II 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. 0.08E, phase I and 0.1E, phase II) will be used). This protocol will make up a draft by the lead laboratory and be finalized by VMT. The criteria to identify immunotoxicants by MITA are provisionally fixed in the protocol ver. 0.08E prior to the phase I. There are two temporary criteria to identify immunotoxicants. The VMT adopted the criteria after the phase I validation study.

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 Chemical 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 management group 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 management group insuring 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 I	5 non- coded	1	Between-lab transferability
Phase I	5 coded	3	Within & between-lab reproducibility
Phase II	20 coded	1	Between-lab reproducibility & predictability

## 5.2 Chemical Acquisition, Coding and Distribution

The 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 December 2017.

Table 3. Schedule of IL-2 Luc assay validation trial

Month	Activity
January 2016	Establish the VMT
	Selection of participating research laboratories
	Deliberation, decision and read-through of draft study plan
	Deliberation and decision of protocol
	Preparation of a tentative list of test chemicals

	Distribution of test chemicals, standard chemicals and positive control chemicals
February,2016	Technical transfer using five known chemicals (non-coded) Start of technical transfer <b>to know between laboratory transferability</b>
	Data collection of technical transfer ( <b><u>Phase 0 study</u></b> )
Phase I study	
September 2016	Coding and distribution of five coded test chemicals
September, 2016	Start of Phase I study
December, 2016	End of Phase I study
February, 2017	<b><u>2<sup>nd</sup> VMT Meeting</u></b> / Phase I results and planning of Phase II study
<b><u>Phase II study to know between- and within-laboratory reproducibility</u></b>	
April, 2017	Coding and distribution of coded test chemicals and positive chemicals
May, 2017	Start of Phase II study using 20 coded test chemicals
August,2017	End of Phase II study
November-December, 2017	<b><u>3<sup>rd</sup> VMT Meeting</u></b> /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

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

Multi-Immuno Tox Assay Validation Phase1研究のデータ解析方法の検討と結果の報告

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研究要旨

**[背景と目的]** Multi-Immuno Tox Assay(以下、MITA)は、化学物質がヒトの免疫系に与える影響を *in vitro* で評価することを目標とする試験法である。MITA の判定方法が十分には確立していないため、3種類の判定方法の候補（方法A、方法B、方法C）について定式化するとともに、バリデーションにおける phase1 研究のデータで検討した。

**[方法]** 方法Aは個々の実験で判定を行い、独立した3回の実験の判定結果をもって最終判定を行う方法である。方法Bは、独立した3回の実験から得られた測定値を統合したデータセットを作成し、このデータセットに基づきデータ解析を行った結果から最終判定を行う方法である。方法Cは、方法Aと同様に3回の実験を統合し、最終判定を行う方法であるが、方法Aのように測定値を使うのではなく、得られる測定値を%換算した値を用いる方法である。MITA のバリデーションにおける phase1 研究は、3施設で、ブラインド化された5物質を3セット行うようにデザインされた。

**[結果]** 施設間再現性の結果はどの方法も同じであった。施設内再現性は、要約結果だけを見ると方法Aが方法B、Cに比べて若干優れているように思われるが、濃度反応関係を見る限り、方法Aが優れているということではなさそうであった。どの方法も適切とはいえず濃度反応関係を考慮すべきであろう。

**[結論]** MITAによる化学物質の評価のために検討した3種類の方法は、まだ検討の余地がある。濃度反応関係を考慮した判定が必要である。

A. 研究目的

Multi-Immuno Tox Assay(以下、MITA)は、化学物質がヒトの免疫系に与える影響を *in vitro* で評価することを目標とする試験法である。MITAでは、ある化学物質の IL2 の発現を評価は独立した3回の実験から得られる測定値を用いて行われ、immunosuppression、immunoaugmentation、no

effect のいずれかの判定がされることが求められている。しかし、その判定方法が十分には確立していなかった。このため判定の方針について研究者の間で議論がなされ、3種類の判定方法の候補の可能性が考えられた。MITA のバリデーションにおける phase1 研究では、研究に入る前に3種類の判定のための候補を適用することにして、研究が実

施された。データ解析を行う際には、研究者の間で議論された方針を解析方法として定式化し、実装させる必要があった。

本報告では方針に基づいた判定方法の説明とそれらを適用した結果を報告する。なお、実装のための各方法の定式化は、分担研究における結果に相当させるべきであるが、バリデーションプロジェクト全体としては方法に相当するため、本報告では B. 研究方法に記載することとする。

## B. 研究方法

### MITA のデータの特徴

MITA では、独立に実施された 3 回の実験の結果を用いて判定には濃度 0(DMSO)群と各濃度群との対比較により、最終的な判定を行う。1 回の実験には、96 穴プレートが用いられ、Control 群に加え、濃度 0(DMSO)、濃度 1~濃度 10 までの計 12 段階の濃度群の測定値が得られる。個々の濃度群では 4 回の繰り返しがある。

### Phase1 研究で検討した 3 種類の方法について

Phase1 研究において検討をおこなった 3 種類の判定方法（方法 1、方法 2、方法 3）についてまとめる。

方法 1 は個々の実験で判定を行い、3 回の実験の判定結果をもって最終判定を行う方法である。3 回の実験それぞれにおいて、濃度 0 群と各濃度群の間に何らかの統計的に有意な違いがみられるかどうかを判定したあと、それら 3 回の判定結果をまとめることで濃度 0 群に対する化学物質の影響を評価する。

それに対して方法 2 は、独立した 3 回の実験で構成されている試験法の特徴を重視し、3 回の実験から得られた測定値を統合したデータセットを作成し、このデータセットに基づきデータ解析を行った結果から最終判定を行う方法である。それぞ

れの実験の違いは、データセットに含める変数の 1 つとすることで、データ解析では実験間の影響の調整を行ったうえで判定を行う。

また、方法 2 は、方法 1 と同様に 3 回の実験を統合し、最終判定を行う方法であるが、方法 1 のように測定値を使うのではなく、得られる測定値を%換算した値を用いてデータ解析し、判定を行う点で方法 1 とは異なる。%換算した値の利用は、実験施設ごとで測定に用いる機械が異なることによって生じる測定値のスケールの大きさの違いの影響を排除することができるかと期待されている。

### 各実験から得られる測定値について

MITA では、内部標準プロモーターである SLR、INF- プロモーターである SLO、IL-2 プロモーターである SLG が導入された細胞を使い、その発光の度合いで免疫の活性の程度が測定される。1 回の実験において、96 穴プレートの各セルから SLG-LA (SLG ルシフェラーゼ活性)、SLO-LA (SLO ルシフェラーゼ活性)、SLR-LA (SLR ルシフェラーゼ活性) の 3 種類の発光に関する測定値が得られる。化学物質の評価において、第 i 番目の実験 (i=1,2,3) の第 j 番目の濃度 (j=0,1,2,...,10) の第 k 番目の繰り返しの測定値をそれぞれ  $SLG_{ijk}$ 、 $SLO_{ijk}$ 、 $SLR_{ijk}$  とする。

これらの測定値を用いて、判定のために以下の 4 種類の値が必要となる。

$$I.I.SLR_{ij} = (\overline{SLR}_{ij}) / (\overline{SLR}_{i0}),$$

$$nSLG_{ijk} = SLG_{ijk} / SLR_{ijk},$$

$$\% \text{supp}_{ij} = \left\{ 1 - \frac{(nSLG_{ij})}{(nSLG_{i0})} \right\} \times 100,$$

ただし、 $\overline{SLG}_{ij} = \sum_{k=1}^4 SLG_{ijk} / 4$ 、

$$\overline{SLR}_{ij} = \sum_{k=1}^4 SLR_{ijk} / 4 \text{ である。}$$

I.I.SLR<sub>0j</sub> = ( $\overline{SLR}_{0j}$ ) / ( $\overline{SLR}_{00}$ ) は細胞の状態が正常かどうかを判断するための指標である。nSLG<sub>ijk</sub> は



SLG<sub>ijk</sub> をプレート内の各セルにおいて標準化した値である。また、%supp<sub>ij</sub> は各濃度での 4 回の繰り返しの平均値を使って算出され、各濃度での免疫毒性の程度を%換算した値である。

## 方法

方法 は個々の実験で判定を行い、3 回の実験の判定結果をもって最終判定を行う方法である。

各実験において、 $I.I.SLR_j \geq 0.05$  ( $j=0, \dots, J$ )をみたく濃度の nSLG<sub>ijk</sub> を以降のデータ解析に採用する。nSLG<sub>ijk</sub> を用いて、いずれかの濃度群の間に差があるかどうかを知るために有意水準 5%で 1way ANOVA を行う。この検定の帰無仮説は、「第 i 番目の変数におけるすべての濃度群における nSLG の母平均は等しい」である。その結果、統計的に有意な差がみられた場合は、濃度 0 群と各濃度群の対比較である両側 Dunnett 検定を有意水準 5%で行う。この検定の帰無仮説は「第 i 番目の実験における濃度 0 群と第 j 濃度群のそれぞれにおける nSLG の母平均は等しい」である。

1way ANOVA と Dunnett 検定の結果に基づいて、まず個々の実験の判定を suppression(-)、augmentation(+)、no effect(0)のいずれかとする。3 回の実験の判定結果が 3 回とも suppression(-)もしくは augmentation(+)で一致した場合は、最終判定はそれぞれ immunosuppression、immunoaugmentation となる。

3 回の実験の判定結果が一致しなかった場合は、それぞれの実験で、絶対値が最大となる

$\max_{0 \leq j \leq 10} |\%supp_{ij}|$  の値を算出し、それら 3 つの値

を用いて両側 t 検定を有意水準 5%で行う。この検定の帰無仮説は「 $\max_{0 \leq j \leq 10} |\%supp_{ij}|$  の母平均は 0

である」である。負の方向に統計的に有意差があれば immunosuppression、正の方向に統計的に有

意差があれば immunoaugmentation、統計的に有意差がなければ no effect と最終判定を行う。

## 方法

方法 は、方法 のように単純に 3 回の実験の測定値から得られる結果を併合するのではなく、3 回の異なる実験の影響を調整した nSLG の平均の差を用いて、濃度 0 群と各濃度群の対比較である両側 Dunnett 検定を有意水準 5%で行うことで最終判定を行う方法である。この検定の帰無仮説は「第 i 番目の実験における濃度 0 群と第 j 濃度群のそれぞれにおける nSLG の母平均は等しい」である。

3 回の独立した実験における  $I.I.SLR_j \geq 0.05$  ( $j=0, \dots, J$ )をみたく濃度を算出し、3 回の実験のうち最も小さい濃度を  $J_{\min}$  とする。この方法では j の範囲を 1 から  $J_{\min}$  となる nSLG<sub>ijk</sub> を用いる。

3 回の異なる実験の影響を調整した Dunnett 検定は、一般線形モデル (GLM) を用いて計算することができる。モデルを

$$nSLG_{ijk} = \mu + \alpha_i + \beta_j + \varepsilon_{ijk}$$

とすると、独立した 3 回の実験の影響を調整した

nSLG<sub>ijk</sub> の平均の差の推定値は  $\hat{\beta}_j$  ( $j=1, \dots, J_{\min}$ )

