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）について平成28年度に実施されたPhase Iバリデーション後の国際VMTミーティング会議（京都）で検討された結果を反映した試験法プロトコール(Multi-ImmunoTox Assay protocol ver. 009.1E)およびデータシート(Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 008.2)を作成しPhase II試験を行った。その結果について国際VMTミーティング会議(大阪）での意見、東北大学での解析を参照とし%suppressionの閾値を±35%としたクライテリアを設定した。

キーワード：試験法プロトコール、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-γプロモーター活性の測定を行った。ヒトTリンパ芽球性白血病由来細胞株JurkatにIL-2プロモーターに制御されたSLGルシフェラーゼ遺伝子（緑色に発色)、IFN-γプロモーターに制御されたSLOルシフェラーゼ遺伝子（橙色に発色)、GAPDHプロモーターに制御されたSLRルシフェラーゼ遺伝子(赤色に発色)を導入した#2H4細胞を1ウェル当たり2×10^5個、黒色の96-well プレート(Greiner bio-one)に播種し化学物質を加え、37°C、5%CO_2下で1時間培養した。つづいて25nM PMAと1μM Ioの混合物(PMA/Io)で刺激し37°C、5%CO_2下で6時間培養した。その後、細胞溶解剤とルシフェラーゼ反応の基質であるルシフェリンの混合剤である Tripluc luciferase assay reagent (TOYOBO)を混合し、室温で10分振盪させたのちマルチプレート対応型ルミノメーターにてルシフェラーゼ活性を測定した。SLG、SLO、 SLRル

シフェラーゼは共通の基質の存在により同時に発光するが、2枚の光学的フィルターにより分離し、各ルシフェラーゼの発光量(SLG-luciferase activity (SLG-LA)、SLO-luciferase activity (SLO-LA)、SLR-luciferase activity (SLR-LA))を検出した。また細胞数の違いや各種刺激後の生存率の違いを勘案しSLG-LA、SLO-LAをSLR-LAで除することによりそれぞれnormalized SLG-luciferase activity(nSLG-LA), normalized SLO- luciferase activity(nSLO-LA)を算出した。さらに以下の式に%suppression抑制率を計算した。

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

C. 結果

1）国際バリデーションPhase 2用の試験法プロトコール、データシート、記録用紙の作成

平成28年度に実施されたPhase Iバリデーション後の国際VMTミーティング会議（H29. 2月、京都）で検討された結果を反映した国際バリデーションPhase 2用の試験法プロトコール、Multi-ImmunoTox Assay protocol ver. 009.1E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. 008.2を作成した（添付文書２）。さらに参加施設用の記録用紙、Multi-ImmunoTox Assay 記録用紙 Ver. 003.1を作成した（添付文書３）。

2）国際バリデーションPhase 2の結果の解析および新クライテリアの設定

国際バリデーションPhase 2の結果について国際VMTミーティング会議（大阪）での意見、東北大学で作成されたMITA data setの各化学物質における%suppressionの最大値、最小値を参考とし%suppressionの閾値を±35%と設定したクライテリア（クライテリア５）を設定した。クライテリア５を用いて国際バリデーションPhase 2の結果を再評価したところ施設間再現性が80%(16/20)であった。2018年3月29日に開催された国際スカイプ会議でクライテリア５は国際バリデーション実行委員に承認された。

D. 考察

クライテリア５による評価では、施設内、施設間再現性についてPhase I、Phase II共にstudy planに記載された基準を満たした。今後、試験に使用された被験物質について*in vitro, in vivo*のデータを集積しMITAアッセイの予測性について検討する。

E. 結論

国際バリデーションPhase IIに向けて試験法プロトコール、データシート、記録用紙を作成した。また、その結果に基づき%suppressionの閾値を±35%とした新しいクライテリアを設定した。

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-ImmunoTox Assay protocol ver. 009.1E

２）Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 008.2

３）Multi-ImmunoTox Assay 記録用紙 Ver. 003.1

G. 研究発表

1. 論文発表

1） Kimura, Y., Fujimura, C., Ito, Y., Takahashi, T., Terui, H. and Aiba, S. Profiling the immunotoxicity of chemicals based on in vitro evaluation by a combination of the Multi-ImmunoTox assay and the IL-8 Luc assay. Arch Toxicol in press.

2） Aiba, S., Kimura, Y. In vitro test methods to evaluate the effects of chemicals on innate and adaptive immune responses. Curr Opin Toxicol 2017 5:6-12

2. 学会発表

1）Kimura Y. et al. Dimethyl sulfoxide is not necessary to dissolve most sensitizers for their *in vitro* stimulation of dendritic cells. 47th Annual European Society for Dermatological Research Meeting. Salzburg, Austria, September 27-30, 2017

2）Kimura Y. et al. Multi-ImmunoTox Assay (MITA): the creation of its data set and the results of validation studies. 10th World Congress Alternatives and Animal Use in the Life Science. Seattle, Washington, USA August 20-24, 2017

3）Aiba S. et al. A novel in vitro assay for sensitisers in purely aqueous system: the modified IL-8 Luc assay using X-VIVO™ 15 as a solvent. 10th World Congress Alternatives and Animal Use in the Life Science. Seattle, Washington, USA August 20-24, 2017

4）木村裕他：Multi-ImmunoTox Assay (MITA) データセットの作成およびバリデーション研究の結果　日本動物実験代替法学会　第30回大会（東京）2017年11月

5）相場節也他：DMSOを用いない *in vitro* 感作性試験　日本動物実験代替法学会　第30回大会（東京）2017年11月

H. 知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得
なし

2. 実用新案登録
なし

3.その他
なし

添付文書 1

Multi-ImmunoTox Assay protocol ver. 009.1E

May. 8th, 2017

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1. Introduction

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

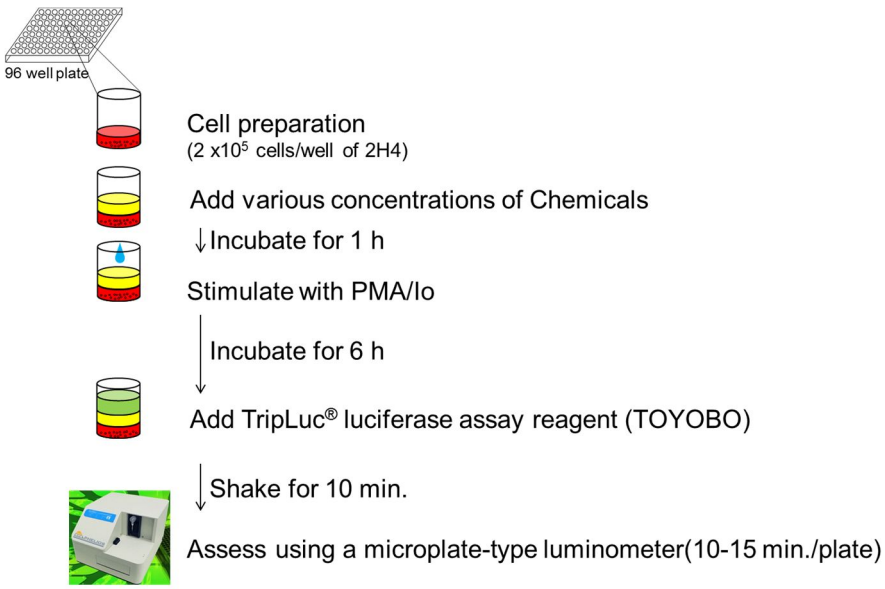
(Kimura Y. et al. Evaluation of the Multi-Immuno Tox Assay composed of 3 human cytokine reporter cells by examining immunological effects of drugs *Toxicol in Vitro*, 28, 759-768, 2014)

Figure 1

Assay design (2 chemicals per one plate)

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

PMA/Io or LPS



2. Materials

2-1 Cells

- #2H4 (IL2-SLG、IFNγ-SLO、G3PDH-SLR)

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

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

2-2 Reagents and equipment

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

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

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

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

2-2-3 For measurement of the luciferase activity

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

2-2-4 Expendable supplies

- T-75 flask tissue culture treated (e.g., Corning Cat#353136)
- 96 well μclear black plate (flat-bottom, for measurement of the luciferase activity, e.g. Greiner Bio-one Cat#655090)
- 96 well clear plate (round-bottom, for preparation of chemicals and stimulants)

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

2-2-5 Equipment for measurement of luciferase activity

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

2-2-6 Others

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

2-3 Culture medium

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

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

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

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

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

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

2-4 Preparation of the stimulant of #2H4

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

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

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

2-4-2 Ionomycin

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

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

3. Cell culture

3-1 Thawing of #2H4 cells

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

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

3-2 Maintenance of #2H4 cells

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

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

4. Preparation of cells for assay

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

Use cells between 1 and 6 weeks after thawing.

