IL-2 Luc leukocyte toxicity test (IL-2 Luc LTT)

protocol ver. 001.8

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1. Introduction

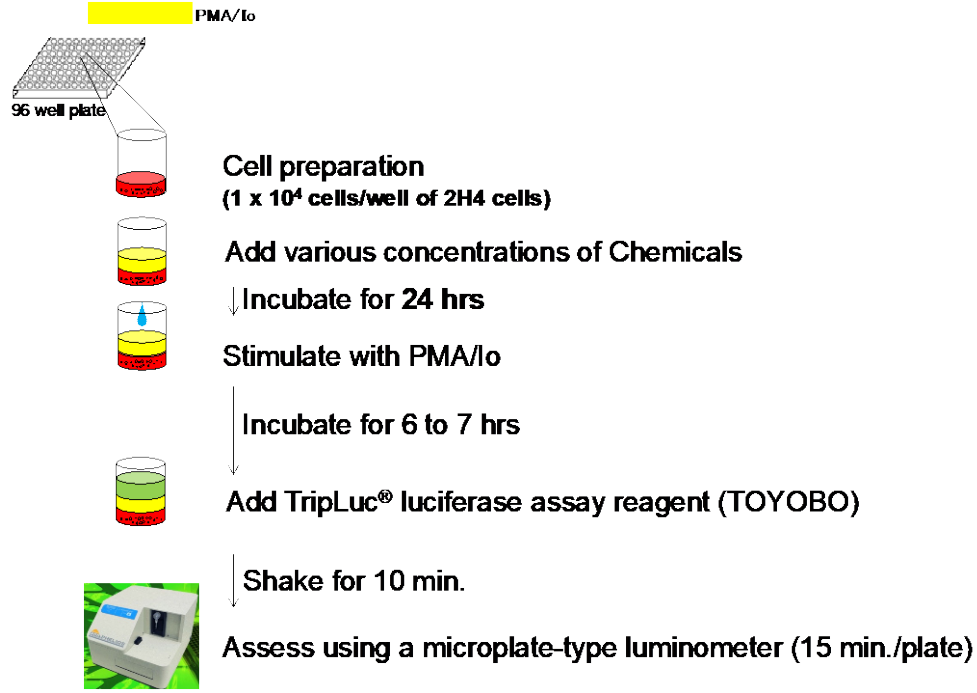
The IL-2 Luc leukocyte toxicity test (IL-2 Luc LTT) is aimed to detect immunosuppressive chemicals the mechanism of which is mostly due to suppression of cell proliferation. The IL-2 Luc LTT protocol is similar to that of the IL-2 Luc assay established previously, except for the duration of chemistry and cell seeding concentration.

(Kimura Y. et al. Optimization of the IL-2 Luc assay for immunosuppressive drugs: a novel in vitro immunotoxicity test with high sensitivity and predictivity *Arch Toxicol*, 95, 2755-2768, 2021)

Figure 1

Assay design (2 chemicals per one plate)

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



2. Materials

2-1 Cells

・ 2H4 (IL2-SLG, IFNγ-SLO, G3PDH-SLR)

A Jurkat-derived IL-2 and IFNγ reporter cell line, 2H4, that harbors the SLG, SLO and SLR luciferase genes under the control of the IL-2, IFNγ and GAPDH promoters, respectively, was established by Tsuruga Institute of Biotechnology, TOYOBO Co. Ltd.

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

2-2 Reagents and equipment

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

・ RPMI-1640 (GIBCO Cat#11875-093, 500 mL)
・ FBS (Biological Industries Cat#04-001-1A Lot: 1524129)
・ Antibiotic-Antimycotic (GIBCO Cat#15240-062)
・ HygromycinB (CAS:31282-04-9, Invitrogen Cat#10687-010)
・ G418 (CAS:108321-42-2, WAKO Cat#074-06801)
・ 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 1~5 sec/well measuring time

2-2-6 Others

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

2-3 Culture medium

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

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	440 mL
FBS	Biological Industries Cat#04-001-1A Lot: 1524129	-	10 %	50 mL
Antibiotic-Antimycotic	GIBCO #15240-062	100×	1×	5 mL
Puromycin	InvivoGen # ant-pr-1	10 mg/mL	0.15 μg/mL	7.5 μL
G418	WAKO Cat#074-06801	50 mg/mL	300 μg/mL	3 mL
HygromycinB	Invitrogen #10687-010	50 mg/mL	200 μg/mL	2 mL

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

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	27 mL
FBS	Biological Industries Cat#04-001-1A Lot: 1524129	-	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#04-001-1A Lot: 1524129	-	10 %	3 mL
Antibiotic-Antimycotic	GIBCO #15240-062	100×	1×	0.3 mL

2-4 Preparation of the stimulant of 2H4

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

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

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

2-4-2 Ionomycin

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

Dissolve 1mg Ionomycin using ethanol 669.3 μL, dispend at 15 μL/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% CO_2 incubator (for culture).

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

3-2 Maintenance of 2H4 cells

Pre-warm the A medium in a T-75 Flask at 37°C in a 5% CO_2 incubator. The culture medium should be changed to the A medium 3 or 4 days after thawing. At that time, count the number of cells, centrifuge the tube at 120-350 x *g* at room temperature for 5 min, discard the supernatant, and resuspend in pre-warmed the A medium in a T-75 Flask. Cells are passaged at $1\sim3x10^5$/mL and incubated at 37°C, 5% CO_2.

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

4. Preparation of cells for assay

A cell passage should be done 3-4 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 (1.0 x 10^6 cells for two chemicals are required, but to have some leeway, 1.5 x 10^6 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 2 x 10^5/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 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
B	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
C	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
D	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
E	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
F	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
G	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
H	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL

5. Preparation of chemicals and cell treatment with chemicals

5-1 Dissolution by vehicle

Dissolve the chemical first in distilled water. Weigh 10 mg of the test chemical in a volumetric flask and add distilled water up to 1 mL. If the chemical is soluble at 10 mg/mL, use 10 mg/ml solution for the stock solution.

If the chemical is not soluble at 10 mg/ml in water, the chemical should be dissolved in DMSO at 200 mg/mL. For example, weigh 200 mg of the test chemical in volumetric flask and add DMSO up to 1 mL. (cf. Figure 3). If the chemical does not dissolve in DMSO at 200 mg/ml, use the highest concentration possible after diluting with DMSO at a dilution factor of 2.

For expensive chemicals, prepare the highest concentration possible instead of 10 mg/mL distilled water. If the chemical is not soluble at 10 mg/ml, prepare the highest concentration possible in DMSO.

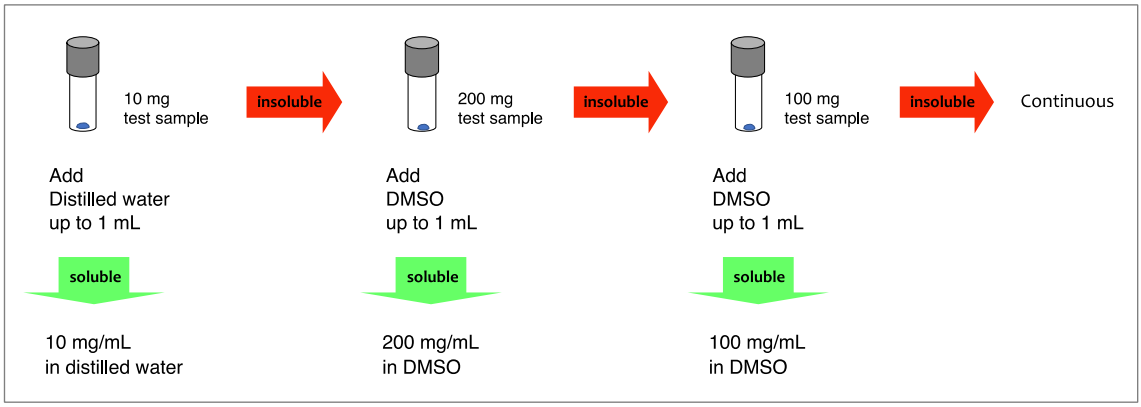
Sonication and vortex may be used if needed, and attempt to dissolve the chemical for at least 5 minutes. Being soluble should be confirmed by centrifugation at 15,000 rpm (≈20,000 x g) for 5 min and confirm the absence of precipitation. The chemical should be used within 4 hours after being dissolved 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 dilution factor of 2 from the highest concentration using distilled water. When the chemical is prepared as a DMSO solution, conduct 10 serial dilutions at a dilution factor of 2 from the highest concentration using DMSO.

In the second and third experiment (2nd and 3rd experiment), determine the minimum concentration at which Inh-GAPLA (mentioned later in **10**) becomes 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 dilution factor of 2 from the highest concentration. (cf. Figure 4) 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 dilution factor of 2 from the highest concentration in the 1st experiment. (cf. Figure 5)

In addition, if the chemical gives Inh-GAPLA < 0.7 at the lowest concentration, use the concentration two step (4-times) higher than the lowest concentration in the first experiment as the highest concentration of the chemical to examine (cf. Figure 6)

Figure 3



For example, in Figure 4 below, the minimum concentration at which Inh-GAPLA becomes lower than 0.05 is 6.25 μg/ml. The highest concentration of the chemical to examine is the concentration one step (2-times) higher than 6.25 μg/ml, which is 12.5 μg/ml.

In Figure 5 below, Inh-GAPLA does 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 25 μg/ml.

In Figure 6 below, since the chemical gives Inh-GAPLA < 0.7 at the lowest concentration, use the concentration two step (4-times) higher than the lowest concentration (0.39 μg/ml) in the first experiment as the highest concentration of the chemical to examine, which is 1.56 μg/ml.

