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Applicability of an Integrated Testing Strategy consisting of *in silico*, *in chemico* and *in vitro* assays for evaluating the skin sensitization potencies of isocyanates

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Abstract

The skin sensitization potential of chemicals has been traditionally assessed using regulatory accepted *in vivo* methods, such as guinea pig or mouse local lymph node assays (LLNAs). A huge effort to refine and reduce the use of animals for safety assessments of chemicals because of regulatory requirements and ethical issues is presently underway, and alternative non-animal methods have been greatly developed. So far, a few studies have investigated the sensitization potencies of isocyanates which is a group of highly reactive chemicals that are known to be occupational allergens. The present study evaluated nine commonly used isocyanates using an *in vivo* LLNA and assessed the applicability of an Integrated Testing Strategy (ITS) consisting of an *in silico* Derek Nexus prediction, an *in chemico* direct peptide reactivity assay (DPRA), and an *in vitro* human Cell Line Activation Test (h-CLAT) to isocyanates. All nine isocyanates were evaluated as positive using the LLNA, Derek Nexus and DPRA, whereas seven chemicals tested positive using the h-CLAT: hexamethylene diisocyanate tested negative, and 1,5-diisocyanatonaphthalene could not be examined because of a solubility issue. When assessed using the ITS, the positive/negative evaluations of skin sensitization hazard were consistent with those assessed using the LLNA for all nine chemicals. However, the potency prediction results of the ITS tended to be underestimated, compared with those of the LLNA. The data presented in this work provide insights into the performance of non-animal testing approaches for evaluating the skin sensitization potencies

of isocyanates.

Keywords: isocyanates; skin sensitization; LLNA; ITS

Abbreviations: GPMT, guinea pig maximization test; LLNA, local lymph node assay; OECD, Organisation for Economic Cooperation and Development; REACH, Registration, Evaluation, Authorisation and Restriction of Chemicals; AOP, adverse outcome pathway; KE, key event; DPRA, direct peptide reactivity assay; h-CLAT, human cell line activation test; IATA, integrated approaches to testing and assessment; ITS, integrated testing strategy; TG, test guideline; EC, effective concentration; MIT, minimum induction threshold.

1. Introduction

Isocyanates, a type of highly reactive chemicals with the functional group $[N=C=O]$, are used as raw materials for polyurethanes and are known to be occupational allergens (Redlich, 2010). Although most cases of isocyanate exposure occur through inhalation and dermal absorption at workplaces (Bello et al., 2007; Jones et al., 2017), non-occupational exposure can also reportedly occur through the use of consumer products containing isocyanates, such as spray foam insulation (Wilder et al., 2011). As part of the process for managing and assessing the skin sensitization risk of chemicals, evaluating the sensitizing potential of chemicals is essential to ensure that human exposure is managed in accordance with each chemical's potency.

The skin sensitization potential of chemicals has been traditionally assessed using animal tests, such as the guinea pig maximization test (GPMT), the Buehler test (OECD, 1992) and the murine local lymph node assay (LLNA) (OECD, 2010). In the last decade, there has been a strong driving force to refine and reduce the use of animal tests for safety assessments of chemicals because of regulatory requirements and ethical issues. For instance, the European regulation for the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) stipulates that animal experiments can be used only if alternative methods are not applicable or if the results obtained from studies using alternative methods are not adequate for classification and risk assessment (European Parliament, 2016). However, the “gold

standard” test methods for the prediction of skin sensitization are still recognized as animal tests, such as the LLNA and the GPMT.

