IL-8 Luc assay protocol

This protocol describes how to maintain the cells, how to prepare test chemicals, and how to measure the luciferase activity of a THP-1-derived IL-8 reporter cell line, THP-G8, for the IL-8 Luc assay.

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1. Materials

1-1 Cells

The human macrophage-like cell line THP-1 was obtained from the American Type Culture Collection (Manassas, VA, USA). A THP-1-derived IL-8 reporter cell line, THP-G8, that harbors the SLO and SLR luciferase genes under the control of the IL-8 and GAPDH promoters, respectively, was established by the Dept. of Dermatology, Tohoku University School of Medicine.

(Takahashi T. et al. An *in vitro* test to screen skin sensitizers using a stable THP-1-derived IL-8 reporter cell line, THP-G8. *Toxicol Sci,* 124(2), 359-369, 2011)

(International patent publication No. WO2012/002507A1)

1-2 Reagents and equipment

For maintenance of the THP-G8 cells

- RPMI-1640 (GIBCO Cat#11875-093, 500 mL)
- FBS (Biological Industries Cat#04-001-1E Lot:715004)
- 100 X concentrated antibiotic and antimycotic (10,000 U/mL of penicillin G, 10,000 μg/mL of streptomycin and 25 μg/mL of amphotericin B in 0.85 % saline) (e.g., GIBCO Cat#15240-062)
- G418 (CAS:108321-42-2, Nacalai Tesque Cat#16513-84)
- Puromycin (CAS:58-58-2, InvivoGen Cat#ant-pr-1)

For chemical exposure, positive control, negative control, solvents

- 4-Nitrobenzyl bromide (CAS:100-11-8, Aldrich Cat#N13054)
- Lactic acid (CAS:50-21-5, Sigma Cat#L6661)
- X-VIVO™ 15 (Lonza, 04-418Q): Chemically defined, serum-free hematopoietic cell medium.

For measurement of the luciferase activity

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

Expendable supplies

- T-75 flask tissue culture treated (e.g., Corning Cat#353136)
- 96-well flat-bottom black plate (for measurement of the luciferase activity, e.g., Greiner Bio-one Cat#655090, Nunc Cat#165305)
- 96 well assay block, 2 mL (e.g., Costar Cat#3960)
- Reservoir
- Pipette

Equipment for measurement of luciferase activity

- Measuring device: a microplate-type luminometer with a multi-color detection system that can accept an optical filter
  e.g.: Phelios AB-2350 (ATTO), ARVO (PerkinElmer), Tristar LB941 (Berthold)
- Optical filter: 600 nm long-pass filter, 600～700 nm band-pass filter
- Measuring time: set at 1～5 sec/well measuring time

Others

- Pipetman
- 8 channel or 12 channel pipetman (optimized for 20~100 μL and 0.5~10 μL)
- Plate shaker (for 96 well plate)
- CO_2 incubator (37°C, 5% CO_2)
- Water bath
- Cell counter: hemocytometer, trypan blue

1-3 Culture medium

1-3-1 A medium: for maintenance of THP-G8 cells (500 mL, stored at 2-8°C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	445 mL
FBS	Biological Industries Cat#04-001-1E Lot:715004	-	10 %	50 mL
Antibiotic and antimycotic	e.g., GIBCO #15240-062	100×	1×	5 mL
Puromycin (CAS:58-58-2)	InvivoGen # ant-pr-1	10 mg/mL	0.15 μg/mL	7.5 μL
G418 (CAS:108321-42-2)	Nacalai tesque #16513-84	50 mg/mL	300 μg/mL	3 mL

1-3-2 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

1-3-3 C medium: for thawing THP-G8 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

1-4 Thawing of THP-G8 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.

1-5 Maintenance of THP-G8 cells

- Pre-warm A medium in a T-75 Flask at 37°C in a 5% CO_2 incubator. The culture medium should be changed to A medium 3 or 4 days after thawing. At that time, count the number of cells, centrifuge the tube at 350 x *g* for 5 min, discard the supernatant, and resuspend in pre-warmed A medium in a T-75 Flask. Cells are passaged at 2～$5x10^5$/mL, depending on the condition of the cells, 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.

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 B medium in a 37°C water bath. Count the number of cells and collect the number of cells needed (2.5 x 10^6 cells for one chemical are required, but to have some leeway, 3.75 x 10^6 cells for one chemical should be prepared), centrifuge the tube at 350 x *g*, 5 min. Resuspend in pre-warmed B medium at a cell density of 1×10^6/mL. Transfer the cell suspension to a reservoir, and add 50 μL of cell suspension to each well of a 96-well flat-bottom black plate using an 8 channel or 12 channel pipetman. (cf. Figure 1, row C-F)

Figure 1.

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

3. Preparation of chemicals and cell treatment with chemicals

Prepare chemicals and add them to wells after preparing the cells.

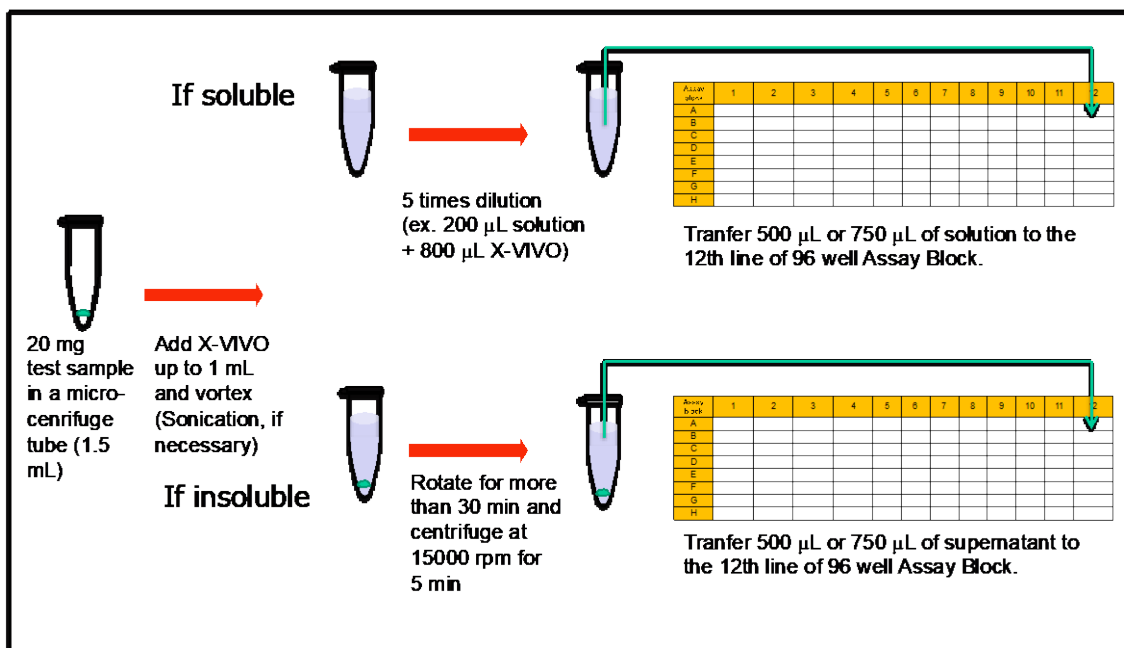
3-1 Dissolution in X-VIVO™ 15 (cf. Figure 2)

Prewarm X-VIVO™ 15 (5 mL) for each chemical at room temperature. Weigh 20 mg of a test chemical in a microfuge tube (1.5 mL) and add X-VIVO™ 15 up to 1 mL. Vortex the microcentrifuge tube vigorously. If necessary, sonicate until the chemical is completely dispersed.

If the chemical is soluble at 20 mg/mL, dilute the solution 5 times with X-VIVO™ 15, and transfer 500 μL (1st experiment) or 750 μL (2nd, 3rd and 4th experiments, dilute with X-VIVO™ 15 if necessary as described on the next page) of the diluted solution to the 12th line of a 96 well assay block.

If the chemical is not soluble at 20 mg/mL, shake the microfuge tube on a rotor (e.g., WKN-2210, WAKEN B TECH Co. Ltd, Kyoto, Japan) at a maximum speed of 8 rpm for more than 30 min, until just before centrifugation. After centrifugation at 15,000 rpm (≈20,000 x g) for 5 min, transfer 500 μL (1st experiment) or 750 μL (2nd, 3rd and 4th experiments, dilute with X-VIVO™ 15 if necessary as described on the next page) of the diluted solution to the 12th line of a 96 well assay block. If undissolved chemical either precipitates or floats, remove the solution carefully so that no undissolved chemical is present in the solution.

Figure 2.



In the first experiment (1st experiment), conduct 11 serial dilutions at a common ratio of 2 from the highest concentration using X-VIVO™ 15.

In the second, third and fourth experiments (2nd, 3rd and 4th experiments), determine the minimum concentration at which Inh-GAPLA (mentioned later in **7-1**) is lower than 0.05 in the 1st experiment (CV05). Use the concentration one step (2-times) higher than this determined concentration as the highest concentration of the chemical to test, and conduct 11 serial dilutions at a common ratio of 1.5 from this highest concentration. If Inh-GAPLA does not decrease to less than 0.05 or is less than 0.05 at the highest concentration in the 1st experiment, conduct 11 serial dilutions at a common ratio of 1.5 from the highest concentration in the 1st experiment.

For example, in Figure 3 below, the minimum concentration at which Inh-GAPLA is less than 0.05 is 1/128. The highest concentration of the chemical to test is the concentration one step (2-times) higher than 1/128, which is 1/64.

In Figure 4 below, Inh-GAPLA did not decrease to less than 0.05. In such cases, the highest concentration of the chemical to test is the highest concentration in the 1st experiment, namely 1/2.

Figure 3.

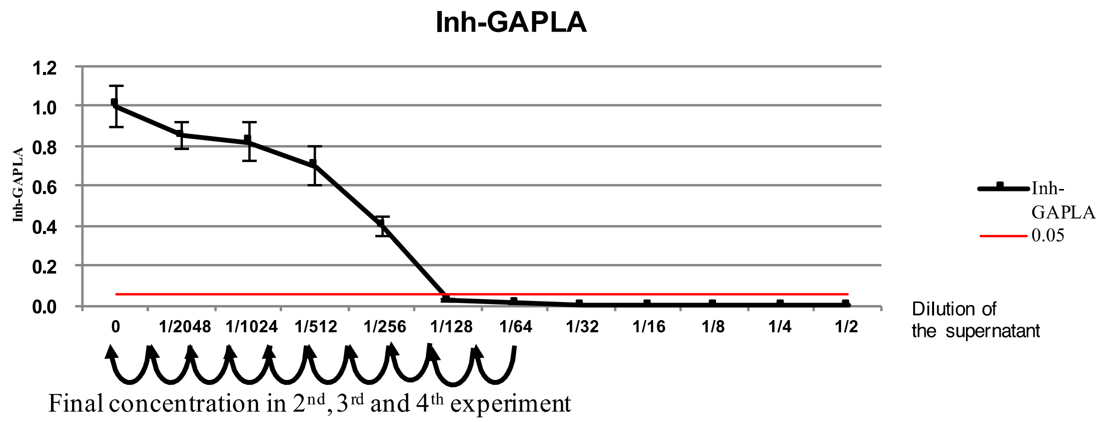
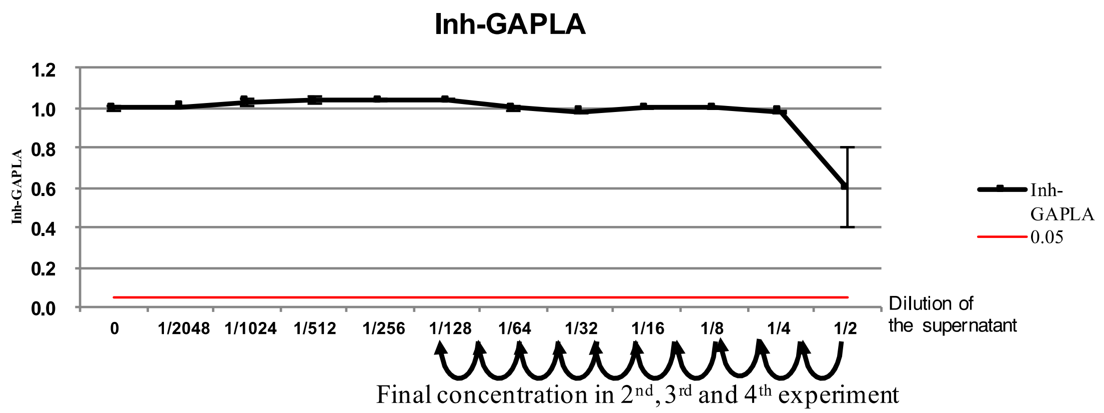