Figure 4

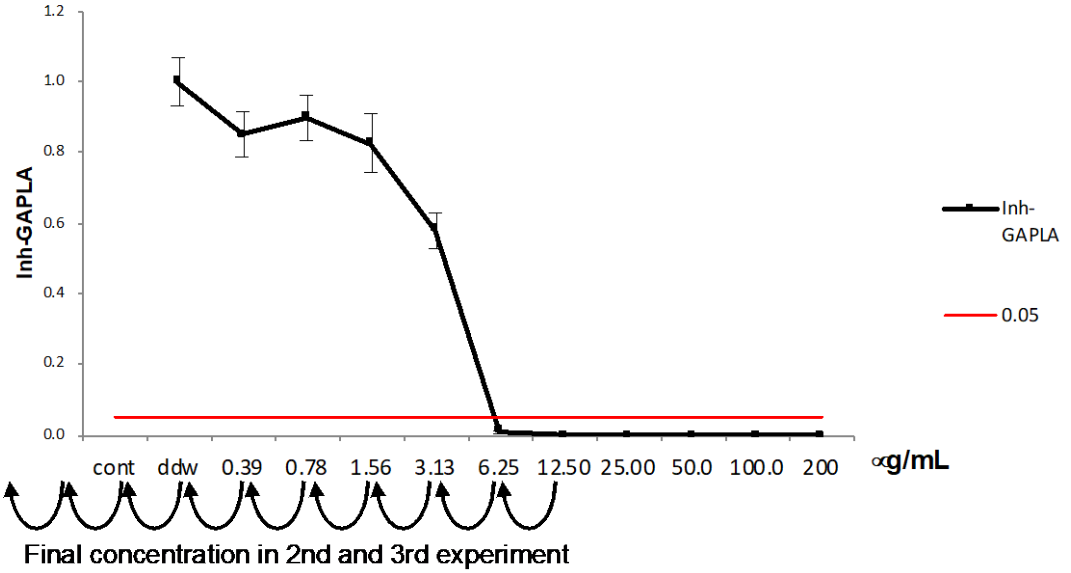


Figure 5

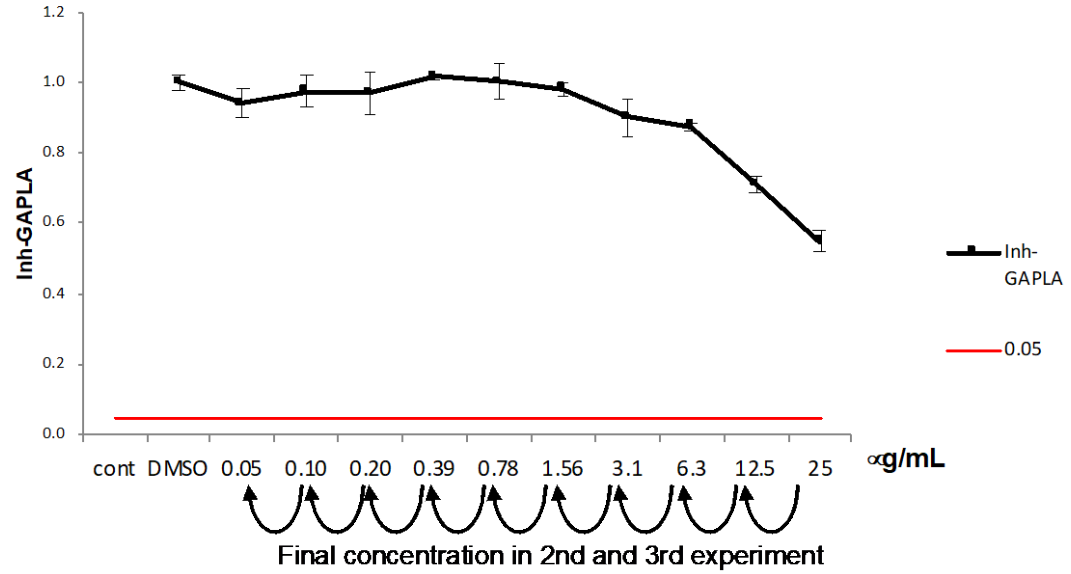
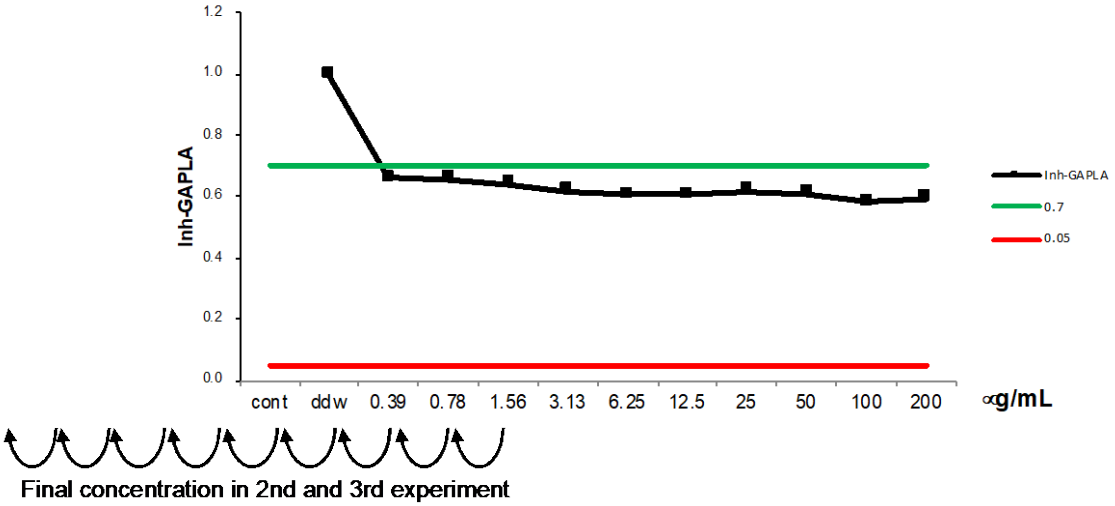


Figure 6



Final concentration in 2nd and 3rd experiment

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 10 mg/mL distilled water solution.

5-2-1 Arrangement of chemicals and vehicle

Add 100 μL of the 10 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 7)

Figure 7

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 10 mg/mL in distilled water 100uL
B												
C												
D												
E												
F												
G												
H												

2-fold dilution : transfer 50 μL (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 0.02 mg/mL in distilled water 100uL	Chemical 0.04 mg/mL in distilled water 50uL	Chemical 0.08 mg/mL in distilled water 50uL	Chemical 0.16 mg/mL in distilled water 50uL	Chemical 0.31 mg/mL in distilled water 50uL	Chemical 0.63 mg/mL in distilled water 50uL	Chemical 1.3 mg/mL in distilled water 50uL	Chemical 2.5 mg/mL in distilled water 50uL	Chemical 5 mg/mL in distilled water 50uL	Chemical 10 mg/mL in distilled water 50uL
B												
C												
D												
E												
F												
G												
H												

5-2-3 2 step dilution

Add 20 μL of the diluted chemical to 480 μL of the B medium prepared in the assay block. And add 50 μL to 2H4 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 24 hours (37°C, CO_2, 5%) (cf. Figure 8-10).

Figure 8

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 50uL	Distilled water 50uL	Chemical 0.02 mg/mL in distilled water 100uL	Chemical 0.04 mg/mL in distilled water 50uL	Chemical 0.08 mg/mL in distilled water 50uL	Chemical 0.16 mg/mL in distilled water 50uL	Chemical 0.31 mg/mL in distilled water 50uL	Chemical 0.63 mg/mL in distilled water 50uL	Chemical 1.3 mg/mL in distilled water 50uL	Chemical 2.5 mg/mL in distilled water 50uL	Chemical 5 mg/mL in distilled water 50uL	Chemical 10 mg/mL in distilled water 50uL
B												
C												
D												
E												
F												
G												
H												

20μL

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 9

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 500uL	B medium 500uL	Chemical 0.8 ug/mL in B medium 500uL	Chemical 1.6 ug/mL in B medium 500uL	Chemical 3.1 ug/mL in B medium 500uL	Chemical 6.3 ug/mL in B medium 500uL	Chemical 12.5 ug/mL in B medium 500uL	Chemical 25 ug/mL in B medium 500uL	Chemical 50 ug/mL in B medium 500uL	Chemical 100 ug/mL in B medium 500uL	Chemical 200 ug/mL in B medium 500uL	Chemical 400 ug/mL in B medium 500uL
B												
C												
D												
E												
F												
G												
H												

50µL

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

Figure 10 Final constituents of each well of the plate

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	Chemical 0 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 0 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 0.4 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 0.8 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 1.6 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 3.1 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 6.3 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 12.5 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 25 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 50 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 100 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 200 ug/mL 2H4 1x10^4 B medium 100uL
B	Chemical 0 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 0 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 0.4 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 0.8 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 1.6 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 3.1 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 6.3 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 12.5 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 25 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 50 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 100 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 200 ug/mL 2H4 1x10^4 B medium 100uL
C	Chemical 0 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 0 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 0.4 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 0.8 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 1.6 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 3.1 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 6.3 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 12.5 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 25 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 50 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 100 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 200 ug/mL 2H4 1x10^4 B medium 100uL
D	Chemical 0 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 0 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 0.4 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 0.8 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 1.6 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 3.1 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 6.3 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 12.5 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 25 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 50 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 100 ug/mL 2H4 1x10^4 B medium 100uL	Chemical 200 ug/mL 2H4 1x10^4 B medium 100uL
E	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
F	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
G	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
H	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 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 200 mg/mL DMSO solution.

