Multi-Immuno Tox Assay protocol ver. 011.1E May 14th, 2020

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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, 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

96 well plate

Assay	design	(Z chen	ncais p	er one I	nale)	

(A 1

flat- bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	cont	PMA/I										
В	(distilled	0	A∕2 ⁹	A/2 ⁸	A/2 ⁷	A/2 ⁶	A/2 ⁵	A/2 ⁴	A/2 ³	A/2 ²	A/2 ¹	А
С	water or	only	∞g⁄ ml	∞g⁄ml	∞g⁄ml	∞g∕ml	∞g⁄ml	∞g⁄ ml	∞g⁄ml	∞g⁄ml	∞g∕ml	∞g⁄ml
D	DMSO)	C	Chemic	al A(c	ommo	n ratio	of 2, 1	0 conc	entrati	ions, n	=4)	
E	cont	PMA/I										
F	(distilled	0	B∕2 ⁹	B∕2 ⁸	B/2 ⁷	B∕2 ⁶	B∕2 ⁵	B/2 ⁴	B∕2 ³	B/2 ²	B/2 ¹	В
G	water or	only	∞g⁄ ml	∞g⁄ml	∞g∕ml	∞g∕ml	∞g∕ml	∞g⁄ml	∞g∕ml	∞g∕ml	∞g⁄ml	∞g∕ml
н	DMSO)	C	Chemic	al B (c	ommo	n ratio	of 2, 1	0 conc	entrat	ions, n	=4)	

PMA/Io

Cell preparation (2 x10⁵ cells/well of 2H4) Add various concentrations of Chemicals ↓Incubate for 1 h Stimulate with PMA/Io

Incubate for 6 h



Shake for 10 min.

Assess using a microplate-type luminometer(10-15 min./plate)

Add TripLuc[®] luciferase assay reagent (TOYOBO)

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 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-κB activation in T cell signaling *Toxicology and Applied Pharmacology*, 254, 245–255, 2011)

2-2 Reagents and equipment

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

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

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

2-2-3 For measurement of the luciferase activity

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

2-2-4 Expendable supplies

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

- 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 $1 \sim 5$ sec/well measuring time

2-2-6 Others

- Pipetman
- 8 channel or 12 channel pipetman (optimized for $10 \sim 100 \ \mu$ L)
- Plate shaker (for 96 well plate)
- CO₂ incubator $(37^{\circ}C, 5\% CO_2)$
- 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	BiologicalIndustriesCat#04-001-1ELot: 715004	-	10 %	50 mL
Antibiotic-Antimycotic	GIBCO #15240-062	100×	$1 \times$	5 mL
Puromycin	InvivoGen # ant-pr-1	10 mg/mL	0.15 μ g/mL	7.5 μL
G418	Nacalai 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	-	10 %	3 mL
	Lot: 715004			

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	BiologicalIndustriesCat#04-001-1ELot: 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-	Sigma #P8139		
myristate 13-			
acetate		2 mM	25 nM
(PMA)			
DMSO	Sigma #D5789		

Dissolve 1 mg PMA using DMSO 811 μ L, dispend 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 # 10634			
Ethanol	Wako #057	- 2 mM	1 μM	
	00456			

Dissolve 1mg Ionomycin using ethanol 669.3 μ L, dispend 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 $(2x10^6 \text{ cells} / 0.5 \text{ mL of freezing medium})$ 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 <u>120-350 x g</u> 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 <u>120-350 x g</u> 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% 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 x 10^7 cells for two chemicals are required, but to have some leeway, 3.0 x 10^7 cells for two chemicals should be prepared), centrifuge the tube at <u>120-350 x g</u>, 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)

flat– bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	2H4	2H4	2H 4	2H 4	2H4	2H4	2H4	2H 4	2H4	2H4	2H4	2H4
	2x10^5	2x10^5	2x10^5	2x10^5	2x10 ^x 5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5
	B medium	B medium	B medium	B medium	Bmedium	B medium	B medium	B medium				
	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
В	2H4	2H4	2H4	2H 4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4
	2x10^5	2x10^5	2x10^5	2x10^5	2x10 ^x 5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5
	B medium	B medium	B medium	B medium	Bmedium	B medium	B medium	B medium				
	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
С	2H4	2H4	2H4	2H 4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4
	2x10^5	2x10^5	2x10^5	2x10^5	2x10 ⁺⁵	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5
	B medium	B medium	B medium	B medium	Bmedium	B medium	B medium	B medium				
	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
D	2H4	2H4	2H4	2H 4	2H4	2H4	2H4	2H 4	2H4	2H4	2H4	2H4
	2x10^5	2x10^5	2x10^5	2x10^5	2x10 ⁺⁵	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5
	B medium	B medium	B medium	B medium	Bmedium	B medium	B medium	B medium				
	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
E	2H4	2H4	2H4	2H 4	2H4	2H4	2H4	2H 4	2H4	2H4	2H4	2H4
	2x10^5	2x10^5	2x10^5	2x10^5	2x10 ⁺⁵	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5
	B medium	B medium	B medium	B medium	Bmedium	B medium	B medium	B medium				
	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
F	2H4	2H4	2H 4	2H 4	2H4	2H4	2H4	2H 4	2H4	2H4	2H4	2H4
	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5
	B medium	B medium	B medium	B medium	Bmedium	B medium	B medium	B medium				
	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
G	2H4	2H4	2H 4	2H 4	2H4	2H4	2H4	2H 4	2H4	2H4	2H4	2H4
	2x10^5	2x10^5	2x10^5	2x10^5	2x10 ⁺⁵	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5
	B medium	B medium	B medium	B medium	Bmedium	B medium	B medium	B medium				
	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
н	2H4	2H4	2H 4	2H 4	2H4	2H4	2H4	2H 4	2H4	2H4	2H4	2H4
	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5
	B medium	B medium	B medium	B medium	Bmedium	B medium	B medium	B medium				
	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL

5. Preparation of chemicals and cell treatment with chemicals

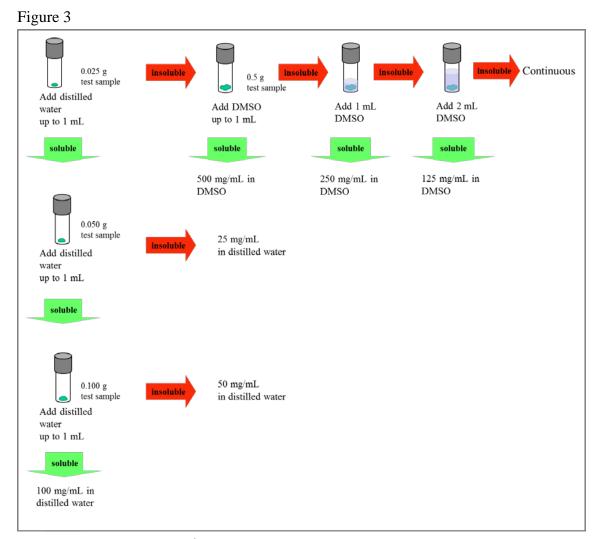
5-1 Dissolution by vehicle

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 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 concentration. If the chemical is not soluble at 100 mg/mL, 50 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 \rightarrow 125 mg/mL \rightarrow continued if needed) with DMSO. (cf. Figure 3)

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 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.



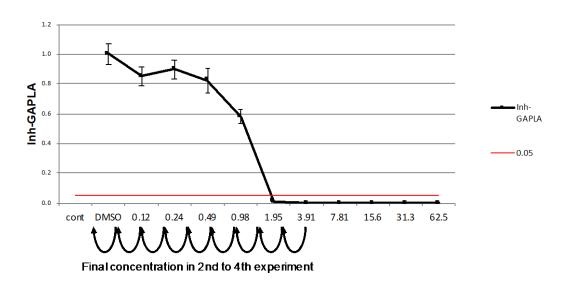
In the first experiment (1st experiment), when the chemical is prepared in distilled water, conduct 10 serial dilutions at a common ratio of 2 from the highest concentration using distilled water. When the chemical is prepared as a DMSO solution, conduct 10 serial dilutions at a common ratio of 2 from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

In the second to fourth experiment $(2^{nd} \text{ to } 4^{th} \text{ experiment})$, determine the minimum concentration at which Inh-GAPLA (mentioned later in <u>10</u>) 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 Inh-GAPLA 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, conduct 10 serial dilutions at a common ratio of 2 from the highest concentration in the 1st experiment.

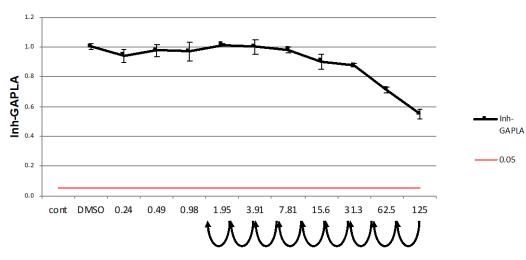
For example, in Figure 4 below, the minimum concentration at which Inh-GAPLA

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

In Figure 5 below, Inh-GAPLA 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 1^{st} experiment, namely 125 µg/ml.