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

Figure 2

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

5. Preparation of chemicals and cell treatment with chemicals

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

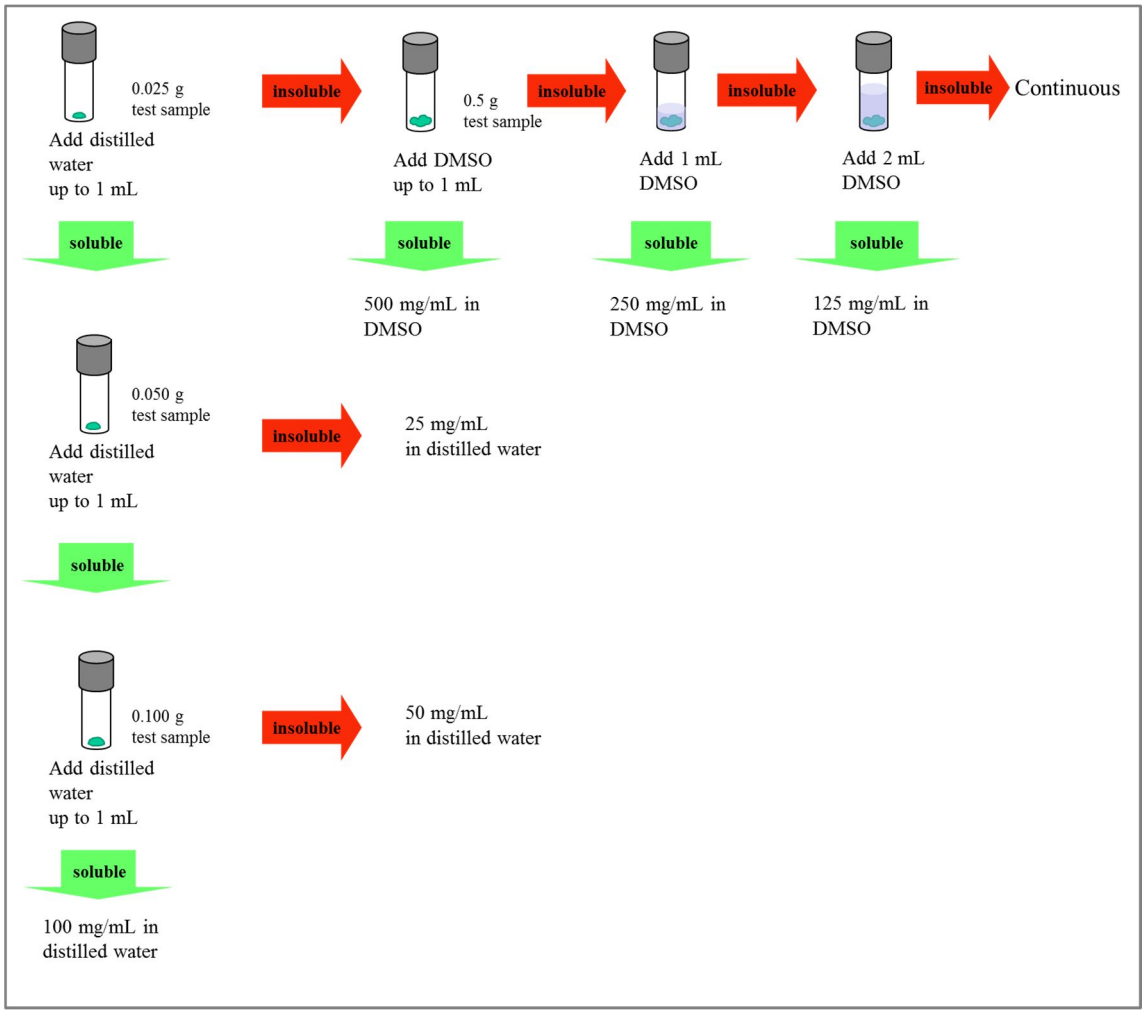
Dissolve the chemical first in distilled water. Namely, weigh 0.025 g of the test chemical in a volumetric flask and add distilled water up to 1 mL. If the chemical is soluble at 25 mg/mL, weigh 0.050 g of the test chemical in a volumetric flask and add distilled water up to 1 mL. If the chemical is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

If the chemical is not soluble in water, the chemical should be dissolved in DMSO at 500 mg/mL. Namely, weigh 0.5 g of the test chemical in volumetric flask and add DMSO up to 1 mL.

If the chemical is not soluble at 500 mg/mL, the highest soluble concentration should be determined by diluting the solution from 500 mg/mL at a common ratio of two (250 mg/mL → 125 mg/mL → continued if needed) with DMSO.

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

Figure 3



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

In the second or third experiment (2nd or 3rd experiment), determine the minimum concentration at which I.I.-SLR-LA (mentioned later in **10**) became lower than 0.05 in the 1st experiment, use the concentration one step (2-times) higher than this determined concentration as the highest concentration of the chemical to examine, and conduct 10 serial dilutions at a common ratio of 2 from the highest concentration. If I.I.-SLR-LA did not become lower than 0.05 or became lower than 0.05 at the highest concentration in the 1st experiment, conduct 10 serial dilutions at a common ratio of 2 from the highest concentration in the 1st experiment.

For example, in Figure 3 below, the minimum concentration at which I.I.-SLR-LA became lower

than 0.05 is 1.95 μg/ml. The highest concentration of the chemical to examine is the concentration one step (2-times) higher than 1.95 μg/ml, which is 3.91 μg/ml.

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

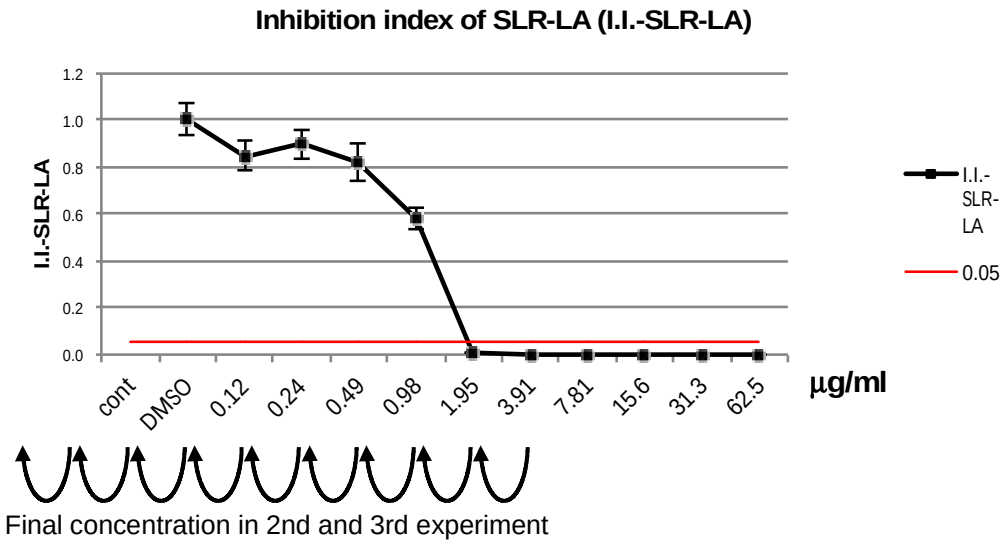


Figure 3.

