**A Dissertation for the Degree of Doctor of Philosophy**

# The Development of Spectrophotometric Direct Peptide Reactivity Assay for Predicting Skin Sensitization Potentials as Animal Alternatives

## 동물실험 대체법으로서 피부 감작성을 예측하기 위한 Spectrophotometric Direct Peptide Reactivity Assay 개발

By

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# ABSTRACT

## The Development of Spectrophotometric Direct Peptide Reactivity Assay for Predicting Skin Sensitization Potentials as Animal Alternatives

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Some ingredients used in cosmetics or topical ointments can act as chemical haptens, which can cause sensitization of the skin. Therefore, in terms of product safety management, it is important to evaluate and exclude the use of ingredients that have the risk of skin sensitization during the product development process. However,

due to the ethical issues of animal toxicity testing and the ban on animal testing of cosmetic ingredients and products in the cosmetic industry, alternative animal testing methods need to be developed to assess the skin sensitization of new ingredients. To meet this need, many animal alternatives have been actively developed around the world, and some alternative assays have been adopted as OECD TGs to assess skin sensitization through validation studies. In a previous preliminary study, I developed a novel *in chemico* Spectrophotometric Direct Peptide Reactivity Assay (Spectro-DPRA), which replaces the local lymph node assay (LLNA), an animal skin sensitization assay. Based on previous research, I expanded the test materials to confirm the effectiveness of the Spectro-DPRA method for predicting the animal skin sensitization potential, and further determined the feasibility of the method for estimating the human skin sensitization potential. Spectro-DPRA showed 83.1% or 90.9% accuracy compared to a conventional LLNA or prediction based on human data, respectively, with a combination model using both a cysteine peptide and lysine peptide cut-off.

Due to the complexity of the mechanism of skin sensitization, a single alternative approach may not be able to achieve high predictivity. Therefore, multiple prediction assays were investigated and combined, including both chemical and in vitro test methods, to determine skin sensitization based on the Adverse Outcome Pathway (AOP) skin sensitization. When Spectro-DPRA and in vitro assays (KeratinoSens™ and h-CLAT) tests were combined, animal or human data predictivity improved compared to a single assay. Especially for the approach in which sensitization potential was determined by the Spectro-DPRA assay followed

by final determination using the results of KeratinoSens$^{TM}$ and h-CLAT assays in comparison with the LLNA animal test and human test results, the highest predictivity was achieved.

In conclusion, the Spectro-DPRA showed a high predictivity for skin sensitization potentials in animals, similar to the existing Direct Peptide Reactivity Assay (DPRA) adopted by the OECD TG. Furthermore, Spectro-DPRA also showed a high predictivity for human skin sensitization potentials. Spectro-DPRA is easier, faster, low cost (use of 1/100 peptide, use of 1/5 to 1/25 chemicals, use of inexpensive optical instruments), and high throughput screening-available compared to DPRA. In addition, the advantages of Spectro-DPRA are likely to develop into animal alternatives that can be used for a wide range of predictive approaches combined with other test methods.

**Keywords**: Skin sensitization, Spectrophotometric DPRA, Integrated approaches, Adverse Outcome Pathway (AOP), Animal alternative

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## LITERATURE REVIEW



## CHAPTER I

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# LIST OF ABBREVIATION

| | |
|---|---|
| **ACD** | Allergic Contact Dermatitis |
| **KCs** | Keratinocytes |
| **LCs** | Langerhans Cells |
| **IL** | Interleukin |
| **APCs** | Antigen-Presenting Cells |
| **MHC** | Major Histocompatibility Complex |
| **iDC** | Immature Dendritic Cell |
| **CD** | Cluster of Differentiation |
| **mDC** | Mature Dendritic Cell |
| **GPMT** | Guinea Pig Maximization Test |
| **OECD TG** | Organization for Economic Co-operation and Development Test Guideline |
| **LLNA** | Local Lymph Node Assay |
| **EC3** | Effective Concentration for a stimulation index of 3 |
| **GHS** | Globally Harmonised System |
| **HRIPT** | Human Repeat Insult Patch Test |
| **BT** | Buehler Test |
| **AOP** | Adverse Outcome Pathway |

| | |
|---|---|
| **REACH** | European regulation of Registration, Evaluation, Authorization, and Restriction of Chemical substances |
| **CLP** | Classification, Labeling, and Packaging |
| **DPRA** | Direct Peptide Reactivity Assay |
| **h-CLAT** | Human cell line activation test |
| **U-SENS** | U937 Cell Line Activation Test |
| **IL-8 Luc Assay** | Interleukin-8 Reporter Gene Assay |
| **KE** | Key Events |
| **MIE** | Molecular Initiation Event |
| **HPLC** | High-Performance Liquid Chromatography |
| **RFI** | Relative Fluorescence Intensity |
| **SLO** | Stable Luciferase Orange |
| **SLR** | Stable Luciferase Red |
| **GAPDH** | Glyceraldehyde 3-phosphate dehydrogenase |
| **IATA** | Integrated Approach and Test and Assessment |
| **ITS** | Integrated Test Strategy |
| **STS** | Sequential Test Strategy |
| **EURL ECVAM** | European Union Reference Laboratory for Alternatives to Animal Testing |
| **DA** | Defined Approach |
| **DIP** | Data Interpretation Procedure |
| **ID** | Identification |
| **HTS** | High-Throughput Screening |

| | |
|---|---|
| **Spectro-DPRA** | Spectrophotometric Direct Peptide Reactivity Assay |
| **UV** | Ultraviolet |
| **LC/MS** | Liquid Chromatography-Mass Spectrometry |
| **DTNB** | 5,5′-Dithiobis-2-nitrobenzoic acid |
| **Log $K_{ow}$** | Log partition coefficient n-octanol/water |
| **CAS** | Chemical Abstract System |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **DMSO** | Dimethylsulfoxide |
| **PPV** | Positive Predictive Value |
| **NPV** | Negative Predictive Value |
| **SCCS** | Scientific Committee on Consumer Safety |
| **ICCVAM** | Interagency Coordinating Committee on the Validation of Alternative Methods |
| **ECETOC CL** | European Centre for Ecotoxicology and Toxicology of Chemicals Classification |
| **MTT** | (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide |
| **EC** | Effective Concentration |
| **FBS** | Fetal Bovine Serum |
| **FACS** | Fluorescence-Activated Cell Sorting |
| **PBS** | Phosphate-Buffered Saline |
| **FITC** | Fluorescein Isothiocyanate |
| **MFI** | Mean Fluorescence Intensity |

# LITERATURE REVIEW

# 1.The mechanism of skin sensitization

Skin sensitizers are substances that cause T cell-mediated delayed-type hypersensitivity responses in the skin and clinically cause allergic contact dermatitis (ACD). ACD is one of the important consumer health problems in cosmetics [1]. ACD elicited after skin contact with such as organic chemicals in cosmetics and topical applicants. Globally, the prevalence of ACD is estimated to be about 15-20% [2].

ACD results from repeated exposure to a non-immunogenic low molecular weight chemical called hapten [1, 3]. The development of ACD is divided into two stages (Figure 1) [4]. The first stage, that is, the sensitization phase, begins after the first exposure to allergens as a result of which antigen-specific T cells are produced. There may be no clinical symptoms at this phase, and in order to bind epidermal protein residues, skin sensitizers must penetrate the stratum corneum of the epidermis. For this reason, most skin sensitizers have hydrophobic properties. Skin sensitizers also have the property of electrophilic low molecular weight and can bind to nucleophilic regions of cellular macromolecules, such as skin proteins (haptenization). It is now known that over 4000 substances can act as hapten worldwide [5]. Some chemicals may require metabolic or abiotic activation for immunologically reactive conversion. These chemicals are called as pro-haptens or pre-haptens, respectively [6]. The major binding sites of skin proteins are widely known as the sulfhydryl, imidazole, and amino groups of cysteine, histidine, and lysine, respectively [7].

Chemical peptide adducts interact with keratinocytes (KC) and Langerhans cells (LC) in the epidermis. KC, the most predominant cell type of the epidermis, acts as an effective barrier to exogenous substances and is activated. Oxidative stress response through Keap1 / Nrf2-ARE (Antioxidant Response Factor) is a well-known toxic pathway in KCs [8, 9]. KCs also secrete cytokines of IL-1α, IL-1β, and IL-18. This cytokine induces the activation of peripheral inflammation-related cells, namely DC and T cells [4].

Cutaneous DC acts as the most potent antigen-presenting cell (APC) and presents processed peptide fragments on major histocompatibility complex (MHC) molecules, and cutaneous DC is mainly present in an immature form (immature dendritic cell; iDC). When DCs ingest antigen, molecular cell stimulus is produced in DCs, which induce activation and maturation of DCs. Subsequently, DCs migrate to local lymph nodes and prime antigen-specific CD4 and CD8 T cells within lymph nodes (Figure 1) [10]. During activation of DC, the production and release of a second messenger such as IL-12 [11], and the expression of adhesion molecules and co-stimulatory molecules such as CD54 (e.g., CD86, CD80, CD83, and CD40) results in a phenotypic change in DCs. This phenotypic alteration of DCs ends after activating naïve T cells in lymph nodes and promoting complete maturation (mature dendritic cell; mDC) in lymph nodes [10, 11]. When re-exposed to the sensitizer (elicitation phase), antigen-specific T effector cells migrate to the affected tissues, and some are transformed into memory T cells in the lymph nodes. Antigen-specific T effector cells that infiltrate affected tissues produce inflammatory responses, such as erythema and itching [12].

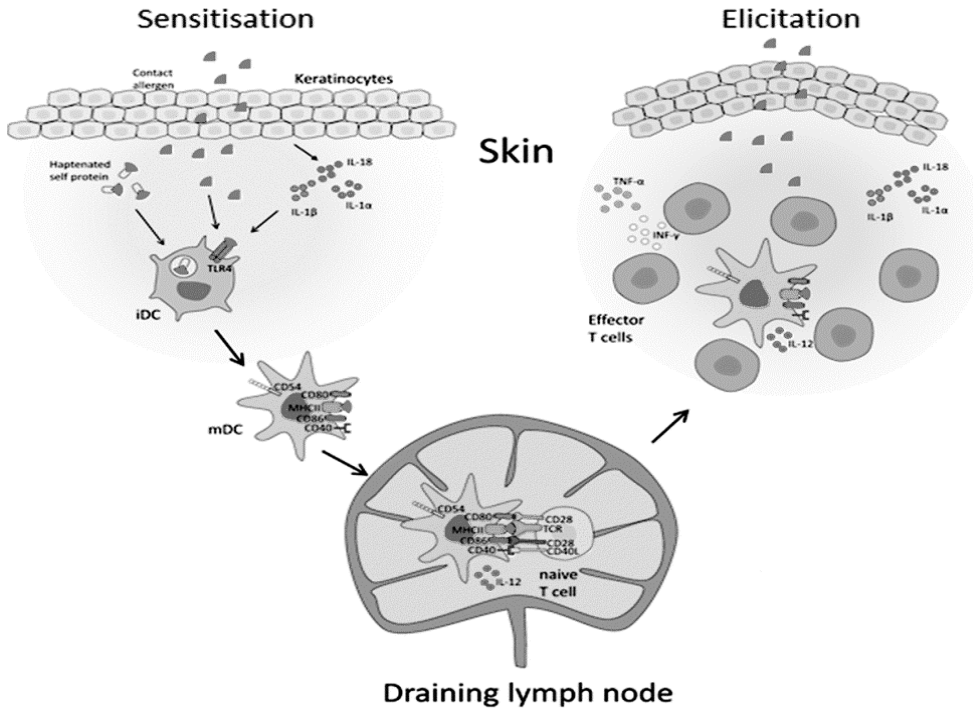


**Fig. 1 The mechanism of skin sensitization [4].**

# 2. The animal tests for skin sensitization

Until now, legislative frameworks for skin sensitization rely on information from animal models. To predict the skin sensitization potential in new chemical and its contains formulations, the animal test such as the guinea pig maximization test (GPMT) and Buehler test (OECD TG 406) [13] have been traditionally used [14]. These *in vivo* assays evaluate the degree of erythema and edema according to a visual scoring system in the elicitation phase [15].

The radioactive and non-radioactive local lymph node assay (LLNA) (OECD TG429, 442A, and 442B) [16-18] is used as an alternative to the traditional guinea pig method for estimating skin sensitization potential. LLNA can present dose-response quantitative data called EC3 by lymphocyte proliferation. EC3 values refer to effective concentrations that induce a threefold increase in lymphocyte proliferation compared to vehicle control. EC3 values were highly correlated with human thresholds for skin sensitization [19-21]. In addition, LLNA EC3 values can refine the potency of skin sensitizers (e.g. GHS subcategories 1A and 1B) (Table 1).

**Table 1. The classification of skin sensitization *in vivo* assays.**

| Category | Classification criteria | LLNA EC3 | HRIPT | GPMT | BT |
|---|---|---|---|---|---|
| Category 1: Skin sensitizer | Evidence in humans that the substance can induce sensitization by skin contact in a substantial number of persons, or positive results from appropriate animal tests. | NA | NA | NA | NA |
| Category 1A: Strong Skin sensitizer | High frequency of occurrence in humans, and/or high potency in animals. May consider severity. | ≤ 2% | Positive response at ≤ 500mg/cm$^3$ | ≥ 30% responders at ≤ 0.1% intradermal induction dose or ≥ 60% responders at > 0.1% to ≤ 1% intradermal induction dose | ≥ 15% responders at ≤ 0.2% topical induction dose or ≥ 60% responders at > 0.2% to ≤ 20% topical induction dose |
| Category 1B: Oher Skin sensitizer | Low to moderate frequency of occurrence in humans, and /or low to moderate in animals. May consider severity. | > 2% | Positive response at > 500mg/cm$^3$ | ≥ 30% to <60% responders at >0.1% to ≤ 1% intradermal induction dose or ≥ 30% responders at > 1% intradermal induction dose | ≥ 15% to <60% responders at >0.2% to ≤ 20% topical induction dose or ≥ 15% responders at > 20% topical induction dose |

# 3. Current status of developing animal alternatives for skin sensitization assessment

## 3.1. The Adverse Outcome Pathway (AOP) based developing animal alternatives and for skin sensitization

The starting of the ban on testing cosmetics or their ingredients on animals in the EU market [22-24] affected the cosmetic industry. However, the toxicity data of skin sensitization for new chemicals is required of European regulation of Registration, Evaluation, Authorization, and Restriction of Chemical substances (REACH; EC 1907/2006) [22] and under the United Nations Globally Harmonized System for Classification, Labeling, and Packaging (GHS/CLP). In addition, the EU Cosmetics Directive (EC 1223/2009)[24] demand information on skin sensitization potentials for newly developed cosmetic ingredients to guarantee the safety of cosmetic ingredients. For these regulatory trends, a reliable toxicity test that did not involve animal experiments, but ensured the safety of cosmetics and their ingredients needed to be established instead of animal dates.

To assess the potential for skin sensitization, many assays have been developed that rely on known mechanisms of skin sensitization, many of which have recently been adopted as OECD test guidelines. Currently, six non-animal test methods have been accepted as OECD test guidelines for skin sensitization tests: (1)

chemical hapten-peptide reactivity (DPRA) (OECD TG442C), (2) KCs activation (KeratinoSens ™ and LuSens ™) (OECD TG442C) and (3) DCs Activation (h-CLAT, U-SENS ™, and IL-8 Luc Assay) [25-27].

Because toxic responses in vivo are induced by several steps, it is difficult to fully represent the results of animal testing as a result of one in vitro assay. Therefore, the concept of adverse outcome pathway (AOP) was developed to understand the toxic response as a whole and well established by the OECD Working Group. AOP consists of a series of key events (KE) triggered by chemicals. AOP begins with the molecular initiation event (MIE), the first KE to represent an event initiated by a chemical. Then several KEs occur in succession to cellular and/ or organ level, eventually causing adverse effects. Among several AOPs, AOP of skin sensitization was well established and each non-animal test method for skin sensitization was developed in accordance with KE of skin sensitization [28] (Figure 2). For skin sensitization, MIE and KE1 covalently bind to skin peptides of chemical or metabolite conjugates to form immunogenic conjugates (haptenization). This immunogenic conjugate elicits two cellular events, a second key event (KE2) and a third key event (KE3). The activation of keratinocytes is the KE2 of skin sensitization AOP, the activation of an inflammatory response and antioxidant response factor (ARE) -dependent pathways are occurred [28]. Dermal DCs and/or LCs take up the immunogenic conjugate and then activate and migrate to the draining lymph nodes (KE3). In the lymph node, epitopes of DC conjugated are presented to T cells, which then induce the activation and proliferation of T cells (KE4), and immunological memory is acquired [29, 30].

