T., Aiba, S., 2014. Evaluation of the Multi-ImmunoTox Assay composed of 3 human cytokine reporter cells by examining immunological effects of drugs. Toxicol In Vitro 28, 759-768.

F. 添付文書

1) Multi-Immuno Tox Assay protocol ver. 008.1E
2) Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 006
3) Multi-ImmunoTox Assay 記録用紙 Ver. 001

G. 研究発表

1. 論文発表

19. Watanabe, M., Noma, H., Kurai, J., Sano, H., Kitano, H., Saito, R., ○Kimura, Y., Aiba, S., Oshimura, M., Shimizu, E. Variation in the Effect of Particulate Matter on Pulmonary Function in Schoolchildren in Western Japan and Its Relation with Interleukin-8. Int J Environ Res Public Health. 2015; 12, 14229-14243.
20. Watanabe, M., Noma, H., Kurai, J., Sano, H., Saito, R., Abe, S., ○Kimura, Y., Aiba, S., Oshimura, M., Yamasaki, A., Shimizu, E. Decreased pulmonary function in school children in Western Japan after exposures to Asian desert dusts and its association with interleukin-8. Biomed Res Int. 2015 ; 583293.
21. ○Kimura, Y., Fujimura, C., Ito, Y., Takahashi, T., Nakajima, Y., Ohmiya, Y. Aiba, S. Optimization of the IL-8 Luc assay as an in vitro test for skin sensitization. Toxicol In Vitro. 2015; 29, 1816-1830.

2. 学会発表

1. ○Kimura, Y., Shimada-Omori, R., Takahashi, T., Tsuchiyama, K., Kusakari, Y., Yamasaki, K., Aiba, S. An interleukin-8 reporter cell line, THP-G8, can evaluate anti-TNF-α neutralizing activity of patients' sera and predict drug effectiveness during anti-TNF-α antibody therapy. 23rd World Congress of Dermatology, (2015, 6) (Vancouver, Canada)
2. ○木村裕：IL-8 Luc assayバリデーション試験. 日本動物実験代替法学会　第28回大会（横浜）2015年12月

**H. 知的財産権の出願・登録状況（予定を含む。）**

1. 特許取得

なし

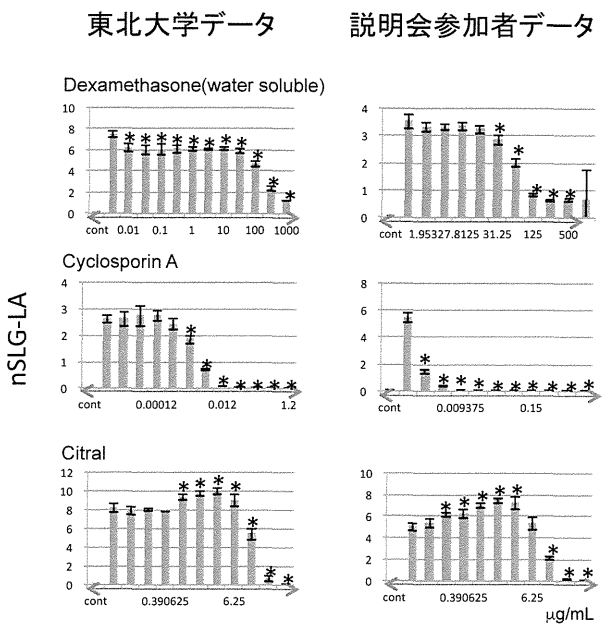
2. 実用新案登録

なし

3.その他

なし

図１　説明会データ


東北大学データ
説明会参加者データ
Dexamethasone(water soluble)
Cyclosporin A
Citral
nSLG-LA
cont 0.01 0.1 1 10 100 1000
cont 1.95327.8125 31.25 125 500
cont 0.00012 0.012 1.2
cont 0.009375 0.15
cont 0.390625 6.25
cont 0.390625 6.25
μg/mL

# Multi-Immuno Tox Assay protocol ver. 008.1E
# Feb. 2, 2016

Department of Dermatology, Tohoku University Graduate School of Medicine
Yutaka Kimura, M.D., Ph.D.
Setsuya Aiba, M.D., Ph.D.