5-3-1 Arrangement of chemicals and vehicle

Add 100 μL of the 200 mg/mL DMSO solution of the chemical to well #A12, 50 μL of DMSO to wells #A1-#A11, and 90 μL of the B medium to wells #B1-#B12 of the 96 well clear plate (round bottom)

5-3-2 Serial dilution

Conduct 9 serial dilutions at a common ratio of 2 as indicated in Figure 8 from well #A11 to well #A3. Transfer 50 μL to the next (left) well. (cf. Figure 11)

Figure 11

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 200 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 μL (pipetman, yellow tip)

↓

round botto m clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 50uL	DMSO 100% 50uL	Chemical 0.39 mg/mL in DMSO 100uL	Chemical 0.78 mg/mL in DMSO 50uL	Chemical 1.6 mg/mL in DMSO 50uL	Chemical 3.1 mg/mL in DMSO 50uL	Chemical 6.3 mg/mL in DMSO 50uL	Chemical 12.5 mg/mL in DMSO 50uL	Chemical 25 mg/mL in DMSO 50uL	Chemical 50 mg/mL in DMSO 50uL	Chemical 100 mg/mL in DMSO 50uL	Chemical 200 mg/mL in DMSO 50uL
B	B mediu m 90uL	B mediu m 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL	B medium 90uL
C												
D												
E												
F												
G												
H												

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

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

Figure 12

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 50uL	DMSO 100% 50uL	Chemical 0.39 mg/mL in DMSO 100uL	Chemical 0.78 mg/mL in DMSO 50uL	Chemical 1.6 mg/mL in DMSO 50uL	Chemical 3.1 mg/mL in DMSO 50uL	Chemical 6.3 mg/mL in DMSO 50uL	Chemical 12.5 mg/mL in DMSO 50uL	Chemical 25 mg/mL in DMSO 50uL	Chemical 50 mg/mL in DMSO 50uL	Chemical 100 mg/mL in DMSO 50uL	Chemical 200 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												

10μL

↓

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 40uL	DMSO 100% 40uL	Chemical 0.39 mg/mL in DMSO 90uL	Chemical 0.78 mg/mL in DMSO 40uL	Chemical 1.6 mg/mL in DMSO 40uL	Chemical 3.1 mg/mL in DMSO 40uL	Chemical 6.3 mg/mL in DMSO 40uL	Chemical 12.5 mg/mL in DMSO 40uL	Chemical 25 mg/mL in DMSO 40uL	Chemical 50 mg/mL in DMSO 40uL	Chemical 100 mg/mL in DMSO 40uL	Chemical 200 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 0.039 mg/mL DMSO 10% in B medium 100uL	Chemical 0.078 mg/mL DMSO 10% in B medium 100uL	Chemical 0.16 mg/mL DMSO 10% in B medium 100uL	Chemical 0.31 mg/mL DMSO 10% in B medium 100uL	Chemical 0.63 mg/mL DMSO 10% in B medium 100uL	Chemical 1.25 mg/mL DMSO 10% in B medium 100uL	Chemical 2.5 mg/mL DMSO 10% in B medium 100uL	Chemical 50 mg/mL DMSO 10% in B medium 100uL	Chemical 10 mg/mL DMSO 10% in B medium 100uL	Chemical 20 mg/mL DMSO 10% in B medium 100uL
C												
D												
E												
F												
G												
H												

5-3-4　2 step dilution

Add 10 μL of the diluted chemical to 490 μL of the B medium prepared in the assay block. And add 50 μL to 2H4 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Manipulate the procedures from 5-3-3 to 5-3-4 as quickly as you can, and do not leave a long time at step after 5-3-3 or Figure 11. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 24 hours (37°C, CO_2, 5%) (cf. Figure 13-15).

Figure 13

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 40uL	DMSO 100% 40uL	Chemical 0.39 mg/mL in DMSO 90uL	Chemical 0.78 mg/mL in DMSO 40uL	Chemical 1.6 mg/mL in DMSO 40uL	Chemical 3.1 mg/mL in DMSO 40uL	Chemical 6.3 mg/mL in DMSO 40uL	Chemical 12.5 mg/mL in DMSO 40uL	Chemical 25 mg/mL in DMSO 40uL	Chemical 50 mg/mL in DMSO 40uL	Chemical 100 mg/mL in DMSO 40uL	Chemical 200 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 0.039 mg/mL DMSO 10% in B medium 100uL	Chemical 0.078 mg/mL DMSO 10% in B medium 100uL	Chemical 0.16 mg/mL DMSO 10% in B medium 100uL	Chemical 0.31 mg/mL DMSO 10% in B medium 100uL	Chemical 0.63 mg/mL DMSO 10% in B medium 100uL	Chemical 1.25 mg/mL DMSO 10% in B medium 100uL	Chemical 2.5 mg/mL DMSO 10% in B medium 100uL	Chemical 50 mg/mL DMSO 10% in B medium 100uL	Chemical 10 mg/mL DMSO 10% in B medium 100uL	Chemical 20 mg/mL DMSO 10% in B medium 100uL
C												
D												
E												
F												
G												
H												

10μL

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 14

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	Chemical 0ug/mL DMSO 0.2% in B medium 500uL	Chemical 0ug/mL DMSO 0.2% in B medium 500uL	Chemical 0.78ug/mL DMSO 0.2% in B medium 500uL	Chemical 1.6ug/mL DMSO 0.2% in B medium 500uL	Chemical 3.1ug/mL DMSO 0.2% in B medium 500uL	Chemical 6.3ug/mL DMSO 0.2% in B medium 500uL	Chemical 12.5ug/mL DMSO 0.2% in B medium 500uL	Chemical 25ug/mL DMSO 0.2% in B medium 500uL	Chemical 50ug/mL DMSO 0.2% in B medium 500uL	Chemical 100ug/mL DMSO 0.2% in B medium 500uL	Chemical 200ug/mL DMSO 0.2% in B medium 500uL	Chemical 400ug/mL DMSO 0.2% in B medium 500uL
B												
C												
D												
E												
F												
G												
H												

50µL

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

Figure 15 Final constituents of each well of the plate

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	Chemical 0ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 0ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 0.39ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 0.78ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 1.6ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 3.1ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 6.3ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 12.5ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 25ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 50ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 100ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 200ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL
B	Chemical 0ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 0ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 0.39ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 0.78ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 1.6ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 3.1ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 6.3ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 12.5ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 25ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 50ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 100ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 200ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL
C	Chemical 0ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 0ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 0.39ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 0.78ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 1.6ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 3.1ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 6.3ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 12.5ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 25ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 50ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 100ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 200ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL
D	Chemical 0ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 0ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 0.39ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 0.78ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 1.6ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 3.1ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 6.3ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 12.5ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 25ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 50ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 100ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL	Chemical 200ug/mL 0.1% DMSO 2H4 1x10^4 B medium 100uL
E	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
F	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
G	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
H	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL

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

6-1 Material

・2 mM PMA stock

・2 mM Ionomycin stock

・B medium

・Ethanol

6-2 Preparation of 100 μM PMA

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

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

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

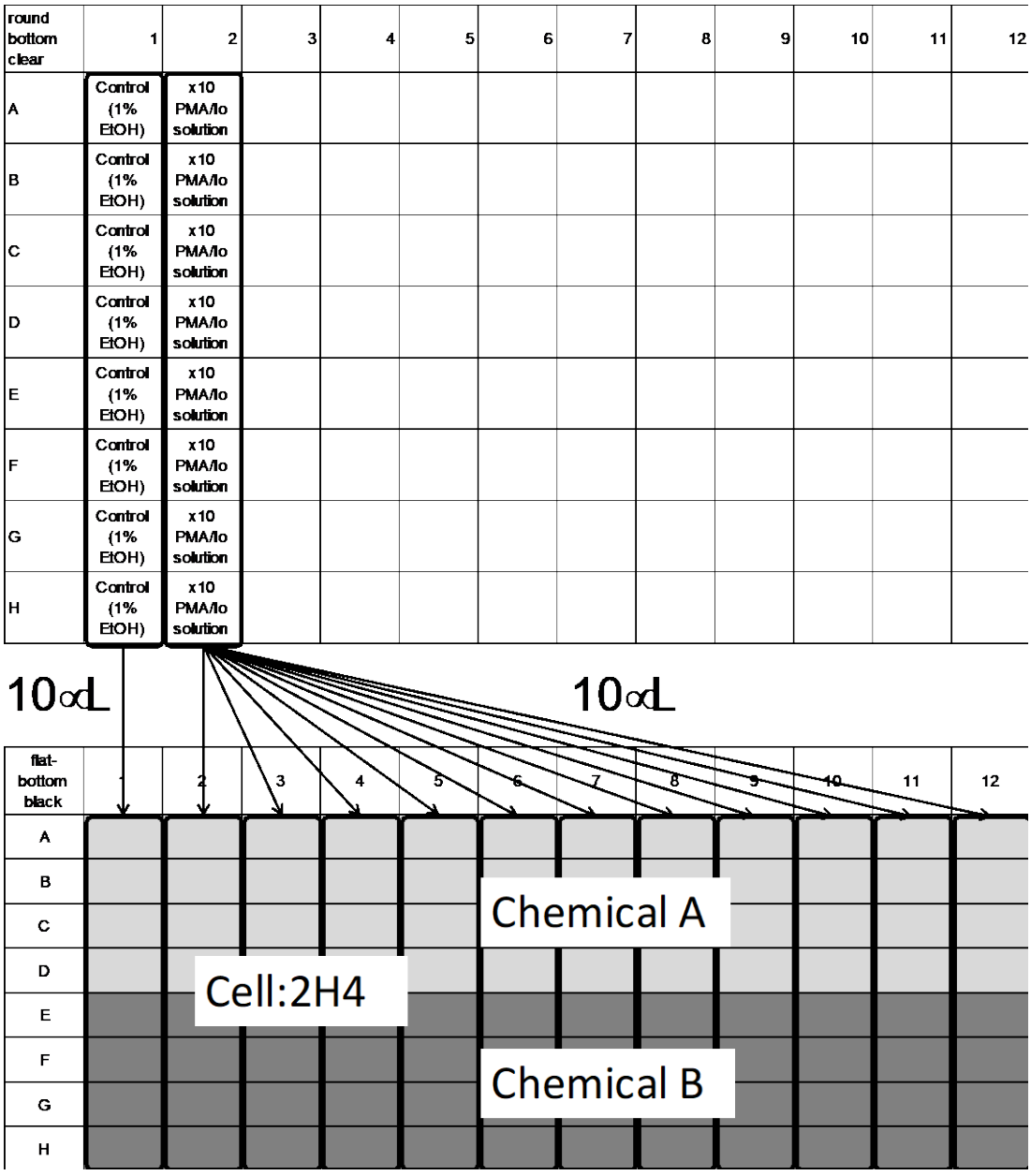
Dilute ethanol, 2 mM ionomycin and 100 μM PMA with the B medium to prepare control or x10 PMA/ionomycin solution. Add the control to well #A1-#H1 of the 96 well clear plate (round bottom), and add x10 PMA/ionomycin solution to wells #A2-#H2 of the 96 well clear plate (round bottom).

