ICCVAM Test Method Evaluation Report on the Murine Local Lymph Node Assay: BrdU-ELISA
A Nonradioactive Alternative Test Method to Assess the Allergic Contact Dermatitis Potential of Chemicals and Products

Interagency Coordinating Committee on the Validation of Alternative Methods

National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

National Institute of Environmental Health Sciences
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Table of Contents

List of Tables

List of Figures

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List of Abbreviations and Acronyms

ACD	Allergic contact dermatitis
ACE	Acetone
AOO	Acetone: olive oil (4:1 by volume)
BRD	Background review document
BrdU	Bromodeoxyuridine
CASRN	Chemical Abstracts Service Registry Number
CI	Confidence interval
CMI	5-Chloro-2-methyl-4-isothiazolin-3-one
CPSC	U.S. Consumer Product Safety Commission
CV	Coefficient of variation
DMF	*N,N*-dimethylformamide
DMSO	Dimethyl sulfoxide
DNCB	Dinitrochlorobenzene
DPCP	Diphenylcyclopropanone
dpm	Disintegrations per minute
EC1.6	Estimated concentration needed to produce a stimulation index of 1.6
EC3	Estimated concentration needed to produce a stimulation index of 3
ECVAM	European Centre for the Validation of Alternative Methods
EGDA	Ethylene glycol dimethacrylate
ELISA	Enzyme-linked immunosorbent assay
EPA	U.S. Environmental Protection Agency
FR	*Federal Register*
GP	Guinea pig
GPMT	Guinea Pig Maximization Test
^{3}H	Tritiated
HCA	Hexyl cinnamic aldehyde
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ILS	Integrated Laboratory Systems
IWG	Immunotoxicity Working Group
JaCVAM	Japanese Center for the Validation of Alternative Methods
LLNA	Murine local lymph node assay
LLNA: BrdU-ELISA	Murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine
LNC	Lymph node cells
MAPS	4-methyl aminophenol sulfate
MBT	2-Mercaptobenzothiazole

MEK	Methyl ethyl ketone
NA	Not available
NC	Not calculated
Ni	Nickel
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences
No.	Number
OECD	Organisation for Economic Co-operation and Development
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SD	Standard deviation
SEM	Standard error of the mean
SI	Stimulation index
TG	Test Guideline
U.K.	United Kingdom
U.S.	United States
U.S.C.	United States Code

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[2] Dr. Pieters was unable to attend the public meeting on April 28-29, 2009. However, he was involved in the peer review of the documents and concurred with the conclusions and recommendations included in the *Independent Scientific Peer Review Panel Report – Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products.*

[3] Dr. Richmond was unable to attend the public meeting on March 4-6, 2008. However, he was involved in the peer review of the documents and concurred with the conclusions and recommendations included in the *Independent Scientific Peer Review Panel Report – Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products.*

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Preface

Allergic contact dermatitis (ACD) is an adverse health effect that frequently develops in workers and consumers exposed to skin sensitizing chemicals and products. ACD results in lost workdays[1] and can significantly diminish quality of life (Hutchings et al. 2001; Skoet et al. 2003). To minimize the occurrence of ACD, regulatory authorities require testing to identify substances that may cause skin sensitization. Sensitizing substances must be labeled with a description of the potential hazard and the precautions necessary to avoid development of ACD.

Skin sensitization testing has typically required the use of guinea pigs (Buehler 1965; Magnusson and Kligman 1970). However, in 1998, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) evaluated and recommended an alternative test method known as the murine (mouse) local lymph node assay ("traditional LLNA").[2] The traditional LLNA provides several advantages compared to guinea pig test methods, including elimination of potential pain and distress, use of fewer animals, less time to perform, and availability of dose-response information. Based on the validation database and performance, ICCVAM recommended the LLNA as an alternative test method for assessing the skin sensitization potential of most types of substances (ICCVAM 1999). United States and international regulatory agencies subsequently accepted the traditional LLNA as a valid alternative test method for ACD testing.

In 2007, the U.S. Consumer Product Safety Commission (CPSC) requested that ICCVAM evaluate several modifications of the traditional LLNA, including a nonradioactive version of the LLNA that measures bromodeoxyuridine (BrdU) incorporation into proliferating lymphocytes by an enzyme-linked immunosorbent assay (ELISA) (referred to hereafter as the "LLNA: BrdU-ELISA"), instead of using a radioactive marker to measure lymphocyte proliferation. The BrdU-ELISA was developed by Dr. Masahiro Takayoshi at the Chemicals Evaluation and Research Institute in Saitama, Japan and validation studies were completed in coordination with the Japanese Center for the Validation of Alternative Methods (JaCVAM) at the National Institute of Health Sciences. ICCVAM assigned this activity a high priority after considering comments from the public and ICCVAM's Scientific Advisory Committee on Alternative Toxicological Methods (SACATM). As part of their ongoing collaboration with ICCVAM, scientists from the European Centre for the Validation of Alternative Methods (ECVAM) and JaCVAM served as liaisons to the ICCVAM Immunotoxicity Working Group (IWG). A detailed timeline of the LLNA: BrdU-ELISA evaluation is included with this report.

This Test Method Evaluation Report provides ICCVAM's recommendations regarding the LLNA: BrdU-ELISA for assessing the ACD potential of chemicals and products. Since the LLNA: BrdU-ELISA does not require a radioactive marker, it can be used by laboratories that currently cannot use the traditional LLNA because they do not have a license for using radioisotopes and in countries that discourage or severely limit the use of radioactive materials. The report also summarizes the validation status of the LLNA: BrdU-ELISA and provides the ICCVAM-recommended LLNA: BrdU-ELISA test method protocol.

Following independent scientific peer reviews in 2008 and 2009, ICCVAM submitted a proposed draft Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) for the LLNA: BrdU-ELISA that was circulated in July 2009 to the 30 OECD member countries

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[1] http://www.blf.gov/IIF

[2] The "traditional LLNA" refers to the validated ICCVAM-recommended LLNA test method protocol, which measures lymphocyte proliferation based on incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine into the cells of the draining auricular lymph nodes (ICCVAM 1999; Dean et al. 2001).

for review and comment. The U.S. CPSC and NICEATM-ICCVAM hosted an OECD Expert Consultation meeting on October 20-22, 2009, to evaluate the comments. A revised TG was distributed to the 30 OECD member countries in December 2009 for comment and then the final draft was forwarded to the OECD Working Group of National Co-ordinators of the Test Guidelines Programme, which was approved as TG 442B at their March 23-25, 2010 meeting.

ICCVAM solicited and considered public comments and stakeholder involvement throughout the LLNA: BrdU-ELISA evaluation process. ICCVAM considered the SACATM comments, the conclusions of the Panel and the OECD Expert Consultation, and all public comments before finalizing the ICCVAM test method recommendations for the LLNA: BrdU-ELISA. The recommendations and the background review document (BRD), which is provided as an appendix to this report, are incorporated in this ICCVAM Test Method Evaluation Report. As required by the ICCVAM Authorization Act, ICCVAM will forward its recommendations to U.S. Federal agencies for consideration. Federal agencies must respond to ICCVAM within 180 days after receiving the ICCVAM test method recommendations. ICCVAM recommendations are available to the public on the NICEATM-ICCVAM website,[3] and agency responses will also be made available on the website as they are received.

We gratefully acknowledge the many individuals who contributed to the preparation, review, and revision of this report. We especially recognize the Panel members for their thoughtful evaluations and generous contributions of time and effort. Special thanks are extended to Dr. Michael Luster for serving as the Panel Chair and to Dr. Michael Woolhiser, Dr. Michael Olson, Dr. Stephen Ullrich, and Kim Headrick for their service as Evaluation Group Chairs. We thank the IWG for assuring a meaningful and comprehensive review. We especially thank Dr. Joanna Matheson (Consumer Product Safety Commission) and Dr. Abigail Jacobs (U.S. Food and Drug Administration Center for Drug Evaluation and Research) for serving as Co-Chairs of the IWG. We also acknowledge Integrated Laboratory Systems, Inc., the NICEATM support contractor, for providing excellent scientific and operational support, including Dr. David Allen, Thomas Burns, Michael Paris, Dr. Eleni Salicru, Frank Stack, and Dr. Judy Strickland. Finally, we thank Dr. Silvia Casati and Dr. Hajime Kojima, the IWG liaisons from ECVAM and JaCVAM, respectively, for their participation and contributions.

This comprehensive ICCVAM evaluation of the LLNA: BrdU-ELISA should facilitate regulatory agency decisions on the acceptability of the method. Use of the method by industry can be expected to significantly reduce and refine animal use for ACD testing while continuing to support the protection of human health.

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[3] Available at http://iccvam.niehs.nih.gov/methods/immunotox/llna-ELISA/TMER.htm

Executive Summary

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recently evaluated the validation status of a nonradioactive version of the murine local lymph node assay (LLNA) called the LLNA: BrdU-ELISA. The LLNA is used to identify chemicals and products that may cause allergic contact dermatitis (ACD), an allergic skin reaction characterized by redness, swelling, and itching. The LLNA: BrdU-ELISA uses bromodeoxyuridine (BrdU) uptake to measure proliferating lymphocytes. The BrdU in this version is quantified with an enzyme-linked immunosorbent assay (ELISA) kit, while the traditional LLNA uses ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine uptake to measure lymphocyte proliferation.[4] This Test Method Evaluation Report provides ICCVAM's recommendations regarding the usefulness and limitations of the LLNA: BrdU-ELISA as an alternative to the traditional LLNA. The report includes the ICCVAM-recommended LLNA: BrdU-ELISA test method protocol, the final LLNA: BrdU-ELISA background review document (BRD) describing the validation status of the test method, and recommendations for future studies and performance standards.

Following nomination of the LLNA: BrdU-ELISA by the U.S. Consumer Product Safety Commission (CPSC), the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), ICCVAM, and the ICCVAM Immunotoxicity Working Group prepared an initial draft BRD and draft test method recommendations. The drafts were provided to an independent international scientific peer review panel (Panel) and to the public for comment. The Panel met twice in public session to review the initial and revised draft BRD and draft ICCVAM recommendations. The initial draft BRD evaluated data for 24 substances. The Panel initially met in public session on March 4-6, 2008, to discuss its peer review of the ICCVAM draft BRD and to provide conclusions and recommendations regarding the validation status of the LLNA: BrdU-ELISA test method. The Panel also reviewed how well the information in the draft BRD supported ICCVAM's draft test method recommendations. The Panel concluded that definitive test method recommendations could not be made until a detailed protocol and individual animal data were obtained and an evaluation of interlaboratory reproducibility was conducted.

NICEATM revised the draft BRD with additional information and data. The revised draft BRD evaluated data for 31 substances. The Panel reconvened in public session on April 28-29, 2009, to review the ICCVAM revised draft BRD and to finalize its conclusions and recommendations on the current validation status of the LLNA: BrdU-ELISA test method.

Based on the revised draft ICCVAM recommendations and Panel reports, NICEATM submitted a proposed draft Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) for the LLNA: BrdU-ELISA. The draft TG was circulated in July 2009 to the 30 OECD member countries for review and comment. The U.S. CPSC and NICEATM-ICCVAM hosted an OECD Expert Consultation meeting on October 20-22, 2009, to evaluate the comments. The expert group reviewed the draft OECD TG for the LLNA: BrdU-ELISA, proposed responses to comments from member countries, and evaluated LLNA: BrdU-ELISA results for 12 additional substances tested and submitted to NICEATM after the April 2009 Panel evaluation. A revised TG was distributed to the 30 OECD member countries in December 2009 for comment and then the final draft was forwarded to the OECD Working Group of National Co-ordinators of

[4] *The traditional LLNA* refers to the validated ICCVAM-recommended LLNA protocol, which measures lymphocyte proliferation based on incorporation of ^{3}H methyl thymidine or ^{125}I-iododeoxyuridine into the cells of the draining auricular lymph nodes (ICCVAM 1999; Dean et al. 2001).

the Test Guidelines Programme, which approved the LLNA: BrdU ELISA as TG442B at their March 23-25, 2010 meeting.

In finalizing this Test Method Evaluation Report and the BRD, which is included as an appendix, ICCVAM considered (1) the conclusions and recommendations of the Panel and the OECD Expert Consultation, (2) comments from ICCVAM's Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), and (3) public comments.

ICCVAM Recommendations: Test Method Usefulness and Limitations

ICCVAM concludes that the accuracy and reliability of the LLNA: BrdU-ELISA support use of the test method to identify substances as potential skin sensitizers or nonsensitizers. For the validation database of 43 substances, the LLNA: BrdU-ELISA correctly identified all 32 LLNA sensitizers (0% [0/32] false negatives), and nine of the 11 LLNA nonsensitizers (18% [2/11] false positives). ICCVAM recommends that a stimulation index (SI) $\geq$ 1.6 be used as the decision criterion to identify substances as potential sensitizers. ICCVAM bases this recommendation on the fact that no false negatives, relative to the traditional LLNA, result with the current validation database when SI $\geq$ 1.6 is used.

A limitation of the LLNA: BrdU-ELISA is the potential for false positive results when borderline positive responses between an SI of 1.6 and 1.9 are obtained (see **Section 3.4**). ICCVAM considers the applicability domain for the LLNA: BrdU-ELISA to be the same as the traditional LLNA unless there are properties associated with a class of materials that may interfere with the accuracy of the LLNA: BrdU-ELISA. One exception would be nickel compounds. Unlike the traditional LLNA, the LLNA: BrdU-ELISA can be used for testing nickel compounds based on its ability to correctly identify them as potential sensitizers.

ICCVAM Recommendations: Test Method Protocol

ICCVAM recommends a LLNA: BrdU-ELISA test method protocol that is based on the protocol developed by Takeyoshi et al. (2001) and refined during an interlaboratory validation study (Kojima et al. 2008). The ICCVAM-recommended LLNA: BrdU-ELISA protocol incorporates all aspects of the ICCVAM-recommended traditional LLNA test method protocol, except for those procedures unique to the conduct of the LLNA: BrdU-ELISA. In testing situations where dose-response information is not required, or negative results are anticipated, ICCVAM recommends that the reduced LLNA: BrdU-ELISA should be considered and used where determined appropriate. The reduced LLNA tests only the high dose, thus further reducing animal use by up to 40%.

ICCVAM Recommendations: Future Studies

ICCVAM recommends the following future studies to further characterize the usefulness and limitations of the LLNA: BrdU-ELISA test method:

- Efforts should be made to identify additional human data and human experience for test substances. These data may be used to further assess the usefulness and limitations of this and other versions of the LLNA for identifying human sensitizing substances. Such efforts might include post-marketing surveillance of consumers for allergic reactions and occupational surveillance of potentially exposed workers.
- Additional substances that are nonsensitizing skin irritants should be tested to determine the impact of such substances on the false positive rate of the LLNA: BrdU-ELISA.
- Efforts should be made to further characterize the sensitization potential of borderline positive substances (those that produce an SI between 1.6 and 1.9) in the LLNA: BrdU-ELISA to determine if such results might be false positives. This could include evaluations of peptide reactivity, determination of molecular weight, identification of

results from related chemicals, human studies where ethically and scientifically justified, review of occupational exposures and postmarketing experience or monitoring, or *in vitro* testing data. All decision criteria should be reassessed as additional discriminators and data become available.

ICCVAM Recommendations: Performance Standards

ICCVAM concludes that the ICCVAM-recommended performance standards (ICCVAM 2009a) for the traditional LLNA can be used to evaluate any future modifications of the LLNA: BrdU-ELISA. The ICCVAM-recommended performance standards for the traditional LLNA apply to the LLNA: BrdU-ELISA because the test method is functionally and mechanistically similar to the traditional LLNA.

Validation Status of the LLNA: BrdU-ELISA

The mechanistic basis of the LLNA: BrdU-ELISA is identical to that of the traditional LLNA. The traditional LLNA measures the lymphocyte proliferation in the draining lymph nodes for the skin area where the test article is applied. In the traditional LLNA, lymphocyte proliferation more than three-fold or higher than the vehicle control is considered a positive response indicative of a skin sensitizing substance. The only difference between the test method protocols for the traditional LLNA and the LLNA: BrdU-ELISA is the procedure for measuring lymphocyte proliferation. The traditional LLNA assesses lymphocyte proliferation by measuring the incorporation of radioactivity into the DNA of dividing cells in the draining auricular lymph nodes. The LLNA: BrdU-ELISA assesses cell proliferation by measuring the incorporation of a nonradioactive thymidine analog, BrdU, into the DNA of dividing cells using an ELISA.

The accuracy of the LLNA: BrdU-ELISA was compared to that of the traditional LLNA using the current validation database of 43 test substances. Optimal LLNA: BrdU-ELISA performance was achieved using SI $\geq$ 1.6 to classify sensitizers versus nonsensitizers. Compared to the traditional LLNA, accuracy was 95% (41/43), with a false positive rate of 18% (2/11) and a false negative rate of 0% (0/32). The two false positive substances produced SI values between 1.6 and 1.9 in the LLNA: BrdU-ELISA. Therefore, other available information such as dose-response, evidence of systemic toxicity or excessive local irritation, and where appropriate, statistical significance together with SI values should be considered to confirm that such borderline positive results are potential skin sensitizers. Consideration should also be given to various properties of the test substance, including whether it is structurally similar to known skin sensitizers.

An evaluation to determine the robustness of the SI $\geq$ 1.6 decision criterion indicated that the SI was quite stable. Taking different samples of the data as training and validation sets had relatively little impact on the cutoff SI criteria or on the resulting number of false positives or false negatives.

ICCVAM concludes that the reproducibility of the LLNA: BrdU-ELISA supports the use of the method to identify substances as potential skin sensitizers and nonsensitizers. The validation database supported an assessment of both intra-and interlaboratory reproducibility. One study was conducted to assess interlaboratory reproducibility.

In a qualitative analysis of intralaboratory reproducibility, two to six LLNA: BrdU-ELISA tests yielded 100% concordance for sensitizer/nonsensitizer outcomes for 10/12 substances (10 sensitizers and two nonsensitizers). One of the nonsensitizers with 100% concordance, however, produced false positive results in 2/2 tests. The two discordant substances were traditional LLNA sensitizers that yielded one test with SI < 1.6 and another test with SI > 1.6. Quantitative analyses of EC1.6 values (estimated concentration needed to produce an SI of 1.6) were performed for four substances tested two to five times. The analyses produced coefficient of variation (CV) values from 37% to 118%.

The qualitative interlaboratory reproducibility analysis of 10 substances (seven sensitizers and three nonsensitizers) tested in three to seven laboratories indicated 100% interlaboratory agreement (3/3, 6/6, or 7/7) for nine substances (seven sensitizers and two nonsensitizers). One of the nonsensitizers with 100% concordance, however, produced false positive results in 3/3 laboratories. There was 67% (4/6) agreement among the tests for the remaining nonsensitizer. Interlaboratory CV values for the EC1.6 values of the seven sensitizers ranged from 31% to 93%.

Reproducibility of results for the 18 substances (13 LLNA sensitizers and 5 LLNA nonsensitizers) that had two to 12 test results, regardless of whether the tests were performed in one laboratory or multiple laboratories, was assessed with respect to SI category. When the SI ≥ 1.6 decision criterion was used to classify sensitizers and nonsensitizers, the results for 78% (14/18) of the substances were 100% concordant. The results for 85% (11/13) of the LLNA sensitizers were 100% concordant (i.e., all yielded SI ≥ 1.6) for two to 12 tests. The results for 60% (3/5) of the nonsensitizers were 100% concordant for two to three tests. All (3/3) tests for two nonsensitizers had SI < 1.6. All (2/2) tests for the third nonsensitizer yielded SI values between 1.6 and 1.9, the narrow region in which false positive results occurred.

The Panel agreed with ICCVAM that the reproducibility of the LLNA: BrdU-ELISA supported the use of the method to identify substances as potential skin sensitizers and nonsensitizers.

ICCVAM Consideration of Independent Peer Review Panel Report and Other Comments

The ICCVAM evaluation process incorporates a high level of scientific peer review and transparency. The evaluation process for the LLNA: BrdU-ELISA included two public review meetings by an independent scientific peer review panel, multiple opportunities for public comments, consideration of the OECD Expert Consultation on the LLNA, and comments from the SACATM. ICCVAM and the Immunotoxicity Working Group considered the Panel report, conclusions of the OECD Expert Consultation, the SACATM comments, and all public comments before finalizing the ICCVAM Test Method Evaluation Report and final BRD for the LLNA: BrdU-ELISA.

1.0 Introduction

The murine local lymph node assay (traditional LLNA[1]) is an alternative skin sensitization test method that requires fewer animals and less time than currently accepted guinea pig (GP) tests (e.g., the guinea pig maximization test [GPMT] and the Buehler test). It also avoids animal discomfort that can occur in the guinea pig tests when substances cause allergic contact dermatitis (ACD). The LLNA measures cell proliferation in the draining auricular lymph nodes of the mouse by analyzing incorporation of a radioactive marker into newly synthesized DNA. The LLNA was the first alternative test method evaluated and recommended by the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). International regulatory authorities have now recognized the traditional LLNA as an acceptable alternative to GP tests for most testing situations.

The LLNA with detection of bromodeoxyuridine (BrdU) incorporation by an enzyme-linked immunosorbent assay (ELISA) (referred to hereafter as the "LLNA: BrdU-ELISA") was one of several modified versions of the LLNA nominated by the U.S. Consumer Product Safety Commission (CPSC) for evaluation by ICCVAM and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM).[2] It is a nonradioactive version of the LLNA that assesses cell proliferation using the incorporation of BrdU into newly synthesized DNA rather than by quantifying the incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine. The increase in BrdU in lymph nodes from test animals compared to vehicle controls is then quantified using an ELISA kit. The LLNA: BrdU-ELISA can reduce the use of animals for skin sensitization testing when it is used in place of GP tests in countries that severely limit or discourage the use of radioactive materials that are required by the traditional LLNA.

In accordance with the ICCVAM Authorization Act of 2000 (Public Law 106-545, 42 United States Code 285*l*-3), ICCVAM coordinates the technical evaluation of new, revised, and alternative test methods with regulatory applicability. After considering comments from the public and ICCVAM's advisory committee, the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), ICCVAM members unanimously agreed that the LLNA: BrdU-ELISA should have a high priority for evaluation. A detailed timeline of the LLNA: BrdU-ELISA evaluation is provided in **Appendix A**. The ICCVAM-recommended LLNA: BrdU-ELISA test method protocol and the final LLNA: BrdU-ELISA background review document (BRD) are provided in **Appendices B** and **C**, respectively.

The ICCVAM Immunotoxicity Working Group (IWG) was established to work with NICEATM to evaluate the LLNA: BrdU-ELISA and other test methods and applications. The European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Center for the Validation of Alternative Methods (JaCVAM) designated liaison members for the IWG.

To facilitate peer review of the LLNA: BrdU-ELISA test method, the IWG and NICEATM prepared a comprehensive draft BRD that provided information and data from validation studies and the scientific literature. A May 17, 2007, *Federal Register* (FR) notice (72 FR 27815[3]) requested data and information on these test methods and nominations of individuals to serve on an international independent scientific peer review panel (Panel). The request was also disseminated via the ICCVAM electronic mailing list and through direct requests to over 100 stakeholders. In response to this request, one individual submitted LLNA: BrdU-ELISA data and three individuals or organizations nominated members to the Panel (see **Section 4.0**).

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[1] The "traditional LLNA" refers to the validated ICCVAM-recommended LLNA test method protocol, which measures lymphocyte proliferation based on incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine into the cells of the draining auricular lymph nodes (ICCVAM 1999; Dean et al. 2001).

[2] Available at http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf

[3] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf

In the initial draft BRD, ICCVAM examined data for 24 substances (16 sensitizers and eight nonsensitizers, as classified by the traditional LLNA) that were tested in a single laboratory, with results reported among six published studies and one platform presentation. On January 8, 2008, ICCVAM announced the availability of the draft BRD to the public and a public Panel meeting to review the validation status of the LLNA: BrdU-ELISA (and other LLNA-related activities) (73 FR 1360[4]). All of the information provided to the Panel, including the ICCVAM draft BRD, draft test method recommendations, and all public comments received prior to the Panel meeting, were made publicly available via the NICEATM–ICCVAM website.[5]

The first Panel meeting was a public session held on March 4–6, 2008, to review the validation status of the LLNA: BrdU-ELISA and the completeness of the ICCVAM draft BRD (see **Appendix D1**). The Panel evaluated (1) the extent to which the draft BRD addressed established validation and acceptance criteria and (2) the extent to which the BRD supported ICCVAM's draft proposed test method uses, recommended test method protocol, draft test method performance standards, and proposed future studies. Interested stakeholders from the public were provided opportunities to comment at the Panel meeting. The Panel considered these comments as well as those submitted prior to the meeting before concluding their deliberations. The Panel agreed with the draft ICCVAM recommendations that the LLNA: BrdU-ELISA may be useful for identifying substances as potential skin sensitizers and nonsensitizers, but that more information and data were needed before definitive conclusions on the usefulness and limitations of the LLNA: BrdU-ELISA could be made. The Panel noted that the following information was needed before definitive recommendations could be made: 1) a detailed test method protocol; 2) individual animal data on a larger set of balanced reference substances with respect to physicochemical properties and sensitization potency; and 3) an evaluation of interlaboratory reproducibility. On May 20, 2008, ICCVAM posted a report of the Panel's recommendations[6] (see **Appendix D2**) on the NICEATM–ICCVAM website for public review and comment (announced in 73 FR 29136[7]).

ICCVAM provided SACATM with the draft BRD and draft test method recommendations, the Panel report, and all public comments for discussion at their meeting on June 18–19, 2008, where public stakeholders were given another opportunity to comment.

NICEATM subsequently obtained a detailed test method protocol and additional data and revised the draft BRD to include this new information. The revised draft BRD included an accuracy evaluation for the expanded database of individual animal results for 31 substances (22 sensitizers and nine nonsensitizers, as classified by the traditional LLNA) as well as an evaluation of interlaboratory reproducibility. Based on the analyses included in the revised draft BRD, ICCVAM prepared revised draft test method recommendations for proposed test method uses and limitations, recommended test method protocol, test method performance standards, and future studies for the LLNA: BrdU-ELISA. ICCVAM released the revised draft documents to the public for comment on February 27, 2009, and announced a second meeting of the Panel (74 FR 8974[8]). The Panel reconvened on April 27-28, 2009, to reassess the validation status of the LLNA: BrdU-ELISA (see **Appendix D3**). The Panel also reviewed the completeness of the revised draft ICCVAM BRD and the extent to which the information therein supported the revised draft ICCVAM test method recommendations. On June 1, 2009, ICCVAM posted the second report of the Panel's recommendations[9] (see

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[4] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_25553.pdf
[5] http://iccvam.niehs.nih.gov
[6] Available at http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
[7] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR-E8-11195.pdf
[8] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR-E9-4280.pdf
[9] Available at http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2009.pdf

Appendix D4) on the NICEATM-ICCVAM website for public review and comment (announced in 74 FR 26242[10]).

ICCVAM provided SACATM with the revised draft BRD, the second Panel report, and all public comments for discussion at their meeting on June 25-26, 2009, where public stakeholders were given another opportunity to comment.

Based on the revised draft ICCVAM recommendations, NICEATM submitted a proposed draft Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) for the LLNA: BrdU-ELISA that was circulated in July 2009 to the 30 OECD member countries for review and comment via their National Co-ordinators, who distributed the draft TG to interested stakeholders. An OECD Expert Consultation meeting was held on October 20-22, 2009, to evaluate the comments. Scientists from the National Institute of Environmental Health Sciences, the Environmental Protection Agency, the Food and Drug Administration, and CPSC, as well as U.S. and international experts from industry and other stakeholder organizations, participated in the meeting, which was co-hosted by CPSC and NICEATM-ICCVAM. The expert group reviewed the draft OECD TG for the LLNA: BrdU-ELISA, proposed responses to comments from member countries, and evaluated additional LLNA: BrdU-ELISA results for substances tested and submitted to NICEATM after the Panel evaluation. The expert group convened a subsequent teleconference on December 1, 2009, to discuss outstanding issues identified at the October meeting. A revised TG was again distributed to the 30 OECD member countries in December 2009 for review and comment by national experts and interested stakeholders. A final teleconference of the Expert Consultation was convened on January 29, 2010, to discuss the member country comments received during the last round of review, and a final draft TG was developed based on these discussions. This final draft was forwarded to the OECD Working Group of National Co-ordinators of the Test Guidelines Programme to consider for adoption at their March 23-25, 2010, meeting.

ICCVAM and the IWG considered the SACATM comments, the Panel report, conclusions of the OECD Expert Consultation, and all public comments before finalizing ICCVAM test method recommendations for the LLNA: BrdU-ELISA. The recommendations (**Section 2**) and the final BRD **(Appendix C)** are incorporated in this ICCVAM Test Method Evaluation Report. As required by the ICCVAM Authorization Act (2000; Public Law 106-545, 42 United States Code 285*l*-3), ICCVAM will forward its recommendations to U.S. Federal agencies for consideration. Federal agencies must respond to ICCVAM within 180 days after receiving ICCVAM test method recommendations. ICCVAM recommendations are available to the public on the NICEATM-ICCVAM website, and agency responses also will be made available on the website as they are received.

[10] Announced in 74 FR 26242 http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR-E9-12360.pdf

2.0 ICCVAM Recommendations for the Nonradioactive LLNA: BrdU-ELISA Test Method

ICCVAM evaluated the validation status of the LLNA: BrdU-ELISA as a nonradioactive modification of the traditional LLNA (ICCVAM 1999; Sailstad et al. 2001; Dean et al. 2001 Haneke et al. 2001) to identify substances that may cause ACD for regulatory hazard classification and labeling purposes. While the traditional LLNA assesses cellular proliferation by measuring the incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine into the DNA of dividing lymph node cells, the LLNA: BrdU-ELISA assesses cellular proliferation by measuring the incorporation of the thymidine analog BrdU using ELISA detection (see **Appendix B**). NICEATM and ICCVAM prepared a comprehensive report on the data and information supporting the validity of this test method, including its accuracy and reliability compared to the traditional LLNA (see **Section 3.0** and **Appendix C)**.

2.1 ICCVAM Recommendations: Test Method Usefulness and Limitations

ICCVAM concludes that the accuracy and reliability of the LLNA: BrdU-ELISA supports the use of the test method to identify substances as potential skin sensitizers and nonsensitizers. For the validation database of 43 substances,[11] the LLNA: BrdU-ELISA correctly identified all 32 LLNA sensitizers (0% [0/32] false negatives), and nine of the 11 LLNA nonsensitizers (18% [2/11] false positives). ICCVAM recommends that a stimulation index (SI) $\geq$ 1.6 be used as the decision criterion to identify substances as potential sensitizers. ICCVAM bases this recommendation on the fact that no false negatives, relative to the traditional LLNA, result with the current validation database when an SI $\geq$ 1.6 is used.

A limitation of the LLNA: BrdU-ELISA is the potential for false positive results when borderline positive responses between an SI of 1.6 and 1.9 are obtained (see **Section 3.4**). ICCVAM considers the applicability domain for the LLNA: BrdU-ELISA to be the same as the traditional LLNA unless there are properties associated with a class of materials that may interfere with the accuracy of the LLNA: BrdU-ELISA. One exception would be nickel compounds where, unlike the traditional LLNA, the LLNA: BrdU-ELISA can be used for testing nickel compounds based on its ability to correctly identify them as potential sensitizers.

2.2 ICCVAM Recommendations: Test Method Protocol

ICCVAM recommends a LLNA: BrdU-ELISA test method protocol (**Appendix B**) that was based on the protocol developed by Takeyoshi et al. (2001) and refined during an interlaboratory validation study (Kojima et al. 2008). The ICCVAM-recommended LLNA: BrdU-ELISA protocol incorporates all aspects of the ICCVAM-recommended LLNA test method protocol (Appendix A of ICCVAM 2009a), except for those procedures unique to the conduct of the LLNA: BrdU-ELISA. Key aspects included in the ICCVAM-recommended protocol include the following:

- The high dose should be the maximum possible concentration (for liquids, solids, or suspensions) that does not produce systemic toxicity and/or excessive local skin irritation. The measurement of ear thickness is a potentially valuable adjunct for identifying local skin irritation.
- A minimum of four animals per dose group is recommended.
- Collection of individual animal data is recommended.

[11] For the accuracy analyses, results for substances tested multiple times were combined so that each substance was represented by one result. In this case, the single result used for each substance represented the most prevalent outcome. Multiple tests were available for 18 substances tested with the LLNA: BrdU-ELISA.

- Inclusion of a concurrent vehicle control and concurrent positive control in each study is recommended.

Additionally, ICCVAM recommends there should be a measure of variability of the positive control response over time. Laboratories should maintain a historical database of positive control SI values such that results can be compared to the mean historical SI. There could be cause for concern when a negative test substance result is accompanied by a concurrent positive control SI value significantly lower than the mean historical SI.

In testing situations where dose-response information is not required, or negative results are anticipated, ICCVAM recommends that the reduced LLNA: BrdU-ELISA should be considered and used where determined appropriate. The reduced LLNA: BrdU-ELISA protocol uses only the high dose (Kimber et al. 2006; ESAC 2007; ICCVAM 2009b), thus further reducing animal use by up to 40%.

2.3 ICCVAM Recommendations: Future Studies

ICCVAM recommends the following future studies to further characterize the usefulness and limitations of the LLNA: BrdU-ELISA test method:

- Efforts should be made to identify additional human data and human experience for test substances. These data may be used to further assess the usefulness and limitations of this and other versions of the LLNA for identifying human sensitizing substances. Such efforts might include post-marketing surveillance of consumers for allergic reactions and occupational surveillance of potentially exposed workers.
- Additional substances that are nonsensitizing skin irritants should be tested to determine the impact of such substances on the false positive rate of the LLNA: BrdU-ELISA.
- Efforts should be made to further characterize the sensitization potential of borderline positive substances (those that produce an SI between 1.6 and 1.9) in the LLNA: BrdU-ELISA to determine if such results might be false positives. This could include evaluations of peptide reactivity, determination of molecular weight, identification of results from related chemicals, human studies where ethically and scientifically justified, review of occupational exposures and postmarketing experience or monitoring, or *in vitro* testing data. All decision criteria should be reassessed as additional discriminators and data become available.

2.4 ICCVAM Recommendations: Performance Standards

ICCVAM concludes that the ICCVAM-recommended performance standards (ICCVAM 2009a) for the traditional LLNA can be used to evaluate any future modifications of the LLNA: BrdU-ELISA. The ICCVAM-recommended performance standards for the traditional LLNA apply to the LLNA: BrdU-ELISA because the test method is functionally and mechanistically similar to the traditional LLNA. ICCVAM, in conjunction with ECVAM and JaCVAM, developed the internationally harmonized test method performance standards for the traditional LLNA (ICCVAM 2009a) to evaluate the performance of LLNA test methods that incorporate specific protocol modifications (e.g., procedures to measure lymphocyte proliferation) compared to the traditional LLNA. Thus, unique performance standards for the LLNA: BrdU-ELISA are not proposed at this time.

3.0 Validation Status of the LLNA: BrdU-ELISA Test Method

The ICCVAM BRD for the LLNA: BrdU-ELISA test method (**Appendix C**) provides a comprehensive review of the current validation status of the LLNA: BrdU-ELISA test method, including its accuracy and reliability, the substances tested, the rationale for the standardized protocol used for the validation studies, and all available data supporting its validity. This section provides a brief description and summary of the validation status of the LLNA: BrdU-ELISA test method.

3.1 Test Method Description

Originally developed by Takeyoshi et al. (2001) and refined during an interlaboratory validation study (Kojima et al. 2008), the purpose of the LLNA: BrdU-ELISA test method is to identify potential skin sensitizers by quantifying lymphocyte proliferation. Like the traditional LLNA, the magnitude of lymphocyte proliferation measured in the LLNA: BrdU-ELISA correlates with the extent to which sensitization develops after a topical induction exposure to a potential skin-sensitizing substance.

3.1.1 General Test Method Procedures

The test substance is administered topically on three consecutive days to the ears of mice at a concentration that provides maximum solubility of the test substance without systemic toxicity and/or excessive local irritation. Two days after the final application of the test substance, 10 mg/mL BrdU, a thymidine analog, in 0.5 mL physiological saline is administered via intraperitoneal injection to each mouse. Approximately 24 hours later, the draining auricular lymph nodes are excised, and a single-cell suspension from the lymph nodes of each animal is prepared for quantifying the incorporation of BrdU, which correlates with lymph node cell proliferation.

The incorporation of BrdU for each mouse is measured using an ELISA and is expressed in absorbance units. The SI is calculated as the ratio of the mean absorbance/mouse for each treatment group against the mean absorbance/mouse for the vehicle control group. Substances producing an SI greater than a specified threshold are considered to be sensitizers. Based on the accuracy evaluation described in **Section 3.4**, the optimum accuracy was produced by SI $\geq$ 1.6.

3.1.2 Similarities and Differences Between the Protocols for the Traditional LLNA and the LLNA: BrdU-ELISA

The differences between the traditional LLNA (Dean et al. 2001; Sailstad et al. 2001; ICCVAM 1999) and the LLNA: BrdU-ELISA include the marker used to detect lymphocyte proliferation, the route of administration of the marker, and time of lymph node excision. In the traditional LLNA, a radioactive marker such as ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine (in phosphate-buffered saline; 250 µL/mouse) is administered via the tail vein. Then, five hours later, the draining auricular lymph nodes are excised and prepared for quantifying the incorporation of radioactivity. As noted above, in the LLNA: BrdU-ELISA, a BrdU solution is injected intraperitoneally to each mouse, and the draining auricular lymph nodes are excised 24 hrs later. All other procedures for the two methods are identical.

3.2 Validation Database

The current validation database for the LLNA: BrdU-ELISA includes results from studies of 43 substances that had previously been tested in the traditional LLNA. These results were obtained from six published studies (Takeyoshi et al. 2003; 2004a; 2004b; 2005; 2006; 2007a), several unpublished studies (Takeyoshi M, unpublished data), one platform presentation (Takeyoshi 2007b), and one poster presentation (Kojima et al. 2008). The data from Takeyoshi et al. were generated in a

single laboratory while the data from Kojima et al. were generated in multiple laboratories during an interlaboratory validation study. Data for 31 substances were available and reviewed by the independent peer review panel in April 2009. Data for 12 additional substances and additional results for four previously tested substances were submitted after the Panel review. ICCVAM and the OECD Expert Consultation considered these additional data and the LLNA: BrdU-ELISA BRD was updated to include the additional data.

The reference test data for the 43 substances were obtained from traditional LLNA tests. Of the 43 substances, 32 were classified by the traditional LLNA as skin sensitizers and 11 were classified as nonsensitizers. GP skin sensitization data were available for 35 substances and human skin sensitization test data or clinical case report information was available for 41 substances (see **Appendix C, Annex III-1**).

Table 3-1 lists the 43 substances, uses, chemical classifications, traditional LLNA EC3 and maximum stimulation index (SI) values, and LLNA: BrdU-ELISA EC1.6 and maximum SI values. Nineteen chemical classes were represented by the substances tested in the LLNA: BrdU-ELISA; 11 substances were classified in more than one chemical class. The classes with the highest number of substances were carboxylic acids (13 substances) and aldehydes (six substances). Of the 22 chemical classes represented in the NICEATM LLNA database by at least five substances (thereby providing a sufficiently large representation for further analyses), 20 classes had at least 60% of the traditional LLNA results identified as positive. For this database of more than 600 substances, these classes were identified as those most likely to be associated with skin sensitization. Fifteen of these classes were also represented in the LLNA: BrdU-ELISA database (only amides, ethers, ketones, macromolecular substances, and polycyclic compounds were not included). Among the chemical classes that have been previously identified as common skin allergens (e.g., aldehydes, ketones, quinones, and acrylates, [Gerberick et al. 2004]), only ketones were not included in the LLNA: BrdU-ELISA database. Nevertheless, the Panel considered the database of substances tested in the LLNA: BrdU-ELISA to be representative of a sufficient range of chemicals typically tested for skin sensitization potential. The traditional LLNA EC3 values (i.e., estimated concentration needed to produce SI = 3) for the 33 sensitizers ranged from 0.009% to 47.5%.

Physicochemical characteristics for the 43 substances are provided in **Appendix C, Annex II**. Molecular weights ranged from 30.03 to 388.29 g/mole. Twenty-five substances are liquids and 18 substances are solids. Log octanol: water partition coefficients, which were available for 41 substances, ranged from -3 to 3.88. Peptide reactivity, which was available for 22 substances, ranged from high to minimal (Gerberick et al. 2007).

Table 3-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: BrdU-ELISA EC1.6 Values, and Maximum SI Values for 43 Tested Substances

Substance Name	Product Use[1]	Chemical Class[2]	Traditional LLNA EC3 (Maximum SI)[3]	LLNA: BrdU-ELISA EC1.6 (Maximum SI)[3]
5-Chloro-2-methyl-4-isothaizolin-3-one*	Cosmetics; Manufacturing; Pesticides	Sulfur Compounds; Heterocyclic Compounds	0.009 (27.7)	0.065 (4.8)
p-Benzoquinone	Manufacturing; Pesticides; Pharmaceuticals	Quinones	0.010 (52.3)	0.150 (6.9)
2,4-Dinitrochlorobenzene*	Manufacturing; Pesticides	Hydrocarbon, Halogenated; Nitro Compounds; Hydrocarbons, Cyclic	0.049 (43.9)	0.032 (18.8)
Diphenylcyclopropenone	Pharmaceuticals	Hydrocarbons, Cyclic	0.050 (NA)	0.450 (19.1)
Glutaraldehyde	Cosmetics; Disinfectant; Manufacturing; Pesticides	Aldehydes	0.083 (18.0)	0.115 (28.6)
4-Phenylenediamine*	Intermediate in chemical synthesis; Manufacturing	Amines	0.11 (26.4)	0.285 (14.7)
Formaldehyde	Disinfectant; Manufacturing	Aldehydes	0.50 (4.0)	0.163 (16.6)
Cobalt chloride*	Manufacturing; Pesticides	Inorganic Chemical, Elements; Inorganic Chemical, Metals	0.66 (7.2)	0.316 (3.7)
4-Methylaminophenol sulfate	Manufacturing	Amines; Phenols	0.8 (6.7)	1.081 (4.0)
trans-Cinnamaldehyde	Food additive; Fragrance agent	Aldehydes	1.4 (13.1)	1.530 (5.9)
Isoeugenol*	Food additive; Fragrance agent	Carboxylic Acids	1.5 (31.0)	5.156 (8.4)
2-Mercaptobenzothiazole*	Manufacturing; Pesticides	Heterocyclic Compounds	1.7 (8.6)	12.097 (1.6)
Cinnamic aldehyde	Cosmetics; Food additive; Fragrance agent; Intermediate in chemical synthesis; Personal care products; Pesticides	Aldehydes	1.9 (18.4)	4.808 (4.0)
3-Aminophenol	Cosmetics; Pharmaceuticals	Amines; Phenols	3.2 (5.7)	2.990 (3.1)

Substance Name	Product Use[1]	Chemical Class[2]	Traditional LLNA EC3 (Maximum SI)[3]	LLNA: BrdU-ELISA EC1.6 (Maximum SI)[3]
Diethyl maleate	Food additive; Intermediate in chemical synthesis	Carboxylic Acids	3.6 (22.6)	8.049 (6.3)
Trimellitic anhydride	Manufacturing	Anhydrides; Carboxylic Acids	4.7 (4.6)	0.862 (7.9)
Nickel sulfate	Manufacturing	Inorganic Chemicals, Metals; Inorganic Chemicals, Elements	4.8 (3.1)	1.027 (4.5)
4-Chloroaniline	Intermediate in chemical synthesis; Manufacturing; Pesticides; Pharmaceuticals	Amines	9.00 (3.3)	11.029 (2.5)
Sodium lauryl sulfate*	Cosmetics; Food additive; Manufacturing; Personal care products; Pesticides; Pharmaceuticals	Alcohols; Sulfur Compounds; Lipids	8.1 (8.9)	13.334 (2.6)
Citral*	Fragrance agent	Hydrocarbons, Other	9.2 (20.5)	7.143 (16.4)
Hexyl cinnamic aldehyde*	Food additive; Fragrance agent	Aldehydes	9.7 (20.0)	12.920 (13.5)
Eugenol*	Cosmetics; Food additive; Intermediate in chemical synthesis; Manufacturing; Personal care products; Pharmaceuticals	Carboxylic Acids	10.1 (17.0)	8.851 (17.7)
Phenyl benzoate*	Manufacturing; Pesticides	Carboxylic Acids	13.6 (11.1)	16.954 (3.4)
Cinnamic alcohol*	Cosmetics; Food additive; Fragrance agent; Intermediate in chemical synthesis; Personal care products	Alcohols	21.0 (5.7)	24.091 (2.7)
Cyclamen aldehyde	Food additive; Fragrance agent	Aldehydes	22.3 (5.2)	41.496 (5.7)
Hydroxycitronellal	Food additive; Fragrance agent; Personal care products	Hydrocarbons, Other	24.0 (8.5)	13.636 (4.8)
Imidazolidinyl urea*	Cosmetics; Personal care products; Pesticides	Urea	24.0 (5.5)	49.545 (1.6)
Ethylene glycol dimethacrylate*	Manufacturing	Carboxylic Acids	28.0 (7.0)	31.751 (3.1)
Linalool	Cosmetics; Food additive; Fragrance agent; Personal care products; Pesticides	Hydrocarbons, Other	30.0 (8.3)	27.596 (4.7)

Substance Name	Product Use[1]	Chemical Class[2]	Traditional LLNA EC3 (Maximum SI)[3]	LLNA: BrdU-ELISA EC1.6 (Maximum SI)[3]
Ethyl acrylate	Manufacturing	Carboxylic Acids	32.8 (4.0)	33.333 (5.0)
Isopropyl myristate	Cosmetics; Personal care products; Pharmaceuticals	Lipids	44.0 (3.4)	9.404 (4.2)
Aniline	Food additive; Manufacturing; Personal care products; Pesticides; Pharmaceuticals	Amines	47.5 (4.4)	73.596 (2.1)
2-Hydroxypropyl methacrylate	Intermediate in chemical synthesis; Manufacturing	Carboxylic Acids	NC (1.3)	NC (1.1)
Diethyl phthalate	Cosmetics; Manufacturing; Personal care products; Pesticides; Pharmaceuticals	Carboxylic Acids	NC (1.5)	NC (0.9)
Dimethyl isophthalate	Manufacturing; Fragrance agent	Carboxylic Acids	NC (1.0)	NC (1.3)
Glycerol	Cosmetics; Food additive; Intermediate in chemical synthesis; Manufacturing; Personal care products; Pharmaceuticals; Solvent	Alcohols; Carbohydrates	NC (1.1)	NC (1.3)
Hexane	Manufacturing; Solvent	Hydrocarbons, Acyclic	NC (2.2)	56.328 (1.9)
Isopropanol*	Cosmetics; Disinfectant; Food additive; Intermediate in chemical synthesis; Manufacturing; Personal care products; Pharmaceuticals; Solvent	Alcohols	NC (1.7)	5.344 (2.2)[4]
Lactic acid*	Food additive; Manufacturing; Pharmaceuticals	Carboxylic Acids	NC (2.2)	15.177 (2.5)
Methyl salicylate*	Cosmetics; Food additive; Fragrance agent; Personal care products; Pharmaceuticals; Solvent	Carboxylic Acids	NC (2.9)	NC (1.4)
Salicylic acid*	Food additive; Manufacturing; Pharmaceuticals	Phenols; Carboxylic Acids	NC (2.5)	NC (1.3)
Sulfanilamide	Pharmaceuticals	Hydrocarbons, Cyclic; Sulfur Compounds	NC (1.0)	NC (1.3)
Propylene glycol	Cosmetics; Food additive; Intermediate in chemical synthesis; Personal care products; Pharmaceuticals; Solvent	Alcohols	NC (1.6)	NC (1.6)

Abbreviations: EC3 = estimated concentration (expressed as percentage) needed to produce SI = 3; EC1.6 = estimated concentration (expressed as percentage) needed to produce SI = 1.6; LLNA = murine local lymph node assay; LLNA: BrdU-ELISA= local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; NA = not available; NC = not calculated since maximum SI < 3.0 for the traditional LLNA or maximum SI < 1.6 for the LLNA: BrdU-ELISA; SI = stimulation index.

