Organisation for Economic Co-operation and Development

ENV/CBC/MONO(2017)19/REV1

Unclassified

English - Or. English

24 July 2023

ENVIRONMENT DIRECTORATE
CHEMICALS AND BIOTECHNOLOGY COMMITTEE

Validation report for the international validation study on the IL-8 Luc assay as a test evaluating the skin sensitizing potential of chemicals conducted by the IL-8 Luc Assay

Series on Testing and Assessment
No. 267
Second Edition

JT03523606

OECD Environment, Health and Safety Publications
Series on Testing & Assessment
No. 267

Validation report for the international validation study on the IL-8 Luc assay as a test evaluating the skin sensitizing potential of chemicals conducted by the IL-8 Luc Assay

IOMC
INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS
A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

Environment Directorate
ORGANISATION FOR ECONOMIC COOPERATION AND DEVELOPMENT
Paris 2023

About the OECD

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 38 industrialised countries in North and South America, Europe and the Asia and Pacific region, as well as the European Commission, meet to co-ordinate and harmonise policies, discuss issues of mutual concern, and work together to respond to international problems. Most of the OECD's work is carried out by more than 200 specialised committees and working groups composed of member country delegates. Observers from several countries with special status at the OECD, and from interested international organisations, attend many of the OECD's workshops and other meetings. Committees and working groups are served by the OECD Secretariat, located in Paris, France, which is organised into directorates and divisions.

The Environment, Health and Safety Division publishes free-of-charge documents in eleven different series: **Testing and Assessment; Good Laboratory Practice and Compliance Monitoring; Pesticides; Biocides; Risk Management; Harmonisation of Regulatory Oversight in Biotechnology; Safety of Novel Foods and Feeds; Chemical Accidents; Pollutant Release and Transfer Registers; Emission Scenario Documents;** and **Safety of Manufactured Nanomaterials.** More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (www.oecd.org/chemicalsafety/).

This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organizations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

This publication is available electronically, at no charge.

For this and many other Environment,

Health and Safety publications, consult the OECD's

World Wide Web site (www.oecd.org/chemicalsafety/)

or contact:

OECD Environment Directorate,

Environment, Health and Safety Division

2 rue André-Pascal

75775 Paris Cedex 16

France

Fax: (33-1) 44 30 61 80

E-mail: ehscont@oecd.org

FOREWORD

This document is the second edition of the report of the validation study of the IL-8 Luc assay for evaluating the skin sensitising potential of chemicals. It supports the development and subsequent update of this test method for inclusion in Test Guideline (TG) 442E addressing the Key Event on activation of dendritic cells on the Adverse Outcome pathway for Skin Sensitisation.

It has been developed in two phases. PART I of the document, published in 2017 as the first edition of this publication, describes the validation study that supported the first version of the IL8-Luc test method. The validation report and peer review reports were made available as supporting documents during two WNT commenting rounds of the draft test method on the IL8-Luc assay in July and December 2016 respectively. The validation report was augmented in March 2017, following the peer review, with the inclusion of an addendum (a new Appendix 15 and revised Appendices 16 and 17), presenting data in support of revised data interpretation criteria in the IL8-Luc test method. The validation report was endorsed by the WNT at the 29th Meeting of the WNT in April 2017.

The validation report was updated in 2023 with the inclusion of PART II. This second part contains an overview document that supports the update of the prediction model of the IL8-Luc assay to improve its performance. At the 35th WNT meeting in April 2023, the WNT approved the updated version of TG 442E and endorsed PART II of the IL8-Luc validation report.

This second edition of the report is published under the responsibility of the Chemicals and Biotechnology Committee.

PART I

VALIDATION REPORT FOR THE INTERNATIONAL VALIDATION STUDY ON THE IL-8 LUC ASSAY AS A TEST EVALUATING THE SKIN SENSITIZING POTENTIAL OF CHEMICALS CONDUCTED BY THE IL-8 LUC ASSAY

Table of contents

1. Summary

Takahashi et al., (2011) previously reported the evaluation of a dataset of 35 chemicals covering the test chemicals proposed by the European Centre for Validation of Alternative Methods (ECVAM) by the interleukin-8 luciferase (IL-8 Luc) assay. The IL-8 Luc assay assesses the effects of chemicals on IL-8 promoter activity, using THP-1 cells transfected with the IL-8 luciferase reporter gene. In that previous study, the performance of the IL-8 Luc assay was an accuracy of 83% (29/35), a sensitivity of 83% (20/24), and a specificity of 82% (9/11).

In the present validation study, coordinated by the Japanese Center for the Validation of Alternative Methods (JaCVAM), the protocol transferability, within or within-laboratory reproducibility, between or inter-laboratory reproducibility, and predictivity of the IL-8 Luc assay were evaluated by 3 independent laboratories in 6 ring trials (Phase I, Phase IIa, Phase IIb, Phase IIc, Phase III, and phase IV trials). Acceptable criteria for the validation study were set at a within laboratory reproducibility of at least 85% and between laboratory reproducibility of at least 80%.

Several problems with the IL-8 Luc assay became evident during the ocurse of the validation study. With the agreement of the Validation Management Team (VMT) members, each was addressed individually and the protocols were modified as described below.

The Phase I study examined 10 coded chemicals (1 extreme, 1 strong, 3 moderate and 2 weak sensitizers, and 3 non-sensitizers) by one experimental set composed of 2 to 3 independent experiments and produced unsatisfactory between-laboratory reproducibility (70%), poor predictive ability, and an overall accuracy of 63%. Subsequently, the Phase IIa study conducted after a minor modification of the protocol, evaluated 10 coded chemicals (2 extreme, 1 strong, 3 moderate and 1 weak sensitizers, and 3 non-sensitizers) by one experimental set and significantly improved between-laboratory reproducibility (70%) and accuracy (77%) were achieved. However, some laboratories still failed to predict the sensitization potential of DNCB, a well-known strong sensitizer, so the protocol and classification criteria were further modified.

For the Phase IIb study, the incubation period was changed from 6 h to 16 h based on the results of a time course study of the IL-8 Luc assay performed by the lead laboratory. The Phase IIb study examined 5 coded chemicals (1 extreme, 2 moderate and 1 weak sensitizers, and 1 non-sensitizer) by 3 experimental sets and demonstrated a between-laboratory reproducibility of 86%, an average within-laboratory reproducibility of 78%, and an average accuracy of 79%. To further improve within-laboratory reproducibility, one criterion for examining the inhibitory effects of N-acetyl-cysteine on IL-8 luciferase activity after chemical treatment was deleted, based on the consideration that some haptens can stimulate dendritic cells without reacting with cysteine residues.

In the Phase IIc (5 coded chemicals [1 strong and 1 moderate sensitizers and 3 non-sensitizers] by 3 experiment sets) and the Phase III (20 coded chemicals [2 extreme, 3 strong, 5 moderate and 4 weak sensitizers, and 6 non-sensitizers] by one experiment set) studies, the original criterion based on FInSLO-LA $\geq$ 1.4 was compared with the criterion based on FInSLO-LA $\geq$ 1.4 and the lower limit of the 95% confidence interval of FInSLO-LA $\geq$ 1.0. The combined results from the Phase IIb, the Phase IIc , and the Phase III studies demonstrated a between-laboratory reproducibility of 86%, an average within-laboratory reproducibility of 84%, and an average accuracy of 84% in the criteria that combined FInSLO-LA $\geq$ 1.4 and the lower limit of the 95% confidence interval of FInSLO-LA $\geq$ 1.0. This met the criterion set by VMT, and demonstrated the feasibility of IL-8 Luc as an *in vitro* skin sensitization method.

The performance of the IL-8 Luc assay for 122 chemicals examined by the lead laboratory was 72% accuracy, 72% sensitivity, and 74% specificity with the criteria that combined FInSLO-LA ≥ 1.4 and the lower limit of the 95% confidence interval of FInSLO-LA ≥ 1.0. To further improve the performance of the IL-8 Luc assay, the lead laboratory tested a different solvent (X-VIVO^{TM}15) to dissolve chemicals instead of DMSO. The IL-8 Luc assay using X-VIVO^{TM} 15 as a solvent exhibited significantly improved performance, to 80% accuracy, 86% sensitivity, and 64% specificity in the examination of 143 chemicals. Then the lead laboratory designated the IL-8 Luc assay using X-VIVO^{TM} 15 as the modified IL-8 Luc assay. After confirming that the modified IL-8 Luc assay produced essentially identical results as the original protocol for correctly judging chemicals evaluated by the original protocol, in the phase IV study, 3 experiment sets of 5 coded chemicals [2 strong and 2 moderate sensitizers and 1 non-sensitizers] were conducted using the modified IL-8 Luc assay. The criterion used was the combination FInSLO-LA ≥ 1.4 and the lower limit of the 95% confidence interval of FInSLO-LA ≥ 1.0. The results demonstrated an average 93.3% for within-laboratory reproducibility, 91.1% for between-laboratory reproducibility, and an average 84.4% of accuracy; these averages satisfied the acceptance criteria for the validation study. The combined results from the Phase IIb, the Phase IIc, the Phase III, and the Phase IV studies demonstrated an average 87.7% for within-laboratory reproducibility, 87.5% for between-laboratory reproducibility, and 82.4% for accuracy, 79.2% for sensitivity, and 90.0% for specificity thus meeting the criterion set by the VMT, and demonstrating the feasibility of IL-8 Luc as in vitro skin sensitization methods.

The major limitation of the IL-8 Luc assay is that this assay judges detergents as sensitizers irrespective of their classification. Therefore, detergents should be included in an exclusion criterion. If we delete the data for detergents from from the data set of the modified IL-8 Luc assay and evaluate the performance of the modified IL-8 Luc assay considering relative human skin sensitizing potency, the performance of the modified IL-8 Luc provided an accuracy of 83%, a sensitivity of 84%, and a specificity of 80% for 138 chemicals.

2. Background (Introduction)

Allergic contact dermatitis (ACD) is one of the most common skin diseases and affects 15-20% of the general population worldwide (Peiser et al., 2012). ACD results from T cell mediated immune responses induced by small reactive chemicals (haptens) and consists of two phases: the sensitization and the elicitation phase. In the sensitization phase, cutaneous dendritic cells induce the sensitization of naïve T cells specific to particular haptens. 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 in 2009, on cosmetics products and their ingredients. This was accompanied by a concomitant marketing ban, effective March 1, 2013, if animal tests were conducted after 2009, for the purpose of cosmetic legislation (Regulation (EC) No 1223/2009 of the European Parliament and of the Council, 2009). On the other hand, under European chemicals legislation REACH, skin sensitization data for all chemical registered under the European Chemicals Legislation (REACH, EC 1907/2006) produced or imported in quantities > 1 ton is mandatory, but at the same time REACH stated that animal testing should only be performed as a last resort (http://echa.europa.eu/documents/10162/13639/alternatives_test_animals_2014_en.pdf).

In 2012, the Organization for Economic Co-operation and Development (OECD) published the adverse outcome pathway (AOP) for skin sensitization (OECD 2012a, 2012b) defining the key events in the sensitization process (Fig. 1). In brief, the chemical sensitizer penetrates the stratum corneum, the uppermost layer of the skin, and subsequently binds covalently to proteins (key event 1) to form hapten-protein conjugates, which can be immunogenic. In parallel, keratinocytes are stimulated to release danger signals e.g. pro-inflammatory cytokines or ATP and, in addition, to activate anti-oxidative response genes (key event 2). Next, dendritic cells (DC) acquire mature phenotypes, such as induction of several co-stimulatory molecules and production of proinflammatory cytokines and chemokines by the concerted recognition of hapten-protein conjugates by MHC (major histocompatibility complex) molecules (key event 3). The activated DCs mobilize and migrate from the skin to the draining lymph node to present the allergen to T cells. After stimulation by DCs, hapten-peptide-specific T cells expand (key event 4) to elicit the eventual adverse outcome in case of a second exposure to the chemical sensitizer. The establishment of sensitization AOP has promoted the development of a variety of non-animal test methods to distinguish sensitizers from non-sensitizers or to generate potency information (reviewed previously in Adler et al., (2011) and Reisinger et al., (2015).

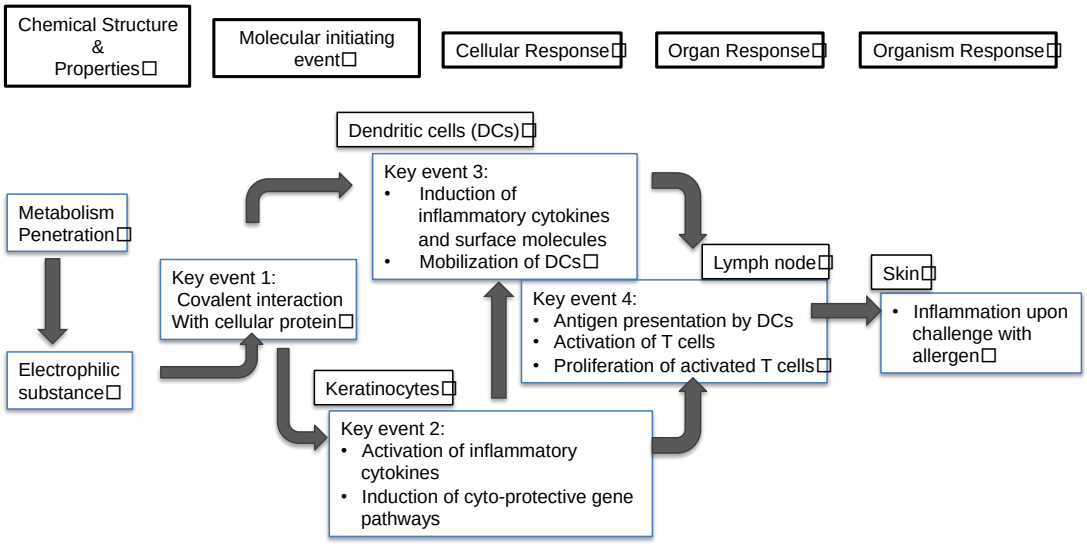
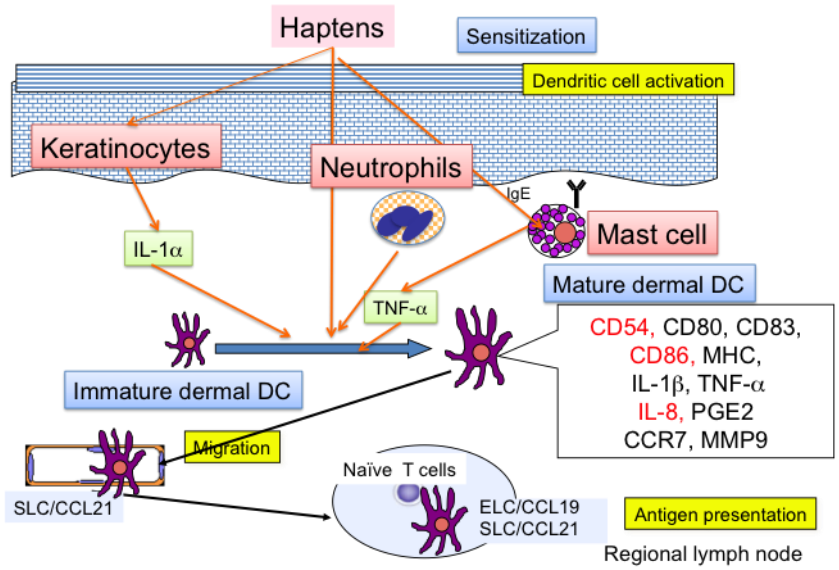


Fig. 1. Adverse outcome pathway of skin sensitization.

Recently, OECD released new test guidelines for skin sensitization testing using the direct peptide reactivity assay (DPRA) (OECD TG 442C) and the KeratinoSens (OECD TG 442D) test methods. DPRA examines protein-binding/haptenization of chemicals and KeratinoSens examines the induction of the Kelch-like ECH-associated protein 1 (Keap-1)/nuclear factor (erythroid-derived 2)-like factor 2 (Nrf2) pathways in keratinocytes. DPRA and KeratinoSens are considered to target two different key events in the AOP, i.e., key event 1 and 2, respectively. 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; Reisinger et al., 2015; Urbisch et al., 2015). In addition to these 2 test guidelines, 3 methods, the human Cell Line Activation Test (h-CLAT: Ashikaga et al., 2006; Sakaguchi et al., 2006), the myeloid U937 skin sensitization test, U-SENS (Piroird et al., 2015), and the current IL-8 Luc assay (Kimura et al., 2015; Takahashi et al., 2011) that target key event 3 underwent formal validation at the European Centre for Validation of Alternative Methods (ECVAM).

It is well known that DCs such as epidermal Langerhans cells (LCs) maintain immature phenotype in the absence of hapten stimulation. Once stimulated by haptens both *in vivo* or *in vitro*, they drastically change their phenotype from immature to mature phenotype (Aiba and Katz, 1990; Enk et al., 1993; Aiba et al., 1997). Mature DCs increase expression of CD40, CD54, CD80, CD83, and CD83 in addition to induction of proinflammatory cytokines, such as IL-1β and TNF-α, and several chemokines including IL-8 (CXCL8) and CCL3 (Steinman, 1991; Caux et al., 1994; Aiba and Tagami, 1999; Aiba, 2007). Several studies have suggested that the basic molecular mechanism of DC maturation is mainly dependent on the activation of p38 mitogen activated protein kinase (p38 MAPK) (Mitjans et al., 2008; Nukada et al., 2008; Sasaki et al., 2007). Therefore, biomarkers of DC activation should not be restricted to the increased expression of CD54 and CD86. The expression of other surface molecules such as CD40 and CD80 and the production of proinflammatory cytokines IL-1β and TNF-α and chemokines IL-8 and CCL3, which are also regulated by the activation of p38 MAPK, can also be good biomarkers for DC activation.

DCs clearly cannot sensitize naïve T cells in lymph nodes solely by their augmented expression of CD54 and CD86. DCs need to produce proinflammatory cytokines to stimulate T cells and chemokines like IL-8 to recruit other inflammatory cells. 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). Recently, Weber et al (Weber et al., 2015) clearly demonstrated that neutrophils are critically involved in both the sensitization and elicitation phase of contact hypersensitivity. The current understanding of the mechanism of DCs are involved in the sensitization phase is illustrated in Fig. 2.



Aiba and Katz, 1990; Enk et al., 1993; Aiba et al., 1997; Steinman, 1991; Caux et al., 1994; Aiba and Tagami, 1999; Aiba, 2007;Weber et al., 2015

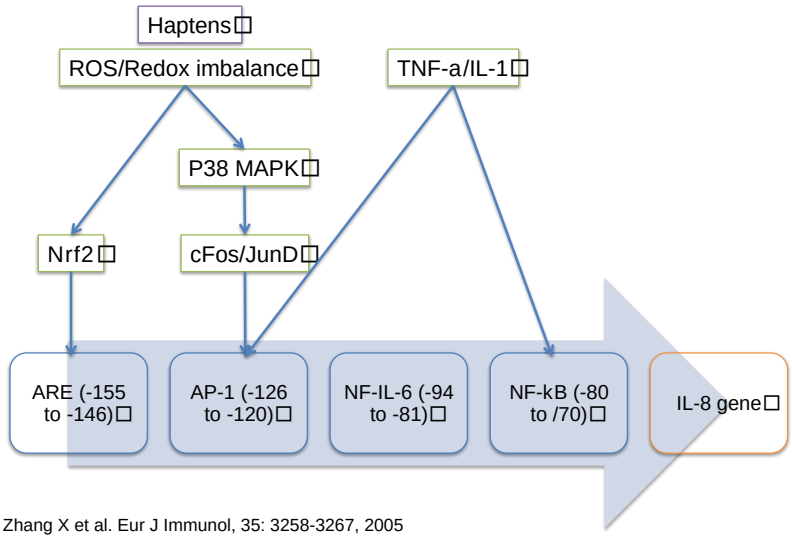
Fig. 2. DC activation in sensitization phase.

An increase in IL-8 mRNA or IL-8 protein may provide a biomarker for discriminating sensitizers from non-sensitizers in monocyte-derived dendritic cells (Toebak et al., 2006), U937 cells (Python et al., 2007), or THP-1 cells (Mitjans et al., 2010; Mitjans et al., 2008; Nukada et al., 2008). In addition, it was recently reported that human immature monocyte-derived dendritic cells (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 to 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, which 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 the crucial role of CXCR2 expression on DCs in both the elicitation phase and the sensitization phase 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 weight and structure, most share common 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). Electrophilicity can be detected by the Keap1-Nrf2 cellular sensor pathway implicated in the antioxidant response of the cell reviewed by Itoh et al. (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 containing an antioxidant response element (ARE) in their promoters (Holland and Fishbein, 2010). These gnes code for proteins mainly involved in detoxification, such as heme oxygenase-1 (HO-

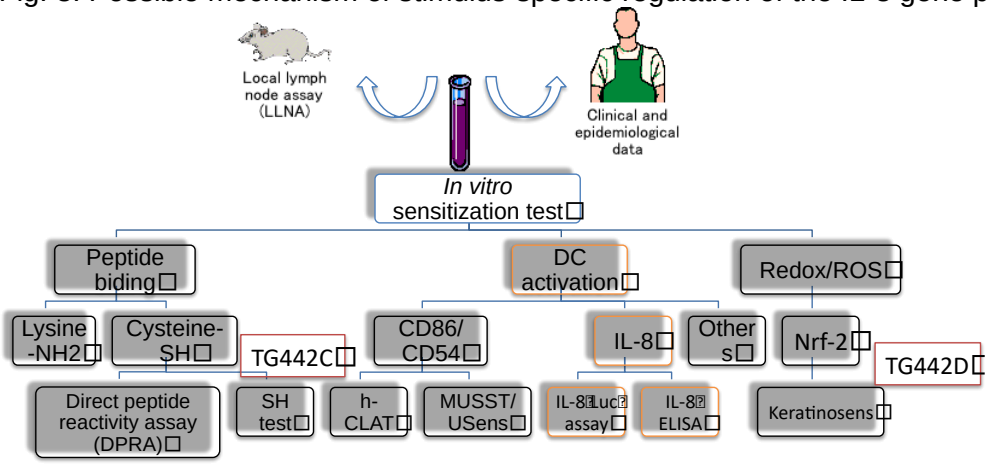
1) or NADPH-quinone oxidoreductase 1 (Nqo1). Interestingly, Zhang et al. reported that the 5' flanking region of IL-8 gene has several areas homologus to the consensus ARE (ATGAC/TnnnGCA/). In addition, Nrf2 only weakly induced IL-8 transcription, but significantly increased the half-life of IL-8 mRNA (Zhang et al., 2005). These data suggested that the induction of IL-8 mRNA by haptens is regulated transcriptionally by p38 MAPK (Mitjans et al., 2008; Nukada et al., 2008) and post-transcriptionally by Nrf2 (Fig. 3). Therefore, the IL-8 Luc assay is a unique screening method for haptens because it detects their effects on p38 MAPK and Keap1-Nrf2. Fig. 4 illustrates the position of the IL-8 Luc assay within the existing portfolio of test methods.

This study reports the validation of a non-animal test method to screen the sensitization potential of chemicals based on the use of THP-G8, a stable IL-8 reporter cell line.



Zhang X et al. Eur J Immunol, 35: 3258-3267, 2005
Kenneth A et al. J Leukocyte Biol, 65: 291-298, 1999
Mitjans M et al. Toxicol in Vitro, 22: 386-3895, 2008, 24: 1803-1809, 2010

Fig. 3. Possible mechanism of stimulus-specific regulation of the IL-8 gene promoter.



The accumulating evidence has suggested that IL-8 production or mRNA expression by either monocyte-derived dendritic cells (MoDCs) (Toebak, 2006), U937 cells (Python, 2007), or THP-1 cells (Mitjans, 2007; Nukada, 2008; Mitjans, 2010; Arkusz, 2010) can provides a promising *in vitro* tool for discrimination between allergens and irritants.

Fig. 4. The current status of development of *in vitro* sensitization test.

3. Objective of the study

The objective of the present validation study was to determine the usefulness and limitations of the IL-8 Luc assay as a non-animal screening method to detect and assess the skin sensitization potential of chemicals.

The specific objectives of the study were to establish:

1) “Transferability,” i.e., the extent to which a laboratory can adapt and easily implement the IL-8 Luc assay;

2) “Between or inter-laboratory reproducibility,” i.e., the extent to which results agree among a number of laboratories;

3) "Within or intra-laboratory reproducibility", i.e., the extent to which results agree in the same laboratory; and

3) “Predictivity,” i.e., the extent to which the *in vitro* results agree with the sensitization potential determined using the murine local lymph node assay or the reported human skin sensitizing potency.

4. Test Method and modification

4-1. The IL-8 assay and refinements of test method by the lead laboratory

4-1-1. First-reported IL-8 Luc assay

To develop a high-throughput method to detect IL-8 mRNA induction, Takahashi et al., (2011) established a stable IL-8 reporter cell line, THP-G8, by transfecting plasmid vectors into THP-1, in which the expression of the stable luciferase orange (SLO) and stable luciferase red (SLR) luciferase genes is regulated by the promoters of IL-8 and GAPDH, respectively. The SLO luciferase activity of THP-G8 cells correlate well with IL-8 mRNA expression by THP-1 after various stimulations, e.g., LPS, TNF-α, 2,4-dinitrochlorobenzene (DNCB), and nickel sulfate ($NiSO_4$).

In the original IL-8 Luc assay, THP-G8 cells were treated for 6 h with a test chemical, then SLO luciferase activity (SLO-LA) and SLR luciferase activity (SLR-LA) were measured and three parameters were defined: (1) normalized SLO luciferase activity (nSLO-LA), which is the quotient obtained by dividing SLO-LA by SLR-LA; (2) the fold induction of nSLO-LA (FInSLO-LA), which is the quotient obtained by dividing nSLO-LA of chemically treated cells by that of non-treated cells; and (3) the inhibition index of SLR-LA (II-SLR-LA), which is the quotient obtained 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 inhibitory effects of pretreatment with N-acetylcysteine (NAC) on FInSLO-LA of chemically treated cells was assessed, and the inhibition index (I.I.) obtained by dividing FInSLO-LA after chemical treatment with NAC pretreatment by FInSLO-LA without NAC pretreatment was calculated.

Using these parameters, the following criteria were chosen to identify possible sensitizers. Chemicals were categorized as sensitizers if the chemicals demonstrated FInSLO-LA **≥** 1.4 and I.I. ≤ 0.8 using the concentration of the chemical at which I.I.-SLR-LA is **≥**0.2 in 2 or 3 of 3 different experiments. Chemicals were categorized as non-sensitizers if they did not fulfill these criteria. According to these criteria, the IL-8 Luc assay for 35 chemicals, including reference chemicals published by ECVAM, further referred to as the “ECVAM list” (Casati et al., 2009) resulted in an overall test accuracy of 82%.

4-1-2. Modification of the IL-8 Luc assay

In the course of the validation study, several problems with the IL-8 Luc assay became evident. Under the agreement of VMT members, each was addressed individually and the protocols were modified as follows.

4-1-3. Determination of the optimal incubation time

The performance of the originally reported IL-8 Luc assay (Takahashi et al., 2011a) was 86% accuracy, 83% sensitivity, and 90% specificity for 35 chemicals. Ring trials with 3 different laboratories identified several problems in the between laboratory reproducibility and correct

classification with this assay as summarized in Table 1. The lead laboratory therefore explored the reason for the poor response of the IL-8 Luc assay to several potent sensitizers, such as DNCB. In previous study performed in the lead laboratory (Takahashi et al., 2011), the time course of FInSLO-LA of THP-G8 cells after LPS stimulation was examined and found maximum induction of FInSLO-LA between 4 and 7 h after stimulation. In this study, the time course of FInSLO-LA after treatment was re-examined with the strong sensitizers DNCB and 4-NBB.

Table 1. Results from Phase I and Phase IIa studies

Study	Chemical name	LLNA	Lab.A	Lab.B	Lab.C
I	2,4-Dinitrochlorobenzene	Extreme	S	S	N
	1,4-Phenylenediamine	Strong	S	N	N
	2-Mercaptobenzothiasole	Moderate	N	N	N
	Cinnamal	Moderate	N	N	N
	Tetramethyl thiuiram disulphide	Moderate	S	S	S
	Eugenol	Weak	S	N	N
	Imidazolidinyl urea	Weak	S	S	S
	Lactic acid	Non-sensitizer	N	N	N
	Salicylic acid	Non-sensitizer	N	N	N
	Sodium lauryl sulphate	Non-sensitizer	N	N	N
	Accuracy (%)		0.8	0.6	0.5
☐	Interlaboratoy reproducibility (%)			0.7	
			☐	☐	☐
IIa	2,4-Dinitrochlorobenzene	Extreme	S	N	S
	4-Nitrobenzylbromide	Extreme	S	S	S
	1,4-Phenylenediamine	Strong	S	S	S
	2-Mercaptobenzothiasole	Moderate	S	S	S
	Glyoxal	Moderate	N	N	N
	Cinnamal	Moderate	S	S	S
	Eugenol	Weak	S	S	N
	Glycerol	Non-sensitizer	N	N	N
	Isopropanol	Non-sensitizer	N	N	N
	Methyl salicylate	Non-sensitizer	S	N	S
	Accuracy (%)		0.8	0.8	0.7
☐	Interlaboratoy reproducibility (%)			0.7	

N: Non-sensitizer, S: sensitizer☐

THP-G8 cells were stimulated with different concentrations of DNCB or 4-NBB for different periods of time and FInSLO-LA was measured (Fig. 5). DNCB and 4-NBB significantly increased 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 a concentration of 1.19 μg/mL and 2.67 μg/mL, respectively. On the other hand, the maximum induction by 4-NBB was observed between 8 h and 10 h at a concentration of 1.19 μg/mL and at 10 h and 16 h at a concentration of 2.67 μg/mL.

Next, SLO-LA and SLR-LA of THP-G8 cells was monitored in real time during DNCB or 4-NBB treatment using a dish-type luminometer (Fig. 6). Consistent with the results obtained by measuring luciferase activity intermittently, real-time monitoring demonstrated an increase in FInSLO-LA from 5 h to 24 h by both 0.8 μg/mL and 1.6 μg/mL of DNCB and 4-NBB, with maximum induction at 12 h and 10 to 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, FInSLO-LA between 6 h incubation and 16 h incubation were compared as shown in Fig. 5 and Fig. 6**.** For both DNCB and 4-NBB, intermittent measurements and real-time monitoring indicated that FInSLO-LA at the optimal concentration was much higher following 16 h incubation than 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).

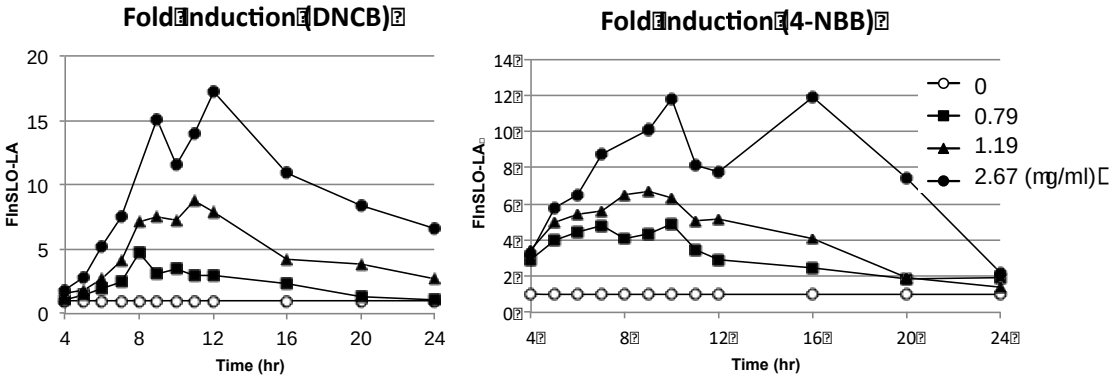


Fig. 5. Determination of the optimal incubation time - Time course study for the IL-8 Luc assay.
THP-G8 cells were stimulated with the indicated dose of DNCB or 4-NBB for various time periods, and luciferase activity was measured using a microplate-type luminometer.

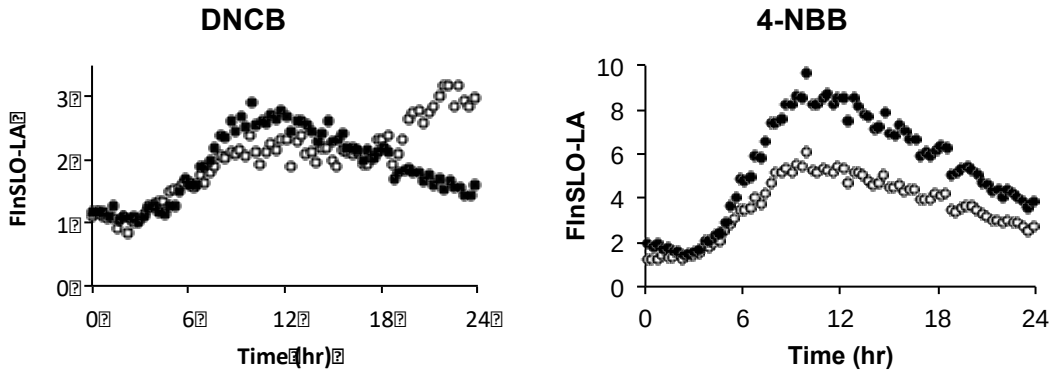


Fig. 6. Determination of the optimal incubation time - Real time monitoring of IL-8 luciferase activity
THP-G8 cells were suspended in RPMI (GIBCO) supplemented with 10% FBS, 0.1 mM D-luciferin, 25 mM Hepes/HCl (pH 7.0) and plated onto 35 mm dishes at $2x10^6$ cells/dish. After 30 min, 0.8 or 1.6 µg/mL DNCB, or 0.8 or 1.6 µg/mL 4-NBB, 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 (AB2500 Kronos). Open circles: 0.8µg/mL, closed circles: 1.6 µg/mL

Finally, the ECVAM list of chemicals was re-evaluated 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 2, 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%.

Table 2. Comparison of the performance between IL-8 Luc assay for 16 h and for 6 h

		The IL-8 Luc assay (6h) Takahashi et al. in Toxicol Sci								The IL-8 Luc assay (16h)							
Chemical	LLNA	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

4-1-4 Comparison between the criteria with I.I.-SLR-LA≥0.2 and those with I.I.-SLR-LA≥0.05

After increasing the incubation time from 6 h to 16 h, the lead laboratory examined additional 89 chemicals used as a dataset for h-CLAT and evaluated their skin sensitization potential (Table S1). In contrast to previous study (Takahashi et al., 2011), Cooper statistics of the IL-8 Luc assay for these 89 chemicals yielded an accuracy of 69% (61/89), a sensitivity of 59% (37/63), and a specificity of 92% (24/26), suggesting that the IL-8 Luc assay using the current criteria produce false negative results for a considerable number of sensitizers tested.

Closer examination of the data showed that the treatment that produced the most false negative results increased FInSLO-LA more than 1.4 at the concentrations providing I.I.-SLR-LA <0.2 (data not shown). The study of the lead laboratory demonstrated that I.I.-SLR-LA did not correlate with 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. They 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 ≥0.05 maintained more than 75% of the PI-excluding cells (Fig. 7). Therefore, the lead laboratory examined the performance of the IL-8 Luc assay using the new criterion I.I.-SLR-LA≥0.05 and obtained Cooper statistics for these 97 chemicals of 74% (72/97) accuracy, 69% (48/70) sensitivity, and 89% (24/27) specificity.

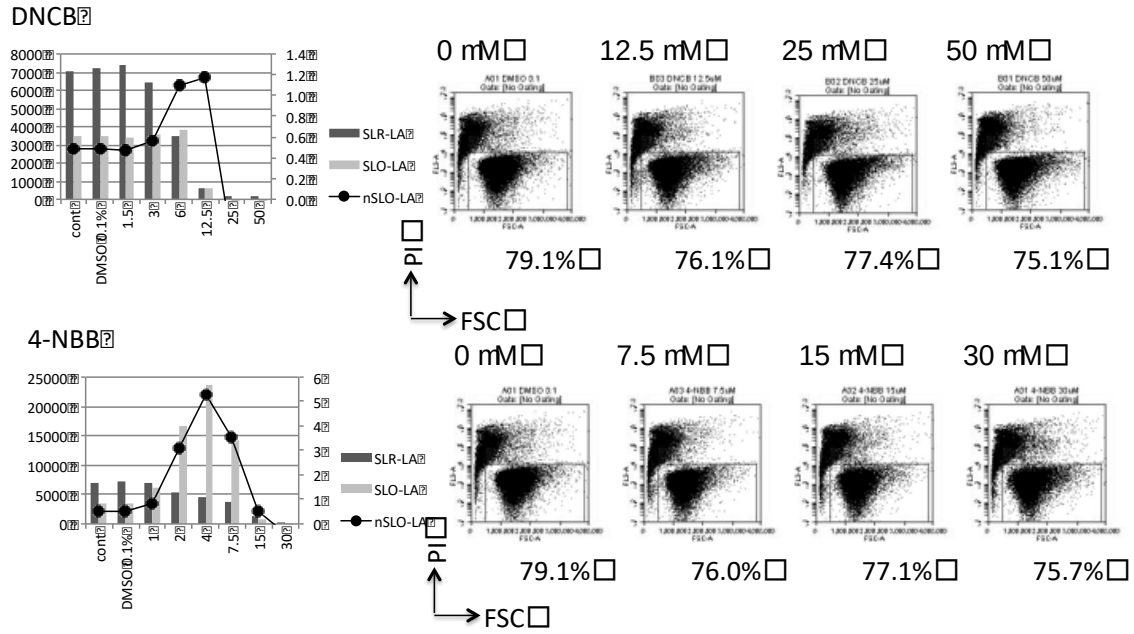


Fig. 7. Comparison between criteria with I.I.-SLR-LA≥0.2 and those with I.I.-SLR-LA≥0.05

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 30μg/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.

4-1-5. Comparison between criteria with and without response to NAC treatment

Despite having accepted the I.I.-SLR-LA≥0.05 criterion, the IL-8 Luc assay still produced false negative results. Therefore, VMT suggested further modification of the classification criteria. Although most haptens react with cysteine residues, there may be some exceptions. For example, Gerberick et al. (2007) reported that phthalic anhydride and trimellitic anhydride significantly reacted with GSH and lysine peptides, but not with cysteine peptides in their direct peptide reactivity assay (DPRA). It was hypothesized that removing the condition that I.I. is ≤ 0.8 (Criterion 1) would increase the accuracy and specificity of the IL-8 Luc assay. Indeed, this 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 3)

Table 3. Comparison of the performance among different criteria

Criteria	With I.I. criterion (I.I. <=0.8)			Without I.I. criterion		
	Without I.I.-SLR-LA criterion	I.I.-SLR-LA>=0.2	I.I.-SLR-LA>=0.05	Criterion 1	Criterion 2	Criterion 3
Chemical numbers	98	89	97	122	122	122
Accuracy	75%	69%	74%	77%	73%	73%
Sensitivity	69%	59%	69%	77%	80%	72%
Specificity	89%	92%	89%	74%	59%	74%

Ninety eight chemicals were evaluated with the criterion of I.I. <= 0.8, while the remaining 24 chemicals were evaluated without it. Among 98 chemicals, some chemicals could not be judged because some experiments did not satisfy the criterion regarding I.I.-SLR-LA.

4-1-6. Comparison between the criterion FInSLO-LA ≥ 1.4, the criterion the lower limit of the 95% confidence interval of FInSLO-LA≥1.0, and their combination

VMT empirically determined the condition of FInSLO-LA ≥ 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 by the KeratinoSens assay (Emter et al., 2010). The lead laboratory likewise tried to evaluate each IL-8 Luc assay experiment statistically. In evaluation of 122 sensitizers including 9 extreme, 17 strong, 34 moderate, and 28 weak sensitizers, and 34 non-sensitizers as classified by the LLNA, using the criterion of the lower limit of the 95% confidence interval of FInSLO-LA $\geq$ 1.0 (Criteria 2) did not necessarily improve the performance of the IL-8 Luc assay (accuracy of 74%, sensitivity of 80%, specificity of 59%). Also the performance of the combination of Criterion 1 and Criterion 2 (Criterion 3) was examined. Both the accuracy and sensitivity of Criterion 3 were lower than those of Criterion 1, although the specificity of Criterion 3 and Criterion 1 were equal (Table S1 and Table 3).

4-1-7. IL-8 Luc assay performance for 122 chemicals.

Following these modifications, the lead laboratory independently examined 122 chemicals, including the chemicals used in the validation study (Table S1 and Table 3). All the chemicals had been previously evaluated and classified with the LLNA (Gerberick et al., 2005). Eighty-eight sensitizers (9 extreme, 17 strong, 34 moderate and 28 weak sensitizers) and 34 non-sensitizers as classified by the LLNA were evaluated. The performance of the IL-8 Luc assay was 76% accuracy, 77% sensitivity, and 74% specificity with Criterion 1; 74% accuracy, 80% sensitivity, and 59% specificity with Criterion 2; and 72% accuracy, 72% sensitivity, and 74% specificity with Criterion 3.

4-1-8. Modification of the IL-8 Luc assay to obtain better performance

Recently, several researchers have demonstrated that most sensitizers can bind to both fetal bovine serum (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 electrophilicity of haptens might be reduced in the presence of FBS, reducing their binding to nucleophilic moieties in cellular proteins.

Therefore, the lead laboratory 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™ 15 (DMSO/ X-VIVO™ 15); and (3) solubilize in X-VIVO™ 15 and then dilute with X-VIVO™ 15 (X-VIVO™ 15 / X-VIVO™ 15) (Figs. 8a, b, and c). As the lead laboratoryhave 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 ≥0.05. In contrast, oxazolone diluted with X-VIVO™ 15 / X-VIVO™ 15 significantly and dose-dependently induced FInSLO-LA at the concentration at which I.I.-SLR-LA showed ≥0.10.

Oxazolone diluted with DMSO/ X-VIVO™ 15 significantly induced FInSLO-LA at the concentration at which I.I.-SLR-LA showed ≥0.6, but significant induction was observed only at this single concentration. These results clearly demonstrated that the dilution of oxazolone with X-VIVO™ 15 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; the lead laboratorytherefore next examined whether changing the culture medium during treatment with the chemicals from RPMI-1640 with 10% FBS to X-VIVO™ 15 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™ 15 than that RPMI-1640 with 10% FBS.

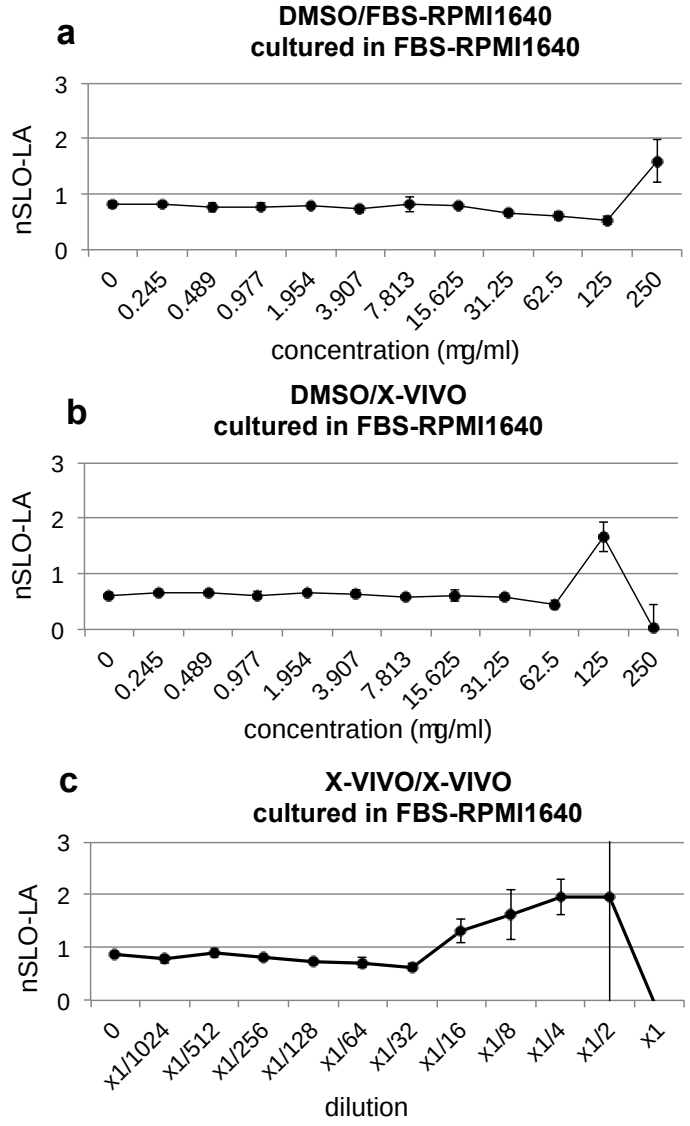


Fig. 8. Effects of FBS on the IL-8 Luc assay

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™ 15 (DMSO/ X-VIVO™ 15); and (c) solubilize in X-VIVO™ 15 and then dilute with X-VIVO™ 15 (X-VIVO™ 15 / X-VIVO™ 15).

Next, the lead laboratory examined whether the other chemicals providing false negative results in the IL-8 Luc assay (Criterion 3) could be judged as sensitizers if diluted with X-VIVO™ 15 / X-VIVO™ 15 (Table 4). Interestingly, 12 of the 20 chemicals that showed false negative results were judged as sensitizers. To confirm the efficacy of solubilization of chemicals with X-VIVO™ 15, the lead laboratory re-evaluated the chemicals in the ECVAM list (Table 5). The IL-8 Luc assay using chemicals diluted with X-VIVO™ 15 / X-VIVO™ 15 increased the FInSLO-LA of most sensitizers, such as 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™ 15 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™ 15 can improve the accuracy and sensitivity, and does not lower the specificity.

Table 4. Re-evaluation of chemicals demonstrating false negative results by the IL-8 Luc assay

using X-VIVO as a solvent

Chemicals	□			□	□	Judgment
		1st	2nd	3rd	4th	
Oxazolone	Extreme	1.66	3.98	□	□	sensitizer
1,4-Phenylenediamine(PPD)	Strong	2.48	4.30			sensitizer
Maleic anhydride	Strong	1.08	1.24	1.08		non-sensitizer
Phthalic anhydride	Strong	1.09	1.02	1.38		non-sensitizer
2-Hydroxyethyl acrylate	Moderate	1.00	1.00	1.00		non-sensitizer
3,4-Dihydrocoumarin	Moderate	1.00	1.00	2.06	1.59	sensitizer
4-Chloroaniline	Moderate	1.81	1.48			sensitizer
Diethyl maleate	Moderate	1.56	2.01	1.68		sensitizer
Diethyl sulfate	Moderate	2.11	3.27			sensitizer
Ethylenediamine	Moderate	1.96	2.38			sensitizer
Glyoxal	Moderate	3.50	2.23			sensitizer
Methyl-2-nonynoate	Moderate	1.93	5.91			sensitizer
Trimellitic anhydride	Moderate	2.28	1.66			sensitizer
1-Bromohexane	Weak	1.07	1.05	1.00		non-sensitizer
4-Allylanisole	Weak	4.67	19.71			sensitizer
Aniline	Weak	1.03	1.12	1.09		non-sensitizer
Benzocaine	Weak	1.11	1.00	1.00		non-sensitizer
Ethyleneglycol dimethacrylate	Weak	4.98	4.36			sensitizer
Penicillin G	Weak	1.35	1.00	1.62	1.10	non-sensitizer
Pyridine	Weak	1.09	1.00	1.00	□	non-sensitizer

Table 5. 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	3rd	4th	
Oxazolone	1.66	3.98	☐	☐	sensitizer
4-NBB	10.03	8.77			sensitizer
Glyoxal	3.5	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	4.3	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	1.29	☐	non-sensitizer
Salicylic acid	1.18	1	1.96	1.36	non-sensitizer
Lactic acid	1	1.08	1.49	1.02	non-sensitizer
SLS	2.94	2.88	☐	☐	sensitizer

Finally, the lead laboratory independently examined 143 chemicals, including 122 chemicals examined by the original IL-8 Luc (Table S2). All the chemicals had been previously evaluated and classified with the LLNA (Gerberick et al., 2005). One hundred seven sensitizers (12 extreme, 18 strong, 40 moderate and 37 weak sensitizers) and 36 non-sensitizers as classified by the LLNA were evaluated. The performance of the modified IL-8 Luc assay was 80% accuracy, 86% sensitivity, and 64% specificity. Comparison of the performance between the IL-8 Luc assay and the IL-8 Luc assay using X-VIVO™ 15 as a solvent (modified IL-8 Luc assay) for 122 chemicals (Table S2) clearly indicated that the modified IL-8 Luc assay significantly improved accuracy and sensitivity (Table 6).

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

Protocols	IL-8 Luc assay	Modified IL-8 Luc assay (X-VIVO)	
Chemical No	122	122	143
Accuracy	72%	80%	80%
Sensitivity	72%	85%	86%
Specificity	74%	65%	64%

4-1-9. Comparability of the modified IL-8 Luc assay to the original IL-8 Luc assay

Next, we examined whether the modified IL-8 Luc assay made false judgment for chemicals correctly evaluated by the original protocol. Of the 122 chemicals, the modified IL-8 Luc assay provided 3 false negative results among 63 sensitizers correctly evaluated by the original protocol, and 4 false positive results among 25 non-sensitizers. The concordance rate between the original and modified IL-8 Luc assays was 92% for chemicals correctly judged by the original IL-8 Luc assay.

4-2. Bioluminescence system

In a typical dual-reporter assay, firefly luciferase from *Photinus pyralis* (FLuc) is used as the experimental reporter and Renilla luciferase is used as the internal control reporter. This internal control reporter connects to a constitutively expressed promoter, such as the herpes simplex virus thymidine kinase promoter, cytomegalovirus (CMV) immediate-early promoter, or simian virus 40 (SV40) promoter. This assay system is commercialized as a Dual-Luciferase Reporter Assay System by Promega Corporation. In this system, both luciferase activities are measured sequentially from single extracts on the basis of their bioluminescent substrate specificity. Firefly luciferase activity is measured first by adding firefly D-luciferin, and then Renilla luciferase activity is measured by adding coelenterazine (another name for Renilla luciferin), with concomitant quenching of firefly luciferase luminescence. Finally, firefly luciferase activity is normalized by Renilla luciferase activity as the promoter activity (Michelini et al., 2014; Nakajima and Ohmiya, 2010; Roda et al., 2004).

An alternative chemical test using a cell-based assay requires the analysis of a large number of samples. It is therefore preferable to use an improved assay system whereby gene expression can be monitored simultaneously in a one-step reaction in single extracts. Beetle luciferases emit red luminescence during reaction, compared to the green emitted by firefly D-luciferin. The two colors can be divided using an optical filter. The dual color-reporter assay is based on the color difference between beetle and firefly luciferases and is sold commercially as the Tripluc Reporter Assay System by TOYOBO (Nakajima et al., 2004; Nakajima et al., 2005).

In the IL-8 Luc assay, the dual-color assay system consisted of an orange-emitting luciferase (SLO; λmax = 580 nm) (Viviani et al., 2001) for the gene expression of the IL-8 promoter, and a red-emitting luciferase (SLR; λmax = 630 nm) (Viviani et al., 1999) for the gene expression of the internal control promoter, GAPDH. The two luciferases emit different colors upon reacting with firefly D-luciferin and their luminescence is measured simultaneously in a one-step reaction by dividing the emission from the assay mixture using an optical filter (Nakajima et al., 2005). First, the total relative light units (F0) are measured in the absence of the filters. Then, the F1 value that passed through the R60 filter (>600-nm long-pass filters) is measured. The two luciferase activities are calculated using the simultaneous equation shown below by substituting the F0 and F1 values. In this equation, O and R are the activities of the orange- and red-emitting luciferases, respectively, and κO_{R60} and κR_{R60} are the transmission coefficients of the orange- and red-emitting luciferases of the R60 filter, respectively.

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

Luminescence activity is measured using a filtered 96-well microplate luminometer (for example, Phelios (ATTO, Tokyo, Japan), Tristan 941 (Berthold, Bad Wildbad, Germany), and the ARVO series (PerkinElmer, Waltham, MA). It is necessary to calibrate the luminometer in each experiment to ensure reproducibility (Niwa et al., 2010). Recombinant orange- and red-emitting luciferases are available for this calibration.

5. Validation Management Structure

5-1. Validation Management Team (VMT)

Chairman:	Noriho Tanaka, the chairman of this validation study, retired from Hatano Research Institute, Food and Drug Safety Center (HRI/FDSC) in 2011. Although he is still attached to a part-time research advisor of HRI, his position in HRI during the validation study is independent from the participating Laboratory.
Management of quality control:	Hajime Kojima (Japanese Center for the Validation of Alternative Methods (JaCVAM), National Institute of Health Sciences (NIHS), Tokyo, Japan)
Chemical selection team:	Hitoshi Sakaguchi (Kao Co. Ltd.), Hiroshi Itagaki (Yokohama National Univ.), Hajime Kojima (NIHS)
Quality assurance team:	Yoshihiro Ohmiya (National Institute of Advanced Industrial Science and Technology (AIST)), Ayako Sakai (HRI) (2011-2013)
Lead laboratory:	Setsuya Aiba (Tohoku University, Miyagi, Japan) Yutaka Kimura (Tohoku University, Miyagi, Japan)
Representative of ECVAM:	Emanuela Corsini (Università degli Studi di Milano, Milan, Italy)
Representative of ICCVAM:	William S. Stokes (Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods, National Institute of Environmental Health Sciences, North Carolina, USA) (2011-2012)
Representative of ICCVAM:	Warren M. Casey (ICCVAM, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods, National Institute of Environmental Health Sciences, North Carolina, USA) (2012-2013)
Representative of ICCVAM:	David G. Allen (NICEATM, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods, National Institute of Environmental Health Sciences, North Carolina, USA)
Representative of KoCVAM:	Ai-Young Lee (Korean Center for the Validation of Alternative Methods (KoCVAM), Dongguk University Medical Center, Gyeonggi-do, Korea)
Representative of ZEBET:	Michael Oelgeschlager (ZEBET-Alternative Methods to Animal Experiments, Berlin, Germany) (2011-2012)
Data management team:	Takashi Omori (Doshisha University, Kyoto, Japan) Aoi Maruya, Mayumi Kobayashi, Azusa Mori (Doshisha University)

5-2. Chemical selection, acquisition, coding and distribution

1) Definition of selection criteria
2) Chemical selection
Members:
Hajime Kojima; JaCVAM
Hitoshi Sakaguchi; Kao Co. Ltd.
Hiroshi Itagaki; Yokohama National Univ.
(1) Liaise with suppliers
(2) Final check of chemicals provided
(3) Acquisition
(4) Coding
(5) Distribution
Member:
Hajime Kojima; JaCVAM

5-3. Independent biostatistician

1) Approve spreadsheets
2) Data collection
3) Data analysis
Members:
Takashi Omori (Doshisha University, Kyoto, Japan)
Aoi Maruya, Mayumi Kobayashi and Azusa Mori (Doshisha University)

5-4. Participating laboratories

The laboratories participating in the validation study are defined as follows:
Laboratory 1: HRI (Mika Watanabe, Kohji Yamakage; Kanagawa, Japan)
Laboratory 2: Sumitomo Chemical Co. Ltd. (Noriyuki Suzuki, Koichi Saito; Osaka, Japan)
Laboratory 3: AIST (Yoshihiro Nakajima; Kagawa, Japan)
All these laboratories had not been involved in the development of the IL-8 Luc assay and were entirely naïve with respect to the IL-8 Luc assay.
Table 7. Participating laboratories, the validation studies to which they contributed, and their identification (ID) codes used in the respective studies.

Laboratory	Pre-validation	Phase I	Phase II	Phase III	Phase IV
HRI	Lab A	Lab A	Lab A	Lab A	Lab A
Sumitomo	Lab B	Lab B	Lab B	Lab B	Lab B
AIST	Lab C	Lab C	Lab C	Lab C	Lab C

5-5. Quality assurance

The present study was conducted in the spirit of Good Laboratory Practice (GLP), although not all the participating laboratories routinely worked under GLP certification. The data presented were inspected by the quality assurance team: Ayako Sakai (HRI) and Yoshihiro Ohmiya (AIST).

5-6. Management office

Shojiro Yamazaki (HRI)
729-5 Ochiai Hadano, Kanagawa, 257-8523
TEL: +81-463-82-4751 FAX: +81-463-82-9627
e-mail: yamazaki.s@fdsc.or.jp

5-7. Meetings held

8/11/2011 (Doshisha Univ., Kyoto)
1st validation management team (VMT) Meeting
Subjects: Technical transfer, plan for the Phase I study, protocol, and test chemicals for the Phase I study.
VMT members: Noriho Tanaka, Hajime Kojima, Yutaka Kimura, Takashi Omori, Yoshihiro Ohmiya
Participating laboratories: HRI, Sumitomo Chemical, AIST

11/11/2011 (Miyagi Prefecture Kensetsu Sangyo Kaikan, Sendai)
1st International VMT Meeting
Subjects: Explanation of the outline of the Phase I study, protocol, and test chemicals for the Phase I study.
VMT members: Noriho Tanaka, Hajime Kojima, Setsuya Aiba, Yutaka Kimura, Takashi Omori, Yoshihiro Ohmiya, Joachim Kreysa, William S. Stokes, Mike Inskip, Hitoshi Sakaguchi, Hiroshi Itagaki
Participating laboratories: HRI, Sumitomo Chemical, AIST
6/12/2011 (E-mail)
E-mail meeting
Subject: Prioritizing the test chemicals used in the Phase I study.
VMT members: Noriho Tanaka, Hajime Kojima, Setsuya Aiba, Yutaka Kimura, Takashi Omori
Participating laboratories: HRI, Sumitomo Chemical, AIST

21-22/2/2012 (Doshisha Univ., Kyoto)
2nd International VMT Meeting
Subjects: Evaluation of the results from the Phase I study and planning the protocol and test chemicals for the Phase II study.
VMT members: Noriho Tanaka, Hajime Kojima, Setsuya Aiba, Yutaka Kimura, Takashi Omori, William S. Stokes, Emanuela Corsini, Ai-Young Lee, Michael Oelgeschlaeger, Hitoshi Sakaguchi, Hiroshi Itagaki, Yoshihiro Ohmiya, Ayako Sakai
Participating laboratories: HRI, Sumitomo Chemical, AIST

6/7/2012 (Tokyo office of Doshisha Univ., Tokyo)
3rd VMT Meeting
Subjects: Modification of the protocol based on the evaluation of the Phase-I study by the lead laboratory, and schedule for the Phase-IIa study.
VMT members: Noriho Tanaka, Hajime Kojima, Setsuya Aiba, Yutaka Kimura, Takashi Omori, Yoshihiro Ohmiya
Participating laboratories: HRI, Sumitomo Chemical, AIST
13-14/9/2012 (Doshisha Univ., Kyoto)
4th International VMT Meeting
Subjects: Progress report following the Phase I study, report from the Phase IIa study, and planning of the Phase IIb study.
VMT members: Noriho Tanaka, Hajime Kojima, Setsuya Aiba, Yutaka Kimura, Takashi Omori, William S. Stokes, Emanuela Corsini, Ai-Young Lee, Hitoshi Sakaguchi, Yoshihiro Ohmiya, Ayako Sakai,
Participating laboratories: HRI, Sumitomo Chemical, AIST

14-15/2/2013 (Doshisha Univ., Kyoto)
5th International VMT Meeting
Subjects: Objective of Phase IIb study and protocol amendments, progress report following the Phase IIb study, and schedule for future validation studies.
VMT members: Noriho Tanaka, Hajime Kojima, Setsuya Aiba, Yutaka Kimura, Takashi Omori, Warren M. Casey, Emanuela Corsini, Ai-Young Lee, Hitoshi Sakaguchi, Hiroshi Itagaki, Yoshihiro Ohmiya, Ayako Sakai,
Participating laboratories: HRI, Sumitomo Chemical, AIST

28/9/2013 (Doshisha Univ., Kyoto)
Meeting to discuss future plans for the validation
Subjects: Future plans for the validation.
VMT members: Noriho Tanaka, Hajime Kojima, Setsuya Aiba, Yutaka Kimura, Takashi Omori, Yoshihiro Ohmiya,
Participating laboratories: HRI, Sumitomo Chemical, AIST

17/10/2013 (Skype-meeting)
Meeting by Skype
Subjects: Future plans for validating and scheduling the Phase IIc study.
VMT members: Noriho Tanaka, Hajime Kojima, Setsuya Aiba, Yutaka Kimura, Takashi Omori, Yoshihiro Ohmiya,
Participating laboratories: HRI, Sumitomo Chemical, AIST

25-26/2/2014 (Doshisha Univ., Kyoto)
6th International VMT Meeting
Subjects: Progress report following the Phase IIc study, and scheduling the Phase III study.
VMT members: Noriho Tanaka, Hajime Kojima, Setsuya Aiba, Yutaka Kimura, Takashi Omori, David G. Allen, Emanuela Corsini, Ai-Young Lee, Hitoshi Sakaguchi, Yoshihiro Ohmiya,
Participating laboratories: HRI, Sumitomo Chemical, AIST

26-27/9/2014 (AIST, Tokyo Waterfront, Tokyo)
7th International VMT Meeting
Subjects: Progress report following the Phase III study, and evaluation of within-laboratory reproducibility, beteen-laboratory reproducibility, and predictivity of the IL-8 Luc Assay, incorporating the results of the Phase IIb, IIc, and III studies.

VMT members: Noriho Tanaka, Hajime Kojima, Setsuya Aiba, Yutaka Kimura, Takashi Omori, David G. Allen, Emanuela Corsini, Ai-Young Lee, Hitoshi Sakaguchi, Hiroshi Itagaki, Yoshihiro Ohmiya,
Participating laboratories: HRI, Sumitomo Chemical, AIST

9/10/2015 (Kobe Univ., Koube)
8th International VMT Meeting and teleconference
Subjects: Progress report following the Phase IV study using X-VIVO^{TM} 15, and evaluation of within-laboratory reproducibility, beteen-laboratory reproducibility, and predictivity of the IL-8 Luc Assay, incorporating the results of the Phase IIb, IIc, III and IV studies.
VMT members: Noriho Tanaka, Hajime Kojima, Setsuya Aiba, Yutaka Kimura, Takashi Omori, Yoshihiro Ohmiya, and David G. Allen, Emanuela Corsini to attend only teleconference
Participating laboratories: HRI, Sumitomo Chemical, AIST

6. Study Design

The objective of this study was to evaluate the within- and between-laboratory reproducibility and predictivity of the IL-8 Luc assay on skin sensitization potential (concordance with the two classifications: sensitizer and non-sensitizer). As a complementary study, the VMT evaluated the predictability of the United Nations Globally Harmonized System of Classification and Labeling of Chemicals (UN GHS) with three classifications (Category 1A, Category 1B and non-sensitizer).

The validation study (Phase I, Phase IIa, Phase IIb, Phase IIc, and Phase III trials) was conducted by three laboratories based on the study design and schedule shown in Tables 8 and 9 and using the test chemicals shown in Tables 10, 11, 12, 13, and 14. The methods were described above in '4. Test Method 4.1 IL-8 Luc assay' and the precise protocol is described below in '8. Protocol 8.2 Protocol for the IL-8 Luc assay'.

Table 8. The number of chemicals analyzed in the validation study

Studies	Within-laboratory	Between-laboratories	Predictivity
I		10	10
IIa		10	10
IIb	5	5	5
IIc	5	5	5
III		20	20
IV	5	5	5
Total	15	55	55

7. Test Chemicals

The selection process for the test chemicals for the IL-8 Luc assay validation study is described below.

In addition, the chemical categories or physical state and chemical properties (e.g., solid: liquid, etc.) were included in the tables of these test chemicals in order to investigate the applicable domain.

Table 9. Breakdown of the IL-8 Luc assay validation study

Phase	The number of the test substances	The number of the repetitions	Examination	Date of experiment start
Pre	3	1	Between- laboratory transferability (Non-coded)	October, 2011
I	10	1	Between- laboratory reproducibility and Transferability (Coded)	November, 2011
IIa	10	1	Within- and between- laboratory reproducibility (Coded)	May, 2012
IIb	5	3	Within- and between- laboratory reproducibility (Coded)	November, 2012
IIc	5	3	Within- and between- laboratory reproducibility (Coded)	November, 2013
III	20	1	Between- laboratory reproducibility and predictivity (Coded)	April, 2014
IV	5	3	Within- and between- laboratory reproducibility (Coded)	July, 2015

7-1. Basic rule for chemical selection

The selection of test chemicals by the Chemical Selection Committee (CSC) in the VMT was based on published papers on *in vivo* skin sensitization tests and validation studies for *in vitro* alternative assays on skin sensitization test methods.

7-1-1. The applied selection criteria

- information on mode/site of action
- coverage of a range of relevant chemical classes and product classes
- quality and quantity of reference data (*in vivo* and *in vitro*)
- high-quality data derived from animal and (if available) from human studies

- information on interspecies variations (for example: variability with regard to the uptake of chemicals, metabolism, etc.)
- coverage of a range of toxic effects/potencies
- chemicals that do not require metabolic activation
- appropriate negative and positive controls
- physical and chemical properties (feasibility of use in the experimental set-up as implicated by the CAS No.)
- single chemical entities or formulations of known high purity
- availability
- cost

In the first phase of the selection procedure, Japanese members of the CSC identified and collected several existing lists of potential chemical sensitizers in order to establish a primary database. The resulting list of chemicals tabulated considered other validation studies, references of other test methods, test chemicals recommended by ECVAM or Sens-it-iv, and reference chemicals in OECD TG 429. An extensive literature search was performed by CSC in order to ensure that all the pre-selected chemicals fulfilled the selection criteria described above.

Emphasis was placed on the fact that different potencies (Category 1A, Category 1B and non-sensitizer) were chosen. In addition, it was decided that at least 20% of the total chemicals to be tested should be negative in order to increase the statistical power of the data analysis.

7-1-2. Chemical Acquisition, Coding and Distribution

The assessment of laboratory transferability, and within- and between-laboratory reproducibility and predictivity, in all test facilities were performed with the coded chemicals. The coding was supervised by JaCVAM, in collaboration with CSC. CSC was responsible for coding and distributing the test chemicals, references and controls for the validation study.

7-1-3. Handling

The chemical master at each test facility received complete information considered essential regarding the test chemicals (physical state, weight or volume of sample, specific density for liquid test chemicals, and storage instructions) by JaCVAM. Moreover, the test facility chemical master stored each chemical at conditions in accordance with the storage instructions and received sealed safety information such as the Material Safety Data Sheet (MSDS) describing the hazards identification and exposure controls/personal protection for each chemical. The test chemicals were delivered directly to the study director and the study director was not shown the MSDSs. The study director was to refer to the MSDSs only in the event of an accident. If the study director referred to the MSDS, he/she was not to reveal the content of the MSDS to the test facility technicians.

No accidents occurred during the course of the validation study, and all test facilities returned the MSDSs for the test chemicals to JaCVAM in their sealed envelope upon completion of the validation study. All test chemicals were disposed of in compliance with the rules and regulations of the test facilities upon completion of the validation study.

7-2. Pre-validation study

Before prevalidation study, the training phase of the IL-8 Luc assay was conducted from October 26, 2010 to January 31, 2011. The participating laboratories, HRI, Sumitomo Chemical,

AIST, and another candidate of participating laboratory, Toyobo Co., Ltd, came together at Department of Dermatology, Tohoku University School of Medicine, Sendai, Japan, on October 26, 2010. In that meeting, the lead laboratory explained the precise procedure of the IL-8 Luc assay and thereafter, demonstrated the methods for chemical preparation, dilution, handling of THP-G8 cells, plating of chemicals and cells, measurement and data handling. After the meeting, these 4 laboratories conduced the IL-8 Luc assay for 2 labeled chemicals, 4-NBB and lactic acid. All laboratories could clearly demonstrate induction of SLO-LA by 4-NBB and its suppression by NAC, and no induction of SLO-LA by lactic acid. The lead laboratory considered the procedure of the IL-8 Luc assay was appropriately transferred to participating laboratories.

Afterwards, transferability of this assay was further checked using three non-coded chemicals (4-NBB: 4-nitrobenzylbromide, TMTD: tetramethyl thiram disulphide and lactic acid) in 4 test facilities, including the lead laboratory. These chemicals were selected by the lead laboratory.

7-3. Validation study -Phase I trial

Between-laboratory reproducibility of this assay was checked using ten coded chemicals in 3 test facilities, as shown in Table 10. These chemicals were selected by CSC based on a table prepared by Drs. Hitoshi Sakaguchi and Hiroshi Itagaki, Japanese members of CSC. The chemicals were coded by JaCVAM as shown in Table 10 and distributed to the test facilities.

Table 10. Chemical code list for the Phase I validation trial for the IL-8 Luc assay

No.	Chemical name	CAS No.	Supplier	LLNA EC3	GHS*	Physical state	Storing
1	1,4-Phenylenediamine	106-50-3	Wako**	0.16	1A	Solid	2-10 °C
2	2-Mercaptobenzothiasole	149-30-4	Wako	1.7	1A	Solid	rt***
3	2,4-Dinitrochlorobenzene	97-00-7	Wako	0.06	1A	Solid	rt
4	Cinnamal	104-55-2	MP Biomedicals, LLC	3	1B	liquid	4°C
5	Tetramethyl thiuram disulphide	137-26-8	Tokyo Chemical Industry	5.2	1B	Solid	rt
6	Eugenol	97-53-0	Wako	13	1B	liquid	rt
7	Imidazolidinyl urea	39236-46-9	MP Biomedicals, LLC	24	1B	Solid	4°C
8	Lactic acid	50-21-5	Alfa Aesar	ND****	NO	liquid	rt
9	Salicylic acid	69-72-7	Wako	ND	NO	Solid	rt
10	Sodium lauryl sulphate	151-21-3	Wako	ND	NO	Solid	rt

* GHS: GHS category, **Wako: Wako Pure Chemical Industries, Ltd,***rt: room temperature, ****ND: Not Detected

7-4. Validation study -Phase II trial

7-4-1. Phase IIa trial

Ten test chemicals were selected by CSC for between-laboratory reproducibility as shown in Table 11. Five chemicals used in the Phase I trial (cinnamal, 2,4-dinitrochlorobenzene, eugenol, 2-mercaptobenzothiasole, and 1,4-penylenediamine) were joined with five new chemicals selected by CSC based on a table prepared by Japanese members of CSC. The chemicals were coded by JaCVAM as shown in Table 11 and distributed to the test facilities.

Table 11. Chemical code list for the Phase IIa validation trial for the IL-8 Luc assay

No.	Chemical name	CAS No.	Supplier	LLNA EC3	GHS*	Physical state	Storing	Phase I
1	1,4-Phenylenediamine	106-50-3	Wako**	0.16	1A	Solid	4°C	Used
2	2-Mercaptobenzothiasole	149-30-4	Wako	1.7	1A	Solid	rt***	Used
3	2,4-Dinitrochlorobenzene	97-00-7	Wako	0.06	1A	Solid	rt	Used
4	4-Nitrobenzylbromide	100-11-8	Sigma-Aldrich	0.05	1A	Solid	rt	
5	Glyoxal	107-22-2	SAJ	0.8	1A	Liquid	rt	
6	Cinnamal	104-55-2	MP Biomedicals ,LLC	3	1B	Liquid	4°C	Used
7	Eugenol	97-53-0	Wako	13	1B	Liquid	rt	Used
8	Glycerol	56-81-5	Wako	ND****	NO	Liquid	rt	
9	Isopropanol	67-63-0	Nakrai Tesque	ND	NO	Liquid	rt	
10	Methyl salicylate	119-36-8	Wako	ND	NO	Liquid	rt	

* GHS: GHS category, **Wako: Wako Pure Chemical Industries, Ltd,***rt: room temperature,
****ND: Not Detected

7-4-2. Phase IIb trial

Five test chemicals were selected by CSC for within- and between-laboratory reproducibility, as shown in Table 12. Three runs were tested separately. These coded chemicals were selected based on their sensitization potential judged from the list used by the ECVAM validation study.

The chemicals were coded by JaCVAM as shown in Table 12 and distributed to the test facilities. Lab.A were also re-tested the same chemicals with the other coded number. In the first study at Lab.A, the data were insufficient with the criteria and the study director at Lab.A considered to be caused by bad conditions of Tripluc kit or measurement instrument. The re-test was

conducted with different batches of Tripluc or another instruments.

Table 12. Chemical code list for the Phase IIb validation trial for the IL-8 Luc assay

No.	Chemical name	CAS No.	Supplier	LLNA EC3	GHS*	Physical state	Storing
1	p-Benzoquinone	106-51-4	Wako**	0.1	1A	Solid	4°C
2	1-Thioglycerol	96-27-5	Wako	3.6	1B	Liquid	4°C
3	Benzyl cinnamate	103-41-3	Wako	18.4	1B	Solid	rt***
4	Diethyl maleate	141-05-9	Wako	5.8	1B	Liquid	rt
5	2,4-Dichloronitrobenzene	611-06-3	Aldrich	ND****	NO	Solid	rt

Re-Test

No.	Chemical name	CAS No.	Supplier
1	p-Benzoquinone	106-51-4	Wako
2	1-Thioglycerol	96-27-5	Wako
3	Benzyl cinnamate	103-41-3	Wako
4	Diethyl maleate	141-05-9	Wako
5	2,4-Dichloronitrobenzene	611-06-3	Aldrich

* GHS: GHS category, **Wako: Wako Pure Chemical Industries, Ltd,***rt: room temperature, ****ND: Not Detected

7-4-3. Phase IIc trial

Five test chemicals were selected by CSC for within- and between-laboratory reproducibility, as shown in Table 13. Three runs were tested separately. These chemicals were selected based on the similarity of their chemical properties and sensitization potentials with the chemicals used in the Phase IIb trial, and based on the list used by the ECVAM validation study. The chemicals was coded by JaCVAM as shown in Table 13 and distributed to the test facilities.

Table 13. Chemical code list for the Phase IIc validation trial for the IL-8Luc assay

No.	Chemical name	CAS No.	Supplier	LLNA EC3	GHS*	Physical state	Storing
1	1,4-Phenylenediamine	106-50-3	Wako**	0.0099	1A	Solid	2-10°C
2	2-Mercaptobenzothiazole	149-30-4	Wako	1.7	1A	Solid	rt***
3	Glycerol	56-81-5	Wako	ND****	NO	Liquid	rt
4	Isopropanol	67-63-0	Wako	ND	NO	Liquid	rt
5	Methyl salicylate	119-36-8	Wako	ND	NO	Liquid	rt

* GHS: GHS category, **Wako: Wako Pure Chemical Industries, Ltd,***rt: room temperature,
****ND: Not Detected

7-5. Validation study -Phase III trial

Twenty chemicals were selected for between-laboratory reproducibility and predictivity by CSC. These chemicals were selected based on their chemical properties, results obtained in earlier phase studies and sensitization potential as judged based on tabulated data from the Phase II trials, the list of chemicals provided by the ECVAM validation study, and the list prepared by Japanese members of CSC at the chemical selection meeting in Kyoto on February 26, 2014 (Table 14). The chemicals were coded by JaCVAM as shown in Table 14 and distributed to the test facilities (Table 15).

Due to the conflicting results obtained in the Phase II studies, 2,4-dichloronitrobenzene was omitted in Table 15.

Table 14. Chemical code list for the Phase III validation trial for the IL-8 Luc assay

No.	Chemical name	CAS No.	Supplier	LLNA EC3	GHS*	Physical state	Storing
1	2,4-Dinitrochlorobenzene	97-00-7	Wako**	0.06	1A	Solid	rt***
2	Beryllium Sulphate	7787-56-6	Wako	0.001	1A	Solid	rt
3	Chloramine T	127-65-1	Wako (MP)	0.4	1A	Solid	4°C
4	Chlorpromazine HCl	69-09-0	Nacalai tesque	0.14	1A	Solid	rt
5	Formaldehyde	50-00-0	Wako	0.61	1A	Liquid	rt
6	Glyoxal	107-22-2	Sigma-Aldrich	0.8	1A	Liquid	rt
7	1,2-Benzisothiazol-3(2H)-one	2634-33-5	Wako	8	1B	Solid	rt
8	Benzyl Salicylate	118-58-1	Wako	2.9	1B	Liquid	rt
9	Citral	5392-40-5	Aldrich	5	1B	Liquid	rt
10	Dihydroeugenol	2785-87-7	Wako	6.8	1B	Liquid	rt
11	Imidazolidinyl urea	39236-46-9	Sigma	24	1B	Solid	4°C
12	Methylmethacrylate	80-62-6	Sigma	90	1B	Liquid	rt
13	R(+)Limonene	5989-27-5	Wako	69	1B	Liquid	rt
14	Nickel Chloride	7718-54-9	Wako	ND****	1B	Solid	rt
15	2,4-Dichloronitrobenzene	611-06-3	Aldrich	ND	NO	Solid	rt
16	4-Aminobenzoic acid	150-13-0	Sigma	ND	NO	Solid	rt
17	4-Hydroxybenzoic acid	99-96-7	Aldrich	ND	NO	Solid	rt
18	Benzyl alcohol	100-51-6	Aldrich	ND	NO	Liquid	rt
19	Dimethyl Isophthalate	1459-93-4	Wako	ND	NO	Solid	rt
20	Xylene	1330-20-7	Wako	ND	NO	Liquid	rt

* GHS: GHS category, **Wako: Wako Pure Chemical Industries, Ltd,***rt: room temperature, ****ND: Not Detected

Table 15. Chemical code list for the validation study for the IL-8 Luc assay

No.	Chemical name	CAS No.	LLNA EC3	GHS category	Physical state	Phase
PC	Cobalt chloride	7646-79-9	0.6	1A	Solid	IIb, IIc & III
1	p-Benzoquinone	106-51-4	0.01	1A	Solid	IIb**
2	1,4-Phenylenediamine	106-50-3	0.11	1A	Solid	IIc**
3	2-Mercaptobenzothiazole	149-30-4	1.7	1A	Solid	IIc**
4	2,4-Dinitrochlorobenzene	97-00-7	0.06	1A	Solid	III
5	Beryllium sulfate	7787-56-6	0.001	1A	Solid	III**
6	Chloramine T	127-65-1	0.4	1A	Solid	III**
7	Chlorpromazine HCl	69-09-0	0.14	1A	Solid	III**
8	Formaldehyde	50-00-0	0.61	1A	Liquid	III**
9	Glyoxal	107-22-2	0.8	1A	Liquid	III
10	1-Thioglycerol	96-27-5	3.6	1B	Liquid	IIb**
11	1,2-Benzosothiazolin-3-one	2634-33-5	8	1B	Solid	III
12	Benzylcinnamate	103-41-3	18.4	1B	Solid	IIb**
13	Benzylsalicylate	118-58-1	2.9	1B	Liquid	III**
14	Citral	5392-40-5	5	1B	Liquid	III
15	Dietyhyl maleate	141-05-9	5.8	1B	Liquid	IIb
16	Dihydroeugenol	2785-87-7	6.8	1B	Liquid	III**
17	Imidazolidinylurea	39236-46-9	24	1B	Solid	III**
18	Methylmethacrylate	80-62-6	90	1B	Liquid	III**
19	R(+)-Limonene	5989-27-5	69	1B	Liquid	III**
20	Nickel chloride	7718-54-9	ND***	1B	Solid	III**
21	2,4-Dichloronitrobenzene	611-06-3	ND	NO	Solid	IIb& III**
22	4-Aminobenzoic acid	150-13-0	ND	NO	Solid	III**
23	4-Hydroxybenzoic acid	99-96-7	ND	NO	Solid	III
24	Benzyl alcohol	100-51-6	ND	NO	Liquid	III**
25	Dimethylisophthalate	1459-93-4	ND	NO	Solid	III**
26	Glycerol	56-81-5	ND	NO	Liquid	IIc**
27	Isopropanol	67-63-0	ND	NO	Liquid	IIc**
28	Methylsalicylate	119-36-8	ND	NO	Liquid	IIc**
29	Xylene	1330-20-7	ND	NO	Liquid	III**

*:PC:Posotove Control, **: Based on the ECVAM validation study, ***ND: Not Detected

7-6. Validation study -Phase IV trial

Five test chemicals were selected by CSC for within- and between-laboratory reproducibility, as shown in Table 16. Three runs were tested separately. These chemicals were selected in consideration of the similarity of their chemical properties and sensitization potentials based on the chemicals and the data of Tohoku University with X-VIVO™ 15 (see Appendix 10.2). The chemicals was coded by JaCVAM as shown in Table 16 and distributed to the test facilities.

Table 16. Chemical code list for the Phase IV validation trial for the IL-8 Luc assay

No.	Chemical	CAS No.	Supplier	GHS	IL-8 Luc results		Physical state	Previous Phase
					DMSO	X-VIVO		
1	Oxazolone	15646-46-5	Sigma-Aldrich	**1A**	**N**	**P**	Solid	Non
2	4-Nitrobenzylbromide	100-11-8	Sigma-Aldrich	**1A**	**P**	**P**	Solid	2A
3	4-Allylanisole	140-67-0	Sigma-Aldrich	**1B**	**N**	**P**	Liquid	Non
4	d-Limonene	5989-27-5	WAKO	**1B**	**N**	**P**	Liquid	3
5	Salicylic acid	69-72-7	WAKO	**Non**	**N**	**N**	Solid	1

7.7. Outcome of validation study

To evaluate the predictivity of this assay, the VMT selected 29 chemicals used in Phase II, III, and IV studies. Twenty-three test chemicals used in the ECVAM validation study are included, but MCI/MI (Kathon CG) was excluded as it is not commercially available in Japan.

8. Protocols

8-1. Overview of the IL-8 Luc assay

An overview of the IL-8 Luc assay is shown in Fig. 9. In addition, the final protocol of the present test (version 023E) is provided as attached Appendix 8, and the procedures are described in detail below.

IL-8 Luc assay as an *in vitro* sensitization test

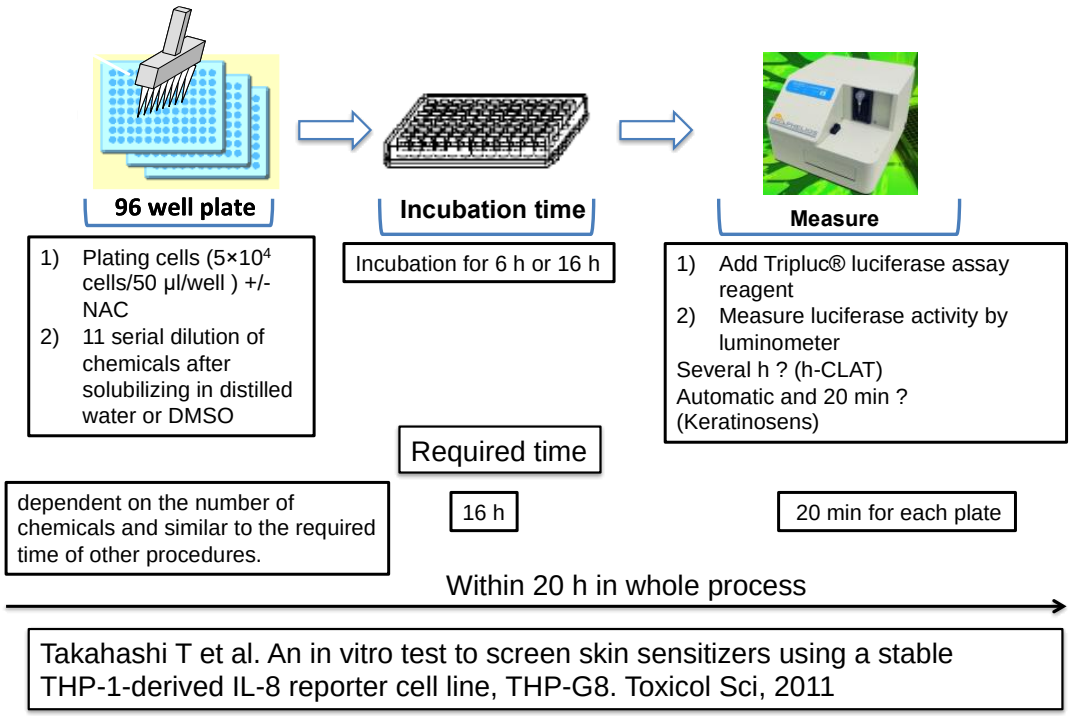


Fig. 9. Overview of the IL-8 Luc assay

8-2. Protocol for the IL-8 Luc assay

8-2-1. Reagents and equipment

The following reagents and equipment were used.
For maintenance of THP-G8 cells

- RPMI-1640 (GIBCO Cat#11875-093, 500 mL)
- FBS (Biological Industries Cat#04-001-1E Lot:715004)
- Antibiotic-Antimycotic (GIBCO Cat#15240-062)
- G418 (Nacalai Tesque Cat#16513-84)
- Puromycin (InvivoGen Cat#ant-pr-1)

For chemical exposure, positive control, and solvents

- Cobalt chloride (hexahydrate) (Sigma Cat#255599)
- 4-Nitrobenzyl bromide (CAS:100-11-8, Aldrich Cat#N13054)
- DMSO (Sigma Cat#D5879)
- Distilled water (GIBCO Cat#10977-015)
- X-VIVO™ 15 (Lonza, 04-418Q): Chemically defined, serum-free hematopoietic cell medium.

