



Intra- and inter-laboratory reproducibility and predictivity of the HaCaSens assay: A skin sensitization test using human keratinocytes, HaCaT

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ABSTRACT

Due to considerable constraints in using animals for risk assessment, much effort has been directed at developing non-animal test methods. Developing assays for skin sensitization, the leading cause of contact dermatitis, is particularly important, but there are currently no *in vitro* skin sensitization tests that completely replace animal tests.

HaCaSens, a simple skin sensitization test using non-transformed HaCaT cells, predicts keratinocyte activation by skin sensitizers with 75% sensitivity, 83% specificity and 77% accuracy in a previous study using 22 coded substances. Although the data show promising results, the number of tested substances is insufficient to prove predictive capacity. Moreover, reproducibility among different laboratories has not been studied.

Here, three laboratories participated in a validation in order to assess HaCaSens feasibility for official validation. To examine transferability, intra- and inter-lab reproducibility and predictive capacity, HaCaSens was assessed on a set of 30 test substances coordinated by the Validation Management Team (VMT). The results showed satisfactory transferability as well as intra- and inter-laboratory reproducibility. Further assessment of its predictive capacity on 20 test substances demonstrated a sensitivity of 81.8% (18/22), specificity of 87.5% (7/8), and accuracy of 83.3% (25/30) in identifying skin sensitizers, which is comparable with presently validated assays, KeratinoSens™ and LuSens.

This validation study shows that the HaCaSens assay is easily transferable, reproducible and highly predictable for identifying skin sensitizers.

1. Introduction

Following contact with the skin, allergens, in particular skin sensitizers, induce immune responses leading to allergic contact dermatitis, one of the most common skin diseases. Every manufactured commercial product must therefore be evaluated for their skin sensitizing potential. However, European Union (EU) regulations prohibit testing using animals (EU, 2006, 2008, 2009). Following the EU's lead, Japan and China have started to restrict animal testing for manufactured products, while the production, marketing and sales of cosmetic products tested using animals are completely banned in Korea (Ministry of Food and Drug

Safety, 2017). Although the mouse local lymph node assay (LLNA; OECD TG 429, 2010; 442A, 2010, 442B, 2010) and the guinea pig maximization test (GPMT; OECD TG 406, 1992) have been widely used as alternatives to animal testing, there is an urgent need for developing a cell-based assay for skin sensitization since both assays can't be full replacement in that they also use animals for testing.

Skin sensitization is a complex process induced by interactions between various cells, so it is not easy to fully replicate every associated mechanism in a simplified *in vitro* system. According to the adverse outcome pathway (AOP) for skin sensitization described by OECD (OECD, 2012, part 1; OECD, 2012, part 2), skin sensitization is

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Table 1
Test substances used in inter-laboratory validation of the HaCaSens assay.