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 μ L of the 100 mg/mL distilled water solution of the chemical to well #A12, and 50 μ L 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 μ L to the next (left) well. (cf. Figure 6)

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Disti ll ed water 50uL	Disti ll ed water 50uL	Distilled water 50uL	Disti ll ed water 50uL	Disti ll ed water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Disti ll ed water 50uL	Disti ll ed water 50uL	Chemical 100 mg/mL in distiled water 100 uL
В						\mathcal{T}				\mathcal{T}		
С						· · ·						
D									I			
E				2-fol	d dilution :	transfer 5	50 uL (pipe	etman, yel	low tip)			
F												
G												
н												

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Disti ll ed water 50uL	Disti ll ed 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 50 uL	Chemical 50 mg/mL in distilled water 50 uL	Chemical 100 mg/mL in distilled water 50 uL
В												
С												
D												
E												
F												
G												
Н												

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 7-9).

Figure 7	7											
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
В												
С												
D							_					
E							20ul					
F							Zoui	-				
G												
Н												
Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
	1 B medium 480uL	2 B medium 480uL	3 B medium 480uL	4 B medium 480uL	5 B medium 480uL	6 B medium 480uL	, 7 B medium 480uL	8 B medium 480uL	9 B medium 480uL	10 B medium 480uL	11 B medium 480uL	12 B medium 480uL
Block	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium
Block A B C	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium
A B C D	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium
Block A B C D E	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium
Block A B C D E F	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium
Block A B C D E	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium

Figure	8
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Assay	1	2	3	4	5	6	7	8	9	10	11	12
Block		2	5	, , , , , , , , , , , , , , , , , , ,	5	0	'	0	J	10		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
В												
С							\mathcal{N}					
D					,							
E		-						50ul				
F						/		x				
G H						ſ	- \					
п												
flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
А	2H4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H4 2x10^5 B medium 50u	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B medium 50uL	2H4 9x10 ⁴⁵ B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B m edium 50uL	2H4 2x10^5 B medium 50uL
В	2H4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 2 medium 50uL	2H4 2x10 ⁴ 5 B medium 50uL	2H 4 2x10^5 8 m edium 50uL	2H 4 2x10^5 B m edium 50uL	2H4 2x10^5 B medium 50uL
с	2H4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B m edium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL
D	2H4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H4 2x10^5 Bmedium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B m edium 50uL	2H4 2x10^5 B medium 50uL
E	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H4 2x10^5 Bmedium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B medium 50uL
F	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B m edium 50uL	2H4 2x10^5 B medium 50uL
G	2H4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B medium 50uL	2H4 2x10 ^{v5} B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B medium 50uL
н	2H4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B medium 50uL	2H4 2x10 ^{v5} B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B m edium 50uL	2H4 2x10^5 B medium 50uL

Figure 9 Final constituents of each well of the plate

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
А	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	Chemic al 0.1 mg/mL 2H4 2x10^5 B medium 100uL	Chemical 0.3 mg/mL 2H4 2x 10^5 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
В	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	Chemic al 0.1 mg/mL 2H4 2x10^5 B medium 100uL	Chemical 0.3 mg/mL 2H4 2x 10^5 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
с	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	Chemic al 0.1 mg/mL 2H4 2x10^5 B medium 100uL	Chemical 0.3 mg/mL 2H4 2x 10^5 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	Chemic al 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	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B medium 50uL	2H4 2x10 ⁴⁵ B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B medium 50uL
F	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H4 2x10^5 Bmedium 50uL	2H4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B medium 50uL	2H4 2x10 ⁴⁵ B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B m edium 50uL	2H4 2x10^5 B medium 50uL
G	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B m edium 50uL	2H4 2x10^5 B medium 50uL
н	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10 ^x 5 B m edium 50uL	2H 4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B medium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 2x10^5 B m edium 50uL	2H 4 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 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 10)

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 50uL	Chemical 500 mg/mL in DMSO 100uL										
В	B medium 90uL											
С						_						
D				2.	fold diluti	on : transfe	er 50 uL (p	oipetman、	yellow tip,)		
E												
F												
G												
н												

						*						
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
в	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
С												
D												
E												
F												
G												
Н												

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 11)

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

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 11. Seal the plate, shake the plate with a plateshaker and incubate in a CO₂ incubator for 1 hour (37°C, CO₂, 5%) (cf. Figure 12-14).

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 40uL	DM SO 100% 40uL	Chemical 1.0 mg/mL in DMSO 90uL	Chemical 2.0 mg/mL in DMSO 40uL	Chemical 3.9 mg/mL in DM SO 40uL	Chemical 7.8 mg/mL in DMSO 40uL	Chemical 16 mg/mL in DMSO 40uL	Chemical 31 mg/mL in DM SO 40uL	Chemical 63 mg/mL in DMSO 40uL	Chemical 125 mg/mL in DMSO 40uL	Chemical 250 mg/mL in DM SO 40uL	Chemical 500 mg/mL in DMSO 40uL
		Chemical 0 mg/mL DMSO 10% in B medium 100uL	Chemical 0.10 mg/mL DMSO 10% in B medium 100uL	DMSO 10%	DMSO 10%					Chemical 12.5 mg/mL DMSO 10% in B medium 100uL		
С												
D												
E												
F												
G							10ul					
Н							- 1001					