For measurement of luciferase activity

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

8-2-2. Culture medium

Various culture media were used depending on the purpose of the cell culture.

Table 17. A medium: for maintenance of THP-G8 cells (500 mL, stored at 2-8°C)

Reagent	Company	Concentration	Final concentration in medium	Required amount
RPMI-1640	GIBCO #11875-093	-	-	445 ml
FBS	Biological Industries Cat#04-001-1E Lot:715004	-	10 %	50 ml
Antibiotic-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

Table 18. 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

Table 19. C medium: for thawing THP-G8 cells (30 mL, stored at 2-8°C)

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

8-2-3. Cell line

The human macrophage-like cell line THP-1 from American Type Culture Collection (Manassas, VA, USA) was cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% Hyclone™ fetal calf serum (Thermo Fisher Scientific Inc. Waltham, MA, USA) and 1% penicillin-streptomycin (Life Technologies, Carlsbad, CA, USA) at 37°C with 5% CO_2.

To establish an IL-8 reporter cell line, a luciferase assay system was constructed using two luciferase genes that emit orange (SLO) or red (SLR) light with a single substrate. Two luciferase vectors, pSLO-test/Neo^r and pSLR-test/Pur^r, were constructed by ligating the *Bam*HI/*Sac*I site of resistant gene vectors containing one of two resistant genes, neomycin (SLO) or puromycin (SLR), the SV40 promoter, and HSV tk polyA signal into the luciferase gene vectors, pSLO-test and pSLR-test (Toyobo, Osaka, Japan), respectively. The activities of the luciferases can be measured simultaneously and quantitatively using optical filters. This system can rapidly and easily monitor multiple gene expression (Nakajima et al., 2005; Noguchi et al., 2008).

Promoter cloning was carried out as follows. IL-8 promoter construct containing nt -5059 to +144 and GAPDH promoter construct containing nt -1373 to +128 from transcription initiation sites identified using DBTSS (http://dbtss.hgc.jp/) were amplified from genomic DNA (Roche Diagnostics GmbH, Mannheim, Germany) by PCR using KOD FX (Toyobo, Osaka, Japan) for the IL-8 promoter or KOD-Plus- (Toyobo) for the GAPDH promoter, and specific primers for IL-8 and GAPDH. The IL-8 or GAPDH promoter was ligated into pSLO-test/Neo^r or pSLR-test/Pur^r vector that had been digested with MluI and SalI, or MluI and EcoRI, respectively. Before transfection, the sequence of the 5' and 3' regions of each promoter using a 3730 DNA Analyzer (Life Technologies Japan, Tokyo, Japan) was confirmed.

IL-8 and GAPDH reporter plasmids were transfected into THP-1 cells using Nucleofector II (Amaxa, Cologne, Germany) according to the manufacturer's instructions. After transfection, cells were cultured in growth medium containing 200 μg/mL G418 (Invitrogen) and 0.15 μg/mL puromycin (Sigma-Aldrich) (A medium) for selection. After repeated limiting dilution, a stable cell line, THP-G8 (Fig. 10) was established.

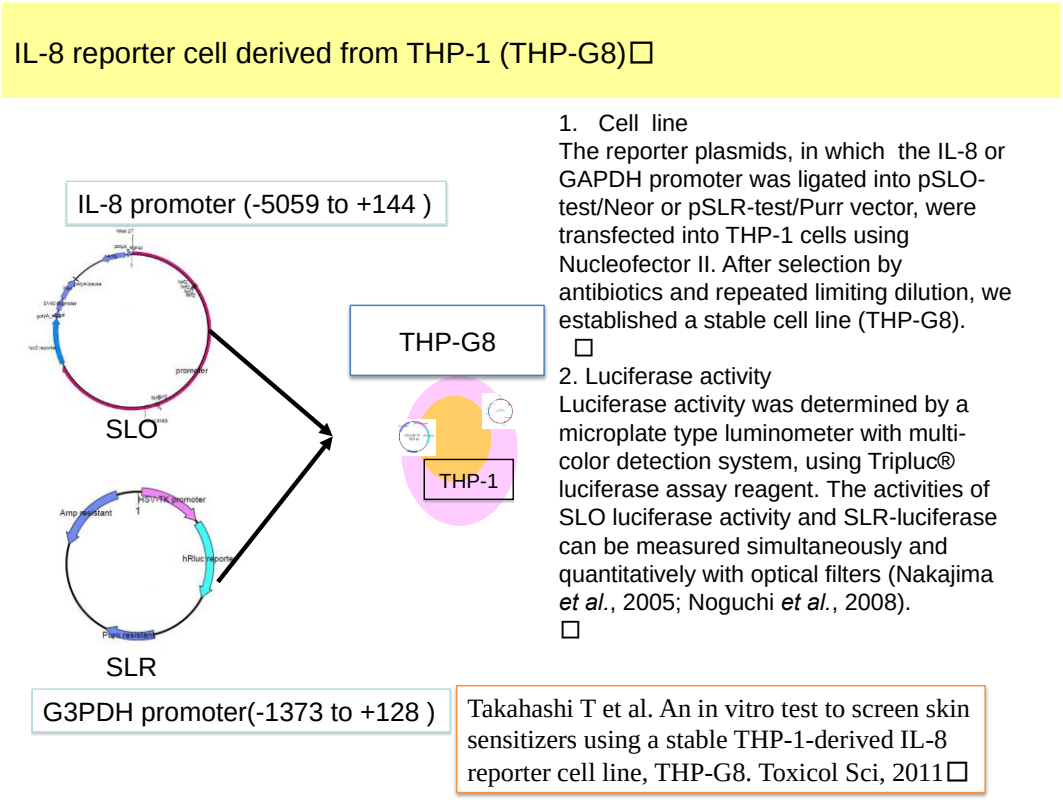


Fig. 10. IL-8 reporter cell, THP-G8

8-2-4. Maintenance of THP-G8 cells

THP-G8 cells are passaged at 2–5×10^5/mL depending on the condition of the cells and are incubated at 37°C in 5% CO_2. The interval between subcultures should be 3–4 days. The cells can be used between one and six weeks after thawing. To subculture THP-G8 cells, A medium in a T-75 flask (BD Falcon Cat#35-3136 **)** was pre-warmed at 37°C in a 5% CO_2 incubator.

The lead laboratory has examined how long THP-G8 cells could be cultured without losing their reactivity to chemicals. THP-G8 cells maintained their response to $CoCl_2$ up to 13 weeks or 30 passages. Therefore, the lead laboratory think that the stock of THP-G8 cells can be cultured without losing activity at least for 13 weeks or even after 30 passages.

In practice, to establish a master cell and working cell back, after thawing, cells from the THP G8 supplier vial are cultured for ~ one week in order to prepare 10 vials designated as the Master Cell Bank. A master cell bank ampoule is thawed, cultured for one week in order to prepare 10 vials designated as the Working Cell Bank, which are used for experiments by at most 20 passages.

8-2-5. Preparation of cells for assay

A cell passage should be conducted 2–4 days before the assay. THP-G8 cells were suspended in prewarmed B medium at a cell density of 1×10^6/mL and transferred to reagent reservoir (Thermo Scientific, Waltham, MA) and 50 µL of cell suspension was added to each well of an opaque black 96-well plate (flat bottom) using an 8 channel or 12 channel pipetman (Gison, Inc, Middleton, WI, USA) (Fig. 11)

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

Fig. 11. Components in each well of two 96-well plates used in the IL-8 Luc assay for screening sensitization chemicals.

8-2-6. Preparation of chemicals and cell treatment with chemicals

Water soluble chemicals were dissolved in distilled water at a concentration of 25 mg/mL. If the chemicals were soluble at 25 mg/mL, then 50 mg/mL solutions were prepared if their solubility was sufficient. If they were not soluble at 50 mg/mL, then 25 mg/mL was judged the highest soluble concentration. If the chemicals were soluble at 50 mg/mL, then 100 mg/mL solutions were prepared if their solubility was sufficient. If they were not soluble at 100 mg/mL, then 50 mg/mL was judged the highest soluble concentration. If they were soluble at 100 mg/mL, then 100 mg/mL was judged 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 minutes. All dissolved chemicals were used within 4 hours of being dissolved in distilled water or DMSO.

X-VIVO™ 15 was used to dissolve chemicals in phase IV. Five millilitters of X-VIVO™ 15 for each chemical is pre-warmed at room temperature. Twenty milligrams of a test chemical was added in a microfuge tube (1.5 mL), the tube was filled up to 1 mL with X-VIVO™ 15, then vigorously voltexed. If necessary, it was sonicated until chemicals are completely dispersed.

If the chemical was soluble at 20 mg/mL, it was diluted 5 times with X-VIVO™ 15, and then, 500 µL (1st experiment) or 750 µL (2nd, 3rd and 4th experiment) of the diluted solution (depending on cytotoxicity of chemicals, as described in the next page) was transferred to the 12th line of a 96 well Assay Block.

If the chemical was not soluble at 20 mg/mL, it was shaken on a rotor (e.g., WKN-2210, WAKEN B TECH Co. Ltd, Kyoto, Japan) at 8 rpm (not exceeding 8 rpm) for more than 30 min, then immediately centrifuged. After centrifugation at 15,000 rpm (≈20,000 x g) for 5 min, 500 µL (1st experiment) or 750 µL (2nd, 3rd and 4th experiments) of the diluted solution (depending on cytotoxicity of chemicals, as described on the next page) was transferred to the 12th line of a 96 well Assay Block. If undissolved chemicals precipitated or floated, particular attention was taken to use solution free undissolved chemical particles

8-2-7. Dilution of chemicals

For water soluble chemicals, 11 serial dilutions were conducted using B medium, 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.

In the experiments using X-VIVO™ 15 as a solvent, 11 serial dilutions of the solution, whether the chemicals were soluble in X-VIVO™ 15 or not, were conducted using X-VIVO™ 15, 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.

8-2-8. Measurements

After incubation with the test chemical for 6 h or 16 h at 37°C in a 5% CO_2 incubator, 100 µL of pre-warmed Tripluc is added to each well in the plate containing reference samples using a pipetman and the plate is shaken for 10 min at room temperature (about 25°C) using a plate shaker. Surface bubbles are removed if present and bioluminescence in each well is measured using a microplate-type luminometer with a multi-color detection system (Phelios; Atto Co., Tokyo, Japan) for 3 sec each in the absence (F0) and presence (F2) of the optical filter. The F0 and F2 data (values are expressed as counts) are processed using an Excel-based Data sheet. In the Data sheet (Appendix 11), SLO-LA and SLR-LA are calculated for each well based on the algorithm to calculate SLO-LA and SLR-LA from the raw luminescence data, which was reported previously (Nakajima et al., 2005; Noguchi et al., 2008). Moreover, in addition to being used to calculate SLO-LA and SLR-LA, this Data sheet can automatically generate the final graph showing the correlation between FInSLO-LA and the concentration of chemicals, between II-SLR-LA and the concentration of chemicals, the 95% confidence interval of FInSLO-LA, and the final judgment based on each criterion.

8-2-9. Luminometer apparatus

Multi-color detection systems such as microplate-type luminometers are available and include Phelios (ATTO, Tokyo, Japan), Tristan 941 (Berthold, Bad Wildbad, Germany), and the ARVO series (PerkinElmer, Waltham, MA). The luminometer detectors must have high sensitivity and low background noise and are usually equipped with optical filters, such as sharp-cut (long-pass) filters and band-pass filters. The transmission coefficients of these filters for each bio-luminescence signal color must be calibrated prior to all experiments following the manufacturer's recommended protocol because the transmittance of the optical filter or the sensitivity of the detector are dependent on the measurement conditions.

8-2-10. Positive control

In each experiment, 8, 16, and 32 dilution of 20 mg/mL solution of 4-NBB that was dissolved according to the protocol, was used as a positive control. If 4-NBB did not satisfy the acceptance criterion at any of the three concentrations, the experimental dataset was rejected.

8-2-11. Calculation and definition of parameters for the IL-8 Luc assay

Some chemicals affect cell viability. Therefore, the lead laboratoryutilized GAPDH promoter

activity (SLA-LA) of THP-G8 cells as an internal control to demonstrate cell viability and viable cell number. 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). On the other hand, there are several reports suggesting that its use as an internal standard is inappropriate in some cases (Oliveira et al., 1999; Thellin et al., 1999). In general, within-tissue variation of GAPDH mRNA expression levels is generally small, whereas between-tissue variation can be substantial, according to tissue types (Barber et al., 2005).

Therefore, it is necessary to determine whether GAPDH mRNA expression or its promoter activity driving luciferase gene is appropriate for normalizing IL-8 mRNA expression or IL-8 promoter activity of THP-1 cells after chemical stimulation. To clarify this issue, the lead laboratory first examined the effects of cell numbers on luciferase activity of THP-G8 cells before and after LPS stimulation. The results clearly demonstrated that GAPDH promoter activity showed linear correlation with the cell number of THP-G8 cells at the cell density less than 50 x 10^{-4}/well irrespective of the presence or absence of LPS. Next, the lead laboratory examined GAPDH promoter activity (SLR-LA) of THP-G8 cells after the stimulation with 122 chemicals including 34 non-sensitizers and 88 sensitizers and analyzed the data statistically. The mean ± SD of (GAPDH promoter activity after chemical treatment/GAPDH promoter activity without treatment) was 1.17 ± 0.43, while the mean ± SD (IL-8 promoter activity after chemical treatment/IL-8 promoter activity without treatment) was 3.37 ± 6.18. Only two chemicals, such as benzocaine and methylisothiazolinone, increased SLR-LO to more than 3, although correct judgments for sensitizer were done for them. These data indicate that GAPDH promoter activity (SLO-LA) can act as an internal control of promoter activity of THP-G8 cells after chemical stimulation.

In the IL-8 Luc assay, the lead laboratorydefined nSLO-LA to represent IL-8 promoter activity by the SLO luciferase activity (SLO-LA) normalized by SLR luciferase activity (SLR-LA). The suppression index of SLR-LA (SI-SLR-LA) was obtained by dividing SLR-LA of THP-G8 treated with chemicals with SLR-LA of non-treated THP-G8. The fold induction of IL-8 promoter activity (FInSLO-LA) was calculated by dividing the nSLO-LA of THP-G8 cells treated with chemicals or LPS by that of non-stimulated THP-G8 cells. The lead laboratory also defined the inhibitory index (I.I) by NAC for each chemical 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 at the concentration in which the chemical induced the largest FInSLO-LA (Table 20).

Table 20. Abbreviations used in the THP-G8 luciferase assay protocol

Abbreviation	Description
SLO-LA	SLO luciferase activity
SLR-LA	SLR luciferase activity
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

8-2-12. Criteria to identify sensitizers using the IL-8 Luc assay

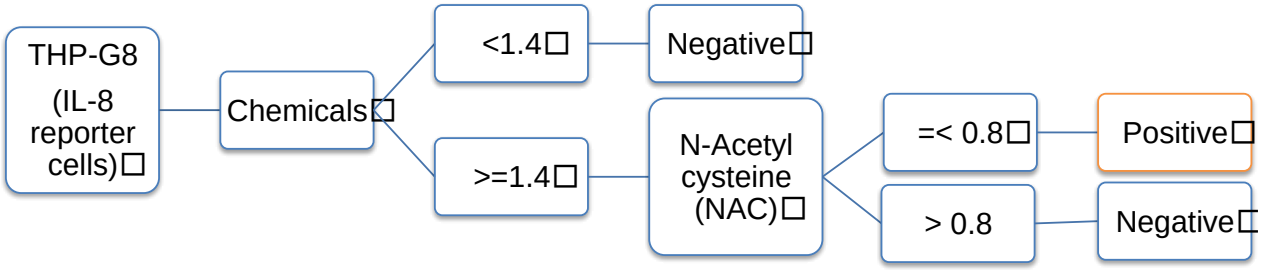
Based on the results of studies and the comments raised by the VMT members, the protocols were changed several times.

In the Phase I study, the VMT used the following criteria.

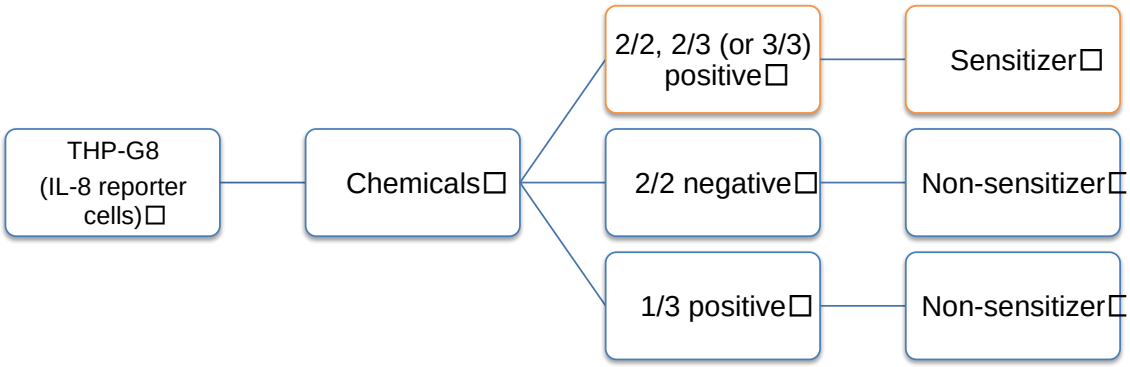
Criteria: In each experiment, chemicals that demonstrate FInSLO-LA ≥ 1.4 and I.I. ≤ 0.8 at the concentration of the chemical at which I.I.-SLR-LA is ≥ 0.2 are judged as positive. Chemicals providing 2 or 3 positive results in 2 or 3 different experiments are judged as sensitizers.

The criterion used in the Phase I study

In each experiment:



In at least two repeated experiments:



In the Phase IIa study, the VMTused the following criteria.

Criteria: Chemicals that demonstrate FInSLO-LA ≥ 1.4 and I.I. ≤ 0.8 are judged as positive in each experiment.

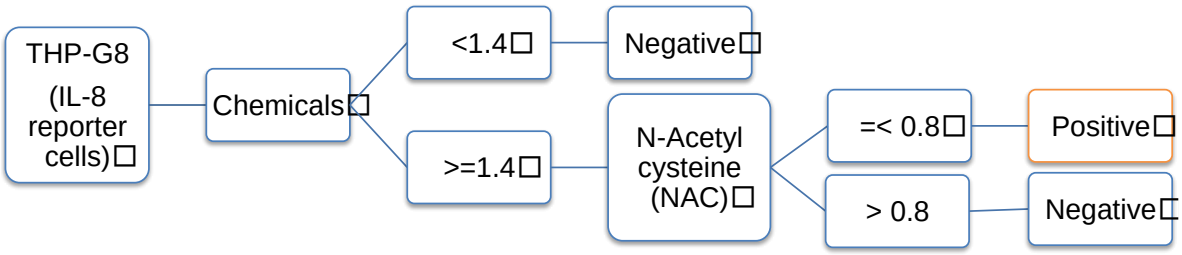
1st experiment: THP-G8 cells are stimulated with chemicals serially using a dilution factor of 2 to a final concentration of 500 µg/mL.

2nd and 3rd experiments: Determine the minimum concentration at which I.I.-SLR-LA is lower than 0.05 in the 1st experiment. Use the concentration one step (2-times) higher than this determined concentration as the highest concentration of the chemical to examine, and conduct 11 serial dilutions diluted by a factor of 1.5 from the highest concentration. If I.I.-SLR-LA is not lower than 0.05, or I.I.-SLR-LA is lower than 0.05 at the highest final concentration in the 1st experiment, conduct 11 serial dilutions diluted by a factor of 1.5 from the highest concentration in the 1st experiment.

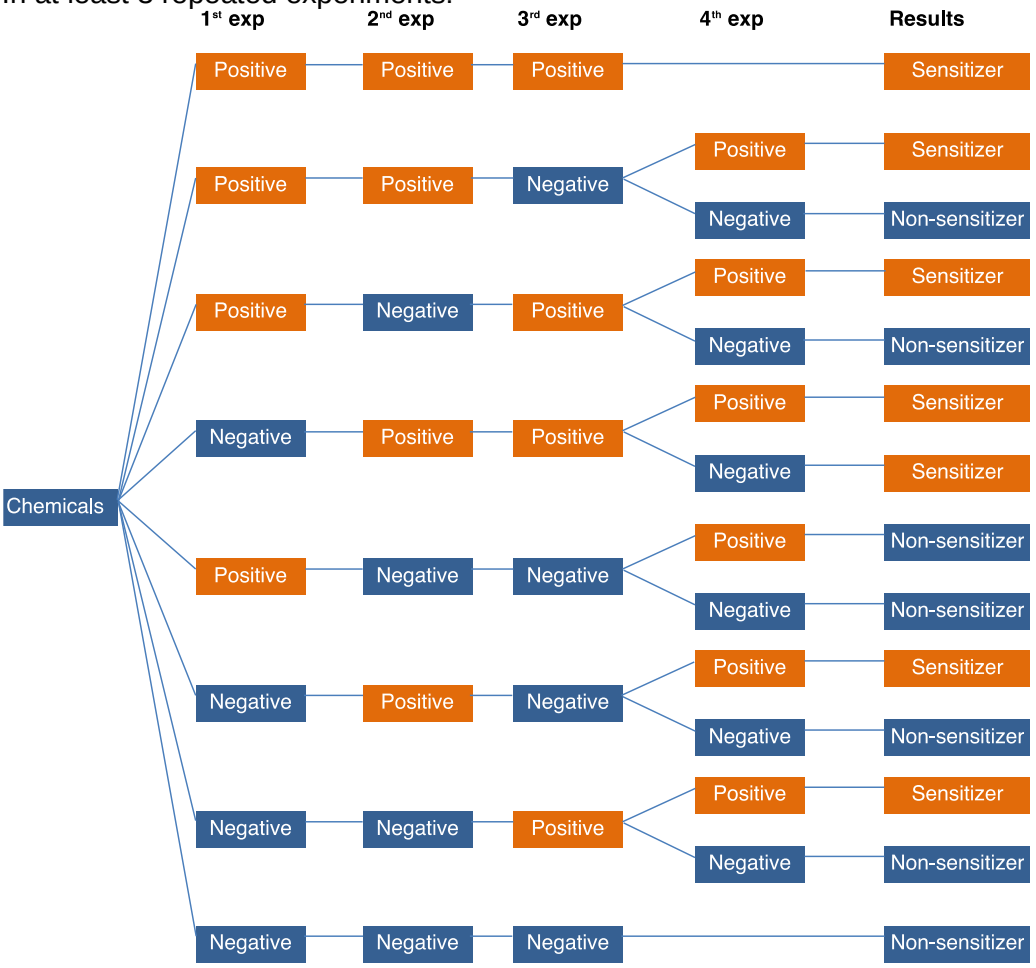
If the results of the three experiments match, this result is the final result. If one result of the three experiments is different from the others, carry out a 4th experiment. Chemicals that provide

2 or 3 positive results in the 2nd, 3rd, or 4th experiments are considered sensitizers and chemicals that provide 0 or 1 positive results in the 2nd, 3rd, and 4th experiments are considered non-sensitizers.

The criterion used in the Phase IIa study
In each experiment:



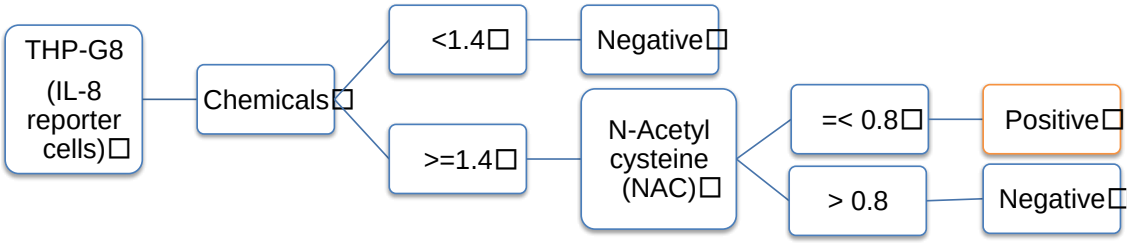
In at least 3 repeated experiments:



In the Phase IIb study, the VMTused the following criteria.
Criteria: Chemicals that demonstrate FInSLO-LA ≥ 1.4 and I.I. ≤ 0.8 are judged as positive in each experiment.
1st experiment: THP-G8 cells are stimulated with chemicals serially diluted by a factor of 2 from the highest soluble concentration.
2nd, 3rd, and 4th experiments:
Determine the minimum concentration at which I.I.-SLR-LA is lower than 0.05 in the 1st experiment. Use the concentration one step (2-times) higher than this determined concentration as the highest concentration of the chemical to examine, and conduct 11 serial dilutions diluted by a factor of 1.5 from the highest concentration. If I.I.-SLR-LA is not lower than 0.05, or I.I.-SLR-LA is lower than 0.05 at the highest final concentration in the 1st experiment, conduct 11 serial dilutions using a dilution factor of 1.5 from the highest concentration in the 1st experiment.

Chemicals that provide 2 positive results in the 1st, 2nd, 3rd, or 4th experiments are considered sensitizers and chemicals that provide 3 negative results in the 1st, 2nd, 3rd, or 4th experiments are considered non-sensitizers.

The criterion used in the Phase IIb study
In each experiment:



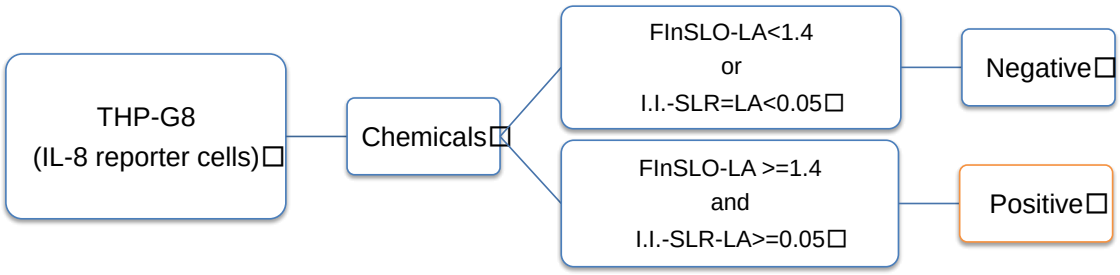
In at least 2 repeated experiments:
The combinations of results correspond with final judgments.

1st	2nd	3rd	4th	Judge
Positive	Positive	-	-	Sensitizer
	Negative	Positive	-	Sensitizer
		Negative	Positive	Sensitizer
			Negative	Non-sensitizer
Negative	Positive	Positive	-	Sensitizer
		Negative	Positive	Sensitizer
			Negative	Non-sensitizer
	Negative	Positive	Positive	Sensitizer
			Negative	Non-sensitizer
		Negative	-	Non-sensitizer

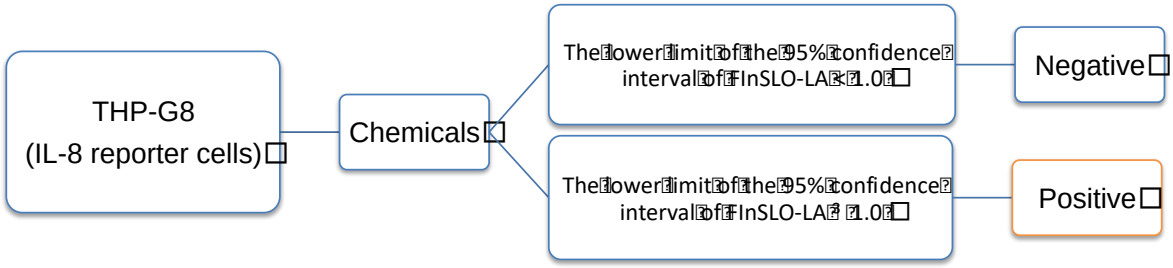
In the Phase IIc and III studies, the VMTused the following criteria.
There are three temporary criteria for identifying sensitizers and one was adopted after the Phase IIc validation. These temporary criteria are:
Criteria 1: Chemicals that demonstrate FInSLO-LA ≥ 1.4 and I.I.-SLR-LA ≥ 0.05 are judged as positive in each experiment.
Criteria 2: Chemicals that demonstrate the lower limit of the 95% confidence interval of FInSLO-LA ≥ 1.0 are judged as positive in each experiment.
Criteria 3: Chemicals that demonstrate FInSLO-LA ≥ 1.4 and the lower limit of the 95% confidence interval of FInSLO-LA ≥ 1.0 are judged as positive in each experiment.
1st experiment: THP-G8 cells are stimulated with chemicals serially diluted by a factor of 2 from the highest soluble concentration.
2nd, 3rd, and 4th experiments: Determine the minimum concentration at which I.I.-SLR-LA is lower than 0.05 in the 1st experiment. Use the concentration one step (2-times) higher than this determined concentration as the highest concentration of the chemical to examine, and conduct 11 serial dilutions using a dilution factor of 1.5 from the highest concentration. If I.I.-SLR-LA is not lower than 0.05, or I.I.-SLR-LA is lower than 0.05 at the highest final concentration in the 1st experiment, conduct 11 serial dilutions using a dilution factor of 1.5 from the highest concentration in the 1st experiment.

Chemicals that provide 2 positive results in the 1st, 2nd, 3rd, or 4th experiments are considered sensitizers and chemicals that provide 3 negative results in the 1st, 2nd, 3rd, or 4th experiments are considered non-sensitizers.

The criteria used in the Phase IIb and III studies
In each experiment:
Criterion 1.



Criterion 2

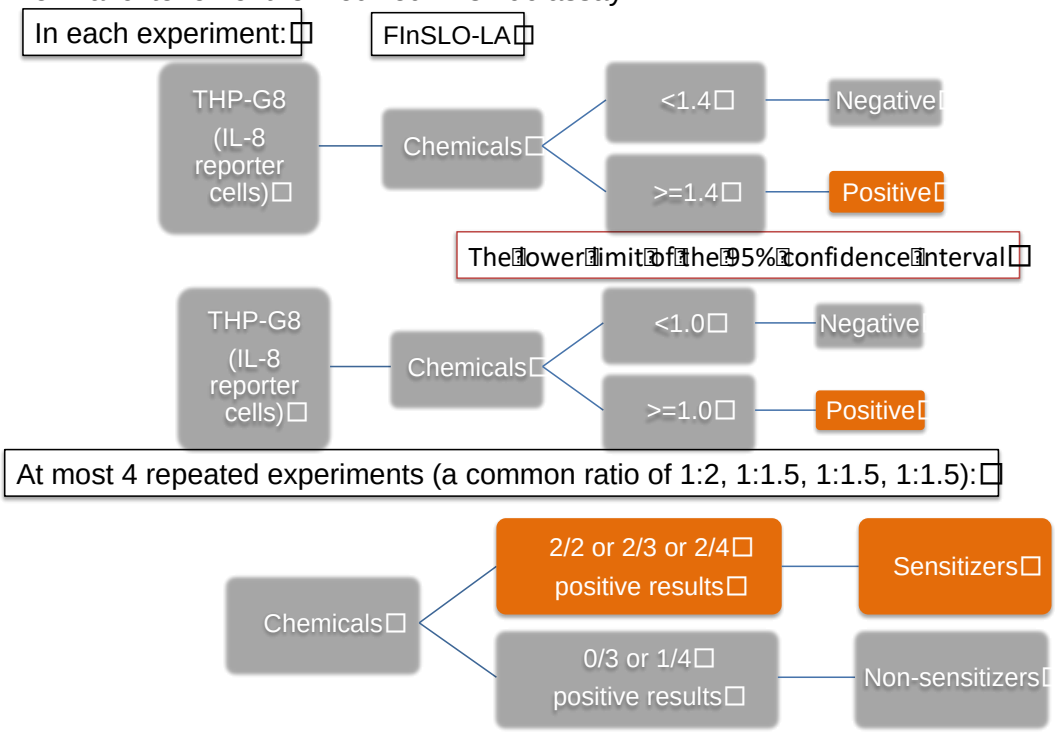


Criterion 3
Fulfill Criteria 1 and 2
In at least 2 repeated experiments:

1st	2nd	3rd	4th	Judgment
Positive	Positive	-	-	Sensitizer
	Negative	Positive	-	Sensitizer
		Negative	Positive	Sensitizer
			Negative	Non-sensitizer
Negative	Positive	Positive	-	Sensitizer
		Negative	Positive	Sensitizer
			Negative	Non-sensitizer
	Negative	Positive	Positive	Sensitizer
			Negative	Non-sensitizer
		Negative	-	Non-sensitizer

In the Phase IV, the VMT decided to use Criterion 3 as the criterion of the IL-8 Luc assay.

The final criterion of the modified IL-8 Luc assay



8-3. Data collection, handling, and criteria

8-3-1. Data collection

8-3-1-1. Operating procedure

The operating procedure of the phase 2b is described in the protocol version 0.15E; one of the phase 2c and 3 is described in the protocol version 0.17E. These protocols are slightly changed in the point of the decision rule for sensitizer, but for the operating procedure, the descriptions of both the protocols are same. In the phase IV trial, the protocol ver.20E was used.

The result of each experiment is judged “Positive” or “Negative”, and the final judgment for “Sensitizer” or “Non-sensitizer” of a tested chemical is based on the results of 2-4 experiments. The rule was decided after phase 2b study in the VMT meeting, and the details were described into the protocol ver. 0.17E.

8-3-1-2. Chemicals

The main aim of Phases IIb, IIc and IV was to evaluate the within- and beteen-laboratory reliability. For each phase, 3 sets of 5 chemicals were distributed to 3 laboratories. A different code for each set of chemicals was used for each laboratory and thus the investigators did not know the same set on code. This document re-coded the chemicals. Each set is indicated by a suffix, providing code names such as P2b01-1, P2b01-2, P2b01-3.

The main aim of the Phase III study was to evaluate the predictively and thus only 1 set of 20 chemicals was used in this phase.

The Table 21 shows the chemical codes used throughout this document.

Table 21. The chemical codes

Phase	Chemicals	Lab.A	Lab.B	Lab.C
IIb	P2b01-1, P2b01-2, P2b01-3, P2b02-1, P2b02-2, P2b02-3, P2b03-1, P2b03-2, P2b03-3, P2b04-1, P2b04-2, P2b04-3, P2b05-1, P2b05-2, P2b05-3	○○○	○○○	○○○
IIc	P2c08-1, P2c08-2, P2c08-3, P2c012-1, P2c012-2, P2c012-3, P2c19-1, P2c19-2, P2c19-3, P2c21-1, P2c21-2, P2c21-3, P2c22-1, P2c22-2, P2c22-3	○○○	○○○	○○○
III	P3-01, P3-02, P3-03, P3-04, P3-05, P3-06, P3-07, P3-08, P3-09, P3-10, P3-11, P3-12, P3-13, P3-14, P3-15, P3-16, P3-17, P3-18, P3-19, P3-20	○	○	○
IV	P4-01-1, P4-01-2, P4-01-3,P4-02-1, P4-02-2, P4-02-3,P4-03-1, P4-03-2, P4-03-3, P4-04-1, P4-04-2, P4-04-3,P4-05-1, P4-05-2, P4-05-3	○○○	○○○	○○○

8-3-2. Data handling

The Excel data sheet developed for this study was distributed to the laboratories. The data management team received data files from the 3 laboratories. The program embedded in each data file can calculate the judgement of “Positive” or “Negative” for each experiment, and the data management team calculated the indexes for the judgments using the observed SLO-LA and SLR-LA values.

JaCVAM provided the list of the 5 chemicals distributed to the 3 laboratories for Phase IIb, the 5 chemicals for Phase IIc, the 20 chemicals for Phase III, and the 5 chemicals for Phase IV. These files included the chemical codes and the chemical names. The files for Phase III and IV had the GHS classification of chemicals whereas the files for Phase IIb and IIc did not. We therefore requested the GHS classification for the 10 chemicals missing this classification data and input the data into the program.

These files were combined for data analysis, and several datasets were constructed for the analysis using SAS ver. 9.3 and 9.4 and used for the data analysis described in this report.

8-3-3. Index from each experiment and decision criteria for judgment

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

$nSLO\text{-}LA_{ij} = SLO\text{-}LA_{ij} / SLR\text{-}LA_{ij.}$

This is the basic unit of measurement in this assay.

8-3-3-1. FInSLO-LA

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

$$\text{FInSLO-LA}_i = \left\{(1/4)\times\sum_j \text{nSLO-LA}_{ij}\right\} / \left\{(1/4)\times\sum_j \text{nSLO-LA}_{0j}\right\}.$$

The lead laboratory has proposed that 1.4 of the value suggest the positive for the tested chemical. This value is based on the investigation of the historical data of the lead laboratory. Data management team followed to use the value through all the phase of present validation study.

The primary outcome measure, FInSLO-LA, is the ratio of 2 arithmetic means as shown in equation (1). The 95% confidence interval (95% CI) based on the ratio can be estimated to show the precision of this primary outcome measure. The lower limit of the 95% CI above 1 is interpreted as that the nSLO-LA with the i-th concentration is greater than it with the 0 concentration significantly.

There are several ways to construct the 95% CI. We used the method kwon as Fieller's theorem in this study. This 95% confidence interval theorem is obtained from the following formula.

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

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

$\bar{x}_0^2 = \left\{(1/4)\times\sum_j \text{nSLO-LA}_{0j}\right\}$, $sd_0^2 = (1/3)\times\sum_j \left(\text{nSLO}-\text{LA}_{0j}-\bar{x}_0\right)^2$, $n_0 = 4$,

$\bar{y}_0^2 = \left\{(1/4)\times\sum_j \text{nSLO-LA}_{ij}\right\}^2$, $sd_{y_i}^2 = (1/3)\times\sum_j \left(\text{nSLO}-\text{LA}_{ij}-\bar{y}_i\right)^2$, $n_{y_i} = 4$,

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

8-3-3-2. I.I.-SLR-LA

The I.I.-SLR-LA is a ratio of the averaged SLR-LA for the repetition of the i-th concentration compared with it of the 0 concentration, and this is written by

$$\text{I.I.-SLR-LA}_i = \left\{(1/4)\times\sum_j \text{SLL-LA}_{ij}\right\} / \left\{(1/4)\times\sum_j \text{SLL-LA}_{0j}\right\}.$$

Since the SLR-LA is the denominator of the nSLO-LA, the extremely smaller value of this is considered to cause the large variation of the nSLO-LA. Therefore, the i-th FInSLO-LA value with extremely smaller value of the I.I.- SLR-LA might be considered to be poor precision.

8-3-3-3. Judgment for "Positive" or "Negative" in each experiment

At the finish the phase IIc trial, the VMT had discussed about the judgment for "Positive" or "Negative". Until the meeting, the VMT had 3 criteria as candidates. These criteria are follows,

Criterion 1: Judged "Positive" if the FInSLO-LA ≥1.4 and I.I.-SLR-LA ≥ 0.5 at a same concentration, and otherwise judged "Negative".

Criterion 2: Judged "Positive" if the lower limit of the 95% confidence interval for FInSLO-LA ≥1, and otherwise judged "Negative".

Criterion 3: Judged "Positive" if the FInSLO-LA ≥1.4 and the lower limit of the 95% confidence interval for FInSLO-LA ≥ 1 at a same concentration, and otherwise judged "Negative".

At the meeting after the phase IIc trial, the VMT did not decide which criterion is superior. Then, the VMT had decided to assess all the 3 criteria in the phase III and IV and had decided to select one of them based on data of the phase IIb, IIc, III and IV studies.

After all, the criterion 3 was superior to others in the view of the inter- and intra-laboratory reproducibility, although the criterion 1 has good performance for relevance. After the discussion, the VMT selected the criterion 3.

Avoiding the redundancy, this report presents the results based on the criterion 3.

8-3-3-4. Final judgment for “Sensitizer” or “Non-sensitizer” using this assay

In this assay, “Sensitizer” is defined as in case that the 2 “Positive” judgments were found in a set of experiments; “Non-sensitizer” is defined as in case that the 3 “Negative” judgments were found in a set of experiments.

Since the distributed Excel data sheet implemented the indication of the “Positive” or “Negative” judgment when the data is entered, the operators in all the laboratories were able to know how many they need to conduct experiments for the final judgment. However, after all the experiments, data management team found the implemented judge in the Excel datasheet was incorrect for the calculation of the 95% CI of the FInSLO-LA. Then they treated as “incomplete (abbreviated as G)” when the assay did not decide the “Sensitizer” or “Non-sensitizer” due to the lack of experiments.

The 95% confidence interval by the Fieller’s theorem required the percentile of the t distribution based on the decimal degree of freedom. Unfortunately, the Excel function “tinv” did not treat the decimal degree of freedom, and can treat only the integer for the degree of freedom even when the decimal degree of freedom value is entered, and no error massage. Data management team found the fact after all the face to face VMT meeting. The 95% confidence interval is critical for the judgment of each experiment. In consequence of this fact, some final judgments were change. In this report, all the results are based on the recalculation by the SAS based on the observations. That is, the judgment used the confidence interval is based on the percentile of the t distribution based on the decimal degree of freedom.

In the phase IV study, the corrected the Excel datasheet was distributed and used. For the correction, the data management team applied an approximation for the 97.5 percentile calculation of the t distribution, as known as the Yamauchi’s method, instead of the Excel tinv function. The approximation is the followings,

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

where $y_1(u) = (u^3 + u)/4$, $y_2(u) = (5u^5 + 16u^3 + 3u)/96$, $y_3(u) = (3u^7 + 19u^5 + 17u^3 - 15u)/384$,
$y_4(u) = (79u^9 + 776u^7 + 1482u^5 - 1920u^3 - 945u)/92160$,
$y_5(u) = (27u^{11} + 339u^9 + 930u^7 - 1782u^5 - 756u^3 + 17955u)/368640$,
and $u = 1.96$.

8-3-4. Reliability

8-3-4-1. Within-laboratory reproducibility for 15 common chemicals

Within-laboratory reproducibility was determined by whether or not tables of 3 sets for the final judgment for each chemical by each laboratory (P2b01, P2b02, P2b03, P2b04, P2b05, P2c08, P2c12, P2c19, P2c21, P4-01, P4-02, P4-03, P4-04, and P4-05) were concordant. The concordance rate was then calculated as a proportion of the concordance of each laboratory.

The concordance rate for within-laboratory reproducibility was based on the results of 3 sets.

Unfortunately, due to the new decision criterion adopted in Phase IIb, the judgments for

several chemicals made by the old criterion were insufficient to allow a decision to be made. These data were labeled “undetermined” for the final criterion. For the Phase IIc study, we found that the 95% confidence interval calculation program implemented in the data file was incorrect. Knowledge of this confidence interval is required for judgment of the experiment. Consequently, the final judgment for several chemicals could not be made and these data were also labeled “undetermined” for the final criterion.

Only the chemicals whose judgment was determined by the 3 sets of experiments were used to calculate the concordance rate.

To summarize, the concordance rate for within-laboratory reproducibility from the 3 laboratories were used to calculate the averaged concordance rate.

8-3-4-2 Between-laboratory reproducibility

Between-laboratory reproducibility was determined using the results from the final judgment from the 3 laboratory for 35 chemicals (P2b01, P2b02, P2b03, P2b04, P2b05, P2c08, P2c12, P2c19, P2c21, P2c22, P3-01, P3-02, P3-03, P3-04, P3-05, P3-06, P3-07, P3-08, P3-09, P3-10, P3-11, P3-12, P3-13, P3-14, P3-15, P3-16, P3-17, P3-18, P3-19, P3-20, P4-1, P4-2, P4-3, P4-4, and P4-5). These judgements were tabulated, then the concordance rate was calculated as a proportion of the concordance in each laboratory. Since P2b-05 and P3-01 were the same chemical, i.e., 2,4-dichloronitrobenzen, the data for P2b-05 were omitted. In addition, as there were results from 3 sets of the Phase IIb, IIc, and III studies, the result for each chemical was decided by a majority.

The concordance rate was calculated using only chemicals for which judgments were made by all 3 laboratories. The data for chemicals for which any laboratory made an ‘undetermined’ result were omitted from the calculation of the concordance rate on between-laboratory reproducibility.

To summarize, the concordance rate for between-laboratory reproducibility from the 3 laboratories were used to calculate the averaged concordance rate.

8-3-5. Relevance

8-3-5-1. Definition of concordance, sensitivity and specificity

The concordance, sensitivity and specificity were estimated as the indexes of relevance. These indexes are estimated using the frequency results obtained from the 2 by 2 contingency table. The definitions of these indexes are summarized in Table 22 below. This calculation was based on the results decided by a majority for the between-laboratory results for each chemical. Again, chemicals labeled as “undetermined” from the evaluation of between-laboratory reproducibility were omitted.

Table 22. Definition of the concordance, sensitivity and specificity

Judgment from the IL8 Luc assay	Chemical category		Total
	Positive	Negative	
Positive	a	b	a+b
Negative	c	d	c+d
Total	a+c	b+d	N

Sensitivity = 100 x a / (a+c)
Specificity = 100 x d / (b+d)
Concordance = 100 x (a+d) / N
False Negative Rate (FN) = 100 x c / (a+c)
False Positive Rate (PN) = 100 x b / (b+d)
Positive Predictive Value (PPV) = 100 x a / (a+b)
Negative Predictive Value (NPV) = 100 x d / (c+d)

8-4. Quality assurance

Assays and quality assurance were carried out in the spirit of GLP, although not all the participating laboratories routinely worked under GLP certification. The participating laboratories conducted the experiments in accordance with the protocol provided by the VMT. All raw data and data analysis sheets were pre-checked for quality by each laboratory and then were reviewed by the VMT quality assurance team. The results accurately reflect the raw data.

9. Results

We conducted Phase I, IIa, IIb, IIc, III, and IV studies in this validation. The incubation time, dilution procedure, and criteria used to judge sensitizers in these ring studies are summarized in Fig. 12. Since the incubation time of the THP-G8 cells with the chemicals was changed from 6 h to 16 h, the within- and between-laboratory reproducibilities were examined based on the Phase IIb, IIc, III, and IV studies.

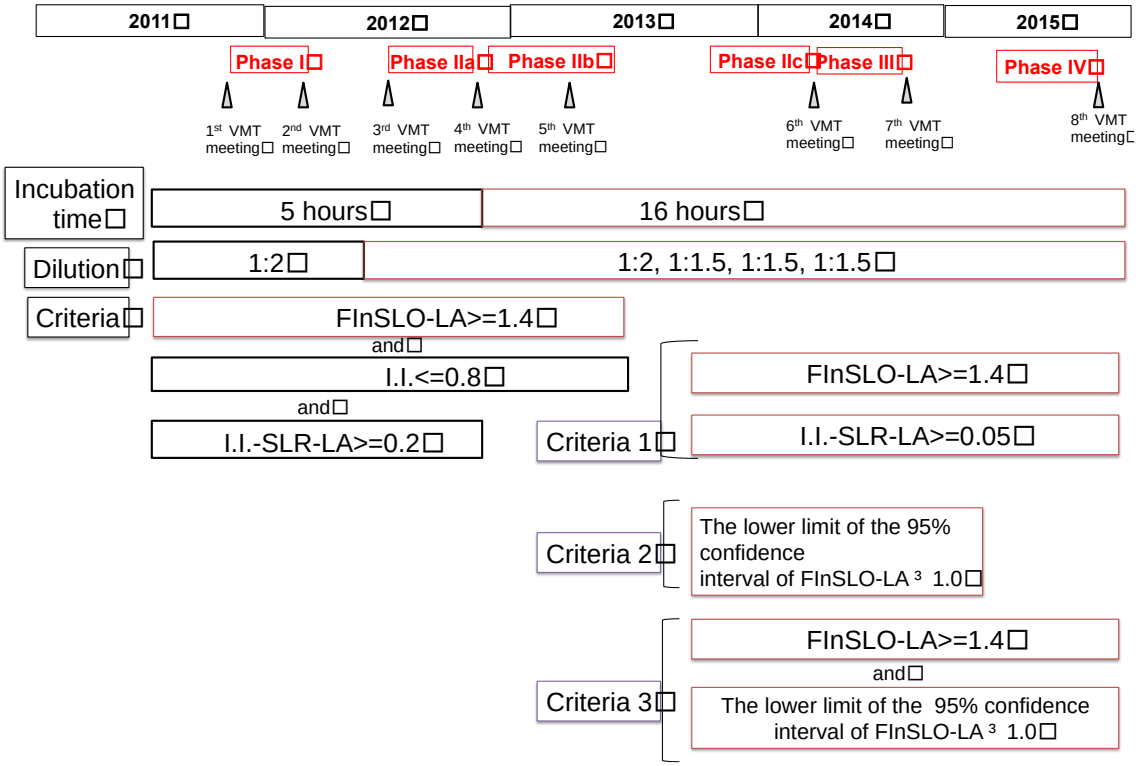


Fig. 12. The modification of the protocols of the IL-8 Luc assay.

9-1. Phase 0 study (for technical transfer)

The preliminary test trial, Phase 0, was performed by the participating laboratories following explicit explanations of the IL-8 Luc assay procedures and protocol Ver. 006 by the Lead laboratory, Tohoku University. In this study, 3 open labeled chemicals, 4-NBB and TMTD as sensitizers and lactic acid as a non-sensitizer, were examined twice by the IL-8 Luc assay in each laboratory. In a total 18 experiments conducted by the three participating laboratories, there was only one misjudgment (Table 23): Lab A judged GHS 1A, 4-NBB, as a non-sensitizer. Based on these results, VMT judged that technical and protocol transfer of the IL-8 Luc assay is acceptable.

Table 23. Results of the Phase 0 study

No.	Chemical name	CAS No.	LLNA EC3	GHS Category	Lab A	Lab B	Lab C
1	4-Nitrobenzylbromide	100-11-8	0.05	1A	1/2[*1]	1/1	3/3
2	Tetramethyl thiuiram disulphide	137-26-8	5.2	1B	2/2	1/1	3/3
3	Lactic acid	50-21-5		NC	0/2	0/1	0/3

*1: One of 2 experiments was misjudged.

9-2. Phase I study (for between-laboratory reproducibility)

9-2-1. Test conditions

A total of 10 coded chemicals (1 extreme, 1 strong, 3 moderate and 2 weak sensitizers, and 3 non-sensitizers) were evaluated by one experimental set in the Phase I study based on IL-8 Luc assay protocol Ver. 009E (originally written in Japanese on 30, Nov, 2011, and translated into English on 19, Dec, 2011)

In each experimental set, two or more experiments are conducted for each chemical. Chemicals that demonstrate FInSLO-LA $\geq$ 1.4 and I.I. $\leq$ 0.8 at the concentration of the chemical at which I.I.-SLR-LA is $\geq$ 0.2 are judged as positive. Chemicals that provide 2 positive results are judged as sensitizers.

9-2-2. Between-laboratory variation assessments in the Phase I study

Between-Lab reproducibility 70%

Table 24. Results of the Phase I study

No.	Chemical name	CAS No.	LLNA EC3	GHS Category	Lab A	Lab B	Lab C
1	1,4-Phenylenediamine	106-50-3	0.16	1A	+(2/3)	-(1/3)	-(0/3)
2	2,4-Dinitrochlorobenzene	97-00-7	0.06	1A	+(2/3)	+(3/3)	-(1/3)
3	2-Mercaptobenzothiasole	149-30-4	1.7	1A	-(0/3)	-(1/3)	-(1/3)
4	Cinnamal	104-55-2	3	1B	-(0/3)	-(0/3)	-(1/3)
5	Tetramethyl thiuiram disulphide	137-26-8	5.2	1B	+(3/3)	+(3/3)	+(3/3)
6	Eugenol	97-53-0	13	1B	+(3/3)	-(0/3)	-(0/3)
7	Imidazolidinyl urea	39236-46-9	24	1B	+(2/3)	+(3/3)	+(3/3)
8	Lactic acid	50-21-5		NC	-(0/3)	-(0/3)	-(0/3)
9	Salicylic acid	69-72-7		NC	-(0/3)	-(0/3)	-(0/3)
10	Sodium lauryl sulphate	151-21-3		NC	-(0/3)	-(0/3)	-(0/3)

9-2-3. Predictivity in the Phase I study

Accuracy of Lab A	80%
Accuracy of Lab B	60%
Accuracy of Lab C	50%
Average	63%

9-2-4. Contingency tables for the Phase I study

Lab A		IL-8 Luc assay		Total
		+	-	
LLNA	+	5	2	7
	-	0	3	3
Total		5	5	10

Sensitivity : 71.4% (5/7)

Specificity : 100% (3/3)

Accuracy : 80% (8/10)

Lab B		IL-8 Luc assay		Total
		+	-	
LLNA	+	3	4	7
	-	0	3	3
Total		3	7	10

Sensitivity : 42.9% (3/7)

Specificity : 100% (3/3)

Accuracy : 60.0% (6/10)

Lab C		IL-8 Luc assay		Total
		+	-	
LLNA	+	2	5	7
	-	0	3	3
Total		2	8	10

Sensitivity : 28.6% (2/7)

Specificity : 100% (3/3)

Accuracy : 50.0% (5/10)

9-2-5. Comments from the VMT members.

All the laboratories could predict non-sensitizers but there were considerable numbers of false negative results. Consequently, the members of the validation management team (VMT) suggested the following improvements for better performance and between- and within-laboratory reproducibility: 1) re-evaluate the positive criteria settings, 2) refine the dose intervals, 3) identify the best incubation period with the chemicals, and 4) change a positive control from LPS to $NiCl_2$.

9-3. Phase IIa study (for between-laboratory reproducibility)

9-3-1. Test conditions

According to the suggestions of the VMT members, the protocol was modified: 1) the initial concentration of water soluble chemicals was changed to 25 mg/mL from 100 mg/mL, 2) the dilution ratio was changed to a common ration of 1.5 in the 2nd, 3rd, and 4th experiments, 3) the highest concentration of chemicals in the 2nd, 3rd, and 4th experiments were changed, 4) the condition "I.I.-SLR-LA is ≥ 0.2" was deleted from the criteria, and 5) the criteria for sensitizers were changed. As a result of implementing these changes, chemicals are diluted 11 times serially using a dilution factor of 2 in the 1st experiment and a dilution factor of 1.5 in the 2nd, 3rd, and 4th experiments. To determine the highest concentration of chemicals to evaluate in the 2nd, 3rd, and 4th experiments, the minimum concentration at which I.I.-SLR-LA is below 0.05 was determined in the 1st experiment. Then, the concentration one step (2-times) higher than this determined concentration was used as the highest concentration of the chemical to examine. Chemicals are classified as sensitizers if judged as positive in 3 experiments among 3 or 4 independent experiments.

The Phase IIa study of between-laboratory reproducibility using a total of 10 coded chemicals (2 extreme, 1 strong, 3 moderate and 4 weak sensitizers, and 3 non-sensitizers) was evaluated by one experiment set based on IL-8 Luc assay protocol Ver. 012E. Five chemicals examined in Phase I were included.

9-3-2. Between-laboratory variation assessments in the Phase IIa study

Between-Lab reproducibility 70%

Table 25. Results of the Phase IIa study

No.	Chemical name	CAS No.	LLNA EC3	GHS Category	Lab.A Judge	Lab.B Judge	Lab.C Judge
5	1,4-Phenylenediamine	106-50-3	0.16	1A	+(2/3)	+(2/3)	+(2/3)
2	2,4-Dinitrochlorobenzene	97-00-7	0.06	1A	+(3/3)	-(1/3)	+(3/3)
4	2-Mercaptobenzothiasole	149-30-4	1.7	1A	+(3/3)	+(3/3)	+(3/3)
7	4-Nitrobenzylbromide	100-11-8	0.05	1A	+(3/3)	+(3/3)	+(3/3)
9	Glyoxal	107-22-2	0.8	1A	-(0/3)	-(0/3)	-(0/3)
1	Cinnamal	104-55-2	3	1B	+(3/3)	+(3/3)	+(3/3)
3	Eugenol	97-53-0	13	1B	+(3/3)	+(2/3)	-(1/3)
8	Glycerol	56-81-5		NC	-(0/3)	-(0/3)	-(0/3)
6	Isopropanol	67-63-0		NC	-(0/3)	-(0/3)	-(0/3)
10	Methyl salicylate	119-36-8	□	NC	+(2/3)	-(1/3)	+(2/3)

9-3-3. Predictivity in the Phase IIa study

Accuracy of Lab A	80%
Accuracy of Lab B	80%
Accuracy of Lab C	70%
Average	77%

9-3-4. Contingency tables for the Phase IIa study

Lab A		IL-8 Luc assay		Total
		+	-	
LLNA	+	6	1	7
	-	1	2	3
Total		7	3	10

Sensitivity : 85.7% (6/7)

Specificity : 66.7% (2/3)

Accuracy : 80.0% (8/10)

Lab B		IL-8 Luc assay		Total
		+	-	
LLNA	+	5	2	7
	-	0	3	3
Total		5	5	10

Sensitivity : 71.4% (5/7)
Specificity : 100% (3/3)
Accuracy : 80.0% (8/10)

Lab C		IL-8 Luc assay		Total
		+	-	
LLNA	+	5	2	7
	-	1	2	3
Total		6	4	10

Sensitivity : 71.4% (5/7)
Specificity : 66.7% (2/3)
Accuracy : 70.0% (7/10)

9-3-5. Comments from the VMT members

The results of the Phase IIa study showed significant improvement in performance regarding within- and between-laboratory reproducibilities. However, the members pointed out the following failures with the IL-8 Luc assay: 1) Lab. B judged an extreme sensitizer, DNCB, as a non-sensitizer and 2) all the laboratories judged glyoxal as a non-sensitizer.

9.4. Phase IIb study (for between- and within- laboratory reproducibility)

9-4-1. Test conditions

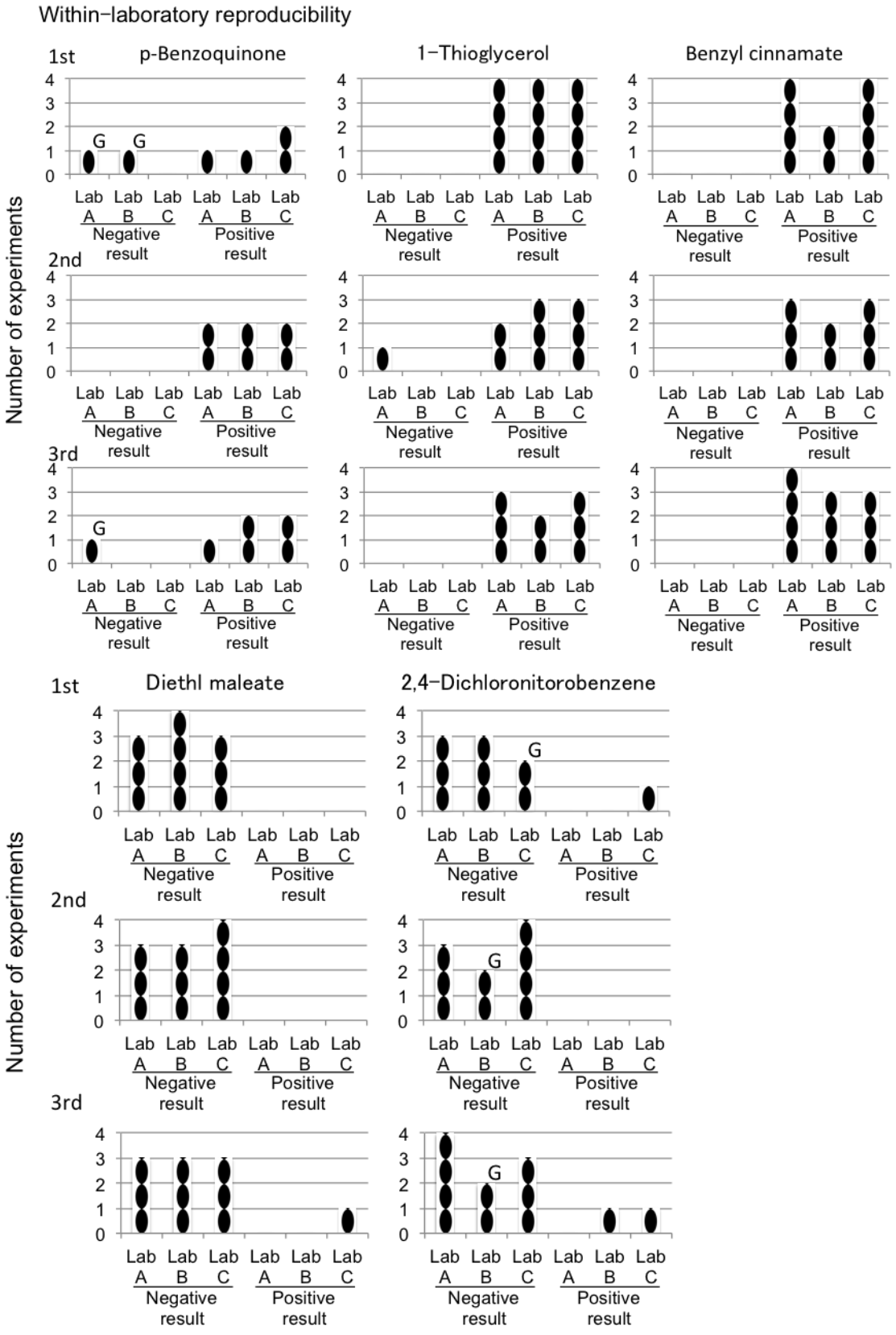
Taking the suggestions of the VMT members into account, the lead laboratory investigated the cause of the poor response of THP-G8 cells to DNCB from all aspects. The optimum incubation period for the IL-8 Luc assay was determined by monitoring FInSLO-LA of THP-G8 cells after stimulation with LPS. Examination of the time course of FInSLO-LA after the addition of the representative haptens, DNCB and 4-NBB, showed the highest induction of FInSLO-LA around 10 h after stimulation. However, a 10 h incubation period is not practical, as then one IL-8 Luc assay takes more than 12 h, including the time for preparation, plating the cells, incubating with the chemicals, and measuring luciferase activity using a luminometer. To find a practical compromise, we compared FInSLO-LA in a variety of chemicals incubated for between 5 h and 16 h, and decided to change the incubation period from 5 h to 16 h. Next, the lead laboratory reexamined the optimal concentration of Glyoxal to stimulate the THP-G8 cells and found that the highest concentration determined by the Phase IIa protocol is insufficient to detect the positive effect of Glyoxal. Accordingly, we changed the highest concentration of chemicals in the Phase IIb protocol.

The Phase IIb study examined within-and between-laboratory reproducibilities using a total of 5 coded chemicals (1 extreme, 2 moderate and 1 weak sensitizer, and 1 non-sensitizer) evaluated by 3 experiment sets based on IL-8 Luc assay protocol Ver. 015E.

9-4-2. Between- and within- laboratory variation assessments

Between-Lab reproducibility	86% ((9+9+9+6+5)/44)
Within-Lab reproducibility	Lab. A 80% (4/5)
	Lab. B 80% (4/5)
	Lab. C 75% (3/4)
	Average 78%

The graphical presentation of between- and within-laboratory variation in Phase IIb study is shown in Fig. 13.


Within-laboratory reproducibility
1st
p-Benzoquinone
1-Thioglycerol
Benzyl cinnamate
2nd
3rd
Diethl maleate
2,4-Dichloronitorobenzene
Number of experiments
G
Lab A Lab B Lab C Lab A Lab B Lab C
Negative result
Positive result

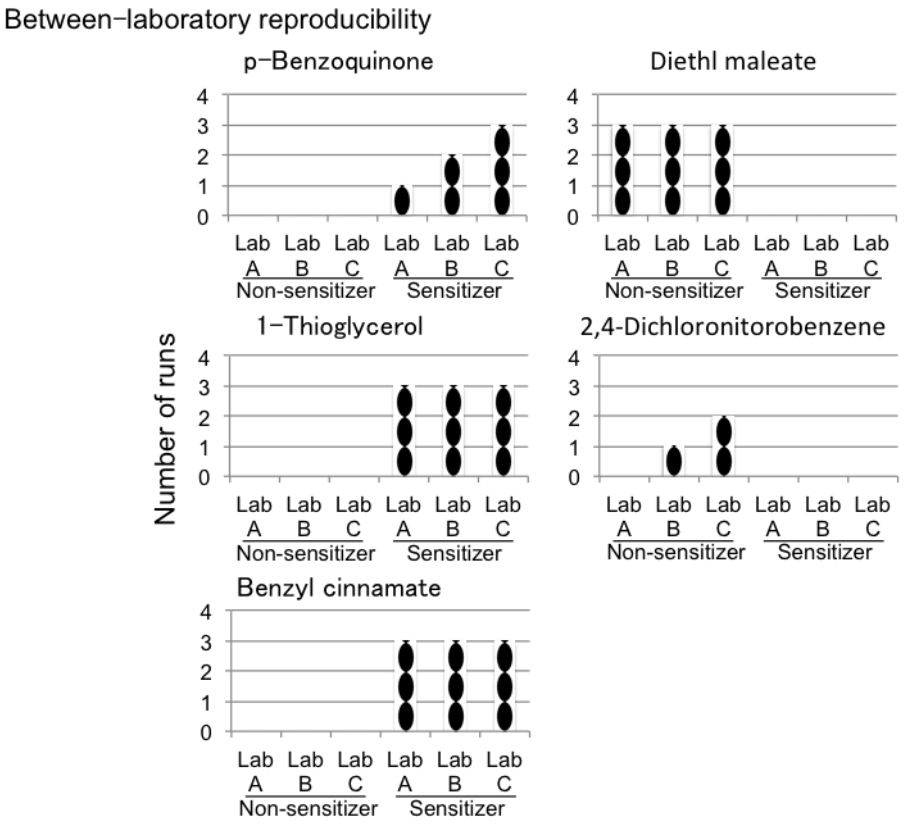


Fig. 13. Between- and within- laboratory variation assessments in Phase IIb study

The Phase IIb study examined within-and between-laboratory reproducibilities using a total of 5 coded chemicals (1 extreme, 2 moderate and 1 weak sensitizer, and 1 non-sensitizer) evaluated by 3 experiment sets based on IL-8 Luc assay protocol Ver. 015E. Closed circles represent the judgments for individual experiments in within-laboratory reproducibility, while those represent the judgments in individual experimental sets for between-laboratory reproducibility. Since Phase IIb studies examined 3 different criteria. Therefore, unnecessary repeats were conducted for judgment by the final criteria.

Table 26. Results of the Phase IIb study

Phase	Chemicals	Repeat	No.	Chemical name	CAS No.	LLNA EC3	GHS Category	Lab A	Lab B	Lab C
IIb	5 coded	3 sets						+(2/2)	+(2/2)	+(2/2)
			1	p-Benzoquinone	106-51-4	0.099	1A	+(2/2)	+(2/2)	+(2/2)
								+(2/2)	+(2/2)	+(2/2)
								+(4/4)	+(4/4)	+(4/4)
			2	1-Thioglycerol	96-27-5	3.6	1B	+(2/3)	+(3/3)	+(3/3)
								+(3/3)	+(2/2)	+(3/3)
								+(4/4)	+(2/2)	+(4/4)
			3	Benzyl cinnamate	103-41-3	18.4	1B	+(3/3)	+(2/2)	+(3/3)
								+(4/4)	+(3/3)	+(3/3)
								-(0/3)	-(1/4)	-(0/3)
			4	Diethyl maleate	141-05-9		1B	+(2/3)	-(0/3)	+(2/4)
								+(3/3)	-(0/3)	-(1/4)
								-(0/3)	-(0/3)	G(1/3)
			5	2,4-Dichloronitrobenzene	611-06-3		NC	-(0/3)	+(2/2)	-(1/4)
								-(1/4)	+(2/3)	+(2/4)

G means that the experiment set was incomplete and did not obtain sufficient data to complete the judgment based on the indicated criterion.

9-4-3. Predictivity in the Phase IIb study

Accuracy of Lab. A	93% (14/15)
Accuracy of Lab. B	67% (10/15)
Accuracy of Lab. C	79% (11/14)
Average	80%

9-4-4. Contingency tables for the Phase IIb results

Lab A		IL-8 Luc assay		Total
		+	-	
LLNA	+	11	1	12
	-	0	3	3
Total		11	4	15

Sensitivity : 91.7% (11/12)
Specificity : 100% (3/3)
Accuracy : 93.3% (14/15)

Lab B		IL-8 Luc assay		Total
		+	-	
LLNA	+	9	3	12
	-	2	1	3
Total		11	4	15

Sensitivity : 75.0% (9/12)
Specificity : 33.3% (1/3)
Accuracy : 66.7% (10/15)

Lab C		IL-8 Luc assay		Total
		+	-	
LLNA	+	10	2	12
	-	1	1	2
Total		11	3	14

Sensitivity : 83.3% (10/12)
Specificity : 50.0% (1/2)
Accuracy : 78.6% (11/14)

9-4-5. Comments from the VMT members

Test method accuracy of Lab. A, Lab. B, and Lab. C was 93.3%, 66.7%, and 78.6%, respectively. In addition, the VMT members considered that the within-laboratory reproducibility

was unsatisfactory.

9-5. Phase IIc study (for between- and within- laboratory reproducibility)

9-5-1. Test conditions

Taking the suggestions of the VMT members into account, the criteria to decide positive or negative in each experiment were refined. The present criteria to decide positive or negative have two indexes: Fold Induction (FInSLO-LA>1.4) and Inhibition Index (Value of I.I.). These complex criteria would result in poor reproducibility. In addition, although most sensitizers exhibit cysteine reactivity, there may be exceptions. For example, Gerberick et al. (2007) reported that phthalic anhydride and trimellitic anhydride demonstrated significant depletion with GSH and lysine peptides, but not with cysteine peptides in their DPRA. Thus, the sole index of using only Fold Induction (FInSLO-LA>1.4) was applied.

On the other hand, since the incubation time of THP-G8 was changed with chemicals from 5 h to 16 h, the magnitude of FInSLO-LA became large enough to be analyzed statistically. Therefore, in Phase IIc, the test was examined for the validity of the following new criteria: chemicals that demonstrate the lower limit of the 95% confidence interval of FInSLO-LA ≥ 1.0 are judged as positive. Therefore, in Phase IIc, the test was examined according to the following three temporary criteria to identify sensitizers with the intent of adopting one after the Phase IIc study.

Criterion 1. The criteria that uses FInSLO-LA and I.I.-SLR-LA
In each experiment:
Criteria: chemicals that demonstrate FInSLO-LA ≥ 1.4 and I.I.-SLR-LA ≥ 0.05 are judged as positive.

Criterion 2. The criteria which use the lower limit of the 95% confidence interval of FInSLO-LA
In each experiment:
Criteria: chemicals that demonstrate the lower limit of the 95% confidence interval of FInSLO-LA ≥ 1.0 are judged as positive.

Criterion 3. The criterion that uses FInSLO-LA and the lower limit of the 95% confidence interval of FInSLO-LA
In each experiment:
Criteria: chemicals that demonstrate FInSLO-LA ≥ 1.4 and the lower limit of the 95% confidence interval of FInSLO-LA ≥ 1.0 are judged as positive.

For the Phase IIc study to examine within- and between-laboratory reproducibilities, a total of 5 coded chemicals (1 strong and 1 moderate sensitizer, and 1 non-sensitizer), using the modified protocol and criteria, were evaluated by 3 experiment sets for each chemical based on IL-8 Luc assay protocol Ver. 017E.

9-5-2. Between- and within- laboratory variation assessments in Phase IIc study

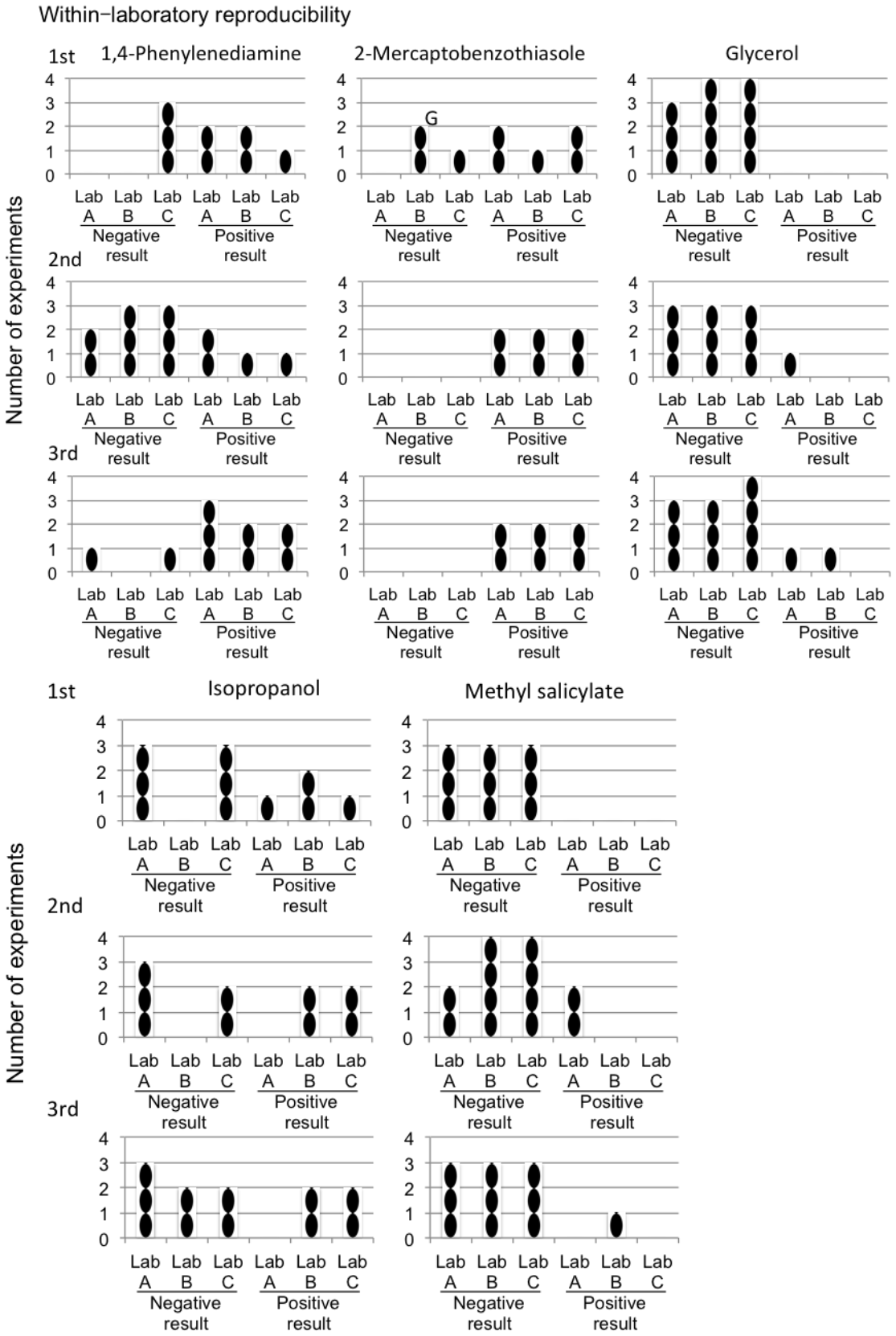
Criterion 1
Between-Lab reproducibility 82% ((7+9+8+5+8)/45)
Within-Lab reproducibility Lab A. 60% (3/5)
Lab B 80% (4/5)
Lab C 60% (3/5)
Average 67%

Criterion 2
Between-Lab reproducibility 91% ((9+8+7+8+8)/44)
Within-Lab reproducibility Lab A 40% (2/5)

Lab B 75% (3/4)
Lab C 100% (5/5)
Average 71%

Criterion 3
Between-Lab reproducibility 86% ((9+9+9+6+5)/44)
Within-Lab reproducibility Lab A 80% (4/5)
Lab B 75% (3/4)
Lab C 60% (3/5)
Average 71%

The graphical presentation of between- and within-laboratory variation in Phase IIc is shown in Fig. 14.


Within−laboratory reproducibility
1st
1,4-Phenylenediamine
2-Mercaptobenzothiasole
Glycerol
2nd
3rd
Isopropanol
Methyl salicylate
Number of experiments
0
1
2
3
4
G
Lab A
Lab B
Lab C
Negative result
Positive result

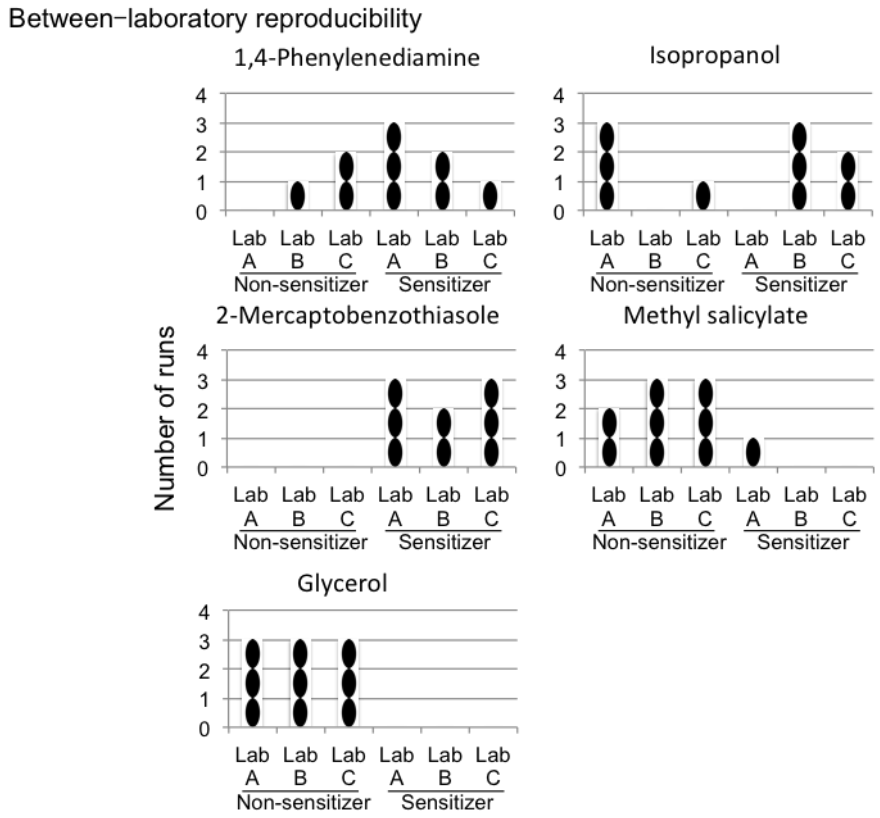


Fig. 14. Between- and within- laboratory variation assessments in Phase IIc study

For the Phase IIc study, to examine within- and between-laboratory reproducibilities, a total of 5 coded chemicals (1 strong and 1 moderate sensitizer, and 3 non-sensitizer), were evaluated using the modified protocol and criteria by 3 experiment sets for each chemical based of the IL-8 Luc assay protocol Ver. 017E. Closed circles represent the judgments for individual experiments in within-laboratory reproducibility, while those represent the judgments in individual experimental sets for between-laboratory reproducibility. Since Phase IIc studies examined 3 different criteria. Therefore, unnecessary repeats were conducted for judgment by the final criteria.

Table 27. Results of the Phase IIc study

No.	Chemical name	CAS No.	LLNA EC3	GHS Category	Criterion 1			Criterion 2			Criterion 3		
					Lab A	Lab B	Lab C	Lab A	Lab B	Lab C	Lab A	Lab B	Lab C
1	1,4-Phenylenediamine	106-50-3	0.0099	1A	+(2/2) +(3/4) -(1/4)	+(2/4) +(2/4) +(2/2)	+(2/4) -(1/4) +(2/3)	+(2/2) +(2/4) +(3/4)	+(3/4) +(2/4) +(2/2)	+(2/4) +(3/4) +(3/3)	+(2/2) +(2/4) +(3/4)	+(2/4) -(1/4) +(2/2)	-(1/4) -(1/4) +(2/3)
2	2-Mercaptobenzothiasole	149-30-4	1.7	1A	+(2/2) +(2/2) +(2/2)	+(3/3) +(2/2) +(2/2)	+(3/3) +(2/2) +(2/2)	+(2/2) +(2/2) +(2/2)	G(1/3) +(2/2) +(2/2)	+(2/3) +(2/2) +(2/2)	+(2/2) +(2/2) +(2/2)	G(1/3) +(2/2) +(2/2)	+(2/3) +(2/2) +(2/2)
3	Glycerol	56-81-5		NC	-(0/3) -(1/4) -(1/4)	-(0/4) -(0/3) +(2/4)	-(1/4) -(0/3) -(0/4)	-(0/3) +(3/4) -(1/4)	+(2/4) -(0/3) -(1/4)	-(0/4) -(0/3) -(1/4)	-(0/3) -(1/4) -(1/4)	-(0/4) -(0/3) -(1/4)	-(0/4) -(0/3) -(0/4)
4	Isopropanol	67-63-0		NC	-(1/4) -(0/3) -(0/3)	+(2/2) +(2/2) +(2/4)	-(1/4) +(2/4) +(2/4)	+(2/4) +(2/3) -(0/3)	+(2/2) +(2/2) +(2/4)	+(2/4) +(4/4) +(2/4)	-(1/4) -(0/3) -(0/3)	+(2/2) +(2/2) +(2/4)	-(1/4) +(2/4) +(2/4)
5	Methyl salicylate	119-36-8		NC	-(0/3) +(2/4) -(0/3)	-(0/3) -(1/4) -(1/4)	-(0/3) -(0/4) -(0/3)	-(0/3) +(2/4) -(0/3)	-(0/3) -(1/4) -(1/4)	-(0/3) -(1/4) -(0/3)	-(0/3) +(2/4) -(0/3)	-(0/3) -(0/4) -(1/4)	-(0/3) -(0/4) -(0/3)

G means that the experiment set was incomplete and did not obtain sufficient data to complete the judgment based on the indicated criterion.

9-5-3. Predictivity in the Phase IIc study

Criterion 1
Accuracy of Lab A 87% (13/15)
Accuracy of Lab B 73% (11/15)
Accuracy of Lab C 80% (12/15)
Average 80%

Criterion 2
Accuracy of Lab A 73% (11/15)
Accuracy of Lab B 71% (10/14)
Accuracy of Lab C 80% (12/15)
Average 75%

Criterion 3
Accuracy of Lab A 93% (14/15)
Accuracy of Lab B 71% (10/14)
Accuracy of Lab C 73% (11/15)
Average 80%

9-5-4. Contingency tables for the Phase IIc results

Criterion 1

Lab A		IL-8 Luc assay		Total
		+	-	
LLNA	+	5	1	6
	-	1	8	9
Total		6	9	15

Sensitivity : 83.3% (5/6)
Specificity : 88.9% (8/9)
Accuracy : 86.7% (13/15)

Lab B		IL-8 Luc assay		Total
		+	-	
LLNA	+	6	0	6
	-	4	5	9
Total		10	5	15

Sensitivity : 100% (6/6)
Specificity : 55.6% (5/9)
Accuracy : 73.3% (11/15)

Lab C		IL-8 Luc assay		Total
		+	-	
LLNA	+	5	1	6
	-	2	7	9
Total		7	8	15

Sensitivity : 83.3% (5/6)
Specificity : 77.8% (7/9)
Accuracy : 80.0% (12/15)

Criterion 2

Lab A	IL-8 Luc assay	Total

		+	-	
LLNA	+	6	0	6
	-	4	5	9
Total		10	5	15

Sensitivity : 100% (6/6)
Specificity : 55.6% (5/9)
Accuracy : 73.3% (11/15)

Lab B		IL-8 Luc assay		Total
		+	-	
LLNA	+	5	0	5
	-	4	5	9
Total		9	5	14

Sensitivity : 100% (5/5)
Specificity : 55.6% (5/9)
Accuracy : 71.4% (10/14)

Lab C		IL-8 Luc assay		Total
		+	-	
LLNA	+	6	0	6
	-	3	6	9
Total		9	6	15

Sensitivity : 100% (6/6)
Specificity : 66.7% (6/9)
Accuracy : 80.0% (12/15)

Criterion 3

Lab A		IL-8 Luc assay		Total
		+	-	
LLNA	+	6	0	6

	-	1	8	9
Total		7	8	15

Sensitivity : 100% (6/6)
Specificity : 88.9% (8/9)
Accuracy : 93.3% (14/15)

Lab B		IL-8 Luc assay		Total
		+	-	
LLNA	+	4	1	5
	-	3	6	9
Total		7	7	14

Sensitivity : 80.0% (4/5)
Specificity : 66.7% (6/9)
Accuracy : 71.4% (10/14)

Lab C		IL-8 Luc assay		Total
		+	-	
LLNA	+	4	2	6
	-	2	7	9
Total		6	9	15

Sensitivity : 66.7% (4/6)
Specificity : 77.8% (7/9)
Accuracy : 73.3% (11/15)

9-5-5. Comments from the VMT members

The average within-laboratory reproducibility and between-laboratory reproducibility were approximately 80% even using these new criteria. Specifically, Criterion 3 produced the highest within-laboratory reproducibility, while the between-laboratory reproducibility was better by Criteria 1 and 2 than by Criterion 3. It remains to be determined which criteria, Criterion 1 or 3, better improves the performance of the IL-8 Luc assay.

