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REPORT OF THE VALIDATION STUDY OF THE LOCAL LYMPH NODE ASSAY BRDU-FCM (LLNA: BRDU-FCM) TEST METHOD

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No. 283

REPORT OF THE VALIDATION STUDY OF THE LOCAL LYMPH NODE ASSAY BRDU-FCM (LLNA: BRDU-FCM) TEST METHOD

IOMC
INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS
A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT
Paris 2018

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Foreword

This document is the report of the international validation study of the Local Lymph Node Assay: BrdU-FCM (LLNA: BrdU-FCM) for evaluating the skin sensitising potential of chemicals. The validation study was coordinated by the Korean Centre for the Validation of Alternative Methods (KoCVAM) between 2012 and 2015. The project for the development of the LLNA: BrdU-FCM was proposed by Korea and included in the WNT Programme of Work in 2016.

The validation study for the LLNA: BrdU-FCM was performed in compliance with the performance standards for assessment of proposed similar or modified LLNA test methods for skin sensitization, in Annex 1 of TG 429 (Skin sensitisation: Local lymph node assay). This validation report supported the development of a new test method for inclusion in the updated Test Guideline 442B (LLNA BrdU-ELISA or –FCM) which describes non-radioactive modifications to the LLNA test method.

Together with the peer review report, the validation report was made available as a supporting document during two commenting rounds of the Working Group of the National Co-ordinators of the Test Guidelines Programme (WNT) on the draft Test Guideline TG 442B, in July and December 2017 respectively. The validation report was endorsed by the WNT at its 30th Meeting in April 2018.

The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to the declassification of the validation report on 30 June 2018. This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

Local Lymph Node Assay:
5-bromo-2-deoxyuridine-flow cytometry method
(LLNA: BrdU-FCM)

Validation Study Report

January 2017

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List of Abbreviations

7-AAD	7-amino-actinomycin D
AOO	Acetone: olive oil
AOP	Adverse Outcome Pathways
ARE	Antioxidant/electrophile response element
AUC	Area under the curve
BLR	Between-laboratory reproducibility
CLP	Classification, Labelling and Packaging
CROs	Contract Research Organisations
CV	Coefficient of variation
DC	Dendritic cells
DMF	*N,N*-dimethylformamide
DNCB	2,4-Dinitrochlorobenzene
DPRA	Direct Peptide Reactivity Assay
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals.
ECt	Estimated concentration
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
EURL ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
GD	Guidance Document
GHS	Globally Harmonized System of Classification and Labelling of Chemicals
GLP	Good Laboratory Practice
HCA	Hexyl cinnamic aldehyde
h-CLAT	Human Cell Line Activation Test
IATA	Integrated Approaches to Testing and Assessment

JaCVAM	Japanese Center for the Validation of Alternative Methods
KoCVAM	Korean Center for the Validation of Alternative Methods
LLNA	Local Lymph Node Assay
LLNA: BrdU-FCM	LLNA: 5-bromo-2-deoxyuridine-flow cytometry method
LNC(s)	Lymph node cell(s)
LNCC	Lymph node cell count
MEK	Methylethyl ketone
MFDS	Ministry of Food and Drug Safety
MSDS	Material Safety Data Sheets
NIER	National Institute of Environment and Research
NIFDS	National Institute of Food and Drug Safety Evaluation
NOEL	No Observed Effect Level
OECD	Organization for Economic Cooperation and Development
PS	Perfornance Standards
QA	Quality assurance
QAU	Quality assurance unit
RDA	Rural Development Administration
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
ROC	Receiver operating characteristic
SI	Stimulation index
SLS	**S**odium lauryl sulphate
SOPs	Standard operating procedures
TLR4	Toll-like receptor 4
TSTD	Toxicological Screening & Testing Division
UN	United Nations
VMT	Validation Management Team
WLR	Within-laboratory reproducibility

Definitions

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of relevance.

Lead laboratory: The laboratory responsible for training other participating facilities involved in standardization, optimization, and validation of a test method.

Project plan: A validation study plan designed to help participants understand the validation study by providing essential information and describing responsibilities and duties of each participating party.

Protocol: A test plan that clearly details each step of a validation method and provides criteria and a process to prepare reagents, supplies, and tools to generate test data.

Reference chemicals: Chemicals that have already been validated in other test systems and species and can be selected for use in the validation process.

Relevance: Description of the relationship between the effect of interest if a test method and whether it is meaningful and useful for a particular purpose. It is the extent to which a test correctly measures or predicts biological effects of interest.

Reliability: The extent to which a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol.

Reproducibility: The closeness of agreement between test method results using the same substances.

Run: A run consists of one or more test chemicals tested concurrently with a vehicle control and positive control.

Sensitivity: The percentage of positive substances correctly classified by a test method.

Specificity: The percentage of negative substances correctly classified by a test method.

Standard Operating Procedures (SOPs): A document that describes specific tests and the process of laboratory operation.

Transferability: The extent to which an independent testing facility can accurately and reliably perform a test procedure.

Validation: A process to demonstrate the reliability and relevance of an alternative test method.

Summary

The murine local lymph node assay (LLNA) has been used worldwide as an alternative test method for evaluating the skin sensitization potential of chemicals since it was adopted as the Organization for Economic Cooperation and Development (OECD) Test Guideline 429 (TG429) in 2002. Since 2010, OECD has adopted two additional generic test methods, the LLNA: BrdU-ELISA and LLNA: DA, which are non-radioisotopic versions of the LLNA. The LLNA: 5-bromo-2-deoxyuridine-flow cytometry method (LLNA: BrdU-FCM) is a new non-radioisotopic version of the LLNA that performs similar to existing LLNA-based test methods. This test measures the proliferation of auricular lymph node cells (LNCs) during the induction phase of skin sensitization by determined the number of BrdU-incorporated LNCs by flow cytometry.

This test method has several benefits. First, the proliferation of living LNCs is quantitatively measured in the LLNA: BrdU-FCM, whereas proliferated cells are indirectly scored based on BrdU content, regardless of whether these cells are alive or dead, in the LLNA: BrdU-ELISA. Second, flow cytometry allows for simultaneous analysis of multiple parameters without sacrificing extra animals (e.g. B/T cell ratio, activation surface marker (CD86 etc.)), which could help to understand the skin sensitization mechanism. Third, the modified pre-screen tests in the LLNA: BrdU-FCM can reduce the number of animals tested and minimize pain and distress in animals by applying a refined dose selection scheme. Fourth, BALB/c mice can be used in the LLNA: BrdU-FCM. BALB/c mice are widely used since they are easy to be obtained and cost-efficient in some countries more than CBA mice.

The validation study for the LLNA: BrdU-FCM was performed in compliance with the performance standards listed in Annex 1 of OECD TG 429 (Skin sensitization: Local lymph node assay) that are used to assess proposed similar or modified LLNA test methods to evaluate skin sensitization by chemicals. The study was performed between 2012 and 2015 and was coordinated by the Validation Management Team (VMT) organized by the Korean Center for the Validation of Alternative Methods (KoCVAM) with the participation of four laboratories. Transferability of the test method was evaluated for all testing sites using hexyl cinnamic aldehyde (HCA) and eugenol. For the evaluation of within-laboratory reproducibility (WLR), the test was repeated four times using 5%, 10%, and 25% HCA, and all three participating laboratories produced results that were within the range of 0.5–2× the estimated concentration (ECt). Between-laboratory reproducibility (BLR) was assessed using 2,4-dinitrochlorobenzene (DNCB) in three repeated runs, and the three participating laboratories generated results that were within the range of 0.5–2× ECt. The predictive capacity of the test method was evaluated in three tests. The 1st and 2nd tests were conducted according to Protocols 1.1 and 1.2, respectively, using the reference chemicals listed in OECD TG 429. The results showed that three chemicals were incorrectly classified by Protocol 1.2. Therefore, to improve the test method's predictive capacity, the protocol was refined to Protocol 1.3 to address viscous substances, and an additional test was performed on falsely predicted viscous substance in accordance with the new protocol. The 3rd test was conducted according to Protocol 1.3 using the 18 reference chemicals listed in OECD TG 429. The final test results showed that the sensitivity of the LLNA: BrdU-FCM was 84.6% (11/13) and the specificity was 100% (5/5), with an overall accuracy of 88.9% (16/18).

The VMT concluded that the transferability, WLR, BLR, and predictive capacity of the LLNA: BrdU-FCM were sufficient, particularly given that a weak sensitizer, methyl methacrylate, which was incorrectly predicted in our study, was classified as a non-sensitizer in a recently published LLNA-related paper. Overall, the VMT propose that the LLNA: BrdU-FCM be considered as a 'me-too' test of the LLNA.

I. Background

Key biological mechanisms related to skin sensitization are described as Adverse Outcome Pathways (AOP) (OECD, 2012). Molecular initiating events are known to progress to adverse effects (i.e. allergic contact dermatitis in humans or contact hypersensitivity in rodents) through a series of intermediate events. The molecular initiating event starts with covalent binding of electrophilic substances to nucleophilic centres in skin proteins. The second key event in AOP takes place in keratinocytes, and includes inflammatory responses as well as gene expression associated with specific cell signalling pathways such as antioxidant/electrophile response element (ARE)-dependent pathways. The third key event is the activation of dendritic cells (DC), typically assessed by the expression of specific cell surface markers or the induction of inflammation-related cytokines. The fourth key event is T cell proliferation, which is indirectly measured in the LLNA (OECD, 2015).

The LLNA, which was adopted as OECD TG 429, has been used to evaluate the skin sensitization potential of chemicals since 2002 (OECD, 2002). In addition, this assay is utilized by the European Union's Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH). Notably, it is also listed as a priority *in vivo* test in Annex VII (REACH, 2006). The United States Environmental Protection Agency (EPA) also adopted the LLNA in its Health Effects Test Guideline and has used it as a method of evaluating skin sensitization since 2003 (EPA, 2003).

However, because ^{3}H-methyl thymidine (an analogue of thymidine) or ^{125}I-iododeoxyuridine is used in the LLNA, the assay requires facilities that allow handling of radioisotopes and poses the risk of radioactive contamination. For this reason, the Japanese Center for the Validation of Alternative Methods (JaCVAM) developed and validated the LLNA: BrdU-ELISA (OECD TG 442B) and the LLNA: DA (OECD TG 442A), for which ^{3}H-methyl thymidine is not needed, with the aim of disseminating the LLNA. Since the adoption as OECD TGs in 2010, these two test methods have been used to evaluate the skin sensitization potential of chemicals (OECD, 2010a & 2010b).

The LLNA: BrdU-FCM was developed with the financial support of Korea's Ministry of Food and Drug Safety (MFDS) in 2009. From 2010 to 2011, the test method's usefulness was assessed, and its protocol was optimized (Jung et al., 2010 and 2012). For three years, beginning in 2012, this validation study was conducted and coordinated by the Validation Management Team.

The LLNA: BrdU-FCM can be used to identify skin-sensitizing chemicals and evaluate skin sensitization potency in the same way as the LLNA, the LLNA: BrdU-ELISA, and the LLNA: DA. The existing LLNA test methods focus on the measurement of proliferating lymphocytes in murine lymph nodes. Each test evaluates the proliferation of lymphocytes using analogues of thymidine (^{3}H-methyl thymidine, ^{125}I-iododeoxyuridine, or 5-bromo-2-deoxyuridine) or by ATP measurement.

The LLNA: BrdU-FCM addresses refinement and reduction, among the 3Rs (Russell and Burch, 1959) as it does not use an adjuvant and needs fewer animals than the traditional skin sensitization test, which uses guinea pigs. In addition, this test method does not require any radioisotopes, which makes it easier to perform and safer for experimenters and the environment.

The basic principle underlying the LLNA: BrdU-FCM is that skin sensitizers induce the proliferation of lymphocytes in the lymph nodes adjacent to where the test substances were applied (OECD, 2010). A test substance is categorized as a skin sensitizer when its stimulation index (SI) is ≥ 2.7. The proliferation of living BrdU-incorporated auricular lymph node cells (LNCs) during the induction phase of skin sensitization is quantitatively measured in the LLNA: BrdU-FCM, whereas proliferated cells are indirectly scored based on BrdU content, regardless of whether these cells are alive or dead in the LLNA: BrdU-ELISA.

This test method has several benefits. First, the proliferation of living LNCs is quantitatively measured in the LLNA: BrdU-FCM, whereas proliferated cells are indirectly scored based on BrdU content, regardless of whether these cells are alive or dead, in the LLNA: BrdU-ELISA. Second, flow cytometry allows for simultaneous analysis of multiple parameters without sacrificing extra animals (e.g. B/T cell ratio, activation surface marker (CD86 etc.)), which could help to understand the skin sensitization mechanism. Our pre-validation study demonstrated the usefulness of the test method as it can also be used to analyse B cell to T cell ratios (cell sub-typing) and cytokine content by flow cytometry and ELISA, respectively, without sacrificing extra animals (Jung et al., 2012). Flow cytometry is commonly used in immunotoxicity tests; therefore, the LLNA: BrdU-FCM, which is more effective in analysing lymphocyte proliferation and B/T cell ratios, can easily be implemented following Good Laboratory Practice (GLP) by CROs. Third, the modified pre-screen tests in the LLNA: BrdU-FCM can reduce the number of animals tested and minimize pain and distress on animals by applying a refined dose selection scheme. Lastly, BALB/c mice can be used in the LLNA: BrdU-FCM. BALB/c mice are being widely used in diverse biological researches since they are easier to obtain and cheaper than CBA strains in some countries. This is beneficial because, in some countries, the price of CBA mice is high when they are imported, making them harder to obtain. It is also noteworthy that pain and distress on laboratory animals would be less and the use of animals would be reduced in the LLNA: BrdU-FCM compared to other existing LLNA test methods, through performing the pre-screen test of the LLNA: BrdU-FCM is performed in two phases. Such an approach is expected to be transferable to the other LLNA-based test methods as well (Ahn et al., 2016).

II. Management of the study

1. Study objectives

The purpose of this validation study was to evaluate the LLNA: BrdU-FCM, proposed as a 'me-too' test of the LLNA because of its predictive capacity. The transferability, within-laboratory and between-laboratory reproducibilities (WLR and BLR, respectively), and predictive capacity of the test method were evaluated to determine whether this test could be as an internationally accepted skin sensitization test method. This validation study was designed and conducted in compliance with OECD Guidance Document (GD) 34 and OECD TG 429 Annex 1 PS (Performance Standards), and the test method's reliability and relevance were assessed with respect to the accepted criteria outline in the PS (OECD, 2010).

2. Project plan

A validation study is generally performed by a government validation agency and is coordinated by a VMT whose role and responsibility is granted by the agency (OECD, 2005). The VMT for this validation

study was supported and operated by KoCVAM. A Project Plan was written and approved by the VMT in April 2012, and this validation study was performed according to the Project Plan. This document describes the study objectives, the composition and roles of the VMT, the procedure for selecting participating laboratories, as well as their roles and quality assurance and GLP systems, details about the study, and test substances. The study timeline is described in Annex 1.

Prior to the initiation of the validation study, the Project Plan was sent to each testing site. The document was constantly updated from 2012 to 2015, and the final version (1.4) is attached to this report as Annex 1.

2.1. Structure of the study

This validation study was conducted and data were generated based on the module approach suggested by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) (Hartung et al., 2004) and in OECD GD 34. However, the overall study procedure and components were established in accordance with OECD TG 429 Annex 1 PS.

The modules in this validation study are as follows:

Module 1. Test definition
Module 2. Within-laboratory reproducibility
Module 3. Transferability
Module 4. Between-laboratory reproducibility
Module 5. Predictive capacity

2.2. Validation Management Team (VMT)

This study was coordinated by the VMT organized by KoCVAM. The VMT and testing laboratories are shown in Figure 1.

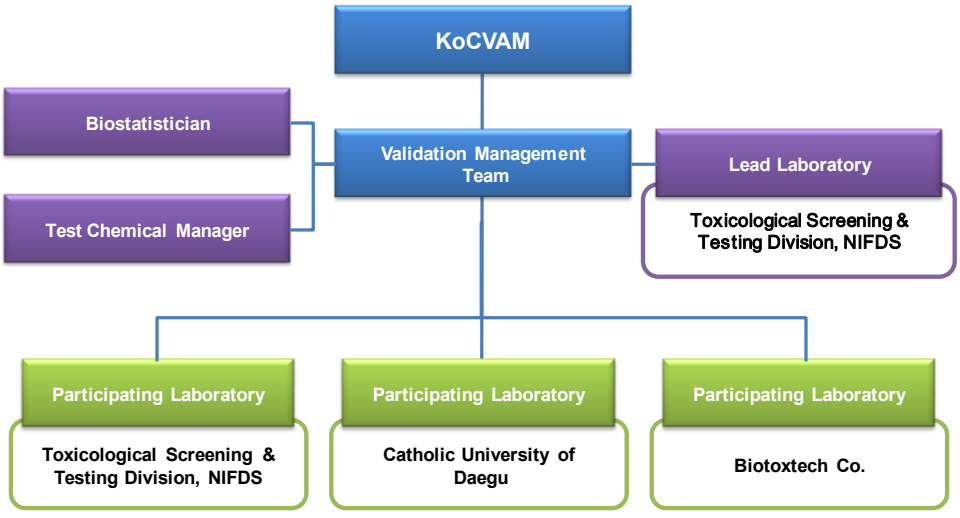

Figure 1. Organisational structure of the VMT and testing laboratories
VMT, validation management team

The Director of KoCVAM served as the chair of the VMT. The VMT coordinated the overall validation process, including reviewing and statistically analysing the test results, selecting and distributing test substances, drawing final conclusions of the validation study, and writing a final validation study report. During this validation study, the members of the VMT changed five times, and such changes are described in the Project Plan (v. 1.4).

The study directors of the lead laboratories participated in VMT meetings to report and discuss study progress, but were not involved in test substance selection and coding.

The chemical manager checked and distributed the test substances used in the WLR, BLR, predictive capacity, and proficiency tests to all testing sites. Among the test substances, the ones selected to evaluate proficiency and predictive capacity were coded using random tables (www.random.org) and distributed.

The biostatistician was responsible for collating Excel spreadsheets with test results from the laboratories, analysing them, and writing a statistical report.

VMT composition (final version):

Chair	Seong, Won Keun (KoCVAM)
Scientific advisory members	Lee, Ai Yeong (Dongguk University, Medical Center Skin Care Clinic)
	Jeong, Tae Cheon (Yeungnam University, College of Pharmacy)
	Lim, Kyung-Min (Ewha Womans University, College of Pharmacy)
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Study director of Lead Laboratory 2	Yi, Jung Sun (National Institute of Food and Drug Safety Evaluation)

2.3. Laboratories

The laboratories that performed the validation study are listed below. The AmorePacific R&D Unit served as the Lead Laboratory from 2012 to 2013 and evaluated the transferability, BLR, WLR, and predictive capacity of the test method (1st test). From 2014 to 2015, the Toxicological Screening & Testing Division (TSTD) at the National Institute of Food and Drug Safety Evaluation (NIFDS) assessed the transferability and predictive capacity (2nd and 3rd tests) in its role as Lead Laboratory.

Lead Laboratory 1 (2012–2013) (Study director: Lim, Kyung-Min; Park, Miyoung)
AmorePacific R&D Unit
Medical Beauty Research Division
314-1, Bora-dong, Giheung-gu, Yongin-si, Gyeonggi province, Republic of Korea

Lead Laboratory 2 (2014–2015) (Study director: Yi, Jung Sun)
National Institute of Food and Drug Safety Evaluation (NIFDS)

Toxicological Screening & Testing Division (TSTD)
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Participating Laboratory 2 (Study director: Jung, Mi Sook)
Biotoxtech Co., Ltd.
Safety Evaluation Team
686-2 Yangcheong-ri, Ochang-eup, Cheongwon-gu, Cheongju-si, Chungbuk province, Republic of Korea

Lead Laboratory 1 declared that the company would ban animal testing in the development of cosmetic products in March 2013. As a consequence, it stopped participating in the validation study, and TSTD became the Lead Laboratory (see Figure 1).

Lead Laboratory 2 has been continuously involved in research on the LLNA and the LLNA: BrdU-FCM since 2009. On 26 February 2014, Lead Laboratory 1 transferred the test method to Lead Laboratory 2. The VMT evaluated the results of a transferability test using HCA and eugenol and accepted the TSTD as Lead Laboratory 2 at the meeting held on 1 May 2014.

2.4. Quality assurance system of the laboratories

Lead Laboratory 1 was designated a GLP facility by regulatory agencies, MFDS, National Institute of Environment and Research (NIER), and Rural Development Administration (RDA), until 2010, and it performed the validation study and collected data in accordance with GLP principles. In addition, it operated its own Quality Assurance Unit (QAU) to ensure the reliability of data generated.

The Catholic University of Daegu, of which Participating Laboratory 1 is a part, has a GLP facility authorized by the regulatory agencies. Participating Laboratory 1 conducted the validation study in accordance with standard operating procedures (SOPs) and GLP operating procedures, and the QAU of the GLP facility biannually inspected the laboratory.

Participating Laboratory 2 is also a GLP facility approved by the regulatory agencies. It complied with the study plan and SOPs, and followed GLP principles. In addition, the laboratory ensured the reliability of data with its own quality assurance (QA) system.

Lead Laboratory 2, which participated in the validation study since March 2014, has continuously conducted R&D projects related to alternative test methods. It is also committed to establishing GLP systems and generating data in accordance with GLP.

The QAU of each laboratory determined whether tests were conducted and data were managed properly. In addition, the QAU of Lead Laboratory 1 inspected each participating laboratory from 2012 to 2013.
(See Attachment 2)

Details on QAU inspection:

- Inspection time:
  Inspection for each participating laboratory by the QAU of Lead Laboratory 1:
  - 16–17 October 2012; 31 May, 14 June, 11 July 2013

  Inspection of each laboratory by its own QAU:
  - Lead Laboratory 1: 30 October 2012
  - Lead Laboratory 2: 20–26 August, 17–23 and 19–25 September 2014; 27 May–1 June 2015
  - Participating Laboratory 1: 3 May 2013; 22 September 2014
  - Participating Laboratory 2: 5–25 August 2014; 27 October–18 November 2015, 26 July–8 August 2016

- List of what was inspected by the QAU of Lead Laboratory 1:
  - Records on the receipt and management of laboratory animals
  - Conduct of experiments in compliance with SOPs
  - Records on the management of lab equipment and the purchase, storage, and management of reagents and test substances
  - Data sheets

- List of what was inspected by the QAU of each laboratory:
  - Conditions in which laboratory animals were kept
  - Records on the receipt, use, and storage of test substances
  - Records on the management of laboratory equipment and SOPs

3. Design of the validation study and selection of sample size

The OECD published a GD on the validation of a new test method (GD 34), which includes the necessary elements for validation (OECD, 2005): (1) test definition, (2) WLR, (3) between-laboratory transferability, (4) BLR, (5) predictive capacity, (6) applicability domain, and (7) performance standards.

The LLNA: BrdU-FCM was validated by considering elements from (1) to (5). The applicability domain of the test method will be determined by peer reviews.

3.1. Module 3. Transferability

3.1.1. Training and transfer of the test method

Lead Laboratory 1 transferred the test method to each participating laboratory, and then a test was performed to see whether the test method was properly implemented. Based on OECD TG 429 paragraph 11, 25% HCA (positive control) and an acetone:olive oil (4:1, v/v, AOO) mixture (vehicle control) were selected. The main purpose of this test was to identify whether the stimulation index (SI) for 25% HCA was ≥3.

Reliability check (from OECD TG 429)
'Inclusion of a concurrent PC is recommended because it demonstrates competency of the laboratory to successfully conduct each assay and allows for an assessment of intra- and inter-laboratory reproducibility and

comparability. … The PC dose should be chosen such that it does not cause excessive skin irritation or systemic toxicity and the induction is reproducible but not excessive (*i.e.* SI>20). Preferred PC test substances are 25% hexyl cinnamic aldehyde (HCA) in acetone: olive oil (4:1, v/v) and 5% mercaptobenzothiazole in *N, N*-dimethylformamide.'

Participating Laboratories 1 and 2 received theoretical and procedural training from Lead Laboratory 1. Lead Laboratory 1 then oversaw and provided advice during test method transfer to ensure that the procedure for performing the LLNA: BrdU-FCM as described in the SOP was clearly understood and properly implemented. Lead Laboratory 1 also transferred the test method to Lead Laboratory 2, and then Lead Laboratory 2 conducted a test to determine whether the method was successfully transferred.

Lead Laboratory 2 trained the participating laboratories on amended protocols (versions 1.2 and 1.3) during the 2nd and 3rd tests to evaluate the predictive capacity of the test method.

3.1.2. Proficiency test

Each participating laboratory's proficiency in the conduct of the assay was assessed using 25% HCA and 5%, 10%, and 25% eugenol. Three concentrations of eugenol were chosen to determine whether ECt values were within the range of 0.5–2×, as described in Table 1 of OECD TG 429 Annex 1.

Eugenol was blinded and distributed to each testing site, but information on concentrations and the vehicle (AOO) were given. Eugenol is known as a weak sensitizer, and the VMT selected it as an appropriate substance for the assessment of each laboratory's competence in performing the assay.

The proficiency test was performed by Participating Laboratories 1 and 2, and Lead Laboratory 2, following Protocol ver 1.0 and ver. 1.2, respectively.

3.2. Module 2. WLR

Evaluation of the WLR was accomplished by tests repeated four times at intervals of more than one week at Lead Laboratory 1 and Participating Laboratories 1 and 2 using 5%, 10%, and 25% HCA, based on OECD TG 429 Annex 1 Paragraph 8.

WLR was assessed according to Protocol 1.0. Lead Laboratory 2 did not conduct a WLR test because it participated in this validation study only after the WLR evaluation, which met the OECD criteria.

Intra-laboratory reproducibility (from OECD TG 429 ANNEX 1)
'To determine intra-laboratory reproducibility, a new or modified LLNA test method should be assessed using a sensitizing substance that is well characterized in the LLNA. Therefore, the LLNA PS is based on the variability of results from repeated tests of hexyl cinnamic aldehyde (HCA). To assess intra-laboratory reliability, threshold estimated concentration (ECt) values for HCA should be derived on four separate occasions with at least one week between tests. Acceptable intra-laboratory reproducibility is indicated by a laboratory's ability to obtain, in each HCA test, ECt values between 5% and 20%, which represents the range of 0.5~2.0 times the mean EC3 specified for HCA (10%) in the LLNA.'

3.3. Module 4. BLR

BLR was evaluated at Lead Laboratory 1 and Participating Laboratories 1 and 2 using 0.05%, 0.1%, and 0.25% DNCB, as described in OECD TG 429 Annex 1 Paragraph 9. As for HCA, the results obtained during the WLR test were used for the BLR evaluation.

BLR was assessed following Protocol 1.1 after the WLR evaluation phase. Lead Laboratory 2 did not conduct a BLR test since it participated in this validation study only after the BLR evaluation, which met OECD criteria.

Inter-laboratory reproducibility (from OECD TG 429 ANNEX 1)
'Inter-laboratory reproducibility of a new or modified LLNA test method should be assessed using two sensitizing substances that are well characterized in the LLNA. The LLNA PS is based on the variability of results from tests of HCA and 2, 4-dinitrochlorobenzene (DNCB) in different laboratories. ECt values should be derived independently from a single study conducted in at least three separate laboratories. To demonstrate acceptable inter-laboratory reproducibility, each laboratory should obtain ECt values of 5% to 20% for HCA and 0.025% to 0.1% for DNCB, which represents the range of 0.5~2.0 times the mean EC3 concentrations specified for HCA (10%) and DNCB (0.05%), respectively, in the LLNA.'

3.4. Module 5. Predictive capacity

After the evaluation of reproducibility was completed, the predictive capacity of the LLNA: BrdU-FCM was assessed to determine whether the assay could reliably distinguish between skin sensitizers and non-sensitizers in accordance with OECD TG 429 Annex 1 Paragraph 7. Reference chemicals with high purity, listed in Table 2, were purchased and distributed to each testing site. These chemicals were coded and tested under blind conditions.

The 1st evaluation test was performed at Lead Laboratory 1 using 18 essential reference chemicals. The 2nd test was conducted by Lead Laboratory 2 and Participating Laboratories 1 and 2 using 12 of the 22 reference chemicals. In the 3rd test, 18 essential reference chemicals were tested at Participating Laboratory 2 (see Table 1).

Defined reliability and accuracy standards (from OECD TG 429 ANNEX 1)
'The accuracy of a similar or modified LLNA test method should meet or exceed that of the LLNA PS when it is evaluated using the 18 minimum reference substances that should be used. The new or modified test method should result in the correct classification based on a "yes/no" decision. However, the new or modified test method might not correctly classify all of the minimum reference substances that should be used. If, for example, one of the weak sensitizers were misclassified, a rationale for the misclassification and appropriate additional data (*e.g.* test results that provide correct classifications for other substances with physical, chemical, and sensitizing properties similar to those of the misclassified reference substance) could be considered to demonstrate equivalent performance. Under such circumstances, the validation status of the new or modified LLNA test method would be evaluated on a case-by-case basis.'

Table 1. Summary of the predictive capacity of the test method

	No. of test substances	Testing facility	Protocol
1st test	18 essential chemicals	Lead Laboratory 1	1.1
2nd test	14 essential and optional chemicals	Lead Laboratory 2, Participating Laboratories 1 and 2	1.2
3rd test	18 essential chemicals	Participating Laboratory 2	1.3
Supplementary test	4 optional chemicals	Participating Laboratory 2	1.3

3.5. Selection of test substances

The VMT selected the reference chemicals listed in OECD TG 429 PS (see Table 2). The lot number, purity, code number, and other information for each chemical are outlined in Annex 2.

Table 2. List of the reference chemicals selected for the evaluation of predictive capacity

No.	Chemical name	CAS No.	Physical State	Veh.	EC3 (%)	0.5x~2.0x EC3	LLNA vs. GP	LLNA vs. Human
18 OECD-recommended essential reference chemicals								
1	CMI/ MI	26172-55-4/ 2682-20-4	Liquid	DMF	0.009	0.0045-0.018	+/+	+/+
2	DNCB	97-00-7	Solid	AOO	0.049	0.025-0.099	+/+	+/+
3	4-Phenylenediamine	106-50-3	Solid	AOO	0.11	0.055-0.22	+/+	+/+
4	Cobalt chloride	7646-79-9	Solid	DMSO	0.6	0.3-1.2	+/+	+/+
5	Isoeugenol	97-54-1	Liquid	AOO	1.5	0.77-3.1	+/+	+/+
6	2-Mercaptobenzothiazole	149-30-4	Solid	DMF	1.7	0.85-3.4	+/+	+/+
7	Citral	5392-40-5	Liquid	AOO	9.2	4.6-18.3	+/+	+/+
8	HCA	101-86-0	Liquid	AOO	9.7	4.8-19.5	+/+	+/+
9	Eugenol	97-53-0	Liquid	AOO	10.1	5.05-20.2	+/+	+/+
10	Phenyl benzoate	93-99-2	Solid	AOO	13.6	6.8-27.2	+/+	+/+
11	Cinnamic alcohol	104-54-1	Solid	AOO	21	10.5-42	+/+	+/+
12	Imidazolidinyl urea	39236-46-9	Solid	DMF	24	12-48	+/+	+/+
13	Methyl methacrylate	80-62-6	Liquid	AOO	90	45-100	+/+	+/+
14	Chlorobenzene	108-90-7	Liquid	AOO	25	NA	-/-	-/*
15	Isopropanol	67-63-0	Liquid	AOO	50	NA	-/-	-/+
16	Lactic acid	50-21-5	Liquid	DMSO	25	NA	-/-	-/*
17	Methyl salicylate	119-36-8	Liquid	AOO	20	NA	-/-	-/-
18	Salicylic acid	69-72-7	Solid	AOO	25	NA	-/-	-/-

OECD optional substances to demonstrate improved performance relative to the LLNA								
19	Sodium lauryl sulphate	151-21-3	Solid	DMF	8.1	4.05-16.2	+/-	+/-
20	Ethylene glycol dimethacrylate	97-90-5	Liquid	MEK	28	14-56	+/-	+/+
21	Xylene	1330-20-7	Liquid	AOO	95.8	47.9-100	+/**	+/-
22	Nickel chloride	7718-54-9	Solid	DMSO	5	NA	-/+	-/+

AOO, acetone: olive oil (4:1, v/v); CAS No., Chemical Abstracts Service Number; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulphoxide; MEK, methyl ethyl ketone; EC3, estimated concentration needed to produce a SI of 3; GP, guinea pig test result (*i.e.* TG 406); CMI/ MI, 5-Chloro-2-methyl-4-isothiazolin-3-one/ 2-methyl-4-isothiazolin-3-one; DNCB, 2,4-dinitrochlorobenzene; HCA, hexyl cinnamic aldehyde; LLNA, murine local lymph node assay result (*i.e.* TG 429); NA, not applicable since SI <3; Veh, test vehicle.

*, Presumed to be a non-sensitizer in humans based on the fact that no clinical patch test results were located, it is not included as a patch test kit allergen, and no case reports of human sensitization were located.
**, GP data not available.

4. Purchase, coding, and distribution of test substances

The HCA used for the transferability evaluation was purchased and tested by each laboratory. The reference chemicals selected for use in the predictive capacity phase and eugenol for the proficiency assessment were purchased and distributed by Lead Laboratory 1 and KoCVAM under blind conditions.

KoCVAM coded and distributed the test substances for the predictive capacity evaluation. Chemical names were unveiled by the chemical manager after each phase was completed, and raw data were sent to the biostatistician. The chemical coding and distribution procedures are described in Annex 3.

Material Safety Data Sheets (MSDS), which can be used when an emergency occurs, were individually sealed and sent to each laboratory. The unopened MSDS were returned and checked by the VMT.

5. Raw data management and statistical analysis

5.1. Raw data management

The raw data obtained from each participating laboratory were recorded in a template (Excel spreadsheet format) created by the VMT (see Annex 4) and were returned to the VMT by e-mail. The template was locked so that laboratories could not change data and data errors were minimized.

5.2. Statistical analysis

Descriptive statistics were used during Modules 2 and 4 to determine whether ECt values were within the range of 0.5–2×, as described in OECD TG 429 Annex 1 PS. In Module 5, sensitivity, specificity, and overall accuracy were calculated based on 'concordance classification'. ECt values were also calculated using the results of the reproducibility and predictive capacity evaluations. Statistical reports are attached as Annex 9.

III. Test definition (Module 1)

1. Purpose of the test method

The LLNA is a skin sensitization test method used to measure the proliferation of murine local LNCs in response to topical exposure to chemicals. This test method evaluates T cell proliferation, which is the 4th event in the skin sensitization AOP (OECD, 2015). The LLNA: BrdU-FCM can be used to identify skin-sensitizing substances and assess the skin sensitization potential of chemicals in the same way as the LLNA, the LLNA: BrdU-ELISA, and the LLNA: DA.

The OECD adopted the LLNA as a test guideline in 2002. The test method was then improved by establishing non-radio-isotopic versions, LLNA: DA and LLNA: BrdU-ELISA, and these two methods were also adopted by the OECD (OECD, 2010a & 2010b). The LLNA was also adopted by the U.S. EPA's Health Effects Test Guideline in 2003 (EPA, 2003). The LLNA: BrdU-FCM is a non-radioisotopic method that is expected to be used widely.

The LLNA: BrdU-FCM can also contribute to the implementation of the REACH program, which highlights the importance of alternative test methods to assess the safety of chemicals, and address the 7th revision of Directive 2003/15/EC of the European Parliament and the Council (European Union, 2003), which states the need to establish alternative test methods for developing cosmetics and to reduce *in vivo* animal testing.

Ultimately, the LLNA: BrdU-FCM is expected to be used in classifying chemicals as skin sensitizers or non-sensitizers, as defined by the United Nations (UN) Globally Harmonized System of Classification and Labelling of Chemicals (GHS) and the Classification, Labelling, and Packaging (CLP) Regulation, and in evaluating the skin sensitization potency of these chemicals based on calculated ECt values.

To assess the possible utility of the LLNA: BrdU-FCM in sub-categorization/potency assessment, we determined ECt values, which are used to sub-categorize sensitizers, from the LLNA. When compared with tLLNA and FCM, 2 among 22 chemicals were falsely predicted. The concordance of FCM compared with the tLLNA was 91% (20/22), while ELISA 82% (18/22) and DA 73% (16/22). When compared with human data and FCM, 6 among 22 chemicals were falsely predicted. The concordance of FCM with human data was 73% (16/22), while tLLNA 77% (17/22), ELISA 68% (15/22) and DA 73% (16/22) (Kim et al., 2016, see Table 14). Preliminary results suggested that the performance of the non-radioisotopic LLNAs in sub-categorization was comparable to that of the radioisotopic LLNA.

2. History of the test method development

The LLNA: BrdU-FCM was developed with financial support from MFDS in 2009. The assay was assessed for suitability in evaluating the skin sensitization potential of chemicals, and its protocol was optimized through research projects conducted from 2010 to 2011 (Jung et al., 2010 and 2012).

This validation study began with the support of MFDS in 2012. The VMT was then organized, the test method was transferred to the participating laboratories, and WLR was evaluated. In 2013, BLR and predictive capacity were assessed using 18 essential reference chemicals. Between 2014 and 2015, the 2nd

and 3rd test of the predictive capacity evaluation was conducted in accordance with OECD TG 429 Annex 1 Paragraph 6.

3. Scientific basis of the test method

Concern over allergic contact dermatitis and immune skin disorders induced by new chemicals or other hazardous substances is growing. Consequently, evaluation of the safety of various substances is increasingly important. This test method was designed to measure LNC proliferation, which is the 4th key event in OECD AOP for skin sensitization, and it reflects an improvement over conventional guinea pig tests by incorporating the 3Rs (refinement, reduction, and replacement).

The scientific basis of the LLNA: BrdU-FCM is similar to that of the LLNA, the LLNA: BrdU-ELISA, and the LLNA: DA. This test method uses mice instead of guinea pigs. In addition, it evaluates skin sensitization responses during the induction phase, rather than directly triggering the responses. Moreover, no radioisotopes are needed in this assay. For these reasons, the LLNA: BrdU-FCM demonstrates refinement and reduction among the 3Rs.

The basic principle underlying the LLNA: BrdU-FCM is that skin sensitizers induce the proliferation of lymphocytes in the lymph nodes adjacent to the site of test substance application (OECD, 2010). The degree of proliferation is proportional to the dose and potency of test substances (OECD, 2010). A test substance is categorized as a sensitizer when the SI is ≥ 2.7. BrdU is an analogue of the DNA precursor thymidine, and when incorporated into DNA during cell proliferation, it is easily detectable by immunological assays. The principle of the [^{3}H] thymidine-based method for detecting cell proliferation applies equally to BrdU incorporation (Takeyoshi et al, 2001). *In vivo* use of radioisotope, ^{3}H-labeled thymidine, during the experimental procedure of the LLNA is prohibitive in some countries due to the difficult disposal of radioactive carcass, which seriously deters its use. BrdU would be a good alternative in that it is non-radioactive and is based on the same assay principle (that is the incorporation of a nucleoside analogue during DNA synthesis). BrdU is incorporated into the DNA of proliferating LNCs. This incorporation is then visualized by fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibodies using fluorescence-activated cell sorting (FACS) techniques. Often, staining with a dye that binds to total DNA such as 7-amino-actinomycin D (7-AAD) is coupled with immunofluorescent BrdU staining. With this combination, two-color flow cytometric analysis enables counting and characterization of cells that are actively synthesizing DNA (BrdU positive) in terms of their cell cycle status (defined by 7-AAD staining intensities). The LLNA: BrdU-FCM quantitatively enumerates BrdU-incorporated auricular LNCs that proliferate during the induction phase of skin sensitization using flow cytometry.

Some substances (e.g. chlorobenzene, salicylic acid, lactic acid) were correctly classified as non-sensitizers in the LLNA: BrdU-FCM, resulting in a high specificity. In contrast, chlorobenzene and salicylic acid were evaluated as false positives in the LLNA: DA and lactic acid was found to be a false positive in the LLNA: BrdU-ELISA (ICCVAM, 2010a; ICCVAM, 2010b).

This test method can prevent excess pain and distress in laboratory animals and reduce the use of animals, because the pre-screen test is performed in two phases. The 1st pre-screen test begins at a concentration of 25%. If no systemic toxicity or excessive skin irritation is found at 25%, 50% and 100%, these concentrations are used in the 2nd test. However, if systemic toxicity or excessive skin irritation is found at

25%, the concentration is reduced. Therefore, severe toxicity or irritation that can be induced at 50% or 100% could be avoided. Such an approach is expected to be transferable to the other LLNA-based test methods as well.

It is also noteworthy that BALB/c mice were chosen for the LLNA: BrdU-FCM instead of CBA mice used in the existing LLNA, LLNA: BrdU-ELISA, and LLNA: DA. In some countries, the price of CBA mice is increased for imports, making them difficult to obtain. Therefore, BALB/c mice are relatively cost-effective compared to CBA mice, allowing this test method to be more widely used.

Importantly, equivalence or compatibility of these two mouse strains in the prediction of skin sensitization potency has been repeatedly demonstrated (Burns et al., 2010, Jung et al., 2010, Hou et al., 2015). DBA/2, B6C3F1, and BALB/c were all identified as appropriate mouse species that could be used in the LLNA (Woolhiser et al., 2000). This study also found that there was no statistically significant difference in the results of the LLNA: BrdU-ELISA using BALB/c and CBA/JN (Hou et al., 2015). Also, Lee et al. (2017) was conducted to compare the test results of the LLNA: BrdU-FCM using BALB/c mice with those using CBA/J mice treated with 13 sensitizers and 5 non-sensitizers, listed in OECD TG 429. As a result, the stimulation index of the LLNA: BrdU-FCM using CBA/J mice was highly correlated to those using BALB/c mice (Lee et al., 2017). However, a further study would be required to determine whether BALB/c mice can also be used in the other LLNA test methods.

Furthermore, the LLNA: BrdU-FCM directly enumerates only living LNCs that proliferate during the induction phase of skin sensitization, whereas the LLNA: BrdU-ELISA indirectly evaluates the proliferation of lymphocytes by measuring BrdU content using an antibody-based ELISA method.

The LLNA: BrdU-FCM facilitates analysis of the expression of various cell surface markers, which may help clarify the mechanism underlying the skin sensitization potential of chemicals. A small aliquot of LNCs are used for the main assay; therefore, remaining cells can be used to score B/T cells by flow cytometry. At the same time, various cytokines generated *ex vivo* by LNCs can be measured. Because of this, the test method facilitates further analyses of lymphocyte subtypes and the expression of cytokines (Lee et al., 2002, Lee et al., 2004, Jung et al., 2010 and 2012).

The LLNA: BrdU-FCM can also accommodate immunophenotyping to evaluate sensitizers. Additional endpoints like surface markers and intracellular cytokine levels, which are widely used to characterize sensitizers, can be measured in the LLNA: BrdU-FCM. Disruption in homeostasis or balance among the T cell sub-populations such as type 1 helper T cells, cytotoxic T cells, or regulatory T cells is recognized as a key event in the manifestation of contact allergies, and the cytokine production profiles from these T cells might be more valuable than simply evaluating the composition of lymphocyte sub-population.

The LLNA and its non-radioisotopic versions are already widely used in many countries to assess the skin sensitization potency of chemicals, and the LLNA: BrdU-FCM could be used for the same purpose.

4. Test method protocol

The LLNA: BrdU-FCM is a ‘me-too’ test, and it complies with the essential test method components described in OECD TG 429 Annex 1 Paragraph 5. In this method, test substances were topically applied to both ears of a mouse in the same way as the other LLNA test methods, and the auricular lymph nodes between

the jugular veins below both ears were harvested at the end of the study period. Proliferation of lymphocytes during the induction phase of skin sensitization was measured. The highest concentrations of the reference chemicals selected for this study were found not to induce systemic toxicity or excessive local irritation. Vehicle control and positive control groups were included in each test. There were at least four mice per group, and data on each mouse were collected. The two additional groups, blank group and non-treatment group, are also needed for setting a flow-cytometer. Therefore, the LLNA: BrdU-FCM is a test method similar to the other LLNA methods in performance and mechanism, and it measures the same biological effects.

All groups of animals needed are as below.

- Blank group (n=1): No BrdU injected
- Non-treatment group (n=1): Injection of BrdU without treatment with any substances
- Vehicle control-treatment group (n≥4): Injection of BrdU and treatment with a vehicle
- Test substance-treatment group (n≥4): Injection of BrdU and treatment with test substances (a minimum of three concentrations are needed)
- Positive control-treatment group (n≥4): Injection of BrdU and treatment with 25% HCA

The protocol for this test method was designed not to include any materials protected by intellectual property rights such as patents (e.g., patented kits) in the testing procedure. The final version of the protocol can be found in Annex 5.

The overall study schedule is outlined in Table 3.

Table 3. Schedule for the LLNA: BrdU-FCM main test

Experiments	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Grouping	O*	O*						
Clinical observation	O	O	O	O	O	O	O	
Treatment		O	O	O				
Measurement of body weight		O					O	
Measurement of ear thickness		O		O			O	
Irritation evaluation		O	O	O	O	O	O	
BrdU solution injection						O		
Sacrifice							O	
Measurement of ear weight							O	
Measurement of lymph node weight							O	
BrdU staining							O	●
Analysis with flow cytometry							O	●

BrdU, 5-bromo-2-deoxyuridine

●: possible to analyse samples

*: possible to group experimental animals on day 0 or day 1

- Observation of general symptoms and erythema

General symptoms were observed and recorded on a daily basis. Erythema in the chemically treated are was scored in accordance with the Draize test method each day prior to the application of test substances. The mice were weighed on days 1 and 6, and the average thickness of each ear was calculated on days 1 (before treatment), 3 (before treatment), and 6 (before autopsy).

- Application of test substances

Doses of 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc. were chosen, based on OECD TG 429. In the pre-screen test, the solubility of each chemical was tested, and the maximum concentrations were determined. The test substance or the vehicle control (25 µL) was applied to the dorsum of each ear three or more times. The substances were applied carefully in a circle using the side of a micropipette tip.

- Injection of BrdU solution

A single intraperitoneal injection of 100 µL BrdU solution (20 mg/mL) was administered to each mouse 24 ± 2 hours before sacrifice.

- Autopsy

A method of euthanasia that minimizes pain and distress in animals and is harmless to experimenters was chosen. In our study, CO_2 gas asphyxiation was used. After sacrifice, the weights of both ears and auricular lymph nodes were measured. Lymph nodes of each mouse were processed separately.

-BrdU staining

The FITC BrdU Flow Kit (Cat. No. 559619, BD Pharmingen™) was used for BrdU staining. The fluorescence staining kit selected for this assay is commercially available.

-Measurement of BrdU by flow cytometry

For the measurement of BrdU incorporated LNCs by flow cytometry (BD FACSCalibur™ or Beckman Coulter Cytomics FC 500), blank, non-treatment, vehicle control-treatment and test substance-treatment samples were prepared before the first measurement. To analyse test results, FSC-SSC and 7-AAD-BrdU graphs were drawn. The Q2 area was then established using blank and non-treatment samples such that the % BrdU-positive LNCs would occupy 1% of that area (see Figure 4). This set-up was used for all tests. The proliferation of lymphocytes was estimated with a gated percent calculated in the Q2 area, and the number of lymphocyte cells was counted.

4.1. Selection of the vehicle and highest concentrations for the main study

AOO, recommended in OECD TG 429, was used as a vehicle for the optimization of the test method and the evaluation of transferability, WLR, and BLR. In the predictive capacity evaluation phase, the participating laboratories were required to follow the vehicle selection procedure as illustrated in Figure 2. The coded test substances were distributed to the performing laboratories, without any information on the vehicle. A vehicle with the highest solubility was selected as the test substance. Lead Laboratory 1 trained the participating laboratories on the procedure (see Figure 2 and Annex 5 for details).

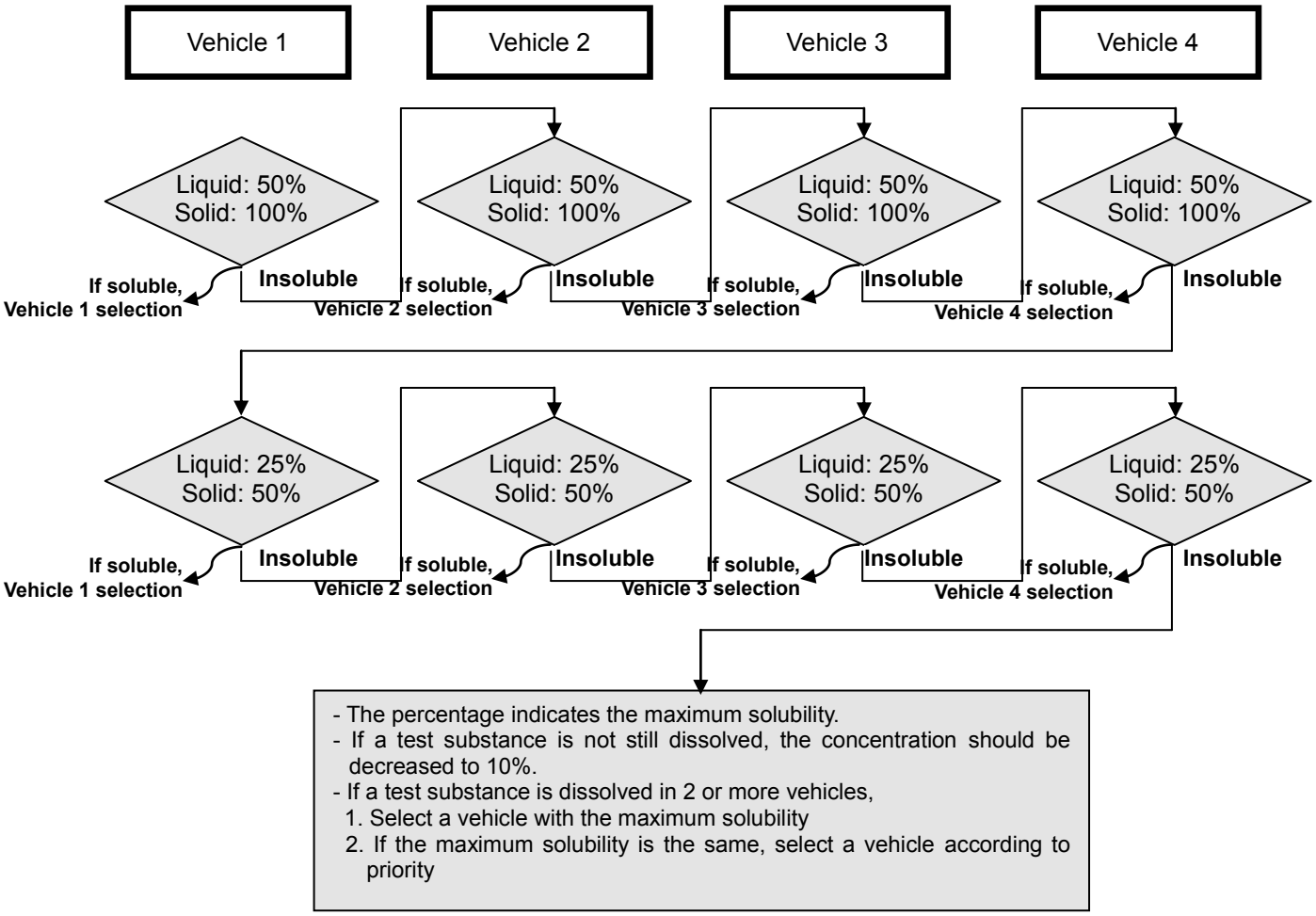


Figure 2. Procedure for selecting vehicle

Test concentrations for the main test are determined in Module 5 (predictive capacity) since it was conducted in coded status. The sufficient number (minimum three) of concentrations that did not induce systemic toxicity or excessive skin irritation in the 1st and 2nd pre-screen tests were selected (see Figure 3 and Annex 5 for details). If a test substance is applied first at a concentration of 100%, extreme toxicity could be induced. For this reason, the 1st pre-screen test was performed at 25%, and in the 2nd test, the highest concentration was selected based on the results of the 1st pre-screen test. If no systemic toxicity or excessive skin irritation is found at 25%, only 50% and 100% concentrations are tested in the 2nd test, and lower concentrations (less than 25%) were not required. If systemic toxicity or excessive skin irritation was found at 25%, the concentrations are decreased, because severe toxicity would be induced at 50% or 100%. This 2-stage strategy could prevents excess pain and distress in laboratory animals and reduces animal testing. In addition, all information on the test substance such as the chemical structure, physicochemical properties and data from any available relevant toxicological studies, including those on structurally related test substances, should be taken into account for the dose selection before conducting pre-screen test.

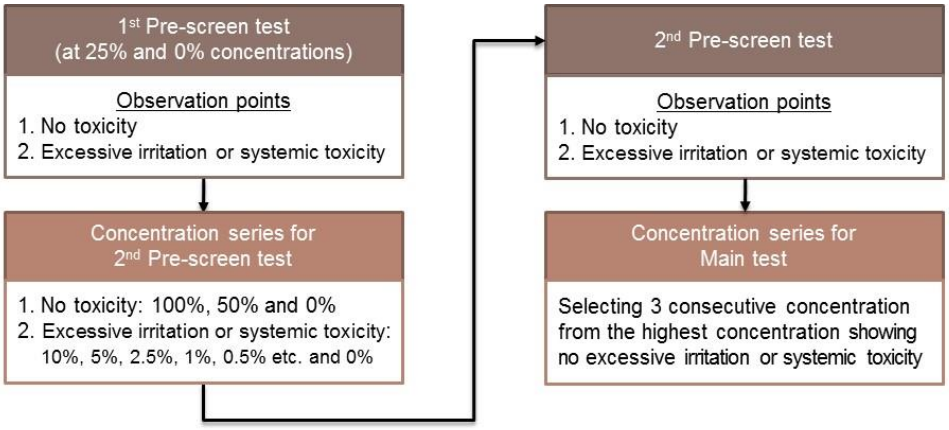


Figure 3. Procedure for selection of the highest concentration to be tested

4.2. Evaluation of skin sensitization potency

Murine auricular lymph nodes were taken from the four different groups (defined below) to determine the proportion of BrdU-incorporated lymphocytes by flow cytometry.

Blank group (n = 1): No BrdU injected or treatment with test substances
Non-treatment group (n = 1): Injection of BrdU without treatment with test substances
Vehicle control-treatment group (n ≥ 4): Injection of BrdU and treatment with a vehicle
Test substance-treatment group (n ≥ 4): Injection of BrdU and treatment with test substances
Positive control-treatment group (n ≥ 4): Injection of BrdU and treatment with a positive substance

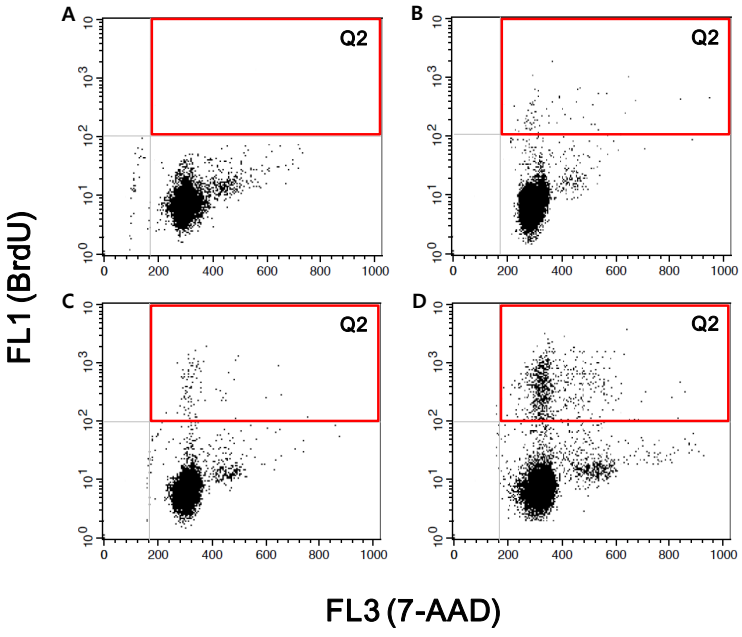


Figure 4. Flow cytometry set-up for the calculation of BrdU-positive lymphocytes

To calculate the SI, flow cytometry was set up as below (see Figure 4):

A: Set up the Q2 area (upper right) using the blank sample so that it contains no cells.
B: Set up the Q2 area again using the non-treatment sample such that the % BrdU-positive LNCs represent about 1% of the cells in it.
C, D: Analyse the vehicle control-treatment samples, the test substance-treatment samples and positive control-treatment samples, and calculate the gated percent in the Q2 area that was set up in steps A and B.

The SI was calculated as follows:

A gated percent in the Q2 area, which was established for each mouse in the vehicle control group, was calculated. The gated percent was then multiplied by the number of LNCs (cells/LN) to determine the number of BrdU-positive LNCs for each mouse. The mean number of BrdU-positive LNCs in all mice relative to the vehicle control group was calculated (1).

A gated percent in the Q2 area, which was established for each mouse in the test substance treatment group, was calculated. The gated percent was then multiplied by the number of LNCs (cells/LN) to determine the number of BrdU-positive LNCs (2).

The SI for each mouse in the test substance treatment group was calculated by dividing (2) by (1).

Number of BrdU-positive LNCs = percentage of BrdU-positive cells (= percent of Q2) × the number of LNCs (cells/LN)

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

4.3. Modifications made to the test protocol

The LLNA: BrdU-FCM protocol was revised three times since 2012, and major revisions are summarized in Tables 3–5.
The tests conducted in each version of the protocol are listed below.

- Version 1.0: Transferability, proficiency, and WLR (Lead Laboratory 1, Participating Laboratories 1 and 2)
- Version 1.1: BLR (Lead Laboratory 1, Participating Laboratories 1 and 2) and the 1st test of the predictive capacity evaluation (Lead Laboratory 1)
- Version 1.2: Proficiency (Lead Laboratory 2) and the 2nd test of the predictive capacity evaluation (Lead Laboratory 2, Participating Laboratories 1 and 2)
- Version 1.3: Additional test (Lead Laboratory 2), the 3rd test of the predictive capacity evaluation and supplementary test on the 4 optional chemicals (Participating Laboratory 2)

Protocol 1.0 was used for the transferability, proficiency, and WLR tests in 2012. The protocol for the pre-screen test to select vehicles, solubility, and maximum concentrations was not included in Protocol 1.0.

Protocol 1.1 was revised on 22 April 2013, and the protocol for the pre-screen test to select vehicles, solubility, and maximum concentrations was included as an annex. The pre-screen test was performed in two phases. To reduce animal testing, the 1st test was conducted at a concentration of 25%, which was expected not to induce skin irritation. The 2nd test was conducted without the 1st test when the maximum soluble concentration was 10% or less. The maximum concentration varied depending on the 1st test results, and four to five concentrations were used in the second test. Two mice were used per concentration. In the 2nd test, three concentrations that did not induce systemic toxicity or excessive irritation were selected as the maximum concentrations for the main test. All participating laboratories were involved in the protocol revision, and Protocol 1.1 was used for the BLR test without pre-screen test and 1st test of the predictive capacity evaluation (see Table 4). Since the protocol revision concerns mostly vehicle selection and solubility steps, it is not expected to affect the WLR and BLR that were assessed using HCA and DNCB. These two substances did not go through the pre-screen test.

Table 4. Major modifications of Protocol 1.0

Version (Approval date)	Comparison of the old and new versions		Rationale for revision
	Old version	New version	
Protocol 1.1 (22 April 2013)	-	**Annex. Pre-Screen Test** 1. Objectives 2. Materials 2.1 Test animals 2.2 Test substances and vehicles 3. Experimental procedure 3.1 Summary of experimental design 3.2 Selection of vehicles 3.3 Dose selection 3.4 Application of test substances 3.5 Observation 3.6 Autopsy (2nd test) 4. Results 4.1 1st pre-screen test 4.2 2nd pre-screen test 5. GLP compliance	Addition of the pre-screen test for the selection of treatment doses and vehicles

The VMT requested an additional test after analysing the results of the predictive capacity evaluation performed according to Protocol 1.1. In response to its request, the participating laboratories tested four optional reference chemicals listed in OECD TG 429 Annex 1, with methyl ethyl ketone (MEK) as the chosen vehicle. In addition, concentrations that were not recommended in the OECD test guidelines were excluded from Protocol 1.1. The solubility test procedure was also modified. All of these revisions were included in Protocol 1.2, along with detailed methods for setting up the FSC-SSC and 7-AAD-BrdU graphs. Protocol 1.2 was approved on 1 May 2014. Protocol 1.2 was used for the proficiency test conducted by Lead Laboratory 2 and the 2nd test of the predictive capacity evaluation in 2014 (see Table 5).