* Reference substance from ICCVAM (2009a).

[1] Information gathered from the following databases:
Hazardous Substances Database (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB)
Haz-Map (http://hazmap.nlm.nih.gov/)
Household Products Database (http://hpd.nlm.nih.gov/index.htm)
International Programme on Chemical Safety INCHEM database (http://www.inchem.org/)
National Toxicology Program (http://ntp.niehs.nih.gov:8080/index.html?col=010stat).

[2] Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs, developed by the National Library of Medicine (http://www.nlm.nih.gov/mesh/meshhome.html).

[3] Mean EC3 (expressed as percent concentration) and maximum SI values are from the NICEATM database of traditional LLNA studies. EC1.6 and SI values for individual LLNA: BrdU-ELISA tests are provided in Annex IV of the BRD (**Appendix C**).

[4] Highest SI of seven tests. Because the majority (five) of the seven tests, had SI values < 1.6, isopropanol is considered to be a nonsensitizer in the LLNA: BrdU-ELISA.

3.3 Reference Test Method Data

Thirty-five of the 43 substances that were tested in the traditional LLNA were considered in the original evaluation of the LLNA by ICCVAM (ICCVAM 1999). The traditional LLNA reference data used for the accuracy evaluation were obtained from ICCVAM (1999) for 33 of these substances. Data for two substances which were negative in the original LLNA evaluation (ICCVAM 1999), aniline and nickel sulfate, were obtained from more recent sources that tested higher concentrations and obtained positive results. The traditional LLNA data for the remaining eight substances that were not considered in the original ICCVAM evaluation were obtained from the scientific literature. The reference data for GP tests (GPMT or Buehler test) and human tests (human maximization test, human patch test allergen, or other human data) were also obtained from the original LLNA evaluation (ICCVAM 1999) and the scientific literature. The LLNA, GP, and human reference data and sources for the 43 substances evaluated are provided in Annex III of the BRD (**Appendix C**).

3.4 Test Method Accuracy

The ICCVAM evaluation of the LLNA: BrdU-ELISA included an assessment of multiple decision criteria including SI ≥ 2.0, the threshold for distinguishing sensitizers and nonsensitizers that was used in the protocol for the interlaboratory validation study (Kojima et al. 2008) (**Table 3-2**). When the optimal decision criterion of SI ≥ 1.6 was used to identify sensitizers vs. nonsensitizers, compared to the traditional LLNA, accuracy was 95% (41/43), with a false positive rate of 18% (2/11) and a false negative rate of 0% (0/32). The two false positive substances, hexane (SI = 1.76 and 1.89) and lactic acid (SI = 1.80, 1.89, and 2.53), produced SI values between 1.6 and 1.9 in the LLNA: BrdU-ELISA. Other available information such as dose-response, evidence of systemic toxicity or excessive local irritation, and (where appropriate) statistical significance together with SI values should be considered to confirm that such borderline results are potential skin sensitizers. Consideration should also be given to various properties of the test substance, including whether it is structurally similar to known skin sensitizers. For example, peptide reactivity (Gerberick et al. 2007) could be used to interpret LLNA: BrdU-ELISA results when borderline positive results (e.g., SI values between 1.6 and 1.9) are produced to confirm that such results are not false positive. Both of the LLNA nonsensitizers with positive results in the LLNA: BrdU-ELISA, lactic acid and hexane, had minimal peptide reactivity. No unique characteristics were identified that could be used as rationale for excluding any particular types of substances from testing in the LLNA: BrdU-ELISA.

An evaluation to determine the robustness of the optimum SI ≥ 1.6 criterion indicated that the SI was quite stable. Taking different samples of the data as training and validation sets had relatively little impact on the cutoff SI criteria or on the resulting number of false positives or false negatives (**Appendix C, Annex VII**).

Figure 3-1 shows that SI values for the LLNA: BrdU-ELISA are generally lower than those for the traditional LLNA at comparable test doses. SI values for substances with more than one test result are represented by the geometric mean with bars to show the overall range of individual study results used to calculate the geometric mean. The purpose of showing the geometric mean and associated ranges is to provide an assessment of variability among results, and the relative sensitivity of the traditional LLNA and LLNA: BrdU-ELISA results. However, the accuracy analyses reported in the BRD are based on individual test results and not on a geometric mean. The SI values for **Figure 3-1** are provided in **Table 3-3**.

Table 3-2 Performance of the LLNA: BrdU-ELISA for 43 Substances in Predicting Skin Sensitizing Potential Using Alternative Decision Criteria to Identify Sensitizers

Alternate Criterion	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate		Positive Predictivity		Negative Predictivity	
	%	(No.[1])	%	(No.[1])	%	(No.[1])	%	(No.[1])	%	(No.[1])	%	(No.[1])	%	(No.[1])
Statistics[2]	91	(39/43)	97	(31/32)	73	(8/11)	27	(3/11)	3	(1/32)	91	(31/34)	89	(8/9)
≥95% CI[3]	88	(38/43)	100	(32/32)	54	(6/11)	46	(5/11)	0	(0/32)	86	(32/37)	100	(6/6)
≥2 SD[4]	91	(39/43)	100	(32/32)	64	(7/11)	36	(4/11)	0	(0/32)	89	(32/36)	100	(7/7)
≥3 SD[5]	91	(39/43)	91	(29/32)	91	(10/11)	9	(1/11)	9	(3/32)	97	(29/30)	77	(10/13)
SI ≥ 5.0	49	(21/43)	31	(10/32)	100	(11/11)	0	(0/11)	69	(22/32)	100	(10/10)	33	(11/33)
SI ≥ 4.5	58	(25/43)	44	(14/32)	100	(11/11)	0	(0/11)	56	(18/32)	100	(14/14)	38	(11/29)
SI ≥ 4.0	63	(27/43)	50	(16/32)	100	(11/11)	0	(0/11)	50	(16/32)	100	(16/16)	41	(11/27)
SI ≥ 3.5	74	(32/43)	66	(21/32)	100	(11/11)	0	(0/11)	34	(11/32)	100	(21/21)	50	(11/22)
SI ≥ 3.0	84	(36/43)	78	(25/32)	100	(11/11)	0	(0/11)	22	(7/32)	100	(25/25)	61	(11/18)
SI ≥ 2.5	93	(40/43)	91	(29/32)	100	(11/11)	0	(0/11)	9	(3/32)	100	(29/29)	79	(11/14)
SI ≥ 2.0	95	(41/43)	94	(30/32)	100	(11/11)	0	(0/11)	6	(2/32)	100	(30/30)	85	(11/13)
SI ≥ 1.9	95	(41/43)	94	(30/32)	100	(11/11)	0	(0/11)	6	(2/32)	100	(30/30)	85	(11/13)
SI ≥ 1.6	95	(41/43)	100	(32/32)	82	(9/11)	18	(2/11)	0	(0/32)	94	(30/32)	100	(9/9)
SI ≥ 1.5	95	(41/43)	100	(32/32)	82	(9/11)	18	(2/11)	0	(0/32)	94	(30/32)	100	(9/9)
SI ≥ 1.3	93	(40/43)	100	(32/32)	73	(8/11)	27	(3/11)	0	(0/32)	91	(32/35)	100	(8/8)

Abbreviations: CI = confidence interval; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine (BrdU); No. = number; SD = standard deviation; SI = stimulation index

[1] The proportion on which the percentage calculation is based.

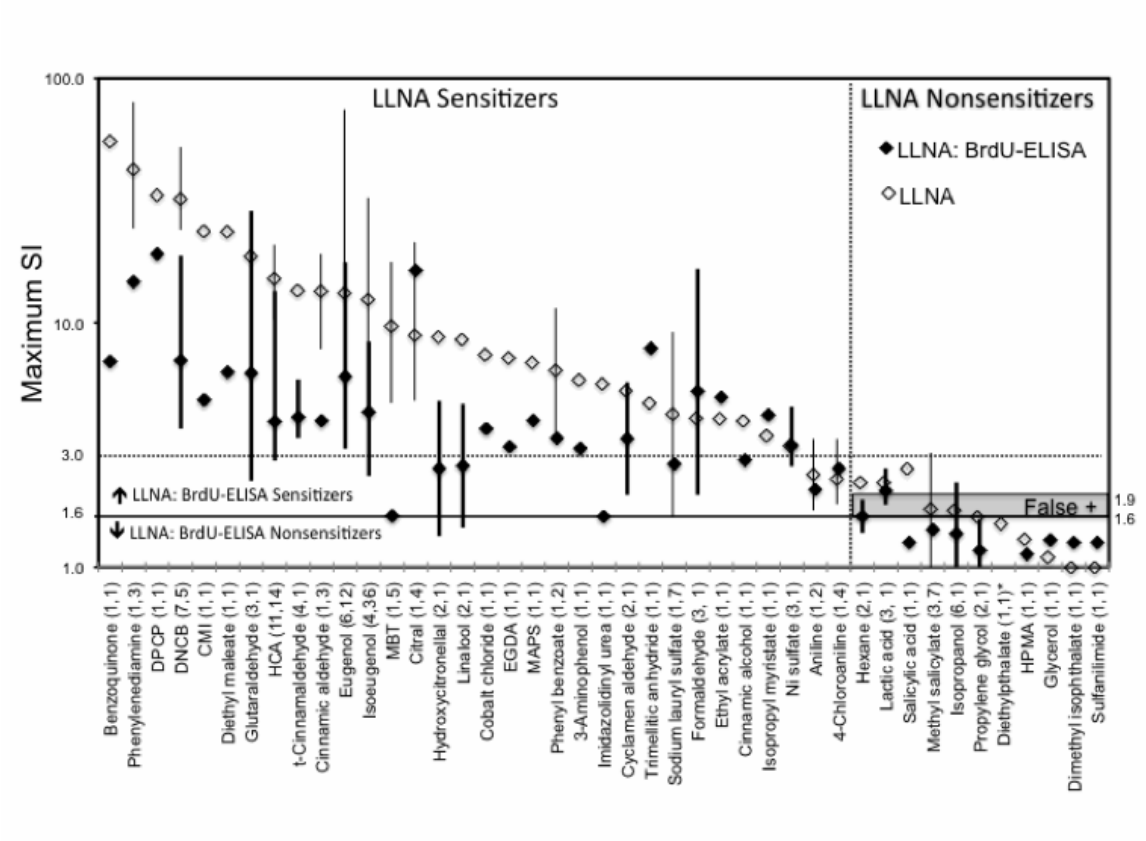
[2] Analysis of variance for difference of group means when substances were tested at multiple doses or *t*-test when substances were tested at one dose. The absorbance data were log-transformed prior to analysis of variance. Significance at $p < 0.05$ was further tested by Dunnett's test.

[3] The mean absorbance of at least one treatment group was outside the 95% confidence interval for the mean absorbance of the vehicle control group.

[4] The mean absorbance of at least one treatment group was greater than 3 SD from the mean absorbance of the vehicle control group.

[5] The mean absorbance of at least one treatment group was greater than 2 SD from the mean absorbance of the vehicle control group.

Figure 3-1 Comparison of LLNA: BrdU-ELISA Stimulation Index with Traditional LLNA Stimulation Index[1]



Abbreviations: CMI = 5-chloro-2-methyl-4-isothiazoline-3-one solution; DPCP = diphenylcyclopropanone; DNCB = 2,4-dinitrochlorobenzene; EGDA = ethylene glycol dimethacrylate; False + = false positive results in the LLNA: BrdU-ELISA (based on most prevalent result for substances with multiple tests) were in the SI range between 1.6 and 1.9; HCA = hexyl cinnamic aldehyde; HPMA = 2-hydroxypropyl methacrylate; LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; MAPS = 4-methyl aminophenol sulfate; MBT = 2-mercaptobenzothiazole; Ni = nickel; SI = stimulation index.

[1] LLNA: BrdU-ELISA and traditional LLNA responses at comparable test doses are shown. Symbols show the SI for substances with one test result or geometric mean maximum SI for substances with more than one test result. **Table 3-3** shows the individual values used. Bars show the range of values reported for multiple test results (heavy bars for LLNA: BrdU-ELISA and light bars for traditional LLNA). Numbers in parentheses beside the chemical names show the number of SI values for the LLNA: BrdU-ELISA and then the number of SI values for the traditional LLNA used in this figure. The number of SI values used in the figure may be different from the total number of SI values available since only comparable test doses and vehicles were used in this figure. The accuracy analyses used individual test results rather than geometric mean SI values. Using individual test results, traditional LLNA nonsensitizers with maximum SI between 1.6 and 1.9 include hexane and lactic acid.

* The LLNA: BrdU-ELISA SI for diethyl phthalate is outside of the displayed data range and is not shown (SI < 1).

Table 3-3 Maximum SI Values of 43 Substances Evaluated in the LLNA: BrdU-ELISA Compared to the Traditional LLNA

Substance Name[1]	Test Vehicle[2]	LLNA: BrdU-ELISA Maximum SI Values[3]	Traditional LLNA Maximum SI Values
Sensitizers (LLNA: BrdU-ELISA SI ≥ 1.6 and Traditional LLNA SI ≥ 3.0)			
Benzoquinone (1,1)	AOO	6.94	52.30
1,4-Phenylenediamine (1,3)	AOO	14.70	23.30, 37.40, 75.30
Diphenylcyclopropenone (1,1)	AOO/ACE	19.10	31.70
2,4-Dinitrochlorobenzene (7,5)	AOO	3.68, 4.50, 5.29, 6.26, 6.53, 12.30, 18.80	23.00, 24.00, 26.80, 36.70, 49.60
CMI (1,1)	DMF	4.83	22.70
Diethyl maleate (1,1)	AOO	6.27	22.60
Glutaraldehyde (3,1)	ACE	2.25, 3.72, 28.60	18.00
HCA (11,14)	AOO	2.72, 2.87, 3.02, 3.27, 3.34, 3.40, 3.60, 3.64, 3.84, 5.90, 13.50	10.00, 11.60, 11.60, 13.40, 14.00, 14.00, 14.10, 14.50, 16.00, 17.00, 17.00, 17.00, 17.60, 20.00
trans-Cinnamaldehyde (4,1)	AOO	3.37, 3.50, 4.11, 5.86	13.10
Cinnamic aldehyde (1,3)	AOO	3.97	7.60, 15.80, 18.40
Eugenol (6,12)	AOO	3.05, 3.17, 3.18, 7.09, 12.30, 17.70	4.01, 6.10, 9.30, 9.60, 10.20, 12.40, 14.10, 16.00, 16.10, 16.10, 17.00, 70.30
Isoeugenol (4,36)	AOO	2.36, 2.43, 7.20, 8.36	4.10, 4.90, 5.00, 5.60, 6.70, 6.80, 7.20, 7.20, 7.50, 7.50, 7.60, 8.70, 10.00, 11.00, 11.10, 11.80, 12.40, 13.80, 13.10, 13.10, 13.10, 14.10, 14.70, 14.70, 15.30, 17.00, 18.40, 19.00, 23.20, 19.20, 19.30, 23.20, 23.60, 24.40, 29.80, 31.00
MBT (1,5)	DMF	1.62	4.60, 9.10, 9.50, 10.80, 17.10
Citral (1,4)	AOO	16.40	4.70, 6.20, 9.30, 20.50
Hydroxycitronellal (2,1)	AOO	1.34, 4.78	8.50
Linalool (2,1)	AOO	1.45, 4.65	8.30
Cobalt chloride (1,1)	DMSO	3.68	7.21
EGDA (1,1)	MEK	3.11	7.00
MAPS (1,1)	DMF	3.98	6.70
Phenyl benzoate (1,2)	DMF/AOO	3.37	3.50, 11.10
3-Aminophenol (1,1)	AOO	3.06	5.70

continued

Table 3-3 Maximum SI Values of 43 Substances Evaluated in the LLNA: BrdU-ELISA Compared to the Traditional LLNA (continued)

Substance Name[1]	Test Vehicle[2]	LLNA: BrdU-ELISA Maximum SI Values[3]	Traditional LLNA Maximum SI Values
Sensitizers (LLNA: BrdU-ELISA SI ≥ 1.6 and Traditional LLNA SI ≥ 3.0)			
Imidazolidinyl urea (1,1)	DMF	1.61	5.50
Cyclamen aldehyde (1,1)	AOO	1.97, 5.71	5.16
Trimellitic anhydride (1,1)	AOO	7.85	4.60
Sodium lauryl sulfate (1,7)	DMF	2.64	1.60, 2.60, 4.10, 5.10, 5.10, 5.40, 8.90
Formaldehyde (3, 1)	ACE	1.97, 4.40, 16.60	4.00
Ethyl acrylate (1,1)	AOO	4.95	3.98
Cinnamic alcohol (1,1)	AOO	2.74	3.90
Isopropyl myristate (1,1)	AOO	4.19	3.40
Ni sulfate (3,1)	DMSO	2.58, 2.66, 4.53	3.10
Aniline (1,2)	AOO	2.07	1.70, 3.30
4-Chloroaniline (1,4)	AOO	2.53	1.80, 1.80, 2.50, 3.30
Traditional LLNA Nonsensitizers (SI <3.0) with Borderline Positive SI Values in LLNA: BrdU-ELISA (1.6 < SI < 1.9; see bold text)			
Hexane (1,1)	AOO	1.38, **1.89**	2.20
Lactic acid (3,1)	DMSO	**1.80**, **1.89**, 2.53	2.20
Nonsensitizers (LLNA: BrdU-ELISA SI < 1.6 and Traditional LLNA SI < 3.0)			
Salicylic acid (1,1)	AOO	1.26	2.50
Methyl salicylate (3,7)	AOO	1.40, 1.44, 1.44	0.90, 1.10, 1.72, 1.90, 2.10, 2.30, 2.90
Isopropanol (6,1)	AOO	0.94, 0.98, 1.01, 1.57, 2.04, 2.22	1.70
Propylene glycol (2,1)	AOO/Water	0.87, 1.57	1.60
Diethyl phthalate (1,1)	AOO	0.88	1.50
HPMA (1,1)	AOO	1.13	1.30
Glycerol (1,1)	Water/DMF	1.29	1.10
Dimethyl isophthalate (1,1)	AOO	1.26	1.00
Sulfanilamide (1,1)	DMF	1.26	1.00

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); CMI = 5-Chloro-2-methyl-4-isothiazoline-3-one solution; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; EGDA = ethylene glycol dimethacrylate; HCA = hexyl cinnamic aldehyde; HPMA = 2-hydroxypropyl methacrylate; LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; MAPS = 4-methyl aminophenol sulfate; MBT = 2-mercaptobenzothiazole; Ni sulfate = nickel (II) sulfate hexahydrate; SI = stimulation index.

[1] Numbers in parentheses beside the substance names indicate the number of tests for the LLNA: BrdU-ELISA followed by the traditional LLNA, which may differ from the total number of tests available since only the most comparable test doses and vehicles were included.

[2] The vehicle used was the same in LLNA: BrdU-ELISA and traditional LLNA tests, except where indicated (e.g., vehicle used in the LLNA: BrdU-ELISA/vehicle used in the traditional LLNA).

[3] The bold text indicates SI values having potential false positive results (1.6 < SI < 1.9) for individual LLNA: BrdU-ELISA tests

3.5 Test Method Reliability (Intra- and Interlaboratory Reproducibility)

The BRD details the evaluation of intra- and interlaboratory reproducibility of the LLNA: BrdU-ELISA test method. Intralaboratory reproducibility was assessed using a concordance analysis of sensitizer/nonsensitizer results, and a coefficient of variation (CV) analysis of SI values and EC1.6 values (estimated concentration needed to produce an SI of 1.6). The qualitative analysis shows that multiple tests of 12 substances (10 LLNA sensitizers and two nonsensitizers) yielded 100% concordance for sensitizer/nonsensitizer outcomes for 83% (10/12) of the substances. The concordant results for one nonsensitizer, hexane, however, were incorrectly positive for both tests (2/2 tests had SI ≥ 1.6). In the quantitative analyses, the CVs for the SI values of 13 substance/concentration combinations that were tested up to five times each ranged from 1% to 80%. In addition, the CVs for the EC1.6 values of four substances that were tested up to five times at multiple doses ranged from 37% to 118%.

When using SI ≥ 1.6 as the threshold to distinguish sensitizers from nonsensitizers, the qualitative interlaboratory reproducibility analysis of 10 substances (seven sensitizers and three nonsensitizers) that were tested in three to seven laboratories indicated 100% agreement (3/3, 6/6, or 7/7) among the laboratories for nine substances (seven sensitizers and two nonsensitizers). However, one of the nonsensitizers, lactic acid, for which there was 100% agreement among the laboratories, was a false positive (i.e., 3/3 laboratories had SI ≥ 1.6). There was 67% (4/6) agreement among the tests for the remaining nonsensitizer. Interlaboratory CVs for the EC1.6 values of the seven sensitizers ranged from 31% to 93%.

When using SI ≥ 1.6 to classify sensitizers, the concordance analysis for the 18 substances with multiple tests indicated that the SI results for 85% (11/13) of the sensitizers (based on traditional LLNA results) were 100% concordant (i.e., all tests yielded SI ≥ 1.6) (**Table 3-4**). The SI results for the remaining two sensitizers included one test with SI < 1.6 and another test with SI > 1.6. The SI results for 60% (3/5) of the nonsensitizers were 100% concordant. All tests for two of the three nonsensitizers yielded SI < 1.6. All tests for the third nonsensitizer yielded SI values between 1.6 and 1.9, the narrow region in which false positive results occurred. The concordance for the other two nonsensitizers was 71% (5/7) for SI < 1.6 and 67% (2/3) for SI values between 1.6 and 1.9.

Table 3-4 Concordance of LLNA: BrdU-ELISA Tests across Maximum SI Categories

Substance	LLNA: BrdU-ELISA Nonsensitizers (Maximum SI ≤ 1.6[1])	LLNA: BrdU-ELISA Sensitizers (Maximum SI ≥ 1.6)		Total Tests
		1.6 < Maximum SI < 1.9[1]	Maximum SI ≥ 1.9[1]	
Sensitizers[2]				
Cyclamen aldehyde	0 (0%)	0 (0%)	0 (100%)	2
2,4-Dinitrochloro-benzene	0 (0%)	0 (0%)	9 (100%)	9
Diphenylcyclopro-penone	0 (0%)	0 (0%)	3 (100%)	3
Eugenol	0 (0%)	0 (0%)	9 (100%)	9
Formaldehyde	0 (0%)	0 (0%)	3 (100%)	3
Glutaraldehyde	0 (0%)	0 (0%)	5 (100%)	5

continued

Table 3-4 Concordance of LLNA: BrdU-ELISA Tests across Maximum SI Categories (continued)

Substance	LLNA: BrdU-ELISA Nonsensitizers (Maximum SI ≤ 1.6[1])	LLNA: BrdU-ELISA Sensitizers (Maximum SI ≥ 1.6)		Total Tests
		1.6 < Maximum SI < 1.9[1]	Maximum SI ≥ 1.9[1]	
Sensitizers[2]				
Hexyl cinnamic aldehyde	0 (0%)	0 (0%)	12 (100%)	12
Hydroxycitronellal	1 (50%)	0 (0%)	1 (50%)	2
Isoeugenol	0 (0%)	0 (0%)	3 (100%)	3
Linalool	1 (50%)	0 (0%)	1 (50%)	2
Nickel sulfate	0 (0%)	0 (0%)	3 (100%)	3
1,4-Phenylenediamine	0 (0%)	0 (0%)	2 (100%)	2
trans-Cinnamaldehyde	0 (0%)	0 (0%)	4 (100%)	4
Nonsensitizers[2]				
Hexane	0 (0%)	2 (100%)	0 (%)	2
Isopropanol	5 (71%)	0 (0%)	2 (29%)	7
Lactic acid	0 (0%)	2 (67%)	1 (33%)	3
Methyl salicylate	3 (100%)	0 (0%)	0 (0%)	3
Propylene glycol	3 (100%)	0 (0%)	0 (0%)	3

Abbreviations: LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SI = stimulation index.

[1] Numbers shown reflect number of tests. Percentage in parentheses reflects percentage of the total number of tests for each substance.

[2] According to traditional murine local lymph node assay results.

3.6 Animal Welfare Considerations: Reduction, Refinement, and Replacement

The LLNA: BrdU-ELISA will use the same number of animals as the updated ICCVAM-recommended traditional LLNA protocol (Appendix A of ICCVAM 2009a). However, since use of the traditional LLNA is restricted in some countries and institutions because of limitations on handling radioactivity, availability and use of the nonradioactive LLNA: BrdU-ELISA may lead to further reduction in use of the GP tests, which would provide for reduced animal use and increased refinement due to the avoidance of pain and distress that occur in the GP tests when substances cause ACD. Additionally, the LLNA: BrdU-ELISA test method protocol requires fewer mice per treatment group (a minimum of four animals/group) than either of the GP tests (10-20 animals/group for the Buehler test and 5-10 animals/group for the GPMT).

4.0 ICCVAM Consideration of Independent Peer Review Panel Report and Other Comments

The ICCVAM evaluation process incorporates a high level of scientific peer review and transparency. The evaluation process for the LLNA: BrdU-ELISA included two public review meetings by an independent scientific peer review panel, multiple opportunities for public comments (see **Section 1.0**), consideration of the OECD Expert Consultation on the LLNA, and comments from the SACATM. ICCVAM and the IWG considered the Panel report, conclusions of the OECD Expert Consultation, the SACATM comments, and all public comments before finalizing the ICCVAM Test Method Evaluation Report and final BRD for the LLNA: BrdU-ELISA. This chapter summarizes the ICCVAM consideration of these reports and comments. The peer review panel reports and public comments are provided as **Appendices D** and **E**, respectively. The report of the OECD Expert Consultation on the LLNA is not publicly available.

4.1 ICCVAM Consideration of Independent Peer Review Panel Report and OECD Comments

4.1.1 Comments on Revised Draft ICCVAM Recommendations: Test Method Usefulness and Limitations

The Panel agreed that the available data and test method performance supported the use of the LLNA: BrdU-ELISA to identify substances as potential sensitizers and nonsensitizers, with certain limitations. The Panel noted that the accuracy analysis they reviewed supported using two decision criteria (i.e., one to identify sensitizers and one to identify nonsensitizers). The Panel emphasized that the decision criteria were empirically derived from the data and produced the best combination of maximum accuracy coupled with the minimum number of results in the range of uncertainty (i.e., the range in which maximum SI results were between the decision criteria for sensitizers and nonsensitizers). Since using two decision criteria allows for a more definitive identification of sensitizers and nonsensitizers, this approach provides animal welfare benefits by reducing further tests that might be required in instances where the hazard classification of a substance is not as clear. In addition, one can use statistical analysis and/or other data and information (e.g., peptide reactivity, quantitative structure-activity relationships, skin penetration information) to provide more information on compounds that fall in the range of uncertainty. However, the Panel questioned how results in the range of uncertainty would be useful for regulatory purposes and emphasized that additional guidance would be needed on how to classify substances with SI values in the range of uncertainty.

The OECD LLNA Expert Consultation viewed that despite certain limitations, the LLNA: BrdU-ELISA is useful as a modified LLNA test method that has the potential to reduce the number of animals required and refine the way in which animals are used for ACD testing. The experts reviewed LLNA: BrdU-ELISA results for 12 additional substances and four substances previously tested that were received by NICEATM after the Panel meeting. Like the Panel, OECD member country experts questioned the regulatory utility of the LLNA: BrdU-ELISA since specific guidance on how to classify substances with SI values in the range of uncertainty had not been developed. Therefore, they recommended instead that a single decision criterion (as was originally proposed by ICCVAM and reviewed by the Panel in 2008) would be more useful to identify substances as potential sensitizers. They agreed with ICCVAM that SI $\geq$ 1.6 provided the optimal test method performance by preventing false negative results. They also agreed with ICCVAM that users may want to consider additional information such as dose-response, evidence of systemic toxicity and/or excessive local skin irritation, and where appropriate, statistical

significance together with SI values to confirm borderline positive results (i.e., SI between 1.6 and 1.9) as potential skin sensitizers.

ICCVAM considered the Panel report and the OECD Expert Consultation recommendations, and concluded that the single SI decision criterion of SI ≥ 1.6 to classify sensitizers would avoid false negative results as well as indeterminate results, which are not useful for regulatory purposes. Borderline results that may occur between 1.6 and 1.9 could be evaluated using other information to confirm the result.

4.1.2 Comments on Revised Draft ICCVAM Recommendations: Test Method Protocol

The Panel concurred with ICCVAM that the validation studies indicated that the standardized protocol was sufficiently transferable and reproducible. The Panel agreed that laboratories should maintain a historical database of positive control SI values and some measure of variability over time. The evaluation of the variation in positive control responses over time has wide applicability to a broad range of test systems.

The Panel agreed with the ICCVAM-recommended protocol, which indicated that 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 three consecutive doses. The OECD Expert Consultation also agreed and emphasized that the highest dose should be the concentration that maximizes exposure while avoiding systemic toxicity and/or excessive local skin irritation after topical application in the mouse. In the absence of such information, and consistent with the updated ICCVAM recommended protocol, a prescreen test should be performed in order to define the appropriate dose level to test in the LLNA: BrdU-ELISA. The Panel and the OECD Expert Consultation agreed in principle with ICCVAM that use of a reduced LLNA: BrdU-ELISA test method protocol instead of the multidose LLNA: BrdU-ELISA test method protocol has the potential to reduce the number of animals used in a test by omitting the middle and low dose groups. However, some members of the OECD Expert Consultation speculated that the reduced LLNA would have limited regulatory use and therefore the extent of potential animal savings is difficult to estimate.

4.1.3 Comments on the Revised Draft ICCVAM Recommendations: Future Studies

The Panel concurred with ICCVAM's revised draft recommendations for future studies, emphasizing that additional decision criteria and guidance should be identified for substances that produce SI values in the range of uncertainty, and that the additional decision criteria should be reassessed as additional discriminators and data become available (e.g., high-quality human ACD data). While the range of uncertainty is eliminated when using the single decision criterion of SI ≥ 1.6, the OECD Expert Consultation recommended that borderline positive results (i.e., SI values between 1.6 and 1.9) be further evaluated to determine if they are correctly identified as potential skin sensitizers.

The Panel recommended further consideration of statistical issues, including how to determine and evaluate classification methods (i.e., classification cutoff points). The Panel also recommended that future interlaboratory validation studies should simultaneously evaluate intralaboratory reproducibility, using appropriate statistics, to evaluate variation both within a laboratory and between laboratories.

ICCVAM considered the Panel report and the OECD Expert Consultation recommendations and concluded that efforts should be made to further characterize the sensitization potential of

borderline positive substances that produce an SI between 1.6 and 1.9 in the LLNA: BrdU-ELISA to confirm that such results are not false positive.

4.1.4 Comments on Revised Draft ICCVAM Recommendations: Performance Standards

The Panel agreed that the ICCVAM-recommended LLNA performance standards state the essential test method requirements, and the LLNA: BrdU-ELISA adheres to them such that it should be considered mechanistically and functionally similar. The only variation with the traditional LLNA is the means by which lymphocyte proliferation during the induction phase is evaluated. Likewise, the OECD Expert Consultation also considered the LLNA: BrdU-ELISA to be mechanistically and functionally similar to the LLNA, and therefore agreed that the LLNA performance standards are applicable.

4.2 ICCVAM Consideration of Public and SACATM Comments

The ICCVAM evaluation process incorporates a high level of transparency. This process is designed to provide numerous opportunities for stakeholder involvement, including submitting written public comments and providing oral comments at ICCVAM independent peer review panel meetings and SACATM meetings. **Table 4-1** lists the 12 different opportunities for public comment that were provided during the ICCVAM evaluation of the validation status of new versions and applications of the LLNA. The number of public comments received in response to each of the opportunities is also indicated. A total of 49 comments were submitted. Comments received in response to or related to the FR notices are available on the NICEATM-ICCVAM website.[12] The following sections, delineated by FR notice, briefly discuss the public comments received.

Table 4-1 Opportunities for Public Comments

Opportunities for Public Comments	Date	Number of Public Comments Received
72 FR 27815: The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data	May 17, 2007	17
72 FR 52130: Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments	September 12, 2007	4
73 FR 1360: Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments	January 8, 2008	7
Independent Scientific Peer Review Panel Meeting Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay	March 4-6, 2008	16
73 FR 25754: Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)	May 7, 2008	1

continued

[12] Available at http://ntp-apps.niehs.nih.gov/iccvampb/searchPubCom.cfm

Table 4-1 Opportunities for Public Comment (continued)

Opportunities for Public Comments	Date	Number of Public Comments Received
73 FR 29136: Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments	May 20, 2008	0
SACATM Meeting, Radisson Hotel, RTP, NC	June 18-19, 2008	0
74 FR 8974: Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments	February 27, 2009	1
Independent Scientific Peer Review Panel Meeting Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Evaluation of the Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay	April 28-29, 2009	2
74 FR 19562: Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)	April 29, 2009	0
74 FR 26242: Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments	June 1, 2009	1
SACATM Meeting, Hilton Arlington Hotel, Arlington, VA	June 25-26, 2009	0

4.2.1 Public Comments in Response to 72 FR 27815 (May 17, 2007): The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data

NICEATM requested the following:

1. Public comments on the appropriateness and relative priority of evaluation of the validation status of
    a. The LLNA as a stand-alone assay for determining potency (including severity) for the purpose of hazard classification
    b. The reduced LLNA approach (Kimber et al. 2006; ESAC 2007; ICCVAM 2009b)
    c. Nonradioactive LLNA methods
    d. The use of the LLNA for testing mixtures, aqueous solutions, and metals
    e. The current applicability domain
2. Nominations of expert scientists to consider as members of a possible peer review panel
3. Submission of data for the LLNA and/or modified versions of the LLNA

In response to this FR notice, NICEATM received 17 comments. Six comments included additional data and information, while two others offered data and information upon request.

Three commenters nominated four potential panelists for consideration. Three commenters suggested reference publications for consideration during the Panel evaluation. The nominees were included in the database of experts from which the Panel was selected. The data and suggested references were included in the draft ICCVAM review documents that were provided to the Panel at the March 2008 meeting.

1. A commenter suggested rearranging the priority sequence of test method evaluation from most to least pressing: a, e, d, b, and c (see list above).

- ICCVAM did not establish a relative priority for these activities because they were all considered to be high-priority activities. Accordingly, all LLNA-related activities described above were discussed at the March 2008 Panel meeting.

One comment pertained to the LLNA: BrdU-ELISA.

1. One commenter indicated that several nonradioactive detection methods for the LLNA (e.g., BrdU incorporation, methods measuring the release of various cytokines, methods using fluorescent markers, and quantification by flow cytometry) have been developed and shown to be as sensitive as protocols involving radiolabeling. The commenter indicated that since both ECVAM and JaCVAM were reviewing some of these types of nonradioactive methods that ICCVAM should collaborate with these ongoing efforts rather than initiate a comprehensive independent review.

- In 2007, the CPSC requested that ICCVAM evaluate several modifications of the LLNA, which included the LLNA: BrdU-ELISA. After considering comments from the public and the SACATM, ICCVAM assigned the activity a high priority. Scientists from ECVAM and JaCVAM served as liaisons to the IWG during the evaluation of the LLNA: BrdU-ELISA and actively participated in the review. Both liaisons nominated scientists to the peer review panel and the JaCVAM liaison provided much of the validation data for the review.

4.2.2 Public Comments in Response to 72 FR 52130 (September 12, 2007): Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments

NICEATM requested public comments on the September 2007 draft ICCVAM-recommended LLNA performance standards developed to facilitate evaluation of modified LLNA test method protocols with regard to the traditional LLNA. In response to this FR notice, NICEATM received four comments, two of which suggested clarifications to the text. Another comment recommended that test substances chosen for testing in the various LLNA methods should be pure, with conclusive structures, and should not be mixtures. Most comments specifically addressed the LLNA performance standards, although one comment pertained to the LLNA in general.

1. One commenter supported the development of performance standards that expedite the validation of new protocols similar to previously validated methods but was disappointed that NICEATM-ICCVAM had chosen to develop performance standards for such a narrow scope of applicability (i.e., modifications of the standard LLNA that involve incorporation of nonradioactive methods of detecting lymphocyte proliferation). The commenter suggested that limited resources available to NICEATM-ICCVAM would be better spent on activities that would have greater impact on the reduction, refinement, or replacement of animal use, such as evaluating the use of human cell lines or *in vitro* skin models as a replacement for the LLNA.

- ICCVAM considered the comment and concluded that the proposed modifications to the LLNA test method protocol and expanded applications have the potential to further reduce and refine animal use. ICCVAM is committed to identifying *in vitro* models and non-animal approaches for assessing ACD and is engaged with ECVAM and JaCVAM in the development of validation studies for such methods.

There were no comments that specifically addressed the LLNA: BrdU-ELISA.

4.2.3 Public Comments in Response to 73 FR 1360 (January 8, 2008): Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments

NICEATM requested public comments on the January 2008 draft BRDs, draft ICCVAM test recommendations, draft test method protocols, and updated draft LLNA performance standards for an international independent scientific peer review panel meeting to evaluate modifications and new applications for the LLNA. NICEATM received 23 comments in response to this FR notice; seven written comments were received in advance of the meeting, and 16 oral comments were offered at the Panel meeting.

Two written comments were relevant to the LLNA: BrdU-ELISA.

1. One commenter noted that the LLNA: BrdU-ELISA was recommended for use by ICCVAM pending receipt of additional information, which the commenter supported, and using alternative decision criteria. The commenter further noted that ICCVAM qualified their acceptance and recommended a weight-of-evidence approach. The commenter indicated that while it is usually good scientific practice to evaluate any test method results in a weight-of-evidence manner, qualifications such as these challenged the recommendations and gave incentive to conduct more testing, when in reality the method evaluated had acceptable performance and should simply be recommended.

- The January 2008 draft ICCVAM recommendations for the LLNA: BrdU-ELISA indicated that the test method may be useful for identifying substances as potential skin sensitizers and nonsensitizers but recommended that more data and information were needed before final recommendations could be made. The January 2008 draft ICCVAM recommendations did not recommend using a weight-of-evidence approach to hazard classification.

2. Another commenter agreed with the January 2008 draft ICCVAM recommendation that more information and data were needed for the LLNA: BrdU-ELISA in order to conduct a meaningful assessment of the procedure's performance relative to the traditional LLNA. The commenter further agreed with the ICCVAM recommendation that it was important to have information regarding the interlaboratory performance of the assay. The commenter also had a suggestion regarding Table 6-2 of the January 2008 draft BRD. Since an alternative SI cutoff for the LLNA: BrdU-ELISA was identified (i.e., SI $\geq$ 1.3) a comparison of LLNA: BrdU-ELISA EC1.3 values to traditional LLNA EC3 values would be helpful.

- A comparison of data for the alternative SI values is included in the final ICCVAM BRD (see **Appendix C**).

Two oral comments were relevant to the LLNA: BrdU-ELISA.

1. One commenter agreed with ICCVAM that the LLNA: BrdU-ELISA and the LLNA: DA should be evaluated separately from one another because they have different treatment schedules. The tests have very little similarity, other than using CBA mice and measuring lymphocyte proliferation.
2. Another commenter explained that the rationale for selection of the CBA/JN strain of mice for the LLNA: BrdU-ELISA was that the sensitivity of the strain to p-benzoquinone was greater than that of the other two strains tested (i.e., BALB/cAnN and CD-1).

4.2.4 Public Comments in Response to 73 FR 25754 (May 7, 2008): Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

NICEATM announced the SACATM meeting and requested written and public oral comments on the agenda topics. One public comment was received in response to this FR notice. The commenter made a general comment that the members of SACATM do not represent a cross-section of the American public.

- The SACATM charter indicates that the Committee shall consist of 15 members, including the Chair. Voting members shall be appointed by the Director, National Institute of Environmental Health Sciences (NIEHS), and include representatives from an academic institution, a State government agency, an international regulatory body, or any corporation developing or marketing new or revised or alternative test methodologies, including contract laboratories. Knowledgeable representatives from public health, environmental communities, or organizations using new or alternative test methodologies may be included as appropriate. There shall be at least one knowledgeable representative having a history of expertise, development, or evaluation of new or revised or alternative test methods from each of the following categories: (1) personal care, pharmaceutical, industrial chemicals, or agricultural industry; (2) any other industry that is regulated by one of the Federal agencies on ICCVAM; and (3) a national animal protection organization established under section 501(c)(3) of the Internal Revenue Code of 1986. The Director, NIEHS, shall select the Chair from among the appointed members of SACATM.

4.2.5 Public Comments in Response to 73 FR 29136 (May 20, 2008): Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments

NICEATM requested submission of written public comments on the Independent Scientific Peer Review Panel Assessment. No public comments were received in response to this FR notice.

4.2.6 Public and SACATM Comments: SACATM Meeting on June 18-19, 2008

The June 18-19, 2008, SACATM meeting included a discussion of the ICCVAM review of the LLNA test method (see **Appendix E3**).

There were no public comments specific to the LLNA: BrdU-ELISA.

Regarding the LLNA: BrdU-ELISA, one SACATM member indicated that the LLNA BrdU-ELISA had potential based on an accuracy of 83% (19/23) but a detailed protocol had not been provided and it was premature to make judgments.

The January 2008 draft ICCVAM recommendations included a statement that a sufficiently detailed protocol of the test method, including a defined and adequately justified decision criterion for distinguishing between sensitizers and nonsensitizers, was required. NICEATM subsequently obtained the detailed protocol, which was included in the revised draft BRD that was evaluated by the Panel in April 2009.

