



The Myeloid U937 Skin Sensitization Test (U-SENS) addresses the activation of dendritic cell event in the adverse outcome pathway for skin sensitization

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ABSTRACT

The U-SENS™ assay, formerly known as MUSST (Myeloid U937 Skin Sensitization Test), is an *in vitro* method to assess skin sensitization. Dendritic cell activation following exposure to sensitizers was modelled in the U937 human myeloid cell line by measuring the induction of the expression of CD86 by flow cytometry. The predictive performance of U-SENS™ was assessed via a comprehensive comparison analysis with the available human and LLNA data of 175 substances. U-SENS™ showed 79% specificity, 90% sensitivity and 88% accuracy. A four laboratory ring study demonstrated the transferability, reliability and reproducibility of U-SENS™, with a reproducibility of 95% within laboratories and 79% between-laboratories, showing that the U-SENS™ assay is a promising tool in a skin sensitization risk assessment testing strategy.

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1. Introduction

The assessment of skin sensitization potential is a major component of the safety evaluation of substances and represents a standard requirement of chemical legislation in the European Union (EU) including the Classification Labelling and Packaging (CLP) of substances and mixtures Regulation (EU, 2008a), the REACH Regulation (EU, 2006), and the Cosmetics Directive (EU, 2009).

Abbreviations: ACD, Allergic Contact Dermatitis; BLR, between-laboratory reproducibility; CRO, Contract Research Organisation; DC, dendritic cells; DPRA, Direct Peptide Reactivity Assay; EC3, estimated concentration that produces a stimulation index of 3 in the LLNA; EC150, estimated concentration that produces a stimulation index of 150 in the U-SENS™ assay; EU, European Union; EU CLP, European Union system of classification of chemicals implemented in the EU classification, labelling and packaging regulation; GPMT, Guinea Pig Maximisation Test; h-CLAT, human-Cell Line Activation Test; LLNA, Local Lymph Node Assay; NS, non sensitizer; OECD, Organisation for Economic Co-operation and Development; PBS, phosphate buffer saline; PI, propidium iodide; PM, prediction model; ROC, Receiver Operating Characteristic; REACH, Registration, Evaluation, Authorization and Restriction of Chemicals; S, sensitizer; SI, stimulation index; SOP, Standard Operating Procedure; TG, Test Guideline; UN GHS, United Nations Globally Harmonized System; WLR, within-laboratory reproducibility.

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Currently, regulatory requirements on skin sensitization can only be met by *in vivo* testing. There are four accepted guidelines in the framework of the Organisation for Economic Cooperation and Development (OECD) and the EU Test Methods Regulation (EU, 2008b): the Buehler Test and Guinea Pig Maximisation Test (GPMT), Test Guideline (TG) 406 and EU test method B.6 (OECD, 1992), the Local Lymph Node Assay (LLNA), TG 429 and EU test method B.42 (OECD, 2010a) and its non-radio-isotopic variants, the LLNA-DA and the LLNA-BrdU Elisa, TG 422A and TG 422B respectively (OECD, 2010b, 2010c). However, the 7th amendment of the EU Cosmetic Directive involving the ban of animal testing for cosmetics ingredients enforces the development and since March 2013, the use of alternative non-animal methods to assess skin sensitization.

The key mechanistic events underpinning the skin sensitization process which lead to Allergic Contact Dermatitis (ACD) in human are well understood. They have been described in the OECD report on “The Adverse Outcome Pathway (AOP) for Skin Sensitization Initiated by Covalent Binding to Proteins” (OECD, 2012). These events include (1) binding of chemicals to a skin protein (haptenation), (2) production of danger signals and release of pro-inflammatory mediators in keratinocytes, (3) activation, maturation, mobilization and migration of cutaneous dendritic cells (DC) to proximal lymph node and (4) antigen presentation to naïve

T-cells and proliferation of specific T-cells. Considerable improvements have been made towards the development of alternative non-animal methods, such as Direct Peptide Reactivity Assay (DPRA; Gerberick et al., 2004), KeratinoSens™ (Emter et al., 2010) and human-Cell Line Activation Test (h-CLAT; Ashikaga et al., 2006) which address the key events 1, 2 and 3 of the AOP, respectively.

There is a general agreement within the scientific community which is that one single alternative method would not be sufficient to replace the animal-based methods for skin sensitization (Adler et al., 2011). Information combining complementary *in silico* and *in vitro* methods may need to be integrated for appropriate alternative assessment (Jowsey et al., 2006; Adler et al., 2011). Such strategies should cover different key events to reflect the mechanistic complexity of skin sensitization. A number of strategies have already been proposed, e.g. by Jaworska et al. (2011), Bauch et al. (2012), Gomes et al. (2012) and Nukada et al. (2013).

As illustrated in Gomes et al. (2012), *in vitro* methods such as DPRA and U-SENS™ were combined with others to define the hazard classification of a test chemical. The U-SENS™ test method, formerly known as MUSST (Myeloid U937 Skin Sensitization Test), was developed to address the key event 3 (activation and mobilization of DC). This assay, using human myeloid cells (U937 cell line) was able to mimic the phenotypical changes occurring during DC activation upon exposure to sensitizers. During DC maturation, biomarkers such as CD40, CD54, CD80, CD83, CD86, and HLA-DR surface antigens are up regulated (Aiba et al., 1997; Coutant et al., 1999; Rousset et al., 2000). Among these, CD86 was reported by Ade et al. (2006) to be the most suitable and robust marker in U937 cells. In this study, the modulation of the CD86 membrane marker in U937 cells was measured by flow cytometry after a 45 h exposure to test chemicals (at least 4 concentrations). Other U937/CD86-based assays were also reported (Python et al., 2007; Bauch et al., 2011; Natsch et al., 2013). Python et al. (2007) proposed an assay based on specific culture conditions (i.e. presence of interleukin-4) and on the analysis of at least two markers out of three (CD86, IL1 beta or IL-8) measured at more than one time point. Such conditions differed from the U937/CD86 assay used by Natsch et al. (2013) as well as from the modified MUSST (mMUSST) described by Bauch et al. (2011). The U-SENS™, which was also developed as a U937/CD86-based assay, varied with respect to the cell line origin, the doses selection strategy, the CD86 positive threshold and/or the prediction model developed to categorize the substances. In addition, another assay addressing the key event 3 has been developed such as the THP-1 cells-based h-CLAT assay, currently under EURL-ECVAM validation and which uses a combination of two markers, CD86 and CD54 without any dose–response needed (Sakaguchi et al., 2010).

Initially, the U-SENS™ assay was developed for internal screening purposes, with the prediction model based on expert judgment. The protocol and the prediction model were then optimized and standardised under the name of MUSST. A successful Cosmetics Europe' ring study allowed the assay to enter in 2009 a formal EURL-ECVAM pre-validation study. However, as inconclusive classifications had a major impact on the reproducibility, the prediction model was further improved using a statistical-based approach (Gomes et al., 2014) which led to the classification of all substances into sensitizers or non-sensitizers. The present study describes a well detailed and standardised alternative non-animal testing method for skin sensitization assessment which is currently shared with EURL-ECVAM and others (Reisinger et al., 2015) for its validation and its integration in the development of combined testing approaches. U-SENS™ data were generated on a consequent set of 175 substances for which human and/or

LLNA *in vivo* skin sensitization data were available. The predictive capacity of the U-SENS™ assay was characterized to determine its added value for hazard and risk assessment purposes. Finally, we report a first transferability and reproducibility assessment resulting from a ring-study including four laboratories, each of them testing the same 14 chemicals according to the modular approach (Hartung et al., 2004).

2. Materials and methods

2.1. Cell line

Human myeloid U937 cell line (CRL-1593.2) from the American Type Culture Collection (ATCC, LGC standards, France) was cultured in RPMI-1640 (Gibco) supplemented with 10% inactivated foetal bovine serum (FCS, BioWest), 2 mM L-Glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma–Aldrich). This complete medium is hereafter referred as RPMI.

Cells were cultured in RPMI in a 37 °C, 5% CO₂ and 95% humidity incubator and used for the test seven weeks maximum after thawing. Their viability and growth were monitored (using trypan blue exclusion) at each passage every two or three days (respectively at 3×10^5 cells/mL or 1.5×10^5 cells/mL).

2.2. U-SENS™ assay

2.2.1. Doses, controls, cells treatment and staining

Solubility assessment of each substance was performed in either RPMI or in dimethyl sulphoxide (DMSO; Sigma–Aldrich) up to 50 mg/mL. Fresh stock solutions of the substances were prepared for each run. The concentrations of the stock solution were 0.4 mg/mL in RPMI or 50 mg/mL in DMSO. DMSO stock solutions were diluted in RPMI to 0.4% of DMSO in the final volume of cell suspension in the sample.

A complete experiment consisted of at least two runs. In each run, four to six concentrations were tested. The concentration ranges of the test substances varied between runs. The first run final concentrations were by default 1, 10, 20, 50, 100, and 200 µg/mL. The concentrations of subsequent runs were chosen from 27 pre-defined options in the range of 0.1–200 µg/mL based on the results of previous runs. The concentrations were selected to (a) show and/or confirm dose-dependency of CD86 increase at non-cytotoxic doses, (b) show and/or confirm the absence of CD86 increase up to the maximum non-cytotoxic concentration and (c) confirm cytotoxicity, if any.

The substance or the appropriate vehicle (100 µL/well, 2× concentrated for the substances) were incubated with U937 cells (100 µL/well, 0.5×10^6 cells/mL) in a 96-well plate, covered by a sealing tape to prevent possible collateral effects of volatile chemicals, for 45 h at 37 °C, 5% CO₂ and 95% humidity. Each treatment condition was prepared in duplicates (one sample for IgG1 and one sample for CD86, see below). In each run, both the negative control lactic acid (Sigma–Aldrich) and the positive control picrylsulphonic acid (Sigma–Aldrich) were included at 200 µg/mL and 50 µg/mL, respectively.

After a 45 h incubation, cells were transferred into a V-shaped microtiter plate and centrifuged (200g, 5 min, 4 °C). Supernatants were discarded. The cells were resuspended in PBS with 5% FCS, transferred into another V-shaped microtiter plate containing 5 µL per well of FITC-conjugated monoclonal antibodies mouse anti-human IgG1 (Clone: MOPC-21 from Becton Dickinson Pharmingen) or 5 µL per well of anti-CD86 (Clone: 2331 (FUN-1), 555657 from Becton Dickinson Pharmingen) for a 30 min incubation in the dark. Cells were then centrifuged (200g, 5 min, 4 °C), washed twice in 100 µL PBS with 5% FCS and once in 100 µL PBS

to be finally resuspended in PBS. During staining and washing steps, all buffers and cells were kept on ice or at 4 °C.

Eight substances could be tested in one run, and two runs could be performed per week. Each substance was evaluated in replicates: two to five runs were necessary to come to a final conclusion.

