# Multi-Immuno Tox Assay protocol ver. 011.1E

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## 1. Introduction

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

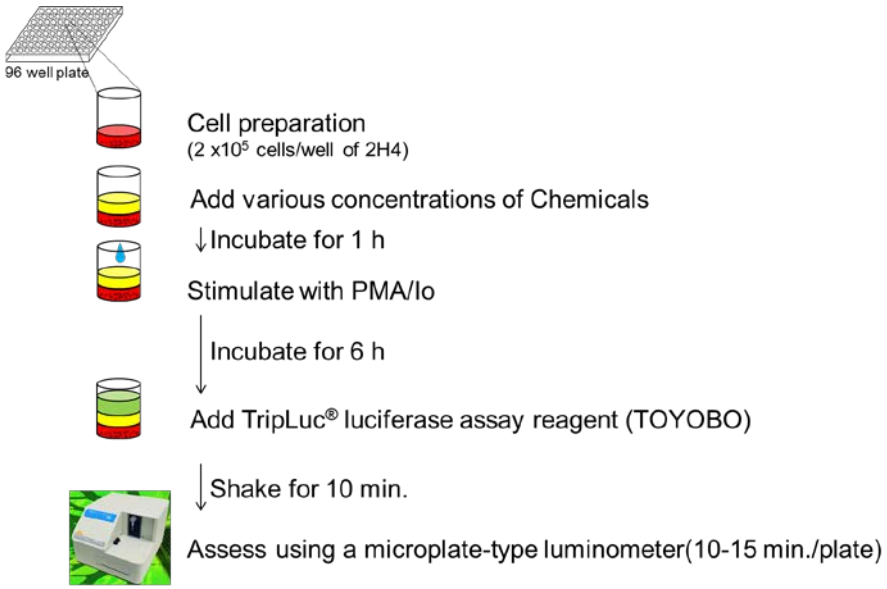
(Kimura Y. et al. Evaluation of the Multi-Immuno Tox Assay composed of 3 human cytokine reporter cells by examining immunological effects of drugs *Toxicol in Vitro*, 28, 759-768, 2014)

Figure 1

Assay design (2 chemicals per one plate)

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

PMA/Io



## 2. Materials

### 2-1 Cells

- 2H4 (IL2-SLG, IFNγ-SLO, G3PDH-SLR)

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

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

### 2-2 Reagents and equipment

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

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

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

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

#### 2-2-3 For measurement of the luciferase activity

- 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: 715004 | - | 10 % | 50 mL |
| Antibiotic-Antimycotic | GIBCO #15240-062 | 100× | 1× | 5 mL |
| Puromycin | InvivoGen # ant-pr-1 | 10 mg/mL | 0.15 μg/mL | 7.5 μL |
| G418 | Nacalai Tesque #16513-84 | 50 mg/mL | 300 μg/mL | 3 mL |
| HygromycinB | Invitrogen #10687-010 | 50 mg/mL | 200 μg/mL | 2 mL |

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

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

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

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

2-4 Preparation of the stimulant of 2H4

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

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

Dissolve 1 mg PMA using DMSO 811 μL, dispend at 5 μL/tube and store at freezer at -30°C. Use these stocks within 6 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 the A medium in a T-75 Flask at 37°C in a 5% $CO_2$ incubator. The culture medium should be changed to the A medium 3 or 4 days after thawing. At that time, count the number of cells, centrifuge the tube at 120-350 x *g* at room temperature for 5 min, discard the supernatant, and resuspend in pre-warmed the A medium in a T-75 Flask. Cells are passaged at $3x10^5$/mL and incubated at 37°C, 5% $CO_2$.

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

## 4. Preparation of cells for assay

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

Use cells between 1 and 6 weeks after thawing.

Pre-warm the B medium in a 37°C water bath. Count the number of cells and collect the number of cells needed (2.0 x $10^7$ cells for two chemicals are required, but to have some leeway, 3.0 x $10^7$ cells for two chemicals should be prepared), centrifuge the tube at 120-350 x *g*, 5 min. Resuspend in pre-warmed the B medium at a cell density of $4\times10^6$/mL. Transfer the cell suspension to a reservoir, and add 50 µL of cell suspension to each well of a 96 well µclear black plate (flat bottom) using an 8 channel or 12 channel pipetman. (cf. Figure 2)

Figure 2

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

## 5. Preparation of chemicals and cell treatment with chemicals

5-1 Dissolution by vehicle

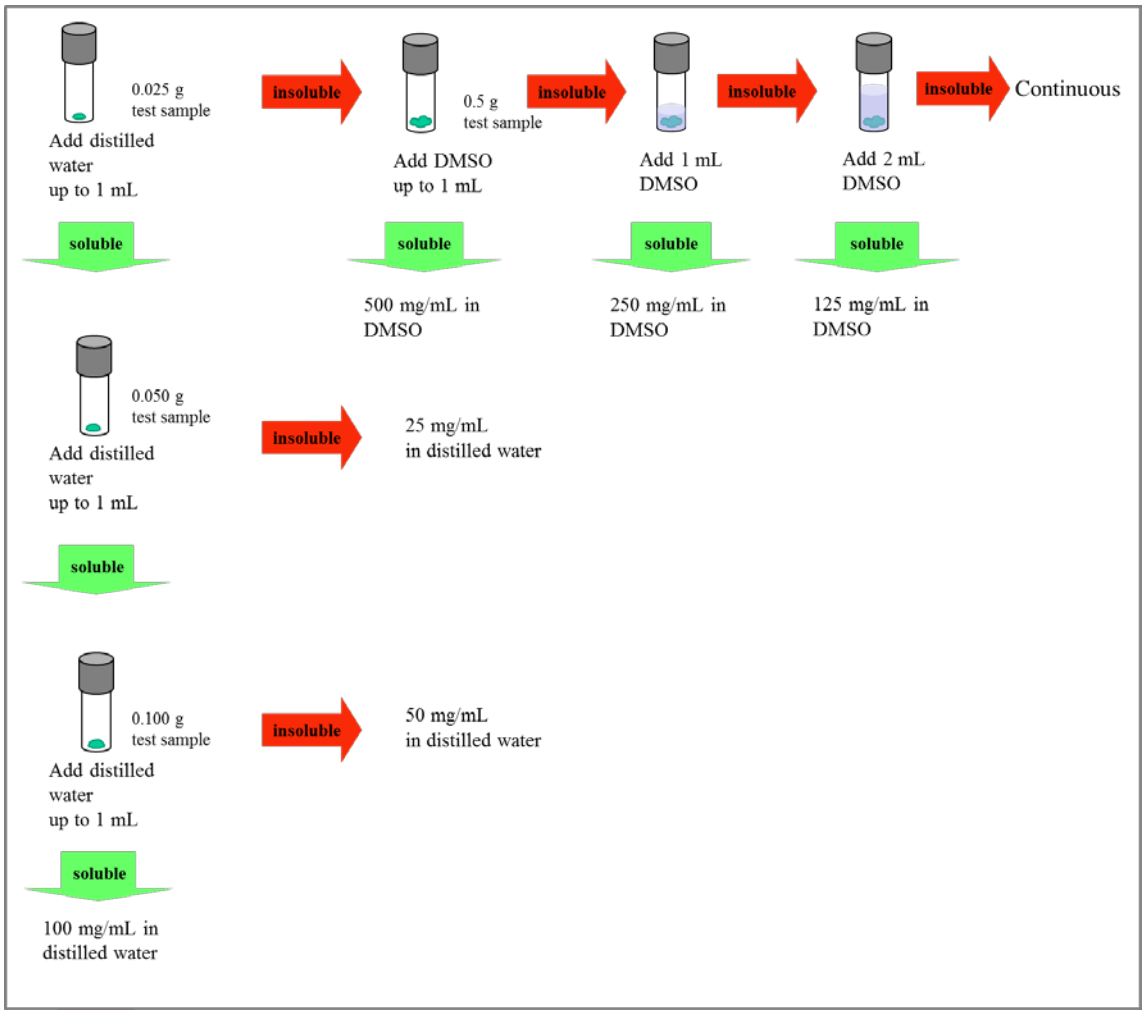
Dissolve the chemical first in distilled water. Namely, weigh 0.025 g of the test chemical in a volumetric flask and add distilled water up to 1 mL. If the chemical is soluble at 25 mg/mL, weigh 0.050 g of the test chemical in a volumetric flask and add distilled water up to 1 mL. If the chemical is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

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

If the chemical is not soluble at 500 mg/mL, the highest soluble concentration should be determined by diluting the solution from 500 mg/mL at a common ratio of two (250 mg/mL → 125 mg/mL → continued if needed) with DMSO. (cf. Figure 3)

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

Figure 3




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

In the second 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 conduct 10 serial dilutions at a common ratio of 2 from the highest concentration. If Inh-GAPLA did not become lower than 0.05 or became lower than 0.05 at the highest concentration in the 1st experiment, conduct 10 serial dilutions at a common ratio of 2 from the highest concentration in the 1st experiment.