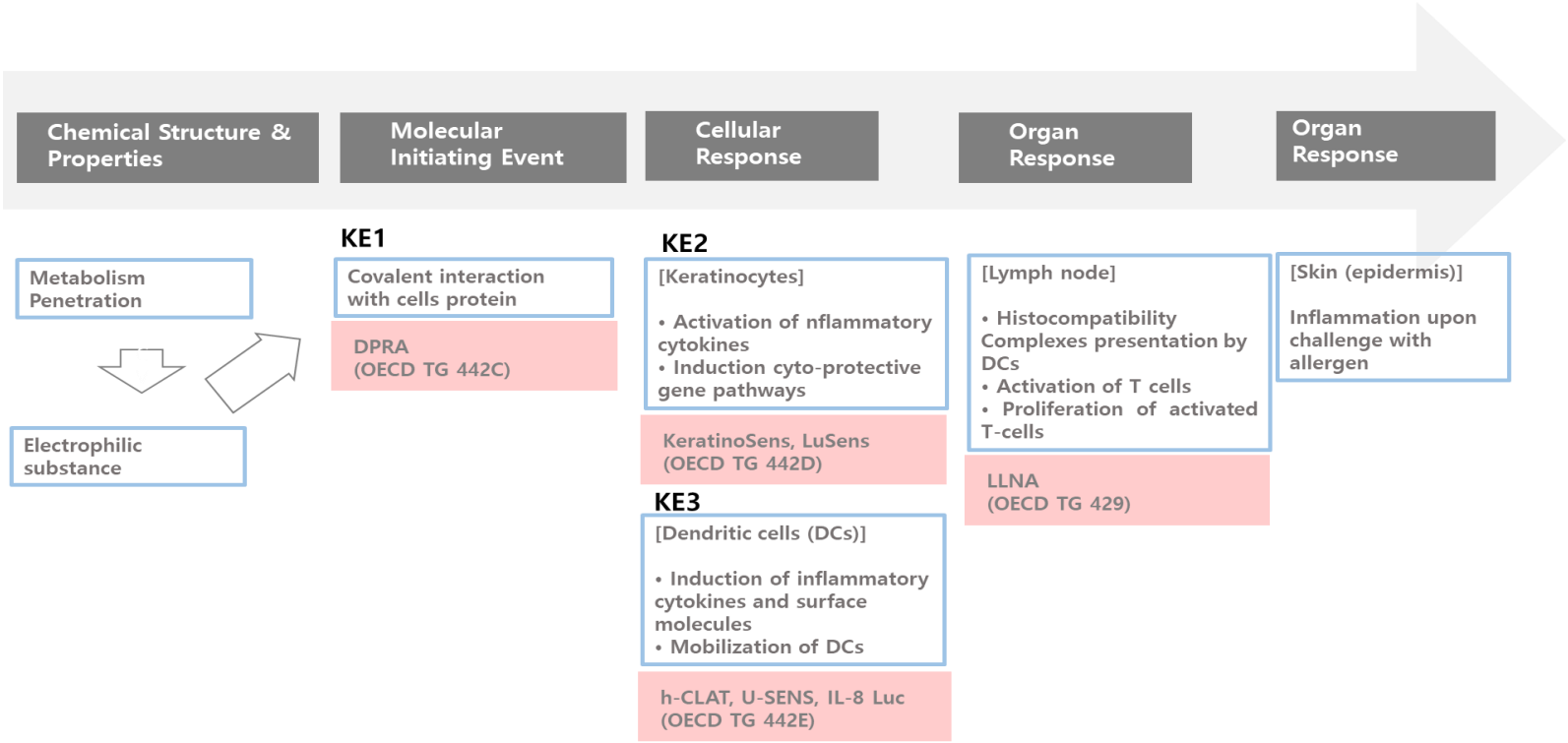


**Fig. 2 OECD AOP for skin sensitization**

(The figure is modified from the OECD AOP wiki: https:// aopwiki.org/Aop:40. Last accessed on 27 June 2016)

### 3.1.1. *In Chemico* Skin Sensitization (KE 1): Covalent interaction between chemical with cell protein

#### 3.1.1.1. Direct Peptide Reactivity Assay (DPRA) (adopted as OECD TG 442C)

Direct Peptide Reactivity Assay (DPRA) is the only validated and regulated method for chemical protein binding (haptenization) by OECD. DPRA assesses the value of haptenization by measuring the depletion of cysteine and lysine using high-performance liquid chromatography (HPLC) [31-33]. The depletion percentage of cysteine and/or lysine is used to distinguish skin sensitizers from non-sensitizers. DPRA can classify chemicals into four classes based on reactivity (no or minimal reactivity, low reactivity, moderate reactivity, high reactivity). Although it cannot be used alone to predict potency, DPRA data can contribute to potency predictions when used with other non-animal test data. DPRA shows predictability with 80% accuracy, 80% sensitivity, and 77% specificity when compared to LLNA results [34, 35]. The DPRA assessment does not include the metabolic system. Chemicals that require enzymatic bioactivation (i.e. hapten) to exert their skin sensitization potential cannot be detected by DPRA [25].

#### 3.1.1.2. Cor1C420 assay

Cor1C420 assay also has a DPRA-like assessment concept, and synthetic peptide Cor1C-420 (Ac-NKKCDLF) derived from the Cys420 sequence of human coronin1 protein is used instead of DPRA. This peptide is

highly reactive with electrophile and contains two Lys residues at the N-terminal side of the Cys residue [36]. Cor1C420 assay assesses the level of haptenization by measuring the depletion of Cor1C-420 peptide using liquid chromatography-mass spectrometry (LC-MS) analysis instead of HPLC. Analysis of LC-MS has advantages in determining peptide depletion, peptide oxidation (auto-dimerization) and actual adduct formation compared with HPLC analysis. Thiol reactivity and additional time (150 min) can also be used to test peptide-binding kinetics, resulting in higher predictivity than DPRA. Cor1C420 test shows predictivity with 88.8% accuracy, 88.5% sensitivity and 89.5% specificity compared to LLNA results [37].

#### 3.1.1.3. Amino acid Derivative Reactivity Assay (ADRA)

Amino acid Derivative Reactivity Assay (ADRA) is also based on the depletion of the peptide by covalent binding of chemical and peptide. Unlike the DPRA, ADRA used specifically modified the reactive cysteine and lysine peptides called N-(2-(1-naphthyl)acetyl)-l-cysteine (NAC) and α-N-(2-(1-naphthyl)acetyl)-l-lysine (NAL) containing naphthalene rings in the N-termini region of cysteine or lysine peptide [38]. This test method can be evaluated at very low concentrations of NAC, NAL (final concentration of 5 μM) and chemicals, thus, making it easy to evaluate hydrophobic materials. In performance studies using 82 chemicals, ADRA shows predictability with 90% accuracy, 87% sensitivity, and 97% specificity when compared to LLNA results [39].

### 3.1.2. *In vitro* keratinocyte activation (KE 2)

The second key event on AOP of skin sensitization takes place in the KCs and includes inflammatory responses as well as gene expression associated with specific cell signaling pathways such as the antioxidant/electrophile response element (ARE)-dependent pathways [9]. The adopted test for KE 2 of skin sensitization by OECD is based on the ARE-Nrf2 luciferase test method (KeratinoSens™ assay and the LuSens assay) (OECD TG 442D).

#### 3.1.2.1. KeratinoSens™ assay (adopted as OECD TG 442D)

The cell line used in the KeratinoSens ™ assay contains the luciferase gene under the transcriptional control of the ARE element derived from the human AKR1C2 gene [40]. When ARE element gene is activated by a skin sensitizer, it activates the luciferase, and this luminescence signal is quantitatively measured using a luminometer [8, 9]. The test substance is determined to be positive if there is an induction of at least 1.5 times the luciferase activity compared to the vehicle control at a concentration of less than 1000 μM with cell viability above 70% [26]. KeratinoSens ™ analysis can identify skin sensitizers with accuracy 77%, sensitivity 78%, and specificity 76% compared to LLNA [26, 35].

#### 3.1.2.2. LuSens assay (adopted as OECD TG 442D)

The LuSens assay has the same evaluation principle as the KeratinoSens ™ assay. Unlike KeratinoSens ™ cell, LuSens cell has the luciferase gene that

controlled by ARE element from the rat NADPH Quinone oxidoreductase (NQO1)[41]. In the LuSens assay, test substances are considered as positive when the luciferase induction is above 1.5-fold compared to the vehicle control at cellular viability above 70 %. The LuSens assay can identify skin sensitizers with 74% for accuracy, 74% for sensitivity and 74% for specificity compare to LLNA [26].

#### 3.1.2.3. In vitro gene expression assay using KC cell

The skin sensitizer can be determined by measuring the gene expression level of the skin's KC after exposure to chemicals. DNA microarray after exposure to skin sensitizer identified 10 signature genes unique to skin sensitizers in the human keratinocyte cell line HaCaT[ [42], mainly related to oxidative stress response and inflammatory response. Further studies with 41 chemicals showed 78% accuracy compared to LLNA [43].

#### 3.1.2.4. In vitro gene expression assay using Reconstructed human epithelial model (RhE models)

The KC gene expression analysis research has also been applied to the Reconstructed human epithelial model (RhE models). RhE models have the advantage that hydrophobic substances can be applied to KCs surface. In Epidermal sensitization assay (EpiSensA), skin sensitizers are identified based on the induction of four genes (as the positive criteria: four-fold increase for encoding activating

transcription factor 3 (ATF3), two-fold increase for DnaJ [Hsp40] homolog subfamily B member 4 (DNAJB4), two-fold increase for glutamate-cysteine ligase modifier subunit (GCLM), and four-fold increase for interleukin -8 (IL-8)) which are all related to oxidative stress response and inflammatory response following topical treatment from the data with over 80% cell viability. EpiSensA showed 94% sensitivity and 90% accuracy compared with LLNA when evaluated using 73 materials, including 29 lipophilic and 11 pre- / pro-haptens [44]. In addition, the EpiSensA showed high transferability and reproducibility in the transferability and within- and between-laboratory reproducibility (WLR and BLR) study performed in three labs using 10 test chemicals (BLR was 90%, 100% WLR was 100%) [45].

Using the Episkin ® RhE model, the SENS-IS assay predicts all potency and skin sensitization hazards. EpiSkin ® tissue models are exposed to different concentrations of chemicals, and the tissues are used for quantitative RT-PCR analysis. The expression levels of the gene set reflecting the potential for irritation (IRR gene group) and the gene sets reflecting the potential for sensitization (REDOX and SENS-IS gene group). The chemical is considered a sensitizer (positive) when more than seven genes are over-expressed in the REDOX or SENS-IS gene (> 1.25). The compound is graded as extreme, moderate, or low sensitizer when detected as a sensitizer at test concentrations of 0.1%, 1%, 10%, or 50%, respectively. The performance study with 50 chemicals, the SENS-IS showed 100% compare to LLNA data [46]. SENS-IS showed 100% of between-laboratory reproducibility (BLR) and 94.5% of within-laboratory reproducibility (WLR) in ring studies in three

laboratories using 19 chemicals [47].

### 3.1.3. In vitro dendritic cell activation (KE 3)

The third major event regarding AOP of skin sensitization is maturation and migration of DC and LC. To identify the activation of DCs, test methods were developed by quantifying changes in expression of cell surface markers (CD54, CD86) or changes in IL-8 expression associated with monocyte and DC activation processes [27].

#### 3.1.3.1. h-CLAT (the human cell line activation test) (adopted as OECD TG 442E)

The h-CLAT evaluates CD54 and CD86 expression in human monocyte leukemia cell line (THP-1) for skin sensitizer [48]. In h-CLAT, the test substance is considered positive if the relative fluorescence intensity (RFI) of CD 86 or CD 54 is at least 150% or at least 200% at concentrations of at least 50% cell viability, respectively. In validation studies compared with LLNA results, h-CLAT showed 85% accuracy, 93% sensitivity, and 66% specificity [27]. Like other non-animal methods, chemicals with low or moderate skin sensitization potency are more likely to make false-negative predictions in h-CLAT. In addition, water-insoluble chemicals (Octanol–water partition coefficient; log $K_{ow}$ > 3.5) tended to produce false-negative results [49].

#### 3.1.3.2. U-SENS (the U937 Cell Line Activation Test) (OECD TG 442E)

U-SENS is a similar DC activation assay to h-CLAT. Unlike h-CLAT, measures only CD86 expression in U937 cell line against skin sensitizer. This assay uses a cut-off of 1.5-fold induction in CD86 expression with cell viability above 70% to identify skin sensitizers [50]. In the validation study, U-SENS showed an accuracy of 86 %, a sensitivity of 91% and a specificity of 65% compared with LLNA results [27].

#### 3.1.3.3. IL-8 Luc assay (the Interleukin-8 Reporter Gene Assay) (OECD TG 442E)

IL8-Luc assays quantify changes in IL-8 expression, a cytokine associated with the activity of DCs. In the IL-8 Luc assay, a THP-1-derived IL-8 reporter cell line (THP-G8, established from the human acute monocyte leukemia cell line THP-1) is used. These cells contain both SLO (Stable Luciferase Orange) and SLR (Stable Luciferase Red) luciferase genes. The SLO gene is under the control of IL-8, and the SLR gene is controlled by the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter [51]. Measure the induction of the two luciferase genes, calculate the ratio of the two inductions, and then divide by the ratio of inductions of untreated cells. Using a cut-off value of $\geq 1.4$ for induction, this test shows with accuracy 86%, sensitivity 96%, and specificity 41% [27].

#### 3.1.3.4. In vitro gene expression assay in the dendritic cell: Genomic Allergen Rapid Detection (GARD) assay

Analysis of Genomic Allergen Rapid Detection (GARD) is a technique for determining changes in the transcription level of genomic biomarkers using the microarray system in the human cell line MUTZ. The evolving genomic biomarkers are called the GARD Prediction Signature (GPS) and are composed of genes related to innate immune response signaling, DC maturation, and exogenous material stress response. A machine learning program called Support Vector Machine (SVM) is used to analyze the microarray data. If the SVM decision value (GARD $^{TM}$ DV) is a positive number (> 0), it is determined as a sensitizer. If a negative number (<0) is obtained, it is determined as a non-sensitizer [52, 53]. In a recent validation study conducted in three laboratories, the inter-laboratory reproducibility was 92.0%, the within-laboratory reproducibility was between 82.1% and 88.9%, and the accuracy was 93.8% [54].

## 3.2. Adverse Outcome Pathway(AOP)-based Integrated Approach and Test and Assessment (IATA) to predict skin sensitization potential

Many alternative methods have been developed based on the Adverse Outcome Pathway (AOP) of skin sensitization [55]. However, due to the complexity

of the biological processes associated with the skin sensitization response, it has been suggested that the evaluation of skin sensitization using every single animal alternative test has its limitations and is not suitable for predicting sensitizer hazard and potency [56-58]. Therefore, there are many efforts to overcome the limitations of a single prediction method by combining and analyzing data from several different methods that target different mechanisms of skin sensitization. Strategies to combine existing data with an experimental analysis and calculation tools are an old and traditional approach and used in various terms. Examples are Integrated Test Strategy (ITS), Sequential Test Strategy (STS), and Integrated Approach and Test and Assessment (IATA) [59].

At present, there are several ongoing approaches to developing so-called defined approaches to achieve higher predictability of skin sensitization potential, combined with the development of OECD TG methods or individual laboratory analyses [58, 60-68]. In addition, the OECD, in collaboration with EURL ECVAM, proposed a roadmap for developing AOP-based IATA/ ITS, so-called defined approach (DA) with a fixed data interpretation procedure (DIP).

Interpret the data generated by a defined set of information sources that can be used alone or in combination with other information sources to meet regulatory requirements; (1) distinguish skin sensitizers from non-sensitizers (hazard IDs), (2) distinguish strong from moderate sensitizers (GHS potency), and (3) provide ongoing quantitative measures used for risk assessment [69]. Currently, 12 defined approaches (DA) are being evaluated by the OECD Working Group for the hazard identification and potency estimation of skin sensitization (Table 2). Some of these

DAs have been validated and will be adopted as one of the OECD test guidelines.

**Table 2. Defined and/or integrated approaches to testing and assessment for assessing skin sensitization potential.**

| OECD study (submitter) | Purpose | Data inputs | Reference |
|---|---|---|---|
| An AOP-based "2 out of 3" Integrated Testing Strategy Approach to Skin Hazard Identification (BASF) | Hazard ID | DPRA, h-CLAT, KeratinoSens™, U-SENS™ | [58] |
| Sequential Testing Strategy (STS) for Hazard Identification of Skin Sensitizers (RIVM) | Hazard ID | DPRA, h-CLAT, KeratinoSens™, HaCaT gene signature, MultiCASE, CAESAR, DEREK, OECD QSAR toolbox | [43] |
| A non-testing pipeline approach for skin sensitization (DuPont/G. Patlewicz) | Hazard ID | Existing data, protein binding profile, physicochemical properties, TIMES-SS, expert judgment | [30] |
| Stacking Meta-model for Skin sensitization Hazard Identification (L'Oréal) | Hazard ID | DPRA, KeratinoSens™, U-SENS™, TIMES-SS, ToxTree, volatility, pH | [70] |
| Integrated decision strategy for skin sensitization hazard (ICCVAM) | Hazard ID | DPRA, h-CLAT, KeratinoSens™, OECD QSAR Toolbox, physicochemical properties | [71] |
| Consensus of Classification Trees for Skin Sensitization Hazard Prediction (EC-JRC) | Hazard ID | TIMES-SS, DRAGON descriptors | [72] |
| Sensitizer Potency Prediction Based on Key Event 1 + 2: Combination of Kinetic Peptide Reactivity Data and KeratinoSens™ Data (Givaudan) | Potency (continuous) | Cor1C420 (kinetic peptide reactivity), KeratinoSens™, TIMES-SS | [73] |
| The Artificial Neural Network Model for Predicting LLNA EC3 (Shiseido) | Potency class/EC3 | DPRA, h-CLAT, ARE (or KeratinoSens™) | [60] |

**Table 2. Defined and/or integrated approaches to testing and assessment for assessing skin sensitization potential (*cont'd*).**

| OECD study (submitter) | Purpose | Data inputs | Reference |
|---|---|---|---|
| Bayesian Network DIP (BN-ITS-3) for Hazard and Potency Identification of Skin Sensitizers (P&G) | Potency class | DPRA, h-CLAT, KeratinoSens™, TIMES-SS, bioavailability (solubility at pH 7, log *D* at pH 7, plasma protein binding, fraction ionized) | [63] |
| Sequential Testing Strategy (STS) for Sensitizing Potency Classification Based on *In Chemico* and *In Vitro* Data (Kao) | Potency class | DPRA, h-CLAT | [68] |
| ITS for Sensitizing Potency Classification Based on *In Silico*, *In Chemico*, and *In Vitro* Data (Kao) | Potency class | DPRA, h-CLAT, DEREK | [68] |
| Data Interpretation Procedure for Skin Allergy Risk Assessment (SARA) (Unilever) | Sensitization probability | Bioavailability, skin protein kinetics, ordinary differential equation model | [29] |

Hazard ID means hazard identification.

### 3.2.1. An AOP-based "2 out of 3" Integrated Testing Strategy Approach to Skin Hazard Identification (BASF)

The "2 out of 3 approach" uses three different dates from *in chemico* and *in vitro* animal alternatives (DPRA, KeratinoSens ™ or LuSens™, and h-CLAT or U-SENS) with different key events (KE 1. 2 and 3) in skin sensitization AOP and determine whether it is a skin sensitizer. Skin sensitizer is determined in the direction in which the results of the two tests are consistent. If a chemical is positive for two of the three tests, it is finally determined to be positive [58]. The predictivity of the "2 out of 3 approach" is mentioned in Table 3, which is widely used in industry and CRO as a relatively simple and integrated predictive approach.

