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# Validation Report of the Alpha-Sens® Test Method Validation Study

## OECD Series on Testing and Assessment

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# Foreword

This document contains the Validation report of the Foetal Bovine Serum (FBS) free ARE Nrf2 Luciferase α-Sens® test method, a me-too method of the KeratinoSens™ assay, an in vitro method for identifying the skin sensitisation potential of chemicals. It supports the development of this test method for inclusion in Appendix ID of Test Guideline 442D, for in vitro skin sensitisation assays, which assesses the Adverse Outcome Pathway Key Event on Keratinocyte activation.

The α-Sens® test method, proposed by Japan, was developed to achieve a serum-free and simple operation procedure for the detection of antioxidant response element (ARE)-mediated transcriptional activity and evaluate the keratinocyte response to the early phase of skin sensitisation. The validation report was peer reviewed by an international Peer Review Panel in 2025. The Peer review report and the Validation report of the α-Sens® test method were circulated for comments to the Working Party of the National Coordinators of the Test Guidelines Programme (WNT) in November 2025. The WNT approved the Validation report at its 38th meeting in April 2026. The WNT also approved the Peer review report of the α-Sens® test method.

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

# Validation Study Report for α-Sens®
# as FBS-free test system for detecting ARE-Nrf2 activation of skin sensitisation

**α-Sens® Validation Management Team**

**September 2025**

# Contents

# 1. Summary

This report provides the results of the 'me-too' validation study for the α-Sens®, the FBS-free test system, as the ARE-Nrf2 luciferase test method in OECD TG442D. The study was conducted basically according to *Performance Standards for Assessment of proposed similar or Modified In Vitro Skin Sensitisation ARE–NRF2 Luciferase Test Method. Series on Testing and Assessment No. 213* (OECD, 2015) (Doc#213). The essential test method components of α-Sens® were confirmed to be equivalent to that of the KeratinoSens™ (the validated reference method (VRM)). The validation study was carried out with three participating laboratories in order to evaluate the transferability of the protocol, within- and between-laboratory reproducibility (WLR and BLR), and its predictive capacity of the α-Sens® as compared to that of human or the Local Lymph Node Assay (LLNA). Following a modular approach of applying the European Centre for the Validation of Alternative Methods (ECVAM) principles on test validity (OECD Series on Testing and Assessment, Number 34, 2005; Hartung *et al*., 2004), the Validation Management Team (VMT) has assessed modules 1 through 4 (test definition, WLR, transferability, and BLR), and has also used the validation study results in order to assess modules 5 and 6 (predictive capacity and applicability domain). After successful completion of the training phase, Phase-I (coded, 9 chemicals for transferability and for part of the data collection for BLR, and 8 chemicals for part of the data collection for WLR) and Phase-II (16 coded chemicals including hydrophobic chemicals (*logP*>3.5), for the remaining part of data collection for WLR and BLR), were conducted under the control of the VMT.

Consequently, the α-Sens® demonstrated the high transferability (100%) and high reproducibility for WLR and BLR (97.2% and 96.0%, respectively), and the predictive capacity was equivalent and/or superior to the existing ARE-Nrf2 Luciferase Test Methods in OECD TG442D, showing accuracy of: 88.3% (85.0% - 90.0%), sensitivity: 91.7% (91.7% - 91.7%) and specificity: 83.3% (75.0% - 85.7%) when compared among 20 reference chemicals in Doc#213. Among 25 test chemicals including hydrophobic chemicals used in this validation study, the predictive capacity for accuracy was: 86.7% (84.0% - 88.0%), sensitivity: 87.5% (87.5% - 87.5%) and specificity: 85.2% (77.8% - 88.9%). In addition, the in-house data of the developer's laboratory for 162 chemicals compared to LLNA results showed the accuracy of 74.1%, sensitivity of 76.3% and specificity of 64.5% (VRM results for the same 162 chemicals: accuracy=70.4%, sensitivity=71.0% and specificity=67.7%). When compared with 112 chemicals for human data, the accuracy, sensitivity and specificity was 83.0%, 84.3% and 79.3%, respectively (VRM results for the same 112 chemicals: accuracy=75.0%, sensitivity=73.5% and specificity=79.3%). All GHS 1A subcategory chemicals within the applicability domain were positive in the α-Sens®. The limitation of the assay is the same as that for the existing ARE-Nrf2 Luciferase Test Methods in OECD TG442D. The possible α-Sens® specific limitation may be noted for chemicals that directly affect *Renilla* luciferase transcription using the cell viability evaluation. The chemical applicability domain of the α-Sens® is the same or indeed extended to that for hydrophobic chemicals, compared to the existing ARE-Nrf2 Luciferase Test Methods in OECD TG442D.

The VMT concluded that the test method definition of the α-Sens® was satisfied and that the successful transferability of the protocol, high WLR and high BLR were confirmed. Furthermore, the VMT concluded that the α-Sens® has good predictive capacity for the reference chemicals, LLNA data and human data.

In addition to the successful performance of the α-Sens® as an additional ARE-Nrf2 Luciferase Test Method in OECD TG442D, many additional advantages are noted for the α-Sens®, such as the animal friendly assay system of serum (FBS) free operation throughout the assay, streamlined operation system, high transcriptional induction of reporter gene product with minimal background induction achieving an extremely high S/N ratio, employing a precise classifier using ARE activity normalised by cell viability, long-time stability of the responsiveness, and reduced plastic waste. These outstanding advantages of the α-Sens® compared to the existing adopted TG methods, strongly encouraged the development of this method to be included in OECD TG442D.

# 2. Introduction

Allergic contact dermatitis resulting from skin sensitisation is a common occupational and environmental health problem (Kimber *et al*., 2002). In order to prevent the health problems caused by skin sensitising chemicals, many efforts have been made to develop the predictive test for skin sensitisers and used by many regulatory authorities around the world. In recent decades, there has been a strong driving force to replace the traditional animal skin sensitisation tests, such as the guinea pig maximisation test (GMPT) and the Buehler test (OECD TG406) (OECD, 2022) and the local lymph node assay (LLNA), known as OECD TG429 (OECD, 2010), with non-animal alternative tests.

OECD launched a programme to develop Adverse Outcome Pathways (AOP) that can describe "the rationale for the use of particular methods" and can be used for "a basis for developing an Integrated Approaches to Testing Assessment (IATA) or an integrated testing strategy (ITS) (OECD, 2013). The skin sensitisation AOP is constructed with the following four key events (KE) recently published (OECD, 2014); KE-1: binding of haptens to endogenous skin proteins; KE-2: keratinocyte activation; KE-3: dendritic cell activation; KE-4: T-cell proliferation, and current non-animal test methods to predict the sensitisation potential of chemicals cover one of these four key events. The cell and artificial skin model-based test methods, such as the KeratinoSens™, LuSens and EpiSensA assays for testing KE-2, the h-CLAT, U-Sens, IL-8Luc and GARD skin assays for testing KE-3, have already been endorsed as OECD TG442D (OECD, 2024a) and TG442E (OECD, 2024b), respectively. In addition, Defined Approaches on Skin Sensitisation (DASS), known as OECD Guideline (GL) 497 (OECD, 2023a), was also developed and enables conclusions on the skin sensitisation potency classes, in addition to determination of the skin sensitisation hazard classes, with combinations of these in vitro and *in silico* prediction methods. However, among these *in vitro* tests, all currently available cell and artificial skin model-based test methods require the use of fetal bovine serum (FBS) to culture the cells and tissues during the assay process, so there is still an aspect of animal tissue use with these methods.

The α-Sens® test method was developed to achieve a serum-free and simple operation procedure with high accuracy for the prediction of skin sensitisers. The α-Sens® employs a dual luciferase assay system to detect both antioxidant response element (ARE)-mediated transcriptional activity and cell viability to evaluate the KE-2 response, the keratinocyte activation of the AOP in the skin sensitisation process (OECD, 2014). The α-Sens® cell line was originally developed from the PHK 16-0b cell line (immortalised human keratinocytes by the HPV16 virus genome) as host cells, and can be cultured in FBS-free culture media and is established by stable transfection of the ARE-inducible *Firefly* luciferase gene and thymidine kinase (TK) promoter-driven *Renilla* luciferase gene for cell viability. Therefore, the α-Sens® can measure ARE-mediated activity and cell viability simultaneously with the same exposure sample. Further, by using the normalised ARE-mediated transcriptional activity with cell viability, named normalised ARE activity (nAA) as a classifier for sensitiser/non-sensitiser is expected to minimise the effects of ARE activity caused by cell viability (Maeda *et al*., 2020).

The α-Sens® has several advantages compared with existing *in vitro* test methods for KE2. Namely,

- Use of FBS-free medium throughout the assay, including cell culture propagation and maintenance. Although the medium contains animal derived pituitary extract, the extract

is obtained during the usual meat production process.

- Time and assay resource saving with the dual luciferase assay system to detect both ARE-mediated transcriptional activity and cell viability with the same exposure sample, and
- Relatively high-performance with 20 chemicals listed in Performance Standard Reference Chemicals (Doc#213, 2015); accuracy (90% [18/20]), sensitivity (92% [11/12]) and specificity (88% [7/8]) for *in vivo* category, on the basis of prior conducted developer's data.

These remarkable features of α-Sens® will contribute to improving the accuracy standards of the DASS, in addition to the animal friendly and streamlining of the assay system aspects (See more details on α-Sens® in the section "4. Test Definition").

This report provides the results of the validation study of the α-Sens® to predict the skin sensitisation potential of chemicals.

# 3. Management of the Study

The detailed validation plan is provided in Appendix-01 Study plan for the validation trial on the α-Sens®.

## 3.1 Study objectives

The aim of this validation study is to validate the α-Sens® method, in order to incorporate this test method as a 'me-too' assay in Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 442D (*in vitro* Skin Sensitisation. ARE-Nrf2 Luciferase Test Method) (OECD, 2024a). The validation study assessed the reliability (reproducibility within and between laboratories) and relevance (predictive capacity) of the α-Sens® assay with a set of test chemicals for which high quality *in vitro* and *in vivo* data are available.

The validation study for the α-Sens® was undertaken as follows:

i) In accordance with the principles and criteria documented in the OECD No. 34 Guidance Document (GD) 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), and

iii) according to the concept discussed on the validation trials with participation of Good Laboratory Practice (GLP) Test Facilities (OECD, 2000) where the whole concept of the validation trials is described in the context of GLP. The validation trial for this assay was performed under the spirit of GLP.

A general conceptual framework (Hartung *et al*., 2004; OECD, 2005) was used for documenting all the study elements to assess the validation status of a test method, termed the "modular approach" to validation. In this approach, the information needed to support the validity of the method is organised 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 (not relevant to this me-too assay)

## 3.2 Study Plan

The Study Plan was drafted, approved and issued by the Validation Management Team (VMT) prior to the start of the testing. The final version is annexed to this report (see Appendix-01).

### *3.2.1 Validation Management Team*

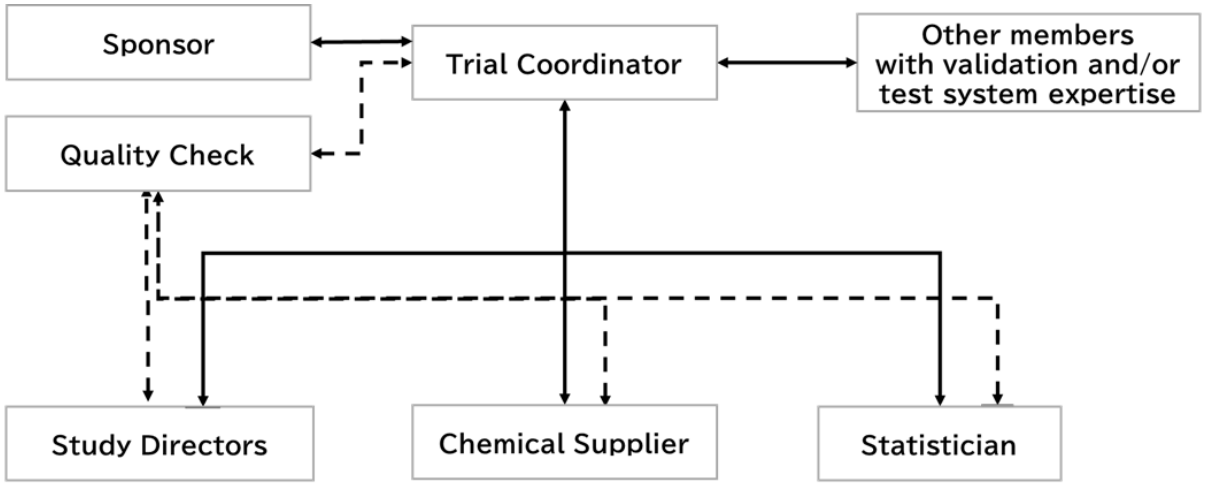
This validation study was coordinated by Japanese Center for the Validation of Alternative Methods (JaCVAM). The VMT encompasses collective expertise with *in vitro* test methods, in the underlying science and the scientific design, management and evaluation of a validation trial.

The VMT, which plays a central role overseeing the conduct of the validation trial, includes members. The members of VMT are listed in Table 3-1.

**Table 3-1 Members of VMT**

| Name | Role and expertise | Affiliation |
|---|---|---|
| Prof. Atsushi Ono | VMT trial coordinator | Okayama University, Japan |
| Prof. Kazuhiko Matsumoto | Data analysis / biostatistician | Nagoya City University, Japan |
| Dr. Miriam N Jacobs | Test system expertise,<br>Validation expertise<br>English proof reading | UK Health Security Agency |
| KoCVAM liaison:<br>Dr. Nam-Hee Kang | Test system expertise | Korean Centre for the Validation of Alternative Methods (KoCVAM), Ministry of Food and Drug Safety (MFDS), Korea |
| Japanese liaison:<br>Prof. Tomoki Fukuyama | Test system expertise | Azabu University, Japan |
| Dr. Hajime Kojima | Quality check (QC) and record receiving / Validation expertise | Japanese Centre for the Validation of Alternative Methods (JaCVAM), National Institute of Health Sciences (NIHS), Japan /<br>Sanyo-Onoda City University, Japan |
| Dr. Akiko Ohno | Quality check (QC) /<br>Validation expertise | JaCVAM, NIHS, Japan |
| Dr. Takao Ashikaga | Chemical supply, sponsor /<br>Validation expertise | JaCVAM, NIHS, Japan |
| Dr. Yumi Akahori | VMT organiser /<br>Validation expertise | CERI, Japan |

The management structure of the validation trial is shown in Fig. 3-1.




*Dash line indicate quality check staff involvement.*

**Fig. 3-1 Management structure of the α-Sens® validation study**

The trial coordinator (as the formal representative of the VMT and the single contact point with the Study Directors (SDs) of each participating laboratory) had responsibilities to coordinate the validation study and to establish the VMT with support from JaCVAM.

JaCVAM organised the quality check (QC) group for checking quality of data (study report, raw data, data spreadsheets and worksheets) from each laboratory and accurate reflection of the validation data in the validation report (see more details in section "8. Quality Check" and Appendix-02).

As a sponsor, JaCVAM supported the financial cost for test chemicals, vehicles and its distribution (including coding), and part of the test consumables for the α-Sens®. The other financial costs were voluntarily covered in-kind, by the respective participating laboratories.

## *3.2.2 Lead Laboratory and participating laboratories*

The lead laboratory is the Chemicals Evaluation and Research Institute, Japan (CERI), which developed the α-Sens®. The participating laboratories in the validation study are listed in Table 3-2. It is the first time the JRF laboratory from India have been involved as a participating laboratory in a validation study for the development of OECD TGs. The other two participating laboratories have prior OECD TG development experience.

Data generation relevant for Between-laboratory transferability, Within-laboratory reproducibility (WLR), and Between-laboratory reproducibility (BLR) was performed by the three participating naïve laboratories and the lead laboratory (CERI). All three naïve laboratories participating in this validation had no previous experience with α-Sens®. Data

obtained by these 3 participating laboratories and the lead laboratory was used to demonstrate the transferability and reproducibility of α-Sens®.

**Table 3-2 Participating laboratories in the validation study**

| | Name of laboratory [GLP facility or not] | Study Director (SD) |
|---|---|---|
| Test laboratory 1 | Food and Drug Safety Center (FDSC)<br>729-5 Ochiai, Hadano, Kanagawa, Japan. 257-8523<br>[GLP facility] | Dr. S. Tachibana |
| Test laboratory 2 | Sumitomo Chemical Company, Limited, Environmental Health Science Laboratory<br>3-1-98 Kasugade-naka, Konohana-ku, Osaka-shi, Japan. 554-8558<br>[non-GLP facility] | M. Sc. R. Kobayashi |
| Test laboratory 3 | Jai Research Foundation (JRF)<br>NH48, Near Daman Ganga Bridge, Valvada, District Valsad, Gujarat, India. 396105<br>[GLP facility] | M. Sc. Priyanka K. Mishra |
| Lead laboratory | Chemicals Assessment and Research Center, Chemicals Evaluation and Research Institute, Japan (CERI)<br>1600 Shimotakano, Sugito-machi, Kitakatsushika-gun, Saitama, Japan 345-0043<br>[CERI has GLP facilities in other areas but the laboratory participating in this validation study, is not a GLP facility] | Dr. Y. Maeda |

## 3.3 Study Design

Before starting the validation study, a training phase was conducted to confirm the testing setup for aspects such as the luminometer and basic protocol transferability (for more details on training see section 5.1 Training). The technician who completed the training phase was qualified to produce the data for the validation (Phase-I and Phase-II). After the training phase, two phases and these validation criteria were set for the validation as shown in Table 3-3. It should be noted that all test chemicals used in Phase-I and Phase-II were coded by JaCVAM, and tested by the participating laboratories in a blinded fashion.

**Table 3-3 The structure of the α-Sens® validation study**

| Phase | Chemicals | | Number of qualified Experiment*1 | Number of qualified Run*2 | Transferability | WLR | BLR |
|---|---|---|---|---|---|---|---|
| | Selection sources | Number of chemicals | | | | | |
| **Phase-I** | Reference chemicals*3 | 8 | 3 | At least 2 | yes | yes | yes |
| | Additional chemicals | 1 | 1 | | | - | |
| **Phase-II** | Reference chemicals*3 | 4 | 3 | | - | yes | yes |
| | Reference chemicals*3 | 8 | 1 | | | - | |
| | Additional chemicals | 4 | | | | | |
| | Total number of test chemicals | **25** | | | | | |

-: not applied.

*1 One “qualified experiment” consists of at least two qualified independent runs in case concordant results are obtained, or of three qualified independent runs in case of discordant results in the first two runs.

*2 Each run consists of three replicates for each concentration tested. Negative (solvent) control and positive control should be tested concurrently with the test chemical in each run.

*3 Reference chemicals listed in Doc#213.

### *3.3.1 α-Sens® Cell line*

The α-Sens® cell line has already been deposited in JCRB Cell Bank (Japanese Collection of Research Bioresources Cell Bank) and JCRB has completed the α-Sens® cell propagation to prepare the cell stocks for worldwide distribution. All laboratories (two participating laboratories and Lead laboratory in Japan, and one participating laboratory in India) received the cell stocks from the JCRB for the training and the validation study.

The cell availability is described in Section "4.4 ".

### *3.3.2 Selection of Test Chemicals*

Before selecting test chemicals for the α-Sens® validation study, the VMT addressed the following considerations.

- The α-Sens® is not optimised using only 20 reference chemicals in Doc#213,
- 20 reference chemicals are needed to compare performance with the existing assays, and
- Test chemicals can be selected from the reference chemicals provided in Doc#213.

Within the task of identifying 20 reference chemicals from Doc#213, the following two key issues were also addressed (see more details in "Appendix-03 Justification for additional test chemical selection").

- The *logP* range among the 20 reference chemicals relatively narrow (*logP:* -1.86 ~ 3.59). Of the reference chemicals, phenyl benzoate (*logP*=3.59) was the only reference chemical with a relatively high *logP* value (>3.5).
  The addition of a hydrophobic chemical also supported the shortcomings identified by the OECD skin sensitisation expert group for the DASS, with respect to addressing issues in relation to borderline chemicals, to distinguish whether they might be positive or negative, as well as expanding the current chemical applicability domain.
- The distribution of GHS subcategories for the 20 reference chemicals had less category 1B chemicals.

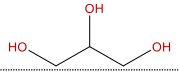
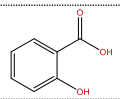
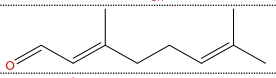
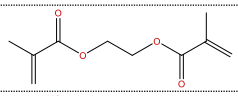
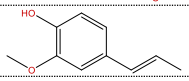
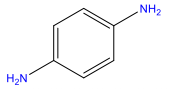
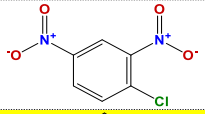
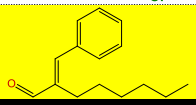
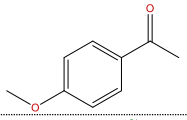
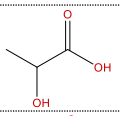
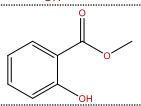
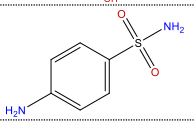
With these considerations in mind, the VMT carefully selected test chemicals for the Phase-I and Phase-II (Table 3-4).

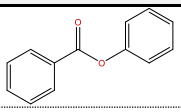
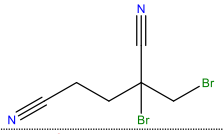
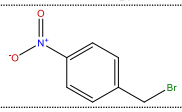
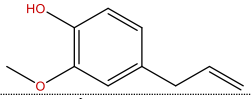
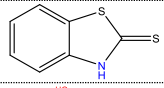
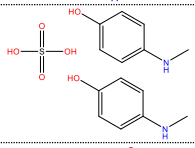
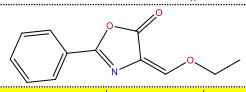
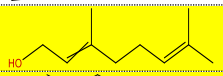
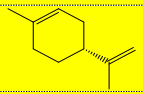
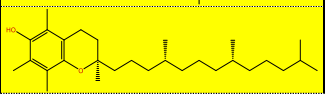
Nine test chemicals for the Phase-I (coded, 9 chemicals for transferability and for part of data collection for BLR, and 8 chemicals for part of data collection for WLR) were selected to cover range of known skin sensitising potency (Extreme, Strong, Moderate, Weak) and negatives, including cytotoxic chemicals. One chemical (as additional chemical) for Phase-I was α-Hexyl cinnamaldehyde (HCA; *logP*: 4.3), which was used to confirm the transferability and the workability of the protocol improvement from the training phase (one of the three participating laboratories initially had produced problematic results during the training phase).

As for test chemicals for Phase-II, the remaining reference chemicals (12 chemicals) were selected. In addition to these chemicals, hydrophobic chemicals (*logP* > 3.5) were selected considering the GHS subcategorisation. The final selection criteria for additional chemicals are described in Appendix-03. It should be noted that the GHS subcategory from human data is based on the report frequency (including induction threshold and exposure levels) and the GHS subcategory from LLNA is based on potency (i.e., EC value). Because of this fact, the selection of additional 1B test chemicals was based on LLNA.

Negative chemicals were selected for approximately 40% of the total test chemicals, in order to increase the predictability of the data analysis.

## Table 3-4 Test chemicals selected for Phase-I and Phase-II study

| No. | Chemical Name | CAS No. | Chemical Structure | Transferability | WLR | BLR | No. of Experiments *1 | *in vivo* category in Doc#213 or in DASS *2 | Physical State | From DASS database (OECD, 2023a) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | *LogP* *3 | *LogP* Source*4 | KS.Call *5, *10 | LLNA.GHS.SUB *6, *11 | HU.GHS.BIN *7, *10 | HU.GHS.SUB *8, *11 | Basketter_human_potency _3class *9, *11 |
| **Phase-I** | | | | | | | | | | | | | | | | |
| 01 | Glycerol | 56-81-5 |  | **yes** | **yes** | **yes** | **3X** | as negative | Liquid | -1.86 | Exp | 0 | NC | NA | NA | NC |
| 02 | Isopropanol | 67-63-0 |  | **yes** | **yes** | **yes** | **3X** | as negative | Liquid | 0.05 | Exp | 0 | NC | NA | NA | NC |
| 03 | Salicylic acid | 69-72-7 |  | **yes** | **yes** | **yes** | **3X** | as negative | Solid | 2.26 | Exp | 0 | 1B | NA | NA | NC |
| 04 | Ethylene glycol dimethacrylate | 97-90-5 |  | **yes** | **yes** | **yes** | **3X** | sensitiser (weak) | Liquid | 1.51 | Pred | 1 | 1B | NA | NA | 1B |
| 05 | Citral | 5392-40-5 |  | **yes** | **yes** | **yes** | **3X** | sensitiser (moderate) | Liquid | 3.00 | Pred | 1 | 1B | 1 | NA | 1B |
| 06 | Isoeugenol | 97-54-1 |  | **yes** | **yes** | **yes** | **3X** | sensitiser (moderate) | Liquid | 3.04 | Exp | 1 | 1A | 1 | 1B | 1A |
| 07 | *p*-Phenylenediamine | 106-50-3 |  | **yes** | **yes** | **yes** | **3X** | sensitiser (strong/extreme) | Solid | -0.30 | Exp | 1 | 1A | 1 | 1A | 1A |
| 08 | 2,4-Dinitrochlorobenzene | 97-00-7 |  | **yes** | **yes** | **yes** | **3X** | sensitiser (extreme) | Solid | 2.17 | Exp | 1 | 1A | 1 | 1A | 1A |
| 09 | α-Hexylcinnamaldehyde (HCA) | 101-86-0 |  | **yes** | **-** | **yes** | **1X** | (Pos) | Liquid | **4.34** | Pred | 1 | 1B | NA | NA | NC |
| **Phase-II** | | | | | | | | | | | | | | | | |
| 10 | 4'-Methoxyacetophenone (=Acetanisole) | 100-06-1 |  | - | **yes** | **yes** | **3X** | as negative | Solid | 1.74 | Exp | 1 | NC | NA | NA | NA |
| 11 | Chlorobenzene | 108-90-7 |  | - | - | **yes** | **1X** | as negative | Liquid | 2.87 | Exp | 0 | NA | NA | NA | NA |
| 12 | Lactic acid | 50-21-5 |  | - | - | **yes** | **1X** | as negative | Liquid | -0.72 | Exp | 0 | NC | NA | NA | NC |
| 13 | Methyl salicylate | 119-36-8 |  | - | - | **yes** | **1X** | as negative | Liquid | | | 0 | - | - | - | - |
| 14 | Sulfanilamide | 63-74-1 |  | - | - | **yes** | **1X** | as negative | Solid | -0.62 | Exp | 0 | NC | 1 | 1B | NA |
| 15 | *n*-Hexane | 110-54-3 |  | - | - | **yes** | **1X** | (Neg) | Liquid | **3.90** | Exp | 0 | NC | NC | NC | NC |

| No. | Chemical Name | CAS No. | Chemical Structure | Transferability | WLR | BLR | No. of Experiments *1 | *in vivo* category in Doc#213 or in DASS *2 | Physical State | From DASS database (OECD, 2023a) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | *LogP* *3 | *LogP*.Source*4 | KS.Call *5, *10 | LLNA.GHS.SUB *6, *11 | HU.GHS.BIN *7, *10 | HU.GHS.SUB *8, *11 | Basketter_human_potency_3class *9, *11 |
| 16 | Phenyl benzoate | 93-99-2 |  | - | **yes** | **yes** | **3X** | sensitiser (weak) | Solid | **3.59** | Exp | 0 | NA | 1 | **1B** | **1B** |
| 17 | Methyldibromo glutaronitrile | 35691-65-7 |  | - | **yes** | **yes** | **3X** | sensitiser (strong) | Solid | 1.89 | Pred | 1 | **1A** | NA | NA | **1A** |
| 18 | 4-Nitrobenzyl bromide | 100-11-8 |  | - | **yes** | **yes** | **3X** | sensitiser (extreme) | Solid | 2.82 | Pred | 1 | **1A** | NA | NA | NA |
| 19 | Eugenol | 97-53-0 |  | - | - | **yes** | **1X** | sensitiser (weak) | Liquid | 2.27 | Exp | 0 | **1B** | 1 | **1B** | **1B** |
| 20 | 2-Mercaptobenzothiazole | 149-30-4 |  | - | - | **yes** | **1X** | sensitiser (moderate) | Solid | 2.42 | Exp | 1 | **1A** | 1 | **1B** | **1B** |
| 21 | 4-Methylaminophenol sulphate | 55-55-0 |  | - | - | **yes** | **1X** | sensitiser (strong) | Solid | 0.63 | Pred | 1 | **1A** | NA | NA | **1B** |
| 22 | Oxazolone | 15646-46-5 |  | - | - | **yes** | **1X** | sensitiser (extreme) | Solid | 1.48 | Pred | 1 | **1A** | NA | NA | NA |
| 23 | Geraniol | 106-24-1 |  | - | - | **yes** | **1X** | (Pos) | Liquid | **3.6** | Exp | 1 | **1B** | 1 | **1B** | **1B** |
| 24 | *D*-Limonene | 5989-27-5 |  | - | - | **yes** | **1X** | (Pos) | Liquid | **4.52** | Exp | 0 | **1B** | NA | NA | NC |
| 25 | α-Tocopherol | 59-02-9 |  | - | - | **yes** | **1X** | (Pos) | Liquid | **9.41** | Pred | 1 | **1B** | NA | NA | NC |

Yellow-highlighted indicates additional chemicals.

*1 "1X" indicates 1 experiment. "3X" indicates 3 experiments.

*2 *in vivo* category in parentheses was based on LLNA data in the DASS database.

*3 Red character indicates *logP*>3.5.

*4 Exp: Experiment data, Pred: Prediction data. According to the Supporting Document (OECD, 2021) of OECD GL497, prediction was made by OPERA (https://comptox.epa.gov/dashboard. Model version was not provided in Supporting Document (OECD, 2021) of OECD GL497.

*5 KS Call: Hazard Prediction from KeratinoSens (TG442D) (cited from DASS database (OECD, 2023a))

*6 LLNA.GHS.SUB: LLNA Potency reference subcategorisation (cited from DASS database (OECD, 2023a))

*7 HU.GHS.BIN: Human Binary hazard reference classification (cited from DASS database (OECD, 2023a))

*8 HU.GHS.SUB: Human Potency reference subcategorisation (cited from DASS database (OECD, 2023a))

*9 Basketter_human_potency_3class: Expert-derived human potency subcategorisation (3-class) from (Basketter *et al*. 2014 cited from DASS database (OECD, 2023a))

*10 1: positive, 0: negative, NA: data not available, NC: not conclusive

*11 1A: GSH subcategory 1A, 1B: GHS subcategory 1B, NA: data not available, NC: not conclusive

.

### *3.3.3 Acquisition, coding, and distribution*

**(1) Acquisition, coding, and distribution**

All test chemicals used for Phase-I and Phase-II were coded and distributed by JaCVAM. The coding was supervised by the Trial Coordinator, responsible for coding and distribution of test chemicals and positive control for the validation study.

For cell culture medium (KGM™ Gold Keratinocyte Growth Medium BulletKit™ (Lonza)) and luciferase assay reagents, it was ensured that the same lot numbers between test laboratories were distributed or purchased by each test laboratory; JRF in India was able to procure the reagents by itself.

**(2) Handling**

Staff members who were independent of those performing the validation study at each test laboratory received test chemicals and essential information about the test chemicals (storage instructions etc.) from JaCVAM. Moreover, the test chemical manager at each test laboratory separately received the Safety Data Sheet (SDS) in a sealed state, including the safety information concerning the hazards identification and exposure controls/personal protection. The SDS was provided as a precaution in case of an emergency.

## 3.4 Data Management

The data spreadsheets (Excel file) for analysing the data at each laboratory and worksheets (Word file) for recording the procedures were formatted and provided for the validation study.

The study report, raw data, data spreadsheets and worksheets at each laboratory were submitted to the trial coordinator after each Phase, and all these data, including the failed data that did not pass the data acceptance criteria, etc., were sent for quality checks and data analysis, respectively.

## 3.5 Statistical Analysis

### *3.5.1 Data analysis*

A biostatistician within the VMT group was responsible for data analysis. The data obtained from this validation study was analysed from the position of an independent third party. The data analysis was mainly used to compare the data with the validation criteria described in the next section. After the Phase-I study, the criteria for checking whether the results of the VC (vehicle control) wells are affected by cross-contamination of chemiluminescence were newly established by the data analysis (see more details in section “5.2.2 (1)”).

## *3.5.2 Validation Criteria*

Performance Standards (PS) for the assessment of proposed similar or modified test methods ("me-too" tests) for *in vitro* ARE-Nrf2 luciferase test methods similar to the KeratinoSens™ were adopted as specified in the OECD Series on Testing and Assessment No. 213 "Performance Standards for Assessment of Proposed Similar or Modified *in vitro* Skin Sensitisation ARE-Nrf2 Luciferase Test Methods" (hereafter referred as "Doc#213") in 2015 (OECD, 2015). This PS comprised the three elements (I) Essential test method components, (II) "Minimum list of reference substances" that should be used to evaluate the reliability and relevance, and (III) defined reliability and accuracy values. The essential test method components were explained in "4.2 Scientific basis: biological and mechanistic relevance".

**(1) Between-laboratory transferability (Transferability)**

The transferability was performed to assess the successful transfer of the assay to a test laboratory without knowledge of the test method to be evaluated until the training phase but having knowledge of similar test systems and endpoint detection methods.

The data for transferability was collected under the Phase-I study using coded 9 chemicals. Three qualified experiments for each 8 chemicals were required in this validation study because these 8 chemicals were selected for WLR in Doc#213. The remaining chemical was tested in one qualified experiment. It should be noted that there are no criteria for transferability in Doc#213. Therefore, the criteria for transferability were set by the VMT.

***Criteria for Transferability:***

All qualitative conclusions of 9 test chemicals from all participating laboratories should meet the same conclusions as those generated by the lead laboratory.

"One qualified experiment" consists of at least two qualified independent runs where concordant results are obtained, or of three qualified independent runs where discordant results were reported for the first two runs. "Run" means each assay including three replicates for each test concentrations of each test chemical.

Three qualitative conclusions per chemical for 8 chemicals and one qualitative conclusion for one hydrophobic chemical were derived from each qualified experiment.

The three qualitative conclusions for each chemical at each lab were summarised by applying the following rules. Thus obtained all qualitative conclusions were used to assess transferability.

| Rules | |
|---|---|
| | >= 2 positive conclusions → Positive |
| | >= 2 negative conclusions → Negative |

**(2) Within-laboratory Reproducibility (WLR)**

Twelve reference chemicals recommended in Doc#213 were used to confirm WLR. Twelve chemicals were separately tested for 8 chemicals in Phase-I and 4 chemicals in Phase-II. These 12 chemicals were tested to produce three qualitative conclusions in each laboratory (one

qualitative conclusion was based on one experiment) since this was one of the requirements in Doc#213.

The calculation rule and criteria for WLR defined in Doc#213 were as described below and these were also agreed by the VMT.

**Calculation rule and Criteria for WLR defined in Doc#213** (OECD, 2015)**:**

- "*For the purpose of evaluating the within-laboratory reproducibility the subset of 12 reference substances …. should be tested by each participating laboratory to produce three qualified experiments to derive three predictions in each laboratory.*" (OECD, 2015).
- **Calculation rule:** "*Within-laboratory reproducibility (WLR) should be calculated based on concordance of classifications* obtained by each participating laboratory for the subset of 12 reference substances …, using three qualified experiments.*" (OECD, 2015).
- **Criteria:** "*An assessment of within-laboratory reproducibility should show a concordance of predictions* (positive versus negative) obtained in three different, independent qualified experiments of the 12 recommended reference substances (shown in bold italics in Table 1) within each participating laboratory equal or higher (≥) than 80.0% (actual for KeratinoSens™ 85.7% based on the validation dataset).*" (OECD, 2015).

*: In this validation report, the kappa coefficients were used for "concordance of classification" and "concordance of predictions"

**(3) Between-laboratory Reproducibility (BLR)**

Except for 12 chemicals used for WLR assessment, the remaining 8 chemicals in the minimum list of reference chemicals in Doc#213 and 5 additional test chemicals were tested in one experiment for each to derive one qualified conclusion for each chemical. A total of 25 test chemicals was also used for the BLR evaluation.

The calculation rule and criteria for BLR defined in Doc#213 were as described below and these were also agreed by the VMT.

***Calculation rule and Criteria for BLR defined in Doc#213:***

- **Calculation rule**: "*Between-laboratory reproducibility (BLR) should be calculated based on concordance of classifications obtained by at least three participating laboratories for the 20 reference substances listed in Table 1. BLR should be calculated based on concordance of classifications using only qualified experiments. For the 12 substances for which each laboratory will generate three classifications (for WLR assessment), one single final classification should be derived per laboratory based on the mode of the three predictions obtained. These single final classifications should then be used for BLR assessment.*" (OECD, 2015).
- **Criteria**: "*For similar or modified test methods, the concordance of predictions (positive versus negative) between a minimum of three laboratories, obtained for the 20 recommended reference substances (omitted), should be equal or higher (≥) than 80.0% (actual for KeratinoSens™: 94.7% based on 19 reference substances (omitted) with the exclusion of 4-methoxy-acetophenone for which no KeratinoSens™ data on BLR is available, and 85.7% based on the validation dataset).*" (OECD, 2015).

For chemicals that have three qualitative conclusions for each chemical at each lab, the following rules were applied to summarise conclusion for each chemical.

| Rules | |
|---|---|
| | >= 2 positive conclusions in qualified experiments → Positive |
| | >= 2 negative conclusions in qualified experiments → Negative |

**(4) Predictive Capacity**

"Minimum list of reference substances" in Doc#213 included 20 reference chemicals to determine reliability and relevance and the proposed methods need to have accuracy, sensitivity and specificity values which are comparable or better than those derived from the validated reference method (VRM), KeratinoSens™. The detailed criteria provided in Doc#213 were provided below.

The VMT agreed to add 5 more chemicals to extend the *logP* range of test chemicals for the validation (see more details in 3.3.2 Selection of Test Chemicals). A total of 25 test chemicals (20 reference chemicals and 5 additional chemicals) were also used to evaluate the predictive capacity of the α-Sens®. In addition, this validation report analysed the predictive capacity using additional in-house data collected in the developer's laboratory (the lead laboratory).

***Calculation rule and Criteria for BLR defined in Doc#213:***

- **Calculation rule**: "*The calculation of the accuracy values should be done using all qualified experiments generated by at least three laboratories with the 20 reference substances. The calculations should be based on the predictions obtained with each qualified experiment. A weighted calculation should be used to take into account the fact that the 20 reference substances will have a different number of experiments, i.e. the 12 substances used for both BLR and WLR assessment will have nine experiments each, whereas the 8 substances used for BLR assessment only will have three experiments each. In summary, the final outcome of each individual qualified experiment obtained for each substance (from all participating laboratories) should be captured as an independent classification in the calculations and correction factors should be applied so that all substances have an equal weight in the calculations. The positive and negative predictions for each substance should thus be divided by the total number of predictions for that substance so that each substance contributes with a final weight of 1 in the calculations*[*1]." (OECD, 2015).
- **Criteria**: *"The accuracy, sensitivity and specificity of the proposed similar or modified test method should be comparable or better to that of the VRM. The accuracy, sensitivity and specificity obtained with the 20 reference substances listed in Table 1 should all be equal or higher (≥) than 80.0% (actual for KeratinoSens™ based on the 20 reference substances and using a weighted calculation: 87.0% accuracy, 86.7% sensitivity and 87.5% specificity. The predictive capacity of KeratinoSens™ calculated on the basis of the full validation dataset is reported in paragraph 10 of the Test Guideline*[*2]*. Furthermore, no strong or extreme sensitisers should be under-predicted as non-sensitiser, unless a clear rationale can be given.*" (OECD, 2015).

  *2: Current Test Guideline (OECD, 2024a) provided "*The accuracy (77% - 155/201), sensitivity (78% - 71/91) and specificity (76% - 84/110) of the KeratinoSens™ test*

*method for discriminating skin sensitisers (i.e. UN GHS Cat. 1) from non-sensitisers when compared to LLNA results were calculated by considering all of the data submitted to EURL ECVAM for evaluation and peer-review of the test method. These figures are similar to those published based on in-house testing of about 145 test substances (77% accuracy, 79% sensitivity, 72% specificity)*." (OECD, 2015).

*1: The explanation "*each substance contributes with a final weight of 1 in the calculations*" (OECD, 2015) is difficult to understand, and the weight calculation (1-i/k) for the Kappa coefficient also cannot be used in the 2X2 contingency table. We recommend that this is clarified by the OECD.

# 4. Test Definition

## 4.1 Development of the test method

The α-Sens® is a 'me-too' method of the ARE-Nrf2 mediated reporter gene assay that is included in OECD TG442D, such as KeratinoSens™ and LuSens, to be used for discrimination between skin sensitisers and non-sensitisers. The α-Sens cell line was originally developed by CERI as a multiclonal assay system to detect ARE-Nrf2 mediated transactivation caused by skin sensitisers (Maeda *et al*., 2020). The host cell of the α-Sens is PHK 16-0b (JCRB cell bank, Osaka, Japan) cell line, the immortalised normal human keratinocyte cell line. The cell line can be cultured in the serum free medium such as KGM™ Gold Keratinocyte Growth Medium BulletKit™ (Lonza) or Epi-Life medium (Thermo Fisher Scientific K. K., Massachusetts, USA) supplemented with HKGS (Thermo Fisher Scientific K. K., Massachusetts, USA) at 37°C under a 5% $CO_2$ and humidified atmosphere. The ARE used for the reporter gene is identical to the other ARE-Nrf2 mediated reporter gene assays included in OECD TG442D corresponding to ARE regulatory sequence from the AKR1C2 gene.

Then the host cell was stably transfected with redesigned reporter gene keeping basic construction, and the optimal clone was established and trademark registered as "α-Sens®".

The differences between the original α-Sens cell system described in Maeda *et al*. (2020) and the current α-Sens® used for the validation study are as follows.

**Reporter gene construct**: Both of α-Sens and α-Sens® cell system employed the rat $\alpha_{2U}$-globulin promoter (AUG)-driven antioxidant response element (ARE)-inducible *Firefly* luciferase reporter gene. However, the α-Sens® employed the quadruplicated same ARE elements to yield high responsiveness and replaced the Luc2 gene with Luc+ gene to avoid the patent restriction.

**Clonality of cell line**: the original α-Sens cell system was developed as multi-clone system. The α-Sens® was redesigned with its reporter construct as noted above, then the optimal cell clone was selected based on the responsiveness to the four negative chemicals (lactic acid, isopropanol, glycerol and salicylic acid and three positive chemicals including weak sensitiser (eugenol, cinnamic aldehyde and dinitrochlorobenzene).

**DMSO concentration**: DMSO is generally used in cell-based assays as a vehicle to dissolve the test chemicals. In the optimisation process of the α-Sens®, we found evidence that 1% DMSO in the final assay /chemical mixture may affect the cell viability and final outcomes of the assay system. Therefore, the final DMSO concentration was changed to 0.1% to avoid the effect of DMSO on the assay system.

**Other assay conditions**: Maximum test concentration of the original α-Sens was 2000 μM (molarity based concentration). The α-Sens® employed gravimetric approach to make applicable to mixtures and multi-constituent substances of unknown composition and set the maximum concentration as 500 μg/mL. Cell density and assay substrate to be added in the

assay well was reduced to half density and volume by confirming the 'no effect' observed in the final outcomes. Although the preincubation and chemical exposure times of the original α-Sens cell system was fixed as 24 h, the α-Sens® allowed some flexibility (24 ± 2 h) after confirming that there was no effect on the outcomes with this flexibility. In addition, the cut-off% for cell viability was set as 50%, which is the lowest cell viability that does not affect the assay outcomes of α-Sens®, based on the results of the optimisation process.

**Table 4-1 Differences between theα-Sens and the α-Sens®**

| | α-Sens (Maeda *et al.*, 2020) | α-Sens® (used for validation study) | Note |
|---|---|---|---|
| Reporter gene construct | ARE-AUG-Luc2 | **4xARE**-AUG-**Luc+** | - |
| Clonality of cell | Multi-clone | **Single-clone** | - |
| Cell density in assay condition | 20,000 cells /well | **10,000** cells /well | Optimised |
| Maximum test concentration | 2000 μM | **500 μg/mL** | KeratinoSens™: 2000 μM or 400 μg/mL LuSens: 2000 μM or 2000 μg/mL |
| Final concentration of vehicle | 1% | **0.1%** | Optimised |
| Preincubation time | 24 h | 24 ± **2 h** | Optimised |
| Duration of chemical exposure | 24 h | 24 ± **2 h** | Optimised |
| Substrate volume to be added | 50 μL/well | **25** μL/well | Optimised |
| Cut-off% for cell viability | Not provided | **50%** | - |
| No. of plate necessary for one assay | 1 | 1 | Multiple plates are required in other existing assays. |

After the optimisation of the assay conditions, the chemicals listed in the DASS database (OECD, 2023a) were tested in the α-Sens®. Then, the optimal cut-off value of nAA, normalised ARE activity by cell viability (see Section 4.3 and Appendix-04 α-Sens® SOP, also available in the Tracking System for Alternative methods towards Regulatory acceptance (TSAR) - https://tsar.jrc.ec.europa.eu/test-method/tm2022-03), to discriminate sensitisers from non-sensitisers was determined as 3.2, giving the highest accuracy for 112 chemicals for which human data are available in the DASS database.

The α-Sens® has some advantages over the existing alternative test methods for skin sensitisation as follows.