9.6. Phase III study (for between- laboratory reproducibility and predictive capacity)

9-6-1. Test conditions

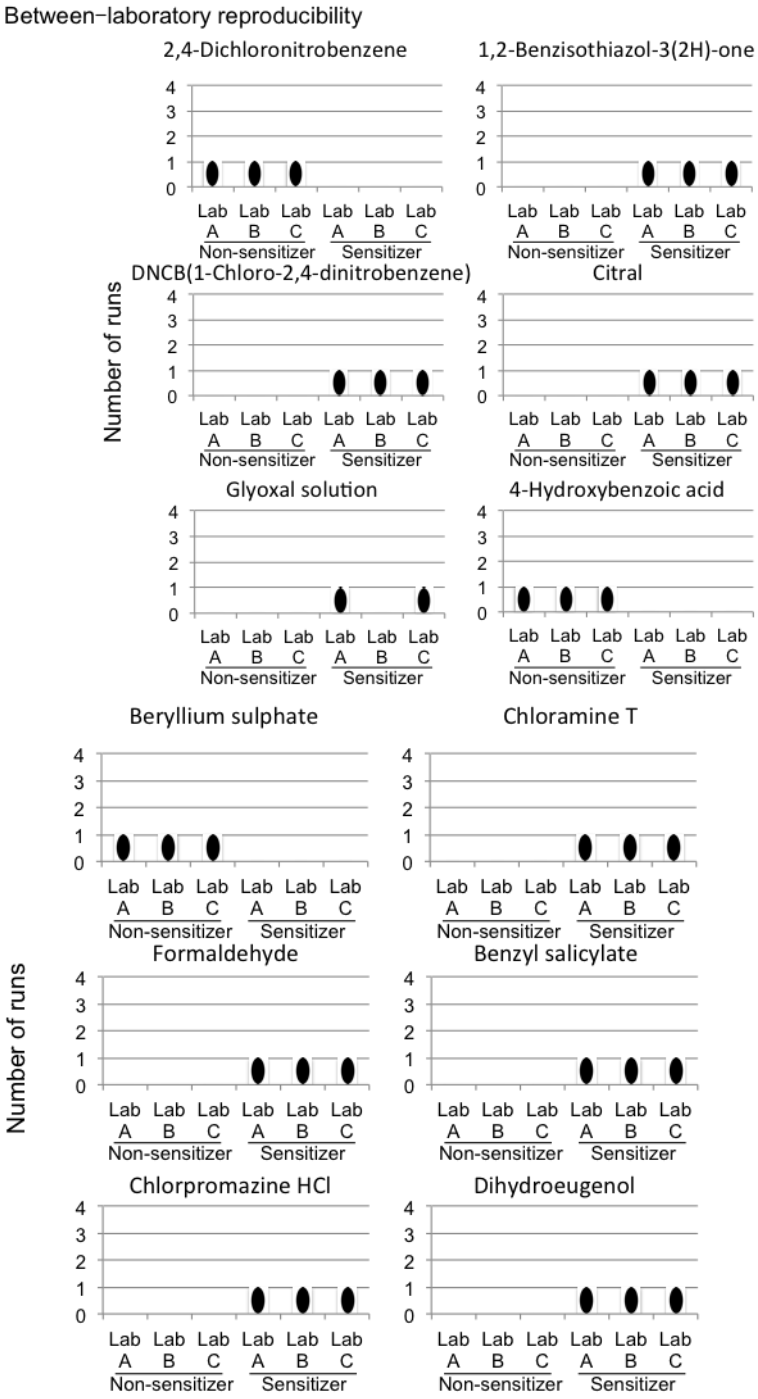
For the Phase III study, a total of 20 coded chemicals (2 extreme, 3 strong, 5 moderate and 4 weak sensitizers, and 6 non-sensitizers) were evaluated by one experiment set for each chemical based on IL-8 Luc assay protocol Ver. 017E. The purpose of the Phase III study was to evaluate the between-laboratory reproducibility and predictivity.

In the Phase III study, to know better criteria for this assay, the data were evaluated by 3 criteria, that is, Criterion 1: chemicals that demonstrate FInSLO-LA ≥ 1.4 and I.I.-SLR-LA ≥ 0.05 are judged as positive; Criterion 2: chemicals that demonstrate the lower limit of the 95% confidence interval of FInSLO-LA ≥ 1.0 are judged as positive; and Criterion 3: chemicals that demonstrate FInSLO-LA ≥ 1.4 and the lower limit of the 95% confidence interval of FInSLO-LA ≥ 1.0 are judged as positive.

9-6-2. Between-laboratory variation assessments in the Phase III study

The graphical presentation of between-laboratory variation in Phase III study is shown in Fig. 15.

Criterion 1
Between-Lab reproducibility 85% (17/20)
Criterion 2
Between-Lab reproducibility 67% (12/18)
Criterion 3
Between-Lab reproducibility 95% (18/19)


Between−laboratory reproducibility
2,4-Dichloronitrobenzene
1,2-Benzisothiazol-3(2H)-one
DNCB(1-Chloro-2,4-dinitrobenzene)
Citral
Glyoxal solution
4-Hydroxybenzoic acid
Beryllium sulphate
Chloramine T
Formaldehyde
Benzyl salicylate
Chlorpromazine HCl
Dihydroeugenol
Number of runs
0
1
2
3
4
Lab A
Lab B
Lab C
Non-sensitizer
Sensitizer

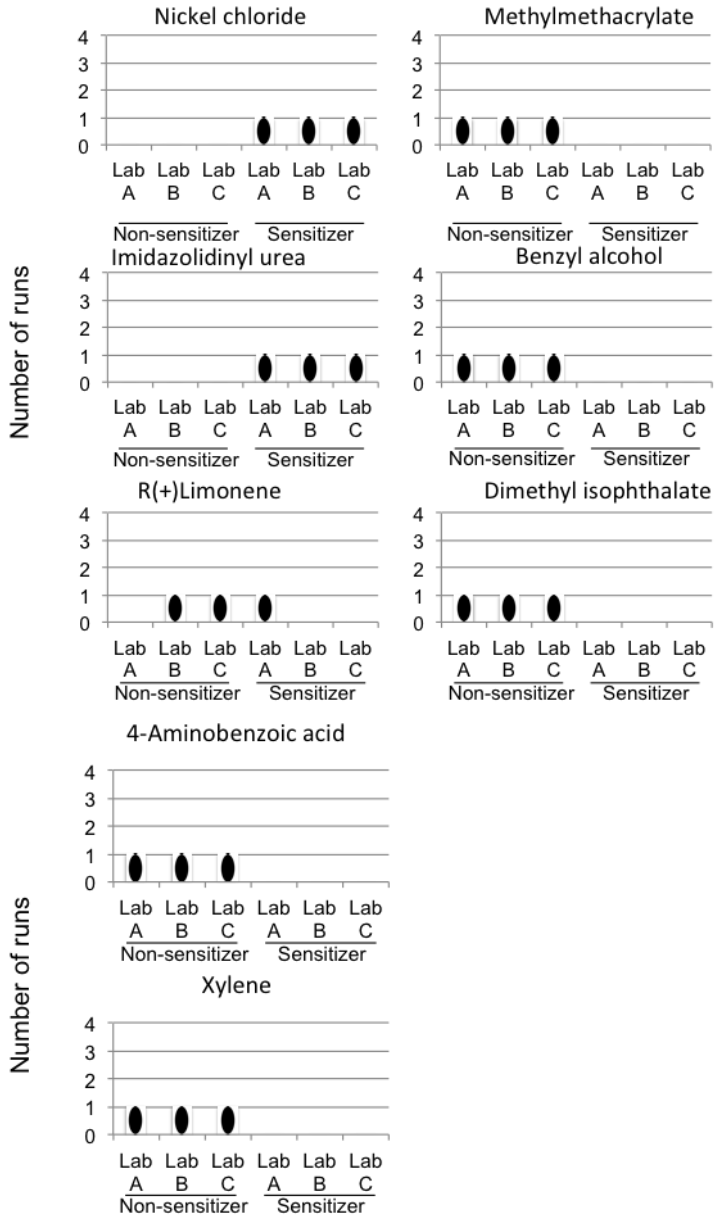


Fig. 15. Between laboratory variation assessments in Phase III study

For the Phase III study, a total of 20 coded chemicals (2 extreme, 3 strong, 5 moderate and 4 weak sensitizers, and 6 non-sensitizers) were evaluated by one experiment set for each chemical based on IL-8 Luc assay protocol Ver. 017E. Closed circles represent the judgments in individual experimental sets for between-laboratory reproducibility.

9-6-3. Predictivity in the Phase III study

Criterion 1
Accuracy of Lab A 90% (18/20)
Accuracy of Lab B 85% (17/20)
Accuracy of Lab C 85% (17/20)
Average 87%

Criterion 2
Accuracy of Lab A 85% (17/20)
Accuracy of Lab B 78% (14/18)
Accuracy of Lab C 75% (15/20)
Average 79%

Criterion 3
Accuracy of Lab A 90% (18/20)
Accuracy of Lab B 84% (16/19)
Accuracy of Lab C 85% (17/20)
Average 86%

Table 28. Results of the Phase III study

No.	Chemical name	CAS No.	LLNA EC3	GHS Category	Criterion 1			Criterion 2			Criterion 3		
					Lab A	Lab B	Lab C	Lab A	Lab B	Lab C	Lab A	Lab B	Lab C
1	2,4-Dichloronitrobenzene	611-06-3		NC	+(2/4)	-(0/3)	-(0/3)	+(2/4)	-(0/3)	-(0/3)	-(1/4)	-(0/3)	-(0/3)
2	DNCB(1-Chloro-2,4-dinitrobenzene)	97-00-7	0.06	1A	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)
3	Glyoxal solution	107-22-2	0.8	1A	+(2/2)	+(2/3)	+(2/2)	+(2/2)	G(1/3)	+(2/2)	+(2/2)	G(1/3)	+(2/2)
4	1,2-Benzisothiazol-3(2H)-one	2634-33-5	8	1B	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)
5	Citral	5392-40-5	5	1B	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)
6	4-Hydroxybenzoic acid	99-96-7		NC	-(0/4)	-(0/3)	-(0/4)	-(0/4)	G(1/3)	-(1/4)	-(0/4)	-(0/3)	-(0/4)
7	Beryllium sulphate	7787-56-6	0.001	1A	+(2/4)	-(0/3)	-(0/3)	+(2/4)	-(0/3)	-(0/3)	-(1/4)	-(0/3)	-(0/3)
8	Formaldehyde	50-00-0	0.61	1A	+(2/3)	+(2/2)	+(2/3)	+(3/3)	+(2/2)	+(2/3)	+(2/3)	+(2/2)	+(2/3)
9	Chlorpromazine HCl	69-09-0	0.14	1A	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)
10	Chloramine T	127-65-1	0.4	1A	+(2/3)	+(2/2)	+(2/2)	+(2/3)	+(2/2)	+(2/2)	+(2/3)	+(2/2)	+(2/2)
11	Benzyl salicylate	118-58-1	2.9	1B	+(2/2)	+(2/2)	+(3/3)	+(2/2)	+(2/2)	+(2/3)	+(2/2)	+(2/2)	+(2/3)
12	Dihydroeugenol	2785-87-7	6.8	1B	+(2/3)	+(2/2)	+(2/2)	+(3/3)	+(2/2)	+(2/2)	+(2/3)	+(2/2)	+(2/2)
13	Nickel chloride	7718-54-9		1B	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)
14	Imidazolidinyl urea	39236-46-9	24	1B	+(2/3)	+(2/3)	+(2/2)	+(2/3)	+(3/3)	+(2/2)	+(2/3)	+(2/3)	+(2/2)
15	R(+)Limonene	5989-27-5	69	1B	+(2/2)	-(0/4)	-(0/3)	+(2/2)	-(1/4)	-(0/3)	+(2/2)	-(0/4)	-(0/3)
16	Methylmethacrylate	80-62-6	90	1B	-(0/3)	-(0/4)	-(0/4)	-(0/3)	-(1/4)	-(1/4)	-(0/3)	-(0/4)	-(0/4)
17	Benzyl alcohol	100-51-6		NC	-(0/4)	-(0/3)	-(0/3)	-(1/4)	-(0/3)	-(0/3)	-(0/4)	-(0/3)	-(0/3)
18	Dimethyl isophthalate	1459-93-4		NC	-(0/3)	-(1/4)	-(1/4)	-(0/3)	+(2/4)	+(4/4)	-(0/3)	-(0/4)	-(1/4)
19	4-Aminobenzoic acid	150-13-0		NC	-(0/3)	-(0/3)	-(0/4)	-(0/3)	-(0/3)	+(2/4)	-(0/3)	-(0/3)	-(0/4)
20	Xylene	1330-20-7	95.8	NC	-(0/4)	-(0/3)	-(0/3)	+(2/4)	-(0/3)	-(0/3)	-(0/4)	-(0/3)	-(0/3)

G means that the experiment set was incomplete and did not obtain sufficient data to complete the judgment based on the indicated criterion.

9-6-4. Contingency tables for the Phase III study

Criterion 1

Lab A		IL-8 Luc assay		Total
		+	-	
LLNA	+	13	1	14
	-	1	5	6
Total		14	6	20

Sensitivity : 92.9% (13/14)
Specificity : 83.3% (5/6)
Accuracy : 90.0% (18/20)

Lab B		IL-8 Luc assay		Total
		+	-	
LLNA	+	11	3	14
	-	0	6	6
Total		11	9	20

Sensitivity : 78.6% (11/14)
Specificity : 100.0% (6/6)
Accuracy : 85.0% (17/20)

Lab C		IL-8 Luc assay		Total
		+	-	
LLNA	+	11	3	14
	-	0	6	6
Total		11	9	20

Sensitivity : 78.6% (11/14)
Specificity : 100.0% (6/6)
Accuracy : 85.0% (17/20)

Criterion 2

Lab A	IL-8 Luc assay	Total

		+	-	
LLNA	+	13	1	14
	-	2	4	6
Total		15	5	20

Sensitivity : 92.9% (13/14)
Specificity : 66.7% (4/6)
Accuracy : 85.0% (17/20)

Lab B		IL-8 Luc assay		Total
		+	-	
LLNA	+	10	3	13
	-	1	4	5
Total		11	7	18

Sensitivity : 76.9% (10/13)
Specificity : 80.0% (4/5)
Accuracy : 77.8% (14/18)

Lab C		IL-8 Luc assay		Total
		+	-	
LLNA	+	11	3	14
	-	2	4	6
Total		13	7	20

Sensitivity : 78.6% (11/14)
Specificity : 66.7% (4/6)
Accuracy : 75.0% (15/20)

Criterion 3

Lab A		IL-8 Luc assay		Total
		+	-	
LLNA	+	12	2	14

	-	0	6	6
Total		12	8	20

Sensitivity : 85.7% (12/14)
Specificity : 100.0% (6/6)
Accuracy : 90.0% (18/20)

Lab B		IL-8 Luc assay		Total
		+	-	
LLNA	+	10	3	13
	-	0	6	6
Total		10	9	19

Sensitivity : 76.9% (10/13)
Specificity : 100.0% (6/6)
Accuracy : 84.2% (16/19)

Lab C		IL-8 Luc assay		Total
		+	-	
LLNA	+	11	3	14
	-	0	6	6
Total		11	9	20

Sensitivity : 78.6% (11/14)
Specificity : 100.0% (6/6)
Accuracy : 85.0% (17/20)

9-7. Combined results of the Phase IIb, IIc, and III studies (for between- and within-laboratory reproducibility and predictive capacity)

9-7-1. Test conditions

The within- and between-laboratory reproducibilities, and the predictivity of the IL-8 Luc assay, were evaluated using all the results from Phases IIb, IIc, and III.

9-7-2. Within- and between-laboratory variation assessments using Phase IIb, IIc, and III studies

Criterion 1
Between-Lab reproducibility 86% (25/29)
Within-Lab reproducibility Lab. A 75% (6/8)
Lab. B 80% (8/10)
Lab. C 78% (7/9)
Average 78%
Criterion 2
Between-Lab reproducibility 77% (20/26)
Within-Lab reproducibility Lab. A 50% (4/8)
Lab. B 86% (6/7)
Lab. C 100% (9/9)
Average 79%
Criterion 3
Between-Lab reproducibility 89% (24/27)
Within-Lab reproducibility Lab. A 89% (8/9)
Lab. B 86% (6/7)
Lab. C 78% (7/9)
Average 84%

9-7-3. Predictivity in the Phases IIb, IIc, and III studies

Criterion 1
Accuracy of Lab. A 90% (26/29)
Accuracy of Lab. B 83% (24/29)
Accuracy of Lab. C 83% (24/29)
Average 85%

Criterion 2
Accuracy of Lab. A 82% (23/28)
Accuracy of Lab. B 82% (22/27)
Accuracy of Lab. C 76% (22/29)
Average 80%

Criterion 3
Accuracy of Lab. A 89% (25/28)
Accuracy of Lab. B 82% (23/28)
Accuracy of Lab. C 79% (23/39)
Average 83%

The data containing 2 Gs in Phase IIb and Phase IIc and those containing one G in Phase III in Table 29 were not used for the analysis of accuracy.

Table 29. Results of the Phase IIb, IIc, and III studies

No.	Chemical name	CAS No.	LLNA EC3	GHS Category	Criterion 1			Criterion 2			Criterion 3		
					Lab A	Lab B	Lab C	Lab A	Lab B	Lab C	Lab A	Lab B	Lab C
1	p-Benzoquinone	106-51-4	0.099	1A	+(2/2) +(2/2) G(1/2)	+(2/2) +(2/2) +(2/2)	+(2/2) +(2/2) +(2/2)	G(1/2) +(2/2) G(1/2)	G(1/2) +(2/2) +(2/2)	+(2/2) +(2/2) +(2/2)	G(1/2) +(2/2) G(1/2)	G(1/2) +(2/2) +(2/2)	+(2/2) +(2/2) +(2/2)
2	1-Thioglycerol	96-27-5	3.6	1B	+(4/4) +(2/3) +(3/3)	+(4/4) +(3/3) +(2/2)	+(4/4) +(3/3) +(3/3)	+(4/4) +(2/3) +(3/3)	+(4/4) +(3/3) +(2/2)	+(4/4) +(3/3) +(3/3)	+(4/4) +(2/3) +(3/3)	+(4/4) +(3/3) +(2/2)	+(4/4) +(3/3) +(3/3)
3	Benzyl cinnamate	103-41-3	18.4	1B	+(4/4) +(3/3) +(4/4)	+(2/2) +(2/2) +(3/3)	+(4/4) +(3/3) +(3/3)	+(4/4) +(3/3) +(4/4)	+(2/2) +(2/2) +(3/3)	+(4/4) +(3/3) +(3/3)	+(4/4) +(3/3) +(4/4)	+(2/2) +(2/2) +(3/3)	+(4/4) +(3/3) +(3/3)
4	Diethyl maleate	141-05-9	5.8	1B	-(0/3) -(0/3) G(1/3)	-(1/4) -(0/3) -(0/3)	-(0/3) -(1/4) -(1/4)	G(1/3) -(0/3) -(0/3)	-(0/4) -(0/3) -(0/3)	-(0/3) -(1/4) -(1/4)	-(0/3) -(0/3) -(0/3)	-(0/4) -(0/3) -(0/3)	-(0/3) -(0/4) -(1/4)
5	2,4-Dichloronitrobenzene	611-06-3		NC	-(0/3) -(0/3) -(1/4)	-(0/3) +(2/2) +(2/3)	G(1/3) -(1/4) +(2/4)	+(2/3) -(0/3) -(1/4)	-(0/3) G(0/2) G(1/3)	G(1/3) -(0/4) -(1/4)	-(0/3) -(0/3) -(0/4)	-(0/3) G(0/2) G(1/3)	G(1/3) -(0/4) -(1/4)
1	1,4-Phenylenediamine	106-50-3	0.0099	1A	+(2/2) +(3/4) -(1/4)	+(2/4) +(2/4) +(2/2)	+(2/4) -(1/4) +(2/3)	+(2/2) +(2/4) +(3/4)	+(3/4) +(2/4) +(2/2)	+(2/4) +(3/4) +(3/3)	+(2/2) +(2/4) +(3/4)	+(2/4) -(1/4) +(2/2)	-(1/4) -(1/4) +(2/3)
2	2-Mercaptobenzothiasole	149-30-4	1.7	1A	+(2/2) +(2/2) +(2/2)	+(3/3) +(2/2) +(2/2)	+(3/3) +(2/2) +(2/2)	+(2/2) +(2/2) +(2/2)	G(1/3) +(2/2) +(2/2)	+(2/3) +(2/2) +(2/2)	+(2/2) +(2/2) +(2/2)	G(1/3) +(2/2) +(2/2)	+(2/3) +(2/2) +(2/2)
3	Glycerol	56-81-5		NC	-(0/3) -(1/4) -(1/4)	-(0/4) -(0/3) +(2/4)	-(1/4) -(0/3) -(0/4)	-(0/3) +(3/4) -(1/4)	+(2/4) -(0/3) -(1/4)	-(0/4) -(0/3) -(1/4)	-(0/3) -(1/4) -(1/4)	-(0/4) -(0/3) -(1/4)	-(0/4) -(0/3) -(0/4)
4	Isopropanol	67-63-0		NC	-(1/4) -(0/3) -(0/3)	+(2/2) +(2/2) +(2/4)	-(1/4) +(2/4) +(2/4)	+(2/4) +(2/3) -(0/3)	+(2/2) +(2/2) +(2/4)	+(2/4) +(4/4) +(2/4)	-(1/4) -(0/3) -(0/3)	+(2/2) +(2/2) +(2/4)	-(1/4) +(2/4) +(2/4)
5	Methyl salicylate	119-36-8		NC	-(0/3) +(2/4) -(0/3)	-(0/3) -(1/4) -(1/4)	-(0/3) -(0/4) -(0/3)	-(0/3) +(2/4) -(0/3)	-(0/3) -(1/4) -(1/4)	-(0/3) -(1/4) -(0/3)	-(0/3) +(2/4) -(0/3)	-(0/3) -(0/4) -(1/4)	-(0/3) -(0/4) -(0/3)
1	2,4-Dichloronitrobenzene	611-06-3		NC	+(2/4)	-(0/3)	-(0/3)	+(2/4)	-(0/3)	-(0/3)	-(1/4)	-(0/3)	-(0/3)
2	DNCB(1-Chloro-2,4-dinitrobenzene)	97-00-7	0.06	1A	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)
3	Glyoxal solution	107-22-2	0.8	1A	+(2/2)	+(2/3)	+(2/2)	+(2/2)	G(1/3)	+(2/2)	+(2/2)	G(1/3)	+(2/2)
4	1,2-Benzisothiazol-3(2H)-one	2634-33-5	8	1B	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)
5	Citral	5392-40-5	5	1B	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)
6	4-Hydroxybenzoic acid	99-96-7		NC	-(0/4)	-(0/3)	-(0/4)	-(0/4)	G(1/3)	-(1/4)	-(0/4)	-(0/3)	-(0/4)
7	Beryllium sulphate	7787-56-6	0.001	1A	+(2/4)	-(0/3)	-(0/3)	+(2/4)	-(0/3)	-(0/3)	-(1/4)	-(0/3)	-(0/3)
8	Formaldehyde	50-00-0	0.61	1A	+(2/3)	+(2/2)	+(2/3)	+(3/3)	+(2/2)	+(2/3)	+(2/3)	+(2/2)	+(2/3)
9	Chlorpromazine HCl	69-09-0	0.14	1A	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)
10	Chloramine T	127-65-1	0.4	1A	+(2/3)	+(2/2)	+(2/2)	+(2/3)	+(2/2)	+(2/2)	+(2/3)	+(2/2)	+(2/2)
11	Benzyl salicylate	118-58-1	2.9	1B	+(2/2)	+(2/2)	+(3/3)	+(2/2)	+(2/2)	+(2/3)	+(2/2)	+(2/2)	+(2/3)
12	Dihydroeugenol	2785-87-7	6.8	1B	+(2/3)	+(2/2)	+(2/2)	+(3/3)	+(2/2)	+(2/2)	+(2/3)	+(2/2)	+(2/2)
13	Nickel chloride	7718-54-9		1B	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)
14	Imidazolidinyl urea	39236-46-9	24	1B	+(2/3)	+(2/3)	+(2/2)	+(2/3)	+(3/3)	+(2/2)	+(2/3)	+(2/3)	+(2/2)
15	R(+)Limonene	5989-27-5	69	1B	+(2/2)	-(0/4)	-(0/3)	+(2/2)	-(1/4)	-(0/3)	+(2/2)	-(0/4)	-(0/3)
16	Methylmethacrylate	80-62-6	90	1B	-(0/3)	-(0/4)	-(0/4)	-(0/3)	-(1/4)	-(1/4)	-(0/3)	-(0/4)	-(0/4)
17	Benzyl alcohol	100-51-6		NC	-(0/4)	-(0/3)	-(0/3)	-(1/4)	-(0/3)	-(0/3)	-(0/4)	-(0/3)	-(0/3)
18	Dimethyl isophthalate	1459-93-4		NC	-(0/3)	-(1/4)	-(1/4)	-(0/3)	+(2/4)	+(4/4)	-(0/3)	-(0/4)	-(1/4)
19	4-Aminobenzoic acid	150-13-0		NC	-(0/3)	-(0/3)	-(0/4)	-(0/3)	-(0/3)	+(2/4)	-(0/3)	-(0/3)	-(0/4)
20	Xylene	1330-20-7	95.8	NC	-(0/4)	-(0/3)	-(0/3)	+(2/4)	-(0/3)	-(0/3)	-(0/4)	-(0/3)	-(0/3)

G means that the experiment set was incomplete and did not obtain sufficient data to complete the judgment based on the Criterion used in Phase III because some chemicals were judged by different Criteria in Phase IIb and IIc.

9-7-4. Contingency tables for the Phase IIb, IIc, and III studies

Criterion 1

Lab A		IL-8 Luc assay		Total
		+	-	
LLNA	+	18	2	20
	-	1	8	9
Total		19	10	29

Sensitivity : 90.0% (18/20)
Specificity : 88.9% (8/9)
Accuracy : 89.7% (26/29)

Lab B		IL-8 Luc assay		Total
		+	-	
LLNA	+	16	4	20
	-	1	8	9
Total		17	12	29

Sensitivity : 80.0% (16/20)
Specificity : 88.9% (8/9)
Accuracy : 82.8% (24/29)

Lab C		IL-8 Luc assay		Total
		+	-	
LLNA	+	16	4	20
	-	1	8	9
Total		17	12	29

Sensitivity : 80.0% (16/20)
Specificity : 88.9% (8/9)
Accuracy : 82.8% (24/29)

Criterion 2

Lab A	IL-8 Luc assay		Total
	+	-	

LLNA	+	17	2	19
	-	3	6	9
Total		20	8	28

Sensitivity : 89.5% (17/19)
Specificity : 66.7% (6/9)
Accuracy : 82.1% (23/28)

Lab B		IL-8 Luc assay		Total
		+	-	
LLNA	+	15	4	19
	-	1	7	8
Total		16	11	27

Sensitivity : 78.9% (15/19)
Specificity : 87.5% (7/8)
Accuracy : 81.5% (22/27)

Lab C		IL-8 Luc assay		Total
		+	-	
LLNA	+	16	4	20
	-	3	6	9
Total		19	10	29

Sensitivity : 80.0% (16/20)
Specificity : 66.7% (6/9)
Accuracy : 75.9% (22/29)

Criterion 3

Lab A		IL-8 Luc assay		Total
		+	-	
LLNA	+	16	3	19
	-	0	9	9

Total	16	12	28

Sensitivity : 84.1% (16/19)
Specificity : 100.0% (9/9)
Accuracy : 89.3% (25/28)

Lab B		IL-8 Luc assay		Total
		+	-	
LLNA	+	15	4	19
	-	1	8	9
Total		16	12	28

Sensitivity : 78.9% (15/19)
Specificity : 88.9% (8/9)
Accuracy : 82.1% (23/28)

Lab C		IL-8 Luc assay		Total
		+	-	
LLNA	+	15	5	20
	-	1	8	9
Total		16	13	29

Sensitivity : 75.0% (15/20)
Specificity : 88.9% (8/9)
Accuracy : 79.3% (23/29)

9-7-5. Comments from the VMT members

Based on the combined results of the Phase IIb, IIc, and III studies, the 3 criteria all showed favorable results. In the within- and between-laboratory reproducibilities, only Criterion 3 satisfied more than 80% as acceptance criteria described in the study plan for the IL-8 Luc Assay. The lead laboratory (Tohoku University) asked for reconsideration of Criterion 3 because the data from the lead laboratory showed best values using Criterion 1. However, considering the poor within-laboratory reproducibility of Criterion 1, the VMT members finally accepted Criterion 3.

9-8. Phase IV study (for between- and within- laboratory reproducibility)

9-8-1. Test conditions

Adoption of the suggestions of the VMT members resulted in the final protocol of the IL-8 Luc assay accepting Criterion 3 as prediction model. In addition, we responded to a comment by the peer review panel that the data on within-laboratory reproducibility was more limited compared to other validation studies, particularly with respect to the number of chemicals tested. To address this, a Phase IV study was conducted to examine between- and within-laboratory reproducibilities of this assay. In this Phase IV study, X-VIVO^{TM} 15 rather than DMSO was used to dilute the chemicals because the peer review panel acknowledged that the modified dilution method improved the sensitivity of the IL-8 Luc assay. The dissolution of chemicals in X-VIVO^{TM} 15 is simpler and produces much smaller concentration variation during dilution than when using DMSO as a solvent. In addition, the lead laboratory confirmed that changing the solvent from DMSO to X-VIVO^{TM} 15 did not alter the judgment of most chemicals correctly evaluated by the IL-8 Luc assay using DMSO, as shown in 4-1-9.

The Phase IV study examined the within- and between-laboratory reproducibilities of a total of 5 coded chemicals (2 strong and 2 moderate sensitizers, and 1 non-sensitizer) by 3 experiment sets for each chemical based on IL-8 Luc assay protocol Ver. 020E.

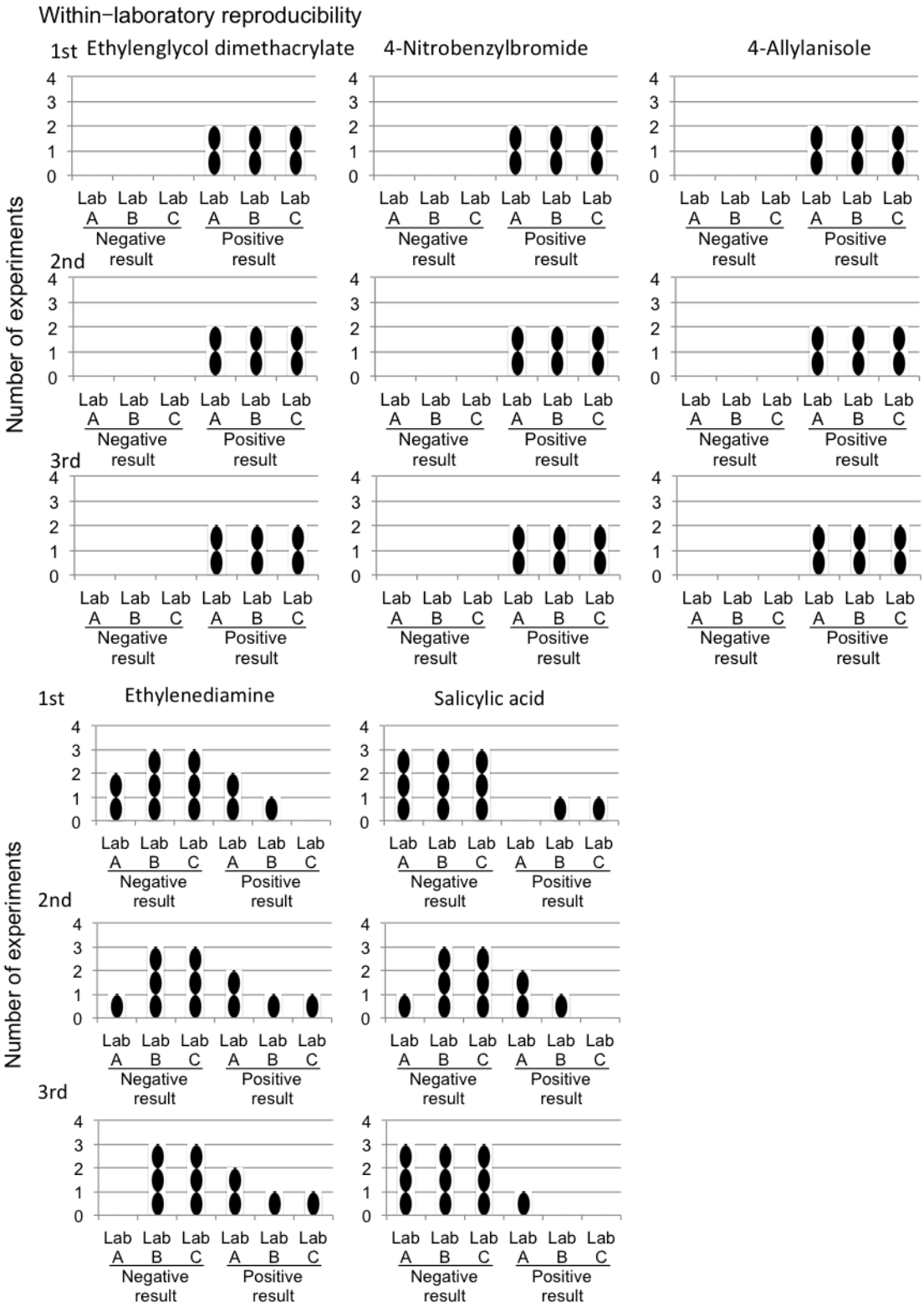
In Phase IV, the test was examined according to the following criterion 3.

Criterion 3.

In each experiment:

Criterion: chemicals that demonstrate FInSLO-LA $\geq$ 1.4 and the lower limit of the 95% confidence interval of FInSLO-LA $\geq$ 1.0 are judged as positive.

A graphical presentation of between- and within-laboratory variation in Phase IV study is shown in Fig. 16.


Within−laboratory reproducibility
1st
2nd
3rd
Ethylenglycol dimethacrylate
4-Nitrobenzylbromide
4-Allylanisole
Ethylenediamine
Salicylic acid
Number of experiments
0
1
2
3
4
Lab A
Lab B
Lab C
Negative result
Positive result

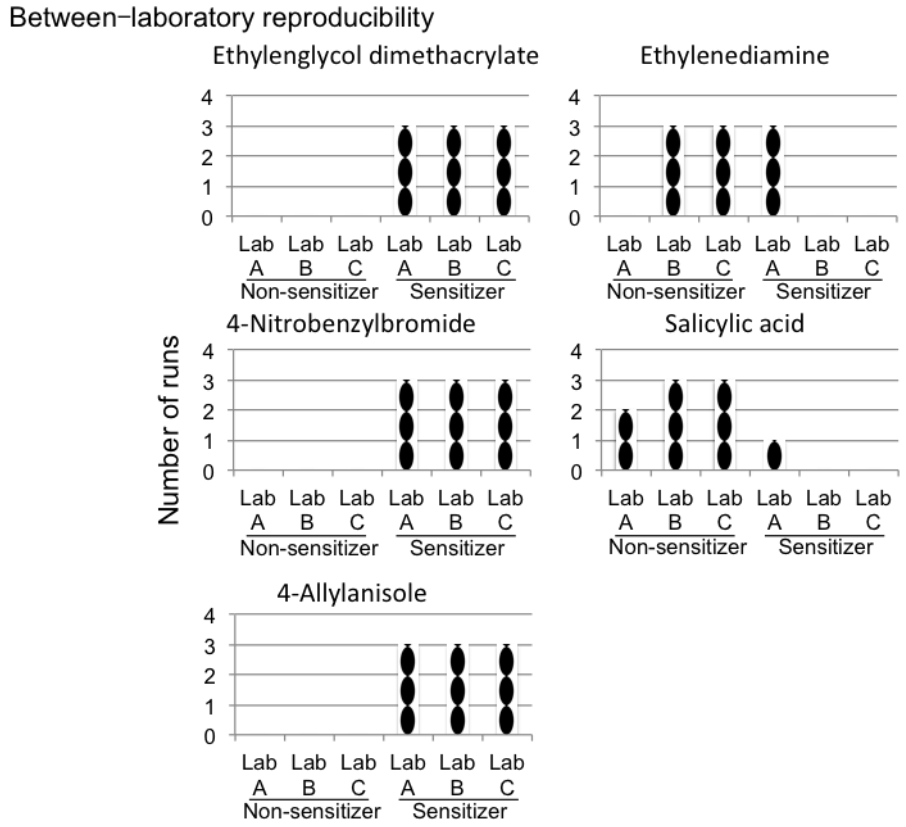


Fig. 16. Between- and within- laboratory variation assessments in Phase IV study

For the Phase IV study to examine within- and between-laboratory reproducibilities, a total of 5 coded chemicals (2 strong and 2 moderate sensitizer, and 1 non-sensitizer), were evaluated by 3 experiment sets for each chemical based on IL-8 Luc assay protocol Ver. 020E. Closed circles represent the judgments for individual experiments in within-laboratory reproducibility, while those represent the judgments in individual experimental sets for between-laboratory reproducibility.

9-8-2. Between- and within- laboratory variation assessments in the Phase IV study

Criterion 3
Between-Lab reproducibility 91.1% ((9+9+9+6+8)/45)
Within-Lab reproducibility Lab A. 80% (4/5)
Lab B. 100% (5/5)
Lab C. 100% (5/5)
Average 93.3%

Table 30. Results of the Phase IV study

No.	Chemical name	CAS No.	LLNA EC3	GHS Category	Criterion 1			Criterion 2			Criterion 3		
					Lab A	Lab B	Lab C	Lab A	Lab B	Lab C	Lab A	Lab B	Lab C
1	Ethyleneglycol dimethacrylate	97-90-5	28	1B	+(2/2)	+(3/4)	+(2/2)	+(2/2)	+(2/4)	+(2/2)	+(2/2)	+(2/4)	+(2/2)
					+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)
					+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)
2	4-Nitrobenzylbromide	100-11-8	0.05	1A	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)
					+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)
					+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)
3	4-Allylanisole	140-67-0	18	1B	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)
					+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)
					+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)	+(2/2)
4	Ethylenediamine	107-15-3	2.2	1B	+(3/4)	-(1/4)	-(0/3)	+(3/4)	+(2/4)	-(0/3)	+(2/4)	-(1/4)	-(0/3)
					+(2/3)	-(1/4)	-(1/4)	+(2/3)	+(4/4)	+(3/4)	+(2/3)	-(1/4)	-(1/4)
					+(2/2)	-(1/4)	-(1/4)	+(2/2)	+(3/4)	+(2/4)	+(2/2)	-(1/4)	-(1/4)
5	Salicylic acid	69-72-7		NC	-(0/3)	-(1/4)	-(1/4)	-(0/3)	-(1/4)	-(1/4)	-(0/3)	-(1/4)	-(1/4)
					+(2/3)	+(2/4)	-(0/3)	+(2/3)	+(2/4)	-(0/3)	+(2/3)	-(1/4)	-(0/3)
					-(1/4)	-(0/3)	-(0/3)	+(2/4)	-(0/3)	-(0/3)	-(1/4)	-(0/3)	-(0/3)

9-8-3. Predictivity in the Phase IV study

Criterion 3
Accuracy of Lab A 93.3% (14/15)
Accuracy of Lab B 80.0% (12/15)
Accuracy of Lab C 80.0% (12/15)

9-8-4. Contingency tables for the Phase IV results

Criterion 3

Lab A		IL-8 Luc assay		Total
		+	-	
LLNA	+	12	0	12
	-	1	2	3
Total		13	2	15

Sensitivity: 100% (12/12)
Specificity: 66.7% (2/3)
Accuracy: 93.3% (14/15)

Lab B		IL-8 Luc assay		Total
		+	-	
LLNA	+	9	3	12
	-	0	3	3
Total		9	6	15

Sensitivity: 75.0% (9/12)
Specificity: 100% (3/3)
Accuracy: 80.0% (12/15)

Lab C		IL-8 Luc assay		Total
		+	-	
LLNA	+	9	3	12
	-	0	3	3
Total		9	6	15

Sensitivity: 75.0% (9/12)
Specificity: 100% (3/3)
Accuracy: 80.0% (12/15)

9-8-5. Comments from the VMT members

The Phase IV study that examined between- and within-laboratory reproducibilities of the IL-8 Luc assay using Criterion 3 and X-VIVO^{TM} 15 as a solvent satisfied more than 80%, the acceptance

criteria set in the study plan for the IL-8 Luc Assay. In addition, the accuracy of the results of each laboratory was more than 80%. These results supported the submission of the validation report to the OECD.

9-9. Combined results of the Phase IIb, IIc, III, and IV studies (for between- and within- laboratory reproducibility and predictive capacity)

9-9-1. The rationale to combine the data obtained from the modified IL-8 Luc assay (Phase IV) with those from the original IL-8 Luc assay (Phase IIb, IIc, and III)

Before examining the combined results of the Phase IIb, IIc, III, and IV studies, the lead laboratory considered the comparability of the reproducibility of DMSO and X-VIVO assay variants. Among 122 chemicals examined by the IL-8 Luc assay, the modified IL-8 Luc assay made 3 false negative results among 63 sensitizers that were correctly evaluated by the original protocol and 4 false positive results among 25 non-sensitizers. The concordance rate between the original and modified IL-8 Luc assays was 92% for the chemicals that were correctly judged by the original IL-8 Luc assay (4-1-9). In addition, the procedure using X-VIVO 15 as a solvent is simpler and less complicated and produces much smaller concentration variation during dilution than that using DMSO as a solvent. These data indicate that the modified IL-8 Luc assay is comparable to the original IL-8 Luc assay.

9-9-2. Test conditions

The within- and between-laboratory reproducibilities, and the predictivity of the IL-8 Luc assay, were evaluated using all the results from Phases IIb, IIc, III, and IV studies.

9-9-3. Within- and between-laboratory variation assessments using Phase IIb, IIc, III and IV studies

Criterion 3
Between-Lab reproducibility 87.5% (28/32)
Within-Lab reproducibility Lab. A 85.7% (12/14)
Lab. B 91.7% (11/12)
Lab. C 85.7% (12/14)
Average 87.7%

9-9-4. Predictivity in the Phase IIb, IIc, III and IV studies

Criterion 3

Accuracy of Lab. A	90.9% (30/33)
Accuracy of Lab. B	81.8% (27/33)
Accuracy of Lab. C	79.4% (27/34)
Based on majority	82.4% (28/34)

The analysis of p-benzoquinone and 2,4-dichloronitrobenzene in IIb was not included in the data of Lab A.
The analysis of 2,4-dichloronitrobenzene in IIb and glyoxal solution in III was not

included in the data of Lab B.
The analysis of 2,4-dichloronitrobenzene in IIb was not included in the data of Lab C.

Table 31. Results of the Phase IIb, IIc, III, and IV studies

Phase	Chemicals	Repeat	No.	Chemical name	CAS No.	LLNA EC3	GHS Category	Lab.A Judge	Lab.B Judge	Lab.C Judge
IIb	5 coded	3 sets	1	p-Benzoquinone	106-51-4	0.099	1A	G(1/2) +(2/2) G(1/2)	G(1/2) +(2/2) +(2/2)	+(2/2) +(2/2) +(2/2)
			2	1-Thioglycerol	96-27-5	3.6	1B	+(4/4) +(2/3) +(3/3)	+(4/4) +(3/3) +(2/2)	+(4/4) +(3/3) +(3/3)
			3	Benzyl cinnamate	103-41-3	18.4	1B	+(4/4) +(3/3) +(4/4)	+(2/2) +(2/2) +(3/3)	+(4/4) +(3/3) +(3/3)
			4	Diethyl maleate	141-05-9	5.8	1B	-(0/3) -(0/3) -(0/3)	-(0/4) -(0/3) -(0/3)	-(0/3) -(0/4) -(1/4)
			5	2,4-Dichloronitrobenzene	611-06-3		NC	-(0/3) -(0/3) -(0/4)	-(0/3) G(0/2) G(1/3)	G(1/3) -(0/4) -(1/4)
IIc	5 coded	3 sets	1	1,4-Phenylenediamine	106-50-3	0.0099	1A	+(2/2) +(2/4) +(3/4)	+(2/4) -(1/4) +(2/2)	-(1/4) -(1/4) +(2/3)
			2	2-Mercaptobenzothiasole	149-30-4	1.7	1A	+(2/2) +(2/2) +(2/2)	G(1/3) +(2/2) +(2/2)	+(2/3) +(2/2) +(2/2)
			3	Glycerol	56-81-5		NC	-(0/3) -(1/4) -(1/4)	-(0/4) -(0/3) -(1/4)	-(0/4) -(0/3) -(0/4)
			4	Isopropanol	67-63-0		NC	-(1/4) -(0/3) -(0/3)	+(2/2) +(2/2) +(2/4)	-(1/4) +(2/4) +(2/4)
			5	Methyl salicylate	119-36-8		NC	-(0/3) +(2/4) -(0/3)	-(0/3) -(0/4) -(1/4)	-(0/3) -(0/4) -(0/3)

III	20 coded	1 set	1	2,4-Dichloronitrobenzene	611-06-3		NC	-(1/4)	-(0/3)	-(0/3)
			2	DNCB(1-Chloro-2,4-dinitrobenzene)	97-00-7	0.06	1A	+(2/2)	+(2/2)	+(2/2)
			3	Glyoxal solution	107-22-2	0.8	1A	+(2/2)	G(1/3)	+(2/2)
			4	1,2-Benzisothiazol-3(2H)-one	2634-33-5	8	1B	+(2/2)	+(2/2)	+(2/2)
			5	Citral	5392-40-5	5	1B	+(2/2)	+(2/2)	+(2/2)
			6	4-Hydroxybenzoic acid	99-96-7		NC	-(0/4)	-(0/3)	-(0/4)
			7	Beryllium sulphate	7787-56-6	0.001	1A	-(1/4)	-(0/3)	-(0/3)
			8	Formaldehyde	50-00-0	0.61	1A	+(2/3)	+(2/2)	+(2/3)
			9	Chlorpromazine HCl	69-09-0	0.14	1A	+(2/2)	+(2/2)	+(2/2)
			10	Chloramine T	127-65-1	0.4	1A	+(2/3)	+(2/2)	+(2/2)
			11	Benzyl salicylate	118-58-1	2.9	1B	+(2/2)	+(2/2)	+(2/3)
			12	Dihydroeugenol	2785-87-7	6.8	1B	+(2/3)	+(2/2)	+(2/2)
			13	Nickel chloride	7718-54-9		1B	+(2/2)	+(2/2)	+(2/2)
			14	Imidazolidinyl urea	39236-46-9	24	1B	+(2/3)	+(2/3)	+(2/2)
			15	R(+)Limonene	5989-27-5	69	1B	+(2/2)	-(0/4)	-(0/3)
			16	Methylmethacrylate	80-62-6	90	1B	-(0/3)	-(0/4)	-(0/4)
			17	Benzyl alcohol	100-51-6		NC	-(0/4)	-(0/3)	-(0/3)
			18	Dimethyl isophthalate	1459-93-4		NC	-(0/3)	-(0/4)	-(1/4)
			19	4-Aminobenzoic acid	150-13-0		NC	-(0/3)	-(0/3)	-(0/4)
			20	Xylene	1330-20-7	95.8	NC	-(0/4)	-(0/3)	-(0/3)
IV	5 coded	3 sets	1	Ethyleneglycol dimethacrylate	97-90-5	28	1B	+(2/2) +(2/2) +(2/2)	+(2/4) +(2/2) +(2/2)	+(2/2) +(2/2) +(2/2)
			2	4-Nitrobenzylbromide	100-11-8	0.05	1A	+(2/2) +(2/2) +(2/2)	+(2/2) +(2/2) +(2/2)	+(2/2) +(2/2) +(2/2)
			3	4-Allylanisole	140-67-0	18	1B	+(2/2) +(2/2) +(2/2)	+(2/2) +(2/2) +(2/2)	+(2/2) +(2/2) +(2/2)
			4	Ethylenediamine	107-15-3	2.2	1B	+(2/4) +(2/3) +(2/2)	-(1/4) -(1/4) -(1/4)	-(0/3) -(1/4) -(1/4)
			5	Salicylic acid	69-72-7		NC	-(0/3) +(2/3) -(1/4)	-(1/4) -(1/4) -(0/3)	-(1/4) -(0/3) -(0/3)

G means that the experiment set was incomplete and did not obtain sufficient data to complete the judgment based on the final criterion because some chemicals were judged by different Criteria in Phase IIb and IIc.

9-9-5. Contingency tables for the Phase IIb, IIc, III, IV studies

Criterion 3

Lab A		IL-8 Luc assay		Total
		+	-	
LLNA	+	20	3	23
	-	0	10	10
Total		20	13	33

Sensitivity: 87.0% (20/23)

Specificity: 100.0% (10/10)

Accuracy: 90.9% (30/33)

Lab B		IL-8 Luc assay		Total
		+	-	
LLNA	+	*18*	*5*	*23*
	-	1	9	10
Total		19	14	33

Sensitivity: 78.3% (18/23)
Specificity: 90.0% (9/10)
Accuracy: 81.8% (27/33)

Lab C		IL-8 Luc assay		Total
		+	-	
LLNA	+	18	6	24
	-	1	9	10
Total		19	15	34

Sensitivity: 75.0% (18/24)
Specificity: 90.0% (9/10)
Accuracy: 79.4% (27/34)

9-9-6. The summarized predictivity of the Phase IIb, IIc, III, and IV

Table 32. The summarized predictivity of the Phase IIb, IIc, III, and IV

Statistics	Based on Majority	A	B	C
Accuracy	**82.4% (28/34) [65.6%, 92.6%]**	90.9% (30/33) [74.9%, 98.4%]	81.8% (27/33) [64.6%, 92.4%]	79.4% (27/34) [62.0%, 90.8%]
Sensitivity	**79.2% (19/24) [59.5%, 90.8%]**	87.0% (20/23) [67.9%, 95.5%]	78.3% (18/23) [58.1%, 90.3%]	75.0% (18/24) [55.1%, 88.0%]
Specificity	**90.0% (9/10) [59.6%, 98.2%]**	100.0%(10/10) [72.2%, 100.0%]	90.0% (9/10) [59.6%, 98.2%]	90.0% (9/10) [59.6%, 98.2%]
False Negative Rate	**20.8% (5/24) [9.2%, 40.5%]**	13.0% (3/23) [4.5%, 32.1%]	21.7% (5/23) [9.7%, 41.9%]	25.0% (6/24) [12.0%, 44.9%]
False Positive Rate	**10.0% (1/10) [1.8%, 40.4%]**	0.0% (0/10) [0.0%, 27.8%]	10.0% (1/10) [1.8%, 40.4%]	10.0% (1/10) [1.8%, 40.4%]
Positive Predictive value	**95.0% (19/20) [76.4%, 99.1%]**	100.0%(20/20) [83.9%, 100.0%]	94.7% (18/19) [75.4%, 99.1%]	94.7% (18/19) [75.4%, 99.1%]
Nevative Predictive value	**64.3% (9/14) [38.8%, 83.7%]**	76.9% (10/13) [49.7%, 91.8%]	64.3% (9/14) [38.8%, 83.7%]	60.0% (9/15) [35.7%, 80.2%]

9-9-7. Comments from the VMT members

The modified IL-8 Luc assay satisfied the acceptable criteria for the validation study with a within-laboratory reproducibility of at least 85% and a between-laboratory reproducibility of at least 80%.

10. Discussion

10-1. Reliability

The IL-8 Luc assay is based on the induction of luciferase activity in the IL-8 reporter cell line, THP-G8. Therefore, it is crucial that THP-G8 cells maintain their ability to induce luciferase activity depending on various stimuli. Before and during this validation study, the response of THP-G8 cells to various haptens, LPS, and TNF-α were carefully observed. We confirmed that the frozen stock of THP-G8 cells can be cultured without losing activity for at least 15 weeks or 30 passages.

The culture of THP-G8 cells is relatively simple and does not use trypsin or EDTA because THP-G8 cells do not adhere to the culture dishes. First, chemicals at graded concentrations are added to the wells of a 96-well culture plate. Then cells adjusted to the optimum concentration are seeded into each well. After 16 h incubation, 100 µL of pre-warmed Tripluc is added to each of the 96 wells. The subsequent process is completely automated, except for calculating the results using the predesigned Excel spreadsheet. Therefore, the IL-8 Luc assay is a test method that can significantly reduce human error.

Moreover, the IL-8 Luc assay does not require the determination of cell viability after chemical treatment. THP-G8 cells can present IL-8 promoter activity as well as promoter activity of GAPDH, a well-known housekeeping gene; therefore, information regarding the effects of the chemical on both IL-8 induction and cell viability is obtained in each experiment. Furthermore, a single experiment requires only 20 h, including the time required for chemical preparation and cell plating, making the IL-8 Luc assay a true high-throughput method.

10-2. Between- and within-laboratory reproducibility

We changed the solvent used to dissolve the chemicals from DMSO/culture medium to X-VIVO™ 15 for the Phase IV study. Therefore, to confirm the reliability of the modified protocol and its equivalence to the original protocol, the lead laboratory produced an additional data set for 143 chemicals (Table S2) and demonstrated higher performance of the modified IL-8 Luc assay. Therefore, we demonstrated between- and within- laboratory reproducibility using the results of the Phase IV study alone and by combining the data from the Phase IIb, IIc, III and IV studies. The obtained results satisfied the acceptance criteria for the validation study with a within-laboratory reproducibility of at least 85% and a between-laboratory reproducibility of at least 80%.

10-3. Predictivity

The performance of the IL-8 Luc assay (Criterion 3) was significantly improved in the modified IL-8 Luc assay. Therefore, to compare the performance of the modified IL-8 Luc assay with that of other test methods, the lead laboratory obtained data for DPRA (Jaworska et al., 2013; Nukada et

al., 2013b), KeratinoSens (Urbisch et al., 2015) and h-CLAT (Ashikaga et al., 2010; Takenouchi et al., 2013; Nukada et al., 2013b) that overlapped with the 143 chemicals (Table 33). DPRA, KeratinoSens, and h-CLAT were used to examine 118, 122, and 134 chemicals, respectively. The results shown in Table 33 demonstrate that the performance of the modified IL-8 Luc assay is comparable with that of h-CLAT and better than that of DPRA or KeratinoSens.

Table 33. The performance of the IL-8 Luc assay and other test methods

Test methods	IL-8 Luc assay	Modified IL-8 Luc assay (X-VIVO)	DPRA	KratinoSens	h-CLAT
Chemical numbers	122	143	118	122	134
Accuracy	73%	80	74%	75%	80%
Sensitivity	72%	86	75%	76%	83%
Specificity	74%	64	70%	71%	70%

10-4. The factors responsible for false negative or positive results in the modified IL-8 Luc assay

10-4-1. Factors responsible for false negative or false positive results in the modified IL-8 Luc assay (1) – physical properties

The modified IL-8 Luc assay examined 143 chemicals in total and produced 15 false negative results for 107 sensitizers identified in the LLNA. To clarify the underlying reason for these false negative results, the lead laboratory first compared 3 physical properties of haptens judged by the IL-8 Luc assay to be sensitizers (correct positive) and those judged to be non-sensitizers (false negative): molecular weight, Log $K_{o/w}$, and water solubility. The results did not demonstrate statistically significant differences in these properties between chemicals judged as false negative and those as correct positive (Fig. 17). Indeed, the modified IL-8 Luc assay correctly evaluated the chemicals with a Log $K_{o/w}$ more than 8 and those with a water solubility of around 0.1 µg /mL.

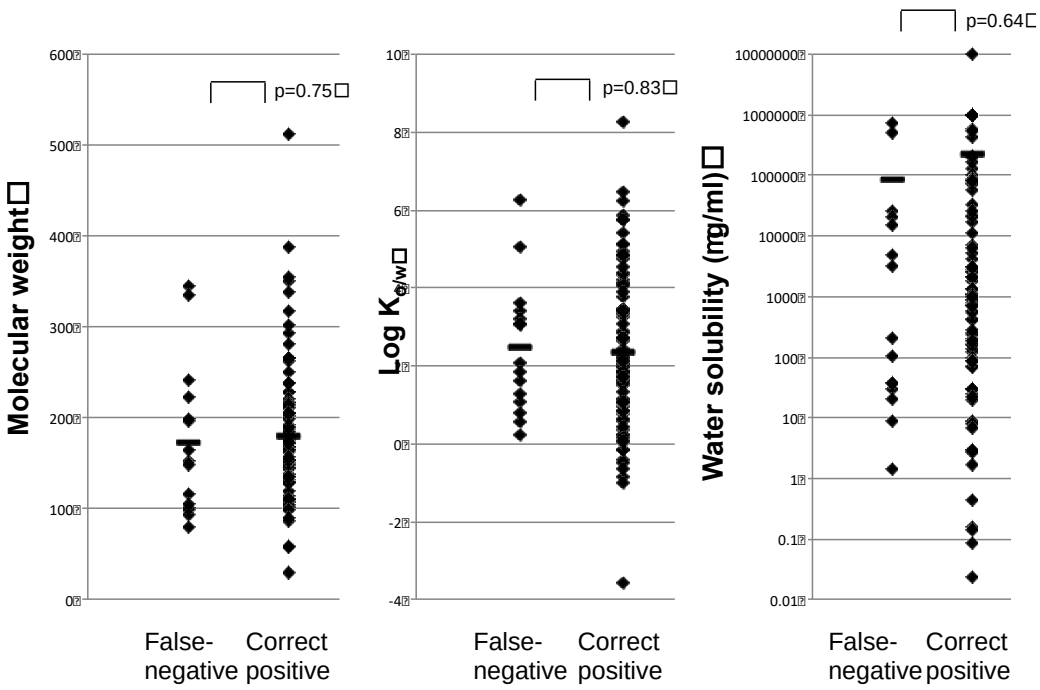


Fig. 17. Factors responsible for false negative results in the IL-8 Luc assay (chemical properties)

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 $K_{o/w}$, 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.

When we examined the water solubility and Min-I.I.-SLR-LA among sensitizers and non-sensitizers, however, 5 sensitizers judged as negative showed a water solubility of less than 100 µg/mL and a Min-I.I-SLR-LA of more than 0.8. Since most sensitizers are generally cytotoxic (i.e., have a low I.I.-SLR-LA) at concentrations above that required to significantly increase Fin-SLO-LA, a Min-I.I-SLR-LA of more than 0.8 suggested that these chemicals did not dissolve using our procedure, as supported by a water solubility of less than 100 µg/mL. These data suggest that the chemicals that satisfy this condition can be haptens that are insoluble in X-VIVO™ 15. Theoretically, non-cytotoxic non-sensitizers that are soluble in X-VIVO™ 15 or cytotoxic non-sensitizers that are not dissolved in X-VIVO™ 15 can satisfy this condition. In addition, there were some chemicals that could be judged as sensitizers by the modified IL-8 Luc assay in spite of satisfying this condition. Therefore, to prevent false negative judgments, we consider that chemicals that demonstrate a water solubility of less than 100 µg/mL and a Min-I.I-SLR-LA of more than 0.8 should not be tested using the IL-8 Luc assay.

10-4-2. Factors responsible for false negative or false positive results in the modified IL-8 Luc assay (2) – detergents

We examined 5 detergents using the IL-8 Luc assay and the modified IL-8 Luc assays: hexadecyltrimethylammonium bromide, benzalkonium chloride, octanoic acid, Tween-80 and SLS. These detergents were found to significantly increase FInSLO-LA, which suggested that they could stimulate IL-8 promoter activity. These data are consistent with previous reports that demonstrated IL-8 production by keratinocytes after stimulation with various detergents (Coquette et al., 1999; White et al., 2011). Detergents can be categorized according to the charge present on 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, Tween-80 is nonionic and SLS and octanoic acid are anionic. Therefore, our data suggested that detergents could stimulate IL-8 promoter activity irrespective of the charges present in the hydrophilic head. It is unclear how detergents induce IL-8 mRNA, although it has been reported that SLS induces IL-8 mRNA, depending on the activation of MEK1/p44/42 ERK (White et al., 2011). This is in contrast to haptens that induce IL-8 mRNA expression in DCs or THP-1 cells, depending on p38 MAPK. Therefore, we decided to include detergents irrespective of the charges present in the hydrophilic head as an exclusion criterion.

10-4-3. Factors responsible for false negative or false positive results in the modified IL-8 Luc assay (3) – relative human skin sensitizing potency

Recently, Basketter and colleagues (Basketter et al., 2014) collected data on the sensitizing potential of chemicals to human skin and classified 131 chemicals into 6 categories based on their relative human skin 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, ethyl vanillin, benzalkonium chloride, isopropanol, methyl salicylate, propyl paraben, p-aminobenzoic acid and propylene glycol are classified as non-sensitizers by LLNA, the relative human skin sensitizing potency included them in category 5, the least potent sensitizers. The IL-8 Luc assay or the modified IL-8 Lu assay also judged vanillin, ethyl vanillin, benzalkonium chloride, and propyl paraben as sensitizers. On the other hand, xylene, judged as a sensitizer by LLNA, is classified by the findings of Basketter and colleagues as a category 6, human non-sensitizers.

10-5. Performance of the modified IL-8 Luc assay after considering the exclusion criteria and human sensitization potential

If we delete the data for hexadecyltrimethylammonium bromide, Tween-80, octanoic acid, SLS and benzalkonium chloride, and consider vanillin, ethyl vanillin, isopropanol, methyl salicylate, p-aminobenzoic acid, propyl paraben and propylene glycol as weak sensitizers (category 5) and xylene as a non-sensitizer, the performance of the modified IL-8 Luc provided an accuracy of 83%, a sensitivity of 84%, and a specificity of 80% for 138 chemicals, while the performance of the original IL-8 Luc assay (Criterion 3) provided an accuracy of 73%, a sensitivity of 69%, and a specificity of 87% for 117 chemicals. We compared the performance of the modified IL-8 Luc assay with that of other test methods for the same chemicals. The results demonstrated that the modified IL-8 Luc assay was better than the other assays (Table 34).

Table 34. The performance of the modified IL-8 Luc assay after deleting the data for detergents and considering human sensitizing potential

Test methods	IL-8 Luc assay	Modified IL-8 Luc assay (X-VIVO)	DPRA	KratinoSens	h-CLAT
Chemical numbers	117	138	115	118	129
Accuracy	73%	83%	72%	74%	77%
Sensitivity	69%	84%	72%	74%	79%
Specificity	87%	80%	72%	73%	64%

Deleting the data for detergents and considering human sensitizing potential

10-6. Known limitations and drawback of the modified IL-8 Luc assay

The IL-8 Luc assay addresses only key event 3 in the skin sensitization AOP, so information obtained by the IL-8 Luc assay is unlikely to be sufficient when used on its own to determine the skin sensitization potentials of chemicals. Therefore, data generated with the present Test Guideline should be considered in the context of integrated approaches, such as IATA, and combine the data with other complementary information (e.g., derived from *in vitro* assays addressing other key events of the skin sensitization AOP, as well as non-testing methods such as read-across from chemical analogues). Examples on how to use the IL-8 Luc assay in combination with other information were reported previously (Kimura et al., 2015) and are described in detail in Sections 10-10, 10-12, and 10-13.

The test method described in this Test Guideline can be used to help discriminate between skin sensitizers (i.e., UN GHS Category 1) and non-sensitizers in the context of the IATA. This Test Guideline cannot be used on its own, neither to sub-categorize skin sensitizers into subcategories 1A and 1B as defined by the UN GHS, nor to predict potency for safety assessment decisions. However, depending on the regulatory framework, a positive result may be used on its own to classify a chemical into UN GHS category 1. Similar to other test methods, the IL-8 Luc assay is more likely to under-predict chemicals showing a low to moderate skin sensitization potency (i.e., UN GHS subcategory 1B) than chemicals showing a high skin sensitization potency (i.e., UN GHS subcategory 1A) (Kimura et al., 2015).

As described in Section 10-4-2, the major limitation of the IL-8 Luc assay is that this assay judges detergents as sensitizers irrespective of their classification. Therefore, detergents should be included in an exclusion criterion. On the other hand, the modified IL-8 Luc assay correctly evaluated the chemicals with a Log $K_{o/w}$ of greater than 8 and those with a water solubility of around 0.1 µg/mL (Fig. 17). When we examined water solubility and Min-I.I.-SLR-LA among sensitizers or non-sensitizers, however, 5 sensitizers judged as negative showed a water solubility of less than 100 µg/mL and a Min-I.I-SLR-LA of more than 0.8 (Fig. 18). Although some chemicals that satisfied this condition could be judged as sensitizers by the modified IL-8 Luc assay, most sensitizers are generally cytotoxic, and thus decrease I.I.-SLR-LA, at a concentration above that at which they significantly increase Fin-SLO-LA. Therefore, we consider that if a chemical has a water solubility of less than 100 µg/mL and a Min-I.I-SLR-LA of more than 0.8, it may not dissolve in the solvent and the data should not be used in the assessment. Theoretically, these conditions can occur for non-cytotoxic non-sensitizers with a water solubility less than 100 µg/mL, or for cytotoxic non-sensitizers that do not dissolve using our procedure. However, none of the 36 non-sensitizers we examined in this study satisfied this criterion.

Of the 3 anhydrides examined by the modified IL-8 Luc assay (phthalic anhydride, trimellitic anhydride, and maleic anhydride), only trimellitic anhydride was judged as a sensitizer. There is a

large body of evidence on differential cytokine induction in the lymph nodes by either respiratory sensitizers such as anhydrides, including phthalic anhydride, trimellitic anhydride, maleic anhydride, hexahydrophthalic anhydride and methyltetrahydrophthalic anhydride, or skin sensitizers such as DNCB (Dearman et al., 2000; Van Och et al., 2002). As a general conclusion, the respiratory sensitizers (most of which are also skin sensitizers) preferentially induced Th2 cytokines such as IL-4 and IL-10, whereas the exclusive skin sensitizers predominantly induced Th1 cytokines such as IFN-γ. The possible reason for this observation, called ‘‘cytokine polarization’’, is that anhydrides such as trimellitic anhydride and phthalic anhydride exclusively react with lysine residues (Gerberick et al., 2007). Therefore, the IL-8 Luc assay can provide only limited information for anhydrides.

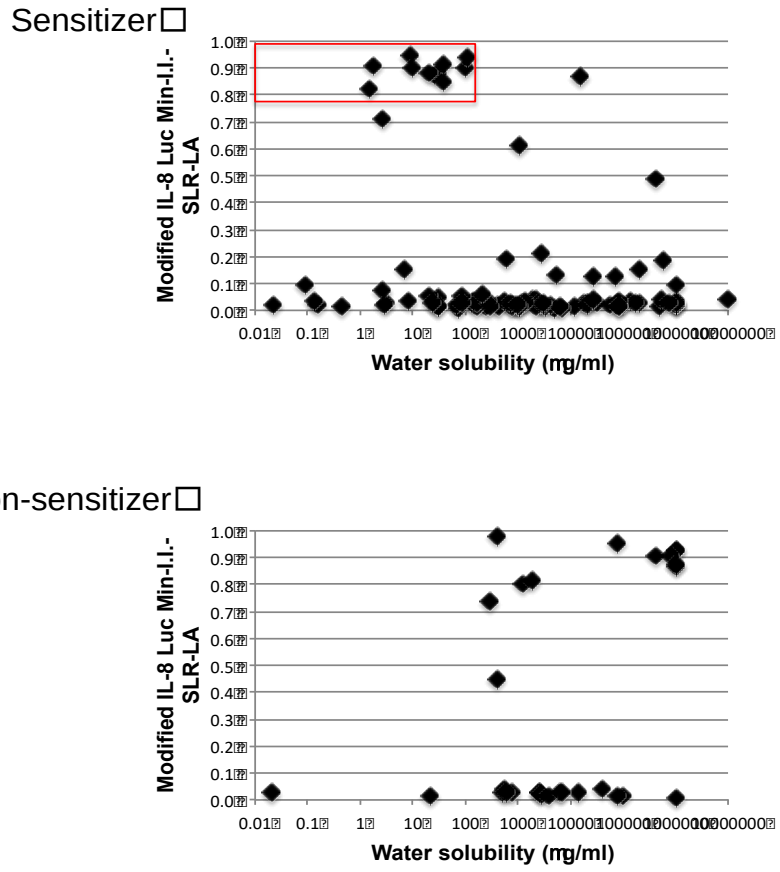


Fig. 18. Correlation between water solubility and Min-I.I.-SLR-LA in sensitizers or non-sensitizers

Water solubility was plotted to Min-I.I.-SLR-LA for every chemical examined by the modified IL-8 Luc assay. The red rectangle surrounds those chemicals with a water solubility less than 100μg /mL and Min-I.I.-FIn-SLR-LA of more than 0.8.

As described in the test guideline of KeratinoSens, chemicals that interfere with the luciferase enzyme can confound the activity of luciferase in cell-based assays causing either apparent inhibition or increased luminescence (Thorne et al., 2010). For example, phytoestrogen concentrations higher than 1μM were reported to interfere with the luminescence signals in other luciferase-based reporter gene assays due to over-activation of the luciferase reporter gene (OECD, 2012). As a consequence, luciferase expression obtained at high concentrations of phytoestrogens or similar compounds suspected of producing phytoestrogen-like over-activation of the luciferase reporter gene must be examined carefully (OECD, 2012).

Finally, the solution of very colorful chemicals that potentially interfere with luciferase measurement are not suited to assessment using the modified IL-8 Luc assay.

10-7. Applicability domain of the modified IL-8 Luc assay

As mentioned, the IL-8 Luc assay cannot evaluate the skin sensitization potential of detergents. Therefore, detergents, irrespective of their classification, fall outside the applicability domain. On the other hand, the IL-8 Luc assay can evaluate a broad range of chemicals other than detergents, even if they are pre/prohaptens or poorly water soluble. However, since it is believed that the metabolic activity of THP-G8 cells is insufficient to metabolize prohaptens, negative results for prohaptens should be interpreted with caution.

As mentioned in the limitation, the negative judgment for chemicals with a water solubility of less than 100 µg/mL and a Min –I.I.-FInSLA-LA of more than 0.8 should be avoided. As indicated in the test guideline for KeratinoSens, the judgment of an anhydride as a non-sensitizer cannot be accepted.

Among 143 chemicals evaluated by the lead laboratory using the modified IL-8 Luc assay, 15 sensitizers were classified as non-sensitizers and 13 non-sensitizers were classified as sensitizers (Table 35). As previously mentioned in the limitation, the modified IL-8 Luc assay cannot provide conclusive information for anhydrides, if they are judged as non-sensitizers by the modified IL-8 Luc assay. Therefore, the negative judgment for maleic anhydride and phthalic anhydride should be omitted. Since benzoyl peroxide, hexyl salicylate, clotrimazole, 1-bromohexane and phenyl benzoate satisfy the condition of water solubility of less than 100 µg/mL and a Min –I.I.-FInSLA-LA of more than 0.8, the judgment should be avoided. One strong sensitizer, 2-nitro-1,4-phenylenediamine, is a pre/prohapten. In general, although the modified IL-8 Luc assay can correctly judge pre/prohaptens, it might be an exception. Benzocaine is yellow and the color may interfere with the luciferase measurement. Xylene is a human non-sensitizer. The reasons for the false judgment of one moderate sensitizer, 2-hydroxyethyl acrylate, and the three weak sensitizers aniline, penicillin G, and pyridine, are not clear.

On the other hand, of the 13 non-sensitizers, 5 were detergents of which 4 have relative human sensitizing potency. We consider detergents as being outside the applicability domain.

Table 35. Chemicals judged as false negative or positive by the modified IL-8 Luc assay

Chemical	Potency category	The modified IL-8 Luc assay	Discussion
2-Nitro-1,4-phenylenediamine	Strong	non-sensitizer	Pre/Prohapten
Benzoyl peroxide	Strong	non-sensitizer	Possibly due to insolubility
Hexyl salicylate	Strong	non-sensitizer	Possibly due to insolubility
Maleic anhydride	Strong	non-sensitizer	Reaction with lysine
Phthalic anhydride	Strong	non-sensitizer	Reaction with lysine
2-Hydroxyethyl acrylate	Moderate	non-sensitizer	
Clotrimazole	Moderate	non-senitizer	Possibly due to insolubility
1-Bromohexane	Weak	non-sensitizer	Possibly due to insolubility
Aniline	Weak	non-sensitizer	
Benzocaine	Weak	non-sensitizer	Interference with luciferase measurement due to its color
Methylmethacrylate	Weak	non-sensitizer	
Penicillin G	Weak	non-sensitizer	
Phenyl benzoate	Weak	non-sensitizer	Possibly due to insolubility
Pyridine	Weak	non-sensitizer	
Xylene	Weak	non-sensitizer	Human non-sensitizer
2-Acetylcyclohexanone	non-sensitizer	sensitizer	
Benzalkonium chloride	non-sensitizer	sensitizer	Detergent, Human sensitizer
Benzyl alcohol	non-sensitizer	sensitizer	
Diethyl phthalate	Non-sensitizer	sensitizer	
Ethyl vaniline	non-sensitizer	sensitizer	Human sensitizer
Hexadecyltrimethylammonium bromide	non-sensitizer	sensitizer	Detergent
Octanoic acid (Caprylic acid)	Non-sensitizer	sensitizer	Detergent
Propyl paraben	Non-sensitizer	sensitizer	Human sensitizer
Saccharin	Non-sensitizer	sensitizer	
Sodium lauryl sulfate	Non-sensitizer	sensitizer	Detergent
Tween-80	non-sensitizer	sensitizer	Detergent
Vanillin	non-sensitizer	sensitizer	Human sensitizer
Zinc sulphate	non-sensitizer	sensitizer	

10-8. Effects of different lots or sources of FBS on the assay

We used a single lot (Lot 715004) of FBS from Biological Industries throughout the validation study. To examine the effects of FBS from different sources or different lots of FBS from the same source on the IL-8 Luc assay, we evaluated 2 lots of FBS from Biological Industries (515269 and 715004), two from Gibco (1088282 and 1085615), and a single lot each from Sigma, Hyclone, and Bovogen. THP-G8 cells were cultured in culture medium containing 10% of one of these FBSs for 4 weeks and the response of the THP-G8 cells to LPS, 4-NBB, 2-MBT, DNCB, cinnamal and $CoCl_2$ was examined. Although there were some differences in their reactivity to these chemicals, THP-G8 cells correctly responded to LPS and these 5 chemicals in the culture medium containing any of examined FCSs, except for the response to 2-MBT by THP-G8 cells cultured with FBSs from Biological Industries (515269) and Sigma (11H481) in Table 36. Based on these data, we consider that FBS from most companies can be used in the IL-8 Luc assay, although it is necessary to test the FBS by examining assay results obtained using appropriate proficiency chemicals before using. Table 36. The effects of different sources or lots of FBS on the IL-8 Luc assay (FlnSLO-LA/I.I)

☐	Biological Industies Cat. 04-001-1E Lot: 515269	Sigma Lot.No. S. 11H481	Hyclone	Gibco lot 1088282	Biological Industies Cat. 04-001-1E Lot: 715004	Gibco lot 1085615	Bovogen
LPS	10.31/0.409	11.45/0.459	13.42/0.656	9.79/0.611	8.96/0.498	9.33/0.617	10.35/0.673
4-NBB	4.37/0.233	4.60/0.224	3.93/0.239	2.99/0.402	5.53/0.181	6.44/0.161	7.13/0.141
2-MBT	1.10/0.866	1.33/0.724	2.08/0.569	1.71/0.583	2.46/0.540	1.95/0.537	2.89/0.496
DNCB	2.91/0.355	2.75/0.393	3.90/0.262	2.20/0.480	4.95/0.186	5.71/0.181	6.95/0.168
CoCl2	2.43/0.444	2.61/0.405	3.34/0.357	2.85/0.362	2.34/0.436	2.23/0.474	2.19/0.464
Cinnamal	3.25/0.302	1.67/0.581	2.10/0.461	1.88/0.593	2.16/0.527	2.37/0.437	2.64/0.403
☐	☐	☐	☐	☐	☐	☐	☐
☐	Good☐sponse☐						
☐	Poor ☐sponse☐						

10-9. The advantages of the modified IL-8 Luc assay

There are several advantages to the modified IL-8 Luc assay:

1. The modified IL-8 Luc assay produced good performance, comparable to or better than other test methods.
2. The culture of THP-G8 cells is relatively simple and does not require trypsin or EDTA as THP-G8 cells grow in suspension.
3. The procedure is simple. In the modified IL-8 Luc assay, chemicals at graded concentrations are added to a 96-well culture plate. Then, cells adjusted to the optimum concentration are seeded into each well. After 16 h incubation, 100 μL of pre-warmed Tripluc is added to each of the 96 wells. The process afterwards is completely automated, except for calculation of the results using the predesigned Excel-based data sheet. Therefore, the modified IL-8 Luc assay is considered as a test method that can significantly reduce human error.
4. In the modified IL-8 Luc assay, X-VIVO^{TM} 15 is used instead of DMSO. After dissolving the chemicals, the sample is centrifuged and the supernatant is used. Therefore, this procedure can avoid variations in the concentration of chemicals added to the wells caused by the addition of undissolved chemicals.
5. The modified IL-8 Luc assay does not require a pre-culture step or the determination of cell viability after chemical treatment. In the modified IL-8 Luc assay, since THP-G8 cells present the promoter activities of both IL-8 and GAPDH promoters, a well-known housekeeping gene, information on the effects of the 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 modified IL-8 Luc assay is a true high-throughput method.
6. The modified IL-8 Luc assay can easily detect chemicals that do not dissolve in the culture medium by using the criterion of a water solubility of the chemical of less than 100 μg/mL and a Min I.I.-FInSLR-LA of more than 0.8. Therefore, the modified IL-8 Luc assay can avoid false negative judgment for the chemicals that are not dissolved in the medium.
7. The modified IL-8 Luc assay is based on a luciferase assay. Therefore, this method can be easily introduced to laboratories that have already conducted a different luciferase assay based-test method, e.g. KeratinoSens.
8. The results obtained by the modified IL-8 Luc assay did not show significant correlation with other test methods, which suggests that the modified IL-8 Luc assay can provide information that cannot be obtained by other test methods. Therefore, when considering integrated approaches, the modified IL-8 Luc assay can be a good partner assay.

11. Conclusion

In the validation study, the results satisfied a within-laboratory reproducibility of at least 85% and between-laboratory reproducibility of at least 80% as acceptance criteria set in the study plan for the IL-8 Luc Assay. Having reached the set criteria, the validation study is concluded.

The IL-8 Luc Assay evaluates dendritic cell activation (Step 3 in the AOP) using an approach different from h-CLAT. There are several advantages of the IL-8 Luc assay over other *in vitro* test methods such the simple procedure, short assay time, automated measurement, the fact that it is a luciferase based assay (Fig. 9) and the fact that the data obtained are independent from those obtained using other test methods. Moreover, even though 4 experiments are required, one set of experiments can be completed within 4 days because neither pre-culture nor viability testing is required. The IL-8 Luc assay is a non-animal high through-put alternative method for screening sensitizers.

Acknowledgement

This validation study was supported by the Grants-in-Aid for the **Ministry of Economy, Trade and Industry (**METI), the Ministry of Health, Labour and Welfare (MHLW) and the Japanese Society for Alternatives to Animal Experiments (JSAAE). We gratefully acknowledge the voluntary works by the participated laboratories and the European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM), the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Korean Center for the Validation of Alternative Methods (KoCVAM) in the validation study.

12. References

Aiba, S., 2007. Dendritic cells: importance in allergy. Allergol Int 56, 201-208.

Aiba, S., Katz, S.I., 1990. Phenotypic and functional characteristics of *in vivo*-activated Langerhans cells. J Immunol 145, 2791-2796.

Aiba, S., Tagami, H., 1999. Dendritic cells play a crucial role in innate immunity to simple chemicals. J Investig Dermatol Symp Proc 4, 158-163.

Aiba, S., Terunuma, A., Manome, H., Tagami, H., 1997. Dendritic cells differently respond to haptens and irritants by their production of cytokines and expression of co-stimulatory molecules. Eur J Immunol 27, 3031-3038.

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. Toxicology In Vitro 20, 767-773.

Ashikaga T., Sakaguchi, H. Sano 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.

Barber, R.D., Harmer, D.W., Coleman, R.A., Clark, B.J., 2005. GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. Physiol Genomics 21, 389-395.

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., Fabian, E., Pachel, C., Ramirez, T., Wiench, B., Wruck, C.J., van Ravenzwaay, B., Landsiedel, R., 2011. Intralaboratory validation of four *in vitro* assays for the prediction of the skin sensitizing potential of chemicals. Toxicol In Vitro 25, 1162-1168.

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. Alternatives to laboratory animals : ATLA 37, 305-312.

Caux, C., Massacrier, C., Vanbervliet, B., Dubois, B., Van Kooten, C., Durand, I., Banchereau, J., 1994. Activation of human dendritic cells through CD40 cross-linking. J Exp Med 180, 1263-1272.

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.

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.

Enk, A.H., Angeloni, V.L., Udey, M.C., Katz, S.I., 1993. An essential role for Langerhans cell-derived IL-1 beta in the initiation of primary immune responses in skin. JI 150, 3698-3704.

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. Toxicological sciences : an official journal of the Society of Toxicology 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.
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 sensitiaiton potency: from theory to practice. J Appl Toxicol 33, 1353-1364.
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. JI 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.
Michelini, E., Cevenini, L., Calabretta, M.M., Calabria, D., Roda, A., 2014. 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 406, 5531-5539.
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.
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.
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., Ikeda, M., Kimura, T., Honma, S., Ohmiya, Y., Honma, K., 2004. Bidirectional role of orphan nuclear receptor RORalpha in clock gene transcriptions demonstrated by a novel reporter assay system. FEBS Lett 565, 122-126.
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.
Nakajima, Y., Ohmiya, Y., 2010. Bioluminescence assays: multicolor luciferase assay, secreted luciferase assay and imaging luciferase assay. Expert Opin Drug Discov 5, 835-849.

Natsch, A., Ryan, C.A., Foertsch, L., Emter, R., Jaworska, J., Gerberick, F., Kern, P., 2013. A dataset on 145 chemicals tested in alternative assays for skin sensitization undergoing prevalidation. J Appl Toxicol 33, 1337-1352.

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.

Niwa, K., Ichino, Y., Kumata, S., Nakajima, Y., Hiraishi, Y., Kato, D., Viviani, V.R., Ohmiya, Y., 2010. Quantum yields and kinetics of the firefly bioluminescence reaction of beetle luciferases. Photochem Photobiol 86, 1046-1049.

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., 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. Toxicology *in vitro* : an international journal published in association with BIBRA 27, 609-618.

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.

Oliveira, J.G., Prados, R.Z., Guedes, A.C., Ferreira, P.C., Kroon, E.G., 1999. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase is inappropriate as internal control in comparative studies between skin tissue and cultured skin fibroblasts using Northern blot analysis. Arch Dermatol Res 291, 659-661.

Peiser, M., Tralau, T., Heidler, J., Api, A.M., Arts, J.H., Basketter, D.A., English, J., Diepgen, T.L., Fuhlbrigge, R.C., Gaspari, A.A., Johansen, J.D., Karlberg, A.T., Kimber, I., Lepoittevin, J.P., Liebsch, M., Maibach, H.I., Martin, S.F., Merk, H.F., Platzek, T., Rustemeyer, T., Schnuch, A., Vandebriel, R.J., White, I.R., Luch, A., 2012. Allergic contact dermatitis: epidemiology, molecular mechanisms, *in vitro* methods and regulatory aspects. Current knowledge assembled at an international workshop at BfR, Germany. Cell Mol Life Sci 69, 763-781.

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.

Roda, A., Pasini, P., Mirasoli, M., Michelini, E., Guardigli, M., 2004. Biotechnological applications of bioluminescence and chemiluminescence. Trends Biotechnol 22, 295-303.

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.

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. Toxicology *in vitro* : an international journal published in association with BIBRA 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. JI 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.

Steinman, R.M., 1991. The dendritic cell system and its role in immunogenicity. Annu Rev Immunol 9, 271-296.

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., Siato K., Ashikaa T., Sakaguchi H. 2013. Predictive performance of the human Cell Line Activation Test (h-CLAT) for lipophilic chemicals with high octanol-water partition coefficients. 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.

Thellin, O., Zorzi, W., Lakaye, B., De Borman, B., Coumans, B., Hennen, G., Grisar, T., Igout, A., Heinen, E., 1999. Housekeeping genes as internal standards: use and limits. J Biotechnol 75, 291-295.

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.