1. Introduction ..... 86
2. Materials ..... 87
2-1 Cells 87
2-2 Reagents and equipment 87
2-2-1 For maintenance of the #2H4 cells ..... 87
2-2-2 For chemical exposure, stimulation and solvents ..... 87
2-2-3 For measurement of the luciferase activity ..... 87
2-2-4 Expendable supplies ..... 87
2-2-5 Equipment for measurement of luciferase activity ..... 87
2-2-6 Others ..... 88
2-3 Culture medium 89
2-3-1 A medium: for maintenance of #2H4 cells (500 mL, stored at 2-8°C) ... 89
2-3-2 B medium: for luciferase assay (30 mL, stored at 2-8°C) ..... 89
2-3-3 C medium: for thawing #2H4 cells (30 mL, stored at 2-8°C) ..... 89
2-4 Preparation of the stimulant of #2H4 90
2-4-1 Phorbol 12-myristate 13-acetate (PMA) ..... 90
2-4-2 Ionomycin ..... 90
3. Cell culture ..... 91
3-1 Thawing of #2H4 cells 91
3-2 Maintenance of #2H4 cells 91
4. Preparation of cells for assay ..... 92
5. Preparation of chemicals and cell treatment with chemicals ..... 93
5-1 Dissolution by vehicle (cf. Figure 3) 93
5-2 When the chemical is prepared as 25, 50 or 100 mg/mL in distilled water 95
5-2-1 Arrangement of chemicals and vehicle ..... 95
5-2-2 Serial dilution ..... 95
5-2-3 2 step dilution ..... 96
5-3 When the chemical is prepared as a 500 mg/mL DMSO solution 98
5-3-1 Arrangement of chemicals and vehicle ..... 98
5-3-2 Serial dilution ..... 98
5-3-3 Dilution of DMSO solution with the B medium ..... 99
5-3-4 2 step dilution ..... 100
6. Preparation of the stimulant (PMA/ionomycin) and addition to #2H4 ..... 102
6-1 Material 102

6-2 Preparation of 100 μM PMA 102
6-3 Preparation of control and x10 PMA/ionomycin solution 102
6-4 Addition of PMA/ionomycin to #2H4 103
7. Control ....104
7-1 Preparing control chemical (dexamethasone, cyclosporine A) 104
7-1-1 Preparing dexamethasone stock ....104
7-1-2 Preparing cyclosporine A stock ....104
7-2 Preparation of cells for assay 105
7-3 Arrangement of chemicals and vehicle 106
7-4 Dilution with the B medium 106
7-5 2 step dilution 107
7-6 Addition of PMA/ionomycin to #2H4 109
8. Calculation of the transmittance factors ....110
8-1 Reagents 110
8-2 Preparation of luminescence reaction solution 110
8-3 Bioluminescence measurement 110
9. Measurement ....114
10. Data analysis ....117
11. Criteria ....117
11. Update record ....119
Appendix 1 Principal of measurement of luciferase activity ....120
Appendix 2 Validation of reagents and equipment ....122

# 1. Introduction

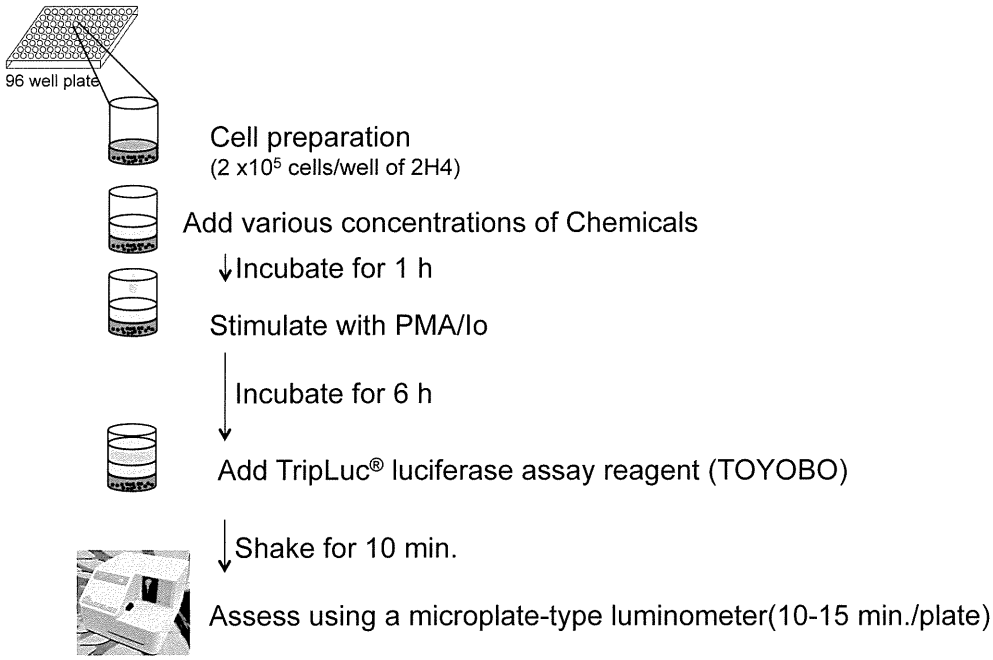
This protocol describes how to maintain the cells, how to prepare the test chemicals, and how to measure the luciferase activity of #2H4 cells transfected with 3 luciferase genes, stable luciferase green (SLG), stable luciferase orange (SLO) and stable luciferase red (SLR), under the control of IL-2, IFNγ and G3PDH promoters, respectively, for the Multi-Immuno Tox Assay.
(Kimura Y. et al. Evaluation of the Multi-ImmunoTox 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 | $A/2^9$ μg/ml | $A/2^8$ μg/ml | $A/2^7$ μg/ml | $A/2^6$ μg/ml | $A/2^5$ μg/ml | $A/2^4$ μg/ml | $A/2^3$ μg/ml | $A/2^2$ μg/ml | $A/2^1$ μg/ml | A μg/ml |
| B | | | | | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | | Chemical A (common ratio of 2、10 concentrations、n=4) | | | | | | | | | |
| E | cont (distilled water or DMSO) | PMA/Io only | $B/2^9$ μg/ml | $B/2^8$ μg/ml | $B/2^7$ μg/ml | $B/2^6$ μg/ml | $B/2^5$ μg/ml | $B/2^4$ μg/ml | $B/2^3$ μg/ml | $B/2^2$ μg/ml | $B/2^1$ μg/ml | B μg/ml |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | Chemical B (common ratio of 2、10 concentrations、n=4) | | | | | | | | | |