No.	Substance	Chemical information			Supplier	Physical state	Purity (%)	LLNA data		Human data	
		Molecular weight (g/mol)	CAS No. ^a					LLNA ^b	Literature	Human	Literature
PC ^c	2,4-Dinitrochlorobenzene (DNCB)	202.55	97-00-7		Sigma-Aldrich	Solid	97	+	OECD TG 429 (2010), TG 442C (2015), TG 442D (2015), TG 442E (2016), ChemSens DB	+	OECD TG 429 (2010), ChemSens DB
NC	Lactic acid	90.08	50-21-5		Sigma-Aldrich	Liquid	≥ 85	–	OECD TG 429 (2010), TG 442C (2015), TG 442D (2015), TG 442E (2016), ChemSens DB	–	OECD TG 429 (2010), ChemSens DB
1	Isopropanol	60.10	67-63-0		Sigma-Aldrich	Liquid	99.5	–	OECD TG 429 (2010), TG 442D (2015), TG 442E (2016), ChemSens DB	–/+ ^d	OECD TG 429 (2010), ChemSens DB
2	Hexyl cinnamic aldehyde	216.32	101-86-0		Sigma-Aldrich	Liquid	85	+	OECD TG 429 (2010), ChemSens DB	+	OECD TG 429 (2010), ChemSens DB
3	Salicylic acid	138.12	69-72-7		Sigma-Aldrich	Solid	> 99	–	OECD TG 429 (2010), TG 442D (2015), ChemSens DB	–	OECD TG 429 (2010), ChemSens DB
4	2-Mercapto benzothiazole	167.25	149-30-4		Sigma-Aldrich	Solid	97	+	OECD TG 429 (2010), TG 442D (2015), TG 442E (2016), ChemSens DB	+	OECD TG 429 (2010), ChemSens DB
5	Formaldehyde	30.03	50-00-0		Sigma-Aldrich	Liquid	36.5–38	+	OECD TG 442C (2015), ChemSens DB	+	ChemSens DB
6	4-Hydroxybenzoic acid	138.12	99-96-7		Sigma-Aldrich	Solid	≥ 99	–	ChemSens DB	–	ChemSens DB
7	Citral	152.23	5392-40-5		Sigma-Aldrich	Liquid	95	+	OECD TG 429 (2010), ChemSens DB	+	OECD TG 429 (2010), ChemSens DB
8	Phenyl benzoate	198.22	93-99-2		TCI	Solid	> 99	+	OECD TG 429 (2010), ChemSens DB	+	OECD TG 429 (2010), ChemSens DB
9	Chlorobenzene	112.56	108-90-7		Sigma-Aldrich	Liquid	99.9	–	OECD TG 429 (2010), ChemSens DB	–	OECD TG 429 (2010), ChemSens DB
10	4-Phenylenediamine	108.14	106-50-3		Sigma-Aldrich	Solid	98	+	OECD TG 429 (2010), TG 442E (2016), ChemSens DB	+	OECD TG 429 (2010), ChemSens DB
11	Methyl salicylate	152.15	119-36-8		TCI	Liquid	> 99	–	OECD TG 429 (2010), ChemSens DB	–	OECD TG 429 (2010), ChemSens DB
12	2,3-Butanedione	86.09	431-03-8		Sigma-Aldrich	Liquid	97	+	OECD TG 442C (2015), ChemSens DB	n.a.	ChemSens DB
13	Isoeugenol	164.20	97-54-1		TCI	Liquid	> 97	+	OECD TG 429 (2010), ChemSens DB	+	OECD TG 429 (2010), ChemSens DB
14	Glutaraldehyde	100.12	111-30-8		Alfa Aesar	Liquid	50	+	ChemSens DB	+	ChemSens DB
15	Cobalt chloride	129.84	7646-79-9		Sigma-Aldrich	Solid	> 98	+	OECD TG 429 (2010), ChemSens DB	+	OECD TG 429 (2010), ChemSens DB
16	Tetramethylthiuram disulfide	240.43	137-26-8		Sigma-Aldrich	Solid	97	+	ChemSens DB	+	ChemSens DB
17	Cinnamic alcohol	134.18	104-54-1		TCI	Solid	> 97	+	OECD TG 429 (2010), TG 442D (2015), ChemSens DB	+	OECD TG 429 (2010), ChemSens DB
18	Vanillin	152.15	121-33-5		Sigma-Aldrich	Solid	99	–	ChemSens DB	+ ^e	ChemSens DB
19	Benzocaine	165.19	94-09-7		Sigma-Aldrich	Solid	–	+	ChemSens DB	+	ChemSens DB
20	Nickel sulfate	262.85	10101-97-0		Sigma-Aldrich	Solid	99	+	OECD TG 442E (2016), ChemSens DB	+	ChemSens DB
21	Cinnamaldehyde	132.16	104-55-2		Sigma-Aldrich	Liquid	> 95	+	ChemSens DB	+	ChemSens DB
22	Eugenol	164.20	97-53-0		TCI	Liquid	99	+	OECD TG 429 (2010), ChemSens DB	+	OECD TG 429 (2010), ChemSens DB
23	Glycerol	92.09	56-81-5		Sigma-Aldrich	Liquid	99	–	OECD TG 442D (2015), TG 442E (2016), ChemSens DB	–	ChemSens DB
24	Methyl methacrylate	100.12	80-62-6		Sigma-Aldrich	Liquid	99	+	OECD TG 429 (2010), ChemSens DB	+	OECD TG 429 (2010), ChemSens DB
25	Ethylene glycol dimethacrylate	198.22	97-90-5		Sigma-Aldrich	Liquid	98	+	OECD TG 429 (2010), TG 442D (2015), ChemSens DB	+	OECD TG 429 (2010), ChemSens DB
26	CMI/MI (Kathon TM CG Preservative)	–	26172-55-4/ 2682-20-4		Dow Chemical Group Company	Liquid	–	+	OECD TG 429 (2010), ChemSens DB	+	OECD TG 429 (2010), ChemSens DB
27	Methyldibromo glutaronitrile	265.94	35691-65-7		Alfa Aesar	Solid	99	+	OECD TG 442D (2015), ChemSens DB	+	ChemSens DB
28	Benzylidenacetone	146.19	122-57-6		TCI	Solid	> 98	+	OECD TG 442C (2015), ChemSens DB	n.a.	ChemSens DB
29	Imidazolidinyl urea	388.29	39236-46-9		Sigma-Aldrich	Solid	–	+	OECD TG 429 (2010), TG 442E (2016), ChemSens DB	+	OECD TG 429 (2010), ChemSens DB

(continued on next page)

Table 1 (continued)

No.	Substance	Chemical information			LLNA data		Human data	
		Molecular weight (g/mol)	CAS No ^a	Supplier	Physical state	Purity (%)	LLNA ^b Literature	Human Literature
30	4-Aminobenzoic acid	1347.14	150-13-0	TCI	Solid	> 99	–	OECD TG 442E (2016), ChemSens DB n.a.

^a CAS No (Chemical Abstracts Service Number).^b Testing results using the LLNA (local lymph node assay) and data taken from OECD Test Guideline (TG 429). +; sensitizer, –; non-sensitizer.^c PC (Positive Control), NC (Negative Control).^d Presumed to be a sensitizer in OECD TG 429, a non-sensitizer based on literature from Bauch et al. (2011) and Ramirez et al. (2016) and classified into human class 5, a weak sensitizer based on the literature from Basketter et al. (2014) and Cottrez et al. (2016).^e Classified into human class 5 based on the literature from Basketter et al. (2014) and Cottrez et al. (2016).

comprised of a series of key events: covalent binding to protein (key event 1), activation of keratinocytes (key event 2) and dendritic cells (key event 3), and T-cells proliferation (key event 4) (Maxwell et al., 2014). A few *in vitro* skin sensitization tests have been developed to address each key event of the AOP, such as the direct peptide reactivity assay (DPRA, OECD TG 442C, 2015), KeratinoSens™ (OECD TG 442D, 2015), LuSens, hCLAT (OECD TG 442E, 2016) and U-SENS (Ashikaga et al., 2006; Emter et al., 2010; Gerberick et al., 2004; Piroird et al., 2015; Ramirez et al., 2014; Sakaguchi et al., 2006).

