IL-2 Luc assay (OECD444A) protocol

(Multi-Immuno Tox Assay protocol ver. 013E)

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1. Introduction

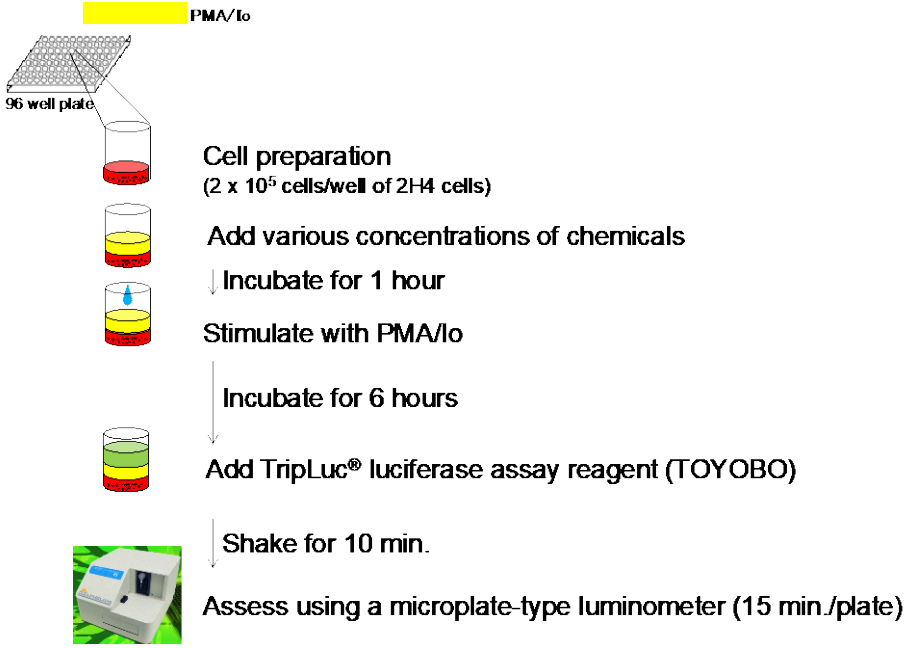
This protocol describes how to maintain the reporter cell line how to prepare the test chemicals, and how to measure the luciferase activity of the cell line. The reporter cell line, 2H4 cell, is transfected with 3 luciferase genes, stable luciferase green (SLG), stable luciferase orange (SLO) and stable luciferase red (SLR), under the control of IL-2, IFN-γ and GAPDH promoters, respectively, for the IL-2 Luc assay.

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

Figure 1

Assay design (2 chemicals per one plate)

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	cont (distilled water or DMSO)	PMA/Io only	$A/2^9$ ○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, IFN-γ-SLO, GAPDH-SLR)

  A Jurkat-derived IL-2 and IFN-γ reporter cell line, 2H4 cells, 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: 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

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

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	440 mL
FBS	Biological Industries Cat#04-001-1E Lot: 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#04-001-1E 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#04-001-1E 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 30 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 ($2x10^6$ cells / 0.5 mL of freezing medium) in a 37°C water bath, then add to a 15 mL polypropylene conical tube containing 9 mL of pre-warmed C medium. Centrifuge the tube at 120-350 x *g* at room temperature for 5 min, discard the supernatant, and resuspend in 15 mL of pre-warmed C medium in a T-75 Flask. Cells are incubated at 37°C, 5% CO_2.

3-2 Maintenance of 2H4 cells

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

The interval between subcultures should be 3~4 days. Cells can only 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 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 give them some leeway, 3.0 x 10^7 cells for two chemicals should be prepared), centrifuge the tube at 120-350 x *g* for5 min. Resuspend in pre-warmed B medium at a cell density of 4×10^6/mL. Transfer the cell suspension to a reservoir, and add 50 µL of cell suspension to each well of a 96 well µclear black plate (flat bottom) using an 8 channel or 12 channel pipetman. (cf. Figure 2)

Figure 2

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

5. Preparation of chemicals and cell treatment with chemicals

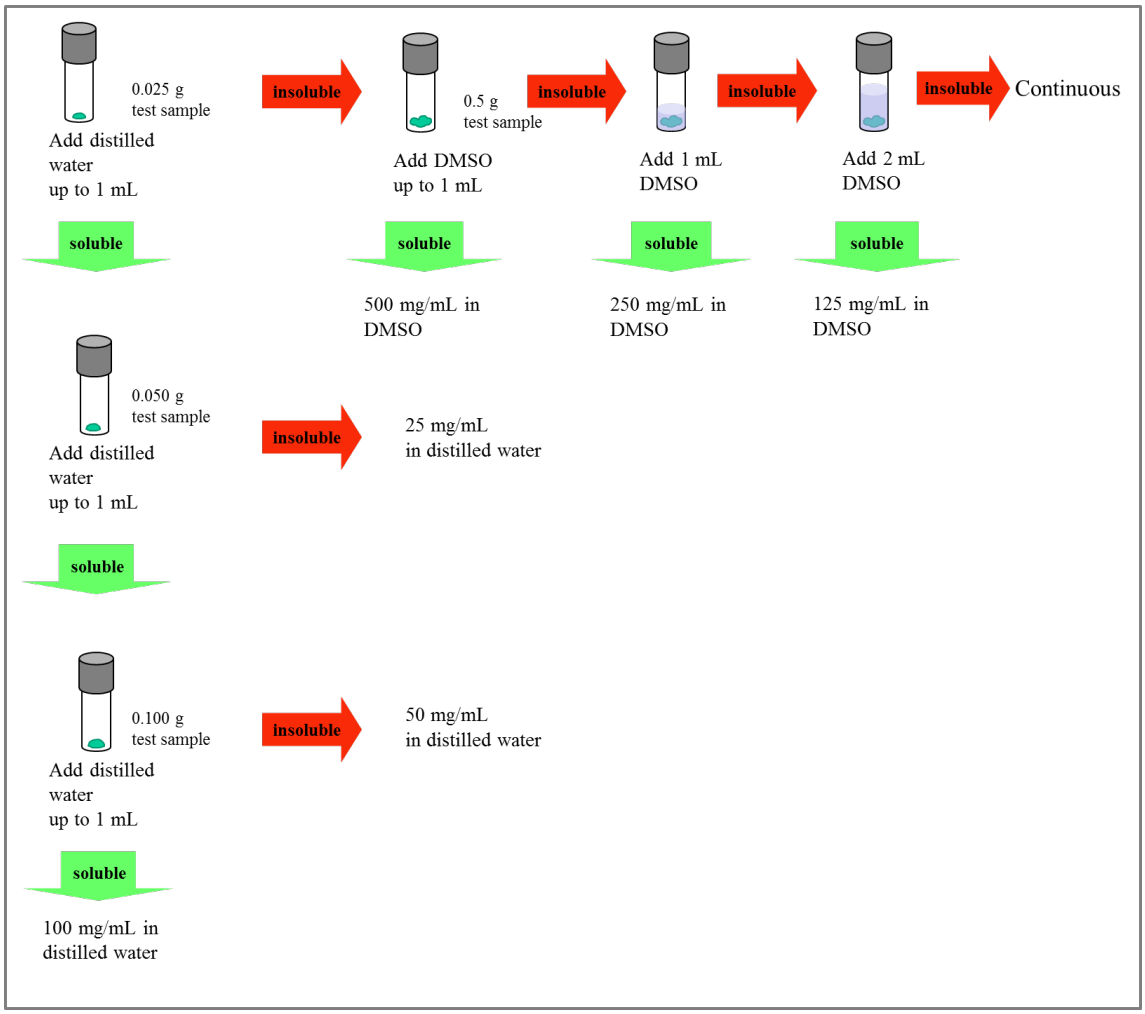
5-1 Dissolution by vehicle

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

If the chemical is not soluble in water, the chemical should be dissolved in DMSO at 500 mg/mL. Namely, weigh 500 mg of the test chemical in volumetric flask and add DMSO up to 1 mL.
If the chemical is not soluble at 500 mg/mL, the highest soluble concentration should be determined by diluting the solution from 500 mg/mL at a common ratio of two (250 mg/mL → 125 mg/mL → continued if needed) with DMSO. (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 the absence of visible precipitation after centrifugation at 15,000 rpm (≈20,000 x g) for 5 min. The chemical should be used within 4 hours after being dissolved in distilled water or DMSO.