PMA/Io or LPS



# 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 μ clear black plate (flat-bottom, for measurement of the luciferase activity, e.g. Greiner Bio-one Cat#655090)
- 96 well clear plate (round-bottom, for preparation of chemicals and stimulants)
- 96 well assay block, 2 mL (e.g., Costar Cat#3960)
- Reservoir
- Pipette

### 2-2-5 Equipment for measurement of luciferase activity

- Measuring device: a microplate-type luminometer with a multi-color detection system that can accept two optical filter
  e.g. Phelios AB-2350 (ATTO), ARVO (PerkinElmer), Tristar LB941 (Berthold)
- Optical filter: 560 nm long-pass filter and 600 nm long-pass filter
- Measuring time: set at 1～5 sec/well measuring time

### 2-2-6 Others

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

## 2-3 Culture medium

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

| Reagent | Company | Concentration | Final concentration in medium | Required amount |
|---|---|---|---|---|
| RPMI-1640 | GIBCO #11875-093 | - | - | 440 mL |
| FBS | Biological Industries<br>Cat#04-001-1E<br>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<br>Cat#04-001-1E<br>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<br>Cat#04-001-1E<br>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 | 1 mM | 25 nM |
| DMSO | Sigma #D5789 | | |

Dissolve 1 mg PMA using DMSO 1338.5 μL, dispend at 10 μL/tube and store at freezer at -30°C. Use these stocks within 6 month after dissolution.

### 2-4-2 Ionomycin

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

Dissolve 1mg Ionomycin using ethanol 1621 μL, dispend at 30 μL/tube and store at freezer at -30°C. Use these stocks within 6 month 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 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 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 chemical are required, but to have some leeway, 3.0 x $10^7$ cells for two chemical should be prepared), centrifuge the tube at 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 |