Keratinocytes are crucial in skin sensitization; they are the first cells to come into contact with chemicals applied to the skin and they produce a number of inflammatory cytokines in response to sensitizers to initiate further immune reactions (Haas et al., 1992). Thus, as the initial step and catalyst for skin sensitization, keratinocyte activation (key event 2) is an attractive target for developing new *in vitro* assays. Among the *in vitro* assays simulating keratinocyte activation, KeratinoSens™ was accepted as an OECD Test Guideline (TG 442D, 2015), and LuSens, which is similar to KeratinoSens™, is undergoing validation study for acceptance as an OECD Test Guideline. These assays assess the skin sensitization potential of a substance by measuring Nrf2/ARE pathway activation in the reporter keratinocyte cell line, HaCaT (Natsch, 2010; Ramirez et al., 2014). However, they showed some limitations in validation studies (Andreas et al., 2011; Ramirez et al., 2016). As these assays identify a sensitizer by measuring the luminescence signal of a specific pathway, sensitizers that interfere with the luminescence signal or activate a pathway other than Nrf2/ARE can result in false negatives.

The HaCaSens assay, a skin sensitization test using non-transformed HaCaT cell line, identifies skin sensitizers by measuring inflammatory cytokine secretion, thereby reducing the possibility of false negatives compared to KeratinoSens™ and LuSens. Previously, we evaluated the predictive capacity of the HaCaSens assay in a coded fashion for 16 sensitizers and 6 non-sensitizers listed in the OECD TG 429 (2010) performed standard (PS). This showed 75% sensitivity, 83% specificity and 77% accuracy, comparable to the results of existing skin sensitization assays (Jung et al., 2016). However along with high predictivity, the assay should be transferable to other laboratories and reproducible in order to be internationally accepted as a test guideline.

Herein, the validation study of the HaCaSens assay is described. The assessment of transferability, within-, between-lab reproducibility and predictive capacity of the HaCaSens was conducted in three laboratories using 22 sensitizers and 8 non-sensitizers. We evaluated the ability of the HaCaSens to detect skin sensitizing potential by assessing transferability and reproducibility among three laboratories and further compare the results of HaCaSens with those of other *in vitro* assay using HaCaT cells, KeratinoSens™ and LuSens.

2. Materials and methods

2.1. Study plan

The chemical selection, coding and distribution of the coded substances in the study were managed by a validation management team (VMT). Three laboratories participated in this validation study of the HaCaSens assay: Seoul National University (Lead laboratory, Lead lab), Seoul National University Hospital (Laboratory 1, Lab1), and Catholic University of Daegu (Laboratory 2, Lab2). Lead lab and Lab2 had previously conducted experiments establishing assay, but this was the first use of the assay by Lab1.

This study was divided into two phases. Phase I assessed the transferability of the assay with 4 test substances, and Phase II assessed the reproducibility and predictivity with 6 and 20 test substances, respectively. In the case of predictivity, to reduce the workload, 8 substances are tested in each laboratory, 2 of which were common between two laboratories, adding to a total 20 substances.

2.2. Test substances

Table 1 describes the 30 test substances used in this study and their properties: molecular weight, CAS number, supplier, physical state, purity, sensitization potential as determined by the LLNA and human data, and respective literature reference. The test substances used in this study are as follows: 2,4-Dinitrochlorobenzene (DNCB), Lactic acid, Isopropanol, Hexyl cinnamic aldehyde, Salicylic acid, 2-Mercapto-benzothiazole, Formaldehyde, 4-Hydroxybenzoic acid, Citral, Chlorobenzene, 4-Phenylenediamine, 2,3-Butanedione, Cobalt chloride, Tetramethylthiuram disulfide, Vanillin, Benzocaine, Nickel sulfate, Glycerol, Methyl methacrylate, Ethylene glycerol dimethacrylate and Imidazolidinyl urea were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phenyl benzoate, Methyl salicylate, Isoeugenol, Cinnamic alcohol, Eugenol, Benzylideneacetone and 4-Aminobenzoic acid were purchased from TCI (Tokyo Chemical Industry, Tokyo, Japan). Glutaraldehyde, Methylbromo glutaronitrile were purchased from Alfa Aesar (Ward Hill, MA, USA) and CMI/MI (Kathon™ CG Preservative) was from Dow Chemical Group Company (Horgen, Switzerland). All test substances used in Phase I and Phase II were acquired, aliquoted and shipped by the VMT.

2.3. Cell culture

The human keratinocyte cell line, HaCaT, was provided by the German Cancer Research Center (Heidelberg, Germany). HaCaT cells were cultured in Dulbecco's modified Eagle medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Life Technologies), 1% penicillin-streptomycin (Gibco, Life Technologies), and 1% non-essential amino acids (Gibco, Life Technologies). Cells were cultured at 37 °C in a humidified incubator containing 5% CO₂. For *in vitro* experiments, chemicals were dissolved in dimethyl sulfoxide (DMSO; the maximum concentration of DMSO in the culture medium was 0.1%) and phosphate-buffered saline (PBS).

2.4. HaCaSens assay

The HaCaSens assay consists of two steps: determination of non-toxic concentration for treatment to cells and calculation of stimulation index (SI) by measuring secretion of IL-1 α and IL-6 in test substance-treated cells.

2.4.1. Determination of lethal concentrations causing death in 25% (LC25)

To determine the concentration of chemical for treatment, lethal concentration causing death in 25% (LC25) was assessed using cell counting kit-8 (CCK-8) (Dojindo, Kumamoto, Japan). Highly water-soluble tetrazolium salt is reduced by dehydrogenase activities in viable cells to give a yellow-color formazan dye. Then, the amount of the formazan dye, generated by the activities of dehydrogenases, indicates the cell viability. According to the manufacturer's protocol, HaCaT cells were seeded in a 96-well plate at a concentration of 1.2×10^5 cells/ml (100 μ l total volume/well) and incubated for 24 h, whereupon the medium was replaced with fresh medium containing specific concentrations of test chemicals (1000, 500, 100, 10, 1 μ g/ml) or vehicle control. At 24 h after incubation, 10 μ l of CCK-8 solution was added to each well and cells were incubated for another 4 h at 37 °C according to reagent instructions. The O.D. value at 450 nm was measured using a VICTOR™ X3 microplate reader (Perkin-Elmer, Waltham, MA, USA). The LC25 was calculated for each chemical by five-parameter non-linear regression analysis of data.