- The serum free operation throughout the assay, including cell culture propagation and maintenance, is a big advantage over the existing methods that use FBS.
- A cell viability can be evaluated using the same cells evaluating ARE activity by using the dual-luciferase test system.
- Rat $\alpha_{2u}$-globurin promoter employed in α-Sens® can induce high transcriptional induction of reporter gene product with minimal background induction to achieve the extremely high S/N ratio (Takeyoshi *et al*, 2003).

- The α-Sens® is confirmed to have a long-time stability of the responsiveness to the chemical of up to 70 passages for 9 months.
- The α-Sens® accomplished a streamlining methodology using the dual luciferase assay system to detect both ARE-mediated transcriptional activity (by ARE-inducible *Firefly* luciferase gene) and cell viability (TK promoter-driven *Renilla* luciferase gene) with the same exposure sample.
- Based on the comparison using 20 reference chemicals listed in "Performance Standards for Assessment of Proposed Similar or Modified *in vitro* Skin Sensitisation ARE-Nrf2 Luciferase Test Methods" (OECD, 2015), the performance of the α-Sens® is equivalent or better than the other assays in TG 442D.

Currently OECD GL497 provides a combined approach of KE based *in vitro* tests to obtain a definitive conclusion, replacing the LLNA. The α-Sens® contributes to the expansion of the test method options for GL497, extending applicability to hydrophobic chemicals, with improved performance.

## 4.2 Scientific basis: biological and mechanistic relevance

The essential test method components for the OECD Test Guideline on an *in vitro* skin sensitisation: ARE-Nrf2 luciferase test method were specified in Doc#213. All components should be evaluated to demonstrate the similarity of the α-Sens® to existing ARE-Nrf2 mediated reporter gene assays.

The status of the α-Sens® for all components are shown in the Table 4-2 below and it was confirmed its similarity to existing ARE-Nrf2 mediated reporter gene assay based on scientific basis including biological and mechanistic relevance.

This confirms the equivalence of the α-Sens® with KeratinoSens™ as a 'me-too' test.

**Table 4-2 Essential test method components of α-Sens®**

| ESSENTIAL TEST METHOD COMPONENTS (OECD, 2015) | α-Sens® |
|---|---|
| Transgenic human cell lines relevant to the skin sensitisation process (e.g. keratinocytes, dendritic cells and other relevant cells) and that have a stable insertion of luciferase reporter gene should be used. | The α-Sens® was established with PHK 16-0b cell line (immortalised human keratinocyte by HPV16 virus genome) and the cell were stably transfected with *firefly* reporter gene to detect ARE-mediated transcriptional activity. |
| The reporter gene must be under the control of the ARE-element of the human AKR1C2 gene or alternative ARE elements. | *Firefly* reporter gene to detect ARE-mediated transcriptional activity was under control of ARE element of AKR1C2 gene. |
| If cell lines containing alternative ARE elements are used, the specific dependence of the chosen ARE element on Nrf2 and the Nrf2 dependence of the reporter gene activity should be demonstrated (e.g. by transiently or permanently inactivating Nrf2 activity in the reporter cell line and by verifying that sensitiser-induced luciferase activity is reduced due to reduced or lost expression of Nrf2). | Not applicable.<br>The α-Sens® employed the ARE element of AKR1C2 gene used in the existing ARE mediated reporter gene assay, KeratinoSens™. |
| It should be demonstrated that the cell line has a stable insertion of the luciferase reporter gene. | The luciferase reporter gene components used in the α-Sens® were stably transfected by using the lentiviral vector system. |
| Clones should be selected on the basis of performance e.g. based on the best signal to noise ratio of the light output of luciferase induction, and on the highest dynamic range of luciferase induction when cells are treated with weak sensitisers. | The α-Sens® was established as the optimal cell clone showing the highest dynamic range of luciferase induction to the seven pilot chemicals containing weak sensitiser; the four negative chemicals (lactic acid, isopropanol, glycerol and salicylic acid) and three positive chemicals (eugenol, cinnamic aldehyde and dinitrochlorobenzene). |
| The optimal cell seeding number as well as media composition (e.g. DMSO, FCS) should be defined to ensure obtaining significant luciferase induction by skin sensitisers, including weak sensitisers. | Assay conditions containing DMSO concentration, cell density, durations of preincubation and chemical treatment and cutoff points to distinguish the sensitiser/non-sensitiser were optimised based on the assay results using the pilot chemicals containing weak sensitisers. |
| The measurement of luciferase reporter gene activity and the appropriate luciferase substrate used should have sufficient light output to ensure sufficient sensitivity and low variability. | Rat $\alpha_{2u}$-globurin promoter employed in the α-Sens® can induce high transcriptional induction of reporter gene product with minimal background induction to achieve the extremely high S/N ratio (Takeyoshi *et al*., 2003), confirmed sufficient light output to ensure sufficient sensitivity and low variability. |
| Finally, cell cytotoxicity should be assessed. | The α-Sens® was stably transfected with the ARE-inducible *Firefly* luciferase gene driven by rat $\alpha_{2u}$-globurin promoter and Thymidine kinase (TK) promoter-driven *Renilla* luciferase gene for cell viability check. Consequently, α-Sens® enable the measurement of ARE mediated activity and cell viability, simultaneously. |

## 4.3 Protocol

The details of the assay protocol are shown in the final version of the Protocol for α-Sens® (Appendix-04). In response to comments from the Scientific Peer Review, simplification of the workflow in the protocol and addition of a list of proficiency chemicals have been included in Appendix-08 (a simplified workflow of Figure 4 in the protocol) and Appendix-09 (Possible proficiency chemicals).

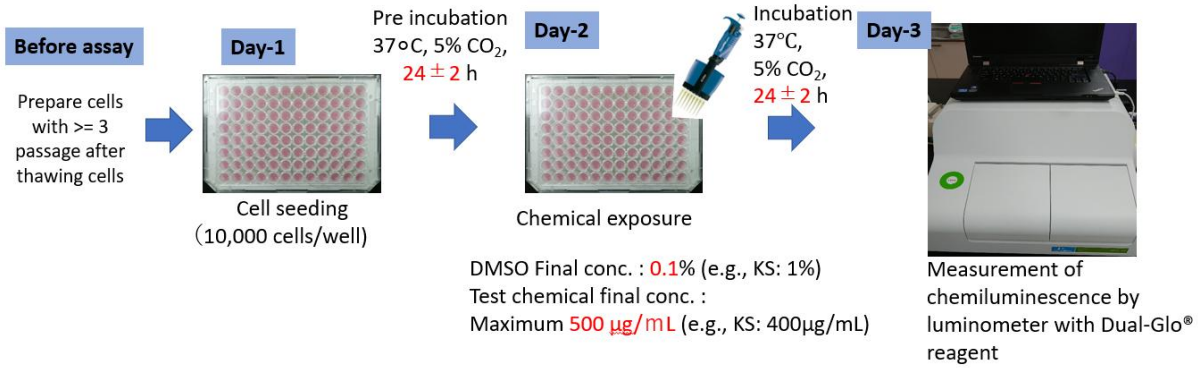



**Fig. 4-1 Protocol outline of the α-Sens®**

Briefly, the α-Sens® cells were seeded on to a 96-well microplate at a density of 10,000 cells/well in 100 µL medium and pre-cultured for 24±2 h. After the pre-cultivation, chemicals were serially diluted from 500 mg/mL in DMSO, acetone or assay medium, then the chemicals were 500 times diluted in assay medium. An aliquot of 100 µL of the diluted test chemical was added to the well and allowed to react for 24±2 h (maximum final concentration: 500 µg/mL with 0.1% vehicle). The vehicle control wells were also prepared with DMSO, acetone or assay medium in the same manner. The medium was removed from the wells, and 50 µL of the Dual-Glo® Reagent (Promega KK., Wisconsin, USA), diluted with PBS (1:1), was added to the wells; the *Firefly* luciferase activity was measured with appropriate luminometer. Then, 25 µL of the Dual-Glo® Stop & Glo® Reagent (Promega KK., Wisconsin, USA) was added to the wells, and the *Renilla* luciferase activity was measured with the luminometer after addition of the first Dual-Glo® Reagent.

The Relative Light Unit (RLU) value normalised by the number of active cells (nRLU) was calculated by dividing the RLU value of ARE mediated *Firefly* luciferase activity of each well by the RLU value of the TK driven *Renilla* luciferase activity in a concurrent well. Then, the normalised ARE Activity (nAA), fold induction for the *Firefly* luciferase activity and cell viability based on the *Renilla* luciferase activity were calculated using the following formulas:

$$\mathrm{nAA} = \frac{\text{nRLU of each well}}{\text{mean nRLU of the vehicle control wells}}$$

$$\text{Fold induction} = \frac{\text{RLU of luciferase in the test well}}{\text{mean RLU of luciferase in the vehicle control wells}}$$

$$\text{Cell viability (\%)} = \frac{\text{RLU of rLuc in the test well}}{\text{mean RLU of rLuc in the vehicle control wells}} \times 100$$

Finally, the cases showing nAA ≥ 3.2 with ≥ 50% cell viability are judged as positive in the α-Sens®.

## 4.4 Cell availability and Licensing issues

The α-Sens® cell has been deposited to the Japanese Collection of Research Bioresources Cell Bank (JCRB, https://cellbank.nibiohn.go.jp/english#). Once, α-Sens® is adopted and included in the OECD TG442D, the α-Sens® cell will be globally available through JCRB.

The developer of the α-Sens® cells promised to provide cells at reasonable cost based on FRAND declaration with MTA. The signed FRAND Declaration has already been submitted to the OECD.

## 4.5 Conclusion on Test Definition

The α-Sens® is a 'me-too' method of KeratinoSens™ as the ARE-Nrf2 mediated luciferase assay in OECD TG442D that addresses a biological mechanism – namely, keratinocyte activation – that is considered to be one of the KEs in the skin sensitisation AOP. The use of the α-Sens® will complement the information generated by the MIE or KE by *in silico, in chemico or in vitro* approaches designed to address the sequence of events leading to the induction of skin sensitisation. This fact supports the use of α-Sens® as a mechanistically relevant element of a testing strategy for skin sensitisation.

The VMT concluded that the α-Sens® Test Method Definition is satisfied.

# 5. Transferability

## 5.1 Training

Before starting the validation study of the α-Sens®, the basic information necessary for conducting the α-Sens® was shared from the lead laboratory to all three participating laboratories by providing an overview of the test method with the SOP for training, and an instruction video file for basic cell handling and experimental procedures.

All technicians participating in the validation study were required to pass three step training trials (Step-1, Step-2-1 and Step-2-2) to ensure that the basic techniques were clearly understood. Equipment setup was also checked during the training phase. The overall goal of the training phase was to ensure that the naïve laboratories were able to obtain the same conclusions as those generated by the lead laboratory.

### *5.1.1 Step-1*

The Step-1 trial was conducted by testing four chemicals with the specified concentrations dissolved in DMSO using "Plate Layout-B" (Table 5-1, Fig. 5-1) according to the SOP for training. The completed spreadsheet(s) (Excel files) were then sent by each participating laboratory to the lead laboratory for review. After the successful completion of the Step-1 trial, the technician(s) could proceed to the Step-2-1.

**Table 5-1 Chemicals used in the Step-1 of the training trials and the concentrations to be prepared for DMSO stock solutions**

| Known category | Test Chemicals | Target Concentration in DMSO |
|---|---|---|
| *Skin sensitisers* | *p*-Benzoquinone (BQ) | 0.5 mg/mL |
| | Butyl Glycidyl Ether (BGE) | 30 mg/mL |
| | α-Hexyl cinnamic aldehyde (HCA) | 50 mg/mL |
| *Non-sensitisers* | Propylene glycol (PG) | 500 mg/mL |

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | BQ 0.50 µg/mL | | | BGE 30.0 µg/mL | | | HCA 50.0 µg/mL | | | PG 500 µg/mL | | |
| B | BQ 0.25 µg/mL | | | BGE 15.0 µg/mL | | | HCA 25.0 µg/mL | | | PG 250 µg/mL | | |
| C | BQ 0.13 µg/mL | | | BGE 7.5 µg/mL | | | HCA 12.5 µg/mL | | | PG 125 µg/mL | | |
| D | BQ 0.06 µg/mL | | | BGE 3.8 µg/mL | | | HCA 6.3 µg/mL | | | PG 62.5 µg/mL | | |
| E | BQ 0.03 µg/mL | | | BGE 1.9 µg/mL | | | HCA 3.2 µg/mL | | | PG 31.3 µg/mL | | |
| F | BQ 0.02 µg/mL | | | BGE 0.9 µg/mL | | | HCA 1.6 µg/mL | | | PG 15.6 µg/mL | | |
| G | BQ 0.01 µg/mL | | | BGE 0.5 µg/mL | | | HCA 0.8 µg/mL | | | PG 7.8 µg/mL | | |
| H | VC (0.1% DMSO) | | | | | Blank | | | | PC (0.5 µg/mL) | | |

**Fig 5-1 Test concentrations and plate layout used for the Step-1**

Yellow wells: Cells and Medium only. Blank: No cells and Medium only.

## *5.1.2 Step-2-1*

In the Step-2-1 trial, one chemical (*p*-benzoquinone) that cause cytotoxic effects was tested using “Plate Layout-A” to ensure the techniques for test concentration setting (Fig. 5-2).

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | BQ 50 µg/mL | BQ 25 µg/mL | BQ 12.5 µg/mL | BQ 6.3 µg/mL | BQ 3.1 µg/mL | BQ 1.6 µg/mL | BQ 0.8 µg/mL | BQ 0.4 µg/mL | BQ 0.2 µg/mL | BQ 0.1 µg/mL | | Blank |
| B | | | | | | | | | | | | VC 0.1% DMSO |
| C | | | | | | | | | | | | |
| D | Medium only wells, for interference check | | | | | | | | | | | |
| E | | | | | | | | | | | | PC 0.5 µg/mL |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | Blank |

**Fig. 5-2 Test concentrations and plate layout used for the Step-2-1**

Yellow wells: Cells and Medium only. Blank: No cells and Medium only.

## *5.1.3 Step-2-2*

The test conditions (maximum test concentration and common ratio) for BQ was decided based on the results of Step-2-1. This information was utilised to conduct the Step-2-2 trial using “Plate Layout-B” to ensure the entire assay steps in each laboratory were correctly conducted (Fig. 5-3). After completing both Step-2-1 and Step-2-2, both results in the spreadsheets were sent to the lead laboratory for review.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | BQ Maximum | | | | | | | | | | | |
| B | | | | | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | BQ Minimum | | | | | | | | | | | |
| H | VC (0.1% DMSO) | | | | | Blank | | | | PC (0.5 µg/mL) | | |

**Fig. 5-3 The plate layout used for the Step-2-2**

Yellow wells: Cells and Medium only, Blank: Medium only wells.

### *5.1.4 Results and outputs of the training trials*

In Step-1 (test with the specified concentration for 4 chemicals), 2 out of 3 participating laboratories could successfully distinguish 3 skin-sensitisers and 1 non-sensitiser. However, another laboratory had some trouble to yield the correct outcome for HCA, which was the most hydrophobic chemical tested for Step-1 (*i.e., logP*=4.3). The lead laboratory troubleshooted the problem and provided some instructions, such as how to stir the test chemical solution just before the exposure to cells in the protocol to avoid the trouble that may be caused by handling hydrophobic chemicals. The laboratory in question conducted several assays for HCA to confirm whether the appropriate outcome could be obtained based on the provided instructions, and it was confirmed that the expected outcomes for HCA were reproducibly and successfully generated in the end.

In Step-2-1 and Step-2-2, all participating laboratories could properly set the test concentrations and provide the correct outcome (positive).

Following the generation of successful results on completion of the training trials, the VMT for the α-Sens® agreed to finalise the training phase and start the official validation study using the modified protocol.

## 5.2 Transferability in Phase-I

### *5.2.1 Results of Transferability*

Transferability was evaluated using the 9 test chemicals that were coded in Phase-1. Since 8 of 9 test chemicals were tested in three experiments, the qualitative conclusions (positive or negative) obtained in two or more experiments were used to evaluate transferability.

The qualitative conclusions of transferability are shown in Table 5-2. Asterisks in Table 5-2 indicate results where the effect of chemiluminescence contamination was observed, and details are given in 5.2.2 (1). When comparing the conclusions from each participating laboratory with those of the lead laboratory, all participating laboratories were in 100% agreement, including the final conclusions for HCA.

It was concluded that the between-laboratory transferability (BLT) was successful, as it met the criteria, “All qualitative conclusions of 9 test chemicals from all participating laboratories should meet its conclusions from the lead laboratory”, set by the VMT.

## Table 5-2 Summary of Transferability

| | No. | Chemical | Known category in Doc#213 | Conclusion | | | Summarised Conclusion *2, *3 | Transferability (Comparison with Lead lab's results) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Exp.-1 *2 | Exp.-2 *2 | Exp.-3 *2 | | Agreement with Lead lab's results | Rate |
| FDSC | 1 | Glycerol | N | N | N | N | N | Yes | 100% ( 9/9 ) |
| | 2 | Isopropanol | N | N | N | N | N | Yes | |
| | 3 | Salicylic acid | N | N | N | N | N | Yes | |
| | 4 | Ethylene glycol dimethacrylate(=EGDMA) | P | P | P | P | P | Yes | |
| | 5 | Citral | P | P | P | P | P | Yes | |
| | 6 | Isoeugenol | P | P | P | P | P | Yes | |
| | 7 | *p*-Phenylenediamine | P | P | P | P | P | Yes | |
| | 8 | 2,4-Dinitrochlorobenzene (=DNCB) | P | P | P | P | P | Yes | |
| | 9 | α-Hexylcinnamaldehyde (=HCA) | P | P | | | P | Yes | |
| Sumitomo Chemical | 1 | Glycerol | N | *1 | N | N | N | Yes | 100% ( 9/9 ) |
| | 2 | Isopropanol | N | N | N | N | N | Yes | |
| | 3 | Salicylic acid | N | N | N | N | N | Yes | |
| | 4 | Ethylene glycol dimethacrylate(=EGDMA) | P | P | P | P | P | Yes | |
| | 5 | Citral | P | P | P | P | P | Yes | |
| | 6 | Isoeugenol | P | P | P | P | P | Yes | |
| | 7 | *p*-Phenylenediamine | P | P | P | P | P | Yes | |
| | 8 | 2,4-Dinitrochlorobenzene (=DNCB) | P | P | P | P | P | Yes | |
| | 9 | α-Hexylcinnamaldehyde (=HCA) | P | P | | | P | Yes | |
| JRF | 1 | Glycerol | N | N | N | N | N | Yes | 100% ( 9/9 ) |
| | 2 | Isopropanol | N | N | N | N | N | Yes | |
| | 3 | Salicylic acid | N | N | N | N | N | Yes | |
| | 4 | Ethylene glycol dimethacrylate(=EGDMA) | P | P | P | P | P | Yes | |
| | 5 | Citral | P | P | P | P | P | Yes | |
| | 6 | Isoeugenol | P | P | P | P | P | Yes | |
| | 7 | *p*-Phenylenediamine | P | P | P | P | P | Yes | |
| | 8 | 2,4-Dinitrochlorobenzene (=DNCB) | P | P | P | P | P | Yes | |
| | 9 | α-Hexylcinnamaldehyde (=HCA) | P | P | | | P | Yes | |
| Lead Lab | 1 | Glycerol | N | N | N | N | N | | |
| | 2 | Isopropanol | N | N | N | N | N | | |
| | 3 | Salicylic acid | N | N | N | N | N | | |
| | 4 | Ethylene glycol dimethacrylate(=EGDMA) | P | P | P | P | P | | |
| | 5 | Citral | P | P | P | P | P | | |
| | 6 | Isoeugenol | P | P | P | P | P | | |
| | 7 | *p*-Phenylenediamine | P | P | P | P | P | | |
| | 8 | 2,4-Dinitrochlorobenzene (=DNCB) | P | P | P | P | P | | |
| | 9 | α-Hexylcinnamaldehyde (=HCA) | P | P | | | P | | |

Food and Drug Safety Center (FDSC), Sumitomo Chemical Company, Limited (Sumitomo Chemical), Jai Research Foundation (JRF)

*1 Results where the effect of chemiluminescence contamination was observed.

*2 N: Negative, P: Positive

*3 Summarised conclusion: Positive (P) if there were more than 2 positive conclusions among 3 experiments. Negative (N) if there were more than 2 negative conclusions among 3 experiments.

## *5.2.2 Modification of the Protocol*

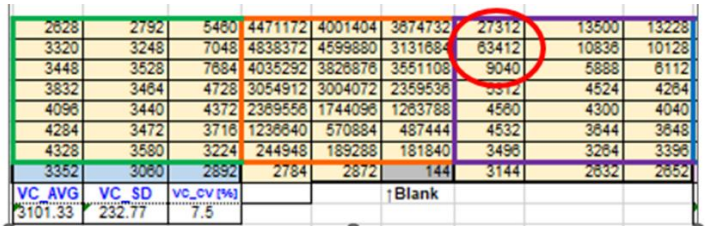
In response to the results of the transferability in Phase-I, the following three points were identified as events that could affect the test results. The possible solutions for these points were investigated, and their solutions were reflected in the protocol and the modified protocol was used for Phase-II study.

- Cross-contamination of luminescence by HiLIC (High Luminesce Inducible Chemicals)
- Potential contamination of volatile chemicals in adjacent wells
- Potential variability due to presence of aggregated cells

**(1) Cross-contamination of luminescence by HiLIC (High Luminesce Inducible Chemicals)**

It was found that one outcome of Glycerol (a non- sensitiser) using Plate Layout-B appeared to be affected (*i.e.*, chemiluminescence value was increased than other replicates) by the presence of a test chemical that emitted strong light in the adjacent well caused light contamination (Fig. 5-4 A). When evaluated using the results of all replicates, it was judged to be positive. However, when evaluated without using the results of the contaminated replicates, it was judged to be negative (Fig. 5-4 B). This fact suggests that contamination by chemiluminescence can have a major effect on the results of assay outcome, especially for negative chemicals, increasing the risk of false positives.

**A**



| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 2628 | 2792 | 5460 | 4471172 | 4001404 | 3874732 | 27312 | 13500 | 13228 |
| 3320 | 3248 | 7048 | 4838372 | 4599880 | 3131684 | 63412 | 10836 | 10128 |
| 3448 | 3528 | 7684 | 4035292 | 3826876 | 3551108 | 9040 | 5888 | 6112 |
| 3832 | 3464 | 4728 | 3054912 | 3004072 | 2359536 | 5512 | 4524 | 4264 |
| 4096 | 3440 | 4372 | 2369556 | 1744096 | 1263788 | 4560 | 4300 | 4040 |
| 4284 | 3472 | 3716 | 1236640 | 570884 | 487444 | 4532 | 3644 | 3648 |
| 4328 | 3580 | 3224 | 244948 | 189288 | 181840 | 3496 | 3264 | 3396 |
| 3352 | 3060 | 2892 | 2784 | 2872 | 144 | 3144 | 2632 | 2652 |
| VC_AVG | VC_SD | vc_CV [%] | | | ↑Blank | | | |
| 3101.33 | 232.77 | 7.5 | | | | | | |

**B**

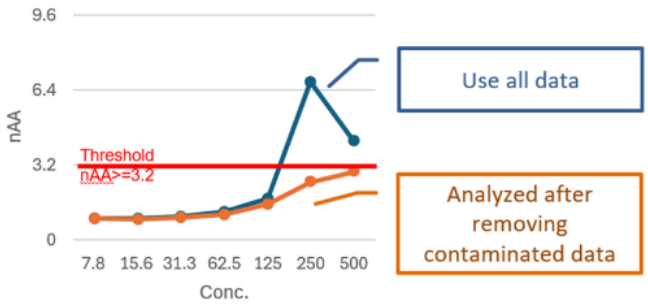



**Fig. 5-4 The effect of chemiluminescence contamination on test results.**

A: Contamination of chemiluminescence observed in test plates. The values indicate the luminescence intensity.

B: Analysis of the effects of chemiluminescence contamination on results. Blue: Concentration-response curve using all results. Orange: Concentration-response curve analysed after removing luminescence contaminated data.

As a part of solution, a method for determining whether a test chemical is HiLIC was established. If the nAA of the adjacent well, which is not exposed to any test chemical in the first test conducted using Plate layout A, is equal to 3.2 or higher, it is considered HiLIC. If a test chemical is identified as HiLIC, no other test chemical is placed next to it. The selection of the maximum concentration and common ratio for the further tests for HiLIC were added to the protocol flowchart (See Appendix-04).

However, it is possible that a test chemical that potentially causes luminescence crosstalk cannot be identified in the initial test by Plate Layout-A. Furthermore, luminescence crosstalk may be sometimes irregular. These cases are called as “suspected HiLIC”. In such cases, there is a possibility that other test chemical(s) are placed in the adjacent well of the suspected HiLIC in Plate Layout-B. Therefore, a step was added to the protocol to avoid a false positive judgement due to contamination of luminescence by (suspected) HiLIC in Plate layout B (See Appendix-05).

There was also a possibility that the luminescence value of the vehicle control (VC) wells could be affected by the (suspected) HiLIC if the (suspected) HiLIC was placed just above the VC wells. Accordingly, the criteria for checking whether the results of the VC wells are affected by cross-contamination of chemiluminescence were newly configured using Phase-I data. This criterion was established by calculating the value, i.e. the average value of the VC was divided by the average value of the 5-well containing only cells without VC or test chemical treatment in Plate Layout-B, and this value should be within the range of 0.82-1.40 (See Appendix-05). This criterion was added in the protocol to be used as one of the data acceptance criteria for Plate Layout-B.

## (2) Potential contamination of volatile chemicals in adjacent wells.

In the case testing test chemicals that have a strong odor like citral and are highly volatile in Plate Layout-A, an increase in luminescence values was observed in wells containing only cells placed next to the high concentration range of volatile chemicals such as citral that did not have strong luminescence, despite the use of plate seal. Since contamination of a volatile chemical via the gas phase could potentially happen, a verification test was conducted to confirm this.

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Conc. | 500 | 250 | 125 | 62.5 | 31.25 | 15.625 | 7.8125 | 3.90625 | 1.95313 | 0.97656 | [ µg/mL ] | | |
| Sample ID | ASD237 | | | 215 | 209 | 246 | 994 | 117136 | 134164 | 7323 | 895 | 533 | 450 | 402 | 191 | ←Blank-2 | |
| Chemical Name | | | Common Ratio | 192 | 224 | 216 | 819 | 76584 | 118800 | 6819 | 881 | 529 | 492 | 408 | 462 | →Vehicle Control (VC) | VC_AVG |
| Max. Conc | 500 | µg/mL | 2 | 166 | 189 | 224 | 423 | 47836 | 76157 | 3809 | 887 | 482 | 494 | 408 | 443 | | 444 |
| | | | | 3342 | 4309 | 797 | 473 | 570 | 650 | 492 | 437 | 379 | 412 | 381 | 427 | | |
| | | | | 359 | 422 | 430 | 505 | 566 | 662 | 581 | 456 | 410 | 417 | 350 | 1102 | →Positive Control (PC) | PC_AVG |
| Sample ID | ASD238 | | | 171 | 182 | 1951 | 14552 | 69280 | 81131 | 36881 | 9884 | 2540 | 1026 | 380 | 1211 | | 1209 |
| Chemical Name | | | Common Ratio | 183 | 172 | 2113 | 15080 | 70034 | 93620 | 41497 | 13028 | 2803 | 1186 | 413 | 1314 | | |
| Max. Conc | 250 | µg/mL | 2 | 191 | 203 | 4012 | 23060 | 83575 | 100641 | 45190 | 11464 | 2295 | 841 | 383 | 167 | ←Blank-1 | Blank AVG |
| | | | Conc. | 250 | 125 | 62.5 | 31.25 | 15.625 | 7.8125 | 3.90625 | 1.953125 | 0.97656 | 0.48828 | [ µg/mL ] | | | 179 |

**Fig. 5-5 Chemical contamination via the gas phase.**

Red box: Increased chemiluminescence values were observed in adjacent wells that had not been exposed chemical to wells where chemiluminescence showed low value in the high concentration range,

ASD237 is citral and the values of luminescence in higher concentration range are decreased due to cytotoxicity. However, an increase of the luminescence values indicated red box are observed.

In the verification test to detect contamination from neighboring wells, serial dilutions of citral in A1-C10 were incubated for 24 h in the same manner as tested in Fig. 5-5. The wells in the green area in Fig. 5-6 contained only 200 µL of water. After 24 h, the water in the wells in the green area was subjected to the HPLC analysis.

The HPLC analysis revealed that citral was detected in the adjacent wells, where the chemical was not added, via the gas phase despite the use of plate seal (Fig. 5-6).

Citral Conc.(μg/mL)

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 500 | 250 | 125 | 62.5 | 31.25 | 15.63 | 7.81 | 3.91 | 1.95 | 0.98 | 0.01 | N.D. |
| B | 500 | 250 | 125 | 62.5 | 31.25 | 15.63 | 7.81 | 3.91 | 1.95 | 0.98 | 0.02 | N.D. |
| C | 500 | 250 | 125 | 62.5 | 31.25 | 15.63 | 7.81 | 3.91 | 1.95 | 0.98 | 0.01 | N.D. |
| D | 4.66 | 2.72 | 1.67 | 0.97 | 0.52 | 0.33 | 0.17 | 0.08 | 0.03 | 0.02 | 0.01 | N.D. |
| E | 0.25 | 0.31 | 0.17 | 0.09 | 0.05 | 0.03 | 0.02 | 0.01 | N.D. | N.D. | N.D. | N.D. |
| F | | | | | | | | | | | N.D. | N.D. |
| G | | | | | | | | | | | N.D. | N.D. |
| H | | | | | | | | | | | | |

**Fig. 5-6 HPLC analysis results for adjacent wells under Citral exposure conditions.**

Before incubation: White wells: Citral solution. Green wells: 200 μL of water only.
After incubation: The citral concentration in green wells were measured by HPLC.
N.D.: not detected.

When investigating the results from each laboratory, the citral contamination was not observed in JRF and it was found that a different type of plate seal (Polyester made) was used in this laboratory (Table 5-3). This suggested that the material of the plate seal may be important to prevent contamination via the gas phase.

**Table 5-3 Details of the plate seals used in each lab.**

| | FDSC | Sumitomo Chemical | JRF | Lead lab. |
|---|---|---|---|---|
| Maker | STEM | STEM | Thermo Scientific | STEM |
| Catalogue # | P96P01S | P96P01S | 236366 | P96P01S |
| Material | PP | PP | Polyester | PP |

PP: Polypropylene

Additional verification test using the 2 different types of plate seals was conducted, and the polyester-made plate seal could reduce the citral contamination via the gas phase (Fig. 5-7). Based on these results, the protocol was changed to specify that polyester plate seals should be used for the assay.

| A | | | | |
|---|---|---|---|---|
| Citral Conc.(μg/mL) | | | | |
| | 1 | 2 | 3 | 4 |
| A | 500 | 250 | 125 | 62.5 |
| B | 500 | 250 | 125 | 62.5 |
| C | 500 | 250 | 125 | 62.5 |
| D | **16.9** | **17.7** | **16.8** | **14.4** |

| B | | | | |
|---|---|---|---|---|
| Citral Conc.(μg/mL) | | | | |
| | 1 | 2 | 3 | 4 |
| A | 500 | 250 | 125 | 62.5 |
| B | 500 | 250 | 125 | 62.5 |
| C | 500 | 250 | 125 | 62.5 |
| D | **4.3** | **2.1** | **1.8** | **1.1** |

| C | | | | |
|---|---|---|---|---|
| Citral Conc.(μg/mL) | | | | |
| | 1 | 2 | 3 | 4 |
| A | 500 | 250 | 125 | 62.5 |
| B | 500 | 250 | 125 | 62.5 |
| C | 500 | 250 | 125 | 62.5 |
| D | **0.4** | **0.7** | **0.5** | **0.8** |

**Fig. 5-7 HPLC analysis results for adjacent wells under citral exposure conditions.**

A: without plate seal. B: using STEM plate seal (PP). C: using Thermo Scientific plate seal (polyester).
Before incubation: White column: Citral solution. Green column: 200 μL of water only.
After incubation: The citral concentration in green wells were measured by HPLC.

**(3) Potential variability due to presence of cell clumps**

During Phase-I study, there were some cases where the coefficient of variation (CV, %) of RLU (Relative Luminescence Units) in the VC wells for both *Firefly* and/or *Renilla* activities exceeded the CV (%) value of data acceptance criteria (should be 20% or less) because of a higher RLU value in one of the three VC wells was observed. It was considered that there was a potential clumping/aggregating of cells in a well, since there was a worksheet record of the observation of cell clumps in a participating laboratory, indicating the potential non-uniform cell seeding. As a countermeasure, the option to use a cell strainer when collecting cells after trypsin treatment to remove cell clumps was added to the protocol.

The usefulness of the cell strainer treatment was demonstrated by Sumitomo Chemical by reducing the failures in the criteria for CV value (*Renilla* Luc of VC wells) (12 failures in Phase-1 → 0 in Phase-2). The cell strainer treatment was shown to be effective in reducing variability due to the presence of cell clumps and ensured uniform seeding of cells.

## 5.3 Conclusion on Transferability

BLT of the three participating laboratories was evaluated in Phase-I and revealed that the results of all participating laboratories were consistent with the results of the lead lab for all chemicals.

Based on this result, the VMT concluded that the transferability of the protocol was successfully confirmed for the α-Sens®.

# 6. Within-Laboratory Reproducibility (WLR)

## 6.1 Results of WLR

The WLR needs to be evaluated based on Doc#213 requirements using the 12 reference chemicals indicated in Doc#213 (see section "3.5.2 (2)"). All participating laboratories conducted three qualified experiments for these 12 test chemicals and the reproducibility of conclusions across three experiments was evaluated. The results of WLR are shown in Table 6-1.

The WLR concordance was 100% (12/12) at FDSC and Sumitomo Chemical, and 91.7% (11/12) at JRF, and the WLR criteria (80% or greater) was sufficiently met or exceeded. The lead laboratory had a 100% (12/12) concordance. The average WLR concordance among three participating laboratories was 97.2% (Table 6-1).

**Table 6-1 Results of WLR for 12 test chemicals**

| No. | Chemical Name | Known category in Doc#213 | FDSC | Sumitomo Chemical | JRF | Lead lab |
|---|---|---|---|---|---|---|
| 1 | Citral | P | P:3/3 | P:3/3 | P:3/3 | P:3/3 |
| 2 | 2,4-Dinitrochlorobenzene (=DNCB) | P | P:3/3 | P:3/3 | P:3/3 | P:3/3 |
| 3 | Ethyleneglycoldimethacrylate (=EGDMA) | P | P:3/3 | P:3/3 | P:3/3 | P:3/3 |
| 4 | Isoeugenol | P | P:3/3 | P:3/3 | P:3/3 | P:3/3 |
| 5 | Methyldibromo glutaronitrile | P | P:3/3 | P:3/3 | P:3/3 | P:3/3 |
| 6 | 4-Nitrobenzyl bromide | P | P:3/3 | P:3/3 | P:3/3 | P:3/3 |
| 7 | Phenyl benzoate | P | N:3/3 | N:3/3 | N:2/3 | P:3/3 |
| 8 | *p*-Phenylenediamine | P | P:3/3 | P:3/3 | P:3/3 | P:3/3 |
| 9 | Glycerol | N | N:3/3 | N:3/3 | N:3/3 | N:3/3 |
| 10 | Isopropanol | N | N:3/3 | N:3/3 | N:3/3 | N:3/3 |
| 11 | 4'-Methoxyacetophenone (=Acetanisole) | N | P:3/3 | P:3/3 | P:3/3 | P:3/3 |
| 12 | Salicylic acid | N | N:3/3 | N:3/3 | N:3/3 | N:3/3 |
| Within Laboratory Reproducibility (WLR) | | | 100% (12/12) | 100% (12/12) | 91.7% (11/12) | 100% (12/12) |
| | | | Average 97.2% | | | |

P: Positive, N: Negative

## 6.2 Discussion on Inconsistent Results

**(1) Phenyl benzoate**

JRF had one positive and two negative results for phenyl benzoate and this was the only inconsistent result among 12 test chemicals in all participating laboratories. Due to this inconsistent result, the WLR concordance for JRF was calculated as 91.7% (11/12). The other two participating laboratories gave three negative judgments, and the WLR concordance for two participating laboratories were calculated as 100% (12/12). The lead laboratory gave three

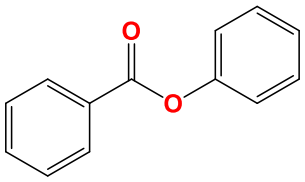
positive judgments, and the WLR concordance for the lead laboratory was calculated as 100% (12/12). It is noted that although the WLR was 100% across the participating laboratories, the skin sensitisation predictions for phenyl benzoate differed across the three participating laboratories and the lead laboratory.

Phenyl benzoate is known as a weak sensitiser. Although positive results were reported in the DPRA used for KE1 detection, and both in the h-CLAT and GARD skin assays for KE3 detection (Fig. 6-1), both KeratinoSens™ and LuSens for KE2 detection reported negative results for phenyl benzoate (Tzutzuy *et al*., 2016). These facts indicate that it would be difficult to discriminate positive or negative for phenyl benzoate using the combined KE1, KE2 and KE3 test methods that use the activation of the ARE-Nrf2 antioxidant pathway as an indicator. The reason for this is discussed below.

Phenyl benzoate is a hydrophobic chemical with a *logP* of 3.59. Of the 8 negatives in independent runs from the three participating laboratories, precipitation in two of the test chemical diluted solutions was observed in 6 runs according to the analysis of worksheets from participating laboratories (Table 6-1). As shown in Fig. 6-2, 6-3, 6-4 and 6-5, in some cases, at the high test concentration range of phenyl benzoate, an increasing trend or an obvious increase (more than 3.2) of nAA were observed in all participating laboratories and the lead laboratory. Therefore, in the case of the observed negative results of phenyl benzoate, it is possible that the test concentrations did not reach the target concentrations.

Focusing on the four positive results generated in JRF and the lead laboratories, the EC3.2[1] and IC50[2] values were close to each other, and these values were also close to the concentrations where precipitation was observed (Table 6-1). Accordingly, when the concentration at which nAA starts to increase, and where the IC50, and the precipitation concentrations are close to each other, it is possible that this gives rise to the ambiguous outcomes observed in α-Sens®, and it is possible that this situation may also occur in other cell-based KE2 assays, such as KeratinoSens™ and LuSens.

**A**




**B**

| | |
|---|---|
| LuSens.Call | Neg |
| GARDskin.call | Pos |
| DPRA.Call | Pos |
| hCLAT.Call | Pos |
| KS.Call | Neg |

**C**

| Type | *logP* | GHS Cat. From DASS | | | Known category in Doc#213 |
|---|---|---|---|---|---|
| | | LLNA | HU.GHS.SUB | Basketter_human_potency_3class | |
| GD213 | 3.59 (Exp) | NA | 1B | 1B | P |

**Fig. 6-1 Details of Phenyl benzoate**

A: Chemical structure of phenyl benzoate,
B: Information on Phenyl benzoate in each non-animal alternative test method for skin sensitisation in the DASS database,
C: Information on phenyl benzoate in *logP*, GHS subcategory and Doc#213.

[1] EC3.2: The concentration calculated by linear regression of the two points of test concentration and nAA across nAA value 3.2.
[2] IC50: The concentration calculated by linear regression of the two points of test concentration and cell viability across 50% cell viability.

**Table 6-1 Summary of Phenyl benzoate in validation studies**

| | FDSC | | | Sumitomo Chemical | | | JRF | | | Lead Lab | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Exp-1 | Exp-2 | Exp-3 | Exp-1 | Exp-2 | Exp-3 | Exp-1 | Exp-2 | Exp-3 | Exp-1 | Exp-2 | Exp-3 |
| **Overall conclusion** | **N** | **N** | **N** | **N** | **N** | **N** | **N** | **N** | **P** | **P** | **P** | **P** |
| Vehicles used | DMSO | DMSO | DMSO | Acetone | DMSO | Acetone | Acetone | DMSO | DMSO | DMSO | DMSO | DMSO |
| Conc. in 2x preparation for exposure (μg/mL) | **500** | **500** | **500** | **500** | **≥694** | **≥250** | **≥136** | **≥125** | **500** | **500** | **500** | **500** |
| Precipitation in 2x preparation | - | - | + | + | + | + | + | + | - | - | - | - |
| Conc. as final conc. | **250** | **250** | **250** | **250** | **≥347** | **≥125** | **≥68** | **≥62.5** | **250** | **250** | **250** | **250** |

P: Positive, N: Negative

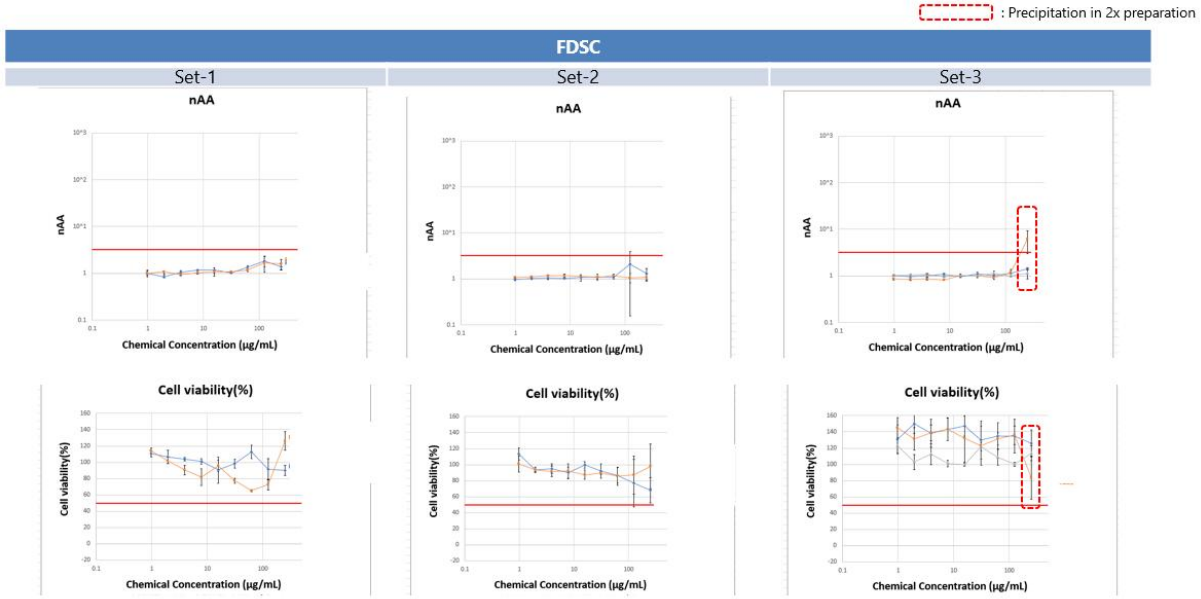



**Fig. 6-2 Concentration-response curves for nAA and cell viability of Phenyl benzoate for FDSC**

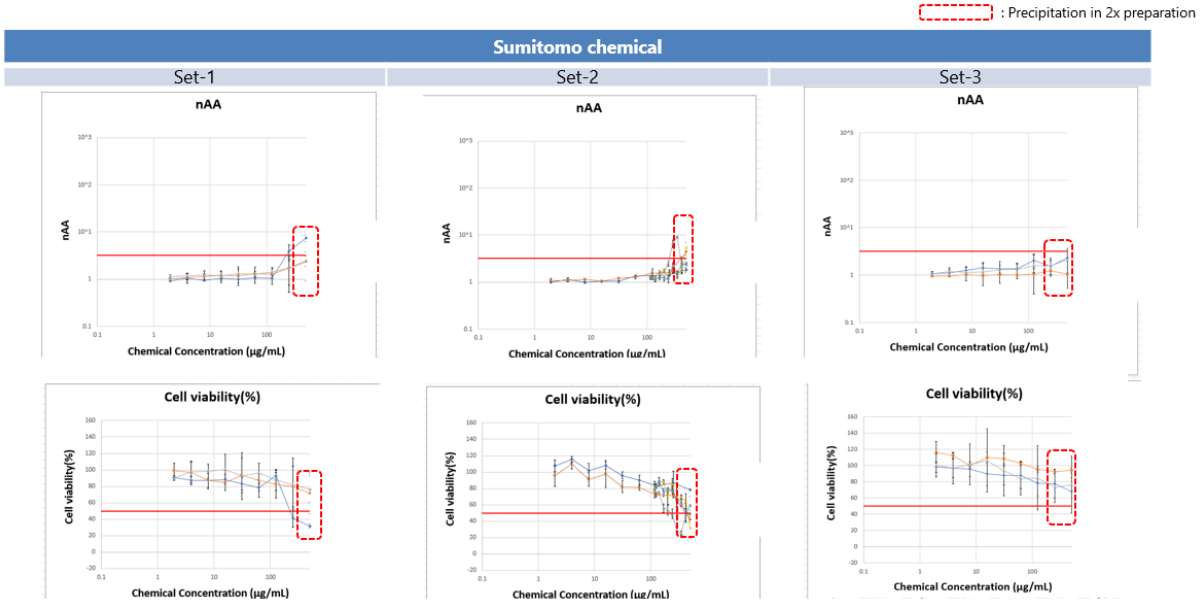



**Fig. 6-3 Concentration-response curves for nAA and cell viability of Phenyl benzoate for Sumitomo Chemical**

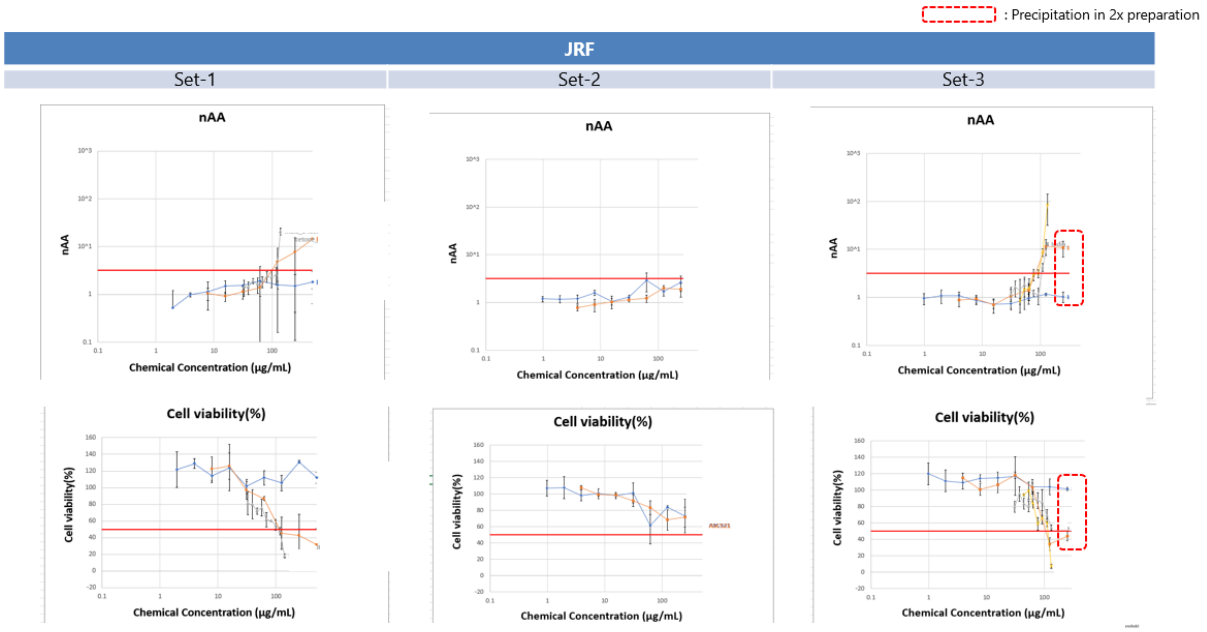



**Fig. 6-4 Concentration-response curves for nAA and cell viability of Phenyl benzoate for JRF**

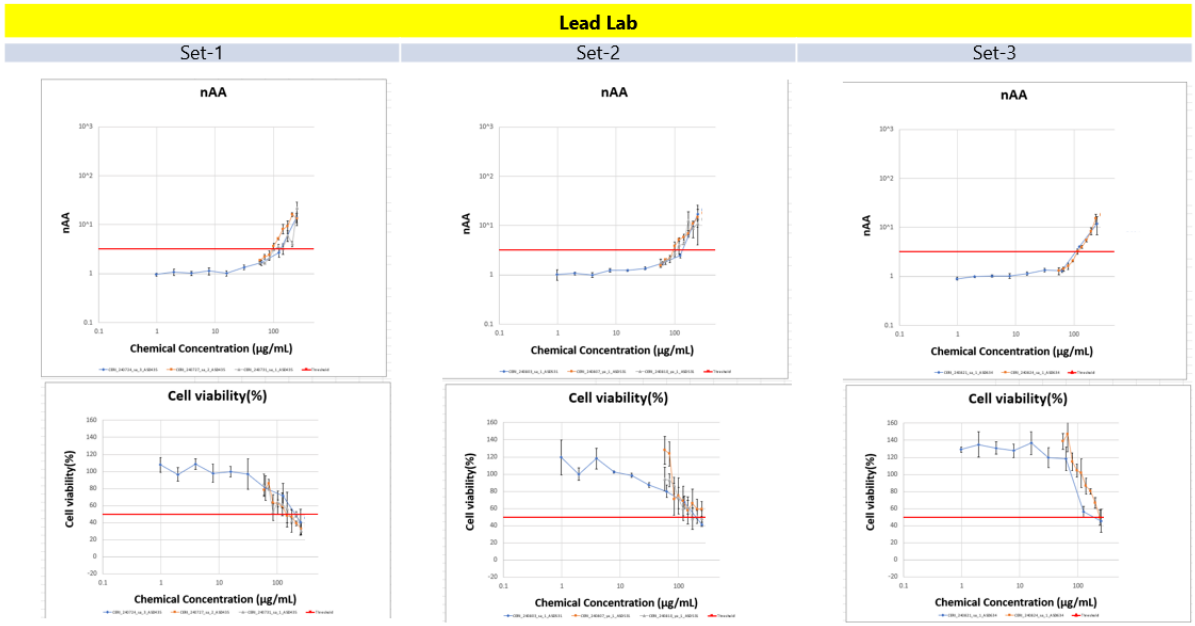



**Fig. 6-5 Concentration-response curves for nAA and cell viability of Phenyl benzoate for Lead lab**

## 6.3 Proposed minor modification of the protocol

During the study, when the stock solution of the test chemical (phenyl benzoate) was prepared for the actual assay, there was a case where the precipitation was observed in the stock solution, even though the maximum concentration and solvent for the stock solution of the test chemical had been previously determined in the solubility test in advance. Therefore, the following contents were added to the section "3.3. Preparation of stock solutions of test chemical(s) and positive control" in the final protocol.

