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およびIL-2 Luc leukocyte toxicity test (IL-2 Luc LTT)クライテリアの設定ならびにプロトコールの作成

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研究要旨
平成３０年度、令和１年度に行われた IL-1β転写活性抑制評価試験(IL-1 luciferase reporter assay; IL-1 Luc assay)の国際バリデーション研究に向けたクライテリアの設定ならびにプロトコールの作成を行った。平成２９年度までに国際バリデーションが終了した IL-2 転写活性抑制評価試験(IL-2 Luciferase reporter assay; IL-2 Luc assay)のプロトコールを参照とし、当教室で作成した 60 化学物質でのデータベースの結果および Phase 0 試験での各施設のデータをもとに｜%suppression｜>=20%を陽性とするクライテリアを設定し Phase Ⅰ用のプロトコールを作成した。そのプロトコールに則り Phase I 試験（平成３０年 12 月～令和１年３月）が行われた。終了後に開催された validation management team (VMT)会議にて改変された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。（添付資料１：Multi-Immuno Tox Assay protocol for THP-G1b(TGCHAC-A4) ver.009E) そのプロトコールに則り令和１年７月より IL-1 Luc assay validation 試験 Phase II が実施された。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。
IL-2 Luc assay を補完する目的で令和２年度より開始された IL-2 Luc leukocyte toxicity test (IL-2 Luc LTT)について、当教室で得られたデータを元に設定されたクライテリアに基づいたプロトコールを作成し、そのプロトコールに則り令和２年 10 月より IL-2 Luc LTT validation 試験 Phase Ⅰが実施された。また、神戸大学の協力を得て Phase Ⅰ試験で使用するデータシートを作成した。(添付資料２：Data sheet for MITA IL-2 Luc LTT Ver.008.8) Phase II 試験に先立ち７８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。

A．研究目的
- IL-1 Luc assayの国際バリデーションの実施に向け､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。
- IL-2 Luc leukocyte toxicity test (IL-2 Luc LTT)の国際バリデーション試験実施に向け､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
IL-1 Luc assayにおいては以下の方法により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

IL-2 Luc LTTにおいては以下の方法によりIL-2およびIFN-γプロモーター活性の測定を行った。ヒトTリンパ芽球性白血病由来細胞株JurkatにIL-2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）、IFN-γプロモーターに制御されたSLOルシフェラーゼ遺伝子（橙色に発色）、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2H4細胞を1ウェル当たり2×10^5個、黒色の96-well プレート(Greiner bio-one)に播種し化学物質を加え、37℃、5%CO_2下で24時間培養した。つづいて25nM PMAと1mM Ioの混合物(PMA/Io)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、SLO、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、2枚の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）、SLO-luciferase activity (SLO-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、SLO-LAをSLR-LAで除することによりそれぞれnormalized SLG-luciferase activity(nSLG-LA), normalized SLO- luciferase activity(nSLO-LA)を算出した。さらに以下の式に%suppression抑制率を計算した。

% suppression = (1-薬物存在下でのnSLG-LAまたはnSLO-LA/薬物非存在下でのnSLG-LAまたはnSLO-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

I.　IL-1 Luc assayアッセイ方法の検討

平成24年度から平成26年度の3年間にわたる厚生労働科学研究費補助金事業「多色発光細胞を用いたhigh-throughput免疫毒性評価試験法の開発」においてTHP-1細胞をベースとしたIL-1βレポーター細胞であるTHP-G1b(TGCHAC-A4)細胞を樹立した。本研究でIL-1β転写活性抑制評価試験(IL-1 luciferase reporter assay; IL-1 Luc assay)に使用するにあたり以下の条件設定を行った。

① LPS、96ウェルプレートの選定

従来のLPS(E.coli 026:B6)ではSLR-LAに比べSLG-LAの値が極端に高くなり結果が不安定になるという問題があったため刺激するLPSについて再検討を行った。入手可能なLPS４種について反応性を測定したところE. coli K12でE.coli 026:B6の３分の１の反

応性を示すことが示された。また従来96ウェルプレートについてはblackプレートを使用していたがウェルの内側を白色、ウェル間を含めた他の部分が黒色のblack & whiteプレートの使用を検討したところシグナルの増強が認められ安定した結果が得られた。以上の結果からLPSはE. coli K12へ、96ウェルプレートはblack & whiteプレートへ変更した。

② 培養条件の検討
THP-G1b(TGCHAC-A4)細胞の培養の際に選択抗生剤としてブラストサイジンを使用していたがブラストサイジンにより細胞のLPSへの反応性が低下する可能性が考えられたためブラストサイジンが入っていない培地を使用することとした。

II. IL-1 Luc assayクライテリアの設定

① 当教室で作成した60化学物質でのデータベースの結果およびPhase 0試験での各施設のデータをもとに｜%suppression｜>=20％を陽性とするクライテリアを設定しPhase I用のプロトコールを作成した。