Table 5. Major modifications of Protocol 1.1

Version (Approval date)	Comparison of the old and new versions		Rationale for Revision
	Old version	New version	
Protocol 1.2 (1 May 2014)	**Main body** 3.13 Measurement of BrdU content by flow cytometry 3.13.1 Preparations prior to measurement 3.13.2 Analysis of flow cytometry results	**Main body** 3.10 Measurement of BrdU content by flow cytometry 3.10.1 Preparations prior to measurement 3.10.2 Analysis of flow cytometry results (1) FSC-SSC graph (2) 7-AAD-BrdU graph (3) Analysis of results	Addition of a more detailed description on BrdU content measurement and construction of the FSC-SSC and 7-AAD-BrdU graphs
	Annex. Pre-Screen Test 2.2 Test substances and vehicles	**Annex. Pre-Screen Test** 2.2 Test substances and vehicles Addition of MEK as a vehicle	Addition of MEK as a vehicle for ethylene glycol dimethacrylate
	3.2 Selection of vehicles A solubility test was conducted (liquid at 75%, solid at 100%) to prioritize vehicles (AOO, DMF, DMSO) in the order of solubility and select optimal vehicles. A liquid should be sufficiently dissolved, using a vortex, because of its density. A vehicle control should be used as a vehicle. The vehicle selection procedure is as follows: <Figure>	3.2 Selection of vehicles As described in 3.2.1, a solubility test was conducted to prioritize vehicles (AOO, DMF, MEK, DMSO) in the order of solubility, and optimal vehicles were chosen. <Figure> 3.2.1 Solubility test	Omission of the 75% dose in vehicle selection following OECD TG 429
	3.3 Dose selection The 1st test was performed at a 25% concentration using the vehicle control group. The 2nd test was implemented without the 1st test when the maximum soluble concentration was 10% or less, and the maximum soluble concentration was used as the maximum concentration. The maximum concentration for the 2nd test was selected based on results of the 1st test, and a vehicle control group was included in the 2nd test. (1) Death or systemic toxicity: 2.5%, 1%, 0.5%, 0.25%, 0.1% (5 doses were chosen, as severe toxicity could be induced) (2) Irritation: 10%, 5%, 2.5%, 1% (3) Non-irritation: 100%, 75%, 50%, 25%	3.3 Dose selection The 1st test was performed at a 25% concentration using the vehicle control group. The 2nd test was implemented without the 1st test when the maximum soluble concentration was 10% or less, and the maximum soluble concentration was used as the maximum concentration. The maximum concentration for the 2nd test was selected based on results of the 1st test, and a vehicle control group was included in the 2nd test. (1) Systemic toxicity or severe irritation: 10%, 5%, 2.5%, 1%, 0.5%, 0.25%, 0.1% (2) Non-irritation: 100%, 50%	Addition of a more detailed description on the test Omission of the 75% dose

BrdU, 5-bromo-2-deoxyuridine; AOO, acetone: olive oil (4:1, v/v); DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulphoxide; MEK, methyl ethyl ketone

SI 2.7, which was calculated based on the results of a statistical analysis of previous data, and an improved solubility test procedure for substances that are viscous and hard to dissolve (i.e. imidazolidinyl urea) were included in Protocol 1.3. Imidazolidinyl urea was tested again according to Protocol 1.3, but these results were not presented in the results of the final predictive capacity evaluation. The VMT approved Protocol 1.3 on 1 June 2015. The 3rd test of the predictive capacity evaluation was conducted using all 18 essential substances according to Protocol 1.3 in 2015 (see Table 6).

Table 6. Major modifications of Protocol 1.2

Version (Approval date)	Comparison of the old and new versions		Rationale for revision
	Old version	New version	
Protocol 1.3 (1 June 2015)	**Main body** 4.3 Criteria for evaluating skin sensitization When the SI is ≥ 3, test substances are classified as sensitizers. When the SI is < 3, test substances are classified as non-sensitizers. **Annex. Pre-Screen Test** 3.2.1 Solubility test (2) Magnetic stir for 30 minutes and then shake for 1 minute to sufficiently dissolve test substances.	**Main body** 4.3 Criteria for evaluating skin sensitization When the SI is ≥ 2.7, test substances are classified as sensitizers. When the SI is <2.7, test substances are classified as non-sensitizers. **Pre-Screen Test** 3.2.1 Solubility test (2) Magnetic stir for 30–60 minutes and then shake for 1 minute to sufficiently dissolve test substances. Add the test substances gradually to avoid lumpiness. If the solution is viscous, do magnetic stirring for 60 minutes.	Revision of the criteria for statistical analysis Modification to improve the solubility test procedure

The protocol version 1.3 was updated (version 1.3.1) to reflect refinement of terminology, information on reagents or equipment, and more detailed descriptions on flow cytometric analysis, and so on, which was recommended by the OECD peer review panels.

4.4. Evaluation of cut-off values

The optimal SI threshold was determined by statistical analysis of the results of WLR and BLR tests and the 1st, 2nd, and 3rd predictive capacity evaluations (see Annex 9).

ECt values of HCA and DNCB for the evaluation of WLR and BLR.

Tests using HCA (5%, 10%, 25%) and DNCB (0.05%, 0.1%, 0.25%) were conducted four times and three times, respectively, by all three laboratories. When the SI threshold was 3.0, the ECt deviated slightly from the OECD criteria. Subsequently, ECt values were re-calculated with thresholds ranging from 2.0–4.0 to obtain an optimal cut-off threshold for WLR and BLR.

As a consequence, both HCA (5–20%) and DNCB (0.025–0.1%) at ECt values of 2.5, 2.6, and 2.7 met OECD TG 429 criteria (Figure 5).

The optimal threshold was then calculated, based on the mean ECt values and standard deviation and whether the mean of ECt values of HCA is close to 10%, the mean value described in TG429 and the mean of ECt values of DNCB is close to 0.05%.

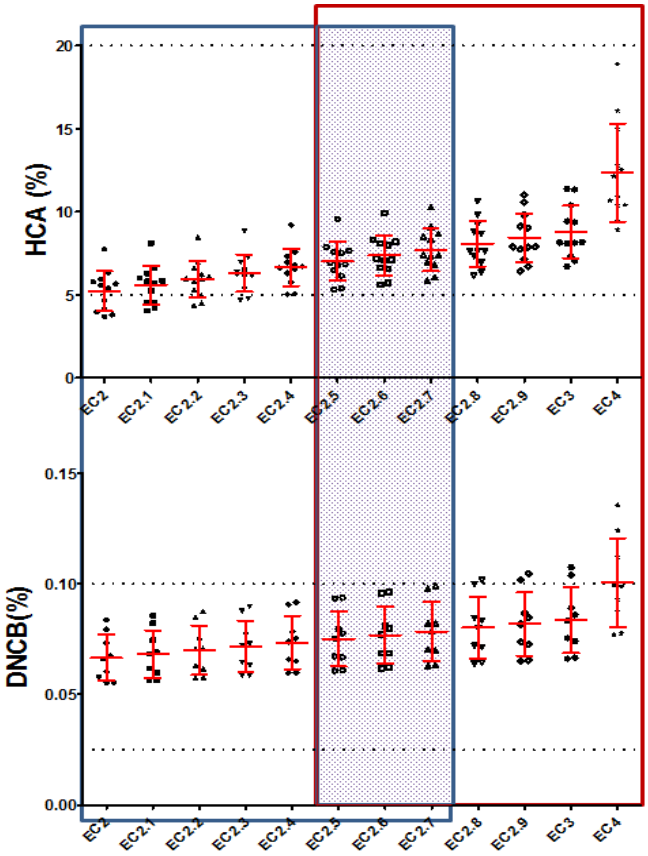


Figure 5. ECt values for HCA and DNCB obtained from all three laboratories when thresholds varied from 2.0 to 4.0. The bars represent means ± standard deviations. Dotted lines: upper and lower limits of acceptable ECt of HCA and DNCB. Red square: ECts of HCA with HCAs that fell within acceptable ranges. Blue square: ECts of DNCB that fell within acceptable ranges for DNCB. Shaded region: ECts that satisfied both criteria for HCA and DNCB.

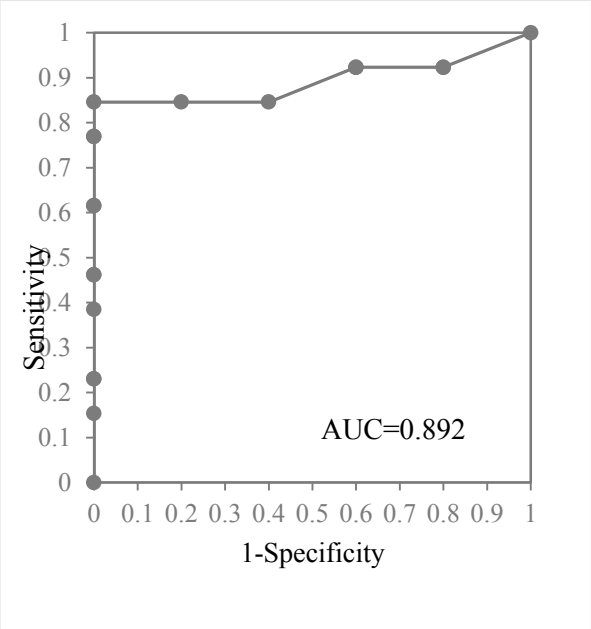
Threshold values for the predictive capacity evaluation

The SI was calculated in compliance with GLP and OECD TG 429 Annex 1 PS. To determine the optimal cut-off value, a receiver operating characteristic (ROC) curve was drawn which has been widely used for diagnosis and alternative test methods (Lin et al., 2002 and Greiner et al., 1995).

The ROC curve was calculated from the maximum SI values at the tested concentrations (low, middle, and high concentrations) of 18 test substances when the 1st and 2nd tests of the predictive capacity evaluation were completed. As a result, optimal sensitivity and specificity were obtained at 2.66 ≤ cut-off value < 4.66 (ROC area under the curve [AUC] = 0.885).

In view of the results of the BLR and WLR evaluation, SI 2.7 was selected from among the optimal SI values (2.5, 2.6, and 2.7), because the highest sensitivity was obtained at SI 2.7. This was reflected in Protocol 1.3.

A ROC analysis of the 3rd test results was conducted. The AUC of ROC was 0.892, which was close to the 0.885 calculated in the 1st and 2nd tests. In addition, the highest accuracy was shown at 2.6 ≤ SI < 2.8 (see Figure 6). Consequently, the predictive capacity was optimized using a cut-off value of SI 2.7 in the 3rd test.



Cut-off	sensitivity	1-specificity	accuracy
0 ≤SI< 0.9	1.00	1.00	0.72
0.9 ≤SI< 1.3	0.92	0.80	0.72
1.3 ≤SI< 1.7	0.92	0.60	0.78
1.7 ≤SI< 1.8	0.85	0.40	0.78
1.8 ≤SI< 2.6	0.85	0.20	0.83
2.6 ≤SI< 2.8	**0.85**	**0.00**	**0.89**
2.8 ≤SI< 4.1	0.77	0.00	0.83
4.1 ≤SI< 8.9	0.62	0.00	0.72
8.9 ≤SI< 12.8	0.46	0.00	0.61
12.8 ≤SI< 13.6	0.38	0.00	0.56
13.6 ≤SI< 34.9	0.23	0.00	0.44
34.9 ≤SI< 47.3	0.15	0.00	0.39
47.3 ≤SI	0.00	0.00	0.28

Figure 6. ROC curve established in the 3rd test of the predictive capacity evaluation, and sensitivity, specificity, and accuracy determined with each cut-off value

4.5. Technical limitations of the test method

Common limitations of the LLNA, LLNA: DA, and LLNA: BrdU-ELISA are as follows:

- Animals are still used in tests, even though mice are used instead of guinea pigs.
- Some test substances could be determined false negatives due to their physicochemical properties (e.g., certain metal salts appear as false negatives because they affect skin penetration rates).
- Some test substances could be determined false positives because of their physicochemical properties (e.g., presence of surfactants).

According to the validation study on the LLNA: BrdU-FCM using 18 essential reference chemicals listed in the TG 429 PS, the LLNA: BrdU-FCM has a limitation since one moderate skin sensitizer, 2-mercaptobenzothiazole, and one borderline skin sensitizer, methyl methacrylate, were misclassified in the LLNA: BrdU-FCM.

5. Conclusion of the VMT on Module 1

The VMT believed that the LLNA: BrdU-FCM could complement existing LLNA test methods because it uses a more accessible mouse strain and provides the opportunity to mechanistically evaluate the skin sensitization potential of chemicals (e.g., B/T cell distribution, intercellular signalling).

The VMT concluded that Module 1 clearly described the purpose, scientific basis, and protocol (Annex 5) for the LLNA: BrdU-FCM, as well as the test method development procedure. The VMT also believed that the protocol was systematically established, its revision history (version 1.0, 1.1, 1.2, 1.3) was well documented, and the criteria for evaluating skin sensitization and pre-screen test results were properly described in the protocol.

In addition, the VMT decided to change the SI from 3.0 to 2.7 as a result of a statistical analysis and so that it could be applied to other test substances.

IV. Transferability (Module 3)

1. Training and transfer of the test method

1.1. Technical training

Lead Laboratory 1 trained Participating Laboratories 1 and 2 on the test method and Protocol 1.0 on 14 May 2012. After the training, a workshop was held by KoCVAM at NIFDS on 15 May 2012 to instruct Lead Laboratory 1 and Participating Laboratories 1 and 2 on flow cytometry and demonstrate the measurement of BrdU content.

Lead Laboratory 1 then visited each testing site during the test method transfer in order to determine whether the assay procedure was clearly understood and properly implemented. (Participating Laboratory 1: 23 May 2012; Participating Laboratory 2: 21 May 2012). Each participating laboratory repeated the transferability confirmation test two to four more times on its own.

Successful transfer of the test method to two Participating Laboratories was confirmed from May to June 2012) in accordance with OECD TG 429 Paragraph 11 using 25% HCA as a positive control and AOO as a vehicle control.

Lead Laboratory 2 received theoretical and procedural training from Lead Laboratory 1 on 26 February 2014. Lead Laboratory 2 then conducted a test using 25% HCA and AOO in order to determine whether the test method was properly transferred (see Annex 6).

Lead Laboratory 2 trained the participating laboratories on Protocols 1.2 and 1.3 during the 2nd and 3rd tests of the predictive capacity evaluation on 26 May 2014 and 15 September 2015, respectively.

1.2. Considerations

Lead Laboratory 1 was required to check each participating laboratory's proficiency in flow cytometry operation. Any personnel who joined in the middle of the validation study had to demonstrate a successful transferability test. The VMT purchased the test substances chosen for the transfer phase and distributed them to each testing site. The SI for 25% HCA should be ≥ 3 compared to the vehicle control group, as described in OECD TG 429 Paragraph 11.

1.3. Results

All laboratories met the transferability criteria because the SIs for 25% HCA were ≥3 (see Table 7).

Table 7. Results of transferability tests

	Test	SI※	SI (Mean ± SD)	Result
Participating Laboratory 1	1	4.8, 8.9, 15.8, 18.1	11.9 ± 6.1	Pass
	2	1.3, 5.5, 14.4, 16.3, 17.7	11.0 ± 7.3	Pass
Participating Laboratory 2	1	10.6, 13.0, 14.3	12.6 ± 1.9	Pass
	2	13.5, 20.9, 25.8, 27.6, 29.8	23.5 ± 6.5	Pass
Lead Laboratory 2	1	3.8, 5.4, 7.0, 8.0	6.1 ± 1.8	Pass

※The SI for 25% HCA was calculated for each mouse. SI, stimulation index.

In October 2013, personnel in Participating Laboratory 1 changed. Transferability was successfully demonstrated because the SI for 25% HCA was 11.15 ± 2.43. Therefore, it was possible for Participating Laboratory 1 to change its study personnel in January 2014.

2. Demonstration of proficiency in the test method

Each participating laboratory conducted a proficiency test using coded test substances. Chemicals were assigned to the laboratories, and their names were not unveiled until the test was completed. The proficiency test was performed at Participating Laboratory 1 on 19–25 October 2012, Participating Laboratory 2 on 27 June–3 July 2012, and Lead Laboratory 2 on 20–26 August 2014. The proficiency of each participating laboratory was evaluated using 25% HCA and 5%, 10%, and 25% eugenol.

Information on the vehicle (AOO) and test doses (5%, 10%, 25%) was given to Participating Laboratories 1 and 2. Lead Laboratory 2 also conducted a proficiency test without knowing the test doses. The skin sensitization potency of eugenol as suggested in OECD TG 429 Annex 1 is as follows:

Eugenol		
Mean EC3	Actual EC3	0.5–2x
10.1	4.9–15	5.05–20.2

The EC3 concentration should be in the range of 5.05–20.2%, as described in OECD TG 429 Annex 1.

2.1. Results of the proficiency test

Because 0.5–2× ECt values for eugenol were within the range of 5.05–20.2% at each participating laboratory (Participating Laboratory 1: 8.2%; Participating Laboratory 2: 11.1%), the VMT concluded that the laboratories were proficient in the test methods (see Table 8).

Lead Laboratory 2 also performed a proficiency test. Its proficiency was demonstrated by ECt values for eugenol (5%, 10%, 25%) that were within the range of 5.05–20.2%.

Table 8. Results of the proficiency test

	Eugenol concentration (%)	SI	EC3※	Result
Participating Laboratory 1	5	1.3	8.2	Pass
	10	4.4		
	25	8.6		
Participating Laboratory 2	5	2.1	11.1	Pass
	10	2.5		
	25	5.8		
Lead Laboratory 2	5	1.3	13.3	Pass
	10	2.8		
	25	4.9		

※EC3: Estimated concentration of a test substance needed to produce a SI of 3

3. Conclusion of the VMT on Module 3

All laboratories met the acceptance criteria for transferability, namely, the SI for 25% HCA was ≥ 3.0. The SI values calculated by Participating Laboratory 1 showed larger variation among the mice than those by Participating Laboratory 2. However, the VMT determined that consistent results were produced, as the means of the SI values were concentrated around 11 in both the 1st and 2nd tests. In addition, a large gap was observed between the means of the SI values generated in the 1st and 2nd tests by Participating Laboratory 2, though a same Lot of FITC BrdU Flow kit (Lot No. 35452) was used throughout the tests. But the means of the SI values obtained by Participating Laboratory 2 in the 1st and 2nd tests exceeded 11. The VMT suggested that the sensitivity of laboratory equipment should be checked as the SI for 25% HCA exceeded 20 at Participating Laboratory 2. The reason for variation was further discussed and explained by the lack of experience of the laboratory with the assay at the initial stages of the validation study.

Proper transfer of the test methods from Lead Laboratory 1 to 2 was confirmed, and the results of testing with HCA and eugenol according to Protocol 1.2 were acceptable.

V. Within-laboratory reproducibility (Module 2)

1. Considerations

WLR was evaluated using 5%, 10%, and 25% HCA four times at intervals of one week or more. The ECt concentration should be within the range of 5–20%, as described in OECD TG 429 Annex 1. Based on a statistical analysis of results from the WLR, BLR, and predictive capacity evaluations, those results improved using an SI threshold of 2.7 rather than at 3.0. Therefore, EC 2.7 was applied in evaluating the test results.

The WLR evaluation was repeated four times at Lead Laboratory 1 and Participating Laboratories 1 and 2 from May 2012 to April 2013 in accordance with Protocol 1.0.

Although the WLR evaluation was conducted according to Protocol 1.0, the WLR results were considered compatible with Protocol 1.3, because the protocol revision mostly concerned the solubilisation of chemicals unrelated to HCA. WLR was analysed using the cut-off SI value of 2.7, which was suggested to be optimal based on a *post hoc* statistical analysis of the WLR, BLR, and predictive capacity (See 4.4).

2. Results of the WLR test

Since the 0.5–2× ECt values for HCA were in the range of 5–20% in the four repeated tests performed by all participating laboratories, the VMT concluded that the WLR of the LLNA: BrdU-FCM was successfully demonstrated (see Table 9, and Attachment 1).

Table 9. Results of the WLR test

	Run	SI for each HCA concentration (%)			EC2.7 (%)	Result
		5	10	25		
Lead Laboratory 1	1	1.8	3.6	10.3	7.40	Pass
	2	2.3	3.1	9.2	7.34	Pass
	3	1.7	4.5	13.9	6.81	Pass
	4	1.8	3.6	9.6	7.38	Pass
Participating Laboratory 1	1	2.5	3.9	9.7	5.95	Pass
	2	1.8	3.3	4.7	9.07	Pass
	3	1.7	3.4	5.4	8.67	Pass
	4	1.8	4.0	7.1	6.96	Pass
Participating Laboratory 2	1	1.4	2.4	6.8	10.28	Pass
	2	1.8	4.7	8.1	6.03	Pass
	3	1.3	3.5	7.9	8.50	Pass
	4	1.8	3.1	7.6	8.34	Pass

HCA, hexyl cinnamic aldehyde.

Lead Laboratory 2, which joined the validation study in May 2014, did not perform a WLR test because the results obtained from 2012 to 2013 met the OECD 429 PS criteria for the WLR.

3. Conclusion of the VMT on Module 2

Because the 0.5–2× ECt values for HCA (5%, 10%, 25%) were in the range of 5–20% in the four repeated tests performed by the two participating laboratories and lead laboratory, the VMT concluded that the WLR of the LLNA: BrdU-FCM was successfully validated.

Lead Laboratory 2, which joined the validation study in May 2014, did not perform a WLR test because results meeting the criteria for WLR, as defined in OECD 429 PS, were already obtained, and unnecessary animal tests were avoided in accordance with the 3R principle. In addition, Lead Laboratory 2 was allowed to participate in the predictive capacity phase without conducting a WLR test because it demonstrated its technical proficiency during the transfer phase.

Aside from the results of the WLR evaluation performed according to the OECD TG 429 PS, lactic acid, methyl salicylate and 2-mercaptobenzothiazole were all classified into non-sensitizers in the 2nd and 3rd predictive capacity tests conducted by Participating Laboratory 2. Consequently, the WLR of the test method could be further supported (See VII. Predictive capacity).

VI. Between-laboratory reproducibility (Module 4)

1. Considerations

The BLR was evaluated using 0.05%, 0.1%, and 0.25% DNCB and 5%, 10%, and 25% HCA. The ECt concentration should be within the range of 0.025–0.1% for DNCB and 5–20% for HCA, as described in OECD TG 429 Annex 1. Based on the statistical analyses of the WLR, BLR, and predictive capacity evaluations, the results were improved using EC2.7 rather than at EC3. Therefore, EC2.7 was applied in the evaluation of test results.

BLR was evaluated at Lead Laboratory 1 and Participating Laboratories 1 and 2 from April to July 2013 in accordance with Protocol 1.1.

Although the BLR evaluation was conducted according to Protocol 1.1, the BLR results were considered compatible with Protocol 1.3, because the protocol revision mostly concerned the solubilisation of chemicals unrelated to HCA or DNCB. BLR was analysed using a cut-off SI value of 2.7, which was suggested to be optimal based on a *post hoc* statistical analysis of WLR, BLR, and predictive capacity (See 4.4).

2. Results of the BLR test

The EC2.7 values for DNCB and HCA can be found in Tables 10 and 9, respectively. The VMT concluded that BLR of the LLNA: BrdU-FCM was successfully demonstrated because all EC2.7 values were in the range of 0.5–2× ECt (see Attachment 1).

Lead Laboratory 2, which joined the validation study in May 2014, did not perform a BLR test because results meeting the guidelines of OECD 429 PS were obtained in 2013.

Table 10. Results of the BLR test

	Test	SI for each DNCB concentration (%)			EC2.7 (%)	Result
		0.05	0.1	0.25		
Lead Laboratory 1	1	1.5	3.0	13.7	0.080	Pass
	2	0.9	2.0	10.5	0.098	Pass
	3	1.5	6.1	19.5	0.063	Pass
Participating Laboratory 1	1	1.8	1.8	8.3	0.099	Pass
	2	1.1	3.7	10.0	0.082	Pass
	3	2.8	4.4	20.5	0.063	Pass
Participating Laboratory 2	1	1.3	2.6	16.2	0.082	Pass
	2	2.1	3.6	13.2	0.070	Pass
	3	1.9	4.0	16.1	0.070	Pass

DNCB, 2,4-dinitrochlorobenzene.

3. Conclusion of the VMT on Module 4

The ECt concentrations for 0.05%, 0.1%, and 0.25% DNCB should be within the range of 0.025–0.1%. The VMT concluded that the BLR of the test method was demonstrated because all EC2.7 values calculated by the Lead Laboratory and the participating laboratories were within the acceptable range and the results for HCA met the guidelines of OECD PS.

Lead Laboratory 2, which joined the validation study in May 2014, did not perform a BLR test because the results that met the acceptance criteria of BLR stated in OECD 429 PS were already produced in 2013 and unnecessary animal tests were avoided in accordance with the 3R principle.

Aside from the results of the BLR evaluation performed according to the OECD TG 429 PS, the BLR could be further supported since many test substances were concurrently used at three predictive capacity tests by three different laboratories. The results for the five test substances including isopropanol, methyl methacrylate, phenyl benzoate, cinnamic alcohol, and chlorobenzene were consistent among the three laboratories (Lead Laboratory 1 in the 1st test, Lead Laboratory 2 or Participating Laboratory 1 or Participating Laboratory 2 in the 2nd test, Participating Laboratory 2 in the 3rd test). Furthermore, 2-mercaptobenzothiazole was consistently predicted as a non-sensitizer at all three laboratories (Lead Laboratory 1 in the 1st predictive capacity test, Lead Laboratory 2, Participating Laboratories 1 and 2 in the 2nd predictive capacity test, Participating Laboratory 2 in the 3rd test) (See VII. Predictive capacity).

VII. Predictive capacity (Module 5)

The predictive capacity of the test was evaluated from 2012 to 2016 (Lead Laboratory 1 from 2012 to 2013 with Protocol 1.1; Lead Laboratory 2 and Participating Laboratories 1 and 2 in 2014 with Protocol 1.2; and Participating Laboratory 2 with Protocol 1.3) based on OECD TG 429 Annex 1, and the 18 reference chemicals listed in Table 1 were tested.

Considerations

Concordance rates between the reference chemicals were calculated. When discordant results were produced, additional supporting documents were submitted. The four optional substances listed in Table 1 were also tested, thereby demonstrating that the performance of the LLNA: BrdU-FCM is comparable to that of the existing LLNA test methods.

1. 1st predictability test (Protocol 1.1)

- Testing facility: Lead Laboratory 1
- Test period: September 2012 to October 2013

Based on results of the 1st test of the predictive capacity evaluation, sensitivity was determined to be 76.9% and specificity was found to be 60%. Therefore, the overall accuracy of the LLNA: BrdU-FCM reached 72.2% (see Attachment 1).

There are 13 skin-sensitizing chemicals among the 18 essential reference chemicals listed in OECD TG 429. Among those 13 skin sensitizers, 2-mercaptobenzothiazole (moderate sensitizer) and imidazolidinyl urea and methyl methacrylate (weak sensitizers) were misclassified. The other 10 substances were correctly classified. Among the five non-sensitizing substances, chlorobenzene and methyl salicylate were misclassified, whereas the other three substances were correctly classified.

In the pre-validation study performed from 2010 to 2011, with the support of MFDS, 2-mercaptobenzothiazole and imidazolidinyl urea that were previously evaluated as false negatives were correctly classified as sensitizers (Jung et al., 2012). In addition, chlorobenzene and methyl salicylate, also determined false positives, were classified as non-sensitizers in the pre-validation study (Jung et al., 2012). Therefore, in 2014 the VMT asked Lead Laboratory 2 and Participating Laboratories 1 and 2 to re-evaluate the predictive capacity after modifying the protocol.

In the pre-validation study with 2-mercaptobenzothiazole, vehicles were found to have an effect on test results (Jung et al., 2012). For that reason, an additional study was conducted to compare DMF and AOO.

In the VMT meeting in May 2014, Protocol 1.2 was approved. A new vehicle (MEK) was included, the solubility test was modified, and the concentration series was selected from those suggested in OECD TG 429 (75% was excluded). In accordance with Protocol 1.2, the 2nd test was conducted at the three laboratories in 2014.

2. 2nd test (Protocol 1.2)

- Testing facility: Lead Laboratory 2, Participating Laboratories 1 and 2
- Test period: August to September 2014

In the 2nd test, chlorobenzene and methyl salicylate were correctly evaluated as negatives, whereas 2-mercaptobenzothiazole, imidazolidinyl urea, and methyl methacrylate were evaluated as false negatives. The results of the 2nd test of isopropanol, lactic acid, phenyl benzoate, and cinnamic alcohol were the same as those of the 1st test.

Ethylene glycol dimethacrylate, xylene, and sodium lauryl sulphate were classified as sensitizers, whereas nickel chloride was classified as a non-sensitizer. The SI for each chemical is presented in Attachment 1 (Figures 10-2 and 10-3).

The predictive capacity of the test was assessed at Lead Laboratory 2 using the four coded substances in accordance with the revised protocol. As a result, the non-sensitizer lactic acid was classified as a non-sensitizer. Nickel chloride and ethylene glycol dimethacrylate showed the same results as those in the LLNA. Weak sensitizer imidazolidinyl urea was classified as a non-sensitizer.

Participating Laboratory 1 performed an evaluation of the predictive capacity using five coded substances. As a result of the test, non-sensitizers chlorobenzene and isopropanol were correctly classified as non-sensitizers, thereby showing improved results for chlorobenzene. In addition, phenyl benzoate and cinnamic alcohol were correctly classified as sensitizers, showing the same results as those obtained by the LLNA. Methyl methacrylate, however, was incorrectly classified as a non-sensitizer.

The five coded substances were chosen at Participating Laboratory 2. As a consequence, non-sensitizers methyl salicylate and lactic acid were correctly classified as non-sensitizers, thereby showing improved results for methyl salicylate. Xylene and sodium lauryl sulphate shoed the same results as those obtained by the LLNA. However, a weak sensitizer, imidazolidinyl-urea was classified as a non-sensitizer, even with the revised protocol. As for sensitizer 2-mercaptobenzothiazole, it was added in the predictive capacity phase to determine whether vehicles had an effect on test results. However, no effect was observed, demonstrating a limitation of this test method.

In conclusion, chlorobenzene and methyl salicylate showed improved results using Protocol 1.2 rather than Protocol 1.1. This was because the 75% concentration of chlorobenzene was excluded from the study, as the solubility test procedure was improved. The results for methyl salicylate were also improved as the protocol was upgraded.

3. Additional test (Imidazolidinyl urea)

- Testing facility: Lead Laboratory 2
- Test period: May to June 2015

Imidazolidinyl urea is considered a sensitizer in OECD TG 429. Its SI is 3.1 at 25% and 5.5 at 50%, so it should be tested at 50% (Basketter et al., 1992). The SI was 1.6 in the LLNA: BrdU-ELISA.

Imidazolidinyl urea was classified as a weak sensitizer by the recently developed Keratinosens™ assay, Human Cell Line Activation Test (h-CLAT assay), and Direct Peptide Reactivity Assay (DPRA). The VMT suggested revising the protocol in order to improve solubility so that imidazolidinyl-urea could be correctly categorized at a 50% concentration.

In response to the VMT's suggestion, Lead Laboratory 2 performed an additional test using imidazolidinyl urea from 27 May to 1 June 2015. DMF was chosen as a vehicle for imidazolidinyl urea. The Lead Laboratory carefully monitored the dissolution process because imidazolidinyl urea is known as a weak sensitizer or a borderline substance that can sensitize skin only when it was completely dissolved. This substance was not completely dissolved with 30 minutes of magnetic stirring. This finding was reflected in Protocol 1.3 (i.e., the magnetic stirring time for solids was extended from 30 minutes (Protocol 1.2) to 30–60 minutes).

The test on imidazolidinyl urea was performed according to Protocol 1.3. Based on results of the test, its SI was found to 2.99 at 25% and 3.52 at 50%. The EC2.7 was 26.8%, so imidazolidinyl urea was classified as a weak sensitizer (see Attachment 1). The experts advised that an overall predictive capacity evaluation based on the final version of the protocol should be performed. Therefore, a 3rd test was conducted using the 18 essential reference chemicals, including imidazolidinyl urea.

4. 3rd test (Protocol 1.3)

- Testing facility: Participating Laboratory 2
- Test period: October to November 2015

The 3rd test was conducted in accordance with Protocol 1.3. The results are described in Table 11 and, Attachment 1. Sensitivity was found to be 84.6%, specificity was 100%, and overall accuracy was 88.9%.

Table 11. Results of the 3rd test

No.	Chemical name	Code	Agree-ment	OECD TG		LLNA: BrdU-FCM		Veh	
				Class	0.5x~2.0x EC3	Class	EC2.7	OECD TG	FCM
1	CMI/ MI	D018	Yes	S	0.0045~0.018	S	1.062*	DMF	DMF
2	DNCB	D009	Yes	S	0.025~0.099	S	0.016*	AOO	AOO
3	4-Phenylenediamine	D015	Yes	S	0.055~0.22	S	0.101	AOO	DMF
4	Cobalt chloride	D003	Yes	S	0.3~1.2	S	0.199	DMSO	DMF
5	Isoeugenol	D014	Yes	S	0.77~3.1	S	1.198*	AOO	AOO
6	2-Mercaptobenzothiazole	D005	No	S	0.85~3.4	N	-	DMF	DMF
7	Citral	D010	Yes	S	4.6~18.3	S	13.08	AOO	AOO
8	HCA	D017	Yes	S	4.8~19.5	S	15.11	AOO	AOO

9	Eugenol	D001	Yes	S	5.05~20.2	S	16.46	AOO	AOO
10	Phenyl benzoate	D008	Yes	S	6.8~27.2	S	5.537*	AOO	AOO
11	Cinnamic alcohol	D004	Yes	S	10.5~42	S	44.28	AOO	AOO
12	Imidazolidinyl urea	D011	Yes	S	12~48	S	32.02	DMF	DMF
13	Methyl methacrylate	D016	No	S	45~100	N	-	AOO	AOO
14	Chlorobenzene	D007	Yes	N	-	N	-	AOO	AOO
15	Isopropanol	D012	Yes	N	-	N	-	AOO	AOO
16	Lactic acid	D006	Yes	N	-	N	-	DMSO	DMF
17	Methyl salicylate	D002	Yes	N	-	N	-	AOO	AOO
18	Salicylic acid	D013	Yes	N	-	N	-	AOO	DMF

S, Sensitizer; N, Non-sensitizer; AOO, Acetone: olive oil (4:1); DMF, N,N-dimethylformamide; DMSO, dimethyl sulphoxide; CMI/ MI, 5-Chloro-2-methyl-4-isothiazolin-3-one/2-methyl-4-isothiazolin-3-one; DNCB, 2,4-Dinitrochlorobenzene; HCA, hexyl cinnamic aldehyde; Veh, test vehicle.
* Set the y-axis to 1 since an EC2.7 value is below 0% or above 100%.

The 18 reference chemicals suggested in OECD TG 429 were used in the 3rd test. The results for the moderate sensitizer 2-mercaptobenzothiazole and weak sensitizer methyl-methacrylate among the 13 skin sensitizers were discordant with those from the LLNA, while the results for the other 11 chemicals concordant with those from the LLNA. All five non-sensitizers indicated the same results as those from the LLNA.

Imidazolidinyl urea, which was falsely predicted in the 2nd test, was correctly classified as a sensitizer with SI 4.1 at 50%. The EC2.7 was 32.0%, which is in the ECt range (12–48) recommended in OECD TG 429. Improved results were seen with imidazolidinyl urea, which becomes viscous when dissolved and was sufficiently dissolved with a concentration of 50% for 60 minutes according to Protocol 1.3.

The supplementary test with 4 optional reference chemicals in the PS was conducted by Participating Laboratory 2 based on protocol 1.3 from June to August 2016. OECD optional substances were tested to see if an improved performance of the LLNA: BrdU-FCM could be demonstrated in comparison with the LLNA. As a result, the LLNA: BrdU-FCM showed the same performance as that of the traditional LLNA (see Table 12 and Attachment 1).

Table 12. Results of the supplementary test with 4 optional reference chemicals

No.	Chemical name	Code	Agree-ment	OECD TG		LLNA: BrdU-FCM		Veh	
				Class	0.5x~2.0x EC3	Class	EC2.7	OECD TG	FCM
19	Sodium lauryl sulphate	D020	Yes	S	4.05~16.2	S	5.2	DMF	DMSO
20	Ethylene glycol dimethacrylate	D022	Yes	S	14~56	S	97.2	MEK	AOO
21	Xylene	D021	Yes	S	47.9~100	S	34.1*	AOO	AOO

22	Nickel chloride	D019	Yes	N	-	N	-	DMSO	DMSO

S, Sensitizer; N, Non-sensitizer; AOO, Acetone: olive oil (4:1); DMF, N,N-dimethylformamide; DMSO, dimethyl sulphoxide; Veh, test vehicle.

* Set the y-axis to 1 since an EC2.7 value is below 0% or above 100%.

5. Discussion about the test results

Experts suggested evaluating the overall predictive capacity in accordance with the final version of the protocol. Therefore, a 3rd test was conducted, based on Protocol 1.3 using the 18 essential reference chemicals. The results showed that the sensitivity was 84.6%, specificity was 100%, and overall accuracy was 88.9% on the basis of the SI 2.7. (see Table 13). The results for imidazolidinyl urea, which were inconsistent with those in the 2nd test by the LLNA, matched those from the LLNA in the 3rd test.

Table 13. Predictive capacity of the test method based on a 3rd test (18 essential chemicals)

		OECD TG 429 PS	
		Sensitizer	Non-sensitizer
LLNA: BrdU-FCM (Sensitizer, SI ≥ 2.7)	Sensitizer	11	0
	Non-sensitizer	2	5
Sensitivity: 84.6%, Specificity: 100%, Overall accuracy: 88.9%			

		OECD TG 429 PS	
		Sensitizer	Non-sensitizer
LLNA: BrdU-FCM (Sensitizer, SI ≥ 1.8)	Sensitizer	11	1
	Non-sensitizer	2	4
Sensitivity: 84.6%, Specificity: 80%, Overall accuracy: 83.3%			

According to the validation study on the LLNA: BrdU-FCM using 18 essential reference chemicals listed in the TG 429 PS, the LLNA: BrdU-FCM has a certain limitation since one moderate skin sensitizer, 2-mercaptobenzothiazole, and one borderline skin sensitizer, methyl methacrylate, were misclassified in the LLNA: BrdU-FCM. Except for two sensitizers, 2-mercaptobenzothiazole and methyl-methacrylate, which were classified as non-sensitizers, 16 among the 18 reference chemicals produced results similar to those from the LLNA.

Methyl methacrylate, a weak sensitizer referred in OECD TG 429, was reported to be a non-sensitizer by the LLNA and LNCC (lymph node cell count) methods (Basketter et al., 2011). Indeed, the chemical is highly likely to be classified as a non-sensitizer because it was evaluated as a sensitizing substance at rather high 90%, 99%, and 79% concentrations in the LLNA, the LLNA: DA and the LLNA: BrdU-ELISA, respectively (ICCVAM, 2010a & 2010b). In addition, human data for methyl methacrylate are insufficient (Basketter et al., 2014b; Teunis et al., 2014), and it was included in the reference chemicals in TG 429 on the basis of only one scientific paper (OECD, 2010). There is also no reliable HRIPT data on this substance, and a few clinical case reports only suggested that methyl methacrylate is a sensitizer (Kim et al., 2016), reflecting

the need to investigate further this substance. Kimber and Pemberton (2014) recently concluded that methyl methacrylate is a contact allergen possessing no more than weak skin-sensitizing potency. In summary, very weak skin sensitizers might be falsely predicted by the LLNA: BrdU-FCM.

OECD TG 429, in which 2-mercaptobenzothiazole was classified as a sensitizer, was also included as a reference chemical on the results of only one scientific paper (OECD, 2010). 2-Mercaptobenzothiazole is categorized as Cat 1A in the LLNA and GPMT, whereas it is categorized as Cat 1B (one level difference) based on the LLNA: DA and ELISA. In contrast, LNCC and FCM categorized 2-mercaptobenzothiazole as No category (two level difference) but in the Buehler test, it was also determined as a non-sensitizer (Frankild et al. 2000), suggesting that this compound produces variable results depending on test methods. In addition, the substance was considered a borderline sensitizer by the LLNA: DA and LLNA: BrdU-ELISA, because its SIs were 2.00 and 1.62, respectively (OECD, 2010a & 2010b). This chemical was also defined as a very weak sensitizing substance as its human No Observed Effect Level (NOEL) was 2269 $\mu g/cm^2$, which is higher than the 2000 $\mu g/cm^2$ of a weak sensitizer, imidazolidinyl urea (Basketter et al., 2014b; Teunis et al., 2014). Based on categorization of chemicals by relative human skin-sensitizing potency (Basketter et al., 2014a), 2-mercaptobenzothiazole was categorized into group 3. Chemicals placed in category 3 are defined as contact allergens with a 500–2500 $\mu g/cm^2$ NOEL, with lower concentrations of these chemicals expected to sensitize only a small proportion or none of the exposed. Based on these results, the LLNA: BrdU-FCM may have difficulty in correctly classifying weak or borderline chemicals as sensitizers.

Imidizolidinyl urea was correctly classified as a sensitizer after the solubility protocol was revised. As for imidazolidinyl urea, which was suggested to be a sensitizer in OECD TG 429, it was classified as a sensitizer at a concentration of 50% because its SI was 3.1 at 25% and 5.5 at 50% (Basketter et al., 1992). In addition, its SI was on the borderline (1.6) in the LLNA: BrdU-ELISA. It was also classified as a weak sensitizer in the recently adopted KeratinoSens assay (EURL ECVAM, 2014) and direct peptide reactivity assay (DPRA) (EURL ECVAM, 2012; Natsch et al., 2013). Its SIs were 2.6 (Lead Laboratory 2) and 2.1 (Participating Laboratory 2) at the highest concentration of 50% in the 2nd test of the predictive capacity evaluation. The VMT suggested revising the protocol in order to improve the solubility of imidazolidinyl urea by extending the magnetic stirring time from 30 minutes to 60 minutes for such a highly viscous chemical. Based on the 3rd test, SI values were found to be 2.11 at 25% and 4.10 at 50%, and the EC2.7 was 32.0%. Thus, it was correctly classified as a weak sensitizer.

Some substances, Chlorobenzene and salicylic acid (false positives in the LLNA: DA) and lactic acid (false positive in the LLNA: BrdU-ELISA), were correctly classified as negatives in the LLNA: BrdU-FCM (see Table 15 and 16).

As a result of the supplementary test for the four optional chemicals, ethylene glycol dimethacrylate, xylene, and sodium lauryl sulfate were classified as sensitizers, whereas nickel chloride was classified as a non-sensitizer. OECD TG 429 PS classifies nickel chloride as a false-negative substance and sodium lauryl sulphate (SLS) and xylene as false-positive substances. The potential for skin sensitization depends on the ability of a test substance to penetrate the skin; therefore, hydrophilic metal compounds, such as nickel chloride, might not be easily absorbed, resulting in a false-negative result (Basketter et al. 1995). In addition, nickel-induced skin sensitization was reported based on activation of human Toll-like receptor 4 (TLR4), whereas mouse TRL4 could not mediate this response (Schmidt et al., 2010). In this regard, the LLNA: BrdU-FCM assay was no better than the traditional LLNA. Sodium lauryl sulphate, which is believed to be a non-sensitizing irritant, induced a weak positive response (EC2.7 = 3.04) in the LLNA: BrdU-FCM. This

misclassification also occurs when using the traditional LLNA (OECD 2010a). Even though the LLNA: BrdU-FCM was no better than the traditional LLNA with respect to the classification of SLS as a skin non-sensitizer, both of these alternative, *in vivo*-based, skin sensitization methods suggest that SLS-mediated positive responses occur through an as-yet-unknown, but probably non-immune-mediated, mechanism (Basketter et al. 1996).

The LLNA: BrdU-FCM was developed primarily to identify skin-sensitizing substances, namely hazard identification. However, the assay can also be used to evaluate skin sensitization potency, which is described in GHS rev 4.0 (UN, 2009). The results of potency sub-categorization by the LLNA, determined for 22 substances in accordance with the GHS (1A or 1B, or NC), indicated that discordant results were produced for only two chemicals (2-mercaptobenzothiazole and methyl methacrylate) in the LLNA: BrdU-FCM. Among the 6 extreme or strong skin sensitizers (1A) mentioned in the OECD TG 429 PS, one substance, 2-mercaptobezothiazole, was wrongly classified as No category. Among the 7 weak skin sensitizers in the OECD TG 429 PS (1B), one substance, methyl methacrylate, was classified as No category. In contrast, all non-sensitizers were correctly classified in the LLNA: BrdU-FCM. The concordance of FCM compared with the tLLNA was 91% (20/22). The concordances of ELISA and DA compared with the tLLNA were 82% (18/22) and 73% (16/22), respectively. When compared with human data and FCM, 6 chemicals were falsely predicted. And the concordance of FCM was 73% (16/22), tLLNA, 77% (17/22), ELISA, 68% (15/22) and DA, 73% (16/22). Preliminary results suggested that the performance of the non-radioisotopic LLNAs in sub-categorization was comparable to that of the radioisotopic LLNA (Kim et al., 2016, see Table 14).

Further analysis of coefficient of variation (CV) for ECt values of HCA was conducted based on the ICCVAM Test Method Evaluation Report (TMER) on the LLNA: DA and the LLNA: BrdU-ELISA (ICCVAM 2010a and ICCVAM 2010b). CV value for EC2.7s of HCA obtained during the WLR, 1st and 3rd predictive capacity evaluation was 9.68% (N=5) for Lead Laboratory 1, 19.09% (N=4) for Participating Laboratory 1, and 35.27 (N=5) for Participating Laboratory 2. Additionally, the Mean± SD value (95% confidence interval) of the positive control group (25% HCA) used in the 3rd predictive capacity evaluation and supplementary test was 7.2 ± 3.9 (2.2, 15.5). These values were produced from one laboratory and thus different values could be generated in other test sites.

6. Conclusion of the VMT on Module 5

The VMT believed that the predictive capacity of the LLNA: BrdU-FCM was fairly high for the reference chemicals suggested in OECD TG 429 PS, except for methyl methacrylate, which was not classified as a sensitizer consistently by the LLNA-based *in vivo* skin sensitization test methods. Notably, the test results were considered reliable, because the predictive capacity was evaluated under blind conditions.

Some substances (e.g., chlorobenzene, salicylic acid, and lactic acid), which were determined to be false positives in the LLNA: DA and LLNA: BrdU-ELISA, were correctly classified in the LLNA: BrdU-FCM. In addition, this test method can evaluate the skin-sensitizing potential of chemicals in combination with newly developed *in vitro* tests based on AOPs and the Integrated Approaches to Testing and Assessment (IATA) (Scott et al., 2010). This assay can also correctly classify test substances that are false negatives in the *in vitro* tests; therefore, it is expected to complement these tests (see Table 15 and 16).

Furthermore, the LLNA: BrdU-FCM uses a new mouse strain (BALB/c) and new analysis method (flow

cytometry). This test method uses BALB/c mice, which are relatively economical compared with CBA mice. In some countries, the price of CBA mice is expensive because they must be imported, making them harder to obtain than the widely used BALB/c mice. In addition, no significant difference in the prediction of skin sensitization potency has been reported between these two mouse strains (Burns et al., 2010; Jung et al., 2010; Hou et al., 2015, Lee et al., 2017). DBA/2, B6C3F1, and BALB/c were all cited as appropriate mouse strains, suggesting alternatives that can be used in the LLNA (Woolhiser et al., 2000). Further, there was no statistical difference in the test results of the LLNA: BrdU-ELISA using BALB/c and CBA/JN, respectively (Hou et al., 2015). Also, Lee et al. (2017) was conducted to compare the test results of the LLNA: BrdU-FCM using BALB/c mice with those using CBA/J mice treated with 13 sensitizers and 5 non-sensitizers, listed in OECD TG 429. As a result, the stimulation index of the LLNA: BrdU-FCM using CBA/J mice was highly correlated to those using BALB/c mice and yielded identical predictivity with BALB/c applying the same SI cut-off 2.7 (Lee et al., 2017, see Table 17). However, it may be reasonable if different cut-off SI value shall be adopted for each species to maximize sensitivity after ROC analysis. Additional quantitative comparison of BALB/c and CBA/J was presented in Figure 7.

In general, before the initiation of LLNA tests, a pre-screen test is required if a chemical's toxicological information (e.g., acute toxicity and dermal irritation) is not available. The concentrations recommended in TG 429 and 442A/B (100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc.) are commonly selected. It is difficult to determine the concentration at which an unknown chemical will induce acute toxicity or dermal irritation without conducting a pre-screen test. However, if information on the quantitative structure-activity relationship or irritation is available, the concentration range can be narrowed so that a pre-screen test is not required. The LLNA: BrdU-FCM employed a newly established pre-screen test that is performed in two phases. If the pre-screen test is conducted following OECD TG 429, 442A/B, 9 mice (n = 1/group) or 18 mice (n = 2/group) are needed for 9 concentrations. However, if the pre-screen test is conducted in two phases, the number of mice will be reduced by 1–8 mice. Specifically, with less-toxic chemicals at high concentration, the number of mice will be reduced by up to 8 times. However, it should be noted that the number of animals depends on the number of selected concentrations. This pre-screen strategy can also be applied to other LLNA-based skin sensitization validation methods (see Figure 8).

In addition, there is no significant difference in the lengths of test periods needed for the LLNA: BrdU-ELISA and LLNA: BrdU-FCM. That is because the process of sample drying is not needed in the LLNA: BrdU-FCM, even though it requires sample counting. Moreover, substrate reactions to reagents last longer in the LLNA: BrdU-ELISA, so stop solutions are sometimes required unlike the LLNA: BrdU-FCM (see Table 18).

Table 14. Potency sub-categorization to UN GHS-compliant based on ECt values

No.	Substances	Human[a] (DSA_{05}[††])		tLLNA[b]			LLNA: BrdU-ELISA			LLNA: DA			LLNA: BrdU-FCM		
		GHS	Sen.	EC3	GHS	Sen.	EC1.6	GHS	Sen.	EC1.8	GHS	Sen.	EC2.7	GHS	Sen.
1	CMI/MI	Cat1A	+	0.009	Cat1A	+	0.065[c]	Cat1A	+	0.009[c]	Cat1A	+	*1.062	Cat1A	+
2	DNCB	Cat1A	+	0.049	Cat1A	+	0.032[c]	Cat1A	+	0.032[c]	Cat1A	+	*0.016	Cat1A	+
3	4-Phenylenediamine	Cat1A	+	0.11	Cat1A	+	0.29[c]	Cat1A	+	0.04[c]	Cat1A	+	0.101	Cat1A	+
4	Cobalt chloride	Cat1A	+	0.6	Cat1A	+	0.3[c]	Cat1A	+	0.9[c]	Cat1A	+	0.199	Cat1A	+
5	Isoeugenol	Cat1A	+	1.5	Cat1A	+	5.2[c]	**Cat1B**	+	1.5[c]	Cat1A	+	*1.2	Cat1A	+
6	2-Mercaptobenzothiazole	Cat1B	+	1.7	**Cat1A**	+	12.1[c]	Cat1B	+	8.0[c]	Cat1B	+	NA	**NC**	-
7	Citral	Cat1B	+	9.2	Cat1B	+	7.1[c]	Cat1B	+	2.1[c]	Cat1B	+	13.1	Cat1B	+
8	HCA	Cat1B	+	9.7	Cat1B	+	12.9[c]	Cat1B	+	6.3[c]	Cat1B	+	15.1	Cat1B	+
9	Eugenol	Cat1B	+	10.1	Cat1B	+	8.9[c]	Cat1B	+	2.6[c]	Cat1B	+	16.5	Cat1B	+
10	Phenyl benzoate	Cat1B	+	13.6	Cat1B	+	17.0[c]	Cat1B	+	0.7[c]	**Cat1A**	+	*5.5	Cat1B	+
11	Cinnamic alcohol	Cat1B	+	21	Cat1B	+	24.1[c]	Cat1B	+	5.2[c]	Cat1B	+	44.3	Cat1B	+
12	Imidazolidinyl urea	Cat1B	+	24	Cat1B	+	49.5[c]	Cat1B	+	6.3[c]	Cat1B	+	32.0	Cat1B	+
13	Methyl methacrylate[†]	Cat1B	+	90	Cat1B	+	79.1[d]	Cat1B	+	99[c]	Cat1B	+	NA	**NC**	-
14	Chlorobenzene[†]	NC	-	NA	NC	-	21.4[d]	**Cat1B**	+	17.9[c]	**Cat1B**	+	NA	NC	-
15	Isopropanol	Cat1B	+	NA	**NC**	-	NA[c]	**NC**	-	NA[c]	**NC**	-	NA	**NC**	-
16	Lactic acid[†]	NC	-	NA	NC	-	15.2[c]	**Cat1B**	+	NA[c]	NC	-	NA	NC	-
17	Methyl salicylate	NC	-	NA	NC	-	NA[c]	NC	-	NA[c]	NC	-	NA	NC	-
18	Salicylic acid	NC	-	NA	NC	-	NA[c]	NC	-	17.7[c]	**Cat1B**	+	NA	NC	-
19	Sodium lauryl sulfate	NC	-	8.1	**Cat1B**	+	13.3[c]	**Cat1B**	+	1.6[c]	**Cat1A**	+	5.2	**Cat1B**	+
20	Ethylene glycol dimethacrylate[†]	Cat1B	+	28	Cat1B	+	31.8[c]	Cat1B	+	19.2[c]	Cat1B	+	97.2	Cat1B	+
21	Xylene	NC	-	95.8	**Cat1B**	+	15.9[d]	**Cat1B**	+	NA[d]	NC	-	34.1	**Cat1B**	+
22	Nickel chloride	Cat 1A	+	NA	**NC**	-	NA[c]	**NC**	-	NA[c]	**NC**	-	NA	**NC**	-

Concordance vs. tLLNA	Cat 1A		67% (4/6)	83% (5/6)	83% (5/6)
	Cat 1B		100% (10/10)	70% (7/10)	90% (9/10)
	NC		67% (4/6)	67% (4/6)	100% (6/6)
	Total		82% (18/22)	73% (16/22)	91% (20/22)
Concordance vs. Human	Cat 1A	83% (5/6)	67% (4/6)	83% (5/6)	83% (5/6)
	Cat 1B	80% (8/10)	90% (9/10)	80% (8/10)	70% (7/10)
	NC	67% (4/6)	33% (2/6)	50% (3/6)	67% (4/6)
	Total	77% (17/22)	68% (15/22)	73% (16/22)	73% (16/22)
Correlation Coefficient (vs. tLLNA)	-	-	0.744	0.848	0.786

+, Sensitizer; -, Non-sensitizer; NA, Not available; NC: No Category; CMI/ MI, 5-Chloro-2-methyl-4-isothiazolin-3-one/ 2-methyl-4-isothiazolin-3-one; DNCB, 2,4-Dinitrochlorobenzene; HCA, hexyl cinnamic aldehyde; Shaded, discordance between LLNA and LLNA variants; **Lined and bolded text**, discordance between Human Result and LLNA variants.

Source, a: ICCVAM LLNA performance standards, 2009 / ICCVAM test method evaluation report, 2011, b: OECD TG 429 d: ICCVAM 2010 review report, d: JaCVAM.

† No HRIPT data but based on clinical case report

†† DSA_{05} : induction dose per skin area, in µg/cm ², in a human repeat-insult patch test or human maximization test that produces a positive response in 5% of the tested population

* Set the y-axis to 1 since an EC2.7 value is below 0% or above 100%

Table 15. Comparison of predictive capacities of LLNA, LLNA: BrdU-FCM, LLNA: DA, LLNA: BrdU-ELISA and LNCC

No.	Chemical	Human/ Guinea pig	LLNA (EC3)	LLNA[b)] (EC3)	LLNA: BrdU-FCM (highest SI)	LLNA: DA* (highest SI)	LLNA: BrdU-ELISA* (highest SI)	LNCC[b)] (EC1.5)
1	CMI/ MI	+/+	+ (0.009)	+ (0.01)	+ (13.55)	+ (7.50)	+ (4.83)	+ (0.011)
2	DNCB	+/+	+ (0.049)	+ (<0.025)	+ (47.29)	+ (9.96)	+ (6.84)	+ (<0.025)
3	4-Phenylenediamine	+/+	+ (0.11)	+ (0.1)	+ (10.02)	+ (5.14)	+ (14.70)	+ (0.1)
4	Cobalt chloride	+/+	+ (0.6)	+ (<0.25)	+ (12.83)	+ (4.25)	+ (3.68)	+ (<0.25)
5	Isoeugenol	+/+	+ (1.5)	+ (2.2)	+ (34.91)	+ (7.09)	+ (6.73)	+ (2.7)
6	2-Mercaptobenzothiazole	+/+	+ (1.7)	+ (4.6)	- (1.44)	+ (2.00)	+a) (1.62)	-
7	Citral	+/+	+ (9.2)	+ (12.6)	+ (8.88)	+ (4.40)	+ (16.35)	+ (9.2)
8	HCA	+/+	+ (9.7)	+ (4.6)	+ (4.34)	+ (5.50)	+ (3.40)	+ (8.7)
9	Eugenol	+/+	+ (10.1)	+ (9.2)	+ (3.94)	+ (7.07)	+ (3.30)	+ (16.2)
10	Phenyl benzoate	+/+	+ (13.6)	+ (8.9)	+ (15.41)	+ (4.24)	+ (3.37)	+ (4.0)
11	Cinnamic alcohol	+/+	+ (21)	+ (25.2)	+ (2.78)	+ (5.66)	+ (2.74)	+ (26.0)
12	Imidazolidinyl urea	+/+	+ (24)	+ (15.9)	+ (4.10)	+ (4.67)	+a) (1.61)	+ (21.5)
13	Methyl methacrylate	+/+	+ (90)	-	- (0.87)	+a) (1.81)	NA	-
14	Chlorobenzene	-/-	-	+ (45.6)	- (1.67)	+ (2.44)	NA	+ (79.0)
15	Isopropanol	+/-	-	-	- (0.89)	- (1.21)	- (1.01)	-

16	Lactic acid	-/-	-	-	- (1.28)	- (0.97)	+ (1.89)	-
17	Methyl salicylate	-/-	-	+ (32.8)	- (1.77)	- (1.55)	- (1.43)	+ (49.0)
18	Salicylic acid	-/-	-	+ (8.0)	- (2.57)	+ (2.00)	- (1.26)	+ (15.8)
19	Sodium lauryl sulphate	-/-	+ (8.1)	+ (2.9)	+ (2.78)	+ (3.39)	+ (2.64)	+ (1.6)
20	Ethylene glycol dimethacrylate	+/-	+ (28)	+ (45.1)	+ (2.85)	+ (4.45)	+ (3.11)	+ (38.0)
21	Xylene	-/	+ (95.8)	+ (39.1)	+ (6.99)	NA	NA	+ (28.2)
22	Nickel chloride	+/+	-	+ (3.5)	- (2.14)	- (1.30)	NA	+ (3.6)

+, Sensitizer; -, Non-sensitizer; NA, Not available; CMI/ MI, 5-Chloro-2-methyl-4-isothiazolin-3-one/ 2-methyl-4-isothiazolin-3-one; DNCB, 2,4-Dinitrochlorobenzene; HCA, hexyl cinnamic aldehyde;

[a)] Borderline value

[b)] Basketter et al., 2011; Kolle et al., 2013

* Source: ICCVAM (2010a), ICCVAM (2010b)

Table 16. Comparison of LLNA, LLNA: BrdU-FCM, LLNA: DA and LLNA: BrdU-ELISA

No.	Classification	LLNA	LLNA[a)]	LLNA: BrdU-FCM	LLNA: DA[b)]	LLNA: BrdU-ELISA[c)]
1	Skin sensitisation phases covered	Induction (T cell proliferation)	Induction (T cell proliferation)	Induction (T cell proliferation)	Induction (T cell proliferation)	Induction (T cell proliferation)
2	Test system	Mouse (CBA)	Mouse (CBA)	Mouse (BALB/c)	Mouse (CBA)	Mouse (CBA)
3	Animal use in treatment group	4	4	4	4	4
4	Test duration (days)	6	6	6-7	8	6
5	Classification criteria	SI > 3	SI > 3	SI ≥ 2.7	SI ≥ 1.8	SI ≥ 1.6
6	**Overall accuracy***	**89% (86/97)**[**]**	**77% (17/22)**	**89% (16/18)**	**93% (41/44)**	**95% (41/43)**
7	**Sensitivity***	**91% (62/68)**[**]**	**94% (15/16)**	**85% (11/13)**	**100% (32/32)**	**100% (32/32)**
8	**Specificity***	**83% (24/29)**[**]**	**33% (2/6)**	**100% (6/6)**	**75% (9/12)**	**82% (9/11)**
9	Analysis tool	Scintillator	Scintillator	Flow cytometry	Luminometer	ELISA
10	Analysis kit needed	No	No	Yes	Yes	Yes

[a)] Basketter et al., 2011; Kolle et al., 2013

[b)] ICCVAM (2010a)

[c)] ICCVAM (2010b)

[d)] OECD (2015a)

[e)] OECD (2015b)

[*] Compared with existing results of the LLNA

[**] LLNA vs. GPMT/BA (ICCVAM Peer Review Report, 1999)

Table 17. Comparison between LLNA: BrdU-FCM with BALB/c and CBA/J

No.	Test chemical	LLNA: BrdU-FCM with BALB/c						LLNA: BrdU-FCM with CBA/J					
		Solvent	Stimulation Index			EC2.7(%)	Class	Solvent	Stimulation Index			EC2.7(%)	Class
			Low	Middle	High				Low	Middle	High		
1	CMI/ MI	DMF	9.99	13.42	13.55	1.062*	+	DMF	1.44	5.46	10.44	1.45	+
2	DNCB	AOO	15.57	38.97	47.29	0.016*	+	AOO	2.03	8.36	24.43	0.05	+
3	4-Phenylenediamine	DMF	3.24	6.48	10.02	0.101	+	DMF	4.44	11.82	7.91	0.04*	+
4	Cobalt chloride	DMF	2.60	7.80	12.83	0.199	+	DMF	5.13	8.55	9.27	0.17*	+
5	Isoeugenol	AOO	7.66	19.30	34.91	1.198*	+	AOO	3.70	8.22	11.07	3.78*	+
6	2-Mercaptobenzothiazole	DMF	1.44	1.13	1.30	NA	-	DMF	1.12	1.95	1.21	NA	-
7	Citral	AOO	1.98	5.05	8.88	13.08	+	AOO	1.53	1.84	3.33	7.71	+
8	HCA	AOO	1.14	1.75	4.34	15.11	+	AOO	2.83	4.36	6.36	2.39	+
9	Eugenol	AOO	0.71	2.01	3.94	16.48	+	AOO	1.65	2.61	3.86	13.28	+
10	Phenyl benzoate	AOO	4.06	5.64	3.19	5.537*	+	AOO	3.81	7.12	9.08	9.37*	+
11	Cinnamic alcohol	AOO	0.49	2.29	2.78	44.28	+	AOO	1.74	2.21	3.11	38.44	+
12	Imidazolidinyl urea	DMF	1.05	2.11	4.10	32.02	+	DMF	1.39	2.08	4.57	28.58	+
13	Methyl methacrylate	AOO	0.57	0.65	0.87	NA	-	AOO	0.96	0.63	0.98	NA	-
14	Chlorobenzene	AOO	1.12	1.25	1.67	NA	-	AOO	1.30	1.49	2.14	NA	-
15	Isopropanol	AOO	0.86	0.83	0.89	NA	-	AOO	0.96	0.77	0.72	NA	-
16	Lactic acid	DMF	1.15	1.18	1.28	NA	-	DMF	1.53	1.21	1.34	NA	-
17	Methyl salicylate	AOO	1.77	1.61	1.14	NA	-	AOO	1.21	1.48	2.17	NA	-
18	Salicylic acid	DMF	1.76	2.26	2.57	NA	-	DMF	0.75	0.60	0.92	NA	-

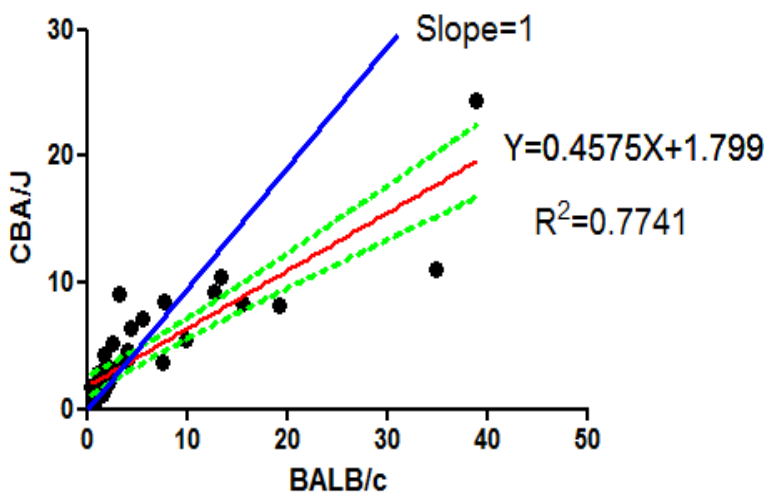
+, Sensitizer; -, Non-sensitizer; AOO, Acetone: olive oil (4:1); DMF, N,N-dimethylformamide; DMSO, dimethyl sulphoxide; NA, Not available; CMI/ MI, 5-Chloro-2-methyl-4-isothiazolin-3-one/ 2-methyl-4-isothiazolin-3-one; DNCB, 2,4-Dinitrochlorobenzene; HCA, hexyl cinnamic aldehyde;

* Set the y-axis to 1 since an EC2.7 value is below 0% or above 100%.