*"Ensure that the test chemical is completely dissolved in the stock solution of the test chemical, even if its solubility has been determined in the solubility test. If precipitation or separation is observed, you may need to start again with an additional solubility test".*

## 6.4 Conclusion on WLR

The average WLR for the three participating laboratories was 97.2% (ranging from 91.7% (11/12) to 100% (12/12)). These WLR results are sufficiently high compared to the criteria set in Doc#213 (see section "3.5.2 (2)").

The VMT concluded that the WLR criteria was passed, and the α-Sens® was highly reproducible within a laboratory.

# 7. Between-Laboratory Reproducibility (BLR)

## 7.1 Results of BLR

The BLR requirement is also described in Doc#213 (see section "3.5.2 (3)"). In addition to the 20 reference chemicals listed in Doc#213, 5 additional chemicals were tested for BLR evaluation (total: 25 test chemicals).

The results of the BLR are summarised in Table 7-1. The BLR for all 25 test chemicals was calculated using the results from the three laboratories. The BLR was calculated to be 96.0% (24/25) and the BLR criteria (80% or greater) was sufficiently exceeded.

**Table 7-1 Summary of BLR**

| No. | Chemical Name | Type | *logP* red: >3.5 $ | GHS Cat. From DASS | | | Known category in #213 | FDSC | Sumitomo Chemical | JRF | BLR | Lead Lab. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | LLNA | HU. GHS. SUB | Basketter human potency 3class | | | | | | |
| 1 | Citral | Reference Chem. | 3.00 Pred | 1B | NA | 1B | P | P | P | P | Yes | P |
| 2 | 2,4-Dinitrochlorobenzene | Reference Chem. | 2.17 Exp | 1A | 1A | 1A | P | P | P | P | Yes | P |
| 3 | Ethylene glycol dimethacrylate | Reference Chem. | 1.51 Pred | 1B | NA | 1B | P | P | P | P | Yes | P |
| 4 | Eugenol | Reference Chem. | 2.27 Exp | 1B | 1B | 1B | P | P | P | P | Yes | P |
| 5 | Geraniol | Additional | 3.56 Exp. | 1B | 1B | 1B | P | P | P | P | Yes | P |
| 6 | α-Hexylcinnamaldehyde | Additional | 4.3 Pred | 1B | NA | NC | P | P | P | P | Yes | P |
| 7 | Isoeugenol | Reference Chem. | 3.04 Exp | 1A | 1B | 1A | P | P | P | P | Yes | P |
| 8 | *D*-Limonene | Additional | 4.52 Exp. | 1B | NA | NC | P | P | P | P | Yes | P |
| 9 | 2-Mercaptobenzothiazole | Reference Chem. | 2.42 Exp | 1A | 1B | 1B | P | P | P | P | Yes | P |
| 10 | 4-Methylaminophenol sulphate | Reference Chem. | 0.63 Pred | 1A | NA | 1B | P | P | P | P | Yes | P |
| 11 | Methyldibromo glutaronitrile | Reference Chem. | 1.89 Pred | 1A | NA | 1A | P | P | P | P | Yes | P |
| 12 | 4-Nitrobenzyl bromide | Reference Chem. | 2.82 Pred | 1A | NA | NA | P | P | P | P | Yes | P |
| 13 | Oxazolone | Reference Chem. | 1.48 Pred | 1A | NA | NA | P | P | P | P | Yes | P |
| 14 | Phenyl benzoate | Reference Chem. | 3.59 Exp | NA | 1B | 1B | P | N | N | N | Yes | P |
| 15 | *p*-Phenylenediamine | Reference Chem. | -0.3 Exp | 1A | 1A | 1A | P | P | P | P | Yes | P |
| 16 | *D*-α-Tocopherol | Additional | 9.4 Pred. | 1B | NA | NC | P *1 | N | N | N | Yes | N |
| 17 | Chlorobenzene | Reference Chem. | 2.87 Exp | NA | NA | NA | N | N | N | N | Yes | N |
| 18 | Glycerol | Reference Chem. | -1.86 Exp | NC | NA | NC | N | N | N | N | Yes | N |
| 19 | *n*-Hexane | Additional | 3.9 Exp. | NC | NC | NC | N | N | N | N | Yes | N |
| 20 | Isopropanol | Reference Chem. | 0.05 Exp | NC | NA | NC | N | N | N | N | Yes | N |
| 21 | Lactic acid | Reference Chem. | -0.72 Exp | NC | NA | NC | N | N | N | N | Yes | N |
| 22 | 4'-Methoxyacetophenone (=Acetanisole) | Reference Chem. | 1.74 Exp | NC | NA | NA | N | P | P | P | Yes | P |
| 23 | Methyl salicylate# | Reference Chem. | 2.55 Exp | | | | N *2 | N | N | P | No | P |
| 24 | Salicylic acid | Reference Chem. | 2.26 Exp | 1B | NA | NC | N | N | N | N | Yes | N |
| 25 | Sulfanilamide | Reference Chem. | -0.62 Exp | NC | 1B | NA | N | N | N | N | Yes | N |
| **Between-Laboratory Reproducibility (BLR)** | | | | | | | | | | | **96.0% (24/25)** | |

$: Exp: Experiment data, Pred: Prediction data. According to the Supporting Document (OECD, 2021) of OECD GL497, prediction was made by OPERA (https://comptox.epa.gov/dashboard. Model version was not provided in Supporting Document (OECD, 2021) of OECD GL497.

#: Methyl salicylate was not included in the DASS database (OECD, 2023a)
*1: See section “9.2 (2)”, *2: See section”7.2 (2)”,
NC: Not Classified, NA: signifies data not available, P: Positive, N: Negative.

## 7.2 Discussion of discordant results

**(1) Phenyl benzoate**

The BLR was evaluated based on the data from three participating laboratories. Phenyl benzoate was negative in the three participating laboratories and therefore these were reproducible results for Phenyl benzoate. However, the result from the lead laboratory was positive. As discussed in section “6.2 (1)”, the concentration at which ARE activity increase, IC50 and the precipitation concentration are very close to each other for phenyl benzoate. As reported that phenyl benzoate was negative in both KeratinoSens™ and LuSens (Tzutzuy *et al*., 2016), it appears that phenyl benzoate is a challenging chemical for test systems that use the activation of the ARE-Nrf2 antioxidant pathway as an indicator.

**(2) Methyl salicylate**

Methyl salicylate was negative for two participating laboratories, and positive for one participating laboratory and the lead laboratory. Investigations of the concentration-response curves for two laboratories with negative results revealed that there was a trend toward an increase in nAA response at the highest test concentration.

Methyl salicylate is not included in the current DASS database (OECD, 2023a) but was reported as negative in Doc#213 (OECD, 2015). A recent the Scientific Committee on Consumer Safety (SCCS) (SCCS, 2021) reported that methyl salicylate is a “weak” sensitiser. As methyl salicylate is a weak sensitiser, this is one of the reasons for inconsistent outcome (positive or negative) in α-Sens®. It should be noted that methyl salicylate was negative in KeratinoSens™ and positive in LuSens (Tzutzuy *et al*., 2016). This fact also indicates that it may be difficult to yield consistent outcomes for methyl salicylate at least in a KE-2 assay. Furthermore, for example, in the validation study of LuSens, it was mentioned that borderline chemicals such as methyl salicylate and eugenol may be classified as both sensitiser and non-sensitiser (ESAC, 2016). The EpiSensA validation report also mentions that there are inherent limitations to the detection of some weak sensitisers in any assay that uses a threshold-based prediction model (OECD, 2023b). Therefore, it is thought that the result of classifying a weak sensitiser such as methyl salicylate can occur in any method that uses a threshold-based prediction model.

As shown in Fig. 7-1, the lead laboratory produced clear positive results for methyl salicylate unlike the other laboratories, with clear cytotoxicity and increasing nAA response at the high concentration range. According to the in-house data of the lead laboratory, the IC50 of methyl salicylate was estimated to be 510-576 µg/mL in the test concentrations range including above 500 µg/mL (based on 2 independent runs), confirming the IC50 of this chemical is very close to the maximum test concentration (500 µg/mL) of the α-Sens®. Accordingly, the very slight difference in the experimental operation could cause the difference in the assay outcomes. This fact suggests that where the IC50 of a test chemical is close to the maximum test concentration, the response may be different due to very slight operation differences.

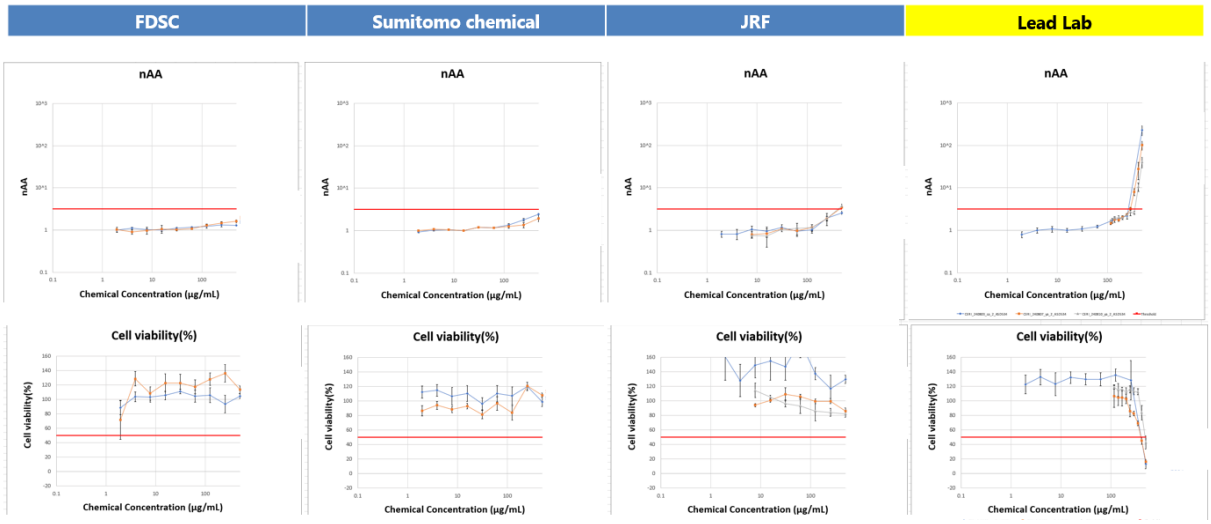



**Fig. 7-1 Concentration-response curves for nAA and cell viability of Methyl salicylate for each laboratory**

## *7.2.2 Conclusion on BLR*

For the BLR evaluated using 25 test chemicals, the BLR rate among three participating laboratories was 96.0% (24/25). The pass criterion for the BLR is 80%.

The VMT concluded that the BLR results fully met the criteria and the α-Sens® was highly reproducible between laboratories.

# 8. Quality Check

Regarding the use of cell culture medium and luciferase assay reagents, the QC personnel of JaCVAM confirmed that the same lots of cell culture medium and luciferase assay reagents were used for all tests and that all tests were conducted within respective expiration dates (detailed information is provided in Appendix 02). In addition, they confirmed that the SDSs sent to each test laboratory for each Phase -I and Phase -II were returned to JaCVAM unopened and was therefore judged that there were no concerns with the conduct of the tests.

The JaCVAM QC conducted a detailed review of the main contents of the test data submitted by the four laboratories (FDSC, JRF, Sumitomo, and CERI (Lead laboratory)) in Phase-I and Phase-II of this validation study. In this review, the test data, Excel spreadsheets, worksheets, raw data, and reports submitted by each laboratory all met the quality standards, and no issues were found that would disrupt the reliability of the test results.

Based on the above, they judged that the data quality of Phase-I and Phase-II of this validation study is sufficiently ensured and that the test results are reliable.

# 9. Predictive Capacity

## 9.1 Predictive Capacity for Refence Chemicals

### (1) Predictive Capacity using 20 Reference Chemicals

When evaluating the predictive capacity using the results for the 20 reference chemicals listed in Doc#213, the accuracy, sensitivity and specificity of each participating laboratory and the lead laboratory are summarised in Table 9-1. The average accuracy for three participating laboratories was calculated to be 88.3%. The average sensitivity and specificity were 91.7% and 83.3%, respectively.

The accuracy, sensitivity and specificity for the lead laboratory were 90.0%, 100% and 75.0%, respectively, and these values were comparable with those of three participating laboratories. It should be noted that the lower specificity is due to a borderline chemical (methyl salicylate).

No strong or extreme sensitisers (classified as 1A in the DASS database (OECD, 2023a)) were under-predicted/misclassified as a non-sensitiser by any of the participating laboratories and the lead laboratory.

**Table 9-1 Predictive capacity using 20 reference chemicals**

| | | Known category | | Accuracy | Sensitivity | Specificity |
|---|---|---|---|---|---|---|
| FDSC | | P | N | (%) | (%) | (%) |
| | P | 11 | 1 | 90.0 | 91.7 | 85.7 |
| | N | 1 | 7 | | | |
| Sumitomo Chemical | | Known category | | Accuracy | Sensitivity | Specificity |
| | | P | N | (%) | (%) | (%) |
| | P | 11 | 1 | 90.0 | 91.7 | 85.7 |
| | N | 1 | 7 | | | |
| JRF | | Known category | | Accuracy | Sensitivity | Specificity |
| | | P | N | (%) | (%) | (%) |
| | P | 11 | 2 | 85.0 | 91.7 | 75.0 |
| | N | 1 | 6 | | | |

| | Accuracy | Sensitivity | Specificity |
|---|---|---|---|
| Average among 3 participating laboratories | 88.3 | 91.7 | 83.3 |

| | | Known category | | Accuracy | Sensitivity | Specificity |
|---|---|---|---|---|---|---|
| Lead Lab | | P | N | (%) | (%) | (%) |
| | P | 12 | 2 | 90.0 | 100.0 | 75.0 |
| | N | 0 | 6 | | | |

P: Positive, N: Negative.

## (2) Predictive Capacity using 19 Reference Chemicals excluding Methyl Salicylate

Since Methyl salicylate is a borderline chemical and it is difficult to evaluate whether it is a skin-sensitiser or not, as discussed in section "7.2 (2)", the predictive capacity (accuracy, sensitivity and specificity) for the 19 reference chemicals excluding Methyl salicylate of each participating laboratory and the lead lab are summarised in Table 9-2. For the three participating laboratories, the average accuracy was calculated to be 89.5%, and the average sensitivity and specificity were 91.7% and 85.7%, respectively. With the exclusion of methyl salicylate, the predictive capacity for the three participating laboratories passed the acceptance criteria specified in Doc#213.

The accuracy, sensitivity and specificity for the lead laboratory was 94.7%, 100% and 85.7%, respectively, and it was confirmed that the predictive capacity among three participating laboratories and the lead laboratory were comparable.

**Table 9-2 Predictive Capacity using 19 Reference Chemicals excluding Methyl Salicylate**

| | | Known category | | Accuracy | Sensitivity | Specificity |
|---|---|---|---|---|---|---|
| **FDSC** | | **P** | **N** | **(%)** | **(%)** | **(%)** |
| | **P** | **11** | **1** | **89.5** | **91.7** | **85.7** |
| | **N** | **1** | **6** | | | |
| **Sumitomo Chemical** | | **Known category** | | **Accuracy** | **Sensitivity** | **Specificity** |
| | | **P** | **N** | **(%)** | **(%)** | **(%)** |
| | **P** | **11** | **1** | **89.5** | **91.7** | **85.7** |
| | **N** | **1** | **6** | | | |
| **JRF** | | **Known category** | | **Accuracy** | **Sensitivity** | **Specificity** |
| | | **P** | **N** | **(%)** | **(%)** | **(%)** |
| | **P** | **11** | **1** | **89.5** | **91.7** | **85.7** |
| | **N** | **1** | **6** | | | |

| | Accuracy | Sensitivity | Specificity |
|---|---|---|---|
| **Average among 3 participating laboratories** | **89.5** | **91.7** | **85.7** |

| | | Known category | | Accuracy | Sensitivity | Specificity |
|---|---|---|---|---|---|---|
| **Lead Lab** | | **P** | **N** | **(%)** | **(%)** | **(%)** |
| | **P** | **12** | **1** | **94.7** | **100** | **85.7** |
| | **N** | **0** | **6** | | | |

P: Positive, N: Negative.

## (3) Comparison of Predictive Capacity among existing KE2 assays for reference chemicals

The predictive capacity for reference chemicals among α-Sens®, KeratinoSens™ and LuSens were compared based on the outcomes among all experiments by all participating laboratories as analysed by ESAC for LuSens (ESAC, 2016). The accuracy, sensitivity and specificity of α-Sens® were demonstrated to be equivalent or superior to that for the KeratinoSens™ and LuSens in both cases for 20 or 19 reference chemicals (Table 9-3).

**Table 9-3 Comparison of Predictive Capacity among existing KE2 assays for 20 reference chemicals**

| Chemical Name | Known category in Doc#213 | No of positive / Total number of experiments | | |
|---|---|---|---|---|
| | | α-Sens® | KeratinoSens™ $ | LuSens $ |
| 4'-Methoxyacetophenone (=Acetanisole) | N | 9/9 | P (Number not available) | 9/9 |
| Glycerol | N | 0/9 | 0/19 | 0/11 |
| Isopropanol | N | 0/9 | 0/17 | 0/11 |
| Salicylic acid | N | 0/9 | 0/17 | 0/11 |
| Chlorobenzene | N | 0/3 | 1/17 | 0/3 |
| Lactic acid | N | 0/3 | 1/19 | 0/3 |
| Methyl salicylate | N* | 1/3 | 3/17 | 3/3 |
| Sulfanilamide | N | 0/3 | 1/17 | 0/3 |
| Ethylene glycol dimethacrylate (=EGDMA) | P | 9/9 | 17/17 | 9/9 |
| Phenyl benzoate | P | 1/9 | 2/19 | 0/9 |
| Citral | P | 9/9 | 17/17 | 9/9 |
| Isoeugenol | P | 9/9 | 19/19 | 9/9 |
| Methyldibromo glutaronitrile | P | 9/9 | 17/17 | 11/11 |
| *p*-Phenylenediamine | P | 9/9 | 17/17 | 9/9 |
| 2,4-Dinitrochlorobenzene (=DNCB) | P | 9/9 | 17/17 | 11/11 |
| 4-Nitrobenzyl bromide | P | 9/9 | 17/17 | 9/9 |
| Eugenol | P | 3/3 | 9/19 | 3/3 |
| 2-Mercaptobenzothiazole | P | 3/3 | 19/19 | 3/3 |
| 4-Methylaminophenol sulphate (=Metol) | P | 3/3 | 17/17 | 3/3 |
| Oxazolone | P | 3/3 | 19/19 | 3/3 |
| For 20 reference chemicals | Accuracy | **90.0%** | **85.0%** | **85.0%** |
| | Sensitivity | **91.7%** | **83.3%** | **91.7%** |
| | Specificity | **87.5%** | **87.5%** | **75.0%** |
| For 19 reference chemicals excluding Methyl Salicylate | Accuracy | **89.5%** | **84.2%** | **89.5%** |
| | Sensitivity | **91.7%** | **83.3%** | **91.7%** |
| | Specificity | **85.7%** | **85.7%** | **85.7%** |

$: from ESAC Opinion No. 2016-14. "ESAC Opinion on the BASF coordinated Performance Standards based validation of the LuSens test method for skin sensitisation testing" https://tsar.jrc.ec.europa.eu/system/files/Published/esac_opinion_2016-04_lusens_final.pdf

*: See section"7.2 (2)"

P: Positive, N: Negative.

## (4) Predictive capacity calculation required by Doc#213

According to Doc#213 for the predictive capacity of the Reference Chemicals requires the following calculation: "*The positive and negative predictions for each chemical should thus be divided by the total number of predictions for that chemical so that each chemical contributes with a final weight of 1 in the calculations* (OECD, 2015)" (See more details in Section "3.5.2 (4)Predictive Capacity"). We understood this explanation as 'each substance ultimately contributes a weight of 1 to the calculation' and analysed the predictive capacity based on this understanding. A detailed analysis is provided in Appendix-06.

The KeratinoSens™ seemed to use the lead lab data for this analysis probably because 4'-methoxyacetophenone (=Acetanisole) (false positive in the KeratinoSens™) was not used in the validation study of the KeratinoSens™ otherwise the KeratinoSens™ can analyse only 19 chemicals. Therefore, we calculated the predictive capacity both with and without the lead laboratory data in the α-Sens® (Table 9-4).

The accuracy, sensitivity and specificity for 20 reference chemicals by following the calculation required in Doc#213 was 88.8%, 93.9% and 81.3%, respectively. All these values

were higher (≥) than 80.0% that set in the success criteria. The accuracy and sensitivity were higher than KeratinoSens™. When compared to KeratinoSens™, the specificity of α-Sens® appears to be lower because methyl salicylate was regarded as negative following the known category described in Doc#213. Kappa coefficient is a measurement of agreement between two outcomes accounting for chance, and the Kappa coefficient of the α-Sens® was the highest ( 0.764 ) among three assay (α-Sens®, KeratinoSens™, and LuSens).

**Table 9-4 Predictive capacity calculation required by Doc#213 for 20 reference chemicals**

| | α-Sens®* | KeratinoSens™ in Doc#213 | LuSens (ESAC, 2016) |
|---|---|---|---|
| Accuracy | 88.8% (88.9%) | 87.0% | 85.0% |
| Sensitivity | 93.9% (92.6%) | 86.7% | 91.7% |
| Specificity | 81.3% (83.3%) | 87.5% | 75.0% |
| Kappa coefficient | 0.764 (0.766) | 0.733 | 0.681 |

*: The percentage in parentheses is based on data from participating labs only.

## 9.2 Predictive Capacity for 25 Test Chemicals in the Validation Study

**(1) Predictive Capacity using all 25 test chemicals**

For evaluating the predictive capacity using the results for all 25 test chemicals (including 20 reference chemicals) in the validation study, the accuracy, sensitivity and specificity of each participating laboratory and the lead laboratory are summarised in Table 9-5. The average accuracy for three participating laboratories was calculated to be 86.7%. The average sensitivity and specificity were 87.5% and 85.2%, respectively.

The accuracy, sensitivity and specificity for the lead laboratory were 88.0%, 93.8% and 77.8%, respectively, and these values were comparable with that of three participating laboratories.

No strong or extreme sensitisers (classified as 1A in the DASS database (OECD, 2023a) were under-predicted as non-sensitisers by any of the participating laboratories and the lead laboratory.

**Table 9-5 Predictive Capacity using all 25 test chemicals**

| | | Known category | | Accuracy | Sensitivity | Specificity |
|---|---|---|---|---|---|---|
| FDSC | | P | N | (%) | (%) | (%) |
| | P | 14 | 1 | 88.0 | 87.5 | 88.9 |
| | N | 2 | 8 | | | |
| Sumitomo Chemical | | Known category | | Accuracy | Sensitivity | Specificity |
| | | P | N | (%) | (%) | (%) |
| | P | 14 | 1 | 88.0 | 87.5 | 88.9 |
| | N | 2 | 8 | | | |
| JRF | | Known category | | Accuracy | Sensitivity | Specificity |
| | | P | N | (%) | (%) | (%) |
| | P | 14 | 2 | 84.0 | 87.5 | 77.8 |
| | N | 2 | 7 | | | |

| | Accuracy | Sensitivity | Specificity |
|---|---|---|---|
| Average | 86.7 | 87.5 | 85.2 |

| | | Known category | | Accuracy | Sensitivity | Specificity |
|---|---|---|---|---|---|---|
| Lead Lab | | P | N | (%) | (%) | (%) |
| | P | 15 | 2 | 88.0 | 93.8 | 77.8 |
| | N | 1 | 7 | | | |

P: Positive, N: Negative.

## (2) Discussion on False positive/false negative

### *False negative: Phenyl benzoate*

A detailed discussion on Phenyl benzoate was provided in section "6.2 (1)". Phenyl benzoate was considered to be a challenging chemical for test systems that use the activation of the ARE-Nrf2 antioxidant pathway as an indicator, because the concentration at which nAA start to increase, the IC50, and the precipitation concentrations are close to each other.

### *False negative: d-α-Tocopherol*

For α-Tocopherol in the DASS database (OECD, 2023a), CAS number '59-02-9' was provided. This CAS number is for '***d***-α-Tocopherol'. The test chemical used in the validation study for α-Sens® was exactly '*d*-α-Tocopherol' with '59-02-9' as CAS number. For this test chemical, negative judgements were reproducibly obtained between three participating laboratories and the lead laboratory (Fig. 8-1).

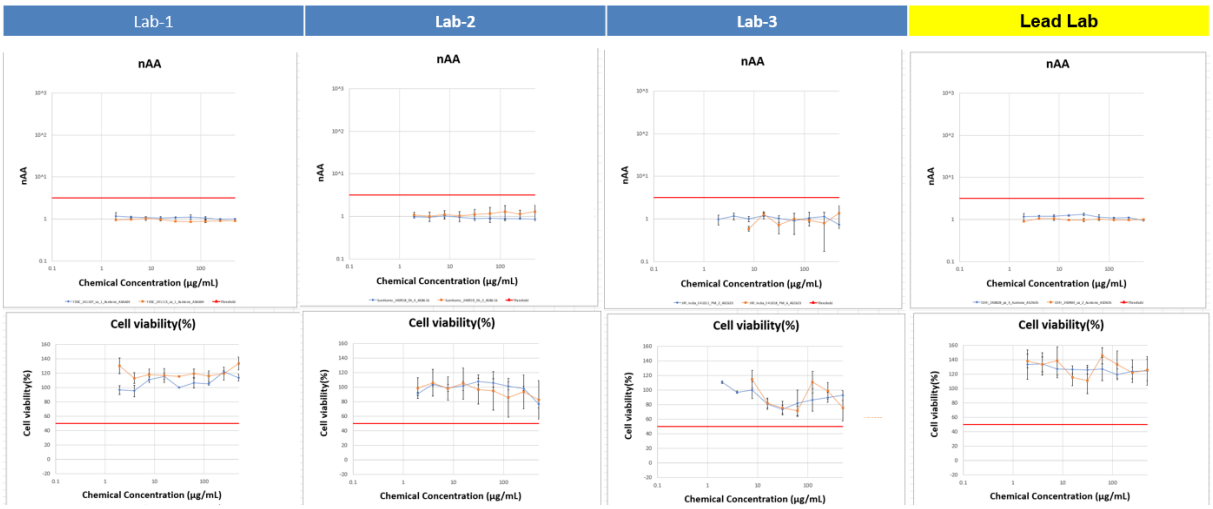



**Fig. 8-1 Concentration-response curves for nAA and cell viability of *d*-α-Tocopherol for each lab**

In this validation study, *d*-α-tocopherols was selected as a positive chemical based on the information in the DASS database (OECD, 2023a) because the data source was accompanying CAS RN of 59-02-9. However recent evaluation documents for α-tocopherols by the Australian Government reported that the synthetically produced *dl*-α-tocopherol (CAS RN of 10191-41-0), which is likely to includes impurities (possibly haptens), was judged as positive based on the positive evidence of LLNA and guinea pig test data, and that the naturally occurring *d*-α-tocopherol was judged as negative based on negative *in silico* data, and animal data using the weight of evidence approach. (Australian Government, 2023).

Furthermore, our investigation found that the positive result for α-tocopherol with CAS RN of 59-02-9 in the DASS database was probably due to the misnumbering and mis identification of the CAS RN for the *dl*-α-tocopherol (Kern PS *et al.,* 2010, Fig. 8-2). Therefore, the *dl*-isomer could be a sensitiser based on the findings from Australian Government (2023) and Kern PS *et al*. (2010). However, the *d*-isomer is considered as a non-sensitiser. In addition, it should be noted that α-tocopherol is vitamin E, which is a well-known antioxidant.

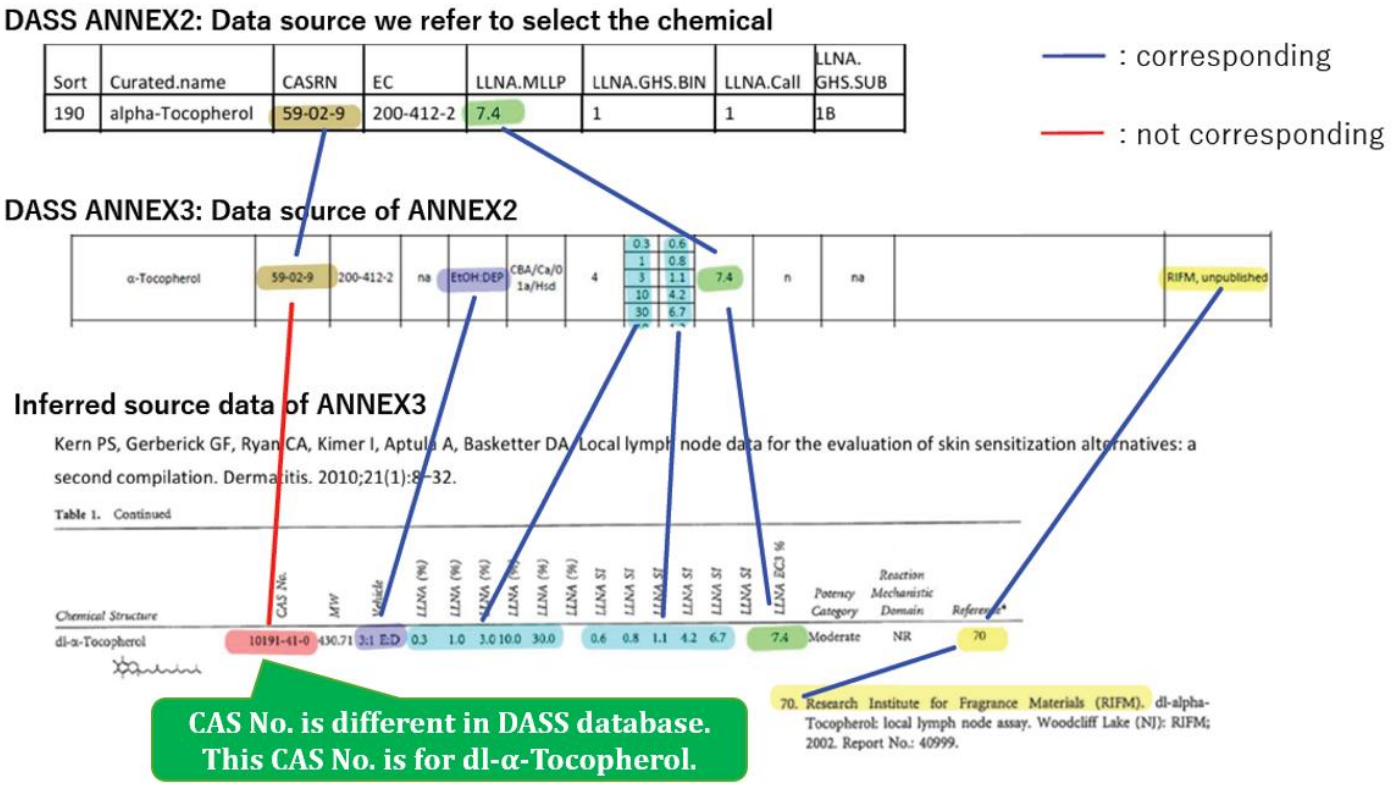



**Fig. 8-2 The LLNA data source of α-Tocopherol in the DASS database (OECD, 2023a)**

***False positive: Methyl salicylate***

The discussion on Methyl salicylate is provided in section "7.2 (2)".

***False positive: 4'-Methoxyacetophenone (=Acetanisole)***

Although 4'-Methoxyacetophenone is a non-sensitiser, all three participating laboratories and the lead laboratory gave positive results in all three experiments (Fig. 8-3, 8-4, 8-5 and 8-6). Since 4'-Methoxyacetophenone has been reported to be positive in both KeratinoSens$^{TM}$ and LuSens (Tzutzuy *et al*., 2016), it was considered that 4'-Methoxyacetophenone could potentially activate the ARE-Nrf2 antioxidant pathway and therefore all test systems utilising the ARE-Nrf2 antioxidant as an indicator yields false positive responses.

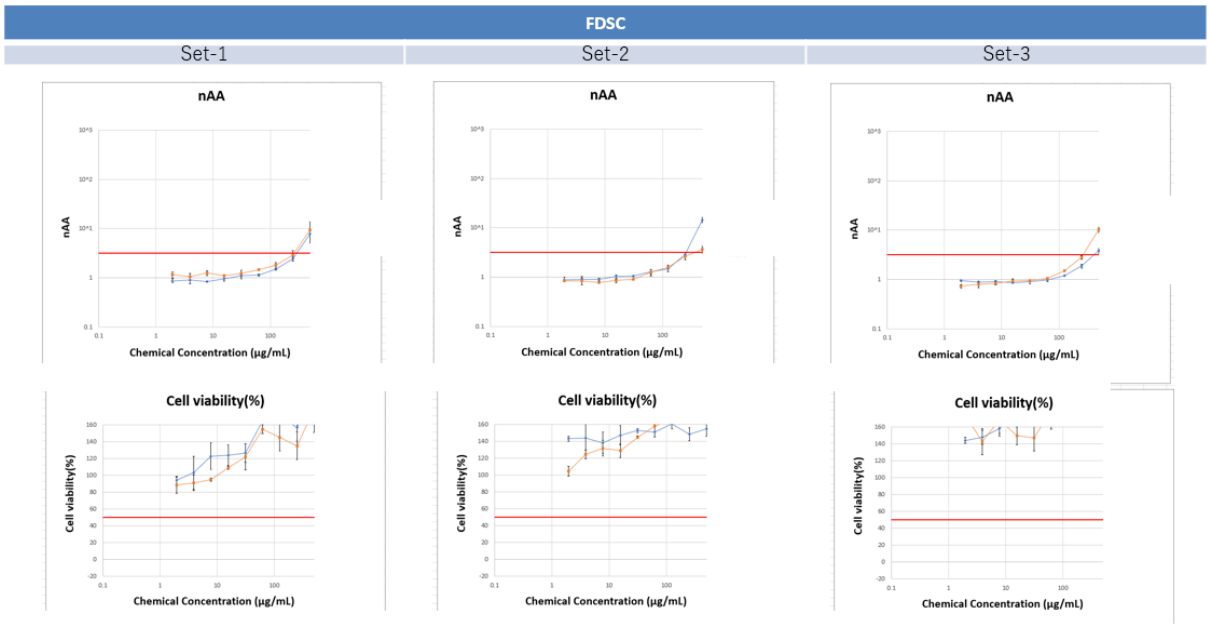



**Fig. 8-3 Concentration-response curves for nAA and cell viability of 4'-Methoxyacetophenone for FDSC**

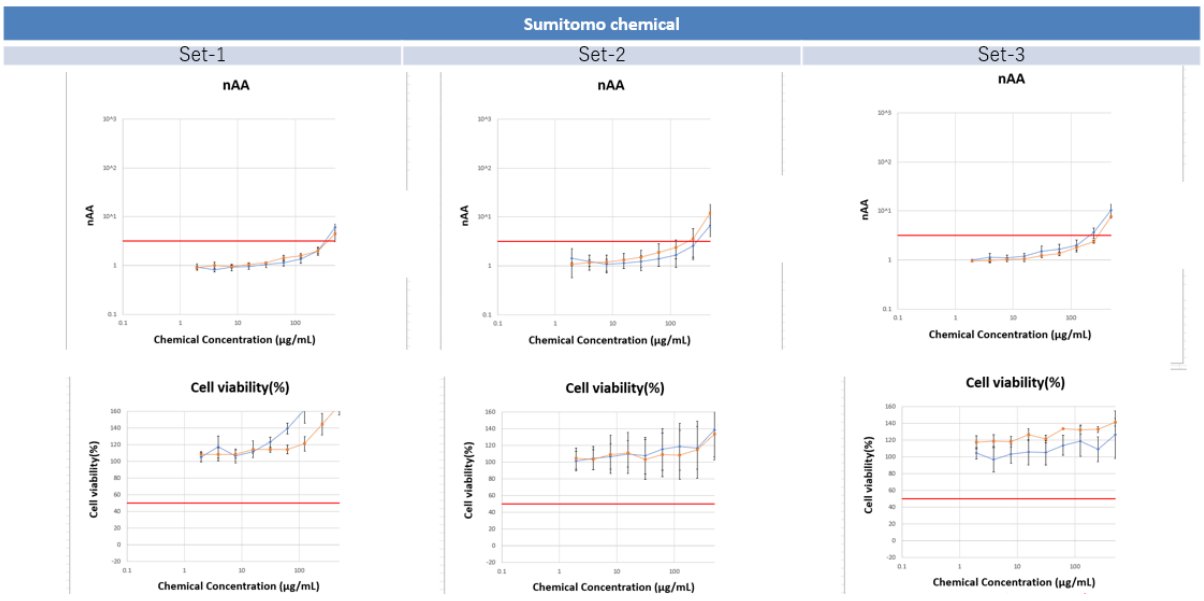



**Fig. 8-4 Concentration-response curves for nAA and cell viability of 4'-Methoxyacetophenone for Sumitomo Chemical**

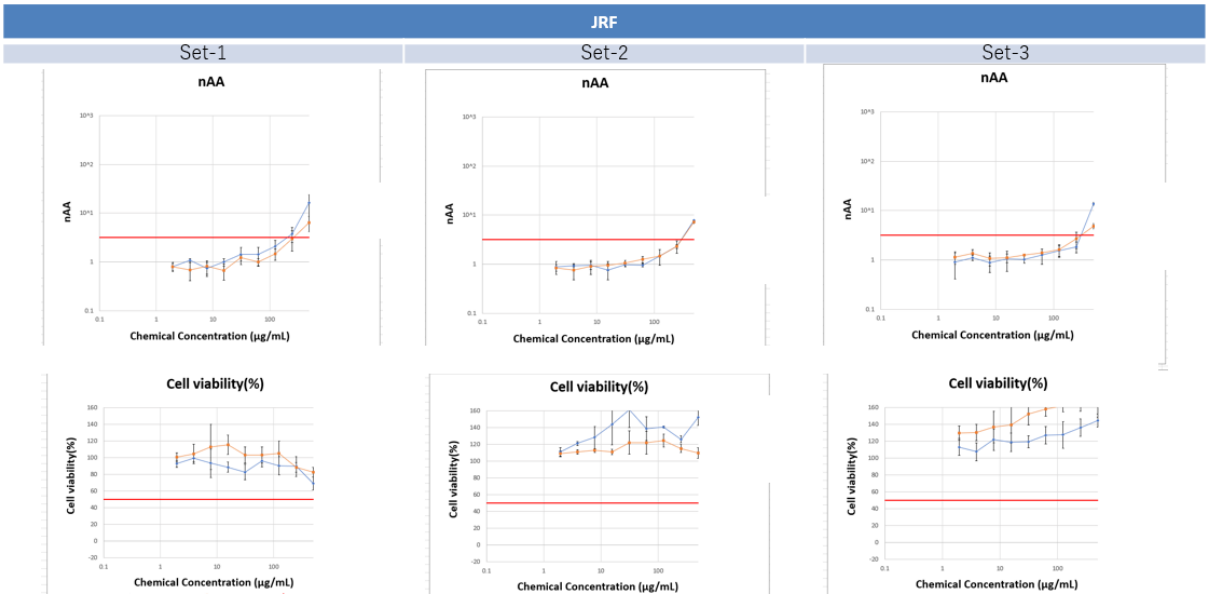



**Fig. 8-5 Concentration-response curves for nAA and cell viability of 4'-Methoxyacetophenone for JRF**

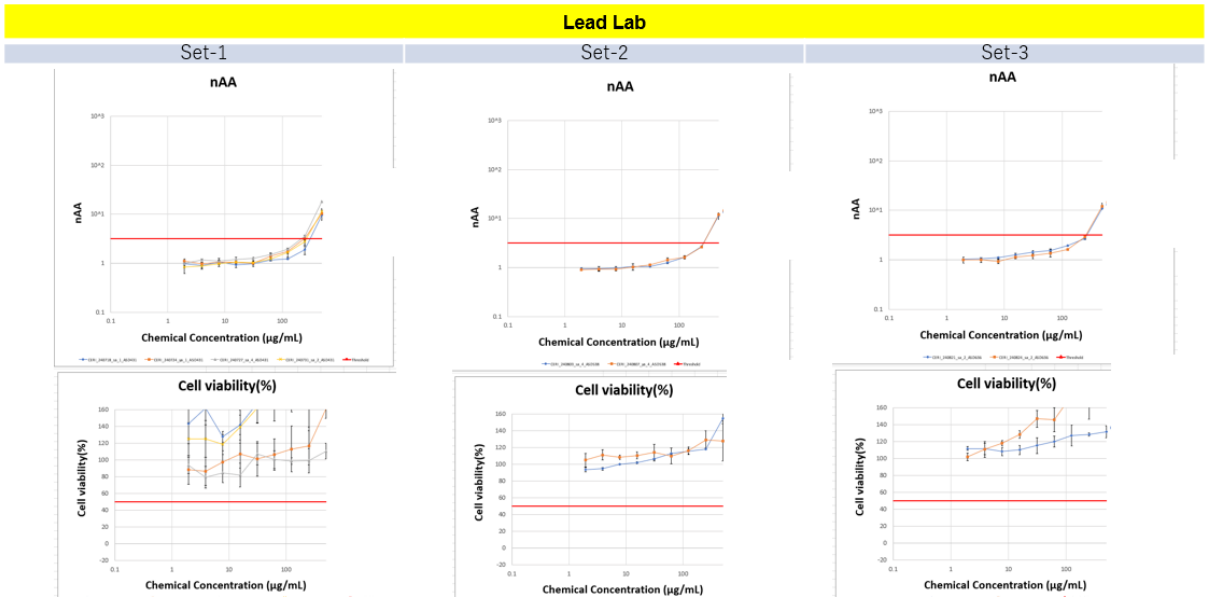



**Fig. 8-6 Concentration-response curves for nAA and cell viability of 4'-Methoxyacetophenone (=Acetanisole) for Lead lab**

## (3) Predictive Capacity using 23 test chemicals excluding Methyl salicylate and α-Tocopherol

As discussed in section "7.2 (2)" and "8.3 (2)", conclusive judgement of Methyl salicylate and α-Tocopherol for their sensitisation categories might be difficult to evaluate for predictive capacity. Therefore, the predictive capacity (accuracy, sensitivity and specificity) for the 23 test chemicals, excluding Methyl salicylate and α-Tocopherol, was also calculated. The results for each participating laboratory and the lead lab are summarised in Table 9-6. Among three participating laboratories, the average accuracy was calculated to be 91.3%, and the average

sensitivity and specificity were 93.3% and 87.5%, respectively. In the case of methyl salicylate and α-Tocopherol exclusion, all parameters of the predictive capacity for three participating laboratories were greater than 80%.