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

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	Chemical	Chemical	Chemical	Chemical	Chemical	Chemical	Chemical	Chemic al	Chemical	Chemical	Chemical	Chemical
	Oug/mL	0ug/mL	2.0ug/mL	3.9ug/mL	7.8ug/mL	15.6ug/mL	31.3ug/mL	62.5ug/mL	125ug/mL	250ug/mL	500ug/mL	1000ug/mL
	DMSO 0.2%	DMSO 0.2%	DMSO 0.2%	DMSO 0.2%	DMSO 0.2%	DMSO 0.2%	DMSO 0.2%					
	in B medium	in B medium	in B medium	in B medium	in B medium	in B medium	in B medium					
	500uL	500uL	500uL	500uL	500uL	500uL	500uL	500uL	500uL	500uL	500uL	500uL
В												
С						-/-	\mathcal{H}					
D						\leftarrow						
F							+					
G							.1 77					
Н						<u></u> 250ι	ᅚ	Δ				
first in attacks												
flat-bottom black	1	2	3	4	5 🖊	6	7	8	9	10	11	12
A	2H4	2H4	2H4	2H4	21/4	2H4	2H4	2H4	2H4	2H4	2H4	2H4
	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10 ⁴ 5	2x10 ⁴ 5	2x 10^5	2x10^5	2x10^5
	B medium	B medium	B medium	B medium	8 medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium
	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
В	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4
	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x1045	2x10^5	2x10^5	2x10^5	2x10^5
	B medium	B medium	B medium	B medium	B medium	B medium	B medium					
	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
с	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4
	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x 10^5	2x10^5	2x10^5
	B medium	B medium	B medium	B medium	B medium	B medium	B medium					
	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
D	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4
	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5
	B medium	B medium	B medium	B medium	B medium	B medium	B medium					
	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
E	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4
	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10 [*] 5	2x10^5	2x10^5	2x10^5	2x10^5
	B medium	B medium	B medium	B medium	B medium	B medium	B medium					
	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
F	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4
	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x 10^5	2x10^5	2x10^5
	B medium	B medium	B medium	B medium	B medium	B medium	B medium					
	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
G	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4
	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x 10^5	2x10^5	2x10^5
	B medium	B medium	B medium	B medium	B medium	B medium	B medium					
	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
н	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4
	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10 ^A 5	2x10^5	2x 10^5	2x10^5	2x10^5
	B medium	B medium	B medium	B medium	B medium	B medium	B medium					
	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	Chemical	Chemical	Chemical	Chemical	Chemical	Chemical	Chemical	Chemic al	Chemical	Chemical	Chemical	Chemical
	0ug/mL	0ug/mL	1.0ug/mL	2.0ug/mL	3.9ug/mL	7.8ug/mL	16ug/mL	31ug/mL	63ug/mL	125ug/mL	250ug/mL	500ug/mL
	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DM SO	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DMSO
	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4
	2x10^5	2x10^5	2x10 ^A 5	2x10^5	2x10^5	2x10 ^A 5	2x10 ^A 5	2x10 ^A 5	2x10^5	2x10 ^A 5	2x10 ^A 5	2x10^5
	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium
	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL
в	Chemical	Chemical	Chemical	Chemical	Chemical	Chemical	Chemical	Chemic al	Chemical	Chemical	Chemical	Chemical
	0ug/mL	0ug/mL	1.0ug/mL	2.0ug/mL	3.9ug/mL	7.8ug/mL	16ug/mL	31ug/mL	63ug/mL	125ug/mL	250ug/mL	500ug/mL
	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DM SO	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DMSO
	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4
	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10 ^A 5	2x10 ^A 5	2x10 ^A 5	2x10^5	2x10^5	2x10 ^A 5	2x10^5
	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium
	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL
с	Chemical	Chemical	Chemical	Chemical	Chemical	Chemical	Chemical	Chemical	Chemical	Chemical	Chemical	Chemical
	0ug/mL	0ug/mL	1.0ug/mL	2.0ug/mL	3.9ug/mL	7.8ug/mL	16ug/mL	31ug/mL	63ug/mL	125ug/mL	250ug/mL	500ug/mL
	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DM SO	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DMSO
	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4
	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10 ^A 5	2x10 ^{^5}	2x10 ^A 5	2x10^5	2x10^5	2x10 ^A 5	2x10^5
	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium
	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL
D	Chemical	Chemical	Chemical	Chemical	Chemical	Chemical	Chemical	Chemic al	Chemical	Chemical	Chemical	Chemical
	Oug/mL	0ug/mL	1.0ug/mL	2.0ug/mL	3.9ug/mL	7.8ug/mL	16ug/mL	31ug/mL	63ug/mL	125ug/mL	250ug/mL	500ug/mL
	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DM SO	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DMSO
	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4
	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10 ^A 5	2x10^5	2x10^5	2x10^5	2x10^5	2x10 ⁴ 5	2x10^5
	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium
	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL	100uL
E	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4
	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x 10^5	2x10^5	2x10^5
	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium
	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
F	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4
	2x10^5	2x10^5	2x10^5	2x10^5	2x10 ^A 5	2x10^5	2x10^5	2x10^5	2x10^5	2x 10^5	2x10^5	2x10^5
	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium
	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
G	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4
	2x10^5	2x10^5	2x10^5	2x10^5	2x10 ^A 5	2x10^5	2x10^5	2x10 ^A 5	2x10^5	2x10^5	2x10 ⁴ 5	2x10^5
	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium
	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL
н	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4	2H4
	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x10^5	2x 10^5	2x10^5	2x10^5
	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium	B medium
	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL	50uL