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

6-4 Addition of PMA/ionomycin to 2H4

Twenty-four hours after the addition of chemicals, add 10 μL of control or PMA/ionomycin solution to the cells (#A1-#H1 or #A2-#H12, respectively) using an 8 channel or 12 channel pipetman after pipetting 20 times. Make sure that the apex of the tip is dipped into the medium. Change tips every line you add. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 6 to 7 hours (37°C, CO_2, 5%). (cf. Figure 16)

Figure 16



7. Control

7-1 Preparing control chemical (bleomycin sulfate, dexamethasone)

7-1-1 Preparing bleomycin sulfate stock

Reagent	Company	Concentration of the stock solution	Preparing concentration	Final concentration
bleomycin sulfate	TOKYO CHEMICAL INDUSTRY B3972	10 mg/mL	10 mg/mL	0.4~200 μg/mL
Distilled water	GIBCO Cat#10977-015			

Dissolve 10 mg of bleomycin sulfate with distilled water 1 mL, dispend at 100 μL/tube and store a freezer at -30°C.

7-1-2 Preparing dexamethasone stock

Reagent	Company	Concentration of the stock solution	Preparing concentration	Final concentration
dexamethasone	Wako 041-18861	500 mg/mL	100 mg/mL	0.2~100 μg/mL
DMSO	Sigma #D5789			

Weigh 1 g of dexamethasone in volumetric flask and add DMSO up to 2 mL, dispend at 100 μL/tube and store a freezer at -30°C.

7-2 Preparation of cells for assay

A cell passage should be done 3-4 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 (1.0×10^6 for two chemicals are required, but to have some leeway, 1.5×10^6 for two controls 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 2×10^5/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 17)

Figure 17

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

7-3 Arrangement of chemicals and vehicle

Add 100 μL of the 10 mg/mL distilled water solution of bleomycin sulfate to well #A12, and 50 μL of the distilled water to wells #A1-#A11 of the 96 well clear plate (round bottom).

Dilute the 500 mg/mL DMSO solution of dexamethasone 5 times with DMSO, add 100 μL of the 100 mg/mL DMSO solution of dexamethasone to well #E12, 50 μL of DMSO to wells #E1-#E11, and 90 μL of the B medium to wells #F1-#F12 of the 96 well clear plate (round bottom).

7-4 Serial dilution

Conduct 9 serial dilutions at a common ratio of 2 as indicated in Figure 17 from well #A11 to well #A3 and #E11 to well #E3. Transfer 50 μL to the next (left) well. (cf. Figure 18)

Figure 18

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	bleomycin sulfate 10 mg/mL in distilled water 100uL
B												
C				2-fold dilution : transfer 50 μL (pipetman, yellow tip)								
D												
E	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	dexa methasone 100 mg/mL in DMSO 100uL
F	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
G				2-fold dilution : transfer 50 μL (pipetman, yellow tip)								
H												

↓

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 50uL	Distilled water 50uL	bleomycin sulfate 0.02 mg/mL in distilled water 100uL	bleomycin sulfate 0.04 mg/mL in distilled water 50uL	bleomycin sulfate 0.08 mg/mL in distilled water 50uL	bleomycin sulfate 0.16 mg/mL in distilled water 50uL	bleomycin sulfate 0.31 mg/mL in distilled water 50uL	bleomycin sulfate 0.63 mg/mL in distilled water 50uL	bleomycin sulfate 1.3 mg/mL in distilled water 50uL	bleomycin sulfate 2.5 mg/mL in distilled water 50uL	bleomycin sulfate 5 mg/mL in distilled water 50uL	bleomycin sulfate 10 mg/mL in distilled water 50uL
B												
C												
D												
E	DMSO 100% 50uL	DMSO 100% 50uL	dexa methasone 0.2 mg/mL in DMSO 100uL	dexa methasone 0.4 mg/mL in DMSO 50uL	dexa methasone 0.8 mg/mL in DMSO 50uL	dexa methasone 1.6 mg/mL in DMSO 50uL	dexa methasone 3.1 mg/mL in DMSO 50uL	dexa methasone 6.3 mg/mL in DMSO 50uL	dexa methasone 13 mg/mL in DMSO 50uL	dexa methasone 25 mg/mL in DMSO 50uL	dexa methasone 50 mg/mL in DMSO 50uL	dexa methasone 100 mg/mL in DMSO 50uL
F	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
G												
H												

7-5 2 step dilution

Add 20 μL of the diluted bleomycin sulfate to 480 μL of the B medium prepared in the assay block. And add 50 μL to 2H4 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. (cf. Figure 19-20)

Figure 19

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 50uL	Distilled water 50uL	bleomycin sulfate 0.02 mg/mL in distilled water 100uL	bleomycin sulfate 0.04 mg/mL in distilled water 50uL	bleomycin sulfate 0.08 mg/mL in distilled water 50uL	bleomycin sulfate 0.16 mg/mL in distilled water 50uL	bleomycin sulfate 0.31 mg/mL in distilled water 50uL	bleomycin sulfate 0.63 mg/mL in distilled water 50uL	bleomycin sulfate 1.3 mg/mL in distilled water 50uL	bleomycin sulfate 2.5 mg/mL in distilled water 50uL	bleomycin sulfate 5 mg/mL in distilled water 50uL	bleomycin sulfate 10 mg/mL in distilled water 50uL
B												
C												
D												
E	DMSO 100% 50uL	DMSO 100% 50uL	dexamethasone 0.2 mg/mL in DMSO 100uL	dexamethasone 0.4 mg/mL in DMSO 50uL	dexamethasone 0.8 mg/mL in DMSO 50uL	dexamethasone 1.6 mg/mL in DMSO 50uL	dexamethasone 3.1 mg/mL in DMSO 50uL	dexamethasone 6.3 mg/mL in DMSO 50uL	dexamethasone 13 mg/mL in DMSO 50uL	dexamethasone 25 mg/mL in DMSO 50uL	dexamethasone 50 mg/mL in DMSO 50uL	dexamethasone 100 mg/mL in DMSO 50uL
F	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
G												
H												

20μL

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	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
F												
G												
H												

Figure 20

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 500uL	B medium 500uL	bleomycin sulfate 0.8 ug/mL in B medium 500uL	bleomycin sulfate 1.6 ug/mL in B medium 500uL	bleomycin sulfate 3.1 ug/mL in B medium 500uL	bleomycin sulfate 6.3 ug/mL in B medium 500uL	bleomycin sulfate 12.5 ug/mL in B medium 500uL	bleomycin sulfate 25 ug/mL in B medium 500uL	bleomycin sulfate 50 ug/mL in B medium 500uL	bleomycin sulfate 100 ug/mL in B medium 500uL	bleomycin sulfate 200 ug/mL in B medium 500uL	bleomycin sulfate 400 ug/mL in B medium 500uL
B												
C												
D												
E	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
F												
G												
H												

50µL

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

7-6 Dilution of DMSO solution with the B medium

Dilute 10 μL of the DMSO solution of dexamethasone in wells #E1-#E12 with 90 μL of the B medium using an 8-12 channel pipetman. (cf. Figure 21)

Figure 21

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 30uL	Distilled water 30uL	bleomycin sulfate 0.02 mg/mL in distilled water 80uL	bleomycin sulfate 0.04 mg/mL in distilled water 30uL	bleomycin sulfate 0.08 mg/mL in distilled water 30uL	bleomycin sulfate 0.16 mg/mL in distilled water 30uL	bleomycin sulfate 0.31 mg/mL in distilled water 30uL	bleomycin sulfate 0.63 mg/mL in distilled water 30uL	bleomycin sulfate 1.3 mg/mL in distilled water 30uL	bleomycin sulfate 2.5 mg/mL in distilled water 30uL	bleomycin sulfate 5 mg/mL in distilled water 30uL	bleomycin sulfate 10 mg/mL in distilled water 30uL
B												
C												
D												
E	DMSO 100% 50uL	DMSO 100% 50uL	dexamethasone 0.2 mg/mL in DMSO 100uL	dexamethasone 0.4 mg/mL in DMSO 50uL	dexamethasone 0.8 mg/mL in DMSO 50uL	dexamethasone 1.6 mg/mL in DMSO 50uL	dexamethasone 3.1 mg/mL in DMSO 50uL	dexamethasone 6.3 mg/mL in DMSO 50uL	dexamethasone 13 mg/mL in DMSO 50uL	dexamethasone 25 mg/mL in DMSO 50uL	dexamethasone 50 mg/mL in DMSO 50uL	dexamethasone 100 mg/mL in DMSO 50uL
F	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
G												
H												