Figure 4



3-2 When the chemical is soluble at 20 mg/mL in X-VIVO™ 15

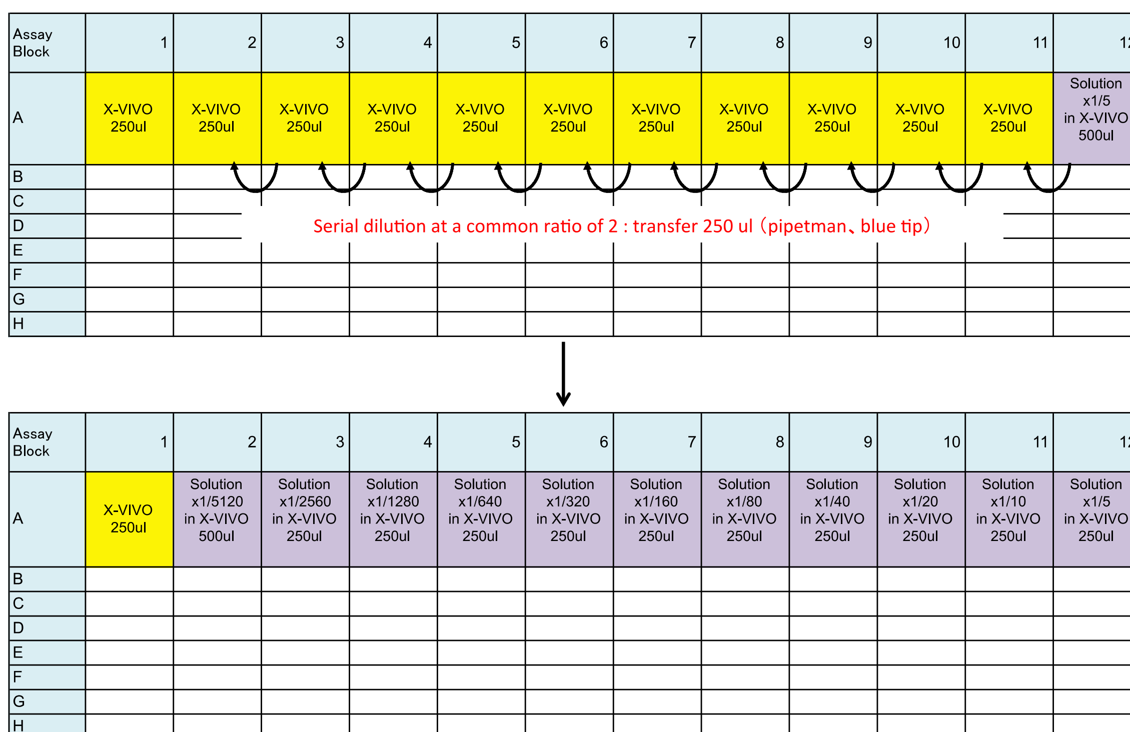
3-2-1 Arrangement of chemicals and vehicle (1st experiment)

Add 500 μL of the 5-fold diluted solution to the #A12 well and 250 μL of X-VIVO™ 15 to wells #A1-#A11 of a 96 well assay block, 2 mL.

3-2-2 Serial dilution (1st experiment)

Conduct 11 serial dilutions at a common ratio of 2 as indicated in Figure 5 from well #A11 to well #A2. (Transfer 250 μL to the next (left) well.) Make sure not to use blue pipette tips previously used for dissolving and transferring chemicals in 3.1.

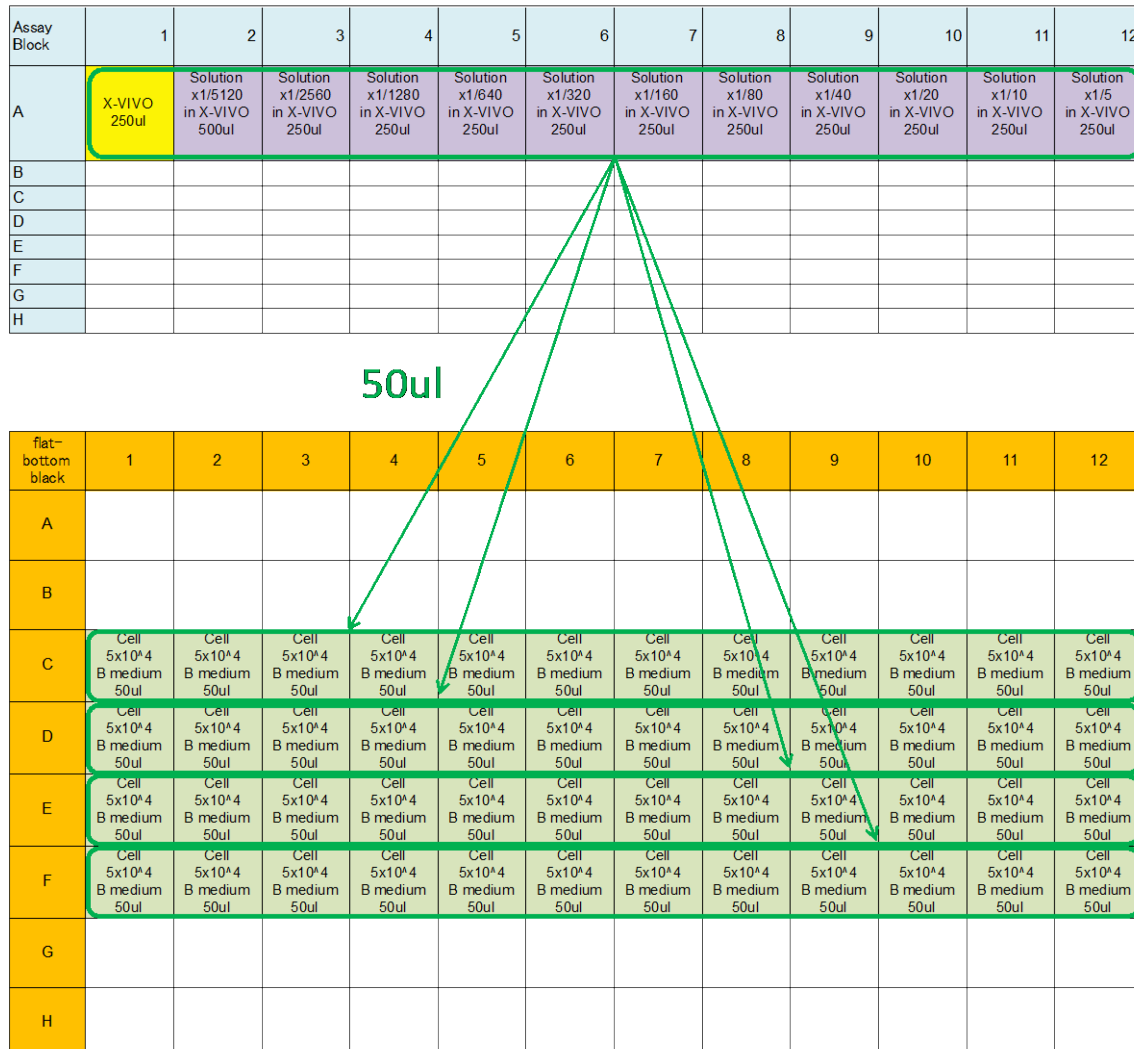
Figure 5.



3-2-3 Addition to the cells (1st experiment)

Add 50 µL to THP-G8 in a 96-well flat-bottom black plate using an 8 channel or 12 channel pipetman. Shake the plate on a plateshaker, and incubate in a CO_2 incubator for 16 hours (37°C, 5% CO_2).

Figure 6



3-2-4. Final constituents of each well of the plate. (1st experiment)

Figure 7

flat- bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	Cell 5x10^4 100ul medium	Cell 5x10^4 Solution x1/10240 100ul medium	Cell 5x10^4 Solution x1/5120 100ul medium	Cell 5x10^4 Solution x1/2560 100ul medium	Cell 5x10^4 Solution x1/1280 100ul medium	Cell 5x10^4 Solution x1/640 100ul medium	Cell 5x10^4 Solution x1/320 100ul medium	Cell 5x10^4 Solution x1/160 100ul medium	Cell 5x10^4 Solution x1/80 100ul medium	Cell 5x10^4 Solution x1/40 100ul medium	Cell 5x10^4 Solution x1/20 100ul medium	Cell 5x10^4 Solution x1/10 100ul medium
D	Cell 5x10^4 100ul medium	Cell 5x10^4 Solution x1/10240 100ul medium	Cell 5x10^4 Solution x1/5120 100ul medium	Cell 5x10^4 Solution x1/2560 100ul medium	Cell 5x10^4 Solution x1/1280 100ul medium	Cell 5x10^4 Solution x1/640 100ul medium	Cell 5x10^4 Solution x1/320 100ul medium	Cell 5x10^4 Solution x1/160 100ul medium	Cell 5x10^4 Solution x1/80 100ul medium	Cell 5x10^4 Solution x1/40 100ul medium	Cell 5x10^4 Solution x1/20 100ul medium	Cell 5x10^4 Solution x1/10 100ul medium
E	Cell 5x10^4 100ul medium	Cell 5x10^4 Solution x1/10240 100ul medium	Cell 5x10^4 Solution x1/5120 100ul medium	Cell 5x10^4 Solution x1/2560 100ul medium	Cell 5x10^4 Solution x1/1280 100ul medium	Cell 5x10^4 Solution x1/640 100ul medium	Cell 5x10^4 Solution x1/320 100ul medium	Cell 5x10^4 Solution x1/160 100ul medium	Cell 5x10^4 Solution x1/80 100ul medium	Cell 5x10^4 Solution x1/40 100ul medium	Cell 5x10^4 Solution x1/20 100ul medium	Cell 5x10^4 Solution x1/10 100ul medium
F	Cell 5x10^4 100ul medium	Cell 5x10^4 Solution x1/10240 100ul medium	Cell 5x10^4 Solution x1/5120 100ul medium	Cell 5x10^4 Solution x1/2560 100ul medium	Cell 5x10^4 Solution x1/1280 100ul medium	Cell 5x10^4 Solution x1/640 100ul medium	Cell 5x10^4 Solution x1/320 100ul medium	Cell 5x10^4 Solution x1/160 100ul medium	Cell 5x10^4 Solution x1/80 100ul medium	Cell 5x10^4 Solution x1/40 100ul medium	Cell 5x10^4 Solution x1/20 100ul medium	Cell 5x10^4 Solution x1/10 100ul medium
G												
H												