② Phase Iの中途で、化学物質の毒性が強い場合に有効なデータが得られず判定ができないという問題が生じたため、有効なデータが６濃度未満でかつ陰性と判定されるときそのアッセイは棄却されるクライテリアに変更した。

以上I、IIを反映したプロトコールを作成した。(Multi-Immuno Tox Assay protocol for THP-G1b ver.008.1E)

III. IL-1 Luc assayデータシートの作成
95%信頼区間の算出法について神戸大学の協力を得てPhase I用のデータシートを作成した。

IV. IL-1 Luc assay Phase I用の記録用紙を作成した。

V. IL-1 Luc assay 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)

VI. IL-1 Luc assay 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。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。

VII. IL-1 Luc assay 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。

VIII. 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。

IX. IL-2 Luc leukocyte toxicity test (IL-2 Luc LTT)試験用プロトコールを作成

当教室で得られたデータを元に設定されVMT会議で承認された以下の様なクライテリアに基づいたプロトコールを作成した(研究代表者_添付資料５)。このプロトコールに則り令和２年１０月よりIL-2 Luc LTT validation試験 Phase Iが実施された。

クライテリア

- %suppression ≦ -35 かつ Min Inh-GAPLA<0.7 を満たす被験物質を leukocyte toxicと判定する。
- -35≤%suppression≤35 かつ Min Inh-GAPLA≧0.7を満たしdistilled waterに不溶の被験物質をindeterminateと判定する。
- それ以外の被験物質をnon-leukocyte toxicと判定する。

X. IL-2 Luc leukocyte toxicity test (IL-2 Luc LTT) Phase II試験７８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７８候補被検物質についてIL-2 Luc assay、IL-2 Luc LTTの判定結果、入手方法をまとめVMTに提出した。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。

XI. IL-2 Luc leukocyte toxicity test (IL-2 Luc LTT) 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２）

D．考察

IL-1 Luc assayについて、培養条件、LPS、96ウェルプレート、クライテリアの変更によりPhase 0における技術移転性が確認された。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。

IL-2 Luc LTTについて、クライテリアを適応したPhase I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。

E．結論

平成３０年度、令和１年度に行われたIL-1 Luc assay validation試験への準備として プロトコール、データシート、記録用紙を作成しそれに則りPhase Iおよび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。

令和２年度に行われたIL-2 Luc leukocyte toxicity test (IL-2 Luc LTT) validation試験への準備として プロトコール、データシート、記録用紙を作成しそれに則りPhase I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、Phase II試験が予定されている。

F．健康危険情報

なし

G．研究発表

1. 論文発表

1) Kimura, Y., Fujimura, C., Ito, Y., Takahashi, T., Terui, H., Aiba, S., 2018. Profiling the immunotoxicity of chemicals based on in vitro evaluation by a combination of the Multi-

ImmunoTox assay and the IL-8 Luc assay. Arch Toxicol 92, 2043-2054.
2) Kimura, Y., Watanabe, M., Suzuki, N., Iwaki, T., Yamakage, K., Saito, K.,Nakajima, Y., Fujimura, C., Ohmiya, Y., Omori, T., Kojima, H., Aiba, S., 2018. The performance of an in vitro skin sensitisation test, IL-8 Luc assay (OECD442E), and the integrated approach with direct peptide reactive assay (DPRA). J Toxicol Sci 43, 741-749.
3) 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 immunotoxic chemicals. Toxicol in Vitro, 2020: 66: 104832
4) Kimura, Y., Fujimura, C., Aiba, S. The modified IL-8 Luc assay, an in vitro skin sensitisation test, can significantly improve the false-negative judgment of lipophilic sensitizers with logKow values > 3.5. Arch Toxicol, 2021:95: 749-758
5) Terui, H., Kimura, Y., Fujimura, C., Aiba, S. The IL-1 promoter-driven luciferase reporter cell line THP-G1b can efficiently predict skin-sensitising chemicals. Arch Toxicol, 2021: 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、小林 眞弓、大森 崇、足利 太可雄、小島 肇、相場 節也：Multi-ImmunoTox Assay (MITA) ：バリデーション研究の結果 日本動物実験代替法学会 第31回大会（熊本）(2018.11)
2) 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)
3) 木村 裕、藤村 千鶴、相場 節也 IL-8 Luc assay (OECD442E) のクライテリアの改変とLogKowが3.5を超える化学物質における偽陰性率の改善（ポスター）日本動物実験代替法学会 第３３回大会 Web開催 (2020.11)
4) 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、岡山 昂祐、高木 佑実、大森 崇、小島 肇、相場 節也 Multi-ImmunoTox Assay (MITA)：IL-1 Luc assay バリデーション試験の結果（ポスター）日本動物実験代替法学会 第３３回大会 Web開催 (2020.11)
5) 木村 裕、藤村 千鶴、相場 節也 IL-8 Luc assay (OECD442E) の改良とLogKow >3.5物質への対応（ポスター）細胞アッセイ研究会 Web開催 (2021.1)
6) 木村 裕、藤村 千鶴、照井 仁、相場 節也 新たな皮膚感作性試験法 IL-1 Luc assayの特性（ポスター） 細胞アッセイ研究会 Web開催 (2021.1)