4.2.7 Public Comments in Response to 74 FR 8974 (February 27, 2009): Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments

NICEATM requested public comments on the revised draft BRDs, revised draft ICCVAM test recommendations, revised draft test method protocols, and revised draft LLNA performance standards for the second international independent scientific peer review panel meeting to evaluate modifications and new applications for the LLNA. NICEATM received three comments in response to this FR notice: one written comment, and two oral comments offered at the Panel meeting.

1. There was a general comment expressing concern that the extensive time and resources that ICCVAM has devoted to this evaluation has detracted from focus on promising *in vitro* methods with potential to have a much greater impact on animal use.

- ICCVAM considers that the evaluations conducted to date have significant potential to further reduce and refine animal use, particularly where the use of the LLNA is precluded due to restrictions associated with the use of radioactivity. ICCVAM is also committed to identifying *in vitro* models and non-animal approaches for assessing ACD and is engaged with ECVAM and JaCVAM in the development of validation studies for such methods.

The commenter further made one written comment relevant to the LLNA: BrdU-ELISA.

1. The commenter supported the revised draft ICCVAM recommendation that the LLNA: BrdU-ELISA can be used for ACD testing with specific defined limitations in the decision criteria. That is, that substances falling within the intermediate SI would be subjected to an integrated decision strategy in conjunction with all other available information (e.g., dose response information, statistical analyses of treated vs. control animals, peptide reactivity, molecular weight, results from related chemicals, other testing data). While the commenter offered general support for this use, they emphasized that it should be made clear that "other testing data" refers to retrospective analyses rather than initiation of additional tests in animals.

- ICCVAM agrees that additional animal tests should be avoided whenever possible. The intermediate SI range was discarded because it was irrelevant for ICCVAM's final recommendation to use a single decision criterion, SI $\geq$ 1.6, to classify sensitizers. However, ICCVAM recommends that borderline positive results (i.e., SI values between 1.6 and 1.9) should be evaluated with other available information (e.g., dose-response information, evidence of systemic toxicity or excessive local irritation, statistical comparison of treated vs. vehicle control groups [where appropriate], peptide reactivity, molecular weight, results from related substances, other testing data) to confirm that such results are positive.

2. The commenter further noted that the Panel recommended that the LLNA: BrdU-ELISA and the two other nonradioactive methods should be evaluated for their ability to assess mixtures, metals, and aqueous solutions concurrently with the assessment of these substances in the traditional LLNA. The commenter viewed that since the only difference between these methods and the traditional LLNA is the method of detection, it is unlikely that there will be any differences in the applicability of these methods and the traditional LLNA with regard to mixtures, metals and aqueous solutions. Therefore, it would be highly inappropriate to perform these redundant studies, especially since there are no available data for comparison.

- As outlined in the test method recommendations, ICCVAM considers the applicability domain for the nonradioactive LLNA methods to be the same as the traditional LLNA unless there are properties associated with a class of materials that may interfere with the accuracy of the LLNA: BrdU-ELISA.

One oral comment was relevant to the LLNA: BrdU-ELISA.

1. One commenter stated that the nonradiolabeled LLNA methods should not be held to a higher standard than the traditional LLNA.

- ICCVAM evaluated the LLNA: BrdU-ELISA test method based on the applicable criteria for validation and acceptance of toxicological test methods in the ICCVAM submission guidelines (ICCVAM 2003). ICCVAM is committed to ensuring that new methods are equivalent to or better than the currently accepted toxicological test methods in order to protect public health.

4.2.8 Public Comments in Response to 74 FR 19562 (April 29, 2009): Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

NICEATM announced the SACATM meeting and requested written and public oral comment on the agenda topics. No public comments were received in response to this FR notice.

4.2.9 Public Comments in Response to 74 FR 26242 (June 1, 2009): Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments

NICEATM requested submission of written public comments on the Independent Scientific Peer Review Panel Assessment. One comment was received in response to this FR notice.

The commenter made one comment relevant to the LLNA: BrdU-ELISA.

1. The commenter did not consider the nonradioactive LLNA methods to provide significant advantages to the traditional LLNA.

- The ICCVAM recommendations for the nonradioactive test methods state that the proposed nonradioactive modifications to the LLNA test method protocol have significant potential to further reduce and refine animal use, given that they will likely increase the use of the LLNA instead of GP test methods where radioactivity is prohibited.

The commenter also indicated that the number of animals used in the LLNA: BrdU-ELISA was eight animals per dose group and for ethical reasons the LLNA: BrdU-ELISA might be avoided.

- The commenter misunderstood the number of animals required by the LLNA: BrdU-ELISA. The ICCVAM-recommended protocol for the LLNA: BrdU-ELISA indicates that four animals per dose group are recommended.

The commenter further indicated that the justification for replacing the GP is not provided for the LLNA: BrdU-ELISA and that it should be mentioned.

- As indicated in Section 10.0 of the final ICCVAM BRD (**Appendix C**), the LLNA: BrdU-ELISA evaluates only the induction phase of skin sensitization and therefore discomfort to animals associated with the elicitation phase is eliminated. Additionally, the LLNA: BrdU-ELISA test method protocol requires fewer mice per treatment group (a minimum of four animals per group) than either of the GP tests (10-20 animals/group for the Buehler test and 5-10 animals/group for GPMT).

4.2.10 Public and SACATM Comments: SACATM Meeting on June 25-26, 2009

The June 25-26, 2009, SACATM meeting included a discussion of the ICCVAM review of the LLNA test method (see **Appendix E4**).

There were no public comments specific to the LLNA: BrdU-ELISA.

In general, SACATM was supportive of the Panel report. However, there was general concern regarding the potential for overlabeling substances that may occur by using LLNA test results. They emphasized the need for developing non-animal test methods for identifying potential skin sensitizers.

One SACATM member commented that many laboratories had moved away from using the LLNA because it used radioactivity. Therefore, the option of LLNA test method protocols that do not use radioactivity would likely increase use of the LLNA.

Regarding the LLNA: BrdU-ELISA, another SACATM member indicated that the use of two SI decision criteria in the LLNA: BrdU-ELISA (i.e., one for determining sensitizers and one for determining nonsensitizers) could potentially place many compounds in the range of uncertainty (i.e., the range in which maximum SI results were between the SI decision criteria for sensitizers and nonsensitizers), so the decision criteria should be reassessed as more data are obtained.

- The final ICCVAM recommendations state that a single decision criterion of SI $\geq$ 1.6 be used to classify substances as potential sensitizers since there were no false negatives in the current validation database, relative to the traditional LLNA, when this criterion is used. However, using an SI $\geq$ 1.6 as the decision criterion results in a false positive rate of 18% (2/11) compared to the traditional LLNA. Since the two false positive substances in the LLNA: BrdU-ELISA produced SI values between 1.6 and 1.9, users may want to consider additional information (e.g., dose-response information, evidence of systemic toxicity and/or excessive local skin irritation, statistical comparison of treated vs. vehicle control groups [where appropriate], peptide reactivity, molecular weight, results from related substances, or other testing data) to confirm that such results in the SI range are positive.

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Appendix A

Timeline for ICCVAM Evaluation of the LLNA: BrdU-ELISA

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January 10, 2007	ICCVAM receives nomination from CPSC for seven LLNA review activities[1], including evaluation of the LLNA: BrdU-ELISA.
January 2007	The ICCVAM IWG is re-established to work with NICEATM to carry out LLNA evaluations.
January 24, 2007	ICCVAM endorses the six CPSC-nominated LLNA review activities and development of ICCVAM LLNA Test Method Performance Standards.
May 17, 2007	*Federal Register* notice (72 *FR* 27815) – *The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data*.
June 12, 2007	SACATM endorses with high priority the six CPSC-nominated LLNA review activities and development of ICCVAM LLNA Test Method Performance Standards.
September 25-26, 2007	ICCVAM participation in ECVAM Workshop: An Evaluation of Performance Standards and Nonradioactive Endpoints for the Local Lymph Node Assay.
January 8, 2008	*Federal Register* notice (73 *FR* 1360) – *Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments*.
March 4–6, 2008	Independent Peer Review Panel Meeting on seven LLNA review activities, CPSC Headquarters, Bethesda, MD; public meeting with opportunity for oral public comments.[2]
May 20, 2008	*Federal Register* notice (73 *FR* 29136) – *Announcement of the Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments*.
June 18–19, 2008	SACATM public meeting for comments on the 2008 Panel report.
February 27, 2009	*Federal Register* notice (74 *FR* 8974) – *Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments*.

[1] http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf
[2] http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel08.htm

April 28–29, 2009	Independent Peer Review Panel Meeting on LLNA review activities, NIH, Bethesda, MD; public meeting with opportunity for oral public comments.[3]
June 1, 2009	*Federal Register* notice (74 *FR* 26242) – *Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments*.
June 25–26, 2009	SACATM public meeting for comments on the 2009 Panel report.
October 20-22, 2009	OECD Expert Consultation Meeting, CPSC Headquarters, Bethesda, MD, on proposed updates to TG 429 and two new TG proposals for nonradioactive LLNA test methods (includes the LLNA: BrdU-ELISA).
December 1, 2009	OECD Expert Consultation Teleconference to discuss remaining issues on proposed updates to TG 429 and two new TG proposals for nonradioactive LLNA test methods, which includes the LLNA: BrdU-ELISA.
March 23-25, 2010	Meeting of the Working Group of National Co-ordinators of the Test Guidelines Programme to approve adoption of proposed updates to TG 429 and two new TG proposals for nonradioactive LLNA test methods, which includes the LLNA: BrdU-ELISA.
March 2010	ICCVAM endorses the TMER for the LLNA: BrdU-ELISA, which includes the final background review document.
2010 (published within two weeks after transmittal)	*Federal Register* notice: Announces availability of ICCVAM TMER for the LLNA: BrdU-ELISA.

Abbreviations: BRD = background review document; CPSC = U.S. Consumer Product Safety Commission; ECVAM = European Centre for the Validation of Alternative Methods; ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; IWG = ICCVAM Immunotoxicity Working Group; LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay based on bromodeoxyuridine detection by enzyme-linked immunosorbent assay; NICEATM = National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods; NIH = National Institutes of Health; OECD = Organisation for Economic Co-operation and Development; SACATM = Scientific Advisory Committee on Alternative Toxicological Methods; TG = Test Guideline; TMER = test method evaluation report.

[3] http://iccvam.niehs.nih.gov/methods/immunotox/llna.htm

Appendix B

ICCVAM-Recommended Test Method Protocol: The Murine Local Lymph Node Assay: 2-Bromodeoxyuridine-ELISA Test Method (LLNA: BrdU-ELISA), a Nonradioactive Alternative Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products

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1.0 General Principle of Detection of Skin Sensitization Using the Nonradiolabelled Murine Local Lymph Node Assay: 2-Bromodeoxyuridine-ELISA Test Method (LLNA: BrdU-ELISA)

The basic principle underlying the murine local lymph node assay (LLNA) is that sensitizers induce proliferation of lymphocytes in the lymph nodes draining the site of substance application. Under appropriate test conditions, this proliferation is proportional to the dose applied, and provides a means of obtaining an objective, quantitative measurement of sensitization. The test measures cell proliferation as a function of *in vivo* radioisotope (^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine) incorporation into the DNA of dividing lymphocytes, and assesses this proliferation in the draining lymph nodes proximal to the application site (see **Annex I**). Due to the use of radioactivity, the LLNA has limited use in regions where the acquisition, use, or disposal of radioactivity is problematic. The LLNA: BrdU-ELISA was therefore developed as a nonradioactive modification to the LLNA (Takeyoshi 2001), which uses nonradiolabelled 5-bromo-2-deoxyuridine (BrdU) (Chemical Abstracts Service Registry Number [CASRN] 59-14-3) with detection by an enzyme-linked immunosorbent assay (ELISA) to assess lymphocyte proliferation. The ability to detect skin sensitizers without the necessity of using a radioactive label for DNA eliminates the potential for occupational exposure to radioactivity and waste disposal issues. Similar to the LLNA, the LLNA: BrdU-ELISA studies the induction phase of skin sensitization and provides quantitative data suitable for dose-response assessment. Lymphocyte proliferation in test groups is compared to that in the concurrent vehicle-treated control group. The proliferation is proportional to the dose and to the potency of the applied allergen and provides a simple means of obtaining a quantitative measurement of sensitization. The LLNA: BrdU-ELISA assesses this proliferation as the proliferation in test groups compared to that in vehicle treated controls. The ratio of the proliferation in treated groups to that in concurrent vehicle treated controls, termed the stimulation index (SI), is determined, and should be $\geq$1.6 before a test substance can be considered as a skin sensitizer, with specific limitations for borderline positive results (i.e., SI between 1.6 and 1.9) as described in Section 3 of this Test Method Evaluation Report.

The methods, described here are based on the use of measuring BrdU content to indicate an increased number of proliferating cells in the draining auricular lymph nodes. BrdU is an analog of thymidine and is similarly incorporated into the DNA of proliferating cells. The incorporation of BrdU is measured by ELISA, which utilizes an antibody specific for BrdU that is also labeled with peroxidase. When the substrate is added, the peroxidase reacts with the substrate to produce a colored product that is quantified at a specific absorbance using a microtiter plate reader. A concurrent positive control is added to each assay to provide an indication of appropriate assay performance.

2.0 Description of the LLNA: BrdU-ELISA

2.1 Sex and strain of animals

The mouse is the species of choice for the LLNA: BrdU-ELISA assay. Validation studies were conducted exclusively with the CBA/JN strain, but other CBA substrains can be used. Young adult female mice (nulliparous and non-pregnant) are used because most data in the existing database were generated using mice of this gender.[1] At the start of the study, mice should be 8-12 weeks of age. All

[1] Male mice may be used if it is sufficiently demonstrated that these animals perform as well as female CBA mice in the LLNA: BrdU-ELISA.

mice should be age matched (preferably within a one-week time frame). Weight variations between the mice should not exceed 20% of the mean weight.

2.2 Preparation of animals

The temperature of the experimental animal room should be 22°C (±3°C) and the relative humidity 30%-70% (although the aim is for 50%-60%). Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, an unlimited supply of standard laboratory mouse diets and drinking water should be used. The mice should be quarantined/acclimatized for at least five days prior to the start of the test (ILAR 1996). Mice should be allocated to small groups by a stratified randomization or other appropriate methods before the start of the study unless adequate scientific rationale for housing mice individually is provided (ILAR 1996). Four animals per cage is the recommended housing arrangement. The mice are uniquely identified prior to being placed in the study. The method used to mark the mice should not involve identification via the ear (e.g., marking, clipping, or punching of the ear). Colored marks on the tail or other appropriate methods should be used. All mice should be examined (e.g., clinical signs, body weights, observation of excrement) prior to the initiation of the test to ensure good health and the absence of skin lesions.

2.3 Preparation of doses

Solid test substances should be dissolved or suspended in appropriate solvents/vehicles and diluted, if appropriate, prior to dosing of the mice. Liquid test substances may be dosed directly (i.e., applied neat) or diluted prior to dosing. Insoluble materials, such as those generally seen in medical devices, should be subjected to an exaggerated extraction in an appropriate solvent to extract all extractable constituents for testing prior to dosing. Fresh preparations of the test substance should be prepared daily unless stability data demonstrate the acceptability of storage.

2.4 Test conditions

2.4.1 Solvent/vehicle

The solvent/vehicle should not interfere with or bias the test result and should be selected on the basis of maximizing 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), *N,N*-dimethylformamide (DMF), methyl ethyl ketone (MEK), propylene glycol, and dimethyl sulfoxide (DMSO) (Van Och et al. 2000; Kimber et al. 1994), but others may be used if sufficient scientific rationale is provided (Kimber and Basketter 1992). Particular care should be taken to ensure that hydrophilic materials are incorporated into a vehicle system that incorporates appropriate solubilizers (e.g., 1% Pluronic® L92) that wet the skin and does not immediately run off. Thus, wholly aqueous vehicles may need to be avoided. In certain situations, it may be necessary for regulatory purposes to test the substance in the clinically relevant solvent or product formulation.

2.4.2 Controls

Concurrent negative (solvent/vehicle) and positive controls should be included in each test to ensure that the test system is functioning properly and that the specific test is valid. In some circumstances (e.g., when using a solvent/vehicle not recommended in **Section 2.4.1**), it may be useful to include a naïve control. Except for treatment with the test substance, the mice in the negative control groups should be handled in an identical manner to the mice of the treatment groups.

Positive controls are used to demonstrate appropriate performance of the assay by responding with adequate and reproducible sensitivity to a sensitizing substance for which the magnitude of the response is well characterized. Inclusion of a concurrent positive control is recommended because it

demonstrates competency of the laboratory to successfully conduct each assay and allows for an assessment of intra- and interlaboratory reproducibility and comparability. The positive control should produce a positive LLNA: BrdU-ELISA response at an exposure level expected to give an increase in the SI ≥ 1.6 over the negative control group. The positive control dose should be chosen such that the induction is reproducible but it does not cause excessive skin irritation or systemic toxicity. Preferred positive control substances are 50% hexyl cinnamic aldehyde (HCA; CASRN 101-86-0) and 50% eugenol (CASRN 97-53-0) in AOO. There may be circumstances in which, given adequate justification, other positive control substances meeting the above criteria may be used.

Although the positive control substance should be tested in the vehicle that is known to elicit a consistent response (e.g., AOO), there may be certain regulatory situations in which testing in a non-standard vehicle (clinically/chemically relevant formulation) will also be necessary. In such situations, the possible interaction of a positive control with this unconventional vehicle should be tested. If the concurrent positive control substance is tested in a different vehicle than the test substance, then a separate vehicle control for the concurrent positive control should be included.

While inclusion of a concurrent positive control group is recommended, there may be situations in which periodic testing (i.e., at intervals ≤6 months) of the positive control substance may be adequate for laboratories that conduct the LLNA: BrdU-ELISA regularly (i.e., conduct the LLNA: BrdU-ELISA at a frequency of no less than once per month) and have an established historical positive control database that demonstrates the laboratory's ability to obtain reproducible and accurate results with positive controls. Adequate proficiency with the LLNA: BrdU-ELISA can be successfully demonstrated by generating consistent results with the positive control in at least 10 independent tests conducted within a reasonable period of time (i.e., less than one year).

A concurrent positive control group should always be included when there is a procedural change to the LLNA: BrdU-ELISA (i.e., change in trained personnel, change in test method materials and/or reagents, change in test method equipment, change in source of test animals), and such changes should be documented in laboratory reports. Consideration should be given to the impact of these changes on the adequacy of the previously established historical database in determining the necessity for establishing a new historical database to document consistency in the positive control results.

Investigators should be aware that the decision to conduct a positive control on a periodic basis instead of concurrently has ramifications on the adequacy and acceptability of negative study results generated without a concurrent positive control during the interval between each periodic positive control study. For example, if a false negative result is obtained in the periodic positive control study, all negative test substance results obtained in the interval between the last acceptable periodic positive control study and the unacceptable periodic positive control study may be questioned. Implications of these outcomes should be carefully considered when determining whether to include concurrent positive controls or to only conduct periodic positive controls. Consideration should also be given to using fewer animals in the concurrent positive control group when this is scientifically justified and if the laboratory demonstrates, based on laboratory-specific historical data, that fewer mice can be used without substantially increasing the failure rate of the positive control (i.e., the rate at which SI < 1.6 and the frequency with which studies will need to be repeated due to positive control failure [Appendix A of ICCVAM 2009a]).

In instances where substances of a specific chemical class or range of responses are being evaluated, benchmark substances may be useful to demonstrate that the test method is functioning properly for detecting the skin sensitization potential of a test substance. Appropriate benchmark substances should have the following properties:

- Structural and functional similarity to the class of the substance being tested
- Known physical/chemical characteristics
- Supporting data from the LLNA: BrdU-ELISA

- Supporting data on known effects in animal models and/or from humans

2.5 Methodology

A minimum of four animals is used per dose group, with a minimum of three concentrations of the test substance, plus a concurrent negative control group treated only with the vehicle for the test substance, and a concurrent positive control. The processing of lymph nodes from individual mice allows for the assessment of interanimal variability and a statistical comparison of the difference between test substance and vehicle control group measurements. In addition, evaluating the possibility of reducing the number of mice in the positive control group is only feasible when individual animal data are collected.

Test substance treatment dose levels should be based on the recommendations given in Kimber and Basketter (1992) and in the ICCVAM Panel Report (ICCVAM 1999). Consecutive doses are normally selected from an appropriate concentration series such as 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc. 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 material of interest (and/or structurally related test materials) should be considered, where available, in selecting the three consecutive concentrations so that the highest concentration maximizes exposure while avoiding systemic toxicity and/or excessive local skin irritation (Kimber et al. 1994; OECD 2002). In the absence of such information, an initial prescreen test may be necessary (**Annex II**).

The LLNA: BrdU-ELISA experimental procedure is performed as follows:

Day 1. Individually identify and record the weight of each animal and any clinical observations. Apply 25 µL of the appropriate dilution of the test substance, the vehicle alone, or the concurrent positive control to the dorsum of each ear.

Days 2 and 3. Repeat the application procedure as carried out on Day 1.

Day 4. No treatment.

Day 5. Inject 0.5 mL (5 mg/mouse) of 10 mg/mL BrdU in physiological saline intraperitoneally.

Day 6. Record the weight of each animal and any clinical observations. Approximately 24 hours (24 h) after BrdU injection, humanely kill the animals. To further monitor the local skin response in the experimental study, additional parameters such as scoring of ear erythema or ear thickness measurements (obtained either by using a thickness gauge, or ear punch weight determinations at necropsy) may be included in the study protocol.

Excise both bilateral draining auricular lymph nodes from each mouse ear (see diagram and description of dissection in **Annex I**) and store in a 1.5 mL centrifuge tube at -20°C until BrdU is measured by ELISA.

For BrdU measurement, a single-cell suspension of lymph node cells (LNC) from each mouse is prepared by adding a small volume of physiological saline (approximately 0.3 mL) to the excised lymph nodes, crushing the lymph nodes with a disposable plastic pestle, and passing through a #70 nylon mesh or another acceptable technique for mechanical disaggregation (e.g., passing through 200 micron-mesh stainless steel gauze) to generate a single-cell suspension. The procedure for preparing the LNC suspension is a critical step of this assay; it is most important to crush the lymph node and suspend the LNC completely. Every technician should establish the skill in advance. The lymph nodes in negative control animals are small, so careful operation is required to avoid an artificial effect on SI values.

In each case, the target volume of the LNC suspension should be adjusted to a pre-determined optimized volume (approximately 15 mL) based on achieving a mean absorbance of the negative control group within 0.1-0.2. Because this absorbance depends on the assay apparatus and the target volume of cell suspension, every laboratory should decide their own optimal volume of LNC suspension in advance.

The incorporation of BrdU into lymph node cells should be determined using a commercial cell proliferation assay kit (Roche Diagnostics GmbH, Roche Applied Science, 68298 Mannheim, Germany; Cat. No. 11 647 229 001) after they are crushed and suspended in physiological saline. The absorbance is defined as the BrdU labeling index. Follow the instructions in the assay kit. Briefly, 100 μL of the LNC suspension is added to the wells of a flat-bottom microplate in triplicate. After fixation and denaturation of the LNC, anti-BrdU antibody is added to each well and allowed to react. Subsequently the anti-BrdU antibody is removed by washing and the substrate solution is then added and allowed to produce chromogen. Absorbance at 370 nm with a reference wavelength of 492 nm is then measured.

2.6 Reduced LLNA

Using this test method protocol, there is also the opportunity to perform a reduced LLNA: BrdU-ELISA (rLLNA: BrdU-ELISA). Use of the rLLNA: BrdU-ELISA has the potential to reduce the number of animals by omitting the middle and low dose groups from the LLNA: BrdU-ELISA (Kimber et al. 2006; ESAC 2007; ICCVAM 2009b). This is the only difference between the LLNA: BrdU-ELISA and the rLLNA: BrdU-ELISA. Thus, the test substance concentration evaluated in the rLLNA: BrdU-ELISA should be the maximum concentration that does not induce overt systemic toxicity and/or excessive local skin irritation in the mouse (**Annex II**). The rLLNA: BrdU-ELISA should be used for the hazard classification of skin sensitizing substances if dose-response information is not needed, provided there is adherence to all other LLNA: BrdU-ELISA protocol specifications.

2.7 Observations

Mice should be carefully observed at least once daily for any clinical signs, either of local irritation at the application site or of systemic toxicity (**Annex II**). Weighing mice prior to treatment and at the time of necropsy will aid in assessing systemic toxicity. All observations are systematically recorded with records maintained for each individual mouse. Animal monitoring plans should include criteria to promptly identify those mice exhibiting systemic toxicity or excessive irritation, or corrosion of skin for euthanasia (OECD 2000).

3.0 Calculation of Results

Results for each treatment group are expressed as the mean SI. The SI is derived by dividing the mean BrdU labeling index/mouse within each test substance group and the concurrent positive control group by the mean BrdU labeling index for the solvent/vehicle control group. The average SI value for vehicle treated controls is then equal to one.

The BrdU labeling index is defined as:

BrdU labeling index = $(ABS_{em} - ABS\ blank_{em}) - (ABS_{ref} - ABS\ blank_{ref})$

where ABS = absorbance, em = emission wavelength and ref = reference wavelength.

The decision process regards a result as positive when SI ≥ 1.6 (see **Section 3** of this Test Method Evaluation Report). However, the strength of the dose response, chemical toxicity, solubility, and, where appropriate, statistical significance should be considered together with SI values to arrive at a final decision (Basketter et al. 1996; ICCVAM 1999; EPA 1998; Kimber et al. 1998).

Collecting data at the level of the individual mouse will enable a statistical analysis for presence and degree of dose response in the data. Any statistical assessment could include an evaluation of the dose-response relationship as well as suitably adjusted comparisons of test groups (e.g., pair-wise dosed group versus concurrent solvent/vehicle control comparisons). Statistical analyses may include, for instance, linear regression or Williams's test to assess dose-response trends, and Dunnett's test for pairwise comparisons. In choosing an appropriate method of statistical analysis, the investigator should maintain an awareness of possible inequalities of variances and other related problems that may necessitate a data transformation or a nonparametric statistical analysis. In any case, the investigator may need to carry out SI calculations and statistical analyses with and without certain data points (sometimes called "outliers").

4.0 Evaluation and Interpretation of Results

Consideration should be given to the possibility of borderline positive results when SI values between 1.6 and 1.9 are obtained. This is based on the validation database of 43 substances using an SI $\geq$ 1.6 for which the LLNA: BrdU-ELISA correctly identified all 32 LLNA sensitizers, but incorrectly identified two of 11 LLNA nonsensitizers with SI values between 1.6 and 1.9 (i.e. borderline positive) (see Section 3.0 of this Test Method Evaluation Report). If an SI value between 1.6 and 1.9 is obtained, other available information such as the nature of the dose-response, evidence of systemic toxicity or excessive local skin irritation, and, where appropriate, statistical significance together with SI values should be considered to confirm that such borderline positive results are potential skin sensitizers (see Section 3.0 of this Test Method Evaluation Report). Consideration should also be given to various properties of the test substance, including whether it has a structural relationship to known skin sensitizers. These and other considerations are discussed in detail elsewhere (Basketter et al. 1998).

Employing the optimized assay condition described previously, the mean SI value for the positive control group (50% HCA) should be equal to or greater than 1.6. If not, data derived from the experiment should not be used for evaluation.

5.0 Data and Reporting

5.1 Data

Data should be summarized in tabular form showing the individual animal BrdU labeling index values, the group mean BrdU labeling index/animal, its associated error term (e.g., standard deviation [SD], standard error of the mean [SEM]), and the mean SI value for each dose group compared against the concurrent solvent/vehicle control group.

5.2 Test report

The test report should contain the following information:

Test Substances and Control Substances

- Identification data (e.g., CASRN, if available; source; purity; known impurities; lot number)
- Physical nature and physicochemical properties (e.g. volatility, stability, solubility, physicochemical properties relevant to the conduct of the study)
- Composition and relative percentages of components, if formulation

Solvent/Vehicle

- Identification data (CASRN; purity; concentration, where appropriate; volume used)

- Justification for choice of vehicle

Test Animals

- Source of CBA mice, housing conditions, diet, etc.
- Microbiological status of the animals, when known
- Number and age of animals

Test Conditions

- Details of test substance preparation and application
- Justification for dose selection (including results from prescreen test, if conducted)
- Vehicle and test substance concentrations used, and total amount of substance applied
- Details of food and water quality (including diet type/source, water source)
- Details of treatment and sampling schedules
- Methods for measurement of toxicity
- Criteria for considering studies as positive or negative
- Details of any protocol deviations and an explanation on how the deviation affects the study design and results

Reliability Check

- Summary of results of latest reliability check, including information on substance, concentration and vehicle used
- Concurrent and/or historical positive and negative (solvent/vehicle) control data for testing laboratory
- Date and laboratory report for the most recent periodic positive control and a report detailing the historical positive control data for the laboratory justifying the basis for not conducting a concurrent positive control, if a concurrent positive control was not included

Results

- Individual weights of mice at start of dosing and at scheduled kill; as well as mean and associated error term (e.g., SD, SEM) for each treatment group
- Time course of onset and signs of toxicity, including dermal irritation at site of administration, if any, for each animal
- Table of individual mouse BrdU labeling indices and SI values for each treatment group
- Mean and associated error term (e.g., SD, SEM) for BrdU labeling index/mouse for each treatment group and the results of outlier analysis for each treatment group
- Calculated SI and an appropriate measure of variability that takes into account the interanimal variability in both the test substance and control groups
- Dose response relationship
- Statistical analysis, where appropriate

Discussion of the Results

- Brief commentary on the results, the dose-response analysis, and statistical analyses, where appropriate, with a conclusion as to whether the test substance should be considered a skin sensitizer

Conclusion

A Quality Assurance Statement for GLP-compliant Studies

- Indicate all inspections made during the study and the dates any results were reported to the Study Director; confirm that the final report reflects the raw data

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Annex I:
An Approach to Dissection and Identification of the Draining ("Auricular") Lymph Nodes

1.0 Background

Although minimal technical training of the LLNA: BrdU-ELISA is required, extreme care must be taken to ensure appropriate and consistent dissection of the lymph nodes. It is recommended that technical proficiency in the dissection and identification of the lymph nodes draining the ear be achieved by practice on mice that have been (a) injected with a colored agent (dye) and/or (b) sensitized with a strong positive sensitizer. Brief descriptions of these practice dissections are provided below. Recognizing that nodes from vehicle-treated and naïve mice are smaller, laboratories performing the LLNA: BrdU-ELISA must also gain proficiency in the dissection of these nodes. It may be helpful for laboratories inexperienced in this procedure to request guidance from laboratories that have successfully performed the LLNA: BrdU-ELISA.

2.0 Training and Preparation for Node Identification

2.1 Identification of the Draining Node – Dye Treatment

Several methods can be used to provide color identification of the draining nodes. These techniques may be helpful for initial identification and should be performed to ensure proper isolation of the appropriate node. Examples of such treatments are listed below. It should be noted that other such protocols might be used effectively.

Evan's Blue Dye treatment:

Inject approximately 0.1 mL of 2% Evan's Blue Dye (prepared in sterile saline) intradermally into the pinna of an ear. Euthanize the mouse after several minutes and continue with the dissection as noted below.

Colloidal carbon and other dye treatments:

Colloidal carbon and India ink are examples of other dye treatments that may be used (Tilney 1971).

2.2 Identification of the Draining Node – Application of Strong Sensitizers

For the purpose of node identification and training, a strong sensitizer is recommended. This agent should be applied in the standard AOO vehicle. Suggested sensitizers for this training exercise include 0.1% oxazolone, 0.1% (w/v) 2,4-dinitrochlorobenzene, and 0.1% (v/v) dinitrofluorobenzene. After treating the ear with a strong sensitizer, the draining node will dramatically increase in size, thus aiding in identification and location of the node.

Using a procedure similar to that described in the test method protocol, apply the agent to the dorsum of both ears (25 μL/ear) for three consecutive days. On the fourth day, euthanize the mouse. Identification and dissection (listed below) of the node should be performed in these animals prior to practice in non-sensitized or vehicle-treated mice, where the node is significantly smaller.

Please note: Due to the exacerbated response, the suggested sensitizers are not recommended as controls for assay performance. They should only be used for training and node identification purposes.

3.0 Dissection Approach

3.1 Lateral Dissection (Figure B-I-1)

Although lateral dissection is not the conventional approach used to obtain the nodes draining the ear, it may be helpful as a training procedure when used in combination with the ventral dissection. Perform this approach bilaterally (on both sides of the mouse). After euthanizing the mouse, place it in a lateral position. Wet the face and neck with 70% ethanol. Use scissors and forceps to make an initial cut from the neck area slightly below the ear. Carefully extend the incision toward the mouth and nose. Angle the tip of the scissors slightly upward during this procedure to prevent the damage of deeper tissue. Gently retract the glandular tissue in the area using the forceps. Using the masseter muscle, facial nerves, blood vessels, and the bifurcation of the jugular vein as landmarks, isolate and remove the draining node (**Figure B-I-1**). The draining node ("auricular") will be positioned adjacent to the masseter muscle and proximal to and slightly above the jugular bifurcation.

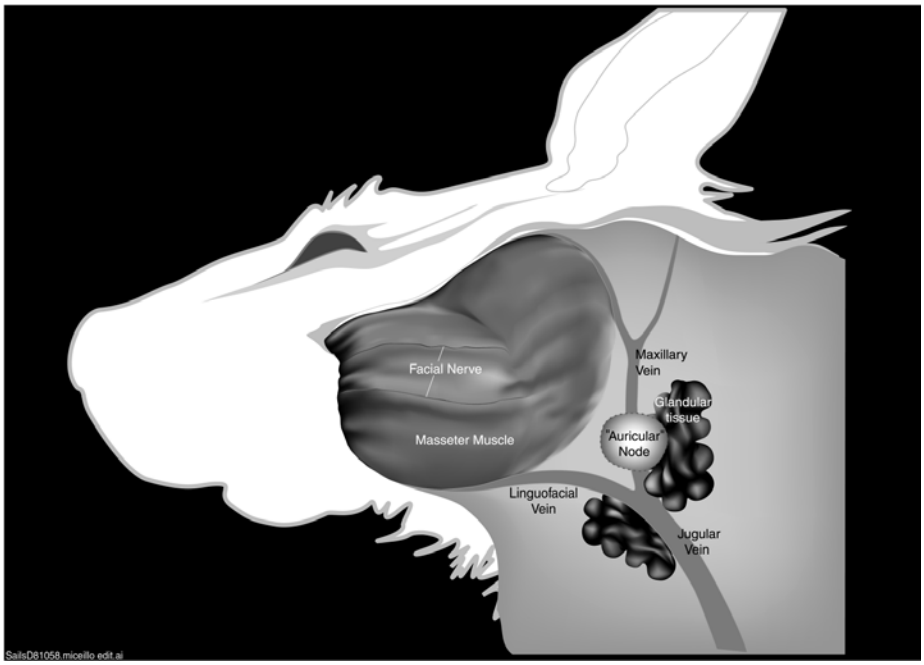
3.2 Ventral Dissection (Figure B-I-2)

The most commonly used dissection approach is from the ventral surface of the mouse. This approach allows both right and left draining nodes to be obtained without repositioning the mouse. With the mouse ventrally exposed, wet the neck and abdomen with 70% ethanol. Use scissors and forceps to carefully make the first incision across the chest and between the arms. Make a second incision up the midline perpendicular to the initial cut, and then cut up to the chin area. Reflect the skin to expose the external jugular veins in the neck area. Take care to avoid salivary tissue at the midline and nodes associated with this tissue. The nodes draining the ear ("auricular") are located distal to the masseter muscle, away from the midline, and near the bifurcation of the jugular veins.

4.0 Accuracy in Identification

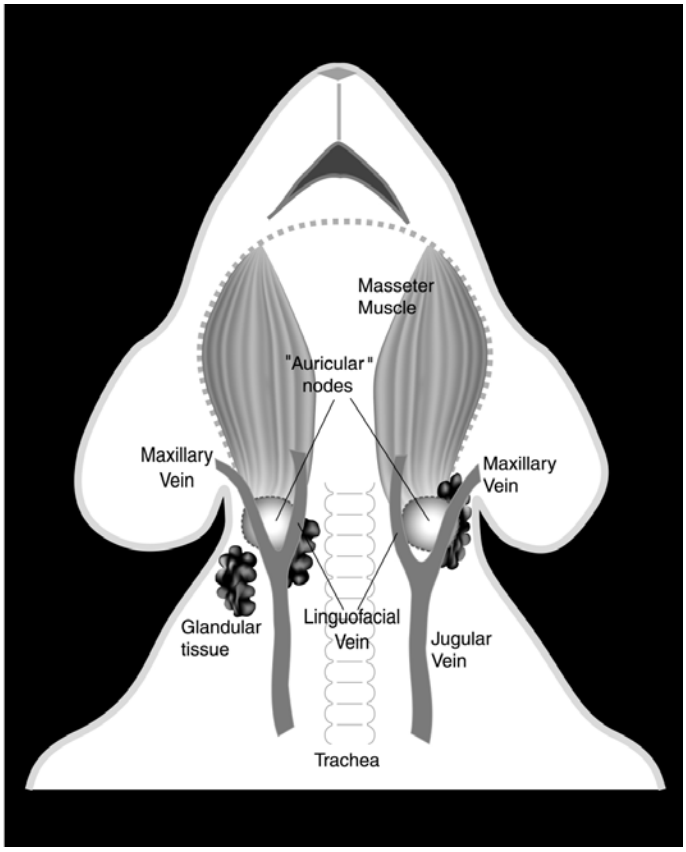
The nodes can be distinguished from glandular and connective tissue in the area by the uniformity of the nodal surface and a shiny translucent appearance. Application of sensitizing agents (especially the strong sensitizers used in training) will cause enlargement of the node size. If a dye is injected for training purposes, the node will take on the tint of the dye.

Figure B-I-1 Lateral Dissection



Credit: Dee Sailstad, U.S. EPA

Figure B-I-2 Ventral Dissection



Credit: Dee Sailstad, U.S. EPA

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Annex II:
Evaluating Local Irritation and Systemic Toxicity in the LLNA: BrdU-ELISA

As noted in the ICCVAM LLNA: BrdU-ELISA test method protocol, the maximum dose tested should be the maximum possible concentration that does not produce systemic toxicity or excessive local irritation after topical application in the mouse. In the absence of information to determine this concentration (e.g., acute toxicity and dermal irritation data, and/or structural and physicochemical information on the test material and/or structurally related test materials), a prescreen test should be performed using three dose levels of the test substance, in order to define the appropriate dose to test in the LLNA: BrdU-ELISA.

The prescreen test is conducted under identical conditions as the main LLNA: BrdU-ELISA study, except there is no assessment of lymph node cell proliferation. The maximum dose tested should be 100% of the test material for liquids or the maximum possible concentration for solids or suspensions. One or two animals per dose group are suggested. All mice will be observed daily for any clinical signs of systemic toxicity or local irritation at the application site. Body weights are recorded pre-test and prior to termination (Day 6). Both ears of each mouse are observed for erythema and scored using **Table B-II-1**. Ear thickness measurements are taken using a thickness gauge (e.g., digital micrometer or Peacock Dial thickness gauge) on Day 1 (predose), Day 3 (approximately 48 hours after the first dose), and Day 6 (termination). Additionally on Day 6, ear thickness could be determined by ear punch weight determinations, which must be performed after the animals are humanely killed. Excessive local irritation is indicated by an erythema score ≥3 and/or an increase in ear thickness of ≥25% on any day of measurement (Reeder et al. 2007; ICCVAM 2009c). The highest dose selected for the main LLNA: BrdU-ELISA study will be the next lower dose in the prescreen concentration series that does not induce systemic toxicity and/or excessive local skin irritation.

Table B-II-1 Erythema Scores

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

In addition to a 25% increase in ear thickness (Reeder et al. 2007; ICCVAM 2009c), a statistically significant increase in ear thickness in the treated mice compared to control mice has also been used to identify irritants in the traditional LLNA (Hayes et al. 1998; Homey et al. 1998; Woolhiser et al. 1998; Hayes and Meade 1999; Ehling et al. 2005; Vohr and Jürgen 2005). While statistically significant increases can occur when ear thickness is less than 25%, they have not been associated specifically with excessive irritation (Woolhiser et al. 1998; Hayes and Meade 1999; Ehling et al. 2005; Vohr and Jürgen 2005; Patterson et al. 2007).

Test guidelines for assessing acute dermal toxicity recommend a number of clinical observations for assessing systemic toxicity (OECD 1987; EPA 1998). The following clinical observations, which are

based on test guidelines and current practices (ICCVAM 2009d), may indicate systemic toxicity when used as part of an integrated assessment and therefore may indicate the maximum dose level to use in the main LLNA: BrdU-ELISA:

- Changes in nervous system function (e.g., piloerection, ataxia, tremors, and convulsions)
- Changes in behavior (e.g., aggressiveness, change in grooming activity, marked change in activity level)
- Changes in respiratory patterns (i.e., changes in frequency and intensity of breathing such as dyspnea, gasping, and rales)
- Changes in food and water consumption
- Lethargy and/or unresponsiveness
- Any clinical signs of more than slight or momentary pain and distress
- Reduction in body weight >5% from Day 1 to Day 6
- Mortality

Moribund animals or animals showing signs of severe pain and distress should be humanely killed (OECD 2000).

Appendix C

Final Background Review Document:

Nonradioactive Murine Local Lymph Node Assay: BrdU-ELISA Test Method Protocol

(LLNA: BrdU-ELISA)

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Background Review Document
Nonradioactive Murine Local Lymph Node Assay: BrdU-ELISA
Test Method Protocol (LLNA: BrdU-ELISA)

Interagency Coordinating Committee on the
Validation of Alternative Methods

National Toxicology Program Interagency Center for the
Evaluation of Alternative Toxicological Methods

National Institute of Environmental Health Sciences
National Institutes of Health
U.S. Public Health Service
Department of Health and Human Services

March 2010

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Table of Contents

List of Tables

List of Figures

List of Abbreviations and Acronyms

ACD	Allergic contact dermatitis
ANOVA	Analysis of variance
AOO	Acetone: olive oil
BRD	Background review document
BrdU	Bromodeoxyuridine
CI	Confidence interval
CASRN	Chemical Abstracts Service Registry Number
Conc.	Concentration tested
CPSC	U.S. Consumer Product Safety Commission
CV	Coefficient of variation
DMF	*N,N*-dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC1.5	Estimated concentration needed to produce a stimulation index of 1.5
EC2	Estimated concentration needed to produce a stimulation index of 2
EC3	Estimated concentration needed to produce a stimulation index of 3
ECt	Estimated concentration needed to produce a stimulation index equaling or greater than a specified threshold
ELISA	Enzyme-linked immunosorbent assay
EPA	U.S. Environmental Protection Agency
GPMT	Guinea pig maximization test
HCA	Hexyl cinnamic aldehyde
HMT	Human maximization test
HPTA	Human patch test allergen
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ISO	International Organization for Standardization
IWG	Immunotoxicity Working Group
JSAAE	Japanese Society for Alternatives to Animal Experiments
K_{ow}	Octanol-water partition coefficient
LLNA	Murine local lymph node assay
LLNA: BrdU-ELISA	LLNA with enzyme-linked immunosorbent assay detection of bromodeoxyuridine
MEK	Methyl ethyl ketone
MeSH	Medical Subject Headings
Min	Minimal
Mod	Moderate
MW	Molecular weight

NA	Not available
NC	Not calculated
NK	Not known
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NT	Not tested
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
Res	Result
SD	Standard deviation
SI	Stimulation index
TG	Test Guideline
Unk	Unknown
Veh.	Vehicle
vs.	Versus
w/v	Weight-to-volume ratio

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Preface

In 1999, the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended the murine (mouse) local lymph node assay (LLNA) as a valid test method to assess the skin sensitization potential of most types of substances (ICCVAM 1999; Sailstad et al. 2001; Dean et al. 2001; Haneke et al. 2001). ICCVAM concluded that the LLNA (referred to herein as the "traditional LLNA") provided several advantages compared to guinea pig test methods, including elimination of potential pain and distress, use of fewer animals, less time required to perform, and availability of dose-response information. United States and international regulatory authorities subsequently accepted the traditional LLNA as an alternative test method for allergic contact dermatitis testing. It is now commonly used around the world.

