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# Local lymph node assay: 5-bromo-d-deoxyuridine-ELISA method for comparative study in assessing chemical potencies and skin sensitization in BALB/c and CBA/J strains

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ABSTRACT

Local lymph node assay (LLNA) is a predictive *in vivo* method to provide estimates of relative potency and to contribute to risk assessment/risk management regarding skin sensitizing potency of chemicals and formulations as a stand-alone alternative test. In addition, LLNA is relatively rapid and cost-effective compared to the Buehler method (Guinea pig test), and confers important animal welfare benefits. CBA/J and BALB/c strains are widely commercially available and have been evaluated by formal LLNA validation studies. However, the LLNA method using BrdU with ELISA, unlike other LLNA methods (OECD TG 429, 442 A, 442B), has not been previously validated. Therefore, in this study a validation method was performed to evaluate if the LLNA:BrdU-ELISA method could also be used to identify sensitizers among chemicals listed in OECD TG 429 using CBA/J and BALB/c strains. Here, we newly found that the LLNA:BrdU-ELISA validation method correctly identified 12 of 13 sensitizers in the BALB/c, 11 of 13 sensitizers in the CBA/J, and 3 of 5 non-sensitizers were identified in the two strains. Collectively, we found that the results of LLNA:BrdU-ELISA method provide a similar level of performance for accuracy and sensitivity in two mouse strains BALB/c and CBA/J.

## 1. Introduction

Local lymph node assay (LLNA) is a novel predictive *in vivo* method to identify chemicals that have skin sensitizing potential and also contributes to the risk assessment/risk management for the evaluation of chemicals and formulations. LLNA measures the ability of topically applied chemicals to induce proliferative responses by draining lymph node cells (LNC) in mice [1,2] and has the advantages of relatively rapid test period and low cost, thus contributing to animal welfare benefits compared to Guinea pig tests [3,4].

LLNA is based on measurement of proliferated responses in corporation of $^{3}$H- methyl thymidine. However, special facilities and disposal procedures are required because it is a radioisotope (RI)-based method [5]. To avoid use of the radioisotope in LLNA, some modified LLNA protocols were developed [6–8]. One of the modified methods utilizes the thymidine analog of 5-bromo-D-deoxyuridine (BrdU) *in vivo*. The Japanese Center for the Validation of Alternative Methods (JaCVAM) has validated the LLNA:BrdU-ELISA method for identification of skin sensitization of chemicals. The LLNA:BrdU-ELISA method has the potential to reduce the number of animals utilized compared to Guinea pig tests and substantially refines the way animals are used in allergic contact sensitization tests [9]. Also, like other LLNA tests, there are certain limitations compared to Guinea pig tests (TG 406) in the testing of certain metals, false positive findings with certain skin irritants (such as some surfactant-type substances), and possible problems regarding solubility of the chemicals (such as practically insoluble or insoluble substances) [9].

Presently, CBA/J strain is the preferred strain recommended in regulatory guidelines [8,9]. Also, BALB/c strain has been used in the LLNA or LLNA:BrdU-ELISA method in some publications [10–12]. In OECD TG 442B, it is stated that "other strains of mouse may be used when sufficient data are generated to demonstrate that significant strain-specific differences in the LLNA:BrdU-ELISA response do not exist." Based on the above sentence in the guideline, BALB/c strain can be used in the LLNA:BrdU-ELISA method as an alternative to CBA/J strain. In addition, BALB/c strain is easier to observe for erythema and

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scoring calculations than CBA/J strain due to their inherent fur color. Above all, BALB/c strain provides a major benefit in terms of animal cost. Therefore, BALB/c strain is relatively cost effective compared to CBA/J strain, and this test method can be used more widely [9].