として得ることができる。有意水準 5%の両側

Dunnett 検定は、 $\beta_j$  に対して上記の GLM モデル

の誤差の自由度を伴う両側 Dunnett の棄却限界値を利用して構成された同時 95%信頼区間を用いて判定を行うことが可能である。この同時 95%信頼区間の下限が 0 より大きな場合に

immunoaugmentation、上限が 0 より小さい場合は immunosuppression、同時信頼区間が 0 を含んでいる場合は no effect と最終判定を行う。

## 方法

方法 は、3 回の異なる実験の影響を調整した  $\%supp_{ij}$  の平均値の差を用いて、Conc.0 群と各濃度群の対比較である両側 Dunnett 検定を有意水準 5%で行うことで最終判定を行う方法である。この検定の帰無仮説は「第 i 番目の実験における濃度 0 群と第 j 濃度群のそれぞれにおける  $\%supp_{ij}$  の母平均は等しい」である。方法 と同様に、一般線形モデル (GLM) を用いて計算することができる。

モデルを

$$\%supp_{.ij} = \mu^* + \alpha_i^* + \beta_j^* + \varepsilon_{ij}^*$$

とすると、独立した 3 回の実験の影響を調整した  $\%supp_{ij}$  の平均の差の推定値は  $\hat{\beta}_j^*$  ( $j=1, \dots, J_{\min}$ ) として得ることができる。有意水準 5%の両側 Dunnett 検定は、 $\hat{\beta}_j^*$  に対して上記の GLM モデル

の誤差の自由度を伴う両側 Dunnett の棄却限界値を利用して構成された同時 95%信頼区間を用いて判定を行うことが可能である。この同時 95%信頼区間の下限が 0 より大きな場合には immunoaugmentation、上限が 0 より小さい場合は immunosuppression、同時信頼区間が 0 を含んでいる場合は no effect と最終判定を行う。

### Phase1 研究のデザイン

Phase1 研究は、MITA の施設内及び施設間再現性を評価するために行われた。この研究では食品薬品安全性センター (FDSC)、産業技術総合研究所つくば本部 (AIST-Tsukuba)、産業技術総合研究所四国センター (AIST-Takamatsu) の 3 施設が参加し、5 種類のブラインド化された化学物質分の試験が 3 セット (1<sup>st</sup> round、2<sup>nd</sup> round、3<sup>rd</sup> round) 行われた。

### C . 研究結果

方法 から方法 のそれぞれで検討した結果を以下の図 1、表 1 から 3 に示す。

( 図 1 は別紙に示す )

図 1 は、各施設の各物質ごとに判定で用いている濃度反応関係を描いている。Criteria1、Criteria2、Criteria3 はそれぞれ方法 、方法 、方法 に対応している。方法 は 3 回の実験の一つ一つについて判定がなされるために、3 つの濃度反応曲線を描いた。一方、方法 、方法 は 3 回の実験を統合したデータセットを作成し、解析を行うために濃度反応曲線は 1 つとなる。

( 表 1 は別紙に示す )

表 1 の S、A、N は、3 回の独立した実験結果をもってそれぞれ「Immunosuppression」「Immunoaugmentation」「No effect」の最終判定結果を示す。また 3 回の独立した実験結果は括弧の中に左から順に示す。また、表 1 の赤色と青色で色をつけた部分は 3 回の独立した実験結果をもってそれぞれ「Immunosuppression」「No effect」と施設内で判定が一致したことを示す。

( 表 2 は別紙に示す )

表 2 中の赤色と緑色で色をつけた部分は 3 セットの実験結果をもってそれぞれ「Immunosuppression」「Immunoaugmentation」と施設内で判定が一致したことを示す。

( 表 3 は別紙に示す )

表 3 中の赤色で色をつけた部分は 3 セットの実

験結果をもって「Immunosuppression」と施設内で判定が一致したことを示す。

#### D. 考察

図 1 からは 3 回の実験で濃度反応関係が異なっても、併合すると濃度反応関係は施設内、施設間ともに似た曲線になっていることがわかる。このことより、MITA は 3 回の実験によって判定するという開発の方針は支持できると言えるであろう。

表 1~3 から施設内再現性については、方法 A は 3/5 となる物質はなかったが、施設間再現性はいずれの方法も 3/5 であったことがわかる。被験物質 1、3、4 はいずれの方法でも判定結果が同じであり、被験物質 2 と 5 の判定が異なっており、これら 2 物質の結果の詳細が必要である。

これらの 2 つの物質について図 1 の特に方法 A、B を見ると濃度反応関係は試験間によって大きく異なっているとは言えず、判定結果の違いは、判定方法に起因する微細な部分であると考えた方がよいであろう。

方法 A は、独立した 3 回の実験の判定結果が異なった場合に行う t 検定において、異なる濃度の値を用いてしまう可能性に対する懸念など、最終判定に至るまでの複雑さがある。また、方法 A、B では Dunnett 検定において濃度 0 群に対してある 1 点の濃度で有意な差があれば immunosuppression もしくは immunoaugmentation と判定されるため、用量依存性を考慮すべきかもしれない。

上記の考察から、どの方法も適切という結論を導くことは難しい。そこで、方法 A、B を修正した判定方法である方法 C' と方法 D' を以下に提案する。これらの方法を採用した場合、Phase1 で得られた結果は改善するが、これは結果を見た後の修正であり、今後の Phase 2 で適用した評価が必要である。また、これらの提案は方針の状態で

あり、今後データを解析するためのデータシートの作成が必要となる。

#### 方法 C'

個々の実験で判定を行い、3 回の実験の判定結果をもって最終判定を行うという方針は方法 A と同様であるが、個々の実験において判定を行う際に Dunnett 検定の有意差の有無だけでなく、その有意となった時の傾向も考慮するという点で方法 A とは異なる。3 回の独立した各実験において、の同時 95%信頼区間の上限が 2 点連続で 0 より小さい場合、もしくは の同時 95%信頼区間の上限が 0 より小さく、かつその付近の 3 濃度点以上の が同じ傾向であれば suppression(-)、下限が 2 点連続で 0 より大きい場合、もしくは の同時 95%信頼区間の上限が 0 より大きく、かつその付近の 3 濃度点以上の が同じ傾向であれば 2 点連続で 0 より大きい場合には augmentation(+)、それ以外の場合は no effect(0)と判定する。

3 回のそれぞれの判定結果の多数決で、その化学物質の最終の毒性判定をおこなう。

#### 方法 D'

3 回の異なる実験の影響を調整した  $\%supp_{ij}$  の平均値の差を用いて最終判定を行うという方針は方法 A と同様である。最終判定を行う際に、Dunnett 検定の有意差の有無だけでなく、その有意となった時の傾向も考慮する。

$\hat{\beta}_i^*$  について得られる同時 95%信頼区間を用いて、この同時 95%信頼区間の下限が 2 点連続で 0 より大きな場合、もしくは下限が 0 より小さく、かつその付近の 3 濃度点以上の  $\hat{\beta}_i^*$  が同じ傾向の場合には Immunosuppression、上限が 2 点連続で 0 より大きな場合、もしくは上限が 0 より小さく、かつその付近の 3 濃度点以上の  $\hat{\beta}_{ij}^*$  が同じ傾向の場合には Immunoaugmentation と最終判定を行う。

また、上記の 2 種類の条件を両方満たす場合には  
Immunosuppression / Immunoaugmentation と  
最終判定を行う。

#### **E . 結論**

MITA による化学物質の評価のために検討した  
3 種類の方法は、まだ検討の余地がある。濃度反応  
関係を考慮した判定が必要であろう。

謝辞：本検討を進めるにあたり解析のためのデー  
タシートの作成とデータの整理を協力してくれた  
神戸大学医学研究科の小林真弓氏に感謝いたしま  
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。

#### **参考文献**

- 1) Littell, R. C., Stroup, W. W., Freund, R.  
J. SAS for linear models. SAS, 2002.
- 2) 永田靖、吉田道弘. 統計的多重比較の基  
礎. サイエンティスト社, 1997.