The adverse outcome pathway (AOP) for skin sensitization was recently published, and the development of non-animal test methods for evaluating the sensitization potential of chemicals has been accelerated, with non-animal test methods now covering three out of four key events (KEs, KE1: binding of haptens to endogenous skin proteins, KE2: keratinocyte activation, and KE3: dendritic cell activation) of the AOP (OECD, 2012). These three KEs are addressed by the following methods, which have been validated and adopted by the Organisation for Economic Cooperation and Development (OECD) for regulatory purposes: the direct peptide reactivity assay (DPRA) addressing KE1 (OECD, 2015a); the ARE-Nrf2 luciferase test method, known as the KeratinoSens™ assay, representing KE2 (OECD, 2015b); and the human Cell Line Activation Test (h-CLAT) describing KE3 (OECD, 2016c). Furthermore, several new assays, such as the LuSens assay (Bauch et al., 2012; Ramirez et al., 2014), the SENS-IS assay (Cottrez et al., 2015, 2016), and the U-SENS™ (Alepee et al., 2015; Piroird et al., 2015), are presently being developed and validated.

Despite the considerable progress in this field, there is no scientific consensus that a single alternative test method can serve as a stand-alone assay to fully replace animal tests because of the complexity of the process involved in the generation of a skin sensitization reaction. Therefore, integrated approaches to testing and assessment (IATA) using combinations of

assays representing different KEs of the AOP have been proposed (OECD, 2016b), and some case studies of IATAs utilizing a combination of *in silico*, *in chemico* and/or *in vitro* tests to evaluate the sensitizing potential of chemicals have been described (OECD, 2016a).

So far, a few studies have investigated the sensitization potencies of isocyanates, especially using non-animal testing approaches. Therefore, we evaluated nine commonly used isocyanates using an *in vivo* LLNA and compared the results with those obtained using a previously proposed Integrated Testing Strategy (ITS) consisting of an *in silico* Derek Nexus prediction, an *in chemico* DPRA, and an *in vitro* h-CLAT (Takenouchi et al., 2015). The ITS used is one of 12 case studies documented in OECD guidance (OECD, 2016a) that can evaluate the skin sensitization potency as well as hazard, and the simple approach consisting of OECD-validated test methods except KE2 assay. We then examined the applicability of this approach to isocyanates. The ITS used is applicable even to highly reactive chemicals such as isocyanates for the qualitative assessment of the skin sensitization hazard. On the other hand, the sensitization potency predictions tended to be underestimated compared with the EC3-based LLNA predictions, because of the underestimated results derived from the h-CLAT. The data presented in this work provide insights into the technical challenges and limitations of a non-animal testing approach to evaluating the skin sensitization potency of isocyanates.

2. Materials and Methods

2.1. Test chemicals

The test chemicals used in this study are listed in Table 1. All the test chemicals were dissolved in vehicles, from which water had been removed using a 3A molecular sieves (Nacalai Tesque Inc., Kyoto, Japan) to prevent the test chemicals from reacting with water.

2.2. Animals

Seven-week-old CBA/J female mice were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). The animals were housed in polycarbonate cages (W x D x H, 215 × 320 × 150 mm) placed in an animal chamber (Toyo-riko Co., Ltd., Tokyo, Japan) maintained at 23°C ± 2°C and a relative humidity of 55% ± 15%. The chambers were ventilated at a frequency of 10–15 cycles/h and were lighted artificially for 12 hours daily. A laboratory diet (MF; Oriental Yeast Co., Tokyo, Japan) and tap water were provided *ad libitum*. All mice were treated in compliance with the Regulation for Animal Experiments at Chemicals Assessment and Research Center, CERI based on the Act on Welfare and Management of Animals (2005) and Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology (2006).