Viviani, V., Uchida, A., Suenaga, N., Ryufuku, M., Ohmiya, Y., 2001. Thr226 is a key residue for bioluminescence spectra determination in beetle luciferases. Biochem Biophys Res Commun 280, 1286-1291.

Viviani, V.R., Bechara, E.J., Ohmiya, Y., 1999. Cloning, sequence analysis, and expression of active Phrixothrix railroad-worms luciferases: relationship between bioluminescence spectra and primary structures. Biochemistry 38, 8271-8279.

Weber, F.C., Nemeth, T., Csepregi, J.Z., Dudeck, A., Roers, A., Ozsvari, B., Oswald, E., Puskas, L.G., Jakob, T., Mocsai, A., Martin, S.F., 2015. Neutrophils are required for both the sensitization and elicitation phase of contact hypersensitivity. J Exp Med 212, 15-22.

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.

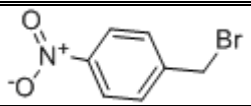
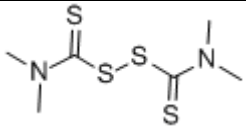
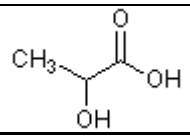
13. List of abbreviations.

4-NBB : 4-Nitrobenzylbromide
95% CI : the 95% confidence interval
AIST : National Institute of Advanced Industrial Science and Technology
AOP : Adverse outcome pathway
ARE: Antioxidant response element
CAS No. : Chemical Abstract Service Number
CMV : Cytomegalovirus
CSC : the Chemical Selection Committee
DC : Dendritic Cell
DMSO : Dimethyl sulphoxide
DNCB : 2,4-Dinitrochlorobenzene
DPRA : the Direct Peptide Reactivity Assay
EC1.5 value : Concentration for a statistically significant induction of 50% above background in the KeratinoSens assay
EC150 : Estimated concentration of RFI = 150 for CD86 in the h-CLAT
EC200 : Estimated concentration of RFI = 200 for CD54 in the h-CLAT
ECVAM : the European Centre for Validation of Alternative Methods
EDTA : Ethylenediaminetetraacetic acid
EGFR : Epidermal growth factor receptor
EGR-1 : Early growth response-1
EU : European Union
FBS : Fetal bovine serum
FInSLO-LA : Fold Induction of SLO-LA
FN : False Negative Rate
GLP : Good laboratory Practice
GSH : Glutathione
h-CLAT : human Cell Line Activation Test
HO-1 : Heme oxygenase-1
HRI/FDSC : Hatano Research Institute, Food & Drug Safety Center
HSV : Herpes simplex viruses
ICCVAM : Interagency Coordinating Committee on the Validation of Alternative Methods
ID : Identification
I.I. : Inhibition index
I.I.-SLR-LA : Inhibition index of SLR-LA
IL-8 : Interleukin-8
JaCVAM : the Japanese Center for the Validation of Alternative Methods
Keap-1 : Kelch-like ECH-associated protein 1
KoCVAM : Korean Center for the Validation of Alternative Methods
LLNA : Local lymph node assay
LPS : Lipopolysaccharide
MAX FInSLO-LA : the maximum value of FInSLO-LA
MDGN : Methylisothiazolinone
MIT : Minimum induction threshold

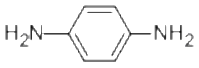
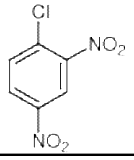
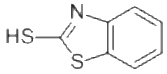
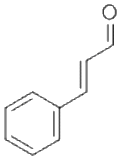
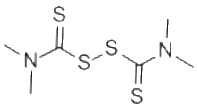
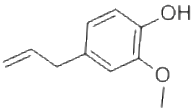
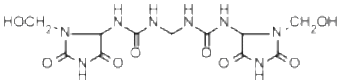
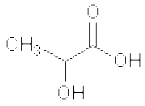
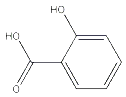
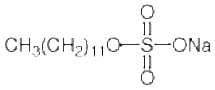
mMUSST : modified myeloid U937 dendritic cell activation test
MoDCs : Monocyte-derived dendritic cells
MOVS: Management Office of Validation Study
mRNA : messenger ribonucleic acid
MSDS : Material safety data sheet
NAC : N-acetyl-cysteine
NICEATM : the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NIHS : National Institute of Health Sciences
NPV : Negative predictive value
Nqo1 : NADPH-quinone oxidoreductase 1
Nrf2 : Nuclear factor (erythroid-derived 2)-like factor 2
nSLO-LA : normalized SLO luciferase activity
OECD : the Organization for Economic Co-operation and Development
PCR : Polymerase chain reaction
PI : Propidium iodide
PN : False Positive Rate
PPD : 1,4-Phenylenediamine
PPV : Positive Predictive Value
QC : Quality Control
REACH : Registration, Evaluation, Authorization and Restriction of CHemicals
RFI : Relative fluorescence intensity
RT : Ring trial
SLO : Stable luciferase orange
SLO-LA : SLO luciferase activity
SLS : Sodium lauryl sulfate
SLR : Stable luciferase red
SLR-LA : SLR luciferase activity
SV40 : Simian virus 40
TG : Test Guideline
TMTD: Tetramethyl thiram disulphide
TNF-α : Tumor necrosis factor-α
UN GHS : the United Nations Globally Harmonized System of Classification and Labeling of Chemicals
VMT : Validation Management Team

14. Appendixes

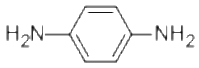
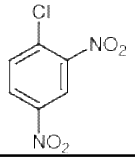
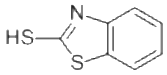
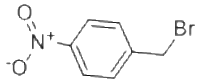
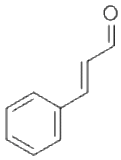
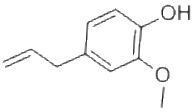
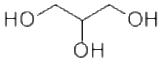
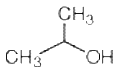
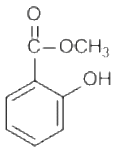
Appendix 1. Chemical structure of the test chemicals for the Phase 0 study

No.	Chemical name	CAS No.	Molecular weight	Chemical structure
0-1	4-Nitrobenzylbromide	100-11-8	216.03	
0-2	Tetramethyl thiuram disulfide	137-26-8	240.43	
0-3	Lactic acid	50-21-5	90.08	

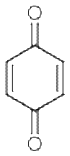
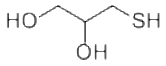
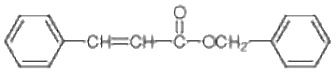
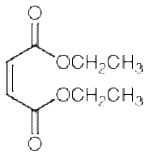
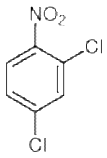
Appendix 2. Chemical structure of the test chemicals for the Phase I study

No.	Chemical name	CAS No.	Molecular weight	Chemical structure
I-1	1,4-Phenylenediamine	106-50-3	108.14	
I-2	2,4-Dinitrochlorobenzene	97-00-7	202.55	
I-3	2-Mercaptobenzothiasole	149-30-4	167.25	
I-4	Cinnamal	104-55-2	132.16	
I-5	Tetramethyl thiuiram disulphide	137-26-8	240.43	
I-6	Eugenol	97-53-0	164.2	
I-7	Imidazolidinyl urea	39236-46-9	388.3	
I-8	Lactic acid	50-21-5	90.08	
I-9	Salicylic acid	69-72-7	138.12	
I-10	Sodium lauryl sulphate	151-21-3	288.38	

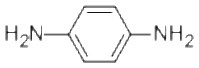
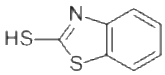
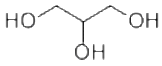
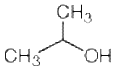
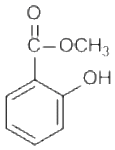
Appendix 3. Chemical structure of the test chemicals for the Phase IIa study

No.	Chemical name	CAS No.	Molecular weight	Chemical structure
IIa-1	1,4-Phenylenediamine	106-50-3	108.14	
IIa-2	2,4-Dinitrochlorobenzene	97-00-7	202.55	
IIa-3	2-Mercaptobenzothiasole	149-30-4	167.25	
IIa-4	4-Nitrobenzylbromide	100-11-8	216.03	
IIa-5	Glyoxal	107-22-2	58.04	
IIa-6	Cinnamal	104-55-2	132.16	
IIa-7	Eugenol	97-53-0	164.2	
IIa-8	Glycerol	56-81-5	92.09	
IIa-9	Isopropanol	67-63-0	60.1	
IIa-10	Methyl salicylate	119-36-8	152.15	

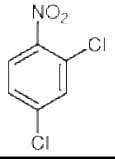
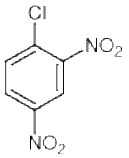
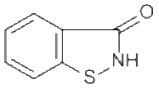
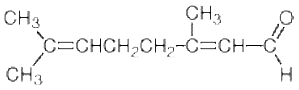
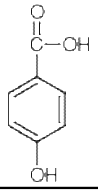
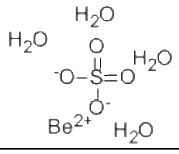
Appendix 4. Chemical structure of the test chemicals for the Phase IIb study

No.	Chemical name	CAS No.	Molecular weight	Chemical structure
IIb-1	p-Benzoquinone	106-51-4	108.1	
IIb-2	1-Thioglycerol	96-27-5	108.16	
IIb-3	Benzyl cinnamate	103-41-3	238.29	
IIb-4	Diethyl maleate	141-05-9	172.18	
IIb-5	2,4-Dichloronitrobenzene	611-06-3	192	

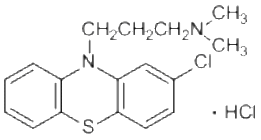
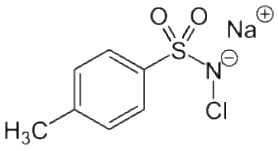
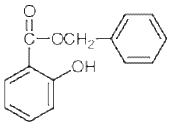
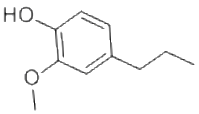
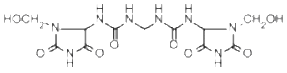
Appendix 5. Chemical structure of the test chemicals for the Phase IIc study

No.	Chemical name	CAS No.	Molecular weight	Chemical structure
IIc-1	1,4-Phenylenediamine	106-50-3	108.14	
IIc-2	2-Mercaptobenzothiasole	149-30-4	167.25	
IIc-3	Glycerol	56-81-5	92.09	
IIc-4	Isopropanol	67-63-0	60.1	
IIc-5	Methyl salicylate	119-36-8	152.15	

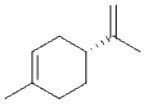
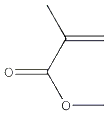
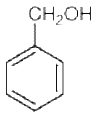
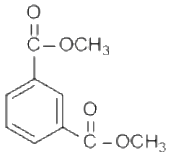
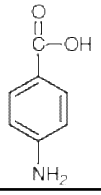
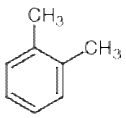
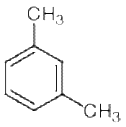
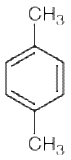
Appendix 6. Chemical structure of the test chemicals for the Phase III study

No.	Chemical name	CAS No.	Molecular weight	Chemical structure
III-1	2,4-Dichloronitrobenzene	611-06-3	192	
III-2	2,4-Dinitrochlorobenzene	97-00-7	202.55	
III-3	Glyoxal solution	107-22-2	58.04	
III-4	1,2-Benzisothiazol-3(2H)-one	2634-33-5	151.18	
III-5	Citral	5392-40-5	152.24	
III-6	4-Hydroxybenzoic acid	99-96-7	138.12	
III-7	Beryllium sulphate	7787-56-6	177.14	

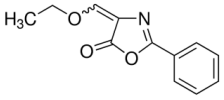
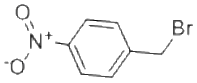
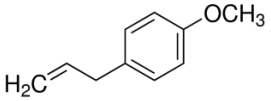
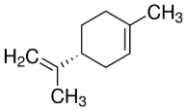
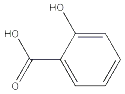
(continued)

No.	Chemical name	CAS No.	Molecular weight	Chemical structure
III-8	Formaldehyde	50-00-0	30.03	
III-9	Chlorpromazine HCl	69-09-0	355.32	
III-10	Chloramine T	127-65-1	227.64	
III-11	Benzyl salicylate	118-58-1	228.25	
III-12	Dihydroeugenol	2785-87-7	166.22	
III-13	Nickel chloride	7718-54-9	129.59	
III-14	Imidazolidinyl urea	39236-46-9	388.3	

(continued)

No.	Chemical name	CAS No.	Molecular weight	Chemical structure
III-15	R(+)Limonene	5989-27-5	136.23	
III-16	Methylmethacrylate	80-62-6	100.12	
III-17	Benzyl alcohol	100-51-6	108.14	
III-18	Dimethyl isophthalate	1459-93-4	194.19	
III-19	4-Aminobenzoic acid	150-13-0	137.14	
III-20	Xylene	1330-20-7	106.17	 o-xylene  m-xylene  p-xylene

Appendix 7. Chemical structure of the test chemicals for the Phase IV study

No.	Chemical name	CAS No.	Molecular weight	Chemical structure
IV-1	Oxazolone	15646-46-5	217.22	
IV-2	4-Nitrobenzylbromide	100-11-8	216.03	
IV-3	4-Allylanisole	140-67-0	148.20	
IV-4	d-Limonene	5989-27-5	136.23	
IV-5	Salicylic acid	69-72-7	138.12	

Appendix 8. Protocol of the IL-8 Luc assay (Ver. 023E)

IL-8 Luc assay protocol ver. 023E
Feb 8, 2016

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

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

Contents

1. Materials

1-1 Cells

The human macrophage-like cell line THP-1 was obtained from the American Type Culture Collection (Manassas, VA, USA). A THP-1-derived IL-8 reporter cell line, THP-G8, that harbors the SLO and SLR luciferase genes under the control of the IL-8 and GAPDH promoters, respectively, was established by the Dept. of Dermatology, Tohoku University School of Medicine.
(Takahashi T. et al. An *in vitro* test to screen skin sensitizers using a stable THP-1-derived IL-8 reporter cell line, THP-G8. *Toxicol Sci,* 124(2), 359-369, 2011)
(International patent publication No. WO2012/002507A1)

1-2 Reagents and equipment

For maintenance of the THP-G8 cells

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

For chemical exposure, positive control, solvents

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

For measurement of the luciferase activity

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

Expendable supplies
- T-75 flask tissue culture treated (e.g., Corning Cat#353136)
- 96 well black plate (flat-bottom, for measurement of the luciferase activity, e.g., Greiner Bio-one Cat#655090, Nunc Cat#165305)
- 96 well clear plate (round-bottom, for preparation of $CoCl_2$)
- 96 well assay block, 2 mL (e.g., Costar Cat#3960)
- Reservoir
- Pipette

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

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

1-3 Culture medium

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

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

1-3-2 B medium: for luciferase assay (30 mL, stored at 2-8°C)

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

1-3-3 C medium: for thawing THP-G8 cells (30 mL, stored at 2-8°C)

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

1-4 Thawing of THP-G8 cells

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

1-5 Maintenance of THP-G8 cells

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

2. Preparation of cells for assay

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

Use cells between 1 and 6 weeks after thawing.

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

Figure 1.

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

3. Preparation of chemicals and cell treatment with chemicals

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

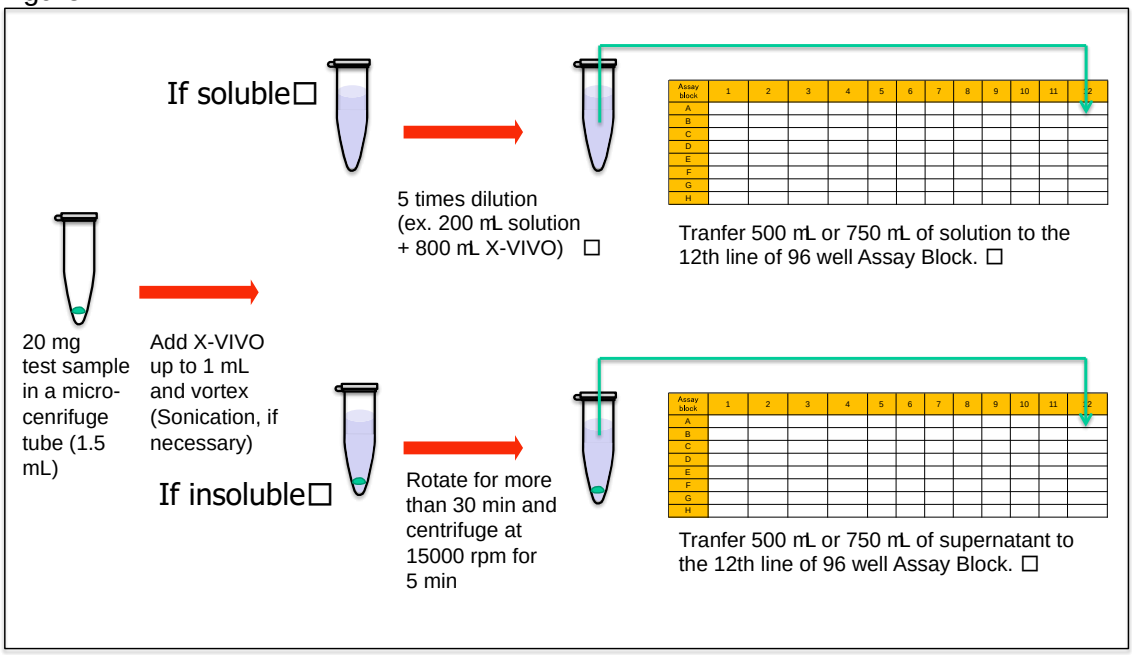
3-1 Dissolution in X-VIVO™ 15 (cf. Figure 2)

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

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

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

Figure 2.



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

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

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

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

Figure 3.

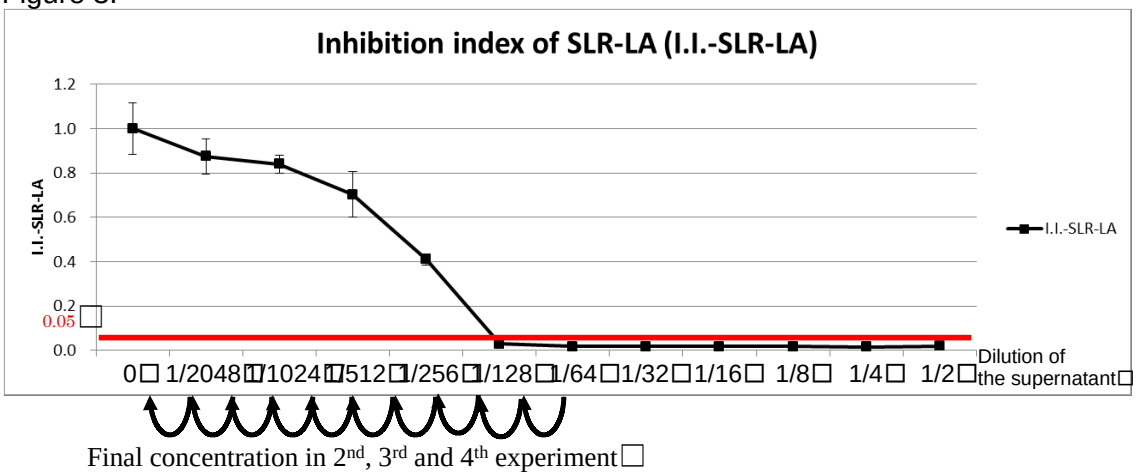
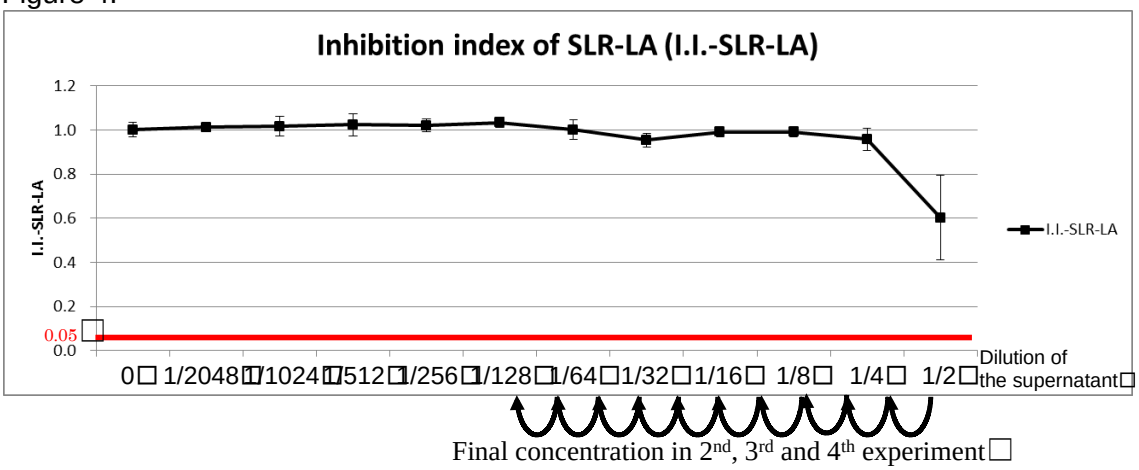


Figure 4.



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

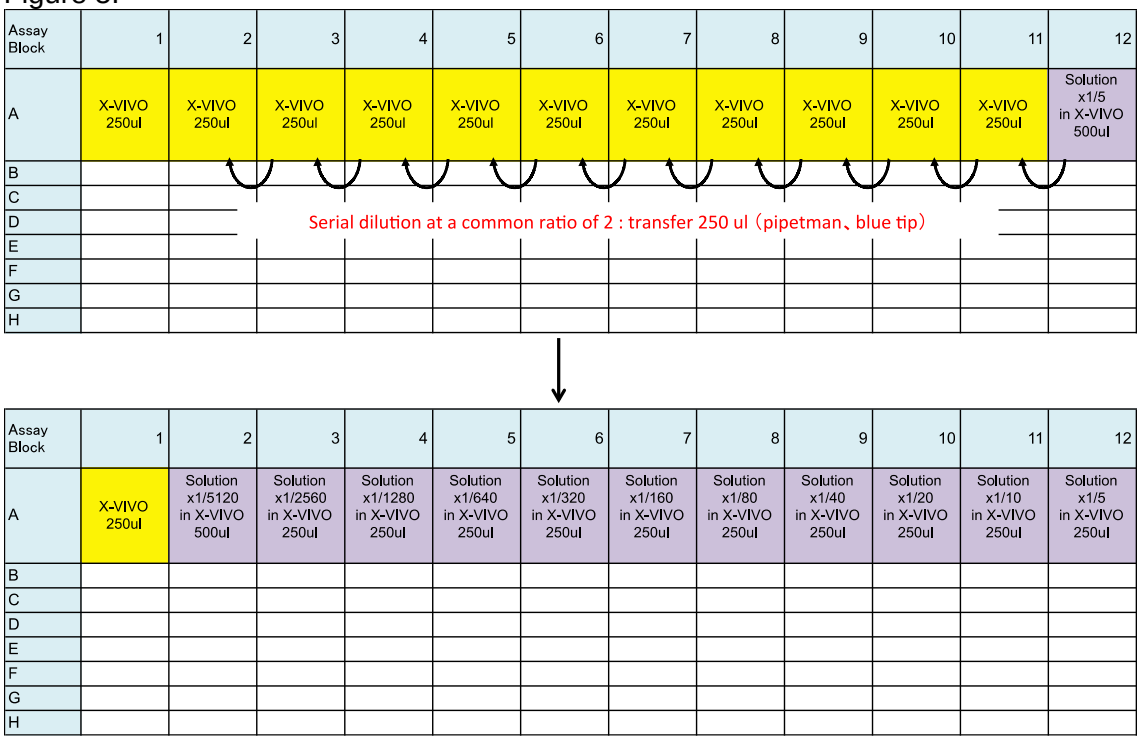
3-2-1 Arrangement of chemicals and vehicle (1st experiment)

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

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

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

Figure 5.



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

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

Figure 6

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	X-VIVO 250ul	Solution x1/5120 in X-VIVO 500ul	Solution x1/2560 in X-VIVO 250ul	Solution x1/1280 in X-VIVO 250ul	Solution x1/640 in X-VIVO 250ul	Solution x1/320 in X-VIVO 250ul	Solution x1/160 in X-VIVO 250ul	Solution x1/80 in X-VIVO 250ul	Solution x1/40 in X-VIVO 250ul	Solution x1/20 in X-VIVO 250ul	Solution x1/10 in X-VIVO 250ul	Solution x1/5 in X-VIVO 250ul
B												
C												
D												
E												
F												
G												
H												

50ul

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

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

Figure 7

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

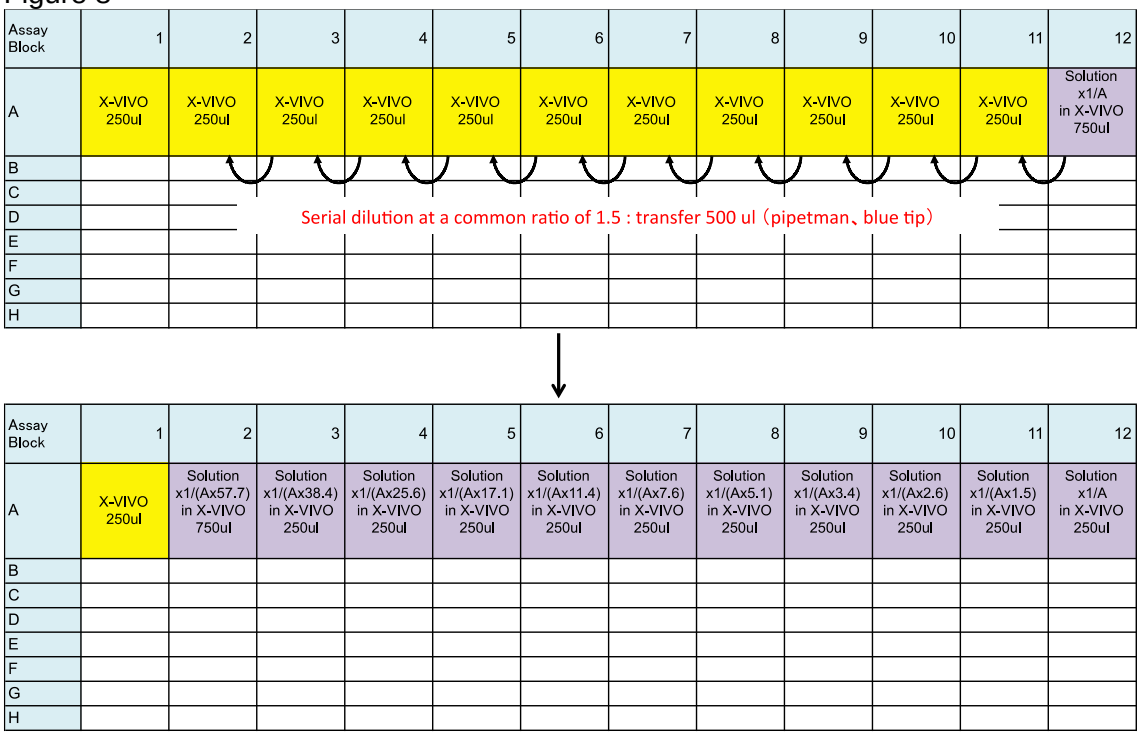
3-2-5 Arrangement of chemicals and vehicle (2nd, 3rd and 4th experiment)

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

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

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

Figure 8



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

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

Figure 9

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	X-VIVO 250ul	Solution x1/(Ax57.7) in X-VIVO 750ul	Solution x1/(Ax38.4) in X-VIVO 250ul	Solution x1/(Ax25.6) in X-VIVO 250ul	Solution x1/(Ax17.1) in X-VIVO 250ul	Solution x1/(Ax11.4) in X-VIVO 250ul	Solution x1/(Ax7.6) in X-VIVO 250ul	Solution x1/(Ax5.1) in X-VIVO 250ul	Solution x1/(Ax3.4) in X-VIVO 250ul	Solution x1/(Ax2.6) in X-VIVO 250ul	Solution x1/(Ax1.5) in X-VIVO 250ul	Solution x1/A in X-VIVO 250ul
B												
C												
D												
E												
F												
G												
H												

50ul

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

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

Figure 10

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

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

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

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

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

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

Figure 11.

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

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

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	X-VIVO 250ul	Supernatant x1/1024 in X-VIVO 500ul	Supernatant x1/512 in X-VIVO 250ul	Supernatant x1/256 in X-VIVO 250ul	Supernatant x1/128 in X-VIVO 250ul	Supernatant x1/64 in X-VIVO 250ul	Supernatant x1/32 in X-VIVO 250ul	Supernatant x1/16 in X-VIVO 250ul	Supernatant x1/8 in X-VIVO 250ul	Supernatant x1/4 in X-VIVO 250ul	Supernatant x1/2 in X-VIVO 250ul	Supernatant x1 in X-VIVO 250ul
B												
C												
D												
E												
F												
G												
H												

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

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

Figure 12.

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	X-VIVO 250ul	Supernatant x1/1024 in X-VIVO 500ul	Supernatant x1/512 in X-VIVO 250ul	Supernatant x1/256 in X-VIVO 250ul	Supernatant x1/128 in X-VIVO 250ul	Supernatant x1/64 in X-VIVO 250ul	Supernatant x1/32 in X-VIVO 250ul	Supernatant x1/16 in X-VIVO 250ul	Supernatant x1/8 in X-VIVO 250ul	Supernatant x1/4 in X-VIVO 250ul	Supernatant x1/2 in X-VIVO 250ul	Supernatant x1 in X-VIVO 250ul
B												
C												
D												
E												
F												
G												
H												

50ul

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

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

Figure 13.

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

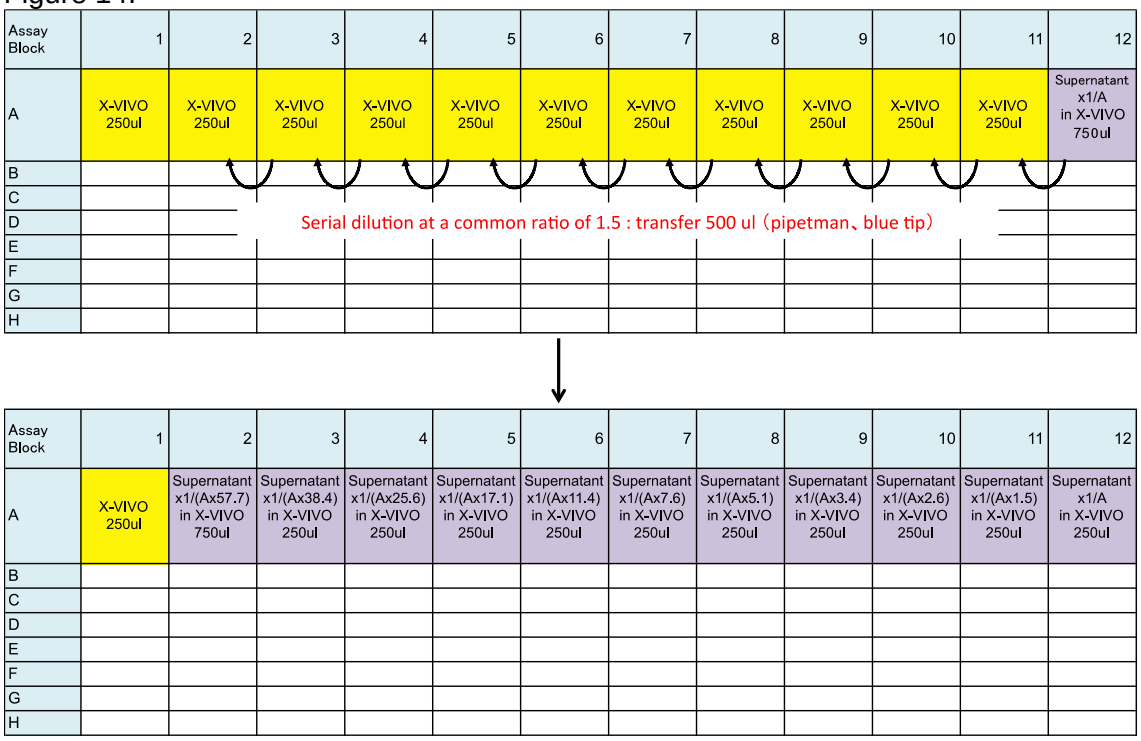
3-3-5 Arrangement of chemicals and vehicle (2nd, 3rd and 4th experiment)

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

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

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

Figure 14.



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

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

Figure 15.

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	X-VIVO 250ul	Supernatant x1/(Ax57.7) in X-VIVO 750ul	Supernatant x1/(Ax38.4) in X-VIVO 250ul	Supernatant x1/(Ax25.6) in X-VIVO 250ul	Supernatant x1/(Ax17.1) in X-VIVO 250ul	Supernatant x1/(Ax11.4) in X-VIVO 250ul	Supernatant x1/(Ax7.6) in X-VIVO 250ul	Supernatant x1/(Ax5.1) in X-VIVO 250ul	Supernatant x1/(Ax3.4) in X-VIVO 250ul	Supernatant x1/(Ax2.6) in X-VIVO 250ul	Supernatant x1/(Ax1.5) in X-VIVO 250ul	Supernatant x1/A in X-VIVO 250ul
B												
C												
D												
E												
F												
G												
H												

50ul

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

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

Figure 16.

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

4. Preparation of positive control using 4-Nitrobenzyl bromide (4-NBB)

4-1 Preparation of cells

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

Figure 17.

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

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

Weigh 20 mg of 4-NBB in a microfuge tube (1.5 mL) and add X-VIVO™ 15 up to 1 mL. Vortex the microfuge tube vigorously and shake on a rotor (e.g., WKN-2210, WAKEN B TECH Co. Ltd, Kyoto, Japan) at a maximum speed of 8 rpm for more than 30 min until just before centrifugation. After centrifugation at 15,000 rpm (≈20,000 x g) for 5 min, dilute the supernatant 4 times with X-VIVO™ 15 and transfer 500 µL of the diluted solution to the 4th line of a 96 well Assay Block. If undissolved chemical either precipitates or floats, remove the solution carefully so that no undissolved chemical is present in the solution. Conduct 2 serial dilutions with X-VIVO™ 15 at a common ratio of 2 (Figure 18) and add 50 µL of the solution to THP-G8 in a 96 well plate using an 8 channel or 12 channel pipetman (Figure 19). Shake the plate on a plateshaker, and incubate in a CO_2 incubator for 16 hours (37°C, 5% CO_2).

Figure 18.

Assay Block	1	2	3	4	5	6	7	8	9	10	11	12
A	X-XIVO 250ul	X-XIVO 250ul	X-XIVO 250ul	4-NBB Supernatant x1/4 in X-VIVO 500ul								
B												
C												
D												
E	Serial dilution at a common ratio of 2 : transfer 250 ml（pipetman、blue tip）☐											
F												
G												
H												

↓

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

Figure 19.

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

50ul

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

4-3 Final constituents of each well of the plate

Figure 20.

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

4-4 Measurement

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

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

Copy the results of the F0 and F1 measurements (values are expressed as counts) and paste them into the appropriate area in the “Data Input” sheet of the data sheet. Calculated factors and graphs will appear on the “4-NBB” sheet of the data sheet. The acceptance criterion is FInSLO-LA ≥ 5.0 at some concentration. Continue the experiment if this criterion is accepted.

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

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

5-1 Reagents

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

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

5-2 Preparation of luminescence reaction solution

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

Add 200 µL of 10 ~ 100 mM Tris/HCl or Hepes/NaOH (pH 7.5 ~ 8.0) supplemented with 10% (w/v) glycerol to each tube of lyophilized purified luciferase sample to dissolve the enzymes, divide into 10 µL aliquots in 1.5 mL disposable tubes and store in a freezer at -80°C. The stored frozen solution of the reference samples can be used for up to 6 months.

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

5-3 Bioluminescence measurement

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

Figure 21.

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

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

Figure 22. An example of the raw output data

Measurement without Filter

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

Measurement with Filter

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

Transmission coefficient of the optical filter were calculated as follow:

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

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

In the case shown above,

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

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

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

6. Measurement of luciferase activity

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

Transfer 100 µL of pre-warmed Tripluc from the reservoir to each well of the plate containing the reference sample using an 8 channel or 12 channel pipetman. Shake the plate for 10 min at room temperature (about 25°C) using a plate shaker. Remove bubbles in the solutions in the wells if they appear. Place the plate in the luminometer to measure the luciferase activity. Bioluminescence is measured for 3 sec each in the absence (F0) and presence (F1) of the optical filter. In case alternative settings are used, e.g., depending on the model of luminometer used, these settings should be justified.

The specific procedure for how to manipulate the raw data is shown below using the analysis of DNCB as an example (Figure 23).

Figure 23. An example of the raw output data (DNCB).

Measurement2 F0

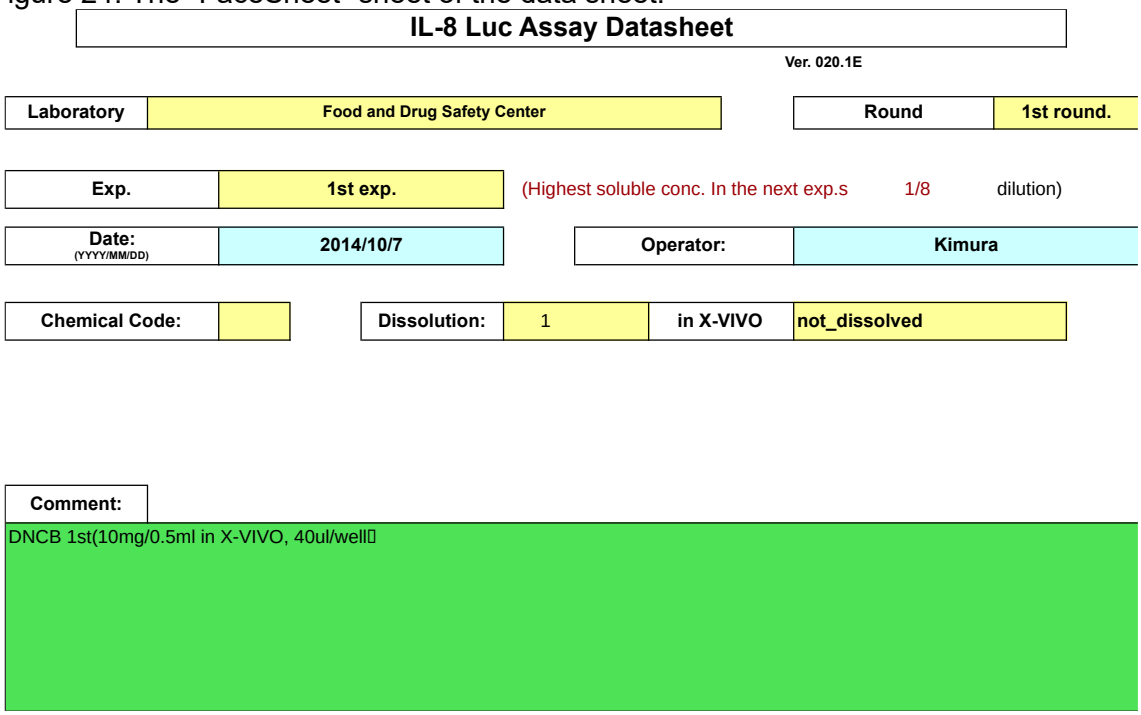
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C	9098	9046	9470	8879	9112	10983	14503	68	63	76	80	67
D	9140	8493	8981	8352	8521	10510	12232	73	78	59	61	51
E	8801	8601	8833	8469	8572	10362	13133	72	74	71	68	74
F	8779	8364	8116	8026	8011	10199	12109	83	66	55	55	72
G												
H												

Measurement2 F2

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

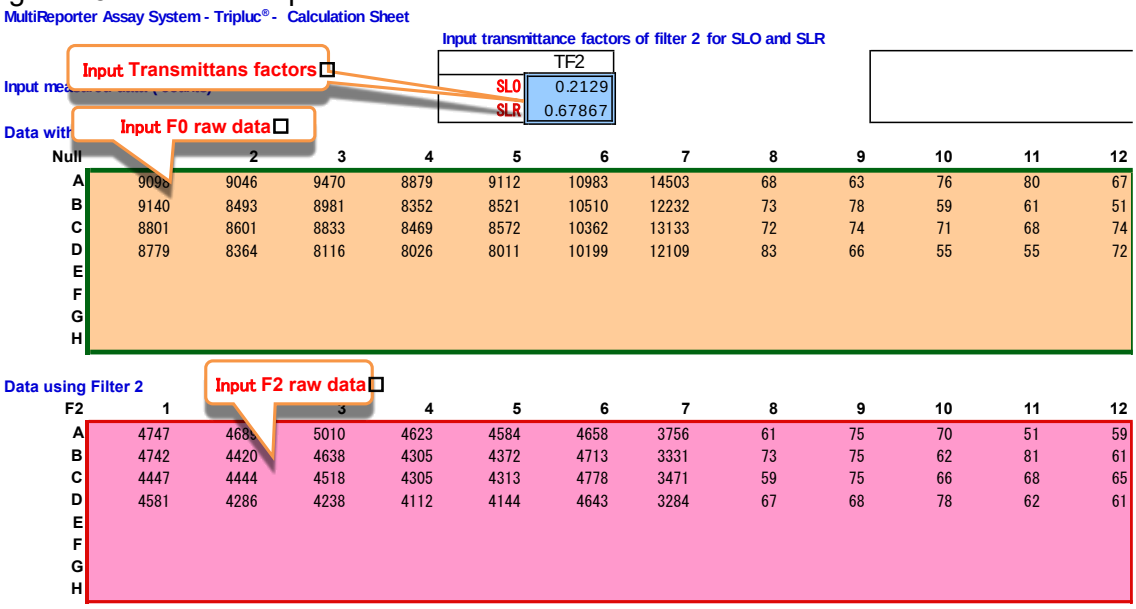
1st. Add the information regarding the name of laboratory, the round of experiments if multiple experimental sets are performed, the experiment number, date, the operator, chemical code, the highest concentration, dissolved or undissolved in X-VIVO™ 15, and comments if any to the "FaceSheet" sheet of the data sheet (Figure 24). In this data sheet, the highest soluble concentrations in the next experiments are automatically shown based on the results.

Figure 24. The "FaceSheet" sheet of the data sheet.



2nd. Copy the results of the F0 and F1 measurements (values are expressed as counts) and paste them into the appropriate area in the “Data Input” sheet of the data sheet shown below (Figure 25). In addition, input the transmittance factors calculated in chapter 5. Calculation of the transmittance factors to TF2 of the “Data Input” sheet (Figure 25).

Figure 25. The “Data Input” sheet of the data sheet.



Next, the calculated results for the parameters of the IL-8 Luc assay for each concentration, e.g., SLO, SLR, nSLO-LA, the mean ± SD of SLO-LA, the mean ± SD of SLR-LA, the mean ± SD of nSLO-LA, the mean ± SD of FInSLO-LA, the mean ± SD of I.I-SLR-LA, and a graphical presentation with the 95% confidence interval and judgment, will automatically appear on the "Result Format" sheet and the "Graph" sheet of the data sheet, respectively (Figure 26, 27).

Figure 26. The "Result Format" sheet of the data sheet.

MultiReporter Assay System -Tripluc®- Calculation Sheet

	Null	T2
SLO	1	0.212902063
SLR	1	0.678670307

inversion matrix

1.457098709	-0.457098709
-2.146990511	2.146990511

Transmittance Data

SLO

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

SLR

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

SLO mod

A	3065	3114	3042	3012	3435	6003	13068	0	0	0	7	0
B	3137	2885	3128	2927	3029	5195	10672	0	0	0	0	0
C	3276	2991	3170	3097	3230	4840	11684	0	0	0	0	0
D	2957	2985	2727	2866	2776	4892	10593	0	0	0	0	0
E	0	0	0	0	0	0	0	0	0	0	0	0
F	0	0	0	0	0	0	0	0	0	0	0	0
G	0	0	0	0	0	0	0	0	0	0	0	0
H	0	0	0	0	0	0	0	0	0	0	0	0

SLR mod

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

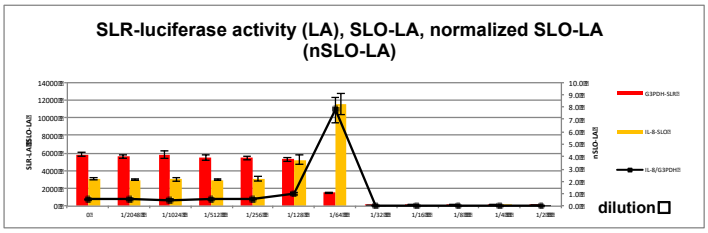
nSLO-LA

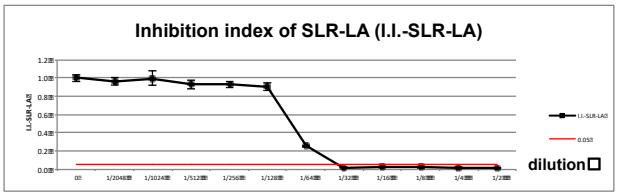
0.508	0.525	0.473	0.513	0.605	1.205	9.108	0.000	0.000	0.000	0.097	0.000
0.523	0.515	0.535	0.540	0.552	0.978	6.839	0.000	0.000	0.000	0.000	0.000
0.593	0.533	0.560	0.577	0.605	0.877	8.063	0.000	0.000	0.000	0.000	0.000
0.508	0.555	0.506	0.556	0.530	0.922	6.989	0.000	0.000	0.000	0.000	0.000
0.000	0.000	0.000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

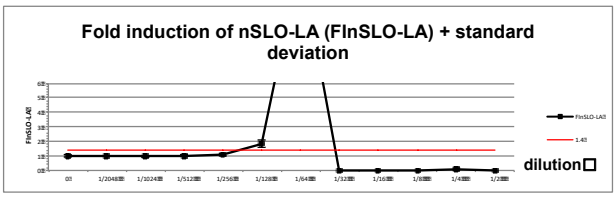
1st exp.

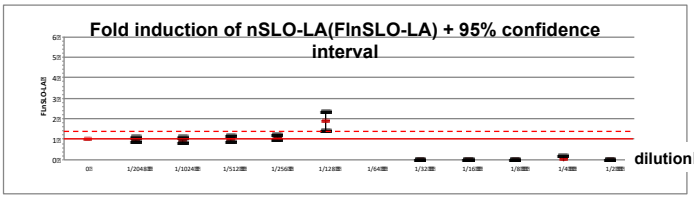
Chemical concentration	0	1/2048	1/1024	1/512	1/256	1/128	1/64	1/32	1/16	1/8	1/4	1/2
Average of SLO-LA	3109	2994	3017	2976	3118	5233	11504	0	0	0	2	0
S.D.	134	93	201	101	282	537	1155	0	0	0	4	0
Average of SLR-LA	5846	5632	5833	5456	5436	5281	1490	106	125	118	110	102
S.D.	233	228	440	297	192	224	59	13	7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

Figure 27. The “Graph” sheet of the data sheet.









	(0)	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
FInSLO-LAi	1	0.998266	0.972978	1.025184	1.075227	1.867987	14.54427	0	0	0	0.045494	0
lower□mit		0.893972	0.855657	0.915809	0.950612	1.433691	11.13383	0	0	0	-0.06676	0
upper□mit		1.125035	1.107502	1.154093	1.218825	2.35017	18.38495	0	0	0	0.158562	0

													Judge
F.I	0	0	0	0	0	1	1	0	0	0	0	0	
95%	0	0	0	0	0	1	1	0	0	0	0	0	Positive
95%+F.I	0	0	0	0	0	1	1	0	0	0	0	0	Positive

Positive

7. Criteria

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

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

Figure 28.

Abbreviation	Definition
SLO-LA	SLO luciferase activity
SLR-LA	SLR luciferase activity
nSLO-LA	Normalized SLO-LA SLO-LA / SLR-LA
I.I.-SLR-LA	Inhibition index for SLR-LA SLR-LA of THP-G8 treated with chemicals / SLR-LA of non-treated THP-G8
FInSLO-LA	Fold induction of nSLO-LA nSLO-LA of THP-G8 cells treated with chemicals / nSLO-LA of non-stimulated THP-G8 cells

7-2 Acceptance criteria

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

- For examining positive control, FInSLO-LA should become more than 5.0 at some concentration of positive chemical, 4-NBB, in every experiment.
- If SLR-LA of control wells without chemicals demonstrate less than 1,000, the results obtained from the plate containing the control wells should be rejected.
- If all concentrations of the chemicals demonstrate less than 0.05 of I.I.-SLR-LA, the experiments are rejected and the first experiment will be repeated from the lowest concentration of the previous experiments.

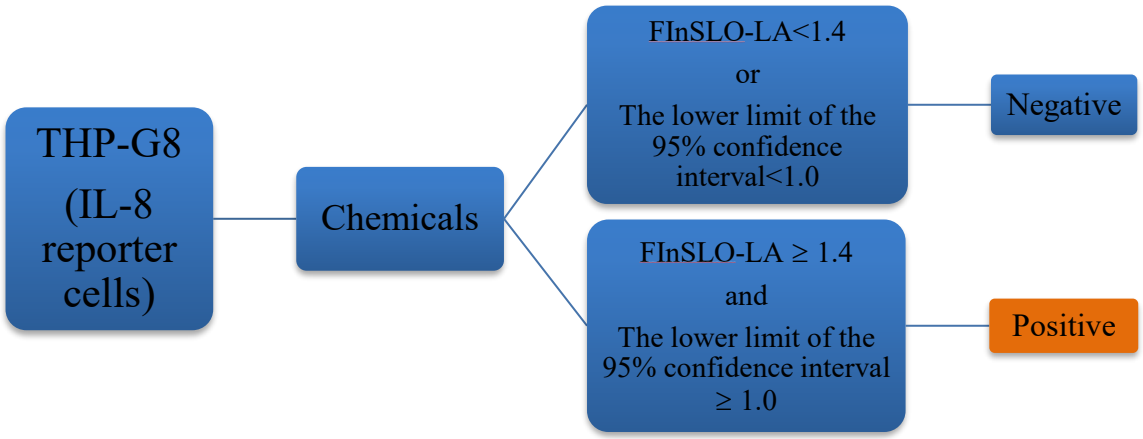
7-3 The criteria to identify sensitizers by the IL-8 Luc assay

The criteria which use FInSLO-LA and the lower limit of the 95% confidence interval of FInSLO-LA. The way of calculation of the 95% confidence interval of FInSLO-LA is described in Annex 3.

In each experiment:

Criteria: chemicals that demonstrate FInSLO-LA $\geq$ 1.4 and the lower limit of the 95% confidence interval of FInSLO-LA $\geq$ 1.0 are judged as positive.

Figure 29.



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

2nd, 3rd and 4th experiments: Determine the minimum concentration at which I.I.-SLR-LA is lower than 0.05 in the 1st experiment. Use the concentration one step (2-times) higher than this determined concentration as the highest concentration of the chemical to test, and conduct 11 serial dilutions at a common ratio of 1.5 from the highest concentration. If I.I.-SLR-LA does not decrease to 0.05 at any concentrations, or I.I.-SLR-LA is lower than 0.05 only at the highest concentration in the 1st experiment, conduct 11 serial dilutions at a common ratio of 1.5 from the supernatant or solution.

Chemicals that provide 2 positive results in the 1st, 2nd, 3rd or 4th experiments are considered sensitizers and chemicals that provide 3 negative results in the 1st, 2nd, 3rd or 4th experiments are considered non-sensitizers. You can skip the follow-on experiment once the judgment by all three criteria is made (Figure 30).

Figure 30. Criteria to determine sensitizer or non-sensitizer.

1st	2nd	3rd	4th	Judge
Positive	Positive	-	-	Sensitizer
	Negative	Positive	-	Sensitizer
		Negative	Positive	Sensitizer
			Negative	Non-sensitizer
Negative	Positive	Positive	-	Sensitizer
		Negative	Positive	Sensitizer
			Negative	Non-sensitizer
	Negative	Positive	Positive	Sensitizer
			Negative	Non-sensitizer
		Negative	-	Non-sensitizer

8. Update record

Ver.023, 2016, Feb 9
Add annex.
Add Acceptance criteria.

Ver. 022E, 2015, Dec. 31
Change positive control to 4-NBB.

Ver. 021E, 2015, Dec. 21
Add schemes showing data transport and delete the graphical presentation of the criteria.

Ver. 020E, 2015, July 3
Change preparation of chemicals (When the chemical is soluble at 20 mg/mL in X-VIVO™ 15, the chemical is diluted 5-fold) and $CoCl_2$.

Ver. 019E, 2015, April 1
Change vehicle to X-VIVO™ 15

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

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

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

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

Ver. 013E, 2012, Aug. 03 distribution

Ver. 012E, 2012, July 12 distribution

Ver. 011E, 2012, June 05 distribution

Ver. 008E, 2011, Dec. 19 distribution

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

Annex 1

Principal of measurement of luciferase activity

MultiReporter Assay System -Tripluc- can be used with a microplate-type luminometer with a multi-color detection system, which can equip an optical filter. (e.g. Phelios AB-2350 (ATTO), ARVO (PerkinElmer), Tristar LB941 (Berthold)) The optical filter used in measurement is 600 ～ 620 nm long or short pass filter, or 600～700 nm band pass filter.

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

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

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

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

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

$F0=O+R$

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

These formulas can be rephrased as follows

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

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

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

This calculation can be performed using the functions "MININVERSE" and "MMULT" in Microsoft Excel. These calculations are integrated in "IL-8_Luc_assay_Data_sheet_20150706_Ver.020.1"

Annex 2
Validation of reagents and equipment

1 Measurement of transmittance of optical filter for multicolor measurement

For color discriminations in the multi-color reporter assay, detectors (luminometer and plate reader) are usually equipped with optical filters, such as sharp-cut (long-pass) filters and band-pass filters. The transmittance factors of these filters for each bioluminescence signal color have to be calibrated prior to all experiments by following the protocols below.

1-1 Reagents

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

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

1-2 Calibration

1-2-1 Preparation of luminescence reaction solution

Thaw Tripluc® Luciferase assay reagent (Tripluc) and keep it at room temperature by bathing in water or ambient air. Start the luminometer 30 min before starting the measurement for stabilization of the photomultiplier.

Add 200 µL of 100 mM Tris-HCl (pH8.0) contains 10% glycerol to each tube of the lyophilized reference samples to dissolve the enzymes, followed by separating them into 1.5 mL disposable tubes at 10 µL each and storing in a freezer at -80°C. The stored frozen solution of the reference samples can be used for one half year.

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

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

1-2-2 Bioluminescence measurement

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

Figure 31.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	SLO 1/1	SLO 1/1	SLO 1/1	SLO 1/4	SLO 1/4	SLO 1/4	SLO 1/16	SLO 1/16	SLO 1/16	SLO 1/64	SLO 1/64	SLO 1/64

C												
D	SLR 1/1	SLR 1/1	SLR 1/1	SLR 1/4	SLR 1/4	SLR 1/4	SLR 1/16	SLR 1/16	SLR 1/16	SLR 1/64	SLR 1/64	SLR 1/64
E												
F												
G												
H												

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

Figure 32.

Measurement without Filter

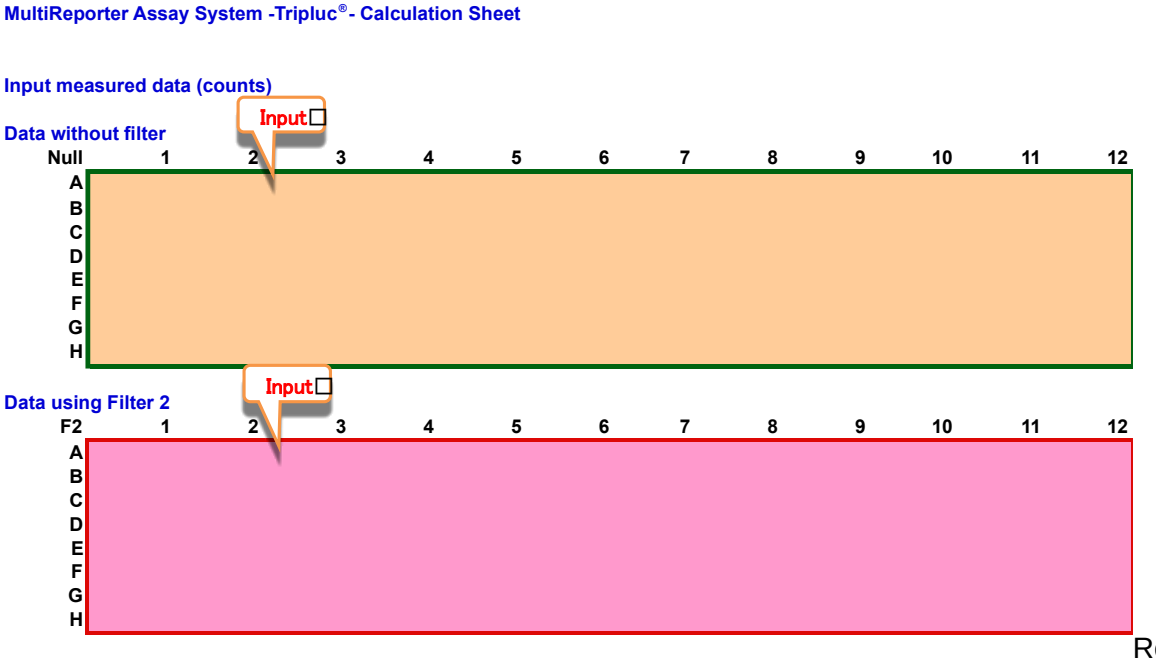
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	9689	9691	9677	2402	2388	2412	704	689	721	177	189	182
C												
D	8588	8444	8462	2281	2128	2239	609	578	690	150	132	129
E												
F												
G												
H												

Measurement with Filter

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

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

Figure 33.



Record all the results for quality control.

2 Quality control of equipment

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

Start LED plate and select “PMT” mode.

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

Place the LED plate into the luminometer. Light intensity is measured for 3 sec each in the absence (F0) and presence (F2) of the optical filter.

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

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

Annex 3

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

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

$nSLO\text{-}LA_{ij} = SLO\text{-}LA_{ij} / SLR\text{-}LA_{ij.}$

1. FInSLO-LA

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

$$FInSLO\text{-}LA_i = \left\{(1/4)\times\sum_j nSLO\text{-}LA_{ij}\right\} / \left\{(1/4)\times\sum_j nSLO\text{-}LA_{0j}\right\}.$$

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

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

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

$\bar{x}_0^2 = \left\{(1/4)\times\sum_j nSLO\text{-}LA_{0j}\right\}$, $sd_0^2 = (1/3)\times\sum_j \left(nSLO - LA_{0j} - \bar{x}_0\right)^2$, $n_0 = 4$,

$\bar{y}_0^2 = \left\{(1/4)\times\sum_j nSLO\text{-}LA_{ij}\right\}^2$, $sd_{y_j}^2 = (1/3)\times\sum_j \left(nSLO - LA_{ij} - \bar{y}_i\right)^2$, $n_{y_i} = 4$,

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

2. I.I.-SLR-LA

The I.I.-SLR-LA is a ratio of the averaged SLR-LA for the repetition of the i-th concentration compared with it of the 0 concentration, and this is written by

$$I.I.\text{-}SLR\text{-}LA_i = \left\{(1/4)\times\sum_j SLL\text{-}LA_{ij}\right\} / \left\{(1/4)\times\sum_j SLL\text{-}LA_{0j}\right\}.$$

Appendix 9. Table S1. Data set of 122 chemicals evaluated by the IL-8 Luc assay

No	Chemical	CAS#	[illegible]				[illegible]		
			Potency category	ver. 14E (FInSLO>=1.4 I.I.<=0.8)	ver. 14E and I.I.-SLR-LA>=0.2	ver. 14E and I.I-SLR-LA>=0.05	ver. 018E Criterion 1	ver. 018E Criterion 2	ver. 018E Criterion 3
1	1-Benzoylacetone(1-BA)	93-91-4	Extreme	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
2	2,4-Dinitrochlorobenzene(DNCB)	97-00-7	Extreme	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
3	4-Nitrobenzyl bromide(4-NBB)	100-11-8	Extreme	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
4	Beryllium sulfate tetrahydrate	7787-56-6	Extreme				non-sensitizer	non-sensitizer	non-sensitizer
5	Diphenylcyclopropenone (DPCP)	886-38-4	Extreme	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
6	Oxazolone	15646-46-5	Extreme	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
7	p-Benzoquinone	106-51-4	Extreme	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
8	Potassium dichromate	7778-50-9	Extreme	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
9	Tetrachlorosalicylanilide	1154-59-2	Extreme	sensitizer		sensitizer	sensitizer	sensitizer	sensitizer
10	1,4-Dihydroquinone	123-31-9	Strong	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
11	1,4-Phenylenediamine(PPD)	106-50-3	Strong	sensitizer	non-sensitizer	sensitizer	sensitizer	sensitizer	non-sensitizer
12	2-Aminophenol	95-55-6	Strong	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
13	2-Nitro-1,4-phenylenediamine	5307-14-2	Strong	non-sensitizer	non-sensitizer	non-sensitizer	sensitizer	sensitizer	sensitizer
14	Benzoyl peroxide	94-36-0	Strong	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
15	Benzyl bromide	100-39-0	Strong	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
16	Chloramine-T	127-65-1	Strong	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
17	Chlorpromazine hydrochloride	69-09-0	Strong				sensitizer	sensitizer	sensitizer
18	Cobalt chloride hexahydrate	7646-79-9	Strong	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
19	Formaldehyde	50-00-0	Strong	sensitizer		sensitizer	sensitizer	sensitizer	sensitizer
20	Glutaraldehyde	111-30-8	Strong	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
21	Iodopropynyl butylca□bamate	55406-53-6	Strong	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
22	Lauryl gallate	1166-52-5	Strong	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
23	Maleic anhydride	108-31-6	Strong	sensitizer	non-sensitizer	sensitizer	sensitizer	non-sensitizer	non-sensitizer
24	Methyldibromoglutaronitrile(MDGN)	35691-65-7	Strong	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
25	Phthalic anhydride	85-44-9	Strong	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
26	Propyl gallate	121-79-9	Strong	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
27	1,2-Benzisothiazolin-3-one	2634-33-5	Moderate	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
28	1-Naphthol	90-15-3	Moderate	sensitizer	non-sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
29	1-Phenyl-1,2-propanedione	579-07-7	Moderate	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
30	1-Thioglycerol(3-mercapto-1,2-propanediol)	96-27-5	Moderate				sensitizer	sensitizer	sensitizer
31	2-Hydroxyethyl acrylate	818-61-1	Moderate	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
32	2-Mercaptobenzothiazole(2-MBT)	149-30-4	Moderate	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
33	2-Methoxy-4-methylphenol	93-51-6	Moderate	non-sensitizer	non-sensitizer	non-sensitizer	sensitizer	sensitizer	sensitizer
34	2-Phenylpropionaldehyde	93-53-8	Moderate	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
35	3,4-Dihydrocoumarin	119-84-6	Moderate	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
36	3-Aminophenol	591-27-5	Moderate	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
37	3-Dimethylaminopropylamine	109-55-7	Moderate	sensitizer		sensitizer	sensitizer	sensitizer	sensitizer

(continued)

38	3-Propylidenephthalide	17369-59-4	Moderate				sensitizer	sensitizer	sensitizer
39	4-Chloroaniline	106-47-8	Moderate	non-sensitizer	non-sensitizer	non-sensitizer	sensitizer	sensitizer	non-sensitizer
40	a-Methylcinnamic aldehyde	101-39-3	Moderate	non-sensitizer	non-sensitizer	non-sensitizer	sensitizer	sensitizer	sensitizer
41	Benzyl salicylate	118-58-1	Moderate				sensitizer	sensitizer	sensitizer
42	Benzylideneacetone	122-57-6	Moderate	sensitizer			sensitizer	sensitizer	sensitizer
43	Cinnamic aldehyde(Cinnamal)	104-55-2	Moderate	sensitizer		sensitizer	sensitizer	sensitizer	sensitizer
44	Diethyl maleate	141-05-9	Moderate	sensitizer	non-sensitizer	sensitizer	sensitizer	non-sensitizer	non-sensitizer
45	Diethyl sulfate	64-67-5	Moderate	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
46	Diethylenetriamine	111-40-0	Moderate				sensitizer	sensitizer	sensitizer
47	Dihydroeugenol(2-Methoxy-4-propylphenol)	2785-87-7	Moderate				sensitizer	sensitizer	sensitizer
48	Ethylenediamine	107-15-3	Moderate	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	sensitizer	non-sensitizer
49	Glyoxal	107-22-2	Moderate	non-sensitizer	non-sensitizer	non-sensitizer	sensitizer	non-sensitizer	non-sensitizer
50	Isoeugenol	97-54-1	Moderate				sensitizer	sensitizer	sensitizer
51	Methyl-2-nonynoate	111-80-8	Moderate	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
52	Methylisothiazolinone, 2-Methyl-2H-Isothiazol-3-	2682-20-4	Moderate	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
53	Nickel(II) chloride	7718-54-9					sensitizer	sensitizer	sensitizer
54	Nickel sulphate hexahydrate	10101-97-0	Moderate	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
55	Perillaldehyde	18031-40-8	Moderate	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
56	Phenylacetaldehyde	122-78-1	Moderate	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
57	Resorcinol	108-46-3	Moderate	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
58	Tetramethylthiuramdisulphide(TMTD)	137-26-8	Moderate	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
59	trans-2-Hexenal	6728-26-3	Moderate	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
60	Trimellitic anhydride	552-30-7	Moderate	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
61	(R)-(+)-Limonene	5989-27-5	Weak				non-sensitizer	non-sensitizer	non-sensitizer
62	1-Bromohexane	111-25-1	Weak	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
63	2,3-Butanedione	431-03-8	Weak	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
64	4-Allylanisole	140-67-0	Weak	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	sensitizer	non-sensitizer
65	5-Methyl-2,3-hexanedione	13706-86-0	Weak	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	sensitizer	non-sensitizer
66	Abietic acid	514-10-3	Weak	sensitizer		sensitizer	sensitizer	sensitizer	sensitizer
67	Amyl cinnamic aldehyde	122-40-7	Weak	non-sensitizer	non-sensitizer	non-sensitizer	sensitizer	sensitizer	sensitizer
68	Aniline	62-53-3	Weak	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
69	Benzocaine	60-09-3	Weak	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
70	Benzyl cinnamate	103-41-3	Weak	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
71	Butyl glycidyl ether	2426-08-6	Weak	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
72	Cinnamic alcohol	104-54-1	Weak				sensitizer	sensitizer	sensitizer
73	Citral	5392-40-5	Weak	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
74	Cyclamen aldehyde	103-95-7	Weak				sensitizer	sensitizer	sensitizer
75	Ethyleneglycol dimethacrylate	97-90-5	Weak	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	sensitizer	non-sensitizer
76	Eugenol	97-53-0	Weak	sensitizer		sensitizer	sensitizer	sensitizer	sensitizer
77	Geraniol	106-24-1	Weak				sensitizer	sensitizer	sensitizer
78	Hexyl cinnamic aldehyde	101-86-0	Weak	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
79	Hydroxycitronellal	107-75-5	Weak	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
80	Imidazolidinyl urea	39236-46-9	Weak	sensitizer		sensitizer	sensitizer	sensitizer	sensitizer

(continued)

81	Lilial	80-54-6	Weak				sensitizer	sensitizer	sensitizer
82	Linalool	78-70-6	Weak				sensitizer	sensitizer	sensitizer
83	Methylmethacrylate	80-62-6	Weak				non-sensitizer	non-sensitizer	non-sensitizer
84	Oxalic acid	144-62-7	Weak	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
85	Penicillin G	61-33-6	Weak	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
86	Phenyl benzoate	93-99-2	Weak	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
87	Pyridine	110-86-1	Weak	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
88	Xylene	1330-20-7	Weak				non-sensitizer	sensitizer	non-sensitizer
89	1-Bromobutane	109-65-9	Non- sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
90	1-Butanol	71-36-3	Non- sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
91	2,4-Dichloro-1-nitrobenzene	611-06-3	Non- sensitizer				non-sensitizer	non-sensitizer	non-sensitizer
92	2-Hydroxypropyl methacrylate	923-26-2	Non- sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
93	4-Hydroxybenzoic acid	99-96-7	Non- sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
94	6-Methylcoumarin	92-48-8	Non- sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
95	Acetoanisole(4-Methoxyacetophenone)	100-06-1	Non- sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	sensitizer	non-sensitizer
96	Benzalkonium chloride	8001-54-5	Non- sensitizer				sensitizer	sensitizer	sensitizer
97	Benzoic acid	65-85-0	Non- sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	sensitizer	non-sensitizer
98	Benzyl alcohol	100-51-6	Non- sensitizer				non-sensitizer	non-sensitizer	non-sensitizer
99	Chlorobenzene	108-90-7	Non- sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
100	Dextran	9004-54-0	Non- sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
101	Diethyl phthalate	84-66-2	Non- sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	sensitizer	sensitizer	sensitizer
102	Dimethyl formamide	68-12-2	Non- sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
103	Dimethyl isophthalate	1459-93-4	Non- sensitizer				non-sensitizer	non-sensitizer	non-sensitizer
104	Ethyl benzoylacetate	94-02-0	Non- sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	sensitizer	sensitizer	sensitizer
105	Ethyl vaniline	121-32-4	Non- sensitizer				sensitizer	sensitizer	sensitizer
106	Glycerol	56-81-5	Non- sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
107	Hexadecyltrimethylammonium bromide	57-09-0	Non- sensitizer	sensitizer		sensitizer	sensitizer	sensitizer	sensitizer
108	Isopropanol	67-63-0	Non- sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
109	Lactic acid	50-21-5	Non- sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
110	Methyl salicylate	119-36-8	Non- sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	sensitizer	non-sensitizer
111	Octanoic acid(Caprylic acid)	124-07-2	Non- sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	sensitizer	non-sensitizer
112	p-Aminobenzoic acid	150-13-0	Non- sensitizer				non-sensitizer	non-sensitizer	non-sensitizer
113	Propyl paraben	94-13-3	Non- sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	sensitizer	non-sensitizer
114	Propylene glycol	57-55-6	Non- sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
115	Saccharin	81-07-2	Non- sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
116	Salicylic acid	69-72-7	Non- sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
117	Sodium lauryl sulfate	151-21-3	Non- sensitizer				sensitizer	sensitizer	sensitizer
118	Streptomycin sulphate	3810-74-0	Non- sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
119	Sulfanilamide	63-74-1	Non- sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
120	Tween-80	9005-65-6	Non- sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	sensitizer	sensitizer	sensitizer
121	Vanillin	121-33-5	Non- sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
122	Zinc sulphate	7733-02-0	Non- sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer	sensitizer

Appendix 10.1. Chemical selection

Report on the selection of test substances for IL-8 Luc assay validation study

2014/11/1
2015/2/10 ver.2

IL-8 Luc assay Validation Management Team (VMT)

Members for Chemical Selection Committee of IL-8 Luc assay Validation Management Team

Noriho Tanaka (HRI, FDSC and OTIP, Japan), Trial Coordinator
Yutaka Kimura (Tohoku Univ., Japan), Lead Lab
Setsuya Aiba (Tohoku Univ., Japan), Lead Lab
Hajime Kojima (JaCVAM, NIHS, Japan), JaCVAM representative
Hitoshi Sakaguchi (Kao Corporation, Japan)
Hiroshi Itagaki (Yokohama National Univ., Japan)
Joachim Kreysa (EURL ECVAM, Italy), EURL ECVAM liaison
Emanuela Corsini (Milan Univ., Italy), EURL ECVAM liaison
William Stokes (NICEATM/ICCVAM, USA), ICCVMA liaison
Warren Casey (NICEATM/ICCVAM, USA), ICCVMA liaison
David Allen (NICEATM/ICCVAM, USA), ICCVMA liaison
Ai-Young Lee (Dept. of Dermatology, Dongguk Univ. South Korea), KoCVAM, liaison

In this report, the selection process of test substances was described for the IL-8 Luc assay validation study.

The objective of this study was to evaluate the within- and between-laboratory reproducibility and predictivity of the IL-8 Luc assay on skin sensitization potential (concordance with the two classifications: sensitizer and non-sensitizer). As a complementary study, the validation management team (VMT) evaluated the predictability of the United Nations Globally Harmonized System of Classification and Labeling of Chemicals (UN GHS) with three classifications (Category 1A, Category 1B and non-sensitizer).

A pre-validation study and validation studies (Phase I, Phase IIa, Phase IIb, Phase IIc, and Phase III trials) were conducted by three laboratories using the test chemicals shown in Table 1.

In addition, the chemical categories or physical state and chemical properties (e.g., solid: liquid, etc.) were included in the tables of these test chemicals in order to investigate the applicable domain.

Table 1. Breakdown of the IL-8 Luc assay validation study

Phase	The number of the test substances	The number of the repetitions	Examination	Date of experiment start
Pre	3	1	Between- laboratory transferability (Non-coded)	October, 2011
I	10	1	Between- laboratory reproducibility and transferability (Coded)	November, 2011
IIa	10	1	Within- and between-laboratory reproducibility (Coded)	May, 2012
IIb	5	3		November, 2012
IIc	5	3		November, 2013
III	20	1	Between- laboratory reproducibility and predictvity (Coded)	April, 2014
IV	5	3	Within- and between-laboratory reproducibility (Coded)	July, 2015

1. Basic rule for chemical selection

The selection of test chemicals by the Chemical Selection Committee (CSC) in the VMT was based on published papers on *in vivo* skin sensitization tests and validation studies for *in vitro* alternative assays on skin sensitization test methods.

1-1 The applied selection criteria

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

- coverage of a range of toxic effects/potencies
- chemicals that do not require metabolic activation
- appropriate negative and positive controls
- physical and chemical properties (feasibility of use in the experimental set-up as implicated by the CAS No.)
- single chemical entities or formulations of known high purity
- availability
- cost

In the first phase of the selection procedure, Japanese members of the CSC identified and collected several existing lists of potential chemical sensitizers in order to establish a primary database. The resulting list of chemicals tabulated considerations from other validation studies, references of other test methods, test chemicals recommended by ECVAM or Sens-it-iv, and reference chemicals in OECD TG 429. All chemicals had originally been compiled by international experts of CSC for various purposes: e.g., as reference compounds for this validation study. An extensive literature search was performed by CSC in order to ensure that all the pre-selected chemicals fulfilled the selection criteria described above.

Emphasis was placed on the fact that different potencies (Category 1A, Category 1B and non-sensitizer) were chosen. In addition, it was decided that at least 20% of the total chemicals to be tested should be negative in order to increase the statistical power of the data analysis.

1-2 Chemical Acquisition, Coding and Distribution

The assessment of between-laboratory transferability, and within- and between-laboratory reproducibility and predictivity, in all test facilities were performed with the coded chemicals. The study plan for the IL-8 Luc assay validation study described the generation of missing datasets under the coded test item. If the results obtained are not very similar to the previous obtained sets of results, the VMT assessed whether the coded chemicals needed to be tested additionally in all the test facilities.

The coding was supervised by JaCVAM, in collaboration with CSC. CSC was responsible for coding and distributing the test chemicals, references and controls for the validation study.

1-3 Handling

The chemical master at each test facility received complete information considered essential regarding the test chemicals (physical state, weight or volume of sample, specific density for liquid test chemicals, and storage instructions) by JaCVAM. Moreover, the test facility chemical master stored each chemical at conditions in accordance with the storage instructions and received sealed safety information such as the Material Safety Data Sheet (MSDS) describing the hazards identification and exposure controls/personal protection for each chemical. The test chemicals were delivered directly to the study director and the study director was not shown the MSDSs. The study director was to refer to the MSDSs only in the event of an accident. If the study director referred to the MSDS, he/she was not to reveal the content of the MSDS to the test facility technicians.

No accidents occurred during the course of the validation study, and all test facilities returned the MSDSs for the test chemicals to JaCVAM in their sealed envelope upon completion of the validation study. All test chemicals were disposed of in compliance with the rules and regulations of the test facilities upon completion of the validation study.

2. Pre-validation study

Transferability of this assay was checked using three non-coded chemicals (4-NBB: 4-nitrobenzylbromide, TMTD: tetramethyl thiram disulphide and lactic acid) in 4 test facilities, including the lead laboratory. These chemicals were selected by the lead laboratory.

3. Validation study -Phase I trial-

Between-laboratory reproducibility and transferability of this assay was checked using ten coded chemicals in 3 test facilities, as shown in Table 2. These chemicals were selected by CSC based on a table prepared by Drs. Hitoshi Sakaguchi and Hiroshi Itagaki, Japanese members of CSC. All chemicals were selected in consideration of valance UN GHS labeling and physical state (e.g., solid: liquid). The chemicals were coded by JaCVAM as shown in Table 2 and distributed to the test facilities.

Table 2. Chemical code list on the phase I validation trial for IL-8 Luc assay

No.	Chemical name	CAS No.	Supplier	Lab. Code			LLNA EC3	GHS*	Physical state	Storing
				LA	LB	LC				
1	1,4-Phenylenediamine	106-50-3	Wako**	LA002	LB009	LC004	0.16	1A	Solid	2-10 °C
2	2-Mercaptobenzothiasole	149-30-4	Wako	LA003	LB010	LC005	1.7	1A	Solid	rt***
3	2,4-Dinitrochlorobenzene	97-00-7	Wako	LA001	LB008	LC003	0.06	1A	Solid	rt
4	Cinnamal	104-55-2	MP Biomedicals, LLC	LA004	LB001	LC006	3	1B	liquid	4°C
5	Tetramethyl thiuram disulphide	137-26-8	Tokyo Chemical Industry	LA005	LB002	LC007	5.2	1B	Solid	rt
6	Eugenol	97-53-0	Wako	LA006	LB003	LC008	13	1B	liquid	rt
7	Imidazolidinyl urea	39236-46-9	MP Biomedicals, LLC	LA007	LB004	LC009	24	1B	Solid	4°C
8	Lactic acid	50-21-5	Alfa Aesar	LA009	LB006	LC001	ND****	NO	liquid	rt
9	Salicylic acid	69-72-7	Wako	LA008	LB005	LC010	ND	NO	Solid	rt
10	Sodium lauryl sulphate	151-21-3	Wako	LA010	LB007	LC002	ND	NO	Solid	rt

* GHS: GHS category, **Wako: Wako Pure Chemical Industries, Ltd,***rt: room temperature, ****ND: Not Detected

4. Validation study -Phase II trial-

4-1) Phase IIa trial

Ten test chemicals were selected by CSC for between-laboratory reproducibility as shown in Table 3. Five chemicals used in the Phase I trial (cinnamal, 2,4-dinitrochlorobenzene, eugenol, 2-mercaptobenzothiasole, and 1,4-penylenediamine) were joined with five new chemicals selected by CSC based on a table prepared by Japanese members of CSC. The chemicals were coded by JaCVAM as shown in Table 3 and distributed to the test facilities.

Table 3. Chemical code list on the phase IIa validation trial for IL-8 Luc assay

No.	Chemical name	CAS No.	Supplier	Lab. Code			LLNA EC3	GHS*	Physical state	Storing	Phase I
				LA	LB	LC					

1	1,4-Phenylenediamine	106-50-3	Wako**	LA2007	LB2008	LC2010	0.16	1A	Solid	4°C	Used
2	2-Mercaptobenzothiasole	149-30-4	Wako	LA2009	LB2006	LC2003	1.7	1A	Solid	rt***	Used
3	2,4-Dinitrochloroben zene	97-00-7	Wako	LA2008	LB2001	LC2002	0.06	1A	Solid	rt	Used
4	4-Nitrobenzylbromide	100-11-8	Sigma-Aldrich	LA2001	LB2010	LC2006	0.05	1A	Solid	rt	
5	Glyoxal	107-22-2	SAJ	LA2004	LB2005	LC2008	0.8	1A	Liquid	rt	
6	Cinnamal	104-55-2	MP Biomedicals ,LLC	LA2005	LB2007	LC2009	3	1B	Liquid	4°C	Used
7	Eugenol	97-53-0	Wako	LA2002	LB2004	LC2005	13	1B	Liquid	rt	Used
8	Glycerol	56-81-5	Wako	LA2010	LB2002	LC2007	ND****	NO	Liquid	rt	
9	Isopropanol	67-63-0	Nakrai Tesque	LA2006	LB2003	LC2001	ND	NO	Liquid	rt	
10	Methyl salicylate	119-36-8	Wako	LA2003	LB2009	LC2004	ND	NO	Liquid	rt	

* GHS: GHS category, **Wako: Wako Pure Chemical Industries, Ltd,***rt: room temperature, **** ND: Not Detected

4-2) Phase IIb trial

Five test chemicals were selected by CSC for within- and between-laboratory reproducibility, as shown in Table 4. Three runs were tested separately, but the order of testing had no impact on the results. These coded chemicals were selected based on their sensitization potential judged from the list used by the ECVAM validation study.

The chemicals were coded by JaCVAM as shown in Table 4 and distributed to the test facilities. Some chemicals were re-coded for the re-testing at Lab. A. (see the validation report).

Table 4. Chemical code list on the phase IIb validation trial for IL-8 Luc assay

No.	Chemical name	CAS No.	Supplier	Lab. Code			LLNA EC3	GHS*	Physical state	Storing
				LA	LB	LC				
1	p-Benzoquinone	106-51-4	Wako**	LA008	LB010	LC011	0.1	1A	Solid	4°C
				LA013	LB001	LC004				
				LA002	LB009	LC006				
2	1-Thioglycerol	96-27-5	Wako	LA001	LB005	LC010	3.6	1B	Liquid	4°C
				LA010	LB013	LC002				
				LA015	LB003	LC015				
3	Benzyl cinnamate	103-41-3	Wako	LA005	LB015	LC008	18.4	1B	Solid	rt***
				LA012	LB007	LC003				
				LA007	LB012	LC014				
4	Diethyl maleate	141-05-9	Wako	LA004	LB002	LC007	5.8	1B	Liquid	rt
				LA011	LB006	LC013				

				LA009	LB014	LC005				
5	2,4-Dichloronitrobenzene	611-06-3	Aldrich	LA014 LA003 LA006	LB011 LB004 LB008	LC009 LC012 LC001	ND****	NO	Solid	rt

Re-Test

No.	Chemical name	CAS No.	Supplier	Lab. Code
1	p-Benzoquinone	106-51-4	Wako	LA003 LA010 LA007
2	1-Thioglycerol	96-27-5	Wako	LA005 LA011 LA008
3	Benzyl cinnamate	103-41-3	Wako	LA014 LA006 LA001
4	Diethyl maleate	141-05-9	Wako	LA009 LA013 LA015
5	2,4-Dichloronitrobenzene	611-06-3	Aldrich	LA012 LA004 LA002

* GHS: GHS category, **Wako: Wako Pure Chemical Industries, Ltd,***rt: room temperature, ****ND: Not Detected

4-3) Phase IIc trial

Five test chemicals were selected by CSC for within- and between-laboratory reproducibility, as shown in Table 5. Three runs were tested separately, but the order of testing had no impact on the results. These chemicals were selected based on the similarity of their chemical properties and sensitization potentials with the chemicals used in the Phase IIb trial, based on the list used by the ECVAM validation study. The chemicals was coded by JaCVAM as shown in Table 5 and distributed to the test facilities.

Table 5. Chemical code list on the phase IIc validation trial for IL-8 Luc assay

No.	Chemical name	CAS No.	Supplier	Lab. Code			LLNA EC3	GHS*	Physical state	Storing
				LA	LB	LC				
1	1,4-Phenylenediamine	106-50-3	Wako**	LA021 LA029 LA032	LB024 LB027 LB035	LC022 LC030 LC033	0.0099	1A	Solid	2-10°C
2	2-Mercaptobenzothiazole	149-30-4	Wako	LA022 LA030 LA033	LB025 LB028 LB031	LC023 LC026 LC034	1.7	1A	Solid	rt***
3	Glycerol	56-81-5	Wako	LA023 LA026 LA034	LB021 LB029 LB032	LC024 LC027 LC035	ND****	NO	Liquid	rt
	Isopropanol	67-63-0	Wako	LA025	LB023	LC021	ND	NO	Liquid	rt

4				LA028 LA031	LB026 LB034	LC029 LC032				
5	Methyl salicylate	119-36-8	Wako	LA024 LA027 LA035	LB022 LB030 LB033	LC025 LC028 LC031	ND	NO	Liquid	rt

* GHS: GHS category, **Wako: Wako Pure Chemical Industries, Ltd,***rt: room temperature, ****ND: Not Detected

5. Validation study -Phase III trial-

Twenty chemicals were selected for between-laboratory reproducibility and predictivity by CSC. These chemicals were selected based on their chemical properties, results obtained in earlier phase studies and sensitization potential as judged based on tabulated data from the Phase II trials, the list of chemicals provided by the ECVAM validation study, and the list prepared by Japanese members of CSC at the chemical selection meeting in Kyoto on February 26, 2014 (Table 6). The chemicals were coded by JaCVAM as shown in Table 6 and distributed to the test facilities.