H．知的財産権の出願・登録状況
（予定を含む。）

なし

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

July 1st, 2019

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1. Introduction

This protocol describes how to maintain the cells, how to prepare the test chemicals, and how to measure the luciferase activity of 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

<table>
<tr><th>flat-bottom B&W</th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th></tr>
<tr><td>A</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>B</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>C</td><td rowspan="4">cont (distilled water or DMSO)</td><td rowspan="4">LPS only</td><td rowspan="3">A/2^9 µg/mL</td><td rowspan="3">A/2^8 µg/mL</td><td rowspan="3">A/2^7 µg/mL</td><td rowspan="3">A/2^6 µg/mL</td><td rowspan="3">A/2^5 µg/mL</td><td rowspan="3">A/2^4 µg/mL</td><td rowspan="3">A/2^3 µg/mL</td><td rowspan="3">A/2^2 µg/mL</td><td rowspan="3">A/2^1 µg/mL</td><td rowspan="3">A µg/mL</td></tr>
<tr><td>D</td></tr>
<tr><td>E</td></tr>
<tr><td>F</td><td colspan="10">Chemical (common ratio of 2, 10 concentrations, n=4)</td></tr>
<tr><td>G</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
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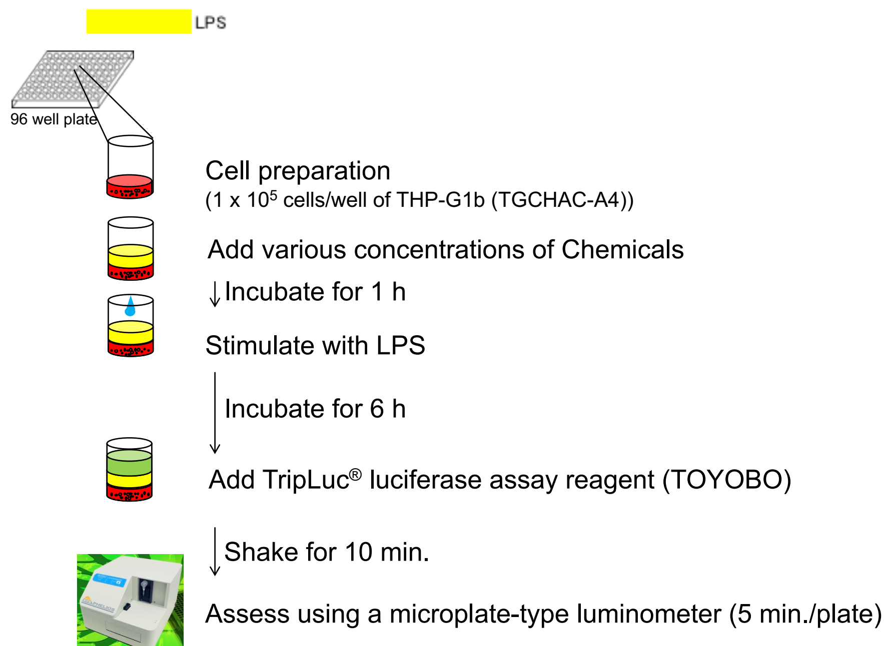
LPS



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 2-5x10^5/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

