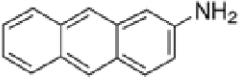
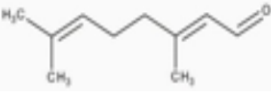
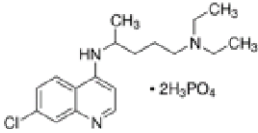
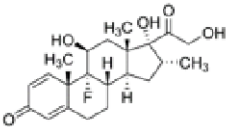


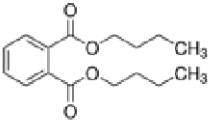
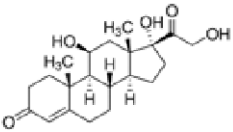
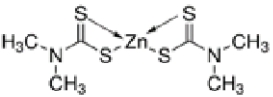
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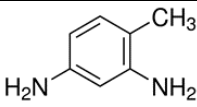
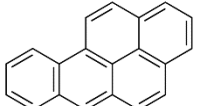
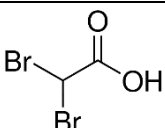
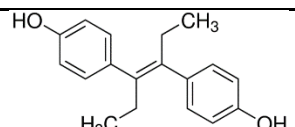
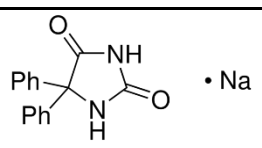
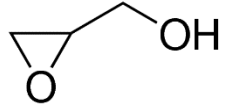
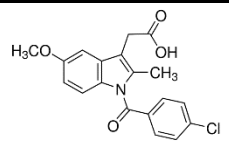
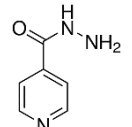
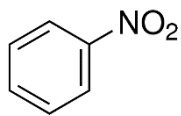
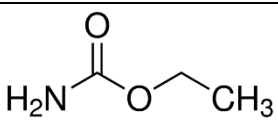
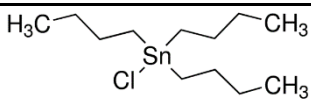
Appendix 1. Chemical structure of the test chemicals for Phase 0 study

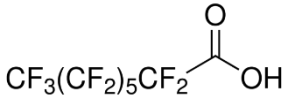
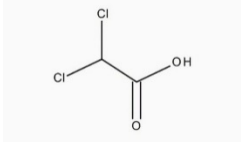
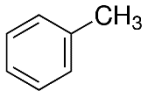
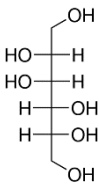
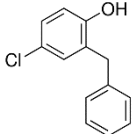
No.	Chemical name	CAS No.	Molecular weight	Chemical structure
0-1	2-Aminoanthracene	613-13-8	193.24	
0-2	Citral	5392-40-5	152.23	
0-3	Chloroquine diphosphate salt	50-63-5	515.86	
0-4	Dexamethasone	50-02-2	392.46	
0-5	Methylmercury(II) chloride	115-09-3	251.08	CH ₃ HgCl

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

No.	Chemical name	CAS No.	Molecular weight	Chemical structure
I-1	Dibutyl phthalate	84-74-2	278.34	
I-2	Hydrocortisone watersoluble	50-23-7	362.46	
I-3	Lead(II)acetate	6080-56-4	379.33	$\left[\text{H}_3\text{C}-\overset{\text{O}}{\parallel}{\text{C}}-\text{O}^- \right]_2 \text{Pb}^{2+} \cdot 3\text{H}_2\text{O}$
I-4	Nickel sulfate hexahydrate	10101-97-0	262.85	$\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$
I-5	Dimethyldithiocarbamate	137-30-4	305.82	