To date, there has been no study comparing the differences between the CBA/J and BALB/c strains using the LLNA:BrdU-ELISA method. Therefore, we have evaluated the accuracy, sensitivity, and correlation of stimulation indices between BALB/c and CBA/J strains for 18 reference chemicals listed in OECD Guideline TG429 using the LLNA:BrdU-ELISA method.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Chemicals were the selected reference chemicals suggested in OECD Guideline TG429 as shown in Table 1. 5-chloro-2-methyl-4-isothiazolin-one/2-methyl-4-isothiazolin-3-one, 2,4-dinitrochlorobenzene, p-phenylenediamine, cobalt (II) chloride, isoeugenol, 2-mercaptobenzothiazole, citral, α-hexylcinnamaldehyde, eugenol, phenyl benzoate, cinnamic alcohol, imidazolidinyl urea, methyl methacrylate, chlorobenzene, isopropanol, lactic acid, methyl salicylate, salicylic acid, olive oil, dimethyl sulfoxide, and N, N-dimethylformamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetone was purchased from DAEJUONG CHEMICALS & METALS CO., LTD (Siheung-si, Republic of Korea). 5-Chloro-2-methyl-4-isothiazolin-one and 2-methyl-4-isothiazolin-3-one were mixed on the based on Kathon™ CG from Dow chemical company. Briefly, the portion of ingredients was 1.15% (both CBA/J and BABL/c strains) for 5-Chloro-2-methyl-4-isothiazolin-one, 0.37 (CBA/J strain) and 0.35 (BABL/c strain) % for 2-methyl-4-isothiazolin-3-one, 26.6 (CBA/J strain) and 26.7 (BABL/c strain) % for Imidazolidinyl urea. α-hexylcinnamaldehyde and eugenol (for α-hexylcinnamaldehyde) were used as positive controls. Acetone:Olive oil (4:1 v/v), dimethyl sulfoxide, and (N,N-dimethylformamide were used as a vehicle. BrdU (Sigma-Aldrich, USA) was dissolved in isotonic sodium chloride for injection (10 mg/mL). ELISA BrdU Kit (Cell Proliferation ELISA, colormetric, Cat. No. 11 647 229 001) was purchased from Roche Applied Science (Mannheim, Germany).

### 2.2. Animals

Specific pathogen-free (SPF) female mice for LLNA-BrdU-ELISA were obtained from Koatech Co. Ltd (Pyeongtaek-si, Republic of Korea). In accordance with the Guide for the Care and Use of Laboratory Animals, 8th edition (NRC 2010) [13], animals were maintained under environmental conditions that remained constant (temperature, 23 ± 3 °C; humidity, 55 ± 15%; ventilation, 10 – 20 air changes/hour; and luminous intensity, 150 – 300 Lux) in the experimental animal facility at the Nonclinical Research Institute, Chemon Inc. (#001333), accredited by the AAALAC International. Throughout the study period, temperature and humidity of the animal room were measured hourly with a computer-based automatic sensor, and environmental conditions such as ventilation frequency and luminous intensity were monitored on a regular basis. Food and water were provided *ad libitum* with a 12-hour light-dark cycle. All procedures and protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Nonclinical Research Institute, Chemon Inc. (Yongin-si, Gyeonggi-do, Republic of Korea), and performed in accordance with the

