



Evaluation of skin sensitization potential of chemicals by local lymph node assay using 5-bromo-2-deoxyuridine with flow cytometry

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ABSTRACT

The local lymph node assay using 5-bromo-2-deoxyuridine with flow cytometry (LLNA: BrdU-FCM) is a modified LLNA used to identify skin sensitizers. This assay measures the proliferation of auricular lymph node cells (LNCs) during the induction phase of skin sensitization and the number of BrdU-positive LNCs using flow cytometry. We determined if LLNA: BrdU-FCM can evaluate the skin sensitization potential of 20 substances, including 16 sensitizers and 4 non-sensitizers, that were tested using LLNA: DA and LLNA: BrdU-ELISA but not listed in OECD TG 429. After selecting appropriate vehicles and conducting pre-screen tests in 2 phases, solvents and test concentrations for the main test were determined. In the main study, we measured changes in LN weight, the number of LNCs, and the proportion of BrdU incorporated into LNCs to calculate stimulation indexes (SI). SI was calculated based on the total number of LNCs and BrdU incorporation in LNCs. We found that all substances were correctly classified as sensitizers or non-sensitizers. Overall, we confirmed that the LLNA: BrdU-FCM can evaluate skin sensitization potential of the 20 substances. Additionally, our results of combining 22 reference substances listed in OECD TG 429 and 20 additional substances showed that concordance of LLNA: BrdU-FCM with the LLNA was higher than before.

1. Introduction

The murine local lymph node assay (LLNA) for evaluating the skin sensitization potential of substances has been used worldwide as an alternative test method to the conventionally used guinea pig test (OECD TG 406, 1992); it was adopted as OECD TG 429 (Skin Sensitization: Local Lymph Node Assay) in 2002 (OECD, 2010a). The LLNA is a skin sensitization test in mice that measures the proliferation of murine local auricular lymph node cells (LNCs) after topical exposure to test substances. However, the use of LLNA has been limited due to the employment of radioisotope-labeled ³H-methyl thymidine and the resultant difficulty in the disposal of radioactive waste in some countries. For this reason, LLNA was modified to replace ³H-methyl thymidine, and two non-radio isotopic LLNA methods (LLNA: DA as OECD TG 442A and LLNA: BrdU-ELISA as OECD TG 442B) were adopted in 2010. LLNA: DA quantifies the ATP content of LNCs and LLNA: BrdU-ELISA measures the incorporation of BrdU, an analogue of thymidine, into

lymph nodes (OECD TG 442A, 2010b; OECD TG 442B, 2010c,d) to evaluate LNC proliferation. *In Chemico* Skin Sensitization (DPRA) as OECD TG 442C and *In Vitro* Skin Sensitization (KeratinSens) as OECD TG 442D were adopted in 2015 and *In Vitro* Skin Sensitization (h-CLAT) as OECD TG 442E was adopted in 2017 (OECD TG 442C, 2015a; OECD TG 442D, 2015b; OECD TG 442E, 2017).

The LLNA: BrdU-FCM is a novel non-radioisotopic version of the LLNA that performs similar to existing LLNA methods. This test measures the proliferation of auricular LNCs during the induction phase of skin sensitization by determining the number of BrdU-positive LNCs by flow cytometry. The LLNA: BrdU-FCM has been optimized and validated (Yang et al., 2015; Kim et al., 2016; Ahn et al., 2016). LLNA: BrdU-FCM can be used to evaluate the skin sensitization potency of test substances in the same way as the conventional LLNA, LLNA: BrdU-ELISA, and LLNA: DA. The LLNA: BrdU-FCM has several additional advantages. First, the proliferation of living LNCs is quantitatively measured in the LLNA: BrdU-FCM, whereas cells are indirectly scored

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Abbreviations

(BrdU)	5-bromo-2-deoxyuridine
(LLNA)	local lymph node assay
(LNCs)	lymph node cells
(BQ)	benzoquinone
(CIN)	cinnamic aldehyde
(CA)	cyclamen aldehyde
(DEM)	diethyl maleate
(HC)	hydroxycitronellal
(IPM)	isopropyl myristate
(LL)	linalool
(BGE)	butyl glycidyl ether
(AN)	aniline
(DEP)	diethyl phthalate

(DPCP)	diphenylcyclopropenone
(GA)	glutaraldehyde
(3-AP)	3-aminophenol
(TMA)	trimellitic anhydride
(AA)	abietic acid
(SA)	sulfanilamide
(PLG)	propylene glycol
(GLY)	glycerol
(PG)	propyl gallate
(MAPS)	4-methylaminophenol sulfate
(DMF)	<i>N,N</i> -dimethylformamide
(MEK)	methyl ethyl ketone
(DMSO)	dimethyl sulfoxide
(AOO)	acetone: olive oil
(PBS)	phosphate-buffered saline.

based on BrdU content, regardless of whether these cells are alive or dead, in the LLNA: BrdU-ELISA (in which the BrdU-content of LNCs is measured). Second, other endpoints, such as cell surface markers or intracellular cytokines, can be measured using a multi-color FACS machine along with specific antibodies. Our pre-validation study demonstrated the usefulness of the test method because it can analyze B cells, T cells, and cytokines by FCM (Jung et al., 2012). Third, the modified pre-screen tests in the LLNA: BrdU-FCM can allow for minimization of pain and distress by refined dose selection scheme. Fourth, BALB/c mice, instead of CBA/J mice, can be used in the LLNA: BrdU-FCM (Lee et al., 2017). BALB/c mice are widely used owing to their easier access and better cost-efficiency in some countries than those of CBA/J mice. Previously, we reported the predictive capacity of LLNA: BrdU-FCM with the 22 reference substances listed in OECD TG 429 using the final protocol version 1.3 (Kim et al., 2016; Ahn et al., 2016), and the results showed 87.5% sensitivity, 100% specificity, and 90.9% accuracy compared with the original LLNA. The data presented are

considered to meet the criteria for the performance standard, and its predictive capacity was also sufficiently validated. In this study, we demonstrate an enhancement in the predictive capacity of LLNA: BrdU-FCM by evaluating 20 test substances whose skin sensitization potentials were evaluated by LLNA: DA and LLNA: BrdU-ELISA but not listed in OECD TG 429.

2. Materials and methods

2.1. Test substances

A total of 20 test substances were selected to include 16 sensitizers and 4 non-sensitizers (Table 1). All of these were selected based on published information about the LLNA: DA and LLNA BrdU-ELISA Test Method Evaluation Report (ICCVAM, 2010a; ICCVAM, 2010b). The 20 test substances were purchased from Sigma-Aldrich (St. Louis, MO, USA). The details of the 20 test substances are presented in Table 1.

Table 1

List of the test substances selected for the predictive capacity evaluation.

No.	Chemical name	Abb.	CAS No.	EC3 (%) ^a	0.5X - 2.0X EC3 ^a	LLNA ^a	Human	Form	Vendor	Purity	S/N
1	<i>p</i> -Benzoquinone	BQ	106-51-4	0.01	0.005–0.02	+	NA	Solid	Sigma-Aldrich	98%	S
2	Diphenylcyclopropenone	DPCP	886-38-4	0.05 ^d	0.025–0.1	+	+	Solid	Sigma-Aldrich	98%	S
3	Glutaraldehyde	GA	111-30-8	0.08	0.04–0.16	+	+	Liquid	Sigma-Aldrich	98%	S
4	Propyl gallate	PG	121-79-9	0.32	0.16–0.64	+	+	Solid	Sigma-Aldrich	98%	S
5	4-Methylaminophenol sulfate	MAPS	55-55-0	0.8	0.4–1.6	+	+	Solid	Sigma-Aldrich	99%	S
6	Cinnamic aldehyde	CIN	104-55-2	1.9	0.95–3.8	+	+	Liquid	Sigma-Aldrich	95%	S
7	Cyclamen aldehyde	CA	103-95-7	1.91	0.955–3.82	+	+	Liquid	Sigma-Aldrich	95%	S
8	3-Aminophenol	3-AP	591-27-5	3.2	1.6–6.4	+	–	Solid	Sigma-Aldrich	98%	S
9	Diethyl maleate	DEM	141-05-9	3.6	1.8–7.2	+	NA	Liquid	Sigma-Aldrich	96%	S
10	Trimellitic anhydride	TMA	552-30-7	4.71	2.355–9.42	+	+	Solid	Sigma-Aldrich	97%	S
11	Abietic acid	AA	514-10-3	11.9	5.95–23.8	+	NA	Solid	Sigma-Aldrich	75%	S
12	Hydroxycitronellal	HC	107-75-5	22.3	11.15–44.6	+	+	Liquid	Sigma-Aldrich	95%	S
13	Isopropyl myristate	IPM	110-27-0	44	22–88	+	–	Liquid	Sigma-Aldrich	98%	S
14	Linalool	LL	78-70-6	30	15–60	+	–	Liquid	Sigma-Aldrich	97%	S
15	Butyl glycidyl ether	BGE	2426-08-6	30.9	15.45–61.8	+	+	Liquid	Sigma-Aldrich	95%	S
16	Aniline	AN	62-53-3	47.5	23.75–95	+	+	Liquid	Sigma-Aldrich	99.5%	S
17	Diethyl phthalate	DEP	84-66-2	NA	NA	–	–	Liquid	Sigma-Aldrich	99.5%	N
18	Sulfanilamide	SA	63-74-1	NA	NA	–	+	Solid	Sigma-Aldrich	99%	N
19	Propylene glycol	PLG	57-55-6	NA	NA	–	–	Liquid	Sigma-Aldrich	99.5%	N
20	Glycerol	GLY	56-81-5	NA	NA	–	–	Liquid	Sigma-Aldrich	99.5%	N

CAS No., Chemical Abstracts Service; EC3, estimated concentration needed to produce a Stimulation Index of 3; LLNA, murine local lymph node assay result (i.e. TG 429); NA, not applicable; Abb., Abbreviations; S, Sensitizer; N, Non-sensitizer; +, Sensitizer; –, Non-sensitizer.