The accuracy, sensitivity and specificity for the lead laboratory were 95.7%, 100% and 87.5%, respectively, and it was confirmed that the predictive capacity between the three participating laboratories and the lead laboratory were comparable.

**Table 9-6 Predictive Capacity using 23 test chemicals excluding Methyl salicylate and α-Tocopherol**

| | | Known category | | Accuracy | Sensitivity | Specificity |
|---|---|---|---|---|---|---|
| FDSC | | P | N | (%) | (%) | (%) |
| | P | 14 | 1 | 91.3 | 93.3 | 87.5 |
| | N | 1 | 7 | | | |
| Sumitomo Chemical | | Known category | | Accuracy | Sensitivity | Specificity |
| | | P | N | (%) | (%) | (%) |
| | P | 14 | 1 | 91.3 | 93.3 | 87.5 |
| | N | 1 | 7 | | | |
| JRF | | Known category | | Accuracy | Sensitivity | Specificity |
| | | P | N | (%) | (%) | (%) |
| | P | 14 | 1 | 91.3 | 93.3 | 87.5 |
| | N | 1 | 7 | | | |

| | Accuracy | Sensitivity | Specificity |
|---|---|---|---|
| Average | 91.3 | 93.3 | 87.5 |

| Lead Lab | | Known category | | Accuracy | Sensitivity | Specificity |
|---|---|---|---|---|---|---|
| | | P | N | (%) | (%) | (%) |
| | P | 15 | 1 | 95.7 | 100 | 87.5 |
| | N | 0 | 7 | | | |

P: Positive, N: Negative.

## 9.3 Discussion on Predictive Capacity for in-house Chemicals

The lead laboratory has also tested 186 commercially available chemicals listed in the DASS database (Appendix-07: α-Sens® in-house dataset). Using this in-house data, the predictive capacity of α-Sens® was further evaluated.

**(1) Sensitisers vs. non-sensitisers**

***Comparison with LLNA data***

Among the 162 chemicals from the LLNA data of "LLNA.Call" in DASS database (OECD, 2023a), the numbers of sensitisers and non-sensitisers were 131 and 31 chemicals, respectively. As shown in Table 9-7, the accuracy, sensitivity and specificity of α-Sens® to LLNA data were 74.1%, 76.3% and 64.5%, respectively. When compared to KeratinoSens™, the accuracy and sensitivity of α-Sens® exceed that of KeratinoSens™. Although the specificity of α-Sens® was slightly lower than that of KeratinoSens™, this was caused by only one chemical as shown in Table 9-7.

**Table 9-7 Predictive Capacity to LLNA data for in-house Chemicals**

| vs LLNA data* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | P | N | Sum | | | P | N | Sum |
| α-Sens® | P | 100 | 11 | 111 | Keratino Sens™ | P | 93 | 10 | 103 |
| | N | 31 | 20 | 51 | | N | 38 | 21 | 59 |
| | Sum | 131 | 31 | 162 | | Sum | 131 | 31 | 162 |
| | Accuracy | 74.1% (120/162) | | | | Accuracy | 70.4% (114/162) | | |
| | Sensitivity | 76.3% (100/131) | | | | Sensitivity | 71.0% (93/131) | | |
| | Specificity | 64.5% (20/31) | | | | Specificity | 67.7% (21/31) | | |

P: Positive, N: Negative.

*: See Appendix-07. Data of LLNA. Call from DASS database (OECD, 2023a) were used.

***Comparison with human data***

112 out of 186 chemicals had human data in the DASS database. The positive and negative calls for human data was summarised from "HU.GHS.BIN" and "Basketter_human_Call" in the DASS database (OECD, 2023a) and is re-named as "Sumarised.Human.Call" in Appendix-07. Among these, the number of skin sensitisers and non-sensitisers for the human data were 83 and 29 chemicals, respectively. The accuracy, sensitivity and specificity of α-Sens® for the human data was calculated to be 83.0%, 84.3% and 79.3%, respectively (Table 9-8). When compared with KeratinoSens™ in the DASS database, the accuracy and sensitivity of α-Sens® was superior to that of KeratinoSens™. Although the specificity of α-Sens® was slightly lower than that of KeratinoSens™, this was only one chemical difference as shown in Table 9-8.

**Table 9-8 Predictive Capacity to Human data for in-house Chemicals**

| vs Human data* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | P | N | Sum | | | P | N | Sum |
| α-Sens® | P | 70 | 6 | 76 | Keratino Sens™ | P | 61 | 6 | 67 |
| | N | 13 | 23 | 36 | | N | 22 | 23 | 45 |
| | Sum | 83 | 29 | 112 | | Sum | 83 | 29 | 112 |
| | Accuracy | 83.0% (93/112) | | | | Accuracy | 75.0% (84/112) | | |
| | Sensitivity | 84.3% (70/83) | | | | Sensitivity | 73.5% (61/83) | | |
| | Specificity | 79.3% (23/29) | | | | Specificity | 79.3% (23/29) | | |

P: Positive, N: Negative.

*: See Appendix-07. Data of "HU.GHS.BIN" and "Basketter_human_Call" in DASS database (OECD, 2023) were used for the analysis. The overall positive and negative judgement of the human data were made as follows. Positive call was assigned first when one of the above-mentioned items in DASS database indicated positive. Negative call was assigned when one of the items was NC (Not conclusive).

***Discussion***

The detailed data can be found in Appendix-07.

Table 9-9 shows 63 chemicals with discordant results in either or both of the test methods α-Sens® and KeratinoSens™ to LLNA. Among the 48 chemicals identified as LLNA positive but negative with either or both α-Sens® and KeratinoSens™, there were 21 chemicals that were negative in both the α-Sens® and KeratinoSens™. This included 8 chemicals (anethole, anisyl alcohol, DMSO, isopropyl myristate, OTNE, pentachlorophenol, salicylic acid, sodium lauryl sulfate) known to be human negative (Non Category in GHS). Although α-Sens® was optimised based upon the concordant accuracy with human data, during the development process, as described in Section 4.1 , in many cases, the negative results of α-Sens® and KeratinoSens™ may be due to the sensitivity to extremely weak sensitisers. The relationship between the concentration causing cytotoxicity and ARE activation as discussed in Section 6.2 (1), for phenyl benzoate, and for differences in the sensitising mechanism other than ARE activation could be the reasons.

For the 38 chemicals classified as negative in the KeratinoSens™ but positive in the LLNA (including 11 human positive chemicals), α-Sens® gave positive results for 17 out of the 38 chemicals. This indicates an improved performance of α-Sens® compared to the KeratinoSens™ for addressing the KE-2. This improved performance has been achieved due to the high sensitivity of the test system because of the customised reporter gene construct and the normalised ARE activity (nAA), the latter being the endpoint.

Although 10 chemicals were positive in LLNA (including 3 human positive chemicals) and KeratinoSens™ but negative in α-Sens®, the reasons for this, including the CAS no. confusion in previous reports around α-Tocopherol is discussed in Section 9.2.(2). It was considered that the two chemicals (Benzyl benzoate and Benzyl salicylate) were negative in α-Sens® and the human data, because the α-Sens® was optimised against the human database. The 5 chemicals (1-Bromohexane, Hydratropaldehyde, Hydroxycitronellal, Oxalic acid and Undec-10-enal) with discordant results were probably due to the relationship between the concentration required to cause cytotoxicity and that needed for ARE activation as discussed in Section 6.2 (1) for phenyl benzoate. The remaining 2 chemicals (Allyl phenoxyacetate and 2-Ethylbutanal) were discordant probably because they are weak (and possibly borderline) sensitisers (both were classified as 1B for LLNA.GHS. SUB).

As for the 14 chemicals with negative results in the LLNA but positive in either KeratinoSens™ or α-Sens®, 7 chemicals (Acetanisole, Ethyl vanillin, 2-Fluoro-5-nitroaniline, 2-Hydroxypropyl methacrylate, Methyl 3-bromoproprionate, 4-Methyl-2-nitroanisole, Methylparaben) were positive in both the KeratinoSens™ and α-Sens®. Among these 7 chemicals, four chemicals (Acetanisole, Ethyl vanillin, 2-Fluoro-5-nitroaniline, Methylparaben) were negative in both the DPRA and hCLAT, therefore these chemicals may be generating false positives due to non-specific induction of ARE activity other than the sensitisation related mechanism, as discussed in Section 9.2 (2) "False positive: 4'-Methoxyacetophenone (=Acetanisole)".

The two out of three chemicals negative in both the LLNA and α-Sens® but positive in KeratinoSens™ (Benzyl butyl phthalate, Dibutyl phthalate), were also negative in both the DPRA and hCLAT, suggesting that these are KeratinoSens™ specific false positives. The other four chemicals that tested positive only in α-Sens®, and two chemicals (DEET, Vanillin) were negative for both DPRA and hCLAT, suggesting that these are α-Sens® specific false positives, although the cause is unclear at present.

Table 9-10 shows 19 chemicals with discordant outcomes between the LLNA and the human data.

Among the 16 chemicals showing human negative and LLNA positive outcomes, 12 chemicals were correctly judged as negative by the α-Sens®. As described above, again this may be because the α-Sens® was optimised based on the accuracy with human data in the development process. The remaining 4 chemicals (Citronellol, alpha-Hexylcinnamaldehyde, alpha-Isomethylionone, *D*-Limonene) produced positive results in the α-Sens®. Of these, Citronellol, alpha-Isomethylionone, *D*-Limonene were positive in either or both of DPRA and hCLAT, alpha-Hexylcinnamaldehyde has been used as a positive control chemical in *in vivo* sensitisation tests, such as the LLNA (OECD TG429 and TG442B), guinea-pig maximisation tests (GPMT) and Buehler test in OECD TG406. *D*-Limonene was selected as one of the additional test chemicals for the validation study as the weakest activity in LLNA MLLP (Median-like location parameter; the mean of EC3 from independent LLNA assay runs) among the 1B candidate chemicals. Considering these observations, it can be concluded that the positive results for these four chemicals in the α-Sens® are plausible.

The remaining three chemicals (Benzyl alcohol, Kanamycin, Sulfanilamide) showing human positive and LLNA negative outcomes, produced negative results in both the α-Sens® and KeratinoSens™, suggesting these chemicals may be challenging chemicals for KE-2 assays in terms of predicting human skin sensitisation.

Table 9-11 shows 9 chemicals for which human data are available, but no LLNA data. Of these 9 chemicals (7 positive and 2 negative), both α-Sens® and KeratinoSens™ could give correct outcomes for 7 chemicals (5 positive and 2 negative). However, there were discordant results for two chemicals (Hydrocortisone, Thioglycerol). As for Hydrocortisone, the negative result from α-Sens® was considered plausible because this chemical was negative in the human data, but positive in KeratinoSens™. Thioglycerol, α-Sens®: positive and KeratinoSens™: negative, is noted as human positive, so the positive result generated by the α-Sens® is also plausible. Neomycin sulfate, which is noted as human positive, was judged as negative in both α-Sens® and KeratinoSens™, suggesting that this chemical is also a challenging chemical for KE-2 assays in terms of predicting human skin sensitisation.

As shown above, the α-Sens® produced a very small number of false positives and false negatives that may have been caused by the test system, and it is concluded that the α-Sens® has better performance than the KeratinoSens™ to address KE-2.

## Table 9-9 Chemicals with discordant results in either or both of α-Sens® and KeratinoSens™ to LLNA

| No.[*1] | Curated.name[*1] | CASRN[*1] | Summarised. Human. Call [*2, *3] | LLNA. Call [*1, *3] | LLNA. MLLP [*1, *4] | Human GHS Subcat. [*5] | LLNA GHS SUB [*1] | α-Sens® Call [*3] | KS. Call [*1, *3] | α-Sens® EC3.2 [*6] | α-Sens® IC50 [*7] | KS. EC1.5 [*1] | KS. EC3[*1] | KS. IC50[*1] | DPRA. Call [*1, *3] | hCLAT. Call [*1, *3] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LLNA: Positive; α-Sens®: Negative; KeratinoSens™: Negative —**21 Chemicals** | | | | | | | | | | | | | | | | |
| 13 | Anethole | 104-46-1 | 0 | 1 | POS | NC | NA | 0 | 0 | N.C | N.C | >2000 | >2000 | 340.3 | 0 | 1 |
| 14 | Aniline | 62-53-3 | 1 | 1 | NC | 1B | 1B | 0 | 0 | N.C | 497.76 | >2000 | >2000 | 195 | 0 | 1 |
| 15 | Anisyl alcohol | 105-13-5 | 0 | 1 | 7.1 | NC | 1B | 0 | 0 | N.C | N.C | >2000 | >2000 | 4000 | 0 | 1 |
| 34 | Bourgeonal | 18127-01-0 | 1 | 1 | 9.5 | 1B | 1B | 0 | 0 | N.C | 13.82 | >2000 | >2000 | 67.73 | 1 | 1 |
| 47 | Chlorpromazine | 50-53-3 | 1 | 1 | POS | 1B | NA | 0 | 0 | N.C | 1.56 | >2000 | >2000 | 7.8 | 0 | 1 |
| 63 | *N,N*-Dibutylaniline | 613-29-6 | NA | 1 | 19.6 | NA | 1B | 0 | 0 | N.C | N.C | >2000 | >2000 | 29.25 | 0 | 0 |
| 69 | 3,4-Dihydrocoumarin | 119-84-6 | 1 | 1 | 5.6 | NA | 1B | 0 | 0 | N.C | 231.05 | 2000 | 2000 | 2000 | 1 | 1 |
| 76 | DMSO | 67-68-5 | 0 | 1 | 72 | NC | 1B | 0 | 0 | N.C | N.C | >2000 | >2000 | 4000 | 0 | 1 |
| 104 | HHPA | 85-42-7 | NA | 1 | 0.84 | NA | 1A | 0 | 0 | N.C | N.C | 2000 | 2000 | 2000 | 1 | 0 |
| 122 | Isopropyl myristate | 110-27-0 | 0 | 1 | 44 | NC | 1B | 0 | 0 | N.C | N.C | >2000 | >2000 | 384.8 | 0 | 1 |
| 141 | Methyl pyruvate | 600-22-6 | NA | 1 | 2.4 | NA | 1B | 0 | 0 | N.C | N.C | >2000 | >2000 | 4000 | 0 | 0 |
| 160 | OTNE | 54464-57-2 | 0 | 1 | 14.2 | NC | 1B | 0 | 0 | N.C | N.C | >2000 | >2000 | 33.01 | 0 | 1 |
| 163 | Penicillin G | 61-33-6 | 1 | 1 | 31.3 | 1B | 1B | 0 | 0 | N.C | N.C | 1308.65 | 4000 | 4000 | 1 | 1 |
| 164 | Pentachlorophenol | 87-86-5 | 0 | 1 | 20 | NC | 1B | 0 | 0 | N.C | 0.12 | >2000 | >2000 | 27.7 | 1 | 1 |
| 171 | Phthalic anhydride | 85-44-9 | NA | 1 | 0.16 | NA | 1A | 0 | 0 | N.C | N.C | 2000 | 2000 | 2000 | 1 | 0 |
| 176 | Pyridine | 110-86-1 | 1 | 1 | 72 | 1B | 1B | 0 | 0 | N.C | N.C | 1300 | 4000 | 4000 | 0 | 1 |
| 180 | Salicylic acid | 69-72-7 | 0 | 1 | 12.2 | NC | 1B | 0 | 0 | N.C | N.C | >2000 | >2000 | 4000 | 0 | 1 |
| 181 | Sodium lauryl sulfate | 151-21-3 | 0 | 1 | 3.7 | NC | 1B | 0 | 0 | N.C | 12.20 | >2000 | >2000 | 44.7 | 0 | 0 |
| 182 | Squaric acid | 2892-51-5 | NA | 1 | POS | NA | NA | 0 | 0 | N.C | N.C | 2000 | 2000 | 2000 | 1 | 0 |
| 187 | 2,2,6,6-Tetramethylheptane-3,5-dione | 1118-71-4 | NA | 1 | 27 | NA | 1B | 0 | 0 | N.C | N.C | 2000 | 2000 | 384.94 | 0 | 1 |
| 192 | Trimellitic anhydride | 552-30-7 | NA | 1 | 9.2 | NA | 1B | 0 | 0 | N.C | N.C | 2000 | 2000 | 2000 | 1 | 1 |
| LLNA Positive; α-Sens®: Positive; KeratinoSens™: Negative —**17 chem** | | | | | | | | | | | | | | | | |
| 4 | 4-Allylanisole | 140-67-0 | NA | 1 | 18 | NA | 1B | 1 | 0 | 21.63 | N.C | 2000 | 2000 | 419.01 | 1 | 1 |
| 10 | 3-Aminophenol | 591-27-5 | NA | 1 | 3.2 | NA | 1B | 1 | 0 | 9.70 | 112.56 | 2000 | 2000 | 2000 | 0 | 1 |
| 12 | alpha-Amylcinnamic alcohol | 101-85-9 | 1 | 1 | NA | 1B | 1B | 1 | 0 | 15.65 | 16.26 | >2000 | >2000 | 44.1 | 0 | 1 |
| 52 | Citronellol | 106-22-9 | 0 | 1 | 43.5 | NC | 1B | 1 | 0 | <167.4 | N.C | >2000 | >2000 | 375 | 1 | 1 |
| 60 | Dibenzoyl peroxide | 94-36-0 | 1 | 1 | 1A | 1B | 1A | 1 | 0 | 7.18 | 11.57 | >2000 | >2000 | 567.63 | 1 | 0 |
| 68 | Diethylenetriamine | 111-40-0 | 1 | 1 | POS | 1A | NA | 1 | 0 | 435.42 | 408.30 | 1259.35 | 2000 | 2000 | 1 | 0 |
| 87 | Eugenol | 97-53-0 | 1 | 1 | 11.6 | 1B | 1B | 1 | 0 | 5.05 | 38.28 | >2000 | >2000 | 1505.69 | 1 | 1 |
| 101 | Hexyl salicylate | 6259-76-3 | 1 | 1 | 0.18 | 1B | NA | 1 | 0 | 73.15 | N.C | >2000 | >2000 | 67.4 | 0 | 1 |
| 120 | alpha-Isomethylionone | 127-51-5 | 0 | 1 | 21.8 | NC | 1B | 1 | 0 | 304.18 | N.C | >2000 | >2000 | 60.03 | 0 | 1 |
| 127 | Lilial | 80-54-6 | 1 | 1 | 8.6 | 1B | 1B | 1 | 0 | 53.57 | 184.92 | >2000 | >2000 | 94.49 | 1 | 1 |
| 128 | *D*-Limonene | 5989-27-5 | 0 | 1 | 52.5 | NC | 1B | 1 | 0 | 47.76 | N.C | >2000 | >2000 | 118.1 | 0 | 1 |
| 129 | Linalool | 78-70-6 | 1 | 1 | 35.5 | 1B | 1B | 1 | 0 | 103.84 | N.C | >2000 | >2000 | 4000 | 0 | 1 |
| 130 | Maleic anhydride | 108-31-6 | NA | 1 | 0.16 | NA | 1A | 1 | 0 | 33.98 | N.C | 1475.85 | 2000 | 2000 | 1 | 1 |
| 132 | 2-Methoxy-*p*-cresol | 93-51-6 | 1 | 1 | 5.4 | 1A | 1B | 1 | 0 | <[1.313] | 48.64 | >2000 | >2000 | 4000 | 0 | 1 |
| 167 | Phenyl benzoate | 93-99-2 | 1 | 1 | POS | 1B | NA | 1 | 0 | 75.85 | N.C | >2000 | >2000 | 191.62 | 1 | 1 |
| 174 | 3-Propylidenephthalide | 17369-59-4 | 1 | 1 | 3.7 | 1B | 1B | 1 | 0 | 19.25 | N.C | >2000 | >2000 | 717.4 | 1 | 1 |

| No.[*1] | Curated.name[*1] | CASRN[*1] | Summarised. Human. Call [*2, *3] | LLNA. Call [*1, *3] | LLNA. MLLP [*1, *4] | Human GHS Subcat. [*5] | LLNA GHS SUB [*1] | α-Sens® Call [*3] | KS. Call [*1, *3] | α-Sens® EC3.2 [*6] | α-Sens® IC50 [*7] | KS. EC1.5 [*1] | KS. EC3[*1] | KS. IC50[*1] | DPRA. Call [*1, *3] | hCLAT. Call [*1, *3] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 177 | Resorcinol | 108-46-3 | 1 | 1 | 6.3 | 1B | 1B | 1 | 0 | 41.66 | N.C | >2000 | >2000 | 4000 | 0 | 1 |
| LLNA: Positive; α-Sens®: Negative; KeratinoSens™ Positive —**10 chem** | | | | | | | | | | | | | | | | |
| 5 | Allyl phenoxyacetate | 7493-74-5 | 1 | 1 | 3.1 | 1B | 1B | 0 | 1 | N.C | N.C | 404.94 | 4000 | 4000 | 0 | 0 |
| 26 | Benzyl benzoate | 120-51-4 | 0 | 1 | 17 | NC | 1B | 0 | 1 | N.C | N.C | 72.49 | 142.47 | 767.19 | 0 | 0 |
| 30 | Benzyl salicylate | 118-58-1 | 0 | 1 | 2.9 | NC | 1B | 0 | 1 | N.C | N.C | 8.42 | 40.88 | 110.98 | 0 | 0 |
| 36 | 1-Bromohexane | 111-25-1 | NA | 1 | 10 | NA | 1B | 0 | 1 | N.C | 26.06 | 128.1 | >2000 | 391.9 | 1 | 0 |
| 83 | 2-Ethylbutanal | 97-96-1 | NA | 1 | 76 | NA | 1B | 0 | 1 | N.C | N.C | 238.26 | 520.37 | >2000 | 1 | 1 |
| 105 | Hydratropaldehyde | 93-53-8 | 1 | 1 | 6.3 | 1B | 1B | 0 | 1 | N.C | 9.40 | 64.76 | 76.67 | 195.06 | 1 | 1 |
| 109 | Hydroxycitronellal | 107-75-5 | 1 | 1 | 21.1 | 1B | 1B | 0 | 1 | N.C | 50.88 | 79.4 | 142.91 | 4000 | 1 | 1 |
| 161 | Oxalic acid | 144-62-7 | NA | 1 | 15 | NA | 1B | 0 | 1 | N.C | N.C | 717.2 | >2000 | >2000 | 0 | 1 |
| 190 | α-Tocopherol | 59-02-9 | 0 | 1 | 7.4 | NC | 1B | 0 | 1 | N.C | N.C | 114.6 | 4000 | 4000 | 0 | 0 |
| 194 | Undec-10-enal | 112-45-8 | NA | 1 | 6.8 | NA | 1B | 0 | 1 | N.C | 28.03 | 66.63 | 4000 | 139.77 | 0 | 0 |
| LLNA: Negative; α-Sens®: Positive; KeratinoSens™: Positive —**7 chem** | | | | | | | | | | | | | | | | |
| 2 | Acetanisole | 100-06-1 | NA | 0 | NC | NA | NC | 1 | 1 | 135.14 | N.C | 377.61 | 1192.39 | >2000 | 0 | 0 |
| 82 | Ethyl vanillin | 121-32-4 | NA | 0 | NC | NA | NC | 1 | 1 | 185.58 | N.C | 161.73 | 700.6 | >2000 | 0 | 0 |
| 90 | 2-Fluoro-5-nitroaniline | 369-36-8 | NA | 0 | NC | NA | NC | 1 | 1 | 40.49 | N.C | 249.8 | 4000 | 4000 | 0 | 0 |
| 111 | 2-Hydroxypropyl methacrylate | 923-26-2 | NA | 0 | NC | NA | NC | 1 | 1 | 7.15 | N.C | 549.68 | >2000 | >2000 | 1 | 0 |
| 135 | Methyl 3-bromoproprionate | 3395-91-3 | NA | 0 | NC | NA | NC | 1 | 1 | 0.70 | 10.85 | 41.13 | 254.47 | 438.55 | 1 | 0 |
| 150 | 4-Methyl-2-nitroanisole | 119-10-8 | NA | 0 | NC | NA | NC | 1 | 1 | <[7.813] | N.C | 359.31 | 4000 | 4000 | 0 | 1 |
| 151 | Methylparaben | 99-76-3 | NA | 0 | NC | NA | NC | 1 | 1 | 58.55 | N.C | 805.35 | >2000 | >2000 | 0 | 0 |
| LLNA Negative; α-Sens®: Negative; KeratinoSens™: Positive —**3 chem** | | | | | | | | | | | | | | | | |
| 28 | Benzyl butyl phthalate | 85-68-7 | NA | 0 | NC | NA | NC | 0 | 1 | N.C | N.C | 9.83 | 4000 | 4000 | 0 | 0 |
| 62 | Dibutyl phthalate | 84-74-2 | NA | 0 | NC | NA | NC | 0 | 1 | N.C | N.C | 56.42 | 4000 | 500 | 0 | 0 |
| 114 | 1-Iodohexane | 638-45-9 | NA | 0 | NC | NA | NC | 0 | 1 | N.C | 11.50 | 25.61 | >2000 | 222.31 | 1 | 1 |
| LLNA: Negative; α-Sens®: Positive; KeratinoSens™: Negative —**4 chem** | | | | | | | | | | | | | | | | |
| 44 | 3-Chloro-*p*-anisaldehyde | 4903-09-7 | NA | 0 | NC | NA | NC | 1 | 0 | 17.84 | 74.33 | 2000 | 2000 | 785.57 | 0 | 1 |
| 57 | DEET | 134-62-3 | 0 | 0 | NC | NC | NC | 1 | 0 | 253.65 | N.C | >2000 | >2000 | 1914 | 0 | 0 |
| 166 | 3-Phenoxypropanenitrile | 3055-86-5 | NA | 0 | NC | NA | NC | 1 | 0 | 274.02 | N.C | >2000 | >2000 | 1753.74 | 1 | 1 |
| 195 | Vanillin | 121-33-5 | 0 | 0 | NC | NC | NC | 1 | 0 | 269.91 | N.C | >2000 | >2000 | 4000 | 0 | 0 |

*1: Data was extracted from DASS database (OECD, 2023a)

*2: See Appendix-07. Data of "HU.GHS.BIN" and "Basketter_human_Call" in DASS database (OECD, 2023) were used for the analysis. The overall positive and negative judgement of the human data were made as follows. Positive call was assigned first when one of the above mentioned items in DASS database indicated positive. Negative call was assigned when one of the items was NC (Not conclusive).

*3: "1": positive. "0": negative, NA: not available, NC: not conclusive

*4: Median-like location parameter; the mean of EC3 from independent LLNA assay.

*5: The GHS subcategories 1A and 1B were assigned using both "HU.GHS.SUB" and "Basketter_human_potency_3class" in the DASS database (OECD, 2023a). If either or both are 1A, 1A was assigned. If both are 1B, or if one is NC or NA and the other is 1B, then the subcategory was 1B. If both are NC, or if one is NA and the other is NC, then NC was assigned.

*6: The concentration calculated by linear regression of the two points of test concentration and nAA across nAA value 3.2 in the α-Sens®. N.C.: Not Calculated.

*7: The concentration calculated by linear regression of the two points of test concentration and cell viability across 50% cell viability. in the α-Sens®. N.C.: Not Calculated.

**Table 9-10 Chemicals with discordant outcomes between LLNA and the human data**

| No.[*1] | Curated.name[*1] | CASRN[*1] | Sumarised. Human. Call [*2, *3] | LLNA. Call [*1, *3] | LLNA. MLLP [*1, *4] | Human GHS Subcat. [*5] | LLNA GHS SUB [*1] | α-Sens® Call [*3] | KS. Call [*1, *3] | α-Sens® EC3.2 [*6] | α-Sens® IC50 [*7] | KS. EC1.5[*1] | KS. EC3[*1] | KS. IC50[*1] | DPRA. Call [*1, *3] | hCLAT. Call [*1, *3] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 13 | Anethole | 104-46-1 | 0 | 1 | POS | NC | NA | 0 | 0 | N.C | N.C | >2000 | >2000 | 340.30 | 0 | 1 |
| 15 | Anisyl alcohol | 105-13-5 | 0 | 1 | 7.1 | NC | 1B | 0 | 0 | N.C | N.C | >2000 | >2000 | 4000.00 | 0 | 1 |
| 26 | Benzyl benzoate | 120-51-4 | 0 | 1 | 17 | NC | 1B | 0 | 1 | N.C | N.C | 72.49 | 142.47 | 767.19 | 0 | 0 |
| 30 | Benzyl salicylate | 118-58-1 | 0 | 1 | 2.9 | NC | 1B | 0 | 1 | N.C | N.C | 8.42 | 40.88 | 110.98 | 0 | 0 |
| 52 | Citronellol | 106-22-9 | 0 | 1 | 43.5 | NC | 1B | 1 | 0 | <167.4 | N.C | >2000 | >2000 | 375.00 | 1 | 1 |
| 76 | DMSO | 67-68-5 | 0 | 1 | 72 | NC | 1B | 0 | 0 | N.C | N.C | >2000 | >2000 | 4000.00 | 0 | 1 |
| 97 | Helional | 1205-17-0 | 0 | 1 | 16.4 | NC | 1B | 1 | 1 | 58.33 | 105.87 | 77.71 | 264.41 | 710.36 | 1 | 1 |
| 102 | alpha-Hexylcinnamaldehyde | 101-86-0 | 0 | 1 | 10.8 | NC | 1B | 1 | 1 | 2.91 | 79.96 | 17.27 | 4000.00 | 26.34 | 0 | 0 |
| 120 | alpha-Isomethylionone | 127-51-5 | 0 | 1 | 21.8 | NC | 1B | 1 | 0 | 304.18 | N.C | >2000 | >2000 | 60.03 | 0 | 1 |
| 122 | Isopropyl myristate | 110-27-0 | 0 | 1 | 44 | NC | 1B | 0 | 0 | N.C | N.C | >2000 | >2000 | 384.80 | 0 | 1 |
| 128 | *D*-Limonene | 5989-27-5 | 0 | 1 | 52.5 | NC | 1B | 1 | 0 | 47.76 | N.C | >2000 | >2000 | 118.10 | 0 | 1 |
| 160 | OTNE | 54464-57-2 | 0 | 1 | 14.2 | NC | 1B | 0 | 0 | N.C | N.C | >2000 | >2000 | 33.01 | 0 | 1 |
| 164 | Pentachlorophenol | 87-86-5 | 0 | 1 | 20 | NC | 1B | 0 | 0 | N.C | 0.12 | >2000 | >2000 | 27.70 | 1 | 1 |
| 180 | Salicylic acid | 69-72-7 | 0 | 1 | 12.2 | NC | 1B | 0 | 0 | N.C | N.C | >2000 | >2000 | 4000.00 | 0 | 1 |
| 181 | Sodium lauryl sulfate | 151-21-3 | 0 | 1 | 3.7 | NC | 1B | 0 | 0 | N.C | 12.20 | >2000 | >2000 | 44.70 | 0 | 0 |
| 190 | α-Tocopherol | 59-02-9 | 0 | 1 | 7.4 | NC | 1B | 0 | 1 | N.C | N.C | 114.60 | 4000.00 | 4000.00 | 0 | 0 |
| 25 | Benzyl alcohol | 100-51-6 | 1 | 0 | NC | 1B | NC | 0 | 0 | N.C | N.C | >2000 | >2000 | 4000.00 | 0 | 1 |
| 123 | Kanamycin | 59-01-8 | 1 | 0 | NC | 1B | NC | 0 | 0 | N.C | N.C | >2000 | >2000 | >2000 | 1 | 0 |
| 183 | Sulfanilamide | 63-74-1 | 1 | 0 | NC | 1B | NC | 0 | 0 | N.C | N.C | 2000.00 | 2000.00 | 2000.00 | 0 | 0 |

*1: Data was extracted from DASS database (OECD, 2023a)

*2: See Appendix-07. Data of "HU.GHS.BIN" and "Basketter_human_Call" in DASS database (OECD, 2023) were used for the analysis. The overall positive and negative judgement of the human data were made as follows. Positive call was assigned first when one of the above-mentioned items in DASS database indicated positive. Negative call was assigned when one of the items was NC (Not conclusive).

*3: "1": positive. "0": negative, NA: not available, NC: not conclusive

*4: Median-like location parameter; the mean of EC3 from independent LLNA assay.

*5: The GHS subcategories 1A and 1B were assigned using both "HU.GHS.SUB" and "Basketter_human_potency_3class" in the DASS database (OECD, 2023a). If either or both are 1A, 1A was assigned. If both are 1B, or if one is NC or NA and the other is 1B, then the subcategory was 1B. If both are NC, or if one is NA and the other is NC, then NC was assigned.

*6: The concentration calculated by linear regression of the two points of test concentration and nAA across nAA value 3.2 in the α-Sens®. N.C.: Not Calculated.

*7: The concentration calculated by linear regression of the two points of test concentration and cell viability across 50% cell viability. in the α-Sens®. N.C.: Not Calculated.

**Table 9-11 Chemicals for which the human data are available, but no LLNA data.**

| No.[*1] | Curated.name[*1] | CASRN[*1] | Summarised. Human. Call [*2, *3] | LLNA. Call [*1, *3] | LLNA. MLLP [*1, *4] | Human GHS Subcat. [*5] | LLNA GHS SUB. [*1] | α-Sens® Call [*3] | KS. Call [*1, *3] | α-Sens® EC3.2 [*6] | α-Sens® IC50 [*7] | KS. EC1.5 [*1] | KS. EC3[*1] | KS. IC50[*1] | DPRA. Call [*1, *3] | hCLAT. Call [*1, *3] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 6 | 4-Aminobenzoic acid | 150-13-0 | 0 | NA | NA | NC | NA | 0 | 0 | N.C | N.C | 4000.00 | 4000.00 | 4000.00 | 0 | 0 |
| 20 | Benzaldehyde | 100-52-7 | 1 | NA | NA | 1A | NA | 1 | 1 | 410.20 | N.C | 443.13 | 4000.00 | 4000.00 | 0 | 1 |
| 22 | Benzocaine | 94-09-7 | 1 | NA | NC | 1B | NA | 1 | 1 | 25.18 | 225.43 | 18.20 | 4000.00 | 4000.00 | 1 | 1 |
| 50 | Cinnamonitrile | 1885-38-7 | 1 | NA | NC | 1B | NA | 1 | 1 | 10.16 | 471.51 | 349.45 | 4000.00 | 4000.00 | 0 | 1 |
| 54 | Coumarin | 91-64-5 | 1 | NA | NC | 1B | NA | 1 | 1 | 52.38 | N.C | 60.03 | 479.96 | 1844.22 | 0 | 0 |
| 106 | Hydrocortisone | 50-23-7 | 0 | NA | NA | NC | NA | 0 | 1 | N.C | 63.73 | 912.20 | 1050.70 | 1559.90 | 1 | 0 |
| 147 | 6-Methylhepta-3,5-dien-2-one | 1604-28-0 | 1 | NA | NA | 1A | NA | 1 | 1 | 1.67 | 14.19 | 60.48 | 221.90 | 1520.44 | 1 | 1 |
| 155 | Neomycin sulfate | 1405-10-3 | 1 | NA | NA | 1B | NA | 0 | 0 | N.C | N.C | 4000.00 | 4000.00 | 4000.00 | NA | 1 |
| 188 | Thioglycerol | 96-27-5 | 1 | NA | NA | 1B | NA | 1 | 0 | 11.75 | 99.54 | 4000.00 | 4000.00 | 4000.00 | 1 | 1 |

*1: Data was extracted from DASS database (OECD, 2023a)

*2: See Appendix-07. Data of "HU.GHS.BIN" and "Basketter_human_Call" in DASS database (OECD, 2023) were used for the analysis. The overall positive and negative judgement of the human data were made as follows. Positive call was assigned first when one of the above-mentioned items in DASS database indicated positive. Negative call was assigned when one of the items was NC (Not conclusive).

*3: "1": positive. "0": negative, NA: not available, NC: not conclusive

*4: Median-like location parameter; the mean of EC3 from independent LLNA assay.

*5: The GHS subcategories 1A and 1B were assigned using both "HU.GHS.SUB" and "Basketter_human_potency_3class" in the DASS database (OECD, 2023a). If either or both are 1A, 1A was assigned. If both are 1B, or if one is NC or NA and the other is 1B, then the subcategory was 1B. If both are NC, or if one is NA and the other is NC, then NC was assigned.

*6: The concentration calculated by linear regression of the two points of test concentration and nAA across nAA value 3.2 in the α-Sens®. N.C.: Not Calculated.

*7: The concentration calculated by linear regression of the two points of test concentration and cell viability across 50% cell viability. in the α-Sens®. N.C.: Not Calculated.

## (2) GHS subcategorisation

### *Comparison with LLNA data*

With respect to GHS subcategories for LLNA, the positive rates in the α-Sens® for 1A and 1B were provided in Table 9-12. The positive rates for 1A and 1B were 94.7% and 67.9%, respectively. As GHS category 1, the positive rate was 76.5%. The negative rate in the α-Sens® for NC was 64.5%. The predictive capacity for KeratinoSens™ was provided in Table 9-12 for reference (See Appendix-07).

**Table 9-12 Predictive Capacity to GHS subcategorisation of LLNA data for in-house Chemicals**

| GHS Subcategory | Number of LLNA data* | Positive in α-Sens® | Positive rates in α-Sens® | Positive in KeratinoSens™ | Positive rates in KeratinoSens™ |
|---|---|---|---|---|---|
| 1A | 38 | 36 | 94.7% (36/38) | 34 | 89.4% (34/38) |
| 1B | 81 | 55 | 67.9% (55/81) | 53 | 65.4% (53/81) |
| 1 (1A+1B) | 119 | 91 | 76.5% (91/119) | 87 | 73.1% (87/119) |
| GHS Subcategory | Number of LLNA data* | Negative in α-Sens® | Negative rates inα-Sens® | Negative in α-Sens® | Negative rates in α-Sens® |
| NC | 31 | 20 | 64.5% (20/31) | 21 | 67.7 % (21/31) |

NC: not conclusive

*: "LLNA.GHS.SUB" in DASS database (OECD, 2023a) was used.

Only two 1A chemicals could not be detected as positive by α-Sens®. These were hexahydrophthalic anhydride (CAS 85-42-7) and phthalic anhydride (CAS 85-44-9), and both were classified as anhydrides. The DPRA results for these two chemicals indicated that both chemicals could bind to lysine rather than cysteine, particularly phthalic anhydride bound only to lysine. The key mechanism leading to the activation of the Keap1-Nrf2-ARE pathway appears to be the electrophilic reaction of stressors with nucleophilic thiols (cysteine sulfhydryl groups) of Keap-1 (OECD, 2024a).Therefore these chemicals give false negatives where binding specificity refers to the cysteine residue in the Keap-1 protein, anhydrides are recognised as out of domain for test systems that mediate the activation of the ARE-Nrf2 antioxidant pathway as an indicator. This includes α-Sens® and KeratinoSens™.

### *Comparison with human data*

With respect to GHS subcategories for human data, the positive rates in α-Sens® for 1A and 1B in the human data are provided in Table 9-13. The positive rates for 1A and 1B were 100% and 66.7%, respectively. For GHS category 1, the positive rate was 80.0%. The negative rate in the α-Sens® for NC was 75.0% (6/8). All 1A chemicals were positive in the α-Sens® and, therefore no human data GHS 1A chemicals were under-predicted as non-sensitisers in the α-Sens®. The predictive capacity for KeratinoSens™ is provided in Table 9-13 for reference (See Appendix-07).

**Table 9-13 Predictive Capacity to GHS subcategorisation of Human data for in-house Chemicals**

| GHS Subcategory | Number of Human data* | Positive in α-Sens® | Positive rates in α-Sens® | Positive in KeratinoSens™ | Positive rates in KeratinoSens™ |
|---|---|---|---|---|---|
| 1A | 33 | 33 | 100% (33/33) | 31 | 93.9% (31/33) |
| 1B | 49 | 37 | 75.5% (37/49) | 30 | 61.2% (30/49) |
| 1 (1A+1B) | 82 | 70 | 85.4% (70/82) | 61 | 74.4% (61/82) |
| GHS Subcategory | Humber of Human data* | Negative in α-Sens® | Negative rates in α-Sens® | Negative in α-KeratinoSens™ | Negative rates in KeratinoSens |
| NC | 29 | 23 | 79.3% (23/29) | 23 | 79.3% (23/29) |

*: The GHS subcategories 1A and 1B were assigned using both "HU.GHS.SUB" and "Basketter_human_potency_3class" in the DASS database (OECD, 2023a). If either or both are 1A, 1A was assigned. If both are 1B, or if one is NC or NA and the other is 1B, then the subcategory was 1B. If both are NC, or if one is NA and the other is NC, then NC was assigned.

## 9.4 Conclusion on predictive capacity

The average accuracy, sensitivity and specificity for participating laboratories were more than 80% in both cases for 20 reference chemicals and 25 test chemicals used in the validation study. Furthermore, the calculation following the Doc#213 for predictive capacity, accuracy, sensitivity and specificity was better than the VRM. Therefore, this validation study has successfully demonstrated that the α-Sens® has sufficient predictive capacity.

Analysis of a larger dataset using in-house data from the developer's laboratory (lead laboratory) suggested that the α-Sens® has successful predictive capacity for both LLNA and humans (Table 9-7 and Table 9-8). The VMT concluded that the α-Sens® has sufficient predictive capacity for the reference chemicals, LLNA data and human data.

# 10. Applicability domain

## 10.1 Limitations

The α-Sens® is a 'Me-Too' test of the cell-based reporter gene assay listed in OECD TG442D employing the same principle as KeratinoSens™ and LuSens, therefore the assay limitations are basically the same as these test methods. Namely, chemicals that bind only to lysine residues and pro/pre-haptens may be misjudged as negative, and chemicals that do not act as sensitisers but as chemical stressors that directly affect luciferase induction, such as phytoestrogens, may be misjudged as positive.

However, the following points are different from the existing test methods and are specific to the α-Sens® test.

**(1) Handling of cases of chemiluminescence contamination**

Strong chemiluminescence can cause light contamination in adjacent wells, which may affect the test results of other test chemicals. This light contamination is an important issue in luciferase reporter gene assays. For KeratinoSens™ and LuSens, no light contamination is allowed; therefore, a laboratory needs to ensure appropriate luminescence measurement prior to testing. On the other hand, the α-Sens® can handle HiLIC in the protocol. Therefore, the starting up the test system for the α-Sens® is simpler than other assays.

**(2) Differences in the principles of detecting cytotoxicity**

KeratinoSens™ and LuSens employ the MTT assay to measure cell viability, which is based on the mitochondrial dehydrogenase activity, requiring a separate plate from the one used for ARE activity measurement. In contrast, α-Sens® uses chemiluminescence from *Renilla* luciferase as an indicator for cell viability, allowing it to measure both cell viability and ARE activity in the same cells. It is also applicable to chemicals that interfere with MTT assay, which is listed as a limitation in the ESAC opinion for LuSens (ESAC, 2016). In addition, *Renilla* luciferase-based cytotoxicity test is reported to reflect the actual effect of the test chemical on the ARE-Nrf2 mediated transcriptional activity compared to the mitochondrial dehydrogenase activity-based cytotoxicity test (Maeda *et al*., 2020). In addition, as reported by Maeda *et al*. (2020), the MTT assay is less sensitive than the *Renilla* luciferase expression-based assay. This suggests that the MTT assay would underestimate the cytotoxic effect on the gene expression capacity of cells when compared to the *Renilla* luciferase expression-based assay. However, *Renilla* luciferase-based cytotoxicity test may not be used for test chemicals that directly affect *Renilla* luciferase activity.

