THP-G8 culture Protocol

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This protocol describes the GPC lab's recommend method for culture and IL-8 Luc assay test using THP-G8 cells. It also includes a Q&A section for frequently asked questions. This document is based on the OECD test guidelines and JaCVAM's published protocol. Please refer to the original document for the details of IL-8 Luc assay.

1. Materials

1-1. Cells

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（SIGMA #172012-500 ML）
- 100×concentrated antibiotic and antimycotic（10000 U/mL of Penicillin G, 10000 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#09380-86）
- Puromycin（CAS:58-58-2, InvivoGen Cat#ant-pr-1）
- T-75 flask tissue culture treated（e.g., Corning Cat#353136）
- CELLBANKER 1（Nippon Zenyaku Kogyo Co., Ltd., Cat# CB011, 100 mL）
- 2 mL Cryotube（Sumitomo Bakelite Co., Ltd., Cat# MS-4502）

For chemical expose, positive control, negative control and 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)
- 96-well flat-bottom black plate (for measurement of the luciferase activity, Nunc Cat#165305)
- 96 well assay block, 2 mL (e.g., Costar Cat#3960)

Equipment of measurement of luciferase activity

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

1-3. Culture medium

A medium：for maintenance of THP-G8 cells

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	445 mL
FBS	SIGMA #172012-500ML	-	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 #09380-86	50 mg/mL	300 μg/mL	3 mL

B medium：for luciferase assay

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	27 mL
FBS	SIGMA #172012-500ML	-	10 %	3 mL

C medium：for thawing THP-G8 cells

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	26.7 mL
FBS	SIGMA #172012-500ML	-	10 %	3 mL
Antibiotic-Antimycotic	GIBCO #15240-062	100×	1×	0.3 mL

2. Cell culture

2-1. Thawing and maintenance of THP-G8 cells

☐ Prepare 30 mL of C medium in a 50 mL polypropylene conical tube.

Reagent	Final concentration in medium	Required amount
RPMI-1640	-	☐ 26.7 mL
FBS	10 %	☐ 3.0 mL
Antibiotic-Antimycotic	1×	☐ 0.3 mL

☐ Pre-warm 9 mL of C medium in a 15 mL polypropylene conical tube in a 37℃ water bath.

☐ 15 mL of C medium in a T-75 Flask at a 37℃ in a 5% CO_2 incubator.

☐ Thaw frozen THP-G8 cells in a 37℃ water bath (Half-thawed, with ice particles remaining), then add 1 mL of C medium from 9 mL of pre-warmed C medium to rapidly dissolve and collect in a 15 mL polypropylene conical tube.

☐ Centrifuge the tube at 350 x *g* at room temperature for 5 min.

☐ Discard the supernatant, resuspend in 1 mL of C medium and count the number of cells.

☐ Add 1 mL of the resuspended cell to 14 mL of C medium and seed the cells into the T-75 flask.

☐ Incubate for 4 days at 37℃, 5% CO_2.

2-2. Medium change (C medium→A medium)

☐ Prepare 20 mL of A medium in a 50 mL polypropylene conical tube.

Reagent	Final concentration in medium	Required amount
RPMI-1640	-	☐ 17.8 mL
FBS	10 %	☐ 2.0 mL
Antibiotic-Antimycotic	1×	☐ 0.2 mL
G418	300 μg/mL	☐ 0.12 mL
Puromycin*	0.15 μg/mL	-

* Dilute 10 mg/mL Puromycin 10-fold in medium and add 3 μL to the above medium.

☐ Pre-warm of A medium in a 50 mL polypropylene conical tube in a 37℃ water bath.

☐ Count the number of cells in culture with T-75 flask and harvest the cells into a 15 mL polypropylene conical tube.

☐ Centrifuge the tube at 350 x *g* at room temperature for 5 min.

☐ Discard the supernatant, resuspend in 4 mL of A medium and count the number of cells.

☐ Adjust with A medium to about 5.0×10^5 cells/mL and seed the cells into T-75 flask (7.5×10^6 cells/15 mL in a T-75 flask).

☐ Incubate for 3-4 days at 37℃, 5% CO_2 (check the cell condition and incubate until $1.5\text{-}2.0\times10^6$ cells/mL).