^a Data taken from ICCVAM LLNA: DA Evaluation Report (2010) and ICCVAM LLNA: BrdU-ELISA Evaluation Report (2010).

^b 25% in H₂O.

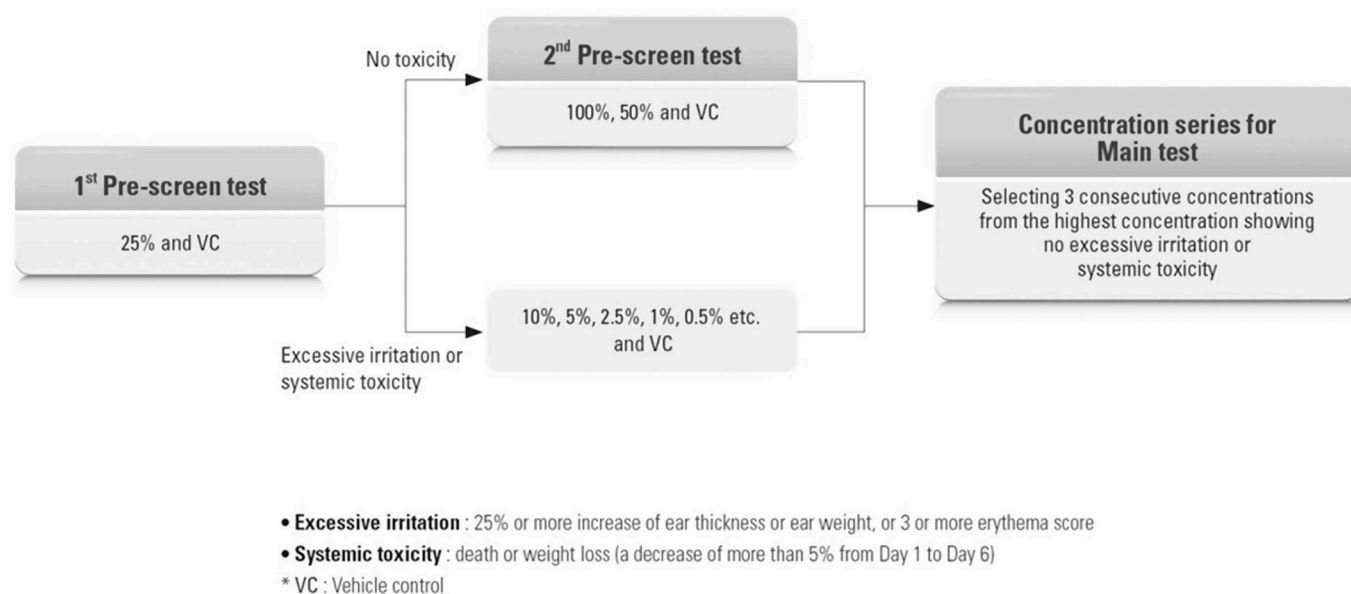


Fig. 1. Procedure for the selection of main test concentrations. Consecutive doses were selected from an appropriate concentration series, such as 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc., following OECD TG 429.

Table 2
Measurement of ear weight and thickness after treatment with 20 test substances.

No.	Chemical name	Conc.(%)	Weight (mg) of ear (mean ± SD)					Ear thickness (% of gain)				
			V	L	M	H	PC	V	L	M	H	PC
1	<i>p</i> -Benzoquinone	0.25, 0.5, 1	13.64 ± 0.15	13.90 ± 0.41	14.98 ± 1.01	14.88 ± 0.50	14.20 ± 0.53	4.1	4.1	8.7	11.2	2.2
2	Diphenylcyclopropanone	0.025, 0.05, 0.1	13.90 ± 0.33	13.76 ± 0.49	13.66 ± 0.70	14.10 ± 0.34	14.14 ± 0.40	6.1	4.8	4.0	5.2	3.8
3	Glutaraldehyde	0.25, 0.5, 1	13.70 ± 0.22	13.78 ± 0.63	13.88 ± 0.40	14.30 ± 0.27	14.26 ± 0.49	1.2	5.3	4.8	5.8	7.9
4	Propyl gallate	5, 10, 25	14.78 ± 0.39	15.08 ± 0.43	14.92 ± 0.89	19.14 ± 2.84	14.70 ± 0.78	6.7	7.2	12.2	20.9	6.2
5	4-Methylaminophenol sulfate	0.5, 1, 2.5	14.78 ± 0.39	14.98 ± 0.52	15.10 ± 0.49	15.24 ± 0.78	14.70 ± 0.78	6.7	7.5	8.5	10.0	6.2
6	Cinnamic aldehyde	25, 50, 100	13.66 ± 0.30	13.70 ± 0.46	13.86 ± 0.44	17.10 ± 3.18	14.58 ± 0.44	1.7	2.9	0.4	11.0	9.9
7	Cyclamen aldehyde	25, 50, 100	13.50 ± 0.37	13.28 ± 0.62	14.04 ± 0.57	14.98 ± 0.88	13.82 ± 0.68	4.8	4.6	9.6	15.9	6.6
8	3-Aminophenol	10, 25, 50	12.84 ± 0.45	13.42 ± 0.39	13.58 ± 0.74	13.40 ± 0.64	13.26 ± 0.24	4.3	9.4	7.8	7.7	5.8
9	Diethyl maleate	25, 50, 100	13.50 ± 0.37	13.80 ± 0.53	14.70 ± 1.38	14.74 ± 0.68	13.82 ± 0.68	4.8	8.0	12.9	11.6	6.7
10	Trimellitic anhydride	1, 2.5, 5	13.90 ± 0.33	13.82 ± 0.39	14.76 ± 0.62	15.62 ± 0.39	14.14 ± 0.40	6.1	5.6	5.0	14.0	3.7
11	Abietic acid	10, 25, 50	12.84 ± 0.45	12.30 ± 0.37	12.56 ± 0.36	13.52 ± 0.34	13.26 ± 0.24	4.3	7.0	5.5	6.8	5.8
12	Hydroxycitronellal	10, 25, 50	13.66 ± 0.18	13.76 ± 0.55	13.90 ± 0.17	13.68 ± 0.62	14.14 ± 0.47	5.1	1.0	2.4	2.8	3.2
13	Isopropyl myristate	25, 50, 100	13.66 ± 0.18	13.58 ± 0.31	13.90 ± 0.49	14.82 ± 0.70	14.14 ± 0.47	5.1	4.5	4.5	8.7	0.3
14	Linalool	25, 50, 100	13.64 ± 0.15	13.66 ± 0.34	13.84 ± 0.66	13.92 ± 0.51	14.20 ± 0.53	2.5	3.0	1.2	1.1	2.4
15	Butyl glycidyl ether	25, 50, 100	13.58 ± 0.65	13.62 ± 0.33	13.46 ± 0.43	13.40 ± 0.43	14.32 ± 0.37	0.8	4.9	5.1	2.3	7.5
16	Aniline	25, 50, 100	13.58 ± 0.65	13.44 ± 0.32	13.34 ± 0.50	13.40 ± 0.75	14.32 ± 0.37	0.8	4.0	5.3	3.6	7.4
17	Diethyl phthalate	25, 50, 100	13.66 ± 0.30	13.46 ± 0.72	13.46 ± 0.53	13.28 ± 0.38	14.58 ± 0.44	1.7	3.2	7.7	3.7	9.9
18	Sulfanilamide	10, 25, 50	13.88 ± 0.47	14.14 ± 0.83	13.96 ± 0.40	14.42 ± 0.61	15.64 ± 0.59	1.0	1.3	1.9	0.6	15.3
19	Propylene glycol	25, 50, 100	13.70 ± 0.22	13.68 ± 0.19	13.76 ± 0.30	13.68 ± 0.54	14.26 ± 0.49	1.2	1.5	4.3	5.5	7.9
20	Glycerol	25, 50, 100	13.88 ± 0.47	13.64 ± 0.15	13.82 ± 0.56	13.44 ± 0.36	15.64 ± 0.59	1.0	1.0	0.6	5.5	15.3

SD, standard deviation; V, vehicle control; L, low dose; M, middle dose; H, high dose; PC, positive control.

* Results of ear weight on day 6.

* Ear thickness increases in percent on day 6 compared to day 1.