2.3. Local lymph node assay (LLNA)

The LLNA was performed in accordance with OECD Test Guideline (TG) 429 (OECD, 2010) using female CBA/J mice. Consecutive three doses (%) were selected by preliminary dose finding tests. Mice were allocated into groups of four, then three dose groups for each test chemical, a concurrent vehicle control group and a positive control group given hexyl cinnamic aldehyde (Wako) were prepared. Test solutions dissolved in 4:1 (v/v) acetone (Wako Pure Chemical Industries, Osaka, Japan)/olive oil (AOO; Wako) were applied to the dorsum of both ears for three consecutive days. Three days after the last application, 20 μ Ci of ^{3} H-methyl thymidine (3H-TdR; Moravek Bio-chemicals, Brea, CA, USA) was injected intravenously into a tail vein. Five hours after the injection of 3H-TdR, the animals were euthanized and the auricular lymph nodes on both sides were removed. A single-cell suspension of lymph node cells was prepared in phosphate-buffered saline (PBS; MP Bio-medicals, LLC, Santa Ana, CA, USA) by gentle mechanical disaggregation through a 200-mesh stainless-steel mesh. The cells were washed twice with PBS and precipitated with 5% trichloroacetic acid (TCA; Wako). After incubation at 4°C for 18 hours, the cells were resuspended in 1 mL of TCA and transferred to 10 mL of scintillant (EcolumeTM; MP Bio-medicals). The incorporation of 3H-TdR was measured using a liquid scintillation counter (TRI-CARB 3110TR; PerkinElmer, Inc., Waltham, MA, USA). The test chemical was classified as positive if the stimulation index (SI) relative to the concurrent AOO-treated

vehicle control value was greater than 3. The effective concentration inducing an SI of 3 (EC3, %) was calculated using a linear interpolation (Basketter et al., 1999) and used to classify the sensitizing potency of the chemical according to the criteria reported by Kimber et al. (2003): extreme ($EC3 < 0.1$), strong ($0.1 \leq EC3 < 1$), moderate ($1 \leq EC3 < 10$), or weak ($10 \leq EC3 \leq 100$).

The EC3 value was calculated by interpolating between 2 points on the SI axis, one immediately above, and one immediately below, the SI value of 3. Where the data points lying immediately above and below the SI value of 3 have the coordinates (a,b) and (c,d) respectively, then the EC3 value may be calculated using the following equation:

$$EC3 (\%) = c + [(3-d)/(b-d)] \times (a-c)$$

2.4. Derek Nexus

Derek Nexus 5.0.2 (Lhasa Limited, Leeds, UK), a knowledge-based expert system, was used to predict the skin sensitizing potential of test chemicals using a previously described *in silico* approach (Langton et al., 2006). When the prediction outcomes were noted as certain, probable, plausible or equivocal, the test substance was evaluated as being positive.

2.5. Direct peptide reactivity assay (DPRA)

The DPRA addresses KE1 or the binding of haptens to endogenous skin proteins in the

skin sensitization AOP. The DPRA was performed in accordance with OECD TG442C (OECD, 2015a). Peptides containing cysteine (Ac-RFAACAA-COOH) or lysine (Ac-RFAAKAA-COOH) were obtained from Scrum Inc. (Tokyo, Japan). Briefly, a 0.67-mM peptide stock solution prepared in buffer and a 100-mM test chemical stock solution prepared in either acetonitrile (Wako) or acetone were added to 100-mM sodium phosphate buffer (pH7.5) for the cysteine peptide or 100-mM ammonium acetate buffer (pH10.2) for the lysine peptide in triplicate. Cinnamic aldehyde (Wako) in acetonitrile was also prepared as positive control with three replicates. The final reaction mixture, containing 0.5 mM of a peptide and 5 mM (for the cysteine assay) or 25 mM (for the lysine assay) of the test chemical, was incubated in the dark for 24 hours at 25°C. After incubation, the peptide was quantified using a Prominence UFLC system (Shimadzu Co., Kyoto, Japan) equipped with a UV/VIS detector SPD-20AV (Shimadzu) and an *L-column2 ODS* (100 mm × 2.1 mm; particle size, 3 μm; CERI, Tokyo, Japan). Peptides were detected at 220 nm. The percentage of depletion was determined based on the decrease in the non-reacted peptide concentration in the sample relative to the average concentration measured in the control. When the mean of the cysteine and lysine % depletion was greater than 6.38%, the test chemical was predicted as positive. The mean % depletion was also used to classify the reactivity class of the chemical according to the criteria described in TG442C: no or minimal reactivity (mean % depletion ≤ 6.38), low reactivity (6.38 < mean % depletion ≤ 22.62), moderate reactivity (22.62 < mean % depletion ≤ 42.47),

or high reactivity ($42.47 < \text{mean \% depletion} \leq 100$).