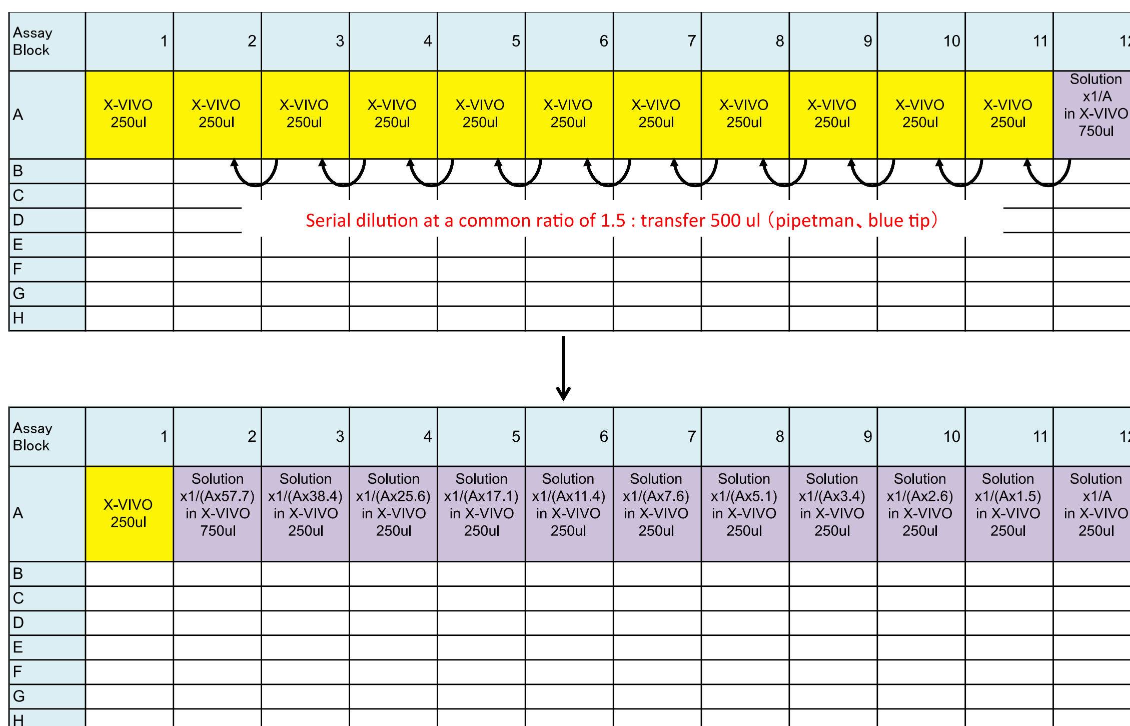
3-2-5 Arrangement of chemicals and vehicle (2nd, 3rd and 4th experiment)

Add 750 μL of the X-VIVO™ 15 solution of the chemical prepared at the highest concentration defined by the 1st experiment (1/A) to well #A12, and 250 μL of X-VIVO™ 15 to wells #A1-#A11of 96 well Assay Block, 2 mL.

3-2-6 Serial dilution (2nd, 3rd and 4th experiment)

Conduct 11 serial dilutions at a common ratio of 1.5 as indicated in Figure 8 from well #A11 to well #A2. (Transfer 500 μL to the next (left) well) Make sure not to use the blue tips previously used for dissolving and transferring chemicals in 3.1.

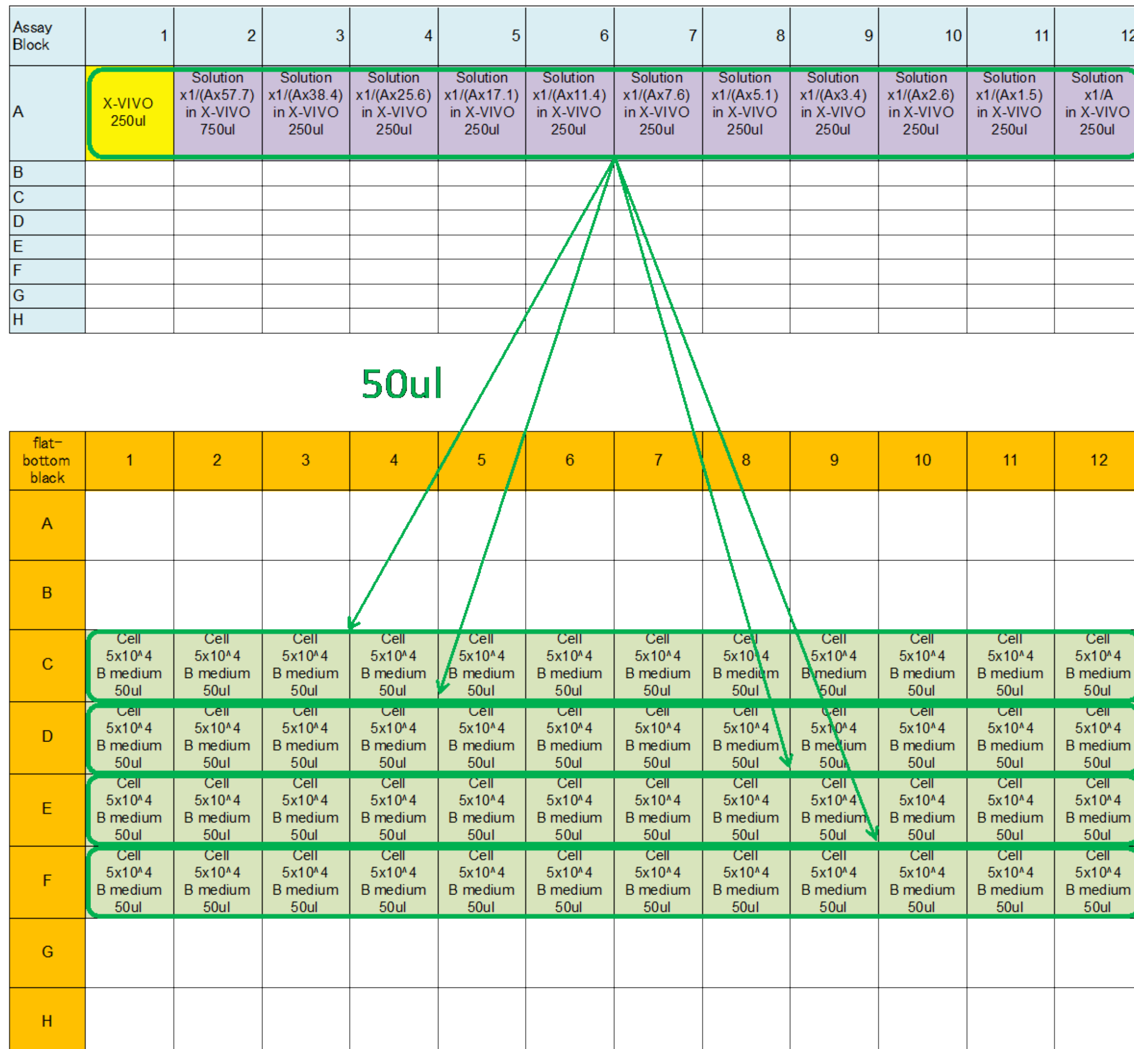
Figure 8



3-2-7 Addition to the cells (2nd, 3rd and 4th experiments)

Add 50 µL to THP-G8 in a 96-well flat-bottom black plate using an 8 channel or 12 channel pipetman. Shake the plate on a plateshaker, and incubate in a CO_2 incubator for 16 hours (37°C, 5% CO_2).

Figure 9



3-2-8. Final constituents of each well of the plate. (2nd, 3rd and 4th experiment)

Figure 10

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	Cell 5x10^4 100ul medium	Cell 5x10^4 Solution x1/(Ax115) 100ul medium	Cell 5x10^4 Solution x1/(Ax76.9) 100ul medium	Cell 5x10^4 Solution x1/(Ax51.3) 100ul medium	Cell 5x10^4 Solution x1/(Ax34.2) 100ul medium	Cell 5x10^4 Solution x1/(Ax22.8) 100ul medium	Cell 5x10^4 Solution x1/(Ax15.2) 100ul medium	Cell 5x10^4 Solution x1/(Ax10.1) 100ul medium	Cell 5x10^4 Solution x1/(Ax6.8) 100ul medium	Cell 5x10^4 Solution x1/(Ax4.5) 100ul medium	Cell 5x10^4 Solution x1/(Ax3) 100ul medium	Cell 5x10^4 Solution x1/(Ax2) 100ul medium
D	Cell 5x10^4 100ul medium	Cell 5x10^4 Solution x1/(Ax115) 100ul medium	Cell 5x10^4 Solution x1/(Ax76.9) 100ul medium	Cell 5x10^4 Solution x1/(Ax51.3) 100ul medium	Cell 5x10^4 Solution x1/(Ax34.2) 100ul medium	Cell 5x10^4 Solution x1/(Ax22.8) 100ul medium	Cell 5x10^4 Solution x1/(Ax15.2) 100ul medium	Cell 5x10^4 Solution x1/(Ax10.1) 100ul medium	Cell 5x10^4 Solution x1/(Ax6.8) 100ul medium	Cell 5x10^4 Solution x1/(Ax4.5) 100ul medium	Cell 5x10^4 Solution x1/(Ax3) 100ul medium	Cell 5x10^4 Solution x1/(Ax2) 100ul medium
E	Cell 5x10^4 100ul medium	Cell 5x10^4 Solution x1/(Ax115) 100ul medium	Cell 5x10^4 Solution x1/(Ax76.9) 100ul medium	Cell 5x10^4 Solution x1/(Ax51.3) 100ul medium	Cell 5x10^4 Solution x1/(Ax34.2) 100ul medium	Cell 5x10^4 Solution x1/(Ax22.8) 100ul medium	Cell 5x10^4 Solution x1/(Ax15.2) 100ul medium	Cell 5x10^4 Solution x1/(Ax10.1) 100ul medium	Cell 5x10^4 Solution x1/(Ax6.8) 100ul medium	Cell 5x10^4 Solution x1/(Ax4.5) 100ul medium	Cell 5x10^4 Solution x1/(Ax3) 100ul medium	Cell 5x10^4 Solution x1/(Ax2) 100ul medium
F	Cell 5x10^4 100ul medium	Cell 5x10^4 Solution x1/(Ax115) 100ul medium	Cell 5x10^4 Solution x1/(Ax76.9) 100ul medium	Cell 5x10^4 Solution x1/(Ax51.3) 100ul medium	Cell 5x10^4 Solution x1/(Ax34.2) 100ul medium	Cell 5x10^4 Solution x1/(Ax22.8) 100ul medium	Cell 5x10^4 Solution x1/(Ax15.2) 100ul medium	Cell 5x10^4 Solution x1/(Ax10.1) 100ul medium	Cell 5x10^4 Solution x1/(Ax6.8) 100ul medium	Cell 5x10^4 Solution x1/(Ax4.5) 100ul medium	Cell 5x10^4 Solution x1/(Ax3) 100ul medium	Cell 5x10^4 Solution x1/(Ax2) 100ul medium
G												
H												