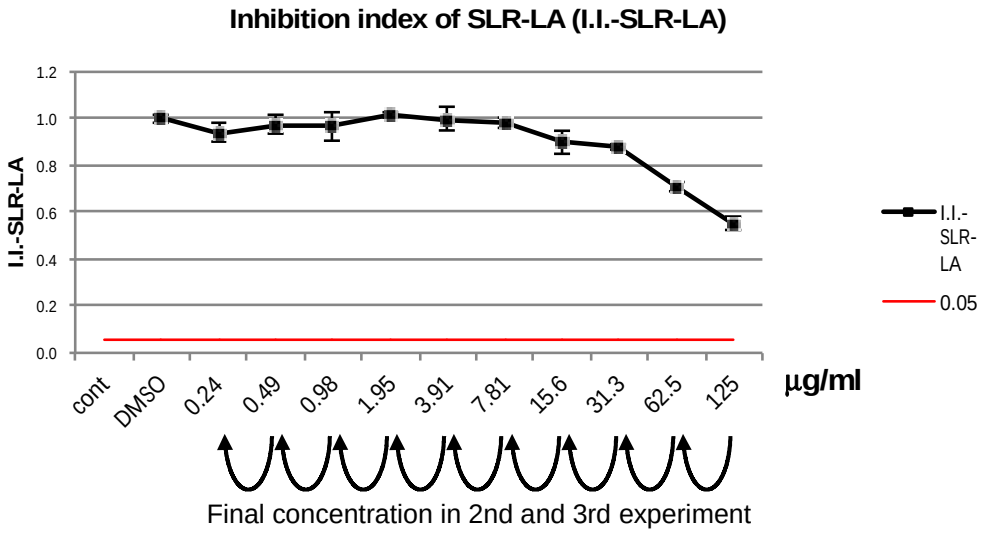


Figure 4

5-2 When the chemical is prepared in distilled water

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

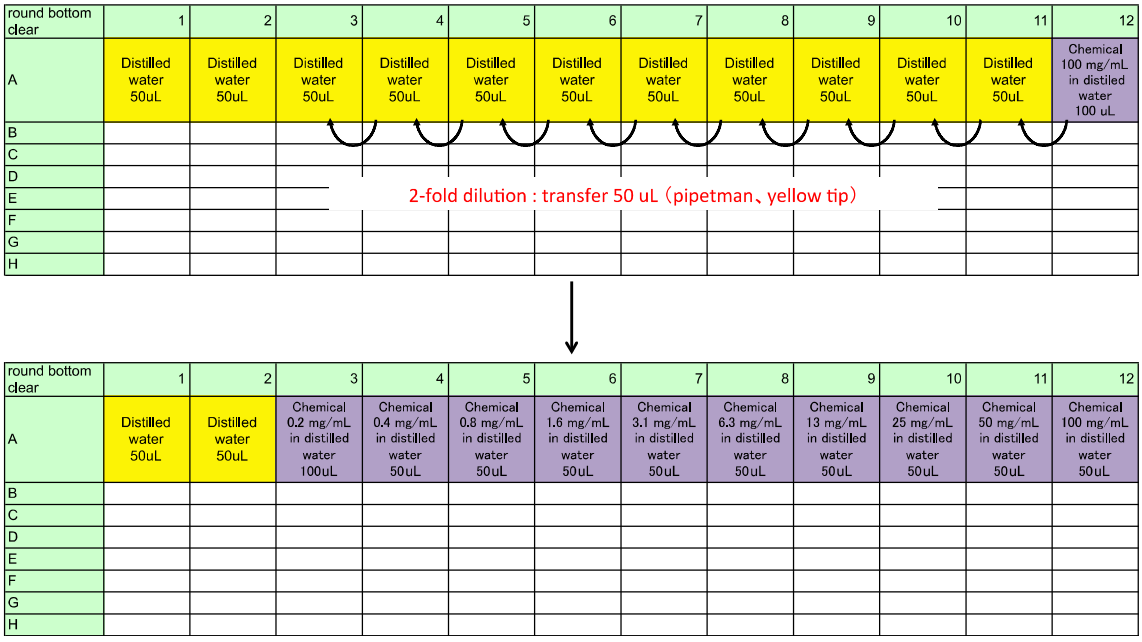
5-2-1 Arrangement of chemicals and vehicle

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

5-2-2 Serial dilution

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

Figure 4



5-2-3　2 step dilution

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

Figure 5

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

20uL

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

Figure 6

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

50uL

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

Figure 7 Final constituents of each well of the plate

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

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

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

5-3-1　Arrangement of chemicals and vehicle

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

5-3-2　Serial dilution

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

Figure 8

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

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

↓

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

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

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

Figure 9

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

10uL

↓

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

5-3-4　2 step dilution

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

Figure 10

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

10uL

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

Figure 11

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

50uL

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

Figure 12 Final constituents of each well of the plate

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

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

6-1 Material

・2 mM PMA stock

・2 mM Ionomycin stock

・B medium

・Ethanol

6-2 Preparation of 100 μM PMA

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

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

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

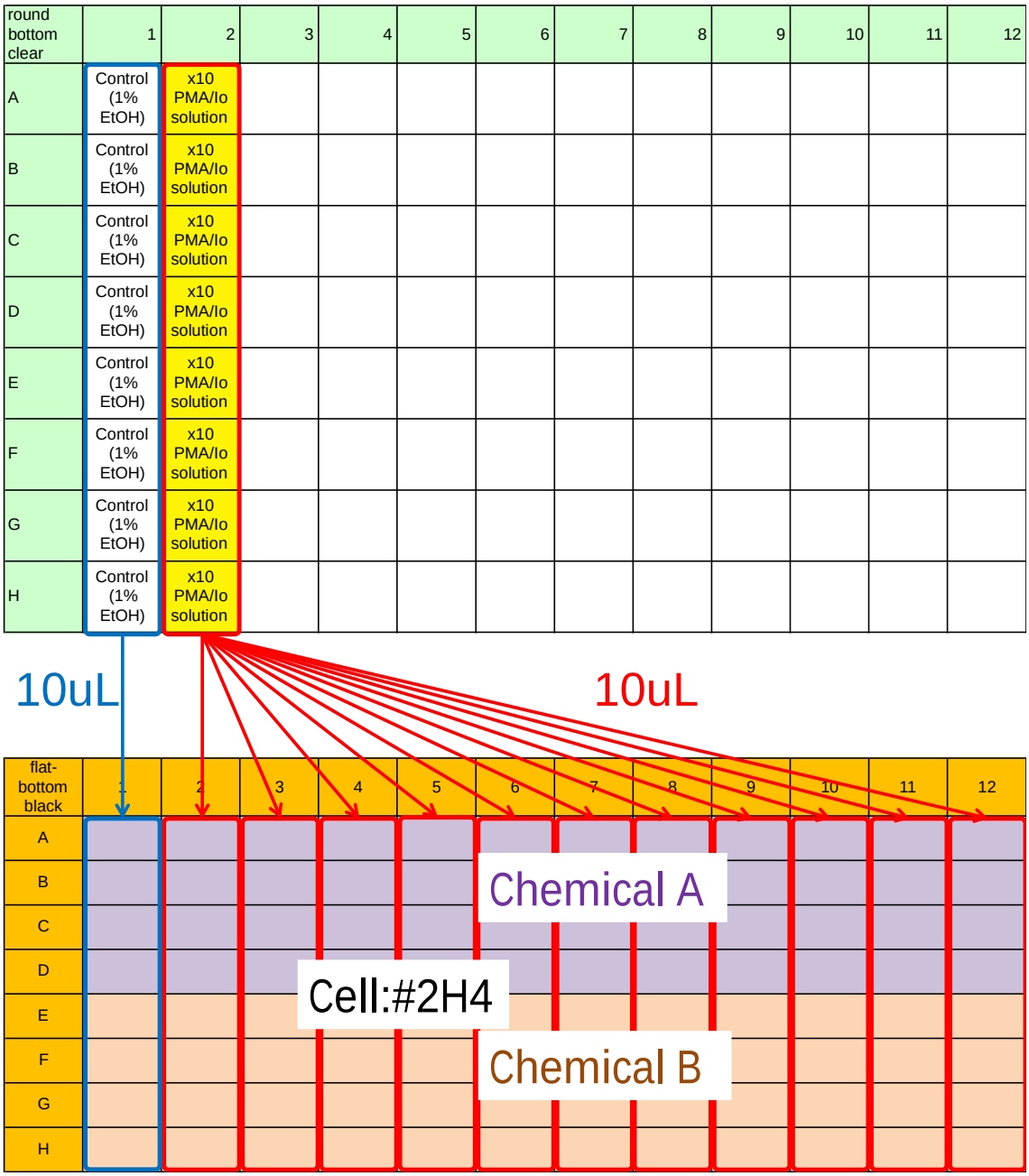
Dilute ethanol, 2 mM ionomycin and 100 μM PMA with the B medium to prepare control or x10 PMA/ionomycin solution. Add the control to well #A1-#H1 of the 96 well clear plate (round bottom), and add x10 PMA/ionomycin solution to wells #A2-#H2 of the 96 well clear plate (round bottom).

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

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

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

Figure 13



7. Control

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

7-1-1 Preparing dexamethasone stock

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

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

7-1-2 Preparing cyclosporine A stock

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

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

7-2 Preparation of cells for assay

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

Use cells between 1 and 6 weeks after thawing.