Figure 3



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

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

For example, Figure 4 below shows that the minimum concentration at which Inh-

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

Figure 5 below shows that Inh-GAPLA did not fall below 0.05. In such a case, the highest concentration of the chemical to examine is the highest concentration in the 1st experiment, namely 125 μg/mL.

Figure 4

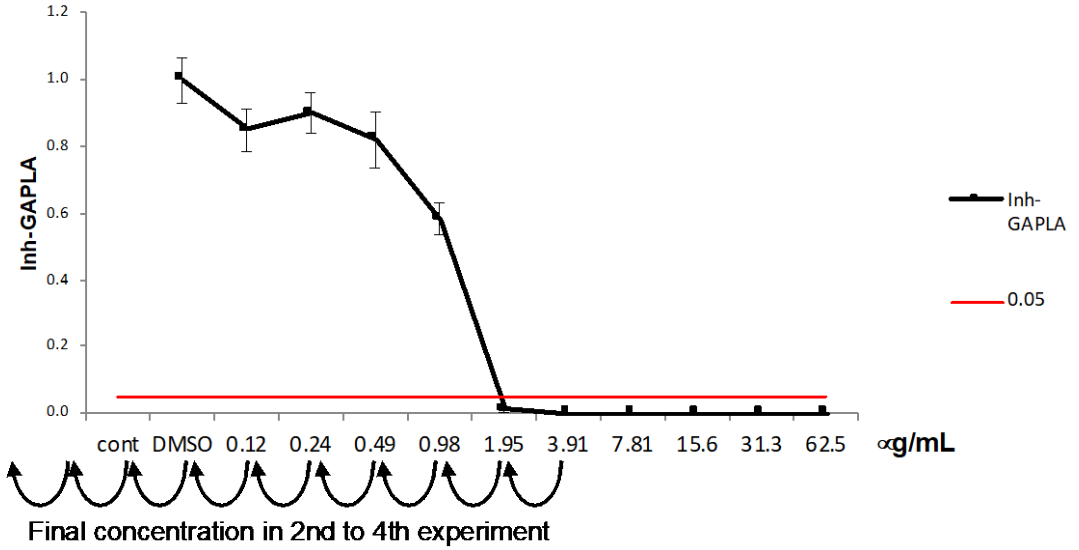
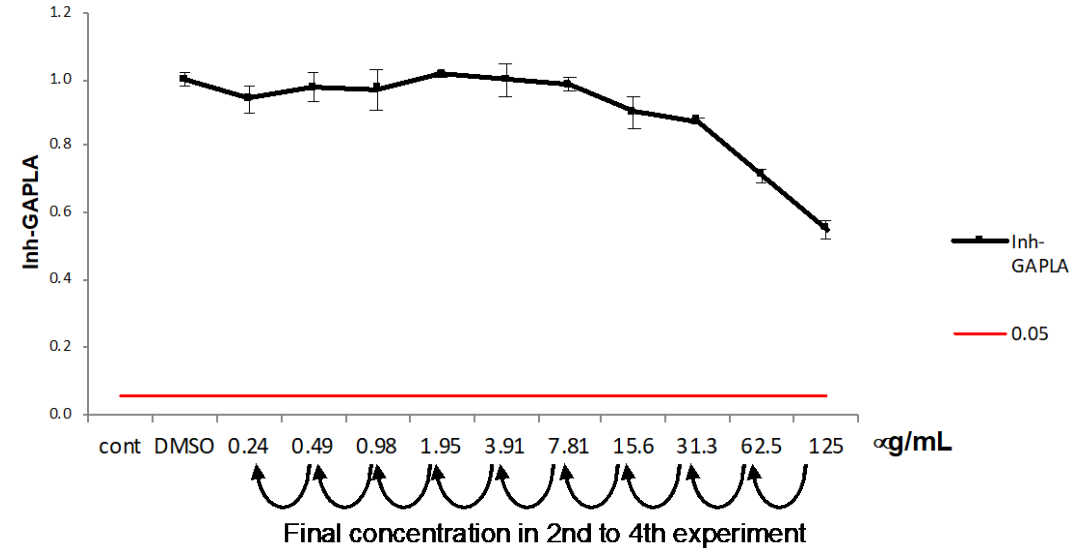


Figure 5



5-2 When the chemical is prepared in distilled water

In the explanation below, if the chemical is prepared at a lower concentration than 100 mg/mL, use the prepared concentration instead of the 100 mg/mL distilled water solution.

5-2-1 Arrangement of chemicals and vehicle

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

5-2-2 Serial dilution

Perform 9 serial dilutions at a factor of 2 as indicated in Figure 6 from well #A11 to well #A3. Indeed, transfer 50 μL to the next (left) well. (cf. Figure 6)

Figure 6

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

5-2-3　2 step dilution

Add 20 μL of the diluted chemical to 480 μL of B medium prepared in the assay block. And add 50 μL to 2H4 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 1 hour (37°C, CO_2, 5%) (cf. Figure 7-9).

Figure 7

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 50μL	Distilled water 50μL	Chemical 0.2 mg/mL in distilled water 100μL	Chemical 0.4 mg/mL in distilled water 50μL	Chemical 0.8 mg/mL in distilled water 50μL	Chemical 1.6 mg/mL in distilled water 50μL	Chemical 3.1 mg/mL in distilled water 50μL	Chemical 6.3 mg/mL in distilled water 50μL	Chemical 13 mg/mL in distilled water 50μL	Chemical 25 mg/mL in distilled water 50μL	Chemical 50 mg/mL in distilled water 50μL	Chemical 100 mg/mL in distilled water 50μL
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 480μL	B medium 480μL	B medium 480μL	B medium 480μL	B medium 480μL	B medium 480μL	B medium 480μL	B medium 480μL	B medium 480μL	B medium 480μL	B medium 480μL	B medium 480μL
B												
C												
D												
E												
F												
G												
H												

Figure 8

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 500µL	B medium 500µL	Chemical 0.008 mg/mL in B medium 500µL	Chemical 0.02 mg/mL in B medium 500µL	Chemical 0.03 mg/mL in B medium 500µL	Chemical 0.06 mg/mL in B medium 500µL	Chemical 0.1 mg/mL in B medium 500µL	Chemical 0.3 mg/mL in B medium 500µL	Chemical 0.5 mg/mL in B medium 500µL	Chemical 1 mg/mL in B medium 500µL	Chemical 2 mg/mL in B medium 500µL	Chemical 4 mg/mL in B medium 500µL
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 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL
B	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL
C	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL
D	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL
E	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL
F	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL
G	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL
H	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL

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
A	Chemical 0 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.004 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.008 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.02 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.03 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.06 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.1 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.3 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.5 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 1 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 2 mg/mL 2H4 2x10^5 B medium 100µL
B	Chemical 0 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.004 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.008 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.02 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.03 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.06 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.1 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.3 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.5 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 1 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 2 mg/mL 2H4 2x10^5 B medium 100µL
C	Chemical 0 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.004 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.008 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.02 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.03 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.06 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.1 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.3 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.5 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 1 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 2 mg/mL 2H4 2x10^5 B medium 100µL
D	Chemical 0 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.004 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.008 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.02 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.03 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.06 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.1 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.3 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 0.5 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 1 mg/mL 2H4 2x10^5 B medium 100µL	Chemical 2 mg/mL 2H4 2x10^5 B medium 100µL
E	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL
F	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL
G	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL
H	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL

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

In the explanation below, if the chemical is prepared at a lower concentration, use the prepared concentration instead of 500 mg/mL DMSO solution.