Vehicles replaced in the original LLNA were selected (OECD TG 429, 2010a). Acetone, olive oil, *N,N*-dimethylformamide (DMF), methyl ethyl ketone (MEK), dimethyl sulfoxide (DMSO), and BrdU were purchased from Sigma-Aldrich. Fetal bovine serum was purchased from Gibco (Gaithersburg, MD, USA). Biopsy punches (6-mm) were purchased from Miltex (York, PA, USA). All other reagents were obtained from Sigma-Aldrich and BD Biosciences (Franklin Lakes, NJ, USA).

2.2. Experimental methods

2.2.1. Mice

Female BALB/c mice (7 weeks old) were purchased from Koatech (Pyeongtaek, Korea) and acclimated for at least 5 days before the experiments. The animals were housed at a temperature of 22 ± 3 °C and

a relative humidity of 30–70% with a 12-h photoperiod in an animal facility at the Korea Ministry of Food and Drug Safety (MFDS; Certification number: 1501MFDS08). The mice were allowed free access to a solid pelletized diet and sterilized drinking water. They were randomized according to body weight into various test groups. All animal experiments were performed in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International Animal Care Policies (Accredited Unit, MFDS: Unit No. 001492).

2.2.2. Pre-screen tests

Pre-screen tests for LLNA: BrdU-FCM were performed as previously described by Ahn et al. (2016). In the pre-screen tests, the solubility of each test substance was tested, and their maximum soluble

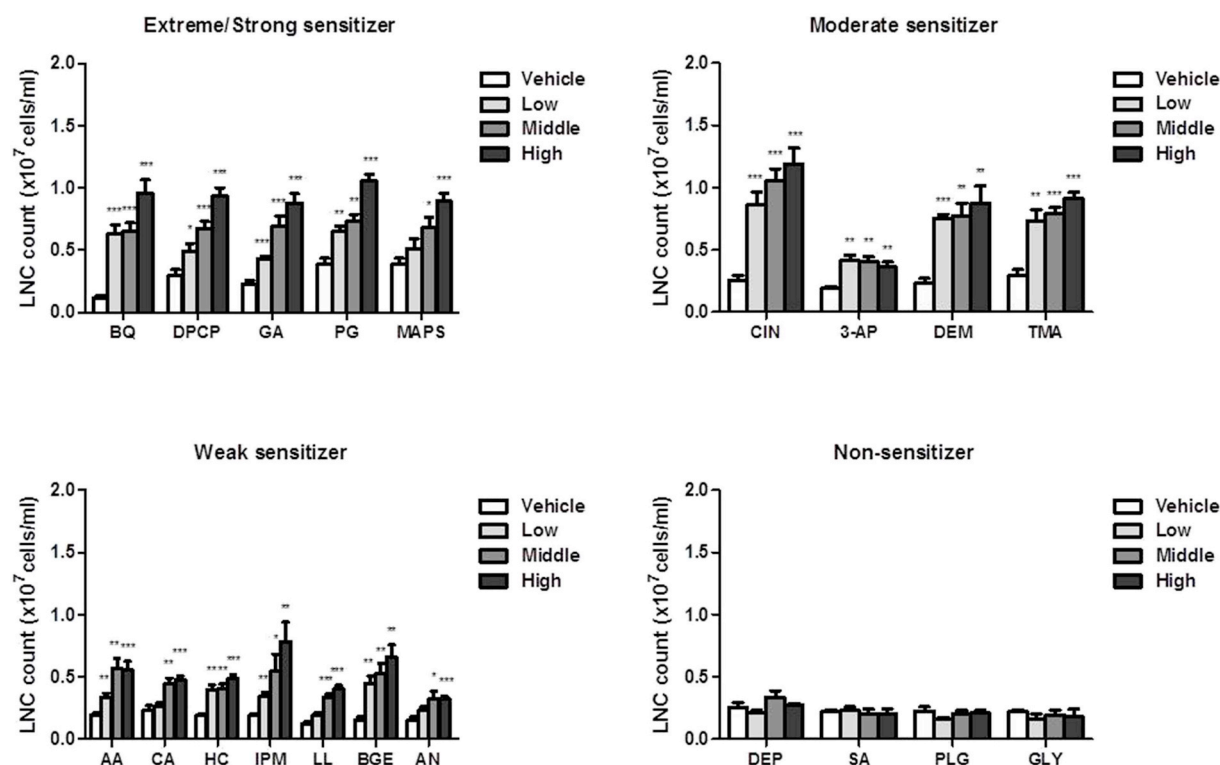


Fig. 2. Changes in the number of lymph node cells (LNCs) after treatment with 20 test substances. Each substance was topically applied to the dorsal skin of the ears of mice on days 1, 2, and 3. After treatment, auricular LNs were collected on day 6, mashed, and LNCs were obtained. After staining them with trypan blue, the number of LNCs was counted by a hemocytometer. Values are the mean $\pm$ standard deviation from 5 mice per group (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus vehicle control; Student's t -test). benzoquinone (BQ), cinnamic aldehyde (CIN), cyclamen aldehyde (CA), diethyl maleate (DEM), hydroxycitronellal (HC), isopropyl myristate (IPM), linalool (LL), butyl glycidyl ether (BGE), aniline (AN), and diethyl phthalate (DEP), diphenylcyclopropanone (DPCP), glutaraldehyde (GA), 3-aminophenol (3-AP), trimellitic anhydride (TMA), abietic acid (AA), sulfanilamide (SA), propylene glycol (PLG), and glycerol (GLY), propyl gallate (PG), and 4-methylaminophenol sulfate (MAPS).

concentrations were determined. Magnetic stirring for 30–60 min was applied to completely dissolve test substances, and the solubility was checked through visual inspection and/or microscopic examination. The vehicle showing the maximum solubility for each test compound was selected among AOO (acetone: olive oil [4:1, v/v]), DMF, MEK, and DMSO. Vehicles that dissolved the test substances completely without forming suspensions, layers, or precipitates were selected. If more than one vehicle was selected, they were prioritized in the order of AOO, DMF, MEK, and DMSO. The first pre-screen tests were performed at 25% concentration. If no toxicity or irritation was found at 25%, only 50% and 100% concentrations were needed in the second test. If toxicity or irritation was found at 25%, the concentrations were lowered to 10%, 5%, 2.5%. If toxicity or irritation was found at 10%, 5%, 2.5%, the concentrations were lowered to 1%, 0.5%, etc. Concentrations of the second pre screen test were tested in a sequential manner. Consecutive doses are normally selected from an appropriate concentration series such as 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc. The following observations were regarded as systemic toxicity or severe skin irritation: an erythema score of over 2, a decrease in body weight by more than 5%, an increase in ear weight or thickness by more than 25% on day 6 compared with day 1, or death during the study.

2.2.3. Application of test substances and injection of BrdU solution

Test concentrations for the main test were determined through the pre-screen tests. On days 1, 2, and 3, 25 μ l of the test substances, vehicle control or the positive control (25% hexyl cinnamic aldehyde in AOO), was applied to the dorsal skin of each ear at a designated time in the

morning. After these substances were fully absorbed within the skin, the mice were moved back to their cages. Test solutions were prepared fresh daily before application. On day 5, the mice were intraperitoneally injected with 100 μ l of BrdU solution (20 mg/ml).

2.2.4. Observation of general symptoms and erythema

General symptoms were observed and recorded daily. Erythema in the treated portions of the skin was scored in accordance with the Draize test method each day prior to the application of test substances. Ear thickness was measured at the center of both ears using a thickness gauge on days 1, 3, and 6. The body weight was measured on days 1 and 6.

2.2.5. Preparation of LNCs and FCM analysis

On day 6, all mice were sacrificed, and their auricular LNs were excised and weighed. Additionally, central parts of both ears were isolated using a 6-mm biopsy punch and weighed. Excised right and left LNs were placed into a cell strainer (BD Biosciences) on a 6-well plate, filled with cold phosphate-buffered saline (PBS) and mashed with a spatula to prepare LNCs. The LNCs were diluted to an appropriate volume and counted using a hemocytometer after staining with trypan blue (Sigma-Aldrich). The LNCs (1.5×10^6 /mL) were filled in tubes and washed with PBS by centrifugation ($300 \times g$) for 7 min at 4 $^{\circ}$ C. After washing, the cells were fixed and permeabilized with Cytoperm Permeabilization Buffer Plus, according to the instructions provided with the BrdU flow kit (BD Pharmingen, Franklin Lakes, NJ, USA). After repeat washing, the cells were treated with DNase at 37 $^{\circ}$ C in a water