Multi-Immuno Tox Assay(MITA)バリデーション Phase1 研究のデータ解析方法の検討と結果の報告 別紙

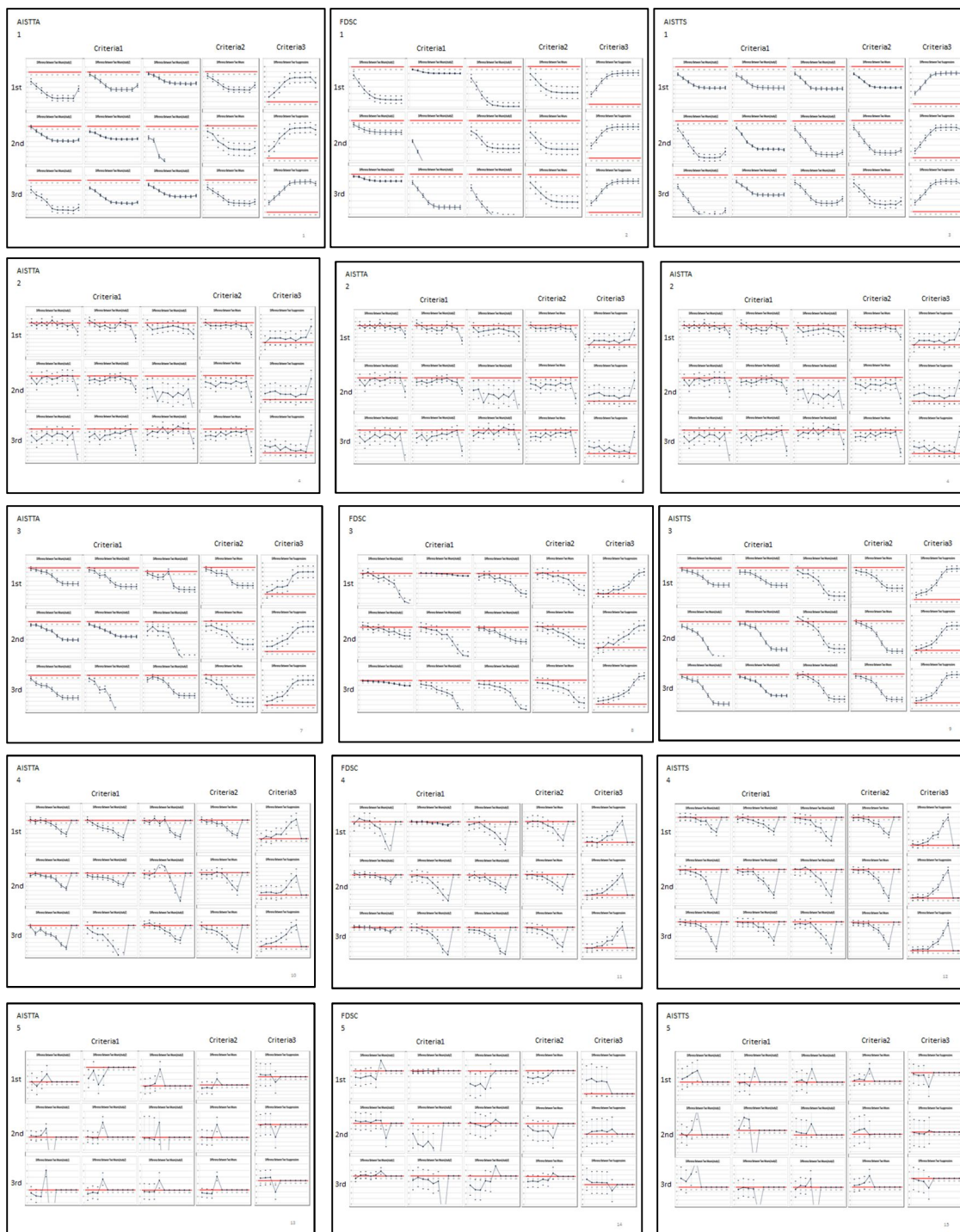


図 1 各判定方法と濃度反応関係

表 1 方法 の 3 施設の結果

		Within				
		4/5	5/5	5/5		
Chemical		AIST-Tsukuba	FDSC	AIST-Takamatsu		
1	solubility	500mg/ml(DMSO)	500mg/ml(DMSO)	500mg/ml(DMSO)	3/5	}
	1st round	S(-/-)	S(-/-)	S(-/-)		
	2nd round	S(-/-)	S(-/-)	S(-/-)		
	3rd round	S(-/-)	S(-/-)	S(-/-)		
2	solubility	125mg/ml(DMSO)	125mg/ml(DMSO)	125mg/ml(DMSO)		
	1st round	N(-/+)	S(-/-)	S(-/-)		
	2nd round	N(+/-/0)	S(-/-)	S(-/-)		
	3rd round	N(-/0/0)	S(-/-)	S(-/-)		
3	solubility	100mg/ml(ddw)	25mg/ml(ddw)	100mg/ml(ddw)		
	1st round	S(-/-)	S(-/-)	S(-/-)		
	2nd round	S(-/-+)	S(-/-)	S(-/-)		
	3rd round	S(-/-)	S(-/-)	S(-/-)		
4	solubility	100mg/ml(ddw)	100mg/ml(ddw)	100mg/ml(ddw)		
	1st round	S(-/-)	S(-/-)	S(-/-)		
	2nd round	S(-/-)	S(-/-)	S(-/-+)		
	3rd round	S(-/-)	S(-/-)	S(-/-)		
5	solubility	62.5mg/ml(DMSO)	15.625mg/ml(DMSO)	62.5mg/ml(DMSO)		
	1st round	A(0/+/+)	N(+/-/0)	N(0/-/+)		
	2nd round	N(+/-/0)	N(-/0)	N(+/-/+)		
	3rd round	N(+/-/+)	N(+/-/+)	N(+/-/+)		

表 2 . 方法 の 3 施設の結果

		Within				
		3/5	4/5	5/5		
Chemical		AIST-Tsukuba	FDSC	AIST-Takamatsu		
1	solubility	500mg/ml(DMSO)	500mg/ml(DMSO)	500mg/ml(DMSO)	3/5	}
	1st round	Immunosuppression	Immunosuppression	Immunosuppression		
	2nd round	Immunosuppression	Immunosuppression	Immunosuppression		
	3rd round	Immunosuppression	Immunosuppression	Immunosuppression		
2	solubility	125mg/ml(DMSO)	125mg/ml(DMSO)	125mg/ml(DMSO)		
	1st round	Immunosuppression	Immunosuppression	Immunosuppression		
	2nd round	Immunoaug./supp.	Immunosuppression	Immunosuppression		
	3rd round	Immunosuppression	Immunosuppression	Immunosuppression		
3	solubility	100mg/ml(ddd)	25mg/ml(ddd)	100mg/ml(ddd)		
	1st round	Immunosuppression	Immunosuppression	Immunosuppression		
	2nd round	Immunosuppression	Immunosuppression	Immunosuppression		
	3rd round	Immunosuppression	Immunosuppression	Immunosuppression		
4	solubility	100mg/ml(ddd)	100mg/ml(ddd)	100mg/ml(ddd)		
	1st round	Immunosuppression	Immunosuppression	Immunosuppression		
	2nd round	Immunosuppression	Immunosuppression	Immunosuppression		
	3rd round	Immunosuppression	Immunosuppression	Immunosuppression		
5	solubility	62.5mg/ml(DMSO)	15.625mg/ml(DMSO)	62.5mg/ml(DMSO)		
	1st round	Immunoaugmentation	Immunosuppression	Immunoaugmentation		
	2nd round	No effect	Immunosuppression	Immunoaugmentation		
	3rd round	Immunoaugmentation	No effect	Immunoaugmentation		

表 3 . 方法 の結果

		Within						
		3/5	4/5	5/5				
Chemical		AIST-Tsukuba	FDSC	AIST-Takamatsu				
1	solubility	500mg/ml(DMSO)	500mg/ml(DMSO)	500mg/ml(DMSO)	3/5	}		
	1st round	Immunosuppression	Immunosuppression	Immunosuppression				
	2nd round	Immunosuppression	Immunosuppression	Immunosuppression				
	3rd round	Immunosuppression	Immunosuppression	Immunosuppression				
2	solubility	125mg/ml(DMSO)	125mg/ml(DMSO)	125mg/ml(DMSO)				
	1st round	Immunosuppression	Immunosuppression	Immunosuppression				
	2nd round	Immunosuppression	Immunosuppression	Immunosuppression				
	3rd round	Immunoaug./supp.	Immunosuppression	Immunosuppression				
3	solubility	100mg/ml(ddw)	25mg/ml(ddw)	100mg/ml(ddw)				
	1st round	Immunosuppression	Immunosuppression	Immunosuppression				
	2nd round	Immunosuppression	Immunosuppression	Immunosuppression				
	3rd round	Immunosuppression	Immunosuppression	Immunosuppression				
4	solubility	100mg/ml(ddw)	100mg/ml(ddw)	100mg/ml(ddw)				
	1st round	Immunosuppression	Immunosuppression	Immunosuppression				
	2nd round	Immunosuppression	Immunosuppression	Immunosuppression				
	3rd round	Immunosuppression	Immunosuppression	Immunosuppression				
5	solubility	62.5mg/ml(DMSO)	15.625mg/ml(DMSO)	62.5mg/ml(DMSO)				
	1st round	Immunoaugmentation	Immunosuppression	No effect				
	2nd round	No effect	No effect	Immunoaugmentation				
	3rd round	No effect	No effect	Immunoaugmentation				



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Multi-ImmunoTox assay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った（Phase 0）。そこで明らかになった問題点を検討し試験法プロトコールを改良、データシート、記録用紙を作成しPhase I試験を行った。

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を行う。

B．研究方法

以下の方法によりIL-2およびIFN- $\gamma$ 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- $\gamma$ 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 $2 \times 10^5$ 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<sub>2</sub>下で1時間培養した。つづいて25nM PMAと1 $\mu$ M I $\alpha$ の混合物(PMA/I $\alpha$ )で刺激し37℃、5%CO<sub>2</sub>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<sup>1)</sup>。

## C. 結果

### C-1. 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<sub>3</sub>HgCl, Chloroquine diphosphate salt, Citral, Dexamethasone)これらの化学物質を 3 回ずつアッセイする Phase 0 試験を実施した。

### C-2. 試験法プロトコール、データシート、記録用紙の作成

Phase 0試験で明らかになった問題点を検討し以下のようにプロトコールを変更した。その変更を反映した試験法プロトコール、Multi-Immuno Tox Assay protocol ver. 008.5Eを作成した(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. 007.2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)。

## 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、1の結果を参照としPhase 2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った。そこで明らかになった問題点を検討し試験法プロトコールを改良、データシート、記録用紙を作成しPhase I試験を行った。

## 引用文献

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. 008.5E
- 2) Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 007.2
- 3) Multi-ImmunoTox Assay 記録用紙 Ver. 002

## G. 研究発表

### 1. 論文発表

Kimura Y, Shimada-Omori R, Takahashi T,

Tsuchiyama K, Kusakari Y, Yamasaki K, Nishikawa R, Nishigori C, Aiba S., Therapeutic drug monitoring of patients with psoriasis during tumour necrosis factor (TNF)-alpha antagonist treatment using a novel interleukin-8 reporter cell line. Br J Dermatol 175, 979-987, 2016..

## 2 . 学会発表

木村裕、相場節也：試験法ワークショップ「IL-2 転写活性抑制をkey eventとするT細胞分化異常誘導に関するAOP」第23回日本免疫毒性学会学術年会（北九州）2016年9月7日

## 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

Multi-Immuno Tox Assay protocol ver. 008.5E  
Sep. 14th, 2016

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1. Introduction .....	5
2. Materials.....	6
2-1 Cells	6
2-2 Reagents and equipment	6
2-2-1 For maintenance of the #2H4 cells .....	6
2-2-2 For chemical exposure, stimulation and solvents .....	6
2-2-3 For measurement of the luciferase activity.....	6
2-2-4 Expendable supplies.....	6
2-2-5 Equipment for measurement of luciferase activity.....	7
2-2-6 Others .....	7
2-3 Culture medium	8
2-3-1 A medium: for maintenance of #2H4 cells (500 mL, stored at 2-8°C).....	8
2-3-2 B medium: for luciferase assay (30 mL, stored at 2-8°C).....	8
2-3-3 C medium: for thawing #2H4 cells (30 mL, stored at 2-8°C) .....	8
2-4 Preparation of the stimulant of #2H4	9
2-4-1 Phorbol 12-myristate 13-acetate (PMA) .....	9
2-4-2 Ionomycin .....	9
3. Cell culture .....	10
3-1 Thawing of #2H4 cells	10
3-2 Maintenance of #2H4 cells	10
4. Preparation of cells for assay .....	11
5. Preparation of chemicals and cell treatment with chemicals.....	12
5-1 Dissolution by vehicle (cf. Figure 3)	12
5-2 When the chemical is prepared as 25, 50 or 100 mg/mL in distilled water	14

5-2-1	Arrangement of chemicals and vehicle.....	14
5-2-2	Serial dilution.....	14
5-2-3	2 step dilution.....	15
5-3	When the chemical is prepared as a 500 mg/mL DMSO solution	17
5-3-1	Arrangement of chemicals and vehicle.....	17
5-3-2	Serial dilution.....	17
5-3-3	Dilution of DMSO solution with the B medium .....	18
5-3-4	2 step dilution.....	19
6.	Preparation of the stimulant (PMA/ionomycin) and addition to #2H4 .....	21
6-1	Material	21
6-2	Preparation of 100 $\mu$ M PMA	21
6-3	Preparation of control and x10 PMA/ionomycin solution	21
6-4	Addition of PMA/ionomycin to #2H4	22
7.	Control .....	23
7-1	Preparing control chemical (dexamethasone, cyclosporine A)	23
7-1-1	Preparing dexamethasone stock .....	23
7-1-2	Preparing cyclosporine A stock.....	23
7-2	Preparation of cells for assay	24
7-3	Arrangement of chemicals and vehicle	25
7-4	Dilution with the B medium	25
7-5	2 step dilution	26
7-6	Addition of PMA/ionomycin to #2H4	28
8.	Calculation of the transmittance factors .....	29
8-1	Reagents	29

8-2	Preparation of luminescence reaction solution	29
8-3	Bioluminescence measurement	29
9.	Measurement.....	33
10.	Data analysis.....	36
11.	Criteria.....	36
11-1	Acceptance criteria	36
11-2	The criteria to identify immunotoxicants by the Multi-Immuno Tox Assay	36
12.	Update record.....	39
Appendix 1	Principle of measurement of luciferase activity.....	41
Appendix 2	Validation of reagents and equipment .....	43

## 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, IFN $\gamma$  and G3PDH promoters, respectively, for the Multi-Immuno Tox Assay.

