厚生労働科学研究費補助金（化学物質リスク研究事業）
化学物質の動物個体レベルの免疫毒性データ集積とそれに基づく Multi-ImmunoTox assay （ MITA ） による予測性試験法の確立と国際標準化
（ H30-化学-一般-001 ）

分担研究報告書
IL-1 Luc assayクライテリアの設定ならびにプロトコールの作成

研究分担者　木村　裕　東北大学病院皮膚科・助教

研究要旨

平成 30 年度に実施された IL-1 Luc assay validation 試験 Phase I 試験後に validation management team (VMT)会議にて改変された IL-1 Luc assay 用クライテリアに沿って Phase II 試験用プロトコールを作成した。（Multi-Immuno Tox Assay protocol for TGCHAC-A4 ver.009E）そのプロトコールに則り令和元年 7 月より IL-1 Luc assay validation 試験 Phase II が実施された。Phase II 試験に先立ち３３候補被検物質の判定を行い VMT の chemical selection team にデータを提出し、そのデータをもとに Phase II における２０被検物質が決定された。また、Phase II 試験で使用するデータシートを作成した。Phase II 試験は１２月に終了し、その後令和２年１月に開催された VMT 会議にて施設内および施設間再現性の結果が承認され IL-1 Luc assay validation 試験が終了されたことを受け IL-1 Luc assay validation report の作成を開始した。

昨年度まで作成してきた６０化学物質による免疫毒性化学物質のデータベース作成を拡充し、９７化学物質からなるデータベースを作成した。

A．研究目的

IL-1 Luc assayの国際バリデーション試験 Phase II実施に向け、その際に使用するクライテリアの設定、プロコトールを作成することを目的とした。

B．研究方法

以下の方法によりIL-1βプロモーター活性の測定を行った。ヒト急性単球性白血病由来細胞株THP-1にIL-1βプロモーターに制御されたSLGルシフェラーゼ遺伝子（緑色に発色）、GAPDHプロモーターに制御されたSLRルシフェラーゼ遺伝子（赤色に発色）を導入したTHP-G1b(TGCHAC-A4)細胞を1ウェル当たり1×10^5個、96-well プレートに播種し化学物質を加え、37℃、5%CO_2下で1時間培養した。つづいてLipopolysaccharide (LPS)で刺激し37℃、5%CO_2下で6時間培養した。その後、細胞溶解剤とルシフェラーゼ反応の基質であるルシフェリンの混合剤であるTripluc luciferase assay reagent (TOYOBO)を混合し、室温で10分振盪させたのちマルチプレート対応型ルミノメーターにてルシフェラーゼ活性を測定した。SLG、SLRルシフェラーゼは共通の基質の存在により同時に発光するが、光学的フィルターにより分離し、各ルシフェラーゼの発光量(SLG-luciferase activity(SLG-LA)、SLR-luciferase activity(SLR-LA))を検出した。また細胞数の違いや各種刺激後の生存率の違いを勘案しSLG-LAをSLR-LAで除することにより normalized SLG-luciferase activity(nSLG-LA)を算出した。さらに以下の式により化学物質によるIL-1βプロモーター活性の抑制率%suppressionを計算した。

% suppression = (1-化学物質存在下でのnSLG-LA/化学物質非存在下でのnSLG-LA) X 100。

（倫理面への配慮）

本研究では主に細胞株を使用しており倫理面の問題はないと判断した。

C．研究結果

1． Phase II試験用プロトコールを作成

平成３０年度にIL-1 Luc assay validation試験 Phase I試験をMulti-Immuno Tox Assay protocol for TGCHAC-A4 ver.008Eに則り実施した。試験終了後、参加施設のデータを検討しPhase II試験に向け下記のようにアクセプタンスクライテリア、クライテリアを変更した。

アクセプタンスクライテリア

- LPS添加時のnIL1LAの誘導の許容下限が5.0であったのを3.0に変更した。
- Inh-GAPLAが0.05以上となる濃度が６未満かつ結果が陰性の場合はそのアッセイを棄却しその後のアッセイは濃度を低くして行うよう変更した。

クライテリア

- %suppressionの閾値を20%から25％に変更した。
- 結果をno effect, suppression, augmentationと３者に分類していたのを、augmentationをno effectに含めてno effect, suppressionの２者への分類に変更した。
- 2000 mg/mLの濃度における結果を除外した。

以上の結果を反映させたプロトコール、Multi-Immuno Tox Assay protocol for TGCHAC-A4 ver.009Eを作成した。(添付資料１)

2． Phase II試験３３候補被検物質の判定

Phase I終了後にVMTのchemical selection teamにより選定されたPhase II試験３３候補被検物質についてIL-1 Luc assayの判定結果、入手方法、IL-1発現への影響についての論文報告をまとめVMTに提出した。(添付資料２) このデータをもとにPhase IIにおける２０被検物質が決定された。

3． Phase II試験で使用するデータシート、記録用紙の作成

神戸大学の協力を得てPhase II試験用のデータシートを作成し、参加施設に配布した。(添付資料３)

4． 免疫毒性化学物質のデータベース作成

昨年度まで作成してきた６０化学物質による免疫毒性化学物質のデータベース作成を拡充し、９７化学物質からなるデータベースを作成した。(添付資料４)

D．考察

Phase I試験後に変更したクライテリアを適応したPhase II試験試験では施設内および施設間再現性共に良好な試験結果が得られた。予測性については今後論文を収集し検討する予定である。

E．結論

令和元年度に行われたIL-1 Luc assay validation試験 Phase II試験への準備として プロトコール、データシート、記録用紙を作成しPhase II試験が実施された。施設内および施設間再現性共に結果は良好でIL-1 Luc assay validation試験は終了しIL-1 Luc assay validation reportの作成を開始した。

F．健康危険情報

なし

G．研究発表

1． 論文発表

1) Kimura Y, Yasuno R, Watanabe M, Kobayashi M, Iwaki T, Fujimura C, Ohmiya Y, Yamakage K, Nakajima Y, Kobayashi M, Mashimo N, Takagi Y, Omori T, Corsini E, Germolec D, Inoue T, Rogen EL, Kojima H, Aiba S An international validation study of the IL-2 Luc assay for evaluating the potential immunotoxic effects of chemicals on T cells and a proposal for reference data for immunotoxicchemicals. Toxicol In Vitro 2020 in press.

2． 学会発表

1) 木村 裕、安野 理恵、渡辺 美香、小林 美和子、岩城 知子、藤村 千鶴、近江谷 克裕、山影 康次、中島 芳浩、真下 奈々、高木 佑実、大森 崇、小島 肇、相場 節也：Multi-ImmunoTox Assay (MITA)の予測性評価に必要な文献に基づく化学物質免疫毒性分類の試み 日本動物実験代替法学会 第32回大会 つくば (2019.11)

（予定を含む。）

H．知的財産権の出願・登録状況　　**なし**

Multi-Immuno Tox Assay protocol for THP-G1b (TGCHAC-A4) ver. 009E

July 1st, 2019

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

1. Introduction

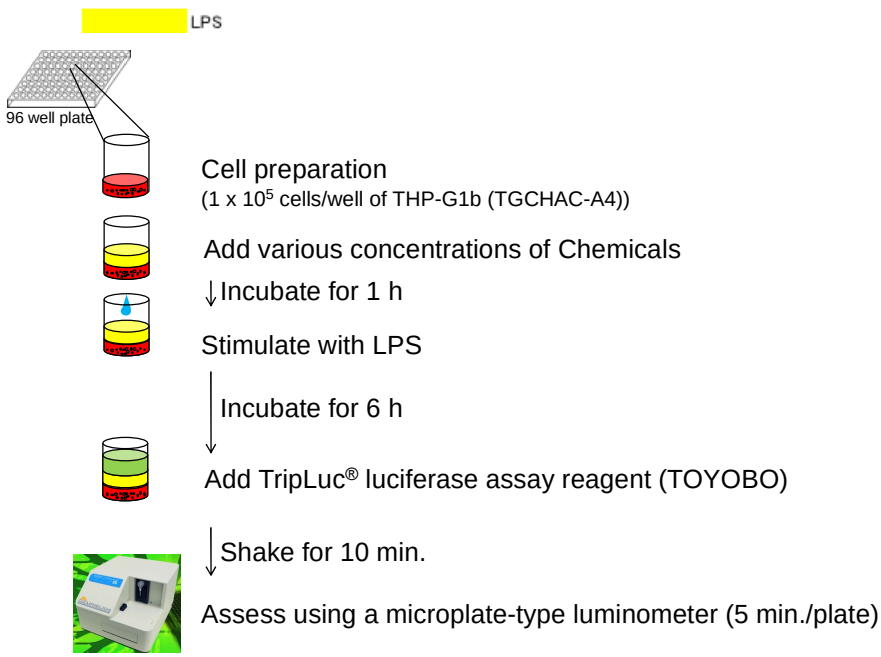
This protocol describes how to maintain the cells, how to prepare the test chemicals, and how to measure the luciferase activity of THP-G1b (TGCHAC-A4), THP-1 cells transfected with 2 luciferase genes, stable luciferase orange (SLG) on the human artificial chromosome (HAC) vector and stable luciferase red (SLR), under the control of IL-1β and G3PDH promoters, respectively, for the Multi-Immuno Tox Assay.

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

Figure 1

Assay design

flat-bottom B&W	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	cont (distilled water or DMSO)	LPS 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
D			Chemical (common ratio of 2, 10 concentrations, n=4)									
E												
F												
G												
H												



2. Materials

2-1 Cells

- THP-G1b (TGCHAC-A4) (IL1β-SLG、G3PDH-SLR)

The human macrophage-like cell line THP-1 was obtained from the American Type Culture Collection (Manassas, VA, USA). A THP-1-derived IL-1β reporter cell line, THP-G1b (TGCHAC-A4), that harbors the SLG and SLR luciferase genes under the control of the IL-1β and G3PDH promoters, respectively, was established by the Dept. of Dermatology, Tohoku University School of Medicine and GPC laboratory Co. Ltd.