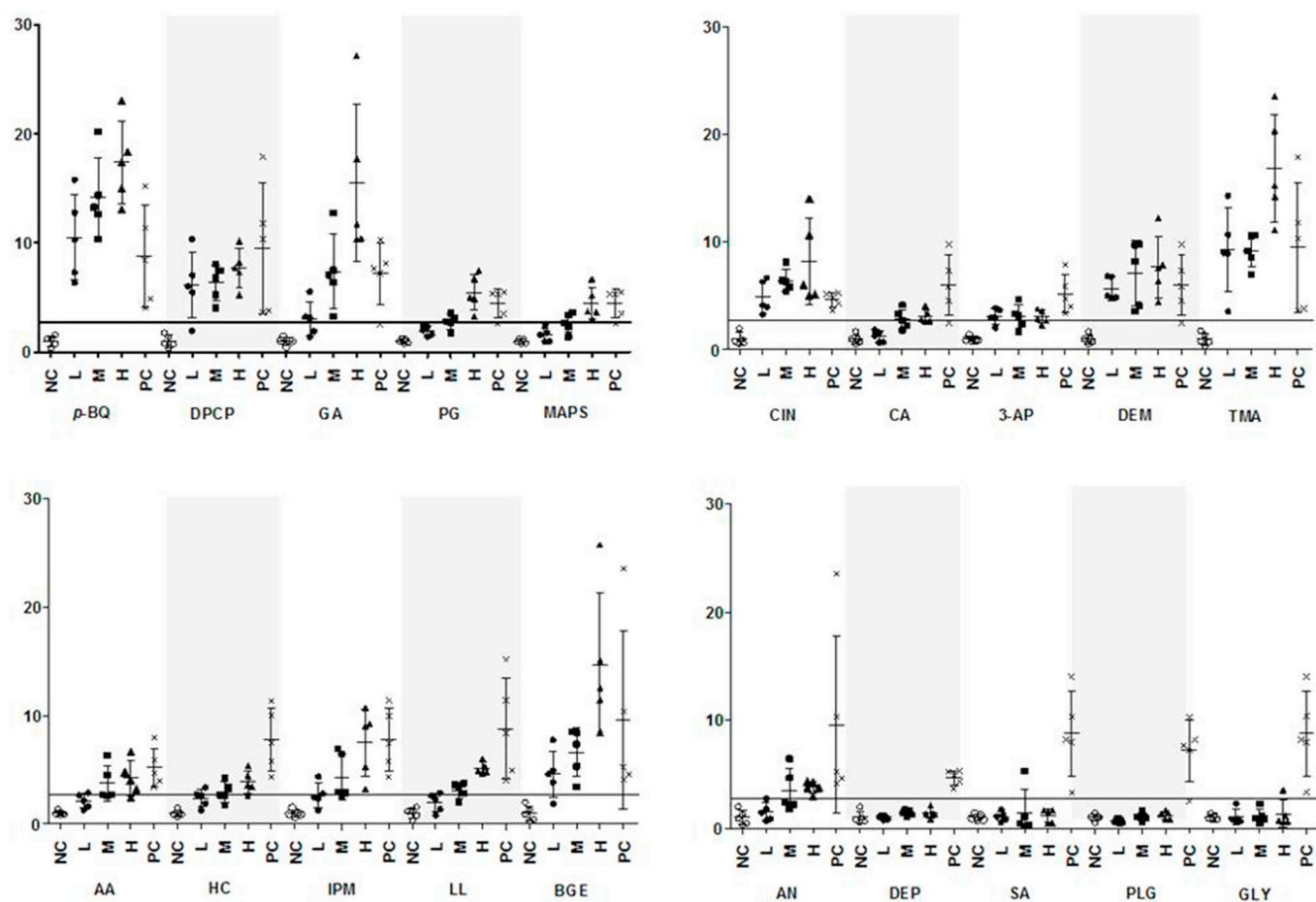


Fig. 3. Stimulation index values of the 20 test substances. Predictive capacity was evaluated using 5 BALB/c mice for each concentration. Symbols are ○ for vehicle control, ● for low concentration, ■ for middle concentration, ▲ for high concentration, and X for positive control.

bath for 1 h to expose BrdU-positive LNCs. After washing, the cells were stained with a FITC-conjugated anti-BrdU antibody for 20 min at room temperature in the dark. Then, the LNCs were transferred to an acquisition tube and resuspended in 7-AAD solution for DNA labeling. Viable LNCs were counted and a total of 10,000 gated cells were analyzed using BD FACS Calibur™ flow cytometry (BD Biosciences), as previously described (Jung et al., 2010, 2012; Yang et al., 2015; Ahn et al., 2016).

2.2.6. Calculation of stimulation indexes (SIs)

The number of BrdU-positive LNCs in each group was obtained by multiplying the number of LNCs by the ratio of cells expressing BrdU among 10,000 LNCs (obtained by flow cytometry). Individual SIs were calculated by dividing the number of BrdU-positive LNCs in the test group by the mean number of BrdU-positive LNCs in the vehicle control group. Results for each group are expressed as the mean SI. A test substance was categorized as a skin sensitizer when its SI was ≥ 2.7 . When the SI was < 2.7 , test substances were classified as non-sensitizers. If an ECt value was below 0% or above 100%, the y-intercept value was set as 1.

Stimulation Index (SI)

$$\text{SI} = \frac{\text{Number of BrdU} - \text{positive LNCs from each mouse exposed to a test substance}}{\text{Mean number of BrdU} - \text{positive LNCs in the vehicle control group}}$$

2.3. Statistical analysis

Statistical analysis of predictive capacity focused on the concordance of predictions (sensitizer/non-sensitizer), allowing the subsequent calculation of sensitivity, specificity, and overall accuracy. Receiver operating curve (ROC) analysis set the optimal cut-off for the SI, which allowed test compounds to be categorized as sensitizers or non-sensitizers during the predictive capacity phase (Hanley and McNeil, 1982; Lin et al., 2002). Statistical analyses of the SIs were performed using Graphpad Prism software (San Diego, CA, USA). A value of $p < 0.05$ was considered statistically significant (Student's *t*-test). The EC 2.7 or 3 describes an estimated concentration needed to produce an SI of 2.7 or 3. Test substances were classified as sensitizers or non-sensitizers by skin sensitization potential (ECETOC, 2003).

Table 3
Agreement between the classification predicted on predictive capacity test and Traditional LLNA.