2-3. Passage

- ☐ Count the number of cells in culture with the T-75 flask.
  * Confirm cell proliferation up to 1.5×10^6 cells/mL and passaging. Do not allow the cell density to exceed 2.0×10^6 cells/mL.
- ☐ Prepare 20 mL of A medium in a 50 mL polypropylene conical tube and pre-warm of A medium in a 37℃ water bath.
- ☐ Count the number of cells in culture with T-75 flask and harvest the cells into a 15 mL polypropylene conical tube.
- ☐ Centrifuge the tube at 350 x *g* at room temperature for 5 min.
- ☐ Discard the supernatant, resuspend in 4 mL of A medium and count the number of cells.
- ☐ Adjust with A medium to about 5.0×10^5 cells/mL and seed the cells into T-75 flask (7.5×10^6 cells/15 mL in a T-75 flask).
- ☐ Incubate for 3-4 days at 37℃, 5% CO_2 (check the cell condition and incubate until 1.5-2.0×10^6 cells/mL).

2-4. Cryopreservation

- ☐ Count the number of cells in culture with the T-75 flask.
  * Confirm cell proliferation up to 1.5×10^6 cells/mL and passaging. Do not allow the cell density to exceed 2.0×10^6 cells/mL.
- ☐ Prepare CELLBANKER 1 on ice.
- ☐ Harvest the cells from T-75 flask into a 15 mL (or 50 mL) polypropylene conical tube.
- ☐ Centrifuge the tube at 350 x *g* at room temperature for 5 min.
- ☐ Discard the supernatant and tapping the tube to loosen the cell pellet.
- ☐ Suspend the cells to 2.0×10^6 cells/500 μL CELLBANKER1, dispense 500 μL into each cryotube, and store immediately at -80℃.
- ☐ Next day, transfer the cells from -80℃ to the gas phase of liquid nitrogen tank.

2-5. Methods for determining transmittance factor

- ☐ Remove TripLuc from -80℃ 1 hour before measurement and thaw it at room temperature (light shielded).
  *The TripLuc should be dispensed at 1 mL/tube at the time of the first thawing to avoid repeated freeze and thaw.
- ☐ Prepare 10 mL of B medium.

Reagent	Final concentration in medium	Required amount
RPMI-1640	-	☐ 9 mL
FBS	10 %	☐ 1 mL

- ☐ Remove SLO and SLR and thaw on ice (store at -80°C until use).
- ☐ Preparation of enzyme solution:
  Add 10 µL of SLO (or SLR) to 1 mL of B medium to prepare 1× enzyme solution. Based on the 1× enzyme solution, prepare 1/4, 1/16, and 1/64×enzyme solutions.
- ☐ The enzyme solution was applied to a 96-well black plate at 100 µL/well (N=3).
- ☐ Add 100 µL of Tripluc to the well containing the enzyme solution and pipette 4 times to mix well.
- ☐ Shake the mixture in a plate shaker for 10 minutes at room temperature (light shielded).
- ☐ Bioluminescence is measured for 3 sec each in the absence (F0) and presence (F1) of the optical filter (The optical filter used in measurement is 600–620 nm long or short pass filter, or 600–700 nm band pass filter).
- ☐ Transmission coefficient of the optical filter was calculated as follows:
  ・Transmission coefficient SLO = F1 / F0
  ・Transmission coefficient SLR = F1 / F0
  Calculated transmittance factors are used for all the measurements executed using the same luminometer.

3. Cells assay

3-1. Preparation of positive control and negative control

Preparation of 4-NBB

- ☐ Put 20 mg of 4-NBB into a 1.5 mL microfuge tube (light shielded) and add up to 1 mL of X-VIVO 15 to it.
- ☐ Using a vortex mixer for 2 minutes, mix well (it will not dissolve, so vortex until there are no remaining particles).
- ☐ Shaken on a rotor at a speed of 8 rpm for at least 30 min.
- ☐ Centrifuge the tube at 20,000 x *g* at room temperature for 5 min.
- ☐ Collect the supernatant in a 1.5 mL microfuge tube and prepare a 4-fold dilution with X-VIVO 15 (the diluted supernatant).
- ☐ Prepare the serial dilutions (1/2, 1/4. 1/8 and 1/16) based on the diluted supernatant.

Preparation of Lactic acid (LA)

- ☐ Prepare a 20 mg/mL solution by diluting the LA stock solution 60.6 times with X-VIVO 15 (the diluted supernatant).
- ☐ Prepare the dilutions (1, 2 and 4 mg/mL) based on the diluted supernatant.