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

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

In Figure 5 below, Inh-GAPLA did not become lower than 0.05. In such a case, the highest concentration of the chemical to examine is the highest concentration in the 1st experiment, namely 125 μg/ml.

Figure 4

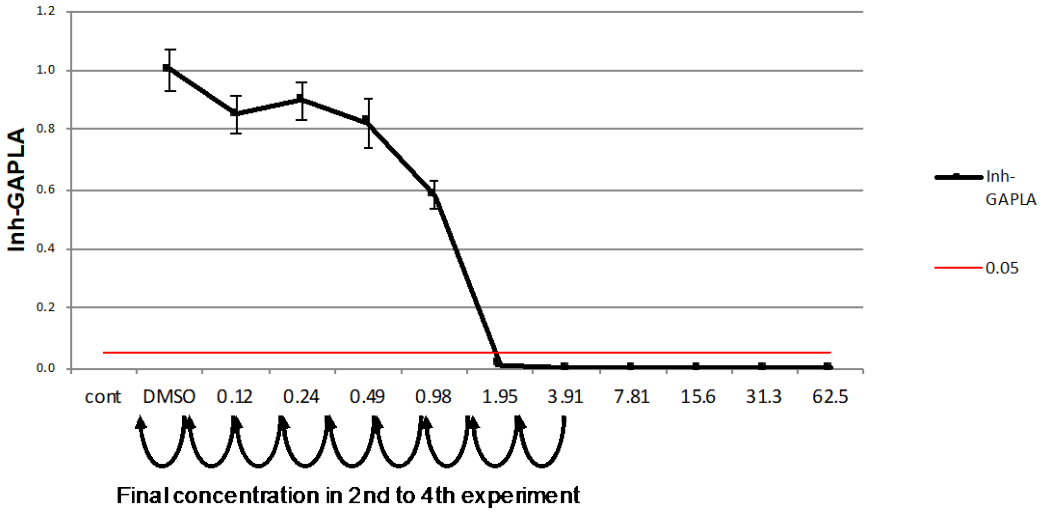
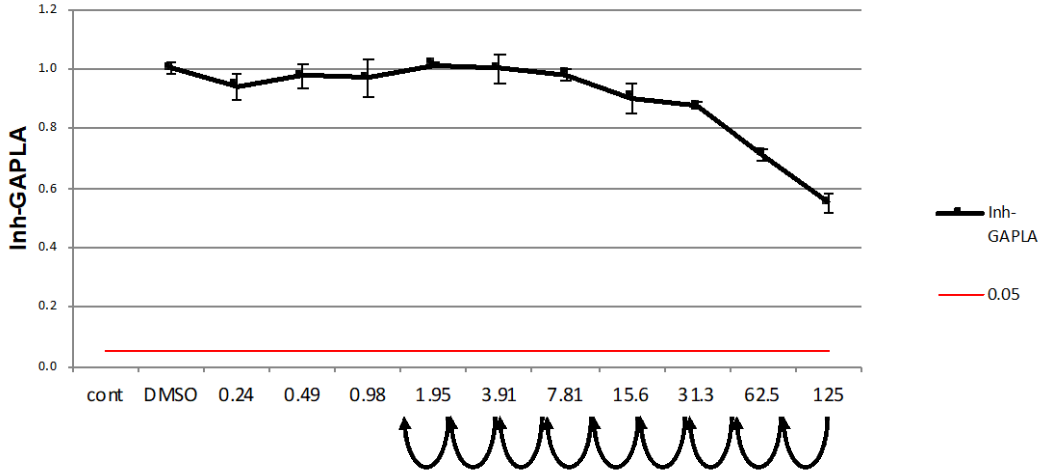



Figure 5



5-2 When the chemical is prepared in distilled water

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

5-2-1 Arrangement of chemicals and vehicle

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

5-2-2 Serial dilution

Conduct 9 serial dilutions at a common ratio of 2 as indicated in Figure 4 from well #A11 to well #A3. Transfer 50 µL to the next (left) well. (cf. Figure 6)

Figure 6

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

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

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

5-2-3　2 step dilution

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

Figure 7

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

20uL

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

Figure 8

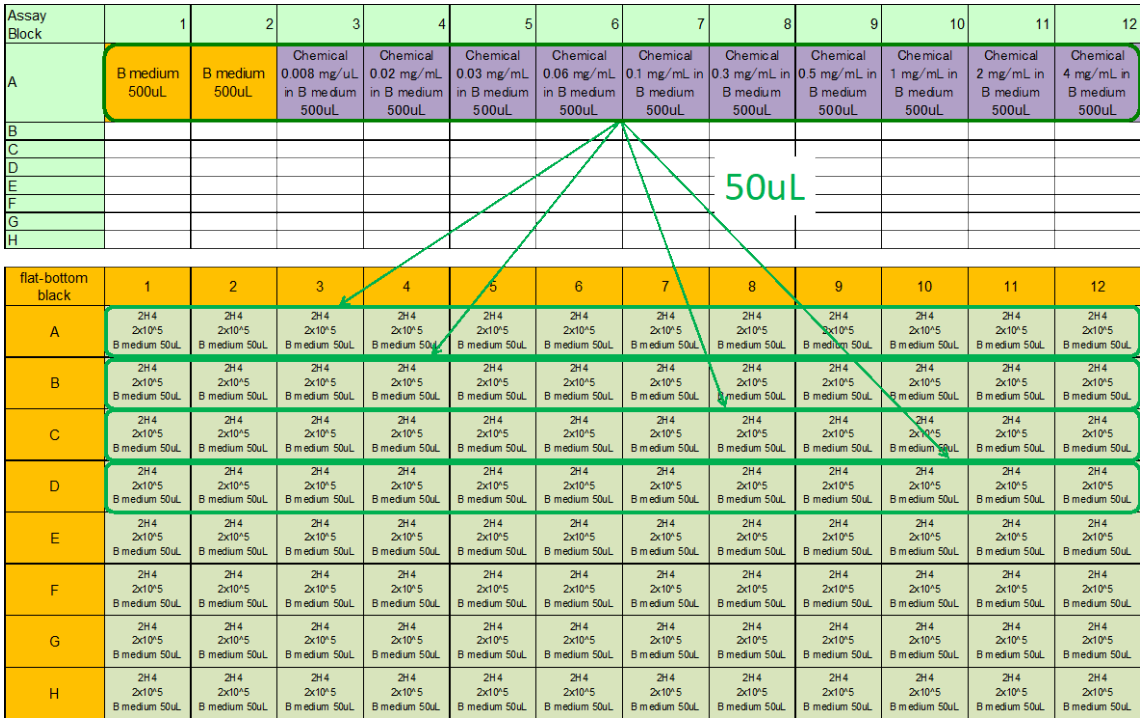



Figure 9 Final constituents of each well of the plate

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

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

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

5-3-1 Arrangement of chemicals and vehicle

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

5-3-2 Serial dilution

Conduct 9 serial dilutions at a common ratio of 2 as indicated in Figure 8 from well #A11 to well #A3. Transfer 50 µL to the next (left) well. (cf. Figure 10)