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

No.	Chemical name	CAS No.	Molecular weight	Chemical structure
II-1	2,4-Diaminotoluene	95-80-7	122.17	
II-2	Benzo(a)pyrene	50-32-8	252.31	
II-3	Cadmium chloride	10108-64-2	183.32	CdCl_2
II-4	Dibromoacetic acid	631-64-1	217.84	
II-5	Diethylstilbestrol	56-53-1	268.35	
II-6	Diphenylhydantoin	630-93-3	274.25	
II-7	Ethylene dibromide	106-93-4	187.86	$\text{BrCH}_2\text{CH}_2\text{Br}$
II-8	Glycidol	556-52-5	74.08	
II-9	Indomethacin	53-86-1	357.79	
II-10	Isoniazid	54-85-3	137.14	
II-11	Nitrobenzene	98-95-3	123.11	
II-12	Urethane, Ethyl carbamate	51-79-6	89.09	
II-13	Tributyltin chloride	1461-22-9	325.51	

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

Appendix 4. Protocol of the Multi-Immuno Tox Assay (ver. 011E)

Multi-Immuno Tox Assay protocol ver. 011E

May. 10th, 2018

Department of Dermatology, Tohoku University Graduate School of Medicine

Yutaka Kimura, M.D., Ph.D.

Setsuya Aiba, M.D., Ph.D.

15. APPENDIXES エラー! ブックマークが定義されていません。

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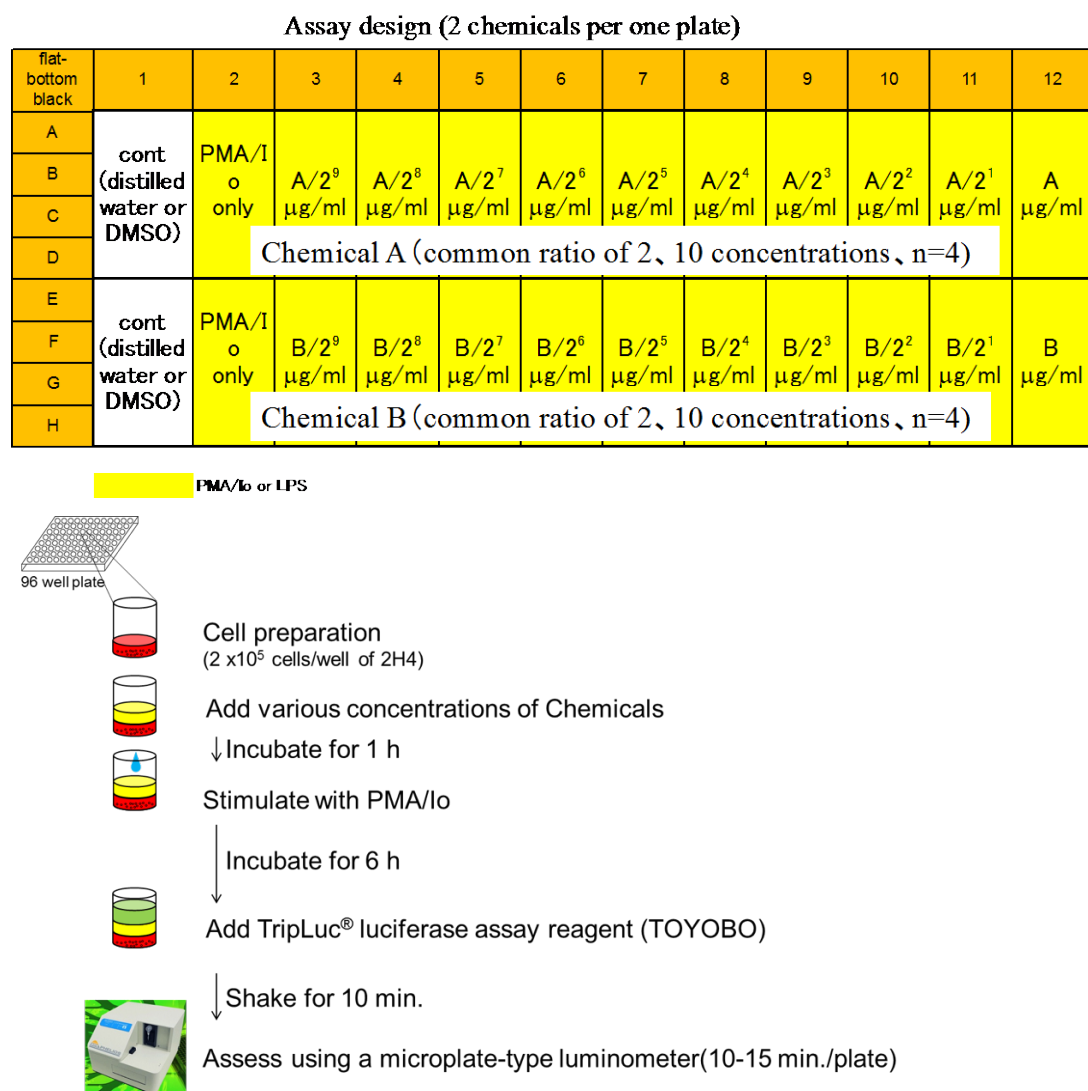
APPENDIX 6 VALIDATION OF REAGENTS AND EQUIPMENT 153