### 3.2.2. Sequential Testing Strategy (STS) for Hazard Identification of Skin Sensitizers (RIVM)

The "RIVM STS approach" is a tiered method that combines *in silico* and *in vitro* methods. First, the combination results of four QSAR models (MultiCASE, CAESAR, DEREK, and OECD QSAR Toolbox) are used to predict LLNA data. Depending on the *in silico* results, DPRA (Tier 1) and KeratinoSens ™ or HaCaT gene signature (Tier 2) evaluations are performed sequentially. If there is a conflict between the results of Tier 1 and Tier 2, an additional h-CLAT test will be carried out to test for the sensitization of the skin. The predictivity of the "RIVM STS approach" is mentioned in Table 3 [43].

### 3.2.3. A non-testing pipeline approach for skin sensitization (DuPont/G. Patlewicz)

The “DuPont IATA-SS approach” is a method that requires a process of expert interpretation rather than a defined approach performed by a fixed method. The knowledge of an expert is required to integrate existing in vivo and non-animal experimental data (mutagenicity, genotoxicity, skin corrosion, irritation, and so on), information for protein binding profiles, metabolites and physicochemical properties. Because of the need for expert opinion, this approach is not quantitative and limited in its use. The predictivity of the “DuPont IATA-SS approach” is mentioned in Table 3 [30].

### 3.2.4. Stacking Meta-model for Skin Sensitization Hazard Identification (L'Oréal)

The “Stacking Meta-model approach” provides a probability-based sensitizer risk prediction by combining the *in vitro* and *in silico* results associated with key events 1 to 3 of skin sensitization AOP. This model is created by the combination of *in silico* prediction (TIMES-SS and Toxtree), *in chemico* prediction (DPRA) and *in vitro* prediction (KeratinoSens ™ and U-SENS ™), the physicochemical properties and five statistical tools (Boosting, Naïve Bayes, SVM, Sparse PLS-DA, and Expert Scoring). This model has advantages that it can be

applied even if data is missing and can be applied to multi-component materials. The predictivity of the “Stacking Meta-model approach” is mentioned in Table 3 [70].

### 3.2.5. Integrated decision strategy for skin sensitization hazard (ICCVAM)

The “ICCVAM SVM model” predicts LLNA results using h-CLAT results, OECD Toolbox (version 3.2) predictions and six physicochemical properties, and uses human data using KeratinoSens ™, h-CLAT, DPRA, log *P* and OECD Toolbox data. Because prediction requires physicochemical properties and information related to the structure, this model has limitations that can only be applied to substances with defined structures. The predictivity of this approach is suggested in Table 3 [71, 74].

### 3.2.6. The consensus of Classification Trees for Skin Sensitization Hazard Prediction (EC-JRC)

The “Joint Research Center (JRC) of the European Commission develops the classification trees consensus (CCT) (EC-JRC) approach” predict skin sensitization hazard by using structural features or protein reactivity descriptors from the TIMES-SS and Dragon software. The machine learning is conducted by both TIMES-SS and Dragon software with existing animal alternative dates and, as a

result, the decision tree is made. The approach could only be applied to organic substances that are well-identified [72]. The predictivity of the "EC-JRC approach" is suggested in Table 3.

### 3.2.7. Sensitizer Potency Prediction Based on Key Event 1 + 2: Combination of Kinetic Peptide Reactivity Data and KeratinoSens™ Data (Givaudan)

Givaudan ITS is an approach that uses a multivariate regression model to predict skin sensitization. To create a regression model, the dates from KeratinoSens ™ assay and Cor1C420 assay, and TIMES-SS mechanistic alert data are required. This method, however, has limitations that cannot be extended to lipophilic chemicals with log *P* exceeding 5 and chemicals with undefined structures [73]. The predictivity of this approach is mentioned in Table 3.

### 3.2.8. The Artificial Neural Network (ANN) Model for Predicting LLNA EC3 (Shiseido)

The ANN-EC3 model is a non-linear statistical model that combines multiple *in vitro* and *in silico* data related to KE 1–3 of the skin sensitization AOP to predicts the LLNA EC3. Multiple Artificial Neural Network (ANN) models were made by physicochemical properties, QSAR predictions and *in chemico/ in vitro* data

from DPRA(KE1), Antioxidant response element (ARE) test or KeratinoSens™ (KE2), and h-CLAT [75]. The predictivity of this approach is mentioned in Table 3 and 4.

### 3.2.9. Bayesian Network DIP (BN-ITS-3) for Hazard and Potency Identification of Skin Sensitizers (P&G)

The BN ITS-3 approach uses quantitative data from DPRA, KeratinoSens ™, and h-CLAT (not negative or positive binary data) associated with KE 1–3 of skin sensitization AOP. TIMES-SS data, bioavailability data calculated in physicochemical properties using ACD Labs software (water solubility, log *D*, fractional ionization, and binding of plasma protein) are also used. The data is integrated using a statistical multi-step process and provides four skin sensitivity classes (Extreme / Strong, Moderate, Weak, Non-sensitizer) [63, 65]. The predictivity of this approach is suggested in Table 3 and 4.

### 3.2.10. Sequential Testing Strategy (STS) for Sensitizing Potency Classification Based on *In Chemico* and *In Vitro* Data (Kao)

The decision of the "Kao-STS approach" is based on data of DPRA and h-CLAT data (represent Key Events 1 and 3, respectively). First, if h-CLAT generates a positive outcome, the test substance is classified as a strong-sensitizer or weak-

sensitizer based on the lowest concentration resulting in a positive reaction (MIT), and no further testing is performed. When the h-CLAT MIT shows a range of more than 10 and less than 5000 μg / ml, the substance is categorized as a weak sensitizer and is classified as a strong sensitizer below 10 μg / ml. If the h-CLAT result is negative, it is necessary to perform additional DPRA. If the result of DPRA is positive, it will be classified as a weak sensitizer; if the result of DPRA is negative, it will be classified as non-sensitizer [66, 68]. Table 3 and 4 suggest the predictivity of this approach.

### 3.2.11. ITS for Sensitising Potency Classification Based on *In Silico*, *In Chemico,* and *In Vitro* Data (Kao)

"Kao ITS approach" uses data from DPRA and h-CLAT and predictions from the Derek Nexus program and present a score-based decision for skin sensitization. Kao ITS can predict three LLNA potencies (strong, weak, and non-sensitizer) similar to Kao STS. Positive response level (% depletion of DPRA, MIT h-CLAT concentration) classifies scores from 0 to 3. Furthermore, the DEREK Nexus alert produced score 0 (no warning) or 1 (warning). The LLNA's potency is predicted by sum of these scores (0-1: non-sensitizer; 2-6: weak; 7: strong) [66, 68]. The performance data of the "Kao ITS approach" is suggested in Table 3 and 4.

### 3.2.12. Data Interpretation Procedure for Skin Allergy Risk Assessment (SARA) (Unilever)

The SARA approach creates a statistical probabilistic model using *in vitro* / *in chemico* test data from DPRA, KeratinoSens™, U-Sens™, and h-CLAT and *in silico* data from Tox-tree and OECD QSAR Toolbox. This probabilistic model predicts EC3 of LLNA or No expected sensitization induction level (NESIL) of Human Repeat Insult Patch Test (HRIPT). SARA is difficult to perform easily because it requires higher qualified statistical analysis than other DAs, but an extrapolation of the results of in vitro / in chemical testing allows quantitative skin sensitization risk assessment (QRA) by predicting a value in actual in vivo testing. The predictability of QRA has the benefit of determining appropriate product concentrations, making it a desirable DA in the industry [29, 76].

**Table 3. Defined approach performance in predicting skin sensitization hazard.**

| OECD case study title (submitter) | Compared to | Sensitivity (%) | Specificity (%) | Accuracy (%) | References |
|---|---|---|---|---|---|
| An Adverse Outcome Pathway-based "2 out of 3" integrated testing strategy approach to skin hazard identification (BASF) | LLNA | 72 | 64 | 70 | [77] |
| | Human | 79 | 73 | 77 | [77] |
| Sequential Testing Strategy (STS) for Hazard Identification of Skin Sensitizers (RIVM) | LLNA | 93 | 64 | 83 | [43] |
| | Human | 96 | 93 | 95 | [43] |
| A non-testing pipeline approach for skin sensitization (DuPont/G. Patlewicz) | LLNA | 86 | 89 | 88 | [30] |
| Stacking Meta-model for Skin Sensitization Hazard Identification (L'Oréal) | LLNA | 85 | 91 | 87 | [70] |
| | Human | 91 | 86 | 86 | [70] |
| Integrated decision strategy for skin sensitization hazard (ICCVAM) | LLNA | 93 | 73 | 88 | [77] |
| | Human | 86 | 72 | 82 | [77] |
| Consensus of Classification Trees for Skin Sensitization Hazard Prediction (EC-JRC) | LLNA | 98 | 85 | 93 | [72] |
| Sensitizer Potency Prediction Based on Key Event 1 + 2: Combination of Kinetic Peptide Reactivity Data and KeratinoSens™ Data (Givaudan) | LLNA | 82 | 78 | 80 | [72] |
| The Artificial Neural Network Model for Predicting LLNA EC3 (Shiseido) | LLNA | 98 | 31 | 81 | [77] |
| | Human | 100 | 31 | 79 | [77] |

**Table 3. Defined approach performance in predicting skin sensitization hazard (*cont'd*).**

| OECD case study title (submitter) | Compared to | Sensitivity (%) | Specificity (%) | Accuracy (%) | References |
|---|---|---|---|---|---|
| Bayesian Network DIP (BN-ITS-3) for Hazard and Potency Identification of Skin Sensitizers (P&G) | LLNA | 83 | 83 | 83 | [77] |
| | Human | 81 | 64 | 76 | [77] |
| Sequential Testing Strategy (STS) for Sensitizing Potency Classification Based on *In Chemico* and *In Vitro* Data (Kao) | LLNA | 93 | 34 | 78 | [77] |
| | Human | 98 | 41 | 80 | [77] |
| ITS for Sensitizing Potency Classification Based on *In Silico*, *In Chemico*, and *In Vitro* Data (Kao) | LLNA | 86 | 60 | 79.2 | [77] |
| | Human | 94 | 67 | 85 | [77] |

**Table 4. Defined approach performance in predicting skin sensitization potency.**

| OECD case study title (submitter) | Compared to | Accuracy (%) | Over-predicted (%) | Under-predicted (%) | References |
|---|---|---|---|---|---|
| The Artificial Neural Network Model for Predicting LLNA EC3 (Shiseido) | LLNA | 70 | 23 | 7 | [77] |
| | Human | 63 | 25 | 12 | [77] |
| Bayesian Network DIP (BN-ITS-3) for Hazard and Potency Identification of Skin Sensitizers (P&G) | LLNA | 68 | 12 | 20 | [77] |
| | Human | 55 | 20 | 25 | [77] |
| Sequential Testing Strategy (STS) for Sensitising Potency Classification Based on in Chemico and *In Vitro* Data (Kao) | LLNA | 68 | 21 | 11 | [77] |
| | Human | 63 | 22 | 14 | [77] |
| ITS for Sensitising Potency Classification Based on *In Silico*, *In Chemico*, and *In Vitro* Data (Kao) | LLNA | 67 | 14 | 19 | [77] |
| | Human | 69 | 13 | 18 | [77] |

# 4. General summary and future forecast

Animal testing on raw materials and products has been banned in the cosmetic industry since March 2013 in the EU and since 2017 in Korea. Toxicological data, however, is needed for newly developed or widely used chemicals in terms of regulation. For skin sensitization, a mechanism-based study called AOP was carried out, animal alternatives corresponding to this mechanism were developed and some alternatives were adopted as a guideline through validation studies. However, each test method has a limitation that some substances cannot be evaluated and therefore cannot be accurately predicted, and most of the currently developed methods focus on the determination of skin sensitizers and non-sensitizers. Defined approaches have been studied to overcome these limitations and increase predictivity, and some DAs have shown good results and are expected to be as guidelines soon.

Development of me-too animal alternatives related to KE 2 or 3 skin sensitization AOP is actively underway and may be added to the OECD TG in the future. However, the development of assays for KE4 related to the proliferation of lymphocytes is likely to be difficult due to the lack of a suitable cell culture system. In addition, the research of predicting sensitization potential and potency with a combination of *in chemico* / *in vitro* methods, *in silico* and statistical / machine learning techniques will be continued worldwide. Chemicals of similar structures, in particular, agrochemicals, have been used in the industry to evaluate skin sensitization using alternative animal testing methods. In addition to predicting

potency categories of skin sensitization, attempts have been made to develop assays or approaches that can predict human NESIL and LLNA EC3 levels. These values are expected to be used in the study to suggest safe concentrations that do not cause skin sensitization by product category reflecting actual exposure to product use.

# CHAPTER I

# High-throughput screening (HTS)-based spectrophotometric direct peptide reactivity assay (Spectro-DPRA) to predict skin sensitization potential

# 1. Introduction

The correlation between skin protein reactivity and skin sensitization reaction is well established. The formation of an adduct between chemical and endogenous proteins or peptides in the skin is critical to trigger the process of skin sensitization [78-80]. The side chains of amino acids carry electron-rich groups that are capable of reacting with nucleophilic allergens in skin proteins. In particular, lysine and cysteine are electron-rich amino acids that strongly react with electrophiles [5, 81]. Haptens are small molecules (molecular weight < 1,000 Da) that may interact and form bonds of various strengths with macromolecules in the skin; this interaction, known as hapten–protein conjugation, is a key phenomenon underlying skin sensitization. For instance, allergic contact dermatitis (ACD) is a skin disorder that results from a hapten-specific delayed-type hypersensitivity reaction [2], which occurs upon repeated exposure to any hapten [1, 3]. The strength and stability of the bond between the chemical and protein are critical factors involved in the first step of skin sensitization [31]. As almost any chemical can form a hapten–protein conjugate and induce skin sensitization, it is essential to determine the skin sensitization potential of new substances in cosmetics and drugs prior to their application to the skin.

The animal guinea pig maximization test (GPMT) and Buehler test (OECD TG 406) have been traditionally used for the prediction of the skin sensitization potential of new chemical formulations [14]. However, these tests are based on the evaluation of the degree of eczema and edema induced by the test substance

according to a visual scoring system in the elicitation phase, and may not provide quantitative data [15]. To obtain quantitative data and to reduce the use of animals in toxicity evaluation studies, local lymph node assay (LLNA) was developed and adopted as OECD Test Guideline 429 [82]. As animal testing for cosmetics was completely banned in the EU [22-24], many alternative methods have been developed to predict the endpoint of animal toxicity testing. For skin sensitization evaluation, many alternative strategies have been designed that rely on the known mechanism of skin sensitization, and many of these methods have been adopted in OECD test guidelines, including hapten-peptide reactivity (direct peptide reactivity assay [DPRA]), keratinocyte activation (KeratinoSens™ and LuSens™), and dendritic cell activation (h-CLAT, U-SENS™, and IL-8 Luc assay) assays [25-27].

In particular, the evaluation of the reactivity between chemicals and proteins/peptides serves as an attractive alternative to animal testing to determine the skin sensitization potential of test compounds [7, 31]. At present, the measurement of reactivity (adduct formation) between a chemical substance and protein/peptide largely relies on two analytical methods, namely, high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [32, 81] and liquid chromatography-mass spectrometry (LC/MS) [37, 83-86]. However, HPLC- or LC/MS-based methods have several disadvantages, including the requirement of relatively expensive equipment for analysis, long sample measurement procedures, and skilled operators. In contrast, spectrophotometric analysis is a conventional method used for the detection of color changes in samples and may be used even with very small quantities of samples. Several studies have reported the development

of spectrophotometric assays for skin sensitization testing, including detection of glutathione (GSH) reactivity by monitoring the free thiol group of GSH following derivatization with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) [87]. In addition, a fluorescence-based high-throughput screening method developed with dansyl cysteamine (DCYA) for the detection of hapten-thiol adduct showed good predictability for skin sensitization in preliminary assessments [88].

I have previously reported a new spectrophotometric assay method (Spectro-DPRA) that was developed to determine the reactivity of chemicals toward two chemical groups, the thiol group of a cysteine-containing peptide (cysteine peptide) detected using DTNB and the amino group of a lysine-containing peptide (lysine peptide) detected using fluorescamine [89]. I also investigated the possibility of using this method as a new *in chemico* sensitization test and reported its relatively good performance. Using a depletion cut-off value for each peptide, I constructed a new prediction model with a high degree of sensitivity (80.0%), specificity (86.7%), and accuracy (82.5%).

In the present study, I sought to confirm the predictive value of Spectro-DPRA by exploring a larger number of chemicals for their animal skin sensitization potential. Furthermore, I validated the use of this method to estimate the human skin sensitization potential of chemicals. To increase the predictive power of the model, I assessed the effect of lipophilicity of chemicals, determined by the log partition coefficient n-octanol/water (Log $K_{ow}$) value, on the prediction of sensitization potential.

# 2. Material and methods

## *2.1. Peptides and test chemicals*

I used two model peptides, a cysteine peptide (Ac-RFAACAA-COOH) and a lysine peptide (Ac-RFAAKAA-COOH) with > 95% purity (Peptron Co., Daejeon, Korea). The chemical name, class, molecular weight, chemical abstract system (CAS) number, Log $K_{ow}$, and LLNA data of 65 test materials are presented in Table 1. The test chemicals included 49 sensitizers (S) and 16 non-sensitizers (NS). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

## *2.2. Measurement of cysteine peptide depletion*

The reaction for the measurement of cysteine peptide was carried out as per the method described in my previous study [89]. The peptide-to-chemical ratio used was 1:5 for cysteine and 1:10 for lysine.

The cysteine peptide working solution was prepared by diluting the cysteine peptide to 400 μM concentration with 100 mM sodium phosphate buffer (pH 8.0, 1 mM ethylenediaminetetraacetic acid [EDTA]) before the experiment. The chemical working solution was prepared by diluting chemicals to 2 mM with 100 mM sodium phosphate buffer (pH 8.0, 1 mM EDTA). Ninety μL of peptide working solution and 90 μL of chemical working solution were reacted in a clear 96-well plate for 24 h at room temperature (22 ℃). After 24 h, 20 μL of 10 mM DTNB solution (Dojindo Laboratories, Kumamoto, Japan) solubilized in 100 mM sodium phosphate buffer

(pH 8.0) was added to each well of the plate. The mixture was incubated for 3 min to complete the reaction between unreacted peptide and DTNB. The optical density was measured before the addition of DTNB solution and after the reaction between unreacted peptide and DTNB using a UV-VIS spectrophotometer (SpectraMAX 190, Molecular Devices, CA, USA) at 412 nm wavelength.

