

RESEARCH ARTICLE

Application of Spectro-DPRA, KeratinoSens™ and h-CLAT to estimation of the skin sensitization potential of cosmetics ingredients

Sun-A Cho^{1,2} | Minseok Choi¹ | Sae-Ra Park¹ | Susun An¹ | Jae-Hak Park² 

¹Safety & Microbiology Research Lab, AmorePacific Corporation R&D Unit, Yongin-si, Republic of Korea

²Department of Laboratory Animal Medicine, Research Institute for Veterinary Science, BK21 PLUS Program for Creative Veterinary Science Research, College of Veterinary Medicine, Seoul National University, Seoul, Republic of Korea

Correspondence

Jae-Hak Park, Laboratory Animal Medicine, College of Veterinary Medicine, Seoul National University, Gwanak-gu, Seoul, 08826, South Korea.

Email: pjhak@snu.ac.kr

Funding information

Amorepacific R&D Unit

Abstract

Ethical issues in animal toxicity testing have led to the search for alternative methods to determine the skin sensitization potential of cosmetic products. The emergence of ethical testing issues has led to the development of many alternative methods that can reliably estimate skin sensitization potentials. However, a single alternative method may not be able to achieve high predictivity due to the complexity of the skin sensitization mechanism. Therefore, several prediction assays, including both in chemico and in vitro test methods, were investigated and integrated based on the skin sensitization adverse outcome pathway. In this study, we evaluated three different integrated approaches to predict a human skin sensitization hazard using data from in vitro assays (KeratinoSens™ and human cell line activation test [h-CLAT]), and a newly developed in chemico assay (spectrophotometric direct peptide reactivity assay [Spectro-DPRA]). When the results of the in chemico and in vitro assays were combined, the predictivity of human data increased compared with that of a single assay. The highest predictivity was obtained for the approach in which sensitization potential was determined by Spectro-DPRA followed by final determination using the result of KeratinoSens™ and h-CLAT assays (96.3% sensitivity, 87.1% specificity, 86.7% positive predictive value, 96.4% negative predictive value and 91.4% accuracy compared with human data). While further optimization is needed, we believe this integrated approach may provide useful predictive data when determining the human skin sensitization potential of chemicals.

KEYWORDS

adverse outcome pathway, integrated approaches, skin sensitization, (AOP), animal alternative, spectrophotometric DPRA

1 | INTRODUCTION

One of the common side effects of cosmetic products, allergic contact dermatitis, is a common health hazard affecting 15%–20% of people worldwide (Adam, Pichler, & Yerly, 2011). It is caused by repeated exposure to a non-immunogenic, low-molecular-weight chemical called hapten (Kimber, Basketter, Gerberick, & Dearman, 2002;

Kimber, Basketter, Gerberick, Ryan, & Dearman, 2011). Allergic contact dermatitis develops in two phases, i.e., a sensitization phase and an elicitation phase (Adler et al., 2011; Basketter et al., 2012). In the sensitization phase, antigen-specific T cells are generated. When the antigen/allergen binds to the peptides of the skin (haptization), these chemical-peptide adducts interact with keratinocytes and Langerhans cells (LCs). Keratinocytes are the main cell population in

the skin epidermis and undergo activated responses when they encounter an antigen/allergen. One example of these responses, the oxidative stress response via Keap1/Nrf2-ARE (antioxidant response element) is a well-known toxicity pathway (Natsch, 2010; Natsch & Emter, 2008). LCs, which are cutaneous immature dendritic cells, recognize the hapten-protein conjugate. These cells take up and process the hapten-protein conjugate and migrate to regional lymph nodes through lymphatics. In the lymph node, these LCs present the antigen to T lymphocytes and trigger an antigen-specific T-cell response. During migration, LCs undergo differentiation and maturation processes, wherein various regulatory cytokines and cell surface maturation biomarkers, such as CD54 and CD86, are expressed (Sakaguchi et al., 2006).

The ban on testing cosmetics or their ingredients on animals in the EU market (EU, 2006; EU, 2008; EU, 2009) affected the cosmetic industry. As a result, a reliable toxicity test that did not involve animal experiments, but ensured the safety of cosmetics and their ingredients needed to be established. Many alternative testing methods have been developed to predict the endpoint of animal toxicity testing for decades. Alternative methods were developed in accordance with the mechanism of skin sensitization. However, due to the complexity of the biological processes involved in skin sensitization reactions, it has been proposed that the assessment of skin sensitization using a single animal alternative test is not adequate (Corsini et al., 2009; dos Santos et al., 2009).

The Organization for Economic Co-operation and Development (OECD) presented the concept of adverse outcome pathway (AOP), which represents a series of processes from the molecular initiation event and key event (OECD, 2012). Currently, there are several ongoing approaches to develop an integrated testing strategy (ITS) for achieving higher predictivity for skin sensitization potential combined with OECD Test Guideline methods or individual laboratory assay development (Hirota, Ashikaga, & Kouzuki, 2018; Jaworska, Dancik, Kern, Gerberick, & Natsch, 2013; Jaworska, Harol, Kern, & Gerberick, 2011; Jaworska et al., 2015; Macmillan, Canipa, Chilton, Williams, & Barber, 2016; Macmillan & Chilton, 2018; Nukada, Miyazawa, Kazutoshi, Sakaguchi, & Nishiyama, 2013; Otsubo et al., 2017; Takenouchi et al., 2015; Urbisch et al., 2015). In addition, the OECD in collaboration with EURL ECVAM, proposed a roadmap for developing an AOP-based integrated approach to testing and assessment (IATA)/ITS and has attempted to develop formally validated and regulated strategies in the approach to skin sensitization predictivity (Kinsner-Ovaskainen et al., 2012).

Based on these research guidelines, we applied AOP-based IATA/ITS to the newly developed in-chemico assay, spectrophotometric direct peptide reactivity assay (Spectro-DPRA), to evaluate hapten-peptide reactivity (Cho et al., 2014; Cho, An, & Park, 2019). We applied this assay in combination with the in vitro assays, KeraTinoSens™ and human cell line activation test (h-CLAT), to evaluate skin cell activation and immune cell activation or maturation, respectively. We compared this with existing approaches to develop a reliable integrated prediction approach for skin sensitization of cosmetic ingredients.

2 | MATERIALS AND METHODS

2.1 | Test chemicals

The chemical names, class, molecular weight, chemical abstract system (CAS) number, log K_{ow} , the classification by human data and local lymph node assay (LLNA) data (animal data) of 58 test materials are presented in Table 1. The test substance was selected based on the results of an in vivo test of the cosmetic ingredient (LLNA and human data: Basketter et al., 2014; Bauch et al., 2012; Cruz et al., 2009; Gerberick et al., 2005; Nukada et al., 2012; Pang & Fiume, 2001; Urbisch et al., 2015; Yamashita, Shinoda, Hagiwara, & Itagaki, 2015). We used most of the test chemicals recommended to confirm the performance standard in skin sensitization evaluations (ICCVAM, 2010). We also included an additional 15 chemicals in this study that are widely used as cosmetic ingredients (α -linolenic acid, squalene, maleic acid, oleic acid, linoleic acid, succinic acid, *p*-anisic acid, caprylyl sulfobetaine, hydroxyacetophenone, apigenin, 2,3-butanediol, caprylic/capric glycerides, 1,2-hexanediol, 1,2-octanediol, 2-phenoxyethanol). There are no published data on LLNA and human data for these 15 substances. However, they are widely used in the cosmetics field and classified as non-sensitizers without any reported sensitization.