One disadvantage of the traditional LLNA is that it requires injection of a radioactive marker to measure cell proliferation in lymph nodes. To avoid the use of radioactive markers, scientists have recently developed several nonradioactive versions of the LLNA. In 2007, the U.S. Consumer Product Safety Commission (CPSC) asked ICCVAM and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to evaluate the scientific validity of these nonradioactive versions. ICCVAM assigned the nomination a high priority, and established the ICCVAM Immunotoxicity Working Group (IWG) to work with NICEATM to review the current literature and evaluate available data to assess the validity of three such test methods. The evaluation process involved two public meetings of an international independent scientific peer review panel (referred to hereafter as "Panel") that reviewed draft and revised draft background review documents (BRDs) and ICCVAM test method recommendations.

A comprehensive draft background review document provided the initial information, data, and analyses supporting the validation status of each of the nonradioactive test methods. ICCVAM also developed draft test method recommendations for each test method regarding its usefulness and limitations, test method protocol, performance standards, and future studies. NICEATM and ICCVAM provided the draft BRDs and draft test method recommendations to the Panel for their consideration at a public meeting on March 4-6, 2008. A report of the Panel meeting was subsequently published on the NICEATM-ICCVAM website.[1] Both the Panel and ICCVAM concluded that more information was needed before a recommendation on the usefulness and limitations of each of the three test methods could be made. The Panel recommended that NICEATM obtain additional data that were not available to the Panel and reanalyze the performance of each nonradioactive LLNA test method. NICEATM subsequently obtained additional data and prepared revised draft BRDs. ICCVAM also prepared revised draft test method recommendations based on the revised draft BRDs. NICEATM and ICCVAM provided the revised draft BRDs and revised draft test method recommendations to the Panel for their consideration at a public meeting on April 28-29, 2009. A report of the Panel meeting was subsequently published on the NICEATM-ICCVAM website.[2]

Based on the revised draft ICCVAM recommendations, NICEATM submitted a proposed draft Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) for the LLNA with enzyme-linked immunosorbent assay detection of bromodeoxyuridine (referred to hereafter as the "LLNA: BrdU-ELISA") that was circulated in July 2009 to the 30 OECD member countries for review and comment. An OECD Expert Consultation Meeting was held on October 20-22, 2009, to evaluate the comments. The expert group reviewed the draft OECD TG for the LLNA: BrdU-ELISA and proposed responses to the comments from member countries. A revised TG was again distributed to the 30 OECD member countries in December 2009 for review and comment,

[1] http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel.htm.
[2] http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel.htm.

and then the final draft was forwarded to the OECD Working Group of National Co-ordinators of the Test Guidelines Programme to consider for adoption at their March 23-25, 2010, meeting.

ICCVAM considered the conclusions and recommendations of the Panel and conclusions from the OECD Expert Consultation, along with comments received from the public and the Scientific Advisory Committee for Alternative Toxicological Methods (i.e., the ICCVAM-NICEATM advisory committee), and then finalized the BRDs and test method recommendations. These will be forwarded to Federal agencies for their consideration and acceptance decisions, where appropriate. This BRD addresses the validation database for the LLNA: BrdU-ELISA.

We gratefully acknowledge the organizations and scientists who provided data and information for this document. We would also like to recognize the efforts of the individuals who contributed to its preparation, review, and revision. We especially recognize the Panel members for their thoughtful evaluations and generous contributions of time and effort. Special thanks are extended to Dr. Michael Luster for serving as the Panel Chair and to Dr. Michael Woolhiser, Dr. Michael Olson, Kim Headrick, and Dr. Stephen Ullrich for their service as Evaluation Group Chairs. We thank the IWG for assuring a meaningful and comprehensive review. We thank Drs. Abigail Jacobs (U.S. Food and Drug Administration) and Joanna Matheson (CPSC) for serving as Co-chairs of the IWG, as well as the members of the IWG and ICCVAM representatives who subsequently reviewed and provided comments throughout the process leading to this final BRD.

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Executive Summary

Background

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended to U.S. Federal agencies that the murine local lymph node assay (LLNA) is a valid substitute for currently accepted guinea pig (GP) test methods to assess the allergic contact dermatitis (ACD) potential of most types of substances. ACD is an allergic skin reaction characterized by redness, swelling, and itching that can result from contact with a sensitizing chemical or product. The recommendation was based on a comprehensive evaluation that included an independent scientific peer review panel (Panel) assessment of the validation status of the LLNA. The Panel report and the ICCVAM recommendations (ICCVAM 1999) are available at the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)-ICCVAM website (http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf). The LLNA was subsequently incorporated into national and international test guidelines for the assessment of skin sensitization (Organisation for Economic Co-operation and Development [OECD] Test Guideline 429 [OECD 2002]; International Organization for Standardization [ISO] 10993-10: Tests for Irritation and Delayed-type Hypersensitivity [ISO 2002]; U.S. Environmental Protection Agency [EPA] Health Effects Test Guidelines on Skin Sensitization [EPA 2003]).

In 2007, the U.S. Consumer Product Safety Commission (CPSC) formally nominated several activities related to the LLNA for evaluation by ICCVAM and NICEATM (available at http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf). One of the nominated activities was an assessment of the validation status of nonradioactive modifications to the current version of the LLNA ([ICCVAM 1999; Sailstad et al. 2001; Dean et al. 2001; Haneke et al. 2001], referred to hereafter as the "traditional LLNA"), which uses radioactivity to detect sensitizers. The information described in this background review document (BRD) was compiled by ICCVAM and NICEATM in response to this nomination. The BRD provides a comprehensive review of data and information regarding the usefulness and limitations of one of these test methods, the LLNA with detection of bromodeoxyuridine (BrdU) incorporation by an enzyme-linked immunosorbent assay (ELISA) (referred to hereafter as the "LLNA: BrdU-ELISA").

Test Method Protocol

The LLNA: BrdU-ELISA was originally developed by Takeyoshi et al. (2001). While the traditional LLNA assesses cellular proliferation by measuring the incorporation of radioactivity into the deoxyribonucleic acid (DNA) of dividing lymph node cells, the LLNA: BrdU-ELISA assesses the same endpoint by measuring the incorporation of the thymidine analog BrdU using an ELISA. A stimulation index (SI), the ratio of the mean BrdU incorporation into the lymph nodes of mice in the test substance group to the mean BrdU incorporation into the lymph nodes of mice in the vehicle control group, is used to identify a substance as a sensitizer. Other than the procedure for measuring lymph node cell proliferation, the protocol for the LLNA: BrdU-ELISA is similar to that of the traditional LLNA (ICCVAM 1999; Sailstad et al. 2001; Dean et al. 2001; Haneke et al. 2001).

Validation Database

The accuracy and reliability of the LLNA: BrdU-ELISA were assessed using the individual animal data for 43 substances from six published studies (Takeyoshi et al. 2003, 2004a, 2004b, 2005, 2006, 2007a), one platform presentation (Takeyoshi 2007b), one poster presentation (Kojima et al. 2008), and unpublished data submitted to NICEATM in 2009. The reference test data for these substances were obtained from the traditional LLNA, GP skin sensitization tests, and/or human skin sensitization tests or clinical information. Of the 43 substances with traditional LLNA data, 32 were classified by the traditional LLNA as skin sensitizers and 11 were classified as nonsensitizers.

Test Method Accuracy

The accuracy evaluation in this BRD includes the evaluation of multiple decision criteria, including the SI ≥ 2.0 recommended in the test method protocol. Based on the evaluation of multiple decision criteria, the optimal performance was achieved using SI ≥ 1.6 to classify sensitizers. Compared with the traditional LLNA, accuracy was 95% (41/43), with a false positive rate of 18% (2/11) and a false negative rate of 0% (0/32). The two false positive substances produced borderline positive SI values between 1.6 and 1.9 in the LLNA: BrdU-ELISA.

When the decision criterion of SI ≥ 2.0 was used to classify sensitizers vs. nonsensitizers, compared to the traditional LLNA, accuracy was 95% (41/43), with a false positive rate of 0% (0/11) and a false negative rate of 6% (2/32). Between the two false negative substances, no unique characteristics were identified that could be used as rationale for excluding any particular types of substances from testing in the LLNA: BrdU-ELISA.

The reduced LLNA: BrdU-ELISA (rLLNA: BrdU-ELISA), which uses only the highest soluble dose of the test substance that does not produce local skin irritation or systemic toxicity, can reduce animal use by 40% for hazard classification purposes where dose-response information is not needed. Using SI ≥ 1.6 to classify sensitizers, the accuracy of the rLLNA: BrdU-ELISA compared with the multiple-dose LLNA: BrdU-ELISA was 95% (82/85 tests), with a false positive rate of 0% (0/11 tests) and a false negative rate of 4% (3/74 tests). The three tests that were false negative in the rLLNA: BrdU-ELISA were weakly positive in the LLNA: BrdU-ELISA at a concentration lower than the highest dose (SI = 1.62, 2.02, and 2.22). The highest dose tested for each of the three tests of two substances was 50%.

Test Method Reliability – Intralaboratory Reproducibility

Intralaboratory reproducibility was assessed using a concordance analysis of sensitizer/nonsensitizer results and a coefficient of variation (CV) analysis of SI values and EC1.6 values (estimated concentration needed to produce an SI of 1.6). The qualitative analysis shows that multiple tests of 12 substances (10 sensitizers and two nonsensitizers) yielded 100% concordance for the sensitizer/nonsensitizer outcomes for 10/12 substances. However, one of the nonsensitizers with 100% concordance produced false positive results in both tests that were conducted for this substance. In the quantitative analyses, the CV values for the SI values of 13 substance/concentration combinations that were tested up to five times each ranged from 1% to 80%. The CV values for the EC1.6 values of four substances that were tested up to five times at multiple doses ranged from 37% to 118%.

Test Method Reliability – Interlaboratory Reproducibility

When using SI ≥ 1.6 to classify sensitizers, the qualitative interlaboratory reproducibility analysis of 10 substances (seven sensitizers and three nonsensitizers), that were tested in up to seven laboratories indicated 100% agreement (3/3, 6/6, or 7/7) among the laboratories for nine substances (seven sensitizers and two nonsensitizers). One of the nonsensitizers with 100% concordance, however, produced false positive results in 3/3 laboratories. There was 67% (4/6) agreement among the tests for the remaining nonsensitizer. Interlaboratory CV values for the EC1.6 values of the seven sensitizers ranged from 31% to 93%.

When using SI ≥ 1.6 to classify sensitizers, the categorical concordance analysis for the 18 substances with multiple tests indicated that the SI results for 85% (11/13) of the LLNA sensitizers were 100% concordant (i.e., all yielded SI ≥ 1.6 and SI ≥ 1.9). Two of the 13 sensitizers produced one test with SI < 1.6 and one test with SI > 1.6. The SI results for 60% (3/5) of the nonsensitizers were 100% concordant. All tests for two nonsensitizers had SI < 1.6, and all tests of the third nonsensitizer yielded SI values between 1.6 and 1.9. The concordance of the other two nonsensitizers was 67% (2/3 tests) for SI values between 1.6 and 1.9 and 71% (5/7 tests) for SI < 1.6.

Animal Welfare Considerations

The LLNA: BrdU-ELISA will use the same number of animals when compared to the updated ICCVAM-recommended LLNA protocol (Appendix A of ICCVAM 2009). However, since use of the traditional LLNA is restricted in some institutions because it involves radioactivity, availability and use of the nonradioactive LLNA: BrdU-ELISA may lead to further reduction in use of the GP tests, which would provide for reduced animal use and increased refinement due to the avoidance of pain and distress in the LLNA procedure.

Further, the LLNA: BrdU-ELISA evaluates the induction phase of sensitization and therefore discomfort to animals associated with the elicitation phase is eliminated. Additionally, the LLNA: BrdU-ELISA protocol requires fewer mice per treatment group (a minimum of four animals per group) than either of the GP tests (10-20 animals/group for the Buehler test and 5-10 animals/group for the GPMT).

Test Method Transferability

The transferability of the LLNA: BrdU-ELISA was demonstrated by an interlaboratory validation study (Kojima et al. 2008). Compared to the traditional LLNA, the LLNA: BrdU-ELISA will not require facilities, equipment, and licensing permits for handling radioactive materials. The level of training and expertise needed to conduct the LLNA: BrdU-ELISA should be similar to the traditional LLNA, except that the understanding and use of the ELISA is required.

1.0 Introduction

1.1 Public Health Perspective

Allergic contact dermatitis (ACD) is a frequent occupational health problem. According to the U.S. Department of Labor Bureau of Labor Statistics, in 2005, 980 cases of ACD involved days away from work.[3] ACD develops in two phases, induction and elicitation. The induction phase occurs when a susceptible individual is exposed topically to a skin-sensitizing substance. Induction depends on the substance passing through the epidermis, where it forms a hapten complex with dermal proteins. The Langerhans cells, the resident antigen-presenting cells in the skin, process the hapten complex. The processed hapten complex then migrates to the draining lymph nodes. Antigen presentation to T-lymphocytes follows, which leads to the clonal expansion of these cells. At this point, the individual is sensitized to the substance (Basketter et al. 2003; Jowsey et al. 2006). Studies have shown that the magnitude of lymphocyte proliferation correlates with the extent to which sensitization develops (Kimber and Dearman 1991, 1996).

The elicitation phase occurs when the individual is again topically exposed to the same substance. As in the induction phase, the substance penetrates the epidermis, is processed by the Langerhans cells, and is presented to circulating T-lymphocytes. The antigen-specific T-lymphocytes are then activated, which causes release of cytokines and other inflammatory mediators. This release produces a rapid dermal immune response that can lead to ACD (ICCVAM 1999; Sailstad 2001; Basketter et al. 2003; Jowsey et al. 2006).

1.2 Historical Background for the Murine Local Lymph Node Assay (LLNA)

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended the LLNA as a valid substitute for currently accepted guinea pig (GP) test methods to assess the ACD potential of most types of substances. The recommendation was based on a comprehensive evaluation that included an independent scientific peer review panel (Panel) assessment of the validation status of the LLNA. The Panel report and the ICCVAM recommendations (ICCVAM 1999) are available at the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)-ICCVAM website (http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf).

ICCVAM forwarded recommendations to U.S. Federal agencies that the LLNA should be considered for regulatory acceptance or other nonregulatory applications for assessing the ACD potential of substances, while recognizing that some testing situations would still require the use of traditional GP test methods (ICCVAM 1999; Sailstad et al. 2001). The LLNA was subsequently incorporated into national and international test guidelines for the assessment of skin sensitization (Organisation for Economic Co-operation and Development [OECD] Test Guideline [TG] 429 [OECD 2002]; International Standards Organization [ISO] 10993-10: Tests for Irritation and Delayed-type Hypersensitivity [ISO 2002]; U.S. Environmental Protection Agency [EPA] Health Effects Test Guidelines on Skin Sensitization [EPA 2003]).

On January 10, 2007, the U.S. Consumer Product Safety Commission (CPSC) formally nominated several activities related to the LLNA for evaluation by ICCVAM and NICEATM (available at http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf). One of the nominated activities was an assessment of the validation status of nonradioactive modifications to the current version of the LLNA ([ICCVAM 1999; Dean et al. 2001], referred to hereafter as the "traditional LLNA"), which uses radioactivity to detect sensitizers. The information described in this background review document (BRD) was compiled by ICCVAM and NICEATM in response to this

[3] Available at http://www.bls.gov/.

nomination. This BRD provides a comprehensive review of available data and information regarding the usefulness and limitations of one of these methods, the LLNA with detection of bromodeoxyuridine (BrdU) incorporation by enzyme-linked immunosorbent assay (ELISA) (referred to hereafter as the "LLNA: BrdU-ELISA"). ICCVAM and its Immunotoxicity Working Group (IWG) evaluated this method in a draft BRD and developed draft test method recommendations based on this initial evaluation. An independent peer review panel (Panel) reviewed the draft BRD in March 2008 to evaluate the extent to which the information contained in the BRD supported the draft test method recommendations. The Panel concluded that additional information was needed to evaluate the test method, including a detailed test method protocol, individual animal data on a larger number of reference substances that cover a wide range of physicochemical properties and sensitization potency, and an evaluation of interlaboratory reproducibility. In response to this recommendation, NICEATM obtained additional LLNA: BrdU-ELISA data and information, which were used to generate a revised draft BRD for review by the Panel in April 2009.

Based on the revised draft ICCVAM test method recommendations, NICEATM submitted a proposed draft OECD TG for the LLNA: BrdU-ELISA that was circulated in July 2009 to the 30 OECD member countries for review and comment via their National Co-ordinators, who distributed the draft TG to interested stakeholders. An OECD Expert Consultation meeting was held on October 20-22, 2009, to evaluate the comments. Scientists from the National Institute of Environmental Health Sciences (NIEHS), the Environmental Protection Agency (EPA), the Food and Drug Administration (FDA), and CPSC, as well as U.S. and international experts from industry and other stakeholder organizations, participated in the meeting, which was co-hosted by CPSC and NICEATM-ICCVAM. The expert group reviewed the draft OECD TG for the LLNA: BrdU-ELISA, proposed responses to comments from member countries, and evaluated additional LLNA: BrdU-ELISA results for 12 substances tested and submitted to NICEATM after the Panel evaluation. The OECD Expert Consultation convened a subsequent teleconference on December 1, 2009, to discuss outstanding issues identified at the October meeting. A revised TG was again distributed to the 30 OECD member countries in December 2009, via their National Co-ordinators, for review and comment by national experts and interested stakeholders. A final teleconference of the OECD Expert Consultation was convened on January 29, 2010 to discuss the member country comments received during the last round of review, and a final draft TG was developed based on these discussions. This final draft was forwarded to the OECD Working Group of National Co-ordinators of the Test Guidelines Programme to consider for adoption at their March 23-25, 2010 meeting.

ICCVAM and the IWG considered the conclusions and recommendations of the Panel, comments received from the public and its advisory committee (i.e., the Scientific Advisory Committee on Alternative Toxicological Methods [SACATM]), along with the conclusions of the OECD Expert Consultation on the LLNA, and developed this final BRD. ICCVAM provides this final BRD to regulatory agencies for consideration as part of the ICCVAM Test Method Evaluation Report.

1.3 The LLNA: BrdU-ELISA

The LLNA: BrdU-ELISA was developed by Takeyoshi et al. (2001) as a nonradioactive alternative to the traditional LLNA. While the traditional LLNA assesses cellular proliferation by measuring the incorporation of radioactivity into the deoxyribonucleic acid (DNA) of dividing lymph node cells, the LLNA: BrdU-ELISA assesses the same endpoint by measuring the incorporation of the thymidine analog BrdU, which is detected and quantified with an ELISA, which is available as a kit commercially from several sources.

This document provides:

- A comprehensive summary of the LLNA: BrdU-ELISA test method protocol
- The substances used in the validation of the test method and the test results

- The performance characteristics (accuracy and reliability) of the test method
- Animal welfare considerations
- Other considerations relevant to the usefulness and limitations of this test method (e.g., transferability, cost of the test method).

2.0 LLNA: BrdU-ELISA Test Method Protocol

The LLNA: BrdU-ELISA protocol (see **Annex I**) is similar to the ICCVAM-recommended protocol for the traditional LLNA (see Appendix A of ICCVAM [2009]), except for the method used to assess lymphocyte proliferation. In both the LLNA: BrdU-ELISA and the traditional LLNA, the test substance is administered on three consecutive days. In the traditional LLNA, ^{3}H- thymidine or ^{125}I-iododeoxyuridine (in phosphate buffered saline; 250 µL/mouse) is administered via the tail vein two days after the final application of the test substance. In the LLNA: BrdU-ELISA, 5 mg BrdU in a volume of 0.5 mL physiological saline (concentration of 10 mg/mL) is administered via intraperitoneal injection two days after the final application of the test substance. Takeyoshi et al. (2001) reported that one injection of 5 mg BrdU was selected over two injections to minimize the incorporation of BrdU in the control group. Injection of BrdU two days after topical treatment with test substance yielded efficient incorporation of BrdU in comparison to injection one day or three days after topical treatment with a test substance (Takeyoshi et al. 2001). On the day following BrdU injection, lymph nodes are excised and a single cell suspension is prepared from the lymph nodes of each animal. A standard aliquot of the cell suspension is added in triplicate to the wells of a flat-bottom 96-well microplate and centrifuged. Supernatants are then removed. FixDenat solution (Roche Applied Science), which fixes the cells and denatures the DNA in one step, is added to each well, and the plate is incubated at room temperature. The FixDenat solution is removed, and the diluted anti-BrdU antibody solution is added to each well. After each well is washed with phosphate buffered saline, an aliquot of substrate solution containing tetramethylbenzidine is added. After incubation at room temperature, the absorbance is measured using a microplate reader.

2.1 Decision Criteria

Like the traditional LLNA, a stimulation index (SI) is used in the LLNA: BrdU-ELISA to distinguish skin sensitizers from nonsensitizers. The SI is the ratio of the mean absorbance of the incorporated BrdU in a lymph node suspension from individual mice in the test substance group to the mean absorbance of the incorporated BrdU in a lymph node suspension from individual mice in the vehicle control group as indicated by the formula below:

$$\text{SI} = \frac{\text{Mean absorbance of the treatment group lymph nodes}}{\text{Mean absorbance of the vehicle control group lymph nodes}}$$

Consistent with the traditional LLNA, an SI $\geq$ 3.0 was initially used as the threshold for labeling a substance as a sensitizer. Takeyoshi et al. (2007b) evaluated the use of other decision criteria such as specific differences in BrdU incorporation between treated and control groups (i.e., greater than the 95% confidence interval [CI] of the control group, greater than the two or three standard deviations [SD] from the control group mean, and statistically significant differences by analysis of variance [ANOVA]) and other SI values to distinguish sensitizers from nonsensitizers and found that lower cutoff values for the SI improved accuracy when compared with the results of the traditional LLNA.

A multilaboratory validation study of the LLNA: BrdU-ELISA organized by the Japanese Society for Alternatives to Animal Experiments (JSAAE) used SI $\geq$ 2 to classify sensitizers (Kojima et al. 2008). The SI $\geq$ 2 criterion was selected for the interlaboratory validation study because prior studies (Takeyoshi et al. 2003, 2004a, 2004b, 2005, 2006, 2007a, 2007b) indicated that the SI $\geq$ 3 criterion was inadequate for reliably distinguishing sensitizers from nonsensitizers (Kojima H, personal communication).

3.0 LLNA: BrdU-ELISA Validation Database

The validation database for the LLNA: BrdU-ELISA includes data that were available for 47 substances; 43 substances had been previously tested in the traditional LLNA. Thirty-nine substances were tested in one laboratory (Takeyoshi et al. 2003, 2004a, 2004b, 2005, 2006, 2007a, 2007b, unpublished data) and four additional substances (along with six of the same substances tested by Takeyoshi et al.) were tested in the multilaboratory validation study coordinated by JSAAE (**Table C-1**). No traditional LLNA data were available for four substances, which include two dimers of eugenol (dihydroxyl-3,3'-dimethoxy-5,5'-diallyl-biphenyl and 4,5'-diallyl-2'-hydroxy-2,3'-dimethoxyphenyl ether) and two dimers of isoeugenol (4-[1-hydroxy-2-(2-methoxy-4-propenyl-phenyoxy)-propyl]-2-methoxy-phenol and 2-methoxy-4-(7-methoxy-3-methyl-5-propenyl-2,3-dihydro-benzofuran-2yl)-phenol) (Takeyoshi et al. 2004a, 2007a). Of the 43 substances with traditional LLNA data, 32 were classified by the traditional LLNA as skin sensitizers and 11 were classified as nonsensitizers. The traditional LLNA EC3 values (i.e., estimated concentration needed to produce an SI = 3) for the 32 sensitizers ranged from 0.009% to 47.5% (**Table C-1)**.

Annex II provides information on physicochemical properties (e.g., physical form tested). For the 43 substances evaluated, the molecular weights ranged from 30.03 to 388.29 g/mole. Twenty-five substances were liquids and 18 substances are solids. Estimated log octanol-water partition coefficients, which were available for 41 substances, ranged from -3 to 3.88. Peptide reactivity, which was available for 22 substances, ranged from high to minimal (Gerberick et al. 2007a).

Annex II further provides information on the Chemical Abstracts Service Registry Number and chemical class for each substance tested. When available, chemical classes for each substance were retrieved from the National Library of Medicine Medical Subject Headings classification system (available at http://www.nlm.nih.gov/mesh/meshhome.html). If chemical classes were unavailable, they were assigned using a standard classification scheme based on the Medical Subject Headings classification system. A substance could be assigned to more than one chemical class; however, no substance was assigned to more than three classes. Chemical class information is presented only to provide an indication of the variety of structural elements that are present in the structures that were evaluated in this analysis. Classification of substances into chemical classes is not intended to indicate the impact of structure on biological activity with respect to sensitization potential.

Table C-1 shows that 19 chemical classes are represented by the 47 substances tested in the LLNA: BrdU-ELISA. Eleven substances are classified in more than one chemical class. The classes with the highest number of substances are carboxylic acids (13 substances) and aldehydes (six substances). Of the 22 chemical classes represented in the NICEATM LLNA database by at least five substances (thereby providing a sufficiently large representation for further analyses), 20 classes had at least 60% of the traditional LLNA results identified as positive. For this database of more than 600 substances, these classes were identified as those most likely to be associated with skin sensitization. Fifteen of these classes were also represented in the LLNA: BrdU-ELISA database (only amides, ethers, ketones, macromolecular substances, and polycyclic compounds were not included). Among the chemical classes that have been previously identified as common skin allergens (e.g., aldehydes, ketones, quinones, and acrylates [Gerberick et al. 2004]), only ketones were not included in the LLNA: BrdU-ELISA database.

Table C-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: BrdU-ELISA EC1.6 Values, and Maximum SI Values for 43 Tested Substances

Substance Name	Product Use[1]	Chemical Class[2]	Traditional LLNA EC3 (Maximum SI)[3]	LLNA: BrdU-ELISA EC1.6 (Maximum SI)[3]
5-Chloro-2-methyl-4-isothaizolin-3-one*	Cosmetics; Manufacturing; Pesticides	Sulfur Compounds; Heterocyclic Compounds	0.009 (27.7)	0.065 (4.8)
p-Benzoquinone	Manufacturing; Pesticides; Pharmaceuticals	Quinones	0.010 (52.3)	0.150 (6.9)
2,4-Dinitrochlorobenzene*	Manufacturing; Pesticides	Hydrocarbon, Halogenated; Nitro Compounds; Hydrocarbons, Cyclic	0.049 (43.9)	0.032 (18.8)
Diphenylcyclopropenone	Pharmaceuticals	Hydrocarbons, Cyclic	0.050 (NA)	0.450 (19.1)
Glutaraldehyde	Cosmetics; Disinfectant; Manufacturing; Pesticides	Aldehydes	0.083 (18.0)	0.115 (28.6)
4-Phenylenediamine*	Intermediate in chemical synthesis; Manufacturing	Amines	0.11 (26.4)	0.285 (14.7)
Formaldehyde	Disinfectant; Manufacturing	Aldehydes	0.50 (4.0)	0.163 (16.6)
Cobalt chloride*	Manufacturing; Pesticides	Inorganic Chemical, Elements; Inorganic Chemical, Metals	0.66 (7.2)	0.316 (3.7)
4-Methylaminophenol sulfate	Manufacturing	Amines; Phenols	0.8 (6.7)	1.081 (4.0)
trans-Cinnamaldehyde	Food Additive; Fragrance Agent	Aldehydes	1.4 (13.1)	1.530 (5.9)
Isoeugenol*	Food Additive; Fragrance Agent	Carboxylic Acids	1.5 (31.0)	5.156 (8.4)
2-Mercaptobenzothiazole*	Manufacturing; Pesticides	Heterocyclic Compounds	1.7 (8.6)	12.097 (1.6)

continued

Table C-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: BrdU-ELISA EC1.6 Values, and Maximum SI Values for 43 Tested Substances (continued)

Substance Name	Product Use[1]	Chemical Class[2]	Traditional LLNA EC3 (Maximum SI)[3]	LLNA: BrdU-ELISA EC1.6 (Maximum SI)[3]
Cinnamic aldehyde	Cosmetics; Food Additive; Fragrance Agent; Intermediate in chemical synthesis; Personal Care Products; Pesticides	Aldehydes	1.9 (18.4)	4.808 (4.0)
3-Aminophenol	Cosmetics; Pharmaceuticals	Amines; Phenols	3.2 (5.7)	2.990 (3.1)
Diethyl maleate	Food Additive; Intermediate in chemical synthesis	Carboxylic Acids	3.6 (22.6)	8.049 (6.3)
Trimellitic anhydride	Manufacturing	Anhydrides; Carboxylic Acids	4.7 (4.6)	0.862 (7.9)
Nickel sulfate	Manufacturing	Inorganic Chemicals, Metals; Inorganic Chemicals, Elements	4.8 (3.1)	1.027 (4.5)
4-Chloroaniline	Intermediate in chemical synthesis; Manufacturing; Pesticides; Pharmaceuticals	Amines	9.00 (3.3)	11.029 (2.5)
Sodium lauryl sulfate*	Cosmetics; Food Additive; Manufacturing; Personal Care Products; Pesticides; Pharmaceuticals	Alcohols; Sulfur Compounds; Lipids	8.1 (8.9)	13.334 (2.6)
Citral*	Fragrance Agent	Hydrocarbons, Other	9.2 (20.5)	7.143 (16.4)
Hexyl cinnamic aldehyde*	Food Additive; Fragrance Agent	Aldehydes	9.7 (20.0)	12.920 (13.5)
Eugenol*	Cosmetics; Food Additive; Intermediate in chemical synthesis; Manufacturing; Personal Care Products; Pharmaceuticals	Carboxylic Acids	10.1 (17.0)	8.851 (17.7)
Phenyl benzoate*	Manufacturing; Pesticides	Carboxylic Acids	13.6 (11.1)	16.954 (3.4)

continued

Table C-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: BrdU-ELISA EC1.6 Values, and Maximum SI Values for 43 Tested Substances (continued)

Substance Name	Product Use[1]	Chemical Class[2]	Traditional LLNA EC3 (Maximum SI)[3]	LLNA: BrdU-ELISA EC1.6 (Maximum SI)[3]
Cinnamic alcohol*	Cosmetics; Food Additive; Fragrance Agent; Intermediate in chemical synthesis; Personal Care Products	Alcohols	21.0 (5.7)	24.091 (2.7)
Cyclamen aldehyde	Food Additive; Fragrance Agent	Aldehydes	22.3 (5.2)	41.496 (5.7)
Hydroxycitronellal	Food Additive; Fragrance Agent; Personal Care Products	Hydrocarbons, Other	24.0 (8.5)	13.636 (4.8)
Imidazolidinyl urea*	Cosmetics; Personal Care Products; Pesticides	Urea	24.0 (5.5)	49.545 (1.6)
Ethylene glycol dimethacrylate*	Manufacturing	Carboxylic Acids	28.0 (7.0)	31.751 (3.1)
Linalool	Cosmetics; Food Additive; Fragrance Agent; Personal Care Products; Pesticides	Hydrocarbons, Other	30.0 (8.3)	27.596 (4.7)
Ethyl acrylate	Manufacturing	Carboxylic Acids	32.8 (4.0)	33.333 (5.0)
Isopropyl myristate	Cosmetics; Personal Care Products; Pharmaceuticals	Lipids	44.0 (3.4)	9.404 (4.2)
Aniline	Food Additive; Manufacturing; Personal Care Products; Pesticides; Pharmaceuticals	Amines	47.5 (4.4)	73.596 (2.1)
2-Hydroxypropyl methacrylate	Intermediate in chemical synthesis; Manufacturing	Carboxylic Acids	NC (1.3)	NC (1.1)
Diethyl phthalate	Cosmetics; Manufacturing; Personal Care Products; Pesticides; Pharmaceuticals	Carboxylic Acids	NC (1.5)	NC (0.9)
Dimethyl isophthalate	Manufacturing; Fragrance Agent	Carboxylic Acids	NC (1.0)	NC (1.3)

continued

Table C-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: BrdU-ELISA EC1.6 Values, and Maximum SI Values for 43 Tested Substances (continued)

Substance Name	Product Use[1]	Chemical Class[2]	Traditional LLNA EC3 (Maximum SI)[3]	LLNA: BrdU-ELISA EC1.6 (Maximum SI)[3]
Glycerol	Cosmetics; Food Additive; Intermediate in chemical synthesis; Manufacturing; Personal Care Products; Pharmaceuticals; Solvent	Alcohols; Carbohydrates	NC (1.1)	NC (1.3)
Hexane	Manufacturing; Solvent	Hydrocarbons, Acyclic	NC (2.2)	56.328 (1.9)
Isopropanol*	Cosmetics; Disinfectant; Food Additive; Intermediate in chemical synthesis; Manufacturing; Personal Care Products; Pharmaceuticals; Solvent	Alcohols	NC (1.7)	5.344 (2.2)[4]
Lactic acid*	Food Additive; Manufacturing; Pharmaceuticals	Carboxylic Acids	NC (2.2)	15.177 (2.5)
Methyl salicylate*	Cosmetics; Food Additive; Fragrance Agent; Personal Care Products; Pharmaceuticals; Solvent	Carboxylic Acids	NC (2.9)	NC (1.4)
Salicylic acid*	Food Additive; Manufacturing; Pharmaceuticals	Phenols; Carboxylic Acids	NC (2.5)	NC (1.3)
Sulfanilamide	Pharmaceuticals	Hydrocarbons, Cyclic; Sulfur Compounds	NC (1.0)	NC (1.3)
Propylene glycol	Cosmetics; Food Additive; Intermediate in chemical synthesis; Personal Care Products; Pharmaceuticals; Solvent	Alcohols	NC (1.6)	NC (1.6)

Abbreviations: EC1.6 = estimated concentration (expressed as percentage) needed to produce SI = 1.6; EC3 = estimated concentration (expressed as percentage) needed to produce SI = 3; LLNA = murine local lymph node assay; LLNA: BrdU-ELISA= local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; NA = not available; NC = not calculated since maximum SI < 3.0 for the traditional LLNA or maximum SI < 1.6 for the LLNA: BrdU-ELISA; SI = stimulation index.

* Reference substance from ICCVAM (2009).

[1] Information gathered from the following databases: Hazardous Substances Database (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB); Haz-Map (http://hazmap.nlm.nih.gov/); Household Products Database (http://hpd.nlm.nih.gov/index.htm); International Programme on Chemical Safety INCHEM database (http://www.inchem.org/); and the National Toxicology Program (http://ntp.niehs.nih.gov:8080/index.html?col=010stat).

[2] Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs, developed by the National Library of Medicine (http://www.nlm.nih.gov/mesh/meshhome.html).

[3] Mean EC3 (expressed as % concentration) and maximum SI values are from the NICEATM database of traditional LLNA studies. EC1.6 and SI values for individual LLNA: BrdU-ELISA tests are provided in Annex IV of the BRD (Appendix C).

[4] Highest SI of seven tests. Because the majority (five) of the seven tests, had SI values < 1.6, isopropanol is considered to be a nonsensitizer in the LLNA: BrdU-ELISA.

4.0 Reference Data

Thirty-five of the 43 substances previously tested in the traditional LLNA were considered in the original evaluation of the LLNA by ICCVAM (ICCVAM 1999). The traditional LLNA reference data used for the accuracy evaluation described in **Section 6.0** were obtained from ICCVAM (1999) for 33 of these substances (**Annex III**). The traditional LLNA data for the two remaining substances included in the original LLNA evaluation (ICCVAM 1999), aniline and nickel sulfate, were obtained from more recent sources, Gerberick et al. (2005) and Ryan et al. (2002), respectively. The traditional LLNA results in ICCVAM (1999) for these two substances were negative, but the subsequent tests at higher concentrations produced positive results. The traditional LLNA data for the remaining eight substances that were not considered in the original ICCVAM evaluation (ICCVAM 1999), *trans*-cinnamaldehyde, cinnamic alcohol, cyclamen aldehyde, diethyl maleate, ethyl acrylate, glutaraldehyde, isopropyl myristate, and linalool, were obtained from Gerberick et al. (2005), Gerberick et al. (2005), Basketter et al. (2005), Gerberick et al. (2005), Gerberick et al. (2005), Hilton et al. (1998), Ryan et al. (2000), and Gerberick et al. (2005), respectively.

The reference data for the GP tests (guinea pig maximization test [GPMT] or Buehler test) and human tests (human maximization test, human patch test allergen, or other human data) were obtained from Marzulli and Maibach (1974), Marzulli and Maibach (1980), Opdyke (1976), Björkner (1984), Gad et al. (1986), Jordan and King (1977), Klecak et al. (1997), ICCVAM (1999), Basketter et al. (1999b, 2005), Basketter and Scholes (1992), Kwon et al. (2003), Robinson et al. (1990), Takeyoshi et al. (2004a), Van der Walle et al. (1982), and Takeyoshi et al. (2007a) (**Annex III**). Although there were no traditional LLNA data available for the eugenol dimers (dihydroxyl-3,3'-dimethoxy-5,5'-diallyl-biphenyl and 4,5'-diallyl-2'-hydroxy-2,3'-dimethoxyphenyl ether) or the isoeugenol dimers (4-[1-Hydroxy-2-(2-methoxy-4-propenyl-phenyoxy)-propyl]-2-methoxy-phenol and 2-Methoxy-4-(7-methoxy-3-methyl-5-propenyl-2,3-dihydro-benzofuran-2yl)-phenol), Takeyoshi et al. (2004a and 2007a, respectively) provided results from the GPMT for these compounds.

An independent quality assurance contractor for the NTP audited the traditional LLNA data provided in ICCVAM (1999). Audit procedures and findings are presented in the quality assurance report on file at the NIEHS. The audit supports the conclusion that the transcribed test data in the submission were accurate, consistent, and complete as compared to the original study records.

5.0 Test Method Data and Results

The LLNA: BrdU-ELISA data evaluated in this technical summary were obtained from individual animal data that were submitted to NICEATM. These data supported six published studies (Takeyoshi et al. 2003, 2004a, 2004b, 2005, 2006, 2007a), one platform presentation (Takeyoshi et al. 2007b), one poster presentation (Kojima et al. 2008), and unpublished data submitted by Dr. Takeyoshi in January 2009. Unpublished data submitted by Dr. Takeyoshi in May and August 2009, after the Panel review, are included in the accuracy (**Section 6**) and reproducibility analyses (**Section 7**) in this final BRD because they were evaluated by the OECD Expert Consultation on the LLNA. Unpublished data for three additional substances (xylene, chlorobenzene, and nickel chloride) and repeat tests of two previously tested substances (2-mercaptobenzothiazole and imidazolidinyl urea) using different vehicles were submitted after the OECD Expert Consultation. Because they could not be considered in an independent peer review, these data are not considered in the accuracy and reproducibility analyses; however, they are included in **Annex V.** The data for the repeat tests are discussed where relevant.

All test results were obtained using the protocol in **Annex I**. The substances tested by Takeyoshi et al. were not coded to prevent the possibility of bias in the interpretation of test results. The interlaboratory validation study reported by Kojima et al. (2008); however, used coded test substances to mask the identity of the test substances from the testing laboratories. **Annex III** contains summary data for the LLNA: BrdU-ELISA and comparative reference data for the 47 substances tested in these studies, and **Annex IV** contains the individual animal data for the LLNA: BrdU-ELISA.

6.0 Test Method Accuracy

A critical component of a formal evaluation of the validation status of a test method is an assessment of the accuracy of the proposed tested method when compared to the current reference test method (ICCVAM 2003). Additional comparisons should also be made against available human data, including experience from testing or accidental exposures. This aspect of assay performance is typically evaluated by calculating:

- *Accuracy* (*concordance*): the proportion of correct outcomes (positive and negative) of a test method
- *Sensitivity*: the proportion of all positive substances that are classified as positive
- *Specificity*: the proportion of all negative substances that are classified as negative
- *False positive rate*: the proportion of all negative substances that are incorrectly identified as positive
- *False negative rate*: the proportion of all positive substances that are incorrectly identified as negative

6.1 LLNA: BrdU-ELISA Database Used for the Accuracy Analysis

Forty-three of the 47 substances listed in **Table C-1** had sufficient LLNA: BrdU-ELISA and traditional LLNA data to conduct an accuracy analysis. The eugenol dimers (dihydroxyl-3,3'-dimethoxy-5,5'-diallyl-biphenyl and 4,5'-diallyl-2'-hydroxy-2,3'-dimethoxyphenyl ether) and the isoeugenol dimers (4-[1-Hydroxy-2-(2-methoxy-4-propenyl-phenyoxy)-propyl]-2-methoxy-phenol and 2-methoxy-4-(7-methoxy-3-methyl-5-propenyl-2,3-dihydro-benzofuran-2yl)-phenol) were excluded from the accuracy analyses because traditional LLNA data for these substances were not identified.

Of the 43 substances tested with both LLNA: BrdU-ELISA and the traditional LLNA, 35 had GP data for a comparison of the performance of the LLNA: BrdU-ELISA vs. GP data with that of the traditional LLNA vs. GP data. No GP data were found for *trans*-cinnamaldehyde, cyclamen aldehyde, diethyl maleate, diphenylcyclopropenone, hexane, isopropyl myristate, or linalool. Additionally, 3-aminophenol was excluded from the accuracy analyses for the dataset with LLNA: BrdU-ELISA, traditional LLNA, and GP data since the available GP data were generated with a nonstandard GPMT protocol.[4]

Of the 43 substances tested with both LLNA: BrdU-ELISA and the traditional LLNA, 41 had human data for a comparison of the performance of the LLNA: BrdU-ELISA vs. human data with that of the traditional LLNA vs. human data. No human data for *trans*-cinnamaldehyde or trimellitic anhydride were located. The complete set of comparative data for each substance is located in **Annex III**.

Multiple tests were available for 18 substances tested with the LLNA: BrdU-ELISA. For the accuracy analyses, results using the same vehicle for multiply tested substances were combined so that each substance was represented by one result for the accuracy analysis. In this case, the single result used for each substance represented the outcome that was most prevalent. For example, using SI $\geq$ 2.0 to identify sensitizers, isopropanol was a nonsensitizer because five of the seven tests for isopropanol had SI < 2. If the number of positive and negative outcomes were equal, the most conservative (i.e., positive) result was used for the accuracy analyses. If there were multiple test results with multiple vehicles for a substance, the vehicle that matched that used in the traditional LLNA was used in the accuracy analysis. For example, of the five tests for glutaraldehyde, two tests used acetone: olive oil

[4] The nonstandard GP protocol did not include the 48-hour topical patch induction that should follow induction by intradermal injection and it replaced the 24-hour skin patch challenge (usually 2 weeks after topical induction) with a 6-hour skin patch challenge (Basketter D, personal communication).

(4:1) (AOO) as the solvent (Takeyoshi et al. 2005), and three tests used acetone as the solvent (Kojima et al. 2008). The tests that used acetone for the solvent were used for the accuracy analyses because the solvent matches that used for the traditional LLNA reference data.

6.2 Accuracy Analysis Using the SI ≥ 2.0 Decision Criterion

The performance characteristics of the LLNA: BrdU-ELISA were first evaluated using the criterion of SI ≥ 2.0 to identify sensitizers, which was the threshold for a positive response used in the interlaboratory validation study (the complete protocol used in the validation study is included in **Annex I)**.

Of the 18 substances with multiple test results, discordant test results were noted among tests for six of the substances with multiple test results: cyclamen aldehyde, hydroxycitronellal, linalool, formaldehyde, isopropanol, and lactic acid. For all six substances, the solvents used for each test were the same. Dr. Takeyoshi tested cyclamen aldehyde (2007b and unpublished), hydroxycitronellal (2007b and unpublished), and linalool (both unpublished) twice; for each substance one test produced SI < 2 and the other test produced SI ≥ 2.

- Cyclamen aldehyde tests yielded SI = 1.97 and 5.71.
- Hydroxycitronellal tests yielded SI = 1.34 and 4.78.
- Linalool tests yielded SI = 1.45 and 4.65.

Other discordances included:

- One of the three laboratories in the interlaboratory validation study reported an SI of 1.97 for formaldehyde, while the others produced SI ≥ 2 (SI = 4.40 and 16.59) (Kojima et al. 2008).
- Two of the seven tests of isopropanol yielded SI ≥ 2 (SI = 2.04 and SI = 2.22), while the others yielded SI < 2 (SI = 0.92, 0.94, 0.98, 1.01, and 1.57). The discordant tests were obtained by two of the six laboratories in the interlaboratory validation study.
- One of the three tests for lactic acid from the interlaboratory validation study produced SI ≥ 2 (i.e., SI = 2.53), while the others yielded SI < 2 (SI = 1.80 and 1.89) (Kojima et al. 2008).

6.2.1 Accuracy vs. the Traditional LLNA

When compared to the traditional LLNA and using a decision criteria of SI ≥ 2.0 to identify sensitizers, the LLNA: BrdU-ELISA had an accuracy of 95% (41/43), a sensitivity of 94% (30/32), a specificity of 100% (11/11), a false positive rate of 0% (0/11), and a false negative rate of 6% (2/32) (**Table C-2**).