5-3-1 Arrangement of chemicals and vehicle

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

5-3-2 Serial dilution

Performe 9 serial dilutions at a factor of 2 as indicated in Figure 10 from well #A11 to well #A3. Indeed, transfer 50 μL to the next (left) well. (cf. Figure 10)

Figure 10

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

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

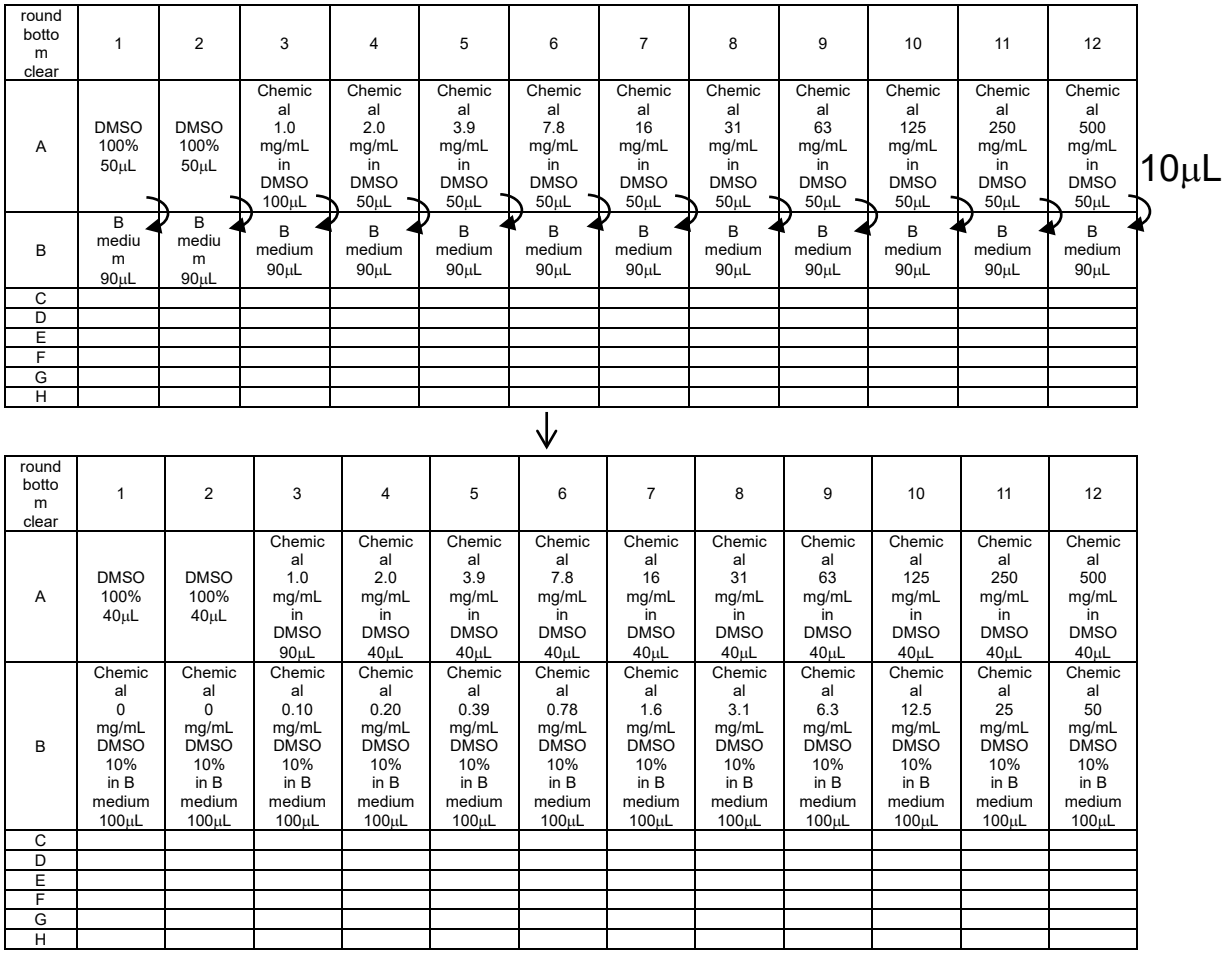
↓

round botto m clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 50μL	DMSO 100% 50μL	Chemical 1.0 mg/mL in DMSO 100μL	Chemical 2.0 mg/mL in DMSO 50μL	Chemical 3.9 mg/mL in DMSO 50μL	Chemical 7.8 mg/mL in DMSO 50μL	Chemical 16 mg/mL in DMSO 50μL	Chemical 31 mg/mL in DMSO 50μL	Chemical 63 mg/mL in DMSO 50μL	Chemical 125 mg/mL in DMSO 50μL	Chemical 250 mg/mL in DMSO 50μL	Chemical 500 mg/mL in DMSO 50μL
B	B mediu m 90μL	B mediu m 90μL	B medium 90μL	B medium 90μL	B medium 90μL	B medium 90μL	B medium 90μL	B medium 90μL	B medium 90μL	B medium 90μL	B medium 90μL	B medium 90μL
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 11)

Figure 11



5-3-4　2 step dilution

Add 10 μL of the diluted chemical to 490 μL of B medium prepared in the assay block. Then, add 50 μL to 2H4 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Steps 5-3-3 to 5-3-4 should be carried out as quickly as possible and do not leave a long time for steps after 5-3-3 or Figure 11. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 1 hour (37°C, CO_2, 5%) (cf. Figure 12-14).