2.2 | Spectrophotometric direct peptide reactivity assay

Spectro-DPRA is the same evaluation principle with standard DPRA (OECD 442C), but unlike DPRA, it determines the reactivity of chemicals toward two chemical groups by spectrophotometric assay instead of high-performance liquid chromatography analysis. Spectro-DPRA was performed according to our previous protocol (Cho et al., 2014; Cho et al., 2019) (Figure 1). We used two model peptides, a cysteine peptide (Ac-RFAACAA-COOH) and a lysine peptide (Ac-RFAAKAA-COOH) with >95% purity (Peptron Co.). The cysteine peptide reaction solution was prepared by diluting the cysteine peptide stock solution to 400 μ M with 100 mM sodium phosphate buffer (pH 8.0, 1 mM ethylenediamine tetraacetic acid [EDTA]). Chemical reaction solutions were prepared by diluting chemical stock solutions to 2 mM in 100 mM sodium phosphate buffer (pH 8.0, 1 mM EDTA). Ninety microliters of peptide reaction solution and 90 μ L of chemical reaction solution were reacted in a 96-well enzyme-linked immunosorbent assay plate for 24 hours at room temperature. After 24 hours, 20 μ L of a 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB; Sigma-Aldrich) solution solubilized in 100 mM sodium phosphate buffer (pH 8.0) was added to each well of the plate. The mixture was incubated for 3 minutes to achieve a complete reaction between the unreacted peptide and DTNB. Both before the addition of the DTNB solution and after the reaction between unreacted peptide and DTNB, the optical density was measured using an ultraviolet-visual spectrophotometer (absorption wavelength: 412 nm, SpectraMAX 190™; Molecular Devices). The lysine peptide reaction solution was prepared by diluting the lysine peptide stock solution to 200 μ M with 100 mM sodium phosphate buffer (pH 10.0, 1 mM EDTA) before the

TABLE 1 List of tested chemicals

Chemical	CAS	MW	Log K_{ow}	Human		LLNA		
				Group	Reference	Group	EC3 (%)	ECETOCCL ^a
5-Chloro-2-methyl-4-isothiazolin-3-on (MCI)	26172-55-4	149.6	-0.34	S	Bauch et al., 2012	S	0.01	Extreme
1-Chloro-2,4-dinitrobenzene (DNCB)	97-00-7	202.55	2.27	S	Bauch et al., 2012	S	0.05	Extreme
Oxazolone	15646-46-5	217.2	1.51	S	Bauch et al., 2012	S	0.003	Extreme
4-phenylenediamine	106-50-3	108.1	-0.39	S	Bauch et al., 2012	S	0.16	Strong
Glutaric dialdehyde	111-30-8	100.1	-0.18	S	Basketter et al., 2014	S	0.1	Strong
Lauryl gallate	1166-52-5	338.4	6.21	S	Basketter et al., 2014	S	0.3	Strong
Hydroquinone	123-31-9	110.1	1.03	S	Basketter et al., 2014	S	0.11	Strong
Propyl gallate	121-79-9	212.2	1.79	S	Basketter et al., 2014	S	0.32	Strong
Cobalt chloride	7646-79-9	129.84	0.85	S	Bauch et al., 2012	S	4.8	Moderate
Isoeugenol	97-54-1	164.2	2.66	S	Basketter et al., 2014	S	1.2	Moderate
2-Mercaptobenzothiazole	149-30-4	167.3	2.86	S	Bauch et al., 2012	S	1.7	Moderate
Cinnamic aldehyde	104-55-2	132.2	1.82	S	Urbisch et al., 2015	S	3	Moderate
Diethyl maleate	141-05-9	172.2	2.2	S	Basketter et al., 2014	S	5.8	Moderate
Diethylenetriamine	111-40-0	103.20	-2.13	S	Urbisch et al., 2015	S	3.28	Moderate
Ammonium persulfate	231-786-5	228.2	NC	S	Pang & Fiume, 2001	S	1.9	Moderate
Citral	5392-40-5	152.2	3.45	S	Bauch et al., 2012	S	13	Weak
Eugenol	97-53-0	164.2	2.73	S	Basketter et al., 2014	S	13	Weak
Phenyl benzoate	93-99-2	198.2	3.04	S	Bauch et al., 2012	S	20	Weak
Cinnamyl alcohol	104-54-1	134.2	1.84	S	Bauch et al., 2012	S	21	Weak
Imidazolidinyl urea	39236-46-9	388.3	-8.28	S	Bauch et al., 2012	S	24	Weak
Methyl methacrylate	80-62-6	100.1	1.28	S	Basketter et al., 2014	S	90	Weak
Ethylene glycol dimethacrylate	97-90-5	198.2	2.21	S	Bauch et al., 2012	S	28	Weak
Benzocaine	94-09-7	165.2	1.8	S	Urbisch et al., 2015	S	22	Weak
Abietic acid	514-10-3	302.5	6.46	S	Basketter et al., 2014	S	15	Weak
Geraniol	106-24-1	154.3	3.47	S	Basketter et al., 2014	S	26	Weak
Linalool	78-70-6	154.3	3.38	S	Basketter et al., 2014	S	30	Weak
Nickel chloride	7718-54-9	129.6	NC	S	Bauch et al., 2012	NS	NC	NS
α -Linolenic acid	463-40-1	278.43	7.3	NS (WU)		S	9.8	Moderate
Squalene	111-02-4	410.73	14.12	NS (WU)		S	6.1	Moderate
Maleic acid	110-16-7	116.07	0.05	NS (WU)		S	4.1	Moderate
Tartaric acid	87-69-4	150.1	-1.00	NS	Bauch et al., 2012	S	8.7	Moderate

(Continues)

TABLE 1 (Continued)

Chemical	CAS	MW	Log K _{ow}	Human		LLNA			
				Group	Reference	Group	EC3 (%)	ECETOC CL ^a	Reference
Dimethyl sulfoxide	67-68-5	78.1	-1.22	NS	Basketter et al., 2014	S	72	Weak	Gerberick et al., 2005
Sodium lauryl sulfate	151-21-3	288.4	2.42	NS	Bauch et al., 2012	S	14	Weak	Gerberick et al., 2005
α-Hexylcinnamaldehyde	101-86-0	216.3	4.82	NS	Basketter et al., 2014	S	11	Weak	Gerberick et al., 2005
Xylene	1330-20-7	106.2	3.09	NS	Bauch et al., 2012	S	95.8	Weak	Urbisch et al., 2015
Pyridine	110-86-1	79.10	0.80	NS	Bauch et al., 2012	S	72	Weak	Urbisch et al., 2015
R-(+)-Limonene	5989-27-5	136.2	4.83	NS	Basketter et al., 2014	S	69	Weak	Gerberick et al., 2005
Isopropanol	67-63-0	60.1	0.28	NS	Bauch et al., 2012	NS	NC	NS	Gerberick et al., 2005
Lactic acid	50-21-5	90.1	-0.65	NS	Bauch et al., 2012	NS	NC	NS	Gerberick et al., 2005
Methyl salicylate	119-36-8	152.2	2.0	NS	Basketter et al., 2014	NS	NC	NS	Gerberick et al., 2005
Salicylic acid	69-72-7	138.1	2.24	NS	Bauch et al., 2012	NS	NC	NS	Gerberick et al., 2005
Benzalkonium chloride	8001-54-5	319.60	2.93	NS	Basketter et al., 2014	NS	NC	NS	Urbisch et al., 2015
Glycerol	56-81-5	152.2	2.0	NS	Bauch et al., 2012	NS	NC	NS	Gerberick et al., 2005
2-Phenoxyethanol	122-99-6	138.2	1.16	NS	Basketter et al., 2014	NS (WU)	NA	NS	
Tween80	9005-65-6	1310.0	4.23	NS	Basketter et al., 2014	NS	NC	NS	Nukada et al., 2012
6-Methylcoumarin	92-48-8	160.2	2.06	NS	Bauch et al., 2012	NS	NC	NS	Gerberick et al., 2005
Fumaric acid	110-17-8	116.1	0.05	NS	Bauch et al., 2012	NS	>25	NS	Urbisch et al., 2015
Oleic acid	112-80-1	282.47	7.73	NS (WU)		S	10.5	Weak	Yamashita et al., 2015
Linoleic acid	60-33-3	280.45	7.51	NS (WU)		S	14.1	Weak	Yamashita et al., 2015
Succinic acid	110-15-6	118.09	0.75	NS (WU)		NS	NC	NS	Yamashita et al., 2015
p-Anisic acid	100-09-4	152.16	1.96	NS (WU)		NS (WU)	NA	NS	
Caprylyl sulfobetaine	15163-36-7	307.5	1.26	NS (WU)		NS (WU)	NA	NS	
Hydroxyacetophenone	99-93-4	136.2	1.35	NS (WU)		NS (WU)	NA	NS	
Apigenin	520-36-5	270.2	3.02	NS (WU)		NS (WU)	NA	NS	
2,3-Butanediol	513-85-9	90.1	-0.92	NS (WU)		NS (WU)	NA	NS	
Caprylic/capric glycerides	85409-09-2	NA	5.29	NS (WU)		NS (WU)	NA	NS	
1,2-Hexanediol	6920-22-5	118.2	0.69	NS (WU)		NS (WU)	NA	NS	
1,2-Octanediol	1117-86-8	188.3	1.67	NS (WU)		NS (WU)	NA	NS	

CAS, chemical abstract system; LLNA, local lymph node assay; MW, molecular weight; NA, not assessed; NC, not calculated; S/NS, sensitizer/non-sensitizer; WU, widely used in cosmetics.