**Table 1**
Test chemical information.

| No. | Test chemical | CAS No. | LLNA (+/- Sensitizer) | Form | Vendor | Lot No. | | Purity (%) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | CBA/J | BALB/c | CBA/J | BALB/c |
| 1 | 5-Chloro-2-methyl-4-isothiazolin-one/ 2-methyl-4-isothiazolin-3-one | 26172–55–4/ 2682–20–4 | + | Liquid | Sigma-Aldrich | LRAB7781 | RAB0761 | 1.13% CMI/ 0.37% MI | 1.15% CMI/ 0.35% MI |
| 2 | 2,4-dinitrochlorobenzene | 97–00–7 | + | Solid | Sigma-Aldrich | BCBZ3828 | BCBS4201V | 99.8% | 99.9% |
| 3 | p-Phenylenediamine | 106–50–3 | + | Solid | Sigma-Aldrich | WXBC8316V | WXBC1642V | 100.0% | 100.0% |
| 4 | Cobalt (II) chloride | 7646–79–9 | + | Solid | Sigma-Aldrich | BCBW4271 | BCBR6930V | 98.5% | 99.2% |
| 5 | Isoeugenol | 97–54–1 | + | Liquid | Sigma-Aldrich | 05622BE | 05622BEV | 99.0% | 99.0% |
| 6 | 2-Mercaptobenzothiazole | 149–30–4 | + | Solid | Sigma-Aldrich | MKCD0898 | MKBX2297V | 99.9% | 99.9% |
| 7 | Citral | 5392–40–5 | + | Liquid | Sigma-Aldrich | STBC5273V | STBG4262V | 96.5% | 98.8% |
| 8 | α-hexylcinnamaldehyde | 101–86–0 | + | Liquid | Sigma-Aldrich | MKCJ7724 | MKCD2910 | 96.7% | 97.2% |
| 9 | Eugenol | 97–53–0 | + | Liquid | Sigma-Aldrich | STBG9481 | STBF3347V | 99.9% | 99.1% |
| 10 | Phenyl benzoate | 93–99–2 | + | Solid | Sigma-Aldrich | STBF6278V | STBF6278V | > 99.9% | > 99.9% |
| 11 | Cinnamic alcohol | 104–54–1 | + | Solid | Sigma-Aldrich | STBH3295 | STBF4901V | > 99.9% | 98.3% |
| 12 | Imidazolidinyl urea | 39236–46–9 | + | Solid | Sigma-Aldrich | BCBV9564 | BCBS1438V | Nitrogen content 26.6% | Nitrogen content 26.7% |
| 13 | Methyl methacrylate | 80–62–6 | + | Liquid | Sigma-Aldrich | STBH1601 | MKBH4379V | > 99.9% | 99.9% |
| 14 | Chlorobenzene | 108–90–7 | – | Liquid | Sigma-Aldrich | STBJ1558 | MKBN7793V | 99.97% | 99.99% |
| 15 | Isopropanol | 67–63–0 | – | Liquid | Sigma-Aldrich | BCBV4036 | BCBR8917V | 99.9% | 99.98% |
| 16 | Lactic acid | 50–21–5 | – | Liquid | Sigma-Aldrich | MKCG0977 | MKBZ2817V | 88.6% | 88.5% |
| 17 | Methyl salicylate | 119–36–8 | – | Liquid | Sigma-Aldrich | MKCG7430 | MKCC0529 | 99.7% | 99.8% |
| 18 | Salicylic acid | 69–72–7 | – | Solid | Sigma-Aldrich | MKCH3688 | MKCB7761 | 99.6% | 100.4% |

guideline published by the OECD [14] as well as the GLP regulations for Nonclinical Laboratory Studies from the Ministry of Food and Drug Safety [15]. The study received 27 animals of each strain and was performed using 25 animals of each strain for evaluation of each chemical. The remaining 2 animals per test were euthanized according to Chemon SOPs and IACUC guidelines.

### 2.3. Skin sensitization, LLNA-BrdU-ELISA

The LLNA-BrdU-ELISA for skin sensitization was performed according to OECD TG 442B [9] under GLP regulations, and the LLNA-BrdU-ELISA was as described previously [16]. As shown in Fig. 1, the animals were exposed *via* ear to chemicals (25 μL/ear), once daily for three consecutive days. On Day 5, animals were intraperitoneally injected with 0.5 mL BrdU (5 mg/mouse) for incorporation into proliferating LNC. After 24 h, the animals were euthanized, and the auricular lymph nodes were collected from each animal. For each mouse, a single-cell suspension of lymph nodes excised bilaterally was prepared by gentle mechanical disaggregation through a glass homogenizer used for shearing cells. Cell proliferation was evaluated by ELISA using the Cell Proliferation ELISA BrdU (colorimetric) kit (Roche Applied Science, Mannheim, Germany) in accordance with the manufacturer's instructions. Absorbance was measured using a spectrophotometer at 370 and 492 nm to obtain the BrdU labeling index (LI) using the following equation:

BrdU LI = [(Abs370 nm) – (Abs370 nm blank)] – [(Abs492 nm) – (Abs492 nm blank)].

Stimulation Index (SI) was calculated as the ratio of the BrdU LI for each treatment group *versus* that of the vehicle control group. If the SI was 1.6 or above (SI $\geq$ 1.6), chemicals were classified as sensitizers. Chemicals with SI between 1.6 and 1.9 were classified as borderlined sensitizers. Chemicals with SI below 1.6 (SI<1.6) were classified as non-sensitizers [3,9].

### 2.4. Statistical analysis

The data presented are expressed as mean ± standard deviation (SD). Statistical analysis was performed using SPSS Statistics version 19 (IBM SPSS Statistics, Armonk, NY, USA), using parametric multiple comparison for comparisons among groups and the level of significance was considered as $p$ values $< 0.05$. Student's t-test was used to test for a difference between means of the negative and positive control groups. If the SI showed a borderline positive response between 1.6 and 1.9 according to the regulatory guideline [9], the dose-responsiveness of the SI was tested by a linear-by-linear association of chi-square test.