2.4.2. Cytokine measurements

To measure the extracellular production of IL-1 α and IL-6, cells were seeded in 24-well plates at a concentration of 1.5×10^5 cells/ml (500 μ l total volume/well). At 24 h post incubation, the culture medium was removed and cells were treated with 0.01, 0.1, 0.5, and

$1 \times$ LC25 concentration of each chemical or vehicle control. After 24 h of incubation, extracellular cytokines produced by cells were measured from supernatants using a DuoSet ELISA kit (R & D Systems, Minneapolis, MN, USA) for IL-6 (DY206, sensitivity: 0.11 pg/ml) and IL-1 α (DY200, sensitivity: 1 pg/ml). The protein content of the cell lysates was measured using the BCA protein assay method (Thermo Scientific, Rockford, IL, USA). The stimulation index (SI) was calculated as follows: SI = cytokine produced in chemical-treated cells/cytokine produced in vehicle control.

2.5. Acceptance criteria for each experiment

All the labs performed experiments in three repetitions consisting of three replicates and sent these data to the VMT. Data quality was assessed by the VMT for whether SI values for the positive control (2,4-Dinitrochlorobenzene, DNCB) were equal to or greater than 3 and those for the negative control (Lactic acid) were below 3 in all three repetitions. If not, data from the experiments were not taken for evaluation.

2.6. Prediction model and statistical evaluation

Three independent experiments (triplicates for each experiment) were performed for 30 test substances for all concentrations (0.01, 0.1, 0.5, and $1 \times$ LC25). For each chemical tested, its sensitizing potential was assessed based on the SI value of interleukin-1 alpha (IL-1 α) and interleukin-6 (IL-6) at each concentration. The following prediction model was applied: 1) a test substance was considered as sensitizer if the highest SI value among all concentrations was greater than or equal to 3 in at least one cytokine. Non-sensitizers produced the SI value under 3 in both cytokines at all concentrations. 2) The final classification of a test substance was determined by the result of at least two concordant repetitions in three valid independent repetitions. This was also applied to triplicate for each independent repetition.

Finally, to assess the predictive capacity of the assay, the sensitivity, specificity, accuracy, positive predictivity and negative predictivity were calculated as described previously (Ramirez et al., 2016).

3. Results

3.1. Phase I. Transferability phase

In Phase I, the HaCaSens assay method was transferred from one laboratory to another participating in this study with a standard operating protocol (SOP) and face-to-face training by the Lead lab. The phase I study was comprised of a training test and proficiency test to assess transferability of the HaCaSens assay. Once the method was transferred, each laboratory performed three repetitive tests with coded chemicals. The training test was conducted with 2 test substances encoded and provided by the Lead lab to each laboratory (data not shown). Only laboratories showing 100% accuracy in the training test could proceed to the proficiency test with 8 test substances including positive control (2,4-Dinitrochlorobenzene, DNCB) and negative control (lactic acid). Unlike the training test, the proficiency test was managed by the VMT, from chemical selection to coding and provision to the three laboratories for final evaluation of transferability. After three successive repetitions, all data were sent to VMT where they evaluated the success of the test according to two acceptance criteria. 1) Correct prediction of the positive control and negative control. If the test results did not meet these criteria, they were considered to be invalid. 2) All test substances should be correctly predicted to proceed further.

All laboratories (Lead lab, Lab1, and Lab2) identified DNCB and lactic acid as positive and negative, respectively. The LC25 and final prediction of each test substance are summarized in Table 2. The positive test substances, hexyl cinnamic aldehyde (HCA) and 2-mercaptobenzothiazole, were positive in all 3 laboratories and the 2 negative

Table 2

Summary of all repetitions performed by each testing laboratory in phase I. Concentrations in $\mu\text{g/ml}$ which reduce cell viability by 75% (LC25) of HaCaT cells and predictions of the three independent experiments with 4 blind-coded test substances (PC: Positive Control; NC: Negative Control; +: positive/sensitizer prediction; -: negative/non-sensitizer prediction).

Test substance		HaCaSens			In vivo prediction			
		LC25 ($\mu\text{g/ml}$) ^a			Exp1	Exp2	Exp3	
Lead lab	DNCB	4 $\pm$ 3.5	10 $\pm$ 6.3	8 $\pm$ 2.8	+	+	+	+
	Lactic acid	> 1000	> 1000	> 1000	–	–	–	–
	Isopropanol	> 1000	> 1000	> 1000	–	–	–	–
	Hexyl cinnamic aldehyde	15 $\pm$ 8.1	52 $\pm$ 12.7	40 $\pm$ 22.5	+	+	+	+
	Salicylic acid	290 $\pm$ 12.3	400 $\pm$ 32.1	360 $\pm$ 55.9	–	–	–	–
Lab1	2-Mercaptobenzothiazole	8 $\pm$ 5.2	4 $\pm$ 3.6	7 $\pm$ 5.7	+	+	+	+
	DNCB	11 $\pm$ 7.6	12 $\pm$ 5.1	6 $\pm$ 3.2	+	+	+	+
	Lactic acid	> 1000	> 1000	> 1000	–	–	–	–
	Isopropanol	> 1000	> 1000	> 1000	–	–	–	–
	Hexyl cinnamic aldehyde	82 $\pm$ 20.1	90 $\pm$ 3.2	98 $\pm$ 25.4	+	–	+	+
Lab2	Salicylic acid	347 $\pm$ 40.2	285 $\pm$ 38.7	783 $\pm$ 70.7	–	–	–	–
	2-Mercaptobenzothiazole	> 1000	> 1000	739 $\pm$ 187.9	+	+	+	+
	DNCB	1 $\pm$ 0.9	1 $\pm$ 1.0	1 $\pm$ 0.7	+	+	+	+
	Lactic acid	> 1000	> 1000	> 1000	–	–	–	–
	Isopropanol	> 1000	> 1000	> 1000	–	–	+	–
	Hexyl cinnamic aldehyde	82 $\pm$ 40.5	39 $\pm$ 27.3	58 $\pm$ 38.9	+	+	+	+
	Salicylic acid	111 $\pm$ 65.8	190 $\pm$ 90.3	76 $\pm$ 12.6	–	–	–	–
	2-Mercaptobenzothiazole	28 $\pm$ 25.8	149 $\pm$ 100.2	36 $\pm$ 9.1	+	+	+	+