Percentage EC3 data and human data were obtained from Basketter et al., 2014; Bauch et al., 2012; Cruz et al., 2009; Gerberick et al., 2005; Nukada et al., 2012; Pang & Fiume, 2001; Urbisch et al., 2015 and Yamashita et al., 2015. Log K_{ow} was estimated by logP KOWWIN v1.68 program. <http://www.epa.gov/oppt/exposure/pubs/episuite.htm>

^aECETOC CL mean potency classification based on the scheme for LLNA EC3 values presented in the ECETOC Technical Report No. 87.

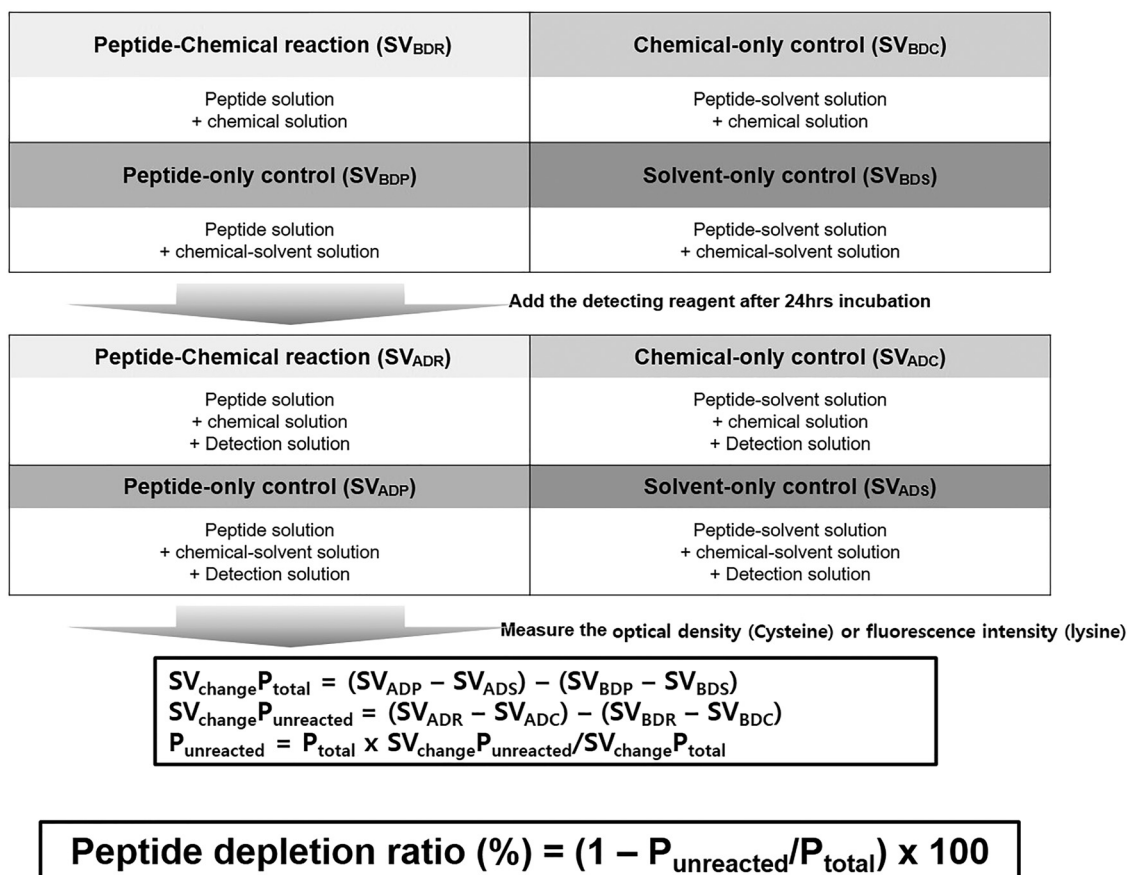


FIGURE 1 Schematic of the Spectro-direct peptide reactivity assay. Every test set was composed of four wells. Change of SV was calculated by measuring optical density or fluorescence intensity just before and after adding the detection reagent. SV_{ADP} and SV_{BDP} are the spectrophotometric values after and before addition of the detection reagent to the peptide-only control, respectively. SV_{ADS} and SV_{BDS} are the spectrophotometric values after and before addition of the detection reagent to the solvent-only control, respectively. SV_{ADR} and SV_{BDR} are the spectrophotometric values after and before addition of the detection reagent to reaction solutions, respectively. SV_{ADC} and SV_{BDC} are the spectrophotometric values after and before addition of the detection reagent to the chemical-only control, respectively. SV, spectrophotometric value

experiment and chemical reaction solutions were prepared by diluting chemical stock solutions to 2 mM with isopropanol. Then, 110 µL of the peptide reaction solution and 110 µL of the chemical reaction solution were reacted in the 96-well clear plate for 24 hours at room temperature. After incubation, 180 µL of the reaction mixtures were transferred to a light-proof black clear-bottom 96-well plate (Greiner Bio-One). Next, 20 µL of a 20 mM fluorescamine solution (prepared in dimethyl sulfoxide) was added to each well of the plate and incubated for 3 minutes to achieve a complete reaction. Fluorescence intensity was measured using a fluorometer before the addition of fluorescamine solution and after the reaction between unreacted peptide and fluorescamine (excitation: 390 nm; emission: 465 nm, 475 nm; Flexstation 3; Molecular Devices). The reactivity of a chemical towards a peptide was expressed as the peptide depletion ratio after 24 hours incubation of the peptide with the chemical, and the peptide depletion rate was calculated as follows: peptide depletion ratio (%) = $(1 - P_{\text{unreacted}}/P_{\text{total}}) \times 100$. If the depletion rate of the cysteine peptide by the test substance exceeded 10% or the depletion rate of the lysine peptide exceeded 30%, it was judged a skin sensitization risk.

2.3 | KeratinoSens™ (ARE-Nrf2 luciferase assay)

The KeratinoSens™ cell line derived from the human keratinocyte cell line, HaCaT, was generously provided by Dr. Andreas Natsch (in vitro toxicology research at Givaudan Fragrances). It contains a stable insertion of a luciferase gene under the control of the ARE-element of the gene AKR1C2. All tests were performed according to the previously published protocol (Natsch et al., 2013). Cells were seeded to three clear bottom white 96-well plates and one clear 96-well plate at a density of 1×10^4 cells/well in Dulbecco's modified Eagle medium (Gibco) with 500 µg/mL G418 (Gibco) and 10% fetal bovine serum (Gibco) for 24 hours at 37°C in a 5% CO₂ atmosphere. The medium was then replaced with medium containing the test substance and a final level of 1% of the solvent vehicle, dimethyl sulfoxide. Each test substance was tested at 12 binary serial dilutions in the range of 0.98–2000 µM. The plates contained five test chemicals with 12 dilutions per column respectively: six wells with the solvent control, one well with no cells for background value and five wells with the positive control, cinnamic aldehyde, in five different concentrations. In each repetition, three parallel replicate plates were prepared for

cytotoxicity measurement using an (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) assay. Cells were incubated in a 37°C and 5% CO₂ atmosphere for 48 hours with the test compounds, and then washed with 200 µL of Dulbecco's phosphate-buffered saline (Gibco). Luciferase activity was measured according to the manufacturer's protocol (One-Glo™ luciferase assay system kit; Promega). After the luciferase activity of each well was measured with luminometer (Promega GloMax® Discover; Promega), fold induction was calculated. Cell viability was also calculated at each test concentration by an MTT assay. Chemicals were classified as positive in the assay if the following three criteria were fulfilled: (1) EC1.5 value is below 1000 µM in all three repetitions or in at least two repetitions; (2) at the lowest concentration with a gene induction above 1.5-fold (i.e., at the EC1.5 determining concentration), the cellular viability is above 70%; and (3) there is an apparent overall dose-response for luciferase induction, which is similar between the repetitions.

