厚生労働科学研究費補助金

化学物質リスク研究事業

免疫毒性評価試験法Multi-ImmunoToxicity assayの国際validationへ向けての検討

平成27年度　総括･分担研究年度終了報告書

研究代表者　相場　節也

平成 28（2016）年　5 月

目　　次

（H27-化学−一般-006））厚生労働科学研究費補助金（化学物質リスク研究事業）
総括･分担研究年度終了報告書

免疫毒性評価試験法Multi-ImmunoToxicity assayの国際validationへ向けての検討

研究代表者　相場　節也
東北大学病院皮膚科

研究要旨
今年度は、Multi-ImmunoToxicity assay (MITA)を用いて更に data set の拡充をはかり合計で 60 化学物質からなる data set を構築した。そのなかで、皮膚感作性物質の多くが LPS で刺激した THP-G8 細胞の IL-8 転写活性を抑制することを見いだし、従来の MITA では単球/樹状細胞に抑制的に作用する免疫抑制物質と皮膚感作性物質を区別できない事が明らかとなった。そこで従来法の MITA に、これまで我々が進めてきた皮膚感作性物質試験法である IL-8 Luc assay を加えた modified MITA を構築し、現在までに 24 物質の data set を作成した。その結果、化学物質が大きく IL-2 レポーター活性抑制物質、IL-8 Luc assay 陽性物質、IL-8 Luc assay 陰性で LPS 刺激下の IL-1β/IL-8 レポーター活性抑制物質、その他（IL-2 あるいは IL-1βレポーター活性増強物質）の４群に分けられることが明らかとなった。次いで、この結果をもとに IL-2 転写活性抑制と IL-8 転写活性増強の 2 つを key event とする adverse outcome pathway (AOP)を作成した。前者では、dimethylthiocarbamate (DTC)の NF-kB 抑制、AG-018986 の p38 MAPK 抑制、メチル水銀(CH_3HgCl)の ERK1/2 抑制、Propanil (3,4-dichloropropionanilide (DCPA)の STIM1、CRAC を介した NFAT 抑制、鉛の calmodurin を介した NFAT 抑制を組み込んだ AOP が作成できた。また後者では、diesel exhaust particle (DEP)、フォルマリン、PM2.5 さらには界面活性剤による IL-8 転写活性亢進作用と adverse outcome としての気道刺激性を組み込んだ AOP を作成した。さらにこれらの結果をもとに、1 月 26 日から 28 日までの三日間の予定で、国内外から免疫毒性の専門家を招き免疫毒性評価系国際化へ向けての kick-off meeting を仙台にて開催する。そこで、MITA の科学的意義、作成した AOP の改良、試験法プロトコルの妥当性などについて議論する予定である。さらに現在、IL-2 レポーター活性抑制物質評価にかかわる技術移転性確認を目的とした 3 施設での施設間差比較試験を実施中である。

研究分担者氏名・所属研究機関名及び所属研究機関における職名
小島　肇・国立医薬品食品衛生研究所安全性生物試験研究センター薬理部・室長
近江谷　克裕・産業技術総合研究所・バイオメディカル部門・部門

山影　康次・食品薬品安全センター秦野研究所代替法試験部・部長
大森　崇・神戸大学医学部附属病院・臨床研究推進センター、生物統計学第二室長

A．研究目的

研究背景：

環境汚染物質、食品添加物、薬剤などの化学物質のなかには免疫系を標的とし、アレルギー、自己免疫疾患、発癌などの健康被害を及ぼすものが少なくない。したがって、外因性化学物質の生体免疫機能への毒性効果として定義される免疫毒性は、公衆衛生行政にとっても重要な課題となっている。しかし現在存在している化学物質の免疫毒性評価系は、極めて多岐にわたる免疫反応に対する化学物質の影響を評価するには不十分であり、さらにその多くが動物実験に依存している。いうまでもなく動物実験には、得られた結果からどこまでヒトに対する影響を類推できるかという科学的問題に加えて費用面、倫理面など多くの問題が存在する。したがって、これらの問題を解決するためには多面的免疫反応を動物実験を用いずに評価する試験系の開発が不可欠である。

我々は，平成18−22年NEDO「高機能簡易型有害性評価手法の開発」プロジェクトにおいて，産業総合研究所が開発した３色発光細胞の技術を応用し，Jurkat細胞におけるINF-γ，IL-2，G3PDHプロモーター活性，THP-１細胞におけるIL-8，IL-1β，G3PDHプロモーター活性をhigh throughputに評価できる長期細胞株を樹立し，化学物質の免疫毒性評価システム(Multi-ImmunoTox assay；MITA)を構築し国内外の特許を取得している．MITAを用いるとヒトT細胞におけるIL-2とIFN-γ、マクロファージ/樹状細胞におけるIL-1βとIL-8の転写に関与するシグナル伝達経路への化学物質の影響を定量的に評価することができる。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、我々はまず作用機序の明らかな種々の免疫抑制剤をMITAを用いて評価するなかで、化学物質毒性評価におけるMITAのプロトコールを作成し、そのプロトコールを用いた薬剤の免疫毒性評価を行った。その結果、代表的な免疫抑制剤であるデキサメサゾン(Dex)、サイクロスポリン(CyA)、タクロリムス（Tac）のT細胞とマクロファージ/樹状細胞に対する薬理効果をMITAが予測できることを明らかにした(Kimura et al., 2014)。さらに、40種類の化学物質を評価したところ、鉛の免疫抑制作用、リチウム、水銀によるによる免疫増強作用を検出できることも明らかとなった。さらに世界に先駆けて、人工染色体を用いたIL-1βレポーター細胞を樹立し、MITA構成細胞の長期安定性を確保した。施設内、施設間再現性も検討し、IL-2とIFN-γレポーター細胞に関しては既に良好な結果が得られている。以上の結果より、MITAが化学物質の免疫毒性を自然免疫と獲得免疫の両面から評価できる新しいhigh-throughput手法となりうることを明らかにした。

計画全体の目的：

図１に示すように、本研究では以下の 4 項目を目的として研究を計画した。

１）自己免疫、免疫抑制、アレルギー (Th1/Th2 不均衡)の３つの adverse outcome (AO)に関して、それらを惹起することが確認されている化学物質各４種類を選び AOP を作成する。

２）作成された AOP を基にして、その中から抽出された Key events (KEs)を網羅し、そのなかで MITA が評価可能な項目を実験的に明らかにする。

３）プロトコールを変更し、MITA により細胞周期に作用する免疫抑制剤、感作性物質なども評価可能とする。

４）MITA の data set の拡充と施設内、施設間再現性を改善し国際的 validation を実施し OECD テストガイドライン化をめざす。

2016 年度の目的：

2016 年度は、特に以下の 6 項目を研究目的とした。

① MITA の最適化と data set の構築

MITA の問題点を明らかにして、MITA を免疫毒性

評価により適した評価系に修正する。

② MITA に適した免疫毒性評価系の探索

③ MITA のパラメーターを key event とする AOP の作成

④ AOPに基づく化学物質評価

⑤ IL-2転写活性抑制試験に関する技術移転性確認

⑥ MITA を用いた免疫毒性評価系国際化へ向けての kick-off meeting の開催

B. 研究方法

試薬

Water-soluble Dexamethasone (Dex), Cyclosporin A (CyA), Tacrolimus (TAC), Rapamycin, Cyclophosphamide, Azathioprine, Mycophenolic acid, Mizoribine, Leflunomide, Methotrexate, 4-Aminophenyl sulfone (Dapsone), Sulfasalazine, Colchicine, Chloroquine, Minocycline, Nicotinamide, Acetaminophen, Digoxin, Warfarin, Cimetidine, Levamizol, Isoniazid, Phorbol 12-myristate 13-acetate (PMA), Ionomycin(Io), Lipopolysaccharides from E. coli 026:B6 (LPS), 2,4-Diaminotoluene, 2-Aminoanthracene, 2-Mercaptobenzothiazole, Amphoterycine B, Benzethonium chloride, Chlorpromazine, Cisplatin, Dibenzo[a,i]pyrene, Dibutyl phthalate, Diethanolamine, Lead acetate, Nitrofurazone, Pentamidine isethionate, p-Nitroaniline, Pyrimethamine, Ribavirin, Sodium bromate, Triethanolamine, Actinomycin D, Cobalt chloride, Dimethyl sulfoxide, Histamine, Hydrocortisone, Isophorone diisocyanate, Mitomycin C は Sigma-Aldrichから購入した。Aluminum chloride, Ethanol, Magnesium sulfate, Methanol, Nickel sulfate, Sodium lauryl sulfate, Lithium carbonate, Mercuric chlorideは和光純薬から購入した。Hydrogen peroxideは三徳化学工業から購入した。Deoxyspergualinは医薬品卸業から購入した。

Jurkat T細胞由来#2H4細胞におけるIL-2，IFN-γ，GAPDHプロモーターアッセイおよびTHP-1 単球細胞由来TGCHAC-A4細胞、THP-G8細胞におけるIL-1β，IL-8，GAPDHプロモーターアッセイ(図2)

IL-2およびIFN-γプロモーター活性の測定には、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細胞を用いた(Saito et al., 2011)。またIL-1βプロモーター活性の測定には、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、GAPDHプロモーターに制御されたSLRルシフェラーゼ遺伝子を導入したTGCHAC-A4細胞を、IL-8プロモーター活性の測定には、THP-1にIL-8プロモーターに制御されたSLOルシフェラーゼ遺伝子およびGAPDHプロモーターに制御されたSLRルシフェラーゼ遺伝子を導入したTHP-G8細胞を用いた(Takahashi et al., 2011b)。なおTGCHAC-A4細胞の樹立には人工染色体技術(Katoh et al., 2004)を用い細胞の安定性を確保した。1ウェル当たり2×10^5個の# 2H4細胞または1ウェル当たり5×10^4個のTGCHAC-A4細胞またはTHP-G8細胞を黒色の96-well プレート(Greiner bio-one)に播種し、薬剤を加え、37℃、5%CO_2下で1時間培養した。つづいて# 2H4細胞については25nM PMAと1μM Ioの混合物(PMA/Io)、TGCHAC-A4細胞、THP-G8細胞については100 ng/ml 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、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

MITAによる免疫毒性評価法（図３）

各実験において得られた結果は、一元配置分散分析を行い、その後 Dunnett 検定により有意な抑制効果, 増強効果があるか否かを検討した。しかし、この実験を 3 回繰り返し検討すると、3 回の実験結果が必ずしも一致していない薬剤が存在した。そこで、一致が見られなかった薬剤に関しては、3 回の繰り返し実験の結果のなかから%suppression の絶対値（免疫抑制物質に関しては正の値、増強物質に関しては負の値となる）が最も大きい値を選び Student's t-test を行い、そこで統計的有意差の得られた場合、その結果を薬剤の最終的判定結果とした。

Jurkat細胞、THP-1細胞におけるmRNA発現

3×10^6 細胞のJurkat細胞またはTHP-1細胞を薬剤で1時間前処理し、その後、それぞれPMA/Ioまたは100 ng/ml LPSで刺激し37℃で6時間培養した。Isogen (Nippon gene)を用いてtotal RNAを抽出した。

定量的 RT-PCR

TaKaRa RNA PCR Kit (AMV) (Takara Bio Inc)を用いてtotal RNAから相補的DNA(cDNA)を合成した。Mx3000p QPCR System (Stratagene)を用いて定量 RT-PCRを行った。プライマーについて、それぞれの遺伝子情報はGenBankより入手し、Primer Express 1.0 (Applied Biosystems)を用いて設計、SIGMA GENOSYSにて合成した。cDNA 10ng、フォワードおよびリバースプライマー400nM、TaqMan probe 60nM、ROX 30nM, Brilliant II Fast QPCR Master Mix (Stratagene)を含む反応液を、95℃で2分間反応させたのち、95℃、5秒間、60℃、20秒間の反応を45サイクル行った。恒常的に発現するGAPDHをコントロール遺伝子とし、ΔΔCt法で各遺伝子発現の解析を行った。

MITAのdata setの拡充

WagnerらがFluorescent Cell Chip assay (FCC)において検討した46種類の化学物質 (Wagner et al., 2006)および免疫毒性が知られている化学物質14種類(WHO 免疫毒性ガイダンスのcase studiesで検討されている物質を含む))に関してMITAを行いdata setを拡充した。この60種類の化学物質は、既に過去の報告をもとにin vivo、in vitroにおいて免疫毒性が報告されていない化学物質(N)、免疫抑制の報告のある化学物質(IT-1)、アレルギー、自己免疫などを誘発する可能性のある化学物質(IT-2)、in vivoにおける影響は明らかではないが何らかの免疫関連パラメーターを変動させる化学物質(M)などに分類されている。

IL-8 Luc assayを組み込んだmodified MITAのdata setの作成

IL-8 Luc assayは、感作性物質が単球細胞に作用した際のIL-8 mRNA発現増強をTHP-G8細胞を用いて評価する試験法で、現在皮膚感作性試験法として国際validationが行われている。具体的には、化学物質をTHP-G8細胞に作用させてその16時間後のIL-8 promoter活性をluminometerにて定量する。すでにプロトコール、陽性、陰性の判定基準も確立している。これまでに改良が加えられ、精度、感度などいずれも80%をこえる評価系である(Takahashi et al., 2011b) (Kimura et al., 2015)。今回は、MITAのdata setの中に含まれる化学物質をIL-8 Luc assay のプロトコールに則り評価した。

（倫理面への配慮）

健常人からの採血に際しては、研究内容、採血における危険性、得られた検査結果により本人の人権が損なわれることのないこと、得られた検査結果は守秘され個人のプライバシーを侵害する可能性がないこと、研究に協力することに同意した後もいつでも自由に辞退できること、この研究によって生じる知的財産権は被験者には帰属しないことについて説明し、本人より同意書を取得している。

C. 研究結果

①MITAの最適化とdata setの構築

1)MITA data setの拡充　(Table 1)

これまでに作成されていたMITA data setの不確定な部分を補い、更にWHOから提出された

Guidance for immunotoxicity risk assessment for chemicals の Case-Studies にて検討されている化学物質、喘息などのアレルギー疾患との関与が想定されている diesel exhaust particles (DEP)、ホルマリン (FA)、dibutyl phthalate を加えた 60 化学物質からなる data set を作成した。WHO Guidance の Case-Studies に含まれる化学物質に関しては、MITA は鉛の免疫抑制、水銀による IFN-γレポーター活性増強作用を、また DEP、FA の Th1 サイトカインである IL-2 レポーター活性抑制作用を検出できた。

2)MITA の問題点

MITA data set (Table 1)から明らかな様に、MITA では $CoCl_2$、$NiCl_2$、isophorone diisocyanate などの感作性物質が IL-8 レポーター活性抑制作用を示し、Dex、hydrocortisone あるいは FR167653 (p38 mitogen activated kinase (MAPK)阻害剤)などの免疫抑制剤との区別できない。そこで MITA を有効に活用するためには、感作性物質評価系との組み合わせが不可欠である。

3)Modified MITA の構築

そこで従来の MITA に、更に LPS 刺激を加えない IL-1β、IL-8 プロモーター活性測定系を加えた modified MITA を構築することにした。特に IL-8 に関しては、すでに OECD test guideline 化をめざして validation が進んでいる感作性試験法、IL-8 Luc assay をそのプロトコール、陽性陰性判定基準も含めて取り込むことにし Table 2 を作成した。IL-1βに関しては、必要に応じて適宜加えることとし、IL-8 Luc assay にほぼ準じたプロトコールを想定して現在試験物質の数を増やしている。

4) Modified MITA による免疫抑制物質と感作性物質の識別

Table 2 を用いて IL-8 レポーター活性抑制物質物質を選び出すと(Table 3)、その中に IL-8 Luc assay で感作性と評価される物質と非感作性と評価される物質が含まれることが分かる。すなわち、IL- 8 Luc assay を加えることにより、Dex、hydrocortisone、FR167653 などの免疫抑制物質と感作性物質との識別が可能となる。

② MITA に適した免疫毒性評価系の探索

Modified MITA の data set の構築により、免疫毒性物質が IL-2 レポーター活性抑制物質、感作性物質、IL-8 Luc assay 陰性かつ LPS 刺激下 IL-8 レポーター活性抑制物質、その他（IL-2 あるいは IL-1βレポーター活性増強物質など）４種類に大きく分類できることが明らかとなった。

１－１）IL-2 レポーター活性抑制物質の分類

Table 2 を用いて、化学物質を IL-2 レポーター活性抑制強度（LOEL(lowest observed effect level)を指標とする）をもとに分類すると（Table 4)、化学物質が IL-2 レポーター活性抑制 LOEL により大きく 1 群 LOEL<0.1、2 群 LOEL<1.0、3 群 LOEL<10、 4 群 LOEL<100、5 群 LOEL<1000、6 群抑制なし、7 群 IL-2 レポーター活性増強に分類できた。そのなかで、代表的な免疫抑制剤 Dex、CyA、Tac、SLE の治療薬である chloroquine は１群、hydrocortisone は 2 群、２価の金属は３群ないし４群に属していた。

1-2)IL-2 レポーター活性と IFN-γレポーター活性との相関

IL-2 と IFN-γは、いずれも Th1 サイトカインとして良く知られている。そこで Table 2 を用いて IL-2 と IFN-γレポーター活性の相関を検討した。IL-2 レポーター活性が抑制された 41 化学物質のうち 12 化学物質に関しては、IFN-γレポーター活性の抑制は認められなかったが、残りの29化学物質に関しては図4に示す様にIL-2 レポーター活性と IFN-γレポーター活性との間に強い正の相関が認められた。また IL-2 レポーター活性が抑制されない化学物質には IFN-γレポーター活性を抑制する物質は含まれなかった。一方、９個の IL-2 レポーター活性増強物質の中には 7 個の IFN-γレポーター活性増強物質が含まれ、IFN-γレポーター活性抑制物質は含まれていなかった。したがって、modified MITA における T 細胞関連パラメーターとしては IL-2 レポーター活性のみで十分であると思われる。

2-１）IL-8 Luc assay による化学物質分類

すでに我々は IL-8 Luc assay が皮膚感作性物質の評価系として有用であることを報告している(Kimura et al., 2015; Takahashi et al., 2011a)。しかし、Table 2 をあらためて IL-8 Luc assay の LOEL をもとに分類すると (Table 5)、感作性

物質以外に hydrogen peroxide、diesel exhaust particles (DEP)など reactive oxygen species 産生物質、sodium lauryl sulfate (SDS)などの界面活性剤も IL-8 Luc assay で陽性と判断されることが明らかとなった。既に感作性物質の評価系としては、これらの物質は applicability domain からはずしている。

2−2）PM2.5 の IL-8 Luc assay を用いた気道刺激性評価

最近、共同研究者の鳥取大学分子制御内科学分野　渡部仁成講師らは、THP-G8 細胞を用いると、実際に日本に飛来してくる PM2.5.や黄砂と中国の黄土高原の砂との生物学的活性の相違を容易に検出できることを明らかにした(Watanabe et al., 2014)。またこの研究では、PM2.5 の喘息、鼻炎などの誘発には、単なる粒子量ではなく、その生物学的活性すなわち THP-G8 レポーター活性が相関することを明らかにした。さらに渡部らは(Watanabe et al., 2015)、島根県松江市の小学生約 400 人を対象に 2012 年と 2013 年の 2 年間、日々の peak expiratory flow (PEF)の変化と大気中の PM2.5 の量およびその THP-G8 細胞を用いた IL-8 レポーター活性を対比した。その結果、大気中の PM2.5 の絶対量ではなくて IL-8 レポ-ター活性が PEF の悪化と相関があることを報告した。また最近、Corsini ら(Corsini et al., 2013)は PM2.5 の炎症惹起活性の指標として THP-1 細胞からの IL-8 産生が気道上皮の IL-8 産生よりも鋭敏であることを報告している。

一方、これまでの研究で、THP-G8 細胞は lipopolysaccharide (LPS)を始めとした種々の Toll-like receptors (TLR 1, 2, 4, 5, 6)および NLR 1, 2 アゴニストに反応し IL-8 レポーター活性を増強すること、したがって IL-8 Luc assay は化学物質のみならず種々の微生物由来毒素の評価系としても応用が可能であることを明らかにしている。

3）IL-8 Luc assay 陰性で IL-8 レポーター活性を抑制する化学物質

Table 5 を参照すると IL-8 レポーター活性抑制物質のなかに IL-8 Luc assay 陰性の化学物質が含まれていることがわかる。この中には p38 MAPK 阻害剤である FR167653 や Dex が含まれていることからも推測できるよう p38 MAPK や NF-kB 阻害作用のある物質が含まれていることが予想される。

4）その他（IL-2 あるいは IL-1βレポーター活性増強物質）

上記１）１）２）３）の分類に含まれない化学物質の中に、IL-2 あるいは IL-1βレポーター活性増強物質が含まれていた。

③ MITAのパラメーターをkey eventとするAOPの作成

上記評価系に関連してAOPを作成した。

1）IL-2 転写活性抑制を key event とした T 細胞分化異常誘導に関する AOP の作成

IL-2 レポーター活性が、MITA で評価可能な T 細胞関連因子の中では最も多くの化学物質で抑制されること、また多くの化学物質で IFN-γレポーター活性と相関が認められることより IL-2 レポーター活性を KE とした AOP を構築することとした。IL-2 はおもに Th1 細胞が分泌するサイトカインであるが、T 細胞の増殖に必須なばかりでなく、IL-12Rß2、IL-4Rα4、gp130 などの発現を介して Th1、Th2 細胞、Treg 細胞の分化に不可欠なサイトカインである。また一方で、Th17 細胞の分化を抑制することにより不必要な自己免疫反応や炎症反応の発症を制御する (Letourneau et al., 2009) (Liao et al., 2011)。そこで化学物質の免疫毒性の指標として、IL-2 の転写制御を評価することは極めて重要な意味を有していると考え、IL-2 転写活性抑制を KE とする T 細胞分化異常誘導に関する AOP を作成することとした。具体的には、文献的に IL-2 の転写活性に影響を及ぼす化学物質を検索し、dimethylthiocarbamate (DTC) (Pyatt et al., 1998)、AG-018986 (Lee and Jessen, 2012)、メチル水銀(CH3HgCl) (Colombo et al., 2004)、Propanil (3,4-dichloropropionanilide (DCPA) (Lewis et al., 2013) (Salazar et al., 2008)、鉛 Colombo *et al.*, 2004) が IL-2 の転写抑制活性を有することを見いだした。またそのメカニズムとして、DTC は NF-kB 抑制、AG-018986 は p38 MAPK 抑制、CH_3HgCl は ERK1/2 抑制、DCPA は STIM1、CRAC を介した NFAT 抑制、鉛は calmodurin を介した NFAT 抑制が報告さ

れていることから、図 5 に示す AOP を作成した。しかし IL-2 欠損マウスが多臓器に重篤な自己免疫疾患を発症することは良く知られているものの、現時点では個々の化学物質による IL-2 転写活性、分泌抑制がどのような免疫異常を臓器レベル、個体レベルで引き起こいうるのかは明らかにされていない。

2) IL-8 転写活性抑制を key event とした化学物質気道刺激性の AOP 作成

IL-8 Luc assay が、感作性物質のみではなく DEP、フォルマリン、PM2.5、界面活性剤さらには微生物由来毒素などにも幅広く反応することを利用し、IL-8 転写活性亢進作用を中心とした気道刺激性 AOP を作成した(図６)。特に、PM2.5 や黄砂のように大気中の化学物質のみならず微生物毒素などもその表面に吸着されている可能性がある物質の評価には有用性が期待できる。しかしこの AOP も IL-2 転写活性抑制を中心とした免疫毒性 AOP と同様に個々の化学物質による IL-8 転写活性、分泌亢進がどのようにして気道過敏に繋がるのかはまだ明らかになっていない。

3)IL-8 レポーター細胞と IL-1βレポーター細胞の組み合わせ

IL-8 Luc assay のみでは、感作性物質と界面活性剤、ROS 刺激を識別できないため IL-1βレポーター細胞との組み合わせを検討した。感作性物質 12 種類と界面活性剤を含む非感作性物質 4 種類で両細胞を刺激したところ、THP-G8 細胞は感作性物質と界面活性剤両者に反応してレポーター活性を増強するが、TGCHAC-A4 細胞は感作性物質には反応するが界面活性剤には反応しないことが明らかになった(Table 6)。

④ AOPに基づく化学物質評価

１）IL-2転写活性抑制を中心とした免疫毒性AOPに基づく化学物質評価

既に鉛に関しては、IL-2レポーター活性を抑制することを明らかにしているので、その他の化学物質に関してIL-2レポーター活性への影響を検討した。その結果、2H4細胞を用いて更に propnail (DCPA)およびメチル水銀のIL-2転写活性に対する影響を検出できることを明らかにした。

２）IL-8転写活性亢進を中心とした免疫毒性AOPに基づく化学物質評価

既にフォルマリン、DEPなどに関しては検討ずみである。また黄砂、PM2.5に関しては渡部らの報告が存在する。

⑤ IL-2転写活性抑制試験に関する技術移転性確認

MITAを構成する2H4細胞を用いた化学物質のIL-2転写活性抑制評価に関する技術移転性確認の目的で、現在東北大、産総研、食薬センターの3施設で5化学物質を用いた施設間差比較試験を施行中である。

⑥ MITA を用いた免疫毒性評価系国際化へ向けての kick-off meeting

平成28年度以降MITAに関するバリデーションを実施するにあたり、その国際的公定化の道筋を明確にすることを目的に、平成28年１月に国際バリデーションのキックオフ会議を企画している。同会議においては、免疫毒性およびその試験法に関する専門家として海外からDr. Emanuel Corsini (Milan Univ.)およびDr. Erwin L. Roggen (3Rs Management and Consulting ApS)を、国内から景山茂樹氏（富士フィルム）および日本免疫毒性学会からの推薦者を外部専門家として招聘し、研究班の班員とともに３日間の予定でMITAの科学的意義、作成したAOPの改良、試験法プロトコルの妥当性などについて討論する予定である。さらに外部専門家の意見をもとに、平成28年度以降に実施するバリデーション計画を立案する。

D．考察

以上の結果から、免疫毒性物質が図 7 に示す様に、modified MITA を用いて IL-2 レポーター活性抑制物質、感作性物質、IL-8 Luc assay 陰性かつ LPS 刺激下 IL-8 レポーター活性抑制物質、その他（IL-2 あるいは IL-1βレポーター活性増強物質など）４種類に分類できることが明らかとなった。

化学物質による免疫毒性はおおきく全身性の免疫毒性と局所性の免疫毒性に分類される。局所性の免疫毒性は、おもに皮膚、気道上皮における刺激性と感作性が、一方全身性免疫毒性は、血中に存在する化学物質による毒性が想定される。化学物質が免疫毒性をどこで発揮するかにより、予想される曝露濃度が大きく異なってくる。皮膚では時に高濃度の化学物質に曝露されることもありえる。一方気道上皮は皮膚と比べればかなり低い濃度の曝露が、また血中はそれらよりさらに低濃度曝露が想定される　(図７)。そこで化学物質の免疫毒性を評価するうえでは、化学物質が何らかの免疫学的作用を発揮する上での最低能渡 (Lowest observed effect level)を明らかにすることが不可欠である。

そこで今年度、我々はdata setをもとにIL-2レポーター活性抑制を指標に、化学物質の分類を試みた。LOELをもとにとりあえず1群 LOEL<0.1、2群LOEL<1.0、3群LOEL<10、 4群LOEL<100、5群LOWEL<1000、6群抑制なし、7群IL-2レポーター活性増強の7群に分類した。その分類では、予想どおり、Dex、CyA、Tac、chloroquine (SLE治療薬)など強力な免疫抑制剤は1群に含まれ、作用の弱いhydrocortisoneは2群に含まれていた。その他、1群、2群にはactinomycin D、cisplatin、mytomycin Cなどの潜在的に免疫抑制作用を有していることが予想される抗がん剤やdibonzopyreneなどの発がん物質も含まれていた。一方これらの薬剤以外に、1群にはpyrimethamineやdigoxinなども含まれていた。Pyrimethamineに関しては、他の1群に含まれる化学物質に比べ抑制効果は弱く、またdigoxinに関しては、LOELの値が通常投与量における到達血中濃度よりもはるかに高値であった。

また本年度の研究により、modified MITAにより評価できる免疫毒性に関与するkey eventが少なくとも２項目見いだせ、これをもとに2種類のAOPを作成することができた。いずれのAOPもまだ不完全なものではあるが、次年度以降これらを更に充実させるとともにIL-2転写抑制に関してはvalidationを実施する予定である。

E. 結論

①IL-8 Luc assay を組み込んだ modified MITA の構築と data set の作成
従来法の MITA の問題点を改善する目的で、MITA と皮膚感作性試験法 IL-8 Luc assay を組み合わせた modified IL-8 Luc assay を構築し data set を作成した。
②化学物質の modified MITA を指標とした免疫毒性別の分類
作成した data set から、化学物質が１）IL-2 転写活性抑制物質、２）IL-8 Luc assay 陽性物質、３）IL-8 Luc assay 陰性、LPS 刺激後の IL-8 転写活性抑制物質、および 4)その他（IL-2 あるいは IL-1βレポーター活性増強物質）の４群に分類できることが明らかとなった。
③AOP の作成
IL-2 転写活性抑制、IL-8 転写活性亢進のそれぞれを key event とした T 細胞分化異常誘導および気道刺激性に関する AOP を作成した。
④国際的視点からの試験法の検討
国際的 validation management 委員会を組織し、MITA の科学的意義、作成した AOP の改良、試験法プロトコルの妥当性などに関して意見を求め試験法の改良に繋げる。

引用文献

1. Colombo, M., Hamelin, C., Kouassi, E., Fournier, M., Bernier, J., 2004. Differential effects of mercury, lead, and cadmium on IL-2 production by Jurkat T cells. Clin Immunol 111, 311-322.
2. Corsini, E., Budello, S., Marabini, L., Galbiati, V., Piazzalunga, A., Barbieri, P., Cozzutto, S., Marinovich, M., Pitea, D., Galli, C.L., 2013. Comparison of wood smoke PM2.5 obtained from the combustion of FIR and beech pellets on inflammation and DNA damage in A549 and THP-1 human cell lines. Arch Toxicol 87, 2187-2199.
3. Katoh, M., Ayabe, F., Norikane, S., Okada, T., Masumoto, H., Horike, S., Shirayoshi, Y.,

Oshimura, M., 2004. Construction of a novel human artificial chromosome vector for gene delivery. Biochem Biophys Res Commun 321, 280-290.

4. Kimura, Y., Fujimura, C., Ito, Y., Takahashi, T., Aiba, S., 2014. Evaluation of the Multi-ImmunoTox Assay composed of 3 human cytokine reporter cells by examining immunological effects of drugs. Toxicol In Vitro 28, 759-768.
5. Kimura, Y., Fujimura, C., Ito, Y., Takahashi, T., Nakajima, S., Ohmiya, Y., Aiba, S., 2015. Optimization of the IL-8 Luc assay as an in vitro test for sin sensitization. Toxicol In Vitro in press.
6. Lee, D.U., Jessen, B., 2012. Off-target immune cell toxicity caused by AG-012986, a pan-CDK inhibitor, is associated with inhibition of p38 MAPK phosphorylation. J Biochem Mol Toxicol 26, 101-108.
7. Letourneau, S., Krieg, C., Pantaleo, G., Boyman, O., 2009. IL-2- and CD25-dependent immunoregulatory mechanisms in the homeostasis of T-cell subsets. J Allergy Clin Immunol 123, 758-762.
8. Lewis, T.L., Holaskova, I., Barnett, J.B., 2013. The toxicity of the N-hydroxy and 6-hydroxy metabolites of 3,4-dichloropropionanilide does not depend on calcium release-activated calcium channel inhibition. Toxicol Sci 131, 395-405.
9. Liao, W., Lin, J.X., Wang, L., Li, P., Leonard, W.J., 2011. Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. Nat Immunol 12, 551-559.
10. Pyatt, D.W., Gruntmeir, J., Stillman, W.S., Irons, R.D., 1998. Dimethyldithiocarbamate inhibits in vitro activation of primary human CD4+ T lymphocytes. Toxicology 128, 83-90.
11. Saito, R., Hirakawa, S., Ohara, H., Yasuda, M., Yamazaki, T., Nishii, S., Aiba, S., 2011. Nickel differentially regulates NFAT and NF-kappaB activation in T cell signaling. Toxicol Appl Pharmacol 254, 245-255.
12. Salazar, K.D., Ustyugova, I.V., Brundage, K.M., Barnett, J.B., Schafer, R., 2008. A review of the immunotoxicity of the pesticide 3,4-dichloropropionanalide. J Toxicol Environ Health B Crit Rev 11, 630-645.
13. Takahashi, T., Kimura, Y., Saito, R., Nakajima, Y., Ohmiya, Y., Yamasaki, K., Aiba, S., 2011a. An in vitro test to screen skin sensitizers using a stable THP-1-derived IL-8 reporter cell line, THP-G8. Toxicol Sci 124, 359-369.
14. Takahashi, T., Kimura, Y., Saito, R., Nakajima, Y., Ohmiya, Y., Yamasaki, K., Aiba, S., 2011b. An in vitro test to screen skin sensitizers using a stable THP-1-derived IL-8 reporter cell line, THP-G8. Toxicological sciences : an official journal of the Society of Toxicology 124, 359-369.
15. Wagner, W., Walczak-Drzewiecka, A., Slusarczyk, A., Biecek, P., Rychlewski, L., Dastych, J., 2006. Fluorescent Cell Chip a new in vitro approach for immunotoxicity screening. Toxicol Lett 162, 55-70.
16. Watanabe, M., Kurai, J., Tomita, K., Sano, H., Abe, S., Saito, R., Minato, S., Igishi, T., Burioka, N., Sako, T., Yasuda, K., Mikami, M., Kurita, S., Tokuyasu, H., Ueda, Y., Konishi, T., Yamasaki, A., Aiba, S., Oshimura, M., Shimizu, E., 2014. Effects on asthma and induction of interleukin-8 caused by Asian

dust particles collected in western Japan. J Asthma 51, 595-602.

17. Watanabe, M., Noma, H., Kurai, J., Sano, H., Saito, R., Abe, S., Kimura, Y., Aiba, S., Oshimura, M., Yamasaki, A., Shimizu, E., 2015. Decreased pulmonary funciton in school children in Western Japan after exposure to Asian desert Dusts and its association with interleukin-8. BioMed Res International in press.

F.研究発表

1．論文発表

1. Fujimura, T., Kambayashi, Y., Furudate, S., Asano, M., Kakizaki, A., ○Aiba, S. Receptor Activator of NF-kappaB Ligand Promotes the Production of CCL17 from RANK+ M2 Macrophages. J Invest Dermatol. 2015; 135, 2884-2887.
2. Kakizaki, A., Fujimura, T., Kambayashi, Y., Furudate, S., Kawano, M., Ogasawara, K., ○ Aiba, S. Comparison of CD163+ Macrophages and CD206+ Cells in Lesional Skin of CD30+ Lymphoproliferative Disorders of Lymphomatoid Papulosis and Primary Cutaneous Anaplastic Large-cell Lymphoma. Acta Derm Venereol. 2015; 95, 600-602.
3. Kambayashi, Y., Fujimura, T., Furudate, S., Asano, M., Kakizaki, A., ○ Aiba, S. The Possible Interaction between Receptor Activator of Nuclear Factor Kappa-B Ligand Expressed by Extramammary Paget Cells and its Ligand on Dermal Macrophages. J Invest Dermatol. 2015; 135, 2547-2550.
4. Watanabe, M., Noma, H., Kurai, J., Sano, H., Kitano, H., Saito, R., Kimura, Y., ○Aiba, S., Oshimura, M., Shimizu, E. Variation in the Effect of Particulate Matter on Pulmonary Function in Schoolchildren in Western Japan and Its Relation with Interleukin-8. Int J Environ Res Public Health. 2015; 12, 14229-14243.
5. Watanabe, M., Noma, H., Kurai, J., Sano, H., Saito, R., Abe, S., Kimura, Y., ○ Aiba, S., Oshimura, M., Yamasaki, A., Shimizu, E. Decreased pulmonary function in school children in Western Japan after exposures to Asian desert dusts and its association with interleukin-8. Biomed Res Int. 2015；583293.
6. Tsutsumi, M., Fukuda, M., Kumamoto, J., Goto, M., Denda, S., Yamasaki, K., ○ Aiba, S., Nagayama, M., Denda, M., 2015a. Abnormal Morphology of Blood Vessels in Erythematous Skin From Atopic Dermatitis Patients. Am J Dermatopathol, in press
7. Tsutsumi, M., Kitahata, H., Fukuda, M., Kumamoto, J., Goto, M., Denda, S., Yamasaki, K., ○Aiba, S., Nagayama, M., Denda, M. Numerical and comparative three-dimensional structural analysis of peripheral nerve fibres in epidermis of patients with atopic dermatitis. Br J Dermatol. 2015, in press.
8. ○Kimura, Y., Fujimura, C., Ito, Y., Takahashi, T., Nakajima, Y., Ohmiya, Y. ○ Aiba, S. Optimization of the IL-8 Luc assay as an in vitro test for skin sensitization. Toxicol In Vitro. 2015; 29, 1816-1830.
9. Watanabe, M., Kurai, J., Minato, S., Noma, H., Sano, H., Saito, R., ○Aiba, S., Oshimura, M., Hatakeyama, K., Yamasaki, A., Shimizu, E. Difference in interleukin-8 transcriptional activity induced in THP-G8 cells by particulate matter collected in winter and summer in western Japan. J Med Invest. 2015; 62, 145-148.
10. ○Ohmiya, Y. Simultaneous multicolor luciferase reporter assays for monitoring of multiple genes expressions. Comb Chem High Throughput Screen. 2015; 18, 937-945.
11. Yasunaga, M., Murotomi, K., Abe, H., Yamazaki, T., Nishii, S., Ohbayashi, T., Oshimura, M., Noguchi, T., Niwa, K., ○Ohmiya, Y., Nakajima, Y. Highly sensitive luciferase reporter assay using a potent destabilization sequence of calpain 3. J Biotechnol. 2015; 194, 115-123.
12. Tabei Y, Sonoda A, ○Nakajima Y, Biju Y, Makita Y, Yoshida Y, and Horie M, Intracellular

accumulation of indium ions released from nanoparticles induces oxidative stress, proinflammatory response and DNA damage. J. Biochem., *in press.*

13. Yoshikawa T, ○ Nakajima Y, Yamada Y, Watanabe K, Yamazaki M, Sakimura K, Honma S, and Honma K. Spatiotemporal profiles of arginine vasopressin transcription in cultured suprachiasmatic nucleus. Eur. J. Neurosci. 2015; 42, 2678-2689.
14. Umeno A, Takashima M, Murotomi K, ○ Nakajima Y, Koike T, Matsuo T, and Yoshida Y. Radical-scavenging activity and antioxidative effect 1 of olive leaf components oleuropein and hydroxytyrosol compared with those of homovanillic alcohol. J. Oleo Sci. 2015; 64, 793-800.
15. Murotomi K, Umeno A, Yasunaga M, Shichiri M, Ishida N, Koike T, Matsuno T, Abe H, Yoshida Y, and ○ Nakajima Y. Oleuropein-rich diet attenuates hyperglycemia and impaired glucose tolerance in type 2 diabetes model mouse. J. Agric. Food. Chem. 2015; 63, 6715-6722.
16. Tabei Y, Sonoda A, ○Nakajima Y, Biju Y, Makita Y, Yoshida Y, and Horie M. *In vitro* evaluation of the cellular influence of indium tin oxide nanoparticles using the human lung epithelial cell line A549. Metallomics,. 2015; 7, 816-827.
17. Murotomi K, Umeno A, Yasunaga M, Shichiri M, Ishida N, Abe H, Yoshida Y, and ○Nakajima Y. Switching from singlet-oxygen-mediated oxidation to free-radical-mediated oxidation in the pathogenesis of type 2 diabetes in model mouse. Free Radic. Res. 2015; 49, 133-138.
18. Yasunaga M, Murotomi K, Abe H, Yamazaki T, Nishii S, Ohbayashi T, Oshimura M, Noguchi T, Niwa K, Ohmiya Y, and ○Nakajima Y. Highly sensitive luciferase reporter assay using a potent destabilization sequence of calpain 3. J. Biotechnol., 2015; 194, 115-123.

2．学会発表

相場節也：皮膚感作性および免疫毒性のadverse outcome pathway (AOP)．日本動物実験代替法学会　第28回大会（横浜）2015年12月

H．知的財産権の出願・登録状況（予定を含む。）

1．特許取得

特願2010-151362; PCT/JP2011/65090

Table 1. MITA data set (60 化学物質)

Chemicals	IL-2		IFN-γ		IL-1β		IL-8	
	Judge	LOEL	Judge	LOEL	Judge	LOEL	Judge	LOEL
2,4-Diaminotoluene	N		A	62.50	N		S	0.98
2-Aminoanthracene	S	0.81	S	5.86	S	2.03	N	
2-Mercaptobenzothiazole	N		N		N		S	125.00
Acetaminophen	A	33.33	A	33.33	A	166.67	A	100.00
Actinomycin D	S	0.00	S	0.01	N		S	0.00
Aluminum chloride	S	3.91	S	62.50	N		N	
Amphoterycin B	S	0.78	S	2.08	A	3.13	A	7.82
Benzethonium chloride	S	1.95	S	1.95	S	3.91	N	
Chlorpromazine	S	3.91	S	3.91	S	7.81	S	7.81
Cisplatin	S	0.24	S	1.22	N		N	
Cobalt chloride	S	3.91	S	9.12	S	3.91	S	125.00
Cyclophosphamide	N		A	168.00	N		N	
Cyclosporine A	S	0.00	S	0.00	N		N	
Dapsone	S	45.01	S	55.14	S	46.88	S	134.75
Dexamethasone	S	0.01	N		S	0.01	S	0.01
Dibenzopyrene	S	0.01	S	0.03	N		N	0.00
Diethanolamin	S	9.12	N		N		N	
Dimethyl sulfoxide	A	3.91	A	625.00	S	66.41	S	3.91
Ethanol	N		N		N		N	
FK 506	S	0.00	S	0.00	A		N	
FR167653	S	0.49	S	0.49	S	145.83	S	125.00
Histamine	S	9.12	A	5.86	N		S	3.91
Hydrocortisone	S	0.34	A	6.27	S	0.34	S	0.34
Hydrogen peroxide	S	7.82	S	31.25	N		N	
Isoniazid	S	1.97	N		N		S	800.00
Isophorone diisocyanate	S	0.98	N		S	0.98	S	0.98
Lead(II) acetate	S	3.91	S	3.91	N		N	

Lithium carbonate	S	0.98	A	116.67	S	0.39	S	0.39
Magnesium sulfate	N		N		S	15.63	N	
Mercuric chloride	N		A	3.91	S	1.95	S	1.95
Methanol	N		N		N		N	
Mitomycin C	S	0.36	N		N		N	
Nickel sulfate	S	14.32	S	32.55	S	250.00	S	250.00
Nitrofurazone	S	0.37	A	3.91	A		A	62.50
Pentamidine isethionate	S	3.91	S	32.55	S	3.91	S	3.91
p-Nitroaniline	S	0.98	S	1.95	S	1.47	S	2.45
Pyrimethamine	S	0.04	N		N		N	
Ribavirin	A	15.63	A	187.50	A	5.86	N	
Sodium bromate	S	125.00	N		N		N	
Sodium dodecyl sulfate	N		N		N		N	
Dibutyl phthalate	S	0.98	S	1.95	S	39.07	S	31.25
Triethanolamine	S	187.50	S	1416.67	N		N	
Hexachlorobenzene	N		N		N		N	
Citral	S	0.36	S	1.37	N		N	
Trichloroethylene	N		N		N		N	
Chloroplatinic acid	N		N		N		S	15.63
Formaldehyde	S	1.71	N		S	15.63	S	15.63
Diesel exhaust particles	S	39.07	A	47.53	N		S	62.50
Azathioprine	N		A	40.01	A	9.23	N	
Chloroquine	S	0.05	S	0.02	S	10.00	S	30.00
Colchicine	A	0.29	A	0.06	A	0.02	A	20.00
Digoxin	S	0.01	S	0.02	N		N	
Methotrexate	A	0.45	A	0.09	N		N	
Minocycline	S	8.33	S	5.00	N		N	
Mizoribine	N		N		A	5.20	A	7.45
Mycophenolic acid	A	0.38	A	6.24	N		N	
Nicotinamide	A	0.10	A	110.03	S	3.00	S	10.00
Rapamycin	A	0.00	N		A	0.91	N	
Sulfasalazine	S	36.00	S	1.20	S	7.80	S	1.20
Warfarin	A	23.33	N		S	30.00	S	0.00

Table 2 Modified MITA data set (60 **化学物質**)

No	Chemicals	IL-2		IFN-γ		IL-1β		IL-8		IL-8 Luc assay	
		Judge	LOEL	Judge	LOEL	Judge	LOEL	Judge	LOEL	Judge	LOEL
1	2,4-Diaminotoluene	N		A	62.50	N		S	0.98	N	
2	2-Aminoanthracene	S	0.81	S	5.86	S	2.03	N		S	0.040
3	2-Mercaptobenzothiazole	N		N		N		S	125.00	S	0.20
4	Acetaminophen	A	33.33	A	33.33	A	166.67	A	100.00		
5	Actinomycin D	S	0.00	S	0.01	N		S	0.00	S	0.05
6	Aluminum chloride	S	3.91	S	62.50	N		N			
7	Amphoterycin B	S	0.78	S	2.08	A	3.13	A	7.82		
8	Benzethonium chloride	S	1.95	S	1.95	S	3.91	N			
9	Chlorpromazine	S	3.91	S	3.91	S	7.81	S	7.81		
10	Cisplatin	S	0.24	S	1.22	N		N			
11	Cobalt chloride	S	3.91	S	9.12	S	3.91	S	125.00	S	0.01
12	Cyclophosphamide	N		A	168.00	N		N			
13	Cyclosporine A	S	0.00	S	0.00	N		N			
14	Dapsone	S	45.01	S	55.14	S	46.88	S	134.75	N	
15	Dexamethasone	S	0.01	N		S	0.01	S	0.01	N	
16	Dibenzopyrene	S	0.01	S	0.03	N		N	0.00		
17	Diethanolamin	S	9.12	N		N		N			
18	Dimethyl sulfoxide	A	3.91	A	625.00	S	66.41	S	3.91	N	
19	Ethanol	N		N		N		N			
20	FK 506	S	0.00	S	0.00	A		N			
21	FR167653	S	0.49	S	0.49	S	145.83	S	125.00	N	
22	Histamine	S	9.12	A	5.86	N		S	3.91	S	0.06
23	Hydrocortisone	S	0.34	A	6.27	S	0.34	S	0.34	N	
24	Hydrogen peroxide	S	7.82	S	31.25	N		N		S	0.01
25	Isoniazid	S	1.97	N		N		S	800.00	N	
26	Isophorone diisocyanate	S	0.98	N		S	0.98	S	0.98	S	0.03
27	Lead(II) acetate	S	3.91	S	3.91	N		N			

28	Lithium carbonate	S	0.98	A	116.67	S	0.39	S	0.39	S	0.25
29	Magnesium sulfate	N		N		S	15.63	N		S	0.002
30	Mercuric chloride	N		A	3.91	S	1.95	S	1.95	S	0.00005
31	Methanol	N		N		N		N			
32	Mitomycin C	S	0.36	N		N		N			
33	Nickel sulfate	S	14.32	S	32.55	S	250.00	S	250.00	S	0.004
34	Nitrofurazone	S	0.37	A	3.91	A		A	62.50		
35	Pentamidine isethionate	S	3.91	S	32.55	S	3.91	S	3.91	N	
36	p-Nitroaniline	S	0.98	S	1.95	S	1.47	S	2.45	N	
37	Pyrimethamine	S	0.04	N		N		N			
38	Ribavirin	A	15.63	A	187.50	A	5.86	N			
39	Sodium bromate	S	125.00	N		N		N			
40	Sodium dodecyl sulfate	N		N		N		N			
41	Dibutyl phthalate	S	0.98	S	1.95	S	39.07	S	31.25	N	
42	Triethanolamine	S	187.50	S	1416.67	N		N			
43	Hexachlorobenzene	N		N		N		N			
44	Citral	S	0.36	S	1.37	N		N		S	0.0025
45	Trichloroethylene	N		N		N		N			
46	Chloroplatinic acid	N		N		N		S	15.63	S	0.0005
47	Formaldehyde	S	1.71	N		S	15.63	S	15.63	S	0.0004
48	Diesel exhaust particles	S	39.07	A	47.53	N		S	62.50	S	0.05
49	Azathioprine	N		A	40.01	A	9.23	N			
50	Chloroquine	S	0.05	S	0.02	S	10.00	S	30.00		
51	Colchicine	A	0.29	A	0.06	A	0.02	A	20.00		
52	Digoxin	S	0.01	S	0.02	N		N			
53	Methotrexate	A	0.45	A	0.09	N		N			
54	Minocycline	S	8.33	S	5.00	N		N			
55	Mizoribine	N		N		A	5.20	A	7.45		
56	Mycophenolic acid	A	0.38	A	6.24	N		N			
57	Nicotinamide	A	0.10	A	110.03	S	3.00	S	10.00		
58	Rapamycin	A	0.00	N		A	0.91	N			
59	Sulfasalazine	S	36.00	S	1.20	S	7.80	S	1.20		
60	Warfarin	A	23.33	N		S	30.00	S	0.00		

Table 3. Modified MITA data set (IL-8 **レポーター活性** vs. IL-8 Luc assay)

Chemicals	IL-2		IFN-γ		IL-1β		IL-8		IL-8 Luc assay	
	Judge	LOEL	Judge	LOEL	Judge	LOEL	Judge	LOEL	Judge	LOEL
Actinomycin D	S	0.00	S	0.01	N		***S***	0.00	***S***	0.05
Dexamethasone	S	0.01	N		S	0.01	***S***	0.01	***N***	
Warfarin	A	23.33	N		S	30.00	***S***	0.10		
Hydrocortisone	S	0.34	A	6.27	S	0.34	***S***	0.34	***N***	
Lithium carbonate	S	0.98	A	116.67	S	0.39	***S***	0.39	***S***	0.25
Isophorone diisocyanate	S	0.98	N		S	0.98	***S***	0.98	***S***	0.03
2,4-Diaminotoluene	N		A	62.50	N		***S***	0.98	***N***	
Sulfasalazine	S	36.00	S	1.20	S	7.80	***S***	1.20		
Mercuric chloride	N		A	3.91	S	1.95	***S***	1.95	***S***	0.00
p-Nitroaniline	S	0.98	S	1.95	S	1.47	***S***	2.45	***N***	
Pentamidine isethionate	S	3.91	S	32.55	S	3.91	***S***	3.91	***N***	
Histamine	S	9.12	A	5.86	N		***S***	3.91	***S***	0.06
Dimethyl sulfoxide	A	3.91	A	625.00	S	66.41	***S***	3.91	***N***	
Chlorpromazine	S	3.91	S	3.91	S	7.81	***S***	7.81		
Nicotinamide	A	0.10	A	110.03	S	3.00	***S***	10.00		
Formaldehyde	S	1.71	N		S	15.63	***S***	15.63	***S***	0.00
Chloroplatinic acid	N		N		N		***S***	15.63	***S***	0.00
Chloroquine	S	0.05	S	0.02	S	10.00	***S***	30.00		
Dibutyl phthalate	S	0.98	S	1.95	S	39.07	***S***	31.25	***N***	
Diesel exhaust particles	S	39.07	A	47.53	N		***S***	62.50	***S***	0.05
FR167653	S	0.49	S	0.49	S	145.83	***S***	125.00	***N***	
Cobalt chloride	S	3.91	S	9.12	S	3.91	***S***	125.00	***S***	0.01
2-Mercaptobenzothiazole	N		N		N		***S***	125.00	***S***	0.20
Dapsone	S	45.01	S	55.14	S	46.88	***S***	134.75	***N***	
Nickel sulfate	S	14.32	S	32.55	S	250.00	***S***	250.00	***S***	0.00
Isoniazid	S	1.97	N		N		***S***	800.00	***N***	
Citral	S	0.36	S	1.37	N		***N***		***S***	0.00
2-Aminoanthracene	S	0.81	S	5.86	S	2.03	***N***		***S***	0.04
Hydrogen peroxide	S	7.82	S	31.25	N		***N***		***S***	0.01
Magnesium sulfate	N		N		S	15.63	***N***		***S***	0.00

Table 4. Modified MITA data (IL-2 **レポーター活性**)

No	Chemicals	IL-2		IFN-γ		IL-1β		IL-8		IL-8 Luc assay	
		Judge	LOEL	Judge	LOEL	Judge	LOEL	Judge	LOEL	Judge	LOEL
21	FK 506	**S**	0.00	S	0.00	A		N			
13	Cyclosporine A	**S**	0.00	S	0.00	N		N			
5	Actinomycin D	**S**	0.00	S	0.01	N		S	0.00	S	0.05
52	Digoxin	**S**	0.01	S	0.02	N		N			
15	Dexamethasone	**S**	0.01	N		S	0.01	S	0.01	N	
16	Dibenzopyrene	**S**	0.01	S	0.03	N		N	0.00		
38	Pyrimethamine	**S**	0.04	N		N		N			
50	Chloroquine	**S**	0.05	S	0.02	S	10.00	S	30.00		
10	Cisplatin	**S**	0.24	S	1.22	N		N			
24	Hydrocortisone	**S**	0.34	A	6.27	S	0.34	S	0.34	N	
33	Mitomycin C	**S**	0.36	N		N		N			
44	Citral	**S**	0.36	S	1.37	N		N		S	0.00
35	Nitrofurazone	**S**	0.37	A	3.91	A		A	62.50		
22	FR167653	**S**	0.49	S	0.49	S	145.83	S	125.00	N	
7	Amphoterycin B	**S**	0.78	S	2.08	A	3.13	A	7.82		
2	2-Aminoanthracene	**S**	0.81	S	5.86	S	2.03	N		S	0.04
29	Lithium carbonate	**S**	0.98	A	116.67	S	0.39	S	0.39	S	0.25
17	Dibutyl phthalate	**S**	0.98	S	1.95	S	39.07	S	31.25	N	
27	Isophorone diisocyanate	**S**	0.98	N		S	0.98	S	0.98	S	0.03
37	p-Nitroaniline	**S**	0.98	S	1.95	S	1.47	S	2.45	N	
47	Formaldehyde	**S**	1.71	N		S	15.63	S	15.63	S	0.00
8	Benzethonium chloride	**S**	1.95	S	1.95	S	3.91	N			
26	Isoniazid	**S**	1.97	N		N		S	800.00	N	
6	Aluminum chloride	**S**	3.91	S	62.50	N		N			
9	Chlorpromazine	**S**	3.91	S	3.91	S	7.81	S	7.81		
11	Cobalt chloride	**S**	3.91	S	9.12	S	3.91	S	125.00	S	0.01
28	Lead(II) acetate	**S**	3.91	S	3.91	N		N			