2.2.2. Flow cytometry data analysis and evaluation

The fluorescence intensities of U937 cell surface markers were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, FACSCanto II, Becton Dickinson or Beckman Navios, Beckman Coulter) using a plate auto-sampler or manually tube by tube. Immediately before cytometry acquisition, a propidium iodide (PI) solution was added to each sample for cell viability determination (final PI concentration 3 µg/mL). Cells were displayed within a size and granularity dot plot set to log scale. A first gate R1 was placed around the cell population and 10,000 events were acquired within this gate. Cells from the R1 gate were displayed within a PI fluorescence and granularity dot plot, and viable cells were delineated by placing a second gate R2 selecting the population of PI-negative cells. Cells from the R2 gate were displayed within a FITC fluorescence and granularity dot plot to analyze the cell surface expression. The analysis marker applied to this dot plot was set so that the percentage of IgG1 positive cells among the viable untreated cells was equal to 0.6%. Then the percentage of IgG1 positive cells in one well and of CD86 positive cells in the second well were measured for each condition, without moving the analysis marker.

For each sample with a viability above or equal to 70%, the percentage of IgG1 isotype positive cells was subtracted from the percentage of CD86 positive cells. Results were expressed as stimulation indexes (SI) calculated as the isotype corrected percentage CD86+ test/isotype corrected percentage CD86+ control × 100.

Note that, for each concentration, CD86 expression was only recorded if the measured viability was at least 70%.

For each vehicle control (RPMI or DMSO), the average % of CD86 and IgG1 was calculated from three replicates. If one replicate was 25% above or below (i.e. SI <75% or >125%) the average, related data were excluded.

The theoretical concentration at which the chemical induces a SI of 150 was calculated by linear regression using the equation $EC150 = C1 + [(150 - SI\ 1)/(SI\ 2 - SI\ 1)(C2 - C1)]$ where C1 and SI 1 are the concentration and SI immediately below SI = 150 and C2 and SI 2 are the data points immediately above SI = 150.

The theoretical concentration at which the chemical induces 30% cytotoxicity (a remaining viability of 70%), i.e. CV70, was calculated similarly.

2.2.3. Acceptance criteria

Several acceptance criteria were defined to ensure the validity of the runs.

At the end of the 45 h incubation treatment period, the mean viability of the triplicate untreated U937 cells had to be >90% and the CD86 basal expression of untreated U937 cells had to be comprised within the range of ≥2% and ≤25%. When DMSO was used as a solvent, the validity of the DMSO vehicle control was assessed by calculating a DMSO SI compared to untreated cells as if DMSO was a treatment. The DMSO vehicle control was valid if the mean value of its triplicate CD86 SI was smaller than 250% of the mean of the triplicate CD86 SI of untreated U937 cells. Furthermore, the runs were considered valid if at least two out of three IgG1 values of untreated U937 cells fell within the range of ≥0.6% and <1.5%. The concurrent tested negative control lactic acid was considered valid if at least two out of the three replicates were negative (CD86 SI < 150%). Similarly, the positive control

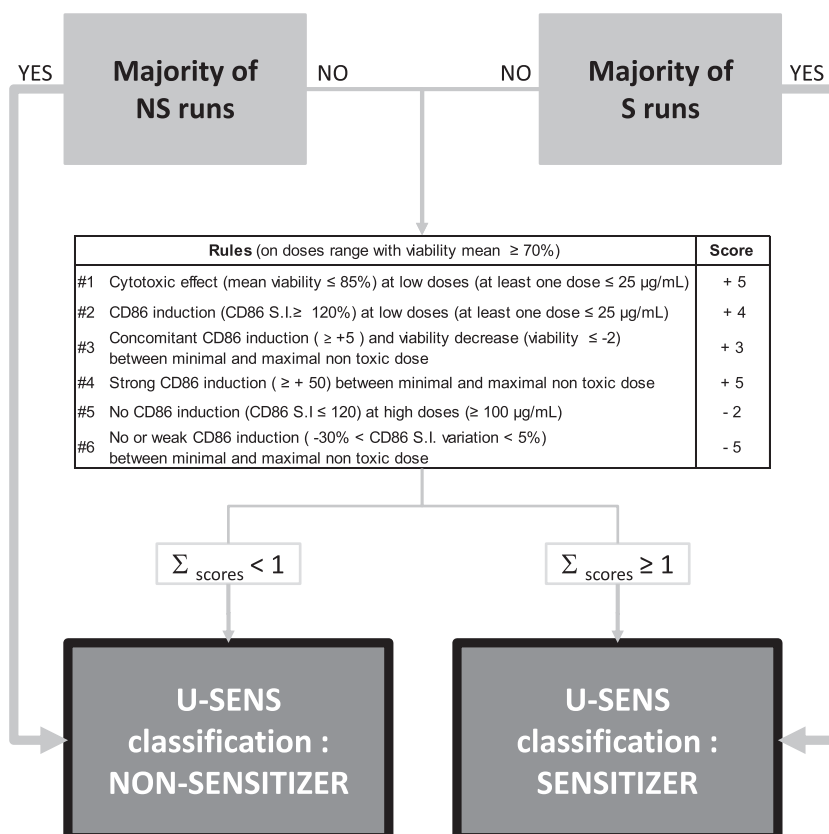


Fig. 1. The U-SENS™ prediction model.

picrylsulphonic acid was considered as valid if at least two out of the three replicates were positive ($\text{CD86 SI} \geq 150\%$).

2.2.4. Prediction model

As reported in Fig. 1, the prediction model (PM) of the U-SENS™ assay classifies all tested substances as Sensitizers (S) or Non-Sensitizers (NS). The PM was applied to an experiment, which itself consisted of at least two valid runs. An S individual run conclusion was drawn if the CD86 SI was at least 150% with a dose–response relationship at non-cytotoxic doses (i.e. viability $\geq 70\%$). If the CD86 SI was less than 150% at non-cytotoxic doses and if no interferences such as poor solubility (e.g. crystals or drops observed under the microscope after the 45 h incubation), colour interference (IgG1 FITC fluorescence mean SI $\geq 150\%$) or cytotoxicity ($\text{CV70} < 100 \mu\text{g/mL}$) were observed, a NS individual run conclusion was assigned. Otherwise no S or NS individual conclusions were attributed to the run. If a majority of S or NS individual conclusions was reached (i.e. generally 2/2 or 2/3 S or NS), the final S or NS conclusion was determined. Otherwise, viability and CD86 mean values of all valid runs of the experiment were used to score the results via the application of six rules, on doses range with viability

mean $\geq 70\%$. The final S or NS conclusion was determined on the basis of the sum of the six scores (Gomes et al., 2014). If a substance induced a very high cytotoxic effect with any or only one dose with viability mean $\geq 70\%$, a very conservative approach was applied, defining the substance as a Sensitizer.

Rules 1 to 4 with positive scores were indicative of S, while rules 5 and 6 with negative scores related to the NS class. Rules 1 and 2 were triggered, if at concentrations up to $25 \mu\text{g/mL}$, a slight effect on respectively CD86 induction ($\text{CD86 SI} \geq 120\%$) or cytotoxicity (viability $\leq 85\%$) was observed. Rule 3 reflected whether the difference in CD86 induction between the highest and the lowest tested concentrations was $\geq 5\%$ and the difference in cytotoxicity of these two concentrations was $\leq -2\%$. Rule 4 considered steep dose–response induction increases ($\geq 50\%$) between the lowest and the highest tested concentrations. Rule 5 was triggered when non-cytotoxic concentrations $\geq 100 \mu\text{g/mL}$ showed no significant CD86 induction ($\text{CD86 SI} \leq 120\%$). Finally, rule 6 compared induction at the highest and lowest test concentration. It indicated when the difference between these two fell in the range of -30% to $+5\%$. The prediction model including the rules is summarised in Fig. 1.

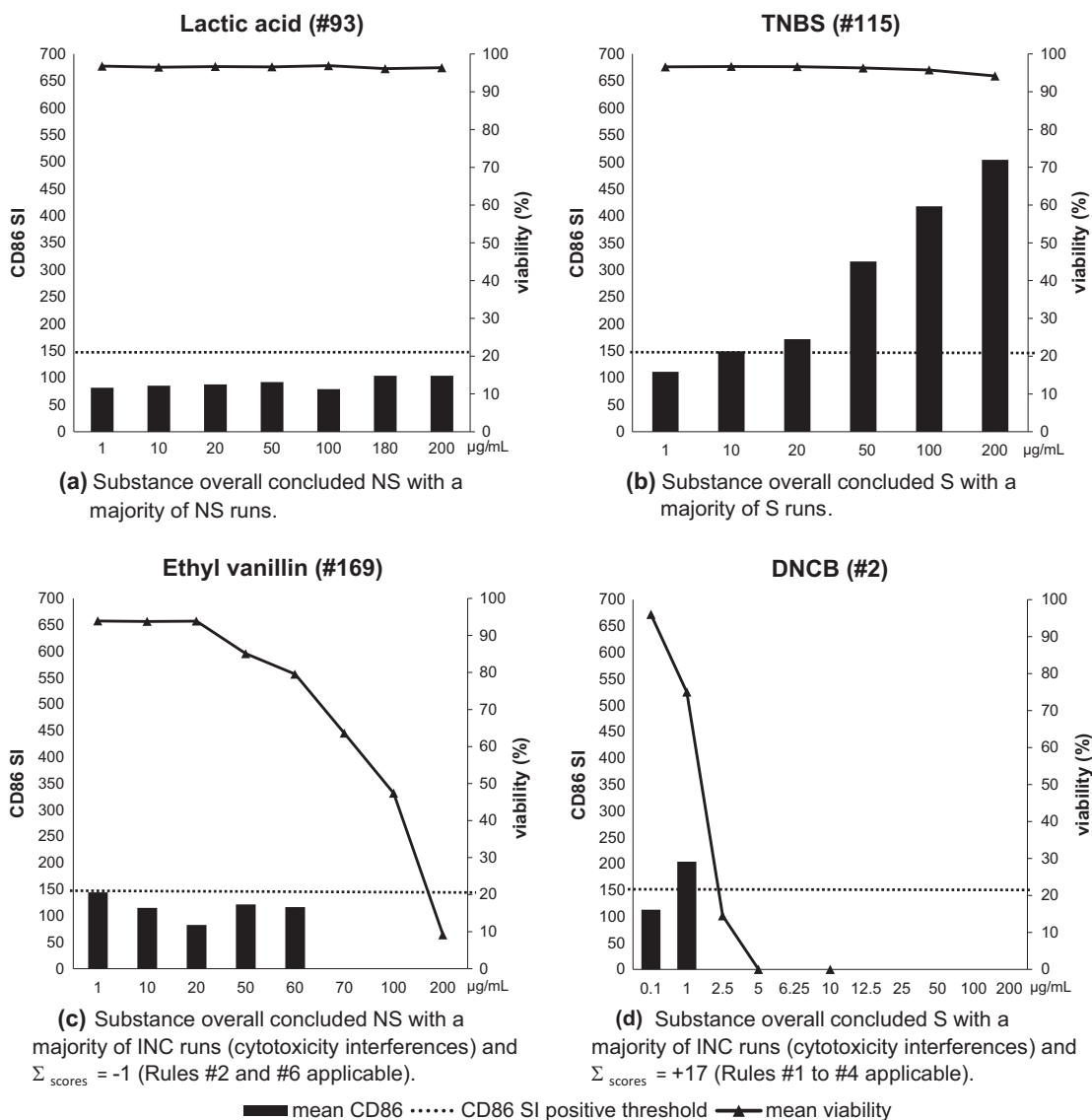


Fig. 2. CD86 S.I. and viability mean values obtained with the negative control (lactic acid), the positive control (TNBS), and two substances (ethyl vanillin and DNCB) classified (respectively NS and S) by applying the rules.