## 3. Results

### 3.1. Skin Sensitization in LLNA through preliminary tests

To check skin sensitization, 18 reference chemicals listed in OECD Guideline TG429 were applied to the ears of mice as shown in Table 1. First, to determine the mean SI index, an appropriate solvent and a non-severe irritation dose range were selected through preliminary tests in Table 2. The dose ranges of the 18 chemicals in the two strains were the same as shown in Table 2.

**Table 2**
Dose.

| No. | Test chemical | Vehicle | Dose (%) | |
|---|---|---|---|---|
| | | | CBA/J | BALB/c |
| 1 | 5-Chloro-2-methyl-4-isothiazolin-one/ 2-methyl-4-isothiazolin-3-one | DMF | 2.5, 5, 10 | 2.5, 5, 10 |
| 2 | 2,4-dinitrochlorobenzene | AOO | 0.05, 0.1, 0.5 | 0.05, 0.1, 0.5 |
| 3 | p-Phenylenediamine | AOO | 0.5, 1.0, 2.5 | 0.5, 1.0, 2.5 |
| 4 | Cobalt (II) chloride | DMSO | 0.25, 0.5, 1.0 | 0.25, 0.5, 1.0 |
| 5 | Isoeugenol | AOO | 5, 10, 25 | 5, 10, 25 |
| 6 | 2-Mercaptobenzothiazole | DMF | 5, 10, 25 | 5, 10, 25 |
| 7 | Citral | AOO | 10, 25, 50 | 10, 25, 50 |
| 8 | α-hexylcinnamaldehyde | DMF | 5, 10, 25 | 5, 10, 25 |
| 9 | Eugenol | AOO | 5, 10, 25 | 5, 10, 25 |
| 10 | Phenyl benzoate | AOO | 10, 25, 50 | 10, 25, 50 |
| 11 | Cinnamic alcohol | AOO | 10, 25, 50 | 10, 25, 50 |
| 12 | Imidazolidinyl urea | DMF | 10, 25, 50 | 10, 25, 50 |
| 13 | Methyl methacrylate | AOO | 25, 50, 100 | 25, 50, 100 |
| 14 | Chlorobenzene | AOO | 10, 25, 50 | 10, 25, 50 |
| 15 | Isopropanol | AOO | 25, 50, 100 | 25, 50, 100 |
| 16 | Lactic acid | DMSO | 5, 10, 25 | 5, 10, 25 |
| 17 | Methyl salicylate | AOO | 10, 25, 50 | 10, 25, 50 |
| 18 | Salicylic acid | AOO | 5, 10, 25 | 5, 10, 25 |

DMF, N,N-dimethylformamide; AOO, acetone: olive oil (4:1); DMSO, dimethyl sulfoxide

### 3.2. Stimulation index values in the BALB/c and CBA/J

As shown in Tables 3 and 4, 5-chloro-2-methyl-4-isothiazolin-one/2-methyl-4-isothiazolin-3-one, 2,4-dinitrochlorobenzene, p-phenylenediamine, cobalt (II) chloride, isoeugenol, citral, α-hexylcinnamaldehyde, eugenol, phenyl benzoate, cinnamic alcohol, imidazolidinyl urea, and methyl salicylate were classified as skin sensitizers (SI over 1.6) while methyl methacrylate, isopropanol, and lactic acid were evaluated as non-sensitizers (SI below 1.6) in both the CBA/J and BALB/c strains under our laboratory conditions. Interestingly, 2-mercaptobenzothiazole (1.98) and chlorobenzene (3.42) were classified as skin sensitizers in BALB/c while they (1.53 and 1.37) were classified as non-skin sensitizers in CBA/J. Salicylic acid (1.20) was classified as a non-skin sensitizer in BALB/c while it (1.70) was classified as a borderlined skin sensitizer in the CBA/J.