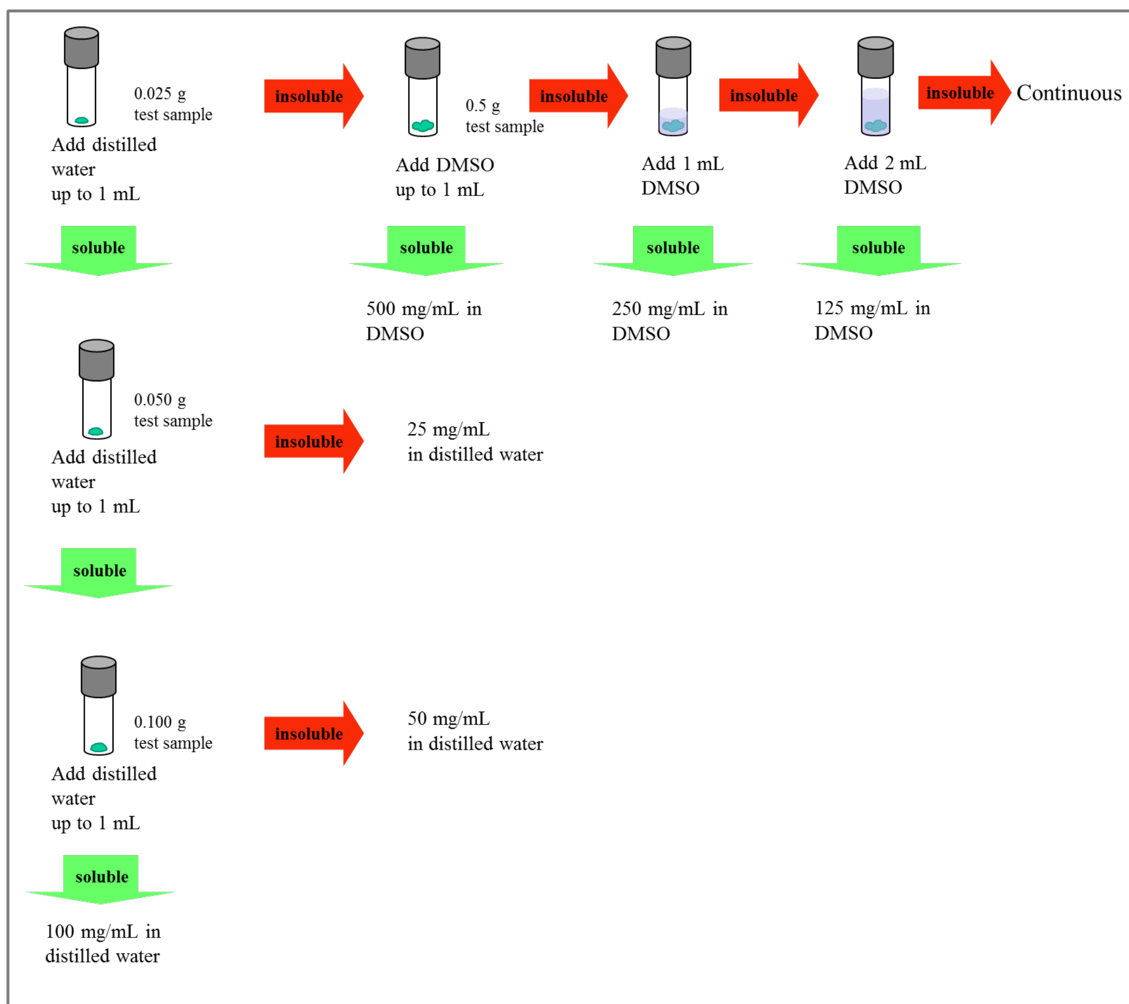
5-1 Dissolution by vehicle (cf. Figure 3)