(Kimura Y. et al. Profiling the immunotoxicity of chemicals based on in vitro evaluation by a combination of the Multi-ImmunoTox assay and the IL-8 Luc assay. Archives of Toxicology, 92, 2043-2054, 2018)

2-2 Reagents and equipment

2-2-1 For maintenance of the THP-G1b (TGCHAC-A4) 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)

2-2-2 For chemical exposure, stimulation, positive control and solvents

- Lipopolysaccharide (LPS) from Escherichia coli K12 (Invivogen Cat#tlrl-eklps, Lot#: LEK-39-01)
- Dexamethasone (CAS:50-02-2, Fujifilm Wako Pure Chemical Cat#041-18861)
- 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 black-flame and white-well plate (flat-bottom, for measurement of the luciferase activity, e.g. PerkinElmer B&W Isoplate-96 TC Cat#6005060)
- 96 well clear plate (round-bottom, for preparation of chemicals and stimulants)
- 96 well assay block, 2 mL (e.g., Costar Cat#3960)

- Seal for 96 well plate (e.g., Perkin Elmer TopSeal-A PLUS Cat#6050185, EXCEL Scientific SealMate Cat#SM-KIT-SP)
- Reservoir
- Pipette

2-2-5 Equipment for measurement of luciferase activity

- Measuring device: a microplate-type luminometer with a multi-color detection system that can accept 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

2-2-6 Others

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

2-3 Culture medium

2-3-1A medium: for maintenance of THP-G1b (TGCHAC-A4) cells (500 mL, stored at 2-8°C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO Cat #11875-093	-	-	445 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	50 mL
Antibiotic-Antimycotic	e.g., GIBCO Cat #15240-062	100×	1×	5 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 Cat #11875-093	-	-	27 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	3 mL

2-4 Preparation of the stimulant of THP-G1b (TGCHAC-A4) cells

2-4-1 Lipopolysaccharide (LPS) from Escherichia coli K12

Reagent	Company	Concentration of the stock solution	Final concentration
Lipopolysaccharide (LPS) from Escherichia coli K12	Invivogen Cat#tlrl-eklps	1 mg/mL	100 ng/mL
Distilled water	GIBCO Cat#10977-015		

Dissolve 5 mg LPS using distilled water 5 mL, dispend at 5 μL/tube and store at freezer at -30°C. Use these stocks within 6 months after dissolution.

3. Cell culture

3-1 Thawing of THP-G1b (TGCHAC-A4) cells

Pre-warm 9 mL of A medium in a 15 mL polypropylene conical tube in a 37°C water bath (for centrifugation) and 15 mL of A 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 A medium. Centrifuge the tube at 120-350 x *g* at room temperature for 5 min, discard the supernatant, and resuspend in 15 mL of pre-warmed A medium in a T-75 Flask. Cells are incubated at 37°C, 5% CO_2.

3-2 Maintenance of THP-G1b (TGCHAC-A4) cells

3 or 4 days after thawing, pre-warm the A medium in a T-75 Flask at 37°C in a 5% CO_2 incubator. Count the number of cells, centrifuge the tube at 120-350 x *g* at room temperature for 5 min, discard the supernatant, and resuspend in the pre-warmed A medium in a T-75 Flask. Cells are passaged at $2\text{-}5x10^5$/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.

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 (5.0 x 10^6 cells are required, but to have some leeway, 7.5 x 10^6 cells should be prepared), centrifuge the tube at 120-350 x *g*, 5 min. Resuspend in pre-warmed the B medium at a cell density of 2×10^6/mL. Transfer the cell suspension to a reservoir, and add 50 µL of cell suspension to each well of a 96 well black-flame and white-well plate (flat bottom) using an 8 channel or 12 channel pipetman. (cf. Figure 2)

Figure 2

flat-bottom B&W	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL
D	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL
E	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL
F	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL
G												
H												

5. Preparation of chemicals and cell treatment with chemicals

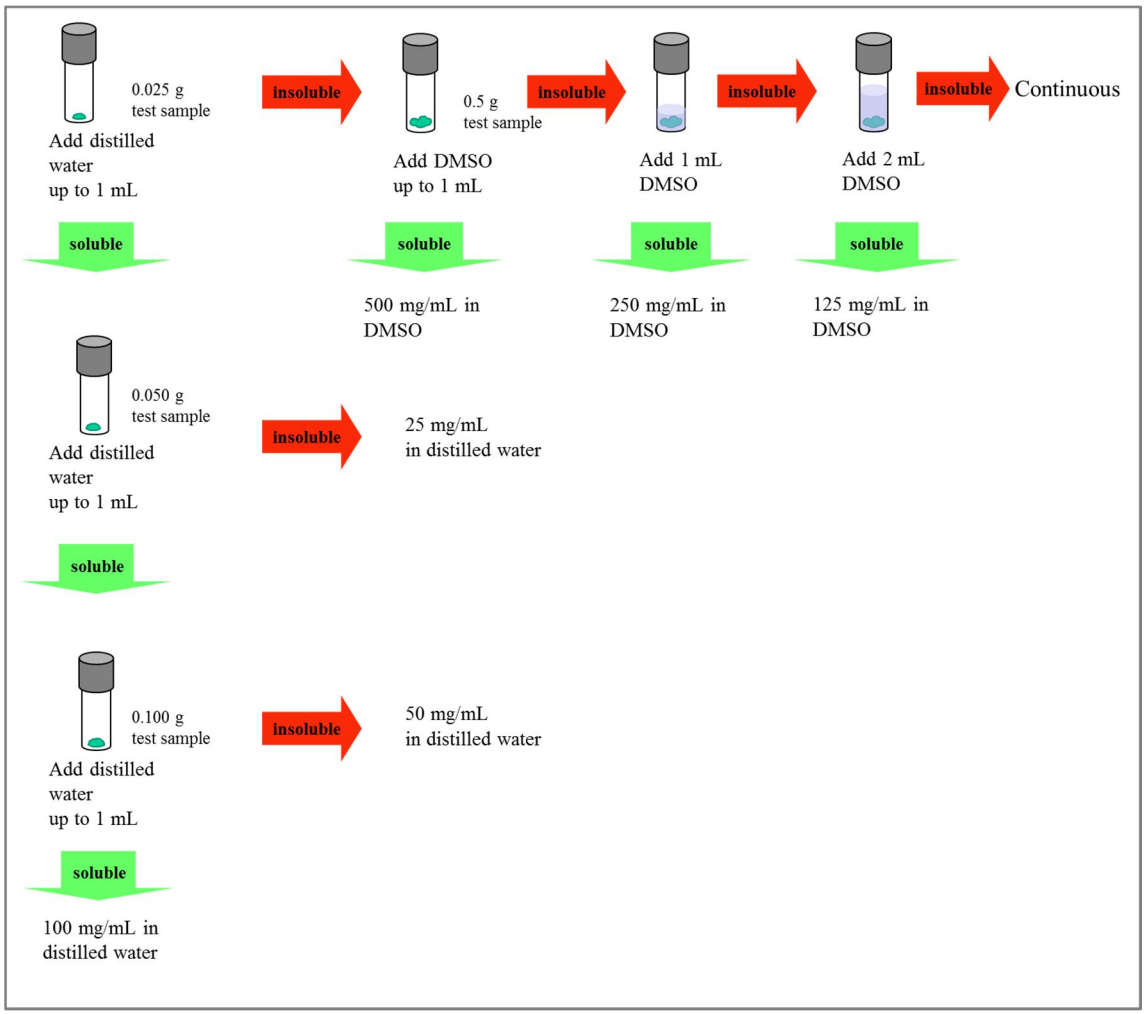
5-1 Dissolution by vehicle (cf. Figure 3)

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. Being soluble should be confirmed by centrifugation at 15,000 rpm (≈20,000 x g) for 5 min and absence of precipitation. The chemical should be used within 4 hours after being dissolved in distilled water or DMSO.

Figure 3



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

In the second to fifth experiment (2nd to 5th experiment), determine the minimum concentration at which I.I.-SLR-LA (mentioned later in **10**) became lower than 0.05 in the 1st experiment, use the concentration one step (2-times) higher than this determined concentration as the highest concentration of the chemical to examine, and conduct 10 serial dilutions at a common ratio of 2 from the highest concentration. If I.I.-SLR-LA did not become lower than 0.05 or became lower than 0.05 at the highest concentration in the 1st experiment, conduct 10 serial dilutions at a common ratio of 2 from the highest concentration in the 1st experiment.

For example, in Figure 4 below, the minimum concentration at which I.I.-SLR-LA became lower than 0.05 is 1.95 μg/ml. The highest concentration of the chemical to examine is the concentration one

step (2-times) higher than 1.95 μg/ml, which is 3.91 μg/ml.

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

Figure 4

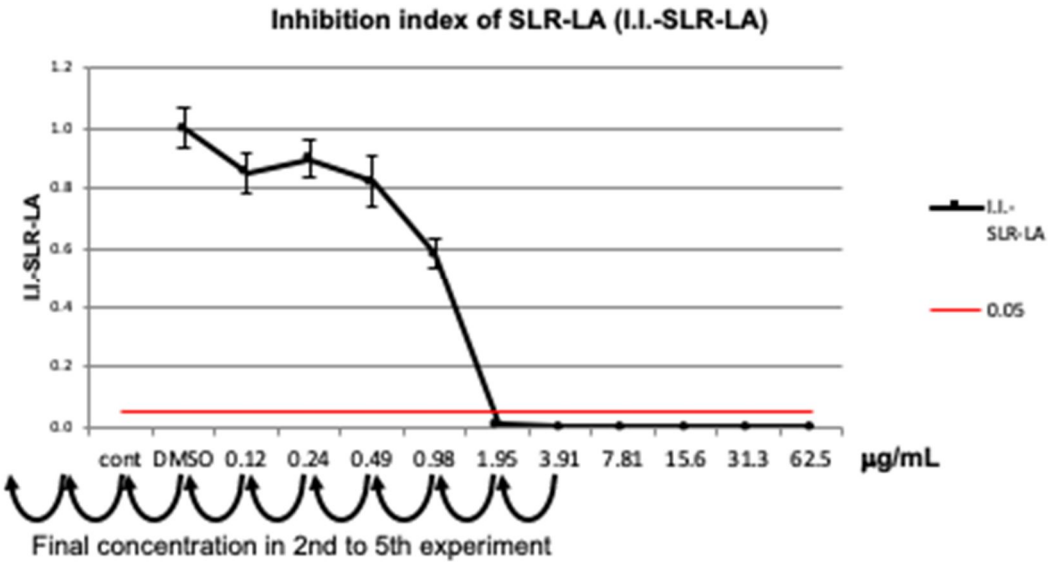
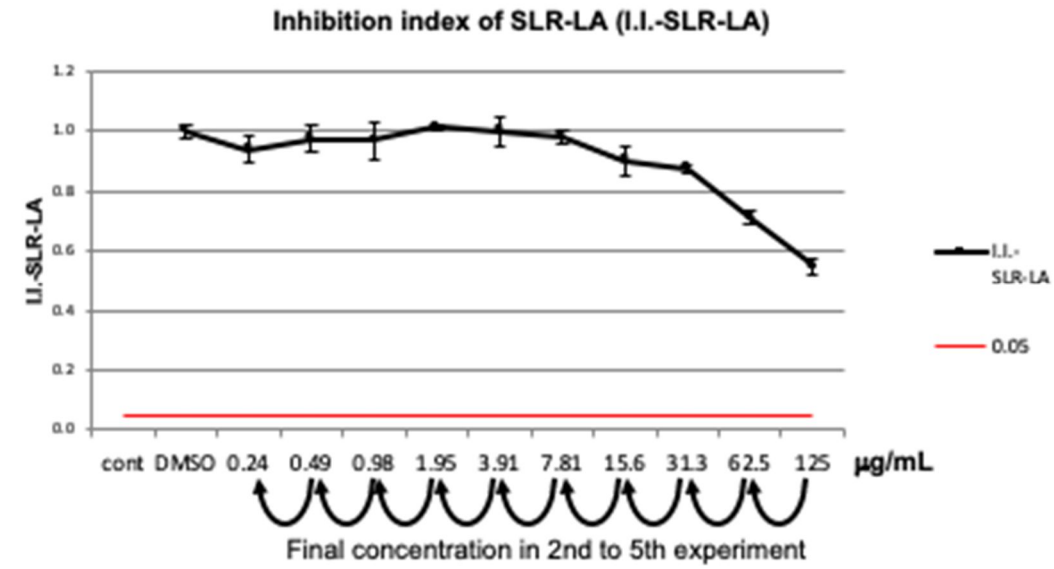