6.2.2 Accuracy vs. Guinea Pig Data

When the accuracy of the LLNA: BrdU-ELISA (SI ≥ 2.0) and the traditional LLNA were compared based on their performance relative to GP tests, the LLNA: BrdU-ELISA had a lower accuracy (86% [30/35] vs. 91% [32/35]) and sensitivity (91% [20/22] vs. 100% [22/22]), and higher false negative rate (9% [2/22] vs. 0% [0/22]; **Table C-2**). The specificity (77% [10/13]) and the false positive rate (23% [3/13]) for the LLNA: BrdU-ELISA and the traditional LLNA were the same when they were compared with GP data.

6.2.3 Accuracy vs. Human Data

When the accuracy of the LLNA: BrdU-ELISA (SI ≥ 2.0) and the traditional LLNA were compared based on their performance relative to the available human data, the LLNA: BrdU-ELISA had a lower

accuracy (73% [30/41] vs. 78% [32/41]) and sensitivity (77% [24/31] vs. 84% [26/31]) and a higher false negative rate (23% [7/31] vs. 16% [5/31]) than the traditional LLNA (**Table C-2**). The specificity (60% [6/10]) and the false positive rate (40% [4/10]) for the LLNA: BrdU-ELISA and the traditional LLNA were the same when they were compared to human data.

Table C-2 Performance of the LLNA: BrdU-ELISA in Predicting Skin-Sensitizing Potential Using the Decision Criterion of SI ≥ 2.0 to Identify Sensitizers

Comparison	n[1]	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate		Positive Predictivity		Negative Predictivity	
		%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]
BrdU-ELISA vs. Traditional LLNA	43	95	41/43	94	30/32	100	11/11	0	0/11	6	2/32	100	30/30	85	11/13
Substances with LLNA: BrdU-ELISA, Traditional LLNA, and GP Data															
BrdU-ELISA vs. Traditional LLNA	35	94	33/35	92	23/25	100	10/10	0	0/10	8	2/25	100	23/23	83	10/12
LLNA: BrdU-ELISA vs. GP[3]	35	86	30/35	91	20/22	77	10/13	23	3/13	9	2/22	87	20/23	83	10/12
Traditional LLNA vs. GP[3]	35	91	32/35	100	22/22	77	10/13	23	3/13	0	0/22	88	22/25	100	10/10
Substances with LLNA: BrdU-ELISA, Traditional LLNA, and Human Data															
BrdU-ELISA vs. Traditional LLNA	41	95	39/41	93	28/30	100	11/11	0	0/11	7	2/30	100	11/11	100	28/28
LLNA: BrdU-ELISA vs. Human[4]	41	73	30/41	77	24/31	60	6/10	40	4/10	23	7/31	86	24/28	46	6/13
Traditional LLNA vs. Human[4]	41	78	32/41	84	26/31	60	6/10	40	4/10	16	5/31	87	26/30	54	6/11

Abbreviations: LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; GP = guinea pig skin sensitization outcomes; LLNA = murine local lymph node assay; No. = number; SI = stimulation index.

[1] n = number of substances included in this analysis.

[2] The data on which the percentage calculation is based.

[3] GP refers to outcomes obtained by studies conducted using either the Guinea Pig Maximization Test or the Buehler Test.

[4] Human refers to outcomes obtained by studies conducted using the human maximization test, the human repeat insult patch test, inclusion of the test substance in a human patch test allergen kit, and/or published clinical case studies/reports.

6.3 Accuracy Analysis (SI ≥ 2.0) Based on the ICCVAM Performance Standards Reference Substances

ICCVAM has developed recommended test method performance standards for the traditional LLNA (ICCVAM 2009[5]), which are proposed to evaluate the performance of modified LLNA test methods that are mechanistically and functionally similar to the traditional LLNA. Because the validation studies for the LLNA: BrdU-ELISA test method were completed prior to the development of LLNA performance standards and because all of the reference substances had not been tested, the LLNA: BrdU-ELISA was not evaluated using the ICCVAM-recommended LLNA performance standards. Thus, the LLNA: BrdU-ELISA test results for the ICCVAM-recommended LLNA performance standards reference substances were evaluated to provide a general comparison of performance. As shown in **Table C-3**, 16 of the 18 required reference substances included in the ICCVAM LLNA Performance Standards have been tested in the LLNA: BrdU-ELISA. Fourteen of the 16 substances yielded the same sensitizer/nonsensitizer outcome in the LLNA: BrdU-ELISA as in the traditional LLNA.

Because all of the required ICCVAM-recommended LLNA performance standards reference substances had not been tested in the LLNA: BrdU-ELISA, selected characteristics of the substances tested in the LLNA: BrdU-ELISA were compared with those of the reference substances. **Table C-4** shows traditional LLNA and other selected characteristics of the 43 substances with traditional LLNA data that were tested in the LLNA: BrdU-ELISA. The characteristics of these substances are compared to the characteristics of the 18 required reference substances from the ICCVAM-recommended LLNA performance standards (ICCVAM 2009). The table indicates that, although not all of the 18 required reference substances from the ICCVAM-recommended performance standards reference substances have been tested, the characteristics of the substances tested in the LLNA: BrdU-ELISA is similar to that included in the performance standards list. In general, there is a proportionally increased number of substances tested in the LLNA: BrdU-ELISA in each of the categories included in the table.

[5] Available at http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm.

Table C-3 Performance of the LLNA: BrdU-ELISA (SI ≥ 2.0) Using the ICCVAM Performance Standards Reference Substances[1]

Substance Name	Recommended Performance Standards[1]				LLNA: BrdU-ELISA[2]			
	Vehicle	Result	EC3 (%) (Max SI)[1]	N[3]	Vehicle	Result	EC2 (%) (Max SI)	N[3]
5-Chloro-2-methyl-4-isothiazolin-3-one	DMF	+	0.009 (22.7)	1	DMF	+	0.12 (4.8)	1
2, 4-Dinitrochlorobenzene	AOO	+	0.049 (43.9)	15	AOO	+	0.044 (18.8)	8
4-Phenylenediamine	AOO	+	0.11 (26.4)	6	AOO	+	NC (14.7)	2
Methyl methacrylate	DMF	+	90 (3.6)	1	NT	NT	NT	NT
Isoeugenol	AOO	+	1.5 (31.0)	47	AOO	+	7.6 (8.4)	2
2-Mercaptobenzothiazole	***DMF***	***+***	***1.7 (8.6)***	***1***	***DMF***	***-***	***NA (1.6)***	***1***
Cobalt chloride	DMSO	+	0.6 (7.2)	2	DMSO	+	0.63 (3.7)	1
Citral	AOO	+	9.2(20.5)	6	AOO	+	NC (16.4)	1
Hexyl cinnamic aldehyde	AOO	+	9.7 (20.0)	21	AOO	+	17.4 (13.5)	11
Eugenol	AOO	+	10.1 (17.0)	11	AOO	+	9.8 (17.7)	8
Phenyl benzoate	AOO	+	13.6 (11.1)	3	DMF	+	28.2 (3.4)	1
Cinnamic alcohol	AOO	+	21 (5.7)	1	AOO	+	33.2 (2.7)	1
Imidazolidinyl urea	***DMF***	***+***	***24 (5.5)***	***1***	***DMF***	***+***	***NA (1.6)***	***1***
Chlorobenzene[4]	AOO	-	NA (1.7)	1	NT	NT	NT	NT
Isopropanol	AOO	-	NA (1.7)	1	AOO	-	NA (2.2)[5]	7
Lactic acid	DMSO	-	NA (2.2)	1	DMSO	-	NA (2.5)[6]	3
Methyl salicylate	AOO	-	NA (2.9)	9	AOO	-	NA (1.4)	3
Salicylic acid	AOO	-	NA (2.5)	1	AOO	-	NA (1.3)	1
Ethylene glycol dimethacrylate	MEK	False +	28 (7.0)	1	MEK	+	49.8 (3.1)	1
Sodium lauryl sulfate	DMF	False +	8.1 (8.9)	5	DMF	+	14.6 (2.6)	1
Nickel chloride[4]	DMSO	False -	NA (2.4)	2	NT	NT	NT	NT
Xylene[4]	AOO	False -	95.8 (3.1)	1	NT	NT	NT	NT

Boldface italic text highlights discordant LLNA: BrdU-ELISA vs. traditional LLNA test results.

Abbreviations: AOO = acetone: olive oil (4:1); LLNA: BrdU-ELISA= murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; EC3 = estimated concentration needed to produce a stimulation index of 3; EC2 = estimated concentration needed to produce a stimulation index of 2; LLNA = murine local lymph node assay; MEK = methyl ethyl ketone; NA = not applicable (i.e., SI outcome was less than decision criterion for sensitizers); NT = not tested; SI = stimulation index.

+ = sensitizer.

- = nonsensitizer.

[1] Mean EC3 values (expressed as % concentration) and maximum SI values (shown in parentheses) are from the NICEATM database of traditional LLNA studies and from *Recommended Performance Standards: Murine Local Lymph Node Assay* (ICCVAM 2009; available: http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm).

[2] Calculated from data supporting Takeyoshi et al. (2003, 2004b, 2005, 2006, 2007a, 2007b, and unpublished) and Kojima et al (2008).

[3] Number of values used to derive the mean EC3 or EC2 values.

[4] Data submitted after conclusion of the independent peer review evaluations (see **Annex V** for data).

[5] Based on the most prevalent outcome (i.e., 5/7 tests yielded SI < 2).

[6] Based on the most prevalent outcome (i.e., 2/3 tests yielded SI < 2).

Table C-4 Characteristics of the Substances Tested in the LLNA: BrdU-ELISA vs. the ICCVAM Performance Standards Reference Substances[1]

EC3 Range (%)	No. Chems	Solid/ Liquid	Actual EC3 Range (%)	Maximum SI Range	Human Data	Peptide Reactivity (Hi/Mod/Min/Lo/Unk)[3]
<0.1	**5**	**3/3**	**0.009 - 0.083**	**18.0 –52.3**	**5**	**5/0/0/0/0**
	2	1/1	0.009 - 0.05	22.6 - 52.3	2	2/0/0/0/0
≥ 0.1 to <1	**4**	**3/1**	**0.11 - 0.8**	**4.0 – 26.4**	**4**	**0/1/0/0/3**
	2	2/0	0.11 - 0.6	6.7 - 75.3	2	0/0/0/0/2
≥ 1 to <10	**12**	**5/7**	**1.4 - 9.7**	**3.1 – 31.0**	**10**	**2/0/1/1/8**
	4	1/3	1.5 - 9.7	8.6 - 29.5	4	1/0/1/0/2
≥ 10 to <100	**11**	**3/8**	**10.1 – 47.5**	**3.4 - 17.0**	**11**	**1/0/1/2/7**
	5	3/2	10.1 - 90	5.5 - 70.3	5	0/1/0/0/4
Negative	**11**	**4/7**	**NC**	**1.0 – 2.9**	**11**	**0/0/7/1/3**
	5	1/4	NC	0.9 - 2.8	3	0/0/2/0/3
Overall	**43**	**18/25**	**0.009 – 47.5**	**0.9 - 52.3**	**41**	**8/1/9/4/21**
	18	10/8	0.009 - 24	0.9 - 75.3	16	3/1/3/0/11

Boldface text represents characteristics of the LLNA: BrdU-ELISA database.

Abbreviations: Chems = chemicals; EC3 = estimated concentration needed to produce SI = 3; Hi = high; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; Lo = low; Min = minimal; Mod = moderate; NC = not calculated because maximum SI < 3; No. = number; SI = stimulation index; Unk = unknown.

[1] From *Recommended Performance Standards: Murine Local Lymph Node Assay* (ICCVAM 2009; available: http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm. Includes the 18 "required" substances for testing.

[2] Data obtained from Gerberick et al. (2007b)

6.4 Discordant Results for Accuracy Analysis Using the SI ≥ 2.0 Decision Criterion

6.4.1 Discordance Between the LLNA: BrdU-ELISA and the Traditional LLNA

When the outcomes for the 43 substances tested in the LLNA: BrdU-ELISA (using SI ≥ 2.0) and the traditional LLNA were compared, the classifications for two substances were different. The LLNA: BrdU-ELISA classified imidazolidinyl urea and 2-mercaptobenzothiazole as nonsensitizers, while the traditional LLNA classified them as sensitizers (i.e., false negative outcome) (**Table C-5**). Both substances were tested in *N,N*-dimethylformamide (DMF) in the LLNA: BrdU-ELISA and the traditional LLNA tests. Both substances are solids. No commonalities in chemical class, size, peptide reactivity (see **Annex II** for physicochemical information), traditional LLNA potency, or potential for skin irritation were noted in these substances.

Imidazolidinyl urea is classified as a urea compound. It has a molecular weight (MW) of 388.39 g/mole. It was originally tested in the LLNA: BrdU-ELISA at 10% and 50% (SI = 0.73 and 1.61, respectively). The EC3 value for the traditional LLNA is 24%. No peptide reactivity information is available. An additional LLNA: BrdU-ELISA test for imidazolidinyl urea that was submitted after the Panel review and OECD Expert Consultation indicated that testing at higher concentrations and in DMSO will increase the response (**Annex V**). The additional test used 50% and 75% imidazolidinyl urea in DMSO and produced SI values of 1.65 and 2.27, respectively.

2-Mercaptobenzothiazole is classified as a heterocyclic compound and has a MW of 167.26 g/mole. It was originally tested in the LLNA: BrdU-ELISA at 12.5%, 25%, and 50% (SI = 1.62, 1.36, and 1.49, respectively). The EC3 value for the traditional LLNA is 1.7%. Peptide reactivity is high. It is labeled as a skin irritant at the concentrations tested in the LLNA: BrdU-ELISA, but imidazolidinyl urea is not. An additional LLNA: BrdU-ELISA test for 2-mercaptobenzothiazole that was submitted after the Panel review and OECD Expert Consultation on the LLNA indicated that testing with dimethyl sulfoxide (DMSO) as the vehicle increases the response (**Annex V**). The additional test used 10% and 25% 2-mercaptobenzothiazole and produced SI values of 1.50 and 2.23, respectively.

Table C-5 Discordant Results for LLNA: BrdU-ELISA (SI ≥ 2.0) Compared to Traditional LLNA and Guinea Pig Reference Data[1]

Substance Name[2]	Vehicle[3]	LLNA: BrdU-ELISA[4]	Traditional LLNA[4]	Guinea Pig Studies	Skin Irritant?
Imidazolidinyl urea (24.0%)	DMF	- (1.61, 50%)	+ (5.5, 50%)	+	Nonirritant at ≤75% (GP)
2-Mercaptobenzo-thiazole (1.7%)	DMF	- (1.62, 50%)[5]	+ (8.6, 10%)	+	Nonirritant at ≤10% (GP); Nonirritant at 25% (humans)
Ethyl acrylate (32.8%)	AOO	+ (4.95, 100%)	+ (4.0, 50%)	−	Nonirritant at 3% (GP)
Ethylene glycol dimethacrylate (28.0%)	MEK	+ (3.11, 100%)	+ (7.0, 50%)	−	Nonirritant at 1% (GP)
Sodium lauryl sulfate (8.1%)	DMF	+ (2.64, 16.7%)	+ (8.9, 20%)	−	Irritant at 20% aq. (rabbits); Irritant at 20% (humans); Irritant at 10% in DMF (mice)

Abbreviations: AOO = acetone: olive oil (4:1); aq = aqueous; DMF = *N,N*-dimethylformamide; LLNA: BrdU-ELISA= murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; GP = guinea pig; LLNA = murine local lymph node assay; MEK = methyl ethyl ketone; SI = stimulation index.

+ = sensitizer.

- = nonsensitizer.

[1] Data sources provided in **Annex III-1**.

[2] Numbers in parentheses are the EC3 values for the traditional LLNA (from **Table C-1**).

[3] Vehicles apply to tests for both the LLNA: BrdU-ELISA and the traditional LLNA.

[4] Numbers in parentheses are highest SI values and maximum concentrations tested.

[5] Highest SI occurred at concentration of 12.5%.

6.4.2 Discordance Among the LLNA: BrdU-ELISA, the Traditional LLNA, and/or the Guinea Pig Test

For the 35 substances with LLNA: BrdU-ELISA, traditional LLNA, and GP test results, five substances produced results that were discordant with GP test results (**Table C-5**). Two substances were negative in the LLNA: BrdU-ELISA and positive in the GP, and three substances were positive in the LLNA: BrdU-ELISA and negative in the GP. The LLNA: BrdU-ELISA results for imidazolidinyl urea and 2-mercaptobenzothiazole were negative, while the GP results were positive. As noted in **Section 6.4.1**, there were few commonalities associated with these two discordant substances.

Ethyl acrylate, ethylene glycol dimethacrylate, and sodium lauryl sulfate (SLS) were classified as sensitizers by the LLNA: BrdU-ELISA and traditional LLNA and as nonsensitizers by GP tests (**Table C-5**). There were a few commonalities among these substances with regard to chemical class, physical form, MW, peptide reactivity (see **Annex II** for physicochemical information), the range of EC3 values (based on traditional LLNA, see **Table C-1**), and potential for skin irritation (**Annex III-1**):

- Ethyl acrylate and ethylene glycol dimethacrylate are carboxylic acids; SLS is an alcohol, sulfur, and lipid compound.
- Ethylene glycol dimethacrylate and ethyl acrylate are liquids; SLS is a solid.
- MWs ranged from 100.10 to 288.38 g/mol.
- Peptide reactivity for ethyl acrylate and ethylene glycol dimethacrylate is high; peptide reactivity data for SLS is not available.
- Ethyl acrylate (EC3 = 32.8%) and ethylene glycol dimethacrylate (EC3 = 28%) are weak sensitizers in the traditional LLNA; SLS (EC3 = 8.1%) is somewhat stronger.
- Ethyl acrylate, ethylene glycol dimethacrylate, and SLS were tested at irritating concentrations in the LLNA: BrdU-ELISA, based on skin irritation data from guinea pigs, humans, or mice.

6.4.3 Discordance Among the LLNA: BrdU-ELISA, the Traditional LLNA, and/or the Human Outcome

When analyses were restricted to the 41 substances with LLNA: BrdU-ELISA, traditional LLNA, and human outcomes, the LLNA: BrdU-ELISA misclassified 11 substances. Both the LLNA: BrdU-ELISA and the traditional LLNA misclassified five human sensitizers (diethyl phthalate, 2-hydroxypropylmethacrylate, isopropanol, propylene glycol, and sulfanilamide) as nonsensitizers (**Table C-6**). The LLNA: BrdU-ELISA also misclassified two other sensitizers as nonsensitizers that were correctly classified by the traditional LLNA (2-mercaptobenzothiazole and imidazolidinyl urea). There were a few commonalities among these seven substances with regard to chemical class, physical form, MW, peptide reactivity (see **Annex II** for physicochemical information), the range of EC3 values (based on traditional LLNA, see **Table C-1**), and potential for skin irritation (**Annex III-1**):

- Diethyl phthalate and 2-hydroxypropylmethacrylate are carboxylic acids; isopropanol and propylene glycol are alcohols; sulfanilamide is a cyclic hydrocarbon and sulfur compound; 2-mercaptobenzothiazole is a heterocyclic compound; and imidazolidinyl urea is a urea.

- Diethyl phthalate, isopropanol, and propylene glycol are liquids; while the other four compounds are solids.
- MWs ranged from 60.1 to 222.2 g/mol.
- Peptide reactivity for diethyl phthalate, isopropanol, and propylene glycol is minimal; the peptide reactivity for 2-hydroxypropylmethacrylate is low; the peptide reactivity for 2-mercaptobenzothiazole is high; and peptide reactivity information for sulfanilamide and imidazolidinyl urea is unavailable.
- 2-Mercaptobenzothiazole is a strong sensitizer in the traditional LLNA (EC3 = 1.7%); imidazolidinyl urea is a weak sensitizers (EC3 = 24%); the other four substances are LLNA nonsensitizers.
- Diethyl phthalate, isopropanol, and imidazolidinyl urea were tested at nonirritating concentrations, but the other four substances were not, based on skin irritation data from guinea pigs, rabbits, and humans.

Four human nonsensitizers were classified as sensitizers by the LLNA: BrdU-ELISA and the traditional LLNA: isopropyl myristate, cyclamen aldehyde, linalool, and SLS. There were a few commonalities among these substances with regard to chemical class, physical form, MW, peptide reactivity (see **Annex II** for physicochemical information), the range of EC3 values (based on traditional LLNA, see **Table C-1**), or potential for skin irritation (**Annex III-1**):

- Isopropyl myristate and SLS are lipids; cyclamen aldehyde is an aldehyde; linalool is a hydrocarbon; and SLS is also an alcohol and sulfur compound.
- Isopropyl myristate, cyclamen aldehyde, and linalool are liquids; and SLS is a solid.
- MWs ranged from 154.2 to 288.4 g/mol.
- Peptide reactivity for isopropyl myristate is minimal; peptide reactivity for cyclamen aldehyde is low; and peptide reactivity information for linalool and SLS is unavailable.
- Isopropyl myristate (EC3 = 44.0%), cyclamen aldehyde (MW = 22.3%), and linalool (EC3 = 30.0%) are weak traditional LLNA sensitizers, while SLS (EC3 = 8.1%) is a stronger sensitizer.
- Isopropyl myristate was tested at nonirritating concentrations; cyclamen aldehyde, linalool, and SLS were tested at irritating concentrations, based on skin irritation data from rabbits, humans, or mice.

Table C-6 Discordant Results for LLNA: BrdU-ELISA (SI ≥ 2.0) When Compared to Traditional LLNA and Human Outcome Data[1]

Substance Name[2]	Vehicle[3]	LLNA: BrdU-ELISA[4]	Traditional LLNA[4]	Human Outcome[5]	Skin Irritant?
Diethyl phthalate	AOO	- (0.88, 50%)	- (1.5, 100%)	+ (HPTA)	Nonirritant at ≤100% (rabbits)
2-Hydroxypro-pylmethacrylate	AOO	- (1.13, 50%)	- (1.3, 50%)	+ (case study, 0.1%)	Nonirritant at ≤10% (GP)
Isopropanol	AOO	- (2.22, 50%)[6]	- (1.7, 50%)[7]	+ (case study, 0.001%)	Nonirritant at ≤100% (rabbits)

continued

Table C-6 Discordant Results for LLNA: BrdU-ELISA (SI ≥ 2.0) When Compared to Traditional LLNA and Human Outcome Data[1] (continued)

Substance Name[2]	Vehicle[3]	LLNA: BrdU-ELISA[4]	Traditional LLNA[4]	Human Outcome[5]	Skin Irritant?
Propylene glycol	AOO[8]	- (1.57, 50%)	- (1.6, 100%)	+ (HPTA)	Nonirritant at ≤25% (humans)
Sulfanilamide	DMF	- (1.26, 50%)	- (1.0, 50%)[9]	+ (5/25, 25%)	Nonirritant at ≤25% (humans)
2-Mercaptoben-zothiazole (1.7%)	DMF	- (1.62, 50%)[10]	+ (8.6, 10%)	+ (5/24, 10%)	Nonirritant at ≤10% (GP); Nonirritant at 25% (humans)
Imidazolidinyl urea (24.0%)	DMF	- (1.34, 100%)	+ (5.5, 50%)	+ (2/150, 2%)	Nonirritant at ≤75% (GP)
Isopropyl myristate (44.0%)	AOO	+ (4.20, 50%)	+ (3.4, 100%)	- (0/25, 20%)	Nonirritant at ≤100% (rabbits)
Cyclamen aldehyde (22.3%	AOO	+ (1.97 and 5.71, 100%)	+ (5.2, 50%)	- (0/64, 4%)	Irritant at 100% (rabbits)
Linalool (30.0%)	AOO	+ (1.45 and 4.65, 100%	+ (8.3, 100%)	- (0/25, 8%)	Irritant at 100% (rabbits)
Sodium lauryl sulfate (8.1%)	DMF	+ (3.4, 10%)	+ (8.9, 20%)[7]	- (0/22 at 10%)	Irritant at 20% aq. (rabbits); Irritant at 20% (humans); Irritant at 10% in DMF (mice)

Abbreviations: AOO = acetone: olive oil (4:1); aq = aqueous; DMF = *N,N*-dimethylformamide; LLNA: BrdU-ELISA= murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; GP = guinea pig; HPTA = human patch test allergen; LLNA = murine local lymph node assay.

+ = sensitizer.

- = nonsensitizer.

[1] Data sources listed in **Annex III-1**.

[2] Numbers in parentheses are EC3 values for the traditional LLNA (from **Table C-1**).

[3] Vehicles apply to tests for both the LLNA: BrdU-ELISA and the traditional LLNA unless otherwise noted.

[4] Numbers in parentheses are highest SI values and maximum concentrations tested.

[5] Information in parentheses indicates the basis for the human outcome. Numbers indicate the incidence of positive human response and the concentration tested.

[6] Negative based on most prevalent outcome. Highest SI of any test is shown (SI = 0.92, 0.94, 0.98, 1.01, 1.57, 2.04, and 2.22). Highest SI values for most tests occurred at <50%.

[7] Highest SI occurred at 10%.

[8] Vehicle for the traditional LLNA was distilled water.

[9] Highest SI occurred at 10% and 25%.

[10] Highest SI occurred at 12.5%.

6.4.4 Discordance Between the LLNA: BrdU-ELISA and the Traditional LLNA When Testing the LLNA Performance Standards Substances

Using SI ≥ 2.0, two discordant substances, 2-mercaptobenzothiazole and imidazolidinyl urea, were noted among the 16 performance standards minimum reference substances tested in the LLNA: BrdU-ELISA. The LLNA: BrdU-ELISA classified both substances as nonsensitizers, while the traditional LLNA, GP, and human tests classified them as sensitizers. The EC3 value for 2-mercaptobenzothiazole in the traditional LLNA, 1.7%, was derived from a test of 1%, 3%, and 10% 2-mercaptobenzothiazole in DMF (Gerberick et al. 2005). The maximum SI was 8.6 at 10%. The LLNA: BrdU-ELISA test used the same vehicle and tested concentrations of 12.5%, 25%, 50% 2-mercaptobenzothiazole, which yielded SI values of 1.62, 1.36, and 1.49, respectively. An additional LLNA: BrdU-ELISA test of 2-mercaptobenzothiazole in DMSO that was submitted after the Panel review and OECD Expert Consultation on the LLNA indicates that testing with DMSO as the vehicle increases the response. The additional test used 10% and 25% 2-mercaptobenzothiazole, which produced SI values of 1.50 and 2.23, respectively (**Annex V**).

The EC3 value for imidazolidinyl urea in the traditional LLNA, 24%, was derived from a test of 10%, 25%, and 50% imidazolidinyl urea in DMF (Gerberick et al. 2005). The maximum SI was 5.5 at 50%. The LLNA: BrdU-ELISA test used the same vehicle and tested concentrations of 10% and 50% imidazolidinyl urea, which yielded SI values of 0.73 and 1.61, respectively. An additional LLNA: BrdU-ELISA test for imidazolidinyl urea that was submitted after the Panel review and OECD Expert Consultation on the LLNA suggests that testing at higher concentrations and/or using DMSO as the vehicle will increase the response. This test used 50% and 75% imidazolidinyl urea in DMSO and produced SI values of 1.65 and 2.27, respectively (**Annex V**).

6.5 LLNA: BrdU-ELISA Accuracy Analysis Using Alternative Decision Criteria

In addition to the accuracy analysis using SI ≥ 2.0 to classify substances as sensitizers, other decision criteria were evaluated for test method performance. The traditional LLNA served as the reference test. The performance characteristics for 15 different decision criteria for determining whether the skin sensitization potential for the substances were positive or negative are reported in this section. The substances evaluated were the 43 substances with both LLNA: BrdU-ELISA and traditional LLNA data discussed in **Section 6.1**. The decision criteria included:

1. SI values ≥1.3, ≥1.5, ≥1.6, ≥1.9, ≥2.0, ≥2.5, ≥3.0, ≥3.5, ≥4.0, ≥4.5, or ≥5.0
2. Statistically significant difference between any treatment group and the vehicle control group. Absorbance values of treated groups were compared with the vehicle control group using ANOVA with a post-hoc Dunnett's test when multiple treatment groups were tested, or Student's *t*-test when there was only one treatment group
3. Mean absorbance values of treated groups ≥95% CI of the control group
4. Mean absorbance values of treated groups ≥2 SD or ≥3 SD from the control group mean

Multiple tests were available for 18 substances tested with the LLNA: BrdU-ELISA. The results for each of these substances in the same vehicle were combined so that each substance was represented by one sensitizer or nonsensitizer result for each criterion evaluated for the accuracy analysis. The results were combined in three ways, and a separate accuracy analysis was performed for each approach.

1. The sensitizer/nonsensitizer outcome for each substance was the most prevalent outcome for each criterion. For example, for the criterion for a statistical difference between control and treatment groups, two of the three lactic acid tests exhibited statistical differences between the control and treated groups (i.e., produced sensitizer results). Thus, the single outcome for lactic acid for the accuracy analysis was a sensitizer result. If the number of positive and negative outcomes were equal, the most conservative (i.e., positive) result was used for the accuracy analyses.
2. The positive/negative outcome for each substance at each criterion was determined by the outcome of the test with the highest maximum SI of the multiple tests.
3. The positive/negative outcome for each substance at each criterion was determined by the outcome of the test with the lowest maximum SI of the multiple tests.

The analysis presented here is based on using the most prevalent outcome for substances with multiple tests, as this is representative of the most likely outcome for a given chemical. The analyses using the highest maximum SI and the lowest maximum SI of the multiple tests for each substance are detailed in **Annex VI**.

As shown in **Section 6.1**, using the most prevalent outcome and the decision criterion of SI ≥ 2.0 resulted in an accuracy of 95% (41/43), a sensitivity of 94% (30/32), a specificity of 100% (11/11), a false positive rate of 0% (0/11), and a false negative rate of 6% (2/32) (**Tables C-2** and **C-7)**. Using higher SI values (i.e., SI ≥ 3.0 to SI ≥ 5.0) as the decision criterion resulted in reduced accuracy and higher false negative rates but the same false positive rates as compared to SI ≥ 2.0 (**Figure C-1** and **Table C-7**). Using SI ≥ 1.9 as the decision criterion produced the same performance statistics as SI ≥ 2.0. Using a lower SI value, down to SI ≥ 1.5, produced the same accuracy as SI ≥ 2.0 (95% [41/43]), but the false positive rate increased to 18% (2/11), and the false negative rate decreased to 0% (0/32). SI ≥ 1.3 is shown for comparison because it was previously recommended by ICCVAM but was considered to be inadequate by the March 2008 Peer Review Panel (ICCVAM 2008). Use of ANOVA and summary statistics (i.e., mean absorbance values of treated groups ≥ 95% confidence interval of the control group, or ≥2 or 3 SD from the control group mean), yielded accuracy values of 9%1 to 93%, with false negative rates of 0% to 6%, and false positive rates of 9% to 36%.

The optimal criterion was considered SI ≥ 1.6 because it produced no false negatives and the accuracy (95% [41/43]) was the highest accuracy produced by any of the criteria examined. Using the most prevalent outcome, SI ≥ 1.6 was the highest SI criterion that yielded no false negatives (0/32). The lowest SI criterion that yielded no false positives (0/11) was SI ≥ 1.9 (**Table C-7**). Analyses to determine the robustness of the optimum SI criterion showed that the optimal SI criterion was stable (**Annex VII**). Taking different samples of the data as training/validation sets had relatively little impact on the cutoff SI criterion or on the resulting number of false positives or false negatives.

Table C-7 Performance of the LLNA: BrdU-ELISA for 43 Substances in Predicting Skin-Sensitizing Potential Using Alternative Decision Criteria to Identify Sensitizers and the Most Prevalent Outcome for Substances with Multiple Tests

Alternative Criterion	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate		Positive Predictivity		Negative Predictivity	
	%	No.[1]	%	No.[1]	%	No.[1]	%	No.[1]	%	No.[1]	%	No.[1]	%	No.[1]
Statistics[2]	91	39/43	97	31/32	73	8/11	27	3/11	3	1/32	91	31/34	89	8/9
≥95% CI[3]	91	39/43	100	32/32	64	7/11	36	4/11	0	0/32	89	32/36	100	7/7
≥2 SD[4]	93	40/43	100	32/32	73	8/11	27	3/11	0	0/32	91	32/35	100	8/8
≥3 SD[5]	93	40/43	94	30/32	91	10/11	9	1/11	6	2/32	97	30/31	83	10/12
SI ≥ 5.0	49	21/43	31	10/32	100	11/11	0	0/11	69	22/32	100	10/10	33	11/33
SI ≥ 4.5	58	25/43	44	14/32	100	11/11	0	0/11	56	18/32	100	14/14	38	11/29
SI ≥ 4.0	63	27/43	50	16/32	100	11/11	0	0/11	50	16/32	100	16/16	41	11/27
SI ≥ 3.5	72	31/43	62	20/32	100	11/11	0	0/11	38	12/32	100	20/20	48	11/23
SI ≥ 3.0	84	36/43	78	25/32	100	11/11	0	0/11	22	7/32	100	25/25	61	11/18
SI ≥ 2.5	93	40/43	91	29/32	100	11/11	0	0/11	9	3/32	100	29/29	79	11/14
SI ≥ 2.0	95	41/43	94	30/32	100	11/11	0	0/11	6	2/32	100	30/30	85	11/13
SI ≥ 1.9	95	41/43	94	30/32	100	11/11	0	0/11	6	2/32	100	30/30	85	11/13
SI ≥ 1.6	95	41/43	100	32/32	82	9/11	18	2/11	0	0/32	94	30/32	100	9/9
SI ≥ 1.5	95	41/43	100	32/32	82	9/11	18	2/11	0	0/32	94	30/32	100	9/9
SI ≥ 1.3	93	40/43	100	32/32	73	8/11	27	3/11	0	0/32	91	32/35	100	8/8

Abbreviations: CI = confidence interval; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; No. = number; SD = standard deviation; SI = stimulation index.

[1] The proportion on which the percentage calculation is based.

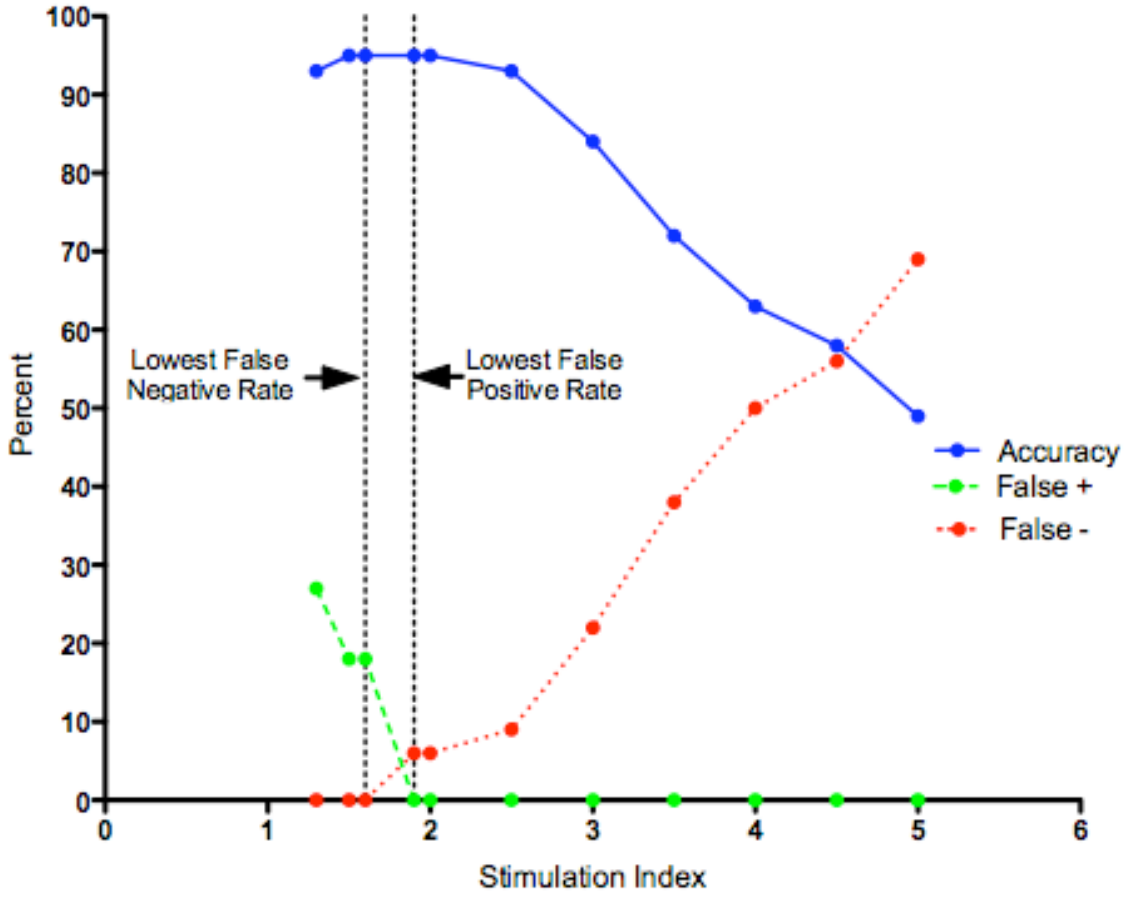
[2] Analysis of variance for difference of group means when substances were tested at multiple doses or *t*-test when substances were tested at one dose. The absorbance data were log-transformed prior to analysis of variance. Significance at $p < 0.05$ was further tested by Dunnett's test.

[3] The mean absorbance of at least one treatment group was outside the 95% confidence interval for the mean absorbance of the vehicle control group.

[4] The mean absorbance of at least one treatment group was greater than 3 SD from the mean absorbance of the vehicle control group.

[5] The mean absorbance of at least one treatment group was greater than 2 SD from the mean absorbance of the vehicle control group.

Figure C-1 Performance of the LLNA: BrdU-ELISA for 43 Substances with SI Compared to the Traditional LLNA Using the Most Prevalent Outcome for Substances with Multiple Tests



As compared to traditional LLNA results, the lines show the change in performance characteristics for the LLNA: BrdU-ELISA with the SI cutoff used to identify sensitizers. This analysis used LLNA: BrdU-ELISA and traditional LLNA results for 32 sensitizers and 11 nonsensitizers. For the 18 substances with multiple test results, the results for each substance were combined using the most prevalent outcome. The solid line shows accuracy, the dashed line shows the false positive rate, and the dotted line shows the false negative rate.

The optimum decision criterion of SI ≥ 1.6 is compared with SI ≥ 2.0 for accuracy of the LLNA: BrdU-ELISA against GP and human data in **Table C-8**. When GP test results were used as the reference data, SI ≥ 1.6 had higher accuracy (89% [31/35]) for SI ≥ 1.6 vs. 86% [30/35]), lower false negative rate (0% [0/32] for SI ≥ 1.6 vs. 9% [2/22]), and increased false positive rate (31% [4/13] for SI ≥ 1.6 vs. 23% [3/13) when compared with SI ≥ 2.0. When results were compared to human data, SI ≥ 1.6 produced the same accuracy (73% [30/41]), decreased the false negative rate (16% [5/31] for SI ≥ 1.6 vs. 23% [7/31]), and increased the false positive rate (60% [6/10] for SI ≥ 1.6 vs. 40% [4/10]) compared with SI ≥ 2.0.

Table C-8 Comparison of Performance for Decision Criteria of SI ≥ 1.6 (Bold) and SI ≥ 2.0 for Predicting Skin Sensitizing Potential with LLNA: BrdU-ELISA

Comparison	n[1]	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate		Positive Predictivity		Negative Predictivity	
		%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]
LLNA: BrdU-ELISA vs. Traditional LLNA	43	**95** 95	**41/43** 41/43	**100** 94	**32/32** 30/32	**82** 100	**9/11** 11/11	**18** 0	**2/11** 0/11	**0** 6	**0/32** 2/32	**94** 100	**32/34** 30/30	**100** 85	**9/9** 11/13
Substances with LLNA: BrdU-ELISA, Traditional LLNA, and GP Data															
LLNA: BrdU-ELISA vs. Traditional LLNA	35	**97** 94	**34/35** 33/35	**100** 92	**25/25** 23/25	**90** 100	**9/10** 10/10	**10** 0	**1/10** 0/10	**0** 8	**0/25** 2/25	**96** 100	**25/26** 23/23	**100** 83	**9/9** 10/12
LLNA: BrdU-ELISA vs. GP[3]	35	**89** 86	**31/35** 30/35	**100** 91	**22/22** 20/22	**69** 77	**9/13** 10/13	**31** 23	**4/13** 3/13	**0** 9	**0/22** 2/22	**85** 87	**22/26** 20/23	**100** 83	**9/9** 10/12
Traditional LLNA vs. GP[3]	35	91	32/35	100	22/22	77	10/13	23	3/13	0	0/22	88	22/25	100	10/10
Substances with LLNA: BrdU-ELISA, Traditional LLNA, and Human Data															
LLNA: BrdU-ELISA vs. Traditional LLNA	41	**95** 95	**39/41** 39/41	**100** 93	**30/30** 28/30	**82** 100	**9/11** 11/11	**18** 0	**2/11** 0/11	**0** 7	**0/30** 2/30	**94** 100	**30/32** 28/28	**100** 85	**9/9** 11/13
LLNA: BrdU-ELISA vs. Human[4]	41	**73** 73	**30/41** 30/41	**84** 77	**26/31** 24/31	**40** 60	**4/10** 6/10	**60** 40	**6/10** 4/10	**16** 23	**5/31** 7/31	**81** 86	**26/32** 24/28	**44** 46	**5/9** 6/13
Traditional LLNA vs. Human[4]	41	78	32/41	84	26/31	60	6/10	40	4/10	16	5/31	87	26/30	54	6/11

Abbreviations: GP = guinea pig skin sensitization outcomes; LLNA: BrdU-ELISA= murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; LLNA = murine local lymph node assay; No. = number.

[1] n = number of substances included in this analysis.

[2] The data on which the percentage calculation is based.

[3] *GP* refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.

[4] *Human* refers to outcomes obtained by studies conducting using the human maximization test, the human repeat insult patch test, inclusion of the test substance in a human patch test allergen kit, and/or published clinical case studies/reports.

6.6 Discordant Results for Accuracy Analysis Using Alternative Decision Criteria

This section discusses the discordant results obtained for the analyses using the alternative decision criteria shown in **Tables C-7** and **C-8** to provide a comparison to the discordant substances identified using the decision criterion of SI ≥ 2.0 to identify sensitizers. Discordant results are first discussed for the alternative decision criteria using the traditional LLNA as the reference test (**Section 6.6.1**). Then discordant results for SI ≥ 1.6, the optimized criterion, are discussed using the traditional LLNA, GP, and human outcomes as references (**Section 6.6.2**).

6.6.1 Discordant Results Using Alternative Decision Criteria Compared with the Traditional LLNA

Using decision criteria of SI ≥ 2.0 and the most prevalent outcome for the substances with multiple tests, the two discordant substances, when compared to the traditional LLNA, were imidazolidinyl urea and 2-mercaptobenzothiazole (**Table C-5**). As indicated in **Section 6.4**, both substances were false negatives when compared to the traditional LLNA.

Table C-9 shows how the number and identity of discordant substances changes with the alternative decision criteria when using the most prevalent outcome for the substances with multiple tests. Use of a statistical test (i.e., ANOVA or *t*-test; "Statistics" in **Table C-7**) or summary statistics (i.e., ≥95% CI, or ≥2 or 3 SD in **Table C-7**) did not result in substantively improved performance relative to using SI ≥ 1.6. SI ≥ 1.3 is shown for comparison because it was previously recommended by ICCVAM. It is not discussed because it was considered to be inadequate by the March 2008 Peer Review Panel (ICCVAM 2008).