Due to the conflicting results obtained in the Phase II studies, 2,4-dichloronitrobenzene was selected again, as shown in Table 7.

Table 6. Chemical code list on the phase III validation study for IL-8 Luc assay

No.	Chemical name	CAS No.	Supplier	Lab. Code			LLNA EC3	GHS*	Physical state	Storing
				LA	LB	LC				
1	2,4-Dinitrochlorobenzene	97-00-7	Wako**	LA102	LB119	LC116	0.06	1A	Solid	rt***
2	Beryllium Sulphate	7787-56-6	Wako	LA107	LB104	LC101	0.001	1A	Solid	rt
3	Chloramine T	127-65-1	Wako (MP)	LA110	LB107	LC104	0.4	1A	Solid	4°C
4	Chlorpromazine HCl	69-09-0	Nacalai tesque	LA109	LB106	LC103	0.14	1A	Solid	rt
5	Formaldehyde	50-00-0	Wako	LA108	LB105	LC102	0.61	1A	Liquid	rt
6	Glyoxal	107-22-2	Sigma-Aldrich	LA103	LB120	LC117	0.8	1A	Liquid	rt
7	1,2-Benzisothiazol-3(2H)-one	2634-33-5	Wako	LA104	LB101	LC118	8	1B	Solid	rt
8	Benzyl Salicylate	118-58-1	Wako	LA111	LB108	LC105	2.9	1B	Liquid	rt
9	Citral	5392-40-5	Aldrich	LA105	LB102	LC119	5	1B	Liquid	rt
10	Dihydroeugenol	2785-87-7	Wako	LA112	LB109	LC106	6.8	1B	Liquid	rt
11	Imidazolidinyl urea	39236-46-9	Sigma	LA114	LB111	LC108	24	1B	Solid	4°C
12	Methylmethacrylate	80-62-6	Sigma	LA116	LB113	LC110	90	1B	Liquid	rt
13	R(+)Limonene	5989-27-5	Wako	LA115	LB112	LC109	69	1B	Liquid	rt
14	Nickel Chloride	7718-54-9	Wako	LA113	LB110	LC107	ND****	1B	Solid	rt
15	2,4-Dichloronitrobenzene	611-06-3	Aldrich	LA101	LB118	LC115	ND	NO	Solid	rt
16	4-Aminobenzoic acid	150-13-0	Sigma	LA119	LB116	LC113	ND	NO	Solid	rt
17	4-Hydroxybenzoic acid	99-96-7	Aldrich	LA106	LB103	LC120	ND	NO	Solid	rt
18	Benzyl alcohol	100-51-6	Aldrich	LA117	LB114	LC111	ND	NO	Liquid	rt
19	Dimethyl Isophthalate	1459-93-4	Wako	LA118	LB115	LC112	ND	NO	Solid	rt
20	Xylene	1330-20-7	Wako	LA120	LB117	LC114	ND	NO	Liquid	rt

* GHS: GHS category, **Wako: Wako Pure Chemical Industries, Ltd,***rt: room temperature, ****ND: Not Detected

6. Outcome of validation study

To evaluate the predictivity of this assay, the VMT selected 29 chemicals used in Phase II and III studies, as shown in Table 7. Twenty-three test chemicals used in the ECVAM validation study are included, but MCI/MI (Kathon CG) was excluded as it is not commercially available in Japan.

Table 7. Chemical code list on the validation study for IL-8 Luc assay

No.	Chemical name	CAS No.	LLNA EC3	GHS category	Physical state	Phase
PC	Cobalt chloride	7646-79-9	0.6	1A	Solid	IIb, IIc & III
1	p-Benzoquinone	106-51-4	0.01	1A	Solid	IIb**
2	1,4-Phenylenediamine	106-50-3	0.11	1A	Solid	IIc**
3	2-Mercaptobenzothiazole	149-30-4	1.7	1A	Solid	IIc**
4	2,4-Dinitrochlorobenzene	97-00-7	0.06	1A	Solid	III
5	Beryllium sulfate	7787-56-6	0.001	1A	Solid	III**
6	Chloramine T	127-65-1	0.4	1A	Solid	III**
7	Chlorpromazine HCl	69-09-0	0.14	1A	Solid	III**
8	Formaldehyde	50-00-0	0.61	1A	Liquid	III**
9	Glyoxal	107-22-2	0.8	1A	Liquid	III
10	1-Thioglycerol	96-27-5	3.6	1B	Liquid	IIb**
11	1,2-Benzosothiazolin-3-one	2634-33-5	8	1B	Solid	III
12	Benzylcinnamate	103-41-3	18.4	1B	Solid	IIb**
13	Benzylsalicylate	118-58-1	2.9	1B	Liquid	III**
14	Citral	5392-40-5	5	1B	Liquid	III
15	Dietyhyl maleate	141-05-9	5.8	1B	Liquid	IIb
16	Dihydroeugenol	2785-87-7	6.8	1B	Liquid	III**
17	Imidazolidinylurea	39236-46-9	24	1B	Solid	III**
18	Methylmethacrylate	80-62-6	90	1B	Liquid	III**
19	R(+)-Limonene	5989-27-5	69	1B	Liquid	III**
20	Nickel chloride	7718-54-9	ND***	1B	Solid	III**
21	2,4-Dichloronitrobenzene	611-06-3	ND	NO	Solid	IIb& III**
22	4-Aminobenzoic acid	150-13-0	ND	NO	Solid	III**
23	4-Hydroxybenzoic acid	99-96-7	ND	NO	Solid	III
24	Benzyl alcohol	100-51-6	ND	NO	Liquid	III**
25	Dimethylisophthalate	1459-93-4	ND	NO	Solid	III**
26	Glycerol	56-81-5	ND	NO	Liquid	IIc**

27	Isopropanol	67-63-0	ND	NO	Liquid	IIc**
28	Methylsalicylate	119-36-8	ND	NO	Liquid	IIc**
29	Xylene	1330-20-7	ND	NO	Liquid	III**

*:PC:Posotove Control, **: Based on the ECVAM validation study, ***ND: Not Detected

References

1) OECD (2005). Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment. OECD Testing Series and Assessment Number 34. 281 ENV/JM/MONO(2005)14, pp 96, Paris, France: OECD. 282

2) Hartung T, Bremer S, Casati S, Coecke S, Corvi R, Fortaner S, Gribaldo L, Halder M, Hoffmann S, 283 Janusch Roi A, Prieto P, Sabbioni E, Scott L, Worth A and Zuang V (2004). A Modular Approach to 284 the ECVAM Principles on Test Validity. ATLA 32, 467–472. 285

Appendix 10.2. List of candidate chemicals for phase IV study

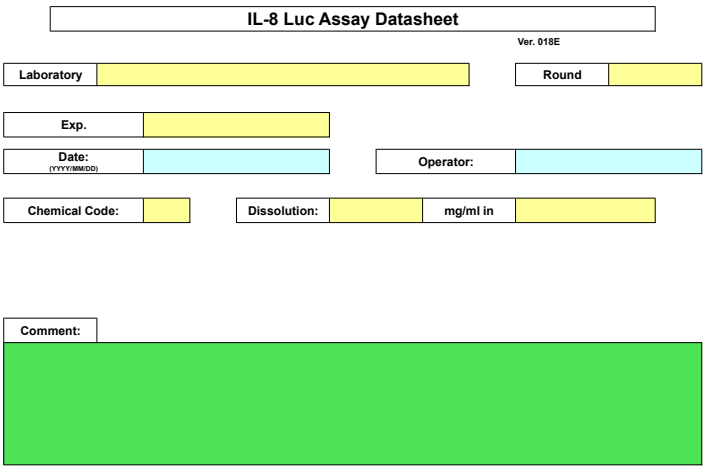
Re-evaluation of chemicals demonstrating false negative results by the IL-8 Luc assay using X-VIVO as a solvent

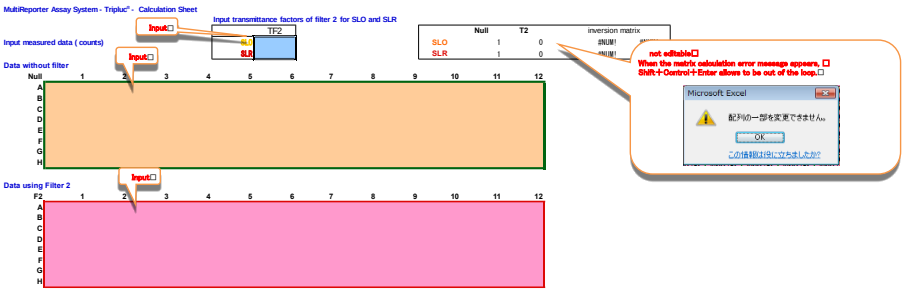
Chemicals	Experiments		Judgment
	1st	2nd	
Oxazolone	1.66	3.98	sensitizer
Phthalic anhydride	1.09	1.60	
2-Hydroxyethyl acrylate	1.00	1.00	
Ethylenediamine	1.96	2.38	sensitizer
Methyl-2-nonynoate	1.93	5.91	sensitizer
3,4-Dihydrocoumarin	1.00	1.00	
Trimellitic anhydride	2.28	1.66	sensitizer
1-Bromohexane	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	

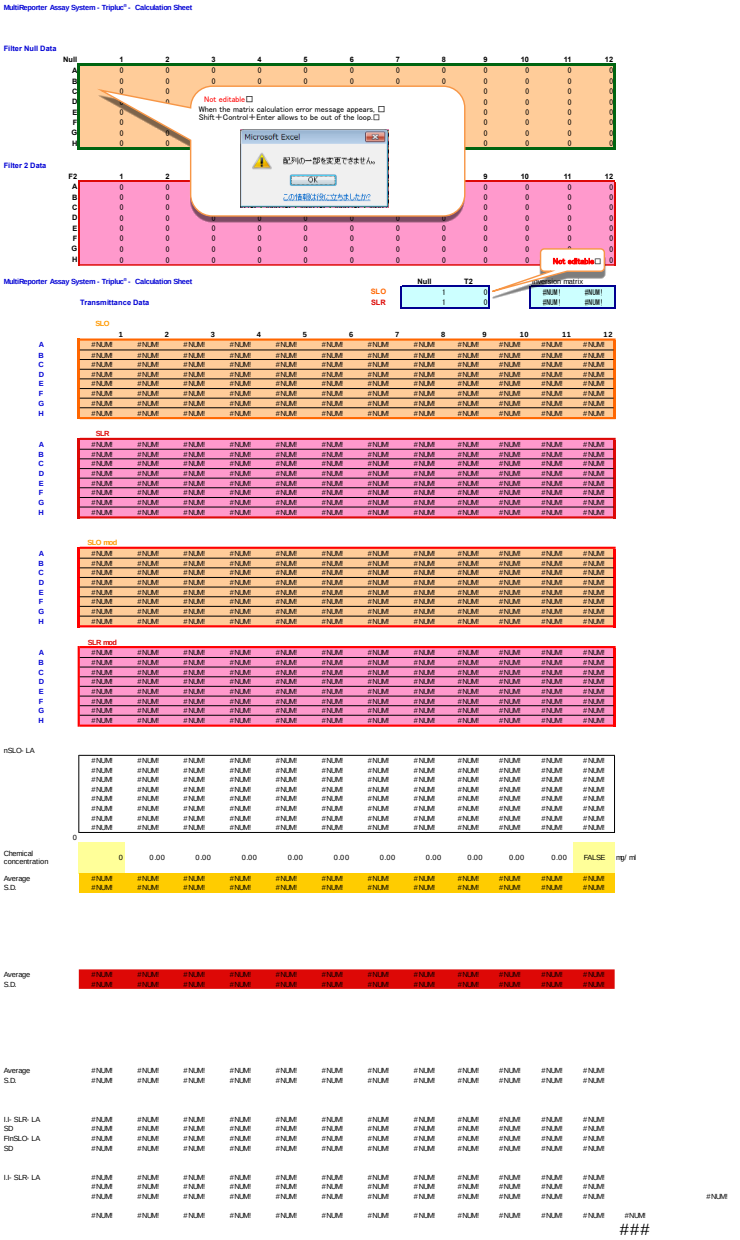
Re-evaluation of chemicals in the ECVAM list by the IL-8 Luc assay using X-VIVO as a solvent

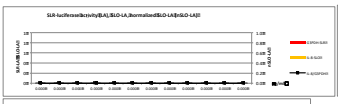
Chemicals	Experiments		Judgment
	1st	2nd	
Oxazolone	7.69	3.97	sensitizer
4-NBB	10.03	8.77	sensitizer
Glyoxal	3.50	2.23	sensitizer
2-MBT	5.17	4.37	sensitizer
DNCB	14.54	9.48	sensitizer
MDGN	4.23	4.35	sensitizer
Cinnamal	1.53	5.57	sensitizer
TMTD	4.58	3.61	sensitizer
PPD	1.05	2.48	sensitizer
Isoeugenol	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	1.49	1.08	non-sensitizer
SLS	2.94	2.88	sensitizer

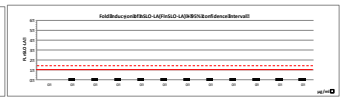
Appendix 11. The IL-8 Luc assay Data sheet

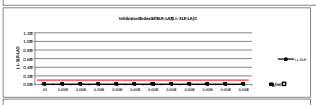


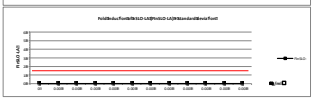

MultiReporter Assay System - Triplus - Calculation Sheet
Input transmittance factors of filter 2 for SLO and SLR
Input
TF2
SLO
SLR
Input measured data (counts)
Input
Data without filter
Null
1 2 3 4 5 6 7 8 9 10 11 12
A B C D E F G H
Input
Data using Filter 2
F2
1 2 3 4 5 6 7 8 9 10 11 12
A B C D E F G H
Null
T2
inversion matrix
SLO 1 0
SLR 1 0
not editable
When the matrix calculation error message appears, Shift+Control+Enter allows to be out of the loop.
Microsoft Excel
配列の一部を変更できません。
OK
この情報は役に立ちましたか?


MultiReporter Assay System - Triplus® - Calculation Sheet
Filter Null Data
Not editable☐
When the matrix calculation error message appears, ☐
Shift+Control+Enter allows to be out of the loop.☐
Microsoft Excel
配列の一部を変更できません。
OK
この情報は役に立ちましたか?
Filter 2 Data
Not editable☐
MultiReporter Assay System - Triplus® - Calculation Sheet
Transmittance Data
SLO
SLR
SLO mod
SLR mod
nSLO- LA
Chemical concentration
Average
S.D.
I.I- SLR- LA
SD
FnSLO- LA
mg/ ml





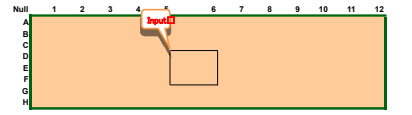


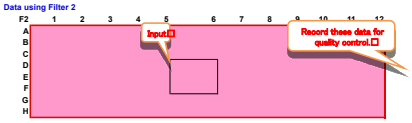


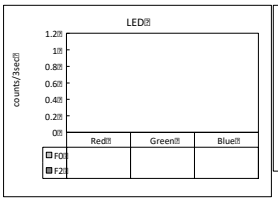
Judge #NUM! 0

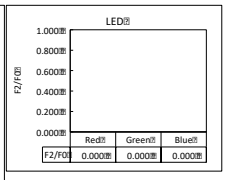
	(0)	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
FInSLO-LAi	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
lower□mit		#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
upper□mit		#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!

													Judge
F. I	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	
95%	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
95%-F. I	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!









	F0	F2	F2/F0
Red	0	0	#DIV/0!
Green	0	0	#DIV/0!
Blue	0	0	#DIV/0!

MultiReporter Assay System - Tripluc® - Calculation Sheet

Filter Null Data

Null	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	0	0	0	0	0	0	0	0	0
B	0	0	0	0	0	0	0	0	0	0	0	0
C	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0	0	0	0
D	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0	0	0	0
E	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0	0	0	0
F	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0	0	0	0
G	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0	0	0	0
H	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0	0	0	0

not editable
When the matrix calculation error message appears,
Shift＋Control＋Enter allows to be out of the loop.

Microsoft Excel
配列の一部を変更できません。
OK
この情報は役に立ちましたか?

Filter 2 Data

F2	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0	0	0	0
B	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0	0	0	0
C	0	0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0	0	0	0
D	0	0	0	0	0	0	0	0	0	0	0	0
E	0	0	0	0	0	0	0	0	0	0	0	0
F	0	0	0	0	0	0	0	0	0	0	0	0
G	0	0	0	0	0	0	0	0	0	0	0	0
H	0	0	0	0	0	0	0	0	0	0	[illegible]	0

not editable

MultiReporter Assay System - Tripluc® - Calculation Sheet

Transmittance Data

	Null	T2
SLO	1	0
SLR	1	0

Inversion matrix

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

SLO

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

SLR

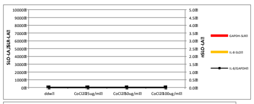
A	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
B	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
C	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
D	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
E	#NUM!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

nSLO- LA

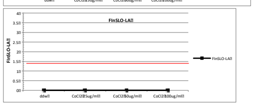
#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
#NUM!	#NUM!	#NUM!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

	NAC(-)						FInSLO- LA	I.I.- SLR- LA	
	Average	STDEV	Average	STDEV	Average	STDEV	NAC(-)	NAC(-)	
ddw	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
CoCl2 25ug	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
CoCl2 50ug	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!
CoCl2 100u	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!	#NUM!

#NUM!



#NUM!



X:without
Y:with

without	#NUM!	without.ave	#NUM!	比	#NUM!
	#NUM!	without.sd	#NUM!	下限	
	#NUM!	n(without)	0	上限	
	#NUM!				
(1)	#NUM!	with.ave	#NUM!	比	#NUM!
	#NUM!	with.sd	#NUM!	下限	#NUM!
	#NUM!	n(with)	0	上限	#NUM!
	#NUM!				
(2)	#NUM!	with.ave	#NUM!	比	#NUM!
	#NUM!	with.sd	#NUM!	下限	#NUM!
	#NUM!	n(with)	0	上限	#NUM!
	#NUM!				
(3)	#NUM!	with.ave	#NUM!	比	#NUM!
	#NUM!	with.sd	#NUM!	下限	#NUM!
	#NUM!	n(with)	0	上限	#NUM!
	#NUM!				
(4)	#NUM!	with.ave	#NUM!	比	#NUM!
	#NUM!	with.sd	#NUM!	下限	#NUM!
	#NUM!	n(with)	0	上限	#NUM!
	#NUM!				
(5)	#NUM!	with.ave	#NUM!	比	#NUM!
	#NUM!	with.sd	#NUM!	下限	#NUM!
	#NUM!	n(with)	0	上限	#NUM!
	#NUM!				
(6)	#NUM!	with.ave	#NUM!	比	#NUM!
	#NUM!	with.sd	#NUM!	下限	#NUM!
	#NUM!	n(with)	0	上限	#NUM!
	#NUM!				
(7)	#NUM!	with.ave	#NUM!	比	#NUM!
	#NUM!	with.sd	#NUM!	下限	#NUM!
	#NUM!	n(with)	0	上限	#NUM!
	#NUM!				
(8)	#NUM!	with.ave	#NUM!	比	#NUM!
	#NUM!	with.sd	#NUM!	下限	#NUM!
	#NUM!	n(with)	0	上限	#NUM!
	#NUM!				
(9)	#NUM!	with.ave	#NUM!	比	#NUM!
	#NUM!	with.sd	#NUM!	下限	#NUM!
	#NUM!	n(with)	0	上限	#NUM!
	#NUM!				
(10)	#NUM!	with.ave	#NUM!	比	#NUM!
	#NUM!	with.sd	#NUM!	下限	#NUM!
	#NUM!	n(with)	0	上限	#NUM!
	#NUM!				
(11)	#NUM!	with.ave	#NUM!	比	#NUM!
	#NUM!	with.sd	#NUM!	下限	#NUM!
	#NUM!	n(with)	0	上限	#NUM!
	#NUM!				

	Judge
I.I-SLR-LA	#NUM!
95%CI	#NUM!
95%CI+F.I	#NUM!

Appendix 12. The summary of the study by the independent biostatistician

Until the present, the phase 4 study following ***the protocol ver. 20E*** was finished. The study attained the success criteria pre-specified in ***the study plan appendix 2***, in the case that the criterion 3 for experiment run was used.

After the phase 4 study, the VMT meeting was held. At the meeting the VMT finally selected the criterion 3 as the criterion of the judgment for each run.

This is the update summary for the results of the IL-8 Luc assay validation study, and the all the results in this report are based on the criterion 3.

1. Basic results

Table 1 shows the judgment of each run for each chemical in the phase IIb, IIc and IV studies. “P” and “N” means judged “Positive” and “Negative”. The column “Judge” lists the final judgment of each chemical in each laboratory. “Undetermined” means the final judge could not met because of the short of the number of experiment to identify sensitizers. The column “Orig. Code” lists the original code labeled to the distributed chemicals to each laboratory. Table 2 is the same table for the phase III study.

Table 1 The results of Phase IIb, IIc and IV studies

Lab. A		Experiment				
Chem. Code	Orig. Code	1	2	3	4	Judge
P2b01-1	3	N	P			Undetermined
P2b01-2	10	P	P			Sens.
P2b01-3	7	N	P			Undetermined
P2b02-1	5	P	P	P	P	Sens.
P2b02-2	11	P	P	N		Sens.
P2b02-3	8	P	P	P		Sens.
P2b03-1	14	P	P	P	P	Sens.
P2b03-2	6	P	P	P		Sens.
P2b03-3	1	P	P	P	P	Sens.
P2b04-1	9	N	N	N		Non-Sens.
P2b04-2	13	N	N	N		Non-Sens.
P2b04-3	15	N	N	N		Non-Sens.
P2b05-1	12	N	N	N		Non-Sens.
P2b05-2	4	N	N	N		Non-Sens.
P2b05-3	2	N	N	N	N	Non-Sens.
P2c08-1	21	P	P			Sens.
P2c08-2	29	P	N	N	P	Sens.
P2c08-3	32	N	P	N	P	Sens.
P2c12-1	22	P	P			Sens.
P2c12-2	30	P	P			Sens.
P2c12-3	33	P	P			Sens.
P2c19-1	23	N	N	N		Non-Sens.
P2c19-2	26	P	N	N	N	Non-Sens.
P2c19-3	34	P	N	N	N	Non-Sens.
P2c21-1	24	N	P	N	N	Non-Sens.
P2c21-2	27	N	N	N		Non-Sens.
P2c21-3	35	N	N	N		Non-Sens.
P2c22-1	25	N	N	N		Non-Sens.
P2c22-2	28	P	N	N	P	Sens.
P2c22-3	31	N	N	N		Non-Sens.
P4-01-1	201	P	P			Sens.
P4-01-2	209	P	P			Sens.
P4-01-3	212	P	P			Sens.
P4-02-1	202	P	P			Sens.
P4-02-2	210	P	P			Sens.
P4-02-3	213	P	P			Sens.
P4-03-1	203	P	P			Sens.
P4-03-2	206	P	P			Sens.
P4-03-3	214	P	P			Sens.
P4-04-1	204	N	N	P	P	Sens.
P4-04-2	207	N	P	P		Sens.
P4-04-3	215	P	P			Sens.
P4-05-1	205	N	N	N		Non-Sens.
P4-05-2	208	N	P	P		Sens.
P4-05-3	211	P	N	N	N	Non-Sens.

Lab. B		Experiment				
Chemical	Orig. Code	1	2	3	4	Judge
P2b01-1	10	P	N			Undetermined
P2b01-2	1	P	P			Sens.
P2b01-3	9	P	P			Sens.
P2b02-1	5	P	P	P	P	Sens.
P2b02-2	13	P	P	P		Sens.
P2b02-3	3	P	P			Sens.
P2b03-1	15	P	P			Sens.
P2b03-2	7	P	P			Sens.
P2b03-3	12	P	P	P		Sens.
P2b04-1	2	N	N	N	N	Non-Sens.
P2b04-2	6	N	N	N		Non-Sens.
P2b04-3	14	N	N	N		Non-Sens.
P2b05-1	11	N	N	N		Non-Sens.
P2b05-2	4	N	N			Undetermined
P2b05-3	8	N	N	P		Undetermined
P2c08-1	24	N	P	N	P	Sens.
P2c08-2	27	N	N	N	P	Non-Sens.
P2c08-3	35	P	P			Sens.
P2c12-1	25	N	N	P		Undetermined
P2c12-2	28	P	P			Sens.
P2c12-3	31	P	P			Sens.
P2c19-1	21	N	N	N	N	Non-Sens.
P2c19-2	29	N	N	N		Non-Sens.
P2c19-3	32	N	P	N	N	Non-Sens.
P2c21-1	22	P	P			Sens.
P2c21-2	30	P	P			Sens.
P2c21-3	33	N	N	P	P	Sens.
P2c22-1	23	N	N	N		Non-Sens.
P2c22-2	26	N	N	N		Non-Sens.
P2c22-3	34	N	N	P	N	Non-Sens.
P4-01-1	204	N	N	P	P	Sens.
P4-01-2	207	P	P			Sens.
P4-01-3	215	P	P			Sens.
P4-02-1	205	P	P			Sens.
P4-02-2	208	P	P			Sens.
P4-02-3	211	P	P			Sens.
P4-03-1	201	P	P			Sens.
P4-03-2	209	P	P			Sens.
P4-03-3	212	P	P			Sens.
P4-04-1	202	P	N	N	N	Non-Sens.
P4-04-2	210	P	N	N	N	Non-Sens.
P4-04-3	213	P	N	N	N	Non-Sens.
P4-05-1	203	N	P	N	N	Non-Sens.
P4-05-2	206	P	N	N	N	Non-Sens.
P4-05-3	214	N	N	N		Non-Sens.

Lab. C		Experiment				
Chemical	Orig. Code	1	2	3	4	Judge
P2b01-1	11	P	P			Sens.
P2b01-2	4	P	P			Sens.
P2b01-3	6	P	P			Sens.
P2b02-1	10	P	P	P	P	Sens.
P2b02-2	2	P	P	P		Sens.
P2b02-3	15	P	P	P		Sens.
P2b03-1	8	P	P	P	P	Sens.
P2b03-2	3	P	P	P		Sens.
P2b03-3	14	P	P	P		Sens.
P2b04-1	7	N	N	N		Non-Sens.
P2b04-2	13	N	N	N	N	Non-Sens.
P2b04-3	5	N	N	P	N	Non-Sens.
P2b05-1	9	N	P	N		Undetermined
P2b05-2	12	N	N	N	N	Non-Sens.
P2b05-3	1	N	P	N	N	Non-Sens.
P2c08-1	22	N	P	N	N	Non-Sens.
P2c08-2	30	N	P	N	N	Non-Sens.
P2c08-3	33	P	N	P		Sens.
P2c12-1	23	N	P	P		Sens.
P2c12-2	26	P	P			Sens.
P2c12-3	34	P	P			Sens.
P2c19-1	24	N	N	N	N	Non-Sens.
P2c19-2	27	N	N	N		Non-Sens.
P2c19-3	35	N	N	N	N	Non-Sens.
P2c21-1	25	N	N	P	N	Non-Sens.
P2c21-2	28	N	N	P	P	Sens.
P2c21-3	31	N	P	N	P	Sens.
P2c22-1	21	N	N	N		Non-Sens.
P2c22-2	29	N	N	N	N	Non-Sens.
P2c22-3	32	N	N	N		Non-Sens.
P4-01-1	202	P	P			Sens.
P4-01-2	210	P	P			Sens.
P4-01-3	213	P	P			Sens.
P4-02-1	203	P	P			Sens.
P4-02-2	206	P	P			Sens.
P4-02-3	214	P	P			Sens.
P4-03-1	204	P	P			Sens.
P4-03-2	207	P	P			Sens.
P4-03-3	215	P	P			Sens.
P4-04-1	205	N	N	N		Non-Sens.
P4-04-2	208	N	P	N	N	Non-Sens.
P4-04-3	211	N	P	N	N	Non-Sens.
P4-05-1	201	N	P	N	N	Non-Sens.
P4-05-2	209	N	N	N		Non-Sens.
P4-05-3	212	N	N	N		Non-Sens.

Table 2 The results of Phase III studies

Lab. A		Experiment				
Chem. Code	Orig. Code	1	2	3	4	Judge
P3-01	101	P	N	N	N	Non-Sens.
P3-02	102	P	P			Sens.
P3-03	103	P	P			Sens.
P3-04	104	P	P			Sens.
P3-05	105	P	P			Sens.
P3-06	106	N	N	N	N	Non-Sens.
P3-07	107	N	P	N	N	Non-Sens.
P3-08	108	N	P	P		Sens.
P3-09	109	P	P			Sens.
P3-10	110	N	P	P		Sens.
P3-11	111	P	P			Sens.
P3-12	112	P	N	P		Sens.
P3-13	113	P	P			Sens.
P3-14	114	N	P	P		Sens.
P3-15	115	P	P			Sens.
P3-16	116	N	N	N		Non-Sens.
P3-17	117	N	N	N	N	Non-Sens.
P3-18	118	N	N	N		Non-Sens.
P3-19	119	N	N	N		Non-Sens.
P3-20	120	N	N	N	N	Non-Sens.

Lab. B		Experiment				
Chem. Code	Orig. Code	1	2	3	4	Judge
P3-01	118	N	N	N		Non-Sens.
P3-02	119	P	P			Sens.
P3-03	120	N	N	P		undetermined
P3-04	101	P	P			Sens.
P3-05	102	P	P			Sens.
P3-06	103	N	N	N		Non-Sens.
P3-07	104	N	N	N		Non-Sens.
P3-08	105	P	P			Sens.
P3-09	106	P	P			Sens.
P3-10	107	P	P			Sens.
P3-11	108	P	P			Sens.
P3-12	109	P	P			Sens.
P3-13	110	P	P			Sens.
P3-14	111	P	N	P		Sens.
P3-15	112	N	N	N	N	Non-Sens.
P3-16	113	N	N	N	N	Non-Sens.
P3-17	114	N	N	N		Non-Sens.
P3-18	115	N	N	N	N	Non-Sens.
P3-19	116	N	N	N		Non-Sens.
P3-20	117	N	N	N		Non-Sens.

Lab. C		Experiment				
Chem. Code	Orig. Code	1	2	3	4	Judge
P3-01	115	N	N	N		Non-Sens.
P3-02	116	P	P			Sens.
P3-03	117	P	P			Sens.
P3-04	118	P	P			Sens.
P3-05	119	P	P			Sens.
P3-06	120	N	N	N	N	Non-Sens.
P3-07	101	N	N	N		Non-Sens.
P3-08	102	N	P	P		Sens.
P3-09	103	P	P			Sens.
P3-10	104	P	P			Sens.
P3-11	105	P	N	P		Sens.
P3-12	106	P	P			Sens.
P3-13	107	P	P			Sens.
P3-14	108	P	P			Sens.
P3-15	109	N	N	N		Non-Sens.
P3-16	110	N	N	N	N	Non-Sens.
P3-17	111	N	N	N		Non-Sens.
P3-18	112	N	P	N	N	Non-Sens.
P3-19	113	N	N	N	N	Non-Sens.
P3-20	114	N	N	N		Non-Sens.

2. Within-laboratory reproducibility

Table 3 shows the final judgment of each set in each chemical, and the concordance based on the results in the phase IIb, IIc and IV.

Table 3 Within-laboratory reproducibility

Lab. A	Set No.			
Chem. Code	1	2	3	Concordance
P2b02	Sens.	Sens.	Sens.	1
P2b03	Sens.	Sens.	Sens.	1
P2b04	Non-Sens.	Non-Sens.	Non-Sens.	1
P2b05	Non-Sens.	Non-Sens.	Non-Sens.	1
P2c08	Sens.	Sens.	Sens.	1
P2c12	Sens.	Sens.	Sens.	1
P2c19	Non-Sens.	Non-Sens.	Non-Sens.	1
P2c21	Non-Sens.	Non-Sens.	Non-Sens.	1
P2c22	Non-Sens.	Sens.	Non-Sens.	0
P4-01	Sens.	Sens.	Sens.	1
P4-02	Sens.	Sens.	Sens.	1
P4-03	Sens.	Sens.	Sens.	1
P4-04	Sens.	Sens.	Sens.	1
P4-05	Non-Sens.	Sens.	Non-Sens.	0

Lab. B	Set No.			
Chem. Code	1	2	3	Concordance
P2b02	Sens.	Sens.	Sens.	1
P2b03	Sens.	Sens.	Sens.	1
P2b04	Non-Sens.	Non-Sens.	Non-Sens.	1
P2c08	Sens.	Non-Sens.	Sens.	0
P2c19	Non-Sens.	Non-Sens.	Non-Sens.	1
P2c21	Sens.	Sens.	Sens.	1
P2c22	Non-Sens.	Non-Sens.	Non-Sens.	1
P4-01	Sens.	Sens.	Sens.	1
P4-02	Sens.	Sens.	Sens.	1
P4-03	Sens.	Sens.	Sens.	1
P4-04	Non-Sens.	Non-Sens.	Non-Sens.	1
P4-05	Non-Sens.	Non-Sens.	Non-Sens.	1

Lab. C	Set No.			
Chem. Code	1	2	3	Concordance
P2b01	Sens.	Sens.	Sens.	1
P2b02	Sens.	Sens.	Sens.	1
P2b03	Sens.	Sens.	Sens.	1
P2b04	Non-Sens.	Non-Sens.	Non-Sens.	1
P2c08	Non-Sens.	Non-Sens.	Sens.	0
P2c12	Sens.	Sens.	Sens.	1
P2c19	Non-Sens.	Non-Sens.	Non-Sens.	1
P2c21	Non-Sens.	Sens.	Sens.	0
P2c22	Non-Sens.	Non-Sens.	Non-Sens.	1
P4-01	Sens.	Sens.	Sens.	1
P4-02	Sens.	Sens.	Sens.	1
P4-03	Sens.	Sens.	Sens.	1
P4-04	Non-Sens.	Non-Sens.	Non-Sens.	1
P4-05	Non-Sens.	Non-Sens.	Non-Sens.	1

Table 4 shows the concordance rate of the within-laboratory reproducibility which is estimated by data of table 3.

Table 4 Within-laboratory concordance rate

Statistics	Lab A	Lab B	Lab C	Average
Intra-laboratory Concodance Rate	85.7% (12/14)	91.7% (11/12)	85.7% (12/14)	87.70%

3. Between-laboratory reproducibility

Table 5 shows the results the final judgment of each chemical in each laboratory, and the concordance in the phase IIb, IIc, III and IV. In the case of the unknown majority for 3 sets, “Undetermined” is listed, and the concordance is missing because the evaluation of the between-laboratory reproducibility should be based on the results of 3 laboratories.

Table 5 Between-laboratory reproducibility

Chem. Code	Lab. A	Lab. B	Lab. C	Concordance
P2b01	Undecided	Sens.	Sens.	.
P2b02	Sens.	Sens.	Sens.	1
P2b03	Sens.	Sens.	Sens.	1
P2b04	Non-Sens.	Non-Sens.	Non-Sens.	1
P2b05	Non-Sens.	Undecided	Non-Sens.	.
P2c08	Sens.	Sens.	Non-Sens.	0
P2c12	Sens.	Sens.	Sens.	1
P2c19	Non-Sens.	Non-Sens.	Non-Sens.	1
P2c21	Non-Sens.	Sens.	Sens.	0
P2c22	Non-Sens.	Non-Sens.	Non-Sens.	1
P3-01	Non-Sens.	Non-Sens.	Non-Sens.	1
P3-02	Sens.	Sens.	Sens.	1
P3-03	Sens.	Gray	Sens.	.
P3-04	Sens.	Sens.	Sens.	1
P3-05	Sens.	Sens.	Sens.	1
P3-06	Non-Sens.	Non-Sens.	Non-Sens.	1
P3-07	Non-Sens.	Non-Sens.	Non-Sens.	1
P3-08	Sens.	Sens.	Sens.	1
P3-09	Sens.	Sens.	Sens.	1
P3-10	Sens.	Sens.	Sens.	1
P3-11	Sens.	Sens.	Sens.	1
P3-12	Sens.	Sens.	Sens.	1
P3-13	Sens.	Sens.	Sens.	1
P3-14	Sens.	Sens.	Sens.	1
P3-15	Sens.	Non-Sens.	Non-Sens.	0
P3-16	Non-Sens.	Non-Sens.	Non-Sens.	1
P3-17	Non-Sens.	Non-Sens.	Non-Sens.	1
P3-18	Non-Sens.	Non-Sens.	Non-Sens.	1
P3-19	Non-Sens.	Non-Sens.	Non-Sens.	1
P3-20	Non-Sens.	Non-Sens.	Non-Sens.	1
P4-01	Sens.	Sens.	Sens.	1
P4-02	Sens.	Sens.	Sens.	1
P4-03	Sens.	Sens.	Sens.	1
P4-04	Sens.	Non-Sens.	Non-Sens.	0
P4-05	Non-Sens.	Non-Sens.	Non-Sens.	1

Table 6 shows the concordance rate of the between-laboratory reproducibility which is estimated by data of Table 5.

Table 6 Concordance rate of the between-laboratory reproducibility

Satistics	
Inter-laboratory Concodance Rate	87.5% (28/32)

4. Predictively (Overall)

Table 7 shows the list of the GHS classification on *in vivo* and the final judgment on the IL-8 Luc assay, which is tabled by the results of the majority between 3 laboratories and these of each laboratory. The “Base on Majority” column means the judgment of the majority for the following 3 columns.

Note: Because of chemical duplication, the P-2B05 (2,4-Dichloronitrobenzene) was eliminated for

evaluation of predictively.

Table 7 Predictivity of the IL-8 Luc assay compared with GHS classification

Chem. Code	Chemical	GHS	Based on Majority	Lab. A	Lab. B	Lab. C
P2b01	Benzoquinone	1A	Sens.	Undetermined	Sens.	Sens.
P2b02	Thioglycerol	1B	Sens.	Sens.	Sens.	Sens.
P2b03	Benzyl cinnamate	1B	Sens.	Sens.	Sens.	Sens.
P2b04	Diethyl maleate	1B	Non-Sens.	Non-Sens.	Non-Sens.	Non-Sens.
P2c08	1,4-Phenylenediamine	1A	Sens.	Sens.	Sens.	Non-Sens.
P2c12	2-MBT(2-mercaptobenzothiazole)	1A	Sens.	Sens.	Sens.	Sens.
P2c19	Glycerol	NC	Non-Sens.	Non-Sens.	Non-Sens.	Non-Sens.
P2c21	Methyl salicylate	NC	Sens.	Non-Sens.	Sens.	Sens.
P2c22	Isopropanol	NC	Non-Sens.	Non-Sens.	Non-Sens.	Non-Sens.
P3-01	2,4-Dichloronitrobenzene	NC	Non-Sens.	Non-Sens.	Non-Sens.	Non-Sens.
P3-02	DNCB(1-Chloro-2,4,-dinitrobenzene)	1A	Sens.	Sens.	Sens.	Sens.
P3-03	Glyoxal solution	1A	Sens.	Sens.	Undetermined	Sens.
P3-04	1,2-Benzisothiazol-3(2H)-one	1B	Sens.	Sens.	Sens.	Sens.
P3-05	Citral	1B	Sens.	Sens.	Sens.	Sens.
P3-06	4-Hydroxybenzoic acid	NC	Non-Sens.	Non-Sens.	Non-Sens.	Non-Sens.
P3-07	Beryllium Sulphate	1A	Non-Sens.	Non-Sens.	Non-Sens.	Non-Sens.
P3-08	Formaldehyde	1A	Sens.	Sens.	Sens.	Sens.
P3-09	Chlorpromazine HCl	1A	Sens.	Sens.	Sens.	Sens.
P3-10	Chloramine T	1A	Sens.	Sens.	Sens.	Sens.
P3-11	Benzyl Salicylate	1B	Sens.	Sens.	Sens.	Sens.
P3-12	Dihydroeugenol	1B	Sens.	Sens.	Sens.	Sens.
P3-13	Nickel Chloride	1B	Sens.	Sens.	Sens.	Sens.
P3-14	Imidazolidinyl urea	1B	Sens.	Sens.	Sens.	Sens.
P3-15	R(+)Limonene	1B	Non-Sens.	Sens.	Non-Sens.	Non-Sens.
P3-16	Methylmethacrylate	1B	Non-Sens.	Non-Sens.	Non-Sens.	Non-Sens.
P3-17	Benzyl alcohol	NC	Non-Sens.	Non-Sens.	Non-Sens.	Non-Sens.
P3-18	Dimethyl Isophthalate	NC	Non-Sens.	Non-Sens.	Non-Sens.	Non-Sens.
P3-19	4-Aminobenzoic acid	NC	Non-Sens.	Non-Sens.	Non-Sens.	Non-Sens.
P3-20	Xylene	NC	Non-Sens.	Non-Sens.	Non-Sens.	Non-Sens.
P4-01	Ethyleneglycol dimethacrylate	1B	Sens.	Sens.	Sens.	Sens.
P4-02	4-Nitrobenzylbromide	1A	Sens.	Sens.	Sens.	Sens.
P4-03	4-Allylanizole(Estragol)	1B	Sens.	Sens.	Sens.	Sens.
P4-04	Ethylenediamine	1B	Non-Sens.	Sens.	Non-Sens.	Non-Sens.
P4-05	Salicylic acid	NC	Non-Sens.	Non-Sens.	Non-Sens.	Non-Sens.

Table 8 shows the 2 by 3 tables by the “Based on Majority” and each laboratory in the table 9-10-4.1.

Table 8 The 2 by 3 table of the IL-8 Luc assay compared with GHS classification based on majority

IL-8 Luc Assay	GHS			
	1A	1B	NC	Total
Sens.	9	10	1	20
Non-Sens.	1	4	9	14
Total	10	14	10	34

Table 9 shows the indexes of relevance based on the table 8.

Table 9 The 2 by 3 tables of the IL-8 Luc assay by each laboratory compared with GHS classification

Lab. A

IL-8 Luc Assay	GHS			
	1A	1B	NC	Total
Sens.	8	12	0	20
Non-Sens.	1	2	10	13
Total	9	14	10	33

Lab. B

IL-8 Luc Assay	GHS			
	1A	1B	NC	Total
Sens.	8	10	1	19
Non-Sens.	1	4	9	14
Total	9	14	10	33

Lab. C

IL-8 Luc Assay	GHS			
	1A	1B	NC	Total
Sens.	8	10	1	19
Non-Sens.	2	4	9	15
Total	10	14	10	34

5. Subgroup analysis

Because of the use of the different solvents, the subgroup analysis for phase IV and others were conducted.

Table 10 and Table 11 show the results of the predictively on the subgroup of the phase IIb, IIc and III study, and phase IV study, respectively.

Table 10 The indexes of predictivity for the subgroup of phase IIb, IIc and III studies

Statistics	Based on Majority	Lab. A	Lab. B	Lab. C
Accuracy	82.8% (24/29) [64.6%, 93.3%]	89.3% (25/28) [70.9%, 98.2%]	82.1% (23/28) [63.4%, 93.1%]	79.3% (23/29) [60.4%, 91.3%]
Sensitivity	80.0% (16/20) [58.4%, 91.9%]	84.2% (16/19) [62.4%, 94.5%]	78.9% (15/19) [56.7%, 91.5%]	75.0% (15/20) [53.1%, 88.8%]
Specificity	88.9% (8/9) [56.5%, 98.0%]	100.0%(9/9) [70.1%, 100.0%]	88.9% (8/9) [56.5%, 98.0%]	88.9% (8/9) [56.5%, 98.0%]
False Negative Rate	20.0% (4/20) [8.1%, 41.6%]	15.8% (3/19) [5.5%, 37.6%]	21.1% (4/19) [8.5%, 43.3%]	25.0% (5/20) [11.2%, 46.9%]
False Positive Rate	11.1% (1/9) [2.0%, 43.5%]	0.0% (0/9) [0.0%, 29.9%]	11.1% (1/9) [2.0%, 43.5%]	11.1% (1/9) [2.0%, 43.5%]
Positive Predictive value	94.1% (16/17) [73.0%, 99.0%]	100.0%(16/16) [80.6%, 100.0%]	93.8% (15/16) [71.7%, 98.9%]	93.8% (15/16) [71.7%, 98.9%]
Nevative Predictive value	66.7% (8/12) [39.1%, 86.2%]	75.0% (9/12) [46.8%, 91.1%]	66.7% (8/12) [39.1%, 86.2%]	61.5% (8/13) [35.5%, 82.3%]

Table 11 The indexes of predictivity for the subgroup of phase IV study

Statistics	Based on Majority	Lab. A	Lab. B	Lab. C
Accuracy	80.0% (4/5) [35.9%, 98.0%]	100.0%(5/5) [56.6%, 100.0%]	80.0% (4/5) [35.9%, 98.0%]	80.0% (4/5) [35.9%, 98.0%]
Sensitivity	75.0% (3/4) [30.1%, 95.4%]	100.0%(4/4) [51.0%, 100.0%]	75.0% (3/4) [30.1%, 95.4%]	75.0% (3/4) [30.1%, 95.4%]
Specificity	100.0%(1/1) [20.7%, 100.0%]	100.0%(1/1) [20.7%, 100.0%]	100.0%(1/1) [20.7%, 100.0%]	100.0%(1/1) [20.7%, 100.0%]
False Negative Rate	25.0% (1/4) [4.6%, 69.9%]	0.0% (0/4) [0.0%, 49.0%]	25.0% (1/4) [4.6%, 69.9%]	25.0% (1/4) [4.6%, 69.9%]
False Positive Rate	0.0% (0/1) [0.0%, 79.3%]	0.0% (0/1) [0.0%, 79.3%]	0.0% (0/1) [0.0%, 79.3%]	0.0% (0/1) [0.0%, 79.3%]
Positive Predictive value	100.0%(3/3) [43.8%, 100.0%]	100.0%(4/4) [51.0%, 100.0%]	100.0%(3/3) [43.8%, 100.0%]	100.0%(3/3) [43.8%, 100.0%]
Nevative Predictive value	50.0% (1/2) [9.5%, 90.5%]	100.0%(1/1) [20.7%, 100.0%]	50.0% (1/2) [9.5%, 90.5%]	50.0% (1/2) [9.5%, 90.5%]

Table 12 shows the indexes of predictivity based on the Tables 10 and 11.

Table 12 The indexes of predictivity

Statistics	Based on Majority	Lab. A	Lab. B	Lab. C
Accuracy	**82.4% (28/34) [65.6%, 92.6%]**	90.9% (30/33) [74.9%, 98.4%]	81.8% (27/33) [64.6%, 92.4%]	79.4% (27/34) [62.0%, 90.8%]
Sensitivity	**79.2% (19/24) [59.5%, 90.8%]**	87.0% (20/23) [67.9%, 95.5%]	78.3% (18/23) [58.1%, 90.3%]	75.0% (18/24) [55.1%, 88.0%]
Specificity	**90.0% (9/10) [59.6%, 98.2%]**	100.0%(10/10) [72.2%, 100.0%]	90.0% (9/10) [59.6%, 98.2%]	90.0% (9/10) [59.6%, 98.2%]
False Negative Rate	**20.8% (5/24) [9.2%, 40.5%]**	13.0% (3/23) [4.5%, 32.1%]	21.7% (5/23) [9.7%, 41.9%]	25.0% (6/24) [12.0%, 44.9%]
False Positive Rate	**10.0% (1/10) [1.8%, 40.4%]**	0.0% (0/10) [0.0%, 27.8%]	10.0% (1/10) [1.8%, 40.4%]	10.0% (1/10) [1.8%, 40.4%]
Positive Predictive value	**95.0% (19/20) [76.4%, 99.1%]**	100.0%(20/20) [83.9%, 100.0%]	94.7% (18/19) [75.4%, 99.1%]	94.7% (18/19) [75.4%, 99.1%]
Nevative Predictive value	**64.3% (9/14) [38.8%, 83.7%]**	76.9% (10/13) [49.7%, 91.8%]	64.3% (9/14) [38.8%, 83.7%]	60.0% (9/15) [35.7%, 80.2%]

Fig. 13a, 13b, and 13c display the estimate of the accuracy, the sensitivity and the specificity, respectively, and these 95% confidence intervals for the overall and the 2 subgroups.

Fig. 13a the estimate of the accuracy and its 95% confidence intervals for the overall and the 2 subgroups.

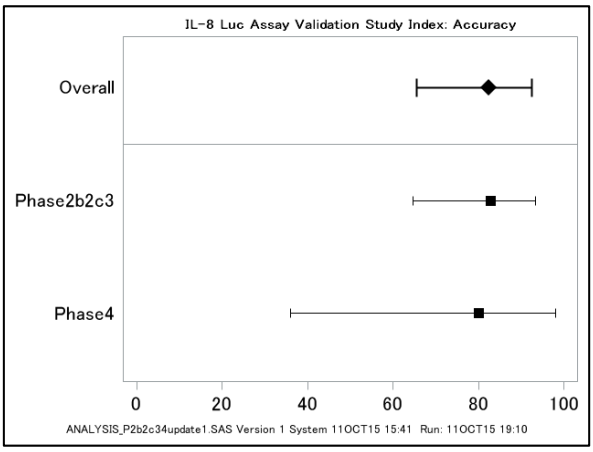


Fig. 13b the estimate of the sensitivity and its 95% confidence intervals for the overall and the 2 subgroups.

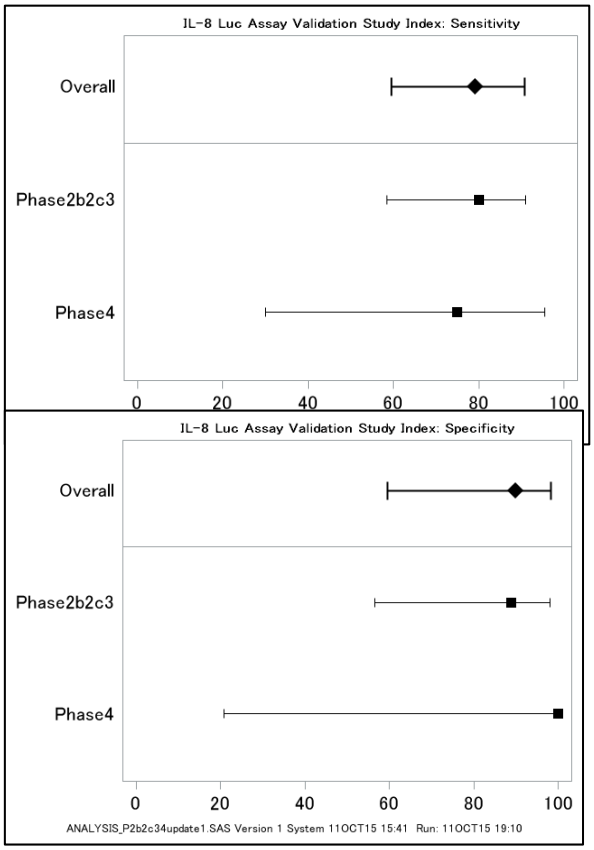


Fig. 13c the estimate of the specificity and its 95% confidence intervals for the overall and the 2 subgroups.

Appendix 13. Study plan

Version 6, June 29th, 2015

Study plan for the validation study on multicolor reporter assay using IL-8 Luc (THP-G8 cells) as a test evaluating the skin sensitizing potential of chemicals

Conducted by:
IL-8 Luc assay Validation Management Team

INDEX

1. Background

The use of multicolor reporter assay using IL-8 Luc (THP-G8 cells) is an important for evaluating the skin sensitizing potential of chemicals because of its technical simplicity, short-term test period and accuracy of test result based on a mechanism of skin sensitization.

The aim of this study is to (pre)validate the IL-8 Luc assay method to assess transferability and between-laboratory variability, in order to incorporate this test for screening the skin sensitizing chemicals. The IL-8 Luc assay for multi-study 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 multi-study validation trials with participation of GLP Test Facilities [Cooper-Hannan et al., 1999] where the whole concept of multi-study 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 multi-study 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-8 Luc assay. This IL-8 Luc assay for multi-study validation trial study has performed under the GLP spirit.

2. Objective of the study

The multi-study validation trial will assess the reliability (reproducibility within and between laboratories) and relevance (predictive capacity) of the IL-8 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**

Name	Role and expertise	Affiliation
Trial Coordinator Noriho Tanaka	VMT Chairperson,	HRI and OTIP, Japan
Lead Lab	*VMT Co-chair	
Yutaka Kimura*	**Developer of this assay	Tohoku Univ., Japan
Setsuya Aiba**	Test method, expertise underlying science	
Hajime Kojima	Management of quality control	JaCVAM, NIHS, Japan (JaCVAM representative)
Takashi Omori	Data analysis, biostatistics dossier	Doshisha Univ., Japan
Liaison members		
ECVAM liaison Emanuela Corcini Joachim Kreysa	Test system expertise, multi-study validation expertise, immunotoxicity expertise	ECVAM, Italy
ICCVAM liaison William Stokes	Test system expertise, multi-study validation expertise	NICEATM, USA
KoCVAM Laiaison Ai-Young Lee	Test system expertise, multi-study validation expertise	KoCVAM, Korea
ZEBET Liaison Michael Oelgeschlaeger	Test system expertise, multi-study validation expertise	ZEBET, Germany

The VMT encompasses collective expertise with the test, in the underlying science and the scientific design, management and evaluation of a multi-study validation trial.
The VMT, which plays a central role overseeing the conduct of the multi-study validation trial, includes:
Table 1. Members for IL-8 Luc assay Validation Management Team

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

Consultation Team

Name	Role and expertise	Affiliation

Hitoshi Sakaguchi* Hiroshi Itagaki**	Management of Chemicals selection, repository, coded and distribution	*Kao Co. Ltd., Japan **Yokohama National Univ., Japan
Yoshihiro Ohmiya* Ayako Sakai**	Document checking for *bioluminescenece measurement by luminometer and for **the other data in data sheets	*AIST, Japan **HRI, FDSC, Japan
Megumi Takada Yumi Tanaka Aya Watanabe Ai Kaneko Aoi Maruya	Data cleaning and data analysis	Doshisha Univ., Japan

The strategic decisions will be taken by the VMT only.

3.1 Participating Test Facilities

The laboratories participating in the study are defined as follow:

Test Facility 1: Hatano Res. Inst., FDSC.: GLP spirit SD : Kohji Yamakage

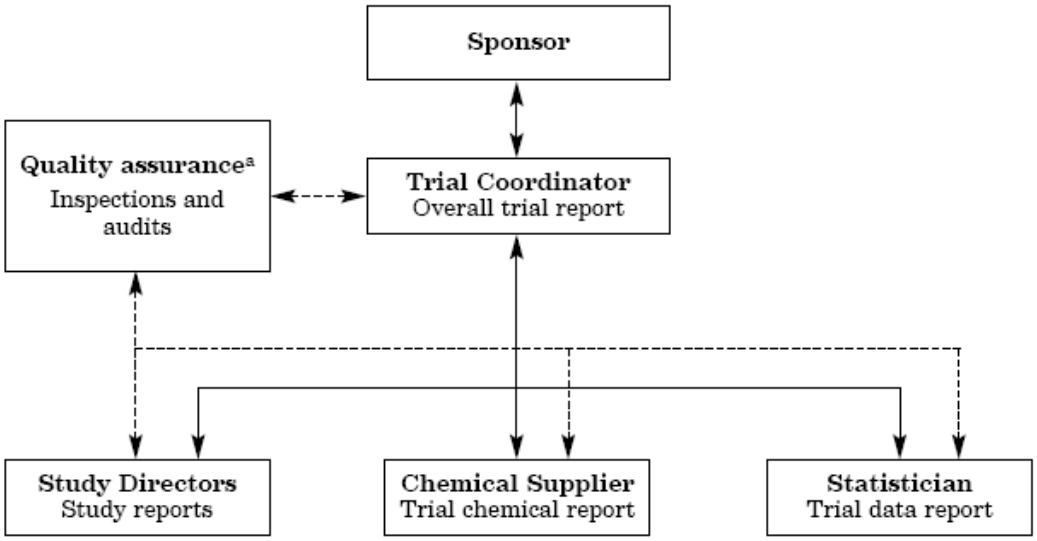
Test Facility 2: Sumitomo Chemical Co. Ltd.: GLP spirit SD : Koichi Saito

Test Facility 3: AIST : GLP spirit SD : Yoshihiro Nakajima

Information relevant for Modules 1, 2, 3 performed by all laboratories. Data obtained by these laboratories have demonstrated that the IL-8 Luc assay is transferable and reproducible between experienced laboratories. The all facility will be the laboratory participating in this multi-study validation trial acting as unexperienced laboratory to assess between laboratory transferability, reliability and relevance of the IL-8 Luc assay method under non-GLP conditions (GLP spirit).

3.2 Trial management structure

The management structure of the multi-study 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-8 Luc assay multi-study trial

1) Chemical management group

The members of chemical management group are elected by recommendation of the IL-8 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 study. 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-8 Luc assay VMT, and check and analyze the data obtained in this validation study from a third-party standpoint. They also take charge of statistical processing in this validation study.

3) Quality assurance group

The members of record management group are elected by recommendation of the IL-8 Luc assay VMT. They prepare protocol, test chemical preparation record forms, blank data sheets, etc. and distributes them to the research laboratories participating in this validation study. They also collect 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 multi-study trial. The lead laboratory is also responsible for providing, if necessary, new versions of the protocols during the entire multi-study trial. The lead lab and the other participating test facilities might be contacted by the VMT for technical issues.

3.3 Sponsor

The IL-8 Luc assay study trial completing the multi-study trial for assessing the validity of IL-8 Luc assay will be financed by the Ministry of Economy, Trade and Industry (METI), Japan.

Contact person: Shinichi Oikawa, Senior Analyst for Chemical Risk
Division of Chemical Management Policy,
Manufacturing Industries Bureau, METI, Japan
Phone: +81-3-3501-0080,
e-mail: oikawa-shinichi@meti.go.jp, fujisawa-hisashi@meti.go.jp

Management Office of Validation Study (MOVS)
Shojiro Yamazaki
Manager, Hadano office,
Organization for Tottori Industrial Promotion (OTIP), Japan
TEL: +81-463-82-4751, FAX:+81- 463- 82- 9627

Management Office of Validation Study (MOVS) will support the IL-8 Luc assay multi-study trial by assuring that reliability is assessed. At the same time, preliminary results of the test method can be evaluated. For this purpose MOVS will support:

- the financial aspects related to the coordination of a multi-study 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 multi-side 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

3.4 Trial coordination

Noriho Tanaka was appointed as the Trial Coordinator with well-defined roles and responsibilities to coordinate the trial and to establishment of a Validation Management Team (VMT) by supporting of JaCVAM.

The name and location of the Trial Coordinator should be identified in each individual study plan. For the IL-8 Luc assay multi-study 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 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 multi-study 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 multi-study validation trial, and

should reflect the overall trial
g) Approval with date and signature of all protocols, Study Plans, Study Reports, Trial Plan and Trial Report
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 Test Facility Managers) 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 Test Facility Managers of the different test facilities. The Test Facility Managers 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.

3.5 Training

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 Study Directors 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-8 Luc assay method protocols. In case any critical observations are made a new version of the IL-8 Luc assay method 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 JaCVAM. The proportion of concordance should be equal or more than 80% as tentative acceptance criteria for phase II 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-8 Luc assay method to the all test facility, the Phase 0 study using non-coded 3 chemicals was performed. A few concentrations of each test item will be tested in triplicate in 3 independent runs according to the IL-8 Luc assay method protocol describing the details of the experimental design.

The ten test items selected for the phase I study are coded as follows: A, B, C, D, E, F, G, H, I and J. The all facility will prepare a study according to internal GLP spirit. 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 spirit, raw data, records and data analysis, study report in GLP spirit).

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-8 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 spirit, raw data, records and data analysis, study report in GLP spirit). 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 study, the protocol (ver. 9E) 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 study.

5. Chemicals

5.1 Chemicals Selection

Test chemicals have been selected by chemical repository based on published papers on *in vivo* skin sensitization test and validation studies for *in vitro* alternative assays on sensitization test.

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-8 Luc assay validation trial with data generation at the test facilities, ten 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, thirty chemicals will be planned in the phase II trial for predictive capacity (Table 2).

Table 2. Outline of test planning in each validation stages.
2013, Nov

Stage	Labs	Chemicals	Information obtained
Phase 0 (finished)	3	5 non-coded	-Between-lab transferability -Within-lab reproducibility(with few chemicals) ?
Phase I (finished)	3	10 coded	-Between-lab reproducibility
Phase IIa (finished)	3	10 coded	-Between-lab & within-lab reproducibility
Phase IIb (finished)	3	5 coded	-Between-lab & within-lab reproducibility
Phase IIc (planning)	3	5 coded	-Between-lab & within-lab reproducibility*
Phase III (planning)	3	20 coded	-Predictive capacity
(Planning of Phase II will be determined after discussion of the results of Phase I)			

*This phase will be performed to make it clear of the criteria to judge sensitizer or non-sensitizer. We have three candidates of the criteria, 1) FInSLO-LA $\geq$ 1.4 and I.I.-SLR-LA$\geq$0.05, 2) FInSLO-LA $\geq$ 1.4 with 95% CI(confidence interval) of FInSLO-LA $\geq$ 1.4 and its lower limit$\geq$1, 3) FInSLO-LA $\geq$ 1.4 with 95% CI of its lower limit$\geq$1, to be judged as positive.

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-8 Luc multi-study 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 multi-study validation trials.

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 Test Facility Manager should receive the safety information concerning the hazards identification and exposure controls/personal protection.

6. Records and archiving

At the end of the trial, a IL-8 Luc assay multi-study 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 a multi-study trial. This represents the whole multi-study 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 Test Facility Managers and Study Directors of the involved test facilities. Although the Test Facility Managers and Study Directors 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 multi-study trial data.

7. Study timeline

An approximate schedule for IL-8 Luc assay validation study is shown in Table 3.

Duration of this validation study is around fifteen -month from Nov 2011 to Feb 2013.

Table 3. Schedule of IL-8 Luc assay validation study

Month	Activity
Aug-Sept, 2011	Establish the VMT
	Selection of participating research laboratories
	Election and approval of the Trial Coordinator and each group
	Deliberation, decision and read-through of draft study plan
	Deliberation and decision of study protocol
	Preparation of a tentative list of test chemicals
	Distribution of test chemicals, standard chemicals and positive control chemicals
Oct ,2011	Technical transfer using three known chemicals (non-coded) Start of technical transfer **to know between laboratory transferability**
	Data collection of technical transfer (**Phase 0 study**)
Phase I study to know between- and within-laboratory reproducibility	

Nov, 2011	**1st VMT Meeting** / chemical selection
	Start of Phase I study using ten coded chemicals
Feb 21, 2012	End of Phase I study
	2nd VMT Meeting / Phase I results and planning of Phase II study
Phase II studies to know between- and within-laboratory reproducibility	
Apr, 2012	Coding and distribution of coded test chemicals and positive chemicals
May, 2012	Start of Phase IIa study using ten coded test chemicals
	3rd VMT Meeting (domestic)/ start of Phase IIa study
Aug, 2012	End of Phase IIa study
Sept, 2012	**4th VMT Meeting** /reviewing of Phase IIa study results and planning of Phase IIb study
Nov, 2012	Start of Phase IIb study using five coded test chemicals
Feb, 2013	**5th VMT Meeting** /reviewing of Phase IIb study results
Nov, 2013	Planning of Phase IIc study using five coded test chemicals
Nov, 2015	Submission of SPSF to OECD, if applicable ?

Abbreviations

CAS: Chemical Abstracts Service
GLP: Good Laboratory Practice
HRI: Hatano Research Institute
FDSC: Food and Drug Safty 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
MOVS: Management Office of Validation Study

ANNEX 1, Apr. 3, 2014

Study plan for the phase III validation trial on multicolor reporter assay using IL-8 Luc (THP-G8 cells) as a test evaluating the skin sensitizing potential of chemicals

The phase III trial will be conducted in accordance with this study plan. Based on the study plan ver.5, this plan was made up by the trial coordinator.

1 Objective of the study

The purpose of phase III validation trial will assess the reliability (between laboratories reproducibility) and relevance (predictive capacity) of the IL-8 Luc assay with a challenging set of test substances.

2 Organization for Validation trial

The validation management team and participated laboratories for validation trial are pursuant by the study plan ver.5.

3 Protocol

In this phase validation trial, the valid protocol of Phase-IIc: ver. 017E (November 11, 2013) will be used. This phase will be performed to make it clear of the criteria to judge sensitizer or non-sensitizer. Three candidates of the criteria are 1) FInSLO-LA ≥ 1.4 and I.I.-SLR-LA≥0.05, 2) FInSLO-LA ≥ 1.4 with 95% CI(confidence interval) of FInSLO-LA ≥ 1.4 and its lower limit≥1, 3) FInSLO-LA ≥ 1.4 with 95% CI of its lower limit≥1, to be judged as positive. Regarding criteria, these 3 criteria will be evaluated in this phase validation trial and best one for between-laboratory reproducibility should be selected after the trial. The proportion of concordance between-laboratory reproducibility using the best one should be equal or more than 80% as acceptance criteria.

Using this best criteria, the between-laboratory reproducibility and predictivity will be calculated with total 29 chemicals including the chemicals used at phase IIb and IIc.

4 Chemicals

Twenty chemicals were selected by the VMT members, David Allen, Emanuela Corsini, Ai-Young Lee, Norio Tanaka, Hitosi Sakaguchi, and Hajime Kojima, considering their chemical properties and sensitization potentials at the chemical selection meeting in Kyoto on February 26, 2014.

20 coded chemicals, 1 test in each chemical. One set of 20 coded chemicals will be distributed from JaCVAM in the first week of April, 2014.

- Valid protocol of Phase-IIc: ver. 017E (November 11, 2013)

5 Records and archiving

At the end of the testing, the test facilities will submit a QC certified copy of whole study dossier to the QC check group.

6 Study timeline

An approximate schedule for IL-8 Luc assay validation trial is shown in Table 1.

Table 1. Schedule of IL-8 Luc assay validation phase III trial

Apr,2014	Start of phase III trial using twenty coded test chemicals
Sep,2014	7th VMT Meeting / reviewing of phase III results
Oct,2014	Submission of SPSF to OECD, if applicable?

ANNEX 2, (drafted at June 29, 2015)

Study plan for the phase IV validation trial on multicolor reporter assay using IL-8 Luc (THP-G8 cells) as a test evaluating the skin sensitizing potential of chemicals

The phase IV trial will be conducted in accordance with this appendix based on the study plan ver. 6. This plan was made up by the trial coordinator and confirmed by VMT members.

1 Objective of the study

The purpose of phase IV validation trial will assess additionally the reliability intra- and inter-laboratory reproducibility of the IL-8 Luc assay with a challenging set of test substances. According the peer reviewer' suggestion, the revised protocol with X-VIVO (supplement for serum-free) will be used in this trial.

2 Organization for Validation trial

The validation management team and participated laboratories for validation trial are pursuant by the study plan ver. 6.

3 Protocol

In this phase validation trial, the revised protocol ver. 020E (June 24, 2015) will be used. This protocol is including the positive criteria and use of X-VIVO.

4 Chemicals

Five chemicals were selected and confirmed by the VMT members, David Allen, Emanuela Corsini, Ai-Young Lee, Noriho Tanaka, Hiroshi Itagaki, Hitoshi Sakaguchi, and Hajime Kojima, considering their chemical properties and sensitization potentials.

Five coded chemicals, 3 test in each chemical in each lab. One set of 5 coded chemicals will be distributed from JaCVAM by the beginning of July 2015.

5 Records and archiving

At the end of the testing, the test facilities will submit a QC certified copy of whole study dossier to the QC check group. And the data will be circulated to data management team.

6 Acceptance criteria

The proportion of concordance intra- and inter-laboratory reproducibility using the best one should be equal or more than 80% as acceptance criteria.

Using this best criteria, the intra- and inter-laboratory reproducibility will be calculated with total 5 chemicals including the chemicals used in previous test phases.

7 Study timeline

An approximate schedule for the Phase IV study of IL-8 Luc assay validation trial is shown in Table 1.

Table 1. Schedule of IL-8 Luc assay validation phase IV trial

July, 2015	Chemical selection and distribution
July, 2015	Start of phase IV trial using 5 coded test chemicals
Beginning of	Submission of test report to VMT from test labs.

Sept, 2015	
Oct., 2015	**8th VMT Meeting** /reviewing of phase IV results

Appendix 14. The list of proficiency chemicals

The list of proficiency chemicals

No.	Chemical name	CAS No.	GHS category	Physical state	Phase
1	2-Mercaptobenzothiazole	149-30-4	1A	Solid	IIc**
2	2,4-Dinitrochlorobenzene	97-00-7	1A	Solid	III
3	4-Nitrobenzylbromide	100-11-8	1A	Solid	IV
4	Ethyleneglycol dimethacrylate	97-90-5	1B	Liquid	IV
5	4-Allylanisole (Estragol)	140-67-0	1B	Liquid	IV
6	Ethylenediamine	107-15-3	1B	Liquid	IV
7	Salicylic acid	69-72-7	NO	Solid	IV
8	4-Aminobenzoic acid	150-13-0	NO	Solid	III**
9	Glycerol	56-81-5	NO	Liquid	IIc**
10	Isopropanol	67-63-0	NO	Liquid	IIc**

The list of performance standard chemicals

No.	Chemical name	CAS No.	GHS category	Physical state	Phase
1	p-Benzoquinone	106-51-4	1A	Solid	IIb**
2	1,4-Phenylenediamine	106-50-3	1A	Solid	IIc**
3	2-Mercaptobenzothiazole	149-30-4	1A	Solid	IIc**
4	2,4-Dinitrochlorobenzene	97-00-7	1A	Solid	III
5	4-Nitrobenzylbromide	100-11-8	1A	Solid	IV
6	Citral	5392-40-5	1B	Liquid	III
7	Imidazolidinylurea	39236-46-9	1B	Solid	III**
8	Methylmethacrylate	80-62-6	1B	Liquid	III**
9	R(+)-Limonene	5989-27-5	1B	Liquid	III**
10	Nickel chloride	7718-54-9	1B	Solid	III**
11	Ethyleneglycol dimethacrylate	97-90-5	1B	Liquid	IV
12	4-Allylanisole (Estragol)	140-67-0	1B	Liquid	IV
13	Ethylenediamine	107-15-3	1B	Liquid	IV
14	2,4-Dichloronitrobenzene	611-06-3	NO	Solid	IIb& III**
15	4-Aminobenzoic acid	150-13-0	NO	Solid	III**
16	4-Hydroxybenzoic acid	99-96-7	NO	Solid	III
17	Glycerol	56-81-5	NO	Liquid	IIc**
18	Isopropanol	67-63-0	NO	Liquid	IIc**
19	Methylsalicylate	119-36-8	NO	Liquid	IIc**
20	Salicylic acid	69-72-7	NO	Solid	IV

Appendix 15. Additional information

Background

The test method as described above has been peer reviewed by an independent Peer Review Panel in 2014-2015 and subsequently submitted for comments to the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) - July and December 2016. Issues raised by the WNT were discussed by the OECD expert group on skin sensitisation in a meeting in November 2016 and via e-mails and teleconferences in 2016-2017.

The WNT and expert group expressed concerns related to the procedure that allows testing of partially soluble and supernatants of insoluble test substances and discussed how to deal with negative results in case of poor or non-soluble substances.

The group agreed that negative results for test chemicals that are not dissolved at 20 mg/ml should be judged as inconclusive, since these test chemicals may produce false negative results due to their inability to dissolve in X-VIVO™ 15.

Annex 15 below presents the adaptations to the protocol in line with this approach and the re-analysis of the data for the calculation of performance of the assay. Annex 16 presents the data set of 143 chemicals evaluated by the IL-8 Luc assay and compares the 3 protocols, including this new prediction model, as applied in the TG.

The data evaluation

1. Criteria for a positive/negative decision require that in each run:

- an IL-8 Luc assay prediction is judged positive if a test chemical has a Ind-IL8LA ≥ 1.4 and the lower limit of the 95% confidence interval of Ind-IL8LA ≥ 1.0
- an IL-8 Luc assay prediction is judged negative if a test chemical has a Ind-IL8LA < 1.4 and/or the lower limit of the 95% confidence interval of Ind-IL8LA < 1.0

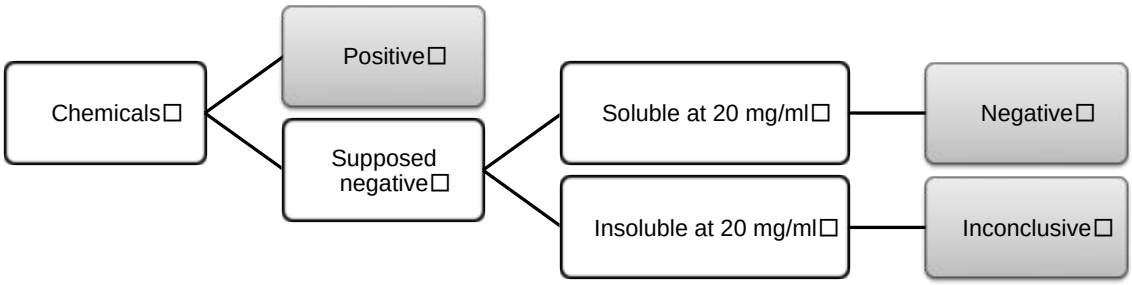
2. Prediction model

Test chemicals that provide two positive results from among the 1st, 2nd, 3rd or 4th runs are identified as positives whereas those that give three negative results from among the 1st, 2nd, 3rd or 4th runs are identified as supposed negative (Table 1). Among supposed negative chemicals, chemicals that are dissolved at 20 mg/ml of X-VOVO™ 15 are judged as negative, while chemicals that are not dissolved at 20 mg/ml of X-VOVO™ 15 are judged as inconclusive (Figure 1).

Table 1. Criteria for identifying positive and supposed negative

1st run	2nd run	3rd run	4th run	Final prediction
Positive	Positive	-	-	Positive
	Negative	Positive	-	Positive
		Negative	Positive	Positive
			Negative	Supposed negative
Negative	Positive	Positive	-	Positive
		Negative	Positive	Positive
			Negative	Supposed negative
	Negative	Positive	Positive	Positive
			Negative	Supposed negative
		Negative	-	Supposed negative

Figure 2. Prediction model for final judgment



The data

Based on the new prediction model, the data set of 143 chemicals evaluated by the IL-8 Luc assay were shown in Appendix 16. Table S2 and the performance of the IL-8 Luc assay was shown in Table 2.

Table 2. The performance of the IL-8 Luc assay based on the new prediction model.

	LLNA	LLNA (considering applicability domain)	Human data	Human (considering applicability domain)
n	143	136	90	84
Inconclusive	25	23	14	13
Sensitivity	96% (92/96)	96% (92/96)	93% (54/58)	93% (54/58)
Specificity	41% (9/22)	53% (9/17)	39% (7/18).	54% (7/13)
Accuracy	86% (101/118)	89% (101/113)	80% (61/76)	86% (61/71)

Relevance and reliability of the IL-8 Luc assay

The within- and between-laboratory reproducibilities, and the predictivity of the IL-8 Luc assay, were evaluated using all the results from Phases IIb, IIc, III, and IV studies. In the Phases IIb, IIc, and III, the IL-8 Luc assay was conducted using DMSO as a solvent. Therefore, the application of the new prediction model did not change the validation results of these studies. On the other hand, the Phase IV study was conducted using X-VIVO^{TM} 15 as a solvent because the peer review panel acknowledged that the modified dilution method improved the sensitivity of the IL-8 Luc assay and the dissolution of chemicals in X-VIVO^{TM} 15 is simpler and produces much smaller concentration variation during dilution when compared with the procedure of the IL-8 Luc assay using DMSO as a solvent. Therefore, chemicals used in Phase IV study potentially contain inconclusive chemicals. However, since the judgment of solubility of chemicals at 20 mg/ml is simple and easy, the judgment for this criterion is correctly made by any laboratories. Therefore, the application of the new prediction model simply change the judgment for supposed negative chemicals insoluble at 20mg/ml from negative to inconclusive, which means that the application of the new prediction model does not affect the within- and between-laboratory reproducibilities judged by the original prediction model. In contrast, the predictivity of the IL-8 Luc assay was a slightly changed and shown in Table 3.

Within- and between-laboratory variation assessments using Phase IIb, IIc, III and IV studies

Between-Lab reproducibility 87.5% (28/32)

Within-Lab reproducibility Lab. A 85.7% (12/14)

Lab. B 91.7% (11/12)
Lab. C 85.7% (12/14)
Average 87.7%

Predictivity in the Phase IIb, IIc, III and IV studies
Accuracy Lab. A 90.6% (29/32)
Lab. B 81.3% (26/32)
Lab C 78.8% (26/36)
Based on majority 81.8% (27/33)
The analysis of p-benzoquinone and 2,4-dichloronitrobenzene in IIb was not included in the data of Lab A.
The analysis of 2,4-dichloronitrobenzene in IIb and glyoxal solution in III was not included in the data of Lab B.
The analysis of 2,4-dichloronitrobenzene in IIb was not included in the data of Lab C.

Table 3. The summarized performance of the Phase IIb, IIc, III, and IV studies

Statistics	Based on Majority	A	B	C
Accuracy	**81.8% (27/33)**	90.6% (29/32)	81.3% (26/32)	78.8% (26/33)
Sensitivity	**79.2% (19/24)**	87.0% (20/23)	78.3% (18/23)	75.0% (18/24)
Specificity	**88.9% (8/9)**	100.0% (9/9)	88.9% (8/9)	88.9% (8/9)
False Negative Rate	**20.8% (5/24)**	13.0% (3/23)	21.7% (5/23)	25.0% (6/24)
False Positive Rate	**11.1% (1/9)**	0.0% (0/9)	11.1% (1/9)	11.1% (1/9)
Positive Predictive value	**95.0% (19/20)**	100.0% (20/20)	94.7% (18/19)	94.7% (18/19)
Negative Predictive value	**61.5% (8/13)**	75.0% (9/12)	61.5% (8/13)	57.1% (8/14)

CONCLUSIONS

After applying the new prediction model, several chemicals are classified as inconclusive by the IL-8 Luc assay. The inconclusive chemicals may contain chemicals that do not dissolve in aqueous phase almost completely. However, by accepting this prediction model, the IL-8 Luc assay significantly improves its sensitivity and accuracy. The within- and between-laboratory reproducibilities were not different from those based on the original prediction model. The performance of the validation study was not significantly different from that based on the original prediction model.