Figure 5



5-2 When the chemical is prepared in distilled water

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

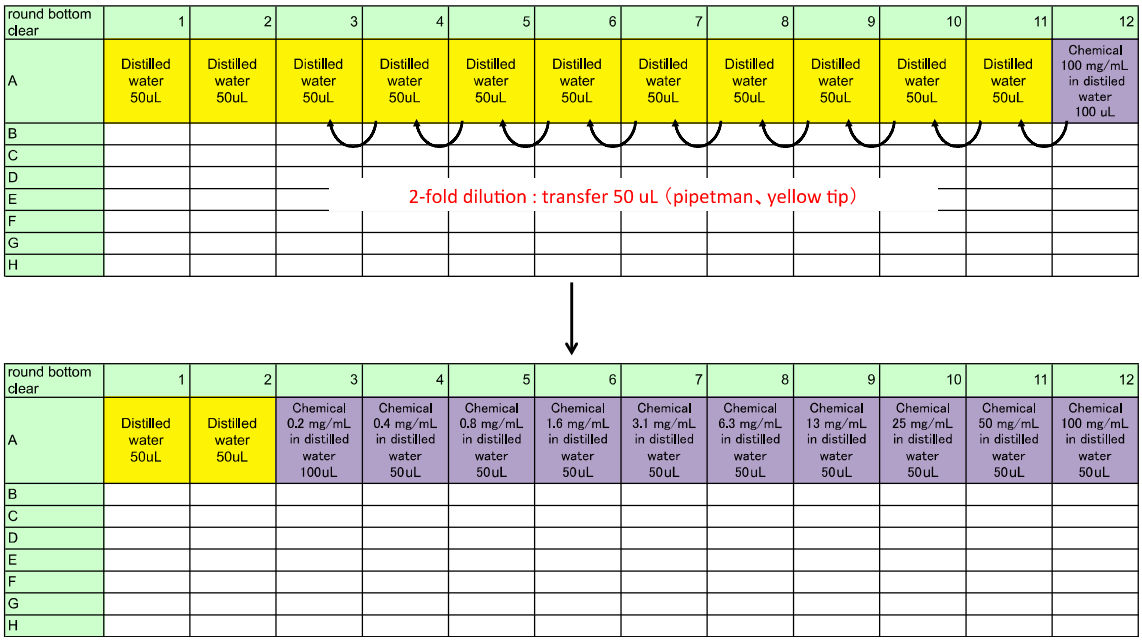
5-2-1 Arrangement of chemicals and vehicle

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

5-2-2 Serial dilution

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

Figure 6



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 THP-G8 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 1 hour (37°C, CO_2, 5%) (cf. Figure 7-9).

Figure 7

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

20uL

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

Figure 8

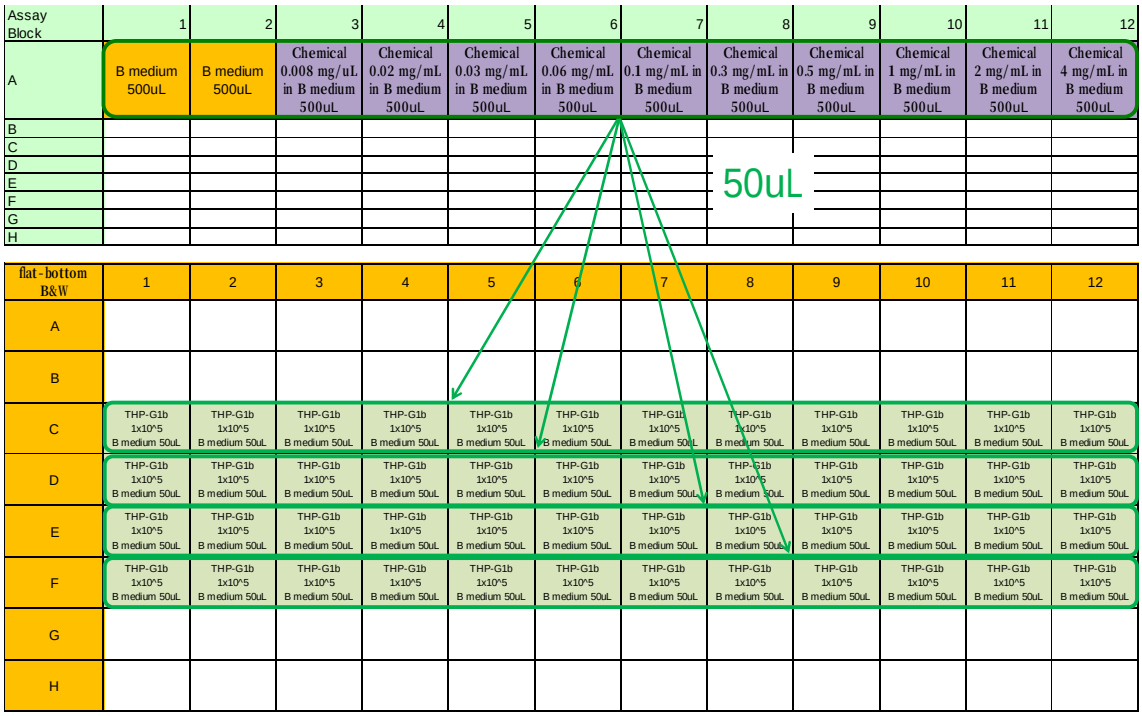


Figure 9 Final constituents of each well of the plate

flat-bottom B&W	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	Chemical 0 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.004 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.008 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.02 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.03 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.06 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.1 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.3 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.5 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 1 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 2 mg/mL THP-G1b 1x10^5 B medium 100uL
D	Chemical 0 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.004 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.008 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.02 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.03 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.06 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.1 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.3 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.5 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 1 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 2 mg/mL THP-G1b 1x10^5 B medium 100uL
E	Chemical 0 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.004 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.008 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.02 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.03 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.06 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.1 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.3 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.5 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 1 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 2 mg/mL THP-G1b 1x10^5 B medium 100uL
F	Chemical 0 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.004 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.008 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.02 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.03 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.06 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.1 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.3 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 0.5 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 1 mg/mL THP-G1b 1x10^5 B medium 100uL	Chemical 2 mg/mL THP-G1b 1x10^5 B medium 100uL
G												
H												

5-3 When the chemical is prepared as a 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 10)

Figure 10

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

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

↓

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

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

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

Figure 11

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 THP-G8 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. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 1 hour (37°C, CO_2, 5%) (cf. Figure 12-14).

Figure 12

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

10uL

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

Figure 13

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

50uL

flat-bottom B&W	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL
D	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL
E	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL
F	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL
G												
H												

Figure 14 Final constituents of each well of the plate

flat-bottom B&W	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	Chemical 0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 1.0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 2.0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 3.9ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 7.8ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 16ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 31ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 63ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 125ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 250ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 500ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL
D	Chemical 0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 1.0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 2.0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 3.9ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 7.8ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 16ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 31ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 63ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 125ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 250ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 500ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL
E	Chemical 0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 1.0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 2.0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 3.9ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 7.8ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 16ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 31ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 63ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 125ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 250ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 500ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL
F	Chemical 0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 1.0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 2.0ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 3.9ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 7.8ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 16ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 31ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 63ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 125ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 250ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL	Chemical 500ug/mL 0.1% DMSO THP-G1b 1x10^5 B medium 100uL
G												
H												

6. Preparation of the stimulant (Lipopolysaccharide (LPS)) and addition to THP-G1b (TGCHAC-A4)

6-1 Material

- 1 mg/mL LPS stock

6-2 Preparation of 1000 ng/mL LPS solution

Dilute 1 mg/mL LPS stock with distilled water as follows (1000 times, final concentration is 1000 ng/mL). Add distilled water as control to well #A1-#D1 of the 96 well clear plate (round bottom), and add 1000 ng/mL LPS solution to wells #A2-#D2 of the 96 well clear plate (round bottom).

1st step

1 mg/mL LPS	distilled water	Total	final concentration
5 μL	995 μL	1000 μL	5 μg/mL