2.4 | Human cell line activation test

Human leukemia monocytic cell line (THP-1) was obtained from the American Type Culture Collection. These cells were cultured in RPMI 1640 medium (Gibco) with 100 U/mL penicillin/100 µg/mL streptomycin (Gibco), 10% fetal bovine serum (v/v) (Gibco) and 0.05 mM 2-mercaptoethanol (Gibco) at 37°C and 5% CO₂ atmosphere. The h-CLAT assay was performed as previously described (Sakaguchi, Ryan, Ovigne, Schroeder, & Ashikaga, 2010). THP-1 cells were plated at 1×10^6 cells/mL in a 24-well plate (1 mL per well) and treated with test chemicals for 24 hours at 37°C and 5% CO₂ atmosphere. After 24 hours, cells were washed twice with FACS buffer (phosphate-buffered saline (PBS; Gibco) solution containing 0.1% (w/v) bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, USA)). Cells were then treated for 15 minutes at 4°C with 0.01% (w/v) globulins in FACS buffer. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated antihuman for CD54 or CD86 (antihuman CD54, ICAM-1/FITC [clone 6.5B5; DAKO] using 6 µL/ 3×10^5 cells/100 µL for the antihuman CD86 monoclonal antibody [mAb], 3 µL/ 3×10^5 cells/100 µL for the antihuman CD54 mAb, 3 µL/ 1×10^5 cells/100 µL for the antihuman IgG mAb) for 30 min at 4°C. FITC-labeled mouse IgG1 (clone DAK-G01; DAKO) was used as an isotype control. Cells are washed twice with FACS buffer and stained with a solution containing 0.625 µg/mL of propidium iodide (Sigma-Aldrich). Cell surface marker expression and cell viability were measured using FACS and analyzed with CellQuest software (Becton-Dickinson). In total, 10 000 living cells were analyzed and the relative fluorescence intensity (RFI) was calculated using the following equation for the expression of surface markers in comparison with the isotype control.

$$\text{RFI} = \text{MFI of chemical-treated cells} - \text{MFI of chemical-treated isotype control cells}$$

$$\text{MFI of vehicle-treated cells} - \text{MFI of vehicle-treated isotype control cells}$$

Where MFI = geometric mean fluorescence intensity. When the cell viability with the test chemical was more than 50%, the RIF value of the cell surface marker CD86 was more than 150%, or when

the RFI value of CD54 was more than 200%, it was judged that there was a risk of skin sensitization.

2.5 | Comparison of skin sensitization potential results for in chemico and in vitro method from peptide depletion and local lymph node assay or human data

The skin sensitization potentials of the 58 chemicals determined using peptide depletion were compared with the in vivo data from the LLNA EC3 values or human data (Basketter et al., 2014; Bauch et al., 2012; Cruz et al., 2009; Gerberick et al., 2005; Nukada et al., 2012; Pang & Fiume, 2001; Urbisch et al., 2015; Yamashita et al., 2015). We evaluated the predictivity of Spectro-DPRA, KeratinoSens™ and h-CLAT by calculating the accuracy, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the results based on LLNA EC3 data or human data of the 58 chemicals as follows (Cooper, Saracci, & Cole, 1979):

$$\text{Sensitivity (\%)} = \text{TP} / (\text{TP} + \text{FN}) \times 100,$$

$$\text{Specificity (\%)} = \text{TN} / (\text{TN} + \text{FP}) \times 100,$$

$$\text{Positive predictive value (\%)} = \text{TP} / (\text{TP} + \text{FP}) \times 100,$$

$$\text{Negative predictive value (\%)} = \text{TN} / (\text{TN} + \text{FN}) \times 100, \text{ and}$$

$$\text{Accuracy (\%)} = (\text{TP} + \text{TN}) / (\text{TP} + \text{TN} + \text{FP} + \text{FN}) \times 100,$$

where TP = true positive, TN = true negative,

FN = false negative, and FP = false positive.

2.6 | Comparing the predictivity of the integrated approach to local lymph node assay and human data

Based on the key event of AOP for skin sensitization (OECD, 2012c), we applied an approach that combined two or three test methods. The test scheme is outlined in Figure 2. Approach 1 (Figure 2A) and has two steps. At the first step, the result of Spectro-DPRA shows as the chemical as a sensitizer. It is therefore judged as a sensitizer. However, if the chemical is determined to be a non-sensitizer, we also conducted the KeratinoSens™ and h-CLAT. If both KeratinoSens™ and h-CLAT predicted the chemical to be positive, the chemical is confirmed as a skin sensitizer. If not, the chemical is considered a non-sensitizer. Approach 2 (Figure 2B) is also composed of two steps. At the first step, if the result of Spectro-DPRA shows the chemical to be a sensitizer, it is judged as a sensitizer. However, if it is shown as a non-sensitizer, we conducted the h-CLAT assay. If h-CLAT predicted the chemical as positive, the chemical is considered a skin sensitizer. Approach 3 (Figure 2C) is carried out similarly to Approach 2; however, instead of h-CLAT, we conducted KeratinoSens™ as the additional assay. If KeratinoSens™ predicted the chemical to be a positive, we conclude the chemical to be a skin sensitizer. The "2 out of 3" prediction (Urbisch et al., 2015) uses any two congruent results of the three tests (Spectro-DPRA, KeratinoSens™ and h-CLAT) to determine

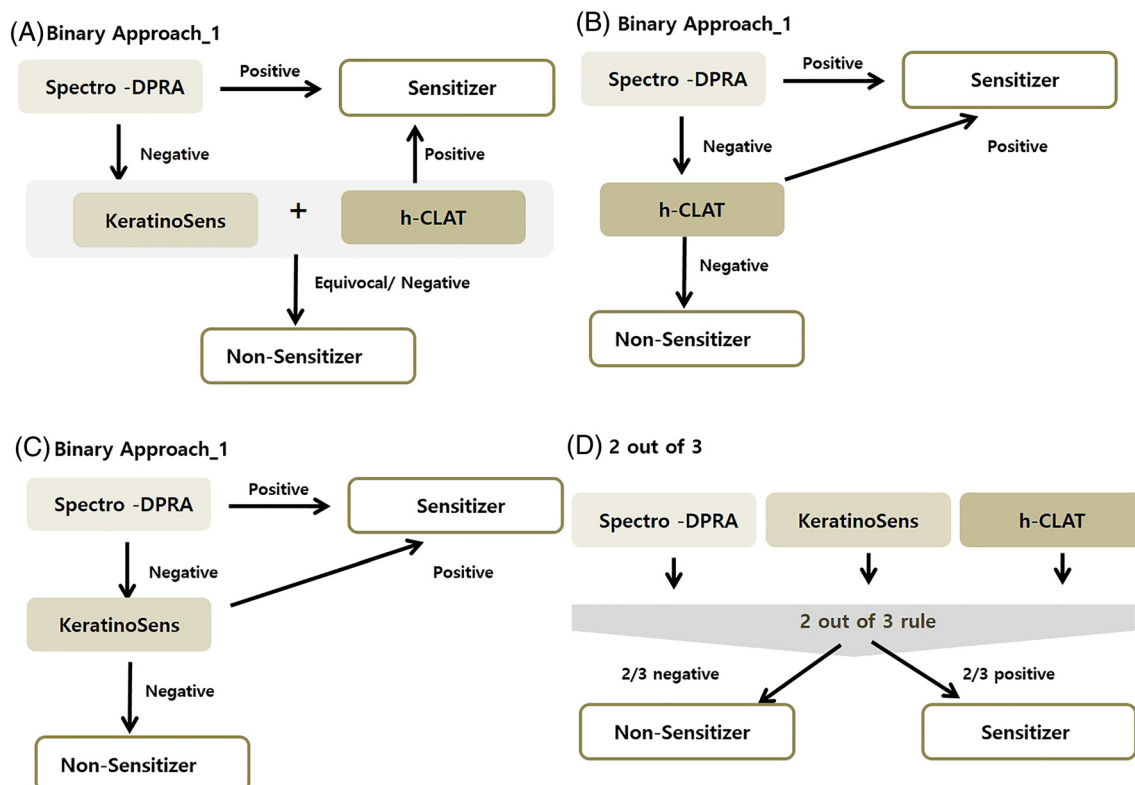


FIGURE 2 Workflow of each integrated approach. A, Binary Approach₁ is composed of two steps. At the first stage, if the result of Spectro-DPRA shows as a sensitizer, it is judged as a sensitizer. However, if it shows as non-sensitizer, we conducted additional tests, both KeratinoSens™ and h-CLAT. If both KeratinoSens™ and h-CLAT predicted the chemical as positive, the chemical is confirmed as a skin sensitizer. If not, the model determines that the chemical is a non-sensitizer. B, Binary Approach₂ is also composed of two steps. At the first stage, if the result of Spectro-DPRA determines that the chemical is a sensitizer, it is judged as a sensitizer. However, if it shows up as a non-sensitizer, we conducted an h-CLAT assay. If h-CLAT predicted the chemical as positive, the chemical was confirmed to be a skin sensitizer. C, Binary Approach₃ is composed the same way as Approach 2, however instead of h-CLAT, we conducted KeratinoSens™. If KeratinoSens™ predicted the chemical as positive, we concluded the chemical was a skin sensitizer. D, “2 out of 3” prediction (Urbisch et al., 2015) uses any two congruent results of the three tests (Spectro-DPRA, KeratinoSens™ and h-CLAT) to determine the overall assessment. If at least two of the three assays were positive, the chemical was classified as a skin sensitizer. If at least two of the three assays were negative, the chemical was classified as a non-sensitizer. h-CLAT, human cell line activation test; Spectro-DPRA, spectrophotometric direct peptide reactivity assay [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

an overall assessment (Figure 2D). If at least two of the three assays were positive, the chemical was classified as a skin sensitizer. If at least two of the three assays were negative, the chemical was classified as a non-sensitizer. We evaluated the predictivity of each approach by calculating the accuracy, sensitivity, specificity, PPV and NPV using Cooper statistics in comparison with LLNA or human data (Cooper et al., 1979).