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 γ 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



2. Materials

2-1 Cells

2H4 (IL2-SLG、 IFN γ -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 γ reporter cell line, 2H4,

that harbors the SLG, SLO and SLR luciferase genes under the control of the IL-2, IFN γ and GAPDH promoters, respectively, was established by Tsuruga Institute of Biotechnology, TOYOBO Co. Ltd.

(Saito R. et al. Nickel differentially regulates NFAT and NF- κ 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[®] Luciferase assay reagent (TOYOBO Cat#MRA-301)

2-2-4 Expendable supplies

T-75 flask tissue culture treated (e.g., Corning Cat#353136)

96 well μ clear black plate (flat-bottom, for measurement of the luciferase activity, e.g. Greiner Bio-one Cat#655090)

96 well clear plate (round-bottom, for preparation of chemicals and stimulants)

96 well assay block, 2 mL (e.g., Costar Cat#3960)

Seal for 96 well plate (e.g., Perkin Elmer TopSeal-A PLUS Cat#6050185, EXCEL Scientific SealMate Cat#SM-KIT-SP)

Reservoir

Pipette

2-2-5 Equipment for measurement of luciferase activity

Measuring device: a microplate-type luminometer with a multi-color detection system that can accept two optical filter

e.g. Phelios AB-2350 (ATTO), ARVO (PerkinElmer), Tristar LB941 (Berthold)

Optical filter: 560 nm long-pass filter and 600 nm long-pass filter

Measuring time: set at 1~5 sec/well measuring time

2-2-6 Others

Pipetman

8 channel or 12 channel pipetman (optimized for 10~100 μ L)

Plate shaker (for 96 well plate)

CO₂ incubator (37°C, 5% CO₂)

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 μ g/mL	75 μ L
G418	Nacal Tesque #16513-84	50 mg/mL	300 μ g/mL	3 mL
HygromycinB	Invitrogen #10687-010	50 mg/mL	200 μ 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 μ L, dispense at 5 μ L/tube and store at freezer at -30°C. Use these stocks within 6 months after dissolution.

2-4-2 Ionomycin

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

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

3. Cell culture

3-1 Thawing of 2H4 cells

Pre-warm 9 mL of C medium in a 15 mL polypropylene conical tube in a 37°C water bath (for centrifugation) and 15 mL of C medium in a T-75 Flask at 37°C in a 5% CO₂ incubator (for culture).

Thaw frozen cells (2×10^6 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% CO₂.

3-2 Maintenance of 2H4 cells

Pre-warm the A medium in a T-75 Flask at 37°C in a 5% CO₂ 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 3×10^5 /mL and incubated at 37°C, 5% CO₂.