No.	Chemical name	Traditional LLNA ^a		LLNA: BrdU-FCM ^{d,e}										Agreement		
		Category	EC3	Vehicle	Category	Conc.(%)	SI (mean ± SD)			V			Max. SI		EC2.7	Vehicle
							L	M	H	PC						
1	<i>p</i> -Benzoquinone	Cat 1A	0.01	AOO	Cat 1A	0.25, 0.5, 1	1.00 ± 0.46	10.50 ± 3.90	14.16 ± 3.69	17.19 ± 3.94	8.76 ± 4.64	17.19	0.09 ^f	AOO	Yes	
2	Diphenylcyclopropenone	Cat 1A	0.05 ^b	NA	Cat 1A	0.025, 0.05, 0.1	1.00 ± 0.56	6.16 ± 3.02	6.33 ± 1.65	7.73 ± 1.76	9.50 ± 5.97	7.73	0.02 ^f	DMF	Yes	
3	Glutaraldehyde	Cat 1A	0.08 ^b	AOO/DMF	Cat 1A	0.25, 0.5, 1	1.00 ± 0.33	3.04 ± 1.60	7.39 ± 3.42	15.47 ± 7.20	7.16 ± 2.85	15.47	0.12 ^f	DMF	Yes	
4	Propyl gallate	Cat 1A	0.32	AOO	Cat 1B	5, 10, 25	1.00 ± 0.21	1.94 ± 0.44	2.78 ± 0.68	5.46 ± 1.64	4.44 ± 1.31	5.46	9.39	DMSO	Yes	
5	4-Methylaminophenol sulfate	Cat 1A	0.8	DMF	Cat 1A	0.5, 1, 2.5	1.00 ± 0.21	1.56 ± 0.63	2.68 ± 0.90	4.45 ± 1.48	4.44 ± 1.31	4.45	1.19	DMSO	Yes	
6	Cinnamic aldehyde	Cat 1A	1.9 ^b	AOO	Cat 1B	25, 50, 100	1.00 ± 0.58	4.89 ± 1.52	6.43 ± 1.06	8.18 ± 4.00	4.66 ± 0.68	8.18	20.56 ^f	AOO	Yes	
7	Cyclamen aldehyde	Cat 1A	22.3	AOO	Cat 1B	25, 50, 100	1.00 ± 0.45	1.23 ± 0.52	2.84 ± 0.92	3.12 ± 0.62	6.00 ± 2.77	3.12	71.71	AOO	Yes	
8	3-Aminophenol	Cat 1B	3.2	AOO	Cat 1B	10, 25, 50	1.00 ± 0.21	3.11 ± 0.71	3.06 ± 1.12	3.05 ± 0.62	5.20 ± 1.79	3.11	31.31 ^f	DMF	Yes	
9	Diethyl maleate	Cat 1B	3.6 ^b	AOO	Cat 1B	25, 50, 100	1.00 ± 0.45	5.66 ± 1.05	7.10 ± 3.04	7.70 ± 2.87	6.00 ± 2.77	7.70	20.43 ^f	AOO	Yes	
10	Trimellitic anhydride	Cat 1B	4.71 ^c	NA	Cat 1A	1, 2.5, 5	1.00 ± 0.56	9.30 ± 3.88	9.20 ± 1.51	16.91 ± 4.99	9.50 ± 5.97	16.91	0.51 ^f	DMF	Yes	
11	Abietic acid	Cat 1B	11.9 ^c	AOO	Cat 1B	10, 25, 50	1.00 ± 0.21	2.13 ± 0.66	3.78 ± 1.63	4.23 ± 1.61	5.20 ± 1.79	4.23	14.47	DMF	Yes	
12	Hydroxycitronellal	Cat 1B	22.3	AOO	Cat 1B	10, 25, 50	1.00 ± 0.31	2.39 ± 0.83	2.93 ± 0.93	3.89 ± 1.05	7.80 ± 2.91	3.89	18.56	AOO	Yes	
13	Isopropyl myristate	Cat 1B	44	AOO	Cat 1B	25, 50, 100	1.00 ± 0.31	2.65 ± 1.13	4.34 ± 2.16	7.50 ± 3.12	7.80 ± 2.91	7.50	25.33	AOO	Yes	
14	Linalool	Cat 1B	30 ^b	AOO	Cat 1B	25, 50, 100	1.00 ± 0.46	1.99 ± 0.87	3.10 ± 0.70	5.14 ± 0.55	8.79 ± 4.64	5.14	41.34	AOO	Yes	
15	Butyl glycidyl ether	Cat 1B	30.9	AOO	Cat 1B	25, 50, 100	1.00 ± 0.69	4.60 ± 2.13	6.63 ± 2.19	14.70 ± 6.61	9.58 ± 8.19	14.70	15.45	AOO	Yes	
16	Aniline	Cat 1B	47.5 ^b	AOO	Cat 1B	25, 50, 100	1.00 ± 0.69	1.51 ± 0.82	3.51 ± 1.97	3.81 ± 0.53	9.58 ± 8.19	3.81	49.39	AOO	Yes	
17	Diethyl phthalate	NC	NA	AOO	NC	25, 50, 100	1.00 ± 0.58	0.99 ± 0.20	1.42 ± 0.25	1.39 ± 0.45	4.66 ± 0.68	1.42	NA	AOO	Yes	
18	Sulfanilamide	NC	NA	DMF	NC	10, 25, 50	1.00 ± 0.27	1.07 ± 0.44	1.45 ± 2.16	1.16 ± 0.66	8.77 ± 3.90	1.45	NA	DMF	Yes	
19	Propylene glycol	NC	NA	Water	NC	25, 50, 100	1.00 ± 0.33	0.66 ± 0.25	1.05 ± 0.38	1.19 ± 0.38	7.16 ± 2.85	1.19	NA	DMF	Yes	
20	Glycerol	NC	NA	DMF	NC	25, 50, 100	1.00 ± 0.27	1.04 ± 0.71	1.13 ± 0.69	1.32 ± 1.25	8.77 ± 3.90	1.32	NA	DMF	Yes	
21	CMI/MI	Cat1A	0.01	DMF	Cat1A	2.5, 5, 10	1.00 ± 0.32	6.62 ± 3.45	9.02 ± 1.85	14.07 ± 1.77	6.24 ± 1.44	14.07	1.06 ^f	DMF	Yes	
22	DNCB	Cat1A	0.05	AOO	Cat1A	0.125, 0.25, 0.5	1.00 ± 0.39	15.57 ± 4.25	38.97 ± 6.12	47.29 ± 9.45	9.01 ± 1.75	47.29	0.02 ^f	AOO	Yes	
23	4-Phenylethylenediamine	Cat1A	0.11	AOO	Cat1A	0.5, 1, 2.5	1.00 ± 0.32	3.24 ± 1.34	6.48 ± 1.48	10.02 ± 3.26	6.24 ± 1.44	10.02	0.10	DMF	Yes	
24	Cobalt chloride	Cat1A	0.6	DMSO	Cat1A	0.25, 0.5, 1	1.00 ± 0.25	2.60 ± 1.00	7.80 ± 4.06	12.83 ± 6.03	11.86 ± 3.15	12.83	0.20	DMF	Yes	
25	Isoeugenol	Cat1A	1.5	AOO	Cat1A	5, 10, 25	1.00 ± 0.32	7.66 ± 1.33	19.30 ± 4.37	34.91 ± 11.73	5.42 ± 1.48	34.91	1.2 ^f	AOO	Yes	
26	2-MBT	Cat1A	1.7	DMF	NC	5, 10, 25	1.00 ± 0.25	1.44 ± 0.58	1.13 ± 0.27	1.30 ± 0.50	11.86 ± 3.15	1.44	NA	DMF	No	
27	Citral	Cat1B	9.2	AOO	Cat1B	10, 25, 50	1.00 ± 0.32	1.98 ± 1.02	5.05 ± 1.85	8.88 ± 4.43	5.42 ± 1.48	8.88	13.1	AOO	Yes	
28	HCA	Cat1B	9.7	AOO	Cat1B	5, 10, 25	1.00 ± 0.27	1.14 ± 0.40	1.75 ± 0.95	4.34 ± 0.74	6.50 ± 3.78	4.34	15.1	AOO	Yes	
29	Eugenol	Cat1B	10.1	AOO	Cat1B	5, 10, 25	1.00 ± 0.38	0.71 ± 0.19	2.01 ± 0.75	3.93 ± 0.74	3.89 ± 3.28	3.93	16.5	AOO	Yes	
30	Phenyl benzoate	Cat1B	13.6	AOO	Cat1B	10, 25, 50	1.00 ± 0.34	4.06 ± 1.39	5.64 ± 2.28	3.19 ± 0.60	3.06 ± 0.79	5.64	5.5 ^f	DMF	Yes	
31	Cinnamic alcohol	Cat1B	21	AOO	Cat1B	10, 25, 50	1.00 ± 0.38	0.49 ± 0.15	2.29 ± 0.89	2.78 ± 0.65	3.89 ± 3.28	2.78	44.3	AOO	Yes	
32	Imidazolidinyl urea	Cat1B	24	DMF	Cat1B	10, 25, 50	1.00 ± 0.35	1.05 ± 0.37	2.11 ± 1.04	4.10 ± 1.52	12.27 ± 4.27	4.10	32.0	DMF	Yes	
33	Methyl methacrylate	Cat1B	90	AOO	NC	25, 50, 100	1.00 ± 0.27	0.57 ± 0.10	0.65 ± 0.19	0.87 ± 0.34	6.50 ± 3.78	0.87	NA	AOO	No	
34	Chlorobenzene	NC	NA	AOO	NC	10, 25, 50	1.00 ± 0.39	1.12 ± 0.49	1.25 ± 0.36	1.67 ± 0.23	9.01 ± 1.75	1.67	NA	AOO	Yes	
35	Isopropanol	NC	NA	AOO	NC	25, 50, 100	1.00 ± 0.32	0.86 ± 0.29	0.83 ± 0.24	0.89 ± 0.27	5.42 ± 1.48	0.89	NA	AOO	Yes	
36	Lactic acid	NC	NA	DMSO	NC	10, 25, 50	1.00 ± 0.25	1.15 ± 0.32	1.18 ± 0.16	1.28 ± 0.50	11.86 ± 3.15	1.28	NA	DMF	Yes	

(continued on next page)

Table 3 (continued)

No.	Chemical name	Traditional LLNA ^a		LLNA: BrdU-FCM ^{d,e}		SI (mean ± SD)					Max. SI	EC2.7	Vehicle	Agreement	
		Category	EC3	Vehicle	Category	Conc.(%)	V	L	M	H					PC
37	Methyl salicylate	NC	NA	AOO	NC	10, 25, 50	1.00 ± 0.38	1.77 ± 0.33	1.61 ± 0.52	1.14 ± 0.27	3.89 ± 3.28	1.77	NA	AOO	Yes
38	Salicylic acid	NC	NA	AOO	NC	1, 2.5, 5	1.00 ± 0.35	1.76 ± 0.49	2.26 ± 0.33	2.57 ± 0.22	12.27 ± 4.27	2.57	NA	DMF	Yes
39	Sodium lauryl sulfate	Cat1B	8.1	DMF	Cat1B	1, 2.5, 5	1.00 ± 0.45	0.94 ± 0.59	1.22 ± 0.68	1.60 ± 0.78	3.95 ± 1.48	1.60	5.2	DMSO	Yes
40	EGD	Cat1B	28	MEK	Cat1B	25, 50, 100	1.00 ± 0.82	1.82 ± 0.79	1.64 ± 0.56	2.85 ± 0.95	8.35 ± 4.32	2.85	97.2	AOO	Yes
41	Xylene	Cat1B	95.8	AOO	Cat1B	25, 50, 100	1.00 ± 0.82	0.90 ± 0.30	2.15 ± 0.63	6.99 ± 2.13	8.35 ± 4.32	6.99	34.1	AOO	Yes
42	Nickel chloride	NC	NC	DMSO	NC	0.5, 1, 2.5	1.00 ± 0.45	1.09 ± 0.49	1.47 ± 0.53	2.14 ± 0.40	3.95 ± 1.48	2.14	NA	DMSO	Yes

AOO, acetone; olive oil (4:1); CMI/MI, 5-Chloro-2-methyl-4-isothiazolin-3-one/2-methyl-4-isothiazolin-3-one; DMF, N,N-dimethylformamide; DMSO, dimethyl sulfoxide; DNCB, 2,4-dinitrochlorobenzene; MBT, Mercaptobenzothiazole; HCA, hexyl cinnamic aldehyde; EGD, Ethylene glycol dimethacrylate; SD, standard deviation; SI, stimulation index; V, vehicle control; L, low dose; M, middle dose; H, high dose; PC, positive control; NC, No Category; NA, Not Applicable.