Figure 10

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

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

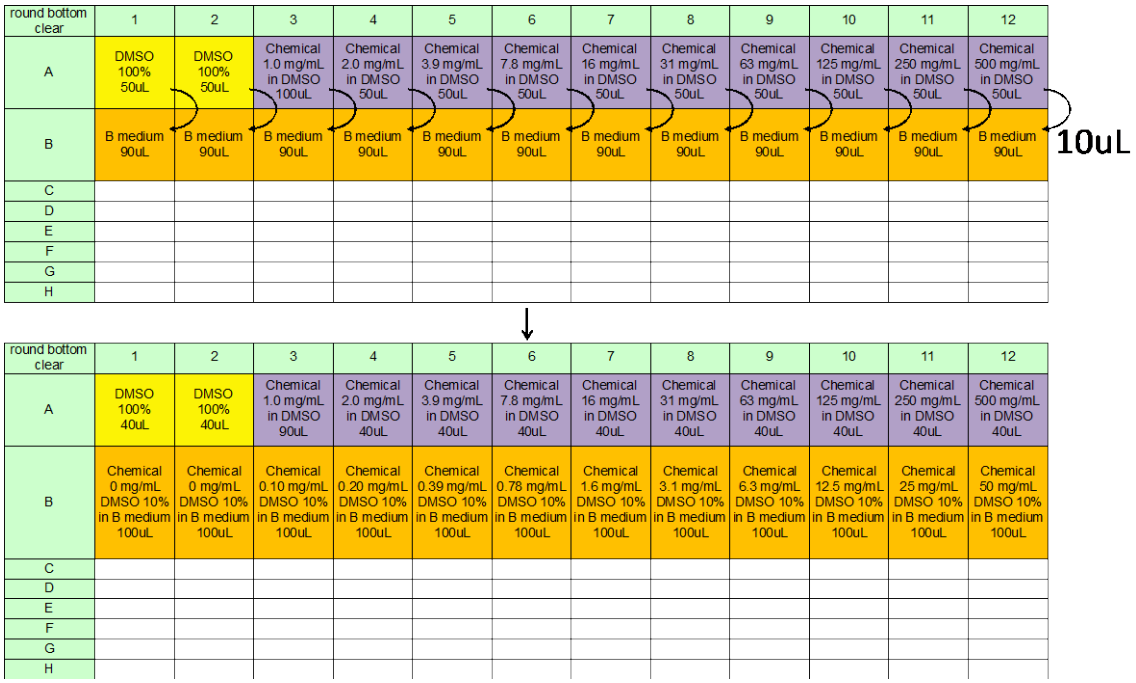
↓

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

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

Dilute 10 μL of the DMSO solution of the chemical in wells #A1-#A12 with 90 μL of the B medium using an 8-12 channel pipetman. (cf. Figure 11)

Figure 11



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 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% 40uL | DMSO 100% 40uL | Chemical 1.0 mg/mL in DMSO 90uL | Chemical 2.0 mg/mL in DMSO 40uL | Chemical 3.9 mg/mL in DMSO 40uL | Chemical 7.8 mg/mL in DMSO 40uL | Chemical 16 mg/mL in DMSO 40uL | Chemical 31 mg/mL in DMSO 40uL | Chemical 63 mg/mL in DMSO 40uL | Chemical 125 mg/mL in DMSO 40uL | Chemical 250 mg/mL in DMSO 40uL | Chemical 500 mg/mL in DMSO 40uL |
| B | Chemical 0 mg/mL DMSO 10% in B medium 100uL | Chemical 0 mg/mL DMSO 10% in B medium 100uL | Chemical 0.10 mg/mL DMSO 10% in B medium 100uL | Chemical 0.20 mg/mL DMSO 10% in B medium 100uL | Chemical 0.39 mg/mL DMSO 10% in B medium 100uL | Chemical 0.78 mg/mL DMSO 10% in B medium 100uL | Chemical 1.6 mg/mL DMSO 10% in B medium 100uL | Chemical 3.1 mg/mL DMSO 10% in B medium 100uL | Chemical 6.3 mg/mL DMSO 10% in B medium 100uL | Chemical 12.5 mg/mL DMSO 10% in B medium 100uL | Chemical 25 mg/mL DMSO 10% in B medium 100uL | Chemical 50 mg/mL DMSO 10% in B medium 100uL |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

10uL

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

Figure 13

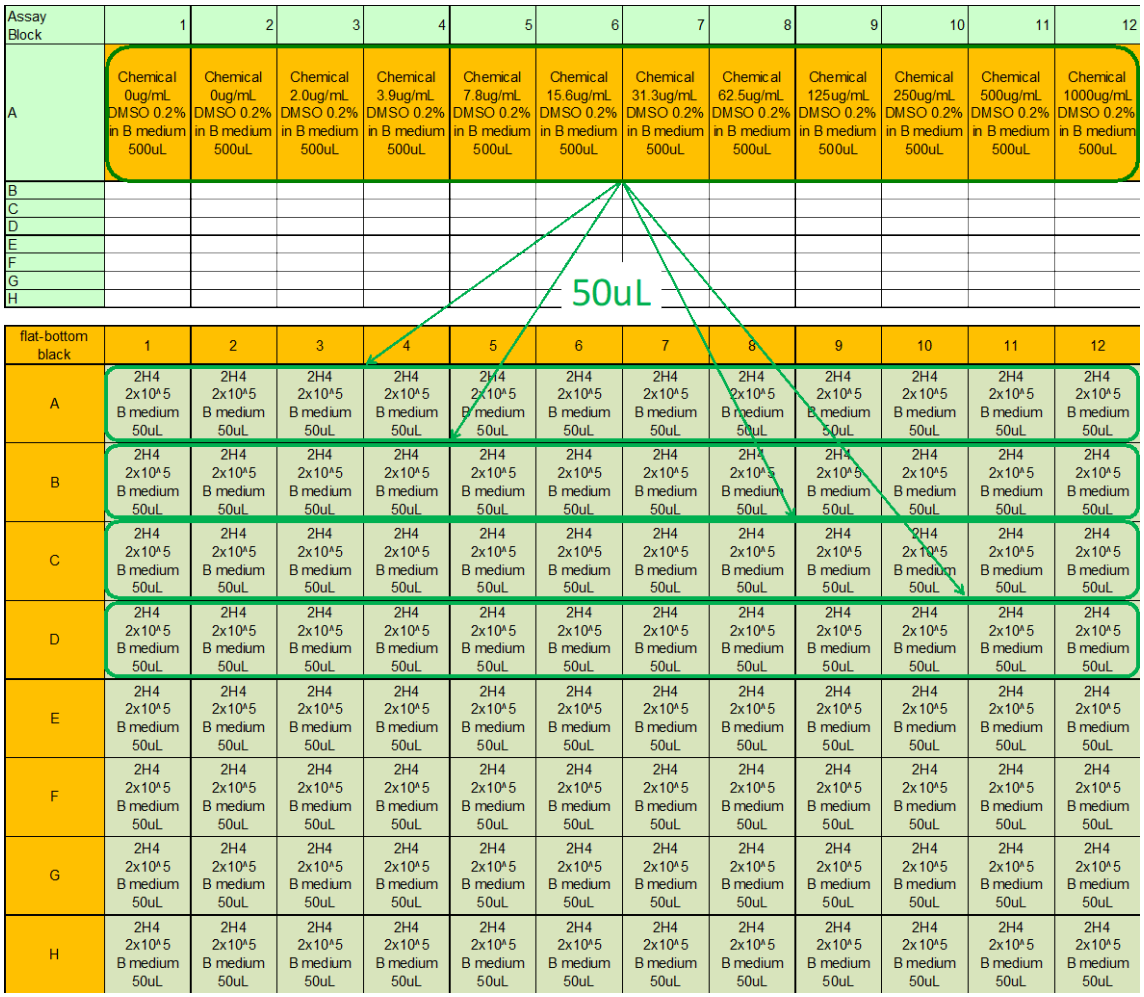

Assay Block
1
2
3
4
5
6
7
8
9
10
11
12
A
Chemical 0ug/mL DMSO 0.2% in B medium 500uL
Chemical 0ug/mL DMSO 0.2% in B medium 500uL
Chemical 2.0ug/mL DMSO 0.2% in B medium 500uL
Chemical 3.9ug/mL DMSO 0.2% in B medium 500uL
Chemical 7.8ug/mL DMSO 0.2% in B medium 500uL
Chemical 15.6ug/mL DMSO 0.2% in B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