^a Results from three replicates per each independent experiment were expressed as means $\pm$ SEM ($\mu\text{g/ml}$) of the concentration of chemical resulting in 25% cell toxicity.

test substances, salicylic acid and isopropanol, were negative in all labs. The results from Phase I showed that all participating labs had 100% accuracy in 3 valid repetitions in both the training test as well as the proficiency test.

3.2. Phase II. Within-/Between-laboratory reproducibility (WLR/BLR)

The assessment of WLR was based on the concordance of predictions of three independent experiments by each participating laboratory. The three laboratories classified six chemicals in three independent experiments and percentages of valid repetition were calculated: 100% (6/6), 100% (6/6) and 83.3% (5/6) for the Lead lab, Lab1 and Lab2, respectively (Table 3).

For the assessment of BLR, the same assay protocol and experimental conditions were maintained as in WLR. Of the six substances tested, phenyl benzoate showed the most inconsistent results among the three laboratories, but was concordant (6/6, 100%) between Lead lab and Lab2 (Table 3). There was a reproducibility rate of more than 80%, suggesting that this assay is considered reproducible across experiments. Upon obtaining these results, the VMT agreed that these participating laboratories (Lead lab, Lab1, and Lab2) were ready to proceed to predictive capacity studies.

3.3. Predictive capacity

The predictive capacity of the HaCaSens assay was determined using a total of 30 test substances, including 22 sensitizers and 8 non-sensitizers. The same number of substances (18 substances/lab) was

assessed by each of the 3 participating laboratory. Discordant substances among the 3 laboratories were ultimately classified based on results of the 2 concordant laboratories. In the case of substances performed by 2 laboratories, results from both must be concordant with *in vivo* prediction.

The predictions for all the 30 test substances are summarized in Table 4, and the sensitivities, specificities and the accuracies are calculated (Table 5). Lead lab was able to identify 83.3% (10/12) sensitizers as true positives, while the Lab1 and Lab2 correctly identified 75% (9/12) and 100% (12/12) respectively. 2,3-Butanedione (Lab1) and benzocaine (Lead lab) were incorrectly predicted and metallic sensitizers such as cobalt chloride (Lab1) and nickel sulfate (Lead lab) were also identified as false negatives. Concerning the true negative substances, Lead lab was able to correctly identify 83.3% (5/6), Lab1 was 83.3% (5/6), and Lab2 was 100% (6/6). Of the non-sensitizers tested in this study, methyl salicylate was not properly identified in Lead lab and Lab1.

The accuracy of the assay was 83.3% (15/18), 77.8% (14/18) and 100% (18/18) for Lead lab, Lab1 and Lab2, respectively. The overall accuracy was 83.3% (25/30) with sensitivity between 75 and 100% and specificity between 80 and 100% (Table 5). Such a high sensitivity, specificity and accuracy suggest that the HaCaSens assay is a reliable *in vitro* skin sensitization test, making it a good screening tool for the identification of skin sensitizers.

When compared with human data, most of the predictions were similar to previous results evaluated on the basis of LLNA data (Supplementary Table 1). For the same set of 26 test substances excluding not applicable (2,3-Butanedione, Benzylideneacetone, 4-

Table 3

Predictions of the 6 blind-coded test substances in three independent experiments for within-/between-laboratory reproducibility assessment of three laboratories (+: positive/sensitizer prediction; -: negative/non-sensitizer prediction)

Test substances		Lead			Lab1			Lab2		
		Exp1	Exp2	Exp3	Exp1	Exp2	Exp3	Exp1	Exp2	Exp3
Sensitizer	Formaldehyde	+	+	+	+	+	+	+	+	+
	4-Phenylenediamine	+	+	+	+	+	+	+	+	+
	Citral	+	+	+	+	+	+	+	+	+
	Phenyl benzoate	+	+	+	–	–	–	+	+	+
Non-sensitizer	Chlorobenzene	–	–	–	–	–	–	–	–	+
	4-Hydroxybenzoic acid	–	–	–	–	–	–	–	–	–

Table 4

The predictive capacity of the 30 blind-coded test substances in three laboratories. In cases with three independent experiments, the mode is reported (S/+ : positive/skin sensitizer prediction (grey shading); NS/– : negative/non-sensitizer prediction; nt: not tested).