3-3 When the chemical is not soluble at 20 mg/mL in X-VIVO™ 15

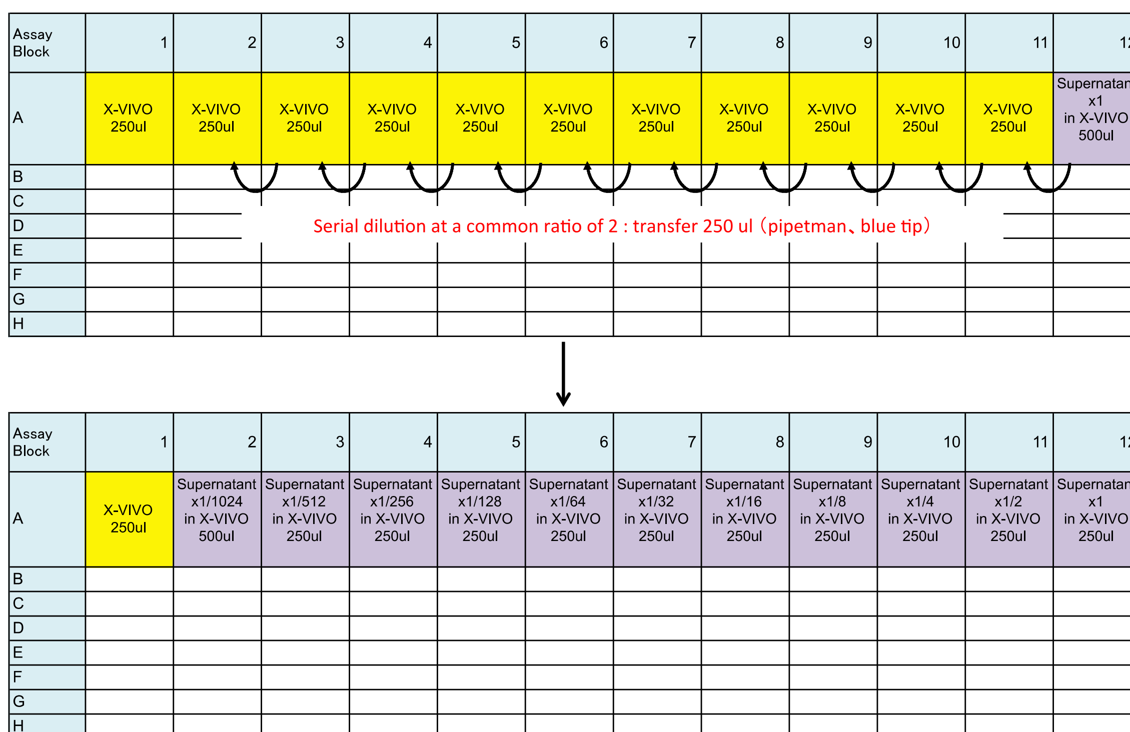
3-3-1 Arrangement of chemicals and vehicle (1st experiment)

Add 500 μL of the supernatant to the #A12 and 250 μL of X-VIVO™ 15 to wells #A1-#A11of a 96 well Assay Block, 2 mL.

3-3-2 Serial dilution (1st experiment)

Conduct 11 serial dilutions at a common ratio of 2 as indicated in Figure 11 from well #A11 to well #A2. (Transfer 250 μL to the next (left) well). Make sure not to use the blue tips previously used for dissolving and transferring chemicals in 3.1.

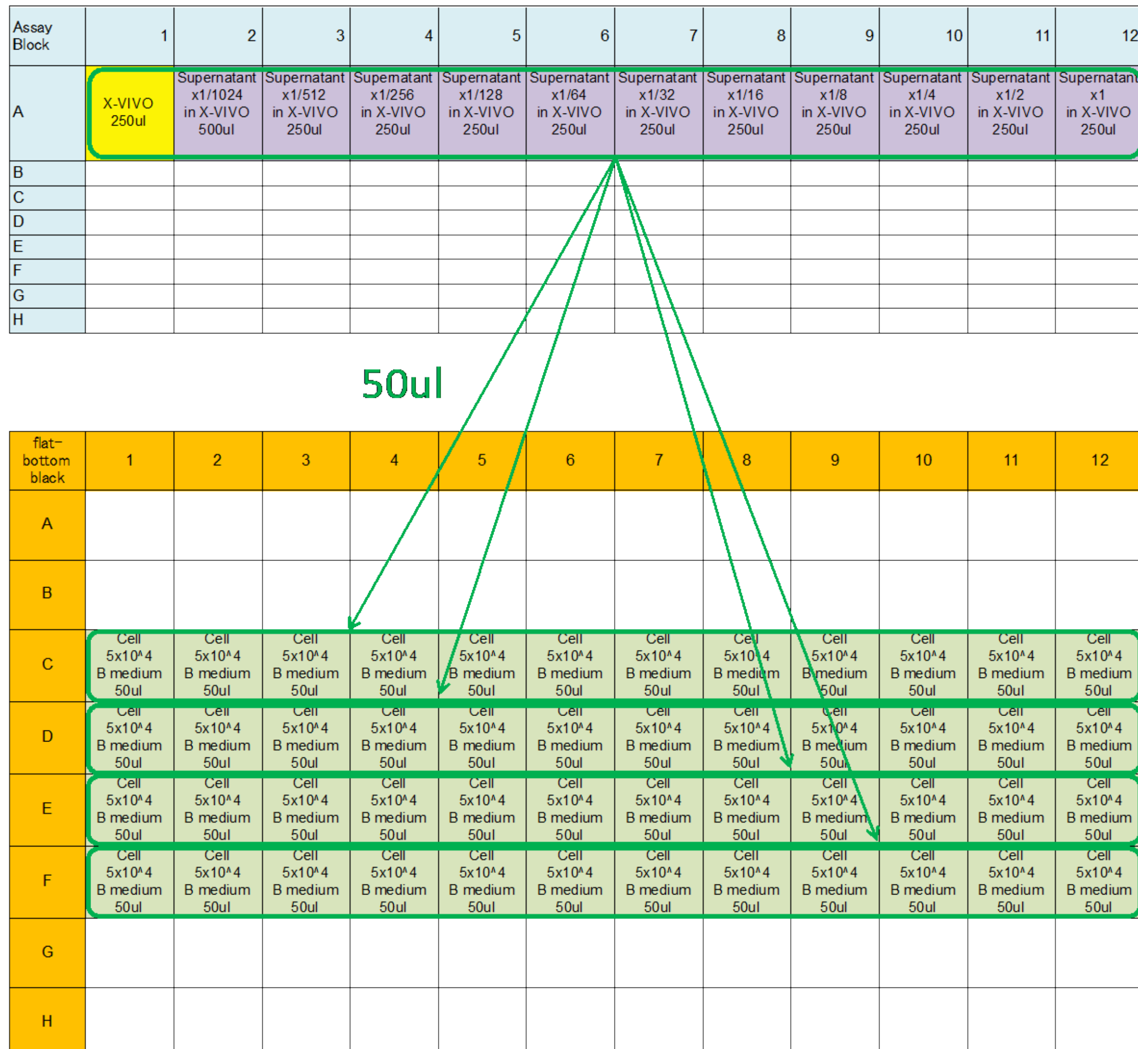
Figure 11.



3-3-3 Addition to the cells (1st experiment)

Add 50 μl to THP-G8 in a 96-well flat-bottom black plate using an 8 channel or 12 channel pipetman. Shake the plate on a plateshaker, and incubate in a CO_2 incubator for 16 hours (37°C, 5% CO_2).

Figure 12.



3-3-4. Final constituents of each well of the plate. (1st experiment)

Figure 13.

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	Cell 5x10^4 100ul medium	Cell 5x10^4 Supernatant x2048 100ul medium	Cell 5x10^4 Supernatant x1/1024 100ul medium	Cell 5x10^4 Supernatant x1/512 100ul medium	Cell 5x10^4 Supernatant x1/256 100ul medium	Cell 5x10^4 Supernatant x1/128 100ul medium	Cell 5x10^4 Supernatant x1/64 100ul medium	Cell 5x10^4 Supernatant x1/32 100ul medium	Cell 5x10^4 Supernatant x1/16 100ul medium	Cell 5x10^4 Supernatant x1/8 100ul medium	Cell 5x10^4 Supernatant x1/4 100ul medium	Cell 5x10^4 Supernatant x1/2 100ul medium
D	Cell 5x10^4 100ul medium	Cell 5x10^4 Supernatant x2048 100ul medium	Cell 5x10^4 Supernatant x1/1024 100ul medium	Cell 5x10^4 Supernatant x1/512 100ul medium	Cell 5x10^4 Supernatant x1/256 100ul medium	Cell 5x10^4 Supernatant x1/128 100ul medium	Cell 5x10^4 Supernatant x1/64 100ul medium	Cell 5x10^4 Supernatant x1/32 100ul medium	Cell 5x10^4 Supernatant x1/16 100ul medium	Cell 5x10^4 Supernatant x1/8 100ul medium	Cell 5x10^4 Supernatant x1/4 100ul medium	Cell 5x10^4 Supernatant x1/2 100ul medium
E	Cell 5x10^4 100ul medium	Cell 5x10^4 Supernatant x2048 100ul medium	Cell 5x10^4 Supernatant x1/1024 100ul medium	Cell 5x10^4 Supernatant x1/512 100ul medium	Cell 5x10^4 Supernatant x1/256 100ul medium	Cell 5x10^4 Supernatant x1/128 100ul medium	Cell 5x10^4 Supernatant x1/64 100ul medium	Cell 5x10^4 Supernatant x1/32 100ul medium	Cell 5x10^4 Supernatant x1/16 100ul medium	Cell 5x10^4 Supernatant x1/8 100ul medium	Cell 5x10^4 Supernatant x1/4 100ul medium	Cell 5x10^4 Supernatant x1/2 100ul medium
F	Cell 5x10^4 100ul medium	Cell 5x10^4 Supernatant x2048 100ul medium	Cell 5x10^4 Supernatant x1/1024 100ul medium	Cell 5x10^4 Supernatant x1/512 100ul medium	Cell 5x10^4 Supernatant x1/256 100ul medium	Cell 5x10^4 Supernatant x1/128 100ul medium	Cell 5x10^4 Supernatant x1/64 100ul medium	Cell 5x10^4 Supernatant x1/32 100ul medium	Cell 5x10^4 Supernatant x1/16 100ul medium	Cell 5x10^4 Supernatant x1/8 100ul medium	Cell 5x10^4 Supernatant x1/4 100ul medium	Cell 5x10^4 Supernatant x1/2 100ul medium
G												
H												

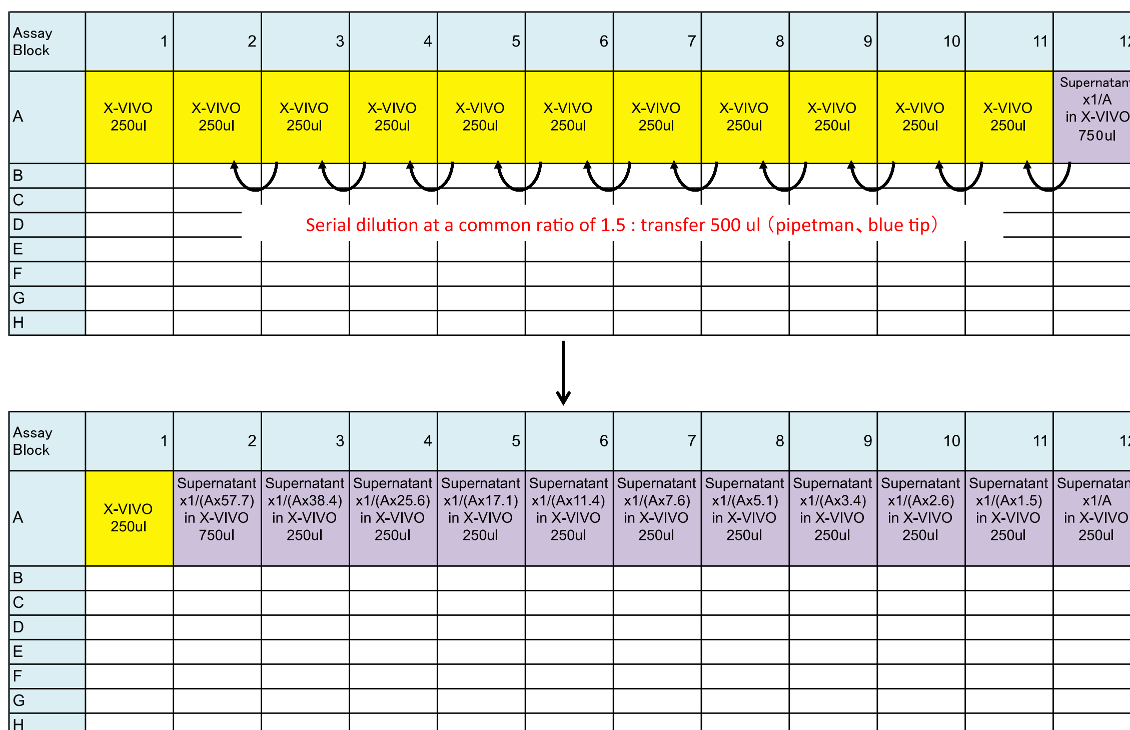
3-3-5 Arrangement of chemicals and vehicle (2nd, 3rd and 4th experiment)

Add 750 μL of the distilled X-VIVO™ 15 solution of the chemical prepared at the highest concentration defined by the 1st experiment (1/A) to well #A12, and 250 μL of X-VIVO™ 15 to wells #A1-#A11 of 96 well Assay Block, 2 mL.