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

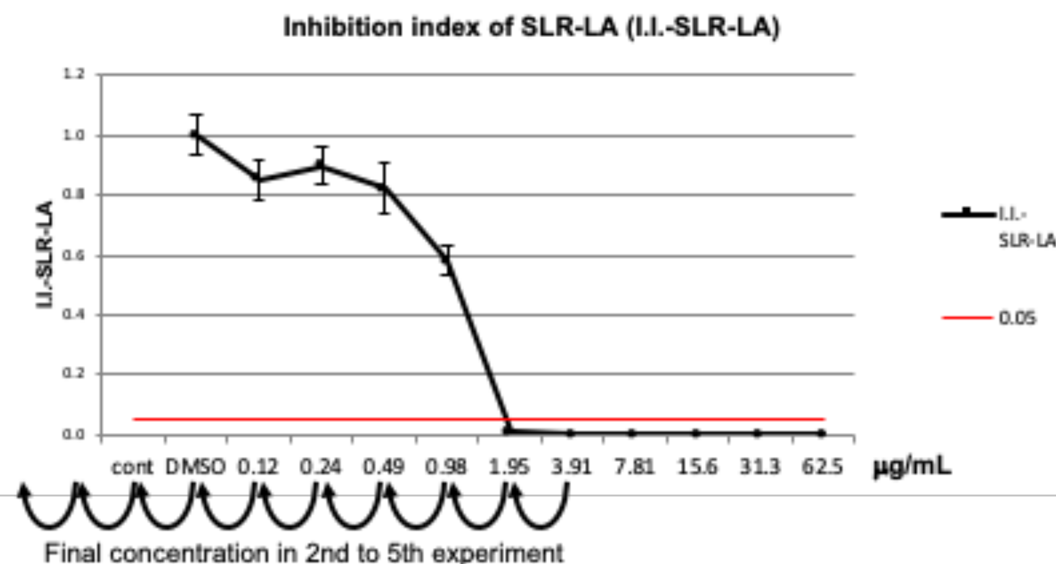
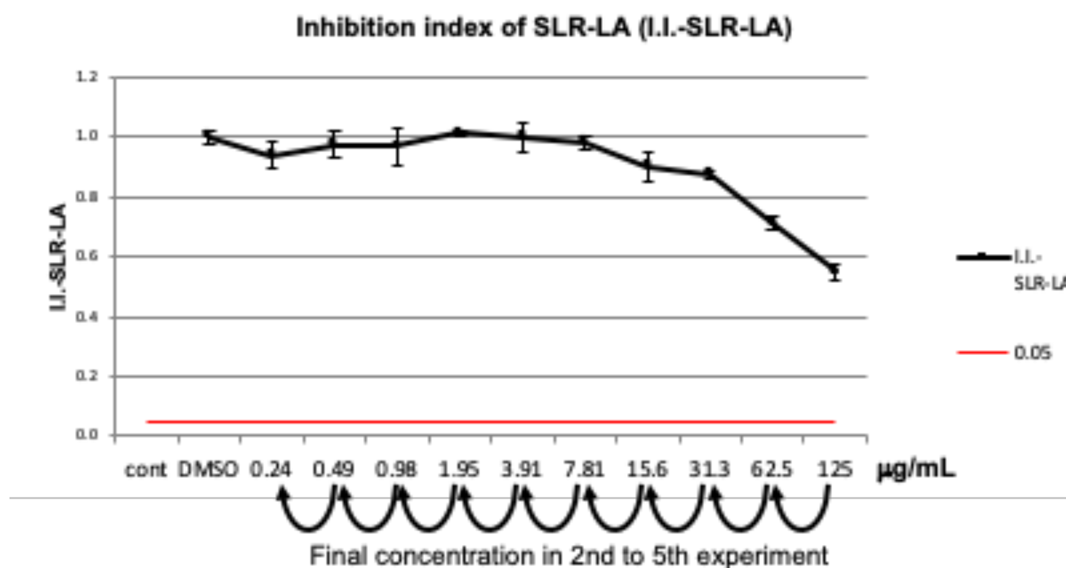


Figure 5



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.

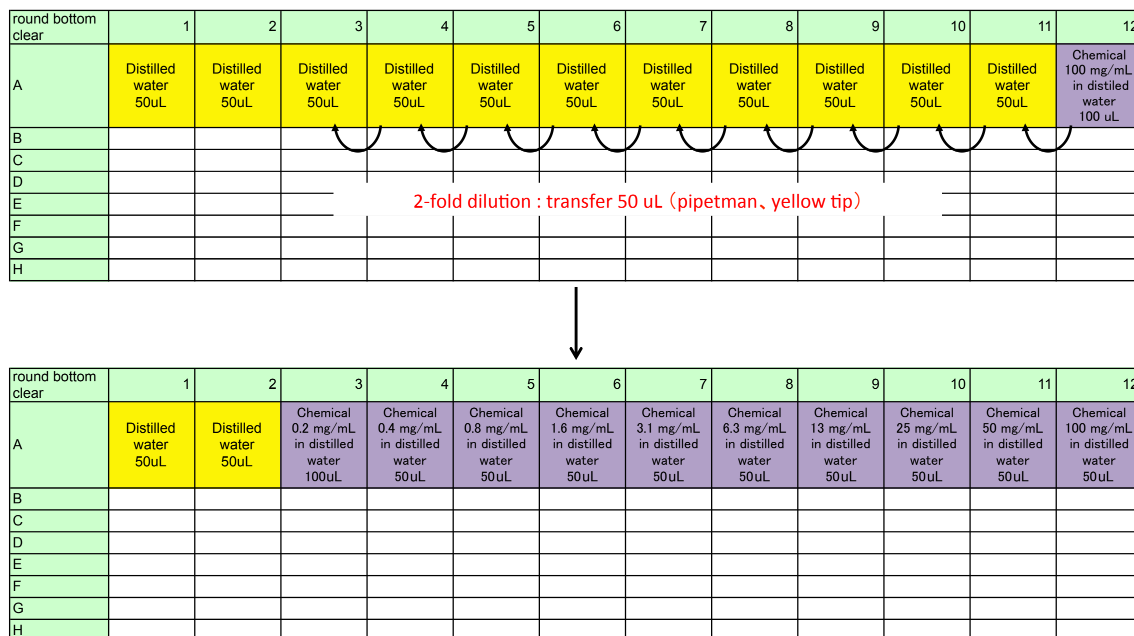
5-2-1 Arrangement of chemicals and vehicle

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

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

50uL

flat-bottom 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 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