36	Pentamidine isethionate	**S**	3.91	S	32.55	S	3.91	S	3.91	N	
25	Hydrogen peroxide	**S**	7.82	S	31.25	N		N		S	0.01
54	Minocycline	**S**	8.33	S	5.00	N		N			
23	Histamine	**S**	9.12	A	5.86	N		S	3.91	S	0.06
18	Diethanolamin	**S**	9.12	N		N		N			
34	Nickel sulfate	**S**	14.32	S	32.55	S	250.00	S	250.00	S	0.00
59	Sulfasalazine	**S**	36.00	S	1.20	S	7.80	S	1.20		
48	Diesel exhaust particles	**S**	39.07	A	47.53	N		S	62.50	S	0.05
14	Dapsone	**S**	45.01	S	55.14	S	46.88	S	134.75	N	
40	Sodium bromate	**S**	125.00	N		N		N			
42	Triethanolamine	**S**	187.50	S	1416.67	N		N			
1	2,4-Diaminotoluene	N		A	62.50	N		S	0.98	N	
3	2-Mercaptobenzothiazole	N		N		N		S	125.00	S	0.20
12	Cyclophosphamide	N		A	168.00	N		N			
20	Ethanol	N		N		N		N			
30	Magnesium sulfate	N		N		S	15.63	N		S	0.00
31	Mercuric chloride	N		A	3.91	S	1.95	S	1.95	S	0.00
32	Methanol	N		N		N		N			
41	Sodium dodecyl sulfate	N		N		N		N			
43	Hexachlorobenzene	N		N		N		N			
45	Trichloroethylene	N		N		N		N			
46	Chloroplatinic acid	N		N		N		S	15.63	S	0.00
49	Azathioprine	N		A	40.01	A	9.23	N			
55	Mizoribine	N		N		A	5.20	A	7.45		
58	Rapamycin	A	0.00	N		A	0.91	N			
57	Nicotinamide	A	0.10	A	110.03	S	3.00	S	10.00		
51	Colchicine	A	0.29	A	0.06	A	0.02	A	20.00		
56	Mycophenolic acid	A	0.38	A	6.24	N		N			
53	Methotrexate	A	0.45	A	0.09	N		N			
19	Dimethyl sulfoxide	A	3.91	A	625.00	S	66.41	S	3.91	N	
39	Ribavirin	A	15.63	A	187.50	A	5.86	N			
60	Warfarin	A	23.33	N		S	30.00	S	0.00		
4	Acetaminophen	A	33.33	A	33.33	A	166.67	A	100.00		

Table 5. Modified MITA (IL-8 Luc assay)

No	Chemicals	IL-2		IFN-γ		IL-1β		IL-8		IL-8 Luc assay	
		Judge	LOEL	Judge	LOEL	Judge	LOEL	Judge	LOEL	Judge	LOEL
31	Mercuric chloride	N		A	3.91	S	1.95	S	1.95	**S**	0.00005
47	Formaldehyde	S	1.71	N		S	15.63	S	15.63	**S**	0.00044
46	Chloroplatinic acid	N		N		N		S	15.63	**S**	0.00052
30	Magnesium sulfate	N		N		S	15.63	N		**S**	0.00200
44	Citral	S	0.36	S	1.37	N		N		**S**	0.00252
34	Nickel sulfate	S	14.32	S	32.55	S	250.00	S	250.00	**S**	0.00449
25	Hydrogen peroxide	S	7.82	S	31.25	N		N		**S**	0.00508
11	Cobalt chloride	S	3.91	S	9.12	S	3.91	S	125.00	**S**	0.00572
27	Isophorone diisocyanat	S	0.98	N		S	0.98	S	0.98	**S**	0.03317
2	2-Aminoanthracene	S	0.81	S	5.86	S	2.03	N		**S**	0.04000
5	Actinomycin D	S	0.00	S	0.01	N		S	0.00	**S**	0.04588
48	Diesel exhaust particles	S	39.07	A	47.53	N		S	62.50	**S**	0.05320
23	Histamine	S	9.12	A	5.86	N		S	3.91	**S**	0.05833
3	2-Mercaptobenzothiazo	N		N		N		S	125.00	**S**	0.19907
29	Lithium carbonate	S	0.98	A	116.67	S	0.39	S	0.39	**S**	0.25024
41	Sodium dodecyl sulfate	N		N		N		N		**S**	9.80000

Table 6. ECVAM **感作性試験開発用試験薬の** IL-1β**および** IL-8 **レポーター細胞による評価**

Chemicals	IL-8 Luc assay			IL-1b reporter assay		
	1st	2nd	Judgment	1st	2nd	Judgment
Oxazolone	P	P	Sensitizer	P	P	Sensitizer
4-NBB	P	P	Sensitizer	P	P	Sensitizer
Glyoxal	P	P	Sensitizer	P	P	Sensitizer
2-MBT	P	P	Sensitizer	P	P	Sensitizer
DNCB	P	P	Sensitizer	P	P	Sensitizer
MDGN	P	P	Sensitizer	P	P	Sensitizer
Cinnamal	P	P	Sensitizer	P	P	Sensitizer
TMTD	P	P	Sensitizer	P	P	Sensitizer
PPD	P	P	Sensitizer	N	N	Non-sensitizer
Isoeugenol	P	P	Sensitizer	N	N	Non-sensitizer
Eugenol	P	P	Sensitizer	P	P	Sensitizer
Cinnamic alcohol	P	P	Sensitizer	P	P	Sensitizer
Glycerol	N	N	Non-sensitizer	N	N	Non-sensitizer
Salicylic acid	N	N	Non-sensitizer	N	N	Non-sensitizer
Lactic acid	N	N	Non-sensitizer	N	N	Non-sensitizer
SLS	P	P	Sensitizer	N	N	Non-sensitizer

図 1． 研究計画

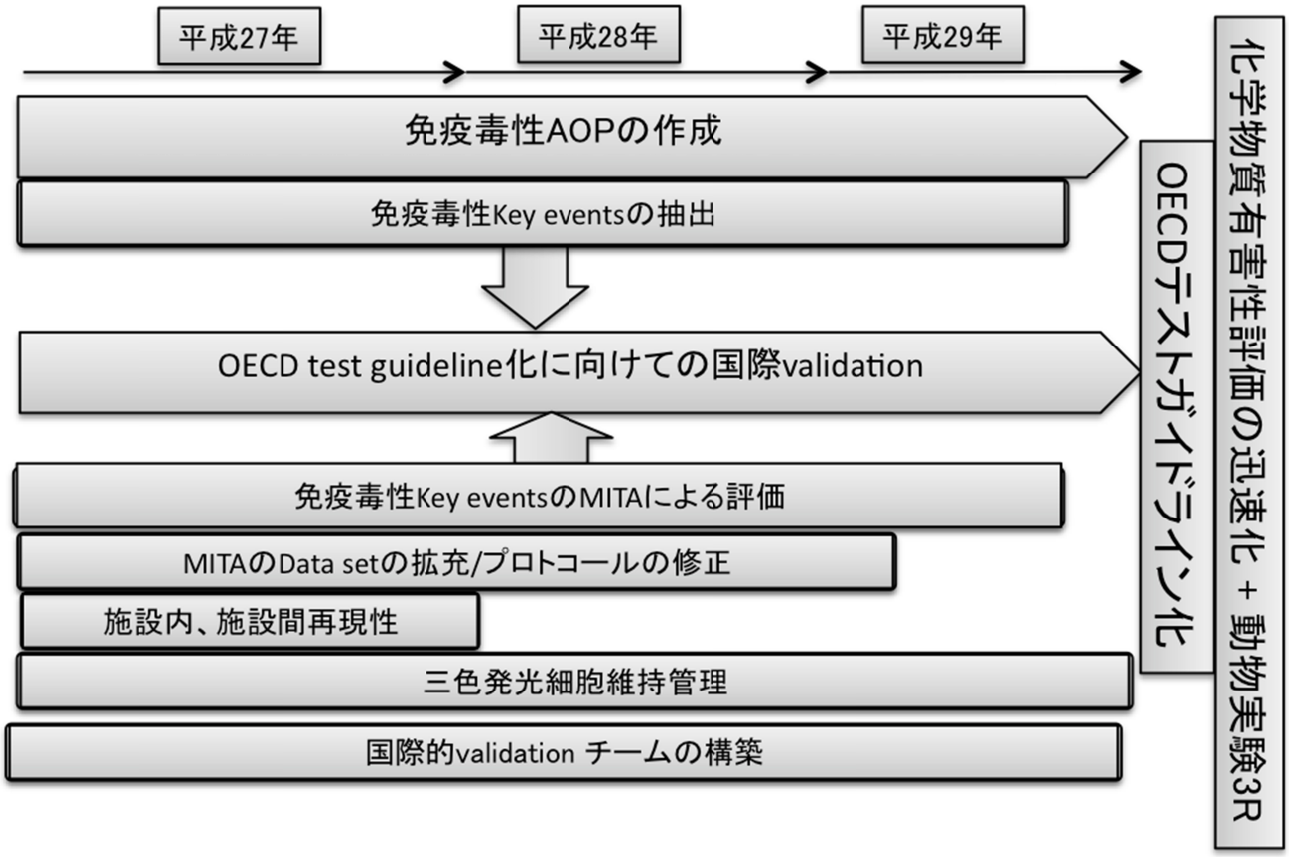

平成27年
平成28年
平成29年
免疫毒性AOPの作成
免疫毒性Key eventsの抽出
OECD test guideline化に向けての国際validation
免疫毒性Key eventsのMITAによる評価
MITAのData setの拡充/プロトコールの修正
施設内、施設間再現性
三色発光細胞維持管理
国際的validation チームの構築
OECDテストガイドライン化
化学物質有害性評価の迅速化 + 動物実験3R

図２．Multi-ImmunoTox assay (MITA)

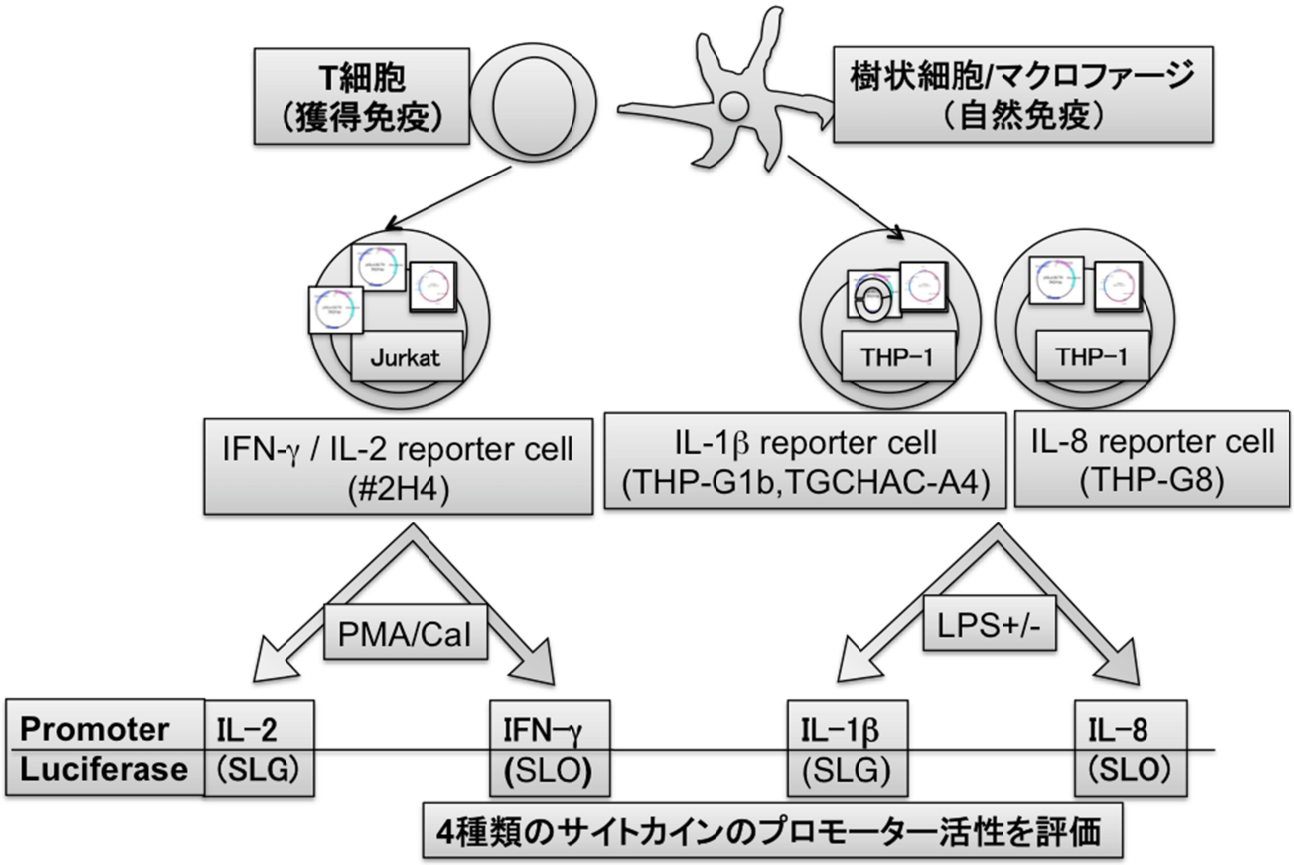


図 3. MITA 判定方法

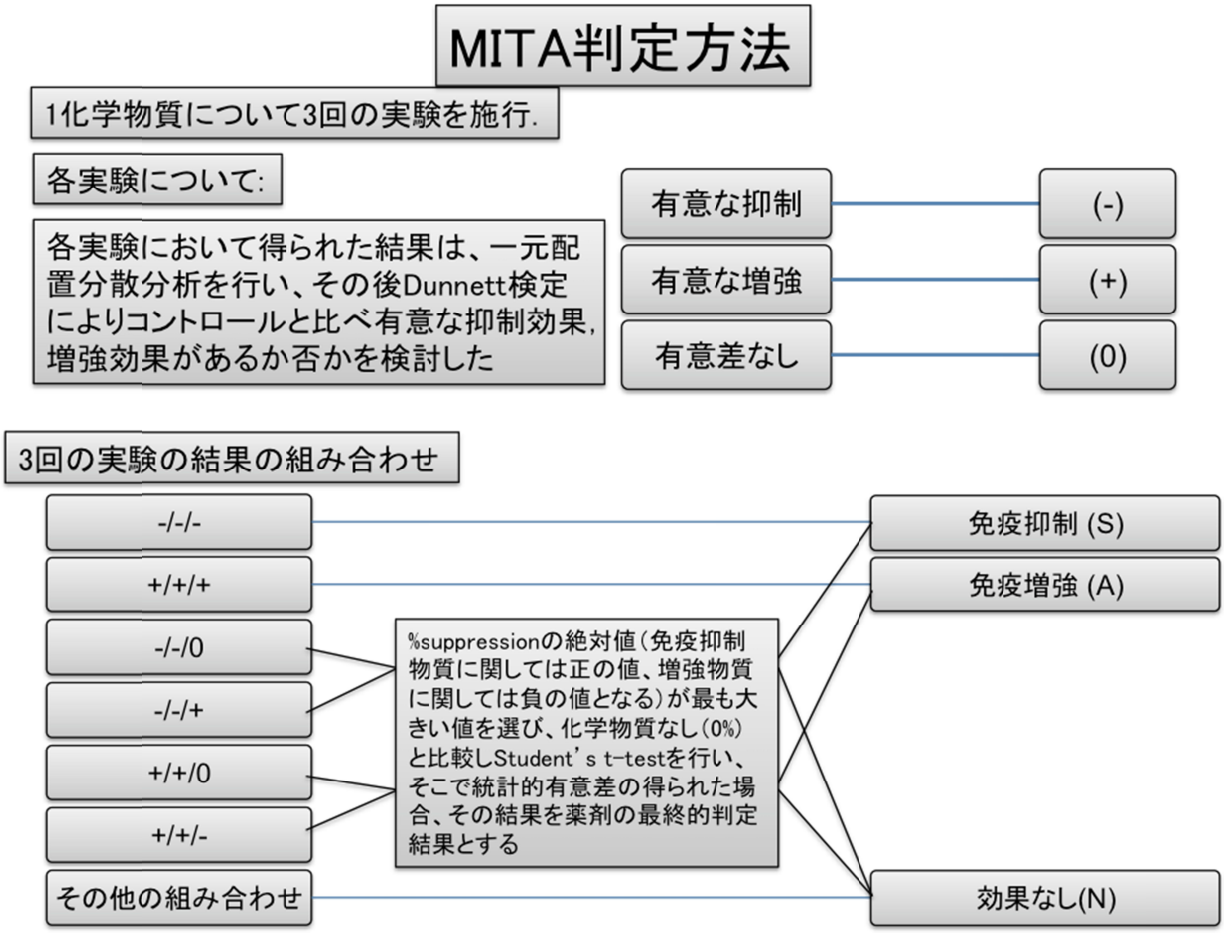

MITA判定方法
1化学物質について3回の実験を施行.
各実験について:
各実験において得られた結果は、一元配置分散分析を行い、その後Dunnett検定によりコントロールと比べ有意な抑制効果,増強効果があるか否かを検討した
有意な抑制
有意な増強
有意差なし
(-)
(+)
(0)
3回の実験の結果の組み合わせ
-/-/-
+/+/+
-/-/0
-/-/+
+/+/0
+/+/-
その他の組み合わせ
%suppressionの絶対値(免疫抑制物質に関しては正の値、増強物質に関しては負の値となる)が最も大きい値を選び、化学物質なし(0%)と比較しStudent's t-testを行い、そこで統計的有意差の得られた場合、その結果を薬剤の最終的判定結果とする
免疫抑制 (S)
免疫増強 (A)
効果なし(N)

図 4.　IL-2 reporter activity と IFN-γ reporter activity との相関

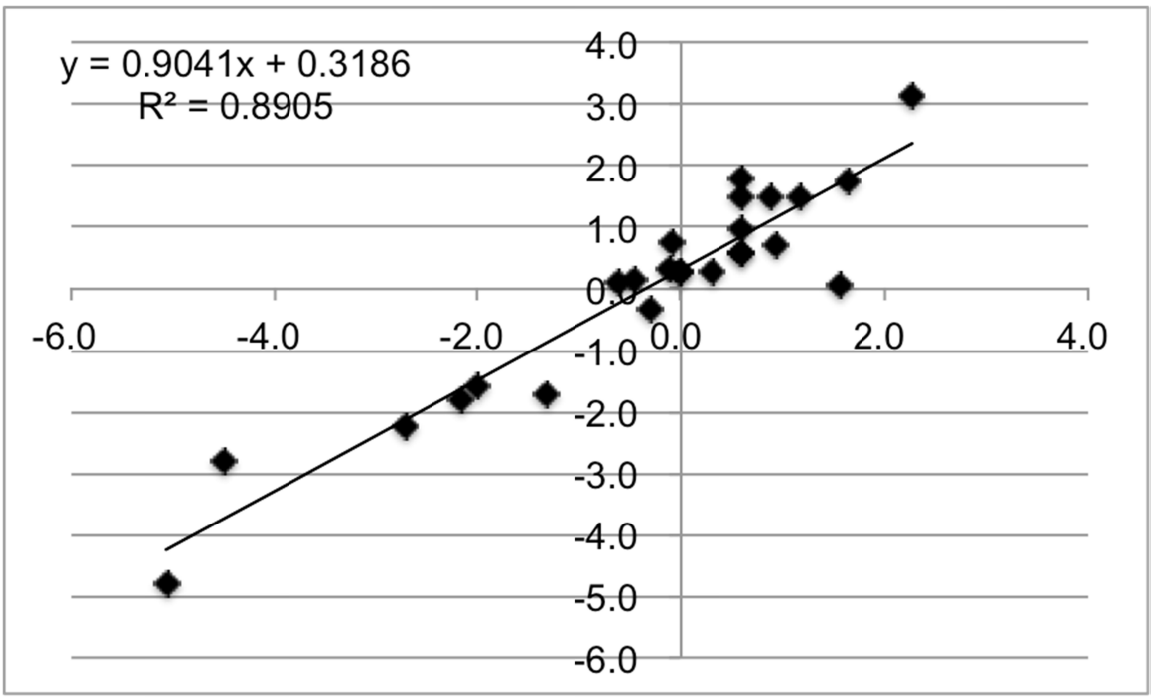


図５．　IL-2 転写活性抑制を KE とする T 細胞分化異常誘導に関する AOP

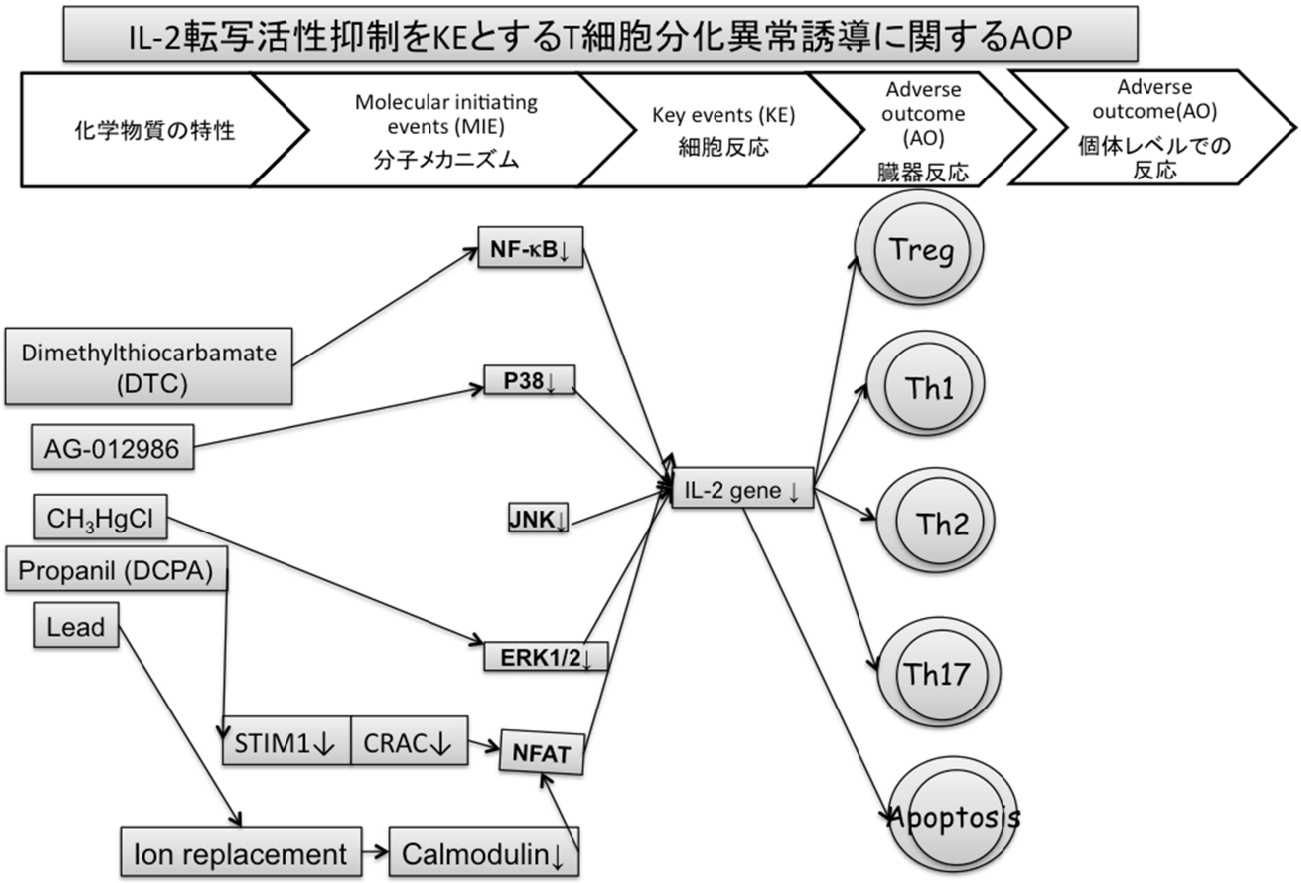


図６．IL-8 転写活性増強を KE として気道刺激性 AOP

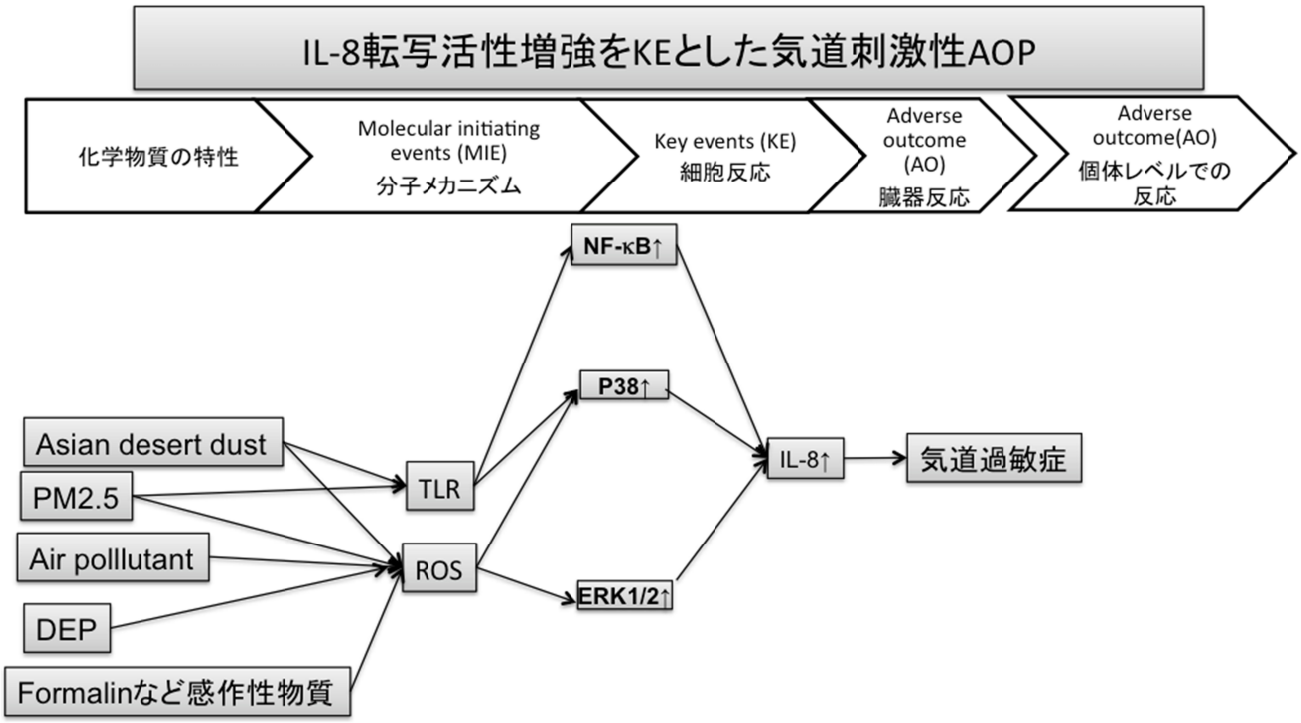


図７MITA を用いた免疫毒性物質の分類

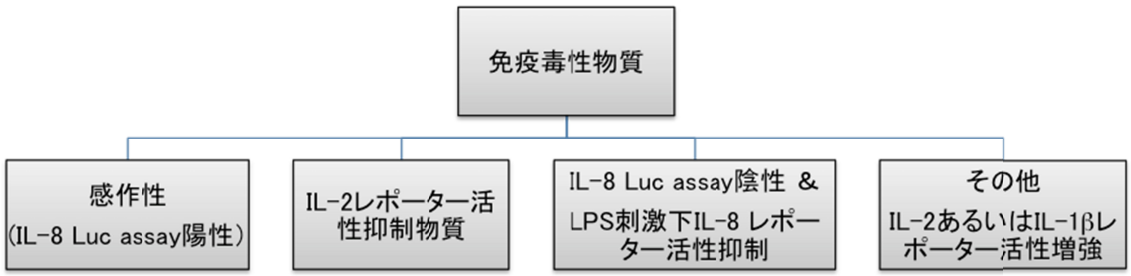
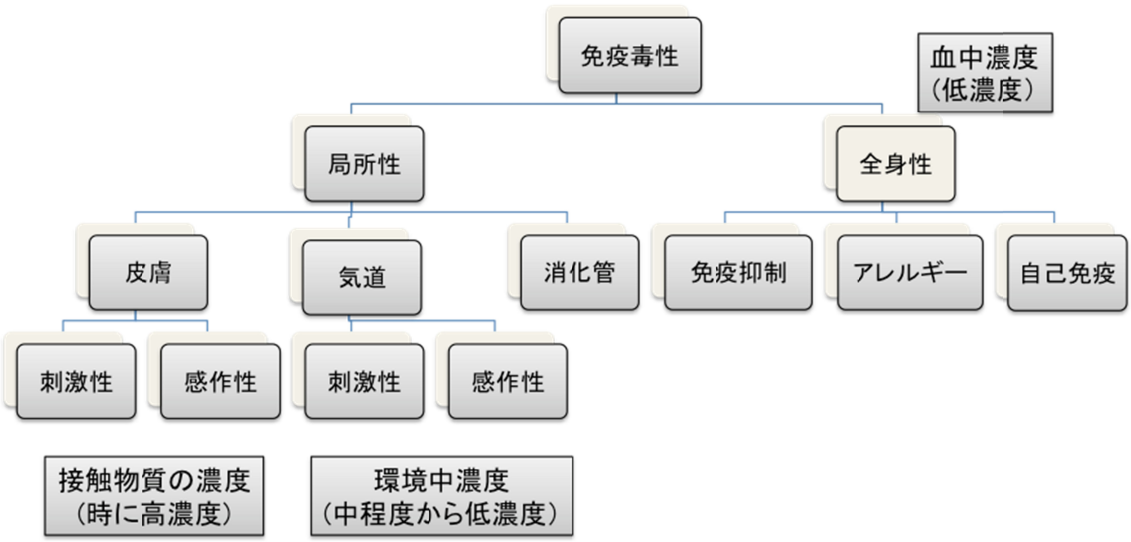


図８．　免疫毒性の発症部位と化学物質の曝露濃度



厚生労働科学研究費補助金（化学リスク研究事業）多色発光細胞を用いたhigh-throughput免疫毒性評価試験法の開発

分担研究報告書

化学物質のMulti-ImmunoTox assayによる解析，精度管理

分担研究者　近江谷克裕
(独)産業技術総合研究所

研究要旨
多色発光タンパク質を用いたMulti-ImmunoTox assay（MITA）だけでは、皮膚感作性物質の多くがLPSで刺激したTHP-G8細胞のIL-8転写活性を抑制し、単球/樹状細胞に抑制的に作用する免疫抑制物質と皮膚感作性物質を区別できないことが明らかとなった。そこで従来法のMITAに、開発を進めていた皮膚感作性物質試験法であるIL-8 Luc assayを加えたmodified MITAを開発、バリデーションに進むためのプロトコールの最適化を行うための5物質のIL-8 Luc assayを実施した。また、免疫毒性試験系として、MITAのIL-2レポーター活性抑制評価系のバリデーション試験を行うための最初の段階である技術移転性を確認するために、5物質のIL-2レポーター活性抑制作用を調べた。

キーワード：免疫毒性、動物実験代替法、*in vitro*

A．研究目的

我々はこれまでに多色発光タンパク質による新たな *in vitro* 免疫毒性評価試験法、いわゆる Multi-ImmunoTox assay（MITA）を確立し各種毒性評価発光細胞を樹立した[1)]。現在、これらの細胞群を用いた化学物質の免疫毒性評価法の確立を目指している。そこで本研究では、化学物質の免疫毒性評価のための MITA 法の OECD ガイドライン化を視野に、ラボ間バリデーション試験の実施と MITA 法の精度管理に必要な周辺技術の開発を目的とした。

より具体的には、東北大学病院で樹立された Jurkat 細胞における INF-γ，IL-2，G3PDH プロモータ活性を測定する細胞株 2H4 及び THP-１細胞における IL-8 と G3PDH プロモータ活性を定量化できる細胞株 TGCHAC-A4、IL-1β と G3PDH プロモータ活性を定量化できる細胞株 THP-G8 をモデル細胞として施設内、施設間バリデーション試験を実施、ガイドライン化するための手法の最適化を目指す。本年度は、IL-8 Luc assay の結果を含む Modified MITA の data set を構築するために 5 物質に関して IL-8 Luc assay を実施した。また、免疫毒性の評価系として IL-2 レポーター活性抑制評価系のバリデーション試験の最初のステップとして、技術移転性確認のため 5 物質の試験を行った。

B．研究方法

B-1）IL-8 Luc assay

IL-8とG3PDHプロモータにそれぞれSLRおよびSLOルシフェラーゼ遺伝子を繋いだ発現ベクターをTHP-1細胞に導入した2色発光細胞株THP-G8を用いて試験を行った。

化学物質の免疫毒性試験法における細胞培養方法、被験物質調整及び添加方法、及

びルシフェラーゼアッセイの方法についてはIL-8 Luc assay protocol Ver. 020E 20150703に準ずる。

試験化学物質として2,4-Diaminotoluene, 2-Aminoanthracene, Dapsone, Dibutyl phthalate, Isoniazidの5物質を供試し、発光測定装置はアトー社製Pheliosを用いた。

B-1) IL2レポーター活性抑制物質評価のためのMITA assay

IL-2とIFN-γ、G3PDHプロモータにそれぞれSLG、SLOおよびSLRルシフェラーゼ遺伝子を繋いだ発現ベクターをJurkat細胞に導入した3色発光細胞株#2H4を用いて試験を行った。

化学物質の免疫毒性試験法における細胞培養方法、被験物質調整及び添加方法、及びルシフェラーゼアッセイの方法についてはMulti-Immuno Tox Assay protocol 案 Ver.008.1E 20160202に準ずる。

試験化学物質として、2-Aminoanthracene, Citral, Chloroquine, Dexamethasone, Methyl mercuric(II) chlorideの5物質とを供試した。

（倫理面への配慮）

倫理的な問題が生じる実験を実施しておらず、特に配慮すべき問題はない。

C.結果

C-1) IL-8 Luc assay

a) 試験開始当初、リードラボである東北大で出された発光値と比べ、約50～60%と低く、反応性の低さが懸念された。そのため、新たに東北大から分与された細胞株を用いて試験を実施したところ反応性の向上が確認され、試験に用いた細胞（当研究機関において凍結保存した株）自体の反応性が落ちている可能性が示唆された。そこで改めて、東北大からの分与株を用いて試験を実施し、その結果（図1）と各Criteriaにおける評価（図2）を示す。

b) 28年度より予定しているMITAバリデーション試験 phaseIの実施に向け、#2H4細胞株を用いた技術移転性の確認試験を実施した。各物質に対し3回繰り返し試験を行った結果を図3に示す。現在、リードラボである東北大にて、試験参加各施設の結果をもって評価、考察を進めている。

D. 考察

従来のMITAでは一部の感作性物質がIL-8レポーター活性抑制作用を示し、一方、デキサメサドンなどの免疫抑制剤との区別ができないため、IL-8プロモータ活性測定系を加えたmodified MITAを構築することは本年度の免疫毒性物質の評価においては重要である。そこで、modified MITAのdata setを構築するために、MITAのdata setから選択した5物質についてIL-8 Luc assayを実施した。その結果、criteriaによって、結果が異なる場合もあるが、概ね再現性良く免疫抑制物質と感作性物質との識別が可能となった。

さらに、今年度の技術移転性結果をもとに、次年度以降は免疫毒性試験のIL-2プロモータ活性評価系のバリデーション試験を行うため、プレバリデーション試験を5つの試験物質について行った。最終的には再現性の高いデータを得ることができたが、細胞を活性化する処理によるバラつきの問題や細胞の維持管理に関しての問題点が浮き彫りになった。来年度以降、これらバラつきの問題を解消し、試験プロトコールの最適化が重要な課題となった。今後、プロトコールの最適化と共に精度管理に関する研究を行う予定である。

E. 結論

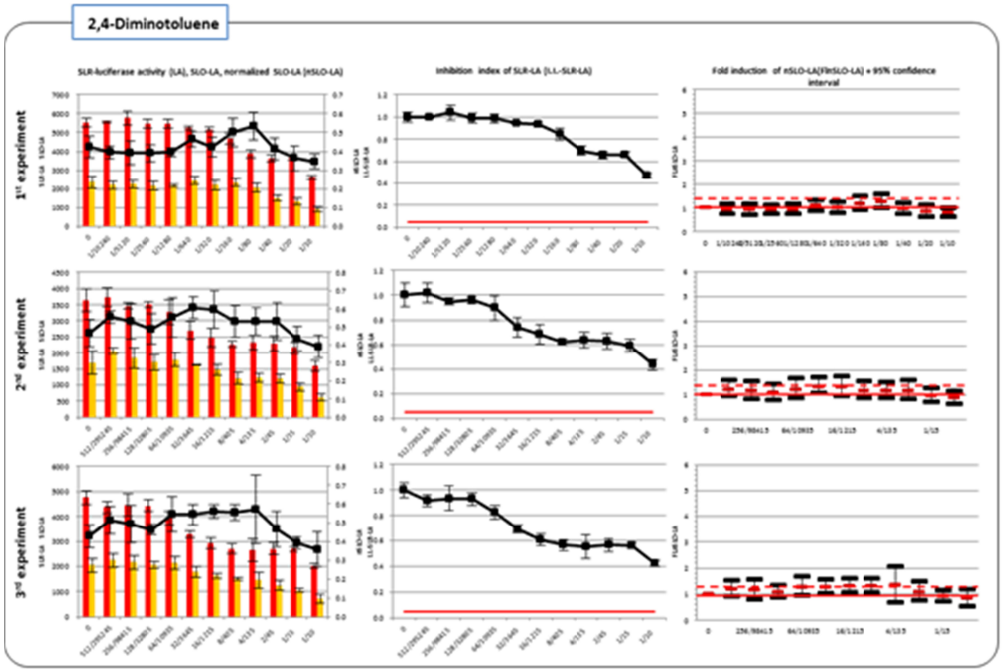
MITAのdata setの構築に協力し、AOP作成に貢献した。また、免疫毒性試験のIL-2プロモータ活性評価系のバリデーション試験に進むための技術移転性の確認実験を終了、プロトコールの最適化のための課題を見出した。

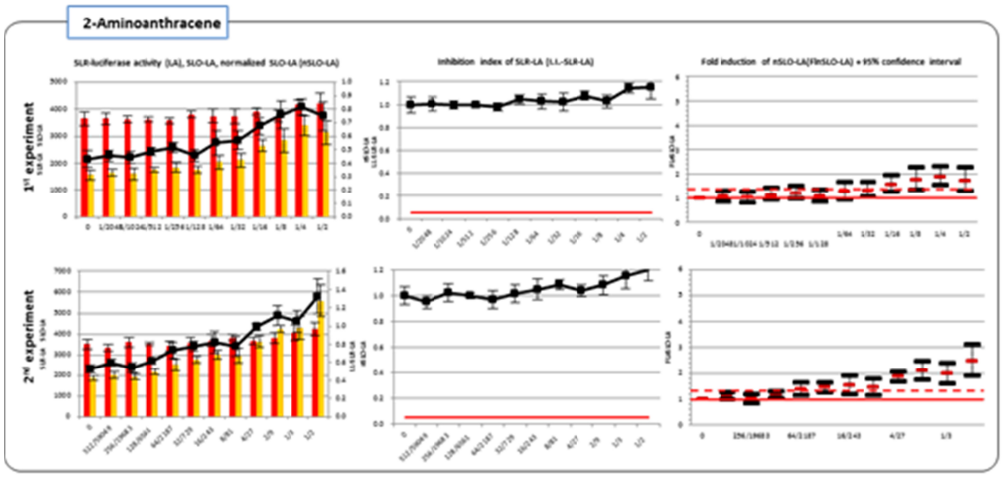
F. 参考文献

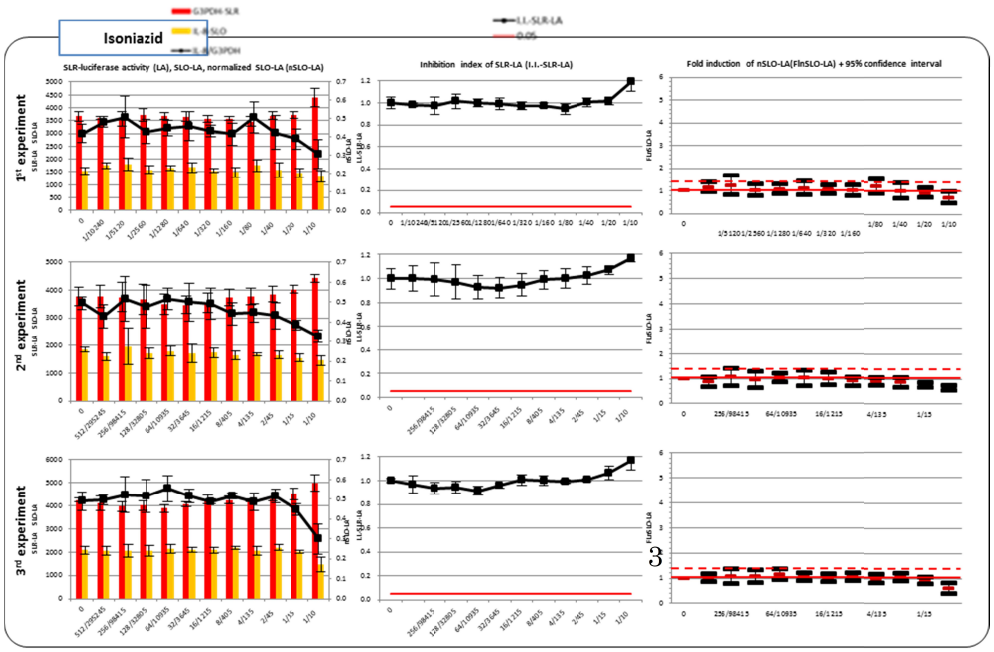
1) Takahashi T, Kimura Y, Saito R, Nakajima Y, Ohmiya Y, Yamasaki K, Aiba S: An in vitro test to screen skin sensitizers using a stable THP-1-derived IL-8 reporter cell line, THP-G8. Toxicol Sci., 124, 359-69, 2011

G. 研究発表

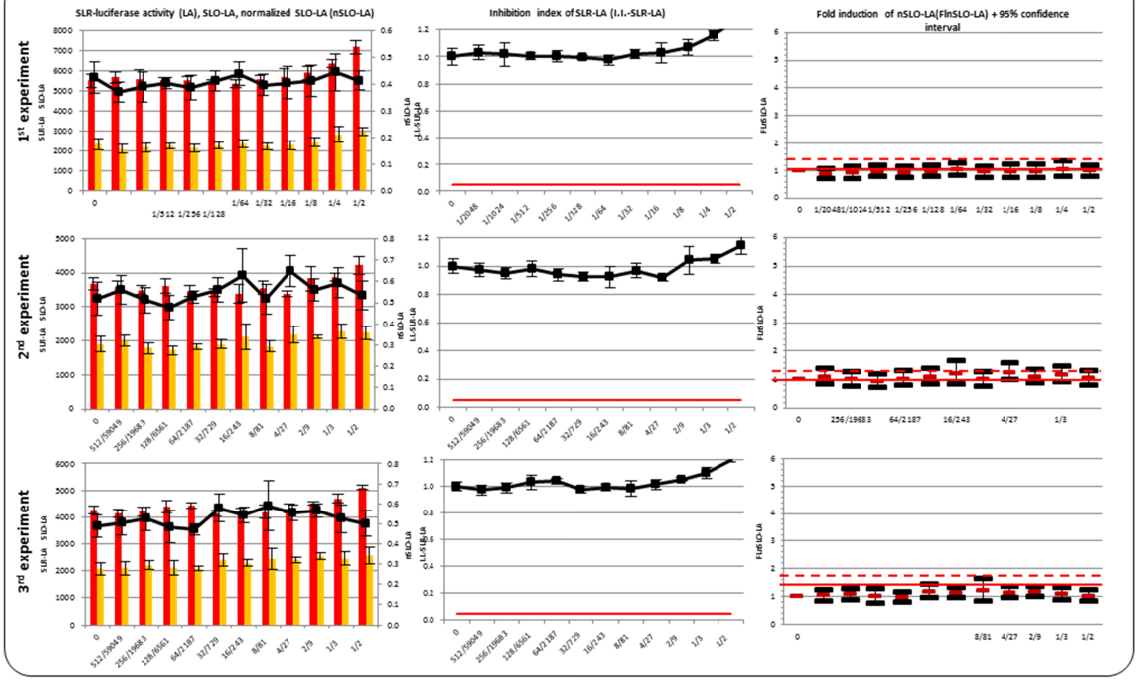
図１ Modified MITA による IL-8 Luc assay の結果







Dapson



Dibutyl phthalate

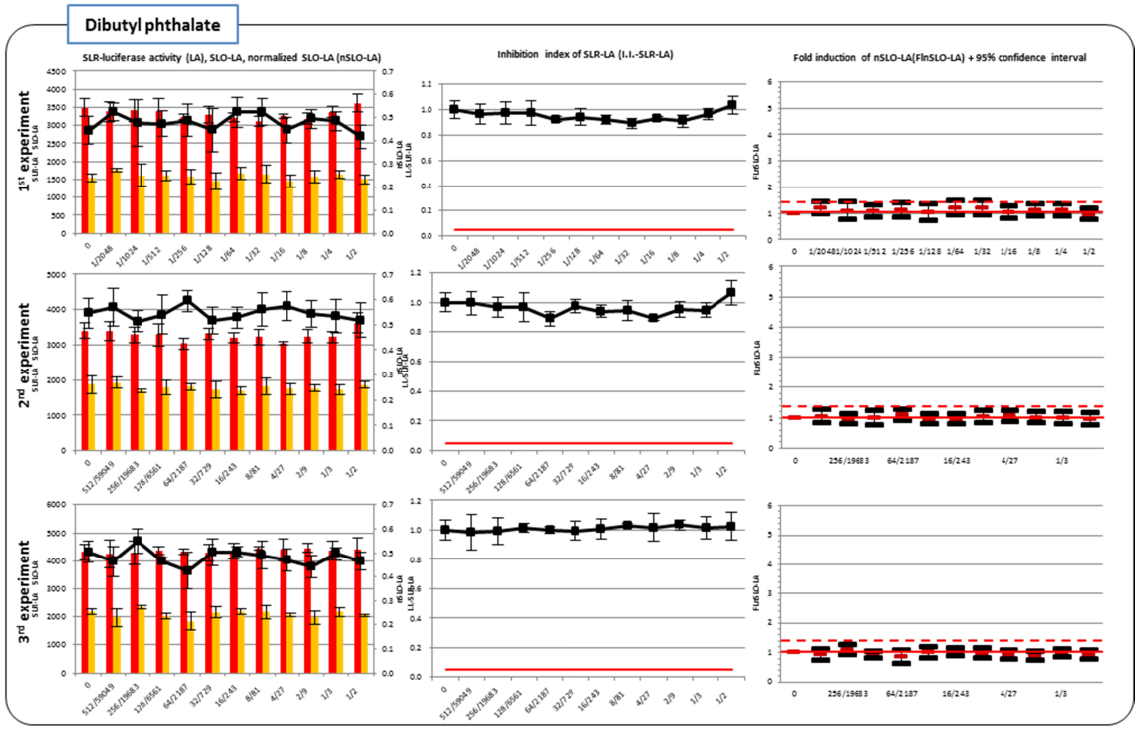


図 2　Modified MITA による IL-8 Luc assay の各 criteria における評価

	1st	2nd	3rd	4th	Judge
2,4-Diaminotoluene	N	N	N	-	Non-sensitizer

図 3　Jurkat 細胞由来株#2H4 における各試験化学物質に対する細胞応答性。

Crite

	1st	2nd	3rd	4th	Judge
Dibutyl phthalate	N	N	N	-	Non-sensitizer
Isoniazid	N	N	N	-	Non-sensitizer

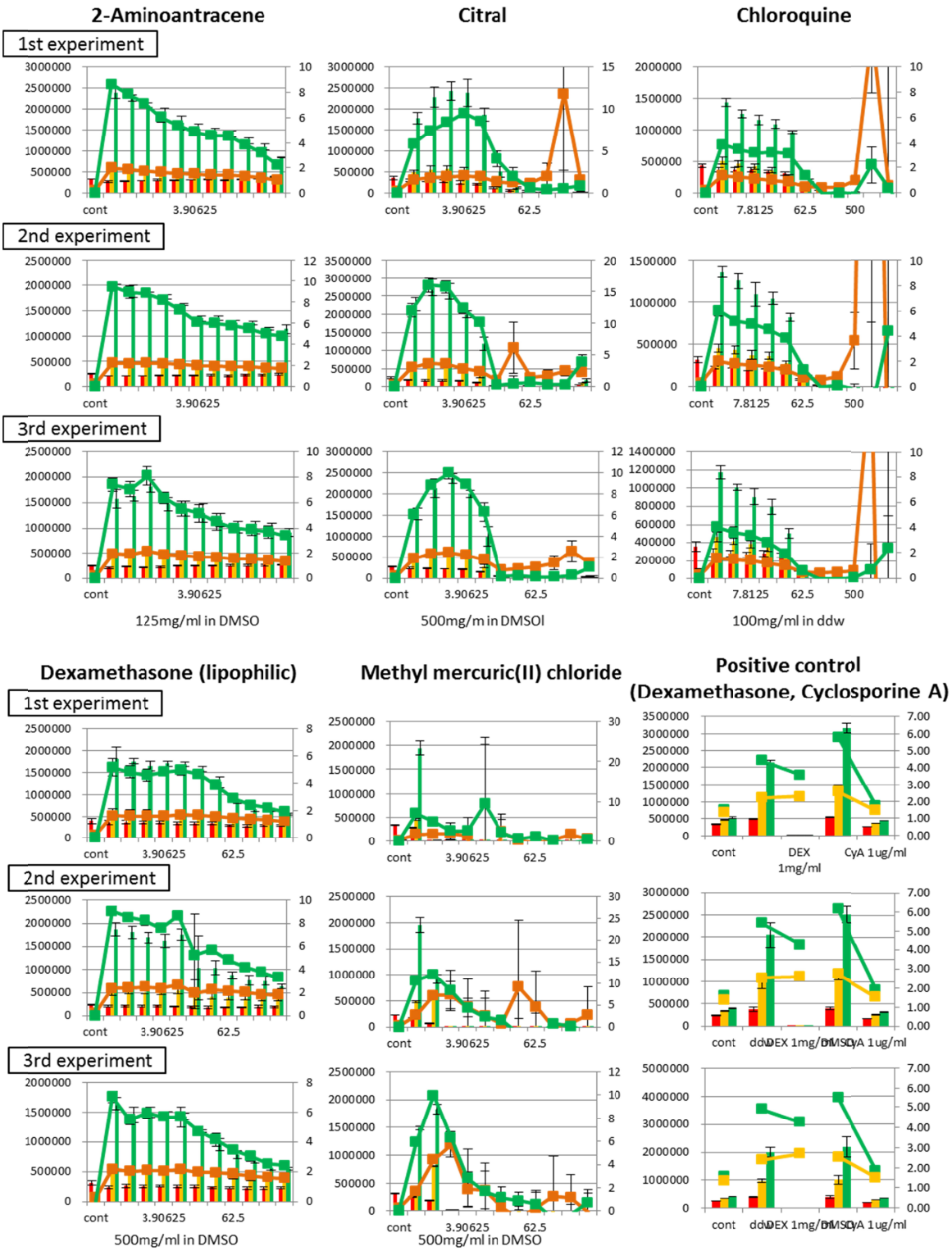
Criteria 2

	1st	2nd	3rd	4th	Judge
2,4-Diaminotoluene	N	P	P	-	sensitizer
2-Aminoanthracene	P	P	-	-	sensitizer
Dapson	N	N	N	-	Non-sensitizer
Dibutyl phthalate	N	N	N	-	Non-sensitizer
Isoniazid	N	N	N	-	Non-sensitizer

Criteria 3

	1st	2nd	3rd	4th	Judge
2,4-Diaminotoluene	N	N	N	-	Non-sensitizer
2-Aminoanthracene	P	P	-	-	sensitizer
Dapson	N	N	N	-	Non-sensitizer
Dibutyl phthalate	N	N	N	-	Non-sensitizer
Isoniazid	N	N	N	-	Non-sensitizer

N;Negative, P;Positive


2-Aminoantracene
Citral
Chloroquine
1st experiment
2nd experiment
3rd experiment
cont
3.90625
62.5
7.8125
500
125mg/ml in DMSO
500mg/m in DMSOl
100mg/ml in ddw
Dexamethasone (lipophilic)
Methyl mercuric(II) chloride
Positive control
(Dexamethasone, Cyclosporine A)
DEX
1mg/ml
CyA 1ug/ml
500mg/ml in DMSO
500mg/ml in DMSO

厚生労働科学研究費補助金（化学リスク研究事業）
免疫毒性評価試験法Multi-ImmunoTox assayの国際validationへ向けての検討
分担研究報告書

化学物質のMITAによる解析,validation

分担研究者　山影康次
一般財団法人食品薬品安全センター　秦野研究所

研究要旨
Multi-ImmunoTox assay（MITA）では、皮膚感作性物質の多くがLPSで刺激したTHP-G8細胞のIL-8転写活性を抑制し、単球/樹状細胞に抑制的に作用する免疫抑制物質と皮膚感作性物質を区別できないことが明らかとなった。そこで従来法のMITAに、これまで我々が進めてきた皮膚感作性物質試験法であるIL-8 Luc assayを加えたmodified MITAを構築するために、5物質のIL-8 Luc assayを実施した。また、免疫毒性試験系として、MITAのIL-2レポーター活性抑制評価系のバリデーション試験を行うための最初の段階である技術移転性を確認するために、5物質のIL-2レポーター活性抑制作用を調べた。

キーワード：IL-8 Luc assay、IL-2 レポーター活性抑制、技術移転性

A．研究目的

免疫毒性評価試験法である Multi-ImmunoToxicity assay (MITA)の data set（60 化学物質）を構築した結果、MITA のプロトコールでハプテンを評価すると $CoCl_2$、$NiCl_2$、isophorone diisocyanate などの感作性物質が IL-8 レポーター活性抑制作用を示し、免疫抑制剤と区別できないことが明らかとなった。真の免疫抑制剤と感作性物質を区別して評価するために、MITA と感作性物質評価系との組み合わせが不可欠であることから、感作性物質の評価系である IL-8 Luc assay の結果を含む Modified MITA の data set を構築するために 5 物質に関して IL-8 Luc assay を実施した。また、免疫毒性の評価系として IL-2 レポーター活性抑制評価系のバリデーション試験の最初のステップとして、技術移転性確認のため 5 物質の試験を行った。