## 10.2 Applicability domain

The α-Sens® validation results showed that highly reproducible results for test chemicals with hydrophobic chemicals ($logP \geq 3.5$) among three participating laboratories. Furthermore, since

α-Sens® was able to detect Bis-GMA (*logP* = 6.07), a skin sensitiser in the DASS database (OECD, 2023a), as positive, the α-Sens® could be applicable up to at least *logP* = 6.

In this validation study, a wide *logP* range (-1.86 ~ 9.4) of test chemicals were tested. The skin-sensitisers and non-sensitisers with the highest *logP* values that could be correctly judged were 4.52 (*D*-Limonene) and 3.9 (*n*-Hexane), respectively. Whereas the d-isomer of α-Tocopherol which has the highest *logP* value (9.4)) was judged as a non-sensitiser and the negative judgement of this chemical is strongly supported by the discussion in 8.3.1 (2).

α-Sens® is designed to use acetone as an alternative vehicle, in addition to DMSO which is frequently used in conventional safety tests using cells. In the validation study, high reproducibility was confirmed even for chemicals that dissolve only in acetone. Because there are many types of vehicles specifically indicated in the protocol, the range of applicable chemicals may also be broad. Therefore, this validation study demonstrated the practical application of the α-Sens® to these hydrophobic chemicals across multiple laboratories.

α-Sens® was validated using chemicals in gravimetric concentration, and high reproducibility was confirmed. As a result, it can be applied not only to a single chemical, but also to mixtures and chemicals with unknown molecular weights.

# 11. VMT overall conclusions and recommendations

## 11.1 Overall conclusions

The α-Sens® is a 'Me-Too' method of the ARE-Nrf2 mediated reporter gene assay listed in OECD TG442D, such as KeratinoSens™ and LuSens.

This validation study for the α-Sens® demonstrated that α-Sens® is highly transferable and reproducible within-laboratory and between-laboratory, and that its predictive capacity is equivalent and/or superior to the existing *in vitro* Skin Sensitisation ARE-Nrf2 Luciferase Test Methods in OECD TG442D, based on the findings below.

| | | | | |
|---|---|---|---|---|
| **Module 1:** | **Test Definition** | The essential test method components of α-Sens® were confirmed to be equivalent to that of the KeratinoSens™. | | |
| **Module 2:** | **WLR:** | 97.2% (91.7% (11/12) to 100% (12/12)) among three participating laboratories | | |
| **Module 3:** | **Transferability:** | 100% using 9 test chemicals in three participating laboratories | | |
| **Module-4:** | **BLR:** | 96.0% (24/25) among three participating laboratories | | |
| **Module 5:** | **Predictive Capacity** | | | |
| | **For 20 reference chemicals:** | Accuracy: 88.3%<br>Sensitivity: 91.7%<br>Specificity: 83.3% | | |
| | **For 25 test chemicals:** | Accuracy: 86.7%<br>Sensitivity: 87.5%<br>Specificity: 85.2% | | |
| | **For larger dataset** | In-house data by the Lead laboratory. | | |
| | | | LLNA (n=162) | Human (n=112) |
| | | Accuracy: | 74.1% | 83.0% |
| | | Sensitivity: | 76.3% | 84.3% |
| | | Specificity: | 64.5% | 79.3% |
| **Module 6:** | **Applicability domain** | The α-Sens® is applicable to mixtures, unknown molecular weight chemicals, chemicals soluble in DMSO, acetone or medium, and chemicals up to *logP* 4.52, and possibly up to 6.07. | | |

The limitation of the assay is basically the same as the existing *in vitro* Skin Sensitisation ARE-Nrf2 Luciferase Test Methods in OECD TG442D. The differences of the limitation could be based on the cell viability evaluation system. A test chemical that directly affects *Renilla* luciferase transcription cannot be evaluated in the α-Sens®.

The advantages of the α-Sens® are outlined below.

- serum (FBS) free operation throughout the assay, including cell culture propagation

and maintenance
- measurement of ARE activity and cell viability in the same exposed cells by using the dual-luciferase assay system
- high transcriptional induction of reporter gene product with minimal background induction, achieving an extremely high S/N ratio
- long-time stability of the responsiveness up to 70 passages for 9 months
- high transferability
- high reproducibility
- high predictive capacity
- efficient workability.
- reduction of plastic waste

Accordingly, the VMT concluded that the α-Sens® is a robust and reliable 'me-too' method compared to the ARE-Nrf2 mediated reporter gene assay included in OECD TG442D.

## 11.2 Recommendations

The predictive capacity for the defined approach for skin sensitisation (DASS) using α-Sens® and other KE1 and/or KE3 assays in OECD guideline No.497 can be updated to include the α-Sens® in the OECD guideline No.497.

# 12. Acknowledgement

This validation study was primarily funded by JaCVAM with the Japan Agency for Medical Research and Development (AMED) under Grant Number JP24mk0101223, which provided most of the funds for the test chemicals, positive control, vehicles, cell culture medium and luciferase assay reagent. The QC personnel at JaCVAM generously supported the QC process for this validation study.

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Version 2.1
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# Appendix-01 Study plan for the validation trial on the α-Sens® as a test method to evaluate the skin sensitisation of chemicals

α-Sens® Validation Management Team

INDEX

## 1. Background

### *Background of α-Sens® development*

Skin sensitization is a hazard endpoint of great importance and the adverse outcome pathway (AOP) initiated by covalent binding of chemicals to skin proteins as molecular initiating event (MIE) has been developed for skin sensitization. As the next step of this MIE, induction of inflammatory responses as well as changes in gene expression relevant to specific cell signaling pathways such as antioxidant/electrophile response element (ARE)-dependent pathways in keratinocytes has been identified in this AOP as Key Event 2 (KE2).

Organization for Economic Co-operation and Development (OECD) Test Guideline (TG) 442D (*in* vitro Skin Sensitisation. ARE-Nrf2 Luciferase Test Method) has been developed to detect for this KE2 as keratinocyte activation [OECD, 2022]. Currently, the following two assays are available in this TG442D.

- The ARE-Nrf2 luciferase KeratinoSens™ test method
- The ARE-Nrf2 luciferase LuSens test method

Although these assays had been validated as OECD TG, the ARE activity and cytotoxicity need to be evaluated on the separate plates and extra time for cytotoxicity is necessary. The predictive performance of skin sensitization of the existing methods has room for improvement. Furthermore, from the animal welfare consideration point of view, KeratinoSens™ with the Xeno-free medium method using human serum has also been adopted in TG442D, but human serum is difficult to obtain in some countries, and there are large lot-to-lot variation in human serum in general. Therefore, defined media are encouraged to replace FBS-free, that are easy-to-use and with better-performance in test methods.

In order to overcome these aspects, the α-Sens®, an assay for assessing skin sensitisation potential, was developed by Chemical Evaluation and Research Institute, Japan (CERI) as an alternative to *in vivo* testing.

α-Sens® cell line was originally developed as "α-Sens (without ®)", the multi-clone cell system of PHK 16-0b cell line (immortalized human keratinocyte by HPV16 virus genome) stably transfected with two reporter constructs using Lentiviral Vector System, Antioxidant response element (ARE) inducible *Firefly* luciferase gene for ARE response and Thymidine Kinase (TK) promoter-driven *Renilla* luciferase gene for cell viability measurement (Maeda et al., 2020). α-Sens was further improved by modifying the reporter gene construct and was established as a single clone cell system, "α-Sens®". The details of the modifications made in the current α-Sens® are provided in Appendix A with comparison with the original multi-clone system.

The advantages of α-Sens® is that the performance of α-Sens® is equivalent or higher than the other existing assays in the primary analysis, and α-Sens® can be conducted with

the simpler operation and resource saving conditions than other KE-2 assay. In addition, the assay can be conducted with less animal welfare issues than the other KE-2 assays by using FBS free media.

## 2. Objective of the validation trial

The aim of this trial is to validate the α-Sens® method, in order to incorporate this test method as a me-too assay in Organization for Economic Co-operation and Development (OECD) Test Guideline (TG) 442D (*in vitro* Skin Sensitisation. ARE-Nrf2 Luciferase Test Method) [OECD, 2022].

The validation trial will assess the reliability (reproducibility within and between laboratories) and relevance (predictive capacity) of the α-Sens® assay with a set of test chemicals for which high quality in vitro and in vivo data are available.

The validation trial for the α-Sens® will be undertaken i) in accordance with the principles and criteria documented in the OECD No. 34 Guidance Document (GD) on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment [OECD, 2005], ii) according to the Modular Approach to validation [Hartung *et al*., 2004] ,iii) according to the concept discussed on the validation trials with participation of Good Laboratory Practice (GLP) Test Facilities [OECD, 2000] where the whole concept of the validation trials is described in the context of GLP. The validation trial for this assay will be performed under the spirit of GLP.

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 the use of datasets from various data sources and studies. This advantage will be used to assess the scientific validity of the α-Sens®.

## 3. Validation Management Team (VMT)

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

The VMT, which plays a central role overseeing the conduct of the validation trial, includes members. Members of VMT including VMT organizer and Observers of VMT are as described in Table 1-1 and Table 1-2, respectively.

**Table 1-1. Members for the α-Sens® assay VMT**

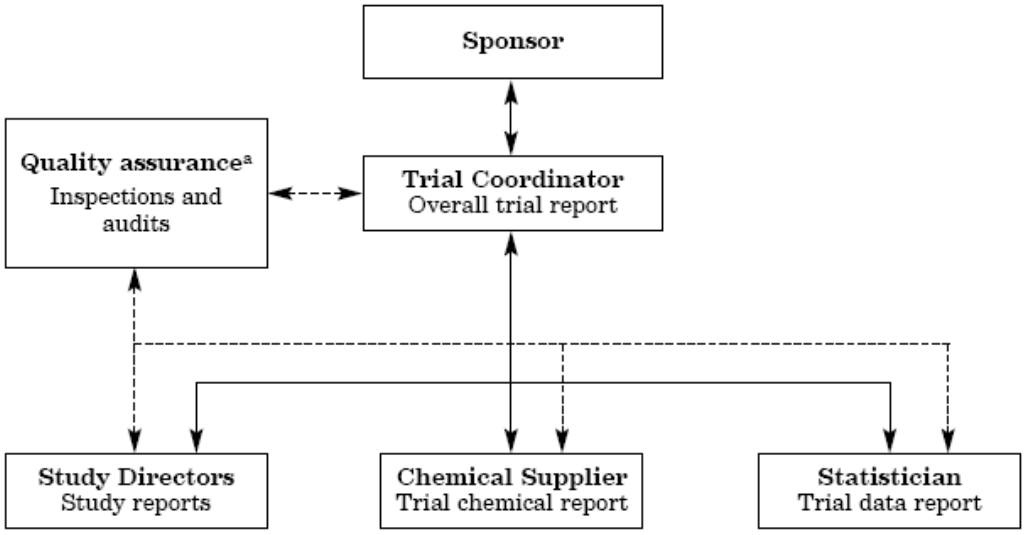
| Name | Role and expertise | Affiliation |
|---|---|---|
| Prof. Atsushi Ono | VMT trial coordinator (see section 3.2) | Okayama University, Japan |
| Prof. Kazuhiko Matsumoto | Data analysis, biostatistics (see section 3.4) | Nagoya City University, Japan |
| Dr. Miriam N Jacobs | Test system expertise, validation expertise | UK Health Security Agency |
| KoCVAM liaison<br>Ms. Namhee Kang | Test system expertise | Korean Centre for the Validation of Alternative Methods (KoCVAM), Ministry of Food and Drug Safety (MFDS), Korea |
| Japanese liaison<br>Prof. Tomoki Fukuyama | Test system expertise | Azabu University, Japan |
| Dr. Hajime Kojima | Quality check (QC) and record receiving. (see section 3.4) Validation expertise | Japanese Centre for the Validation of Alternative Methods (JaCVAM), National Institute of Health Sciences (NIHS), Japan / Sanyo-Onoda City University, Japan |
| Dr. Akiko Ohno | Quality check (QC), Validation expertise | JaCVAM, NIHS, Japan |
| Dr. Takao Ashikaga | Chemical supply, sponsor (see section 3.7), Validation expertise | JaCVAM, NIHS, Japan |
| Dr. Yumi Akahori | VMT organizer Validation expertise | CERI, Japan |

**Table 1-2. Members of Observers of VMT**

| Name | Roles and expertise | Affiliation |
|---|---|---|
| Dr. Masahiro Takeyoshi<br>Dr. Yosuke Maeda | Lead Laboratory (see section 3.5), developers of α-Sens® | CERI, Japan |
| TBD | Participating Laboratories (see section 3.6) | ◆ Food and Drug Safety Center (FDSC)<br>◆ Sumitomo Chemical Company, Limited<br>◆ Jai Research Foundation |

### 3.1. Trial management structure

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




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

*Dashed lines indicate assurance staff involvement.*

**Figure 1: Management Structure of the α-Sens® validation trial**

### 3.2. Trial coordinator

Dr. Ono Atsushi has been appointed as the Trial Coordinator with well-defined roles and responsibilities to coordinate the trial and to establishment of a VMT by supporting of JaCVAM.

For the α-Sens® validation trial, the Trial Coordinator has direct access to the coding of test chemicals.

The Trial Coordinator's responsibilities include:

a) Supports to lead laboratory, including meeting organization
b) Communication and coordination with test facilities for the validation trial
c) Assessing and documenting the impact of any amendments and/or deviations from the trial plan and study plans on the quality and integrity of the validation trial
d) Ensuring that the individual study reports from each participating test facility are forwarded, in a timely manner, for data and statistical analysis
e) Preparing the trial plan and report, which can be based on the study reports from the lead laboratories and other test facilities involved in the validation trial, and should reflect the overall trial
f) Approval with date and signature of all protocols, Study Plans and Study Reports
g) 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 Study Directors (SDs)) is of fundamental importance. The Trial Coordinator is the single critical point of trial control and must ensure clear lines of communication between the involved test facilities in the trial. The communication line of the Trial Coordinator is the SDs of each test facilities. The SDs are the single point of contact with the Trial coordinator (unless otherwise communicated by the participating Test Facilities) to assure a transparent and recorded documentation flow during the trial. The Trial Coordinator should also ensure that appropriate arrangements have been made for the supply of the test systems, and test chemicals, control chemicals and so on, which meet the requirements of the trial. Furthermore, the Trial Coordinator should ensure 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.

### 3.3. Data analysis

A biostatistician within the VMT group will be responsible for data analysis. The data obtained from this validation study will be analyzed from the position of a third party.

### 3.4. Quality assurance

JaCVAM will manage the quality assurance group. JaCVAM will prepare and distribute protocol, test chemical preparation record forms (worksheets), blank spreadsheets, etc. to the laboratories participating in this validation trial. JaCVAM will also collect filled out forms (worksheets) and spreadsheets after completion of experiments, pointing out omissions or flaws in recording, if any, and requesting correction of such errors.

JaCVAM will confirm the accurate reflection of the validation trial data to validation report and will prepare Quality Check (QC) statement.

### 3.5. Lead laboratory

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

The lead laboratory will support the α-Sens® validation trial. Lead laboratory will support:

- the evaluation of preliminary results of the test method
- the availability of the α-Sens® cells to the participating test facilities by supporting the Lead laboratory with the logistics for delivering the test system to the facility
- the independent data analysis and statistical support (biostatistician) for generation of the study reports.

### 3.6. Participating Test Facilities

The laboratories participating in the trial are defined in Table 2.

**Table 2 Participating laboratories in the validation trial**

| | Name of facility [GLP facility or not] | Study Director (SD) |
|---|---|---|
| Test facility 1 | Food and Drug Safety Center (FDSC)<br>729-5 Ochiai, Hadano, Kanagawa, Japan 257-8523<br>[GLP facility] | Dr. S. Tachibana |
| Test facility 2 | Sumitomo Chemical Company, Limited<br>Environmental Health Science Laboratory<br>3-1-98 Kasugade-naka, Konohana-ku, Osaka-shi, Japan 554-8558<br>[not GLP facility] | M. Sc. R. Kobayashi |
| Test facility 3 | Jai Research Foundation<br>NH48, Near Daman Ganga Bridge, Valvada, District Valsad, Gujarat, India 396105<br>[GLP facility] | M. Sc. Priyanka K. Mishra |
| Lead Lab | Chemicals Evaluation and Research Institute, Japan (CERI)<br>Chemicals Assessment and Research Center<br>1600 Shimotakano, Sugito-machi, Kitakatsushika-gun, Saitama, Japan 345-0043<br>[CERI has GLP facility in other area but the laboratory, participating this validation study, is not GLP facility] | Dr. Y. Maeda |

Information relevant for Modules 2, 3 and 4 will be performed by all laboratories above and Lead laboratory (CERI). All 3 laboratories participating in this validation have no experience with α-Sens®. Data obtained by these laboratories and Lead laboratory will be used to demonstrate the transferability and reproducibility of α-Sens®.

All test facilities need to prepare their own study plan according to internal GLP principle, following the validation plan agreed by the VMT. These plans will be submitted to the Trial Coordinator and lead laboratory for approval.

After the each phase, the test facilities will submit a study report including raw data, all the spreadsheets and worksheets required to be filled out to the Trial Coordinator.

### 3.7. Sponsor

JaCVAM will support the financial cost for test chemicals, vehicles and its distribution (including coding), and part of the test consumables for the α-Sens®. Also, JaCVAM may support a part of the financial aspect to coordinate organizing VMT meeting by face-to-face based, if necessary. The other financial cost will be voluntarily covered.

## 4. Modules to be evaluated

This validation trial has 2-phase structure as shown in Table 3.

During the Phase-II, just after the 1st set of experiment was completed in Phase-II, the spreadsheets and worksheets for 1st set of experiment should be submitted to the Trial Coordinator for check that the revised part of the protocol is properly handled at each laboratory.

**Table 3. Outline of test planning at each study in the validation trial.**

| Study | Chemicals | Number of qualified Experiment[*1] | Number of qualified Run[*2] | Between-laboratory transferability | Within-laboratory reproducibility (WLR) | Between-laboratory reproducibility (BLR) |
|---|---|---|---|---|---|---|
| | | | | *Assessed with Phase-I data* | *Assessed with Phase-I and Phase-II data* | *Assessed with Phase-I and Phase-II data* |
| Phase-I | 24 coded chemicals (8 identical chemicals) | 3 | At least 2 | ○ | ○ | ○ |
| | 1 coded chemical | 1 | At least 2 | ○ | | ○ |
| Phase-II | 12 coded chemicals (4 identical chemicals) | 3 | At least 2 | | ○ | ○ |
| | 12 coded chemicals (12 identical chemicals) | 1 | At least 2 | | | ○ |
| Criteria | | | | All qualitative conclusion of each test chemical from all participating laboratories should meet its conclusions from the lead laboratory. | The proportion of concordance among 12 identical chemicals within each participating laboratory against known skin sensitization classification should be equal or higher than 80% as tentative acceptance criteria after completion of for the phase-II validation, as defined in OECD GD213 (OECD, 2015). | The proportion of concordance between-laboratory reproducibility should be equal or higher than 80% as acceptance criteria as described in OECD GD213 (OECD, 2015). |

[*1] One “qualified experiment” consists of at least two qualified independent runs in case concordant results are obtained, or of three qualified independent runs in case of discordant results in the first two runs.

[*2] Each run consists of three replicates for each concentration tested. Negative (solvent) control and positive control should be tested concurrently with the test chemical in each run.

### 4.1. [Module 2] Within-laboratory reproducibility

Twelve specific reference chemicals have been recommended to confirm the between-laboratory reproducibility in GD No.213 (GD213), "Performance standards for assessment of proposed similar or modified *in vitro* skin sensitisation ARE-Nrf2 Luciferase test methods" (OECD, 2015) and **these 12 reference chemicals will be the first candidates** for within-laboratory reproducibility. Each of twelve **coded** chemicals will be tested to produce **three qualified experiments to derive three qualitative conclusions** in each laboratory. Twelve chemicals will be separately tested for 8 in phase-I and 4 chemicals in Phase-II.

"One qualified experiment" consists of at least two qualified independent runs in case concordant results are obtained, or of three qualified independent runs in case of discordant results in the first two runs, for a qualitative conclusion of each test chemical. "Run" means each assay including three replicates for each test concentrations of each test chemical. Therefore, three-experiment will give three qualitative conclusions for each test chemical. OECD GD213 requested that "An assessment of within-laboratory reproducibility should show a concordance of predictions (positive versus negative) obtained in three different, independent qualified experiments of the 12 recommended reference substances within each participating laboratory equal or higher (≥) than 80.0%."

For this assessment, the three qualitative conclusions for each chemical at each lab will be summarized by applying the following rules to calculate its concordance of prediction.

<Rules>

| |
|---|
| >= 2 positive conclusions → Positive |
| >= 2 negative conclusions → Negative |

### 4.2. [Module 3] Between-laboratory transferability

This between-laboratory transferability (Module 3) is performed in order to assess the successful transfer of the assay to a test facility without knowledge of the test method to be evaluated but having knowledge of similar test systems and endpoint detection methods.

The Phase-I study using **coded nine chemicals** will be performed. **Three-experiment for 8 chemicals and one-experiment for one hydrophobic chemical are required** in Phase-I. Three qualitative conclusion per chemical for 8 chemicals and one qualitative

conclusion for one hydrophobic chemical are derived. The definition of "experiment" is same as described in "4.1 [Module 2] Within-laboratory reproducibility". Multiple test concentrations of each test chemical will be tested in each independent run according to the α-Sens® protocol describing the details of the experimental design.

The results of the between-laboratory transferability will be reviewed before proceeding to module 4 on the between-laboratory reproducibility.

The three qualitative conclusions for each chemical at each lab will be summarized by applying the following rules. Thus obtained all qualitative conclusion of 9 test chemicals from all participating laboratories should meet its conclusions from the lead laboratory. If the transferability data do not meet this acceptance criteria, the Trial Coordinator representing the VMT will try to identify the problems and make protocol corrections where needed.

<Rules>

| |
|---|
| >= 2 positive conclusions → Positive |
| >= 2 negative conclusions → Negative |

### 4.3. [Module 4] Between-laboratory reproducibility

Twenty reference chemicals, including 12 test chemicals to be used for the between-laboratory reproducibility, have been recommended to confirm the between-laboratory reproducibility in OECD GD 213 and **these 20 reference chemicals will be the first candidates** for between-laboratory reproducibility. In total up to 25 test chemicals can be selected by VMT. All chemicals for between-laboratory reproducibility shall be **coded**. Twelve coded test chemicals used for within laboratory reproducibility and additional 13 **coded** test chemical (in total, 25 identical chemicals) will be tested. For additional 13 chemicals, **one-experiment** to derive one qualified conclusion for each chemical is required. The definition of experiment is same as described in "4.1 [Module 2] Within-laboratory reproducibility". Multiple test concentrations of each test chemical will be tested in triplicate according to the α-Sens® protocol describing the details of the experimental design.

For the between-laboratory reproducibility, OECD GD213 requested that "the concordance of predictions (positive versus negative) between a minimum of three laboratories, obtained for the 20 recommended reference substances (shown in Table 1), should be equal or higher (≥) than 80.0%".

For chemicals that have three qualitative conclusions for each chemical at each lab, the following rules will be applied to summarize conclusion for each chemical to calculate

the concordance of predictions of each laboratory.

<Rules>

| |
|---|
| >= 2 positive conclusions → Positive |
| >= 2 negative conclusions → Negative |

### 4.4. [Module 5] Predictive capacity

According to OECD GD213, the following are requested,

- "The accuracy, sensitivity and specificity of the proposed similar or modified test method should be comparable or better to that of the VRM".
- "The accuracy, sensitivity and specificity obtained with the 20 reference substances listed in Table 1 should all be equal or higher (≥) than 80.0%".
- "Furthermore no strong or extreme sensitisers should be under-predicted as non-sensitiser, unless a clear rationale can be given".

The necessity for further chemical analysis will be subject to a VMT decision once the data of the between laboratory reproducibility are assessed.

## 5. Protocol

The protocol will be drafted by the lead laboratory and be finalized by VMT.

## 6. Chemicals

### 6.1. Chemicals Selection

For demonstrating the performance of proposed similar or modified *in vitro* skin sensitization ARE-Nrf2 luciferase test methods as OECD TG442D, OECD GD213 have recommended test chemicals, which had been used in the validation and in-house studies of KeratinoSens™ (this method is included in OECD TG442D and is the validated reference method used in OECD GD213). Recommended reference chemicals are selected based on the following considerations,

- Representative of the range of Skin sensitization potency (10 < weak; 1< moderate <10; 0.1< strong < 1; extreme < 0.1)
- Performance characteristics of KeratinoSens™
- Well defined chemical structure

- Variety of mechanism of action (including pro-haptens, oxidising chemicals, adduct forming chemicals, Michael acceptors, Schiff base formation, acyl transfer chemicals, aryl electrophile, electrophile-H-polar chemical)
- Variety of chemical categories based on their functional group
- induce to the extent possible definitive results in the in vivo reference test method
- commercially available
- not associated with prohibitive disposal costs.

In addition to the chemicals that can be selected from the reference chemicals as recommended in OECD GD213, the following considerations are addressed;

- α-Sens® is not optimized using only these reference chemicals,
- The 20 reference chemicals are needed to compare performance with the existing assays.

Keeping in mind these selection considerations, the VMT will carefully select test chemicals for the Phase-I and Phase-II in this validation trial, taking into account skin sensitization potency (including GHS sub-category 1A, 1B) etc.

Nine test chemicals for the Phase-I (coded, 9 chemicals for transferability and for part of data collection for between-laboratory reproducibility, and 8 chemicals for part of data collection for within-laboratory reproducibility) will be selected by the VMT to cover range of known skin sensitizing potency (Extreme, Strong, moderate, weak) and negatives, including chemicals possessing cytotoxicity.

As for test chemicals for Phase-II, the 20 recommended reference chemicals from OECD GD213 will be the first candidates and re-evaluated by the VMT. In addition to these chemicals, hydrophobic chemicals (*logP/Kow* > 3.5) will be selected by VMT. It should be noted that negative chemicals will be selected approximately 40% of the total test chemicals, in order to increase the predictability of the data analysis.

Also, the following information will be confirmed by chemical repository based on published papers on skin sensitization

- 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 Chemical Abstracts Service (CAS) No.)
- single chemical entities or formulations of known high purity
- availability
- costs

After discussion of Phase-I results, detailed test planning of the Phase-II will be determined.

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

### 6.2. Chemicals Acquisition, Coding and Distribution

The assessment of within-laboratory reproducibility (Module 2) and between laboratory reproducibility (Module 4) in all test facilities will be performed using coded chemicals.

Chemicals will be distributed through JaCVAM. The coding will be supervised by the Trial Coordinator, responsible of coding and distribution of test, reference and control chemicals for the validation trial.

### 6.3. Handling

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

## 7. Records and archiving

The recording documents and data from test facilities will be archived at JaCVAM until it will be judged unnecessary.

## 8. Preparation of Validation Report

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

## 9. Study timeline

An approximate schedule for α-Sens® validation trial is shown in Table 4.

Duration of this validation trial until the completion of the validation report is around 2 years from April 2023 to March 2025.

**Table 4. Schedule of α-Sens® validation trial**

| Month | Activity |
|---|---|
| Feb. 2023 | ■ Establish the VMT, **1st VMT Meeting (2/27)**<br>• Discussion, decision and read-through of draft study plan<br>• Discussion on protocol<br>• Discussion on Phase-I and Phase-II chemicals |
| Mar.- 2023 | Training |
| May. 2023 | ■ **2nd VMT Meeting**<br>• Agreement on plan of Phase-I trial<br>• Discussion and decision of test chemicals to be tested in Phase-I<br>• Discussion on Phase-II chemicals |
| July 2023 | ■ **3rd VMT**<br>• Discussion on training result |
| August 2023 | ■ **4th VMT**<br>• Discussion on training result<br>• Discussion and agreement on protocol |
| Oct.-Nov. 2023 | ■ **Phase-I chemical distribution** |
| Nov. 2023 | ■ Start of Phase-I<br>• Discussion and agreement on Phase-II chemicals vis e-mail. |
| March. 2024 | End of Phase-I (Early March) |
| May 2024 | ■ **5th VMT Meeting**<br>• Discussion on Phase-I results<br>• Agreement on plan of Phase-II trial |
| June 2024 | ■ **Phase-II chemical distribution** |
| June-July 2024 | ■ Start of Phase-II |
| Sep.-Oct. 2024 | ■ End of Phase-II |
| Nov.-Dec. 2024 | ■ **6th VMT Meeting**<br>• Discussion on Phase-II result |
| Dec.-Feb. 2024 | ■ **Preparation of Draft Validation Report** |
| Feb. 2024 | ■ **7th VMT Meeting**<br>• Discussion on Draft Validation Report |
| March 2024 | Complete the preparation of draft validation report and draft revised test guideline by VMT. |

## 11. Abbreviations

CAS: Chemical Abstracts Service
GD: Guidance Document
GLP: Good Laboratory Practice
FDSC: Food and Drug Safety Center
JaCVAM: Japanese Centre for the Validation of Alternative Methods
KoCVAM: Korean Centre for the Validation of Alternative Methods
NIHS: National Institute of Health Sciences
OECD: Organization for Economic Co-operation and Development
QC: Quality Check
SD: Study Director
TG: Test Guideline
VMT: Validation Management Team

## 12. Reference

Hartung *et al*.(2004) A modular approach to the ECVAM principles on test validity. Altern Lab Anim; 32(5); 467-72.

Maeda *et al.* (2020) α-Sens®: The improved ARE-Nrf2-based sensitization screening assay using normalized transcriptional activity. Toxicology; 439: 152476. doi: 10.1016/j.tox.2020.152476

OECD (2000) OECD series on principles of good laboratory practice and compliance monitoring No 5, Compliance of Laboratory Suppliers with GLP Principles. Paris, France: Organisation for Economic Cooperation and Development.

OECD (2005) OECD *Series on Testing and Assessment, Number 34: Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment*, OECD ENJ/JM/MONO(2005)14, OECD Publishing: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2005)14&doclanguage=en

OECD (2015) OECD *Series on Testing and Assessment, Number* 213. Performance Standards for assessment of proposed similar or modified in vitro skin sensitisation ARE-NrF2 luciferase test methods.: https://www.oecd.org/env/ehs/testing/Performance-Standards-for-Assessment-of-Proposed-Similar-or-Modified-in-vitro-Skin--Sensitisation-ARE-NRF2-Luciferase-Test-Methods.pdf

OECD (2022) OECD Test Guideline No. 442D In Vitro Skin Sensitisation. ARE-Nrf2

Luciferase Test Method.: https://www.oecd-ilibrary.org/docserver/9789264229822-en.pdf?expires=1666587690&id=id&accname=guest&checksum=F249F7E27CF421CFC5A47EFC871EFECB

**α-Sens® Validation Report**
**Appendix-02**

Appendix-02 - Quality check

This Appendix includes:

- A QC report for α-Sens® validation study (Phase-I and Phase-II)
- A QC Statement
- Signatures by Trial coordinator, Biostatistician and Study Directors from the Laboratories

# *Quality check (QC) Report for α-SENS validation study*

Akiko Ohno and Hajime Kojima
by the QC team in the validation study

May 30, 2025

## 1. Reports and sheets used for QC checking

### 1-1-1 Type of documents

Reports and sheets used in the α-SENS validation study were shown below.

1) Study report (PDF format)
2) Worksheet (for Material Preparation、Cell Handling and α-Sens Assay) (PDF format)
3) Data spreadsheet (EXCEL format)
4) Raw data (plate data): CERI (PDF format), FDSC (EXCEL format), JRF (JPG format), Sumitomo Chemical (CSV format)

### 1-1-2 Distributed by JaCVAM the media, luminescent reagent and test chemicals

The media, luminescent reagents and coded test chemicals were distributed by JaCVAM to each facility. The QC personnel of JaCVAM checked they were quality-assured without any problems within the expiration dates for both Phase I and Phase II (see appendix 1)

Additionally, we clarified there were no problems with the record for preparation and shipment of the coded test chemicals conducted by JaCVAM to each testing facility.

### 1-2 Rename of files

In Phase I, the QC personnel of JaCVAM revised the file names for the data spreadsheets and worksheets sent from four facilities: FDSC, JRF, Sumitomo, and CERI. Similarly, in Phase II, we also revised the file names for the data spreadsheets.

### 1-3 Documentation archiving

All documents were classified and are archived on a JaCVAM-restricted site. Printed materials are archived at JaCVAM until this test method becomes an OECD TG.

## 2. QC check results for sheets and files

### 2-1 Phase I

### 2-1-1 Data spreadsheets

After completion of Phase I at testing facilities, the sheets and files sent from all three participating laboratories and the lead laboratory were checked by the QC personnel of JaCVAM.

The number of data spreadsheets received from each facility was shown below.

FDSC: 33, JRF: 26, Sumitomo: 46, CERI (Lead lab.): 28

### 2-1-2 Results

There were no issues with the essential content of the test data from each testing facility, but the following deficiencies were found. The facilities were contacted and requested for revision.

*FDSC*: There were two corrections. The first correction was a discrepancy between the data in the FDSC data spreadsheet (Plate Name: FDSC_231220B_Na_3, Sheet Name: LayoutB-(3)) and the raw data. This was confirmed by the QC personnel of JaCVAM to be a transcription error from the raw data and was corrected appropriately.
The second correction was a correction to the FDSC worksheet (folder name: 240228_FDSC_AssayWS_03, file name: 240227_Chemical Exposure (page 5), and there was a correction in the worksheets for Chemical Exposure for this plate. The tests conducted on this day should have been ASA305, ASA307, and ASA308. However, ASA301 was listed. The QC personnel of JaCVAM confirmed that this was not a mistake for ASA305 and made the appropriate correction (301 → 305).

*JRF*: All of the worksheets were checked by SD, but there was no signature by SD on each worksheet. In accordance with the proposal from the QC personnel of JaCVAM,‘SD Certification was used in place of a signature’was decided. (→Worksheet folder of JRF,

file name: SD Certification (PDF)).

*Sumitomo, CERI (Lead lab.)*: No deficiency was observed.

## 2-2 Phase II

### 2-2-1 Data spreadsheets

After completion of Phase II, the sheets and files sent from all three participating laboratories were checked by the QC personnel of JaCVAM.

The number of data spreadsheets received from each facility was shown below.
FDSC: 35, JRF: 35, Sumitomo: 40, CERI (Lead lab.):38

### 2-2-1 Results

There were no issues with the essential content of the test data from each testing facility, but the following deficiencies were found. The facilities were contacted and requested for revision.

*CERI*: The Excel file'CERI_S1_240718_sa_5'was missing. Even though precipitation was observed in the stock solution prepared on the day, the exposures were done. Therefore, the CERI's SD responded that the relevant data was rejected. The reason for rejecting the data from'CERI_S1_240718_sa_5' was that, while the first solubility test showed no issues, a slight precipitation was visually observed during the second test, leading to a judgment of potential precipitation.
In response, the CERI's SD confirmed that after re-evaluating the solubility, no precipitation was found, and the test was performed without issues. The JaCVAM QC personnel confirmed that this fact was documented in the raw data sheet by CERI's SD.

*FDSC, Sumitomo, CERI (Lead lab.)*: No deficiency was observed.

## 3. Conclusion

The QC personnel of JaCVAM conducted a detailed review of the main contents of the test data submitted by each of four laboratories (FDSC, JRF, Sumitomo, and CERI) in Phase I and Phase II of this validation study. As a result, the test data, Excel spreadsheets, worksheets, raw data, and reports submitted by each facility met all the quality standards, and no issues were found the reliability of the test results. Based on the above, the QC personnel of JaCVAM concluded that the data quality of Phase I and Phase II was sufficiently ensured and that the test results were reliable.

## 4. Acknowledgements

We would like to express our sincere gratitude to Ms. Tamaki Yoshikawa, Dr. Shigenobu Hagino and Ms. Yasuko Sato for their support as the QC personnel of JaCVAM. The submitted data and related documents were organized into four different folders (test data, Excel spreadsheets, worksheets, raw data, and reports) for each facility in Phase I and Phase II and stored in a state that allowed for smooth review work.

## Appendix 1 Information of medium and reagents supported by JaCVAM

**Phase I**

1) Item code: 00192060
Description: Lonza KGM Gold Keratinocyte growth medium Bulletkit 500mL
Product No: 192151
Lot No.0001232410 (Expired date: 2024/7/15)
Product No: 192152
Lot No.0001223983 (Expired date: 2024/8/27)

2) Item code: E2920
Description: Promega Dual-Glo Luciferase Assay System 10mL (Promega) 10mL
Lot No.0000588952 (Expired date: 2024/8/21)

**Phase II**

3) Item code: 192060
Description: Lonza KGM Gold Keratinocyte growth medium Bulletkit 500mL
Product No: 192151
Lot No.0001288560 (Expired date: 2025/2/19)
Product No: 192152
Lot No.0001281090 (Expired date: 2025/3/11)

4) Item code: E2920
Description: Promega Dual-Glo Luciferase Assay System 10mL (Promega) 10mL
Lot No.0000617563 (Expired date: 2025/6/5)

# Quality Statement for α-SENS validation study

All validation study for α-SENS were performed in non-GLP facilities but adhered to the principles of GLP (OECD, 1998).

Therefore, all the data obtained during the validation process were collected and analyzed by the QC team in validation management team. Specifically, all records (data spreadsheets, raw data, study reports and worksheets) submitted by the four participating facilities (FDSC, JRF, Sumitomo, and CERI) in Phase I and Phase II of this validation study were thoroughly checked by the QC team. The QC team clarified all data to be within acceptable ranges in the validation study plan.

May 30th, 2025

Akiko Ohno, Ph.D.
JaCVAM, NIHS, Japan

Hajime Kojima, Ph.D.
Sanyo-Onoda City University, Japan

Trial Coordinator:

Prof. Atsushi Ono

Okayama University, Japan.

Signature: 小野 敦

Date: June. 10, 2025

**Biostatistician:**

Dr. Kazuhiko Matsumoto

Nagoya City University, Japan.

Signature: Kazuhiko Matsumoto

Date: June 3th. 2025

Participating laboratories

Food and Drug Safety Center (FDSC)
Study director: Dr. S. Tachibana

Signature: S. Tachibana

Date: June 4, 2025

Participating laboratories

Sumitomo Chemical Company, Limited
Study director: M. Sc. R. Kobayashi

Signature: Ryota Kobayashi

Date: June 5, 2025

JAI RESEARCH FOUNDATION

Participating laboratories

Jai Research Foundation (JRF), India
Study director: M. Sc. Priyanka K. Mishra

Signature: P Mishra

Date: June 04, 2025

Lead laboratory

Chemicals Evaluation and Research Institute, Japan (CERI)

Study director: Dr. Y. Maeda

Signature: Yosuke Maeda

Date: June 6, 2025

16th, January 2024

# Justifications for additional test chemical selection

# for Phase-II of the α-Sens validation trial

This document provides the justification for additional test chemicals for Phase-II of the α-Sens validation trial.

## 1. Background

The use of the 20 reference chemicals listed in OECD Guidance Document 213 for the α-Sens validation trial had been agreed on the 2nd VMT meeting. Also, it was agreed that the additional test chemicals should be *logKow* > 3.5 because the range of *logKow* among the 20 reference chemicals are relatively narrow (*logKow*: -1.86 ~ 3.59) and wider range of *logKow* for test chemicals to be used for the validation trial is recommended for the future application of the assay. This addition could be also supported by the needs from the OECD members of the skin sensitization expert group for DASS for understanding borderline chemicals to distinguish positive or negative and developing a broader chemical applicability domain. Due to the budget and time-framework limitation, 5 chemicals are the maximum number for the additional test chemicals.

As the test chemicals for Phase-I test chemicals, α-Hexyl cinnamaldehyde (HCA; *logKow*: 4.3) had been added to confirm the transferability. The reason for HCA selection for Phase-I is as below.

- Under the training phase, one of 3 laboratories participating produced problematic results for HCA. This trouble led the improvements for the protocol and its workability should be confirmed under the transferability phase (i.e., Phase-I study).
- It was not clear whether the trouble was due to the hydrophobic property of HCA or not. Therefore, HCA that produced problematic outcomes at one laboratory was selected as additional test chemical for Phase-I study.

Therefore, 4 additional test chemicals need to be selected.

## 1. Criteria for selection of additional test chemicals

The selection criteria are provided in Table-1.

**Table-1: Criteria for selection of additional test chemicals**

| |
|---|
| *A. Screening Criteria* |
| [1] Chemicals that have *logKow* > 3.5. |
| [2] Chemicals that are listed in DASS database (OECD, 2021. GD336. Annex 1) since the sensitization classification is agreed by OECD experts. |
| [3] Chemicals that are commercially available. |
| [4] Chemicals with their purity >=95%. |
| [5] Chemicals that are **not** Poisonous and Deleterious Substances. |
| [6] Chemicals that do **not** require refrigerated/frozen transport because of the potential chemical stability issue. |
| *B. Final Selection Criteria* |
| [1] Negatives need to be "approximately 40% of the total test chemicals". |
| [2] Consider skin sensitization potency (including GHS sub-category 1A, 1B). Need to have a good distribution of GHS subcategory (Attachment-1: Analysis of GHS sub-category distribution for reference chemicals). |
| [3] Consider to strike the balance with outcomes from KeratinoSens® |
| [4] Select chemicals with clear evidence on positives or negatives classification. Preferably evidences in human data or its skin sensitization potency is well-known. |
| [5] Preferably select chemicals with use of solvent other than DMSO since many of reference chemicals will be tested with DMSO. |
| [6] Select chemicals to increase chemical structure diversity |

## 2. Candidate chemicals

The list of candidate chemicals that met the ***screening criteria*** in Table-1 is provided in Attachment-2.

## 3. Selection of additional test chemicals for Phase-II

Under the analysis of GHS subcategory distribution of the 20 reference chemicals, The subcategory from LLNA data were assigned because human data cannot quantitative data to make subcategory classification. Therefore, GHS subcategory by LLNA was used to count chemicals classified as 1A and 1B.

Among the 20 reference chemicals, chemicals classified as 1A and non-sensitizers were both 8 chemicals. Chemicals classified as 1B were only 5 chemicals. It should be noted that HCA (1B chemical) was already selected for Phase-I. Therefore, three more 1B chemicals were necessary. Since negative rate among test chemicals needs to be "approximately 40% of the total test chemicals" and 1 more non-sensitizer were necessary (9/25 = 36%). Three more chemicals with 1B subcategory were selected.

**For a non-sensitizer selection:**

In general, it is very had to identify true negative. ***n*-Hexane** (*LogKow*: 3.9) was the only chemical with fully negative data in *in vivo* (LLNA) and human data and in vitro (DPRA, KeratinoSens and *h*-CLAT in DASS database (see Attachment-2).

**For 3 chemicals classified as 1B:**

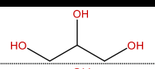
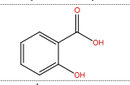
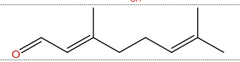
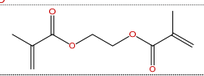
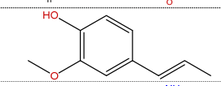
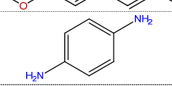
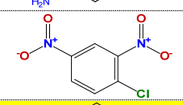
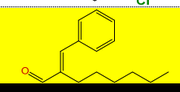
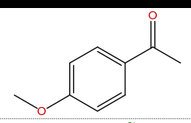
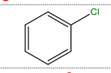
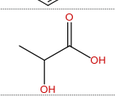
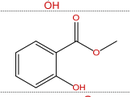
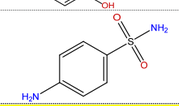
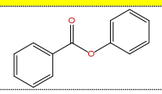
***D*-Limonene** (*LogKow*: 4.5) was selected as the weakest activity in LLNA MLLP (Median-like location parameter. The mean of EC3 from independent LLNA assay) among 1B candidate chemicals. This selection could be useful to demonstrate the sensitivity of the assay. Also, the potential solvent is acetone, and this chemical increases chemical structure diversity.

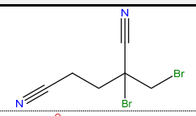
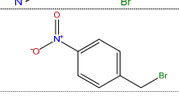
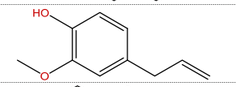
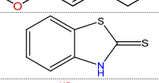
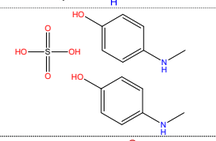
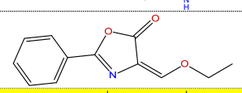
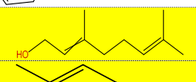
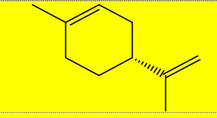
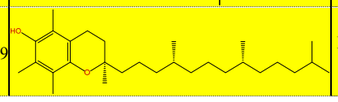
**α-Tocopherol** (*LogKow*: 9.4) was selected as the highest *LogKow* value among 1B candidate chemicals to extend the applicability of the assay. Also, this chemical can chemical structure diversity.

**Geraniol** (*LogKow*: 3.6) was selected among chemicals that have human data. This chemical was the only chemical that was detected by KE2 (KeratinoSens) and KE3 (h-CLAT) assays.

The reasons for selected/not selected are provided in Attachment-2.