Figure 12

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

10μL

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

Figure 13

Figure 14

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	Chemical 0 µg/mL DMSO 0.2% in B medium 500µL	Chemical 0 µg/mL DMSO 0.2% in B medium 500µL	Chemical 2.0 µg/mL DMSO 0.2% in B medium 500µL	Chemical 3.9 µg/mL DMSO 0.2% in B medium 500µL	Chemical 7.8 µg/mL DMSO 0.2% in B medium 500µL	Chemical 15.6 µg/mL DMSO 0.2% in B medium 500µL	Chemical 31.3 µg/mL DMSO 0.2% in B medium 500µL	Chemical 62.5 µg/mL DMSO 0.2% in B medium 500µL	Chemical 125 µg/mL DMSO 0.2% in B medium 500µL	Chemical 250 µg/mL DMSO 0.2% in B medium 500µL	Chemical 500 µg/mL DMSO 0.2% in B medium 500µL	Chemical 1000 µg/mL DMSO 0.2% in B medium 500µL
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 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL
B	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL
C	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL
D	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL
E	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL
F	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL
G	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL
H	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL

Figure 14 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 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 0 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 1.0 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 2.0 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 3.9 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 7.8 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 16 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 31 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 63 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 125 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 250 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 500 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL
B	Chemical 0 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 0 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 1.0 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 2.0 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 3.9 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 7.8 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 16 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 31 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 63 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 125 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 250 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 500 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL
C	Chemical 0 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 0 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 1.0 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 2.0 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 3.9 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 7.8 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 16 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 31 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 63 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 125 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 250 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 500 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL
D	Chemical 0 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 0 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 1.0 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 2.0 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 3.9 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 7.8 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 16 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 31 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 63 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 125 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 250 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL	Chemical 500 µg/mL 0.1% DMSO 2H4 2x10^5 B medium 100µL
E	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL
F	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL
G	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL
H	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL	2H4 2x10^5 B medium 50µL

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

6-1 Material

・2 mM PMA stock

・2 mM Ionomycin stock

・B medium

・Ethanol

6-2 Preparation of 100 μM PMA

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

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

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

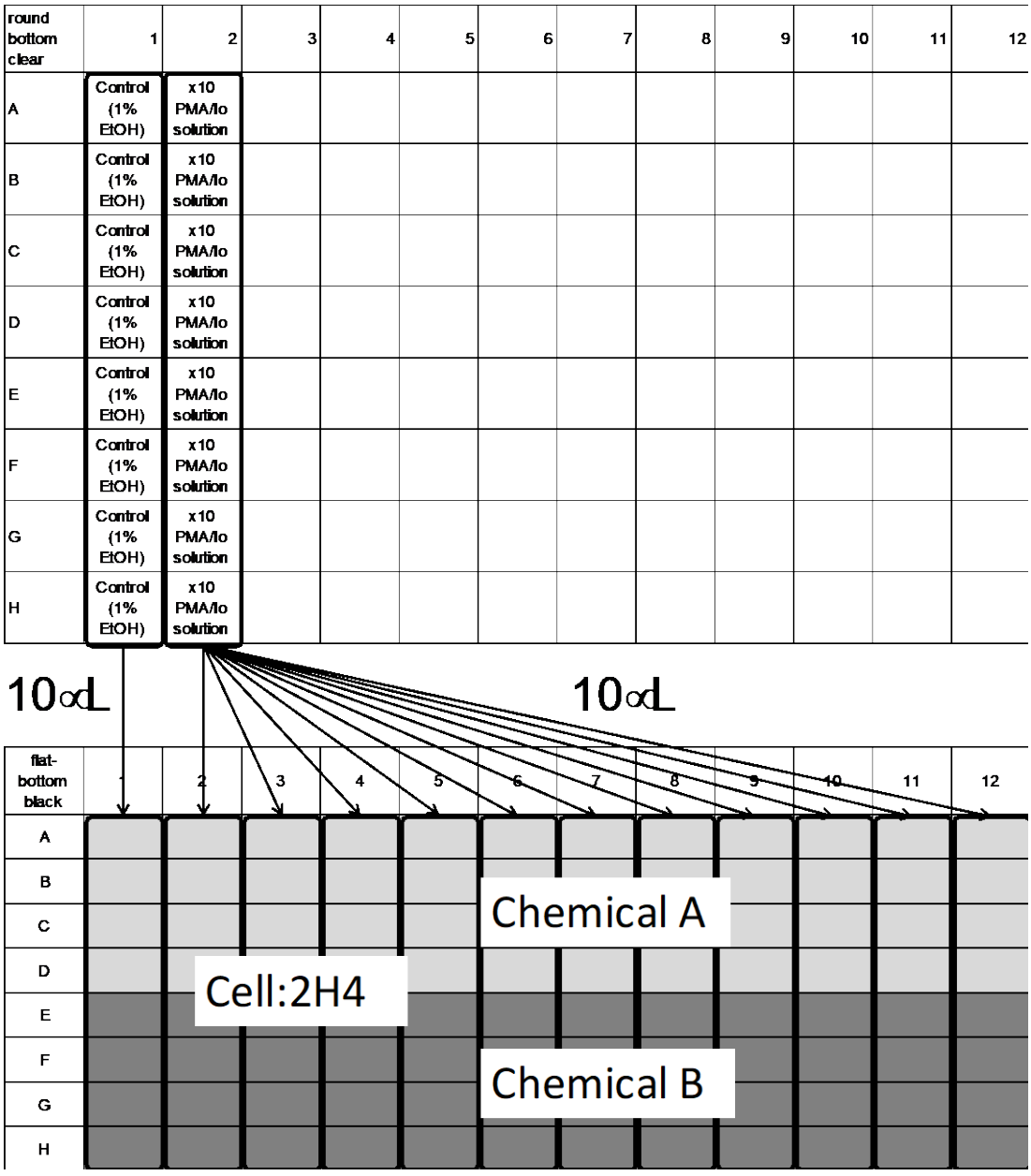
Dilute ethanol, 2 mM ionomycin and 100 μM PMA with the B medium to prepare control or x10 PMA/ionomycin solution. Add the control to well #A1-#H1 of the 96 well clear plate (round bottom), and add x10 PMA/ionomycin solution to wells #A2-#H2 of the 96 well clear plate (round bottom).

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

6-4 Addition of PMA/ionomycin to 2H4

One hour after the addition of chemicals, add 10 µL of control or PMA/ionomycin solution to the cells (#A1-#H1 or #A2-#H12, respectively) using an 8 channel or 12 channel pipetman after pipetting 20 times. Ensure that the apex of the tip is immersed in the medium. Change tips line by line to avoid chemical contamination. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 6 hours (37°C, CO_2, 5%). (cf. Figure 15)

Figure 15



7. Control

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

7-1-1 Preparing dexamethasone stock

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

Weigh 1 g of dexamethasone in volumetric flask and add DMSO up to 2 mL, dispend at 50 μL/tube and store a freezer at -30°C.

7-1-2 Preparing cyclosporine A stock

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

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

7-2 Preparation of cells for assay

A cell passage should be done 3-4 days before the assay.

Use cells between 1 and 6 weeks after thawing.

Pre-warm B medium in a 37°C water bath. Count the number of cells and collect the number of cells needed (3.2 x 10^6 cells are required, but to have some leeway, 4.8 x 10^6 cells should be prepared), centrifuge the tube at 120-350 x *g*, 5 min. Resuspend in pre-warmed 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 (#A1 to #D4) of a 96 well µclear black plate (flat bottom) using an 8 channel or 12 channel pipetman. (cf. Figure 16)

Figure 16

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

7-3 Arrangement of chemicals and vehicle

Add 50 µL of DMSO to #A1-2, 50 µl of 100 mg/mL dexamethasone stock solution to #A3, 50 µl of 100 µg/mL cyclosporine A stock solution to #A4, and 90 µl of B medium to #B1-4 of the 96 well clear plate (round bottom). (cf. Figure 17)

7-4 Dilution with B medium

Add 10 µL of DMSO in #A1-2, 10 µL of dexamethasone solution in #A3 and 10 µL of cyclosporine A solution in #A4 to #B1-4 . (cf. Figure 17)