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

Figure 14

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

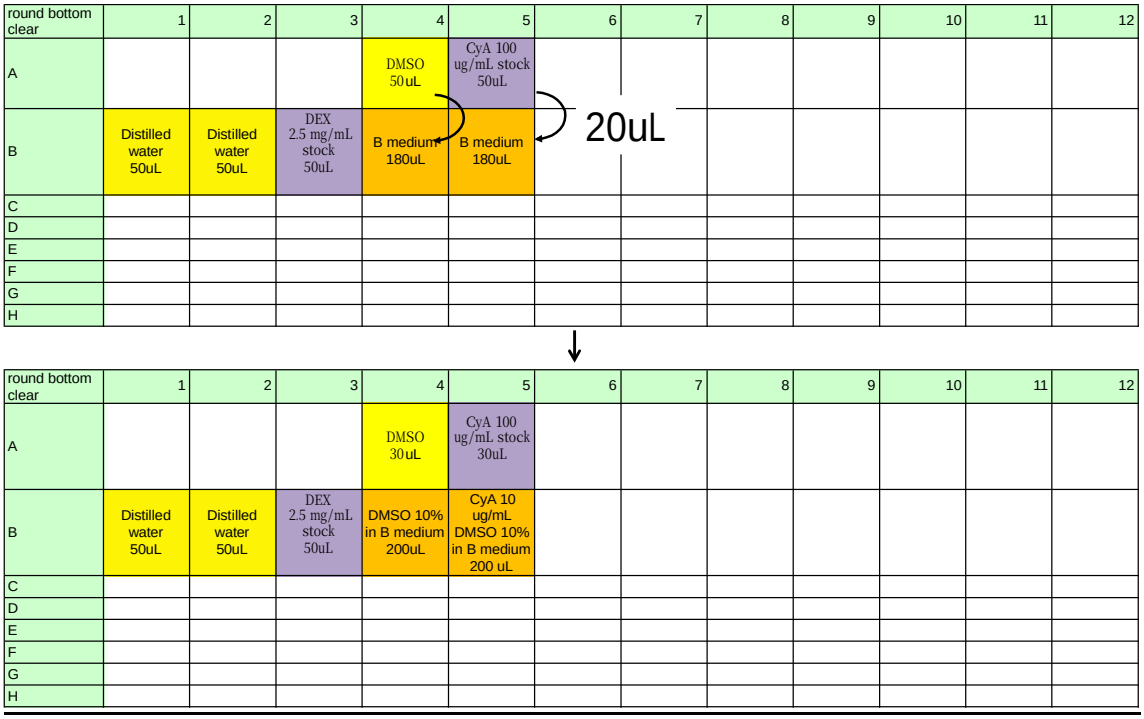
7-3 Arrangement of chemicals and vehicle

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

7-4 Dilution with the B medium

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

Figure 15



7-5　　2 step dilution

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

Figure 16

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				20uL								
H												

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 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
B	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
C	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
D	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
E												
F												
G												
H												

Figure 18 Final constituents of each well of the plate

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

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

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

Figure 19

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

10uL

10uL

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

8. Calculation of the transmittance factors

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

8-1 Reagents

- Single reference samples:

Lyophilized luciferase enzyme reagent for stable luciferase green (SLG)
Lyophilized luciferase enzyme reagent for stable luciferase orange (SLO)
Lyophilized luciferase enzyme reagent for stable luciferase red (SLR)

- Assay reagent:

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

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

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

8-2 Preparation of luminescence reaction solution

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

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

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

8-3 Bioluminescence measurement

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

Figure 20

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

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

Figure 22

Measurement without Filter

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

Measurement with Filter 1

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

Measurement with Filter 2

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

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

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

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

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

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

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

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

In the case shown above,

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

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

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

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

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

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

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

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

Figure 23

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

9. Measurement

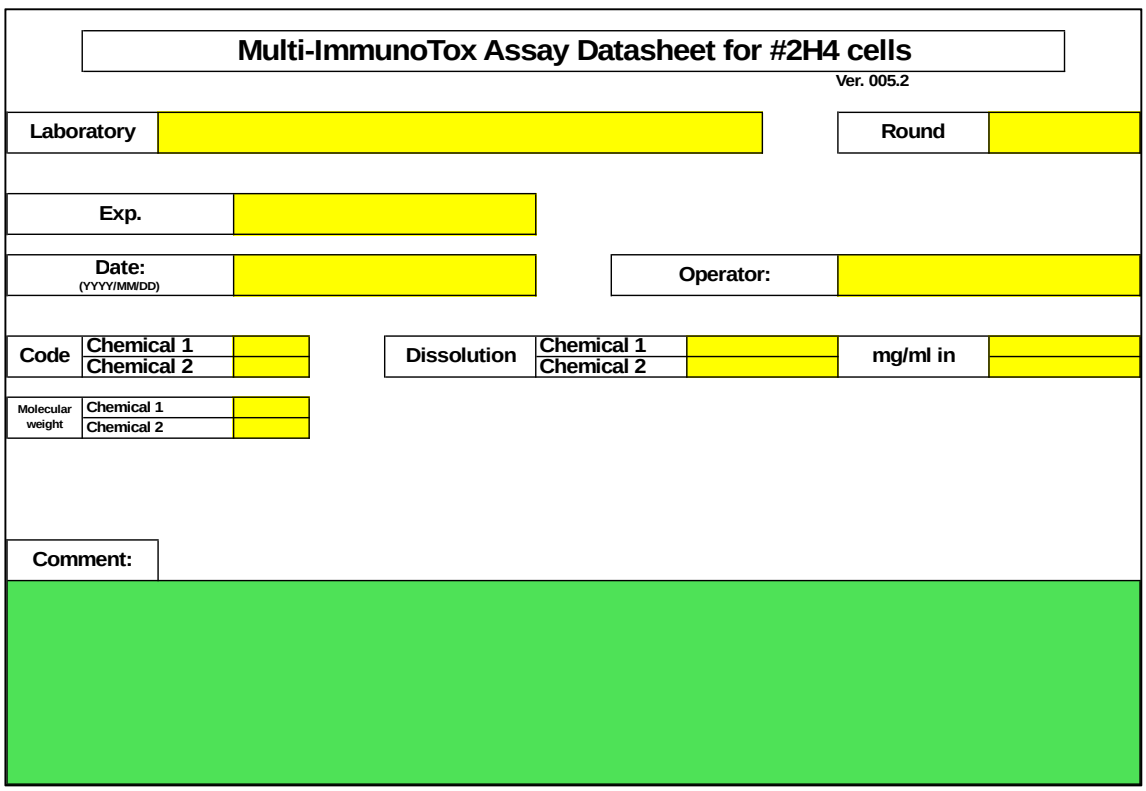
Please refer Appendix 1 for the principle of measurement of luciferase activity.

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

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

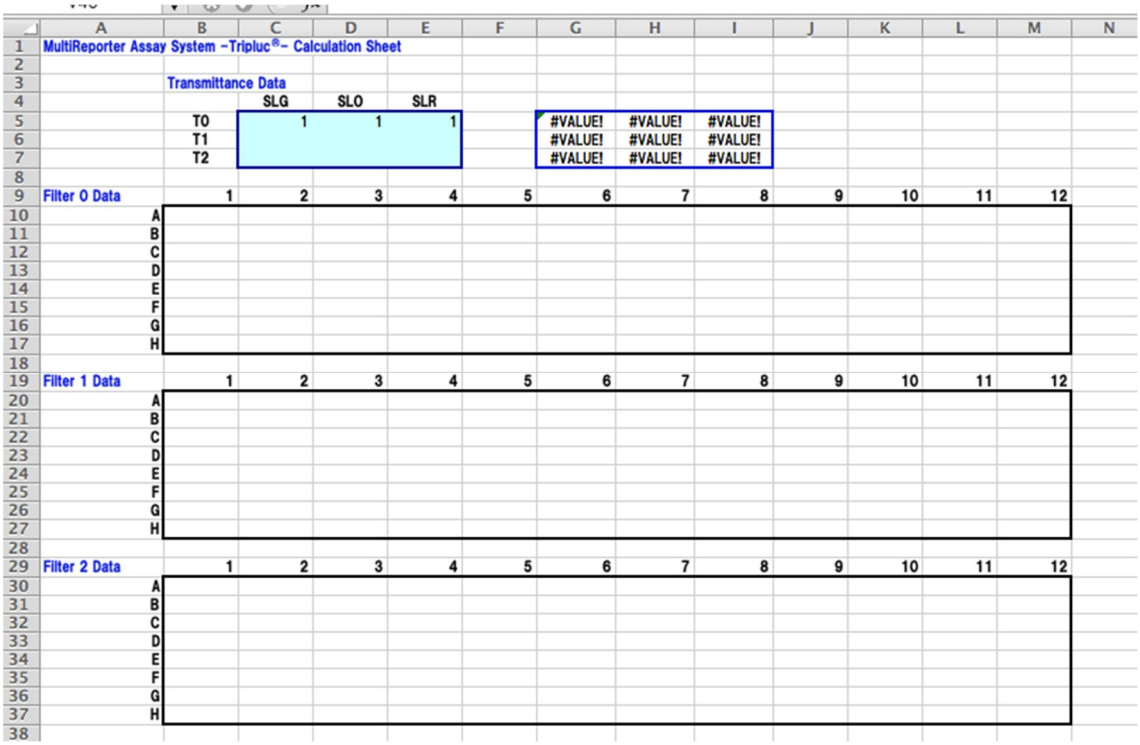
1st. Add the information regarding the name of laboratory, the round of experiments if multiple experimental sets are performed, the experiment number, date, the operator, chemical codes, dissolved in distilled water or DMSO, the prepared concentration, molecular weight of the chemicals and comments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

