



The modified IL-8 Luc assay, an in vitro skin sensitisation test, can significantly improve the false-negative judgment of lipophilic sensitizers with $\log K_{ow}$ values > 3.5

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Abstract

False-negative judgment due to poor chemical solubility is a problem with in vitro skin sensitisation tests. Water-insoluble chemicals are typically dissolved in DMSO in most sensitisation tests but precipitate when diluted with medium beyond their solubility in water. Such tests lack procedures to rule out false-negative judgments due to poor solubility. The IL-8 Luc assay (OECD442E) is unique in that if chemicals do not dissolve at 20 mg/mL in medium and have no effect on IL-8 luciferase activity (IL8LA), they are classified as indeterminate. The purpose of the present study was to reduce the number of indeterminate chemicals and improve assay performance. The IL-8 Luc assay can simultaneously examine glyceraldehyde 3-phosphate dehydrogenase luciferase activity (GAPLA) and IL8LA, and thus we examined the correlation between the reduction of GAPLA (defined as Inh-GAPLA) and the reduction of propidium iodide (PI)-excluding cells for three sensitizers and three non-sensitizers. We observed a significant correlation between luciferase activity driven by the GAPDH promoter of THP-G8 cells and the number of viable cells. Furthermore, chemicals providing an Inh-GAPLA value below 0.8 always reduced the ratio of PI-excluding cells to less than 0.6. Using the modified criteria, indeterminate chemicals are judged as negative if they provide Inh-GAPLA values below 0.8. This modification reduced the number of indeterminate chemicals and increased specificity, highlighting the unique advantage of the IL-8 Luc assay.

Keywords Skin sensitisation test · Luciferase assay · Lipophilic chemicals · Reporter assay

Introduction

There are three reasons for false-negative judgments in in vitro skin sensitisation tests (IVSSTs). Reason 1 is that chemicals classified as prohaptens must be metabolized to acquire biological activity as sensitizers. Reason 2 is that sensitisation may be induced by a unique biological process different from that described in the adverse outcome pathway (AOP) for skin sensitisation, as may be the case with urushiol (Kim et al. 2016). Reason 3 is perhaps the

most critical: sensitizers dissolve poorly in culture medium, resulting in concentrations too low to stimulate the target cells.

Most IVSSTs use DMSO to dissolve chemicals, but the chemicals often precipitate when diluted with medium to beyond their solubility in water. These precipitates are generally ignored in IVSSTs because the precipitates form in the small wells of multiple-well plates. This leads to two potential problems: reducing the performance of the assays (reason 3) and resulting in an unknown concentration of the chemical in each well.

Takenouchi et al. (2013) reported that the sensitivity and predictivity of the human Cell Line Activation Test (h-CLAT) for lipophilic chemicals with log octanol/water partition coefficients ($\log K_{ow}$) > 3.5 was significantly inferior to that for those with $\log K_{ow} < 3.5$, suggesting that h-CLAT is not suitable for examining the sensitizing potential of chemicals with $\log K_{ow} > 3.5$. Other IVSSTs may have similar issues, and thus there is an urgent need to design an IVSST that can detect sensitizers with $\log K_{ow} > 3.5$ with high

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sensitivity. Furthermore, most IVSSTs have no procedure for recognizing a false-negative judgment due to reason 3,

In contrast, the IL-8 Luc assay (OECD442E) avoid judgment if chemicals are not dissolved at 20 mg/ml of X-VOVO™ 15 and have no effects on the IL-8 luciferase activity (IL8LA). These chemicals are classified as indeterminate (Kimura et al. 2018; OECD 2018). Although the elimination of insoluble chemicals from judgment is an advantage of the IL-8 Luc assay over other IVSSTs, the assay cannot evaluate a substantial number of chemicals, which is a major drawback compared to other IVSSTs. Altering the IL-8 Luc assay to reduce the number of indeterminate chemicals is, therefore, an urgent matter.

Fortunately, the IL-8 Luc assay uniquely uses the dual reporter cell line THP-G8, which can evaluate the effects of chemicals on both IL-8 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter activities (Kimura et al. 2015; Takahashi et al. 2011). GAPDH mRNA is ubiquitously expressed at moderately abundant levels. It is frequently used as an endogenous control for quantitative real-time polymerase chain reaction because, in some experimental systems, its expression is constant at different times and after experimental manipulation (Edwards and Denhardt 1985; Mori et al. 2008; Winer et al. 1999). Although there are several reports suggesting that its use as an internal standard is inappropriate in some cases (Oliveira et al. 1999; Thellin et al. 1999), in general, within-tissue variation of GAPDH mRNA expression levels is small whereas between-tissue variation can be substantial, depending on tissue type (Barber et al. 2005). Therefore, we introduced the GAPDH reporter gene into THP-G8 cells.

We previously reported that luciferase activity driven by the GAPDH promoter (GAPLA) of THP-G8 cells shows good correlation with the number of viable cells (Takahashi et al. 2011) and that the parameter Inh-GAPLA, defined as the ratio of GAPLA of THP-G8 treated with chemicals divided by the GAPLA levels of untreated cells after 16 h of chemical treatment, can be used as a marker of cell viability (Kimura et al. 2015; Takahashi et al. 2011). However, there is a discrepancy between Inh-GAPLA and the percent of propidium iodide (PI)-excluding cells as examined by flow cytometry: THP-G8 cells with an Inh-GAPLA value of at least ≥ 0.05 after 16 h of chemical treatment still contained more than 75% of PI-excluding cells (Kimura et al. 2015; Takahashi et al. 2011).

In this study, we examined more precisely the correlation between Inh-GAPLA and the number of PI-excluding cells for three representative sensitizers and non-sensitizers. We found a significant correlation between Inh-GAPLA and the reduction of PI-excluding cells, with strengthened correlation depending on the culture period. Moreover, chemicals providing Inh-GAPLA below 0.8 after 16 h of chemical treatment always reduced PI-excluding cells to less than 0.6

after 48 h, indicating that this is a marker of the cytotoxicity of chemicals and reflects that chemicals are dissolved in the culture medium. We, therefore, modified the criteria for the IL-8 Luc test using the values of Inh-GAPLA, examined the performance of the assay, and further explored the possibility of improving its performance by the combination with another non-animal skin sensitisation test, direct peptide reactivity assay (DPRA).

Materials and methods

Cells and culture

We established the THP-G8 reporter cell line, derived from THP-1 human acute monocytic leukemia cells expressing the genes for SLO regulated by the IL-8 promoter and SLR regulated by the GAPDH promoter (Takahashi et al. 2011; Kimura et al. 2015). THP-G8 cells were maintained in RPMI-1640 medium (Gibco, Carlsbad, CA) containing 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), antibiotic and antimycotic agents (Gibco), 0.15 µg/mL puromycin (InvivoGen, San Diego, CA), and 300 µg/mL G418 (Nacalai Tesque, Kyoto, Japan). Cells were cultured at 37 °C with 5% CO₂. For testing, THP-G8 cells were plated into a 96-well flat-bottom black plate (Greiner Bio-one, Frickenhausen, Germany) at 5×10^4 cells/50 µL/well.

Different protocols for the IL-8 Luc assay

Using THP-G8 cells, we developed the IL-8 Luc assay to detect skin sensitizing chemicals in vitro and modified the protocol of the assay during the course of development. We first published the IL-8 Luc assay using DMSO as a solvent (Takahashi et al. 2011), changed the solvent to X-VIVO™ 15 (Kimura et al. 2015), and finally modified the criteria to determine sensitizers taking the solubilities of the chemicals into consideration (Kimura et al. 2018; OECD 2018). In this study, to aid our understanding of the effects of these modifications, we designated the IL-8 Luc assay using DMSO as a solvent as the D-IL-8 Luc assay, that using X-VIVO™ 15 as the X-IL-8 Luc assay, the X-IL-8 Luc assay together with taking into consideration the solubilities of the chemicals as the tgIL-8 Luc assay (an OECD 442E test guideline), and the X-IL-8 Luc assay using modified criteria as the moIL-8 Luc assay.