3 | RESULTS

3.1 | Spectrophotometric direct peptide reactivity assay has a higher predictivity than the KeratinoSens™ and human cell line activation test assays

The results of Spectro-DPRA, KeratinoSens™ and h-CLAT for 58 chemicals are outlined in Table 2. Based on these results, we calculated the sensitivity, specificity, PPV, NPV and accuracy using Cooper statistics (Cooper et al., 1979) (Tables 3 and 4).

Compared with human data, all single assay tests could predict the strong and extreme sensitizers accurately. However, for predicting the weak and moderate sensitizers, the level of prediction varied based on the test method. Spectro-DPRA showed sensitivity, specificity, PPV, NPV and accuracy of 81.5%, 90.3%, 88.0%, 84.8% and 86.2%, respectively. Cobalt chloride, ammonium persulfate, geraniol, linalool and nickel chloride were predicted as false negatives. α -Linolenic acid, α -hexylcinnamaldehyde and linoleic acid were classified as false positives. The h-CLAT assay showed 88.9%, 74.2%, 75.0%, 88.5% and 81.0% for sensitivity, specificity, PPV, NPV and accuracy, respectively. The h-CLAT assay could not predict seven chemicals (isoeugenol, diethylenetriamine, abietic acid, salicylic acid, 2-phenoxyethanol, linoleic acid and succinic acid). However, unlike other assays, h-CLAT assay could correctly predict linalool as a sensitizer. The KeratinoSens™ test showed similar predictivity to the h-CLAT test (88.9% for sensitivity, 74.2% specificity, 75.0% for PPV, 88.5% for NPV and 81.0% for accuracy (Tables 2 and 3).

The overall predictability compared with the LLNA data was lower than that for human data for all test methods. Spectro-DPRA showed

TABLE 2 Detailed results of the predictions based on the individual test methods

Chemical	Human	Spectro-DPRA			KeratoSense™			h-CLAT		ITS prediction		
		LLNA	Cys dep.	Lys dep.	Prediction (cut-off: Cys >10 or Lys >30)		BL	Prediction (cut-off: BL >1.5)		CD54 RFI	CD86 RFI	Prediction (cut-off: CD54 >200 or CD86 >150)
5-Chloro-2-methyl-4-isothiazolin-3-on (MCI)	S	S	22.14	7.38	S	2.96	S	339.84	88.17	S	S	S
1-Chloro-2,4-dinitrobenzene (DNCEB)	S	S	99.29	6.17	S	2.73	S	508.89	320.59	S	S	S
Oxazolone	S	S	17.78	95.05	S	39.04	S	177.24	222.22	S	S	S
4-phenylenediamine	S	S	98.87	86.10	S	8.20	S	132.20	167.80	S	S	S
Glutaric dialdehyde	S	S	1.97	99.51	S	5.30	S	428.83	193.20	S	S	S
Lauryl gallate	S	S	16.79	49.13	S	2.14	S	1165.03	204.13	S	S	S
Hydroquinone	S	S	101.75	81.03	S	9.76	S	151.65	163.90	S	S	S
Propyl gallate	S	S	101.60	54.87	S	3.53	S	659.56	61.16	S	S	S
Cobalt chloride	S	S	-1.49	-2.46	NS	12.21	S	760.50	68.03	S	S	S
Isoeugenol	S	S	97.10	8.53	S	2.70	S	159.06	87.46	NS	S	S
2-Mercaptobenzothiazole	S	S	88.63	1.65	S	1.80	S	480.67	103.13	S	S	S
Cinnamic aldehyde	S	S	21.31	2.55	S	5.37	S	347.50	206.23	S	S	S
Diethyl maleate	S	S	96.99	5.61	S	6.05	S	149.73	200.83	S	S	S
Diethylenetriamine	S	S	19.98	88.30	S	1.15	NS	8.92	10.14	NS	S	NS
Ammonium persulfate	S	S	-13.69	8.75	NS	1.61	S	190.79	187.33	S	S	S
Citral	S	S	17.35	10.96	S	1.63	S	379.92	93.95	S	S	S
Eugenol	S	S	17.38	8.60	S	1.42	NS	220.34	89.77	S	S	S
Phenyl benzoate	S	S	21.67	6.97	S	5.61	S	268.91	132.29	S	S	S
Cinnamyl alcohol	S	S	42.46	7.51	S	6.37	S	246.76	106.88	S	S	S
Imidazolidinyl urea	S	S	-11.95	38.94	S	2.84	S	323.58	538.89	S	S	S
Methyl methacrylate	S	S	11.05	12.18	S	8.89	S	231.87	80.51	S	S	S
Ethylene glycol dimethacrylate	S	S	44.78	-2.93	S	10.44	S	216.78	106.43	S	S	S
Benzocaine	S	S	0.61	43.73	S	5.30	S	111.48	150.69	S	S	S
Abietic acid	S	S	80.55	4.03	S	10.21	S	110.38	91.18	NS	S	S
Geraniol	S	S	5.56	18.04	NS	1.59	S	210.24	95.56	S	S	S
Linalool	S	S	2.68	-4.11	NS	1.38	NS	299.31	92.76	S	NS	NS
Nickel chloride	S	NS	3.13	17.53	NS	1.61	S	326.37	180.19	S	S	S
α-Linolenic acid	NS (WU)	S	44.20	7.80	S	1.83	S	4800.00	1680.00	S	S	S
Squalene	NS (WU)	S	-3.40	8.10	NS	0.85	NS	218.00	262.00	S	NS	NS
Maleic acid	NS (WU)	S	9.60	-1.24	NS	0.95	NS	270.18	142.26	S	NS	NS
Tartaric acid	NS	S	-4.63	5.86	NS	1.34	S	120.00	93.40	NS	NS	NS

(Continues)

TABLE 2 (Continued)