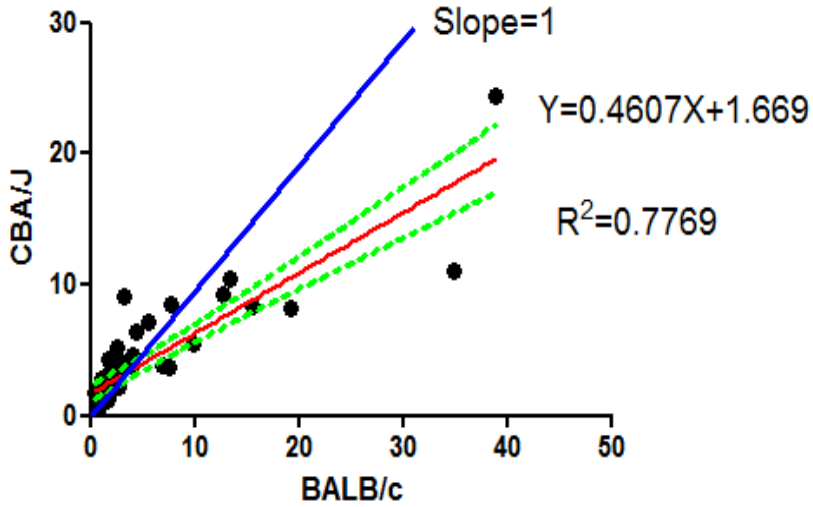
Table 18. Comparison between LLNA: BrdU-ELISA and LLNA: BrdU-FCM

Test	Step	LLNA: BrdU-ELISA	LLNA: BrdU-FCM
Pre-screen test	Grouping	· Test substances (8 doses), vehicle control · Duration: 6 days	· 1st test: Test substances (25%, 1 dose), vehicle control · 2nd test i) No systemic toxicity or excessive irritation: test substances (50%, 100%, 2 doses), vehicle control ii) Systemic toxicity or excessive irritation: test substances (5 doses under 25%), vehicle control · Duration: 12 days
	No. of animals	· 18 mice (8 doses, vehicle control)	· 1st test: 4 mice · 2nd test: With no systemic toxicity or excessive irritation, 6 mice; with systemic toxicity or excessive irritation, 12 mice * No toxicity: total of 10 mice * Toxicity: total of 16 mice
	Body weight, treatment, ear erythema, ear thickness, ear weight		
Main test	Grouping	· Test substances (3 doses), vehicle and positive control	· Test substances (3 doses), vehicle and positive control
	No. of animals	25 mice	27 mice
	Body weight, treatment, ear erythema, ear thickness, ear weight		
	Cell counting	· No counting · Dilution: vehicle control value (0.1–0.2)	· Cell counting: 1.5×10^6 cells · Overnight, if necessary
	Drying	· Aliquot cells on a 96-well plate and dry for **1 hour** · Overnight, if necessary	-
	Kit treatment	· 96 well kit treatment for **3–4 hours**	· Kit treatment for **3–4 hours**
	Cell proliferation	· Measurement by ELISA for **30 minutes** · If necessary, **add <u>stop solution</u>**	· Measurement by flow cytometry for **1–2 hours**
	Data evaluation	· Similar	· Similar

A. Lee et al., 2017 (essential chemicals)



B. Lee et al., 2017 + Unpublished data (optional chemicals)



C. Additional SI analysis (by Irvin & Strickland) (58 pairs)

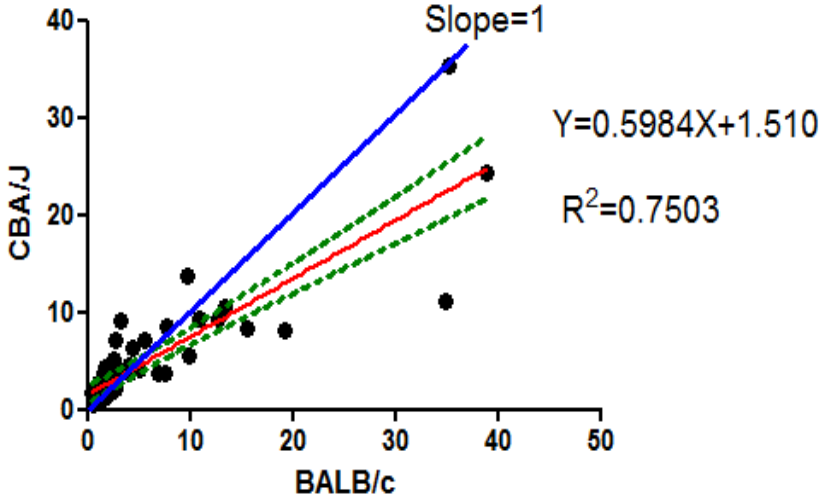


Figure 7. Quantitative comparison of BALB/c and CBA/J for LLNA: BrdU-FCM

A. The graph represents analysis of SI data using LLNA: BrdU-FCM from Lee et al., 2017 (essential chemicals) for the two mouse strains (39 data pairs in all; 15 among 18 chemicals). The data points are all pair matched with respect to test concentrations, so it is a fair and robust comparison between the two strains. As a result, a linear regression equation was generated as y = 0.4575 x + 1.799, and the R2 value of this model was 0.7741, thus demonstrating a high correlation (red line: result of regression analysis, dotted green line: 95% confidence interval); **B** represents the results for the PS reference chemicals used in the LLNA: BrdU-FCM from Lee et al., 2017 (15 essential chemicals) and unpublished experiments (3 among 4 optional chemicals) (48 data pairs in all); **C** indicates the results of additional analysis of the combined data of the LLNA: BrdU-FCM and the tLLNA (Woolhiser 2000 and Strickland poster) (by Irvin & Strickland). All data is included in Attachment 1, Table 14.

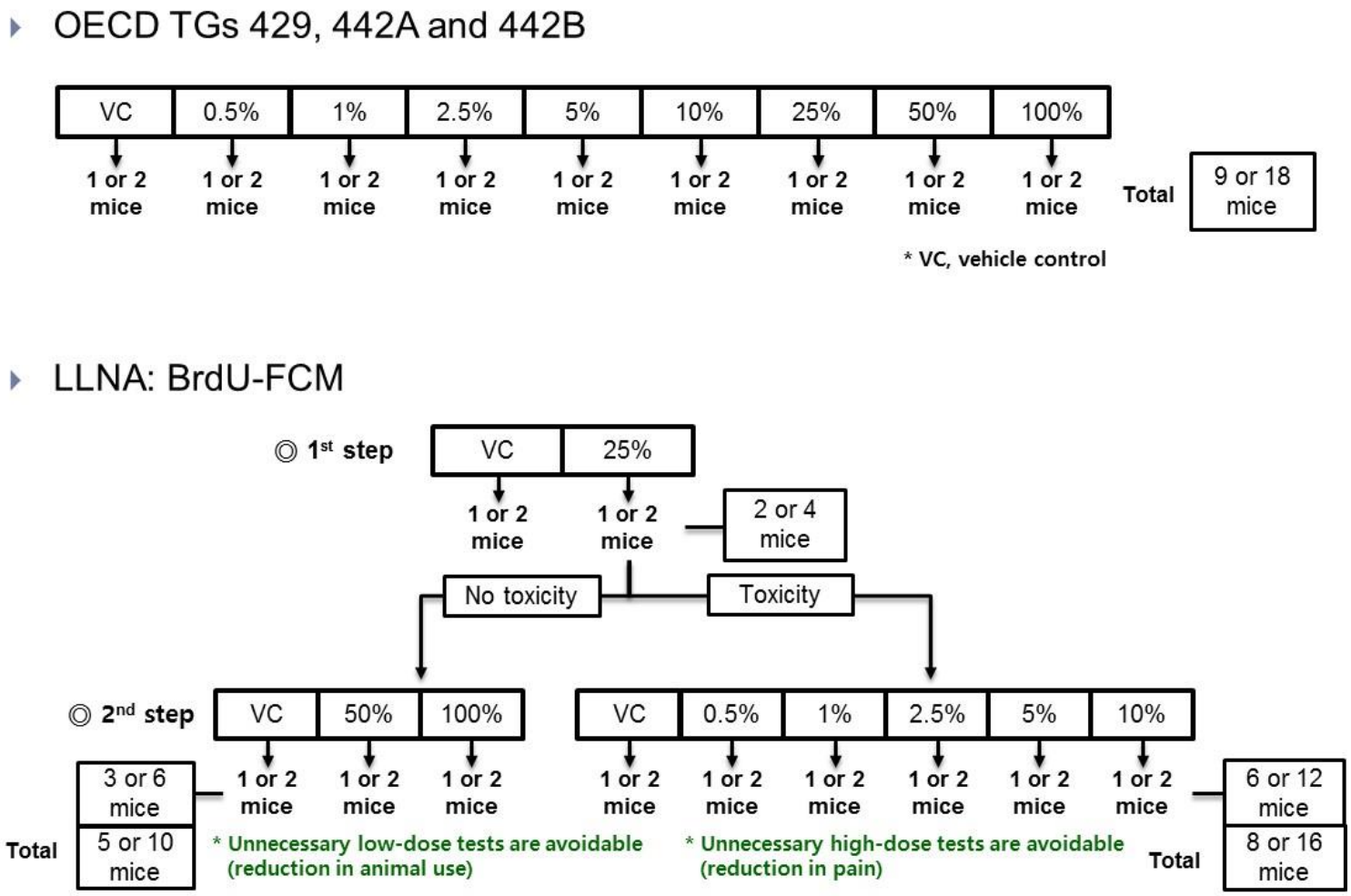


Figure 8. Comparison of pre-screen tests in the LLNA, LLNA: DA, LLNA: BrdU-ELISA, and LLNA: BrdU-FCM (3Rs, refinement and reduction)

VIII. Overall conclusions and recommendations of the VMT

Overall conclusions

The purpose of this validation study was to determine whether the LLNA: BrdU-FCM could serve as an alternative, as a 'me-too' assay, to the LLNA (OECD TG 429), LLNA: BrdU-ELISA (OECD TG 442B), and LLNA: DA (OECD TG 442A) assays by international organizations. To this end, the transferability, WLR, BLR, and predictive capacity of the test method were evaluated. As a result of the evaluation, the VMT concluded that the test method mostly met the acceptance criteria described in OECD TG 429 PS. The VMT decided to propose the LLNA: BrdU-FCM as a 'me-too' test of the LLNA (Ahn et al., 2016).

The major conclusions of each module are as follows:

• **Module 1. Test definition**: The scientific principle underlying the LLNA: BrdU-FCM is similar to that of the existing LLNA test methods. However, what sets the LLNA: BrdU-FCM apart from the other LLNA-based test methods is that it uses relatively inexpensive BALB/c mice (compared to the CBA mouse strain), and it facilitate evaluation of the skin sensitization potential of chemicals.

• **Module 2. WLR**: All three laboratories produced results that met the expectations outlined in OECD TG 429 PS.

• **Module 3. Transferability**: If a testing facility is familiar *in vivo* experimental methods that use mice and the operation of flow cytometry, the LLNA: BrdU-FCM test can easily be adopted in accordance with the protocol used for the predictive capacity evaluation. However, training for the entire test procedure may be required.

• **Module 4. BLR**: All three laboratories produced results that met the expectations outlined OECD TG 429 PS.

• **Module 5. Predictive capacity**: The predictive capacity of the LLNA: BrdU-FCM was high (94% [16/17]) for the reference chemicals suggested in OECD TG 429 PS, except for methyl methacrylate, which was not classified as a sensitizer consistently by LLNA-based *in vivo* skin sensitization test methods. Notably, the test results should be considered reliable, as the predictive capacity was evaluated under blind conditions.

Recommendations

An additional test using chemicals that are similar, in terms of chemical or physical properties and skin sensitization potency, to the falsely classified ones can be performed to further demonstrate that LLNA: BrdU-FCM achieves the accuracy recommended in OECD TG 429 PS. Furthermore, other endpoints for evaluating skin sensitization potential should to be investigated if this method is used for GHS categorization. In addition, further studies (e.g., analysis of specific lymphocyte activation markers related to skin sensitization (T cell-specific surface markers or cytokines)) to evaluate the utility of the LLNA: BrdU-FCM for characterizing the skin-sensitizing potential of test substances would be of value.

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[ANNEX 1]

Project Plan (version 1.4)

for the Performance Standard-Based Validation Study on the LLNA: BrdU-FCM)

(August 31, 2015, final version)

1. Study goal

The purpose of this validation study was to evaluate the LLNA: BrdU-FCM, proposed as a 'me-too' test of the LLNA because of its predictive capacity. The purpose of the LLNA: BrdU-FCM is to evaluate the skin sensitization potential of chemicals. This assay, as well as the LLNA (OECD TG 429), LLNA: DA (OECD TG 442A), LLNA: BrdU-ELISA (OECD TG 442B), is an improvement over skin sensitization tests that use guinea pigs (OECD TG 406), in terms of reduction and refinement of the 3Rs.

2. Study objectives

This validation study was designed to validate the accuracy and reliability of the test method, which represents an upgrade from the LLNA (OECD TG 429).

The evaluation of the accuracy and reliability of the test method was performed according to OECD TG 429 Annex 1, 'Performance Standards (PS) for Assessment of Proposed Similar or Modified LLNA Test Methods for Skin Sensitization'.

The accuracy of the test method was evaluated under blind conditions and was based on the degree of concordance between the results of tests using the reference chemicals listed in the PS and reference values.

Within-laboratory reproducibility (WLR) and between-laboratory reproducibility (BLR) of the test method were evaluated to determine its reliability. The test for the WLR evaluation was repeated four times using hexyl cinnamic aldehyde (HCA) at the three testing sites. The test for the BLR assessment was repeated three times using HCA and 2,4-dinitrochlorobenzene (DNCB) at the three participating laboratories.

The protocol, the selection of test substances, and the evaluation of WLR and BLR should be documented according to the requirements outlined in OECD TG 429 PS and OECD GD 34.

3. Management of the validation study

3.1. Roles of the VMT

The VMT plays a pivotal role throughout a validation study.

- Goal set-up
- Review and approval of a Project Plan
- Review and approval of a protocol
- Selection of test substances
- Interpretation and evaluation of test results (approval for statistical reports)
- Approval of a validation study report
- Assessment of quality assurance inspection results

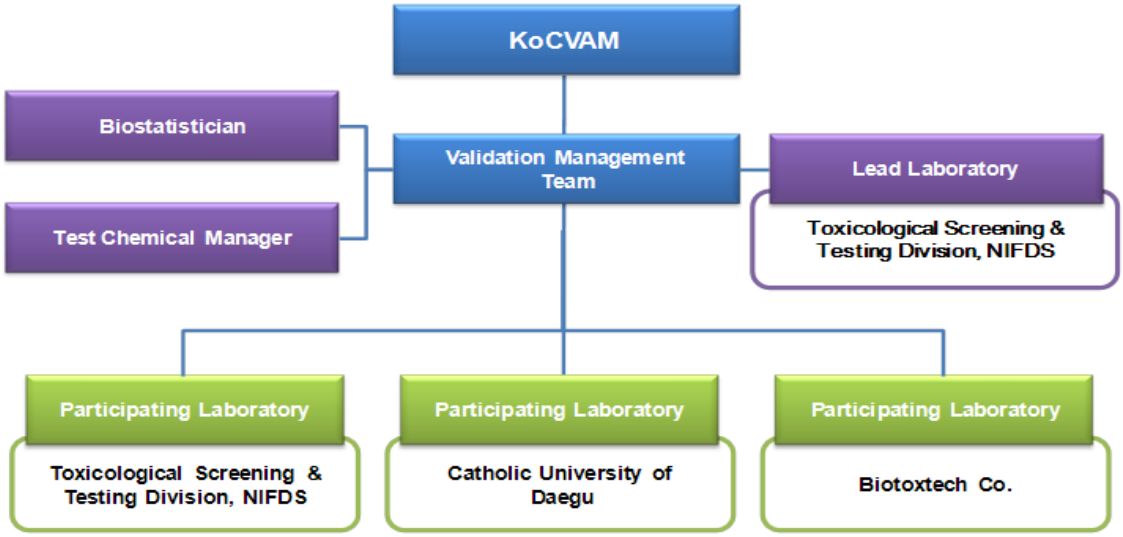


Figure A1-1. Organizational structure of the VMT and testing laboratories

Table A1-1. List of VMT members

Role	Name	Affiliation
Chair	Director of KoCVAM[1]	KoCVAM at NIFDS
Scientific advisor	Sohn, Soojung	KoCVAM at NIFDS
Scientific advisor	Lee, Ai Yeong	Dongguk University Hospital
Scientific advisor	Jeung, Eui Bae	Chungbuk National University
Scientific advisor	Jeong, Tae Cheon	Yeungnam University
Scientific advisor	Chun, Young Jin	Chung-Ang University
Scientific advisor	Lim, Kyung-Min	Ewha Womans University
Biostatistician	Bae, SeungJin	Ewha Womans University

The director of KoCVAM has served as chair of the VMT. The VMT chair has been replaced by a new KoCVAM director (Dr. Han Soon-young→Dr. Seong Won-keun in June 2013 and Dr. Seong Won-keun→Dr. Park Hye-kyung in August 2015).

3.2. Roles of the chemical manager

KoCVAM codes and distributes the reference chemicals selected for this study to the participating laboratories.

- Establishment of criteria for selecting test substances
- Test substance check
- Test substance coding
- Test substance distribution

3.3. Roles of the biostatistician

The biostatistician collates Excel spreadsheets with test results from the laboratories and statistically analyses the data.

- Test result collation (Excel spreadsheet format)
- Test result analysis
- Statistical report writing

3.4. Roles of the lead laboratory

The lead laboratory trains the participating laboratories and transfers the test method to them. It also provides the participating laboratories with a consistent protocol and all forms, including Excel spreadsheets. The lead laboratory modifies the protocol or forms based on comments received from the participating laboratories.

- Lead Laboratory 1: Medical Beauty Research Center at the AmorePacific R&D Unit[1]
- Lead Laboratory 2: Toxicological Screening and Testing Division (TSTD) at the National Institute of Food and Drug Safety Evaluation (NIFDS)

3.5. Roles of participating laboratories

The participating laboratories had no prior experience performing the test method, and transferability of the test method was tested. These laboratories let the lead laboratory know if they have any comments on a protocol or forms.

- Participating Laboratory 1: Catholic University of Daegu (Study director: Professor Yong Heo)
- Participating Laboratory 2: Biotoxtech Co. (Study director: Mi Sook Jung)

4. Budget

This validation study was supported by grants from NIFDS under the Ministry of Food and Drug Safety (12182MFDS791, 13172MFDS987, 15181MFDS457) and KoCVAM (093-2000-2034-300, 093-4000-4032-300).

5. Project plan

5.1. Transfer phase

Transfer of the test method: Lead Laboratory 1 trained the participating laboratories in the performance of the test method on 14 May 2012, and on the use of flow cytometry on 15 May 2012, at a workshop hosted by KoCVAM. The lead laboratory then visited the participating laboratories to see if the test method was successfully transferred (Participating Laboratory 2 on 21 May 2012, and Participating Laboratory 1 on 23 May 2012). Lead Laboratory 1 transferred the test method to Lead Laboratory 2 on 26 February 2014.

[1] Lead Laboratory 1, which participated in this validation study from 2012 to 2013, was replaced by Lead Laboratory 2 in May 2014.

Proficiency test: After self-training two to four times, each participating laboratory performed a proficiency test to determine whether the test method was properly implemented (27 June–3 July 2012, at Participating Laboratory 2; 19–25 October 2012, at Participating Laboratory 1; 20–26 August 2014, at Lead Laboratory 2).

5.2. Evaluation of test method accuracy

1st test: KoCVAM (Dr. Lee Yong-kyung) distributed 18 coded reference chemicals (OECD TG 429) to Lead Laboratory 1 on two separate occasions (18 July 2012, and 11 January 2013). The 1st test was conducted using the 18 chemicals according to Protocols 1.0 and 11 (September 19–25, 2012, with three chemicals and 8 May–22 October 2013, with 16 chemicals).

2nd test: The 2nd test was conducted from August to September 2014 using 14 reference chemicals (OECD TG 429) in accordance with Protocol 1.2, which was upgraded from Protocol 1.1 to enhance the accuracy of the test method (with changes in the solubility test and concentration selection procedures). These chemicals were distributed to Lead Laboratory 2 and Participating Laboratories 1 and 2.

Additional test: Protocol 1.2 was modified to Protocol 1.3 to improve criteria for evaluating results and handling substances that are difficult to dissolve. An additional test on imidazolidinyl urea was completed from May to June 2015.

3rd test: The 3rd test was conducted, using the 18 coded reference chemicals distributed to Participating Laboratory 2, according to Protocol 1.3 to determine the predictive capacity of the test method. This test was conducted from October to November 2015.

5.3. Evaluation of test method reliability

WLR: The evaluation of WLR of the test method was conducted from 30 May 2012, to 23 April 2013, and repeated four times at Lead Laboratory 1 and Participating Laboratories 1 and 2 using HCA.

BLR: The evaluation of BLR of the test method was repeated three times at Lead Laboratory 1 and Participating Laboratories 1 and 2 using HCA and DNCB (16 April–16 July 2013, for DNCB and 30 May 2012–23 April 2013, for HCA).

6. Test substances

The reference chemicals listed in OECD TG 429 were selected for this validation study. The VTM was responsible for the final selection of test substances, and KoCVAM coded and distributed test substances.

Table A1-2. Recommended reference chemicals listed in OECD TG 429

No.	Chemical	CAS No.	Veh.	EC3(%)	0.5x~2.0x EC3	LLNA vs. GP	LLNA vs. Human
18 OECD-recommended essential reference chemicals							
1	5-Chloro-2-methyl-4-isothiazolin-3-one (CMI)/2-methyl-4-isothiazolin-3-one (MI)	26172-55-4/ 2682-20-4	DMF	0.009	0.0045-0.018	+/+	+/+
2	DNCB	97-00-7	AOO	0.049	0.025-0.099	+/+	+/+
3	4-Phenylenediamine	106-50-3	AOO	0.11	0.055-0.22	+/+	+/+
4	Cobalt chloride	7646-79-9	DMSO	0.6	0.3-1.2	+/+	+/+
5	Isoeugenol	97-54-1	AOO	1.5	0.77-3.1	+/+	+/+
6	2-Mercaptobenzothiazole	149-30-4	DMF	1.7	0.85-3.4	+/+	+/+
7	Citral	5392-40-5	AOO	9.2	4.6-18.3	+/+	+/+
8	HCA	101-86-0	AOO	9.7	4.8-19.5	+/+	+/+
9	Eugenol	97-53-0	AOO	10.1	5.05-20.2	+/+	+/+
10	Phenyl benzoate	93-99-2	AOO	13.6	6.8-27.2	+/+	+/+
11	Cinnamic alcohol	104-54-1	AOO	21	10.5-42	+/+	+/+
12	Imidazolidinyl urea	39236-46-9	DMF	24	12-48	+/+	+/+
13	Methyl methacrylate	80-62-6	AOO	90	45-100	+/+	+/+
14	Chlorobenzene	108-90-7	AOO	25	NA	-/-	-/*
15	Isopropanol	67-63-0	AOO	50	NA	-/-	-/+
16	Lactic acid	50-21-5	DMSO	25	NA	-/-	-/*
17	Methyl salicylate	119-36-8	AOO	20	NA	-/-	-/-
18	Salicylic acid	69-72-7	AOO	25	NA	-/-	-/-
OECD optional substances to demonstrate improved performance relative to the LLNA							
19	Sodium lauryl sulphate	151-21-3	DMF	8.1	4.05-16.2	+/-	+/-
20	Ethylene glycol dimethacrylate	97-90-5	MEK	28	14-56	+/-	+/+
21	Xylene	1330-20-7	AOO	95.8	47.9-100	+/**	+/-
22	Nickel chloride	7718-54-9	DMSO	5	NA	-/+	-/+

AOO = acetone: olive oil (4:1, v/v), CAS No. = Chemical Abstracts Service Number, DMF = N,N-dimethylformamide, DMSO = dimethyl sulphoxide, MEK = methyl ethyl ketone, EC3 = estimated concentration needed to produce a SI of 3, GP = guinea pig test result (i.e. TG 406), CMI/ MI = 5-Chloro-2-methyl-4-isothiazolin-3-one/ 2-methyl-4-isothiazolin-3-one; DNCB = 2,4-dinitrochlorobenzene; HCA = hexyl cinnamic aldehyde, Liq = liquid, LLNA = murine local lymph node assay result (i.e. TG 429), NA = not applicable since SI <3, Sol = solid, Veh = test vehicle.

* = Presumed to be a non-sensitizer in humans based on the fact that no clinical patch test results were located, it is not included as a patch test kit allergen, and no case reports of human sensitization were located.

7. Analysis of validation study results and report preparation

Test data were collected and statistically analysed. The final version of the validation study report contains the results of accuracy and reliability evaluations conducted in compliance with the criteria in OECD TG 429 Annex 1.

8. Quality assurance

The participating laboratories performed this validation study in compliance with OECD GLP.

9. Literature

1. OECD_TG 406. 1992. OECD guideline for the testing of chemicals: Skin Sensitization (Guinea Pig Maximization Test; Buehler Test).

2. OECD_TG 429. 2010. OECD guideline for the testing of chemicals: Skin Sensitization: Local lymph node assay.

3. OECD_TG 442A. 2010. Skin Sensitization: Local lymph node assay: DA.

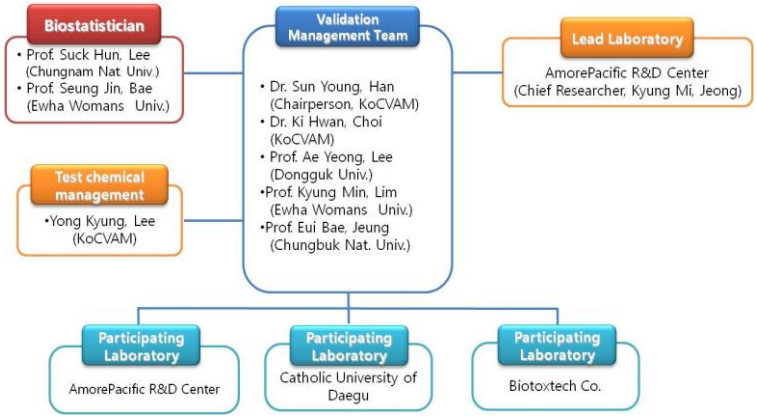
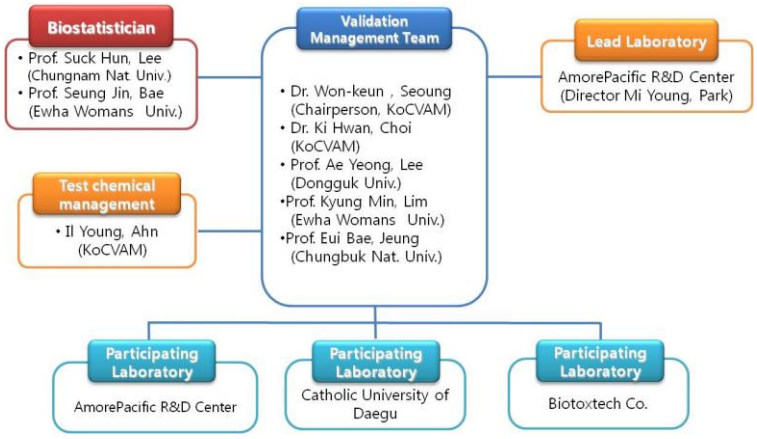
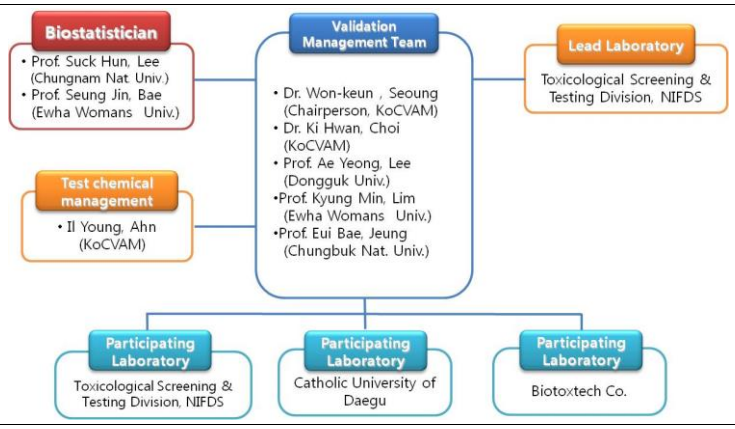
4. OECD_TG 442B. 2010. Skin Sensitization: Local lymph node assay: BrdU-ELISA.

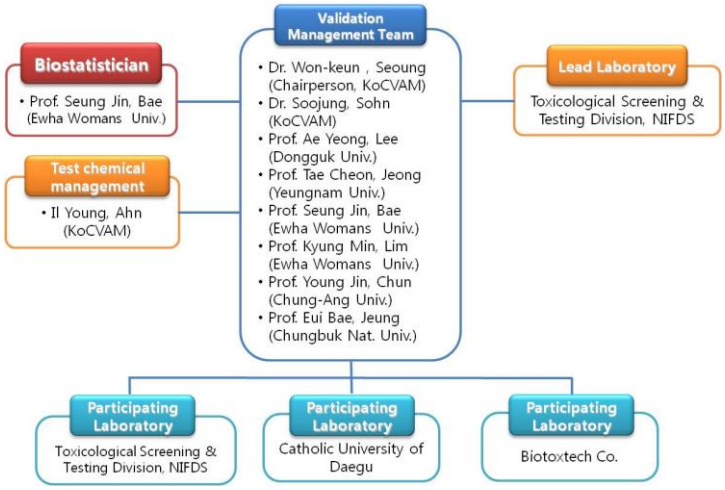
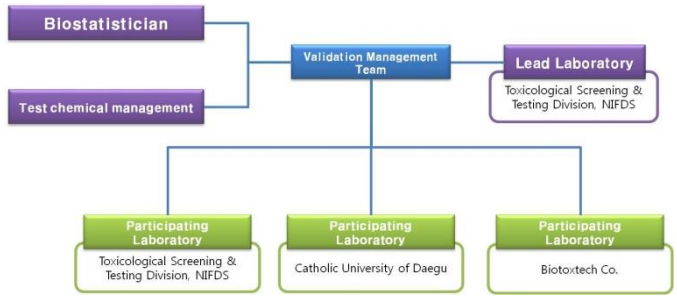
Table A1-3. Timeline for the LLNA: BrdU-FCM validation study

Date	Activity
16 April 2012	**1st VMT meeting** - Organized a VMT - Selected participating laboratories - Chose test substances - Approved the Project Plan (v.1.0) and the protocol (v.1.0)
14–15 May 2012	Trained the participating laboratories on laboratory equipment use and transferred the test method to them
21 and 23 May 2012	Checked whether the test method was properly transferred
27 June–3 July 2012 19–25 October 2012 20–26 August 2014	Performed a proficiency test and collected test results at Participating Laboratory 2 at Participating Laboratory 1 at Lead Laboratory 2
30 May 2012–16 July 2013	Evaluated the WLR and BLR of the test method using two reference chemicals (OECD TG 429)
16 April 2013	**2nd VMT meeting** - Approved the Project Plan (v.1.1) and (on 22 April) the protocol (v.1.1)
12–25 September 2012	Conducted the 1st test of the predictive capacity evaluation using 18 reference chemicals (OECD TG 429)
8 May~ 22 October 2013	
10 October 2013	**3rd VMT meeting** - Reviewed validation study results - Approved the Project Plan (v.1.2)
26 February 2014	Transferred the test method from Lead Laboratory 1 to Lead Laboratory 2
1 May 2014	**4th VMT meeting** - Approved the Project Plan (v.1.3) and the protocol (v.1.2)
26 May 2014	Trained the participating laboratories for Protocol 1.2
August–September 2014	Conducted the 2nd test of the predictive capacity evaluation using 14 reference chemicals (OECD TG 429)
10 November 2014	**5th VMT meeting** - Reviewed validation study results and statistical data
26 February 2015	**6th VMT meeting** - Reviewed a draft validation study report (v.1.0)
2 April 2015	**7th VMT meeting**

	- Reviewed a draft validation study report (v.1.1)
2 June 2015	**8th VMT meeting** - Reviewed a draft validation study report (v.1.2) - Approved the protocol (v.1.3)
10 July 2015	**9th VMT meeting** - Reviewed a draft validation study report (v.1.3)
21 July 2015	Reviewed a draft validation study report (v.1.4)
31 August 2015	Approved the Project Plan (v.1.4)
15 September 2015	Transferred the test method to Participating Laboratory 2 by Lead Laboratory 2
October–November 2015	Conducted the 3rd test of the predictive capacity evaluation using 18 reference chemicals (OECD TG 429)
21 December 2015	**10th VMT meeting** - Reviewed a draft validation study report (v.1.5)

Table A1-4. Summary of changes in VMT members and study personnel

Composition of the VMT and testing laboratories	Details
	**Project Plan version 1.0**
	**Project Plan version 1.1** The chair and some members of the VMT changed in order to coordinate the validation study more efficiently. Biostatisticians were added to the team. A chemical manager and the lead laboratory with study director were more clearly shown.
	**Project Plan version 1.2** The chair and chemical manager were replaced due to personnel changes at NIFDS. The study director of the lead laboratory was changed.
	**Project Plan version 1.3** The lead laboratory was changed from the AmorePacific R&D Unit to the TSTD at NIFDS.

	**Addition of VMT members** Two external experts were added to the VMT to more efficiently coordinate the validation study. A biostatistician was also included in the VMT.
	**Project Plan version 1.4** The chair of the VMT, Won-keun Seoung, was replaced by Hye-kyung Park due to personnel changes at NIFDS.

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[ANNEX 2]

List of Test Substances

Table A2-1. List of test substances used for transferability, WLR and BLR evaluations

No.	Substance	Manufacture	Purity	Cat No.	Remark
1	HCA	Aldrich	85%	291285	Used for Transferability, WLR, BLR
2	DNCB	Aldrich	99%	237329	Used for BLR
3	Eugenol	Fluka	99%	46129	Used for Transferability

HCA = hexyl cinnamic aldehyde.; DNCB = 2,4-dinitrochlorobenzene.

Table A2-2. List of test substances used for predictive capacity evaluation

No.	Substance	Manufacture	Purity	Cat No.	Lot No.	Code (2012-3)	Code (2014)	Code (2015)
1	CMI/ MI	Rohm and Hass	-	KathonCG	-	A010	NT	D018
2	DNCB	Aldrich	99%	237329	BCBD8253V (2012); BCBH7403V (2013); BCBN7826V (2015)	A004	NT	D009
3	4-Phenylenediamine	Sigma	98%	P6001	BCBG1562V (2013); WXBB8077V (2015)	A002	NT	D015
4	Cobalt chloride	Sigma-Aldrich	97% 98%	232696; 60818	BCBG0246V (2013); BCBN3189V (2015)	A005	NT	D003
5	Isoeugenol	Aldrich	98%	I17206	05622BEV (2013); 05622BEV (2015)	A012	NT	D014
6	2-Mercaptobenzothiazole	Aldrich	97%	M3302	MKBH9652V (2013); MKBH9652V (2014); MKBR2057V (2015)	A011	NT	D005
7	Citral	Aldrich	95%	C83007	MKBB9276V (2013); STBC5273V (2015)	A009	NT	D010
8	HCA	Aldrich	85%	291285	MKAA2596 (2012~3); MKBT2800V (2015)	A013	NT	D017
9	Eugenol	Fluka	99%	46129; E51791	MKBH6197V (2012~3); STBD3743V (2014); STBF3347V (2015)	A018	A001	D001
10	Phenyl benzoate	Aldrich	99%	142719	S78617V (2013); S78617V (2014); S78617V (2015)	A015	B003	D008
11	Cinnamic alcohol	Aldrich	98%	108197	STBC2697V (2013); STBD2742V (2014); STBD9743V (2015)	A014	B004	D004
12	Imidazolidinyl urea	Aldrich	95%	I5133	048K0664 (2013); SLBC3830V (2014); BCBL2782V (2015)	A016	A005, C002	D011
13	Methyl methacrylate	Aldrich	99%	M55909	LB70151 (2013); MKBF9569V (2014); MKBF9569V (2015)	A007	B002	D016
14	Chlorobenzene	Sigma-Aldrich	99.8%	284513	SHBB2598V (2013); SHBD3200V (2014); SHBF7216V (2015)	A008	B005	D007
15	Isopropanol	Sigma-Aldrich	99% 99.5%	I9030; I9516	SHBB1347V (2013); BCBL6528V (2014); BCBN7386V (2015)	A017	B001	D012
16	Lactic acid	Fluka	90%	69785	MKBH4952V (2012~3); BCBJ0074V (2014); BCBP5043V (2015)	A001	A002, C004	D006
17	Methyl salicylate	Sigma-Aldrich	99%	M6752	MKBH8725V (2013); MKBP7145V (2014); MKBV2107V (2015)	A006	C001	D002
18	Salicylic acid	Sigma	99%	S5922	MKBH2297V (2013); MKBP1051V (2015)	A003	NT	D013
19	Sodium lauryl sulphate	Sigma	98.5%	L3771	SLBH4318V (2014); SLBL7079L (2015)	NT	C005	NT
20	Ethylene glycol dimethacrylate	Aldrich	98%	335681	SHBC7307V (2014); SHBF8868V (2015)	NT	A003	NT
21	Xylene	Sigma-Aldrich	98.5%	247642	SHBC9884V (2014); SHBG0803V (2015)	NT	C003	NT
22	Nickel chloride	Aldrich	98%	339350	STBD3844V (2014); STBF1083V (2015)	NT	A004	NT

CMI/ MI = 5-Chloro-2-methyl-4-isothiazolin-3-one/ 2-methyl-4-isothiazolin-3-one; DNCB = 2,4-dinitrochlorobenzene; HCA = hexyl cinnamic aldehyde.

Table A2-3. List of test substances used for supplementary test

No.	Substance	Manufacture	Purity	Cat No.	Lot No.	Code
19	Sodium lauryl sulphate	Sigma	98.9%	L4509	SLBJ5023V	D020
20	Ethylene glycol dimethacrylate	Aldrich	97.8%	335681	05604BJ	D022
21	Xylene	Sigma-Aldrich	98.95%	247642	SHBF5480V	D021
22	Nickel chloride	Aldrich	98%	339350	BCBQ4882V	D019

[ANNEX 3]

Chemical Coding and Distribution Procedures

Chemical Coding and Distribution Procedures

1. Purpose

This guideline describes the procedures for coding, distributing, and shipping the test substances selected for the pre- and main validation studies.

2. Chemical coding

A code for the identification of a test substance consists of four or more numbers and letters. The initial letter represents the laboratory using the substance, and the following numbers indicate the test substance. The identifying number for a chemical is three digits. 'A008' can be an example of one of the study codes.

Table A3-1. Examples of laboratory letters

Laboratory 1	Laboratory 2	Laboratory 3	Laboratory 4
A	B	C	D

Table A3-2. Examples of chemical identification numbers

	Laboratory 1	Laboratory 2	Laboratory 3	Laboratory 4
Chemical #1	008	005	008	003
Chemical #2	006	006	004	009
Chemical #3	001	003	007	005
Chemical #4	003	007	002	006
Chemical #5	009	004	005	002
Chemical #6	002	008	006	008
Chemical #7	004	009	003	001
Chemical #8	005	001	001	004
Chemical #9	007	002	009	007

The true random number generator of True Random Number Service (www.random.org) can be used to create an ID number for a chemical. If the same number is generated twice, another number should be generated.

3. Chemical distribution

Chemicals should be obtained from reagent manufacturers and stored in the recommended conditions. Protective equipment must be worn when handling chemicals. The Record Sheet for Chemical Distribution is provided in A3-5.

Liquid or solid test substances should be weighed by an analytical balance and stored in amber glass bottles. A spatula should be used to remove solid chemicals from storage containers and a pipette for liquid ones. The bottles should be sealed by parafilm.

To prevent cross-contamination of chemicals, tools must be cleaned between uses or new ones used.

4. Chemical labelling

Information on the potential hazards of test substances, based on the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) must be included on chemical labels so that experimenters can be aware of precautions before handling them. In addition, Material Safety Data Sheets (MSDS), which are provided along with test substances, enable experimenters to respond to emergencies.

The purity, molecular weight, and other information for a test substance can also be included, depending on the needs for a particular test method.

Examples of chemical labels can be found below:

Ex. 1

Study title
Code
Storage temperature
Quantity
Hazard

LLNA: BrdU-FCM

CODE: A018

Storage:
Quantity: mL
Hazard:

Ex. 2

Study title
Code
Purity
Molecular weight
Storage temperature
Hazard

LLNA: BrdU-FCM

CODE: A018

Purity:
Molecular weight:
Storage:
Hazard:

One person, in addition to the experimenter, must be involved in the distribution and labelling of test substances. The items checked, including the weight of each chemical, should be documented in the Record Sheet for Chemical Distribution (see A3-5).

5. Shipping and receipt of chemicals

For potentially hazardous test substances, information on chemical shipping as specified in MSDS should be included on labels.

A list of chemicals and MSDS, stored together in an envelope, should be provided so that experimenters can respond to emergencies. The envelope should be sealed with tape, and a chemical manager should sign the envelope in order to verify whether it has been opened.

Test substances are transported in containers that are packed to prevent damage. The test substances should be stored at 4°C and packed and shipped on wet ice packs. Substances that should be maintained at -20°C must be transported on dry ice.

If test substances are shipped abroad, the shipper must comply with regulations of carriers and other countries.

The Record Sheet for Chemical Shipping (A3-6) should be completed.

If test substances are transported by a carrier service, a recipient should be informed of the shipment by e-mail. In addition, a Verification of Receipt (A3-7) for chemicals and relevant documents should be attached to the e-mail. After completing the Verification of Receipt, a recipient should return the document to KoCVAM.

6. Completing the forms

One experimenter should be charged with completing the forms in A3-5 and A3-6 for confirmation by KoCVAM.

※ Ref.: Standards for Classification and Labelling of Chemicals and Material Safety Data Sheets (MoL public notice no. 2012-14)

Record Sheet for Chemical Distribution

Date:

Laboratory	Code	Chemical	Storage Conditions	Weight (g)	Comments
Laboratory 1	A008	Chemical #1	RT		
Laboratory 2	B005	Chemical #1	RT		
Laboratory 3	C008	Chemical #1	RT		
Laboratory4	D003	Chemical #1	RT		

Balance used:

Name (person who distributed): _____ (signature) Date: _____

Name (person who confirmed): _____ (signature) Date: _____

Record Sheet for Chemical Shipping

Date:

Laboratory	Code	Chemical	Storage Conditions	Comments
Laboratory 1	A008	Chemical #1	RT	
	A006	Chemical #2	4°C	
	A001	Chemical #3	RT	
	A003	Chemical #4	RT	

Name (person who shipping): _____(signature) Date: _____

Name (person who confirmed): _____(signature) Date: _____

Verification of Receipt

Date of receipt:

Laboratory	Code	Storage Conditions	Receipt of chemicals (Yes/No)	Sealing of MSDS envelope (Yes/No)	Comments
Laboratory 1	A008	RT			
	A006	4°C			
	A001	RT			
	A003	RT			

Name (recipient): _____ (signature) Date: _____

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[ANNEX 4]

Template for the Study Result Report

Table A4-1.

Preliminary study 1 - Test Material [ ] - DATA SHEET_version 1.0

Test No.		IACUC No.		Study Director	
Receipt of animals		Acclimatization		Period of study	

Date		Operator						
		GP	BW	ET	Treat	CO	EW	Sac.
Day 1								
Day 2								
Day 3								
Day 4								
Day 5								
Day 6								

Group	TM(%)	Animal No.	Death
VC			
T1			

Group	ID	BW (g)		Ear thickness (mm)														EW (mg)
				Day 1						Day 3				Day 6				
		Day 1	Day 6	L			R			L			R	L		R		

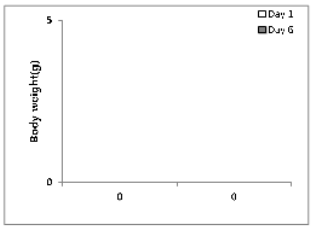
Result (Mean & SD)

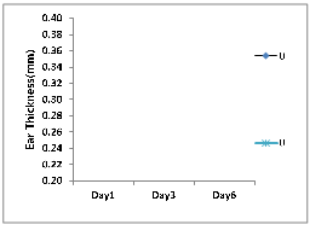
Mean	체중			Ear thickness						EW
	Day 1	Day 6	gain	Day1	Day3	Day6	D3-D1	D6-D1	3-D1(%)	6-D1(%
0	#####	#DIV/0!	#DIV/0!	#####	#####	#####	#####	#####	#####	#####
0	#####	#DIV/0!	#DIV/0!	#####	#####	#####	#####	#####	#####	#####

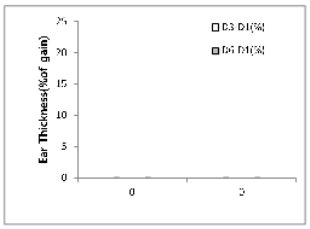
SD	체중			Ear thickness						EW
	Day 1	Day 6	gain	D1	D3	D6	D3-D1	D6-D1	3-D1(%)	6-D1(%
0	#####	#DIV/0!	0.00	#####	#####	#####	#####	#####	#####	#####
0	#####	#DIV/0!	0.00	#####	#####	#####	#####	#####	#####	#####

		체중	D3-D1	D6-D1	3-D1(%	6-D1(%)
0	0	0.0	#DIV/0!	#####	#####	#####
	0	0.0	#DIV/0!	#####	#####	#####
0	0	0.0	#DIV/0!	#####	#####	#####
	0	0.0	#DIV/0!	#####	#####	#####

Graph







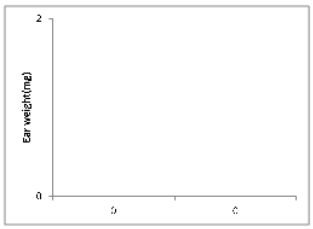


Table A4-2.

Preliminary study 2 - Test Material [ ] - DATA SHEET_version 1.0

Test No.		IACUC No.		Study Director	
Receipt of animals		Acclimatization		Period of study	

Date		Operator						
		GP	BW	ET	Treat	CO	EW	Sac.
Day 1								
Day 2								
Day 3								
Day 4								
Day 5								
Day 6								

Group	TM(%)	Animal No.	Death
VC			
T1			
T2			
T3			
T4			
T5			

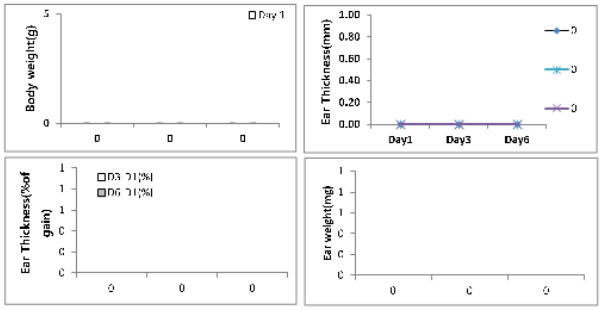
Group	ID	BW (g)		Ear thickness (mm)																		EW (mg)
				Day 1						Day 3						Day 6						
		Day 1	Day 6	L			R			L			R			L			R			

Result (Mean & SD)

Mean	체중			Ear thickness							EW
	Day 1	Day 6	gain	Day1	Day3	Day6	D3-D1	D6-D1	D3-D1(%)	D6-D1(%	
0	#####	#DIV/0!	#DIV/0!	#DIV/0!	#####	#DIV/0!	#####	#####	#####	#####	#####
0	#####	#DIV/0!	#DIV/0!	#DIV/0!	#####	#DIV/0!	#####	#####	#####	#####	#####
0	#####	#DIV/0!	#DIV/0!	#DIV/0!	#####	#DIV/0!	#####	#####	#####	#####	#####

SD	체중			Ear thickness							EW
	Day 1	Day 6	gain	D1	D3	D6	D3-D1	D6-D1	D3-D1(%)	D6-D1(%	
0	#####	#DIV/0!	0.00	#DIV/0!	#####	#DIV/0!	#####	#####	#####	#####	#####
0	#####	#DIV/0!	0.00	#DIV/0!	#####	#DIV/0!	#####	#####	#####	#####	#####
0	#####	#DIV/0!	0.00	#DIV/0!	#####	#DIV/0!	#####	#####	#####	#####	#####

Graph



		체중	D3-D1	D6-D1	3-D1(%	D6-D1(%)
0	0	0.0	#DIV/0!	#DIV/0!	#####	#DIV/0!
	0	0.0	#DIV/0!	#DIV/0!	#####	#DIV/0!
0	0	0.0	#DIV/0!	#DIV/0!	#####	#DIV/0!
	0	0.0	#DIV/0!	#DIV/0!	#####	#DIV/0!
0	0	0.0	#DIV/0!	#DIV/0!	#####	#DIV/0!
	0	0.0	#DIV/0!	#DIV/0!	#####	#DIV/0!

Table A4-3.

Main study - Test Material [ ] - DATA SHEET_version 1.0

Test No.			IACUC No.			Study Director	
Receipt of animals			Acclimatization			Period of study	

Date		Operator											
		GP	BW	ET	Treat	CO	EW	LW	LNC	BrdU	Sac.	Stain	FACs
Day 1													
Day 2													
Day 3													
Day 4													
Day 5													
Day 6													
Day 7													

Group	Test material (%)	Conc.	Animal No.	Death
VC				
TL				
TM				
TH				
PC				

Group	ID	BW (g)		Ear thickness (mm)																		EW (mg)	LW (mg)	LNC ($\times10^7$)	FACS		
				Day 1						Day 3						Day 6									BrdU	#BrdU	SI
		Day 1	Day 6	L			R			L			R			L			R								

Count	Dilution	Total ($\times10^6$ cells/mL)	㎖ for 1.5×10^6 cells/mL

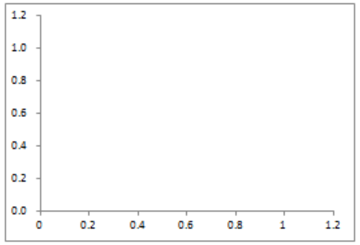


Table A4-4.

Main study - Test Material [ ] - DATA SHEET_version 1.0

Test No.		IACUC No.		Study Director	
Receipt of animals		Acclimatization		Period of study	

Date		Operator										
		BW	ET	Treat	CO	EW	LW	LNC	BrdU	Sac.	Stain	FACs
Day 1												
Day 2												
Day 3												
Day 4												
Day 5												
Day 6												
Day 7												

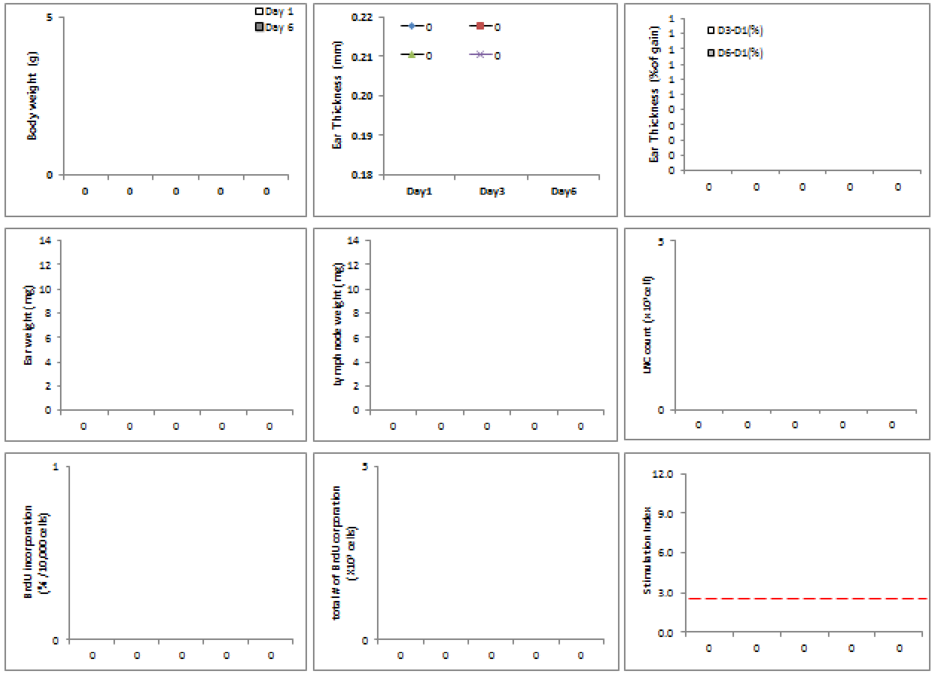
#VALUE!				
Group	Test material (%)	Conc.	Animal No.	Death
VC				
TL				
TM				
TH				
PC				

Result (Mean & SD)

Mean	체중			Ear thickness							EW	LW	LNC	FACS		
	Day 1	Day 6	gain	Day1	Day3	Day6	D3-D1	D6-D1	3-D1(%	6-D1(%				BrdU	#BrdU	SI
0	#####	#DIV/0!	#DIV/0!	####	####	####	####	####	####	####	####	####	####	####	####	####
0	#####	#DIV/0!	#DIV/0!	####	####	####	####	####	####	####	####	####	####	####	####	####
0	#####	#DIV/0!	#DIV/0!	####	####	####	####	####	####	####	####	####	####	####	####	####
0	#####	#DIV/0!	#DIV/0!	####	####	####	####	####	####	####	####	####	####	####	####	####
0	#####	#DIV/0!	#DIV/0!	####	####	####	####	####	####	####	####	####	####	####	####	####

SD	체중			Ear thickness							EW	LW	LNC	FACS		
	Day 1	Day 6	gain	D1	D3	D6	D3-D1	D6-D1	3-D1(%	6-D1(%				BrdU	#BrdU	SI
0	#####	#DIV/0!		####	####	####					####	####	####	####	####	####
0	#####	#DIV/0!		####	####	####					####	####	####	####	####	####
0	#####	#DIV/0!		####	####	####					####	####	####	####	####	####
0	#####	#DIV/0!		####	####	####					####	####	####	####	####	####
0	#####	#DIV/0!		####	####	####					####	####	####	####	####	####

Graph



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[ANNEX 5]

Protocol (version 1.3.1)

Protocol for the Local Lymph Node Assay Using the Flow Cytometry Method
Version: 1.3.1

Date of revision: November 25, 2016
Written by: Toxicological Screening and Testing Division at the National Institute of Food and Drug Safety Evaluation (NIFDS)

1. Objective

The Local Lymph Node Assay using a flow cytometry method (LLNA-FCM) is a modified version of the LLNA, which employs radioactive isotopes such as thymidine or iodine to measure lymph cell proliferation. The purpose of this protocol is to describe the test method procedures to predict skin sensitization potential of chemicals by measuring a non-radioactive substance of 5-bromo-2'-deoxyuridine (BrdU) and lymph cells by flow cytometry.

2. Materials

2.1 Test animals

Female BALB/c mice are the animals of choice for this test. Seven-week-old mice should be purchased and acclimated for at least 5 days prior to the start of the experiment. Healthy mice, 8 to 12 weeks old, with normal body weights and showing no abnormal clinical signs, should be used in experiments. Mice whose weight exceeds the mean weight by 20% should not be used. At least four mice are randomly allocated to the vehicle control-treatment group and the positive control-treatment group and the test substance-treatment group, and the average body weight of each group is similar. The two additional groups, blank group and non-treatment group, are also needed for setting a flow-cytometer.

All groups of animals needed are as below:

- Blank group (n=1): No BrdU injected
- Non-treatment group (n=1): Injection of BrdU without treatment with any substances
- Vehicle control-treatment group (n≥4): Injection of BrdU and treatment with a vehicle
- Test substance-treatment group (n≥4): Injection of BrdU and treatment with test substances (a minimum of three concentrations are needed)
- Positive control-treatment group (n≥4): Injection of BrdU and treatment with 25% Hexyl cinnamic aldehyde (HCA)

The temperature of the experimental animal room should be 22 ± 3°C. The relative humidity should be between 30% and 70%. Lighting should be artificial with a cycle of 12 hours light and 12 hours dark. Test animals should be fed on a solid diet for mouse chow and have free access to UV-sterilized or boiled tap water.

2.2 Reagents and equipment

Step	Equipment and reagents	Details
Treatment	Glass vial	For chemical preparation
	Balance 1	For chemical weight measurement, readable to 0.1 mg e.g. BS224S, Sartorius, Germany; XT200A, Precisa, Switzerland
	Balance 2	For body weight measurement, readable to 0.1 g e.g. CP622, Sartorius, Germany; MWP-300H, CAS, U.S.A.
	Skin marker	For animal identification
	Micropipette and tips	For applying test solution (25 µL), for dissolved liquid chemical and vehicles e.g. 200 µL, Gilson, Inc., U.S.A.; Nichipet EX CE 200, Nichiryo Co., Ltd., Japan
	Cage set	Capable of housing five mice, with food and water dispensers
	Thickness gauge	For ear thickness measurement, readable to 0.01 mm e.g. Mitutoyo corporation, Japan (Product Code 700-118-20 or 543-

		681B)
	Syringe	For BrdU injection (1 mL, 26GX1/2")
	5-Bromo-2′-deoxy-Uridine (BrdU)	e.g. Cat. No. Sigma B5002 (1 g)
Sacrifice	CO_2 gas system	For humane sacrifice
	Biopsy punch	For ear punch (6 mm) e.g. Stiefel Laboratories, Inc., U.S.A.; Miltex Inc., Germany
	Dissecting instruments	For lymph node excision, scissors and tweezers
	Micropipette and tips	For handling liquids (20 µL, 100 µL, and 1000 µL) e.g. 20 µL, 200 µL, and 1000 µL, Gilson, Inc., U.S.A.; Nichipet EX CE 10, 20, 200, 1000, Nichiryo Co., Ltd., Japan
	Phosphate buffered saline	pH 7.2, sterilized e.g. Cat. No. 10010-023, Lonza Walkersville Inc., U.S.A.
	6-well plate	Cell culture plate, sterilized e.g. Cat. No. 3516, Corning, Inc., U.S.A.
	15 mL tube	Polypropylene, sterilized
	70-µm nylon mesh	70-µm pore size, white e.g. sterile cell strainer, Ref. 352350, Falcon, Inc., U.S.A.
	Spatula	e.g. Cat. No. US.3282, Usbeck Inc., Germany
	Vortex mixer	
	Trypan blue	For live-cell staining e.g. Cat. No. Sigma T-8154 (100 mL)
	Hemocytometer	For counting living cells e.g. DHC-N01, NanoEnTek, Inc., Korea
	Microscope	
Staining	BrdU flow kit	For BrdU special staining Cat. No. BD 559619, BD, Inc., U.S.A.
	Foetal bovine serum	For preparing staining buffer, heat inactivated e.g. Cat. No. 16000, Gibco Inc., U.S.A.
	Dulbecco's Phosphate-Buffered Saline	e.g. Cat. No. 14190-250, Lonza Walkersville Inc., U.S.A.
	Timer	
	Ice box	
	Centrifuge	For cell collection, temperature range 4–25°C e.g. Micro 17TR, Hanil Science Industrial. Korea
	Water bath	For incubation of DNase at 37℃. e.g. DK-CB001, Daiki Sciences, Korea; KMC-1205W, Vision Scientific, Korea
	Flow cytometry tube	e.g. Cat. No. BD 352008 (5 mL) , BD, Inc., U.S.A.
Flow cytometry	Flow cytometer	e.g. BD FACS Calibur™ or, Beckman coulter Cytomics FC 500
Etc.	General laboratory materials	Test tube rack, microtube rack, gloves, cotton, paper towels, 70% EtOH, etc.

3. Experimental procedures

3.1 Experimental design

Schedule for the LLNA: BrdU-FCM main test

Experiments	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Grouping	O*	O*						
Clinical observation	O	O	O	O	O	O	O	
Treatment		O	O	O				
Measurement of body weight		O					O	
Measurement of ear thickness		O		O			O	
Irritation evaluation		O	O	O	O	O	O	
BrdU solution injection						O		
Sacrifice							O	
Measurement of ear weight							O	
Measurement of lymph node weight							O	
BrdU staining							O	●
Analysis with flow cytometry							O	●

BrdU, 5-bromo-2-deoxyuridine

●: possible to analyse samples

*: possible to group experimental animals on day 0 or day 1

3.2 Preparation of test substances

Collect information on the chemicals and, if applicable, keep chemical reports. The vehicle should not interfere with or bias the test result and should be selected on the basis of maximising the solubility in order to obtain the highest concentration achievable while producing a solution/suspension suitable for application of the test substance. Recommended vehicles are acetone:olive oil (4:1 v/v, AOO, acetone (Cat. No. 650501 or 270725, Sigma Inc., U.S.A.); olive oil (Cat. No. O1514, Sigma Inc., U.S.A.)), *N,N*-dimethylformamide (DMF, Cat. No. D4551, Sigma Inc., U.S.A.), methyl ethyl ketone (MEK, Cat. No. 360473, Sigma Inc., U.S.A.), propylene glycol (Cat. No. P4347, Sigma-Aldrich Inc., U.S.A.) and dimethyl sulphoxide (DMSO, Cat. No. D5879 or D2650, Sigma Inc., U.S.A.) but others may be used if sufficient scientific rationale is provided, as suggested in OECD TG 429. For test substances without known solutions, tests for the selection of the vehicles should be performed. Vehicle selection tests, if necessary, is following the pre-screen test described in pages A5-16~21.