Figure 17

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

↓

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 40µL	DMSO 100% 40µL	DEX 100 mg/mL in DMSO 40µL	CyA 100 µg/mL in DMSO 40µL								
B	DMSO 10% in B medium 100µL	DMSO 10% in B medium 100µL	DEX 10 mg/mL DMSO 10% in B medium 100µL	CyA 10 µg/mL DMSO 10% in B medium 100µL								
C												
D												
E												
F												
G												
H												

7-5 2 step dilution

Add 10 μL of DMSO, Dex, and CyA solution in #B1-4 of the 96 well clear plate to 490 μL of 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. Steps 7-4 to 7-5 should be carried out as quickly as possible and do not take a long time for steps 7-4 or Figure 17 onwards. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 1 hour (37°C, CO_2, 5%). (cf. Figure 18-20)

Figure 18

Round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100% 40μL	DMSO 100% 40μL	DEX 100 mg/mL in DMSO 40μL	CyA 100 μg/mL in DMSO 40μL								
B	DMSO 10% in B medium 100μL	DMSO 10% in B medium 100μL	DEX 10 mg/mL DMSO 10% in B medium 100μL	CyA 10 μg/mL DMSO 10% in B medium 100μL								
C												
D												
E												
F												
G												
H												

10μL

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

Figure 19

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 0.2% in B medium 500μL	DMSO 0.2% in B medium 500μL	DEX 200 μg/mL DMSO 0.2% in B medium 500μL	CyA 200 ng/mL DMSO 0.2% in B medium 500μL								
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 2x10^5 B medium 50μL	2H4 2x10^5 B medium 50μL	2H4 2x10^5 B medium 50μL	2H4 2x10^5 B medium 50μL								
B	2H4 2x10^5 B medium 50μL	2H4 2x10^5 B medium 50μL	2H4 2x10^5 B medium 50μL	2H4 2x10^5 B medium 50μL								
C	2H4 2x10^5 B medium 50μL	2H4 2x10^5 B medium 50μL	2H4 2x10^5 B medium 50μL	2H4 2x10^5 B medium 50μL								
D	2H4 2x10^5 B medium 50μL	2H4 2x10^5 B medium 50μL	2H4 2x10^5 B medium 50μL	2H4 2x10^5 B medium 50μL								
E												
F												
G												
H												

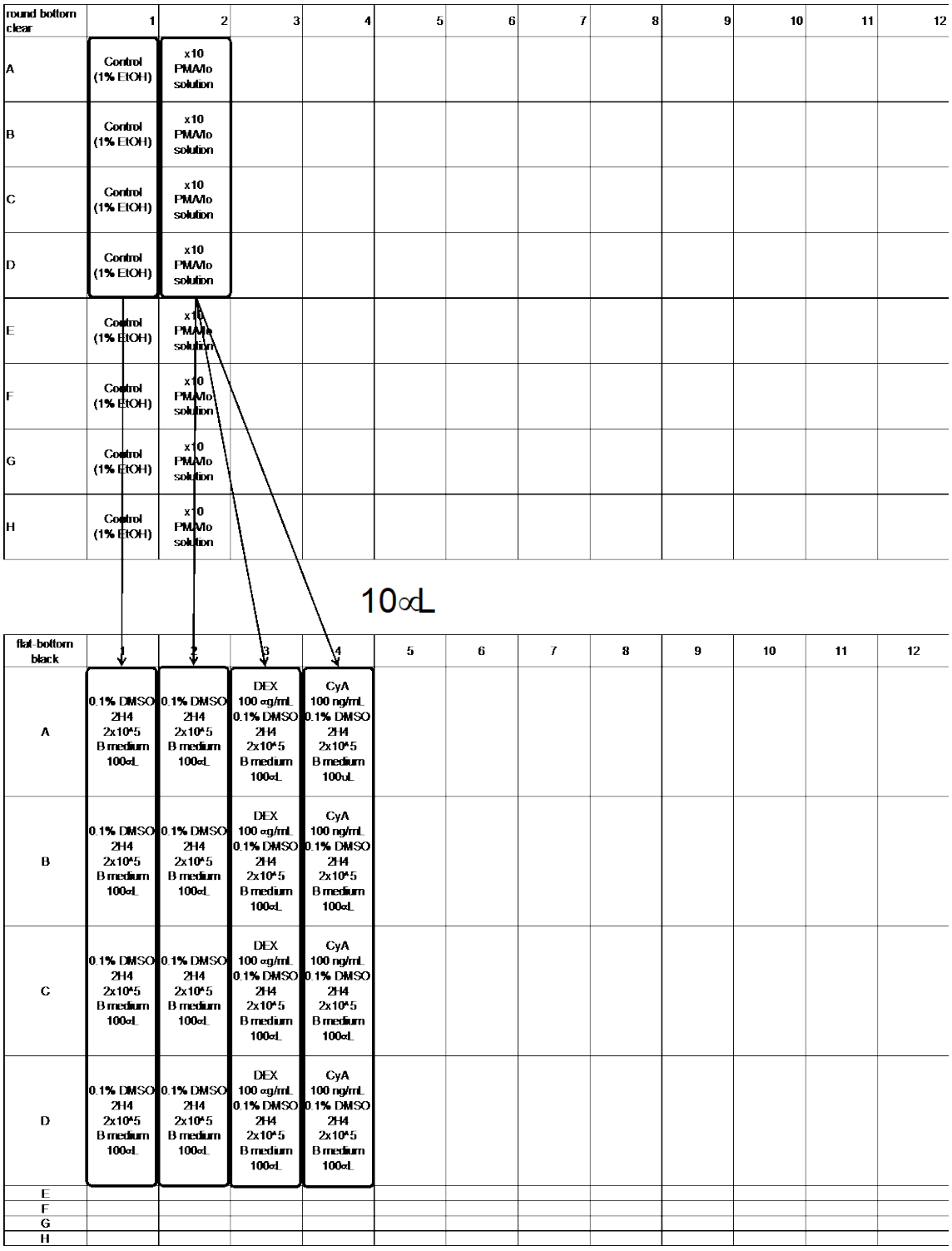
Figure 20 Final constituents of each well of the plate

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

7-6 Addition of PMA/ionomycin to 2H4

One hour after the addition of dexamethasone and cyclosporine A, add 10 μL of control or PMA/ionomycin solution prepared in §6-3 to the cells (#A1-#D1 or #A2-#D4, 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 hours (37°C, CO_2, 5%). (cf. Figure 21)

Figure 21


round bottom clear
1
2
3
4
5
6
7
8
9
10
11
12
A
B
C
D
E
F
G
H
Control (1% EtOH)
x10 PMAMo solution
10∝L
flat bottom black
0.1% DMSO 2H4 2x10^5 B medium 100∝L
DEX 100 ∝g/mL 0.1% DMSO 2H4 2x10^5 B medium 100∝L
CyA 100 ng/mL 0.1% DMSO 2H4 2x10^5 B medium 100uL

8. Calculation of the transmittance factors

Color discrimination in multi-color reporter assays can generally performed 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

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.

8-3 Bioluminescence measurement

Switch on the luminometer 30 minutes before the start of the measurement to allow the photomultiplier to stabilise. Thaw Tripluc® Luciferase assay reagent (Tripluc) and keep it at room temperature either in a water bath or at ambient air temperature. Add 1 mL of B medium to each tube of frozen reference sample (10 μL sample per tube). Keep the reference samples on ice to prevent deactivation.