## Table-2: Proposed Test chemical list for α-Sens validation trial

| No. | Chemical Name | CASRN | Chemical Structure | Status | Required No. of Experiments | in vivo category | Possible vehicle | Physical State | LogKow | LogKow.Source | LogS | LogS.Source | BP | BP.Source | MP | MP.Source | LogVP | LogVP.Source | MW | pre/prohapten | DPRA.Call | LuSens | KS.Call | hCLAT.Call | LLNA.MLLP | LLNA.Call | LLNA.GHS.SUB | HU.GHS.BIN | HU.GHS.SUB | Basketter_human_Call | Basketter_human_potency_3class |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Phase-I** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 01 | Glycerol | 56-81-5 |  | **BLR** and **WLR** from Reference Chemicals | **3X** | as negative | DMSO | Liquid | -1.86 | Exp | 1.08 | Exp | 290.00 | Exp | 18.20 | Exp | -3.77 | Exp | 92.09 | | 0 | Neg | 0 | 0 | NC | 0 | NC | NA | NA | 0 | NC |
| 02 | Isopropanol | 67-63-0 |  | **BLR** and **WLR** from Reference Chemicals | **3X** | as negative | DMSO | Liquid | 0.05 | Exp | 0.96 | Exp | 82.30 | Exp | -89.50 | Exp | 1.66 | Exp | 60.10 | | 0 | Neg | 0 | 0 | NC | 0 | NC | NA | NA | 0 | NC |
| 03 | Salicylic acid | 69-72-7 |  | **BLR** and **WLR** from Reference Chemicals | **3X** | as negative | DMSO | Solid | 2.26 | Exp | -1.83 | Exp | 255.16 | Pred | 158.00 | Exp | -4.09 | Exp | 138.12 | | 0 | Neg | 0 | 1 | 12.2 | 1 | 1B | NA | NA | 0 | NC |
| 04 | Ethylene glycol dimethacrylate | 97-90-5 |  | **BLR** and **WLR** from Reference Chemicals | **3X** | sensitiser (weak) | DMSO | Liquid | 1.51 | Pred | -0.86 | Pred | 225.00 | Pred | 58.43 | Pred | -1.36 | Pred | 198.22 | | 1 | Pos | 1 | 1 | 28 | 1 | 1B | NA | NA | 1 | 1B |
| 05 | Citral | 5392-40-5 |  | **BLR** and **WLR** from Reference Chemicals | **3X** | sensitiser (moderate) | DMSO | Liquid | 3.00 | Pred | -2.06 | Exp | 228.00 | Exp | 12.15 | Pred | -0.19 | Pred | 152.23 | | 1 | Pos | 1 | 1 | 5.8 | 1 | 1B | 1 | NA | 1 | 1B |
| 06 | Isoeugenol | 97-54-1 |  | **BLR** and **WLR** from Reference Chemicals | **3X** | sensitiser (moderate) | DMSO | Liquid | 3.04 | Exp | -2.27 | Pred | 266.00 | Exp | 11.75 | Exp | -1.92 | Exp | 164.20 | pre\ | 1 | Pos | 1 | 0 | 1.3 | 1 | 1A | 1 | 1B | 1 | 1A |
| 07 | p-Phenylenediamine | 106-50-3 |  | **BLR** and **WLR** from Reference Chemicals | **3X** | sensitiser (strong/extreme) | DMSO | Solid | -0.30 | Exp | -0.42 | Exp | 267.00 | Exp | 146.00 | Exp | -2.89 | Pred | 108.14 | pre | 1 | Pos | 1 | 1 | 0.11 | 1 | 1A | 1 | 1A | 1 | 1A |
| 08 | 2,4-Dinitrochlorobenzene | 97-00-7 |  | **BLR** and **WLR** from Reference Chemicals | **3X** | sensitiser (extreme) | DMSO | Solid | 2.17 | Exp | -3.65 | Pred | 315.00 | Exp | 53.00 | Exp | -4.07 | Exp | 202.55 | | 1 | Pos | 1 | 1 | 0.054 | 1 | 1A | 1 | 1A | 1 | 1A |
| 09 | alpha-Hexylcinnamaldehyde (HCA) | 101-86-0 |  | **BLR_as Additional Chemical** | **1X** | Pos | DMSO | Liquid | **4.34** | Pred | -3.72 | Pred | 278.92 | Pred | 39.20 | Exp | -2.57 | Pred | 216.32 | | 0 | Pos | 1 | 0 | 10.8 | 1 | 1B | NA | NA | 0 | NC |
| **Phase-II** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 10 | 4'-Methoxyacetophenone | 100-06-1 |  | **BLR** and **WLR** from Reference Chemicals | **3X** | as negative | DMSO | Solid | 1.74 | Exp | -1.92 | Pred | 258.00 | Exp | 38.50 | Exp | -2.19 | Exp | 150.17 | | 0 | Pos | 1 | 0 | NC | 0 | NC | NA | NA | NA | NA |
| 11 | Chlorobenzene | 108-90-7 |  | **BLR** from Reference Chemicals | **1X** | as negative | DMSO | Liquid | 2.87 | Exp | -2.37 | Exp | 131.70 | Exp | -45.20 | Exp | 1.08 | Exp | 112.56 | | 0 | Neg | 0 | 1 | NA | NA | NA | NA | NA | NA | NA |
| 12 | Lactic acid | 50-21-5 |  | **BLR** from Reference Chemicals | **1X** | as negative | DMSO | Liquid | -0.72 | Exp | 1.05 | Exp | 208.69 | Pred | 40.87 | Exp | -1.09 | Exp | 90.08 | | 0 | Neg | 0 | 0 | NC | 0 | NC | NA | NA | 0 | NC |
| 13 | Methyl salicylate | 119-36-8 |  | **BLR** from Reference Chemicals | **1X** | as negative | DMSO | Liquid | | | | | | | | | | | | | - | Pos | 0 | - | - | - | - | - | - | - | - |
| 14 | Sulfanilamide | 63-74-1 |  | **BLR** from Reference Chemicals | **1X** | as negative | DMSO | Solid | -0.62 | Exp | -1.36 | Exp | 284.04 | Pred | 165.50 | Exp | -4.68 | Pred | 172.20 | | 0 | Neg | 0 | 0 | NC | 0 | NC | 1 | 1B | NA | NA |
| 15 | n-Hexane | 110-54-3 |  | **BLR_as Additional Chemicals** | **1X** | Neg (candidate) | Acetone | Liquid | **3.90** | Exp | -3.90 | Exp | 68.70 | Exp | -95.30 | Exp | 2.18 | Exp | 86.18 | | 0 | Neg | 0 | 0 | NC | 0 | NC | NC | NC | 0 | NC |
| 16 | Phenyl benzoate | 93-99-2 |  | **BLR** and **WLR** from Reference Chemicals | **3X** | sensitiser (weak) | DMSO | Solid | **3.59** | Exp | -3.72 | Exp | 314.00 | Exp | 71.00 | Exp | -2.77 | Exp | 198.22 | | 1 | Neg | 0 | 1 | POS | 1 | NA | 1 | 1B | 1 | 1B |

| No. | Chemical Name | CASRN | Chemical Structure | Status | Required No. of Experiments | in vivo category | Possible vehicle | Physical State | LogKow | LogKow.Source | LogS | LogS.Source | BP | BP.Source | MP | MP.Source | LogVP | LogVP.Source | MW | pre/prohapten | DPRA.Call | LuSens | KS.Call | hCLAT.Call | LLNA.MLLP | LLNA.Call | LLNA.GHS.SUB | HU.GHS.BIN | HU.GHS.SUB | Basketter_human_Call | Basketter_human_potency_3class |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 17 | Methyldibromo glutaronitrile | 35691-65-7 |  | **BLR** and **WLR** from Reference Chemicals | **3X** | sensitiser (strong) | DMSO | Solid | 1.89 | Pred | -1.06 | Pred | 256.84 | Pred | 52.00 | Exp | -2.55 | Pred | 265.93 | pre | 1 | Pos | 1 | 1 | 0.9 | 1 | **1A** | NA | NA | 1 | **1A** |
| 18 | 4-Nitrobenzyl bromide | 100-11-8 |  | **BLR** and **WLR** from Reference Chemicals | **3X** | sensitiser (extreme) | DMSO | Solid | 2.82 | Pred | -2.43 | Pred | 262.87 | Pred | 99.00 | Exp | -2.03 | Pred | 216.03 | | 1 | Pos | 1 | 1 | 0.05 | 1 | **1A** | NA | NA | NA | NA |
| 19 | Eugenol | 97-53-0 |  | **BLR** from Reference Chemicals | **1X** | sensitiser (weak) | DMSO | Liquid | 2.27 | Exp | -2.29 | Exp | 253.20 | Exp | -9.10 | Exp | -1.65 | Exp | 164.20 | pre/pro | 1 | Pos | 0 | 1 | 11.6 | 1 | **1B** | 1 | **1B** | 1 | **1B** |
| 20 | 2-Mercaptobenzothiazole | 149-30-4 |  | **BLR** from Reference Chemicals | **1X** | sensitiser (moderate) | DMSO | Solid | 2.42 | Exp | -3.17 | Exp | 289.39 | Pred | 181.00 | Exp | -5.37 | Pred | 167.25 | | 1 | Pos | 1 | 1 | 1.35 | 1 | **1A** | 1 | **1B** | 1 | **1B** |
| 21 | 4-Methylaminophenol sulphate | 55-55-0 |  | **BLR** from Reference Chemicals | **1X** | sensitiser (strong) | DMSO | Solid | 0.63 | Pred | -0.92 | Pred | 276.73 | Pred | 159.91 | Pred | -2.52 | Pred | 123.15 | pre/pro | 1 | Pos | 1 | 1 | 0.8 | 1 | **1A** | NA | NA | 1 | **1B** |
| 22 | Oxazolone | 15646-46-5 |  | **BLR** from Reference Chemicals | **1X** | sensitiser (extreme) | DMSO | Solid | 1.48 | Pred | -2.18 | Pred | 316.04 | Pred | 60.25 | Pred | -3.56 | Pred | 217.22 | | 1 | Pos | 1 | 1 | 0.002 | 1 | **1A** | NA | NA | NA | NA |
| 23 | Geraniol | 106-24-1 |  | **BLR as Additional Chemicals** | **1X** | Pos | DMSO | Liquid | 3.6 | Exp | -2.83 | Exp | 227.5 | Exp | 6.78 | Pred | -1.5 | Exp | 154.3 | pre/pro | 0 | - | 1 | 1 | 16.1 | 1 | **1B** | 1 | **1B** | 1 | **1B** |
| 24 | D-Limonene | 5989-27-5 |  | **BLR_as Additional Chemicals** | **1X** | Pos (**Pair-A** candidate) | Acetone | Liquid | **4.52** | Exp | -4.04 | Exp | 176.00 | Exp | -86.43 | Exp | 0.19 | Exp | 136.23 | pre | 0 | - | 0 | 1 | 52.5 | 1 | **1B** | NA | NA | 0 | NC |
| 25 | alpha-Tocopherol | 59-02-9 |  | **BLR_as Additional Chemicals** | **1X** | Pos (**Pair-A** candidate) | DMSO | Liquid | **9.41** | Pred | -7.55 | Pred | 394.20 | Pred | 33.10 | Pred | -6.63 | Pred | 430.71 | | 0 | - | 1 | 0 | 7.4 | 1 | **1B** | NA | NA | 0 | NC |

Chemicals highlighted in yellow are the additional test chemicals other than 20 reference chemicals listed in OECD Guidance Document 213.

Attachment-1: Analysis of GHS sub-category distribution for reference chemicals

| | | 20 Reference Chemicals | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Phase-I Chemicals forα-Sens validation | | | | Phase-II Chemicals forα-Sens validation | | | | Total | | | |
| GD 213 in vivo cate-gory | GHS Subcategory | LLNA.GHS.SUB \| HU.GHS.SUB \| Basketter_human *1 | total | Possible Vehicle | Physical state | LLNA.GHS.SUB \| HU.GHS.SUB \| Basketter_human *1 | total | Possible vehicle | Physical state | LLNA.GHS.SUB \| HU.GHS.SUB \| Basketter_human *1 | Total | Possible Vehicle | Physical state |
| Pos *2 | 1B | 1B \| NA \| 1B 2 | 2 | DMSO: 8 | Liq.: 3 Solid: 2 | 1B \| 1B \| 1B 1<br>NA \| 1B \| 1B 1 | 2 | DMSO: 12 | Liq.: 3 Solid: 2 | 1B \| NA \| 1B 2<br>1B \| 1B \| 1B 1<br>NA \| 1B \| 1B 1 | 4 | DMSO: 20 | Liq.: 6 Solid: 4 |
| | 1A | 1A \| 1A \| 1A 2<br>1A \| 1B \| 1A 1 | 3 | | | 1A \| 1B \| 1B 1<br>1A \| NA \| NA 2<br>1A \| NA \| 1B 1<br>1A \| NA \| 1A 1 | 5 | | | 1A \| 1A \| 1A 2<br>1A \| 1B \| 1A 1<br>1A \| 1B \| 1B 1<br>1A \| NA \| NA 2<br>1A \| NA \| 1B 1<br>1A \| NA \| 1A 1 | 8 | | |
| Neg | 1B | 1B \| NA \| NC 1 | 1 | | Liq.: 2 Solid: 1 | NC \| 1B \| NA 1 | 1 | | Liq.: 1 Solid: 6 | 1B \| NA \| NC 1<br>NC \| 1B \| NA 1 | 2 *3 | | Liq.: 3 Solid: 7 |
| | NC | NC \| NA \| NC 2 | 2 | | | NC \| NA \| NC 1<br>NC \| NA \| NA 1 | 2 | | | NC \| NA \| NC 2<br>NC \| NA \| NC 1<br>NC \| NA \| NA 1 | 4 | | |
| | NA | 0 | 0 | | | NA \| NA \| NA 1 | 1 | | | NA \| NA \| NA 1 | 1 | | |
| | No data on DASS | 1 | 0 | | | 1 | 1 *2 | | | 1 | 1 *3 | | |
| Total | | 8 | | | | 12 | | | | 20 | | | |

*1: Basketter_human_potency_3class in DASS, *2: LLNA has quantitative classification criteria, but human data does not have such criteria. Therefore LLNA-based classification was given priority.

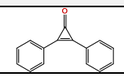
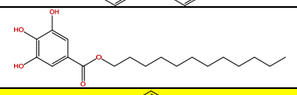
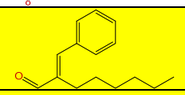
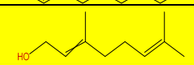
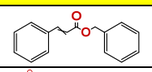
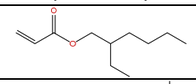
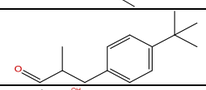
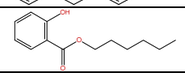
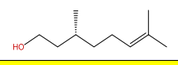
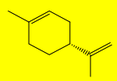
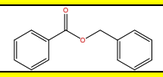
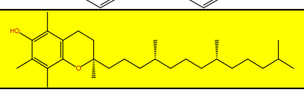
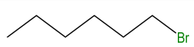
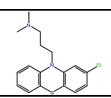
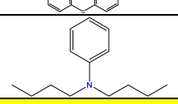
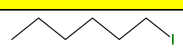
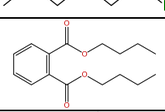
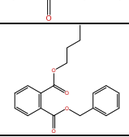
*3: Classified as non-sensitizer in GD213. it should be noted that α-Sens and KS gave same outcome for these chemicals.

| | Phase-I | | | Phase-II | | | Total number of test chemicals | | |
|---|---|---|---|---|---|---|---|---|---|
| | Pos | | Neg | Pos | | Neg | Pos | | Neg |
| | 1A | 1B | non | 1A | 1B | non | 1A | 1B | non |
| Subcategory distribution for 20 reference chemicals | 3 | 2 | 3 | 5 | 2 | 5 | 8 | 4 | 8 |
| | 8 | | | 12 | | | 20 | | |

Addition of 5 chemicals

| | No addition | Add 4 chem. | Add 1 chem |
|---|---|---|---|
| Subcategory distribution for 20 reference chemicals + 5 additional chemicals | 8 | 8 | 9 |
| | 25 | | |

## Attachment-2: List of Candidate Chemicals that met the criteria in Table-1.

| DASS No. | Chemical Name | CASRN | Chemical Structure | Reasons | Solvent in α-Sens | Physical State | LogKow | LogKow.Source | LogS | LogS.Source | BP | BP.Source | MP | MP.Source | LogVP | LogVP.Source | MW | pre/prohapten | DPRA.Call | LuSens | KS.Call | hCLAT.Call | LLNA.MLLP | LLNA.Call | LLNA.GHS.SUB | HU.GHS.BIN | HU.GHS.SUB | Basketter_human_Call | Basketter human_potency_3class |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 74 | Diphenylcyclopropenone | 886-38-4 |  | Not selected because of 1A subcategory | DMSO | Solid | 3.5 | Pred | -3.84 | Pred | 345.4 | Pred | 119.00 | Exp | -5.0 | Pred | 206.2 | | 1 | - | 1 | 1 | 1A | 1 | 1A | NA | NA | 1 | 1A |
| 126 | Lauryl gallate | 1166-52-5 |  | Not selected because of 1A subcategory | DMSO | Solid | 4.6 | Pred | -3.30 | Pred | 386.7 | Pred | 96.50 | Exp | -8.9 | Pred | 338.4 | pre | 1 | - | 1 | 1 | 1A | 1 | 1A | NA | NA | 1 | 1A |
| 102 | α-Hexylcinnamaldehyde | 101-86-0 |  | **Already selected for Phase-I** chemical | DMSO | Liquid | 4.3 | Pred | -3.72 | Pred | 278.9 | Pred | 39.20 | Exp | -2.6 | Pred | 216.3 | | 0 | 1 | 1 | 0 | 10.8 | 1 | 1B | NA | NA | 0 | NC |
| 93 | Geraniol | 106-24-1 |  | Human data are available, and detected by KeratinoSens and hCLAT, | DMSO | Liquid | 3.6 | Exp | -2.83 | Exp | 227.5 | Exp | 6.78 | Pred | -1.5 | Exp | 154.3 | pre/pro | 0 | - | 1 | 1 | 16.1 | 1 | 1B | 1 | 1B | 1 | 1B |
| 29 | Benzyl cinnamate | 103-41-3 |  | Not selected because logKow is in the range already selected for sensitizers. | DMSO | Liquid | 3.6 | Pred | -4.48 | Pred | 350.0 | Exp | 39.00 | Exp | -5.0 | Exp | 238.3 | | 0 | - | 1 | 0 | 18.4 | 1 | 1B | NA | NA | 1 | 1B |
| 86 | 2-Ethylhexyl acrylate | 103-11-7 |  | Not selected because human data is not available. | Acetone | Liquid | 4.2 | Pred | -3.26 | Exp | 213.5 | Exp | -90.00 | Exp | -0.7 | Exp | 184.3 | | 1 | - | 1 | 1 | 36.8 | 1 | 1B | NA | NA | NA | NA |
| 127 | Lilial | 80-54-6 |  | Not selected since other high priority chemicals were selected. | DMSO | Liquid | 3.9 | Pred | -3.65 | Pred | 278.4 | Pred | 21.52 | Pred | -0.7 | Pred | 204.3 | | 1 | - | 0 | 1 | 8.6 | 1 | 1B | 1 | 1B | 1 | 1B |
| 101 | Hexyl salicylate | 6259-76-3 |  | Not selected because 2 salicylates were included in reference chemicals. | DMSO | Liquid | 5.5 | Exp | -3.55 | Pred | 271.8 | Pred | 25.54 | Pred | -4.6 | Pred | 222.3 | | 0 | - | 0 | 1 | 0.18 | 1 | NA | NC | NC | 1 | 1B |
| 52 | Citronellol | 106-22-9 |  | Not selected because this chemical will not increase chemical structure diversity | DMSO | Liquid | 3.9 | Exp | -2.68 | Pred | 224.3 | Exp | 9.67 | Pred | -1.4 | Exp | 156.3 | | 1 | - | 0 | 1 | 43.5 | 1 | 1B | NC | NC | 0 | NC |
| 128 | D-Limonene | 5989-27-5 |  | **Selected** that have **weakest LLNA** MLLP among 1B candidates. Also, the potential solvent is acetone. This chemical could increase structural diversity. | Acetone | Liquid | 4.5 | Exp | -4.04 | Exp | 176.0 | Exp | -86.43 | Exp | 0.2 | Exp | 136.2 | pre | 0 | - | 0 | 1 | 52.5 | 1 | 1B | NA | NA | 0 | NC |
| 26 | Benzyl benzoate | 120-51-4 |  | Not selected because logKow is in the range already selected for sensitizers. | DMSO | Liquid | 4 | Exp | -4.14 | Exp | 323.5 | Exp | 21.00 | Exp | -3.6 | Exp | 212.2 | | 0 | - | 1 | 0 | 17 | 1 | 1B | NC | NC | 0 | NC |
| 190 | alpha-Tocopherol | 59-02-9 |  | **Selected** because **logKow is the highest** among 1 B candidate | DMSO | Liquid | 9.4 | Pred | -7.55 | Pred | 394.2 | Pred | 33.10 | Pred | -6.6 | Pred | 430.7 | | 0 | - | 1 | 0 | 7.4 | 1 | 1B | NA | NA | 0 | NC |
| 36 | 1-Bromohexane | 111-25-1 |  | Not selected because logKow is in the range already selected for sensitizers. | DMSO | Liquid | 3.8 | Exp | -3.81 | Exp | 155.3 | Exp | -84.70 | Exp | 0.6 | Exp | 165.1 | | 1 | - | 1 | 0 | 10 | 1 | 1B | NA | NA | NA | NA |
| 122 | Isopropyl myristate | 110-27-0 |  | Not selected since other high priority chemicals were selected. | Acetone | Liquid | 6.9 | Pred | -7.29 | Exp | 292.4 | Pred | 3.00 | Exp | -4.0 | Exp | 270.5 | | 0 | - | 0 | 1 | 44 | 1 | 1B | NA | NA | 0 | NC |
| 47 | Chlorpromazine | 50-53-3 |  | Not selected since other high priority chemicals were selected. | DMSO | Solid | 5.4 | Exp | -5.16 | Exp | 361.1 | Pred | 89.07 | Pred | -6.8 | Pred | 318.9 | pre/pro | 0 | - | 0 | 1 | POS | 1 | NA | 1 | 1B | 1 | 1B |
| 63 | N,N-Dibutylaniline | 613-29-6 |  | Not selected because logKow is in the range already selected for sensitizers. | Acetone | Liquid | 3.9 | Pred | -3.13 | Pred | 274.8 | Exp | -32.20 | Exp | -1.6 | Pred | 205.3 | | 0 | - | 0 | 0 | 19.6 | 1 | 1B | NA | NA | NA | NA |
| 99 | n-Hexane | 110-54-3 |  | **Selected** with **fully negative data in LLNA and human data.** | Acetone | Liquid | 3.9 | Exp | -3.90 | Exp | 68.7 | Exp | -95.30 | Exp | 2.2 | Exp | 86.2 | | 0 | 0 | 0 | 0 | NC | 0 | NC | NC | NC | 0 | NC |
| 114 | 1-Iodohexane | 638-45-9 |  | Not selected because no negative human data to judge negative | Acetone | Liquid | 4 | Pred | -4.27 | Pred | 181.0 | Exp | -74.20 | Exp | 0.1 | Exp | 212.1 | | 1 | - | 1 | 1 | NC | 0 | NC | NA | NA | NA | NA |
| 62 | Dibutyl phthalate | 84-74-2 |  | Not selected because no negative human data to judge negative | DMSO | Liquid | 4.6 | Exp | -4.40 | Exp | 340.0 | Exp | -35.00 | Exp | -4.7 | Exp | 278.3 | | 0 | - | 1 | 0 | NC | 0 | NC | NA | NA | NA | NA |
| 28 | Benzyl butyl phthalate | 85-68-7 |  | Not selected because no negative human data to judge negative | DMSO | Liquid | 4.8 | Exp | -5.25 | Exp | 370.0 | Exp | 77.39 | Pred | -5.1 | Exp | 312.4 | | 0 | - | 1 | 0 | NC | 0 | NC | NA | NA | NA | NA |

# Protocol for α-Sens®

Ver. P2.2

July 2024

# Table of Contents

## Abbreviations and Definitions

| | |
|---|---|
| ARE | Antioxidant Response Element |
| AUG | a partial rat $\alpha_{2U}$-Globlin promotor fragment |
| DMSO | Dimethyl sulfoxide |
| EC3.2 | Interpolated concentration resulting in a 3.2 of nAA |
| EDTA | Ethylenediaminetetraacetic acid |
| FBS | Fetal Bovine Serum |
| HiLIC | High Luminescence Inducible Chemical |
| nAA | normalized Antioxidant response element (ARE)-mediated transcriptional Activity |
| PC | Positive Control |
| PBS | Phosphate Buffered Saline |
| RLU | Relative Luminescence Units |
| TK | Thymidine Kinase |
| VC | Vehicle Control |

# 1. Overview

## 1.1. About α-Sens®

α-Sens® is an assay system to evaluate the skin sensitization potential of chemicals by distinguishing skin-sensitizers or non-skin sensitizers. α-Sens® cell line was originally developed as "α-Sens (without ®)", the multi-clone cell system of PHK 16-0b cell line (immortalized human keratinocyte by HPV16 virus genome) stably transfected with two reporter constructs using Lentiviral Vector System, Antioxidant response element (ARE) inducible *Firefly* luciferase gene for ARE response and Thymidine Kinase (TK) promoter-driven *Renilla* luciferase gene for cell viability measurement (Maeda *et al*., 2020). α-Sens was further improved by modifying the reporter gene construct and was established as a single clone cell system, "α-Sens® (with ®)". The details of the modifications made in the α-Sens® are provided in Appendix A with comparison to the original multi-clone system.

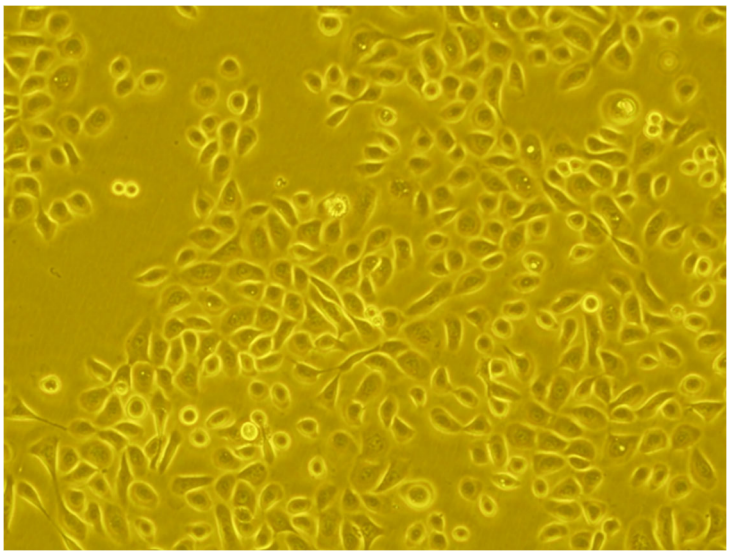


**Fig.-1 Representative image of α-Sens® cell line**

Host cells of α-Sens®: Immortalized normal human keratinocyte PHK 16-0b (Catalogue#: JCRB0141)
Medium for α-Sens®: Serum free medium is used throughout the assay procedures of α-Sens®.
α-Sens® employs a dual- luciferase reporter system.
1st Luc: *Firefly* luciferase reactions measuring ARE mediated activity by using original reporter construct (4ARE-AUG-*Luc*+) (AUG: a partial rat $\alpha_{2U}$-Globlin promotor fragment)
2nd Luc: Thymidine Kinase (TK) promoter driven-*Renilla* luciferase activity is used for monitoring cytotoxicity.

By using α-Sens®, ARE mediated activity and cell viability can be measured simultaneously with the same cell and the normalized ARE-mediated transcriptional Activity, named nAA, is used as a parameter to evaluate the skin sensitization potential.

## 1.2. Overview of α-Sens®

The general procedures of α-Sens® is provided in Fig.-2.

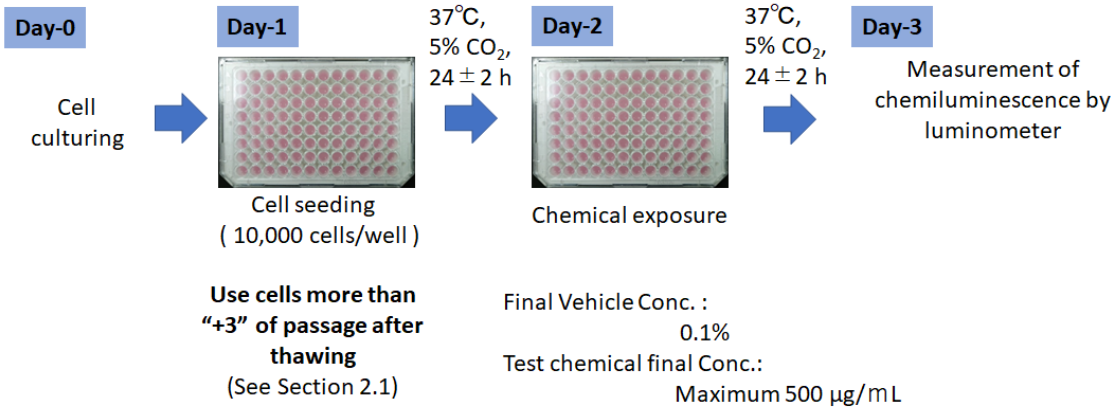



**Fig.-2 General procedures of α-Sens®**

α-Sens® uses 2 different plate layouts, "Plate Layout-A" and "Plate Layout-B" (Fig.-3). The recommendable use of each plate layouts are provided in "3.4 Chemical Exposure using "Plate Layout-A"" and "3.6 Chemical Exposure using "Plate Layout-B"". It should be noted that "Plate Layout-A" is the first choice for the first test of test chemicals.

Both assay outcomes from "Plate Layout-A" and "Plate Layout-B" can be used for positive or negative judgement for each run if all data acceptance criteria and data interpretation criteria are met.

The flowchart for test-concentration setting, plate layout and determination of the common ratio for the assay is as provided in Fig.-4.

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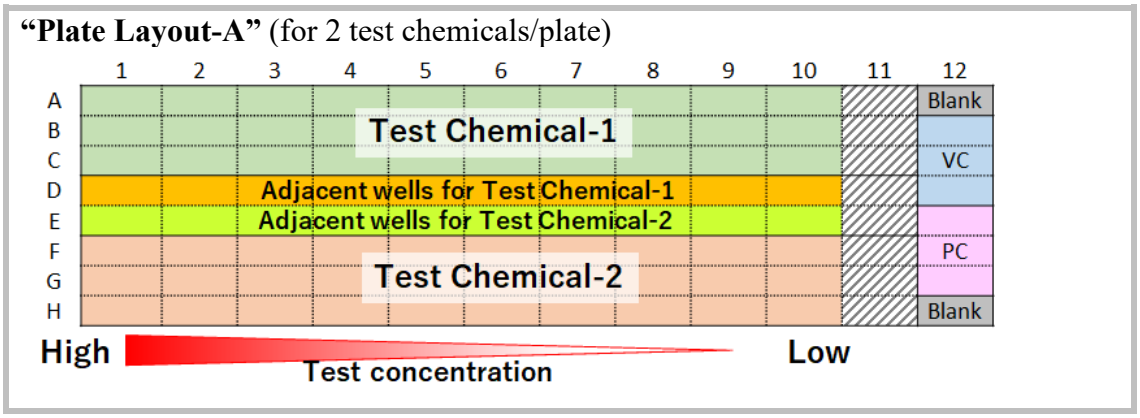


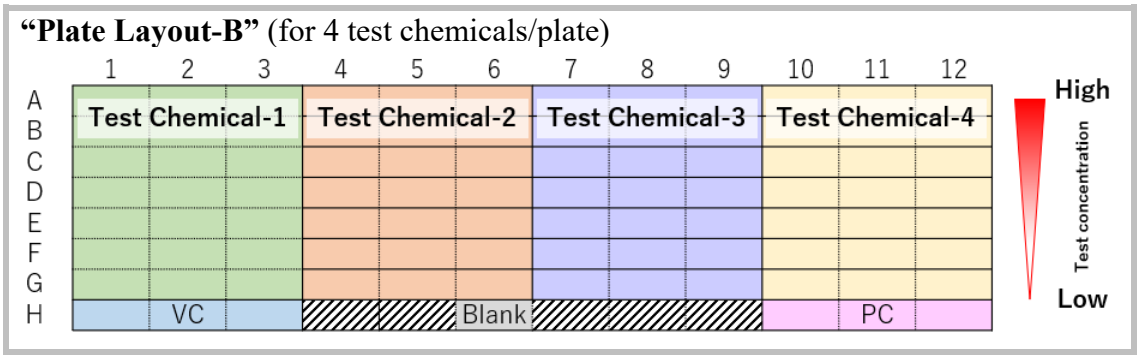



**Fig.-3 Representative plate layouts for α-Sens®**

VC: Vehicle Control, PC: Positive Control at 0.5 μg/mL of Cinnamic aldehyde,
Blank: no cells, α-Sens® medium only,
Wells with diagonal line: Containing cells and α-Sens® medium, to be treated with both *Firefly* and *Renilla* luciferase reagents.
Adjacent wells in "Plate Layout-A": Containing cells and α-Sens® medium, to be treated with both *Firefly* and *Renilla* luciferase reagents. These adjacent wells are used to identify a chemical that have high luminescence inducing potential.

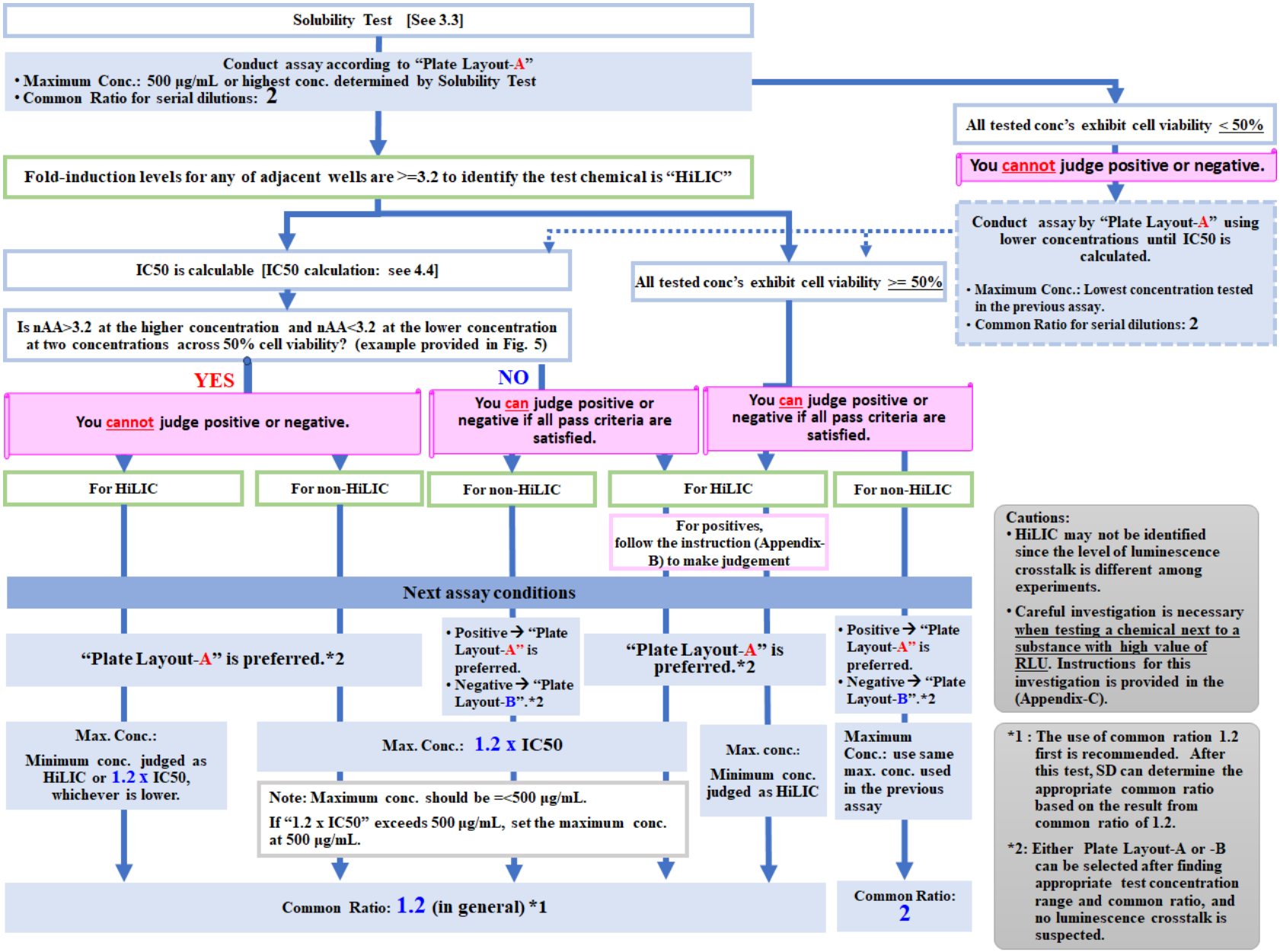



**Fig.-4 Flowchart for the concentration-setting, plate layout and determination of the common ratio for the assay**

| | nAA | Cell viability |
|---|---|---|
| Higher conc. | 5.1 | 40% |
| Lower conc. | 3.0 | 65% |

| (μg/mL) | Chemical X | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | nAA | | | | | | Judge | Adoption | EC3.2 | Cell viability (%) | | | | | | |
| | Well-1 | Well-2 | Well-3 | Mean | SD | CV | | | | Well-1 | Well-2 | Well-3 | Mean | SD | CV | IC50 Calc. |
| 500 | 5.40 | 4.80 | 5.20 | 5.13 | 0.31 | 6.0% | Positive | Fail | X | 37.0 | 40.0 | 43.0 | 40.0 | 3.0 | 7.5% | 400.3 |
| 250 | 3.30 | 2.80 | 2.95 | 3.02 | 0.26 | 8.5% | Negative | Pass | X | 67.0 | 59.2 | 69.0 | 65.1 | 5.2 | 8.0% | X |
| 125 | 2.32 | 2.05 | 1.54 | 1.97 | 0.39 | 20.0% | Negative | Pass | X | 105.3 | 115.3 | 119.0 | 113.2 | 7.1 | 6.3% | X |
| 62.5 | 1.45 | 1.11 | 1.15 | 1.23 | 0.19 | 15.0% | Negative | Pass | X | 108.7 | 119.2 | 120.3 | 116.1 | 6.4 | 5.5% | X |
| 31.25 | 1.52 | 1.36 | 1.17 | 1.35 | 0.18 | 13.3% | Negative | Pass | X | 123.4 | 106.1 | 99.3 | 109.6 | 12.4 | 11.3% | X |
| 15.625 | 0.92 | 1.05 | 0.97 | 0.98 | 0.07 | 7.1% | Negative | Pass | X | 118.8 | 115.4 | 131.3 | 121.9 | 8.4 | 6.9% | X |
| 7.8125 | 1.15 | 1.12 | 1.10 | 1.12 | 0.02 | 2.1% | Negative | Pass | X | 104.0 | 94.0 | 108.4 | 102.10 | 7.4 | 7.2% | X |
| 3.90625 | 0.97 | 1.06 | 0.89 | 0.97 | 0.09 | 8.8% | Negative | Pass | X | 110.4 | 102.6 | 93.1 | 102.0 | 8.7 | 8.5% | X |
| 1.953125 | 1.13 | 0.94 | 0.87 | 0.98 | 0.14 | 13.9% | Negative | Pass | X | 105.4 | 98.2 | 96.2 | 99.9 | 4.8 | 4.8% | X |
| 0.976563 | 0.92 | 1.11 | 0.89 | 0.98 | 0.12 | 12.4% | Negative | Pass | | 80.2 | 97.6 | 92.0 | 89.9 | 8.9 | 9.9% | |

**Imax:** 3.02 **Judgement:** NEGATIVE **EC3.2:** Not calculable (μg/mL) **IC50=** 400.3 (μg/mL)

**Fig.-5 Example where positive or negative judgement cannot be made.**

For data interpretation refer to “5.2 Data interpretation” for data interpretation for each test chemical.

## 1.3. Key Considerations in the Assay

- If a test chemical is a High Luminescence inducible chemical ("HiLIC"),
  - Interference of the luminescence crosstalk may affect its neighboring wells. However, this luminescence crosstalk may be sometimes irregular.

**What To Do:**

- Follow the flowchart in Section "5.3 Identification of High Luminescence inducible chemical ("HiLIC") by "Plate Layout-A"". If a test chemical is identified as a HiLIC, one can use "Layout-A" for further evaluation for positive or negative judgement.
- It is possible that a chemical that potentially causes luminescence crosstalk **cannot** be identified in the initial assay by Layout-A and suspected to cause luminescence crosstalk in further testing (*e.g.,* the fold-induction of adjacent well is close to 3.2). Furthermore, luminescence crosstalk may be sometimes irregular as described above. In this protocol, these cases are called as "suspected HiLIC".
- When testing HiLIC or suspected HiLIC in Plate Layout-B, HiLIC or suspected HiLIC should be placed in A7-G9 wells and no other test chemical should be tested in A4-G6 wells and A10- G12 wells. In this case, it is necessary to confirm that luminescence crosstalk by such chemical does not affect the RLUs of vehicle control (VC) or adjacent other test chemical(s).
  - The effect on VC is evaluated by the additional data acceptance criteria for "Layout-B" which is the ratio of the averaged RLU values between VC and 5 wells media control in H row (ratio = $RLU_{avg}$ of VC / $RLU_{avg}$ of media control) (See more details in Section 4.4).
  - In some cases, HiLIC may not be suspected during first testing by Layout-A, but when tested with Layout-B a luminescence cross talk may be observed. In such case, the effect on adjacent chemical(s) is investigated according to the "Investigation instruction for high RLU values" shown in Appendix-C.

- The protocol of α-Sens® is developed to use Glo-type substrate reagents, Flash-type substrate reagents are not applicable.

**What To Do:**

- Only Glo-type substrate reagents are applicable for α-Sens®.

- In general, the number of cells can vary depending on the cell counting equipment (*i.e.*, maker or type of equipment *etc*.).

**What To Do:**

- The number of cells should be counted by hemocytometer or the cell counting equipment that can provide the equivalent cell number to hemocytometer.

## 1.4. Proprietary and License Information of α-Sens®

α-Sens® is a registered trademark in Japan. α-Sens® is patented as JP2022059283A. To use α-Sens® cell line, Material Transfer Agreement (MTA), including fee issue, is required between user and cell line developer (*i.e.*, CERI). CERI have agreed on the implementation of Fair, Reasonable And Non-Discriminatory (FRAND) declaration.

While KeratinoSens™ and LuSens employ *Luc2* sequence patented by Promega as *Firefly* Luciferase in their assay and the use of Promega reagent must be used for license reasons, α-Sens® uses *Luc+* sequence that has no conflict with licensing issues as *Firefly* luciferase (this fact is confirmed by contacting Promega).

## 1.5. How to Obtain α-Sens® Cell Line

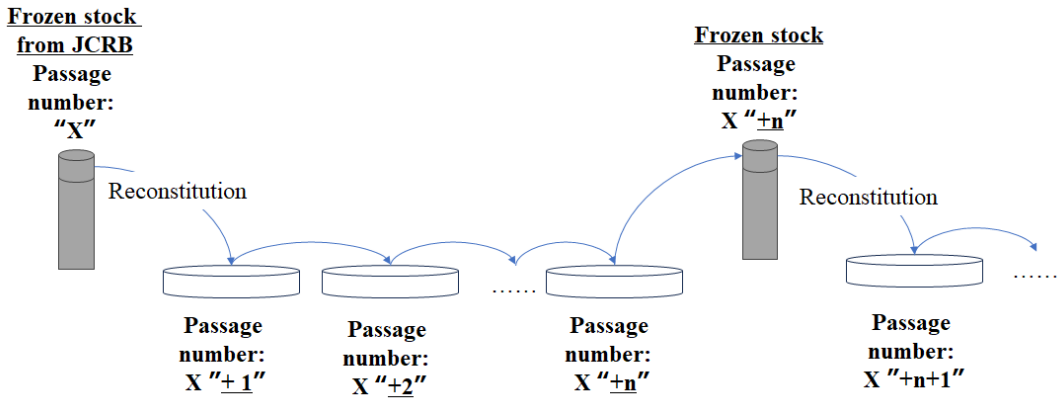
α-Sens® cell line has been deposited at JCRB Cell Bank (Japanese Collection of Research Bioresources Cell Bank: https://cellbank.nibiohn.go.jp/english/). α-Sens® cell line is available via JCRB for the purpose of the validation study.

Once α-Sens® is accepted as OECD Test Guideline, the cell line will be available via JCRB for the purpose other than the validation study, after agreeing on the MTA between a user and CERI.

## 2. Cell maintenance

### 2.1. Counting method for passage.

Just after the cell reconstitution, the number of passages should be counted as "+1" of passage.




"+30" is recommended as the maximum passage number from JCRB stock (cell culturing period is around 3.5 months). However, as long as the data acceptance criteria are fully met, more passages are acceptable for the assay.