Vehicles used to dissolve test substances should be used as vehicle controls for the test. 25% HCA (Cat. No. 921285, Sigma Inc., U.S.A.) in AOO should be used as positive controls. All test substances mentioned above should be prepared daily.

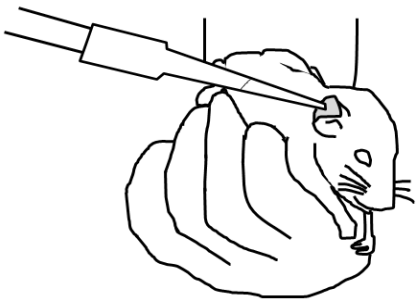
3.3 Dose selection

Consecutive doses are normally selected from an appropriate concentration series such as 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc. as suggested in OECD TG 429. In the absence of information needed to determine the highest dose to be tested, pre-screen tests should be performed in order to define an appropriate dose level in the LLNA. Adequate scientific rationale should accompany the selection of the concentration series used. All existing toxicological information (e.g. acute toxicity and dermal irritation) and structural and physicochemical information on the test substance of interest (and/or structurally related test substances) should be considered where available, in selecting the three consecutive concentrations so that the highest concentration maximises exposure while avoiding systemic toxicity and/or excessive local skin irritation.

In this protocol, doses for the main test could be determined by performing two pre-screen tests to avoid extreme toxicity, a 1st pre-screen test with a relative low single dose (25% which is not expected to cause skin irritation) and vehicle controls, and a 2nd pre-screen test with two or more doses and vehicle controls. After the pre-screen test, minimum three consecutive doses, the maximum that did not cause excessive local skin irritation or systemic toxicity, are selected. For details, follow the pre-screen test procedure described on pages A5-16~21.

3.4 Application of test substances

A test solution, vehicle control, or positive control, all 25 μL, is applied to the dorsum of each ear of a mouse in three or more rounds. Test substances should be slowly spread on the surface in a circle using the side of a micropipette tip. Care should be taken with the pipette so that a large amount of the test substances is not applied at once. After application, the mouse should be placed in a temporary cage with no litter in order for the test substances to be fully absorbed and then returned to its cage. Application of chemicals should be performed on days 1, 2, and 3 at a designated time in the morning.



3.5 Injection of BrdU solution

5-Bromo-2′-deoxy-Uridine solution (BrdU, Cat. No. B5002, Sigma Inc., U.S.A.) should be prepared, with BrdU reagents, in pre-warmed and sterilized phosphate buffered saline (PBS, Cat. No. 10010-023, Lonza Walkersville Inc., U.S.A.) at 37^{o}C for 30 minutes at 20 mg/mL. BrdU solution should be prepared daily.

A single intraperitoneal injection of 100 μL of a BrdU solution (20 mg/mL) prepared daily in PBS (2 mg/mouse) should be administered to the mouse 24 ± 2 hours before sacrifice.

3.6 Observations

3.6.1 General symptoms and death

Check daily on mice for general symptoms and death and record any event.

3.6.2 Erythema

Check on erythema where the chemical substance was applied and record observations daily. The evaluation of erythema should be performed in accordance with the Draize test method and assigned an erythema score prior to the application of test substances (see Table A5-1).

Table A5-1. Erythema scores

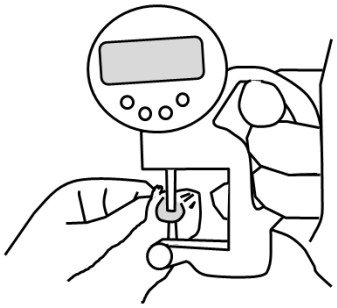
Observation	Score
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet-redness) to eschar formation interfering with the grading of erythema	4

3.6.3 Body weight

Body weights of animals should be measured at the beginning of the experiment (day 1) and at sacrifice (day 6). Mice whose weight variation exceeds the mean body weight by 20% prior to day 1 should be excluded from the test. If the weight variation of a mouse between day 1 and day 6 is greater than 5%, it should be evaluated for systemic toxicity.

3.6.4 Ear thickness

Ear thickness is measured at the centre of each ear using a digital thickness gauge (Cat. No. 700-118-20, Mitutoyo corporation, Japan; Cat. No. 543-681B, Mitutoyo corporation, Japan) on days 1, 3, and 6. Mean values are used as a barometer of changes in thickness.



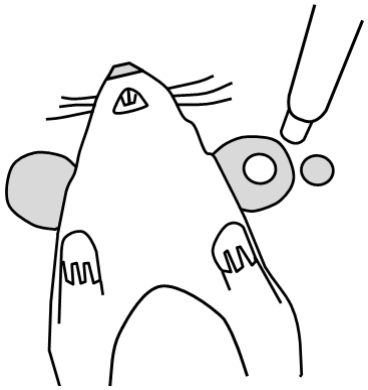
3.7 Autopsy

3.7.1 Humane killing

Humane killing should be employed to minimize pain and stress in animals and should be harmless to experimenters. Humane killing by CO_2 gas asphyxiation is recommended for this test.

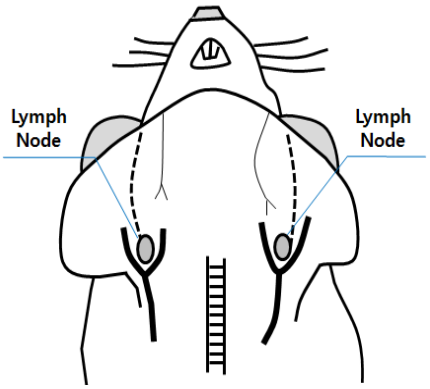
3.7.2 Measurement of ear weight

After sacrifice, spread ears evenly on a rubber pad and sample the centre of both ears using a 6-mm punch (Cat. No. BI-3000, Stiefel Laboratories, Inc., U.S.A.; Cat. No. 33-36, Miltex Inc., Germany). When measuring ear weight, measure both ears together.



3.7.3 Harvesting of lymph nodes (LNs) and measurement of LN weight

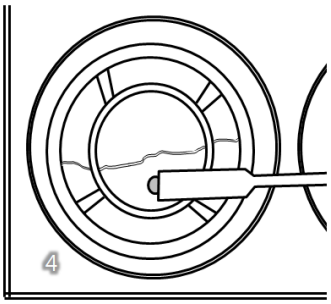
Auricular LN between the jugular veins under ears should be harvested separately. For the locations of auricular lymph nodes, refer to the figure on the right. Weigh both auricular lymph nodes together and place them into the well of a flat-bottom 6-well plate (Cat. No. 3516, Corning, Inc., U.S.A.) filled with a cold PBS (1 mL). The 6-well plate should be maintained on ice.



3.8 Preparation of lymph node cells (LNCs)

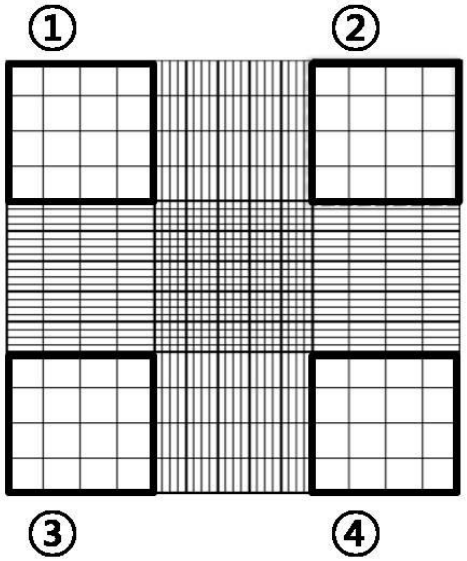
3.8.1 Preparation of single LNCs

From each mouse, a single-cell suspension of lymph node cells (LNC) excised bilaterally is prepared by gentle mechanical disaggregation through a 70-μm nylon mesh. The procedure for preparing the LNC suspension is critical in this assay and therefore, every operator should acquire relevant skills in advance. Further, the lymph nodes in NC animals are small, so special care should be taken to avoid any artificial effects on SI values. Fill a 6-well plate with cold PBS (1 mL) and harvest LNs in a 70-μm cell strainer (e.g. Falcon sterile cell strainer, Ref. 352350). Then, mash the LNs with a spatula until there is only a white lymph node membrane left. Transfer the single cell suspension to a 15-mL tube. Wash inside and outside of the well and strainer previously filled with the suspension with 1 mL of cold PBS. This entire process should be performed on ice. The LNC suspension can be diluted to an appropriate volume (e.g. 1/10 dilution). The LNs in each mouse should be processed separately.



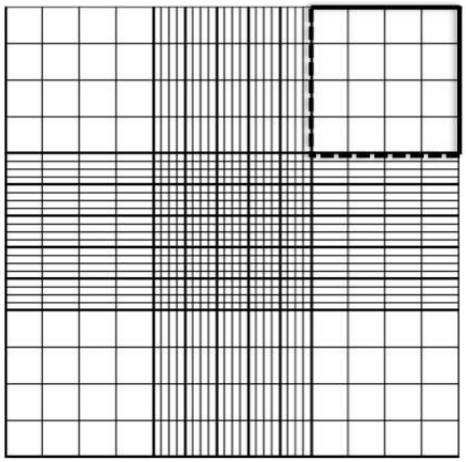
3.8.2 Count of LNCs

Mix 20 μL of a diluted cell suspension and another 20 μL of trypan blue (Cat. No. T-8154, Sigma Inc., U.S.A.) together, and then load 10 μL of the mixture into a haemocytometer. Because only viable cells should be scored, stained and dark-looking cells should not be counted; only cells that are not stained should be scored. Scoring LNCs using a haemocytometer (Cat. No. DHC-N01, NanoEnTek, Inc., Korea) as shown below. Score cells in sections 1–4 (in numerical order) in the figure below.



Areas to be scored on a haemocytometer

For cells on borders, score those in solid lines and those on dotted lines (the bottom horizontal line and the left vertical line) should not be scored. Cells should not be counted using devices other than a haemocytometer.



Acceptable range of cell scoring on borders when using a haemocytometer

Total cell number/mL = (Haemocytometer counting/4 $^{a)}$) x $2^{b)}$ x $A^{c)}$ x 10^4

a) Average haemocytometer count
b) Trypan blue dilution
c) Cell dilution factor

3.9 BrdU staining

The FITC BrdU Flow Kit (Cat. No. 559619, BD Pharmingen™, U.S.A.) should be used for BrdU staining.

The kit contains the following components:

- Fluorochrome-conjugated anti-BrdU Antibody

- BD Cytofix/Cytoperm™ Buffer
- BD Perm/Wash™ Buffer (10X)
- BD Cytoperm™ Permeabilization Buffer Plus (Cat. No. 561651, BD biosciences Inc., U.S.A.)
- 7-amino-actinomycin D (7-AAD)
- DNase (Cat. No. D-4513, BD biosciences Inc., U.S.A.)

3.9.1 Preparation of reagents

(1) Prepare a BD Perm/Wash™ Buffer (10X) at a 10-fold dilution. Based on 6 mL of Perm/Wash™ Buffer needed per reagent, prepare a sufficient quantity for the assay.
(2) Prepare a BD Falcon™ 12 × 75-mm sample acquisition tube (Cat. No. 352008, BD biosciences Inc., U.S.A.) to measure fluid cells, because one is not included in the kit.
(3) Prepare staining buffer [BD Pharmingen Stain Buffer (FBS) (Cat. No. 554656) or 1× Dulbecco's Phosphate-Buffered Saline (DPBS, Cat. No. 14190-250, Lonza Walkersville Inc., U.S.A.) + 3% heat-inactivated FBS (Cat. No. 16000, Gibco Inc., U.S.A.) + 0.09% sodium azide (Cat. No. S2002 or S8032, Sigma Inc., U.S.A), because it is not included in the kit.

3.9.2 Process

(1) Tubes filled with LNCs (1.5×10^6/mL) should be separated by centrifugation (500 × *g*) for 7 minutes at 4°C.
(2) After removing the supernatant, 100 µL of a Cytofix/Cytoperm Buffer should be added and incubated for 20 minutes on ice.
(3) Then, 1 mL of Perm/Wash buffer is added. After centrifugation (500 × *g*) for 7 minutes at 4°C, the supernatant should be removed. When removing supernatant liquid, care should be taken not to damage pellets. After this step, overnight storage is possible. Add 1 mL of staining buffer, mix, and store the solution at 4°C. The next day, after centrifugation (500 × *g*) for 7 minutes at 4°C, remove the buffer and proceed to step 4.
(4) Add 100 µL of Cytoperm Permeabilization Buffer Plus, mix, and leave the solution on ice for 10 minutes.
(5) Add 1 mL of 1× Perm/Wash Buffer, and after centrifugation (500 × *g*) for 7 minutes at 4°C, remove the supernatant.
(6) Add 100 µL of Cytofix/Cytoperm Buffer, mix, and place the solution on ice for 5 minutes.
(7) Add 1 mL of 1× Perm/Wash Buffer, and after centrifugation (500 × *g*) for 7 minutes at 4°C, remove the supernatant.
(8) After combining 700 µL of PBS with 300 µg of DNase (300 µg/mL), add 100 µL of the solution and mix it sufficiently. Then, incubate the mixture in a 37°C water bath for 1 hour.
(9) Add 1 mL of 1× Perm/Wash Buffer, and after centrifugation (500 × *g*) for 7 minutes at 4°C, remove the supernatant.
(10) Dilute Anti-BrdU 50 fold with 1× Perm/Wash Buffer. Add 50 µL of Anti-BrdU and incubate the solution at room temperature for 20 minutes in the dark.
(11) Add 1 mL of 1× Perm/Wash Buffer, and after centrifugation (500 × *g*) for 7 minutes at 4°C, remove the supernatant.
(12) Add 100 µL of staining buffer, and after tapping to mix, add another 900 µL of the staining buffer. Add 20 µL of 7-AAD and mix.
(13) Measure fluorescence-stained cells by flow cytometry.

3.10 Measurement of BrdU by flow cytometry

3.10.1 Preparation prior to measurement

To measure incorporated BrdU, the following samples should be prepared prior to the first measurement.

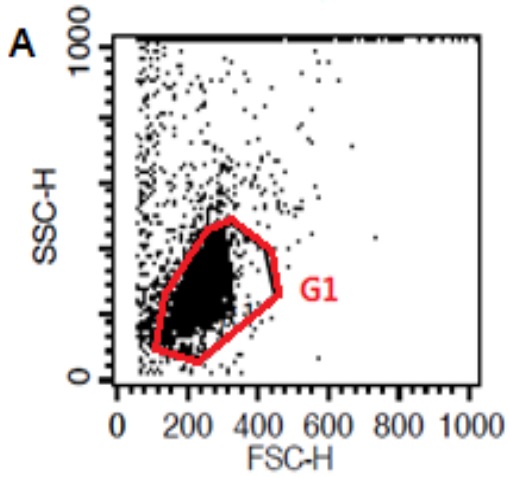
- Blank sample (n=1): LNCs from the mouse that was not injected with BrdU.
- Non-treatment sample (n=1): LNCs from the mouse that was not treated with any substances, but that received a BrdU injection.
- Vehicle control-treatment sample (n≥4): LNCs from the mouse that was treated with the vehicle control and received a BrdU injection.
- Test substance-treatment sample (n≥4, a minimum of three concentrations): LNCs from the mouse that was treated with test substances and received a BrdU injection.
- Positive control-treatment sample (n≥4): LNCs from the mouse that was treated with the positive control and received a BrdU injection.

3.10.2 Analysis of flow cytometric results

A flow cytometer should be calibrated using appropriate tools (e.g. 'BD FACSComp' for FACS Calibur™ or 'Beckman coulter FlowCheck' for Cytomics FC500) prior to testing or regularly.

(1) Forward scatter-side scatter (FSC-SSC) graph

1) Both the X (FSC) and Y (SSC) axes are on a linear scale (graph A).
2) Set up a zone (gate) with a flock of viable lymph nodes at its centre in the FSC-SSC graph (G1).
3) Outline G1 such that it has 10,000 cells.



(2) 7-AAD-BrdU graph

1) The X axis (7-AAD, FL3) is on a linear scale, whereas the Y (BrdU, FL1) axis is a log scale (graph B-E).
2) Compensation should be set once using unstained, only BrdU-stained, only 7-AAD stained samples, and double stained with both anti-BrdU and 7-AAD at the time of beginning this assay, and the compensation can be saved for future use.

① Unstained samples: Samples that are not stained at all

→ Use these samples for cornering cells into the bottom left of the FL1 vs FL3 graph.

② BrdU-stained samples: Samples stained with only fluorescein isothiocyanate (FITC)-conjugated anti-BrdU

→ Use these samples to properly locate BrdU positive cells along the Y axis. Adjust 'FL3-FL1' compensation parameter or if 'FL3-FL1' not available, use 'FL2-FL1' and 'FL3-FL2' compensation parameter.

③ 7-AAD-stained samples: Samples stained only with 7-AAD

→ Use these samples to properly locate 7-AAD positive cells along the X axis. Adjust 'FL1-

FL2' and 'FL2-FL3' compensation parameter.

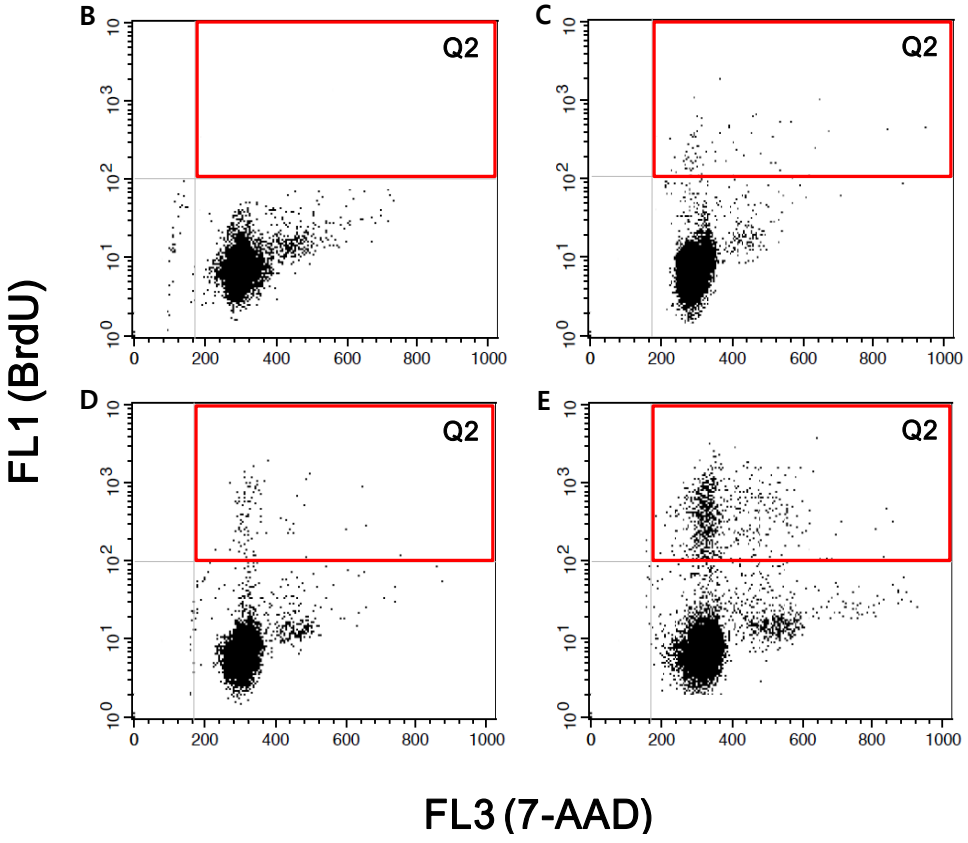
④ (Double-stained sample): Samples stained with both FITC-conjugated anti-BrdU and 7-AAD
→ Use these samples to finalize the compensation (Figure B~E).

3) Set up Q2 following the steps below.

- Using the blank sample, set up Q2 (upper right) where no cells are present (B).
- Using the non-treatment sample, set up Q2 so that % BrdU-positive cells are about 1% of all cells (C).
- The Q2 region percentage indicates the proportion of FITC conjugated anti-BrdU-Antibody positive live lymphocyte in 10,000 LNCs

(3) Count of % BrdU-positive cells

1) Perform flow cytometric operation for the vehicle control-treatment samples (D), the test substance-treatment samples and the positive control-treatment samples (E).
2) Obtain the gated percentage data (Q2 region %) from 'Quadrant Statistics' for the each samples.



4. Results

4.1 General symptoms and evaluation of the SI

4.1.1 General symptoms and death rate

Record general symptoms and death caused by test substances and positive- and vehicle-control chemicals.

4.1.2 Irritation evaluation criteria

Dermal irritation should be evaluated in each test substance dose group. Excessive skin irritation is determined by the erythema score, ear thickness, and ear weight.

Erythema: Erythema is evaluated according to the Draize method. Level 3 erythema constitutes excessive skin irritation.

Ear thickness and weight: An increase of 25% or more is considered excessive skin irritation.

4.1.3 Weight of LNs

Immediately after both auricular lymph nodes have been harvested, weigh them together.

4.2 Calculation of the SI

The number of BrdU-positive LNCs in the LNs of the vehicle control-treatment group is obtained by multiplying the number of LNCs by the ratio of cells expressing BrdU in 10,000 LNCs (obtained by flow cytometry). The number of BrdU-positive LNCs in the LNs of the test substance-treatment group is obtained by the method described above. Individual SIs are calculated by dividing the number of BrdU-positive LNCs in the test substance-treatment group by the mean number of BrdU-positive LNCs in the vehicle control-treatment group. The mean SI of each test substance group is calculated based on individual SIs.

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

With the SIs of test substance groups per dose, determine the dose-response curve and then predict the dose for SI 2.7 (EC2.7).

EC2.7: estimated concentration needed to produce an SI of 2.7

[Y (SI) = aX(concentration) + b → EC2.7 = (2.7-b)/a]

4.3 Interpretation of results

If the SI is 2.7 or above (SI ≥ 2.7), a chemical is classified as a sensitizer. If the SI is below 2.7 (SI < 2.7), a chemical is classified as a non-sensitizer.

4.4 Acceptance criteria

The positive control, 25% HCA, should produce a positive LLNA response at an exposure level where the SI $\geq$ 2.7 occurs, compared with the vehicle control group.

4.5 Statistical analysis

The results from the vehicle control and those from test substances should be compared by one-way ANOVA. The significance of the difference between groups should be evaluated using a *post-hoc* test.

5. Reduced LLNA: BrdU-FCM

In certain situations, when there is a regulatory need to confirm a negative prediction of skin sensitizing potential an optional rLLNA: BrdU-FCM protocol using fewer animals may be used in a way similar to rLLNA in TG 429, provided there is adherence to all other LLNA: BrdU-FCM protocol specifications in this protocol. Before applying the rLLNA: BrdU-FCM approach, clear justifications and scientific rationale for its use should be provided. If a positive or equivocal result is obtained, additional testing may be needed in order to interpret or clarify the finding.

The reduction in number of dose groups is the only difference between the LLNA: BrdU-FCM and the rLLNA: BrdU-FCM test method protocols and for this reason the rLLNA: BrdU-FCM does not provide dose-response information. Therefore, the rLLNA: BrdU-FCM should not be used when dose-response information is needed. Like the multi-dose LLNA: BrdU-FCM, the test substance concentration evaluated in the rLLNA: BrdU-FCM should be the maximum concentration that does not induce overt systemic toxicity and/or excessive local skin irritation in the mouse.

6. GLP compliance

This study must be performed in compliance with OECD Principle of Good Laboratory Practice (GLP).

7. References

Ahn IY, Kim TS, Jung ES, Yi JS, Jang WH, Kung KM, Park MY, Jung MS, Jeaon EY, Yeo KW, Jo JH, Park JE, Kim CY, Park YC, Seong WK, Lee AY, Chun YJ, Jeong TC, Jeung EB, Lim KM, Bae SJ, Heo Y. 2016. Performance standard-based validation study for local lymph node assay:5-bromo-2-deoxyuridine-flow cytometry method. Regulatory Toxicology and Pharmacology 80: 183-194.

Jung KM, Jang WH, Lee YK, Yum YN, Sohn SJ, Kim BH, Chung JH, Park YH, Lim KM. 2012. B cell increases and ex vivo IL-2 production as secondary endpoints for the detection of sensitizers in non-radioisotopic local lymph node assay using flow cytometry. Toxicol. Lett. 209(3):255-63.

Jung KM, Bae IH, Kim BH, Kim WK, Chung JH, Park YH, and Lim KM. 2010. Comparison of flow cytometry and immunohistochemistry in non-radioisotopic murine lymph node assay using bromodeoxyuridine. Toxicol. Lett. 192:229-237.

Yang H, Na J, Jang WH, Jung MS, Jeon JY, Heo Y, Yeo KW, Jo JH, Lim KM, Bae SJ. 2015. Appraisal of WLR and BLR of non-radioisotopic local lymph node assay using flow cytometry, LLNA: BrdU-FCM: Comparison of OECD TG429 performance standard and statistical evaluation. Toxicol. Lett. 2015 May 5; 234(3):172-9.

OECD. 2010. OECD Guideline for the testing of chemicals, Skin sensitization: Local lymph node assay (OECD TG 429). Annex 1 Performance standards for assessment of proposed similar or modified LLNA test methods for skin sensitization.

OECD. 2010a. OECD guideline for the testing of chemicals. Skin sensitization: Local lymph node assay: BrdU-ELISA (OECD TG 442B).

List of data sheets (for main study)

- Data Sheet - Delivery and Acclimation
- Data Sheet - Grouping
- Data Sheet - Day 1 (Main Study)
- Data Sheet - Day 2 (Main Study)
- Data Sheet - Day 3 (Main Study)
- Data Sheet - Day 4 (Main Study)
- Preparation of BrdU Sol. – Day 5 (Main Study)
- Data Sheet - Day 5 (Main Study)
- Data Sheet - Day 6-1 (Main Study)
- Data Sheet - Day 6-2 (Main Study)
- Data Sheet – Day 6-3 (Main Study)
- Data Sheet – Day 6-4 (Main Study)
- Record of test substance
- Record of equipment setting values and maintenance

Pre-Screen Test

1. Objective

Pre-screen test is used to determine an appropriate dose range and vehicle for the main LLNA study. Basically, dose and vehicle selection of LLNA: BrdU-FCM is same with TG 429 and other LLNA test guidelines.

- Dose selection (Paragraph 18 of TG 429)

  Consecutive doses are normally selected from an appropriate concentration series such as 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc. In the absence of information needed to determine the highest dose to be tested, pre-screen tests should be performed in order to define an appropriate dose level in the LLNA. Adequate scientific rationale should accompany the selection of the concentration series used. All existing toxicological information (e.g. acute toxicity and dermal irritation) and structural and physicochemical information on the test substance of interest (and/or structurally related test substances) should be considered where available, in selecting the three consecutive concentrations so that the highest concentration maximises exposure while avoiding systemic toxicity and/or excessive local skin irritation.

- Vehicle selection (Paragraph 19 of TG 429)

  The vehicle should not interfere with or bias the test result and should be selected on the basis of maximising the solubility in order to obtain the highest concentration achievable while producing a solution/suspension suitable for application of the test substance. Recommended vehicles are acetone: olive oil (4:1, AOO), *N,N*-dimethylformamide (DMF), methyl ethyl ketone (MEK), propylene glycol (PG), and dimethyl sulphoxide (DMSO) but others may be used if sufficient scientific rationale is provided. In certain situations I may be necessary to use a clinically relevant solvent or the commercial formulation in which the test substance is marketed as an additional control. Particular care should be taken to ensure that hydrophilic substances are incorporated into a vehicle system, which wets the skin and does not immediately run off, by incorporation of appropriate solubilisers (e.g. 1% Pluronic® L92). Thus, wholly aqueous vehicles are to be avoided.

Pre-screen test suggested in this protocol is one example following the above principles. In this protocol, doses for the main test are determined by performing two pre-screen tests to avoid extreme toxicity, a 1st pre-screen test with a relative low single dose (25%) and vehicle controls, and a 2nd pre-screen test with two or more doses and vehicle controls.

2. Materials

2.1 Test animals

All procedures are the same as those used in the main test, except for the number of animals per group. Here, the maximum number of animals per dose group is 2.

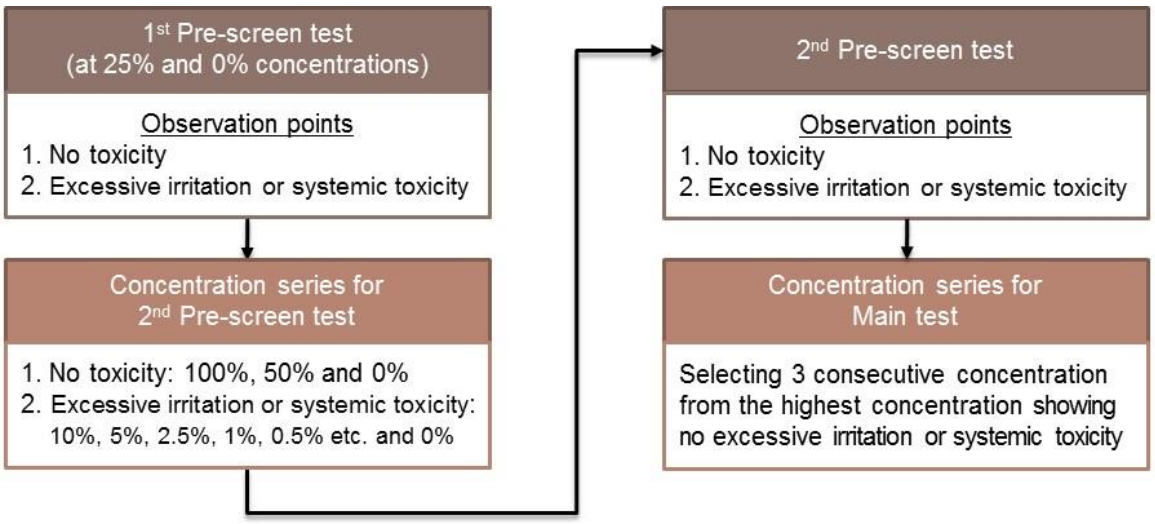
2.2 Test substances and vehicles

Collect information on test substances and, if applicable, keep chemical reports. Information on storage conditions, mass, density, safety, etc. for test substances and vehicles used in the pre-screen test should be recorded. AOO, DMF, MEK, PG and DMSO, described in OECD TG 429 LLNA (TG No.429), are recommended as vehicles. If vehicles other than those described in OECD TG 429 are used, a scientific rationale should be given for the selection of the vehicles.

3. Experimental procedure

3.1 Experimental design

The pre-screen test is summarized as follows.



- Excessive irritation: 25% or more increase of ear thickness or ear weight, or 3 or more erythema score
- Systemic toxicity: death or weight loss (a decrease of more than 5% from Day 1 to Day 6)

If a test substance is applied first at a concentration of 100%, extreme toxicity could be induced. For this reason, the 1st pre-screen test is performed at 25%, and in the 2nd test, the highest concentration is selected based on the results of the 1st pre-screen test.

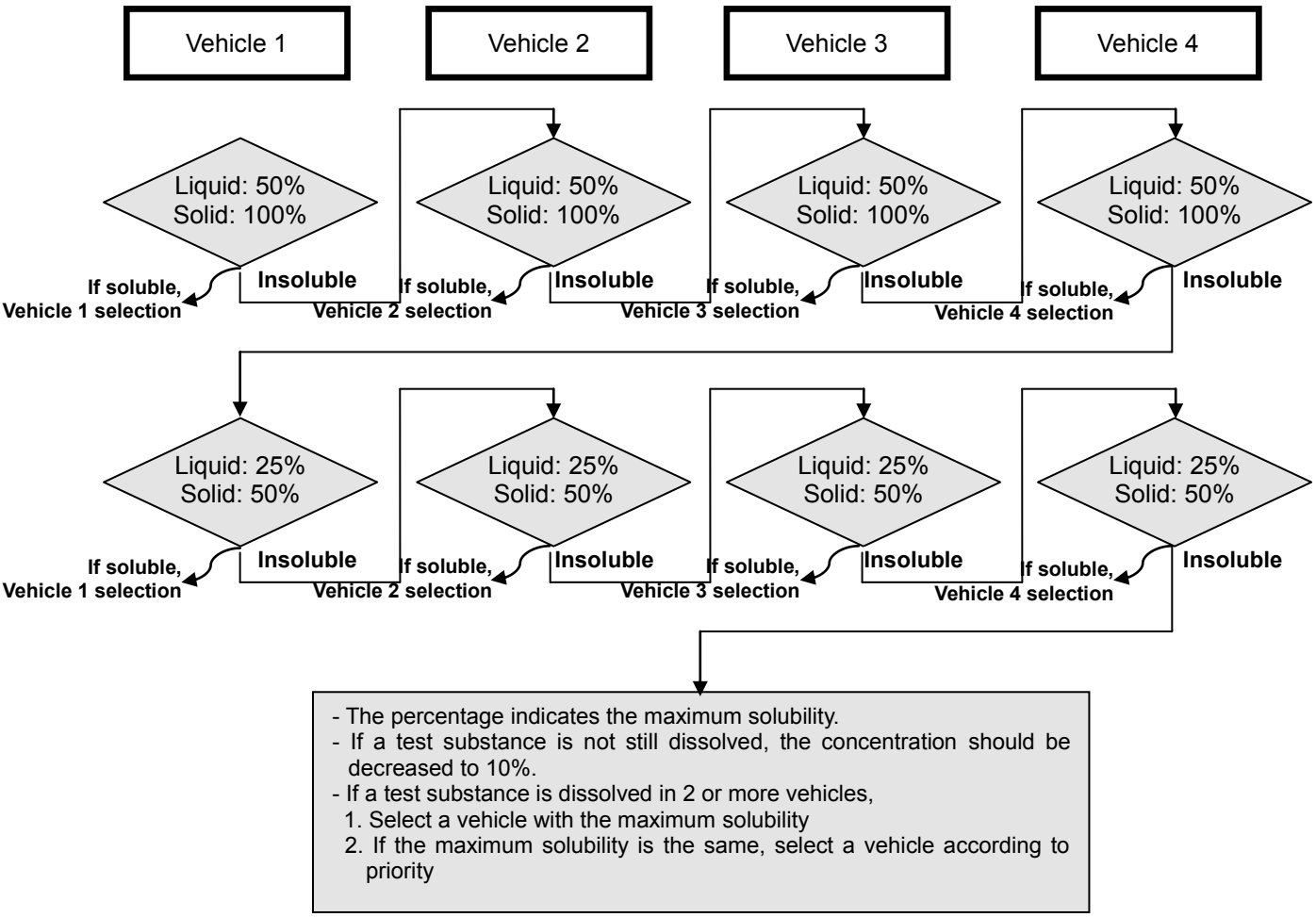
The schedule for the pre-screen test is summarized below.
(The experiments that are marked as '2nd' should be performed during the 2nd pre-screen test.)

Experiments	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Grouping	O					
Clinical observation	O	O	O	O	O	O
Treatment	O	O	O			
Measurement of Body weight	O					O
Measurement of Ear thickness	O		O			O
Irritation evaluation	O	O	O	O	O	O
Sacrifice						O

3.2 Selection of vehicles

To find a vehicle that best dissolves test substances, solubility tests should be performed using AOO, DMF, MFK, PG and DMSO consecutively as described in 3.2.1.

The procedure for vehicle selection is as follows.



3.2.1 Solubility test

(1) Prepare vehicles (e.g. AOO, DMF, MEK, PG and DMSO). Following the figure above, weigh test substances and allow them to dissolve. In the case of solid test substances, place them in a beaker and stir them with a magnetic bar. In the case of liquid test substances, place them into a glass vial and shake them.
(2) Stir for 30–60 minutes and shake for 1 minute at 10-minute intervals so that all test substances are sufficiently dissolved. If a substance is viscous, magnetic stir for 60 minutes.
(3) Solutions with completely dissolved test substances look transparent, with no visible suspensions or precipitates. Once test substances are dissolved, allow them to rest for 30 minutes to ensure that there are no precipitates or visible suspensions. In the case of liquid test substances, let them stand for 5 minutes.
(4) In the case of liquid test substances, vehicles that dissolved test substances completely and did not show layers should be selected as a vehicle. If more than one vehicle is selected, prioritize them in the solvent order AOO, DMF, MEK, PG and DMSO, and then finish the solubility tests.
(5) If no vehicle completely dissolves the test substances, lower the doses, and try again. Doses include 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc. (For liquid test substances, doses start at 50%.)

3.3 Dose selection

Basically, consecutive doses are normally selected from an appropriate concentration series such as 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc. following TG 429.

In this protocol, the 1st pre-screen test is performed at 25% and 0% (vehicle control).

If no systemic toxicity or excessive irritation is found at 25% in the 1st pre-screen test, only 50% and 100% concentrations are needed in the 2nd test, and low concentrations are not required. However, if systemic toxicity or excessive irritation is found at 25%, concentrations can be decreased to less than 25% to avoid severe toxicity induced at 50% and 100%.

Concentration series for the 2nd pre-screen test.

(1) If no systemic toxicity or excessive irritation is observed at 25% in the 1st pre-screen test
: 100%, 50% and 0% (vehicle control)

** if n=1/group, total 3/3 groups; if n=2/group, total 6/3 groups*

(2) If systemic toxicity or excessive irritation is observed at 25% in the 1st pre-screen test
: 0% (vehicle control), 10%, 5%, 2.5%, 1%, 0.5%, etc.

** if n=1/group, total 6 or more/6 groups; if n=2/group, total 12 or more/6 groups*

(* If a test substance has a maximum solubility below 10%, the 2nd pre-screen test should be performed without the 1st pre-screen test.)

This 2-stage strategy could prevent serious pain and distress in laboratory animals and reduce animal testing. The minimum three consecutive concentrations (highest concentration and two consecutive lower concentrations) that did not induce systemic toxicity and excessive irritation in the main test are selected.

3.4 Application of test substances

Chemicals are applied to animals in the same way as the main test, but without BrdU injection.

3.5 Observations

Clinical signs, death, and erythema should be observed and recorded daily until sacrifice. Body weights and ear thickness measurements should also be recorded.

3.6 Autopsy

Animals should be killed humanely, and ear weights measured the same way as in the main test.

4. Results

4.1 1st pre-screen test

The 1st pre-screen test evaluates clinical symptoms and death rate. Excessive skin irritation is determined by collective measures of erythema, ear thickness, and ear weight.

4.2 2nd pre-screen test

The evaluation of clinical symptoms, death rate, and excessive skin irritation is carried out in the same way as the 1st pre-screen test.

5. GLP compliance

These tests are performed in compliance with Good Laboratory Practice (GLP).

List of data sheets (for pre-screen test)

- Data Sheet - Delivery and Acclimation (1st pre-screen test)
- Data Sheet - Grouping (1st pre-screen test)
- Data Sheet - Day 1 (1st pre-screen test)
- Data Sheet - Day 2 (1st pre-screen test)
- Data Sheet - Day 3 (1st pre-screen test)
- Data Sheet - Day 4 (1st pre-screen test)
- Data Sheet - Day 5 (1st pre-screen test)
- Data Sheet - Day 6 (1st pre-screen test)
- Data Sheet - Delivery and Acclimation (2nd pre-screen test)
- Data Sheet - Grouping (2nd pre-screen test)
- Data Sheet - Day 1 (2nd pre-screen test)
- Data Sheet - Day 2 (2nd pre-screen test)
- Data Sheet - Day 3 (2nd pre-screen test)
- Data Sheet - Day 4 (2nd pre-screen test)
- Data Sheet - Day 5 (2nd pre-screen test)
- Data Sheet - Day 6 (2nd pre-screen test)
- Preparation of materials - Selection of vehicle

Protocol Revision History

Version	Protocol: LLNA: BrdU-FCM_Ver1.0			
	Written by	© AmorePacific R&D Unit	Date of establishment	16 April 2012
1.0	Description	© AmorePacific R&D Unit, the lead laboratory, established the protocol (ver. 1.0) for the validation study on the LLNA: BrdU-FCM.		
Version	Previous ver.	Protocol: LLNA: BrdU-FCM_Ver1.0	Revised ver.	Protocol: LLNA: BrdU-FCM_Ver1.1
	Written by	© AmorePacific R&D Unit	Date of revision	22 April 2013
1.1	Description	- Modifications made to protocol: LLNA: BrdU-FCM_Ver1.0 - Addition of the pre-screen test for the selection of treatment doses - Addition of figures that make it easier to understand the test method - Correction of typos and grammatical errors		
Version	Previous ver.	Protocol: LLNA: BrdU-FCM_Ver1.1	Revised ver.	Protocol: LLNA: BrdU-FCM_Ver1.2
	Written by	NIFDS	Date of revision	1 May 2014
1.2	Description	- Modifications made to protocol: LLNA: BrdU-FCM_Ver1.1 - Changes in data sheets for easier use - Addition of a detailed description of the solubility test - Omission of 75% from treatment doses - Addition of MEK as a vehicle - Correction of typos and grammatical errors		
Version	Previous ver.	Protocol: LLNA: BrdU-FCM_Ver1.2	Revised ver.	Protocol: LLNA: BrdU-FCM_Ver1.3
	Written by	NIFDS	Date of revision	1 June 2015
1.3	Description	- Modifications made to the protocol: LLNA: BrdU-FCM_Ver1.2 - Change in SI from 3 to 2.7 - Improvement in the solubility test procedures for viscous substances (i.e., the time for magnetic stirring was extended from 30 minutes to 30–60 minutes)		
Version	Previous ver.	Protocol: LLNA: BrdU-FCM_Ver1.3	Revised ver.	Protocol: LLNA: BrdU-FCM_Ver1.3.1
	Written by	NIFDS	Date of revision	25 November 2016
1.3.1	Description	- Addition of a detailed description of the main test and pre-screen test.		

[ANNEX 6]

Training and Transfer Report

LLNA: BrdU-FCM

Training and Transfer Protocol

1. General information

- Training laboratory: AmorePacific R&D Unit
- Trainers: Kyung-min Lim, Kyoung-Mi Jung, Won-hee Jang
- Participating laboratory: Catholic University of Daegu, Biotoxtech Co. Ltd.
- Trainees: Yong Heo, Kyung-Wook Yeo (Catholic University of Daegu)
  Mi-Sook Jung, Eun-Young Jeon (Biotoxtech Co., Ltd.)

2. Training Program

1) Training on SOP

- Date: 14 May 2012
- Location: AmorePacific R&D Unit, Yongin-si, Gyeonggi-do, Korea
- AM: 1. Outline of the test, 2. LLNA: BrdU-FCM SOP ver1.0
- PM: Demonstration by trainers, exercise by trainees (all steps of the LLNA: BrdU-FCM)

2) Training on flow cytometry

- Date: 15 May 2012
- Location: National Institute of Food and Drug Safety Evaluation, 187, Osongsaengmyeong 2-ro, Osong-eup, Heungdeok-gu, Cheongju-si, Chungcheongbuk-do, Korea
- PM: Training on flow cytometer from Becton Dickinson (1. overview of flow cytometry, 2. demonstration of the use of flow cytometry)

3) Training of Lead Laboratory 2

- Date: 26 February 2014
- Location: National Institute of Food and Drug Safety Evaluation, 187, Osongsaengmyeong 2-ro, Osong-eup, Heungdeok-gu, Cheongju-si, Chungcheongbuk-do, Korea
- AM: Discussions (1. Outline of the test, 2. LLNA: BrdU-FCM SOP ver1.1)
- PM: Demonstration by trainers, exercise by trainees (all steps of the LLNA: BrdU-FCM), training on flow cytometry (demonstration on the use of flow cytometry)

3. Transfer plan

1) Test substance for transfer

- The following chemicals should be used in the transfer phase and acquired by the trained laboratories:

Chemical name	CAS No.	Physical state	Veh.	EC3 (%)	0.5x~2.0x EC3	LLNA vs. GP	LLNA vs. Human
Hexyl cinnamic aldehyde (HCA)	101-86-0	Liquid	AOO	9.7	4.8-19.5	+/+	+/+

2) Inspection of transfer

(1) Participating Laboratory 1 (Catholic University of Daegu)
- Date: 23 May 2012
- Inspection location: Catholic University of Daegu, 13-13, Hayang-ro, Hayang-eup, Gyeongsan-si, Gyeongsangbuk-do, Korea
- Inspectors: Kyoung-Mi Jung, Won-hee Jang (AmorePacific)
- Trainees: Kyung-Wook Yeo (Catholic University of Daegu)
- Content: After transfer of the test method, inspectors visited the trained laboratory and confirmed that all steps of the test were performed correctly.

(2) Participating Laboratory 2 (Biotoxtech Ltd.)
- Date: 21 May 2012
- Inspection location: Biotoxtech, 53, Yeongudanji-ro, Ochang-eup, Cheongwon-gu Cheongju-si, Chungcheongbuk-do, Korea
- Inspectors: Kyoung-Mi Jung, Won-hee Jang (AmorePacific)
- Trainee: Eun-Young Jeon (Biotoxtech Ltd.)
- Content: After transfer of the test method, inspectors visited the trained laboratory and confirmed that all steps of the steps were performed correctly.

3) Confirmation of transfer

(1) Participating Laboratory 1 (Catholic University of Daegu)
- Date: 31 May 2012–6 June 2012
- Location: Catholic University of Daegu
- Test substances: AOO, HCA (25%)

(2) Participating Laboratory 2 (Biotoxtech)
- Date: 21–27 June 2012
- location: Biotoxtech
- Test substances: AOO, HCA (25%)

4) Success criteria

The SI for 25% HCA should be ≥ 3 compared with that of the vehicle control group, as described in OECD TG 429 Paragraph 11. The values for ear thickness, LNC count, % BrdU-positive LNCs, and SI were compared values from the lead laboratory.

4. Proficiency test plan:

1) Test substances for the proficiency test
- The following chemicals are coded and distributed for the proficiency test.

Chemical name	CAS No.	Physical state	Veh.	EC3 (%)	0.5x~2.0x EC3	LLNA vs. GP	LLNA vs. Human
Eugenol	97-53-0	Liquid	AOO	10.1	5.05-20.2	+/+	+/+

- Test group: AOO, A (eugenol) 5%, 10%, 25%; HCA 25%

2) Proficiency test

(1) Participating Laboratory 1 (Catholic University of Daegu)
- Date: 19–25 October 2012

(2) Participating Laboratory 2 (Biotoxtech)
- Date: 27 June 2012–3 July 2012

(3) Lead Laboratory 2 (NIFDS)
- Date: 20–26 August 2014

3) Success criteria

The EC3 concentration of eugenol should be in the range of 5.05–20.2%, as described in OECD TG 429 Annex 1. The values for ear thickness, LNC count, % BrdU-positive LNCs, and SI were compared with values from the lead laboratory.

LLNA: BrdU-FCM

Transfer report

1. Transfer Results

* Vehicle control: AOO/Positive control: HCA 25%

1) Biotoxtech

- Date: 21–27 June 2012
- Ear thickness, LNC count, % BrdU-positive LNCs, and SI were measured (Figure A6-1) and compared with data from the lead laboratory (Table A6-1).
- Ear thickness was comparable with that of the lead laboratory.
- The increase in LNC count in the positive control was lower than that in the lead laboratory.
- % BrdU-positive LNCs of the vehicle and positive controls were lower than those of the lead laboratory.
- The SI of the positive control was higher than that of the lead laboratory.

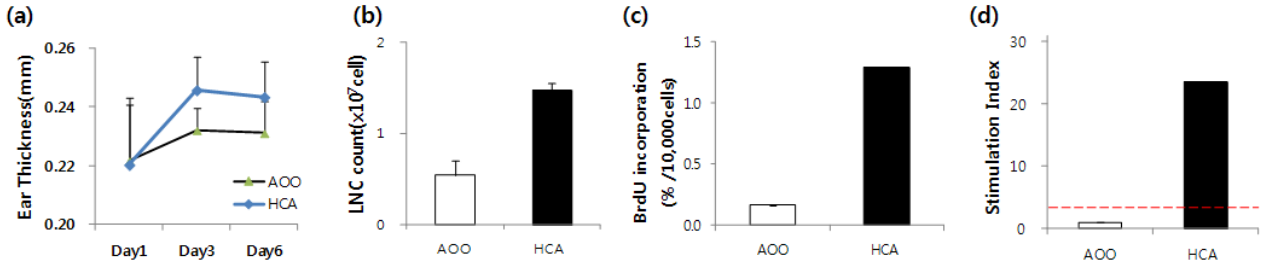


Figure A6-1. Results of transfer to Biotoxtech. (a) Ear thickness, (b) lymph node cell count, (c) % BrdU-positive LNCs, (d) stimulation index

Table A6-1. Comparison of results from Biotoxtech (BT) and AmorePacific (AP).

Lab.	Test chemical	Ear thickness			LNC count ($x10^7$ cell)	BrdU incorporation rates (%/10,000cells)	Stmulation index
		Day1	Day3	Day6			
BT	AOO	0.222±0.021	0.232±0.008	0.231±0.011	0.54±0.16	0.17±0.13	1.00±0.67
	HCA	0.220±0.020	0.246±0.011	0.243±0.012	1.47±0.08	1.30±0.38	23.52±6.51
AP	AOO	0.215±0.009	0.232±0.008	0.228±0.004	1.40±0.34	1.02±0.06	1.00±0.19
	HCA	0.216±0.005	0.231±0.005	0.244±0.013	5.68±1.31	2.53±0.34	10.33±3.46

* Data are presented as means ± SDs
* Digital ear thickness gage: Mitutoyo Corporation, Japan (Product Code 543-681B)

2) Catholic University of Daegu

- Date: 31 May 2012–6 June 2012
- Ear thickness, LNC count, % BrdU-positive LNCs, and SI were measured (Figure A6-2) and compared with data from the lead laboratory (Table A6-2).
- Ear thickness and the increase in LNC count in the positive control were comparable with that of the lead laboratory.
- % BrdU-positive LNCs of the vehicle and positive controls were lower than those of the lead laboratory.
- The SI of the positive control was comparable with that of the lead laboratory.

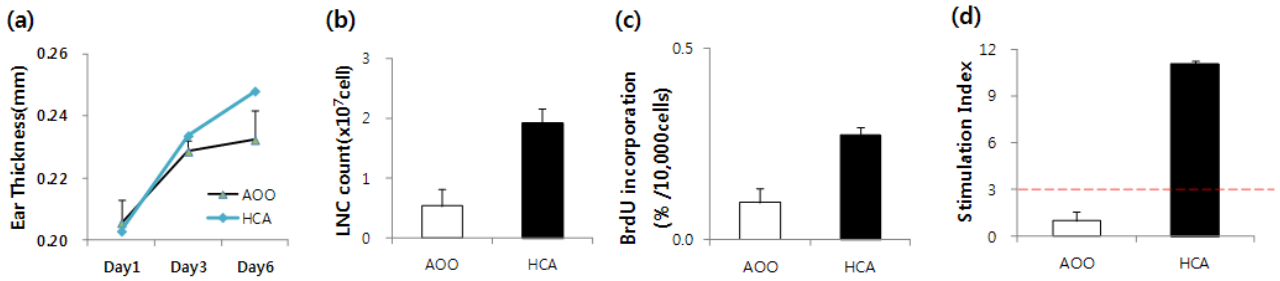


Figure A6-2. Results of transfer to Catholic University of Daegu. (a) Ear thickness, (b) lymph node cell count, (c) % BrdU-positive LNCs, (d) stimulation index

Table A6-2. Comparison of results from Catholic University of Daegu (DCU) and AmorePacific (AP).

Lab.	Test chemical	Ear thickness			LNC count ($x10^7$ cell)	BrdU incorporation rates (%/10,000cells)	Stmulation index
		Day1	Day3	Day6			
DCU	AOO	0.206±0.007	0.229±0.003	0.232±0.009	0.54±0.28	0.10±0.04	1.00±0.57
	HCA	0.203±0.007	0.234±0.005	0.248±0.010	1.93±1.05	0.27±0.06	11.04±7.25
AP	AOO	0.215±0.009	0.232±0.008	0.228±0.004	1.40±0.34	1.02±0.06	1.00±0.19
	HCA	0.216±0.005	0.231±0.005	0.244±0.013	5.68±1.31	2.53±0.34	10.33±3.46

* Data are presented as means ± SDs
* Digital ear thickness gage: Mitutoyo Corporation, Japan (Product Code 543-681B)

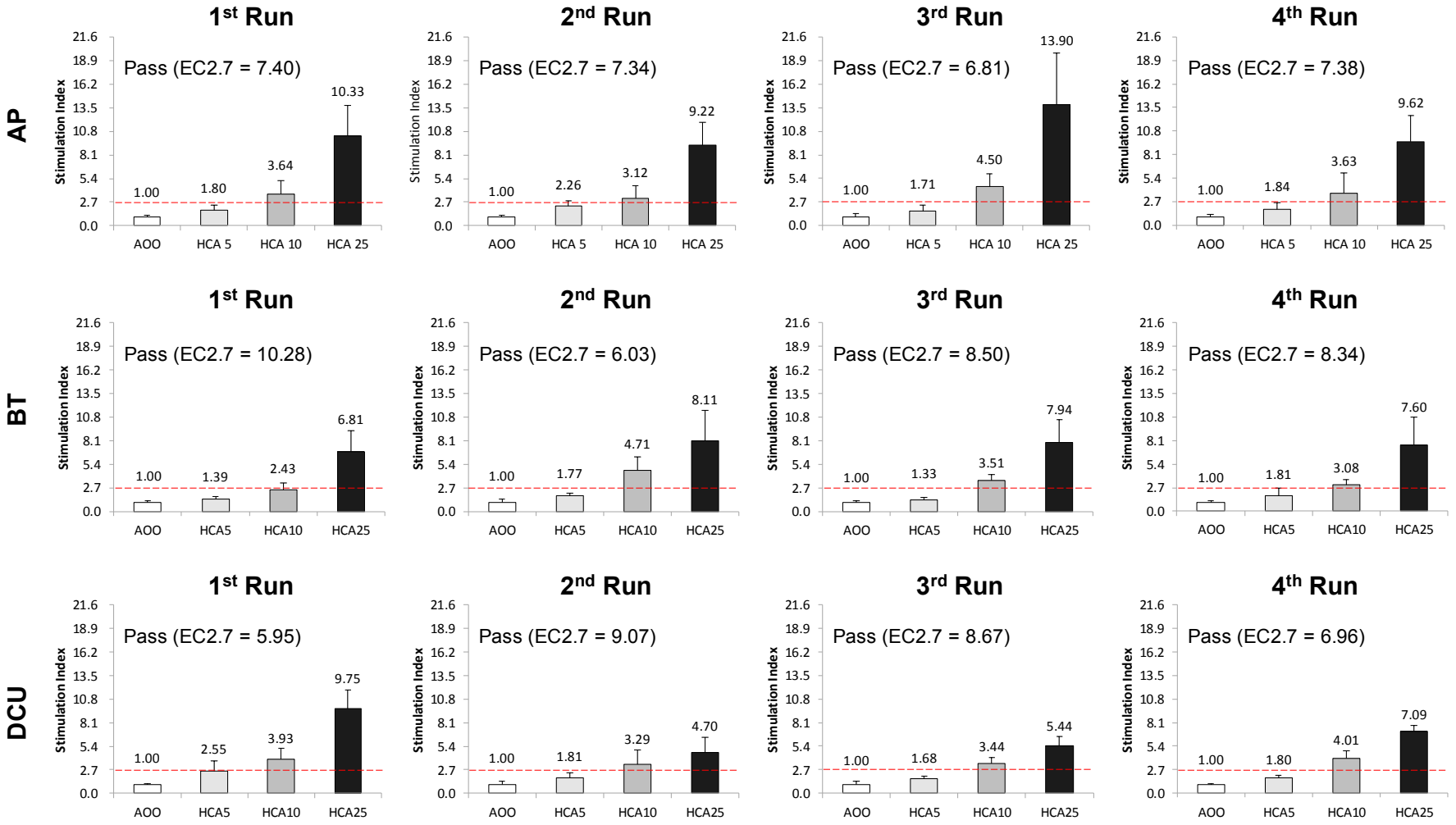
2. Discussion

- The peritoneal injection volume of BrdU should be confirmed because the % BrdU-positive LNCs was lower than that of the lead laboratory.
- Samples should be sufficiently vortexed so that LNCs are re-suspended during staining with the BrdU flow kit.