Examples of dose response curves obtained with two sensitizers and two non-sensitizers are presented in Fig. 2.

2.3. Test substances and predictive capacity

In order to evaluate the predictive performance of the U-SENSTM assay, 175 commercially available substances – most of them purchased from Sigma–Aldrich – have been tested by L'Oréal (Table 1); all being mono-constituent, except the preservative MCI/MI (#104).

In the work presented herein, the primary substance selection criterion was the availability of existing human and/or LLNA data to allow a comparative evaluation of the results. As reported in Table 1 and Fig. 3, this 175 substances *in vivo* database was composed of 101 human and 166 LLNA data respectively, with a common set of 92 substances. With regards to human data, we chose the classification proposed by Basketter et al. (2014) as the unique reference source among others (Schlede et al., 2003; De Groot and Weyland, 1988). This classification is based on the combination of clinical data and consideration of frequency of ACD in the occupational and general population, some historical human test data, and analysis of human exposure and allows to distinguish six potency classes. In the present study, substances belonging to category 1–5 were considered as S, whereas category 6 substances were considered as NS (category 5 being a border line category, a conservative safety approach made us consider a positive call).

The source references for the 166 LLNA data are provided in Table 1 and classified as S and NS. Among these, 74 substances have LLNA data only.

When available, LLNA EC3 values, i.e. the estimated concentration that produces a stimulation index of 3, and the derived classifications were reported. There are five EC3 potency classes: extreme when EC3 values were below 0.1%; strong for EC3 values comprised between 0.1% and 1.0%, moderate for EC3 values between 1.0% and 10.0%, weak for EC3 values higher than 10.0% and NS when no EC3 value can be calculated due to insufficient stimulation in the testing conditions.

The EU CLP classification (EU, 2011) was based on three tentative classes: Cat. 1A (EC3 ≤ 2%); Cat. 1B (EC3 > 2%) and No Category when no EC3 can be calculated due to insufficient stimulation.

Protein binding classes were determined for each substance according to Toxtree v2.6.0 application (Patlewicz et al., 2008), where the reaction mechanistic domains proposed by Aptula and Roberts (2006) were implemented.

Finally, substances were grouped into appropriate cosmetic or industrial use.

The predictive performance was described as sensitivity (the proportion of true sensitizers predicted as positive), specificity (the proportion of true non-sensitizers predicted as negative), accuracy (overall proportion of correct predictions) and kappa coefficient (measure of “true” agreement), which were calculated from contingency tables. Receiver Operation Characteristics (ROC) curves were used to characterize the predictive capacity of the U-SENSTM assay in more detail.

2.4. Ring study

As a preparatory step for a formal validation study, a ring trial focusing on the U-SENSTM assay's transferability as well as within- and between-laboratory reproducibility (WLR and BLR, respectively) was conducted according to the Standard Operating Procedure (SOP) established by L'Oréal. Therefore, 21 substances issued from the 175 data set were selected. These are listed in Table 3. With regards to the assessment of the BLR and thus selection of the test substances, the rationale applied consisted of primarily considering substances (in bold in Table 3) for which

potential interfering factors could be observed (e.g. colour interferences with cytometry parameters or poor solubility) on the basis of our expert judgment and lessons learnt from historical testing programs. Most of these substances were sensitizers as predicted by the LLNA. Therefore, the seven substances that were added to evaluate the within-laboratory reproducibility (WLR) in the lead laboratory only, were all non-sensitizers in the LLNA.

The WLR was studied in the lead laboratory L'Oréal (Aulnay-sous-Bois, France) by testing the aforementioned 21 substances in three independent experiments. Fourteen of these were coded. The BLR reproducibility was assessed by testing the same 14 coded substances in one experiment in the three additional laboratories: Bioassay (Heidelberg, Germany), CiToxLAB (Evreux, France) and WIL Research (Saint Germain sur l'Arbresle, France). The lead laboratory L'Oréal that had developed the method, trained the personnel of the other testing laboratories. In addition, it provided advice during the test method transfer in order to ensure proper implementation of the SOP. All laboratories used the same SOP version as well as data documentation and reporting files. The U-SENSTM assay's reliability was primarily assessed by concordance of final conclusions, regardless of the *in vivo* reference results. Additionally, CV70 and EC150 values were compared.

3. Results

3.1. Predictive capacity

3.1.1. The U-SENSTM database

A 175 substances database, including both cosmetic and non-cosmetic ingredients (70:30), was established to characterize the predictive capacity of U-SENSTM (Table 1). All potency classes, from extreme to non-sensitizer in accordance with the human, LLNA and EU-CLP classifications were represented. Their respective distributions are summarised in Fig. 3. A tentative EU-CLP classification could be established based on LLNA data which resulted in: 50 Category 1A substances, 79 Category 1B and 37 No Category.

The substances were assigned to various ingredient classes according to their use. The most prevalent ingredient classes were fragrances (29%), preservatives (16%) and dyes (8%) (Fig. 4a). Finally, several reactivity classes were also represented; these included Michael acceptor, Schiff base formation, acyl transfer agent, SN2 (substitution nucleophilic bi-molecular) or SNAr (nucleophilic aromatic substitution) based upon Toxtree classification schema (Patlewicz et al., 2008, Fig. 4b) but also pre- or pro-haptens. For example, the database contains presumed pre-haptens such as 1,4-dihydroquinone (#27), 3-methylcatechol (#107), isoeugenol (#18) and 1,4-phenylenediamine (#5), and pro-haptens such as geraniol (#54), cinnamic alcohol (#40), aniline (#57), 2-aminophenol (#14), 1-naphthol (#120), eugenol (#38), and 2-methoxy-4-methylphenol (#136) (Bertrand et al., 1997; Smith and Hotchkiss, 2001; Smith-Pease et al., 2003).

3.1.2. Predictive capacity of the U-SENSTM test method

The predictive capacity of the U-SENSTM test method was comprehensively explored by comparing the results obtained to the human (Basketter et al., 2014) and LLNA data, and their combination (Table 1). LLNA models early events in the sensitization process and was formally validated for the prediction of skin sensitization (NIH, 1999; Balls and Hellsten, 2000). It is widely used in regulations and constitutes the pivotal reference standard for the assessment of non-animal test methods. On the other hand, human skin sensitization data are not as standardised since they have to include, the frequency of reported cases related to human exposure in the general population or in occupational situations unless

Table 1
Database of 175 substances used in the evaluation of the predictive performance of the U-SENS™ assay.

N°	Substance	CAS number	Ingredient class	Toxtree protein binding class ^a	Human potency cat. ^b	LLNA ^c				In vivo ref class	U-SENS ^f				Human versus LLNA/U-SENS ^g
						EC3 (%)	Potency cat. ^d	CLP cat.	Data source ^e		Vehicle	Class	EC150 (µg/mL)	CV70 (µg/mL)	
1	Tetrachlorosalicylanilide	1154-59-2	Preservatives	Acyl transfer agent	1	0.04	Extreme	Cat. 1A	6	S	DMSO	S*	2	2	✓
2	2,4-Dinitrochlorobenzen DNCB	97-00-7	Non cosmetic	SNAr	1	0.05	Extreme	Cat. 1A	6	S	DMSO	S*	0.5	1.1	✓
3	Diphenylcyclopropanone	886-38-4	Non cosmetic	Michael acceptor	1	0.05	Extreme	Cat. 1A	3	S	DMSO	S*	0.2	1.4	✓
4	Potassium dichromate	7778-50-9	Non cosmetic	No binding	1	0.08	Extreme	Cat. 1A	6	S	RPMI	S*	0.9	0.9	✓
5	1,4-Phenylenediamine	106-50-3	Dyes (oxidation)	Michael acceptor	1	0.11	Strong	Cat. 1A	3	S	RPMI	S	3	21	✓
6	Dimethyl fumarate	624-49-7	Non cosmetic	Michael acceptor	1	0.35	Strong	Cat. 1A	3	S	RPMI	S	1	27	✓
7	Methylisothiazolinone	2682-20-4	Preservatives	Michael acceptor/SN2	1	0.4	Strong	Cat. 1A	3	S	RPMI	S	1	8	✓
8	Glutaraldehyde (act. 50%)	111-30-8	Preservatives	Schiff base formation	2	0.1	Strong	Cat. 1A	6	S	RPMI	S	83	>200	✓
9	Toluene diamine sulphate	615-50-9	Dyes (oxidation)	Michael acceptor	2	0.2	Strong	Cat. 1A	3	S	RPMI	S	2	12	✓
10	Gold chloride	13453-07-1	Non cosmetic	No binding	2	0.3	Strong	Cat. 1A	3	S	RPMI	S*	0.1	0.1	✓
11	Lauryl gallate	1166-52-5	Others	Michael acceptor	2	0.3	Strong	Cat. 1A	6	S	DMSO	S*	0.7	0.7	✓
12	Propyl gallate	121-79-9	Fragrances	Michael acceptor	2	0.32	Strong	Cat. 1A	9	S	RPMI	S	2	33	✓
13	2-Nitro-1,4-phenylenediamine	5307-14-2	Dyes (direct)	Michael acceptor	2	0.4	Strong	Cat. 1A	6	S	DMSO	S	5	115	✓
14	2-aminophenol	95-55-6	Dyes (oxidation)	Michael acceptor	2	0.4	Strong	Cat. 1A	9	S	DMSO	S	0.1	10	✓
15	Formaldehyde (act. 37%)	50-00-0	Preservatives	Schiff base formation	2	0.4	Strong	Cat. 1A	3	S	DMSO	S*	6	6	✓
16	Methyl heptine carbonate	111-12-6	Fragrances	Michael acceptor	2	0.5	Strong	Cat. 1A	7	S	DMSO	S	39	70	✓
17	Methyldibromo glutaronitrile	35691-65-7	Preservatives	Michael acceptor/SN2	2	0.9	Strong	Cat. 1A	6	S	DMSO	S	3	5	✓
18	Isoeugenol	97-54-1	Fragrances	Michael acceptor	2	1.2	Moderate	Cat. 1A	6	S	DMSO	S	45	115	✓
19	Glyoxal (act. 40%)	107-22-2	Preservatives	No binding	2	1.4	Moderate	Cat. 1A	6	S	RPMI	S	33	93	✓
20	Cinnamic aldehyde	104-55-2	Fragrances	Michael acceptor	2	2	Moderate	Cat. 1B	3	S	DMSO	S	1	8	✓
21	Diethyl maleate	141-05-9	Non cosmetic	Michael acceptor	2	2.1	Moderate	Cat. 1B	3	S	DMSO	S	7	30	✓
22	3-Dimethylaminopropylamine	109-55-7	Non cosmetic	Schiff base formation	2	2.2	Moderate	Cat. 1B	6	S	RPMI	S	103	>200	✓
23	1,2-Benzisothiazolin-3-one	2634-33-5	Preservatives	Acyl transfer agent/SN2	2	2.3	Moderate	Cat. 1B	6	S	DMSO	S	0.4	3	✓
24	Thioglycerol	96-27-5	Others	No binding	2	3.6	Moderate	Cat. 1B	3	S	RPMI	S*	82	>200	✓
25	Lylal	31906-04-4	Fragrances	Schiff base formation	2	17.1	Weak	Cat. 1B	6	S	DMSO	S	6	24	✓
26	Metol	55-55-0	Dyes (oxidation)	Michael acceptor	3	0.8	Strong	Cat. 1A	6	S	RPMI	S	0.3	3	✓
27	1,4-Dihydroquinone	123-31-9	Actives	Michael acceptor	3	0.11	Strong	Cat. 1A	6	S	RPMI	S	1	6	✓
28	Chlorpromazine	50-53-3	Non cosmetic	Schiff base formation	3	0.14	Strong	Cat. 1A	3	S	DMSO	S*	6	6	✓
29	Benzoyl peroxide	94-36-0	Actives	Acyl transfer agent	3	0.3	Strong	Cat. 1A	3	S	DMSO	S*	28	64	✓
30	Hydroxyethyl acrylate	868-77-9	Others	Michael acceptor	3	1.4	Moderate	Cat. 1A	3	S	RPMI	S	40	>200	✓
31	Bisphenol A-diglycidyl ether	1675-54-3	Non cosmetic	SN2	3	1.5	Moderate	Cat. 1A	6	S	DMSO	S	9	23	✓
32	2-Mercaptobenzothiazole	149-30-4	Non cosmetic	Acyl transfer agent	3	1.7	Moderate	Cat. 1A	6	S	DMSO	S	40	80	✓
33	Ethylene diamine	107-15-3	Others	Schiff base formation	3	2.2	Moderate	Cat. 1B	6	S	RPMI	S	16	58	✓
34	Farnesol	4602-84-0	Fragrances	Schiff base formation	3	4.8	Moderate	Cat. 1B	3	S	DMSO	S	8	34	✓
35	Phenyl benzoate	93-99-2	Preservatives	Acyl transfer agent	3	5.2	Moderate	Cat. 1B	3	S	DMSO	S	101	>200	✓
36	Tetramethylthiuramdisulphide	137-26-8	Non cosmetic	No binding	3	6	Moderate	Cat. 1B	3	S	DMSO	S	0.1	3	✓
37	Citral	5392-40-5	Fragrances	Schiff base formation	3	13	Weak	Cat. 1B	6	S	DMSO	S	4	13	✓
38	Eugenol	97-53-0	Fragrances	Michael acceptor	3	13	Weak	Cat. 1B	6	S	DMSO	S	29	142	✓
39	Abietic acid	514-10-3	Non cosmetic	No binding	3	15	Weak	Cat. 1B	6	S	DMSO	S	36	72	✓
40	Cinnamic alcohol	104-54-1	Fragrances	Michael acceptor	3	21	Weak	Cat. 1B	6	S	DMSO	S	21	110	✓
41	Imidazolidinyl urea	39236-46-9	Preservatives	Acyl transfer agent	3	24	Weak	Cat. 1B	6	S	RPMI	S	11	28	✓
42	Coumarin	91-64-5	Fragrances	Michael acceptor	3	30	Weak	Cat. 1B	3	S	DMSO	S	104	>200	✓
43	Butyl glycidyl ether	2426-08-6	Non cosmetic	SN2	3	31	Weak	Cat. 1B	6	S	RPMI	S	149	>200	✓
44	Chlorhexidine gluconate	55-56-1	Preservatives	Acyl transfer agent	4	na	na	na	na	S	DMSO	S*	4	4	✓
45	Bronopol	52-51-7	Preservatives	No binding	4	na	na	na	na	S	RPMI	S*	2	6	✓
46	Hexyl salicylate	6259-76-3	Fragrances	No binding	4	0.18	Strong	Cat. 1A	7	S	DMSO	S*	27	27	✓
47	Iodopropynyl butylcarbamate	55406-53-6	Preservatives	Acyl transfer agent	4	0.87	Strong	Cat. 1A	7	S	DMSO	S*	2	4	✓
48	Resorcinol	108-46-3	Dyes (oxidation)	Michael acceptor	4	5.5	Moderate	Cat. 1B	7	S	RPMI	S	5	173	✓
49	Amyl cinnamic aldehyde	122-40-7	Fragrances	Michael acceptor	4	11	Weak	Cat. 1B	3	S	DMSO	S	13	27	✓
50	Lillial	80-54-6	Fragrances	Schiff base formation	4	19	Weak	Cat. 1B	6	S	DMSO	S	9	34	✓
51	Hydroxycitronellal	107-75-5	Fragrances	Schiff base formation	4	20	Weak	Cat. 1B	3	S	DMSO	S*	6	9	✓
52	Benzocaine	94-09-7	Preservatives	No binding	4	22	Weak	Cat. 1B	3	S	DMSO	S	103	>200	✓