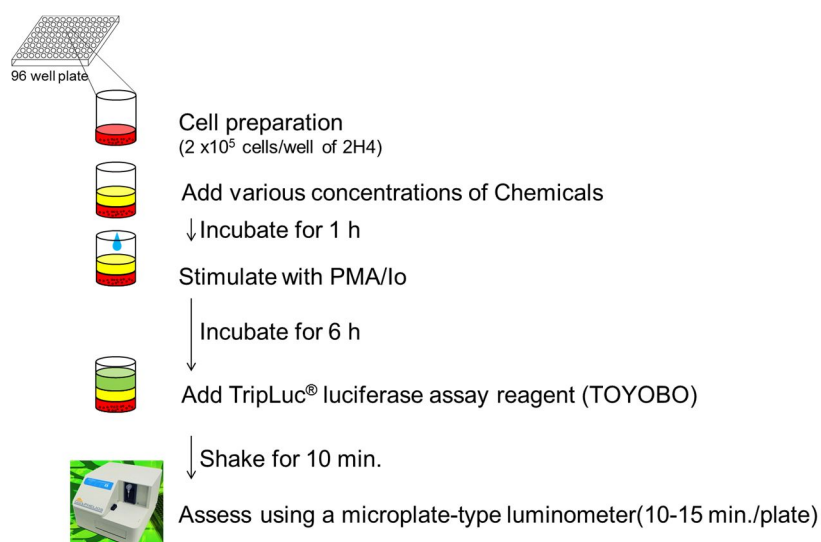
(Kimura Y. et al. Evaluation of the Multi-ImmunoTox Assay composed of 3 human cytokine reporter cells by examining immunological effects of drugs *Toxicol in Vitro*, 28, 759-768, 2014)

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/I o only	A/2 <sup>9</sup> $\mu\text{g/ml}$	A/2 <sup>8</sup> $\mu\text{g/ml}$	A/2 <sup>7</sup> $\mu\text{g/ml}$	A/2 <sup>6</sup> $\mu\text{g/ml}$	A/2 <sup>5</sup> $\mu\text{g/ml}$	A/2 <sup>4</sup> $\mu\text{g/ml}$	A/2 <sup>3</sup> $\mu\text{g/ml}$	A/2 <sup>2</sup> $\mu\text{g/ml}$	A/2 <sup>1</sup> $\mu\text{g/ml}$	A $\mu\text{g/ml}$
B												
C												
D												
Chemical A (common ratio of 2, 10 concentrations, n=4)												
E	cont (distilled water or DMSO)	PMA/I o only	B/2 <sup>9</sup> $\mu\text{g/ml}$	B/2 <sup>8</sup> $\mu\text{g/ml}$	B/2 <sup>7</sup> $\mu\text{g/ml}$	B/2 <sup>6</sup> $\mu\text{g/ml}$	B/2 <sup>5</sup> $\mu\text{g/ml}$	B/2 <sup>4</sup> $\mu\text{g/ml}$	B/2 <sup>3</sup> $\mu\text{g/ml}$	B/2 <sup>2</sup> $\mu\text{g/ml}$	B/2 <sup>1</sup> $\mu\text{g/ml}$	B $\mu\text{g/ml}$
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、IFN $\gamma$ -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 IFN $\gamma$  reporter cell line, #2H4, that harbors the SLG, SLO and SLR luciferase genes under the control of the IL-2, IFN $\gamma$  and GAPDH promoters, respectively, was established by Tsuruga Institute of Biotechnology, TOYOBO Co. Ltd.

(Saito R. et al. Nickel differentially regulates NFAT and NF- $\kappa$ B 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<sup>®</sup> 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  $\mu$ clear black plate (flat-bottom, for measurement of the luciferase)

activity, e.g. Greiner Bio-one Cat#655090)

- 96 well clear plate (round-bottom, for preparation of chemicals and stimulants)
- 96 well assay block, 2 mL (e.g., Costar Cat#3960)
- Seal for 96 well plate (e.g., Perkin Elmer TopSeal-A PLUS Cat#6050185, EXCEL Scientific SealMate Cat#SM-KIT-SP)
- Reservoir
- Pipette

#### 2-2-5 Equipment for measurement of luciferase activity

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

#### 2-2-6 Others

- Pipetman
- 8 channel or 12 channel pipetman (optimized for 10~100  $\mu\text{L}$ )
- Plate shaker (for 96 well plate)
- CO<sub>2</sub> incubator (37°C, 5% CO<sub>2</sub>)
- 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 $\mu$ g/mL	7.5 $\mu$ L
G418	Nacalai tesque #16513-84	50 mg/mL	300 $\mu$ g/mL	3 mL
HygromycinB	Invitrogen #10687-010	50 mg/mL	200 $\mu$ g/mL	2 mL

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

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

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

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

## 2-4 Preparation of the stimulant of #2H4

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

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

Dissolve 1 mg PMA using DMSO 811  $\mu$ L, dispense at 5  $\mu$ L/tube and store at freezer at  $-30^{\circ}\text{C}$ . Use these stocks within 6 month after dissolution.

## 2-4-2 Ionomycin

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

Dissolve 1mg Ionomycin using ethanol 669.3  $\mu$ L, dispense at 30  $\mu$ L/tube and store at freezer at  $-30^{\circ}\text{C}$ . 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<sub>2</sub> incubator (for culture).

Thaw frozen cells ( $2 \times 10^6$  cells / 0.5 mL of freezing medium) in a 37°C water bath, then add to a 15 mL polypropylene conical tube containing 9 mL of pre-warmed C medium. Centrifuge the tube at 120-350 x g at room temperature for 5 min, discard the supernatant, and resuspend in 15 mL of pre-warmed C medium in a T-75 Flask. Cells are incubated at 37°C, 5% CO<sub>2</sub>.

#### 3-2 Maintenance of #2H4 cells

Pre-warm the A medium in a T-75 Flask at 37°C in a 5% CO<sub>2</sub> incubator. The culture medium should be changed to the A medium 3 or 4 days after thawing. At that time, count the number of cells, centrifuge the tube at 120-350 x g at room temperature for 5 min, discard the supernatant, and resuspend in pre-warmed the A medium in a T-75 Flask. Cells are passaged at  $3 \times 10^5$ /mL and incubated at 37°C, 5% CO<sub>2</sub>.

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 ( $2.0 \times 10^7$  cells for two chemical are required, but to have some leeway,  $3.0 \times 10^7$  cells for two chemical should be prepared), centrifuge the tube at  $120\text{-}350 \times g$ , 5 min. Resuspend in pre-warmed the B medium at a cell density of  $4 \times 10^6/\text{mL}$ . Transfer the cell suspension to a reservoir, and add 50  $\mu\text{L}$  of cell suspension to each well of a 96 well  $\mu\text{clear}$  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 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL
B	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL
C	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL
D	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL
E	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL
F	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL
G	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL
H	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL

## 5. Preparation of chemicals and cell treatment with chemicals

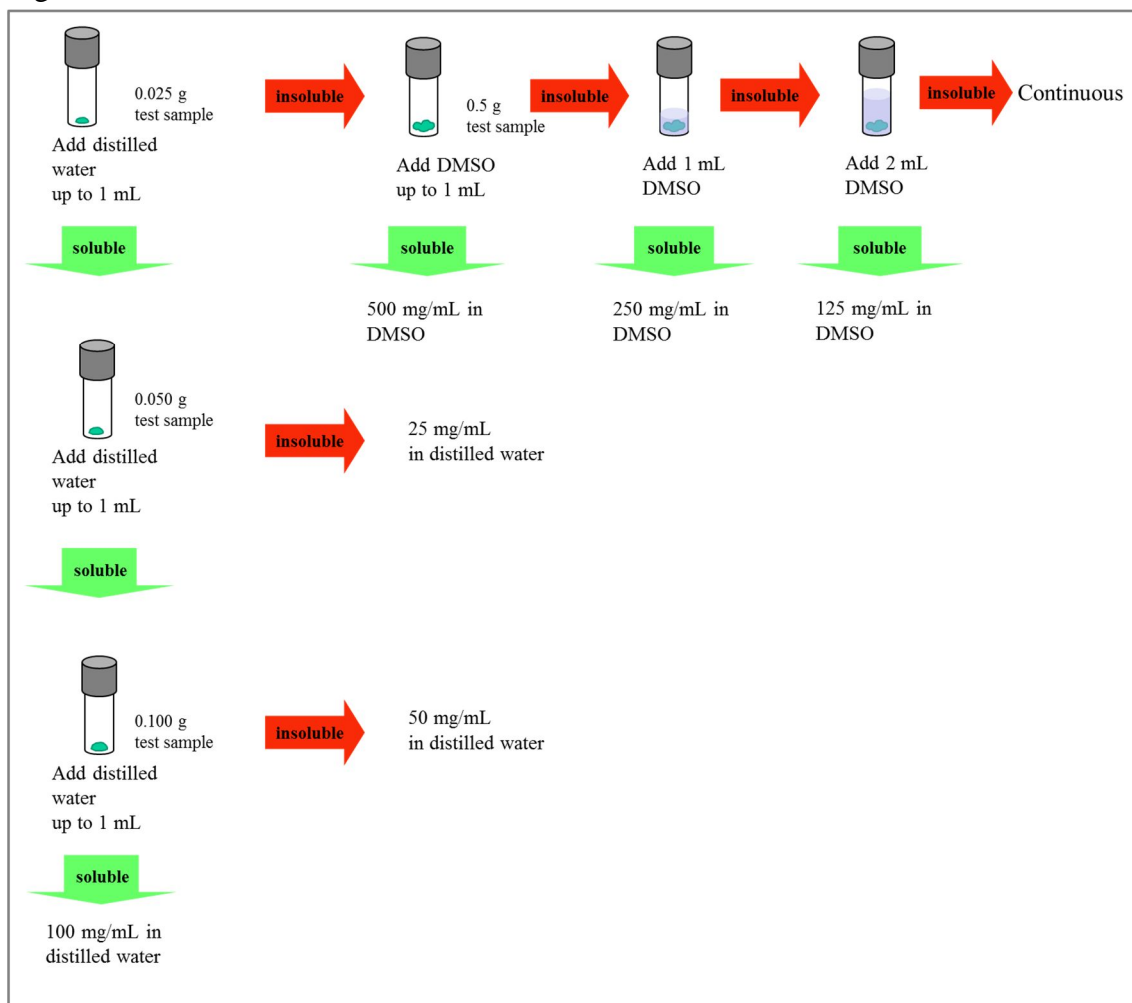
### 5-1 Dissolution by vehicle (cf. Figure 3)

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





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.

