Received: 13 February 2018 | Revised: 23 March 2018 | Accepted: 26 March 2018

DOI: 10.1002/jat.3634

RESEARCH ARTICLE

WILEY Journal of Applied Toxicology

Transferability and within- and between-laboratory reproducibilities of EpiSensA for predicting skin sensitization potential in vitro: A ring study in three laboratories

Hideyuki Mizumachi[1] | Megumi Sakuma[2] | Mayu Ikezumi[3] | Kazutoshi Saito[1] | Midori Takeyoshi[2] | Noriyasu Imai[2] | Hiroko Okutomi[3] | Asami Umetsu[3] | Hiroko Motohashi[3] | Mika Watanabe[3] | Masaaki Miyazawa[1]

[1] Kao Corporation, R&D, Safety Science Research, 2606 Akabane, Ichikai-Machi, Haga-Gun, Tochigi 321-3497, Japan

[2] KOSÉ Corporation, Research Laboratories, 1-18-4 Azusawa, Itabashi-ku, Tokyo 174-0051, Japan

[3] Food and Drug Safety Center, Hatano Research Institute, 729-5 Ochiai, Hadano-shi, Kanagawa 257-8523, Japan

Correspondence
Hideyuki Mizumachi, Kao Corporation, 2606, Akabane, Ichikai-Machi, Haga-Gun, Tochigi 321-3497, Japan
Email: mizumachi.hideyuki@kao.com

Abstract

The epidermal sensitization assay (EpiSensA) is an in vitro skin sensitization test method based on gene expression of four markers related to the induction of skin sensitization; the assay uses commercially available reconstructed human epidermis. EpiSensA has exhibited an accuracy of 90% for 72 chemicals, including lipophilic chemicals and pre−/pro-haptens, when compared with the results of the murine local lymph node assay. In this work, a ring study was performed by one lead and two naive laboratories to evaluate the transferability, as well as within- and between-laboratory reproducibilities, of EpiSensA. Three non-coded chemicals (two lipophilic sensitizers and one non-sensitizer) were tested for the assessment of transferability and 10 coded chemicals (seven sensitizers and three non-sensitizers, including four lipophilic chemicals) were tested for the assessment of reproducibility. In the transferability phase, the non-coded chemicals (two sensitizers and one non-sensitizer) were correctly classified at the two naive laboratories, indicating that the EpiSensA protocol was transferred successfully. For the within-laboratory reproducibility, the data generated with three coded chemicals tested in three independent experiments in each laboratory gave consistent predictions within laboratories. For the between-laboratory reproducibility, 9 of the 10 coded chemicals tested once in each laboratory provided consistent predictions among the three laboratories. These results suggested that EpiSensA has good transferability, as well as within- and between-laboratory reproducibility.

KEYWORDS

alternative method, gene expression, lipophilic chemicals, reconstructed human epidermis, skin sensitization

1 | INTRODUCTION

Allergic contact dermatitis resulting from skin sensitization causes a decreased quality of life for patients, and is a condition that is highly associated with significant social impact (Peiser et al., 2010). In addition, skin sensitization is a significant toxicological endpoint for cosmetic ingredients. The evaluation of skin sensitization has conventionally been dependent on animal tests such as the local lymph node assay (LLNA; OECD, 2010) and the guinea pig maximization test (Organization for Economic Cooperation and Development [OECD], 1992), which are test guidelines in the framework of the OECD. On the other hand, the development of non-animal tests for identifying

the skin sensitization potential of chemicals has recently been prioritized because of ethical issues and the 7th Amendment to the Cosmetics Directive (EU, 2009).

While skin sensitization consists of highly complex multifactorial events, the chemical and biological key events related to the induction of skin sensitization have been investigated in detail. These processes have been described in the OECD report on "The Adverse Outcome Pathway (AOP) for Skin Sensitization Initiated by Covalent Binding to Proteins" (OECD, 2012). Briefly, these events include: (i) binding of small reactive chemicals (called haptens) to skin proteins (key event 1); (ii) inflammatory response and induction of cytoprotective gene pathways in keratinocytes (key event 2); (iii) induction of surface molecules, with mobilization and migration to the lymph node by dendritic cells (key event 3); and (iv) presentation of the resulting histocompatibility complexes to naive T cells by dendritic cells, with subsequent differentiation and proliferation of activated T cells (key event 4). To define the skin sensitization potential of various chemicals, several kinds of in silico, in chemico and in vitro methods focusing on these key events have been developed. Most recently, the direct peptide reactivity assay (key event 1), the KeratinoSens™ (key event 2), and human cell line activation test (h-CLAT, key event 3) have been adopted as OECD Test Guidelines (OECD, 2015a, 2015b, 2016). No in vitro assay that addresses key event 4 has been adopted yet. Each of these three adopted tests (direct peptide reactivity assay, KeratinoSens™ and h-CLAT) have been reported to each exhibit an accuracy and a balanced accuracy of approximately 80% (compared with LLNA results, for a panel of over 160 chemicals) in discriminating sensitizers from non-sensitizers (Urbisch et al., 2015).

However, there are two shared limitations to the application of these three tests. First, lipophilic chemicals that are not soluble in an appropriate solvent are difficult to evaluate correctly, as these three tests employ aqueous-phase systems. For KeratinoSens™, test chemicals that are not soluble or fail to form a stable dispersion cannot be tested at the highest concentrations. Therefore, a positive result could still be used to support the identification of the test chemical as a skin sensitizer, whereas a negative result should be considered inconclusive (OECD, 2015). Similarly, for the h-CLAT, test chemicals with log $K_{ow} \geq 3.5$ tend to produce false-negative results, and therefore a negative result for test chemicals with log $K_{ow} \geq 3.5$ should not be considered conclusive (OECD, 2016). Second, pre- and pro-haptens, which become reactive haptens through either a biological oxidation or metabolic conversion (Aptula, Roberts, & Pease, 2007), are also difficult to evaluate due to the limited oxidative or metabolic activity of in vitro assays (OECD, 2015b, 2016) and the limited oxidative activity or the absence of the metabolic activation of in chemico assays (OECD, 2015a).

To overcome these two limitations, we focused on a model that employs a reconstructed human epidermis (RhE) consisting of normal, human-derived epidermal keratinocytes. Even a lipophilic chemical that is soluble in a vehicle used in animal tests can be directly applied to the RhE model, just as such a chemical would be applied in an animal test. Moreover, RhE models have been reported to show a metabolic capability like that of human skin (Oesch, Fabian, Guth, & Landsiedel, 2014; Tokudome, Katayanagi, & Hashimoto, 2015), suggesting that pre- and pro-haptens might be correctly evaluated using an RhE model. Recently, some RhE model-based skin sensitization test methods have been reported. For instance, the SenCeeTox® assay, which employs EpiDerm™ or SkinEthic™ RhE, measures the expression of 11 genes whose products are primarily involved in the cytoprotective response (McKim Jr, Keller 3rd, & Gorski, 2012). Compared with LLNA, SenCeeTox® exhibited an accuracy of 90% and a balanced accuracy of 84% when tested with a limited panel of 10 chemicals (Reisinger et al., 2015). In contrast, the SENS-IS assay, which employs EpiSkin, measures the expression of 61 genes whose products are related primarily to redox, inflammation and tissue repair processes (Cottrez et al., 2016). Compared with LLNA, SENS-IS exhibited an accuracy of 93% and a balanced accuracy of 94% against a panel of 28 lipophilic chemicals with log $K_{ow} \geq 3.5$, and an accuracy of 100% when tested against a panel of 14 pre−/pro-haptens. The challenge in using SENS-IS is the potential difficulty in analyzing expression for a large number of marker genes; this observation suggests that the combination of an RhE model-based method and use of a small number of marker genes may overcome the above two limitations and the difficulty in analysis.