Figure 14 Final constituents of each well of the plate

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 μ M).

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

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

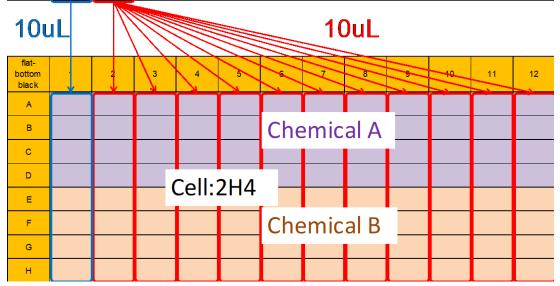
Dilute ethanol, 2 mM ionomycin and 100 μ M PMA with the B medium to prepare control or x10 PMA/ionomycin solution. Add the control to well #A1-#H1 of the 96 well clear plate (round bottom), and add x10 PMA/ionomycin solution to wells #A2-#H2 of the 96 well clear plate (round bottom).

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

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 hours (37°C, CO₂, 5%). (cf. Figure 15)

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Control (1% EtOH)	x 10 PMA/Io solution										
в	Control (1% EtOH)	x 10 PMA/Io solution										
с	Control (1% EtOH)	x 10 PMA/Io solution										
D	Control (1% EtOH)	x 10 PMA/Io solution										
E	Control (1% EtOH)	x 10 PMA/Io solution										
F	Control (1% EtOH)	x 10 PMA/Io solution										
G	Control (1% EtOH)	x 10 PMA/Io solution										
н	Control (1% EtOH)	x 10 PMA/Io solution										



7. Control

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

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

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

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

Descent	Compony	Concentration of	Preparing	Final
Reagent	Company	the stock solution	concentration	concentration
Cyclosporine	Sigma #C1832-			
А	5MG	100 μg/mL	100 μg/mL	100 ng/mL
DMSO	Sigma #D5789	100 µg/IIIL	100 µg/IIIL	100 lig/lil2

Dissolve 5 mg of cyclosporine A with DMSO 50 mL, dispend at 50 μ L/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 4×10⁶/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 16)

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

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 17)

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 17)

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A				DMSO 50uL	CyA 100 ug/mL stock 50uL	(
В	Distilled water 50uL	Distilled water 50uL	DEX 2.5 mg/mL stock 50uL	B medium * 180uL	B medium 180uL	∕20)uL					
С												
D												
E												
F												
G												
Н												
\downarrow												
				_		¥						
round bottom clear	1	2	3	4	5	↓ 6	7	8	9	10	11	12
	1	2	3	4 DMSO 30uL	5 CyA 100 ug/mL stock 30uL	•	7	8	9	10	11	12
	1 Distilled water 50uL	2 Distilled water 50uL	3 DEX 2.5 mg/mL stock 50uL	DMSO 30uL DM SO 10% in B medium	CyA 100 ug/mL stock 30uL CyA 10 ug/mL	6	7	8	9	10	11	12
clear A	Distilled water	Distilled water	DEX 2.5 mg/mL stock	DMSO 30uL DM SO 10% in B medium	CyA 100 ug/mL stock 30uL CyA 10 ug/mL DMSO 10% in B medium	6	7	8	9	10	11	12
A B C D	Distilled water	Distilled water	DEX 2.5 mg/mL stock	DMSO 30uL DM SO 10% in B medium	CyA 100 ug/mL stock 30uL CyA 10 ug/mL DMSO 10% in B medium	6	7	8	9	10	11	12
A B C D E	Distilled water	Distilled water	DEX 2.5 mg/mL stock	DMSO 30uL DM SO 10% in B medium	CyA 100 ug/mL stock 30uL CyA 10 ug/mL DMSO 10% in B medium	6	7	8	9	10	11	12
A B C D	Distilled water	Distilled water	DEX 2.5 mg/mL stock	DMSO 30uL DM SO 10% in B medium	CyA 100 ug/mL stock 30uL CyA 10 ug/mL DMSO 10% in B medium	6	7	8	9	10	11	12
A B C D E	Distilled water	Distilled water	DEX 2.5 mg/mL stock	DMSO 30uL DM SO 10% in B medium	CyA 100 ug/mL stock 30uL CyA 10 ug/mL DMSO 10% in B medium	6	7	8	9	10	11	12