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)

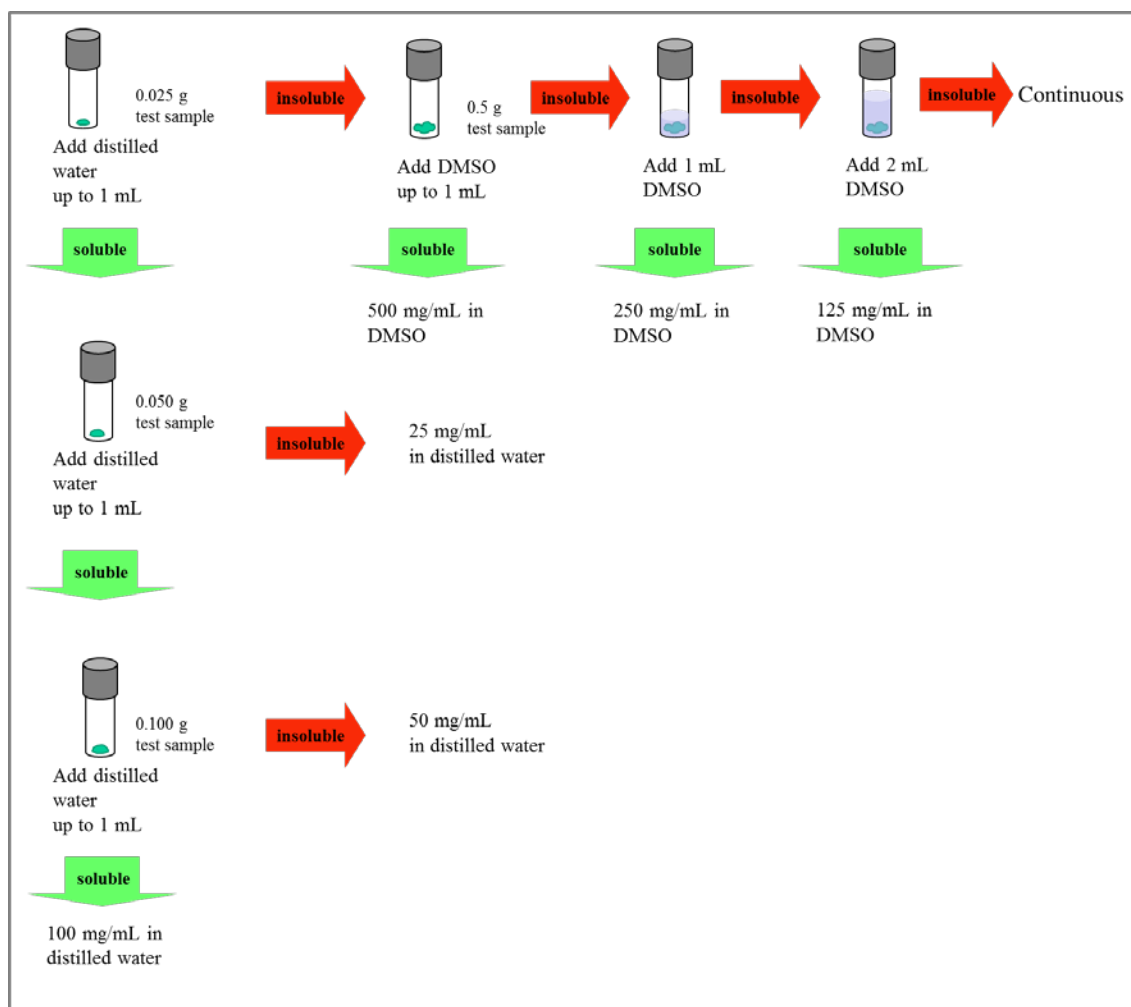
Dissolve the chemical first in distilled water. Namely, weigh 0.025 g of the test chemical in a volumetric flask and add distilled water up to 1 mL. If the chemical is soluble at 25 mg/mL, weigh 0.050 g of the test chemical in a volumetric flask and add distilled water up to 1 mL. If the chemical is 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 ($\approx 20,000 \times g$) for 5 min and absence of precipitation. The chemical should be used within 4 hours after being dissolved in distilled water or DMSO.

Figure 3



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

In the second to fourth experiment (2nd to 4th experiment), determine the minimum concentration at which I.I.-SLR-LA (mentioned later in **10**) became lower than 0.05 in the 1st experiment, use the concentration one step (2-times) higher than this determined concentration as the highest concentration of the chemical to examine, and conduct 10 serial dilutions at a common ratio of 2 from the highest concentration. If I.I.-SLR-LA did not become lower than 0.05 or became lower than 0.05 at the highest concentration in the 1st experiment, conduct 10 serial dilutions at a common ratio of 2 from the highest concentration in the 1st experiment.