10μL

↓

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 30uL	Distilled water 30uL	bleomycin sulfate 0.02 mg/mL in distilled water 80uL	bleomycin sulfate 0.04 mg/mL in distilled water 30uL	bleomycin sulfate 0.08 mg/mL in distilled water 30uL	bleomycin sulfate 0.16 mg/mL in distilled water 30uL	bleomycin sulfate 0.31 mg/mL in distilled water 30uL	bleomycin sulfate 0.63 mg/mL in distilled water 30uL	bleomycin sulfate 1.3 mg/mL in distilled water 30uL	bleomycin sulfate 2.5 mg/mL in distilled water 30uL	bleomycin sulfate 5 mg/mL in distilled water 30uL	bleomycin sulfate 10 mg/mL in distilled water 30uL
B												
C												
D												
E	DMSO 100% 40uL	DMSO 100% 40uL	dexamethasone 0.2 mg/mL in DMSO 90uL	dexamethasone 0.4 mg/mL in DMSO 40uL	dexamethasone 0.8 mg/mL in DMSO 40uL	dexamethasone 1.6 mg/mL in DMSO 40uL	dexamethasone 3.1 mg/mL in DMSO 40uL	dexamethasone 6.3 mg/mL in DMSO 40uL	dexamethasone 13 mg/mL in DMSO 40uL	dexamethasone 25 mg/mL in DMSO 40uL	dexamethasone 50 mg/mL in DMSO 40uL	dexamethasone 100 mg/mL in DMSO 40uL
F	dexamethasone 0 mg/mL DMSO 10% in B medium 100uL	dexamethasone 0 mg/mL DMSO 10% in B medium 100uL	dexamethasone 0.02 mg/mL DMSO 10% in B medium 100uL	dexamethasone 0.04 mg/mL DMSO 10% in B medium 100uL	dexamethasone 0.08 mg/mL DMSO 10% in B medium 100uL	dexamethasone 0.16 mg/mL DMSO 10% in B medium 100uL	dexamethasone 0.31 mg/mL DMSO 10% in B medium 100uL	dexamethasone 0.63 mg/mL DMSO 10% in B medium 100uL	dexamethasone 1.3 mg/mL DMSO 10% in B medium 100uL	dexamethasone 2.5 mg/mL DMSO 10% in B medium 100uL	dexamethasone 5 mg/mL DMSO 10% in B medium 100uL	dexamethasone 10 mg/mL DMSO 10% in B medium 100uL
G												
H												

7-7 2 step dilution

Add 10 μL of the diluted dexamethasone to 490 μL of the B medium prepared in the assay block. And add 50 μL to 2H4 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Manipulate the procedures from 7-6 to 7-7 as quickly as you can, and do not leave a long time at step after 7-6 or Figure 20. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 24 hours (37°C, CO_2, 5%). (cf. Figure 22-24).

Figure 22

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 30uL	Distilled water 30uL	bleomycin sulfate 0.02 mg/mL in distilled water 80uL	bleomycin sulfate 0.04 mg/mL in distilled water 30uL	bleomycin sulfate 0.08 mg/mL in distilled water 30uL	bleomycin sulfate 0.16 mg/mL in distilled water 30uL	bleomycin sulfate 0.31 mg/mL in distilled water 30uL	bleomycin sulfate 0.63 mg/mL in distilled water 30uL	bleomycin sulfate 1.3 mg/mL in distilled water 30uL	bleomycin sulfate 2.5 mg/mL in distilled water 30uL	bleomycin sulfate 5 mg/mL in distilled water 30uL	bleomycin sulfate 10 mg/mL in distilled water 30uL
B												
C												
D												
E	DMSO 100% 40uL	DMSO 100% 40uL	dexamethasone 0.2 mg/mL in DMSO 90uL	dexamethasone 0.4 mg/mL in DMSO 40uL	dexamethasone 0.8 mg/mL in DMSO 40uL	dexamethasone 1.6 mg/mL in DMSO 40uL	dexamethasone 3.1 mg/mL in DMSO 40uL	dexamethasone 6.3 mg/mL in DMSO 40uL	dexamethasone 13 mg/mL in DMSO 40uL	dexamethasone 25 mg/mL in DMSO 40uL	dexamethasone 50 mg/mL in DMSO 40uL	dexamethasone 100 mg/mL in DMSO 40uL
F	dexamethasone 0 mg/mL DMSO 10% in B medium 100uL	dexamethasone 0 mg/mL DMSO 10% in B medium 100uL	dexamethasone 0.02 mg/mL DMSO 10% in B medium 100uL	dexamethasone 0.04 mg/mL DMSO 10% in B medium 100uL	dexamethasone 0.08 mg/mL DMSO 10% in B medium 100uL	dexamethasone 0.16 mg/mL DMSO 10% in B medium 100uL	dexamethasone 0.31 mg/mL DMSO 10% in B medium 100uL	dexamethasone 0.63 mg/mL DMSO 10% in B medium 100uL	dexamethasone 1.3 mg/mL DMSO 10% in B medium 100uL	dexamethasone 2.5 mg/mL DMSO 10% in B medium 100uL	dexamethasone 5 mg/mL DMSO 10% in B medium 100uL	dexamethasone 10 mg/mL DMSO 10% in B medium 100uL
G												
H												

10μL

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 300uL	B medium 300uL	bleomycin sulfate 0.8 ug/mL in B medium 300uL	bleomycin sulfate 1.6 ug/mL in B medium 300uL	bleomycin sulfate 3.1 ug/mL in B medium 300uL	bleomycin sulfate 6.3 ug/mL in B medium 300uL	bleomycin sulfate 12.5 ug/mL in B medium 300uL	bleomycin sulfate 25 ug/mL in B medium 300uL	bleomycin sulfate 50 ug/mL in B medium 300uL	bleomycin sulfate 100 ug/mL in B medium 300uL	bleomycin sulfate 200 ug/mL in B medium 300uL	bleomycin sulfate 400 ug/mL in B medium 300uL
B												
C												
D												
E	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
F												
G												
H												

Figure 23

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 300uL	B medium 300uL	bleomycin sulfate 0.8 ug/mL in B medium 300uL	bleomycin sulfate 1.6 ug/mL in B medium 300uL	bleomycin sulfate 3.1 ug/mL in B medium 300uL	bleomycin sulfate 6.3 ug/mL in B medium 300uL	bleomycin sulfate 12.5 ug/mL in B medium 300uL	bleomycin sulfate 25 ug/mL in B medium 300uL	bleomycin sulfate 50 ug/mL in B medium 300uL	bleomycin sulfate 100 ug/mL in B medium 300uL	bleomycin sulfate 200 ug/mL in B medium 300uL	bleomycin sulfate 400 ug/mL in B medium 300uL
B												
C												
D												
E	dexamethasone 0ug/mL DMSO 0.2% in B medium 500uL	dexamethasone 0ug/mL DMSO 0.2% in B medium 500uL	dexamethasone 0.4ug/mL DMSO 0.2% in B medium 500uL	dexamethasone 0.8ug/mL DMSO 0.2% in B medium 500uL	dexamethasone 1.6ug/mL DMSO 0.2% in B medium 500uL	dexamethasone 3.1ug/mL DMSO 0.2% in B medium 500uL	dexamethasone 6.3ug/mL DMSO 0.2% in B medium 500uL	dexamethasone 12.5ug/mL DMSO 0.2% in B medium 500uL	dexamethasone 25ug/mL DMSO 0.2% in B medium 500uL	dexamethasone 50ug/mL DMSO 0.2% in B medium 500uL	dexamethasone 100ug/mL DMSO 0.2% in B medium 500uL	dexamethasone 200ug/mL DMSO 0.2% in B medium 500uL
F												
G												
H												

50µL

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.4 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.8 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 1.6 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 3.1 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 6.3 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 12.5 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 25 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 50 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 100 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 200 ug/mL 2H4 1x10^4 B medium 100uL
B	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.4 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.8 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 1.6 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 3.1 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 6.3 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 12.5 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 25 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 50 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 100 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 200 ug/mL 2H4 1x10^4 B medium 100uL
C	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.4 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.8 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 1.6 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 3.1 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 6.3 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 12.5 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 25 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 50 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 100 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 200 ug/mL 2H4 1x10^4 B medium 100uL
D	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.4 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.8 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 1.6 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 3.1 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 6.3 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 12.5 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 25 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 50 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 100 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 200 ug/mL 2H4 1x10^4 B medium 100uL
E	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
F	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
G	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL
H	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL	2H4 1x10^4 B medium 50uL

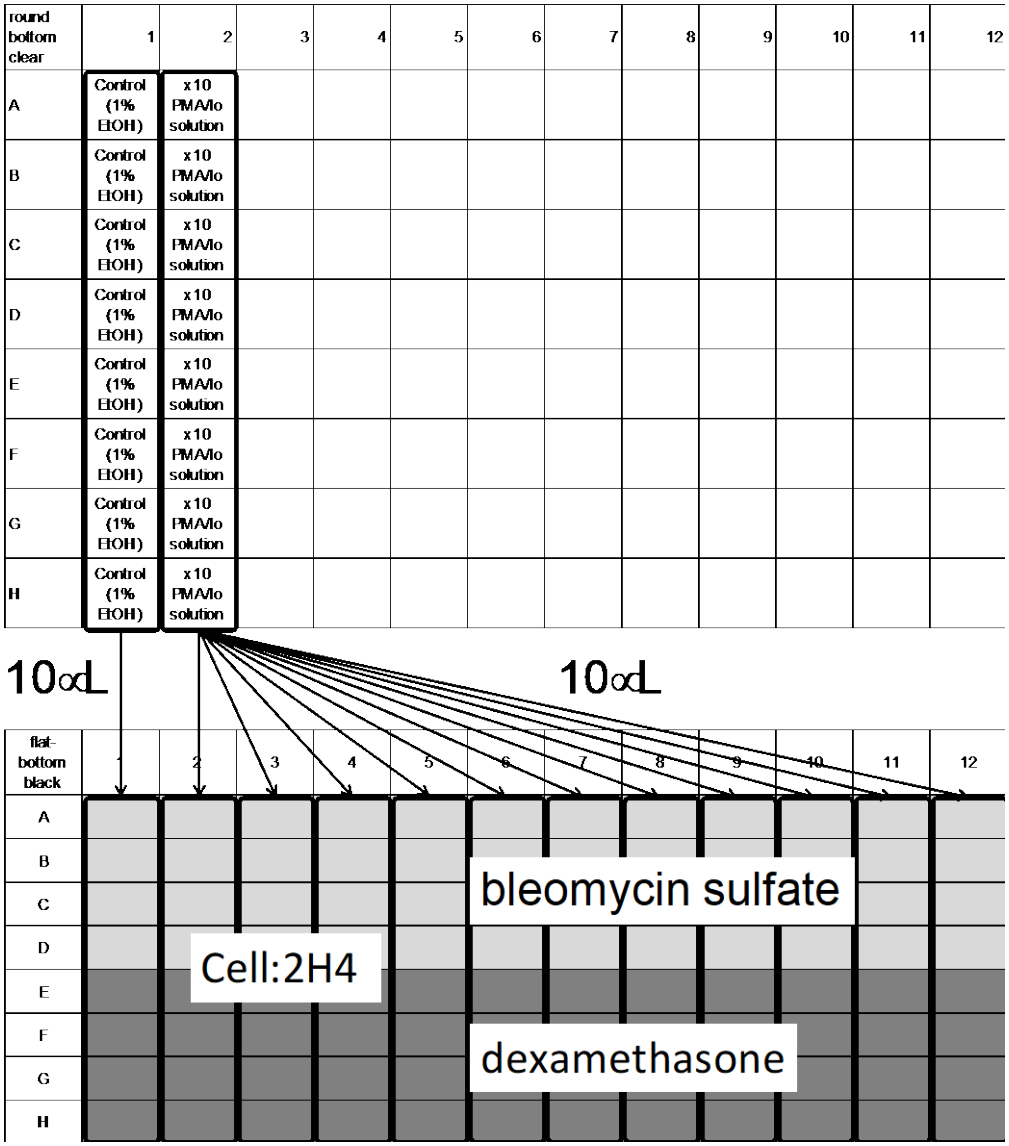
Figure 24 Final constituents of each well of the plate