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

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

6-1 Material

・2 mM PMA stock

・2 mM Ionomycin stock

・B medium

・Ethanol

6-2 Preparation of 100 μM PMA

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

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

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

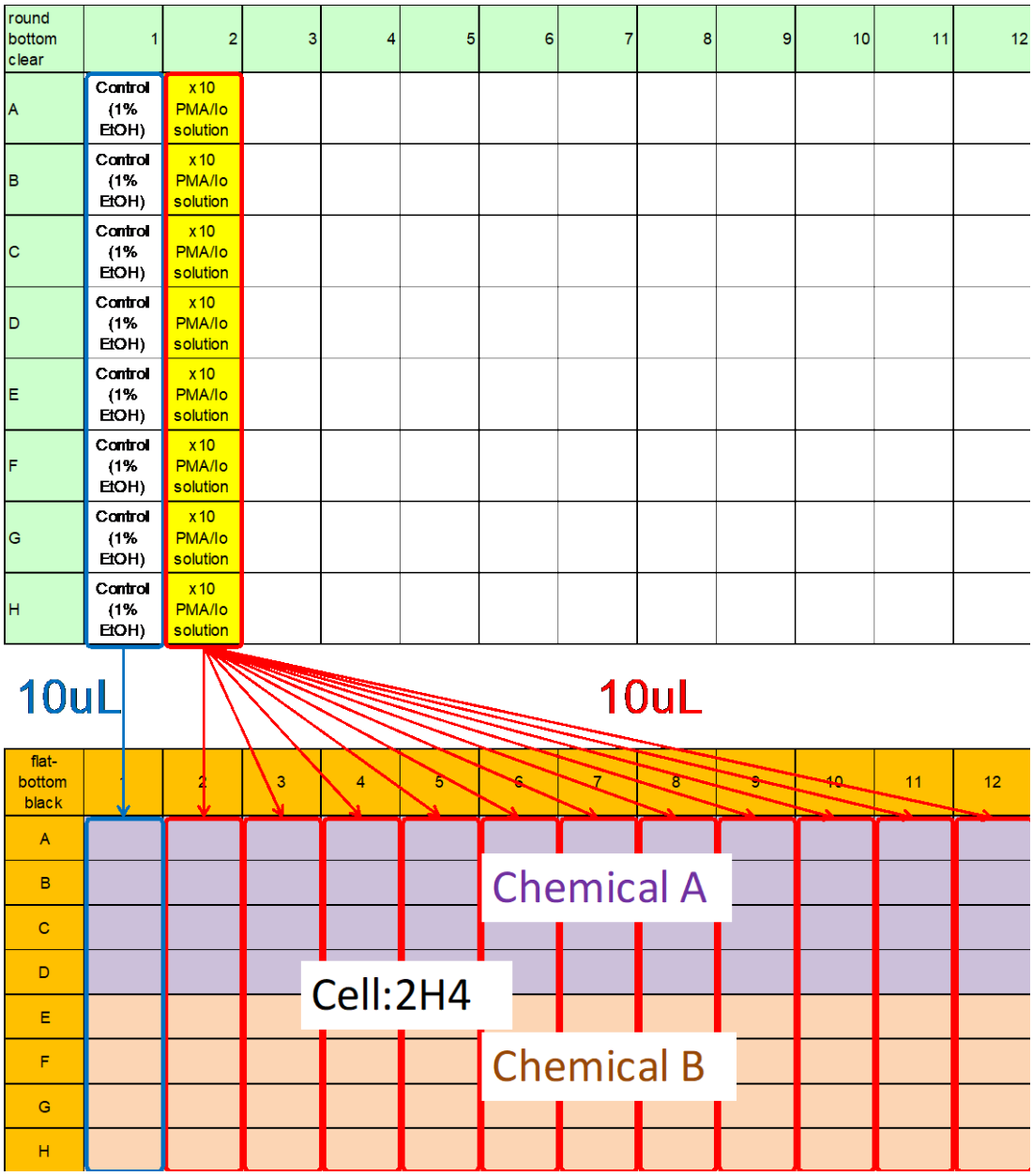
Dilute ethanol, 2 mM ionomycin and 100 μM PMA with the B medium to prepare control or x10 PMA/ionomycin solution. Add the control to well #A1-#H1 of the 96 well clear plate (round bottom), and add x10 PMA/ionomycin solution to wells #A2-#H2 of the 96 well clear plate (round bottom).

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

6-4 Addition of PMA/ionomycin to 2H4

One hour after the addition of chemicals, add 10 µL of control or PMA/ionomycin solution to the cells (#A1-#H1 or #A2-#H12, respectively) using an 8 channel or 12 channel pipetman after pipetting 20 times. Make sure that the apex of the tip is dipped into the medium. Change tips every line you add. Seal the plate, shake the plate with a plateshaker and incubate in a $CO_2$ incubator for 6 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-water soluble | Sigma #D2915-100MG | 2.5 mg/mL | 2.5 mg/mL | 50 μg/mL |
| Distilled water | GIBCO Cat#10977-015 | | | |

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

7-1-2 Preparing cyclosporine A stock

| Reagent | Company | Concentration of the stock solution | Preparing concentration | Final concentration |
|---|---|---|---|---|
| Cyclosporine A | Sigma #C1832-5MG | 100 μg/mL | 100 μg/mL | 100 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 2-4 days before the assay.

Use cells between 1 and 6 weeks after thawing.

Pre-warm the B medium in a 37°C water bath. Count the number of cells and collect the number of cells needed (5.0 x $10^6$ cells are required, but to have some leeway, 7.5 x $10^6$ cells should be prepared), centrifuge the tube at 120-350 x *g*, 5 min. Resuspend in pre-warmed the B medium at a cell density of $4\times10^6$/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 16)