The epidermal sensitization assay (EpiSensA) is an in vitro test method based on the gene expression of four mechanistically relevant markers (encoding activating transcription factor 3 [ATF3]; glutamate–cysteine ligase, modifier subunit [GCLM]; DnaJ [Hsp40] homolog, subfamily B, member 4 [DNAJB4]; and interleukin [IL]-8). Expression of these genes reflects keratinocyte responses in the early phase of skin sensitization, including (i) the inflammatory response (e.g., induction of inflammatory cytokines), and (ii) the induction of cytoprotective gene pathways (e.g., antioxidant response element-dependent pathways) (OECD, 2012). For a panel of 72 test chemicals, including 29 lipophilic chemicals and 11 putative pre−/pro-haptens, EpiSensA provided a sensitivity of 94% (51 of 54 sensitizers), accuracy of 90% (65 of 72 chemicals) and balanced accuracy of 86% when compared with LLNA (Saito, Takenouchi, Nukada, Miyazawa, & Sakaguchi, 2017). Moreover, for a subset of 27 lipophilic sensitizers and 11 pre- and pro-haptens, EpiSensA yielded sensitivities of 93% and 100%, respectively, compared with LLNA.

Thus, the purpose of the present study was to provide a preliminary evaluation of the transferability, as well as the within- (WLR) and between-laboratory reproducibilities (BLR), of EpiSensA at three laboratories, in advance of the official validation study. Transferability was confirmed based on testing with two sensitizers and one non-sensitizer after training of two naive laboratories by the lead laboratory. WLR was assessed using the results generated with three coded chemicals tested in three independent experiments in each laboratory. BLR was assessed using the results generated with 10 coded chemicals tested in each of the three laboratories. The evaluation of the information obtained in the present study is expected to facilitate the optimization and standardization of the protocol to be employed in the official validation process.

2 | MATERIALS AND METHODS

2.1 | Test chemicals and preparation

For the transferability phase, two chemicals that are known sensitizers in LLNA (bisphenol A diglycidyl ether [BADGE] and clotrimazole) and

one that is a non-sensitizer in LLNA (lactic acid) were purchased from Sigma-Aldrich (St. Louis, MO, USA) (Gerberick et al., 2005). BADGE and clotrimazole were selected as test chemicals by the Kao Corporation (Tochigi, Japan), the lead laboratory, based on relatively low water solubility (giving log K_{ow} values of 3.84 and 6.26, respectively, in KOWWIN ver.1.68 in EPI suite; Environmental Protection Agency, Washington, DC, USA), and consistent induction of the expression of marker genes in historical data (data not shown). Lactic acid was selected because this chemical has been used historically as a representative non-sensitizer (Casati et al., 2009). BADGE, clotrimazole and lactic acid were transferred (as non-coded materials) to two naive laboratories (KOSÉ Corporation, Tokyo, Japan and Food and Drug Safety Center [FDSC], Kanagawa, Japan) for testing at prescribed concentrations of 6.25%, 3.13% and 1.56% respectively. The EpiSensA protocol also was sent to the naive laboratories and face-to-face training was performed regarding chemical exposure, tissue washing and RNA extraction.

For the WLR and BLR phases, 10 test chemicals were selected (Table 1), taking into account the skin sensitization potentials reported in the literature (Gerberick et al., 2005; Gerberick, House, Fletcher, & Ryan, 1992; Kern et al., 2010; Kimber et al., 1998), commercial availabilities, diversities of chemical structure and physicochemical properties. These 10 chemicals included four lipophilic chemicals (log K_{ow} ≥3.5) that have yielded false predictions or inconclusive results in existing in vitro tests (Urbisch et al., 2015). All tested chemicals, except for lilial (Wako Pure Chemicals, Osaka, Japan), were purchased from Sigma-Aldrich. These chemicals were coded and divided into two groups: For a first set of three test chemicals (i.e., metol, dibutyl aniline and cetrimide), three different vials with different codes for each chemical were sent from the lead laboratory to the naive laboratories for use in assessing the WLR. Then, the remaining seven test chemicals were also coded and sent to the naive laboratories for use in assessing the BLR.

2.2 | EpiSensA test protocol

The EpiSensA test protocol has been described in Saito et al. (2017). A brief outline is provided below.

2.2.1 | RhE culture

An RhE model "LabCyte EPI-MODEL 24" (Japan Tissue Engineering Co. Ltd, Aichi, Japan) was pre-cultured overnight at 37°C (5% CO_2) in 0.5 ml per well of the culture medium provided by the manufacturer.

2.2.2 | Chemical exposure

For a dose-finding study, each test chemical was dissolved in an appropriate vehicle, consisting of either AOO (acetone/olive oil at 4:1, *v*/v), distilled water (DW), or 50% ethanol in DW (50% EtOH). In particular, the assay was performed using chemical dissolved in the vehicle that permitted the test chemical to be dissolved to the highest concentration. Working solutions of each test chemical were prepared as fourfold serial dilutions from the highest concentration to concentrations of 0.02% or below. An aliquot (5 μl) of working solution was applied to each tissue surface (one tissue per group). One tissue for non-treated control and two tissues for killed control (treated with 10 μl of 10% Triton X-100; Sigma-Aldrich) were prepared for cell viability measurements. The tissues were incubated for 6 hours at 37°C under 5% CO_2. Cell viability was measured by the lactate dehydrogenase (LDH) assay using an LDH cytotoxicity detection kit (Takara Bio, Inc., Tokyo, Japan) according to the manufacturer's instructions. The lowest concentration showing less than 80% cell viability was used for the subsequent main study.

For the main study investigation of each test chemical, twofold serial dilutions were prepared to range from the lowest concentration showing less than 80% cell viability to the highest concentration showing more than 90% cell viability (basically from three to five working solutions at twofold serial dilutions). When the viability was not less than 80% in the dose-finding study, the chemical was tested in the main study by preparing at least three working solutions at twofold serial dilutions from the highest soluble concentration. Positive controls consisted of BADGE and clotrimazole dissolved (separately) in AOO at 6.25% and 3.13%, respectively. Aliquots (5 μl) of working solutions of each chemical were applied to three tissues per group; the exposed tissues then were incubated for 6 hours. One tissue for non-treated control and two tissues for killed controls were prepared

TABLE 1 Test chemicals used to evaluate within- and between-laboratory reproducibilities

Chemical name	CAS no.	LLNA EC3 (%)	LogK_{ow}[a]	Physical state
Metol	55–55-0	0.78	2.34	Solid
Dibutyl aniline	613–29-6	20	**5.12**	Liquid
Cetrimide	57–09-0	–	3.18	Solid
2,4-Dinitrochlorobenzene	97–00-7	0.05	2.27	Solid
2-Mercaptobenzothiazole	149–30-4	1.7	2.86	Solid
Undec-10-enal	112–45-8	6.8	**4.12**	Liquid
Lilial	80–54-6	19	**4.36**	Liquid
Imidazolidinyl urea	39236–46-9	24	−8.28	Solid
Benzyl butyl phthalate	85–68-7	–	**4.84**	Liquid
Glycerol	56–81-5	–	−1.65	Liquid

LLNA, local lymph node assay.

[a]Calculated by KOWWIN ver. 1.68 in EPI Suite™. Lipophilic chemicals with log K_{ow} ≥3.5 are indicated in bold.

for cell viability measurements. Cell viability was measured by the LDH assay.