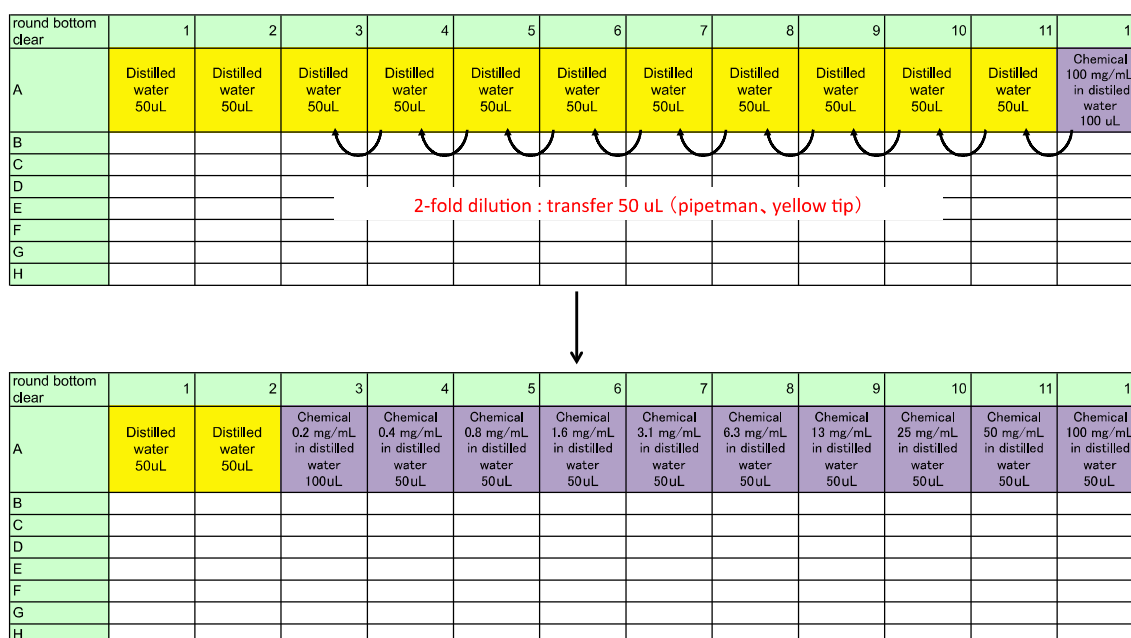
5-2-1 Arrangement of chemicals and vehicle

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



5-2-3 2 step dilution

Add 20  $\mu\text{L}$  of the diluted chemical to 480  $\mu\text{L}$  of the B medium prepared in the assay block. And add 50  $\mu\text{L}$  to #2H4 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Seal the plate, shake the plate with a plateshaker and incubate in a  $\text{CO}_2$  incubator for 1 hour ( $37^\circ\text{C}$ ,  $\text{CO}_2$ , 5%)(cf. Figure 5-7).

Figure 5

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

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 480 $\mu\text{L}$	B medium 480 $\mu\text{L}$	B medium 480 $\mu\text{L}$	B medium 480 $\mu\text{L}$	B medium 480 $\mu\text{L}$	B medium 480 $\mu\text{L}$	B medium 480 $\mu\text{L}$	B medium 480 $\mu\text{L}$	B medium 480 $\mu\text{L}$	B medium 480 $\mu\text{L}$	B medium 480 $\mu\text{L}$	B medium 480 $\mu\text{L}$
B												
C												
D												
E												
F												
G												
H												

20 $\mu\text{L}$



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  $\mu$ L of the 500 mg/mL DMSO solution of the chemical to well #A12, 50  $\mu$ L of DMSO to wells #A1-#A11, and 90  $\mu$ L 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  $\mu$ L 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  $\mu$ L of the DMSO solution of the chemical in wells #A1-#A12 with 90  $\mu$ L 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% 50 $\mu$ L	DMSO 100% 50 $\mu$ L	Chemical 1.0 mg/mL in DMSO 100 $\mu$ L	Chemical 2.0 mg/mL in DMSO 50 $\mu$ L	Chemical 3.9 mg/mL in DMSO 50 $\mu$ L	Chemical 7.8 mg/mL in DMSO 50 $\mu$ L	Chemical 16 mg/mL in DMSO 50 $\mu$ L	Chemical 31 mg/mL in DMSO 50 $\mu$ L	Chemical 63 mg/mL in DMSO 50 $\mu$ L	Chemical 125 mg/mL in DMSO 50 $\mu$ L	Chemical 250 mg/mL in DMSO 50 $\mu$ L	Chemical 500 mg/mL in DMSO 50 $\mu$ L
B	B medium 90 $\mu$ L	B medium 90 $\mu$ L	B medium 90 $\mu$ L	B medium 90 $\mu$ L	B medium 90 $\mu$ L	B medium 90 $\mu$ L	B medium 90 $\mu$ L	B medium 90 $\mu$ L	B medium 90 $\mu$ L	B medium 90 $\mu$ L	B medium 90 $\mu$ L	B medium 90 $\mu$ L
C												
D												
E												
F												
G												
H												

10 $\mu$ L

↓

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

5-3-4 2 step dilution

Add 10  $\mu\text{L}$  of the diluted chemical to 490  $\mu\text{L}$  of the B medium prepared in the assay block. And add 50  $\mu\text{L}$  to #2H4 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Manipulate the procedures from 5-3-3 to 5-3-4 as quickly as you can, and do not leave a long time at step after 5-3-3 or Figure 10. Seal the plate, shake the plate with a platemaker and incubate in a  $\text{CO}_2$  incubator for 1 hour ( $37^\circ\text{C}$ ,  $\text{CO}_2$ , 5%) (cf. Figure 10-12).

Figure 10

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

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												

10uL



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

### 6-1 Material

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

### 6-2 Preparation of 100 $\mu$ M PMA

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

2 mM PMA	B medium	Total	final concentration
5 $\mu$ L	95 $\mu$ L	100 $\mu$ L	100 $\mu$ M

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

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

	B medium	2 mM Ionomycin	100 $\mu$ M PMA	Ethanol	Total
Control	995 $\mu$ L	-		5 $\mu$ L	1000 $\mu$ L
x10 PMA/ionomycin solution	2382 $\mu$ L	12 $\mu$ L	6 $\mu$ L	-	2400 $\mu$ L



6-4 Addition of PMA/ionomycin to #2H4

One hour after the addition of chemicals, add 10  $\mu$ L of control or PMA/ionomycin solution to the cells (#A1-#H1 or #A2-#H12, respectively) using an 8 channel or 12 channel pipetman after pipetting 20 times. Make sure that the apex of the tip is dipped into the medium. Change tips every line you add. Seal the plate, shake the plate with a plateshaker and incubate in a CO<sub>2</sub> incubator for 6 hour (37°C, CO<sub>2</sub>, 5%). (cf. Figure 13)

Figure 13

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

10uL

10uL

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A												
B						Chemical A						
C						Chemical A						
D					Cell:#2H4							
E						Chemical B						
F						Chemical B						
G						Chemical B						
H						Chemical B						

## 7. Control

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

#### 7-1-1 Preparing dexamethasone stock

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

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

#### 7-1-2 Preparing cyclosporine A stock

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

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

## 7-2 Preparation of cells for assay

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

Use cells between 1 and 6 weeks after thawing.

Pre-warm the B medium in a 37°C water bath. Count the number of cells and collect the number of cells needed ( $5.0 \times 10^6$  cells are required, but to have some leeway,  $7.5 \times 10^6$  cells should be prepared), centrifuge the tube at 120-350 x g, 5 min. Resuspend in pre-warmed the B medium at a cell density of  $4 \times 10^6$ /mL. Transfer the cell suspension to a reservoir, and add 50  $\mu$ L of cell suspension to each well of a 96 well  $\mu$ clear 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 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL							
B	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL							
C	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL							
D	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL	#2H4 2x10 <sup>5</sup> B medium 50uL							
E												
F												
G												
H												

7-3 Arrangement of chemicals and vehicle

Add DMSO 50  $\mu$ L to #A4, 100  $\mu$ g/mL cyclosporine A stock 50  $\mu$ L to #A5, distilled water 50  $\mu$ L to #B1 and #B2, 2.5 mg/ml dexamethasone stock 50  $\mu$ L to #B3 and the B medium 180  $\mu$ L 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  $\mu$ L 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 100 ug/mL stock 50uL							
B	Distilled water 50uL	Distilled water 50uL	DEX 2.5 mg/mL stock 50uL	B medium 180uL	B medium 180uL							
C												
D												
E												
F												
G												
H												

↓

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A				DMSO 30uL	CyA 100 ug/mL stock 30uL							
B	Distilled water 50uL	Distilled water 50uL	DEX 2.5 mg/mL stock 50uL	DMSO 10% in B medium 200uL	CyA 10 ug/mL DMSO 10% in B medium 200 uL							
C												
D												
E												
F												
G												
H												

7-5 2 step dilution

Add 20  $\mu\text{L}$  of the diluted chemical or vehicle to 480  $\mu\text{L}$ (1-3 lanes) or 980  $\mu\text{L}$ (4-5 lanes) of the B medium prepared in the assay block. And add 50  $\mu\text{L}$  to #2H4 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Manipulate the procedures from 7-4 to 7-5 as quickly as you can, and do not leave a long time at step after 7-4 or Figure 16. Seal the plate, shake the plate with a plateshaker and incubate in a CO<sub>2</sub> incubator for 1 hour (37°C, CO<sub>2</sub>, 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 100 ug/mL stock 30uL							
B	Distilled water 50uL	Distilled water 50uL	DEX 2.5 mg/mL stock 50uL	DMSO 10% in B medium 200uL	CyA 10 ug/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 100 ug/mL B medium 500uL	DMSO 0.2% B medium 1000uL	CyA 200 ng/mL DMSO 0.2% B medium 1000uL							
B												
C												
D												
E												
F												
G												
H												

50uL

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

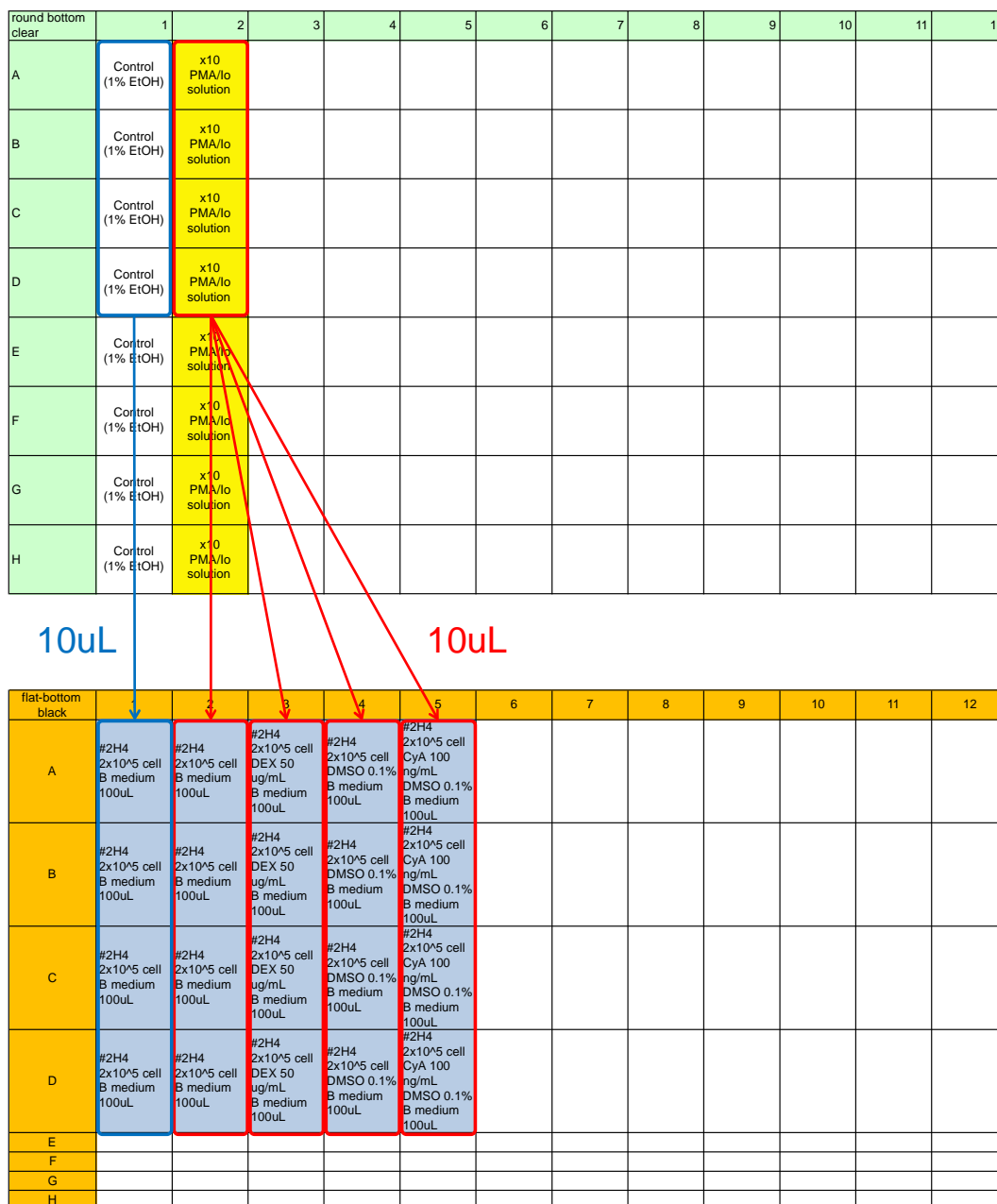
Figure 18 Final constituents of each well of the plate