Figure 16

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

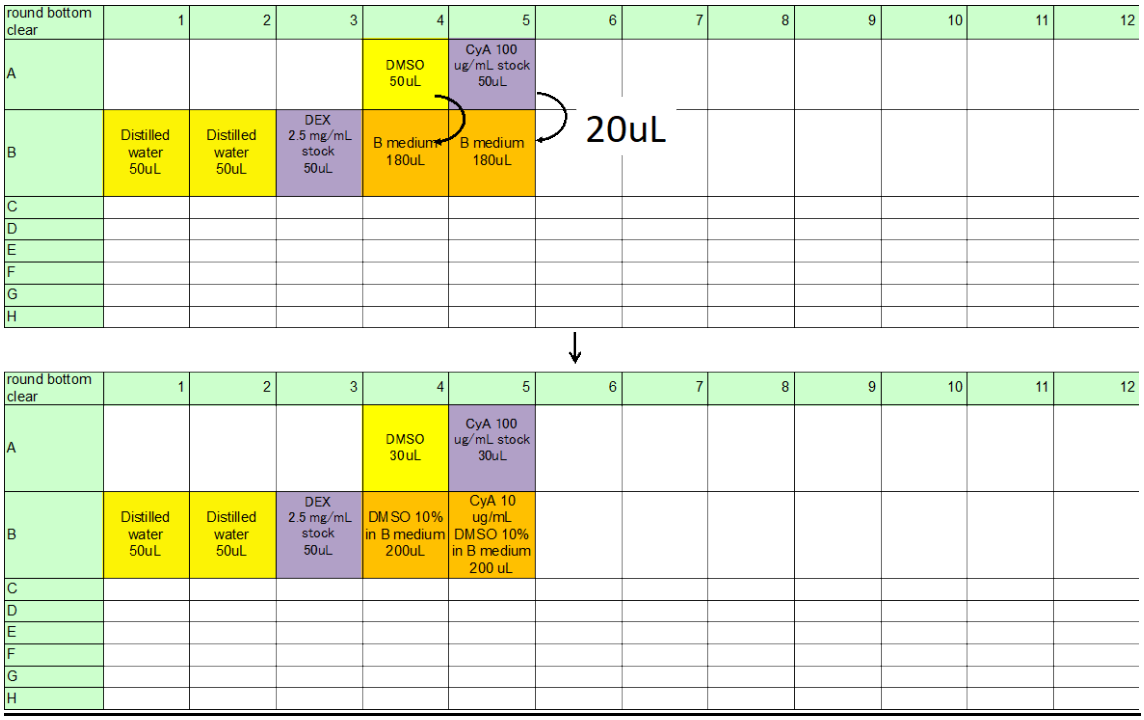
7-3 Arrangement of chemicals and vehicle

Add DMSO 50 µL to #A4, 100 µg/mL cyclosporine A stock 50 µl to #A5, distilled water 50 µl to #B1 and #B2, 2.5 mg/ml dexamethasone stock 50 µl to #B3 and the B medium 180 µl to #B4 and #B5 of the 96 well clear plate (round bottom). (cf. Figure 17)

7-4 Dilution with the B medium

Dilute DMSO in #A4 and cyclosporine A DMSO solution in #A5 by adding 20 µL to the B medium in #B4 and #B5, respectively. (cf. Figure 17)

Figure 17



7-5　　2 step dilution

Add 20 μL of the diluted chemical or vehicle to 480 μL (1-3 lanes) or 980 μL (4-5 lanes) of the B medium prepared in the assay block. And add 50 μL to 2H4 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Manipulate the procedures from 7-4 to 7-5 as quickly as you can, and do not leave a long time at step after 7-4 or Figure 17. 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