Figure 24 “Face Sheet” of the data sheet



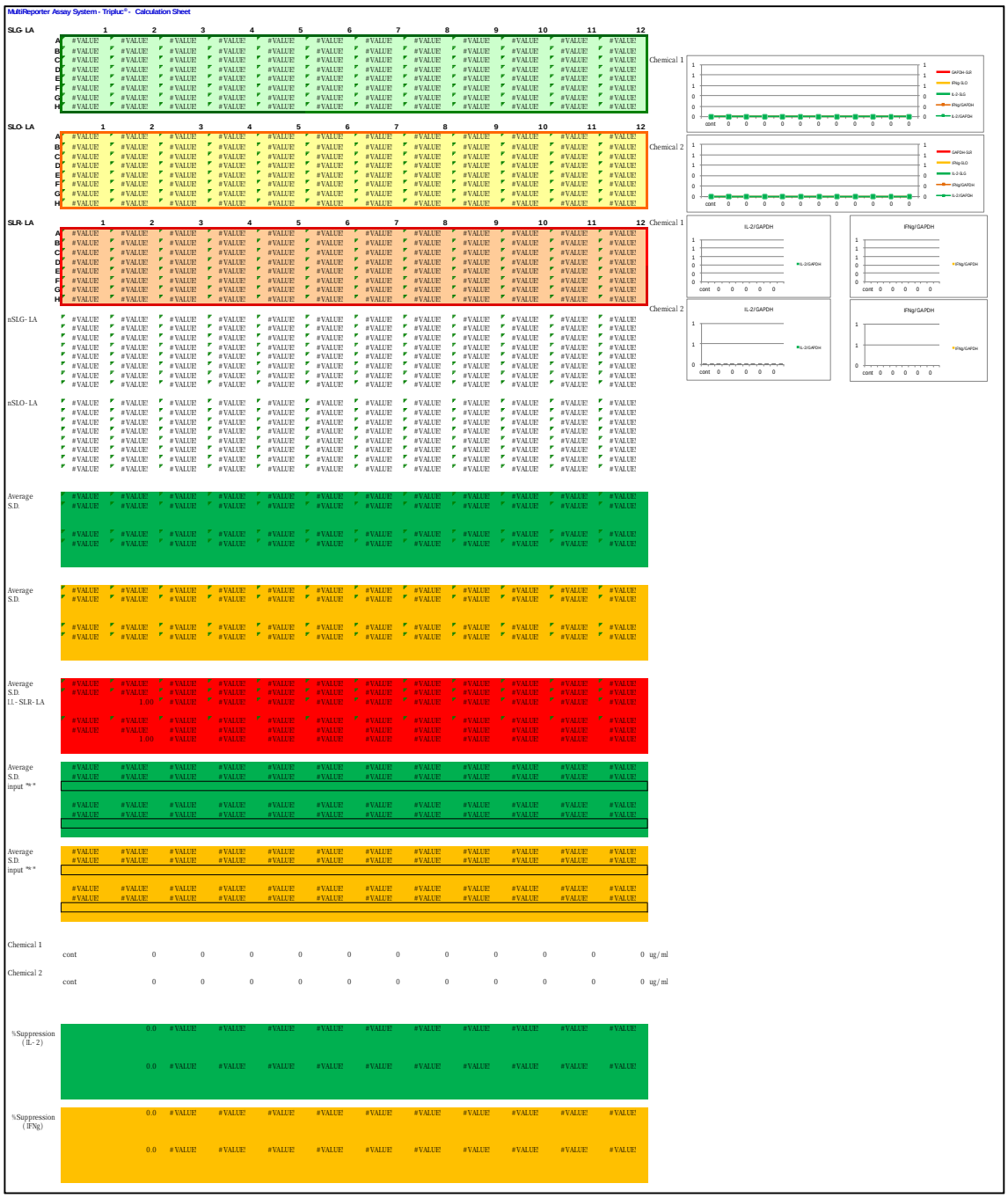
2nd. Copy the results of the F0, F1 and F2 measurements (values are expressed as counts) and paste them into the appropriate area in the “Data Input” sheet of the data sheet shown below. In addition, input the transmittance factors calculated in "§5. Calculation of the transmittance factors" to #C6-#E7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

Figure 25 “Data Input” sheet of the data sheet



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

11. Criteria

11-1 Acceptance criteria

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

- If Fold induction of nSLO-LA of PMA/Ionomycin wells without chemicals (=(nSLO-LA of #2H4 cells treated with PMA/Ionomycin) / (nSLO-LA of non-treated #2H4 cells)) demonstrate less than 3.0, the results obtained from the plate containing the control wells should be rejected.
- If the number of concentration which satisfy I.I.-SLR-LA>=0.05 is less than 4, the results should be rejected, and the following experiments should be done using the concentration described in **5-1**.

11-2 Criterion

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 %suppression 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 result with two or more consecutive statistically significant positive (negative) points or one or more statistically significant positive (negative) point as well as a trend in which 3 or more points above (below) the red line are increasing (decreasing) is regarded as immunosuppression (immunoaugmentation). The result simultaneously satisfies the two conditions is regarded as immunosuppression / immunoaugmentation. Other result is regarded as no effect.

In the second experiment, If the number of concentration which satisfy I.I.-SLR-LA>=0.05 is less than 6, determine the minimum concentration at which I.I.-SLR-LA became lower than 0.05 in the second experiment, use the concentration one step (2-times) higher than this determined concentration as the highest concentration of the chemical to examine, and conduct 10 serial dilutions at a common ratio of 2 from the highest concentration in the following experiments.

If the number of concentration which satisfy I.I.-SLR-LA>=0.05 in common by 3 experiments is less than 6, conduct 4^{th} or 5^{th} experiment.

If there are plural combinations of at least 6 concentrations which satisfy I.I.-SLR-LA>=0.05, adopt the combination with higher concentration.

12. Update record

Ver. 009.1E 2017.5.8 distribution
Change the criteria

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

Ver. 008.5E 2016.9.14 distribution
Change the criteria

Ver. 008.4E 2016.9.9 distribution
Change the criteria

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

Ver. 008.1E 2016.2.2 distribution
Changes after the VMT meeting

Ver. 008.0E 2016.1.19
Translation to English
Addition of appendix

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

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

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

Ver. 004.0J 2014.11.17　distribution
For the validation study at AIST, FDSC and Tohoku university (chemicals: Sodium Bromate ($NaBrO_3$), Nickel (II) sulfate ($NiSO_4$), Dibutyl phthalate (DP), 2-Mercaptobenzothiazole (2-MBT))
Change THP-G1b cells to TGCHAC-A4 cells
Change cell number of THP-G8 and TGCHAC-A4 5x10^4/well to 1x10^5/well
Change concentration of chemicals 11 steps to 10 steps
Change final concentration of LPS (THP-G8 : 25 ng/mL, TGCHAC-A4 : 1 ng/mL)
Change the way of addition of LPS (2 mL/well to 10 mL/well)
Change the criteria

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

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

Ver. 001J 2012. Nov. 09 distribution

Appendix 1 Principle of measurement of luciferase activity

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

(1) Measurement of three-color luciferase with two optical filters.
This is an example using Phelios AB-2350 (ATTO). This luminometer equips a 560 nm long-pass filter (560 nm LP, Filter 1) and a 600 nm long pass filter (600 nm LP, Filter 2) for optical isolation.