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

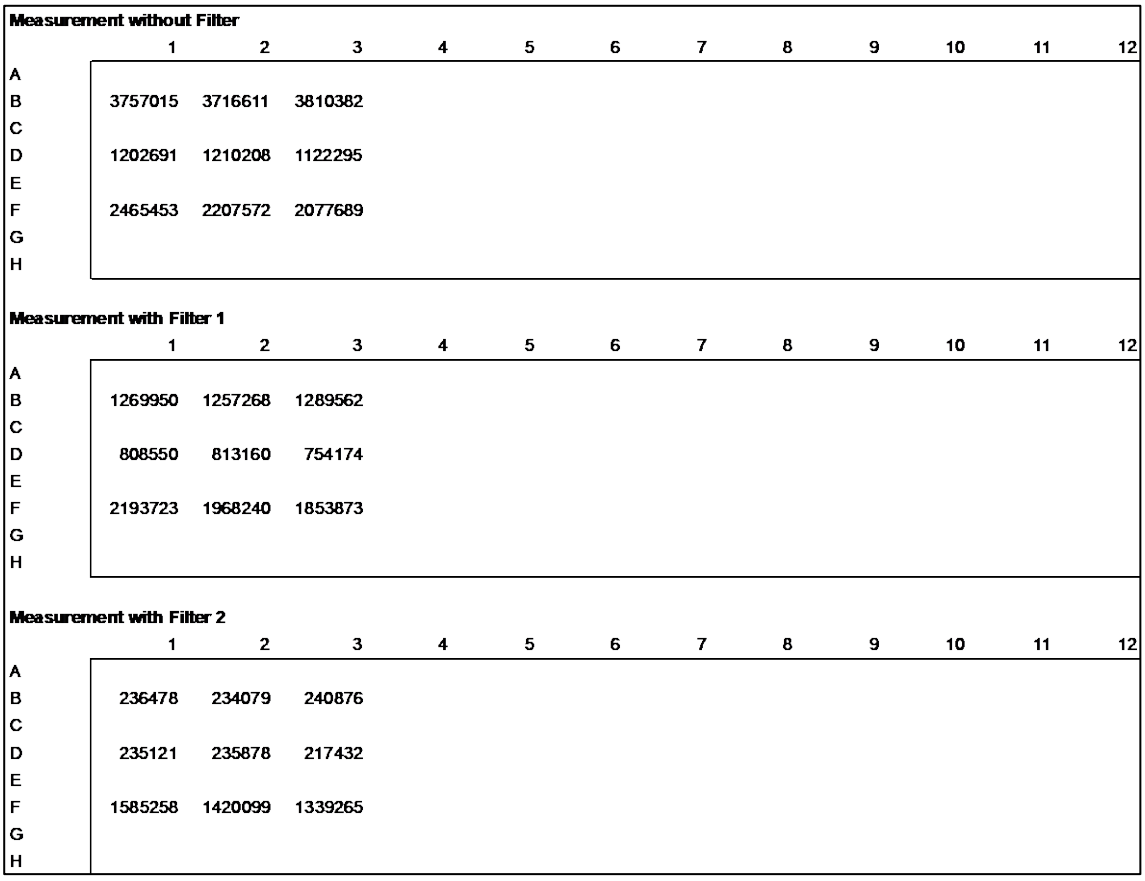
as shown below.

Figure 22

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

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

Figure 23



Measurement without Filter

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

Measurement with Filter 1

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

Measurement with Filter 2

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

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

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

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

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

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

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

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

In the case shown above,

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

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 Calculation Sheet as follow.

Figure 24

	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

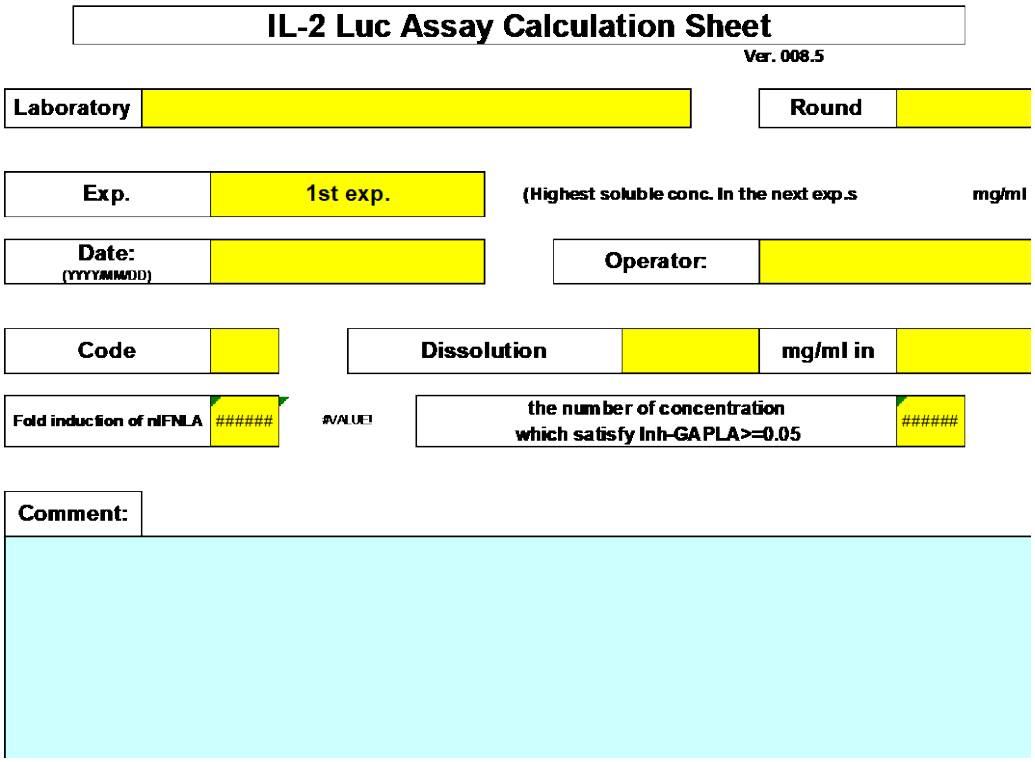
Please refer Appendix 1 for the principle of measurement of luciferase activity.