53	Amylcinnamyl alcohol	101-85-9	Fragrances	Michael acceptor	4	25	Weak	Cat. 1B	3	S	DMSO	S	15	40	✓
54	Geraniol	106-24-1	Fragrances	Schiff base formation	4	26	Weak	Cat. 1B	6	S	RPMI	S	62	159	✓
55	Linalool	78-70-6	Fragrances	No binding	4	30	Weak	Cat. 1B	6	S	DMSO	S	41	>200	✓
56	Ethyleneglycol dimethacrylate	97-90-5	Non cosmetic	Michael acceptor	4	35	Weak	Cat. 1B	3	S	DMSO	S	81	>200	✓
57	Aniline	62-53-3	Dyes (oxidation)	No binding	4	89	Weak	Cat. 1B	6	S	RPMI	S	63	>200	✓
58	Methylmethacrylate	80-62-6	Non cosmetic	Michael acceptor	4	90	Weak	Cat. 1B	7	S	DMSO	S	157	>200	✓
59	Anethole	104-46-1	Fragrances	Michael acceptor	5	2.3	Moderate	Cat. 1B	6	S	DMSO	S*	>200	>200	✓
60	Benzyl salicylate	118-58-1	Fragrances	Acyl transfer agent	5	2.9	Moderate	Cat. 1B	7	S	DMSO	S*	63	63	✓
61	Anisyl alcohol	105-13-5	Fragrances	Michael acceptor/SN2	5	5.9	Moderate	Cat. 1B	7	S	DMSO	NS	>200	>200	M
62	Hexyl cinnamic aldehyde	101-86-0	Fragrances	Michael acceptor	5	8	Moderate	Cat. 1B	3	S	DMSO	S	4	25	✓
63	Benzyl benzoate	120-51-4	Fragrances	Acyl transfer agent	5	17	Weak	Cat. 1B	6	S	DMSO	S*	>200	>200	✓
64	Pentachlorophenol	87-86-5	Non cosmetic	SNAr	5	20	Weak	Cat. 1B	6	S	DMSO	S*	21	22	✓
65	Benzaldehyde	100-52-7	Fragrances	Schiff base formation	5	25	Weak	Cat. 1B	3	S	DMSO	S	85	175	✓
66	Diethanolamine	111-42-2	Others	No binding	5	40	Weak	Cat. 1B	3	S	RPMI	S	26	>200	✓
67	Isopropyl myristate	110-27-0	Others	No binding	5	44	Weak	Cat. 1B	6	S	DMSO	NS	>200	>200	M
68	Citronellol	106-22-9	Fragrances	No binding	5	43.5	Weak	Cat. 1B	7	S	DMSO	S	25	>200	✓
69	Limonene (not oxidised)	138-86-3	Fragrances	No binding	5	69	Weak	Cat. 1B	3	S	DMSO	S*	43	48	✓
70	Pyridine	110-86-1	Non cosmetic	No binding	5	72	Weak	Cat. 1B	6	S	RPMI	NS	>200	>200	M
71	Methyl salicylate	119-36-8	Preservatives	No binding	5	NC	NS	no cat.	6	S	DMSO	NS	>200	>200	L&M
72	Vanillin	121-33-5	Fragrances	Schiff base formation/ michael ACCEPTOR	5	NC	NS	no cat.	6	S	DMSO	S	121	>200	L
73	4-Aminobenzoic acid	150-13-0	UV filter	No binding	5	NC	NS	no cat.	4	S	DMSO	NS	>200	>200	L&M
74	Propyl paraben	94-13-3	Preservatives	No binding	5	NC	NS	no cat.	9	S	RPMI	S	24	121	L
75	Benzalkonium chloride	8001-54-5	Preservatives	NA	5	NC	NS	no cat.	3	S	RPMI	S*	0.1	1	L
76	Hydrocortisone	50-23-7	Actives	Schiff base formation	5	NC	NS	no cat.	3	S	DMSO	NS	>200	>200	L&M
77	Propylene glycol	57-55-6	Others	No binding	5	NC	NS	no cat.	6	S	RPMI	NS	>200	>200	L&M
78	Isopropanol	67-63-0	Others	No binding	5	NC	NS	no cat.	6	S	RPMI	NS	>200	>200	L&M
79	Phenoxyethanol	122-99-6	Preservatives	No binding	5	NC	NS	no cat.	3	S	DMSO	S*	183	>200	L
80	Sorbic acid	110-44-1	Preservatives	Michael acceptor	5	na	na	na	na	S	DMSO	S	115	>200	✓
81	Triclosan	3380-34-5	Preservatives	No binding	5	na	na	na	na	S	DMSO	S*	4	5	✓
82	Triethanolamine	102-71-6	Actives	No binding	5	na	na	na	na	S	RPMI	S*	112	>200	✓
83	Butylene glycol	107-88-0	Others	No binding	5	na	na	na	na	S	RPMI	NS	>200	>200	M
84	Cetrimide	57-09-0	Non cosmetic	No binding	5	na	na	na	na	S	RPMI	S*	0.8	0.8	✓
85	Tocopherol	59-02-9	Others	No binding	6	7.4	Moderate	Cat. 1B	3	NS	RPMI	NS	>200	>200	L
86	Sodium lauryl sulphate	151-21-3	Surfactants	SN2	6	14	Weak	Cat. 1B	6	NS	RPMI	S*	54	74	L&M
87	DMSO	67-68-5	Non cosmetic	No binding	6	72	Weak	Cat. 1B	6	NS	RPMI	NS	>200	>200	L
88	Xylene	1330-20-7	Non cosmetic	No binding	6	96	Weak	Cat. 1B	5	NS	DMSO	NS	>200	>200	L
89	Salicylic acid	69-72-7	Preservatives	No binding	6	NC	NS	No cat.	6	NS	DMSO	NS	>200	>200	✓
90	Octanoic acid	124-07-2	Surfactants	No binding	6	NC	NS	No cat.	6	NS	RPMI	S	59	>200	M
91	Hexane	110-54-3	Non cosmetic	No binding	6	NC	NS	No cat.	6	NS	RPMI	NS	>200	>200	✓
92	Diethyl phthalate	84-66-2	Fragrances	No binding	6	NC	NS	No cat.	6	NS	DMSO	S*	167	>200	M
93	Lactic acid	50-21-5	Actives	No binding	6	NC	NS	No cat.	6	NS	RPMI	NS	>200	>200	✓
94	Glycerol/glycerin	56-81-5	Actives	No binding	6	NC	NS	No cat.	6	NS	RPMI	NS	>200	>200	✓
95	1-Butanol	71-36-3	Others	No binding	6	NC	NS	No cat.	6	NS	RPMI	NS	>200	>200	✓
96	Tween 80	9005-65-6	Surfactants	NA	6	NC	NS	No cat.	3	NS	RPMI	S	9	>200	M
97	Phenol	108-95-2	Preservatives	No binding	6	NC	NS	No cat.	3	NS	DMSO	S	22	102	M
98	Dextran	9004-54-0	Non cosmetic	Schiff base formation	6	NC	NS	No cat.	3	NS	RPMI	NS	>200	>200	✓
99	Diethyl toluamide	94271-03-1	Non cosmetic	No binding	6	NC	NS	No cat.	3	NS	DMSO	S	53	>200	M
100	Sorbitol	50-70-4	Others	No binding	6	na	na	na	na	NS	RPMI	NS	>200	>200	✓
101	Glucose	50-99-7	Others	No binding	6	na	na	na	na	NS	RPMI	NS	>200	>200	✓
102	2,4-Dinitrofluorobenzene	70-34-8	Non cosmetic	SNAr	na	0.032	Extreme	Cat. 1A	13	S	RPMI	S*	2	4	-
103	Oxazolone	15646-46-5	Non cosmetic	Acyl transfer agent	na	0.003	Extreme	Cat. 1A	6	S	DMSO	S	145	>200	-
104	MCl/MI (act. 1.5%)	55965-84-9	Preservatives	Michael acceptor/SN2	na	0.005	Extreme	Cat. 1A	12	S	RPMI	S	11	34	-
105	7,12-Dimethylbenz[α]anthracene	57-97-6	Non cosmetic	SN2	na	0.006	Extreme	Cat. 1A	6	S	RPMI	S	33	>200	-
106	p-Benzquinone	106-51-4	Dyes (direct)	Michael acceptor	na	0.01	Extreme	Cat. 1A	6	S	RPMI	S	2	15	-
107	3-Methylcatechol	488-17-5	Fragrances	Michael acceptor	na	0.02	Extreme	Cat. 1A	14	S	RPMI	S	0.3	4	-
108	Bandrowski's base	20048-27-5	Dyes (direct)	No binding	na	0.04	Extreme	Cat. 1A	7	S	DMSO	S	1	3	-
109	4-Nitrobenzyl bromide	100-11-8	Non cosmetic	SN2	na	0.05	Extreme	Cat. 1A	6	S	DMSO	S*	0.4	0.4	-
110	Methylnitrosourea	684-93-5	Non cosmetic	SN2	na	0.05	Extreme	Cat. 1A	6	S	RPMI	S	13	>200	-