### 2.2. Preparation of α-Sens® medium

[Material]

- KGM-Gold™ BulletKit™ (Lonza, #00192060), containing following materials:
  - KBM-Gold™ Keratinocyte Basal Medium
  - KGM-Gold™ Keratinocyte Growth Medium SingleQuots™ Supplements and Growth Factors
    1 x KGM-Gold™ SingleQuots™ Supplement pack containing:
    - Hydrocortisone
    - Transferrin
    - Epinepherine
    - GA-1000 (antibiotic) # This is not used for medium preparation. As GA-1000 is not used in this protocol, you can discard GA-1000.
    - BPE
    - RhEGF
    - Insulin
- 5-mL disposable pipette
- Electric pipettor

[Method]

# ***Procedure shall be performed under sterile condition in the biosafety cabinet.***

1. Add all Supplements and Growth Factors except GA-1000 to the bottle of KBM-Gold™ Keratinocyte Basal Medium using a disposable pipette.
2. Add 1 mL of medium to each vial of Supplements and Growth Factors and rinse the vial to increase recovery.
3. After adding all Supplements and Growth Factors, tightly cap the bottle of the medium and entirely mix the medium. (No sterile filtration is required)

# ***Prepared medium, hereafter referred to as "α-Sens® medium", should be stored at 4°C, and should be used within one month.***

# ***α-Sens® cells can be also maintained with medium for primary cultured human keratinocytes supplemented with HKGS (Thermo Scientific) in EpiLife (Thermo Scientific) other than KGM-Gold, or equivalent.***

## 2.3. Reconstitution of α-Sens® cell from the frozen cell stock

[Material]

- Frozen α-Sens® in a cryotube
- α-Sens® medium
- 15-mL Sterile centrifuge Tube
- 100-mm Cell Culture Dish
- 2-mL disposable pipette
- 10-mL disposable pipette
- Electric pipettor

[Method]

***# Although the use of 60 mm dish is recommended in JCRB document, 100 mm dish is used for this protocol.***

1. Allow the α-Sens® Medium to stand for 1-h to bring to room temperature or warm to room temperature (approx. 20-25℃).
2. Dispense 9 mL of α-Sens® medium into a 15-mL sterile centrifuge tube.
3. Thaw the α-Sens® frozen cell stock in water bath set at 37℃ with gentle agitation until a half of ice remains.
   (The remaining frozen part of cells will be thawed during preparation.)
4. Transfer the cell into α-Sens® medium in the 15-mL sterile centrifuge tube, and pipette gently.
5. Centrifuge the tube at 300 x g for 5 minutes at room temperature (approx. 20-25℃).
6. Carefully remove the supernatant while avoiding sucking out the cells. Tap the tube gently to break the cell pellet.
7. Resuspend the cells with 10 mL α-Sens® medium.
8. Transfer the suspension to a 100 mm dish.
9. Incubate the dish in a 5% $CO_2$ incubator at 37℃ with humidified conditions.
10. After 24 ± 2 hours, check if the cells are attached to the dish and replace medium with α-Sens® medium at room temperature (approx. 20-25℃).

## 2.4. Preparation of frozen cell stock of α-Sens®

[Material]

- α-Sens® cell propagated at approx. 50-90% confluency in a culture dish
- α-Sens® medium
- PBS (-) (GIBCO, product #10010023) or equivalent
- (10X) 0.5% Trypsin-EDTA (GIBCO, product #15400-054) or equivalent
- CELL BANKER 2 (ZENOGEN PHARMA, #CB031), CELL BANKER 1 (ZENOGEN PHARMA, #CB011) or equivalent
- 15- or 50-mL Sterile centrifuge Tube
- 1.5-mL cryotube
- 5-mL disposable pipette
- 10-mL disposable pipette
- 25-mL disposable pipette
- Electric pipettor
- Cell strainer (70 um, if necessary)

[Method]

***# Before handling cells for the preparation of frozen cell stock, prepare the cryotubes labelled with name of cell, number of cells, number of cell passages, date and person who prepare the stock.***

1. Allow the α-Sens® Medium to stand for 1-h to bring to room temperature or warm to room temperature (approx. 20-25℃).
2. Check the cell conditions.
3. Remove the medium from the dish.
4. Rinse the cells with 10 mL of PBS (-) and remove the PBS (-).
5. Add 2 mL/dish of 0.05% Trypsin-EDTA solution and spread with gentle tilting to cover throughout the dish.
6. Incubate the dish for 10 minutes in a 5% $CO_2$ incubator with humidified conditions at 37°C.
7. After 10 minutes, monitor the cells under a microscope. The cells should begin to detach from the dish-bottom when they appear rounded.
8. Tap the dish-side gently to detach the cells from the dish-bottom.
9. Add 8 mL/dish of α-Sens® medium into the dish and perform mixing by pipetting the medium up and down about 10 times or more in the dish to completely detach the cells.
10. Transfer the cell suspension to a 15- or 50-mL sterile centrifuge tube.

***# If cell clumps are visually observed or suspected in cell suspension, use a cell strainer to avoid incorporating them according to the instructions of the product manual.***

11. Count the number of cells using the protocol at each laboratory.
12. Centrifuge the tube at 300 x g for 5 minutes at 25℃ or below.
13. Calculate appropriate volume of Cell-Banker cryopreservation solution to prepare 2 x $10^6$ cells/mL.
14. After centrifugation, carefully remove the supernatant while avoiding sucking out the cells. Tap the tube gently to break the cell pellet.
15. Add appropriate volume of Cell-Banker and resuspend the cells to prepare 2 x $10^6$ cells/mL.
16. Dispense 1 mL of the suspension into labelled-cryotubes.
17. Store the cryotubes in a deep freezer at -80℃ or in cell storage container in a liquid nitrogen.

## 2.5. Propagation of α-Sens cell lines

[Material]

- α-Sens® cell propagated at approx. 50-90% confluency in a culture dish
- α-Sens® medium
- PBS (-) (GIBCO, product #10010023) or equivalent
- (10X) 0.5% Trypsin-EDTA (GIBCO, product #15400-054) or equivalent
- 100-mm Tissue Culture Dish
- 15- or 50-mL Sterile centrifuge Tube
- 5-mL disposable pipette
- 10-mL disposable pipette
- 25-mL disposable pipette
- Electric pipettor
- Cell strainer (70um, if necessary)

[Method]

1. Allow the α-Sens® Medium to stand for 1-h to bring to room temperature or warm to room temperature (approx. 20-25°C).
2. Check the cell conditions.
3. Remove the medium from the dish.
4. Rinse the cells with 10 mL of PBS (-) and remove the PBS (-).
5. Add 2 mL/dish of 0.05% Trypsin-EDTA solution and spread with gentle tilting to cover throughout the dish.
6. Incubate the dish for 10 minutes in a 5% $CO_2$ incubator with humidified conditions at 37°C.
7. After 10 minutes, monitor the cells under a microscope.
   The cells should begin to detach from the dish-bottom when they appear rounded.
8. Tap the dish-side gently to detach the cells from the dish-bottom.
9. Add 8 mL/dish of α-Sens® medium into the dish and perform mixing by pipetting the medium up and down about 10 times or more in the dish to completely detach the cells.
10. Transfer the cell suspensions to a 15- or 50-mL sterile centrifuge tube.
11. Centrifuge the tube at 300 x g for 5 minutes at room temperature (approx. 20-25°C).
12. After centrifugation, carefully remove the supernatant while avoiding sucking out the cells. Tap the tube gently to break the cell pellet.
    ***# If cell clumps are visually observed or suspected in cell suspension, use a cell strainer to avoid incorporating them according to the instructions of the product manual.***

13. Add appropriate volume of α-Sens® medium and re-suspend the cells.
14. Count the number of cells using the protocol at each laboratory.
15. Add appropriate volume of α-Sens® Medium to the tube.

***# For 2-day interval cell passage, cells should be prepared at a concentration of 1.5 x $10^5$ cells/mL and seeded at 10 mL/100-mm Dish.***

| 2-day interval cell passage | Cell passage | Day-1 | Day-2 | Cell passage |
|---|---|---|---|---|
| | | Medium change | | |

***# For 3-day interval cell passage, cells should be prepared at a concentration of 1.0 x $10^5$ cells/mL and seeded at 10 mL/100-mm Dish.***

| 3-day interval cell passage | Cell passage | Day-1 | Day-2 | Day-3 | Cell passage |
|---|---|---|---|---|---|
| | | Medium change | | Medium change | |

16. Gently mix the cell suspension with pipette.
17. Add 10 mL of cell suspension in a 100-mm cell-culture dish.
18. Incubate the dish in a 5% $CO_2$ incubator with humidified conditions at 37°C.

***# After 24 ± 2 hours of cell propagation, check if the cells are attached to the dish and replace medium with fresh and prewarmed α-Sens® medium at room temperature (approx. 20-25℃).***
***# The interval of the medium change as described above is the basis of this protocol. In case of weekend (Saturday and Sunday), the medium may be changed to 2-day interval (e.g., Friday: passage, Saturday & Sunday: no medium change, Monday: medium change), as long as the data acceptance criteria are fulfilled.***

## 3. α-Sens® assay

### 3.1. Solubility test of the test chemical

[Material]

- α-Sens® medium
- DMSO (FUJIFILM Wako Pure Chemical corporation, product #045-24511) or equivalent
- Acetone (FUJIFILM Wako Pure Chemical corporation, product #016-00346) or equivalent
- 0.5- or 2-mL sample tube (Eppendorf Safe-Lock® Tubes with volume marking) or equivalent
- Electronic balance
- Small desktop centrifuge
- Appropriate volume of calibrated single pipette
- Vortex
- Ultrasonic equipment

[Method]

The priority for selecting vehicles for solubility test is as follows.

1. DMSO
2. α-Sens® medium
   # No filter sterilization is required in case of α-Sens® medium use as vehicle to avoid the loss of chemical.
3. Acetone

***# The purpose of this solubility test is as follows.***

- ***Estimation of a test chemical solubility to set first maximum test concentration for α-Sens® assay, and***
- ***Selection of suitable vehicle for the test chemical.***

***# Under the first solubility test for the test chemical, the target highest concentration to be tested should be 500 mg/mL.***

***#: Before starting the solubility test, set 3 target concentrations (the highest, second highest and lowest) to be tested in one solubility test, using common ratio "2" from the highest concentration.***

1. Prepare 0.5- or 2-mL sample tubes.
2. By using calibrated single pipette, check the volume marking on the tube to be used or make the appropriate volume marking.

(*e.g.*, Check 0.1 mL, 0.25 mL or 0.5 mL for 0.5-mL tube, 0.5 mL, 1.0 mL or 2.0 mL for 2-mL tube)

3. Before weighing test chemicals, make sure that no residual liquid used in the volume check is remaining.
4. Accurately weigh appropriate amount of the test chemical in the sample tube. For 500 mg/mL as the highest target concentration, 50 mg or 250 mg is recommended to weigh for 0.5- or 2-mL sample tube, respectively.
5. Add the selected vehicle up to the appropriate volume marking of the tube to prepare the highest target concentration (e.g., 500 mg/mL).
6. Vortex the sample tube and/or use ultrasonic treatment, then confirm the volume after spin-down.

   If the volume is below the appropriate volume marking of the sample tube, add the selected vehicle until the content volume is as expected.
7. Examine the solubility visually.

   **For solid chemical,**

   - Vortex again to disperse insoluble solid, if any, observe the tube under the room light and check whether visible solids are remaining.
   - If visible solids are remaining, repeat vortexing the tube and/or use ultrasonic treatment. Check again for any remaining visible solids as described above.

   **For liquid chemical,**

   - Spin-down the tube and observe the tube under the room light to check for phase separation.
   - If the phase separation is observed, repeat vortexing the tube and/or use ultrasonic treatment. Check the phase separation as described above.

***#: At the highest target concentration in DMSO, if the remaining solid or phase separation is not observed, no further procedures are required. if the remaining solid or phase separation is observed, try α-Sens® Medium.***

***#: At the highest target concentration in α-Sens® Medium, if the remaining solid or phase separation is not observed, no further procedures are required. if the remaining solid or phase separation is observed, try acetone.***

***#: If the highest target concentration is prepared without the remaining solid or phase separation in any of the vehicles, no further procedures are required.***

***# If the remaining solid or phase separation is observed in all vehicles, proceed following procedures in the order of vehicle selection priority.***

8. Add the vehicle up to the appropriate volume marking of the sample tube to prepare the second highest target concentration (e.g., 250 mg/mL), and vortex the tube and/or use ultrasonic treatment.
9. Examine the solubility visually as described in step 7.

***#: If the remaining solid or phase separation is not observed in any of the vehicles, no further procedures are required.***

***#: If the test chemical does not dissolve at the second highest concentration, proceed following procedures in the order of vehicle selection priority.***

10. Add the vehicle to prepare the lowest target concentration, and vortex the tube and/or use ultrasonic treatment.
11. Examine the solubility visually as described in step 7.
12. Then, decide the vehicle and the maximum dissolved concentration for the test chemical for the assay. The vehicle for the assay should be the one for which the highest concentration can be prepared.

***#: If remaining solid or phase separation is observed at the lowest concentration in all vehicles, try another solubility test for lower concentration.***
***By dividing the lowest concentration by common ratio 2, the target highest concentration for another solubility test is decided.***
***(e.g., Lowest concentration in this solubility test =125 mg/mL. 125 mg/mL / 2 = 62.5 mg/mL. 62.5 mg/mL will be the highest target concentration for another solubility test.).***

## 3.2. Seeding of cells into assay plate

[Material]

- 96well plate Nunclon Delta-Treated, Flat-Bottom, White (Thermo Fisher Scientific KK. #136101) or equivalent
- α-Sens® medium
- PBS (-)
- (10X) 0.5% Trypsin-EDTA (GIBCO, product #15400-054) or equivalent
- 15- or 50-mL Sterile centrifuge Tube
- 1.5-mL cryotube
- 5-mL disposable pipette
- 10-mL disposable pipette
- 25-mL disposable pipette
- Electric pipettor
- 12-multichannel pipette or 8-multichannel pipette *[1]
- Pipette tip for using with multichannel pipette
- Reservoir for 12-multichannel pipette or 8-multichannel pipette
- Cell strainer (70um, if necessary)

[Method]

***# α-Sens® cells passaged more than "+3" (>= "+3") from the frozen cell stock can be used for the assay.***

***# The confluency of the α-Sens® cells in the dish are microscopically examined. If the cells in the dish are at 50%-80% confluency, the cells can be used for seeding to prepare the assay plate.***

1. Allow the α-Sens® medium to stand for 1-h to bring to room temperature or warm to room temperature (approx. 20-25°C).
2. Check the cell conditions.
3. Remove the medium from the dish.
4. Rinse the cells with 10 mL of PBS (-) and remove the PBS (-).
5. Add 2 mL/dish of 0.05% Trypsin-EDTA solution and spread with gentle tilting to cover throughout the dish.

[1] *When using a multi-channel pipette, ENSURE that the volumes in tips are uniform. If you have any concerns for uniformity, record what operation was performed at which step.*

6. Incubate the dish for 10 minutes in a 5% $CO_2$ incubator with humidified conditions at 37°C.
7. After 10 minutes, monitor the cells under a microscope. The cells should begin to detach from the dish-bottom when they appear rounded.
8. Tap the dish-side gently to detach the cells from the dish-bottom.
9. Add 8 mL/dish of α-Sens® medium into the dish and perform mixing by pipetting the medium up and down about 10 times or more in the dish to completely detach the cells.
10. Transfer the cell suspensions in a 15- or 50-mL sterile centrifuge tube.
11. Centrifuge the tube at 300x g for 5 minutes at room temperature (approx. 20-25°C).
12. After centrifugation, carefully remove the supernatant avoiding sucking out cells. Tap the tube gently to break the cell pellet.

    ***# If cell clumps are visually observed or suspected in cell suspension, use a cell strainer to avoid incorporating them according to the instructions of the product manual.***

13. Add appropriate volume of α-Sens® medium and re-suspend the cells for cell counting.
14. Count the number of cells using the protocol at each laboratory.
15. Dilute the cell suspension with α-Sens® medium to prepare 1.0 x $10^5$ cells/mL.
16. Add 100 µL of the cell suspension into each well of 96-well white plate (10,000 cells/well) using multi pipettor, ensuring that the cell suspension is homogenized.

    ***# Keep in mind that "Blank" well in "Plate Layout-A" or "Plate Layout-B" must be blank without cells and 100 µL of α-Sens® Medium needs to be added in "Blank".***

17. Incubate the assay plate in a 5% $CO_2$ incubator with humidified conditions at 37°C, for 24 ± 2 hours before Chemical Exposure.

## 3.3. Preparation of stock solutions of test chemical(s) and positive control

[Material]

- *trans*-Cinnamic aldehyde (as positive control, CAS. 14371-10-9, Fujifilm Wako, #031-03453, 98.0+% or equivalent)
- Suitable vehicle determined by solubility test, such as DMSO (as dilution solution)
- α-Sens® medium
- 1-mL or appropriate volume of volumetric flask
- 2-mL sampling tube

[Method]

***# The stock solutions of test chemical(s) and positive control must be prepared on the day of chemical exposure.***

***# The appropriate volume of volumetric flasks should be used for stock solution preparation because the "volume" of test chemical cannot be ignored in many cases to prepare the stock solution especially at the high concentration.***

**For test chemical(s)**

1. By using a 1-mL or appropriate volume of volumetric flask, prepare the maximum concentration of the chemical solution, which was determined by the solubility test (see "3.1 Solubility test of the test chemical").

OR

1. By using a 1-mL or appropriate volume of volumetric flask, prepare 1,000-fold concentration of the final maximum test concentration decided to be tested.
   For example, in case the final maximum test concentration is 0.2 μg/mL, prepare 0.2 mg/mL of the test chemical with the vehicle.

**For positive control**

1. Accurately weigh approx. 50 mg of Cinnamic aldehyde in a 1-mL volumetric flask.
2. Solubilize Cinnamic aldehyde by diluting in the volumetric flask using DMSO to prepare a 50 mg/mL DMSO solution.
3. Confirm that Cinnamic aldehyde is completely dissolved.
4. Transfer 10 μL of 50 mg/mL DMSO stock solution into a 2-mL sampling tube and add 990 μL of DMSO.
5. Mix the solution to prepare a 0.5 mg/mL DMSO solution (to be used for PC).

## 3.4. Chemical Exposure using “Plate Layout-A”

- "Plate Layout-A” can test more concentrations in triplicate for two test chemicals than “Plate Layout-B” and is efficient for the test-concentration finding of test chemicals. Therefore, "Plate Layout-A” should be the first choice for the first test of test chemicals.
- When all tested concentrations exhibit cell viability <50%, conduct next assay using “Plate Layout-A”.
- For further evaluation of High Luminescence Inducible Chemical (HiLIC) or suspected HiLIC, “Plate Layout-A” is the preferred choice (See details about HiLIC “1.3 Key Considerations in the Assay”) since “Plate Layout-A” is originally designed to avoid luminescence crosstalk to Blank, VC and PC wells, also to the other chemicals tested in the same plate, by placing the wells of only cells and α-Sens® medium between each test chemical, and Blank, VC and PC (See Fig.-3).
- For the following cases, the use of “Plate Layout-A” is the preferred choice for the next run to find the appropriate test concentration range and common ratio.
  - When you cannot make positive or negative judgement because of the case of Fig.-5 in the previous run. This is because IC50 value may be varied and "Plate Layout-A” can accommodate more test concentrations.
  - When the 1st run using “Plate Layout-A” gives positive. This is because to find the test concentration range for EC3.2 calculation. When the test concentration range covers the test concentrations to calculate EC3.2, “Plate Layout-B” may also be used instead of “Plate Layout-A”.

**#: Either Plate Layout-A or -B can be selected after finding appropriate test concentration range and common ratio, and if no luminescence crosstalk is suspected.**

**#: It should be noted that outcomes from “Plate Layout-A” can be used for positive or negative judgement for each run if all data acceptance criteria and data interpretation criteria are met.**

***#: Note: Refer Appendix-D, in case a vehicle other than DMSO has been selected for the test chemical(s).***

**(1) Preparation of serial dilution of test chemical(s)**

[Material]

- Suitable vehicle determined by solubility test, such as DMSO (as dilute solution)
- α-Sens® medium
- 2-mL sampling tube
- 96-well plate, PP (for dilution), U- or V- bottom
- Deepwell plate, 96 well, 2-mL (for purpose of mixing, 2-mL-deepwell is required)
- 10-mL disposable pipette
- 25-mL disposable pipette
- Electric pipettor
- 12-multichannel pipette or 8-multichannel pipette *[1]
- Pipette tip
- Single pipette, as appropriate
- Reservoir for 12-multichannel pipette or 8-multichannel pipette (If acetone is used as vehicle, use a glass reservoir.)

**(1)-1) Preparation of 1000 x solution plate for "Plate Layout-A"**

[Method]

***# The concentration setting (maximum concentration and common ratio for dilution) should be followed in "3.5 Setting of the top-concentration and concentration range"***

***# In case using different common ratio, following amounts can be used to prepare serial dilutions, as examples.***

| Common ratio | Vehicle volume in a well from column 2 to 10 | Amount to transfer from the previous well |
|---|---|---|
| 2 | 50 µL | 50 µL |
| 1.2 | 30 µL | 150 µL |

1. Add appropriate volume of vehicle to the corresponding Column-2 to Column-10 wells of 96-well PP-plate using a multi-channel pipette set at appropriate volume.
2. Add 100 µL (or 200 µL) of the stock solution of each test chemical in a corresponding well of Column-1 using pipette set at 100 µL (or 200 µL).
3. Transfer appropriate volume of test chemical solution from Column-1 to the corresponding wells of Column-2 using a multi-channel pipette and mix gently about 10 times or more by pipetting.
4. Repeat the same treatment up to Column-10 well to prepare 10 serial dilutions of test chemicals.

5. Prepare the vehicle only and 0.5 mg/mL *trans*-Cinnamic aldehyde wells to be used as vehicle control (VC) and positive control (PC).

***# If acetone is used as a vehicle and its volatilization is as a concern, the serial dilutions of the test chemical with acetone may be prepared, e.g. in volumes of 500 µL or 1 mL, using acetone-resistant PP microtubes or similar. The preparation procedure of the serial dilutions should be recorded.***

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | In case of common ratio "2"<br>Chemical-1 Stock soln. 100µL | Vehicle 50µL<br>+50 µL | Vehicle 50µL<br>+50 µL | Vehicle 50µL<br>+50 µL | Vehicle 50µL<br>+50 µL | Vehicle 50µL<br>+50 µL | Vehicle 50µL<br>+50 µL | Vehicle 50µL<br>+50 µL | Vehicle 50µL<br>+50 µL | Vehicle 50µL<br>+50 µL | | |
| B | In case of common ratio "1.2"<br>Chemical-2 Stock soln. 100µL | Vehicle 30µL<br>+150 µL | Vehicle 30µL<br>+150 µL | Vehicle 30µL<br>+150 µL | Vehicle 30µL<br>+150 µL | Vehicle 30µL<br>+150 µL | Vehicle 30µL<br>+150 µL | Vehicle 30µL<br>+150 µL | Vehicle 30µL<br>+150 µL | Vehicle 30µL<br>+150 µL | | |
| C | … | … | … | … | … | … | … | … | … | … | | |
| G | … | … | … | … | … | … | … | … | … | … | | |
| H | … | … | … | … | … | … | … | … | … | … | | |

**Example layout for 1000 x solution plate**

**(1)-2) Preparation of 2x solution plate for "Plate Layout-A"**

***# This procedure shall be performed in the biosafety cabinet.***

***# Important!! Immediately after preparing 2x solution for one assay plate, you have to conduct chemical exposure to cells to avoid precipitation of chemicals in 2x solution plate.***

***Therefore, it is important to prepare 2x solution and perform cell exposure by one assay plate each. (i.e., if you have 2 assay plate, prepare 2x solution for one assay plate and perform cell exposure for the first assay plate, then prepare next 2x solution and perform cell exposure for the next assay plate.)***

[Method]

1. Allow the α-Sens® Medium to stand for 1-h to bring to room temperature or warm to room temperature (approx. 20-25°C).
2. Add 998 µL of α-Sens® medium to necessary wells in a deepwell plate using a multichannel pipette set at 998µL by a single action (Preferable).
   ***#: If you do not have a high-volume multichannel pipette, you may perform***

***dispensing multiple times to bring final volume to 998 µL in the deepwell plate. In such case, the dispensing procedure should be recorded.***

3. Also add 998 µL of α-Sens® medium to "A12" – "H12" wells using the multichannel pipette.

***#: Important!! When adding 2 µL of 2x solution to the 96-deepwell plate, just eject the whole volume of the aspirated solutions in a single action without pipetting to avoid the precipitation or adhesion in/on the pipette tip.***
***Any observation such as precipitation or any other observation should be recorded.***

4. Add 2 µL of vehicle to B12, C12 and D12 wells (3 wells) in the 96-deepwell plate for vehicle control (VC) using the (multi-channel) pipette set at 2 µL.
5. Add 2 µL of 0.5 mg/mL Cinnamic aldehyde solution to E12, F12 and G12 wells (3 wells) in the 96-deepwell plate for positive control (PC) using the (multi-channel) pipette set at 2 µL.
6. Transfer 2 µL of chemical solutions from Column-1 – Column-10 wells in 1000x solution plate to the corresponding Column-1 – Column- 10 wells in the 96-deepwell plate using the multichannel pipette set at 2 µL.
   ***# If you have additional test chemicals, use the subsequent rows (i.e., Row B to H), where it is appropriate, to apply the same procedures. At maximum, serial dilutions for six test chemicals can be prepared per plate.***

***#: Important!! The following mixing process is critically important for the assay outcome. Ensure that this process is properly conducted as described in the protocol.***

7. After completion of 2 µL addition for one assay plate (*i.e.*, addition of 2x solution for 2 chemicals, VC and PC), pipette* the solution 20 times or more with a multi-channel pipette set at 300 µL, to ensure complete mixing.
   ***[Pipetting*: turn round and round while pipetting at the well-bottom (3 rounds), then take the liquid from the well-bottom and release it around 3/4 height of the solution in deep-wells.***
   ***Repeat this mixing 20 times or more to ensure the solution homogeneous.]***

***#: In case of the chemical precipitation or phase-separation is observed when adding 2x solution to the medium in a deepwell, if it can be homogenously dispersed and can be pipette properly without tip clogging, the test concentration can be used for data analysis.***

**Example Plate layout for 2X solution plate for "Plate Layout A"**

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Chemical-1 ( ) | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | … | Medium 998 µL |
| B | Chemical-2 ( ) | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | … | Medium 998 µL + 2 µL vehicle |
| C | Chemical-3 ( ) | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | … | Medium 998 µL + 2 µL vehicle |
| D | Chemical-4 ( ) | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | … | Medium 998 µL + 2 µL vehicle |
| E | Chemical-5 ( ) | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | … | Medium 998 µL + 2 µL PC |
| F | Chemical-6 ( ) | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | Medium 998 µL +2 µL | … | Medium 998 µL + 2 µL PC |
| G | … | … | … | … | … | … | … | … | … | … | … | … | Medium 998 µL + 2 µL PC |
| H | … | … | … | … | … | … | … | … | … | … | … | … | Medium 998 µL |

***# If acetone is used as the vehicle and its volatilization is as a concern, the x2 solutions can be prepared in larger volumes. For example, add 9.98 mL of α-Sens® medium to the required number of 15-mL tubes or Spitz tubes (hereafter referred to as tubes), transfer 20 µL of x1000 solution to the corresponding tube. Transfer these x2 solutions to a deep-well plate along the plate layout for chemical exposure using a multichannel pipette. The preparation procedure of the x2 solutions should be recorded.***

**(2) Chemical Exposure to Cells for "Plate Layout-A"**

[Material]

- α-Sens® medium
- Sterile Polyester Plate Seal (Thermo Scientific 236366PK or equivalent)
- 12-multichannel pipette or 8-multichannel pipette *[1]
- Pipette tip for multichannel pipette

[Method]

In brief, after 24 ± 2 h incubation of the assay plate prepared in the section "3.2 Seeding of cells into assay plate", add 100 µL of diluted chemicals from the 2x solution plate (See 3.4(1)-2) Preparation of 2x solution plate for "Plate Layout-A") to the assay plate (final volume will be 200 µL/well).

***# This procedure shall be performed in the biosafety cabinet.***

***#: Use the assay plate that was incubated for 24± 2 h after cell seeding to the plate.***

***# Important!! Even if you have multiple assay plates to run on the same day, the preparation of 2x solution and exposure to cells must be performed each assay plate. This operation is for avoiding precipitation of chemicals in the medium in 2x solution plate, especially at high concentration.***

1. Just before 100 μL addition in triplicate of the diluted chemical solution from 2x solution plate to the corresponding Column of the assay plate, gently mix the corresponding Column wells (e.g., A1-A10 wells) **of 2x solution plate** by 5 times or more pipetting with a multichannel pipette.
2. Then, transfer 100 μL of the diluted chemical solution from 2x solution plate to the corresponding wells in triplicate (e.g., A1 -C1 wells) of the assay plate at each test concentration, using the multichannel pipette [*2].

   (If you have another test chemical in the same plate) Repeat this treatment for the next test chemical by changing the corresponding Column of 2x solution plate.)

3. Add 100μL of α-Sens® medium to D1-D10, E1-E10 as adjacent wells for test chemical wells, using the multichannel pipette *[2].
4. Add 100μL of α-Sens® medium to Row-11 (A11-H11) wells, using the multichannel pipette*[2].
5. Take 100 μL Row 12 (A12-H12) solution from 2x solution plate and add the solution to the corresponding Row (A12-H12) of the assay plate, using the multichannel pipette*[2].
6. After the chemical exposure to the cells, the plate is sealed properly and is covered with the plate lid.
7. Incubate the plate for 24±2 hours in a 5% $CO_2$ incubator in humidified conditions at 37°C.
8. After exposure, carefully observe the deepwell plate to check the presence of precipitation *etc*.

***# Important!! Polyester plate seals can reduce chemical contamination through the air phase compared to Polypropylene plate seals.***

*[2] ***Important!! Do not pipette the solution in wells containing cells, in order to avoid the detachment of cells from the well bottom. This will affect the value of cell viability and the coefficient variation (CV) of triplicate wells.***

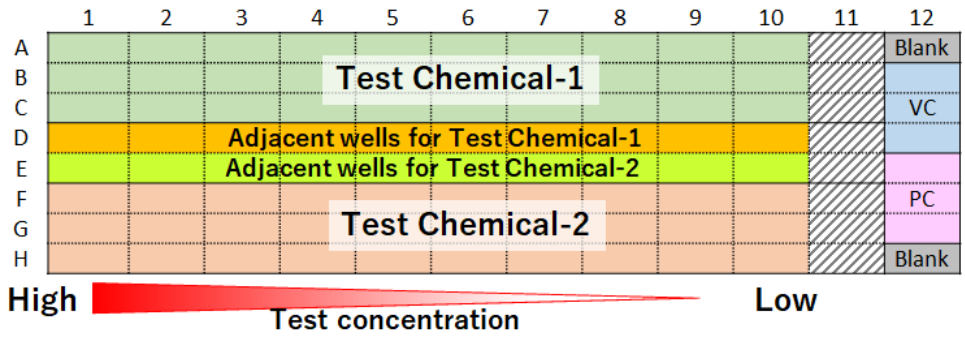



**Fig.- 6 Representative plate layout for Plate layout-A**

VC: Vehicle Control, PC: Positive Control at 0.5 µg/mL of Cinnamic aldehyde as final conc., Blank: no cells, α-Sens® medium only,
Wells shaded by diagonal line: Containing cells and α-Sens® medium, to be treated with both *Firefly* and *Renilla* luciferase reagents.
Adjacent wells in "Plate Layout-A": Containing cells and α-Sens® medium, to be treated with both *Firefly* and *Renilla* luciferase reagents. These adjacent wells are used to identify a chemical that have strong luminescence.

***#: Note: Refer Appendix-D, in case a vehicle other than DMSO has been selected for the test chemical(s).***

### 3.5. Setting of the top-concentration and concentration range

After the first assay by "Plate Layout-A", follow the flowchart in Fig.-4. to identify preferable plate layout, maximum concentration and common ratio for test chemicals.

## 3.6. Chemical Exposure using “Plate Layout-B”

- “Plate Layout-B” can use 7 test concentrations in triplicates for four test chemicals.
- When the 1st run using “Plate Layout-A” gives negative judgement, “Plate Layout-B” is used since it is not necessary to set many test concentrations to give negative as far as the appropriate maximum test concentration and common ratio is set by following Fig.-4.
- When the test concentration range covers the test concentrations to calculate EC3.2, “Plate Layout-B” is used for the second or subsequent tests of test chemicals.
- After finding appropriate test concentration range and common ratio, and if no luminescence crosstalk is suspected, either Plate Layout-A or -B can be selected.
- When HiLIC or Suspected HiLIC chemical is tested in “Plate Layout-B”, such chemical should be placed in A7-G9 wells and no other test chemical should not be tested in A4-G6 and A10-G12 wells.
- ***#: Note: Refer Appendix-D, in case a vehicle other than DMSO has been selected for the test chemical(s).***

### (1) Preparation of serial dilution of test chemical(s)

[Material]

- Suitable vehicle determined by solubility test, such as DMSO (as dilute solution)
- α-Sens® medium
- 2-mL sampling tube
- 96-well plate, PP (for dilution), U- or V-bottom
- Deepwell plate, 96 well, 2-mL (for purpose of mixing, 2-mL deepwell is required)
- 10-mL disposable pipette
- 25-mL disposable pipette
- Electric pipettor
- 12-multichannel pipette or 8-multichannel pipette *[1]
- Pipette tip
- Single pipette, as appropriate
- Reservoir for 12-multichannel pipette or 8-multichannel pipette（If acetone is used as vehicle, use a glass reservoir）

**(1)-1) Preparation of 1000 x solution plate for "Plate Layout-B"**

[Method]

***# The concentration setting (maximum concentration and common ratio for dilution) should be followed in "3.5 Setting of the top-concentration and concentration range"***

***# In case using different common ratio, following amounts can be used to prepare serial dilutions, as examples.***

| Common ratio | Vehicle volume to be added in wells from Row-B to G | Transfer volume from the previous well |
|---|---|---|
| 2 | 50 µL | 50 µL |
| 1.2 | 30 µL | 150 µL |

1. Add appropriate volume of vehicle to corresponding wells of Row-"B" – Row-"G" in 96-well PP-plate using a multi-channel pipette set at appropriate volume.
2. Add 100 µL (or 200 µL) of the stock solution of each test chemical in a corresponding well of Row-"A" using pipette set at 100 µL.
3. Transfer appropriate volume of test chemical stock solution from Row-"A" to Row-"B" using the multi-channel pipette and mix gently by pipetting about 10 times or more.
4. Repeat this dilution procedures upto Row-"G".
5. Add 50 µL of vehicle to "H1" well (and "H5", "H9", if necessary) for vehicle control (VC) using pipette set at 50 µL.
6. Add 50 µL of 0.5 mg/mL *trans*-Cinnamic aldehyde (for PC) to "H4" (and "H8", "H12", if necessary) using pipette set at 50 µL.

***# If you have additional test chemicals, use the subsequent columns (i.e., 2 to 12), where it is appropriate, to apply the same procedures. At maximum, serial dilutions for 12 test chemicals can be prepared per 1000x solution plate.***

| | In case of common ratio = **2** | | In case of common ratio = **1.2** | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 12 |
| A | 100 µL of stock soln. of chem -1. | 100 µL of stock soln. of chem -2. | 200 µL of stock soln. of chem -3. | 100 µL of stock soln. of chem -4 | … |
| B | Vehicle 50 µL + 50 µL of A1 soln. | … | Vehicle 30 µL + 150 µL of A3 soln. | … | … |
| C | Vehicle 50 µL + 50 µL of B1 soln. | … | Vehicle 30 µL + 150 µL of B3 soln. | … | … |
| D | Vehicle 50 µL + 50 µL of C1 soln. | … | Vehicle 30 µL + 150 µL of C3 soln. | … | … |
| E | Vehicle 50 µL + 50 µL of D1 soln. | … | Vehicle 30 µL + 150 µL of D3 soln. | … | … |
| F | Vehicle 50 µL + 50 µL of E1 soln. | … | Vehicle 30 µL + 150 µL of E3 soln. | … | … |
| G | Vehicle 50 µL + 50 µL of F1 soln. | … | Vehicle 30 µL + 150 µL of F3 soln. | … | … |
| H | Vehicle | | | PC | … |

***# If acetone is used as a vehicle and its volatilization is as a concern, the serial dilutions of the test chemical with acetone may be prepared, e.g. in volumes of 500 µL or 1 mL, using acetone-resistant PP microtubes or similar. The preparation procedure of the serial dilutions should be recorded.***

**(1)-2) Preparation of 2x solution plate for "Plate Layout-B"**

***# This procedure shall be performed in the biosafety cabinet.***
***# Important!! Immediately after preparing 2x solution for one assay plate, you have to conduct chemical exposure to cells to avoid precipitation of chemicals in 2x solution plate.***

***Therefore, it is important to prepare 2x solution and perform cell exposure by one assay plate each. (i.e., if you have 2 assay plate, prepare 2x solution for one assay plate and perform cell exposure for the first assay plate, then prepare next 2x solution and perform cell exposure for the next assay plate.)***

[Method]

In brief, prepare a 2x solution plate using α-Sens® medium from a 1000x solution plate.

1. Allow the α-Sens® Medium to stand for 1-h to bring to room temperature or warm to room temperature (Approx. 20-25°C).
2. Add 998 µL of α-Sens® medium to the necessary wells of Row "A"-"H" of the deepwell plate using a multichannel pipette set at 998µL by a single action (Preferable). ***#: If you do not have a high-volume multichannel pipette, you may perform dispensing multiple times to bring 998 µL in the deepwell plate, the dispensing procedure should be recorded***.

***#: Important!! When adding 2 µL of 2x solution to the 96-deepwell plate, just eject the whole volume of the aspirated solutions in a single action without pipetting to avoid the precipitation or adhesion in/on the pipette tip.***
***Any observation such as precipitation or any other observation should be recorded.***

3. Transfer 2 µL of 2x solutions from the appropriate Row of "A"-"H" wells (*e.g.*, A1-H1) in the 1000x solution to the corresponding Row of "A" – "H" wells (*e.g.*, A1-H1) in the deepwell plate (2x solution plate) using a multichannel pipette set at 2 µL.

***#: Important!! The following mixing process is critically important for the assay outcome.***

***Ensure that this process is properly conducted as described in the protocol.***

4. After completion of 2 µL addition for one assay plate, pipette* the solution 20 times or more with a multi-channel pipette set at 300 µL (Eppendorf multichannel pipette can set 300 µL), to ensure complete mixing.

   ***[Pipetting*: turn round and round while pipetting at the well-bottom (3 rounds), then take the liquid from the well-bottom and release it around 3/4 height of solution in the deep-wells.***

   ***Repeat this mixing 20 times or more to ensure the solution homogeneous.]***

***# In case chemical precipitation or phase-separation is observed when adding 2x solution to the medium in a deepwell, if it can be homogenously dispersed and can be pipette properly without tip clogging, the test concentration can be used for data analysis.***

***# If you have additional test chemicals, use the subsequent columns (i.e., 2 to 12), where it is appropriate, to apply the same procedures. At maximum, serial dilutions for 12 test chemicals can be prepared per plate.***

**Example Plate layout for 2X solution plate for "Plate Layout B"**

| | For the 1st assay plate | | | | For the 2nd assay plate | | | | For the 3rd assay plate | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| | Chemical-1 | Chemical-2 | Chemical-3 | Chemical-4 | Chemical-5 | Chemical-6 | Chemical-7 | Chemical-8 | Chemical-9 | Chemical-10 | Chemical-11 | Chemical-12 |
| A | Medium 998 µL + 2 µL | Medium 998 µL + 2 µL | … | … | … | … | … | … | … | … | … | … |
| B | Medium 998 µL + 2 µL | Medium 998 µL + 2 µL | … | … | … | … | … | … | … | … | … | … |
| C | Medium 998 µL + 2 µL | Medium 998 µL + 2 µL | … | … | … | … | … | … | … | … | … | … |
| D | Medium 998 µL + 2 µL | Medium 998 µL + 2 µL | … | … | … | … | … | … | … | … | … | … |
| E | Medium 998 µL + 2 µL | Medium 998 µL + 2 µL | … | … | … | … | … | … | … | … | … | … |
| F | Medium 998 µL + 2 µL | Medium 998 µL + 2 µL | … | … | … | … | … | … | … | … | … | … |
| G | Medium 998 µL + 2 µL | Medium 998 µL + 2 µL | … | … | … | … | … | … | … | … | … | … |
| H | Medium 998 µL + 2 µL Vehicle | Medium 998 µL | Medium 998 µL | Medium 998 µL + 2 µL PC | Medium 998 µL + 2 µL Vehicle | Medium 998 µL | Medium 998 µL | Medium 998 µL + 2 µL PC | Medium 998 µL + 2 µL Vehicle | Medium 998 µL | Medium 998 µL | Medium 998 µL + 2 µL PC |

***# If acetone is used as the vehicle and its volatilization is as a concern, the x2 solutions can be prepared in larger volumes. For example, add 9.98 mL of α-Sens® medium to the required number of 15-mL tubes or Spitz tubes (hereafter referred to as tubes), transfer 20 µL of x1000 solution to the corresponding tube. Transfer these x2 solutions to a deep-well plate along the plate layout for chemical exposure using a multichannel pipette. The preparation procedure of the x2 solutions should be recorded.***

**(2) Chemical Exposure to Cells for "Plate Layout-B"**

[Material]

- α-Sens® medium
- Sterile Polyester Plate Seal (Thermo Scientific 236366PK or equivalent)
- 12-multichannel pipette or 8-multichannel pipette *[1]
- Pipette tip for multichannel pipette

[Method]

In brief, after 24 ± 2 h incubation of the assay plate prepared in the section "3.2 Seeding of cells into assay plate", add 100 µL of diluted chemicals from the 2x solution plate (See 3.6(1)-2)Preparation of 2x solution plate for "Plate Layout-B") to the assay plate.

***# This procedure shall be performed in the biosafety cabinet.***

***#: Use the assay plate that was incubated for 24± 2 h after cell seeding to the plate.***

***# Important!! Even if multiple assay plates are tested on the same day, the preparation of 2x solution and exposure to cells must be performed each assay plate. This operation is for avoiding precipitation of chemicals in the medium in 2x solution plate, especially at high concentration.***

1. Just before 100 µL addition in triplicate (e.g., A1-H1, A2-H2 and A3-H3) of the diluted chemical solution from 2x solution plate to the corresponding Row of the assay plate, gently mix the solution in the corresponding Row wells (e.g., A1-H1) **of 2x solution plate** by 5 times or more pipetting with a multi-channel pipette.
2. Then transfer 100 µL of diluted solution from appropriate Row wells (e.g., A1-H1) in 2x solution plate to the corresponding well in triplicate (e.g., A1-H1, A2-H2 and A3-H3) in the assay plate using the multi-channel pipette *[3].
   (When testing multiple chemicals, the same treatment should be done according to the plate layout below.)
3. Seal the assay plate properly and cover the plate with lid.
4. Incubate for 24±2 hours in a 5% $CO_2$ incubator in humidified conditions at 37°C.
5. After exposure, carefully observe the deepwell plate to check for the presence of precipitation *etc*.

*[3] ***Important!! Do not pipette the solution in wells containing cells, in order to avoid the detachment of cells from the well bottom. This will affect the value of cell viability and the coefficient variation (CV) of triplicates wells.***

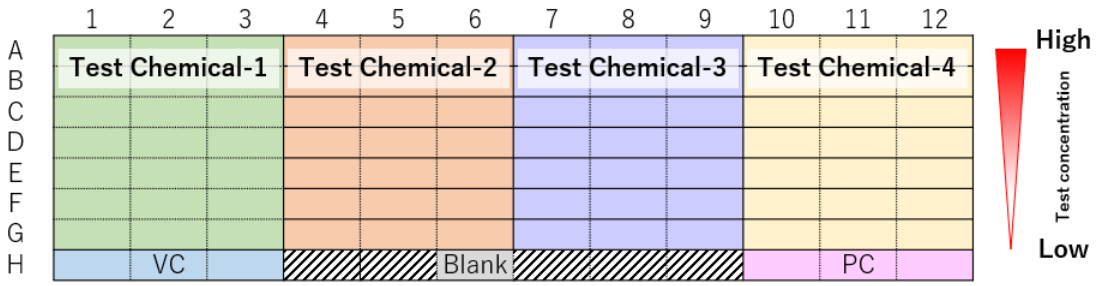



**Fig.-7 Representative plate layout for Plate layout-B**

VC: Vehicle Control, PC: Positive Control at 0.5 µg/mL of Cinnamic aldehyde, Blank: no cells, α-Sens® medium only,
Wells shaded by diagonal line: Containing cells, to be treated with both *Firefly* and *Renilla* luciferase reagents. These wells will be used for one of data acceptance criteria.

***#: Note: Refer Appendix-D, in case a vehicle other than DMSO has been selected for the test chemical(s).***

## 3.7. Luciferase assay

[Material]

- Dual-Glo® Luciferase Assay System (Promega、E2920) or equivalent

  *Dual-Glo® Luciferase Reagent for Dual-1 working solution (for measuring Firefly luciferase activity):*

  ・Dual-Glo® Luciferase Substrate

  ・Dual-Glo® Luciferase Buffer

  *For Dual-2 working solution (for measuring Renilla luciferase activity):*

  ・Dual-Glo® Stop & Glo Substrate

  ・Dual-Glo® Stop & Glo Buffer

  ***# Check the recommended storage conditions described by the manufacturer's instruction and store the reagents as per the instruction since the conditions of the luciferase reagents affects the assay outcomes, in general.***
- PBS (-)
- Luminometer
- 5-mL disposable pipette
- 10-mL disposable pipette
- Electric pipettor
- 12-multichannel pipette or 8-multichannel pipette *[1]
- Pipette tip for using multichannel pipette
- Reservoir for 12-multichannel pipette or 8-multichannel pipette
- 15-mL conical tube (if necessary for storage)
- Items necessary for light-shielding preservation

[Method]

1. Prepare "Dual-Glo® Luciferase Reagent" for Dual-1 (for measuring Firefly luciferase activity) and Dual-2 working solution according to the manufacturer's instruction.