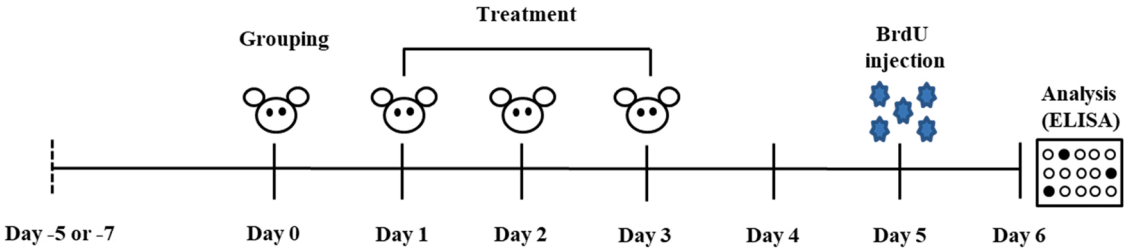


**Fig. 1.** Schematic procedure for main study.

**Table 3**
Comparison of stimulation index values in CBA/J and BALB/c mice.

| No. | Test chemical | CBA/J | | | | | | + /- sensitizer | BALB/c | | | | | | + /- sensitizer |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Dose (%) | Mean SI | | | | | | Dose (%) | Mean SI | | | | | |
| | | | VC | L | M | H | PC | | | VC | L | M | H | PC | |
| 1 | 5-Chloro-2-methyl-4-isothiazolin-one/ 2-methyl-4-isothiazolin-3-one | 2.5, 5, 10 | 1.00 ± 0.69 | 1.87 ± 0.58 | 3.79 ± 1.33 | 4.85 ± 2.39 | 3.48 ± 1.16 | + | 2.5, 5, 10 | 1.00 ± 0.14 | 1.42 ± 0.46 | 2.30 ± 0.65 | 2.81 ± 0.35 | 2.93 ± 0.27 | + |
| 2 | 2,4-dinitrochlorobenzene | 0.05, 0.1, 0.5 | 1.00 ± 0.29 | 1.01 ± 0.18 | 1.59 ± 0.16 | 4.00 ± 0.44 | 2.65 ± 1.09 | + | 0.05, 0.1, 0.5 | 1.00 ± 0.33 | 1.62 ± 0.38 | 1.77 ± 0.47 | 3.44 ± 0.86 | 2.54 ± 0.42 | + |
| 3 | p-Phenylenediamine | 0.5, 1.0, 2.5 | 1.00 ± 0.13 | 1.67 ± 0.06 | 2.15 ± 0.10 | 2.35 ± 0.11 | 3.08 ± 1.25 | + | 0.5, 1.0, 2.5 | 1.00 ± 0.19 | 1.30 ± 0.36 | 1.93 ± 0.81 | 4.20 ± 0.82 | 3.65 ± 1.52 | + |
| 4 | Cobalt (II) chloride | 0.25, 0.5, 1.0 | 1.00 ± 0.62 | 2.64 ± 1.44 | 6.51 ± 1.80 | 5.81 ± 1.15 | 6.76 ± 1.07 | + | 0.25, 0.5, 1.0 | 1.00 ± 0.12 | 1.32 ± 0.07 | 1.40 ± 0.18 | 2.04 ± 0.25 | 2.03 ± 0.10 | + |
| 5 | Isoeugenol | 5, 10, 25 | 1.00 ± 0.12 | 3.23 ± 0.64 | 4.09 ± 0.94 | 4.65 ± 0.69 | 2.99 ± 0.80 | + | 5, 10, 25 | 1.00 ± 0.38 | 1.81 ± 0.33 | 1.59 ± 0.30 | 2.57 ± 0.31 | 1.70 ± 0.58 | + |
| 6 | 2-Mercaptobenzothiazole | 5, 10, 25 | 1.00 ± 0.15 | 1.18 ± 0.17 | 1.43 ± 0.08 | 1.53 ± 0.25 | 3.46 ± 0.89 | – | 5, 10, 25 | 1.00 ± 0.80 | 1.06 ± 0.32 | 1.81 ± 0.81 | 1.98 ± 1.00 | 5.95 ± 0.61 | + |
| 7 | Citral | 10, 25, 50 | 1.00 ± 0.33 | 2.04 ± 0.70 | 2.33 ± 0.52 | 3.05 ± 0.30 | 3.47 ± 0.81 | + | 10, 25, 50 | 1.00 ± 0.32 | 1.29 ± 0.33 | 2.14 ± 0.24 | 3.41 ± 0.45 | 4.39 ± 0.68 | + |
| 8 | α-hexylcinnamaldehyde | 5, 10, 25 | 1.00 ± 0.26 | 1.03 ± 0.06 | 1.28 ± 0.19 | 1.83 ± 0.27 | 2.45 ± 0.42 | + | 5, 10, 25 | 1.00 ± 0.17 | 1.20 ± 0.12 | 1.35 ± 0.13 | 1.97 ± 0.43 | 2.46 ± 0.13 | + |