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

7-6 Addition of PMA/ionomycin to #2H4

One hour after the addition of dexamethasone and cyclosporine A, add 10  $\mu$ L of control or PMA/ionomycin solution prepared in §6-3 to the cells (#A1-#D1 or #A2-#D5, respectively) using an 8 channel or 12 channel pipetman after pipetting 20 times. Make sure that the apex of the tip is dipped into the medium. Change tips every line you add. Seal the plate, shake the plate with a platemaker and incubate in a CO<sub>2</sub> incubator for 6 hour (37°C, CO<sub>2</sub>, 5%). (cf. Figure 19)

Figure 19





## 8. Calculation of the transmittance factors

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

### 8-1 Reagents

- Single reference samples:

Lyophilized luciferase enzyme reagent for stable luciferase green (SLG)

Lyophilized luciferase enzyme reagent for stable luciferase orange (SLO)

Lyophilized luciferase enzyme reagent for stable luciferase red (SLR)

- Assay reagent:

Tripluc<sup>®</sup> 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<sup>®</sup> 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  $\mu$ L of 100 mM Tris-HCl (pH8.0) contains 10 % glycerol to each tube of lyophilized reference sample to dissolve the enzymes, divide into 10  $\mu$ L 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  $\mu$ L sample per

tube). Keep the reference samples on ice to prevent deactivation.

### 8-3 Bioluminescence measurement

Transfer 100  $\mu\text{L}$  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 $\mu\text{L}$	SLG 100 $\mu\text{L}$	SLG 100 $\mu\text{L}$									
C												
D	SLO 100 $\mu\text{L}$	SLO 100 $\mu\text{L}$	SLO 100 $\mu\text{L}$									
E												
F	SLR 100 $\mu\text{L}$	SLR 100 $\mu\text{L}$	SLR 100 $\mu\text{L}$									
G												
H												

Transfer 100  $\mu\text{L}$  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:

$$\text{Transmittance factor } (\kappa_{GR56}) = \frac{\#B1 \text{ of F1} + \#B2 \text{ of F1} + \#B3 \text{ of F1}}{\#B1 \text{ of F0} + \#B2 \text{ of F0} + \#B3 \text{ of F0}}$$

$$\text{Transmittance factor } (\kappa_{OR56}) = \frac{\#D1 \text{ of F1} + \#D2 \text{ of F1} + \#D3 \text{ of F1}}{\#D1 \text{ of F0} + \#D2 \text{ of F0} + \#D3 \text{ of F0}}$$

$$\text{Transmittance factor } (\kappa_{RR56}) = \frac{\#F1 \text{ of F1} + \#F2 \text{ of F1} + \#F3 \text{ of F1}}{\#F1 \text{ of F0} + \#F2 \text{ of F0} + \#F3 \text{ of F0}}$$

$$\text{Transmittance factor } (\kappa_{GR60}) = \frac{\#B1 \text{ of F2} + \#B2 \text{ of F2} + \#B3 \text{ of F2}}{\#B1 \text{ of F0} + \#B2 \text{ of F0} + \#B3 \text{ of F0}}$$

$$\text{Transmittance factor } (\kappa_{OR60}) = \frac{\#D1 \text{ of F2} + \#D2 \text{ of F2} + \#D3 \text{ of F2}}{\#D1 \text{ of F0} + \#D2 \text{ of F0} + \#D3 \text{ of F0}}$$

$$\text{Transmittance factor } (\kappa_{RR60}) = \frac{\#F1 \text{ of F2} + \#F2 \text{ of F2} + \#F3 \text{ of F2}}{\#F1 \text{ of F0} + \#F2 \text{ of F0} + \#F3 \text{ of F0}}$$

In the case shown above,

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

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

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

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

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

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

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

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

Figure 23

	A	B	C	D	E	F
1	<b>MultiReporter Assay System -Tripluc<sup>®</sup>- Calculation Sheet</b>					
2						
3		<b>Transmittance Data</b>				
4			<b>SLG</b>	<b>SLO</b>	<b>SLR</b>	
5		<b>F0</b>	1	1	1	
6		<b>F1</b>	$\kappa_{G_{R56}}$	$\kappa_{O_{R56}}$	$\kappa_{R_{R56}}$	
7		<b>F2</b>	$\kappa_{G_{R60}}$	$\kappa_{O_{R60}}$	$\kappa_{R_{R60}}$	
8						

## 9. Measurement

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

Thaw Tripluc<sup>®</sup> 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  $\mu$ L 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.

1<sup>st</sup>. 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

Multi-ImmunoTox Assay Datasheet for #2H4 cells						
Ver. 005.2						
Laboratory					Round	
Exp.						
Date: (YYYY/MM/DD)			Operator:			
Code	Chemical 1		Dissolution	Chemical 1		
	Chemical 2			Chemical 2	mg/ml in	
Molecular weight	Chemical 1					
	Chemical 2					
Comment:						

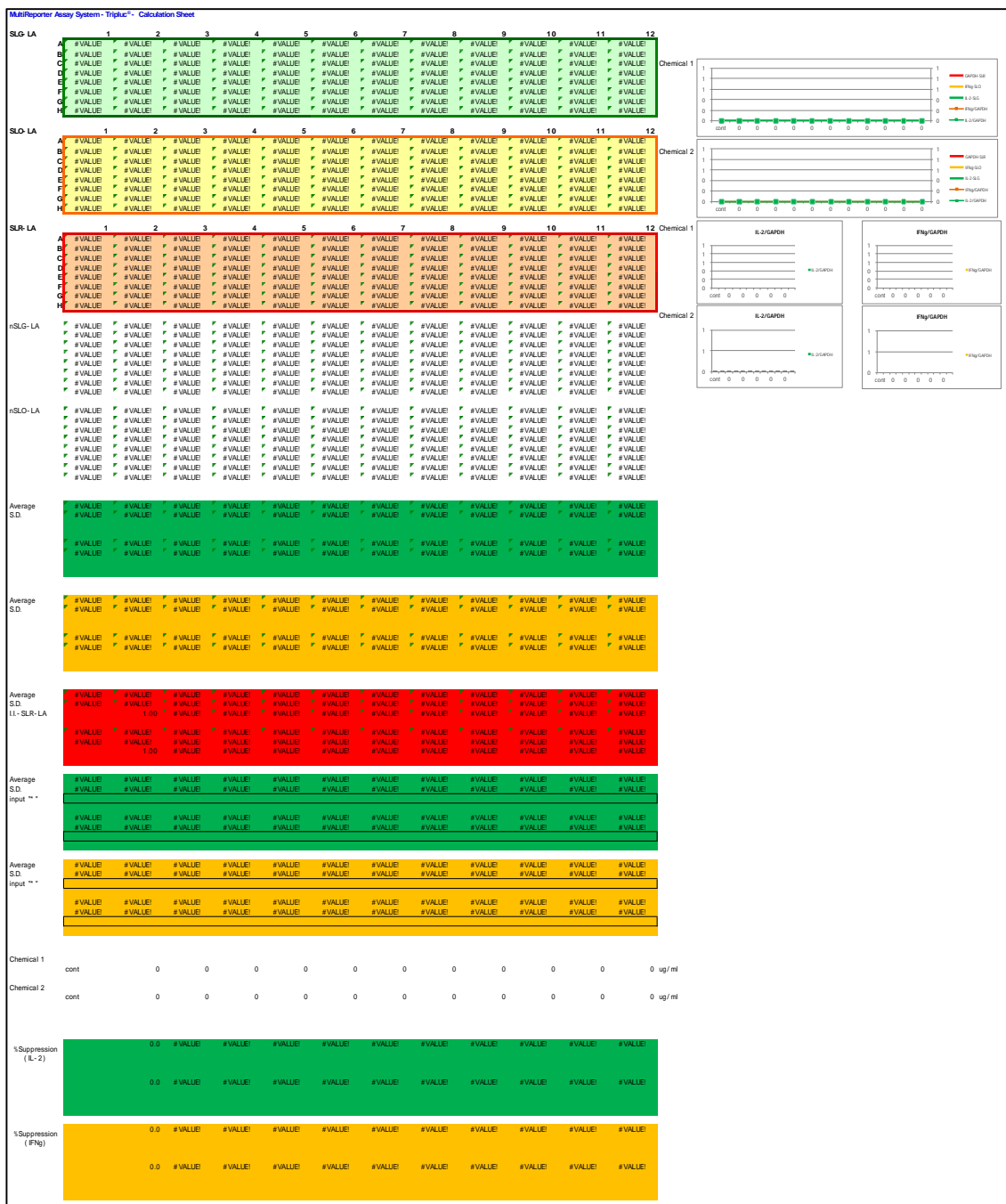
2<sup>nd</sup>. Copy the results of the F0, F1 and F2 measurements (values are expressed as counts) and paste them into the appropriate area in the “Data Input” sheet of the data sheet shown below. In addition, input the transmittance factors calculated in "§5. Calculation of the transmittance factors" 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 <sup>®</sup> - 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- $\gamma$  promoter)
- SLR-luciferase activity (SLR-LA): Luciferase activity of stable luciferase red  
(Under the control of G3PDH promoter)
- Normalized SLG-LA (nSLG-LA):  $=(\text{SLG-LA})/(\text{SLR-LA})$
- Normalized SLO-LA (nSLO-LA):  $=(\text{SLO-LA})/(\text{SLR-LA})$
- Inhibition index of SLR-LA (I.I.-SLR-LA): The cytotoxic effect of chemicals  
 $=(\text{SLR-LA of \#2H4 treated with chemicals})/(\text{SLR-LA of untreated \#2H4})$
- % suppression: The effect of chemicals on IL-2 or IFN- $\gamma$  promoter  
 $=(1-(\text{nSLG-LA or nSLO-LA of \#2H4 treated with chemicals})/(\text{nSLG-LA or nSLO-LA of non-treated \#2H4})) \times 100$

## 11. Criteria

### 11-1 Acceptance criteria

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

- If Fold induction of nSLO-LA of PMA/Ionomycin wells without chemicals ( $=(\text{nSLO-LA of \#2H4 cells treated with PMA/Ionomycin}) / (\text{nSLO-LA of non-treated \#2H4 cells}))$ ) demonstrate less than 1.5, the results obtained from the plate containing the control wells should be rejected.