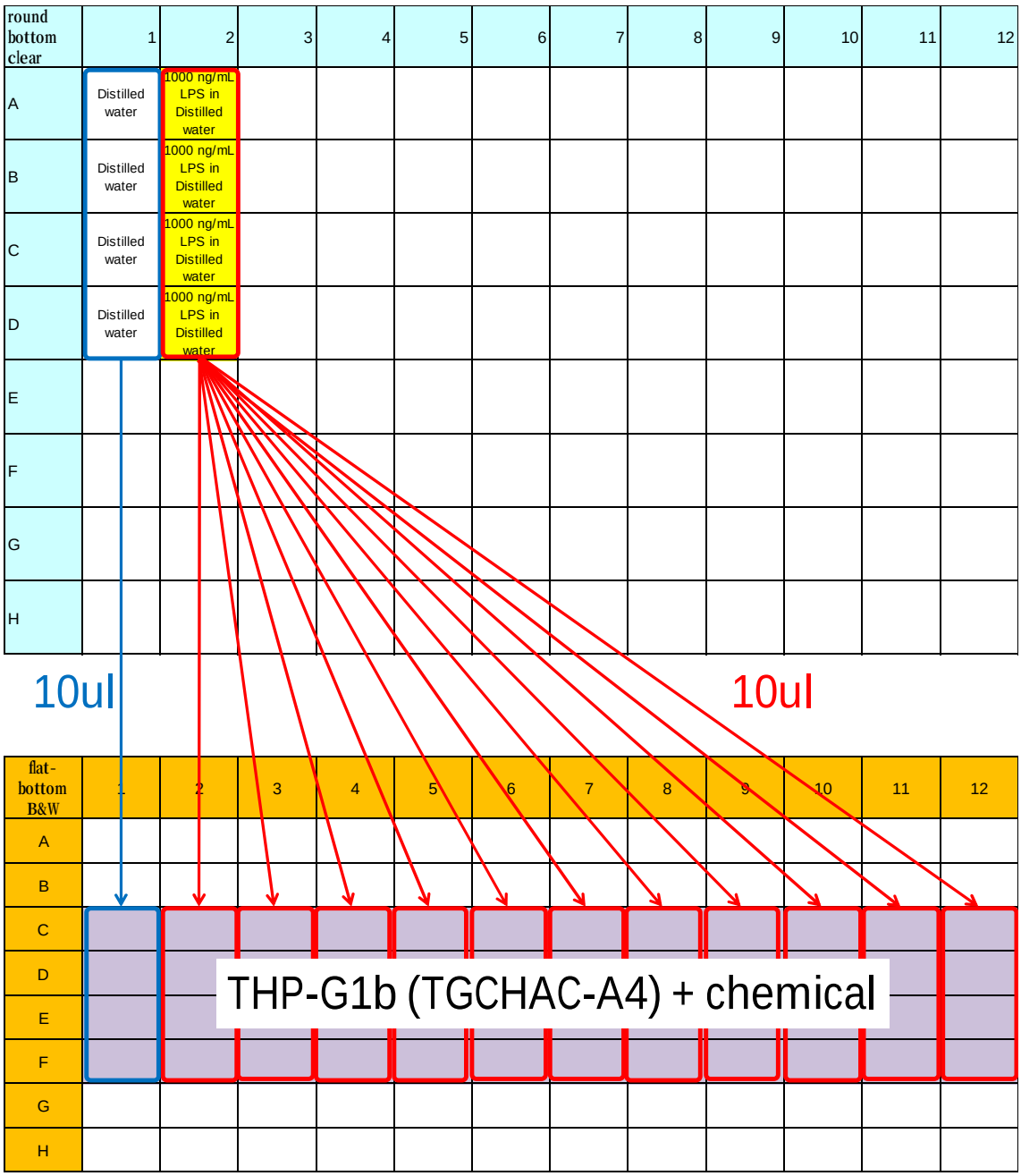
2nd step

5 μg/mL LPS	distilled water	Total	final concentration
250 μL	1000 μL	1250 μL	1000 ng/mL

6-3 Addition of LPS to THP-G1b (TGCHAC-A4)

One hour after the addition of chemicals, add 10 μL of control or 1000 ng/mL LPS solution to the cells (#C1-#F1 or #C2-#F12, respectively) using an 8 channel or 12 channel pipetman after pipetting 20 times. Make sure that the apex of the tip is dipped into the medium. Change tips every line you add. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 6 hours (37°C, CO_2, 5%). (cf. Figure 15)

Figure 15



7. Positive control

7-1 Preparing control chemical (dexamethasone)

7-1-1 Preparing dexamethasone stock

Reagent	Company	Concentration of the stock solution	Preparing concentration	Final concentration
Dexamethasone	Fujifilm Wako Pure Chemical Cat#041-18861	100 mg/mL	10, 50, 100 mg/mL	10, 50, 100 μg/mL
Dimethyl sulfoxide (DMSO)	Sigma Cat#D5879			

Dissolve 1 g of Dexamethasone with DMSO 10 mL, dispend at 100 μL/tube and store at freezer at -30°C.

7-2 Preparation of cells for assay

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

Use cells between 1 and 6 weeks after thawing.

Pre-warm the B medium in a 37°C water bath. Count the number of cells and collect the number of cells needed (2.0 x 10^6 cells are required, but to have some leeway, 3 x 10^6 cells should be prepared), centrifuge the tube at 120-350 x *g*, 5 min. Resuspend in pre-warmed the B medium at a cell density of 2×10^6/mL. Transfer the cell suspension to a reservoir, and add 50 μL of cell suspension to each well of a 96 well black-flame and white-well plate (flat bottom) using an 8 channel or 12 channel pipetman. (cf. Figure 16)

Figure 16

flat-bottom B&W	1	2	3	4	5	6	7	8	9	10	11	12
A	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL							
B	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL							
C	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL							
D	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL	THP-G1b 1x10^5 B medium 50uL							
E												
F												
G												
H												

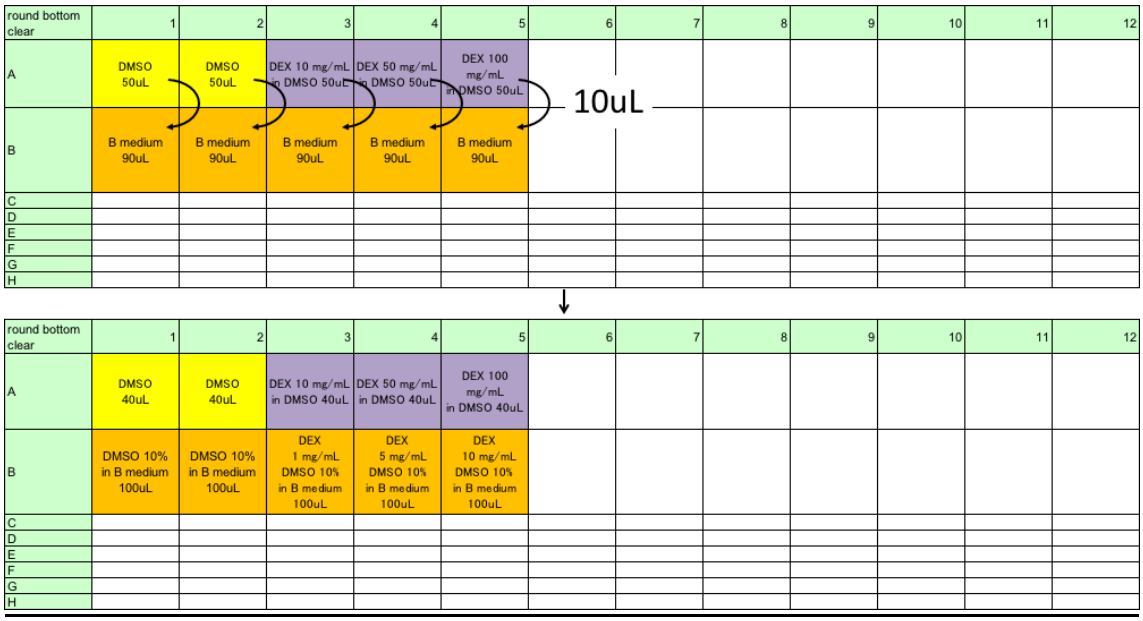
7-3 Arrangement of chemicals and vehicle

Add DMSO 50 μL to #A1-2, 10 mg/mL dexamethasone 50 μl to #A3, 50 mg/mL dexamethasone 50 μl to #A4, 100 mg/mL dexamethasone 50 μl to #A5 and B medium 90 μl to #B1-5 of the 96 well clear plate (round bottom). (cf. Figure 17)

7-4 Dilution with the B medium

Dilute DMSO in #A1-2 and dexamethasone DMSO solution in #A3-5 by adding 10 μL to the B medium in #B1-5. (cf. Figure 17)

Figure 17



7-5 2 step dilution

Add 10 μL of the diluted DMSO or dexamethasone to 490 μL of the B medium prepared in the assay block. And add 50 μL to THP-G1b (TGCHAC-A4) in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Manipulate the procedures from 7-4 to 7-5 as quickly as you can, and do not leave a long time at step after 7-4 or Figure 16. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 1 hour (37°C, CO_2, 5%). (cf. Figure 18-20)