B. 研究方法

B-1)用いた細胞

IL-8 Luc assayには、IL-8およびG3PDHの各プロモーター領域にそれぞれ橙および赤色のルシフェラーゼ遺伝子を繋いだベクターをTHP-1細胞に導入した安定細胞株THP-G8を使用した。

IL-2レポーター活性抑制試験には、緑、橙、赤色の発光色の異なるルシフェラーゼ遺伝子をIL-2, IFN-γ, G3PDHの各プロモーター領域に繋いだベクター（それぞれ緑、橙、赤色）をJurkat細胞に導入した安定細胞株#2H4を使用した。

B-2) 使用した化学物質

IL-8 Luc assay には、isophorone diisocyanate、pentamidine isethionate salt、4-nitroaniline、magnesium sulfate heptahydrate、lithium carbonateの5物質を用いた。

IL-2レポーター活性抑制試験の技術移転性には、2-aminoanthracece、chloroquine、citral、dexamethasone、methylmercury(II) chlorideを用いた。

B-3) 実験方法

IL-8 Luc assay については、バリデーション試験で実施した方法に従った。すなわち、1×10⁶/mLに調整した THP-G8細胞の50 μLを96 wellプレートに播種し、X-VIVO™に溶解した原液または懸濁液を遠心した原液とそれを希釈した各濃度の化学物質溶液50 μLを添加し、16時間処理(37°C、 5%CO_2)した。処理終了後、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、各色ルシフェラーゼ活性をPherios（アトー社製）で測定し、色分離式により各プロモーター活性を算出した。

IL-2レポーター活性抑制試験は、MITAプロトコールに準じて行った。概要としては、#2H4細胞を96 wellプレートに播種し、各種濃度の化学物質を添加した。1時間後にPMA/ionomycin (#2H4細胞)による活性化処理を行い、6時間処理(37°C、 5%CO_2)後にTripluc luciferase assay reagentを用いて各色ルシフェラーゼ活性をPheriosで測定し、IL-2プロモーター活性を算出した。

C.結果

C-1) IL-8 Luc assay

実施した5物質の結果を図1に示した。化学物質処理群のG3PDHプロモーター活性（SLR-LA）を陰性対照のその活性で割った阻害指数（I.I.-SLR-LA）が0.05以上で、標準化した（G3PDHプロモーター活性で割った）IL-8プロモーター活性（SLO-LA）を化学物質処理群と陰性対照群とで比較したfold induction（FInSLO-LA）が1.4以上でかつ、その95%信頼限界の下限が1.0以上の場合を陽性とし、2回以上陽性結果が得られた場合を感作性有りと判定した。

試験した5物質中、pentamidine isethionate saltと4-nitroanilineは非感作性物質と判定され、残りの3物質は感作性物質と判定された。

C-2) IL-2レポーター活性抑制試験の技術移転性

5物質の実験を3回繰り返し、その結果を東北大へ送付した。バリデーション試験の試験実施施設である3施設（秦野研究所、産総研バイオメディカル研究部門、産総研健康工学研究部門）の結果を東北大で比較検討している。

D. 考察

MITA data setの解析の結果、MITAでは$CoCl_2$、$NiCl_2$、isophorone diisocyanateなどの感作性物質がIL-8 レポーター活性抑制作用を示し、Dex、hydrocortisoneあるいはFR167653 (p38 mitogen activated kinase (MAPK)阻害剤)などの免疫抑制剤との区別ができないことが明らかとなった。この問題点を解決するために、IL-8プロモーター活性測定系を加えたmodified MITAを構築することは免疫毒性物質の評価において重要であると考えられる。そこで、modified MITAのdata setを構築するために、MITAのdata setから選択した5物質についてIL-8 Luc assayを実施した。これにより免疫抑制物質と感作性物質との識別が可能となった。

また、今年度の技術移転性結果をもとに、次年度以降は免疫毒性試験のIL-2プロモーター活性評価系のバリデーション試験を継続する予定である。

E. 結論

MITAのdata set（60化学物質）を構築に協力し、AOP作成に貢献した。また、免疫毒性試験のIL-2プロモーター活性評価系のバリデーション試験の最初のステップである技術移転性の確認実験を終了した。

F. 参考文献

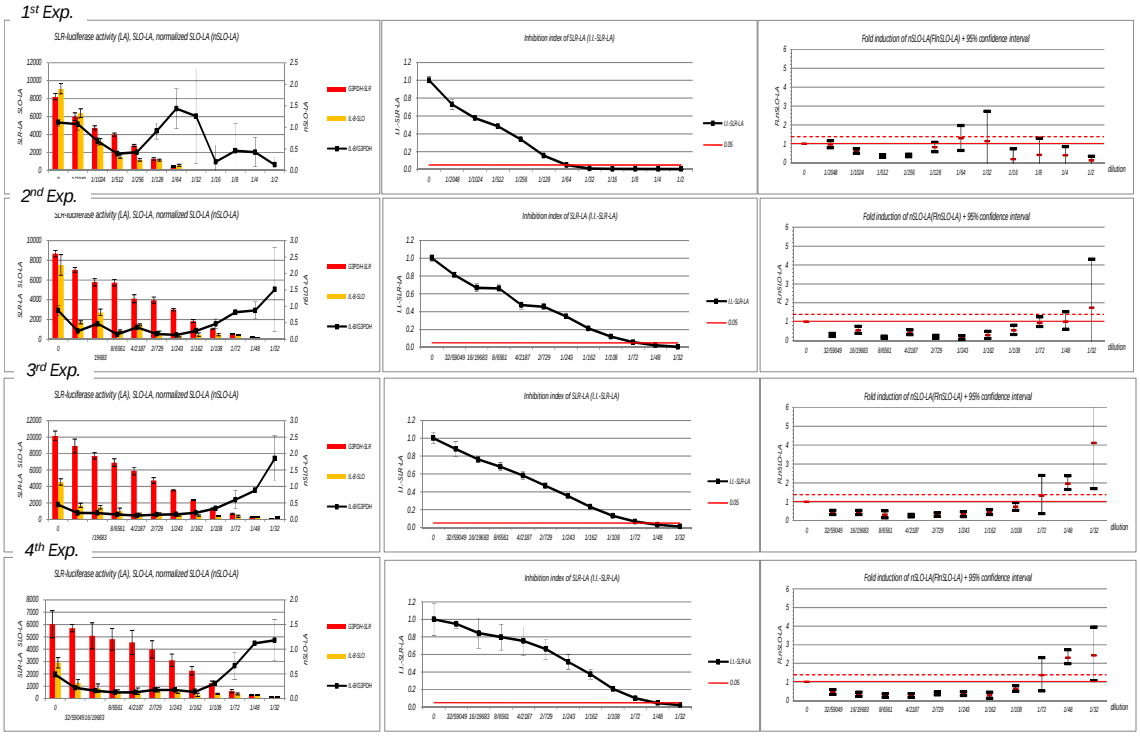
なし

G. 研究発表

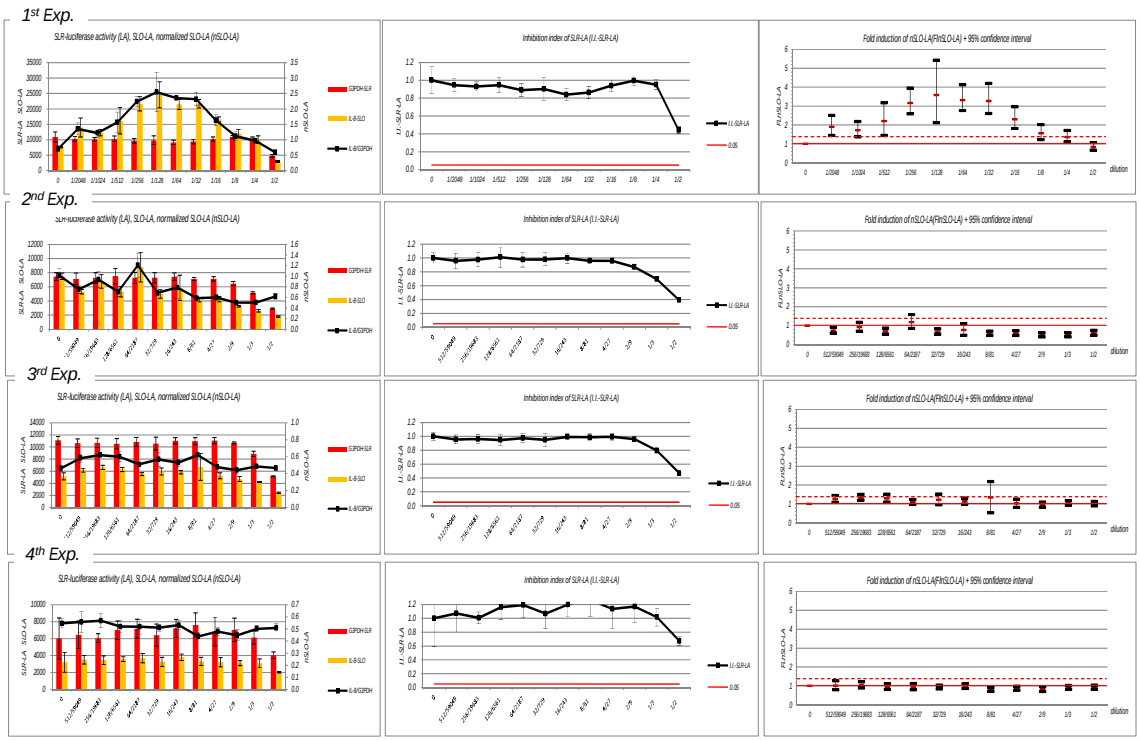
なし

図１　Modified MITA による data set 構築のための IL-8 Luc assay の結果

<Pentamidine isethionate salt>

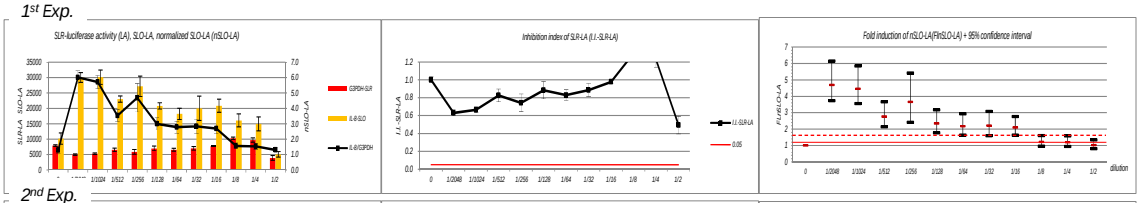


< 4-Nitroaniline >

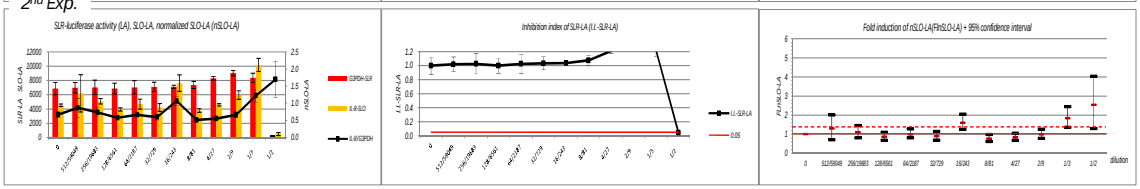


<Isophorone diisocyanate>

1st Exp.

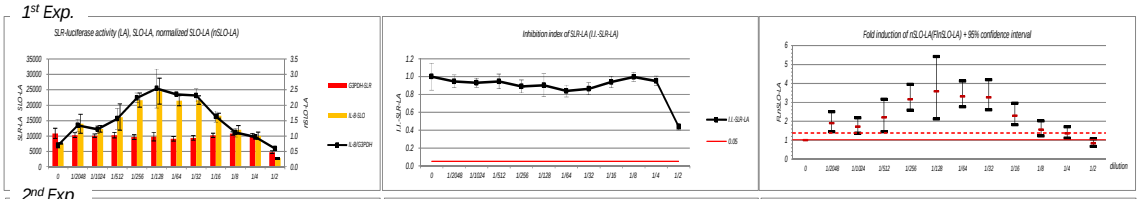


2nd Exp.

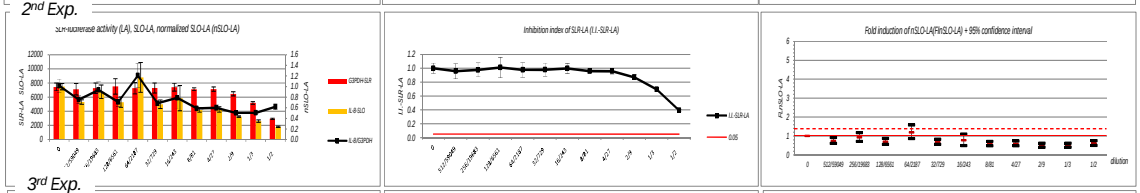


<Magnesium sulfate heptahydrate>

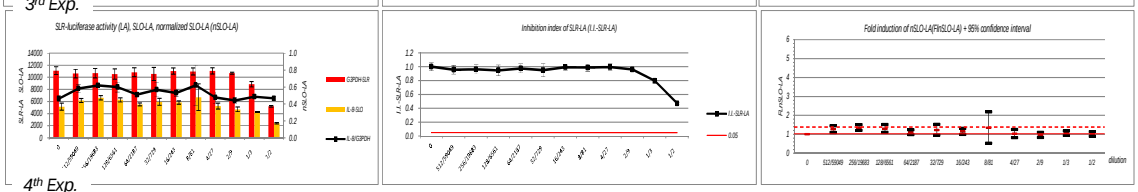
1st Exp.



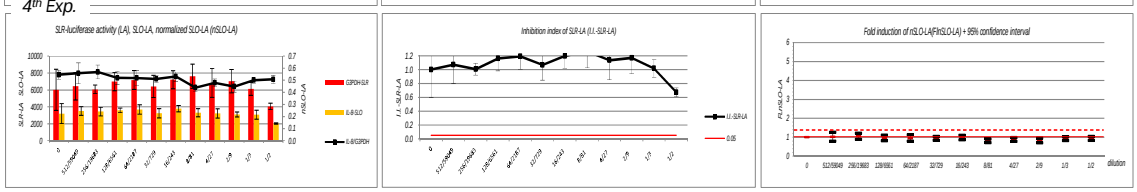
2nd Exp.



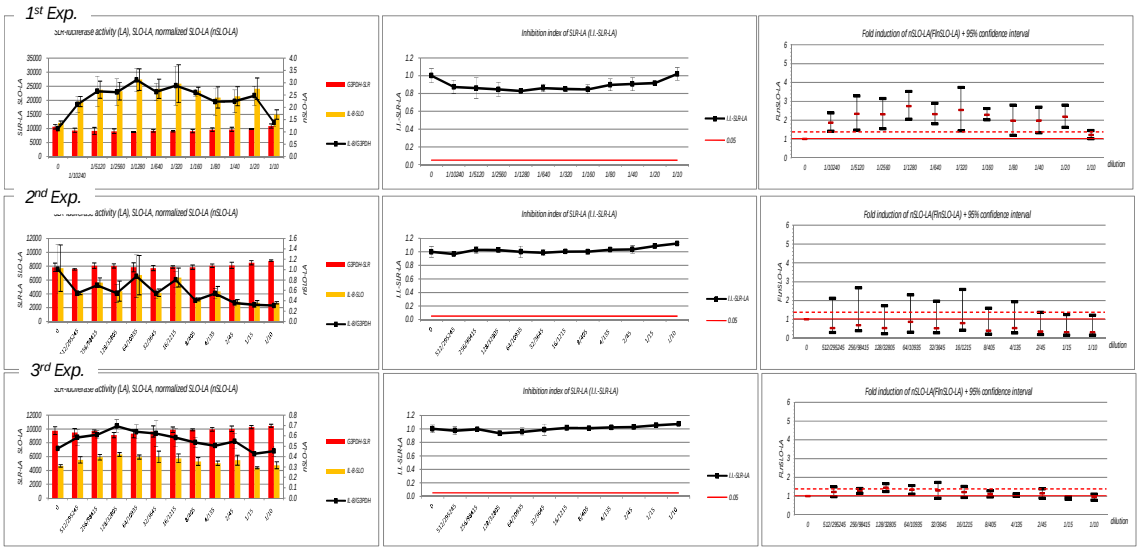
3rd Exp.



4th Exp.



<Lithium carbonate>



厚生労働科学研究費補助金（化学リスク研究事業）
免疫毒性評価試験法Multi-ImmunoTox assayの国際validationへ向けての検討
分担研究報告書

国際バリデーションの施行

分担研究者　小島　肇
国立医薬品食品衛生研究所

研究要旨

新たな*in vitro*免疫毒性評価試験法（Multi-ImmunoTox assay：MITA)のOECD（Organisation for Economic Co-operation and Development）試験法ガイドラインとしての公定化を目指し、国際バリデーションに向けた活動を行った。国際的な専門家を招聘して本試験法に対して意見を求めた結果、平成28年度以降、MITAに関する国際バリデーションを開始することになった。

キーワード：免疫毒性、動物実験代替法、バリデーション

A．研究目的

新たに開発された *in vitro* 免疫毒性評価試験法（Multi-ImmunoTox assay：MITA）の OECD（Organisation for Economic Co-operation and Development）における試験法ガイドライン（Test Guideline：TG）を目指し、国際バリデーションの開始を模索する。

B．研究方法

B-1. 国際的な専門家との意見交換

平成28年度以降、MITAに関する国際バリデーションを開始し、国際的なTGへの道程を明確にすることを予定している。その第一歩として、平成28年１月国際バリデーションのキックオフ会議を企画した。

B-2.バリデーション研究の被験物質選択

キックオフ会議において、Phase0として、トレーニングに用いる被験物質およびphase I として施設内再現性を評価するための被験物質の選択を行った。

B-3.IL-8 Lucアッセイの公定化

平成27年10月、OECDの皮膚感作性試験専門家会議に参加し、日本で開発されたin vitro皮膚感作性試験のうち、IL-8 LucアッセイのTGとしての意義、必要性について意見交換した。

C．結果

C-1.　国際的な専門家との意見交換

国際バリデーションのキックオフ会議には、免疫毒性およびその試験法に関する専門家として、海外から Dr. Emanual Corsini (Milan Univ.)、Dr. Erwin L. Roggen（3Rs Management and Consulting ApS）および Dr. Dori Germolec（NTP/NIEHS：電話でのみ参加）を、国内からは、景山茂樹博士（富士フィルム）および日本免疫毒性学会の推薦者である井上智彰博士（中外製薬）を外部専門家とし

て招聘し、研究班の班員を含む表１に示すメンバーにて 2 日間掛けて、MITA の科学的意義、試験法プロトコルの妥当性などについて討論した。会議の議事次第を添付文書 1 に示す。

会議に先立ち、研究代表者の相場は、当初の計画を一部変更する以下の提案を示した。

１）細胞の選択と測定指標の妥当性

平成 28 年度からの 2 年間は IL-2 レポーター細胞の 2H4 を用いてバリデーション研究を実施する。この細胞は今年度に作成した 60 種類の化学物質からなるデータセットにおいても鋭敏に IL-2 転写活性抑制物質を検出することができている。したがって今年度に作成した IL-2 転写抑制を指標としたＴ細胞サブセット分化異常の AOP に則った評価も可能である。この IL-2 の結果とすでにバリデーション研究が終了している IL-8　Luc アッセイの組み合わせにより、免疫毒性を評価することとした。

２）一つ一つの試験法を独立してバリデーション研究を行うべきであるが、そのスケジュール

これから行うバリデーション研究においては予算の関係もあり、上記 IL-2 転写活性抑制に関する試験法に関してのみ施行する。

３）アンタゴニスト試験系に対する懸念（これまでの経験ではアンタゴニストのプロトコルがよく練られていないと頓挫する可能性が高い）

Lipopolysaccharide (LPS)に対するアンタゴニストを用いる評価系は、現時点では再現性が不十分である。現在、種々検討しているところである。

４）試験法データを組み合わせた判別式の確立

今後、作成する予定である。

この案をもとに表１に示すメンバーにて議論を重ねた。その議事録を添付文書2に示す。主な論点を以下に示す。

・免疫毒性は多岐に渡り、IL-2やIL-8にエンドポイントを特定することが妥当か。
・IL-2は免疫毒性に重要であり、事実的なエンドポイントである。作用機構の点から有用である。
・細胞毒性は免疫抑制の作用機構の一つであり、IL-2を細胞毒性の代用として測定すべきである。
・IL-2が関与したAOP(Adverse Outcome Pathway)の開発も必要である。
・評価には他の試験結果との組み合わせが必要である。
・バリデーション研究の開始は早すぎる。バリデーション研究を行う理由が見当たらない。
・偽陰性の原因を明確にすべきほうが重要である。
・今すべきことは、ヒトや動物の免疫毒性データを整備することである。
・MITAはスクリーニングツールとして有用であり、真の陰性結果を見つけられることを確かめるためにバリデーション研究は必要である。
・IL-2レポーター細胞のもとであるJarkat細胞は他の細胞よりは再現性が得られやすい。
・バリデーション研究を実施するなら、再現性の確認のために実施すべきである。80%の施設内再現性が必要である。
・被験物質の選択が重要である。
・数年前に作成した免疫毒性物質リストと比較して、相場らの選択物質は主な免疫毒性物質を網羅している。
・過去の経験から、陰性物質を見つけることが難しい。
・まず明らかな陽性、陰性物質を含む5物質で結果の一致性を確認すべきである。
・プロトコルに細胞の管理や最大適用濃度を明記することが重要である。

以上の議論の末、再現性を確認するための国際バリデーション研究の実施に合意が得られた。

早速、添付文書3に示すバリデーション計画案を示し、外部専門家の意見をもとに、平成28年度以降に実施するバリデーション計画を検討した。まずphase0としてトレーニングを実施すること、次に

phase1として施設内再現性を確認する計画に合意を得た。

C-2.バリデーション研究の被験物質選択

まずはphase 0 として、3施設のトレーニングを行う5物質を選定した。表2にそのリストを示す。

施設内再現性を確認するphase I の5物質も選定したが、コード化して実施することもあり、本報告書には記載していない。

C-3.IL-8 Luc アッセイの公定化

OECD における専門家会議において、日本からOECD に提案している皮膚感作性試験代替法 IL-8 Luc アッセイが専門家会議で議論された。議事概要を添付文書 4 に示す。

日本から開発者の相場らが本会議に参加し、概要説明を行うとともに、内容の詳細について議論した。まだ第三者評価が進行中であるが、TG 化が期待される IL-8 Luc アッセイの国際的な理解が深まった。

D. 考察

免疫毒性は多岐に渡り、作用機構がわかっているものは少ない。そのような状況下でIL-2やIL-8に特化した試験法の開発には懐疑的な意見が外部専門家からあった。

とはいえ、IL-2が免疫毒性の重要なエンドポイントであることは間違いなく、この試験法の開発を中断するほどの大きな理由は見当たらない。そこで、再現性の確認を目的としたバリデーション研究を行うことで外部専門家の合意が得られた。その際の被験物質の選択も重要であり、phase 0を経て、phase Iの5物質で施設内再現性を確認した後、施設間再現性を確認するためのPhase IIの被験物質選択が重要との見解で一致した。

E. 結論

新たな*in vitro*免疫毒性評価試験法（MITA）のOECDにおける公定化の道筋を明確にするため、国際的な専門家を招聘して意見を求めた。

その結果、平成28年度以降、MITAに関する国際バリデーション研究を開始することになった。

F. 添付文書

1) Agenda：Kick-off meeting for the MITA assay
2) Minutes of MITA Kick-off meeting, Jan. 27 & 28, 2016
3) Study plan for the validation trial on multicolor reporter assay using IL-2 Luc (IL-2 Luc assay) as a test evaluating the immunotoxic potential of chemicals
4) Draft Summary Record: Expert Group Meeting on Skin Sensitisation, 14-15 October 2015, Paris, France

表１． MITA**第一回国際バリデーション会議　参加者リスト**

No.	Name	Affiliation	Country
1	Emanuela Corsini	Universit.AN` degli Studi di Milano	Italy
2	Erwin L. Roggen	3Rs Management and Consulting ApS	Denmark
3	Shigeki Kageyama	Fujifilm Corporation	Japan
4	Tomoaki Inoue	Chugai Pharmaceutical Co., Ltd.	Japan
5	Setsuya Aiba	Tohoku University Graduate School of Medicine	Japan
6	Yutaka Kimura	Tohoku University Graduate School of Medicine	Japan
7	Yoshihiro Ohmiya	National Institute of Advanced Industrial Science and Technology	Japan
8	Rie Yasuno	National Institute of Advanced Industrial Science and Technology	Japan
9	Kohji Yamakage	Food and Drug Safety Center, Hatano Research Institute	Japan
10	Takashi Omori	Kobe University	Japan
11	Shihori Tanabe	National Institute of Health Sciences	Japan
12	Hajime Kojima	JaCVAM, National Institute of Health Sciences	Japan
13	Steven Venti	Translator	Japan

表２． Phase 0 **トレーニング用物質**

Chemical	CAS No.	MW	Physical state	MITA IL-2 result
2-Aminoanthracene	613-13-8	193.24	Solid	S(-/-/-)
CH_3HgCl	115-09-3	251.08	Solid	+-
Chloroquine diphosphate salt	50-63-5	515.86	Solid	S(-/-/-)
Citral	5392-40-5	152.23	Liquid	S(+-/+-/+-*)
Dexamethasone	50-02-2	392.46	Solid	S(-/-/-)

G. 研究発表

1. **論文発表**

1) Speit G, Kojima H, Burlinson B, Collins AR, Kasper P, Plappert-Helbig U, Uno Y, Vasquez M, Beevers C, De Boeck M, Escobar PA, Kitamoto S, Pant K, Pfuhler S, Tanaka J, Levy DD : Critical issues with the in vivo comet assay: A report of the comet assay working group in the 6th International Workshop on Genotoxicity Testing (IWGT),Mutat Res Genet Toxicol Environ Mutagen. 2015 May 1;783:6-12

2) **小島　肇**：**化粧品原料に対する安全性規制の世界動向**, Cosmetic Stage, 9(4), 1-9 (2015)

3) **小島　肇**：**化粧品等の接触皮膚炎を起こす物質を評価するための動物実験代替法について**，**アレルギーの臨床,** 35(5)471，36-40 (2015)

4) Kojima H, Kasamatsu T: Regulatory science - JEMS symposium in 2014. Genes and Environment. 2015; 37:12

5) **小島　肇：経皮吸収型製剤開発における基礎と実務への応用**，**情報機構**, 71-83 (2015)

6) **小島　肇：昨今の皮膚毒性評価法の動向**, **谷本学校毒性質問箱,** 17，8-14 (2015)

7) **小島　肇：動物実験代替法の変遷と最新動向**，**日本香粧品学会誌　創立 40 周年記念,** 39，72-75 (2015)

8) Sewell F, Ragan I, Marczylo T, Anderson B, Braun A, Casey W, Dennison N, Griffiths D, Guest R, Holmes T, van Huygevoort T, Indans I, Kenny T, Kojima H, Lee K, Prieto P, Smith P, Smedley J, Stokes WS, Wnorowski G, Horgan G: A global initiative to refine acute inhalation studies through the use of 'evident toxicity' as an endpoint: Towards adoption of the fixed concentration procedure, Regul Toxicol Pharmacol. 2015;73(3):770-779.

9) Uno Y, Kojima H, Omori T, Corvi R, Honma M, Schechtman LM, Tice RR, Beevers C, De Boeck M, Burlinson B, Hobbs CA, Kitamoto S, Kraynak AR, McNamee J, Nakagawa Y, Pant K, Plappert-Helbig U, Priestley C, Takasawa H, Wada K, Wirnitzer U, Asano N, Escobar PA, Lovell D, Morita T, Nakajima M, Ohno Y, Hayashi M: JaCVAM-organized international validation study of the in vivo rodent alkaline comet assay for detection of genotoxic carcinogens: II. Summary of definitive validation study results, Mutat Res Genet Toxicol Environ Mutagen. 2015; Jul;786-788, 45-76

10) Morita T, Uno Y, Honma M, Kojima H, Hayashi M, Tice RR, Corvi R, Schechtman L: The JaCVAM international validation study on the in vivo comet assay: Selection of test chemicals, Mutat Res Genet Toxicol Environ Mutagen. 2015; Jul;786-788, 14-44

11) Uno Y, Kojima H, Omori T, Corvi R, Honma M, Schechtman LM, Tice RR, Burlinson B, Escobar PA, Kraynak AR, Nakagawa Y, Nakajima M, Pant K, Asano N, Lovell D, Morita T, Ohno Y, Hayashi M: JaCVAM-organized international validation study of the in vivo rodent alkaline comet assay for the detection of genotoxic carcinogens: I. Summary of pre-validation study results, Mutat Res Genet Toxicol Environ Mutagen. 2015; Jul;786-788, 3-13

12) Uno Y, Kojima H, Hayashi M: The JaCVAM-organized international validation study of the in vivo rodent alkaline comet assay, Mutat Res Genet Toxicol Environ Mutagen. 2015; Jul;786-788, 2
13) Yamaguchi H, Kojima H, Takezawa T: Predictive performance of the Vitrigel-eye irritancy test method using 118 chemicals, J Appl Toxicol. 2015; Oct 15.
14) **小島　肇：日本動物実験代替法評価センター**（JaCVAM)**の紹介並びに我が国における動物実験代替法の現状**, LABIO62, 42-44 (2015)
15) Kojima H.: The use of 3-D models as alternatives to animal testing. Altern Lab Anim. 2015 Sep;43(4): P40-3.
16) **小島　肇，西川秋佳**: **日本動物実験代替法評価センター**（JaCVAM)**平成** 26 **年度報告**, AATEX-JaCVAM, 4(1), 30-38 (2015)
17) **小島　肇**: **機能性化粧品素材，シーエムシー出版**, 62-69 (2016)
18) **小島　肇**: VICH **セッション５所感**, JVPA Digest 55, 19 (2016)
19) **小島　肇**: **日本で開発または評価された** OECD **テストガイドライン**, **生物化学的測定研究会年報**, 20 (2016)

2. **口頭発表**

1） **山口宏之，小島　肇，竹澤俊明**: Vitrigel-Eye Irritancy Test (EIT)method: Assessment of predicitive performance using 118 test chemilcas, **日本組織培養学会　第** 88 **回大会**(2015.5)(**広島**)
2） Kojima H, Kleinstreuer N, Schaeffer MW, Kim TS, Chen W, Sozu T, Watanabe M, Niitsma T, Yamashita K, Miyazaki H, Fukuda T, Yamaguchi N, Fujiwara S, Yamaguchi H, Takezawa T: Validation study of Vitrigel-EIT (Eye Irritancy Test) method, II International Conference of Alternatives to Animal Experimentation (2015.5)(Lisbon, Portugal)
3） **小島　肇**: **動物実験代替法についての各国の動向および最新研究**, 2015 **年日中化粧品国際交流協会年度交流イベント** (2015.6)(**東京**)
4） **小島　肇**: **日本で開発または評価された** OECD **テストガイドライン**, **生物化学的測定研究会　第** 20 **回学術集会** (2015.6)(**東京**)
5） **細井一弘、小島　肇**: **シンポジウム６トランスレーションに有用な試験系構築の基本的考え方：**ROS **アッセイを例にして**, **第** 42 **回日本毒性学会学術年会** (2015.6)(**金沢**)
6） Kojima H, Kleinstreuer N, Schaeffer MW, Kim TS, Chen W, Sozu T, Watanabe M, Niitsma T, Yamashita K, Miyazaki H, Fukuda T, Yamaguchi N, Fujiwara S, Yamaguchi H, Takezawa T: **眼刺激性試験代替法** Vitrigel-EIT **のバリデーション研究**, **第** 42 **回日本毒性学会学術年会** (2015.6)(**金沢**)
7） **山口宏之，小島　肇，竹澤俊明**: **高感度な** in vitro **眼刺激性試験法** Vitrigel-EIT **の予測性評価**, **第** 42 **回日本毒性学会学術年会**(2015.6)(**金沢**)
8） **伊藤浩太，榊原隆史，越田　美，奥村宗平, 中山拓生, 平賀　武夫, 小島　肇, 松浦正男**: **牛角膜を用いた混濁度度および透過性試験法**(BCOP **法**)**による** GHS **分**

類２B物質の測定結果, 第42回日本毒性学会学術年会(2015.6)(金沢)

9） 小島　肇, 西川秋佳: JaCVAM update(日本動物実験代替法評価センター)進捗, International Cooperation on Alternative Test Methods (ICATM) Coordination meeting (2015.6)(韓国・済州島)

10）小島　肇: JaCVAM activities update(日本動物実験代替法評価センター)活動報告, The 7th International Congress of Asian Society of Toxicology (2015.6)(韓国・済州島)

11）小島　肇: 動物実験代替法の国内外の最新動向, 皮膚基礎研究クラスターフォーラム　第10回教育セミナー (2015.7)(東京)

12）小島　肇:国立衛研の立場, 第3期　日本化学工業協会　LRI研究報告会 (2015.8)(東京)

13）Kojima H：New SPSF for OECD Test guideline on the in vitro skin sensitization assay，12th Annual meeting of Korean Society for Alternative to Animal Experiments (2015.8)(韓国・ソウル)

14）Kojima H：The JaCVAM/OECD activities on the comet assay，11th International Workshop of Comet assay Workshop (2015.9)(Antwerp, Belgium)

15）Kojima H：Development and validation of alternative to animal testing，ITRI seminar (2015.9)(Shinchiku, Taiwan)

16）Kojima H：The Asian Congress on Alternatives and Animal Use in the Life Sciences 2016，16th Annual Congress of European Society of Alternatives to Animal Testing (2015.9)(Linz, Austria)

17）Kojima H, Oshimura M, and Imatanaka N: Update for Japanese Project “ARCH-Tox” for the Future Chemicals Management Policy: Research and Development of in vitro and in vivo Assays for Internationally Leading Hazard Assessment and Test Methods, 4th annual meeting of the American Society for Cellular and Computional Toxicology (2015.10)(EPA, NC, U.S.A.)

18）小島　肇：シンポジウム７：経済産業省プロジェクト 「石油精製物質等の新たな化学物質規制に必要な国際先導的有害性試験法の開発： ARCH-Tox」の計画概要, 日本動物実験代替法学会　第28回大会(2015.12)(横浜)

19）中山 拓人, 寒水 孝司, 山口 宏之, 竹澤 俊明，小島 肇: Vitrigel-EIT（Eye Irritancy Test）法による眼刺激性の判定法の提案, 日本動物実験代替法学会　第28回大会(2015.12)(横浜)

20）古川 正敏, 榊原 隆史, 伊藤 浩太, 佐々木 啓, 越田 美, 奥村 宗平， 河村 公太郎, 松浦 正男，小島 肇: 牛摘出角膜を用いた混濁度及び透過性試験法（BCOP法：眼刺激性代替法試験）における角膜の病理所見による弱刺激性物質の評価について, 日本動物実験代替法学会　第28回大会 (2015.12)(横浜)

21）加藤 義直, 山本 直樹, 佐藤 淳, 中田 悟，小島 肇: 不死化ヒト角膜細胞株（iHCE-NY）を用いた三次元角膜再構築モデルにおける 眼刺激性評価方法の検討　～後培養による回復性の評価～, 日本動物実験代替法学会　第28回大会 (2015.12)(横浜)

22）内野 正, 宮﨑 洋, 山下 邦彦, 小島 肇, 竹澤 俊明，秋山 卓美，五十嵐 良明：

ビトリゲルチャンバーを用いた皮膚感作性試験代替法（Vitirgel-SST法）の室内再現性, 日本動物実験代替法学会　第28回大会(2015.12)(横浜)

23）宮﨑 洋, 山下 邦彦, 内野 正, 竹澤 俊明, 小島 肇：ビトリゲルチャンバーを用いた皮膚感作性試験代替法（Vitrigel-SST法）による 感作性物質の評価（第2報）, 日本動物実験代替法学会第28回大会 (2015.12)(横浜)

24）成田 和人, Vo Thihong Phuc, 中川 史子, 小島 肇, 板垣 宏：皮膚感作性試験の偽陰性評価解消に向けた検討：無水フタル酸に対する LP溶媒暴露法, 日本動物実験代替法学会　第28回大会 (2015.12)(横浜)

25）九十九 英恵, 松成 夏美, 小島 肇, 板垣 宏：タンパク質のアレルギー性を評価するin vitro試験法の開発, 日本動物実験代替法学会　第28回大会 (2015.12)(横浜)

26）小島　肇：OECDで検討されてきたin vitroスクリーニング法, 環境ホルモン学会　第18回研究発表会 (2015.12)(栃木)

27）Furukawa M, Sakakibara T, Ito K, Sasaki S, Koshita M, Okumura S, Kawamura K, Matsuura M, Kojima H: Histopathological Findings on the Cornea in the Bovine Corneal Opacity and Permeability Test (BCOP Test) for Alternative to Eye Irritation Test, 55th annual meeting of the Society of Toxicology (2016.3) (New Orleans, U.S.A.)

28）Narita K, Vo P.T, Nakagawa F, Kojima H, Itagakai H: Reducing False Negatives of Chemicals in the in vitro Skin Sensitization Test, 55th annual meeting of the Society of Toxicology (2016.3) (New Orleans, U.S.A.)

29）Tsukumo H, Matsunari N, Sugiyama M, Toyoda A, Kojima H, Itagakai H: Development of an in vitro test for Allergenic Potency of Proteins, 55th annual meeting of the Society of Toxicology (2016.3) (New Orleans, U.S.A.)

H. 知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得
なし

2. 実用新案登録
なし

3.その他
なし

添付文書 1

Agenda
Kick-off meeting for the MITA assay

Date ： January 27, 2016, 13:00 - 17:00
January 28, 2016, 9:00 - 13:00
Venue：New Mitoya, Sendai, Japan (http://www.mitoya-group.co.jp/access/)
Participants: Corsini, E., Rogen, E., Kageyama, S.
Aiba, S., Kimura, Y., Yamakage, K., Ohmiya, Y., Yasuno, R., Omori, T., Kojima, H., Tanabe, S.

January 27
Introduction (13:00-13:10)
1. Welcome address and house keeping (Kojima, H.)

Chair: Ohmiya, Y.

Presentations (13:10-17:00)
2. Development of AOP on immunosuppression in EGMST, OECD (Kojima, H.)
3. Outline of the MITA assay (Aiba, S.)
4. Research on the immunotoxicity (E. Corsini)
5. *Applying Toxicogenomics for In Vitro Assessment of Immunotoxicants.(E.L Roggen)*

Coffee Break

Chair: Yamakage, K.

6. Research on the immunotoxicity (Kageyama, S.)
7. Research on the immunotoxicity (Inoue T.)
8. Research on the immunotoxicity (S. Tanabe)
9. Development of reporter gene assay (Ohmiya, Y.)

January 28
Proposal of validation study (9:00-11:00)

Chair: Omori, T.

10. Outline of the IL-2 Luc assay (Aiba, S.)
11. Protocol (Kimura, Y.)
12. Results of preliminary test by three laboratories (Kimura, Y.)
13. Study plan (Kojima, H.)

Coffee Break

Chair: Dr. Kojima, H.

14. Discussion and suggestion (11:30-12:30)

Closing session (12:30-13:00)
15. Wrap-up on discussion
16. Future plan
17. Any other business
18. Closing remark (Aiba, S.)

MITA Kick-off meeting **添付資料２**

Jan. 27, 2016

Kojima	Opening remarks and review of the agenda, followed by self-introductions Review of new and pending OECD Test Guidelines Increased emphasis on identifying AOP, because of the benefits of using AOP as a framework for development of IATA for skin sensitization testing.
Corsini	Do we need to discuss which aspects immunosuppressive or immunomodulatory aspects are to be tested?
Roggen	Need to keep it simple but also ensure that all essential elements are included.
Aiba	Presentation on Multi-Immuno Tox (MITA) assay (See presentation.)
Corsini	Presentation on Research in Immunotoxicity (See presentation.)
Roggen	Presentation on Applying Toxicogenomics for In Vitro Assessment of Immunotoxicants (See presentation.)
Kageyama	Presentation on Research in Immunotoxicity (See presentation.)
Tanabe	Presentation on Research in Immunotoxicity (See presentation.)

MITA Kick-off meeting, Day Two

Jan. 28, 2016

Kojima	Agenda will change a little. Dr. Aiba will summarize the proposed protocol and then we will discuss. If everyone agrees, we will discuss whether to pursue this approach or not.
Aiba	(Summary of proposed protocol)
Omori	I would like to discuss whether this is a viable test or not. MITA appears to be valuable as an immunotoxicological assay. If you agree, I suggest we discuss a validation study. We have completed a validation for IL=8, and we should validate IL-2.
Roggen	Although not a general test for immunotoxicity, it could be useful for some specific things, which we need to identify.
Aiba	I think this modified MITA could be useful to regulators. It should help identify the characteristics of immunotoxicants.
Germolec	Very hard to hear what is being said.
Inoue	With regard to differentiating T-helper cells, IL-2 is related but so is IL-10 and others, so perhaps the results will differ from in vivo testing.
Aiba	Yes, this is not a complete method. But it could be useful in screening chemicals.
Corsini	I agree that as a screening tool it could be useful. Negative results will require additional testing. But immunotoxicity will require weight of evidence, so combined with other tests it will be useful.
Ohmori	These comments seen to suggest that a validation study is needed. Does anyone disagree?
Aiba	Is it possible apply to OECD for a guideline?
Kojima	Immunosuppression is an important factor in safety assessment of chemicals.
Tanabe	What will the endpoint be?
Aiba	We will need to combine with other cell lines, so we would like to validate for 2H4 cell line alone not just MITA.
Roggen	From ECVAM point of view, I am afraid that reproducibility needs to be improved. And from this data, I'm not sure I understand the limits in terms of applicability domain. Also, it seems there were some false negatives, so it seems there is something we don't yet understand.
Aiba	There are so many different chemicals but there is limited information about their toxicity. Most chemicals lack information about immunotoxicity.
Roggen	Yes, we need tests to identify immunotoxins that are not sensitizers, which is

why it is too early for a validation study. Still need more information about reproducibility and how it will be used for screening, etc.

Corsini Perhaps you can comment more about table on page 24.

Aiba The data shows that IL-2 gives consistent results but is still difficult to get results for THP cells.

Corsini If I understand, Jurkat gives good between-lab reproducibility, but the other cell lines are more difficult. Do you have figures for the 60 chemicals for accuracy, sensitivity, and specificity?

Aiba Looking at page 23, there are chemicals that don't give clear results. For example, acetaminophen. Some of these chemicals have known characteristics, but for many, it is not so clear.

Corsini It is difficult to find a compound that is always negative. So the chemical selection will be crucial and not so easy for this validation.

Aiba There are many chemicals that do not have clear information about immunosuppression.

Germolec We are working to find some true negatives, but it has been a struggle. It is difficult to find a chemical that is active but doesn't have an effect on something. So I wonder if we can find a true negative.

Aiba We thought that we had a good negative control, but when we increased the doses, we found it was an immunosuppressant.

Most of the chemicals that affect the pathways can be detected by IL-2 and with just these two tests in the modified MITA, we can detect the characteristics of most chemicals.

Kageyama Compared with other assays, for example, incubation time is longer and concentrations are higher, so I think that this is important. On page 23 these problems occur when time is short or concentration is low.

Roggen With regard to the Fluorescent cell chip method, which uses mouse cells that would be extensively used. We are focusing more and more on human cells now than 10 years ago. It is difficult to find negative compounds, but cytotoxicity is one mechanism of immunosuppression, so perhaps IL-2 upregulation should be measured in the absence of cytotoxicity.

Aiba We do check to see if viability of our cell line contains luciferase activity stimulated by GADPH promoters.

Inoue On page 23, not just sensitivity or cytotoxicity, there are some substances here that give opposite results between Fluorescent cell chip method and MITA.

Ohmori Is the purpose of the validation study to establish the test method? Perhaps we should discuss whether or not there is a need for this test method.

Aiba If we decide that the method is not sufficiently established, we will have to give up this study. I think I have done almost everything necessary to establish the method and categorize chemicals with it. But the situation surrounding MITA is still immature. Perhaps there is no clear need to screen immunotoxicity and there is not clear target. Pharmaceutical makers have a clear need for skin sensitizer test methods but I don't know if there is a need for screening immunotoxicity.

It is probably just a small step to where a need will be established, but we are not there yet.

Roggen I understand your reasoning, but I also wish that work on immunotoxicity will continue. Unfortunately, I do not see a rationale for proceeding with a validation now. Perhaps we need more human and in vivo data about immunotoxicity.

Corsini I see your point, and I think immunotoxicity should be given much more importance. But we are not studying it enough. I agree that we should find some rationale for this study to proceed.

Germolec It is a constantly moving thing. We have a list of 25 compounds. Mostly environmental chemicals. We are still working on making a larger list, so let

me update you all about the status of that list.

Roggen You said the biggest challenge is finding negative substances.

Germolec It is difficult to run through all the assays and find a substance that is truly negative across all parameters. It is often based on cytotoxicity. We were looking at very wide dose-response curves to try to find cytotoxicity and then refine that immunological responses. It has been hard to find immunotoxicity in the absence of cytotoxicity.

Roggen Do you know the Jerka test using 25 genes to identify immunotoxicants? Maybe you need to look at the gene level to achieve that.

Aiba Can we reach a conclusion? The situation is difficult, I know. But I would like to submit an AOP for airway hyper sensitivity or contaminated water. But it is difficult to select chemicals. I would be happy to hear ideas. There is little info on chemicals, key events, and outcome. What chemicals might be useful?

Corsini There are probably close to 100 compounds that are immunotoxic or non-immunotoxic. There was work in the 90s listing non-immunotoxic chemicals. How many do we need?

Germolec There is crossover between the list we are making and the MITA list.

Roggen And to validate, you will need chemicals that affect IL-2 expression.

Corsini Or chemical that affect T-cell expression. You have tested 60 chemicals. How did you select them?

Ohmori One purpose of validation study is to validate reliability. How about that? Most of this discussion is about relevance.

Roggen I think this is a pragmatic approach, since we know IL-2 is important. But to validate that for regulatory use, we have to identify the chemicals that follow that mechanism, and we might not have enough to do that. From a mechanistic point of view, this is an important approach. Reliability is a technical aspect that has to be done but can be improved.

Aiba How can we examine chemicals for immunotoxicity? Is there a method that can be applied to the compounds? It would require a high concentration. How do people examine the effects of pesticides? Skin sensitizers can be tested in a high concentration, but working with low concentrations makes it hard to discern immunotoxicity.

Corsini Pesticides are immunotoxic in animal experiments. So you compare in vivo and in vitro data. Most pesticides today are not immunotoxic, but some old ones are.

Kageyama On page 23, discrepancies are important. By evaluating cytotoxicity, you can examine the effect on T-cells. So combining IL-2 and cytotoxicity is important. So page 23 shows a mixture of two assays.

Roggen All different aspects are important. Here we have two assays that can be combined eventually with other assays.

Ohmori I think this test method is not yet established. We should discuss whether or not this method is valuable enough to establish.

Aiba I think the method is established. We can still modify the assay. We have to optimize certain aspects, but the issue here is that we cannot find a good positive control. But we have no information about a chemical that is clearly immunotoxic.

Roggen I think we agree the tests are important and address specific mechanistic events that are related to immunosuppression. So the test is valuable. The next step is a project to improve reproducibility. Then establish a list of reliable positive and negative controls. It won't be easy but you can do it. And after you have that, you can think about a validation. Chemical selection is crucial to validation planning, and will take time. These are the three steps needed to proceed with this project.

Corsini I agree with Erwin.

Kojima I also agree. It is also necessary to establish AOP.

Roggen AOPs are never finished, so do that in parallel with other activities.
Aiba It is a chicken and egg issue. The modified MITA can provide some insight into the characteristics of chemicals. This method will be useful to people who want to identify the mechanism of immunotoxicity.
Ohmori So, the conclusion is to continue the project. So what is the next step? Should we discuss the plan?
Corsini It is important to come up with a good list of chemicals that we are confident of in terms of immunotoxicity.
Germolec Yes we are willing to share what we have and what we will find in the future.
Corsini We started a few years ago with making a list.
Germolec Yes, there have been many stops and starts. But we are willing to share.

Break

Aiba (Explanation of protocol)
Corsini PMA has a shelf life. Is there is shelf life to the stock solution? From my own experience, PMA stops working after 6 months, so please indicate a shelf life.
Will you include a positive and negative control?
Aiba See page 23.
Corsini What is the cost of this cell line?
Aiba Will be determined in the future.
Roggen I have a comment about the cells. Cell lines lose functionality, especially in transfected parts. It is important to define the No. of permissible passages.
Aiba We are now confirming that. But I think more than 30 times. This line is already 5 years old but we are still using it.
Roggen I understand but you need to define that in the procedure.
Kageyama What about interference from FCS?
Aiba We have used this line for more than 5 years, and have changed a couple of times. I don't think it is a factor.
Inoue On pg 23, DMSO could affect the performance of the cells.
Aiba Page 27 shows DMSO control
Kojima Regarding the criteria, the student t-test is perhaps not the best method. Could you change it to a different method?
Aiba This criteria was accepted for my paper, but if we need to change it for validation, we can do so.
Roggen From the reviewer's point of view, dose dependent response is important. It would be better to say "and" instead of "or" on pg 36.
Kageyama Why don't you use beta mercaptoethanol for the T-cell culture?
Ohmiya Perhaps use a different name rather than 2H4.
Tanabe How many chemicals per plate?
Aiba Can test two chemicals on each plate.
Omori Please send other questions by mail.

Kojima (Presents draft study plan)
Lead lab is responsible for training. Is there a plan for training?
Aiba Yes, this is similar to earlier assays and we do not anticipate any problem.
Roggen For within-lab reproducibility, you have 80% acceptance criteria, but I think a rationale for that target is necessary. I always have this question. This figure is very strict.
Why is there no target for between-lab transferability?
Kojima We expect 100% transferability.
As to the 80% target, we have five chemicals. So we allow one chemical to be out of concordance.
Roggen Maybe better to say "4 of 5" instead of "80%."
Kojima Do you think we need more than 5 chemicals?

Roggen	5 is enough, depending on how clear cut the results are.
Corsini	Is five chemicals enough for within-lab reproducibility with blind chemicals?
Roggen	Five is a common number for within-lab reproducibility.
Kojima	The Phase II will add 20 chemicals, other than the five used for the within-lab reproducibility.
Roggen	The more IL-2 immunotoxicants that you have, the better.
Kojima	(review of schedule) End of Phase 1 study is at the end of this 2016. In the Table 3 it is says Phase 2, but that is a typo. It should be Phase 1.
Aiba	You need 100% concordance?
Kojima	For Phase 0 Study, yes.
Aiba	What happens if there is a non-concordance?
Kojima	Then solve the technical problem.
Roggen	For the five chemicals, the number of positive or negative is not that important. Concordance of results is what is important. (discussion of chemicals)
Aiba	I propose these chemicals for Phase 0: 2-aminoanthracere (DMSO), CH_3HgCl (DMSO), chloroquine (ddw), citral (DMSO), and dexamethansone (water insoluble, DMSO)
Kojima	Is DMSO for positive control?
Aiba	I don't think so.
Roggen	We are looking for five chemicals that are clearly positive? But to challenge the labs, you will want to use chemicals with differing levels of effect.
Aiba	Many of these chemicals are not so toxic, so we can increase the concentration. We have to think about the effect of the chemicals on blood.
Roggen	Is the maximum concentration the concentration that gives the highest toxicity? That is one way of defining it.
Corsini	Max. concentration is 1 mg/mM for Keratinsens and HCLAT.
Roggen	Here we are limited to drugs. We want this test to work for everything. But we need to provide information that will enable people to find the correct concentration for any chemical. There should be some that have only a weak effect, either positive or negative. To see if they can clearly identify it. And then others that are clearly positive or negative.
Aiba	Finding two negatives is a problem.
Corsini	Can we select substances that haven't been tested yet but are known immunotoxicants and affect T-cells.
Roggen	It's better to select chemicals for Phase 1 that have been tested already. For the real validation, you can expand (in Phase 2).
Corsini	So better to select chemicals listed on pages 32 and 33.
Aiba	For Phase 2, I propose: lead acetate ++ (ddw), hydrocortisone + (ddw), dibutyl phthalate +, DMDTC - (DMSO), and nickel sulfate + (ddw)
Roggen	It is good to have one clear positive and one clear negative and then the others that are not so clear. Even if you have to repeat this Phase a couple of times, it is time well spent.
Omori	After checking the properties of these chemicals, Dr. Aiba will discuss the final selection by email. Time to close the meeting now.
Aiba	Thanks for participating. There is still a lot of information that has to be collected and many decisions to make. Thank you for your support.

添付資料３

Version 1,1 January, 2016

Study plan for the validation trial on multicolor reporter assay using IL-2 Luc (IL-2 Luc assay) as a test evaluating the immunotoxic potential of chemicals

IL-2 Luc assay Validation Management Team

INDEX

1. Background

The use of multicolor reporter assay using IL-2 Luc, Jurkat cell (IL-2 assay) is an important for evaluating the immunotoxic potential of chemicals as a part of Multi-ImmunoTox assay (MITA), because of its technical simplicity, short-term test period and accuracy of test result based on a mechanism of immunotoxicity.

The aim of this trial is to (pre)validate the IL-2 Luc assay method to assess transferability and inter-laboratory variability, in order to incorporate this test for screening the immunotoxic chemicals. The IL-2 Luc assay for the validation trial will be undertaken i) in accordance with the principles and criteria documented in the OECD No. 34 Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment [OECD, 2005], ii) according to the Modular Approach to validation [Hartung et al., 2004] ,iii) according to the concept discussed on the validation trials with participation of GLP Test Facilities [Cooper-Hannan et al., 1999] where the whole concept of the validation trials is described in the context of GLP, iv) and in line with the ISO procedure JRC.I.03.GP.01v.01 (http://ihcpnet.jrc.it/quality-safety/quality-documents/unit-03-ivm/doc/JRC.I.03.GP.01v.01.pdf).

The studies part of a validation trial should ideally be performed in accordance with GLP [OECD, 1998-2007; FDA, 1999; EPA, 1998a&b; JSQA, 2010; SCC, 2010]. As a minimum, but not necessary limited, use of standard operating procedures (SOP), adequate data recording, reporting and record keeping are essential.