Test substances	Laboratory									<i>In vitro</i> data	<i>In vivo</i> data
	Lead			Lab 1			Lab 2				
	IL–6	IL–1α	S vs NS	IL–6	IL–1α	S vs NS	IL–6	IL–1α	S vs NS		
Isopropanol	NS(3/3)	NS(3/3)	NS(3/3)	NS(3/3)	NS(3/3)	NS(3/3)	NS(3/3)	NS(3/3)	NS(3/3)	NS	NS
Hexyl cinnamic aldehyde	S(3/3)	S(3/3)	S(3/3)	S(2/3)	S(2/3)	S(2/3)	S(3/3)	S(3/3)	S(3/3)	S	S
Salicylic acid	NS(3/3)	NS(3/3)	NS(3/3)	NS(3/3)	NS(3/3)	NS(3/3)	NS(3/3)	NS(3/3)	NS(3/3)	NS	NS
2–Mercapto benzothiazole	S(3/3)	S(3/3)	S(3/3)	S(3/3)	S(3/3)	S(3/3)	S(3/3)	S(3/3)	S(3/3)	S	S
Formaldehyde	S(3/3)	NS(3/3)	S(3/3)	S(3/3)	NS(2/3)	S(3/3)	NS(3/3)	S(3/3)	S(3/3)	S	S
4–Hydroxybenzoic acid	NS(3/3)	NS(3/3)	NS(3/3)	NS(3/3)	NS(3/3)	NS(3/3)	NS(3/3)	NS(3/3)	NS(3/3)	NS	NS
Citral	S(Z/3)	S(3/3)	S(3/3)	S(2/3)	S(2/3)	S(2/3)	S(2/3)	S(3/3)	S(3/3)	S	S
Phenyl benzoate	S(3/3)	S(2/3)	S(3/3)	NS(3/3)	NS(3/3)	NS(3/3)	S(2/3)	S(3/3)	S(3/3)	S	S
Chlorobenzene	NS(3/3)	NS(3/3)	NS(3/3)	NS(3/3)	NS(3/3)	NS(3/3)	NS(2/3)	NS(2/3)	NS(2/3)	NS	NS
4–Phenylenediamine	S(2/3)	S(2/3)	S(3/3)	S(2/3)	S(3/3)	S(3/3)	S(3/3)	S(2/3)	S(3/3)	S	S
Methyl salicylate	S(3/3)	NS(3/3)	S (3/3)	S(3/3)	S(2/3)	S (3/3)	nt	nt	–	S	NS
2,3–Butanedione	nt	nt	–	NS(3/3)	NS(3/3)	NS(3/3)	nt	nt	–	NS	S
Isoeugenol	nt	nt	–	S(2/3)	NS(2/3)	S(2/3)	nt	nt	–	S	S
Glutaldehyde	S(3/3)	S(2/3)	S(3/3)	S(2/3)	S(2/3)	S (3/3)	nt	nt	–	S	S
Cobalt chloride	nt	nt	–	NS(3/3)	NS(2/3)	NS(2/3)	nt	nt	–	NS	S
Tetramethylthiuram disulfide	nt	nt	–	S(2/3)	S(2/3)	S(3/3)	nt	nt	–	S	S
Cinnamic alcohol	nt	nt	–	S(3/3)	NS(3/3)	S(3/3)	nt	nt	–	S	S
Vanillin	nt	nt	–	NS(3/3)	NS(3/3)	NS(3/3)	nt	nt	–	NS	NS
Benzocaine	NS(3/3)	NS(3/3)	NS(3/3)	nt	nt	–	nt	nt	–	NS	S
Nickel sulfate	NS(2/3)	NS(2/3)	NS(2/3)	nt	nt	–	nt	nt	–	NS	S
Cinnamaldehyde	S(3/3)	S(2/3)	S(3/3)	nt	nt	–	nt	nt	–	S	S
Eugenol	S(3/3)	NS(3/3)	S(3/3)	nt	nt	–	nt	nt	–	S	S
Glycerol	nt	nt	–	nt	nt	–	NS(3/3)	NS(3/3)	NS(3/3)	NS	NS
Methyl methacrylate	nt	nt	–	nt	nt	–	NS(3/3)	S(3/3)	S(3/3)	S	S
Ethylene glycol dimethacrylate	nt	nt	–	nt	nt	–	NS(3/3)	S(3/3)	S(3/3)	S	S
CMI/MI (Kathon™ CG Preservative)	nt	nt	–	nt	nt	–	NS(3/3)	S(3/3)	S(3/3)	S	S
Methyldibromo glutaronitrile	S(2/3)	NS(2/3)	S(2/3)	nt	nt	–	S(2/3)	S(3/3)	S(3/3)	S	S
Benzylideneacetone	nt	nt	–	nt	nt	–	NS(3/3)	S(3/3)	S(3/3)	S	S
Immidazolidinyl urea	nt	nt	–	nt	nt	–	S(2/3)	S(3/3)	S(3/3)	S	S
4–Aminobenzoic acid	NS(3/3)	NS(3/3)	NS(3/3)	nt	nt	–	NS(3/3)	NS(2/3)	NS(2/3)	NS	NS

Table 5
Accuracy of HaCaSens for 30 test substances.

	Laboratory			Overall accuracy (%)
	Lead	Lab1	Lab2	
N	18	18	18	30
Sensitivity (%)	83.3 (10/12)	75.0 (9/12)	100.0 (12/12)	81.8 (18/22)
Specificity (%)	83.3 (5/6)	83.3 (5/6)	100.0 (6/6)	87.5 (7/8)
Positive predictive value (%)	90.9 (10/11)	90.0 (9/10)	100.0 (12/12)	94.7 (18/19)
Negative predictive value (%)	71.4 (5/7)	62.5 (5/8)	100.0 (6/6)	63.6 (7/11)
Accuracy (%)	83.3 (15/18)	77.8 (14/18)	100.0 (18/18)	83.3 (25/30)

Table 6
Comparison of predictivity among HaCaSens, LuSens and KeratinoSens™ for a set of 14 test substances (+: positive/sensitizer prediction; -: negative/non-sensitizer prediction; n.a.: not applicable)