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

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

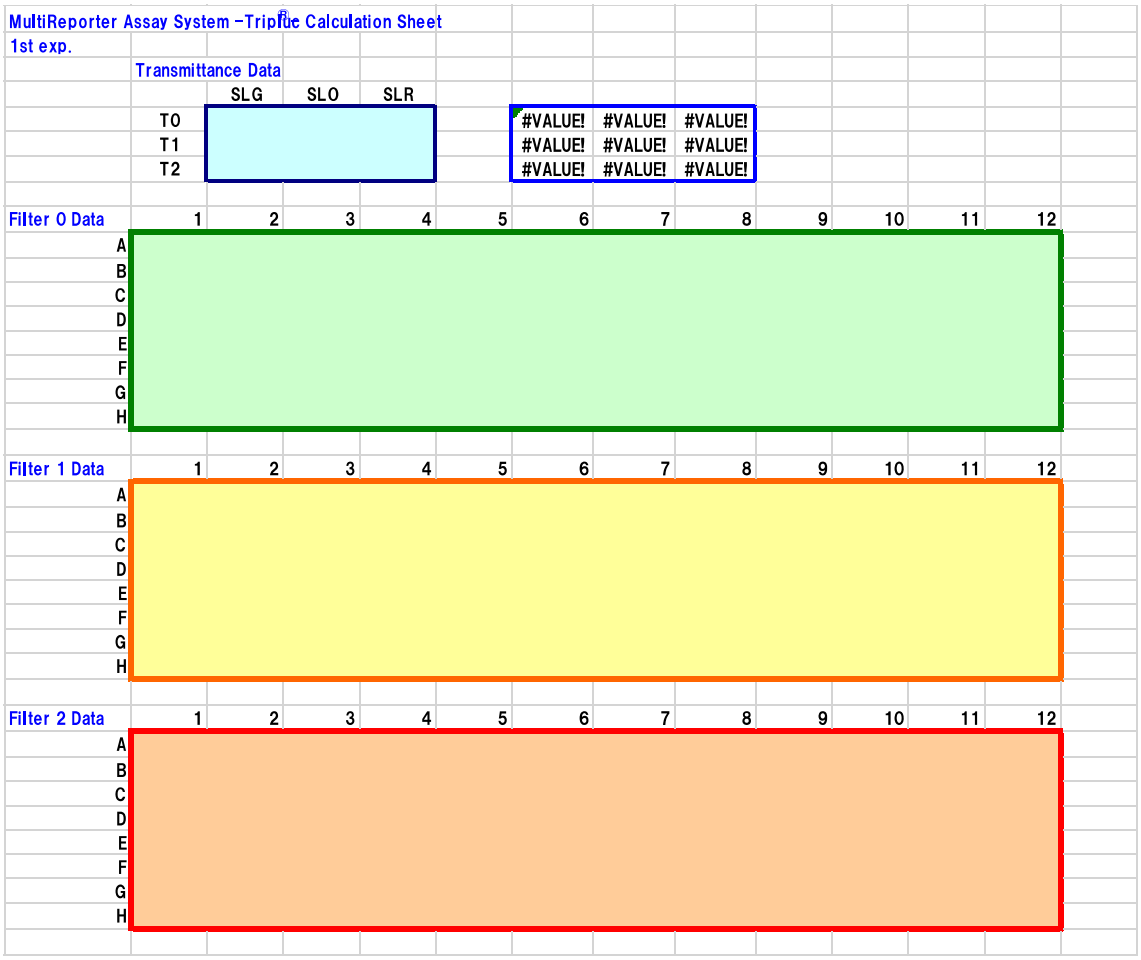
1st. Put the information regarding the name of laboratory, the round of experiments in the “Face Sheet” of the Calculation Sheet if multiple experimental sets are performed, the experiment number, date, the operator, chemical codes, dissolved in distilled water or DMSO, the prepared concentration, molecular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

Figure 25 “Face Sheet" of the Calculation Sheet



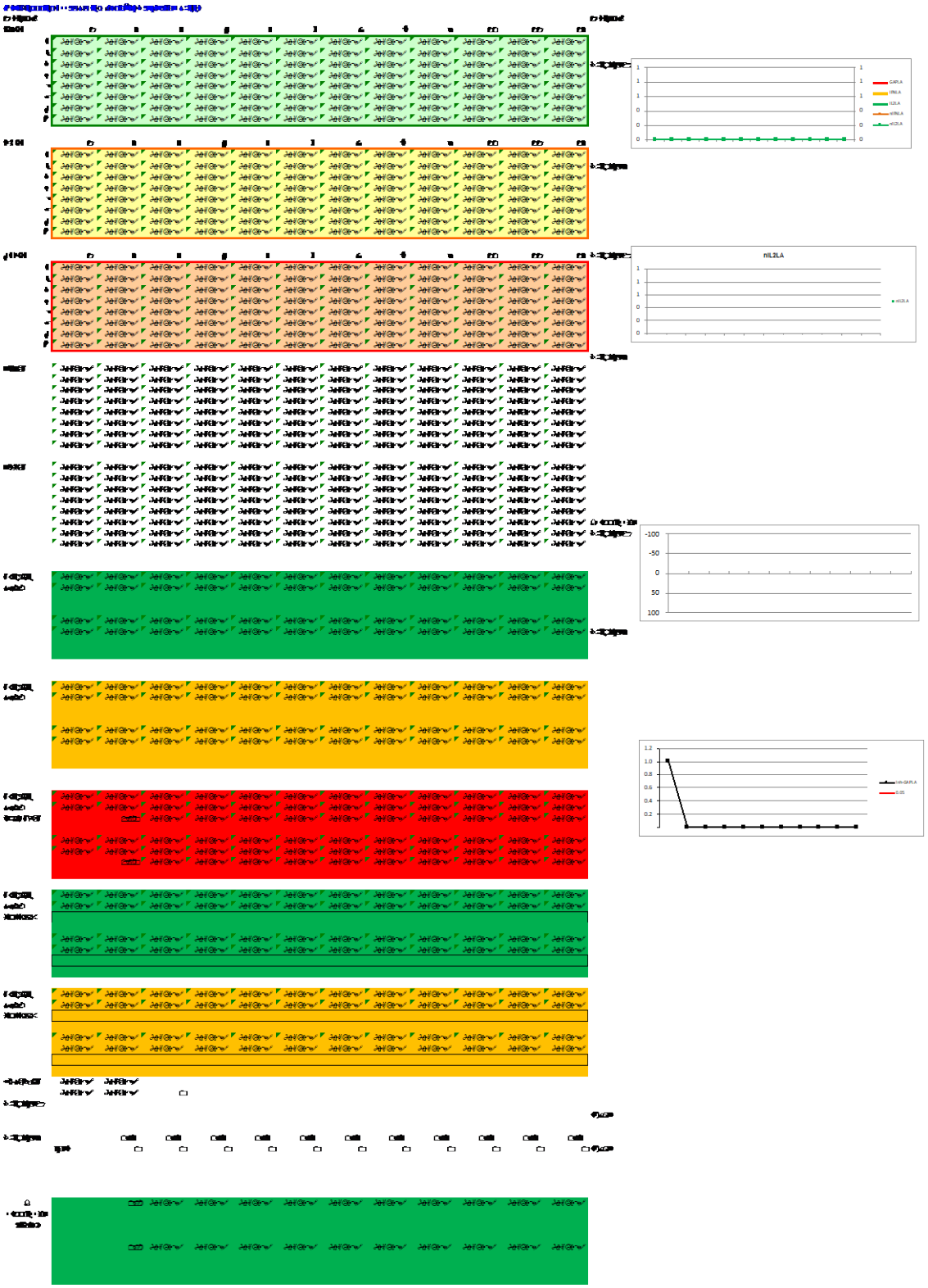
2nd. Copy the results of the F0, F1 and F2 measurements (values are expressed as counts) and paste them into the appropriate area in the “Data Input” sheet of the Calculation Sheet shown below. In addition, put the transmittance factors calculated in "§5. Calculation of the transmittance factors" to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

Figure 26 “Data Input” sheet of the Calculation 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 Calculation Sheet.

Figure 27 “Result Format” sheet of the Calculation Sheet



10. Data analysis

Definition of the parameters used in the IL-2 Luc 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 IL-2 Luc assay.

- 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

1. The runs are repeated until two consistent positive (or negative) runs are obtained. A maximum of three runs is possible. When two consistent results are obtained, the chemicals are judged as the obtained consistent results.