A general conceptional framework [Hartung et al., 2004; OECD, 2005] will be used for documenting all the study to assess the validation status of a test method, called “modular approach” to validation. In this approach, the information needed to support the validity of the method is organized into modules that provide the following information:

Module 1: Test Definition

Module 2: Within-laboratory repeatability and reproducibility

Module 3: Between-laboratory transferability

Module 4: Between-laboratory reproducibility

Module 5: Predictive capacity

Module 6: Applicability domain

Module 7: Performance standards

The Modular approach as introduced by Hartung et al., allows using datasets from various data sources and studies. This advantage is used in the following proposal to assess the scientific validity of the IL-2 Luc assay. This IL-2 Luc assay for the validation trial has performed under the GLP principle.

2. Objective of the trial

The validation trial will assess the reliability (reproducibility within and between laboratories) and relevance (predictive capacity) of the IL-2 Luc assay with a challenging set of test substances (test items) for which high quality *in vitro and in vivo* data are available.

3. Validation Management Team (VMT)

The VMT encompasses collective expertise with the test, in the underlying science and the scientific design, management and evaluation of a validation trial.

The VMT, which plays a central role overseeing the conduct of the validation trial, includes:

Table 1. Members for IL-2 Luc assay Validation Management Team

Name	Role and expertise	Affiliation
Trial Coordinator Hajime Kojima	VMT trial coordinator , Chemical supplier	JaCVAM, NIHS, Japan (JaCVAM representative)
Lead Lab Yutaka Kimura* Setsuya Aiba*	*Developer of this assay Test method, expertise underlying science	Tohoku Univ., Japan
Shihori Tanabe	Management of quality control	JaCVAM, NIHS, Japan (JaCVAM representative)
Takashi Omori	Data analysis, biostatistics dossier	Kobe Univ., Japan
International expert members		
EU liaison Emanuela Corcini	Test system expertise, validation expertise, immunotoxicity expertise	Milan Univ., Italy
EU liaison Erwin L. Roggen,	Test system expertise, validation expertise, immunotoxicity expertise	3Rs Management and Consulting ApS, Denmark
ICCVAM liaison Dori Germolec	Immunotoxicity expertise	NTP/NIEHS, USA
Japan liaison Shigeki Kageyama	Immunotoxicity expertise	Fujifilm Corporation, Japan
JSIT liason Tomoaki Inoue	Immunotoxicity expertise	Chugai Pharmaceutical Co., Ltd.

3.1Participating Test Facilities

The laboratories participating in the trial are defined as follow:

Test Facility 1: Hatano Res. Inst., FDSC. Study Director (SD) : Kohji Yamakage

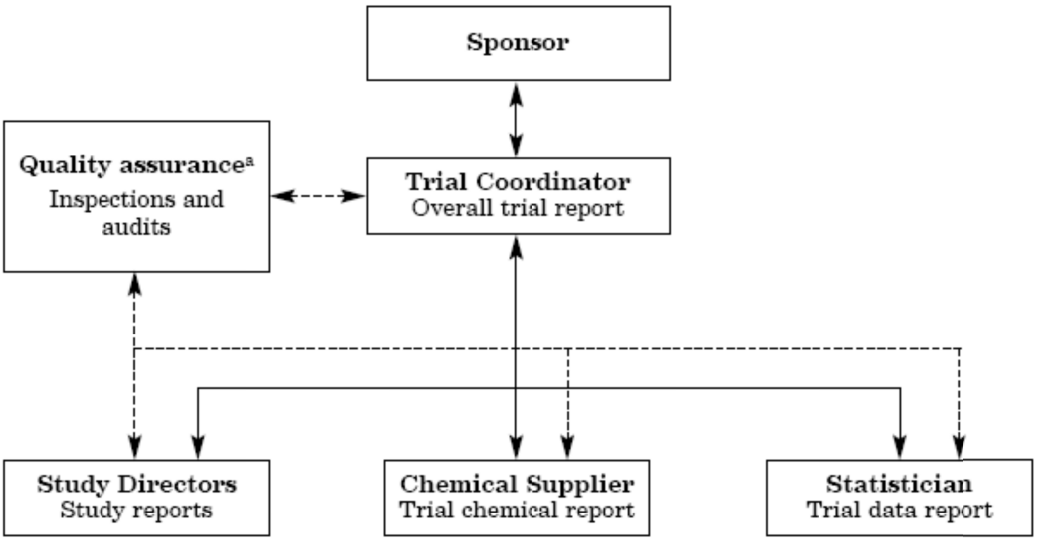
Test Facility 2: AIST, Tsukuba SD : Yoshihiro Ohmiya

Test Facility 3: AIST, Takamatsu SD : Yoshihiro Nakajima

Information relevant for Modules 1, 2, 3 performed by all laboratories. Data obtained by these laboratories have demonstrated that the IL-2 Luc assay is transferable and reproducible between experienced laboratories. The all facility will be the laboratory participating in this validation trial acting as unexperienced laboratory to assess between laboratory transferability, reliability and relevance of the IL-2 Luc assay method under non-GLP conditions (GLP principle).

3.2 Trial management structure

The management structure of the validation trial is shown in **Figure 1**



[a]Several Quality Assurance units might be involved in a multi-study trial.

Dashed lines indicate assurance staff involvement.

Figure 1: Management Structure of the IL-2 Luc assay validation trial

1) Chemical management group

The members of chemical management group are elected by recommendation of the IL-2 Luc assay VMT. They prepare a tentative list of test chemicals and works with the VMT to make a final decision on the test chemicals to be used in the validation trial. The coded test chemicals listed are distributed by JaCVAM.

2) Data analysis group

The members of data analysis group are elected by recommendation of the IL-2 Luc assay VMT, and check and analyze the data obtained in this validation trial from a third-party standpoint. They also take charge of statistical processing in this validation trial.

3) Quality assurance group

The members of record management group are elected by recommendation of the IL-2 Luc assay VMT. They prepares protocol, test chemical preparation record forms, blank data sheets, etc. and distributes them to the research laboratories participating in this validation trial. They also collects filled out forms and data sheets after completion of experiments, pointing out omissions or flaws in recording, if any, and requesting correction of such errors.

4) Lead laboratory

The lead laboratory representing the test method is responsible for providing the test method protocol and the eventually necessary data recording or calculation templates. The Trial Coordinator has to ensure that such data recording or calculation templates have been validated before distribution to the test facilities involved in the validation trial. The lead laboratory is also responsible for providing, if necessary, new versions of the protocols during the entire validation trial. The lead lab and the other participating test facilities might be contacted by the VMT for technical issues.

3.3Sponsor

The validation trial for assessing the validity of IL-2 Luc assay will be financed by the Ministry of Health, Labour and Welfare (MHLW), Japan.

The lead laboratory will support the IL-2 Luc assay validation trial by assuring that reliability is assessed. At the same time, preliminary results of the test method can be evaluated. For this purpose Lead laboratory will support:

- the financial aspects related to the coordination of a validation trial (e.g. organization of VMT meetings where also the involved test facilities can be invited for technical clarifications to the VMT, the publication of the validation trial results)
- the test, reference and control item purchase, coding and distribution to the test facility
- the availability of the test systems to the participating laboratories by supporting the Lead laboratory with the logistics for delivering the test system to the facility
- the independent data analysis and statistical support (biostatistician) based on the study reports generated
- the other costs for participating laboratories

3.4Trial coordination

Dr. Hajime Kojima was appointed as the Trial Coordinator with well-defined roles and responsibilities to coordinate the trial and to establishment of a VMT by supporting of JaCVAM.

The name and location of the Trial Coordinator should be identified in each individual study plan. For the IL-2 Luc assay validation trial, the Trial Coordinator has direct access to the test item coding.

The Trial Coordinator's responsibilities include:

a) Establishment of/support to lead laboratory, including meeting organization

b) Trial communication and coordination with test facilities

c) Recording of document and data flow between test facilities

d) Assessing and documenting the impact of any amendments and/or deviations from the trial plan and study plans on the quality and integrity of the validation trial

e) Ensuring that the individual study reports are forwarded, in a timely manner, for data and statistical analysis

f) Preparing the trial plan and report, which can be based on the study reports from the lead laboratories and other test facilities involved in the validation trial, and should reflect the overall trial

g) Approval with date and signature of all protocols, Study Plans and Study Reports

h) The communication of the results of the trial into the public domain

The role of Trial Coordinator (as the formal representative of the VMT and the single contact point with the SDs) is of fundamental importance. The Trial Coordinator is the single critical point of trial control and must ensure clear lines of communication between the involved test facilities in the trial. The communication line of the Trial Coordinator is with the SDs of the different test facilities. The SDs are the single point of contact with the Trial coordinator (unless otherwise communicated by the participating Test Facilities) to assure a transparent and recorded documentation flow during the trial. The Trial Coordinator should also ensure that appropriate arrangements have been made for the supply of the test systems, and test, control and reference items, which meet the requirements of the trial, and that there are appropriate test method protocols (dated signature by the trial coordinator and the Lead Laboratories) and, if appropriate, validated data recording, data analysis, data reporting sheets for the test method.

It is the responsibility of the Trial Coordinator to approve the study plans send for approval by the test facilities, and any amendments to the study plan, by dated signature.

3.5Training

The lead laboratory will be responsible for issuing a training agenda to the Trial Coordinator for further distribution to the all test facility giving details what training aspects will be covered during the training of the other SDs and Study Personnel at the lead laboratory. Furthermore, after the training, the lead laboratory will issue to the Trial Coordinator a training report and indicating if critical observations are made by the other test facilities regarding the IL-2 Luc assay protocols. In case any critical observations are made a new version of the IL-2 Luc assay protocols might necessary be issued to the other test facilities before initiating the between-laboratory transferability.

3.6 [Module 2] Within-laboratory reproducibility

The within-laboratory reproducibility of the all test facility has been done by an independent biostatistical analysis, under the VMT. The proportion of concordance should be equal or more than 80% as tentative acceptance criteria for phase I validation.

3.7[Module 3] Between-laboratory transferability

This between-laboratory transferability (Module 3, identical to ICCVAM proficiency testing phase) is performed in order to assess the successful transfer of the assay to a test facility unexperienced with that particular test method but having knowledge of similar test systems and endpoint detection methods.

For the transfer of IL-2 Luc assay to the all test facility, the Phase 0 study using non-coded five chemicals was performed. A few concentrations of each test item will be tested in triplicate in 3 independent runs according to the IL-2 Luc assay protocol describing the details of the experimental design.

The five test items selected for the phase I study are coded as follows: A, B, C, D, and E. The all facility will prepare a study according to internal GLP principle. This plan will be submitted to the Trial Coordinator and lead laboratory for approval.

The results of the between-laboratory transferability will be reviewed before progressing with module 4 on the between laboratory reproducibility. If the transferability data do not meet test acceptance criteria, the Trial Coordinator representing the VMT will try to identify the problems and make corrections where needed. At the end of the testing, the test facilities will submit a QC certified copy of whole study dossier to the Trial Coordinator (study plan in GLP principle, raw data, records and data analysis, study report in GLP principle).

3.8 [Module 4] Between-laboratory reproducibility

Ten coded test items have been selected to confirm the between-laboratory reproducibility in the phase I study. A few concentrations of each test item will be tested in triplicate according to the IL-2 Luc assay method protocol describing the details of the experimental design.

At the end of the testing, the test facilities will submit a QC certified copy of whole study dossier to the trial coordinator (study plan in GLP principle, raw data, records and data analysis, study report in GLP principle). The proportion of concordance between-laboratory reproducibility should be equal or more than 80% as acceptance criteria,

3.9[Module 5] Predictive capacity

The necessity for further chemical analysis will be subject to a VMT decision once the data of the between laboratory reproducibility has been assessed. Depending on the statistical analysis the lean design for validation as well as the automatisation of the test leading to an increased dataset will be considered.

4. Protocol

In this validation trial, the protocol (ver. 1E) will be used (attached Document #2). This protocol will make up a draft by the lead laboratory and be finalized by VMT.

A measurement of bioluminescence intensity induced with chemical treatment will be measured by luminometer (Phelios: ATTO, Cat #:AB-2350) calibrated using stabilized SLG, SLO and SLR enzymes in this validation trial.

5. Chemicals

5.1 Chemicals Selection

Test chemicals have been selected by chemical repository based on published papers on in vivo immunotoxicity

The applied selection criteria were:

- information on mode/site of action
- coverage of range of relevant chemical classes and product classes quality and quantity of reference data (*in vivo* and *in vitro*)
- high quality data derived from animals and (if available) also humans
- knowledge on interspecies variations (for example: variability with regard to the uptake of chemicals, metabolism, etc.)
- coverage of range of toxic effects/potencies

- chemicals that do not need metabolic activation
- appropriate negative and positive controls
- physical and chemical properties (feasibility of use in the experimental set-up as defined by the CAS No.)
- single chemical entities or formulations of known high purity
- availability
- costs

In the first phase of the selection procedure, the Chemical Selection Committee identified and collected several existing lists of potential chemical sensitizing in order to establish a primary database. These chemicals had originally been compiled by international experts for various purposes e.g. as reference compounds for validation studies. An extensive literature research was performed by the Chemical Selection Committee in order to insure that the preselected chemical fulfilled the selection criteria described above.

Emphasis was laid on the fact that different potencies (strong, weak and no activity) have been chosen. In addition, it was decided that at least 20% of the total substances to be tested should be negative in order to increase the statistical power of the data analysis.

In the first phase IL-2 Luc assay validation trial with data generation at the test facilities, five chemicals will be tested three times in each test chemical for between-laboratory reproducibility and to confirm transferability. After discussion of Phase I results, detailed test planning of the Phase II will be determined. At this moment, twenty chemicals will be planned in the phase II trial for predictive capacity (Table 2).

Table 2. Outline of test planning at each study in the validation trial.

Study		Chemicals	Test Number	Information obtained
Phase (planning)	0	5 non- coded	1	Between-lab transferability
Phase (planning)	I	5 coded	3	Within & between-lab reproducibility
Phase (planning)	II	20 coded	1	Between-lab reproducibility & predictability

(Planning of Phase II will be determined after discussion of the results of Phase I)

5.2 Chemicals Acquisition, Coding and Distribution

The assessment of within-laboratory reproducibility (Module 2), between laboratory transferability (Module 3) in the all test facilities have been performed with coded chemicals. This IL-2 Luc validation trial plan describes the generation of the missing data sets under coded test item. If the results obtained are not very similar to the previous obtained sets, the VMT has to assess if coded chemicals need to be tested in the all test facilities.

The coding will be supervised by the Trial Coordinator, in collaboration with the chemical repository responsible of coding and distribution of test, reference and control items for the validation trial.

5.3 Handling

Each test facility shall receive through the Trial Coordinator essential information about the test chemicals (physical state, weight or volume of sample, specific density for liquid test chemicals, and storage instructions). Moreover, the SD should receive the safety information concerning the hazards identification and exposure controls/personal protection.

6. Records and archiving

At the end of the trial, the IL-2 Luc assay validation trial report is prepared by the Trial Coordinator or the VMT personnel who appointed by the Trial Coordinator. The trial report summarizes the trial goals, procedures, results and conclusions of the validation trial. This represents the whole validation trial, including archiving and, as such, will cover several study reports, as well as reports for test item supply, data management and statistics. The Trial Coordinator oversees the preparation of the trial report. The Trial Coordinator will be representing the VMT discussions responsible for preparation of the scientific conclusions. Signatories to the trial report include the Trial Coordinator, the statistician, and the SDs of the involved test facilities. Although the SDs may not be involved with the preparation of the trial report, their signatures confirm that the trial report is an accurate reflection of the management and study events. The trial report should contain a statement, signed by the Trial Coordinator, commenting on the accuracy and completeness of the trial report and identifying any significant issues which could have affected the integrity of the trial, including matters of GLP compliance. A QC statement will be included in the trial report, in order to identify what QC monitoring was done and to confirm whether or not the trial report is an accurate reflection of the validation trial data.

7. Study timeline

An approximate schedule for IL-2 Luc assay validation trial is shown in Table 3.

Duration of this validation trial is around twenty -month from May 2016 to Nov 2017.

Table 3. Schedule of IL-2 Luc assay validation trial

Month	Activity
January 2016	Establish the VMT
	Selection of participating research laboratories
	Deliberation, decision and read-through of draft study plan
	Deliberation and decision of protocol
	Preparation of a tentative list of test chemicals
	Distribution of test chemicals, standard chemicals and positive control chemicals
February,2016	Technical transfer using five known chemicals (non-coded) Start of technical transfer **to know between laboratory transferability**
	Data collection of technical transfer (**Phase 0 study**)
Phase I study	
May 2016	Coding and distribution of five coded test chemicals
June, 2016	Start of Phase I study
September, 2016	End of Phase I study
January, 2017	**2nd VMT Meeting** / Phase I results and planning of Phase II study
Phase II study to know between- and within-laboratory reproducibility	
2017	Coding and distribution of coded test chemicals and positive chemicals
2017	Start of Phase II study using 20 coded test chemicals
2017	End of Phase II study
January, 2018	**3rd VMT Meeting** /reviewing of Phase II study results
2018	Completed validation report

Abbreviations

CAS: Chemical Abstracts Service
GLP: Good Laboratory Practice
HRI: Hatano Research Institute
FDSC: Food and Drug Safety Center
JaCVAM: Japanese Centre for the Validation of Alternative Methods
NIHS: National Institute of Health Sciences
OECD: Organization for Economic Co-operation and Development
QC: Quality Control
TG: Test Guideline
VMT: Validation Management Team

ENV/JM/TG/M(2015)6

Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development

English - Or. English

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

Test Guidelines Programme

Draft Summary Record: Expert Group Meeting on Skin Sensitisation

14-15 October 2015 Paris,
France

Contact(s):
Nathalie DELRUE, Administrator, Test Guidelines, Nathalie.DELRUE@oecd.org, +(33-1) 45 24 98 44

ENV/JM/TG/M(2015)6
For Official Use
English - Or English

Draft Summary Record: Expert Group Meeting on Skin Sensitisation

14-15 October 2015

Paris, France

1. Opening of the meeting

1. The meeting was chaired by the OECD Secretariat. Participants from Denmark, France, Japan, Korea, Netherlands, Spain, Switzerland, United Kingdom, European Commission (European Chemicals Agency and Joint Research Center), BIAC and ICAPO attended the meeting. The list of participants is available in Annex 1. The Chair introduced the draft agenda, indicating that the main objective was to address comments received from the WNT in July 2015 on the draft Test Guidelines for human Cell Line Activation test (h-CLAT). The second objective was to discuss the status and issues related to other assays on skin sensitisation, either on-going projects included in the workplan or potential new projects.

Part 1: human Cell Line Activation test (h-CLAT)

2. Discussion of the main issues raised by the WNT comments

2. Joao Barroso (EC) presented the main issues with h-CLAT, raised by WNT comments, mainly h-CLAT reproducibility, applicability and limitations. This was followed by a presentation from Roman Liška (EC) of the re- analysis of the within and between laboratory reproducibility of the h-CLAT. He explained how new approaches were used to assess the within and between laboratory reproducibility of the assay in the validation study, i.e. the permutation of runs' predictions that takes into account all possible sequences of events and the bootstrap probabilistic approach.

3. It was clarified that these new approaches can be used since the runs conducted in the test are fully independent: the runs are conducted on different days and if done on the same day, all preparations have to be re-done. How they happen in time is thus random and the runs are interchangeable. The group agreed on the methodology for calculation of the WLR. Based on the new approaches, the value of the WLR in the validation study was found to be between 82.4 and 84.8% (while the WLR in the h-CLAT validation report was of 80.0% and the target WLR was of 85%). Despite the fact that the level of WLR is slightly below the target, it was agreed that the level of WLR is transparently reflected in the TG, which specifies that "The level of reproducibility in predictions that can be expected from the test method is in the order of 80% within and between laboratories". It was noted that the 'Report on re-analysis of the within and between laboratory reproducibility of the h-CLAT' would be made available together with the validation report (after approval process) in the Series on Testing and Assessment on the OECD public website.

4. To better control certain parameters that could be a source of variability and thus to reduce the variability of the assay, some revisions to the draft Test Guideline had been proposed before the meeting. The optimisation work conducted throughout the development of the test method to minimise sources of variability were presented by Masaki Miyazawa and Takao Ashikaga (BIAC). It was in particular proposed to better control cell density (relevant changes brought to paragraph 19 of the draft TG) and the exposure time (paragraphs 24, 25 and 29 revised). In addition, as the reactivity check using controls is regularly performed, the group also agreed to emphasise in the draft TG, on the need to use the data from the reactivity check as a mean of verification of laboratory proficiency. For this purpose, a new paragraph was added in the section on laboratory proficiency and the test report was also revised accordingly.

5. It was agreed that since the result of a test is based on 2 concordant runs, when the first two runs are concordant, it is not necessary to run a third one. The group agreed on the wording of the draft TG that says: "each test chemical is tested in at least two independent runs to derive a single prediction". "If however, the first two runs are not concordant for at least one of the markers (CD54 or CD86), a third run is needed and the final prediction will be

based on the majority result of the three individual runs (i.e., 2 out of 3)" (paragraphs 29 and 33). A figure of the prediction model used in the h-CLAT test method (figure 1 of draft TG) was considered to be a very useful addition to the draft TG.

6. The group also discussed the number of replicates that should be used in the TG and it was agreed that because a prediction is obtained from at least two independent runs, one replicate could be enough. Paragraph 29 was revised to provide this rationale.

7. Regarding the limitation of the assay and description of the applicability domain, it was recognised that the assay presents limitations for the detection of three types of chemicals, which were described in the TG: (i) pro/pre- haptens, (ii) substances of low water solubility and (iii) strong fluorescent chemicals. The group agreed with the proposed text (paragraph 12), after further harmonised with the wording used in the Keratinosens TG. It was acknowledged that the detection of pro/pre-haptens is an area where progress needs to be made (an upcoming workshop dedicated to discussing this topic was mentioned), but the current state of knowledge doesn't allow to provide more details in the TG.

3. Other issues

8. A few other topics were discussed such as the use of the word 'solvent' vs 'vehicle' in the TG. It was agreed to replace 'solvent' by 'solvent/vehicle' throughout the text of the TG to acknowledge the fact that some test chemicals can be dispersed (and 'solvent' would not be appropriate in this case).

9. There was some discussion and request for clarification about the sentence in paragraph 11 relative to the testing of mixture: "However, before use of this Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed when there is a regulatory requirement for the testing of the mixture." This sentence was developed and agreed by the WNT at the meeting in April 2014. It was clarified that it should be understood in a context of Classification and Labelling, to express that when a mixture is tested and the result is negative, the substances that are part of the mixture can't be declassified on the basis of the results obtained from the mixture.

10. It was suggested to provide the historical relative fluorescence activity (RFI) value and the CV75 value (75% of cell viability) obtained for each proficiency substance included in Table 1 of Annex 2 of the TG. Having the ranges for these values, derived from the 4 laboratories which participated in the validation study, was considered particularly useful to facilitate comparison of data in case other antibodies and cytotoxicity markers are used. Japan/ EURL ECVAM will go back to the data obtained from the laboratories involved in the validation study and investigate the relevance of providing this information. If relevant, these data will be included in the revised version of the draft TG.

11. In terms of next steps, the Secretariat indicated that Japan/EURL ECVAM will now prepare the next version of the draft TG, based on the outcome of the meeting, and update the responses to comments received from the previous WNT commenting round, as appropriate. The revised draft TG will be circulated to the WNT for written comments before the end of the year. It is expected that the draft TG is then submitted for approval at the next meeting of the WNT, in April 2016.

Part 2: Other assays on the OECD Test Guideline workplan

4. Myeloid U937 Skin Sensitization Test (U-SENS) for identifying skin sensitization potential of chemicals

12. Nathalie Alépée (France) presented the history of the U-SENS™ skin sensitisation test, the outcome of the validation study and the statistical analysis that has been conducted. Like the h-CLAT, this assay addresses the 3rd key event in the skin sensitisation pathway, i.e. activation of dendritic cells. Following pre-validation of the assay, the validation phase started in 2013 and the validation test report was submitted to EURL ECVAM in July 2014. Following EURL ECVAM initial review, responses from France and the revised validation report are expected to be available in November 2015. In view of its subsequent peer review by ESAC, a new ESAC group for skin sensitisation is being established.

13. It was noted that the BLR and WLR values (respectively 84.2% and 91.7%) were above the h-CLAT values. It was suggested that this could come from a few differences between the 2 assays, such as the difference in cell line or the fact that this assay only looks at the expression of one type of cell surface markers (CD86). Some participants considered that the prediction model in case of inconclusive result is complex and might be difficult to use.

14. The possibility to develop a Performance-Based Test Guideline (PBTG) including h-CLAT and the U-SENS test methods was mentioned. It was noted however, that for the time being, considering the respective stages of development of the 2 draft TGs, the h-CLAT would be developed as a stand-alone TG and when the U-SENS is ready, the TG would be adapted and turned into a PBTG that would include both test methods. At that time, Performance Standards (PS) would also need to be developed. It was clarified that PS are a document that accompany a PBTGs and are intended to be a guide for the developers of new or modified test methods, similar to the validated reference methods. They communicate the basis by which new test methods can be determined to have sufficient accuracy and reliability for a specific testing purpose.

5. IL-8 Luc assay: An In Vitro Method for Identifying the Skin Sensitisation Potential of Chemicals

15. Setsuya Aiba (Japan) presented the validation report of the IL-8 Luc assay. This assay is based on IL-8 production, which is also a marker of dendritic cell activation in allergic contact dermatitis. The assay was optimised over the various phases of the validation, leading to significant improvement of the performance of the system.

16. Its peer review started in March 2015 and the final peer review meeting was expected to be held in Japan, on 23/24 October. Initial comments from the peer review panel (PRP) were presented by the chair of the PRP, David Basketter (UK). The validation report was well received by the panel. Regarding the BLR and WLR, the PRP concluded that the "data on BLR, with a sufficient number of test chemicals, exceeded the success criterion". However, although the "average WLR met the success criterion of 80%", "the data on WLR was more limited than comparable validation studies, particularly in respect to the number of chemicals tested". "Consequently, the PRP recommends additional assessment of within laboratory reproducibility with more and different test chemicals using the final protocol and prediction model". It is expected that the PRP report is available early 2016.

Part 3: New projects, not yet on the OECD workplan

6. LLNA: BrdU-FCM

17. Korea plans to submit a Standard Project Submission Form (SPSF) in November for inclusion in the TG workplan of a new project for the development of a TG on non-radioactive Mouse Local Lymph Node Assay using Flow-Cytometry Method (LLNA: BrdU-FCM). The method and outcome of the validation study were presented by Ilyoung Ahn (Korea). The method is a modified method of the LLNA: BrdU-ELISA (TG 442B) which enables to reduce the number of animals tested in pre-screen tests, compared with the existing LLNA test methods. The validation study has been conducted to evaluate its reliability and relevance based on the performance standards available in Annex I of TG 429 and Guidance Document 34. The validation study is almost finished and is planned to be completed in December.

18. The presentation was well received and the pre-screen step of the protocol considered an interesting development. However, as this assay is an in vivo assay it was uncertain if the WNT would consider it as a priority for inclusion in the workplan. As an alternative option, it was suggested that the project could consist in an update TG 442B to include also the BrdU-FCM method, rather than to create another LLNA TG.

7. SENS-IS assay

19. France plans to submit an SPSF in November for inclusion in the TG workplan of a new project for the development of the SENS-IS™ assay. The method and outcome of the validation study were

presented by Hervé Groux (France). The assay is a new approach for the identification of skin sensitisers where genes specifically modulated in sensitised skins allow the detection of sensitisers in a reconstructed human skin model. The results of the validation study, conducted in 3 laboratories were very promising and although it is a patented method the group showed interest and supported further development.

Part 4: Guidance documents for reporting IATA and IATA for skin sensitisation

8. Task Force on Hazard Assessment (TFHA) and skin sensitisation activities

20. Joop De Knecht (Secretariat) updated the Expert Group on the activities of the Task Force for Hazard Assessment (TFHA) related to Integrated Approaches to Testing and Assessment (IATA) and their application. He presented the scope of 2 draft documents currently under development and discussion by the TFHA: a Guidance Document on the reporting of IATA and a Guidance Document on the reporting of structured approaches to data integration and individual information sources used within IATA for skin sensitisation. The objective of developing these documents is to provide a consistent approach to the documentation of IATA, which is a preliminary step towards harmonisation. It was noted that a lot of strategies are currently proposed and questioned if the objective is to tend towards a final one with time. It was indicated that the objective is to provide tools for assessment but today it is hardly possible to say if one strategy is better than the other.

平成 27 年度厚生労働科学研究補助金（化学物質リスク事業）

分担研究報告書

データシートでの毒性判定結果提示のための平均値の比の95％信頼区間の計算の検討

分担研究者：大森崇

研究要旨

[*背景と目的*]　化学物質免疫毒性評価系として modified MITA が構築されつつある。modified MITA のバリデーション研究を行うにあたり、プロトコルに適したデータシートを作成する必要がある。試験施設の実験者がデータシートにデータを入力した段階で判定結果を知る必要が望ましい。毒性判定結果を平均値の比の 95%信頼区間を用いる場合、Excel の関数である t.inv 関数ではうまく結果を返すことができないことがわかっている。本研究では、t.inv 関数を用いずに山内の近似式として知られる近似式によって適切に平均値の比の 95%信頼区間を得ることができるかどうかを検討した。

[*方法*]　これまでに実施された計 4168 の実データを用い、山内の近似式による 97.5%点と比の 95%信頼区間の下限の値を統計解析ソフト R で算出したこれらの値と比較することを行った。

[*結果*]　検討に用いた実データの小数自由度が 3~6 の範囲であった。この範囲においてパーセント点も 95%区間の下限も R と山内の近似のどちらもほぼ同様な値を取っていることがわかった。

[*結論*]　Excel によるデータシートで平均値の比の 95%信頼区間を算出する際には、Excel の t 分布のパーセント点を計算する関数である t.inv 関数を用いるのではなく、山内の近似式である(1)式によってパーセント点を計算し、平均値の比の 95%信頼区間を得ればよい。

A. *研究目的*

Multi-ImmunoToxicity assay (MITA)は化学物質免疫毒性評価系として開発された試験法である。現在この試験法の改良がなされ IL-8 Luc assay を加えた modified MITA が構築されている。今後国内外から免疫毒性の専門家を招き、MITA の科学的意義、作成した adverse outcome pathway ならびに試験法プロトコルの妥当性などについて議論する予定である。本研究班では、modified MITA を用いた IL-2 転写活性抑制を指標とした T 細胞の分化異常誘導化学物質評価系と、IL-8 転写活性増強を指標とした気道刺激性物質評価系による試験法ガイドラインをめざしており、その目的のために多施設のバリデーション研究を計画する必要がある。

多施設バリデーション研究では、施設内/施設間再現性の評価ならびに関連性の評価が必要となる。その際、各試験実施施設に被験物質が送付され、これらの物質を用いて提案されたプロトコルに基づき実施された試験の結果が試験法のプロトコルに沿って作成されたデータシートに入力される。最終的な施設内/施設間再現性や関連性の評価は、

入力されたデータは解析施設に集められ、作成されたデータセットの下で行われる。

ところで提案される試験法は複数の繰り返し実験の結果で判定されることが多い。modified MITA においてもこの試験法を構成する一つである IL-8 Luc assay は複数の実験結果によって毒性の判定が行われる。IL-8 Luc assay は 4 回の実験結果の中で 2 回の陽性結果が得られれば毒性ありと判定することとなっている。このことはすべての被験物質について 4 回の実験を行うことをしなくても最低 2 回の実験結果で毒性を判定できる場合があることを意味する。必要最小限の実験でバリデーション研究を行うことができるならばコスト削減や期間短縮が期待できる。しかし、そのためには試験実施施設における実験実施者が、各実験の実験データをデータシートに入力した段階で実験結果が陽性であるのか陰性であるのかを知る必要がある。つまり、入力したデータシートは実験結果が提示されるように設計する必要がある。

データシートは多施設で試験を行うどのような施設でも簡便に扱うことができ、後の試験法の普及を考えると特別なソフトウエアを必要としないことが望ましいであろう。我々は Microsoft 社の Excel が広く普及している現状を考慮し、Excel を用いたデータシートを作成してきた。modified MITA の場合もその予定である。

毒性試験法はある被験物質について複数の濃度とその反応の関係で毒性を評価することが多い。ある 1 回の実験において、被験物質がの濃度が 0 である反応の測定値の平均値と特定の濃度の反応の測定値の平均値の比の大きさで陽性/陰性の判断を行うことが多く、modified MITA の場合も例外ではない。2 つの平均値の比の値に基づく判定では、陽性か陰性かの判定を行うための基準値の大きさは試験系が開発される過程で決められることが多い。一方で、比を算出する際に用いた個々の測定値のばらつきを考慮して、統計的な差によって判定することも可能であり、これは 95%信頼区間を用いることで行うことができる。

平均値の比の 95%信頼区間には、t 分布の 97.5%点が必要となる。[1] t 分布の 97.5%値は自由度と呼ばれるパラメータの関数であるため、自由度を与える必要がある。平均値の比の場合、比を構成するそれぞれの平均値を得るために用いたデータで得られるそれぞれの分散が等しいと仮定を置く場合には、自由度は整数となるが、そのような仮定ができない場合は、小数自由度を求めて使うことなる。[2] 毒性試験の場合、被験物質の濃度が 0 の測定値のばらつきは、特定での測定値のばらつきより小さくなることが観察されることが多いため、分散が等しいという仮定を置くことは避けることが望ましいであろう。つまり、小数自由度を用いた t 分布の 97.5%点が必要となる。ところが、Excel に組み込まれている t 分のパーセント点を計算する関数である t.inv 関数は、小数自由度を適切に反映しておらず、小数の値を入力しても整数に切り上げた自由度でのパーセント点を返すものになっている。先に記述したよう、データシートは Excel を用いることが望ましいとなると、小数自由度に対応した平均値の比の 95%信頼区間をシート上で計算できる必要がある。通常の統計ソフトに導入されているパーセント点の計算方法は公開されていないため知ることができない。一方、t 分布のパーセント点の近似式として山内の近似式として知られる方法がある。[3] 山内の近似式は Excel に組み込むことができる程度に簡便な近似式である。

そこで、本研究ではすでに研究結果が得られている modified MITA を構成する一つの試験法である IL-8 Luc assay のデータを用いて、山内の近似式により算出される 97.5%点と 95%信頼区間の下限について、統計解析ソフトである R で計算され

る結果の比較を行い、山内の近似式のデータシートへの適用可能性を検討することを目的とした。

B. *研究方法*

B.1. *検討に用いたデータ*

これまでに IL-8 Luc assay は、バリデーション研究を通して得られた 379 実験分データを用いた。この試験は 0 濃度と 11 の濃度の試験からなるため、計 4168 のパーセントが得られることになる。

B.2. *山内の近似式*

自由度 ν の t 分布の 97.5%点を $t_{0.975}(\nu)$ とすると、山内の近似式は $t_{0.975}(\nu)$ を

$$t_{0.975}(\nu) \approx u + \frac{y_1(u)}{\nu} + \frac{y_2(u)}{\nu^2} + \cdots + \frac{y_5(u)}{\nu^5} \qquad (1)$$

で近似する。ただし $y_1(u) = (u^3 + u)/4$,

$y_2(u) = (5u^5 + 16u^3 + 3u)/96$,

$y_3(u) = (3u^7 + 19u^5 + 17u^3 - 15u)/384$,

$y_4(u) = (79u^9 + 776u^7 + 1482u^5 - 1920u^3 - 945u)/92160$,

$y_5(u) = (27u^{11} + 339u^9 + 930u^7 - 1782u^5 - 756u^3 + 17955u)/368640$,

$u = 1.96$ である。[3)]

B.3. *パーセント点と 95%信頼区間の下限の比較*

1468 の比のデータについて、上記の式で得られる山内の近似式により算出される 97.5%点の値と 95%信頼区間の下限の値を、統計解析ソフトである R で計算されるそれぞれの値と比較した。

C. *研究結果*

C.1. *小数自由度の要約*

検討に用いた 1468 の比のデータについての要約統計量を表 1 に示す。

表 1　小数自由度の要約統計量

サイズ(n)	平均値	標準偏差	最小値	中央値	最大値
4169	4.49	1.12	3.00	4.58	6.00

自由度の範囲は 3～6 の範囲にあることがわかる。

C.2. *97.5%点の比較結果*

検討に用いた 1468 の比のデータについて R により得られる 97.5%を横軸に、山内の近似式により得られる 97.5%を縦軸にとった散布図を図 1 に示す。

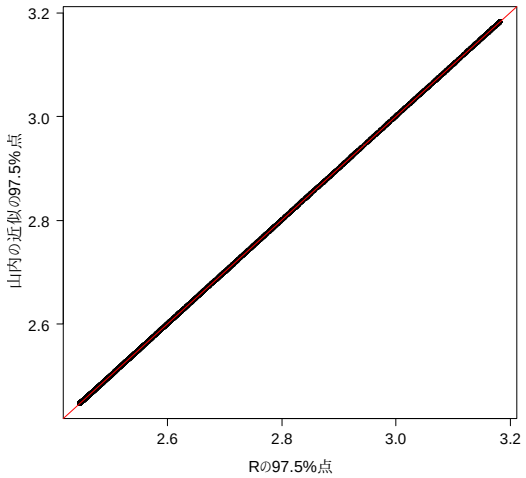


図 1　R と山内の近似の 97.5%点

C.3. *95%信頼区間の下限の比較結果*

検討に用いた 1468 の比のデータについて R により得られる 95%信頼区間の下限を横軸に、山内の近似式により得られる 95%信頼区間の下限を縦軸にとった散布図を図 2 に示す。図において点線で示した参照線は、それぞれ 1 の値のところであり、これは 95%信頼区間の下限を用いたときの統計的有意差の基準となる値である。

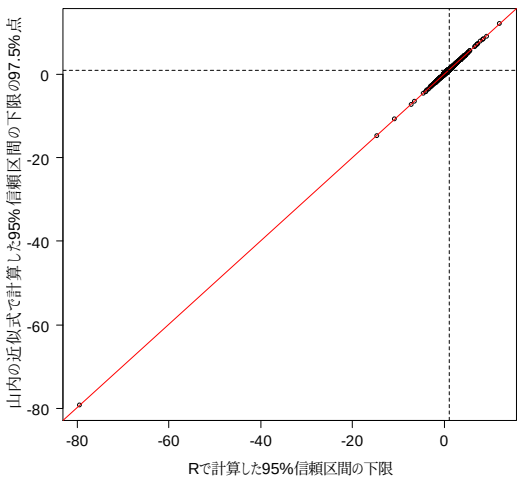


図 2　R と山内の近似の 95%信頼区間の下限

D. *考察*

表 1 では 1468 の比のデータの自由度の中央値は 4.58 である。この値を用いて Excel2010 の t.inv 関数で、97.5%点を計算するとエラーもなく 2.776445 という値が返される。しかし、この値は自由度を 4 として t.inv 関数で計算しても同じ値である 2.776445 が返されてしまう。一方、R のパーセント点を計算する関数 qt を用いた場合、自由度が 4.58 の場合には 2.643129 が、自由度が 4 の場合には 2.776445 が返され、小数自由度に対して適切な値を返していることがわかる。

図 1 よりパーセント点は R と山内の近似のどちらもほぼ同様な値を取っていることがわかる。また、図 2 より 95%区間の下限も R と山内の近似のどちらもほぼ同様な値を取っていることがわかる。よって、山内の近似式は統計解析ソフト R を用いて解析するのと同様の結果を得ることができることがわかった。

よって、Excel でデータシートを構築する場合には、Excel の t 分布のパーセント点を計算関数である t.inv 関数を用いるのではなく、山内の近似式である(1)式によって計算し、比の 95%信頼区間を得ればよい。この結果を今後 modified MITA のバリデーション研究で行う際に作成するデータシートを反映させることにする。

Excel は広く普及しているソフトウエアである。R はフリーのソフトウエアであるという利点があるものの、実験実施者にとって統計ソフトは馴染みがあるソフトウエアではないため、R での解析を実験実施者に求めるべきではないであろう。また、Excel の t.inv 関数の問題は Excel のバージョンが更新されることで問題は解消されるかもしれない。しかしながら、どのような実験施設も常に最新の Excel を有し、利用しているとは考えにくい。

E. *結論*

以上より、Excel によるデータシートで平均値の比の 95%信頼区間を算出する際には、Excel の t 分布のパーセント点を計算する関数である t.inv 関数を用いるのではなく、山内の近似式である(1)式によってパーセント点を計算し、平均値の比の 95%信頼区間を得ればよい。

謝辞：本検討を進めるにあたりデータの整理を協力してくれた同志社大学文化情報学部の丸谷あおいさんに感謝いたします。

F. *健康危険情報*

なし。

G. *研究発表*

なし。

H. *知的財産権の出願・登録状況*

なし。

参考文献

1) Fieller, E. C. (1944). A fundamental formula in the statistics of biological assays and some applications. *Quarterly Journal of Pharmacy and Pharmacology*, **17**, 117–123.

2) **永田靖.統計的方法のしくみ-正しく理解するための 30 の急所-.日科技連出版社，1996.**

3) **芳賀敏郎、橋本茂司. 統計解析プログラムの基礎. 日科技連出版社，1980.**

厚生労働科学研究費補助金（化学リスク研究事業）
免疫毒性評価試験法Multi-ImmunoTox assayの国際validationへ向けての検討
分担研究報告書

化学物質のMITAによる解析、validation、プロトコール作成

分担研究者　木村　裕
東北大学病院皮膚科

研究要旨
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」にて開発した新たな*in vitro*免疫毒性評価試験法（Multi-ImmunoTox assay：MITA）について現時点で得られたdata setおよび相場によるIL-2転写活性抑制を中心とした免疫毒性AOPをもとに国際バリデーション用の試験法プロトコール、データシート、記録用紙を作成した。施設間試験の実施者に対し試験法の説明会を実施し技術移転を図った。国際バリデーションに先立ち技術移転性を確認するため5物質でのトレーニングを行い、プロトコールの問題点を改良しPhase I試験に臨む予定である。

キーワード：試験法プロトコール、技術移転性、バリデーション

A．研究目的

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」にて開発した新たな *in vitro* 免疫毒性評価試験法（Multi-ImmunoTox assay：MITA）のOECD（Organisation for Economic Co-operation and Development）における試験法ガイドライン（Test Guideline：TG）化を目的とし、試験法プロトコールを作成し国際バリデーションの準備を行う。

B．研究方法

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1時間培養した。つづいて25nM PMAと1μM 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

各実験において得られた結果は、一元配置分散分析を行い、その後Dunnett検定により有意な抑制効果，増強効果があるか否かを検討した。しかし、この実験を3回繰り返し検討すると、3回の実験結果が必ずしも一致していない薬剤が存在した。そこで、一致が見られなかった薬剤に関しては、3回の繰り返し実験の結果のなかから%suppressionの絶対値（免疫抑制物質に関しては正の値、増強物質に関しては負の値となる）が最も大きい値を選びStudent's t-testを行い、そこで統計的有意差の得られた場合、その結果を薬剤の最終的判定結果とした[1]。

C．結果

C-1. 試験法プロトコール、データシート、記録用紙の作成

現時点で得られたdata setおよび相場によるIL-2転写活性抑制を中心とした免疫毒性AOPを参考とし、IL-2，IFN-γレポーター細胞である#2H4細胞を用いた試験法プロトコール、Multi-Immuno Tox Assay protocol ver. 008.1Eを作成し、国際バリデーションに向け英訳した（添付文書１）。データ入力、結果表示用にエクセルファイルをベースとしたdata sheet、Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 006を作成した（添付文書２）。さらに参加施設用の記録用紙を作成し各施設に配布した（添付文書３）。

C-2. 試験法の説明会を実施

初めて MITA を行う参加施設の実施者を対象とし当研究室にて説明会を 2015 年 8 月と 2016 年 2 月の計２回開催した。当研究室において参加者の手技により PMA/Io に対する#2H4 細胞の反応および陽性コントロール化学物質による抑制が認められることを確認した（図１）。

C-3. Phase 0 試験の実施

MITA の国際バリデーション Phase I 試験に先立ち技術移転性を確認するため Phase 0 試験用に以下の化学物質を参加 3 施設に送付した。(2-Aminoanthracene, CH_3HgCl, Chloroquine diphosphate salt, Citral, Dexamethasone) 現在、これらの化学物質を３回ずつアッセイする Phase 0 試験を施行中である。

D. 考察

現時点でのクライテリアでは、低濃度で亢進し、高濃度で抑制が見られる化学物質については最終的な結果がばらつくことが予測される。今後Phase 0の結果を参照としPhase Iへ向けたクライテリアの改変を検討する。

E．結論

国際バリデーション用の試験法プロトコール、データシート、記録用紙を作成した。施設間試験の実施者に対し試験法の説明会を実施し技術移転を図った。国際バリデーションに先立ち技術移転性を確認するため5物質でのトレーニングを行い、プロトコールの問題点を改良しPhase I試験に臨む予定である。

引用文献

1. Kimura, Y., Fujimura, C., Ito, Y., Takahashi,

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

F. **添付文書**

1) Multi-Immuno Tox Assay protocol ver. 008.1E

2) Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 006

3）Multi-ImmunoTox Assay **記録用紙** Ver. 001

G. 研究発表

1. 論文発表

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

２．学会発表

1. ○Kimura, Y., Shimada-Omori, R., Takahashi, T., Tsuchiyama, K., Kusakari, Y., Yamasaki, K., Aiba, S. An interleukin-8 reporter cell line, THP-G8, can evaluate anti-TNF-α neutralizing activity of patients' sera and predict drug effectiveness during anti-TNF-α antibody therapy. 23rd World Congress of Dermatology, (2015, 6) (Vancouver, Canada)

2. ○木村裕：IL-8 Luc assayバリデーション試験．日本動物実験代替法学会　第28回大会（横浜）2015年12月

H．知的財産権の出願・登録状況（予定を含む。）

1. 特許取得

なし

2. 実用新案登録

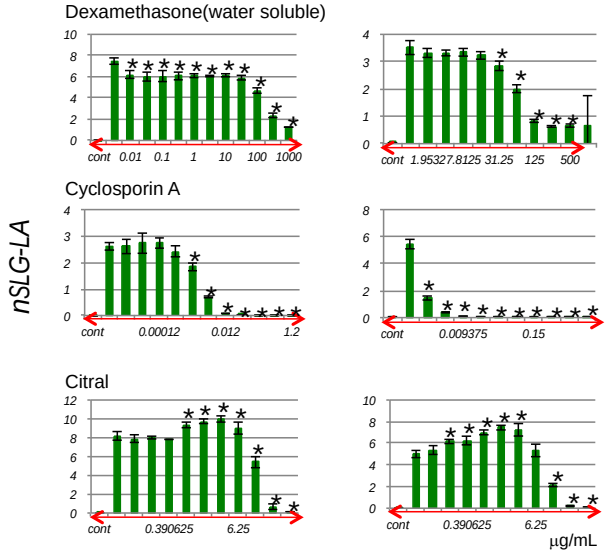
なし

3.その他

なし

図１　説明会データ

東北大学データ　　説明会参加者データ



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

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

1. Introduction

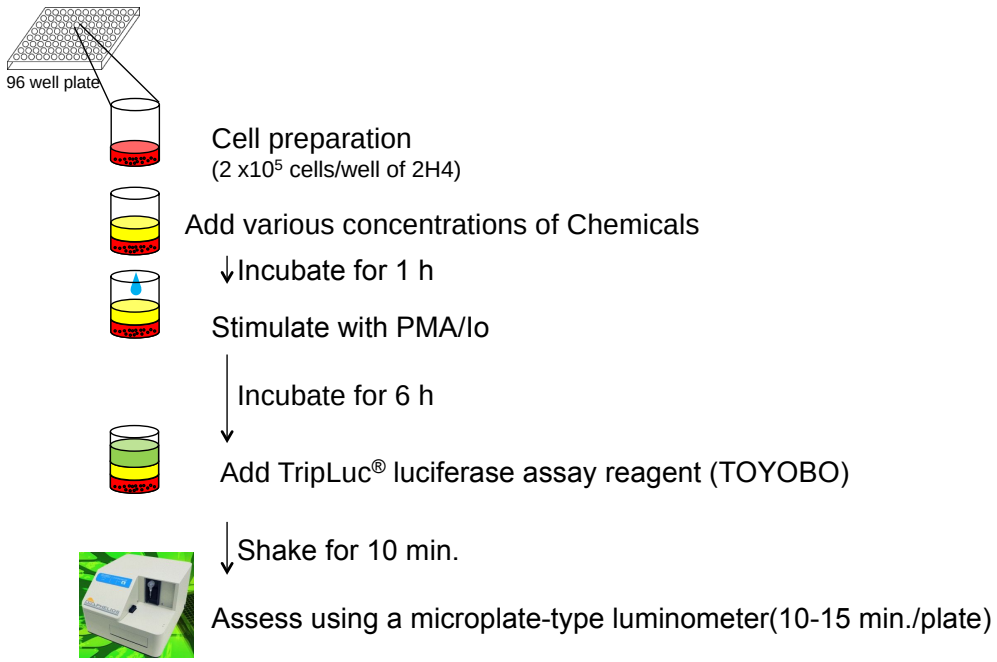
This protocol describes how to maintain the cells, how to prepare the test chemicals, and how to measure the luciferase activity of #2H4 cells transfected with 3 luciferase genes, stable luciferase green (SLG), stable luciferase orange (SLO) and stable luciferase red (SLR), under the control of IL-2, IFNγ□□□□□G3PDH promoters, respectively, for the Multi-Immuno Tox Assay.
(Kimura Y. et al. Evaluation of the Multi-ImmunoTox Assay composed of 3 human cytokine reporter cells by examining immunological effects of drugs *Toxicol in Vitro*, 28, 759-768, 2014)

Figure 1

Assay design (2 chemicals per one plate)

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	cont (distilled water or DMSO)	PMA/Io only	A/2^9 μg/ml	A/2^8 μg/ml	A/2^7 μg/ml	A/2^6 μg/ml	A/2^5 μg/ml	A/2^4 μg/ml	A/2^3 μg/ml	A/2^2 μg/ml	A/2^1 μg/ml	A μg/ml
B												
C												
D			Chemical A (common ratio of 2、10 concentrations、n=4)									
E	cont (distilled water or DMSO)	PMA/Io only	B/2^9 μg/ml	B/2^8 μg/ml	B/2^7 μg/ml	B/2^6 μg/ml	B/2^5 μg/ml	B/2^4 μg/ml	B/2^3 μg/ml	B/2^2 μg/ml	B/2^1 μg/ml	B μg/ml
F												
G												
H			Chemical B (common ratio of 2、10 concentrations、n=4)									

PMA/Io or LPS



2. Materials

2-1 Cells

- #2H4 (IL2-SLG、IFNγ-SLO、G3PDH-SLR)

  The human acute T lymphoblastic leukemia cell line Jurkat was obtained from the American Type Culture Collection (Manassas, VA, USA). A Jurkat-derived IL-2 and IFNγ reporter cell line, #2H4, that harbors the SLG, SLO and SLR luciferase genes under the control of the IL-2, IFNγ and GAPDH promoters, respectively, was established by Tsuruga Institute of Biotechnology, TOYOBO Co. Ltd.

  (Saito R. et al. Nickel differentially regulates NFAT and NF-κB activation in T cell signaling *Toxicology and Applied Pharmacology,* 254, 245–255, 2011)

2-2 Reagents and equipment

2-2-1 For maintenance of the #2H4 cells

- RPMI-1640 (GIBCO Cat#11875-093, 500 mL)
- FBS (Biological Industries Cat#04-001-1E Lot: 715004)
- Antibiotic-Antimycotic (GIBCO Cat#15240-062)
- HygromycinB (CAS:31282-04-9, Invitrogen Cat#10687-010)
- G418 (CAS:108321-42-2, Nacalai Tesque Cat#16513-84)
- Puromycin (CAS:58-58-2, InvivoGen Cat#ant-pr-1)

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

- Ionomycin (CAS:56092-82-1, Sigma Cat#I0634)
- Phorbol 12-myristate 13-acetate (PMA) (CAS:16561-29-8, Sigma Cat#P8139)
- Ethanol (e.g., Wako Cat#057-00456)
- Dimethyl sulfoxide (DMSO) (CAS:67-68-5, Sigma Cat#D5879)
- Distilled water (GIBCO Cat#10977-015)

2-2-3 For measurement of the luciferase activity

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

2-2-4 Expendable supplies

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

2-2-5 Equipment for measurement of luciferase activity

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

2-2-6 Others

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

2-3 Culture medium

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

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	440 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	50 mL
Antibiotic-Antimycotic	GIBCO #15240-062	100×	1×	5 mL
Puromycin	InvivoGen # ant-pr-1	10 mg/mL	0.15 μg/mL	7.5 μL
G418	Nacalai tesque #16513-84	50 mg/mL	300 μg/mL	3 mL
HygromycinB	Invitrogen #10687-010	50 mg/mL	200 μg/mL	2 mL

2-3-2 B medium: for luciferase assay (30 mL, stored at 2-8°C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	27 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	3 mL

2-3-3 C medium: for thawing #2H4 cells (30 mL, stored at 2-8°C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	26.7 mL
FBS	Biological Industries Cat#04-001-1E Lot: 715004	-	10 %	3 mL
Antibiotic-Antimycotic	GIBCO #15240-062	100×	1×	0.3 mL

2-4 Preparation of the stimulant of #2H4

2-4-1 Phorbol 12-myristate 13-acetate (PMA)

Reagent	Company	Concentration of the stock solution	Final concentration
Phorbol 12-myristate 13-acetate (PMA)	Sigma #P8139	1 mM	25 nM
DMSO	Sigma #D5789		

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

2-4-2 Ionomycin

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

Dissolve 1mg Ionomycin using ethanol 1621 μL, dispend at 30 μL/tube and store at freezer at -30°C. Use these stocks within 6 month after dissolution.

3. Cell culture

3-1 Thawing of #2H4 cells

Pre-warm 9 mL of C medium in a 15 mL polypropylene conical tube in a 37°C water bath (for centrifugation) and 15 mL of C medium in a T-75 Flask at 37°C in a 5% CO_2 incubator (for culture).

Thaw frozen cells ($2x10^6$ cells / 0.5 mL of freezing medium) in a 37°C water bath, then add to a 15 mL polypropylene conical tube containing 9 mL of pre-warmed C medium. Centrifuge the tube at 350 x *g* at room temperature for 5 min, discard the supernatant, and resuspend in 15 mL of pre-warmed C medium in a T-75 Flask. Cells are incubated at 37°C, 5% CO_2.

3-2 Maintenance of #2H4 cells

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

The interval between subcultures should be 3~4 days. Cells can be used between one and six weeks after thawing.

4. Preparation of cells for assay

A cell passage should be done 2-4 days before the assay.
Use cells between 1 and 6 weeks after thawing.