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.4 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.8 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 1.6 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 3.1 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 6.3 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 12.5 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 25 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 50 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 100 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 200 ug/mL 2H4 1x10^4 B medium 100uL
B	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.4 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.8 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 1.6 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 3.1 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 6.3 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 12.5 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 25 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 50 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 100 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 200 ug/mL 2H4 1x10^4 B medium 100uL
C	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.4 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.8 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 1.6 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 3.1 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 6.3 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 12.5 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 25 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 50 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 100 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 200 ug/mL 2H4 1x10^4 B medium 100uL
D	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.4 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 0.8 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 1.6 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 3.1 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 6.3 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 12.5 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 25 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 50 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 100 ug/mL 2H4 1x10^4 B medium 100uL	bleomycin sulfate 200 ug/mL 2H4 1x10^4 B medium 100uL
E	dexamethasone 0ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 0ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 0.2ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 0.4ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 0.8ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 1.6ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 3.1ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 6.3ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 12.5ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 25ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 50ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 100ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL
F	dexamethasone 0ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 0ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 0.2ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 0.4ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 0.8ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 1.6ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 3.1ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 6.3ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 12.5ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 25ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 50ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 100ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL
G	dexamethasone 0ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 0ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 0.2ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 0.4ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 0.8ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 1.6ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 3.1ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 6.3ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 12.5ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 25ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 50ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 100ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL
H	dexamethasone 0ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 0ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 0.2ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 0.4ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 0.8ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 1.6ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 3.1ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 6.3ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 12.5ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 25ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 50ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL	dexamethasone 100ug/mL DMSO 0.1% 2H4 1x10^4 B medium 100uL

7-8　Addition of PMA/ionomycin to 2H4

Twenty-four hours after the addition of bleomycin sulfate and dexamethasone, add 10 µL of control or PMA/ionomycin solution prepared in §6-3 to the cells (#A1-#H1 or #A2-#H12, respectively) using an 8 channel or 12 channel pipetman after pipetting 20 times. Make sure that the apex of the tip is dipped into the medium. Change tips every line you add. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 6 to 7 hours (37°C, CO_2, 5%). (cf. Figure 25)

Figure 25



8. Calculation of the transmittance factors

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

8-1 Reagents

- Single reference samples:

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

- Assay reagent:

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

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

Reagent	Company	Conc.	Final conc. in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	27 mL
FBS	Biological Industries Cat#04-001-1A Lot: 1524129	-	10 %	3 mL

8-2 Preparation of luminescence reaction solution

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

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

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

8-3 Bioluminescence measurement

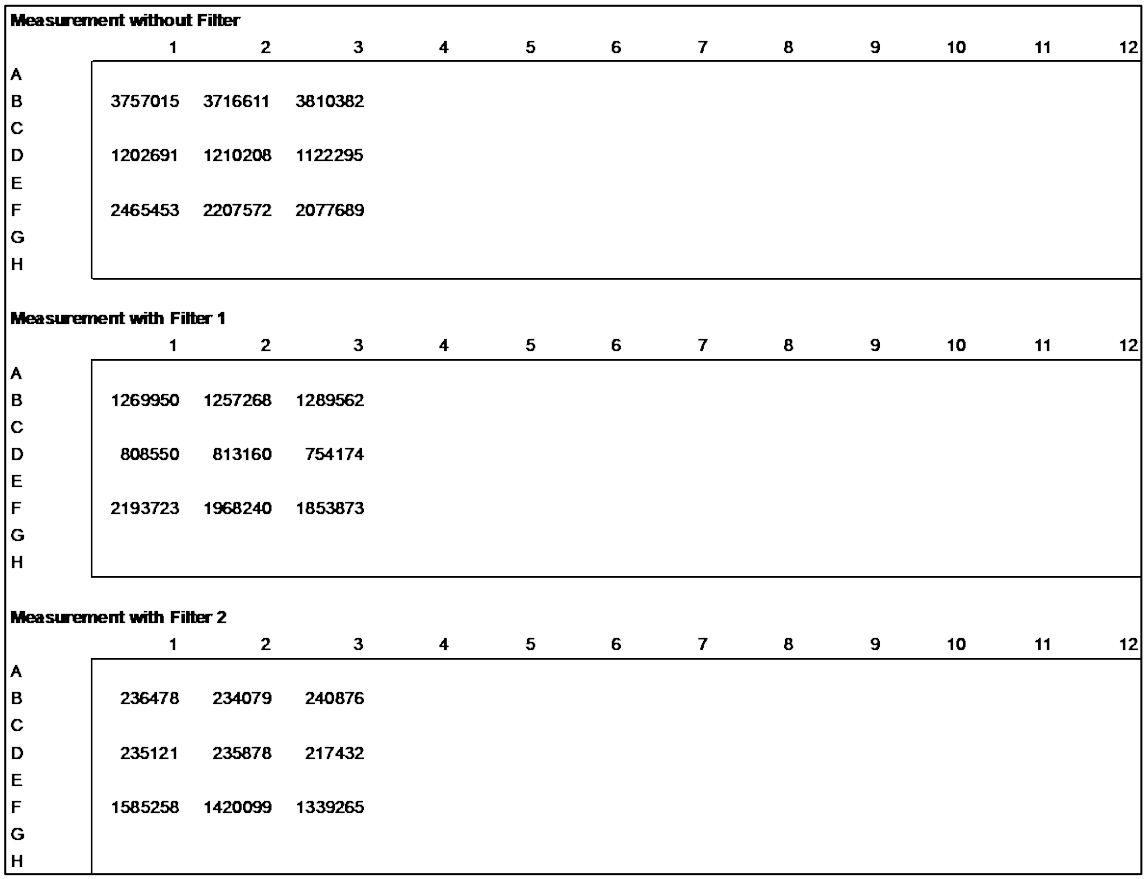
Transfer 100 µL of the diluted reference samples to a black 96 well plate (flat bottom) as shown below.

Figure 26

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

Transfer 100 µL of pre-warmed Tripluc to each well of the plate containing the reference sample using a pipetman. Shake the plate for 10 min at room temperature 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 27



Measurement without Filter

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

Measurement with Filter 1

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

Measurement with Filter 2

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

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

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

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

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

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

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

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

In the case shown above,

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

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

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

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

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

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

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

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

Figure 28

	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

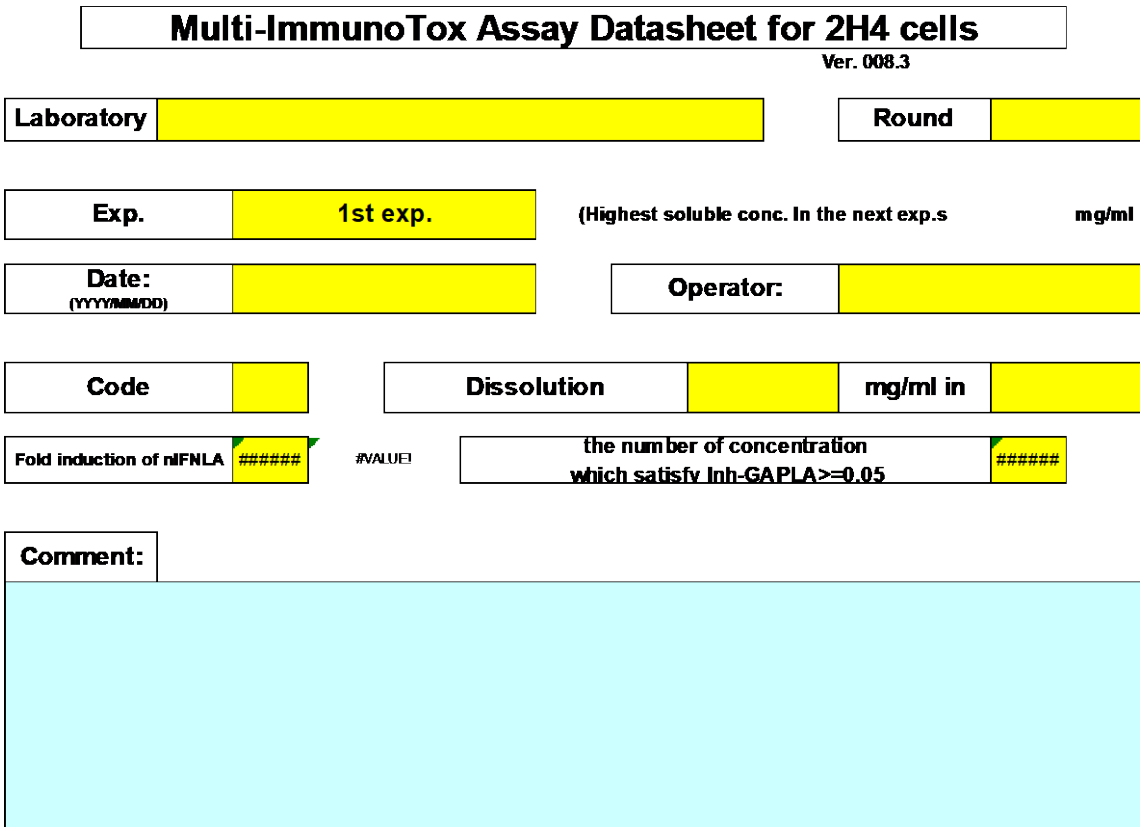
Please refer Appendix 1 for the principle of measurement of luciferase activity.