[ANNEX 7]

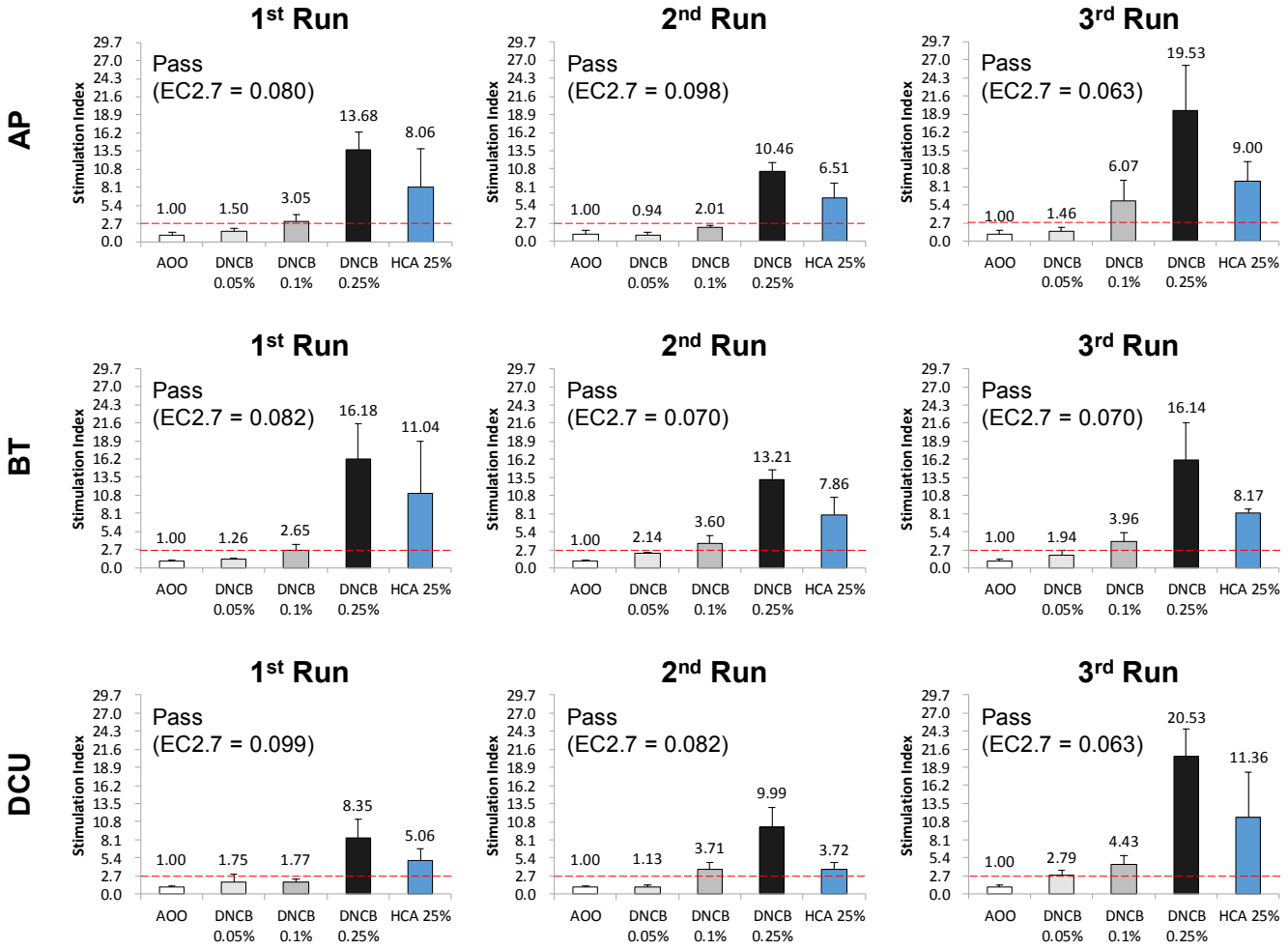
WLR and BLR Evaluation

Figure A7-1. Results of the WLR (HCA)



*Pass: ECt value for HCA is in the range of 5%~20%.
AP, AmorePacific R&D Unit; AOO, acetone: olive oil (4:1, v/v) mixture; BT, Biotoxtech Co., Ltd; DCU, Catholic University of Daegu; HCA, Hexyl cinnamic aldehyde

Figure A7-2. Results of the BLR (DNCB)



*Pass: ECt value for DNCB is in the range of 0.025% to 0.1%.
AP, AmorePacific R&D Unit; AOO, acetone: olive oil (4:1, v/v) mixture; BT, Biotoxtech Co., Ltd; DCU, Catholic University of Daegu; DNCB, 2,4-dinitrochlorobenzene

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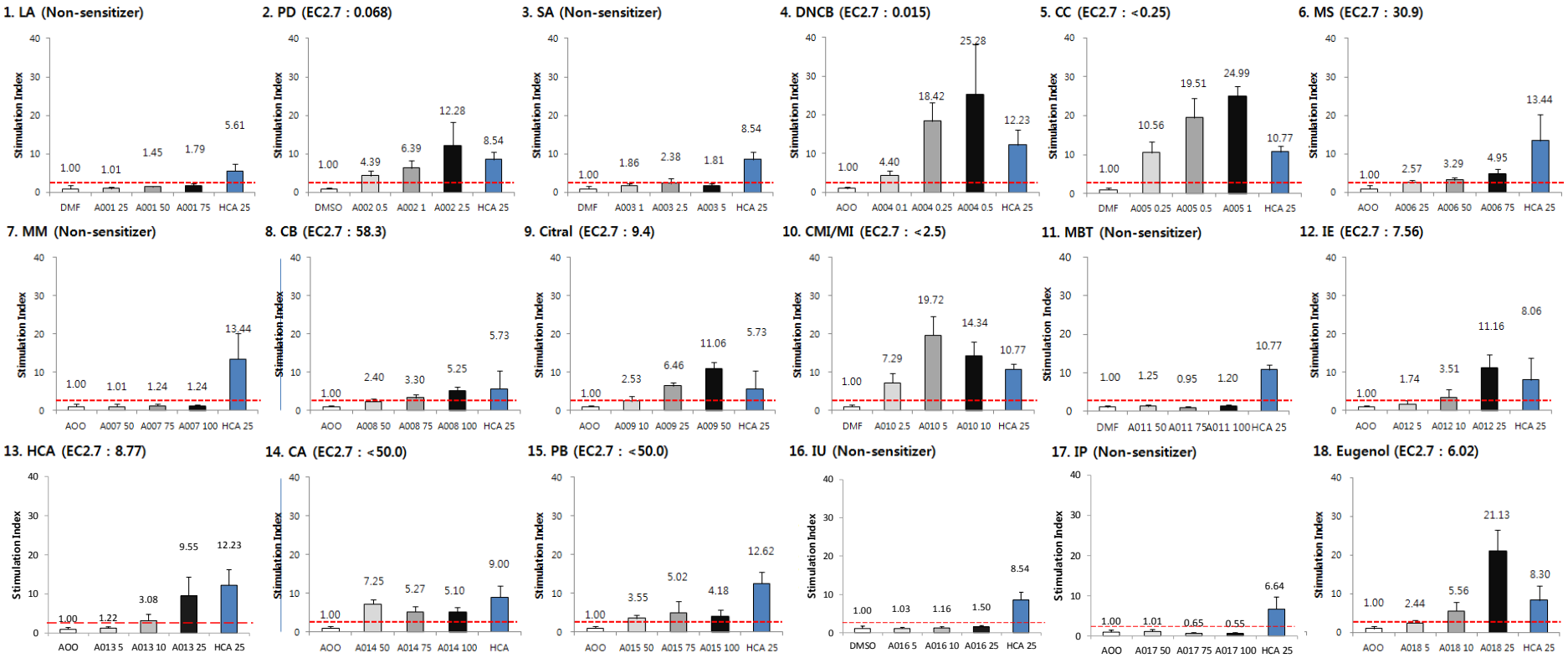
[ANNEX 8]

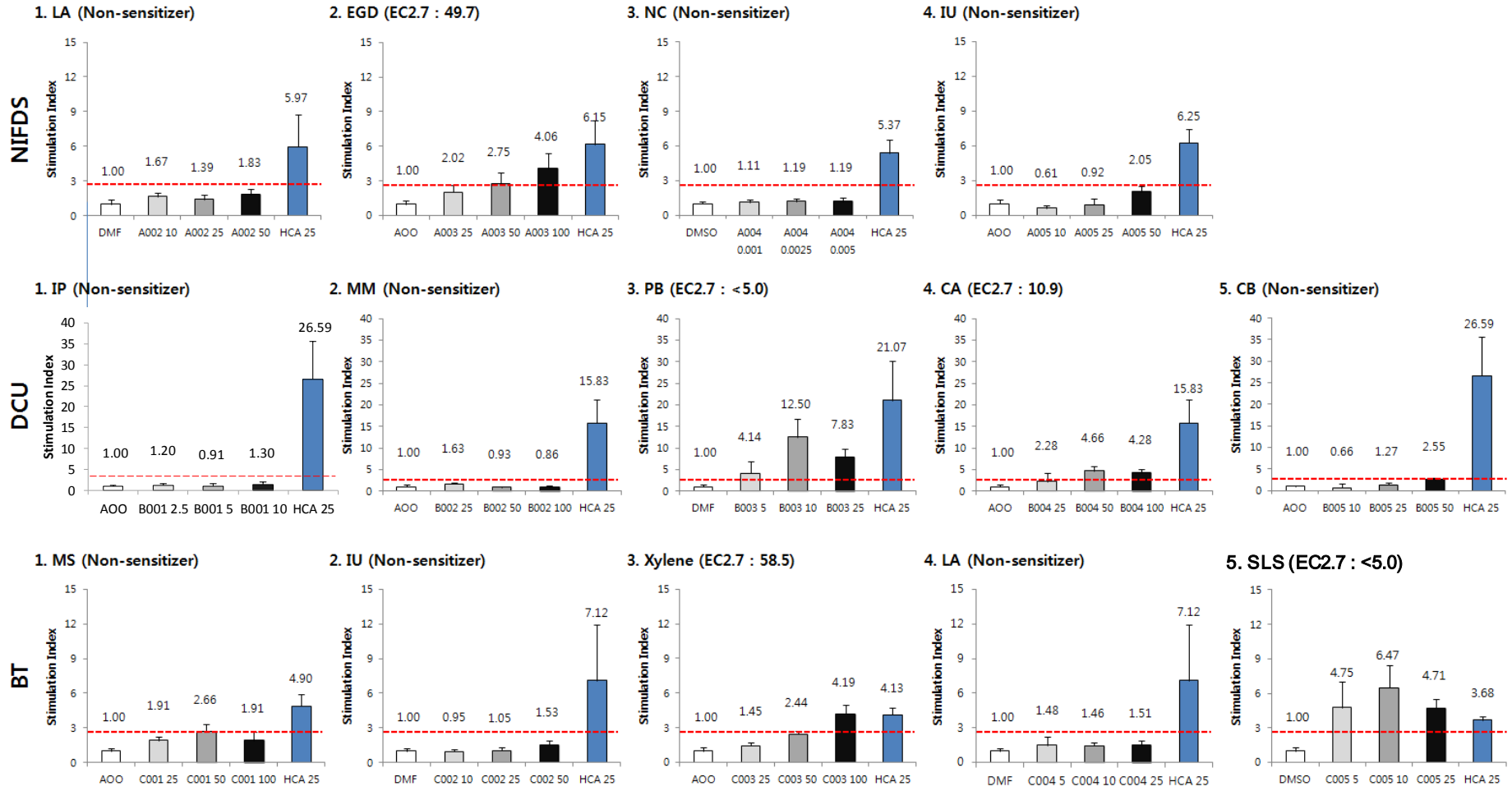
Predictive Capacity Evaluation

Figure A8-1. Results of the 1st test (red-dotted line represents SI=2.7) (Protocol 1.1)



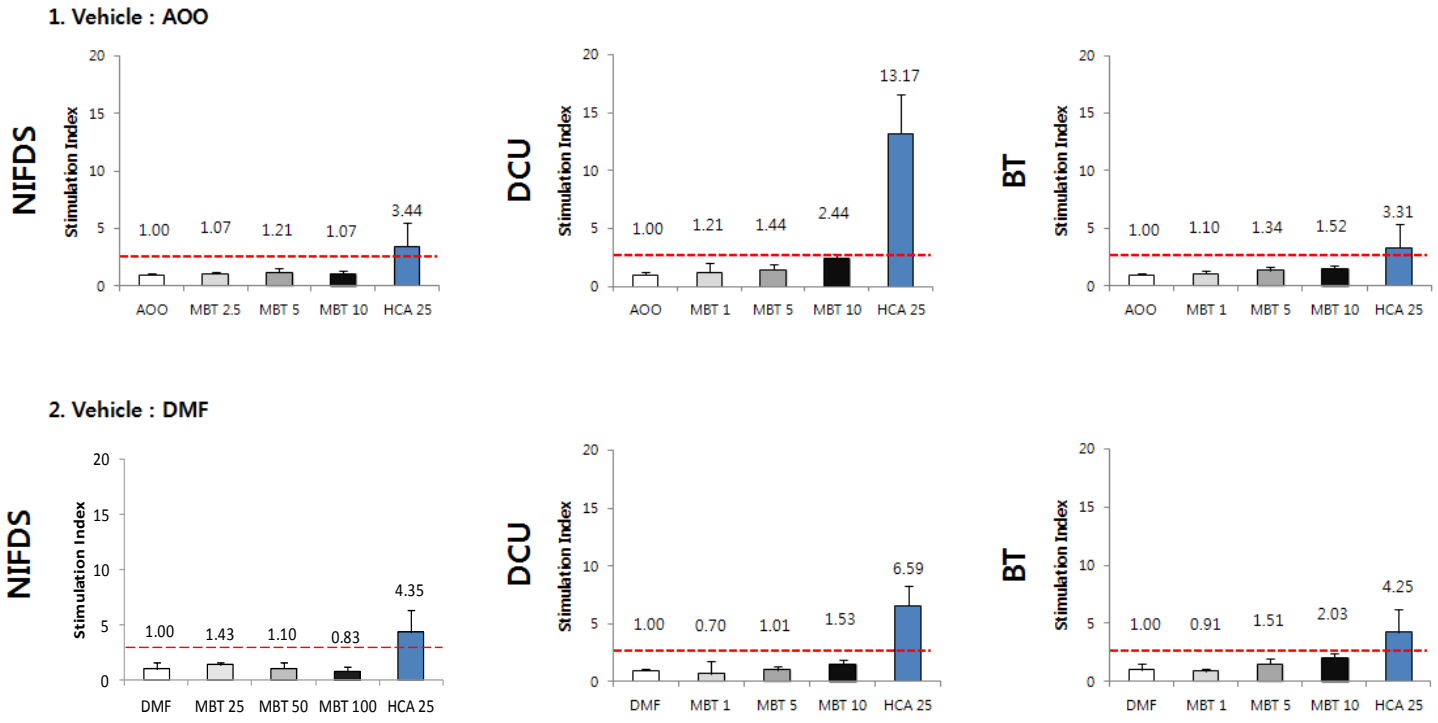
CMI/MI, 5-Chloro-2-methyl-4-isothiazolin-3-one/2-methyl-4-isothiazolin-3-one; DNCB, 2,4-dintrochlorobenzene; PD, 4-Phenylenediamine; CC, Cobalt chloride; IE, Isoeugenol; MBT, 2-Mercaptobenzothiazole; HCA, Hexyl cinnamic aldehyde; PB, Phenyl benzoate; CA, Cinnamic alcohol; IU, Imidazolidinyl urea; MM, Methyl methacrylate; CB, Chlorobenzene; IP, Isopropanol; LA, Lactic acid; MS, Methyl salicylate; SA, Salicylic acid

Figure A8-2. Results of the 2nd test (red-dotted line represents SI=2.7) (Protocol 1.2)



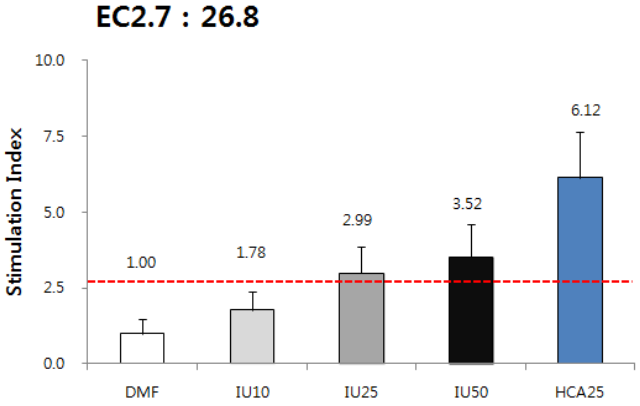
PB, Phenyl benzoate; CA, Cinnamic alcohol; IU, Imidazolidinyl urea; MM, Methyl methacrylate; CB, Chlorobenzene; IP, Isopropanol; LA, Lactic acid; MS, Methyl salicylate; EGD, Ethylene glycol dimethacrylate; NC, Nickel chloride; SLS, Sodium lauryl sulphate

Figure A8-3. Results of the 2nd test (2-Mercaptobenzothiazole) (red-dotted line represents SI=2.7) (Protocol 1.2)



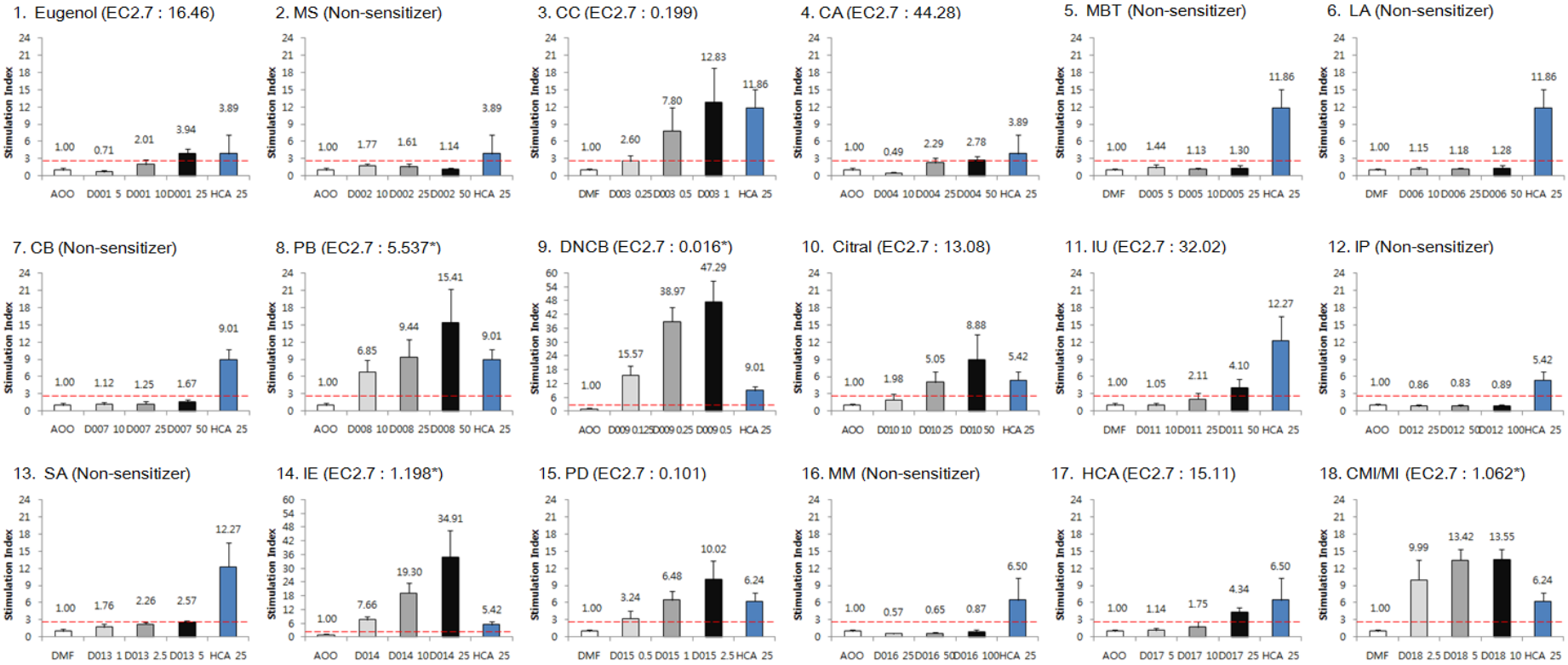
AOO = acetone: olive oil (4:1, v/v), DMF = N,N-dimethylformamide, MBT, 2-Mercaptobenzothiazole; HCA, Hexyl cinnamic aldehyde

Figure A8-4. Results of the additional test for Imidazolidinyl Urea (red-dotted line represents SI=2.7) (Protocol 1.3)



DMF = N,N-dimethylformamide, IU, Imidazolidinyl urea, HCA, Hexyl cinnamic aldehyde

Figure A8-5. Results of the 3rd test (red-dotted line represents SI=2.7) (Protocol 1.3)



CMI/MI, 5-Chloro-2-methyl-4-isothiazolin-3-one/2-methyl-4-isothiazolin-3-one; DNCB, 2,4-dintrochlorobenzene; PD, 4-Phenylenediamine; CC, Cobalt chloride; IE, Isoeugenol; MBT, 2-Mercaptobenzothiazole; HCA, Hexyl cinnamic aldehyde; PB, Phenyl benzoate; CA, Cinnamic alcohol; IU, Imidazolidinyl urea; MM, Methyl methacrylate; CB, Chlorobenzene; IP, Isopropanol; LA, Lactic acid; MS, Methyl salicylate; SA, Salicylic acid

* Set the y-axis to 1 since an EC2.7 value is below 0% or above 100%.

[ANNEX 9]

Statistical Report

Contents

1. Background

The murine local lymph node assay (LLNA) is an alternative test to evaluate the skin sensitization (OECD, 2010a), replacing conventional guinea pig tests (OECD, 1992). To quantify the proliferation of lymph node cells (LNCs), original LLNA method uses a radioisotopic 3H-thymidine upon exposure to test substances. However many countries have been enforced the regulation to limit the use of radiation because of its long physical half-life. To avoid this issues, newly developed LLNA: BrdU-FCM uses a non-radioisotopic thymidine analogue, 5-bromo-2-deoxyuridine (BrdU) and detects the BrdU incorporated LNCs through antibody-assisted flow cytometric method (Jung et al., 2012, 2010). LLNA: BrdU- FCM may provide additional advantages over other non-radioisotopic LLNAs that include high sensitivity and capacity to accommodate multiple endpoints like *ex vivo* cytokine releases, cell sub-typing and surface marker expression.

When developing new assays corresponding to the conventional test method, OECD test guideline recommends the equivalence assessment to the original test method in reproducibility and predictive capacity based on the pre-determined criteria described in performance standard (PS).

Therefore, the goal of this study is to evaluate the reliability of the LLNA: BrdU-FCM test method with reproducibility assessment (within- and between laboratories) in accordance with OECD TG 429 guideline.
And the predictive capacity evaluation of the test method based on the OECD TG was another goal of this study.

1) To evaluate the WLR of a new test method to LLNA, one reference (positive) compound (hexylcinnamaldehyde, HCA) shall be tested repeatedly four times. And then ECt values, an estimate of the test substance concentration required to produce a SI of threshold (cut-off) for determination of sensitizers, must fall within pre-determined acceptable range (5–20%) in accordance with OECD TG429 PS.

2) To evaluate the BLR, two positive reference compounds (HCA and 2,4-dinitrochlorobenzene, DNCB) shall be tested by 3 independent laboratories and ECt values obtained must fall within acceptable range (5–20% for HCA and 0.025–0.1% for DNCB).

+ We employed both parametric (one-way ANOVA and student t-test) and non-parametric (Kruskal–Wallis and Wilcoxon rank sum test) methods to evaluate the WLR and BLR along with examining assumptions behind parametric approach (such as test of normality and equal variance assumption), and results obtained from both approaches were compared and discussed.

3) To evaluate the predictive capacity, SI values of 22 reference substances (18 mandatory and 4 optional substances) listed in OECD TG 429 were obtained from 3 independent laboratories. And then SI values were examined its predictive capacity by comparing with GHS classification.

2. Methods

2.1 Production of data for the study

For evaluation of the reproducibility and predictive capacity of the LLNA: BrdU-FCM, 3 laboratories (AmorePacific R&D Unit; a lead laboratory, Biotoxtech Co. and Catholic University of Daegu; 2 participating laboratories) participated in this study.

2.1.1 Data for WLR and BLR

To evaluate WLR and BLR, HCA and DNCB known as skin sensitizers were tested four times and three times respectively in every laboratories. And an SI value which represents the fold increase of BrdU-positive LNCs over vehicle control was obtained.

2.1.2 Data for predictive capacity

To evaluate the predictive capacity of the LLNA: BrdU-FCM, 22 reference substances were tested with a concurrent positive control HCA 25% and SI values were also obtained in 3 laboratories (test substances were divided accordingly into 3 laboratories).

2.2 Data management

For statistical analysis of SI values, the results were transferred to the statistics team and managed by them

2.3 Statistical analysis of WLR and BLR

2.3.1 Analysis of WLR and BLR based on performance standard of OECD test guideline 429

OECD guideline advises that ECt values of HCA be between 5 to 20% in order to satisfy the WLR and also ECt values of HCA must be between 5-20% and those of DNCB also be between 0.025-0.1% for satisfaction of the BLR.

The reproducibility was evaluated based on ECt value (defined as the estimated concentration that yields an SI value of predetermined threshold) in accordance with OECD TG 429.

First, ECt values were estimated from SI values using the linear interpolation method that matches an x (concentration) where the y (SI) equals threshold.

Second, descriptive statistics like a scatterplot and a box-whisker plot were used to demonstrate the mean ± standard deviation(SD) of SI values and ECt values estimated from SI values.

And then these ECt values' ranges of HCA and DNCB were evaluated whether or not they satisfy the WLR and BLR respectively.

2.3.2 Analysis of WLR and BLR using inferential statistics with SI values of HCA 25%

Additionally SI values of the positive reference compounds (HCA) were investigated based on inferential statistics to assess WLR and BLR. Both parametric (one-way ANOVA and student t-test) and non-parametric (Kruskal-Wallis and Wilcoxon rank sum test) methods were performed and results were compared and discussed.

- null hypothesis for WLR: H_0: $\mu_{test1} = \mu_{test2} = \mu_{test3} = \mu_{test4}$
- null hypothesis for BLR: H_0: $\mu_{laboratory1} = \mu_{laboratory2} = \mu_{laboratory3}$

If the null hypothesis of equal means is rejected ($p<0.05$), a post-hoc analysis was performed based on parametric Tukey method for one-way ANOVA and non-parametric Dwass-Steel-Critchlow-Fligner method (Critchlow and Fligner, 1991)

Before the analysis, normality assumption was tested based on Shapiro-Wilk test and Kolmogorov-Smimov test for one-way ANOVA and student t-test (Shapiro et al., 1968) and homoscedasticity (equal variance) assumption was examined based on Bartlett's test & Levene's test (Brown and Forsythe, 1974), too. If these assumptions fail and the conclusions between parametric and non-parametric analysis are different, then non-parametric approach was preferred for decision.

2.4 Statistical analysis of the predictive capacity

2.4.1 Analysis based on optimal cut-off values using ROC curve

The predictive capacity of the LLNA: BrdU-FCM was evaluated by comparing the traditional LLNA performances (gold standard). It is necessary to determine the optimal cut-off to define as sensitizer or non-sensitizer for predictive capacity evaluation. So we utilized ROC (Receiver operating characteristic) curve analysis frequently used to obtain optimal cut-off value in diagnostic tests (Hanley and McNeil, 1982). ROC curve is shown by the x(1-specificity, false positive rate) and y(sensitivity, true positive rate) axes then we can decide what is the optimal cut-off for predictive capacity.

Table A9-1. 2X2 contingency table

	Prediction	sensitizer	non-sensitizer	
Gold Standard	sensitizer	A	B	A+B
	non-sensitizer	C	D	C+D
		A+C	B+D	TOTAL

· Sensitivity: A/A+B · Specificity: D/C+D

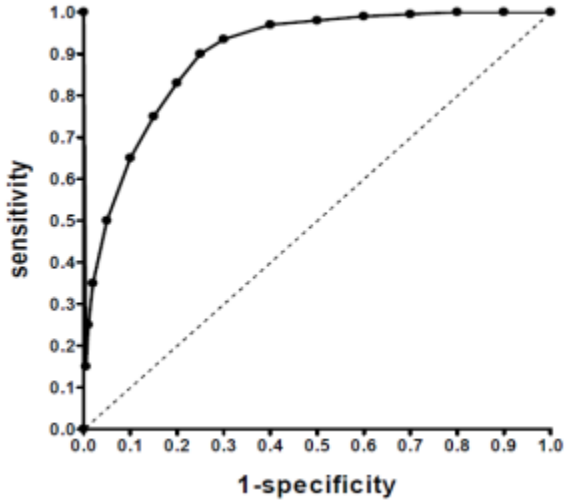


Figure A9-1. Example of ROC curve

- Using absolute SI values

Maximum mean SI value of each (18 obligatory) chemicals was used to plot ROC curve, since the test substance is defined as sensitizer if the maximum SI value is greater than the cut-off value, otherwise as non-

sensitizer.

- *Using standardized SI values*
We realized that there are substantial inter-test variations between the groups. To reduce these variations, SI values were standardized with that of the corresponding concurrent positive control as can be often used elsewhere (Bennett and Briggs, 2011). So the maximum SI values of each chemical was standardized as follows;

*(SI values of the chemical – SI value of the vehicle control)*100*
/ (SI value of the positive control – SI value of the vehicle control)

And also these standardized SI values were used to obtain the optimal cut-off value using the ROC curve.

2.4.2 Analysis based on inferential statistics.

One-sided t-test/ Wilcoxon Rank Sum Test
Inferential statistics were employed for further evaluation of predictive capacity. Because cut-off approach to classify test substance into sensitizer or non-sensitizer don't take into account variances of them. However inferential statistics considering SD (standard deviation) can overcome this limitation. Since the sample size is small we used both parametric (t-test) and non-parametric (wilcoxon rank sum test) method like 2.3.2. Also normality assumption (Kolmogorov-smirnov test) was examined and non-parametric approach was considered when that fail.

We assumed that mean of maximum SI value of a sensitizer group will be statistically significantly bigger than mean of the vehicle control group.

· null hypothesis: H_0: $\mu_{\text{maximum SI}} = \mu_{\text{control}}$ (α=0.05)

Based on analysis of this hypothesis, the substance is determined as a sensitizer when the p-value is less than 0.05. Otherwise the substance is determined as a non-sensitizer.

One-way Analysis of Variance (ANOVA) or Kruskal-Wallis test
Also we assumed that the means of each group (vehicle control, low, middle and high concentration) will be statistically significantly different and the null hypothesis is as follows;

· null hypothesis: H_0: $\mu_{\text{vehicle}} = \mu_{\text{low}} = \mu_{\text{middle}} = \mu_{\text{high}}$ (α=0.05)

If the p-value exceeds 0.05 it is diagnosed as a non-sensitizer. If not, we conducted *post hoc* analysis to make sure which group is different from vehicle group. Normality and homoscedasticity (Levene's test) assumption was tested. If Levene's test are satisfied, parametric approach is preferred for decision. And then parametric *post hoc* analysis (Tukey's test) is applied. If not, non-parametric *post hoc* analysis (DSCF, Dwass–Steel–Critchlow–Fligner) was applied. All statistical analyses were conducted using SAS version 9.3 (SAS Inc., Cary, NC, USA)

3. Result

3.1 Statistical analysis of WLR and BLR

3.1.1 Determination of optimal ECt

HCA (5, 10 and 25%) and DNCB (0.05, 0.1 and 0.25%), reference skin sensitizer substances, were tested four and three times respectively by 3 laboratories. In order to evaluate reproducibility, we determined optimal ECt through calculation from EC2.0 to EC4.0. ECt values were estimated from SI values using the linear interpolation method such that matches an x(concentration) where the y (SI) equals threshold.

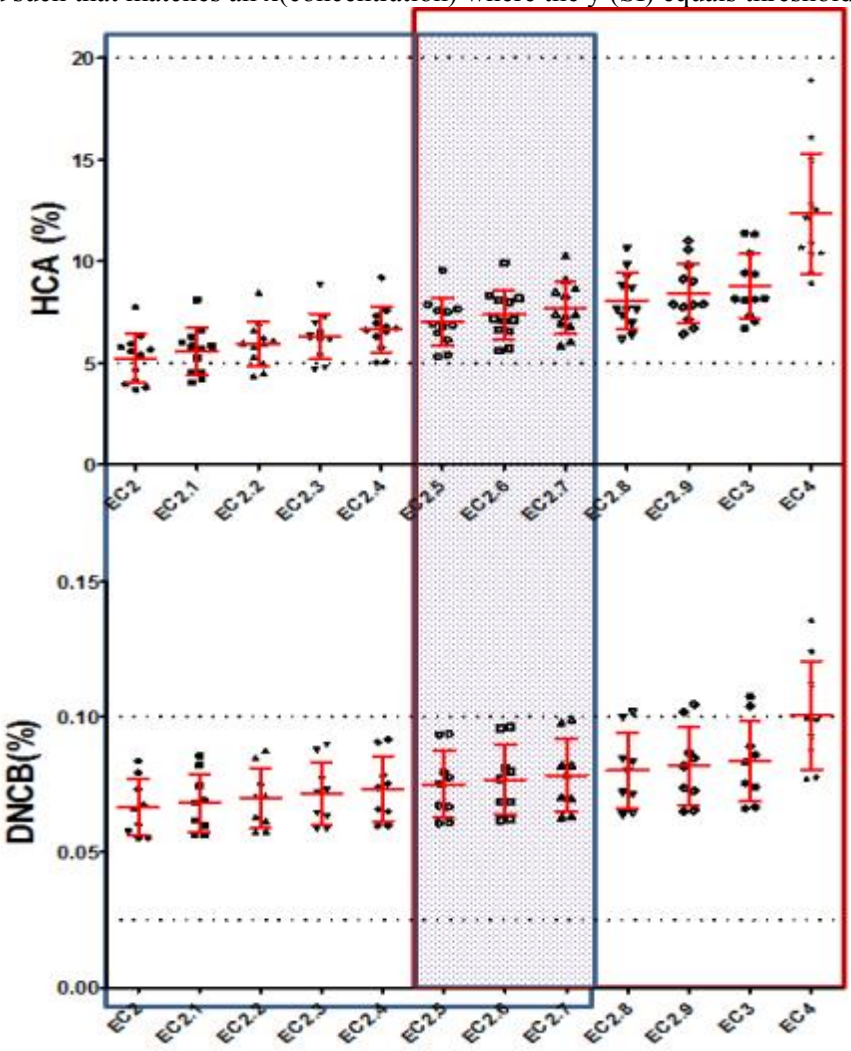


Figure A9-2. ECt values for HCA and DNCB obtained from all three laboratories when threshold varies from 2.0 to 4.0.

In the result, EC2.5, EC2.6 and EC2.7 satisfied acceptable range described in OECD TG 429. Of those ECt values, cut-off 2.7 had the highest accuracy in ROC curve analysis. Therefore, EC2.7 was selected as optimal value.

3.1.2 WLR and BLR based on performance standard of OECD test guideline 429

Within-laboratory reproducibility(WLR)

Each EC2.7 value of HCA is shown in Table A9-2. OECD guideline advises that ECt values of HCA be between 5 to 20% in order to satisfy the WLR.

Therefore, the WLR of the LLNA: BrdU-FCM was confirmed.

Table A9-2. EC2.7 values of HCA

HCA	Laboratory 1				Laboratory 2				Laboratory 3			
EC2.7	7.4	7.3	6.8	7.4	10.3	6.0	8.5	8.3	5.9	9.1	8.7	7.0

HCA, Hexyl cinnamic aldehyde

Between-laboratory reproducibility(BLR)
Each EC2.7 value of HCA is shown in Table A9-3. ECt values of HCA must be between 5-20% and those of DNCB also be between 0.025-0.1% for satisfaction of the BLR.
Therefore, the BLR of the LLNA: BrdU-FCM was confirmed.

Table A9-3. EC2.7 values of DNCB

DNCB	Laboratory1			Laboratory 2			Laboratory 3		
EC2.7	0.079	0.098	0.063	0.082	0.07	0.07	0.099	0.082	0.063

DNCB, 2,4-dintrochlorobenzene

3.1.3 Descriptive statistics for SI values (Raw data)

As mentioned earlier, HCA (5, 10 and 25%) and DNCB (0.05, 0.1 and 0.25%), skin sensitizer substances, were tested four and three times respectively by 3 laboratories. The scatterplot with mean±SD of SI values is as follows:

The visual presentation helps to estimate variance and reproducibility of SI values.

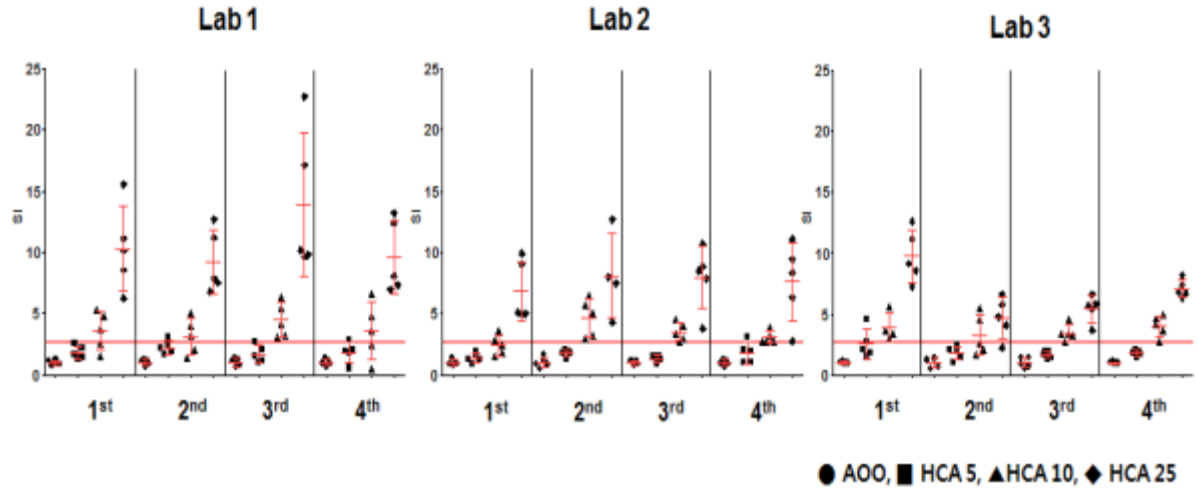


Figure A9-3. Dose-dependent SI values for HCA obtained from all laboratories
*Red lines : cut-off 2.7
In Figure A9-4, we were able to see the substantial level of variance clearly.

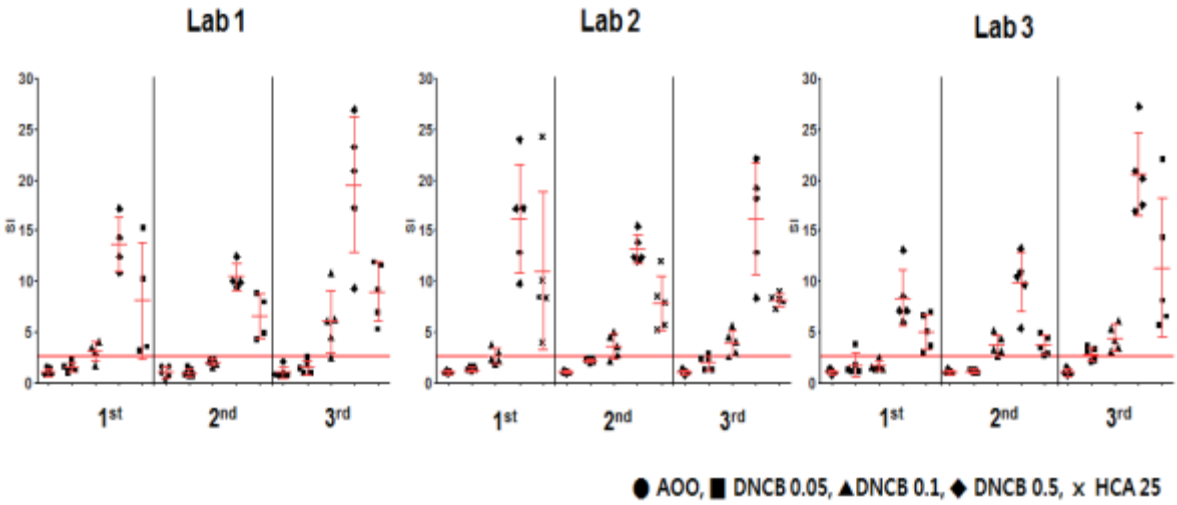


Figure A9-4. Dose-dependent SI values for DNCB and a concurrent positive control (HCA 25 %) obtained from all laboratories.

Next, Figure A9-5 is the scatterplot and box-whisker plot with mean±SD of SI values in HCA 25%. A value (2.25) smaller than cut-off value 2.7 was noted in the 2nd test at laboratory 3.

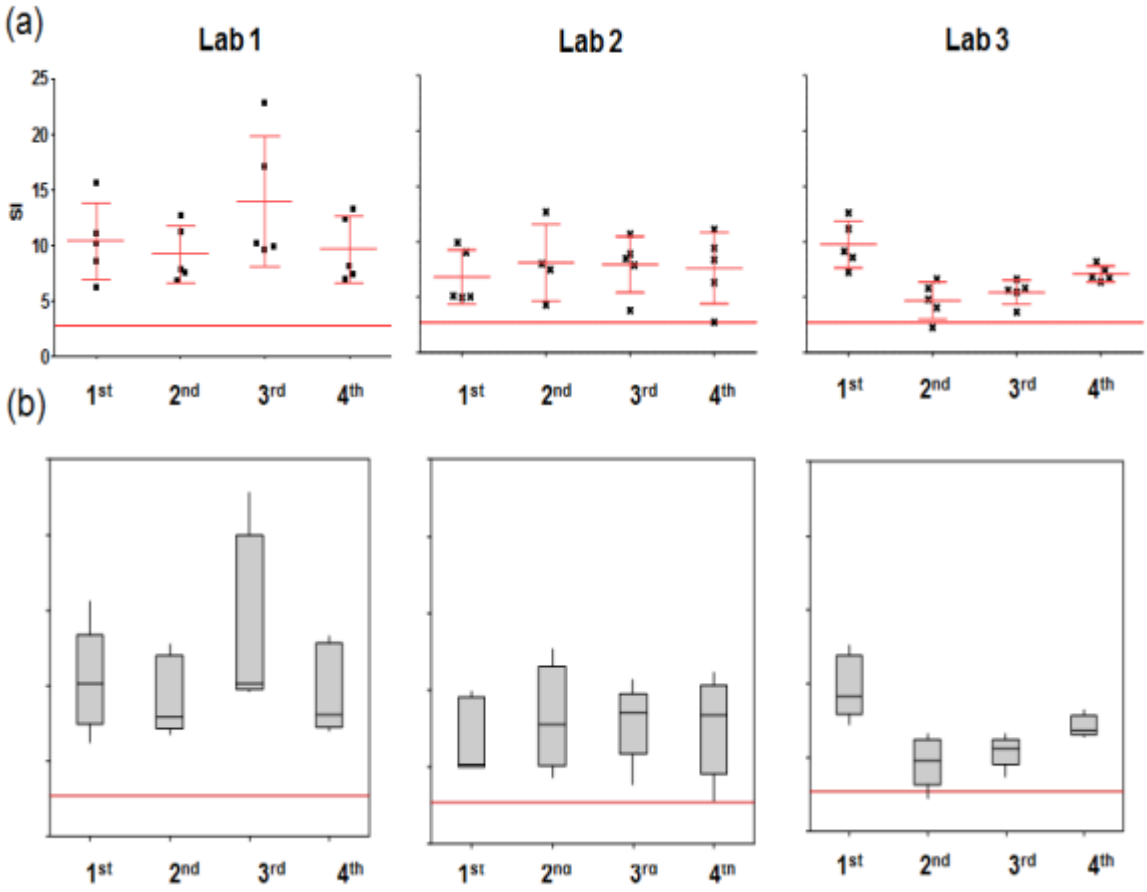


FIgure A9-5. SI values of a concurrent positive control (HCA 25%) obtained during WLR.

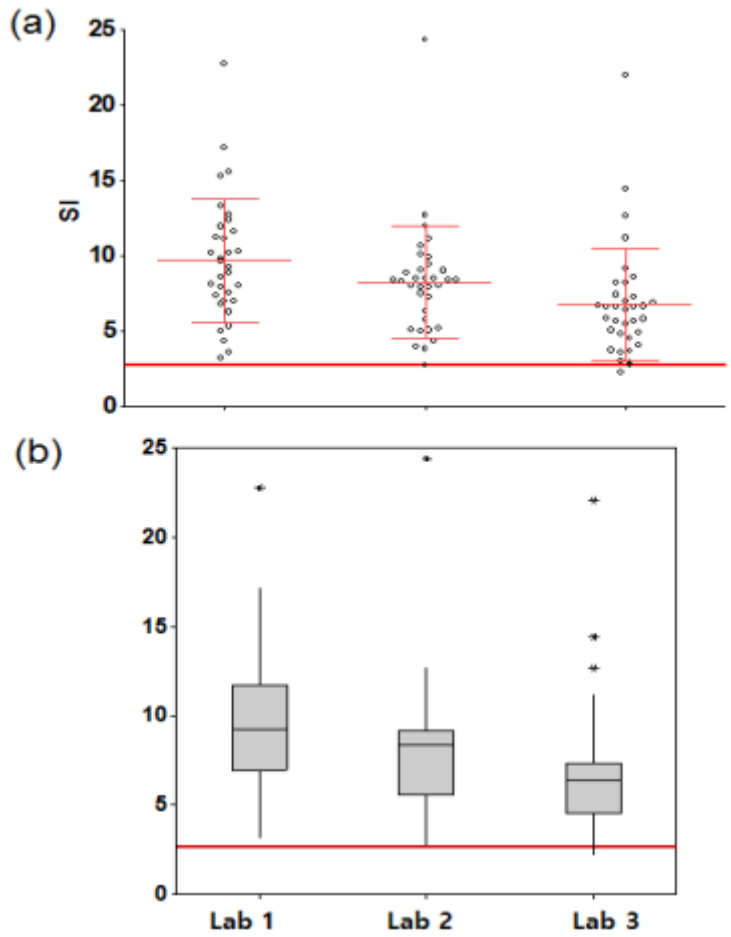


Figure A9-6. BLR of SI values of a concurrent positive control (HCA 25 %).

ECt value is estimated with a set of SI values consisting of three concentration points. So, as we showed above, some information including dispersion of values is ignored. Therefore, an ECt value is not the proper indicator sometimes.

3.1.4 Analysis of WLR and BLR using inferential statistics with SI values of HCA 25%

Additionally SI values of the positive reference compounds(HCA) were investigated based on inferential statistics to assess WLR and BLR. Both parametric (one-way ANOVA and student t-test) and non-parametric (Kruskal-Wallis and Wilcoxon rank sum test) methods were utilized for analysis.

Within-laboratory reproducibility(WLR)
The hypothesis of WLR was as follows:

· null hypothesis for WLR: H_0: $\mu_{test1} = \mu_{test2} = \mu_{test3} = \mu_{test4}$

We used both parametric method (one-way ANOVA) and non-parametric method (Kruskal-wallis). Before the analysis, equal variance of SI values (HCA 25%) were confirmed through Levene's test and Bartlett's test. However normality assumption test failed in the 1st test at laboratory 2 using Shapiro-Wilk (p=0.0309) and Komogorov- smimov (p=0.0359) test method.

Table A9-4 is a summary table of analysis results. WLR was found at laboratory 1 and 2 by both parametric (p=0.9255 and 0.9040) and non-parametric (p=0.9722 and 0.9709) method. However significant difference

was observed between laboratory 3 tests based on both methods (p=0.0004 and 0.0020). So, laboratory 3 failed to WLR through inferential statistics approach.

As a result of the *post hoc* test between laboratory 3 tests, test 1 was significantly different compared to test 2 and test 3. Also mean value of test 2 was significantly smaller than the other laboratories.

Table A9-4. Summary and statistical analysis of WLR in SI values of HCA 25% obtained during WLR.

Laborator	Test	Mean	SD	N	Median (Min, Max)	Significance (p value)	
						ANOVA	Kruskal-Wallis
Laboratory 1	1	10.3	3.5	5	10.1(6.3, 15.6)	0.9255	0.9722
	2	9.2	2.6	5	7.9(6.8, 12.7)	0.9255	0.9722
	3	10.3	3.5	5	10.2(9.6, 22.8)	0.9255	0.9722
	4	9.6	3.0	5	8.1(7.0, 13.3)	0.9255	0.9722
Laboratory 2	1	6.8	2.5	5	5.1(5.0, 9.9)	0.9040	0.9709
	2	8.1	3.5	4	7.8(4.3, 12.7)	0.9040	0.9709
	3	7.9	2.5	5	8.5(3.8, 10.7)	0.9040	0.9709
	4	7.6	3.2	5	8.4(2.7, 11.1)	0.9040	0.9709
Laboratory 3	1	9.7^{a}	2.1	5	$9.1(7.2,12.6)^{c}$	**0.0004***	**0.0020***
	2	4.7^{b}	1.7	5	$4.8(2.3, 6.6)^{d}$	**0.0004***	**0.0020***
	3	5.4^{b}	1.1	5	$5.6(3.7, 6.6)^{d}$	**0.0004***	**0.0020***
	4	$7.1^{a,b}$	0.7	5	$6.8(6.4, 7.8)^{c,d}$	**0.0004***	**0.0020***

*p < 0.05:
a, b Grouped by Tukey's post hoc analysis.
c, d Grouped by Dwass, Steel, Critchlow–Fligner method.

Between-laboratory reproducibility(BLR)
The hypothesis of BLR was as follows:

· null hypothesis for BLR: H_0: $\mu_{laboratory1} = \mu_{laboratory2} = \mu_{laboratory3}$

BLR was performed between laboratories 1 and 2 excluding laboratory 3 who failed in WLR. Analysis methods were t-test(parametric) and Kruskal-wallis (non-parametric) test.

As a result of the analysis, laboratories 1 and 2 were not statistically significantly different by both approaches (p=0.138 and 0.1004). Therefore BLR between laboratories 1 and 2 was demonstrated.

Table A9-5. Overall summary and statistical analysis of BLR in SI values of a concurrent positive control (HCA 25%).

Laboratory	MEAN	SD	N	Median (Min, Max)	Significance(p value)		
					Comparison	t-test	Wilcoxon rank sum
Laboratory 1	9.7	4.1	33	9.2(3.2,11.8)	Laboratory 1 vs. Laboratory 2	0.138	0.1004
Laboratory 2	8.2	3.7	34	8.3(2.7, 24.3)			
Laboratory 3	6.7	3.8	35	6.4(2.3, 22.0)			

Table A9-6. SI values for 22 reference substances obtained with the LLNA: BrdU-FCM

No.	Substances	CAS No.	Vendor	Vehicle	Conc.(%)	SI values (mean±SD)					MAX SI
						NC	L	M	H	PC	
1	5-Chloro-2-methyl-4-isothiazolin-3-one(CMI)/2-methyl-4-isothiazolin-3-one(MI)	26172-55-4/ 2682-20-4	Rohm and Hass	DMF	2.5,5,10	1.00±0.51	7.29±2.39	19.72±4.95	14.34±3.55	10.77±1.34	19.72
2	DNCB	97-00-7	Aldrich	AOO	0.1,0.25,0.5	1.00±0.35	4.40±1.16	18.42±4.75	25.28±12.87	12.23±3.94	25.28
3	4-Phenylenediamine	106-50-3	Sigma	DMSO	0.5,1,2.5	1.00±0.20	4.39±1.32	6.39±1.89	12.28±5.89	8.54±2.01	12.28
4	Cobalt chloride	7646-79-9	Sigma-Aldrich	DMF	0.25,0.5,1.0	1.00±0.51	10.56±2.80	19.51±5.00	24.99±2.70	10.77±1.34	24.99
5	Isoeugenol	97-54-1	Aldrich	AOO	5,10,25	1.00±0.38	1.74±0.86	3.51±2.02	11.16±3.41	8.06±5.80	11.16
6	2-Mercaptobenzothiazole	149-30-4	Aldrich	DMF	25,50,100	1.00±0.58	1.43±0.17	1.10±0.52	0.83±0.41	4.35±1.95	1.43
7	Citral	5392-40-5	Aldrich	AOO	10,25,50	1.00±0.32	2.53±1.21	6.46±0.78	11.06±1.66	5.73±4.63	11.06
8	HCA	101-86-0	Aldrich	AOO	5,10,25	1.00±0.35	1.22±0.41	3.08±1.64	9.55±4.76	12.23±3.94	9.55
9	Eugenol	97-53-0	Fluka	AOO	5,10,25	1.00±0.70	2.44±0.77	5.56±2.32	21.13±5.31	8.30±3.80	21.13
10	Phenyl benzoate	93-99-2	Aldrich	DMF	5,10,25	1.00±0.47	4.14±1.90	12.50±4.16	7.83±2.77	21.07±9.12	12.50
11	Cinnamic alcohol	104-54-1	Aldrich	AOO	25,50,100	1.00±0.58	2.28±0.85	4.66±1.05	4.28±1.98	15.83±5.45	4.66
12	Imidazolidinyl urea	39236-46-9	Aldrich	DMF	10,25,50	1.00±0.46	1.78±0.60	2.99±0.85	3.52±1.09	6.12±1.51	3.52
13	Methyl methacrylate	80-62-6	Aldrich	AOO	25,50,100	1.00±0.58	1.63±0.46	0.93±0.23	0.86±0.28	15.83±5.45	1.63
14	Chlorobenzene	108-90-7	Sigma-Aldrich	AOO	10,25,50	1.00±0.17	0.66±0.28	1.27±0.54	2.55±0.84	26.59±9.06	2.55
15	Isopropanol	67-63-0	Sigma-Aldrich	AOO	2.5,5,10	1.00±0.17	1.20±0.29	0.91±0.72	1.30±0.58	26.59±9.06	1.30

16	Lactic acid	50-21-5	Fluka	DMF	5,10,25	1.00±0.25	1.48±0.71	1.46±0.28	1.51±0.41	7.12±4.78	1.51
17	Methyl salicylate	119-36-8	Sigma-Aldrich	AOO	25,50,100	1.00±0.21	1.91±0.28	2.66±0.67	1.91±0.72	4.90±1.01	2.66
18	Salicylic acid	69-72-7	Sigma	DMF	1,2.5,5	1.00±0.70	1.86±0.54	2.38±1.39	1.81±0.47	8.54±2.01	2.38
19	Sodium lauryl sulphate	151-21-3	Sigma	AOO	5,10,25	1.00±0.31	4.75±2.29	6.47±1.94	4.71±0.83	3.68±0.34	6.47
20	Ethylene glycol dimethacrylate	97-90-5	Aldrich	DMSO	25,50,100	1.00±0.34	2.02±0.64	2.75±0.99	4.06±1.34	6.15±2.06	4.06
21	Xylene	1330-20-7	Sigma-Aldrich	AOO	25,50,100	1.00±0.28	1.45±0.26	2.44±0.31	4.19±0.82	4.13±0.60	4.19
22	Nickel chloride	7718-54-9	Aldrich	DMSO	0.001,0.0025,0.005	1.00±0.15	1.11±0.26	1.19±0.27	1.19±0.34	5.37±1.19	1.19

DNCB, 2,4-dintrochlorobenzene; HCA, Hexyl cinnamic aldehyde; AOO, acetone: olive oil (4:1, v/v); DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulphoxide; SI, stimulation index; MAX, maximum mean SI value among treated groups; NC, vehicle control; PC, positive control (hexylcinnamaldehyde, 25%); Test substance treated with L, low; M, middle; H, high concentrations. SI values were obtained using the LLNA: BrdU-FCM method
Grey shaded : non-sensitizer substances in traditional LLNA

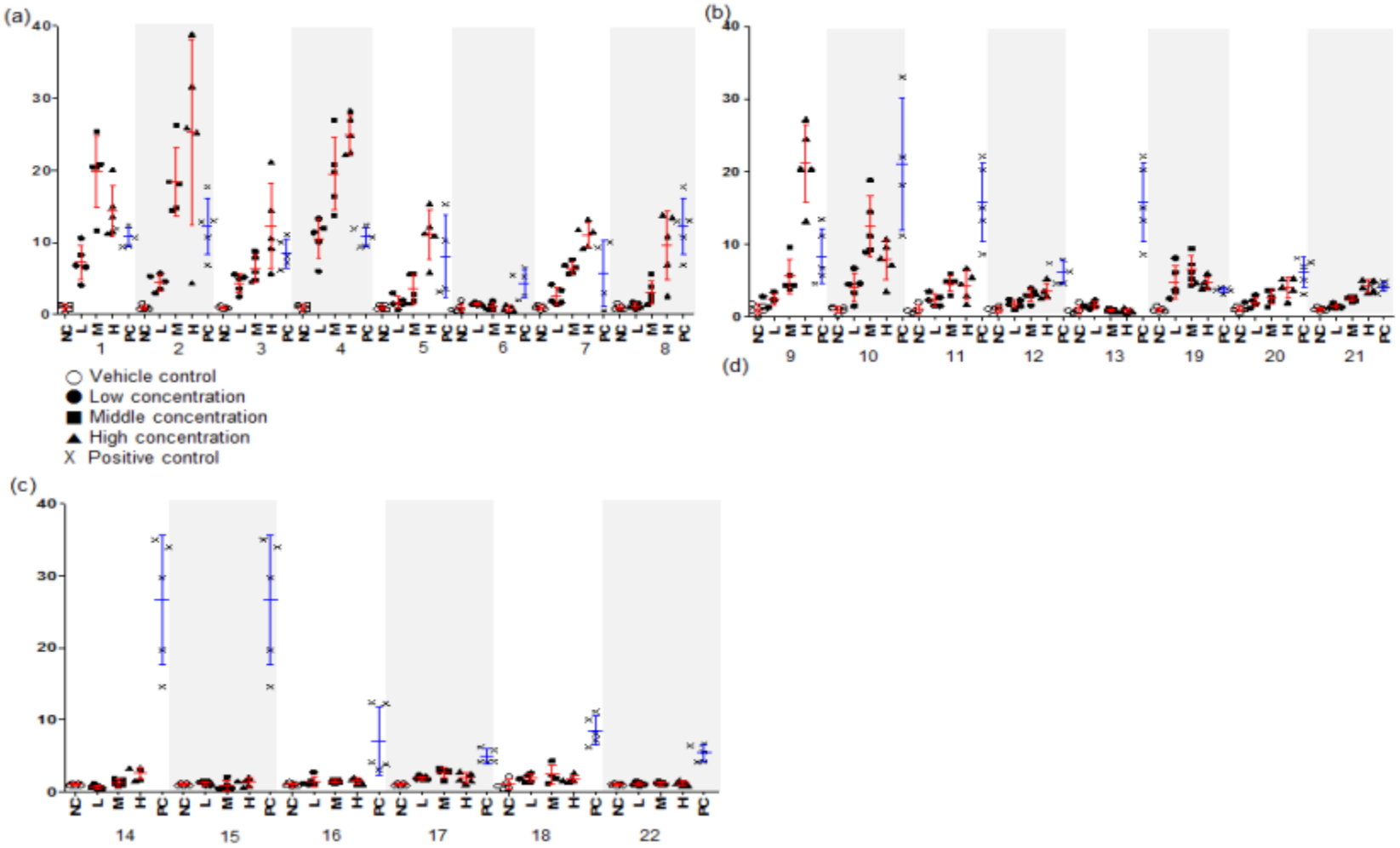


Figure A9-7. SI value of 22 substances.(N=4,5) (a) sensitizers (1~8) (b) sensitizers (9~13, 19~21), (c) non-sensitizers (14~18, 22)

3.2 Statistical analysis of the predictive capacity

3.2.1 Summaries of SI values obtained from the tests for predictive capacity

The results obtained through the experiments from 2012 until 2014 were summarized as follows (Table A9-6):

SI values of 22 reference substances (18 mandatory and 4 optional substances) listed in OECD TG 429 were obtained from 3 independent laboratories.

SI values of 22 substances(N=4,5) were visually demonstrated by scatterplot

3.2.2 Analysis based on optimal cut-off values using ROC curve
As previously stated, SI value 2.7 was determined as an optimal cut-off, through the ROC curve analysis as well as WLR/BLR for ECt values. Max SI values of 18 obligatory substances were used to obtain optimal cut-off value using a ROC analysis.

Figure A9-8 shows a result of a ROC analysis. AUC (Area Under the Curve) was 0.8846.

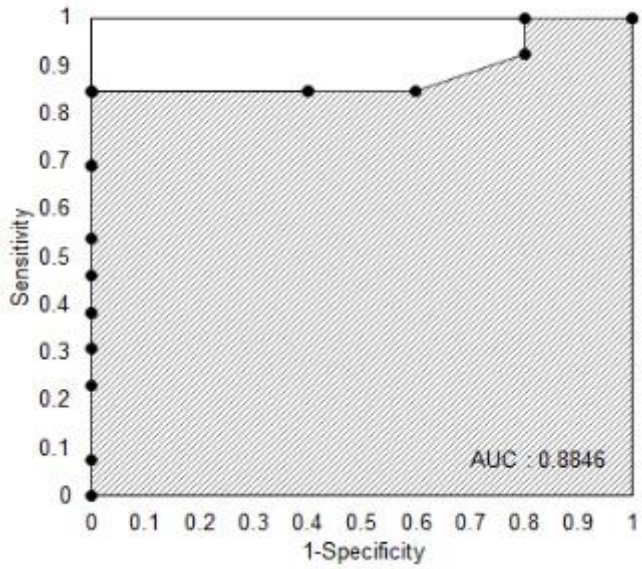


Figure A9-8. ROC curve of 18 obligatory substances' SI values

Table A9-7 was the sensitivity, 1-specificity and accuracy of each cut-off. Accuracy was the highest from cut-off 2.7 to 4.6. So we could conclude 2.7 as an optimal cut-off value among 2.5, 2.6 and 2.7 that fulfilled WLR/BLR

Table A9-7. Sensitivity, 1-specificity and accuracy of each cut off.

Cut-off	Sensitivity	1-Specificity	Accuracy
0≤SI<1.43	1.00	1.00	0.72
1.43 ≤SI< 1.63	1.00	0.80	0.78
1.63 ≤SI< 1.67	0.92	0.80	0.72
1.67 ≤SI< 2.38	0.85	0.60	0.78
2.38 ≤SI< 2.66	0.85	0.40	0.78
2.66 ≤SI< 4.66	**0.85**	**0.00**	**0.89**
4.66 ≤SI< 11.06	0.69	0.00	0.78

11.06 ≤SI< 11.16	0.54	0.00	0.67
11.16 ≤SI< 12.28	0.46	0.00	0.61
12.28 ≤SI< 19.72	0.38	0.00	0.56
:	:	:	:
25.28 ≤SI	0.00	0.00	0.28

We classified 22 substances to sensitizer or non-sensitizer by cut-off 2.7, and then results were compared with OECD TG 429.

Table A9-8 shows the result. 2-Mercaptobenzothiazole and Methyl methacrylate were a false negative when compared to the LLNA ref. Non-sensitizer was shaded to be easily identifiable in Table A9-8.

The sensitivity, specificity and accuracy were 87.5% (14/16), 100% (6/6), 90.9% (20/22) respectively for all 22 substances.

Table A9-8. Comparison of results between LLNA ref.(OECD TG429) and LLNA:BrdU- FCM

No.	Substances	LLNA ref.	LLNA: BrdU-FCM
1	CMI/ MI	+	+
2	DNCB	+	+
3	4-Phenylenediamine	+	+
4	Cobalt chloride	+	+
5	Isoeugenol	+	+
6	2-Mercaptobenzothiazole	+	-
7	Citral	+	+
8	HCA	+	+
9	Eugenol	+	+
10	Phenyl benzoate	+	+
11	Cinnamic alcohol	+	+
12	Imidazolidinyl urea	+	+
13	Methyl methacrylate	+	-
14	Chlorobenzene	-	-
15	Isopropanol	-	-
16	Lactic acid	-	-
17	Methyl salicylate	-	-
18	Salicylic acid	-	-
19	Sodium lauryl sulphate	+	+
20	Ethylene glycol dimethacrylate	+	+
21	Xylene	+	+
22	Nickel chloride	-	-

CMI/MI, 5-Chloro-2-methyl-4-isothiazolin-3-one/2-methyl-4-isothiazolin-3-one; DNCB, 2,4-dintrochlorobenzene; HCA, Hexyl cinnamic aldehyde; Decision, + stands for the positive and – stands for the negative decision.

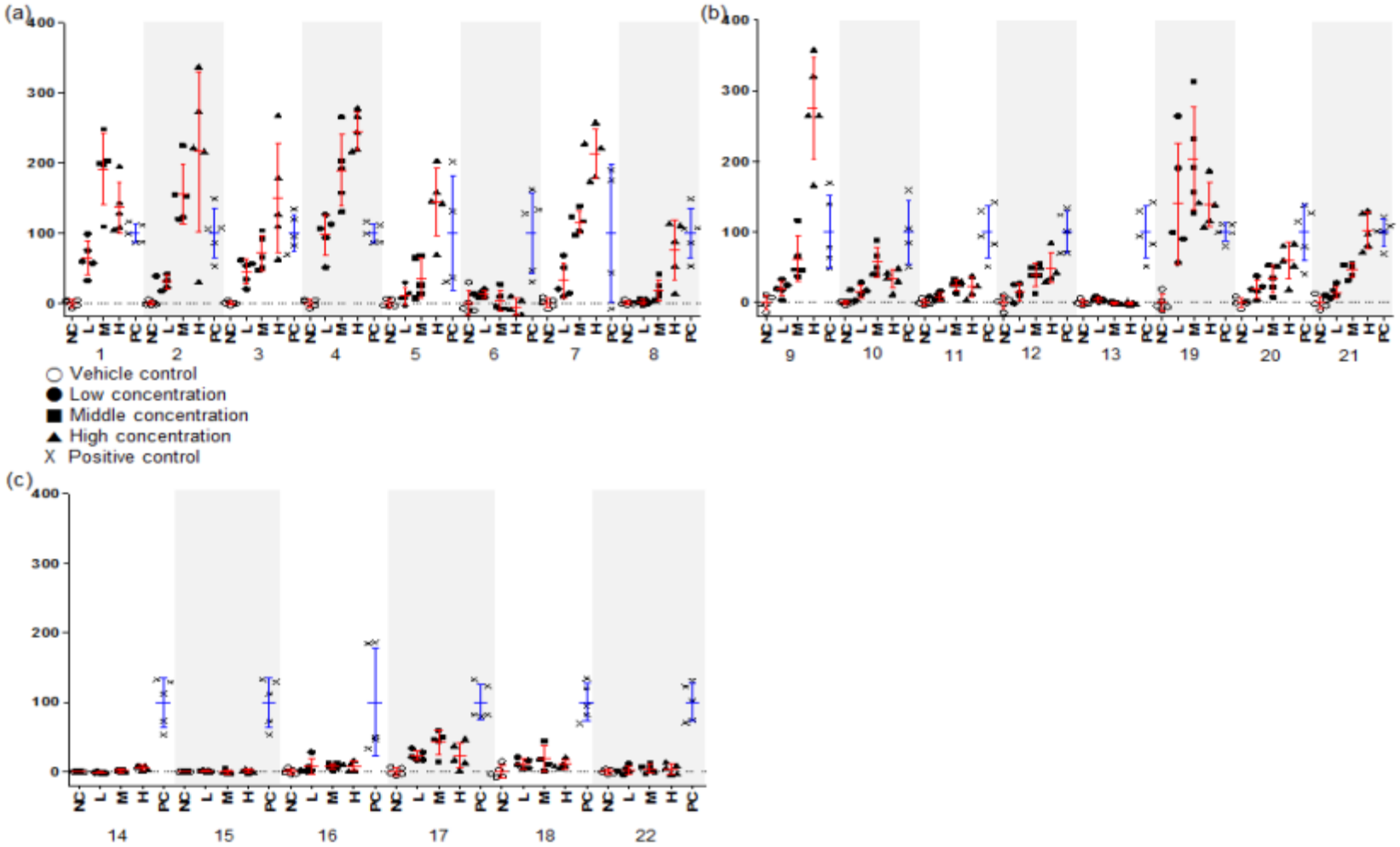


Figure A9-9. Standardized SI value of 22 substances.(N=4,5) (a) sensitizers (1~8), (b) sensitizers (9~13, 19~21), (c) non-sensitizers (14~18, 22)

- *Using standardized SI values*

To reduce variations between the groups, SI values were standardized with that of the corresponding concurrent positive control. And then a ROC analysis was performed again to obtain optimal cut-off that was 42.6. Sensitivity, specificity and accuracy were 81.3% (13/16), 100% (6/6) and 86.4% (19/22) respectively. The accuracy was slightly fallen due to the false negative prediction for cinnamic alcohol.