Appendix 16. Table S2. Data set of 143 chemicals evaluated by the IL-8 Luc assay based on the new prediction model (TG protocol applied)

No	CAS#	Chemical	GHS	LLNA Potency category	IL-8 Luc assay	IL-8 Luc assay (X-VIVO)	IL-8 Luc assay (X-VIVO, Test Guideline protocol)
1	93-91-4	1-Benzoylacetone(1-BA)	1A	Extreme	sensitizer	sensitizer	sensitizer
2	1086-00-6	1-Chloromethylpyrene	1A	Extreme	sensitizer	sensitizer	sensitizer
3	97-00-7	2,4-Dinitrochlorobenzene(DNCB)	1A	Extreme	sensitizer	sensitizer	sensitizer
4	100-11-8	4-Nitrobenzyl bromide(4-NBB)	1A	Extreme	sensitizer	sensitizer	sensitizer
5	20048-27-5	Bandrowski's base	1A	Extreme	sensitizer	sensitizer	sensitizer
6	7787-56-6	Beryllium sulfate tetrahydrate	1A	Extreme	non-sensitizer	sensitizer	sensitizer
7	70-34-8	Dinitrofluorobenzene	1A	Extreme	sensitizer	sensitizer	sensitizer
8	886-38-4	Diphenylcyclopropenone (DPCP)	1A	Extreme	sensitizer	sensitizer	sensitizer
9	15646-46-5	Oxazolone	1A	Extreme	non-sensitizer	sensitizer	sensitizer
10	106-51-4	p-Benzoquinone	1A	Extreme	sensitizer	sensitizer	sensitizer
11	7778-50-9	Potassium dichromate	1A	Extreme	sensitizer	sensitizer	sensitizer
12	1154-59-2	Tetrachlorosalicylanilide	1A	Extreme	sensitizer	sensitizer	sensitizer
13	123-31-9	1,4-Dihydroquinone	1A	Strong	sensitizer	sensitizer	sensitizer
14	106-50-3	1,4-Phenylenediamine(PPD)	1A	Strong	non-sensitizer	sensitizer	sensitizer
15	95-55-6	2-Aminophenol	1A	Strong	sensitizer	sensitizer	sensitizer
16	5307-14-2	2-Nitro-1,4-phenylenediamine	1A	Strong	sensitizer	non-sensitizer	inconclusive
17	94-36-0	Benzoyl peroxide	1A	Strong	sensitizer	non-sensitizer	inconclusive
18	100-39-0	Benzyl bromide	1A	Strong	sensitizer	sensitizer	sensitizer
19	127-65-1	Chloramine-T	1A	Strong	sensitizer	sensitizer	sensitizer
20	69-09-0	Chlorpromazine hydrochloride	1A	Strong	sensitizer	sensitizer	sensitizer
21	7646-79-9	Cobalt chloride hexahydrate	1A	Strong	sensitizer	sensitizer	sensitizer
22	50-00-0	Formaldehyde	1A	Strong	sensitizer	sensitizer	sensitizer
23	111-30-8	Glutaraldehyde	1A	Strong	sensitizer	sensitizer	sensitizer
24	6259-76-3	Hexyl salicylate	1A	Strong	sensitizer	non-sensitizer	inconclusive
25	55406-53-6	Iodopropynyl butylcarbamate	1A	Strong	sensitizer	sensitizer	sensitizer
26	1166-52-5	Lauryl gallate	1A	Strong	sensitizer	sensitizer	sensitizer
27	108-31-6	Maleic anhydride	1A	Strong	non-sensitizer	non-sensitizer	inconclusive
28	35691-65-7	Methyldibromoglutaronitrile(MDGN)	1A	Strong	sensitizer	sensitizer	sensitizer
29	85-44-9	Phthalic anhydride	1A	Strong	non-sensitizer	non-sensitizer	inconclusive
30	121-79-9	Propyl gallate	1A	Strong	sensitizer	sensitizer	sensitizer
31	90-15-3	1-Naphthol	1A	Moderate	sensitizer	sensitizer	sensitizer
32	579-07-7	1-Phenyl-1,2-propanedione	1A	Moderate	sensitizer	sensitizer	sensitizer
33	96-27-5	1-Thioglycerol(3-mercapto-1,2-propanediol)	1B	Moderate	sensitizer	sensitizer	sensitizer
34	2634-33-5	1,2-Benzisothiazolin-3-one	1B	Moderate	sensitizer	sensitizer	sensitizer
35	3344-77-2	12-Bromo-1-dodecanol	1B	Moderate	sensitizer	sensitizer	sensitizer
36	818-61-1	2-Hydroxyethyl acrylate	1A	Moderate	non-sensitizer	non-sensitizer	non-sensitizer
37	149-30-4	2-Mercaptobenzothiazole(2-MBT)	1A	Moderate	sensitizer	sensitizer	sensitizer
38	93-51-6	2-Methoxy-4-methylphenol	1B	Moderate	sensitizer	sensitizer	sensitizer
39	93-53-8	2-Phenylpropionaldehyde	1B	Moderate	sensitizer	sensitizer	sensitizer
40	4313-03-5	2,4-Heptadienal	1B	Moderate	sensitizer	sensitizer	sensitizer
41	591-27-5	3-Aminophenol	1B	Moderate	sensitizer	sensitizer	sensitizer
42	109-55-7	3-Dimethylaminopropylamine	1B	Moderate	sensitizer	sensitizer	sensitizer
43	17369-59-4	3-Propylidenephthalide	1B	Moderate	sensitizer	sensitizer	sensitizer
44	119-84-6	3,4-Dihydrocoumarin	1B	Moderate	non-sensitizer	sensitizer	sensitizer
45	106-47-8	4-Chloroaniline	1B	Moderate	non-sensitizer	sensitizer	sensitizer
46	21834-92-4	5-Methyl-2-phenyl-2-hexenal	1B	Moderate	sensitizer	sensitizer	sensitizer
47	101-39-3	a-Methylcinnamic aldehyde	1B	Moderate	sensitizer	sensitizer	sensitizer
48	118-58-1	Benzyl salicylate	1B	Moderate	sensitizer	sensitizer	sensitizer
49	122-57-6	Benzylideneacetone	1B	Moderate	sensitizer	sensitizer	sensitizer
50	104-55-2	Cinnamic aldehyde(Cinnamal)	1B	Moderate	sensitizer	sensitizer	sensitizer

(continued)

51	23593-75-1	Clotrimazole	1B	Moderate	sensitizer	non-sensitizer	inconclusive
52	141-05-9	Diethyl maleate	1B	Moderate	non-sensitizer	sensitizer	sensitizer
53	64-67-5	Diethyl sulfate	1B	Moderate	non-sensitizer	sensitizer	sensitizer
54	111-40-0	Diethylenetriamine	1B	Moderate	sensitizer	sensitizer	sensitizer
55	2785-87-7	Dihydroeugenol(2-Methoxy-4-propylphenol)	1B	Moderate	sensitizer	sensitizer	sensitizer
56	107-15-3	Ethylenediamine	1B	Moderate	non-sensitizer	sensitizer	sensitizer
57	107-22-2	Glyoxal	1A	Moderate	non-sensitizer	sensitizer	sensitizer
58	97-54-1	Isoeugenol	1A	Moderate	sensitizer	sensitizer	sensitizer
59	111-80-8	Methyl-2-nonynoate	1B	Moderate	non-sensitizer	sensitizer	sensitizer
60	2682-20-4	ıiazolinone, 2-Methyl-2H-Isothiazol-3-one(Ker	1A	Moderate	sensitizer	sensitizer	sensitizer
61	10101-97-0	Nickel sulphate hexahydrate	1B	Moderate	sensitizer	sensitizer	sensitizer
62	7718-54-9	Nickel(II) chloride		Moderate	sensitizer	sensitizer	sensitizer
63	18031-40-8	Perillaldehyde	1B	Moderate	sensitizer	sensitizer	sensitizer
64	122-78-1	Phenylacetaldehyde	1B	Moderate	sensitizer	sensitizer	sensitizer
65	108-46-3	Resorcinol	1B	Moderate	sensitizer	sensitizer	sensitizer
66	133-37-9	Tartaric acid	1B	Moderate	sensitizer	sensitizer	sensitizer
67	137-26-8	Tetramethylthiuramdisulphide(TMTD)	1B	Moderate	sensitizer	sensitizer	sensitizer
68	6728-26-3	trans-2-Hexenal	1B	Moderate	sensitizer	sensitizer	sensitizer
69	552-30-7	Trimellitic anhydride	1B	Moderate	non-sensitizer	sensitizer	sensitizer
70	112-45-8	Undec-10-enal	1B	Moderate	sensitizer	sensitizer	sensitizer
71	5989-27-5	(R)-(+)-Limonene	1B	Weak	non-sensitizer	sensitizer	sensitizer
72	111-25-1	1-Bromohexane	1B	Weak	non-sensitizer	non-sensitizer	inconclusive
73	103-11-7	2-Ethylhexyl acrylate	1B	Weak	sensitizer	sensitizer	sensitizer
74	431-03-8	2,3-Butanedione	1B	Weak	sensitizer	sensitizer	sensitizer
75	140-67-0	4-Allylanisole	1B	Weak	non-sensitizer	sensitizer	sensitizer
76	13706-86-0	5-Methyl-2,3-hexanedione	1B	Weak	non-sensitizer	sensitizer	sensitizer
77	514-10-3	Abietic acid	1B	Weak	sensitizer	sensitizer	sensitizer
78	122-40-7	Amyl cinnamic aldehyde	1B	Weak	sensitizer	sensitizer	sensitizer
79	62-53-3	Aniline	1B	Weak	non-sensitizer	non-sensitizer	non-sensitizer
80	60-09-3	Benzocaine	1B	Weak	non-sensitizer	non-sensitizer	inconclusive
81	103-41-3	Benzyl cinnamate	1B	Weak	sensitizer	sensitizer	sensitizer
82	1565-94-2	Bis-GMA	1B	Weak	sensitizer	sensitizer	sensitizer
83	2426-08-6	Butyl glycidyl ether	1B	Weak	sensitizer	sensitizer	sensitizer
84	104-54-1	Cinnamic alcohol	1B	Weak	sensitizer	sensitizer	sensitizer
85	5392-40-5	Citral	1B	Weak	sensitizer	sensitizer	sensitizer
86	103-95-7	Cyclamen aldehyde	1B	Weak	sensitizer	sensitizer	sensitizer
87	97-90-5	Ethyleneglycol dimethacrylate	1B	Weak	non-sensitizer	sensitizer	sensitizer
88	97-53-0	Eugenol	1B	Weak	sensitizer	sensitizer	sensitizer
89	19317-11-4	Farnesal	1B	Weak	sensitizer	sensitizer	sensitizer
90	106-24-1	Geraniol	1B	Weak	sensitizer	sensitizer	sensitizer
91	101-86-0	Hexyl cinnamic aldehyde	1B	Weak	sensitizer	sensitizer	sensitizer
92	107-75-5	Hydroxycitronellal	1B	Weak	sensitizer	sensitizer	sensitizer
93	39236-46-9	Imidazolidinyl urea	1B	Weak	sensitizer	sensitizer	sensitizer
94	80-54-6	Lilial	1B	Weak	sensitizer	sensitizer	sensitizer
95	78-70-6	Linalool	1B	Weak	sensitizer	sensitizer	sensitizer
96	31906-04-4	Lyral	1B	Weak	sensitizer	sensitizer	sensitizer
97	80-62-6	Methylmethacrylate	1B	Weak	non-sensitizer	non-sensitizer	inconclusive
98	613-29-6	N,N-Dibutylaniline	1B	Weak	sensitizer	sensitizer	sensitizer
99	112-05-0	Nonanoic acid	1B	Weak	sensitizer	sensitizer	sensitizer
100	144-62-7	Oxalic acid	1B	Weak	sensitizer	sensitizer	sensitizer

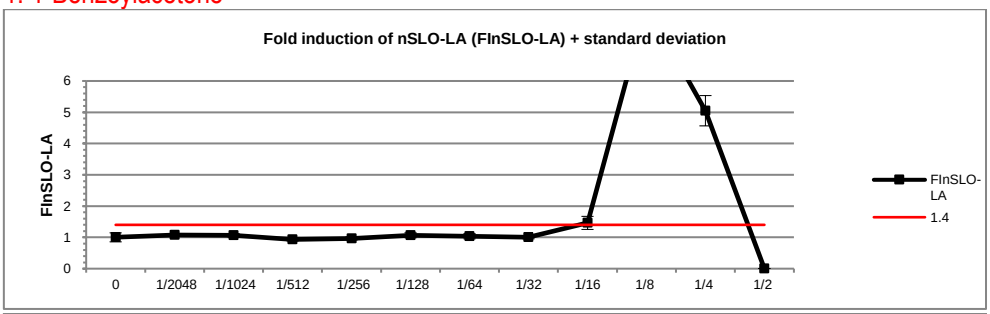
(continued)

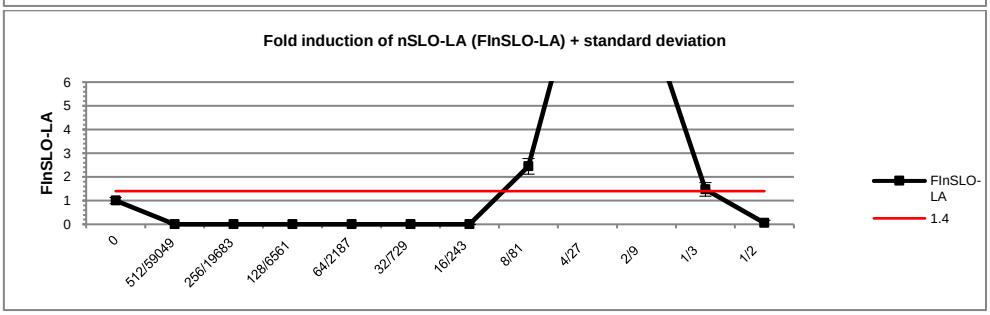
101	61-33-6	Penicillin G	1B	Weak	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
102	87-86-5	Pentachlorophenol	1B	Weak	sensitizer	sensitizer	sensitizer	sensitizer
103	2050-14-8	Phenol, 2,2-azobis-	1B	Weak	sensitizer	sensitizer	sensitizer	sensitizer
104	93-99-2	Phenyl benzoate	1B	Weak	sensitizer	non-sensitizer	inconclusive	inconclusive
105	110-86-1	Pyridine	1B	Weak	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
106	1330-20-7	Xylene	1B	Weak	non-sensitizer	non-sensitizer	inconclusive	inconclusive
107	127-51-5	α-iso-Methylionone	1B	Weak	sensitizer	sensitizer	sensitizer	sensitizer
108	109-65-9	1-Bromobutane	Non	Non-sensitizer	non-sensitizer	non-sensitizer	inconclusive	non-sensitizer
109	71-36-3	1-Butanol	Non	Non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
110	874-23-7	2-Acetylcyclohexanone	Non	Non-sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
111	923-26-2	2-Hydroxypropyl methacrylate	Non	Non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
112	611-06-3	2,4-Dichloro-1-nitrobenzene	Non	Non-sensitizer	non-sensitizer	non-sensitizer	inconclusive	inconclusive
113	99-96-7	4-Hydroxybenzoic acid	Non	Non-sensitizer	non-sensitizer	non-sensitizer	inconclusive	non-sensitizer
114	92-48-8	6-Methylcoumarin	Non	Non-sensitizer	non-sensitizer	non-sensitizer	inconclusive	inconclusive
115	100-06-1	Acetoanisole(4-Methoxyacetophenone)	Non	Non-sensitizer	non-sensitizer	non-sensitizer	inconclusive	non-sensitizer
116	8001-54-5	Benzalkonium chloride	Non	Non-sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
117	65-85-0	Benzoic acid	Non	Non-sensitizer	non-sensitizer	non-sensitizer	inconclusive	non-sensitizer
118	100-51-6	Benzyl alcohol	Non	Non-sensitizer	non-sensitizer	sensitizer	sensitizer	sensitizer
119	108-90-7	Chlorobenzene	Non	Non-sensitizer	non-sensitizer	non-sensitizer	inconclusive	inconclusive
120	9004-54-0	Dextran	Non	Non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
121	84-66-2	Diethyl phthalate	Non	Non-sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
122	68-12-2	Dimethyl formamide	Non	Non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
123	1459-93-4	Dimethyl isophthalate	Non	Non-sensitizer	non-sensitizer	non-sensitizer	inconclusive	inconclusive
124	94-02-0	Ethyl benzoylacetate	Non	Non-sensitizer	sensitizer	non-sensitizer	inconclusive	non-sensitizer
125	121-32-4	Ethyl vanilline	Non	Non-sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
126	492-94-4	Furil	Non	Non-sensitizer	sensitizer	non-sensitizer	inconclusive	inconclusive
127	56-81-5	Glycerol	Non	Non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
128	57-09-0	Hexadecyltrimethylammonium bromide	Non	Non-sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
129	67-63-0	Isopropanol	Non	Non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
130	50-21-5	Lactic acid	Non	Non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
131	119-36-8	Methyl salicylate	Non	Non-sensitizer	non-sensitizer	non-sensitizer	inconclusive	inconclusive
132	124-07-2	Octanoic acid(Caprylic acid)	Non	Non-sensitizer	non-sensitizer	sensitizer	sensitizer	sensitizer
133	150-13-0	p-Aminobenzoic acid	Non	Non-sensitizer	non-sensitizer	non-sensitizer	inconclusive	non-sensitizer
134	94-13-3	Propyl paraben	Non	Non-sensitizer	non-sensitizer	sensitizer	sensitizer	sensitizer
135	57-55-6	Propylene glycol	Non	Non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
136	81-07-2	Saccharin	Non	Non-sensitizer	non-sensitizer	sensitizer	sensitizer	sensitizer
137	69-72-7	Salicylic acid	Non	Non-sensitizer	non-sensitizer	non-sensitizer	inconclusive	non-sensitizer
138	151-21-3	Sodium lauryl sulfate	Non	Non-sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
139	3810-74-0	Streptomycin sulphate	Non	Non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer	non-sensitizer
140	63-74-1	Sulfanilamide	Non	Non-sensitizer	non-sensitizer	non-sensitizer	inconclusive	non-sensitizer
141	9005-65-6	Tween-80	Non	Non-sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
142	121-33-5	Vanillin	Non	Non-sensitizer	sensitizer	sensitizer	sensitizer	sensitizer
143	7733-02-0	Zinc sulphate	Non	Non-sensitizer	sensitizer	sensitizer	sensitizer	sensitizer

Appendix 17. Dose-response curves on IL-8 Luc assay

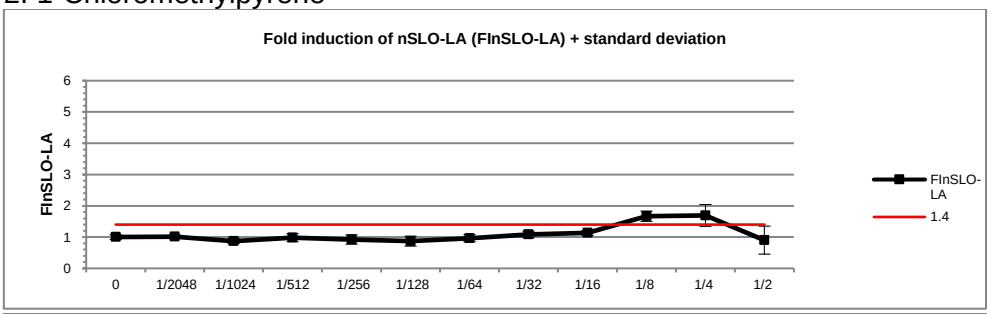
When the first experiments clearly indicated the optimal concentration to induce FIn-SLO-LA, we deleted the experiments in which the concentration of chemicals for stimulation is lower than the estimated optimal concentration. These chemicals make an entry in red.

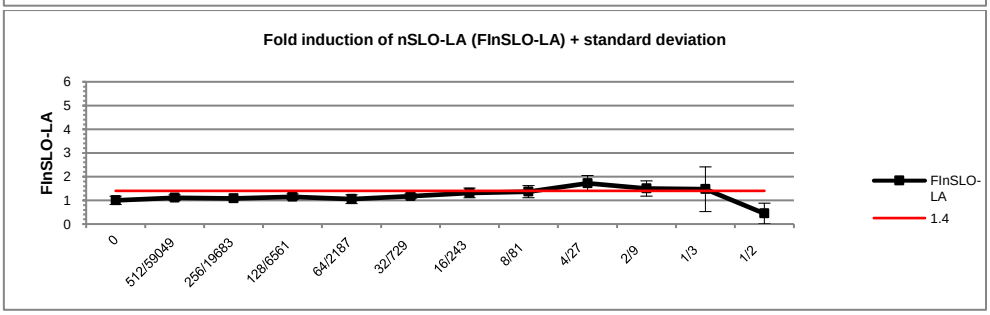
1. 1-Benzoylacetone



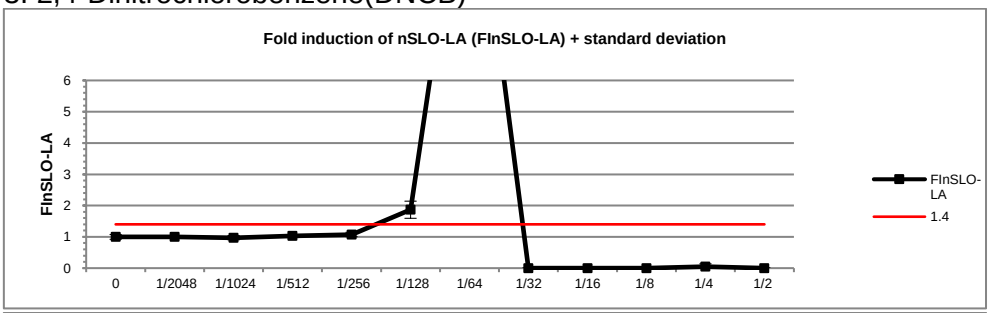


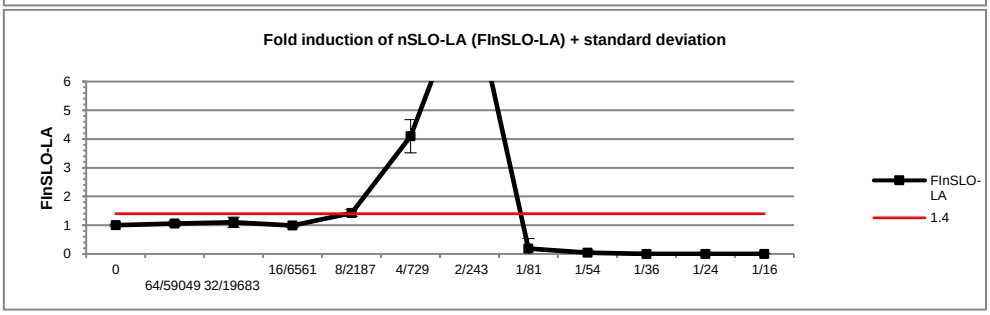
2. 1-Chloromethylpyrene



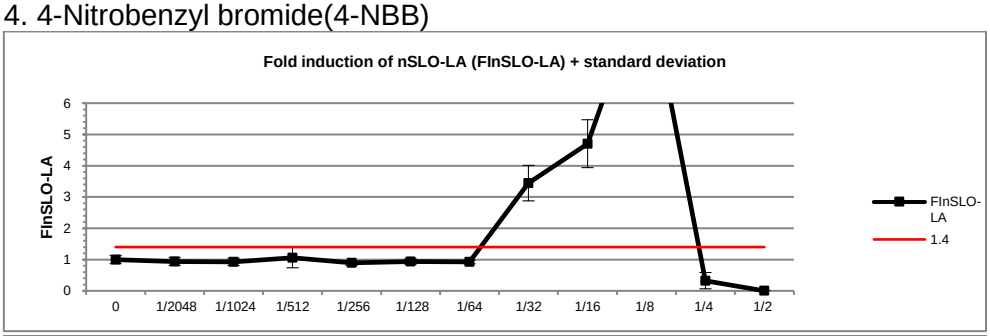


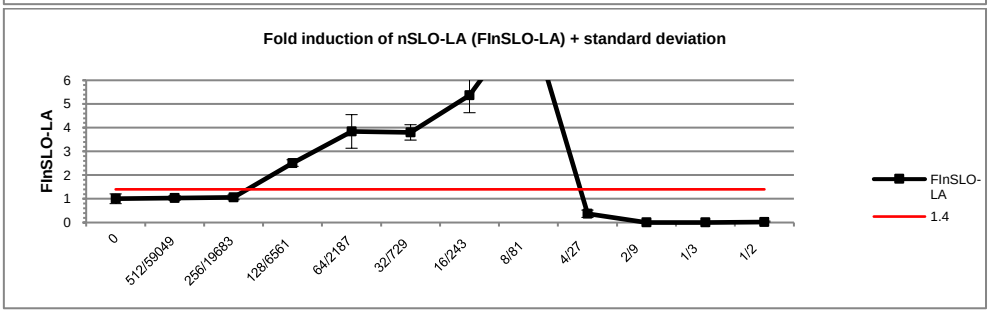
3. 2,4-Dinitrochlorobenzene(DNCB)



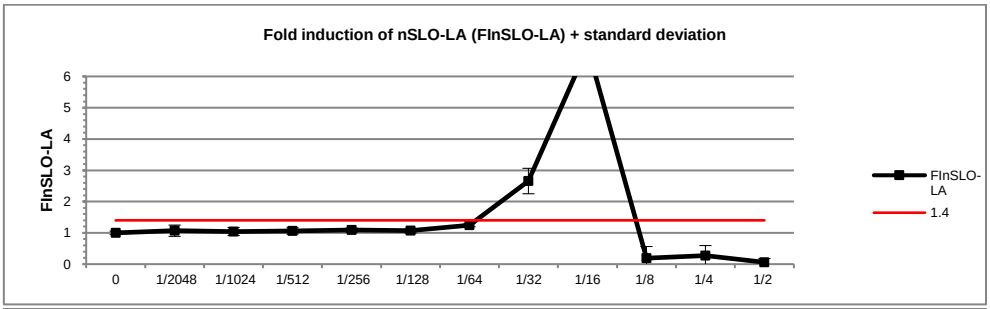


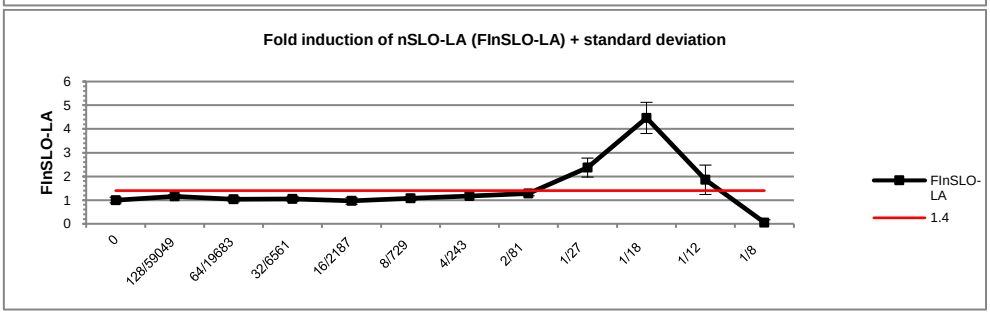
4. 4-Nitrobenzyl bromide(4-NBB)



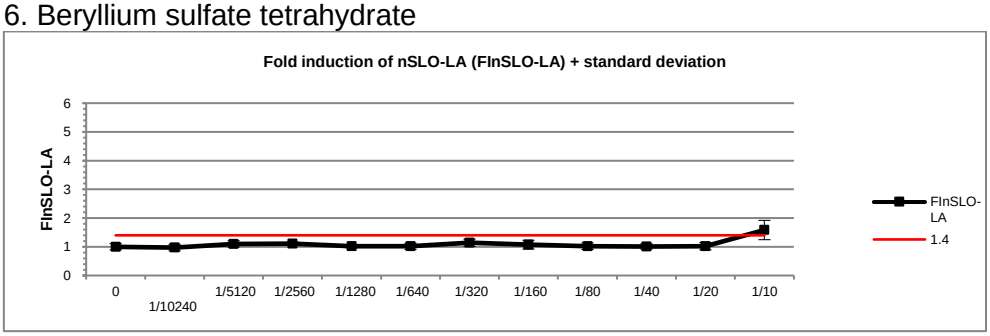


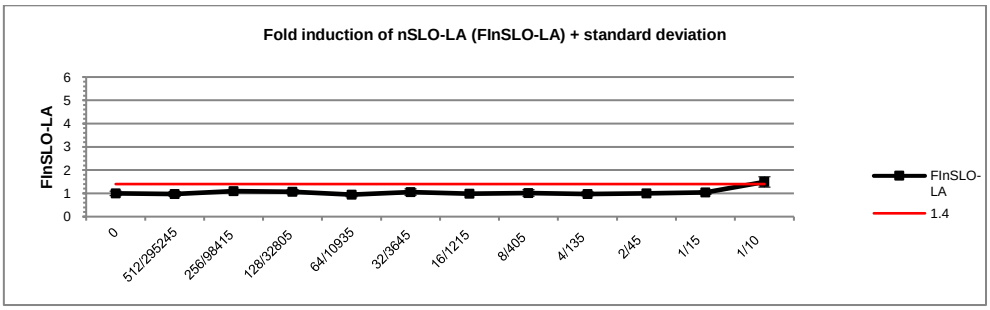
5. Bandrowski's base



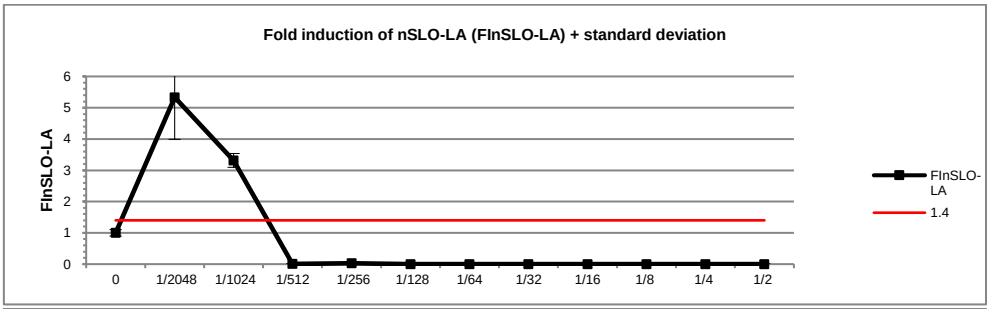


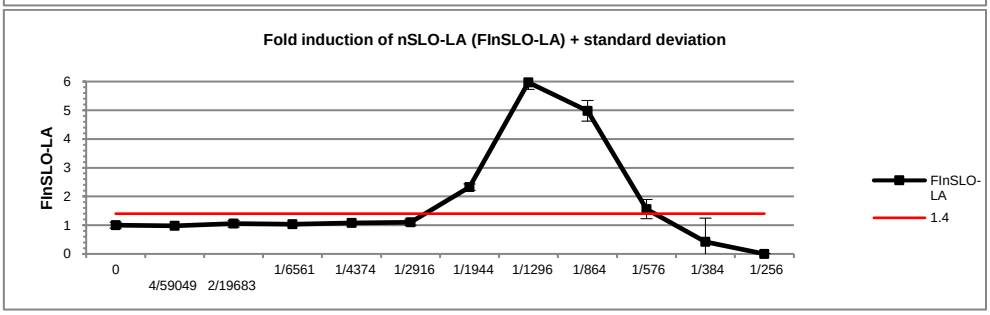
6. Beryllium sulfate tetrahydrate



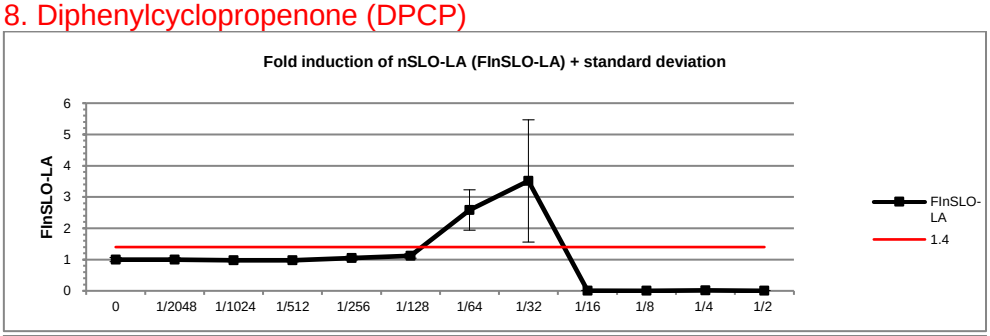


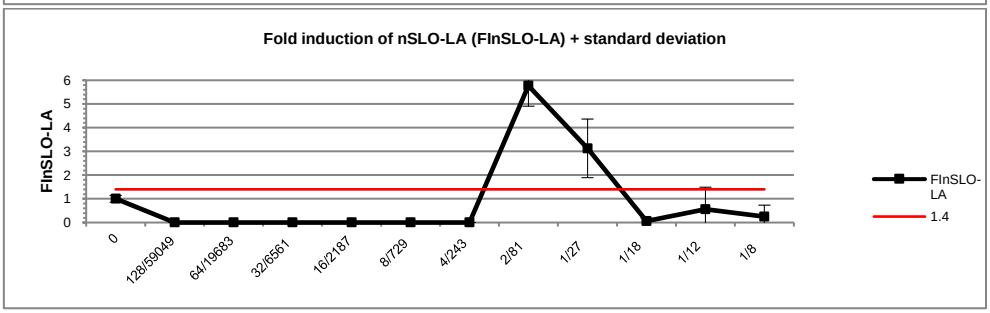
7. Dinitrofluorobenzene



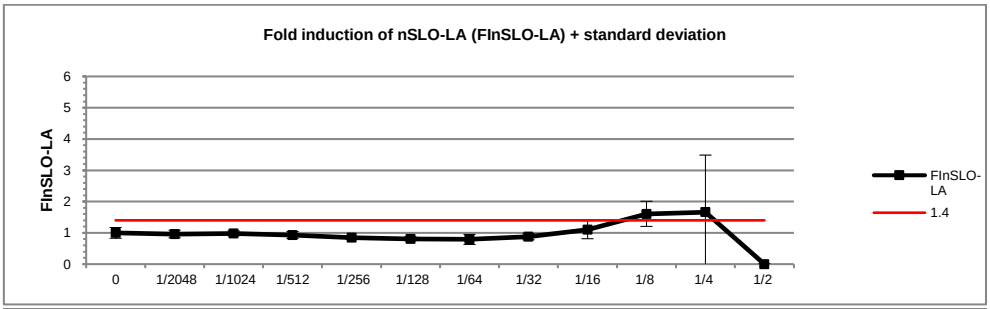


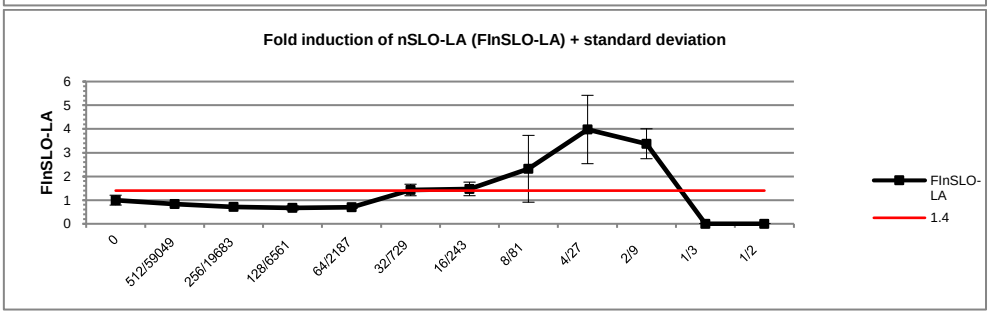
8. Diphenylcyclopropenone (DPCP)



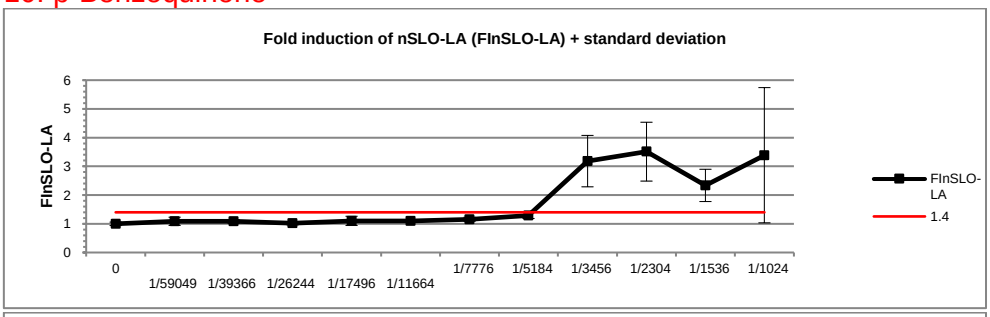


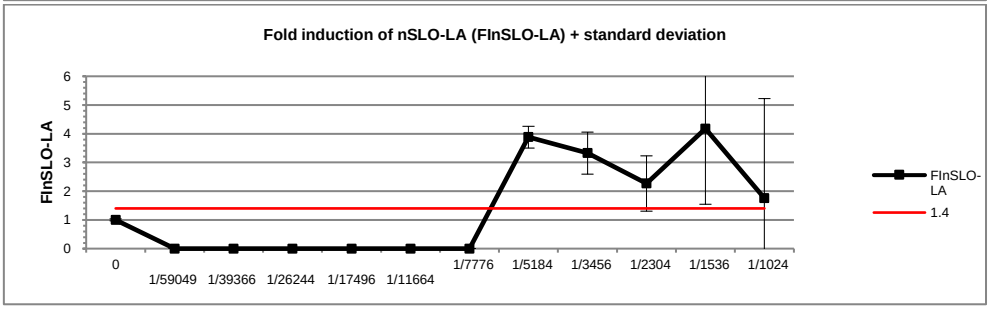
9. Oxazolone



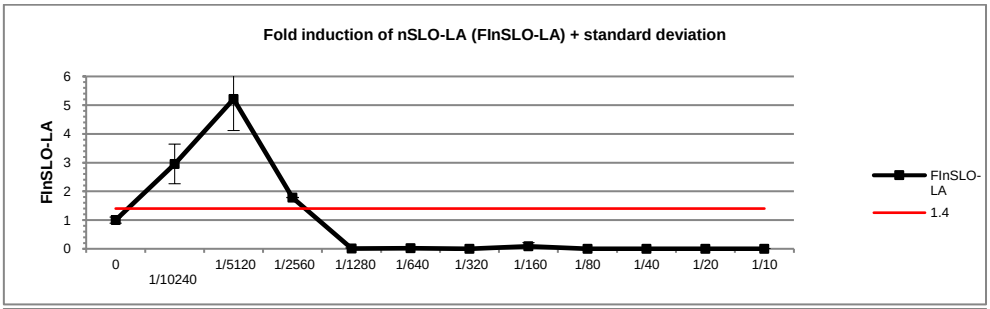


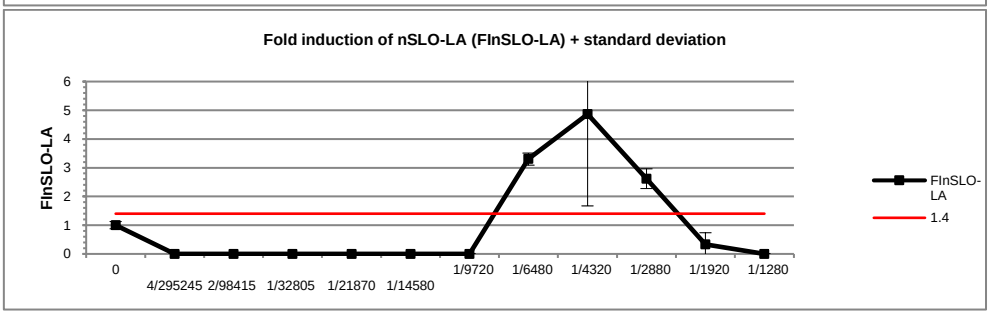
10. p-Benzoquinone



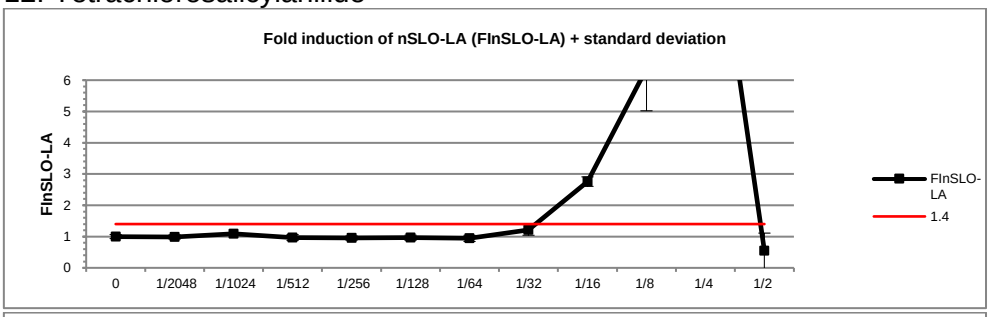


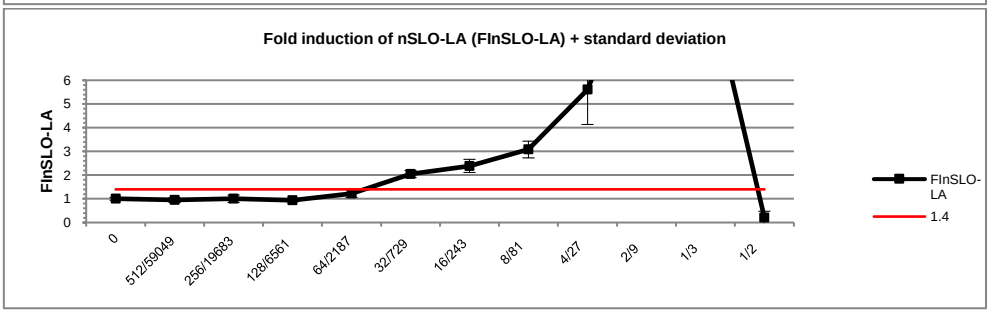
11. Potassium dichromate



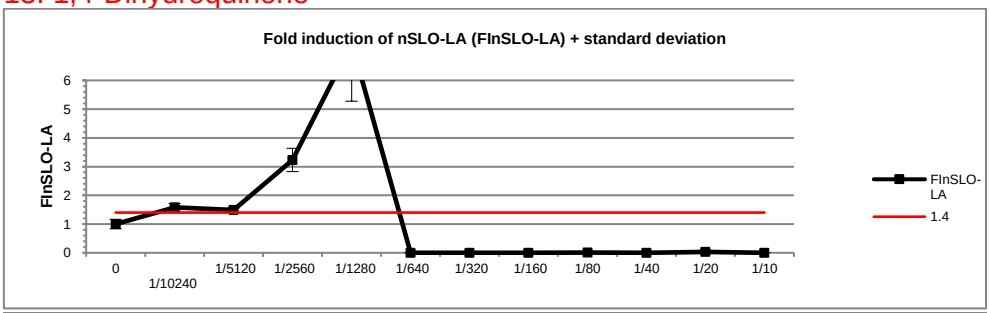


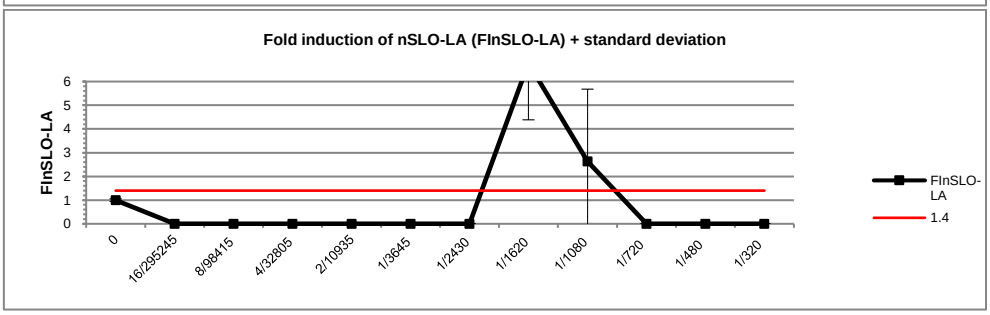
12. Tetrachlorosalicylanilide



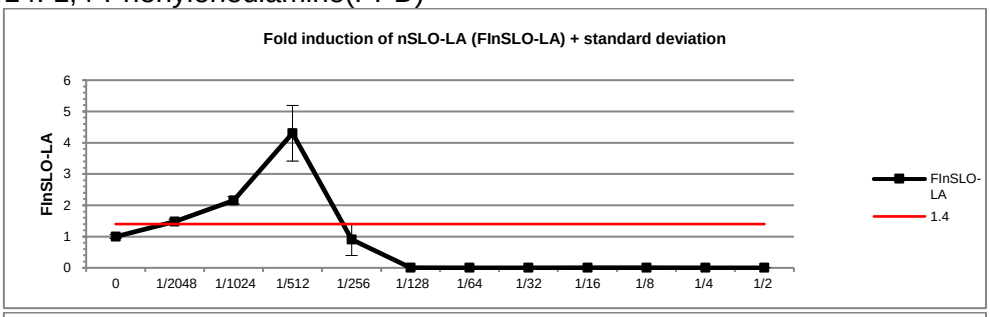


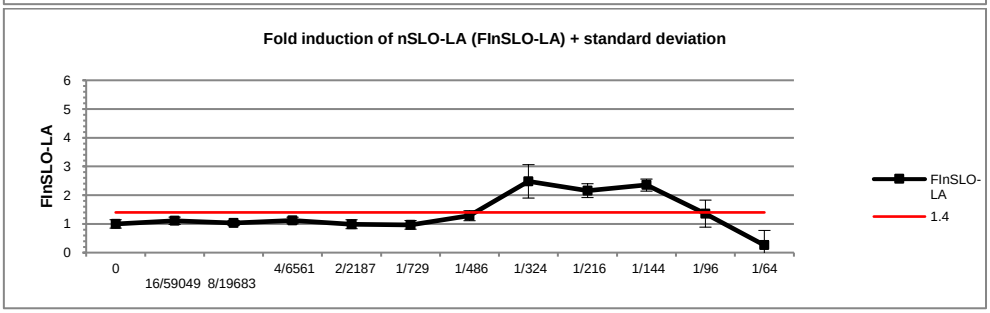
13. 1,4-Dihydroquinone



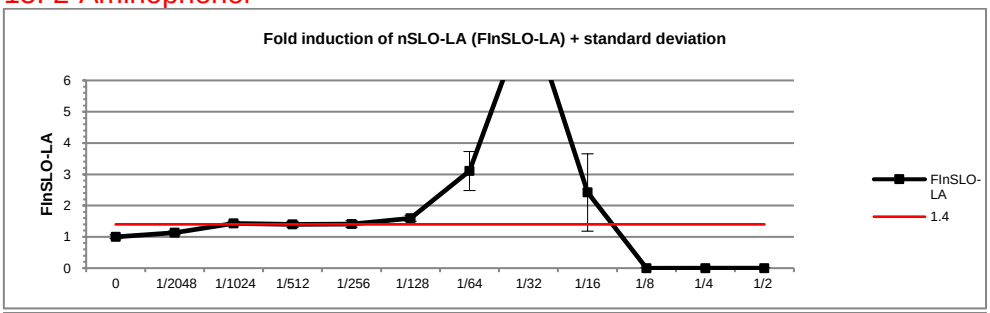


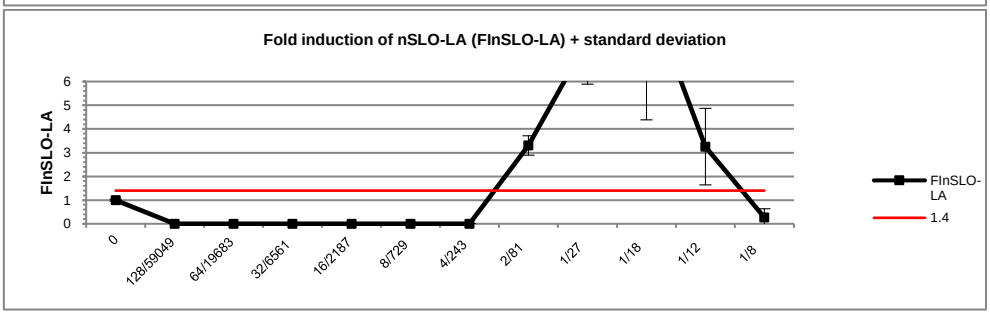
14. 1,4-Phenylenediamine(PPD)



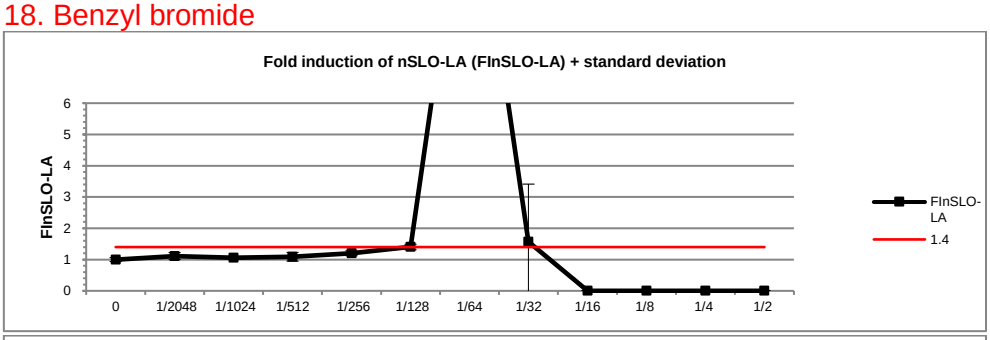


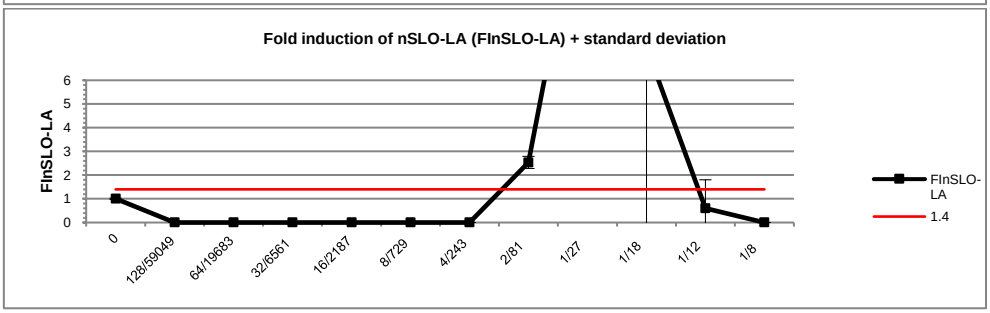
15. 2-Aminophenol



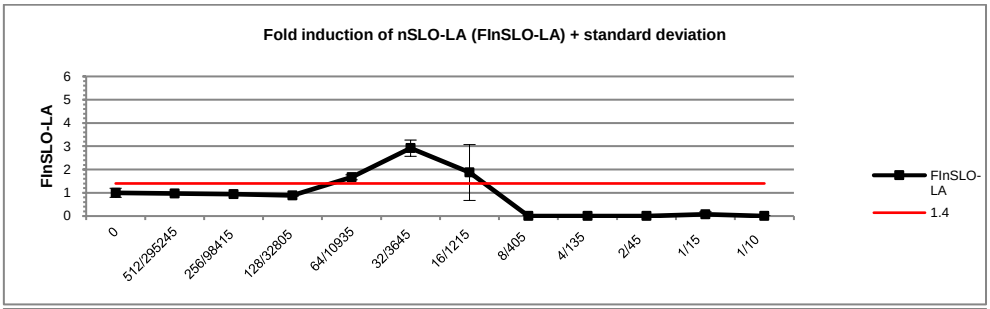


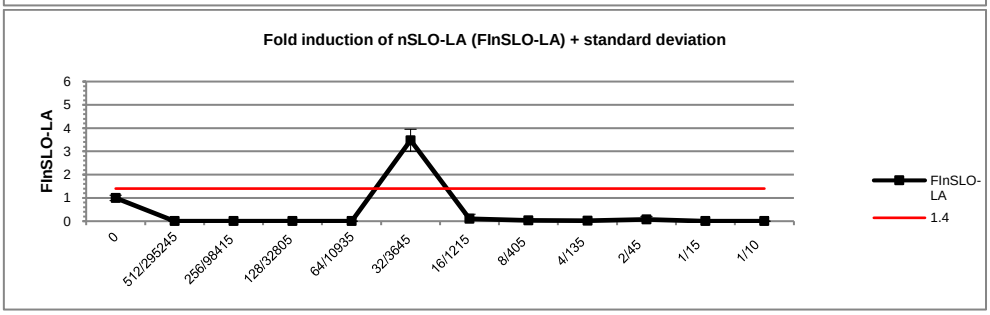
18. Benzyl bromide



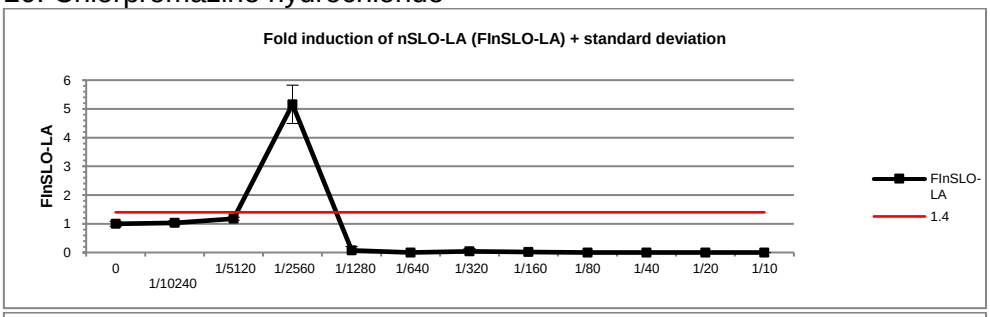


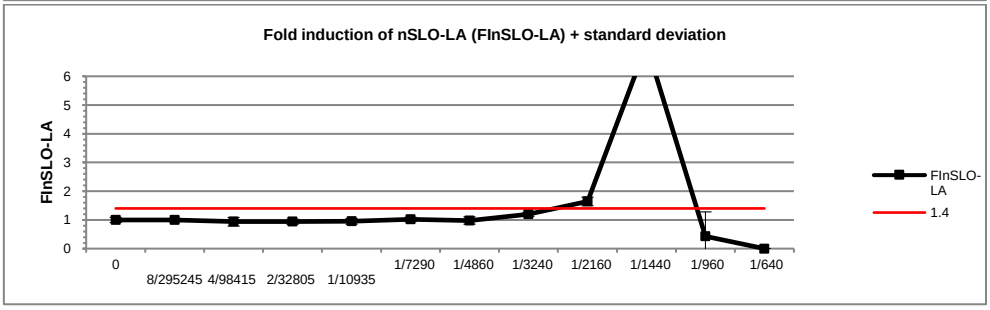
19. Chloramine-T



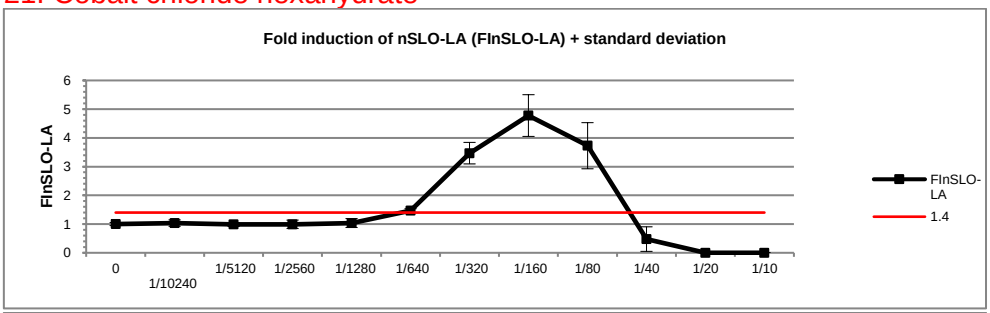


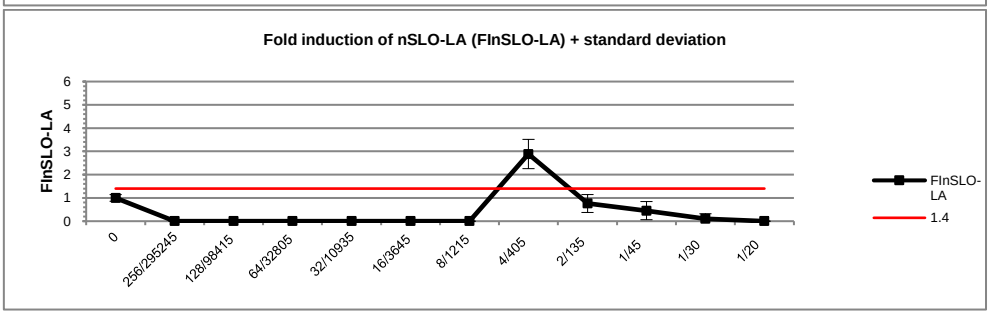
20. Chlorpromazine hydrochloride



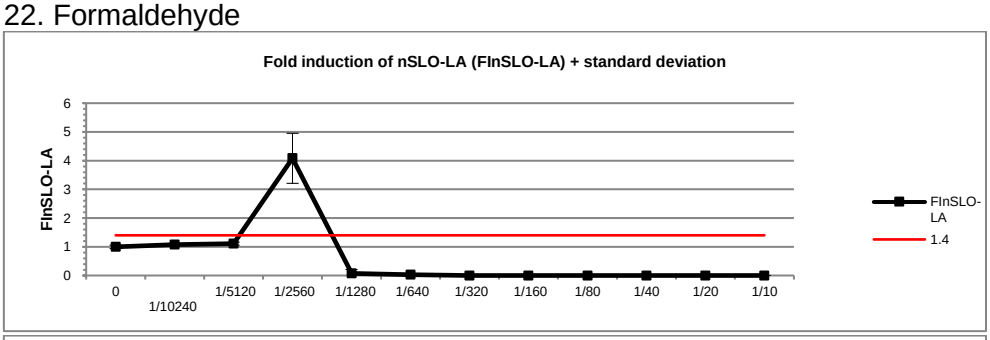


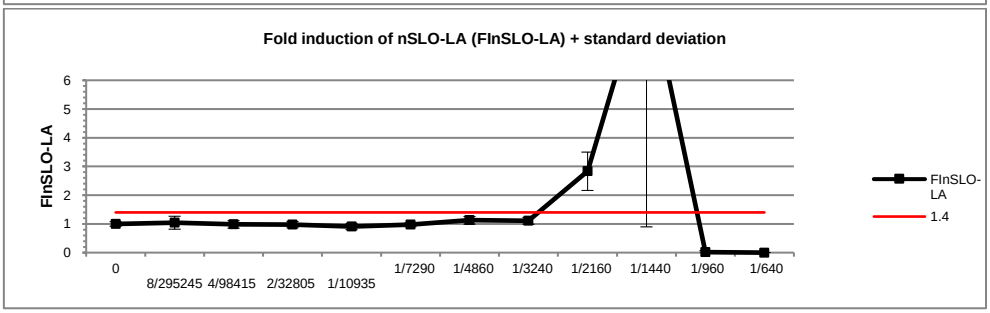
21. Cobalt chloride hexahydrate



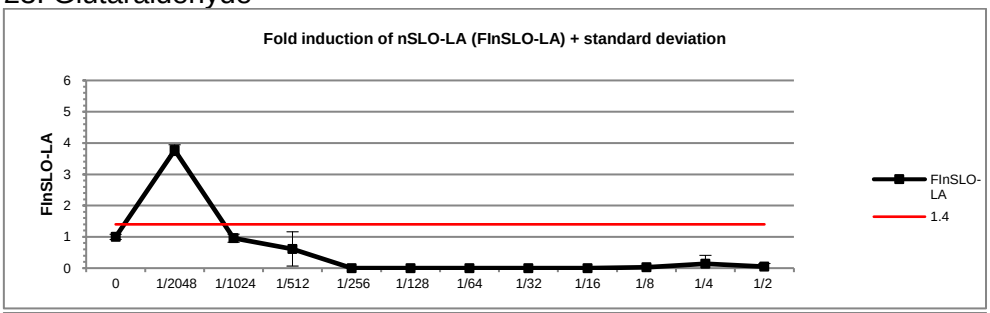


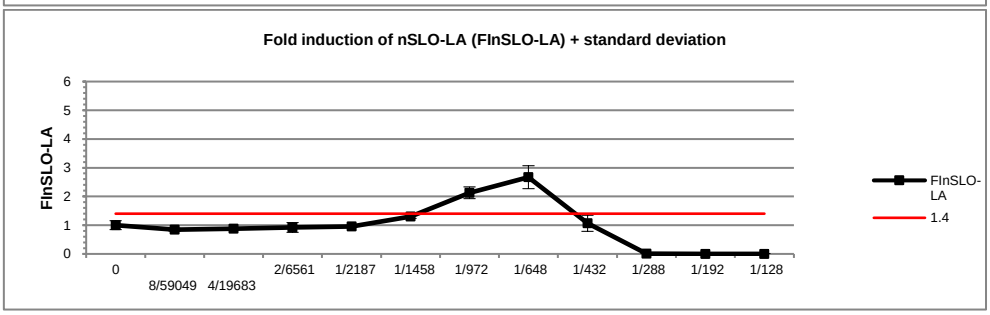
22. Formaldehyde



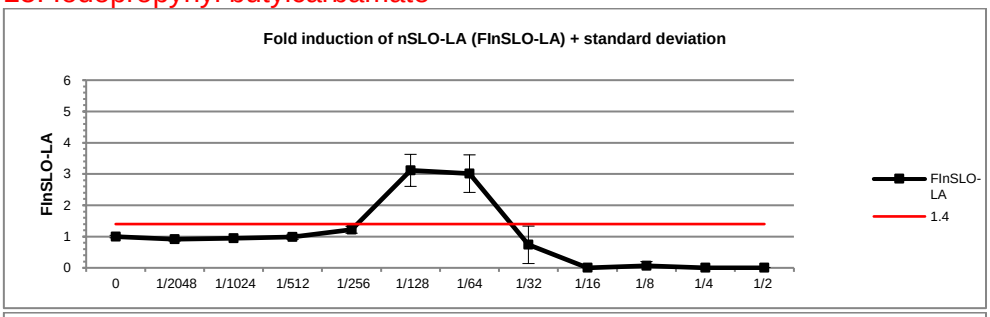


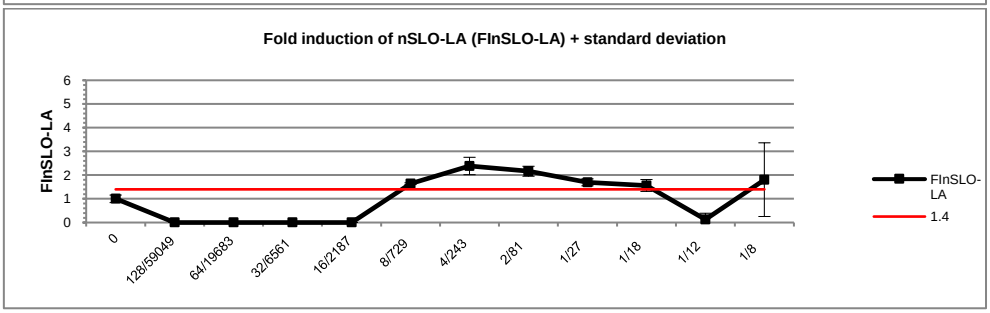
23. Glutaraldehyde



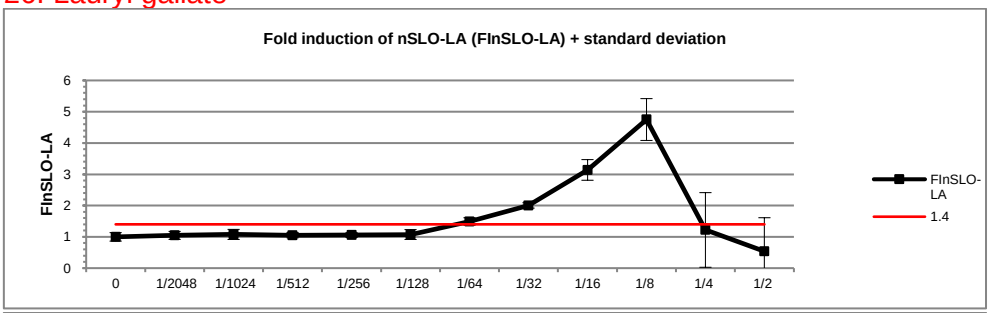


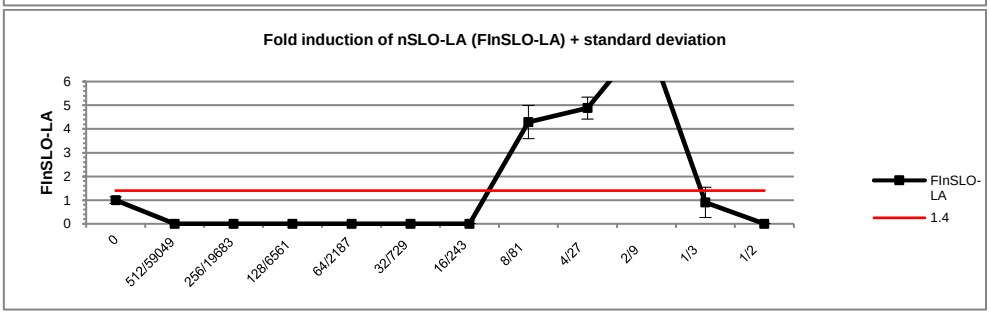
25. Iodopropynyl butylcarbamate



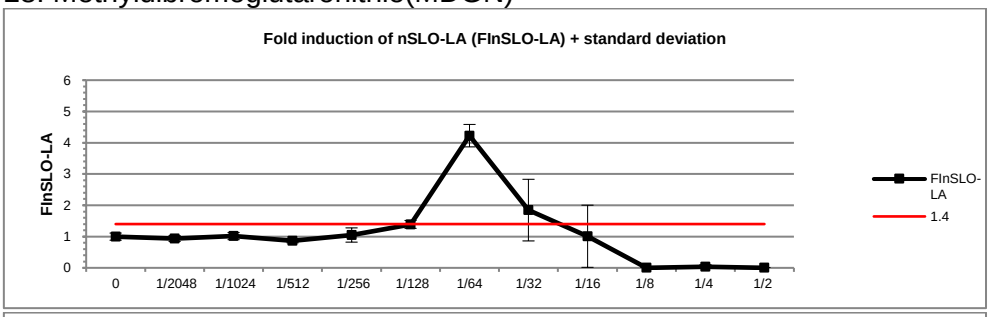


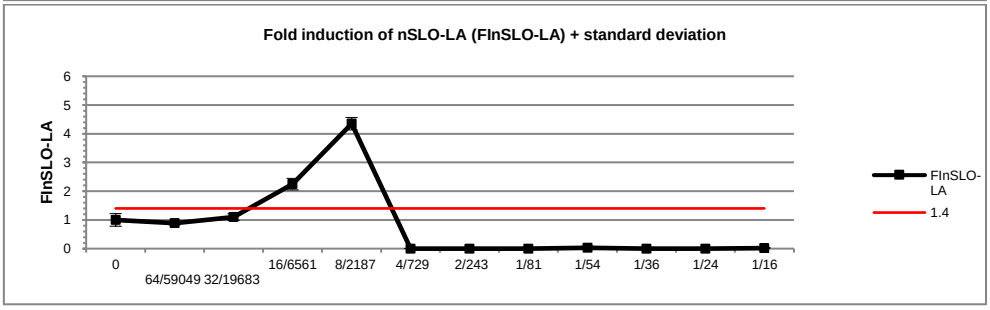
26. Lauryl gallate



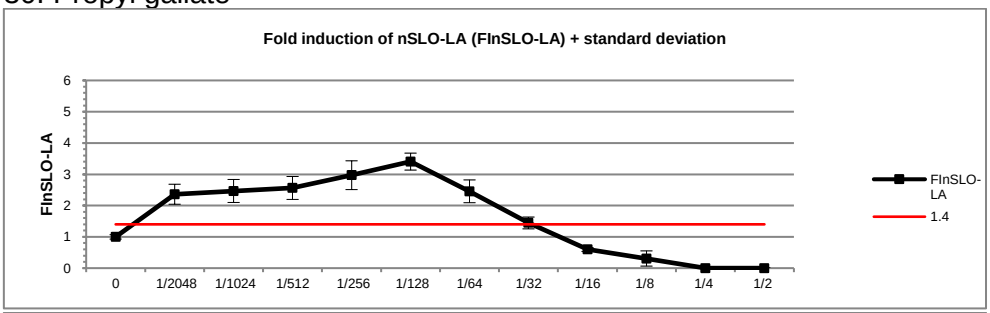


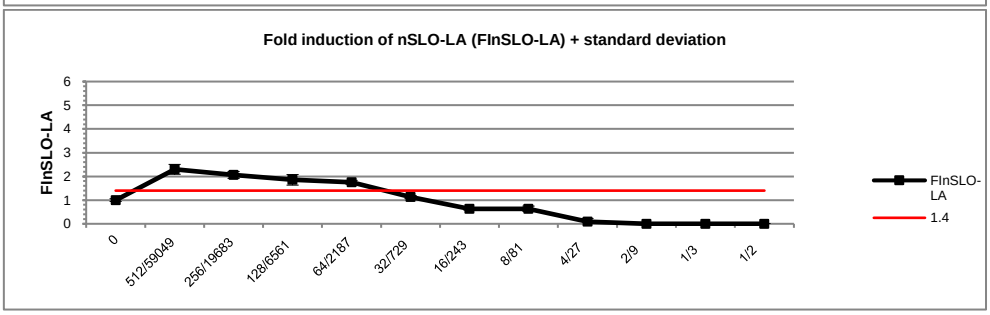
28. Methyldibromoglutaronitrile(MDGN)



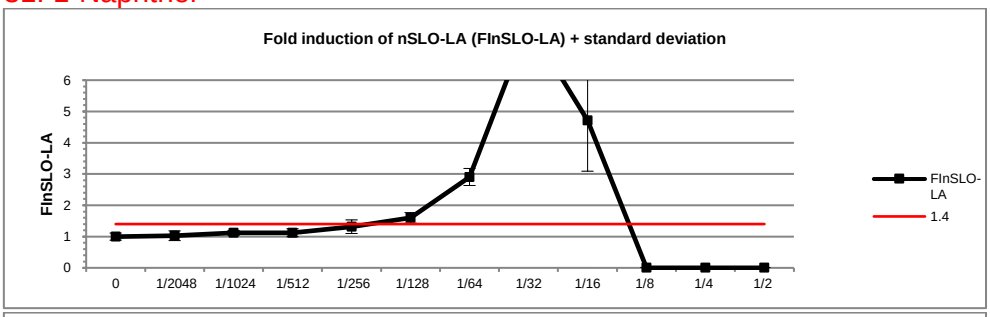


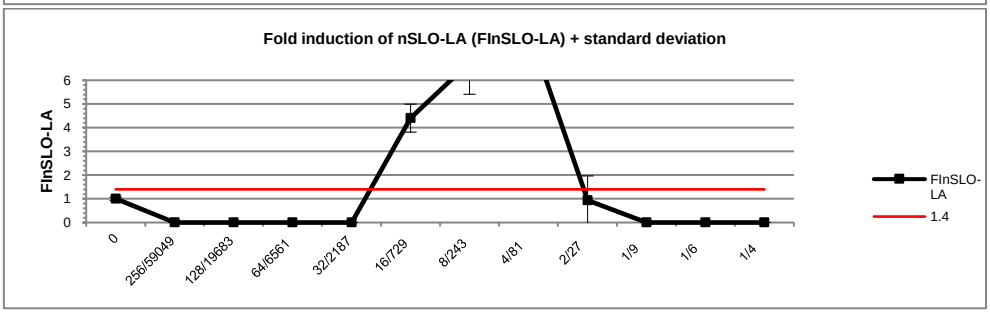
30. Propyl gallate



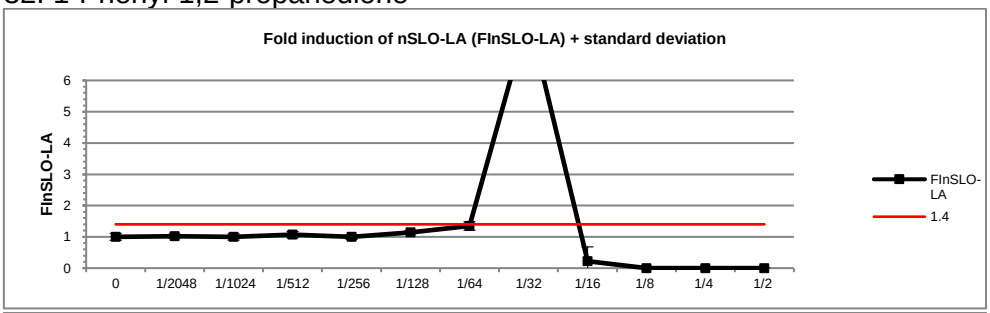


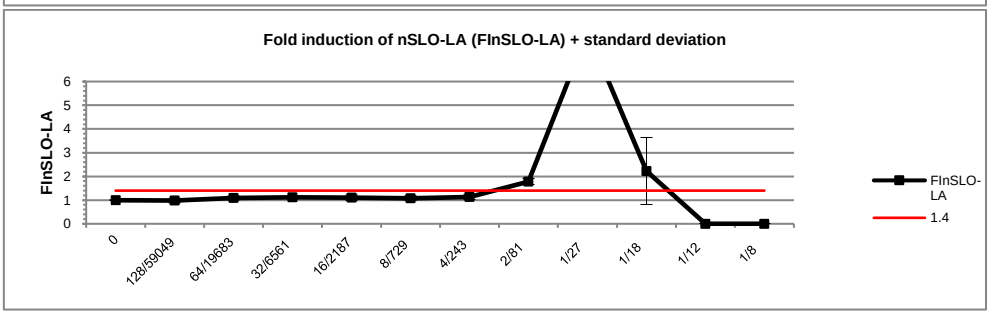
31. 1-Naphthol



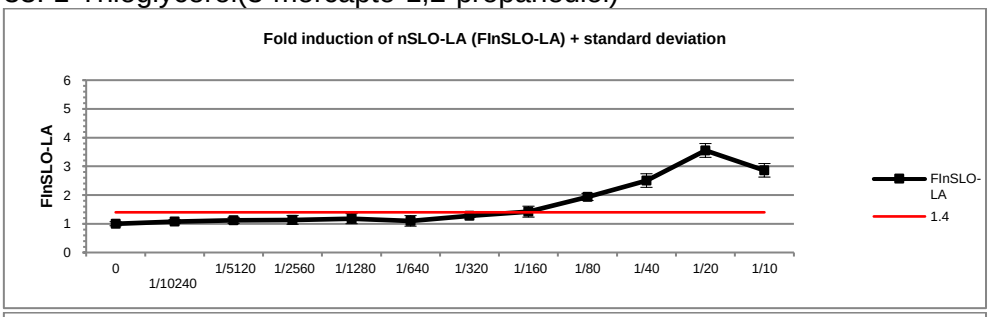


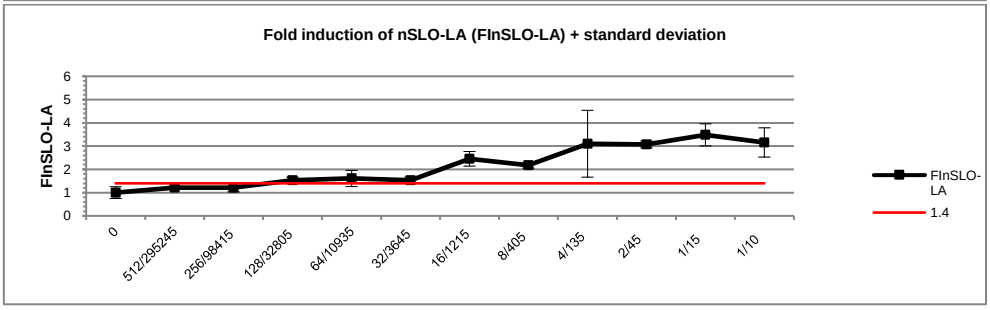
32. 1-Phenyl-1,2-propanedione



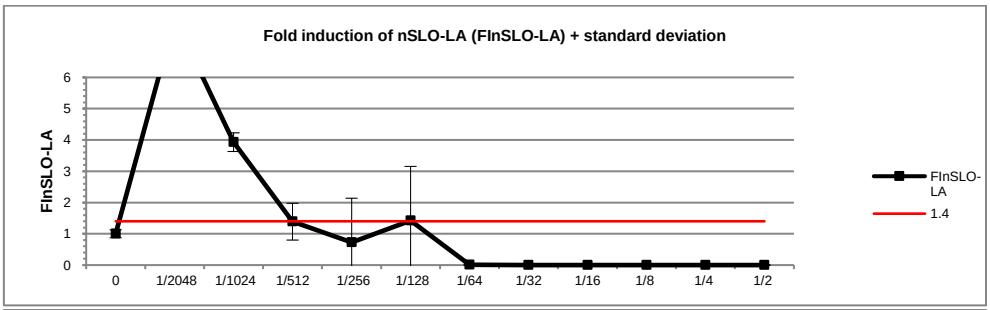


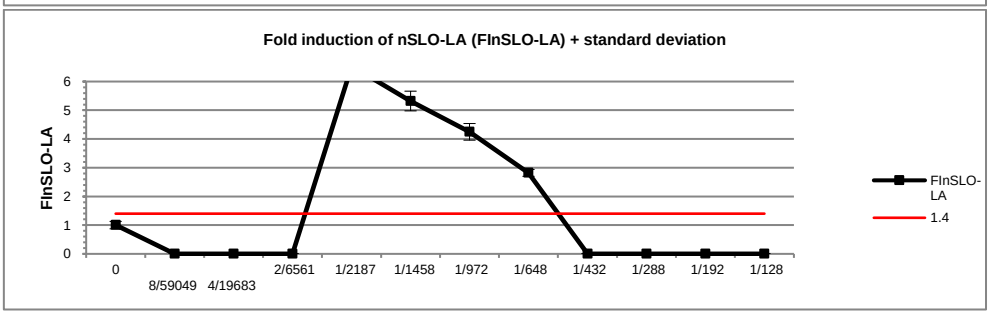
33. 1-Thioglycerol(3-mercapto-1,2-propanediol)



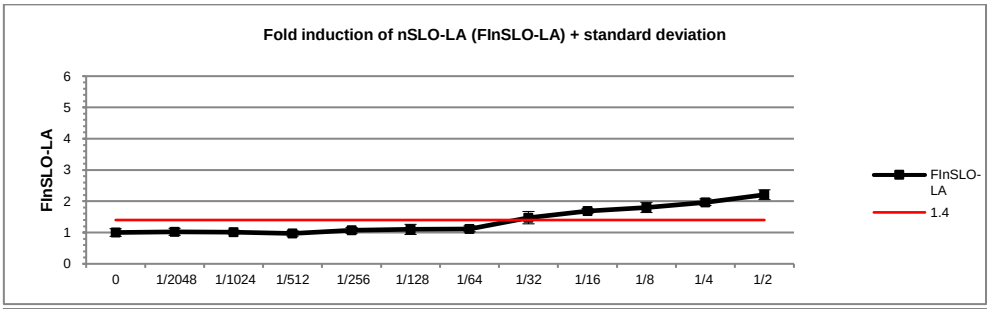


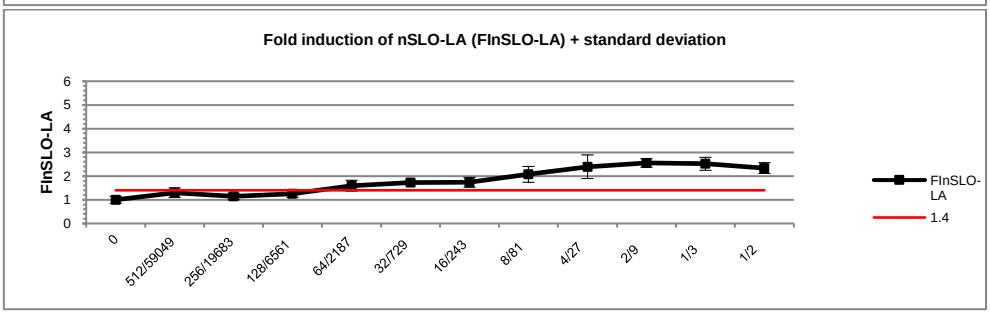
34. 1, 2-Benzisothiazolin-3-one



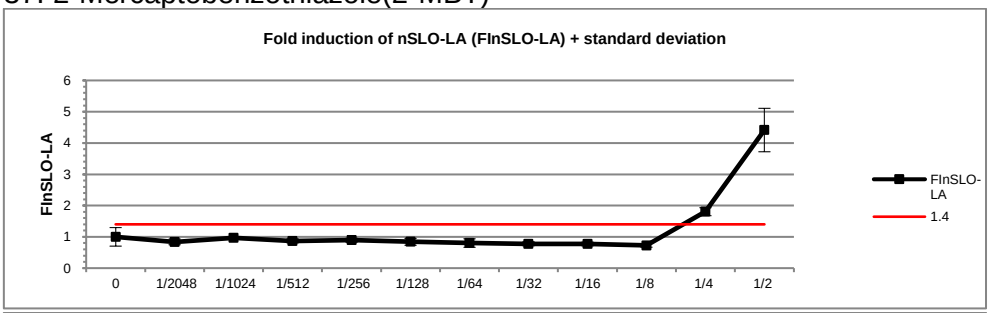


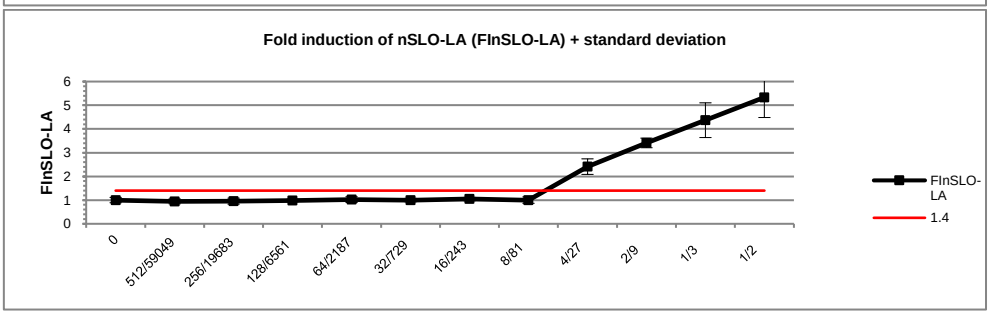
35. 12-Bromo-1-dodecanol



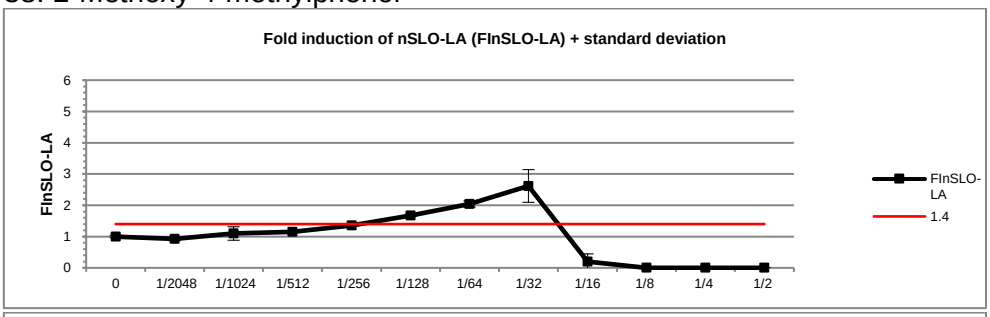


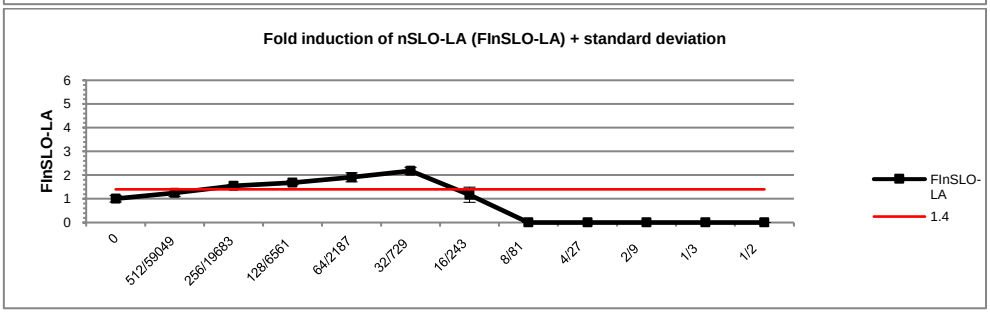
37. 2-Mercaptobenzothiazole(2-MBT)