Chemical	Spectro-DPRA				KeratiNoSens™		h-CLAT		ITS prediction					
	Human	LLNA	Cys dep.	Lys dep.	Prediction (cut-off: Cys >10 or Lys >30)	BL	Prediction (cut-off: BL >1.5)	CD54 RFI	CD86 RFI	Prediction (cut-off: CD54 >200 or CD86 >150)				
										Approach_1	Approach_2	Approach_3	2 out of 3	
Dimethyl sulfoxide	NS	S	0.38	-3.16	NS	1.16	NS	163.74	92.97	NS	NS	NS	NS	NS
Sodium lauryl sulfate	NS	S	7.36	4.21	NS	1.16	NS	119.78	119.43	NS	NS	NS	NS	NS
α-Hexylcinnamaldehyde	NS	S	41.05	0.84	S	2.14	S	67.23	66.77	NS	S	S	S	S
Xylene	NS	S	-0.53	4.63	NS	1.27	NS	157.75	100.40	NS	NS	NS	NS	NS
Pyridine	NS	S	-1.87	12.19	NS	1.43	NS	132.34	93.64	NS	NS	NS	NS	NS
R-(+)-Limonene	NS	S	1.95	2.44	NS	1.37	NS	154.56	94.97	NS	NS	NS	NS	NS
Isopropanol	NS	NS	-3.02	9.58	NS	1.15	NS	72.27	79.94	NS	NS	NS	NS	NS
Lactic acid	NS	NS	9.13	-5.72	NS	1.25	NS	95.60	91.37	NS	NS	NS	NS	NS
Methyl salicylate	NS	NS	9.79	2.37	NS	1.04	NS	186.81	136.77	NS	NS	NS	NS	NS
Salicylic acid	NS	NS	0.48	-9.03	NS	1.06	NS	249.45	60.50	S	NS	S	NS	NS
Benzalkonium chloride	NS	NS	8.63	0.16	S	1.06	NS	104.37	26.45	NS	S	NS	NS	NS
Glycerol	NS	NS	0.62	9.89	NS	1.36	NS	121.85	95.54	NS	NS	NS	NS	NS
2-Phenoxyethanol	NS	NS (WU)	8.67	15.35	NS	1.34	NS	251.65	156.87	S	NS	S	NS	NS
Tween80	NS	NS	5.86	15.20	NS	1.74	S	151.75	51.01	NS	NS	NS	S	NS
6-Methylcoumarin	NS	NS	0.78	-8.36	NS	5.42	S	102.73	53.99	NS	NS	NS	S	NS
Fumaric acid	NS	NS	3.67	5.16	NS	0.86	NS	101.75	96.23	NS	NS	NS	NS	NS
Oleic acid	NS (WU)	S	1.84	8.40	NS	1.29	NS	147.00	88.00	NS	NS	NS	NS	NS
Linoleic acid	NS (WU)	S	38.60	7.50	S	1.92	S	480.00	168.00	S	S	S	S	S
Succinic acid	NS (WU)	NS	7.05	14.31	NS	0.85	NS	124.00	154.00	S	NS	S	NS	NS
p-Anisic acid	NS (WU)	NS (WU)	-6.10	-8.96	NS	1.22	NS	112.50	132.00	NS	NS	NS	NS	NS
Caprylyl sulfobetaine	NS (WU)	NS (WU)	-14.9	-14.33	NS	0.99	NS	116.39	114.70	NS	NS	NS	NS	NS
Hydroxyacetophenone	NS (WU)	NS (WU)	-2.94	-10.89	NS	1.15	NS	143.10	62.18	NS	NS	NS	NS	NS
Apigenin	NS (WU)	NS (WU)	-17.87	0.21	NS	18.89	S	114.89	54.17	NS	NS	NS	S	NS
2,3-Butanediol	NS (WU)	NS (WU)	3.69	-0.30	NS	1.18	NS	80.00	97.13	NS	NS	NS	NS	NS
Caprylic/capric glycerides	NS (WU)	NS (WU)	5.20	12.66	NS	1.18	NS	147.22	94.30	NS	NS	NS	NS	NS
1,2-Hexanediol	NS (WU)	NS (WU)	-6.90	-0.71	NS	1.12	NS	124.24	114.14	NS	NS	NS	NS	NS
1,2-Octanediol	NS (WU)	NS (WU)	8.89	-0.34	NS	1.17	NS	104.55	110.10	NS	NS	NS	NS	NS

Cys dep., % of cysteine depletion; h-CLAT, human cell line activation test; LLNA, local lymph node assay; Lys dep., % of lysine depletion; BL 1.5, 1.5 fold of luciferase induction; RFI, relative fluorescence intensity; S/NS, sensitizer/non-sensitizer; Spectro-DPRA, Spectro-direct peptide reactivity assay; WU, widely used in cosmetics.

TABLE 3 Performance of animal alternative individually for the prediction of human data

Human	Spectro-DPRA		KeratinoSens™		h-CLAT	
	Positive	Negative	Positive	Negative	Positive	Negative
Sensitizer (27)	22	5	24	4	24	3
Non-sensitizer (31)	3	28	8	23	8	23
Sensitivity (%)	81.5		88.9		88.9	
Specificity (%)	90.3		74.2		74.2	
PPV (%)	88.0		75.0		75.0	
NPV (%)	84.8		88.5		88.5	
Accuracy (%)	86.2		81.0		81.0	

h-CLAT, human cell line activation test; NPP, negative predictive value; PPV, positive predictive value; Spectro-DPRA, spectrophotometric direct peptide reactivity assay.

TABLE 4 Performance of animal alternative individually for the prediction of LLNA data

LLNA	Spectro-DPRA		KeratinoSens™		h-CLAT	
	Positive	Negative	Positive	Negative	Positive	Negative
Sensitizer (38)	25	13	28	10	28	10
Non-sensitizer (20)	0	20	5	15	4	16
Sensitivity (%)	65.8		73.0		73.7	
Specificity (%)	100.0		76.2		80.0	
PPV (%)	100.0		84.4		87.5	
NPV (%)	60.6		61.5		61.5	
Accuracy (%)	77.6		74.1		75.9	

h-CLAT, human cell line activation test; LLNA, local lymph node assay; NPP, negative predictive value; PPV, positive predictive value; Spectro-DPRA, spectrophotometric direct peptide reactivity assay.

TABLE 5 Performance of binary and “2 out of 3” approaches in different data sets for the prediction of human data

Human	Binary Approach_1		Binary Approach_2		Binary Approach_3		2 out of 3	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Sensitizer (27)	26	1	27	0	26	1	25	2
Non-sensitizer (31)	4	27	8	23	8	23	4	27
Sensitivity (%)	96.3		100.0		96.3		92.6	
Specificity (%)	90.3		74.2		74.2		87.1	
PPV (%)	89.7		77.1		78.8		86.2	
NPV (%)	96.6		100.0		85.8		93.1	
Accuracy (%)	93.1		86.2		84.5		89.7	

NPP, negative predictive value; PPV, positive predictive value.

sensitivity, specificity, PPV, NPV and accuracy percentages of 65.8%, 100.0%, 100.0%, 60.6% and 77.6%, respectively. Spectro-DPRA could not predict 13 chemicals accurately (cobalt chloride, ammonium persulfate, geraniol, linalool, squalene, maleic acid, tartaric acid, dimethyl sulfoxide, sodium lauryl sulfate, xylene, pyridine, R-(+)-limonene and oleic acid). The Spectro-DPRA also showed the highest predictivity of any other assays and aligned most similarly to human data.

3.2 | Improvement of predictivity through integrated testing

A brief scheme of each integrated approach is given in Figure 2. Based on data sets of Spectro-DPRA, KeratinoSens™ and h-CLAT (Table 2), we applied four integrated approaches (Figure 2) and evaluated the predictivity compared with human or LLNA data using Cooper statistics (Cooper et al., 1979). The sensitivity, specificity, PPV, NPV and accuracy of each approach is suggested in Tables 5 and 6.

TABLE 6 Performance of binary and “2 out of 3” approaches in different data sets for the prediction of to LLNA data

LLNA	Binary Approach_1		Binary Approach_2		Binary Approach_3		2 out of 3	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Sensitizer (38)	29	9	32	6	31	7	28	10
Non-sensitizer (20)	1	19	4	16	4	16	1	19
Sensitivity (%)	76.3		83.8		81.1		73.7	
Specificity (%)	95.0		81.0		80.1		95.0	
PPV (%)	96.7		88.6		88.2		96.4	
NPV (%)	67.9		73.9		70.8		65.5	
Accuracy (%)	82.8		82.8		81.0		81.0	

LLNA, local lymph node assay; NPP, negative predictive value; PPV, positive predictive value.

Compared with the human data, prediction rates were greater than 84.5% in all integrated approaches except for the specificity and PPV of Binary Approach_2 and 3. Compared with the single test method, the overall predictability of each test increased when using the combined approach with the exception of Binary Approach_2 & 3. Among the approaches, Binary Approach_1 showed the highest predictivity value. Most notably, four chemicals (cobalt chloride, ammonium persulfate, geraniol, nickel chloride) that previously showed false negative results using the Spectro-DPRA were predicted correctly as sensitizers by using a combination of tests.

By using an integrated approach, we were able to increase the predictivity compared with a single assay approach. We were able to attain more accurate predictivity that was close to the human and LLNA data. Binary Approach_2 showed the highest accuracy (82.8%) to LLNA data.