Pre-warm the B medium in a 37°C water bath. Count the number of cells and collect the number of cells needed (2.0 x 10^7 cells for two chemical are required, but to have some leeway, 3.0 x 10^7 cells for two chemical should be prepared), centrifuge the tube at 350 x *g*, 5 min. Resuspend in pre-warmed the B medium at a cell density of 4×10^6/mL. Transfer the cell suspension to a reservoir, and add 50 µL of cell suspension to each well of a 96 well µclear black plate (flat bottom) using an 8 channel or 12 channel pipetman. (cf. Figure 2)

Figure 2

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
B	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
C	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
D	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
E	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
F	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
G	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
H	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL

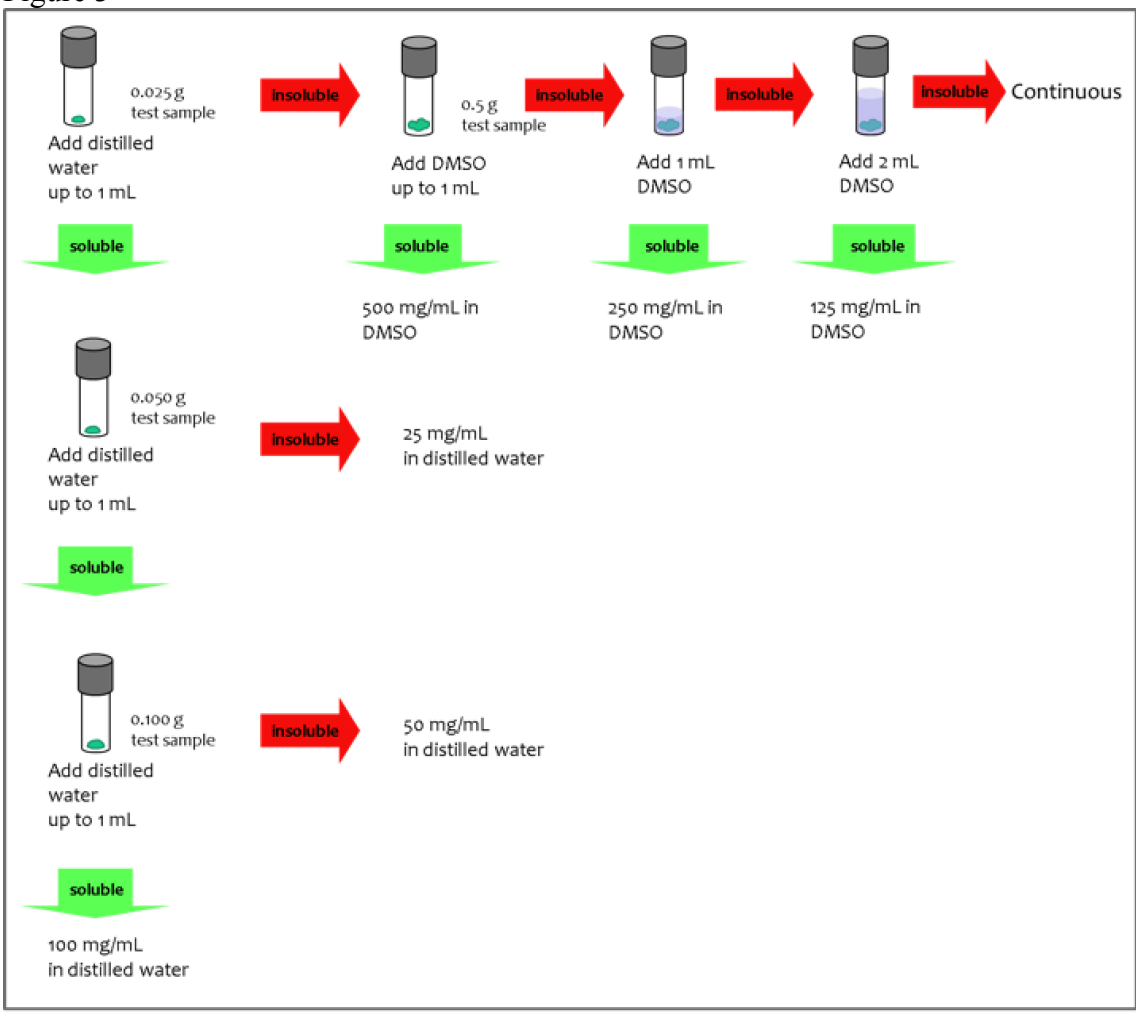
5. Preparation of chemicals and cell treatment with chemicals

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

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

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

Figure 3


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

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

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

5-2-1 Arrangement of chemicals and vehicle

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

5-2-2 Serial dilution

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

Figure 4

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Chemical 100 mg/mL in distiled water 100 uL
B												
C												
D												
E												
F												
G												
H												

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

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

5-2-3 2 step dilution

Add 20 µL of the diluted chemical to 480 µL of the B medium prepared in the assay block. And add 50 µL to #2H4 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Shake the plate with a plateshaker, and incubate in a CO_2 incubator for 1 hour (37°C, 5%)(cf. Figure 5-7).

Figure 5

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

20uL

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

Figure 6

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

50uL

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
B	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
C	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
D	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
E	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
F	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
G	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
H	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL

Figure 7 Final constituents of each well of the plate

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	Chemical 0 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.004 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.008 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.02 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.03 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.06 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.1 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.3 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.5 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 1 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 2 mg/mL #2H4 2x10^5 B medium 100uL
B	Chemical 0 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.004 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.008 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.02 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.03 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.06 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.1 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.3 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.5 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 1 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 2 mg/mL #2H4 2x10^5 B medium 100uL
C	Chemical 0 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.004 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.008 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.02 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.03 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.06 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.1 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.3 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.5 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 1 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 2 mg/mL #2H4 2x10^5 B medium 100uL
D	Chemical 0 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.004 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.008 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.02 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.03 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.06 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.1 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.3 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 0.5 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 1 mg/mL #2H4 2x10^5 B medium 100uL	Chemical 2 mg/mL #2H4 2x10^5 B medium 100uL
E	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
F	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
G	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
H	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL

5-3 When the chemical is prepared as a 500 mg/mL DMSO solution

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

5-3-1 Arrangement of chemicals and vehicle

Add 100 µL of the 500 mg/mL DMSO solution of the chemical to well #A12, 50 µL of DMSO to wells #A1-#A11, and 90 µL of the B medium to wells #B1-#B12 of the 96 well clear plate (round bottom)

5-3-2 Serial dilution

Conduct 9 serial dilutions at a common ratio of 2 as indicated in Figure 8 from well #A11 to well #A3. Transfer 50 µL to the next (left) well. (cf. Figure 8)

Figure 8

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

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

↓

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

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

Dilute 10 µL of the DMSO solution of the chemical in wells #A1-#A12 with 90 µL of the B medium using an 8-12 channel pipetman. (cf. Figure 9)

Figure 9

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

10uL

↓

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

5-3-4 2 step dilution

Add 10 µL of the diluted chemical to 490 µL of the B medium prepared in the assay block. And add 50 µL to #2H4 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Manipulate the procedures from 5-3-3 to 5-3-4 as quickly as you can, and do not leave a long time at step after 5-3-3 or Figure 10. Shake the plate with a plateshaker and incubate in a CO_2 incubator for 1 hour (37°C, 5%) (cf. Figure 10-12).

Figure 10

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

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 black	1	2	3	4	5	6	7	8	9	10	11	12
A	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
B	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
C	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
D	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
E	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
F	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
G	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
H	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL

Figure 12 Final constituents of each well of the plate

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	Chemical 0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 1.0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 2.0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 3.9ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 7.8ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 16ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 31ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 63ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 125ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 250ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 500ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL
B	Chemical 0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 1.0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 2.0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 3.9ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 7.8ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 16ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 31ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 63ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 125ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 250ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 500ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL
C	Chemical 0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 1.0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 2.0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 3.9ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 7.8ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 16ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 31ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 63ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 125ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 250ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 500ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL
D	Chemical 0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 1.0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 2.0ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 3.9ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 7.8ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 16ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 31ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 63ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 125ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 250ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL	Chemical 500ug/mL 0.1% DMSO #2H4 2x10^5 B medium 100uL
E	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
F	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
G	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL
H	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL

6. Preparation of the stimulant (PMA/ionomycin) and addition to #2H4

6-1 Material

- 1 mM PMA stock
- 1 mM Ionomycin stock
- B medium
- Ethanol

6-2 Preparation of 100 μM PMA

Dilute 1mM PMA stock with the B medium as follows (10 times, final concentration is 100 μM).

1 mM PMA	B medium	Total	final concentration
10 μL	90 μL	100 μL	100 μM

6-3 Preparation of control and x10 PMA/ionomycin solution

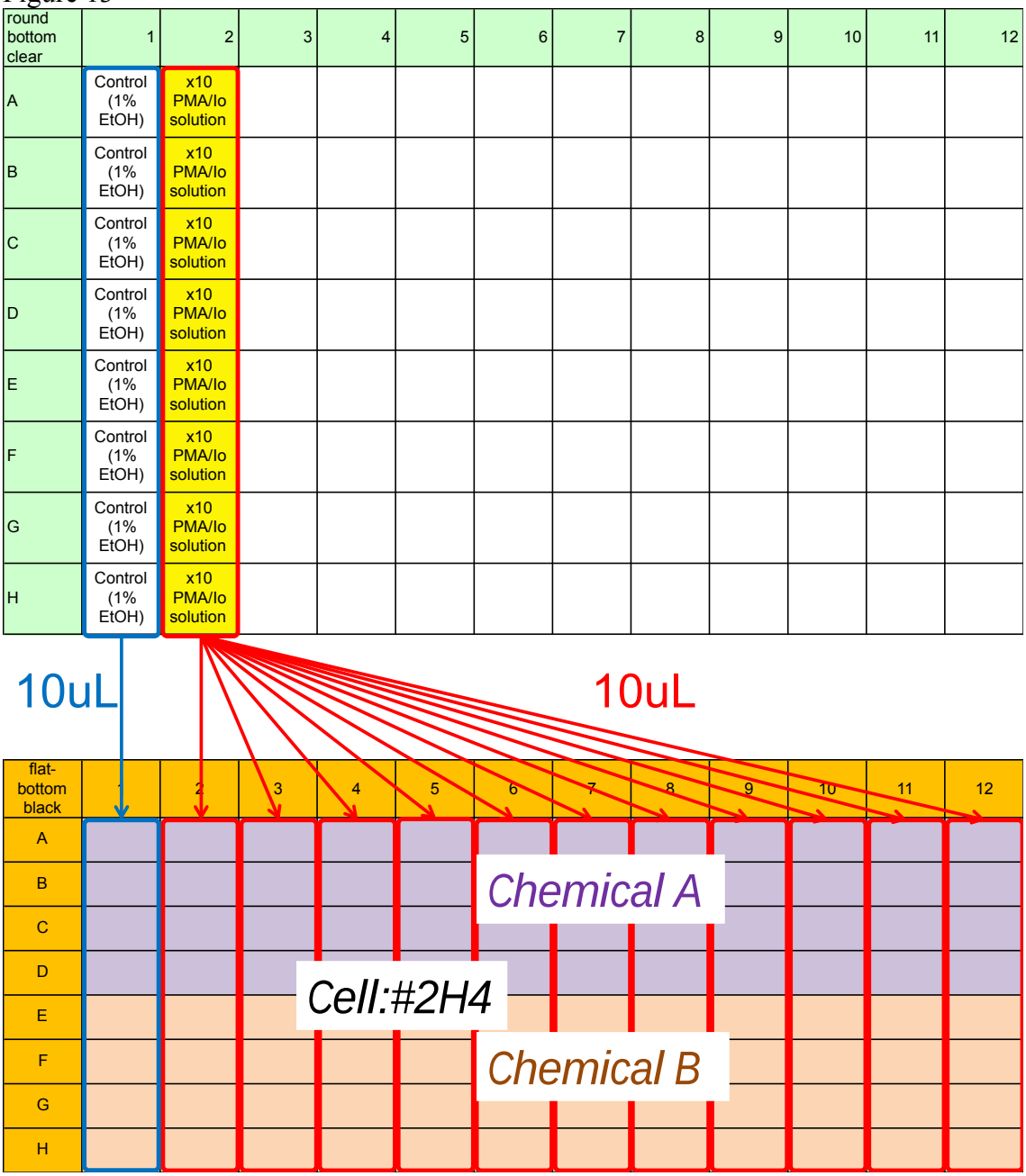
Dilute ethanol, 1mM ionomycin and 100 μM PMA with the B medium to prepare control or x10 PMA/ionomycin solution. Add the control to well #A1-#H1 of the 96 well clear plate (round bottom), and add x10 PMA/ionomycin solution to wells #A2-#H2 of the 96 well clear plate (round bottom).

	B medium	1 mM Ionomycin	100 μM PMA	Ethanol	Total
Control	990 μL	-		10 μL	1000 μL
x10 PMA/ionomycin solution	2370 μL	24 μL	6 μL	-	2400 μL

6-4 Addition of PMA/ionomycin to #2H4

One hour after the addition of chemicals, add 10 µL of control or PMA/ionomycin solution to the cells (#A1-#H1 or #A2-#H12, 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. Shake the plate with a plateshaker and incubate in a CO_2 incubator for 6 hour (37°C, 5%). (cf. Figure 13)

Figure 13



7. Control

7-1 Preparing control chemical (dexamethasone, cyclosporine A)

7-1-1 Preparing dexamethasone stock

Reagent	Company	Concentration of the stock solution	Preparing concentration	Final concentration
Dexamethasone-water soluble	Sigma #D2915-100MG	50 mg/mL	50 mg/mL	1 mg/mL
Distilled water	GIBCO Cat#10977-015			

Dissolve 100 mg of Dexamethasone-water soluble with distilled water 2000 μL, dispend at 50 μL/tube and store a freezer at -30°C.

7-1-2 Preparing cyclosporine A stock

Reagent	Company	Concentration of the stock solution	Preparing concentration	Final concentration
Cyclosporine A	Sigma #C1832-5MG	12 mg/mL	1 mg/mL	1 μg/mL
DMSO	Sigma #D5789			

Dissolve 5 mg of cyclosporine A with DMSO 416 μL, dispend at 10 μL/tube and store a 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 (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 350 x *g*, 5 min. Resuspend in pre-warmed the B medium at a cell density of 4×10^6/mL. Transfer the cell suspension to a reservoir, and add 50 µL of cell suspension to each well of a 96 well µclear black plate (flat bottom) using an 8 channel or 12 channel pipetman. (cf. Figure 14)

Figure 14

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
B	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
C	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
D	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
E												
F												
G												
H												

7-3 Arrangement of chemicals and vehicle

Add DMSO 50 µL to #A4、12 mg/mL cyclosporine A stock 10 µl + DMSO 110 µL to #A5, distilled water 50 µl to #B1 and #B2, 50 mg/ml dexamethasone stock 50 µl to #B3 and the B medium 180 µl to #B4 and #B5 of the 96 well clear plate (round bottom). (cf. Figure 15)

7-4 Dilution with the B medium

Dilute DMSO in #A4 and cyclosporine A DMSO solution in #A5 by adding 20 µL to the B medium in #B4 and #B5, respectively. (cf. Figure 15)

Figure 15

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A				DMSO 50uL	CyA 12 mg/mL stock 10uL + DMSO 110uL							
B	Distilled water 50uL	Distilled water 50uL	DEX 50 mg/mL stock 50uL	B medium 180uL	B medium 180uL							
C												
D												
E												
F												
G												
H												

20uL

↓

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A				DMSO 30uL	CyA 1 mg/mL in DMSO 100uL							
B	Distilled water 50uL	Distilled water 50uL	DEX 50 mg/mL stock 50uL	DMSO 10% in B medium 200uL	CyA 100ug/mL DMSO 10% in B medium 200 uL							
C												
D												
E												
F												
G												
H												

7-5　　2 step dilution

Add 20 µL of the diluted chemical or vehicle to 480 µL(1-3 lanes) or 980 µL(4-5 lanes) of the B medium prepared in the assay block. And add 50 µL to #2H4 in a 96 well plate using an 8 channel or 12 channel pipetman after pipetting 20 times. Manipulate the procedures from 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. Shake the plate with a plateshaker and incubate in a CO_2 incubator for 1 hour (37°C, 5%). (cf. Figure 16-18)

Figure 16

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A				DMSO 30uL	CyA 1 mg/mL in DMSO 100uL							
B	Distilled water 50uL	Distilled water 50uL	DEX 50 mg/mL stock 50uL	DMSO 10% in B medium 200uL	CyA 100ug/mL DMSO 10% in B medium 200 uL							
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 980uL	B medium 980uL							
B												
C												
D												
E												
F												
G												
H												

Figure 17

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	B medium 500uL	B medium 500uL	DEX 2mg/mL B medium 500uL	DMSO 0.2% B medium 1000uL	CyA 2ug/mL DMSO 0.2% B medium 1000uL							
B												
C												
D												
E												
F												
G												
H												

50ul

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
B	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
C	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
D	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL	#2H4 2x10^5 B medium 50uL							
E												
F												
G												
H												

Figure 18 Final constituents of each well of the plate

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell DEX 1mg/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 1ug/mL DMSO 0.1% B medium 100uL							
B	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell DEX 1mg/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 1ug/mL DMSO 0.1% B medium 100uL							
C	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell DEX 1mg/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 1ug/mL DMSO 0.1% B medium 100uL							
D	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell DEX 1mg/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 1ug/mL DMSO 0.1% B medium 100uL							
E												
F												
G												
H												

7-6 Addition of PMA/ionomycin to #2H4

One hour after the addition of dexamethasone and cyclosporine A, add 10 µL of control or PMA/ionomycin solution prepared in §6-3 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. Shake the plate with a plateshaker and incubate in a CO_2 incubator for 6 hour (37°C, 5%). (cf. Figure 19)

Figure 19

round bottom clear	1	2	3	4	5	6	7	8	9	10	11	12
A	Control (1% EtOH)	x10 PMA/Io solution										
B	Control (1% EtOH)	x10 PMA/Io solution										
C	Control (1% EtOH)	x10 PMA/Io solution										
D	Control (1% EtOH)	x10 PMA/Io solution										
E	Control (1% EtOH)	x10 PMA/Io solution										
F	Control (1% EtOH)	x10 PMA/Io solution										
G	Control (1% EtOH)	x10 PMA/Io solution										
H	Control (1% EtOH)	x10 PMA/Io solution										

10uL 10uL

flat-bottom black	1	2	3	4	5	6	7	8	9	10	11	12
A	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell DEX 1mg/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 1ug/mL DMSO 0.1% B medium 100uL							
B	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell DEX 1mg/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 1ug/mL DMSO 0.1% B medium 100uL							
C	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell DEX 1mg/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 1ug/mL DMSO 0.1% B medium 100uL							
D	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell B medium 100uL	#2H4 2x10^5 cell DEX 1mg/mL B medium 100uL	#2H4 2x10^5 cell DMSO 0.1% B medium 100uL	#2H4 2x10^5 cell CyA 1ug/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 orange (SLO)
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	Conc.	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

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 black 96 well plate (flat bottom) as shown below.

Figure 20

flat-bottom black	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	SLO 100 µL	SLO 100 µL	SLO 100 µL									
E												
F	SLR 100 µL	SLR 100 µL	SLR 100 µL									
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, F2) of the optical filters. An example of the raw output data is shown below.

Figure 22

Measurement without Filter

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	3757015	3716611	3810382									
C												
D	1202691	1210208	1122295									
E												
F	2465453	2207572	2077689									
G												
H												

Measurement with Filter 1

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	1269950	1257268	1289562									
C												
D	808550	813160	754174									
E												
F	2193723	1968240	1853873									
G												
H												

Measurement with Filter 2

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	236478	234079	240876									
C												
D	235121	235878	217432									
E												
F	1585258	1420099	1339265									
G												
H												

Six transmittance factors of the optical filters were calculated as follow:

Transmittance factor (κG_{R56})=$\frac{\#B1 \text{ of } F1+ \#B2 \text{ of } F1+ \#B3 \text{ of } F1}{\#B1 \text{ of } F0+ \#B2 \text{ of } F0+ \#B3 \text{ of } F0}$

Transmittance factor (κO_{R56})=$\frac{\#D1 \text{ of } F1+\#D2 \text{ of } F1+\#D3 \text{ of } F1}{\#D1 \text{ of } F0+\#D2 \text{ of } F0+\#D3 \text{ of } F0}$

Transmittance factor (κR_{R56})= $\frac{\#F1 \text{ of } F1+\#F2 \text{ of } F1+\#F3 \text{ of } F1}{\#F1 \text{ of } F0+\#F2 \text{ of } F0+\#F3 \text{ of } F0}$

Transmittance factor (κG_{R60})=$\frac{\#B1 \text{ of } F2+ \#B2 \text{ of } F2+ \#B3 \text{ of } F2}{\#B1 \text{ of } F0+ \#B2 \text{ of } F0+ \#B3 \text{ of } F0}$

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

Transmittance factor (κR_{R60})=$\frac{\#F1 \text{ of } F2+\#F2 \text{ of } F2+\#F3 \text{ of } F2}{\#F1 \text{ of } F0+\#F2 \text{ of } F0+\#F3 \text{ of } F0}$

In the case shown above,

Transmittance factors (κG_{R56})=$\frac{1269550+1257268+1289562}{3757015+3716611+3810382}$=0.338

Transmittance factors (κO_{R56})=$\frac{808550+813160+754174}{1202691+1210208+1122295}$=0.672

Transmittance factors (κR_{R56})=$\frac{2193723+1968240+1853873}{2465453+2207572+2077689}$=0.891

Transmittance factors (κG_{R60})= $\frac{236478+234079+240876}{3757015+3716611+3810382}$=0.06

Transmittance factors (κO_{R60})=$\frac{235121+235878+217432}{1202691+1210208+1122295}$=0.195

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

Calculated transmittance factors are used for all the measurements executed using the same **添付**

luminometer.
Input the transmittance factors to #C6-#E7 of the “Data Input” sheet of the Data sheet as follow.

Figure 23

	A	B	C	D	E	F
1	MultiReporter Assay System -Tripluc®- Calculation Sheet					
2						
3		Transmittance Data				
4			SLG	SLO	SLR	
5		F0	1	1	1	
6		F1	κG_{R56}	κO_{R56}	κR_{R56}	
7		F2	κG_{R60}	κO_{R60}	κR_{R60}	
8						

9. Measurement

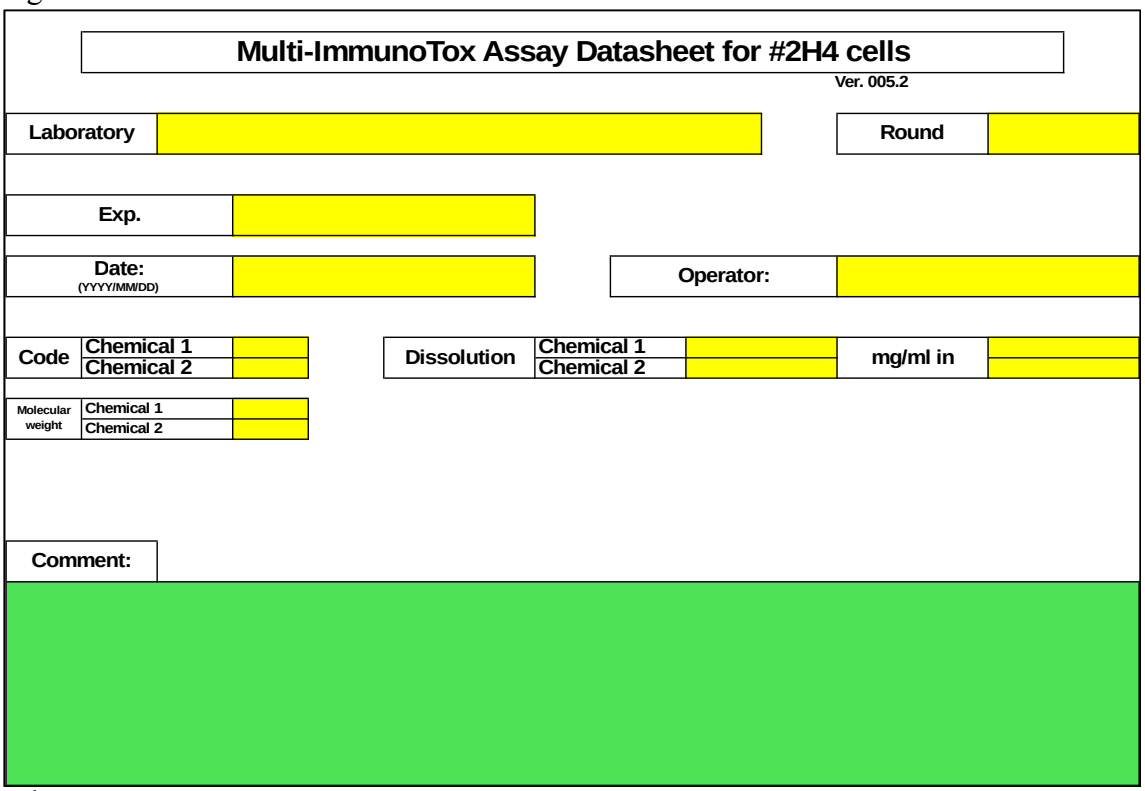
Please reffer Appendix 1 for the principal of measurement of luciferase activity.

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) on 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 of (F0) and presence (F1, F2) of the optical filters.

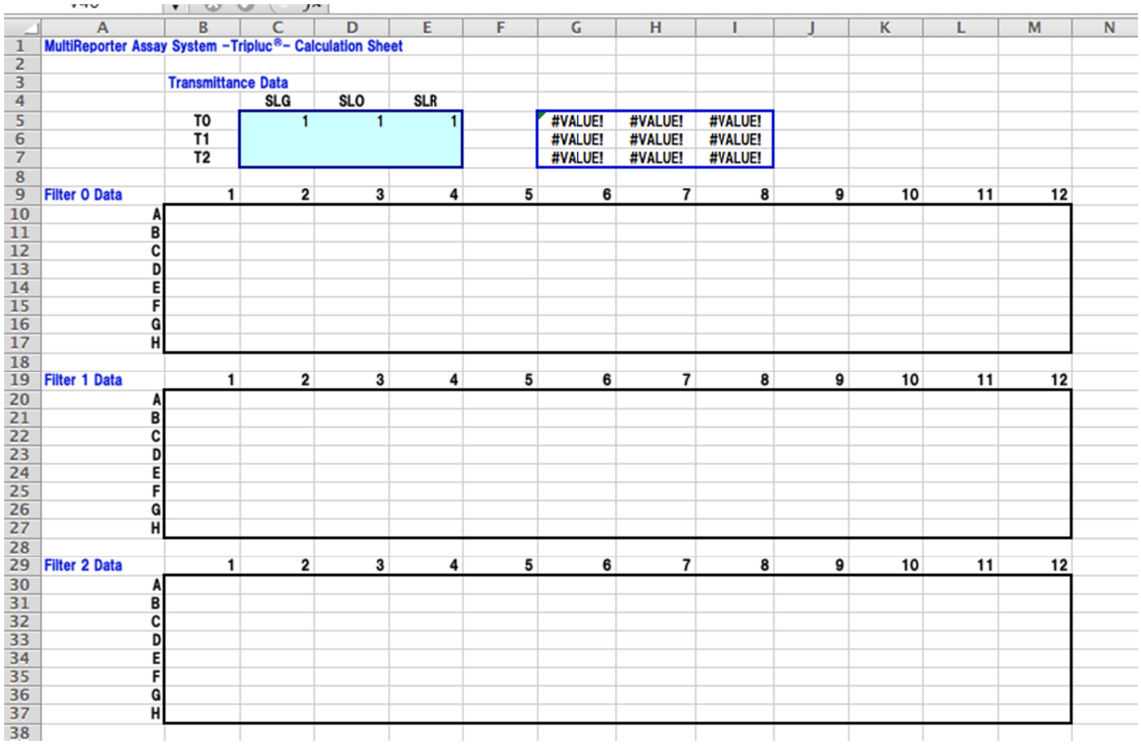
1st. Add the information regarding the name of laboratory, the round of experiments if multiple experimental sets are performed, the experiment number, date, the operator, chemical codes, dissolved in distilled water or DMSO, the prepared concentration, molecular weight of the chemicals and comments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

Figure 24 “Face Sheet” of the data sheet



2nd. Copy the results of the F0, F1 and F2 measurements (values are expressed as counts) and paste them into the appropriate area in the “Data Input” sheet of the data sheet shown below. In addition, input the transmittance factors calculated in "§5. Calculation of the transmittance factors" to #C6-#E7 of the “Data Input” sheet.

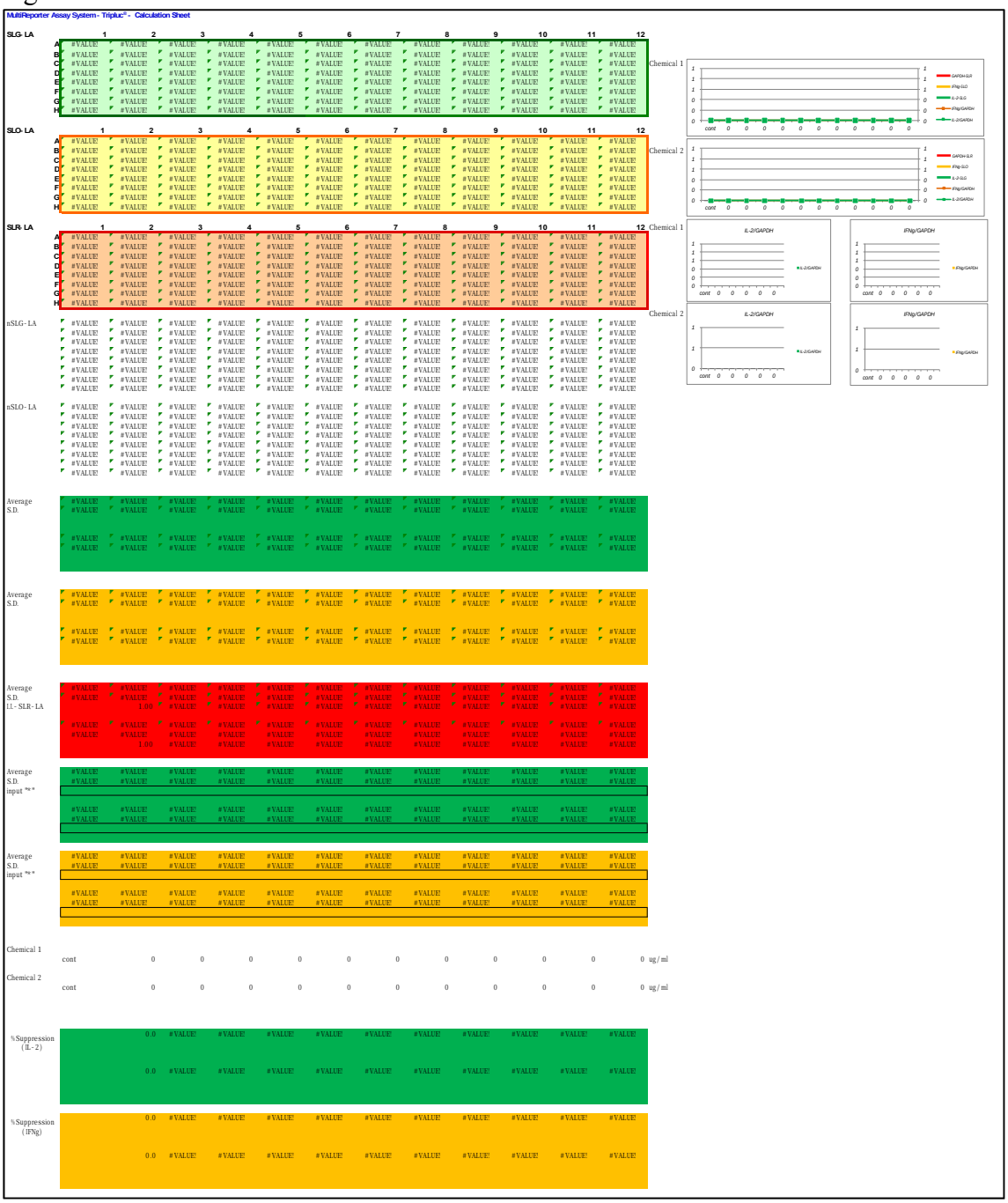
Figure 25 “Data Input” sheet of the data sheet



	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	MultiReporter Assay System –Tripluc®– Calculation Sheet													
2														
3		Transmittance Data												
4			SLG	SLO	SLR									
5		T0	1	1	1		#VALUE!	#VALUE!	#VALUE!					
6		T1					#VALUE!	#VALUE!	#VALUE!					
7		T2					#VALUE!	#VALUE!	#VALUE!					
8														
9	Filter 0 Data	1	2	3	4	5	6	7	8	9	10	11	12	
10	A													
11	B													
12	C													
13	D													
14	E													
15	F													
16	G													
17	H													
18														
19	Filter 1 Data	1	2	3	4	5	6	7	8	9	10	11	12	
20	A													
21	B													
22	C													
23	D													
24	E													
25	F													
26	G													
27	H													
28														
29	Filter 2 Data	1	2	3	4	5	6	7	8	9	10	11	12	
30	A													
31	B													
32	C													
33	D													
34	E													
35	F													
36	G													
37	H													
38														

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

Figure 26 “Result Format” sheet of the data sheet



10. Data analysis

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

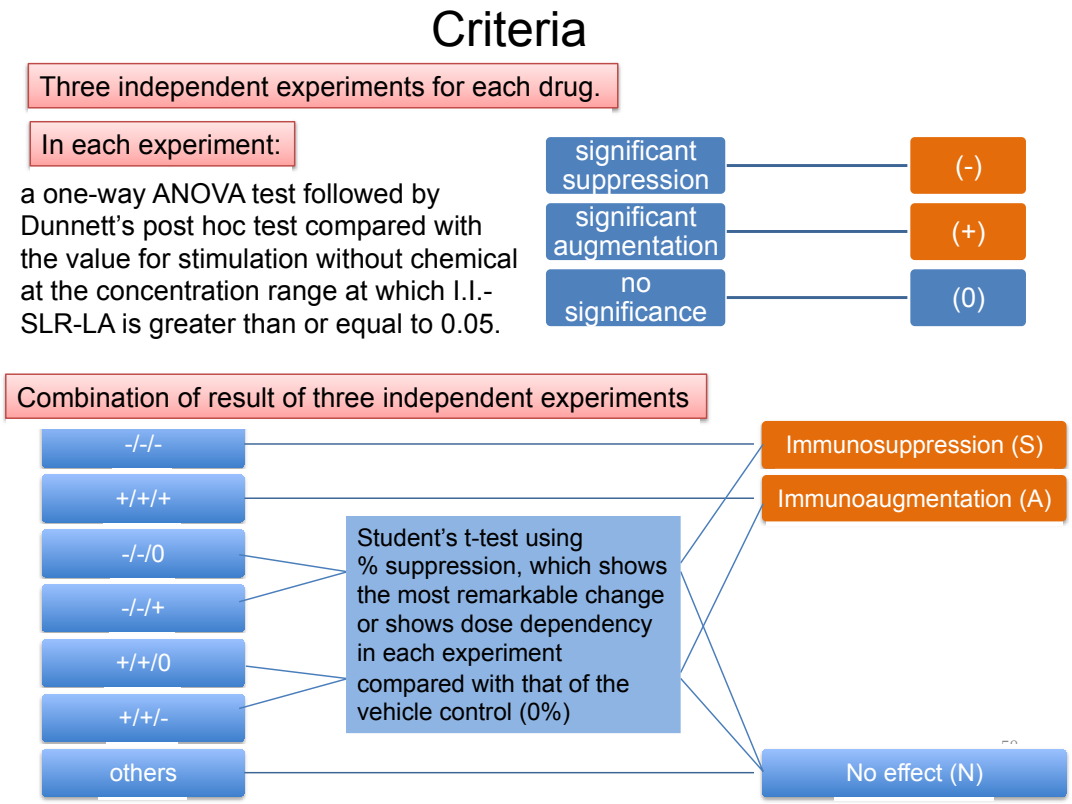
- SLG-luciferase activity (SLG-LA)：Luciferase activity of stable luciferase green (Under the control of IL-2 promoter)
- SLO-luciferase activity (SLO-LA)：Luciferase activity of stable luciferase orange (Under the control of IFN-□ promoter)
- SLR-luciferase activity (SLR-LA)：Luciferase activity of stable luciferase red (Under the control of G3PDH promoter)
- Normalized SLG-LA (nSLG-LA)：=(SLG-LA)/(SLR-LA)
- Normalized SLO-LA (nSLO-LA)：=(SLO-LA)/(SLR-LA)
- Inhibition index of SLR-LA (I.I.-SLR-LA)：The cytotoxic effect of chemicals
  =(SLR-LA of #2H4 treated with chemicals)/(SLR-LA of untreated #2H4)
- % suppression：The effect of chemicals on IL-2 or IFN-γ promoter
  =(1-(nSLG-LA or nSLO-LA of #2H4 treated with chemicals)
  /(nSLG-LA or nSLO-LA of non-treated #2H4)) x 100

11. Criteria

Conduct three independent experiments for each chemical.
Determined in each experiment whether the chemicals induce statistically significant suppression or augmentation or no significant effects by a one-way ANOVA test followed by Dunnett's post hoc test at the concentration at which I.I.-SLR-LA is greater than or equal to 0.05.

If chemicals showed statistically significant immunosuppression or immunostimulation in three experiments, they were judged as immunosuppressive or immunostimulatory chemicals, respectively. If chemicals showed statistically significant immunosuppression or immunostimulation in only two independent experiments, they were judged as potential immunosuppressive or immunostimulatory drugs, respectively. If not, they were judged as "no effect". Then, for potential immunosuppressive or immunostimulatory chemicals, we selected their percent suppression or percent augmentation (negative percent suppression) in three experiments that showed the most remarkable change and that showed dose dependency and statistically compared suppression or augmentation of chemicals with that of vehicle control in three independent experiments by the Student's t-test. Only when chemicals demonstrated statistical significance, they were judged as immunosuppressive or immunostimulatory, respectively.

Figure 27


Criteria
Three independent experiments for each drug.
In each experiment:
a one-way ANOVA test followed by Dunnett's post hoc test compared with the value for stimulation without chemical at the concentration range at which I.I.-SLR-LA is greater than or equal to 0.05.
significant suppression
significant augmentation
no significance
(-)
(+)
(0)
Combination of result of three independent experiments
-/-/-
+/+/+
-/-/0
-/-/+
+/+/0
+/+/-
others
Student's t-test using % suppression, which shows the most remarkable change or shows dose dependency in each experiment compared with that of the vehicle control (0%)
Immunosuppression (S)
Immunoaugmentation (A)
No effect (N)

11. Update record

Ver. 008.1J 2016.2.2 distribution
Changes after the VMT meeting

Ver. 008.0J 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 □L□□□□□ to 10 □L□□□□□)
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　Principal of measurement of luciferase activity

MultiReporter Assay System -Tripluc- can be used with a microplate-type luminometer with a multi-color detection system, which can equip two optical filters (e.g. Phelios AB-2350 (ATTO), ARVO (PerkinElmer), Tristar LB941 (Berthold)). The optical filters used in measurement are a 560 nm long-pass filter and a 600 nm long-pass filter.

(1) Measurement of three-color luciferase with two optical filters.
This is an example using Phelios AB-2350 (ATTO). This luminometer equips a 560 nm long-pass filter (560 nm LP, Filter 1) and a 600 nm long pass filter (600 nm LP, Filter 2) for optical isolation.

First, using luciferase enzyme reagent of SLG (λmax = 550 nm), SLO (λmax = 580 nm) and SLR (λmax = 630 nm), measure i) the intensity of light without filter (all optical), ii) the intensity of 560 nm LP (Filter 1) transmitted light iii) the intensity of 600 nm LP (Filter 2) transmitted light, and calculate the coefficient factor listed below.

Coefficient factor		Abbreviation	Definition
SLG	Filter 1 transmittance factor	κG_{R56}	The intensity of 560 nm LP (Filter 1) transmitted SLG / the intensity of SLG without filter (all optical)
	Filter 2 transmittance factor	κG_{R60}	The intensity of 600 nm LP (Filter 2) transmitted SLG / the intensity of SLG without filter (all optical)
SLO	Filter 1 transmittance factor	κO_{R56}	The intensity of 560 nm LP (Filter 1) transmitted SLO / the intensity of SLO without filter (all optical)
	Filter 2 transmittance factor	κO_{R60}	The intensity of 600 nm LP (Filter 2) transmitted SLO / the intensity of SLO without filter (all optical)
SLR	Filter 1 transmittance factor	κR_{R56}	The intensity of 560 nm LP (Filter 1) transmitted SLR / the intensity of SLR without filter (all optical)
	Filter 2 transmittance factor	κR_{R60}	The intensity of 600 nm LP (Filter 2) transmitted SLR / the intensity of SLR without filter (all optical)

When the intensity of SLG, SLO and SLR in test sample are defined as G, O and R, respectively, i) the intensity of light without filter (all optical): F0, ii) the intensity of 560 nm LP (Filter 1) transmitted light and iii) the intensity of 600 nm LP (Filter 2) transmitted light are described as below.

$$F0=G+O+R$$
$$F1=\kappa G_{R56} \times G + \kappa O_{R56} \times O + \kappa R_{R56} \times R$$
$$F2=\kappa G_{R60} \times G + \kappa O_{R60} \times O + \kappa R_{R60} \times R$$

These formulas can be rephrased as follows

$$\begin{pmatrix} F0 \\ F1 \\ F2 \end{pmatrix} = \begin{pmatrix} 1 & 1 & 1 \\ \kappa G_{R56} & \kappa O_{R56} & \kappa R_{R56} \\ \kappa G_{R60} & \kappa O_{R60} & \kappa R_{R60} \end{pmatrix} \begin{pmatrix} G \\ O \\ R \end{pmatrix}$$

Then using calculated coefficient factors and measured F0, F1 and F2, you can calculate G, O and R-value as follows.

$$\begin{pmatrix} \mathrm{G} \\ \mathrm{O} \\ \mathrm{R} \end{pmatrix} = \begin{pmatrix} 1 & 1 & 1 \\ \kappa\mathrm{G}_{R56} & \kappa\mathrm{O}_{R56} & \kappa\mathrm{R}_{R56} \\ \kappa\mathrm{G}_{R60} & \kappa\mathrm{O}_{R60} & \kappa\mathrm{R}_{R60} \end{pmatrix}^{-1} \begin{pmatrix} \mathrm{F0} \\ \mathrm{F1} \\ \mathrm{F2} \end{pmatrix}$$

This calculation can be performed using the functions "MININVERSE" and "MMULT" in Microsoft Excel. These calculations are integrated in the Data Sheet.

Appendix 2　Validation of reagents and equipment

5-1 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.

5-1-1　Reagents

- Single reference samples:
  Lyophilized luciferase enzyme reagent of SLG
  Lyophilized luciferase enzyme reagent of SLO
  Lyophilized luciferase enzyme reagent of SLR
- Assay reagent:
  Tripluc® Luciferase assay reagent（TOYOBO Cat#MRA-301）
- B medium: for luciferase assay (30 mL, stored at 2 – 8°C)

Reagent	Company	Conc.	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

5-1-2 Calibration

5-1-2-1 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 the the B medium to each tube of the frozen reference sample (10 μL in a tube) and label them as SLG1/1, SLO1/1 and SLR1/1. Keep the reference samples on ice to prevent deactivation.

Prepare dilution series of the single reference samples of SLG, SLO and SLR as follows. Dilute 0.3 mL of each 1/1 solution with 0.9 mL of the B medium to make SLG1/4, SLO1/4 and SLR1/4. In the same manner, prepare 1/16 and 1/64 solution of each. Keep diluted reference samples on ice.

5-1-2-2 Bioluminescence measurement

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

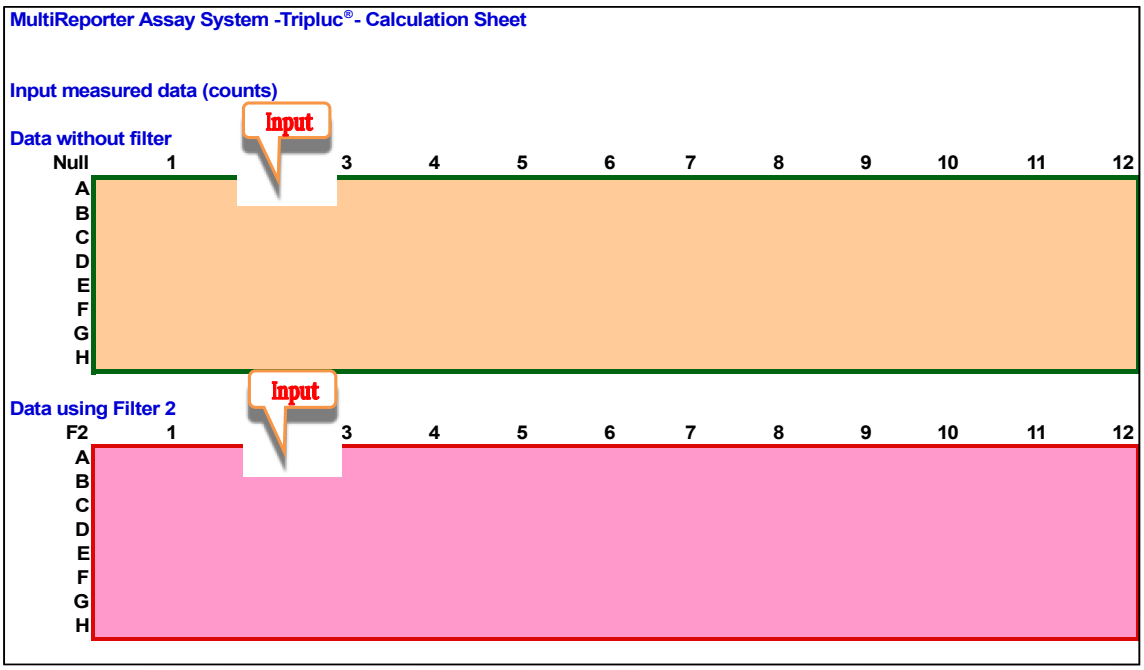
Figure 28

flat-bottom black	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	SLO 1/1	SLO 1/1	SLO 1/1	SLO 1/4	SLO 1/4	SLO 1/4	SLO 1/16	SLO 1/16	SLO 1/16	SLO 1/64	SLO 1/64	SLO 1/64
E												
F	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
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 25°C) with a plate shaker. Remove bubbles on the solutions in wells if they appear. Place the plate into the luminometer to measure the luciferase activity. Bioluminescence is measured for 3 sec each in the absence (F0) and presence (F1, F2) of the optical filters.

Copy the results of the F0, F1 and F2 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 29



Record all the results for quality control.

5-2 Quality control of equipment

In order to confirm the detector stability as the quality control, the reference luciferase sample, optical property, the protocol described here should be performed at the beginning of the experiments every day.

5-2-1 Light source

LED Plate: Reference LED light source plates equipped with stabilized red, green, and blue LEDs are commercially available. For example,

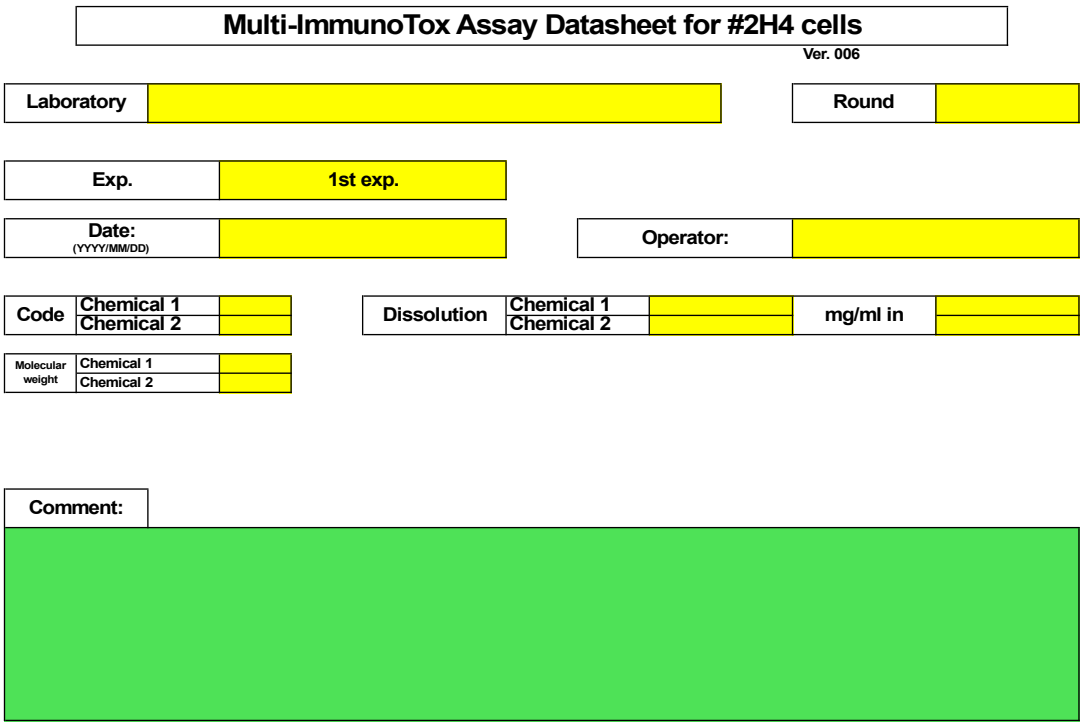
TRIANT® (wSL-0001) by ATTO (Tokyo, Japan)

L12367 by Hamamatsu Photonics (Shizuoka, Japan)

5-2-2 Data collection (an example using TRIANT® by ATTO)

1) Start luminometer 30 min before starting the measurement for stabilization of the photomultiplier.
2) Start LED plate and select “PMT” mode.
3) Select three-color (BRG) mode and adjust light intensity to 1/10 (10E-1).
4) 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.
5) Blue, green, and red LEDs are located at the position of #F6, #E6, and #D6, respectively. Copy the collected data of each position to the appropriate area on Sheet “LED” in the excel file of the data sheet.
6) Check the photo-detector performance by comparing with old data of the LED plate. For quality control purpose, every collected data should be recorded.
7) LED plate data typically fluctuates up to 1.5% (σ). Disagreement to the old data should be less than 3×σ (= 4.5%).

Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 006, Face sheet



Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 006, Data imput sheet

MultiReporter Assay System -Tripluc®- Calculation Sheet

Transmittance Data

	SLG	SLO	SLR
T1	1	1	1
T2			
T3			

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

Filter 1 Data

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

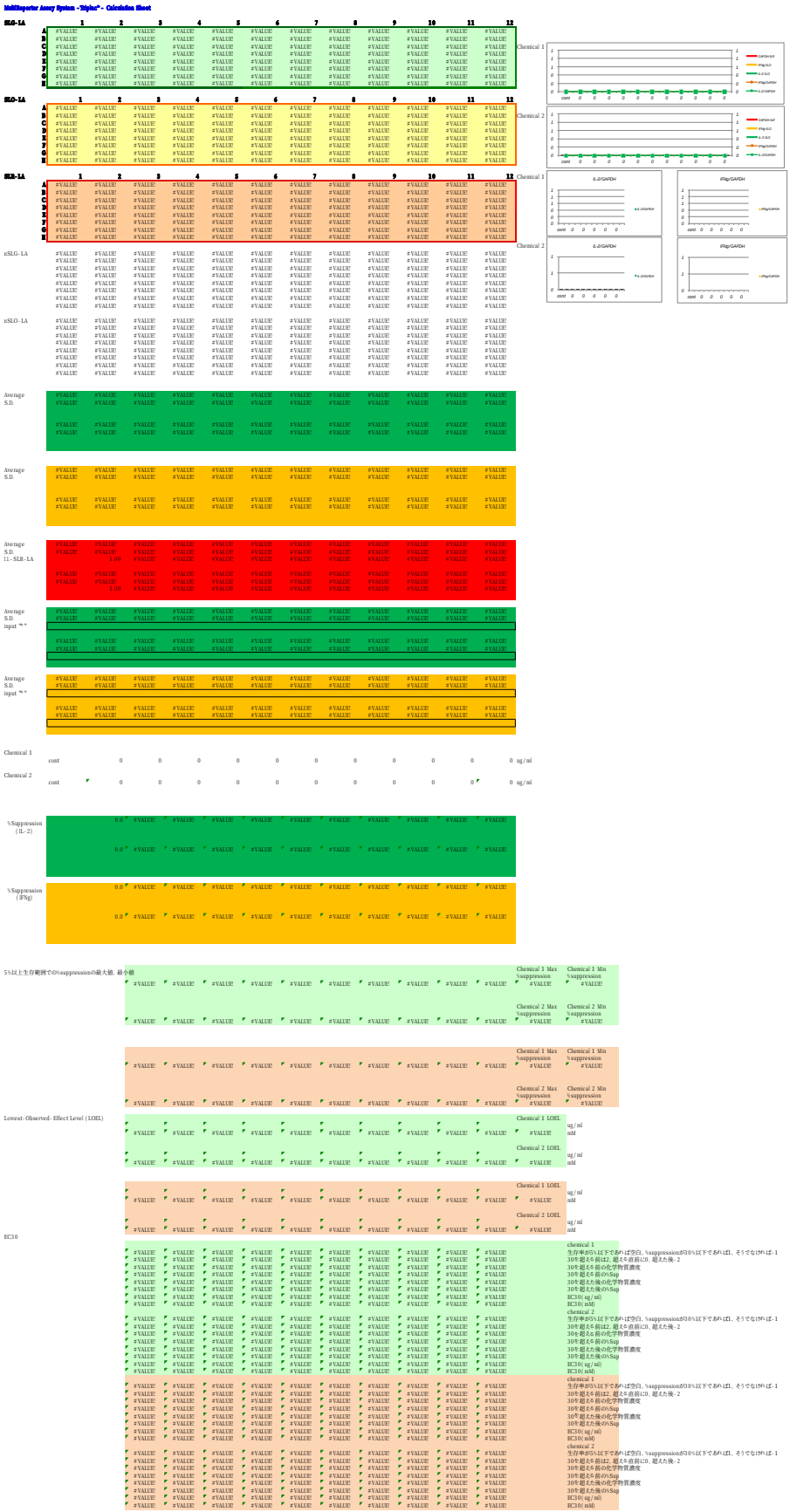
Filter 2 Data

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

Filter 3 Data

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

Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 006, Result format sheet



Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 006, Control sheet

MultiReporter Assay System - Tripluc® - Calculation Sheet

SLG-LA	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!

SLO-LA	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!

SLR-LA	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!

nSLG-LA												
	#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!
	#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!

nSLO-LA												
	#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!
	#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!

	cont	ddw	DEX 1mg/ml	DMSO	CyA 1ug/ml							
Average	#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!	

Average	#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!	