Table 1 (continued)

N°	Substance	CAS number	Ingredient class	Toxtree protein binding class ^a	Human potency cat. ^b	LLNA ^c				<i>In vivo</i> ref class	U-SENS ^f				Human versus LLNA/U-SENS ^g
						EC3 (%)	Potency cat. ^d	CLP cat.	Data source ^e		Vehicle	Class	EC150 (µg/mL)	CV70 (µg/mL)	
111	Cyanuric chloride	108-77-0	Non cosmetic	SNAr	na	0.09	Extreme	Cat. 1A	6	S	RPMI	S	15	68	–
112	p-Aminophenol	123-30-8	Dyes (Oxidation)	Michael acceptor	na	–	Strong	Cat. 1A	10	S	RPMI	S	2	6	–
113	Phthalic anhydride	85-44-9	Non cosmetic	Acyl transfer agent	na	0.16	Strong	Cat. 1A	1	S	DMSO	NS	>200	>200	D
114	Benzyl bromide	100-39-0	Non cosmetic	SN2	na	0.2	Strong	Cat. 1A	6	S	DMSO	S*	14	16	–
115	2,4,6-Trinitrobenzenesulphonic acid	2508-19-2	Non cosmetic	No binding	na	0.36	Strong	Cat. 1A	13	S	RPMI	S	10	>200	–
116	3-Phenylenediamine	108-45-2	Dyes (oxidation)	Michael acceptor	na	0.49	Strong	Cat. 1A	6	S	RPMI	S	4	137	–
117	CD3	25646-71-3	Non cosmetic	Michael acceptor	na	0.6	Strong	Cat. 1A	6	S	RPMI	S	1	4.4	–
118	Squaric acid diethyl ester	5231-87-8	Non cosmetic	Schiff base formation	na	0.9	Strong	Cat. 1A	10	S	DMSO	S	32	71	–
119	1-Phenyl-1,2-propanedione	579-07-7	Others	Schiff base formation	na	1.3	Moderate	Cat. 1A	6	S	DMSO	S	35	77	–
120	1-Naphthol	90-15-3	Dyes (oxidation)	Michael acceptor	na	1.3	Moderate	Cat. 1A	6	S	DMSO	S	3	31	–
121	2-Hydroxyethyl acrylate	818-61-1	Non cosmetic	Michael acceptor	na	1.4	Moderate	Cat. 1A	6	S	RPMI	S	2.1	10	–
122	Nonanoyl chloride	764-85-2	Surfactants	Acyl transfer agent	na	1.8	Moderate	Cat. 1A	6	S	DMSO	S	19	56	–
123	N,N-bis(2-hydroxyethyl)-p-phenylenediamine sulphate	54381-16-7	Dyes (oxidation)	Michael acceptor	na	1.04	Moderate	Cat. 1A	16	S	RPMI	S	8	29	–
124	Methyl-2-nonynoate	111-80-8	Fragrances	Michael acceptor	na	2.5	Moderate	Cat. 1B	6	S	DMSO	S	18	105	–
125	3,3,5-trimethylhexanoyl chloride	36727-29-4	Non cosmetic	Acyl transfer agent	na	2.7	Moderate	Cat. 1B	6	S	DMSO	S	17	48	–
126	Phenylacetaldehyde	122-78-1	Fragrances	Schiff base formation	na	3	Moderate	Cat. 1B	6	S	DMSO	S	7	18	–
127	3-Aminophenol	591-27-5	Dyes (oxidation)	Michael acceptor	na	3.2	Moderate	Cat. 1B	9	S	DMSO	S	11	128	–
128	Diethyl sulphate	64-67-5	Non cosmetic	SN2	na	3.3	Moderate	Cat. 1B	6	S	RPMI	S	22	>200	–
129	Benzylideneacetone	122-57-6	Fragrances	Michael acceptor	na	3.7	Moderate	Cat. 1B	6	S	RPMI	S	3	7	–
130	3-Propylidenephthalide	17369-59-4	Fragrances	Acyl transfer agent	na	3.7	Moderate	Cat. 1B	6	S	DMSO	S	32	>200	–
131	Squaric acid	2892-51-5	Non cosmetic	Schiff base formation	na	4.3	Moderate	Cat. 1B	14	S	RPMI	S	63	>200	–
132	a-Methyl cinnamic aldehyde	101-39-3	Fragrances	Michael acceptor	na	4.5	Moderate	Cat. 1B	6	S	DMSO	S	9	>200	–
133	Nickel sulphate	10101-97-0	Others	No binding	na	4.8	Moderate	Cat. 1B	11	S	RPMI	S	16	55	–
134	trans-2-Hexenal	6728-26-3	Fragrances	Michael acceptor	na	5.5	Moderate	Cat. 1B	6	S	RPMI	S	4	51	–
135	3,4-Dihydrocoumarin	119-84-6	Fragrances	Acyl transfer agent	na	5.6	Moderate	Cat. 1B	6	S	DMSO	NS	>200	>200	D
136	2-Methoxy-4-methyl-phenol	93-51-6	Fragrances	Michael acceptor	na	5.8	Moderate	Cat. 1B	6	S	DMSO	S	13	159	–
137	Diethylenetriamine	111-40-0	Others	Schiff base formation	na	5.8	Moderate	Cat. 1B	6	S	RPMI	NS	>200	>200	D
138	1-Bromoeicosane	4276-49-7	Non cosmetic	SN2	na	6.1	Moderate	Cat. 1B	6	S	RPMI	NS	>200	>200	D
139	2-Phenylpropionaldehyde	93-53-8	Fragrances	Schiff base formation	na	6.3	Moderate	Cat. 1B	6	S	DMSO	S	4	12	–
140	4-Chloroaniline	106-47-8	Non cosmetic	No binding	na	6.5	Moderate	Cat. 1B	2	S	DMSO	S	1	182	–
141	Dihydroeugenol	2785-87-7	Fragrances	Michael acceptor/SN2	na	6.8	Moderate	Cat. 1B	6	S	DMSO	S	47	122	–
142	Undec-10-enal	112-45-8	Fragrances	Schiff base formation	na	6.8	Moderate	Cat. 1B	6	S	RPMI	S	9	29	–
143	12-Bromo-1-dodecanol	3344-77-2	Non cosmetic	SN2	na	6.9	Moderate	Cat. 1B	6	S	DMSO	S	9	28	–
144	Safranal	116-26-7	Fragrances	Schiff base formation/ Michael acceptor	na	7.5	Moderate	Cat. 1B	6	S	DMSO	S	12	39	–
145	Methyl methanesulphonate	66-27-3	Non cosmetic	SN2	na	8.1	Moderate	Cat. 1B	6	S	RPMI	S	2	33	–
146	Tween 21	9005-64-5	Surfactants	NA	na	–	Weak	Cat. 1B	10	S	RPMI	S	68	>200	–
147	Farnesal	19317-11-4	Fragrances	Schiff base formation	na	12	Weak	Cat. 1B	6	S	DMSO	S*	5	6	–
148	1-Bromohexane	111-25-1	Non cosmetic	SN2	na	10	Weak	Cat. 1B	6	S	DMSO	S*	>200	183	–
149	2-Ethylhexyl acrylate	103-11-7	Non cosmetic	Michael acceptor	na	10.0	Weak	Cat. 1B	7	S	DMSO	S	15	>200	–
150	2,3-Butanedion	431-03-8	Fragrances	Schiff base formation	na	11	Weak	Cat. 1B	6	S	RPMI	S	11	103	–
151	Oxalic acid	144-62-7	Others	No binding	na	15	Weak	Cat. 1B	6	S	DMSO	NS	>200	>200	D
152	4-Allylanisole	140-67-0	Fragrances	Michael acceptor	na	18	Weak	Cat. 1B	6	S	DMSO	S*	99	>200	–
153	Benzyl cinnamate	103-41-3	Fragrances	Michael acceptor/SN2	na	18.4	Weak	Cat. 1B	7	S	DMSO	S*	177	>200	–
154	4,4,4-Trifluoro-1-phenylbutane-1,3-dione	326-06-7	Non cosmetic	Schiff base formation	na	20	Weak	Cat. 1B	6	S	DMSO	S	18	41	–
155	Alpha-iso-Methylionone	127-51-5	Fragrances	Michael acceptor/SN2	na	21.8	Weak	Cat. 1B	7	S	DMSO	S	23	37	–
156	Cyclamen aldehyde	103-95-7	Fragrances	Schiff base formation	na	22	Weak	Cat. 1B	6	S	DMSO	S	20	116	–
157	Undecylenic acid	112-38-9	Preservatives	No binding	na	25	Weak	Cat. 1B	10	S	DMSO	S	18	75	–
158	R(+)-Limonene	5989-27-5	Fragrances	No binding	na	22	Weak	Cat. 1B	17	S	RPMI	S	30	>200	–
159	Tartaric acid	87-69-4	Actives	No binding	na	NC	NS	No cat.	15	NS	RPMI	NS	>200	>200	–