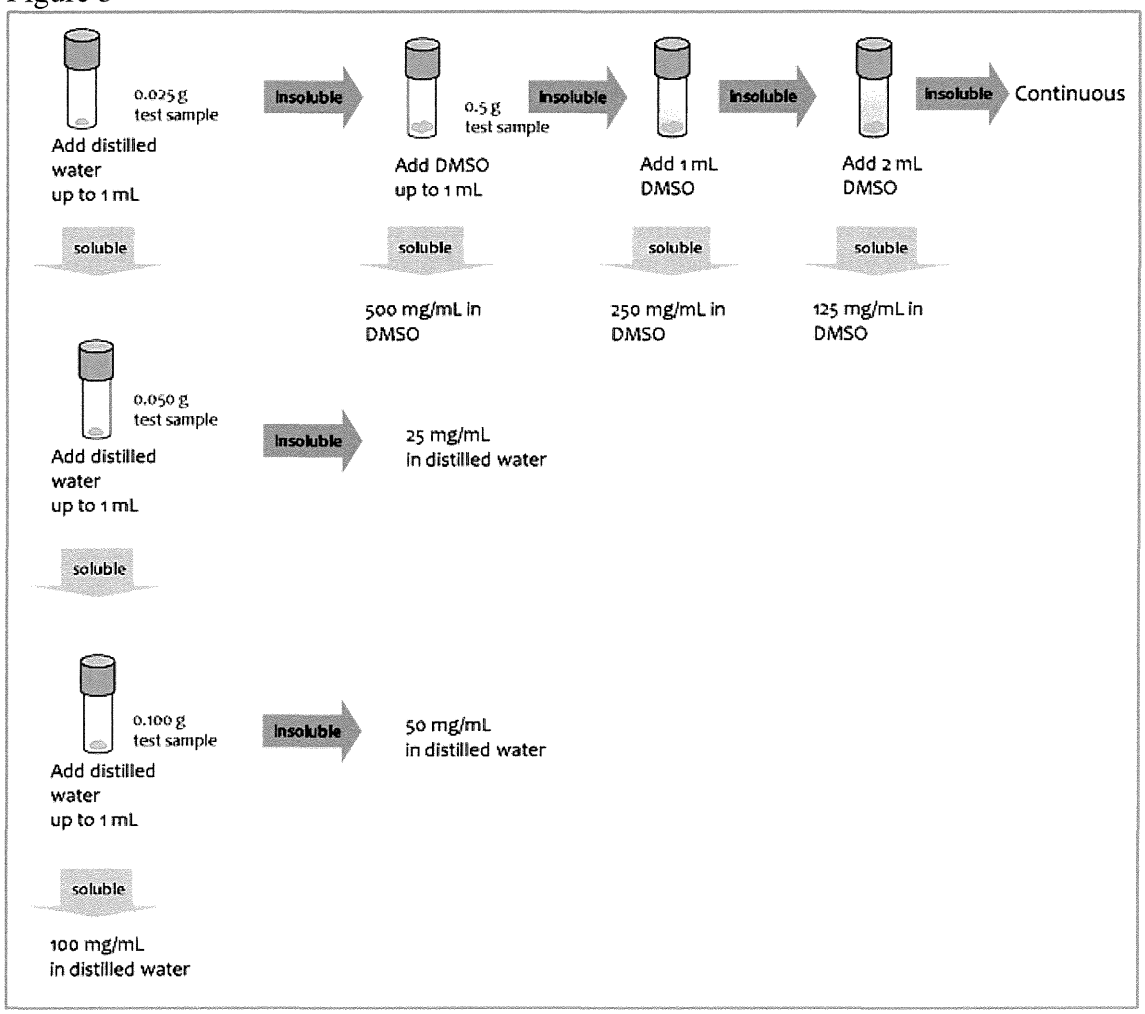
# 5. Preparation of chemicals and cell treatment with chemicals

5-1 Dissolution by vehicle (cf. Figure 3)

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

If the chemical is not soluble in water, the chemical should be dissolved in DMSO at 500 mg/mL. Namely, weigh 0.5 g of the test chemical in volumetric flask and add DMSO up to 1 mL.
If the chemical is not soluble at 500 mg/mL, the highest soluble concentration should be determined by diluting the solution from 500 mg/mL at a common ratio of two (250 mg/mL → 125 mg/mL → continued if needed) with DMSO. Sonication and vortex may be used if needed, and attempt to dissolve the chemical for at least 5 minutes. The chemical should be used within 4 hours after being dissolved in distilled water or DMSO.

Figure 3


0.025 g
test sample
Add distilled
water
up to 1 mL
Insoluble
0.5 g
test sample
Add DMSO
up to 1 mL
Insoluble
Add 1 mL
DMSO
Insoluble
Add 2 mL
DMSO
Insoluble
Continuous
soluble
soluble
soluble
soluble
500 mg/mL in
DMSO
250 mg/mL in
DMSO
125 mg/mL in
DMSO
0.050 g
test sample
Add distilled
water
up to 1 mL
Insoluble
25 mg/mL
in distilled water
soluble
0.100 g
test sample
Add distilled
water
up to 1 mL
Insoluble
50 mg/mL
in distilled water
soluble
100 mg/mL
in distilled water

5-2　When the chemical is prepared as 25, 50 or 100 mg/mL in distilled water

If the chemical is prepared at 25 or 50 mg/mL in distilled water, use the prepared concentration instead of the 100 mg/mL distilled water solution.

5-2-1　Arrangement of chemicals and vehicle

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

5-2-2　Serial dilution

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

Figure 4

| round bottom clear | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Distilled water 50uL | Distilled water 50uL | Distilled water 50uL | Distilled water 50uL | Distilled water 50uL | Distilled water 50uL | Distilled water 50uL | Distilled water 50uL | Distilled water 50uL | Distilled water 50uL | Distilled water 50uL | Chemical 100 mg/mL in 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. Shake the plate with a plateshaker, and incubate in a $CO_2$ incubator for 1 hour (37°C, 5%)(cf. Figure 5-7).

Figure 5

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

20uL

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

Figure 6

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

50uL

| 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 |

Figure 7 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 500 mg/mL DMSO solution

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

5-3-1 Arrangement of chemicals and vehicle

Add 100 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 8)

Figure 8

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

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

↓

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

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

Dilute 10 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 9)

Figure 9

| 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 | | | | | | | | | | | | |

10uL

↓

| 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 | | | | | | | | | | | | |

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 10. Shake the plate with a plateshaker and incubate in a $CO_2$ incubator for 1 hour (37°C, 5%) (cf. Figure 10-12).

Figure 10

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

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