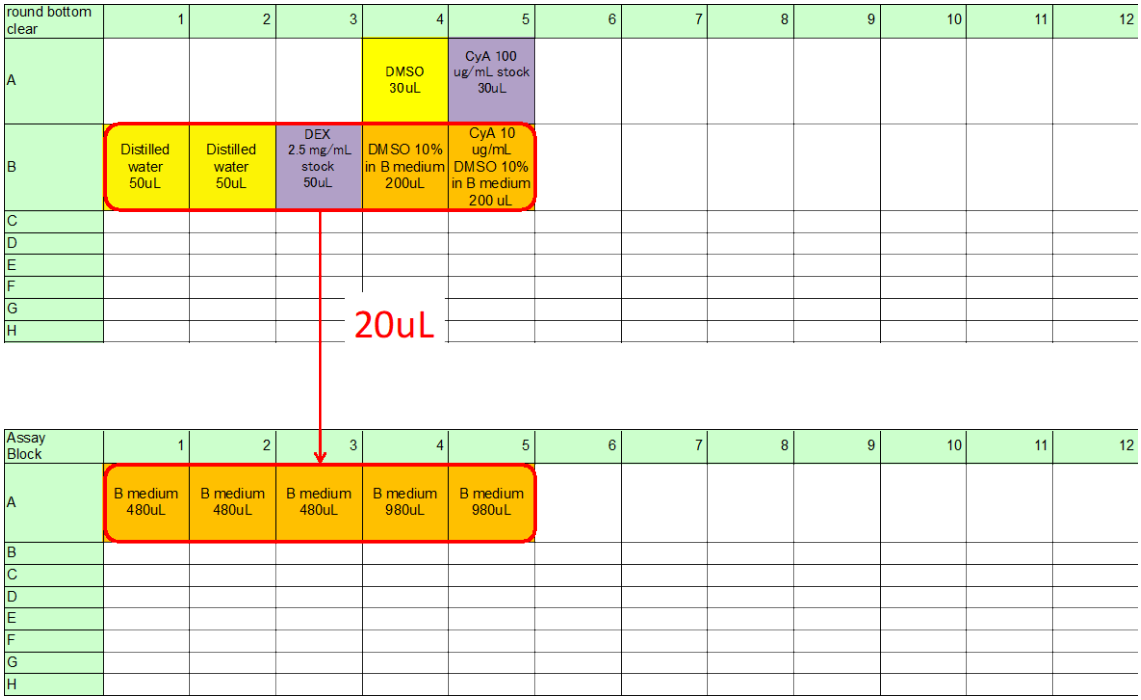


Figure 19

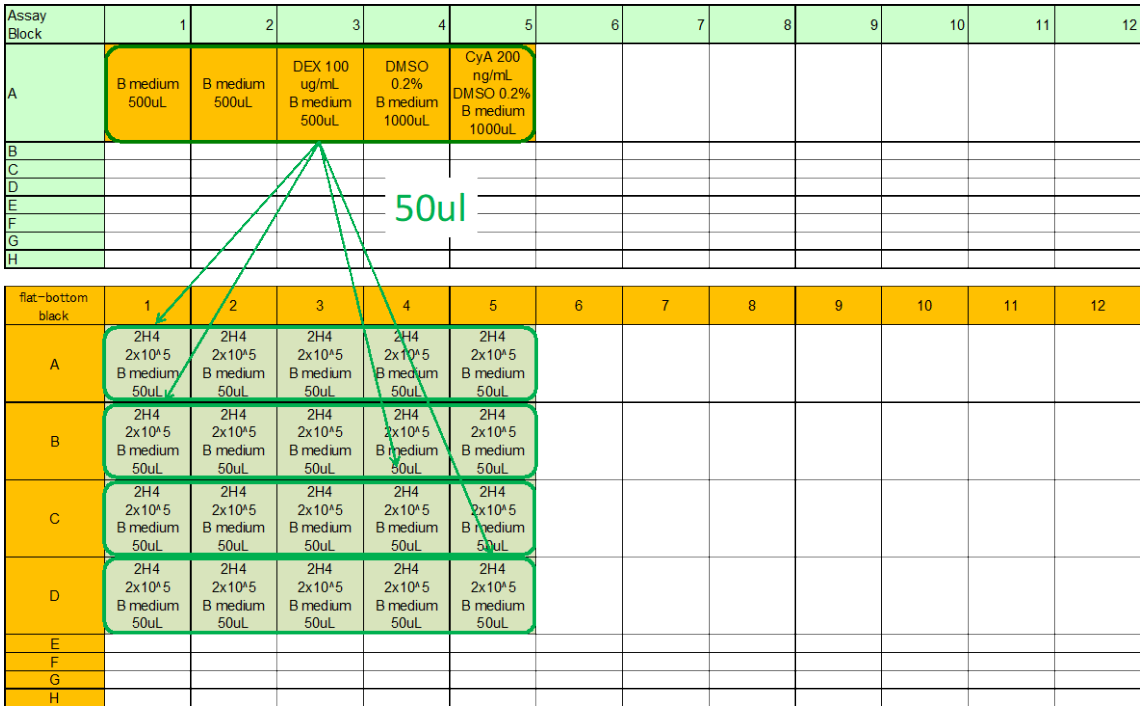


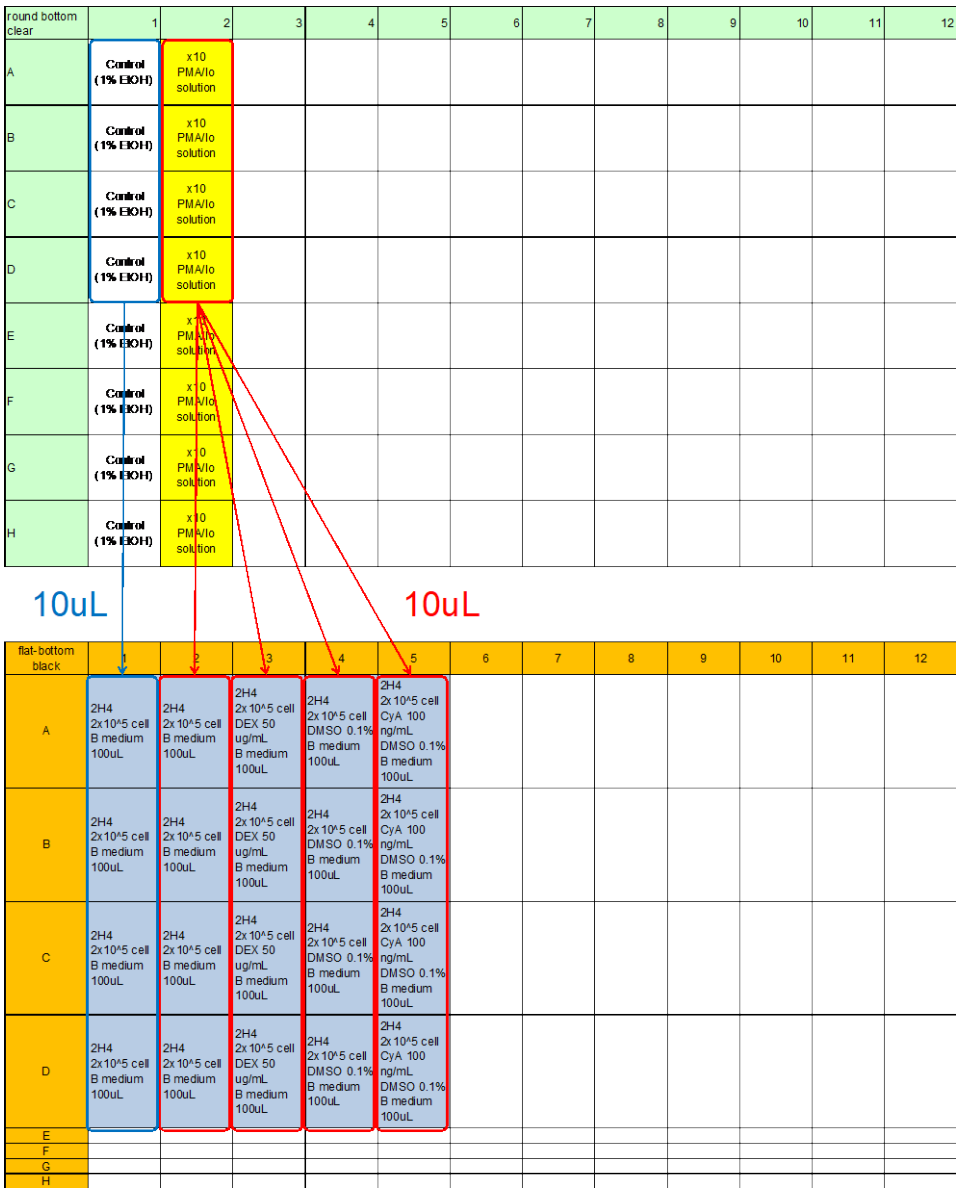

Figure 20 Final constituents of each well of the plate

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

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

One hour after the addition of dexamethasone and cyclosporine A, add 10 µL of control or PMA/ionomycin solution prepared in §6-3 to the cells (#A1-#D1 or #A2-#D5, respectively) using an 8 channel or 12 channel pipetman after pipetting 20 times. Make sure that the apex of the tip is dipped into the medium. Change tips every line you add. Seal the plate, shake the plate with a plateshaker and incubate in a $CO_2$ incubator for 6 hours (37°C, $CO_2$, 5%). (cf. Figure 21)

Figure 21



## 8. Calculation of the transmittance factors

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

### 8-1 Reagents

- Single reference samples:

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

- Assay reagent:

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

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

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

### 8-2 Preparation of luminescence reaction solution

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

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

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

### 8-3 Bioluminescence measurement

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

as shown below.

Figure 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 | | | | | | | | | |
| C | | | | | | | | | | | | |
| D | SLO 100 µL | SLO 100 µL | SLO 100 µL | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | SLR 100 µL | SLR 100 µL | SLR 100 µL | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

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

Figure 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 ($\kappa O_{R56}$)=$\frac{808550+813160+754174}{1202691+1210208+1122295}$=0.672

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

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

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

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

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

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

Figure 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 | $\kappa G_{R56}$ | $\kappa O_{R56}$ | $\kappa R_{R56}$ | |
| 7 | | F2 | $\kappa G_{R60}$ | $\kappa O_{R60}$ | $\kappa R_{R60}$ | |
| 8 | | | | | | |

## 9. Measurement

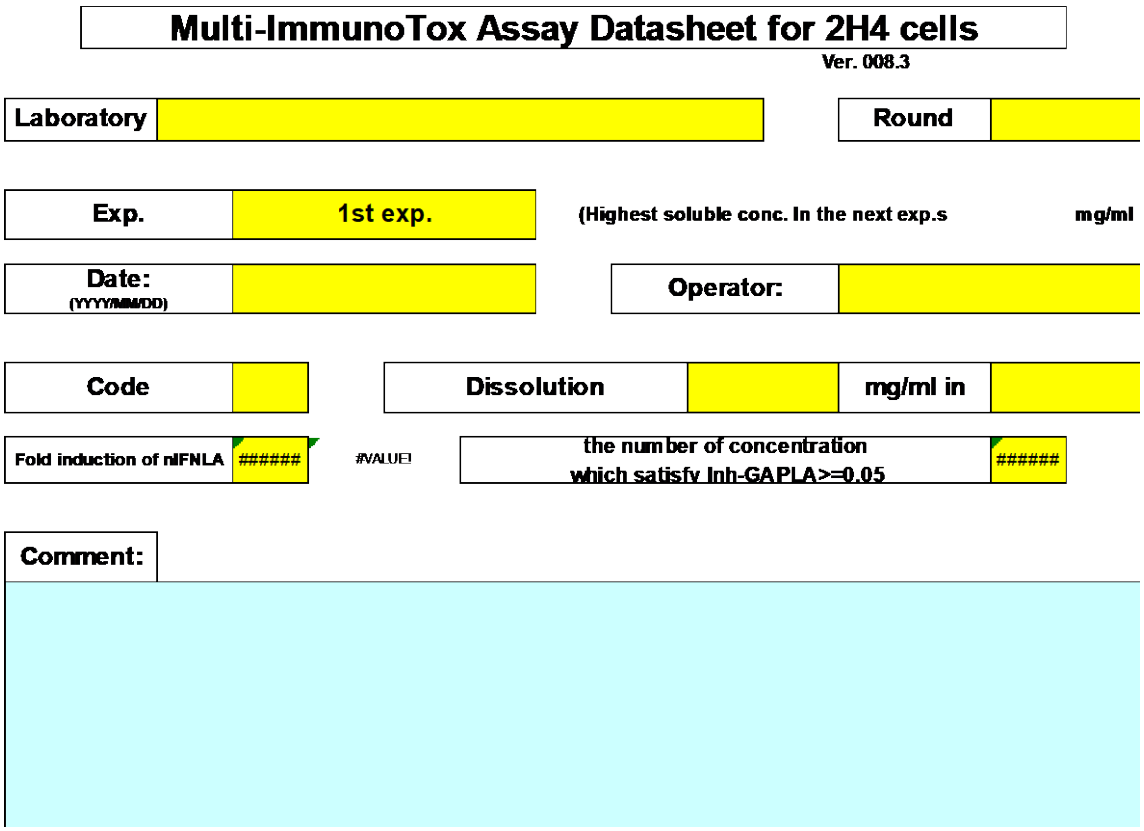
Please refer Appendix 1 for the principle of measurement of luciferase activity.