For example, in Figure 3 below, the minimum concentration at which I.I.-SLR-LA became lower than 0.05 is 1.95 µg/ml. The highest concentration of the chemical to examine is the concentration one step (2-times) higher than 1.95 µg/ml, which is 3.91 µg/ml.

In Figure 4 below, I.I.-SLR-LA did not become lower than 0.05. In such a case, the highest

concentration of the chemical to examine is the highest concentration in the 1st experiment, namely 125 µg/ml.

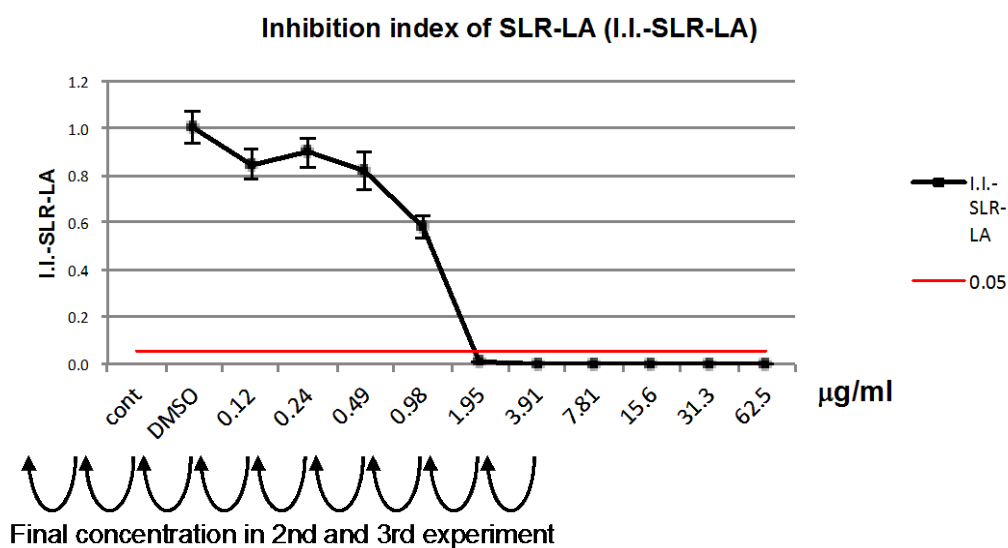


Figure 3.