Table C-9 Discordant Results for LLNA: BrdU-ELISA Using Alternative Decision Criteria Compared to the Traditional LLNA and the Most Prevalent Outcome for Substances with Multiple Tests

Discordant Substance[1]	Alternative Decision Criterion[2]														
	Statistics[3]	≥95% CI[4]	≥2 SD[5]	≥3 SD[6]	SI ≥ 5.0	SI ≥ 4.5	SI ≥ 4.0	SI ≥ 3.5	SI ≥ 3.0	SI ≥ 2.5	SI ≥ 2.0	SI ≥ 1.9	SI ≥ 1.6	SI ≥ 1.5	SI ≥ 1.3
5-Chloro-2-methyl-4-isothiazolin-3-one solution (0.009%)					-										
Formaldehyde (0.50%)					-	-									
Cobalt chloride (0.6%)					-	-	-								
4-Methylaminophenol sulfate (0.8%)					-	-	-								
trans-Cinnamic aldehyde (1.4%)					-	-	-								
2-Mercaptobenzothiazole (1.7%)	-				-	-	-	-	-	-	-	-			
Cinnamic aldehyde (1.9%)					-	-	-								
3-Aminophenol (3.2%)					-	-	-	-							
Diethyl maleate (3.6%)					-	-	-	-							
Nickel sulfate (4.8%)					-	-	-	-	-						
4-Chloroaniline (6.5%)					-	-	-	-	-						
Sodium lauryl sulfate (8.1%)					-	-	-	-	-						
Hexyl cinnamic aldehyde (9.7%)					-	-	-	-							
Eugenol (10.1%)					-	-	-	-							
Phenyl benzoate (13.6%)					-	-	-	-							

continued

Table C-9 Discordant Results for LLNA: BrdU-ELISA Using Alternative Decision Criteria Compared to the Traditional LLNA and the Most Prevalent Outcome for Substances with Multiple Tests (continued)

Discordant Substance[1]	Alternate Decision Criterion[2]														
	Statistics[3]	≥95% CI[4]	≥2 SD[5]	≥3 SD[6]	SI ≥ 5.0	SI ≥ 4.5	SI ≥ 4.0	SI ≥ 3.5	SI ≥ 3.0	SI ≥ 2.5	SI ≥ 2.0	SI ≥ 1.9	SI ≥ 1.6	SI ≥ 1.5	SI ≥ 1.3
Cinnamic alcohol (21.0%)				-	-	-	-	-	-						
Hydroxycitronellal (24.0%)					-										
Imidazolidinyl urea (24.0%)				-	-	-	-	-	-	-	-	-			
Ethylene glycol dimethacrylate (28.0%)					-	-	-	-							
Linalool (30.0%)					-										
Ethyl acrylate (32.8%)					-										
Isopropyl myristate (44.0%)					-	-									
Aniline (47.5%)					-	-	-	-	-	-					
Glycerol (-)	+	+	+												
Hexane (-)	+	+	+	+									+	+	+
Lactic acid (-)	+	+	+										+	+	+
Methyl salicylate (-)		+													+

Abbreviations: CI = confidence interval; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SD = standard deviation; SI = stimulation index.

[1] Compared to the traditional LLNA outcome. Traditional LLNA result in parentheses: "-" for nonsensitizers and EC3 values (%) for sensitizers.

[2] LLNA: BrdU-ELISA outcomes are indicated by "+" for sensitizer results and "-" for nonsensitizer results.

[3] Analysis of variance for difference of group means when substances were tested at multiple doses or *t*-test when substances were tested at one dose. The absorbance data were log-transformed prior to analysis of variance. Significance at $p < 0.05$ was further tested by Dunnett's test.

[4] The mean absorbance of at least one treatment group was outside the 95% confidence interval for the mean absorbance of the vehicle control group.

[5] The mean absorbance of at least one treatment group was greater than 3 SD from the mean absorbance of the vehicle control group.

[6] The mean absorbance of at least one treatment group was greater than 2 SD from the mean absorbance of the vehicle control group.

Ten of the ICCVAM performance standards required reference substances were discordant for the analysis of alternative decision criteria using the most prevalent outcome for substances with multiple tests (**Table C-7**). Eight sensitizers (5-chloro-2-methyl-4-isothiazolin-3-one, cobalt chloride, 2-mercaptobenzothiazole, hexyl cinnamic aldehyde, eugenol, phenyl benzoate, cinnamic alcohol, and imidazolidinyl urea) were misclassified by some criteria as nonsensitizers, and two nonsensitizers (lactic acid and methyl salicylate) were misclassified as sensitizers by some criteria. The criteria that yielded the correct results for most of the sensitizers included summary statistics (i.e., ≥95% CI, ≥2 SD, or ≥3 SD), statistical tests (i.e., ANOVA or *t*-test), and SI ≥ 3.0 to ≥1.6. The exceptions were:

- 2-mercaptobenzothiazole, which was incorrectly negative by a statistical test (i.e., ANOVA) and at SI ≥ 5.0 to ≥1.9.
- Cinnamic alcohol, which was incorrectly negative at SI ≥ 3.0.
- 5-chloro-2-methyl-4-isothiazolin-3-one, which was also correctly positive at SI ≥ 4.5 to ≥3.5.
- Cobalt chloride, which was also correctly positive at SI ≥ 3.5.

The criteria that yielded the correct results for the nonsensitizers were generally SI criterion greater than 1.9. For lactic acid, the criteria that yielded the correct results included treatment group mean ≥3 SD from the vehicle control, and SI ≥ 5.0 to 1.9. All criteria yielded the correct results for methyl salicylate except for treatment group absorbance ≥95% CI of vehicle control mean.

6.6.2 Discordant Results for Accuracy Analysis of the SI ≥ 1.6 Decision Criterion

When the outcomes for the 43 substances tested in the LLNA: BrdU-ELISA (using SI ≥ 1.6) and the traditional LLNA were compared, the classifications for two substances were different. Hexane and lactic acid, nonsensitizers in the traditional LLNA, were misclassified as sensitizers in the LLNA: BrdU-ELISA. In the LLNA: BrdU-ELISA and the traditional LLNA, hexane was tested in AOO and lactic acid was tested in DMSO. Chemical class, physical form, MW, peptide reactivity (see **Annex II** for physicochemical properties), and potential for skin irritation were examined to identify commonalities among the discordant substances. Hexane is a hydrocarbon, and lactic acid is a carboxylic acid. Both substances are liquids and have low MW (hexane MW = 86.18 g/mol and lactic acid MW = 90.08 g/mol) and minimal peptide reactivity. Both substances were tested in the LLNA: BrdU-ELISA at concentrations expected to produce skin irritation based on data in humans (hexane) or rabbits (lactic acid).

When the outcomes for the 35 substances tested in the LLNA: BrdU-ELISA (using SI ≥ 1.6) and GP tests were compared, the classifications for four substances were different. Ethyl acrylate, ethylene glycol dimethacrylate, lactic acid, and SLS were classified as nonsensitizers in GP tests but were misclassified as sensitizers in the LLNA: BrdU-ELISA. The LLNA: BrdU-ELISA test result was concordant with the traditional LLNA for three of the four substances (i.e., all except lactic acid) (**Table C-10**). Chemical class, physical form, MW, peptide reactivity (see **Annex II** for physicochemical properties), and potential for skin irritation were examined to identify the following commonalities among the discordant substances:

- Ethyl acrylate, ethylene glycol dimethacrylate, and lactic acid are carboxylic acids; SLS is an alcohol, lipid, and sulfur compound.
- MWs range from 90.08 to 288.38 g/mol.
- Ethyl acrylate, ethylene glycol dimethacrylate, and lactic acid are liquids; SLS is a solid.
- Peptide reactivity for ethylene glycol dimethacrylate is high; peptide reactivity for lactic acid is minimal; peptide reactivity data for ethyl acrylate and SLS are not available.

- Ethyl acrylate (EC3 = 32.8%) and ethylene glycol dimethacrylate (EC3 = 28%) are weak sensitizers in the traditional LLNA; SLS (EC3 = 8.1%) is somewhat stronger. Lactic acid is a nonsensitizer in the traditional LLNA.
- Ethyl acrylate, ethylene glycol dimethacrylate, lactic acid, and SLS were tested at irritating concentrations in the LLNA: BrdU-ELISA, based on skin irritation data from guinea pigs, rabbits, mice, or humans.

Table C-10 Discordant Results for LLNA: BrdU-ELISA (SI ≥ 1.6) Compared to Traditional LLNA and Guinea Pig Reference Data[1]

Substance Name[2]	Vehicle[3]	LLNA: BrdU-ELISA[4]	Traditional LLNA[4]	Guinea Pig Studies	Skin Irritant?
Ethyl acrylate (32.8%)	AOO	+ (4.95, 100%)	+ (4.0, 50%)	-	Nonirritant at 3% (GP)
Ethylene glycol dimethacrylate (28.0%)	MEK	+ (3.11, 100%)	+ (7.0, 50%)	-	Nonirritant at 1% (GP)
Lactic acid	DMSO	+ (1.80, 1.89, and 2.53, 50%)	- (2.2, 25%)	-	Slightly irritating at 10% (rabbits)
Sodium lauryl sulfate (8.1%)	DMF	+ (2.64, 16.7%)	+ (8.9, 20%)[5]	-	Irritant at 20% aq. (rabbits); Irritant at 20% (humans); Irritant at 10% in DMF (mice)

Abbreviations: AOO = acetone: olive oil (4:1); aq = aqueous; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; GP = guinea pigs; LLNA: BrdU-ELISA= murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; LLNA = murine local lymph node assay; MEK = methyl ethyl ketone; NA = not available; SI = stimulation index.

+ = sensitizer.

- = nonsensitizer.

[1] Data sources provided in **Annex III-1**.

[2] Numbers in parentheses are the EC3 values (estimated concentration needed to produce a stimulation index [SI] of 3) for the traditional LLNA (from **Table C-1**).

[3] Vehicles apply to tests for both the LLNA: BrdU-ELISA and the traditional LLNA.

[4] Numbers in parentheses are highest SI values and maximum concentrations tested.

[5] Highest SI occurred at 10%.

When the outcomes for the 41 substances with LLNA: BrdU-ELISA (using SI ≥ 1.6) and human outcome data were compared, the classifications for 11 substances were different (**Table C-11**). The LLNA: BrdU-ELISA results for two of these substances (hexane and lactic acid) were discordant with the traditional LLNA. The LLNA: BrdU-ELISA classified five human sensitizers as nonsensitizers (diethyl phthalate, 2-hydroxypropylmethacrylate, isopropanol, propylene glycol, and sulfanilamide) and six human nonsensitizers as sensitizers (hexane, lactic acid, isopropyl myristate, cyclamen aldehyde, linalool, and SLS).

Table C-11 Discordant Results for LLNA: BrdU-ELISA (SI ≥ 1.6) When Compared to Traditional LLNA and Human Outcome Data[1]

Substance Name[2]	Vehicle[3]	LLNA: BrdU-ELISA[4]	Traditional LLNA[4]	Human Outcome[5]	Skin Irritant?
Diethyl phthalate	AOO	- (0.88, 50%)	- (1.5, 100%)	+ (HPTA)	Nonirritant at ≤ 100% (rabbits)
2-Hydroxypro-pylmethacrylate	AOO	- (1.13, 50%)	- (1.3, 50%)	+ (case study, 0.1%)	Nonirritant at ≤ 10% (GP)
Isopropanol	AOO	- (2.22, 50%)[6]	- (1.7, 50%)[7]	+ (case study, 0.001%)	Nonirritant at ≤ 100% (rabbits)
Propylene glycol	AOO[8]	- (1.57, 50%)	- (1.6, 100%)	+ (HPTA)	Nonirritant at ≤ 25% (humans)
Sulfanilamide	DMF	- (1.26, 50%)	- (1.0, 50%)[9]	+ (5/25, 25%)	Nonirritant at ≤ 25% (humans)
Hexane	AOO	+ (1.76, 100%)[10]	- (2.2, 100%)	- (0/25, 100%)	Irritant at 100% (humans)
Lactic acid	DMSO	+ (1.80, 1.89, and 2.53, 100%)	- (2.5, 25%)	- (no data located)[11]	Slightly irritating at ≤ 10% (rabbits)
Isopropyl myristate (44.0%)	AOO	+ (4.20, 50%)	+ (3.4, 100%)	- (0/25, 20%)	Nonirritant at ≤ 100% (rabbits)
Cyclamen aldehyde (22.3%	AOO	+ (1.97 and 5.71, 100%)	+ (5.2, 50%)	- (0/64, 4%)	Irritant at 100% (rabbits)
Linalool (30.0%)	AOO	+ (1.45 and 4.65, 100%)[12]	+ (8.3, 100%)	- (0/25, 8%)	Irritant at 100% (rabbits)
Sodium lauryl sulfate (8.1%)	DMF	+ (3.4, 10%)	+ (8.9, 20%)[13]	- (0/22 at 10%)	Irritant at 20% aq. (rabbits); Irritant at 20% (humans); Irritant at 10% in DMF (mice)

Abbreviations: AOO = acetone: olive oil (4:1); aq = aqueous; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; GP = guinea pigs; HPTA = human patch test allergen; LLNA = murine local lymph node assay; LLNA: BrdU-ELISA= murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SI = stimulation index.

+ = sensitizer.

- = nonsensitizer.

[1] Data sources provided in **Annex III-1**.

[2] Numbers in parentheses are EC3 values (estimated concentration needed to produce a stimulation index [SI] of 3) for substances that are sensitizers in the traditional LLNA; from **Table C-1**.

[3] Vehicles apply to tests for both the LLNA: BrdU-ELISA and the traditional LLNA unless otherwise noted.

[4] Numbers in parentheses are highest SI values and maximum concentrations tested.

[5] Information in parentheses indicates the basis for the human outcome. Numbers indicate the incidence of positive human response and concentration tested.

[6] Negative based on most prevalent call. Highest SI of any test is shown. Highest SI values for most tests occurred at <50%.

[7] Highest SI occurred at 10%.

[8] The vehicle for the traditional LLNA was distilled water.

[9] Highest SI occurred at 10% and 25%.

[10] An additional test yielded SI = 1.89 at 50%.

[11] Presumed to be a nonsensitizer in humans because no clinical patch test results were located, it is not a patch test kit allergen, and no case reports of human sensitization were located.

[12] When the number of positive and negative outcomes were equal for a substance, the most conservative result was used in the accuracy analysis (see **Section 6.5**).

[13] Highest SI occurred at 10% and 25%.

Few commonalities in chemical class, physical form, MW, peptide reactivity, traditional LLNA range of EC3 values, and potential for skin irritation were noted among the discordant substances. For the five human sensitizers that were misclassified as nonsensitizers:

- Four different chemical classes were represented: carboxylic acids (diethyl phthalate and 2-hydroxypropylmethacrylate), alcohols (isopropanol and propylene glycol), sulfur compounds (sulfanilamide) and cyclic hydrocarbons (sulfanilamide) (**Tables C-1** and **C-11**).
- Three substances were liquids (diethyl phthalate, isopropanol, and propylene glycol), and two were solids (2-hydroxypropylmethacrylate and sulfanilamide).
- MWs ranged from 60.10 (isopropanol) to 222.24 g/mole (diethyl phthalate).
- Four substances exhibited low peptide reactivity; no peptide reactivity information was available for sulfanilamide.
- All five substances were also classified as nonsensitizers by the traditional LLNA.
- Although 2-hydroxypropylmethacrylate, propylene glycol, and sulfanilamide are skin irritants at the concentrations tested in the LLNA: BrdU-ELISA (based on data from humans, rabbits, or guinea pigs), the other two substances were not irritating to skin at the concentrations tested (**Table C-11**).

There were few commonalities in chemical class, physical form, MW, peptide reactivity, range of EC3 values (based on the traditional LLNA), and potential for skin irritation noted among the six human nonsensitizers that were misclassified as sensitizers by the LLNA: BrdU-ELISA:

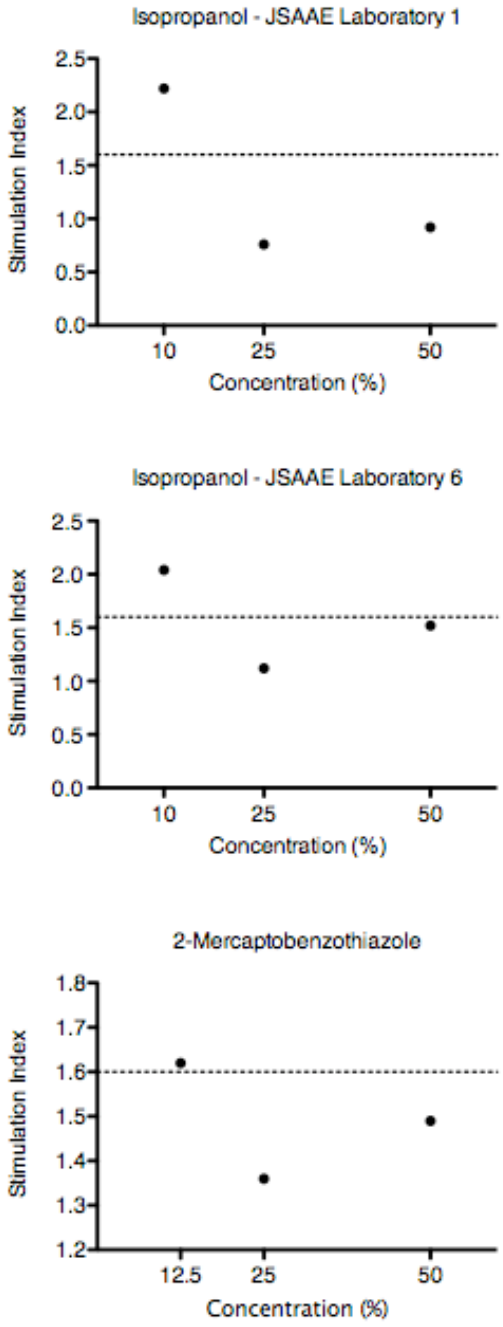
- The six substances represented six different chemical classes: carboxylic acids (cyclamen aldehyde and lactic acid), lipids (isopropyl myristate and SLS), acyclic hydrocarbons (hexane), sulfur compounds (SLS), alcohols (SLS), and hydrocarbons, other (linalool) (**Tables C-1** and **C-11**).
- Five substances are liquids, and SLS is a solid.
- Four substances have minimal to low peptide reactivity, but no peptide reactivity data are available for linalool or SLS.
- MWs ranged from 86.15 g/mole for hexane to 288.38 g/mole for SLS.

- Isopropyl myristate, cyclamen aldehyde, linalool, and SLS were also classified as sensitizers by the traditional LLNA (EC3 values were 44.0%, 22.3%, 30.0%, and 8.1%, respectively); but hexane and lactic acid were classified as nonsensitizers by the traditional LLNA.
- Five of the substances misclassified as sensitizers (hexane, lactic acid, cyclamen aldehyde, linalool, and SLS) were tested at concentrations that are irritating to skin, but one was not (isopropyl myristate), based on skin irritation data from humans, mice, or rabbits (**Table C-11**).

6.7 Accuracy Analysis for the Reduced LLNA: BrdU-ELISA (rLLNA: BrdU-ELISA)

An accuracy analysis for the rLLNA: BrdU-ELISA was performed using the optimized SI ≥ 1.6 criterion to identify sensitizers. The rLLNA: BrdU-ELISA uses only the highest soluble dose of the test substance that does not produce local skin irritation or systemic toxicity; the two lower dose groups are not used. The available validation database for the rLLNA: BrdU-ELISA analysis included 85 individual tests that used multiple doses. The performance of the rLLNA: BrdU-ELISA was evaluating by comparing the outcome of the highest dose for each test to the outcome of the same test when considering all doses tested. Using SI ≥ 1.6 to identify sensitizers, the accuracy of the rLLNA: BrdU-ELISA was 95% (82/85), with a false positive rate of 0% (0/11) and a false negative rate of 4% (3/74). The three tests that were false negative in the rLLNA: BrdU-ELISA were weakly positive in the multiple-dose LLNA: BrdU-ELISA. Two tests of 10%, 25%, and 50% isopropanol produced maximum SI values of 2.04 and 2.22 at the lowest dose tested (**Figure C-2**). The third false negative was the test of 12.5%, 25%, and 50% 2-mercaptobenzothiazole, which produced the maximum SI of 1.62 at the lowest dose tested (**Figure C-2**).

Figure C-2 Dose-Response Curves for Substances Identified as Nonsensitizers by the rLLNA: BrdU-ELISA and Sensitizers by the LLNA: BrdU-ELISA



The horizontal dashed line shows the stimulation index of 1.6, which is the threshold for a positive response. Points above the line indicate sensitizer responses and points below the line indicate nonsensitizer responses.

Abbreviations: JSAAE = Japanese Society for Alternatives to Animal Experiments.

6.8 Accuracy Analysis Using Multiple Alternative Decision Criteria

As detailed in **Section 6.5**, the accuracy of the LLNA: BrdU-ELISA when using a number of alternative decision criteria was evaluated using the traditional LLNA as the reference test. Using the database of 31 substances that was available for the Panel review in April 2009, **Annex VII** provides an accuracy and reproducibility analysis that uses two SI decision criteria: one to identify sensitizers and another to identify nonsensitizers. The lowest SI decision criterion that produced no false positives was used to identify sensitizers, and the SI decision criterion that produced no false negatives was used to identify nonsensitizers. **Annex VII** also includes an evaluation of additional information that could be used in an integrated decision strategy for classifying indeterminate substances and an analysis of the effect of sample size on the indeterminate range of SI values.

7.0 Test Method Reliability

An assessment of test method reliability (intra- and inter-aboratory reproducibility) is an essential element of any evaluation of the performance of an alternative test method (ICCVAM 2003). Intralaboratory reproducibility refers to the extent to which qualified personnel within the same laboratory can replicate results using a specific test protocol at different times. Interlaboratory reproducibility refers to the extent to which different laboratories can replicate results using the same protocol and test substances, and indicates the extent to which a test method can be transferred successfully among laboratories.

The available LLNA: BrdU-ELISA data were amenable to both intralaboratory and interlaboratory reproducibility analyses. This section provides an assessment of reproducibility for the decision criterion of SI ≥ 1.6 to identify sensitizers. In **Section 6.5**, this criterion was identified as the optimum criterion for producing no false negatives and minimal false positives, compared with the traditional LLNA. **Annex IX** describes the evaluation of reproducibility for additional decision criteria to identify sensitizers that were evaluated in **Section 6.5**: SI ≥ 1.5 and SI ≥ 2.0 (used in the JSAAE interlaboratory validation study).

7.1 Intralaboratory Reproducibility

The test results for the LLNA: BrdU-ELISA were amenable to intralaboratory reproducibility analyses for three endpoints: sensitizer or nonsensitizer classification, SI values, and EC1.6 values. Analyses of intralaboratory reproducibility were performed using a concordance analysis for the qualitative results (sensitizer vs. nonsensitizer) (**Section 7.1.1**) and a coefficient of variation (CV) analysis for the quantitative results (SI values and EC1.6 values) (**Sections 7.1.2** and **7.1.3**, respectively).

7.1.1 Intralaboratory Reproducibility – Qualitative Results

The dataset available for an intralaboratory concordance analysis of the qualitative test results for the LLNA: BrdU-ELISA included 12 substances that were tested multiple times by Takeyoshi et al. (2003, 2004a, 2005, 2006, 2007a, 2007b, unpublished). Hexyl cinnamic aldehyde and eugenol were tested six times; isoeugenol, diphenycyclopropenone, and propylene glycol were tested three times; and cyclamen aldehyde, 2,4-dinitrochlorobenzene, glutaraldehyde, hexane, hydroxycitronellal, linalool, and 4-phenylenendiamine were each tested twice (Takeyoshi et al. 2003, 2004a, 2005, 2006, 2007a, unpublished) (**Table C-12**). All substances were sensitizers in the traditional LLNA except for propylene glycol and hexane. The multiple test results for 10/12 substances were 100% concordant when SI ≥ 1.6 was used to classify substances as sensitizers. However, the concordant tests for one nonsensitizer, hexane, were incorrectly positive. The substances with disconcordant results were the sensitizers hydroxycitronellal and linalool, which produced one positive (SI ≥ 1.6) and one negative (SI < 1.6) result in the LLNA: BrdU-ELISA.

By comparison, the qualitative intralaboratory concordance analysis for the traditional LLNA (ICCVAM 1999) was based on a dataset of six substances that included six results each for benzocaine and hexyl cinnamic aldehyde, five results for eugenol, four results each for isoeugenol and methyl salicylate, and three results for 2,4-dinitrochlorobenzene. Intralaboratory results for each substance were 100% concordant with the exception of benzocaine. One of the six benzocaine (5/6 or 83% concordance) results for the traditional LLNA was reported as equivocal because SI increased with dose but did not reach the criterion of SI ≥ 3.0. Thus, the proportion of substances for which intralaboratory concordance of qualitative results was 100% was identical for LLNA: BrdU-ELISA (10/12) and the traditional LLNA (5/6).

Table C-12 Intralaboratory Reproducibility for the LLNA: BrdU-ELISA Outcome of Substances Tested Multiple Times

Substance Name	Highest Concentration Tested (%)	Highest SI	Outcome[1]	Takeyoshi et al. Reference
Cyclamen aldehyde	100	1.97	+	2007b
	100	5.71	+	Unpublished
2,4-Dinitro-chlorobenzene	2	17.90	+	2005
	2	6.84	+	2006, 2007b
Diphenylcyclopro-penone	2	19.10	+	2005; 2007b
	10	9.34	+	2005
	10	11.62	+	2007b
Eugenol	10	3.18	+	2003
	30	3.30	+	2004a
	30	3.83	+	2007a
	50	12.30	+	2005
	50	3.10	+	2006
	50	17.70	+	2007b
Glutaraldehyde	2	14.60	+	2005, 2007b
	10	15.50	+	2005, 2007b
Hexane	50	1.89	+	2005
	100	1.76	+	Unpublished
Hexyl cinnamic aldehyde	25	2.41	+	2003
	50	3.60	+	2003
	50	5.90	+	2005
	50	3.64	+	2006
	50	2.72	+	2006
	50	3.02	+	2007b
Hydroxycitronellal	100	1.34	-	2007b
	100	4.78	+	Unpublished
Isoeugenol	10	8.40	+	2005
	10	2.40	+	2006, 2007b
	30	6.73	+	2007a
Linalool	100	1.45	-	Unpublished
	100	4.65	+	Unpublished

continued

Table C-12 Intralaboratory Reproducibility for the LLNA: BrdU-ELISA Outcome of Substances Tested Multiple Times (continued)

Substance Name	Highest Concentration Tested (%)	Highest SI	Outcome[1]	Takeyoshi et al. Reference
4-Phenylenediamine	2	11.70	+	2005, 2007b
	10	14.70	+	2005, 2007b
Propylene glycol	10	1.20	-	2005
	50	1.57	-	2005
	50	0.91	-	2006, 2007b

Abbreviations: LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SI = stimulation index.

[1] (+) = sensitizer; (-) = nonsensitizer.

7.1.2 Intralaboratory Reproducibility – SI

Ten substances were tested multiple times by Takeyoshi et al. (2003, 2004a, 2005, 2006, 2007a, 2007b, unpublished data) at the same concentrations. Because two substances had multiple tests for more than one concentration, there were 13 substance/concentration combinations that were tested two to five times in separate experiments. The multiple SI values for each substance/concentration were used to calculate a CV for the assessment of intralaboratory variability. As shown by **Table C-13**, the CV values ranged from 1% (25% hexyl cinnamic aldehyde) to 80% (100% hydroxycitronellal and 10% isoeugenol). There are no data for comparison with the traditional LLNA because the intralaboratory reproducibility of the traditional LLNA was not assessed by CV analysis of SI values (ICCVAM 1999).

7.1.3 Intralaboratory Reproducibility – EC1.6 Values

CV values were also calculated for the EC1.6 values for the four sensitizers that were tested more than once using multiple doses by Takeyoshi et al. (2003, 2004a, 2005, 2006, 2007a, 2007b) and repeatedly yielded positive LLNA: BrdU-ELISA results. The individual animal data for cyclamen aldehyde, eugenol, hexyl cinnamic aldehyde, and isoeugenol were used to calculate EC1.6 values for the LLNA: BrdU-ELISA. The methods for calculating EC1.6 values for each sensitizer were modified from those used by Ryan et al. (2007) to calculate EC3 values. Linear interpolation was used to calculate EC1.6 values for each test with SI values higher or lower than 1.6, and extrapolation was used to calculate EC1.6 values for tests with no SI values below 1.6. The equation for linear interpolation was:

$$EC1.6 = c + \left[\frac{(1.6 - d)}{(b - d)}\right] \times (a - c)$$

The linear interpolation equation uses the points immediately above and below SI = 1.6, with the (dose, SI) coordinates of (a, b) immediately above SI = 1.6 and (c, d) immediately below SI = 1.6. The equation for extrapolation was:

$$EC1.6_{ex} = 2^{\left\{\log_2(c) + \frac{(1.6-d)}{(b-d)} \times [\log_2(a) - \log_2(c)]\right\}}$$

Table C-13 Intralaboratory Reproducibility for the SI of Substances Tested in LLNA: BrdU-ELISA – Coefficient of Variation

Substance Name	Concentration Tested (%)	SI	Mean	SD	CV (%)	Takeyoshi et al. Reference
Cyclamen aldehyde	100	1.97	3.84	2.64	69	2007b
		5.71				Unpublished
2,4-Dinitrochlorobenzene	2	17.86	12.35	7.79	63	2005
		6.84				2006, 2007b
Diphenylcyclopropenone	10	9.34	10.48	1.61	15	2005; 2007b
		11.62				2007b
Eugenol	30	3.33	3.58	0.35	10	2004a
		3.83				2007a
Eugenol	50	12.28	11.01	7.40	67	2005
		3.05				2006
		17.69				2007b
Hexane	50	1.89	1.64	0.36	22	2005
		1.38				Unpublished
Hexyl cinnamic aldehyde	12.5	1.88	1.74	0.21	12	2003
		1.59				2003
Hexyl cinnamic aldehyde	25	2.44	2.42	0.02	1	2003
		2.41				2003
Hexyl cinnamic aldehyde	50	3.64	3.78	1.25	33	2003
		5.90				2005
		3.64				2006
		2.72				2006
		3.02				2007b
Hydroxycitronellal	100	1.34	3.06	2.43	80	2007b
		4.78				Unpublished
Isoeugenol	10	8.36	5.09	3.15	80	2005
		7.20				2005
		2.36				2006, 2007b
		2.43				2007a
Linalool	100	1.45	3.05	2.26	74	Unpublished
		4.65				Unpublished

continued

Table C-13 Intralaboratory Reproducibility for the SI of Substances Tested in LLNA: BrdU-ELISA – Coefficient of Variation (continued)

Substance Name	Concentration Tested (%)	SI	Mean	SD	CV (%)	Takeyoshi et al. Reference
Propylene glycol	50	1.57	1.14	0.62	54	2005
		0.70				2006, 2007b

Abbreviations: CV = coefficient of variation; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SD = standard deviation; SI = stimulation index.

The extrapolation equation uses the two points immediately above SI = 1.6, with the coordinates of (a, b) for the point closest to SI = 1.6, and (c, d) for the higher point. As shown in **Table C-14**, there were five EC1.6 values for hexyl cinnamic aldehyde, four EC1.6 values for eugenol, and two EC1.6 values for cyclamen aldehyde and isoeugenol. The CV values were 118% for cyclamen aldehyde, 67% for eugenol, 37% for hexyl cinnamic aldehyde, and 42% for isoeugenol. The ICCVAM LLNA *Performance Standards* criteria for demonstrating adequate intralaboratory reproducibility is based on results from at least four independent tests of hexyl cinnamic aldehyde (ICCVAM 2009). Intralaboratory reproducibility is considered adequate when each test yields an ECt value (i.e., the estimated concentration needed to produce an SI of a specific threshold value; in this case, SI = 1.6) within 5% to 20% (ICCVAM 2009). All of the five EC1.6 values for hexyl cinnamic aldehyde were within the acceptable range for intralaboratory reproducibility.

Table C-14 Intralaboratory Reproducibility for the EC1.6 Values of Substances Tested in LLNA: BrdU-ELISA - Coefficient of Variation

Substance Name	EC1.6 (%)	Mean	SD	CV (%)	Takeyoshi et al. Reference
Cyclamen aldehyde	76.0	41.5	48.8	118	2007b
	7.0				Unpublished
Eugenol	7.0	8.2	5.5	67	2004a
	13.5				2006
	1.1				2007b
	11.2				2007a
Hexyl cinnamic aldehyde	6.3	14.5	5.4	37	2003
	12.7				2003
	18.7				2006
	19.6				2006
	15.5				2007b
Isoeugenol	6.7	5.2	2.2	42	2006; 2007b
	3.6				2007a

Abbreviations: CV = coefficient of variation; EC1.6 = estimated concentration needed to produce a stimulation index of 1.6; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SD = standard deviation.

The intralaboratory reproducibility of the traditional LLNA was assessed by CV analysis of EC3 values using a larger dataset (ICCVAM 1999) than that available for the LLNA: BrdU-ELISA analysis. Two EC3 values were reported by each of five laboratories for 2,4-dinitrochlorobenzene, five EC3 values were reported by one laboratory for isoeugenol, six EC3 values were reported for

hexyl cinnamic aldehyde by two laboratories, and five EC3 values were reported for eugenol by one laboratory (**Table C-15**).

Table C-15 Intralaboratory Reproducibility for the EC3 Values of Substances Tested in the Traditional LLNA[1]

Substance Name	Number of Laboratories	Number of Tests per Laboratory	CV (%)
2, 4-Dinitrochlorobenzene	5	2	13-47
Isoeugenol	1	5	26
Hexyl cinnamic aldehyde	2	6	19-27
Eugenol	1	5	18

Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of 3; LLNA = murine local lymph node assay.

[1]From ICCVAM (1999).

The intralaboratory CV values for the EC1.6 values from LLNA: BrdU-ELISA tests were higher than EC3 values for the same substances from the traditional LLNA reported in ICCVAM (1999). The intralaboratory EC1.6 CV value from the LLNA: BrdU-ELISA tests of eugenol was higher that that reported by ICCVAM (1999) (67% vs. 18%). The intralaboratory EC1.6 CV value from the LLNA: BrdU-ELISA tests of isoeugenol was greater than that from ICCVAM (1999) (42% vs. 26%). The intralaboratory EC1.6 CV value for hexyl cinnamic aldehyde was greater than those from ICCVAM (1999) (37% vs. 19% to 27%).

7.2 Interlaboratory Reproducibility

The interlaboratory reproducibility of the LLNA: BrdU-ELISA was assessed using the individual animal data from the multilaboratory validation study organized by the JSAAE (Kojima et al. 2008). Phase I of the study evaluated the reliability and transferability of the test method protocol by testing 12 substances in three to nine laboratories. With the exception of the positive control data, neither the summary results nor the individual animal data from Phase I of the validation study have been released. Phase II of the study tested 10 substances in three to seven laboratories as shown in **Table C-16**. All the laboratories that participated in the validation study used the same experimental protocol (**Annex I**) and participated in a 1-day seminar that explained the protocol and execution of the test method. The same commercial ELISA kit, test materials, and the same doses of the test substances were used in all of the laboratories. The Validation Management Team determined the doses and vehicles for testing and coded the identity of the test substances prior to distribution to the test laboratories. Seven substances were sensitizers and three substances were nonsensitizers according to the traditional LLNA. Six substances were ICCVAM *Recommended Performance Standards* reference substances: 2,4-dinitrochlorobenzene, eugenol, hexyl cinnamic aldehyde, lactic acid, isopropanol, and methyl salicylate (ICCVAM 2009).

Table C-16 Substances and Test Allocation for the Phase II Interlaboratory Validation Study of the LLNA: BrdU-ELISA

Substance Name[1]	Vehicle	Concentrations Tested			Laboratory[2]						
					1	2	3	4	5	6	7
Nickel sulfate (+)	DMSO	1%	3%	10%			X	X			X
Isopropanol (-)	AOO	10%	25%	50%	X	X	X	X	X	X	X
Eugenol (+)	AOO	10%	25%	50%		X				X	X
Cinnamic aldehyde (+)	AOO	1%	3%	10%		X		X	X		
2,4-Dinitrochlorobenzene (+)	AOO	0.1%	0.3%	1%	X	X	X	X	X	X	X
Glutaraldehyde (+)	ACE	0.1%	0.3%	1%	X				X	X	
Methyl salicylate (-)	AOO	10%	25%	50%	X	X	X				
Hexyl cinnamic aldehyde (+)	AOO	10%	25%	50%	X	X	X	X	X	X	X
Lactic acid (-)	DMSO	10%	25%	50%			X	X			X
Formaldehyde (+)	ACE	1%	3%	10%	X				X	X	

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.

[2] X indicates that a substance was tested in a particular laboratory: 1 = Daicel Chemical Industries Ltd.; 2 = Food and Drug Safety Center; 3 = Otsuka Pharmaceutical Co. Ltd.; 4 = Taisho Pharmaceutical Co. Ltd.; 5 = Fuji Film Co. Ltd.; 6 = Biosafety Research Center, Foods, Drugs and Pesticides; 7 = National Institute of Health Sciences.

The LLNA: BrdU-ELISA test results from the JSAAE validation study were used for interlaboratory reproducibility analyses for three endpoints: sensitizer or nonsensitizer classification and EC1.6 values. Analyses of interlaboratory reproducibility were performed using a concordance analysis for the qualitative results (sensitizer vs. nonsensitizer) (**Section 7.2.1**) and a CV analysis for the quantitative results (EC1.6 values) (**Section 7.2.2**).

7.2.1 Interlaboratory Reproducibility – Qualitative Results

The available quantitative absorbance data for interlaboratory reproducibility analysis were used to calculate SI values for each substance and dose tested. Substances with SI $\geq$ 1.6 at any dose were classified as sensitizers. Substances with SI < 1.6 at all doses were classified as nonsensitizers. The qualitative (sensitizer/nonsensitizer) interlaboratory concordance analysis for the 10 substances tested during Phase II of the JSAAE interlaboratory validation study is shown in **Table C-17**. The qualitative comparison evaluated the consistency of LLNA: BrdU-ELISA results (i.e., positive vs. negative) for 10 substances tested among up to 7 laboratories. The concordance results show that interlaboratory concordance was 100% (3/3, 6/6, or 7/7) for nine substances. However, one nonsensitizer, lactic acid, yielded concordant sensitizer results (SI = 1.80, 1.89, and 2.53). The discordant substance was isopropanol, for which interlaboratory concordance was 67% (4/6). Two of

the six tests of isopropanol yielded SI ≥ 1.6 (SI = 2.04 and SI = 2.22), while the others yielded negative results (i.e., SI < 1.6). The Validation Management Team considered the interlaboratory reproducibility to be acceptable using SI ≥ 2.0 to identify sensitizers (Kojima et al. 2008). Because the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), there were no traditional concordance data for comparison with the LLNA: BrdU-ELISA concordance.

Table C-17 Qualitative Results for the Phase II Interlaboratory Validation Study on the LLNA: BrdU-ELISA[1]

Substance Name	Laboratory							Concordance
	1	2	3	4	5	6	7	
2,4-Dinitrochlorobenzene	+ (4.30)	+ (8.37)	+ (6.26)	+ (5.50)	+ (18.80)	+ (4.83)	+ (12.98)	7/7
Glutaraldehyde	+ (3.72)				+ (28.64)	+ (2.25)		3/3
Nickel sulfate			+ (2.58)	+ (4.53)			+ (2.66)	3/3
trans-Cinnamic aldehyde		+ (3.37)		+ (3.50)	+ (4.11)			3/3
Formaldehyde	+ (4.40)				+ (16.59)	+ (1.97)		3/3
Eugenol		+ (3.17)				+ (3.18)	+ (7.09)	3/3
Hexyl cinnamic aldehyde	+ (3.40)	-[3]	+ (2.87)	+ (3.34)	+ (13.50)	+[4] (3.27)	+ (3.84)	6/6
Isopropanol	+[2] (2.22)	-[3]	- (0.98)	- (1.57)	- (0.94)	+[2,5] (2.04)	- (1.01)	4/6
Lactic acid			+ (1.80)	+ (1.89)			+ (2.53)	3/3
Methyl salicylate	- (1.43)	- (1.44)	- (1.40)					3/3

Abbreviation: LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine.

[1] (+) indicates sensitizer result; (-) indicates nonsensitizer result. Highest stimulation index value for each test is shown in parentheses.

[2] Stimulation index (SI) ≥ 1.6 at lowest dose tested but <1.6 at the higher doses. The Validation Management Team considered these to be nonsensitizer results using the SI ≥ 2.0 criterion (Kojima et al. 2008).

[3] Test failed because concurrent positive control failed (i.e., SI < 1.6). Result not included in the concordance analysis.

[4] Three mice tested at highest dose.

[5] Three mice per dose group.

7.2.2 Interlaboratory Reproducibility – EC1.6 Values

The SI values from the interlaboratory validation study were used to calculate EC1.6 values for each sensitizer according to the methods reported in **Section 7.1.3**. The EC1.6 values from each laboratory were then used to calculate CV values for each substance. The resulting values are shown in **Table C-18**. CV values ranged from 31% (*trans*-cinnamic aldehyde) to 93% (glutaraldehyde). The mean CV was 69%.

The ICCVAM LLNA performance standards indicate that interlaboratory reproducibility should be evaluated with at least two sensitizing chemicals with well-characterized activity in the traditional LLNA (ICCVAM 2009). Acceptable reproducibility is attained when each laboratory obtains ECt values within 0.025% to 0.1% for 2,4-dinitrochlorobenzene and within 5% to 20% for hexyl cinnamic aldehyde (ICCVAM 2009). EC1.6 values from five laboratories were outside the range for 2,4-dinitrochlorobenzene, and the EC1.6 values from two laboratories were outside the range for hexyl cinnamic aldehyde. Laboratories 2 through 6 reported EC1.6 values that were lower than the specified acceptance range for 2,4-dinitrochlorobenzene (0.011%, 0.023%, 0.023%, 0.0022%, and 0.017%, respectively). For hexyl cinnamic aldehyde, Laboratories 4 and 5 obtained EC1.6 values that were lower than the acceptance range (4.80% and 3.64%, respectively).

Table C-18 EC1.6 Values from the Phase II Interlaboratory Validation Study on the LLNA: BrdU-ELISA[1]

Substance Name	Laboratory							Mean ± SD	% CV
	1	2	3	4	5	6	7		
2,4-Dinitro-chlorobenzene	0.062 (4.3 @ 1%)	***0.011***[1] (8.37 @ 1%)	***0.023*** (5.99 @ 0.3%)	***0.023*** (5.50 @ 1%)	***0.0022*** (18.80 @ 0.3%)	***0.017*** (4.83 @ 0.3%)	0.050 (12.18 @ 1%)	0.027 ± 0.021	80
Hexyl cinnamic aldehyde	10.43 (3.4 @ 50%)	-[2] (1.83 @ 50%)	16.98 (2.87 @ 50%)	***4.80*** (3.34 @ 50%)	***3.64*** (13.5 @ 50%)	8.60[3] (3.27 @ 50%)	10.10 (3.84 @ 50%)	11.78 ± 8.33	71
Glutaraldehyde	0.079	NT	NT	NT	0.031	0.24	NT	0.12 ± 0.11	93
Nickel sulfate	NT	NT	1.84	0.57	NT	NT	0.67	1.03 ± 0.70	68
trans-Cinnamic aldehyde	NT	1.88	NT	1.04	1.96	NT	NT	1.63 ± 0.51	31
Formaldehyde	0.29	NT	NT	NT	0.19	0.010	NT	0.16 ± 0.14	88
Eugenol	NT	13.82	NT	NT	NT	11.65	3.77	9.75 ± 5.29	54

Note: Boldface indicates substances recommended for assessing interlaboratory reproducibility in *Recommended Performance Standards* (ICCVAM 2009). Boldface italic EC1.6 values are outside of the acceptable range from the ICCVAM LLNA performance standards: 5%-20% for hexyl cinnamic aldehyde and 0.025%-0.1% for 2,4-dinitrochlorobenzene. Values in parentheses are the highest SI values achieved.

Abbreviations: CV = coefficient of variation; EC1.6 = estimated concentration needed to produce a stimulation index of 1.6; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; NT = not tested; SI = stimulation index.

[1] EC1.6 values interpolated using lowest dose and SI = 1 at 0% concentration because the dose response at the two lowest doses (0.1% and 0.3%) was flat (SI = 6.39 and 6.53, respectively).

[2] Test failed because associated positive control failed (i.e., SI < 1.6; vehicle control absorbance was unusually high). Result not included in the mean EC1.6 and CV values.

[3] Three mice tested at highest dose.

The interlaboratory CV values for the LLNA: BrdU-ELISA EC1.6 values were higher than those for the traditional LLNA EC3 values. The analysis of interlaboratory variation of EC3 values for the traditional LLNA reported CV values of 7% to 84% for five substances tested in five laboratories (**Table C-19**; ICCVAM 1999). Three of the same substances were evaluated in the traditional LLNA and the LLNA: BrdU-ELISA. All interlaboratory CV values for the LLNA: BrdU-ELISA were greater than those for the traditional LLNA. The CV of 80% for 2,4-dinitrochlorobenzene was greater than the two CV values of 37% and 27%, calculated from five values each, reported by ICCVAM (1999). The CV of 71% for hexyl cinnamic aldehyde tested in the LLNA: BrdU-ELISA was greater than the 7% reported by ICCVAM (1999). The CV of 54% for eugenol tested in the LLNA: BrdU-ELISA was greater than the 42% reported by ICCVAM (1999).