The precise procedures for the different IL-8 Luc assays were described in our previous publications (Kimura et al. 2015, 2018; OECD 2018; Takahashi et al. 2011) and their differences are summarized in Table 1.

Table 1 Differences in the protocols of different IL-8 Luc assays

Condition	D-IL-8 Luc	X-IL-8 Luc	tgIL-8 Luc	moIL-8 Luc
Solvent	DMSO	X-VIVO™ 15	X-VIVO™ 15	X-VIVO™ 15
Criteria for a positive/negative decision	- An IL-8 Luc assay prediction is judged positive if a test chemical has an Ind-IL8LA ≥ 1.0 - An IL-8 Luc assay prediction is judged negative if a test chemical has an Ind-IL8LA < 1.0	- An IL-8 Luc assay prediction is judged positive if a test chemical has an Ind-IL8LA ≥ 1.4 and the lower limit of the 95% confidence interval of Ind-IL8LA ≥ 1.0 - An IL-8 Luc assay prediction is judged negative if a test chemical has an Ind-IL8LA < 1.4 and/or the lower limit of the 95% confidence interval of Ind-IL8LA < 1.0		
Prediction model	Test chemicals that provide two positive results are identified as positive whereas those that give three negative results are identified as negative	Test chemicals that provide two positive results are identified as positive whereas those that give three negative results are identified as negative	Test chemicals that provide two positive results are identified as positive whereas those that give three negative results are identified as negative	Test chemicals that provide two positive results are identified as positive whereas those that give three negative results are identified as negative
Criteria for negative chemicals	Not applicable	Supposed negative chemicals that dissolve at 20 mg/mL in X-VIVO™ 15 are identified as negative	Supposed negative chemicals that dissolve at 20 mg/mL in X-VIVO™ 15 are identified as negative	Supposed negative chemicals that dissolve at 20 mg/mL in X-VIVO™ 15 or do not dissolve at 20 mg/mL in X-VIVO™ 15 and have no effect on IL8LA but show Inh-GAPLA < 0.8 are identified as negative
Criteria for indeterminate chemicals	Not applicable	Supposed negative chemicals that do not dissolve at 20 mg/mL in X-VIVO™ 15 and have no effect on IL8LA are identified as indeterminate	Supposed negative chemicals that do not dissolve at 20 mg/mL in X-VIVO™ 15 and have no effect on IL8LA are identified as indeterminate	Supposed negative chemicals that do not dissolve at 20 mg/mL in X-VIVO™ 15 and have no effect on IL8LA and show Inh-GAPLA ≥ 0.8 are identified as indeterminate

Luciferase gene reporter assay

The activity of SLO driven by the IL-8 promoter (IL-8 luciferase activity: IL8LA) and that of SLR driven by the GAPDH promoter (GAPDH luciferase activity: GAPLA) in THP-G8 cells can be measured simultaneously and quantitatively using optical filters (Nakajima et al. 2005; Noguchi et al. 2008). In this study, luciferase activity was determined using a microplate-type luminometer with a Phelios multi-color detection system (Atto Co., Tokyo, Japan) and Tripluc® luciferase assay reagent (Toyobo Co., Ltd., Osaka, Japan), according to the manufacturer's instructions.

Some chemicals affected cell viability and thus we defined the parameter nIL8LA to represent IL-8 promoter activity. This parameter is calculated by normalizing IL8LA divided by GAPLA. We also calculated Inh-GAPLA by dividing the GAPLA of THP-G8 cells treated with chemicals by that of non-treated cells. Ind-IL8LA was calculated by dividing the nIL8LA of THP-G8 cells treated with chemicals by that of non-stimulated cells. The parameters used in the moIL-8 Luc assay are defined in Table 2.

PI-excluding cells

Cell viability was determined by a propidium iodide (PI) exclusion assay using flow cytometry. THP-G8 cells after 16 h, 24 h, and 48 h of chemical treatment were mixed with 30 $\mu\text{g/mL}$ of PI (Sigma), then dead cells permeable to PI were counted using a fluorescence-activated cell sorter (C6 flow cytometer, Accuri Cytometers Inc., Ann Arbor, MI). Since THP-G8 cells without chemical treatment also decreased PI-excluding cells, depending on the culture period, we defined the ratio of PI-excluding cells as: the reduction of PI-excluding cells (PI reduction) = % of PI-excluding cells among THP-G8 cells treated with chemicals / % of PI-excluding cells among THP-G8 cells without chemical treatment.

Correlation between Inh-GAPLA and PI-excluding cells

To examine the correlation between Inh-GAPLA and PI-excluding cells in different time periods after chemical treatment, we first conducted the tg-IL-8 Luc assay for 2,4-dinitrochlorobenzene (DNCB), 4-nitrobenzyl bromide (4-NBB), diethyl maleate, and limonene, and 2 non-sensitisers, sodium lauryl sulfate (SLS) and streptomycin sulfate. The precise procedure of the tg-IL-8 Luc was described in our previous publication (Kimura et al. 2018; OECD 2018).

Briefly, the chemicals were dissolved by adding X-VIVO™ 15, a commercially available serum-free medium (Lonza, 04-418Q), to 20 mg of test chemical (regardless of the chemical's solubility) in a microcentrifuge tube, brought to a volume

Table 2 Parameters used in the IL-8 Luc assay

Parameters	Definition
GAPLA	SLR luciferase activity driven by the GAPDH promoter
IL8LA	SLO luciferase activity driven by the IL-8 promoter
nIL8LA	IL8LA / GAPLA
Ind-IL8LA	nIL8LA of THP-G8 cells treated with chemicals/nIL8LA of untreated cells
Inh-GAPLA	GAPLA of THP-G8 treated with chemicals/GAPLA of untreated cells
CV05	The lowest concentration of the chemical at which Inh-GAPLA is < 0.05
Css	The concentration of an X-VIVO™ 15 stock solution of the test chemical

of 1 mL, vortexed vigorously, and then shaken on a rotor at a maximum speed of 8 rpm for 30 min. For test chemicals soluble in X-VIVO™ 15, the solution was diluted by a factor of 5 with X-VIVO™ 15 and used as an X-VIVO™ 15 stock solution of the test chemical. For test chemicals not soluble in X-VIVO™ 15, the mixture was rotated again for at least 30 min, and then centrifuged at 15,000 rpm ($\approx 20,000$ g) for 5 min; the resulting supernatant was used as an X-VIVO™ 15 stock solution of the test chemical. In this study, the concentration of stock solution was designated as C_{ss}.

In the first test run, 10 serial dilutions of the X-VIVO™ 15 stock solutions of the test chemicals were diluted by a factor of two. In subsequent test runs (i.e., the second, third, and fourth replicates), each X-VIVO™ 15 stock solution was made at a concentration four times higher than the concentration of cell viability 05 (CV05; the lowest concentration at which the Inh-GAPLA becomes < 0.05) in the first experiment. Ten serial dilutions of the X-VIVO™ 15-s stock solutions were prepared at a dilution factor of 1.5. Next, 50 μ L/well of diluted solution was added to 50 μ L of cell suspension in the wells of a 96-well flat-bottom black plate. The samples were then mixed on a plate shaker and incubated for 16 h at 37 °C and 5% CO₂, then the luciferase activity was measured as described below. Each concentration of each test chemical was tested in four wells. The IL-8 Luc assay requires at most four test runs to determine whether a chemical has a positive or a negative prediction.