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 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.

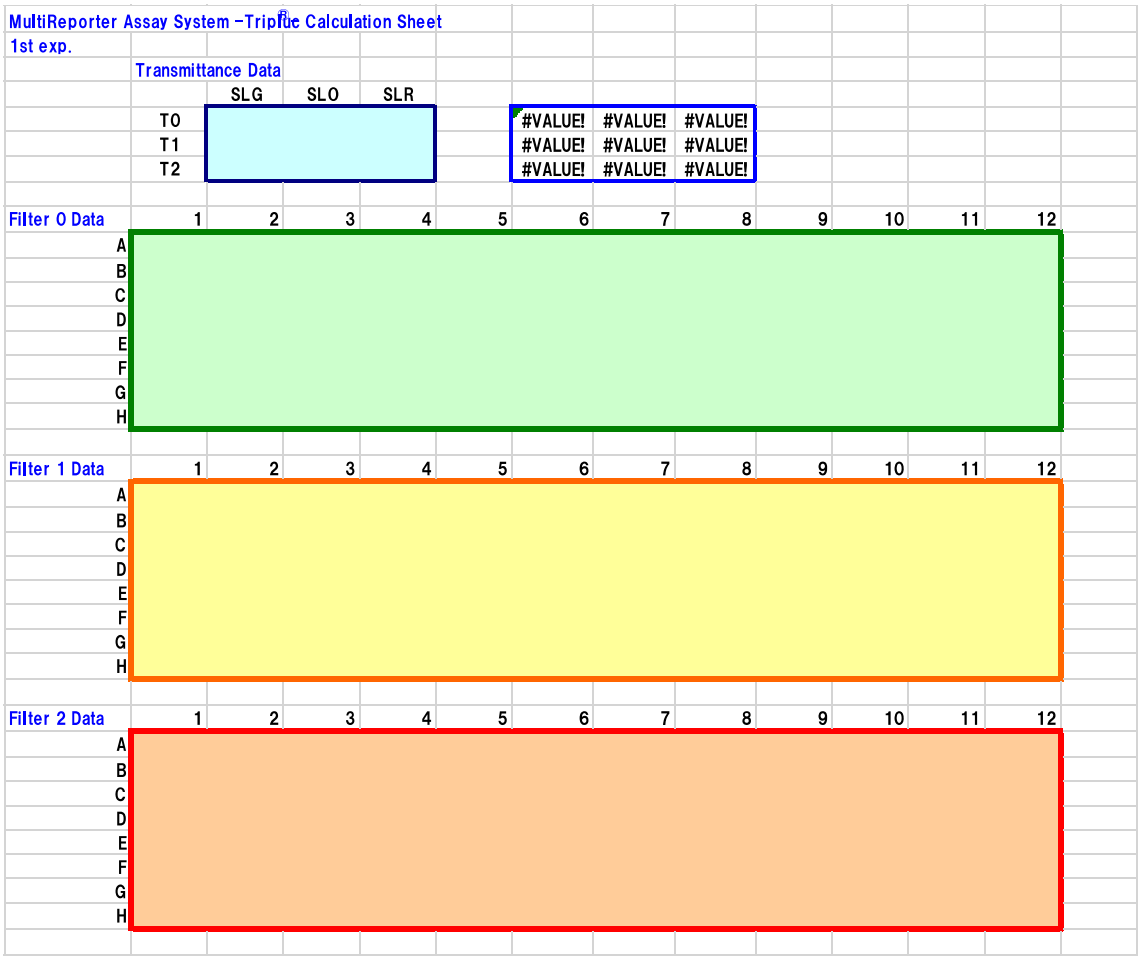
1st. Add the information regarding the name of laboratory, the round of experiments if multiple experimental sets are performed, the experiment number, date, the operator, chemical codes, dissolved in distilled water or DMSO, the prepared concentration, molecular weight of the chemicals and comments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

Figure 29 “Face Sheet” of the data sheet



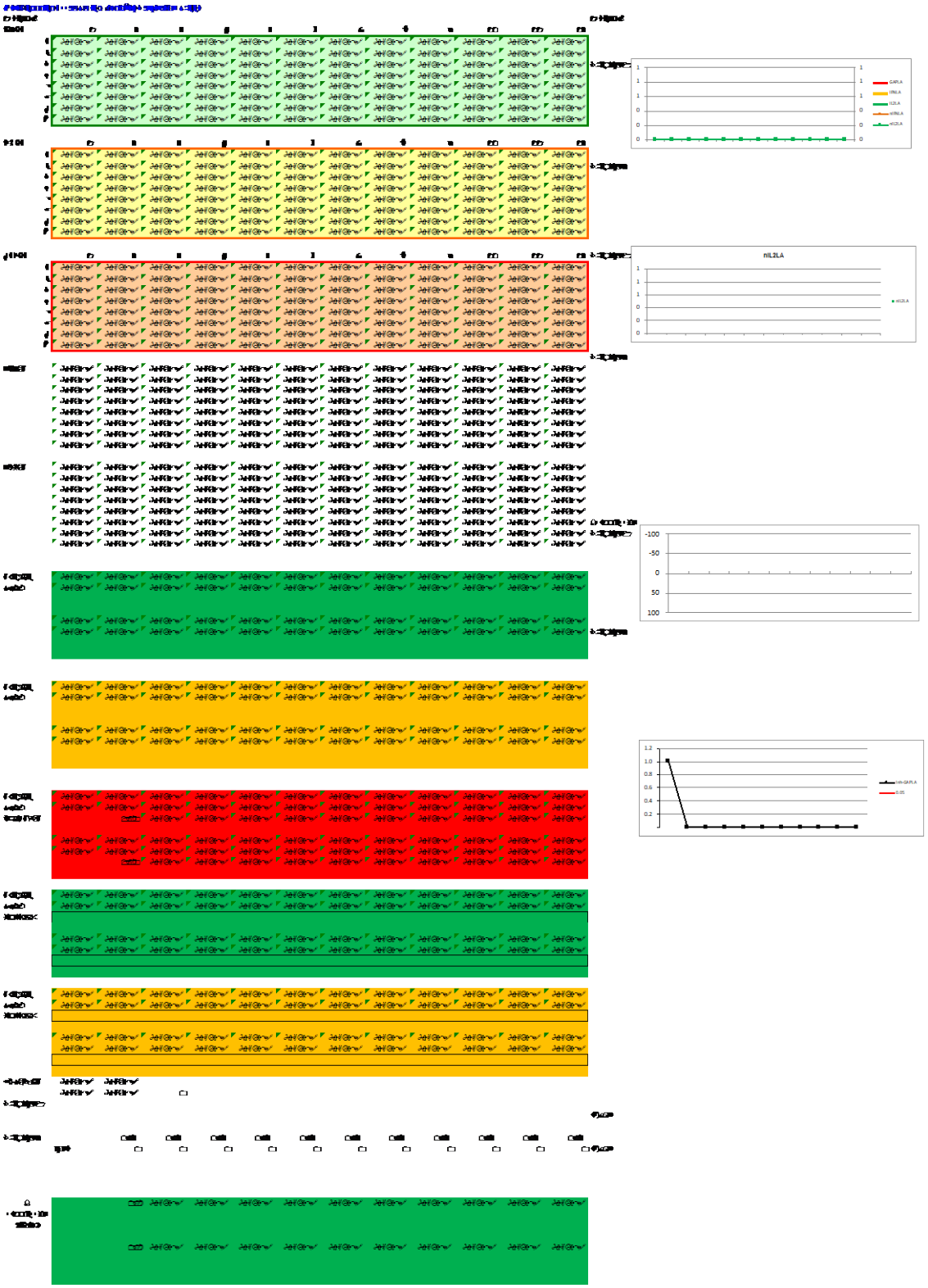
2nd. Copy the results of the F0, F1 and F2 measurements (values are expressed as counts) and paste them into the appropriate area in the “Data Input” sheet of the data sheet shown below. In addition, input the transmittance factors calculated in "§5. Calculation of the transmittance factors" to #C6-#E7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

Figure 30 “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 ± SD of IL2LA, the mean ± SD of IFNLA, the mean ± SD of GAPLA, % suppression and graphs will automatically appear on the “Result Format” sheet of the data sheet.

Figure 31 “Result Format” sheet of the data sheet



10. Data analysis

In the IL-2 Luc LTT, chemicals are first determined to be suppressive, stimulatory, or no effect based on the values of %suppression, which is defined as (nIL2LA of 2H4 cells treated with chemicals / nIL2LA of non-treated 2H4 cells) x 100. Then, considering the values of Inh-GAPLA, which is defined as GAPLA of 2H4 cells treated with chemicals / GAPLA of untreated cells, chemicals classified as suppressive, stimulatory, or no effect were further classified into leukocyte toxic, suppressive, stimulatory or no effect.

Definition of the parameters used in IL-2 Luc leukocyte toxicity test (IL-2 Luc LTT).