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

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

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

$F0=G+O+R$

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

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

These formulas can be rephrased as follows

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

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

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

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

Appendix 2 Validation of reagents and equipment

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

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

5-1-1 Reagents

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

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

5-1-2 Calibration

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

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

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

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

ice to prevent deactivation.

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

5-1-2-2 Bioluminescence measurement

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

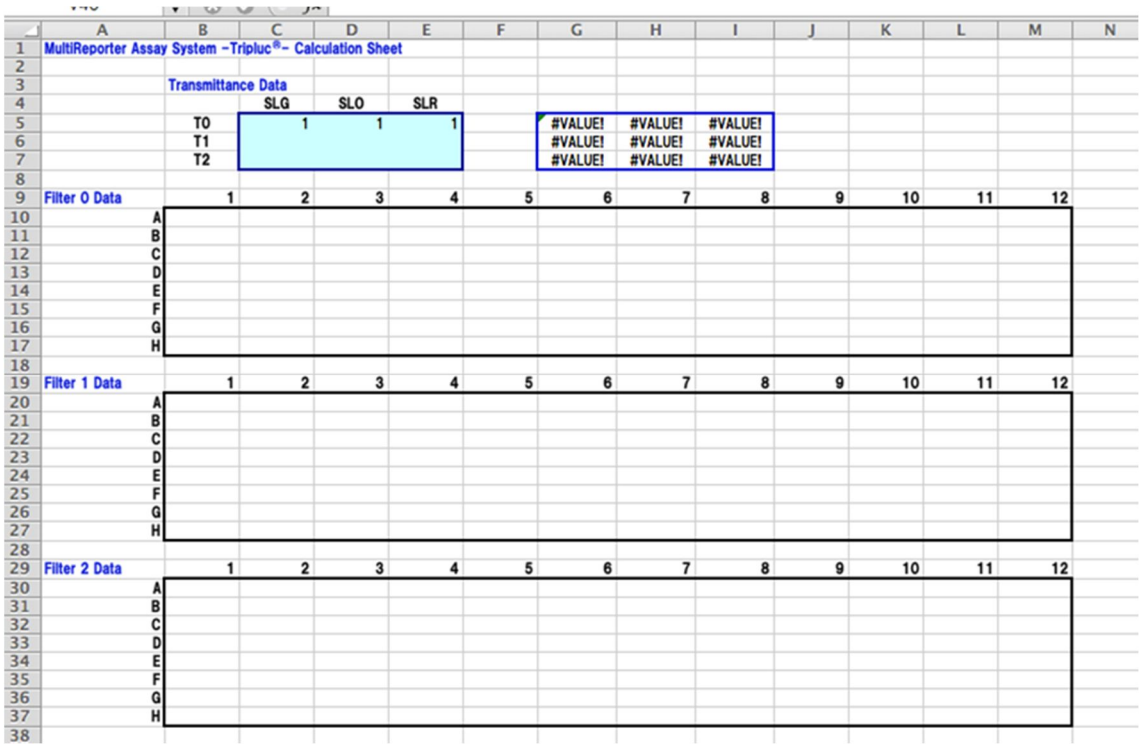
Figure 27

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

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

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

Figure 28



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

Record all the results for quality control.

5-2 Quality control of equipment

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

5-2-1 Light source

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

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

L12367 by Hamamatsu Photonics (Shizuoka, Japan)

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

1) Start luminometer 30 min before starting the measurement for stabilization of the photomultiplier.
2) Start LED plate and select “PMT” mode.
3) Select three-color (BRG) mode and adjust light intensity to 1/10 (10E-1).

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

Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 008.2

Face Sheet

Multi-ImmunoTox Assay Datasheet for #2H4 cells

Ver. 008.2

Laboratory		Round	

Exp.	1st exp.	(Highest soluble conc. In the next exp.s	mg/ml

Date: (YYYY/MM/DD)		Operator:	

Code		Dissolution		mg/ml in	

FInSLO-LA	#######	#VALUE!	the number of concentration which satisfy I.I.-SLR-LA>=0.05	#######

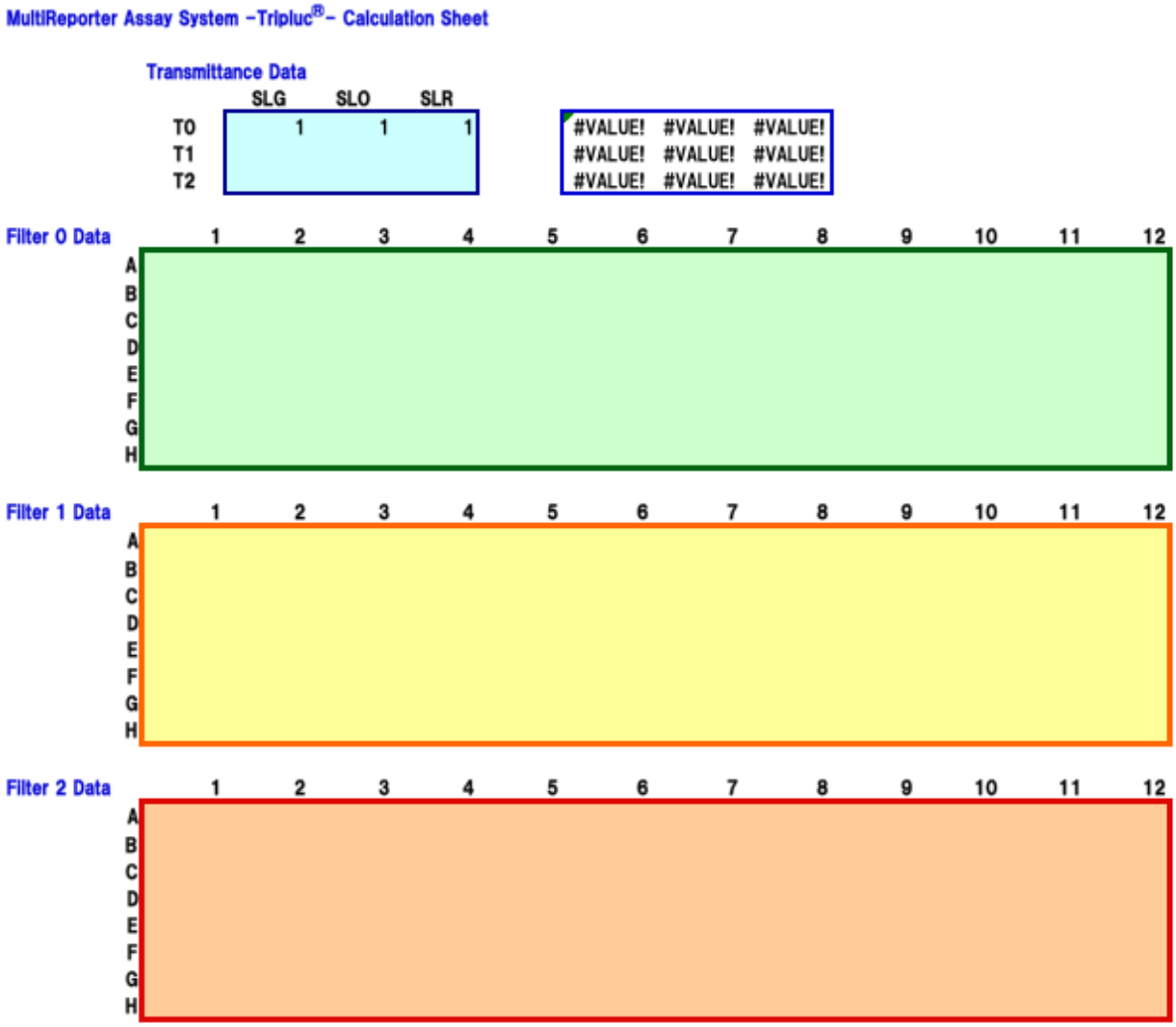
Comment:

																									ug/mL
1st																									
2nd																									
3rd																									
4th																									
5th																									
6th																									

1st Exp.	
2nd Exp.	
3rd Exp.	
4th Exp.	
5th Exp.	
6th Exp.	