Test substance	LLNA reference results		HaCaSens	LuSens ^a	KeratinoSens™ ^a
	+	LLNA vs potency			
Isopropanol	–	n.a.	–	–	–
2-Mercapto benzothiazole	+	Moderate	+	+	+
Citral	+	Moderate	+	+	+
Phenyl benzoate	+	Weak	+	–	–
Chlorobenzene	–	n.a.	–	–	–
Methyl salicylate	–	n.a.	+	+	–
Lactic acid	–	n.a.	–	–	–
Isoeugenol	+	Moderate	+	+	+
Eugenol	+	Weak	+	+	–
Glycerol	–	n.a.	–	–	–
DNCB	+	Extreme	+	+	+
Ethylene glycol dimethacrylate	+	Weak	+	+	+
Salicylic acid	–	n.a.	–	–	–
Methyldibromoglutaronitrile	+	Strong	+	+	+
Total number			14	14	14
Sensitivity (%)			100.0 (8/8)	87.5 (7/8)	75.0 (6/8)
Specificity (%)			83.3 (5/6)	83.3 (5/6)	100.0 (6/6)
Accuracy (%)			92.9 (13/14)	85.7 (12/14)	85.7 (12/14)

^a Data from Andreas et al. (2011) and Ramirez et al. (2016).

Aminobenzoic acid) or equivocally predicted chemicals (isopropanol), the HaCaSens acquired 81% sensitivity, 80% specificity and 80.8% accuracy against human data.

3.4. Comparison with KeratinoSens™ and LuSens

For the same set of 8 sensitizers and 6 non-sensitizers, the results of HaCaSens showed higher accuracy (92.9%) than those of LuSens (85.7%) and KeratinoSens™ (85.7%) as shown in Table 6 (Andreas et al., 2011; Ramirez et al., 2016). HaCaSens correctly predicted 13 of the 14 test substances and wrongly predicted 1 substance (methyl salicylate). LuSens had 87.5% (7/8) sensitivity and 83.3% specificity (5/6). That is, LuSens led to one false positive prediction (methyl salicylate) and one false negative prediction (phenylbenzoate). KeratinoSens™ had 75% (6/8) sensitivity and 100% (6/6) specificity and led to two false negative (phenylbenzoate and eugenol) (Table 6). Clearly,

HaCaSens has superior accuracy than the other assays, with 13 correct predictions out of 14 test substances. Moreover, our results showed that HaCaSens correctly predicted all sensitizers (weak, moderate, strong or extreme) and only over predicted one non-sensitizer, methyl salicylate.

4. Discussion

Along with developing new *in vitro* skin sensitization assays, transferability and reproducibility should be demonstrated for OECD acceptance as a new method. To this end, we performed a validation study consisting of two steps, phase I and phase II, of HaCaSens under VMT oversight.

In phase I, the transferability of the HaCaSens assay was evaluated using four concentrations based on LC25. The results from Phase I showed that all participating labs had 100% accuracy in 3 valid repetitions in the training test as well as the proficiency test (Table 2). These indicate the assay is easily transferable to other laboratories. In phase II, three laboratories (Lead lab, Lab1, and Lab2) performed the assay to assess within-/between-laboratory reproducibility and predictive capacity (Tables 3 and 4) and obtained satisfactory results.

Although the overall data from all three laboratories correctly predicted sensitizers with SI greater than 3, reproducibility appeared to be limited when taking into account high deviation of SI among laboratories. In particular, LC25 of test substances varied due to different cell conditions and differences in manually making test solutions from chemical stock, especially poorly soluble substances. These factors could lead to misclassification and thus poor reproducibility.

We also assessed predictive capacity and as shown in Table 5, the HaCaSens assay attained an overall good sensitivity (81.8%), specificity (87.5%) and accuracy (83.3%), with one false positive (methyl salicylate) and four false negatives (benzocaine, 2,3-butanedione, nickel sulfate and cobalt chloride). Regarding the chemicals predicted incorrectly as non-sensitizer, we can discuss as follows for the 4 chemicals, nickel sulfate, cobalt chloride, benzocaine and 2,3-butanedione. As previously reported, sensitizers containing a metal cation such as nickel sulfate and cobalt chloride, were misclassified due to their lower cell membrane permeability (Jung et al., 2016). For benzocaine, it was reported to have equivocal results in prediction using LLNA (Cho et al., 2014; Idehara et al., 2008; Natsch et al., 2013; Suzuki et al., 2009). Finally, the reason for the FN prediction of 2,3-butanedione in HaCaSens was the very low LC25 value of the chemical measured in Lab1. Considering IC50 for the chemical in the study of Natsch and coworkers (859.9 µM), the LC25 value in Lab 1 (9 µg/ml) was too low to sensitize keratinocytes to produce inflammatory cytokines (Natsch et al., 2013). Also, it is possible that two chemicals (benzocaine, 2,3-butanedione) are borderline chemicals which have very weak sensitizing potentials and prone to misclassification in terms of cytokine production.

Methyl salicylate, incorrectly predicted as a sensitizer by both Lead lab and Lab1, is considered a non-sensitizer according to LLNA. However, there is some evidence of sensitizing potential in humans according to report from The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, 2010), so misclassification of the chemical may be partly due to human origin of cells used in HaCaSens. This discrepancy of skin sensitizing potential between mouse and human base assays is worth noting since classification of most sensitizers is currently based on the data from test using animals like LLNA and GPMT and therefore could be inaccurate when applied to humans.