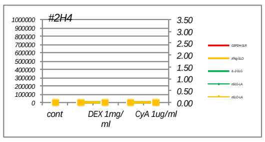
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!							
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!							
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!		#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!		#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	

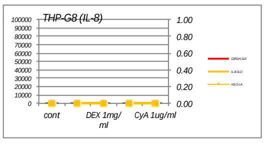
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!							
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!							
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!		#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!		#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	

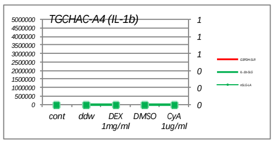
Average	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!							
S.D.	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!							
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!		#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	
	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!		#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	

IL-2 抑制率(%)	0	0	#VALUE!	0	#VALUE!							
IL-1b 抑制率(%)	0	0	#VALUE!	0	#VALUE!							

IFN-g 抑制率(%)	0	0	#VALUE!	0	#VALUE!							
IL-8 抑制率(%)							0	0	#VALUE!	0	#VALUE!	







添付文書２

Multi-ImmunoTox Assay Datasheet for #2H4 cells Ver. 006, Update record

2016年2月2日 Ver.006
phase 0用

2015年11月17日 Ver.005.2
FaceSheetを追加
EC30、Lowest-Observed-Effect Level (LOEL)、Max %suppression, Min %suppressionが算出されるように改変

2014年11月26日 Ver.005.1
コントロール用のシートを修正

2014年11月17日 Ver.005
Multi-Immuno Tox Assayバリデーションプロトコール20141117 Ver.004J案の変更に合わせ、コントロール等のプレート配置を変更
%suppressionのグラフを削除

2013/11/6 Ver.004
統計処理用のシートを追加
Result Format2シートの化学物質の濃度表示を修正（公比2になるように）

未配布 Ver.003.1
グラフの大きさを縮小（パワーポイントにコピーペーストしやすくするため）

2013年9月19日 Ver.003
コントロール用のシートを追加（dexamethasone, cyclosporin A）

2012年11月13日 Ver.002.1
%Suppressionのグラフを追加

2012年8月31日 Ver.002
抑制率の計算方法を変更（バックグランドの値を引き算する方法）

2012年8月28日 Ver.001

添付文書３

Multi-ImmunoTox Assay **記録用紙** Ver. 001, **試薬管理シート**

実験名　MITA バリデーション研究

被試験試薬コード

被試験試薬管理

受領日　　年　月　日　　受領者氏名

保管場所　　温度 (℃)　　℃

備考

受領量 (容器込)　　g

月 日	使用量(g)	残存量(g)	実験担当者名	備考	Exp. No.	溶解性検討
H. / /						
/						
/						
/						
/						
/						
/						
/						
/						
/						
/						
/						

添付文書３

Multi-ImmunoTox Assay **記録用紙** Ver. 001, **試験者シート**

実験名　MITA バリデーション研究

実験日 _____

施設名 _____

実験責任者名 _____

実験担当者名 _____

実験担当者名 _____

実験担当者名 _____

実験担当者名 _____

試験物質コード

_____	_____ 回目
_____	_____ 回目
_____	_____ 回目
_____	_____ 回目
_____	_____ 回目
_____	_____ 回目
_____	_____ 回目
_____	_____ 回目
_____	_____ 回目
_____	_____ 回目

Multi-ImmunoTox Assay **記録用紙** Ver. 001, **細胞継代シート**

3-1 #2H4培養方法

3-1-1 細胞蘇生(P1)

□ あらかじめ、#2H4用C培地15 mLを37° C恒温槽で温めておく(培養用)。
□ 凍結細胞を37° C恒温槽で融解し、#2H4用C培地9 mLを入れておいた15 mLの遠沈管に加える
(細胞液0.5 mL+C培地 9 mL=計9.5 mL)
□ 遠心して細胞を集める (350 x *g*, 5分程度)。
□ 上清を吸引除去し、先に温めておいた#2H4用C培地15 mLに細胞を懸濁してT-75 Flaskで培養を
開始する (37℃, 5%CO_2)。
□上記より一部細胞浮遊液を採取し、培養開始時の細胞生存率を計測する。
(計算)

生細胞数:
死細胞数:

実施日: 年 月 日、実施者:

3-1-2 選択抗生剤での培養開始(P2)

□ あらかじめ、#2H4用A培地必要量を37° C恒温槽で温めておく。
□ 細胞蘇生して3日～4日後に、選択抗生剤を入れた培養(#2H4用A培地)を開始する。
フラスコ中の細胞塊を滅菌ピペットでピペッティングしてほぐし、細胞数を計測する。
(+)/ × = ×10^4/mL－A液

□ 必要細胞量を取り、遠心して細胞を集める (350 x *g*, 5分程度)。上清を吸引除去し、先に温めておいた
#2H4用A培地15mLに3×10^5/mLで細胞を懸濁してT-75 Flaskで培養する。
※ 継代濃度が同一であれば、容量の変更や維持本数変更は構わない。

A液の採取量: mL

実施日: 年 月 日、実施者:

3-1-3 通常の継代培養(P3以降)

P-
□ あらかじめ、#2H4用A培地必要量を37° C恒温槽で温めておく。
□ フラスコ中の細胞塊を滅菌ピペットでピペッティングしてほぐし、細胞数を計測する。
継代細胞濃度は3×10^5/mL、継代間隔は3~4日程度で行う。
(+)/ × = ×10^4/mL－A液
□ 必要細胞量を取り、遠心して細胞を集める (350 x g, 5分程度)。上清を吸引除去し、先に温めておいた
A培地15mLに3×10^5/mLで細胞を懸濁してT-75 Flaskで培養する。
※ 継代濃度が同一であれば、容量の変更や維持本数変更は構わない。

A液の採取量: mL

実施日: 年 月 日、実施者:

P-
□ あらかじめ、#2H4用A培地必要量を37° C恒温槽で温めておく。
□ フラスコ中の細胞塊を滅菌ピペットでピペッティングしてほぐし、細胞数を計測する。
継代細胞濃度は3×10^5/mL、継代間隔は3~4日程度で行う。
(+)/ × = ×10^4/mL－A液
□ 必要細胞量を取り、遠心して細胞を集める (350 x g, 5分程度)。上清を吸引除去し、先に温めておいた
A培地15mLに3×10^5/mLで細胞を懸濁してT-75 Flaskで培養する。
※ 継代濃度が同一であれば、容量の変更や維持本数変更は構わない。

A液の採取量: mL

実施日: 年 月 日、実施者:

P-
□ あらかじめ、#2H4用A培地必要量を37° C恒温槽で温めておく。
□ フラスコ中の細胞塊を滅菌ピペットでピペッティングしてほぐし、細胞数を計測する。
継代細胞濃度は2×10^5/mL、継代間隔は3~4日程度で行う。
(+)/ × = ×10^4/mL－A液
□ 必要細胞量を取り、遠心して細胞を集める (350 x g, 5分程度)。上清を吸引除去し、先に温めておいた
A培地15mLに3×10^5/mLで細胞を懸濁してT-75 Flaskで培養する。
※ 継代濃度が同一であれば、容量の変更や維持本数変更は構わない。

A液の採取量: mL

実施日: 年 月 日、実施者:

P-
□ あらかじめ、#2H4用A培地必要量を37° C恒温槽で温めておく。
□ フラスコ中の細胞塊を滅菌ピペットでピペッティングしてほぐし、細胞数を計測する。
継代細胞濃度は2×10^5/mL、継代間隔は3~4日程度で行う。
(+)/ × = ×10^4/mL－A液
□ 必要細胞量を取り、遠心して細胞を集める (350 x g, 5分程度)。上清を吸引除去し、先に温めておいた
A培地15mLに3×10^5/mLで細胞を懸濁してT-75 Flaskで培養する。
※ 継代濃度が同一であれば、容量の変更や維持本数変更は構わない。

A液の採取量: mL

実施日: 年 月 日、実施者:

添付文書３

Multi-ImmunoTox Assay **記録用紙** Ver. 001, **細胞調製シート**

実験名　MITA バリデーション研究

実験日　＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

施設名　＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

細胞調製

室温　＿＿＿＿℃

予定プレート数　＿＿＿＿枚　x　2.0x10^{7} cells/枚 x1.5=＿＿＿＿cells（必要細胞数）

細胞調製（試験物質用）

細胞蘇生年月日　＿＿＿＿年＿＿＿＿月＿＿＿＿日

前回継代年月日　＿＿＿＿年＿＿＿＿月＿＿＿＿日

前回継代時
細胞濃度･培養液量　＿＿＿＿cells/mL　X　＿＿＿＿mL

実験当日細胞濃度　＿＿＿＿cells/mL －①

遠心した細胞数　＿＿＿＿＿＿＿＿＿＿cells[*1]　①を＿＿＿＿mLを採取

再懸濁した培地量　＿＿＿＿＿＿＿＿＿＿mL（[*1]の細胞数÷(4x10^{6})）

それぞれのプレートに50 μL /wellで分注　□　（　：　）

細胞調製（コントロール(dexamethasone, cyclosporine A)用）

上で調製した細胞を別のプレートの#A1-#D5に50 μL/wellで分注　□　（　：　）

添付文書３

Multi-ImmunoTox Assay **記録用紙** Ver. 001, **被試験試薬の調製①シート**

実験名　MITA バリデーション研究

実験日　＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

施設名　＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

被試験試薬コード　＿＿＿＿　＿＿＿＿＿＿回目

被試験試薬の調製①（溶媒への溶解）

25mg/mL水溶液で	□完全に溶解せず	□完全に溶解	
		↓	
50mg/mL水溶液で		□完全に溶解せず	→25mg/mL水溶液を調製
		□完全に溶解	
		↓	
100mg/mL水溶液で		□完全に溶解せず	→50mg/mL水溶液を調製
		□完全に溶解	→100mg/mL水溶液として調製を継続
500mg/mL DMSO溶液で	□完全に溶解	→500mg/mL DMSO溶液として調製を継続	
	□完全に溶解せず		
	↓		
250mg/mL DMSO溶液で	□完全に溶解	→250mg/mL DMSO溶液として調製を継続	
	□完全に溶解せず		
	↓		
125mg/mL DMSO溶液で	□完全に溶解	→125mg/mL DMSO溶液として調製を継続	
	□完全に溶解せず		
	↓		
62.5mg/mL DMSO溶液で	□完全に溶解	→62.5mg/mL DMSO溶液として調製を継続	
	□完全に溶解せず		
	↓		
31.25mg/mL DMSO溶液で	□完全に溶解	→31.25mg/mL DMSO溶液として調製を継続	
	□完全に溶解せず		
	↓		

Multi-ImmunoTox Assay 記録用紙 Ver. 001, 被試験試薬の調製②(DW)シート

実験名 MITA バリデーション研究

実験日 _____

施設名 _____

被試験試薬コード _____ _____回目

水溶液に調製された場合

試験液の調製と細胞への処理

被試験試薬_____mgをDistilled waterに溶解し_____mLとする。 →_____mg/mL　　調製時間 (　:　)

96 well clear plate (丸底) に下図のようにDistilled water、被試験試薬水溶液を分注する。

丸底･透明	1	2	3	4	5	6	7	8	9	10	11	12
A	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	Distilled water 50uL	被試験試薬水溶液 100uL
B												
C												
D												
E												
F												
G												
H												

□

well#A11から#A3までDistilled waterで公比2で段階希釈を9段階おこなう。

添加時間

アッセイブロックにB培地480 µLを分注し、上図の希釈液を20 µL添加して25倍希釈し、これを50 µL/wellずつ細胞に添加する。 □ (　:　)

プレートシェーカーを使用し、撹拌して混合する。 □

細胞をインキュベーターへ入れ、1時間反応させる。 □

賦活剤(PMA/ionomycin)の調製と細胞への処理

1mM PMAストックをB培地で10倍希釈し100 µM溶液を作製する。(1mM PMA 10 µL + B培地 90 µL) □

下図のようにコントロール溶液と x10 PMA/ionomycin溶液を作製する。 □

	B medium	1 mM Ionomycin	100 µM PMA	Ethanol	Total
Control	990 µL	-		10 µL	1000 µL
x10 PMA/ ionomycin solution	2370 µL	24 µL	6 µL	-	2400 µL

添加時間

コントロール溶液を#A1-#H1、x10 PMA/ionomycin溶液を#A2-#H12に10 µLずつ分注する。 □ (　:　)

プレートシェーカーを使用し、撹拌して混合する。

細胞をインキュベーターへ入れ、6時間反応させる。

測定(被試験物質)

Tripluc® Luciferase assay reagentを溶解させる。 □

光電子増倍管を安定させるため、ルミノメータは測定開始30 分前には電源を入れる。 □

添加時間

リザーバーにTriplucを移し、8チャンネルもしくは12チャンネルピペットマンを使用して、
反応終了後のアッセイプレートに100 µL/wellずつ分注する。 □ (　:　)

Tripluc添加後、プレートシェーカーを使用して室温 (23-27 ℃) で10分間 (30分間まで可) 撹拌し、
細胞を溶解させる。 □ 撹拌中温度 (　　℃)

ルミノメータでLuciferase活性を測定する。(測定中温度が26-31℃であることを確認する。)
フィルタ無し、フィルタ有りで各々3 秒/well測定する (アトー社製Pheliosの場合はF0、F1、F2を使用)。 □ 測定時間 (　:　)

測定中温度 (　　℃)

Multi-ImmunoTox Assay 記録用紙 Ver. 001, 被試験試薬の調製③(DMSO)シート

実験名 MITA バリデーション研究

実験日 _____

施設名 _____

被試験試薬コード _____ _____回目

DMSO溶液に調製された場合

試験液の調製と細胞への処理

被試験試薬_____mgをDMSOに溶解し_____mLとする。 → _____mg/mL　　調製時間 (　:　)

96 well clear plate(丸底)に下図のようにDMSO、B培地、被試験試薬DMSO溶液を分注する。 ☐

丸底・透明	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 50uL	DMSO 50uL	DMSO 50uL	DMSO 50uL	DMSO 50uL	DMSO 50uL	DMSO 50uL	DMSO 50uL	DMSO 50uL	DMSO 50uL	DMSO 50uL	被試験試薬 DMSO溶液 100uL
B	B培地 90uL	B培地 90uL	B培地 90uL	B培地 90uL	B培地 90uL	B培地 90uL	B培地 90uL	B培地 90uL	B培地 90uL	B培地 90uL	B培地 90uL	B培地 90uL
C												
D												
E												
F												
G												
H												

well#A11から#A3までDMSOで公比2で段階希釈を10段階おこなう。 ☐

段階希釈した被試験試薬DMSO溶液 10 μLを8チャンネルもしくは12チャンネルピペットマンを使用して下のB培地90 μLにうつし10倍に希釈する。 ☐

希釈した段階での沈殿の有無、性状

#B1	#B2	#B3	#B4	#B5	#B6	#B7	#B8	#B9	#B10	#B11	#B12
有☐無☐	有☐無☐	有☐無☐	有☐無☐	有☐無☐	有☐無☐	有☐無☐	有☐無☐	有☐無☐	有☐無☐	有☐無☐	有☐無☐

沈殿の性状 (例:粉状、泥状、膜状、ミセル様　　)

アッセイブロックにB培地490 μLを分注し、上図の希釈液を10 μL添加して50倍希釈し、これを50 μL/wellずつ細胞に添加する。 ☐　添加時間 (　:　)

プレートシェーカーを使用し、撹拌して混合する。 ☐

細胞をインキュベーターへ入れ、1時間反応させる。 ☐

賦活剤(PMA/ionomycin)の調製と細胞への処理

1mM PMAストックをB培地で10倍希釈し100 μM溶液を作製する。(1mM PMA 10 μL + B培地 90 μL) ☐

下図のようにコントロール溶液と x10 PMA/ionomycin溶液を作製する。 ☐

	B medium	1 mM Ionomycin	100 μM PMA	Ethanol	Total
Control	990 μL	-		10 μL	1000 μL
x10 PMA/ ionomycin solution	2370 μL	24 μL	6 μL	-	2400 μL

コントロール溶液を#A1–#H1、x10 PMA/ionomycin溶液を#A2–#H12に10 μLずつ分注する。 ☐　添加時間 (　:　)

プレートシェーカーを使用し、撹拌して混合する。

細胞をインキュベーターへ入れ、6時間反応させる。

測定(被試験物質)

Tripluc® Luciferase assay reagentを溶解させる。 ☐

光電子増倍管を安定させるため、ルミノメータは測定開始30 分前には電源を入れる。 ☐

リザーバーにTriplucを移し、8チャンネルもしくは12チャンネルピペットマンを使用して、
反応終了後のアッセイプレートに100 μL/wellずつ分注する。 ☐　添加時間 (　:　)

Tripluc添加後、プレートシェーカーを使用して室温(23–27 ℃)で10分間(30分間まで可)撹拌し、
細胞を溶解させる。 ☐　撹拌中温度 (　　℃)

ルミノメータでLuciferase活性を測定する。(測定中温度が26–31℃であることを確認する。)
フィルタ無し、フィルタ有りで各々3 秒/well測定する(アトー社製Phelios の場合はF0、F1、F2を使用)。 ☐　測定時間 (　:　)
測定中温度 (　　℃)

Multi-ImmunoTox Assay 記録用紙 Ver. 001, 被試験試薬の調製④（コントロール）シート

実験名 MITA バリデーション研究

実験日 _____

施設名 _____

被試験試薬コード _____ _____回目

コントロールの調製と細胞への処理

dexamethasone, cyclosporine Aの調製

96 well clear plate（丸底）に下図のようにDMSO 50 μL (#A4)、12 mg/mL cyclosporine A stock 10 μL + DMSO 110 μL (#A5)、Distilled water 50 μL (#B1、 #B2)、50 mg/mL dexamethasone stock 50 μL (#B3)、B培地 180 μl (#B4、 #B5)を分注する。 ☐ 添加時間 （ ： ）

丸底・透明	1	2	3	4	5	6	7	8	9	10	11	12
A				DMSO 50uL	CyA 12 mg/mL ストック 10uL + DMSO 110uL							
B	Distilled water 50uL	Distilled water 50uL	DEX 60 mg/mL ストック 50 uL	B培地 180uL	B培地 180uL							
C												
D												
E												
F												
G												
H												

#A4のDMSOと#A5のcyclosporine A DMSO溶液 20μLを下のB培地180 μLにうつし10倍に希釈する。 ☐

アッセイブロックの#A1–#A3にB培地480 μL、#A1–#A3にB培地980 μLを分注し、上図の希釈液を20 μL添加して混合し、50 μL/wellずつ細胞に添加する。 ☐ 添加時間 （ ： ）

プレートシェーカーを使用し、撹拌して混合する。 ☐

細胞をインキュベーターへ入れ、1時間反応させる。 ☐

賦活剤(PMA/ionomycin)の調製と細胞への処理

1mM PMAストックをB培地で10倍希釈し100 μM溶液を作製する。（1mM PMA 10 μL + B培地 90 μL） ☐

下図のようにコントロール溶液と x10 PMA/ionomycin溶液を作製する。 ☐

	B medium	1 mM Ionomycin	100 μM PMA	Ethanol	Total
Control	990 μL	-		10 μL	1000 μL
x10 PMA/ ionomycin solution	2370 μL	24 μL	6 μL	-	2400 μL

コントロール溶液を#A1–#D1、x10 PMA/ionomycin溶液を#A2–#D5に10 μLずつ分注する。 ☐ 添加時間 （ ： ）

プレートシェーカーを使用し、撹拌して混合する。

細胞をインキュベーターへ入れ、6時間反応させる。

測定（コントロール）

Tripluc® Luciferase assay reagentを溶解させる。 ☐

光電子増倍管を安定させるため、ルミノメータは測定開始30 分前には電源を入れる。 ☐

リザーバーにTripluc を移し、8チャンネルもしくは12チャンネルピペットマンを使用して、
反応終了後のアッセイプレートに100 μL/wellずつ分注する。 ☐ 添加時間 （ ： ）

Tripluc添加後、プレートシェーカーを使用して室温（23–27 ℃）で10分間（30分間まで可）撹拌し、
細胞を溶解させる。 ☐ 撹拌中温度 （ ℃）

ルミノメータでLuciferase活性を測定する。（測定中温度が26–31℃であることを確認する。）
フィルタ無し、フィルタ有りで各々3 秒/well測定する（アトー社製Phelios の場合はF0、F1、F2を使用）。 ☐ 測定時間 （ ： ）
測定中温度 （ ℃）

Multi-ImmunoTox Assay **記録用紙** Ver. 001, **更新履歴シート**

Ver. 001J　2016年02月02日配布

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kimura, Y., Fujimura, C., Ito, Y., Takahashi, T., Nakajima, Y., Ohmiya, Y. Aiba, S.	Optimization of the IL-8 Luc assay as an in vitro test for skin sensitization	Toxicol in Vitro	29	1816-1830	2015
Ohmiya, Y	Simultaneous multicolor luciferase reporter assays for monitoring of multiple genes expressions	Comb Chem High Throughput Screen	18	937-945	2015
Watanabe, M., Noma, H., Kurai, J., Sano, H., Kitano, H., Saito, R., Kimura, Y., Aiba, S., Oshimura, M., Shimizu, E	Variation in the Effect of Particulate Matter on Pulmonary Function in Schoolchildren in Western Japan and Its Relation with Interleukin-8.	Int J Environ Res Public Health	12	14229-14243	2015
Watanabe, M., Noma, H., Kurai, J., Sano, H., Saito, R., Abe, S., Kimura, Y., Aiba, S., Oshimura, M., Yamasaki, A., Shimizu, E.	Decreased pulmonary function in school children in Western Japan after exposures to Asian desert dusts and its association with interleukin-8.	Biomed Res Int	2015	583293	2015

Watanabe, M., Kurai, J., Minato, S., Noma, H., Sano, H., Saito, R., Aiba, S., Oshimura, M., Hatakeyama, K., Yamasaki, A., Shimizu, E.	Difference in interleukin-8 transcriptional activity induced in THP-G8 cells by particulate matter collected in winter and summer in western Japan	J Med Invest	62	145-148	2015
Yasunaga, M., Murotomi, K., Abe, H., Yamazaki, T., Nishii, S., Ohbayashi, T., Oshimura, M., Noguchi, T., Niwa, K., Ohmiya, Y., Nakajima, Y	Highly sensitive luciferase reporter assay using a potent destabilization sequence of calpain 3	J Biotechnol	194	115-123	2015

Toxicology in Vitro 29 (2015) 1816–1830

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Optimization of the IL-8 Luc assay as an *in vitro* test for skin sensitization

Yutaka Kimura, Chizu Fujimura, Yumiko Ito, Toshiya Takahashi, Yoshihiro Nakajima, Yoshihiro Ohmiya, Setsuya Aiba

Department of Dermatology, Tohoku University Graduate School of Medicine, Sendai 980-8574, Japan
Health Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Takamatsu, Kagawa 761-0395, Japan
Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566, Japan

article info

Article history:
Received 18 February 2015
Revised 25 June 2015
Accepted 6 July 2015
Available online 15 July 2015

Keywords:
Contact dermatitis
Sensitization test
Luciferase assay
Alternative method
Skin sensitization

abstract

We previously reported a dataset of the IL-8 Luc assay covering reference chemicals published by ECVAM, in which the effects of chemicals on IL-8 promoter activity were evaluated by an IL-8 reporter cell line, THP-G8 cells. To clarify its performance, we created another dataset of 88 sensitizers and 34 non-sensitizers. Simultaneously, to improve its performance, we changed the incubation time from 5 h to 16 h, deleted the criterion regarding the effects of N-acetylcysteine, and set an exclusion criterion for detergents. These modifications significantly improved its performance. In addition, we examined the following three criteria to judge chemicals as sensitizers: Criterion 1: Fold induction of SLO luciferase activity (FInSLO-LA) **P** 1.4, Criterion 2: the lower limit of the 95% confidence interval of FInSLO-LA **P** 1.0, Criterion 3: the intersection of criteria 1 and 2. Among them, Criterion 1 produced the best performance, demonstrating that the accuracy, sensitivity and specificity were 81%, 79%, and 90%, respectively. In addition, we found that the IL-8 Luc assay solubilizing chemicals with X-VIVO substantially improved its performance. Finally, the IL-8 Luc assay combined with DPRA and DEREK could improve substantially its performance. These data suggest that the IL-8 Luc assay is a promising test method to screen skin sensitizers.

1. Introduction

In the regulatory context, currently only data from animal experiments are acceptable to assess the skin sensitizing potential of chemicals. The European Union (EU) imposed an animal testing ban effective 2009 on both cosmetics products and their ingredients. This was accompanied by a concomitant marketing ban effective March 1, 2013 if animal tests were conducted after this date for the purpose of cosmetics legislation (Regulation (EC) No. 1223/2009 of the European Parliament and of the Council, 2009). On the other hand, under the European chemicals legislation REACH, skin sensitization data for any chemical registered under the European Chemicals Legislation (REACH, EC 1907/2006) is mandatory, and animal testing should only be performed as a last resort (http://echa.europa.eu/documents/10162/13639/alternatives_test_animals_2014_en.pdf).

Pushed by these ethical and legislative demands, various promising methods have been developed as alternative methods to detect the skin sensitizing potential of chemicals and four methods are currently undergoing formal validation at the European Centre for Validation of Alternative Methods (ECVAM). In 2012, the Organization for Economic Co-operation and Development (OECD) published the adverse outcome pathway (AOP) for skin sensitization (OECD, 2012) in which the key steps in the sensitization process are defined. According to the AOP, 4 methods are considered to target three different steps in the skin sensitization process: protein-binding/haptenization (e.g., the Direct Peptide Reactivity Assay, DPRA) (Gerberick et al., 2004), induction of the Kelch-like ECH-associated protein 1 (Keap-1)/nuclear factor (erythroid-derived 2)-like factor 2 (Nrf2) pathways in keratinocytes (e.g., the KeratinoSens™ assay) (Emter et al., 2010), and the activation of antigen presenting cells such as dendritic cell-like cell lines (The Myeloid U937 Skin Sensitization Test (U-SENS) (Piroird et al., 2015) or the human Cell Line Activation Test, h-CLAT) (Ashikaga et al., 2006; Sakaguchi et al., 2006). However, it is unlikely that a single assay will be sufficient to adequately assess the sensitization potential because of the complexity of the sensitization process (Bauch et al., 2012).

Dendritic cell activation is one of the key steps in sensitization indicated in the AOP for skin sensitization published by the OECD

Corresponding author at: Department of Dermatology, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Sendai 980-8574, Japan.
E-mail address: saiba@med.tohoku.ac.jp (S. Aiba).

http://dx.doi.org/10.1016/j.tiv.2015.07.006
0887-2333/

(2012). In this step, CD54 and CD86 expression is augmented; an increase in interleukin-8 (IL-8) mRNA or IL-8 protein has been suggested as another biomarker for discriminating sensitizers from non-sensitizers in monocyte-derived dendritic cells (MoDCs) (Toebak et al., 2006), U937 cells (Python et al., 2007), or THP-1 cells (Mitjans et al., 2008, 2010; Nukada et al., 2008). IL-8 is well established as a potent chemotactic peptide for neutrophils, T lymphocytes, basophils (Leonard et al., 1990), and NK cells (Sebok et al., 1993). It was recently reported that human immature MoDCs express the IL-8 receptors CXCR1 and CXCR2, which are down-regulated in mature MoDCs (Gouwy et al., 2014) and that accordingly, human immature MoDCs are chemoattracted by IL-8 (Feijoo et al., 2005). It is impossible to demonstrate the exact role of IL-8 in contact hypersensitivity using IL-8 knockout mice because of the lack of a mouse counterpart of IL-8. Regardless, several studies suggest the importance of IL-8 in the DC activation step in the AOP for skin sensitization. Specifically, CCL2 that is coordinately regulated with IL-8 (Singha et al., 2014) plays a crucial role in dendritic cell maturation (Jimenez et al., 2010). On the other hand, Natsuaki et al. have reported DC clusters around macrophages in both the elicitation phase and the sensitization phase in murine contact sensitivity, suggesting the crucial role of CXCR2 expression on DCs in murine contact sensitivity (Natsuaki et al., 2014). Since CXCR2 is a receptor for IL-8 in humans (Marchese et al., 1995; Murphy and Tiffany, 1991), a murine counterpart of IL-8 produced by dermal macrophages may play a crucial role in murine contact sensitization.

In addition, it is now well-recognized that skin sensitization and chemical protein reactivity are linked. Although chemical sensitizers are extremely diverse in molecular weight and structure, most share electrophilic properties and possess intrinsic reactivity toward various amino acids containing nucleophilic heteroatoms (i.e., cysteine, lysine, histidine, arginine, and methionine). Indeed, a correlation between the reactivity of chemicals with cysteine or lysine residues in peptides and their sensitization potential has been demonstrated (Gerberick et al., 2007). Electrophiles can be detected by the Keap1-Nrf2 cellular sensor pathway implicated in the antioxidant response of the cell and recently reviewed by Itoh et al. (2010). Under normal conditions, Keap1 sequesters the transcriptional regulator nuclear Nrf2 in the cytoplasm, provoking its proteasomal degradation. In the presence of electrophiles, the highly reactive cysteine residues of Keap1 are modified, leading to the dissociation of Keap1 from Nrf2. Nrf2 translocates to the nucleus, forms heterodimers with small Maf proteins, and then induces the transcription of genes with an antioxidant response element (ARE) in their promoters (Holland and Fishbein, 2010). These genes code for proteins mostly involved in detoxification, such as heme oxygenase-1 (HO-1) and NADPH-quinone oxidoreductase 1 (Nqo1). Interestingly, Zhang et al. reported that the 5^0 flanking region of the IL-8 gene has several areas homologous to the consensus ARE (ATGAC/TnnnGCA/); in addition, Nrf2 caused only a weak induction of IL-8 transcription but significantly increased the half-life of IL-8 mRNA (Zhang et al., 2005). These data suggested that the induction of IL-8 mRNA by haptens is regulated transcriptionally by p38 MAPK and post-transcriptionally by Nrf2. Therefore, the IL-8 Luc assay is a unique screening method for haptens since it detects their effects on p38 MAPK and Keap1-Nrf2.

We established the IL-8 reporter cell assay (IL-8 Luc assay) using a stable THP-1-derived IL-8 reporter cell line, THP-G8, which harbors SLO and SLR luciferase genes under the control of IL-8 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoters, respectively (Takahashi et al., 2011). This previous study demonstrated that use of the IL-8 Luc assay to examine 35 chemicals, including reference chemicals published by ECVAM (referred to as the ''ECVAM list''; (Casati et al., 2009), resulted in an overall test accuracy of 82%.

In the current study, we increased the number of chemicals for evaluation by the IL-8 Luc assay, explored the reason for false results, modified the procedure and protocol (including the criteria), examined the correlation of parameters between the IL-8 Luc assay and other test methods, and proposed the test battery system.

2. Materials and methods

2.1. Cells and culture

We previously established a reporter cell line, THP-G8 cells derived from the human acute monocytic leukemia cell line THP-1 cells containing stable luciferase orange (SLO) regulated by IL-8 promoter and stable luciferase red (SLR) by GAPDH promoter (Takahashi et al., 2011). THP-G8 cells were cultured in RPMI-1640 (Gibco, Carlsbad, CA) with antibiotic–antimycotic (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (FBS) (Biological Industries, Kibbutz Beit Haemek, Israel) at 37 °C with 5% CO_2.

2.2. Test chemicals and chemical treatment

We examined 122 chemicals that are listed with their Chemical Abstract Service (CAS) numbers in Table S1. All chemicals had been previously evaluated and classified with the LLNA (Gerberick et al., 2005). Seventy-two sensitizers were evaluated, including 8 extreme, 16 strong, 25 moderate, and 23 weak sensitizers, as classified by the local lymph node assay (LLNA); 28 non-sensitizers were also evaluated, one of which (sodium lauryl sulfate; SLS) was false positive in the LLNA. All the chemicals were purchased from Sigma–Aldrich, St. Louis, MO, at the highest available purity.

Water soluble chemicals were dissolved in distilled water at a concentration of 25 mg/ml, 50 mg/ml, or 100 mg/ml to determine the highest soluble concentration. Chemicals not soluble in water were dissolved in DMSO at 500 mg/ml. If they were not soluble at 500 mg/ml, the highest soluble concentration was determined by diluting the suspension from 500 mg/ml by a factor of two with DMSO. Sonication and vortex mixing were used if needed and the attempt to dissolve the chemical continued for at least 5 min. All dissolved chemicals were used within 4 h of being dissolved in distilled water or DMSO.

To examine the effects of FBS on the IL-8 Luc assay, three methodologies were examined to solubilize haptens: (1) solubilize in DMSO, and then dilute with RPMI-1640 containing 10% FBS, as used in the IL-8 Luc assay (DMSO/FBS); (2) solubilize in DMSO and then dilute with X-VIVO 15 (Lonza, Walkersville, MD) (DMSO/X-VIVO); and (3) solubilize in X-VIVO and then dilute with X-VIVO (X-VIVO/X-VIVO).

For water soluble chemicals, 11 serial dilutions were conducted using RPMI-1640 with 10% FBS diluting by a factor of 2, in the 1st experiment. In the 2nd, 3rd, or 4th experiment, 11 serial dilutions were conducted, diluting by a factor of 1.5. For water insoluble chemicals, 11 serial dilutions were conducted using DMSO as the solvent, diluting by a factor of 2 in the 1st experiment and by a factor of 1.5 in the 2nd, 3rd, and 4th experiments.

Based on the previous report (Saito et al., 2011; Takahashi et al., 2011), THP-G8 cells (5×10^4 cells/50 ll/well) in 96-well black plates (Greiner bio-one GmbH, Frickenhausen, Germany) were cultured for varying time periods. The optimum cell numbers at seeding were based on the previous reports. In some experiments, the cells were pretreated with 25 mM N-acetyl-L-cysteine (NAC) for 30 min.

2.3. IL-8 promoter-luciferase gene reporter assay

The luciferase reporter assay system was constructed using 2 luciferase genes, SLO and SLR, that emit orange and red light, respectively, with a single substrate. The activities of these luciferases can be measured simultaneously and quantitatively with optical filters. This system can rapidly and easily monitor multiple gene expression (Nakajima et al., 2005; Noguchi et al., 2008). In this study, luciferase activity was determined using a microplate-type luminometer with a multi-color detection system, Phelios (Atto Co., Tokyo, Japan), using the Triplu[®] luciferase assay reagent (TOYOBO Co., Ltd., Osaka, Japan) according to the manufacturer's instructions.

Since some chemicals affected cell viability, we defined the parameter nSLO-LA to represent IL-8 promoter activity. This parameter is calculated by normalizing SLO luciferase activity (SLO-LA) to SLR luciferase activity (SLR-LA). We also calculated the inhibition index of SLR-LA (I.I.-SLR-LA) by dividing the SLR-LA of THP-G8 cells that were treated with chemicals by the SLR-LA of non-treated THP-G8 cells. The fold induction of IL-8 promoter activity (FInSLO-LA) was calculated by dividing the nSLO-LA of THP-G8 cells that were treated with chemicals by that of non-stimulated THP-G8 cells.

We further evaluated the suppressive effect of NAC co-treatment with each chemical by calculation of the inhibition index (I.I.). The I.I. was obtained by dividing the FInSLO-LA of THP-G8 cells stimulated with the chemical in the presence of NAC by the FInSLO-LA stimulated with the chemical alone, using the concentration of chemical at which the chemical induced the largest FInSLO-LA.

The parameters used in the IL-8 Luc assay are shown in Table 1.

Using these parameters, we defined the criteria to identify possible sensitizers. Each criterion is composed of two conditions. Condition 1 defines positive induction or negative induction of SLO-LA in each experiment and condition 2 defines sensitizers or non-sensitizers based on the repeated experiments. In our previous paper (Takahashi et al., 2011), chemicals that demonstrate FInSLO-LA ≥ 1.4 and I.I. ≥ 0.8 at the concentration of the chemical at which I.I.-SLR-LA is ≥0.2 in two or three of three different experiments are categorized as sensitizers and those that do not fulfill these criteria are classified as non-sensitizers. In this study, we optimized the IL-8 Luc assay by examining its performance using six criteria. The precise definition of each criterion is summarized in Table 2. Briefly, these six criteria are different from the original criterion in condition 2: chemicals are categorized as sensitizers when they fulfill condition 1 in two or three of three different experiments in the original criterion, while they are categorized as sensitizers when they fulfill condition 1 in two of two to four different experiments in the new six criteria. Among them, Criterion A, B, and C include the condition I.I. ≥ 0.8, while Criterion 1, 2, and 3 do not.

Table 1
Parameters used in the IL-8 Luc assay.

Parameters	Description
SLO-LA	SLO luciferase activity regulated by IL-8 promoter
SLR-LA	SLR luciferase activity regulated by G3PDH promoter
nSLO-LA	SLO-LA/SLR-LA
I.I.-SLR-LA	SLR-LA of THP-G8 treated with chemicals/SLR-LA of non-treated THP-G8
FInSLO-LA	nSLO-LA of THP-G8 cells treated with chemicals/nSLO-LA of non-stimulated THP-G8 cells
I.I.	FInSLO-LA of THP-G8 cells stimulated with the chemical and NAC/FInSLO-LA stimulated with the chemical alone

2.4. Real-time monitoring of luciferase activity of THP-G8 cells after chemical stimulation

To clarify the time-dependent change of SLO-LA and SLR-LA of THP-G8 cells after chemical stimulation, THP-G8 was suspended with RPMI1640 supplemented with 10% FBS, 0.1 mM D-luciferin 25 mM HEPES/HCl (pH 7.0), and plated onto 35 mm dish at 2×10^6 cells/dish. After 30 min, 1.6 or 0.8 μg/ml DNCB, 1.6 or 0.8 μg/ml 4-NBB was added to the culture. Bioluminescence was in real-time under a 5% CO_2 atmosphere at 37 °C using the dish-type luminometer AB2500 Kronos (ATTO, Tokyo, Japan).

2.5. Statistics

To demonstrate statistical significance, representative data from at least three independent experiments for each analysis is shown. A one-way ANOVA test followed by Dunnett's post hoc test was used to evaluate statistical significance. p values <0.05 were considered statistically significant. We performed Pearson's product–moment correlation analysis to determine the strength of a correlation.

3. Results

3.1. Determination of the optimal incubation time

The performance of the IL-8 Luc assay, when we first reported (Takahashi et al., 2011), was an accuracy of 86%, a sensitivity of 83%, and a specificity of 90% for 35 chemicals including ECVAM list

Table 2
Criteria used in the IL-8 Luc assay.

Criteria		Condition 1	Condition 2
Original	Sensitizer	FInSLO-LA ≥ 1.4 and I.I. ≥ 0.8 at the concentration of the chemical at which I.I.-SLR-LA is ≥0.2	Fulfill the condition 1 in 2 or 3 of 3 different experiments
Original	Non-sensitizer		Do not fulfill the condition 1 in 2 of 3 different experiments
A	Sensitizer	FInSLO-LA ≥ 1.4 and I.I. ≥ 0.8 at the concentration of the chemical at which I.I.-SLR-LA is ≥0.2	Fulfill the condition 1 in 2 of 2–4 different experiments
B	Sensitizer	FInSLO-LA ≥ 1.4 and I.I. ≥ 0.8 at any concentrations	
C	Sensitizer	FInSLO-LA ≥ 1.4 and I.I. ≥ 0.8 at the concentration of the chemical at which I.I.-SLR-LA is ≥0.05	
1	Sensitizer	FInSLO-LA ≥ 1.4 at the concentration of the chemical at which I.I.-SLR-LA is ≥0.05	
2	Sensitizer	The lower limit of the 95% confidence interval of FInSLO-LA ≥ 1.0	
3	Sensitizer	1 and 2	
Either criteria	Non-sensitizer		Do not fulfill the condition 1 in 3 of 3–4 different experiments

(Casati et al., 2009). When we conducted ring trials with 3 different laboratories using 10 coded chemicals, they revealed the accuracy and the intralaboratory reproducibility were not necessarily high enough to conduct further validation studies. Indeed, the accuracy of each laboratory was 80% by Laboratory A, 60% by Laboratory B, and 63% by Laboratory C, respectively, and the intralaboratory reproducibility was 70%. Therefore, we first explored the reason for the poor response of the IL-8 Luc assay to some potent sensitizers, such as DNCB. In our previous study, we examined the time course of FInSLO-LA of THP-G8 cells after LPS stimulation and found the maximum induction of FInSLO-LA between 4 and 7 h after stimulation. In this study, we re-examined the time course of FInSLO-LA after the treatment with strong sensitizers, DNCB and 4-NBB.

We stimulated THP-G8 cells with different concentrations of DNCB or 4-NBB for different time periods and FInSLO-LA was measured (Fig. 1a and b). DNCB and 4-NBB significantly augmented FInSLO-LA dose-dependently from 5 h to 24 h and from 4 h to 24 h after stimulation, respectively. The maximum induction by DNCB was observed between 9 h and 12 h at the concentration of 1.19 lg/ml and 2.67 lg/ml. On the other hand, the maximum induction by 4-NBB was observed between 8 h and 10 h at the concentration of 1.19 lg/ml and at 10 h and 16 h at the concentration of 2.67 lg/ml.

Next, we monitored SLO-LA of THP-G8 cells for 1 min at intervals of 19 min during DNCB or 4-NBB treatment under a dish type luminometer (Fig. 2a and b). Consistent with the results obtained by measuring luciferase activity intermittently, real-time monitoring also demonstrated the increase in FInSLO-LA from 5 h to 24 h by both 0.8 lg/ml and 1.6 lg/ml of DNCB and 4-NBB with maximum induction at 12 h and 10–12 h, respectively.

These results demonstrated that the optimal incubation period with chemicals is around 10 h. This is not practical because one IL-8 Luc assay requires more than 12 h, including general preparation, plating the cells, applying the chemicals and measuring luciferase activity using a luminometer. Therefore, from the practical standpoint, we compared FInSLO-LA between 6 h incubation and 16 h incubation in Figs. 1 and 2. For both DNCB and 4-NBB, intermittent measurement and real-time monitoring indicated that FInSLO-LA at the optimal concentration was much higher in 16 h incubation than in 6 h incubation. This was confirmed by stimulating THP-G8 cells with different concentrations of DNCB and 4-NBB for 6 h and 16 h (data not shown).

Finally, we evaluated the ECVAM list of chemicals by the IL-8 Luc assay with a 16 h incubation period and with the IL-8 Luc assay with a 6 h incubation period and compared the results. As shown in Table 3, most sensitizers increased FInSLO-LA in the 16 h incubation IL-8 Luc assay more than in the 6 h incubation IL-8 Luc assay. In addition, the data from the 16 h incubation period moved isoeugenol from the non-sensitizer category to the sensitizer category, increasing accuracy to 94%.

3.2. Comparison between the criterion with I.I.-SLR-LA **P** *0.2 and that with I.I.-SLR-LA* **P** *0.05*

After increasing the incubation time from 6 h to 16 h, we examined 89 chemicals that were used as a data set of h-CLAT (Ashikaga et al., 2010) and evaluated their skin sensitization potential (Table S1). The dataset for the IL-8 Luc assay was created following examination of the IL-8 Luc assay's performance using the six criteria described in Table 2. When we examined its performance using Criterion A, which used the same condition 1 as the original criterion, Cooper statistics of the IL-8 Luc assay for these 89 chemicals yielded an accuracy of 69%, a sensitivity of 59%, and a specificity of 92%, suggesting that the IL-8 Luc assay using the current criterion produce false negative results for a considerable number of sensitizers tested. Closer examination of the data showed that most of the treatments that produced false negative results increased FInSLO-LA more than 1.4 at the concentrations providing I.I.-SLR-LA < 0.2 (data not shown). SLR-LA corresponds with promoter activity of GAPDH gene. GAPDH mRNA is a ubiquitously expressed at moderately abundant levels. It is frequently used as an endogenous control for quantitative real-time polymerase chain reaction because, in some experimental systems, its expression is constant at different times and after various experimental manipulations (Edwards and Denhardt, 1985; Mori et al., 2008; Winer et al., 1999). Indeed, in this study, all but two (benzocaine and methylisothiazolinone) of the 122 chemicals examined did not increase SLR-LA, and most dose-dependently decreased SLR-LA at toxic concentrations, suggesting that SLR-LA can act as an internal control to indicate cell number and viability. Our previous study demonstrated that I.I.-SLR-LA is more sensitive in detecting dying cells than the percentage of PI-excluding cells, and cells showing less than 0.2 of I.I.-SLR-LA retained more than 80% of PI-excluding cells. In this study, we further examined the correlation between the percentage of PI-excluding cells and I.I.-SLR-LA, and confirmed that THP-G8 cells treated with chemicals whose I.I.-SLR-LA showed P0.05 maintained more than 75% of the PI-excluding cells (Fig. 3). We therefore examined the performance of the IL-8 Luc assay using Criterion C that included I.I.-SLR-LA **P** 0.05 instead of I.I.-SLR-LA **P** 0.2 in the condition 1 and obtained Cooper statistics for these 89 chemicals of 74% accuracy, 69% sensitivity, and 89% specificity.

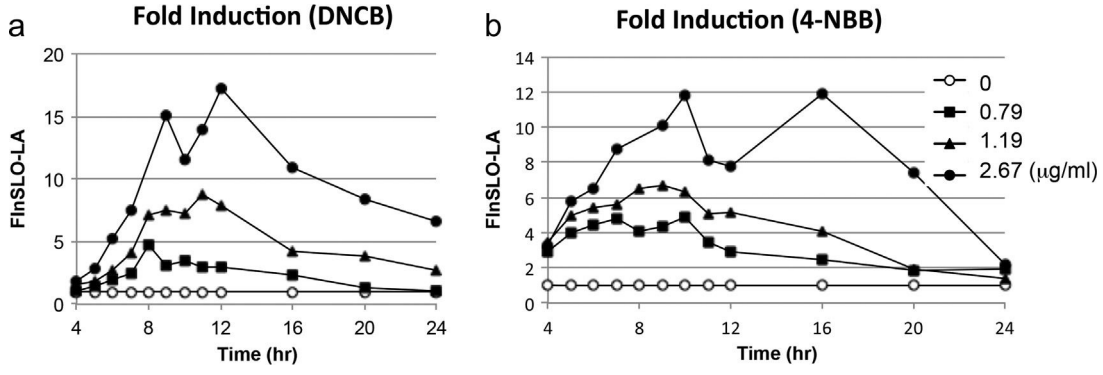


Fig. 1. Determination of the optimal incubation time – time course study for the IL-8 Luc assay. THP-G8 cells were stimulated with the indicated dose of DNCB (a) or 4-NBB (b) for various time periods, and luciferase activity was measured using a microplate-type luminometer.

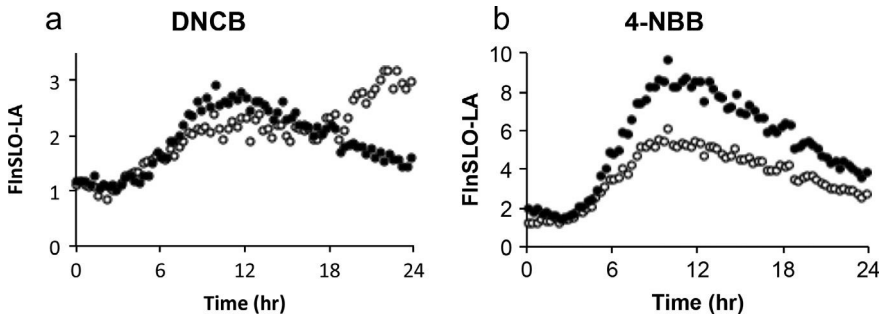


Fig. 2. Determination of the optimal incubation time – real time monitoring of IL-8 luciferase activity. THP-G8 cells were suspended in RPMI-1640 supplemented with 10% FBS, 0.1 mM D-luciferin, 25 mM HEPES/HCl (pH 7.0) and plated onto 35 mm dishes at 2×10^6 cells/dish. After 30 min, 0.8 or 1.6 lg/ml DNCB (a), or 0.8 or 1.6 lg/ml 4-NBB (b), was added to the culture. Bioluminescence was continuously recorded at intervals of 19 min under a 5% CO_2 atmosphere at 37 °C using a dish-type luminometer. Open circles: 0.8 lg/ml, closed circles: 1.6 lg/ml.

3.3. Comparison between the criterion with and without the response to NAC treatment

Despite having accepted the I.I.-SLR-LA P 0.05 criterion, the IL-8 Luc assay still produced significant numbers of false negative results. Therefore, we further modified the criterion. Although most haptens react with cysteine residues, there may be some exceptions. For example, Gerberick et al. reported that phthalic anhydride and trimellitic anhydride significantly reacted with glutathione and lysine peptides, but not with cysteine peptides in their direct peptide reactivity assay (DPRA) (Gerberick et al., 2007). We hypothesized that removing the condition that I.I. is 60.8 (Criterion 1) would increase the accuracy and specificity of the IL-8 Luc assay. Indeed, this modification significantly improved the performance of the IL-8 Luc assay and significantly improved accuracy and sensitivity (i.e., accuracy of 78% and sensitivity of 77%) but decreased the specificity to 74% (Table S1 and Table 4), when we examined the 122 chemicals (9 extreme, 17 strong, 34 moderate, and 28 weak sensitizers, and 34 non-sensitizers as classified by the LLNA (Gerberick et al., 2005)) as a dataset for h-CLAT (Ashikaga et al., 2010; Nukada et al., 2012; Takenouchi et al., 2013).

3.4. Comparison between the criterion with FInSLO-LA P 1.4, the criterion with the lower limit of the 95% confidence interval of FInSLO-LA P 1.0, and their combination

We empirically determined the condition of FInSLO-LA P 1.4 (Takahashi et al., 2011), similar to the condition used in h-CLAT (Sakaguchi et al., 2006). In contrast, each independent repetition was statistically evaluated in the KeratinoSens assay (Emter et al., 2010). We likewise tried to evaluate each IL-8 Luc assay experiment statistically. When we evaluated 122 chemicals using the criterion of the lower limit of the 95% confidence interval of FInSLO-LA P 1.0 (Criterion 2) did not necessarily improve the performance of the IL-8 Luc assay (accuracy of 74%, sensitivity of 80%, and 59% of specificity). We also examined the performance of the combination of Criterion 1 and Criterion 2 (Criterion 3). Both the accuracy and sensitivity of Criterion 3 was inferior to those of Criterion 1, although the specificity of Criterion 3 and Criterion 1 was equal (Table S1 and Table 4).

3.5. The factors that produce false negative or positive results in the IL-8 Luc assay (1) – physical properties

The IL-8 Luc assay conducted according to Criterion 1 produced 9 false negative results among 88 haptens determined by LLNA. To clarify the underlying reason for these false negative results, we first compared two physical properties of haptens judged by the IL-8 Luc assay to be sensitizers (true positive) and those judged to be non-sensitizers (false negative): molecular weight, and Log Ko/*w* or water solubility. The results could not demonstrate statistically significant differences (Fig. 4). Indeed, 8 sensitizers had Log Ko/*w* values above 3.5. The accuracy and sensitivity of these chemicals by the IL-8 Luc assay were both 87.5%.

3.6. The factors that produce false negative or positive results in IL-8 Luc assay (2) – the effects of FBS

The effects of FBS in the culture medium were considered next. Recently, several researchers have demonstrated that most sensitizers can bind to both FBS and cellular proteins, although the distribution of covalent binding to cellular or FBS protein varies depending on the hapten (Divkovic et al., 2005; Hopkins et al., 2005; Saito et al., 2013). These reports suggested that the amount of reactive electrophiles in haptens to bind to nucleophiles of cellular protein might be reduced in the presence of FBS.

Therefore, we examined whether a reduction in FBS concentration in the IL-8 Luc assay can reduce false negative results. First, THP-G8 cells were stimulated with oxazolone, which was judged as a non-sensitizer by the IL-8 Luc assay. Three methodologies were examined to solubilize oxazolone: (1) solubilize in DMSO, and then dilute with RPMI-1640 containing 10% FBS, as used in the IL-8 Luc assay (DMSO/FBS); (2) solubilize in DMSO and then dilute with X-VIVO (DMSO/X-VIVO); and (3) solubilize in X-VIVO and then dilute with X-VIVO (X-VIVO/X-VIVO) (Fig. 5a–c). As we have repeatedly demonstrated, oxazolone diluted with DMSO/FBS did not induce significant induction of FInSLO-LA at the concentration at which I.I.-SLR-LA showed P0.05. In contrast, oxazolone diluted with X-VIVO/X-VIVO significantly and dose-dependently induced FInSLO-LA at the concentration at which I.I.-SLR-LA showed P0.10. Oxazolone diluted with DMSO/X-VIVO significantly induced FInSLO-LA at the concentration at which I.I.-SLR-LA showed P0.6, but significant induction was observed only at this single concentration. These results clearly demonstrated that the dilution of oxazolone with X-VIVO significantly improved the response of THP-G8 cells. Furthermore, contrary to our expectation, even solubilization of oxazolone with DMSO did not necessarily improve THP-G8 response.

Clearly, FBS perturbed the response of THP-G8 cells for oxazolone; we therefore next examined whether changing the culture medium during treatment with the chemicals from RPMI-1640 with 10% FBS to X-VIVO improved the response of THP-G8 cells to haptens. The results clearly showed that the response of THP-G8 cells to oxazolone became far weaker in X-VIVO than that RPMI-1640 with 10% FBS (data not shown).

Table 3
Comparison of the performance between IL-8 Luc assay (16 h) and IL-8 Luc assay (6 h).