^a Data taken from ICCVAM Test Method Evaluation Report (2011).

^b Data taken from ICCVAM LLNA: BrdU-ELISA Evaluation Report (2010).

^c Data taken from ICCVAM LLNA: DA Evaluation Report (2010).

^d Data taken from Ahn et al. (2016) (No.21–38).

^e Data taken from Ha et al. (2017) (No.39–42).

^f Set y-axis = 1 because an EC2.7 value is below 0% or above 100%.

3. Results

3.1. Results of the pre-screen tests for 20 substances

The 20 test substances (ICCVAM, 2010a; ICCVAM, 2010b) were selected to evaluate their predictive capacity and listed in Table 1. We selected 20 test substances, including 16 sensitizers and 4 non-sensitizers. First, we selected an appropriate solvent. In the vehicle selection test, AOO was chosen as the vehicle for p-benzoquinone (BQ), cinnamic aldehyde (CIN), cyclamen aldehyde (CA), diethyl maleate (DEM), hydroxycitronellal (HC), isopropyl myristate (IPM), linalool (LL), butyl glycidyl ether (BGE), aniline (AN), and diethyl phthalate (DEP). DMF was selected as the vehicle for diphenylcyclopropanone (DPCP), glutaraldehyde (GA), 3-aminophenol (3-AP), trimellitic anhydride (TMA), abietic acid (AA), sulfanilamide (SA), propylene glycol (PLG), and glycerol (GLY). DMSO was selected as the vehicle for propyl gallate (PG) and 4-methylaminophenol sulfate (MAPS). The pre-screen tests were performed to determine concentrations of the substances to be used in the main test (Fig. 1). The main test concentrations without systemic toxicity or severe irritation were selected through pre-screen tests conducted in 2 phases (Table 2).

3.2. Changes in LNC count

Using the 3 concentrations selected through the pre-screen tests, the sensitization potential of 20 test substances was evaluated with LLNA: BrdU-FCM in BALB/c mice. The ear weight and thickness were measured. Results of ear weight and thickness were presented in Table 2. The auricular lymph nodes were weighed and LNCs were counted. LN weights in the group treated with BQ, DPCP, GA, PG, MAPS, CLN, CA, 3-AP, DEM, TMA, AA, HC, IPM, LL, BGE, and AN were higher than those in the vehicle control group (data not shown). The number of LNCs significantly increased in the group treated with sensitizers. A dose-dependent increase was observed in cases of BQ, DPCP, GA, PG, MAPS, CLN, DEM, TMA, CA, HC, IPM, LL, and BGE. However, there was no change in the number of LNCs after treatment with DEP, SA, PLG, and GLY (Fig. 2).

3.3. SI and predictive capacity

In the main study, SIs were calculated based on the total number of LNCs and BrdU incorporation in LNCs (Figs. 2 and 3). The optimal SI threshold was determined by statistically analyzing the results of predictive capacity evaluation (Kim et al., 2016; Yang et al., 2015). When the cut-off SI value was set at 2.7, LLNA: BrdU-FCM could successfully identify all 16 sensitizers and 4 non-sensitizers out of the 20 test substances (Table 3). Table 4 shows the results of human, guinea pig, conventional LLNA, LLNA: BrdU-FCM, LLNA: DA, and LLNA: BrdU-ELISA analysis for 20 test substances and all 42 test substances, sorted according to their sensitization potentials. Considering the predictive capacity of LLNA: BrdU-FCM with other LLNA methods or human classification, Cooper statistics were applied to the 20 test substances (Table 5). When compared with the results of human classification, sensitivity was 90%, specificity was 50%, and overall accuracy was 75%. Compared with the results of variants of the LLNA, sensitivity was 100%, specificity was 100%, and overall accuracy was 100%. In a previous study, concordance analysis for binary decision (sensitizer/non-sensitizer) for 22 reference substances listed in OECD TG 429 revealed 91% accuracy compared with conventional LLNA, 73% accuracy compared with the human classification, 81% accuracy compared with LLNA: DA, and 89% accuracy compared with LLNA: BrdU-ELISA (Ahn et al., 2016; Ha et al., 2017). Taking 22 reference substances listed in OECD TG 429 and 20 additional substances together, the concordance of LLNA: BrdU-FCM with human data was 74% (28/38), 95% (40/42) with conventional LLNA, 94% (33/35) with LLNA: BrdU-ELISA, and 88% (29/33) with LLNA: DA.

Table 4

Comparison of the predictive capacities of the LLNA, the LLNA: DA, the LLNA: BrdU-ELISA, and the LLNA: BrdU-FCM for the 42 test substances.

No.	Chemical name	Human/Guinea pig ^a	LLNA ^a	LLNA: BrdU-FCM	LLNA: DA ^b	LLNA: BrdU-ELISA ^c
1	<i>p</i> -Benzoquinone	NA/+	+	+	+	+
2	Diphenylcyclopropenone	+ /NA	+	+	NA	+
3	Glutaraldehyde	+ / +	+	+	+	+
4	Propyl gallate	+ / +	+	+	+	NA
5	4-Methylaminophenol sulfate	+ / +	+	+	NA	+
6	Cinnamic aldehyde	+ / +	+	+	+	+
7	Cyclamen aldehyde	- /NA	+	+	NA	+
8	3-Aminophenol	NA/+	+	+	+	+
9	Diethyl maleate	+ /NA	+	+	+	+
10	Trimellitic anhydride	NA/+	+	+	+	+
11	Abietic acid	NA/+	+	+	+	NA
12	Hydroxycitronellal	+ / +	+	+	+	+
13	Isopropyl myristate	- /NA	+	+	NA	+
14	Linalool	- /NA	+	+	NA	+
15	Butyl glycidyl ether	+ / +	+	+	+	NA
16	Aniline	+ / +	+	+	NA	+
17	Diethyl phthalate	- / -	-	-	-	-
18	Sulfanilamide	+ / -	-	-	-	-
19	Propylene glycol	- / -	-	-	NA	-
20	Glycerol	- / -	-	-	NA	-
21	CMI/MI	+ / +	+	+	+	+
22	DNCB	+ / +	+	+	+	+
23	4-Phenylenediamine	+ / +	+	+	+	+
24	Cobalt chloride	+ / +	+	+	+	+
25	Isoeugenol	+ / +	+	+	+	+
26	2-MBT	+ / +	+	-	+ ^d	+ ^d
27	Citral	+ / +	+	+	+	+
28	HCA	+ / +	+	+	+	+
29	Eugenol	+ / +	+	+	+	+
30	Phenyl benzoate	+ / +	+	+	+	+
31	Cinnamic alcohol	+ / +	+	+	+	+
32	Imidazolidinyl urea	+ / +	+	+	+	+ ^d
33	Methyl methacrylate	+ / +	+	-	+ ^d	NA
34	Chlorobenzene	- / -	-	-	+	NA
35	Isopropanol	+ / -	-	-	-	-
36	Lactic acid	- / -	-	-	-	+
37	Methyl salicylate	- / -	-	-	-	-
38	Salicylic acid	- / -	-	-	+ ^d	-
39	Sodium lauryl sulfate	- /	+	+	+	+
40	EGD	+ / -	+	+	+	+
41	Xylene	- /NA	+	+	NA	NA
42	Nickel chloride	+ / +	-	-	-	NA

NA, not available; CMI/MI, 5-Chloro-2-methyl-4-isothiazolin-3-one/2-methyl-4-isothiazolin-3-one; DNCB, 2,4-dinitrochlorobenzene; MBT, Mercaptobenzothiazole; HCA, hexyl cinnamic aldehyde; EGD, Ethylene glycol dimethacrylate; +, Sensitizer; -, Non-sensitizer.

^a Baskett et al., 2001 and ICCVAM Test Method Evaluation Report (2009, 2010).

^b ICCVAM, 2010a.

^c ICCVAM, 2010b.

^d Borderline value.

3.4. Evaluation of cut-off values

A previous study showed that the optimum cut-off value of the test method is 2.7 (Ahn et al., 2016). In addition, the area under curve (AUC) of ROC was 0.877 for 18 reference substances listed in OECD TG 429 (Ahn et al., 2016). ROC curve compare sensitivity versus specificity across a range of values to predict a dichotomous outcome. Area under the ROC curve is another measure of test performance (Florkowski, 2008). ROCs are considered excellent, good, fair, poor, and failures when the AUCs were 0.91, 0.80.9, 0.70.8, 0.60.7, and 0.50.6, respectively (El Khoul et al., 2009). ROC analysis of results of the 42 test substances was conducted (Fig. 4). The AUC of the ROC was 0.96 for 42 test substances, which is higher than 0.877 calculated for 18 reference substances listed in OECD TG 429. Moreover, the highest accuracy was produced at $2.6 \leq SI < 2.8$, which includes 2.7, further supporting

that the predictive capacity was optimized at the cut-off value of 2.7.