2.6. Human cell line activation test (h-CLAT)

The h-CLAT addresses the KE3 or dendritic cell (DC) activation. The CD86 and the CD54 are known as a co-stimulatory molecule and an adhesion molecule with important role in antigen presentation by DC, and these molecules are upregulated in activated DC. A human monocyte cell line THP-1 also reported to show induction of CD86 and CD54 molecules after stimulation of skin sensitizers and serves as a DC surrogate (Ashikaga et al., 2002, 2006). Thus, the h-CLAT quantitatively measures CD86 and CD54 expressions on the cell surface of THP-1 cells. The h-CLAT was performed in accordance with OECD TG442E (OECD, 2016c). THP-1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640 (Thermo Fisher Scientific, San Jose, CA, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Thermo Fisher Scientific), 0.05 mM 2-mercaptoethanol (Wako), and 1% (v/v) penicillin-streptomycin (Thermo Fisher Scientific) at 37°C under 5% CO₂ and humidified atmosphere. Test chemicals were dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA). THP-1 cells were plated at 1×10^6 cells/mL in a 24-well plate (1 mL/well) and treated for 24 hours with test chemicals at eight concentrations ($\mu\text{g/mL}$) of a 1.2-fold serial dilution, a positive control well treated with 4 $\mu\text{g/mL}$ 2,4-dinitrochlorobenzene (Wako) was also prepared. Test chemical-treated cells were

washed twice with staining buffer (PBS containing 0.1% bovine serum albumin; Sigma-Aldrich), then incubated for 15 min at 4°C with 0.01% (w/v) globulins (Cohn fraction II and III, Human; Sigma-Aldrich) to block FcR. The cells were then stained with fluorescein isothiocyanate-conjugated anti-human CD86 antibody (clone Fun-1; BD Biosciences, San Diego, CA, USA), CD54 antibody (clone 6.5B5; DAKO, Glostrup, Denmark), or mouse IgG1 (DAKO) at 4°C for 30 min. After washing and resuspending, the cells were stained with propidium iodide (Sigma-Aldrich), and the fluorescence intensity of the viable cells was measured using flow cytometry (Cytomics FC 500; Beckman Coulter, Brea, CA, USA). The relative fluorescence intensities (RFI) of CD86 and CD54 were calculated at cell viabilities exceeding 50%, as described previously (OECD, 2016c). If the RFI of CD86 or CD54 was greater than 150 or 200 (respectively) at any dose in at least two of three independent runs, the test chemical was regarded as being positive. The thresholds for CD86 and CD54 expression (EC150 for CD86 and EC200 for CD54) were calculated using a linear interpolation. Then, the final EC150 and EC200 were determined as the median value (not the average) of the ECs calculated from the three independent runs as described in OECD TG442E. When only two of three independent runs met the positive criteria, the higher calculated value was adopted. When the EC150 and EC200 were calculated, the smaller value was determined as the minimum induction threshold (MIT) of the test substance in the h-CLAT.

2.7. Integrated testing strategy (ITS)

A score-based Integrated Testing Strategy (hereinafter referred to as Kao ITS) consisting of an *in silico* Derek Nexus prediction, an *in chemico* DPRA, and an *in vitro* h-CLAT was used as described previously (Takenouchi et al., 2015). As shown in Table 2, the results from the DPRA and h-CLAT were converted to scores from 0 to 3, and the Derek Nexus results were converted to scores of 0 or 1. A total score of 2 or greater was defined as positive according to the scoring criteria and classified into predicted potency classes (Takenouchi et al., 2015). Namely, the total score was used to sub-categorize the skin sensitizer into two sub-categories: a strong sensitizer (corresponding to extreme and strong sensitizers by the LLNA) if the total score was 7, or a weak sensitizer (corresponding to moderate and weak sensitizers by the LLNA) if the total score was between 2 and 6.