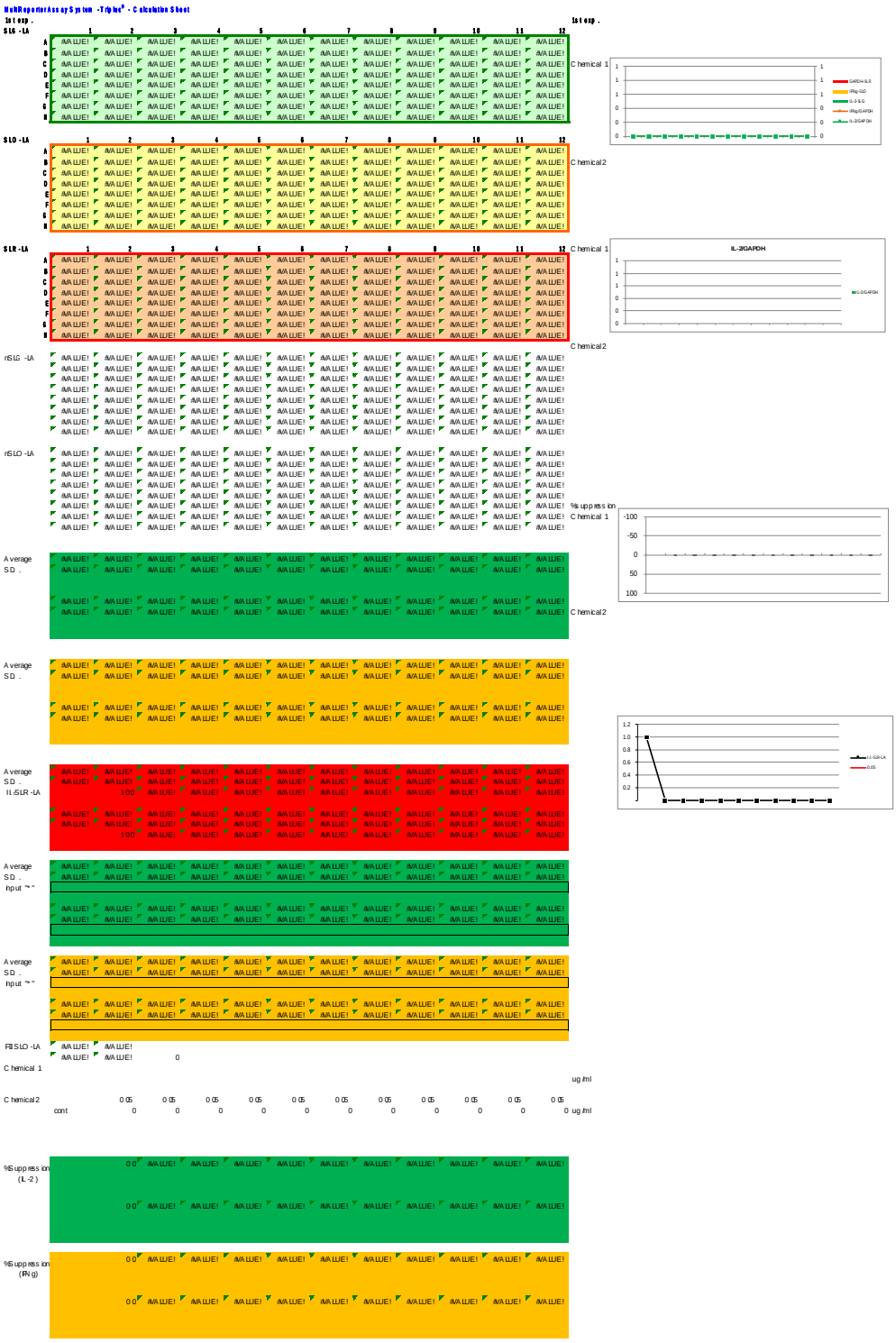
Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 008.2

Data Imput Sheet



Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 008.2

Result Format Sheet



添付文書２

Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 008.2

Control Sheet

MultiReporter Assay System -Tripluc®- Calculation Sheet

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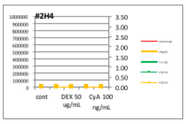

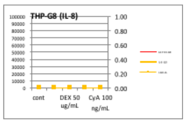

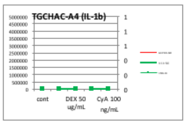

	cont	ddw	DEX 50 ug/ı	DMSO	CyA 100 ng/mL							
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!							
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!							
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!		#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
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Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!							
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!							
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Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!							
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!							
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Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!							
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!							
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Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!							
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!							
FlnSLO-LA	#VALUE!	#VALUE!	ddw	#VALUE!	DMSO							
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!		#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!		#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
IL-2 抑制率 (%)	0	0	#VALUE!	0	#VALUE!							
IL-1b 抑制率 (%)	0	0	#VALUE!	0	#VALUE!							
IFN-g 抑制率 (%)	0	0	#VALUE!	0	#VALUE!							
IL-8 抑制率 (%)							0	0	#VALUE!	0	#VALUE!	

添付文書２

Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 008.2
Update Record Sheet

2017年5月8日	Ver.008.2 判定を追加
2017年4月19日	Ver.008.1 6回のアッセイを１シートにまとめる
2017年4月11日	Ver.008 phase 2用
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年11月6日	Ver.004 統計処理用のシートを追加 Result Format2シートの化学物質の濃度表示を修正（公比2になるように）
未配布	Ver.003.1 グラフの大きさを縮小（パワーポイントにコピーペーストしやすくするため）
2013年9月19日	Ver.003 コントロール用のシートを追加（dexamethasone, cyclosporin A）
2012年11月13日	Ver.002.1 %Suppressionのグラフを追加
2012年8月31日	Ver.002 抑制率の計算方法を変更（バックグランドの値を引き算する方法）
2012年8月28日	Ver.001

添付文書３

Multi-ImmunoTox Assay 記録用紙 Ver. 003.1
試薬管理シート

実験名　MITA バリデーション研究

被試験試薬コード

被試験試薬管理

受領日　　年　月　日　　受領者氏名

保管場所　　温度（℃）　　℃

備考

受領量（容器込）　　g

月 日	使用量(g)	残存量(g)	実験担当者名	備考	Exp. No.	溶解性検討
H. / /						
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添付文書３

Multi-ImmunoTox Assay 記録用紙 Ver. 003.1

試験者シート

実験名　MITA バリデーション研究

実験日

施設名

実験責任者名

実験担当者名

実験担当者名

実験担当者名

実験担当者名

試験物質コード

	回目
	回目
	回目
	回目
	回目
	回目
	回目
	回目
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	回目

添付文書３

Multi-ImmunoTox Assay 記録用紙 Ver. 003.1

細胞継代シート

3-1 #2H4培養方法

3-1-1 細胞蘇生(P1)

□ あらかじめ、#2H4用C培地15 mLを37°C恒温槽で温めておく(培養用)。
□ 凍結細胞を37°C恒温槽で融解し、#2H4用C培地9 mLを入れておいた15 mLの遠沈管に加える
（細胞液0.5 mL+C培地 9 mL=計9.5 mL）
□ 遠心して細胞を集める（120-350 x g, 5分程度）。
□ 上清を吸引除去し、先に温めておいた#2H4用C培地15 mLに細胞を懸濁してT-75 Flaskで培養を
開始する（37°C, 5%CO_2）。
□上記より一部細胞浮遊液を採取し、培養開始時の細胞生存率を計測する。
（計算）

生細胞数:
死細胞数:

実施日:　　年　月　日、実施者:

3-1-2 選択抗生剤での培養開始(P2)

□ あらかじめ、#2H4用A培地必要量を37°C恒温槽で温めておく。
□ 細胞蘇生して3日～4日後に、選択抗生剤を入れた培養(#2H4用A培地)を開始する。
フラスコ中の細胞塊を滅菌ピペットでピペッティングしてほぐし、細胞数を計測する。
（　＋　）/　×　＝　×10⁴/mL－A液

□ 必要細胞量を取り、遠心して細胞を集める（120-350 x g, 5分程度）。上清を吸引除去し、先に温めておいた
#2H4用A培地15mLに3×10^5/mLで細胞を懸濁してT-75 Flaskで培養する。
※　継代濃度が同一であれば、容量の変更や維持本数変更は構わない。

A液の採取量:　　mL

実施日:　　年　月　日、実施者:

3-1-3 通常の継代培養(P3以降)

P-

□ あらかじめ、#2H4用A培地必要量を37°C恒温槽で温めておく。
□ フラスコ中の細胞塊を滅菌ピペットでピペッティングしてほぐし、細胞数を計測する。
継代細胞濃度は3×10^5/mL、継代間隔は3~4日程度で行う。
（　＋　）/　×　＝　×10⁴/mL－A液
□ 必要細胞量を取り、遠心して細胞を集める（120-350 x g, 5分程度）。上清を吸引除去し、先に温めておいた
A培地15mLに3×10^5/mLで細胞を懸濁してT-75 Flaskで培養する。
※　継代濃度が同一であれば、容量の変更や維持本数変更は構わない。