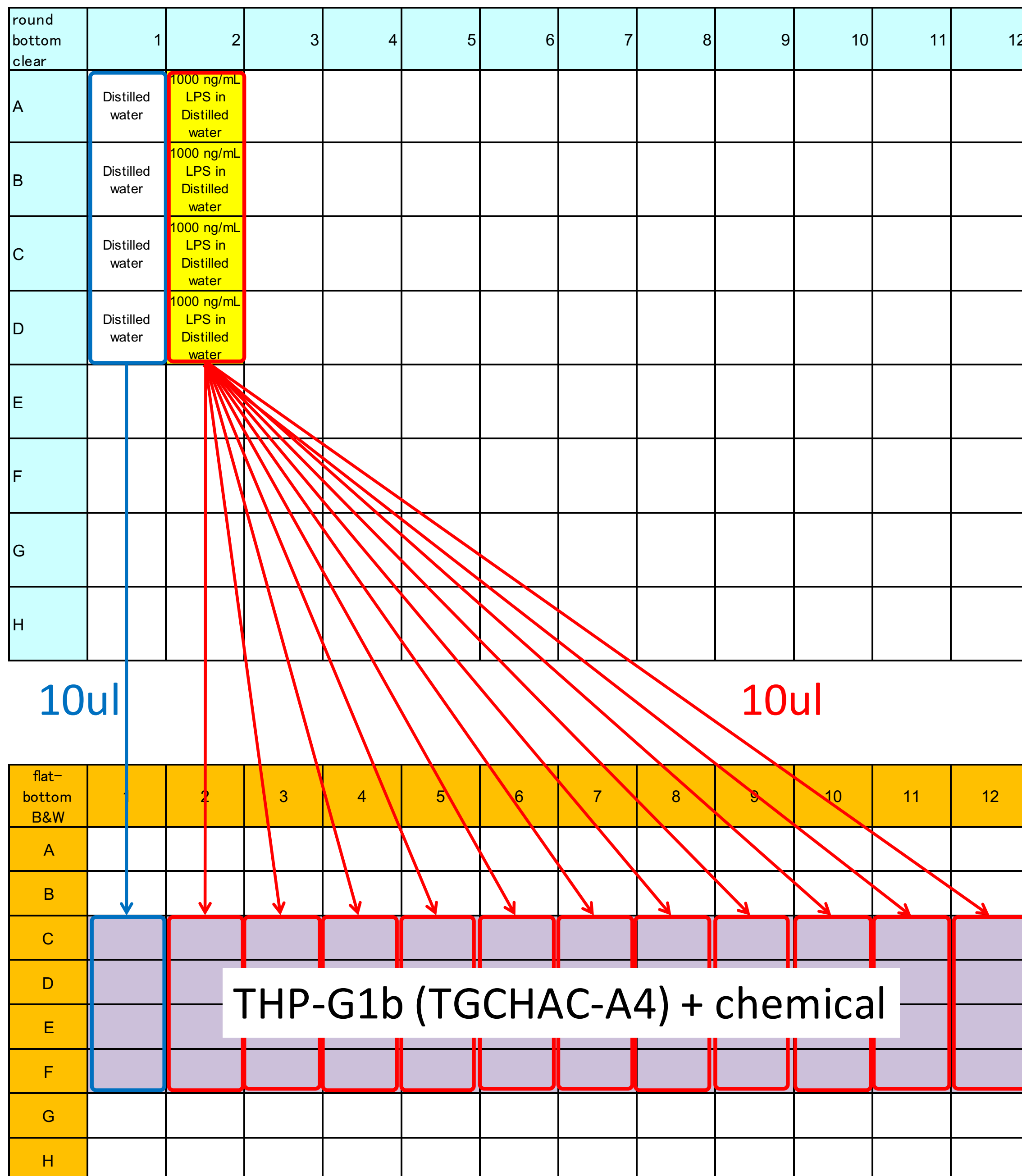
2nd step

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

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 50uL	DMSO 50uL	DEX 10 mg/mL in DMSO 50uL	DEX 50 mg/mL in DMSO 50uL	DEX 100 mg/mL in DMSO 50uL							
B	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 40uL	DMSO 40uL	DEX 10 mg/mL in DMSO 40uL	DEX 50 mg/mL in DMSO 40uL	DEX 100 mg/mL in DMSO 40uL							
B	DMSO 10% in B medium 100uL	DMSO 10% in B medium 100uL	DEX 1 mg/mL DMSO 10% in B medium 100uL	DEX 5 mg/mL DMSO 10% in B medium 100uL	DEX 10 mg/mL DMSO 10% in B medium 100uL							
C												
D												
E												
F												
G												
H												

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

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 40uL	DMSO 40uL	DEX 10 mg/mL in DMSO 40uL	DEX 50 mg/mL in DMSO 40uL	DEX 100 mg/mL in DMSO 40uL							
B	DMSO 10% in B medium 100uL	DMSO 10% in B medium 100uL	DEX 1 mg/mL DMSO 10% in B medium 100uL	DEX 5 mg/mL DMSO 10% in B medium 100uL	DEX 10 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												
C												
D												
E												
F												
G												
H												