Since we previously examined these six chemicals using the tg-IL-8 Luc assay (Kimura et al. 2018), THP-G8 cells were stimulated using concentrations of each chemical at which the values of Inh-GAPLA distributed from 0.02 to 1.0. Inh-GAPLA was determined only 16 h after initiating chemical treatment whereas PI reduction was examined after 16 h, 24 h, and 48 h. The correlation between Inh-GAPLA and PI reduction was examined using the same concentration of test chemical.

The examination of non-sensitisers with $\log K_{ow} > 3.5$ by the IL-8 Luc assay

Since there were no non-sensitisers with $\log K_{ow} > 3.5$ among the 143 chemicals we have already examined by the IL-8

Luc assay, we additionally examined 4 non-sensitisers with $\log K_{ow} > 3.5$, 1-iodohexane, butylbenzylphthalate, clofibrate, and dibutyl phthalate (Sigma-Aldrich, St Louis, MO) by the tgIL-8 Luc assay.

Results

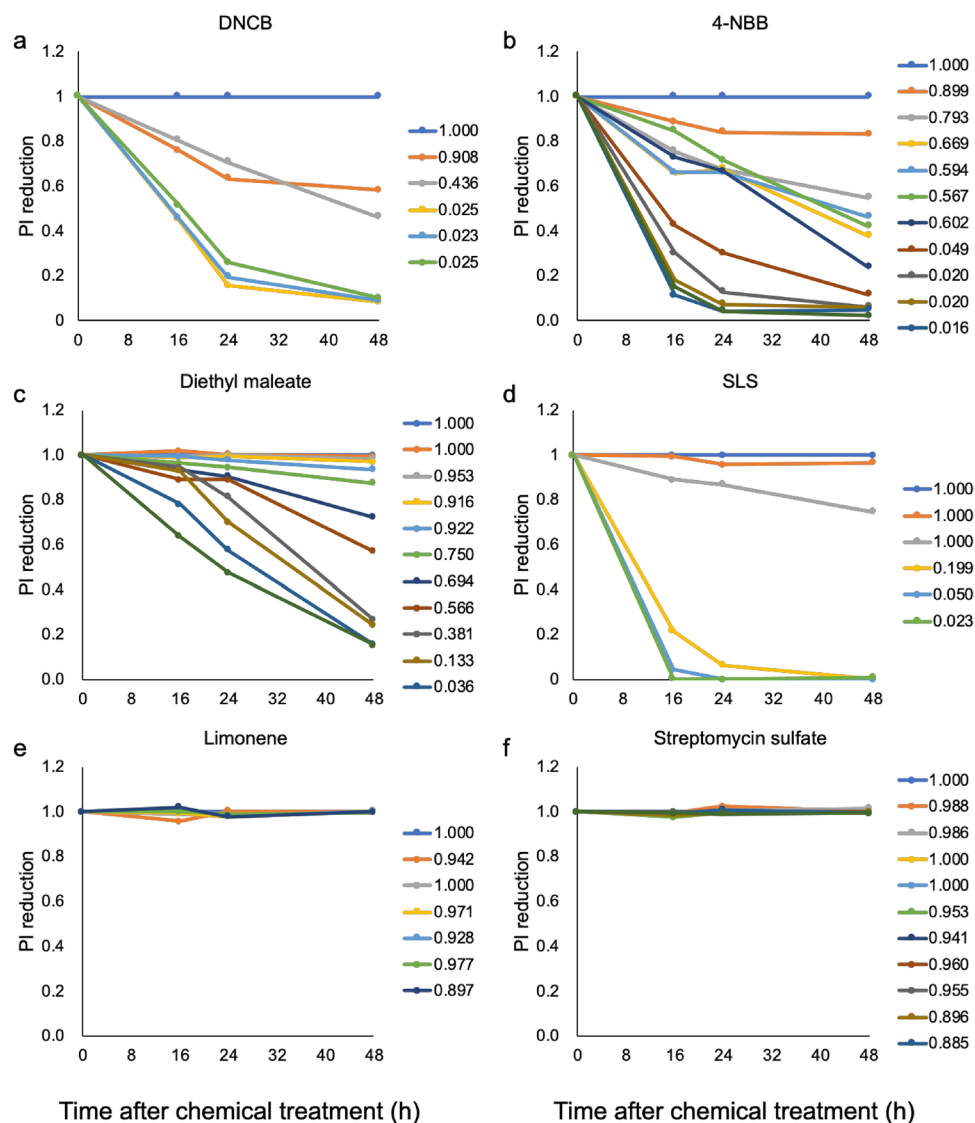
The correlation between Inh-GAPLA and PI-excluding cells

THP-G8 cells were treated with various concentrations of the three sensitizers DNCB, 4-NBB, diethyl maleate, and limonene, and the two non-sensitizers SLS and streptomycin sulfate. The cells were examined for Inh-GAPLA after 16 h of chemical treatment, and the number of PI-excluding cells was determined after 16 h, 24 h, and 48 h of chemical treatment. All the chemicals were dissolved and diluted according to the protocol for the tg-IL-8 Luc assay, then THP-G8 cells were stimulated using the concentration of each chemical at which the values of Inh-GAPLA distributed from 0.02 to 1.0. These concentrations of chemicals were determined based on in-house data. When these cells were cultured during 48 h, the number of PI-excluding cells in THP-G8 cells treated with most concentrations of chemicals decreased gradually depending on the culture period (Fig. 1). However, the chemicals providing more than 0.8 of Inh-GAPLA maintained more than 0.8 of PI reduction except for DNCB. Indeed, THP-G8 cells treated with DNCB that gave 0.908 of Inh-GAPLA reduced PI reduction to 0.6 after 48 h of culture. In contrast, THP-G8 cells that gave less than 0.8 of Inh-GAPLA reduced PI reduction dose-dependently.

Inh-GAPLA and PI reduction values for all chemicals were summarized and examined for correlation. Chemical treatment for 16 h, 24 h, and 48 h gave a significant correlation with Inh-GAPLA (Fig. 2). Interestingly, the correlation strengthened with increased culture period, with correlation coefficients after 16 h, 24 h, and 48 h of 0.708, 0.828, and 0.912, respectively.

These data suggest that Inh-GAPLA is a good marker for cell viability, with a strong correlation with PI-excluding cells, and that Inh-GAPLA below 0.8 indicates

Fig. 1 PI reduction in THP-G8 cells treated with chemicals decreases as the level of Inh-GAPLA decreases and the culture period increases. THP-G8 cells treated with various concentrations of the four sensitizers DNCB, 4-NBB, diethylmaleate, and limonene, and the two non-sensitizers SLS and streptomycin sulfate, were examined for Inh-GAPLA after 16 h and for the number of PI-excluding cells after 16 h, 24 h, and 48 h of chemical treatment. THP-G8 cells were stimulated using concentrations of chemicals at which the values of Inh-GAPLA distributed from 0.02 to 1.0. The reduction in PI of THP-G8 cells treated with each concentration of the chemical is shown at 0 h, 16 h, 24 h, and 48 h. Each line is labeled with an Inh-GAPLA value for THP-G8 cells treated with the concentration of the chemical at which PI reduction was measured



cytotoxicity of the test chemical, which in turn suggests that the chemical dissolved in X-VOVO™ 15.

Distribution of the lowest Inh-GAPLA values of 143 chemicals examined by the IL-8 Luc assay

We next plotted the lowest Inh-GAPLA of 143 chemicals examined in the tgIL-8 Luc assay in our previous publication (Kimura et al. 2018) (Fig. 3). The plot indicates that chemicals can be classified into three groups: chemicals showing Inh-GAPLA values ≥ 0.80 , from 0.20 to 0.80, and ≤ 0.20 . These data suggest that Inh-GAPLA values < 0.8 is a reasonable marker indicating that the test chemical is dissolved sufficiently to be cytotoxic.