3-3-6 Serial dilution (2nd, 3rd and 4th experiment)

Conduct 11 serial dilutions at a common ratio of 1.5 as indicated in Figure 14 from well #A11 to well #A2. Transfer 500 μL to the next (left) well. Make sure not to use the blue tips previously used for dissolving and transferring chemicals in 3.1.

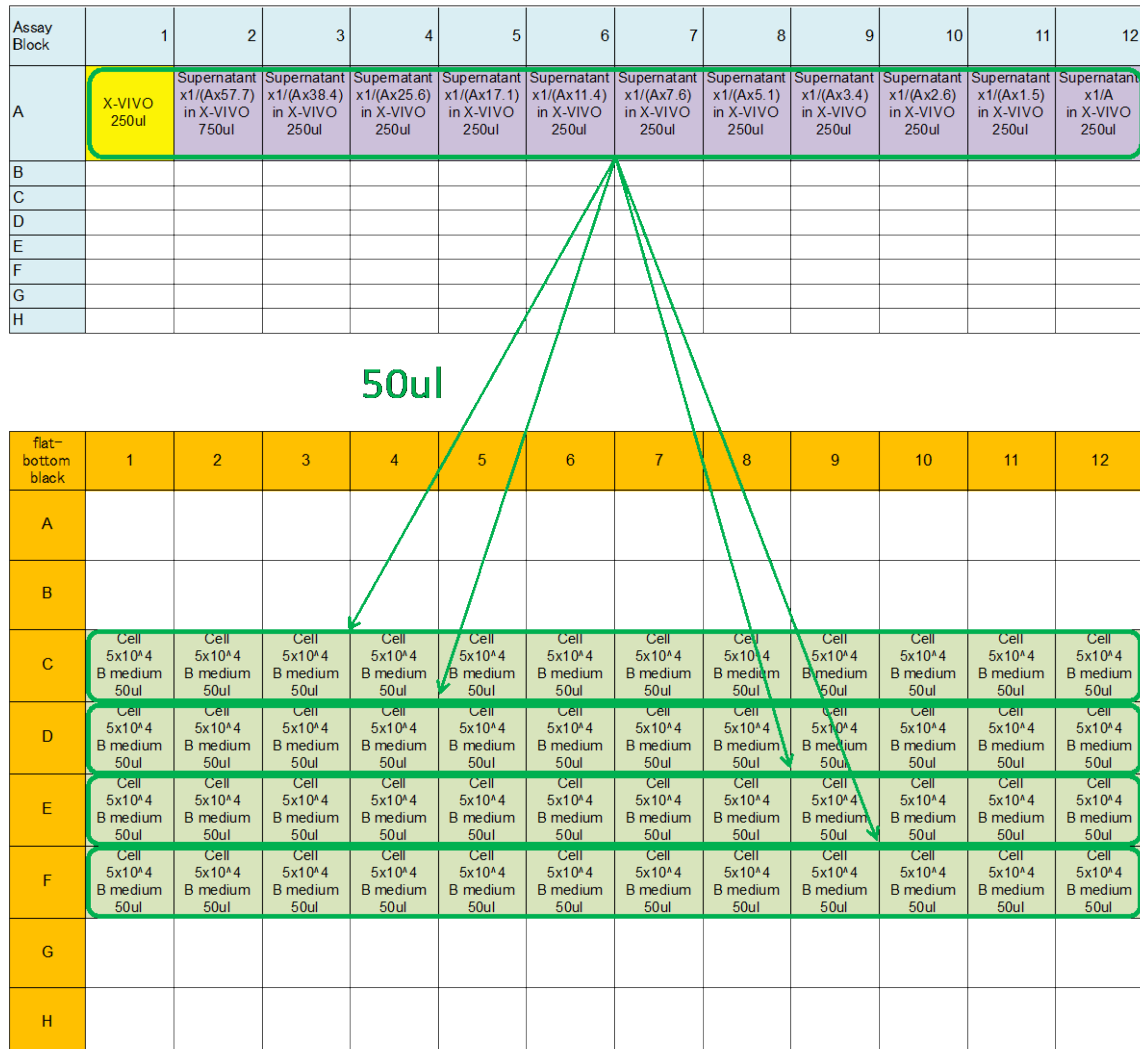
Figure 14.



3-3-7 Addition to the cells (2nd, 3rd and 4th experiments)

Add 50 μL to THP-G8 in a 96-well flat-bottom black plate using an 8 channel or 12 channel pipetman. Shake the plate on a plateshaker, and incubate in a CO_2 incubator for 16 hours (37°C, 5% CO_2).

Figure 15.



3-3-8. Final constituents of each well of the plate. (2nd, 3rd and 4th experiments)

Figure 16.

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	Cell 5x10^4 100ul medium	Cell 5x10^4 Supernatant x1/(Ax115) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax76.9) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax51.3) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax34.2) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax22.8) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax15.2) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax10.1) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax6.8) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax4.5) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax3) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax2) 100ul medium
D	Cell 5x10^4 100ul medium	Cell 5x10^4 Supernatant x1/(Ax115) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax76.9) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax51.3) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax34.2) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax22.8) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax15.2) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax10.1) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax6.8) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax4.5) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax3) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax2) 100ul medium
E	Cell 5x10^4 100ul medium	Cell 5x10^4 Supernatant x1/(Ax115) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax76.9) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax51.3) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax34.2) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax22.8) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax15.2) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax10.1) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax6.8) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax4.5) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax3) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax2) 100ul medium
F	Cell 5x10^4 100ul medium	Cell 5x10^4 Supernatant x1/(Ax115) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax76.9) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax51.3) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax34.2) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax22.8) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax15.2) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax10.1) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax6.8) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax4.5) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax3) 100ul medium	Cell 5x10^4 Supernatant x1/(Ax2) 100ul medium
G												
H												

4. Preparation of positive control and negative control

4-1 Preparation of cells

Add 50 μL of the cell suspension to wells #C1-#C4, #D1-#D4, #E1-#E4, #F1-#F4 of a 96-well flat-bottom black plate (flat bottom) (cf. Figure 17).

Figure 17.

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

4-2 Preparation of 4-nitrobenzyl bromide (4-NBB) and treatment of THP-G8 cells

20 mg of 4-NBB is prepared in a 1.5-mL microfuge tube, to which X-VIVO™ 15 is added up to 1 mL. The tube is vortexed vigorously and shaken on a rotor (e.g., WKN-2210, WAKEN B TECH Co. Ltd, Kyoto, Japan) at a maximum speed of 8 rpm for at least 30 min. After centrifugation at 20,000g for 5 min, the supernatant is diluted by a factor of 4 with X-VIVO™ 15, and 500 μl of the diluted supernatant is transferred to a well in a 96-well assay block. The diluted supernatant is further diluted with X-VIVO™ 15 at factors of 2 and 4, and 50 μl of the solution is added to 50 μl of THP-G8 cell suspension in the wells of a 96-well flat-bottom black plate. (cf. Figure 18, 19) Each concentration of the positive control should be tested in 4 wells. The plate is agitated on a plateshaker, and incubated in a CO_2 incubator for 16 hours (37°C, 5% CO_2)

Figure 18.

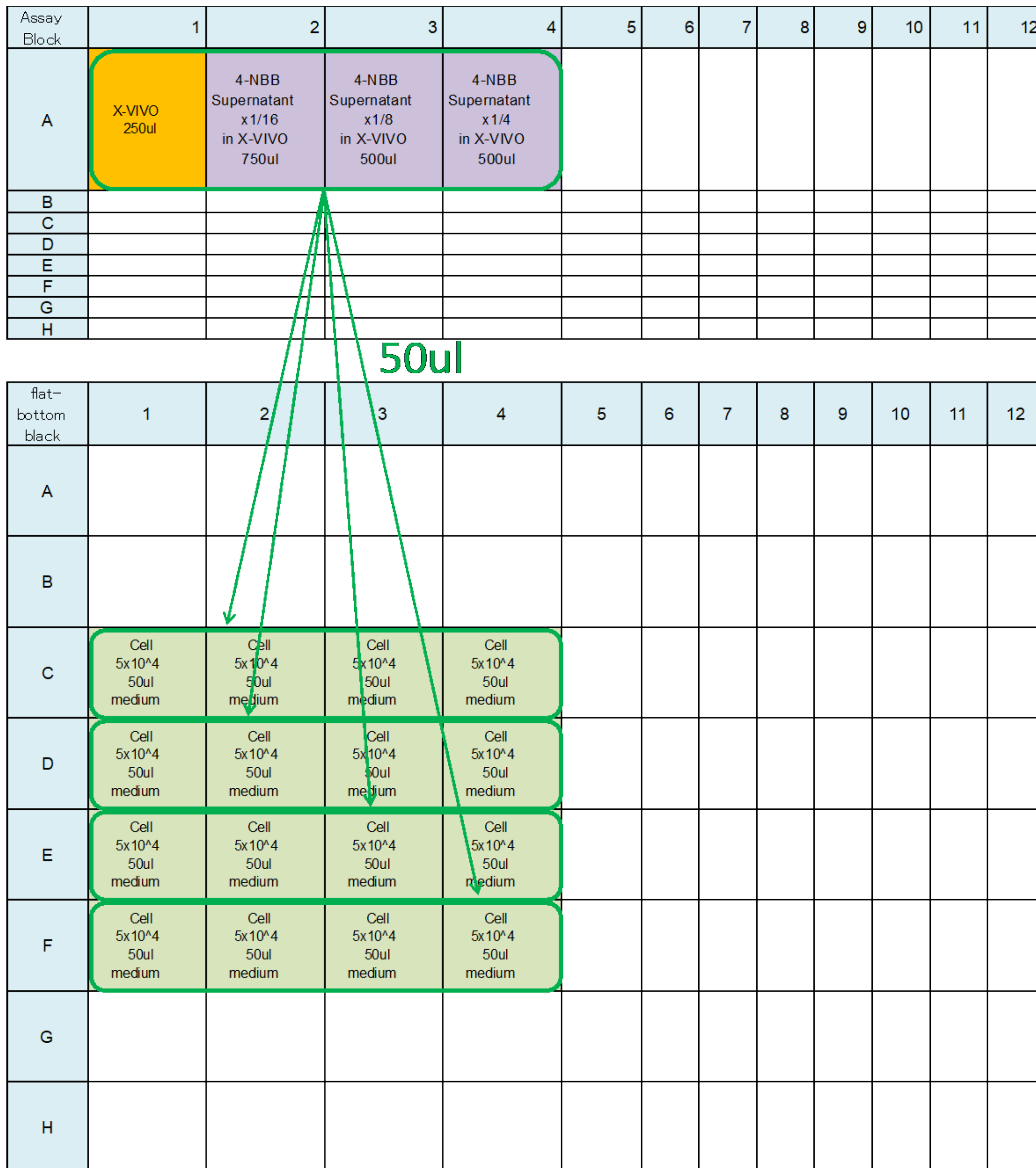
Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	X-XIVO 250ul	X-XIVO 250ul	X-XIVO 250ul	4-NBB Supernatant x1/4 in X-VIVO 500ul								
B												
C												
D												
E												
F												
G												
H												

Serial dilution at a common ratio of 2 : transfer 250 ㎕（pipetman、blue tip）

↓

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	X-VIVO 250ul	4-NBB Supernatant x1/16 in X-VIVO 750ul	4-NBB Supernatant x1/8 in X-VIVO 500ul	4-NBB Supernatant x1/4 in X-VIVO 500ul								
B												
C												
D												
E												
F												
G												
H												

Figure 19.