2.2.3 | RNA extraction, cDNA synthesis and real-time polymerase chain reaction

After test chemical exposure, the tissue surface was rinsed three times with Dulbecco's phosphate-buffered saline (Life Technologies, Carlsbad, CA, USA) and the tissue was gently collected into a 1.5 ml microtube containing 0.5 ml TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and homogenized by vortex mixing for at least 90 seconds. Chloroform (100 µl; Tokyo Chemical Industry, Tokyo, Japan) was added to the homogenized sample and the sample was centrifuged at 12 000 *g* for 15 minutes at 4°C. The aqueous phase was transferred to another 1.5 ml microtube and total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

Reverse transcription of total RNA was performed using the Superscript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions and then stored at −20°C.

Quantitative reverse transcription–polymerase chain reaction (reverse transcription–PCR) was performed using the TaqMan Gene Expression Assay (Applied Biosystems, Waltham, MA, USA) and TaqMan Universal PCR Master Mix (Applied Biosystems). Cycle threshold (C_t) values of four skin sensitization marker genes (*ATF3*, *GCLM*, *DNAJB4* and *IL-8*) and one endogenous control gene (*GAPDH*, encoding the housekeeping protein glyceraldehyde 3-phosphate dehydrogenase) were measured using the 7500 Fast Real-Time PCR System (Applied Biosystems; as used at Kao Corp.), the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA; as used at KOSÉ Corp.) or an ABI PRISM 7900HT machine (Applied Biosystems; as used at FDSC). Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and expressed as fold-change normalized to expression of the control (*GAPDH*) gene.

2.2.4 | Prediction model

The mean value (three tissues per group) of maximum fold induction (I_{max}) was obtained using the data from the concentrations with over 80% cell viability. When the I_{max} of at least one of the four marker genes exceeded the respective cut-off value (*ATF3*, 15-fold; *GCLM*, 2-fold; *DNAJB4*, 2-fold; and *IL-8*, 4-fold), the chemical was judged as positive in EpiSensA. The data were accepted when the following criteria were fulfilled: (i) the mean cell viability of both positive controls (6.25% BADGE and 3.13% clotrimazole) exceeded 80%, and (ii) the mean value of fold induction for all four marker genes exceeded the cut-off value for either or both positive controls (6.25% BADGE or 3.13% clotrimazole).

2.3 | Data analysis

The success of the transfer was judged based on whether the two sensitizers and one non-sensitizer were correctly classified as positive and negative, respectively, at the two naive laboratories. The WLR was assessed based on the concordance of predictions, positive or negative, between three independent experiments performed in each laboratory for the three coded chemicals. An additional analysis was

TABLE 2 Transferability analysis based on marker gene induction exceeding the respective cut-off values and fold-changes in each of the three laboratories

Chemicals	LLNA results	Conc. (%)	Judgment		ATF3			GCLM			DNAJB4			IL-8			Viability		
			KOSÉ	FDSC	Historical	KOSÉ	FDSC	Historical	KOSÉ	FDSC	Historical	KOSÉ	FDSC	Historical	KOSÉ	FDSC	Historical	KOSÉ	FDSC
BADGE	Sensitizer	6.25	P	P	15.2–151	24.3	71.1	2.0–7.2	2.8	7.4	6.4–31.9	7.2	23.5	12.6–78.8	9.9	29.9	81.4–100	97.7	93.4
Clotrimazole	Sensitizer	3.13	P	P	101–3293	437	1102	1.1–1.9	1.5	1.5	17.7–48.4	13.9	48.8	10.7–375	108	338	82.2–97.3	95.9	84.3
Lactic acid	Non-sensitizer	1.56	N	N	0.6–2.4	1.5	1.8	0.9–1.4	1.3	1.3	1.1–1.4	1.1	1.4	0.8–1.6	2.8	1.4	95.8–98.0	94.6	98.0

ATF3, activating transcription factor 3; DNAJB4, DnaJ homolog, subfamily B, member 4; GCLM, glutamate–cysteine ligase, modifier subunit; IL, interleukin; LLNA, local lymph node assay.

Test chemicals (6.25% BADGE, 3.13% clotrimazole and 1.56% lactic acid) were evaluated at the naive laboratories; mean values of fold inductions and cell viability, and ranges of historical fold induction in the lead laboratory, are shown. Historical ranges of gene induction by BADGE, clotrimazole and lactic acid were obtained from four, three and three experiments, respectively. Fold inductions that exceeded the respective cut-off values are highlighted in gray.

performed to evaluate the reproducibility of the three respective inductions for each marker gene in each laboratory. The BLR was assessed based on the results generated with a total of 10 chemicals, including seven chemicals that were tested once and three chemicals that were tested independently three times in each laboratory. The final prediction for the chemicals that were tested three times in each laboratory was based on the third experiment. An additional analysis was performed to evaluate the reproducibility among the three laboratories in the inductions for each marker gene. This additional analysis was used for informational purposes only, and was not used to draw conclusions regarding the WLR and BLR.

The EC values for each marker gene, i.e., the concentrations at which the induction exceeded a cut-off value (EC15 for *ATF3*, EC2 for *DNAJB4*, EC2 for *GCLM* and EC4 for *IL-8*), were calculated using linear interpolation from the dose–response curve. The EC values of EpiSensA have been reported to show good correlation with the LLNA EC3 that is used as an indicator of skin sensitization potency (Saito et al., 2017). If the fold induction at the lowest tested concentration exceeded the cut-off value and was less than twice the cut-off value, the EC values were extrapolated from gene expressions at the lowest and second lowest concentrations, based on the dose–response curve. Additionally, the 20% inhibitory concentration (IC_{20}) effecting a 20% reduction of cell viability was estimated by linear interpolation from the dose–response curve of cell viability.

3 | RESULTS

3.1 | Transferability phase

Table 2 shows the fold inductions of the four marker genes and cell viability in the two naive laboratories, and the historical ranges of the fold inductions in the lead laboratory, when two sensitizers, 6.25% BADGE and 3.13% clotrimazole, and one non-sensitizer, 1.56% lactic acid, were tested. The historical values of BADGE, clotrimazole and lactic acid were obtained from four, three and three independent experiments, respectively. The two sensitizers and one non-sensitizer were correctly classified at the two naive laboratories. Moreover, slightly higher or lower fold inductions than historical data were observed for *GCLM* with BADGE at FDSC, for *DNAJB4* with

TABLE 3 Within-laboratory reproducibility analysis based on marker gene induction exceeding the respective cut-off values, fold-changes and EC values in each of the three laboratories