Figure 19

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 0.2% B medium 500uL	DMSO 0.2% B medium 500uL	DEX 20 ug/mL DMSO 0.2% in B medium 500uL	DEX 100 ug/mL DMSO 0.2% in B medium 500uL	DEX 200 ug/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	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												

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

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 μL	SLG 100 μL	SLG 100 μL									
C												
D	SLR 100 μL	SLR 100 μL	SLR 100 μL									
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:

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

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

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

MultiReporter Assay System –Tripluc® – Calculation Sheet

	Input transmittance factors of filter for SLG and SLR		
	TF		
Input measured data (counts)	SLG	κG_{R60}	SLG
	SLR	κR_{R60}	SLR

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

Multi-ImmunoTox Assay Datasheet for THP-G1b cells

Ver. 007

Laboratory		Round	
Exp.			
Date: (YYYY/MM/DD)		Operator:	
Code		Dissolution		mg/mL in	
FInSLO-LA	#NUM!	#NUM!	
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 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

MultiReporter Assay System –Tripluc®– Calculation Sheet

Input transmittance factors of filter for SLG and SLR

Input measured data (counts)

	TF
SLG	
SLR	

	Null	TF	inversion matrix	
SLG	1	0	#NUM!	#NUM!
SLR	1	0	#NUM!	#NUM!

Data without filter

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

Data using Filter

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

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

MultiReporter Assay System -Tripluc®- Calculation Sheet

Filter Null Data

Null	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	0	0	0	0	0	0	0	0	0
B	0	0	0	0	0	0	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0	0
D	0	0	0	0	0	0	0	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

Filter 2 Data

F2	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	0	0	0	0	0	0	0	0	0
B	0	0	0	0	0	0	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0	0
D	0	0	0	0	0	0	0	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

50000
40000
30000
20000
10000
0
15
10
5
0
cont. 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0.00 0 FALSE
GAPDH-SLR
IL-1b-SLO
IL-1b/GAPDH

100
50
0
-50
-100
%Suppression
0 0 0 0 0 0 0 0 0 0 0
%Suppression

1.2
1.0
0.8
0.6
0.4
0.2
0.0
I.I.-SLR-LA
0 0 0 0 0 0 0 0 0 0 0
I.I.-SLR-LA
0.05

MultiReporter Assay System -Tripluc®- Calculation Sheet

	Null	T
SLG	1	0
SLR	1	0

inversion matrix

#NUM!	#NUM!
#NUM!	#NUM!

Transmittance Data

SLG

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

SLR

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

SLG mod

A	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
B	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
C	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
D	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
E	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
F	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
G	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
H	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!

SLR mod

A	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
B	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
C	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
D	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
E	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
F	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
G	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
H	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!

nSLG-LA

#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!

Chemical concentration	cont.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	FALSE	μg/ml
SLG-LA Average	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	
S.D.	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	
SLR-LA Average	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	
S.D.	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	
nSLG-LA Average	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	
S.D.	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	
I.I-SLR-LA	#NUM!	1.000	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	
%suppression (IL-1β)		0.000	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	
FInSLO-LA	#NUM!	#NUM!											

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 ≥ 25 of the % suppression or one statistically significant data with ≥ 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 ≥ 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.

$$F0=G+R$$

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

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：Data sheet for MITA IL-2 Luc LTT Ver.008.8

①フェイスシート

Multi-ImmunoTox Assay Datasheet for 2H4 cells

Ver. 008.8

Laboratory		Round	

Exp.		(Highest soluble conc. In the next exp.s	mg/ml
Date: (YYYY/MM/DD)		Operator:	

Code		Dissolution		mg/ml in	
Fold induction of nIFNLA		Rejected	the number of concentration which satisfy Inh-GAPLA>=0.05		

Comment:

②データ入力シート

MultiReporter Assay System -Triple Calculation Sheet

1st exp.

Transmittance Data

	SLG	SLO	SLR
T0			
T1			
T2			

#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!

Filter 0 Data

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

Filter 1 Data

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

Filter 2 Data

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

③計算結果シート

MultiReporter Assay System -Tripluc Calculation Sheet

1st exp.

IL2LA

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

1st exp.

Chemical 1

1 1 1 0 0 0 | 1 0.8 0.6 0.4 0.2 0
GAPLA IFNLA IL2LA nIFNLA nIL2LA

Chemical 2

IFNLA

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

GAPLA

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

Chemical 1

nIL2LA
1 0.8 0.6 0.4 0.2 0
nIL2LA

Chemical 2

nIL2LA

1	2	3	4	5	6	7	8	9	10	11	12
#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!
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#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!

nIFNLA

1	2	3	4	5	6	7	8	9	10	11	12
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
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#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!