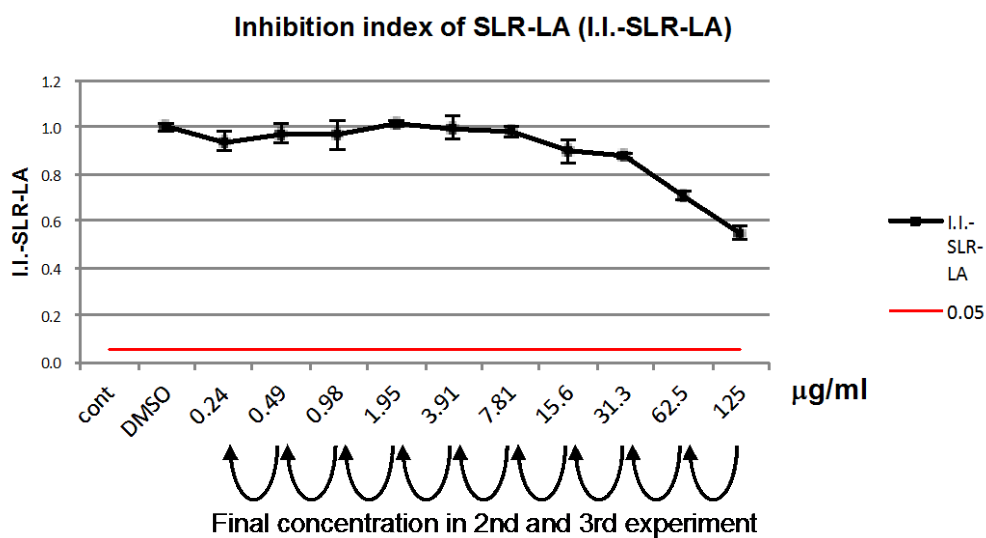


Figure 4

5-2 When the chemical is prepared in distilled water

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

5-2-1 Arrangement of chemicals and vehicle

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

5-2-2 Serial dilution

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

Figure 4

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

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

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

5-2-3 2 step dilution

Add 20 μ L of the diluted chemical to 480 μ L of the B medium prepared in the assay block. And add 50 μ 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 CO₂ incubator for 1 hour (37°C, CO₂, 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

50uL[illegible]

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

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

10 μ L

↓

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

5-3-4 2 step dilution

Add 10 μ L of the diluted chemical to 490 μ L of the B medium prepared in the assay block. And add 50 μ 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 plateshaker and incubate in a CO₂ incubator for 1 hour (37°C, CO₂, 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												

Figure 11

Figure 12 Final constituents of each well of the plate

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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 μ M PMA

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

2 mM PMA	B medium	Total	final concentration
5 μ L	95 μ L	100 μ L	100 μ M

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

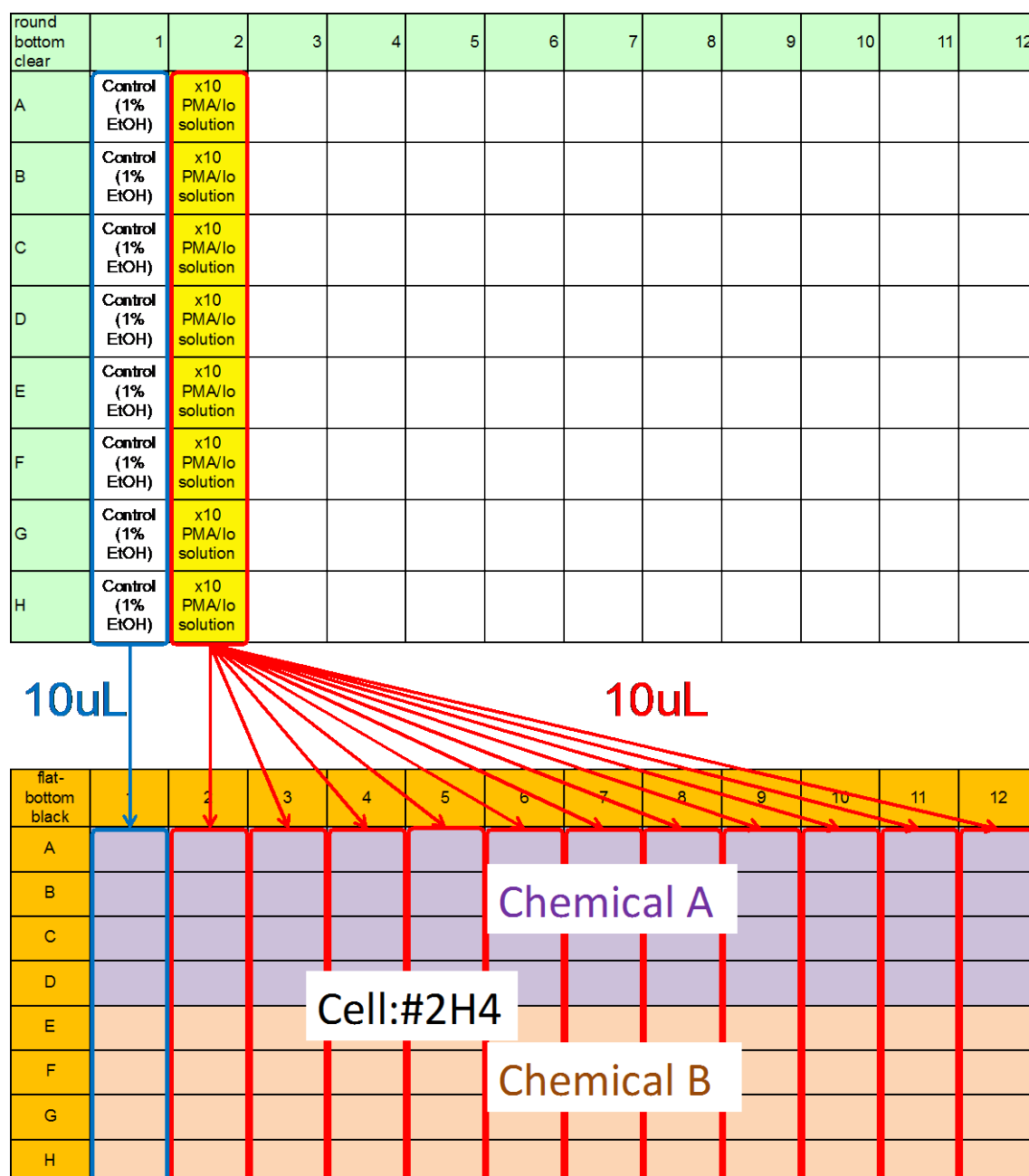
Dilute ethanol, 2 mM ionomycin and 100 μ M PMA with the B medium to prepare control or x10 PMA/ionomycin solution. Add the control to well #A1-#H1 of the 96 well clear plate (round bottom), and add x10 PMA/ionomycin solution to wells #A2-#H2 of the 96 well clear plate (round bottom).