							I_{max} (cut-off value)				EC value (%)			
Chemicals	Laboratory		Vehicle	Dose range (%)	Judgment	IC_{20} (%)	ATF3 (15-fold)	GCLM (2-fold)	DNAJB4 (2-fold)	IL-8 (4-fold)	ATF3	GCLM	DNAJB4	IL-8
Metol	Kao	Replicate 1	DW	0.78–3.13	P	>3.13	395.1	22.6	31.2	8.0	<0.78	<0.78	<0.78	<0.78
		Replicate 2	DW	0.78–3.13	P	>3.13	448.0	21.7	35.1	6.8	<0.78	<0.78	<0.78	<0.78
		Replicate 3	DW	0.78–3.13	P	>3.13	460.5	20.9	32.1	6.9	<0.78	<0.78	<0.78	<0.78
	KOSÉ	Replicate 1	DW	0.78–3.13	P	2.17	213.2	11.6	19.3	4.2	<0.78	<0.78	<0.78	<0.78
		Replicate 2	DW	0.39–3.13	P	>3.13	130.7	13.9	21.9	5.1	<0.39	<0.39	<0.39	<0.39
		Replicate 3	DW	0.78–3.13	P	>3.13	202.1	14.3	16.6	4.8	<0.78	<0.78	<0.78	<0.78
	FDSC	Replicate 1	DW	0.39–3.13	P	1.43	183.6	17.4	27.1	3.2	**0.37**	<0.78	<0.78	–
		Replicate 2	DW	0.39–3.13	P	0.90	200.1	16.7	35.9	5.9	<0.39	<0.39	<0.39	<0.39
		Replicate 3	DW	0.39–3.13	P	1.48	142.8	6.0	31.2	7.0	<0.78	<0.78	<0.78	<0.78
Dibutyl aniline[a]	Kao	Replicate 1	AOO	25–100	P	–	10.7	1.3	1.1	5.9	–	–	–	44.1
		Replicate 2	AOO	25–100	P	–	34.4	1.3	1.3	23.0	64.8	–	–	42.0
		Replicate 3	AOO	25–100	P	–	8.4	1.1	1.1	8.9	–	–	–	33.7
	KOSÉ	Replicate 1	AOO	25–100	P	–	18.8	1.6	1.3	22.5	86.9	–	–	**23.8**
		Replicate 2	AOO	12.5–100	P	–	5.4	1.2	1.3	8.4	–	–	–	39.6
		Replicate 3	AOO	25–100	P	–	30.2	1.3	1.0	14.3	73.9	–	–	57.3
	FDSC	Replicate 1	AOO	25–100	P	–	7.1	0.8	1.1	7.4	–	–	–	65.0
		Replicate 2	AOO	25–100	P	–	38.8	0.9	1.4	45.6	43.1	–	–	25.2
		Replicate 3	AOO	25–100	P	–	13.7	0.7	1.7	11.3	–	–	–	38.1
Cetrimide	Kao	Replicate 1	50% EtOH	0.20–1.56	N	1.43	7.5	1.2	1.2	2.5	–	–	–	–
		Replicate 2	50% EtOH	0.20–1.56	N	1.03	8.3	1.1	1.3	2.2	–	–	–	–
		Replicate 3	50% EtOH	0.20–1.56	N	0.91	6.6	1.1	1.4	2.3	–	–	–	–
	KOSÉ	Replicate 1	50% EtOH	0.20–1.56	N	0.81	2.7	1.4	1.1	3.0	–	–	–	–
		Replicate 2	50% EtOH	0.20–1.56	N	0.87	5.8	0.8	1.0	2.8	–	–	–	–
		Replicate 3	50% EtOH	0.20–1.56	N	1.04	5.0	0.9	0.8	3.0	–	–	–	–
	FDSC	Replicate 1	50% EtOH	0.20–1.56	N	0.99	2.5	0.6	0.7	1.8	–	–	–	–
		Replicate 2	50% EtOH	0.20–1.56	N	0.85	7.8	0.8	1.4	2.9	–	–	–	–
		Replicate 3	50% EtOH	0.20–1.56	N	1.02	1.2	0.8	0.6	1.6	–	–	–	–

AOO, acetone/olive oil; ATF3, activating transcription factor 3; DNAJB4, DnaJ homolog, subfamily B, member 4; DW, distilled water; EtOH, ethanol; GCLM, glutamate–cysteine ligase, modifier subunit; IL, interleukin; N, negative; P, positive.

Three coded test chemicals were transferred and tested in triplicate in each of the three laboratories (Kao, KOSÉ and FDSC). Vehicles, the dose ranges of main study, judgment, IC_{20}, I_{max} and EC values are shown. In addition, the I_{max} values that exceeded the respective cut-off values are indicated by gray highlighting. When the I_{max} of at least one of the four marker genes exceeded the respective cut-off value, the chemical was judged as "P" rather than "N". EC values were calculated using linear interpolation (indicated as fine letters) or linear extrapolation (indicated as bold letters; only when the fold induction at the lowest concentration was smaller than twice the respective cut-off value).

[a]Lipophilic chemicals with log K_{ow} ≥3.5.

clotrimazole at KOSÉ and FDSC, and for *IL-8* with BADGE or lactic acid at KOSÉ. However, almost all fold inductions of each marker gene for each test chemical fell within the ranges of historical data. In addition, as with marker gene expression, almost all cell viability results fell within ranges of historical data. These results suggested that technical transfer to the two naive laboratories was successful.

3.2 | Within-laboratory reproducibility phase

For the WLR phase, three coded chemicals (metol, dibutyl aniline and cetrimide) were tested in three independent experiments at each of the three laboratories. Table 3 shows selected vehicles, dose ranges of the main study, judgment, IC_{20}, I_{max} and EC values obtained via EpiSensA. For each chemical in each laboratory, the vehicles selected were concordant among three experiments. However, although the highest tested concentrations of all tested chemicals were the same among the three experiments performed in each of the laboratories, the lowest concentrations were not always the same. Regarding WLR, in each of the laboratories, concordant predictions (positive/negative) in each of the three independent experiments (replicates 1–3) were obtained for the respective chemicals, indicating a WLR of 100%.

For metol, the dose-responses of fold induction and cell viability among three experiments were similar in all of the laboratories (Figure 1). For all experiments, FDSC reported cell viabilities of less than 80% when testing metol at the highest and second highest concentrations. All four marker genes were consistently positive in each of the three experiments performed at both Kao and KOSÉ, whereas only three of the four (*ATF3*, *GCLM* and *DNAJB4*) were consistently positive in the three experiments performed at FDSC. For dibutyl aniline, the IC_{20} values could not be calculated at any of the laboratories, as the viabilities in all experiments exceeded 80%. Moreover, *IL-8* was consistently positive in each of the three experiments performed in each of the three laboratories, whereas *GCLM* and *DNAJB4* were consistently negative. For cetrimide, comparable IC_{20} values were obtained in all experiments performed in each of the three laboratories. In addition, cetrimide consistently yielded negative results for all four marker genes in all three experiments performed in each of the laboratories.

For dibutyl aniline, it was possible to calculate EC values for *IL-8* induction in all three experiments performed in each of the laboratories. The corresponding EC values obtained in each laboratory were generally of the same magnitude, indicating good reproducibility. The EC values for metol could not be calculated due to the high levels of induction observed with *ATF3*, *GCLM* and *DNAJB4*, and a lack of dose–response for all four marker genes.

3.3 | Between-laboratory reproducibility phase

BLR was assessed using the results for three test chemicals in the third experiment in the WLR phase along with results for an additional seven test chemicals. Table 4 shows selected vehicles, the dose ranges of main study, judgment, IC_{20}, I_{max} and EC values obtained via EpiSensA. Regarding the BLR in relation to prediction, nine of the 10 chemicals were consistently classified as positive or negative by all three laboratories, indicating a BLR of 90%. The chemical that was not consistently classified by the laboratories was glycerol. Kao and KOSÉ reported positive results for glycerol, but the marker genes