### 11-2 The criteria to identify immunotoxicants by the Multi-Immuno Tox Assay

There are two temporary criteria to identify sensitizers. We are going to adopt one of these criteria after phase I validation.



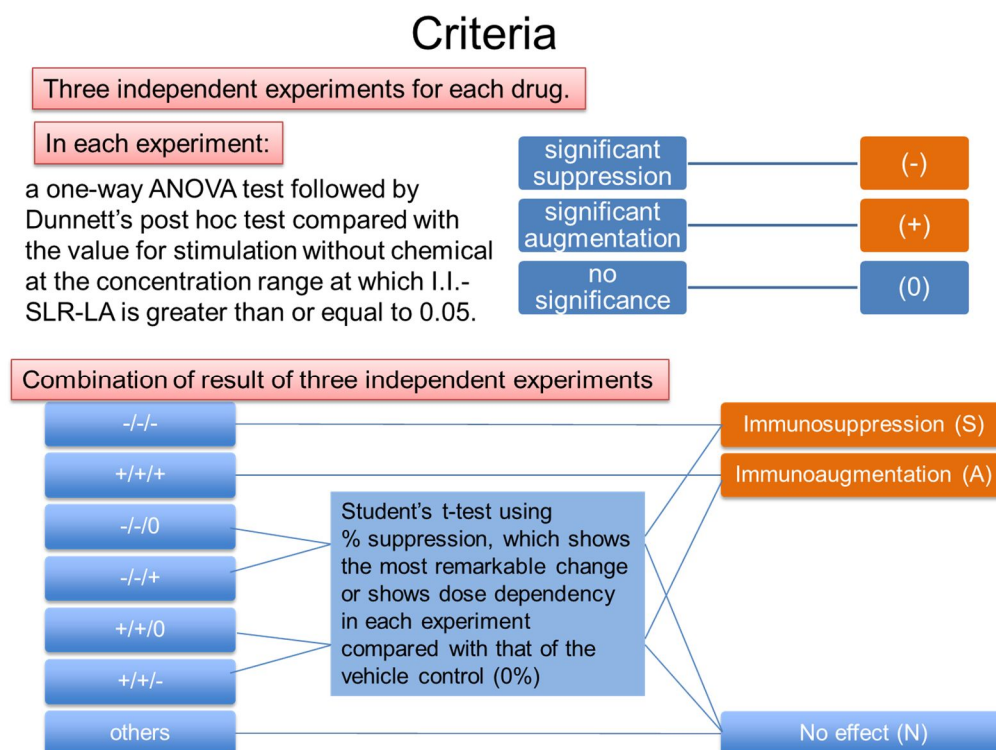
## 1. Criterion 1

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



## 2. Criterion 2

Conduct three independent experiments for each chemical.

Identification of immunotoxicant is evaluated by the 95% simultaneous confidence interval of the difference of two adjusted means of nSLG-LA between a concentration group of a tested chemical and the control group, which is based on the Dunnett's test adjusted three independent experiments and can be calculated by the framework of the general linear model.

For the concentration at which I.I.-SLR-LA is greater than or equal to 0.05 at all three experiments, the behavior of the chemical at the concentration is regarded as immunoaugmentation in case of the lower limit of the simultaneous 95% confidence interval is greater than 0, the behavior of the chemical at the concentration is regarded as immunosuppression in case of the upper limit of the simultaneous 95% confidence interval is less than 0, and the behavior of the chemical at the concentration is no effect in case of the simultaneous 95% confidence interval contains 0.

## 12. Update record

Ver. 008.5E 2016.9.14 distribution

Change the criteria

Ver. 008.4E 2016.9.9 distribution

Change the criteria

Ver. 008.3E 2016.8.1 distribution

Correction of the preparation of PMA and ionomycin

Change the preparation of PMA and ionomycin

Change the preparation of controls

Addition of Acceptance criteria

Ver. 008.1E 2016.2.2 distribution

Changes after the VMT meeting

Ver. 008.0E 2016.1.19

Translation to English

Addition of appendix

Ver. 006.0J 2015.8.17

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

Delete the alteration in Ver. 005.0J

Ver. 005.0J 2015.1.9 distribution

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

Ver. 004.1J 2014.12.10 distribution

Change the cellular 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 ( $\text{NaBrO}_3$ ), Nickel (II) sulfate ( $\text{NiSO}_4$ ), Dibutyl phthalate (DP),  
2-Mercaptobenzothiazole (2-MBT))  
Change THP-G1b cells to TGCHAC-A4 cells  
Change cell number of THP-G8 and TGCHAC-A4  $5 \times 10^4$ /well to  $1 \times 10^5$ /well  
Change concentration of chemicals 11 steps to 10 steps  
Change final concentration of LPS (THP-G8 : 25 ng/mL, TGCHAC-A4 : 1 ng/mL)  
Change the way of addition of LPS (2 mL/well to 10 mL/well)  
Change the criteria

Ver. 002.0J 2013.08.19 distribution

For the validation study at AIST and FDSC (chemicals:  $\text{CoCl}_2$ ,  $\text{NiSO}_4$ , Isophorone diisocyanate, 2-Mercaptobenzothiazole )  
Change the common ratio 3 to 2  
Change the concentration of LPS 100 ng/mL to 25 ng/mL  
Add description about the control (dexamethasone)  
Delete the appendix about THP-G8 cell

Ver. 001.1J 2012. Nov. 13 distribution

Add the appendix about THP-G8 cell

Ver. 001J 2012. Nov. 09 distribution

## Appendix 1 Principle of measurement of luciferase activity

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

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

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

First, using luciferase enzyme reagent of SLG ( $\lambda_{\max} = 550$  nm), SLO ( $\lambda_{\max} = 580$  nm) and SLR ( $\lambda_{\max} = 630$  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	$\kappa G_{R56}$	The intensity of 560 nm LP (Filter 1) transmitted SLG / the intensity of SLG without filter (all optical)
	Filter 2 transmittance factor	$\kappa G_{R60}$	The intensity of 600 nm LP (Filter 2) transmitted SLG / the intensity of SLG without filter (all optical)
SLO	Filter 1 transmittance factor	$\kappa O_{R56}$	The intensity of 560 nm LP (Filter 1) transmitted SLO / the intensity of SLO without filter (all optical)
	Filter 2 transmittance factor	$\kappa O_{R60}$	The intensity of 600 nm LP (Filter 2) transmitted SLO / the intensity of SLO without filter (all optical)
SLR	Filter 1 transmittance factor	$\kappa R_{R56}$	The intensity of 560 nm LP (Filter 1) transmitted SLR / the intensity of SLR without filter (all optical)
	Filter 2 transmittance factor	$\kappa R_{R60}$	The intensity of 600 nm LP (Filter 2) transmitted SLR / the intensity of SLR without filter (all optical)

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

$$F0=G+O+R$$

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

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

These formulas can be rephrased as follows

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

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

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

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

## Appendix 2 Validation of reagents and equipment

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

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

#### 5-1-1 Reagents

- Single reference samples:

Lyophilized luciferase enzyme reagent of SLG

Lyophilized luciferase enzyme reagent of SLO

Lyophilized luciferase enzyme reagent of SLR

- Assay reagent:

Tripluc<sup>®</sup> 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<sup>®</sup> 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  $\mu$ L 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  $\mu$ L each and storing in a freezer at  $-80^{\circ}\text{C}$ . 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  $\mu$ L 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  $\mu$ L of the diluted reference samples to a black 96 well plate (flat bottom) as shown below.

Figure 27

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

Transfer 100  $\mu$ L 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  $25^{\circ}\text{C}$ ) with a plate shaker. Remove bubbles on the solutions in wells if they appear. Place the plate into the luminometer to measure the luciferase activity. Bioluminescence is measured for 3 sec each in the absence (F0) and presence (F1, F2) of the optical filters.

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

Figure 28

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

Record all the results for quality control.

## 5-2 Quality control of equipment

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

### 5-2-1 Light source

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

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

L12367 by Hamamatsu Photonics (Shizuoka, Japan)

### 5-2-2 Data collection (an example using TRIAN® 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% ( ). Disagreement to the old data should be less than  $3 \times$  (= 4.5%).

Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 007.2, Face sheet

<b>Multi-ImmunoTox Assay Datasheet for #2H4 cells</b>
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Ver. 007.2

<b>Laboratory</b>		<b>Round</b>	
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<b>Exp.</b>	1st exp.
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<b>Date:</b> <small>(YYYY/MM/DD)</small>		<b>Operator:</b>	
---	--	------------------	--

<b>Code</b>	Chemical 1	
	Chemical 2	

<b>Dissolution</b>	Chemical 1		<b>mg/ml in</b>	
	Chemical 2			

<b>Molecular weight</b>	Chemical 1	
	Chemical 2	

<b>FluSLO-LA</b>	Chemical 1	#VALUE!	#VALUE!
	Chemical 2	#VALUE!	

<b>Comment:</b>	
-----------------	--

Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 007.2, Data input sheet

MultiReporter Assay System -Tripluc<sup>®</sup>- Calculation Sheet

Transmittance Data

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

Filter 0 Data

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

Filter 1 Data

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

Filter 2 Data

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





## Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 007.2, Update record

2016年9月16日	Ver. 007. 2 FaceSheet#F23の数式を修正
2016年9月14日	Ver. 007. 1 FInSLO-LAが算出されるよう変更 %suppressionのグラフを追加
2016年7月21日	Ver. 007 phase 1用
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 (バックグラウンドの値を引き算する方法)
2012年8月28日	Ver. 001



Multi-ImmunoTox Assay 記録用紙 Ver. 002, 試薬管理シート

実験名 <u>MITA バリデーション研究</u>						
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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Multi-ImmunoTox Assay 記録用紙 Ver. 002, 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
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	_____	回目