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

6-4 Addition of PMA/ionomycin to 2H4

One hour after the addition of chemicals, add 10 μ 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 CO₂ incubator for 6 hour (37°C, CO₂, 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, 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 µg/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 (5.0×10^6 cells are required, but to have some leeway, 7.5×10^6 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 μ L to #A4, 100 μ g/mL cyclosporine A stock 50 μ L to #A5, distilled water 50 μ L to #B1 and #B2, 2.5 mg/mL dexamethasone stock 50 μ L to #B3 and the B medium 180 μ 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 μ 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 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												
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												

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 μ 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 plateshaker and incubate in a CO₂ incubator for 6 hour (37°C, CO₂, 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/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										

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												

10uL

10uL

8. Calculation of the transmittance factors

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

8-1 Reagents

Single reference samples:

Lyophilized luciferase enzyme reagent for stable luciferase green (SLG)

Lyophilized luciferase enzyme reagent for stable luciferase orange (SLO)

Lyophilized luciferase enzyme reagent for stable luciferase red (SLR)

Assay reagent:

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

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

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

8-2 Preparation of luminescence reaction solution

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

Add 200 μ 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 μ 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 μ L sample per tube). Keep the reference samples on ice to prevent deactivation.

8-3 Bioluminescence measurement

Transfer 100 μ 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 μ L	SLG 100 μ L	SLG 100 μ L									
C												
D	SLO 100 μ L	SLO 100 μ L	SLO 100 μ L									
E												
F	SLR 100 μ L	SLR 100 μ L	SLR 100 μ L									
G												
H												

Transfer 100 μ 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,

$$\text{Transmittance factors } (\kappa_{GR56}) = \frac{1269550 + 1257268 + 1289562}{3757015 + 3716611 + 3810382} = 0.338$$

$$\text{Transmittance factors } (\kappa_{OR56}) = \frac{808550 + 813160 + 754174}{1202691 + 1210208 + 1122295} = 0.672$$

$$\text{Transmittance factors } (\kappa_{RR56}) = \frac{2193723 + 1968240 + 1853873}{2465453 + 2207572 + 2077689} = 0.891$$

$$\text{Transmittance factors } (\kappa_{GR60}) = \frac{236478 + 234079 + 240876}{3757015 + 3716611 + 3810382} = 0.06$$

$$\text{Transmittance factors } (\kappa_{OR60}) = \frac{235121 + 235878 + 217432}{1202691 + 1210208 + 1122295} = 0.195$$

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

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	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

<div style="border: 1px solid black; display: inline-block; padding: 2px 10px;">Multi-ImmunoTox Assay Datasheet for #2H4 cells</div> <div style="float: right; font-size: small;">Ver. 005.2</div>													
Laboratory				Round									
Exp.													
Date: <small>(YYYYMMDD)</small>				Operator:									
Code		Chemical 1		Chemical 2		Dissolution		Chemical 1		Chemical 2		mg/ml in	
Molecular weight		Chemical 1		Chemical 2		Dissolution		Chemical 1		Chemical 2		mg/ml in	
Comment:													

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

#E7 of the “Data Input” sheet.