Abbreviations	Definition
GAPLA	SLR luciferase activity reflecting GAPDH promoter activity
IL2LA	SLG luciferase activity reflecting IL-2 promoter activity of 2H4 cells
IFNLA	SLO luciferase activity reflecting IFN-γ promoter activity of 2H4 cells
nIL2LA	IL2LA/GAPLA of 2H4 cells
nIFNLA	IFNLA/GAPLA of 2H4 cells
% suppression	(1-(nIL2LA of 2H4 cells treated with chemicals / nIL2LA of non-treated 2H4 cells)) x 100
Inh-GAPLA	GAPLA of 2H4 cells treated with chemicals / GAPLA of untreated cells
CV05	The lowest concentration of the chemical at which Inh-GAPLA becomes < 0.05
Min Inh-GAPLA	The minimum value of Inh-GAPLA of each experiment

11. Criteria

11-1 Acceptance criteria

The following acceptance criteria should be met when using the MITA method. Control experiment should be done once in a month. Dexamethasone should be judged as non-leukocyto toxic or indeterminate, and bleomycin sulfate should be judged as leukocyto toxic by following criterion.

In each time of the experiments, a control experiment examining nIFNLA of 2H4 cells treated with PMA/Io and nIFNLA of non-treated 2H4 cells must be conducted. Then, the fold induction of nIFNLA of 2H4 cells treated with PMA/Ionomycin to nIFNLA of non-

treated 2H4 cells is calculated. If the fold induction is less than 3.0, the results obtained from these experiments should be rejected.

11-2 Criterion to determine leukocyte toxic or non-leukocyte toxic in the IL-2 Luc LTT

The experiments are repeated until 2 consistent leukocyte toxic results, indeterminate results, or non-leukocyte toxic results are obtained. When 2 consistent results are obtained, the chemicals are judged as indicated by the obtained consistent results.

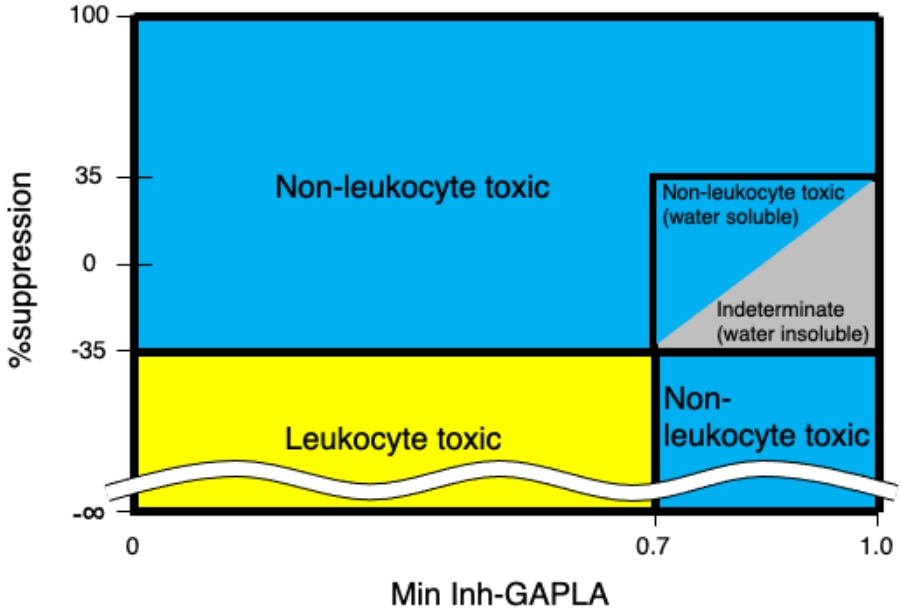
In each experiment, if chemicals meet the following criteria described below and give Min Inh-GAPLA < 0.7, they are judged as leukocyte toxic. Otherwise, they are judged as provisional non-leukocyte toxic.

The criteria for stimulatory:

1. The mean of % suppression is ≤-35 (stimulatory) with statistical significance. The statistical significance is judged by its 95% confidence interval.
2. The result shows 2 or more consecutive statistically significant stimulatory data points or 1 statistically significant stimulatory data point with a trend in which at least 3 consecutive data points decrease in a dose-dependent manner. In the latter case, the trend can cross 0, as long as only 1 data point shows the opposite effect without statistical significance.
3. The results are judged using only data obtained at the concentration at which Inh-GAPLA is ≥0.05.

Of chemicals that are not judged as provisional non-leukocyte toxic, if chemicals do not give statistically significant suppressive or stimulatory data points, give Min Inh-GAPLA ≥ 0.7, and they are insoluble at 10 mg/mL in distilled water, they are judged as indeterminate because they may be not dissolved in the vehicle at the concentration sufficient to show the effects. Otherwise, they are judged as non-leukocyte toxic.

Figure 32


100
35
0
-35
-∞
%suppression
Non-leukocyte toxic
Non-leukocyte toxic
(water soluble)
Indeterminate
(water insoluble)
Leukocyte toxic
Non-
leukocyte toxic
0
0.7
1.0
Min Inh-GAPLA

12. Update record

Ver. 001.8 2021. September. 17th distribution
change the incubate time

Ver. 001.7 2021. September. 7th distribution
change the incubate time
change the reference

Ver. 001.6 2021. July. 26th distribution
change the acceptance criteria

Ver. 001.5 2021. June. 15th distribution
change the concentration of dexamethasone
correct definition of the parameters table

Ver. 001.4 2021. January. 12th distribution
change the preparation of chemical

Ver. 001.3 2020. September. 16th distribution
change cell culture
change the preparation of chemical
change the criterion

Ver. 001.2 2020. August. 7 distribution
change the preparation of chemical
change the criterion

Ver. 001.1 2020. July. 20 distribution
delete the description about D0-IL-2 Luc assay
change the criterion

Ver. 001 2020. June. 19 distribution

Appendix 1 Principle of measurement of luciferase activity

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

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

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

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

			intensity of SLR without filter (all optical)
	Filter 2 transmittance factor	κR_{R60}	The intensity of 600 nm LP (Filter 2) transmitted SLR / the intensity of SLR without filter (all optical)

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

F0=G+O+R

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

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

These formulas can be rephrased as follows

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

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

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

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

Appendix 2　Validation of reagents and equipment

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

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

5-1-1 Reagents

・Single reference samples:

Lyophilized luciferase enzyme reagent of SLG

Lyophilized luciferase enzyme reagent of SLO

Lyophilized luciferase enzyme reagent of SLR

・Assay reagent:

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

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

Reagent	Company	Conc.	Final conc. in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	27 mL
FBS	Biological Industries Cat#04-001-1A Lot: 1524129	-	10 %	3 mL

5-1-2 Calibration

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

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

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

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

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

5-1-2-2 Bioluminescence measurement

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

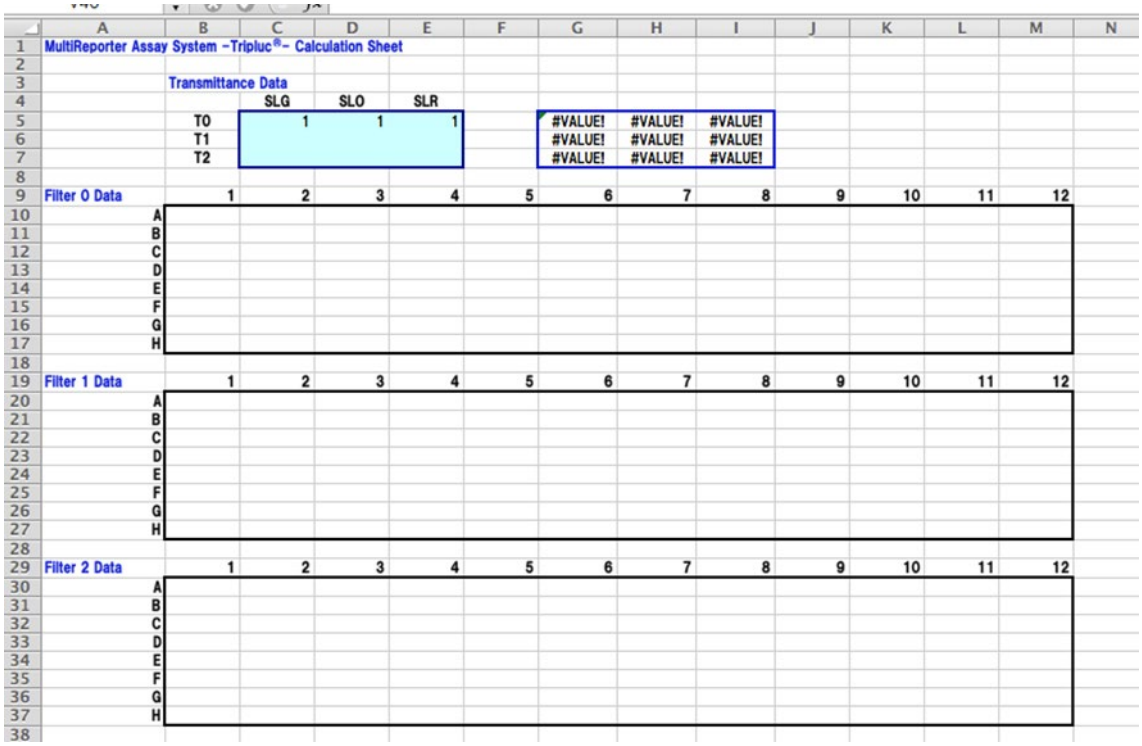
Figure 33

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

Transfer 100 µL of pre-warmed Tripluc to each well containing the reference samples of the plate using a pipetman. Shake the plate for 10 min at room temperature 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 34



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

Record all the results for quality control.

5-2 Quality control of equipment

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

5-2-1 Light source

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

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

L12367 by Hamamatsu Photonics (Shizuoka, Japan)

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

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

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