Table A9-9. Comparison of results among LLNA ref.(OECD TG429) and LLNA:BrdU- FCMs (cut-off 2.7 and standardized 42.6)

No.	Substances	LLNA ref.	LLNA: BrdU-FCM cut-off 2.7	Standized SI cut-off 42.6
1	CMI/ MI	+	+	191.6
2	DNCB	+	+	216.1
3	4-Phenylenediamine	+	+	149.6
4	Cobalt chloride	+	+	245.5
5	Isoeugenol	+	+	143.8
6	2-Mercaptobenzothiazole	+	-	12.5
7	Citral	+	+	212.7
8	HCA	+	+	76.1
9	Eugenol	+	+	275.6
10	Phenyl benzoate	+	+	57.3
11	Cinnamic alcohol	+	+	24.7
12	Imidazolidinyl urea	+	+	49.2
13	Methyl methacrylate	+	-	4.3
14	Chlorobenzene	-	-	6.0
15	Isopropanol	-	-	1.2
16	Lactic acid	-	-	8.3
17	Methyl salicylate	-	-	42.6
18	Salicylic acid	-	-	18.3
19	Sodium lauryl sulphate	+	+	204.3
20	Ethylene glycol dimethacrylate	+	+	59.5
21	Xylene	+	+	102.0
22	Nickel chloride	-	-	4.4

CMI/MI, 5-Chloro-2-methyl-4-isothiazolin-3-one/2-methyl-4-isothiazolin-3-one; DNCB, 2,4-dintrochlorobenzene; HCA, Hexyl cinnamic aldehyde; Decision, + stands for the positive and – stands for the negative decision.

3.2.3 Analysis based on inferential statistics.

Inferential statistics were employed for further evaluation of predictive capacity in order to take into account variances of SI values.

One-sided t-test/ Wilcoxon Rank Sum Test

We assumed that mean of maximum SI value of a sensitizer group will be statistically significantly bigger than mean of the vehicle control group.

· null hypothesis: H_0: $\mu_{maximum\ SI} = \mu_{control}$ (α=0.05)

Based on analysis of this hypothesis, the substance is determined as a sensitizer when the p-value is less than 0.05. Otherwise is determined as a non-sensitizer.

Except for 2-Mercaptobenzothiazole, sensitizer substances based on LLNA ref. were equally decided as sensitizer, so the sensitivity was improved to 93.8% compared with the optimal cut-off approach. However specificity (33.3%) and accuracy (77.3%) was worse than cut-off method.

Table A9-10. Summary of one-sided t-test or Wilcoxon Rank Sum Test

No.	Substances	LLNA ref.	LLNA: BrdU-FCM (2.7)	Comparison of the group with MAX mean SI vs NC		
				T-test	Wilcoxon	Decision
1	CMI/ MI	+	+	0.0000	0.004*	+
2	DNCB	+	+	0.0015	0.004	+
3	4-Phenylenediamine	+	+	0.0014	0.004	+
4	Cobalt chloride	+	+	0.0000	0.004	+
5	Isoeugenol	+	+	0.0001	0.004	+
6	2-Mercaptobenzothiazole	+	-	0.0757	0.075	-
7	Citral	+	+	0.0000	0.004	+
8	HCA	+	+	0.0020	0.004	+
9	Eugenol	+	+	0.0000	0.004	+
10	Phenyl benzoate	+	+	0.0001	0.004	+
11	Cinnamic alcohol	+	+	0.0001	0.004	+
12	Imidazolidinyl urea	+	+	0.0007	0.004	+
13	Methyl methacrylate	+	-	0.0455	0.048	+
14	Chlorobenzene	-	-	0.0019	0.004	+
15	Isopropanol	-	-	0.1486	0.274	-
16	Lactic acid	-	-	0.0226	0.048	+
17	Methyl salicylate	-	-	0.0004	0.004	+
18	Salicylic acid	-	-	0.0455	0.056	+
19	Sodium lauryl sulphate	+	+	0.0001	0.004	+
20	Ethylene glycol dimethacrylate	+	+	0.0006	0.004	+
21	Xylene	+	+	0.0000	0.004	+
22	Nickel chloride	-	-	0.1405	0.226	-

CMI/MI, 5-Chloro-2-methyl-4-isothiazolin-3-one/2-methyl-4-isothiazolin-3-one; DNCB, 2,4-dintrochlorobenzene; HCA, Hexyl cinnamic aldehyde; Decision, + stands for the positive and – stands for the negative decision. one-sided t-test; Wilcoxon, Wilcoxon rank sum test. * normality assumption failed by Kolmogorov–Smirnov test

One-way Analysis of Variance (ANOVA) or Kruskal-Wallis test

Also we assumed that the means of each group (vehicle control, low, middle and high concentration) will be statistically significantly different if the test substance is a sensitizer and the null hypothesis is as follows;

· null hypothesis: H_0: $\mu_{vehicle} = \mu_{low} = \mu_{middle} = \mu_{high}$ (α=0.05)

If the p-value is bigger than 0.05 it is diagnosed as a non-sensitizer. If not, we conducted post-hoc analysis to identify the different group from the vehicle group.

Table A9-11 is a summary of ANOVA or Kruskal-Wallis with *post hoc*. Regardless of the Levene's test, both parametric (ANOVA) and non-parametric (Kruskal-wallis) methods all agreed.

Also like the result of the one-sided t-test/ Wilcoxon Rank Sum Test, specificity (66.7%) and accuracy (81.8%) were worse than the optimal cut-off approach except for sensitivity (87.5%).

Overall, analysis based on optimal cut-off values had the highest accuracy. Table A9-12 is the result of overall analysis.

Table A9-11. Summary of ANOVA or Kruskal-Wallis with post hoc

No.	Substances	LLNA ref. (OECDTG)	LLNA: BrdU-FCM(2.7)	Levene's Test	ANOVA	Dunnett	Kruskal-Wallis	DSCF	Decision
1	5-Chloro-2-methyl-4-isothiazolin-3-one(CMI)/2-methyl-4-isothiazolin-3-one (MI)	+	+	0.2880	<.0001	L,M,H	0.0007	L,M,H	+
2	DNCB	+	+	0.1146	0.0001	M,H	0.0018	L,M,H	+
3	4-Phenylenediamine	+	+	0.0541	0.0003	M,H	0.0014	L,M,H	+
4	Cobalt chloride	+	+	0.1320	<.0001	L,M,H	0.0007	L,M,H	+
5	Isoeugenol	+	+	0.1334	<.0001	H	0.0014	M,H	+
6	2-Mercaptobenzothiazole	+	-	0.4394	0.2274	-	0.1405	-	-
7	Citral	+	+	0.0329	<.0001	M,H	0.0005	L,M,H	+
8	HCA	+	+	0.0183	0.0002	H	0.0024	H	+
9	Eugenol	+	+	0.0704	<.0001	H	0.0006	M,H	+
10	Phenyl benzoate	+	+	0.1236	<.0001	M,H	0.0011	L,M,H	+
11	Cinnamic alcohol	+	+	0.0472	0.0007	M,H	0.0045	M	+
12	Imidazolidinyl urea	+	+	0.4496	0.0004	M,H	0.0031	M,H	+
13	Methyl methacrylate	+	-	0.4117	0.0327	-	0.0533	-	-
14	Chlorobenzene	-	-	0.0025	0.0002	H	0.0056	H	+
15	Isopropanol	-	-	0.2595	0.5818	-	0.4515	-	-
16	Lactic acid	-	-	0.3085	0.2635	-	0.1438	-	-
17	Methyl salicylate	-	-	0.1666	0.0014	M	0.0083	L,M	+
18	Salicylic acid	-	-	0.1272	0.1185	-	0.1316	-	-
19	Sodium lauryl sulphate	+	+	0.0877	0.0004	L,M,H	0.0054	L,M,H	+
20	Ethylene glycol dimethacrylate	+	+	0.1679	0.0006	H	0.0043	H	+
21	Xylene	+	+	0.0013	<.0001	M,H	0.0007	M,H	+
22	Nickel chloride	-	-	0.2660	0.6319	-	0.5968	-	-

DNCB, 2,4-dintrochlorobenzene; HCA, Hexyl cinnamic aldehyde; Test substance treated with L, low; M, middle; H, high concentrations; Decision, + stands for the positive and – stands for the negative decision.

Table A9-12. Overall Summary of prediction models for the LLNA: BrdU-FCM

No.	Substances	LLNA ref.	LLNA:BrdU-FCM (2.7)	(Max SI-NC SI)/ (PC SI-NC SI)x100		T-test/Wilcoxon (NC vs MAX SI)		ANOVA/ Kruskal-Wallis
				Max SI %	42.6	group	Decision	Decision
1	5-Chloro-2-methyl-4-isothiazolin-3-one(CMI)/2-methyl-4-isothiazolin-3-one (MI)	+	+	191.56	+	Middle	+	+
2	DNCB	+	+	216.14	+	High	+	+
3	4-Phenylenediamine	+	+	149.64	+	High	+	+
4	Cobalt chloride	+	+	245.48	+	High	+	+
5	Isoeugenol	+	+	143.82	+	High	+	+
6	2-Mercaptobenzothiazole	+	-	12.75	-	Low	-	-
7	Citral	+	+	212.65	+	High	+	+
8	HCA	+	+	76.09	+	High	+	+
9	Eugenol	+	+	275.59	+	High	+	+
10	Phenyl benzoate	+	+	57.31	+	Middle	+	+
11	Cinnamic alcohol	+	+	24.66	-	Middle	+	+
12	Imidazolidinyl urea	+	+	49.24	+	High	+	+
13	Methyl methacrylate	+	-	4.26	-	Low	+	-
14	Chlorobenzene	-	-	6.04	-	High	+	+
15	Isopropanol	-	-	1.18	-	High	-	-
16	Lactic acid	-	-	8.30	-	High	+	-
17	Methyl salicylate	-	-	42.58	-	Middle	+	+
18	Salicylic acid	-	-	18.30	-	Middle	+	-
19	Sodium lauryl sulphate	+	+	204.32	+	Middle	+	+
20	Ethylene glycol dimethacrylate	+	+	59.48	+	High	+	+
21	Xylene	+	+	101.95	+	High	+	+
22	Nickel chloride	-	-	4.38	-	High	-	-
	Sensitivity		87.5% (14/16)		81.3% (13/16)		93.8% (15/16)	87.5% (14/16)
	Specificity		100% (6/6)		100% (6/6)		33.3% (2/6)	66.7% (4/6)
	Accuracy		90.9% (20/22)		86.4% (19/22)		77.3% (17/22)	81.8% (18/22)

DNCB, 2,4-dintrochlorobenzene; HCA, Hexyl cinnamic aldehyde; Decision, + stands for the positive and – stands for the negative decision.

3.3 The predictive capacity analysis. (2015 data)

Data used for the predictive capacity evaluation was collected from 2012 to 2014 by 3 laboratories including retest results. So we had a risk of data selection. To avoid it, a predictive capacity study was conducted in a single laboratory and replaced the above results.

Data are as follows:

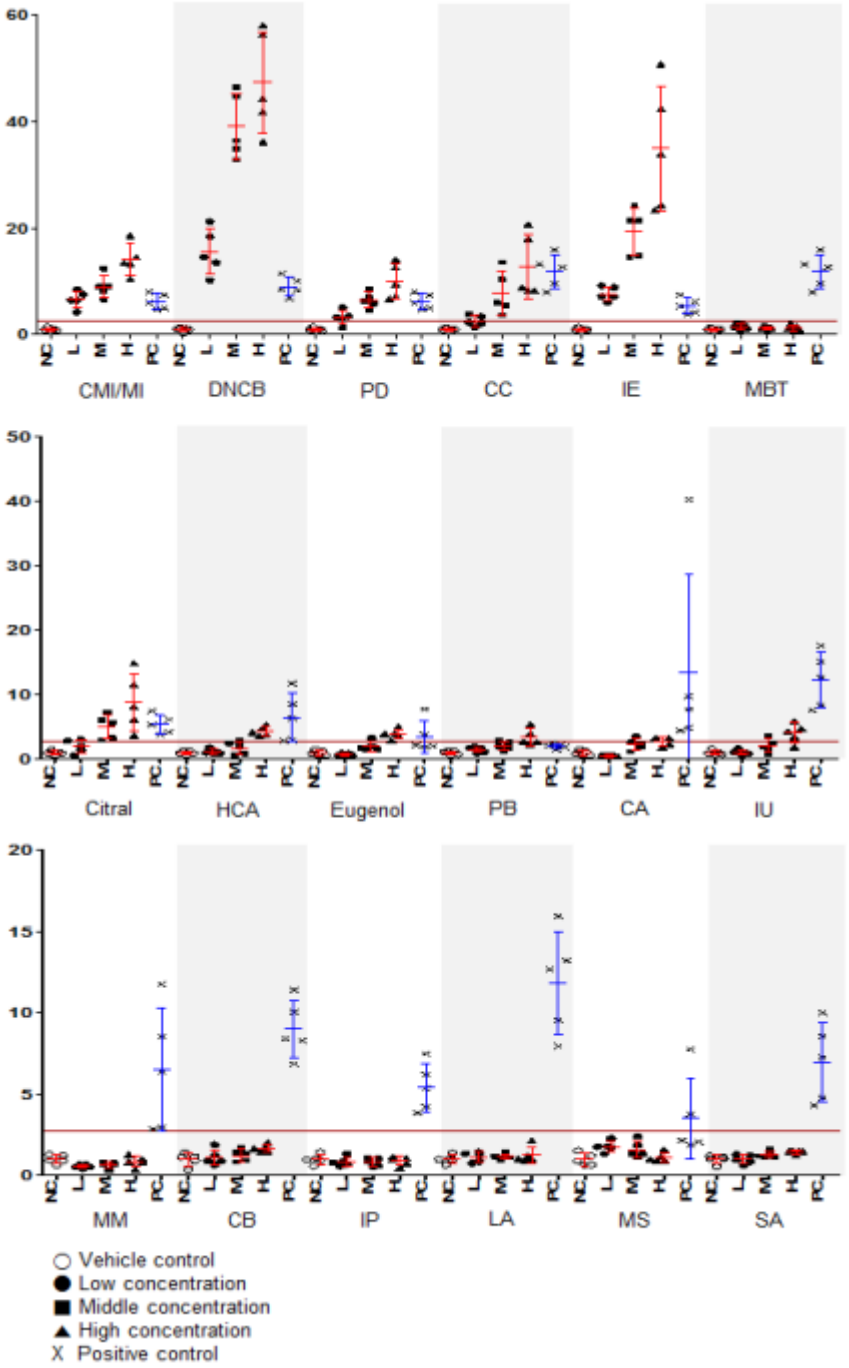


Figure A9-10. SI values of 18 substances (N=5)

Table A9-13. SI values for 18 obligatory substances obtained with the LLNA: BrdU-FCM in 2015

No.	Substances	CAS No.	Vendor	Vehicle	Conc.(%)	SI values (mean±SD)					MAX SI
						NC	L	M	H	PC	
1	5-Chloro-2-methyl-4-isothiazolin-3-one(CMI)/2-methyl-4-isothiazolin-3-one(MI)	26172-55-4/ 2682-20-4	Rohm and Hass	DMF	2.5,5,10	1.00±0.32	9.99±3.45	13.42±1.85	13.55±1.77	6.24±1.44	13.55
2	DNCB	97-00-7	Aldrich	AOO	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
3	4-Phenylenediamine	106-50-3	Sigma	DMSO	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
4	Cobalt chloride	7646-79-9	Sigma-Aldrich	DMF	0.25,0.5,1.0	1.00±0.25	2.60±1.00	7.80±4.06	12.83±6.03	11.86±3.15	12.83
5	Isoeugenol	97-54-1	Aldrich	AOO	5,10,25	1.00±0.32	7.66±1.33	19.3±4.37	34.91±11.73	5.42±1.48	34.91
6	2-Mercaptobenzothiazole	149-30-4	Aldrich	DMF	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
7	Citral	5392-40-5	Aldrich	AOO	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
8	HCA	101-86-0	Aldrich	AOO	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
9	Eugenol	97-53-0	Fluka	AOO	5,10,25	1.00±0.38	0.71±0.19	2.01±0.75	3.94±0.74	3.89±3.28	3.94
10	Phenyl benzoate	93-99-2	Aldrich	DMF	10,25,50	1.00±0.39	6.85±1.96	9.44±3.00	15.41±5.82	9.01±1.75	15.41
11	Cinnamic alcohol	104-54-1	Aldrich	AOO	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
12	Imidazolidinyl urea	39236-46-9	Aldrich	DMF	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
13	Methyl methacrylate	80-62-6	Aldrich	AOO	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
14	Chlorobenzene	108-90-7	Sigma-Aldrich	AOO	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
15	Isopropanol	67-63-0	Sigma-Aldrich	AOO	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
16	Lactic acid	50-21-5	Fluka	DMF	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
17	Methyl salicylate	119-36-8	Sigma-Aldrich	AOO	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
18	Salicylic acid	69-72-7	Sigma	DMF	1.0,2.5,5.0	1.00±0.35	1.76±0.49	2.26±0.33	2.57±0.22	12.27±4.27	2.57

AOO, acetone: olive oil (4:1, v/v); DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulphoxide; DNCB, 2,4-dintrochlorobenzene; HCA, Hexyl cinnamic aldehyde; Test substance treated with L, low; M, middle; H, high concentrations; PC, positive control.

Table A9-14. Predictive capacity was analysed with the same procedure at 3.2.2. and the same result appeared.

No.	Substances	Conc.(%) (2012-2014)	Conc.(%) (2015 new)	Max SI (2012-2014)	Max SI (2015 new)	LLNA Ref.	Decision (2012-2014)	Decision (2015 new)
1	5-Chloro-2-methyl-4-isothiazolin-3-one (CMI)/2-methyl-4-isothiazolin-3-one (MI)	2.5,5,10	2.5,5,10	19.72±4.95	13.55±1.77	+	+	+
2	DNCB	0.125,0.25,0.5	0.125,0.25,0.5	25.28±12.87	47.29±9.45	+	+	+
3	4-Phenylenediamine	0.5,1,2.5	0.5,1,2.5	12.28±5.89	10.02±3.26	+	+	+
4	Cobalt chloride	0.25,0.5,1.0	0.25,0.5,1.0	24.99±2.70	12.83±6.03	+	+	+
5	Isoeugenol	5,10,25	5,10,25	11.16±3.41	34.91±11.73	+	+	+
6	2-Mercaptobenzothiazole	25,50,100	5,10,25	1.43±0.17	1.44±0.58	+	-	-
7	Citral	10,25,50	10,25,50	11.06±1.66	8.88±4.43	+	+	+
8	HCA	5,10,25	5,10,25	9.55±4.76	4.34±0.74	+	+	+
9	Eugenol	5,10,25	5,10,25	21.13±5.31	3.94±0.74	+	+	+
10	Phenyl benzoate	5,10,25	10,25,50	12.50±4.16	15.41±5.82	+	+	+
11	Cinnamic alcohol	25,50,100	10,25,50	4.66±1.05	2.78±0.65	+	+	+
12	Imidazolidinyl urea	10,25,50	10,25,50	3.52±1.09	4.10±1.52	+	+	+
13	Methylmethacrylate	25,50,100	25,50,100	1.63±0.46	0.87±0.34	+	-	-
14	Chlorobenzene	10,25,50	10,25,50	2.55±0.84	1.67±0.23	-	-	-
15	Isopropanol	2.5,5.0,10	25,50,100	1.30±0.58	0.89±0.27	-	-	-
16	Lactic acid	5,10,25	10,25,50	1.51±0.41	1.28±0.50	-	-	-
17	Methyl salicylate	25,50,100	10,25,50	2.66±0.67	1.77±0.33	-	-	-
18	Salicylic acid	1.0,2.5,5.0	1.0,2.5,5.0	2.38±1.39	2.57±0.22	-	-	-

DNCB, 2,4-dintrochlorobenzene; HCA, Hexyl cinnamic aldehyde; Decision, + stands for the positive and – stands for the negative decision.

4. Conclusion

The within- and between laboratory reproducibility of the LLNA: BrdU-FCM method satisfied the acceptance criteria given by OECD guidelines.

And the result of a predictive capacity analysis based on OECD TG 429 was 89% or 91% accuracy for 18 or 22 ref. substances, respectively, with an optimal cut-off SI value of 2.7.

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[Attachment 1]

Raw Data

- Data Used for WLR Evaluation (Protocol 1.0)
- Data Used for BLR Evaluation (Protocol 1.1)
- Data Used for 1st Predictive Capacity Evaluation (Protocol 1.1)
- Data Used for 2nd Predictive Capacity Evaluation (Protocol 1.2)
- Data Used for Additional Test (Protocol 1.3)
- Data Used for 3rd Predictive Capacity Evaluation (Protocol 1.3)
- Data Used for Supplementary Test (4 Optional Chemicals - Protocol 1.3)
- Data Used for Comparison of BALB/c and CBA/J mice
- The mean± SD, CV values and quantiles of the EC2.7 values of the HCA used in the WLR, 1st and 3rd predictive capacity evaluation
- The mean± SD values and quantiles of the vehicle control group and positive control group (25% HCA) used in the 3rd predictive capacity evaluation and supplementary test

Data Used for WLR Evaluation
(Protocol 1.0)

Attachment 1-2

Table 1. Individual Animal Data for the LLNA: BrdU-FCM (Within Laboratory Reproducibility Data - Lead Lab 1.)

Test	Substance	Vehicle	Conc. (%)	Animal No.	LNC ($x10^6$/ml)	BrdU (%)	SI	EC2.7
1st	VC	AOO	0	1	5.30	1.03	0.77	NA
				2	9.20	0.93	1.21	
				3	7.40	1.05	1.10	
				4	6.10	1.07	0.92	
				Mean	**7.00**	**1.02**	**1.00**	
	HCA	AOO	5	1	7.10	1.23	1.23	7.40
				2	11.00	1.09	1.69	
				3	7.50	1.32	1.40	
				4	14.00	1.08	2.14	
				5	15.00	1.20	2.54	
				Mean	**10.92**	**1.18**	**1.80**	
			10	1	9.10	1.24	1.59	
				2	13.00	1.43	2.63	
				3	15.00	1.79	3.79	
				4	24.00	1.42	4.81	
				5	22.00	1.72	5.35	
				Mean	**16.62**	**1.52**	**3.64**	
			25	1	22.00	2.01	6.25	
				2	24.00	2.53	8.58	
				3	26.00	2.76	10.14	
				4	32.00	2.46	11.12	
				5	38.00	2.90	15.57	
				Mean	**28.40**	**2.53**	**10.33**	
2nd	VC	AOO	0	1	6.70	0.56	1.10	NA
				2	4.20	0.68	0.84	
				3	6.50	0.36	0.69	
				4	6.10	0.70	1.26	
				5	5.30	0.71	1.11	
				Mean	**5.76**	**0.60**	**1.00**	
	HCA	AOO	5	1	7.80	1.04	2.39	7.34
				2	10.00	1.06	3.12	
				3	7.80	0.74	1.70	
				4	11.00	0.68	2.20	
				5	8.60	0.74	1.87	
				Mean	**9.04**	**0.85**	**2.26**	
			10	1	7.90	0.58	1.35	
				2	15.00	0.92	4.06	
				3	10.00	0.68	2.00	
				4	11.00	0.98	3.17	
				5	12.00	1.42	5.02	
				Mean	**11.18**	**0.92**	**3.12**	
			25	1	16.00	1.44	6.78	
				2	22.00	1.22	7.90	
				3	20.00	1.90	11.19	
				4	16.00	1.60	7.54	
				5	28.00	1.54	12.70	
				Mean	**20.40**	**1.54**	**9.22**	
3rd	VC	AOO	0	1	5.70	0.80	1.27	NA
				2	5.80	0.44	0.71	
				3	4.10	0.50	0.57	
				4	5.20	0.76	1.10	
				5	7.90	0.61	1.34	
				Mean	**5.74**	**0.62**	**1.00**	

3rd	HCA	AOO	5	1	8.70	0.86	2.09	6.81
				2	8.80	1.10	2.70	
				3	5.00	0.84	1.17	
				4	6.10	0.92	1.56	
				5	5.00	0.74	1.03	
				Mean	**6.72**	**0.89**	**1.71**	
			10	1	11.00	1.37	4.20	
				2	12.00	0.94	3.15	
				3	16.00	1.44	6.42	
				4	16.00	1.23	5.49	
				5	11.00	1.06	3.25	
				Mean	**13.20**	**1.21**	**4.50**	
			25	1	25.00	1.38	9.62	
				2	40.00	2.04	22.75	
				3	28.00	1.30	10.15	
				4	24.00	1.47	9.84	
				5	30.00	2.05	17.15	
				Mean	**29.40**	**1.65**	**13.90**	
4th	VC	AOO	0	1	9.00	0.45	0.95	NA
				2	10.00	0.60	1.40	
				3	6.00	0.45	0.63	
				4	5.40	0.73	0.92	
				5	6.90	0.68	1.10	
				Mean	**7.46**	**0.58**	**1.00**	
	HCA	AOO	5	1	9.80	0.86	1.97	7.38
				2	9.90	0.88	2.04	
				3	8.10	0.97	1.84	
				4	4.50	0.53	0.56	
				5	9.90	1.21	2.80	
				Mean	**8.44**	**0.89**	**1.84**	
			10	1	9.60	1.09	2.45	
				2	11.00	0.21	0.54	
				3	15.00	1.05	3.68	
				4	15.00	1.90	6.66	
				5	16.00	1.29	4.83	
				Mean	**13.32**	**1.11**	**3.63**	
			25	1	24.00	2.21	12.40	
				2	30.00	1.89	13.26	
				3	24.00	1.31	7.35	
				4	22.00	1.57	8.08	
				5	22.00	1.36	7.00	
				Mean	**24.40**	**1.67**	**9.62**	

- Flow cytometer: BD FACS Calibur™
- Setting values of the flow cytometer

Detector	Voltage	Amp Gain	Mode
FSC	E00	5.08	Lin
SSC	474	1.00	Lin
FL1	412	1.00	Log
FL3	650	1.00	Lin

Table 2. Individual Animal Data for the LLNA: BrdU-FCM (Within Laboratory Reproducibility Data - Participating Lab. 1)

Test	Substance	Vehicle	Conc. (%)	Animal No.	LNC ($x10^6$/ml)	BrdU (%)	SI	EC2.7
1st	VC	AOO	0	1	3.10	0.60	1.04	NA
				2	2.50	0.72	1.01	
				3	2.30	0.68	0.87	
				4	2.70	0.64	0.97	
				5	2.70	0.74	1.12	
				Mean	**2.66**	**0.68**	**1.00**	
	HCA	AOO	5	1	5.30	0.72	2.13	5.95
				2	7.20	1.12	4.51	
				3	6.00	0.83	2.78	
				4	4.50	0.72	1.81	
				5	3.40	0.79	1.50	
				Mean	**5.28**	**0.84**	**2.55**	
			10	1	8.90	1.14	5.67	
				2	7.20	0.85	3.42	
				3	4.30	1.22	2.93	
				4	7.70	0.86	3.70	
				Mean	**7.03**	**1.02**	**3.93**	
			25	1	10.10	1.62	9.14	
				2	10.40	2.17	12.61	
				3	9.90	2.02	11.17	
				4	9.20	1.41	7.25	
				5	10.10	1.52	8.58	
				Mean	**9.94**	**1.75**	**9.75**	
2nd	VC	AOO	0	1	6.60	0.68	1.25	NA
				2	4.10	0.44	0.50	
				3	6.40	0.78	1.39	
				4	5.50	0.80	1.23	
				5	4.20	0.53	0.62	
				Mean	**5.36**	**0.65**	**1.00**	
	HCA	AOO	5	1	8.50	0.58	1.38	9.07
				2	5.00	0.73	1.06	
				3	8.90	0.82	2.04	
				4	9.20	0.83	2.13	
				5	11.30	0.78	2.46	
				Mean	**8.58**	**0.75**	**1.81**	
			10	1	9.10	0.81	2.06	
				2	7.30	0.83	1.69	
				3	15.40	1.27	5.46	
				4	14.90	1.11	4.62	
				5	13.50	0.69	2.60	
				Mean	**12.04**	**0.94**	**3.29**	
			25	1	17.40	1.19	5.78	
				2	18.00	1.32	6.63	
				3	15.60	0.93	4.05	
				4	12.50	1.37	4.78	
				5	12.80	0.63	2.25	
				Mean	**15.26**	**1.09**	**4.70**	
3rd	VC	AOO	0	1	7.00	0.72	1.41	NA
				2	3.00	0.68	0.57	
				3	3.80	0.67	0.71	
				4	4.20	0.74	0.87	
				5	6.80	0.75	1.43	
				Mean	**4.96**	**0.71**	**1.00**	

3rd	HCA	AOO	5	1	5.30	0.87	1.29	8.67
				2	6.00	1.13	1.90	
				3	5.50	1.13	1.74	
				4	5.00	1.04	1.46	
				5	6.00	1.18	1.98	
				Mean	**5.56**	**1.07**	**1.68**	
			10	1	10.70	1.52	4.56	
				2	8.40	1.14	2.68	
				3	8.20	1.45	3.33	
				4	8.10	1.43	3.25	
				5	8.80	1.37	3.38	
				Mean	**8.84**	**1.38**	**3.44**	
			25	1	13.20	1.79	6.62	
				2	10.30	1.89	5.46	
				3	8.60	1.52	3.66	
				4	11.80	1.70	5.62	
				5	12.20	1.70	5.81	
				Mean	**11.22**	**1.72**	**5.44**	
4th	VC	AOO	0	1	5.40	0.64	0.98	NA
				2	4.50	0.72	0.92	
				3	5.10	0.78	1.13	
				4	4.40	0.81	1.01	
				5	4.90	0.70	0.97	
				Mean	**4.86**	**0.73**	**1.00**	
	HCA	AOO	5	1	7.40	0.71	1.49	6.95
				2	7.50	0.89	1.89	
				3	6.00	1.10	1.87	
				4	6.20	0.96	1.68	
				5	6.30	1.16	2.07	
				Mean	**6.68**	**0.96**	**1.80**	
			10	1	7.10	1.37	2.75	
				2	11.10	1.33	4.18	
				3	10.90	1.50	4.63	
				4	9.60	1.32	3.59	
				5	10.40	1.67	4.92	
				Mean	**9.82**	**1.44**	**4.01**	
			25	1	11.70	2.02	6.69	
				2	12.00	2.01	6.83	
				3	12.20	2.14	7.39	
				4	11.20	2.01	6.37	
				5	12.00	2.41	8.18	
				Mean	**11.82**	**2.12**	**7.09**	

- Flow cytometer: BD FACS Calibur™
- Setting values of the flow cytometer

Detector	Voltage	Amp Gain	Mode
FSC	E00	2.00	Lin
SSC	341	1.00	Lin
FL1	618	1.00	Log
FL3	804	1.00	Lin

Table 3. Individual Animal Data for the LLNA: BrdU-FCM (Within Laboratory Reproducibility Data - Participating Lab. 2)

Test	Substance	Vehicle	Conc. (%)	Animal No.	LNC ($x10^6$/ml)	BrdU (%)	SI	EC2.7
1st	VC	AOO	0	1	3.60	0.82	0.82	NA
				2	5.70	0.85	1.35	
				3	5.00	0.76	1.06	
				4	5.70	0.55	0.87	
				5	5.30	0.61	0.90	
				Mean	**5.06**	**0.72**	**1.00**	
	HCA	AOO	5	1	7.70	0.61	1.31	10.28
				2	5.80	0.58	0.94	
				3	7.80	0.87	1.89	
				4	8.00	0.67	1.49	
				5	7.70	0.61	1.31	
				Mean	**7.40**	**0.67**	**1.39**	
			10	1	11.00	0.79	2.42	
				2	10.90	0.93	2.82	
				3	8.90	0.61	1.51	
				4	13.10	0.99	3.61	
				5	8.40	0.76	1.78	
				Mean	**10.46**	**0.82**	**2.43**	
			25	1	21.80	1.49	9.04	
				2	16.70	1.07	4.97	
				3	15.70	1.15	5.03	
				4	26.40	1.35	9.92	
				5	14.80	1.24	5.11	
				Mean	**19.08**	**1.26**	**6.81**	
2nd	VC	AOO	0	1	4.50	0.78	0.90	NA
				2	5.70	1.10	1.61	
				3	6.10	0.71	1.12	
				4	3.90	0.54	0.54	
				5	3.90	0.82	0.82	
				Mean	**4.82**	**0.79**	**1.00**	
	HCA	AOO	5	1	6.00	0.83	1.28	6.03
				2	8.80	0.89	2.02	
				3	7.30	0.96	1.80	
				4	7.10	0.99	1.81	
				5	8.80	0.85	1.93	
				Mean	**7.60**	**0.90**	**1.77**	
			10	1	16.20	1.22	5.09	
				2	10.00	1.17	3.01	
				3	16.00	1.39	5.73	
				4	16.00	1.59	6.55	
				5	9.80	1.26	3.18	
				Mean	**13.60**	**1.33**	**4.71**	
			25	1	17.00	1.71	7.49	
				2	10.40	1.60	4.29	
				3	17.00	1.83	8.01	
				4	23.00	2.14	12.68	
				Mean	**16.85**	**1.82**	**8.11**	
3rd	VC	AOO	0	1	4.00	0.82	0.85	NA
				2	3.60	0.79	0.74	
				3	4.30	0.98	1.09	
				4	4.70	0.98	1.17	
				5	4.90	0.89	1.13	
				Mean	**4.30**	**0.89**	**1.00**	

3rd	HCA	AOO	5	1	6.00	0.99	1.54	8.50
				2	5.50	0.91	1.30	
				3	4.10	1.39	1.48	
				4	6.60	0.86	1.47	
				5	4.40	0.75	0.85	
				Mean	**5.32**	**0.98**	**1.33**	
			10	1	12.80	1.35	4.48	
				2	9.60	1.10	2.74	
				3	9.60	1.20	2.98	
				4	11.60	1.32	3.97	
				5	9.80	1.33	3.38	
				Mean	**10.68**	**1.26**	**3.51**	
			25	1	11.20	1.31	3.80	
				2	18.00	1.69	7.88	
				3	16.40	2.09	8.88	
				4	20.30	2.03	10.67	
				5	16.80	1.95	8.48	
				Mean	**16.54**	**1.81**	**7.94**	
4th	VC	AOO	0	1	4.40	0.75	0.69	NA
				2	3.80	1.16	0.93	
				3	4.00	1.13	0.95	
				4	4.60	1.22	1.18	
				5	5.80	1.02	1.25	
				Mean	**4.52**	**1.06**	**1.00**	
	HCA	AOO	5	1	5.10	0.89	0.96	8.34
				2	4.80	1.05	1.06	
				3	7.30	1.23	1.89	
				4	8.90	1.65	3.09	
				5	6.70	1.44	2.03	
				Mean	**6.56**	**1.25**	**1.81**	
			10	1	8.80	1.48	2.74	
				2	9.00	1.44	2.73	
				3	10.00	1.56	3.28	
				4	9.80	1.33	2.74	
				5	11.70	1.58	3.89	
				Mean	**9.86**	**1.48**	**3.08**	
			25	1	12.40	1.05	2.74	
				2	14.30	2.10	6.32	
				3	20.00	1.99	8.38	
				4	20.00	2.24	9.43	
				5	22.00	2.40	11.11	
				Mean	**17.74**	**1.96**	**7.60**	

- Flow cytometer: Beckman coulter Cytomics FC 500
- Setting values of the flow cytometer

Detector	Voltage	Amp Gain	Mode
FSC	203	10.0	Lin
SSC	662	50.0	Lin
FL1	411	1.0	Log
FL3	768	2.0	Lin

Data Used for BLR Evaluation
(Protocol 1.1)

Attachment 1-9

Table 4. Individual Animal Data for the LLNA: BrdU-FCM (Between Laboratory Reproducibility Data - Lead Lab 1.)

Test	Substance	Vehicle	Conc. (%)	Animal No.	LNC ($x10^6$/ml)	BrdU (%)	SI	EC2.7
1st	VC	AOO	0	1	7.50	0.66	1.37	NA
				2	4.60	0.54	0.69	
				3	5.60	0.46	0.71	
				4	5.40	0.52	0.78	
				5	9.00	0.58	1.45	
				Mean	**6.42**	**0.55**	**1.00**	
	DNCB	AOO	0.05	1	7.70	0.58	1.24	0.080
				2	8.70	0.66	1.59	
				3	8.40	0.68	1.58	
				4	5.40	0.66	0.99	
				5	8.90	0.86	2.12	
				Mean	**7.82**	**0.69**	**1.50**	
			0.1	1	14.00	1.04	4.04	
				2	13.00	0.86	3.10	
				3	8.90	0.70	1.73	
				4	14.00	0.86	3.34	
				Mean	**12.48**	**0.87**	**3.05**	
			0.25	1	30.00	1.72	14.30	
				2	32.00	1.22	10.82	
				3	34.00	1.82	17.15	
				4	34.00	1.32	12.44	
				Mean	**32.50**	**1.52**	**13.68**	
	HCA	AOO	25	1	23.00	0.56	3.57	NA
				2	19.00	0.60	3.16	
				3	25.00	1.48	10.26	
				4	27.00	2.04	15.27	
				Mean	**23.50**	**1.17**	**8.06**	
2nd	VC	AOO	0	1	6.40	0.58	0.91	NA
				2	5.30	0.46	0.60	
				3	2.50	0.50	0.31	
				4	8.30	0.78	1.59	
				5	9.80	0.66	1.59	
				Mean	**6.46**	**0.60**	**1.00**	
	DNCB	AOO	0.05	1	6.60	0.72	1.17	0.098
				2	6.70	0.46	0.76	
				3	7.10	0.36	0.63	
				4	4.80	0.58	0.68	
				5	8.40	0.70	1.45	
				Mean	**6.72**	**0.56**	**0.94**	
			0.1	1	8.50	0.73	1.59	
				2	13.00	0.70	2.24	
				3	11.00	0.66	1.78	
				4	14.00	0.68	2.34	
				5	13.00	0.66	2.11	
				Mean	**11.90**	**0.69**	**2.01**	
			0.25	1	25.00	1.62	9.95	
				2	25.00	1.54	9.46	
				3	26.00	1.94	12.40	
				4	30.00	1.36	10.03	
				Mean	**26.50**	**1.62**	**10.46**	
	HCA	AOO	25	1	25.00	1.30	7.99	NA
				2	21.00	0.96	4.96	
				3	26.00	1.38	8.82	
				4	19.00	0.92	4.30	
				Mean	**22.75**	**1.14**	**6.51**	

	VC	AOO	0	1	6.90	0.38	0.67	NA
				2	5.30	0.56	0.76	
				3	5.60	0.44	0.63	
				4	8.40	0.92	1.97	
				5	5.30	0.72	0.97	
				Mean	**6.30**	**0.60**	**1.00**	
3rd	DNCB	AOO	0.05	1	5.90	0.92	1.38	0.063
				2	11.00	0.88	2.47	
				3	12.00	0.54	1.65	
				4	5.50	0.62	0.87	
				5	6.40	0.58	0.95	
				Mean	**8.16**	**0.71**	**1.46**	
			0.1	1	9.30	1.06	2.52	
				2	14.00	1.28	4.57	
				3	15.00	1.64	6.28	
				4	21.00	2.02	10.82	
				5	13.00	1.86	6.17	
				Mean	**14.46**	**1.57**	**6.07**	
			0.25	1	32.00	3.30	26.94	
				2	22.00	3.72	20.88	
				3	34.00	2.68	23.25	
				4	26.00	2.60	17.25	
				5	22.00	1.66	9.32	
				Mean	**27.20**	**2.79**	**19.53**	
	HCA	AOO	25	1	32.00	1.46	11.92	NA
				2	13.00	1.60	5.31	
				3	22.00	1.64	9.21	
				4	34.00	1.34	11.62	
				5	34.00	0.80	6.94	
				Mean	**27.00**	**1.37**	**9.00**	

- Flow cytometer: BD FACS Calibur™
- Setting values of the flow cytometer

Detector	Voltage	Amp Gain	Mode
FSC	E00	5.08	Lin
SSC	474	1.00	Lin
FL1	412	1.00	Log
FL3	650	1.00	Lin

Table 5. Individual Animal Data for the LLNA: BrdU-FCM (Between Laboratory Reproducibility Data - Participating Lab. 1)

Test	Substance	Vehicle	Conc. (%)	Animal No.	LNC ($x10^6$/ml)	BrdU (%)	SI	EC2.7
1st	VC	AOO	0	1	4.10	0.45	0.98	NA
				2	3.00	0.64	1.02	
				3	3.10	0.63	1.04	
				4	2.20	0.51	0.60	
				5	3.70	0.69	1.36	
				Mean	**3.22**	**0.58**	**1.00**	
	DNCB	AOO	0.05	1	3.50	0.54	1.01	0.099
				2	4.00	0.61	1.30	
				3	4.20	0.75	1.68	
				4	2.90	0.65	1.00	
				5	6.00	1.18	3.77	
				Mean	**4.12**	**0.75**	**1.75**	
			0.1	1	4.30	0.59	1.35	
				2	4.40	0.71	1.66	
				3	4.10	0.70	1.53	
				4	5.50	0.62	1.82	
				5	5.10	0.91	2.47	
				Mean	**4.68**	**0.71**	**1.77**	
			0.25	1	7.80	1.71	7.10	
				2	10.50	2.34	13.08	
				3	9.20	1.73	8.47	
				4	7.80	1.44	5.98	
				5	8.40	1.59	7.11	
				Mean	**8.74**	**1.76**	**8.35**	
	HCA	AOO	25	1	6.30	0.90	3.02	NA
				2	7.10	1.33	5.03	
				3	12.00	1.09	6.96	
				4	6.50	1.07	3.70	
				5	9.00	1.38	6.61	
				Mean	**8.18**	**1.15**	**5.06**	
2nd	VC	AOO	0	1	3.50	0.47	0.86	NA
				2	2.90	0.57	0.86	
				3	3.60	0.74	1.39	
				4	3.10	0.53	0.85	
				5	3.40	0.59	1.04	
				Mean	**3.30**	**0.58**	**1.00**	
	DNCB	AOO	0.05	1	4.50	0.37	0.87	0.082
				2	3.60	0.47	0.88	
				3	5.00	0.50	1.30	
				4	4.50	0.56	1.31	
				5	5.00	0.50	1.30	
				Mean	**4.52**	**0.48**	**1.13**	
			0.1	1	8.40	1.01	4.41	
				2	8.90	1.10	5.09	
				3	7.20	0.87	3.26	
				4	6.80	0.88	3.11	
				5	6.00	0.85	2.65	
				Mean	**7.46**	**0.94**	**3.71**	
			0.25	1	13.50	1.56	10.96	
				2	12.90	1.97	13.22	
				3	7.20	1.43	5.36	
				4	11.70	1.72	10.47	
				5	12.00	1.59	9.93	
				Mean	**11.46**	**1.65**	**9.99**	

2nd	HCA	AOO	25	1 2 3 4 5 **Mean**	9.70 9.00 6.60 5.50 6.40 **7.44**	0.89 1.05 1.03 0.96 0.88 **0.96**	4.49 4.92 3.54 2.75 2.93 **3.72**	NA
3rd	VC	AOO	0	1 2 3 4 5 **Mean**	2.90 3.90 2.10 2.90 2.40 **2.84**	0.49 0.47 0.35 0.30 0.42 **0.41**	1.21 1.56 0.63 0.74 0.86 **1.00**	NA
	DNCB	AOO	0.05	1 2 3 4 5 **Mean**	3.90 4.20 5.40 4.90 5.10 **4.70**	0.66 0.57 0.78 0.77 0.67 **0.69**	2.19 2.04 3.59 3.22 2.91 **2.79**	0.063
			0.1	1 2 3 4 5 **Mean**	4.50 7.20 6.00 4.40 6.80 **5.78**	0.81 0.86 0.83 0.90 1.06 **0.89**	3.11 5.28 4.24 3.37 6.14 **4.43**	
			0.25	1 2 3 4 5 **Mean**	12.90 10.10 11.20 12.00 14.00 **12.04**	1.83 2.04 1.77 2.04 2.28 **1.99**	20.12 17.56 16.89 20.86 27.20 **20.53**	
	HCA	AOO	25	1 2 3 4 5 **Mean**	8.30 6.50 14.20 7.20 8.00 **8.84**	0.80 1.19 1.82 1.33 2.11 **1.45**	5.66 6.59 22.02 8.16 14.39 **11.36**	NA

- Flow cytometer: BD FACS Calibur™
- Setting values of the flow cytometer

Detector	Voltage	Amp Gain	Mode
FSC	E00	2.00	Lin
SSC	341	1.00	Lin
FL1	618	1.00	Log
FL3	804	1.00	Lin

Table 6. Individual Animal Data for the LLNA: BrdU-FCM (Between Laboratory Reproducibility Data - Participating Lab. 2)

Test	Substance	Vehicle	Conc. (%)	Animal No.	LNC ($x10^6$/ml)	BrdU (%)	SI	EC2.7
1st	VC	AOO	0	1	6.80	0.69	1.10	NA
				2	4.80	0.76	0.86	
				3	5.40	0.78	0.99	
				4	6.40	0.83	1.25	
				5	4.70	0.72	0.80	
				Mean	**5.62**	**0.76**	**1.00**	
	DNCB	AOO	0.05	1	5.90	0.73	1.01	0.082
				2	5.50	0.90	1.16	
				3	5.70	0.88	1.18	
				4	6.60	0.95	1.48	
				5	8.40	0.75	1.48	
				Mean	**6.42**	**0.84**	**1.26**	
			0.1	1	10.00	1.29	3.04	
				2	8.20	1.24	2.39	
				3	12.20	1.32	3.79	
				4	10.90	0.75	1.92	
				5	6.50	1.37	2.10	
				Mean	**9.56**	**1.19**	**2.65**	
			0.25	1	24.40	2.24	12.86	
				2	30.00	2.44	17.23	
				3	29.80	2.44	17.11	
				4	33.80	3.01	23.94	
				5	19.00	2.18	9.75	
				Mean	**27.40**	**2.46**	**16.18**	
	HCA	AOO	25	1	21.80	1.65	8.46	NA
				2	8.80	1.91	3.96	
				3	42.00	2.46	24.31	
				4	21.20	2.02	10.08	
				5	18.40	1.94	8.40	
				Mean	**22.44**	**2.00**	**11.04**	
2nd	VC	AOO	0	1	3.20	0.82	0.79	NA
				2	4.26	0.73	0.94	
				3	4.20	0.76	0.96	
				4	3.80	0.95	1.09	
				5	4.40	0.93	1.23	
				Mean	**3.97**	**0.84**	**1.00**	
	DNCB	AOO	0.05	1	6.80	1.03	2.11	0.070
				2	6.30	1.09	2.06	
				3	7.40	1.05	2.34	
				4	8.30	0.92	2.30	
				5	6.30	1.01	1.91	
				Mean	**7.02**	**1.02**	**2.14**	
			0.1	1	8.20	0.86	2.12	
				2	10.40	1.17	3.66	
				3	11.00	1.36	4.50	
				4	8.70	1.04	2.72	
				5	10.10	1.64	4.98	
				Mean	**9.68**	**1.21**	**3.60**	
			0.25	1	22.20	2.07	13.82	
				2	17.00	2.42	12.37	
				3	20.60	2.49	15.42	
				4	19.00	2.11	12.06	
				5	21.80	1.89	12.39	
				Mean	**20.12**	**2.20**	**13.21**	

2nd	HCA	AOO	25	1 2 3 4 5 **Mean**	12.00 15.00 12.00 19.60 17.10 **15.14**	1.59 1.75 1.44 2.03 1.65 **1.69**	5.74 7.89 5.20 11.96 8.48 **7.86**	NA
3rd	VC	AOO	0	1 2 3 4 5 **Mean**	3.50 4.50 3.10 3.50 2.60 **3.44**	0.77 0.79 0.87 0.70 0.60 **0.75**	1.05 1.35 1.05 0.95 0.61 **1.00**	NA
	DNCB	AOO	0.05	1 2 3 4 5 **Mean**	6.40 8.60 3.50 6.90 3.90 **5.86**	0.85 0.84 0.85 0.90 0.81 **0.85**	2.11 2.80 1.16 2.41 1.23 **1.94**	0.070
			0.1	1 2 3 4 5 **Mean**	8.40 8.10 6.30 6.50 8.60 **7.58**	1.74 1.29 1.18 1.02 1.38 **1.32**	5.67 4.06 2.89 2.57 4.61 **3.96**	
			0.25	1 2 3 4 5 **Mean**	22.00 18.40 9.70 22.80 14.60 **17.50**	2.59 2.69 2.21 2.05 2.27 **2.36**	22.12 19.22 8.32 18.15 12.87 **16.14**	
	HCA	AOO	25	1 2 3 4 5 **Mean**	13.40 13.20 11.20 13.40 10.60 **12.36**	1.73 1.63 1.66 1.59 1.94 **1.71**	9.00 8.35 7.22 8.27 7.98 **8.17**	NA

- Flow cytometer: Beckman coulter Cytomics FC 500
- Setting values of the flow cytometer

Detector	Voltage	Amp Gain	Mode
FSC	203	10.0	Lin
SSC	662	50.0	Lin
FL1	411	1.0	Log
FL3	768	2.0	Lin

Data Used for 1st Predictive Capacity Evaluation (Protocol 1.1)

Attachment 1-16

Table 7. Individual Animal Data for the LLNA: BrdU-FCM (1st Predictive Capacity Data - Lead Lab. 1)

No.	Substance	Vehicle	Conc. (%)	Animal No.	LNC ($x10^6$/ml)	BrdU (%)	SI	EC2.7
1	VC	DMF	0	1	5.80	0.72	0.69	NA
				2	7.30	0.22	0.27	
				3	9.00	0.96	1.44	
				4	8.00	0.88	1.17	
				5	10.00	0.86	1.43	
				Mean	**8.02**	**0.73**	**1.00**	
	CMI/MI	DMF	2.5	1	20.00	1.98	6.59	<2.5
				2	28.00	2.28	10.62	
				3	30.00	1.36	6.79	
				4	18.00	1.38	4.13	
				5	22.00	2.28	8.34	
				Mean	**23.60**	**1.86**	**7.29**	
			5	1	38.00	4.00	25.28	
				2	36.00	3.42	20.48	
				3	35.00	3.58	20.84	
				4	40.00	3.06	20.36	
				5	24.00	2.92	11.66	
				Mean	**34.60**	**3.40**	**19.72**	
			10	1	40.00	3.02	20.09	
				2	44.00	1.86	13.61	
				3	50.00	1.36	11.31	
				4	34.00	2.06	11.65	
				5	40.00	2.26	15.04	
				Mean	**41.60**	**2.11**	**14.34**	
	PC	AOO	25	1	28.00	2.04	9.50	NA
				2	30.00	2.14	10.68	
				3	30.00	2.48	12.37	
				4	22.00	2.58	9.44	
				5	30.00	2.38	11.88	
				Mean	**28.00**	**2.32**	**10.77**	
2	VC	AOO	0	1	6.20	0.74	1.11	NA
				2	8.10	0.8	1.56	
				3	5.80	0.5	0.70	
				4	7.50	0.46	0.83	
				5	5.70	0.58	0.80	
				Mean	**6.66**	**0.62**	**1.00**	
	DNCB	AOO	0.1	1	16.00	1.18	4.56	0.015
				2	12.00	1.96	5.67	
				3	12.00	1.02	2.95	
				4	17.00	1.3	5.33	
				5	11.00	1.32	3.50	
				Mean	**13.60**	**1.36**	**4.40**	
			0.25	1	27.00	2.22	14.46	
				2	34.00	2.24	18.37	
				3	40.00	2.72	26.25	
				4	36.00	2.10	18.24	
				5	34.00	1.80	14.77	
				Mean	**34.20**	**2.22**	**18.42**	
			0.5	1	64.00	2.52	38.91	
				2	46.00	2.86	31.74	
				3	56.00	1.92	25.94	
				4	40.00	0.46	4.44	
				5	52.00	2.02	25.34	
				Mean	**51.60**	**1.96**	**25.28**	
	PC	AOO	25	1	31.00	0.92	6.88	NA
				2	36.00	2.04	17.72	
				3	34.00	1.30	10.66	
				4	31.00	1.72	12.86	
				5	25.00	2.16	13.03	
				Mean	**31.40**	**1.63**	**12.23**	

	VC	DMSO	0	1	11.00	0.64	1.01	NA
				2	11.00	0.63	0.99	
				3	15.00	0.61	1.31	
				4	9.10	0.57	0.74	
				5	11.00	0.60	0.95	
				Mean	**11.42**	**0.61**	**1.00**	
3	PPD	DMSO	0.5	1	32.00	1.10	5.04	0.068
				2	23.00	1.71	5.63	
				3	22.00	0.79	2.49	
				4	32.00	1.14	5.23	
				5	29.00	0.86	3.57	
				Mean	**27.60**	**1.12**	**4.39**	
			1	1	30.00	1.84	7.91	
				2	30.00	1.37	5.89	
				3	20.00	1.57	4.50	
				4	26.00	1.30	4.84	
				5	32.00	1.92	8.80	
				Mean	**27.60**	**1.60**	**6.39**	
			2.5	1	36.00	2.06	10.62	
				2	30.00	2.16	9.28	
				3	22.00	1.83	5.77	
				4	46.00	3.22	21.22	
				5	34.00	2.98	14.51	
				Mean	**33.60**	**2.45**	**12.28**	
	PC	AOO	25	1	33.00	1.09	10.02	NA
				2	21.00	1.39	8.13	
				3	16.00	1.40	6.24	
				4	20.00	1.29	7.19	
				5	30.00	1.33	11.11	
				Mean	**24.00**	**1.30**	**8.54**	
	VC	DMF	0	1	5.80	0.72	0.69	NA
				2	7.30	0.22	0.27	
				3	9.00	0.96	1.44	
				4	8.00	0.88	1.17	
				5	10.00	0.86	1.43	
				Mean	**8.02**	**0.73**	**1.00**	
4	Cobalt chloride	DMF	0.25	1	24.00	1.50	5.99	<0.25
				2	32.00	2.14	11.39	
				3	33.00	2.18	11.97	
				4	24.00	2.54	10.14	
				5	28.00	2.86	13.32	
				Mean	**28.20**	**2.24**	**10.56**	
			0.5	1	44.00	2.24	16.39	
				2	40.00	2.96	19.69	
				3	44.00	3.68	26.93	
				4	35.00	2.36	13.74	
				5	42.00	2.98	20.82	
				Mean	**41.00**	**2.84**	**19.51**	
			1	1	54.00	3.02	27.12	
				2	46.00	2.94	22.49	
				3	62.00	2.74	28.25	
				4	42.00	3.56	24.87	
				5	40.00	3.34	22.22	
				Mean	**48.80**	**3.12**	**24.99**	
	PC	AOO	25	1	28.00	2.04	9.50	NA
				2	30.00	2.14	10.68	
				3	30.00	2.48	12.37	
				4	22.00	2.58	9.44	
				5	30.00	2.38	11.88	
				Mean	**28.00**	**2.32**	**10.77**	

	VC	AOO	0	1	7.50	0.66	1.37	NA
				2	4.60	0.54	0.69	
				3	5.60	0.46	0.71	
				4	5.40	0.52	0.78	
				5	9.00	0.58	1.45	
				Mean	**6.42**	**0.55**	**1.00**	
5	Isoeugenol	AOO	5	1	3.40	0.76	0.72	7.56
				2	5.20	1.04	1.50	
				3	9.60	0.74	1.97	
				4	11.00	1.00	3.05	
				5	7.10	0.74	1.46	
				Mean	**7.26**	**0.86**	**1.74**	
			10	1	19.00	1.06	5.58	
				2	14.00	0.38	1.47	
				3	9.80	0.72	1.96	
				4	17.00	1.22	5.75	
				5	11.00	0.92	2.81	
				Mean	**14.16**	**0.86**	**3.51**	
			25	1	19.00	2.14	11.27	
				2	24.00	1.66	11.04	
				3	15.00	1.42	5.90	
				4	25.00	1.76	12.20	
				5	19.00	2.92	15.38	
				Mean	**20.40**	**1.98**	**11.16**	
	PC	AOO	25	1	23.00	0.56	3.57	NA
				2	19.00	0.60	3.16	
				3	25.00	1.48	10.26	
				4	27.00	2.04	15.27	
				Mean	**23.50**	**1.17**	**8.06**	
	VC	DMF	0	1	5.80	0.72	0.69	NA
				2	7.30	0.22	0.27	
				3	9.00	0.96	1.44	
				4	8.00	0.88	1.17	
				5	10.00	0.86	1.43	
				Mean	**8.02**	**0.73**	**1.00**	
6	MBT	DMF	50	1	6.90	0.78	0.90	NC
				2	8.10	0.60	0.81	
				3	9.00	0.88	1.32	
				4	10.00	1.10	1.83	
				5	8.70	0.96	1.39	
				Mean	**8.54**	**0.86**	**1.25**	
			75	1	5.40	0.80	0.72	
				2	9.00	0.82	1.23	
				3	6.00	0.74	0.74	
				4	8.30	0.82	1.13	
				5	4.90	1.14	0.93	
				Mean	**6.72**	**0.86**	**0.95**	
			100	1	8.70	1.22	1.77	
				2	9.20	0.90	1.38	
				3	8.70	0.64	0.93	
				4	7.00	0.56	0.65	
				5	8.40	0.90	1.26	
				Mean	**8.40**	**0.84**	**1.20**	
	PC	AOO	25	1	28.00	2.04	9.50	NA
				2	30.00	2.14	10.68	
				3	30.00	2.48	12.37	
				4	22.00	2.58	9.44	
				5	30.00	2.38	11.88	
				Mean	**28.00**	**2.32**	**10.77**	

	VC	AOO	0	1	6.60	0.32	0.57	NA
				2	6.40	0.60	1.04	
				3	6.10	0.50	0.82	
				4	7.40	0.70	1.40	
				5	7.50	0.58	1.17	
				Mean	**6.80**	**0.54**	**1.00**	
7	Citral	AOO	10	1	7.30	0.84	1.65	9.4
				2	7.20	0.74	1.44	
				3	10.00	0.72	1.94	
				4	13.00	1.20	4.21	
				5	14.00	0.90	3.40	
				Mean	**10.30**	**0.88**	**2.53**	
			25	1	17.00	1.42	6.51	
				2	17.00	1.22	5.60	
				3	17.00	1.48	6.79	
				4	20.00	1.40	7.55	
				5	19.00	1.14	5.84	
				Mean	**18.00**	**1.33**	**6.46**	
			50	1	30.00	1.46	11.82	
				2	26.00	1.64	11.50	
				3	26.00	1.36	9.54	
				4	36.00	1.36	13.21	
				5	30.00	1.14	9.23	
				Mean	**29.60**	**1.39**	**11.06**	
	PC	AOO	25	1	11.00	1.02	3.03	NA
				2	19.00	0.12	0.62	
				3	23.00	1.50	9.31	
				4	24.00	1.54	9.97	
				Mean	**19.25**	**1.05**	**5.73**	
8	VC	AOO	0	1	6.20	0.74	1.11	NA
				2	8.10	0.80	1.56	
				3	5.80	0.50	0.70	
				4	7.50	0.46	0.83	
				5	5.70	0.58	0.80	
				Mean	**6.66**	**0.62**	**1.00**	
	HCA	AOO	5	1	9.30	0.44	0.99	8.77
				2	8.70	0.76	1.60	
				3	7.50	0.92	1.66	
				4	5.30	0.54	0.69	
				5	8.80	0.54	1.15	
				Mean	**7.92**	**0.64**	**1.22**	
			10	1	11.00	0.54	1.43	
				2	18.00	1.28	5.56	
				3	13.00	0.82	2.57	
				4	13.00	1.22	3.83	
				5	12.00	0.70	2.03	
				Mean	**13.40**	**0.91**	**3.08**	
			25	1	21.00	2.66	13.48	
				2	22.00	2.06	10.93	
				3	28.00	2.04	13.78	
				4	28.00	0.38	2.57	
				5	19.00	1.52	6.97	
				Mean	**23.60**	**1.73**	**9.55**	
	PC	AOO	25	1	31.00	0.92	6.88	NA
				2	36.00	2.04	17.72	
				3	34.00	1.30	10.66	
				4	31.00	1.72	12.86	
				5	25.00	2.16	13.03	
				Mean	**31.40**	**1.63**	**12.23**	