3. Results

3.1. Assay results

The results of the LLNA, Derek Nexus, DPRA and h-CLAT are summarized in Table 3. Among them, mean % depletion in DPRA and MIT value in h-CLAT are expressed without standard deviation (SD) because it is not required in the corresponding TGs, and it is not used for the discrimination between sensitizers and non-sensitizers. All the chemicals were

evaluated as positive in the LLNA and Derek Nexus, and the EC3 values for each chemical were all less than 0.1%, indicating that they were all classified as extreme sensitizers. Similarly, all the chemicals were evaluated as positive by the DPRA, and the % depletion of cysteine and lysine peptides ranged from 52.8% to 81.0%, indicating that all the chemicals were assigned to a high reactivity class. Regarding the h-CLAT results, seven chemicals tested positive: the chemical HDI was evaluated as negative, and the chemical NDI could not be tested using the h-CLAT because of its low solubility in the vehicles.

3.2. Potency prediction using Kao ITS

The individual scores for each assay were determined using the Kao ITS criteria (Table 2) and are shown in Table 4. The total scores for the eight chemicals excluding the chemical HMDI ranged from 4 to 6; these chemicals were predicted to be weak sensitizers. Meanwhile, the chemical HMDI had a total score of 7 and was predicted to be a strong sensitizer.

4. Discussion

The skin sensitization potential of chemicals has traditionally been evaluated using regulatory accepted animal methods, and the current first choice method for skin sensitization potential is the LLNA, e.g. REACH registration. It can provide a quantitative endpoint that allows for prediction of potency classification and use for GHS sub-categorization for skin

sensitization. Recently, however, non-animal methods need to provide information allowing both hazard identification and the potency sub-categorization of skin sensitizers because of regulatory requirements and animal welfare.

Some clinical observations of isocyanate-induced allergic contact dermatitis have been made, whereas non-clinical studies examining skin sensitization potencies are too limited, especially with the non-animal testing approaches used so far. Potency data are necessary for some regulatory authorities and for risk assessments to determine safe levels of chemical use. Thus, we attempted to obtain skin sensitization potency data for isocyanates using *in vivo* LLNA and compared the results with predictions based on the Kao ITS, consisting of an *in silico* Derek Nexus prediction, an *in chemico* DPRA, and an *in vitro* h-CLAT. We then evaluated the applicability of this ITS approach to isocyanates.

In the present study, all nine isocyanates were evaluated as positive in the LLNA and were classified as extreme sensitizers based on their EC3 values. The prediction results for isocyanates tested using Derek Nexus were “probable” or “plausible” because these chemicals were recognized as having structural alerts. Therefore, all the chemicals were predicted to be positive according to Derek Nexus, and their ITS scores for Derek Nexus were 1 based on the scoring criteria of the Kao ITS. Furthermore, all the chemicals were predicted to be positive and were assigned to a high reactivity class using DPRA because of their high reactivity to active-hydrogen containing groups, such as the amino and thiol groups contained in peptides

(Wisnewski et al., 1999). Therefore, the ITS scores for DPRA were 3 for all nine chemicals.

In the h-CLAT, seven of the nine chemicals tested positive. Among them, six chemicals had an MIT of over 10 $\mu\text{g/mL}$ and were scored as 2, whereas the chemical HMDI had an MIT of 5.7 $\mu\text{g/mL}$ and was scored as 3. Meanwhile, the chemical HDI was evaluated as negative in the h-CLAT and was scored as 0. The remaining chemical, NDI, could not be tested using the h-CLAT because of a solubility issue.