## *2.3. Measurement of lysine peptide depletion*

The reaction for measurement of lysine peptide was performed according to the previously described method [89]. The lysine peptide working solution was prepared by diluting the lysine peptide to 200 μM with 100 mM sodium phosphate buffer (pH 10.0, 1 mM EDTA) before the experiment, and the chemical working solutions were prepared following dilution of chemicals to 2 mM concentration with isopropanol. One hundred μL of lysine peptide working solution and 100 μL of chemical working solution were reacted in a 96-well polypropylene plate for 24 h at room temperature, and 180 μL of the reaction mixture was transferred to a light-proof black clear-bottom 96-well plate (Greiner Bio-One, Frickenhausen, Germany). Twenty μL of 20 mM fluorescamine solution (prepared in DMSO) was added to each well of the plate and the plate was incubated for 3 min to allow reaction between unreacted peptide and fluorescamine. Fluorescence intensity was measured before the addition of fluorescamine solution and after the reaction between unreacted peptide and fluorescamine using a fluorometer (FlexStation 3, Molecular Devices; excitation: 390 nm, emission: 465 and 475 nm).

## *2.4. Calculation of peptide depletion ratio*

The reactivity of a chemical with a peptide was expressed as the peptide depletion ratio after 24 h of incubation together. The peptide depletion ratio was calculated as follows (Fig. 1): Peptide depletion ratio (%) = $(1 - P_{unreacted}/P_{total}) \times 100$ Some chemicals have a unique color or may interfere with the spectrophotometric property changes upon reaction with the peptide and/or detection reagent. For the exact evaluation, background signals must be monitored. Thus, I used three different controls and measured the spectrophotometric values before and after the addition of detection reagents to avoid any interference from the background signal (Fig. 1) [89]. The calculated peptide depletion ratios are presented as means ± standard deviation (SD) obtained from three independent experiments carried out in triplicates.

## *2.5. Comparison of skin sensitization potential results from peptide depletion ratio and LLNA*

The skin sensitization potentials of 66 chemicals determined from peptide depletion ratio were compared with the in vivo data from the EC3 of LLNA [15, 58, 66, 90, 91]. I evaluated the predictivity of Spectro-DPRA by calculating the accuracy, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the results based on LLNA data for 65 chemicals as follows:

Sensitivity (%) = TP/(TP + FN) × 100,

Specificity (%) = TN/(TN + FP) × 100,

PPV (%) = TP/(TP + FP) × 100,

NPV (%) = TN/(TN + FN) × 100, and

Accuracy (%) = (TP + TN)/(TP + TN + FP + FN) × 100, where TP = true positive, TN = true negative, FN = false negative, and FP = false positive.

## Table 1. List of tested chemicals.

| Chemical | CAS no. | M.W. | Log $K_{ow}$ | LLNA (Animal) | | | | Human | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Group | EC3 (%) | ECETOC CL | Reference | Group | Reference |
| 1-Chloro-2,4-dinitrobenzene (DNCB) | 97-00-7 | 202.6 | 2.27 | S | 0.05 | Extreme | [91] | S | [92] |
| 5-Chloro-2-methyl-4-isothiazolin-3-on (MCI) | 26172-55-4 | 149.6 | -0.34 | S | 0.009 | Extreme | [91] | S | [92] |
| Oxazolone | 15646-46-5 | 217.2 | 1.51 | S | 0.003 | Extreme | [91] | S | [92] |
| Diphenylcyclopropenone | 886-38-4 | 206.2 | 3.25 | S | 0.003 | Extreme | [15] | S | [93] |
| Potassium dichromate | 7778-50-9 | 294.2 | -3.59 | S | 0.08 | Extreme | [91] | S | [93] |
| Glutaric dialdehyde | 111-30-8 | 100.1 | -0.18 | S | 0.1 | Strong | [91] | S | [92] |
| Propyl gallate | 121-79-9 | 212.2 | 1.79 | S | 0.32 | Strong | [15] | S | [93] |
| Hydroquinone | 123-31-9 | 110.1 | 1.03 | S | 0.11 | Strong | [91] | S | [93] |
| *p*-Phenylenediamine | 106-50-3 | 108.1 | -0.39 | S | 0.16 | Strong | [91] | S | [93] |
| Lauryl gallate | 1166-52-5 | 338.4 | 6.21 | S | 0.3 | Strong | [91] | S | [93] |
| Methyldibromoglutaronitrile | 35691-65-7 | 265.9 | 1.63 | S | 0.9 | Strong | [91] | S | [93] |
| Dinitroflurobenzene (DNFB) | 70-34-8 | 186.1 | 1.83 | S | 0.016 | Strong | [58] | S | [93] |
| Phthalic anhydride | 85-44-9 | 148.1 | 2.07 | S | 0.16 | Strong | [15] | S | [92] |
| Isoeugenol | 97-54-1 | 164.2 | 2.66 | S | 1.2 | Moderate | [91] | S | [92] |
| Diethylenetriamine | 111-40-0 | 103.2 | -2.13 | S | 5.8 | Moderate | [91] | S | [58] |
| 2-Vinyl pyridine | 100-69-6 | 105.1 | 1.71 | S | 1.6 | Moderate | [66] | n.a. | . |

**Table 1. List of tested chemicals (*cont'd*).**

| Chemical | CAS no. | M.W. | Log $K_{ow}$ | LLNA (Animal) | | | | Human | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Group | EC3 (%) | ECETOC CL | Reference | Group | Reference |
| Ammonium persulfate | 231-786-5 | 228.2 | n.c. | S | 1.9 | Moderate | [90] | S | [94] |
| 3-Aminophnol | 591-27-5 | 109.1 | 0.24 | S | 3.2 | Moderate | [91] | n.a. | . |
| Tartaric acid | 87-69-4 | 150.1 | -1 | S | 8.7 | Moderate | [91] | NS | [92] |
| Cinnamic aldehyde | 104-55-2 | 132.2 | 1.82 | S | 3 | Moderate | [91] | S | [92] |
| 2-Mercaptobenzothiazole | 149-30-4 | 167.3 | 2.86 | S | 1.7 | Moderate | [15] | S | [93] |
| Resorcinol | 108-46-3 | 110.1 | 1.03 | S | 5.7 | Moderate | [15] | S | [92] |
| Diethyl maleate | 141-05-9 | 172.2 | 2.2 | S | 5.8 | Moderate | [91] | S | [93] |
| Nickel II sulfate heptahydrate | 7786-81-4 | 154.8 | n.c. | S | 4.8 | Moderate | [15] | S | [92] |
| 2-Methyl-4-isothiazolin-3-one | 2682-20-4 | 115.2 | -0.83 | S | 1.9 | Moderate | [91] | S | [93] |
| 1-Naphthol | 90-15-3 | 144.2 | 2.69 | S | 1.3 | Moderate | [91] | n.a. | . |
| 3-Dimethylamino-1-propylamine | 109-55-7 | 102.2 | -0.45 | S | 2.2 | Moderate | [91] | S | [93] |
| 4-Chloroaniline | 106-47-8 | 127.6 | n.c. | S | 6.5 | Moderate | [15] | n.a. | . |
| Ethylenediamine | 107-15-3 | 60.1 | -1.62 | S | 2.2 | Moderate | [91] | S | [92] |
| Oxalaldehyde | 107-22-2 | 58 | -1.66 | S | 1.4 | Moderate | [91] | n.a. | . |
| Geraninol | 106-24-1 | 154.3 | 3.47 | S | 26 | Weak | [91] | S | [93] |

**Table 1. List of tested chemicals (*cont'd*).**

| Chemical | CAS no. | M.W. | Log $K_{ow}$ | LLNA (Animal) | | | | Human | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Group | EC3 (%) | ECETOC CL | Reference | Group | Reference |
| R(+) Limonene (not oxidized) | 5989-27-5 | 136.2 | 4.83 | S | 69 | Weak | [91] | NS (not oxidized) | [93] |
| Linalool | 78-70-6 | 154.3 | 3.38 | S | 30 | Weak | [91] | S | [93] |
| 2-Ethylbutyraldehyde (diethyl acetaldehyde) | 97-96-1 | 100.2 | 1.73 | S | 76 | Weak | [91] | n.a. | . |
| 2-(4-tert-Butylbenzyl)propionaldehyde (Lilial) | 80-54-6 | 204.3 | 4.36 | S | 19 | Weak | [91] | S | [93] |
| Sodium lauryl sulfate (SLS) | 151-21-3 | 288.4 | 2.42 | S | 14 | Weak (FP) | [91] | NS | [92] |
| Eugenol | 97-53-0 | 164.2 | 2.73 | S | 13 | Weak | [91] | S | [92] |
| Phenyl benzoate | 93-99-2 | 198.2 | 3.04 | S | 20 | Weak | [91] | S | [92] |
| Cinnamic alcohol | 104-54-1 | 134.2 | 1.84 | S | 21 | Weak | [91] | S | [92] |
| Imidazolidinyl urea | 39236-46-9 | 388.3 | -8.28 | S | 24 | Weak | [91] | S | [92] |
| Methyl methacrylate | 80-62-6 | 100.1 | 1.28 | S | 90 | Weak | [58] | S | [92] |
| Ethylene glycol dimethacrylate | 97-90-5 | 198.2 | 2.21 | S | 28 | Weak | [91] | S | [92] |
| Xylene | 1330-20-7 | 106.2 | 3.09 | S | 95.8 | Weak | [58] | NS | [92] |
| Benzocaine | 1994-09-07 | 165.2 | 1.8 | S | 22 | Weak | [15] | S | [58] |
| Dimethylsulfoxide | 67-68-5 | 78.1 | -1.22 | S | 72 | Weak | [91] | NS | [93] |
| Abietic acid | 514-10-3 | 302.5 | 6.46 | S | 15 | Weak | [91] | S | [93] |

**Table 1. List of tested chemicals (*cont'd*).**

| Chemical | CAS no. | M.W. | Log $K_{ow}$ | LLNA (Animal) | | | | Human | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Group | EC3 (%) | ECETOC CL | Reference | Group | Reference |
| Hexylcinnamic aldehyde (HCA) | 101-86-0 | 216.3 | 4.82 | S | 11 | Weak | [91] | NS | [93] |
| Citral | 5392-40-5 | 152.2 | 3.45 | S | 13 | Weak | [91] | S | [92] |
| Isopropyl myristate | 110-27-0 | 270.5 | 7.17 | S | 44 | Weak (FP) | [91] | NS | [92] |
| Lactic acid | 50-21-5 | 90.1 | -0.65 | NS | n.c. | NS | [91] | NS | [93] |
| Methyl salicylate | 119-36-8 | 152.2 | 2 | NS | n.c. | NS | [91] | NS | [92] |
| Glycerol | 56-81-5 | 92.1 | -1.65 | NS | n.c. | NS | [91] | NS | [92] |
| Tween80 | 9005-65-6 | 1310 | 4.23 | NS | n.c. | NS | [15] | NS | [92] |
| Isopropanol | 67-63-0 | 60.1 | 0.28 | NS | n.c. | NS | [91] | NS | [93] |
| Salicylic acid | 69-72-7 | 138.1 | 2.24 | NS | n.c. | NS | [91] | NS | [92] |
| 6-Methyl coumarin | 92-48-8 | 160.2 | 2.06 | NS | n.c. | NS | [91] | NS | [92] |
| Benzoic acid | 65-85-0 | 122.1 | 1.87 | NS | > 20 | NS | [58] | n.a. | . |
| Nonanoic acid | 112-05-0 | 158.2 | 3.52 | NS | > 50 | NS | [58] | n.a. | . |
| Octanoic acid | 124-07-2 | 144.2 | 3.03 | NS | n.c. | NS | [91] | NS | [93] |
| *n*-Hexane | 110-54-3 | 86.2 | 3.29 | NS | n.c. | NS | [91] | NS | [93] |
| Chlorobenzene | 108-90-7 | 112.6 | 2.64 | NS | n.c. | NS | [91] | n.a. | . |

**Table 1. List of tested chemicals (*cont'd*).**

| Chemical | CAS no. | M.W. | Log $K_{ow}$ | LLNA (Animal) | | | | Human | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Group | EC3 (%) | ECETOC CL | Reference | Group | Reference |
| 1-Butanol | 71-36-3 | 74.1 | 0.84 | NS | n.c. | NS | [91] | NS | [93] |
| Propylene glycol | 57-55-6 | 76.1 | -0.78 | NS | n.c. | NS | [91] | NS | [93] |
| Vanillin | 121-33-5 | 152.2 | 1.05 | NS | n.c. | NS | [91] | NS | [93] |
| Saccharin | 1981-07-02 | 183.2 | 0.45 | NS | n.c. | NS | [91] | n.a. | . |

Log $K_{ow}$ was estimated by the KOWWIN v1.68 program. http://www.epa.gov/oppt/exposure/pubs/episuite.htm
n.c. - Not calculated
n.a. - Not Assessed
S/NS- Sensitizer/ Non-Sensitizer

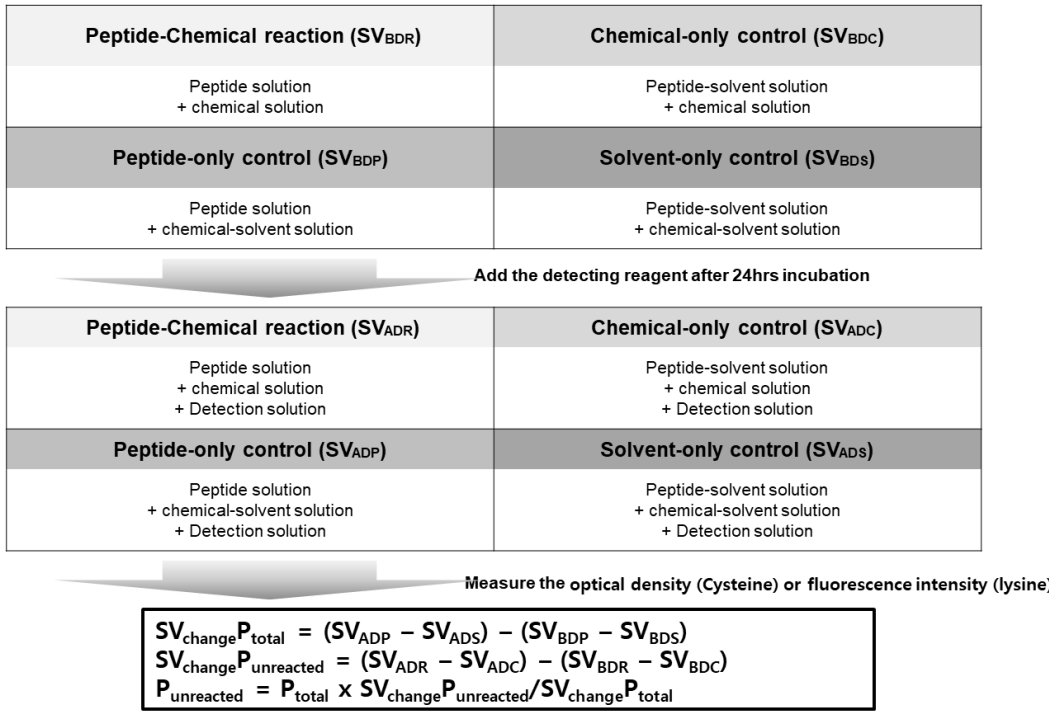


**Figure 1. Scheme of the Spectrophotometric direct peptide reactivity assay and formula for calculation the depletion ratio.**

Every test set was composed of 4 wells. The change of spectrophotometric value (SV) was calculated by measurement of optical density (OD) or fluorescence intensity (FI) just before and after adding the detection reagent. $SV_{ADP}$ and $SV_{BDP}$ are the spectrophotometric values after and before the addition of the detection reagent to the peptide-only control, respectively. $SV_{ADS}$ and $SV_{BDS}$ are the spectrophotometric values after and before the addition of the detection reagent to the solvent-only control, respectively. $SV_{ADR}$ and $SV_{BDR}$ are the spectrophotometric values after and before the addition of the detection reagent to reaction solutions, respectively. $SV_{ADC}$ and $SV_{BDC}$ are the spectrophotometric values after and before the addition of the detection reagent to the chemical-only control, respectively.

# 3. Results

## *3.1. Chemical reactivity toward cysteine or lysine peptides as compared with LLNA*

The reactivity of the 65 chemicals toward the peptides (peptide depletion ratio) is shown in Table 2. Overall, the reactivity results between the chemicals and peptides correlated with LLNA results. Except for a few, most chemicals classified as strong and extreme sensitizers in LLNA showed high reactivity with cysteine or lysine peptide. For the majority of the extreme and strong sensitizer in LLNA, over 40% of depletion was observed for cysteine or lysine peptide. DNCB, diphenylcyclopropenone, and methyldibromoglutaronitrile demonstrated a significant depletion of cysteine peptide but not lysine peptide. On the contrary, oxazolone, glutaric dialdehyde, and phthalic anhydride only demonstrated significant depletion for lysine peptide. The chemicals classified as weak sensitizers in LLNA showed very low reactivity toward cysteine and lysine peptides.