4. Discussion

The LLNA is a validated method for evaluating the skin sensitization potential of chemicals. Similar to conventional LLNA, LLNA: DA, and LLNA: BrdU-ELISA, the LLNA: BrdU-FCM method was developed to evaluate the potential of skin sensitization. LLNA: BrdU-FCM measures the proliferation of auricular LNCs during the induction phase of skin sensitization and the number of BrdU-positive LNCs using flow cytometry.

The LLNA methods address refinement among the 3Rs (Russell and Burch, 1959) as it does not use an adjuvant in the traditional skin sensitization test in guinea pigs. In general, before the initiation of LLNA tests, a pre-screen test is required to obtain toxicological

Table 5
Cooper's statistics on LLNA: BrdU-FCM vs. LLNA, and LLNA: BrdU-FCM vs. Human data.

(A)							
		LLNA Sensitizer	Non-sensitizer	Total	Human Sensitizer	Non-sensitizer	Total
LLNA: BrdU-FCM	Sensitizer	16	0	16	9	3	12
	Non-sensitizer	0	4	4	1	3	4
	Total	16	4	20	10	6	16
	Overall accuracy: 100%				Overall accuracy: 75%		
	Sensitivity: 100%				Sensitivity: 90%		
	Specificity: 100%				Specificity: 50%		
		LLNA:DA Sensitizer	Non-sensitizer	Total	LLNA: BrdU-ELISA Sensitizer	Non-sensitizer	Total
LLNA: BrdU-FCM	Sensitizer	10	0	10	13	0	13
	Non-sensitizer	0	2	2	0	4	4
	Total	10	2	12	13	4	17
	Overall accuracy: 100%				Overall accuracy: 100%		
	Sensitivity: 100%				Sensitivity: 100%		
	Specificity: 100%				Specificity: 100%		
		LLNA Sensitizer	Non-sensitizer	Total	Human Sensitizer	Non-sensitizer	Total
LLNA: BrdU-FCM	Sensitizer	30	0	30	21	5	26
	Non-sensitizer	2	10	12	5	7	12
	Total	32	10	42	26	12	38
	Overall accuracy: 95.2%				Overall accuracy: 73.7%		
	Sensitivity: 93.8%				Sensitivity: 80.8%		
	Specificity: 100%				Specificity: 58.3%		
		LLNA:DA Sensitizer	Non-sensitizer	Total	LLNA: BrdU-ELISA Sensitizer	Non-sensitizer	Total
LLNA: BrdU-FCM	Sensitizer	23	0	23	26	0	26
	Non-sensitizer	4	6	10	2	7	9
	Total	27	6	33	28	7	35
	Overall accuracy: 87.9%				Overall accuracy: 94.3%		
	Sensitivity: 85.2%				Sensitivity: 92.9%		
	Specificity: 100%				Specificity: 100%		

OECD TG 429 22 reference substances data taken from [Ahn et al. \(2016\)](#) and [Ha et al. \(2017\)](#).

information (e.g., acute toxicity and dermal irritation), if not available. The concentrations recommended in OECD TG 429 and 442A/B (100%, 50%, 25%, 10%, 5%, 2.5%, 1%, and 0.5%) are commonly chosen. It is difficult to identify the concentration at which an unknown chemical might induce acute toxicity or dermal irritation without conducting a pre-screen test. However, if information on quantitative structure-activity relationship or irritation is available, the concentration range can be narrowed. Pre-screen tests for LLNA: BrdU-FCM were performed in 2 phases ([Figs. 1 and 5](#); [Table 6](#)). When systemic toxicity or severe skin irritation was found in a second pre-screen test, additional pre-screen tests were carried out.

In this study, we aimed to confirm the enhanced predictive capacity of LLNA: BrdU-FCM after selecting and evaluating 20 additional test substances whose sensitization potentials were evaluated previously by LLNA: DA and LLNA: BrdU-ELISA, but were not listed in OECD TG 429. The 20 test substances ([Basketter et al., 1995](#); [ICCVAM, 1999](#); [Gerberick et al., 2004 2007](#); [ICCVAM, 2010a, 2010b, 2011](#)) included 16 sensitizers and 4 non-sensitizers. The skin sensitization potentials of BQ, 3-AP, TMA, and AA were not confirmed in humans.

Skin sensitization potentials of cyclamen aldehyde, isopropyl myristate, linalool, and sulfanilamide based on human data and LLNA were inconsistent. cyclamen aldehyde, isopropyl myristate, and linalool (human non-sensitizers) were classified as sensitizers by the LLNA: BrdU-ELISA and the traditional LLNA. Cyclamen aldehyde is suggested as a non-sensitizer based on human data in the ICCVAM Test Method

Evaluation Report by the murine LLNA: BrdU-ELISA ([RIFM, 2007](#)); however, it is also classified as a human sensitizer ([Basketter et al., 2001](#); [Schneider et al., 2004](#)). This substance was suggested as a sensitizer in the performed LLNA test ([Gerberick et al., 2005](#); [ICCVAM, 2010b](#)). Isopropyl myristate is suggested as a sensitizer by LLNA ([Ryan et al., 2000](#); [Basketter et al., 2003](#); [Kimber et al., 2003](#); [Gerberick et al., 2005](#); [Urbisch et al., 2014](#)). Linalool is suggested as a non-sensitizer based on human data in the ICCVAM Test Method Evaluation Report by murine LLNA: BrdU-ELISA ([Greif et al., 1967](#); [Ryan et al., 2000](#)); however, it is also classified as a human sensitizer ([Gerberick et al., 2001](#); [Basketter et al., 2014](#)). Linalool is suggested as a sensitizer by LLNA ([Ryan et al., 2000](#); [Gerberick et al., 2004](#); [Tswjita-Inoue et al., 2014](#); [Hou et al., 2015](#)). Sulfanilamide was also predicted as a non-sensitizer by the LLNA: DA and the traditional LLNA; however, the results were discordant when compared to the positive results in a human maximization test (DSA₀₅) ([Kligman AM et al., 1966](#)). It was also suggested as a non-sensitizer by LLNA ([Basketter et al., 1999](#); [Nukada et al., 2013](#); [Yamashita et al., 2015](#); [Hou et al., 2015](#); [Yamamoto et al., 2015](#); [Ramirez et al., 2016](#); [Saito et al., 2016](#)).

The predictive capacity of LLNA: BrdU-FCM was based on the evaluation of 20 test substances ([ICCVAM, 2010a](#); [ICCVAM, 2010b](#)). First, we selected an appropriate solvent and determined test concentrations for the main test. The main test concentrations without systemic toxicity or severe irritation were selected through the pre-screen tests conducted in 2 phases. DPCP-treated mice showed systemic toxicity or