The scoring criteria in the Kao ITS shown in Table 2 was determined with the dataset of 139 substances by Takenouchi et al. (2015), and an sensitivity, specificity and accuracy were 89, 70 and 84%, respectively, compared with the LLNA. Moreover, the over-prediction rate, under-prediction rate and overall accuracy of three-rank classification of the Kao ITS were 12%, 18% and 71%, respectively. As for extreme sensitizers by the LLNA, 10 out of 10 chemicals were estimated as positive and 8 out of 10 were classified as strong sensitizers by the Kao ITS. In the present study, all nine isocyanates classified as extreme sensitizers by the LLNA were evaluated as positive whereas only one chemical was classified as strong sensitizer by the Kao ITS.

Since the ITS scores of eight chemicals in the h-CLAT were less than 2, these chemicals were classified as weak sensitizers by the Kao ITS. Quantitative data derived from the h-CLAT tended to be underestimated, compared with the results of the other tests. We hypothesized that highly reactive isocyanates readily react with water and various proteins in

the culture medium during the preparation of test chemical solutions at several concentrations of serial dilution using culture medium before exposure to cells, resulting in decreases in the actual concentrations of residual unreacted isocyanates below the theoretical values. Consequently, this property possibly leads to underestimated MIT values in the h-CLAT, and this limitation could occur in other *in vitro* cell-based assays as well.

When THP-1 cells were treated with the chemical HDI in serum-free culture medium (X-VIVO™ 15; Lonza, Walkersville, USA), as described previously (Kimura et al., 2015), HDI tested positive in the h-CLAT (MIT, 13.7 µg/mL). One hypothesis is that the chemical HDI reacted rapidly with proteins in the serum-supplemented culture medium, since isocyanate groups are much reactive with amines and thiols.

Skin sensitization reaction has the quantitative aspect same as other toxicological reactions, and this aspect is directly relevant to the sensitization risk assessment in humans. So the prediction of the sensitization potency is important approach to conduct risk assessments. To date, huge efforts have been made to predict the skin sensitization potential using non-animal testing strategies and the combination of *in silico*, *in chemico* and *in vitro* can be used to support the hazard identification, hazard characterization and/or safety assessment of chemicals (OECD, 2016b); however, there is still no regulatory acceptance of integrated approaches to testing and assessments (IATA) for hazard identification and potency prediction. Thus, this study was aimed to evaluate the prediction ability of the Kao ITS, one of 12

proposed IATAs by OECD guidance (OECD, 2016a), when assessing the skin sensitization potencies as well as hazards of isocyanates. The limitation may be existing because only isocyanates were used. Non-animal assays tend to underestimate chemicals showing a low to moderate skin sensitization potency than chemicals showing a high skin sensitization potency (OECD, 2015a, 2015b, 2016c). In the Kao ITS, 29 out of 29 extreme and strong sensitizers by the LLNA were estimated as positive, whereas 62 out of 73 moderate and weak sensitizers by the LLNA were evaluated as positive. Qualitative results presented here were concordant between the Kao ITS and the LLNA possibly because all isocyanates were classified as extreme sensitizers by the LLNA. Note that false negative results may be occurred by the Kao ITS if the weak skin sensitizing chemicals are used.

In this study, we evaluated skin sensitization potential of nine isocyanates using an *in vivo* LLNA, an *in silico* Derek Nexus prediction, an *in chemico* DPRA, and an *in vitro* h-CLAT. This type of isocyanate compounds are well-known sensitizers but had limited information on skin sensitization using the test methods described above. Importantly, this is the first report to evaluate isocyanates using the h-CLAT. Thus, the data presented here may be used for a reference database when developing a complete weight of evidence-based ITS/IATA approaches to increase the classes of chemicals.

Conflict of interest

The authors did not report any conflict of interest.

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Table 1. Chemicals used in this study.