4-3 Final constituents of each well of the plate

Figure 20.

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	Cell 5x10^4 100ul medium	Cell 5x10^4 4-NBB x1/32 100ul medium	Cell 5x10^4 4-NBB x1/16 100ul medium	Cell 5x10^4 4-NBB x1/8 100ul medium								
D	Cell 5x10^4 100ul medium	Cell 5x10^4 4-NBB x1/32 100ul medium	Cell 5x10^4 4-NBB x1/16 100ul medium	Cell 5x10^4 4-NBB x1/8 100ul medium								
E	Cell 5x10^4 100ul medium	Cell 5x10^4 4-NBB x1/32 100ul medium	Cell 5x10^4 4-NBB x1/16 100ul medium	Cell 5x10^4 4-NBB x1/8 100ul medium								
F	Cell 5x10^4 100ul medium	Cell 5x10^4 4-NBB x1/32 100ul medium	Cell 5x10^4 4-NBB x1/16 100ul medium	Cell 5x10^4 4-NBB x1/8 100ul medium								
G												
H												

4-4 Preparation of lactic acid (LA) and treatment of THP-G8 cells

20 mg of LA prepared in a 1.5-mL microfuge tube, to which X-VIVO™ 15 is added up to 1 mL (20 mg/ mL). Twenty mg/mL of LA solution is diluted by a factor of 5 with X-VIVO™ 15 (4 mg/mL); 500 μl of this 4 mg/mL LA solution is transferred to a well of a 96-well assay block. This solution is diluted by a factor of 2 with X-VIVO™ 15 and then diluted again by a factor of 2 to produce 2 mg/mL and 1 mg/mL solutions. 50 μl of these 3 solutions and vehicle control (X-VIVO™ 15) are added to THP-G8 in a 96-well flat-bottom black plate. (cf. Figure 21, 22) Each concentration of the negative control is tested in 4 wells. The plate is agitated on a plateshaker and incubated in a CO_2 incubator for 16 hours (37°C, 5% CO_2).

Figure 21.

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	X-XIVO 250ul	X-XIVO 250ul	X-XIVO 250ul	Lactic acid 4 mg/ml in X-VIVO 500ul								
B												
C												
D												
E												
F												
G												
H												

Serial dilution at a common ratio of 2 : transfer 250 μl（pipetman、blue tip）

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	X-VIVO 250ul	Lactic acid 1 mg/ml in X-VIVO 750ul	Lactic acid 2 mg/ml in X-VIVO 500ul	Lactic acid 4 mg/ml in X-VIVO 500ul								
B												
C												
D												
E												
F												
G												
H												

Figure 22.

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	X-VIVO 250ul	Lactic acid 1 mg/ml in X-VIVO 750ul	Lactic acid 2 mg/ml in X-VIVO 500ul	Lactic acid 4 mg/ml in X-VIVO 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												
B												
C	Cell 5x10^4 50ul medium	Cell 5x10^4 50ul medium	Cell 5x10^4 50ul medium	Cell 5x10^4 50ul medium								
D	Cell 5x10^4 50ul medium	Cell 5x10^4 50ul medium	Cell 5x10^4 50ul medium	Cell 5x10^4 50ul medium								
E	Cell 5x10^4 50ul medium	Cell 5x10^4 50ul medium	Cell 5x10^4 50ul medium	Cell 5x10^4 50ul medium								
F	Cell 5x10^4 50ul medium	Cell 5x10^4 50ul medium	Cell 5x10^4 50ul medium	Cell 5x10^4 50ul medium								
G												
H												

4-5 Final constituents of each well of the plate

Figure 23.

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	Cell 5x10^4 100ul medium	Cell 5x10^4 Lactic acid 0.5 mg/ml 100ul medium	Cell 5x10^4 Lactic acid 1 mg/ml 100ul medium	Cell 5x10^4 Lactic acid 2 mg/ml 100ul medium								
D	Cell 5x10^4 100ul medium	Cell 5x10^4 Lactic acid 0.5 mg/ml 100ul medium	Cell 5x10^4 Lactic acid 1 mg/ml 100ul medium	Cell 5x10^4 Lactic acid 2 mg/ml 100ul medium								
E	Cell 5x10^4 100ul medium	Cell 5x10^4 Lactic acid 0.5 mg/ml 100ul medium	Cell 5x10^4 Lactic acid 1 mg/ml 100ul medium	Cell 5x10^4 Lactic acid 2 mg/ml 100ul medium								
F	Cell 5x10^4 100ul medium	Cell 5x10^4 Lactic acid 0.5 mg/ml 100ul medium	Cell 5x10^4 Lactic acid 1 mg/ml 100ul medium	Cell 5x10^4 Lactic acid 2 mg/ml 100ul medium								
G												
H												

4-6 Measurement

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 to each well of the plate containing the reference samples using a pipetman. Shake the plate for 10 min at room temperature (about 20° C) using 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 (F0) and presence (F1) of the optical filter.

Copy the results of the F0 and F1 measurements (values are expressed as counts) and paste them into the appropriate area in the "Data Input" sheet of the Calculation Sheet. Calculated factors and graphs will appear on the "4-NBB, Lactic acid" sheet of the Calculation Sheet. The acceptance criterion is that Ind-IL8LA should be more than 5.0 at least in one concentration of the positive control, 4-NBB, in each run and that Ind-IL8LA should be less than 1.4 at any concentration of the negative control, lactic acid, in each run. Continue the experiment if this criterion is accepted.

5. Determination of the transmission coefficients of optical filter for SLO and SLR

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.

5-1 Reagents

- Single reference samples:
  Lyophilized purified SLO enzyme
  Lyophilized purified SLR enzyme
- Assay reagent:
  Tripluc® Luciferase assay reagent（TOYOBO Cat#MRA-301）
- 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

5-2 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 10 ~ 100 mM Tris/HCl or Hepes/NaOH (pH 7.5 ~ 8.0) supplemented with 10% (w/v) glycerol to each tube of lyophilized purified luciferase 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 B medium to each tube of frozen reference sample (10 µL sample per tube). Keep the reference samples on ice to prevent deactivation.

5-3 Bioluminescence measurement

Transfer 100 µL of the diluted reference samples to a 96-well flat-bottom black plate as shown below (the SLO reference sample to #B1, #B2, #B3, the SLR reference sample to #D1, #D2, #D3)..

Figure 24.

	1	2	3	4	5	6	7	8	9	10	11	12
	SLO 1/1	SLO 1/1	SLO 1/1									
	SLR 1/1	SLR 1/1	SLR 1/1									

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 20°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) of the optical filter. An example of the raw output data is shown below.

Figure 25. An example of the raw output data

Measurement without Filter

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	9567	9782	9621									
C												
D	8561	8469	8495									
E												
F												
G												
H												

Measurement with Filter

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	2043	1998	2018									
C												
D	5749	5765	5784									
E												
F												
G												
H												

Transmission coefficient of the optical filter were calculated as follow:

Transmission coefficient (SLO (κO_{R60}))= (#B1 of F1+ #B2 of F1+ #B3 of F1) / (#B1 of F0+ #B2 of F0+ #B3 of F0)

Transmission coefficient (SLR (κR_{R60}))= (#D1 of F1+ #D2 of F1+ #D3 of F1) / (#D1 of F0+ #D2 of F0+ #D3 of F0)

In the case shown above,

Transmission coefficient (SLO (κO_{R60}))=(2043+1998+2018) / (9567+9782+9621)=0.21,

Transmission coefficient (SLR (κR_{R60}))=(5749+5765+5784) / (8561+8469+8495)=0.68

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

6. 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 20°C) using 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 (F0) and presence (F1) of the optical filter. In case alternative settings are used, e.g., depending on the model of luminometer used, these settings should be justified.

The specific procedure for how to manipulate the raw data is shown below using the analysis of DNCB as an example (Figure 26).

Figure 26. An example of the raw output data (DNCB).

Measurement2 F0

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	9098	9046	9470	8879	9112	10983	14503	68	63	76	80	67
D	9140	8493	8981	8352	8521	10510	12232	73	78	59	61	51
E	8801	8601	8833	8469	8572	10362	13133	72	74	71	68	74
F	8779	8364	8116	8026	8011	10199	12109	83	66	55	55	72
G												
H												

Measurement2 F2

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	4747	4689	5010	4623	4584	4658	3756	61	75	70	51	59
D	4742	4420	4638	4305	4372	4713	3331	73	75	62	81	61
E	4447	4444	4518	4305	4313	4778	3471	59	75	66	68	65
F	4581	4286	4238	4112	4144	4643	3284	67	68	78	62	61
G												
H												

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 code, the highest concentration, dissolved or undissolved in X-VIVO™ 15, and comments if any to the "FaceSheet" sheet of the Calculation Sheet (Figure 27). In this Calculation Sheet, the highest soluble concentrations in the next experiments are automatically shown based on the results.

Figure 27. The "FaceSheet" sheet of the Calculation Sheet.

IL-8 Luc Assay Calculation Sheet

Ver. 021.1

Laboratory		Round	

Exp.	

Date: (YYYY/MM/DD)		Operator:	

Chemical Code:		Dissolution:		in X-VIVO	

25
500
250 dissolved ### ### ### ### 1/80 ### ###
125 not_diss: # ### ### ### 1/16 ### ###
62.5
31.25
15.625

Comment:

2nd. Copy the results of the F0 and F1 measurements (values are expressed as counts) and paste them into the appropriate area in the "Data Input" sheet of the Calculation Sheet shown below (Figure 28). In addition, input the transmittance factors calculated in chapter 5. Calculation of the transmittance factors to TF2 of the "Data Input" sheet (Figure 28).

Figure 28. The "Data Input" sheet of the Calculation Sheet.

MultiReporter Assay System -Tripluc® - Calculation Sheet

Input transmittance factors of filter 2 for SLO and SLR

	TF
SLO	0.2129
SLR	0.6787

Input measured data (counts)

Data without filter

Null	1	2	3	4	5	6	7	8	9	10	11	12
A	9098	9046	9470	8879	9112	10983	14503	68	63	76	80	67
B	9140	8493	8981	8352	8521	10510	12232	73	78	59	61	51
C	8801	8601	8833	8469	8572	10362	13133	72	74	71	68	74
D	8779	8364	8116	8026	8011	10199	12109	83	66	55	55	72
E												
F												
G												
H												

Data using Filter

Filter	1	2	3	4	5	6	7	8	9	10	11	12
A	4747	4689	5010	4623	4584	4658	3756	61	75	70	51	59
B	4742	4420	4638	4305	4372	4713	3331	73	75	62	81	61
C	4447	4444	4518	4305	4313	4778	3471	59	75	66	68	65
D	4581	4286	4238	4112	4144	4643	3284	67	68	78	62	61
E												
F												
G												
H												

Next, the calculated results for the parameters of the IL-8 Luc assay for each concentration, e.g., IL8LA, GAPLA, nIL8LA, the mean ± SD of IL8LA, the mean ± SD of GAPLA, the mean ± SD of nIL8LA, the mean ± SD of Ind-IL8LA, the mean ± SD of Inh-GAPLA, and a graphical presentation with the 95% confidence interval and judgment, will automatically appear on the "Result Format" sheet and the "Graph" sheet of the Calculation Sheet, respectively (Figure 29, 30).