160	Chlorobenzene	108-90-7	Non cosmetic	No binding	na	NC	NS	No cat.	6	NS	DMSO	NS	>200	>200	-
161	Sulphamic acid	121-57-3	Non cosmetic	No binding	na	NC	NS	No cat.	6	NS	RPMI	NS	>200	>200	-
162	4-Hydroxybenzoic acid	99-96-7	Preservatives	No binding	na	NC	NS	No cat.	6	NS	DMSO	NS	>200	>200	-
163	Saccharin	81-07-2	Non cosmetic	Acyl transfer agent	na	NC	NS	No cat.	6	NS	RPMI	NS	>200	>200	-
164	1-Bromobutane	109-65-9	Non cosmetic	SN2	na	NC	NS	No cat.	6	NS	DMSO	NS	>200	>200	-
165	6-Methyl coumarin	92-48-8	Fragrances	Michael acceptor	na	NC	NS	No cat.	6	NS	DMSO	S	94	187	D
166	Ethyl benzoylacetate	94-02-0	Fragrances	No binding	na	NC	NS	No cat.	6	NS	DMSO	NS	>200	>200	-
167	Vinylidene dichloride	75-35-4	Non cosmetic	Michael acceptor	na	NC	NS	No cat.	6	NS	DMSO	NS	>200	>200	-
168	Benzoic acid	65-85-0	Preservatives	No binding	na	NC	NS	No cat.	9	NS	RPMI	NS	>200	>200	-
169	Ethyl vanillin	121-32-4	Fragrances	Schiff base formation	na	NC	NS	No cat.	6	NS	DMSO	NS*	66	66	-
170	Sulphanilamide	63-74-1	Non cosmetic	No binding	na	NC	NS	No cat.	6	NS	RPMI	NS	>200	>200	-
171	Kanamycin (sulphate)	25389-94-0	Non cosmetic	No binding	na	NC	NS	No cat.	6	NS	RPMI	NS	>200	>200	-
172	Streptomycin sulphate	3810-74-0	Non cosmetic	Schiff base formation	na	NC	NS	No cat.	6	NS	RPMI	NS	>200	>200	-
173	Citric acid	77-92-9	Preservatives	No binding	na	NC	NS	No cat.	10	NS	RPMI	NS	>200	>200	-
174	Polyethylene glycol	25322-68-3	Others	No binding	na	NC	NS	No cat.	10	NS	RPMI	NS	>200	>200	-
175	Benzyl alcohol	100-51-6	Preservatives	No binding	na	NC	NS	No cat.	9	NS	DMSO	NS	>200	>200	-

^a SN2: substitution nucleophilic bi-molecular; SNAr: nucleophilic aromatic substitution (Patlewicz et al., 2008).

^b Human skin sensitizing potency category (Basketter et al., 2014); na: not available.

^c LLNA (TC429 and EU test method B.42 (OECD, 2010a; UN, 2011)); na: not available.

^d LLNA potency category based on the EC3 value as proposed by Kimber et al. (2003).

^e Data sources: 1: Arts et al. (2008); 2: Basketter et al. (2003); 3: Basketter et al. (1999); 4: Dearman et al. (1999); 5: Estrada et al. (2003); 6: Gerbenick et al. (2005); 7: Kern et al. (2010); 8: Kimber et al. (1995); 9: Natsch et al. (2009); 10: proprietary data; 11: Ryan et al. (2002); 12: Warbrick et al. (1999); 13: Roberts et al. (2011); 14: Natsch et al. (2013); 15: ICCVAM, 2008; 16: SCCP/0983/06 opinion on COLIPA N° A50.

^f S* or NS* U-SENSTM classifications were obtained through the sum of scores determined by the application of the rules. Substances with EC150 and CV70 > 200 µg/mL were usually classified as NS. Application of the 6 rules strategy might exceptionally conduct to an S classification for substances (# 59 and # 63) that did not reach the positive (CD86 SI ≥ 150%) and cytotoxic (viability < 70%) thresholds. For the needs of analysis, the overall CV70 value was also attributed by default to the overall EC150 (values highlighted in italic) for substances reaching the viability threshold but not the positive threshold.

^g Human versus LLNA and/or U-SENSTM S/NS classifications; ✓: concordant hazard classifications between LLNA and human; ✗: discordant hazard classifications between U-SENSTM and human; -: discordant hazard classifications between LLNA and U-SENSTM without any human data available; D: discordant hazard classifications between LLNA and U-SENSTM without any human data available.

specific tests are known. The recent and thorough review by Basketter et al. (2014) constitutes the most accurate and complete human reference set to date.

Of the 175 substances constituting the present database, 166 substances had LLNA data whilst 101 had human data. A total of 92 substances had both human and LLNA data (Fig. 3 and Table 1).

The hazard identification of potent and frequent sensitizers (human categories 1–4) was perfectly performed by the U-SENSTM test method, with a sensitivity of 100%. Irrespective of the data set ($N \geq 74$) analyzed, the sensitivity of the U-SENSTM test method against either human or LLNA data was equal to or above to 89%. When confronted to the 101 human data set, with S ranging from class 1 to 5 and NS in class 6 (Basketter et al., 2014), the U-SENSTM test method showed a high sensitivity of 89%, a specificity of 65% and an overall accuracy of 85% (Fig. 5).

Next, we focussed on the 92 substances subset for which both human and LLNA data were available. The predictive capacity of the U-SENSTM test method was comparable with respect to both referentials, with of 90% sensitivity, 60% specificity and 85% accuracy with regards to human data and 92% sensitivity, 55% specificity, and 84% accuracy with regards to LLNA (Fig. 5). Despite the lower specificity, we concluded that the predictive ability of U-SENSTM is comparable for both human and LLNA data.

To evaluate the overall predictive capacity of U-SENSTM, all 175 substances present in the database were examined. The *in vivo* reference classification was defined as follows: priority to human data (i.e. for substances where both human and LLNA data were available, human data were taken as reference). Consequently, in the absence of human data, the reference classification was given by the LLNA (Table 2). On this global set, U-SENSTM showed high sensitivity of 90% and 79% specificity giving an overall accuracy of 88% and a kappa of 64% indicating a good agreement despite the unbalanced data set (81% S *in vivo* versus 19% NS *in vivo*).

The considerable increase in specificity compared to that calculated for the 92 substances can be explained by the human dataset; the predictive values for the 9 substances having only human data were of 86% for sensitivity and 100% for specificity. In parallel, sensitivity for the 74 substances with only LLNA data was 91% and specificity 94%. Indeed, 6 out of 7 *in vitro* false positive relative to the 175 *in vivo* reference classifications were concentrated in the 92 substances common set. Moreover, 13 out of 14 *in vitro* false negative fall into human potency class 5 as defined by Basketter et al. (a rare cause of ACD except perhaps in special circumstances, e.g. use in topical medicaments) or EU-CLP Cat. 1B.

3.1.3. Graduation of the potency

The correlation between the U-SENSTM quantitative parameters EC150 for CD86 reactivity and CV70 for cytotoxicity (alone or in combination) and the EU-CLP categories, to allow to discriminate the more potent sensitizers from the weaker and non-sensitizers, was evaluated. Fig. 6 shows the distribution of the plots (EC150 against CV70) in respect to their EU-CLP classifications. ROC curves (not shown) were used to evaluate the predictivity of the individual readouts (EC150 and CV70) as well as of their (equally weighted and linear) combination.

This statistical analysis resulted in the definition of two cut-off levels: 40 µg/mL for EC150 and 100 µg/mL for CV70 where substances with EC150 ≤ 40 µg/mL and CV70 ≤ 100 µg/mL correspond to Cat. 1A and all others as Cat. 1B/No Cat. When applied, this rule correctly predicted 82% of Cat. 1A (41/50) and 73% of Cat. 1B/No Cat (85/116).

Next, the informative value of both EC150 and CV70 parameters for human potency evaluation was investigated (Fig. 7). Two groups were defined upon considerations of the 6 human potency classes: group A composed of classes 1 and 2 (25 strong sensitizers), and group B composed of classes 3–6 (76 weak sensitizers

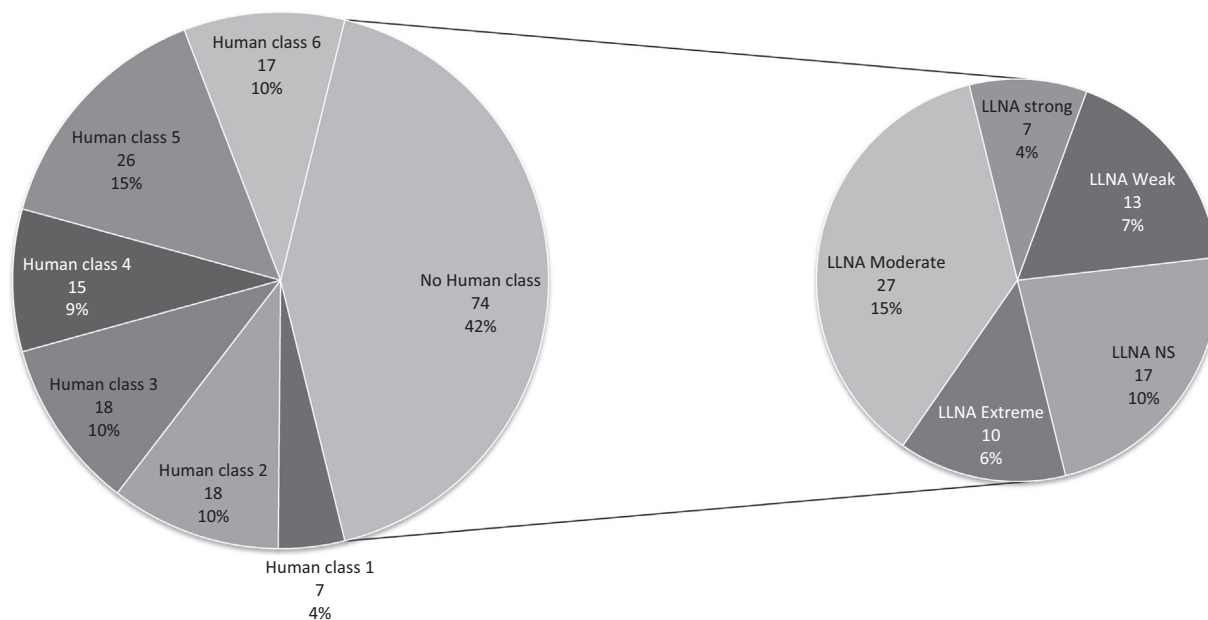


Fig. 3. Distribution of human and LLNA potency classes of 175 substances. The number below each class corresponds to the number of substances in the respective class. The percentage reflects the class representation within the overall data set of 175 substances.