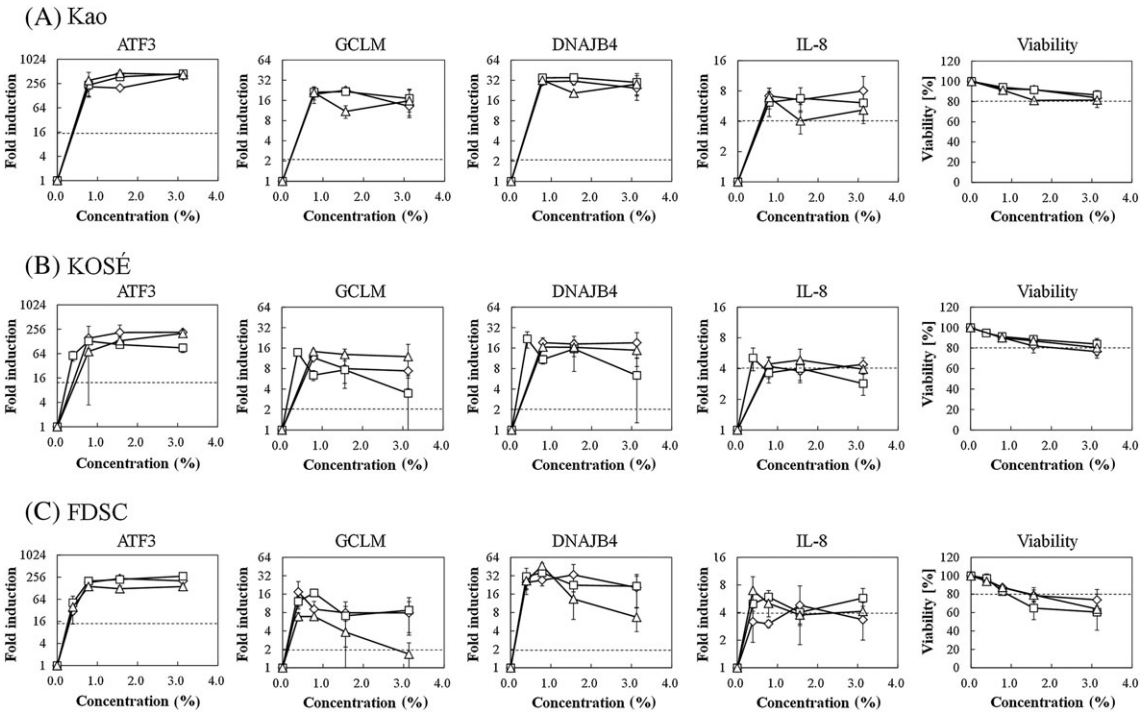

FIGURE 1 Fold-induction of four marker genes and cell viability for metol in within-laboratory reproducibility phase. First to third replicates are indicated as open diamonds, open squares and open triangles, respectively, as performed at Kao (A), KOSÉ (B) and FDSC (C). Values are presented as mean ± SD (n = 3). Dashed lines indicate the cut-off values of the marker genes. ATF3, activating transcription factor 3; DNAJB4, DnaJ homolog, subfamily B, member 4; GCLM, glutamate–cysteine ligase, modifier subunit; IL, interleukin

TABLE 4 BLR analysis based on marker gene inductions exceeding the respective cut-off values, fold-changes and EC values in each of the three laboratories

Chemicals	Laboratory	Vehicle	Dose range (%)	Judgment	IC_{20} (%)	I_{max} (cut-off value) ATF3 (15-fold)	GCLM (2-fold)	DNAJB4 (2-fold)	IL-8 (4-fold)	EC value (%) ATF3	GCLM	DNAJB4	IL-8
Metol	Kao	DW	0.78–3.13	P	>3.13	460.5	20.9	32.1	6.9	<0.78	<0.78	<0.78	<0.78
	KOSÉ	DW	0.78–3.13	P	>3.13	202.1	14.3	16.6	4.8	<0.78	<0.78	<0.78	<0.78
	FDSC	DW	0.39–3.13	P	1.48	142.8	6.9	46.7	7.0	<0.39	<0.39	<0.39	<0.39
Dibutyl aniline[a]	Kao	AOO	25–100	P	–	8.4	1.1	1.1	8.9	–	–	–	33.7
	KOSÉ	AOO	25–100	P	–	30.2	1.3	1.0	14.3	73.9	–	–	57.3
	FDSC	AOO	25–100	P	–	13.7	0.5	1.0	11.3	–	–	–	38.1
Cetrimide	Kao	50% EtOH	0.20–1.56	N	0.91	6.6	1.1	1.4	2.3	–	–	–	–
	KOSÉ	50% EtOH	0.20–1.56	N	1.04	5.0	0.9	0.8	3.0	–	–	–	–
	FDSC	50% EtOH	0.20–1.56	N	1.02	1.2	0.8	0.6	1.6	–	–	–	–
2,4-Dinitrochlorobenzene	Kao	AOO	0.10–0.78	P	0.23	145.2	7.3	19.4	5.1	<0.024	<0.024	<0.024	0.15
	KOSÉ	AOO	0.02–0.20	P	0.17	70.0	5.8	8.3	3.8	0.028	<0.024	**0.021**	–
	FDSC	AOO	0.02–0.20	P	0.11	136.1	14.1	26.9	2.7	0.038	**0.024**	**0.022**	–
2-Mercaptobenzothiazole	Kao	AOO	0.20–1.56	P	1.12	233.0	2.4	2.0	79.2	0.36	<0.20	0.20	0.39
	KOSÉ	AOO	0.10–0.78	P	>0.78	254.2	1.9	2.2	144.9	0.18	–	0.12	0.14
	FDSC	AOO	0.39–3.13	P	1.91	427.4	1.6	15.3	175.7	<0.39	–	<0.39	<0.39
Undec-10-enal[a]	Kao	AOO	0.39–6.25	P	3.02	214.0	2.8	3.5	81.9	0.59	0.48	**0.32**	0.44
	KOSÉ	AOO	0.78–6.25	P	5.45	259.3	1.8	4.3	220.0	0.90	–	**0.37**	**0.78**
	FDSC	AOO	3.13–25	P	7.74	648.8	1.4	24.4	238.0	<3.13	–	<3.13	<3.13
Lilial[a]	Kao	AOO	0.39–6.25	P	2.08	23.2	1.6	2.6	13.1	1.24	–	<0.39	0.85
	KOSÉ	AOO	0.78–3.13	P	3.01	23.8	1.2	1.5	19.9	1.23	–	–	0.80
	FDSC	AOO	0.39–3.13	P	1.63	54.3	1.7	4.6	30.5	0.93	–	**0.31**	0.80
Imidazolidinyl urea	Kao	DW	12.5–50	P	>50	76.4	1.0	1.8	6.3	<12.5	–	–	<12.5
	KOSÉ	DW	6.25–25	P	>25	26.8	1.0	1.5	5.4	7.75	–	–	8.13
	FDSC	DW	12.5–50	P	>50	22.8	1.1	3.1	8.0	<12.5	–	<12.5	<12.5
Benzyl butyl phthalate[a]	Kao	AOO	25–100	N	–	2.6	1.0	1.4	2.2	–	–	–	–
	KOSÉ	AOO	25–100	N	–	1.3	1.0	1.6	2.0	–	–	–	–
	FDSC	AOO	25–100	N	–	0.4	0.5	1.2	0.8	–	–	–	–
Glycerol	Kao	DW	25–100	P	–	26.5	0.8	1.1	3.4	67.1	–	–	–
	KOSÉ	DW	12.5–50	P	>50	13.8	0.8	1.0	4.7	–	–	–	34.1
	FDSC	DW	25–100	N	–	9.6	0.5	1.0	3.5	–	–	–	–

AOO, acetone/olive oil; ATF3, activating transcription factor 3; BLR, between-laboratory reproducibility; DNAJB4, DnaJ homolog, subfamily B, member 4; DW, distilled water; EtOH, ethanol; GCLM, glutamate–cysteine ligase, modifier subunit; IL, interleukin; N, negative; P, positive.

Seven coded test chemicals were transferred and tested in the three laboratories (Kao, KOSÉ and FDSC). BLR was assessed using the results for replicate three of the assays performed on the three chemicals tested in the within-laboratory reproducibility phase, along with assays performed on an additional seven test chemicals. Vehicles, the dose ranges of main study, judgment, IC_{20}, I_{max} and EC values are shown. In addition, the I_{max} values that exceeded the respective cut-off values are indicated by gray highlighting. When the I_{max} of at least one of the four marker genes exceeded the respective cut-off value, the chemical was judged as "P" rather than "N". EC values were calculated using linear interpolation (indicated as fine letters) or linear extrapolation (indicated as bold letters; only when the fold induction at the lowest concentration was smaller than twice the respective cut-off value).

[a]Lipophilic chemicals with log K_{ow} ≥3.5.

classified as positive in these two laboratories were not consistent, with Kao detecting induction of *ATF3* and KOSÉ detecting induction of *IL-8*. Notably, four lipophilic chemicals (dibutyl aniline, undec-10-enal, lilial and benzyl butyl phthalate) yielded concordant results at the three laboratories.