Chemical Name	Abbreviation	CAS No.	Supplier	Purity
Toluene 2,4-diisocyanate	TDI	584-84-9	TCI	> 98.0%
Methylenediphenyl 4,4'-diisocyanate	MDI	101-68-8	TCI	> 97.0%
Hexamethylene diisocyanate	HDI	822-06-0	TCI	> 98.0%
Isophorone diisocyanate	IPDI	4098-71-9	TCI	> 99.0%
1,5-Diisocyanatonaphthalene	NDI	3173-72-6	TCI	> 98.0%
m-Xylylene diisocyanate	XDI	3634-83-1	TCI	> 98.0%
Dicyclohexylmethane 4,4'-diisocyanate (mixture of isomers)	HMDI	5124-30-1	TCI	> 90.0%
2-Isocyanatoethyl acrylate	AOI	13641-96-8	TCI	> 98.0%
2-Isocyanatoethyl methacrylate	MOI	30674-80-7	Sigma-Aldrich	> 98.0%

TCI: Tokyo Chemical Industry (Tokyo, Japan).

Table 2. Scoring criteria for the outcomes in the Derek Nexus, DPRA and h-CLAT in the Kao ITS.

Score	Derek Nexus alert	Mean depletion in the DPRA (%)	MIT in the h-CLAT (µg/mL)
0	No alert	< 6.376	Not calculated
1	Alert	$\geq 6.376, < 22.62$	$> 150, \leq 5000$
2	-	$\geq 22.62, < 42.47$	$> 10, \leq 150$
3	-	≥ 42.47	≤ 10

MIT: minimum induction threshold

Table 3. Summary of results from the LLNA, Derek Nexus, DPRA and h-CLAT.

Chemical	LLNA			Derek Nexus	DPRA			h-CLAT			
	Decision	EC3 (%)	Potency category		Decision	Depletion (%)	Reactivity class	Decision	EC150 (µg/mL)	EC200 (µg/mL)	MIT (µg/mL)
Tolylene-2,4-diisocyanate	P	0.02	Extreme	P (Plausible)	P	55.2	High	P	NC	127.9	127.9
Methylenediphenyl 4,4'-diisocyanate	P	0.02	Extreme	P (Plausible)	P	52.8	High	P	NC	71.5	71.5
Hexamethylene diisocyanate	P	0.005	Extreme	P (Probable)	P	54.6	High	N	NC	NC	NC
Isophorone diisocyanate	P	0.01	Extreme	P (Probable)	P	63.1	High	P	17.4	NC	17.4
1,5-Diisocyanatonaphthalene	P	0.01	Extreme	P (Plausible)	P	60.3	High	-	-	-	-
m-Xylylene diisocyanate	P	0.01	Extreme	P (Plausible)	P	66.9	High	P	NC	62.7	62.7
Dicyclohexylmethane 4,4'-diisocyanate	P	0.06	Extreme	P (Probable)	P	56.4	High	P	5.7	13.8	5.7
2-Isocyanatoethyl Acrylate	P	0.02	Extreme	P (Plausible)	P	81.0	High	P	12.6	24.0	12.6
2-Isocyanatoethyl methacrylate	P	0.05	Extreme	P (Plausible)	P	60.0	High	P	NC	78.1	78.1

EC3: effective concentration inducing a stimulation index of 3, EC150: effective concentration threshold for CD86, EC200: effective concentration threshold for CD54, MIT: minimum induction threshold, P: positive, N: negative, -: not applicable, NC: not calculated.

Table 4. The individual scores for each assay and potency estimation using the Kao ITS

Chemical	ITS score				Decision	Potency class
	Derek Nexus	DPRA	h-CLAT	Total score		
Toluene-2,4-diisocyanate	1	3	2	6	P	Weak
Methylenediphenyl 4,4'-diisocyanate	1	3	2	6	P	Weak
Hexamethylene diisocyanate	1	3	0	4	P	Weak
Isophorone diisocyanate	1	3	2	6	P	Weak
1,5-Diisocyanatonaphthalene	1	3	-	4	P	Weak
m-Xylylene diisocyanate	1	3	2	6	P	Weak
Dicyclohexylmethane 4,4'-diisocyanate	1	3	3	7	P	Strong
2-Isocyanatoethyl Acrylate	1	3	2	6	P	Weak
2-Isocyanatoethyl methacrylate	1	3	2	6	P	Weak

P: positive, -: not applicable.