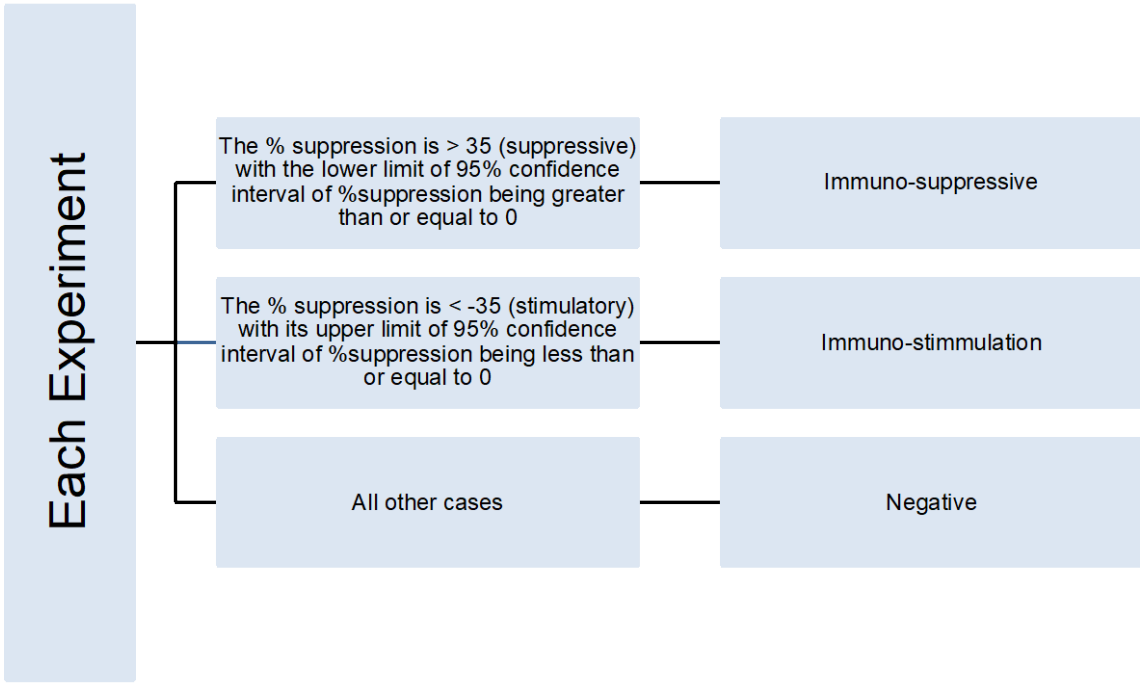
Identification of immunotoxicant is evaluated by the mean of %suppression and its 95% simultaneous confidence interval.

In each run, the test chemical is judged positive (immune-suppressive or -stimulatory) when all three following criteria are fulfilled:

1. Each data point (% suppression) is judged as a positive result (suppressive or stimulatory) if the % suppression is > 35 (suppressive) with the lower limit of 95% confidence interval of %suppression being greater than or equal to 0 or < -35 of % suppression (stimulatory) with its upper limit being less than or equal to 0.
2. The outcome shows two or more consecutive statistically significant results (increase [=suppression] or decrease [=stimulation]); alternatively one positive data point (increase or decrease) with the same trend for at least 3 consecutive data points (i.e. concentration dependent trend), in which case the trend can cross the zero line, as long as the 95% confidence interval of the data point indicating the opposite response crosses 0.
3. The outcome is judged using only data obtained in the concentration range at which Inh-GAPLA is $\geq$ 0.05.

In all other cases the test chemical is judged as not active (negative).

Graphs illustrating these criteria are shown below.



12. Update record

Ver. 0013E 2023.5.7
Change the control, water soluble dexamethasone to water insoluble dexamethasone
Change the appearance of figures.

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 ($NaBrO_3$), Nickel (II) sulfate ($NiSO_4$), Dibutyl phthalate (DP), 2-Mercaptobenzothiazole (2-MBT))
Change THP-G1b cells to TGCHAC-A4 cells
Change cell number of THP-G8 and TGCHAC-A4 5x10^4/well to 1x10^5/well
Change concentration of chemicals 11 steps to 10 steps
Change final concentration of LPS (THP-G8 : 25 ng/mL, TGCHAC-A4 : 1 ng/mL)
Change the way of addition of LPS (2 mL/well to 10 mL/well)
Change the criteria

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

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

Ver. 001J 2012. Nov. 09 distribution

Appendix 1 Principle of measurement of luciferase activity

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

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

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

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

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

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

$F0=G+O+R$

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

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

These formulas can be rephrased as follows

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

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

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

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

Appendix 2　Validation of reagents and equipment

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

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

5-1-1 Reagents

・Single reference samples:
Lyophilized luciferase enzyme reagent of SLG
Lyophilized luciferase enzyme reagent of SLO
Lyophilized luciferase enzyme reagent of SLR

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

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

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

5-1-2 Calibration

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

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

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

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

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

5-1-2-2 Bioluminescence measurement

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

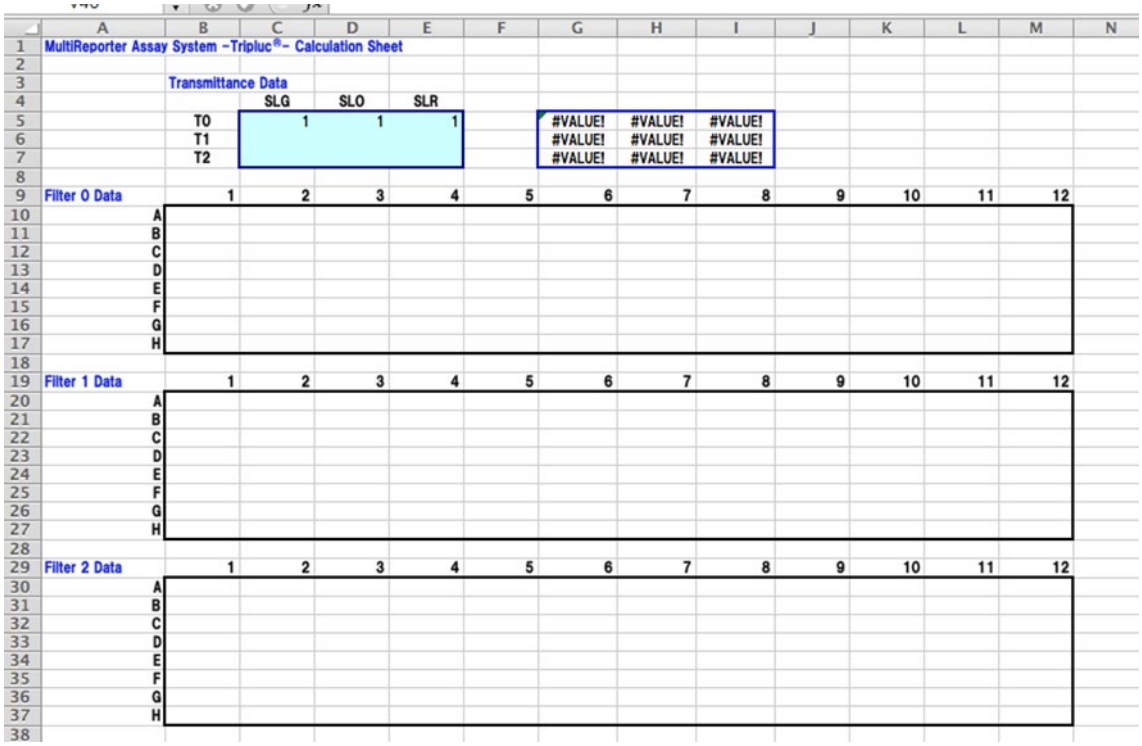
Figure 28

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

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

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

Figure 29



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 Calculation 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%).