7-5 2 step dilution

Add 20 μ L of the diluted chemical or vehicle to 480 μ L (1-3 lanes) or 980 μ L (4-5 lanes) 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 7-4 to 7-5 as quickly as you can, and do not leave a long time at step after 7-4 or Figure 17. Seal the plate, shake the plate with a plateshaker and incubate in a CO₂ incubator for 1 hour (37°C, CO₂, 5%). (cf. Figure 18-20)

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A				DMSO 30uL	CyA 100 ug/mL stock 30uL							
в	Distilled water 50uL	Distilled water 50uL	DEX 2.5 mg/mL stock 50uL	DM SO 10% in B medium 200uL	CyA 10 ug/mL DMSO 10% in B medium 200 uL							
С												
D												
E												
F												
G				20uL ;								
Н				ZUUL								
Assay Block	1	2										
· · · · · · · · · · · · · · · · · · ·			3	4	5	6	7	8	9	10	11	12
A	B medium 480uL	B medium 480uL	B medium 480uL	4 B medium 980uL	5 B medium 980uL	6	7	8	9	10	11	12
В	B medium 480uL	B medium 480uL	B medium	B medium	B medium	6	7	8	9	10	11	12
А	B medium 480uL	B medium 480uL	B medium	B medium	B medium	6	7	8	9	10	11	12
B C D	B medium 480uL	B medium 480uL	B medium	B medium	B medium	6	7	8	9	10	11	12
B C D E	B medium 480uL	B medium 480uL	B medium	B medium	B medium	6	7	8	9	10	11	12
B C D E F	B medium 480uL	B medium 480uL	B medium	B medium	B medium	6	7	8	9	10	11	12
B C D E	B medium 480uL	B medium 480uL	B medium	B medium	B medium	6	7	8	9	10	11	12

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			A	- 50u								
G H		\square										
Π												
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							
В	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL	2H4 2x10^5 B medium 50uL							
с	2H4 2x10^5 B medium 50uL	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												
Н												

Figure 20 Final constituents of each well of the plate

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

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 hours (37°C, CO₂, 5%). (cf. Figure 21)



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	Required
	r y	medium		amount
RPMI-1640	GIBCO #11875-093	-	-	27 mL
FBS	Biological Industries			
	Cat#04-001-1E	-	10 %	3 mL
	Lot: 715004			

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 22

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
А												
В	SLG 100 µL	SLG 100 µL	SLG 100 µL									
С												
D	SLO 100 µL	SLO 100 μL	SLO 100 µL									
Е												
F	SLR 100 µL	SLR 100 µL	SLR 100 µL									
G												
Н												

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 23

	ent without	i Finter										
	1	2	3	4	5	6	7	8	9	10	11	12
A [
в	3757 0 15	3716611	3810382									
с												
D	1202691	1210208	1122295									
E												
F	2465453	2207572	2077689									
G												
н												
Measurem	ent with Fil	ter 1										
1	1	2	3	4	5	6	7	8	9	10	11	12
A [
в	1269950	1257268	1289562									
c												
D	808550	813160	754174									
E												
F	2193723	1968240	1853873									
G												
н												
Measurem	ent with Fil	ter 2										
		2	3	4	5	6	7	8	9	10	11	12
A [· · ·				_		-	-				
в	236478	234079	240876									
c												
D	235121	235878	217432									
E												
F	1585258	1420099	1339265									
G												
н												