For some chemicals (in particular, metol and 2-mercaptobenzothiazole), an IC_{20} value was not obtained in all laboratories. On the other hand, dibutyl aniline, imidazolidinyl urea, benzyl butyl phthalate and glycerol were not toxic even at the highest tested concentrations. The respective chemicals yielded IC_{20} values of similar magnitudes when tested at each of the three laboratories, with the exception of some chemicals that did not decrease cell viability to less than 80%.

Table 4 shows that some chemicals provided inconsistent inductions of marker genes among the three laboratories, with examples including the induction of: *IL-8* by 2,4-dinitrochlorobenzene (DNCB); *GCLM* by undec-10-enal; and *DNAJB4* by lilial. The dose–responses of marker genes and cell viability for these three chemicals (DNCB, undec-10-enal and lilial) are provided in Figure 2. For DNCB (Figure 2A), the fold inductions of *ATF3* and *IL-8* yielded similar dose–response curves in each of the three laboratories when DNCB were tested at low cytotoxic concentrations (with viability ≥80%). Likewise, undec-10-enal (Figure 2B) and lilial (Figure 2C) also yielded similar (among the three laboratories, for the respective chemicals) dose–response curves for fold induction and cell viability, even though the tested concentrations were not consistent. For some chemicals, it was possible to calculate EC values in all laboratories, including values for induction of *IL-8* by dibutyl aniline, and those for induction of *ATF3* and *IL-8* by lilial. The EC values obtained for the respective chemicals in all three laboratories were of similar magnitude, indicating good BLR.

3.4 | Analysis of positive controls

The positive controls, 6.25% BADGE and 3.13% clotrimazole, were tested a total of six times in the WLR and BLR phases in each laboratory. For the WLR and BLR assays, all fold inductions for each marker gene in all laboratories are presented in Figure 3 as box plots. As shown using open diamonds in Figure 3(A), 6.25% BADGE yielded inductions exceeding the respective cut-off values for *GCLM*, *DNAJB4* and *IL-8* in all experiments (six independent experiments in each laboratory) and exceeding the cut-off value for *ATF3* in all experiments except for one (i.e., in 17 of 18 experiments across three laboratories). That exception consisted of an experiment (performed at FDSC) in which low fold induction (4.6-fold) of *ATF3* was observed in one tissue, resulting in a low mean value (11-fold) when averaged across three tissues. In contrast, 3.13% clotrimazole yielded inductions exceeding the respective cut-off values for *ATF3*, *DNAJB4* and *IL-8* in all experiments, but exceeding the cut-off value for *GCLM* in two of 18 experiments across the three laboratories (Figure 3B). As indicated by the "whiskers" shown in Figure 3, the ranges of fold induction roughly overlapped for all four marker genes among the three testing laboratories. These results suggested that 6.25% BADGE provided more consistent positive expression of all marker genes in each of the three laboratories than did 3.13% clotrimazole.

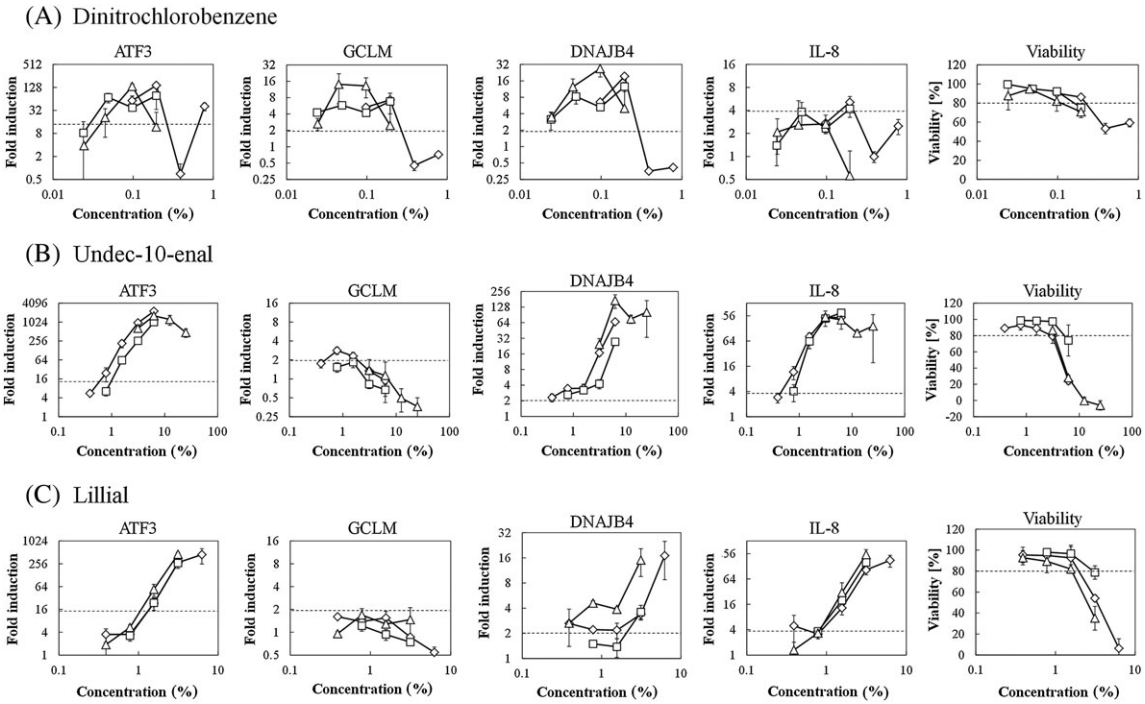

FIGURE 2 Fold induction of four marker genes and cell viability for three chemicals in between-laboratory reproducibility phase. Results obtained at Kao, KOSÉ and FDSC are indicated as open diamonds, open squares and open triangles, respectively, for 2,4-dinitrochlorobenzene (A), undec-10-enal (B) and lilial (C). Values are presented as mean ± SD (n = 3). Dashed lines indicate the cut-off values of the marker genes. ATF3, activating transcription factor 3; DNAJB4, DnaJ homolog, subfamily B, member 4; GCLM, glutamate–cysteine ligase, modifier subunit; IL, interleukin