   ***# Must be followed as per the manufacturer's instruction since the conditions of the luciferase reagents affects the assay outcomes, in general.***

   ***# Dual-2 working solution should be prepared just before use. 2.5 mL per plate.***

   ***# Keep Dual-2 working solution in the dark until use.***

2. Dilute "Dual-Glo® Luciferase reagent" for Dual-1 with the same volume of PBS (-) to prepare Dual-1 working solution (5 mL of Dual-1 working solution is required for a

plate).

***# Keep Dual-1 working solution in the dark until use.***

***# The procedures, from the medium removal to the addition of Dual-1 working solution in the assay plate, shall be done for each assay plate.***

3. Bring out an assay plate from $CO_2$ incubator and carefully remove the lid and seal from the plate, avoiding splashing the solution in wells.
4. Remove all medium from each well by using multichannel pipette.
5. ***# When removing the medium, do not touch the cells in the well.*** Add 200 μL of PBS (-) to all wells and remove PBS (-) by using multichannel pipette for washing (same caution above described is applied).
6. Add 50 μL of Dual-1 working solution to all wells of the assay plate.
   ***#: No additional pipetting or mixing is required after the addition of Dual-1 working solution.***
7. Allow to stand for 10 minutes at room temperature (approx. 20-25°C) in the plate reader or under the light-protected condition. (Do not shake or tap the plate)
8. After 10 minutes, measure the *Firefly* luminescence signal with the luminometer.
9. After the measurement of the *Firefly* luminescence, retrieve the plate from the luminometer and add 25 μL of Dual-2 working solution to all wells of the assay plate.
   ***#: No additional pipetting or mixing is required after the addition of Dual-2 working solution.***
10. Allow to stand for 10 minutes at room temperature (approx. 20-25°C) in the plate reader or under the light-protected condition. (Do not shake or tap the plate)
11. After 10 minutes, measure the *Renilla* luminescence signal with the luminometer.

## 4. Data Analysis

*: If a vehicle other than DMSO was used for the test chemical(s), parameters, such as fold induction, cell viability and nAA, for the test chemical(s) for data analysis should be based on the same vehicle used for the test chemical(s) (*i.e.*, Acetone or α-Sens® medium). Parameters for the positive control (PC) should be based on the vehicle control for positive control ($VC_{PC}$) (*i.e.*, DMSO).

### 4.1. Calculation of Fold induction

1. Subtract the blank RLU signal from the *Firefly* activity of all wells.
2. Then, calculate the average of RLU of *Firefly* activity for the vehicle control (VC) wells.
3. Calculate the fold induction dividing RLU of each well by the averaged RLU of *Firefly* activity of VC.
4. Calculate the averaged fold induction for three-well of each concentration, VC and positive control (PC).

*: Calculate the coefficient of variation (CV) for vehicle control (VC) from RLU signal from the *Firefly* activity.

### 4.2. Calculation of cell viability

1. Subtract the blank RLU signal from the *Renilla* activity.
2. Then, calculate the average of RLU of *Renilla* activity for Vehicle Control (VC) wells.
3. Cell viability (%) is calculated from the signal values by dividing the RLU of *Renilla* activity of each well by the averaged RLU of VC.
4. Calculate the averaged cell viability for each three-wells for each concentration, VC and positive control (PC).

*: Calculate the coefficient of variation (CV) for vehicle control (VC) from RLU signal from the *Renilla* activity.

### 4.3. Calculation of nAA

1. Subtract the blank RLU signal from the *Firefly* and *Renilla* activities of all wells.
2. Calculate nRLU for each well of assay plate by dividing the *Firefly* luciferase signal by the corresponding *Renilla* luciferase signal.
3. Calculate the averaged of nRLU for VC.
4. Calculate the nAA for each well by dividing the nRLU of each well by the averaged nRLU of VC.

5. Calculate the average of nAA for each concentration and positive control.
6. Only accept nAA if a cell viability is greater than 50% as calculated by section 4.2, and the judgement should be conducted by using the nAA values.

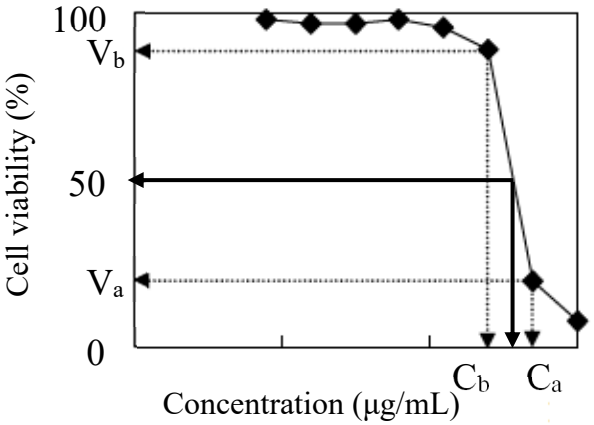
## 4.4. Calculation of ratio of RLU between vehicle control and medium control

1. Calculate the RLUs average of VC (n=3) and RLUs average of H4-5 and H7-9 well (n=5).
2. Calculate $RLU_{avg\_VC}$ / $RLU_{avg\_H(n=5)}$ as ratio to be used for data acceptance criteria in "Plate Layout-B".

*: The same vehicle used for the test chemical(s) should be used for the vehicle control (VC).

## 4.5. Calculation of IC50 for cell viability

IC50 (50% Inhibitory Concentration of cell viability) can be calculated using the equation bellow, using data points lying immediately above and below low dose ($C_b$, $V_b$) and high dose ($C_a$, $V_a$).

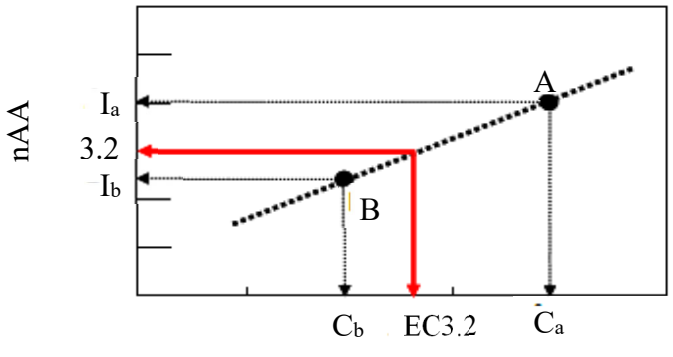



$$IC50 = C_a + \frac{(C_b-C_a) * (50-V_a)}{(V_b-V_a)}$$

## 4.6. Calculation of EC3.2

EC3.2 can be calculated by the following equation using data points lying immediately above and below (high dose (Ca, Ia) and low dose (Cb, Ib)).

$$EC3.2 = Ca + [(3.2-Ia) / (Ib-Ia) * (Cb-Ca)]$$



# 5. Data interpretation

## 5.1. Data Acceptance Criteria for assay

The assay results need to fulfill the following conditions to be qualified for further evaluation for data interpretation:

1. nAA for Positive control (0.5 μg/mL Cinnamic aldehyde): 3.2 or more.
2. Fold induction for Positive control (0.5 μg/mL Cinnamic aldehyde): 3.2 or more.
3. Cell viability for Positive control (0.5 μg/mL Cinnamic aldehyde): 50% or more.
4. The coefficient of variation (CV) of the RLU of *Firefly* and *Renilla* activities in the vehicle control: 20% or less.
   If a vehicle other than DMSO was used for the test chemical(s), this criterion should be applied to both the vehicle controls for the test chemical(s) and for the positive control ($VC_{PC}$).

For plate Layout-B, following additional criteria should be passed.

5. $RLU_{avg\_VC}$ / $RLU_{avg\_H(n=5)}$: within 0.82～1.40. (see the calculation in "4.4 Calculation of ratio of RLU between vehicle control and medium control")

## 5.2. Data interpretation

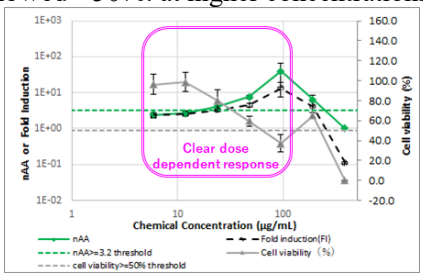
### (1) Positive / negative judgement for each run

It is important to check the data visually with checking the graph. If biologically ambiguous response is observed, it is recommended to repeat the assay.

Judgement can be made based on the guides in Table 1.

**Table 1. Judgement guides for outcome from each run**

| | |
|---|---|
| inconclusive | **Case-[1]:** Biologically ambiguous response is observed by visual inspection : need to repeat the assay.<br>e.g., If cell viability >= 50% at a concentration higher than the concentration at which cell viability showed <50%.<br>**You cannot make any conclusions from this outcome.**<br><br>**Case-[2]:** All test concentrations are below 50% (<50%) cell viability: need further testing by lowering the maximum test concentration.<br>**You cannot make any conclusions from this outcome.**<br><br>**Case-[3]:** Cell viability for lower and higher concentrations of 2 concentrations crossing 3.2 of nAA are >=50% and <50%, respectively, and the common ration used is other than 1.2: need further testing using smaller common ratio. **You cannot make any conclusions from this outcome.**<br><br>**Case-[4]:** Multiple EC3.2 values are calculated where cell viability where cytotoxicity is not observed.: need further testing. If this phenomenon is repeated, no additional testing is required. |

| | |
|---|---|
| | Note: You can ignore the EC3.2 where cell viability is below 50% and no need for further testing in such case.<br><br>**Case-[5]:** Cell viability for lower and higher concentrations of 2 concentrations crossing 3.2 of nAA are >=50% and <50%, respectively. **You cannot make any conclusions from this outcome.** If the same results are generated, you need to use lower common ratio.<br><br>**Case-[6]: Luminescence crosstalk**<br>◆ In case of chemical identified as HiLIC in Plate Layout-A, or in case of a test chemical that showed luminescence crosstalk in Plate Layout-B by the investigation in Appendix-C, follow the instruction in Appendix-B.<br>◆ When a chemical was not identified as HiLIC or suspected HiLIC by Plate layout-A, but luminescence crosstalk was observed in the following run in Plate layout-B and was considered to have affected the judgement for the adjacent test chemical(s). **You cannot make any conclusions for the adjacent test chemical(s) from this outcome (also see Appendix-C).** |
| positive | **Case-[7]:** nAA is >=3.2 and its cell viability is >=50% at the test concentration.<br><br>**Case-[8]:** In case of HiLIC tested in Plate Layout-A, or in case of a test chemical that showed luminescence crosstalk in Plate Layout-B by the investigation in Appendix-C, follow the instruction in Appendix-B.<br><br>**Case-[9]:** When a clear positive response is observed in the lower concentration range, even if the spreadsheet says "Inconclusive" due to pattern [3] (If cell viability >= 50% at a concentration higher than the concentration at which cell viability showed <50%. at higher concentrations).<br>Example for Case-[9]:  |
| negative | **Case-[10]:** None of the above conditions are applied and nAA values at all test concentration >=50% cell viability is <3.2. |

## (2) Overall conclusion from 2 or 3 qualified runs

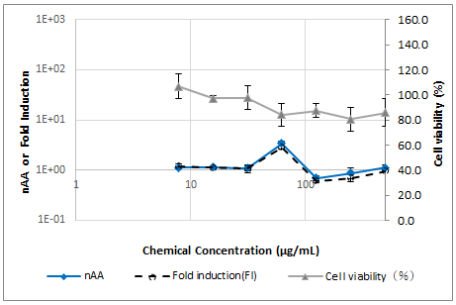
Overall conclusion can be "Positive" or "Negative".

At least two runs should be performed to make overall conclusion.

- if the concordant results obtained, judgement can be accepted for these two runs.
- if the results are discordant in the first two runs, the additional run should be conducted, and the first two concordant results should be used for judgments.

Note:

- **Case-[11]:** If the first two concordant results are both “inconclusive” by Case-[4] (Multiple EC3.2 values are calculated) in Table 1, the overall conclusion is “Positive”.
- **Case-[12]:** In the non-cytotoxic (cell viability>=50%) test concentration range, the results that nAA ≥ 3.2 in the lower concentration and nAA <3.2 in the higher concentration are repeatedly obtained. In such cases (as below), this test chemical is considered as “Positive”.

Example of Case-[12]: 

## 5.3. Identification of High Luminescence inducible chemical ("HiLIC") by "Plate Layout-A"

In the first run by "Plate Layout-A", the fold-induction level of the adjacent wells of each test chemical (Fig.-8) must be checked to identify the strong luminescence chemical. If the fold-induction level of the adjacent wells is >=3.2, the test chemical is identified as "High Luminescence inducible chemical (HiLIC)" under α-Sens® assay conditions.

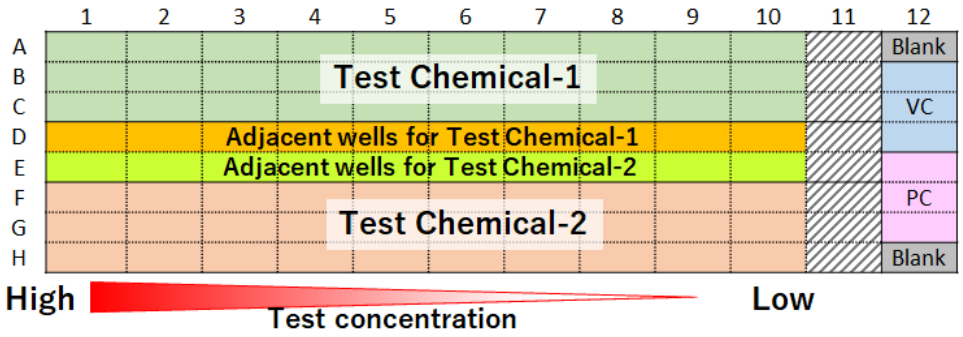



**Fig.-8 Plate Layout-A for α-Sens®**

VC: Vehicle Control, PC: 0.5 µg/mL Cinnamic aldehyde, Blank: no cells, α-Sens® medium only,
Wells by diagonal line: Containing cells, to be treated with both *Firefly* and *Renilla* luciferase reagents.
Adjacent wells: Containing cells, to be treated with both *Firefly* and *Renilla* luciferase reagents. These adjacent wells are used to identify a chemical that have strong luminescence.

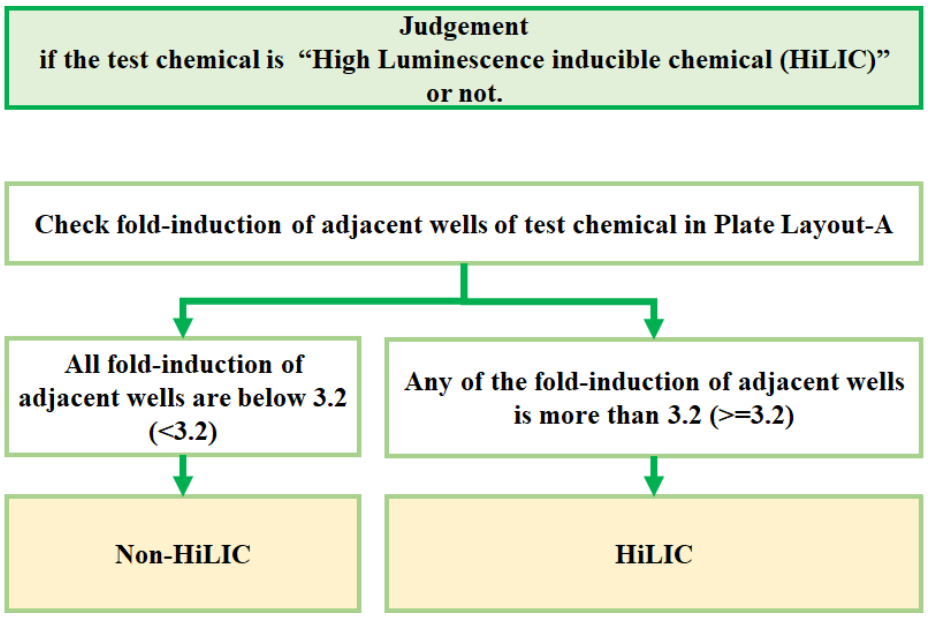



**Fig.-9 Flowchart to identify "High Luminescence inducible chemical (HiLIC)"**

## 5.4. Investigation of interference of the luminescence crosstalk from HiLIC (See Appendix-C)

Study Directors should determine whether a luminescence crosstalk from high luminescence affected the judgement from the assay by following three steps in Plate Layout-B (also see Appendix-C).

Step-1: Cautions should be paid for “positive” judged chemical (Chem-A) that have a few well(s) with nAA >= 3.2 in only one side.

Step-2: Check nAA values of positive judged chemical next to the chemical of interest in Step-1 (Chem-A).

Step-3: Check RLU of interest in Step-1 (Chem-A) whether these RLU value are affected by the adjacent chemicals or not. If it seemed affected, discard this data for the chemical interested in Step-1 (Chem-A) and retest should be conducted.

## Appendix-A: Details of the changes from Maeda et al. (2020) to the current α-Sens® assay

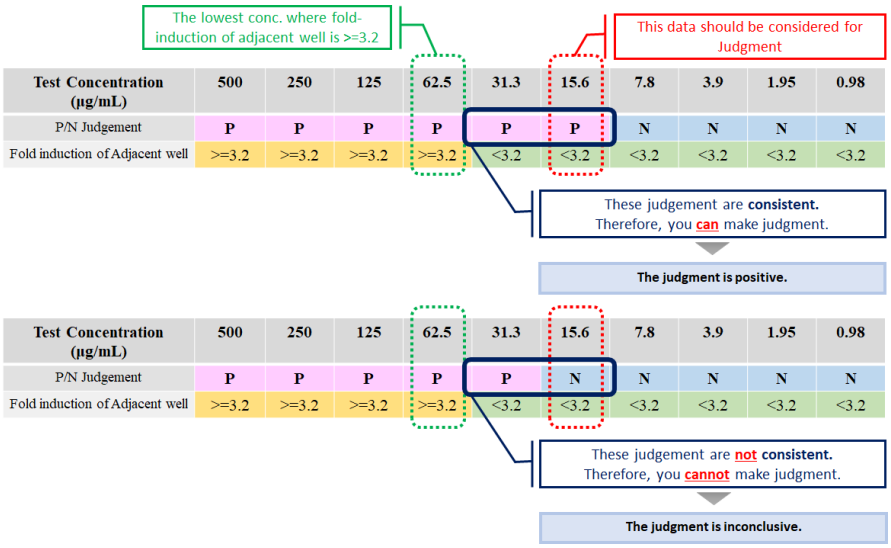
| | **Meada *et al.*, 2020** | **Current α-Sens®** | **Note** |
|---|---|---|---|
| Reporter gene construct | ARE-AUG-*Luc2* | 4xARE-AUG-*Luc+* | |
| Clonality of cell | Multi-clone | Single-clone | |
| Cell density in assay condition | 20,000 cells/well | 10,000 cells/well | Confirmed By optimization |
| Maximum test concentration | 2000 μM | 500 μg/mL | KeratinoSens™:<br>2000 μM or 400 μg/mL<br>LuSens:<br>2000 μM or 2000 μg/ mL |
| DMSO final concentration | 1% | 0.1% | Confirmed By optimization |
| Pre-incubation time (seeding ~~to~~ before exposure) | 24 h | 24±2 h | Confirmed By optimization |
| Chemical exposure time | 24 h | 24±2 h | Confirmed By optimization |
| Substrate volume to be added | 50 μL/well | 25 μL/well | Confirmed By optimization |
| Cut-off % for cell viability | Not set | 50% | |

AUG: a partial rat $\alpha_{2U}$-Globlin promotor fragment

## Appendix-B: Instruction for positive judgement for HiLIC in Plate Layout-A, and for test chemicals that gave luminescence crosstalk in Plate Layout-B.

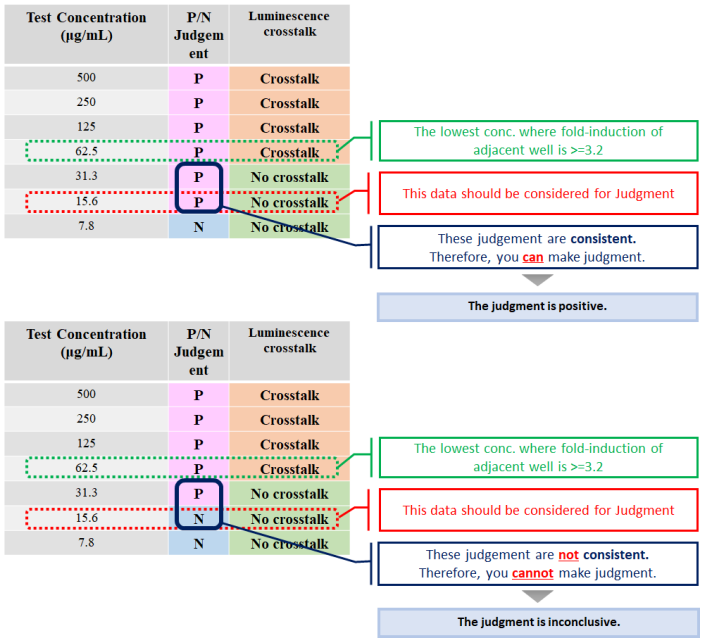
For positive judgment for HiLIC in Plate Layout-A, consider the data at the concentration of HiLIC or test chemicals corresponding to two dilution steps lower of adjacent well from the lowest concentration where fold-induction nAA >=3.2 was observed.

■ **for HiLIC in Plate Layout-A**




For positive judgment for test chemicals that gave luminescence crosstalk in Plate Layout-B, consider the data at the concentration of HiLIC or test chemicals corresponding to two dilution steps lower from the lowest concentration where crosstalk was observed.

■ **for test chemicals that gave luminescence crosstalk in Plate Layout-B**



**When the test chemicals were judged as inconclusive by above mentioned conditions**

The following points should be considered for the next test.

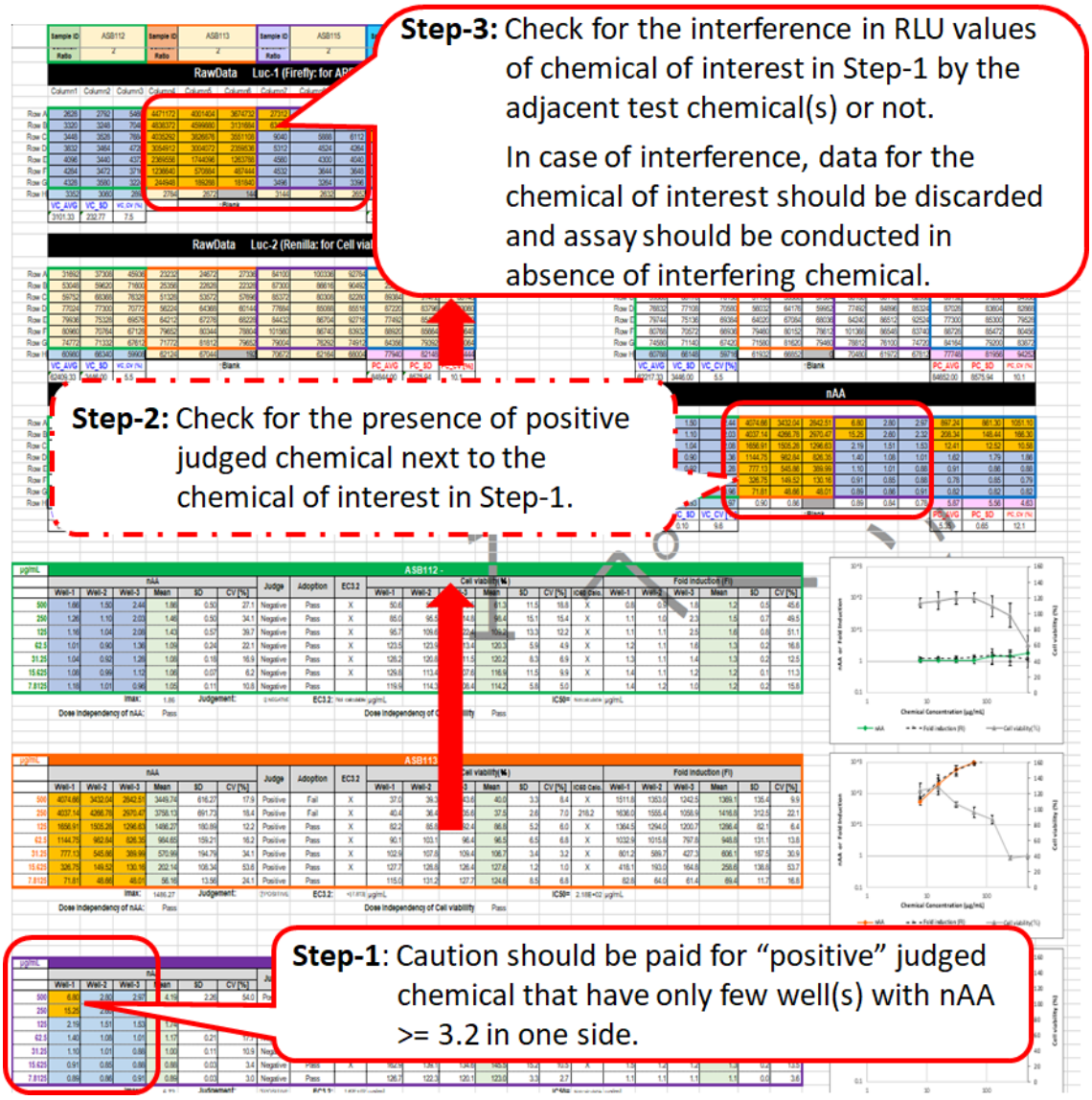
1. If test chemicals were judged as inconclusive using Plate Layout-B, change to use Plate Layout-A.
2. According to Fig.-4, the maximum concentration should be the minimum concentration judged as HiLIC, and the common ratio should be 1.2.

Chemicals that are judged as HiLIC or suspected HiLIC should have high ARE activity and are likely to be positive in α-Sens®, which uses ARE activity as an indicator.

Therefore, if the result of the next test is negative, there is a possibility that the test concentration setting is inappropriate. In such case, the study director can determine the test conditions for the subsequent test, such as including test concentration one step higher than the previous test using Plate Layout-A.

If the result of the next test is also inconclusive (*i.e.*, you cannot make a positive/negative judgment), for the subsequent test, the maximum test concentration and common ratio can be determined by the study director (*e.g.* the test concentration may be set one step higher/lower than the previous test and/or with a lower common ratio such as 1.1, depending upon and justified by the data previously observed).

**Appendix-C: Instruction for investigation of a chemical adjacent to a high RLU value chemical.**



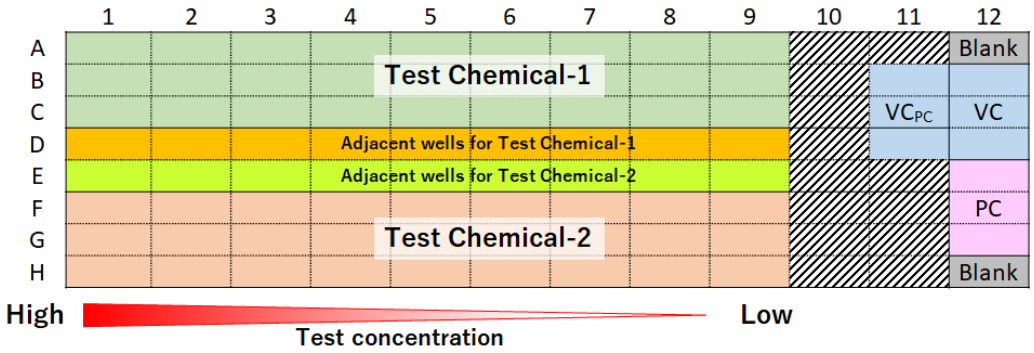
## Appendix-D: Plate layouts for chemical exposure if a vehicle other than DMSO is selected as the vehicle for the test chemical(s).

If a vehicle other than DMSO is selected, assay plates should be separated for each vehicle used the test chemical(s) for the reasons of the spreadsheet (*e.g.*, if one test chemical uses acetone and another test chemical uses DMSO, these test chemicals should be tested in the different assay plate).

The test chemical(s) using the vehicle other than DMSO should use the following plate layouts. It should be noted that the vehicle of PC ($VC_{PC}$) is DMSO.

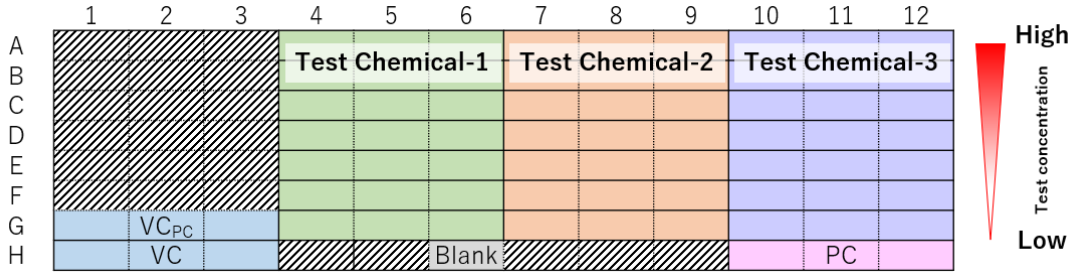
For data analysis, the dedicated spreadsheet should be used if the vehicle other than DMSO was used.

■ **Plate Layout-A**




VC: Vehicle Control (a vehicle used for test chemicals), $VC_{PC}$: Vehicle of PC (DMSO), PC: Positive Control at 0.5 µg/mL of Cinnamic aldehyde as final conc., Blank: no cells, α-Sens® medium only,
Wells shaded by diagonal line: Containing cells and α-Sens® medium, to be treated with both *Firefly* and *Renilla* luciferase reagents.
Adjacent wells in "Plate Layout-A": Containing cells and α-Sens® medium, to be treated with both *Firefly* and *Renilla* luciferase reagents. These adjacent wells are used to identify a chemical that have strong luminescence.

■ **Plate Layout-B**




VC: Vehicle Control (a vehicle used for test chemicals), $VC_{PC}$: Vehicle of PC (DMSO), PC: Positive Control at 0.5 µg/mL of Cinnamic aldehyde as final conc., Blank: no cells, α-Sens® medium only,
Wells shaded by diagonal line: Containing cells and α-Sens® medium, to be treated with both *Firefly* and *Renilla* luciferase reagents.

## Appendix-05: Derivation of ratio ($RLU_{avg\_VC}$ / $RLU_{avg\_H(n=5)}$) criteria for Plate Layout-B

The data used for the derivation of ratio ($RLU_{avg\_VC}$ / $RLU_{avg\_H(n=5)}$) criteria for Plate Layout-B were as below.

- ✓ Excluded the plate data marked "Not acceptable" by any of other data acceptance criteria.
- ✓ Excluded the plate data that have tested known HiLIC (Ethylene glycol dimethacrylate (EGDMA))
- ✓ Excluded the plate data missing data in H row

The data used for the analysis (N=25) was provided in Table.

[1] Calculated the RLUs average of VC (n=3) ($RLU_{avg\_VC}$) and RLUs average of H4-5 and H7-9 well (n=5) ($RLU_{avg\_H(n=5)}$) for each plate.

[2] Calculated the ratio by $RLU_{avg\_VC}$ / $RLU_{avg\_H(n=5)}$ for each plate.

[3] Calculated the average value and SD of ratio ($RLU_{avg\_VC}$ / $RLU_{avg\_H(n=5)}$) acquired in Phase-I (N=25).

[4] Used this average ± 2SD to set ratio ($RLU_{avg\_VC}$ / $RLU_{avg\_H(n=5)}$) criteria for Plate Layout-B (calculated value：0.82~1.40).

Confirmed that all data used in the analysis was within this range.

**α-Sens® Validation Report**
**Appendix-05**

**Table. RLU of VC wells and H4, H5, H7, H8, and H9 wells in all labs in Phase-I**

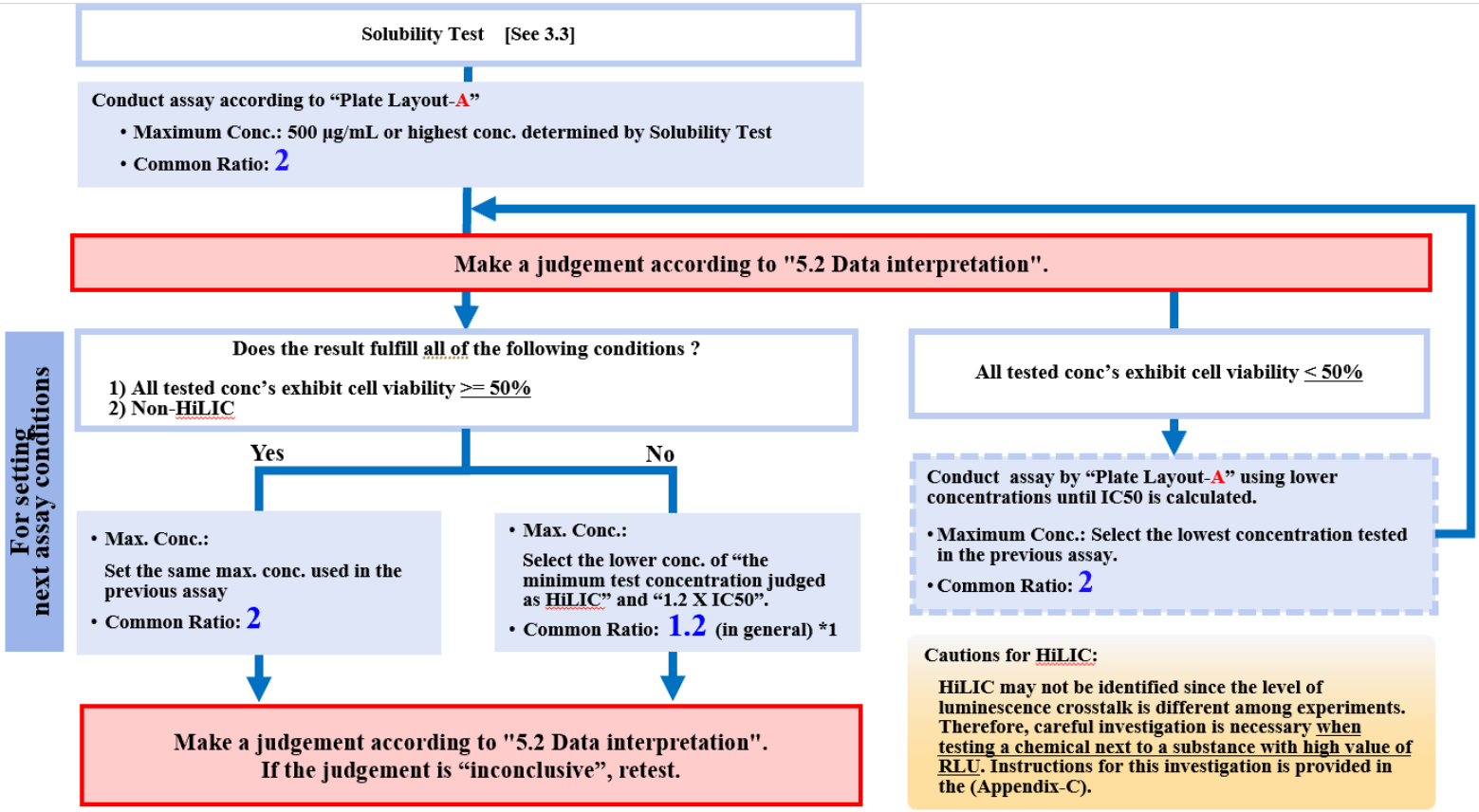
| No. | Lab name | RLU of Vehicle Control (VC) | | | | | | RLU of wells with medium and cells only (H4, H5, H7, H8 and H9) | | | | | | | | Ratio of RLU $= \frac{RLUavg\_VC}{RLUavg\_H(n=5)}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | VC-1 | VC-2 | VC-3 | $RLU_{avg\_VC}$ | SD | CV | Well-H4 | Well-H5 | Well-H7 | Well-H8 | Well-H9 | $RLU_{avg\_H(n=5)}$ | SD | CV | |
| 1 | CERI | 474 | 459 | 494 | 475.7 | 17.6 | 3.7% | 429 | 436 | 457 | 429 | 435 | 437.2 | 11.5 | 2.6% | 1.09 |
| 2 | CERI | 604 | 586 | 463 | 551.0 | 76.7 | 13.9% | 487 | 483 | 547 | 588 | 563 | 533.6 | 46.7 | 8.8% | 1.03 |
| 3 | CERI | 474 | 560 | 483 | 505.7 | 47.3 | 9.3% | 433 | 517 | 578 | 499 | 442 | 493.8 | 59.2 | 12.0% | 1.02 |
| 4 | FDSC | 1975 | 2483 | 2410 | 2289.3 | 274.7 | 12.0% | 2084 | 2028 | 1705 | 1585 | 1337 | 1747.8 | 311.7 | 17.8% | 1.31 |
| 5 | FDSC | 2081 | 1692 | 1676 | 1816.3 | 229.3 | 12.6% | 1585 | 1275 | 1851 | 1957 | 2219 | 1777.4 | 361.3 | 20.3% | 1.02 |
| 6 | FDSC | 1539 | 2046 | 1640 | 1741.7 | 268.4 | 15.4% | 1552 | 1550 | 1858 | 1776 | 2108 | 1768.8 | 233.4 | 13.2% | 0.98 |
| 7 | FDSC | 1511 | 1341 | 1300 | 1384.0 | 111.9 | 8.1% | 1247 | 994 | 1027 | 840 | 1139 | 1049.4 | 153.7 | 14.6% | 1.32 |
| 8 | FDSC | 950 | 1028 | 874 | 950.7 | 77.0 | 8.1% | 826 | 869 | 1152 | 1196 | 1067 | 1022.0 | 166.6 | 16.3% | 0.93 |
| 9 | FDSC | 1529 | 1161 | 1424 | 1371.3 | 189.6 | 13.8% | 1113 | 1437 | 1069 | 1115 | 1580 | 1262.8 | 230.7 | 18.3% | 1.09 |
| 10 | FDSC | 2595 | 2655 | 2456 | 2568.7 | 102.1 | 4.0% | 2191 | 2142 | 1921 | 2120 | 2808 | 2236.4 | 335.7 | 15.0% | 1.15 |
| 11 | FDSC | 1913 | 2075 | 1980 | 1989.3 | 81.4 | 4.1% | 2394 | 2421 | 2499 | 2110 | 2436 | 2372.0 | 151.5 | 6.4% | 0.84 |
| 12 | sumitomo | 3064 | 3248 | 2948 | 3086.7 | 151.3 | 4.9% | 2920 | 2748 | 2360 | 2012 | 3000 | 2608.0 | 414.5 | 15.9% | 1.18 |
| 13 | sumitomo | 2348 | 2728 | 2648 | 2574.7 | 200.3 | 7.8% | 1776 | 2152 | 1456 | 1716 | 2712 | 1962.4 | 487.3 | 24.8% | 1.31 |
| 14 | sumitomo | 2012 | 2120 | 2172 | 2101.3 | 81.6 | 3.9% | 1808 | 1972 | 1920 | 1844 | 3992 | 2307.2 | 944.0 | 40.9% | 0.91 |
| 15 | sumitomo | 2796 | 2676 | 2820 | 2764.0 | 77.1 | 2.8% | 2820 | 2896 | 3080 | 3900 | 3076 | 3154.4 | 431.9 | 13.7% | 0.88 |
| 16 | sumitomo | 2772 | 2860 | 2816 | 2816.0 | 44.0 | 1.6% | 2472 | 2576 | 2544 | 2320 | 2628 | 2508.0 | 119.3 | 4.8% | 1.12 |
| 17 | sumitomo | 2444 | 2476 | 2512 | 2477.3 | 34.0 | 1.4% | 2300 | 2360 | 2176 | 2160 | 2280 | 2255.2 | 85.1 | 3.8% | 1.10 |
| 18 | sumitomo | 2328 | 2620 | 3004 | 2650.7 | 339.0 | 12.8% | 2296 | 2284 | 2360 | 2144 | 1820 | 2180.8 | 216.5 | 9.9% | 1.22 |
| 19 | JRF, India | 7770 | 10325 | 8637 | 8910.7 | 1299.3 | 14.6% | 6551 | 8079 | 8751 | 6937 | 5653 | 7194.2 | 1231.0 | 17.1% | 1.24 |
| 20 | JRF, India | 7559 | 7879 | 6816 | 7418.0 | 545.3 | 7.4% | 7566 | 6743 | 9136 | 8039 | 7679 | 7832.6 | 869.4 | 11.1% | 0.95 |
| 21 | JRF, India | 10077 | 9684 | 9962 | 9907.7 | 202.1 | 2.0% | 8094 | 8419 | 6988 | 6585 | 8340 | 7685.2 | 841.3 | 10.9% | 1.29 |
| 22 | JRF, India | 8572 | 6775 | 8481 | 7942.7 | 1012.3 | 12.7% | 5358 | 6051 | 7135 | 6717 | 7350 | 6522.2 | 817.9 | 12.5% | 1.22 |
| 23 | JRF, India | 8406 | 10001 | 11190 | 9865.7 | 1396.9 | 14.2% | 7834 | 9015 | 7110 | 8105 | 7805 | 7973.8 | 688.4 | 8.6% | 1.24 |
| 24 | JRF, India | 12525 | 9200 | 11363 | 11029.3 | 1687.4 | 15.3% | 8596 | 11273 | 8438 | 7452 | 7492 | 8650.2 | 1557.6 | 18.0% | 1.28 |
| 25 | JRF, India | 9992 | 7111 | 8172 | 8425.0 | 1457.1 | 17.3% | 7724 | 6967 | 7582 | 7227 | 9310 | 7762.0 | 914.9 | 11.8% | 1.09 |
| | | | | | | | | | | | | | | | Average | **1.112** |
| | | | | | | | | | | | | | | | SD | **0.146** |
| | | | | | | | | | | | | | | | AVG - 2SD | **0.82** |
| | | | | | | | | | | | | | | | AVG +2SD | **1.40** |

Appendix-07: α-Sens® in-house dataset

The lead laboratory has tested 186 commercially available chemicals listed in the DASS database. Data are presented in Appendix-07 available as an Excel file at this link: https://www.oecd.org/content/dam/oecd/en/topics/policy-sub-issues/testing-of-chemicals/test-guidelines-documents/Appendix_07_alpha_sens_validation_study_in_house_dataset.xlsx

**α-Sens® Validation Report**
**Appendix-08**

Appendix-08 Simplified workflow (Fig.-4 in the SOP)




*1 : The use of common ratio 1.2 first is recommended. After this test, Study Directo can determine the appropriate common ratio based on the result from common ratio of 1.2.

Note :
- Plate layout B can be selected if the following conditions are met:
  - A suitable test concentration range and common ratio have been found, and
  - Negative and
  - non-HiLIC
- Maximum conc. should be =<500 µg/mL. If "1.2 x IC50" exceeds 500 µg/mL, set the maximum conc. at 500 µg/mL.

Appendix-09 Possible proficiency chemicals

| Chemical | CAS | Vehicle | Physical state | *logP* from DASS *1 | α-Sens _Call *2 | Human (from DASS DB)*1 | | LLNA (from DASS DB)*1 | | | Explanation |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | HU.GHS. SUB *3 | Basketter_ human_Call *2 | LLNA.GHS. SUB*3 | LLNA. MLLP *4 | potency | |
| Glycerol | 56-81-5 | DMSO | Liquid | -1.86 | 0 | NC | 0 | NC | NC | non-Sensitizer | • Clear negative in both Human and LLNA |
| Lactic acid | 50-21-5 | DMSO | Liquid | -0.72 | 0 | NA | 0 | NC | NC | non-Sensitizer | • Clear negative in both Human and LLNA |
| Isopropanol | 67-63-0 | DMSO | Liquid | 0.05 | 0 | NA | 0 | NC | NC | non-Sensitizer | • Clear negative in both Human and LLNA |
| *n*-Hexane | 110-54-3 | Acetone | Liquid | 3.90 | 0 | NC | 0 | NC | NC | non-Sensitizer | • Clear negative in both Human and LLNA<br>• higher *logP* (>3.5)<br>• use of vehicle other than DMSO |
| *D*-Limonene | 5989-27-5 | Acetone | Liquid | 4.52 | 1 | NA | 0 | 1B | 52.5 | Weak | • higher *logP* (>3.5)<br>• use of vehicle other than DMSO |
| EGDMA | 97-90-5 | DMSO | Liquid | 1.51 | 1 | NA | 1 | 1B | 28 | Weak | • High luminescence inducible chemical (HiLIC) |
| Citral | 5392-40-5 | DMSO | Liquid | 3.00 | 1 | 1B | 1 | 1B | 5.8 | Moderate | • Volatile chemical |
| 2-Mercaptobenzothiazole | 149-30-4 | DMSO | Solid | 2.42 | 1 | 1B | 1 | 1A | 1.35 | Moderate | • Clear positive in both Human and LLNA |
| *p*-Phenylenediamine | 106-50-3 | DMSO | Solid | -0.30 | 1 | 1A | 1 | 1A | 0.11 | Strong | • Clear positive in both Human and LLNA |
| DNCB | 97-00-7 | DMSO | Solid | 2.17 | 1 | 1A | 1 | 1A | 0.054 | Extreme | • Strong cytotoxicity |

*1: Data was extracted from DASS database (OECD, 2023)

*2: “1”: positive. “0”: negative

*3: NA: not available, NC: not conclusive

*4: Median-like location parameter; the mean of EC3 from independent LLNA assay.