Table C-19 Interlaboratory Reproducibility of the EC3 Values for Substances Tested in the Traditional LLNA[1]

Substance Name	Laboratory					CV (%)
	1	2	3	4	5	
2, 4-Dinitrochlorobenzene	0.3	0.5	0.6	0.9	0.6	37
	0.5	0.6	0.4	0.6	0.3	27
Hexyl cinnamic aldehyde	7.9	7.6	8.4	7.0	8.1	7
Isoeugenol	1.3	3.3	1.8	3.1	1.6	41
Eugenol	5.8	14.5	8.9	13.8	6.0	42
Sodium lauryl sulfate	13.4	4.4	1.5	17.1	4.0	84

Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of 3; LLNA = murine local lymph node assay.

[1] From ICCVAM (1999).

7.3 Reproducibility Analysis for Substances With Multiple Tests

This section examines the reproducibility of the tests for the 18 substances that had multiple test results, regardless of whether the tests were performed in one laboratory or multiple laboratories. For the 18 substances, two to 12 tests were available. The frequency with which SI values for the 18 substances occurred in one of three SI categories was considered. The three SI categories were:

- LLNA: BrdU-ELISA nonsensitizers with SI < 1.6
- LLNA: BrdU-ELISA sensitizers with SI between 1.6 and 1.9 (borderline positive results with potential to be false positives with respect to classification by the traditional LLNA)
- LLNA: BrdU-ELISA sensitizers with SI ≥ 1.9

Table C-20 shows the proportion of the tests for each substance that produced SI values in each category. When using SI ≥ 1.6 to classify sensitizers, the categorical concordance analysis for the 18 substances with multiple tests indicated that the SI results for 85% (11/13) of the LLNA sensitizers were 100% concordant (i.e., all yielded SI ≥ 1.6 and SI ≥ 1.9). For the 13 traditional LLNA sensitizers with multiple test results, there were two tests that produced SI < 1.6: one test of linalool (SI = 1.45) and one test of hydroxycitronellal (SI = 1.34). The other tests of linalool and hydroxycitronellal produced SI ≥ 1.6 and SI ≥ 1.9. Both tests of these substances were performed in the same laboratory. None of the tests for the 13 sensitizers produced 1.6 < SI < 1.9.

The LLNA: BrdU-ELISA tests for traditional LLNA nonsensitizers were more variable than those for traditional LLNA sensitizers. The results for isopropanol were particularly variable: 71% (5/7) of the tests produced SI ≤ 1.6 (SI = 0.92, 0.94, 0.98, 1.01, and 1.57) and 29% (2/7) produced SI ≥ 1.9 (SI = 2.04 and 2.22). All isopropanol tests were performed in different laboratories. Lactic acid tests produced SI values in two categories: 67% (2/3) of the tests had 1.6 ≤ SI < 1.9 (SI = 1.80 and 1.89), and 33% (1/3) of the tests had SI ≥ 1.9 (SI = 2.53). All isopropanol tests were performed in different laboratories. The multiple test results for hexane, methyl salicylate, and propylene glycol were 100% concordant. However, the two hexane tests produced SI values in the 1.6 ≤ SI < 1.9 category (SI = 1.76 and 1.89) (i.e., sensitizer). Both tests were performed in the same laboratory. The three methyl salicylate (SI = 1.40, 1.43, and 1.44) tests performed in different laboratories and the three propylene glycol (SI = 1.20, 1.57, and 0.91) tests performed in the same laboratory produced SI values in the SI < 1.6 category (i.e., nonsensitizer).

Table C-20 Concordance of LLNA: BrdU-ELISA Tests for Substances with Multiple Tests by Maximum SI Category

Substance	LLNA: BrdU-ELISA Nonsensitizers (Maximum SI < 1.6[1])	LLNA: BrdU-ELISA Sensitizers (Maximum SI ≥ 1.6)		Total Tests
		1.6 ≤ Maximum SI < 1.9[1]	Maximum SI ≥ 1.9[1]	
Sensitizers[2]				
Cyclamen aldehyde	0 (0%)	0 (0%)	0 (100%)	2
2,4-Dinitrochloro-benzene	0 (0%)	0 (0%)	9 (100%)	9
Diphenylcyclopro-penone	0 (0%)	0 (0%)	3 (100%)	3
Eugenol	0 (0%)	0 (0%)	9 (100%)	9
Formaldehyde	0 (0%)	0 (0%)	3 (100%)	3
Glutaraldehyde	0 (0%)	0 (0%)	5 (100%)	5
Hexyl cinnamic aldehyde	0 (0%)	0 (0%)	12 (100%)	12
Hydroxycitronellal	1 (50%)	0 (0%)	1 (50%)	2
Isoeugenol	0 (0%)	0 (0%)	3 (100%)	3
Linalool	1 (50%)	0 (0%)	1 (50%)	2
Nickel sulfate	0 (0%)	0 (0%)	3 (100%)	3
1,4-Phenylenediamine	0 (0%)	0 (0%)	2 (100%)	2
trans-Cinnamaldehyde	0 (0%)	0 (0%)	4 (100%)	4
Nonsensitizers[2]				
Hexane	0 (0%)	2 (100%)	0 (%)	2
Isopropanol	5 (71%)	0 (0%)	2 (29%)	7
Lactic acid	0 (0%)	2 (67%)	1 (33%)	3
Methyl salicylate	3 (100%)	0 (0%)	0 (0%)	3
Propylene glycol	3 (100%)	0 (0%)	0 (0%)	3

Abbreviations: LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SI = stimulation index.

[1] Numbers shown reflect number of tests. Percentage in parentheses reflects percentage of the total number of tests for each substance.

[2] According to traditional LLNA results.

8.0 Data Quality

The data submitted by Dr. Takeyoshi were generated at the Hita Laboratory and the Tokyo Laboratory of the Chemicals Evaluation and Research Institute, Japan (Takeyoshi M, personal communication). Although the laboratories conduct studies routinely that conform to Good Laboratory Practices (GLP), the studies on the LLNA: BrdU-ELISA did not conform fully with GLP guidelines since they were not intended for regulatory purposes. However, all systems employed for these studies (i.e., test facilities, study staff, reagents, and the other study elements) were reportedly the same as those employed in the fully GLP-compliant studies conducted in the laboratory. Although multiple staff members checked the reported data for consistency with the raw data, no audit report is available (Takeyoshi M, personal communication). The raw data are also not available for audit.

The data from the interlaboratory validation study (Kojima et al. 2008) were generated in GLP laboratories, but the LLNA: BrdU-ELISA studies were not fully GLP compliant. The data from each laboratory were reviewed by the chief of the Validation Management Team and the biostatistician.

9.0 Other Scientific Reports and Reviews

The Validation Management Team for the multilaboratory validation study concluded that the LLNA: BrdU-ELISA, using the SI ≥ 2 criterion to identify sensitizers, had sufficient relevance compared with the traditional LLNA and acceptable interlaboratory reproducibility (Kojima et al. 2008). The validation study has been peer reviewed in Japan. The peer review report is expected to be released in 2010 (Kojima H, personal communication).

A set of studies was conducted by Yamano et al. using a similar LLNA: BrdU-ELISA-based method (Yamano et al. 2003, 2004, 2005, 2006, 2007). The test method protocol (e.g., application of test substance to ear of mouse) was similar to what was described in the Takeyoshi et al. studies discussed in this BRD. Compared to the method of Takeyoshi et al., which administered 5 mg BrdU/mouse, the concentration of BrdU administered (via intraperitoneal injection) by Yamano et al. was 150 mg/kg/15 mL saline, which would be approximately 3 mg BrdU/mouse (based on a 20 g mouse). The studies discussed the use of a BrdU-ELISA-based method to assess the skin sensitization potential of a variety of substances, including metal salts of napthenic acid, methylated phenols, industrial biocides, and preservatives. The outcomes of these studies were not included in this evaluation since comparative traditional LLNA data were not available for the substances tested. Therefore, a comparison of the accuracy of the LLNA: BrdU-ELISA of Yamano et al. with the traditional LLNA could not be conducted.

10.0 Animal Welfare Considerations

The LLNA: BrdU-ELISA evaluates only the induction phase of skin sensitization; therefore, the discomfort to animals that can occur in the guinea pig tests with the elicitation phase of ACD is eliminated. Additionally, the LLNA: BrdU-ELISA test method protocol requires fewer mice per treatment group (a minimum of four animals per group) than either of the GP tests (10-20 animals/group for Buehler and 5-10 animals/group for GPMT).

The LLNA: BrdU-ELISA will require the use of the same number of animals as the updated ICCVAM LLNA protocol (ICCVAM 2009). However, since the traditional LLNA uses radioactivity, which is restricted in some countries and institutions, broader use of the nonradioactive LLNA: BrdU-ELISA protocol in place of the GP test could further reduce the number of guinea pigs that are still being used to assess skin sensitization.

10.1 Rationale for the Need to Use Animals

The rationale for the use of animals in the LLNA: BrdU-ELISA is the same as that for the traditional LLNA; there are no valid and accepted non-animal ways to determine the ACD potential of substances and products, except for situations where human studies could be conducted ethically and where such studies would meet regulatory safety assessment requirements. The most detailed information about the induction and regulation of immunological responses are available for mice (ICCVAM 1999).

10.2 Basis for Determining the Number of Animals Used

The number of animals used for the experimental, vehicle, and positive control groups is based on the number of animals used in the development (Takeyoshi et al. 2003, 2004a, 2004b, 2005, 2006, 2007a, 2007b, unpublished data) and interlaboratory validation (Kojima et al. 2008) of the LLNA: BrdU-ELISA test method, which is the same as that specified in the updated ICCVAM LLNA protocol (Appendix A of ICCVAM 2009).

10.3 Reduction Considerations

A further reduction of 40% (12 vs. 20) could be achieved by using the rLLNA: BrdU-ELISA in cases where dose-response information is not needed for hazard identification purposes. In such an approach, only the highest soluble dose of the test article that does not produce skin irritation or systemic toxicity would be administered, and the two lower dose groups would not be used. Additional reductions could be achieved by testing more substances concurrently, so that the same vehicle and positive control group could be used for multiple substances, thus further reducing the number of animals for each additional substance by eight animals, or 40% (12 vs. 20).

11.0 Practical Considerations

Several issues are taken into account when assessing the practicality of using an alternative to an existing test method. In addition to performance evaluations, assessments of the laboratory equipment and supplies needed to conduct the alternative test method, level of personnel training, labor costs, and the time required to complete the test method relative to the existing test method are necessary. The time, personnel cost, and effort required to conduct the proposed test method(s) must be considered to be reasonable when compared to the existing test method it is intended to replace.

11.1 Transferability of the LLNA: BrdU-ELISA

Test method transferability addresses the ability of a method to be accurately and reliably performed by multiple laboratories (ICCVAM 2003), including those experienced in the particular type of procedure as well as laboratories with less or no experience in the particular procedure. The transferability of the LLNA: BrdU-ELISA was demonstrated by the interlaboratory validation study (Kojima et al. 2008) (**Section 7.2**).

11.2 Facilities and Major Fixed Equipment Required to Conduct the LLNA: BrdU-ELISA

Compared to the traditional LLNA, the LLNA: BrdU-ELISA will not require facilities, equipment, or licensing permits for handling radioactive materials. The remaining facilities (e.g., animal care facilities) are the same for the two methods.

11.3 LLNA: BrdU-ELISA Training Considerations

The level of training and expertise needed to conduct the LLNA: BrdU-ELISA should be similar to the traditional LLNA. Additionally, individuals will need to understand and know how to perform ELISAs.

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13.0 Glossary

Accuracy[6]: (a) The closeness of agreement between a test method result and an accepted reference value. (b) The proportion of correct outcomes of a test method. It is a measure of test method performance and one aspect of *relevance*. The term is often used interchangeably with concordance (see also *two-by-two table*). Accuracy is highly dependent on the prevalence of positives in the population being examined.

Allergic contact dermatitis (ACD): A Type IV allergic reaction of the skin that results from repeated skin contact with a skin sensitizer. Clinical signs of ACD include the development of erythema (redness) and edema (swelling), blistering, and itching. Also referred to as skin sensitization.

Assay[6]: The experimental system used. Often used interchangeably with *test* and *test method*.

Coded substances: Substances labeled by code rather than name so that they can be tested and evaluated without knowledge of their identity or anticipation of test results. Coded substances are used to avoid intentional or unintentional bias when evaluating laboratory or test method performance.

Concordance[6]: The proportion of all substances tested that are correctly classified as positive or negative. It is a measure of test method performance and one aspect of *relevance*. The term is often used interchangeably with *accuracy* (see also *two-by-two table*). Concordance is highly dependent on the prevalence of positives in the population being examined.

EC1.6: The estimated concentration needed to produce a stimulation index of 1.6, as compared to the concurrent vehicle control.

EC3: The estimated concentration needed to produce a stimulation index of 3, as compared to the concurrent vehicle control.

ECt: The estimated concentration needed to produce a stimulation index of a specific threshold.

False negative[6]: A substance incorrectly identified as negative by a test method.

False negative rate[6]: The proportion of all positive substances falsely identified by a test method as negative (see *two-by-two table*). It is one indicator of test method accuracy.

False positive[6]: A substance incorrectly identified as positive by a test method.

False positive rate[6]: The proportion of all negative substances that are falsely identified by a test method as positive (see *two-by-two table*). It is one indicator of test method accuracy.

Good Laboratory Practices (GLP)[6]: Regulations promulgated by the U.S. Food and Drug Administration and the U.S. Environmental Protection Agency, and principles and procedures adopted by the Organization for Economic Cooperation and Development and Japanese authorities, that describe record keeping and quality assurance procedures for laboratory records that will be the basis for data submissions to national regulatory agencies.

Hazard[6]: The potential for an adverse health or ecological effect. A hazard potential results only if an exposure occurs that leads to the possibility of an adverse effect being manifested.

Interlaboratory reproducibility[6]: A measure of whether different qualified laboratories using the same protocol and test substances can produce qualitatively and quantitatively similar results.

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[6] Definition used by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM 2003).

Interlaboratory reproducibility is determined during the prevalidation and validation processes and indicates the extent to which a test method can be transferred successfully among laboratories.

Intralaboratory repeatability[6]: The closeness of agreement between test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions within a given time period.

Intralaboratory reproducibility[6]: The first stage of validation; a determination of whether qualified people within the same laboratory can successfully replicate results using a specific test protocol at different times.

Immunological: Relating to the immune system and immune responses.

In vivo: In the living organism. Refers to assays performed in multicellular organisms.

Lymphocyte: A white blood cell found in the blood, lymph, and lymphoid tissues, which regulates and plays a role in acquired immunity.

Murine local lymph node assay (LLNA): An *in vivo* test method used to assess the skin sensitization potential of a substance by measuring the proliferation of lymphocytes in the lymph nodes draining the ears (i.e., auricular lymph nodes) of mice, subsequent to topical exposure of the ear to the substance. The traditional LLNA measures lymphocyte proliferation by quantifying the amount of ^{3}H-thymidine or ^{125}I-iododeoxyuridine incorporated into the cells of the draining lymph nodes.

Murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine (LLNA: BrdU-ELISA): An *in vivo* test method used to assess the skin sensitization potential of a substance by measuring the proliferation of lymphocytes in the lymph nodes draining the ears (i.e., auricular lymph nodes) of mice, subsequent to topical exposure of the ear to the substance. The LLNA: BrdU-ELISA is a nonradioactive modification of the traditional LLNA and assesses lymphocyte proliferation by quantifying the amount of bromodeoxyuridine (BrdU) incorporated into the cells of the draining lymph nodes using an enzyme-linked immunosorbent assay (ELISA).

Negative predictivity[6]: The proportion of correct negative responses among substances testing negative by a test method (see *two-by-two table*). It is one indicator of test method accuracy. Negative predictivity is a function of the sensitivity of the test method and the prevalence of negatives among the substances tested.

Nonsensitizer: A substance that does not cause skin sensitization following repeated skin contact.

Performance[6]: The accuracy and reliability characteristics of a test method (see *accuracy*, *reliability*).

Positive control: A substance known to induce a positive response, which is used to demonstrate the sensitivity of the test method and to allow for an assessment of variability in the conduct of the assay over time. For most test methods, the positive control substance is tested concurrently with the test substance and the vehicle/solvent control. However, for some *in vivo* test methods, periodic studies using a positive control substance is considered adequate by the OECD.

Positive predictivity[6]: The proportion of correct positive responses among substances testing positive by a test method (see *two-by-two table*). It is one indicator of test method accuracy. Positive predictivity is a function of the sensitivity of the test method and the prevalence of positives among the substances tested.

Prevalence[6]: The proportion of positives in the population of substances tested (see *two-by-two table*).

Protocol[6]: The precise, step-by-step description of a test, including the listing of all necessary reagents, criteria, and procedures for the evaluation of the test data.

Quality assurance[6]: A management process by which adherence to laboratory testing standards, requirements, and record keeping procedures is assessed independently by individuals other than those performing the testing.

Reduction alternative[6]: A new or modified test method that reduces the number of animals required.

Reference test method[6]: The accepted *in vivo* test method used for regulatory purposes to evaluate the potential of a test substance to be hazardous to the species of interest.

Refinement alternative[6]: A new or modified test method that refines procedures to lessen or eliminate pain or distress in animals or enhances animal well-being.

Relevance[6]: The extent to which a test method correctly predicts or measures the biological effect of interest in humans or another species of interest. Relevance incorporates consideration of the *accuracy* or *concordance* of a test method.

Reliability[6]: A measure of the degree to which a test method can be performed reproducibly within and among laboratories over time. It is assessed by calculating intra- and interlaboratory reproducibility and intralaboratory repeatability.

Replacement alternative[6]: A new or modified test method that replaces animals with non-animal systems or one animal species with a phylogenetically lower one (e.g., a mammal with an invertebrate).

Reproducibility[6]: The consistency of individual test results obtained in a single laboratory (intralaboratory reproducibility) or in different laboratories (interlaboratory reproducibility) using the same protocol and test substances (see intra- and inter-laboratory reproducibility).

rLLNA: BrdU-ELISA (reduced LLNA: BrdU-ELISA): A variant of the LLNA: BrdU-ELISA that employs a single, high dose of the test substance rather than multiple doses to determine its skin sensitization potential, thus using fewer animals.

Sensitivity[6]: The proportion of all positive substances that are classified correctly as positive in a test method. It is a measure of test method accuracy (see *two-by-two table*).

Skin sensitizer: A substance that induces an allergic response following skin contact (UN 2005).

Specificity[6]: The proportion of all negative substances that are classified correctly as negative in a test method. It is a measure of test method accuracy (see *two-by-two table*).

Stimulation index (SI): A value calculated for the LLNA, to assess the skin sensitization potential of a test substance. The value is calculated as the ratio of the bromodeoxyuridine incorporated into the auricular lymph nodes of a group of treated mice to the bromodeoxyuridine incorporated into the corresponding lymph nodes of a group of vehicle control mice. For the traditional LLNA: BrdU-ELISA and the rLLNA: BrdU-ELISA, an SI $\geq$ 1.6 classifies a substance as a skin sensitizer.

Test[6]: The experimental system used; used interchangeably with *test method* and *assay*.

Test method[6]: A process or procedure used to obtain information on the characteristics of a substance or agent. Toxicological test methods generate information regarding the ability of a substance or agent to produce a specified biological effect under specified conditions. Used interchangeably with *test* and *assay*. See also *validated test method* and *reference test*.

Transferability[6]: The ability of a test method or procedure to be accurately and reliably performed in different, competent laboratories.

Two-by-two table[6]: The two-by-two table can be used for calculating accuracy (concordance) ([a+d]/[a+b+c+d]), negative predictivity (d/[c+d]), positive predictivity (a/[a+b]), prevalence ([a+c]/[a+b+c+d]), sensitivity (a/[a+c]), specificity (d/[b+d]), false positive rate (b/[b+d]), and false negative rate (c/[a+c]).

		New Test Outcome		
		Positive	**Negative**	**Total**
Reference Test Outcome	**Positive**	a	c	a + c
	Negative	b	d	b + d
	Total	a + b	c + d	a + b + c + d

Validated test method[6]: An accepted test method for which validation studies have been completed to determine the relevance and reliability of this method for a specific proposed use.

Validation[6]: The process by which the reliability and relevance of a procedure are established for a specific purpose.

Vehicle control: An untreated sample containing all components of a test system, including the vehicle that is processed with the test substance-treated and other control samples to establish the baseline response for the samples treated with the test substance dissolved in the same vehicle.

Weight-of-evidence (process): The strengths and weaknesses of a collection of information are used as the basis for a conclusion that may not be evident from the individual data.

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Annex I

LLNA: BrdU-ELISA Protocol

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1.0 Introduction

This document describes the recommended standard operating procedure for the non-radioisotopic modification of the LLNA, which is based on 2-Bromodeoxyuridin (BrdU) incorporation in place of ^{3}H-thymidine or ^{125}I-iodoeoxyuridine to measure lymph node cell proliferation. This document is based on the protocol used in the JSAAE multilaboratory validation study of the LLNA: BrdU-ELISA, *Recommended Standard Operating Procedure for the Non-Radioisotopic Local Lymph Node Assay using BrdU-ELISA (Non-RI LLNA), version 1.20, July 31, 2008*, by Masahiro Takeyoshi, Ph.D., Chemicals Evaluation and Research Institute, Japan.

2.0 Description of the Method

The method is practically identical to the standard LLNA methodology excluding the use of BrdU and colorimetric detection. A single intraperitoneal injection (5 mg/mouse per injection) of BrdU is made on day 4. This administration schedule was decided as the most effective labeling protocol to yield maximum SI values based on preliminary study data with several different protocols (Takeyoshi et al. 2001). Approximately 24 hours after the BrdU injection, the auricular lymph nodes are removed, weighed, and stored at -20˚C until analysis using an enzyme-linked immunosorbent assay to measure the level of BrdU incorporation.

The cell proliferation response is measured by a commercial BrdU detection kit (i.e., Roche Diagnostics GmbH, Roche Applied Science, 68298 Mannheim, Germany; Cat. No. 11 647 229 001). To perform the BrdU-ELISA, the lymph nodes are crushed, passed through a #70 nylon mesh. The lymph node cells (LNC) from individual animals are suspended in 15 mL of physiological saline. The cell suspension is added to the wells of a flat-bottom microplate in triplicate. After fixation and denaturation of the LNC, anti-BrdU antibody is added to each well, and after rinsing, substrate solution containing tetramethylbenzidine (TMB) is added and allowed to produce chromogen. Absorbance at 370 nm with a reference wavelength of 492 nm is defined as the BrdU labeling index.

2.1 Animals

2.1.1 *Animal source*

Young adult female mice (nulliparous and nonpregnant) of the CBA/JN or other recommended mouse strains, such as CBA/Ca or CBA/J strain, should be used at age 8-12 weeks. All animals should be age matched (preferably within a 1-week time frame).

2.1.2 *Quarantine and Acclimation*

Healthy animals in good general condition on arrival should be quarantined for more than 5 days. During the quarantine and acclimation period, clinical signs, body weights, and excrement of the animals should be observed.

2.1.3 *Grouping*

Animals confirmed to be in good health with favorable body weight gains during the quarantine and acclimation period should be allocated to groups by a stratified randomization or other appropriate methods before the start of the study.

2.1.4 *Identification*

Animals should be identified by colored marks on the tails, ear tags, or other appropriate methods.

2.1.5 *Animal Husbandry*

The animals should be housed in an animal room maintained at a temperature of 22 ± 3°C and a relative humidity of 30%-70%. The rooms should be artificially lighted for 12 hours daily, and the animals should be given free access to conventional laboratory diet and drinking water.

2.2 Chemicals and Vehicle

2.2.1 *Vehicle*

The solvent/vehicle should be selected on the basis of maximizing the test concentrations while producing a solution/suspension suitable for application of the test substance. In order of preference, recommended solvents/vehicles are AOO, DMF, methyl ethyl ketone, propylene glycol, and DMSO, but others may be used.

2.2.2 *Test Chemicals*

Solid test substances should be dissolved in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance should be prepared daily unless stability data demonstrate the acceptability of storage.

2.2.3 *Controls*

Concurrent negative (vehicle) and positive controls should be included in each test. The positive control (50% hexyl cinnamic aldehyde, CASRN 101-86-0) should be used to ensure the appropriate performance of the assay. The positive control should produce a positive LLNA response at an exposure level expected to give an increase in the stimulation index (SI) >2 over the negative (vehicle) control group.

2.2.4 *Dose selection*

Doses are selected from the concentration series 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc. The maximum concentration tested should be the highest achievable level while avoiding overt systemic toxicity and excessive local irritation. All test solutions should be prepared on the day of application unless the stability is confirmed in advance.

2.2.5 *Preparation of BrdU*

BrdU should be accurately weighed and dissolved in physiological saline for injection to make a 10 mg/mL solution. The BrdU solution should be sterilized by a commercial filtration system (i.e. MILLEX®-HV, MILLIPORE etc.). The BrdU solution can be prepared before administration and stored in a freezer below -20°C until use.

2.3 Animal Experiment

2.3.1 *Grouping*

A minimum of four successfully treated animals is used per dose group, with a minimum of three consecutive concentrations of the test substance plus a negative (vehicle) control and a positive control group.

Table C-I-1 Structure of LLNA: BrdU-ELISA Test Groups

Group	Number of Animals
Negative (vehicle) control	4
Positive control (50% hexyl cinnamic aldehyde)	4
Test substance-low dose	4
Test substance-middle dose	4
Test substance-high dose	4

2.3.2 *Sensitization Procedure*

Apply 25 μL of test solution to the dorsum of both ears of the mice using microvolume pipette daily for 3 consecutive days.

2.3.3 *BrdU Administration*

A single intraperitoneal injection of 0.5 mL of BrdU solution (5 mg/mouse/injection) should be given to the mice 48 hours after the topical application.

2.3.4 *General Condition*

Clinical signs should be observed at least once a day.

2.3.5 *Body Weights*

Body weights should be measured on the day of the first test substance application and on the day that lymph nodes are collected.

2.3.6 *Collection of Lymph Nodes And Measurement of Lymph Node Weight*

Approximately 24 hours after BrdU injection, the auricular lymph nodes should be removed. The lymph nodes should be carefully dissected and trimmed of fascia and fat, weighed, and stored individually in a 1.5 mL centrifuge tube at -20°C until the ELISA is performed.

2.4 BrdU-ELISA

The incorporation of BrdU into lymph node cells should be determined using a commercial cell proliferation assay kit (Roche Diagnostics GmbH, Roche Applied Science, 68298 Mannheim, Germany; Cat. No. 11 647 229 001) after they are crushed and suspended in physiological saline. The absorbance is defined as the BrdU labeling index. Follow the instructions in the assay kit.

2.5 Preparation of Reagents in the BrdU-ELISA Kit

The assay method should be according to the instruction manual in the assay kit excluding preparation of the BrdU labeling solution.

2.5.1 *Peroxidase (POD) Conjugated Anti-BrdU Antibody (Anti-BrdU-POD) Stock Solution*

Dissolve anti-BrdU-POD (bottle 3) in 1.1 mL double-distilled water for 10 minutes, and mix thoroughly. This solution can be stored at 2-8°C for several months. For long-term storage it is recommended to store the solution in aliquots at -15 to -25°C.

2.5.2 *Anti-BrdU-POD Working Solution*

Dilute anti-BrdU-POD stock solution 1:100 with antibody dilution solution (bottle 4). For one 96-well microtiter plate, dilute 100 mL anti-BrdU-POD stock solution in 10 mL antibody dilution solution (bottle 4). Prepare shortly before use.

2.5.3 *Washing Solution*

Dilute washing buffer concentrate (bottle 5) 1:10 with double distilled water. For one 96-well microtiter plate, dilute 10 mL washing buffer concentrate (bottle 5) with 90 mL double-distilled water. This solution can be stored at 2-8°C for several weeks.

2.6 Preparation of Cell Suspension of Lymph Nodes

The procedure for preparing the lymph node cell (LNC) suspension is a critical step of this assay. It is most important to crush the lymph nodes and suspend the LNC completely. Every technician should establish this skill in advance. The lymph nodes in negative control animals are very small, so careful operation is required to avoid an artificial effect on SI values.

2.6.1 *Optimizing Assay Condition*

Mean absorbance of negative (vehicle) control group should be within 0.1-0.2. Because the absorbance depends on the combination of assay apparatus and the target volume of the LNC suspension, every laboratory should decide their own optimal target volume of LNC suspension in advance so that the absorbance of the negative control is within 0.1-0.2. The volume is expected to be approximately 15 mL. The volume of the LNC suspension for all test animals should be adjusted to the optimized volume.

2.6.2 *Preparation of LNC Suspension*

A small amount (approximately 0.3 mL) of physiological saline should be added to the centrifuge tube that contains the collected lymph nodes. The lymph nodes should be crushed with a disposable plastic pestle to make the LNC suspension. The LNC suspension should be passed through a #70 nylon mesh and adjusted to the optimal target volume in a 50 mL Falcon tube.

[Note: Although a crushing apparatus other than a plastic pestle can be used to prepare the LNC, the target volume of the LNC suspension should be adjusted to the optimized volume.]

2.7 Assay Flow (BrdU-ELISA)

1. The cell suspension (100 µL) is added to the wells of a flat-bottom microplate (three wells per sample) after mixing thoroughly with a vortex. Simultaneously, three blank wells should be prepared by adding 100 µL of physiological saline.

2. After filling all sample wells and blank wells, the plate should be centrifuged at 300 x *g* for 10 minutes.
3. Remove 3/4 of the supernatant volume. Great care should be taken so that the LNC are not aspirated.
4. The assay plate should be dried completely in a hot-air oven.
5. Add 200 µL of Fix-Denat solution and allow plate to stand for 30 minutes at room temperature.
6. Remove the Fix-Denat solution completely.
7. Add 100 µL of anti-BrdU-POD antibody working solution and allow it to react for 1 hour.
8. Remove the anti-BrdU-POD antibody solution completely.
9. Add 200 µL of wash solution into each well, and wash the well by pippetting 10 times. Discard the wash solution completely.
10. The wash step (Step 9) should be repeated twice (three times total).
11. Add 100 µL of TMB substrate solution and let it stand for 15 minutes at room temperature in a dark place.
12. Measure an absorbance (ABS) at 370 nm with a reference wavelength of 492 nm. When using stop solution (1 M sulfuric acid, 25 µL/well), measure ABS at 450 nm with a reference wavelength of 690 nm.

3.0 Calculation of Results

BrdU labeling index and SI are defined as follows:

3.1 Without Stop Solution

$$\text{BrdU labeling index} = (ABS_{370}\text{-}ABS_{blank370}) - (ABS_{490}\text{-}ABS_{blank490})$$

3.2 With Stop Solution

$$\text{BrdU labeling index} = (ABS_{450}\text{-}ABS_{blank450}) - (ABS_{650}\text{-}ABS_{blank650})$$

3.3 Stimulation Index

$$SI = \frac{\text{BrdU labeling index for each test animal}}{\text{Mean BrdU labeling index for concurrent vehicle control group}}$$

4.0 Evaluation of Results

4.1 Success Criteria for Each Experiment

Employing the optimized assay condition described previously, the mean SI for the positive control group (50% hexyl cinnamic aldehyde) should be equal to or greater than 2. If not, any data derived from the experiment should not be used for evaluation.

4.2 Evaluation of the Results

The mean BrdU labeling index for each animal should be calculated based on the results of BrdU ELISA. The SI for each animal should be calculated by dividing of the mean BrdU labeling index for each treated animal by the mean BrdU labeling index of the concurrent vehicle control group. A positive response is defined as mean SI of the test group ≥2.

5.0 References

EPA. 2003. Health Effects Test Guidelines: OPPTS 870.2600 - Skin Sensitization. EPA 712–C–98–197. Washington, DC:U.S. Environmental Protection Agency. Available: http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Series/870-2600.pdf.

Kimber I, Dearman RJ, Scholes EW, Basketter DA. 1994. The local lymph node assay: developments and applications. Toxicology 93:13-31.

OECD. 2002. Guideline For Testing of Chemicals - Test Guideline 429: Skin Sensitisation: Local Lymph Node Assay. Paris:OECD. Available: http://puck.sourceoecd.org/vl=4134815/cl=32/nw=1/rpsv/cw/vhosts/oecdjournals/1607310x/v1n4/contp1-1.htm.

Takeyoshi M, Yamasaki K, Yakabe Y, Takatsuki M, Kimber I. 2001. Development of non-radio isotopic endpoint of murine local lymph node assay based on 5-bromo-2'-deoxyuridine (BrdU) incorporation. Toxicology Letters 119:203-208.

Annex II

Physicochemical Properties of Substances Tested Using the LLNA: BrdU-ELISA

(Alphanumeric Order)

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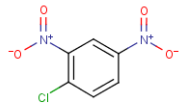
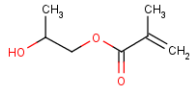
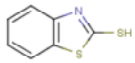
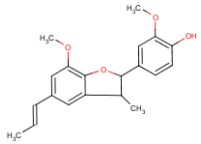
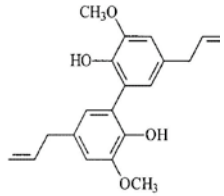
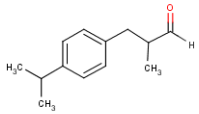
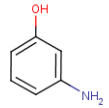
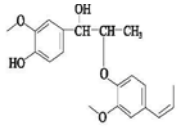
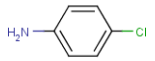
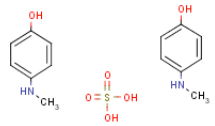
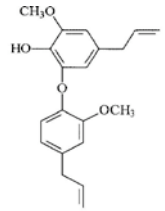
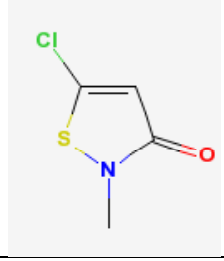
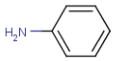
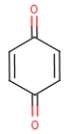
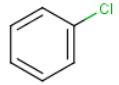
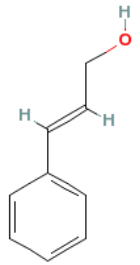
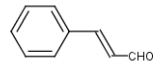
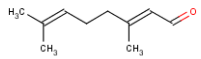
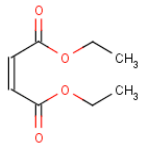
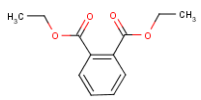
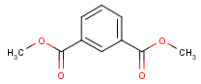
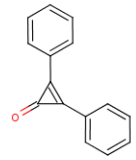
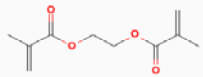
Physicochemical Properties of Substances Tested Using the LLNA: BrdU-ELISA								
Chemical Name	**Synonyms**	**CASRN**	**Mol. Weight (g/mol)**	**Log Kow[1,2]**	**Peptide Reactivity[3]**	**Physical Form**	**Chemical Class[4]**	**Structure**
1-Chloro-2-dinitrobenzene	2,4-Dinitrochloro-benzene	97-00-7	202.55	-0.057	High	Solid	Hydrocarbon, halogenated; Nitro compounds; Hydrocarbons, cyclic	
1,4-Phenylenediamine	p-PDA; p-Phenylenediamine	106-50-3	108.141	1.17	NA	Solid	Amines	
2-Hydroxypropyl methacrylate	2-HPMA	923-26-2	144.168	1.03	Low	Solid	Carboxylic acids	
2-Mercaptobenzothiazole	Captax	149-30-4	167.253	1.8	High	Solid	Heterocyclic compounds	
2-Methoxy-4-(7-methoxy-3-methyl-5-propenyl-2,3-dihydro-benzofuran-2yl)-phenol	Dehydrodiisoeugenol	2680-81-1	326.39	NA	NA	NA	Carboxylic acids	

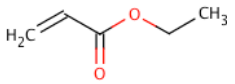
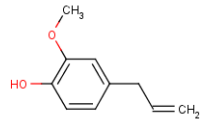
Physicochemical Properties of Substances Tested Using the LLNA: BrdU-ELISA								
Chemical Name	**Synonyms**	**CASRN**	**Mol. Weight (g/mol)**	**Log Kow[1,2]**	**Peptide Reactivity[3]**	**Physical Form**	**Chemical Class[4]**	**Structure**
2,2'-Dihydroxyl-3,3'-dimethoxy-5,5'-diallyl-biphenyl	DHEA	NA	326.39	NA	NA	NA	Carboxylic acids	
3-(4-Isopropylphenyl) isobutyraldehyde	Cyclamen aldehyde	103-95-7	190.28	3.28	Low	Liquid	Carboxylic acids	
3-Aminophenol	m-Aminophenol; 3-Hydroxyaniline	591-27-5	109.126	1.17	NA	Solid	Amines; Phenols	
4-[1-Hydroxy-2-(2-methoxy-4-propenyl-phenyoxy)-propyl]-2-methoxy-phenol	β-O-4-Dilignol	NA	327.39	NA	NA	NA	Carboxylic acids	

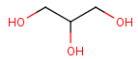
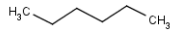
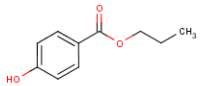
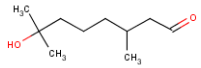
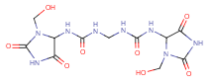
Physicochemical Properties of Substances Tested Using the LLNA: BrdU-ELISA								
Chemical Name	**Synonyms**	**CASRN**	**Mol. Weight (g/mol)**	**Log Kow[1,2]**	**Peptide Reactivity[3]**	**Physical Form**	**Chemical Class[4]**	**Structure**
4-Chloroaniline	4-Chlorobenzeneamine; Aniline, p-chloro-; Benzenamine, 4-chloro-	106-47-8	127.57	1.8	NA	Liquid	Amines	
4-Methylaminophenol sulfate	Metol p-Methylaminophenol sulfate	55-55-0	344.386	-0.13	NA	Solid	Amines; Phenols	
4,5'-Diallyl-2'-hydroxy-2,3'-dimethoxyphenyl ether	DHEB	NA	326.39	NA	NA	NA	Carboxylic acids	
5-Chloro-2-methyl-4-isothiazolin-3-one solution	CMI MCI Kathon CG	26172-55-4	149.599	0.92	High	Liquid	Sulfur compounds Heterocyclic compounds	

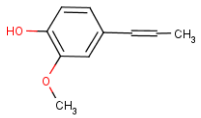
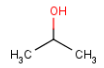
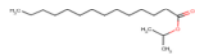
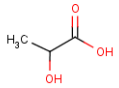
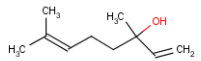
Physicochemical Properties of Substances Tested Using the LLNA: BrdU-ELISA								
Chemical Name	**Synonyms**	**CASRN**	**Mol. Weight (g/mol)**	**Log Kow[1,2]**	**Peptide Reactivity[3]**	**Physical Form**	**Chemical Class[4]**	**Structure**
Aniline	Benzenamine	62-53-3	93.1265	1.56	NA	Liquid	Amines	
Benzoquinone	p-Quinone; 1,4-Cyclohexadienedione	106-51-4	108.095	1.17	High	Solid	Quinones	
Chlorobenzene	Benzene chloride Monochlorbenzene Phenyl chloride	108-90-7	112.557	2.19	NA	Liquid	Hydrocarbons, cyclic; Hydrocarbons, halogenated	
Cinnamic alcohol	Cinnamyl alcohol 3-Phenyl-2-propen-1-ol	104-54-1	134.18	2.29	NA	Solid	Alcohols	

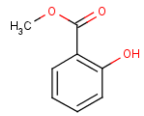
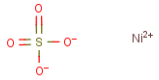
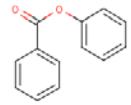
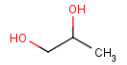
Physicochemical Properties of Substances Tested Using the LLNA: BrdU-ELISA								
Chemical Name	**Synonyms**	**CASRN**	**Mol. Weight (g/mol)**	**Log Kow[1,2]**	**Peptide Reactivity[3]**	**Physical Form**	**Chemical Class[4]**	**Structure**
Cinnamic aldehyde	Cinnamal; cinnamaldehyde; 3-phenyl-2-propenal	104-55-2	132.16	2.29	High	Liquid	Aldehydes	
Citral	3,7-Dimethyl-2,6-octadienal; Geranial-neral mixture	5392-40-5	152.233	2.54/ 3.45	NA	Liquid	Hydrocarbons, other	
Cobalt chloride	Cobaltous chloride	7646-79-9	129.84	NA	NA	Solid	Inorganic chemicals	
Diethyl maleate	Ethyl maleate	141-05-9	172.18	0.89	NA	Liquid	Carboxylic acids	

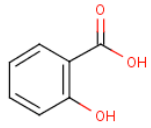
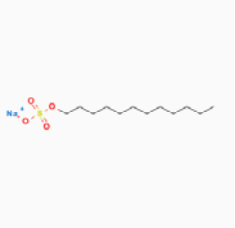
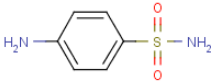
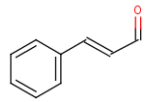
Physicochemical Properties of Substances Tested Using the LLNA: BrdU-ELISA								
Chemical Name	**Synonyms**	**CASRN**	**Mol. Weight (g/mol)**	**Log Kow[1,2]**	**Peptide Reactivity[3]**	**Physical Form**	**Chemical Class[4]**	**Structure**
Diethyl phthalate	1,2-Benzenedicarboxylic acid, diethyl ester; Diethyl 1,2-benzenedicarboxylate	84-66-2	222.24	1.87	Minimal	Liquid	Carboxylic acids	
Dimethyl isophthalate	Dimethyl m-phthalate	1459-93-4	194.19	1.66	NA	Solid	Carboxylic acids	
Diphenylcyclopropenone	2,3-Diphenylcyclo-propenone	886-38-4	206.24	3.25	High	Solid	Hydrocarbons, cyclic	
Ethlene glycol dimethacrylate	EGDMA	97-90-5	198.216	1.38	High	Liquid	Carboxylic acids	

Physicochemical Properties of Substances Tested Using the LLNA: BrdU-ELISA								
Chemical Name	**Synonyms**	**CASRN**	**Mol. Weight (g/mol)**	**Log Kow[1,2]**	**Peptide Reactivity[3]**	**Physical Form**	**Chemical Class[4]**	**Structure**
Ethyl acrylate	2-Propenoic acid, ethyl ester Acrylic acid, ethyl ester	140-88-5	100.10	1.22	NA	Liquid	Acrylates	
Eugenol	2-Methoxy-4-(2-propenyl)phenol; 4-Allyl-2-methoxyphenol; 4-Allylguaiacol	97-53-0	164.201	2.15/ 2.73	NA	Liquid	Carboxylic acids	
Formaldehyde	Formalin	50-00-0	30.03	0.33	Moderate	Liquid	Aldehydes	
Glutaraldehyde	Glutaral	111-30-8	100.12	0.92	High	Liquid	Aldehydes	

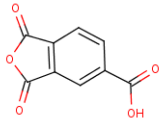
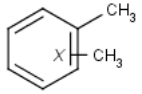
Physicochemical Properties of Substances Tested Using the LLNA: BrdU-ELISA								
Chemical Name	**Synonyms**	**CASRN**	**Mol. Weight (g/mol)**	**Log Kow[1,2]**	**Peptide Reactivity[3]**	**Physical Form**	**Chemical Class[4]**	**Structure**
Glycerol	Glycerin	56-81-5	92.09	0.05	Minimal	Liquid	Alcohols; Carbohydrates	
Hexane	Hexyl hydride; n-Hexane	110-54-3	86.1754	1.94	Minimal	Liquid	Hydrocarbons, acyclic	
Hexyl cinnamic aldehyde	HCA; alpha-Hexylcinnamaldehyde; 2-(Phenylmethylene) octanal	101-86-0	216.319	3.77/ 4.82	Minimal	Liquid	Aldehydes	
Hydroxycitronellal	7-Hydroxy-3,7-dimethyloctanol	107-75-5	172.26	2.15	Low	Liquid	Hydrocarbons, other	
Imidazolidinyl urea	Germall 115 Imidurea	39236-46-9	388.294	-3	NA	Solid	Urea	

Physicochemical Properties of Substances Tested Using the LLNA: BrdU-ELISA								
Chemical Name	**Synonyms**	**CASRN**	**Mol. Weight (g/mol)**	**Log Kow[1,2]**	**Peptide Reactivity[3]**	**Physical Form**	**Chemical Class[4]**	**Structure**
Isoeugenol	2-Methoxy-4-propenylphenol; 4-Propenylguaiacol	97-54-1	164.201	2.15	NA	Liquid	Carboxylic acids	
Isopropanol	Isopropyl alcohol, 2-Propanol	67-63-0	60.095	0.82	Minimal	Liquid	Alcohols	
Isopropyl myristate	1-Methylethyl tetradecanoate	110-27-0	270.46	3.88	Minimal	Liquid	Lipids	
Lactic acid	2-Hydroxypropanoic acid	50-21-5	90.08	0.05	Minimal	Liquid	Carboxylic acids	
Linalool	3,7-dimethylocta-,6-dien-3-ol	78-70-6	154.25	2.54	NA	Liquid	Hydrocarbons	

Physicochemical Properties of Substances Tested Using the LLNA: BrdU-ELISA								
Chemical Name	**Synonyms**	**CASRN**	**Mol. Weight (g/mol)**	**Log Kow[1,2]**	**Peptide Reactivity[3]**	**Physical Form**	**Chemical Class[4]**	**Structure**
Methyl salicylate	Oil of wintergreen; 2-Hydroxybenzoic acid methyl ester	119-36-8	152.15	1.28	Minimal	Liquid	Phenols; Carboxylic acids	
Nickel chloride	Nickelous chloride	7718-54-9	129.60	NA	NA	Solid	Inorganic chemicals, metals; Inorganic chemicals, elements	
Nickel sulfate	Nickelous sulfate	7786-81-4	154.76	NA	NA	Solid	Inorganic chemicals, metals; Inorganic chemicals, elements	
Phenyl benzoate	Diphenylcarboxylate	93-99-2	198.22	2.89	NA	Solid	Carboxylic acids	
Propylene glycol	1,2-Dihydroxypropane; 1,2-Propanediol	57-55-6	76.0944	0.43	Minimal	Liquid	Alcohols	

Physicochemical Properties of Substances Tested Using the LLNA: BrdU-ELISA								
Chemical Name	**Synonyms**	**CASRN**	**Mol. Weight (g/mol)**	**Log Kow[1,2]**	**Peptide Reactivity[3]**	**Physical Form**	**Chemical Class[4]**	**Structure**
Salicylic acid	2-Hydroxybenzoic acid	69-72-7	138.121	1.03	NA	Solid	Phenols; Carboxylic acids	
Sodium lauryl sulfate	Irium Sodium dodecyl sulfate SDS SLS	151-21-4	288.38	1.87	NA	Solid	Alcohols; Lipids; Sulfur compounds	
Sulfanilamide	4-Aminobenzenesulfon-amide 4-Aminophenylsulfon-amide 4-Sulfamoylaniline	63-74-1	172.21	0.4	NA	Solid	Amides; Sulfur compounds; Amines	
trans-Cinnamaldehyde	3-Phenylpropenal	14371-10-9	132.6	1.82	NA	Liquid	Aldehydes	

Physicochemical Properties of Substances Tested Using the LLNA: BrdU-ELISA

Chemical Name	Synonyms	CASRN	Mol. Weight (g/mol)	Log Kow[1,2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Trimellitic anhydride	1,2,4-Benzenetricarboxylic acid, cyclic 1,2-anhydride (8CI); 1,3-Dihydro-1,3-dioxo-5-isobenzofurancarboxylic acid; 5-Isobenzofurancarboxylic acid; 1,3-dihydro-1,3-dioxo-Benzene-1,2,4-tricarboxylic acid 1,2-anhydride	552-30-7	192.13	1.95	Low	Solid	Anhydrides; Carboxylic acids	
Xylene	Dimethylbenzene Methyl toluene	1330-20-7	106.17	3.09	NA	Liquid	Hydrocarbons, aromatic	

Abbreviations: CASRN=Chemical Abstracts Registry Number; g/mol=grams per mole; NA = not available.