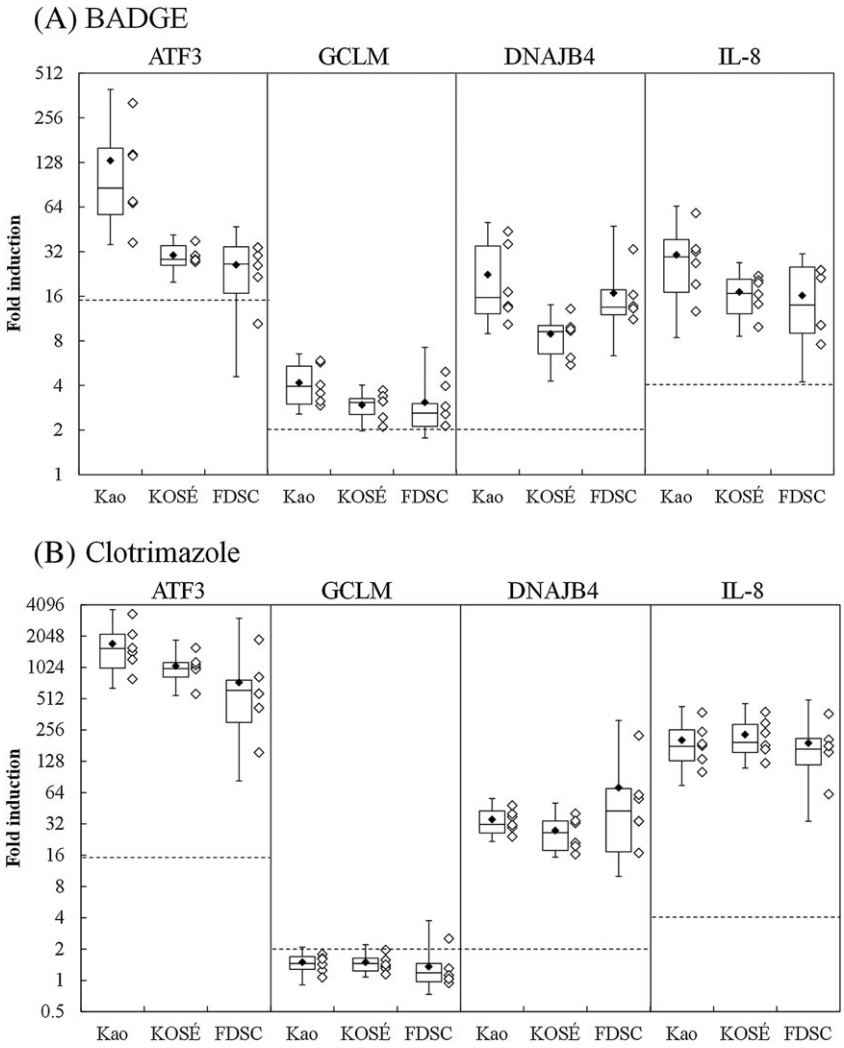

FIGURE 3 Reproducibility in fold induction of positive controls among the three laboratories. All obtained data for positive controls (6.25% BADGE in A and 3.13% clotrimazole in B) were plotted as box plots for each marker gene. These positive controls were tested in six experiments in each of the three laboratories. Open diamonds indicate the averages across three tissues in each experiment. Horizontal bars indicate median values; upper and lower values of the boxes indicate the upper and lower quartiles (respectively) of the values; whiskers indicate the maximal and minimal fold inductions; and the closed diamonds indicate the mean values. Dashed lines indicate the cut-off values of the marker genes. ATF3, activating transcription factor 3; DNAJB4, DnaJ homolog, subfamily B, member 4; GCLM, glutamate–cysteine ligase, modifier subunit; IL, interleukin

4 | DISCUSSIONS

4.1 | Transferability and reproducibility

The transferability and reproducibility of EpiSensA were assessed from a ring study performed at three laboratories. The transferability was evaluated by testing three non-coded test chemicals at prescribed concentrations. This phase provided correct classifications of two sensitizers and one non-sensitizer at the two naive laboratories. Although each laboratory used different real-time PCR systems, the EpiSensA protocol was successfully transferred to the two naive laboratories, suggesting that the difference in real-time PCR systems does not affect the prediction ability of EpiSensA. For the WLR phase, 100% concordant results (positive or negative) were obtained in each laboratory for the three coded chemicals. For the BLR phase, nine of 10 coded chemicals (including four lipophilic chemicals) were classified consistently by the three laboratories, indicating a BLR of 90%.

EpiSensA requires a specific range of test concentrations for each chemical to identify correctly the sensitizing potential. The determination of test concentration is based on the IC_{20} calculated in the dose-finding study. Notably, the dose ranges were highly consistent within and between laboratories, despite the use of distinct lots of the RhE model in the three laboratories. This observation suggested that the procedure for dose-range determination described in the EpiSensA protocol is highly reproducible when performed in different laboratories.

According to the validation study report of h-CLAT in four laboratories using 15 test chemicals (EC EURL ECVAM, 2012), the WLR in three independent experiments was 73.3% to 86.7% for each laboratory. The BLR comparing all four laboratories was 79.2% for 24 chemicals. Moreover, in a ring study of SENS-IS performed in three laboratories using 19 test chemicals, the WLR in three independent experiments was 100% at each of the three laboratories, and the BLR comparing the three laboratories was 100% (Cottrez et al., 2016). Compared with the results obtained in the present work, EpiSensA has sufficient WLR and BLR.

Potency prediction is important for the Globally Harmonized System classification and it was widely reported that LLNA EC3 showed a quantitative potency index for skin sensitizers (Api, Basketter, & Lalko, 2015; Basketter et al., 2000; Schneider & Akkan, 2004). EpiSensA permits the classification of skin sensitization potency into two categories (extreme/strong or moderate/weak based on LLNA EC3) by using EC values of multiple marker genes (Saito et al., 2017). Although this ring study was not designed to obtain EC values correctly, the corresponding EC values obtained in each laboratory

were generally of the same magnitude within and between laboratories, indicating good reproducibility.

4.2 | False-positive result for glycerol

In this study, glycerol yielded false-positive results with *ATF3* at the lead laboratory and with *IL-8* at one of the naive laboratories. Glycerol previously has been shown to upregulate *ATF3* and *IL-8* gene expression at levels approaching the respective cut-off values (yielding I_{max} values of 12.3-fold for *ATF3* and 3.1-fold for *IL-8*) when tested at high concentrations, but this chemical was nonetheless predicted as negative (Saito et al., 2017). Likewise, when tested in EpiSensA, propylene glycol yielded increased expression of *ATF3* (with an I_{max} value of 13.7-fold) but was predicted as negative (Saito et al., 2017). Considering the hydrophilicity of these polyols, high osmotic stress may occur under conditions of high-concentration exposure (Thornit, Vinten, Sander, Lund-Andersen, & la Cour, 2010). Notably, osmotic stress has been reported to induce phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α) (Bevilacqua et al., 2010), and phosphorylation of eIF2α has been associated with increased expression of *ATF3* (Cai & Brooks, 2011). Therefore, non-specific expression of *ATF3* may occur with exposure to hydrophilic and low-cytotoxicity chemicals such as polyols. As gene expression of *ATF3* and *IL-8* could be partly upregulated through a shared signal pathway (Saito et al., 2017), the expression of *IL-8* also might be induced by polyols through a mechanism unrelated to skin sensitization. Further evaluation will be necessary to determine whether chemicals such as polyols fall outside the applicability domain of EpiSensA.

4.3 | Positive control

Whereas the induction of all four marker genes by BADGE met the positive criteria in 17 of the 18 experiments, the induction by clotrimazole met the positive criteria for all four markers in only two of the 18 experiments, while it consistently induced the three markers ATF3, DNAJB4 and IL-8 in all 18 experiments. These results suggested that 6.25% BADGE is a more robust positive control for confirmation that the experiment is being performed correctly and that acceptable data have been obtained.

5 | CONCLUSION AND FUTURE PLANS

This investigation was an initial, small-scale study to assess the transferability and reproducibility of the EpiSensA test method across three laboratories using 10 coded test chemicals. The EpiSensA protocol was found to be easily transferable. As confirmed by the results obtained by testing three chemicals in three independent experiments in each laboratory, the WLR was 100%. Testing of 10 chemicals in the three laboratories revealed a BLR of 90%. Together, these results suggested that the EpiSensA test method has very good transferability and reproducibility. This high reproducibility of EpiSensA encourages us to proceed with a larger-scale official validation study.

CONFLICT OF INTEREST

The authors did not report any conflict of interest.