A液の採取量:　　mL

実施日:　　年　月　日、実施者:

P-

□ あらかじめ、#2H4用A培地必要量を37°C恒温槽で温めておく。
□ フラスコ中の細胞塊を滅菌ピペットでピペッティングしてほぐし、細胞数を計測する。
継代細胞濃度は3×10^5/mL、継代間隔は3~4日程度で行う。
（　＋　）/　×　＝　×10⁴/mL－A液
□ 必要細胞量を取り、遠心して細胞を集める（120-350 x g, 5分程度）。上清を吸引除去し、先に温めておいた
A培地15mLに3×10^5/mLで細胞を懸濁してT-75 Flaskで培養する。
※　継代濃度が同一であれば、容量の変更や維持本数変更は構わない。

A液の採取量:　　mL

実施日:　　年　月　日、実施者:

P-

□ あらかじめ、#2H4用A培地必要量を37°C恒温槽で温めておく。
□ フラスコ中の細胞塊を滅菌ピペットでピペッティングしてほぐし、細胞数を計測する。
継代細胞濃度は2×10^5/mL、継代間隔は3~4日程度で行う。
（　＋　）/　×　＝　×10⁴/mL－A液
□ 必要細胞量を取り、遠心して細胞を集める（120-350 x g, 5分程度）。上清を吸引除去し、先に温めておいた
A培地15mLに3×10^5/mLで細胞を懸濁してT-75 Flaskで培養する。
※　継代濃度が同一であれば、容量の変更や維持本数変更は構わない。

A液の採取量:　　mL

実施日:　　年　月　日、実施者:

P-

□ あらかじめ、#2H4用A培地必要量を37°C恒温槽で温めておく。
□ フラスコ中の細胞塊を滅菌ピペットでピペッティングしてほぐし、細胞数を計測する。
継代細胞濃度は2×10^5/mL、継代間隔は3~4日程度で行う。
（　＋　）/　×　＝　×10⁴/mL－A液
□ 必要細胞量を取り、遠心して細胞を集める（120-350 x g, 5分程度）。上清を吸引除去し、先に温めておいた
A培地15mLに3×10^5/mLで細胞を懸濁してT-75 Flaskで培養する。
※　継代濃度が同一であれば、容量の変更や維持本数変更は構わない。

A液の採取量:　　mL

実施日:　　年　月　日、実施者:

添付文書３

Multi-ImmunoTox Assay 記録用紙 Ver. 003.1
細胞調整シート

実験名　MITA バリデーション研究

実験日　＿＿＿＿＿＿＿＿＿＿＿＿

施設名　＿＿＿＿＿＿＿＿＿＿＿＿

細胞調製

室温　＿＿＿℃

予定プレート数　＿＿＿枚　x　$2.0x10^7$ cells/枚 x1.5=＿＿＿＿cells（必要細胞数）

細胞調製（試験物質用）

細胞蘇生年月日　＿＿＿年＿＿＿月＿＿＿日

前回継代年月日　＿＿＿年＿＿＿月＿＿＿日

前回継代時
細胞濃度･培養液量　＿＿＿cells/mL　X　＿＿＿mL

実験当日細胞濃度　＿＿＿cells/mL －①

遠心した細胞数　＿＿＿＿＿＿＿＿＿＿cells[*1]　①を＿＿＿mLを採取

再懸濁した培地量　＿＿＿＿＿＿＿＿＿＿mL（[*1]の細胞数÷($4x10^6$)）

それぞれのプレートに50 ∝L /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 ∝L/wellで分注　□　（　：　）

添付文書３

Multi-ImmunoTox Assay 記録用紙 Ver. 003.1
被試験試薬の調製①シート

実験名　MITA バリデーション研究

実験日　＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

施設名　＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

被試験試薬コード　＿＿＿＿　＿＿＿＿＿＿回目

被試験試薬の調製①（溶媒への溶解）

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

Multi-ImmunoTox Assay 記録用紙 Ver. 003.1

被試験試薬の調製②(DW)シート

実験名 MITA バリデーション研究

実験日 _____

施設名 _____

被試験試薬コード _____ 1 2 3 4 5 6 回目

Distilled water溶液に調製された場合

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

試験液の調製と細胞への処理

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

調製時間
(:)

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

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

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

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

プレートをシールし、プレートシェーカーを使用し、撹拌して混合する。 □

細胞をインキュベーターへ入れ、1時間反応させる。 □

賦活剤(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

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

プレートをシールし、プレートシェーカーを使用し、撹拌して混合する。

細胞をインキュベーターへ入れ、6時間反応させる。

測定(被試験物質)

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

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

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

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

ルミノメータでLuciferase活性を測定する。(測定中温度が26-31℃であることを確認する。)
フィルタ無し、フィルタ有りで各々3 秒/well測定する(アトー社製Pheliosの場合はF0、F1、F2を使用)。 □ 測定時間
(:)
測定中温度
(℃)

Multi-ImmunoTox Assay 記録用紙 Ver. 003.1

被試験試薬の調製③(DMSO)シート

実験名 MITA バリデーション研究

実験日 _____

施設名 _____

被試験試薬コード _____ 1 2 3 4 5 6 回目

DMSO溶液に調製された場合

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

試験液の調製と細胞への処理

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

調製時間
(:)

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

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

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

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

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

沈殿の性状 (例:粉状、泥状、膜状、ミセル様)

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

プレートをシールし、プレートシェーカーを使用し、撹拌して混合する。 □

細胞をインキュベーターへ入れ、1時間反応させる。 □

賦活剤(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 (:)

プレートをシールし、プレートシェーカーを使用し、撹拌して混合する。

細胞をインキュベーターへ入れ、6時間反応させる。

測定(被試験物質)

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

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

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

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

ルミノメータでLuciferase活性を測定する。(測定中温度が26-31℃であることを確認する。)
フィルタ無し、フィルタ有りで各々3 秒/well測定する(アトー社製Pheliosの場合はF0、F1、F2を使用)。 □ 測定時間 (:)
測定中温度 (℃)

Multi-ImmunoTox Assay 記録用紙 Ver. 003.1

被試験試薬の調製④（コントロール）シート

実験名 MITA バリデーション研究

実験日 _____

施設名 _____

被試験試薬コード _____ _____回目

コントロールの調製と細胞への処理

dexamethasone, cyclosporine Aの調製
96 well clear plate（丸底）に下図のようにDMSO 50 µL (#A4)、100 µg/mL cyclosporine A stock 50 µL (#A5)、Distilled water 50 µL (#B1、#B2)、2.5 mg/mL dexamethasone stock 50 µL (#B3)、B培地 180 µl (#B4、#B5)を分注する。 ☐ 添加時間 （ ： ）

丸底・透明	1	2	3	4	5	6	7	8	9	10	11	12
A				DMSO 50 uL	CyA 100 µg/mL ストック 50 uL							
B	Distilled water 50 uL	Distilled water 50 uL	DEX 2.5 mg/mL ストック 50 uL	B培地 180 uL	B培地 180 uL							
C												
D												
E												
F												
G												
H												

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

アッセイブロックの#A1–#A3にB培地480 µL、#A1–#A3にB培地980 µLを分注し、上図の希釈液を20 µL添加して混合し、50 µL/wellずつ細胞に添加する。 ☐ 添加時間 （ ： ）

プレートをシールし、プレートシェーカーを使用し、撹拌して混合する。 ☐

細胞をインキュベーターへ入れ、1時間反応させる。 ☐

賦活剤(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–#D1、x10 PMA/ionomycin溶液を#A2–#D5に10 µLずつ分注する。 ☐ 添加時間 （ ： ）

プレートをシールし、プレートシェーカーを使用し、撹拌して混合する。

細胞をインキュベーターへ入れ、6時間反応させる。

測定（コントロール）

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

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

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

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

ルミノメータでLuciferase活性を測定する。（測定中温度が26–31℃であることを確認する。）
フィルタ無し、フィルタ有りで各々3 秒/well測定する（アトー社製Pheliosの場合はF0、F1、F2を使用）。 ☐ 測定時間 （ ： ）
測定中温度 （ ℃）

添付文書３

Multi-ImmunoTox Assay 記録用紙 Ver. 003.1
更新履歴シート

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