3-2. Preparation of cells for assay

* THP-G8 cells for assay should be passaged 2 times before the assay.

- ☐ Count the number of cells in culture with the T-75 flask.
  * Confirm cell proliferation up to 1.5×10^6 cells/mL and passaging. Do not allow the cell density to exceed 2.0×10^6 cells/mL.
- ☐ Prepare 10 mL of B medium in a 15 mL polypropylene conical tube and pre-warm of B medium in a 37℃ water bath.
- ☐ Count the number of cells in culture with T-75 flask and harvest the number of required cells into a 15 mL polypropylene conical tube.
- ☐ Centrifuge the tube at 350 x *g* at room temperature for 5 min.
- ☐ Discard the supernatant and tapping the tube to loosen the cell pellet.
- ☐ Resuspend in 1 mL of B medium and count the number of cells.
- ☐ Adjust with B medium to 1.0×10^6 cells/mL and applied 50 µL into each well of a 96well black plate (5.0×10^4 cells/well).

3-3. Treatment of THP-G8 cells

- ☐ Add 50 µL of the prepared 4-NBB and LA to each well containing cells (no pipetting).
- ☐ Incubate for 16 hours at 37℃, 5% CO_2.

3-4. Measurement

- ☐ Remove TripLuc from -80℃ 1 hour before measurement and thaw it at room temperature (light shielded).
  *The TripLuc should be dispensed at 1 mL/tube at the time of the first thawing to avoid repeated freeze and thaw.
- ☐ Add 100 µL of Tripluc to the well using an 8 channel or 12 channel pipet man and pipette 4 times to mix well.
- ☐ Shake the mixture in a plate shaker for 10 minutes at room temperature (light shielded).
- ☐ 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.

3-5. Data evaluation

- ☐ Use the Data sheet (ver.021.1) and put the values F0 in the “Data without filter” and F2 in the “Data using filter” of the Data Input sheet.
- ☐ The results of the assay are automatically calculated on the “4NBB, Lactic acid” sheet.
- ☐ Assay criteria are Ind-IL8LA greater than or equal to 5.0 for at least one concentration of 4-NBB and Ind-IL8LA less than or equal to 1.4 for all concentrations of LA.
  * Only THP-G8 cells that have passed this criteria can be used for IL-8 Luc assay.

4. Prediction model (for test chemicals)

4-1. Preparation of cells for assay

* THP-G8 cells for assay should be passaged 2 times before the assay.

** Use THP-G8 cells until 6 weeks after thawing or 12 passage.

- ☐ Count the number of cells in culture with the T-75 flask.
  * Confirm cell proliferation up to 1.5×10^6 cells/mL and passaging. Do not allow the cell density to exceed 2.0×10^6 cells/mL.
- ☐ Prepare 10 mL of B medium in a 15 mL polypropylene conical tube and pre-warm of B medium in a 37°C water bath.
- ☐ Count the number of cells in culture with T-75 flask and harvest the number of required cells into a 15 mL polypropylene conical tube.
- ☐ Centrifuge the tube at 350 x *g* at room temperature for 5 min.
- ☐ Discard the supernatant and tapping the tube to loosen the cell pellet.
- ☐ Resuspend in 1 mL of B medium and count the number of cells.
- ☐ Adjust with B medium to 1.0×10^6 cells/mL and applied 50 µL into each well of a 96well black plate (5.0×10^4 cells/well).
  * 2.5×10^6 cells are required for each reagent to be tested.

4-2. Preparation of test chemicals

Soluble chemicals

- ☐ Put 20 mg into a 1.5 mL microfuge tube (light shielded) and add up to 1 mL of X-VIVO 15 to it. In case the test substance is a liquid, calculate the specific gravity and put the volume required for 20 mg in a 1.5 mL microfuge tube (light shielded) and add up to 1 mL.
- ☐ Using a vortex mixer for 2 minutes, mix well.
- ☐ Shaken on a rotor at a speed of 8 rpm for at least 30 min.
- ☐ Prepare 4 mg/mL solution (500 µL) with X-VIVO15 and apply into #12 well of a 96well assay plate.
- ☐ Apply 250 µL X-VIVO 15 into #1~11 wells.
- ☐ Repeat 2-fold dilution to prepare serial dilutions (250 µL each transferred to the next well to the left).