Modification of the IL-8 Luc assay

Of the 143 chemicals examined by the tgIL-8 Luc assay, 23 were classified as indeterminate. As mentioned above, Inh-GAPLA of the IL-8 Luc assay can be used as a marker for dissolved chemicals. We, therefore, modified the criteria using the values for Inh-GAPLA: of the indeterminate chemicals (those that do not dissolve at 20 mg/mL in X-VIVO™ 15 and have no effect on IL8LA), chemicals are judged as negative if they provide Inh-GAPLA values < 0.8 whereas those with values ≥ 0.8 are judged as indeterminate. We demonstrated LLNA potency category, solubility in 20 mg/ml of X-VIVO™ 15, the average of maximum values of Ind-IL8LA and the average of minimum values of Inh-GAPLA in repeated experiments, the judgments by the tgIL-8 Luc

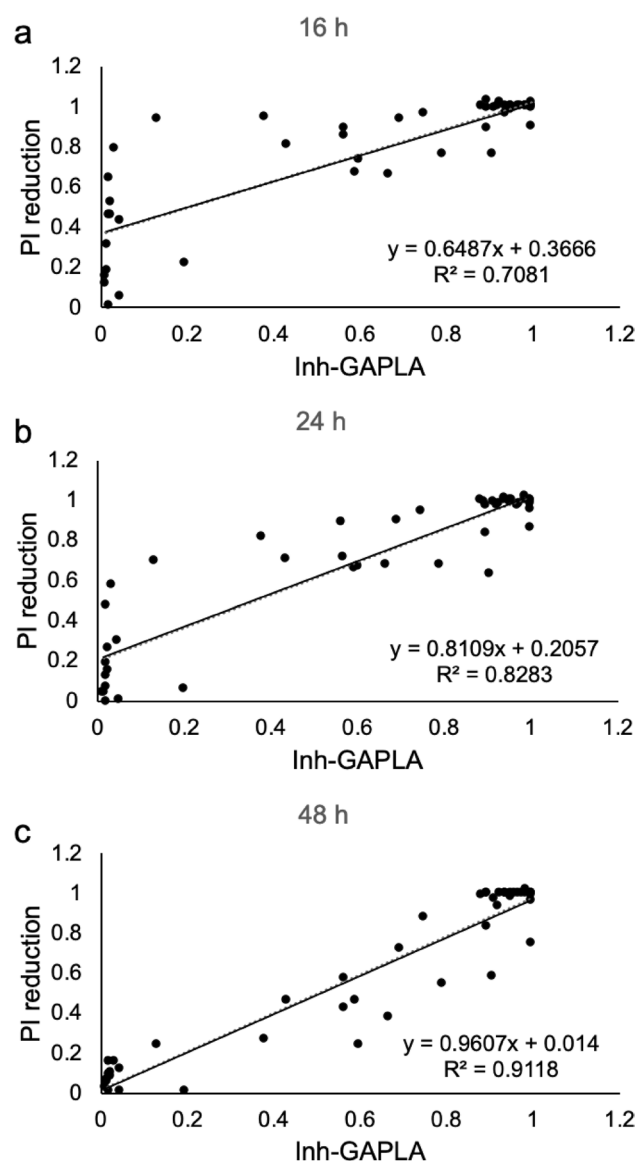


Fig. 2 There is a significant correlation between Inh-GAPLA and PI reduction values with strengthened correlation, depending on the culture period. Inh-GAPLA and PI reduction values for all chemicals were examined for correlation at three time points (16 h, 24 h, and 48 h) of chemical treatment

assay and by the moIL-8 Luc assay, and $\log K_{ow}$ of the 143 chemicals (Supplementary Table 1).

By applying these criteria, 10 of the 23 indeterminate chemicals were classified as non-sensitizers. Consequently, the performance of the moIL-8 Luc assay was 93.9% for sensitivity, 68.0% for specificity, and 88.6% for accuracy when 143 chemicals were examined, while that of the tgIL-8 Luc assay was 95.8% for sensitivity, 53.0% for specificity, and 89.4% for accuracy. In addition, the number of chemicals classified as indeterminate is 13 by the moIL-8 Luc assay and 23 by the tgIL-8 Luc assay (Table 3).

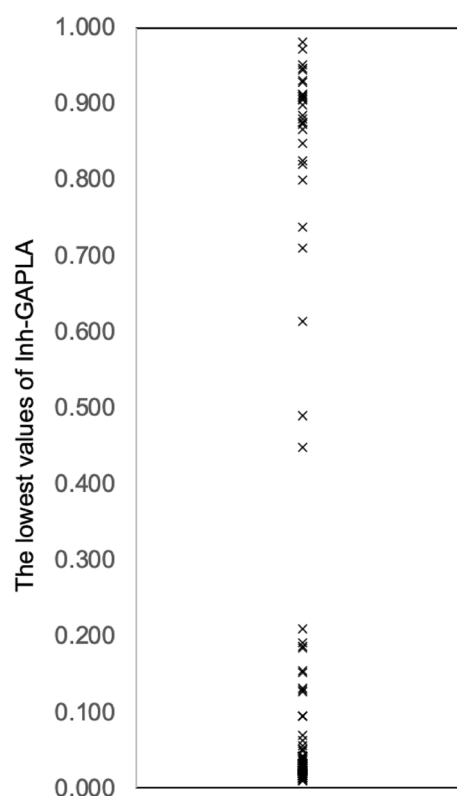


Fig. 3 The distribution of the lowest values of Inh-GAPLA of chemicals examined by the IL-8 Luc assay ($n = 143$). The lowest values of Inh-GAPLA of 143 chemicals examined by the IL-8 Luc assay, published previously, are plotted

Next, we plotted the $\log K_{ow}$ values of sensitizers, non-sensitizers, false-negative, false-positive and indeterminate chemicals as classified by the tgIL-8 Luc assay and the moIL-8 Luc assay. Of 23 chemicals classified as indeterminate by the tgIL-8 Luc assay, 10 were classified as negative and 13 as indeterminate by the moIL-8 Luc assay. Interestingly, the $\log K_{ow}$ values of these 13 indeterminate chemicals were much higher than those of the 10 negative chemicals (Fig. 4a, b).

The effects of the $\log K_{ow}$ values of chemicals on the performance of the tgIL-8 Luc and moIL-8 Luc assays