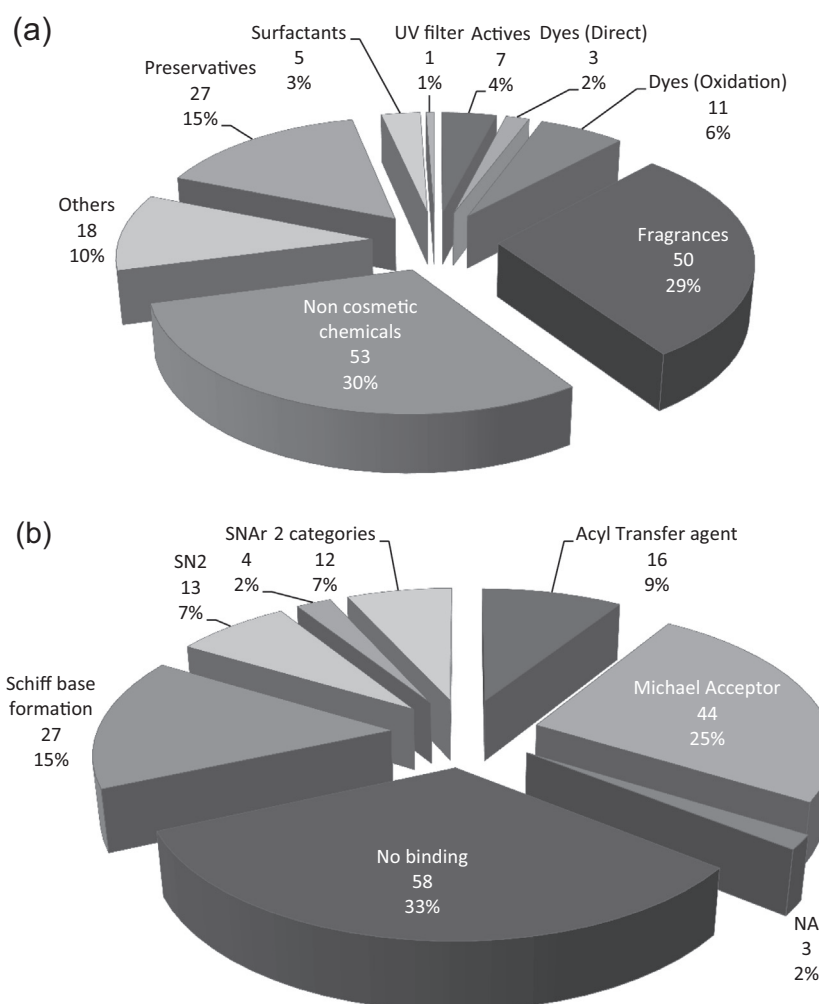


Fig. 4. Distribution of the evaluation set related to 175 substances grouped by (a) use categories and (b) the corresponding reactivity classes according to Toxtree v2.6.0 (Patlewicz et al., 2008). SN2 and SNAr correspond to the nucleophilic substitutions (bi-molecular and aromatic, respectively).

to non-sensitizers). ROC curve analysis led to determination of a cut-off level of 7 µg/mL for EC150 where substances with $EC150 \leq 7$ µg/mL fall into group A and all others in group B. With this cut-off, 76% of human group A (19/25) and 82% of human group B (62/76) were correctly predicted. In this case, the CV70 did not provide additional discriminative information.

Altogether, these results show the interesting capacity of the U-SENSTM test method to inform potency evaluation.

3.2. Ring trial results

The reproducibility and transferability of the U-SENSTM test method in a ring trial were assessed before submitting it to formal validation.

The within-laboratory reproducibility (WLR) was evaluated by testing 21 substances (14 coded + 7 non-coded) in three independent experiments, in one laboratory (L'Oréal). Consistent predictions for 20 out of 21 substances resulted in a WLR of 95% (Table 3, Lead Lab). Furthermore, similar levels of EC150 values and CV70 values were observed for all substances (Table 3). These highly consistent results indicated that the U-SENSTM delivered reliable and reproducible data within a laboratory.

The between-laboratory reproducibility (BLR) was assessed in 4 laboratories: Bioassay (Lab A), CiToxLab (Lab B) and WIL Research (Lab C) and L'Oréal (Lead Laboratory). Labs A to C assessed 14 substances in a single experiment whilst the Lead Laboratory conducted 3 experiments per substance, from which the majority classification was used for the BLR evaluation. Cross comparisons of the results showed consistency between the 4 testing laboratories; the same prediction was made for 11 of the 14 substances, i.e. a BLR of 79% (Table 3). The BLR for the pair-wise laboratory comparisons ranged between 86% (12/14 substances) and 93% (13/14 substances). Similar levels of CV70 values were observed for all substances, EC150 values were comparable for the majority of substances with the exception of phenyl benzoate (#116), 12-bromo-1-dodecanol (#143) and 6-methylcoumarin (#153) (Table 3). The transferability, within- and between-laboratory

Table 2

2 × 2 contingency table including predictive parameters comparing the U-SENSTM classification with the *in vivo* reference classification i.e. a combination of human and LLNA classifications.

		Non-sensitizer	Sensitizer	Sum
Human & LLNA	Non-sensitizer	27	7	34
	Sensitizer	14	127	141
	Sum	41	134	175

Specificity: 79.4%.

Sensitivity: 90.1%.

Accuracy: 88.0%.

Kappa: 64.4%.

reproducibility of the U-SENSTM are comparable (EURL ECVAM, 2013, 2014) to those obtained with positively validated *in vitro* skin sensitization test methods, such as the DPRA or the KeratinoSensTM. These promising results encouraged us to further explore the WLR and BLR. A formal and larger multi-laboratory validation study is currently ongoing.

4. Discussion

Evaluating skin sensitization is one of the major concerns in the safety assessment of cosmetic ingredients. With the ban on animal testing, there is a need to define an efficient strategy that is able to predict with high accuracy not only the skin sensitization hazard of a chemical but also its related potency. The U-SENSTM assay is an *in vitro* test method employing the U937 cell line to measure, by flow cytometry, both the cytotoxicity and CD86 expression induced by test substances. Its experimental procedures have been described in detail, including an optimized data analysis and a model allowing the prediction of two classes, S or NS.

Based on a global set of 175 substances, the U-SENSTM test method showed a high sensitivity of 90%, a specificity of 79% and an overall accuracy of 88% with a kappa of 64%, in good agreement with the *in vivo* profiles despite the unbalanced data set (81% S

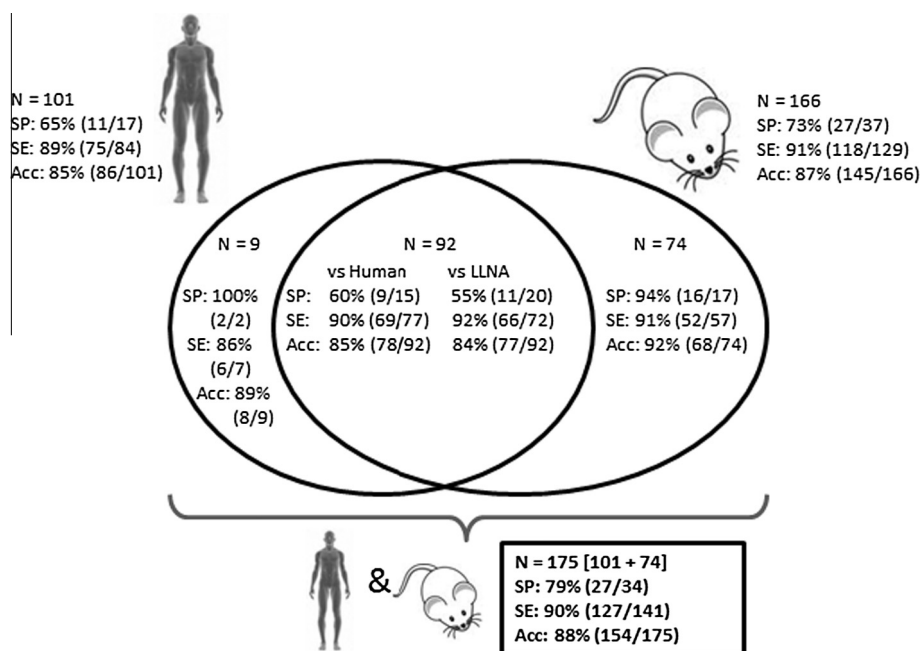


Fig. 5. Predictive performance of the U-SENSTM assay compared to human and LLNA data: 175 substances with *in vivo* data (101 human, 166 LLNA and 92 for the common subset). Human categories were dichotomised by considering category 6 (Basketter et al., 2014) as non-sensitizers and the categories 1–5 as sensitizers. N: number of substances. The values in parenthesis are the number on substances correctly predicted out of the evaluated substances. SP: specificity; SE: sensitivity; Acc: Accuracy.

Table 3

WLR and BLR for U-SENSTM assay: EC150 and CV70 values for 21 chemicals (3 experiments) in 1 laboratory for the WLR and for 14 substances (1 experiment) in 4 laboratories. L'Oréal was the lead laboratory (Lead Lab). The other laboratories involved were Bioassay (Lab A), CToxLab (Lab B) and WIL Research (Lab C).

No ^a	INCI name ^b	U-SENS TM classification						EC150 (µg/mL)						CV70 (µg/mL)					
		Lead Lab			Lab A	Lab B	Lab C	Lead Lab			Lab A	Lab B	Lab C	Lead Lab			Lab A	Lab B	Lab C
		Exp 1	Exp 2	Exp 3	Exp 1	Exp 1	Exp 1	Exp 1	Exp 2	Exp 3	Exp 1	Exp 1	Exp 1	Exp 1	Exp 2	Exp 3	Exp 1	Exp 1	Exp 1
11	Lauryl gallate	S	S	S	S	S	S	0.7	0.4	0.1	0.3	0.3	0.3	1	2	1	3	1	2
17	MDBGN	S	S	S	S	S	S	3	1	1	2	2	1	5	4	3	2	5	4
21	Diethyl maleate	S	S	S	S	S	S	7	0.1	2	10	3	3	30	33	31	27	26	31
35	Phenyl benzoate	S	S	S	S	S	S	101	96	75	192	7	61	>200	>200	>200	>200	151	>200
36	TMTD	S	S	S	S	S	S	0.1	0.1	0.1	0.1	0.1	0.1	3	3	3	3	4	0.1
63	Benzyl benzoate	S	S	S	NS	S	NS	>200	>200	>200	>200	180	>200	>200	>200	>200	>200	>200	>200
89	Salicylic acid	NS	NS	NS	NS	NS	NS	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200
109	4-Nitrobenzyl bromide	S	S	S	S	S	S	0.4	0.4	0.4	0.5	0.4	0.4	0.4	0.4	0.4	0.5	0.4	0.4
116	m-Phenylenediamine	S	S	S	S	S	S	4	4	5	3	7	2	>200	137	>200	135	>200	>200
127	m-Aminophenol	S	S	S	S	S	S	11	3	10	9	4	7	128	141	141	123	131	184
143	12-Bromo-1-dodecanol	S	S	S	S	S	S	9	11	15	11	1	17	28	28	36	34	24	31
153	4-Allylanisole	S	S	S	S	S	S	99	29	25	62	74	54	>200	>200	129	>200	>200	>200
160	Chlorobenzene	NS	NS	S	NS	S	NS	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200
165	6-Methylcoumarin	S	S	S	NS	S	S	94	138	64	>200	24	65	187	>200	>200	>200	>200	>200
71	Methyl salicylate ^c	NS	NS	NS				>200	>200	>200				>200	>200	>200			
72	Vanillin ^c	S	S	S				121	49	149				>200	>200	>200			
94	Glycerol ^c	NS	NS	NS				>200	>200	>200				>200	>200	>200			
161	Sulphanilic acid ^c	NS	NS	NS				>200	>200	>200				>200	>200	>200			
162	4-Hydroxybenzoic acid ^c	NS	NS	NS				>200	>200	>200				>200	>200	>200			
164	1-Bromobutane ^c	NS	NS	NS				>200	>200	>200				>200	>200	>200			
173	Citric acid ^c	NS	NS	NS				>200	>200	>200				>200	>200	>200			

^a Numbers refer to Table 1.