%suppression

Chemical 1

-100 -50 0 50 100

Chemical 2

	1	2	3	4	5	6	7	8	9	10	11	12
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#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!

	1	2	3	4	5	6	7	8	9	10	11	12
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
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1.2 1.0 0.8 0.6 0.4 0.2 0.0
Inh-GAPLA 0.05 0.7

	1	2	3	4	5	6	7	8	9	10	11	12
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
Inh-GAPLA		1.00	#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!
		1.00	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

	1	2	3	4	5	6	7	8	9	10	11	12
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
input "*"												
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

	1	2	3	4	5	6	7	8	9	10	11	12
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
input "*"												
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

	1	2	3	4	5	6	7	8	9	10	11	12	
Fold induction	#VALUE!	#VALUE!											
	#VALUE!	#VALUE!	0										
Chemical 1													
													ug/ml
Chemical 2		0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	
	cont	0	0	0	0	0	0	0	0	0	0	0	ug/ml

	1	2	3	4	5	6	7	8	9	10	11	12
% suppression (IL-2)		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
% suppression (IFNg)		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		0.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
濃度の数 (n)		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

2	3	4	5	6	7	8	9	10	11	12	13	14
1	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!		
	1	#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!	
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1	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!		
	1	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!		
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!		
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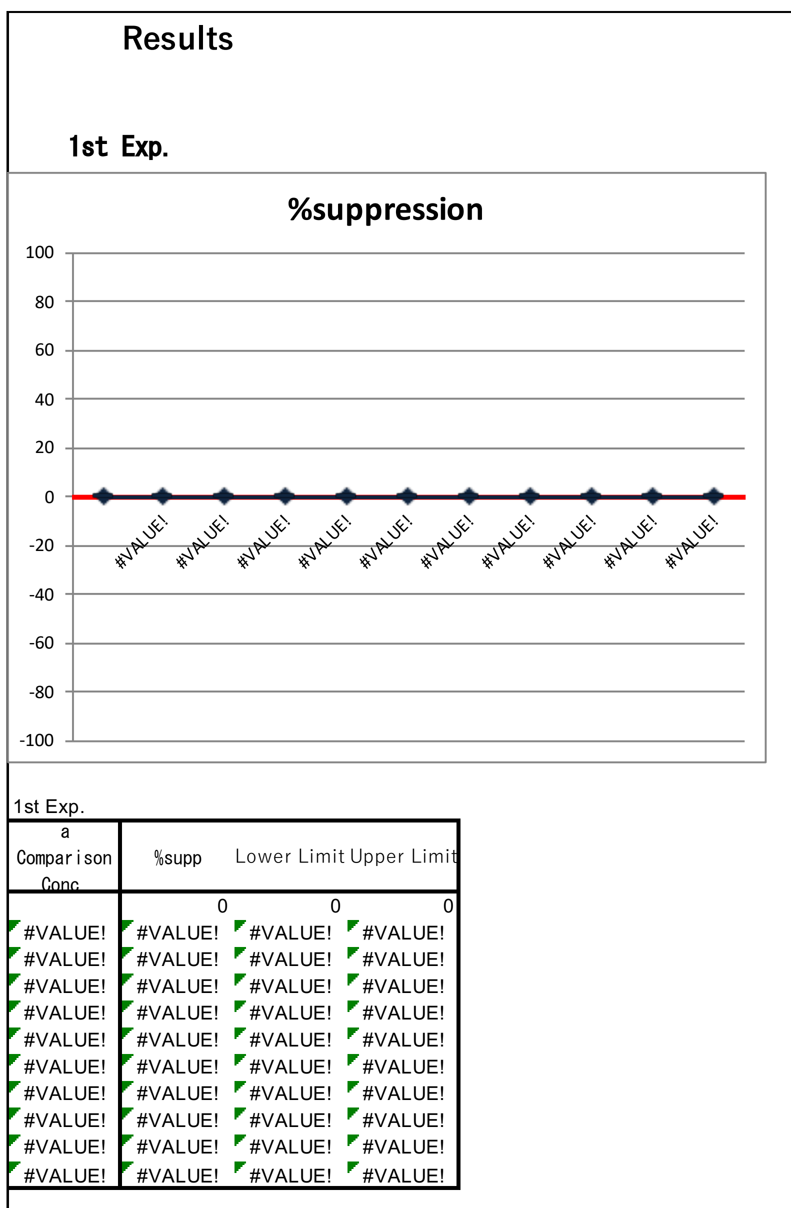
④グラフシート



1st Exp.

a Comparison Conc	%supp	Lower Limit	Upper Limit
	0	0	0
#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!
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#VALUE!	#VALUE!	#VALUE!	#VALUE!
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