**Table 2. The depletion percent of cysteine peptide or lysine peptide.**

| Group | | Test chemical | Cysteine depletion (%) | | | | | Lysine depletion (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LLNA | Human | | Run 1 | Run 2 | Run 3 | Mean | SD | Run 1 | Run 2 | Run 3 | Mean | SD |
| S | S | 1-Chloro-2,4-dinitrobenzene (DNCB) | 91.19 | 89.1 | 86.12 | 88.8 | 2.55 | 9.82 | 8.11 | 10.82 | 9.59 | 1.37 |
| S | S | 5-Chloro-2-methyl-4-isothiazolin-3-on (MCI) | 22.64 | 22.63 | 24.07 | 23.12 | 0.83 | 3.86 | 2.13 | 9.99 | 5.33 | 4.13 |
| S | S | Oxazolone | 20.22 | 19.94 | 27.13 | 22.43 | 4.08 | 93.27 | 92.97 | 93.37 | 93.2 | 0.21 |
| S | S | Diphenylcyclopropenone | 90.03 | 90.41 | 68.26 | 82.9 | 12.68 | -2.73 | -3 | 3.77 | -0.65 | 3.83 |
| S | S | Potassium dichromate | 33.26 | 35.61 | 40.53 | 36.47 | 3.71 | 45.78 | 46.93 | 49.17 | 47.3 | 1.72 |
| S | S | Glutaric dialdehyde | 8.33 | 8.52 | 16.89 | 11.25 | 4.89 | 97.02 | 97.46 | 97.54 | 97.34 | 0.28 |
| S | S | Propyl gallate | 105.29 | 95.79 | 101.03 | 100.7 | 4.76 | 58.53 | 58.21 | 59.35 | 58.7 | 0.59 |
| S | S | Hydroquinone | 91.25 | 89.99 | 91.72 | 90.99 | 0.89 | 85.87 | 86.14 | 86.43 | 86.15 | 0.28 |
| S | S | *p*-Phenylenediamine | 97.18 | 97.62 | 98.56 | 97.79 | 0.71 | 89.13 | 89.51 | 89.69 | 89.44 | 0.29 |
| S | S | Lauryl gallate | 24.27 | 50 | 78.59 | 50.95 | 27.17 | 43.9 | 44.83 | 46.42 | 45.05 | 1.27 |
| S | S | Methyldibromoglutaronitrile | 95.74 | 94.76 | 97.97 | 96.16 | 1.64 | -9.61 | -9.98 | -2.81 | -7.47 | 4.04 |
| S | S | Dinitroflurobenzene (DNFB) | 102.48 | 99.2 | 102.73 | 101.47 | 1.97 | 95.07 | 94.69 | 95.03 | 94.93 | 0.21 |
| S | S | Phthalic anhydride | -3.81 | -4.4 | -3.73 | -3.98 | 0.36 | 51.23 | 52.06 | 54.33 | 52.54 | 1.61 |
| S | S | Isoeugenol | 91.36 | 98.08 | 98.6 | 96.01 | 4.04 | 24.14 | 24.38 | 25.17 | 24.56 | 0.54 |
| S | S | Diethylenetriamine | 86.35 | 83.51 | 80.6 | 83.49 | 2.88 | 92.32 | 88.53 | 84.63 | 88.49 | 3.84 |
| S | n.a | 2-Vinyl pyridine | 64.72 | 56.94 | 54.31 | 58.65 | 5.41 | 0.97 | 0.22 | 0.66 | 0.62 | 0.38 |
| S | S | Ammonium persulfate | -1.52 | 1.35 | 4.34 | 1.39 | 2.93 | -4.18 | -5.25 | -2.59 | -4.01 | 1.34 |
| S | n.a | 3-Aminophnol | -2.27 | 2.29 | 6.99 | 2.34 | 4.63 | 90.18 | 86.25 | 92.74 | 89.72 | 3.27 |

**Table 2. The depletion percent of cysteine peptide or lysine peptide (*cont'd*).**

| Group | | Test chemical | Cysteine depletion (%) | | | | | Lysine depletion (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LLNA | Human | | Run 1 | Run 2 | Run 3 | Mean | SD | Run 1 | Run 2 | Run 3 | Mean | SD |
| S | NS | Tartaric acid | -0.28 | -3.75 | -1.13 | -1.72 | 1.81 | 1.54 | 1.48 | 2.54 | 1.85 | 0.6 |
| S | S | Cinnamic aldehyde | 34.42 | 21.68 | 16.95 | 24.35 | 9.04 | 6.71 | 6.94 | 8.22 | 7.29 | 0.82 |
| S | S | 2-Mcaptobenzothiazole | 53.77 | 54 | 54 | 53.93 | 0.13 | 9.98 | 8.54 | 12.74 | 10.42 | 2.13 |
| S | S | Resorcinol | 21.35 | 20.79 | 20.79 | 20.98 | 0.33 | 3.84 | 2.35 | 7.27 | 4.49 | 2.52 |
| S | S | Diethyl maleate | 97.77 | 98.44 | 98.44 | 98.22 | 0.39 | -4.46 | -8.55 | 0.68 | -4.11 | 4.63 |
| S | S | Nickel II sulfate heptahydrate | 23.74 | 24.32 | 24.32 | 24.12 | 0.33 | -12 | -15.31 | -6.83 | -11.38 | 4.28 |
| S | S | 2-Methyl-4-isothiazolin-3-one | 57.6 | 65.31 | 65.31 | 62.74 | 4.45 | -4.16 | -5.14 | -0.47 | -3.26 | 2.46 |
| S | n.a | 1-Naphthol | 10.76 | 11.07 | 11.07 | 10.97 | 0.18 | -1.64 | -5.36 | 1.25 | -1.92 | 3.31 |
| S | S | 3-Dimethylamino-1-propylamine | 4.42 | 3.88 | 3.88 | 4.06 | 0.31 | 36.34 | 35.18 | 37.62 | 36.38 | 1.22 |
| S | n.a | 4-Chloroaniline | 3.92 | 3.25 | 3.25 | 3.47 | 0.39 | 82.78 | 58.6 | 80.53 | 73.97 | 13.36 |
| S | S | Ethylenediamine | 34.66 | 34.95 | 34.95 | 34.85 | 0.17 | 43.36 | 41.27 | 46.02 | 43.55 | 2.38 |
| S | n.a | Oxalaldehyde | 4.26 | 3.84 | 3.84 | 3.98 | 0.24 | 4.59 | 0.48 | 8.6 | 4.56 | 4.06 |
| S | S | Geraninol | 2.34 | 9.55 | 0.19 | 4.03 | 4.91 | 11.45 | 9.23 | 13.84 | 11.51 | 2.31 |
| S | NS (not oxidized) | R(+) Limonene (not oxidized) | -2.24 | 2.25 | 4.31 | 1.44 | 3.35 | 4.2 | 4.68 | 5.9 | 4.93 | 0.88 |
| S | S | Linalool | 3 | 4.71 | 9.22 | 5.64 | 3.21 | 11.97 | 12.3 | 13.3 | 12.52 | 0.69 |
| S | n.a | 2-Ethylbutyraldehyde (diethyl acetaldehyde) | 20.53 | 7.6 | 1.93 | 10.02 | 9.53 | 13.77 | 14.78 | 17.21 | 15.25 | 1.77 |
| S | S | 2-(4-tert-Butylbenzyl)propionaldehyde (Lilial) | 4.43 | 5.72 | 5.89 | 5.34 | 0.8 | 2.99 | 2.97 | 5.99 | 3.98 | 1.74 |
| S | NS | Sodium lauryl sulfate (SLS) | 0.54 | 1.63 | 1.74 | 1.3 | 0.66 | 10.48 | 9.17 | 11.91 | 10.52 | 1.37 |

**Table 2. The depletion percent of cysteine peptide or lysine peptide (*cont'd*).**

| Group | | Test chemical | Cysteine depletion (%) | | | | | Lysine depletion (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LLNA | Human | | Run 1 | Run 2 | Run 3 | Mean | SD | Run 1 | Run 2 | Run 3 | Mean | SD |
| S | S | Eugenol | 18.18 | 17.96 | 17.96 | 18.03 | 0.12 | 8.26 | 8.02 | 10.46 | 8.91 | 1.34 |
| S | S | Phenyl benzoate | 14.24 | 13.22 | 13.22 | 13.56 | 0.59 | 1.85 | 2.02 | 4.98 | 2.95 | 1.76 |
| S | S | Cinnamic alcohol | 44.98 | 41.59 | 40.81 | 42.46 | 2.21 | 10.61 | 11.42 | 9.16 | 10.4 | 1.14 |
| S | S | Imidazolidinyl urea | 0.31 | -12.1 | -10.24 | -7.34 | 6.69 | 39.26 | 39.55 | 38.01 | 38.94 | 0.82 |
| S | S | Methyl methacrylate | 11.68 | 11.24 | 13.97 | 12.3 | 1.46 | 4.27 | 5.27 | 4.21 | 4.58 | 0.6 |
| S | S | Ethylene glycol dimethacrylate | 61.21 | 59.74 | 60.49 | 60.48 | 0.74 | -0.78 | 0.78 | -2 | -0.67 | 1.39 |
| S | NS | Xylene | 9.17 | 7.03 | 7.04 | 7.75 | 1.23 | 4.3 | 3.86 | 3.48 | 3.88 | 0.41 |
| S | S | Benzocaine | 0.2 | -0.36 | 0.46 | 0.1 | 0.42 | 33.01 | 43.84 | 42.34 | 39.73 | 5.87 |
| S | NS | Dimethylsulfoxide | -2.73 | -2.09 | -2.42 | -2.41 | 0.32 | 0.1 | 1.48 | -1.21 | 0.12 | 1.35 |
| S | S | Abietic acid | 91.5 | 90.29 | 89.49 | 90.42 | 1.01 | 14.69 | 16.15 | 12.4 | 14.42 | 1.89 |
| S | NS | Hexylcinnamic aldehyde (HCA) | 46.9 | 54.68 | 40.35 | 47.31 | 7.18 | 10.14 | 9.27 | 10.88 | 10.1 | 0.81 |
| S | S | Citral | 97.89 | 98.23 | 99.14 | 98.42 | 0.65 | 9.28 | 9.54 | 9.08 | 9.3 | 0.24 |
| S | NS | Isopropyl myristate | -24.12 | -11.16 | -8.24 | -14.51 | 8.45 | -6.55 | -6.43 | -7.35 | -6.78 | 0.5 |
| NS | NS | Lactic acid | -3.12 | -2.68 | -3.07 | -2.96 | 0.24 | 0.92 | 1.1 | 5.31 | 2.45 | 2.48 |
| NS | NS | Methyl salicylate | -2.99 | -1.76 | -2.4 | -2.38 | 0.62 | 6.07 | 5.43 | 9.61 | 7.03 | 2.25 |
| NS | NS | Glycerol | 2.96 | 3.7 | 2.75 | 3.13 | 0.5 | 5.29 | 6.22 | 5.84 | 5.78 | 0.47 |
| NS | NS | Tween80 | 0.88 | -0.08 | -0.57 | 0.08 | 0.74 | 17.93 | 16.56 | 19.98 | 18.16 | 1.72 |
| NS | NS | Isopropanol | 1.04 | 0.1 | 0.23 | 0.46 | 0.51 | -2.29 | -1.81 | -2.6 | -2.23 | 0.39 |

**Table 2. The depletion percent of cysteine peptide or lysine peptide (*cont'd*).**

| Group | | Test chemical | Cysteine depletion (%) | | | | | Lysine depletion (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LLNA | Human | | Run 1 | Run 2 | Run 3 | Mean | SD | Run 1 | Run 2 | Run 3 | Mean | SD |
| NS | NS | Salicylic acid | 1.78 | 0.63 | 1.09 | 1.17 | 0.58 | -2.75 | -2.39 | -3.7 | -2.95 | 0.67 |
| NS | NS | 6-Methyl coumarin | 1.93 | 2.59 | 0.87 | 1.8 | 0.87 | -5.44 | -6.27 | -4.64 | -5.45 | 0.82 |
| NS | n.a | Benzoic acid | 5.58 | 2.54 | 1.03 | 3.05 | 2.32 | -9.78 | -9.53 | -9.17 | -9.49 | 0.31 |
| NS | n.a | Nonanoic acid | 3.51 | 2.29 | 1.27 | 2.36 | 1.12 | -6.61 | -7.14 | -6.33 | -6.69 | 0.41 |
| NS | NS | Octanoic acid | 1.41 | 1.2 | -0.23 | 0.79 | 0.89 | -6.14 | -6.09 | -6.48 | -6.23 | 0.21 |
| NS | NS | *n*-Hexane | 2.38 | 2.81 | 2.37 | 2.52 | 0.25 | -9.35 | -11.27 | -8.78 | -9.8 | 1.3 |
| NS | n.a. | Clorobenzene | 0.96 | 1.39 | 0.68 | 1.01 | 0.36 | 4.55 | 3.7 | 4.68 | 4.31 | 0.53 |
| NS | NS | 1-Btanol | 2.05 | 2.38 | 1.69 | 2.04 | 0.34 | -12.79 | -14.22 | -12.39 | -3.14 | 0.96 |
| NS | NS | Propylene glycol | 0.34 | 1.1 | 1.2 | 0.88 | 0.47 | -4.23 | -6.3 | -2.91 | -4.48 | 1.7 |
| NS | NS | Vanillin | 0.51 | 1.62 | 0.85 | 0.99 | 0.57 | 12.49 | 11.3 | 12.89 | 12.22 | 0.83 |
| NS | n.a. | Saccharin | 1.42 | 2.12 | 1.54 | 1.69 | 0.38 | 1.59 | -0.65 | 1.62 | 0.86 | 1.3 |

*Data were obtained from 3 independent experiments carried out in triplicate.

n.a. - Not Assessed

S/NS- Sensitizer/ Non-Sensitizer

## *3.2. Classification based on the chemical reactivity toward cysteine or lysine peptide as compared to LLNA*

In the cysteine depletion cut-off model, 17 chemicals (phthalic anhydride, ammonium persulfate, 3-aminophnol, tartaric acid, 3-dimethylamino-1-propylamine, 4-chloroaniline, oxalaldehyde, geraniol, R(+) limonene, linalool, 2-(4-tert-butylbenzyl)propionaldehyde [lilial], sodium lauryl sulfate [SLS], imidazolidinyl urea, xylene, benzocaine, dimethyl sulfoxide, and isopropyl myristate) were estimated as false negative at > 10% cut-off. In the lysine depletion cut-off model, 35 chemicals were estimated as false negative at a cut-off of > 30%. Six chemicals (phthalic anhydride, 3-aminophnol, 3-dimethylamino-1-propylamine, 4-chloroaniline, imidazolidinyl urea, and benzocaine) deemed as false negative in the cysteine depletion cut-off model were estimated as true positive in the lysine depletion cut-off model. There was no false positive in both cysteine and lysine depletion cut-off model (Table 3).

## *3.3. Classification based on chemical reactivity toward cysteine or lysine peptide as compared to human data*

For correlation analysis with human data, I used 55 chemicals that were already analyzed in human studies (Table 3). In the cysteine depletion cut-off model, 10 chemicals were predicted as false negative at > 10% cut-off (Table 3). Six chemicals (tartaric acid, R(+) limonene, xylene, sodium lauryl sulfate (SLS), dimethyl sulfoxide, and Isopropyl myristate) deemed false-negative following

comparison with LLNA data were estimated as true negative. Hexyl cinnamic aldehyde (HCA) was found as a false positive in the cysteine depletion cut-off model. In the lysine depletion cut-off model, 25 chemicals were estimated as false negative at > 30% cut-off (Table 3). However, four chemicals (phthalic anhydride, 3-dimethylamino-1-propylamine, imidazolidinyl urea, and benzocaine) estimated as false negative in the cysteine depletion cut-off model were predicted as true positive in lysine depletion cut-off model. There was no false-positive was noted in the lysine depletion cut-off model.

**Table 3. Categorization of chemicals for skin sensitization potential using different cut-offs of peptide depletion.**

| Group | | Log $K_{ow}$ | Test chemical | Peptide depletion | | Criteria of classification | | |
|---|---|---|---|---|---|---|---|---|
| LLNA | Human | | | Cys | Lys | Cys > 10 | Lys > 30 | Cys > 10 or Lys > 30 |
| S | S | 2.27 | 1-Chloro-2,4-dinitrobenzene (DNCB) | 88.8 | 9.59 | S | NS | S |
| S | S | -0.34 | 5-Chloro-2-methyl-4-isothiazolin-3-on (MCI) | 23.12 | 5.33 | S | NS | S |
| S | S | 1.51 | Oxazolone | 22.43 | 93.2 | S | S | S |
| S | S | 3.25 | Diphenylcyclopropenone | 82.9 | -0.65 | S | NS | S |
| S | S | -3.59 | Potassium dichromate | 36.47 | 47.3 | S | S | S |
| S | S | -0.18 | Glutaric dialdehyde | 11.25 | 97.34 | S | S | S |
| S | S | 1.79 | Propyl gallate | 100.7 | 58.7 | S | S | S |
| S | S | 1.03 | Hydroquinone | 90.99 | 86.15 | S | S | S |
| S | S | -0.39 | *p*-Phenylenediamine | 97.79 | 89.44 | S | S | S |
| S | S | 6.21 | Lauryl gallate | 50.95 | 45.05 | S | S | S |
| S | S | 1.63 | Methyldibromoglutaronitrile | 96.16 | -7.47 | S | NS | S |
| S | S | 1.83 | Dinitroflurobenzene (DNFB) | 101.47 | 94.93 | S | S | S |
| S | S | 2.07 | Phthalic anhydride | -3.98 | 52.54 | NS | S | S |
| S | S | 2.66 | Isoeugenol | 96.01 | 24.56 | S | NS | S |
| S | S | -2.13 | Diethylenetriamine | 83.49 | 88.49 | S | S | S |
| S | n.a | 1.71 | 2-Vinyl pyridine | 58.65 | 0.62 | S | NS | S |
| S | S | n.c | Ammonium persulfate | 1.39 | -4.01 | NS | NS | NS |
| S | n.a | 0.24 | 3-Aminophnol | 2.34 | 89.72 | NS | S | S |