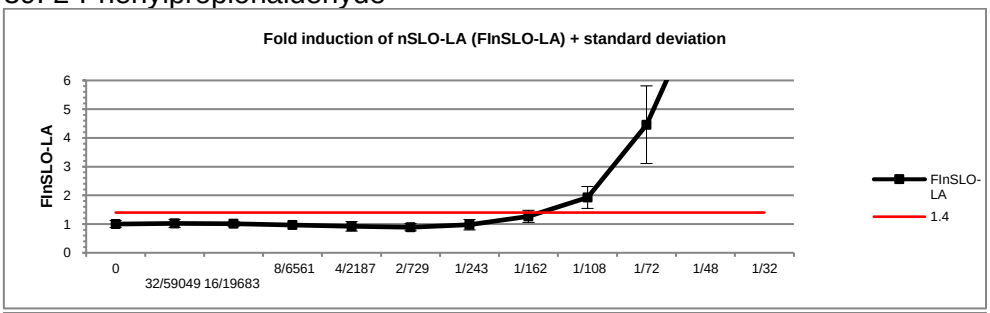


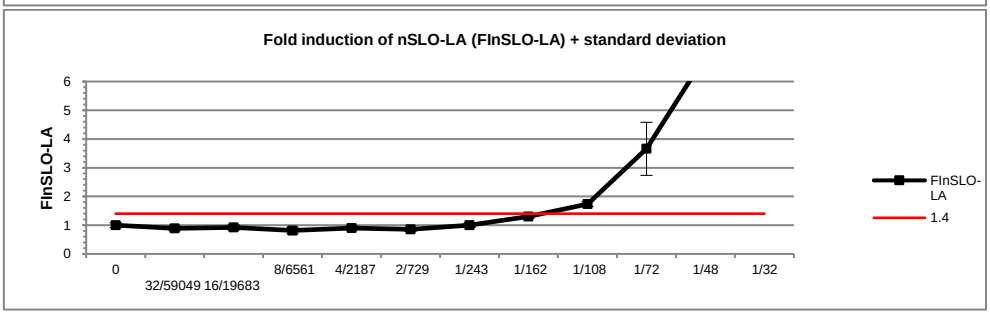
38. 2-Methoxy-4-methylphenol



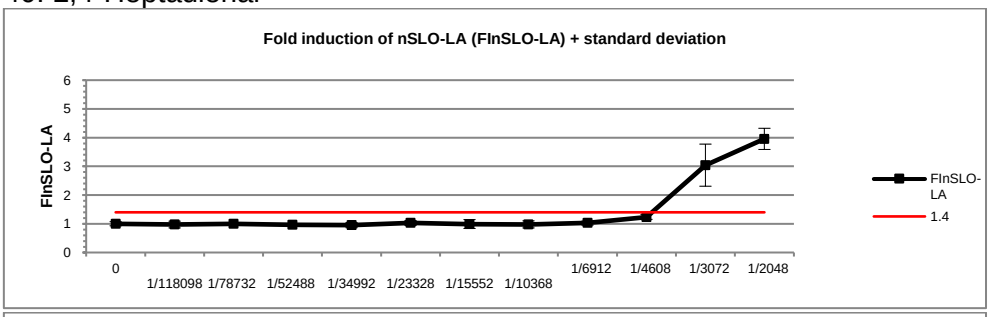


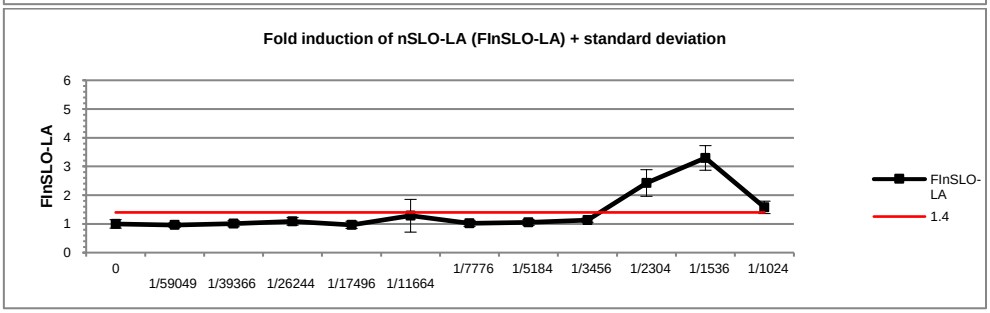
39. 2-Phenylpropionaldehyde



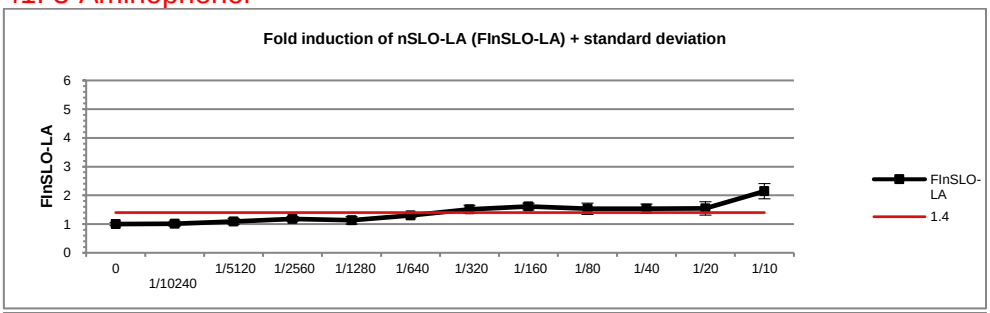


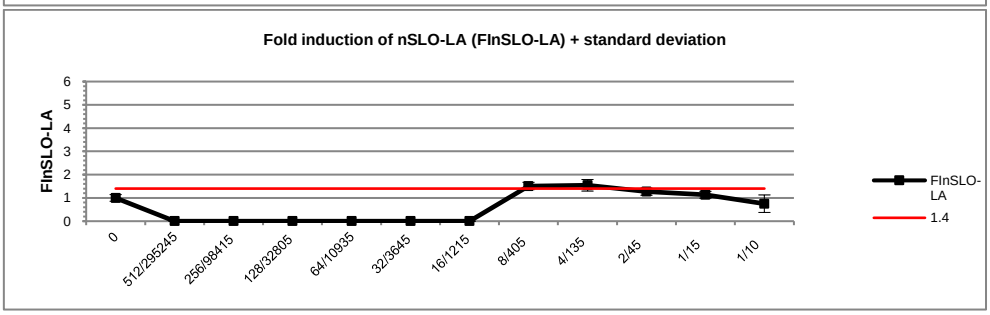
40. 2,4-Heptadienal



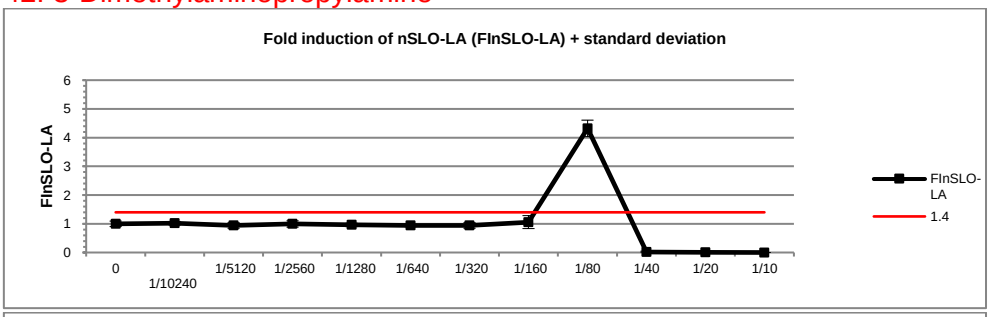


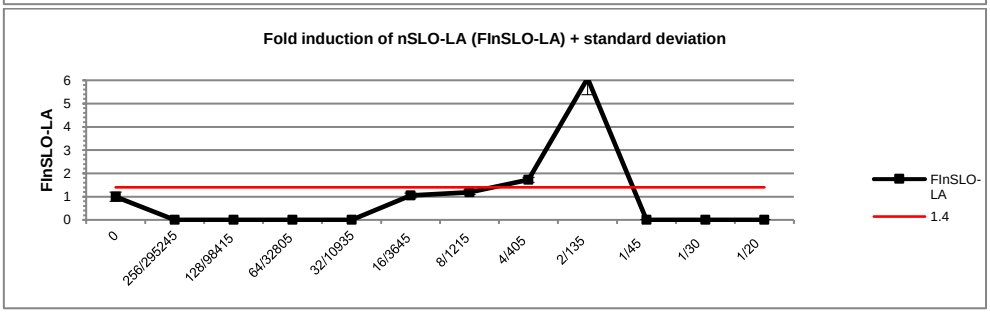
41. 3-Aminophenol



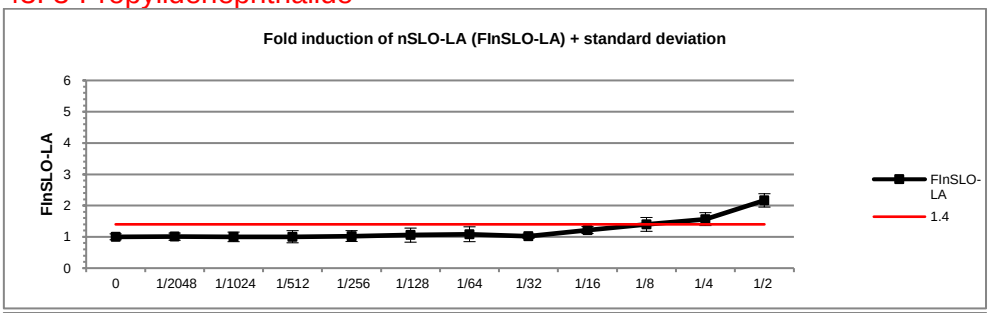


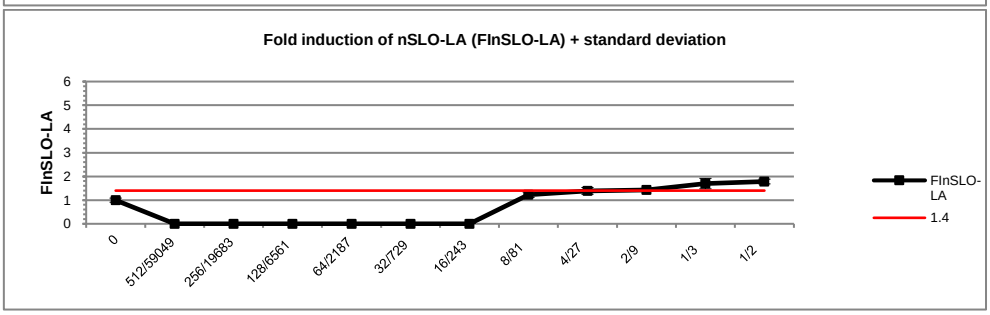
42. 3-Dimethylaminopropylamine



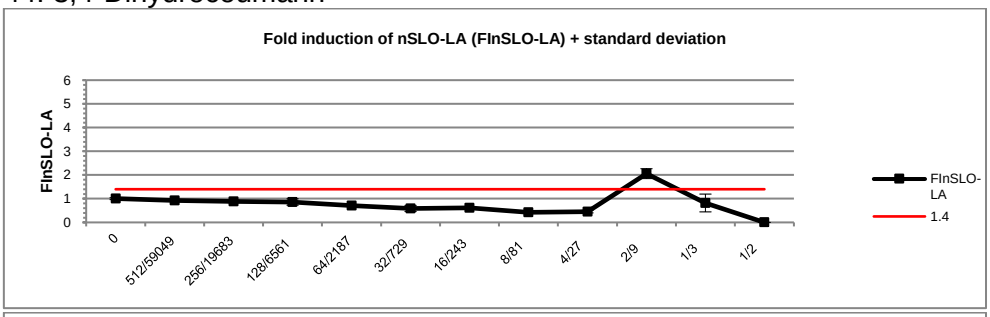


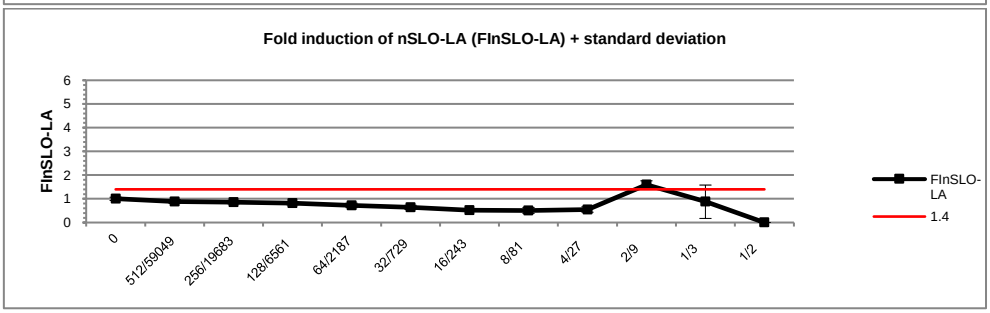
43. 3-Propylidenephthalide



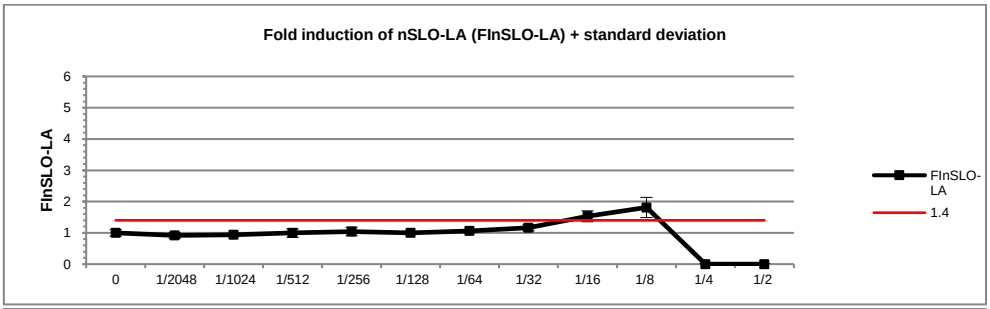


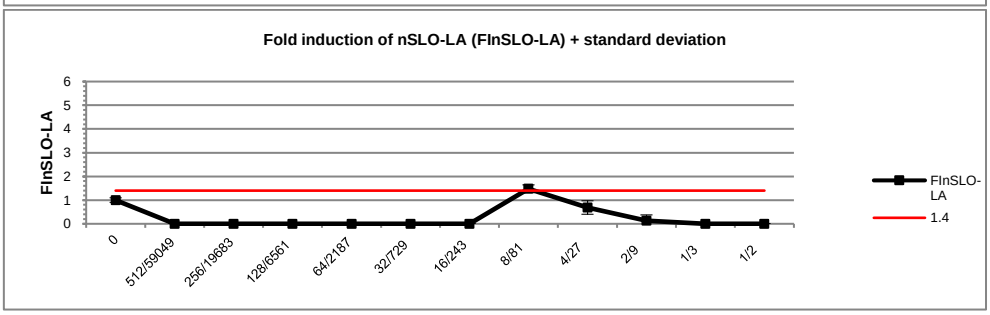
44. 3,4-Dihydrocoumarin



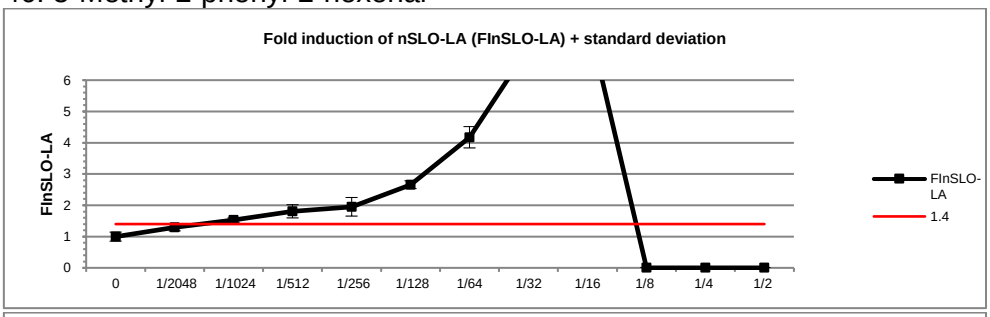


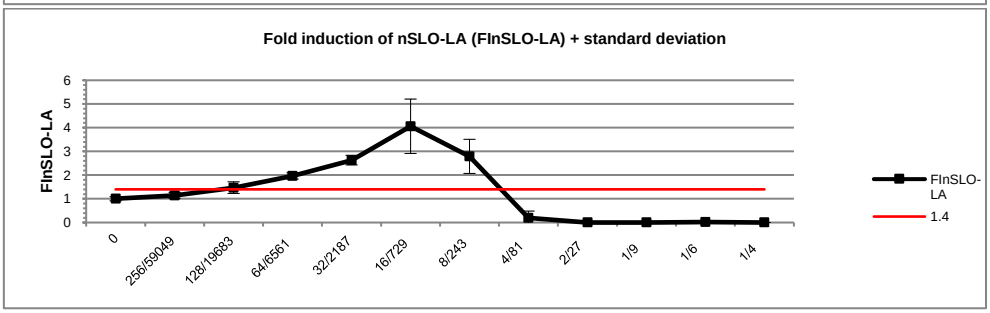
45. 4-Chloroaniline



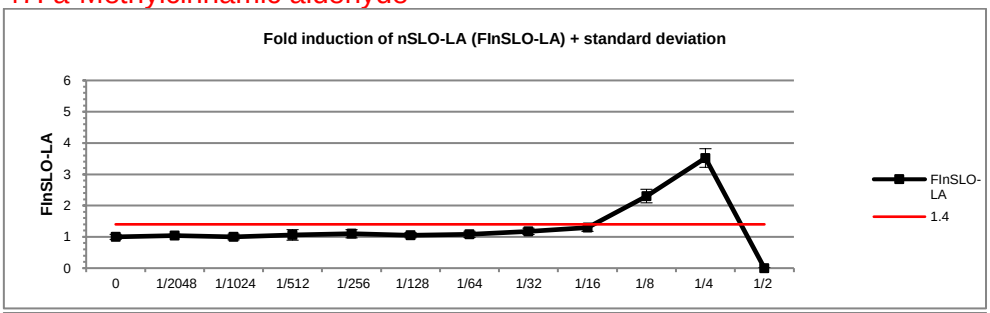


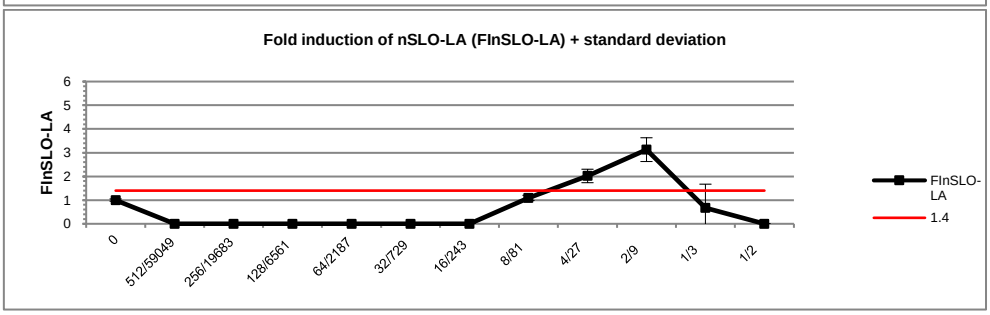
46. 5-Methyl-2-phenyl-2-hexenal



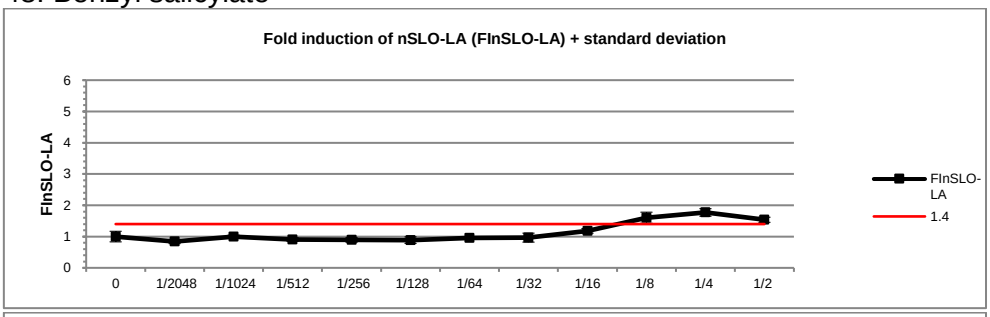


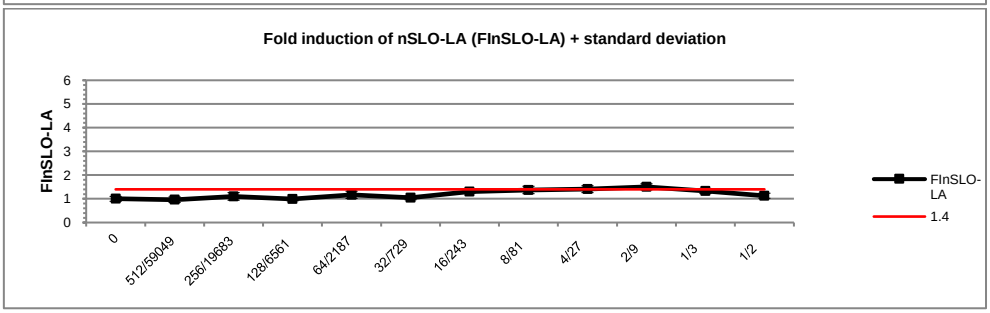
47. a-Methylcinnamic aldehyde



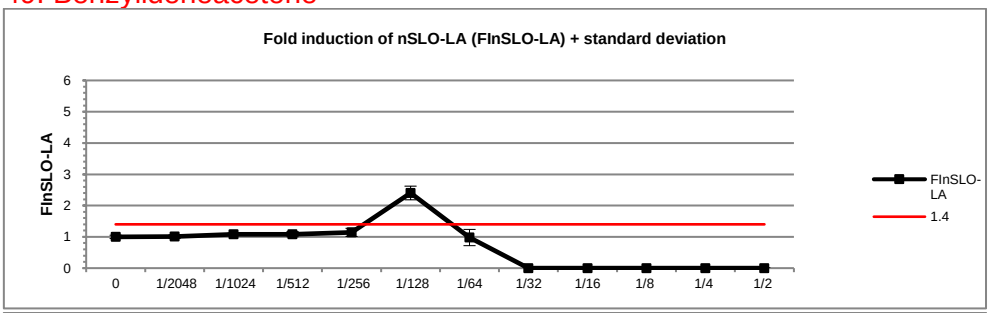


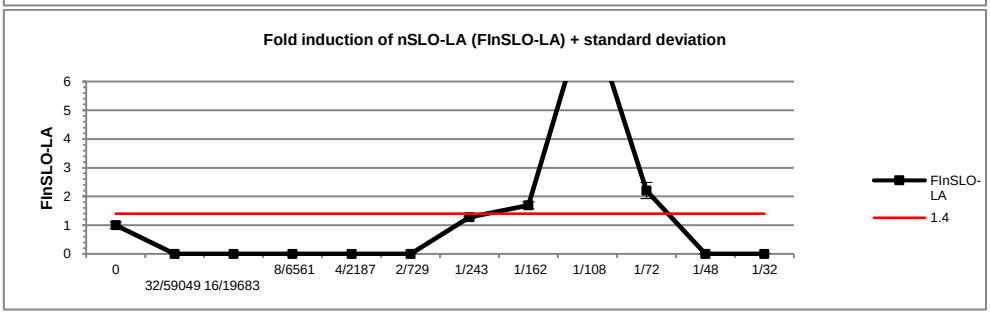
48. Benzyl salicylate



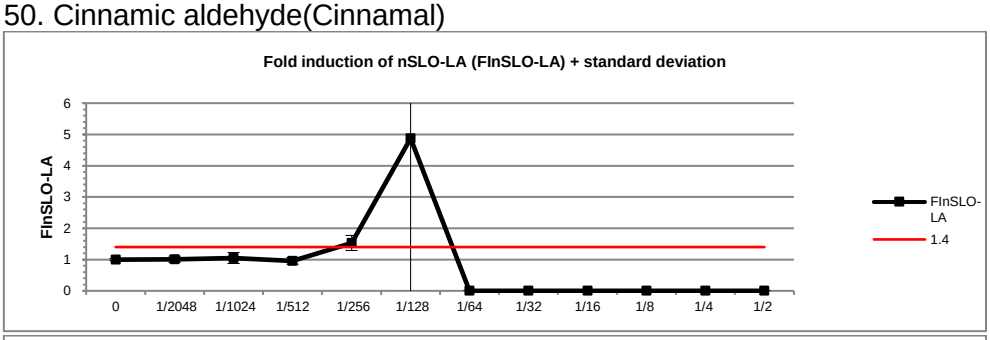


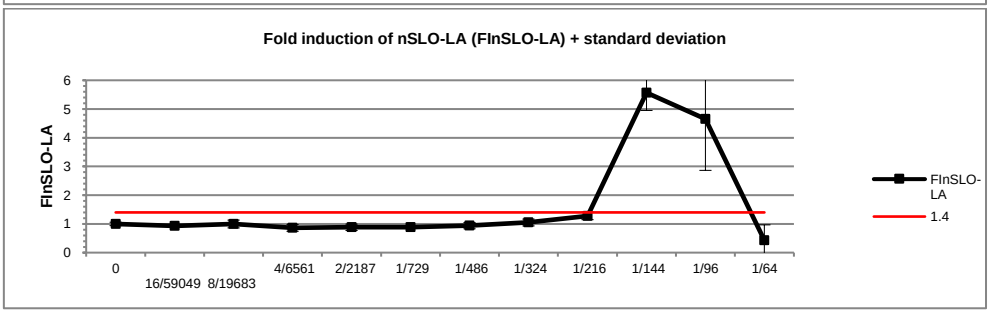
49. Benzylideneacetone



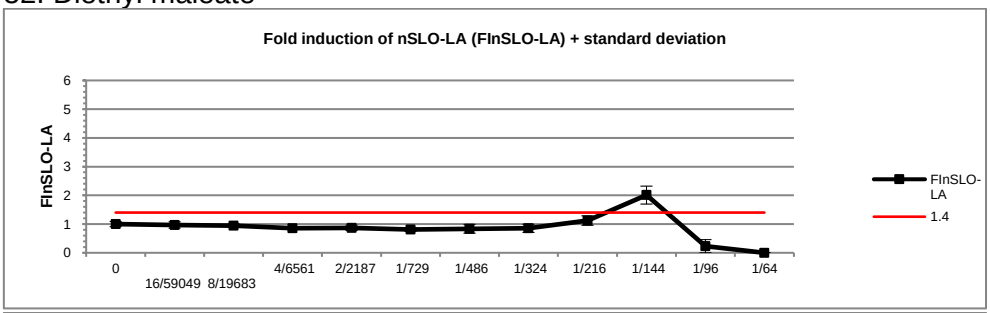


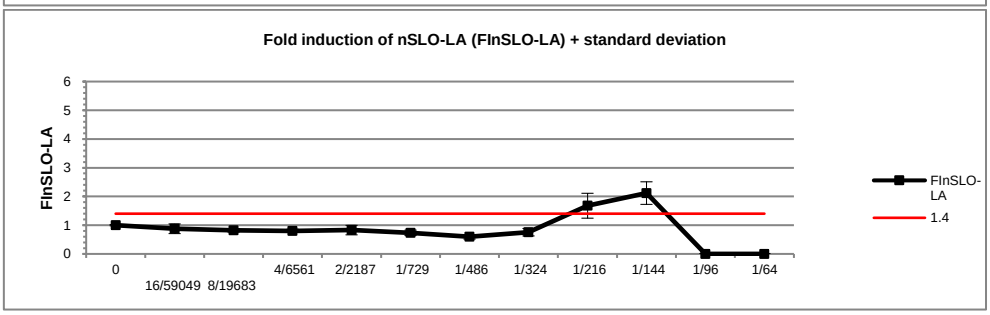
50. Cinnamic aldehyde(Cinnamal)



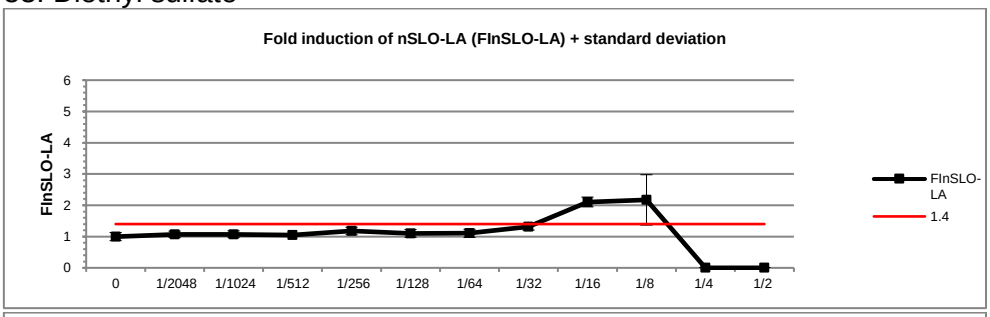


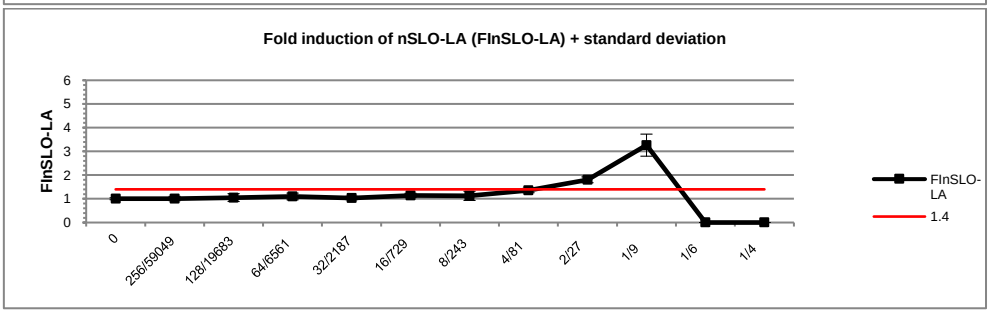
52. Diethyl maleate



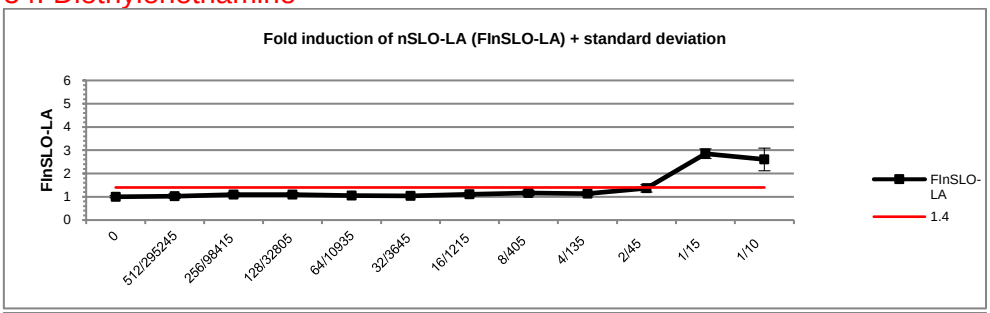


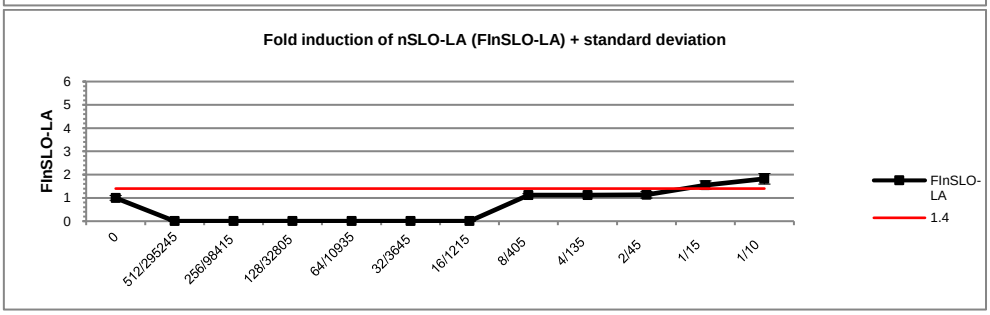
53. Diethyl sulfate



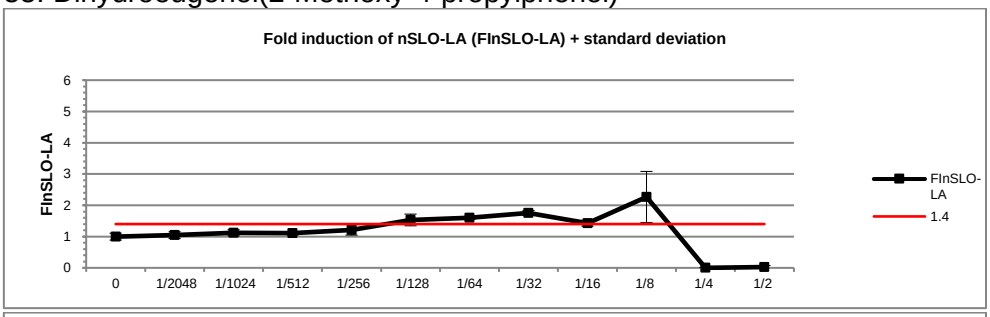


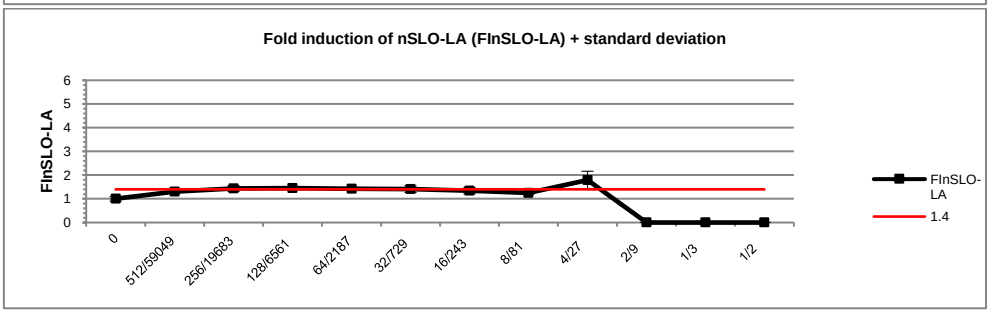
54. Diethylenetriamine



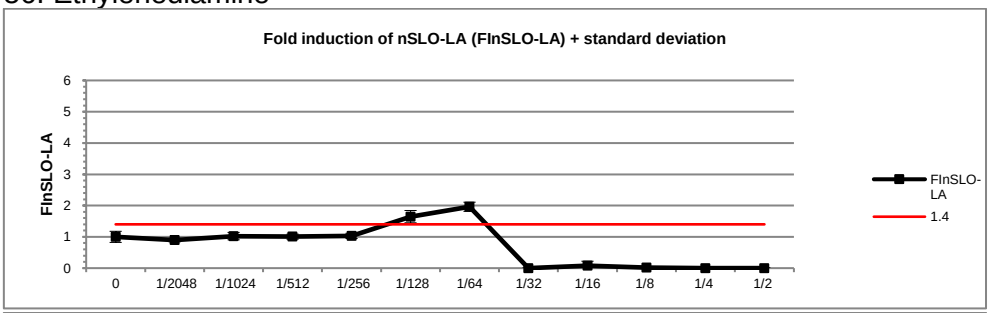


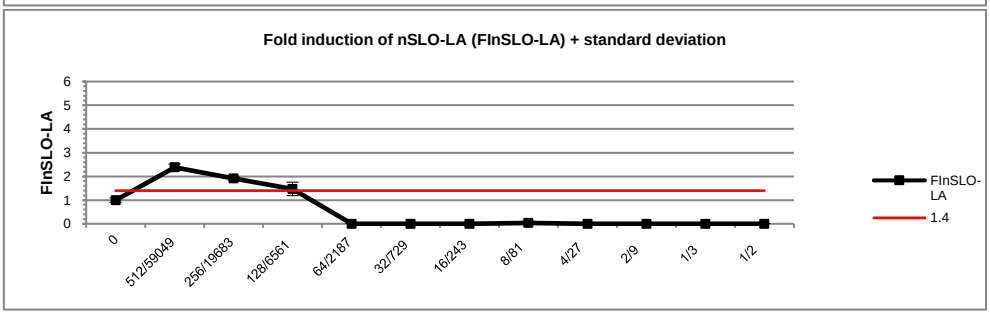
55. Dihydroeugenol(2-Methoxy-4-propylphenol)



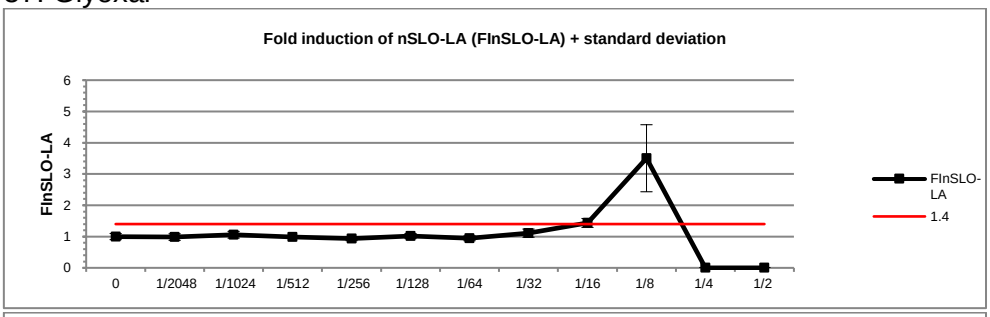


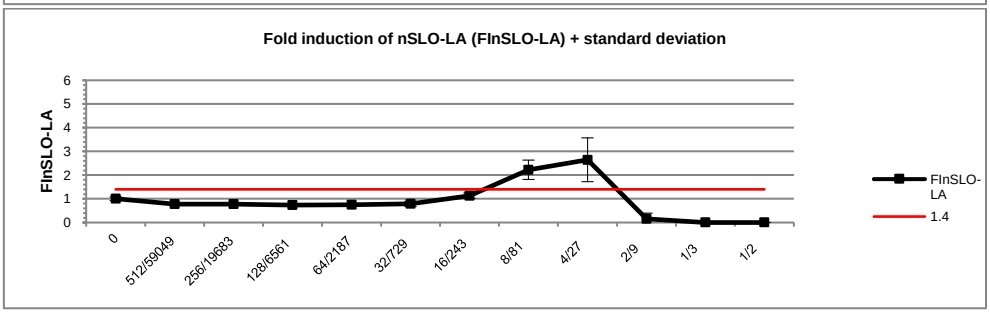
56. Ethylenediamine



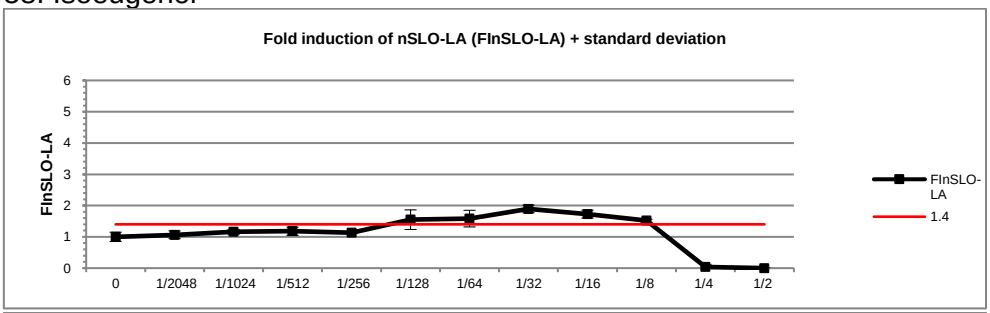


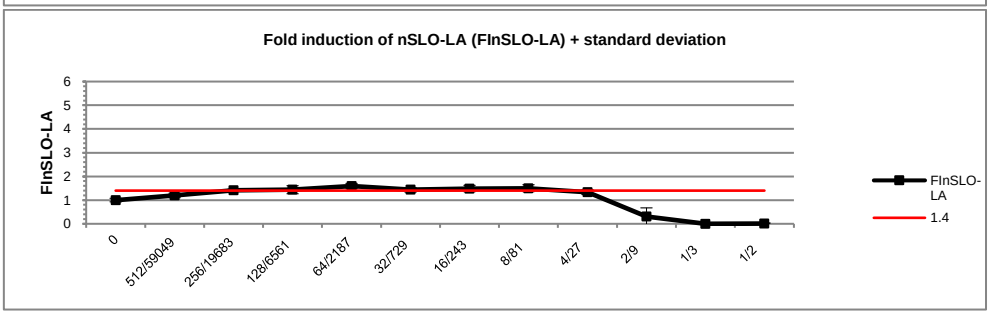
57. Glyoxal



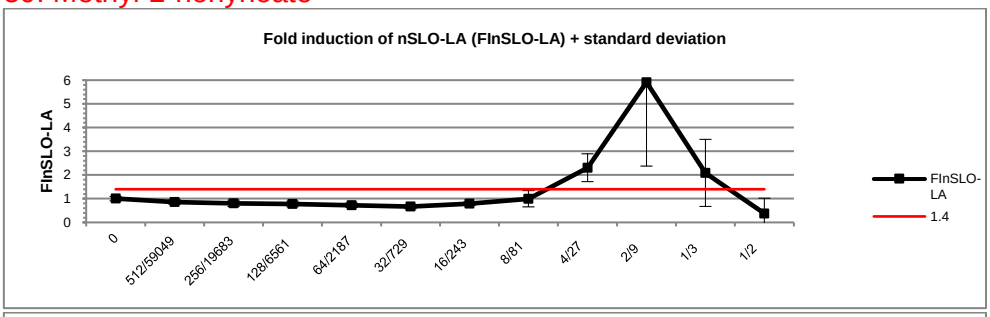


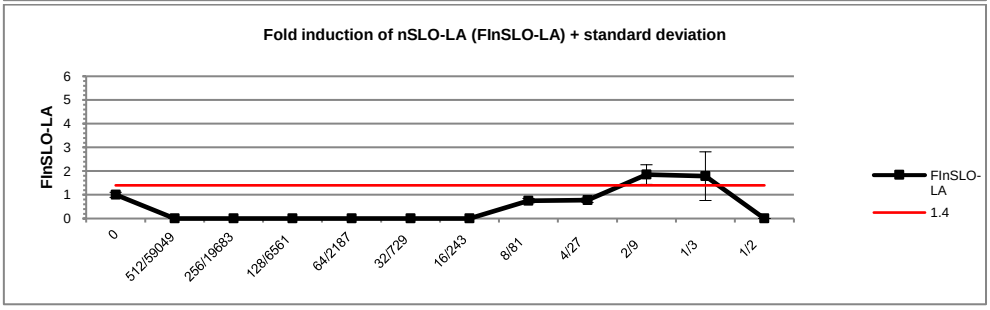
58. Isoeugenol



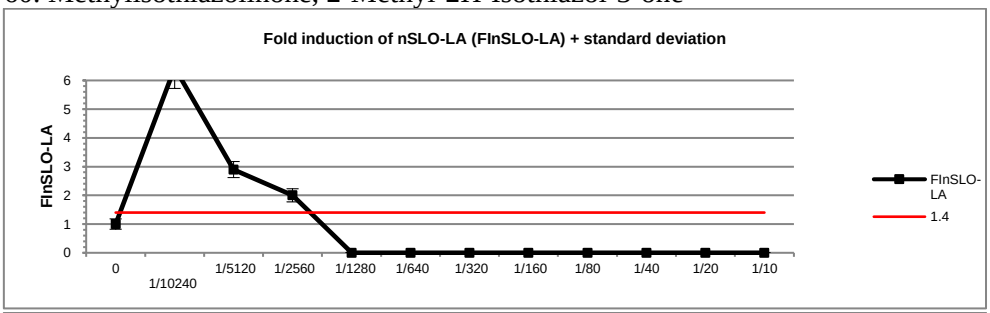


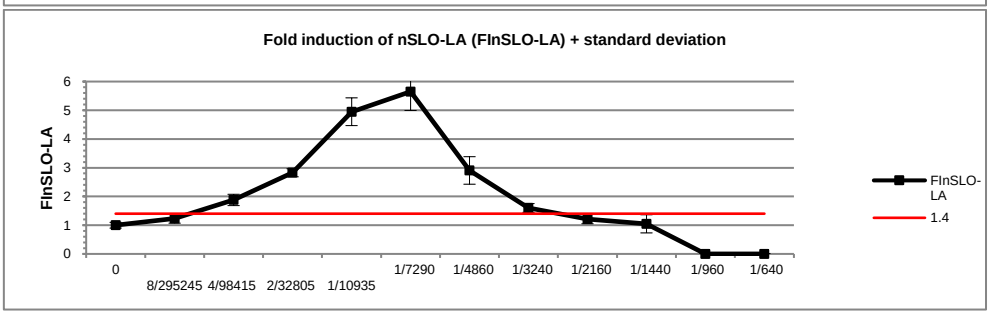
59. Methyl-2-nonynoate



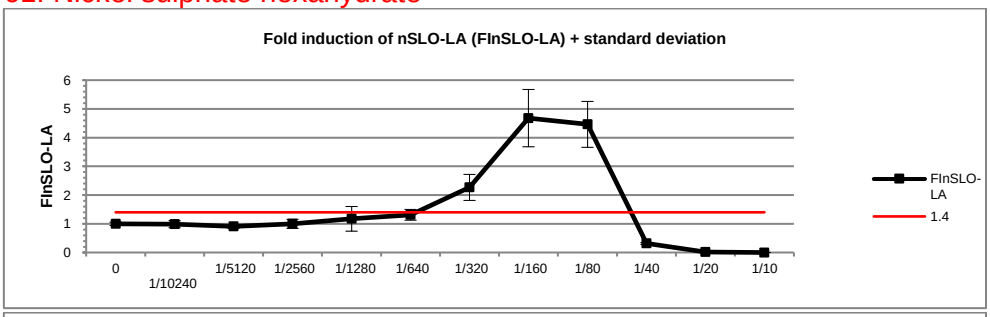


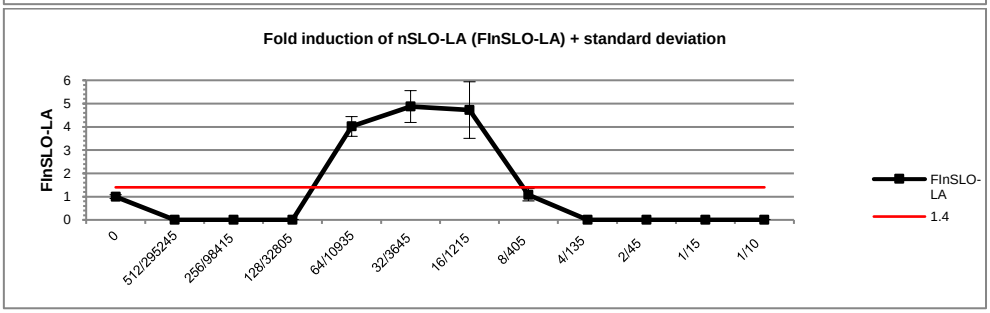
60. Methylisothiazolinone, 2-Methyl-2H-Isothiazol-3-one



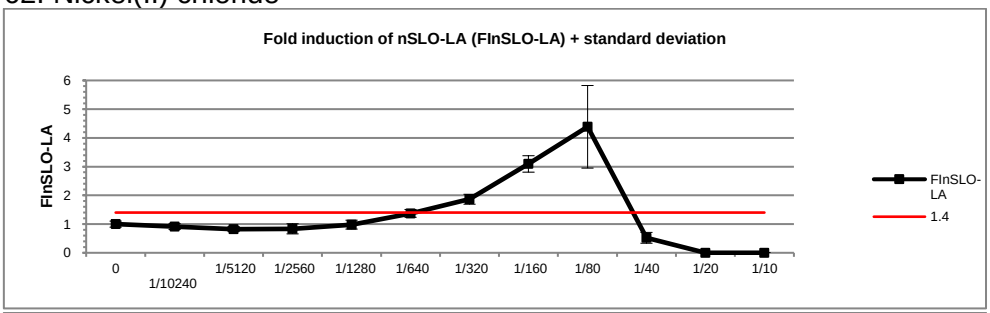


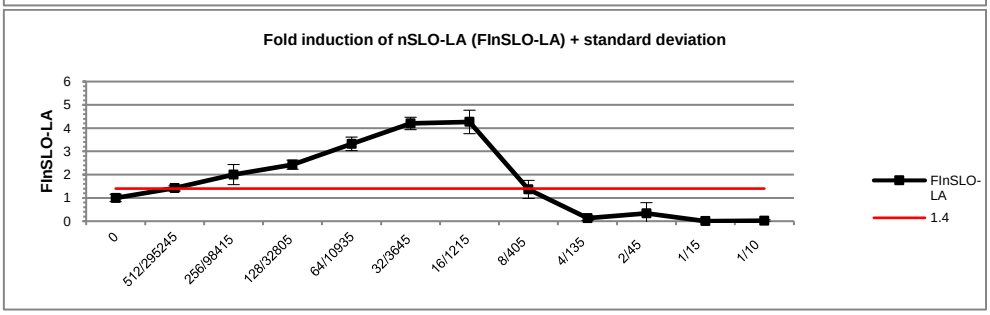
61. Nickel sulphate hexahydrate



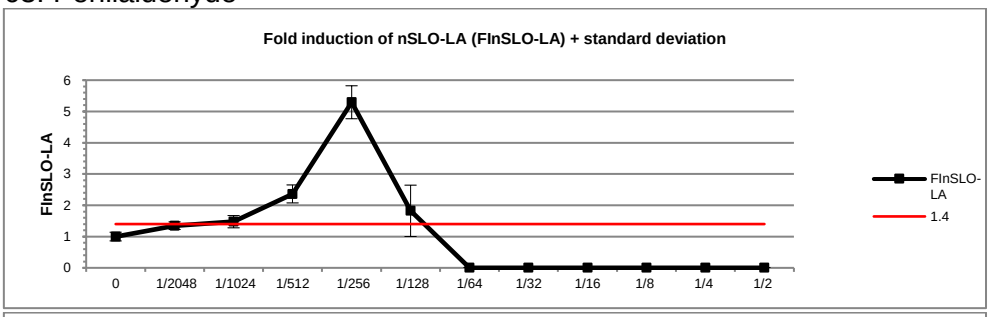


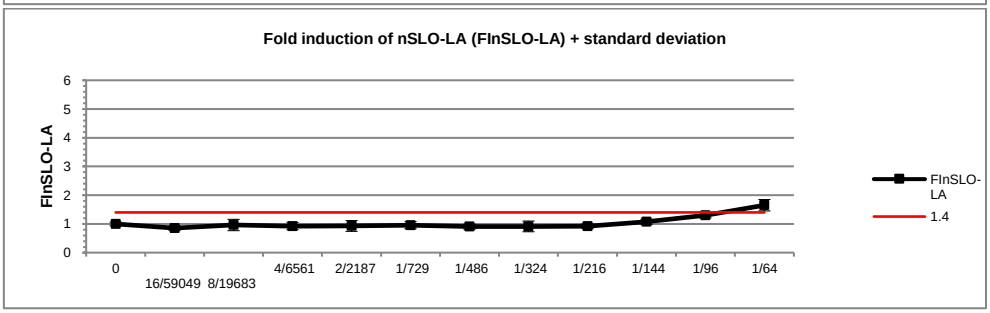
62. Nickel(II) chloride



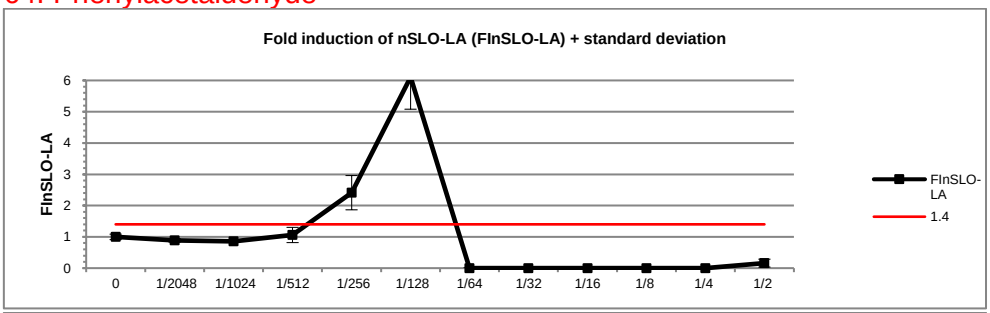


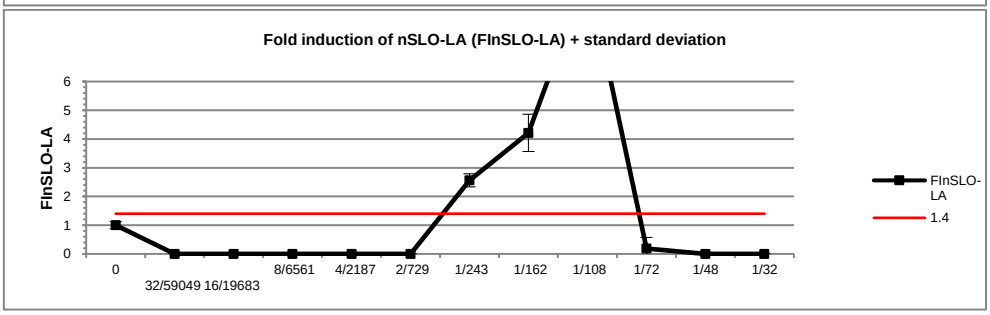
63. Perillaldehyde



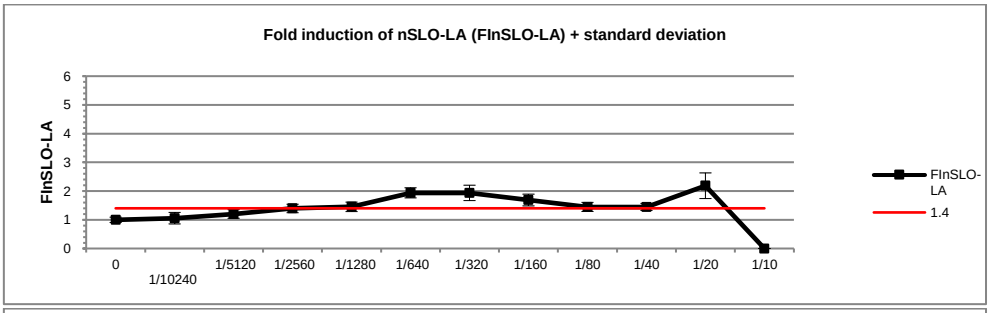


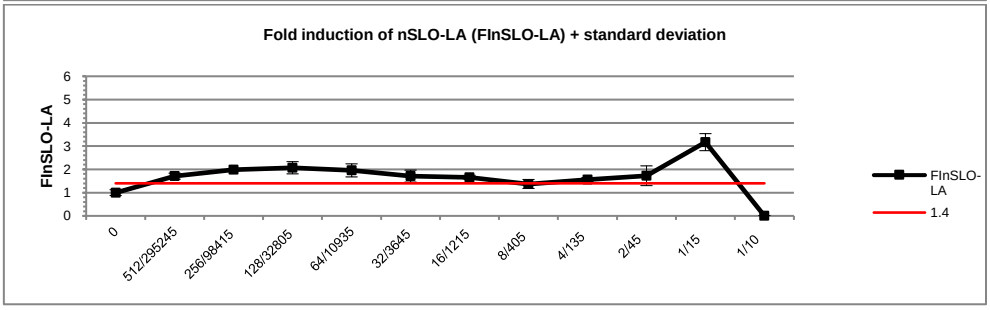
64. Phenylacetaldehyde



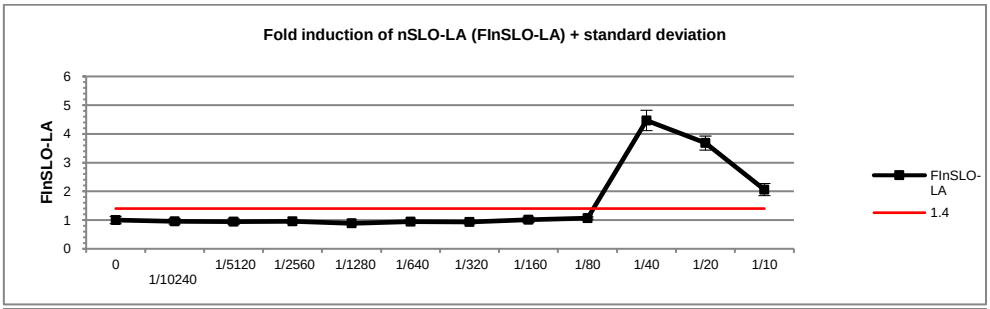


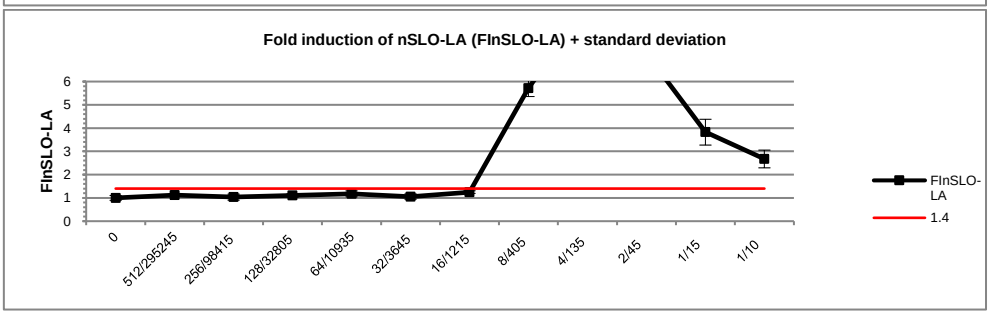
65. Resorcinol



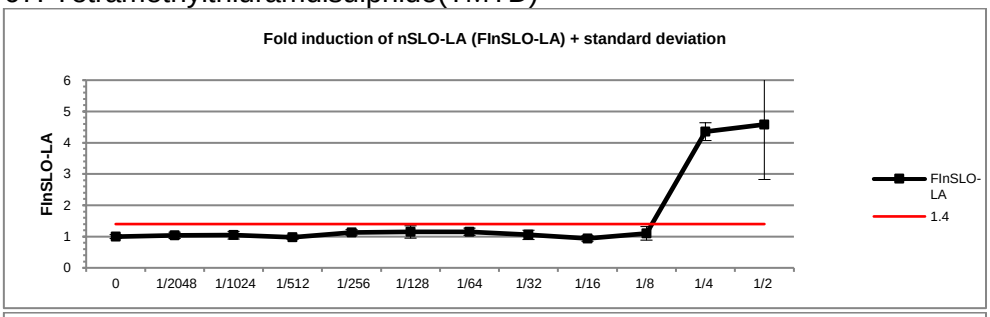


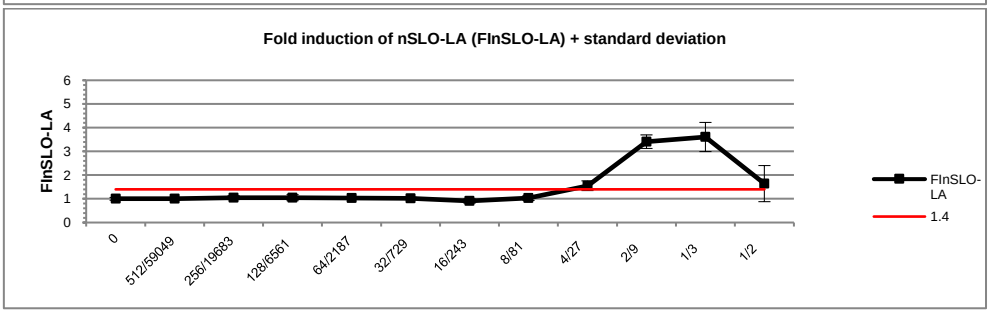
66. Tartaric acid



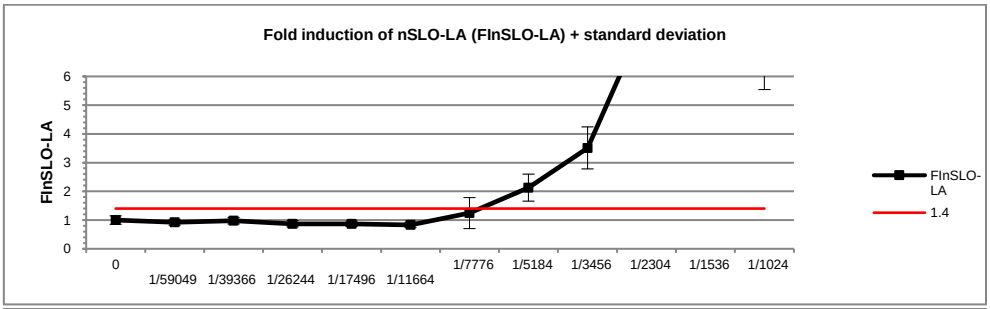


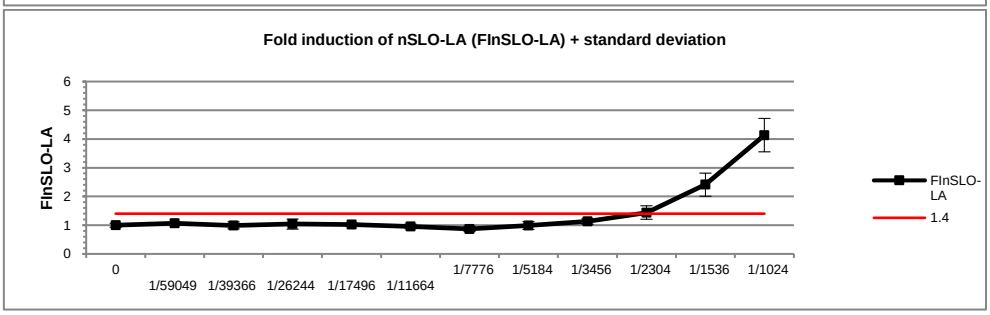
67. Tetramethylthiuramdisulphide(TMTD)