ORCID

Hideyuki Mizumachi http://orcid.org/0000-0001-8778-1951

REFERENCES

Api, A. M., Basketter, D., & Lalko, J. (2015). Correlation between experimental human and murine skin sensitization induction thresholds. *Cutaneous and Ocular Toxicology*, *34*, 298–302. https://doi.org/10.3109/15569527.2014.979425

Aptula, A. O., Roberts, D. W., & Pease, C. K. (2007). Haptens, prohaptens and prehaptens, or electrophiles and proelectrophiles. *Contact Dermatitis*, *56*, 54–56. https://doi.org/10.1111/j.1600-0536.2007.00944.x

Basketter, D. A., Balikie, L., Dearman, R. J., Kimber, I., Ryan, C. A., Gerberick, G. F., … Rycroft, R. J. (2000). Use of the local lymph node assay for the estimation of relative contact allergenic potency. *Contact Dermatitis*, *42*, 344–348. https://doi.org/10.1034/j.1600-0536.2000.042006344.x

Bevilacqua, E., Wang, X., Majumder, M., Gaccioli, F., Yuan, C. L., Wang, C., … Hatzoglou, M. (2010). eIF2alpha phosphorylation tips the balance to apoptosis during osmotic stress. *The Journal of Biological Chemistry*, *285*, 17098–17111. https://doi.org/10.1074/jbc.M110.109439

Cai, Q., & Brooks, H. L. (2011). Phosphorylation of eIF2α via the general control kinase, GCN2, modulates the ability of renal medullary cells to survive high urea stress. *American Journal of Physiology. Renal Physiology*, *301*, F1202–F1207. https://doi.org/10.1152/ajprenal.00272.2011

Casati, S., Aeby, P., Kimber, I., Maxwell, G., Ovigne, J. M., Roggen, E., … Basketter, D. (2009). Selection of chemicals for the development and evaluation of *in vitro* methods for skin sensitisation testing. *Alternatives to Laboratory Animals*, *37*, 305–312. https://doi.org/10.1016/j.toxlet.2007.05.255

Cottrez, F., Boitel, E., Ourlin, J. C., Peiffer, J. L., Fabre, I., Henaoui, I. S., … Groux, H. (2016). SENS-IS, a 3D reconstituted epidermis based model for quantifying chemical sensitization potency: Reproducibility and predictivity results from an inter-laboratory study. *Toxicology In Vitro*, *32*, 248–260. https://doi.org/10.1016/j.tiv.2016.01.007

EC EURL ECVAM. (2012). human Cell Line Activation Test (h-CLAT) Validation Study Report. Accessible at: https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-recommendations

EU (2009). Regulation (EC) No. 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. *Official Journal of European Union, L*, *342*, 59–209.

Gerberick, G. F., House, R. V., Fletcher, E. R., & Ryan, C. A. (1992). Examination of the local lymph node assay for use in contact sensitization risk assessment. *Fundamental and Applied Toxicology*, *19*, 438–445. https://doi.org/10.1016/0272-0590(92)90183-I

Gerberick, G. F., Ryan, C. A., Kern, P. S., Schlatter, H., Dearman, R. J., Kinber, I., … Basketter, D. A. (2005). Compilation of historical local lymph node data for evaluation of skin sensitization alternative methods. *Dermatitis*, *16*, 157–202. https://doi.org/10.2310/6620.2005.05040

Kern, P. S., Gerberick, G. F., Ryan, C. A., Kimber, I., Aptula, A., & Basketter, D. A. (2010). Local lymph node data for the evaluation of skin sensitization alternatives: a second compilation. *Dermatitis*, *21*, 8–32. https://doi.org/10.2310/6620.2009.09038

Kimber, I., Hilton, J., Dearman, R. J., Gerberick, G. F., Ryan, C. A., Basketter, D. A., … Hastings, K. L. (1998). Assessment of the skin sensitization potential of topical medicaments using the local lymph node assay: an interlaboratory evaluation. *Journal of Toxicology and Environmental Health, Part A*, *53*, 567–579. https://doi.org/10.1080/009841098159141

McKim, J. M. Jr., Keller, D. J. 3rd, & Gorski, J. R. (2012). An in vitro method for detecting chemical sensitization using human reconstructed skin models and its applicability to cosmetic, pharmaceutical, and medical device safety testing. *Cutaneous and Ocular Toxicology*, *31*, 292–305. https://doi.org/10.3109/15569527.2012.667031

OECD. (1992). Guideline for Testing of Chemicals No. 406: Skin Sensitization, adopted 17 July.

OECD. (2010). Guideline for Testing of Chemicals No. 429: Skin Sensitization: Local Lymph Node Assay, adopted 22 July.

OECD. (2012). The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins. Part 1: Scientific Evidence. Series on Testing and Assessment No. 168. ENV/JM/MONO(2012)10/PART1.

OECD. (2015a). Guideline for Testing of Chemicals No. 442C: In Chemico Skin Sensitisation: direct peptide reactivity assay (DPRA), adopted 5 February 2015.

OECD. (2015b). Guideline for Testing of Chemicals No. 442D: In Vitro Skin Sensitisation: ARE-Nrf2 Luciferase Test Method, adopted 5 February 2015.

OECD. (2016). Guideline for Testing of Chemicals No. 442E: In Vitro Skin Sensitisation: human Cell Line Activation Test (h-CLAT), adopted 29 July 2016.

Oesch, F., Fabian, E., Guth, K., & Landsiedel, R. (2014). Xenobiotic-metabolizing enzymes in the skin of rat, mouse, pig, guinea pig, man, and in human skin models. *Archives of Toxicology*, *88*, 2135–2190. https://doi.org/10.1007/s00204-014-1382-8

Peiser, M., Tralau, T., Heidler, J., Api, A. M., Arts, J. H., Basketter, D. A., … Luch, A. (2010). Allergic contact dermatitis: epidemiology, molecular mechanisms, in vitro methods and regulatory aspects. Current knowledge assembled at an international workshop at BfR, Germany. *Cellular and Molecular Life Sciences*, *69*, 763–781. https://doi.org/10.1007/s00018-011-0846-8

Reisinger, K., Hoffmann, S., Alépée, N., Ashikaga, T., Barroso, J., Elcombe, C., … Maxwell, G. (2015). Systematic evaluation of non-animal test methods for skin sensitisation safety assessment. *Toxicology In Vitro*, *29*, 259–270. https://doi.org/10.1016/j.tiv.2014.10.018

Saito, K., Takenouchi, O., Nukada, Y., Miyazawa, M., & Sakaguchi, H. (2017). An *in vitro* skin sensitization assay termed EpiSensA for broad sets of chemicals including lipophilic chemicals and pre/pro-haptens. *Toxicology In Vitro*, *40*, 11–25. https://doi.org/10.1016/j.tiv.2016.12.005

Schneider, K., & Akkan, Z. (2004). Quantitative relationship between the local lymph node assay and human skin sensitization assays. *Regulatory Toxicology and Pharmacology*, *39*, 245–255. https://doi.org/10.1016/j.yrtph.2004.02.002

Thornit, D. N., Vinten, C. M., Sander, B., Lund-Andersen, H., & la Cour, M. (2010). Blood-retinal barrier glycerol permeability in diabetic macular edema and healthy eyes: estimations from macular volume changes after peroral glycerol. *Investigative Ophthalmology & Visual Science*, *51*, 2827–2834. https://doi.org/10.1167/iovs.09-4172

Tokudome, Y., Katayanagi, M., & Hashimoto, F. (2015). Esterase activity and intracellular localization in reconstructed human epidermal cultured skin models. *Annals of Dermatology*, *27*, 269–274. https://doi.org/10.5021/ad.2015.27.3.269

Urbisch, D., Mehling, A., Guth, K., Ramirez, T., Honarvar, N., Kolle, S., … Sakaguchi, H. (2015). Assessing skin sensitization hazard in mice and men using non-animal test methods. *Regulatory Toxicology and Pharmacology*, *71*, 337–351. https://doi.org/10.1016/j.yrtph.2014.12.008

How to cite this article: Mizumachi H, Sakuma M, Ikezumi M, et al. Transferability and within- and between-laboratory reproducibilities of EpiSensA for predicting skin sensitization potential in vitro: A ring study in three laboratories. *J Appl Toxicol*. 2018;38:1233–1243. https://doi.org/10.1002/jat.3634