**Table 3. Categorization of chemicals for skin sensitization potential using different cut-offs of peptide depletion (*cont'd*).**

| Group | | Log $K_{ow}$ | Test chemical | Peptide depletion | | Criteria of classification | | |
|---|---|---|---|---|---|---|---|---|
| LLNA | Human | | | Cys | Lys | Cys > 10 | Lys > 30 | Cys > 10 or Lys > 30 |
| S | NS | -1 | Tartaric acid | -1.72 | 1.85 | NS | NS | NS |
| S | S | 1.82 | Cinnamic aldehyde | 24.35 | 7.29 | S | NS | S |
| S | S | 2.86 | 2-Mercaptobenzothiazole | 53.93 | 10.42 | S | NS | S |
| S | S | 1.03 | Resorcinol | 20.98 | 4.49 | S | NS | S |
| S | S | 2.2 | Diethyl maleate | 98.22 | -4.11 | S | NS | S |
| S | S | n.c | Nickel II sulfate heptahydrate | 24.12 | -11.38 | S | NS | S |
| S | S | -0.83 | 2-Methyl-4-isothiazolin-3-one | 62.74 | -3.26 | S | NS | S |
| S | n.a | 2.69 | 1-Naphthol | 10.97 | -1.92 | S | NS | S |
| S | S | -0.45 | 3-Dimethylamino-1-propylamine | 4.06 | 36.38 | NS | S | S |
| S | n.a | n.c | 4-Chloroaniline | 3.47 | 73.97 | NS | S | S |
| S | S | -1.62 | Ethylenediamine | 34.85 | 43.55 | S | S | S |
| S | n.a | -1.66 | Oxalaldehyde | 3.98 | 4.56 | NS | NS | NS |
| S | S | 3.47 | Geraninol | 4.03 | 11.51 | NS | NS | NS |
| S | NS (not oxidized) | 4.83 | R(+) Limonene (not oxidized) | 1.44 | 4.93 | NS | NS | NS |
| S | S | 3.38 | Linalool | 5.64 | 12.52 | NS | NS | NS |
| S | n.a | 1.73 | 2-Ethylbutyraldehyde (diethyl acetaldehyde) | 10.02 | 15.25 | S | NS | S |

**Table 3. Categorization of chemicals for skin sensitization potential using different cut-offs of peptide depletion (*cont'd*).**

| Group | | Log $K_{ow}$ | Test chemical | Peptide depletion | | Criteria of classification | | |
|---|---|---|---|---|---|---|---|---|
| LLNA | Human | | | Cys | Lys | Cys > 10 | Lys > 30 | Cys > 10 or Lys > 30 |
| S | S | 4.36 | 2-(4-tert-Butylbenzyl)propionaldehyde (Lilial) | 5.34 | 3.98 | NS | NS | NS |
| S | NS | 2.42 | Sodium lauryl sulfate (SLS) | 1.3 | 10.52 | NS | NS | NS |
| S | S | 2.73 | Eugenol | 18.03 | 8.91 | S | NS | S |
| S | S | 3.04 | Phenyl benzoate | 13.56 | 2.95 | S | NS | S |
| S | S | 1.84 | Cinnamic alcohol | 42.46 | 10.4 | S | NS | S |
| S | S | -8.28 | Imidazolidinyl urea | 0 | 38.94 | NS | S | S |
| S | S | 1.28 | Methyl methacrylate | 12.3 | 4.58 | S | NS | S |
| S | S | 2.21 | Ethylene glycol dimethacrylate | 60.48 | -0.67 | S | NS | S |
| S | NS | 3.09 | Xylene | 7.75 | 3.88 | NS | NS | NS |
| S | S | 1.8 | Benzocaine | 0.1 | 39.73 | NS | S | S |
| S | NS | -1.22 | Dimethylsulfoxide | 0 | 0.12 | NS | NS | NS |
| S | S | 6.46 | Abietic acid | 90.42 | 14.42 | S | NS | S |
| S | NS | 4.82 | Hexylcinnamic aldehyde (HCA) | 47.31 | 10.1 | S | NS | S |
| S | S | 3.45 | Citral | 98.42 | 9.3 | S | NS | S |
| S | NS | 7.17 | Isopropyl myristate | 0 | -6.78 | NS | NS | NS |
| NS | NS | -0.65 | Lactic acid | 0 | 2.45 | NS | NS | NS |
| NS | NS | 2 | Methyl salicylate | 0 | 7.03 | NS | NS | NS |
| NS | NS | -1.65 | Glycerol | 3.13 | 5.78 | NS | NS | NS |

**Table 3. Categorization of chemicals for skin sensitization potential using different cut-offs of peptide depletion (*cont'd*).**

| Group | | Log $K_{ow}$ | Test chemical | Peptide depletion | | Criteria of classification | | |
|---|---|---|---|---|---|---|---|---|
| LLNA | Human | | | Cys | Lys | Cys > 10 | Lys > 30 | Cys > 10 or Lys > 30 |
| NS | NS | 4.23 | Tween80 | 0.08 | 18.16 | NS | NS | NS |
| NS | NS | 0.28 | Isopropanol | 0.46 | -2.23 | NS | NS | NS |
| NS | NS | 2.24 | Salicylic acid | 1.17 | -2.95 | NS | NS | NS |
| NS | NS | 2.06 | 6-Methyl coumarin | 1.8 | -5.45 | NS | NS | NS |
| NS | n.a | 1.87 | Benzoic acid | 3.05 | -9.49 | NS | NS | NS |
| NS | n.a | 3.52 | Nonanoic acid | 2.36 | -6.69 | NS | NS | NS |
| NS | NS | 3.03 | Octanoic acid | 0.79 | -6.23 | NS | NS | NS |
| NS | NS | 3.29 | *n*-Hexane | 2.52 | -9.8 | NS | NS | NS |
| NS | n.a | 2.64 | Chlorobenzene | 1.01 | 4.31 | NS | NS | NS |
| NS | NS | 0.84 | 1-Butanol | 2.04 | -3.14 | NS | NS | NS |
| NS | NS | -0.78 | Propylene glycol | 0.88 | -4.48 | NS | NS | NS |
| NS | NS | 1.05 | Vanillin | 0.99 | 12.22 | NS | NS | NS |
| NS | n.a | 0.45 | Saccharin | 1.69 | 0.86 | NS | NS | NS |

S/NS – Sensitizer/Non-Sensitizer

n.a. -Not Assessed

## *3.4. Comparison of the chemical classification based on a single or combined cut-off prediction model*

Based on the cut-off criteria established in the previous study, I categorized the compounds into sensitization and non-sensitization groups based on their reactivity to each peptide alone or in combination (Table 3). The predictivity was calculated following comparison with LLNA or human data. The accuracy of the combined cut-offs (10% cut-off for cysteine peptide and 30% cut-off for lysine peptide) was higher than that of the classification by a single cut-off of either the cysteine peptide method or the lysine peptide method (Table 4 and 5).

## *3.5. Variation in predictivity according to* Log $K_{ow}$

I applied the same prediction cut-off approach to 47 (for LLNA) and 39 (for human) chemicals with Log $K_{ow}$ physical properties above −1 and below 4; the results are shown in Tables 4 and 5. The prediction of skin sensitization potential was relatively higher as compared with the data without Log $K_{ow}$.

The sensitivity, specificity, PPV, NPV, and accuracy were 75.8%, 100.0%, 100.0%, 63.6%, and 83.0%, respectively, after the application of 10% cut-off to the cysteine peptide method as compared with LLNA data. At 30% cut-off for the lysine peptide method, the sensitivity, specificity, PPV, NPV, and accuracy were 27.3%, 100.0%, 100.0%, 36.8%, and 48.9%, respectively, and the predictivity was lower than that with the cysteine peptide method. The predictivity value for the combination of cut-offs for two peptides was higher than that for the cysteine or

lysine peptide cut-off method (87.9% sensitivity, 100.0% specificity, 100.0% PPV, 77.8% NPV, and 91.5% accuracy).

The sensitivity, specificity, PPV, NPV, and accuracy were 81.5%, 100.0%, 100.0%, 70.6%, and 87.2%, respectively, at 10% cut-off for the cysteine peptide method as compared with the human data. I applied a cut-off of 30% to the lysine peptide method, and found that the sensitivity, specificity, PPV, NPV, and accuracy were 33.3%, 100.0%, 100.0%, 40.0%, and 53.8%, respectively; the predictivity was lower than that reported with the animal data. The predictivity value was higher for the combination of cut-offs for the two peptides than that for cysteine or lysine peptide cut-off method (92.6% sensitivity, 100.0% specificity, 100.0% PPV, 85.7% NPV, and 94.9% accuracy).

**Table 4. Sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of prediction models using single cut-off or combination cut-offs compare to LLNA data.**

| LLNA | 65 chemicals | | | | | |
|---|---|---|---|---|---|---|
| | Cys > 10 | | Lys > 30 | | Cys > 10 or Lys > 30 | |
| | Positive | Negative | Positive | Negative | Positive | Negative |
| **Sensitizer (49)** | 32 | 17 | 14 | 35 | 38 | 11 |
| **Non-sensitizer (16)** | 0 | 16 | 0 | 16 | 0 | 16 |
| **Sensitivity (%)** | 65.3 | | 28.6 | | 77.6 | |
| **Specificity (%)** | 100 | | 100 | | 100 | |
| **PPV (%)** | 100 | | 100 | | 100 | |
| **NPV (%)** | 48.5 | | 31.4 | | 59.3 | |
| **Accuracy (%)** | 73.8 | | 46.2 | | 83.1 | |

| LLNA | 47 chemicals (w/o Log $K_{ow}$ ≤ -1 or ≥ 4) | | | | | |
|---|---|---|---|---|---|---|
| | Cys > 10 | | Lys > 30 | | Cys > 10 or Lys > 30 | |
| | Positive | Negative | Positive | Negative | Positive | Negative |
| **Sensitizer (33)** | 25 | 8 | 9 | 24 | 29 | 4 |
| **Non-sensitizer (14)** | 0 | 14 | 0 | 14 | 0 | 14 |
| **Sensitivity (%)** | 75.8 | | 27.3 | | 87.9 | |
| **Specificity (%)** | 100 | | 100 | | 100 | |
| **PPV (%)** | 100 | | 100 | | 100 | |
| **NPV (%)** | 63.6 | | 36.8 | | 77.8 | |
| **Accuracy (%)** | 83 | | 48.9 | | 91.5 | |

*PPV: Positive predictive value / NPV: Negative predictive value

**Table 5. Sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of prediction models using single cut-off or combination cut-offs compare to human data.**

| Human | 55 chemicals | | | | | |
|---|---|---|---|---|---|---|
| | Cys > 10 | | Lys > 30 | | Cys > 10 or Lys > 30 | |
| | Positive | Negative | Positive | Negative | Positive | Negative |
| **Sensitizer (36)** | 28 | 8 | 12 | 24 | 32 | 4 |
| **Non-sensitizer (19)** | 1 | 18 | 0 | 19 | 1 | 18 |
| **Sensitivity (%)** | 77.8 | | 33.3 | | 88.9 | |
| **Specificity (%)** | 94.7 | | 100 | | 94.7 | |
| **PPV (%)** | 96.6 | | 100 | | 97 | |
| **NPV (%)** | 69.2 | | 44.2 | | 81.8 | |
| **Accuracy (%)** | 83.2 | | 56.4 | | 90.9 | |

| Human | 39 chemicals (w/o Log $K_{ow} \leq$ -1 or $\geq$ 4) | | | | | |
|---|---|---|---|---|---|---|
| | Cys > 10 | | Lys > 30 | | Cys > 10 or Lys > 30 | |
| | Positive | Negative | Positive | Negative | Positive | Negative |
| **Sensitizer (27)** | 22 | 5 | 9 | 18 | 25 | 2 |
| **Non-sensitizer (12)** | 0 | 12 | 0 | 12 | 0 | 12 |
| **Sensitivity (%)** | 81.5 | | 33.3 | | 92.6 | |
| **Specificity (%)** | 100 | | 100 | | 100 | |
| **PPV (%)** | 100 | | 100 | | 100 | |
| **NPV (%)** | 70.6 | | 40 | | 85.7 | |
| **Accuracy (%)** | 87.2 | | 53.8 | | 94.9 | |

*PPV: Positive predictive value / NPV: Negative predictive value

# 4. Discussion

Numerous *in vitro* or *in chemico* assays have been developed as per the Adverse Outcome Pathway (AOP) concept for skin sensitization as alternatives to animal testing. Several of these methods have been adopted as OECD test guidelines to predict the skin sensitization potential of new substances, including DPRA for chemical-peptide reactivity (KE 1), KeratinoSens™ and LuSens™ for keratinocyte activation (KE2), and h-CLAT, U-SENS™, and IL-8 Luc assays for dendritic cell activation (KE3) [25-27].

Of these, DPRA is the only OECD test guideline based on the binding of haptens to skin proteins as the first key event in skin sensitization reactions [31]. However, the main disadvantage of DPRA is the requirement for specialized analytical instruments for the detection of residual peptides, such as HPLC with UV detection system [32, 81]. The process is time-consuming and requires 22-26 h to obtain results after reaction initiation. Furthermore, only a maximum of 10 samples can be assessed per day.

To overcome these limitations, I attempted to develop a new high-throughput screening method to predict the sensitization potentials of new chemicals based on the conventional spectrophotometric analysis. In comparison with the previous spectrophotometric method developed to assess the reactivity of chemicals toward GHS [87], my method could predict the reactivity of chemicals beyond those associated with thiol-reactive compounds.

My method can also determine the reactivity of chemicals toward two chemical groups, cysteine and lysine peptides with DTNB (for free thiol group detection) and fluorescamine (for free amine group detection). I validated this method by expanding test chemicals and demonstrated its usefulness as an animal alternative for skin sensitization screening. The false-positive rate for the lysine peptide method was higher than that for the conventional LLNA method. However, in the combination cut-off prediction model, the false-predicted chemicals (especially false negative in the lysine reaction) were correctly predicted as sensitizers. These results suggest that the combination of both cysteine and lysine peptide reactivity could allow a more accurate prediction of the skin sensitization potentials of chemicals. In my previous study, the combination of two cut-off prediction methods for each type of peptide in Spectro-DPRA achieved a high degree of sensitivity (80.0%), specificity (86.7%), and accuracy (82.5%) compared to those of the DPRA (80%, 77%, and 80%, respectively, compared to the LLNA) [89]. I expanded the test chemicals to confirm whether my prediction model shows the constant level of predictivity compared to the previous study. Like the previous study, the combination of two cut-off prediction methods in Spectro-DPRA achieved a high degree of sensitivity (77.6.0%), specificity (100.0%), and accuracy (83.1%) compared with LLNA results in this study.

The need for the use of alternative testing methods has arisen from the ethical issues related to animal toxicity tests; thus, most of these alternatives are also used to predict the results of animal tests. However, the ultimate goal of animal toxicity testing is to determine the human toxicity potential of a substance; therefore,

the ideal test method would be the one that could directly predict the outcomes in humans. Accordingly, I evaluated the correlation between animal and human data with my test method. Consistent with LLNA results, combined cut-offs showed higher accuracy than the classification cut-off using either cysteine or lysine peptide as compared to human data. Furthermore, a high correlation (88.9% sensitivity, 94.7% specificity, 97% PPV, 81.8% NPV, and 90.9% accuracy) was noted.

Log $K_{ow}$ is widely accepted as a key physicochemical parameter, reflective of the degree of skin absorption of a substance. Cosmetic ingredients with an MW > 500 Da and Log $K_{ow}$ (Log $P_{ow}$) $\leq -1$ or $\geq 4$ tend to show very low dermal absorption [95]. To confirm this observation, I applied my prediction model to 47 (LLNA) and 39 (human) chemicals with a Log $K_{ow}$ (Log $P_{ow}$) range of −1 to 4. The predictability of the combination cut-off prediction model increased regardless of the Log $K_{ow}$ value and showed higher accuracy.

Aside from the higher predictivity than the *in vivo* data, my spectrophotometric analysis could measure the chemical-peptide reactivity of 12 chemicals simultaneously on a 96-well plate within only 10 min, indicative of the significant reduction in the measurement time as compared to DPRA (22-26 h) that allows analysis of several samples in a day. However, my spectrophotometric analysis method also has limitations similar to those of the previously developed alternative assays [25-27]. This method could not estimate pre/pro-hapten and was unable to predict highly lipophilic chemicals.

In conclusion, the prediction model combining the classification cut-offs of the two peptide methods (cysteine and lysine) showed a high degree of sensitivity,

specificity, PPV, NPV, and accuracy for skin sensitization reactions as compared with *in vivo* data, especially human data. Although these methods need further optimization, the results of the present study highlight the applicability of Spectro-DPRA as a good animal test alternative. It is an easy, rapid, and high-throughput screening method for the prediction of the skin sensitization potential of chemicals.

# CHAPTER II

# Application of Spectro-DPRA, KeratinoSens™, and h-CLAT to an estimation of the skin sensitization potential.

# 1. Introduction

One of the common side effects of cosmetic products, ACD, is a common health hazard affecting 15-20% of people worldwide [2]. It is caused by repeated exposure to a non-immunogenic, low-molecular-weight chemical called hapten [1, 3]. ACD develops in two phases—a sensitization phase and an elicitation phase [96, 97]. In the sensitization phase, antigen-specific T-cells are generated. When the antigen/allergen binds to the peptides of the skin (haptenization), these chemical-peptide adducts interact with KCs and LCs. KCs 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 [8, 9]. 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 [98].

The ban on testing cosmetics or their ingredients on animals in the EU market [22-24] 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 [56, 57].

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 (MIE) and Key event [28]. Currently, there are several ongoing approaches to develop an Integrated Testing Strategy (ITS) for achieving higher predictivity for skin sensitization potential combined with OECD TG methods or individual laboratory assay development [58, 60-68]. In addition, the OECD in collaboration with EURL ECVAM proposed a roadmap for developing AOP-based IATA and has attempted to develop formally validated and regulated strategies in the approach to skin sensitization predictivity [69].

Based on these research guidelines, I applied AOP-based IATA to the newly developed *in-chemico* assay, Spectro-Direct peptide reactivity assay (Spectro-DPRA), to evaluate hapten-peptide reactivity [89]. I applied this assay in combination with the *in vitro* assays, KeratinoSens$^{TM,}$ and h-CLAT, to evaluate skin cell activation and immune cell activation or maturation, respectively. I compared this to existing approaches to develop a reliable integrated prediction approach for skin sensitization of cosmetic ingredients.

# 2. Material 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 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 from LLNA and human test [15, 58, 90-94, 99]. I used most of the test chemicals recommended to confirm the performance standard in skin sensitization evaluations [100]. I 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.