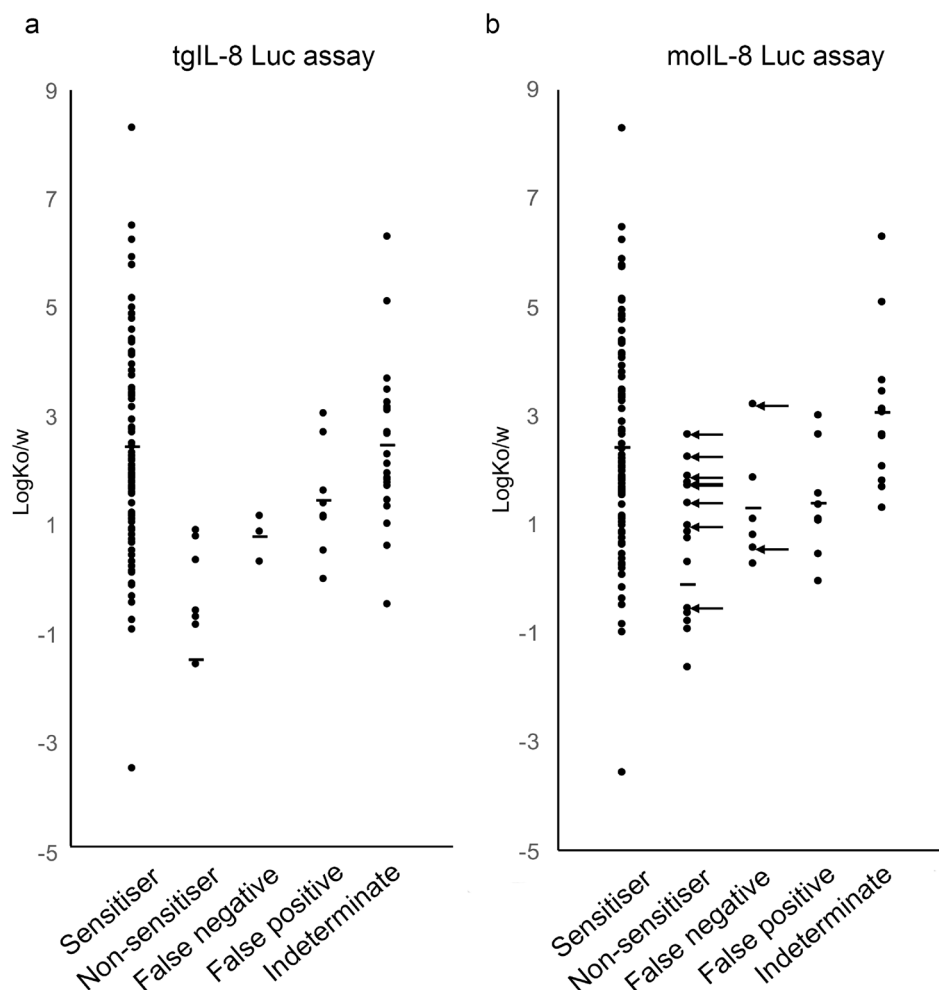
Takenouchi et al. (2013) reported that the sensitivity and predictivity of h-CLAT for lipophilic chemicals with $\log K_{ow} > 3.5$ was significantly inferior to that for those with $\log K_{ow} < 3.5$. We, therefore, examined the difference in the performance of the tgIL-8 Luc and moIL-8 Luc assays for chemicals with $\log K_{ow} < 3.5$ and those with $\log K_{ow} \geq 3.5$. As shown in Table 4, the performance of the moIL-8 Luc assay for 100 chemicals with $\log K_{ow} < 3.5$, which were composed of 75 sensitizers and 25 non-sensitizers, was

Table 3 Performance of the IL-8 Luc assay using different protocols and criteria

	Single assay				IATA	
	D-IL-8 Luc	X-IL-8 Luc	tgIL-8 Luc	moIL-8 Luc	tgIL-8 Luc + DPRA	tgIL-8 Luc + DPRA
Chemical number	143	143	143	143	143	143
Out of AD	7	7	7	7	0	0
"indeterminate"	n.a	n.a	23	13	0	0
Accuracy	77.9%	84.6%	89.4%	88.6%	87.3%	86.7%
Sensitivity	78.1%	87.6%	95.8%	93.9%	93.5%	91.6%
Specificity	77.4%	74.2%	53.0%	68.0%	68.6%	72.2%

n.a: not applicable, D-IL-8 Luc assay: DMSO as the solvent, X-IL-8 Luc assay: X-VIVO15 as the solvent, tgIL-8 Luc assay: X-VIVO15 as the solvent and the criteria of the OECD test guideline, moIL-8 Luc assay: X-VIVO15 as the solvent and the modified criteria, IATA: integrated approaches to testing and assessment

Fig. 4 The distribution of the $\log K_{ow}$ values of chemicals with different judgments in the tgIL-8 Luc assay and the moIL-8 Luc assay. The values of $\log K_{ow}$ of sensitizers, non-sensitizers, false negatives, false positives and indeterminates classified by the tgIL-8 Luc assay (a) and the moIL-8 Luc assay (b) are plotted. Twenty-three chemicals classified as indeterminate by the tgIL-8 Luc assay were further classified as 10 negatives (arrows) and 13 indeterminates by the moIL-8 Luc assay. Of the ten negative chemicals, eight were non-sensitizers and two were false negatives (a and b)



92.0% for sensitivity, 68.0% for specificity, and 86.0% for accuracy, while that for 26 chemicals with $\log K_{ow} \geq 3.5$, which were only sensitizers, was 100.0% for sensitivity and 100.0% for accuracy. There were 10 and 3 indeterminate judgments for chemicals with $\log K_{ow} < 3.5$ and those with $\log K_{ow} \geq 3.5$, respectively. Unfortunately, since there were

no non-sensitizers with $\log K_{ow} \geq 3.5$ of the 36 non-sensitizers, we could not obtain sensitivity for chemicals with $\log K_{ow} \geq 3.5$.

Therefore, in this study, we examined 4 non-sensitizers with $\log K_{ow} \geq 3.5$, 1-iodohexane, butylbenzylphthalate, clofibrate, and dibutyl phthalate. None of them dissolved at

Table 4 Performance of the moIL-8 Luc assay for chemicals with $\log K_{ow} < 3.5$ and those with $\log K_{ow} > 3.5$

	$\log K_{ow} < 3.5$	$\log K_{ow} \geq 3.5$
No. of chemicals	110	26
No. of sensitizers	75	23
No. of Non-sensitizers	25	0
Accuracy	86.0%	100.0%
Sensitivity	92.0%	100.0%
Specificity	68.0%	Not determined
No. of indeterminate	10	3

20 mg/mL in X-VIVO™ 15, satisfied the criteria for positive or gave less than 0.8 of Inh-GAPLA. So, they were judged as indeterminate.

The combination with the DPRA

There is a wide consensus that because of the complexity of the skin sensitisation pathway, no single non-animal method can replace animal testing. Instead, it is necessary to develop integrated approaches to testing and assessment (IATA) using combinations of assays representing different key events of the Adverse Outcome Pathway (AOP) [reviewed by Roberts and Patlewicz (2018)]. Based on this concept, various combinations of testing have been proposed. In this study, we proposed a combination with the DPRA. First, chemicals are examined by the moIL-8 Luc assay because it is characterized by high sensitivity. Then, the chemicals excluded by the applicability domain, or judged as being indeterminate, are evaluated by the DPRA. The obtained results were 91.6% for sensitivity, 72.2% for specificity and 86.7% for accuracy (Table 3). Performance was not significantly improved by the combination with DPRA, but the combination approach succeeded in reducing chemicals that could not be evaluated by the moIL-8 Luc assay.

Discussion

Many sensitizers do not dissolve in culture medium and thus most IVSSTs use DMSO as a solvent. Although sensitizers dissolved in DMSO can be miscible with water, DMSO does not change the solubility of a chemical. Chemicals dissolved in DMSO precipitate after dilution with culture medium. Fortunately, since most sensitizers are potent, even a small amount of dissolved sensitizer can usually stimulate target cells. In contrast, the X-IL-8, tgIL-8 and moIL-8 Luc assays use X-VIVO™ 15 instead of DMSO. Unexpectedly, as shown in Table 3, the sensitivity of the X-IL-8 Luc assay is much greater than that of the D-IL-8 Luc assay, which we attribute to the vigorous dissolution process for the X-IL-8

Luc assay, as opposed to just pipetting in a small well as with the D-IL-8 Luc assay. A significant number of chemicals do not dissolve at 20 mg/mL in the IL-8 Luc assay using X-VIVO™ 15, as seen with other IVSSTs using DMSO. However, most of them appear to dissolve in X-VIVO™ 15 to concentrations sufficient to stimulate IL8LA in THP-G8 cells. Even though, it is thought that some chemicals cannot dissolve to sufficient concentrations.

Indeed, the sensitivity and predictivity of h-CLAT for lipophilic chemicals with $\log K_{ow} > 3.5$ was significantly inferior to those with $\log K_{ow} < 3.5$, suggesting that h-CLAT is not suitable for detecting sensitizers among chemicals with $\log K_{ow} > 3.5$ (Takenouchi et al. 2013). This is due to the solubility of the chemical and is likely an issue in other IVSSTs using DMSO as a solvent. In addition, although $\log K_{ow} > 3.5$ appears to be a good indicator for poor solubility, h-CLAT can judge a substantial number of chemicals with $\log K_{ow} > 3.5$ as sensitizers (Takenouchi et al. 2013), suggesting that $\log K_{ow} > 3.5$ is not necessarily a clear marker for the solubility of a chemical in medium. If this is the case, most IVSSTs cannot determine if a chemical is dissolved in the medium.