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

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

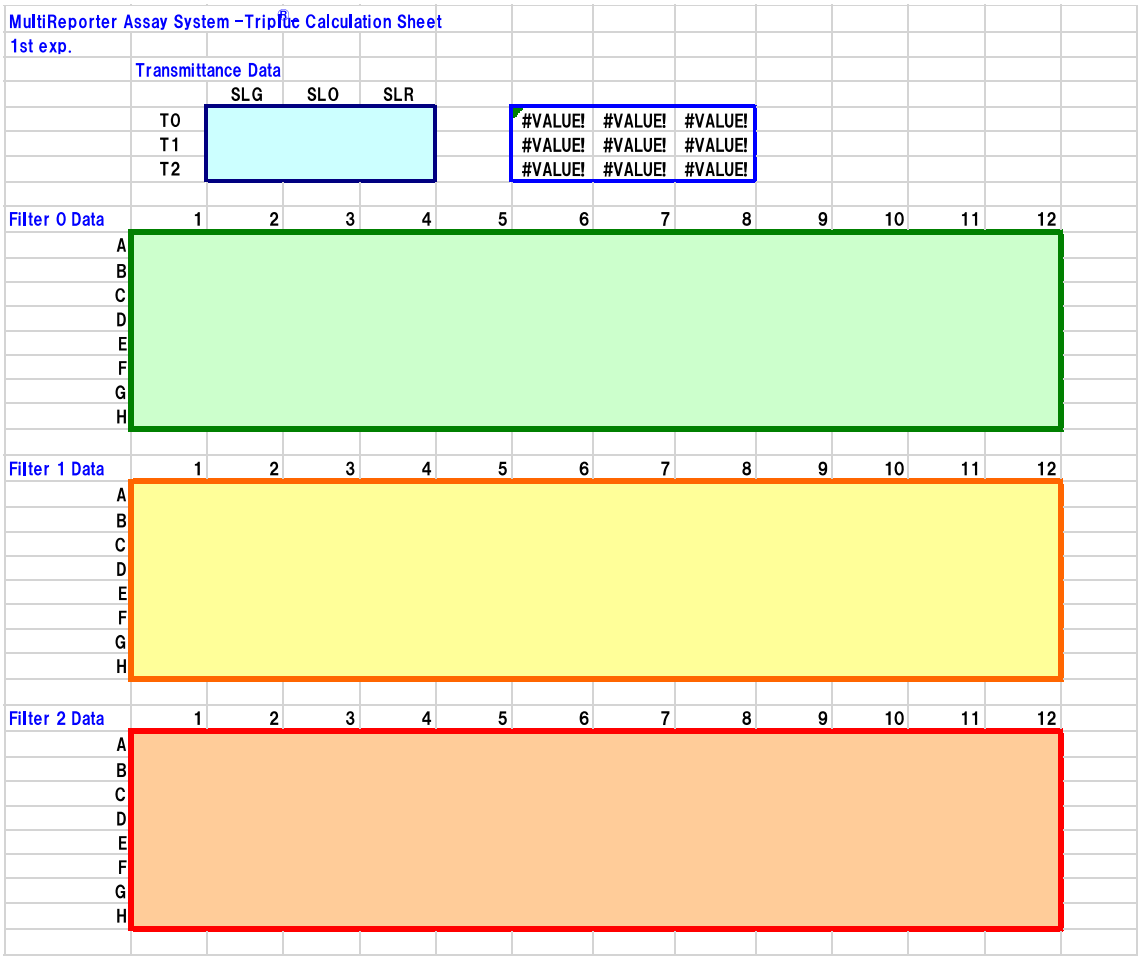
1st. Add the information regarding the name of laboratory, the round of experiments if multiple experimental sets are performed, the experiment number, date, the operator, chemical codes, dissolved in distilled water or DMSO, the prepared concentration, molecular weight of the chemicals and comments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

Figure 25 “Face Sheet” of the data sheet



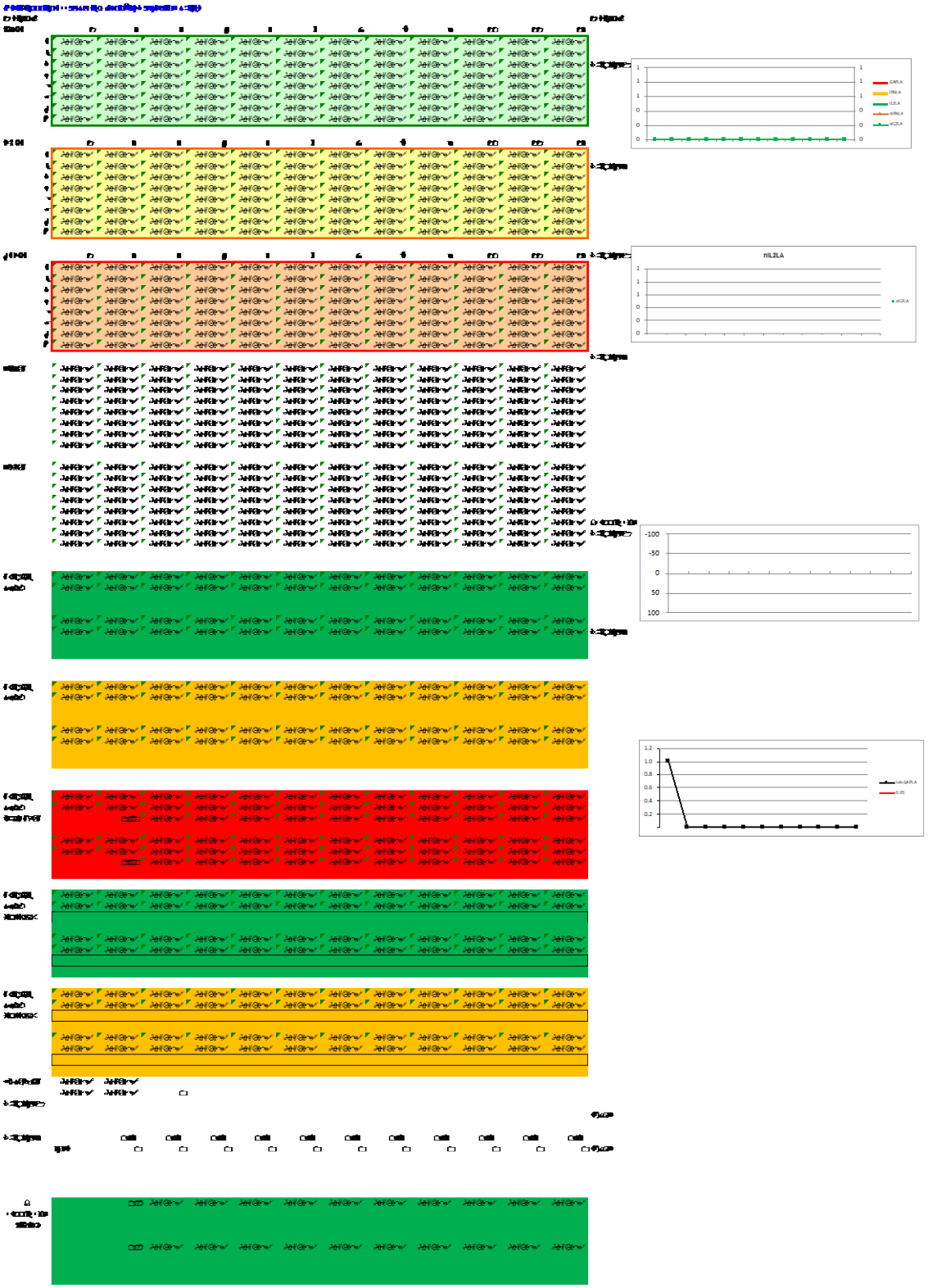
2nd. Copy the results of the F0, F1 and F2 measurements (values are expressed as counts) and paste them into the appropriate area in the “Data Input” sheet of the data sheet shown below. In addition, input the transmittance factors calculated in "§5. Calculation of the transmittance factors" to #C6-#E7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