| 9 | Eugenol | 5, 10, 25 | 1.00 ± 0.33 | 0.95 ± 0.11 | 1.18 ± 0.37 | 1.76 ± 0.04 | 4.94 ± 1.61 | + | 5, 10, 25 | 1.00 ± 0.27 | 1.17 ± 0.21 | 1.64 ± 0.41 | 2.97 ± 0.17 | 2.45 ± 0.95 | + |
| 10 | Phenyl benzoate | 10, 25, 50 | 1.00 ± 0.29 | 1.26 ± 0.21 | 1.40 ± 0.16 | 1.72 ± 0.43 | 3.86 ± 1.01 | + | 10, 25, 50 | 1.00 ± 0.94 | 1.10 ± 0.50 | 1.14 ± 0.37 | 1.84 ± 0.60 | 3.42 ± 0.90 | + |
| 11 | Cinnamyl alcohol | 10, 25, 50 | 1.00 ± 0.34 | 1.25 ± 0.52 | 1.88 ± 0.57 | 2.44 ± 0.21 | 2.72 ± 0.43 | + | 10, 25, 50 | 1.00 ± 0.17 | 1.10 ± 0.31 | 1.94 ± 0.50 | 2.58 ± 0.54 | 3.85 ± 0.62 | + |
| 12 | Imidazolidinyl urea | 10, 25, 50 | 1.00 ± 0.23 | 1.27 ± 0.23 | 1.49 ± 0.16 | 1.71 ± 0.34 | 2.76 ± 0.74 | + | 10, 25, 50 | 1.00 ± 0.22 | 1.42 ± 0.18 | 1.29 ± 0.24 | 2.02 ± 0.24 | 2.03 ± 0.23 | + |
| 13 | Methyl methacrylate | 25, 50, 100 | 1.00 ± 0.09 | 1.15 ± 0.17 | 0.80 ± 0.34 | 0.86 ± 0.15 | 1.71 ± 0.36 | – | 25, 50, 100 | 1.00 ± 0.29 | 1.06 ± 0.20 | 1.12 ± 0.25 | 1.28 ± 0.26 | 3.76 ± 0.63 | – |
| 14 | Chlorobenzene | 10, 25, 50 | 1.00 ± 0.57 | 1.17 ± 0.48 | 1.59 ± 0.62 | 1.37 ± 0.57 | 3.11 ± 0.87 | – | 10, 25, 50 | 1.00 ± 0.85 | 2.40 ± 0.89 | 2.53 ± 0.51 | 3.42 ± 1.02 | 5.88 ± 1.49 | + |
| 15 | Isopropanol | 25, 50, 100 | 1.00 ± 0.20 | 0.88 ± 0.20 | 1.36 ± 0.18 | 1.30 ± 0.17 | 2.66 ± 0.67 | – | 25, 50, 100 | 1.00 ± 0.11 | 1.00 ± 0.21 | 0.86 ± 0.20 | 1.00 ± 0.32 | 2.11 ± 0.63 | – |
| 16 | Lactic acid | 5, 10, 25 | 1.00 ± 0.34 | 0.83 ± 0.39 | 0.86 ± 0.57 | 1.05 ± 0.50 | 2.89 ± 0.43 | – | 5, 10, 25 | 1.00 ± 0.13 | 1.30 ± 0.34 | 0.78 ± 0.08 | 0.84 ± 0.21 | 2.11 ± 0.67 | – |
| 17 | Methyl salicylate | 10, 25, 50 | 1.00 ± 0.27 | 1.35 ± 0.34 | 1.34 ± 0.49 | 1.89 ± 0.37 | 2.74 ± 0.89 | + | 10, 25, 50 | 1.00 ± 0.69 | 2.08 ± 0.72 | 2.52 ± 0.60 | 2.57 ± 1.52 | 3.43 ± 1.70 | + |
| 18 | Salicylic acid | 5, 10, 25 | 1.00 ± 0.15 | 1.29 ± 0.16 | 1.48 ± 0.58 | 1.70 ± 0.30 | 2.58 ± 0.59 | + | 5, 10, 25 | 1.00 ± 0.16 | 0.95 ± 0.16 | 1.19 ± 0.33 | 1.20 ± 0.38 | 2.510.10 | – |

VC, Vehicle control; L, Low dose; M, Medium dose; H, High dose; PC, Positive Control