^b In bold: based on the experimenter's observations on our historical data set, chemicals for which potential interfering factors could be observed (e.g. colour interferences with cytometry parameters or poor solubility).

^c Non coded substances.

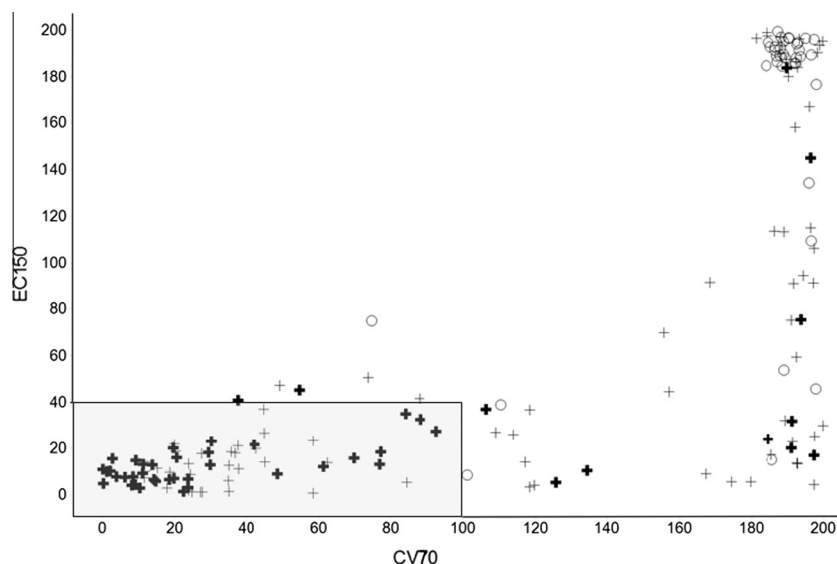


Fig. 6. Distribution of EC150 and CV70 U-SENSTM results of 166 substances according to the EU-CLP classification based on LLNA data: Cat. 1A (+), Cat. 1B (+) and No Cat. (○). 39 plots with EC150 and CV70 > 200 µg/mL: 38 Cat. 1B/No Cat. and 1 Cat. 1A.

in vivo versus 19% NS *in vivo*). This information indicates the ability of the U-SENSTM assay to discriminate sensitizers from non-sensitizers. However, this assay is not recommended as a standalone method as it addresses only DC activation which is one of the events describing the skin sensitization pathway.

As the applicability domain is an important feature of alternative assays, a substance set covering a wide range of industrial applications and chemical reactivities was selected in order to characterize the applicability domain of the U-SENSTM assay. Several classes of cosmetics ingredients (fragrances, dyes, preservatives, actives, surfactants and UV filters) could potentially induce interferences in the U-SENSTM assay (volatility, colour, cytotoxicity, solubility, reproducibility). When focusing on the false negative and false positive predictions, no specific class could be singled out or excluded from the applicability domain, the misclassified

substances being assigned to different classes. Interestingly, substances reported to be pre-haptens or pro-haptens were all correctly predicted by the U-SENSTM assay.

The applicability of water-based assays for lipophilic compounds characterized by a high octanol–water partition coefficient was investigated by Takenouchi et al. (2013); these authors concluded that non-water soluble compound could be excluded. In the study presented herein, the U-SENSTM test method was able to correctly predict such test substances: abietic acid (#39), hexyl cinnamic aldehyde (#62), benzyl benzoate (#63), undec-10-enal (#142), 12-bromo-1-dodecanol (#143), 1-bromohexane (#148), benzyl cinnamate (#153) and cyclamen aldehyde (#156).

Another source of false negative results is the possible colour interference of dyes and fluorescence used in flow cytometry. However, the dyes examined did not cause such interference,

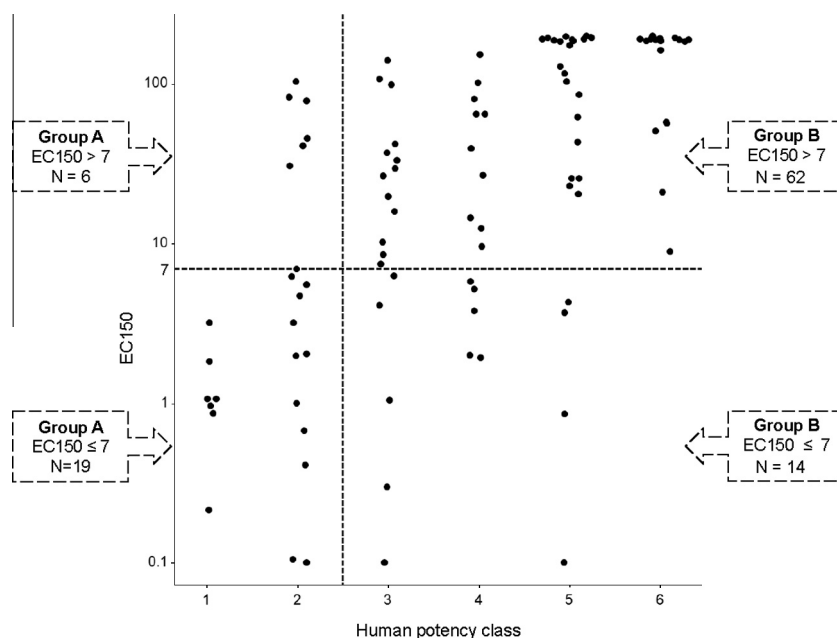


Fig. 7. EC150 U-SENSTM results of 101 substances according to the human potency classes 1–6.

and were correctly identified as sensitizers. Furthermore, of the 15 ascertained false negatives none were dyes. Colour interference was therefore not a concern for the substance set studied herein.

Phthalic anhydride (#113), a strong LLNA sensitizer and a human respiratory sensitizer, featured none of the characteristics known to potentially cause false negative results in the U-SENSTM assay. Upon contact with water, phthalic anhydride is known to undergo rapid hydrolysis (half-life less than a minute at 25 °C) and lead to the formation of the presumed non-sensitizer phthalic acid (OECD, 2005). This hydrolysis, which, supposedly takes place in the U-SENSTM cell culture medium incubated at 37 °C, but not during the topical application in the LLNA (in a acetone/olive oil-based vehicles), may account for the false-negative result obtained with phthalic anhydride in the U-SENSTM test method.

Membrane disrupting substances can theoretically lead to false positive results due to a non-specific increase of CD86 expression. In this study, 3 out of 7 false positives relative to the *in vivo* reference classification were surfactants (#86: sodium lauryl sulphate; #90: octanoic acid, #96: Tween 80). Also, phenol (#97), which is known to interact with cell membranes and therefore also used as a disinfectant, was classified as false positive. As a result, the U-SENSTM assay is at best only partially suitable for membrane-disrupting substances.

Altogether, results obtained with the dataset of 175 substances appear promising and showed that the U-SENSTM assay offers a large applicability domain. However, as for any *in vitro* method, technical limitations as mentioned above and the physico-chemical properties of the substances have to be considered when interpreting the data.

Accurate hazard identification of skin sensitizers represents the first step in a much more challenging process of safety and risk assessment for which an appreciation of the sensitizing potency is needed. Therefore, the ultimate target of current efforts aimed at developing integrated alternative approaches to assess skin sensitization is to deliver quantitative information. This could then allow to fine tune the classification into potency classes, from weak, moderate to strong or extreme sensitizer. In this respect, the relationship between the EC3 value of the LLNA and an appreciation of human potency based on clinical experience has been established (Basketter et al., 2005, 2007) and proposed for quantitative risk assessment (Kimber et al., 2008; Api et al., 2008). In order to evaluate the ability of the U-SENSTM assay to provide information with regard to the sensitizing potency of a specific compound, we analyzed the relationship between the 2 quantitative parameters of the U-SENSTM assay, the EC150 and the CV70 values, and the potency classes defined on the basis of LLNA EU-CLP classification or clinical data. A tentative approach based on ROC curves for the determination of cut off values for EC150s (and to a lesser extent for CV70s) was developed to identify the extreme and strong sensitizers (EU-CLP classification Cat. 1A based on LLNA or Human potency classes 1 and 2 according to Basketter et al., 2014). As already proposed by Jaworska et al. (2013), this type of information could complement the quantitative information provided by other assays, like the trend for induction at lower concentrations for strong sensitizers with the KeratinoSensTM assay (Emter et al., 2010), or the level of peptide depletion (Gerberick et al., 2007) in addition to kinetic measurements of reactivity (Roberts et al., 2008) with the DPRA.

In the context of the AOP integrated approaches to testing and assessment (IATA) and in particular the integrated testing strategies (ITS), the question of the interchangeability of the tests methods addressing the same key event of the AOP was raised (Urbisch et al., 2014). For their analysis, Urbisch and co-workers compiled the results of the U937-CD86 (Natsch et al., 2013), the mMUSST (Bauch et al., 2011) and the h-CLAT assays. Data for both U937 based assays (U937-CD86 and mMUSST) were available for 44

substances; it appeared that 12 out of the 44 compounds were differently predicted by the two assays resulting in an interchangeability of 73%. Regarding the h-CLAT and the two U937-CD86/mMUSST assays, an interchangeability of 72% was reported based on a common set of 105 substances. To get the most complete analysis, the U-SENS results were also compared to the results of the 3 assays used as surrogates for dermal dendritic cells reported by Urbisch et al. (2014), namely the U937-CD86, the mMUSST and the h-CLAT assays. Interchangeabilities of 78% (84/108), 73% (37/51) and 78% (94/120) were obtained respectively. The differences observed can be explained by positive outcomes observed in the U-SENSTM assay while negative ones were obtained in at least one of the 3 other assays. The U-SENSTM appeared to be more sensitive than the other methods as it allowed the correct classification of a number of known LLNA strong to extreme (e.g. oxazolone, 7,12-dimethylbenz[α]anthracene, 4-nitrobenzylbromide, squaric acid diethyl ester) or human class 1 and 2 sensitizers, (e.g. tetrachlorosalicylanilide, lauryl gallate, isoeugenol, methyl dibromoglutaronitrile), which were misclassified by at least one of the other assays. In addition, the majority of borderline substances classified in human potency class 5 were predicted as Sensitizer with the U-SENSTM test method (9 out of 13 substances) and Non-Sensitizers with the h-CLAT assay (8 out of 13 substances).

Overall, the U-SENSTM assay, under an EURL-ECVAM validation process, relies on a more conservative approach than other assays do.

In summary, with a sensitivity of about 90% and only slightly lower specificity and accuracy in predicting skin sensitization potential of substances, the U-SENSTM test method could complement the already validated and approved battery of assays to assess skin sensitization once formally validated. The extensive U-SENSTM database presented herein can already be used in any initiative aimed at developing integrated testing strategies for skin sensitization.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The [Transparency document](#) associated with this article can be found in the online version.

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