Figure 25 “Data Input” sheet of the data sheet

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	MultiReporter Assay System - Tripluc® - Calculation Sheet													
2		Transmittance Data												
3			SLG	SLO	SLR									
4		T0	1	1	1		#VALUE!	#VALUE!	#VALUE!					
5		T1					#VALUE!	#VALUE!	#VALUE!					
6		T2					#VALUE!	#VALUE!	#VALUE!					
7														
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 \pm SD of SLG-LA, the mean \pm SD of SLO-LA, the mean \pm 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) : $=(\text{SLG-LA})/(\text{SLR-LA})$

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- γ 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 3.0, the results obtained from the plate containing the control wells should be rejected.

11-2 Criterion

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

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

In each experiment, when the chemicals clear the following 3 criteria, they are judged as suppressive

or stimulatory. Otherwise, they are judged as no effect chemicals.

1. The mean of %suppression is ≥ 35 (suppressive) or ≤ -35 (stimulatory) with statistical significance.

The statistical significance is judged by its 95% confidence interval.

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

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

12. Update record

Ver. 0011.0E 2018.5.10

Change the criteria

Ver. 0010.0E 2018.1.15 distribution

Change the criteria

Ver. 009.1E 2017.5.8 distribution

Change the criteria

Ver. 009.0E 2017.4.7 distribution

Change the preparation of chemicals

Change the acceptance criteria

Change the criteria

Ver. 008.5E 2016.9.14 distribution

Change the criteria

Ver. 008.4E 2016.9.9 distribution

Change the criteria

Ver. 008.3E 2016.8.1 distribution

Correction of the preparation of PMA and ionomycin

Change the preparation of PMA and ionomycin

Change the preparation of controls

Addition of Acceptance criteria

Ver. 008.1E 2016.2.2 distribution

Changes after the VMT meeting

Ver. 008.0E 2016.1.19

Translation to English

Addition of appendix

Ver. 006.0J 2015.8.17

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

Delete the alteration in Ver. 005.0J

Ver. 005.0J 2015.1.9 distribution

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

Ver. 004.1J 2014.12.10 distribution

Change the 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 (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 5×10^4 /well to 1×10^5 /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: 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 5. 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 ($\lambda_{\text{max}} = 550 \text{ nm}$), SLO ($\lambda_{\text{max}} = 580 \text{ nm}$) and SLR ($\lambda_{\text{max}} = 630 \text{ nm}$), 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_{K56}	The intensity of 560 nm LP (Filter 1) transmitted SLG / the intensity of SLG without filter (all optical)
	Filter 2 transmittance factor	κG_{K60}	The intensity of 600 nm LP (Filter 2) transmitted SLG / the intensity of SLG without filter (all optical)
SLO	Filter 1 transmittance factor	κO_{K56}	The intensity of 560 nm LP (Filter 1) transmitted SLO / the intensity of SLO without filter (all optical)
	Filter 2 transmittance factor	κO_{K60}	The intensity of 600 nm LP (Filter 2) transmitted SLO / the intensity of SLO without filter (all optical)
SLR	Filter 1 transmittance factor	κR_{K56}	The intensity of 560 nm LP (Filter 1) transmitted SLR / the intensity of SLR without filter (all optical)
	Filter 2 transmittance factor	κR_{K60}	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 "MINVERSE" and "MMULT" in Microsoft Excel. These calculations are integrated in the Data Sheet.

Appendix 6. 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 μ 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 μ L 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 μ 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 μ 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 μ 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°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% (σ). Disagreement to the old data should be less than $3 \times \sigma$ (= 4.5%).