Figure 29. The "Result Format" sheet of the Calculation Sheet.

MultiReporter Assay System® Calculation Sheet

Transmittance Data

	Null	T
SLO	1	0.212902063
SLR	1	0.678670307

inversion matrix	
1.457098709	-0.457098709
-2.146990511	2.146990511

IL8LA

	1	2	3	4	5	6	7	8	9	10	11	12
A	3065	3114	3042	3012	3435	6003	13068	-32	-69	-40	7	-29
B	3137	2885	3128	2927	3029	5195	10672	-50	-47	-47	-85	-57
C	3276	2991	3170	3097	3230	4840	11684	-22	-53	-38	-47	-32
D	2957	2985	2727	2866	2776	4892	10593	-23	-50	-87	-53	-26
E	0	0	0	0	0	0	0	0	0	0	0	0
F	0	0	0	0	0	0	0	0	0	0	0	0
G	0	0	0	0	0	0	0	0	0	0	0	0
H	0	0	0	0	0	0	0	0	0	0	0	0

GAPLA

	1	2	3	4	5	6	7	8	9	10	11	12
A	6033	5932	6428	5867	5677	4980	1435	100	132	116	73	96
B	6003	5608	5853	5425	5492	5315	1560	123	125	106	146	108
C	5525	5610	5663	5372	5342	5522	1449	94	127	109	115	106
D	5822	5379	5389	5160	5235	5307	1516	106	116	142	108	98
E	0	0	0	0	0	0	0	0	0	0	0	0
F	0	0	0	0	0	0	0	0	0	0	0	0
G	0	0	0	0	0	0	0	0	0	0	0	0
H	0	0	0	0	0	0	0	0	0	0	0	0

IL8LA mod

	1	2	3	4	5	6	7	8	9	10	11	12
A	3065	3114	3042	3012	3435	6003	13068	0	0	0	7	0
B	3137	2885	3128	2927	3029	5195	10672	0	0	0	0	0
C	3276	2991	3170	3097	3230	4840	11684	0	0	0	0	0
D	2957	2985	2727	2866	2776	4892	10593	0	0	0	0	0
E	0	0	0	0	0	0	0	0	0	0	0	0
F	0	0	0	0	0	0	0	0	0	0	0	0
G	0	0	0	0	0	0	0	0	0	0	0	0
H	0	0	0	0	0	0	0	0	0	0	0	0

GAPLA mod

	1	2	3	4	5	6	7	8	9	10	11	12
A	6033	5932	6428	5867	5677	4980	1435	100	132	116	73	96
B	6003	5608	5853	5425	5492	5315	1560	123	125	106	146	108
C	5525	5610	5663	5372	5342	5522	1449	94	127	109	115	106
D	5822	5379	5389	5160	5235	5307	1516	106	116	142	108	98
E	0	0	0	0	0	0	0	0	0	0	0	0
F	0	0	0	0	0	0	0	0	0	0	0	0
G	0	0	0	0	0	0	0	0	0	0	0	0
H	0	0	0	0	0	0	0	0	0	0	0	0

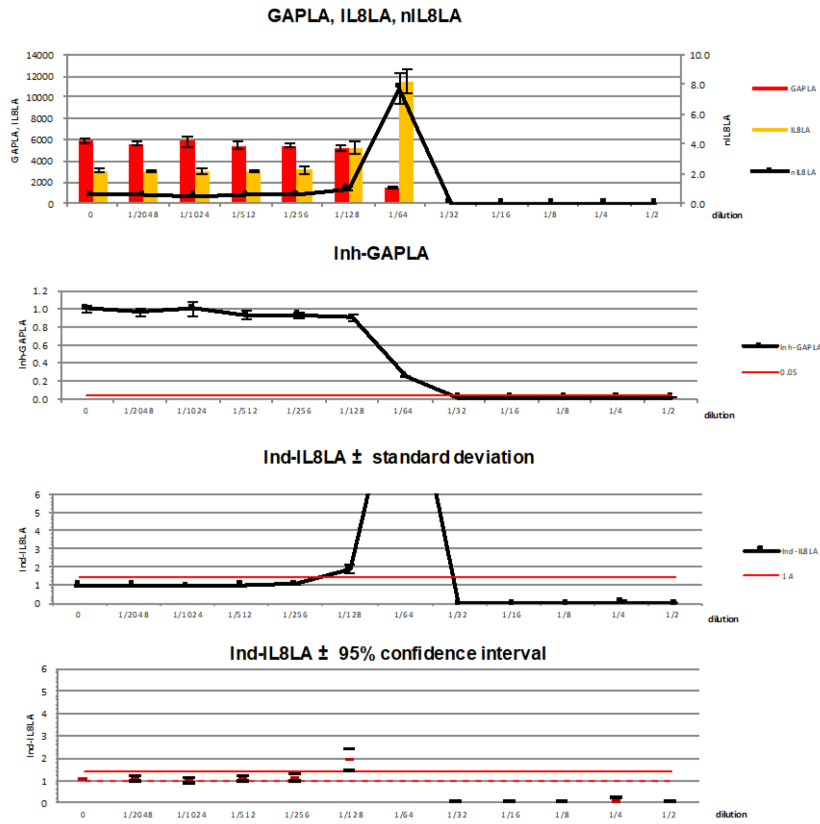
nIL8LA

0.508	0.525	0.473	0.513	0.605	1.205	9.108	0.000	0.000	0.000	0.097	0.000
0.523	0.515	0.535	0.540	0.552	0.978	6.839	0.000	0.000	0.000	0.000	0.000
0.593	0.533	0.560	0.577	0.605	0.877	8.063	0.000	0.000	0.000	0.000	0.000
0.508	0.555	0.506	0.556	0.530	0.922	6.989	0.000	0.000	0.000	0.000	0.000
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

1st exp.

Chemical concentration	0	1/2048	1/1024	1/512	1/256	1/128	1/64	1/32	1/16	1/8	1/4	1/2	dilution
Average	3109	2994	3017	2976	3118	5233	11504	0	0	0	2	0	
S.D.	134	93	201	101	282	537	1155	0	0	0	4	0	
Average	5846	5632	5833	5456	5436	5281	1490	106	125	118	110	102	
S.D.	233	228	440	297	192	224	59	13	7	16	30	6	
Average	0.533	0.532	0.518	0.546	0.573	0.995	7.750	0.000	0.000	0.000	0.024	0.000	
S.D.	0.041	0.017	0.037	0.027	0.038	0.146	1.057	0.000	0.000	0.000	0.048	0.000	
Inh-GAPLA	1.000	0.963	0.998	0.933	0.930	0.903	0.255	0.018	0.021	0.020	0.019	0.017	
SD	0.040	0.039	0.075	0.051	0.033	0.038	0.010	0.002	0.001	0.003	0.005	0.001	
Ind-IL8LA	1.000	0.998	0.973	1.025	1.075	1.868	14.544	0.000	0.000	0.000	0.045	0.000	
SD	0.076	0.032	0.070	0.050	0.071	0.274	1.984	0.000	0.000	0.000	0.091	0.000	

Figure 30. The “Graph” sheet of the Calculation Sheet.



	(0)	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
Ind-IL8LA	1	0.998	0.973	1.025	1.075	1.868	14.54	0	0	0	0.045	0
lower limit		0.894	0.856	0.916	0.951	1.434	11.13	0	0	0	-0.067	0
upper limit		1.125	1.108	1.154	1.219	2.35	18.38	0	0	0	0.159	0

												Judge
Ind-IL8LA +95%	0	0	0	0	1	1	0	0	0	0	0	Positive

Judge	Positive

7. Criteria

7-1 Definition of the parameters used in the IL-8 Luc assay

The way of calculation of these parameters are described in Appendix 3.

Figure 31.

Abbreviations	Definition
GAPLA	SLR luciferase activity reflecting GAPDH promoter activity
IL8LA	SLO luciferase activity reflecting IL-8 promoter activity
nIL8LA	IL8LA / GAPLA
Ind-IL8LA	nIL8LA of THP-G8 cells treated with chemicals / nIL8LA of untreated cells
Inh-GAPLA	GAPLA of THP-G8 treated with chemicals / GAPLA of untreated cells
CV05	Minimum concentration at which chemicals show less than 0.05 of Inh-GAPLA

7-2 Acceptance criteria

The following acceptance criteria should be satisfied when using the IL-8 Luc assay method.

- In the assay of positive control, Ind-IL8LA should be more than 5.0 at least in one concentration in each experiment.
- In the assay of negative control, Ind-IL8LA should be less than 1.4 at any concentrations in each experiment.
- Data from plates in which the GAPLA of control wells with cells is less than 5 times of that of wells without cells should be rejected.
- Data from plates in which the Inh-GAPLA of all concentrations of the test or control chemicals is less than 0.05 should be rejected. In this case, the first test should be repeated from the lowest final concentration of the previous test.

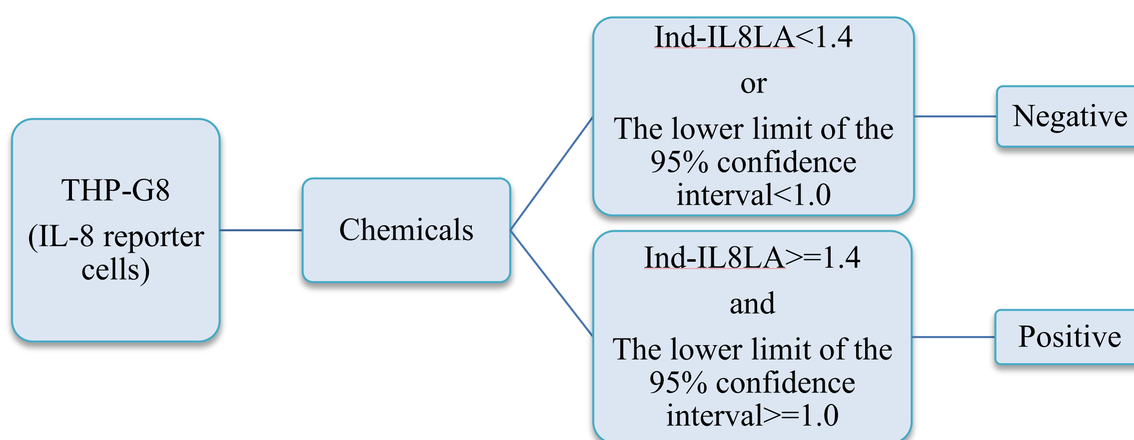
7-3 The criteria to identify sensitizers by the IL-8 Luc assay

1. <u>The criterion to determine positive or negative in each experiment:</u>

Chemicals that demonstrate Ind-IL8LA ≥ 1.4 and the lower limit of the 95% confidence interval of Ind-IL8LA ≥ 1.0 are judged as positive.

The way of calculation of the 95% confidence interval of Ind-IL8LA is described in Appendix 3.

Figure 32.Judgment for each run



2. The criterion to determine sensitiser, supposed non-sensitiser, non-sensitiser or inconclusive

To determine the judgment of the IL-8 Luc assay, the experiments are conducted at most 4 times. To repeat the experiments, the highest concentration in the 2nd, 3rd, and the 4th experiment is determined as follows.

1st experiment: THP-G8 cells are stimulated with chemicals serially diluted at a common ratio of 2 using the supernatant or completely dissolved solution.