Figure 18

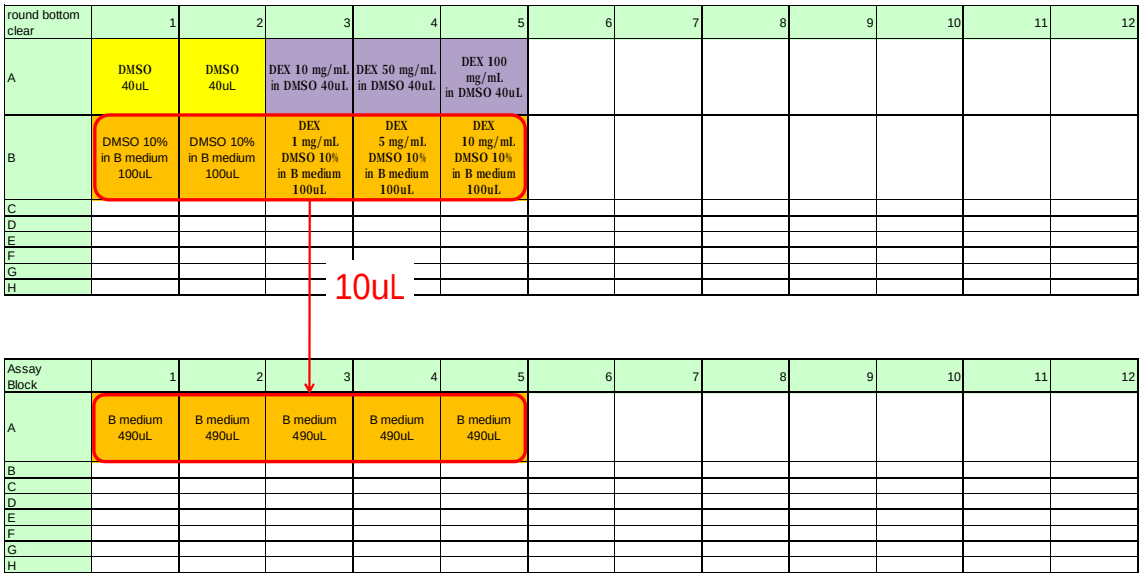


Figure 19

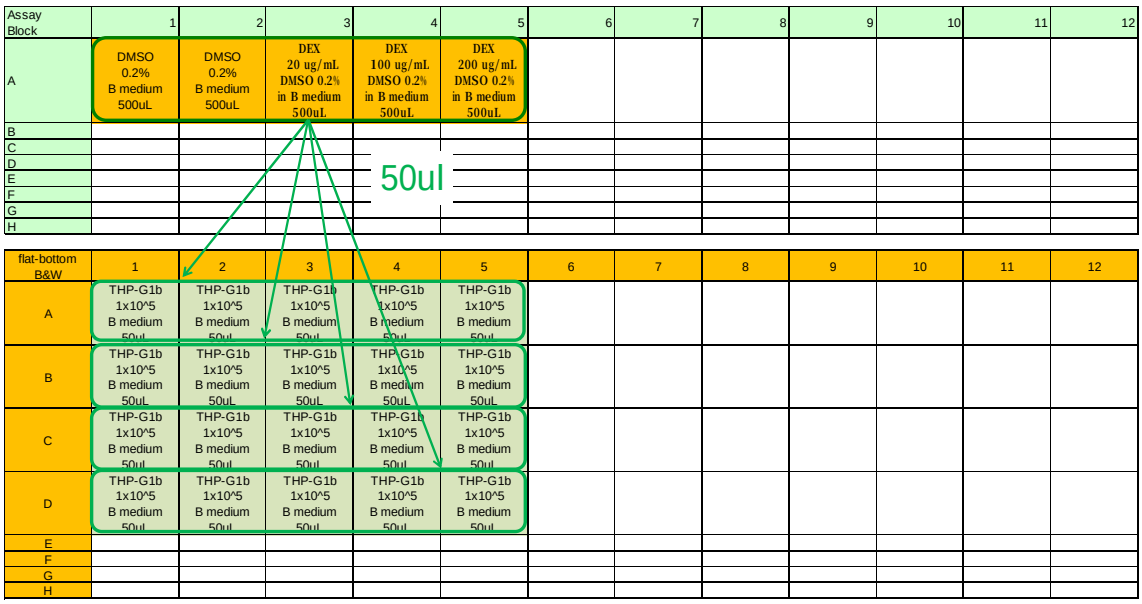


Figure 20 Final constituents of each well of the plate

flat-bottom B&W	1	2	3	4	5	6	7	8	9	10	11	12
A	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 10 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 50 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 100 ug/mL DMSO 0.1% B medium 100uL							
B	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 10 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 50 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 100 ug/mL DMSO 0.1% B medium 100uL							
C	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 10 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 50 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 100 ug/mL DMSO 0.1% B medium 100uL							
D	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 10 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 50 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 100 ug/mL DMSO 0.1% B medium 100uL							
E												
F												
G												
H												

7-6 Addition of LPS to THP-G1b (TGCHAC-A4)

One hour after the addition of dexamethasone, add 10 µL of distilled water or 1000 ng/mL LPS solution prepared in §6 to the cells (#A1-#D1 or #A2-#D5, respectively) using an 8 channel or 12 channel pipetman after pipetting 20 times. Make sure that the apex of the tip is dipped into the medium. Change tips every line you add. Seal the plate, shake the plate with a plateshaker and incubate in a CO_2 incubator for 6 hours (37°C, CO_2, 5%). (cf. Figure 21)

Figure 21

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water	1000 ng/mL LPS in Distilled water										
B	Distilled water	1000 ng/mL LPS in Distilled water										
C	Distilled water	1000 ng/mL LPS in Distilled water										
D	Distilled water	1000 ng/mL LPS in Distilled water										
E												
F												
G												
H												

10uL

10uL

flat-bottom B&W	1	2	3	4	5	6	7	8	9	10	11	12
A	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 10 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 50 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 100 ug/mL DMSO 0.1% B medium 100uL							
B	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 10 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 50 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 100 ug/mL DMSO 0.1% B medium 100uL							
C	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 10 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 50 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 100 ug/mL DMSO 0.1% B medium 100uL							
D	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 10 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 50 ug/mL DMSO 0.1% B medium 100uL	THP-G1b 1x10^5 cell DEX 100 ug/mL DMSO 0.1% B medium 100uL							
E												
F												
G												
H												

8. Calculation of the transmittance factors

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

8-1 Reagents

- Single reference samples:

  Lyophilized luciferase enzyme reagent for stable luciferase green (SLG)

  Lyophilized luciferase enzyme reagent for stable luciferase red (SLR)

- Assay reagent:

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

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

Reagent	Company	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

8-2 Preparation of luminescence reaction solution

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

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

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

8-3 Bioluminescence measurement

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

Figure 22.

flat-bottom B&W	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	SLG 100 ∝L	SLG 100 ∝L	SLG 100 ∝L									
C												
D	SLR 100 ∝L	SLR 100 ∝L	SLR 100 ∝L									
E												
F												
G												
H												

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

Figure 23. An example of the raw output data

Measurement without Filter

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

Measurement with Filter

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

Two transmittance factors of the optical filter were calculated as follow:

$$\text{Transmittance factor } (\kappa G_{R60}) = \frac{\#B1 \text{ of } F1 + \#B2 \text{ of } F1 + \#B3 \text{ of } F1}{\#B1 \text{ of } F0 + \#B2 \text{ of } F0 + \#B3 \text{ of } F0}$$

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

In the case shown above,

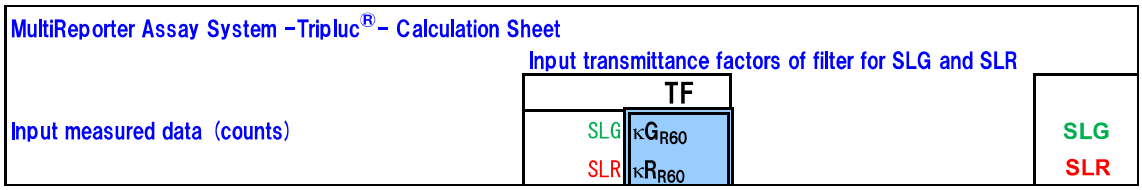
$$\text{Transmittance factors } (\kappa G_{R60}) = \frac{236478+234079+240876}{3757015+3716611+3810382} = 0.063$$

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

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

Input the transmittance factors to #G4-5 of the “Data Input” sheet of the Data sheet as follow.

Figure 24



9. Measurement

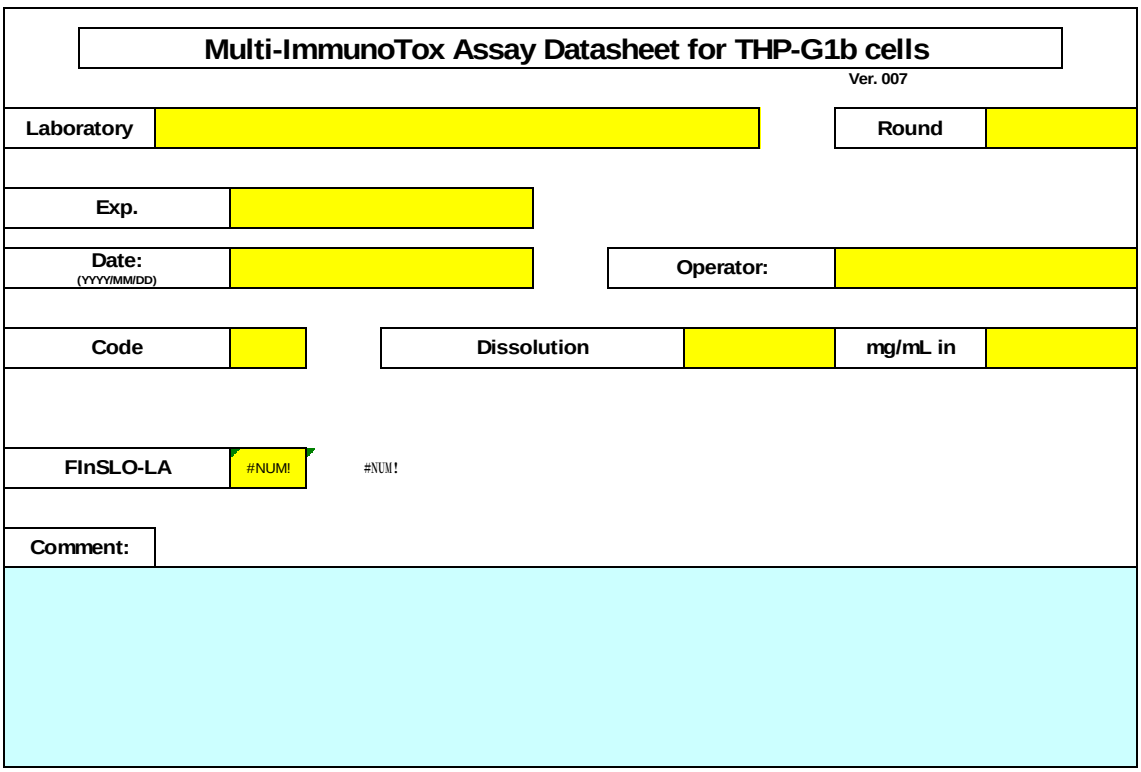
Please refer Appendix 1 for the principle of measurement of luciferase activity.

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

Transfer 100 μL of pre-warmed Tripluc from the reservoir to each well of the plate containing the reference sample using an 8 channel or 12 channel pipetman. Shake the plate for 10 min at room temperature (about 25°C) 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.

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

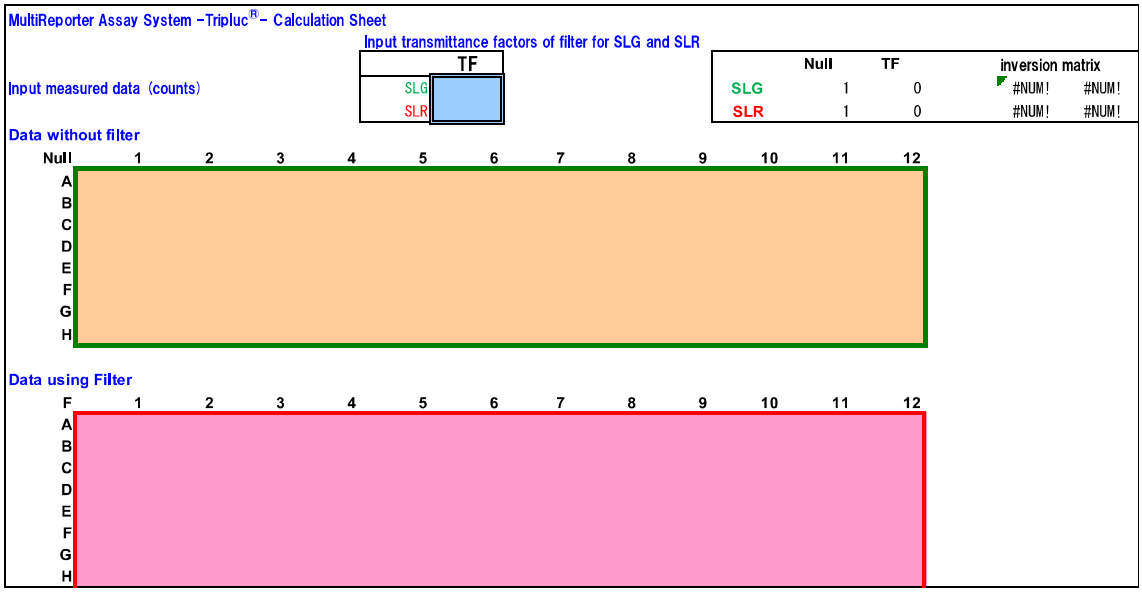
Figure 25 "Face Sheet" of the data sheet



2nd. Copy the results of the F0 and F1 measurements (values are expressed as counts) and paste them into the appropriate area in the "Data Input" sheet of the data sheet shown below (Figure 28). In

addition, input the transmittance factors calculated in chapter 5. Calculation of the transmittance factors to TF of the “Data Input” sheet (Figure 26).