	VC	AOO	0	1	5.10	0.60	0.87	NA
				2	9.90	outlier	outlier	
				3	8.60	0.74	1.80	
				4	6.90	0.78	1.53	
				5	4.30	0.66	0.80	
				Mean	**6.96**	**0.70**	**1.00**	
9	Eugenol	AOO	5	1	13.00	0.66	2.43	6.02
				2	6.90	1.16	2.27	
				3	8.10	0.56	1.29	
				4	13.00	0.92	3.39	
				5	15.00	0.66	2.81	
				Mean	**11.20**	**0.79**	**2.44**	
			10	1	19.00	1.08	5.82	
				2	16.00	0.96	4.35	
				3	13.00	1.20	4.42	
				4	11.00	1.20	3.74	
				5	25.00	1.34	9.49	
				Mean	**16.80**	**1.16**	**5.56**	
			25	1	31.00	2.32	20.38	
				2	36.00	2.40	24.48	
				3	40.00	2.40	27.20	
				4	23.00	2.02	13.17	
				5	30.00	2.40	20.40	
				Mean	**32.00**	**2.31**	**21.13**	
	PC	AOO	25	1	26.00	1.82	13.41	NA
				2	22.00	0.73	4.55	
				3	27.00	1.46	11.17	
				4	21.00	0.95	5.65	
				5	22.00	1.08	6.73	
				Mean	**23.60**	**1.21**	**8.30**	
10	VC	AOO	0	1	4.20	0.44	0.88	NA
				2	3.10	0.62	0.92	
				3	4.10	0.44	0.86	
				4	7.40	0.52	1.83	
				5	3.60	0.30	0.51	
				Mean	**4.48**	**0.46**	**1.00**	
	Phenyl benzoate	AOO	50	1	8.10	1.14	4.40	<50.0
				2	7.50	0.60	2.14	
				3	9.60	0.84	3.84	
				4	9.80	0.68	3.17	
				5	8.20	1.08	4.22	
				Mean	**8.64**	**0.87**	**3.55**	
			75	1	15.00	1.36	9.71	
				2	7.60	0.64	2.32	
				3	12.00	1.04	5.94	
				4	7.80	0.80	2.97	
				5	9.10	0.96	4.16	
				Mean	**10.30**	**0.96**	**5.02**	
			100	1	7.50	0.82	2.93	
				2	9.10	1.14	4.94	
				3	6.10	0.68	1.97	
				4	10.00	1.26	6.00	
				5	13.00	0.82	5.08	
				Mean	**9.14**	**0.94**	**4.18**	
	PC	AOO	25	1	24.00	1.54	17.60	NA
				2	25.00	0.88	10.47	
				3	21.00	1.10	11.00	
				4	25.00	1.08	12.85	
				5	24.00	0.98	11.20	
				Mean	**23.80**	**1.12**	**12.62**	

	VC	AOO	0	1	6.90	0.38	0.67	NA
				2	5.30	0.56	0.76	
				3	5.60	0.44	0.63	
				4	8.40	0.92	1.97	
				5	5.30	0.72	0.97	
				Mean	**6.30**	**0.60**	**1.00**	
11	Cinnamic alcohol	AOO	50	1	21.00	1.48	7.93	<50.0
				2	17.00	1.66	7.20	
				3	17.00	1.92	8.33	
				4	16.00	1.34	5.47	
				5	18.00	1.60	7.35	
				Mean	**17.80**	**1.60**	**7.25**	
			75	1	17.00	1.74	7.55	
				2	18.00	1.14	5.24	
				3	17.00	1.20	5.20	
				4	18.00	0.84	3.86	
				5	21.00	0.84	4.50	
				Mean	**18.20**	**1.15**	**5.27**	
			100	1	20.00	0.88	4.49	
				2	16.00	0.86	3.51	
				3	21.00	1.18	6.32	
				4	22.00	1.08	6.06	
				Mean	**19.75**	**1.00**	**5.10**	
	PC	AOO	25	1	32.00	1.46	11.92	NA
				2	13.00	1.60	5.31	
				3	22.00	1.64	9.21	
				4	34.00	1.34	11.62	
				5	34.00	0.80	6.94	
				Mean	**27.00**	**1.37**	**9.00**	
12	VC	DMSO	0	1	6.00	0.45	0.75	NA
				2	8.60	0.51	1.22	
				3	11.00	0.69	2.11	
				4	3.40	0.53	0.50	
				5	4.10	0.36	0.41	
				Mean	**6.62**	**0.51**	**1.00**	
	Imidazolidinyl urea	DMSO	5	1	7.70	0.70	1.50	NC
				2	6.50	0.58	1.05	
				3	5.00	0.60	0.84	
				4	5.70	0.62	0.98	
				5	4.90	0.57	0.78	
				Mean	**5.96**	**0.61**	**1.03**	
			10	1	4.30	0.52	0.62	
				2	8.50	0.54	1.28	
				3	4.60	0.67	0.86	
				4	7.50	0.61	1.27	
				5	11.00	0.57	1.75	
				Mean	**7.18**	**0.58**	**1.16**	
			25	1	8.70	0.58	1.41	
				2	12.00	0.60	2.01	
				3	9.70	0.52	1.40	
				4	9.20	0.58	1.49	
				5	7.40	0.59	1.22	
				Mean	**9.40**	**0.57**	**1.50**	
	PC	AOO	25	1	33.00	1.09	10.02	NA
				2	21.00	1.39	8.13	
				3	16.00	1.40	6.24	
				4	20.00	1.29	7.19	
				5	30.00	1.33	11.11	
				Mean	**24.00**	**1.30**	**8.54**	

	VC	AOO	0	1	4.80	0.69	1.45	NA
				2	4.60	0.04	0.08	
				3	9.70	0.50	2.12	
				4	3.60	0.57	0.90	
				5	5.10	0.20	0.45	
				Mean	**5.56**	**0.40**	**1.00**	
13	Methyl methacrylate	AOO	50	1	3.20	0.55	0.77	NC
				2	7.50	0.65	2.13	
				3	4.40	0.54	1.04	
				4	3.90	0.46	0.79	
				5	4.50	0.15	0.30	
				Mean	**4.70**	**0.47**	**1.01**	
			75	1	6.30	0.52	1.43	
				2	4.10	0.67	1.20	
				3	7.70	0.55	1.85	
				4	4.30	0.47	0.89	
				5	4.80	0.40	0.84	
				Mean	**5.44**	**0.52**	**1.24**	
			100	1	5.70	0.49	1.22	
				2	3.40	0.53	0.79	
				3	5.60	0.70	1.72	
				4	5.50	0.59	1.42	
				5	3.40	0.72	1.07	
				Mean	**4.72**	**0.61**	**1.24**	
	PC	AOO	25	1	19.00	0.62	5.16	NA
				2	21.00	1.11	10.21	
				3	27.00	1.60	18.92	
				4	36.00	1.38	21.76	
				5	23.00	1.11	11.18	
				Mean	**25.20**	**1.16**	**13.44**	
	VC	AOO	0	1	6.60	0.32	0.57	NA
				2	6.40	0.60	1.04	
				3	6.10	0.50	0.82	
				4	7.40	0.70	1.40	
				5	7.50	0.58	1.17	
				Mean	**6.80**	**0.54**	**1.00**	
14	Chloro-benzene	AOO	50	1	9.50	0.96	2.46	58.3
				2	12.00	0.84	2.72	
				3	12.00	0.62	2.01	
				4	9.10	0.68	1.67	
				5	15.00	0.78	3.16	
				Mean	**11.52**	**0.78**	**2.40**	
			75	1	17.00	0.90	4.13	
				2	11.00	0.88	2.61	
				3	18.00	0.86	4.18	
				4	13.00	0.80	2.81	
				5	11.00	0.94	2.79	
				Mean	**14.00**	**0.88**	**3.30**	
			100	1	18.00	1.30	6.31	
				2	18.00	1.18	5.73	
				3	12.00	1.24	4.01	
				4	16.00	1.04	4.49	
				5	17.00	1.24	5.69	
				Mean	**16.20**	**1.20**	**5.25**	
	PC	AOO	25	1	11.00	1.02	3.03	NA
				2	19.00	0.12	0.62	
				3	23.00	1.50	9.31	
				4	24.00	1.54	9.97	
				Mean	**19.25**	**1.05**	**5.73**	

	VC	AOO	0	1	5.10	0.60	0.69	NA
				2	9.90	outlier	outlier	
				3	8.60	0.74	1.44	
				4	6.90	0.78	1.22	
				5	4.30	0.66	0.64	
				Mean	**6.96**	**0.70**	**1.00**	
15	Isopropanol	AOO	50	1	6.00	0.66	0.90	NC
				2	6.20	1.24	1.74	
				3	7.50	0.74	1.26	
				4	5.40	0.52	0.64	
				5	3.70	0.60	0.50	
				Mean	**5.76**	**0.75**	**1.01**	
			75	1	5.80	0.40	0.53	
				2	4.70	0.44	0.47	
				3	4.80	0.76	0.83	
				4	4.20	0.72	0.69	
				5	4.70	0.70	0.75	
				Mean	**4.84**	**0.60**	**0.65**	
			100	1	4.30	0.70	0.68	
				2	6.20	0.56	0.79	
				3	3.80	0.56	0.48	
				4	5.90	0.31	0.41	
				5	2.90	0.60	0.39	
				Mean	**4.62**	**0.55**	**0.55**	
	PC	AOO	25	1	26.00	1.82	10.73	NA
				2	22.00	0.73	3.64	
				3	27.00	1.46	8.94	
				4	21.00	0.95	4.52	
				5	22.00	1.08	5.39	
				Mean	**23.60**	**1.21**	**6.64**	
	VC	DMF	0	1	9.40	1.76	2.26	NA
				2	10.00	0.11	0.15	
				3	6.30	0.73	0.63	
				4	8.50	0.80	0.93	
				5	7.20	1.06	1.04	
				Mean	**8.28**	**0.89**	**1.00**	
16	Lactic acid	DMF	25	1	11.00	0.77	1.15	NC
				2	10.00	0.87	1.19	
				3	8.20	0.73	0.82	
				4	13.00	0.26	0.46	
				5	12.00	0.89	1.46	
				Mean	**10.84**	**0.70**	**1.01**	
			50	1	13.00	0.90	1.60	
				2	11.00	0.81	1.21	
				3	12.00	0.85	1.39	
				4	13.00	0.73	1.29	
				5	13.00	0.99	1.75	
				Mean	**12.40**	**0.86**	**1.45**	
			75	1	13.00	0.86	1.52	
				2	17.00	1.06	2.46	
				3	9.70	0.74	0.98	
				4	12.00	1.03	1.69	
				5	14.00	1.22	2.33	
				Mean	**13.14**	**0.98**	**1.79**	
	PC	AOO	25	1	24.00	2.21	7.23	NA
				2	30.00	1.89	7.73	
				3	24.00	1.31	4.29	
				4	22.00	1.57	4.71	
				5	22.00	1.36	4.08	
				Mean	**24.40**	**1.67**	**5.61**	

	VC	AOO	0	1	4.80	0.69	1.45	NA
				2	4.60	0.04	0.08	
				3	9.70	0.50	2.12	
				4	3.60	0.57	0.90	
				5	5.10	0.20	0.45	
				Mean	**5.56**	**0.40**	**1.00**	
17	Methyl salicylate	AOO	25	1	11.00	0.65	3.13	30.9
				2	10.00	0.68	2.98	
				3	7.90	0.91	3.15	
				4	8.90	0.41	1.60	
				5	7.80	0.59	2.02	
				Mean	**9.12**	**0.65**	**2.57**	
			50	1	10.00	0.55	2.41	
				2	14.00	0.50	3.07	
				3	11.00	0.77	3.71	
				4	12.00	0.79	4.15	
				5	12.00	0.59	3.10	
				Mean	**11.80**	**0.64**	**3.29**	
			75	1	11.00	0.73	3.52	
				2	14.00	0.84	5.15	
				3	14.00	0.70	4.29	
				4	13.00	0.97	5.52	
				5	14.00	1.02	6.25	
				Mean	**13.20**	**0.85**	**4.95**	
	PC	AOO	25	1	19.00	0.62	5.16	NA
				2	21.00	1.11	10.21	
				3	27.00	1.60	18.92	
				4	36.00	1.38	21.76	
				5	23.00	1.11	11.18	
				Mean	**25.20**	**1.16**	**13.44**	
	VC	DMF	0	1	6.00	0.45	0.75	NA
				2	8.60	0.51	1.22	
				3	11.00	0.69	2.11	
				4	3.40	0.53	0.50	
				5	4.10	0.36	0.41	
				Mean	**6.62**	**0.51**	**1.00**	
18	Salicylic acid	DMF	1	1	7.90	0.80	1.76	NC
				2	11.00	0.84	2.57	
				3	9.20	0.54	1.38	
				4	9.30	0.52	1.35	
				5	9.30	0.87	2.25	
				Mean	**9.34**	**0.71**	**1.86**	
			2.5	1	18.00	0.86	4.31	
				2	9.30	0.41	1.06	
				3	10.00	0.66	1.84	
				4	12.00	0.69	2.31	
				Mean	**12.33**	**0.66**	**2.38**	
			5	1	13.00	0.72	2.61	
				2	10.00	0.65	1.81	
				3	6.30	0.93	1.63	
				4	10.00	0.58	1.62	
				5	10.00	0.50	1.39	
				Mean	**9.86**	**0.68**	**1.81**	
	PC	AOO	25	1	33.00	1.09	10.02	NA
				2	21.00	1.39	8.13	
				3	16.00	1.40	6.24	
				4	20.00	1.29	7.19	
				5	30.00	1.33	11.11	
				Mean	**24.00**	**1.30**	**8.54**	

- Flow cytometer: BD FACS Calibur™
- Setting values of the flow cytometer

Detector	Voltage	Amp Gain	Mode
FSC	E00	5.08	Lin
SSC	474	1.00	Lin
FL1	412	1.00	Log
FL3	650	1.00	Lin

Data Used for 2nd Predictive Capacity Evaluation (Protocol 1.2)

Table 8. Individual Animal Data for the LLNA: BrdU-FCM (2nd Predictive Capacity Data - Lead Lab. 2)

No.	Substance	Vehicle	Conc. (%)	Animal No.	LNC ($x10^6$/ml)	BrdU (%)	SI	EC2.7
1	VC	AOO	0	1	4.85	0.88	1.38	NA
				2	4.00	0.64	0.83	
				3	2.45	0.64	0.51	
				4	4.55	0.62	0.91	
				5	4.55	0.94	1.38	
				Mean	**4.08**	**0.74**	**1.00**	
	Imidazolidinyl urea	AOO	10	1	2.30	0.59	0.44	NC
				2	5.80	0.51	0.95	
				3	3.55	0.52	0.60	
				4	3.75	0.49	0.59	
				5	2.45	0.60	0.47	
				Mean	**3.57**	**0.54**	**0.61**	
			25	1	5.20	0.51	0.86	
				2	4.00	0.58	0.75	
				3	6.40	0.63	1.30	
				4	1.65	0.40	0.21	
				5	5.05	0.91	1.48	
				Mean	**4.46**	**0.61**	**0.92**	
			50	1	8.25	0.83	2.21	
				2	6.35	0.66	1.35	
				3	7.20	0.86	2.00	
				4	8.50	0.95	2.61	
				5	7.25	0.88	2.06	
				Mean	**7.51**	**0.84**	**2.05**	
	PC	AOO	25	1	16.05	1.47	7.61	NA
				2	14.30	1.54	7.11	
				3	12.00	1.31	5.07	
				4	12.90	1.56	6.49	
				5	8.70	1.77	4.97	
				Mean	**12.79**	**1.53**	**6.25**	
2	VC	DMF	0	1	2.80	0.41	0.42	NA
				2	5.70	0.57	1.19	
				3	4.70	0.44	0.76	
				4	5.20	0.73	1.39	
				5	7.00	0.49	1.25	
				Mean	**5.08**	**0.53**	**1.00**	
	Lactic acid	DMF	10	1	5.55	0.59	1.20	NC
				2	6.05	0.86	1.90	
				3	5.65	0.83	1.71	
				4	6.90	0.67	1.69	
				5	6.35	0.79	1.83	
				Mean	**6.10**	**0.75**	**1.67**	
			25	1	4.70	0.78	1.34	
				2	3.95	0.82	1.18	
				3	3.80	0.61	0.85	
				4	6.05	0.91	2.01	
				5	4.30	1.01	1.59	
				Mean	**4.56**	**0.83**	**1.39**	
			50	1	8.25	0.76	2.29	
				2	6.75	0.72	1.77	
				3	4.95	0.78	1.41	
				4	5.55	0.64	1.30	
				5	5.90	1.11	2.39	
				Mean	**6.28**	**0.80**	**1.83**	
	PC	AOO	25	1	10.15	1.23	4.56	NA
				2	9.75	1.16	4.13	
				3	7.00	1.30	3.32	
				4	14.25	1.62	8.43	
				5	13.90	1.85	9.39	
				Mean	**11.01**	**1.43**	**5.97**	

	VC	AOO	0	1	3.50	0.73	1.10	NA
				2	3.55	0.63	0.96	
				3	3.10	0.72	0.96	
				4	2.55	0.48	0.53	
				5	4.10	0.83	1.46	
				Mean	**3.36**	**0.68**	**1.00**	
3	EGD	AOO	25	1	7.65	0.90	2.95	49.7
				2	4.95	0.55	1.17	
				3	6.55	0.77	2.16	
				4	4.95	0.86	1.83	
				5	4.30	1.07	1.97	
				Mean	**5.68**	**0.83**	**2.02**	
			50	1	4.25	0.77	1.40	
				2	9.70	0.90	3.75	
				3	5.80	0.86	2.14	
				4	8.30	1.02	3.63	
				5	9.35	0.70	2.81	
				Mean	**7.48**	**0.85**	**2.75**	
			100	1	9.60	1.29	5.31	
				2	11.15	0.86	4.12	
				3	10.30	1.17	5.17	
				4	10.10	0.46	1.99	
				5	7.00	1.24	3.73	
				Mean	**9.63**	**1.00**	**4.06**	
	PC	AOO	25	1	7.70	0.93	3.07	NA
				2	15.80	1.20	8.14	
				3	13.10	1.34	7.53	
				4	13.90	1.16	6.92	
				5	9.35	1.27	5.10	
				Mean	**11.97**	**1.18**	**6.15**	
	VC	DMSO	0	1	6.55	0.58	1.14	NA
				2	5.55	0.70	1.16	
				3	5.25	0.54	0.85	
				4	4.10	0.79	0.97	
				5	4.95	0.59	0.88	
				Mean	**5.28**	**0.64**	**1.00**	
4	Nickel chloride	DMSO	0.001	1	5.20	0.67	1.04	NC
				2	4.50	0.61	0.82	
				3	5.50	0.59	0.97	
				4	4.35	0.92	1.20	
				5	6.55	0.77	1.51	
				Mean	**5.22**	**0.71**	**1.11**	
			0.0025	1	4.15	0.69	0.86	
				2	3.55	0.93	0.99	
				3	6.05	0.72	1.31	
				4	6.45	0.80	1.55	
				5	6.20	0.67	1.25	
				Mean	**5.28**	**0.76**	**1.19**	
			0.005	1	5.15	0.57	0.88	
				2	6.00	0.69	1.24	
				3	6.55	0.71	1.39	
				4	3.80	0.72	0.82	
				5	6.85	0.79	1.62	
				Mean	**5.67**	**0.70**	**1.19**	
	PC	AOO	25	1	12.15	1.50	5.46	NA
				2	11.85	1.15	4.09	
				3	15.45	1.45	6.72	
				4	10.40	1.36	4.24	
				5	15.35	1.38	6.35	
				Mean	**13.04**	**1.37**	**5.37**	

	VC	AOO	0	1	3.95	0.48	0.94	NA
				2	2.75	0.66	0.90	
				3	3.40	0.69	1.16	
				4	3.30	0.57	0.93	
				5	3.70	0.58	1.06	
				Mean	**3.42**	**0.60**	**1.00**	
5	MBT	AOO	2.5	1	3.50	0.67	1.16	NC
				2	4.75	0.52	1.22	
				3	3.20	0.55	0.87	
				4	4.35	0.59	1.27	
				5	3.85	0.44	0.84	
				Mean	**3.93**	**0.55**	**1.07**	
			5	1	3.65	0.52	0.94	
				2	3.00	0.52	0.77	
				3	4.40	0.72	1.57	
				4	4.80	0.68	1.62	
				5	3.60	0.64	1.14	
				Mean	**3.89**	**0.62**	**1.21**	
			10	1	3.15	0.49	0.77	
				2	3.20	0.57	0.90	
				3	3.60	0.47	0.84	
				4	4.20	0.68	1.42	
				5	4.10	0.70	1.42	
				Mean	**3.65**	**0.58**	**1.07**	
	PC	AOO	25	1	5.95	0.74	2.18	NA
				2	6.80	1.01	3.41	
				3	4.35	0.88	1.90	
				4	5.45	0.99	2.68	
				5	10.75	1.32	7.04	
				Mean	**6.66**	**0.99**	**3.44**	
	VC	DMF	0	1	3.55	0.47	0.64	NA
				2	3.40	0.78	1.01	
				3	5.70	0.92	2.00	
				4	2.60	0.75	0.74	
				5	4.25	0.38	0.62	
				Mean	**3.90**	**0.66**	**1.00**	
6	MBT	DMF	25	1	5.25	0.64	1.28	NC
				2	4.60	0.72	1.26	
				3	5.90	0.74	1.66	
				4	5.00	0.75	1.43	
				5	6.70	0.59	1.51	
				Mean	**5.49**	**0.69**	**1.43**	
			50	1	7.70	0.65	1.91	
				2	4.60	0.41	0.72	
				3	3.85	0.50	0.73	
				4	3.40	0.63	0.82	
				5	6.00	0.58	1.33	
				Mean	**5.11**	**0.55**	**1.10**	
			100	1	2.65	0.47	0.47	
				2	3.05	0.61	0.71	
				3	4.00	0.75	1.14	
				4	4.75	0.75	1.36	
				5	2.00	0.59	0.45	
				Mean	**3.29**	**0.63**	**0.83**	
	PC	AOO	25	**1**	**6.20**	**0.85**	**2.01**	NA
				2	**12.45**	**1.36**	**6.45**	
				3	**10.65**	**0.63**	**2.56**	
				4	**11.65**	**1.19**	**5.28**	
				5	**10.80**	**1.33**	**5.47**	
				Mean	**10.35**	**1.07**	**4.35**	

- Flow cytometer: BD FACS Calibur™
- Setting values of the flow cytometer

Detector	Voltage	Amp Gain	Mode
FSC	E00	2.14	Lin
SSC	393	1.34	Lin
FL1	660	1.00	Log
FL3	860	1.00	Lin

Table 9. Individual Animal Data for the LLNA: BrdU-FCM (2nd Predictive Capacity Data - Participating Lab. 1)

No.	Substance	Vehicle	Conc. (%)	Animal No.	LNC ($x10^6$/ml)	BrdU (%)	SI	EC2.7
1	VC	DMF	0	1	1.73	0.08	0.21	NA
				2	3.38	0.26	1.35	
				3	2.25	0.27	0.93	
				4	2.15	0.40	1.32	
				5	2.10	0.37	1.19	
				Mean	**2.32**	**0.28**	**1.00**	
	Phenyl benzoate	DMF	5	1	6.45	0.68	6.73	<5.0
				2	3.60	0.60	3.31	
				3	4.33	0.70	4.64	
				4	4.28	0.68	4.46	
				5	2.58	0.39	1.54	
				Mean	**4.25**	**0.61**	**4.14**	
			10	1	7.05	1.33	14.38	
				2	5.95	0.98	8.94	
				3	10.88	1.13	18.85	
				4	5.68	1.06	9.23	
				5	6.30	1.15	11.11	
				Mean	**7.17**	**1.13**	**12.50**	
			25	1	5.60	0.84	7.21	
				2	6.43	1.09	10.74	
				3	6.25	1.00	9.59	
				4	5.93	0.89	8.09	
				5	3.83	0.60	3.52	
				Mean	**5.61**	**0.88**	**7.83**	
	PC	AOO	25	1	7.75	1.52	18.07	NA
				2	10.05	1.43	22.04	
				3	6.95	1.05	11.19	
				4	11.50	1.87	32.98	
				Mean	**9.06**	**1.47**	**21.07**	
2	VC	AOO	0	1	2.40	0.38	0.93	NA
				2	2.53	0.34	0.88	
				3	2.93	0.66	1.97	
				4	1.70	0.44	0.76	
				5	2.18	0.20	0.45	
				Mean	**2.35**	**0.40**	**1.00**	
	Cinnamic alcohol	AOO	25	1	3.50	0.63	2.26	10.9
				2	4.35	0.78	3.47	
				3	3.55	0.40	1.45	
				4	4.58	0.58	2.71	
				5	4.40	0.33	1.48	
				Mean	**4.08**	**0.54**	**2.28**	
			50	1	8.10	0.57	4.72	
				2	7.08	0.82	5.91	
				3	5.05	0.58	3.00	
				4	9.15	0.52	4.89	
				5	5.90	0.79	4.77	
				Mean	**7.06**	**0.66**	**4.66**	
			100	1	7.55	0.59	4.53	
				2	3.20	0.52	1.70	
				3	15.15	0.43	6.66	
				4	5.05	0.58	2.99	
				5	6.30	0.86	5.54	
				Mean	**7.45**	**0.60**	**4.28**	
	PC	AOO	25	1	8.08	1.61	13.29	NA
				2	10.10	1.96	20.24	
				3	9.95	1.47	14.95	
				4	12.50	1.73	22.11	
				5	7.83	1.07	8.56	
				Mean	**9.69**	**1.57**	**15.83**	

	VC	AOO	0	1	2.40	0.38	0.93	NA
				2	2.53	0.34	0.88	
				3	2.93	0.66	1.97	
				4	1.70	0.44	0.76	
				5	2.18	0.20	0.45	
				Mean	**2.35**	**0.40**	**1.00**	
3	Methyl methacrylate	AOO	25	1	4.45	0.39	1.77	NC
				2	2.73	0.49	1.36	
				3	4.30	0.35	1.55	
				4	2.78	0.40	1.14	
				5	4.08	0.56	2.33	
				Mean	**3.67**	**0.44**	**1.63**	
			50	1	2.88	0.41	1.21	
				2	2.33	0.47	1.11	
				3	2.55	0.28	0.73	
				4	2.20	0.31	0.70	
				5	2.55	0.34	0.89	
				Mean	**2.50**	**0.36**	**0.93**	
			100	1	1.43	0.48	0.70	
				2	4.03	0.31	1.28	
				3	3.30	0.23	0.78	
				4	2.30	0.43	1.01	
				5	3.05	0.18	0.56	
				Mean	**2.82**	**0.33**	**0.86**	
	PC	AOO	25	1	8.08	1.61	13.29	NA
				2	10.10	1.96	20.24	
				3	9.95	1.47	14.95	
				4	12.50	1.73	22.11	
				5	7.83	1.07	8.56	
				Mean	**9.69**	**1.57**	**15.83**	
	VC	AOO	0	1	1.55	0.47	0.90	NA
				2	1.73	0.44	0.93	
				3	2.10	0.45	1.16	
				4	1.45	0.46	0.82	
				5	1.90	0.51	1.19	
				Mean	**1.75**	**0.47**	**1.00**	
4	Chloro-benzene	AOO	10	1	2.75	0.33	1.12	NC
				2	1.80	0.18	0.40	
				3	1.43	0.40	0.70	
				4	1.85	0.20	0.45	
				5	1.83	0.28	0.63	
				Mean	**1.93**	**0.28**	**0.66**	
			25	1	2.33	0.60	1.71	
				2	1.78	0.31	0.68	
				3	2.73	0.58	1.94	
				4	2.15	0.43	1.14	
				5	2.25	0.32	0.88	
				Mean	**2.25**	**0.45**	**1.27**	
			50	1	4.10	0.57	2.87	
				2	4.00	0.67	3.29	
				3	2.83	0.43	1.49	
				4	3.35	0.44	1.81	
				5	3.20	0.83	3.26	
				Mean	**3.50**	**0.59**	**2.55**	
	PC	AOO	25	1	11.50	2.10	29.68	NA
				2	13.50	2.11	35.01	
				3	9.15	1.75	19.68	
				4	13.25	2.09	34.03	
				5	7.95	1.49	14.56	
				Mean	**11.07**	**1.91**	**26.59**	

	VC	AOO	0	1	1.55	0.47	0.90	NA
				2	1.73	0.44	0.93	
				3	2.10	0.45	1.16	
				4	1.45	0.46	0.82	
				5	1.90	0.51	1.19	
				Mean	**1.75**	**0.47**	**1.00**	
5	Isopropanol	AOO	2.5	1	2.05	0.61	1.54	NC
				2	2.35	0.32	0.92	
				3	2.13	0.34	0.89	
				4	2.90	0.39	1.39	
				5	2.33	0.45	1.29	
				Mean	**2.35**	**0.42**	**1.20**	
			5	1	2.65	0.66	2.15	
				2	1.68	0.23	0.47	
				3	1.53	0.24	0.45	
				4	2.33	0.33	0.94	
				5	1.55	0.28	0.53	
				Mean	**1.95**	**0.35**	**0.91**	
			10	1	1.78	0.68	1.48	
				2	1.90	0.68	1.59	
				3	1.28	0.36	0.56	
				4	3.20	0.51	2.01	
				5	1.90	0.37	0.86	
				Mean	**2.01**	**0.52**	**1.30**	
	PC	AOO	25	1	11.50	2.10	29.68	NA
				2	13.50	2.11	35.01	
				3	9.15	1.75	19.68	
				4	13.25	2.09	34.03	
				5	7.95	1.49	14.56	
				Mean	**11.07**	**1.91**	**26.59**	
	VC	AOO	0	1	2.50	0.56	1.18	NA
				2	2.38	0.54	1.08	
				3	2.10	0.64	1.13	
				4	2.23	0.48	0.90	
				5	2.10	0.41	0.72	
				Mean	**2.26**	**0.53**	**1.00**	
6	MBT	AOO	1	1	2.25	0.68	1.28	NC
				2	2.33	0.50	0.98	
				3	3.03	0.47	1.19	
				4	3.73	0.56	1.75	
				5	2.33	0.42	0.82	
				Mean	**2.73**	**0.53**	**1.21**	
			5	1	4.25	0.57	2.03	
				2	2.95	0.44	1.09	
				3	1.68	0.56	0.79	
				4	3.28	0.61	1.68	
				5	3.18	0.60	1.60	
				Mean	**3.07**	**0.56**	**1.44**	
			10	1	3.23	0.63	1.71	
				2	4.10	0.95	3.27	
				3	3.45	0.65	1.88	
				4	3.30	0.70	1.94	
				5	5.15	0.79	3.42	
				Mean	**3.85**	**0.74**	**2.44**	
	PC	AOO	25	1	8.83	1.33	9.85	NA
				2	9.50	1.82	14.52	
				3	8.63	2.07	14.99	
				4	12.15	1.69	17.24	
				5	7.40	1.49	9.26	
				Mean	**9.30**	**1.68**	**13.17**	

	VC	DMF	0	1 2 3 4 **Mean**	3.48 3.38 3.15 3.35 **3.34**	0.67 0.79 0.62 0.77 **0.71**	0.98 1.12 0.82 1.08 **1.00**	NA
7	MBT	DMF	1	1 2 3 4 5 **Mean**	2.88 4.18 1.85 4.40 4.48 **3.56**	0.31 0.46 0.35 0.51 0.59 **0.44**	0.37 0.81 0.27 0.94 1.11 **0.70**	NC
			5	1 2 3 4 5 **Mean**	3.28 3.63 4.45 5.30 4.98 **4.33**	0.42 0.47 0.61 0.66 0.54 **0.54**	0.58 0.72 1.14 1.47 1.13 **1.01**	
			10	1 2 3 4 5 **Mean**	7.50 2.60 8.65 3.95 4.48 **5.44**	0.61 0.46 0.88 0.42 0.72 **0.62**	1.92 0.50 3.20 0.70 1.35 **1.53**	
	PC	AOO	25	1 2 3 4 5 **Mean**	8.83 9.50 8.63 12.15 7.40 **9.30**	1.33 1.82 2.07 1.69 1.49 **1.68**	4.93 7.26 7.50 8.62 4.63 **6.59**	NA

- Flow cytometer: BD FACS Calibur™
- Setting values of the flow cytometer

Detector	Voltage	Amp Gain	Mode
FSC	E00	2.14	Lin
SSC	341	1.00	Lin
FL1	618	1.00	Log
FL3	804	1.00	Lin

Table 10. Individual Animal Data for the LLNA: BrdU-FCM (2nd Predictive Capacity Data - Participating Lab. 2)

No.	Substance	Vehicle	Conc. (%)	Animal No.	LNC ($x10^6$/ml)	BrdU (%)	SI	EC2.7
1	VC	DMF	0	1	9.40	0.52	0.81	NA
				2	9.80	0.58	0.94	
				3	11.20	0.74	1.37	
				4	8.93	0.52	0.77	
				5	10.40	0.65	1.12	
				Mean	**9.95**	**0.60**	**1.00**	
	Imidazolidinyl urea	DMF	10	1	9.40	0.57	0.89	NC
				2	10.20	0.48	0.81	
				3	11.80	0.66	1.29	
				4	10.20	0.49	0.83	
				5	10.60	0.54	0.95	
				Mean	**10.44**	**0.55**	**0.95**	
			25	1	11.93	0.53	1.04	
				2	14.27	0.53	1.25	
				3	11.40	0.68	1.28	
				4	14.87	0.24	0.59	
				5	10.80	0.61	1.09	
				Mean	**12.65**	**0.52**	**1.05**	
			50	1	13.67	0.93	2.10	
				2	16.20	0.47	1.26	
				3	14.40	0.46	1.09	
				4	13.27	0.66	1.45	
				5	16.60	0.64	1.76	
				Mean	**14.83**	**0.63**	**1.53**	
	PC	AOO	25	1	18.20	1.01	3.04	NA
				2	52.40	1.43	12.38	
				3	21.40	1.15	4.07	
				4	19.80	1.17	3.83	
				5	48.00	1.55	12.29	
				Mean	**31.96**	**1.26**	**7.12**	
2	VC	DMF	0	1	9.40	0.52	0.81	NA
				2	9.80	0.58	0.94	
				3	11.20	0.74	1.37	
				4	8.93	0.52	0.77	
				5	10.40	0.65	1.12	
				Mean	**9.95**	**0.60**	**1.00**	
	Lactic acid	DMF	5	1	14.20	1.16	2.72	NC
				2	13.00	0.67	1.44	
				3	10.47	0.59	1.02	
				4	11.80	0.58	1.13	
				5	11.80	0.57	1.11	
				Mean	**12.25**	**0.71**	**1.48**	
			10	1	12.27	0.66	1.34	
				2	14.20	0.75	1.76	
				3	16.20	0.65	1.74	
				4	12.73	0.65	1.37	
				5	10.40	0.65	1.12	
				Mean	**13.16**	**0.67**	**1.46**	
			25	1	14.00	0.87	2.01	
				2	15.07	0.67	1.67	
				3	14.40	0.71	1.69	
				4	14.60	0.44	1.06	
				5	11.00	0.61	1.11	
				Mean	**13.81**	**0.66**	**1.51**	
	PC	AOO	25	1	18.20	1.01	3.04	NA
				2	52.40	1.43	12.38	
				3	21.40	1.15	4.07	
				4	19.80	1.17	3.83	
				5	48.00	1.55	12.29	
				Mean	**31.96**	**1.26**	**7.12**	

	VC	AOO	0	1	11.80	0.41	0.77	NA
				2	13.20	0.57	1.20	
				3	12.80	0.44	0.90	
				4	10.60	0.73	1.24	
				5	11.20	0.49	0.88	
				Mean	**11.92**	**0.53**	**1.00**	
3	Methyl salicylate	AOO	25	1	17.60	0.59	1.66	NC
				2	15.20	0.86	2.09	
				3	17.40	0.83	2.31	
				4	17.00	0.67	1.82	
				5	13.20	0.79	1.67	
				Mean	**16.08**	**0.75**	**1.91**	
			50	1	22.20	0.78	2.77	
				2	22.60	0.82	2.97	
				3	15.80	0.61	1.54	
				4	18.40	0.91	2.68	
				5	20.60	1.01	3.33	
				Mean	**19.92**	**0.83**	**2.66**	
			100	1	20.20	0.76	2.46	
				2	13.20	0.72	1.52	
				3	13.40	0.78	1.67	
				4	10.40	0.64	1.07	
				5	16.00	1.11	2.84	
				Mean	**14.64**	**0.80**	**1.91**	
	PC	AOO	25	1	21.40	1.23	4.22	NA
				2	22.00	1.16	4.09	
				3	20.20	1.30	4.21	
				4	23.80	1.62	6.17	
				5	19.60	1.85	5.81	
				Mean	**21.40**	**1.43**	**4.90**	
	VC	DMSO	0	1	6.60	1.58	0.71	NA
				2	11.40	1.08	0.84	
				3	8.80	1.76	1.06	
				4	12.20	1.81	1.51	
				5	8.00	1.61	0.88	
				Mean	**9.40**	**1.57**	**1.00**	
4	SLS	DMSO	5	1	12.20	3.01	2.51	<5.0
				2	30.00	2.98	6.11	
				3	31.80	3.72	8.08	
				4	23.60	2.27	3.66	
				5	21.40	2.33	3.41	
				Mean	**23.80**	**2.86**	**4.75**	
			10	1	21.80	3.48	5.18	
				2	36.00	2.93	7.21	
				3	30.40	2.96	6.15	
				4	22.00	2.94	4.42	
				5	38.00	3.62	9.40	
				Mean	**29.64**	**3.19**	**6.47**	
			25	1	22.00	2.75	4.13	
				2	24.00	2.37	3.89	
				3	29.40	2.39	4.80	
				4	28.00	3.15	6.03	
				5	25.80	2.68	4.72	
				Mean	**25.84**	**2.67**	**4.71**	
	PC	AOO	25	1	23.00	2.34	3.68	NA
				2	21.40	2.15	3.14	
				3	24.80	2.35	3.98	
				4	23.60	2.45	3.95	
				5	21.20	2.51	3.64	
				Mean	**22.80**	**2.36**	**3.68**	

	VC	AOO	0	1	11.80	0.72	1.11	NA
				2	13.20	0.81	1.40	
				3	12.80	0.57	0.96	
				4	10.60	0.48	0.67	
				5	11.20	0.59	0.87	
				Mean	**11.92**	**0.63**	**1.00**	
5	Xylene	AOO	25	1	13.80	0.73	1.32	58.5
				2	12.60	0.81	1.34	
				3	13.40	0.87	1.53	
				4	14.00	1.02	1.87	
				5	11.80	0.78	1.21	
				Mean	**13.12**	**0.84**	**1.45**	
			50	1	18.80	0.81	1.99	
				2	16.20	1.26	2.67	
				3	13.00	1.31	2.23	
				4	17.20	1.20	2.70	
				5	17.40	1.14	2.60	
				Mean	**16.52**	**1.14**	**2.44**	
			100	1	20.60	1.21	3.26	
				2	25.20	1.54	5.08	
				3	18.80	1.65	4.06	
				4	19.60	1.94	4.98	
				5	20.60	1.32	3.56	
				Mean	**20.96**	**1.53**	**4.19**	
	PC	AOO	25	1	21.40	1.13	3.17	NA
				2	22.00	1.66	4.78	
				3	20.20	1.66	4.39	
				4	23.80	1.33	4.15	
				5	19.60	1.62	4.16	
				Mean	**21.40**	**1.48**	**4.13**	
	VC	AOO	0	1	9.60	1.03	1.10	NA
				2	10.20	0.95	1.07	
				3	8.60	1.26	1.20	
				4	9.40	0.89	0.93	
				5	7.80	0.81	0.70	
				Mean	**9.12**	**0.99**	**1.00**	
6	MBT	AOO	1	1	11.20	0.96	1.19	NC
				2	10.60	1.13	1.33	
				3	10.20	0.73	0.83	
				4	9.60	1.20	1.28	
				5	9.40	0.86	0.90	
				Mean	**10.20**	**0.98**	**1.10**	
			5	1	11.80	0.66	0.86	
				2	15.00	0.90	1.50	
				3	12.80	1.18	1.67	
				4	13.80	1.07	1.64	
				5	11.80	0.80	1.05	
				Mean	**13.04**	**0.92**	**1.34**	
			10	1	12.20	1.16	1.57	
				2	14.00	0.94	1.46	
				3	11.20	0.70	0.87	
				4	17.80	1.12	2.21	
				5	14.60	0.92	1.49	
				Mean	**13.96**	**0.97**	**1.52**	
	PC	AOO	25	1	23.60	1.57	4.11	NA
				2	16.00	0.95	1.69	
				3	25.60	1.30	3.69	
				4	20.40	1.08	2.44	
				5	24.20	1.73	4.64	
				Mean	**21.96**	**1.33**	**3.31**	

7	VC	DMF	0	1	10.20	1.08	1.57	NA
				2	6.40	0.69	0.63	
				3	8.20	0.61	0.71	
				4	9.20	0.88	1.15	
				5	8.40	0.79	0.94	
				Mean	**8.48**	**0.81**	**1.00**	
	MBT	DMF	1	1	8.80	0.80	1.00	NC
				2	9.20	0.65	0.85	
				3	11.40	0.28	0.45	
				4	11.00	0.81	1.27	
				5	10.60	0.65	0.98	
				Mean	**10.20**	**0.64**	**0.91**	
			5	1	12.20	0.72	1.25	
				2	12.20	0.97	1.68	
				3	15.80	0.74	1.66	
				4	11.20	0.99	1.58	
				5	8.00	1.21	1.38	
				Mean	**11.88**	**0.93**	**1.51**	
			10	1	12.20	1.69	2.93	
				2	10.60	1.09	1.64	
				3	12.80	1.14	2.07	
				4	13.40	0.85	1.62	
				5	14.20	0.92	1.86	
				Mean	**12.64**	**1.14**	**2.03**	
	PC	AOO	25	1	23.60	1.57	5.27	NA
				2	16.00	0.95	2.16	
				3	25.60	1.30	4.73	
				4	20.40	1.08	3.13	
				5	24.20	1.73	5.95	
				Mean	**21.96**	**1.33**	**4.25**	

- Flow cytometer: Beckman coulter Cytomics FC 500
- Setting values of the flow cytometer

Detector	Voltage	Amp Gain	Mode
FSC	203	10.0	Lin
SSC	662	50.0	Lin
FL1	411	1.0	Log
FL3	768	2.0	Lin

Data Used for Additional Test
(Protocol 1.3)

Table 11. Individual Animal Data for the LLNA: BrdU-FCM (additional test (imidazolidinyl urea)- Lead Lab. 2)

No.	Substance	Vehicle	Conc. (%)	Animal No.	LNC ($x10^6$/ml)	BrdU (%)	SI	EC2.7
1	VC	DMF	0	1	3.10	0.51	0.92	NA
				2	4.20	0.49	1.20	
				3	3.03	0.15	0.26	
				4	4.98	0.38	1.10	
				5	4.33	0.60	1.51	
				Mean	**3.93**	**0.43**	**1.00**	
	Imidazolidinyl urea	DMF	10	1	6.03	0.49	1.72	26.79
				2	6.53	0.63	2.40	
				3	4.33	0.38	0.96	
				4	6.33	0.63	2.32	
				5	4.03	0.64	1.50	
				Mean	**5.45**	**0.55**	**1.78**	
			25	1	8.08	0.75	3.53	
				2	7.53	0.67	2.94	
				3	9.73	0.68	3.85	
				4	7.03	0.73	2.99	
				5	7.60	0.37	1.64	
				Mean	**7.99**	**0.64**	**2.99**	
			50	1	9.13	0.54	2.87	
				2	9.15	1.00	5.33	
				3	8.63	0.72	3.62	
				4	7.35	0.60	2.57	
				5	8.88	0.62	3.21	
				Mean	**8.63**	**0.70**	**3.52**	
	PC	AOO	25	1	10.15	1.04	6.15	NA
				2	14.13	0.56	4.61	
				3	7.40	1.07	4.62	
				4	10.70	1.26	7.86	
				5	12.50	1.01	7.36	
				Mean	**10.98**	**0.99**	**6.12**	

- Flow cytometer: BD FACS Calibur™
- Setting values of the flow cytometer

Detector	Voltage	Amp Gain	Mode
FSC	E00	1.25	Lin
SSC	440	1.00	Lin
FL1	555	1.00	Log
FL3	900	1.00	Lin

Data Used for 3rd Predictive Capacity Evaluation (Protocol 1.3)

Attachment 1-42

Table 12. Individual Animal Data for the LLNA: BrdU-FCM (3rd Predictive Capacity Data - Participating Lab. 2)

No.	Substance	Vehicle	Conc. (%)	Animal No.	LNC ($x10^6$/ml)	BrdU (%)	SI	EC2.7
1	VC	DMF	0	1	7.45	0.69	0.71	NA
				2	10.60	1.02	1.49	
				3	4.60	1.18	0.75	
				4	5.95	1.16	0.95	
				5	7.00	1.13	1.09	
				Mean	**7.12**	**1.04**	**1.00**	
	CMI/MI	DMF	2.5	1	27.55	1.35	5.14	1.062*
				2	34.20	1.90	8.98	
				3	41.20	2.57	14.63	
				4	37.00	2.19	11.19	
				5	27.70	2.61	9.99	
				Mean	**33.53**	**2.12**	**9.99**	
			5	1	41.50	2.50	14.33	
				2	31.20	2.83	12.20	
				3	41.30	2.85	16.26	
				4	29.60	3.01	12.31	
				5	31.10	2.79	11.99	
				Mean	**34.94**	**2.80**	**13.42**	
			10	1	39.70	1.97	10.80	
				2	38.80	2.61	13.99	
				3	40.60	2.65	14.86	
				4	45.40	2.06	12.92	
				5	33.20	3.31	15.18	
				Mean	**39.54**	**2.52**	**13.55**	
	PC	AOO	25	1	23.35	1.55	5.00	NA
				2	20.25	1.71	4.78	
				3	21.10	2.05	5.98	
				4	25.90	2.24	8.01	
				5	29.75	1.81	7.44	
				Mean	**24.07**	**1.87**	**6.24**	
2	VC	AOO	0	1	3.95	0.20	0.37	NA
				2	7.40	0.34	1.17	
				3	7.45	0.33	1.14	
				4	5.85	0.34	0.92	
				5	7.55	0.40	1.40	
				Mean	**6.44**	**0.32**	**1.00**	
	DNCB	AOO	0.125	1	14.85	1.49	10.27	0.016*
				2	17.35	2.28	18.36	
				3	12.85	2.45	14.61	
				4	11.05	2.63	13.49	
				5	17.00	2.68	21.14	
				Mean	**14.62**	**2.31**	**15.57**	
			0.25	1	2.95	3.07	32.70	
				2	31.20	3.20	46.34	
				3	36.30	2.65	44.64	
				4	24.90	3.14	36.29	
				5	23.70	3.17	34.87	
				Mean	**23.81**	**3.05**	**38.97**	
			0.5	1	32.85	2.74	41.77	
				2	31.30	2.49	36.17	
				3	44.20	2.16	44.31	
				4	37.60	3.33	58.11	
				5	39.75	3.04	56.08	
				Mean	**37.14**	**2.75**	**47.29**	
	PC	AOO	25	1	20.10	0.89	8.30	NA
				2	20.25	0.73	6.86	
				3	26.25	0.69	8.41	
				4	20.05	1.08	10.05	
				5	22.55	1.09	11.41	
				Mean	**21.84**	**0.90**	**9.01**	

	VC	DMF	0	1	7.45	0.69	0.71	NA
				2	10.60	1.02	1.49	
				3	4.60	1.18	0.75	
				4	5.95	1.16	0.95	
				5	7.00	1.13	1.09	
				Mean	**7.12**	**1.04**	**1.00**	
3	PPD	DMF	0.5	1	19.30	1.11	2.96	0.101
				2	15.00	1.60	3.32	
				3	8.65	1.13	1.35	
				4	15.35	1.65	3.50	
				5	21.80	1.69	5.09	
				Mean	**16.02**	**1.44**	**3.24**	
			1	1	28.80	1.57	6.25	
				2	21.80	1.54	4.64	
				3	23.40	2.18	7.05	
				4	21.40	1.98	5.85	
				5	30.05	2.08	8.63	
				Mean	**25.09**	**1.87**	**6.48**	
			2.5	1	27.30	1.90	7.17	
				2	19.50	2.51	6.76	
				3	28.75	2.38	9.45	
				4	34.55	2.96	14.13	
				5	28.75	3.17	12.59	
				Mean	**27.77**	**2.58**	**10.02**	
	PC	AOO	25	1	23.35	1.55	5.00	NA
				2	20.25	1.71	4.78	
				3	21.10	2.05	5.98	
				4	25.90	2.24	8.01	
				5	29.75	1.81	7.44	
				Mean	**24.07**	**1.87**	**6.24**	
	VC	DMF	0	1	6.75	0.40	0.66	NA
				2	6.80	0.59	0.98	
				3	7.00	0.58	0.99	
				4	7.30	0.77	1.37	
				5	6.05	0.69	1.01	
				Mean	**6.78**	**0.61**	**1.00**	
4	Cobalt Chloride	DMF	0.25	1	9.25	1.03	2.32	0.199
				2	14.30	0.98	3.41	
				3	8.15	1.04	2.06	
				4	13.70	1.15	3.83	
				5	12.05	0.48	1.41	
				Mean	**11.49**	**0.94**	**2.60**	
			0.5	1	31.40	1.78	13.59	
				2	15.75	1.42	5.44	
				3	18.60	1.32	5.97	
				4	12.60	1.20	3.68	
				5	27.60	1.54	10.33	
				Mean	**21.19**	**1.45**	**7.80**	
			1	1	37.20	2.29	20.71	
				2	24.55	3.01	17.96	
				3	21.65	1.70	8.95	
				4	20.90	1.62	8.23	
				5	22.10	1.54	8.27	
				Mean	**25.28**	**2.03**	**12.83**	
	PC	AOO	25	1	24.40	1.34	7.95	NA
				2	33.10	1.98	15.93	
				3	34.50	1.51	12.66	
				4	35.95	1.51	13.20	
				5	29.75	1.32	9.55	
				Mean	**31.54**	**1.53**	**11.86**	

	VC	AOO	0	1	5.50	0.55	0.94	NA
				2	5.30	0.58	0.96	
				3	2.55	0.73	0.58	
				4	5.70	0.60	1.06	
				5	7.60	0.62	1.46	
				Mean	**5.33**	**0.62**	**1.00**	
5	Isoeugenol	AOO	5	1	21.00	1.10	7.18	1.198*
				2	26.60	1.11	9.17	
				3	18.80	1.52	8.88	
				4	14.85	1.30	6.00	
				5	17.80	1.28	7.08	
				Mean	**19.81**	**1.26**	**7.66**	
			10	1	28.50	1.67	14.79	
				2	42.20	1.64	21.50	
				3	24.35	1.93	14.60	
				4	39.70	1.97	24.30	
				5	32.80	2.09	21.30	
				Mean	**33.51**	**1.86**	**19.30**	
			25	1	36.40	2.15	24.32	
				2	28.40	2.65	23.38	
				3	50.70	3.22	50.72	
				4	42.40	2.57	33.86	
				5	45.50	2.99	42.27	
				Mean	**40.68**	**2.72**	**34.91**	
	PC	AOO	25	1	12.65	0.98	3.85	NA
				2	22.10	0.90	6.18	
				3	24.60	0.98	7.49	
				4	16.75	1.03	5.36	
				5	23.35	0.58	4.21	
				Mean	**19.89**	**0.89**	**5.42**	
	VC	DMF	0	1	6.75	0.40	0.66	NA
				2	6.80	0.59	0.98	
				3	7.00	0.58	0.99	
				4	7.30	0.77	1.37	
				5	6.05	0.69	1.01	
				Mean	**6.78**	**0.61**	**1.00**	
6	MBT	DMF	5	1	8.15	0.71	1.41	NC
				2	3.75	0.82	0.75	
				3	8.25	1.09	2.19	
				4	6.70	0.64	1.04	
				5	10.00	0.75	1.82	
				Mean	**7.37**	**0.80**	**1.44**	
			10	1	7.35	0.70	1.26	
				2	5.75	0.63	0.88	
				3	7.15	0.59	1.03	
				4	7.85	0.81	1.55	
				5	5.70	0.69	0.96	
				Mean	**6.76**	**0.68**	**1.13**	
			25	1	9.85	0.88	2.11	
				2	5.40	0.83	1.09	
				3	5.85	0.72	1.02	
				4	6.45	0.92	1.44	
				5	8.10	0.43	0.85	
				Mean	**7.13**	**0.76**	**1.30**	
	PC	AOO	25	1	24.40	1.34	7.95	NA
				2	33.10	1.98	15.93	
				3	34.50	1.51	12.66	
				4	35.95	1.51	13.20	
				5	29.75	1.32	9.55	
				Mean	**31.54**	**1.53**	**11.86**	

	VC	AOO	0	1	5.50	0.55	0.94	NA
				2	5.30	0.58	0.96	
				3	2.55	0.73	0.58	
				4	5.70	0.60	1.06	
				5	7.60	0.62	1.46	
				Mean	**5.33**	**0.62**	**1.00**	
7	Citral	AOO	10	1	7.65	0.83	1.97	13.08
				2	5.75	0.32	0.57	
				3	13.60	0.72	3.04	
				4	7.15	0.65	1.44	
				5	11.70	0.79	2.87	
				Mean	**9.17**	**0.66**	**1.98**	
			25	1	19.15	1.02	6.07	
				2	16.85	1.38	7.22	
				3	10.00	0.96	2.98	
				4	15.25	1.20	5.69	
				5	10.95	0.96	3.27	
				Mean	**14.44**	**1.10**	**5.05**	
			50	1	31.80	1.17	11.56	
				2	21.85	0.91	6.18	
				3	18.75	1.39	8.10	
				4	18.50	0.64	3.68	
				5	25.50	1.88	14.90	
				Mean	**23.28**	**1.20**	**8.88**	
	PC	AOO	25	1	12.65	0.98	3.85	NA
				2	22.10	0.90	6.18	
				3	24.60	0.98	7.49	
				4	16.75	1.03	5.36	
				5	23.35	0.58	4.21	
				Mean	**19.89**	**0.89**	**5.42**	
	VC	AOO	0	1	8.45	1.24	1.29	NA
				2	9.30	1.08	1.23	
				3	5.90	0.87	0.63	
				4	6.90	1.12	0.95	
				5	8.90	0.82	0.90	
				Mean	**7.89**	**1.03**	**1.00**	
8	HCA	AOO	5	1	9.00	1.04	1.15	15.11
				2	8.55	0.97	1.02	
				3	10.05	1.47	1.82	
				4	7.35	0.99	0.89	
				5	8.05	0.84	0.83	
				Mean	**8.60**	**1.06**	**1.14**	
			10	1	16.05	1.25	2.47	
				2	11.20	0.67	0.92	
				3	14.90	1.54	2.82	
				4	11.55	0.44	0.62	
				5	13.20	1.17	1.90	
				Mean	**13.38**	**1.01**	**1.75**	
			25	1	15.80	1.85	3.59	
				2	16.75	1.84	3.79	
				3	22.50	1.94	5.36	
				4	14.55	2.32	4.15	
				5	28.80	1.36	4.81	
				Mean	**19.68**	**1.86**	**4.34**	
	PC	AOO	25	1	18.85	1.29	2.99	NA
				2	29.00	2.39	8.52	
				3	14.00	1.66	2.86	
				4	28.00	1.85	6.37	
				5	38.70	2.47	11.75	
				Mean	**25.71**	**1.93**	**6.50**	

	VC	AOO	0	1	4.70	0.47	0.65	NA
				2	4.90	0.47	0.67	
				3	6.00	0.69	1.21	
				4	4.50	0.69	0.91	
				5	5.10	1.04	1.55	
				Mean	**5.04**	**0.67**	**1.00**	
9	Eugenol	AOO	5	1	4.40	0.71	0.92	16.46
				2	4.00	0.47	0.55	
				3	4.90	0.44	0.63	
				4	4.10	0.44	0.53	
				5	4.60	0.68	0.92	
				Mean	**4.40**	**0.55**	**0.71**	
			10	1	8.00	0.63	1.48	
				2	10.80	0.55	1.74	
				3	9.90	0.69	2.00	
				4	8.90	0.59	1.54	
				5	12.70	0.89	3.31	
				Mean	**10.06**	**0.67**	**2.01**	
			25	1	15.80	0.80	3.70	
				2	17.70	0.97	5.03	
				3	10.80	0.94	2.98	
				4	11.80	1.15	3.98	
				5	13.00	1.05	4.00	
				Mean	**13.82**	**0.98**	**3.94**	
	PC	AOO	25	1	8.70	0.81	2.07	NA
				2	9.10	0.70	1.87	
				3	11.50	0.64	2.16	
				4	32.40	1.01	9.59	
				5	12.90	1.00	3.78	
				Mean	**14.92**	**0.83**	**3.89**	
10	VC	AOO	0	1	3.95	0.20	0.37	NA
				2	7.40	0.34	1.17	
				3	7.45	0.33	1.14	
				4	5.85	0.34	0.92	
				5	7.55	0.40	1.40	
				Mean	**6.44**	**0.32**	**1.00**	
	Phenyl benzoate	AOO	10	1	8.65	1.27	5.10	5.537*
				2	10.40	1.68	8.11	
				3	7.90	1.20	4.40	
				4	10.45	1.80	8.73	
				5	8.05	2.12	7.92	
				Mean	**9.09**	**1.61**	**6.85**	
			25	1	8.00	1.56	5.79	
				2	16.45	1.74	13.28	
				3	10.10	1.82	8.53	
				4	8.90	1.92	7.93	
				5	10.45	2.40	11.64	
				Mean	**10.78**	**1.89**	**9.44**	
			50	1	13.10	2.07	12.59	
				2	13.60	1.91	12.06	
				3	14.10	2.74	17.93	
				4	16.40	3.21	24.43	
				5	6.90	3.14	10.06	
				Mean	**12.82**	**2.61**	**15.41**	
	PC	AOO	25	1	20.10	0.89	8.30	NA
				2	20.25	0.73	6.86	
				3	26.25	0.69	8.41	
				4	20.05	1.08	10.05	
				5	22.55	1.09	11.41	
				Mean	**21.84**	**0.90**	**9.01**	

	VC	AOO	0	1	4.70	0.47	0.65	NA
				2	4.90	0.47	0.67	
				3	6.00	0.69	1.21	
				4	4.50	0.69	0.91	
				5	5.10	1.04	1.55	
				Mean	**5.04**	**0.67**	**1.00**	
11	Cinnamic alcohol	AOO	10	1	3.60	0.63	0.66	44.28
				2	2.40	0.51	0.36	
				3	2.50	0.44	0.32	
				4	3.70	0.50	0.54	
				5	3.90	0.50	0.57	
				Mean	**3.22**	**0.52**	**0.49**	
			25	1	9.90	0.78	2.26	
				2	8.30	0.48	1.17	
				3	22.60	0.39	2.58	
				4	16.00	0.76	3.56	
				5	10.50	0.61	1.88	
				Mean	**13.46**	**0.60**	**2.29**	
			50	1	17.50	0.36	1.85	
				2	17.00	0.65	3.24	
				3	12.50	0.64	2.34	
				4	16.00	0.66	3.09	
				5	13.00	0.88	3.35	
				Mean	**15.20**	**0.64**	**2.78**	
	PC	AOO	25	1	8.70	0.81	2.07	NA
				2	9.10	0.70	1.87	
				3	11.50	0.64	2.16	
				4	32.40	1.01	9.59	
				5	12.90	1.00	3.78	
				Mean	**14.92**	**0.83**	**3.89**	
	VC	DMF	0	1	7.90	0.92	1.61	NA
				2	5.95	0.69	0.91	
				3	9.25	0.38	0.78	
				4	5.20	0.65	0.75	
				5	5.55	0.78	0.96	
				Mean	**6.77**	**0.68**	**1.00**	
12	Imidazolidinyl urea	DMF	10	1	6.50	0.73	1.05	32.02
				2	6.90	0.69	1.05	
				3	5.65	0.54	0.68	
				4	7.45	1.00	1.65	
				5	8.05	0.45	0.80	
				Mean	**6.91**	**0.68**	**1.05**	
			25	1	7.55	1.11	1.85	
				2	9.95	0.37	0.81	
				3	15.75	1.05	3.66	
				4	9.80	1.10	2.39	
				5	9.00	0.92	1.83	
				Mean	**10.41**	**0.91**	**2.11**	
			50	1	22.15	1.22	5.98	
				2	21.85	0.98	4.74	
				3	16.10	1.22	4.35	
				4	11.85	1.34	3.51	
				5	9.75	0.88	1.90	
				Mean	**16.34**	**1.13**	**4.10**	
	PC	AOO	25	1	32.80	2.42	17.56	NA
				2	29.30	1.96	12.71	
				3	24.90	1.53	8.43	
				4	24.80	1.38	7.57	
				5	37.80	1.80	15.05	
				Mean	**29.92**	**1.82**	**12.27**	

	VC	AOO	0	1	8.45	1.24	1.29	NA
				2	9.30	1.08	1.23	
				3	5.90	0.87	0.63	
				4	6.90	1.12	0.95	
				5	8.90	0.82	0.90	
				Mean	**7.89**	**1.03**	**1.00**	
13	Methyl methacrylate	AOO	25	1	4.10	0.83	0.42	NC
				2	4.75	1.14	0.67	
				3	5.05	0.88	0.55	
				4	5.90	0.93	0.67	
				5	4.25	1.06	0.55	
				Mean	**4.81**	**0.97**	**0.57**	
			50	1	8.60	0.65	0.69	
				2	5.30	0.50	0.33	
				3	6.95	0.94	0.80	
				4	6.10	1.03	0.77	
				5	6.50	0.86	0.69	
				Mean	**6.69**	**0.80**	**0.65**	
			100	1	7.95	1.05	1.03	
				2	6.15	1.11	0.84	
				3	5.35	1.24	0.82	
				4	5.30	1.98	1.29	
				5	9.90	0.30	0.37	
				Mean	**6.93**	**1.14**	**0.87**	
	PC	AOO	25	1	18.85	1.29	2.99	NA
				2	29.00	2.39	8.52	
				3	14.00	1.66	2.86	
				4	28.00	1.85	6.37	
				5	38.70	2.47	11.75	
				Mean	**25.71**	**1.93**	**6.50**	
	VC	AOO	0	1	3.95	0.20	0.37	NA
				2	7.40	0.34	1.17	
				3	7.45	0.33	1.14	
				4	5.85	0.34	0.92	
				5	7.55	0.40	1.40	
				Mean	**6.44**	**0.32**	**1.00**	
14	Chlorobenzene	AOO	10	1	4.80	0.56	1.25	NC
				2	4.90	0.29	0.66	
				3	5.30	0.36	0.89	
				4	5.30	0.37	0.91	
				5	5.70	0.72	1.90	
				Mean	**5.20**	**0.46**	**1.12**	
			25	1	6.70	0.30	0.93	
				2	7.15	0.52	1.73	
				3	7.70	0.37	1.32	
				4	4.80	0.38	0.85	
				5	6.20	0.49	1.41	
				Mean	**6.51**	**0.41**	**1.25**	
			50	1	5.40	0.60	1.50	
				2	5.10	0.71	1.68	
				3	5.45	0.67	1.69	
				4	5.15	0.60	1.43	
				5	5.20	0.84	2.03	
				Mean	**5.26**	**0.68**	**1.67**	
	PC	AOO	25	1	20.10	0.89	8.30	NA
				2	20.25	0.73	6.86	
				3	26.25	0.69	8.41	
				4	20.05	1.08	10.05	
				5	22.55	1.09	11.41	
				Mean	**21.84**	**0.90**	**9.01**	