**Table 1. List of tested chemicals.**

| Chemical | CAS | M.W. | Log $K_{ow}$ | Human | | LLNA | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Group | Reference | Group | EC3 (%) | ECETOC CL* | Reference |
| 5-Chloro-2-methyl-4-isothiazolin-3-on (MCI) | 26172-55-4 | 149.6 | -0.34 | S | [92] | S | 0.01 | Extreme | [92] |
| 1-Chloro-2,4-dinitrobenzene (DNCB) | 97-00-7 | 202.55 | 2.27 | S | [92] | S | 0.05 | Extreme | [91] |
| Oxazolone | 15646-46-5 | 217.2 | 1.51 | S | [92] | S | 0.003 | Extreme | [91] |
| 4-phenylenediamine | 106-50-3 | 108.1 | -0.39 | S | [92] | S | 0.16 | Strong | [91] |
| Glutaric dialdehyde | 111-30-8 | 100.1 | -0.18 | S | [93] | S | 0.1 | Strong | [91] |
| Lauryl gallate | 1166-52-5 | 338.4 | 6.21 | S | [93] | S | 0.3 | Strong | [91] |
| Hydroquinone | 123-31-9 | 110.1 | 1.03 | S | [93] | S | 0.11 | Strong | [91] |
| Propyl gallate | 121-79-9 | 212.2 | 1.79 | S | [93] | S | 0.32 | Strong | [15] |
| Cobalt chloride | 7646-79-9 | 129.84 | 0.85 | S | [92] | S | 4.8 | Moderate | [92] |
| Isoeugenol | 97-54-1 | 164.2 | 2.66 | S | [93] | S | 1.2 | Moderate | [91] |
| 2-Mercaptobenzothiazole | 149-30-4 | 167.3 | 2.86 | S | [92] | S | 1.7 | Moderate | [91] |
| Cinnamic aldehyde | 104-55-2 | 132.2 | 1.82 | S | [58] | S | 3 | Moderate | [91] |
| Diethyl maleate | 141-05-9 | 172.2 | 2.2 | S | [93] | S | 5.8 | Moderate | [91] |
| Diethylenetriamine | 111-40-0 | 103.20 | -2.13 | S | [58] | S | 3.28 | Moderate | [58] |
| Ammonium persulfate | 231-786-5 | 228.2 | n.c. | S | [94] | S | 1.9 | Moderate | [90] |
| Citral | 5392-40-5 | 152.2 | 3.45 | S | [92] | S | 13 | Weak | [91] |
| Eugenol | 97-53-0 | 164.2 | 2.73 | S | [93] | S | 13 | Weak | [91] |
| Phenyl benzoate | 93-99-2 | 198.2 | 3.04 | S | [92] | S | 20 | Weak | [91] |

**Table 1. List of tested chemicals (*cont'd*).**

| Chemical | CAS | M.W. | Log $K_{ow}$ | Human | | LLNA | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Group | Reference | Group | EC3 (%) | ECETOC CL* | Reference |
| Cinnamyl alcohol | 104-54-1 | 134.2 | 1.84 | S | [92] | S | 21 | Weak | [91] |
| Imidazolidinyl urea | 39236-46-9 | 388.3 | -8.28 | S | [92] | S | 24 | Weak | [91] |
| Methyl methacrylate | 80-62-6 | 100.1 | 1.28 | S | [93] | S | 90 | Weak | [58] |
| Ethylene glycol dimethacrylate | 97-90-5 | 198.2 | 2.21 | S | [92] | S | 28 | Weak | [91] |
| Benzocaine | 94-09-7 | 165.2 | 1.8 | S | [58] | S | 22 | Weak | [15] |
| Abietic acid | 514-10-3 | 302.5 | 6.46 | S | [93] | S | 15 | Weak | [91] |
| Geraniol | 106-24-1 | 154.3 | 3.47 | S | [93] | S | 26 | Weak | [91] |
| Linalool | 78-70-6 | 154.3 | 3.38 | S | [93] | S | 30 | Weak | [91] |
| Nickel chloride | 7718-54-9 | 129.6 | n.c. | S | [92] | NS | NC | NS | [58] |
| α-Linolenic acid | 463-40-1 | 278.43 | 7.3 | NS (W.U.) | - | S | 9.8 | Moderate | [99] |
| Squalene | 111-02-4 | 410.73 | 14.12 | NS (W.U.) | - | S | 6.1 | Moderate | [99] |
| Maleic acid | 110-16-7 | 116.07 | 0.05 | NS (W.U.) | - | S | 4.1 | Moderate | [99] |
| Tartaric acid | 87-69-4 | 150.1 | -1.00 | NS | [92] | S | 8.7 | Moderate | [91] |
| Dimethylsulfoxide | 67-68-5 | 78.1 | -1.22 | NS | [93] | S | 72 | Weak | [91] |
| Sodium Lauryl sulfate | 151-21-3 | 288.4 | 2.42 | NS | [92] | S | 14 | Weak | [91] |
| α-Hexylcinnamaldehyde | 101-86-0 | 216.3 | 4.82 | NS | [93] | S | 11 | Weak | [91] |
| Xylene | 1330-20-7 | 106.2 | 3.09 | NS | [92] | S | 95.8 | Weak | [58] |
| Pyridine | 110-86-1 | 79.10 | 0.80 | NS | [92] | S | 72 | Weak | [58] |
| R-(+)-Limonene | 5989-27-5 | 136.2 | 4.83 | NS | [93] | S | 69 | Weak | [91] |

**Table 1. List of tested chemicals (*cont'd*).**

| Chemical | CAS | M.W. | Log $K_{ow}$ | Human | | LLNA | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Group | Reference | Group | EC3 (%) | ECETOC CL* | Reference |
| Isopropanol | 67-63-0 | 60.1 | 0.28 | NS | [92] | NS | n.c. | NS | [91] |
| Lactic acid | 50-21-5 | 90.1 | -0.65 | NS | [92] | NS | n.c. | NS | [91] |
| Methyl salicylate | 119-36-8 | 152.2 | 2.0 | NS | [93] | NS | n.c. | NS | [91] |
| Salicylic acid | 69-72-7 | 138.1 | 2.24 | NS | [92] | NS | n.c. | NS | [91] |
| Benzalkonium chloride | 8001-54-5 | 319.60 | 2.93 | NS | [93] | NS | n.c. | NS | [58] |
| Glycerol | 56-81-5 | 152.2 | 2.0 | NS | [92] | NS | n.c. | NS | [91] |
| 2-Phenoxyethanol | 122-99-6 | 138.2 | 1.16 | NS | [93] | NS (W.U.) | n.a. | NS | . |
| Tween80 | 9005-65-6 | 1310.0 | 4.23 | NS | [93] | NS | n.c. | NS | [15] |
| 6-Methylcoumarin | 92-48-8 | 160.2 | 2.06 | NS | [92] | NS | n.c. | NS | [91] |
| Fumaric acid | 110-17-8 | 116.1 | 0.05 | NS | [92] | NS | >25 | NS | [58] |
| Oleic acid | 112-80-1 | 282.47 | 7.73 | NS (W.U.) | - | S | 10.5 | Weak | [99] |
| Linoleic acid | 60-33-3 | 280.45 | 7.51 | NS (W.U.) | - | S | 14.1 | Weak | [99] |
| Succinic acid | 110-15-6 | 118.09 | 0.75 | NS (W.U.) | - | NS | n.c. | NS | [99] |
| *p*-Anisic acid | 100-09-4 | 152.16 | 1.96 | NS (W.U.) | - | NS (W.U.) | n.a. | NS | - |
| Caprylyl sulfobetaine | 15163-36-7 | 307.5 | 1.26 | NS (W.U.) | - | NS (W.U.) | n.a. | NS | - |
| Hydroxyacetophenone | 99-93-4 | 136.2 | 1.35 | NS (W.U.) | - | NS (W.U.) | n.a. | NS | - |
| Apigenin | 520-36-5 | 270.2 | 3.02 | NS (W.U.) | - | NS (W.U.) | n.a. | NS | - |
| 2,3-Butanediol | 513-85-9 | 90.1 | -0.92 | NS (W.U.) | . | NS (W.U.) | n.a. | NS | . |

**Table 1. List of tested chemicals (*cont'd*).**

| Chemical | CAS | M.W. | Log $K_{ow}$ | Human | | LLNA | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Group | Reference | Group | EC3 (%) | ECETOC CL* | Reference |
| Caprylic/capric glycerides | 85409-09-2 | n.a. | 5.29 | NS (W.U.) | - | NS (W.U.) | n.a. | NS | - |
| 1,2-Hexanediol | 6920-22-5 | 118.2 | 0.69 | NS (W.U.) | - | NS (W.U.) | n.a. | NS | - |
| 1,2-Octanediol | 1117-86-8 | 188.3 | 1.67 | NS (W.U.) | - | NS (W.U.) | n.a. | NS | - |

Log $K_{ow}$ was estimated by the KOWWIN v1.68 program. http://www.epa.gov/oppt/exposure/pubs/episuite.htm

n.c. means not calculated

n.a. means not assessed

W.U. means widely used in cosmetics

S/NS means Sensitizer/ non-Sensitizer

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

## *2.2. Spectrophotometric – Direct Peptide Reactivity Assay (Spectro-DPRA)*

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 HPLC analysis. Spectro-DPRA was performed according to my previous protocol [89, 101].I used 2 model peptides, a cysteine peptide (Ac-RFAACAA-COOH) and a lysine peptide (Ac-RFAAKAA-COOH) with > 95% purity (Peptron Co., Daejeon, Korea). The cysteine peptide reaction solution was prepared by diluting the cysteine peptide stock solution to 400 µM with 100 mM sodium phosphate buffer (pH 8.0, 1 mM 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). 90 µL of the peptide reaction solution and 90 µL of chemical reaction solution were reacted in a 96-well ELISA plate for 24 h at room temperature. After 24 h, 20 µL of a 10 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB; Sigma-Aldrich, St. Louis, USA) 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 min 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 (OD) was measured using a UV-VIS spectrophotometer (SpectraMAX 190$^{TM}$, Molecular Devices, CA, USA; absorption wavelength: 412 nm). The lysine peptide reaction solution was prepared by diluting the lysine peptide stock solution to 200 µM with 100 mM sodium phosphate buffer (pH 10.0, 1 mM EDTA) before

the 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 chemical reaction solution were reacted in the 96-well clear plate for 24 h 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, Frickenhausen, Germany). Next, 20 µL of a 20 mM fluorescamine solution (prepared in DMSO) was added to each well of the plate and incubated for 3 min 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 24h incubation of the peptide with the chemical, and the peptide depletion rate was calculated as follows: Peptide depletion ratio (%) = $(1 - P_{unreacted} / P_{total}) \times 100$. 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 to be a skin sensitization risk.

## *2.3. ARE-Nrf2 luciferase assay: KeratinoSens™*

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 [35]. Cells were seeded to three clear

bottom white 96-well plates and one clear 96-well plate at density of $1 \times 10^4$ cells /well in DMEM (Dulbecco's Modified Eagle Medium; Gibco, Grand Island, USA) with 500 μg/ml G418 (Gibco) and 10% FBS Gibco) for 24 h at 37°C in a 5% $CO_2$ atmosphere. The medium was then replaced with medium containing the test substance and a final level of 1% of the solvent vehicle, DMSO. Each test substance was tested at 12 binary serial dilutions in the range of 0.98 to 2,000 μM. The plates contained 5 test chemicals with 12 dilutions per column respectively: 6 wells with the solvent control, 1 well with no cells for background value and 5 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 MTT ((3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) (Sigma-Aldrich) assay. Cells were incubated in a 37°C and 5% $CO_2$ atmosphere for 48 h with the test compounds and then washed with 200 μl of DPBS (Gibco, Grand Island, USA). Luciferase activity was measured according to the manufacturer's protocol (One-Glo™ luciferase Assay system kit, Promega, WI, USA). After the luciferase activity of each well was measured with a luminometer (Promega GloMax® Discover, Promega, WI, USA), 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: (i) The EC1.5 value is below 1,000 μM in all three repetitions or at least two repetitions, (ii) 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 (iii) there is an apparent overall dose-response for luciferase induction, which is similar

between the repetitions

## *2.4. Human Cell Line Activation Test (h-CLAT )*

Human leukemia monocytic cell line (THP-1) was obtained from the American Type Culture Collection (ATCC: Manassas, VA, USA). These cells were cultured in RPMI 1640 medium (Gibco) with 100 U $ml^{-1}$ Penicillin / 100 µg $ml^{-1}$ Streptomycin (Gibco), 10% fetal bovine serum (FBS) (v/v) (Gibco) and 0.05 mM 2-mercaptoethanol (GIBCO) at 37℃ and 5% $CO_2$ atmosphere. The h-CLAT assay was performed as previously described [48]. THP-1 cells were plated at $1 \times 10^6$ cells/ml in a 24-well plate (1 ml per well) and treated with test chemicals for 24 h at 37℃ and 5% $CO_2$ atmosphere. After 24 h, cells were washed twice with FACS (Fluorescence Activated Cell Sorter) 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 min at 4℃ with 0.01% (w/v) globulins in FACS buffer. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated antihuman for CD 54 or CD 86 (anti-human CD54, ICAM-1 / FITC (clone 6.5B5) (DAKO, Glostrup, Denmark) Using (6 µl / 3 x $10^5$ cells / 100 µl for the anti-human CD86 mAb, 3 µl / 3 x $10^5$ cells / 100 µl for the anti-human CD54 mAb, 3 µl / 1 x $10^5$ cells / 100 µl for the anti-human 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 Cell-Quest software (Becton-Dickinson, San Diego, CA, USA). A total of 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.

MFI = Geometric Mean Fluorescence Intensity

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

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 LLNA 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 [15, 58, 90-94, 99]. I evaluated the predictivity of Spectrophotometric DPRA,

KeratinoSens™ and h-CLAT by calculating the accuracy, sensitivity, specificity, positive predictive value and negative predictive value of the results based on LLNA EC3 data or human data of the 58 chemicals as follows [102]:

Sensitivity (%) = TP / (TP + FN) × 100,

Specificity (%) = TN / (TN + FP) × 100,

Positive predictive value (%) = TP / (TP + FP) × 100,

Negative predictive value (%) = TN / (TN + FN) × 100, and

Accuracy (%) = (TP + TN) / (TP + TN + FP + FN) × 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 LLNA and human data.*

Based on the key event of AOP for skin sensitization [28], I applied an approach that combined two or three test methods. The test scheme is outlined in Figure 1. Approach 1 (A) and has two steps. In 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, I conducted the KeratinoSens™ and h-CLAT in addition. 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 to be a non-sensitizer. Approach 2 (B) is also composed of two steps. In the first step, if the result of Spectro-DPRA shows the chemical to be a sensitizer, it is judged as a sensitizer. However, if shows as non-sensitizer, I conducted the h-

CLAT assay. If h-CLAT predicted the chemical as positive, the chemical is considered a skin sensitizer. Approach 3 (C) 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 positive, we conclude the chemical to be a skin sensitizer. (D) The '2 out of 3' prediction [58] uses any two congruent results of the three tests (Spectro-DPRA, KeratinoSens™, and h-CLAT) to determine an 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 non-sensitizer. We evaluated the predictivity of each approach by calculating the accuracy, sensitivity, specificity, positive predictive value, and negative predictive value using Cooper statistics in comparison to LLNA or human data [102].

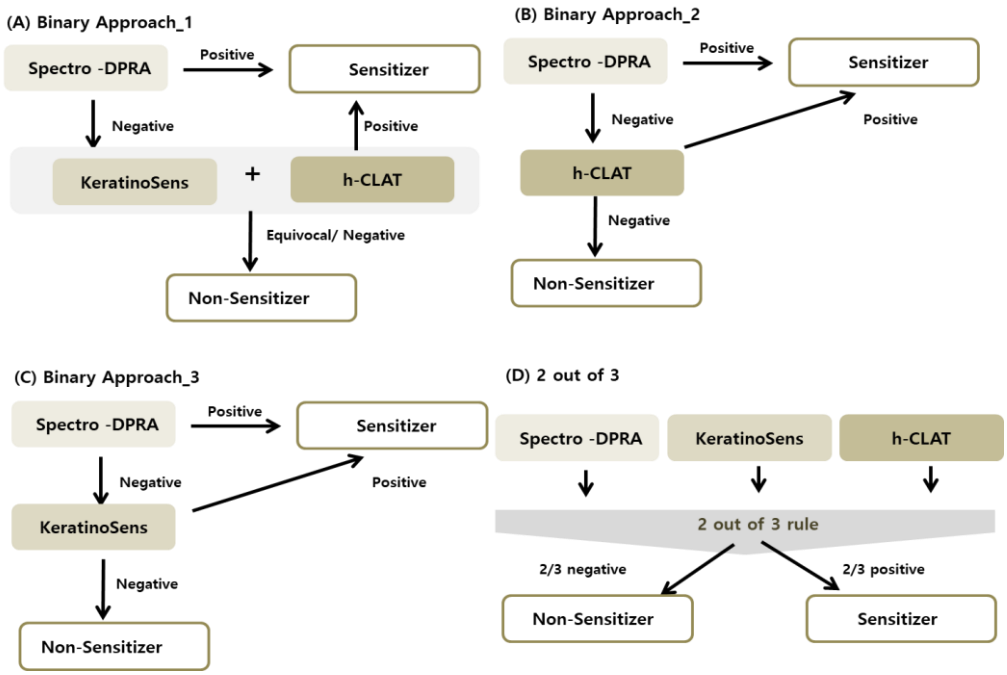


**Figure 1. The workflow of each integrated approach.**

Binary Approach _1(A) 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 shows as non-sensitizer, I 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. Binary Approach _2 (B) 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 shows up as a non-sensitizer, I conducted an h-CLAT assay. If h-CLAT predicted the chemical as positive, the chemical was confirmed to be a skin sensitizer. Binary Approach _3 (C) is composed the same way as Approach 2, however, instead of h-CLAT, we conducted KeratinoSens™. If KeratinoSens™ predicted the chemical as positive, I concluded the chemical was a skin sensitizer. (D), The '2 out of 3' prediction [58] 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 classified as a skin sensitizer. If at least two of the three assays were negative, the chemical classified as non-sensitizer.

# 3. Results

## *3.1. Spectro-DPRA has a higher predictivity than the KeratinoSens ™ and h-CLAT assays*

The results of Spectro-DPRA, KeratinoSens ™ and h-CLAT for 58 chemicals are outlined in Table 2. Based on these results, I calculated the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy using Cooper statistics [102] (Table 3 and 4).

Compared to 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, positive predictive value, negative predictive value 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, positive predictive value, negative predictive value 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 positive predictive value, 88.5% for negative predictive value

and 81.0% for accuracy (Table 2 and 3).