In contrast, the moIL-8 Luc assay can avoid false-negative judgment caused by poor solubility. Since THP-G8 is a dual reporter cell line that can assess the effects of chemicals on both IL-8 promoter activity and GAPDH promoter activity, the moIL-8 Luc assay can determine if a chemical is solubilized at a concentration sufficient to exert cytotoxicity, even in case of no effect on IL8LA. In this study, we demonstrated that Inh-GAPLA is a good marker for cell viability and that Inh-GAPLA below 0.8 indicates that the chemical is cytotoxic, which suggests that the chemical dissolved in X-VIVO™ 15. So, we modified the criteria. This simple modification of the criteria using the data inevitably obtained by the moIL-8 Luc assay succeeded in reducing the number of chemicals labeled with indeterminate by the tgIL-8 Luc assay and significantly improving its specificity.

Moreover, the sensitivity of the moIL-8 Luc assay for 26 chemicals with $\log K_{ow} > 3.5$ was 100.0%, although three chemicals were excluded from judgment. This high sensitivity, and the ability to exclude judgment for chemicals that are not dissolved at a high enough concentration to affect IL8LA or GAPLA, are significant advantages of the moIL-8 Luc assay over other IVSSTs.

Even by the moIL-8 Luc assay, however, it is impossible to discriminate non-sensitisers, which do not affect either IL8LA or GAPLA, from sensitizers that cannot dissolve sufficiently to stimulate either IL8LA or GAPLA. This issue is shared by other IVSSTs. Namely, the chemicals that IVSSTs judged as non-sensitisers can be sensitizers with poor solubility. Narita et al. (2018) recently have proposed a unique procedure to prevent the false-negative judgments due to poor water solubility: dispersing chemicals in liquid paraffin,

then exposing THP-8 cells to these dispersions, apparently prevents false-negative judgments for at least some lipophilic chemicals. However, as previously mentioned, it is not easy for most IVSSTs to know whether a false-negative judgment is due to poor solubility. To overcome this problem, Narita et al. (2018) proposed applying their method if the $\log K_{ow}$ of a chemical is over 2.0, but many chemicals with $\log K_{ow} > 0.2$ are correctly judged as sensitizers by h-CLAT. On the other hand, since the moIL-8 Luc assay can discriminate chemicals that do not dissolve in medium to a concentration sufficient to stimulate IL8LA or decrease GAPLA, it is clear for the moIL-8 Luc assay which chemicals need to be tested in this procedure. Applying their procedure to chemicals that are judged as indeterminate can reduce false-negative results by the moIL-8 Luc assay.

It is widely accepted that no single non-animal method can replace animal testing and that IATA is mandatory. However, it is not easy to determine which assays should be combined, and how to use and weight the data. For the tgIL-8 Luc assay or moIL-8 Luc assay, however, the purpose of the IATA is straightforward: to reduce the number of chemicals classified as out of the applicability domain or indeterminate. It is, therefore, not difficult to select a partner assay; since the classification as indeterminate is dependent on the solvent used in the assay, the partner should be an assay using a solvent other than water. We, therefore, selected DPRA, in which chemicals can be dissolved in acetonitrile, isopropanol, or acetone (Gerberick et al. 2007). Taking advantage of the high sensitivity of the moIL-8 Luc assay, we used DPRA to judge chemicals classified as out of the applicability domain or indeterminate. The results were very satisfactory, in particular in reducing the number of unevaluated chemicals.

Finally, compared with other in vitro assays, the tgIL-8 Luc assay is unique because it can eliminate the false-negative results due to extremely poor chemical solubility in water. In this study, we demonstrated that the moIL-8 Luc assay can decrease chemicals classified as indeterminate using GAPLA as a marker for cell viability. Moreover, by excluding false judgments due to poor solubility, we may be able to consider the reason for the false-negative judgment which is not due to solubility.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by CF, YK and SA. The first draft of the manuscript was written by SA and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability All the data are available from the corresponding author.

Compliance with ethical standards

Conflict of interest The authors declare that they do not have any conflict of interest.

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Supplementary Table 1. The summary of LLNA category, chemical property, solubility, and judgment by the IL-8 Luc assay

Chemical	CAS#	LLNA (Potency category)	Pre- /Prohaptent	Dissolved at 20 mg/ml of X- VIVO 15	Ave. of Max Ind-IL8LA	Ave. of Min Inh-GAPLA	tgIL-8 Luc assay	molL-8 Luc assay	LogKow
1-Benzoylacetone(1-BA)	93-91-4	Extreme		No	10.041	0.030	sensitizer	sensitizer	0.61
1-Chloromethylpyrene	1086-00-6	Extreme		No	1.706	0.023	sensitizer	sensitizer	5.73
2,4-Dinitrochlorobenzene(DNCB)	97-00-7	Extreme		No	12.013	0.016	sensitizer	sensitizer	2.27
4-Nitrobenzyl bromide(4-NBB)	100-11-8	Extreme		No	9.399	0.010	sensitizer	sensitizer	2.7
Bandrowski's base	20048-27-5	Extreme	Pre/Pro-haptent	No	3.561	0.014	sensitizer	sensitizer	0.74
Beryllium sulfate tetrahydrate	7787-56-6	Extreme		Yes	1.709	0.489	sensitizer	sensitizer	-0.17
Dinitrofluorobenzene	70-34-8	Extreme		No	5.651	0.020	sensitizer	sensitizer	1.83
Diphenylcyclopropenone (DPCP)	886-38-4	Extreme		No	4.642	0.023	sensitizer	sensitizer	3.25
Oxazolone	15646-46-5	Extreme		No	2.793	0.042	sensitizer	sensitizer	1.51
p-Benzoquinone	106-51-4	Extreme		No	3.846	0.027	sensitizer	sensitizer	0.25
Potassium dichromate	7778-50-9	Extreme		Yes	5.039	0.028	sensitizer	sensitizer	-3.59
Tetrachlorosalicylanilide	1154-59-2	Extreme		No	9.165	0.023	sensitizer	sensitizer	5.87
1,4-Dihydroquinone	123-31-9	Strong	Pre-haptent	Yes	7.036	0.031	sensitizer	sensitizer	1.03
1,4-Phenylenediamine(PPD)	106-50-3	Strong	Pre-haptent	Yes	3.393	0.026	sensitizer	sensitizer	-0.39
2-Aminophenol	95-55-6	Strong	Pre-haptent	No	9.064	0.028	sensitizer	sensitizer	0.6
2-Nitro-1,4-phenylenediamine	5307-14-2	Strong	Pre/Pro-haptent	No	1.311	0.130	inconclusive	non-sensitizer	0.55
Benzoyl peroxide	94-36-0	Strong		No	1.074	0.884	inconclusive	inconclusive	3.43
Benzyl bromide	100-39-0	Strong		No	14.947	0.030	sensitizer	sensitizer	2.88
Chloramine-T	127-65-1	Strong		Yes	3.198	0.019	sensitizer	sensitizer	-0.5
Chlorpromazine hydrochloride	69-09-0	Strong	Pre/Pro-haptent	Yes	6.128	0.019	sensitizer	sensitizer	3.69
Cobalt chloride hexahydrate	7646-79-9	Strong		Yes	3.832	0.027	sensitizer	sensitizer	0.85
Formaldehyde	50-00-0	Strong		Yes	4.281	0.019	sensitizer	sensitizer	0.35
Glutaraldehyde	111-30-8	Strong		Yes	3.220	0.031	sensitizer	sensitizer	-0.18
Hexyl salicylate	6259-76-3	Strong		No	1.309	0.946	inconclusive	inconclusive	5.06
Iodopropynyl butylcarbamate	55406-53-6	Strong		No	2.751	0.026	sensitizer	sensitizer	2.45
Lauryl gallate	1166-52-5	Strong	Pre-haptent	No	6.332	0.033	sensitizer	sensitizer	6.21
Maleic anhydride	108-31-6	Strong		No	1.134	0.011	non-sensitizer ^{out of AE}	non-sensitizer ^{out of AD}	1.62
Methyldibromoglutaronitrile(M DGN)	35691-65-7	Strong	Pre-haptent	No	4.288	0.017	sensitizer	sensitizer	1.63
Phthalic anhydride	85-44-9	Strong		No	1.163	0.016	non-sensitizer ^{out of AE}	non-sensitizer ^{out of AD}	2.07
Propyl gallate	121-79-9	Strong	Pre-haptent	No	3.914	0.024	sensitizer	sensitizer	1.79
1-Naphthol	90-15-3	Moderate	Pre-haptent	No	7.982	0.028	sensitizer	sensitizer	2.69
1-Phenyl-1,2-propanedione	579-07-7	Moderate		No	8.080	0.021	sensitizer	sensitizer	1.11
1-Thioglycerol(3-mercaptopropanediol)	96-27-5	Moderate		Yes	3.518	0.184	sensitizer	sensitizer	-0.84
1,2-Benzisothiazolin-3-one	2634-33-5	Moderate		No	7.209	0.030	sensitizer	sensitizer	0.64
12-Bromo-1-dodecanol	3344-77-2	Moderate		No	2.381	0.710	sensitizer	sensitizer	5.11
2-Hydroxyethyl acrylate	818-61-1	Moderate		Yes	1.000	0.015	non-sensitizer	non-sensitizer	0.25
2-Mercaptobenzothiazole(2-MBT)	149-30-4	Moderate		No	4.393	0.038	sensitizer	sensitizer	2.86
2-Methoxy-4-methylphenol	93-51-6	Moderate	Pre/Pro-haptent	No	2.399	0.043	sensitizer	sensitizer	1.88
2-Phenylpropionaldehyde	93-53-8	Moderate		No	11.050	0.094	sensitizer	sensitizer	1.96
2,4-Heptadienal	4313-03-5	Moderate		No	3.627	0.209	sensitizer	sensitizer	1.86
3-Aminophenol	591-27-5	Moderate	Pro-haptent	Yes	1.844	0.126	sensitizer	sensitizer	0.24
3-Dimethylaminopropylamine	109-55-7	Moderate	Pro-haptent	Yes	5.204	0.033	sensitizer	sensitizer	0.45
3-Propylenephthalide	17369-59-4	Moderate		No	1.975	0.614	sensitizer	sensitizer	2.03
3,4-Dihydrocoumarin	119-84-6	Moderate		No	1.825	0.016	sensitizer	sensitizer	0.97
4-Chloroaniline	106-47-8	Moderate		No	1.644	0.027	sensitizer	sensitizer	1.72
5-Methyl-2-phenyl-2-hexenal	21834-92-4	Moderate		No	6.019	0.016	sensitizer	sensitizer	3.77