When assessing the predictive capacity of HaCaSens on a set of 30 test substances, it obtained values above 80% in all aspects (sensitivity, specificity and accuracy). Besides, it is important to note that most of the sensitizers tested in this study, in particular highly hazardous strong sensitizers, were correctly identified, while a few borderline sensitizers and non-sensitizers were misclassified. This indicates that the assay may be a good screening method for identifying skin sensitizing potential. The assay showed satisfactory results against human data as

well (Supplementary Table 1). Although the most of the predictions were same as those against LLNA data in terms of sensitivity, specificity and accuracy, there was one more false negative, vanillin. It is considered as a non-sensitizer in LLNA data and sensitizer in human data. However, according to literature from Basketter et al., 2014 and Cottrez et al., 2016, it is specifically classified into group “human class 5” containing substances that have a very low intrinsic ability to cause skin sensitization. With the previously reported data from LLNA as well as human, we can conclude that vanillin exhibits very low capacity in sensitizing the skin enough and thus fail to stimulate the HaCaT cells to produce cytokines significantly (Basketter et al., 2001).

Moreover, when compared with the same set of test substances tested in KeratinoSens™ and LuSens previously, the HaCaSens was shown to have better accuracy of 92.9% (13/14) with one false positive, methyl salicylate (Table 6) (Andreas et al., 2011; Ramirez et al., 2016). As mentioned above, methyl salicylate yielded different prediction as a sensitizer in human (ICCVAM, 2010; Emter et al., 2010) and was also misclassified in LuSens (Ramirez et al., 2016). Thus, misclassification of methyl salicylate is not specific to the HaCaSens assay, but appears to be inherently associated with the chemical properties of this compound.

In the present validation study, we show that the HaCaSens assay is easily transferable to other laboratories and reproducible within-/between laboratories. Moreover, the HaCaSens assay acquired satisfactory accuracy, sensitivity and specificity that are comparable to currently used *in vitro* assays using HaCaT cells. Taken together, HaCaSens is a promising *in vitro* skin sensitization assay in that the assay uses a non-genetically transformed cell line that is similar to normal keratinocytes (van der Veen et al., 2015) and utilizes a common laboratory method, ELISA, for measuring cytokine production. In addition, further comparing the results of HaCaSens with those of other similar assays (KeratinoSens™ and LuSens) suggests that unlike these assays targeting specific pathway for sensitization, HaCaSens can be a more broadly applicable assay. As one of the examples, the prediction of phenyl benzoate using each assay is very interesting. In fact, phenyl benzoate was incorrectly predicted as non-sensitizer in KeratinoSens™ and LuSens as the chemical is an acyl transfer agent acting with lysine residues and thus doesn't activate Keap1-Nrf2-ARE pathway (Andreas et al., 2011; Ramirez et al., 2016). That's why HaCaSens which has no specific target for identification of sensitizers could predict sensitizing potential of phenyl benzoate while ARE activating assays tend to misclassify such type of agents. Of course, this assay needs to be further tested with a wider range of chemicals, such as respiratory sensitizers, to more clearly examine advantages and limitations of HaCaSens over other *in vitro* assays addressing same or different key events of skin sensitization such as DPRA and hCLAT. With more extensive validation studies of various kinds of substances and many participating laboratories including international collaborations, the HaCaSens assay can be proposed as an *in vitro* skin sensitization assay to the OECD test guideline.

5. Conclusion

In the present study, skin sensitizing potentials of 30 coded chemicals were assessed by the HaCaSens assay conducted by 3 participating laboratories (Lead lab, Lab1, and Lab2). Results demonstrated satisfactory transferability as well as within-/between-laboratory reproducibility indicating that this method is sufficiently robust and easily transferable to other laboratories. Assessment of the 22 sensitizers and 8 non-sensitizers showed an accuracy of 83.3%, 77.8% and 100% in Lead lab, Lab1 and Lab2, respectively. In addition, when comparing HaCaSens with other assays targeting keratinocyte activation, its accuracy of 92.9% was superior to LuSens and KeratinoSens™ (85.7% for both). Most importantly, the data presented in this study provides reliable support for proposing HaCaSens as an *in vitro* assay comparable with other skin sensitization assays.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tiv.2017.10.018>.

Transparency document

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

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Supplementary Table 1. Comparison of predictivity of the HaCaSens with human data (data from the literature) (+: positive/sensitizer prediction; -: negative/non-sensitizer prediction; n.a.: not applicable; * equivocal prediction in human data)

Test substances	Reference data		HaCaSens vs. Human
	LLNA	Human	
Isopropanol	-	-/+*	-/*
Hexyl cinnamic aldehyde	+	+	+/+
Salicylic acid	-	-	-/-
2-Mercapto benzothiazole	+	+	+/+
Formaldehyde	+	+	+/+
4-Hydroxybenzoic acid	-	-	-/-
Citral	+	+	+/+
Phenyl benzoate	+	+	+/+
Chlorobenzene	-	-	-/-
4-Phenylenediamine	+	+	+/+
Methyl salicylate	-	-	+/-
2,3-Butanedione	+	n.a	
Isoeugenol	+	+	+/+
Glutaldehyde	+	+	+/+
Cobalt chloride	+	+	-/+
Tetramethylthiuram disulfide	+	+	+/+
Cinnamic alcohol	+	+	+/+
Vanillin	-	+	-/+
Benzocaine	+	+	-/+
Nickel sulfate	+	+	-/+

Cinnamaldehyde	+	+	+/+
Eugenol	+	+	+/+
Glycerol	-	-	-/-
Methyl methacrylate	+	+	+/+
Ethylene glycol dimethacrylate	+	+	+/+
CMI/MI (Kathon™ CG Preservative)	+	+	+/+
Methyldibromo glutaronitrile	+	+	+/+
Benzylideneacetone	+	n.a	
Imidazolidinyl urea	+	+	+/+
4-Aminobenzoic acid	-	n.a	

Sensitivity (%)		81.0 (17/21)
Specificity (%)		80.0 (4/5)
Accuracy (%)		80.8 (21/26)