Figure 26 “Data Input” sheet of the data sheet



Next, the calculated results for the parameters of the Multi-Immuno Tox assay for each concentration, e.g., SLG-LA, SLR-LA, nSLG-LA, the mean ± SD of SLG-LA, the mean ± SD of SLR-LA, %suppression and graphs will automatically appear on the “Result Format” sheet of the data sheet.

Figure 27 “Result Format” sheet of the data sheet

10. Data analysis

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

11. Criteria

11-1 Acceptance criteria

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

- If Fold induction of nSLG-LA of LPS wells without chemicals (=(nSLG-LA of THP-G1b cells treated with LPS) / (nSLG-LA of non-treated THP-G1b cells)) demonstrate less than 3, the results obtained from the plate containing the control wells should be rejected.
- If the number of concentrations which satisfy I.I.-SLR-LA>=0.05 is less than 6, the experiment, is accepted only when viable wells satisfy the following positive criteria and then, the following experiments should be done using the concentration described in **5-1**.

11-2 Criterion

The experiments are repeated until two consistent positive results or two consistent "non-suppression" are obtained. When two consistent results are obtained, the chemicals are judged as the obtained consistent results.

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

In each experiment, when the chemicals clear the following 4 criteria, they are judged as suppression. Otherwise, they are judged as non-suppression.

1. The %suppression is $\geq$ 25 with statistical significance. The statistical significance is judged when the 95% confidence interval does not include 0.
2. The result shows two or more consecutive statistically significant positive data or one statistically significant positive data with a trend in which at least 3 consecutive data increase in a dose dependent manner. In the latter case, the trend can cross 0, as long as only one data point shows the opposite effect without statistical significance.
3. The results are judged using only data obtained in the concentration at which I.I.-SLR-LA is $\geq$ 0.05
4. The results at 2000 μg/ml is excluded.

The acceptance criteria and the criterion for judgment can be described in the following with the same meaning and possibly more understandable way.

11-1 Acceptance criteria

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

- If Fold induction of nSLG-LA of LPS wells without chemicals (=(nSLG-LA of THP-G1b cells treated with LPS) / (nSLG-LA of non-treated THP-G1b cells)) as a control demonstrates less than 3, the results obtained from the plate containing the control wells should be rejected.
- If the number of concentrations which satisfies I.I.-SLR-LA>=0.05 is less than 6, the experiment is accepted only when viable wells satisfy the following positive criteria and then, the following experiments should be conducted using the concentration described in **5-1**.
- When chemicals are dissolved at 100 mg/ml in water, the data of the wells treated with chemicals at the highest concentration, i.e. 2 mg/ml, was omitted.

11-2 Criterion

The experiments are repeated until two consistent suppression or two consistent non-suppression are obtained. When two consistent results are obtained, the chemicals are judged as the obtained consistent results.

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

In each experiment, when the chemicals clear the following 3 criteria, they are judged as suppression Otherwise, they are judged as non-suppression.

1. The result shows two or more consecutive statistically significant positive data with $\geq$ 25 of the % suppression or one statistically significant data with $\geq$ 25 of the % suppression and a trend in which at least 3 consecutive data increase in a dose dependent manner. In the latter case, the trend can cross 0, as long as only one data point shows negative data without statistical significance. The statistical significance is judged when the 95% confidence interval does not include 0.
2. The results are judged using only data obtained in the concentration at which I.I.-SLR-LA is $\geq$ 0.05

12. Update record

Ver. 009E for THP-G1b (TGCHAC-A4) 2019.7.1
Change the Acceptance criteria
Change the criteria

Ver. 008.1E for THP-G1b (TGCHAC-A4) 2019.2.7
Change the Acceptance criteria
Change the criteria

Ver. 008E for THP-G1b (TGCHAC-A4) 2018.12.3
Addition of thresholds to the criteria.
Change the composition of the culture medium
Change the prepareation of the dexamethasone solution

Ver. 007E for THP-G1b (TGCHAC-A4) 2018.7.12

Ver. 0011.0E 2018.5.10
Change the criteria

Ver. 0010.0E 2018.1.15 distribution
Change the criteria

Ver. 009.1E 2017.5.8 distribution
Change the criteria

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

Ver. 008.5E 2016.9.14 distribution
Change the criteria

Ver. 008.4E 2016.9.9 distribution
Change the criteria

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

Ver. 008.1E 2016.2.2 distribution
Changes after the VMT meeting

Ver. 008.0E 2016.1.19
Translation to English
Addition of appendix

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

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

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

Ver. 004.0J 2014.11.17 distribution
For the validation study at AIST, FDSC and Tohoku university (chemicals: Sodium Bromate ($NaBrO_3$), Nickel (II) sulfate ($NiSO_4$), Dibutyl phthalate (DP), 2-Mercaptobenzothiazole (2-MBT))
Change THP-G1b cells to TGCHAC-A4 cells
Change cell number of THP-G8 and TGCHAC-A4 5x10^4/well to 1x10^5/well
Change concentration of chemicals 11 steps to 10 steps
Change final concentration of LPS (THP-G8 : 25 ng/mL, TGCHAC-A4 : 1 ng/mL)
Change the way of addition of LPS (2 mL/well to 10 mL/well)
Change the criteria

Ver. 002.0J 2013.08.19 distribution
For the validation study at AIST and FDSC (chemicals: $CoCl_2$, $NiSO_4$, Isophorone diisocyanate, 2-

Mercaptobenzothiazole）
Change the common ratio 3 to 2
Change the concentration of LPS 100 ng/mL to 25 ng/mL
Add description about the control (dexamethasone)
Delete the appendix about THP-G8 cell

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

Ver. 001J 2012. Nov. 09 distribution

Appendix 1 Principle of measurement of luciferase activity

MultiReporter Assay System -Tripluc- can be used with a microplate-type luminometer with a multi-color detection system, which can equip 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 SLG and SLR luminescence.

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

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

When the intensity of SLG and SLR in test sample are defined as G 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.

$$F0=G+R$$

$$F1=\kappa G_{R60} \times G + \kappa R_{R60} \times R$$

These formulas can be rephrased as follows

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

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

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

This calculation can be performed using the functions "MININVERSE" and "MMULT" in Microsoft Excel. These calculations are integrated in the Data sheet for MITA THP-G1b.

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 SLG
  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 SLG1/1, SLR1/1, and SLG/SLR1/1. Keep the reference samples on ice to prevent deactivation.

Prepare dilution series of the single reference samples of SLG and SLR as follows. Dilute 0.3 mL of each 1/1 solution with 0.9 mL of B medium to make SLG1/4 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 28.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	SLG 1/1	SLG 1/1	SLG 1/1	SLG 1/4	SLG 1/4	SLG 1/4	SLG 1/16	SLG 1/16	SLG 1/16	SLG 1/64	SLG 1/64	SLG 1/64
C												
D	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 29.

Measurement without Filter

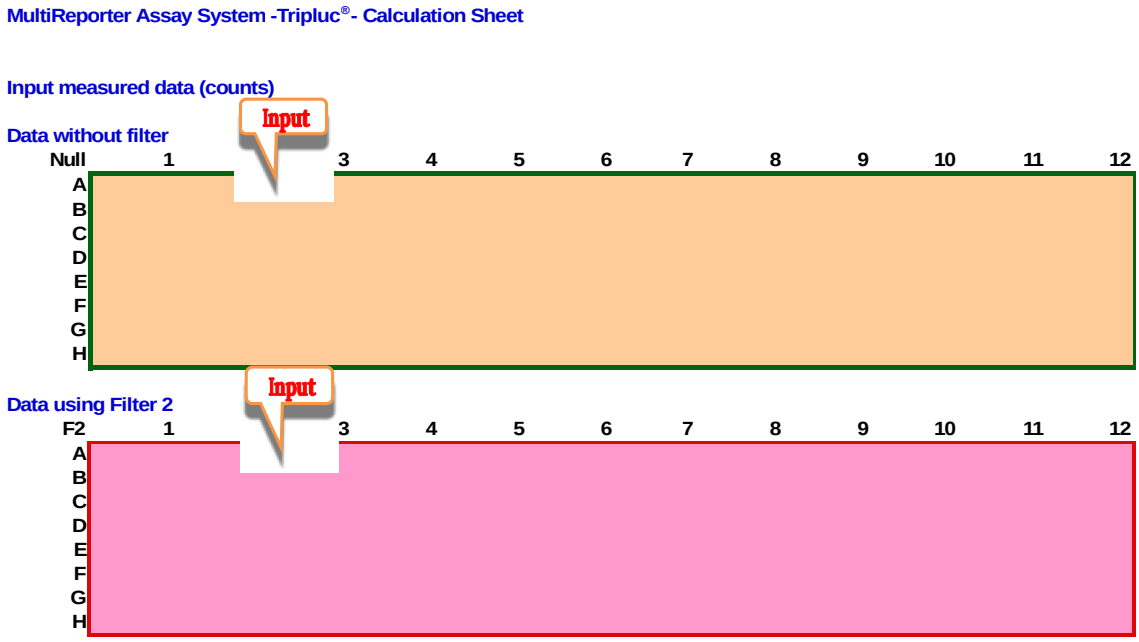
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	9689	9691	9677	2402	2388	2412	704	689	721	177	189	182
C												
D	8588	8444	8462	2281	2128	2239	609	578	690	150	132	129
E												
F												
G												
H												

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 data sheet for data analyses shown below.

Figure 30.



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.