## Multi-ImmunoTox Assay 記録用紙 Ver. 002, 細胞継代シート

<p>3-1 #2H4培養方法</p> <p>3-1-1 細胞継代 (P1)</p> <p><input type="checkbox"/> あらかじめ、#2H4用C培地15 mLを37℃恒温槽で温めておく(培養用)。</p> <p><input type="checkbox"/> 凍結細胞を37℃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)</p> <p><input type="checkbox"/> 速心して細胞を集める (350 x g 5分程度)。</p> <p><input type="checkbox"/> 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<sub>2</sub>)。</p> <p><input type="checkbox"/> 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。</p> <p>(計算)</p>          <p>生細胞数:</p> <p>死細胞数:</p> <p>実施日: _____ 年 月 日、実施者: _____</p>
<p>3-1-2 選択抗生剤での培養開始 (P2)</p> <p><input type="checkbox"/> あらかじめ、#2H4用A培地必要量を37℃恒温槽で温めておく。</p> <p><input type="checkbox"/> 細胞継代して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減菌ピペットでベネツピングしてほくし、細胞数を計測する。</p> <p>( + ) / x = × 10<sup>4</sup>/mL—A液</p> <p><input type="checkbox"/> 必要細胞量を取り、速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5mLに3 × 10<sup>4</sup>/mLで細胞を懸濁してT-75 Flaskで培養する。</p> <p>※ 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。</p> <p>A液の採取量: _____ mL</p> <p>実施日: _____ 年 月 日、実施者: _____</p>
<p>3-1-3 通常の継代培養 (P3以降)</p> <p>P-</p> <p><input type="checkbox"/> あらかじめ、#2H4用A培地必要量を37℃恒温槽で温めておく。</p> <p><input type="checkbox"/> フラスコ中の細胞塊を減菌ピペットでベネツピングしてほくし、細胞数を計測する。</p> <p>継代細胞濃度は3 × 10<sup>4</sup>/mL、継代間隔は3~4日程度で行う。</p> <p>( + ) / x = × 10<sup>4</sup>/mL—A液</p> <p><input type="checkbox"/> 必要細胞量を取り、速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<sup>4</sup>/mLで細胞を懸濁してT-75 Flaskで培養する。</p> <p>※ 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。</p> <p>A液の採取量: _____ mL</p> <p>実施日: _____ 年 月 日、実施者: _____</p>
<p>P-</p> <p><input type="checkbox"/> あらかじめ、#2H4用A培地必要量を37℃恒温槽で温めておく。</p> <p><input type="checkbox"/> フラスコ中の細胞塊を減菌ピペットでベネツピングしてほくし、細胞数を計測する。</p> <p>継代細胞濃度は3 × 10<sup>4</sup>/mL、継代間隔は3~4日程度で行う。</p> <p>( + ) / x = × 10<sup>4</sup>/mL—A液</p> <p><input type="checkbox"/> 必要細胞量を取り、速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<sup>4</sup>/mLで細胞を懸濁してT-75 Flaskで培養する。</p> <p>※ 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。</p> <p>A液の採取量: _____ mL</p> <p>実施日: _____ 年 月 日、実施者: _____</p>
<p>P-</p> <p><input type="checkbox"/> あらかじめ、#2H4用A培地必要量を37℃恒温槽で温めておく。</p> <p><input type="checkbox"/> フラスコ中の細胞塊を減菌ピペットでベネツピングしてほくし、細胞数を計測する。</p> <p>継代細胞濃度は2 × 10<sup>4</sup>/mL、継代間隔は3~4日程度で行う。</p> <p>( + ) / x = × 10<sup>4</sup>/mL—A液</p> <p><input type="checkbox"/> 必要細胞量を取り、速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<sup>4</sup>/mLで細胞を懸濁してT-75 Flaskで培養する。</p> <p>※ 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。</p> <p>A液の採取量: _____ mL</p> <p>実施日: _____ 年 月 日、実施者: _____</p>
<p>P-</p> <p><input type="checkbox"/> あらかじめ、#2H4用A培地必要量を37℃恒温槽で温めておく。</p> <p><input type="checkbox"/> フラスコ中の細胞塊を減菌ピペットでベネツピングしてほくし、細胞数を計測する。</p> <p>継代細胞濃度は2 × 10<sup>4</sup>/mL、継代間隔は3~4日程度で行う。</p> <p>( + ) / x = × 10<sup>4</sup>/mL—A液</p> <p><input type="checkbox"/> 必要細胞量を取り、速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<sup>4</sup>/mLで細胞を懸濁してT-75 Flaskで培養する。</p> <p>※ 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。</p> <p>A液の採取量: _____ mL</p> <p>実施日: _____ 年 月 日、実施者: _____</p>

## Multi-ImmunoTox Assay 記録用紙 Ver. 002, 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 <sup>7</sup> 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 -		
遠心した細胞数	_____ cells <sup>*1</sup>	を	_____ mLを採取
再懸濁した培地量	_____ mL ( <sup>*1</sup> の細胞数 ÷ (4x10 <sup>6</sup> ))		
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	( : )		

Multi-ImmunoTox Assay 記録用紙 Ver. 002, 被試験試薬の調製 シート

実験名 <u>MITA バリデーション研究</u>		
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	<input type="checkbox"/> 完全に溶解せず	<input type="checkbox"/> 完全に溶解
	↓	↓
50mg/mL水溶液で		<input type="checkbox"/> 完全に溶解せず <input type="checkbox"/> 完全に溶解
		→25mg/mL水溶液を調製
		↓
100mg/mL水溶液で		<input type="checkbox"/> 完全に溶解せず <input type="checkbox"/> 完全に溶解
		→50mg/mL水溶液を調製 →100mg/mL水溶液として調製を継続
	↓	
500mg/mL DMSO溶液で	<input type="checkbox"/> 完全に溶解 <input type="checkbox"/> 完全に溶解せず	→500mg/mL DMSO溶液として調製を継続
	↓	
250mg/mL DMSO溶液で	<input type="checkbox"/> 完全に溶解 <input type="checkbox"/> 完全に溶解せず	→250mg/mL DMSO溶液として調製を継続
	↓	
125mg/mL DMSO溶液で	<input type="checkbox"/> 完全に溶解 <input type="checkbox"/> 完全に溶解せず	→125mg/mL DMSO溶液として調製を継続
	↓	
62.5mg/mL DMSO溶液で	<input type="checkbox"/> 完全に溶解 <input type="checkbox"/> 完全に溶解せず	→62.5mg/mL DMSO溶液として調製を継続
	↓	
31.25mg/mL DMSO溶液で	<input type="checkbox"/> 完全に溶解 <input type="checkbox"/> 完全に溶解せず	→31.25mg/mL DMSO溶液として調製を継続
	↓	

Multi-ImmunoTox Assay 記録用紙 Ver. 002, 被試験試薬の調製 (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。

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。

添加時間  
( : )

プレートをしールし、プレートシェーカーを使用し、攪拌して混合する。

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

2 mM PMAストックをB培地で20倍希釈し100 μM溶液を作製する。(2 mM PMA 5 μL + B培地 95 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	2 mM Ionomycin	100 μM PMA	Ethanol	Total
Control	995 μL	-	-	5 μL	1000 μL
x10 PMA/ionomycin solution	2382 μL	12 μL	6 μL	-	2400 μL

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  
( : )

プレートをしールし、プレートシェーカーを使用し、攪拌して混合する。

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 °C)で10分間(30分間まで可)攪拌し、細胞を溶解させる。

攪拌中温度  
( °C)

ルミノメータでLuciferase活性を測定する。(測定中温度が26-31°C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  
( °C)

Multi-ImmunoTox Assay 記録用紙 Ver. 002, 被試験試薬の調製 (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 50 $\mu$ L	DMSO 50 $\mu$ L	DMSO 50 $\mu$ L	DMSO 50 $\mu$ L	DMSO 50 $\mu$ L	DMSO 50 $\mu$ L	DMSO 50 $\mu$ L	DMSO 50 $\mu$ L	DMSO 50 $\mu$ L	DMSO 50 $\mu$ L	DMSO 50 $\mu$ L	被試験試薬 DMSO 溶液 100 $\mu$ L
B	B 培地 90 $\mu$ L	B 培地 90 $\mu$ L	B 培地 90 $\mu$ L	B 培地 90 $\mu$ L	B 培地 90 $\mu$ L	B 培地 90 $\mu$ L	B 培地 90 $\mu$ L	B 培地 90 $\mu$ L	B 培地 90 $\mu$ L	B 培地 90 $\mu$ L	B 培地 90 $\mu$ L	B 培地 90 $\mu$ L
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  $\mu$ L 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  $\mu$ L にうつつし 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
有口無口	有口無口	有口無口	有口無口	有口無口	有口無口	有口無口	有口無口	有口無口	有口無口	有口無口	有口無口

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  $\mu$ L を分注し、上図の希釈液を 10  $\mu$ L 添加して 50 倍希釈し、これを 50  $\mu$ L/well ずつ細胞に添加する。

プレートをシールし、プレートシェーカーを使用し、攪拌して混合する。

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

2 mM PMA ストックを B 培地で 20 倍希釈し 100  $\mu$ M 溶液を作製する。 (2 mM PMA 5  $\mu$ L + B 培地 95  $\mu$ L)

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	2 mM Ionomycin	100 $\mu$ M PMA	Ethanol	Total
Control	995 $\mu$ L	-	-	5 $\mu$ L	1000 $\mu$ L
x10 PMA/ionomycin solution	2382 $\mu$ L	12 $\mu$ L	6 $\mu$ L	-	2400 $\mu$ L

コントロール溶液を #A1-#H1、x10 PMA/ionomycin 溶液を #A2-#H12 に 10  $\mu$ L ずつ分注する。

プレートをシールし、プレートシェーカーを使用し、攪拌して混合する。

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<sup>®</sup> 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  $\mu$ L/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  $^{\circ}$ C) で 10 分間 (30 分間まで可) 攪拌し、細胞を溶解させる。

ルミノメータで Luciferase 活性を測定する。(測定中温度が 26-31  $^{\circ}$ C 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)。

添加時間

( : )

添加時間

( : )

添加時間

( : )

攪拌中温度

(  $^{\circ}$ C )

測定時間

( : )

測定中温度

(  $^{\circ}$ C )

Multi-ImmunoTox Assay 記録用紙 Ver. 002, 被試験試薬の調製 (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  $\mu$ L (#A4)、100  $\mu$ g/mL cyclosporine A stock 50  $\mu$ L (#A5)、Distilled water 50  $\mu$ L (#B1、#B2)、2.5 mg/mL dexamethasone stock 50  $\mu$ L (#B3)、B培地 180  $\mu$ L (#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 50 $\mu$ L	CyA 100 $\mu$ g/mL ストック 50 $\mu$ L							
B	Distilled water 50 $\mu$ L	Distilled water 50 $\mu$ L	DEX 2.5 mg/mL ストック 50 $\mu$ L	B培地 180 $\mu$ L	B培地 180 $\mu$ L							
C												
D												
E												
F												
G												
H												

#A4のDMSOと#A5のcyclosporine A DMSO溶液 20  $\mu$ Lを下のB培地 180  $\mu$ Lにうつつし10倍に希釈する。

アッセイブロックの#A1-#A3にB培地480  $\mu$ L、#A1-#A3にB培地980  $\mu$ Lを分注し、上図の希釈液を20  $\mu$ L添加して混合し、50  $\mu$ L/wellずつ細胞に添加する。

添加時間  
( : )

プレートをシールし、プレートシェーカーを使用し、攪拌して混合する。

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

2 mM PMAストックをB培地で20倍希釈し100  $\mu$ M溶液を作製する。(2 mM PMA 5  $\mu$ L + B培地 95  $\mu$ L)

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	2 mM Ionomycin	100 $\mu$ M PMA	Ethanol	Total
Control	995 $\mu$ L	-	-	5 $\mu$ L	1000 $\mu$ L
x10 PMA/ ionomycin solution	2382 $\mu$ L	12 $\mu$ L	6 $\mu$ L	-	2400 $\mu$ L

コントロール溶液を#A1-#D1、x10 PMA/ionomycin溶液を#A2-#D5に10  $\mu$ Lずつ分注する。

添加時間  
( : )

プレートをシールし、プレートシェーカーを使用し、攪拌して混合する。

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  $\mu$ L/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  $^{\circ}$ C)で10分間(30分間まで可)攪拌し、細胞を溶解させる。

攪拌中温度  
(  $^{\circ}$ C )

ルミノメータでLuciferase活性を測定する。(測定中温度が26-31 $^{\circ}$ C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  
(  $^{\circ}$ C )



Multi-ImmunoTox Assay 記録用紙 Ver. 002, 更新履歴シート

Ver. 002J 2016年07月21日配布

Ver. 001J 2016年02月02日配布