4 | DISCUSSION

The overall predictivity of a single test was higher for human data than for LLNA. The accuracy of a single test was in the range of 81.0%–86.2% for human data and in the range of 74.1%–77.6% for LLNA data. We applied an integrated approach using Spectro-DPRA as the first step for Approach 1–3. Spectro-DPRA represents a molecular initiating event or key event 1 haptenization in the AOP of skin sensitization similar to standard DPRA (OECD TG 442C). Skin sensitization is a hapten-specific delayed-type hypersensitivity (Adam et al., 2011). Therefore, skin sensitizers cannot induce skin sensitization directly, and the formation of adducts between chemical and endogenous proteins in the skin is essential for the initiation of skin sensitization (Dupuis, Benezra, Schlewer, & Stampf, 1980; Landsteiner & Jacobs, 1935; Patlewicz, Basketter, Smith, Hotchkiss, & Roberts, 2001). In the four integrated approaches, the range of accuracy was 84.4%–91.4% compared with human data and 81.0%–82.8% compared with LLNA data. All three integrated approaches showed a higher predictivity to human data. The accuracy of Spectro-DPRA, KeratinoSens™ and h-CLAT was better for human data than for LLNA data, and the overall accuracy was higher than that of single tests. The

integrated approach resulted in fewer false positive and negative sensitivity predictions. A previous study using the “2 out of 3” prediction model with data from DPRA, KeratinoSens™ and h-CLAT showed a similar accuracy to our study, with 90% accuracy for human data and 79% accuracy for LLNA data (our data showed 89.7% and 81.0% accuracy for human and LLNA data, respectively) (Urbisch et al., 2015). In a binary combination study with KeratinoSens™ and h-CLAT, the accuracy was 94.4% for human data and 93.4% for LLNA data, which was somewhat higher than our results (Otsubo et al., 2017). Another binary combination study with DPRA and h-CLAT had an accuracy of 93% (human) and 85% (LLNA) with 93% (human) non-sensitizer predictions being correct (Roberts & Patlewicz, 2018). However, the above study did not include unsaturated lipid compounds, which are well known to cause false positives in LLNA and are highly lipophilic. Thus, one advantage of our integrated approach, particularly in reference to Binary Approach_1, is that we can evaluate the skin sensitization potential of cosmetic ingredients with high lipophilicity.

The combination of three assay methods (Spectro-DPRA, KeratinoSens™ and h-CLAT) helped to improve the overall prediction power compared with the binary approaches that used two test methods (Spectro-DPRA + KeratinoSens™ or Spectro-DPRA + h-CLAT) (Tables 5 and 6). Among the three integrated approaches, Binary Approach_1 (Figure 2.) showed the highest sensitivity, specificity, PPV, NPV and accuracy. This high prediction power seems to be due to the high predictivity of Spectro-DPRA itself. Spectro-DPRA could not predict cobalt chloride, ammonium persulfate, geraniol and nickel chloride as sensitizers; however, by combining with other tests that have other key events of skin sensitization AOP, we were able to predict these chemicals as sensitizers correctly. As previously described, Spectro-DPRA showed high positive predictivity and was easy to evaluate. If Spectro-DPRA is performed and followed up with binary testing of KeratinoSens™ and h-CLAT, we can predict skin sensitization hazard for cosmetic ingredients more accurately. However, this approach also has limitations. This approach cannot estimate all pre-/pro-haptens accurately. Similarly, to previous studies examining an integrated approach, as pro-hapten is outside the domain of the Spectro-DPRA, two negative results could falsely classify a chemical as a non-sensitizer. However, as most fragrance chemicals used in

cosmetics have pre-/pro-hapten properties, further research is needed to improve prediction for these chemicals.

In conclusion, following a binary approach, our method posits that if at the first stage Spectro-DPRA shows the chemical to be a sensitizer, it is considered a sensitizer. If this first stage shows the chemical to be a non-sensitizer, we conducted both KeratinoSens™ and h-CLAT as additional tests. If both KeratinoSens™ and h-CLAT predicted the chemical as positive, the chemical is confirmed to be a skin sensitizer. If the chemical is still not predicted to be a sensitizer, it is confirmed to be a non-sensitizer. Overall, the order of our integrated approach performed better than individual assays and our combination of assays was accurate at predicting the sensitization outcome in both the LLNA and human data. Although further optimization are needed, the approach in which skin sensitization potential was determined by the Spectro-DPRA followed by the final determination that using the result of the KeratinoSens™ and h-CLAT assays could be a reliable and convenient approach to predict the human outcome.

CONFLICT OF INTERESTS

The authors have no conflict of interest to report.

ACKNOWLEDGMENTS

This research was supported by Amorepacific R&D Unit.

ORCID

Jae-Hak Park  <https://orcid.org/0000-0002-4971-4640>

REFERENCES

- Adam, J., Pichler, W. J., & Yerly, D. (2011). Delayed drug hypersensitivity: models of T-cell stimulation. *British Journal of Clinical Pharmacology*, 71, 701–707. <https://doi.org/10.1111/j.1365-2125.2010.03764.x>
- Adler, S., Basketter, D., Creton, S., Pelkonen, O., van Benthem, J., Zuang, V., ... Zaldivar, J. M. (2011). Alternative (non-animal) methods for cosmetics testing: current status and future prospects-2010. *Archives of Toxicology*, 85, 367–485. <https://doi.org/10.1007/s00204-011-0693-2>
- Basketter, D., Crozier, J., Hubesch, B., Manou, I., Mehling, A., & Scheel, J. (2012). Optimised testing strategies for skin sensitization—the LLNA and beyond. *Regulatory Toxicology and Pharmacology*, 64, 9–16. <https://doi.org/10.1016/j.yrtph.2012.06.003>
- Basketter, D. A., Alepee, N., Ashikaga, T., Barroso, J., Gilmour, N., Goebel, C., ... Templier, M. (2014). Categorization of chemicals according to their relative human skin sensitizing potency. *Dermatitis*, 25, 11–21. <https://doi.org/10.1097/DER.0000000000000003>
- Bauch, C., Kolle, S. N., Ramirez, T., Eltze, T., Fabian, E., Mehling, A., ... Landsiedel, R. (2012). Putting the parts together: combining in vitro methods to test for skin sensitizing potentials. *Regulatory Toxicology and Pharmacology*, 63, 489–504. <https://doi.org/10.1016/j.yrtph.2012.05.013>
- Cho, S. A., An, S., & Park, J. H. (2019). High-throughput screening (HTS)-based spectrophotometric direct peptide reactivity assay (Spectro-DPRA) to predict human skin sensitization potential. *Toxicology Letters*, 314, 27–36. <https://doi.org/10.1016/j.toxlet.2019.07.014>
- Cho, S. A., Jeong, Y. H., Kim, J. H., Kim, S., Cho, J. C., Heo, Y., ... An, S. (2014). Method for detecting the reactivity of chemicals towards peptides as an alternative test method for assessing skin sensitization potential. *Toxicology Letters*, 225, 185–191. <https://doi.org/10.1016/j.toxlet.2013.12.007>
- Cooper, J. A. 2nd, Saracci, R., & Cole, P. (1979). Describing the validity of carcinogen screening tests. *British Journal of Cancer*, 39, 87–89. <https://doi.org/10.1038/bjc.1979.10>
- Corsini, E., Mitjans, M., Galbiati, V., Lucchi, L., Galli, C. L., & Marinovich, M. (2009). Use of IL-18 production in a human keratinocyte cell line to discriminate contact sensitizers from irritants and low molecular weight respiratory allergens. *Toxicology in Vitro*, 23, 789–796. <https://doi.org/10.1016/j.tiv.2009.04.005>
- Cruz, M. J., De Vooght, V., Munoz, X., Hoet, P. H., Morell, F., Nemery, B., & Vanoirbeek, J. A. (2009). Assessment of the sensitization potential of persulfate salts used for bleaching hair. *Contact Dermatitis*, 60, 85–90. <https://doi.org/10.1111/j.1600-0536.2008.01477.x>
- Dupuis, G., Benezra, C., Schlewer, G., & Stampf, J. L. (1980). Allergic contact dermatitis to alpha-methylene-gamma-butyrolactones. Preparation of alantolactone-protein conjugates and induction of contact sensitivity in the guinea pig by an alantolactone-skin protein conjugate. *Molecular Immunology*, 17, 1045–1051. [https://doi.org/10.1016/0161-5890\(80\)90099-1](https://doi.org/10.1016/0161-5890(80)90099-1)
- EU. (2006). Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. Official J. No L, 396, 1–1355.
- EU. (2008). Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending 15 and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. Off. J. No L, 353, 1–1355.
- EU. (2009). Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. Off. J. No L, 342, 1–59.
- Gerberick, G. F., Ryan, C. A., Kern, P. S., Schlatter, H., Dearman, R. J., Kimber, I., ... Basketter, D. A. (2005). Compilation of historical local lymph node data for evaluation of skin sensitization alternative methods. *Dermatitis*, 16, 157–202.
- Hirota, M., Ashikaga, T., & Kouzuki, H. (2018). Development of an artificial neural network model for risk assessment of skin sensitization using human cell line activation test, direct peptide reactivity assay, KeratinoSens and in silico structure alert parameter. *Journal of Applied Toxicology*, 38, 514–526. <https://doi.org/10.1002/jat.3558>
- ICCVAM. (2010). ICCVAM Test Method Evaluation Report on the Murine Local Lymph Node Assay: DA A Nonradioactive Alternative Test Method to Assess the Allergic Contact Dermatitis Potential of Chemicals and Products. NIH Publication Number 10-7551. National Toxicology Program.
- Jaworska, J., Dancik, Y., Kern, P., Gerberick, F., & Natsch, A. (2013). Bayesian integrated testing strategy to assess skin sensitization potency: from theory to practice. *Journal of Applied Toxicology*, 33, 1353–1364.
- Jaworska, J., Harol, A., Kern, P. S., & Gerberick, G. F. (2011). Integrating non-animal test information into an adaptive testing strategy—skin sensitization proof of concept case. *ALTEX*, 28, 211–225. <https://doi.org/10.14573/altex.2011.3.211>
- Jaworska, J. S., Natsch, A., Ryan, C., Strickland, J., Ashikaga, T., & Miyazawa, M. (2015). Bayesian integrated testing strategy (ITS) for skin sensitization potency assessment: a decision support system for quantitative weight of evidence and adaptive testing strategy. *Archives*