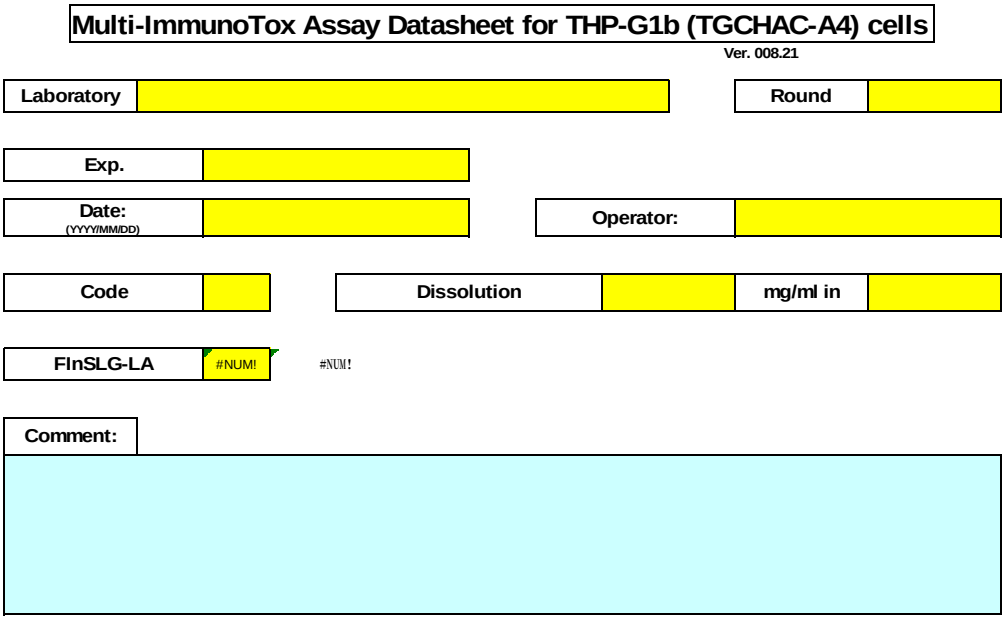
LED plate data typically fluctuates up to 1.5% (σ). Disagreement to the old data should be less than 3×σ (= 4.5%).

添付資料２ ： Phase II 試験３３候補被検物質の判定ならびに入手経路、論文報告

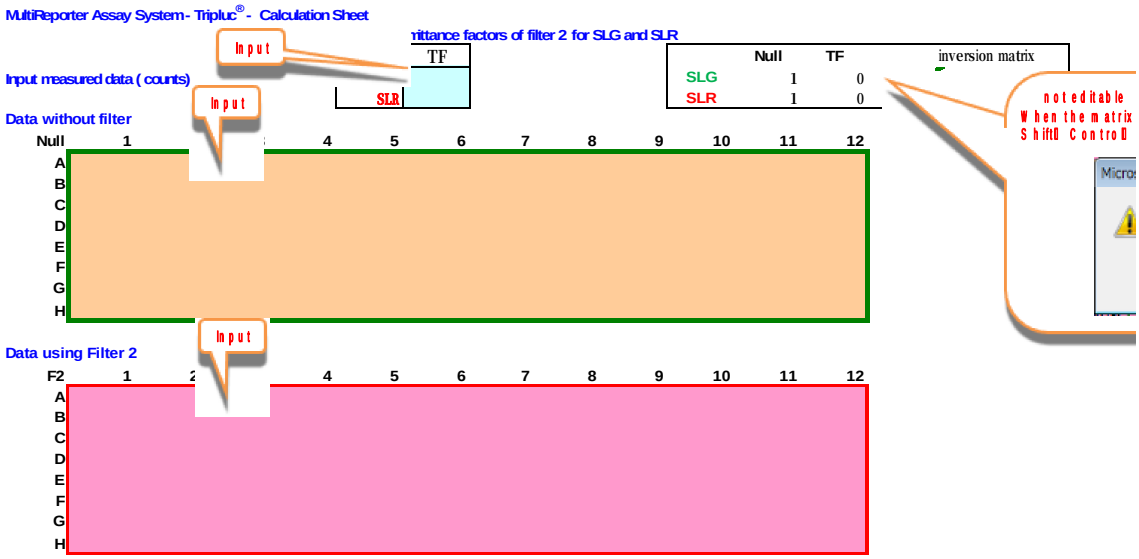
No.	Chemical	Cas.no.	Physical state	store	conc. (mg/ml)	vehicle	THP-G1b (HACA4)	Supplier	Ref. no.	Volume	Price	The effects of chemicals on IL-1 mRNA expression or IL-1 production in the literature			
1	2,4-Diaminotoluene	95-80-7	solid	r.t.	25	ddw	A	sigma	101915	50g	¥ 5,300	X			
2	Cadmium chloride	10108-64-2	solid	暗	100	ddw	S	aldrich	655198	5g	¥ 6,100	Odewumi et al. 2016 Li et al. 2016 Hemdan et al. 2006 Marth et al. 2000	human mice human human	cell line (A549, adenocarcinomic human alveolar basal epithelial cells) testis PBMC PBMC	A A S A (low), no effect (high)
3	Diethylstilbestol	56-53-1	solid	r.t.	62.5	DMSO	S	sigma	D4628	1g	¥ 20,300	Yamashita et al. 2005		peritoneal exudate macrophages	A
4	5,5-Diphenylhydantoin sodium salt	630-93-3	solid	r.t.	31.25	DMSO	S	sigma	D4505	25g	¥ 11,700	Okada et al. 2001 Modeer et al. 1989	mice human	spleen adherent cells PBMC, cell line (U937)	S A
5	Indomethacin	53-86-1	solid	r.t.	500	DMSO	S	sigma	I7378	5g	¥ 12,500	Muñoz-Miralles et al. 2018 Utsunomiya et al. 1994 Chang et al. 1990 Rordorf-Adam et al.1989	mice rat human human	intestine pleural exudate monocyte monocyte	A A S A
6	Isonicotinic Acid Hydrazide (Isoniazid)	54-85-3	solid	r.t.	50	ddw	N	fulka	I3377	5g	¥ 5,000	Metushi and Uetrecht 2014 Kucharz and Sierakowski 1992	mice human	serum (in vivo) monocyte	S (il-1a), no effect (IL-1b) S
7	Pentachlorophenol	87-86-5	solid	暗	125	DMSO	S	aldrich	P2604	5g	¥ 5,300	Martin and Whalen 2017 Chen et al. 2005	human crucian carp	immune cell macrophage	A S
8	Urethane, Ethyl carbamate	51-79-6	solid	r.t.	100	ddw	N	sigma	U2500	100g	¥ 9,100	Balharry et al. 2008 Bette et al. 2004	human rat	in vitro model of human upper respiratory tract epithelium (EpiAirway-100) spleen (in vivo)	A/S S
9	Tributyltin chloride	1461-22-9	liquid	暗	500	DMSO	A	aldrich	T50202	5g	¥ 3,400	Kimura et al. 2005 Tryphonas et al. 2004 Corsini et al. 1996	mice rat mice	macrophages, neutrophils (ex vivo) serum (in vivo) ear, keratinocyte cell line (HEL30)	no effect no effect A
10	Perfluorooctanoic acid	335-67-1	solid	r.t.	500	DMSO	S	TCI	P0764	25g	¥ 17,000	Son et al. 2009	mice	spleen, thymus	A (spleen), no effect (thymus)
11	Dichloracetic acid	79-43-6	liquid	4c暗	100	ddw	S	sigma	D54702	100ml	¥ 4,600	X			
12	Toluene	108-88-3	liquid	暗	500	DMSO	N	sigma	244511	100ml	¥ 4,500	X			
13	Hydroquinone(1,4-Dihydroquinone)	123-31-9	solid	r.t.	50	ddw	N	sigma	H9003	100g	¥ 4,400	Heluany et al. 2018 Thomas et al. 1989	rat mice	synoviocytes (ex vivo) macrophages (in vitro)	A S
14	4,4'-Sulfonyldianiline (Dapsone)	80-08-0	solid	r.t.	500	DMSO	SSS	sigma	A74807	100g	¥ 4,400	Abe et al. 2008 Interview form of dapsone	human human	PBMC (in vitro) Macrophages (in vitrio)	no effect S
15	Ethanol	64-17-5	liquid	r.t.	100	ddw	N	WAKO	057-00456	500ml	¥ 2,160	Tornier et al. 2006	human	reconstituted epidermis	No effect
16	Nitrofurazone	59-87-0	solid	r.t.	125	DMSO	N	aldrich	73340	100g	¥ 6,800	X			
17	Pentamidine isethionate	140-64-7	solid	-20	100	ddw	S	sigma	P0547	1g	¥ 31,900	Van Wauwe et al. 1996	human	whole blood	no effect
18	Trichloroethylene	79-01-6	liquid	r.t.	500	DMSO	S	WAKO	206-18575	500ml	¥ 1,400	Jia et al. 2012	human	serum, cell line (HaCaT)	A
19	Ziram(DMDTC)	137-30-4	solid	r.t.	125	DMSO	S	aldrich	329711	1kg	¥ 12,744	Muroi and Tanamoto 2015	mice	macrophage-like cell line J774A.1	S
20	Acetaminophen	103-90-2	solid	r.t.	500	DMSO	N	sigma	A7085	100g	¥ 9,800	Williams et al. 2010 Dambach et al. 2002 Blazka et al. 1995 Chang et al. 1990	mice mice mice human	plasma, liver liver serum (in vivo) monocyte	A A A S (1mg/ml)
21	Citral	5392-40-5	liquid	r.t.	500	DMSO	S	aldrich	C83007	5ml	¥ 4,100	Bachiega et al. 2011	mice	Peritoneal macrophages	S
22	Dibutyl phthalate	84-74-2	liquid	r.t.	500	DMSO	S	aldrich	524980	25ml	¥ 1,800	Ni et al. 2016 Couleau et al. 2015 Hansen et al. 2015 Li et al. 2013 Kruger et al. 2012	human human human mice human	HepG2 and L02 (normal human liver) cell lines cell line (THP-1) monocytes/macrophages F4/80+ macrophages corneal endothelial cell line B4G12	A no effect no effect S A
23	Hexachlorobenzene	118-74-1	solid	r.t.	3.9	DMSO	S	TCI	H0053	25 g	¥ 3,400	Fu et al. 2016	Rat	cell line (pheochromocytoma, PC12)	A
24	Lead(II) acetate	6080-56-4	solid	暗	100	ddw	N	aldrich	316512	5g	¥ 4,700	Hemdan et al. 2005	human	PBMC	S
25	Mercuric chloride	7487-94-7	solid	暗	50	ddw	S	WAKO	138-01152	25g	¥ 3,300	Zdolsek et al. 1994	mice	peritoneal macrophage	A
26	Triethanolamine	102-71-6	liquid	r.t.	100	ddw	S	sigma	90279	100ml	¥ 4,900	Tornier et al. 2006 Muller-Decker et al. 1998 Muller-Decker et al. 1994	human human human	reconstituted epidermis suction blister fluid cell line (keratinocyte)	A, no effect no effect A
27	t-Butylhydroquinone	1948-33-0	solid	r.t.	250	DMSO	S	aldrich	112941	5g	¥ 4,900	Hao et al. 2018 Bai et al. 2017 Sukumari-Ramesh and Alleyne 2016	rat rat mice	bone marrow mast cells paraventricular nucleus brain	A S S
28	5,5-Diphenylhydantoin	57-41-0	solid	r.t.	500	DMSO	S	sigma	D4007	100g	¥ 11,700	Okada et al. 2001 Modeer et al. 1989	mice human	spleen adherent cells PBMC, cell line (U937)	S A
29	Bisphenol A	80-05-7	solid	r.t.	500	DMSO	S	aldrich	239658	50g	¥ 10,400	Teixeira et al. 2016 Couleau et al. 2015 Yang et al. 2015	human human carp	PBMC-derived Macrophage cell line (differentiated THP-1) macrophage primary macrophages	no effect A A
30	Butylated hydroxytoluene	128-37-0	solid	r.t.	500	DMSO	S	aldrich	B1378	100g	¥ 2,900	Eugui et al. 1994	human	PBMC	no effect
31	D-Penicillamine	52-67-5	solid	4c	100	ddw	N	sigma	P4875	5g	¥ 21,900	Chang et al. 1990 Rordorf-Adam et al. 1989 DiMartino et al. 1987 Brisset et al. 1986	human human rat rabbit	monocyte monocyte peritoneal exudate mononuclear cells	S no effect S S
32	Nonylphenol	84852-15-3	liquid	r.t.	100	ddw	N	aldrich	290858	25ml	¥ 2,000	Gu et al. 2018 Yu et al. 2016	rat rat	hippocampus liver	A A
33	Sodium chlorite	7758-19-2	solid	暗	100	ddw	A/S	sigma	244155	100g	¥ 6,800	X			