Chemical	LLNA	The IL-8 Luc assay (6 h) Takahashi et al. in Toxicol. Sci.								The IL-8 Luc assay (16 h)							
		1st		2nd		3rd		Positive exp	Decision	1st		2nd		3rd		Positive exp	Decision
		FInSLO-LA	I.I.	FInSLO-LA	I.I.	FInSLO-LA	I.I.			FInSLO-LA	I.I.	FInSLO-LA	I.I.	FInSLO-LA	I.I.		
Oxazolone	Sensitizer	1	N.D.	1	N.D.			0	Non-sens	1.43	1.15	1.29	1.03			0	Non-sens
4-NBB	Sensitizer	3.9	0.27	4.4	0.21			2	Sens	7.4	0.14	6.63	0.14			2	Sens
DNCB	Sensitizer	2.3	0.44	1.8	0.53			2	Sens	5.83	0.15	10.38	0.08			2	Sens
MDGN	Sensitizer	1.6	0.65	2.7	0.5			2	Sens	3.68	0.28	1.87	0.47			2	Sens
Glyoxal	Sensitizer	1.7	0.77	1.4	0.76			2	Sens	2.97	0.71	3.76	0.3			2	Sens
2-MBT	Sensitizer	1.9	0.7	1.9	0.76			2	Sens	2.29	0.78	6.36	0.56			2	Sens
Cinnamal	Sensitizer	2	0.54	2.7	0.42			2	Sens	5.09	0.19	8.05	0.15			2	Sens
TMTD	Sensitizer	1.7	0.63	3.6	0.44			2	Sens	7.28	0.14	3.95	0.28			2	Sens
PPD	Pre/pro hapten	1.5	0.66	2.3	0.57			2	Sens	1.61	0.61	1.76	0.6			2	Sens
Isoeugenol	Pre/pro hapten	1.8	0.79	1.8	0.86	1.55	1.04	1	Non-sens	2.94	0.74	4.07	0.44			2	Sens
Eugenol	Pre/pro hapten	2.1	0.64	1.7	0.77			2	Sens	4.85	0.69	2.72	1.17	1.69	0.72	2	Sens
Cinnamic alcohol	Pre/pro hapten	2.1	0.47	2.3	0.76			2	Sens	7.68	0.54	8.52	0.54			2	Sens
Glycerol	Irritant	1.5	0.82	1.4	0.83			0	Non-sens	1.12	0.95	1.03	1.25			0	Non-sens
Salicylic acid	Irritant	1.3	1.27	1.1	0.98			0	Non-sens	1	1	1	1			0	Non-sens
Lactic acid	Irritant	1.2	0.82	1.1	1.32			0	Non-sens	1.35	1.95	1.54	2.18			0	Non-sens
SLS	Irritant	3.1	0.88	4.6	0.86			0	Non-sens	2.84	1.18	4.49	1.04			0	Non-sens

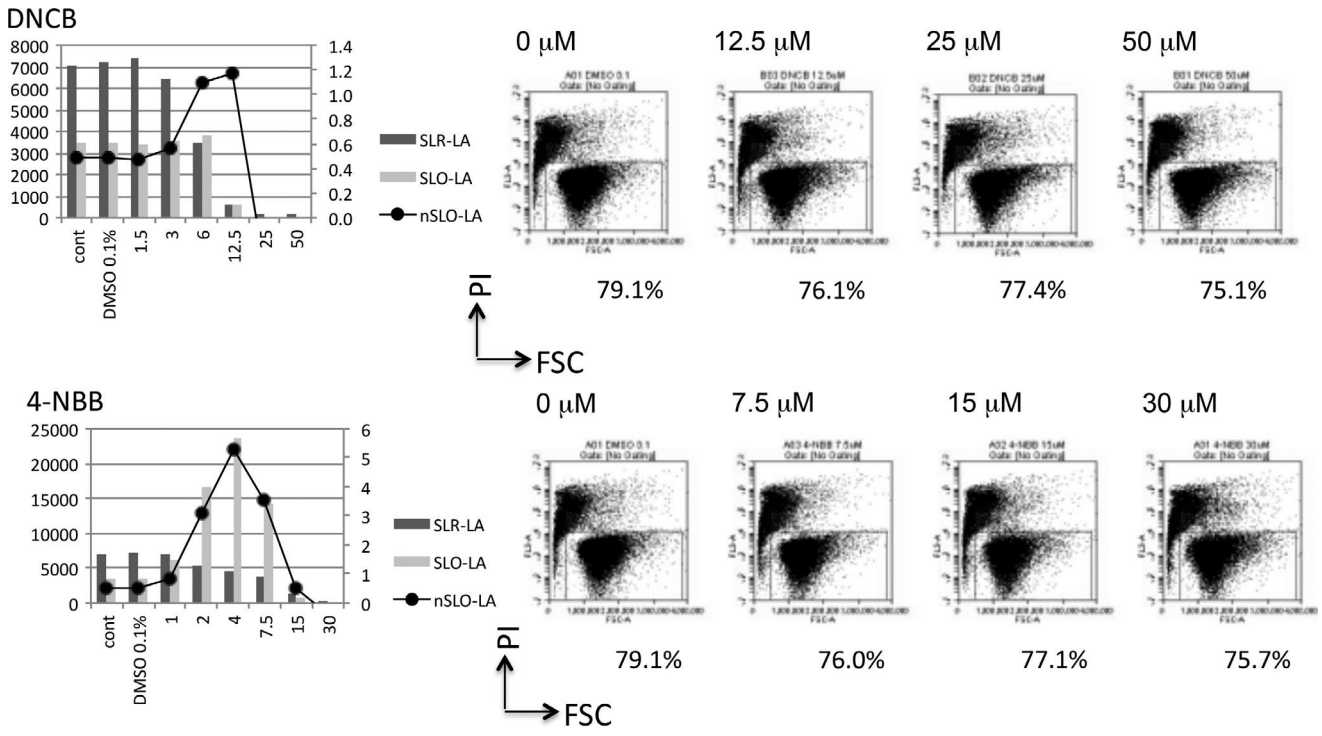


Fig. 3. Comparison between criterion with I.I.-SLR-LA P 0.2 and that with I.I.-SLR-LA P 0.05. Cell viability was determined by a PI exclusion assay using flow cytometry. THP-G8 cells were stimulated with the indicated dose of DNCB or 4-NBB. In this PI exclusion assay, THP-G8 cells after chemical treatment were mixed with 30 lg/ml of PI, and the live cells (which are not permeable to PI) were counted using flow cytometry. The results of the luciferase assay are shown on the left.

Table 4
Comparison of the performance among different criterion.

Criteria	With I.I. criterion (I.I. 6 0.8)			Without I.I. criterion		
	Without I.I.-SLR-LA criterion	I.I.-SLR-LA P 0.2	I.I.-SLR-LA P 0.05	Criterion 1	Criterion 2	Criterion 3
Chemical numbers	98	89	97	122	122	122
Accuracy	0.75	0.69	0.74	0.77	0.73	0.73
Sensitivity	0.69	0.59	0.69	0.77	0.80	0.72
Specificity	0.89	0.92	0.89	0.74	0.59	0.74

Next, we examined whether the other chemicals providing false negative results in the IL-8 Luc assay could be judged as sensitizers if diluted with X-VIVO/X-VIVO (Table 5). Interestingly, 5 of the 14 chemicals that showed false negative results were judged as sensitizers. To confirm the efficacy of solubilization of chemicals with X-VIVO, we re-evaluated the chemicals in the ECVAM list (Table 6). The IL-8 Luc assay using chemicals diluted with X-VIVO/X-VIVO increased the FInSLO-LA of all sensitizers, i.e., oxazolone, 4-NBB, DNCB, MDGN, eugenol, and PPD, and changed the judgment of oxazolone. As a result, the Cooper statistics of the IL-8 Luc assay using chemicals diluted with X-VIVO yielded an accuracy of 94%, a sensitivity of 100%, and a specificity of 75%. These data suggested that the IL-8 Luc assay diluted with X-VIVO can improve the accuracy and sensitivity, while it does not lower the specificity.

3.7. The factors that produce false negative or positive results in IL-8 Luc assay (3) – detergents

The IL-8 Luc assay conducted according to Criterion 1 produced 8 false positive results among 28 non-sensitizers, of which hexadecyltrimethylammonium bromide, benzalkonium chloride, Tween-80, and SLS are well-known detergents. It has been reported that the treatment of reconstructed human epidermis with detergents, such as sodium lauryl sulfate, triton, and benzalkonium chloride, increased IL-8 mRNA levels in and IL-8 release from the cells (Coquette et al., 1999). Moreover, White et al. have demonstrated that SLS induced early growth response-1 (EGR-1) depending on the activation of MEK1/p44/42 ERK and EGFR (White et al., 2011). In contrast to these observations on epidermal cells, several researchers examined IL-8 release by THP-1 cells and demonstrated the lack of IL-8 production when stimulated with SLS (Mitjans et al., 2008; Trompezinski et al., 2008). However, the concentration of SLS used in these studies was 30 lg/ml or less. Since the optimal concentration of SLS to induce FInSLO-LA is 50 lg/ml, and the concentration range that significantly augmented FInSLO-LA was narrow, the previous authors either did not stimulate THP-1 with the optimal concentration of SLS or there was a discrepancy between IL-8 release and IL-8 mRNA induction that corresponds with FInSLO-LA. Regardless, we propose not to evaluate detergents by the IL-8 Luc assay.

3.8. The factors that produce false negative or positive results in the IL-8 Luc assay (4) – relative human skin sensitizing potency

Recently, Basketter et al. (2014) collected data regarding the sensitizing potential of chemicals to human skin and classified 131 chemicals into 6 categories based on their relative human skin

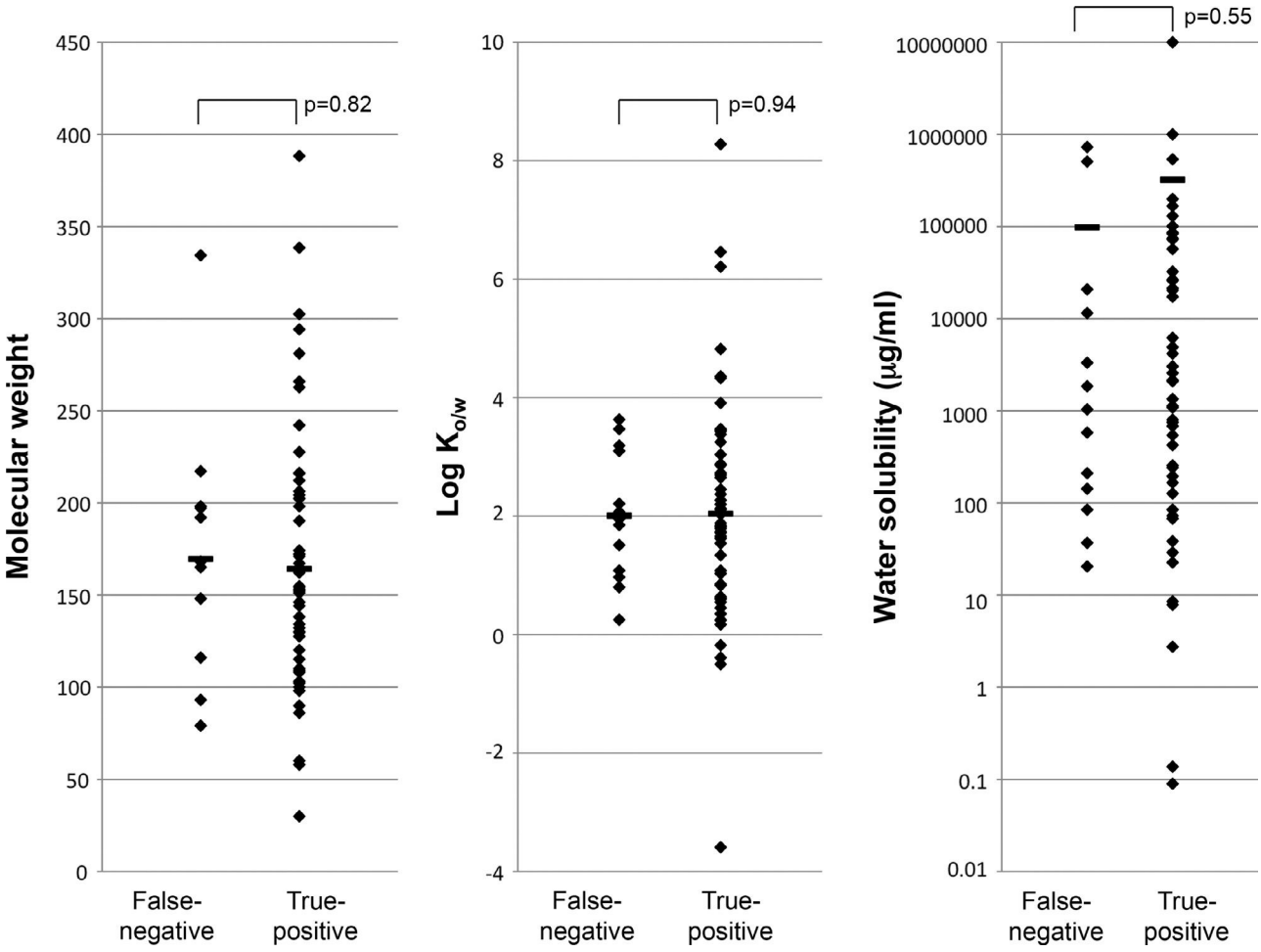


Fig. 4. Factors responsible for false negative or positive results in the IL-8 Luc assay (chemical properties). Sensitizers judged by the LLNA are divided into false negative and true positive groups according to the results of the IL-8 Luc assay. Molecular weight, Log Ko/*w*, and water solubility of each chemical are plotted on the *y*-axis for each group. The mean and *p* value compared with Student's *t*-test are shown.

sensitizing potency, with category 1 being the most potent, category 5 being the least potent, and category 6 being true non-sensitizers. Comparison of the relative human skin sensitizing potency of these chemicals with their LLNA evaluation provided excellent correlation in that all sensitizers judged by relative human skin sensitizing potency are also classified as sensitizers by LLNA. However, there were some differences between the two categorization schemes. For example, although vanillin and benzalkonium chloride are classified as non-sensitizers by LLNA, relative human skin sensitizing potency included them in category 5, the least potent sensitizer. The IL-8 Luc assay also judged vanillin as sensitizers.

3.9. The performance of the IL-8 Luc assay after considering the exclusion criterion and human sensitization potential, and using X-VIVO as a solvent

If we delete the data of hexadecyltrimethylammonium bromide, Tween-80, SLS and benzalkonium chloride and consider vanillin as a sensitizer, the performance of the IL-8 Luc assay was accuracy of 81%, sensitivity of 79%, and specificity of 90% in Criterion 1, accuracy of 77%, sensitivity of 80%, and specificity of 69% in Criterion 2, and accuracy of 77%, sensitivity of 73%, and specificity of 90 in Criterion 3 (Table 7). Furthermore, if we consider the results using X-VIVO as a solvent, the performance of the IL-8 Luc assay was accuracy of 90%, sensitivity of 90%, and specificity of 90% in Criterion 1, accuracy of 82%, sensitivity of 87%, and specificity of 69% in Criterion 2, and accuracy of 86%, sensitivity of 84%, and specificity of 90% in Criterion 3 (Table 8).

3.10. Correlation between the IL-8 Luc assay and other screening methods

Next, we examined the correlation of the parameters between the IL-8 Luc assay and other assays. Statistical analysis of the correlation between FInSLO-LA, and the percent depletion of peptides containing either lysine or cysteine in DPRA, provided no significant correlation (Fig. 6). Similarly, we also examined the correlation between the minimum concentration required to induce more than 1.4 of FInSLO-LA in the IL-8 Luc assay (IL-8 Luc assay minimum induction threshold (MIT)) and that required to induce more than 150% of CD86 augmentation (h-CLAT MIT (CD86 EC150)) (Fig. 7a) or more than 200% of CD54 in h-CLAT (h-CLAT MIT (CD54 EC200)) (Fig. 7b). The results demonstrated weak correlation between them (Fig. 7a and b). In contrast, there was no significant correlation between the IL-8 Luc assay MIT and EC 1.5 of KeratinoSens (Fig. 7c).

3.11. Score-based battery system

To improve the performance of the IL-8 Luc assay for predicting sensitizing potential and the potency of chemicals, we developed a

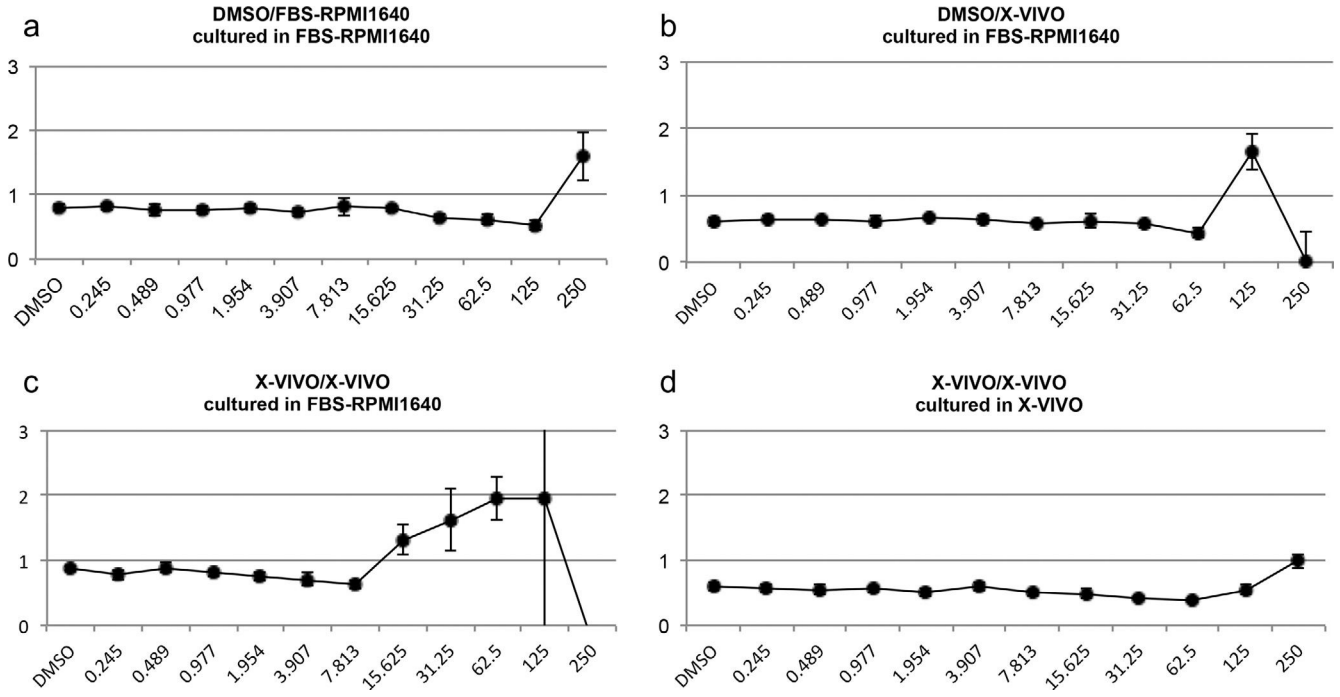


Fig. 5. The effects of FBS on the IL-8 Luc assay. To stimulate THP-G8 cells, we solubilized oxazolone in the following 3 ways: (a) solubilize in DMSO and then dilute with RPMI-1640 containing 10% FBS as used in the IL-8 Luc assay (DMSO/FBS); (b) solubilize in DMSO and then dilute with X-VIVO (DMSO/X-VIVO); and (c) solubilize in X-VIVO and then dilute with X-VIVO (X-VIVO/X-VIVO).

Table 5
Re-evaluation of chemicals demonstrating false negative results by the IL-8 Luc assay using X-VIVO as a solvent.

Chemicals	Experiments		Judgment
	1st	2nd	
Oxazolone	1.66	3.98	Sensitizer
Phthalic anhydride	1.09	1.60	
2-Hydroxyethyl acrylate	1.00	1.00	
Ethylenediamine	1.96	2.38	Sensitizer
Methyl-2-nonynoate	1.93	5.91	Sensitizer
3,4-Dihydrocoumarin	1.00	1.00	
Trimellitic anhydride	2.28	1.66	Sensitizer
1-Bromohexxane	1.07	1.05	
4-Allylanisole	4.67	19.71	Sensitizer
Benzocaine	1.11	1.00	
Ethyleneglycol dimethacrylate	4.98	4.36	Sensitizer
Penicillin G	1.35	1.00	
Pyridine	1.09	1.00	
Aniline	1.03	1.12	

test battery by assigning scores to the outcomes in each single test, based on the concept reported by Jowsey et al. (2006). We converted the results of the IL-8 Luc assay and DPRA into a score from 0 to 2 based on the sensitizing potency classification. In the IL-8 Luc assay, we first obtained the maximum value of FInSLO-LA (MAX FInSLO-LA) and the MIT for each chemical. MAX FInSLO-LA was the largest value of FInSLO-LA in all repeated experiments. The MIT was defined as the lowest value among the concentrations in all repeated experiments in which the chemical induced FInSLO-LA more than 1.4. MAX FInSLO-LA/MIT was calculated by dividing MAX FInSLO-LA by MIT and each chemical was given a score of 0, 1, or 2 based on the criterion shown in Table 9.

The mean ± SEM of MAX FInSLO-LA/MIT of chemicals in each group with different allergenicity was shown in Fig. 8a. Generally, the MAX FInSLO-LA/MIT values were higher in the group of chemicals containing more potent sensitizers. The data and the scoring system published by Nukada et al. (2013) and Jaworska et al. (2013) were used for DPRA and DEREK. The positive results in DPRA were classified as strong if the average depletion score of cysteine and lysine peptide was above 22.62% and weak if it was above 6.376% and less than or equal to 22.62%. Similarly, if the analysis outcome by DEREK was defined as probable or plausible, the test chemical was judged as a sensitizer. If defined as doubtful or no report, the test chemical was judged as a non-sensitizer. For the 103 test chemicals, the total battery score between 0 and 5 was then calculated by the sum of the individual scores. The box plot indicated the resulting scores split up for the five LLNA potency classifications (i.e., extreme, strong, moderate, weak, and not classified) (Fig. 8b). The median values in the box plot of the IL-8 Luc assay combined with DPRA and DEREK decreased with the corresponding LLNA potency classes with a better linear correlation ($R^2 = 0.90$) than that of the IL-8 Luc assay alone.

Table 6
Re-evaluation of the chemicals in the ECVAM List by the IL-8 Luc assay using X-VIVO as a solvent.

Chemicals	Experiments		Judgment
	1st	2nd	
Oxazolone	7.69	3.98	Sensitizer
4-NBB	10.03	8.77	Sensitizer
Glyoxal	3.50	2.23	Sensitizer
2-MBT	5.17	4.37	Sensitizer
DNCB	14.54	9.48	Sensitizer
MDGN	4.23	4.35	Sensitizer
Cinnamal	1.53	5.57	Sensitizer
TMTD	4.58	3.61	Sensitizer
PPD	4.30	2.48	Sensitizer
Isoeugenol	1.89	1.59	Sensitizer
Eugenol	2.57	2.52	Sensitizer
Cinnamic alcohol	7.24	7.13	Sensitizer
Glycerol	1.09	1.04	Non-sensitizer
Salicylic acid	1.18	1.00	Non-sensitizer
Lactic acid	1.00	1.08	Non-sensitizer
SLS	2.94	2.88	Sensitizer

Table 7
Performance of the IL-8 Luc assay after deleting the data for detergents and considering human sensitizing potential.

Criteria	IL-8 Luc assay			Modified IL-8 Luc assay		
	Criterion 1	Criterion 2	Criterion 3	Criterion 1	Criterion 2	Criterion 3
Chemical numbers	122	122	122	118	118	118
Accuracy	0.77	0.73	0.73	0.81	0.77	0.77
Sensitivity	0.77	0.80	0.72	0.79	0.80	0.73
Specificity	0.74	0.59	0.74	0.90	0.69	0.90

Modified IL-8 Luc assay: Deleting the data for detergents and considering human sensitizing potential.

Table 8
Performance of the IL-8 Luc assay after deleting the data for detergents, considering human sensitizing potential, and changing to X-VIVO as a solvent.

Criteria	IL-8 Luc assay			Modified IL-8 Luc assay		
	Criterion 1	Criterion 2	Criterion 3	Criterion 1	Criterion 2	Criterion 3
Chemical numbers	122	122	122	118	118	118
Accuracy	0.77	0.73	0.73	0.90	0.82	0.86
Sensitivity	0.77	0.80	0.72	0.90	0.87	0.84
Specificity	0.74	0.59	0.74	0.90	0.69	0.90

Modified IL-8 Luc assay: Deleting the data for detergents, considering human sensitizing potential, and changing to X-VIVO as a solvent.

Next, we set the positive criterion as a score of above 2 for hazard identification. Furthermore, to determine the sensitizing potency using a three-rank classification (strong, weak, and not classified) from the total battery scores, we set the strong score as 5, the weak score as 4, 3, and 2, and not classified as 1 and 0. Table 10 demonstrates the results of hazard identification and shows that the sensitivity, the specificity and the overall accuracy of the score-based battery system was 87.1%, 80.8%, and 85.4%, respectively, according to the Cooper statistics. All these parameters were improved compared to either DPRA or the IL-8 Luc assay alone for the examined chemical sets.

In the potency classification (Table 11), the strong class in this battery system included 14 of 23 extreme and strong sensitizers in the LLNA. Likewise, the weak class included 38 of 54 moderate and weak sensitizers in the LLNA. Thus, the accuracy in the potency prediction with the battery system was 70.9%, the over-prediction rate was 10.7%, and the under-prediction rate was 18.4%. These data suggested that the potential and potency of sensitizing chemicals were identified with good reliability by the score-based battery system.

3.12. Tiered system with h-CLAT and DPRA

We next developed a tiered approach, weighing the predictive performance of the IL-8 Luc assay and DPRA for the 103 evaluated chemicals. The IL-8 Luc assay provided high sensitivity and sufficiently detected extreme and strong sensitizers (Fig. 8a), indicating that the IL-8 Luc assay was a good first step for a tiered approach. The positive results in the IL-8 Luc assay were classified into two classes, strong or weak, based on Max-FInSLO-LA/MIT values. Then, to reliably predict weak and moderate sensitizers, which the IL-8 Luc assay failed to detect, we determined that DPRA was a good second step. The positive results in DPRA were classified into the weak class regardless of the average depletion score. If the chemical scored as negative in both tests, it was considered as not classified. As shown in Table 12, the tiered system provided a relatively high sensitivity of 96.1% (74 of 77 sensitizers) and an accuracy of 87.4% (90 of 103 test chemicals).

The potency classification by the tiered system is summarized in Table 13. All tested chemicals, except for 1 chemical classified as strong in the tiered system, were sensitizers in the LLNA, suggesting a positive predictivity of 98.0% in this class. Moreover, 16 of 19 chemicals categorized as not classified by the tiered system were non-sensitizers in the LLNA, suggesting a negative predictivity of 84.2%. The false negative rate in the weak class was 5.5% (3 of 54 sensitizers). Importantly, the tiered system with the IL-8 Luc assay and DPRA could detect 51 of 54 sensitizers classified as moderate and weak in the LLNA. The strong, weak, and non-classified classes have good correlation with the extreme/strong, moderate/weak, and non-classified class in the LLNA. Thus, the accuracy of potency prediction with the battery system was 62.1%, the over-prediction rate was 28.2%, and the under-prediction rate was 9.7%.

4. Discussion

In parallel with conducting the inter- and intra-laboratory reproducibility tests, we tried to improve the performance of the IL-8 Luc assay. Consequently, we found that the modification of the original protocol of the IL-8 Luc assay by increasing the incubation time, revising the lower limit of I.I.-SLR-LA, deleting the process to examine the effects of NAC, and setting the exclusion criterion could substantially improve the performance of the IL-8 Luc assay. In final, by taking these modifications into account, the performance of the IL-8 Luc assay was accuracy of 81%, sensitivity of 79%, and specificity of 90% in the evaluation of 122 chemicals.

In addition, we here examined the difference in determining positive induction of SLO-LA between Criterion 1 and Criterion 2. Condition 1 used in Criterion 1 was determined empirically, while that used in Criterion 2 was based on the statistical significance. After examining the 122 chemicals, the concordance rate was 90%, which suggests that condition 1 used in Criterion 1, FInSLO-LA $\mathbf{P}$ 1.4 at the concentration of the chemical at which I.I.-SLR-LA is P0.05, is statistically supported. We also accepted the new condition 2 to judge sensitizers based on the repeated experiments: chemicals are categorized as sensitizers when they fulfill condition 1 in two of two to four different experiments, while they were categorized as sensitizers when they fulfilled condition 1 in two or three of three different experiments in our previous report (Takahashi et al., 2011). In this new condition 2, some experiments showing positive induction are repeated only twice to judge sensitizers, while those showing negative induction are repeated three times to judge non-sensitizers. Therefore, to verify this condition, we repeated three experiments for 21 chemicals

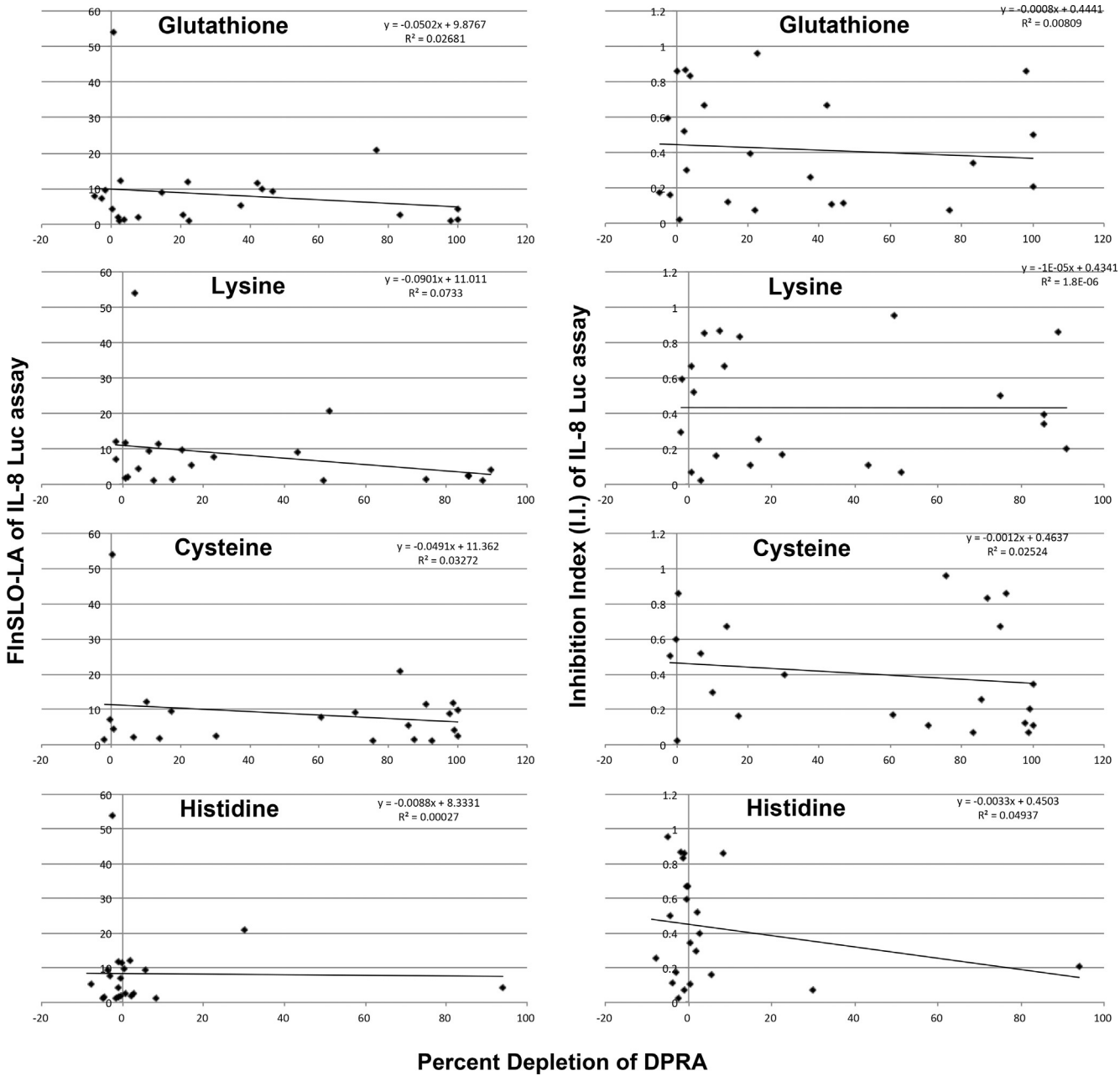


Fig. 6. Correlation between the IL-8 Luc assay and other screening methods (DPRA). The correlation between FInSLO-LA and the percent depletion of peptides containing either lysine or cysteine in DPRA was examined.

that showed positive induction in the first two experiments. The results demonstrated that the third experiment also showed positive induction in all 21 chemicals, verifying the new condition 2.

In this study, we examined 4 detergents, i.e., hexadecyltrimethylammonium bromide, benzalkonium chloride, Tween-80 and SLS and found that these detergents significantly increased FInSLO-LA, which suggested that they could stimulate IL-8 promoter activity. These data are consistent with the previous reports that have demonstrated IL-8 production by keratinocytes after the stimulation of various detergents (Coquette et al., 1999; White et al., 2011). Detergents can be categorized according to the charge present in the hydrophilic head (after dissociation in aqueous solution) into four primary groups: anionic, cationic, amphoteric (dual charge) and nonionic (Corazza et al., 2010). Quaternary ammonium compounds, such as hexadecyltrimethylammonium bromide and benzalkonium chloride, are cationic, while Tween-80 and SLS are nonionic and anionic, respectively. Therefore, our data suggested that detergents could stimulate IL-8 promoter activity irrespective of kinds of the charges present in the hydrophilic head. It is not necessarily clear how detergents induce IL-8 mRNA. In contrast to haptens that induce IL-8 mRNA expression by DCs or THP-1 cells depending on p38 MAPK, however, at least SLS has been demonstrated to induce IL-8 mRNA depending on the activation of MEK1/p44/42 ERK (White et al., 2011). Indeed, our previous study demonstrated NAC could not suppress FInSLO-LA induced by Tween-80, SLS, and benzalkonium chloride, while it significantly attenuated it induced by most haptens (Takahashi et al., 2011). Therefore, it is plausible to include the criterion for examining the effects of NAC on FInSLO-LA. However, since the IL-8 Luc assay including the criterion to examine the effects of NAC makes the test

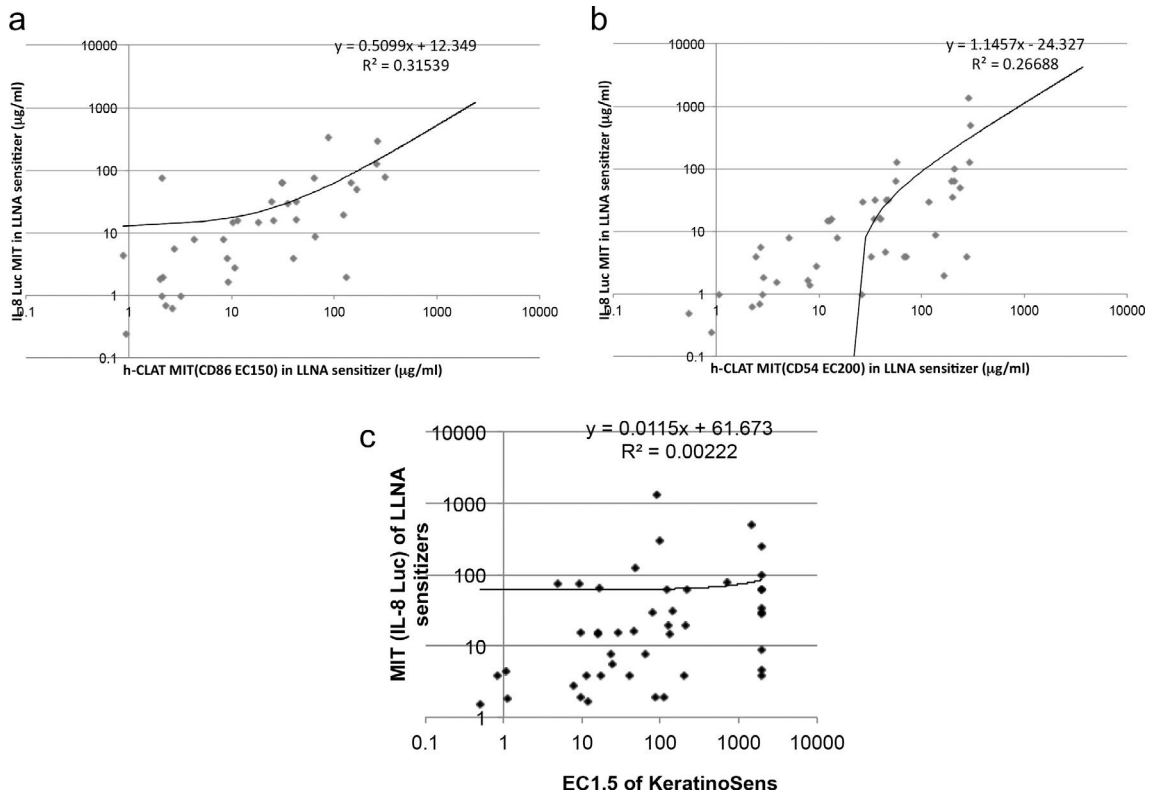


Fig. 7. Correlation between the IL-8 Luc assay and other screening methods (h-CLAT and KeratinoSens). The correlation between the minimum concentration required to induce more than 1.4 of FInSLO-LA in the IL-8 Luc assay (IL-8 Luc assay MIT) and that required to induce more than 150% of CD86 augmentation (h-CLAT MIT (CD86 EC150)) (a), that to induce more than 200% of CD54 in h-CLAT (h-CLAT MIT (CD54 EC200)) (b), and EC 1.5 of KeratinoSens (c).

Table 9
Conversion of the outcome in each single test into scores.

Score	MAX FInSLO-LA/minimum induction threshold (MIT) in IL-8 Luc assay	Avg. score in DPRA	DEREK or times
2	P0.1 (Strong positive)	>22.62% (Strong positive)	
1	0.01–0.1 (Weak positive)	6.376–22.62% (Weak positive)	Alert (Positive)
0	<0.01 (Negative)	<6.376% (Negative)	No alert (Negative)

The outcome of each single test was converted to a score, based on the previously reported concept by Jowsey et al. (2006).

method complicated and reduces intra- and inter-laboratory reproducibilities, we would like to include detergents in an exclusion criterion.

It is widely recognized that a single *in vitro* test is insufficient to replace animal testing and that integration of results from different *in vitro* tests, as well as *in silico* methods, is needed for prediction of the skin sensitization potential of chemicals, Since Jowsey et al. (2006) first proposed the integration framework based on the scoring system, weighing the evidence from structure–activity relationships in skin sensitization, penetration, peptide reactivity, and dendritic cell and T-cell activation to evaluate the sensitizing potential as well as the relative potency, a variety of test batteries integrated with different *in vitro* tests and/or *in silico* methods have been reported (Bauch et al., 2012; Jaworska et al., 2011; Natsch et al., 2009; Nukada et al., 2013; Tsujita-Inoue et al., 2014). Most of these approaches substantially improved the accuracy and sensitivity for the potential and potency prediction, compared with LLNA. Therefore, in this study, we also tried to combine the IL-8 Luc assay with other *in vitro* methods to test for skin sensitizing potentials.

Before determining the best combination of test methods, we first examined whether any of the parameters in the IL-8 Luc assay correlate with LLNA potency. Although we could not recognize significant correlation between MIT or FInSLO-LA of the IL-8 Luc assay and LLNA EC3 (data not shown), we found that the MAX FInSLO-LA/MIT values were higher in the group of chemicals with more potent sensitizers. Therefore, we decided to use the MAX FInSLO-LA/MIT with the battery approach.

When we tried score-based battery system and tiered battery system according to the procedure conducted by Nukada et al. (2013), both systems increased accuracy, 87.9% and 90.1%, respectively and the score-based system increased specificity while the tiered-system improved sensitivity. In particular, the chemicals judged as false negative by the IL-8 Luc assay due to the reactivity of chemicals with FBS can be judged as positive by DPRA except for ethylenediamine (Jaworska et al., 2013).

In addition to the yes/no prediction, *in vitro* approach to detect allergenicity of chemicals is required to predict the potency. However, the accuracy in the potency prediction with the scored battery system and the tiered battery system was 67% and 58.2%,

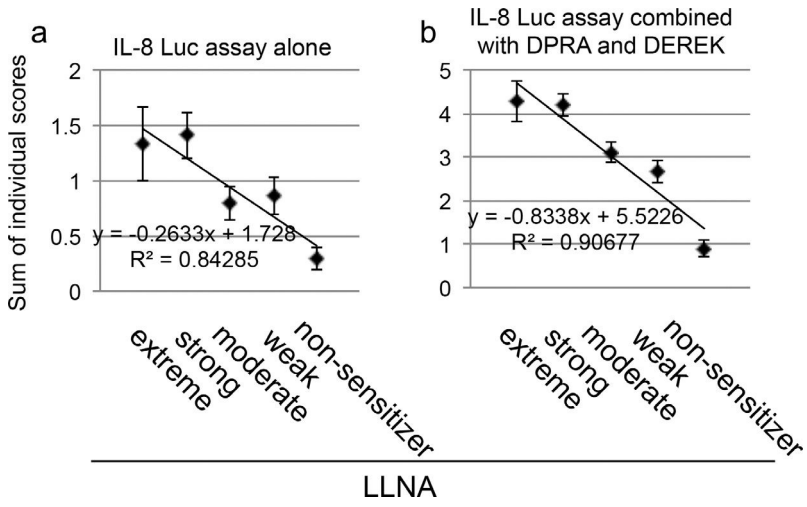


Fig. 8. Score-based battery system based on the IL-8 Luc assay. The mean ± SEM of MAX FInSLO-LA/MIT of chemicals in each group was plotted for different allergenicity groups in the LLNA after conversion of the raw data into a score based on Table 9 (a). The data and the scoring system published by Nukada et al. (2013) and Jaworska et al. (2013) were used for DPRA and DEREK. Then, for the 103 test chemicals, the total battery score between 0 and 5 was calculated by the sum of individual scores (b).

Table 10
Hazard identification by the score-based battery system based on the IL-8 Luc assay.

LLNA	Score-based battery system	
	Positive	Negative
Sensitizers (77)	67	10
Non-sensitizers (26)	5	21
	Sensitivity (%)	87.1
	Specificity (%)	80.8
	Accuracy (%)	85.4

respectively. It is still far from perfect. There is a significant spread for the quantitative data within individual potency classes. LLNA and human data correlate partly with each other, with an R^2 in log–log linear regression between LLNA and human data between 0.45 and 0.75 reported in different studies (ICCVAM, 2011). It means that EC3 in LLNA cannot correctly predict the potency classification of chemicals in humans. In addition, so far, it is not clear how the potency of haptens is determined. Most screening methods do not take T cell response into account. To improve the accuracy, it may need to construct a battery system with a screening method based on T cell response.

Finally, in this study, we explored the factors that impair the performance of the IL-8 Luc assay. Among them, the impacts of FBS on the performance of the IL-8 Luc assay was not negligible. It is a well-known procedure to solubilize water insoluble chemicals in DMSO and dilute the solution with culture medium. This procedure was also employed by h-CLAT and KeratinoSens. Indeed, most of water insoluble haptens we examined in this study significantly increased FInSLO-LA after diluted by this procedure, although we must admit that the IL-8 Luc assay with this procedure produced considerable numbers of false negative results. Surprisingly, the judgment of 5 among 14 haptens in 122 chemical lists we examined was corrected by diluting chemicals with X-VIVO. Saito et al. have reported the similar observation, in which hapten-induced ROS production by THP-1 was significantly attenuated in the presence of FBS (Saito et al., 2013). It is conceivable that FBS suppresses the binding of some haptens with cysteine or lysine residues. Therefore, our novel procedure in which chemicals are diluted with X-VIVO may significantly improve the performance of the IL-8 Luc assay.

There are several advantages in the IL-8 Luc assay. At first, the culture of THP-G8 cells are relatively simple and does not use trypsin or EDTA because THP-G8 cells do not stick to the culture dishes. The second is its simple procedure. At first, chemicals in graded concentrations are added into 96-well culture plate as required in every *in vitro* test method. Then, the cells adjusted to the optimum concentration are seeded to each plate. After 16 h incubation, the plates are set in the luminometer. The process afterward is completely automated except calculating the obtained results in the predesigned Excel sheet. Therefore, the IL-8 Luc assay is considered as a test method that can significantly reduce human errors.

Moreover, the IL-8 Luc assay does not need the step to preculture or determine cell viability after chemical treatment. In the IL-8 Luc assay, since THP-G8 cells can present the promoter activities of IL-8 promoter and GAPDH, a well known house keeping gene, the information of the effects of chemicals on both IL-8 induction and cell viability is obtained simultaneously in each experiment. Therefore, even though 4 experiments are required, one set of experiments can be completed within 4 days. Therefore, the IL-8 Luc assay is a truly high-through method.

Finally, this study succeeded in optimizing the IL-8 Luc assay to predict allergenicity of chemicals and demonstrated that the IL-8

Table 11
Potency classification by the score-based battery system based on the IL-8 Luc assay.

LLNA	Score-based battery system		
	Strong	Weak	Not-classified
Extreme + strong (23)	14	9	0
Moderate + weak (54)	6	38	10
Non-sensitizer (26)	0	5	21
Over prediction rate (%)		10.7% (11/91)	
Under prediction rate (%)		18.4% (19/103)	
Accuracy (%)		70.9% (73/103)	

Table 12
Hazard identification by the tiered system based on the IL-8 Luc assay.

LLNA	Tiered system	
	Positive	Negative
Sensitizers (77)	74	3
Non-sensitizers (26)	10	16
	Sensitivity (%)	96.1
	Specificity (%)	61.5
	Accuracy (%)	87.4

Table 13
Potency classification by tiered battery system based on the IL-8 Luc assay.

LLNA	Tiered system		
	Strong	Weak	Not-classified
Extreme + strong (23)	16	7	0
Moderate + weak (54)	19	32	3
Non-sensitizer (26)	1	9	16
Over prediction rate (%)		28.2% (29/103)	
Under prediction rate (%)		9.7% (10/103)	
Accuracy (%)		62.1% (64/103)	

Luc assay is a promising *in vitro* alternative method. Furthermore, the battery approach of the IL-8 Luc assay combined with DPRA and DEREK could significantly improve the performance.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

Acknowledgments

This work was supported in part by the New Energy and Industrial Development Organization (NEDO), by Grant-in-Aid for Challenging Exploratory Research of JSPS KAKENHI Grant Number 26670473, by Health Labor Sciences Research in Japan, and by Ministry Economy, Science and Industry in Japan, and Japanese Society for Alternatives to Animal Experiments.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tiv.2015.07.006.

References

Ashikaga, T., Sakaguchi, H., Sono, S., Kosaka, N., Ishikawa, M., Nukada, Y., Miyazawa, M., Ito, Y., Nishiyama, N., Itagaki, H., 2010. A comparative evaluation of in vitro skin sensitisation tests: the human cell-line activation test (h-CLAT) versus the local lymph node assay (LLNA). ATLA 38, 275–284.

Ashikaga, T., Yoshida, Y., Hirota, M., Yoneyama, K., Itagaki, H., Sakaguchi, H., Miyazawa, M., Ito, Y., Suzuki, H., Toyoda, H., 2006. Development of an in vitro skin sensitization test using human cell lines: the human Cell Line Activation Test (h-CLAT). I. Optimization of the h-CLAT protocol. Toxicol. In Vitro 20, 767–773.

Basketter, D.A., Alepee, N., Ashikaga, T., Barroso, J., Gilmour, N., Goebel, C., Hibatallah, J., Hoffmann, S., Kern, P., Martinozzi-Teissier, S., Maxwell, G., Reisinger, K., Sakaguchi, H., Schepky, A., Tailhardat, M., Templier, M., 2014. Categorization of chemicals according to their relative human skin sensitizing potency. Dermatitis 25, 11–21.

Bauch, C., Kolle, S.N., Ramirez, T., Eltze, T., Fabian, E., Mehling, A., Teubner, W., van Ravenzwaay, B., Landsiedel, R., 2012. Putting the parts together: combining in vitro methods to test for skin sensitizing potentials. Regul. Toxicol. Pharmacol. 63, 489–504.

Casati, S., Aeby, P., Kimber, I., Maxwell, G., Ovigne, J.M., Roggen, E., Rovida, C., Tosti, L., Basketter, D., 2009. Selection of chemicals for the development and evaluation of in vitro methods for skin sensitisation testing. ATLA 37, 305–312.

Coquette, A., Berna, N., Vandenbosch, A., Rosdy, M., Poumay, Y., 1999. Differential expression and release of cytokines by an in vitro reconstructed human epidermis following exposure to skin irritant and sensitizing chemicals. Toxicol. In Vitro 13, 867–877.

Corazza, M., Lauriola, M.M., Zappaterra, M., Bianchi, A., Virgili, A., 2010. Surfactants, skin cleansing protagonists. J. Eur. Acad. Dermatol. Venereol. 24, 1–6.

Divkovic, M., Pease, C.K., Gerberick, G.F., Basketter, D.A., 2005. Hapten-protein binding: from theory to practical application in the in vitro prediction of skin sensitization. Contact Dermatitis 53, 189–200.

Edwards, D.R., Denhardt, D.T., 1985. A study of mitochondrial and nuclear transcription with cloned cDNA probes. Changes in the relative abundance of mitochondrial transcripts after stimulation of quiescent mouse fibroblasts. Exp. Cell Res. 157, 127–143.

Emter, R., Ellis, G., Natsch, A., 2010. Performance of a novel keratinocyte-based reporter cell line to screen skin sensitizers in vitro. Toxicol. Appl. Pharmacol. 245, 281–290.

Feijoo, E., Alfaro, C., Mazzolini, G., Serra, P., Penuelas, I., Arina, A., Huarte, E., Tirapu, I., Palencia, B., Murillo, O., Ruiz, J., Sangro, B., Richter, J.A., Prieto, J., Melero, I., 2005. Dendritic cells delivered inside human carcinomas are sequestered by interleukin-8. Int. J. Cancer 116, 275–281.

Gerberick, G.F., Ryan, C.A., Kern, P.S., Schlatter, H., Dearman, R.J., Kimber, I., Patlewicz, G.Y., Basketter, D.A., 2005. Compilation of historical local lymph node data for evaluation of skin sensitization alternative methods. Dermatitis 16, 157–202.

Gerberick, G.F., Vassallo, J.D., Bailey, R.E., Chaney, J.G., Morrall, S.W., Lepoittevin, J.P., 2004. Development of a peptide reactivity assay for screening contact allergens. Toxicol. Sci. 81, 332–343.

Gerberick, G.F., Vassallo, J.D., Foertsch, L.M., Price, B.B., Chaney, J.G., Lepoittevin, J.P., 2007. Quantification of chemical peptide reactivity for screening contact allergens: a classification tree model approach. Toxicol. Sci. 97, 417–427.

Gouwy, M., Struyf, S., Leutenez, L., Portner, N., Sozzani, S., Van Damme, J., 2014. Chemokines and other GPCR ligands synergize in receptor-mediated migration of monocyte-derived immature and mature dendritic cells. Immunobiology 219, 218–229.

Holland, R., Fishbein, J.C., 2010. Chemistry of the cysteine sensors in Kelch-like ECH-associated protein 1. Antioxid. Redox Signal. 13, 1749–1761.

Hopkins, J.E., Naisbitt, D.J., Kitteringham, N.R., Dearman, R.J., Kimber, I., Park, B.K., 2005. Selective haptenation of cellular or extracellular protein by chemical allergens: association with cytokine polarization. Chem. Res. Toxicol. 18, 375–381.

ICCVAM, 2011. ICCVAM Test Method Evaluation Report: Usefulness and Limitations of the Murine Local Lymph Node Assay for Potency Categorization of Chemicals Causing Allergic Contact Dermatitis in Humans. <http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNA-pot/TMER.pdf>.

Itoh, K., Mimura, J., Yamamoto, M., 2010. Discovery of the negative regulator of Nrf2, Keap1: a historical overview. Antioxid. Redox Signal. 13, 1665–1678.

Jaworska, J., Dancik, Y., Kern, P., Gerberick, F., Natsch, A., 2013. Bayesian integrated testing strategy to assess skin sensitization potency: from theory to practice. J. Appl. Toxicol. 33, 1353–1364.

Jaworska, J., Harol, A., Kern, P.S., Gerberick, G.F., 2011. Integrating non-animal test information into an adaptive testing strategy – skin sensitization proof of concept case. ALTEX 28, 211–225.

Jimenez, F., Quinones, M.P., Martinez, H.G., Estrada, C.A., Clark, K., Garavito, E., Ibarra, J., Melby, P.C., Ahuja, S.S., 2010. CCR2 plays a critical role in dendritic cell maturation: possible role of CCL2 and NF-kappa B. J. Immunol. 184, 5571–5581.

Jowsey, I.R., Basketter, D.A., Westmoreland, C., Kimber, I., 2006. A future approach to measuring relative skin sensitising potency: a proposal. J. Appl. Toxicol. 26, 341–350.

Leonard, E.J., Skeel, A., Yoshimura, T., Noer, K., Kutvirt, S., Van Epps, D., 1990. Leukocyte specificity and binding of human neutrophil attractant/activation protein-1. J. Immunol. 144, 1323–1330.

Marchese, A., Heiber, M., Nguyen, T., Heng, H.H., Saldivia, V.R., Cheng, R., Murphy, P.M., Tsui, L.C., Shi, X., Gregor, P., et al., 1995. Cloning and chromosomal mapping of three novel genes, GPR9, GPR10, and GPR14, encoding receptors related to interleukin 8, neuropeptide Y, and somatostatin receptors. Genomics 29, 335–344.

Mitjans, M., Viviani, B., Lucchi, L., Galli, C.L., Marinovich, M., Corsini, E., 2008. Role of p38 MAPK in the selective release of IL-8 induced by chemical allergen in naive THP-1 cells. Toxicol. In Vitro 22, 386–395.

Mitjans, M., Galbiati, V., Lucchi, L., Viviani, B., Marinovich, M., Galli, C.L., Corsini, E., 2010. Use of IL-8 release and p38 MAPK activation in THP-1 cells to identify allergens and to assess their potency in vitro. Toxicol. In Vitro 24, 1803–1809.

Mori, R., Wang, Q., Danenberg, K.D., Pinski, J.K., Danenberg, P.V., 2008. Both beta-actin and GAPDH are useful reference genes for normalization of quantitative RT-PCR in human FFPE tissue samples of prostate cancer. Prostate 68, 1555–1560.

Murphy, P.M., Tiffany, H.L., 1991. Cloning of complementary DNA encoding a functional human interleukin-8 receptor. Science 253, 1280–1283.

Nakajima, Y., Kimura, T., Sugata, K., Enomoto, T., Asakawa, A., Kubota, H., Ikeda, M., Ohmiya, Y., 2005. Multicolor luciferase assay system: one-step monitoring of multiple gene expressions with a single substrate. Biotechniques 38, 891–894.

Natsch, A., Emter, R., Ellis, G., 2009. Filling the concept with data: integrating data from different in vitro and in silico assays on skin sensitizers to explore the battery approach for animal-free skin sensitization testing. Toxicol. Sci. 107, 106–121.

Natsuaki, Y., Egawa, G., Nakamizo, S., Ono, S., Hanakawa, S., Okada, T., Kusuba, N., Otsuka, A., Kitoh, A., Honda, T., Nakajima, S., Tsuchiya, S., Sugimoto, Y., Ishii, K.J., Tsutsui, H., Yagita, H., Iwakura, Y., Kubo, M., Ng, L.G., Hashimoto, T., Fuentes, J., Guttman-Yassky, E., Miyachi, Y., Kabashima, K., 2014. Perivascular leukocyte clusters are essential for efficient activation of effector T cells in the skin. Nat. Immunol. 15, 1064–1069.