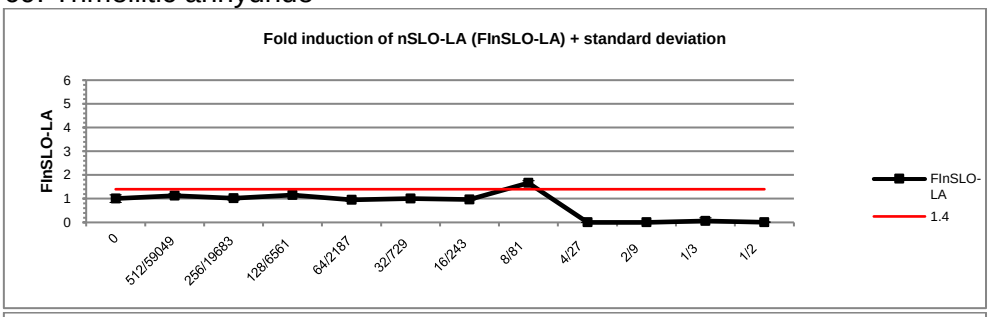


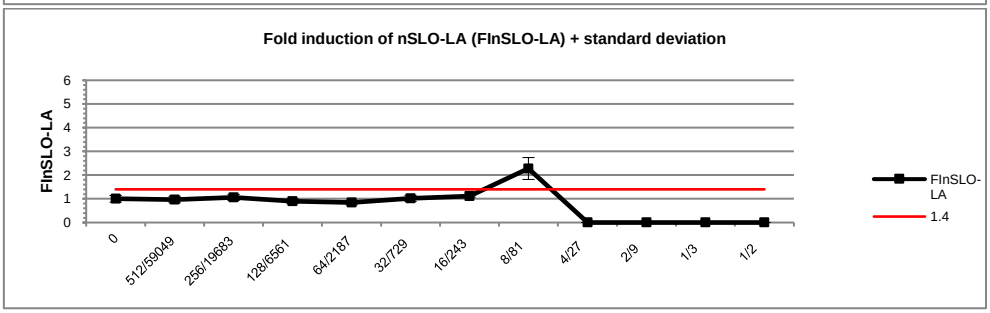
68. trans-2-Hexenal



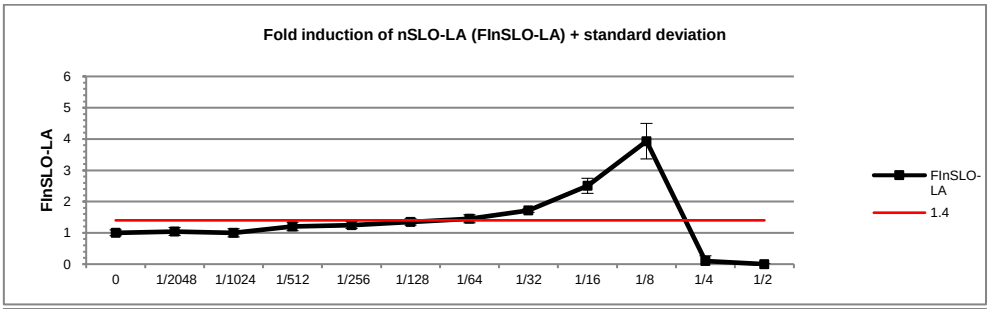


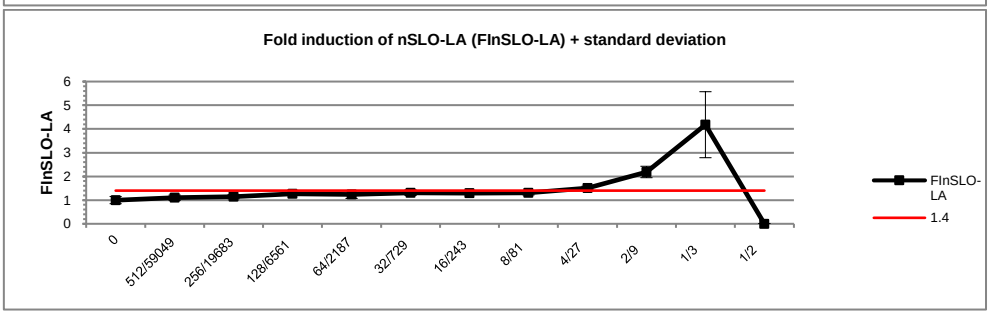
69. Trimellitic anhydride



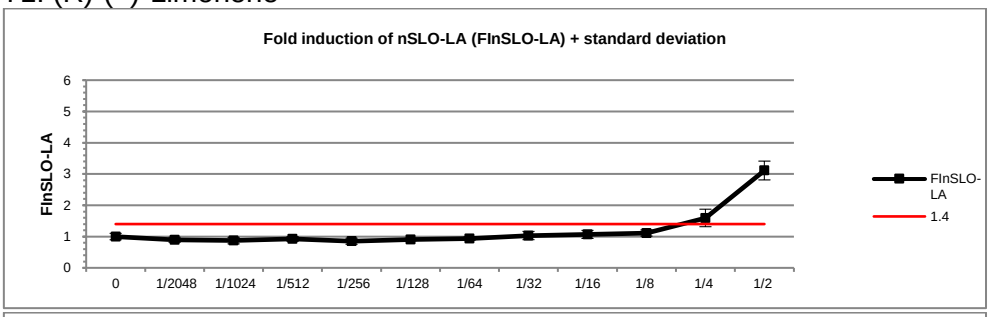


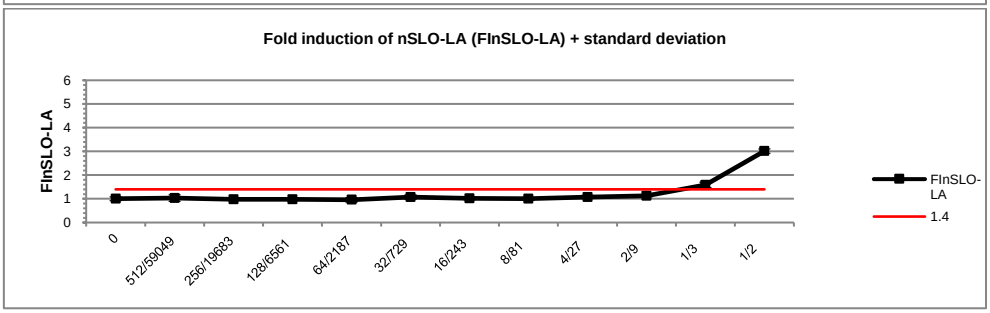
70. Undec-10-enal



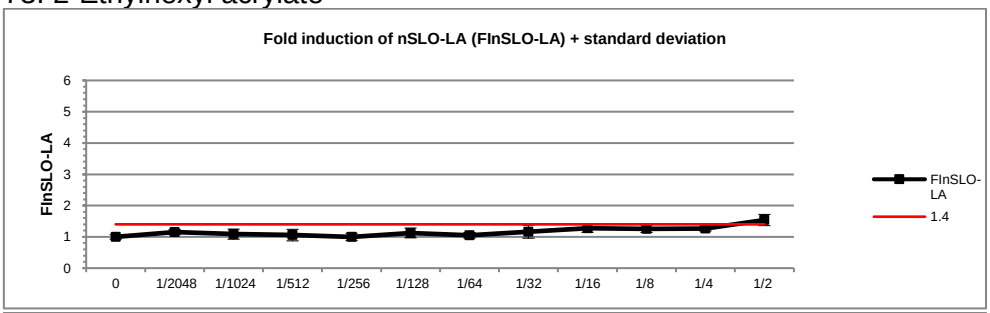


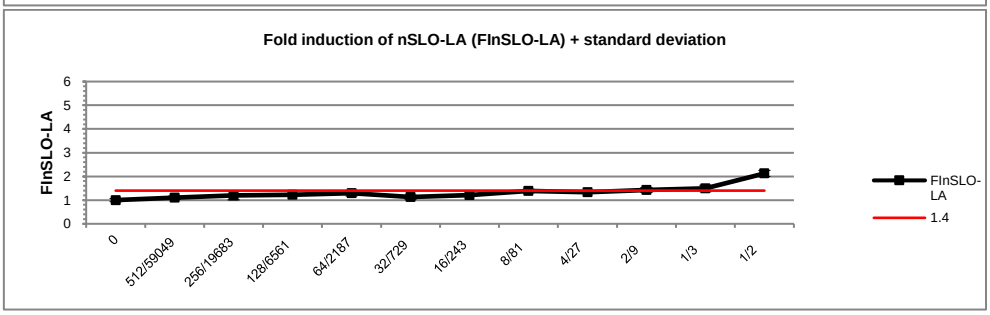
71. (R)-(+)-Limonene



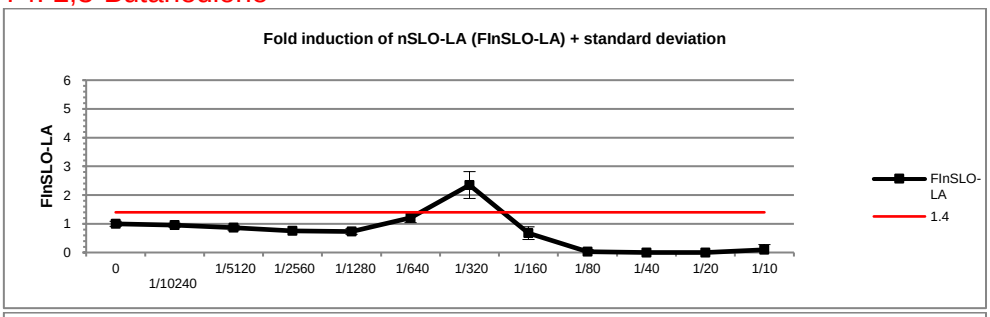


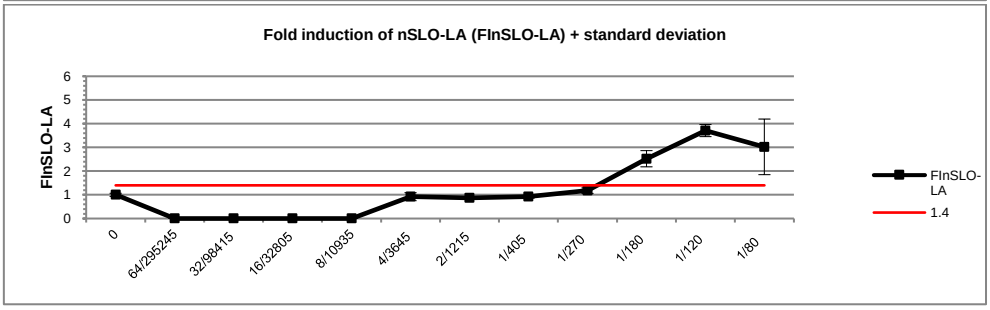
73. 2-Ethylhexyl acrylate



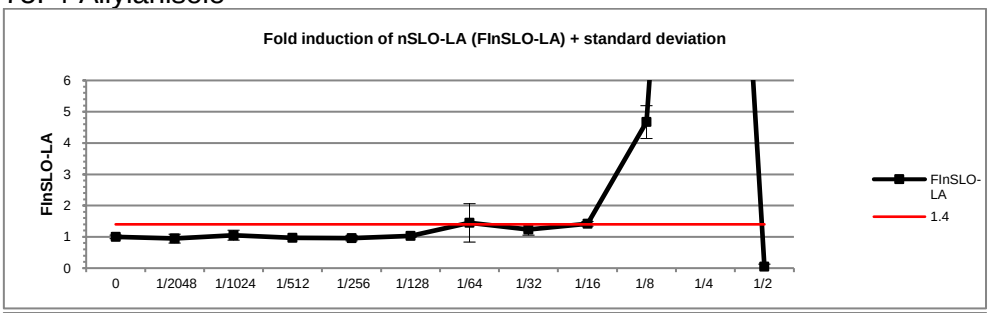


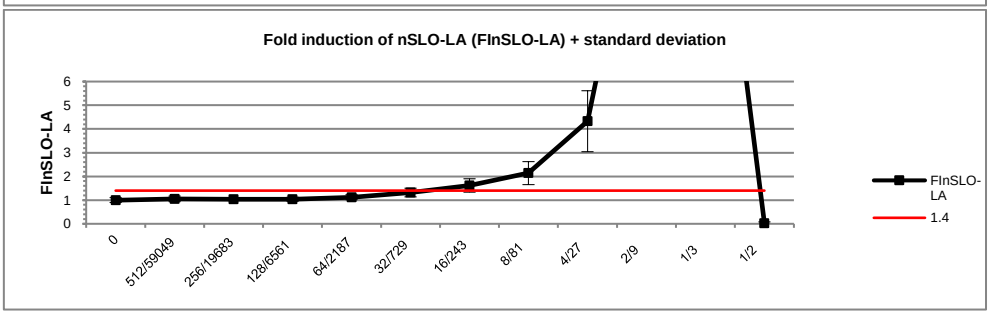
74. 2,3-Butanedione



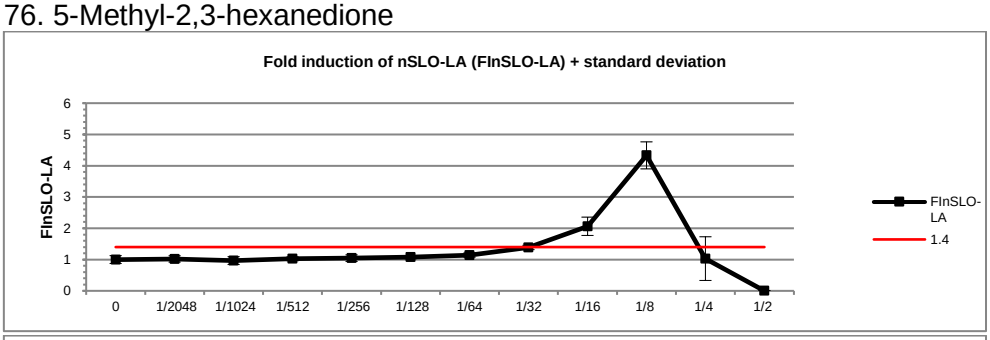


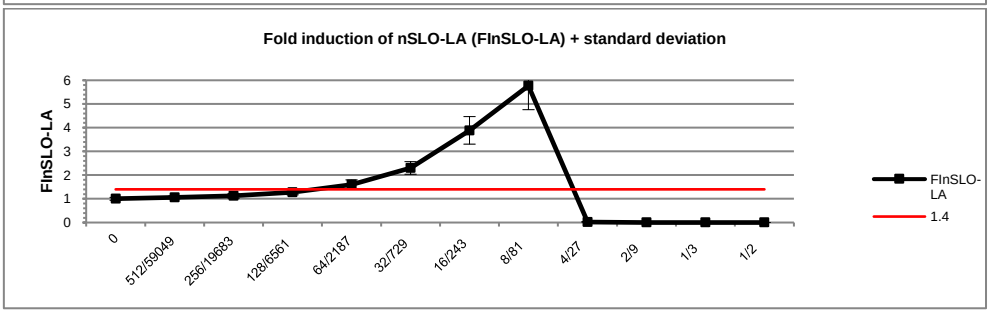
75. 4-Allylanisole



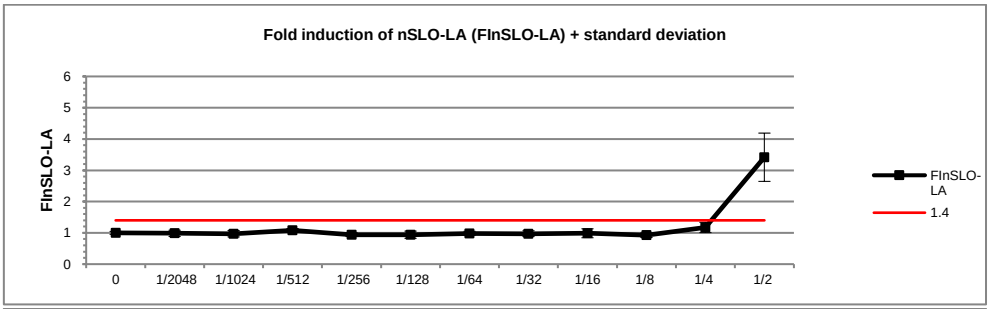


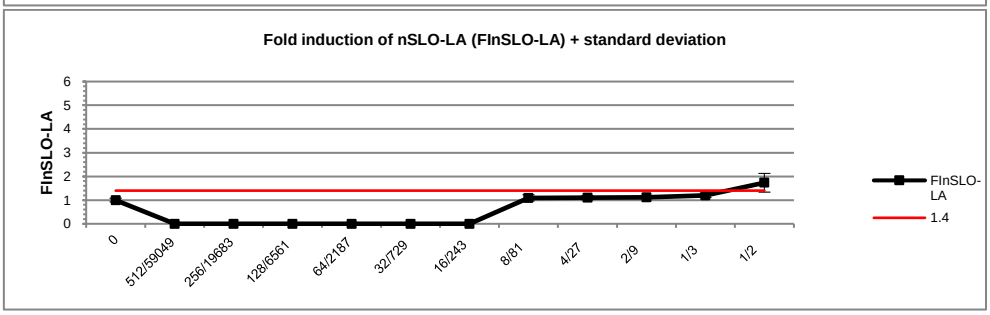
76. 5-Methyl-2,3-hexanedione



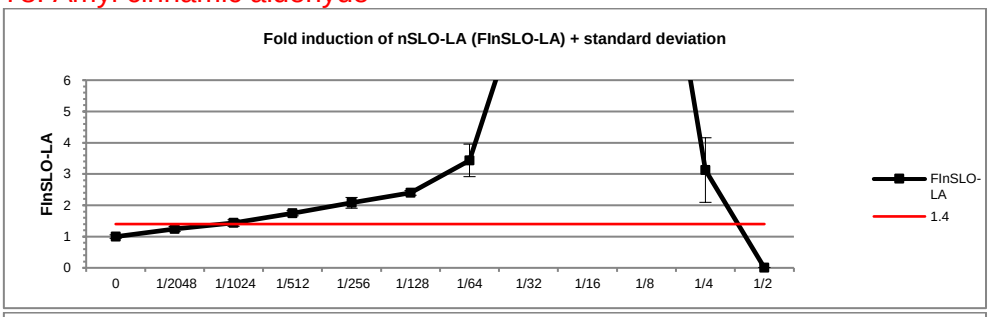


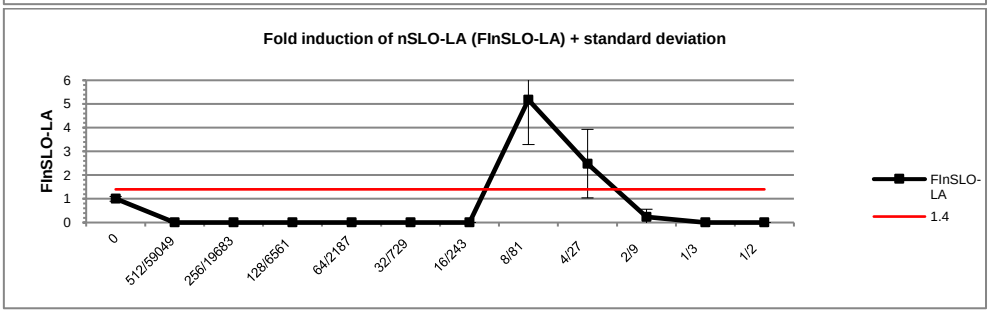
77. Abietic acid



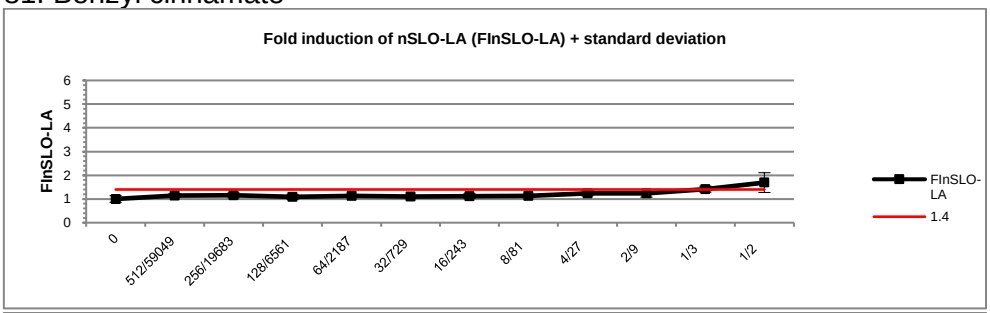


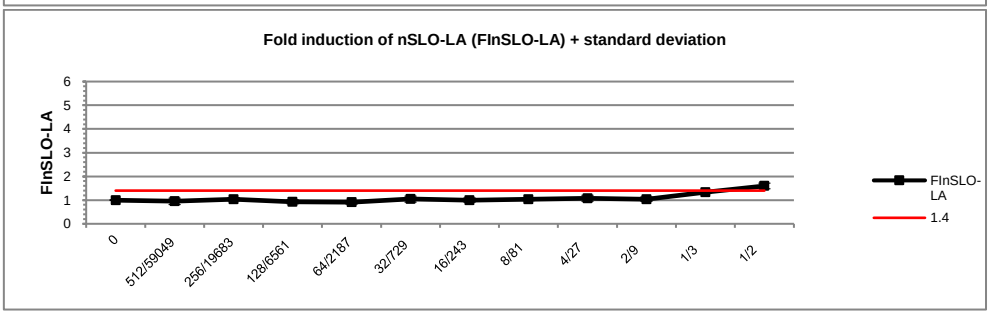
78. Amyl cinnamic aldehyde



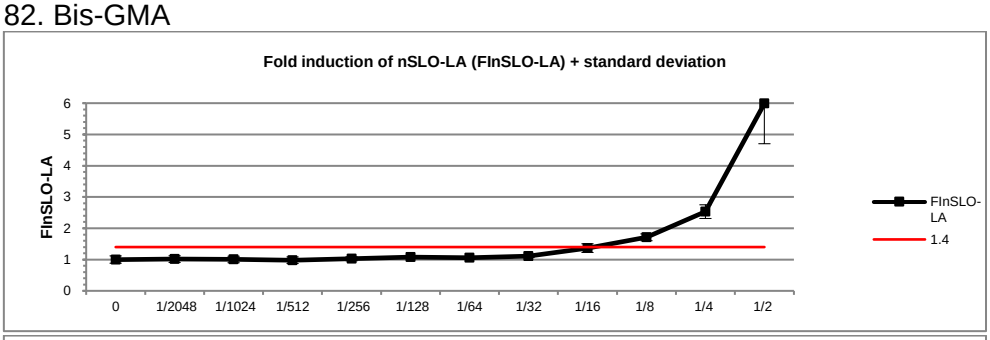


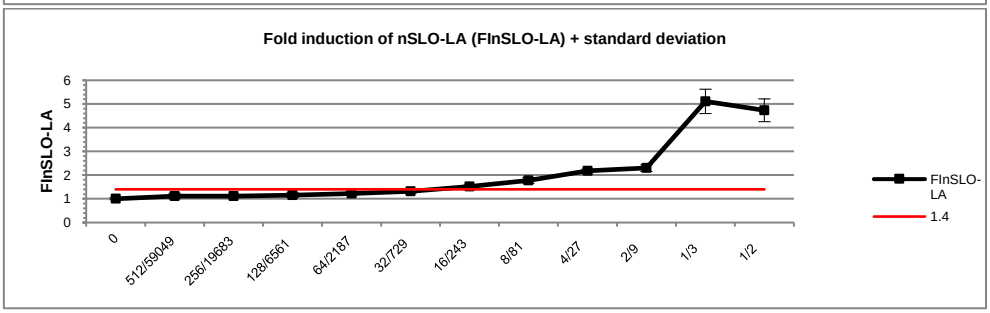
81. Benzyl cinnamate



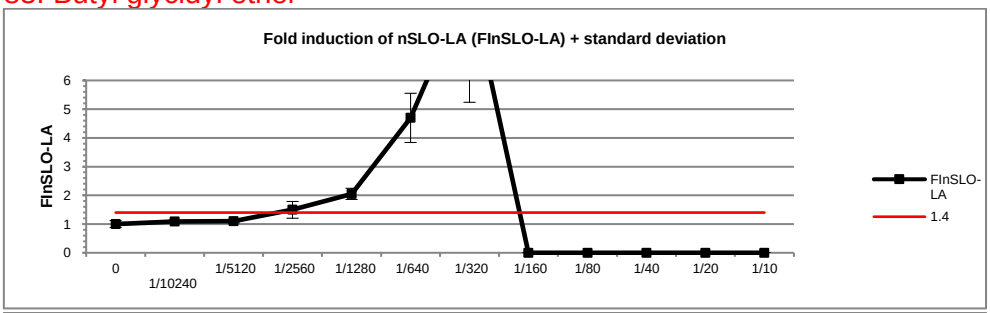


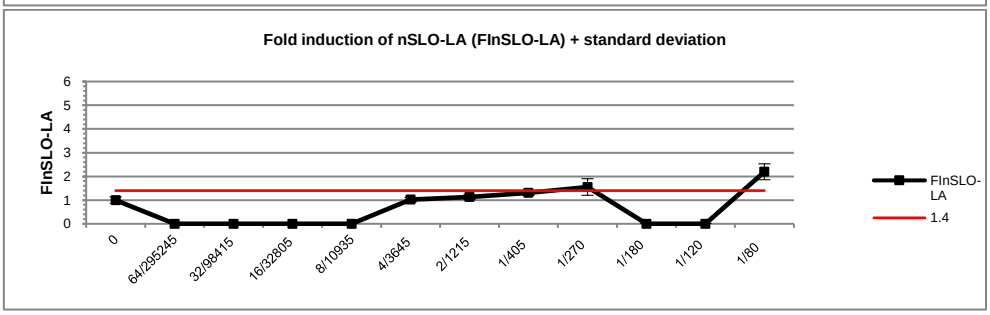
82. Bis-GMA



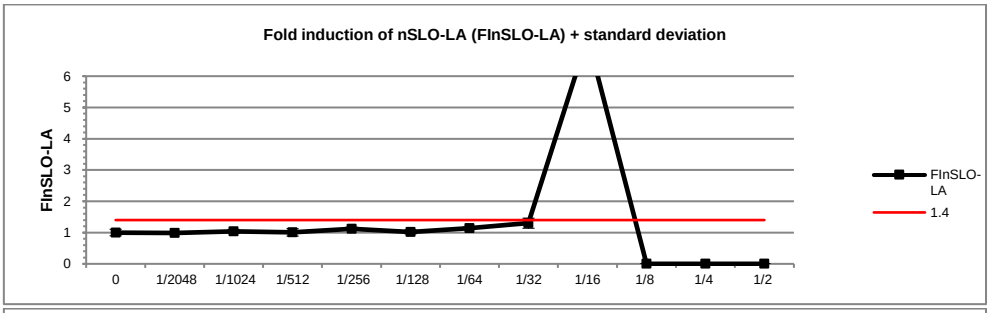


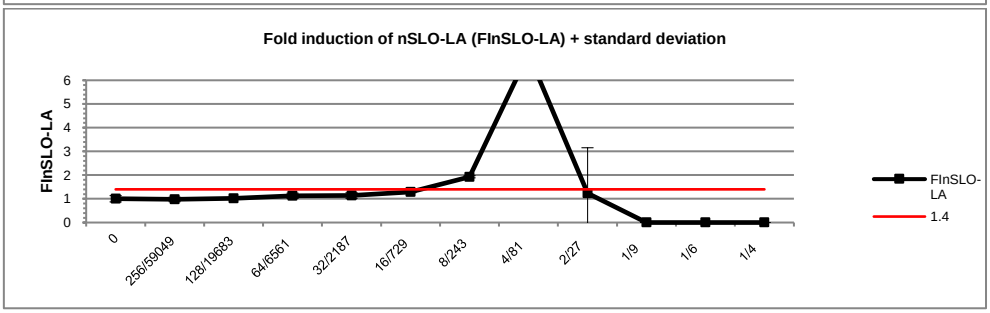
83. Butyl glycidyl ether



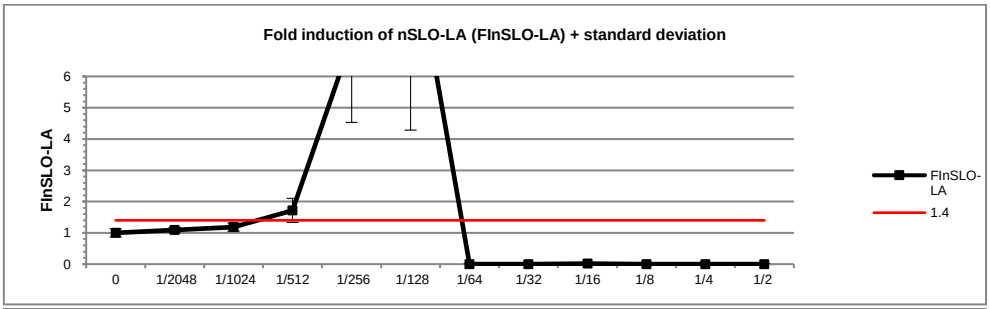


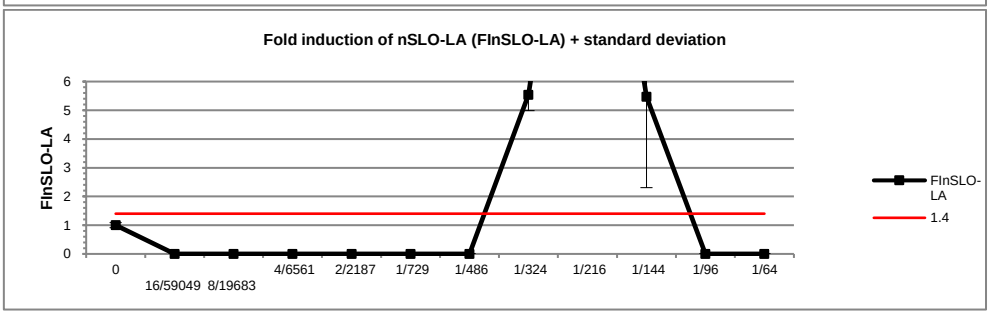
84. Cinnamic alcohol



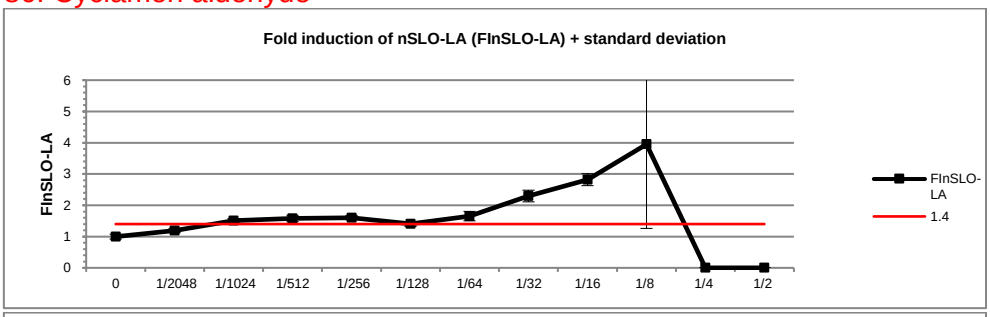


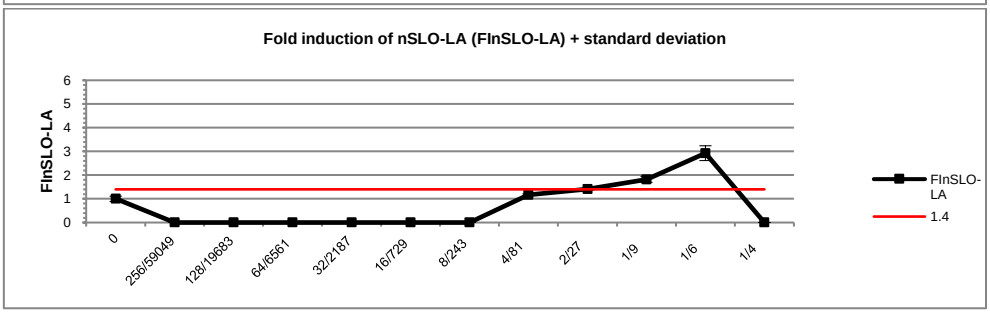
85. Citral



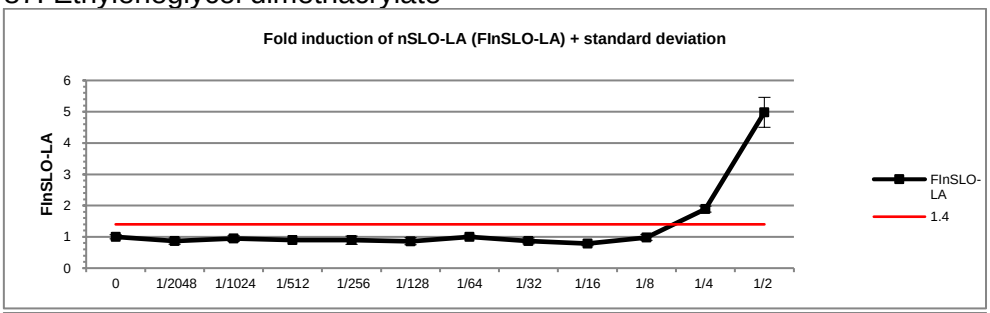


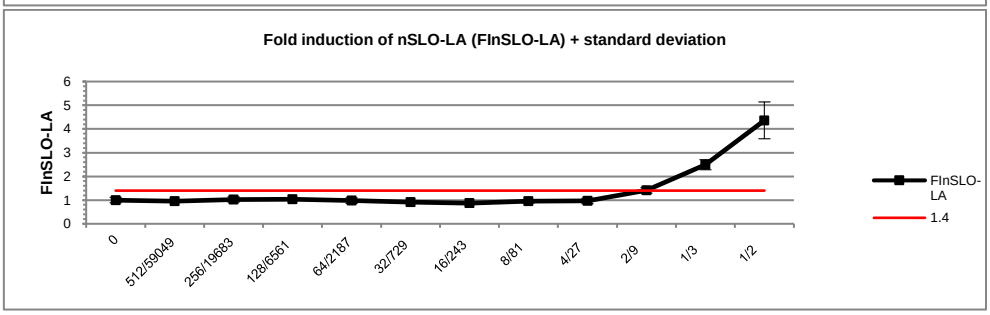
86. Cyclamen aldehyde



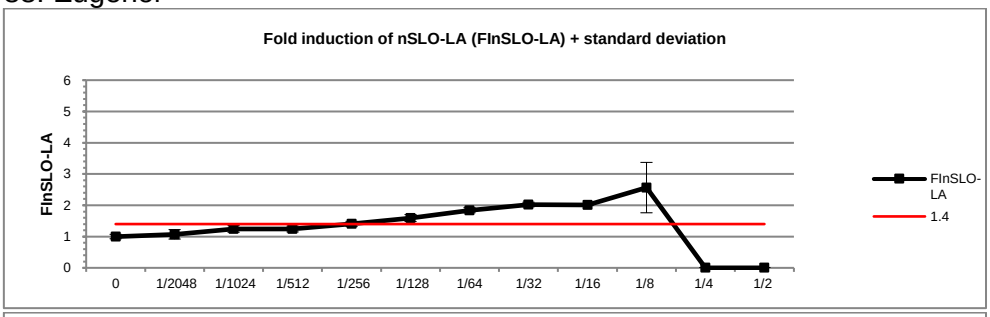


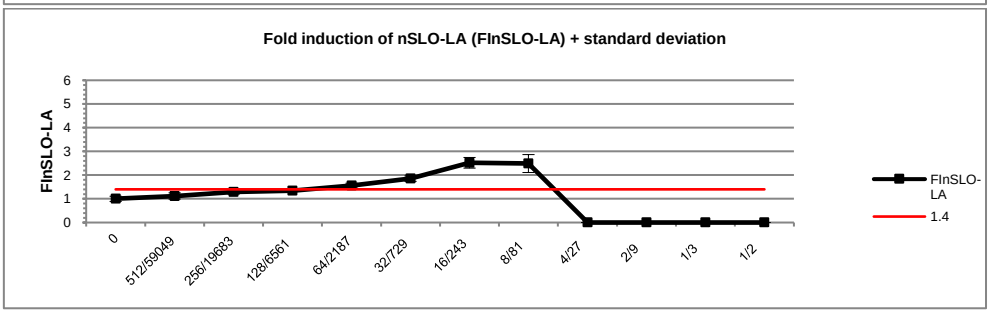
87. Ethyleneglycol dimethacrylate



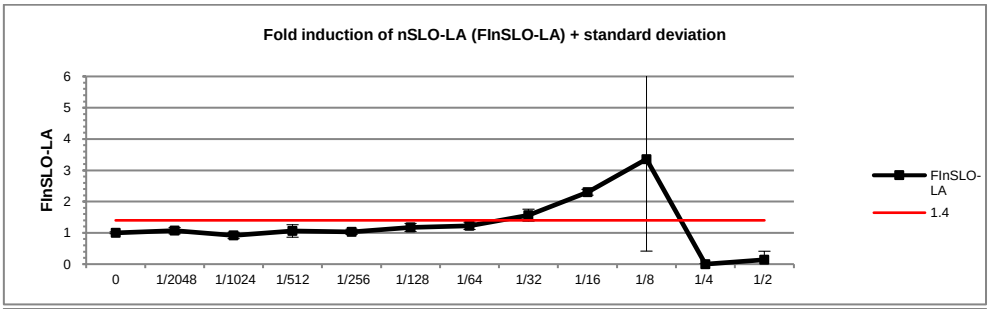


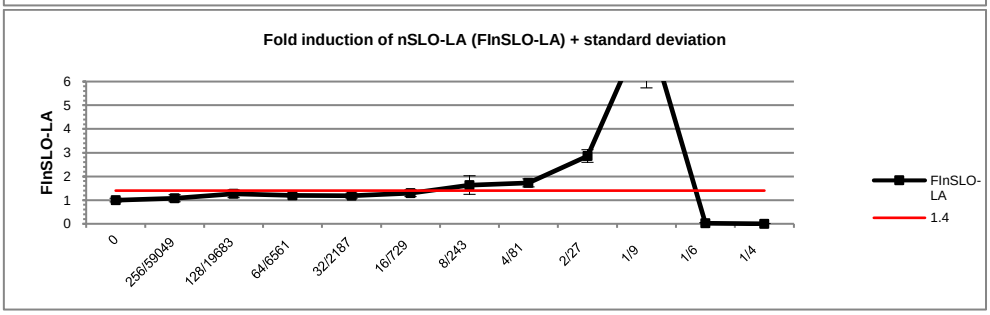
88. Eugenol



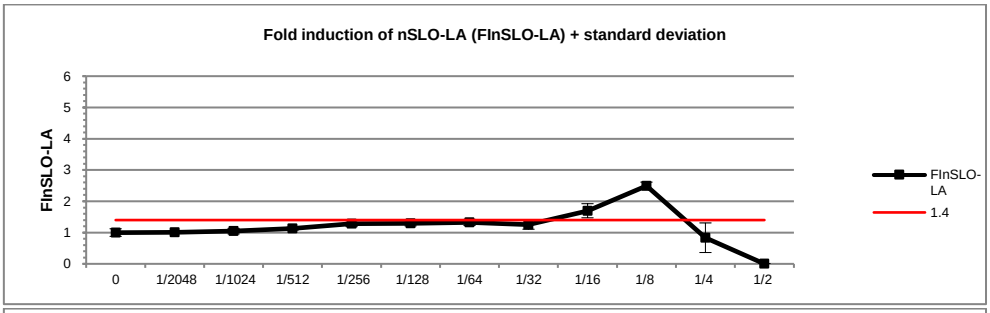


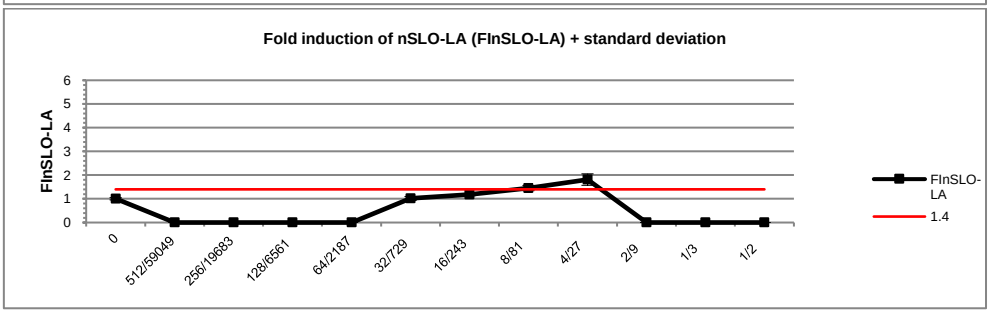
89. Farnesal



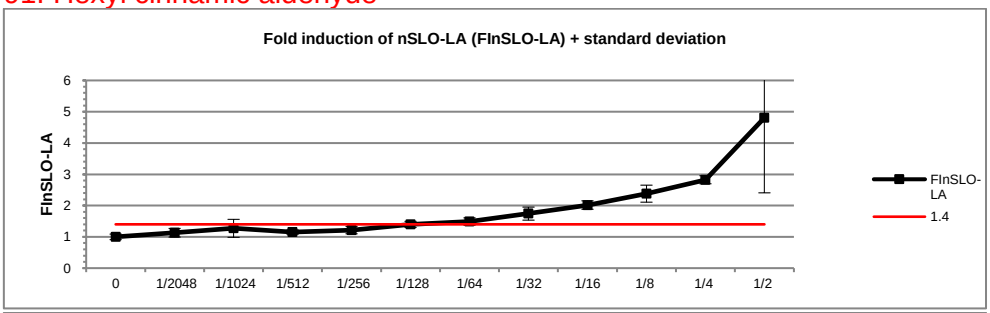


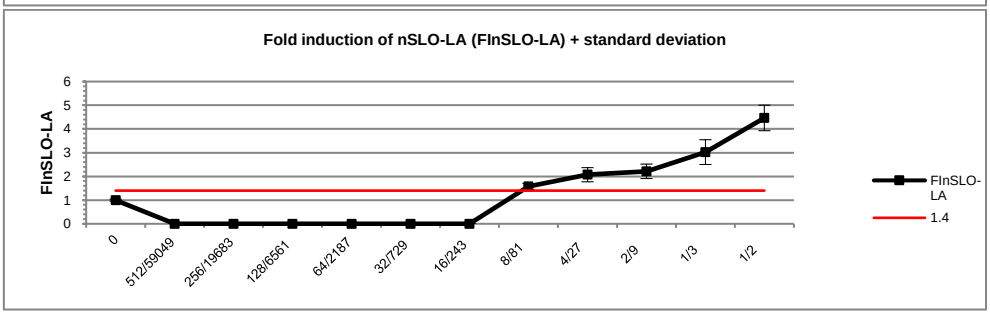
90. Geraniol



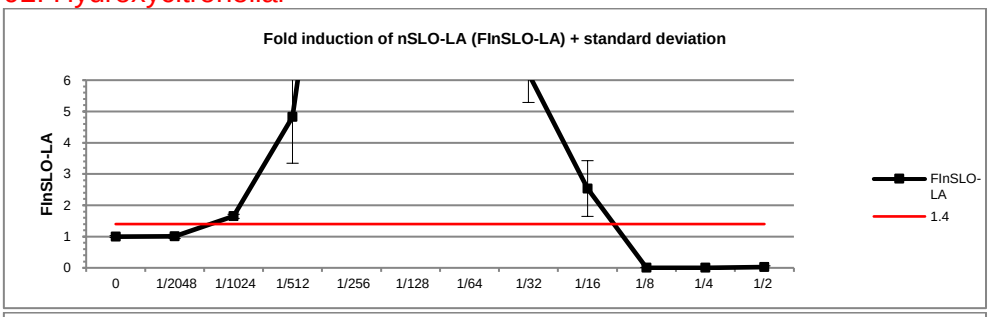


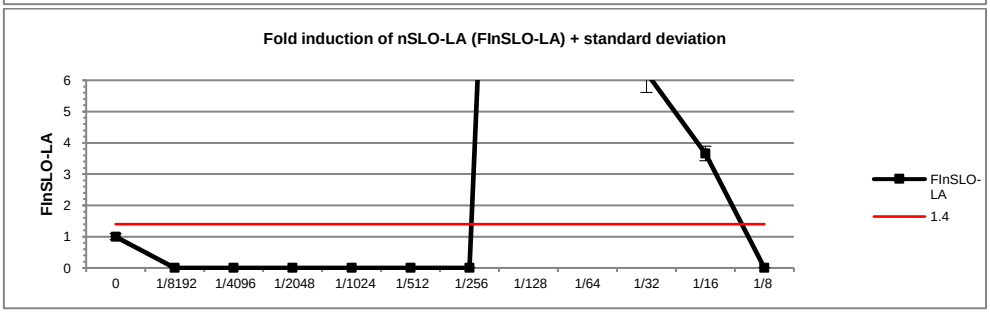
91. Hexyl cinnamic aldehyde



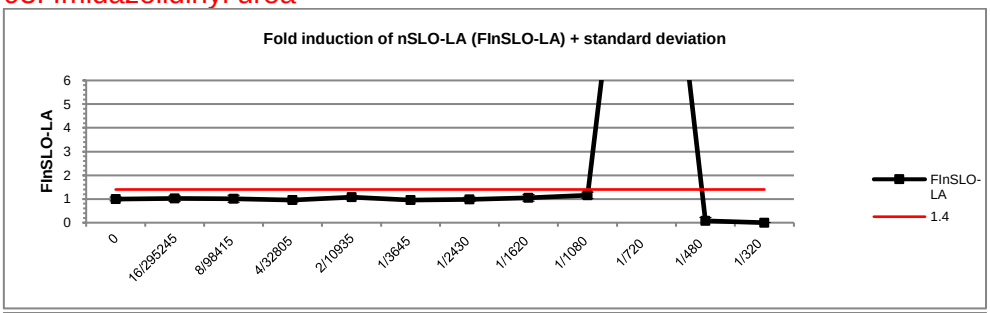


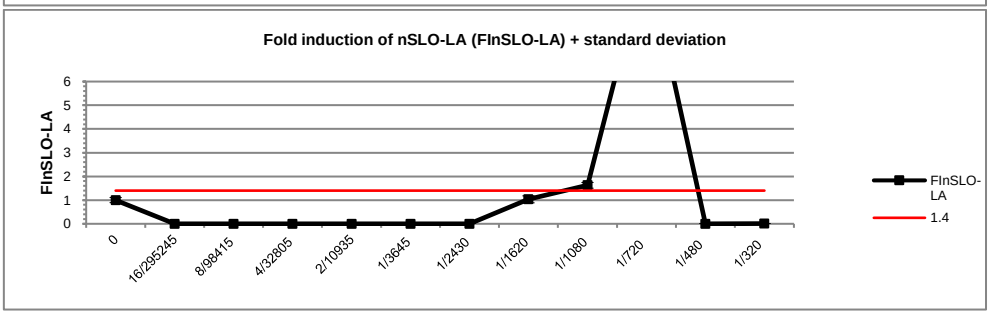
92. Hydroxycitronellal



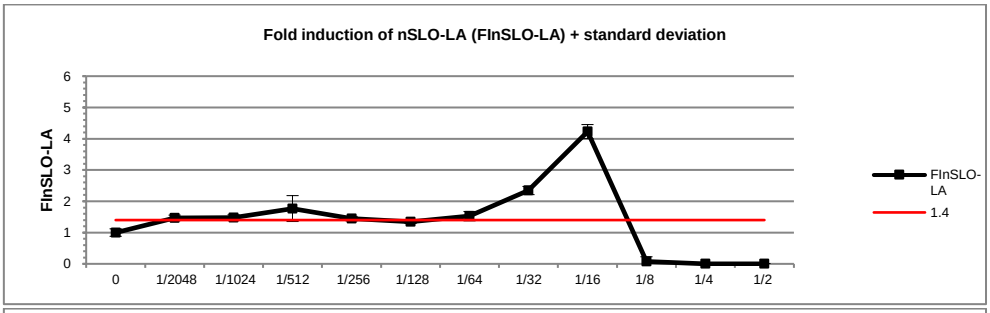


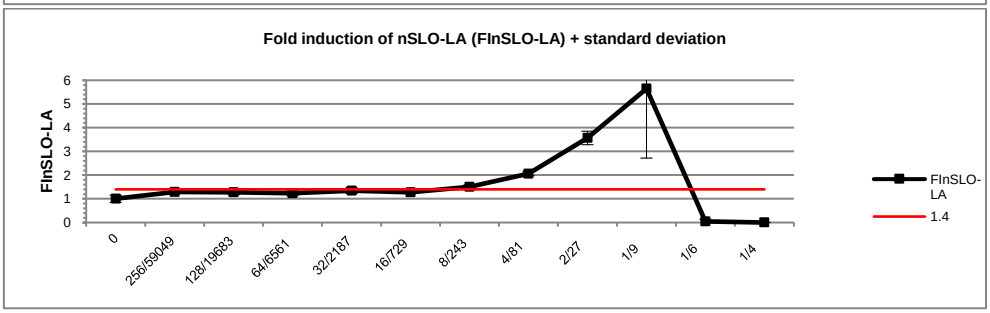
93. Imidazolidinyl urea



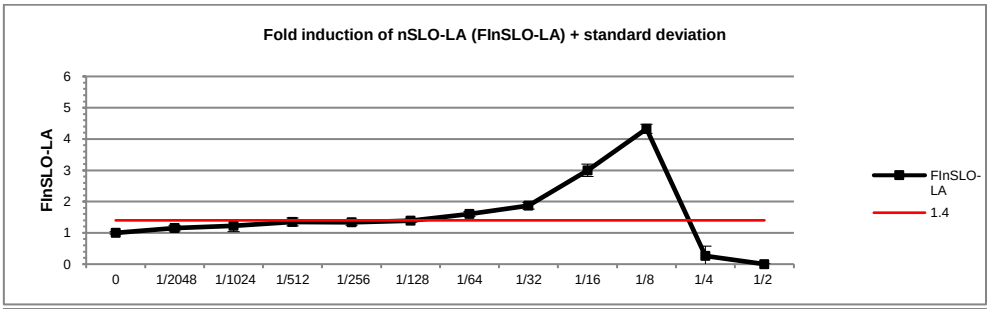


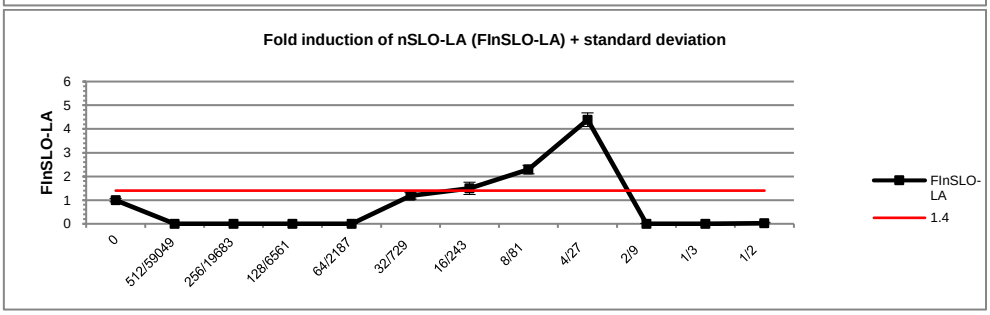
94. Lilial



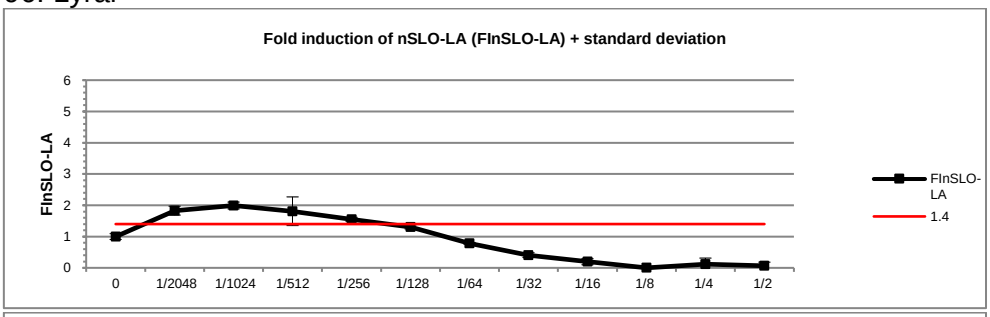


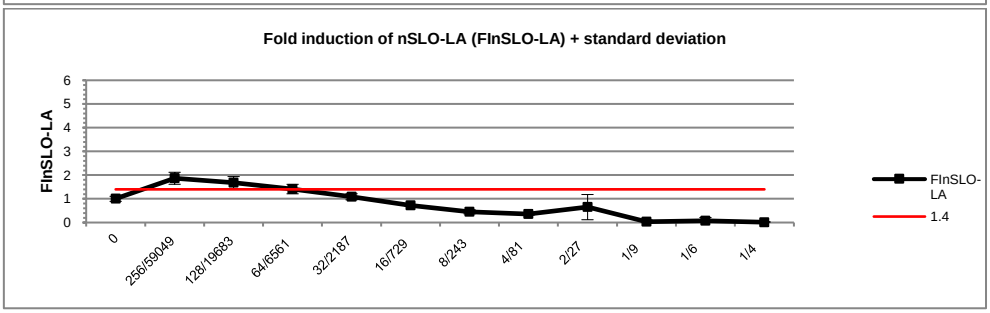
95. Linalool



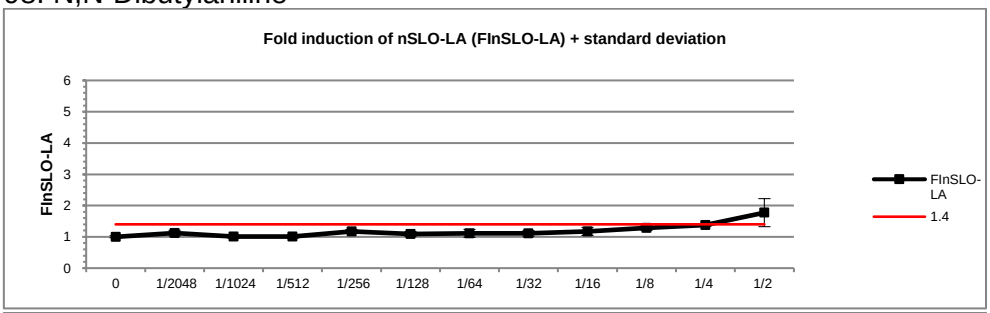


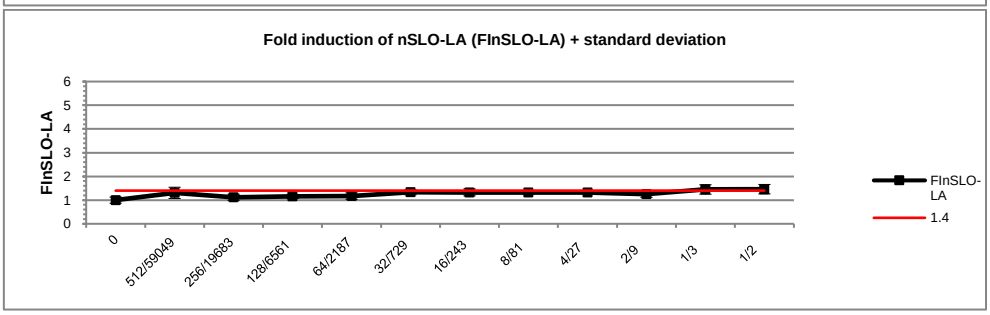
96. Lyral



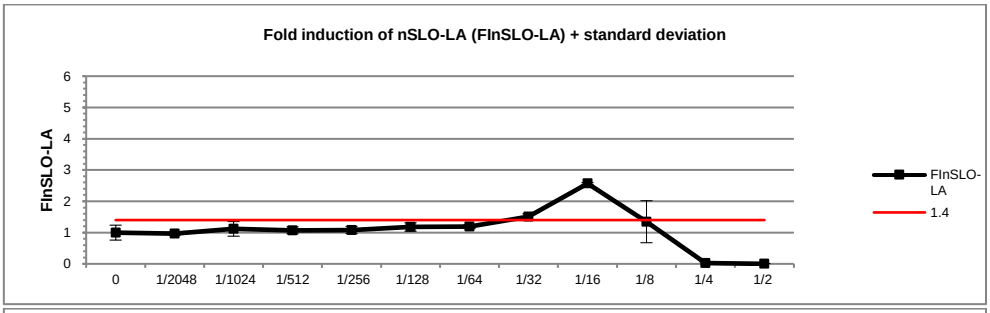


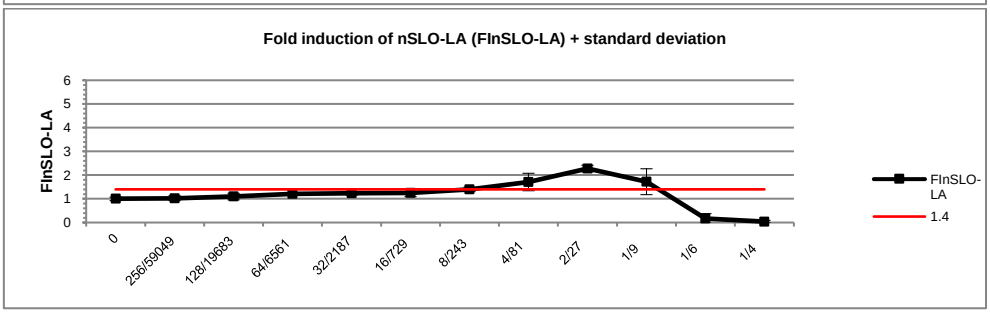
98. N,N-Dibutylaniline



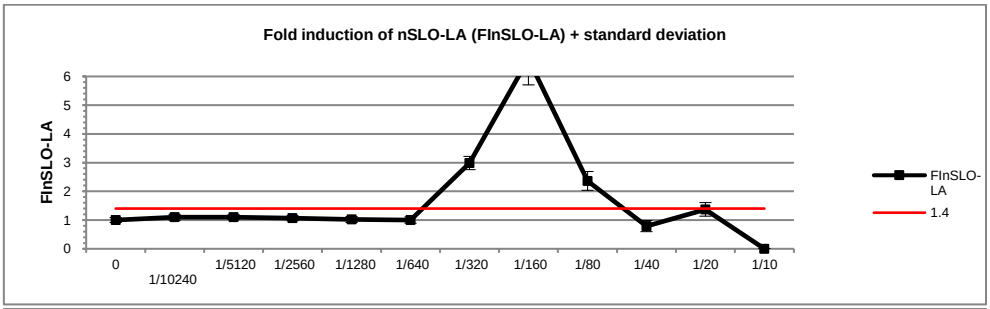


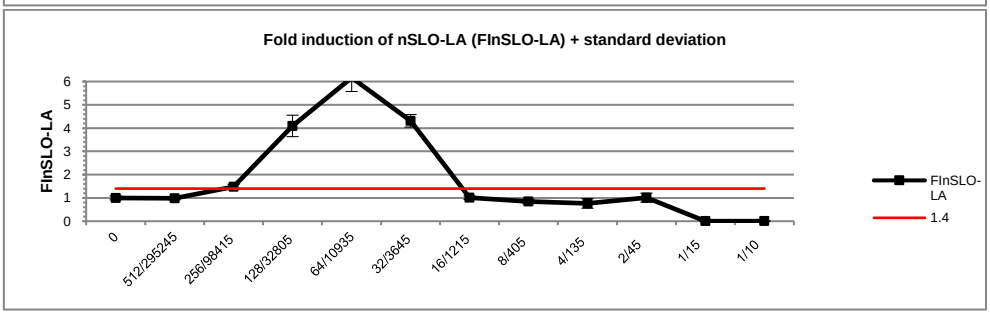
99. Nonanoic acid



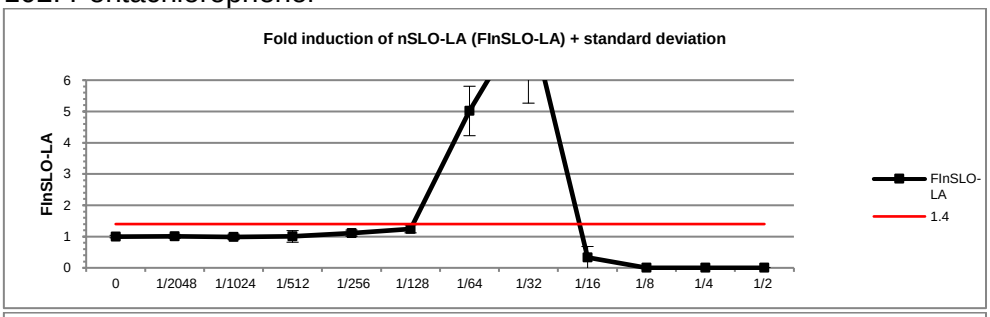


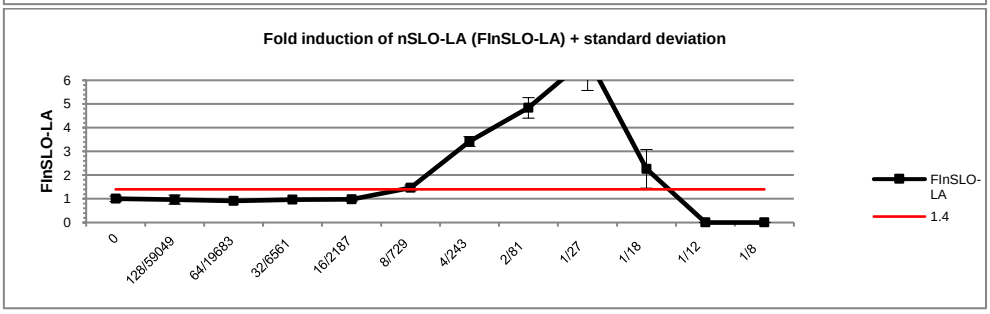
100. Oxalic acid



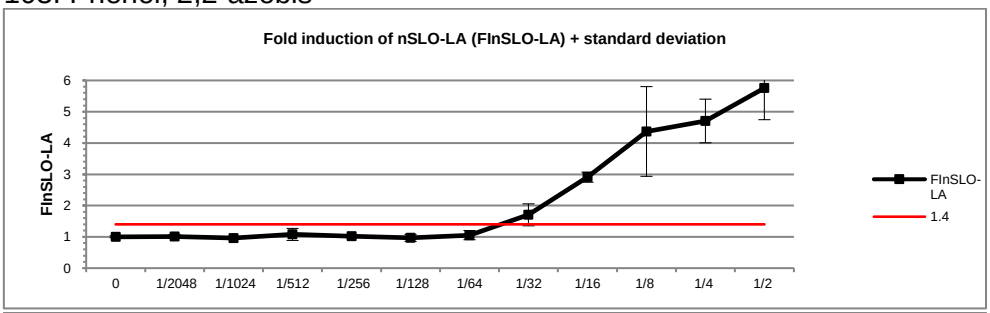


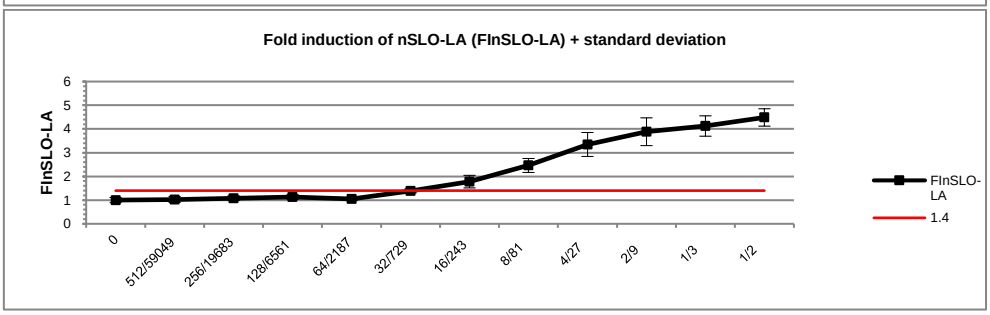
102. Pentachlorophenol



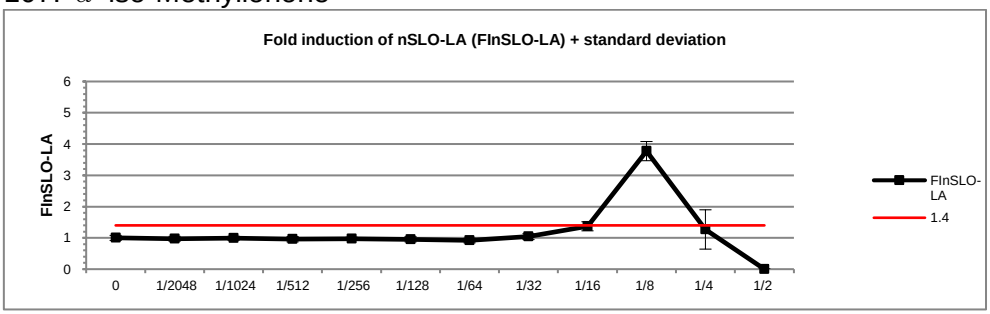


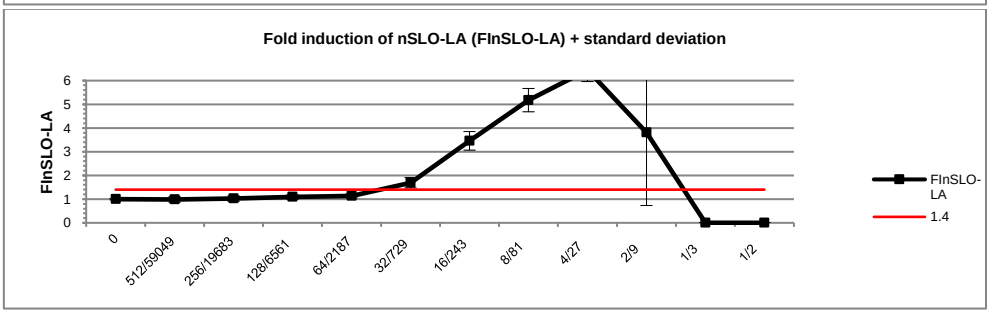
103. Phenol, 2,2-azobis-





107. α-iso-Methylionone





PART II

Overview report supporting the modified IL-8 Luc assay

The validation management team of the IL-8 Luc assay
Setsuya Aiba, M.D., PhD.
Yutaka Kimura, M.D., PhD.
Hajime Kojima, PhD.

February 2023

Contents

20	Xylene	7	95.8	NC	-(0/4)	-(0/3)	-(0/3)	+(2/4)	-(0/3)	-(0/3)	-(0/4)	-(0/3)	-(0/3)

Summary

The purpose of the modification

The proposed revision aims to improve the performance of the IL-8 Luc assay (TG442E), which is designated as the original IL-8 Luc assay, and to reduce the number of inconclusive chemicals.

The modification of the protocol

The modification of the protocol is to simply modify the prediction model.
The modified prediction model is as follows.
Among supposed non-sensitizers, if chemicals are dissolved at 20 mg/ml in X-VIVO™ 15, they are judged as non-sensitizers. If chemicals are not dissolved at 20 mg/ml in X-VIVO™ 15, or there is cause for uncertainty regarding the extent of dissolution or miscibility, chemicals that give Inh-less than 0.8 of GAPLA are judged as non sensitizers, while those that give 0.8 or higher of Inh-GAPLA are judged as inconclusive.

The performance of the IL-8 Luc assay with the modified prediction model

Of the 143 chemicals examined by the tgIL-8 Luc assay, 23 were classified as inconclusive. On the other hand, by applying the modified criteria, 10 of the 23 inconclusive chemicals were classified as non-sensitizers. As a result, the performance of the modified IL-8 Luc assay was 93.9% for sensitivity, 68.0% for specificity, and 88.6% for accuracy, while that of the original IL-8 Luc assay was 95.8% for sensitivity, 52.9% for specificity, and 89.4% for accuracy.

Conclusion

The modification of IL-8 Luc assay increased specificity and decreased the number of inconclusive chemicals, although sensitivity and accuracy were somewhat reduced. Even after modification, however, the modified IL-8 Luc assay maintains the sensitivity higher than other TGs. In addition, this modification shed light on the important advantages of the modified IL-8 Luc assay over other *in vitro* skin sensitization tests due to its ability to exclude the false negative judgment of chemicals due to poor solubility in the medium. When the modified IL-8 Luc assay is combined with other in vitro test methods, it is required to reduce the number of inconclusive chemicals and to increase its specificity. By the modification, the modified IL-8 Luc assay is now well suited for use

in integrated approaches.

2 Introduction

The positioning of the original IL-8 Luc assay

The performance of the original IL-8 Luc assay is characterized by high sensitivity and accuracy (Kimura et al., 2018). Apparently, however, the disadvantage of the original IL-8 Luc assay is to contain a substantial number of inconclusive chemicals and low specificity (Table 1).

Table.1 The performance of the origial IL-8 Luc assay (DASS data base for LLNA)

	tgIL-8 Luc assay	h-CLAT	KeratinoSens
Positive	69	66	59
False negative	4	15	22
Negative	6	12	12
False positive	5	3	3
Inconclusive	8		
Out of applicability domain	4		
Sensitivity (%)	94.5%	81.5%	72.8%
Specificity (%)	54.5%	80.0%	80.0%
Accuracy (%)	89.3%	81.3%	74.0%
Balanced Accuracy (%)	74.5%	80.8%	76.4%

tgIL-8 Luc assay: the IL-8 Luc assay described in TG442E

4

The potential problem with *in vitro* skin sensitization tests using DMSO as a solvent

There are several problems with *in vitro* skin sensitization tests using DMSO (Fig. 1). Water-insoluble chemicals are usually dissolved in DMSO in most sensitization tests but they precipitate when diluted with medium beyond their solubility in culture medium. Therefore, the problem 1 is that hydrophobic skin sensitizing substances would

theoretically have a higher probability of not being detected or to be under-predicted compared with hydrophilic skin sensitizing substances. The problem 2 is that i*n vitro* tests using DMSO cannot distinguish between negatives due to the chemicals not dissolving in the medium and, as a result, failing to meet the positive criteria (false negative), and negatives due to lack of activity as a sensitizer. Finally, the problem 3 is that it is difficult for these tests to calculate the accurate performance (Fig 1). When they judge chemicals as negative, negative chemical include c: false negative chemicals because the sensitizing potential of chemicals cannot be detected by the assay; e: false negative chemicals because they do not dissolve in the culture medium; d: true negative chemicals that do not meet the positive criteria; and f: true negative chemicals that do not dissolve in the culture medium. Thus, e and f should not be judged. Since the test methods using DMSO as a solvent in TG442E cannot exclude chemicals that should not be judged due to poor solubility, the exact performance of these test methods is unknown. As a result, accuracy and sensitivity appear to be lower than they actually are, while specificity appears to be higher. To get the actual performance, e and f need to be determined and excluded from the calculation.

Fig. 1. The problem with i*n vitro* skin sensitization tests using DMSO as a solvent

Current estimation of the performance of the assay

***In vitro* skin sensitization tests using DMSO**	**LLNA (+)**	**LLNA (-)**
Positive	a	b
Negative	c + e	d + f

e: false negative chemicals that are not dissolved
f: negative chemicals that are not dissolved

Performance	Parameters
Accuracy	$\frac{a+d+f}{a+b+c+d+e+f}$
Sensitivity	$\frac{a}{a+c+e}$
Specificity	$\frac{d+f}{b+d+f}$

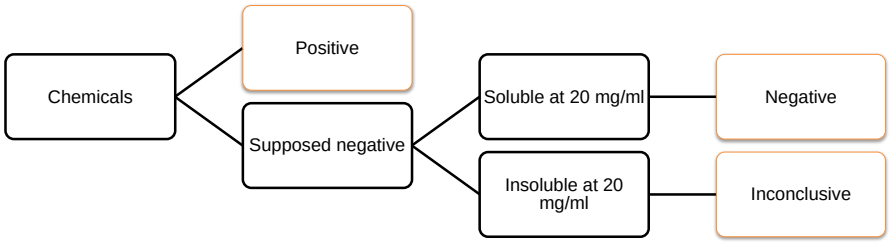
e and f should be excluded from judgment.

As a result, the accuracy and sensitivity appear to be lower than they actually are, and the specificity appears to be higher.

The prediction model of the IL-8 Luc assay and its advantage

On the other hand, in the IL-8 Luc assay (OECD442E), if a chemical does not dissolve in the medium, the decision is withheld with the classification of inconclusive. The prediction model is shown in Fig. 2.

Fig. 2. The prediction model of the original L-8 Luc assay



The Original IL-8 Luc assay can avoid judgement chemicals are not disolved in medium.

As a result, the IL-8 Luc assay can avoid the problem 2. In addition, the IL-8 Luc assay can correctly estimate its performance (Fig. 3).

Fig. 3. The original IL-8 Luc assay performance is unaffected by inconclusive chemicals.

IL-8 Luc assay	LLNA (+)	LLNA (-)
Positive	a	b
Negative	c	d
Inconclusive	e	f

Performance	Parameters
Accuracy	$\frac{a+d}{a+b+c+d}$
Sensitivity	$\frac{a}{a+c}$
Specificity	$\frac{d}{b+d}$

e and f are excluded from judgment.

As a result, the IL-8 Luc assay can correctly estimate its performance.

3 The purpose of the modification

The purpose of the modification of the IL-8 Luc assay is to increase specificity and reduce the number of inconclusive chemicals by simply modifying the prediction model.

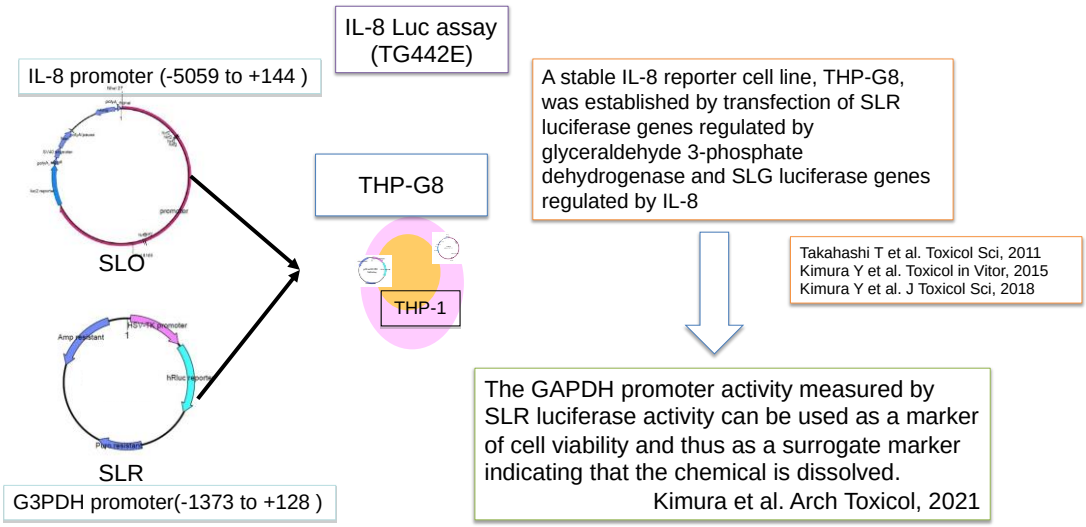
4 The modification of the original IL-8 Luc assay

The unique character of the original IL-8 Luc assay

In order to reduce the number of inconclusive chemicals, in addition to the positive/negative judgment indicator, another indicator of the dissolution of the chemical in the medium should be incorporated. So, we took advantage of the dual luciferase reporter cell nature of THP-G8 cells. Namely, the cell line used in the original IL-8 Luc assay, THP-G8 cells are a dual luciferase reporter cell with SLR luciferase gene regulated by glycerol-3-phosphate dehydrogenase (GAPDH) promoter and SLG luciferase gene regulated by IL-8 promoter (Fig. 4)(Takahashi et al., 2011). So, we think that GAPDH promoter activity measured by SLR luciferase activity can be used as a marker of cell viability and thus as a surrogate marker for the dissolution of chemicals in the medium.

GAPDH mRNA is 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 experimental manipulation (Edwards and Denhardt, 1985; Mori et al., 2008; Winer et al., 1999). Although there are several reports suggesting that its use as an internal standard is inappropriate in some cases (Oliveira et al., 1999; Thellin et al., 1999), in general within-tissue variation of GAPDH mRNA expression levels is small whereas between-tissue variation can be substantial, depending on tissue type (Barber et al., 2005).

Fig. 4. THP-G8 cell is a dual reporter cell line



The rationale of the modified IL-8 Luc assay

To create the modified IL-8 Luc assay, we defined the following parameters to examine the induction of IL-8 promoter-driven luciferase activity and GAPDH promoter-driven luciferase activity (Table 2). Ind-IL8LA is used for positive/negative judgment, while Inh-GAPLA is aimed to be used for cytotoxicity. Propidium iodide (PI) reduction was obtained using flow cytometry as used in h-CLAT or U-SENS (Sakaguchi et al., 2009).

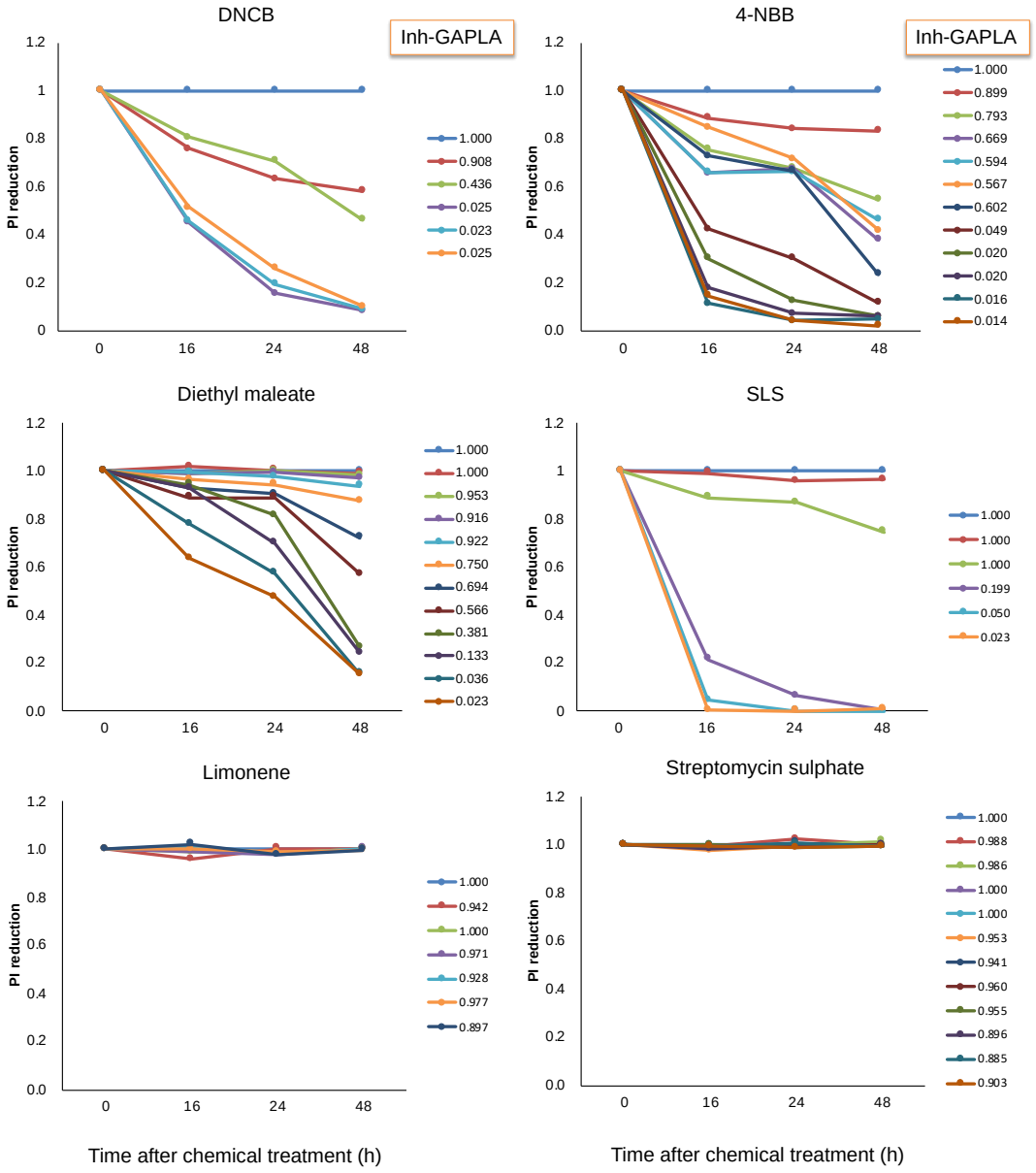
Table 2. Parameters

Parameters	Definition
GAPLA	SLR luciferase activity driven by GAPDH promoter
IL8LA	SLO luciferase activity riven by IL-8 promoter
nIL8LA	IL8LA / GAPLA
Ind-IL8LA	nIL8LA of THP-G8 cells treated with chemicals / nIL8LA of untreated cells
Inh-GAPLA	GAPLA of THP-G8 treated with chemicals / GAPLA of untreated cells
PI reduction	% of PI-excluding cells among THP-G8 cells treated with chemicals / % of PI-excluding cells among THP-G8 cells without chemical treatment.

The following experiments are aimed to demonstrate the correlation between Inh-GAPLA and PI-excluding cells (Kimura, Fujimura and Aiba, 2021). THP-G8 cells were treated with various concentrations of 4 sensitizers DNCB, 4-NBB, diethylmaleate, and limonene, and 2 non-sensitizers SLS and streptomycin sulfate. The cells were examined for Inh-GAPLA after 16 h of chemical treatment, and for the number of PI-excluding cells, namely PI reduction, after 16 h, 24 h, and 48 h of chemical treatment (Fig. 5).

In this experiment, THP-G8 cells were stimulated using the concentration of each chemical at which the values of Inh-GAPLA distributed from 0.02 to 1.0. These concentrations of chemicals were determined based on in-house data. When these cells were cultured, PI reduction in THP-G8 cells treated with the chemicals providing less than 0.8 of Inh-GAPLA gradually decreased dose-dependently and depending on the culture period. However, the chemicals providing more than 0.8 of Inh-GAPLA maintained more than 0.8 of PI reduction.

Fig. 5. PI reduction of THP-G8 cells treated with chemicals decreases as the level of Inh-GAPLA decreases and the culture period increases.

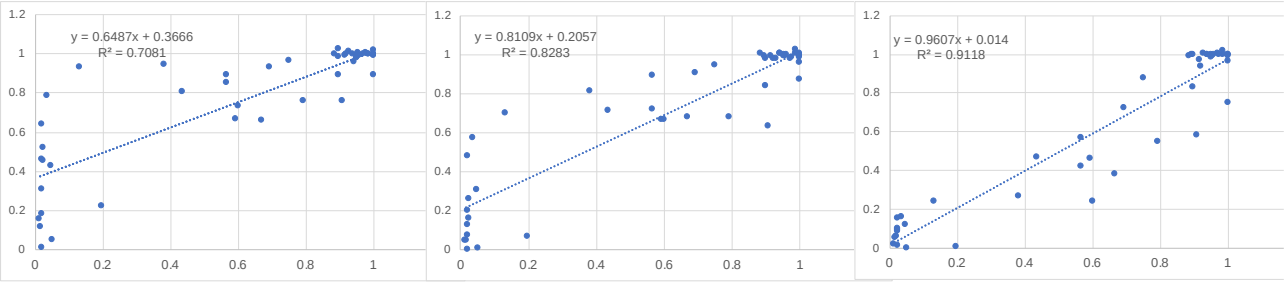


Kimura et al. The modified IL-8 Luc assay can significantly improve the false-negative judgment of lipophilic sensitizers with logK $_{ow}$ values > 3.5 Arch Toxicol 95:749-758, 2021

Inh-GAPLA and PI reduction values for all chemicals were summarized and examined for correlation. The time point after 16 h, 24 h, and 48 h of chemical treatment gave a significant correlation between Inh-GAPLA and PI reduction. Interestingly, the correlation strengthened with increased culture period, with correlation coefficients after 16 h, 24 h, and 48 h of 0.71, 0.83, and 0.91, respectively.

These data suggest that Inh-GAPLA after 16 h of chemical treatment correlates well with PI reduction values 48 h after chemical treatment. In addition, the chemicals providing more than 0.8 of Inh-GAPLA maintained more than 0.8 of PI reduction even 48 h after chemical treatment (Fig. 6).

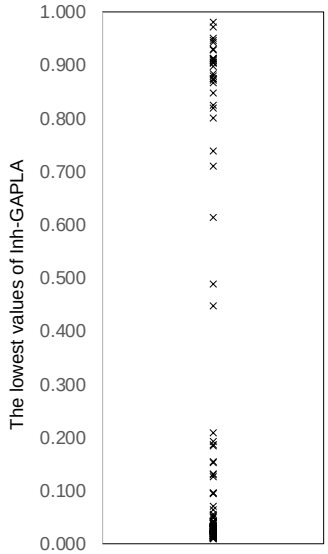
Fig. 6. The significant correlation between Inh-GAPLA and PI reduction



Kimura et al. Arch Toxicol 95:749-758, 2021

Next, to determine the cut-off value of Inh-GAPLA, we plotted the lowest Inh-GAPLA among 143 chemicals examined in the IL-8 Luc assay in our previous publication (Kimura et al. 2018). This plot shows that the chemicals can be clearly classified into two groups: chemicals showing Inh-GAPLA values ≥ 0.80 and chemicals showing Inh-GAPLA values < 0.80 (Fig. 7). We think that Inh-GAPLA values < 0.8 is a reasonable marker indicating that the test chemical is dissolved sufficiently to be cytotoxic.

Fig. 7. The distribution of the lowest values of Inh-GAPLA of chemicals examined by the original IL-8 Luc assay (n = 143)



Kimura et al. Arch Toxicol 95:749-758, 2021

14

Further consideration of the cut-off value of Inh-GAPLA by the biostatistician.

To further validate the cut-off value of Inh-GAPLA of 0.8, Dr. Omori added a statistical study to determine the cut-off value for Inh-GAPLA in the modified IL-8 Luc assay.

A statistical investigation to determine cut-off value for Inh-GAPLA in modified IL-8 Luc assay

Takashi Omori, Ph D.
Division of biostatistics Department of Social/Community Medicine and Health Science, Kobe University School of Medicine

Introduction

Dr. Aiba et al. conducted experiments to grasp relationship between PI reduction and Inh-GAPLA, in which THP-G8 cells were treated with various concentrations of 6 chemicals. From the experiments, they obtained PI reduction values after 48 hr with related Inh-GAPLA values. Although they discussed and suggested the cut-off value of 0.8 for Inh-GAPLA, it is not clearly explanation to set the value. They also showed the Inh-GAPLA distribution using their published 143 chemical data with the cut-off value. They also showed the Inh-GAPLA distribution using their published 143 chemical data (Kimura et al. 2018), with the cut-off value of 0.8.

The aim of this report was to determine the cut-off value from the view based on the statistical data analysis.

Methods

We used two datasets from Dr. Aiba and Dr. Kimura. One is the dataset consist of the independent 54 observations of the pair of PI reduction and Inh-GAPLA obtained from 6 chemicals. The other is the dataset 143 observations of Inh-GAPLA from 143 chemicals.

First, we fitted the regression line of the PI reduction as dependent variable with Inh-GAPLA as independent variable using the first dataset, and estimated the 95% confidence interval for the fitted line considering errors of data. As the reference value for PI reduction, we focused CV75 for the h-CLAT, that is, the cell viability of 75%. Using the lower limit of 95% confidence interval of the fitted linear regression line, we inversely estimated the value correspond to Inh-GAPLA for PI of 0.75.

Nest, we draw a dot chart to grasp the distribution of the Inh-GAPLA with the reference line of the obtained cut-off value. In the chart, we divided the distribution into a group whether conclusive or inconclusive when the original IL-8 Luc assay applied.

Results

The scatter plot of PI reduction after 48 hr with Inh-GAPLA with the linear regression line is shown in Fig. 9. For the fitted regression line in Fig. 8, the R square statistics was 0.9174 and the estimated intercept and slope were 0.014 and 0.964 respectively, and the mean square error, which is the estimate of the standard deviation of the error term, was 0.115. The reference line in horizontal axis is 0.75 and 0.79819.

Fig. 8. The correlation between Inh-GAPLA and PI reduction

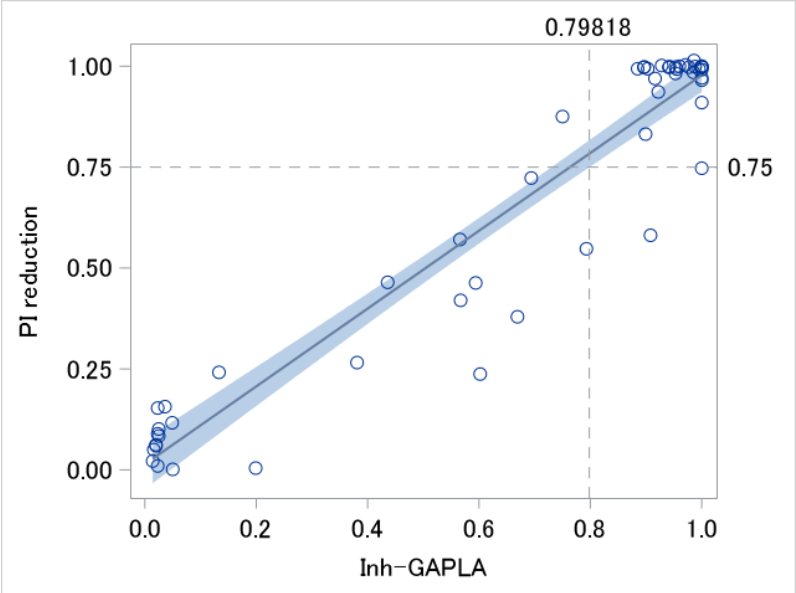
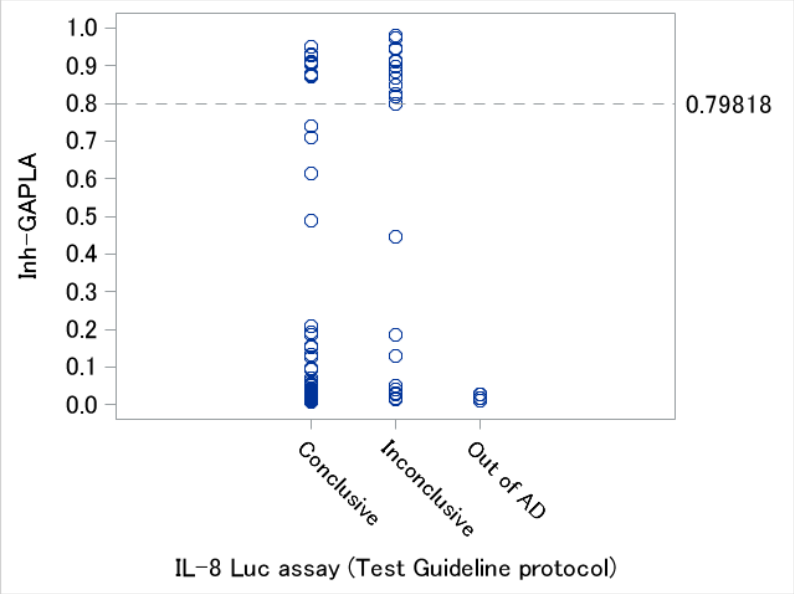


Fig. 9 shows the distribution of the Inh-GAPLA of chemicals that were classified as sensitizers or non-sensitizers and those that were classified as inconclusive. In this plot, the reference line is 0.79819, which is the obtained value from the above examination.

Fig. 9. Distribution of the lowest values of Inh-GAPLA of conclusive chemicals, i.e., sensitizers or non-sensitizers and inconclusive chemicals.



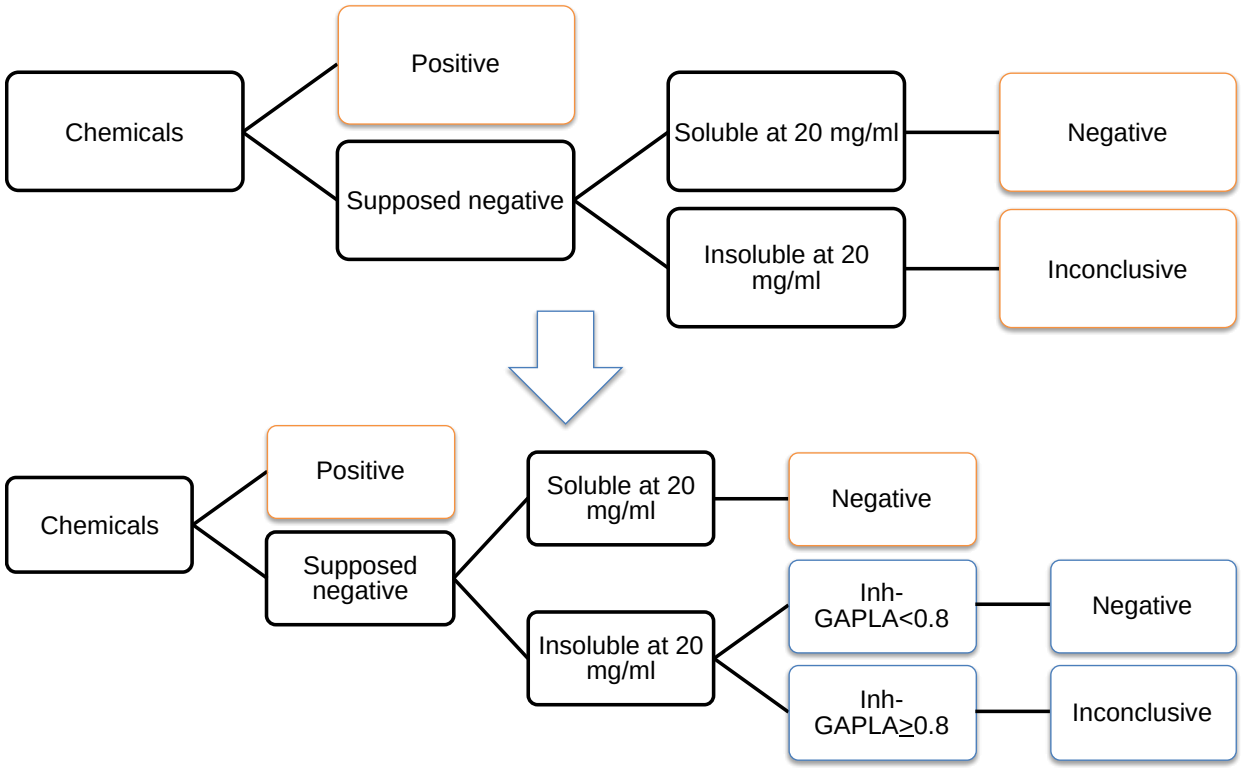
Discussion and Conclusion

The estimated value of our investigation was around 0.8. The result supports the cut-off value of 0.8 which Aiba et. al. suggested. Fig. 10 showed that the distribution of the Inh-GAPLA is not unimodal for both conclusive group and inconclusive group. In both groups, the density of the lowest Inh-GAPLA values are high around 0.9 and around 0 to 0.1, which also supports the validity of the cut-off value.

The prediction model of the modified IL-8 Luc assay

So, we propose a new prediction model. In this model, chemicals that do not dissolve in X-VIVO 15 at 20 mg/ml are determined to be negative if Inh-GAPLA is less than 0.8, and to be inconclusive if Inh-GAPLA is greater than 0.8 (Fig.10).

Fig. 10. Modification of the prediction model



The performance of the modified IL-8 Luc assay

This is the performance of the modified IL-8 Luc assay. This simple modification reduced the number of inconclusive chemicals and significantly improved specificity, although it slightly reduced sensitivity and accuracy. In addition, the balanced accuracy was also significantly improved by the modification (Kimura et al. 2021) (Table 3).

Table 3. The performance of the original IL-8 Luc assay and the modified IL-8 Luc assay for the LLNA data set

	tgIL-8 Luc assay	moIL-8 Luc assay
Positive	92	92
False negative	4	6
Negative	9	17
False positive	8	8
Inconclusive	23	13
Out of applicability domain	7	7
Sensitivity (%)	95.8 (92/96)	93.9 (92/98)
Specificity (%)	52.9 (9/17)	68.0 (17/25)
Accuracy (%)	89.3 (101/113)	88.6 (109/123)
Balanced Accuracy (%)	74.4	81.0

tgIL-8 Luc assay: the IL-8 Luc assay described in TG442E
moIL-8 Luc assay: the IL-8 Luc assay with the modified prediction model

Table 4 demonstrates the performance of the IL-8 Luc assay for human sensitizers. For human sensitizers, the modified IL-8 Luc assay showed the reasonable performance for the chemicals in the IL-8 Luc assay data set (Table 4). The modification of the IL-8 Luc assay improved specificity and slightly decreased sensitivity and accuracy. (Supplementary file 1)

Table 4. The performance of the original IL-8 Luc assay and the modified IL-8 Luc assay for human sensitizers in the IL-8 Luc assay data set

	tgIL-8 Luc assay	moIL-8 Luc assay
Positive	52	52
False negative	4	6
Negative	7	12
False positive	8	8
Inconclusive	13	6
Out of applicability domain	6	6
Sensitivity (%)	92.9 (52/56)	89.7 (52/58)
Specificity (%)	46.7 (7/15)	60.0 (12/20)
Accuracy (%)	83.1 (59/71)	82.1 (64/78)
Balanced Accuracy (%)	69.8	74.8

tgIL-8 Luc assay: the IL-8 Luc assay described in TG442E
moIL-8 Luc assay: the IL-8 Luc assay with the modified prediction model

The comparison of the performance of the modified IL-8 Luc assay with other *in vitro* skin sensitisation test methods

Since the sensitivity of the modified IL-8 Luc assay became a little lower, we compared the performance of the modified IL-8 Luc assay with that of other *in vitro* skin sensitisation test methods, such as the h-CLAT and the KeratinoSens, using DASS data base (Table 5). Although the modified IL-8 Luc assay slightly decreased sensitivity, its sensitivity is still high compared with that of other *in vitro* skin sensitisation test methods. In contrast to sensitivity, the modified IL-8 Luc assay significantly increased specificity. In this table, the specificity of the modified IL-8 Luc assay seems to be lower than that of other *in vitro* skin sensitisation test methods. However, it is important to emphasize that the specificity of the test method using DMSO as solvent is higher than it actually is, for the reasons mentioned in section 2.

Table 5. The performance of the modified IL-8 Luc assay (DASS data base for LLNA)

	tgIL-8 Luc assay	moIL-8 Luc assay	h-CLAT	KeratinoSens
Positive	69	69	66	59
False negative	4	6	15	22
Negative	6	9	12	12
False positive	5	5	3	3
Inconclusive	8	3		
Out of applicability domain	4	4		
Sensitivity (%)	94.5%	92.0%	81.5%	72.8%
Specificity (%)	54.5%	64.3%	80.0%	80.0%
Accuracy (%)	89.3%	87.6%	81.3%	74.0%
Balanced Accuracy (%)	74.5%	78.2%	80.8%	76.4%

tgIL-8 Luc assay: the IL-8 Luc assay described in TG442E
moIL-8 Luc assay: the IL-8 Luc assay with the modified prediction model

21

The comparison of the performance of the modified IL-8 Luc assay with other *in vitro* skin sensitisation test methods for human sensitizers

The performance of the modified IL-8 Luc assay against human sensitizers was compared with that of other *in vitro* skin sensitisation test methods for human sensitizers in the DASS data base (Supplementary file 1) (Table 6). The data showed that the performance of the modified IL-8 Luc assay was similar to that of h-CLAT and KeratinoSens. In fact, the sensitivity is slightly inferior to that of the h-CLAT and superior

to that of KeratinoSens. The specificity is superior to that of the h-CLAT and inferior to that of KeratinoSens. The accuracy is slightly superior to that of the h-CLAT and significantly superior to that of the KeratinoSens.

Table 6. The performance of the modified IL-8 Luc assay for human sensitizers (DASS database)

	tgIL-8 Luc assay	moIL-8 Luc assay	h-CLAT	KeratinoSens
Positive	30	30	32	24
False negative	3	5	5	13
Negative	1	2	3	5
False positive	1	1	2	0
Inconclusive	6	3		
Out of applicability domain	1	1		
Sensitivity (%)	90.9%	85.7%	86.5%	64.9%
Specificity (%)	50.0%	66.7%	60.0%	100.0%
Accuracy (%)	88.6%	84.2%	83.3%	69.0%
Balanced Accuracy (%)	70.5%	76.2%	73.3%	82.5%

tgIL-8 Luc assay: the IL-8 Luc assay described in TG442E
moIL-8 Luc assay: the IL-8 Luc assay with the modified prediction model

The comparison of the performance of the modifed IL-8 Luc assay with other *in vitro* skin sensitisation test methods for poorly water-soluble chemicals.

Since this modification of the IL-8 Luc assay targets poorly water-soluble chemicals, we compared the performance of the modified IL-8 Luc assay for the poorly water-soluble chemicals with that of other 2 *in vitro* skin sensitisation test methods, such as the h-CLAT and the KeratinoSens, using DASS data base (Table 7). In the IL-8 Luc assay, poorly water-soluble chemicals are defined as chemicals that are insoluble in water at 20 mg/ml. Again, the sensitivity of the modified IL-8 Luc assay was superior to that of other 2 *in vitro* skin sensitisation test methods. The sensitivity was superior to that of the h-CLAT or the KeratinoSens. The specificity was inferior to that of the h-CLAT or the KeratinoSens. The accuracy was superior to that of the h-CLAT or that of the Keratinosens. We thought that the low specificity was partly due to the too small number of poorly water-soluble non-sensitizers that we examined. In addition, it should be stressed that the h-CLAT or the KeratinoSens using DMSO as a solvent, are likely to include false negative results due to its low solubility in water, as discussed above (Introduction p5). (Supplementary file 2)

Table 7. The performance of the modified IL-8 Luc assay for poorly water-soluble chemicals (DASS data base)

	tgIL-8 Luc assay	moIL-8 Luc assay	h-CLAT	KeratinoSens
Positive	56	56	50	47
False negative	0	2	13	16
Negative	0	3	6	6
False positive	4	4	2	2
Inconclusive	8	3		
Out of applicability domain	3	3		
Sensitivity (%)	100.0%	96.6%	79.4%	74.6%
Specificity (%)	0.0%	42.9%	75.0%	75.0%
Accuracy (%)	93.3%	90.8%	78.9%	74.6%
Balanced Accuracy (%)	50.0%	69.8%	77.2%	74.8%

tgIL-8 Luc assay: the IL-8 Luc assay described in TG442E
moIL-8 Luc assay: the IL-8 Luc assay with the modified prediction model

26

We next compared the performance of the modified IL-8 Luc assay with other 2 *in vitro* skin sensitisation test methods for inconclusive chemicals. In the IL-8 Luc assay, an inconclusive chemical is defined as a chemical that is insoluble in water at 20 mg/ml and has a maximum Ind-IL8LA value of less than 1.4. Table 8 shows the evaluation results of each chemical by each test method, along with its LogKow. Table 9 shows the performance of each assay. Needless to say, the sensitivity of the modified IL-8 Luc assay for inconclusive chemicals is 0%, since inconclusive chemicals are collected with a maximum Ind-IL8LA value of less than 1.4. Notably, the sensitivity of the h-CLAT or the KeratinoSens for inconclusive chemicals is not high.

Table 8. The evaluation of inconclusive chemicals by different TGs

Chemical	tgIL-8 Luc assay	moIL-8 Luc assay	LogKow	DPRA	h-CLAT	KeratinoSens
2-Nitro-1,4-phenylenediamine	inconclusive	non-sensitizer	0.55	P	P	P
Benzoyl peroxide	inconclusive	inconclusive	3.43	P	N	N
Hexyl salicylate	inconclusive	inconclusive	5.06	N	P	N
Clotrimazole	inconclusive	inconclusive	6.26	P	N	
1-Bromohexane	inconclusive	inconclusive	3.63	P	N	P
Benzocaine	inconclusive	non-sensitizer	3.19	P	P	P
Methylmethacrylate	inconclusive	inconclusive	1.28		P	
Phenyl benzoate	inconclusive	inconclusive	3.04	P	P	N
Xylene	inconclusive	inconclusive	3.09	N	N	N
1-Bromobutane	inconclusive	non-sensitizer	2.65	N/P	P	N
2,4-Dichloro-1-nitrobenzene	inconclusive	inconclusive	3.1			
4-Hydroxybenzoic acid	inconclusive	non-sensitizer	1.39	N	N	N
6-Methylcoumarin	inconclusive	inconclusive	2.06	N	N	P
Acetoanisole(4-Methoxyacetophenone)	inconclusive	non-sensitizer	1.75	N	N	P
Benzoic acid	inconclusive	non-sensitizer	1.87	P	P/N	N
Chlorobenzene	inconclusive	inconclusive	2.64	N	P	N
Dimethyl isophthalate	inconclusive	inconclusive	1.66			
Ethyl benzoylacetate	inconclusive	non-sensitizer	1.71	N	N	P
Furil	inconclusive	inconclusive	1.8	P	N	P
Methyl salicylate	inconclusive	inconclusive	2.6	N	N	N
p-Aminobenzoic acid	inconclusive	non-sensitizer	0.96	N		N
Salicylic acid	inconclusive	non-sensitizer	2.24	P	P	N
Sulfanilamide	inconclusive	non-sensitizer	-0.55	N	N	N

tgIL-8 Luc assay: IL-8 Luc assay (OECD TG442E)

moIL-8 Luc assay: Modified IL-8 Luc assay

Table 9. The performance of different TGs for inconclusive chemicals

	moIL-8 Luc assay	h-CLAT	KeratinoSens
Positive	0	6	3
False negative	2	5	6
True negative	8	12	11
False positive	0	4	5
Inconclusive	13		
Sensitivity	0.0%	54.5%	33.3%
Specificity	100.0%	75.0%	68.8%
Accuracy	80.0%	66.7%	56.0%

moIL-8 Luc assay: Modified Il-8 Luc assay

5 Discussion

The reason for the false negative judgment by the modified IL-8 Luc assay

The modified IL-8 Luc assay determined two chemicals that were determined to be inconclusive by the original IL-8 Luc assay, 2-nitro-1,4-phenylenediamine and benzocaine, as a non-sensitizer.

There is limited information on 2-nitro-1,4-phenylenediamine (NPPD), but it too is classified as a pre/pro-hapten (Patlewicz et al., 2016). So, we would like to change the description regarding the evaluation of pre/pro-hapten in the test guideline from *"However, although negative results for suspected pre/prohaptens should be interpreted with caution, the IL-8 Luc assay correctly judged 11 out of 11 pre-haptens, 6/6 pro-haptens, and 6/8 pre/pro-haptens in the IL-8 Luc assay data set (2)."* to

"The IL-8 Luc assay correctly judged 10 out of 11 pre-haptens, 6/6 pro-haptens, and 6/8 pre/pro-haptens in the IL-8 Luc assay data set (2), negative results for suspected pre/prohaptens should be interpreted with caution."

Regarding benzocaine, the modified IL-8 Luc is supposed to make a false negative judgment by its weak sensitizing potential.

The performance of the modified IL-8 Luc assay for chemicals with logKow ≥ 3.5.

Finally, we examined the difference in the performance of the modified IL-8 Luc assays for chemicals with logKow < 3.5 and those with logKow ≥ 3.5. As shown in Table 10, the performance of the modified IL-8 Luc assay for 110 chemicals with logKow < 3.5, which were determined as 75 sensitizers, 25 non-sensitizers, and 10 inconclusive, 92.0% for sensitivity, 68.0% for specificity, and 86.0% for accuracy, while that for 26 chemicals with logKow ≥ 3.5, which were only sensitizers, was 100.0% for sensitivity and 100.0% for accuracy. There were 10 and 3 indeterminate judgments for chemicals with logKow < 3.5 and those with logKow ≥ 3.5, respectively. Unfortunately, since there were no non-sensitizers with logKow ≥ 3.5 of the 36 non-sensitizers, we could not obtain specificity for chemicals with logKow ≥ 3.5. Therefore, in this study, we further examined 4 non-

sensitizers with logKow ≥ 3.5, 1-iodohexane, butylbenzylphthalate, clofibrate, and dibutyl phthalate. None of them dissolved at 20 mg/mL in X-VIVO™ 15, satisfied the criteria for positive or gave less than 0.8 of Inh-GAPLA. So, they were judged as inconclusive. Therefore, although the specificity of the modified IL-8 Luc assay is still unknown, its high sensitivity to chemicals with logKow ≥ 3.5 and ability to avoid false negative results due to low solubility are advantage over other in vitro skin sensitization tests.

Table 10. The performance of the modified IL-8 Luc assay for chemicals with logKow<3.5 and those with logKow≥ 3.5

	Chemical	Potency category	LogKow	modified IL-8 Luc assay
1	1-Chloromethylpyrene	Extreme	5.73	positive
2	Tetrachlorosalicylanilide	Extreme	5.87	positive
3	Chlorpromazine hydrochloride	Strong	3.69	positive
4	Hexyl salicylate	Strong	5.06	inconclusive
5	Lauryl gallate	Strong	6.21	positive
6	12-Bromo-1-dodecanol	Moderate	5.11	positive
7	5-Methyl-2-phenyl-2-hexenal	Moderate	3.77	positive
8	Benzyl salicylate	Moderate	4.31	positive
9	Clotrimazole	Moderate	6.26	inconclusive
10	Undec-10-enal	Moderate	4.12	positive
11	(R)-(+)-Limonene	Weak	4.83	positive
12	1-Bromohexane	Weak	3.63	inconclusive
13	2-Ethylhexyl acrylate	Weak	4.09	positive
14	Abietic acid	Weak	6.46	positive
15	Amyl cinnamic aldehyde	Weak	4.33	positive
16	Benzyl cinnamate	Weak	4.06	positive
17	Bis-GMA	Weak	4.94	positive
18	Cyclamen aldehyde	Weak	3.91	positive
19	Farnesal	Weak	5.74	positive
20	Hexyl cinnamic aldehyde	Weak	4.82	positive
21	Imidazolidinyl urea	Weak	8.28	positive
22	Lilial	Weak	4.36	positive
23	N,N-Dibutylaniline	Weak	5.12	positive
24	Pentachlorophenol	Weak	4.74	positive
25	Phenol, 2,2-azobis-	Weak	4.55	positive
26	α-iso-Methylionone	Weak	4.84	positive

	$\log K_{ow} < 3.5$	$\log K_{ow} \geq 3.5$
No. of chemicals	110	26
No. of sensitizers	75	23
No. of Non-sensitizers	25	0
Accuracy	86.0%	100.0%
Sensitivity	92.0%	100.0%
Specificity	68.0%	Not determined
No. of indeterminate	10	3

Kimura et al. Arch Toxicol 95:749-758, 2021

6 Conclusion

Finally, the IL-8 Luc assay (TG442E) is characterized by 1) high sensitivity and accuracy, 2) high sensitivity for poorly water-soluble sensitizers and sensitizers with logK_{ow} >3.5, 3) the ability to exclude false negative results for sensitizers due to poor solubility, 4) low sensitivity, and 5) a substantial number of chemicals that are determined to be inconclusive.

The modification was intended to increase specificity and reduce inconclusive chemicals and was to just change the prediction model. In fact, the modification of IL-8 Luc assay increased specificity and decreased the number of inconclusive chemicals, although sensitivity and accuracy were somewhat reduced. Even after modification, however, the modified IL-8 Luc assay maintains the sensitivity higher than other TGs. In addition, this modification shed light on the important advantages of the IL-8 Luc assay over other *in vitro* skin sensitization tests due to its ability to exclude the false negative judgment of chemicals due to poor solubility in the medium.

If in vitro skin sensitization is used as a standalone test, sensitivity is very important and any modification that reduces sensitivity may not be allowed. However, it is not the case now. When the IL-8 Luc assay is combined with other in vitro test methods, it is required to reduce the number of inconclusive chemicals and to increase its specificity. By the modification, the IL-8 Luc assay is well suited for use in integrated approaches.

7 References

Barber, R.D., Harmer, D.W., Coleman, R.A., et al. (2005), GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. *Physiol Genomics* 21: 389-395, 10.1152/physiolgenomics.00025.2005

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,

Kimura, Y., Fujimura, C., Aiba, S. (2021), The modified IL-8 Luc assay, an in vitro skin sensitisation test, can significantly improve the false-negative judgment of lipophilic sensitizers with logKow values > 3.5. *Arch Toxicol* 95: 749-758, 10.1007/s00204-020-02934-9

Kimura, Y., Watanabe, M., Suzuki, N., et al. (2018), The performance of an in vitro skin sensitisation test, IL-8 Luc assay (OECD442E), and the integrated approach with direct peptide reactive assay (DPRA). *J Toxicol Sci* 43: 741-749, 10.2131/jts.43.741

Kimura, Y., Fujimura, C., Aiba, S. (2021) The modified IL-8 Luc assay, an invitro skin sensitisation test, can significantly improve the false-negative judgment of lipophilic sensitizers with log Kow values>3.5. *Arch Toxicol* 95: 749-758, 0.1007/s00204-020-02934-9

Mori, R., Wang, Q., Danenberg, K.D., et al. (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, 10.1002/pros.20815

Oliveira, J.G., Prados, R.Z., Guedes, A.C., et al. (1999), The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase is inappropriate as internal control in comparative studies between skin tissue and cultured skin fibroblasts using Northern blot analysis. *Arch Dermatol Res* 291: 659-661,

Patlewicz, G., Casati, S., Basketter, D.A., et al. (2016), Can currently available non-animal methods detect pre and pro-haptens relevant for skin sensitization? *Regul Toxicol Pharmacol* 82: 147-155, 10.1016/j.yrtph.2016.08.007

Sakaguchi, H., Ashikaga, T., Miyazawa, M., et al. (2009), The relationship between CD86/CD54 expression and THP-1 cell viability in an in vitro skin sensitization test--human cell line activation test (h-CLAT). *Cell Biology and Toxicology* 25: 109-126, 10.1007/s10565-008-9059-9 [doi]

Takahashi, T., Kimura, Y., Saito, R., et al. (2011), An in vitro test to screen skin sensitizers using a stable THP-1-derived IL-8 reporter cell line, THP-G8. *Toxicological Sciences* 124: 359-369, 10.1093/toxsci/kfr237

Thellin, O., Zorzi, W., Lakaye, B., et al. (1999), Housekeeping genes as internal standards: use and limits. *J Biotechnol* 75: 291-295,

Winer, J., Jung, C.K., Shackel, I., et al. (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, 10.1006/abio.1999.4085