**Table 4**
The EC1.6 values.

| No. | Test chemical | Traditional LLNA (OECD TG429) | | | LLNA:BrdU-ELISA CBA/J | | | LLNA:BrdU-ELISA BALB/c | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Vehicle | EC3 | Category[a] | Vehicle | EC1.6 | Category[a] | Vehicle | EC1.6 | Category[a] |
| 1 | 5-Chloro-2-methyl-4-isothiazolin-one/ 2-methyl-4-isothiazolin-3-one | DMF | 0.009 | Extreme | DMF | 2.14 | Moderate | DMF | 3.01 | Moderate |
| 2 | 2,4-dinitrochlorobenzene | AOO | 0.049 | Extreme | AOO | 0.10 | Strong | AOO | 0.49 | Strong |
| 3 | p-Phenylenediamine | AOO | 0.11 | Strong | AOO | 0.48 | Strong | AOO | 0.74 | Strong |
| 4 | Cobalt (II) chloride | DMSO | 0.6 | Strong | DMSO | 0.15 | Strong | DMSO | 0.66 | Strong |
| 5 | Isoeugenol | AOO | 1.5 | Moderate | AOO | 2.48 | Moderate | AOO | 4.42 | Moderate |
| 6 | 2-Mercaptobenzothiazole | DMF | 1.7 | Moderate | DMF | – | Negative | DMF | 8.60 | Moderate |
| 7 | Citral | AOO | 9.2 | Moderate | AOO | 7.84 | Moderate | AOO | 15.47 | Weak |
| 8 | α-hexylcinnamaldehyde | DMF | 9.7 | Moderate | DMF | 18.73 | Weak | DMF | 16.05 | Weak |
| 9 | Eugenol | AOO | 10.1 | Weak | AOO | 20.86 | Weak | AOO | 9.57 | Moderate |
| 10 | Phenyl benzoate | AOO | 13.6 | Weak | AOO | 40.63 | Weak | AOO | 41.43 | Weak |
| 11 | Cinnamyl alcohol | AOO | 21 | Weak | AOO | 18.33 | Weak | AOO | 18.93 | Weak |
| 12 | Imidazolidinyl urea | DMF | 24 | Weak | DMF | 37.50 | Weak | DMF | 35.62 | Weak |
| 13 | Methyl methacrylate | AOO | 90 | Weak | AOO | – | Negative | AOO | – | Negative |
| 14 | Chlorobenzene | AOO | – | Negative | AOO | – | Negative | AOO | 6.67 | Moderate |
| 15 | Isopropanol | AOO | – | Negative | AOO | – | Negative | AOO | – | Negative |
| 16 | Lactic acid | DMSO | – | Negative | DMSO | – | Negative | DMSO | – | Negative |
| 17 | Methyl salicylate | AOO | – | Negative | AOO | 36.82 | Weak | AOO | 7.69 | Moderate |
| 18 | Salicylic acid | AOO | – | Negative | AOO | 18.18 | Weak | AOO | – | Negative |

DMF, N, N-dimethylformamide; AOO, acetone: olive oil (4:1); DMSO, dimethyl sulfoxide
* EC3: Estimated concentration of a test substance needed to produce a Stimulation Index of 3, Ref [8].
EC1.6: Estimated concentration of a test substance needed to produce a Stimulation Index of 1.6.
[a] ECETOC, 2003. Contact Sensitization: Classification According to Potency a Commentary. Technical Report No. 87, ECETOC, Brussels, Belgium

## 4. Discussion

In this study, the LLNA:BrdU-ELISA method was performed using BALB/c and CBA/J strains, to evaluate potential differences in the classification of 18 chemicals (13 sensitizers and 5 non-sensitizers) listed in OECD Guideline TG429 regarding the use of mouse strains [8]. Reproducibility of the LLNA:BrdU-ELISA method was evaluated using the EC threshold calculation and stimulation index with the two strains of the mice in this study.