Insoluble chemicals

- ☐ Put 20 mg into a 1.5 mL microfuge tube (light shielded) and add up to 1 mL of X-VIVO 15 to it. In case the test substance is a liquid, calculate the specific gravity and put the volume required for 20 mg in a 1.5 mL microfuge tube (light shielded) and add up to 1 mL.
- ☐ Using a vortex mixer for 2 minutes, mix well (it will not dissolve, so vortex until there are no remaining particles).

- ☐ Shaken on a rotor at a speed of 8 rpm for at least 30 min (light shielded).
- ☐ Sonicate the sample for 30 seconds in an ultrasonic disruptor while cooling and keep on ice for 30 seconds.
- ☐ Repeat the sonication and incubation for about 5 times until the test substance is completely dissolved or stably dispersed.
- ☐ Then, shaken on a rotor at a speed of 8 rpm for at least 30 min again (light shielded).
- ☐ Centrifuge the tube at 20,000 x *g* at room temperature for 5 min.
- ☐ Transfer the supernatant to a new 1.5 mL tube (be careful not to absorb the dissolved substance. If you absorb them, repeat centrifuge).
- ☐ Apply 500 µL of the supernatant into the #12 well of a 96well assay plate.
- ☐ Apply 250 µL X-VIVO 15 into #1~11 wells.
- ☐ Repeat 2-fold dilution to prepare serial dilutions (250 µL each transferred to the next well to the left).

4-3. Data evaluation

- ☐ Use the Data sheet (ver.021.1) and put the values F0 in the “Data without filter” and F2 in the “Data using filter” of the Data Input sheet.
- ☐ The results of the assay are automatically calculated on the “Result Format” and “Graph” sheet.
  * In the first test, determine the lowest concentration (CV05) at which Inh-GAPLA is less than 0.05. For the second test, use a concentration one step higher than this determined one as the highest concentration (#12 well). From this highest concentration, prepare a serial dilution by 1.5-fold. If the Inh-GAPLA value of the highest concentration of the test substance does not decrease to less than 0.05 or is less than 0.05, the second test is prepared as a serial dilution by 1.5-fold from the highest concentration of the first test.

5. Q&A

Q1. What are the storage conditions for the cells?

A1. Cells should be stored in the gas phase of liquid nitrogen as soon as possible after arrival, and cells should be thawed, quality check test and stock production should be carried out within 30 days after shipment.

Q2. What is the shipping condition?

A2. The cells will be packed in dry ice for delivery. In the case that the cells are not in a proper condition upon arrival, please contact us.

Q3. Do you check for viral infections?

A3. We have checked for 9 types of viruses: HIV1, HIV2, HTLV1&2, HCV, HBV, Parvovirus B19, EBV, CMV, and WNY, and have confirmed negative results.
In addition, mycoplasma negation test and germ-free test are performed for each lot of products, and negative results have been confirmed.

Q4. How do you stock cells?

A4. When the cell density reaches 1.5 to 2×106 cells/mL, cells are stored at 2×105 cells/0.5 mL/vial using CELLBANKER 1. It is possible to store cells in 10% DMSO/culture medium instead of CELLBANKER 1.

Q5. After thawing frozen cells and starting culture, the number of cells does not increase.

A5. It might be that cells were warmed too long in the water bath when thawing. When thawing cells, we recommend that cells be half-thawed and added 1 mL from 9 mL of warmed C medium in a 15-mL tube while ice cubes remain, and then collected in a 15-mL tube and centrifuged.

Q6. Cells do not increase.

A6. It is possible that the cells are seeded at a low density. At our company, we seed cells at 5×10^5 cells/mL and passage cells when they proliferate to $1.5\text{-}2\times10^6$ cells/mL in 3 to 4 days.
We recommend that cells be passaged when they have grown well. In addition, we suggest that the cell pellets after centrifugation should be loosened by tapping to reduce damage to cells.

Q7. I have trouble checking the reactivity of cells in 4-NBB and LA.

A7. It is suggested that the cell condition affects the results of the assay. We recommend that cells be applied to the assay plate when the cells have proliferated to 1.5 to 2×10^6 cells/mL. It is also recommended to perform steady pipetting after the addition of Tripluc.

Q8. The measured luminescence value is too low.

A8. The sensitivity of the equipment you use is not sufficient. The measured values of the standard enzyme solution are useful for reference, so please contact us with the measured values.

Q9. I do not have much experience in handling cells and worry about the technical skill.

A9. Our technical staff will answer your questions, so please feel free to contact us.