a-Methylcinnamic aldehyde	101-39-3	Moderate		No	3.324	0.023	sensitizer	sensitizer	2.37
Benzyl salicylate	118-58-1	Moderate		No	1.638	0.875	sensitizer	sensitizer	4.31
Benzylideneacetone	122-57-6	Moderate		No	5.501	0.032	sensitizer	sensitizer	2.04
Cinnamic aldehyde(Cinnamal)	104-55-2	Moderate		No	3.550	0.012	sensitizer	sensitizer	1.82
Clotrimazole	23593-75-1	Moderate		No	1.260	0.824720848	inconclusive	inconclusive	6.26
Diethyl maleate	141-05-9	Moderate		No	1.783	0.022	sensitizer	sensitizer	2.2
Diethyl sulfate	64-67-5	Moderate		No	2.686	0.011	sensitizer	sensitizer	1.14
Diethylenetriamine	111-40-0	Moderate		Yes	3.158	0.096	sensitizer	sensitizer	2.13
Dihydroeugenol(2-Methoxy-4-propylphenol)	2785-87-7	Moderate	Pro-hapten	No	2.029	0.019	sensitizer	sensitizer	2.87
Ethylenediamine	107-15-3	Moderate	Pro-hapten	Yes	2.172	0.043	sensitizer	sensitizer	1.62
Glyoxal	107-22-2	Moderate		Yes	2.866	0.020	sensitizer	sensitizer	1.66
Isoeugenol	97-54-1	Moderate	Pre-hapten	No	1.743	0.013	sensitizer	sensitizer	2.65
Methyl-2-nonynoate	111-80-8	Moderate		No	3.923	0.033	sensitizer	sensitizer	3.1
Methylisothiazolinone, 2-Methyl-2H-Isothiazol-3-one(KeratinoSens)	2682-20-4	Moderate		Yes	6.047	0.041	sensitizer	sensitizer	0.83
Nickel sulphate hexahydrate	10101-97-0	Moderate		Yes	4.776	0.025	sensitizer	sensitizer	2.04
Nickel(II) chloride	7718-54-9	Moderate		Yes	4.326	0.016	sensitizer	sensitizer	0.05
Perillaldehyde	18031-40-8	Moderate		No	3.472	0.027	sensitizer	sensitizer	3.34
Phenylacetaldehyde	122-78-1	Moderate		No	7.948	0.024	sensitizer	sensitizer	1.54
Resorcinol	108-46-3	Moderate	Pro-hapten	Yes	2.677	0.032	sensitizer	sensitizer	1.03
Tartaric acid	133-37-9	Moderate		Yes	6.874	0.154606673	sensitizer	sensitizer	-1
Tetramethylthiuramdisulphide(TMTD)	137-26-8	Moderate		No	4.096	0.049	sensitizer	sensitizer	0.17
trans-2-Hexenal	6728-26-3	Moderate		No	8.479	0.132	sensitizer	sensitizer	1.58
Trimellitic anhydride	552-30-7	Moderate		No	1.971	0.015	sensitizer	sensitizer	1.95
Undec-10-enal	112-45-8	Moderate		No	4.055	0.018	sensitizer	sensitizer	4.12
(R)-(+)-Limonene	5989-27-5	Weak	Pre-hapten	No	3.063	0.879	sensitizer	sensitizer	4.83
1-Bromohexane	111-25-1	Weak		No	1.041	0.913	inconclusive	inconclusive	3.63
2-Ethylhexyl acrylate	103-11-7	Weak		No	1.831	0.904	sensitizer	sensitizer	4.09
2,3-Butanedione	431-03-8	Weak		Yes	3.030	0.024	sensitizer	sensitizer	1.34
4-Allylanisole	140-67-0	Weak	Pro-hapten	No	25.439	0.053	sensitizer	sensitizer	3.47
5-Methyl-2,3-hexanedione	13706-86-0	Weak		No	5.049	0.013	sensitizer	sensitizer	0.06
Abietic acid	514-10-3	Weak	Pre-hapten	No	2.576	0.095	sensitizer	sensitizer	6.46
Amyl cinnamic aldehyde	122-40-7	Weak		No	12.379	0.034	sensitizer	sensitizer	4.33
Aniline	62-53-3	Weak	Pre/Pro-hapten	Yes	1.078	0.021	non-sensitizer	non-sensitizer	1.08
Benzocaine	60-09-3	Weak		No	1.036	0.051	inconclusive	non-sensitizer	3.19
Benzyl cinnamate	103-41-3	Weak		No	1.249	0.904731565	sensitizer	sensitizer	4.06
Bis-GMA	1565-94-2	Weak		No	5.551	0.029	sensitizer	sensitizer	4.94
Butyl glycidyl ether	2426-08-6	Weak		Yes	5.811	0.041	sensitizer	sensitizer	1.08
Cinnamic alcohol	104-54-1	Weak	Pre/Pro-hapten	No	7.185	0.013	sensitizer	sensitizer	1.84
Citral	5392-40-5	Weak		No	12.797	0.027	sensitizer	sensitizer	3.45
Cyclamen aldehyde	103-95-7	Weak		No	2.871	0.026	sensitizer	sensitizer	3.91
Ethyleneglycol dimethacrylate	97-90-5	Weak		No	4.672	0.192	sensitizer	sensitizer	2.21
Eugenol	97-53-0	Weak	Pre/Pro-hapten	No	2.544	0.013	sensitizer	sensitizer	2.73
Farnesal	19317-11-4	Weak		No	5.383	0.017	sensitizer	sensitizer	5.74
Geraniol	106-24-1	Weak	Pre/Pro-hapten	No	2.153	0.035	sensitizer	sensitizer	3.47
Hexyl cinnamic aldehyde	101-86-0	Weak		No	4.636	0.071	sensitizer	sensitizer	4.82
Hydroxycitronellal	107-75-5	Weak		No	42.067	0.026	sensitizer	sensitizer	2.11
Imidazolidinyl urea	39236-46-9	Weak		Yes	16.558	0.020	sensitizer	sensitizer	8.28
Lilial	80-54-6	Weak		No	3.898	0.011	sensitizer	sensitizer	4.36