Noguchi, T., Ikeda, M., Ohmiya, Y., Nakajima, Y., 2008. Simultaneous monitoring of independent gene expression patterns in two types of cocultured fibroblasts with different color-emitting luciferases. BMC Biotechnol. 8, 40.

Nukada, Y., Miyazawa, M., Kosaka, N., Ito, Y., Sakaguchi, H., Nishiyama, N., 2008. Production of IL-8 in THP-1 cells following contact allergen stimulation via mitogen-activated protein kinase activation or tumor necrosis factor-alpha production. J. Toxicol. Sci. 33, 175–185.

Nukada, Y., Ashikaga, T., Miyazawa, M., Hirota, M., Sakaguchi, H., Sasa, H., Nishiyama, N., 2012. Prediction of skin sensitization potency of chemicals by human Cell Line Activation Test (h-CLAT) and an attempt at classifying skin sensitization potency. Toxicol. In Vitro 26, 1150–1160.

Nukada, Y., Miyazawa, M., Kazutoshi, S., Sakaguchi, H., Nishiyama, N., 2013. Data integration of non-animal tests for the development of a test battery to predict the skin sensitizing potential and potency of chemicals. Toxicol. In Vitro 27, 609–618.

OECD, 2012. The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins, Part 1: Scientific Evidence. OECD Environment, Health and Safety Publications, Series on Testing and Assessment No. 168.

Piroird, C., Ovigne, J.M., Rousset, F., Martinozzi-Teissier, S., Gomes, C., Cotovio, J., Alepee, N., 2015. The Myeloid U937 Skin Sensitization Test (U-SENS) addresses the activation of dendritic cell event in the adverse outcome pathway for skin sensitization. Toxicol. In Vitro 29, 901–916.

Python, F., Goebel, C., Aeby, P., 2007. Assessment of the U937 cell line for the detection of contact allergens. Toxicol. Appl. Pharmacol. 220, 113–124.

Saito, K., Miyazawa, M., Nukada, Y., Sakaguchi, H., Nishiyama, N., 2013. Development of an in vitro skin sensitization test based on ROS production in THP-1 cells. Toxicol. In Vitro 27, 857–863.

Saito, R., Hirakawa, S., Ohara, H., Yasuda, M., Yamazaki, T., Nishii, S., Aiba, S., 2011. Nickel differentially regulates NFAT and NF-kappaB activation in T cell signaling. Toxicol. Appl. Pharmacol. 254, 245–255.

Sakaguchi, H., Ashikaga, T., Miyazawa, M., Yoshida, Y., Ito, Y., Yoneyama, K., Hirota, M., Itagaki, H., Toyoda, H., Suzuki, H., 2006. Development of an in vitro skin sensitization test using human cell lines; human Cell Line Activation Test (h-CLAT). II. An inter-laboratory study of the h-CLAT. Toxicol. In Vitro 20, 774–784.

Sebok, K., Woodside, D., Al-Aoukaty, A., Ho, A.D., Gluck, S., Maghazachi, A.A., 1993. IL-8 induces the locomotion of human IL-2-activated natural killer cells. Involvement of a guanine nucleotide binding (Go) protein. J. Immunol. 150, 1524–1534.

Singha, B., Gatla, H.R., Manna, S., Chang, T.P., Sanacora, S., Poltoratsky, V., Vancura, A., Vancurova, I., 2014. Proteasome inhibition increases recruitment of IkappaB kinase beta (IKKbeta), S536P-p65, and transcription factor EGR1 to interleukin-8 (IL-8) promoter, resulting in increased IL-8 production in ovarian cancer cells. J. Biol. Chem. 289, 2687–2700.

Takahashi, T., Kimura, Y., Saito, R., Nakajima, Y., Ohmiya, Y., Yamasaki, K., Aiba, S., 2011. An in vitro test to screen skin sensitizers using a stable THP-1-derived IL-8 reporter cell line, THP-G8. Toxicol. Sci. 124, 359–369.

Takenouchi, O., Miyazawa, M., Saito, K., Ashikaga, T., Sakaguchi, H., 2013. Predictive performance of the human Cell Line Activation Test (h-CLAT) for lipophilic chemicals with high octanol-water partition coefficients. J. Toxicol. Sci. 38, 599–609.

Toebak, M.J., Pohlmann, P.R., Sampat-Sardjoepersad, S.C., von Blomberg, B.M., Bruynzeel, D.P., Scheper, R.J., Rustemeyer, T., Gibbs, S., 2006. CXCL8 secretion by dendritic cells predicts contact allergens from irritants. Toxicol. In Vitro 20, 117–124.

Trompezinski, S., Migdal, C., Tailhardat, M., Le Varlet, B., Courtellemont, P., Haftek, M., Serres, M., 2008. Characterization of early events involved in human dendritic cell maturation induced by sensitizers: cross talk between MAPK signalling pathways. Toxicol. Appl. Pharmacol. 230, 397–406.

Tsujita-Inoue, K., Hirota, M., Ashikaga, T., Atobe, T., Kouzuki, H., Aiba, S., 2014. Skin sensitization risk assessment model using artificial neural network analysis of data from multiple in vitro assays. Toxicol. In Vitro 28, 626–639.

White, K.J., Maffei, V.J., Newton-West, M., Swerlick, R.A., 2011. Irritant activation of epithelial cells is mediated via protease-dependent EGFR activation. J. Invest. Dermatol. 131, 435–442.

Winer, J., Jung, C.K., Shackel, I., Williams, P.M., 1999. Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. Anal. Biochem. 270, 41–49.

Zhang, X., Chen, X., Song, H., Chen, H.Z., Rovin, B.H., 2005. Activation of the Nrf2/antioxidant response pathway increases IL-8 expression. Eur. J. Immunol. 35, 3258–3267.

Combinatorial Chemistry & High Throughput Screening, **2015,** *18,* **000-000**

Simultaneous Multicolor Luciferase Reporter Assays for Monitoring of Multiple Genes Expressions

Yoshihiro Ohmiya*

DAILAB, Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan

Yoshihiro Ohmiya

Abstract: Multicolor luciferase reporter assays are powerful tools for simultaneously monitoring the expression of several genes. Dual-color luciferase reporter assays were established in 2004 and have been used in broad range of application from basic researches to applications in clinical research, e.g., drug screening and alternative toxicological tests. The advantage of this method is that it can be used to simultaneously monitor sequential and complex changes in the expression of multiple genes from the single-cell level to the tissue level. In this review, I focus on the simultaneous analysis of multiple genes using multicolor beetle luciferases.

Keywords: Beetle luciferase, bioluminescence, multicolor luciferase assay, imaging luciferase assay, reporter assay.

INTRODUCTION

Bioluminescence is a form of "cold light" in living organisms, based on the chemical reaction; that is, the measured light activity depends on the amount of enzyme, substrate, and cofactor(s). Bioluminescence reporters, such as luciferase (enzyme) and luciferin (substrate), are widely used for the analysis of various biological functions, such as protein-protein interactions, and gene expression [1-3]. The structural relationship between luciferin and luciferase is restricted in each bioluminescence reaction. Additionally, bioluminescence can be reproduced *in vitro* using a chemically synthesized luciferin reporter and a genetically produced luciferase enzyme. The bioluminescence system comprising these two components produces a unique light source used widely in the field of biotechnology for visualizing biological functions. Thus, luciferases are good candidate reporter enzymes in cell-based assays. Advanced biotechnologies using multicolor luciferases permit the quantitative analysis of multiple genes from the single-cell level to the tissue level using a luminometer/charged-coupled device (CCD) camera equipped with optical filters. Bioluminescent protein techniques are sensitive approaches for understanding cell physiology, whereas fluorescence imaging that use fluorescent proteins (e.g., green fluorescent protein [GFP]) as probes have contributed to the understanding of cell physiology researches. Especially, bioluminescent probes are highly sensitive, display good linearity, and are easy to perform compared with other fluorescent probes. Beetle luciferases are particularly suitable as reporter enzyme because they produce stable color lights ranging from green to red under any condition; in contrast, the light produced by firefly luciferases can change depending on pH or temperature. In this review, I discuss the simultaneous analysis of multiple genes using multicolor beetle luciferases.

*Address correspondence to this author at the Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Higashi, Tsukuba, Ibaraki, Japan;
Tel: ??????????????????????????????; Fax: ??????????????????????????????;
E-mail: y-ohmiya@aist.go.jp

FIREFLY AND BEETLE BIOLUMINESCENCE

Bioluminescence is a well-known phenomenon. In the presence of firefly and beetle luciferases as a catalytic enzyme and ATP and Mg^{2+} as cofactors, the bioluminescence reactions produc photons (Fig. **1**) [4-6]. In light-emitting reactions, the emission maxima of firefly D-luciferin-type bioluminescence are found at around 535-630 nm (Fig. **2A**, **B**). Beetle bioluminescence is also known to produce much higher quantum yield (QY) values than other types of luminescence [7, 8]. The QY value of native firefly D-luciferin varies from 0.15 to 0.60 depending on the luciferase enzyme; for example, the green-yellow-emitting luciferase of the American firefly (*Photinus pyralis*) has a QY value of 0.41. Moreover, the QY value of the firefly luciferase is correlated with the luminescence peak wavelength. The red-emitting luciferase of the Brazilian railroad worm (Phrixothrix hirtus), which has the reddish peak wavelength, has the lowest QY (0.15), whereas the green-emitting luciferase of the Brazilian click beetle (Pyrearinus termitilluminans), whose luminescence spectrum has the greenish peak of all reported luciferases, possesses the highest quantum yield (0.61) [9].

Interestingly, beetle bioluminescence, including that of fireflies, can be classed into two large groups according to bioluminescent spectral sensitivity to pH: 1) pH-sensitive luciferases (fireflies) that exhibit a red color shift with increasing temperature or decreasing pH [4, 5]; and 2) pH insensitive (railroad worm and click beetle) luciferases that are not affected by these changes in pH and temperature [9, 10]. Additionally, the spectra of pH-insensitive beetle luciferases are narrower than those of pH-sensitive firefly luciferases, although many color mutants have still been constructed. For example, Fig. (**2**) shows the corrected luminescence spectra for pH-insensitive (Fig. **2A**) and -sensitive luciferases (Fig.

1386-2073/15 $58.00+.00

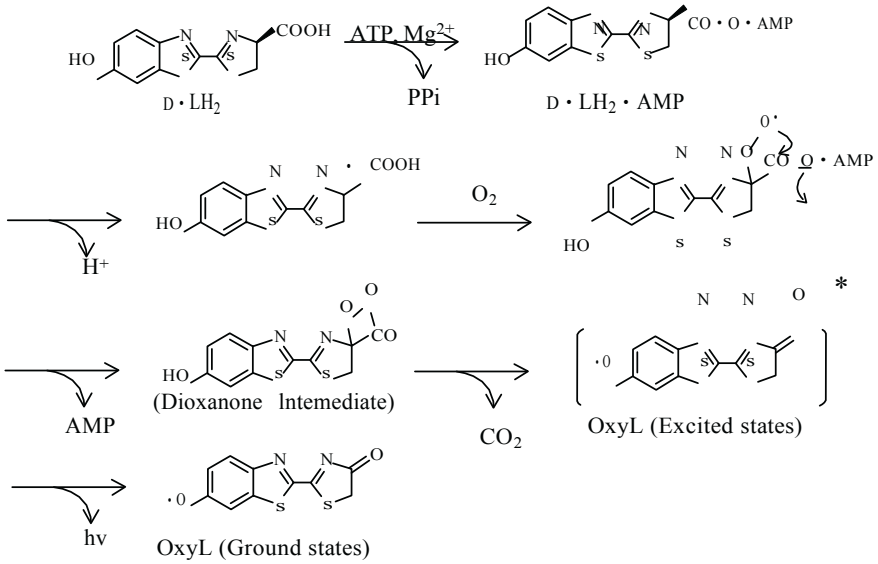


Fig. (1). Proposal mechanism of chemical reaction in firefly bioluminescence. Luciferin (D-LH_2) is converted into an adenylate (D-LH_2-AMP) in the presence of ATP, which is oxidized in the presence of oxygen, forming a peroxide intermediate by splitting of AMP. Decomposition of the intermediate *via* the dioxetanone intermediate is sufficiently energetic to produce the excited state of the oxyluciferin (OxyL) monomer or dianion, and then to produce the light from excited state to ground state.

2B). The spectra of the multicolor luciferase mutants are broader than those of pH-insensitive red and green light-emitting luciferases [9]. For the various beetle luciferases, the QY values and spectra of the bioluminescence reaction have been analyzed in order to explain their relationships.

The mechanisms mediating the different color emissions of beetle bioluminescence were proposed based on active site determined by the 3D-structure of the *Luciola cruciata* firefly luciferase [11]. For example, Hirano *et al*. investigated the spectroscopic properties of the phenolate anion of firefly luciferin and proposed that the excited luciferin as a light emitter was modulated by the polarity of the active-site environment of firefly luciferase and protonated basic moiety in the active site [12]. On the other hand, the emitter of firefly bioluminescence may relate to the lability of firefly oxyluciferin. Maltsev *et al*. explained that its lability is due to autodimerization of the coexisting enol and keto forms in a Mannich-type reaction based on NMR spectroscopy and X-ray crystallography data of a side product [13]. Nazivet *et al*. demonstrated that emitting light depends on the micro-environmental polarity at the phenolate/phenol of the benzothiazole fragment in oxyluciferin, and furthermore, denied that the color modulation of the emitting light depends on the size of the compact luciferase protein which is a cavity embedding the excited oxyluciferin molecule [14]. Nazivet *et al*. also demonstrated that based on a model of Cypridina oxyluciferin and coelenteramide, carbonyl group of firefly oxyluciferin or the different chemical environment of the dioxetanone is more rigid in the firefly bioluminescence

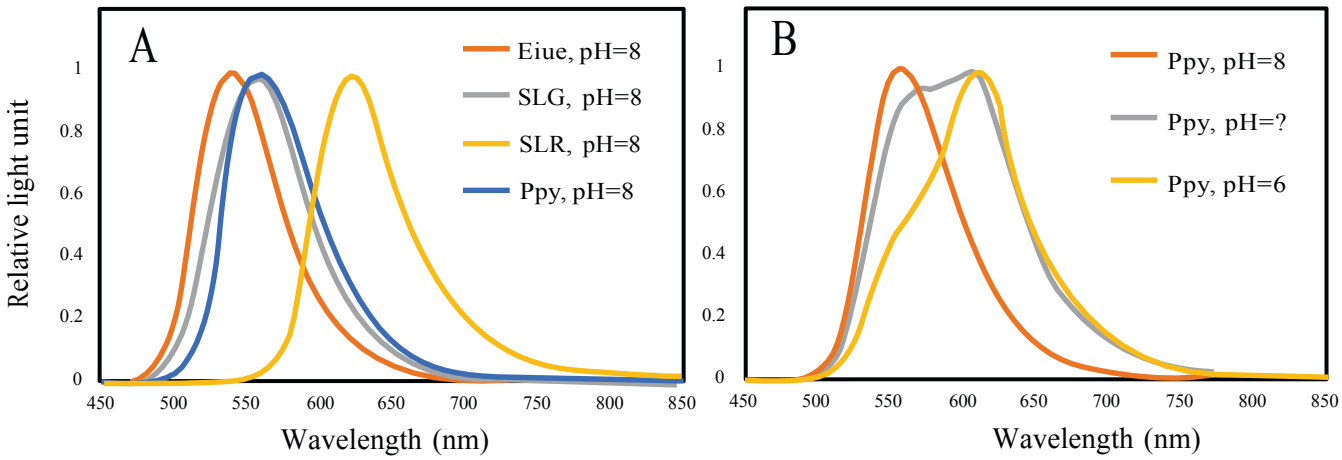


Fig. (2). Luminescence spectra of bioluminescence reactions for various beetle luciferases. Spectra were measured using PicaGene reagent at 24°C, corrected for spectral sensitivity of the sphere spectrometer, and normalized at each luminescence maximum [9]. Ppy; *Photinus pyralis* luciferase, ELuc, SLG, SLR (see Table **1**).

system [15]. However, the mechanisms mediating the pH sensitivity (or insensitivity) of the beetle luciferase enzyme remain unknown. In particular, the mechanism of pH insensitivity in the beetle luciferases is not clear because the 3D-structure of pH-insensitive luciferases has not been determined. Thus, improving our understanding of the mechanisms of beetle bioluminescence could facilitate the development of novel luciferase assays and biological applications of luciferases in a variety of research fields.

CELL-BASED MULTICOLOR LUCIFERASE REPORTER ASSAYS

Fig. (**3**) demonstrates a simple luciferase reporter assay. In general, the reporter plasmid containing the luciferase gene plus the target promoter region of interest is transfected into target cells, and luciferase-expressing cells are lysed after an appropriate period, e.g., 1–2 days. We can measure the amount of expressed luciferase as a light signal *in vitro*, and we can estimate the target promoter activity as a light intensity. Table **1** summarizes the characteristic properties of bioluminescence systems based on commercially available luciferases from beetles, sea pansy, copepods, and ostracods. Commercially available luciferins comprise only three types: firefly D-luciferin, coelenterazine and *Cypridina* luciferin, although other luciferins, including dinoflagellate [16] and *Latia* luciferins [17], have been identified. In light-emitting reactions, the emission maxima of firefly D-luciferin-type, coelenterazine-type, and *Cypridina* luciferin-type bioluminescence are found at around 535–630, 460–480, and 460 nm, respectively. The molecular weights of luciferases vary widely (20–62 kDa), and their molecular structures, which originate from phylogenetically distant systems, belong to different super families. *Cypridina* [18], *Gaussia* [19], and *Metridia* [20] luciferases (Cluc, GLuc, and MetLuc, respectively) are secreted enzymes. The luciferin-luciferase reaction is triggered by adding luciferin, although the bioluminescence of firefly, click beetle and railroad worm luciferases requires ATP and magnesium ions as cofactors.

Advances in the luciferase assay system have an additional luciferase as an internal control reporter (dual-reporter assay), thereby minimizing inherent experimental variability that can undermine experimental accuracy, such as differences in the number and viability of cells used and the efficiency of cell transfection and lysis. Thus, the first generation of dual-reporter assays is a combination of firefly luciferase and sea pansy *Renilla* luciferase using firefly D-luciferin-type and coelenterazine-type bioluminescence. Of the luciferases identified to date, the firefly luciferase from *P. pyralis* is the commonly used bioluminescent reporter in commercial vectors. Only the expression of one gene or one target event can be monitored at a time, although this luciferase has been extensively used to monitor cellular events in cell-based assays and *in vivo* imaging [21-23].

Gene expression events are both complex and sequential because of the elaborated regulatory pathways found in living cells. Many researchers have sought new reporter assay systems, focusing on the characteristics of multicolor beetle luciferases. Advanced luciferase technology, involving progressives in both the luciferase and the detection equipment, as well as newly cloned luciferase genes, have allowed us to simultaneously monitor the expressions of multiple genes when luciferases are used that induce different color emission spectra in the catalysis of a single D-luciferin substrate. The advantages of beetle luciferases producing multiple colors are as follows: (1) the colors are separable with an optical filter; (2) the number of substrates is minimal; (3) the temperature dependences are similar; and (4) the half-lives are similar (Fig. **4**). Thus, these mixed emission spectra are measured simultaneously. Each intensities can be quantified by splitting them with optical filter(s) [24].

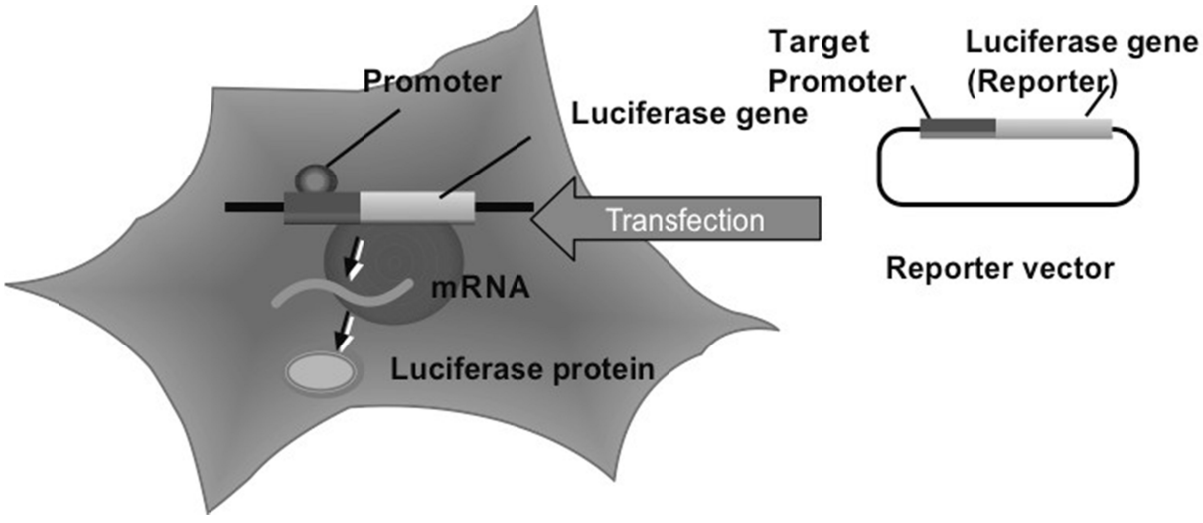


Fig. (3). Principle of a simple luciferase reporter assay. The reporter plasmid vector consists of the target promoter sequence and luciferase gene sequence. After transfection of the plasmid into target cells, the promoter region regulates the expression of luciferase gene in living cells. The expressed luciferase protein catalyzes a reaction with luciferin to produce light. In the transient transfection luciferase assay, luciferase-expressing cells are lysed for an appropriate period. The amount of expressed luciferase protein can be estimated from the light intensity which indicates the promoter activity in living cells. In this case, the promoter activity is normalized by cell numbers or cellular enzymatic activity.

Table 1. Summary of characteristic properties of commercialized bioluminescence system.

Organism	Gene Symbol	Luciferin	Mass (kDa)	λmax (nm)	Main Company
Non-Secreted					
Firefly	luc(+), luc2	firefly luciferin	61	562	Promega
Sea pansy	Rluc	coelenterazine	36	480	Promega
Click beetle (Jamaica)	CBGluc	firefly luciferin	60	537	Promega
Click beetle (Jamaica)	CBRluc	firefly luciferin	60	613	Promega
Click beetle (Brazil)	ELuc	firefly luciferin	61	638	TOYOBO
Railroad-worm	SLR	firefly luciferin	60	630	TOYOBO
Railroad-worm (Japan)	SLG	firefly luciferin	60	550	TOYOBO
Railroad-worm (Japan)	SLO	firefly luciferin	60	580	TOYOBO
Secreted					
Copepoda	GLuc	coelenterazine	20	480	NEB
Copepoda	MetLuc	coelenterazine	24	480	Clontech
Ostracod	Cluc	cypridinid luciferin	61	465	NEB

APPLICATION OF CELL-BASED MULTICOLOR LUCIFERASE REPORTER ASSAY

In the first example of dual-color luciferase assays in 2004, Kitayama *et al*. constructed a simple dual-reporter system monitored simultaneously two promoter activities in living cyanobacterial cells [24]. Two Phrixothrix railroad-worm luciferases [10] catalyzing the generation of different color emissions served as the dual reporters; each emissions was separated by interference filters to estimate the individual emission signals using photomultiplier tubes. Using this system, they clearly demonstrated the expression profiles between promoters in the same cells.

As a second example, Nakajima *et al*. developed a simultaneous monitoring system [25] in mammalian cell line using green light emitting luciferase (SLG, see Table **1**) and red light-emitting luciferase (SLR, see Table **1**) from the Phrixothrix railroad worm. The two spectral mixed emissions were divided using a > 600-nm long-pass filter, and the respective luciferase activities were calculated. Splitting the emissions with a long-pass filter is advantageous in that the emission loss is less than when interference filters are used. The linear response range of this system using cell extracts which are expressed the green and red light-emitting luciferases could be estimated to be more than two orders of magnitude.

Furthermore, Nakajima *et al*. established a tricolor reporter assay using SLG, orange-emitting luciferase (SLO, see Table **1**), and SLR [26]. Because all of these enzymes emit light with D-luciferin, their respective activities can be detected in a one-step reaction from a single sample. Using this method, they estimated that the linear response range of the system exceeds two orders of magnitude, although the low-threshold light intensities require one order of magnitude higher intensity than those estimated in the dual-color luciferase assay. Using this system, they introduced SLG as an internal control reporter and measured its activity separately from those of SLO for *Bmal1* and SLR for the part of *Bmal1* promoters for retinoic acid receptor-related orphan receptor α response element (RORE) (Fig. **5**). They demonstrated a simultaneous monitoring of the clock genes expressions *in vitro* and clarified the role of retinoic acid receptor-related orphan receptor α (RORα) in the transcriptional regulation of the clock genes *Bmal1* and RORE in mammalian cell.

Since 2005, many researchers have published papers describing the application of the dual-color luciferase assay. Branchini *et al*. developed a model system for the dual-color luciferase assay by combination of green light-(Val241Ile/Gly246Ala/Phe250Ser, λmax = 549 nm) and red (Ser284Thr, λmax = 615 nm) emitting firefly luciferase mutants. They divided the emissions using two band-pass filters and confirmed that the green light-emitting GST fusion protein could be measured over a 10,000-fold range from about 20 amol to 200 fmol when the amount (10 fmol) of red light-emitting GST fusion protein was kept constant in the crude cell extracts [27]. Moreover, based on the results, they generated thermostable firefly luciferase mutants and quantified both luciferase activities at 1.0 fmol in a mixture using the microplate luminometer format [28]. Using a similar approach, Michelini *et al*. simultaneously monitored the expression of two genes in cell extracts using two beetle luciferases (green light-emitting luciferase from *P. pyralis* [λmax = 560 nm] and red light-emitting luciferase from *Luciola italica* [λmax = 613 nm]), which emit light *via* reaction with firefly D-luciferin as the experimental reporter. The latter enzyme is a mutant of the wild-type form, displaying a red-shift emission and better thermostability [29].

Furthermore, in plant cells, Ogura *et al*. used reporter plasmids harboring the green light-emitting luciferase from the Jamaican click beetle *Pyrophorus plagiophthalamus* (CBG; λmax = 537 nm) connected with the chlorophyll *a/b* binding protein (*Cab*) promoter and the red light-emitting luciferase from *P. plagiophthalamus* (CBR; λmax = 613 nm) connected with the cauliflower mosaic virus promoter as a control. They co-injected these plasmids into plant cells

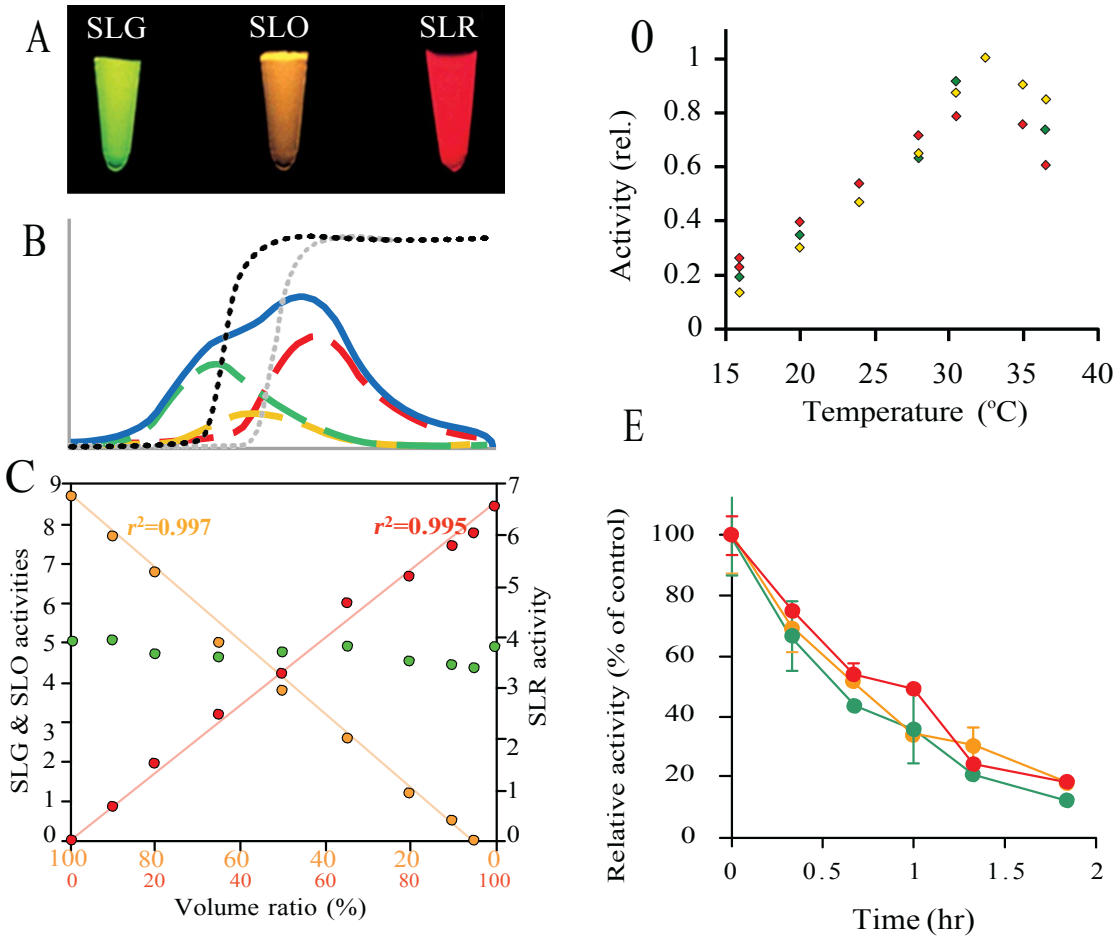


Fig. (4). Characteristic properties of SLG, SLO, and SLR. **A**; Bioluminescence color of SLG, SLO, and SLR. **B**; Bioluminescence spectra example (blue line) of mixture of SLG, SLO, and SLR and individual spectra of SLG (green dotted line), SLO (orange dotted line) and SLR (red dotted line) luciferases, and the transmission spectra of > 560 nm (O56, black dotted line) and > 600 nm (R60, gray dotted line) long-pass filters. **C**; Quantitative relationship among SLG (green circle), SLO (orange circles), and SLR (red circles) activities in a mixture of each proteins expressed in silkworm. Each samples were diluted with PicaGene Dual lysis buffer (Toyo B-net, JP) at the indicated volume ratio were mixed. The respective luciferase activities were measured with an AB2250 luminometer (ATTO, JP) after injecting PicaGene. RLU, relative light unit. **D**; Temperature dependence of SLG (green circle), SLO (orange circles), and SLR (red circles) activities of each proteins. Luminescence activities under different temperatures were measured as followings. 5 ∝L of each luciferases solution (0.1 ∝g/mL) at 4°C was mixed with 50 uL of Tripluc Luciferase Assay Reagent (TOYOBO, JP) that was pre-incubated at 16, 20, 24, and 28°C for 10 min. All the apparatus except for the luminometer at room temperature were also incubated at each temperatures in advance. **E**; Half-lives of PEST (rapid degradation sequence) sequence fused-SLG (green circle), -SLO (orange circles), and -SLR (red circles) activities. Functional half-life of PEST-fused SLG, SLO and SLR luciferases in NIH3T3 cells. Expression plasmids were independently transfected into NIH3T3 cells. Forty hours after transfection, the culture medium was replaced with DMEM supplemented with 10% FBS and 100 µM cycloheximide, and incubated for 30 min to block protein synthesis. After 30 min (time = 0), incubation was continued in the same medium. At the indicated periods, the cells were disrupted and measured their activities in Tripluc Luciferase Assay Reagent (TOYOBO, JP).

using the microprojectile bombardment system and demonstrated the simultaneous measurement of dual-color luciferase activities in extracts of spinach leaves [30]. Respective light intensities were measured simultaneously with two interference filters using a CCD camera and demonstrated the light-induced expression of the *Cab* promoter.

PRACTICAL APPLICATIONS OF CELL-BASED MULTICOLOR LUCIFERASE REPORTER ASSAYS

For the purposes of diagnostics, drug discovery and alternatives for animal models, the reliability and high-throughput features of reporter assays need to be improved. Assays must also save time and money, reduce the amount of sample needed, and facilitate the interpretation of data. The dual-color reporter assay is a simple method in which only one luciferin is used and the similar characteristic properties of luciferases are exploited; however, special equipment is required for measuring the different color emissions. The internal control reporter as a simultaneous dual-reporter assay can be optimized for inherent experimental reliability in order to improve experimental accuracy.

Indeed, to establish a practical drug screening, Davis *et al.* developed a high-throughput dual-color luciferase assay system using a 1,536-well plate format for screening small

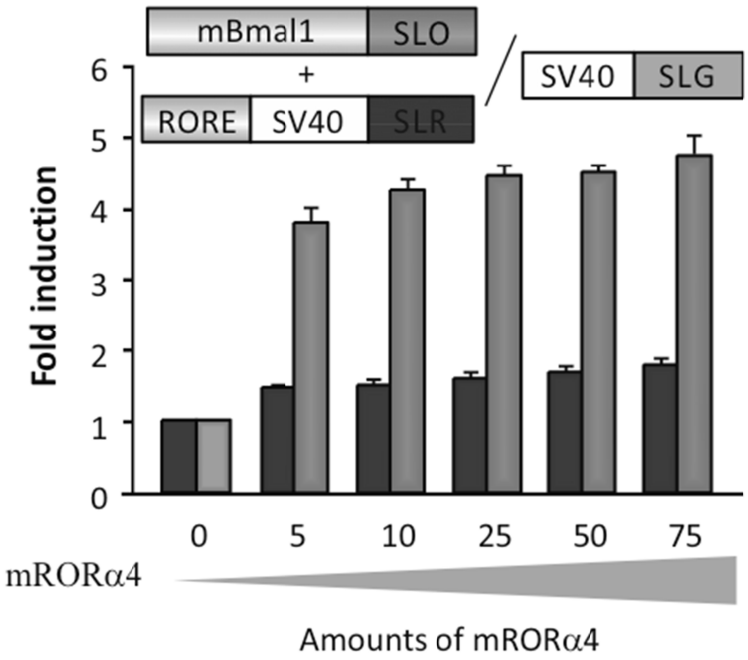


Fig. (5). Simultaneous monitoring of mRORα4-dose-dependent induction of RORE-mediated SLR gene (red bars) and *mBmal1* promoter mediated SLO gene (orange bars) transcription as a control of simultaneous expression of SV40 derived SLG gene. Reporter plasmids pRORE-SLR, Bp915-SLO and pSV40-SLG were co-transfected with or without indicated amounts of expression plasmids carry mRORa4 into NIH/3T3 cells. The amount of DNA added per well was kept constant by adding control plasmid. All values are shown as multiples. The diagram of the reporter plasmids shown location of elements. RORE, Rev-Erb/ROR response element in the *mBmal1* promoter; mBmal1 915, 915-bp fragment of the *mBmal1* promoter region; SV40, simian virus 40 promoter.

molecules that stabilize inhibitor of kappaB alpha (IκBα), a critical repressor of nuclear factor (NF-κB) [31]. They generated a dual luciferase cell line (OCI-Ly3), in which IκBα was fused to the CBG and CBR. Both luciferases were placed under an inducible promoter and integrated into the chromosomes. In screening of drug candidates, the IκBα-fused green light-emitting luciferase as an experimental reporter and the red light-emitting luciferase as an internal control reporter. By measuring their activities simultaneously in cell extracts with two band-pass filters using a CCD-based plate reader, they succeeded in screening and identifying known and unknown inhibitors of NFκB signaling from a collection of bioactive molecules.

As a toxicological test using a dual-color luciferase system, Takahashi *et al*. established a stable THP-1-derived interleukin (IL)-8 reporter cell line, THP-G8, which harbors SLO gene under IL-8 promoter and SLR gene under glyceraldehyde 3-phosphate dehydrogenase promoter as a control, respectively [32]. They evaluated the performance of this assay using values in at least two of three independent experiments as the criteria of a sensitizer. These experiments showed the test accuracies of 82% for the selected 22 chemicals and of 88% for the chemicals proposed by the European Center for the Validation of Alternative Methods. This assay is a candidate replacement for the animal tests of skin sensitization according to OECD Guidelines because of its accuracy, high-throughput performance, reliability and convenience.

On the other hand, the dual-color luciferase system is a powerful and simple technique that can be used to analysis the complex interactions of two genes, even at the whole-organism level. Noguchi *et al*. established a dual color transgenic mice and monitoring simultaneously expression of two genes at the tissue or whole-organism level [33]. They clearly monitored antiphasic oscillations of *Bmal1* and *Per2* genes names should be italicized, consistent with their endogenous mRNA profiles in the superchiasmatic nucleus and in peripheral tissues. The demonstrating results clearly show that the system allows the long-term, quantitative, and simultaneous monitoring of the expressions of the two genes.

CELL-BASED MULTICOLOR LUCIFERASE REPORTER IMAGING ASSAYS

Bioluminescence imaging at the single cell level using multicolor luciferases is a sensitive approach for understanding cellular physiology. This advance in luciferase technology has enabled quantitative and long-term visualization of cellular events at single-cell resolution using a luminescence microscope equipped with a highly sensitive cooled CCD camera [34]. However, it is possible to visualize single events only in living cells. Zhang *et al*. performed a real-time imaging of ATP release from a single cell using immobilized firefly luciferase and acquired time-lapse images of ATP release and diffusion from the cell surface, allowing determination of the actual ATP concentration at the surface of single living cells [35]. Hoshino *et al*. also developed a luciferase fused fluorescent protein composed of Rluc and EYFP (BAF-Y) for real-time single cell imaging [36]. They showed that BAF-Y exhibits the enhancement of Rluc emission intensity and appropriate subcellular distribution when fused to target-signal peptides, thus permitting the use of highly spatial and temporal resolution

microscopy. However, bioluminescence imaging using coelenterazine is unsuitable for long-term experiments because coelenterazine is degraded by auto-oxidation in living cells.

For the spatiotemporal resolution of bioluminescence long-term imaging at the single-cell level, beetle bioluminescence is advantageous because firefly D-luciferin is stable for auto-oxidation and degradation in living cells. Moreover, the background of beetle bioluminescence is lower than that of other systems, and a CCD camera can detect weak signals at the single-cell level. Nakajima *et al*. developed an enhanced green light-emitting beetle luciferase from *P. termitilluminans* (ELuc; see Table **1**), whose light signal intensity in mammalian cells is more than 10-fold greater than that of the firefly luciferase [37]. Although the current luciferase reporter is used for bioluminescence imaging, it is difficult to perform subcellular level imaging due to the inadequate signal intensity in these viable cells; indeed, higher bioluminescence intensity is required for subcellular imaging with a higher-magnification lens. They demonstrated the subcellular localization of ELuc with high resolution in mammalian cells.

In the first example of dual-color luciferase imaging in a single cell, Kwon *et al*. simultaneously visualized the expression patterns of two genes for several days in a single cell using ELuc and SLR luciferases [38]. They constructed a new dual-path optical luminescence imaging system. The light signal generated in a target cell is collimated by the objective lens and is divided by the dichroic mirror into green and red lights. Dual-color bioluminescence imaging clarified that the expression levels of genes encoding two circadian proteins (*mBmal1* and *mPer2*) oscillate in antiphase, with a circadian period of ~24 h in individual cells over 4 days. However, disorders such as 'blinking' and varying periodicities were observed several times at the single-cell level, although the bioluminescence monitoring data from the cell population showed robust and a stable circadian rhythm within the same timeframe.

Moreover, current researches in bioluminescence technique have made it possible to visualize the subcellular localization of color difference luciferases and monitor simultaneously the expression levels of two genes at subcellular resolution. Yasunaga *et al*. visualized simultaneously the subcellular localization of ELuc in the peroxisome and SLR in the nucleus in a single cell using a high-magnification objective lens with a 3-min exposure time using a combination of optical filters without binning [39]. They simultaneously quantified the kinetics of activation of NFκB using nuclear-targeted SLR and transcriptional changes in the internal control promoter using peroxisome-targeted ELuc at a single cell level and showed that the activation kinetics, including activation rate and amplitude (Fig. **6**).

FUTURE SCOPE

In the postgenomic era, we must clarify quantitatively and spatially the complex phenomena of biological systems in real time. Bioluminescence is a unique light source and

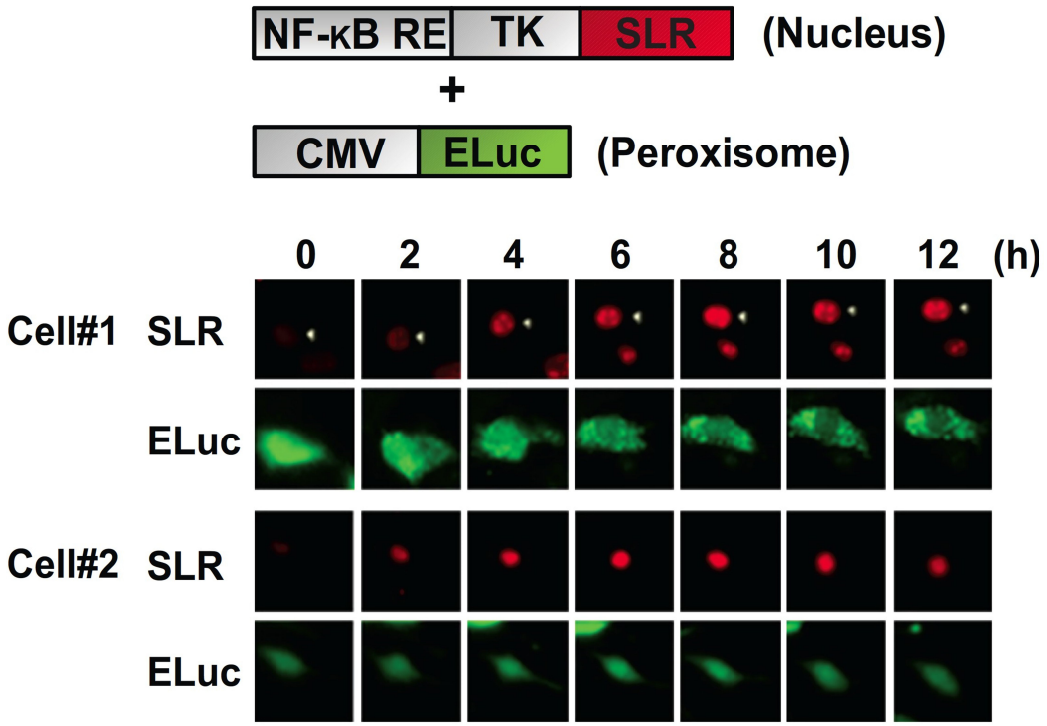


Fig. (6). Time-lapse dual-color bioluminescence imaging of tumor necrosis factor α (TNFα)-induced nuclear factor κB response element (NF-κB RE)-dependent transactivation using reporter plasmid pCMV-ELuc (peroxisome) and pNFκB-TK-SLR (nuclear) in NIH/3T3 cells. NIH/3T3 cells were transfected with pCMV-ELuc and pNFκB-TK-SLR plasmids. One day after the transfection, the culture medium was replaced with Dulbecco's modified Eagle's medium supplement with TNFα. CCD images were acquired using 3-min exposure time at 2 h intervals for 12 h with a x40 objective lens without binning. Representative serial luminescence images acquired using R62 (red, indicated as SLR) and BG39 (green, indicated ELuc) filters.

luciferase is a good candidate reporter enzyme in the field of bioresearch. The sensitivity and linearity of bioluminescence assays are superior to that of other reporter enzymes. The combination of beetle luciferases and firefly D-luciferin produce stable spectra from green to red under different buffer conditions, and their different color lights can be measured by equipment for measuring light intensity using color separation techniques. Thus, the use of different color beetle luciferases can provide new methods to analyze multiple genes both *in vitro, in cellulo* and *in vivo*. The multicolor luciferase assay is a powerful tool that will, in the near future, reveal the relationship between the core biological clock gene and cell cycle-related or metabolic-related genes, and the balance between the expressions of different genes in the immune system. However, it is a big limitation to establish the bioluminescent reporter cell, tissue, and animal. For instance, we need a hard effort to establish several genes expressed cell line. Furthermore, it is also too hard to keep the potential of reporter cell line for a long time. At the next stage, we have to develop new fusion technologies with bioluminescent reporter system for gene engineering or cell-tissue-animal engineering including artificial chromosome technology, genome editing system and etc.

CONFLICT OF INTEREST

The author confirms that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

I wish to thank my colleagues who supported our research: Drs. Y. Nakajima and K. Niwa.

REFERENCES

[1] Bronstein, I.; Fortin, J.; Stanley, P.E.; Stewart, G.S.; Kricka, L.J. Chemiluminescent and bioluminescent reporter gene assays. *Anal. Biochem.,* **1994**, *219*, 169-181.

[2] Wilson, T.; Hastings, J.W. Bioluminescence. *Annu. Rev. Cell Dev. Biol.,* **1998**, *14*, 197-230.

[3] Greer, L.F.; Szalay A.A. Imaging of light emission from the expression of luciferases in living cells and organisms:a review. *Luminescence*, **2002**, *17*, 43-74.

[4] DeLuca, M. Firefly luciferase. *Adv. Enzymol*., **1976**, *44*, 37-68.

[5] Wood, K.W. The chemical mechanism and evolutionary development of beetle bioluminescence. *Photochem. Photobiol.,* **1995**, *62*, 662-673.

[6] Fraga, H. Firefly luminescence: A historical perspective and recent developments. *Photochem. Photobiol. Sci.,* **2008**, *7*, 146-158.

[7] Seliger, H. H.; McElroy, W.D. Quantum yield in oxidation of firefly luciferin. *Biochem. Biophys. Res. Commun.,* **1959**, *1*, 21-24.

[8] Ando, Y.; Niwa, K.; Yamada, N.; Enomoto, T.; Irie, T.; Kubota, H.; Ohmiya, Y.; Akiyama, H. Firefly bioluminescence quantum yield and colour change by pH-sensitive green emission. *Nat. Photonics,* **2008**, *2*, 44-47.

[9] Niwa, K.; Ichino, Y.; Kumata, S.; Nakajima, Y.; Hiraishi, Y.; Kato, D.; Viviani V.R.; Ohmiya, Y. Quantum yields and kinetics of the Firefly bioluminescence reaction of beetle luciferases. *Photobiology & Photochemistry*, **2010**, *86*, 1046-1049.

[10] Viviani, V. R.; Bechara, E.J.; Ohmiya, Y. Cloning, sequence analysis, and expression of active *Phrixothrix* railroadworms luciferases: Relationship between bioluminescence spectra and primary structures. Biochemistry, **1999**, *38*, 8271-8279.

[11] Nakatsu, T.; Ichiyama, S.; Hiratake, J.; Saldanha, A.; Kobashi, N.; Sakata, K.; Kato, H. Structural basis for the spectral difference in luciferase bioluminescence. *Nature,* **2006**, *440*, 372-376.

[12] Hirano, T.; Hasumi, Y.; Ohtsuka, K.; Maki, S.; Niwa, H.; Yamaji, M.; Hashizume, D. Spectroscopic studies of the light-color modulation mechanism of firefly (beetle) bioluminescence. *J. Am. Chem. Soc.,* **2009**, *131*, 2385-2396.

[13] Maltsev, O.V.; Nath, N.K.; Naumov, P.; Hintermann, L. Why is firefly oxyluciferin a notoriously labile substance? *Angew. Chem. Int.,* **2014**, *53*, 847-850.

[14] Navizet, I.; Liu, Y.; Ferre, N.; Xiao, H.; Fang, W.; Lindh, R. Color-tuning mechanism of firefly investigated by multi-configurational perturbation method. *J. Am. Chem. Soc.,* **2010**, *132*, 706-712.

[15] Navizet, I.; Roca-Sanjuan, D.; Yue, L.; Liu, Y.; Ferre, N.; Lindh, R. Are the bio- and chemiluminescence states of the firefly oxyluciferin the same as the fluorescence state? *Photochem. Photobiol*., **2013**, *89*, 319-325.

[16] Nakamura, H.; Kishi, Y.; Shimomura, O.; Morse, D.; Hastings, J. W. Structure of dinoflagellate luciferin and its enzymatic and nonenzymatic air-oxidation products. *J. Am. Chem. Soc.*, **1989**, *111*, 7607-7611.

[17] Shimomura, O.; Johnson S.H. The structure of *Latia* luciferin. *Biochemistry*, **1968**, *7*, 1734-1738.

[18] Nakajima, Y.; Kobayashi, K.; Yamagishi, K.; Enomoto, T.; Ohmiya, Y. cDNA cloning and characterization of a secreted luciferase from the luminous Japanese ostracod, *Cypridina noctiluca*. *Biosci. Biotechnol. Biochem*., **2004**, *68*, 565-570.

[19] Verhaegent, M.; Christopoulos, T.K. Recombinant *Gaussia* luciferase. Overexpression, purification, and analytical application of a bioluminescent reporter for DNA hybridization. *Anal. Chem*., **2002**, *74*, 4378-4385.

[20] Markova, S.V.; Golz, S.; Frank, L.A.; Kalthof, B.; Vysotski, E.S. Cloning and expression of cDNA for a luciferase from the marine copepod *Metridia longa*. A novel secreted bioluminescent reporter enzyme. *J. Biol. Chem*., **2004**, *279*, 3212-3217.

[21] Roda, A.; Guardigi, M.; Michelini, E.; Mirasoli, M. Bioluminescence in analytical chemistry and *in vivo* imaging. *Trend Anal. Chem.,* **2009**, *28*, 307-322.

[22] Nakajima, Y.; Ohmiya, Y. Bioluminescence assays: multicolor luciferase assay, secreted luciferase assay and imaging luciferase assay. *Expert Opin. Drug Discov.,* **2010**, *5*, 835-849.

[23] Michelini, E.; Calabretta, M.M.; Calabria, D.; Mirasoli, M.; Roda, A. Exploiting *in vitro* and *in vivo* bioluminescence for the implementation of the three Rs principle (replacement, reduction, and refinement) in drug discovery. *Anal. Bioanal. Chem.*, **2014**, *406*, 5531-5539.

[24] Kitayama, Y.; Kondo, T.; Nakahira, Y.; Nishimura, H.; Ohmiya, Y.; Oyama, T. An *in vivo* dual-reporter system of cyanobacteria using two railroad-worm luciferases with different color emissions. *Plant Cell Physiol.*, **2004**, *45*, 109-113.

[25] Nakajima, Y.; Ikeda, M.; Kimura, T.; Honma, S.; Ohmiya, Y.; Honma, K. Role of orphan nuclear receptor ROR a in clock gene transcriptions demonstrated by a novel reporter assay system. *FEBS Lett.,* **2004**, *565*, 122-126.

[26] Nakajima, Y.; Kimura, T.; Sugata, K.; Enomoto, T.; Asakawa, T, Kubota H, Ikeda M, Ohmiya, Y. A multicolor luciferase assay system, one-step monitoring of multiple gene expressions with a single substrate. *Biotechniques*, **2005**, *38*, 891-894.

[27] Branchini, B.R.; Southworth, T.L.; Khattak, N.F.; Michelini, E.; Roda, A. Red- and green-emitting firefly luciferase mutants for bioluminescent reporter applications. *Anal. Biochem.,* **2005**, *345*, 140-148.

[28] Branchini, B.R.; Ablamsky, D.M.; Murtiashaw, M.H.; Uzasci, L.; Fraga, H.; Southworth, T.L. Thermostable red and green light-producing firefly luciferase mutants for bioluminescent reporter applications. *Anal. Biochem.,* **2007**, *361*, 253-262.

[29] Michelini, M.; Cevenini, L.; Mezzanotte, L.; Ablamsky, D.; Southworth, T.; Branchini, B.; Roda, A. Spectral-resolved gene technology for multiplexed bioluminescence and high-content screening. *Anal. Chem.,* **2008**, *80*, 260-267.

[30] Ogura, R.; Matsuo, N.; Wako, N.; Tanaka, T.; Ono, S.; Hiratsuka, K. Multi-color luciferase as reporters for monitoring transient gene expression in higher plants. *Plant Biotech.,* **2005**, *22*, 151-155.

[31] Davis, R.E.; Zhang, Y-Q.; Southall, N.; Staudt, L.M.; Austin, C.P.; Inglese, J.; Auld, DS. A cell-based assay for IκBα stabilization using a two-color dual luciferase-based sensor. *Assay Drug Dev. Technol.,* **2007**, *5*, 85-103.

[32] Takahashi, T.; Kimura, Y.; Saito, R.; Nakajima, Y.; Ohmiya, Y.; Yamasaki, K.; Aiba, S. An *in vitro* test to screen skin sensitizers

using a stable THP-1-derived IL-8 reporter cell line, THP-G8. *Toxicol. Sci.*, **2011**, *124*, 359-369.

[33] Noguchi, T.; Michihata, T.; Nakamura, W.; Takumi, T.; Shimizu, R.; Yamamoto, M.; Ikeda, M.; Ohmiya, Y.; Nakajima, Y. Dual-color luciferase mouse directly demonstrates coupled expression of two clock genes. *Biochemistry*, **2010**, 4i9, 8053-8061.

[34] Welsh, D.K.; Kay, S.A. Bioluminescence imaging in living organisms. *Curr. Opin. Biotechnol.*, **2005**, *16*, 73-78.

[35] Zhang, Y.; Phillips, G.J.; Li, Q.; Yeung, E.S. Imaging localized astrocyte ATP release with firefly luciferase beads attached to the cell surface. *Anal. Chem.*, **2008**, *80*, 9316-9325.

[36] Hoshino, H.; Nakajima, Y.; Ohmiya, Y. Single-cell imaging with fluorescent protein excited by luciferin-luciferase reaction. *Nature Methods*, **2007**, *8*, 637-639.

[37] Nakajima, Y.; Yamazaki, T.; Nishii, S.; Noguchi, T.; Hoshino, H.; Niwa, K.; Viviani, V.R.; Ohmiya, Y. Enhanced beetle luciferase for high-resolution bioluminescence imaging. *PLoS One,* **2010**, *5*(4), e10011.

[38] Kwon, H.J.; Enomoto, T.; Shimogawara, M.; Yasuda, K.; Nakajima, Y.; Ohmiya, Y. Bioluminescence imaging of dual gene expression at the single-cell level. *BioTechniques*, **2010**, *48*(6), 460-462.

[39] Yasunaga, M.; Nakajima, Y.; Ohmiya, Y. Dual-color bioluminescence imaging assay using green- and red-emitting beetle luciferases at subcellular resolution. *Anal. Bioanal. Chem.,* **2014**, *406*, 5735-5742.

Received: May 28, 2015 **Revised: July 28, 2015** **Accepted: September 15, 2015**