Figure 26 “Data Input” sheet of the data sheet




Next, the calculated results for the parameters of the Multi-Immuno Tox assay for each concentration, e.g., IL2LA, IFNLA, GAPLA, nIL2LA, nIFNLA, the mean ± 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 27 “Result Format” sheet of the data sheet



## 10. Data analysis

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

- SLG-luciferase activity (IL2LA)：Luciferase activity of stable luciferase green (Under the control of IL-2 promoter)
- SLO-luciferase activity (IFNLA)：Luciferase activity of stable luciferase orange (Under the control of IFN-γ promoter)
- SLR-luciferase activity (GAPLA)：Luciferase activity of stable luciferase red (Under the control of G3PDH promoter)
- Normalized SLG-LA (nIL2LA)：= (IL2LA)/(GAPLA)
- Normalized SLO-LA (nIFNLA)：= (IFNLA)/(GAPLA)
- Inhibition index of GAPLA (Inh-GAPLA)：The cytotoxic effect of chemicals
  = (GAPLA of 2H4 treated with chemicals) / (GAPLA of untreated 2H4)
- % suppression：The effect of chemicals on IL-2 promoter
  = (1-(nIL2LA of 2H4 treated with chemicals) / (nIL2LA of non-treated 2H4)) x 100

## 11. Criteria

11-1 Acceptance criteria

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

- If Fold induction of nIFNLA of PMA/Ionomycin wells without chemicals (=(nIFNLA of 2H4 cells treated with PMA/Ionomycin) / (nIFNLA of non-treated 2H4 cells)) demonstrate less than 3.0, the results obtained from the plate containing the control wells should be rejected.

11-2 Criterion

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

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

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

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

significance. The statistical significance is judged by its 95% confidence interval.
2. The result shows two or more consecutive statistically significant positive (negative) data or one statistically significant positive (negative) data with a trend in which at least 3 consecutive data increase (decrease) in a dose dependent manner. In the latter case, the trend can cross 0, as long as only one data point shows the opposite effect without statistical significance.
3. The results are judged using only data obtained in the concentration at which Inh-GAPLA is $\geq$ 0.05

## 12. Update record

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

Ver. 0011.0E 2018.5.10
Change the criteria

Ver. 0010.0E 2018.1.15 distribution
Change the criteria

Ver. 009.1E 2017.5.8 distribution
Change the criteria

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

Ver. 008.5E 2016.9.14 distribution
Change the criteria

Ver. 008.4E 2016.9.9 distribution
Change the criteria

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

Ver. 008.1E 2016.2.2 distribution
Changes after the VMT meeting

Ver. 008.0E 2016.1.19
Translation to English

Addition of appendix

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

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

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

Ver. 004.0J 2014.11.17　distribution
For the validation study at AIST, FDSC and Tohoku university (chemicals: Sodium Bromate ($NaBrO_3$), 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 | $\kappa G_{R56}$ | The intensity of 560 nm LP (Filter 1) transmitted SLG / the intensity of SLG without filter (all optical) |
| | Filter 2 transmittance factor | $\kappa G_{R60}$ | The intensity of 600 nm LP (Filter 2) transmitted SLG / the intensity of SLG without filter (all optical) |
| SLO | Filter 1 transmittance factor | $\kappa O_{R56}$ | The intensity of 560 nm LP (Filter 1) transmitted SLO / the intensity of SLO without filter (all optical) |
| | Filter 2 transmittance factor | $\kappa O_{R60}$ | The intensity of 600 nm LP (Filter 2) transmitted SLO / the intensity of SLO without filter (all optical) |
| SLR | Filter 1 transmittance factor | $\kappa R_{R56}$ | The intensity of 560 nm LP (Filter 1) transmitted SLR / the intensity of SLR without filter (all optical) |
| | Filter 2 transmittance factor | $\kappa R_{R60}$ | The intensity of 600 nm LP (Filter 2) transmitted SLR / the intensity of SLR without filter (all optical) |

When the intensity of SLG, SLO and SLR in test sample are defined as G, O and R,

respectively, i) the intensity of light without filter (all optical): F0, ii) the intensity of 560 nm LP (Filter 1) transmitted light and iii) the intensity of 600 nm LP (Filter 2) transmitted light are described as below.

$$F0=G+O+R$$

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

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

These formulas can be rephrased as follows

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

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

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

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

## Appendix 2　Validation of reagents and equipment

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

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

5-1-1 Reagents

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

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

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

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

5-1-2 Calibration

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

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

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

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

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

5-1-2-2 Bioluminescence measurement

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

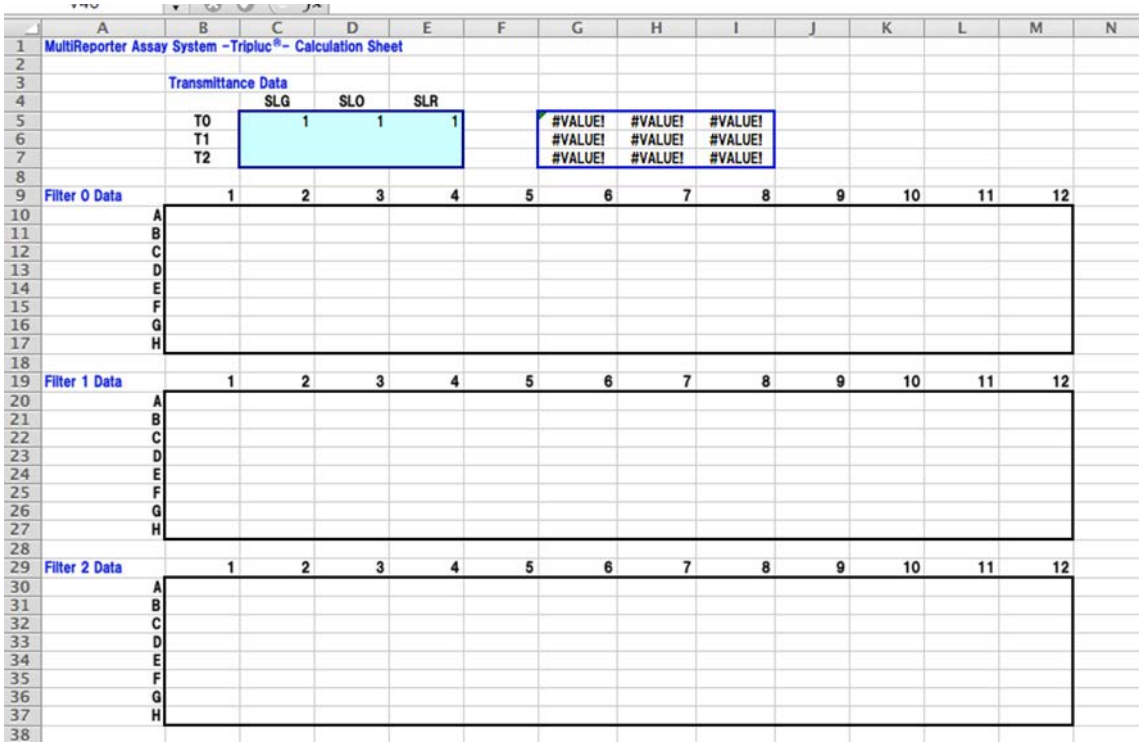
Figure 28

| 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 data 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 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%).