添付資料３：Multi-ImmunoTox Assay Datasheet for THP-G1b (TGCHAC-A4) cells ver.008.21E

①フェイスシート



②データ入力シート



③計算結果シート

MultiReporter Assay System- Triplus® - Calculation Sheet

Filter Null Data

Filter 2 Data

MultiReporter Assay System - Triplus® - Calculation Sheet

Transmittance Data

SLG

SLR

SLG mod

SLR mod

nSLG-LA

Chemical concentration

SLG-LA Average

S.D.

SLR-LA Average

S.D.

nSLG-LA Average

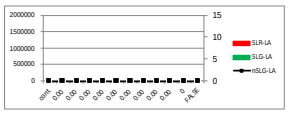
S.D.

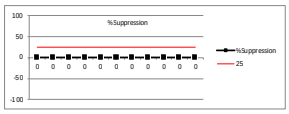
濃度の数(n)

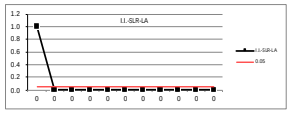
Il-SLR-LA

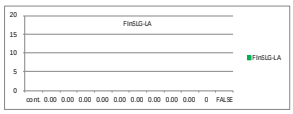
%suppression (IL-1 β)

FlnSLG-LA





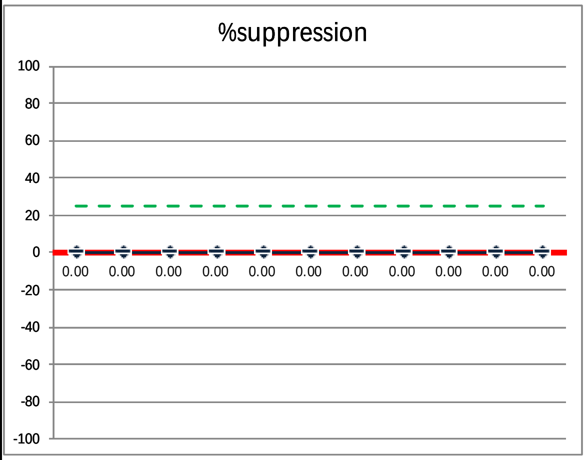




④グラフシート

Results

0 LINE 入力 25



a Comparison Conc.	%supp	Lower Limit	Upper Limit	Sig./N.S.	Eliminate
0	0	0	0		
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

添付資料４ ： MITAデータベース（97化学物質）

No.	Chemicals	CAS No.	IL-2		IL-1β		IL-8 Luc
			Judge	LOEL (ug/mL)	Judge	LOEL (ug/mL)	Judge
1	2-Aminoanthracene	613-13-8	S	5.86	S	11.72	P
2	2-Mercaptobenzothiazole	149-30-4	A	16.11	S	93.75	P
3	2,4-Diaminotoluene	95-80-7	N		N		N
4	Acetaminophen	103-90-2	A	100.00	N		N
5	Actinomycin D	50-76-0	S	0.02	S	0.13	P
6	Aluminum chloride	7784-13-6	S	104.17	N		N
7	Amphoterycin B	1397-89-3	S	2.60	S	1.17	P
8	Azathioprine	446-86-6	S	58.48	S	41.55	N
9	Benzethonium chloride	121-54-0	S	1.63	N		P
10	Chloroplatinic acid	18497-13-7	S	250.00	S	23.44	P
11	Chloroquine	50-63-5	S	17.83	S	39.06	P
12	Chlorpromazine	69-09-0	S	1.95	S	3.91	P
13	Cisplatin	15663-27-1	S	16.93	S	46.88	P
14	Citral	5392-40-5	S	25.00	S	4.88	P
15	Cobalt chloride	7791-13-1	S	16.93	S	46.88	P
16	Colchicine	64-86-8	S	0.27	N		P
17	Cyclophosphamide	6055-19-2	N		N		P
18	Cyclosporine A	59865-13-3	S	0.00	N		N
19	4-Aminophenyl sulfone, Dapsone	80-08-0	S	72.92	S	125.00	N
20	Dexamethasone	50-02-2	S	41.67	S	0.98	N
21	Dibenzopyrene	191-30-0	N		N		N
22	Dibutyl phthalate	84-74-2	S	2.60	S	15.63	N
23	Diesel exhaust particles		S	62.50	S	39.06	P
24	Diethanolamin	111-42-2	S	250.00	S	333.33	P
25	Digoxin	20830-75-5	S	0.07	S	0.59	P
26	Dimethyl sulfoxide	67-68-5	A	2000.00	N		N
27	Ethanol	64-17-5	N		N		N
28	FK506	109581-93-3	S	0.00	N		N
29	Formaldehyde	50-00-0	S	7.81	N		P
30	FR167653	158876-65-4	S	1.30	S	0.49	N
31	Hexachlorobenzene	118-74-1	N		N		N
32	Histamine	51-45-6	S	750.00	N		P
33	Hydrocortisone	50-23-7	N		S	0.24	N
34	Hydrogen peroxide	7722-84-1	S	23.44	S	375.00	P
35	Isoniazid	54-85-3	S	1000.00	N		N
36	Isophorone diisocyanate	4098-71-9	S	7.81	S	3.91	P
37	Lead(II) acetate	6080-56-4	S	57.29	N		N
38	Lithium carbonate	554-13-2	N		N		P
39	Magnesium sulfate	10034-99-8	S	2000.00	N		N
40	Mercuric chloride	7487-94-7	S	1.95	S	1.95	P
41	Methanol	67-56-1	N		N		N
42	Methotrexate	13307-73-1	N		N		P
43	Minocycline	13614-98-7	S	18.52	S	62.50	P
44	Mitomycin C	50-07-7	S	20.00	N		P
45	Mizoribine	50924-49-7	N		N		N
46	Mycophenolic acid	24280-93-1	A	0.40	S	72.00	P
47	Nickel sulfate	10101-97-0	S	104.17	S	375.00	P
48	Nicotinamide	98-92-0	A	288.07	N		N
49	Nitrofurazone	59-87-0	S	83.33	N		P
50	*p* -Nitroaniline	100-01-6	S	83.33	S	125.00	N
51	Pentamidine isethionate	140-64-7	S	52.08	S	64.45	P
52	Pyrimethamine	58-14-0	S	7.81	N		P
53	Rapamycin	53123-88-9	N		N		N
54	Ribavirin	36791-045	A	26.04	S	750.00	N
55	Sodium bromate	7789-38-0	S	500.00	S	500.00	P
56	Sodium dodecyl sulfate	151-21-3	S	62.50	S	62.50	P
57	Sulfasalazine	599-79-1	S	92.94	S	44.81	N
58	Trichloroethylene	79-01-6	N		N		N
59	Triethanolamine	102-71-6	S	1333.33	S	1000.00	P
60	Warfarin	81-81-2	N		N		N
61	4-Chloro-o-phenylenediamine	95-83-0	S	0.98	S	0.98	P
62	4,4-Thiobis(6-t-butyl-m-cresol)	96-69-5	S	3.91	S	3.91	P
63	Acetonitrile	75-05-8	N		N		N
64	Benzo(a)pyrene	50-32-8	S	5.86	N		N
65	Cadmium chloride	10108-64-2	N		S	15.63	P
66	Diethylstilbestrol	56-53-1	S	1.95	N		P
67	Dimethylbenz(a)-anthracene	57-97-6	S	6.41	S	25.63	N
68	Dimethylvinyl chloride	513-37-1	N		N		N
69	Diphenylhydantoin	630-93-3	N		N		P
70	Urethane, Ethyl carbamate	51-79-6	A	666.67	N		N
71	Ethylene dibromide	106-93-4	N		N		P
72	Indomethacin	53-86-1	A	31.25	N		P
73	m-Nitrotoluene	99-08-1	N		N		N
74	Methyl carbamate	598-55-0	A	750.00	N		N
75	n-Nitrosodimethylamine	62-75-9	A	1500.00	N		N
76	Nitrobenzene	98-95-3	N		N		N
77	o-Benzyl-p-chlorophenyl	120-32-1	S	2.93	S	2.93	P
78	o-Phenylphenol	90-43-7	S	7.81	S	46.88	P
79	Oxymetholone	434-07-1	S	7.81	S	0.98	N
80	p-Nitrotoluene	99-99-0	N		N		N
81	Pentachlorophenol	87-86-5	S	31.25	S	23.44	P
82	Phorbol myristate acetate (TPA)	16561-29-8	S		S	0.04	P
83	t-Butylhydroquinone	1948-33-0	N		S	4.88	P
84	Toluene	108-88-3	N		N		N
85	Vanadium pentoxide	1314-62-1	N		N		P
86	Zinc dimethyldithiocarbamate	137-30-4	N		N		P
87	Dibromoacetic acid	631-64-1	A	156.25	N		P
88	Glycidol	556-52-5	A	82.59	S	500	P
89	Tributyltin chloride	1461-22-9	S	0.19	N		P
90	Perfluorooctanoic acid	335-67-1	A	19.97	S	125.00	P
91	Dichloracetic acid	79-43-6	S	1285.71	N		P
92	Mannitol	69-65-8	N		N		N
93	Hydroquinone	123-31-9	S	3.91	N		P
94	Bisphenol A	80-05-7	S	7.81	S	31.25	P
95	2,6-Di-tert-butyl-4-methylpheno	128-37-0	S	7.81	N		N
96	Nonylphenol	84852-15-3	N		N		P
97	Sodium chlorite	7758-19-2	S	2.93	S	41.67	P