Linalool	78-70-6	Weak	Pre-hapten	No	4.359	0.028	sensitizer	sensitizer
Lyrar	31906-04-4	Weak		No	1.930	0.015	sensitizer	sensitizer
Methylmethacrylate	80-62-6	Weak		No	1.014	0.866680106	inconclusive	inconclusive
N,N-Dibutylaniline	613-29-6	Weak		No	2.092	0.911	sensitizer	sensitizer
Nonanoic acid	112-05-0	Weak		No	2.423	0.015	sensitizer	sensitizer
Oxalic acid	144-62-7	Weak		Yes	6.389	0.029	sensitizer	sensitizer
Penicillin G	61-33-6	Weak		Yes	1.325	0.064	non-sensitizer	non-sensitizer
Pentachlorophenol	87-86-5	Weak		No	7.554	0.020	sensitizer	sensitizer
Phenol, 2,2-azobis-	2050-14-8	Weak		No	5.122	0.153	sensitizer	sensitizer
Phenyl benzoate	93-99-2	Weak		No	1.139	0.848	inconclusive	inconclusive
Pyridine	110-86-1	Weak		Yes	1.046	0.027	non-sensitizer	non-sensitizer
Xylene	1330-20-7	Weak		No	1.057	0.943076035	inconclusive	inconclusive
α-iso-Methylionone	127-51-5	Weak		No	5.110	0.020	sensitizer	sensitizer
1-Bromobutane	109-65-9	Non-sensitizer		No	1.056	0.447	inconclusive	non-sensitizer
1-Butanol	71-36-3	Non-sensitizer		Yes	1.037	0.951	non-sensitizer	non-sensitizer
2-Acetylcyclohexanone	874-23-7	Non-sensitizer		No	4.742	0.027	sensitizer	sensitizer
2-Hydroxypropyl methacrylate	923-26-2	Non-sensitizer		Yes	1.188	0.039	non-sensitizer	non-sensitizer
2,4-Dichloro-1-nitrobenzene	611-06-3	Non-sensitizer		No	1.269	0.912	inconclusive	inconclusive
4-Hydroxybenzoic acid	99-96-7	Non-sensitizer		No	1.021	0.031	inconclusive	non-sensitizer
6-Methylcoumarin	92-48-8	Non-sensitizer		No	1.071	0.800	inconclusive	inconclusive
Acetoanisole(4-Methoxyacetophenone)	100-06-1	Non-sensitizer		No	1.139	0.029	inconclusive	non-sensitizer
Benzalkonium chloride	8001-54-5	Non-sensitizer		Yes	3.807	0.016033451	sensitizer ^{out of AD}	sensitizer ^{out of AD}
Benzoic acid	65-85-0	Non-sensitizer		No	1.218	0.030	inconclusive	non-sensitizer
Benzyl alcohol	100-51-6	Non-sensitizer		Yes	3.535	0.056	sensitizer	sensitizer
Chlorobenzene	108-90-7	Non-sensitizer		No	1.045	0.981	inconclusive	inconclusive
Dextran	9004-54-0	Non-sensitizer		Yes	1.169	0.872	non-sensitizer	non-sensitizer
Diethyl phthalate	84-66-2	Non-sensitizer		No	1.480	0.738	sensitizer	sensitizer
Dimethyl formamide	68-12-2	Non-sensitizer		Yes	1.002	0.928	non-sensitizer	non-sensitizer
Dimethyl isophthalate	1459-93-4	Non-sensitizer		No	1.147	0.972	inconclusive	inconclusive
Ethyl benzoylacetate	94-02-0	Non-sensitizer		No	1.611	0.042	inconclusive	non-sensitizer
Ethyl vanillin	121-32-4	Non-sensitizer		No	2.203	0.024	sensitizer	sensitizer
Furil	492-94-4	Non-sensitizer		No	1.304	0.898	inconclusive	inconclusive
Glycerol	56-81-5	Non-sensitizer		Yes	1.304	0.930	non-sensitizer	non-sensitizer
Hexadecyltrimethylammonium bromide	57-09-0	Non-sensitizer		Yes	32.008	0.015889997	sensitizer ^{out of AD}	sensitizer ^{out of AD}
Isopropanol	67-63-0	Non-sensitizer		Yes	1.084	0.909	non-sensitizer	non-sensitizer
Lactic acid	50-21-5	Non-sensitizer		Yes	1.033	0.009	non-sensitizer	non-sensitizer
Methyl salicylate	119-36-8	Non-sensitizer		No	1.258	0.819	inconclusive	inconclusive
Octanoic acid(Caprylic acid)	124-07-2	Non-sensitizer		No	1.863	0.029	sensitizer ^{out of AD}	sensitizer ^{out of AD}
p-Aminobenzoic acid	150-13-0	Non-sensitizer		No	1.057	0.018	inconclusive	non-sensitizer
Propyl paraben	94-13-3	Non-sensitizer		No	1.478	0.038	sensitizer	sensitizer
Propylene glycol	57-55-6	Non-sensitizer		Yes	1.088	0.906	non-sensitizer	non-sensitizer
Saccharin	81-07-2	Non-sensitizer		No	2.251	0.028	sensitizer	sensitizer
Salicylic acid	69-72-7	Non-sensitizer		No	1.381	0.015	inconclusive	non-sensitizer
Sodium lauryl sulfate	151-21-3	Non-sensitizer		Yes	2.911	0.028	sensitizer ^{out of AD}	sensitizer ^{out of AD}
Streptomycin sulphate	3810-74-0	Non-sensitizer		Yes	1.110	0.874	non-sensitizer	non-sensitizer
Sulfanilamide	63-74-1	Non-sensitizer		No	1.027	0.186	inconclusive	non-sensitizer
Tween-80	9005-65-6	Non-sensitizer		Yes	2.233	0.027	sensitizer ^{out of AD}	sensitizer ^{out of AD}
Vanillin	121-33-5	Non-sensitizer		No	2.560	0.025	sensitizer	sensitizer
Zinc sulphate	7733-02-0	Non-sensitizer		No	2.397	0.013148412	sensitizer	sensitizer
out of AD: out of applicability domain, n.d.: not determined								
Ave. of Max Ind-IL8LA: Average of the maximum values of Ind-IL8LA in two experiments								
Ave. of Min Inh-GAPLA: Average of the minuimu values of Ind-GAPLA in three experiments								