- of *Toxicology*, 89, 2355–2383. <https://doi.org/10.1007/s00204-015-1634-2>
- Kimber, I., Basketter, D. A., Gerberick, G. F., & Dearman, R. J. (2002). Allergic contact dermatitis. *International Immunopharmacology*, 2, 201–211. [https://doi.org/10.1016/S1567-5769\(01\)00173-4](https://doi.org/10.1016/S1567-5769(01)00173-4)
- Kimber, I., Basketter, D. A., Gerberick, G. F., Ryan, C. A., & Dearman, R. J. (2011). Chemical allergy: translating biology into hazard characterization. *Toxicological Sciences*, 120(Suppl 1), S238–S268. <https://doi.org/10.1093/toxsci/kfq346>
- Kinsner-Ovaskainen, A., Maxwell, G., Kreysa, J., Barroso, J., Adriaens, E., Alepee, N., ... Zuang, V. (2012). Report of the EPAA-ECVAM workshop on the validation of Integrated Testing Strategies (ITS). *Alternatives to Laboratory Animals*, 40, 175–181. <https://doi.org/10.1177/026119291204000310>
- Landsteiner, K., & Jacobs, J. (1935). Studies on the sensitization of animals with simple chemical compounds. *The Journal of Experimental Medicine*, 61, 643–656. <https://doi.org/10.1084/jem.61.5.643>
- Macmillan, D. S., Canipa, S. J., Chilton, M. L., Williams, R. V., & Barber, C. G. (2016). Predicting skin sensitisation using a decision tree integrated testing strategy with an in silico model and in chemico/in vitro assays. *Regulatory Toxicology and Pharmacology*, 76, 30–38. <https://doi.org/10.1016/j.yrtph.2016.01.009>
- Macmillan, D. S., & Chilton, M. L. (2018). A defined approach for predicting skin sensitisation hazard and potency based on the guided integration of in silico, in chemico and in vitro data using exclusion criteria. *Regulatory Toxicology and Pharmacology*, 101, 35–47.
- Natsch, A. (2010). The Nrf2-Keap1-ARE toxicity pathway as a cellular sensor for skin sensitizers—functional relevance and a hypothesis on innate reactions to skin sensitizers. *Toxicological Sciences: An Official Journal of the Society of Toxicology*, 113, 284–292. <https://doi.org/10.1093/toxsci/kfp228>
- Natsch, A., & Emter, R. (2008). Skin sensitizers induce antioxidant response element dependent genes: application to the in vitro testing of the sensitization potential of chemicals. *Toxicological Sciences*, 102, 110–119. <https://doi.org/10.1093/toxsci/kfm259>
- Natsch, A., Ryan, C. A., Foertsch, L., Emter, R., Jaworska, J., Gerberick, F., & Kern, P. (2013). A dataset on 145 chemicals tested in alternative assays for skin sensitization undergoing prevalidation. *Journal of Applied Toxicology*, 33, 1337–1352.
- Nukada, Y., Ashikaga, T., Miyazawa, M., Hirota, M., Sakaguchi, H., Sasa, H., & Nishiyama, N. (2012). Prediction of skin sensitization potency of chemicals by human Cell Line Activation Test (h-CLAT) and an attempt at classifying skin sensitization potency. *Toxicology in Vitro*, 26, 1150–1160. <https://doi.org/10.1016/j.tiv.2012.07.001>
- Nukada, Y., Miyazawa, M., Kazutoshi, S., Sakaguchi, H., & Nishiyama, N. (2013). Data integration of non-animal tests for the development of a test battery to predict the skin sensitizing potential and potency of chemicals. *Toxicology in Vitro*, 27, 609–618. <https://doi.org/10.1016/j.tiv.2012.11.006>
- OECD. (2012). The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins. Part 1: Scientific Evidence Series on Testing and Assessment No.168 JT03321047. Organisation for Economic Co-operation and Development (OECD). Paris, France.
- Otsubo, Y., Nishijo, T., Miyazawa, M., Saito, K., Mizumachi, H., & Sakaguchi, H. (2017). Binary test battery with KeratinoSens and h-CLAT as part of a bottom-up approach for skin sensitization hazard prediction. *Regulatory Toxicology and Pharmacology*, 88, 118–124. <https://doi.org/10.1016/j.yrtph.2017.06.002>
- Pang, S., & Fiume, M. Z. (2001). Final report on the safety assessment of ammonium, potassium, and sodium persulfate. *International Journal of Toxicology*, 20(Suppl 3), 7–21.
- Patlewicz, G., Basketter, D. A., Smith, C. K., Hotchkiss, S. A., & Roberts, D. W. (2001). Skin-sensitization structure-activity relationships for aldehydes. *Contact Dermatitis*, 44, 331–336. <https://doi.org/10.1034/j.1600-0536.2001.044006331.x>
- Roberts, D. W., & Patlewicz, G. (2018). Non-animal assessment of skin sensitization hazard: Is an integrated testing strategy needed, and if so what should be integrated? *Journal of Applied Toxicology*, 38, 41–50. <https://doi.org/10.1002/jat.3479>
- Sakaguchi, H., Ashikaga, T., Miyazawa, M., Yoshida, Y., Ito, Y., Yoneyama, K., ... Suzuki, H. (2006). Development of an in vitro skin sensitization test using human cell lines; human Cell Line Activation Test (h-CLAT). II. An inter-laboratory study of the h-CLAT. *Toxicology in Vitro*, 20, 774–784. <https://doi.org/10.1016/j.tiv.2005.10.014>
- Sakaguchi, H., Ryan, C., Ovigne, J. M., Schroeder, K. R., & Ashikaga, T. (2010). Predicting skin sensitization potential and inter-laboratory reproducibility of a human Cell Line Activation Test (h-CLAT) in the European Cosmetics Association (COLIPA) ring trials. *Toxicology in Vitro*, 24, 1810–1820. <https://doi.org/10.1016/j.tiv.2010.05.012>
- dos Santos, G. G., Reinders, J., Ouwehand, K., Rustemeyer, T., Scheper, R. J., & Gibbs, S. (2009). Progress on the development of human in vitro dendritic cell based assays for assessment of the sensitizing potential of a compound. *Toxicology and Applied Pharmacology*, 236, 372–382. <https://doi.org/10.1016/j.taap.2009.02.004>
- Takenouchi, O., Fukui, S., Okamoto, K., Kurotani, S., Imai, N., Fujishiro, M., ... Miyazawa, M. (2015). Test battery with the human cell line activation test, direct peptide reactivity assay and DEREK based on a 139 chemical data set for predicting skin sensitizing potential and potency of chemicals. *Journal of Applied Toxicology*, 35, 1318–1332. <https://doi.org/10.1002/jat.3127>
- Urbisch, D., Mehling, A., Guth, K., Ramirez, T., Honarvar, N., Kolle, S., ... Sakaguchi, H. (2015). Assessing skin sensitization hazard in mice and men using non-animal test methods. *Regulatory Toxicology and Pharmacology*, 71, 337–351. <https://doi.org/10.1016/j.yrtph.2014.12.008>
- Yamashita, K., Shinoda, S., Hagiwara, S., & Itagaki, H. (2015). Further development of LLNA:DAE method as stand-alone skin-sensitization testing method and applied for evaluation of relative skin-sensitizing potency between chemicals. *The Journal of Toxicological Sciences*, 40, 137–150. <https://doi.org/10.2131/jts.40.137>

How to cite this article: Cho S-A, Choi M, Park S-R, An S, Park J-H. Application of Spectro-DPRA, KeratinoSens™ and h-CLAT to estimation of the skin sensitization potential of cosmetics ingredients. *J Appl Toxicol*. 2020;40:300–312. <https://doi.org/10.1002/jat.3904>