	VC	AOO	0	1	5.50	0.55	0.94	NA
				2	5.30	0.58	0.96	
				3	2.55	0.73	0.58	
				4	5.70	0.60	1.06	
				5	7.60	0.62	1.46	
				Mean	**5.33**	**0.62**	**1.00**	
15	Isopropanol	AOO	25	1	5.40	0.54	0.91	NC
				2	4.15	0.46	0.59	
				3	4.15	0.62	0.80	
				4	3.70	0.59	0.68	
				5	6.35	0.68	1.34	
				Mean	**4.75**	**0.58**	**0.86**	
			50	1	7.85	0.43	1.05	
				2	3.80	0.51	0.60	
				3	4.10	0.44	0.56	
				4	5.65	0.51	0.90	
				5	6.60	0.52	1.07	
				Mean	**5.60**	**0.48**	**0.83**	
			100	1	5.30	0.29	0.48	
				2	6.90	0.50	1.07	
				3	4.10	0.76	0.97	
				4	6.70	0.38	0.79	
				5	7.25	0.51	1.15	
				Mean	**6.05**	**0.49**	**0.89**	
	PC	AOO	25	1	12.65	0.98	3.85	NA
				2	22.10	0.90	6.18	
				3	24.60	0.98	7.49	
				4	16.75	1.03	5.36	
				5	23.35	0.58	4.21	
				Mean	**19.89**	**0.89**	**5.42**	
	VC	DMF	0	1	6.75	0.40	0.66	NA
				2	6.80	0.59	0.98	
				3	7.00	0.58	0.99	
				4	7.30	0.77	1.37	
				5	6.05	0.69	1.01	
				Mean	**6.78**	**0.61**	**1.00**	
16	Lactic acid	DMF	10	1	5.70	0.62	0.86	NC
				2	5.45	0.57	0.76	
				3	8.20	0.73	1.46	
				4	6.30	0.88	1.35	
				5	7.10	0.78	1.35	
				Mean	**6.55**	**0.72**	**1.15**	
			25	1	7.60	0.60	1.11	
				2	7.90	0.51	0.98	
				3	6.20	0.93	1.40	
				4	6.70	0.75	1.22	
				5	7.70	0.63	1.18	
				Mean	**7.22**	**0.68**	**1.18**	
			50	1	7.70	0.56	1.05	
				2	8.25	0.50	1.00	
				3	7.60	0.66	1.22	
				4	6.20	0.64	0.96	
				5	7.90	1.12	2.15	
				Mean	**7.53**	**0.70**	**1.28**	
	PC	AOO	25	1	24.40	1.34	7.95	NA
				2	33.10	1.98	15.93	
				3	34.50	1.51	12.66	
				4	35.95	1.51	13.20	
				5	29.75	1.32	9.55	
				Mean	**31.54**	**1.53**	**11.86**	

	VC	AOO	0	1	4.70	0.47	0.65	NA
				2	4.90	0.47	0.67	
				3	6.00	0.69	1.21	
				4	4.50	0.69	0.91	
				5	5.10	1.04	1.55	
				Mean	**5.04**	**0.67**	**1.00**	
17	Methyl salicylate	AOO	10	1	4.30	1.81	2.28	NC
				2	5.10	1.18	1.76	
				3	4.10	1.47	1.77	
				4	6.10	0.96	1.72	
				5	4.40	1.04	1.34	
				Mean	**4.80**	**1.29**	**1.77**	
			25	1	3.90	1.63	1.86	
				2	4.30	1.91	2.41	
				3	3.10	1.45	1.32	
				4	2.70	1.64	1.30	
				5	3.30	1.19	1.15	
				Mean	**3.46**	**1.56**	**1.61**	
			50	1	5.60	0.62	1.02	
				2	5.10	0.79	1.18	
				3	4.20	0.77	0.95	
				4	4.30	0.75	0.95	
				5	5.10	1.07	1.60	
				Mean	**4.86**	**0.80**	**1.14**	
	PC	AOO	25	1	8.70	0.81	2.07	NA
				2	9.10	0.70	1.87	
				3	11.50	0.64	2.16	
				4	32.40	1.01	9.59	
				5	12.90	1.00	3.78	
				Mean	**14.92**	**0.83**	**3.89**	
	VC	DMF	0	1	7.90	0.92	1.61	NA
				2	5.95	0.69	0.91	
				3	9.25	0.38	0.78	
				4	5.20	0.65	0.75	
				5	5.55	0.78	0.96	
				Mean	**6.77**	**0.68**	**1.00**	
18	Salicylic acid	DMF	1	1	12.85	0.81	2.30	NC
				2	6.55	0.77	1.12	
				3	8.40	0.92	1.71	
				4	11.10	0.89	2.19	
				5	7.10	0.95	1.49	
				Mean	**9.20**	**0.87**	**1.76**	
			2.5	1	12.45	0.75	2.07	
				2	13.95	0.90	2.78	
				3	10.30	1.04	2.37	
				4	12.35	0.72	1.97	
				5	10.05	0.95	2.11	
				Mean	**11.82**	**0.87**	**2.26**	
			5	1	10.25	1.15	2.61	
				2	8.80	1.36	2.65	
				3	10.40	1.18	2.72	
				4	13.50	0.91	2.72	
				5	7.70	1.28	2.18	
				Mean	**10.13**	**1.18**	**2.57**	
	PC	AOO	25	1	32.80	2.42	17.56	NA
				2	29.30	1.96	12.71	
				3	24.90	1.53	8.43	
				4	24.80	1.38	7.57	
				5	37.80	1.80	15.05	
				Mean	**29.92**	**1.82**	**12.27**	

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

- Flow cytometer: Beckman coulter Cytomics FC 500
- Setting values of the flow cytometer

Detector	Voltage	Amp Gain	Mode
FSC	203	10.0	Lin
SSC	662	50.0	Lin
FL1	411	1.0	Log
FL3	768	2.0	Lin

Data Used for Supplementary Test
(4 Optional Chemicals – Protocol 1.3)

Table 13. Individual Animal Data for the LLNA: BrdU-FCM (4 optional test chemicals - Participating Lab. 2)

No.	Substance	Vehicle	Conc. (%)	Animal No.	LNC ($x10^6$/ml)	BrdU (%)	SI	EC2.7
19	VC	DMSO	0	1	7.08	0.64	1.42	NA
				2	4.13	0.55	0.71	
				3	4.85	0.73	1.11	
				4	4.23	0.74	0.98	
				5	3.53	0.70	0.77	
				Mean	**4.76**	**0.67**	**1.00**	
	SLS	DMSO	0.5	1	4.88	0.69	1.06	5.2
				2	5.48	0.67	1.15	
				3	5.58	0.80	1.40	
				4	6.98	0.92	2.01	
				5	5.83	0.91	1.66	
				Mean	**5.75**	**0.80**	**1.46**	
			1	1	6.45	0.68	1.38	
				2	6.35	0.69	1.38	
				3	5.38	0.77	1.30	
				4	5.68	0.97	1.73	
				5	4.88	0.60	0.92	
				Mean	**5.75**	**0.74**	**1.34**	
			2.5	1	9.03	1.18	3.34	
				2	13.28	1.23	5.12	
				3	8.43	0.99	2.62	
				4	8.65	0.92	2.50	
				5	1.63	0.60	0.31	
				Mean	**8.20**	**0.98**	**2.78**	
	PC	AOO	25	1	11.58	1.72	6.25	NA
				2	8.40	1.61	4.24	
				3	8.53	1.37	3.67	
				4	9.53	1.35	4.04	
				5	9.28	1.74	5.07	
				Mean	**9.46**	**1.56**	**4.65**	
20	VC	AOO	0	1	5.63	0.75	2.45	NA
				2	2.40	0.44	0.61	
				3	2.80	0.51	0.83	
				4	1.45	0.64	0.54	
				5	2.10	0.46	0.56	
				Mean	**2.88**	**0.56**	**1.00**	
	EGD	AOO	25	1	4.80	1.11	3.10	97.20
				2	2.50	1.06	1.54	
				3	2.30	0.70	0.94	
				4	4.93	0.58	1.66	
				5	3.68	0.87	1.86	
				Mean	**3.64**	**0.86**	**1.82**	
			50	1	3.30	0.56	1.07	
				2	3.93	0.95	2.17	
				3	2.98	0.79	1.37	
				4	5.43	0.73	2.30	
				5	2.83	0.78	1.28	
				Mean	**3.69**	**0.76**	**1.64**	
			100	1	5.03	0.95	2.78	
				2	3.90	0.84	1.91	
				3	6.30	0.82	3.00	
				4	8.63	0.87	4.36	
				5	3.43	1.11	2.21	
				Mean	**5.46**	**0.92**	**2.85**	
	PC	AOO	25	1	19.95	0.66	7.66	NA
				2	14.08	0.71	5.81	
				3	9.88	0.76	4.37	
				4	13.30	1.08	8.35	
				5	15.45	1.73	15.55	
				Mean	**14.53**	**0.99**	**8.35**	

	VC	AOO	0	1	5.63	0.75	2.45	NA
				2	2.40	0.44	0.61	
				3	2.80	0.51	0.83	
				4	1.45	0.64	0.54	
				5	2.10	0.46	0.56	
				Mean	**2.88**	**0.56**	**1.00**	
21	Xylene	AOO	25	1	2.98	0.43	0.74	34.14*
				2	2.35	0.52	0.71	
				3	3.60	0.40	0.84	
				4	3.43	0.40	0.80	
				5	3.58	0.69	1.43	
				Mean	**3.19**	**0.49**	**0.90**	
			50	1	3.83	0.61	1.36	
				2	5.53	0.69	2.22	
				3	4.55	1.00	2.65	
				4	5.58	0.52	1.69	
				5	5.60	0.88	2.87	
				Mean	**5.02**	**0.74**	**2.15**	
			100	1	12.83	0.81	6.04	
				2	9.48	0.80	4.41	
				3	11.68	0.96	6.52	
				4	11.98	1.13	7.87	
				5	14.23	1.22	10.09	
				Mean	**12.04**	**0.98**	**6.99**	
	PC	AOO	25	1	19.95	0.66	7.66	NA
				2	14.08	0.71	5.81	
				3	9.88	0.76	4.37	
				4	13.30	1.08	8.35	
				5	15.45	1.73	15.55	
				Mean	**14.53**	**0.99**	**8.35**	
	VC	DMSO	0	1	6.53	0.92	1.66	NA
				2	4.08	1.11	1.25	
				3	1.98	1.38	0.75	
				4	3.13	0.90	0.78	
				5	2.05	0.98	0.56	
				Mean	**3.55**	**1.06**	**1.00**	
22	Nickel chloride	DMSO	0.5	1	6.93	0.54	1.03	NC
				2	3.30	0.82	0.75	
				3	6.58	0.73	1.33	
				4	3.08	0.65	0.55	
				5	6.08	1.06	1.78	
				Mean	**5.19**	**0.76**	**1.09**	
			1	1	7.90	1.08	2.36	
				2	6.20	0.88	1.51	
				3	5.23	0.89	1.29	
				4	4.03	1.08	1.20	
				5	6.08	0.60	1.01	
				Mean	**5.89**	**0.91**	**1.47**	
			2.5	1	11.28	0.84	2.62	
				2	9.38	0.81	2.10	
				3	11.28	0.79	2.46	
				4	6.65	1.01	1.86	
				5	7.80	0.77	1.66	
				Mean	**9.28**	**0.84**	**2.14**	
	PC	AOO	25	1	6.33	1.26	2.20	NA
				2	11.28	1.91	5.96	
				3	11.80	1.50	4.90	
				4	8.85	1.32	3.23	
				5	7.55	1.65	3.45	
				Mean	**9.16**	**1.53**	**3.95**	

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

- Flow cytometer: Beckman coulter Cytomics FC 500
- Setting values of the flow cytometer

Detector	Voltage	Amp Gain	Mode
FSC	203	10.0	Lin
SSC	662	50.0	Lin
FL1	411	1.0	Log
FL3	768	2.0	Lin

Data Used for Comparison of BALB/c and CBA/J mice

Table 14. Data set for BALB/c vs. CBA/J

Test chemical	Dose	BALB/c	CBA/J	Remark
CMI/MI	2.5	9.99	5.46	Lee et al., 2016 from LLNA: BrdU-FCM
	5	13.42	10.44	
DNCB	0.1	15.57	8.36	
	0.25	38.97	24.43	
CC	0.25	2.6	5.13	
	0.5	7.8	8.55	
	1	12.83	9.27	
IE	5	7.66	3.7	
	10	19.3	8.22	
	25	34.91	11.07	
MBT	5	1.44	1.12	
	10	1.13	1.95	
	25	1.3	1.21	
CT	10	1.98	3.33	
HCA	5	1.14	2.83	
	10	1.75	4.36	
	25	4.34	6.36	
EUG	5	0.71	1.65	
	10	2.01	2.61	
	25	3.94	3.86	
PB	10	4.06	3.81	
	25	5.64	7.12	
	50	3.19	9.08	
CA	10	0.49	1.74	
	25	2.29	2.21	
	50	2.78	3.11	
IU	10	1.05	1.39	
	25	2.11	2.08	
	50	4.1	4.57	
MMA	25	0.57	0.96	
	50	0.65	0.63	
	100	0.87	0.98	
CB	25	1.25	1.3	
	50	1.67	1.49	
IP	25	0.86	0.96	
	50	0.83	0.77	
	100	0.89	0.72	
MS	25	1.61	1.21	
	50	1.14	1.48	
SLS	0.5	1.46	1.18	Unpublished data from LLNA: BrdU-FCM
	1	1.34	1.99	
	2.5	2.78	2.15	
EGD	25	1.82	1.36	
	50	1.64	1.89	
	100	2.85	4.17	
XY	25	0.9	2.22	
	50	2.15	2.75	
	100	6.99	3.81	
Nickel sulfate	2.5	2.19	1.8	Burns Strickland Poster from radioisotopic LLNA (*CBA/N)
	5	2.46	3.1	
Cobalt chloride	1	1.5	3.5	
	2.5	1.6	3.8	
	5	2.7	7.2	
HCA	5	1.7	2.5	M.R.Woolhiser, 2000 Table 2. from radioisotopic LLNA (**CBA)
	25	5	4.1	
	50	10.9	9.4	
TDI	1	9.8	13.7	
DNFB	0.15	35.3	35.3	

Table 15. The mean± SD, CV values and quantiles of the EC2.7 values of the HCA used in the WLR, 1st and 3rd predictive capacity evaluation

	N	Mean	SD	CoV (%)	Quantile						
					Min	5%	25%	Median	75%	95%	Max
Lead Lab.1	5	7.54	0.73	9.68	6.81	6.11	7.08	7.38	8.09	8.97	8.77
Part. Lab.1	4	7.66	1.46	19.09	5.95	4.79	6.20	7.82	8.97	10.53	9.07
Part. Lab.2	5	9.65	3.40	35.27	6.03	2.98	7.19	8.50	12.70	16.32	15.11

Table 16. The mean± SD values and quantiles of the vehicle control group and positive control group (25% HCA) used in the 3rd predictive capacity evaluation and supplementary test

Variable	N	Mean	SD	Quantile						
				Min	5%	25%	Median	75%	95%	Max
NC	50	1.0	0.4	0.4	0.6	0.7	1.0	1.2	1.6	2.5
PC	50	7.2	3.9	1.9	2.2	4.2	6.3	8.5	15.5	17.6

[Attachment 2]

QA inspection reports

Inspection for each participating laboratory by the QAU of Lead Laboratory 1

- Participating Laboratory 1: 17 October 2012 (Transferability)

2012-10-31 10:14/AP49197

AMOREPACIFIC

QAU Audit Report

시험기관	대구카톨릭대학교	기관책임자	허용
시험책임자	허용	시험담당자	여경욱
점검일	2012 년 10 월 17 일		

주요 점검 결과(QAU)

LLNA 시험 중 3건에 대해 점검 진행하였고 보완 필요한 사항은 다음과 같습니다.

1) 관련문서 점검사항
- 2건 시험에 대한 시험번호 누락
- 물질수령기록서 누락 : HCA, acetone, olive oil, substance A, BrdU, BrdU kit
- 장비사용기록지 누락 : 체중측정용 저울, 물질측정용 저울, 귀/림프절측정용 저울, 귀두께측정기, FACS
- 각 장비 SOP 누락
- 장비 검교정기록 누락 : FACS
- 개체식별카드 실물 보관 필요

2) 실험 과정 및 데이터 sheet 작성 관련 점검사항
- 거래명세서와 acclimation data sheet 기재 내용 간 불일치 항목에 대한 점검 필요
- 측정 프린트지와 data sheet 수기기재 사항, excel sheet 기록 간 불일치 항목에 대한 점검 필요

본 점검 사항을 바탕으로 향후 연구에서 개선이 된다면 좀더 좋은 연구결과를 도출할 수 있을 것으로 보입니다.

점검 결과 답변 및 대응 (시험책임자)

1) 시험번호 : 기록하였음 2) 물질수령 기록서 : [illegible] 이후 수령기록이 작성되었음 3) 장비사용기록지 : 추후 작성하겠음
4) 각 장비 SOP : 각 기기의 manual은 [illegible] 이후 SOP 작성[illegible]
5) FACS 정도[illegible] 기록 : GLP 센터 FACS는 FACS Comp[illegible]
6) 개체식별카드 : [illegible] 7) 거래명세서 [illegible]
8) 수기기재 사항 : [illegible]

점검 결과 답변 및 대응 (과제책임자)

GLP 기관인 대구카톨릭대학교 GLP센터의 시험에 대한 GLP 원칙을 준수하여 GLP센터의 자료 감독하에 [illegible] [illegible]

신뢰성보증업무담당자	박양희 (서명)	날 짜	2012년 10 월 31 일
신뢰성보증업무책임자	정경미 (서명)	날 짜	2012 년 10 월 31 일
시험책임자	허용 (서명)	날 짜	2012년 11 월 16일
과제책임자	허용 (서명)	날 짜	2012년 11 월 16일

[illegible] GLP 센터 [illegible]

- Participating Laboratory 1: 31 May 2013 (WLR, BLR)

AMOREPACIFIC

QAU Audit Report

시험기관	대구카톨릭대학교	기관책임자	허용
시험책임자	허용	시험담당자	여경욱
점검일	2013 년 05 월 31 일		

주요 점검 결과(QAU)

LLNA 시험 총 7건에 대해 점검 진행하였고 보완 필요한 사항은 다음과 같습니다.

1) 관련문서 점검사항
   - 장비사용기록지 누락 : 2013년 4월 이전의 5건
   - 순화기간 개체식별카드(빨강) 누락 : 2013년 4월 이전의 시험 5건
   - 내부 QAU 점검 리포트 누락 : 2013년 4월 시험 이외의 6건
   - 장비사용기록지 상 시간 기재 필요(날짜만 확인 가능)
2) 실험 과정 및 데이터 sheet 작성 관련 점검사항
   - 측정 프린트지에 대한 복사물 확보 필요
   - 일반증상 확인여부 알 수 없음(확인여부 표기 필요)

전년도 점검에 비해 많은 부분이 개선되어 보완 요구 사항이 현저히 줄어든 것으로 보입니다. 본 중간점검 사항을 바탕으로 향후 연구에서 개선이 된다면 좀더 좋은 연구결과를 도출할 수 있을 것으로 보입니다.

점검 결과 답변 및 대응 (시험책임자)

- 특이 증상이 있는 경우만 확인하기에 별도 증상은 기록하지 않았음
- 프린트물 원본이 없기에 복사해 놓았음. - QAU는 중간점검 작성하였음
- 장비 사용 기록은 날짜만 적었는데 시간까지도 적겠음

점검 결과 답변 및 대응 (과제책임자)

각 지적 내용이 Data의 신뢰도나 정확성에 영향을 미치지는 않지만 GLP 원칙에 의거 관리하겠음.

신뢰성보증업무담당자	정경미 (서명)	날 짜	2013 년 05 월 31 일
신뢰성보증업무책임자	정경미 (서명)	날 짜	2013 년 05 월 31 일
시험책임자	허용 (서명)	날 짜	2013 년 05 월 31 일
과제책임자	허용 (서명)	날 짜	2013 년 05 월 31 일

- Participating Laboratory 2: 16 October 2012 (Transferability, WLR)

2012-10-31 10:13/AP49197

AMOREPACIFIC

QAU Audit Report

시험기관	㈜ 바이오톡스텍	기관책임자	전태원
시험책임자	정미숙	시험담당자	전은영
점검일	2012 년 10 월 16 일		

주요 점검 결과(QAU)

LLNA 시험 중 5건에 대해 점검 진행하였고 보완 필요한 사항은 다음과 같습니다.

1) 관련문서 점검사항
   - 물질수령기록서 누락 : substance A, BrdU, BrdU kit
   - 물질사용기록지 누락 : substance A
   - 장비사용기록지 누락 : 귀두께측정기
   - 개체식별카드 실물 보관 필요
2) 실험 과정 및 data sheet 작성 관련 점검사항
   - 귀두께측정 프린트지 확보 필요
   - 시험번호 D12025 (2012.8.22 개시)의 acclimation data sheet가 누락
   - 시험번호 D12028 (2012.9.19 개시)의 data sheet 수기기재 내용과 프린트지, excel sheet 기록 간의 불일치 항목이 있어 문제점 기재하므로 이에 보완이 필요합니다.

본 점검 사항을 바탕으로 향후 연구에서 개선한다면 좀더 좋은 연구결과를 도출할 수 있을 것으로 보입니다.

점검 결과 답변 및 대응 (시험책임자)

1) ① Substance A 수령기록서는 최초시험에서 누락되었으나 그 이후의 시험은 수령기록서를 작성하였음.
② BrdU, BrdU Kit는 시약으로 당사에서는 시약은 수령기록서 대신 일반물질 사용기록서를 작성해서 입수부터 사용 및 폐기까지 관리하고 있음
- Substance A 물질사용기록지는 최초시험에서 누락되었으나, 그 이후 시험은 수령기록서를 작성하였음
- 귀두께측정기는 SOP에서 일반체중계 [illegible] 점검을 실시하게 되어 있어 별도의 사용기록서는 작성하지 않음
- 개체식별카드 실물은 보관하지 않고 전자파일로 보관하기로 SOP에 변경되어 있기 때문에 폐기하였음
2) - 귀두께측정 프린트지는 초기에 확보하지 않았으나, 그 이후는 모두 보관하고 있음
- acclimation data sheet는 추가하였음
- 불일치한 항목은 수정하였음

점검 결과 답변 및 대응 (과제책임자)

초기 누락된 부분은 추후 보강하는 것이 필요하나 이에 대한 적절한 QAU 답변이 타당함.

신뢰성보증업무담당자	박양희 (서명)	날 짜	2012년 10월 31일
신뢰성보증업무책임자	성경미 (서명)	날 짜	2012년 10월 31일
시험책임자	정미숙 (서명)	날 짜	2012년 11월 5일
과제책임자	(서명)	날 짜	2012년 11월 16일

- Participating Laboratory 2: 14 June 2013 (WLR, BLR)

AMOREPACIFIC

QAU Audit Report

시험기관	㈜ 바이오톡스텍	기관책임자	전태원
시험책임자	정미숙	시험담당자	전은영
점검일	2013 년 06 월 14 일		

주요 점검 결과(QAU)

LLNA 시험 중 4건에 대해 점검 진행하였고 보완 필요한 사항은 다음과 같습니다.

1) 관련문서 점검사항
   - 장비사용기록지 상 시간 기재 필요(날짜만 확인 가능)
   - 개체식별카드 실물 보관 필요(전자 문서 보관)
2) 실험 과정 및 data sheet 작성 관련 점검사항
   - 귀두께측정 프린트지 확보 필요 : 시험번호 D12025(2012.10.10 개시)의 1건
   - 시험번호 D13023(2013.04.17 개시)의 preparation of materials[1] sheet 누락
   - LNC 기재 내용 관련하여 sheet 기재란 단위와 맞지 않게 작성되고 있어 점검 시 혼동할 수 있는 문제점 기재하므로, 이에 대한 확인 및 보완 필요합니다.

본 점검 사항을 바탕으로 향후 연구에서 개선한다면 좀더 좋은 연구결과를 도출할 수 있을 것으로 보입니다.

점검 결과 답변 및 대응 (시험책임자)

위에서 기재된 모든 사항은 기록에 대한 사항으로 시험의 완결성과 신뢰성에는 문제가 없는것으로 판단함

점검 결과 답변 및 대응 (과제책임자)

주요 3개 결과에 대해 보완 지적된 내용은 확인해 보완토록 조치하였습니다

신뢰성보증업무담당자	정 경 미 (서명)	날 짜	2013 년 06 월 14 일
신뢰성보증업무책임자	정 경 미 (서명)	날 짜	2013 년 06 월 14 일
시험책임자	정미숙 (서명)	날 짜	2013 년 06 월 14 일
과제책임자	[illegible] (서명)	날 짜	2013 년 6 월 14 일

Inspection of each laboratory by its own QAU

- Lead Laboratory 1: 30 October 2012 (WLR, Predictive capacity)

2012-10-31 10:14/AP49197

AMORE PACIFIC

QAU Audit Report

시험기관	아모레퍼시픽 기술연구원	기관책임자	임경민
시험책임자	임경민	시험담당자	장원희
점검일	2012 년 10 월 30 일		

주요 점검 결과(QAU)

LLNA 시험 총 5건에 대해 점검 진행하였고 보완 필요한 사항은 다음과 같습니다.

1) 관련문서 점검사항
   - 물질사용기록지 누락 : HCA, substance A, BrdU
   - 장비사용기록지 누락 : 체중측정용 저울, 물질측정용 저울, 귀/림프절측정용 저울, 귀두께측정기, FACS
   - 각 장비 SOP 누락
   - 장비 검교정기록 누락 : 귀두께 측정기
   - 개체식별카드 실물 보관 필요
2) 실험 과정 및 데이터 sheet 작성 관련 점검사항
   - 거래명세서와 사용 동물수가 일치하지 않을 경우 사유 명시 필요
   - Acclimation data sheet에 grouping시 개체 ID 및 BW 기재 필요
   - FACS 결과 출력물 확보 필요

본 점검 사항을 바탕으로 향후 연구에서 개선한다면 좀더 좋은 연구결과를 도출할 수 있을 것으로 보입니다.

점검 결과 답변 및 대응 (시험책임자)

위 A 항을 확인하였으며 충분히 개선하도록 하겠습니다.

점검 결과 답변 및 대응 (과제책임자)

앞서 내용 확인 보완치 내용은 추후 관계 기관 요청시 제시할 수 있도록 보완 바람

신뢰성보증업무담당자	박양희 (서명)	날 짜	2012년 10 월 31 일
신뢰성보증업무책임자	정경미 (서명)	날 짜	2012 년 10 월 31 일
시험책임자	[illegible] (서명)	날 짜	2012년 10 월 31 일
과제책임자	[illegible] (서명)	날 짜	2012년 11 월 16 일

- Lead Laboratory 1: 11 July 2013 (BLR, Predictive capacity)

AMOREPACIFIC

QAU Audit Report

시험기관	아모레퍼시픽 기술연구원	기관책임자	박미영
시험책임자	박미영	시험담당자	장원희
점검일	2013 년 07 월 11 일		

주요 점검 결과(QAU)

LLNA 시험 중 10건에 대해 점검 진행하였고 보완 필요한 사항은 다음과 같습니다.

실험 과정 및 데이터 sheet 작성 관련 점검사항

- 거래명세서와 사용 동물수가 일치하지 않을 경우 사유 명시 필요
- 실험 중단 시, 특정 부분 이탈 시 사유 명시 필요
- 각 측정프린트지 복사물 확보 필요

본 점검 사항을 바탕으로 향후 연구에서 개선한다면 좀더 좋은 연구결과를 도출할 수 있을 것으로 보입니다.

점검 결과 답변 및 대응 (시험책임자)

위 내용은 결과에 영향을 미치지 않는 항목이나 질적 관리를 위해 해당 점검사항을 시험에 반영하겠음.

점검 결과 답변 및 대응 (과제책임자)

이탈의 경우 GLP에 준한 시험이기에 작성 sheet에 기입하도록 하겠습니다.

신뢰성보증업무담당자	정경미 (서명)	날 짜	2013 년 07 월 11 일
신뢰성보증업무책임자	정경미 (서명)	날 짜	2013 년 07 월 11 일
시험책임자	박미영 (서명)	날 짜	2013 년 07 월 11 일
과제책임자	허용 (서명)	날 짜	2013 년 7 월 12 일

- Lead Laboratory 2: 20–26 August 2014 (Transferability)

피부감작성시험의 신뢰성보증 점검 목록

시 험 번 호	숙련도시험 - A001
점 검 실 시	20 14년 8 월 20 일 이름 이 진 하 (서명)
점검 통보일	20 14년 8 월 20 일

	점검 내용	YES	NO	N/A	비고
1	실험동물의 입수 및 순화 기록 확인	V			
2	시험물질 조제 기록 확인	V			
3	시험물질 처리 기록 확인	V			
4					
5					
6					

문제점 : 없음

신뢰성보증업무 책임자 20 14 년 8 월 20 일 이름 이 진 하 (서명)
운영책임자 20 14 년 8 월 20 일 이름 김 태 성 (서명)

개선 및 회답 :

신뢰성보증업무 책임자 20 년 월 일 이름 (서명)
해당담당자 20 년 월 일 이름 (서명)

- 재점검일 : 20 년 월 일

- 상기 점검의 종료를 확인하였음.

신뢰성보증업무 책임자 20 14 년 8 월 20 일 이름 이 진 하 (서명)

피부감작성시험의 신뢰성보증 점검 목록

시 험 번 호	숙련도시험 - A001
점 검 실 시	2014 년 8 월 22 일 이름 이 진 하 (서명)
점검 통보일	2014 년 8 월 22 일

	점검 내용	YES	NO	N/A	비고
1	시험물질 조제 기록 확인	V			
2	시험물질 처리 기록 확인	V			
3					
4					
5					
6					

문제점 : 없음

신뢰성보증업무 책임자 2014 년 8 월 22 일 이름 이 진 하 (서명)
운영책임자 2014 년 8 월 22 일 이름 김 태 성 (서명)

개선 및 회답 :

신뢰성보증업무 책임자 20 년 월 일 이름 (서명)
해당담당자 20 년 월 일 이름 (서명)

- 재점검일 : 20 년 월 일

- 상기 점검의 종료를 확인하였음.

신뢰성보증업무 책임자 2014 년 8 월 22 일 이름 이 진 하 (서명)

피부감작성시험의 신뢰성보증 점검 목록

시 험 번 호	숙련도시험 - A001
점 검 실 시	2014 년 8 월 25 일 이름 이진하 (서명)
점검 통보일	2014 년 8 월 25 일

	점검 내용	YES	NO	N/A	비고
1	체중, 귀두께, 일반증상 기록 확인(일자별)	V			
2	귀무게, 림프절 무게 기록 확인	V			
3	림프세포 계수 결과 확인	V			
4	Anti-BrdU 염색 절차 수행 확인	V			
5	BrdU 등 시약 조제 기록 확인	V			
6					

문제점 : 없음

신뢰성보증업무 책임자 20 14 년 8 월 25 일 이름 이진하 (서명)
운영책임자 20 14 년 8 월 25 일 이름 김태성 (서명)

개선 및 회답 :

신뢰성보증업무 책임자 20 년 월 일 이름 (서명)
해당담당자 20 년 월 일 이름 (서명)

- 재점검일 : 20 년 월 일

- 상기 점검의 종료를 확인하였음.

신뢰성보증업무 책임자 20 14 년 8 월 25 일 이름 이진하 (서명)

피부감작성시험의 신뢰성보증 점검 목록

시 험 번 호	숙련도시험 - A001
점 검 실 시	20 14 년 8 월 26 일 이름 이 진 하 (서명)
점검 통보일	20 14 년 8 월 26 일

	점검 내용	YES	NO	N/A	비고
1	유세포 측정 절차 및 결과 기록 확인				
2	실험실 기기 사용 기록 확인				
3	실험실 기기 SOP 보관 확인				
4					
5					
6					

문제점 :
특이사항 없음

신뢰성보증업무 책임자 20 14 년 8 월 26 일 이름 이 진 하 (서명)
운영책임자 20 14 년 8 월 26 일 이름 김 태 성 (서명)

개선 및 회답 :

신뢰성보증업무 책임자 20 년 월 일 이름 (서명)
해당담당자 20 년 월 일 이름 (서명)

- 재점검일 : 20 년 월 일

- 상기 점검의 종료를 확인하였음.

신뢰성보증업무 책임자 20 14 년 8 월 26 일 이름 이 진 하 (서명)

- Lead Laboratory 2: 17–23 September 2014 (Predictive capacity-2nd test)

피부감작성시험의 신뢰성보증 점검 목록

시 험 번 호	2차 예측력시험 - A002, A003
점 검 실 시	20 14 년 9 월 17 일 이름 이 진 하 (서명)
점검 통보일	20 14 년 9 월 17 일

	점검 내용	YES	NO	N/A	비고
1	실험동물의 입수 및 순화 기록 확인	V			
2	시험물질 조제 기록 확인	V			
3	시험물질 처리 기록 확인	V			
4					
5					
6					

문제점 : 없음

신뢰성보증업무 책임자 20 14 년 9 월 17 일 이름 이 진 하 (서명)
운영책임자 20 14 년 9 월 17 일 이름 김 태 성 (서명)

개선 및 회답 :

신뢰성보증업무 책임자 20 년 월 일 이름 (서명)
해당담당자 20 년 월 일 이름 (서명)

- 재점검일 : 20 년 월 일

- 상기 점검의 종료를 확인하였음.

신뢰성보증업무 책임자 20 14 년 9 월 17 일 이름 이 진 하 (서명)

피부감작성시험의 신뢰성보증 점검 목록

시 험 번 호	2차 예측력시험 - A002, A003
점 검 실 시	2014년 9 월 19 일 이름 이 진 하 이(서명)
점검 통보일	2014 년 9 월 19 일

	점검 내용	YES	NO	N/A	비고
1	시험물질 조제 기록 확인	V			
2	시험물질 처리 기록 확인	V			
3					
4					
5					
6					

문제점 : 특이 사항 없음

신뢰성보증업무 책임자 20 14 년 9 월 19 일 이름 이 진 하 이(서명)
운영책임자 20 14 년 9 월 19 일 이름 김 태 성 (서명)

개선 및 회답 :

신뢰성보증업무 책임자 20 년 월 일 이름 (서명)
해당담당자 20 년 월 일 이름 (서명)

- 재점검일 : 20 년 월 일

- 상기 점검의 종료를 확인하였음.

신뢰성보증업무 책임자 20 14 년 9 월 19 일 이름 이 진 하 이(서명)

피부감작성시험의 신뢰성보증 점검 목록

시 험 번 호	2차 예측력시험 - A002, A003
점 검 실 시	20 14년 9 월 22 일 이름 이 진 하 (서명)
점검 통보일	20 14 년 9 월 22일

	점검 내용	YES	NO	N/A	비고
1	체중, 귀두께, 일반증상 기록 확인(일자별)	✓			
2	귀무게, 림프절 무게 기록 확인	✓			
3	림프세포 계수 결과 확인	✓			
4	Anti-BrdU 염색 절차 수행 확인	✓			
5	BrdU 등 시약 조제 기록 확인	✓			
6					

문제점 : 특이 사항 없음

신뢰성보증업무 책임자 20 14 년 9 월 22 일 이름 이 진 하 (서명)
운영책임자 20 14 년 9 월 22 일 이름 전 태 성 (서명)

개선 및 회답 :

신뢰성보증업무 책임자 20 년 월 일 이름 (서명)
해당담당자 20 년 월 일 이름 (서명)

- 재점검일 : 20 년 월 일

- 상기 점검의 종료를 확인하였음.

신뢰성보증업무 책임자 20 14 년 9 월 22 일 이름 이 진 하 (서명)

피부감작성시험의 신뢰성보증 점검 목록

시 험 번 호	2차 예측력시험 - A002, A003
점 검 실 시	2014년 9 월 23 일 이름 이 진 하 (서명)
점검 통보일	2014년 9 월 23 일

	점검 내용	YES	NO	N/A	비고
1	유세포 측정 절차 및 결과 기록 확인				
2	실험실 기기 사용 기록 확인				
3	실험실 기기 SOP 보관 확인				
4					
5					
6					

문제점 : 없음

신뢰성보증업무 책임자 2014년 9 월 23 일 이름 이 진 하 (서명)
운영책임자 2014년 9 월 23 일 이름 김 태 성 (서명)

개선 및 회답 :

신뢰성보증업무 책임자 20 년 월 일 이름 (서명)
해당담당자 20 년 월 일 이름 (서명)

- 재점검일 : 20 년 월 일

- 상기 점검의 종료를 확인하였음.

신뢰성보증업무 책임자 2014년 9 월 23 일 이름 이 진 하 (서명)

- Lead Laboratory 2: 19–25 September 2014 (Predictive capacity-2nd test)

피부감작성시험의 신뢰성보증 점검 목록

시 험 번 호	2차 예측력시험 - A004, A005
점 검 실 시	2014년 9 월 19 일 이름 이 진 희 (서명)
점검 통보일	2014년 9 월 19 일

	점검 내용	YES	NO	N/A	비고
1	실험동물의 입수 및 순화 기록 확인	V			
2	시험물질 조제 기록 확인	V			
3	시험물질 처리 기록 확인	V			
4					
5					
6					

문제점 : 없음

신뢰성보증업무 책임자 20 14 년 9 월 19 일 이름 이 진 희 (서명)
운영책임자 20 14 년 9 월 19 일 이름 김 태 성 (서명)

개선 및 회답 :

신뢰성보증업무 책임자 20 년 월 일 이름 (서명)
해당담당자 20 년 월 일 이름 (서명)

- 재점검일 : 20 년 월 일

- 상기 점검의 종료를 확인하였음.

신뢰성보증업무 책임자 20 14 년 9 월 19 일 이름 이 진 희 (서명)

피부감작성시험의 신뢰성보증 점검 목록

시 험 번 호	2차 예측력시험 - A004, A005
점 검 실 시	2014년 9 월 21 일 이름 이 진하 (서명)
점검 통보일	2014 년 9 월 21 일

	점검 내용	YES	NO	N/A	비고
1	시험물질 조제 기록 확인	V			
2	시험물질 처리 기록 확인	V			
3					
4					
5					
6					

문제점 : 없음

신뢰성보증업무 책임자 20 14 년 9 월 21 일 이름 이 진하 (서명)
운영책임자 20 14 년 9 월 21 일 이름 김태성 (서명)

개선 및 회답 :

신뢰성보증업무 책임자 20 년 월 일 이름 (서명)
해당담당자 20 년 월 일 이름 (서명)

- 재점검일 : 20 년 월 일

- 상기 점검의 종료를 확인하였음.

신뢰성보증업무 책임자 20 14 년 9 월 21 일 이름 이 진하 (서명)

피부감작성시험의 신뢰성보증 점검 목록

시 험 번 호	2차 예측력시험 - A004, A005
점 검 실 시	20 14 년 9 월 24일 이름 이 진 하 (서명)
점검 통보일	20 14 년 9 월 24 일

	점검 내용	YES	NO	N/A	비고
1	체중, 귀두께, 일반증상 기록 확인(일자별)	V			
2	귀무게, 림프절 무게 기록 확인	V			
3	림프세포 계수 결과 확인	V			
4	Anti-BrdU 염색 절차 수행 확인	V			
5	BrdU 등 시약 조제 기록 확인	V			
6					

문제점 : 없음

신뢰성보증업무 책임자 20 14 년 9 월 24 일 이름 이 진 하 (서명)
운영책임자 20 14 년 9 월 24 일 이름 김 태 오 (서명)

개선 및 회답 :

신뢰성보증업무 책임자 20 년 월 일 이름 (서명)
해당담당자 20 년 월 일 이름 (서명)

- 재점검일 : 20 년 월 일

- 상기 점검의 종료를 확인하였음.

신뢰성보증업무 책임자 20 14 년 9 월 24 일 이름 이 진 하 (서명)

피부감작성시험의 신뢰성보증 점검 목록

시 험 번 호	2차 예측력시험 - A004, A005
점 검 실 시	2014 년 9 월 25 일 이름 이 진 하 (서명)
점검 통보일	2014 년 9 월 25 일

	점검 내용	YES	NO	N/A	비고
1	유세포 측정 절차 및 결과 기록 확인	V			
2	실험실 기기 사용 기록 확인	V			
3	실험실 기기 SOP 보관 확인	V			
4					
5					
6					

문제점 : 없음

신뢰성보증업무 책임자 20 14 년 9 월 25 일 이름 이 진 하 (서명)
운영책임자 20 14 년 9 월 25 일 이름 김 태 성 (서명)

개선 및 회답 :

신뢰성보증업무 책임자 20 년 월 일 이름 (서명)
해당담당자 20 년 월 일 이름 (서명)

- 재점검일 : 20 년 월 일

- 상기 점검의 종료를 확인하였음.

신뢰성보증업무 책임자 20 14 년 9 월 25 일 이름 이 진 하 (서명)

- Lead Laboratory 2: 27 May–1 June 2015 (Predictive capacity-additional test)

피부감작성시험의 신뢰성보증 점검 목록

시 험 번 호	Addtional test - Imidazolidinyl Urea
점 검 실 시	2015 년 5 월 27 일 이름 김 주환
점검 통보일	2015 년 5 월 27 일

	점검 내용	YES	NO	N/A	비고
1	실험동물의 입수 및 순화 기록 확인	V			
2	시험물질 조제 기록 확인	√			
3	시험물질 처리 기록 확인	√			
4					
5					
6					

문제점 :

없음

신뢰성보증업무 책임자 2015 년 5 월 27 일 이름 김주환

운영책임자 2015 년 5 월 27 일 이름 [illegible]

개선 및 회답 :

신뢰성보증업무 책임자 20 년 월 일 이름 (서명)

해당담당자 20 년 월 일 이름 (서명)

- 재점검일 : 20 년 월 일

- 상기 점검의 종료를 확인하였음.

신뢰성보증업무 책임자 2015 년 5 월 27 일 이름 김 주환

식품의약품안전평가원 SOP-신뢰-03-F01

피부감작성시험의 신뢰성보증 점검 목록

시 험 번 호	Addtional test - Imidazolidinyl Urea
점 검 실 시	2015 년 5 월 29 일 이름 김 수 환 (서명)
점검 통보일	20 년 월 일

	점검 내용	YES	NO	N/A	비고
1	시험물질 조제 기록 확인	√			
2	시험물질 처리 기록 확인	√			
3					
4					
5					
6					

문제점 :

없음

신뢰성보증업무 책임자 20 15 년 5 월 29 일 이름 김수환 (서명)
운영책임자 2015 년 5 월 29 일 이름 김태성 (서명)

개선 및 회답 :

신뢰성보증업무 책임자 20 년 월 일 이름 (서명)
해당담당자 20 년 월 일 이름 (서명)

- 재점검일 : 20 년 월 일

- 상기 점검의 종료를 확인하였음.

신뢰성보증업무 책임자 20 15 년 5 월 29 일 이름 김수환 (서명)

피부감작성시험의 신뢰성보증 점검 목록

시 험 번 호	Addtional test - Imidazolidinyl Urea
점 검 실 시	2015 년 6 월 1 일 이름 김 주 한
점검 통보일	2015년 6 월 1 일

	점검 내용	YES	NO	N/A	비고
1	체중, 귀두께, 일반증상 기록 확인(일자별)	✓			
2	귀무게, 림프절 무게 기록 확인	✓			
3	림프세포 계수 결과 확인	✓			
4	Anti-BrdU 염색 절차 수행 확인	✓			
5	BrdU 등 시약 조제 기록 확인	✓			
6					

문제점 :

없음

신뢰성보증업무 책임자 2015 년 6 월 1 일 이름 김 주 한

운영책임자 2015 년 6 월 1 일 이름 김 태 성

개선 및 회답 :

신뢰성보증업무 책임자 20 년 월 일 이름 (서명)

해당담당자 20 년 월 일 이름 (서명)

- 재점검일 : 20 년 월 일

- 상기 점검의 종료를 확인하였음.

신뢰성보증업무 책임자 2015 년 6 월 1 일 이름 김 주 한

피부감작성시험의 신뢰성보증 점검 목록

시 험 번 호	Addtional test - Imidazolidinyl Urea
점 검 실 시	2015 년 6 월 1 일 이름 김주환
점검 통보일	2015 년 6 월 1 일

	점검 내용	YES	NO	N/A	비고
1	유세포 측정 절차 및 결과 기록 확인	V			
2	실험실 기기 사용 기록 확인	V			
3	실험실 기기 SOP 보관 확인	V			
4					
5					
6					

문제점 :

신뢰성보증업무 책임자 2015 년 6 월 1 일 이름 김주환
운영책임자 2015 년 6 월 1 일 이름 김태성

개선 및 회답 :

신뢰성보증업무 책임자 20 년 월 일 이름 (서명)
해당담당자 20 년 월 일 이름 (서명)

- 재점검일 : 20 년 월 일

- 상기 점검의 종료를 확인하였음.

신뢰성보증업무 책임자 2015 년 6 월 1 일 이름 김주환

- Participating Laboratory 1: 3 May 2013 (WLR, BLR)

LLNA 시험 - 내부 QAU Check list

시험번호 : CUD-2-12-009-LLNA-9	시험기관 : 대구가톨릭대학교			
순번	점검내용	Y	N	비고
1	동물입수기록	v		
	동물거래명세서	v		
	- 입수동물 정보의 SOP와의 일치 확인	v		마우스, F, 8주령, 17-19g, 30마리
	검역 중 일반증상관찰 기록	v		
	군구성 방법 및 개체식별카드 확인	v		군당 6마리, 개체식별카드
	동물 체중측정 및 귀두께 측정 기록	v		
	- 프린트지 확인	v		
	- 프린트지 복사여부	v		
	- Excel 시트와 Data 시트 간 기록 비교	v		
	동물 사육환경관련 기록	v		대구가톨릭대학교 GLP센터 SPF사육실 사육실1번
	- 온도: 22±3 ℃, 습도: 30~70%, 명암주기 : 12시간			23.4±0.37℃, 48.75 1.69%, 명암주기 12hr
2	물질수령기록			
	- HCA	v		2013. 4. 3
	- DNCB	v		2013. 4. 3
	- Aceton	v		2013. 4. 3
	- Olive oil	v		2013. 4. 3
	- BrdU	v		2013. 3. 26
	BrdU Kit	v		2013. 4. 3
3	물질사용기록			
	- HCA	v		
	- DNCB	v		
	- Aceton	v		
	- Olive oil	v		
	- BrdU	v		
	- 물질 조제기록	v		
4	BrdU 보관상태			
	- 냉동 : BrdU	v		-20℃ (VS -120C)
	- 냉장 : 5개 시약	v		4℃ (KMC -1302L)
	- Deep freezer : DNase	v		-70℃ (MDF -792)

순번	점검내용	Y	N	비고
5	장비사용기록			
	- Chemical microbalance(XT220A)	V		
	- 설치류 체중 측정용 전자저울(MWP-300H)	V		
	- Ear thickness guage(C1012B)	V		
	- Flow cytometry(BD FACSCalibur)	V		
	- Water bath(KMC-1205W)	V		
	- Laminar flow cabinet		V	미보유
6	장비 검교정 / 점검 기록			
	- Chemical microbalance(XT220A)	V		2012.5.17
	- 설치류 체중 측정용 전자저울(MWP-300H)		V	2012.3.29 (교정기간경과)
	- Ear thickness guage(C1012B)	V		2012.5.29
	- Flow cytometry(BD FACSCalibur)	V		QC 사용 측정, 2013.4.12 외부교정실시
	- 마이크로 피펫 4점	V		사용전 점검실시
	- 냉장고, 냉동고 측정용 온도계 점검	V		GLP센터에서 실시
	- Laminar flow cabinet		V	낙하균 검사 필요
7	각 장비 SOP 확인		V	Laminar flow cabinet 미보유

QAU 점검내용

① 시험담당자의 사인이 통일되어야 합니다
② 시험물질 수령기록 2013년 3월 ~ 4월 시험책임자 사인이 누락되어 있습니다.
③ 체중측정용 저울의 교정주기가 지나 교정이 필요합니다
④ Laminar flow cabinet 의 SOP, 사용기록, 낙하균 점검이 필요합니다
⑤ 수시 통보 부재시 부재사유를 명확히 작성해주세요.
위의 사항들을 보완 부탁드립니다.

점검실시	2013 년 5 월 3 일	내부 QAU	이기용 (인)
점검확인	2013 년 5 월 3 일	기관책임자	허 등 (인)

- Participating Laboratory 1: 22 September 2014 (Predictive capacity-2nd test)

LLNA 시험 - 내부 QAU Check list

시험번호 : CUD-2-12-009-LLNA-9 **시험기관 :** 대구가톨릭대학교

순번	점검내용	Y	N	비고
1	동물입수기록	V		
	동물거래명세서	V		
	- 입수동물 정보의 SOP와의 일치 확인	V		
	검역 중 일반증상관찰 기록	V		
	군구성 방법 및 개체식별카드 확인	V		
	동물 체중측정 및 귀두께 측정 기록	V		
	- 프린트지 확인	V		
	- 프린트지 복사여부	V		
	- Excel 시트와 Data 시트 간 기록 비교	V		
	동물 사육환경관련 기록	V		이상없음
	- 온도: 22±3 ℃, 습도: 30~70%, 명암주기 : 12시간	V		
2	물질수령기록			
	- HCA	V		확인
	- DNCB	V		확인
	- Aceton	V		확인
	- Olive oil	V		확인
	- BrdU	V		확인
	- BrdU Kit	V		확인
3	물질사용기록			
	- HCA	V		
	- DNCB	V		
	- Aceton	V		
	- Olive oil	V		
	- BrdU	V		
	- 물질 조제기록	V		
4	BrdU 보관상태			
	- 냉동 : BrdU	V		양호
	- 냉장 : 5개 시약	V		양호
	- Deep freezer : DNase	V		양호

순번	점검내용	Y	N	비고
5	장비사용기록			
	- Chemical microbalance(XT220A)	V		
	- 설치류 체중 측정용 전자저울(MWP-300H)	V		
	- Ear thickness guage(C1012B)	V		
	- Flow cytometry(BD FACSCalibur)	V		
	- Water bath(KMC-1205W)	V		
	- Laminar flow cabinet	V		
6	장비 검교정 / 점검 기록			
	- Chemical microbalance(XT220A)	V		
	- 설치류 체중 측정용 전자저울(MWP-300H)	V		
	- Ear thickness guage(C1012B)	V		
	- Flow cytometry(BD FACSCalibur)	V		
	- 마이크로 피펫 4점	V		
	- 냉장고, 냉동고 측정용 온도계 점검		V	온도계 점검 미실시
	- Laminar flow cabinet	V		
7	각 장비 SOP 확인	V		

QAU 점검내용

① 시험 담당자의 서명이 통일되어야 합니다. 일관성이 결여되네요

② 장비 사용 기록지의 책임자 서명이 일부 누락되어 있습니다.

③ 측정용 온도계의 점검이 실시되지 않았습니다. 교정 받은 온도계를 활용하여 점검이 필요합니다.

④ 기록지 수정시 수정사유를 명확하게 작성해 주세요.

점검실시	2014 년 9 월 22 일	내부 QAU	이 기용
점검확인	2014 년 [illegible] 월 [illegible] 일	기관책임자	허 (인)

- Participating Laboratory 2: 5–25 August 2014 (Predictive capacity-2nd test)

QA audit report

Study director	정미숙
Study No.	R14008
Study title	5 종 시험물질의 Balb/c 마우스를 이용한 피부감작성 시험 (Local lymph node assay: BrdU-FCM법)
QAP	박유리
Audit phase	시험계획서
Audit date	2014-08-05
Reporting date (Management and Study director)	2014. 08. 05.
QA audit results	☑ No comment ☐ Comment (Please refer to the QA audit comment record)

Written by (Signature)	140805
Confirmation	2014.8.8

QA audit report

Study director	정미숙
Study No.	R14008
Study title	5 종 시험물질의 Balb/c 마우스를 이용한 피부감작성 시험 (Local lymph node assay: BrdU-FCM법)
QAP	박유리
Audit phase	시험물질의 조제 투여 피부반응의 평가 시험계획서의 변경 (1)
Audit date	2014-08-19
Reporting date (Management and Study director)	2014-08-19
QA audit results	☑ No comment ☐ Comment (Please refer to the QA audit comment record)

Written by (Signature)	2014.08.19 2014.08.19
Confirmation	2014.8.20

QA audit report

Study director	정미숙
Study No.	R14008
Study title	5 종 시험물질의 Balb/c 마우스를 이용한 피부감작성 시험 (Local lymph node assay: BrdU-FCM법)
QAP	박유리
Audit phase	측정 부검
Audit date	2014-08-24
Reporting date (Management and Study director)	2014-08-25
QA audit results	☑ **No comment** ☐ **Comment** (Please refer to the QA audit comment record)

Written by (Signature)	140824 2014.08.25
Confirmation	2014.8.26

QA audit report

Study director	정미숙
Study No.	R14008
Study title	5 종 시험물질의 Balb/c 마우스를 이용한 피부감작성 시험 (Local lymph node assay: BrdU-FCM법)
QAP	박유리
Audit phase	측정
Audit date	2014-08-25
Reporting date (Management and Study director)	2014. 08. 25
QA audit results	☑ **No comment** ☐ **Comment** (Please refer to the QA audit comment record)

Written by (Signature)	14.08.25 2014.08.25
Confirmation	2014.8.26

- Participating Laboratory 2: 27 October–18 November 2015 (Predictive capacity-3rd test)

QA audit report

Study director	정미숙
Study No.	R15012
Study title	한국형 피부감작성대체시험법 검증연구 (유세포분석을 이용한 국소림프절시험법)
QAP	정우용
Audit phase	시험계획서
Audit date	2015-10-27
Reporting date (Management and Study director)	2015-10-28
QA audit results	☑ No comment ☐ Comment (Please refer to the QA audit comment record)

Written by (Signature)	정우용 2015. 10. 27
Confirmation	2015. 10. 27

QA audit report

Study director	정미숙
Study No.	R15012
Study title	한국형 피부감작성대체시험법 검증연구 (유세포분석을 이용한 국소림프절시험법)
QAP	정우용
Audit phase	시험물질의 보관 시험물질의 조제(나) 투여 피부반응의 평가 측정
Audit date	2015-11-02
Reporting date (Management and Study director)	2015.11.02
QA audit results	☑ No comment ☐ Comment (Please refer to the QA audit comment record)

Written by (Signature)	정우용 2015.11.02
Confirmation	2015.11.03

QA audit report

Study director	정미숙
Study No.	R15012
Study title	한국형 피부감작성대체시험법 검증연구 (유세포분석을 이용한 국소림프절시험법)
QAP	정우용
Audit phase	시험물질의 조제 (1차)
Audit date	2015-11-04
Reporting date (Management and Study director)	2015. 11. 04
QA audit results	☑ No comment ☐ Comment (Please refer to the QA audit comment record)

Written by (Signature)	정우용 2015. 11. 04
Confirmation	2015. 11. 06

QA audit report

Study director	정미숙
Study No.	R15012
Study title	한국형 피부감작성대체시험법 검증연구 (유세포분석을 이용한 국소림프절시험법)
QAP	정우용
Audit phase	시험물질의 조제 (2차) 투여 시험물질의 조제 (3rd)
Audit date	2015-11-06
Reporting date (Management and Study director)	2015.11.06
QA audit results	☑ No comment ☐ Comment (Please refer to the QA audit comment record)

Written by (Signature)	정우용 2015.11.06
Confirmation	2015.11.06

QA audit report

Study director	정미숙
Study No.	R15012
Study title	한국형 피부감작성대체시험법 검증연구 (유세포분석을 이용한 국소림프절시험법)
QAP	정우용
Audit phase	부검 측정
Audit date	2015-11-07
Reporting date (Management and Study director)	2015.11.09
QA audit results	☑ No comment ☐ Comment (Please refer to the QA audit comment record)

Written by (Signature)	이정현 2015.11.07 정우용 2015-11-09
Confirmation	2015.11.09

QA audit report

Study director	정미숙
Study No.	R15012
Study title	한국형 피부감작성대체시험법 검증연구 (유세포분석을 이용한 국소림프절시험법)
QAP	정우용
Audit phase	측정
Audit date	2015-11-08
Reporting date (Management and Study director)	2015.11.09
QA audit results	☑ No comment ☐ Comment (Please refer to the QA audit comment record)

Written by (Signature)	이은현 2015.11.08 정우용 2015.11.09
Confirmation	2015.11.09

QA audit report

Study director	정미숙
Study No.	R15012
Study title	한국형 피부감작성대체시험법 검증연구 (유세포분석을 이용한 국소림프절시험법)
QAP	정우용
Audit phase	시험물질의 조제 (4차)
Audit date	2015-11-09
Reporting date (Management and Study director)	2015. 11. 09
QA audit results	☑ No comment ☐ Comment (Please refer to the QA audit comment record)

Written by (Signature)	정우용 2015. 11. 09
Confirmation	2015. 11. 09

QA audit report

Study director	정미숙
Study No.	R15012
Study title	한국형 피부감작성대체시험법 검증연구 (유세포분석을 이용한 국소림프절시험법)
QAP	정우용
Audit phase	시험물질의 조제 (5차)
Audit date	2015-11-11
Reporting date (Management and Study director)	2015. 11. 12
QA audit results	☑ No comment ☐ Comment (Please refer to the QA audit comment record)

Written by (Signature)	이동헌 2015. 11. 11 정우용 2015. 11. 12
Confirmation	2015. 11. 12

QA audit report

Study director	정미숙
Study No.	R15012
Study title	한국형 피부감작성대체시험법 검증연구 (유세포분석을 이용한 국소림프절시험법)
QAP	정우용
Audit phase	시험물질의 조제 (6차)
Audit date	2015-11-16
Reporting date (Management and Study director)	2015. 11. 16
QA audit results	☑ No comment ☐ Comment (Please refer to the QA audit comment record)

Written by (Signature)	정우용 2015. 11. 16
Confirmation	2015. 11. 17

QA audit report

Study director	정미숙
Study No.	R15012
Study title	한국형 피부감작성대체시험법 검증연구 (유세포분석을 이용한 국소림프절시험법)
QAP	정우용
Audit phase	시험물질의 조제 (1차)
Audit date	2015-11-18
Reporting date (Management and Study director)	2015. 11. 18
QA audit results	☑ No comment ☐ Comment (Please refer to the QA audit comment record)

Written by (Signature)	정우용 2015-11-18
Confirmation	2015.11.19

- Participating Laboratory 2: 26 July ~ 8 August 2016 (Supplementary test)

QA audit report

Study director	정미숙
Study No.	R16006
Study title	한국형 피부감작성대체시험법 검증연구 (유세포분석을 이용한 국소림프절시험법)
QAP	설미라
Audit phase	시험계획서
Audit date	2016-07-26
Reporting date (Management and Study director)	2016.07.26
QA audit results	☑ No comment ☐ Comment (Please refer to the QA audit comment record)

Written by (Signature)	2016.07.26
Confirmation	2016.07.26

QA audit report

Study director	정미숙
Study No.	R16006
Study title	한국형 피부감작성대체시험법 검증연구 (유세포분석을 이용한 국소림프절시험법)
QAP	설미라
Audit phase	시험물질의 보관 시험물질의 조제 투여 피부반응의 평가 측정
Audit date	2016-08-01
Reporting date (Management and Study director)	2016.08.01
QA audit results	☑ **No comment** ☐ **Comment** (Please refer to the QA audit comment record)

Written by (Signature)	2016.08.01
Confirmation	2016.08.08

QA audit report

Study director	정미숙
Study No.	R16006
Study title	한국형 피부감작성대체시험법 검증연구 (유세포분석을 이용한 국소림프절시험법)
QAP	설미라
Audit phase	시험물질의 조제
Audit date	2016-08-04
Reporting date (Management and Study director)	2016.08.04
QA audit results	☑ **No comment** ☐ **Comment** (Please refer to the QA audit comment record)

Written by (Signature)	2016.08.04
Confirmation	2016.08.04

QA audit report

Study director	정미숙
Study No.	R16006
Study title	한국형 피부감작성대체시험법 검증연구 (유세포분석을 이용한 국소림프절시험법)
QAP	설미라
Audit phase	시험물질의 조제 투여
Audit date	2016-08-05
Reporting date (Management and Study director)	2016.08.05
QA audit results	☑ **No comment** ☐ **Comment** (Please refer to the QA audit comment record)

Written by (Signature)	2016.08.05
Confirmation	2016.08.05

QA audit report

Study director	정미숙
Study No.	R16006
Study title	한국형 피부감작성대체시험법 검증연구 (유세포분석을 이용한 국소림프절시험법)
QAP	설미라
Audit phase	부검 측정
Audit date	2016-08-06
Reporting date (Management and Study director)	2016.08.08
QA audit results	☑ **No comment** ☐ **Comment** (Please refer to the QA audit comment record)

Written by (Signature)	정주리 2016.08.08 설미라 2016.08.08
Confirmation	2016.08.08

이) 연구계 정주리 2016.08.06

QA audit report

Study director	정미숙
Study No.	R16006
Study title	한국형 피부감작성대체시험법 검증연구 (유세포분석을 이용한 국소림프절시험법)
QAP	설미라
Audit phase	측정
Audit date	2016-08-07
Reporting date (Management and Study director)	2016.08.08
QA audit results	☑ No comment ☐ Comment (Please refer to the QA audit comment record)

Written by (Signature)	김주희 2016.08.01 [illegible] 2016.08.08
Confirmation	[illegible] 2016.08.08