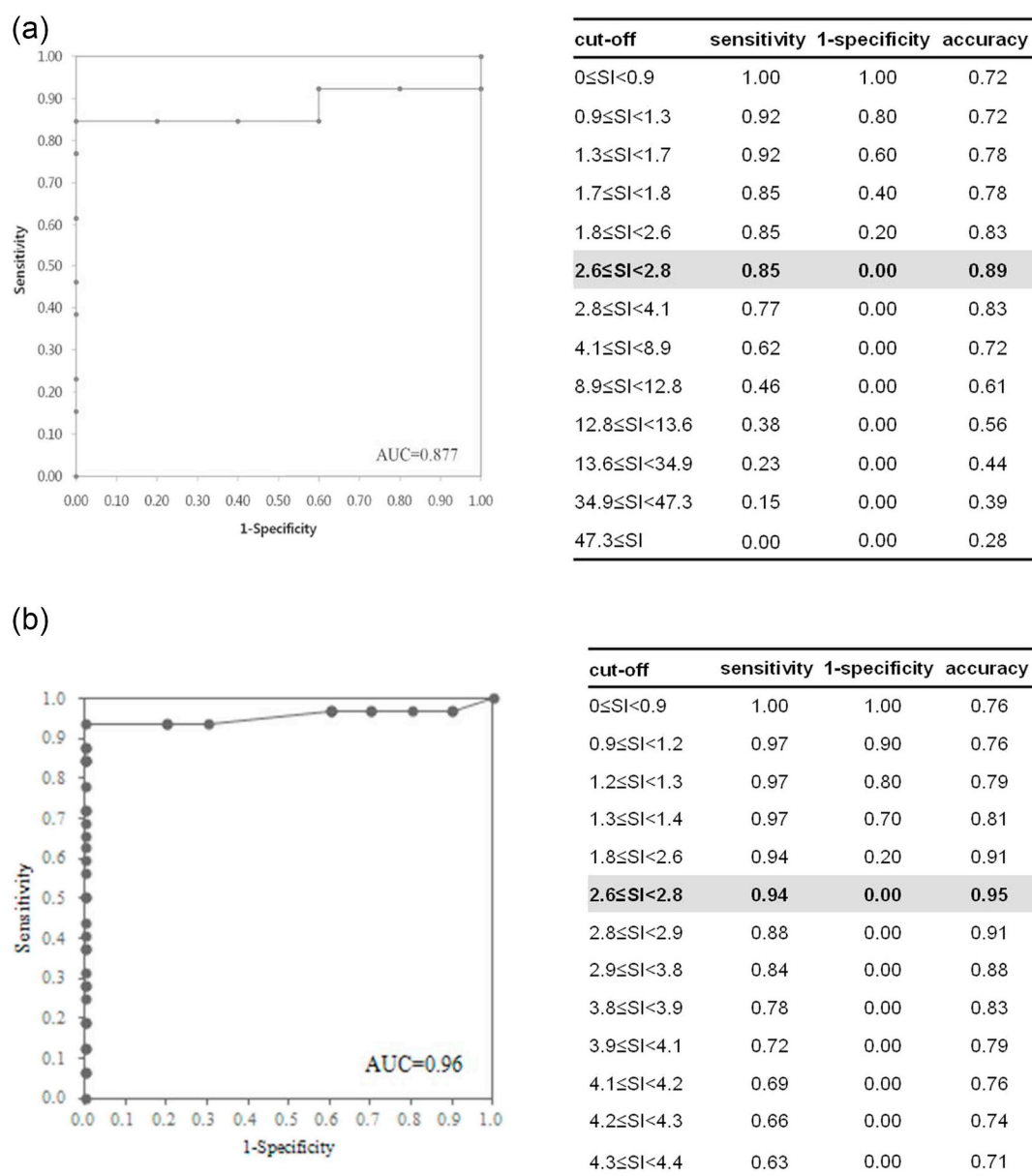


Fig. 4. Receiver operating curve (ROC) for evaluating the predictive capacity. (A) Maximum stimulation indexes (SIs) of 18 reference chemicals were used to obtain the cut-off value (AUC = 0.877). Data taken from [Ahn et al. \(2016\)](#). (B) ROC for evaluating the predictive capacity using 42 test substances, predictive capacity parameters at each cut-off (AUC = 0.96). The 18 reference chemicals were taken from [Ahn et al. \(2016\)](#), and 4 optional chemicals were based on [Ha et al. \(2017\)](#).

excessive irritation at 10, 5, 2.5, 1, 0.5, and 0.25% concentrations, whereas GA-treated mice showed systemic toxicity or excessive irritation at 10, 5, and 2.5% concentrations. Mice were found to exhibit an increase in ear thickness by more than 25% on day 6 compared with day 1 after treatment with DPCP and GA. Therefore, additional pre-screen tests were carried out.

In the main test, the number of LNCs and the proportion of BrdU incorporated into LNs were measured, and SIs were obtained according to a previous method described by [Ahn et al. \(2016\)](#). The 20 additional test substances were correctly classified as sensitizers or non-sensitizers by maintaining a cut-off of 2.7. Compared with results of the conventional LLNA and its variant methods, sensitivity was 100%, specificity was 100%, and overall accuracy was 100%. When compared with the results of human classification, sensitivity was 90%, specificity was

50%, and overall accuracy was 75%. On [Table 5](#), the specificity of the LLNA: BrdU-FCM and the LLNA methods was 100%. In addition to the LLNA: BrdU-FCM, the LLNA methods were inconsistent with the human data.

In a previous study, predictive capacity for the 22 reference chemicals listed in OECD TG 429 showed 75% sensitivity, 100% specificity, and 90.9% accuracy compared with the conventional LLNA ([Ahn et al., 2016](#); [Ha et al., 2017](#)) and 75% sensitivity, 67% specificity, and 73% accuracy compared with the results of human classification ([Ahn et al., 2016](#); [Ha et al., 2017](#)). In comparison with LLNA: DA, sensitivity was 77%, specificity was 100%, and accuracy was 81% ([Ahn et al., 2016](#); [Ha et al., 2017](#)), and 87% sensitivity, 100% specificity, and 89% accuracy in comparison with LLNA: BrdU-ELISA ([Ahn et al., 2016](#); [Ha et al., 2017](#)).

► OECD TGs 429, 442A and 442B

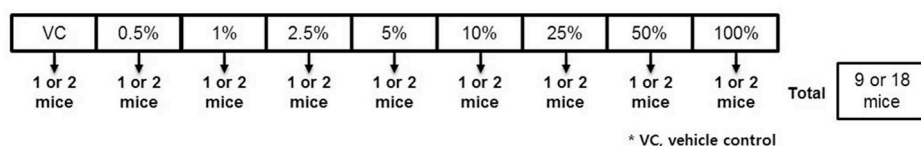


Fig. 5. Numbers of mice used for the pre-screen tests in the local lymph node assay (LLNA) (OECD TG 429), the LLNA: DA (TG 442A), the LLNA: BrdU-ELISA (TG 442B), and the LLNA: BrdU-FCM. When systemic toxicity or severe skin irritation was found in pre-screen tests, concentrations of the pre-screen test were tested in a sequential manner.

► LLNA: BrdU-FCM

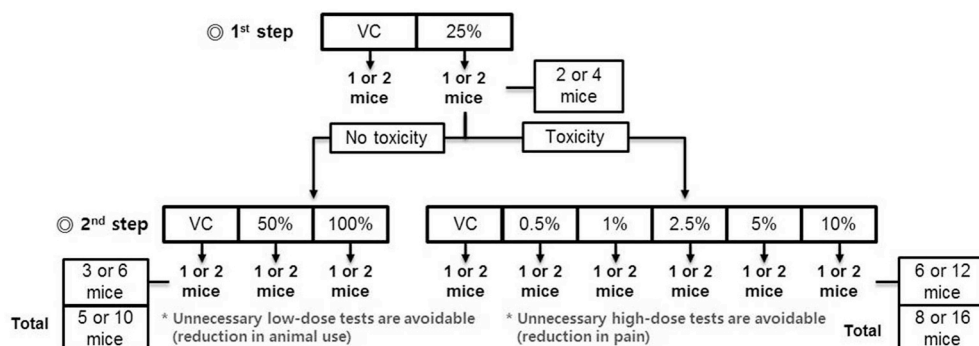


Table 6

Concentration series and number of animals used for the pre-screen test in the predictive capacity evaluation with LLNA: BrdU-FCM.

No.	Chemical name	Concentration series (%) in pre-screen test		No. of animals (n = 2 for each concentration) ^a
		1st step	2nd step	
1	Benzoquinone	Insoluble	5, 2.5, 1	8
2	Diphenylcyclopropenone	Insoluble	10, 5, 2.5/1, 0.5, 0.25 (3rd)/0.1, 0.05 (4th)	22
3	Glutaraldehyde	25	10, 5, 2.5/1, 0.5 (3rd)	18 (100% & 50% avoided)
4	Propyl gallate	25	50	8
5	4-Methylaminophenol sulfate	Insoluble	2.5, 1, 0.5	8
6	Cinnamic aldehyde	25	100, 50	10
7	Cyclamen aldehyde	25	100, 50	10
8	3-Aminophenol	25	50	8
9	Diethyl maleate	25	100, 50	10
10	Trimellitic anhydride	Insoluble	10, 5, 2.5	8
11	Abietic acid	25	50	8
12	Hydroxycitronellal	25	100, 50	10
13	Isopropyl myristate	25	100, 50	10
14	Linalool	25	100, 50	10
15	Butyl glycidyl ether	25	100, 50	10
16	Aniline	25	100, 50	10
17	Diethyl phthalate	25	100, 50	10
18	Sulfanilamide	25	50	8
19	Propylene glycol	25	100, 50	10
20	Glycerol	25	100, 50	10

^a Negative control group (n = 2) was included in each step of the pre-screen test.

Predictive capacity for 42 test substances, including the 22 listed in OECD TG 429 and the 20 additional ones, was evaluated by LLNA: BrdU-FCM and compared with the conventional LLNA, human classification, LLNA: DA, and LLNA: BrdU-ELISA, with corresponding values for sensitivity as 94% (30/32), 81% (21/26), 85% (23/27), and 93% (26/28), specificity as 100% (10/10), 59% (7/12), 100% (6/6), and 100% (7/7), and accuracy as 95% (40/42), 74% (28/38), 88% (29/33),

and 94% (33/35). We identified that accuracy was enhanced with an increase in the number of tested substances.

In conclusion, we examined the performance of the LLNA: BrdU-FCM in BALB/c mice with 20 additional test substances, including 16 sensitizers and 4 non-sensitizers. Results for all 20 additional test substances were consistent with those reported by the conventional LLNA and its non-radioisotopic variant methods. Our results using LLNA: BrdU-FCM after combining 22 reference substances listed in OECD TG 429 with 20 additional substances showed high concordance with those by conventional LLNA (Ahn et al., 2016). Additionally, the accuracy of LLNA: BrdU-FCM was higher than those of other LLNA methods and human classification (Ahn et al., 2016). In addition, the LLNA: BrdU-FCM can also be modified to include immunophenotyping to evaluate sensitizers (Jung et al., 2012). Further studies (e.g., analysis of specific lymphocyte activation related to skin sensitization [T cell-specific surface markers or cytokines]) may be performed to check the utility of LLNA: BrdU-FCM for characterizing the skin sensitization potential of test substances.

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