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

 $Transmittance factor (\kappa G_{R56}) = \frac{\#B1 \text{ of } F1 + \#B2 \text{ of } F1 + \#B3 \text{ of } F1}{\#B1 \text{ of } F0 + \#B2 \text{ of } F0 + \#B3 \text{ of } F0}$ $Transmittance factor (\kappa O_{R56}) = \frac{\#D1 \text{ of } F1 + \#D2 \text{ of } F1 + \#D3 \text{ of } F1}{\#D1 \text{ of } F0 + \#D2 \text{ of } F0 + \#D3 \text{ of } F0}$ $Transmittance factor (\kappa R_{R56}) = \frac{\#F1 \text{ of } F1 + \#F2 \text{ of } F1 + \#F3 \text{ of } F1}{\#F1 \text{ of } F0 + \#F2 \text{ of } F0 + \#F3 \text{ of } F0}$ $Transmittance factor (\kappa G_{R60}) = \frac{\#B1 \text{ of } F2 + \#B2 \text{ of } F2 + \#B3 \text{ of } F2}{\#B1 \text{ of } F0 + \#B2 \text{ of } F0 + \#B3 \text{ of } F2}$ $Transmittance factor (\kappa O_{R60}) = \frac{\#D1 \text{ of } F2 + \#D2 \text{ of } F2 + \#B3 \text{ of } F2}{\#D1 \text{ of } F0 + \#D2 \text{ of } F0 + \#D3 \text{ of } F0}$ $Transmittance factor (\kappa R_{R60}) = \frac{\#D1 \text{ of } F2 + \#D2 \text{ of } F2 + \#D3 \text{ of } F2}{\#D1 \text{ of } F0 + \#D2 \text{ of } F0 + \#D3 \text{ of } F0}$ $Transmittance factor (\kappa R_{R60}) = \frac{\#F1 \text{ of } F2 + \#F2 \text{ of } F2 + \#F3 \text{ of } F2}{\#F1 \text{ of } F0 + \#F2 \text{ of } F0 + \#F3 \text{ of } F0}$ In the case shown above, $Transmittance factors (\kappa G_{R56}) = \frac{1269550 + 1257268 + 1289562}{3757015 + 3716611 + 3810382} = 0.338$

Transmittance factors (
$$\kappa O_{R56}$$
)= $\frac{808550+813160+754174}{1202691+1210208+1122295}$ =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.

	Α	В	С	D	E	F
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2						
3		Transmittan	ce Data			
4			SLG	SLO	SLR	
5		F0	1	1	1	
6		F1	к G ₈₅₆	κ Ο _{R56}	κ R _{R56}	
7		F2	к G _{R56} к G _{R60}	к О _{R60}	κ R _{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 μ L of pre-warmed Tripluc from the reservoir to each well of the plate containing the reference sample using an 8 channel or 12 channel pipetman. Shake the plate for 10 min at room temperature (about 25°C) on a plate shaker. Remove bubbles in the solutions in the wells if they appear. Place the plate in the luminometer to measure the luciferase activity. Bioluminescence is measured for 3 sec each in the absence of (F0) and presence (F1, F2) of the optical filters.

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.

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Figure 25 "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 26 "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., IL2LA, IFNLA, GAPLA, nIL2LA, nIFNLA, the mean \pm SD of IL2LA, the mean \pm SD of IFNLA, the mean \pm SD of GAPLA, % suppression and graphs will automatically appear on the "Result Format" sheet of the data sheet.

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Figure 27 "Result Format" sheet of the data sheet

10. Data analysis

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

- SLG-luciferase activity (IL2LA) : Luciferase activity of stable luciferase green (Under the control of IL-2 promoter)
- SLO-luciferase activity (IFNLA) : Luciferase activity of stable luciferase orange (Under the control of IFN-γ promoter)
- SLR-luciferase activity (GAPLA) : Luciferase activity of stable luciferase red (Under the control of G3PDH promoter)
- Normalized SLG-LA (nIL2LA) : = (IL2LA)/(GAPLA)
- Normalized SLO-LA (nIFNLA) : = (IFNLA)/(GAPLA)
- Inhibition index of GAPLA (Inh-GAPLA) : The cytotoxic effect of chemicals = (GAPLA of 2H4 treated with chemicals) / (GAPLA of untreated 2H4)
- % suppression : The effect of chemicals on IL-2 promoter
 = (1-(nIL2LA of 2H4 treated with chemicals) / (nIL2LA 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 nIFNLA of PMA/Ionomycin wells without chemicals (=(nIFNLA of 2H4 cells treated with PMA/Ionomycin) / (nIFNLA of non-treated 2H4 cells)) demonstrate less than 3.0, the results obtained from the plate containing the control wells should be rejected.

11-2 Criterion

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

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

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

1. The mean of % suppression is \geq 35 (suppressive) or \leq -35 (stimulatory) with statistical

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

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

3. The results are judged using only data obtained in the concentration at which Inh-GAPLA is ≥ 0.05

12. Update record

Ver. 0011.1E 2020.5.13 Update the parameters Change the name of the cell line, #2H4 to 2H4

Ver. 0011.0E 2018.5.10 Change the criteria

Ver. 0010.0E 2018.1.15 distribution Change the criteria

Ver. 009.1E 2017.5.8 distribution Change the criteria

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

Ver. 008.5E 2016.9.14 distribution Change the criteria

Ver. 008.4E 2016.9.9 distribution Change the criteria

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

Ver. 008.1E 2016.2.2 distribution Changes after the VMT meeting

Ver. 008.0E 2016.1.19 Translation to English Addition of appendix

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

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

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

Ver. 004.0J 2014.11.17 distribution For the validation study at AIST, FDSC and Tohoku university (chemicals: Sodium Bromate (NaBrO₃), Nickel (II) sulfate (NiSO₄), Dibutyl phthalate (DP), 2-Mercaptobenzothiazole (2-MBT)) Change THP-G1b cells to TGCHAC-A4 cells Change cell number of THP-G8 and TGCHAC-A4 5x10⁴/well to 1x10⁵/well