2nd, 3rd and 4th experiments: Determine the minimum concentration at which Inh-GAPLA is 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 test, and conduct 11 serial dilutions at a common ratio of 1.5 from the highest concentration. If Inh-GAPLA does not decrease to 0.05 at any concentrations, or Inh-GAPLA is lower than 0.05 only at the highest concentration in the 1st experiment,

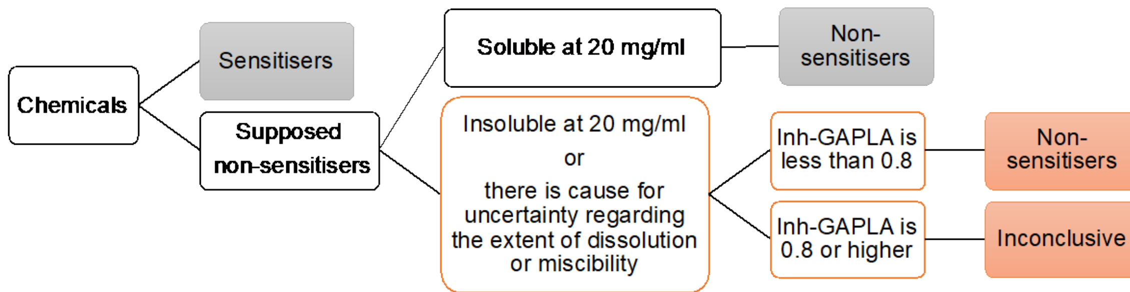
conduct 11 serial dilutions at a common ratio of 1.5 from the supernatant or solution.

Based on positive or negative judgment in each experiment, the judgment of the IL-I Luc assay was determined by the following criterion.
Test chemicals that provide two positive results from among the 1st, 2nd, 3rd or 4th experiment are identified as sensitisers whereas those that give three negative results from among the 1st, 2nd, 3rd or 4th runs are identified as supposed non-sensitisers (Table 32). Among supposed non-sensitisers, if chemicals are dissolved at 20 mg/ml in X-VOVO™ 15, they are judged as non-sensitisers. If chemicals are not dissolved at 20 mg/ml in X-VOVO™ 15, or there is cause for uncertainty regarding the extent of dissolution or miscibility, chemicals that give less than 0.8 of Inh-GAPLA are judged as non-sensitisers, while those that give 0.8 or higher of Inh-GAPLA are judged as inconclusive (Figure 33).

Table 32. Criteria for identifying positive and supposed negative

<table>
<tr><th>1st run</th><th>2nd run</th><th>3rd run</th><th>4th run</th><th>Final prediction</th></tr>
<tr><td rowspan="4">Positive</td><td>Positive</td><td>-</td><td>-</td><td>Sensitiser</td></tr>
<tr><td rowspan="3">Negative</td><td>Positive</td><td>-</td><td>Sensitiser</td></tr>
<tr><td rowspan="2">Negative</td><td>Positive</td><td>Sensitiser</td></tr>
<tr><td>Negative</td><td>Supposed non-sensitiser</td></tr>
<tr><td rowspan="6">Negative</td><td rowspan="3">Positive</td><td>Positive</td><td>-</td><td>Sensitiser</td></tr>
<tr><td rowspan="2">Negative</td><td>Positive</td><td>Sensitiser</td></tr>
<tr><td>Negative</td><td>Supposed non-sensitiser</td></tr>
<tr><td rowspan="3">Negative</td><td rowspan="2">Positive</td><td>Positive</td><td>Sensitiser</td></tr>
<tr><td>Negative</td><td>Supposed non-sensitiser</td></tr>
<tr><td>Negative</td><td>-</td><td>Supposed non-sensitiser</td></tr>
</table>

Figure 33. Prediction model for final judgment


Chemicals
Sensitisers
Supposed non-sensitisers
Soluble at 20 mg/ml
Non-sensitisers
Insoluble at 20 mg/ml
or
there is cause for uncertainty regarding the extent of dissolution or miscibility
Inh-GAPLA is less than 0.8
Non-sensitisers
Inh-GAPLA is 0.8 or higher
Inconclusive

8. Update record

Ver.025, 2021, May 27

Change the criteria for the proposal of the modification of the prediction model of the IL-8 Luc assay (OECD TG442E)

Ver.024.2, 2017, March 10

Change the criteria.

Ver.024.1, 2017, Feb 28

Change the acceptance criteria.

Change the name of the parameters.

Add negative control, lactic acid.

Ver.024, 2017, Feb 27

Change rt, 25 to 20.

Ver.023, 2016, Feb 9

Add appendix.

Add Acceptance criteria.

Ver. 022E, 2015, Dec. 31

Change positive control to 4-NBB.

Ver. 021E, 2015, Dec. 21

Add schemes showing data transport and delete the graphical presentation of the criteria.

Ver. 020E, 2015, July 3

Change preparation of chemicals (When the chemical is soluble at 20 mg/mL in X-VIVO™ 15, the chemical is diluted 5-fold) and $CoCl_2$.

Ver. 019E, 2015, April 1

Change vehicle to X-VIVO™ 15

Ver. 017E, 2013, Nov. 14 distribution
Modify the criteria

Ver. 016E, 2013, Oct. 29 distribution
Delete the criteria I.I.≤0.80

Ver. 015E, 2012, Nov. 12 distribution
Change preparation of chemicals

Ver. 014E, 2012, Oct. 26 distribution
Change preparation of water-soluble chemicals
Change preparation of $CoCl_2$
Delete the description concerning LPS
Change incubation time (5 hours to 16 hours)
Change the quality control of the equipment
Change the criteria

Ver. 013E, 2012, Aug. 03 distribution

Ver. 012E, 2012, July 12 distribution

Ver. 011E, 2012, June 05 distribution

Ver. 008E, 2011, Dec. 19 distribution

IL-8 Luc assay detailed protocol, 2011, Nov. 11 distribution

Appendix 1

Principal 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 an optical filter. (e.g. Phelios AB-2350 (ATTO), ARVO (PerkinElmer), Tristar LB941 (Berthold)) The optical filter used in measurement is 600 ～ 620 nm long or short pass filter, or 600～700 nm band pass filter.

(1) Measurement of two-color luciferases with an optical filter.

This is an example using Phelios AB-2350 (ATTO). This luminometer equips a 600 nm long pass filter (600 nm LP (Filter 1); R60 HOYA Co.), for splitting SLO and SLR luminescence.

To determine transmission coefficients of the 600 nm LP, first, using purified SLO and SLR luciferase enzymes, measure i) the intensity of SLO and SLR bioluminescence intensity without filter (F0), ii) the SLO and SLR bioluminescence intensity that passed through 600 nm LP (Filter 1), and calculate the transmission coefficients of 600 nm LP for SLO and SLR listed below.

Transmission coefficients		Abbreviation	Definition
SLO	Filter 1 Transmission coefficients	κO_{R60}	The filter's transmission coefficient for the SLO
SLR	Filter 1 Transmission coefficients	κR_{R60}	The filter's transmission coefficient for the SLR

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

$$F0=O+R$$

$$F1=\kappa O_{R60} \times O + \kappa R_{R60} \times R$$

These formulas can be rephrased as follows

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

Then using calculated coefficient factors (κO_{R60} and κR_{R60}) and measured F0 and F1, you can calculate O and R-value as follows.

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

This calculation can be performed using the functions "MININVERSE" and "MMULT" in Microsoft Excel. These calculations are integrated in "IL-8_Luc_assay_Data_sheet_20170227_Ver.021.1".

Appendix 2

Validation of reagents and equipment

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.

1-1 Reagents

・Single reference samples:

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		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

1-2 Calibration

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

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

1-2-2 Bioluminescence measurement

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

Figure 31.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	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
C												
D	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
E												
F												
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 20°C) with a plate shaker. Remove bubbles on the solutions in wells if they appear. Place the plate into the luminometer of the under-test to measure the luciferase activity.

Bioluminescence is measured for 3 sec each in the absence (F0) and presence (F1) of the optical filter. The example of the raw output data was shown below.

Figure 32.

Measurement without Filter

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	9689	9691	9677	2402	2388	2412	704	689	721	177	189	182
C												
D	8588	8444	8462	2281	2128	2239	609	578	690	150	132	129
E												
F												
G												
H												

Measurement with Filter

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	2022	1945	2067	502	496	510	143	149	153	37	49	45
C												
D	5722	5756	5721	1523	1459	1589	413	397	468	102	108	97
E												
F												
G												
H												

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

Figure 33.

MultiReporter Assay System -Tripluc®- Calculation Sheet

Input measured data (counts)

Data without filter

Input

Null	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Data using Filter 2

Input

F2	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Record all the results for quality control.

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 hereafter should be performed at the beginning of the experiments every day using reference LED light source plates equipped with stabilized LEDs. LED plate data typically fluctuates up to 1.5% (σ). Disagreement to the old data should be less than 3×σ (= 4.5%). Here describes an example using a commercially available reference light source, TRIANT® (wSL-0001) by ATTO (Tokyo, Japan).

Start LED plate and select "PMT" mode.

Select three-color (BRG) mode and adjust light intensity to 1/10 (10E-1).

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.

Read and record the data at the position of #F6, #E6, and #D6 that are LEDs of blue, green, and red LEDs, respectively.

LED plate data typically fluctuates up to 1.5% (σ). Disagreement to the old data should be less than 3×σ (= 4.5%).

Appendix 3

Calculation of the parameters used in the IL-8 Luc assay

The j-th repetition (j = 1 to 4) of the i-th concentration (j = 0 to 11) is measured for IL8LA and GAPLA respectively. The normalized IL8LA is referred as nIL8LA, and is defined as

$nIL8LA_{ij} = IL8LA_{ij} / GAPLA_{ij}$.

1. Ind-IL8LA

The fold increase of the averaged nIL8LA for the repetition on the i-th concentration compared with it on the 0 concentration, Ind-IL8LA, is the primary measure of this assay. This ratio is able to write by the following formula,

$\text{Ind-IL8LA}_i = \{(1/4) x \Sigma_j \, nIL8LA_{ij}\} \ / \ \{(1/4) x \Sigma_j \, nIL8LA_{0j}\}$.

The 95% confidence interval theorem known as Fieller's theorem is obtained from the following formula.

$$\left[\frac{-B-\sqrt{B^2-4AC}}{2A}, \frac{-B+\sqrt{B^2-4AC}}{2A}\right],$$

where $A = \overline{x}_0^2 - t^2_{0.975(\nu)} \times \frac{sd_0^2}{n_0}$, $B = -2 \times \overline{x} \times \overline{y}$, $C = \overline{y}_i^2 - t^2_{0.975(\nu)} \times \frac{sd_{y_i}^2}{n_{y_i}}$, and

$\overline{x}_0^2 = \{(1/4) x \Sigma_j \, nIL8LA_{0j}\}$, $sd_0^2 = (1/3) x \Sigma_j \, (nIL8LA_{0j} - \overline{x}_0)^2$, $n_0 = 4$,

$\overline{y}_0^2 = \{(1/4) x \Sigma_j \, nIL8LA_{ij}\}^2$, $sd_{yj}^2 = (1/3) x \Sigma_j \, (nIL8LA_{ij} - \overline{y}_i)^2$, $n_{y_i} = 4$,

$t_{0.975(\nu)}$ is 97.5 percentile of the central t distribution with the ν of the degree of freedom.

2. Inh-GAPLA

The Inh-GAPLA is a ratio of the averaged GAPLA for the repetition of the i-th concentration compared with it of the 0 concentration, and this is written by

$$\text{Inh-GAPLA}_i = \{(1/4) \times \Sigma_j \text{GAPLA}_{ij}\} / \{(1/4) \times \Sigma_j \text{GAPLA}_{0j}\}$$