According to the acceptance criteria described in OECD Guideline TG 429 performance standards, the predictive capacity using the LLNA: BrdU-ELISA method indicated that 12 of 13 sensitizers in the BALB/c, 11 of 13 sensitizers in the CBA/J, and 3 of 5 non-sensitizers were identified based on the SI 1.6 in the two strains as shown in Table 3. Only one chemical 2-mercaptobenzothiazole, which is reported to be a skin sensitizer by LLNA [8], was classified as a non-sensitizer in the CBA/J, while the same chemical was classified as a sensitizer in the BALB/c. However, 2-mercaptobenzothiazole has low skin sensitizing potential and is often misclassified as a non-sensitizer [17–22]. Even in one mouse strain, it is not clear whether 2-mercaptobenzothiazole has been distinguished either as a sensitizer or a non-sensitizer because of the characteristics of 2-mercaptobenzothiazole itself. However, 2-mercaptobenzothiazole is certainly more sensitizing in BALB/c than in the CBA/J under our present conditions. It is confirmed that the BALB/c strain showed a similar, but not equal, response to the CBA/J, the preferred strain in LLNA [18]. In addition, the following were classified as sensitizers: chlorobenzene and methyl salicylate in BALB/c and methyl salicylate and salicylic acid in CBA/J. However, chlorobenzene, methyl salicylate, and salicylic acid are usually accepted as non-sensitizers by LLNA [8] and LLNA:BrdU-FCM method [18]. Although there were some differences in the EC1.6 values, all were of similar magnitude except for chlorobenzene and methyl salicylate in the BALB/c strain. Chlorobenzene and methyl salicylate were classified as sensitizers in this study. The BALB/c strain showed higher SI values than those of the CBA/J under our laboratory conditions especially for chlorobenzene's EC1.6. methyl salicylate and salicylic acid were also classified as sensitizers in the CBA/J strain. Although methyl salicylate and salicylic acid are classified as non-sensitizers, methyl salicylate and salicylic acid were classified as borderlined sensitizers under our present conditions according to the Guideline [9]. These SI variations, for some chemicals in this study might come from many other factors such as differences in lymph node cell proliferation resulting from exposure to chemicals in the absence of excessive local irritation. The many steps in the ELISA procedure are the primary source of these variations. In addition, the smaller SI values gained may relate to inter-laboratory variations in the 18 chemicals used under our experimental conditions. Some research articles have reported that some chemical classes induce false positives in the LLNA [23–25]. To clarify such controversial results for regulatory purposes, further studies should be performed to provide support and evidence for the variations. Nevertheless, the obtained data revealed that the BALB/c strain apparently has a higher responsiveness to some chemicals than the CBA/J strain. Also, the data suggest that the BALB/c strain is considered to be a promising candidate strain for use in the LLNA: BrdU-ELISA method as shown in the results under our laboratory conditions.

Guinea pig test (maximization and Buehler test) [26,27] were the standard methods to assess the skin sensitizing potential of chemicals. Murine LLNA test [10,28,29] is widely accepted as a stand-alone skin sensitization test and offers several significant advantageous animal welfare benefits compared to traditional Guinea pig test methods. Developed and introduced the non-RI BrdU-based modified LLNA method is still less sensitive than the traditional LLNA method [30]. Here, we did evaluate a set of the 18 minimum references chemicals as specified in OECD Guideline TG429 in two strains, BALB/c and CBA/J, using the LLNA:BrdU-ELISA method. We newly found that LLNA: BrdU-ELISA validation method correctly identified sensitizers and non-sensitizers in two strains BALB/c and CBA/J. As a result in this study, the performance of the BALB/c strain could support its use as an alternative mouse strain since the skin sensitization potential of the chemicals in the list of OECD Guideline TG429 was correctly identified (12 of 13 chemicals) in terms of sensitizers and equivalent or better than that of the CBA/J strain (11 of 13 chemicals) although there was no difference in non-sensitizers identification (3 of 5 chemicals) between BALB/c and CBA/J strains. Moreover, several research articles have shown that the skin sensitizers were correctly determined in the LLNA: BrdU-ELISA method in BALB/c [12,31,32]. In terms of animal price and experimentation, it is more advantageous to use BALB/c mice since they are comparatively more cost effective (BALB/c mice are usually

approximately 3 times cheaper than the CBA/J strain) and erythema and scoring calculation are simplified in BALB/c mice compared to the CBA/J mice due to the fur color. In addition to the proven, presently preferred strain, CBA/J, our data suggested that the BALB/c strain can be used to accurately predict the sensitization or non-sensitization potential of chemicals using the LLNA:BrdU-ELISA method.

## 5. Conclusion

We performed the evaluation of LLNA:BrdU-ELISA method in both the BALB/c and CBA/J strains with 13 sensitizers and 5 non-sensitizers listed in the OECD TG429. The results of LLNA:BrdU-ELISA method achieved a similar level of performance and sensitivity in two strains BALB/c and CBA/J.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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