Change 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: CoCl₂, NiSO₄, 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 (λ max = 550 nm), SLO (λ max = 580 nm) and SLR (λ max = 630 nm), measure i) the intensity of light without filter (all optical), ii) the intensity of 560 nm LP (Filter 1) transmitted light iii) the intensity of 600 nm LP (Filter 2) transmitted light, and calculate the coefficient factor listed below.

Coeffici	ient factor	Abbreviation	Definition
SLG	Filter 1 transmittance factor	кGr56	The intensity of 560 nm LP (Filter 1)
			transmitted SLG / the intensity of SLG
			without filter (all optical)
	Filter 2 transmittance factor	κGr60	The intensity of 600 nm LP (Filter 2)
			transmitted SLG / the intensity of SLG
			without filter (all optical)
SLO	Filter 1 transmittance factor	кOr56	The intensity of 560 nm LP (Filter 1)
			transmitted SLO / the intensity of SLO
			without filter (all optical)
	Filter 2 transmittance factor	кО R60	The intensity of 600 nm LP (Filter 2)
			transmitted SLO / the intensity of SLO
			without filter (all optical)
SLR	Filter 1 transmittance factor	KRR56	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.

These formulas can be rephrased as follows

$$\begin{pmatrix} F0\\F1\\F2 \end{pmatrix} = \begin{pmatrix} 1 & 1 & 1\\\kappa G_{R56} & \kappa O_{R56} & \kappa R_{R56}\\\kappa G_{R60} & \kappa O_{R60} & \kappa R_{R60} \end{pmatrix} \begin{pmatrix} G\\0\\R \end{pmatrix}$$

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

$$\begin{pmatrix} G \\ 0 \\ R \end{pmatrix} = \begin{pmatrix} 1 & 1 & 1 \\ \kappa G_{R56} & \kappa O_{R56} & \kappa R_{R56} \\ \kappa G_{R60} & \kappa O_{R60} & \kappa R_{R60} \end{pmatrix}^{-1} \begin{pmatrix} F0 \\ F1 \\ F2 \end{pmatrix}$$

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)

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

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.

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
А												
В	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
С												
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												
Н												

Figure 28

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 29

1	A	В	C	D	E	F	G	Н	1		K	L	M	N
1	MultiReporter Assa	y System -Tr	ipluc®- Calc	ulation Shee	t									
2							1							
2		Transmittan	ce Data											
4			SLG	SLO	SLR									
4		TO	1	1	1		#VALUE!	#VALUE!	#VALUE!					
6		T1					#VALUE!	#VALUE!	#VALUE!					
7		T2					#VALUE!	#VALUE!	#VALUE!					
8							WINLOL.	W THEFE	THE CE.			-		
9	Filter O Data	1	2	3	4	5	6	7	8	9	10	11	12	
0	A		-											
1	B													
2	c		-										-	
23	D				-								-	
4	E													
5	Ē	-					-							
6	G													
7	H				1		2						-	
8														
9	Filter 1 Data	1	2	3	4	5	6	7	8	9	10	11	12	
0	A													
1	B													
2	c						÷							
3	D													
4	Ē													
5	Ē													
6	G													
7	H													
8							2							
9	Filter 2 Data	1	2	3	4	5	6	7	8	9	10	11	12	
0	A	· ·												
1	B													
2	c													
3	D													
4 5 6	Ē													
5	Ē													
6	G						-							
7	Ĥ										-			
8														

Record all the results for quality control.

5-2 Quality control of equipment

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

5-2-1 Light source

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

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

L12367 by Hamamatsu Photonics (Shizuoka, Japan)

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

- 1) Start luminometer 30 min before starting the measurement for stabilization of the photomultiplier.
- 2) Start LED plate and select "PMT" mode.
- 3) Select three-color (BRG) mode and adjust light intensity to 1/10 (10E-1).
- 4) Place the LED plate into the luminometer. Light intensity is measured for 3 sec each

in the absence (F0) and presence (F2) of the optical filter.

- 5) Blue, green, and red LEDs are located at the position of #F6, #E6, and #D6, respectively. Copy the collected data of each position to the appropriate area on Sheet "LED" in the excel file of the data sheet.
- 6) Check the photo-detector performance by comparing with old data of the LED plate. For quality control purpose, every collected data should be recorded.
- 7) LED plate data typically fluctuates up to 1.5% (σ). Disagreement to the old data

should be less than $3 \times \sigma$ (= 4.5%).