The overall predictability compared to the LLNA data was lower than that for human data for all test methods. Spectro-DPRA showed sensitivity, specificity, positive predictive value, negative predictive value 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, dimethylsulfoxide, sodium lauryl sulfate, xylene, pyridine, R-(+)-limonene, and oleic acid). The Spectro-DPRA assay also showed the highest predictivity of any other assays and aligned most similarly to human data.

**Table 2. Detailed results of the predictions based on the individual test methods.**

| Chemical | Human | LLNA | Spectro-DPRA | | | Keratinosens™ | | h-CLAT | | | ITS prediction | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 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 |
| 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 | S | S |
| 1-Chloro-2,4-dinitrobenzene (DNCB) | S | S | 99.29 | 6.17 | S | 2.73 | S | 508.89 | 320.59 | S | S | S | S | S |
| Oxazolone | S | S | 17.78 | 95.05 | S | 39.04 | S | 177.24 | 222.22 | S | S | S | S | S |
| 4-phenylenediamine | S | S | 98.87 | 86.10 | S | 8.20 | S | 132.20 | 167.80 | S | S | S | S | S |
| Glutaric dialdehyde | S | S | 1.97 | 99.51 | S | 5.30 | S | 428.83 | 193.20 | S | S | S | S | S |
| Lauryl gallate | S | S | 16.79 | 49.13 | S | 2.14 | S | 1165.03 | 204.13 | S | S | S | S | S |
| Hydroquinone | S | S | 101.75 | 81.03 | S | 9.76 | S | 151.65 | 163.90 | S | S | S | S | S |
| Propyl gallate | S | S | 101.60 | 54.87 | S | 3.53 | S | 659.56 | 61.16 | S | S | S | S | S |
| Cobalt chloride | S | S | -1.49 | -2.46 | NS | 12.21 | S | 760.50 | 68.03 | S | S | S | S | S |
| Isoeugenol | S | S | 97.10 | 8.53 | S | 2.70 | S | 159.06 | 87.46 | NS | S | S | S | S |
| 2-Mercaptobenzothiazole | S | S | 88.63 | 1.65 | S | 1.80 | S | 480.67 | 103.13 | S | S | S | S | S |
| Cinnamic aldehyde | S | S | 21.31 | 2.55 | S | 5.37 | S | 347.50 | 206.23 | S | S | S | S | S |
| Diethyl maleate | S | S | 96.99 | 5.61 | S | 6.05 | S | 149.73 | 200.83 | S | S | S | S | S |
| Diethylenetriamine | S | S | 19.98 | 88.30 | S | 1.15 | NS | 8.92 | 10.14 | NS | S | S | S | NS |
| Ammonium persulfate | S | S | -13.69 | 8.75 | NS | 1.61 | S | 190.79 | 187.33 | S | S | S | S | S |
| Citral | S | S | 17.35 | 10.96 | S | 1.63 | S | 379.92 | 93.95 | S | S | S | S | S |
| Eugenol | S | S | 17.38 | 8.60 | S | 1.42 | NS | 220.34 | 89.77 | S | S | S | S | S |
| Phenyl benzoate | S | S | 21.67 | 6.97 | S | 5.61 | S | 268.91 | 132.29 | S | S | S | S | S |

**Table 2. Detailed results of the predictions based on the individual test methods (*cont'd*).**

| Chemical | Human | LLNA | Spectro-DPRA | | | Keratinosens™ | | h-CLAT | | | ITS prediction | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 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 |
| Cinnamyl alcohol | S | S | 42.46 | 7.51 | S | 6.37 | S | 246.76 | 106.88 | S | S | S | S | S |
| Imidazolidinyl urea | S | S | -11.95 | 38.94 | S | 2.84 | S | 323.58 | 538.89 | S | S | S | S | S |
| Methyl methacrylate | S | S | 11.05 | 12.18 | S | 8.89 | S | 231.87 | 80.51 | S | S | S | S | S |
| Ethylene glycol dimethacrylate | S | S | 44.78 | -2.93 | S | 10.44 | S | 216.78 | 106.43 | S | S | S | S | S |
| Benzocaine | S | S | 0.61 | 43.73 | S | 5.30 | S | 111.48 | 150.69 | S | S | S | S | S |
| Abietic acid | S | S | 80.55 | 4.03 | S | 10.21 | S | 110.38 | 91.18 | NS | S | S | S | S |
| Geraniol | S | S | 5.56 | 18.04 | NS | 1.59 | S | 210.24 | 95.56 | S | S | S | S | S |
| Linalool | S | S | 2.68 | -4.11 | NS | 1.38 | NS | 299.31 | 92.76 | S | NS | S | NS | NS |
| Nickel chloride | S | NS | 3.13 | 17.53 | NS | 1.61 | S | 326.37 | 180.19 | S | S | S | S | S |
| α-Linolenic acid | NS (W.U.) | S | 44.20 | 7.80 | S | 1.83 | S | 4800.00 | 1680.00 | S | S | S | S | S |
| Squalene | NS (W.U.) | S | -3.40 | 8.10 | NS | 0.85 | NS | 218.00 | 262.00 | S | NS | S | NS | NS |
| Maleic acid | NS (W.U.) | S | 9.60 | -1.24 | NS | 0.95 | NS | 270.18 | 142.26 | S | NS | S | NS | NS |
| Tartaric acid | NS | S | -4.63 | 5.86 | NS | 1.34 | S | 120.00 | 93.40 | NS | NS | NS | S | NS |
| Dimethylsulfoxide | 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 |

**Table 2. Detailed results of the predictions based on the individual test methods (*cont'd*).**

| Chemical | Human | LLNA | Spectro-DPRA | | | Keratinosens™ | | h-CLAT | | | ITS prediction | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 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 |
| 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 (W.U.) | 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 (W.U.) | S | 1.84 | 8.40 | NS | 1.29 | NS | 147.00 | 88.00 | NS | NS | NS | NS | NS |
| Linoleic acid | NS (W.U.) | S | 38.60 | 7.50 | S | 1.92 | S | 480.00 | 168.00 | S | S | S | S | S |
| Succinic acid | NS (W.U.) | NS | 7.05 | 14.31 | NS | 0.85 | NS | 124.00 | 154.00 | S | NS | S | NS | NS |
| *p*-Anisic acid | NS (W.U.) | NS (W.U.) | -6.10 | -8.96 | NS | 1.22 | NS | 112.50 | 132.00 | NS | NS | NS | NS | NS |
| Caprylyl sulfobetaine | NS (W.U.) | NS (W.U.) | -14.9 | -14.33 | NS | 0.99 | NS | 116.39 | 114.70 | NS | NS | NS | NS | NS |
| Hydroxyacetophenone | NS (W.U.) | NS (W.U.) | -2.94 | -10.89 | NS | 1.15 | NS | 143.10 | 62.18 | NS | NS | NS | NS | NS |

**Table 2. Detailed results of the predictions based on the individual test methods (*cont'd*).**

| Chemical | Human | LLNA | Spectro-DPRA | | | Keratinosens™ | | h-CLAT | | | ITS prediction | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 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 |
| Apigenin | NS (W.U.) | NS (W.U.) | -17.87 | 0.21 | NS | 18.89 | S | 114.89 | 54.17 | NS | NS | NS | S | NS |
| 2,3-Butanediol | NS (W.U.) | NS (W.U.) | 3.69 | -0.30 | NS | 1.18 | NS | 80.00 | 97.13 | NS | NS | NS | NS | NS |
| Caprylic/capric glycerides | NS (W.U.) | NS (W.U.) | 5.20 | 12.66 | NS | 1.18 | NS | 147.22 | 94.30 | NS | NS | NS | NS | NS |
| 1,2-Hexanediol | NS (W.U.) | NS (W.U.) | -6.90 | -0.71 | NS | 1.12 | NS | 124.24 | 114.14 | NS | NS | NS | NS | NS |
| 1,2-Octanediol | NS (W.U.) | NS (W.U.) | 8.89 | -0.34 | NS | 1.17 | NS | 104.55 | 110.10 | NS | NS | NS | NS | NS |

S/NS means Sensitizer/ Non-Sensitizer

n.e. means Not Estimated

W.U. means 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 | 3 | 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 | |

PPV means Positive predictive value.

NPV means Negative predictive value

**Table 4. Performance of animal alternatives 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 | |

PPV means Positive predictive value.

NPV means Negative predictive value

## *3.2. The improvement of predictivity through Integrated Testing*

A brief scheme of each integrated approach is given in Figure 1. Based on data sets of Specto-DPRA, KeratinoSens™, and h-CLAT (Table 2), I applied four integrated approaches (Figure 1) and evaluated the predictivity compared to human or LLNA data using Cooper statistics [102]. The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of each approach is suggested in Table 5 and 6.

Compared with the human data, prediction rates were higher than 84.5% in all integrated approaches except for the specificity and positive predictive value of Binary Approach_2 and 3. Compared to the single test method, the overall predictability of each test increased when using the combined approach except Binary Approach_2 and 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 assay were predicted correctly as sensitizers by using a combination of tests.

By using an integrated approach, I was able to increase the predictivity compared to a single assay approach. I was able to attain more accurate predictivity that was close to the human and LLNA data. Binary Approach_1 and 2 showed the highest accuracy (82.8%) to LLNA data

**Table 5. Performance of binary and '2 out of 3' approaches in different datasets 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 | | 92.6 | | 92.6 | |
| Specificity (%) | 87.1 | | 74.2 | | 74.2 | | 87.1 | |
| PPV (%) | 86.7 | | 77.1 | | 75.8 | | 86.2 | |
| NPV (%) | 96.4 | | 100.0 | | 95.8 | | 93.1 | |
| Accuracy (%) | 91.4 | | 86.2 | | 84.5 | | 89.7 | |

PPV means Positive predictive value.

NPV means Negative predictive value

**Table 6. Performance of binary and '2 out of 3' approaches in different datasets 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 | | 81.0 | | 95.0 | |
| PPV (%) | 96.7 | | 88.6 | | 88.2 | | 96.6 | |
| NPV (%) | 67.9 | | 73.9 | | 70.8 | | 65.5 | |
| Accuracy (%) | 82.8 | | 82.8 | | 81.0 | | 81.0 | |

PPV means Positive predictive value.

NPV means Negative predictive value

# 4. Discussion

The overall predictivity of the single test was higher for human data than for LLNA. The accuracy of the 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. I applied an integrated approach using Spectro-DPRA as the first step for Approach 1-3. Spectro-DPRA represents a molecular initiating event (MIE) or key event 1 (KE1) haptenization in the AOP of skin sensitization similar to standard DPRA (OECD TG 442C). Skin sensitization is a hapten-specific delayed-type hypersensitivity [2]. 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 [78-80]. In the four integrated approaches, the range of accuracy was 84.5-91.4% compared to human data and 81.0-82.8% compared to LLNA data. All three integrated approaches showed a higher predictivity to human data. The accuracy of Spectro-DRPA, 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 my study, with 90% accuracy for human data and 79% accuracy for LLNA data (My data showed 89.7% and 81.0% accuracy for human and LLNA data, respectively) [58]. 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 my results [67]. 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 [103]. 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 my integrated approach, especially in reference to Binary approach_1, is that I can evaluate the skin sensitization potential of cosmetic ingredients with high lipophilicity.

The combination of 3 assay methods (Spectro-DPRA, KeratinoSens ™ and h-CLAT) helped to improve the overall prediction power compared to the binary approaches that used 2 test methods (Spectro-DPRA + KeratinoSens ™ or Spectro-DPRA + h-CLAT) (Table 5 and 6). Among the three integrated approaches, Binary Approach_1 (Fig. 2.) showed the highest sensitivity, specificity, positive predictive value, negative predictive value, 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 KE of skin sensitization AOP, I was able to correctly predict these chemicals as sensitizers. 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, I can more accurately predict skin sensitization hazard for cosmetic ingredients. However, this approach also has limitations. This approach cannot estimate all pre/pro-haptens accurately. Similar to previous studies examining an integrated approach, since pro-hapten is outside the domain of the Spectro-DPRA, two negative results could falsely classify a chemical as a non-sensitizer. However,

since 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, my 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, I 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 my integrated approach performed better than individual assays and my combination of assays was accurate at predicting the sensitization outcome in both the LLNA and human data. Although further optimization is needed, the approach in which skin sensitization potential was determined by the Spectro-DPRA assay followed by final determination using the result of KeratinoSens™ and h-CLAT assays could be a reliable and convenient approach to predict the human outcome.

# GENERAL CONCLUSION

Allergic contact dermatitis is a symptom that causes the main customer claims for cosmetics. The pre-screening the possibility of skin sensitization on cosmetic ingredients is essential to prevent these side effects caused by cosmetic ingredients. In general, animal experiments were conducted to evaluate skin sensitization potential on cosmetic ingredients, but due to ethical issues, the cosmetics industry has been banned from toxicological evaluations, and therefore, reliable animal alternative methods and effective hazard identification strategies are needed.

In chapter I, the novel spectrophotometric evaluation method for peptide reactivity (Spectro-DPRA) was used to evaluate the predictivity of the data of animals and humans. Spectro-DPRA showed 83.1% or 90.9% accuracy compared to a conventional LLNA or prediction based on human data, respectively, with a combination model using both a cysteine peptide and lysine peptide cut-off. The Spectro-DPRA could serve as high-throughput available *in chemico* screening method with high accuracy to predict the animal and human skin sensitization potential of chemicals.

In chapter II, to improve the predictivity and setup the integrated evaluation strategy for skin sensitization, I combined the results with novel developed spectrophotometric evaluation method (Spectro-DPRA) and two other AOP-based in vitro assays (KeratinoSens$^{TM}$ and h-CLAT). When the results of the *in chemico*

and *in vitro* assays were combined, the predictivity of human data increased compared to that of a single assay. The highest predictivity was obtained for the approach in which sensitization potential was determined by the Spectro-DPRA assay 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 to human data). I believe this integrated approach may provide useful predictive data when determining the human skin sensitization potential of cosmetic ingredients.

In conclusion, the Spectro-DPRA could serve as an easy, rapid, low-cost, high-throughput available *in chemico* screening animal alternatives with high accuracy to predict the animal and human skin sensitization potential of chemicals compared to standard DPRA (OECD TG 442C). In addition, the advantages of Spectro-DPRA are likely to evolve into animal alternatives that can be used for a wide variety of predictive approaches integrated with other test methods.

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# 국문 초록

# 동물실험 대체법으로서 피부 감작성을 예측하기 위한 Spectrophotometric Direct Peptide Reactivity Assay 개발

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화장품 혹은 피부 외용제에 사용되는 성분 중 일부는 화학적 햅텐으로 작용하여 피부 감작 반응을 일으킬 수 있다. 따라서 이 들 성분에 의한 피부 감작성 유발 가능성을 사전에 평가하여 제외하는 것으로 제품의 안전성 관리 측면에서 필수 적이다. 그러나 동물을 이용한 독성 시험에 대

한 윤리적인 이슈 및 화장품 산업계에서 화장품 원료 및 제품에 대한 동물시험 전면 금지 이슈로 인하여 신규 성분에 대한 피부 감작성을 평가하기 위한 동물대체 시험법의 개발이 필요하게 되었다. 이러한 필요성에 부합하여 전세계적으로 수많은 동물 대체 시험법의 개발이 활발히 진행되었고 일부 시험 법들은 과학적 검증을 통해 피부 감작성 관련 동물 대체 OECD 가이드라인 시험법으로 채택되었다. 이전 예비 연구에서 동물 피부 감작성 시험법인 LLNA (Local Lymph Node Assay)를 대체하는 광학적으로 평가 가능한 신규 *in chemico* Spectrophotometirc Direct Peptide Reactivity Assay (Spectro-DPRA)을 구축하였다. 이러한 이전 연구를 바탕으로 평가 물질을 확장 평가함으로써 단독 동물 대체 시험법으로서의 효용성을 확인하고 더 나아가 인체 피부 감작 데이터를 예측 할 수 있는지에 대해 확인하고자 하였다. 신규 시험 법인 Spectro-DPRA는 시스테인 펩타이드 혹은 라이신 펩타이드의 소실 율을 조합한 기준 (cut-off) 예측 모델에서 피부 감작성 유무에 대해 LLNA 동물 시험 결과와 비교하여 83.1%, 인체 시험 결과와 비교하여 90.9%의 정확성 (accuracy)의 높은 예측율을 보였다.

또한 피부 감작성 메커니즘의 복잡성으로 인해 단일 동물 대체 시험법만으로는 높은 예측력을 달성하기 어려우므로 전세계적으로 피부 감작성 독성 발현 경로 (Adverse Outcome Pathway; AOP)에 근거한 여러 화학적

방법 (in chemico)과 시험관 방법 (in vitro)의 조합으로 통합적으로 예측하여 각 시험법의 검출 한계를 보완하여 예측율을 높이는 연구가 이루어지고 있다. Spectro-DPRA를 다른 시험관 방법 (KeratinoSens™ 과 h-CLAT)의 결과를 여러 형태로 조합한 접근법으로 분석 시, 각각의 단독 시험 법에 비해 동물과 인체의 피부 감작성 예측력이 향상 되었다. 특히 Spectro-DPRA 방법으로 예측을 먼저 한 후 음성의 결과에 대해 KeratinoSens™ 과 h-CLAT의 결과로 확증하는 접근법이 LLNA 동물 시험 및 인체 시험 결과와 비교하여 가장 높은 피부 감작성 예측력을 보였다.

결론적으로 Spectro-DPRA 시험 법은 단독 적용시 기존의 OECD TG 채택된 Direct Peptide Reactivity Assay (DPRA)와 유사 수준의 높은 동물의 피부 감작성 예측력을 보였다. 또한 더 나아가 Spectro-DPRA은 인체의 피부 감작성에 대해서도 높은 예측율을 보였다. DPRA에 비해 Spectro-DPRA는 시험 과정이 간편하고 저비용이며 (DPRA에 비해 1/100 수준의 펩타이드 사용, 1/5 ~ 1/25 용량의 케미컬 사용, 고가의 분석 기기가 아닌 보편화된 광학 기기 사용), 다량 평가가 가능한 동물 대체 시험법이다. 또한 Spectro-DPRA은 타 동물 대체 시험법과 조합하여 피부 감작성 통합적 예측 접근에도 활용성이 높은 동물 대체 시험법으로 발전될 수 있을 것으로 사료된다.

주요어: 피부 감작성, Spectrophotometric DPRA, 통합적 접근법, 독성 발현 경로, 동물대체 시험법

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