[1] Physicochemical properties were obtained from PubChem (http://pubchem.ncbi.nlm.nih.gov/), ChemID (http://chem.sis.nlm.nih.gov/chemidplus/chemidheavy.jsp), or the Sigma Chemical Catalog.

[2] K_{ow} represents the octanol-water partition coefficient (expressed on log scale). When two numbers are shown, the first number is the value calculated by the method of Moriguchi et al. (1994 Chem Pharm Bull 42:976-978) and provided in Gerberick et al. (2005 Dermatitis 16:157-202). The second number was calculated by the method of Meylan and Howard (1995 J Pharm Science 84:83-92) and obtained from the website: http://www.srcinc.com/what-we-do/databaseforms.aspx?id=385 .

[3] Peptide reactivity data obtained from: Gerberick et al. 2007a.

[4] Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs developed by the National Library of Medicine found at http://www.nlm.nih.gov/mesh/meshhome.html.

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Annex III

Comparative LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Skin Sensitization Data

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Annex III-1

LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA (Alphanumeric Order)

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LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
1,4-Phenylene-diamine	AOO	2	11.70	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+ (26.4, 1%)	+	+	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.5% (GP)	Basketter et al. 2007
1,4-Phenylene-diamine	AOO	10	14.70	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+ (26.4, 1%)	+	+	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.5% (GP)	Basketter et al. 2007
2-Hydroxypropyl-methacrylate	AOO	50	1.13	Takeyoshi et al. 2007b	- (1.3, 50%)	-	+ (case study, 0.1%)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	Bjorkner 1984	No at ≤ 10% (GP)	Scholes et al. 1992
2-Mercaptobenzo-thiazole	DMF	50	1.62[7]	Takeyoshi et al. 2007b	+ (8.6, 10%)	+	+ (5/24, 10%)	ICCVAM 1999 (Ryan et al. 2000)	ICCVAM 1999	ICCVAM 1999 (Kligman 1966)	No at ≤ 10% (GP)	Basketter et al. 2007
2-Mercaptobenzo-thiazole	DMSO[8]	25	2.23[9,10]	Takeyoshi unpublish-ed 2009	+ (8.6, 10%)	+	+ (5/24, 10%)	ICCVAM 1999 (Ryan et al. 2000)	ICCVAM 1999	ICCVAM 1999 (Kligman 1966)	No at ≤ 10% (GP)	Basketter et al. 2007

LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
2-Mercaptobenzo-thiazole	DMSO[8]	25	2.51[10,11]	Takeyoshi unpublish-ed 2009	+ (8.6, 10%)	+	+ (5/24, 10%)	ICCVAM 1999 (Ryan et al. 2000)	ICCVAM 1999	ICCVAM 1999 (Kligman 1966)	No at ≤ 10% (GP)	Basketter et al. 2007
2-Methoxy-4-(7-methoxy-3-methyl-5-propenyl-2,3-dihydro-benzofuran-2yl)-phenol (Synonym: Dehydrodiisoeugenol)	AOO	30	5.37	Takeyoshi et al. 2007a	NA	+	NA	NA	Takeyoshi et al. 2007a	NA	No at ≤ 5% (GP)	Takeyoshi et al. 2007a
2,2'-Dihydroxyl-3,3'-dimethoxy-5,5'-diallyl-biphenyl (DHEA)	AOO	30	2.30	Takeyoshi et al. 2004a	NA	-	NA	NA	Takeyoshi et al. 2004a	NA	No at ≤ 5% (GP)	Takeyoshi et al. 2004a
2,4-Dinitrochloro-benzene	AOO	1	4.30	Kojima et al. 2008 1	+ (43.9, 0.25%)	+	+	ICCVAM 1999 (Kimber et al. 1995)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.1% (GP)	Basketter et al. 2007

LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
2,4-Dinitrochlorobenzene	AOO	1	8.37	Kojima et al. 2008 2	+ (43.9, 0.25%)	+	+	ICCVAM 1999 (Kimber et al. 1995)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.1% (GP)	Basketter et al. 2007
2,4-Dinitrochlorobenzene	AOO	1	6.26[12]	Kojima et al. 2008 3	+ (43.9, 0.25%)	+	+	ICCVAM 1999 (Kimber et al. 1995)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.1% (GP)	Basketter et al. 2007
2,4-Dinitrochlorobenzene	AOO	1	5.50	Kojima et al. 2008 4	+ (43.9, 0.25%)	+	+	ICCVAM 1999 (Kimber et al. 1995)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.1% (GP)	Basketter et al. 2007
2,4-Dinitrochlorobenzene	AOO	1	18.80[12]	Kojima et al. 2008 5	+ (43.9, 0.25%)	+	+	ICCVAM 1999 (Kimber et al. 1995)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.1% (GP)	Basketter et al. 2007
2,4-Dinitrochlorobenzene	AOO	1	4.83	Kojima et al. 2008 6	+ (43.9, 0.25%)	+	+	ICCVAM 1999 (Kimber et al. 1995)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.1% (GP)	Basketter et al. 2007
2,4-Dinitrochlorobenzene	AOO	1	12.98	Kojima et al. 2008 7	+ (43.9, 0.25%)	+	+	ICCVAM 1999 (Kimber et al. 1995)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.1% (GP)	Basketter et al. 2007

LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
2,4-Dinitrochlorobenzene	AOO	2	17.90	Takeyoshi et al. 2005	+ (43.9, 0.25%)	+	+	ICCVAM 1999 (Kimber et al. 1995)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.1% (GP)	Basketter et al. 2007
2,4-Dinitrochlorobenzene	AOO	2	6.84	Takeyoshi et al. 2006; Takeyoshi et al. 2007b	+ (43.9, 0.25%)	+	+	ICCVAM 1999 (Kimber et al. 1995)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.1% (GP)	Basketter et al. 2007
4-[1-Hydroxy-2-(2-methoxy-4-propenyl-phenyoxy)-propyl]-2-methoxy-phenol (Synonym: β-O-4-Dilignol)	AOO	30	1.19[13]	Takeyoshi et al. 2007a	NA	-	NA	NA	Takeyoshi et al. et al. 2007a	NA	No at ≤ 5% (GP)	Takeyoshi et al. et al. 2007a
4-Chloroaniline	AOO	25	2.53	Takeyoshi et al. 2007b	+ (3.3)	+	+	Basketter et al. 2000	ICCVAM 1999	Basketter et al. 1999	No at 2.5% (GP)	Basketter and Scholes 1992

LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
4-Methylamino-phenol sulfate	DMF	10	3.98	Takeyoshi unpublish-ed 2009	+ (6.7, 2.5%)	+	+ (HPTA)	Basketter and Scholes, 1992	Basketter and Scholes, 1992	Basketter et al. 1999b	No at ≤ 5% (GP)	Basketter et al. 2007
4,5'-Diallyl-2'-hydroxy-2,3'-dimethoxyphenyl ether (DHEB)	AOO	20	7.30	Takeyoshi et al. 2004a	NA	+	NA	NA	Takeyoshi et al. 2004a	NA	No at ≤ 5% (GP)	Takeyoshi et al. 2004a
5-Chloro-2-methyl-4-isothiazolin-3-one solution	DMF	0.5	4.83	Takeyoshi unpublish-ed 2009	+ (0.1, 27.7)	+	+	ICCVAM 1999	Gerberick et al. 2005	ICCVAM 1999	Nonirritant at 0.1% (GP)	Basketter et al. 2007g
Aniline	AOO	100	2.07	Takeyoshi unpublish-ed 2009	+ (3.6, 100%)[14]	+	+ (7/25 at 20%)	ICCVAM 1999 (Basketter et al. 1991)	ICCVAM 1999	ICCVAM 1999 (Kligman 1966)	No at ≤ 100% (GP); Irritant at 20% in humans	Basketter et al. 2007; Kligman 1966

LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA												
Chemical Name	**Veh.[1]**	**Highest Conc. Tested (%)**	**Highest SI**	**LLNA: BrdU Ref.[2]**	**Trad. LLNA Result[3]**	**GP Result**	**Human Result[4]**	**Ref. Trad. LLNA[5]**	**Ref. GP**	**Ref. Human[6]**	**Skin Irritant?**	**Reference Skin Irritation**
Chlorobenzene	AOO	100	4.43[10]	Takeyoshi unpublished 2009	- (1.7, 10%)	-	NA	ICCVAM 1999	ICCVAM 1999	NA	No data. Low irritancy potential assumed based on clinical literature.	Basketter et al. 1998
Cinnamic alcohol	AOO	50	2.74	Takeyoshi unpublished 2009	+ (5.7, 100%)	+	+	Gerberick et al. 2005	Robinson et al. 1990	Jordan and King 1977	Nonirrit. at 1% (GP)	Robinson et al. 1990
Cinnamic aldehyde	AOO	50	3.97	Takeyoshi et al. 2007b	+ (18.4, 25%)[13]	+	+	ICCVAM 1999 (Basketter et al. 1992)	ICCVAM 1999	ICCVAM 1999	Mild irritant at 100% (rabbits)	ECETOC 1995
Citral	AOO	50	16.35	Takeyoshi et al. 2007b; Takeyoshi et al. 2005	+ (20.5, 20%)	+	+	ICCVAM 1999 (Basketter et al. 1991)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.5% (GP)	Basketter et al. 2007
Cobalt chloride	DMSO	5	3.68	Takeyoshi unpublished 2009	+ (7.5, 5)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes 1992

LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
Cyclamen aldehyde	AOO	100	1.97	Takeyoshi et al. 2007b	+ (5.2, 50%)	NA	- (0/64, 4%)	Gerberick et al. 2005	NA	Basketter et al. 2005	Yes at 100% (rabbits)	ECETOC 1995
Cyclamen aldehyde	AOO	100	5.71	Takeyoshi unpublish-ed 2009	+ (5.2, 50%)	NA	- (0/64, 4%)	Gerberick et al. 2005	NA	Basketter et al. 2005	Yes at 100% (rabbits)	ECETOC 1995
Diethyl maleate	AOO	25	6.27	Takeyoshi unpublish-ed 2009	+ (22.6, 50%)	NA	+	Gerberick et al. 2005	NA	Marzulli and Maibach 1980	Nonirrit. at 100% (GP)	Basketter et al. 2007b
Diethyl phthalate (Phthallic acid diethylester)	AOO	50	0.88	Takeyoshi et al. 2007b	- (1.5, 100%)	-	+ (HPTA)	ICCVAM 1999 (Gerberick et al. 2005)	Klecak et al. 1977	ICCVAM 1999	No at ≤ 100% (rabbits)	ECETOC 1995
Dimethyliso-phthalate	AOO	50	1.26	Takeyoshi et al. 2007b	- (1, 25%)	-	-	ICCVAM 1999 (Basketter and Scholes 1992)	ICCVAM 1999	Basketter et al. 1999	NA	NA

LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
Diphenylcyclo-propenone	AOO	2	19.10	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+ (NA)	NA	+	ICCVAM 1999	NA	ICCVAM 1999	NA	NA
Diphenylcyclo-propenone	AOO	10	9.34	Takeyoshi et al. 2005	+ (NA)	NA	+	ICCVAM 1999	NA	ICCVAM 1999	NA	NA
Diphenylcyclo-propenone	AOO	10	11.62	Takeyoshi et al. 2007b	+ (NA)	NA	+	ICCVAM 1999	NA	ICCVAM 1999	NA	NA
Ethyl acrylate	AOO	100	4.95	Takeyoshi unpublish-ed 2009	+ (4, 50%)	-	+	Gerberick et al. 2005	Van der Walle et al. 1982	Marzulli and Maibach 1974	Nonirrit. at 3% (GP)	Van der Walle et al. 1982
Ethylene glycol dimethacrylate	MEK	100	3.11	Takeyoshi unpublish-ed 2009	+ (7, 50%)	-	+	ICCVAM 1999	ICCVAM 1999; Gerberick 1992	ICCVAM 1999; Basketter et al. 1999b	Nonirrit. at 1% (GP)	Wahlberg and Boman 1985
Eugenol	AOO	10	3.18	Takeyoshi et al. 2005	+ (17, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	ICCVAM 1999	No at ≤ 25% (GP)	Basketter et al. 2007

LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
Eugenol	AOO	30	3.30	Takeyoshi et al. 2004a	+ (17, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	ICCVAM 1999	No at ≤ 25% (GP)	Basketter et al. 2007
Eugenol	AOO	30	3.83	Takeyoshi et al. 2007a	+ (17, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	ICCVAM 1999	No at ≤ 25% (GP)	Basketter et al. 2007
Eugenol	AOO	50	12.30	Takeyoshi et al. 2005	+ (17, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	ICCVAM 1999	No at ≤ 25% (GP)	Basketter et al. 2007
Eugenol	AOO	50	3.10	Takeyoshi et al. 2006	+ (17, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	ICCVAM 1999	No at ≤ 25% (GP)	Basketter et al. 2007
Eugenol	AOO	50	7.09	Kojima et al. 2008 7	+ (17, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	ICCVAM 1999	No at ≤ 25% (GP)	Basketter et al. 2007
Eugenol	AOO	50	3.17	Kojima et al. 2008 2	+ (17, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	ICCVAM 1999	No at ≤ 25% (GP)	Basketter et al. 2007

LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
Eugenol	AOO	50	3.18	Kojima et al. 2008 6	+ (17, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	ICCVAM 1999	No at ≤ 25% (GP)	Basketter et al. 2007
Eugenol	AOO	50	17.70	Takeyoshi et al. 2007b	+ (17, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	ICCVAM 1999	No at ≤ 25% (GP)	Basketter et al. 2007
Formaldehyde	ACE	10	16.59	Kojima et al. 2008 5	+ (11.9, 25%)	+	+	ICCVAM 1999 (Kimber et al. 1991)	ICCVAM 1999	ICCVAM 1999	No at ≤ 2% (GP)	Basketter et al. 2007
Formaldehyde	ACE	10	4.40	Kojima et al. 2008 1	+ (11.9, 25%)	+	+	ICCVAM 1999 (Kimber et al. 1991)	ICCVAM 1999	ICCVAM 1999	No at ≤ 2% (GP)	Basketter et al. 2007
Formaldehyde	ACE	10	1.97	Kojima et al. 2008 6	+ (11.9, 25%)	+	+	ICCVAM 1999 (Kimber et al. 1991)	ICCVAM 1999	ICCVAM 1999	No at ≤ 2% (GP)	Basketter et al. 2007
Glutaraldehyde	ACE	1	28.64	Kojima et al. 2008 5	+ (18, 2.5%)	+	+	Hilton et. al 1998 (Gerberick et al. 2005)	Gad et al. 1986	Schneider and Akkan 2004	NA	NA

LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
Glutaraldehyde	ACE	1	3.72	Kojima et al. 2008 1	+ (18, 2.5%)	+	+	Hilton et. al 1998 (Gerberick et al. 2005)	Gad et al. 1986	Schneider and Akkan 2004	NA	NA
Glutaraldehyde	ACE	1	2.25	Kojima et al. 2008 6	+ (18, 2.5%)	+	+	Hilton et. al 1998 (Gerberick et al. 2005)	Gad et al. 1986	Schneider and Akkan 2004	NA	NA
Glutaraldehyde	AOO[15]	2	14.60	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+ (18, 2.5%)	+	+	Hilton et. al 1998 (Gerberick et al. 2005)	Gad et al. 1986	Schneider and Akkan 2004	NA	NA
Glutaraldehyde	AOO[15]	10	15.50	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+ (18, 2.5%)	+	+	Hilton et. al 1998 (Gerberick et al. 2005)	Gad et al. 1986	Schneider and Akkan 2004	NA	NA
Glycerol	None[8]	50	1.29	Takeyoshi et al. 2007b	- (1.1, 100%)[16]	-	-	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	ICCVAM 1999	NA	NA

LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
Hexane	AOO	100	1.76	Takeyoshi unpublish-ed 2009	- (2.2, 100%)	NA	- (0/25, 100%)	ICCVAM 1999 (Gerberick et al. 2005)	NA	ICCVAM 1999 (Kligman 1966)	Yes at 100% (humans)	Kligman 1966
Hexane	AOO	50	1.89	Takeyoshi et al. 2005	- (2.2, 100%)	NA	- (0/25, 100%)	ICCVAM 1999 (Gerberick et al. 2005)	NA	ICCVAM 1999 (Kligman 1966)	Yes at 100% (humans)	Kligman 1966
Hexyl cinnamic aldehyde	AOO	25	2.44	Takeyoshi et al. 2003	+ (20, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10% (GP)	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	3.64	Takeyoshi et al. 2003	+ (20, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10% (GP)	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	5.90	Takeyoshi et al. 2005	+ (20, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10% (GP)	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	3.64	Takeyoshi et al. 2006	+ (20, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10% (GP)	Basketter et al. 2007

LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
Hexyl cinnamic aldehyde	AOO	50	2.72	Takeyoshi et al. 2006	+ (20, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10% (GP)	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	3.02	Takeyoshi et al. 2007b	+ (20, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10% (GP)	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	3.40	Kojima et al. 2008 1	+ (20, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10% (GP)	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	2.87	Kojima et al. 2008 3	+ (20, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10% (GP)	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	3.34	Kojima et al. 2008 4	+ (20, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10% (GP)	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	13.50	Kojima et al. 2008 5	+ (20, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10% (GP)	Basketter et al. 2007

LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
Hexyl cinnamic aldehyde	AOO	50	3.27	Kojima et al. 2008 6	+ (20, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10% (GP)	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	3.84	Kojima et al. 2008 7	+ (20, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10% (GP)	Basketter et al. 2007
Hydroxy-citronellal	AOO	100	1.34	Takeyoshi et al. 2007b	+ (8.5, 100%)	+	+ (14/73, 20%)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	ICCVAM 1999 (Marzulli and Maibach 1980)	No at ≤ 50% (GP)	Basketter et al. 2007
Hydroxy-citronellal	AOO	100	4.78	Takeyoshi unpublish-ed 2009	+ (8.5, 100%)	+	+ (14/73, 20%)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	ICCVAM 1999 (Marzulli and Maibach 1980)	No at ≤ 50% (GP)	Basketter et al. 2007
Imidazolidinyl urea	DMF	50	1.61	Takeyoshi unpublish-ed 2009	+ (5.5, 50%)	+	+	Gerberick et al. 2005	ICCVAM 1999	ICCVAM 1999	No at ≤ 75% (GP)	Basketter and Scholes 1992

LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
Imidazolidinyl urea	DMSO	75	2.27[10]	Takeyoshi unpublish-ed 2009	+ (5.5, 50%)	+	+	Gerberick et al. 2005	ICCVAM 1999	ICCVAM 1999	No at ≤ 75% (GP)	Basketter and Scholes 1992
Isoeugenol	AOO	10	8.40	Takeyoshi et al. 2005	+ (31, 5%)	+	+	ICCVAM 1999 (Basketter and Cadby 2004)	ICCVAM 1999	ICCVAM 1999	No at ≤ 5% (GP)	Basketter et al. 2007
Isoeugenol	AOO	10	2.40	Takeyoshi et al. 2006; Takeyoshi et al. 2007b	+ (31, 5%)	+	+	ICCVAM 1999 (Basketter and Cadby 2004)	ICCVAM 1999	ICCVAM 1999	No at ≤ 5% (GP)	Basketter et al. 2007
Isoeugenol	AOO	30	6.73	Takeyoshi et al. 2007a	+ (31, 5%)	+	+	ICCVAM 1999 (Basketter and Cadby 2004)	ICCVAM 1999	ICCVAM 1999	No at ≤ 5% (GP)	Basketter et al. 2007
Isopropanol	AOO	50	2.22[13]	Kojima et al. 2008 1	- (1.7, 50%)[13]	-	+ (case study, 0.001%)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	Kwon et al. 2003	No at ≤ 100% (rabbits)	ECETOC 1995

LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA												
Chemical Name	**Veh.[1]**	**Highest Conc. Tested (%)**	**Highest SI**	**LLNA: BrdU Ref.[2]**	**Trad. LLNA Result[3]**	**GP Result**	**Human Result[4]**	**Ref. Trad. LLNA[5]**	**Ref. GP**	**Ref. Human[6]**	**Skin Irritant?**	**Reference Skin Irritation**
Isopropanol	AOO	50	0.98[13]	Kojima et al. 2008 3	- (1.7, 50%)[13]	-	+ (case study, 0.001%)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	Kwon et al. 2003	No at ≤ 100% (rabbits)	ECETOC 1995
Isopropanol	AOO	50	1.57	Kojima et al. 2008 4	- (1.7, 50%)[13]	-	+ (case study, 0.001%)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	Kwon et al. 2003	No at ≤ 100% (rabbits)	ECETOC 1995
Isopropanol	AOO	50	0.94[13]	Kojima et al. 2008 5	- (1.7, 50%)[13]	-	+ (case study, 0.001%)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	Kwon et al. 2003	No at ≤ 100% (rabbits)	ECETOC 1995
Isopropanol	AOO	50	2.04[13]	Kojima et al. 2008 6	- (1.7, 50%)[13]	-	+ (case study, 0.001%)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	Kwon et al. 2003	No at ≤ 100% (rabbits)	ECETOC 1995
Isopropanol	AOO	50	1.01	Kojima et al. 2008 7	- (1.7, 50%)[13]	-	+ (case study, 0.001%)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	Kwon et al. 2003	No at ≤ 100% (rabbits)	ECETOC 1995

LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA												
Chemical Name	**Veh.[1]**	**Highest Conc. Tested (%)**	**Highest SI**	**LLNA: BrdU Ref.[2]**	**Trad. LLNA Result[3]**	**GP Result**	**Human Result[4]**	**Ref. Trad. LLNA[5]**	**Ref. GP**	**Ref. Human[6]**	**Skin Irritant?**	**Reference Skin Irritation**
Isopropanol	AOO	100	0.92[14]	Takeyoshi et al. 2007b	- (1.7, 50%)[13]	-	+ (case study, 0.001%)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	Kwon et al. 2003	No at ≤ 100% (rabbits)	ECETOC 1995
Isopropyl myristate	AOO	50	4.20	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+ (3.4, 100%)	NA	- (0/25)	Ryan et al. 2000 (Gerberick et al. 2005)	NA	Opdyke 1976	No at ≤ 100% (rabbits)	ECETOC 1995
Lactic acid	DMSO	50	2.53	Kojima et al. 2008 7	- (2.2, 25%)	-	- (no data)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	Basketter et al. 1999	Slightly irritating at 10% (rabbits)	Cosmetic Ingredient Review Panel 1998
Lactic acid	DMSO	50	1.89	Kojima et al. 2008 4	- (2.2, 25%)-	-	- (no data)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	Basketter et al. 1999	Slightly irritating at 10% (rabbits)	Cosmetic Ingredient Review Panel 1998
Lactic acid	DMSO	50	1.80	Kojima et al. 2008 3	- (2.2, 25%)-	-	- (no data)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	Basketter et al. 1999	Slightly irritating at 10% (rabbits)	Cosmetic Ingredient Review Panel 1998

LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
Linalool	AOO	100	1.45[14]	Takeyoshi unpublish-ed 2009	+ (8.3, 100%)	NA	-	Gerberick et al. 2005	NA	Basketter et al. 2001	Mild irritant at 100% (rabbits)	ECETOC 1995
Linalool	AOO	100	4.65	Takeyoshi unpublish-ed 2009	+ (8.3, 100%)	NA	-	Gerberick et al. 2005	NA	Basketter et al. 2001	Mild irritant at 100% (rabbits)	ECETOC 1995
m-Aminophenol	AOO	25	3.06	Takeyoshi et al. 2007b	+ (5.7, 10%)	NA	+	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999; GP was + nonstd	ICCVAM 1999	No at ≤ 5% (GP)	Basketter et al. 2007
Methyl salicylate	AOO	50	1.43	Kojima et al. 2008 1	- (2.9, 20%)	-	-	ICCVAM 1999 (Kimber et al. 1995)	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (mice)	Gerberick et al. 2002
Methyl salicylate	AOO	50	1.44	Kojima et al. 2008 2	- (2.9, 20%)	-	-	ICCVAM 1999 (Kimber et al. 1995)	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (mice)	Gerberick et al. 2002
Methyl salicylate	AOO	50	1.40	Kojima et al. 2008 3	- (2.9, 20%)	-	-	ICCVAM 1999 (Kimber et al. 1995)	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (mice)	Gerberick et al. 2002

LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
Nickel chloride	DMSO	5	2.39[9,10]	Takeyoshi unpublish-ed 2009	- (2.4, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	Vanden-berg and Epstein 1963	No at ≤ 0.15% (GP)	Basketter and Scholes 1992
Nickel sulfate	DMSO	10	2.58	Kojima et al. 2008 3	+ (3.1, 5%)	+	+	Ryan et al 2002	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.15% (GP); Yes at 10% (humans)	Basketter and Scholes 1992; Kligman 1966
Nickel sulfate	DMSO	10	4.53	Kojima et al. 2008 4	+ (3.1, 5%)	+	+	Ryan et al 2002	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.15% (GP); Yes at 10% (humans)	Basketter and Scholes 1992; Kligman 1966
Nickel sulfate	DMSO	10	2.66	Kojima et al. 2008 7	+ (3.1, 5%)	+	+	Ryan et al 2002	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.15% (GP); Yes at 10% (humans)	Basketter and Scholes 1992; Kligman 1966

LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
p-Benzoquinone	AOO	1	6.90	Takeyoshi et al. 2004b; Takeyoshi et al. 2007b	+ (52.3, 2.5%)	+	+	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 2.5% (GP)	Basketter et al. 2007
Phenyl benzoate	AOO	66.7	3.37	Takeyoshi unpublish-ed 2009	+ (11.1, 25%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al 2005a	NA	NA
Propylene glycol	AOO[17]	10	1.20	Takeyoshi et al. 2005	- (1.6, 100%)	-	+ (HPTA)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	ICCVAM 1999	No at ≤ 25% (humans)	Kligman 1966
Propylene glycol	AOO[17]	50	1.57	Takeyoshi et al. 2005	- (1.6, 100%)	-	+ (HPTA)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	ICCVAM 1999	No at ≤ 25% (humans)	Kligman 1966
Propylene glycol	AOO[17]	50	0.91[13]	Takeyoshi et al. 2006; Takeyoshi et al. 2007b	- (1.6, 100%)	-	+ (HPTA)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	ICCVAM 1999	No at ≤ 25% (humans)	Kligman 1966

LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA												
Chemical Name	**Veh.[1]**	**Highest Conc. Tested (%)**	**Highest SI**	**LLNA: BrdU Ref.[2]**	**Trad. LLNA Result[3]**	**GP Result**	**Human Result[4]**	**Ref. Trad. LLNA[5]**	**Ref. GP**	**Ref. Human[6]**	**Skin Irritant?**	**Reference Skin Irritation**
Salicylic acid	AOO	25	1.26	Takeyoshi unpublished 2009	- (2.5, 25%)	-	-	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Irritant at 20% aq (mice)	Gerberick et al. 2002
Sodium lauryl sulfate	DMF	16.7	2.64	Takeyoshi unpublished 2009	+ (8.9, 20%)	-	-	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Irritant at 20% aq (rabbits); Irritant at 10% in DMF (mice); Irritant at 20% (humans)	ECETOC #66, 1995; Antonopoulus et al. 2008; Kligman 1966
Sulfanilamide	DMF	50	1.26	Takeyoshi unpublished 2009	- (1, 50%)	-	-	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Kligman 1966	No at ≤ 25% (humans)	Kligman 1966
trans-Cinnamaldehyde	AOO	10	5.90	Takeyoshi et al. 2005	+ (13.1, 25%)	NA	NA	Gerberick et al. 2005	NA	NA	NA	NA

LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
trans-Cinnamaldehyde	AOO	10	4.11	Kojima et al. 2008 5	+ (13.1, 25%)	NA	NA	Gerberick et al. 2005	NA	NA	NA	NA
trans-Cinnamaldehyde	AOO	10	3.50	Kojima et al. 2008 4	+ (13.1, 25%)	NA	NA	Gerberick et al. 2005	NA	NA	NA	NA
trans-Cinnamaldehyde	AOO	10	3.37	Kojima et al. 2008 2	+ (13.1, 25%)	NA	NA	Gerberick et al. 2005	NA	NA	NA	NA
Trimellitic anhydride	AOO	10	7.85	Takeyoshi unpublished 2009	+ (4.6, 25%)	+	NA	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	NA	No at ≤ 10% (GP)	Basketter and Scholes 1992
Xylene	AOO	100	4.09[10]	Takeyoshi unpublished 2009	+ (3.1, 100%)	NA	-	ICCVAM 1999	NA	ICCVAM 1999	Irritant at 100% (humans)	Kligman 1966

Abbreviations: ACE = acetone; aq = aqueous; AOO = acetone: olive oil (4:1); DMF = *N,N*-dimethyl formamide; DMSO = dimethyl sulfoxide; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; Conc.= concentration; GP = guinea pig; LLNA = murine local lymph node assay; NA = not available; nonstd = nonstandard; PC = positive control; Ref. = reference; SI = stimulation index; Trad. = traditional; Veh. = Vehicle.

+ = sensitizer; - = nonsensitizer

[1] Applies to both traditional LLNA and LLNA: BrdU-ELISA unless otherwise noted.

[2] Number after Kojima et al. 2008 represents the laboratory that submitted the test.

[3] Numbers in parentheses indicate the maximum SI and the highest concentration tested.

[4] Information in parentheses provides the evidence for the human result, usually as incidence of a positive human response at the challenge concentration.

[5] Reference in parentheses applies to the maximum SI and the highest concentration tested, if it is different from the reference for the traditional LLNA result.

[6] Reference in parentheses applies to the evidence for the human result if different from the sensitizer/nonsensitizer outcome.

[7] Maximum SI occurred at 12.5%.

[8] Vehicle for the traditional LLNA was DMF.

[9] An outlier in the vehicle control group was excluded for the calculation of the SI values for this test. See **Annex IV** for the results of the test with and without the outlier.

[10] This test was not used in the accuracy analysis because the test results were submitted after all independent peer reviews had been completed.

[11] Mouse strain, CBA/J, is different from that specified in the protocol, CBA/JN.

[12] Maximum SI occurred at 0.3%.

[13] Maximum SI occurred at 10%.

[14] Maximum SI occurred at 50%.

[15]Vehicle for the traditional LLNA was acetone.

[16]Maximum SI occurred at 25%.

[17]Vehicle for the traditional LLNA was distilled water.

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Annex III-2

Comparison of Multiple LLNA: BrdU-ELISA Decision Criteria and Traditional LLNA Results (Alphanumeric Order)

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Comparison of Multiple LLNA: BrdU-ELISA Decision Criteria and Traditional LLNA Results

Chemical Name	CASRN	Highest Conc. Tested (%)	Highest SI	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.	SI≥ 5.0	SI≥ 4.5	SI≥ 4.0	SI≥ 3.5	SI≥ 3.0	SI≥ 2.5	SI≥ 2.0	SI≥ 1.9	SI≥ 1.6	SI≥ 1.5	SI≥ 1.3	Ref. LLNA: BrdU-ELISA[1]	Trad. LLNA Result[2]
1,4-Phenylene-diamine	106-50-3	2	11.70	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+
1,4-Phenylene-diamine	106-50-3	10	14.70	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+
2-Hydroxy-propylmetha-crylate	923-26-2	50	1.13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Takeyoshi et al. 2007b	-
2-Mercaptoben-zothiazole	149-30-4	50	1.62	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+	Takeyoshi et al. 2007b	+
2,4-Dinitrochloro-benzene	97-00-7	1	4.30	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 1	+
2,4-Dinitrochloro-benzene	97-00-7	1	8.37	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 2	+
2,4-Dinitrochloro-benzene	97-00-7	1	6.26	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 3	+

Comparison of Multiple LLNA: BrdU-ELISA Decision Criteria and Traditional LLNA Results

Chemical Name	CASRN	Highest Conc. Tested (%)	Highest SI	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.	SI≥ 5.0	SI≥ 4.5	SI≥ 4.0	SI≥ 3.5	SI≥ 3.0	SI≥ 2.5	SI≥ 2.0	SI≥ 1.9	SI≥ 1.6	SI≥ 1.5	SI≥ 1.3	Ref. LLNA: BrdU-ELISA[1]	Trad. LLNA Result[2]
2,4-Dinitrochloro-benzene	97-00-7	1	5.50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 4	+
2,4-Dinitrochloro-benzene	97-00-7	1	18.80	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 5	+
2,4-Dinitrochloro-benzene	97-00-7	1	4.83	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 6	+
2,4-Dinitrochloro-benzene	97-00-7	1	12.98	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 7	+
2,4-Dinitrochloro-benzene	97-00-7	2	17.90	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2005	+
2,4-Dinitrochloro-benzene	97-00-7	2	6.84	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2006; Takeyoshi et al. 2007b	+
4-Chloroaniline	106-47-8	25	2.53	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	Takeyoshi et al. 2007b	+
4-Methylamino-phenol sulfate	55-55-0	10	3.98	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Takeyoshi unpublished 2009	+

Comparison of Multiple LLNA: BrdU-ELISA Decision Criteria and Traditional LLNA Results

Chemical Name	CASRN	Highest Conc. Tested (%)	Highest SI	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.	SI≥ 5.0	SI≥ 4.5	SI≥ 4.0	SI≥ 3.5	SI≥ 3.0	SI≥ 2.5	SI≥ 2.0	SI≥ 1.9	SI≥ 1.6	SI≥ 1.5	SI≥ 1.3	Ref. LLNA: BrdU-ELISA[1]	Trad. LLNA Result[2]
5-Chloro-2-methyl-4-isothiazolin-3-one solution	55965-84-9	0.5	4.83	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Takeyoshi unpublished 2009	+
Aniline	62-53-3	100	2.07	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	Takeyoshi unpublished 2009	+
Cinnamic alcohol	104-54-1	50	2.74	+	-	+	+	-	-	-	-	-	+	+	+	+	+	+	Takeyoshi unpublished 2009	+
Cinnamic aldehyde	104-55-2	50	3.97	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Takeyoshi et al. 2007b	+
Citral	5392-40-5	50	16.35	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2007b; Takeyoshi et al. 2005	+
Cobalt chloride	7646-79-9	5	3.68	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Takeyoshi unpublished 2009	+
Cyclamen aldehyde	103-95-7	100	1.97	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+	Takeyoshi et al. 2007b	+
Cyclamen aldehyde	103-95-7	100	5.71	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi unpublished 2009	+

Comparison of Multiple LLNA: BrdU-ELISA Decision Criteria and Traditional LLNA Results

Chemical Name	CASRN	Highest Conc. Tested (%)	Highest SI	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.	SI≥ 5.0	SI≥ 4.5	SI≥ 4.0	SI≥ 3.5	SI≥ 3.0	SI≥ 2.5	SI≥ 2.0	SI≥ 1.9	SI≥ 1.6	SI≥ 1.5	SI≥ 1.3	Ref. LLNA: BrdU-ELISA[1]	Trad. LLNA Result[2]
Diethyl maleate	141-05-9	25	6.27	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi unpublished 2009	+
Diethyl phthalate (Phthallic acid diethylester)	84-66-2	50	0.88	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Takeyoshi et al. 2007b	-
Dimethyliso-phthalate	1454-93-4	50	1.26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Takeyoshi et al. 2007b	-
Diphenylcy-clopropenone	886-38-4	2	19.10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+
Diphenylcy-clopropenone	886-38-4	10	11.62	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2007b	+
Diphenylcy-clopropenone	886-38-4	10	9.34	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+
Ethyl acrylate	140-88-5	100	4.95	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Takeyoshi unpublished 2009	+

Comparison of Multiple LLNA: BrdU-ELISA Decision Criteria and Traditional LLNA Results

Chemical Name	CASRN	Highest Conc. Tested (%)	Highest SI	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.	SI≥ 5.0	SI≥ 4.5	SI≥ 4.0	SI≥ 3.5	SI≥ 3.0	SI≥ 2.5	SI≥ 2.0	SI≥ 1.9	SI≥ 1.6	SI≥ 1.5	SI≥ 1.3	Ref. LLNA: BrdU-ELISA[1]	Trad. LLNA Result[2]
Ethylene glycol dimethacrylate	97-90-5	100	3.11	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	Takeyoshi unpublished 2009	+
Eugenol	97-53-0	10	3.18	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	Takeyoshi et al. 2004a	+
Eugenol	97-53-0	30	3.30	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	Takeyoshi et al. 2004a	+
Eugenol	97-53-0	30	3.83	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Takeyoshi et al. 2007a	+
Eugenol	97-53-0	50	12.30	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2005	+
Eugenol	97-53-0	50	3.10	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	Takeyoshi et al. 2006	+
Eugenol	97-53-0	50	7.09	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 7	+
Eugenol	97-53-0	50	3.17	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	Kojima et al. 2008 2	+
Eugenol	97-53-0	50	3.18	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	Kojima et al. 2008 6	+
Eugenol	97-53-0	50	17.70	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2007b	+

Comparison of Multiple LLNA: BrdU-ELISA Decision Criteria and Traditional LLNA Results

Chemical Name	CASRN	Highest Conc. Tested (%)	Highest SI	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.	SI≥ 5.0	SI≥ 4.5	SI≥ 4.0	SI≥ 3.5	SI≥ 3.0	SI≥ 2.5	SI≥ 2.0	SI≥ 1.9	SI≥ 1.6	SI≥ 1.5	SI≥ 1.3	Ref. LLNA: BrdU-ELISA[1]	Trad. LLNA Result[2]
Formaldehyde	50-00-0	10	16.59	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 5	+
Formaldehyde	50-00-0	10	4.40	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 1	+
Formaldehyde	50-00-0	10	1.97	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+	Kojima et al. 2008 6	+
Glutaralde-hyde	111-30-8	1	28.64	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 5	+
Glutaralde-hyde	111-30-8	1	3.72	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Kojima et al. 2008 1	+
Glutaralde-hyde	111-30-8	1	2.25	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	Kojima et al. 2008 6	+
Glutaralde-hyde	111-30-8	2	14.60	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+
Glutaralde-hyde	111-30-8	10	15.50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+
Glycerol	56-81-5	50	1.29	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	Takeyoshi et al. 2007b	-

Comparison of Multiple LLNA: BrdU-ELISA Decision Criteria and Traditional LLNA Results

Chemical Name	CASRN	Highest Conc. Tested (%)	Highest SI	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.	SI≥ 5.0	SI≥ 4.5	SI≥ 4.0	SI≥ 3.5	SI≥ 3.0	SI≥ 2.5	SI≥ 2.0	SI≥ 1.9	SI≥ 1.6	SI≥ 1.5	SI≥ 1.3	Ref. LLNA: BrdU-ELISA[1]	Trad. LLNA Result[2]
Hexane	110-54-3	100	1.76	+	-	-	+	-	-	-	-	-	-	-	-	+	+	+	Takeyoshi unpublished 2009	-
Hexane	110-54-3	50	1.89	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	Takeyoshi et al. 2005	-
Hexyl cinnamic aldehyde	101-86-0	50	3.64	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Takeyoshi et al. 2003	+
Hexyl cinnamic aldehyde	101-86-0	25	2.44	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	Takeyoshi et al. 2003	+
Hexyl cinnamic aldehyde	101-86-0	50	5.90	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2005	+
Hexyl cinnamic aldehyde	101-86-0	50	3.64	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Takeyoshi et al. 2006	+
Hexyl cinnamic aldehyde	101-86-0	50	2.72	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	Takeyoshi et al. 2006	+
Hexyl cinnamic aldehyde	101-86-0	50	3.02	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	Takeyoshi et al. 2007b	+

Comparison of Multiple LLNA: BrdU-ELISA Decision Criteria and Traditional LLNA Results

Chemical Name	CASRN	Highest Conc. Tested (%)	Highest SI	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.	SI≥ 5.0	SI≥ 4.5	SI≥ 4.0	SI≥ 3.5	SI≥ 3.0	SI≥ 2.5	SI≥ 2.0	SI≥ 1.9	SI≥ 1.6	SI≥ 1.5	SI≥ 1.3	Ref. LLNA: BrdU-ELISA[1]	Trad. LLNA Result[2]
Hexyl cinnamic aldehyde	101-86-0	50	3.40	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	Kojima et al. 2008 1	+
Hexyl cinnamic aldehyde	101-86-0	50	2.87	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	Kojima et al. 2008 3	+
Hexyl cinnamic aldehyde	101-86-0	50	3.34	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	Kojima et al. 2008 4	+
Hexyl cinnamic aldehyde	101-86-0	50	13.50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 5	+
Hexyl cinnamic aldehyde	101-86-0	50	3.27	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	Kojima et al. 2008 6	+
Hexyl cinnamic aldehyde	101-86-0	50	3.84	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Kojima et al. 2008 7	+
Hydroxycit-ronellal	107-73-5	100	1.34	+	-	+	+	-	-	-	-	-	-	-	-	-	-	+	Takeyoshi et al. 2007b	+
Hydroxycit-ronellal	107-73-5	100	4.78	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Takeyoshi unpublished 2009	+

Comparison of Multiple LLNA: BrdU-ELISA Decision Criteria and Traditional LLNA Results

Chemical Name	CASRN	Highest Conc. Tested (%)	Highest SI	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.	SI≥ 5.0	SI≥ 4.5	SI≥ 4.0	SI≥ 3.5	SI≥ 3.0	SI≥ 2.5	SI≥ 2.0	SI≥ 1.9	SI≥ 1.6	SI≥ 1.5	SI≥ 1.3	Ref. LLNA: BrdU-ELISA[1]	Trad. LLNA Result[2]
Imidazolidinyl urea	39236-46-9	50	1.61	+	-	+	+	-	-	-	-	-	-	-	-	+	+	+	Takeyoshi unpublished 2009	+
Isoeugenol	97-54-1	10	8.40	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2005	+
Isoeugenol	97-54-1	10	2.40	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	Takeyoshi et al. 2006; Takeyoshi et al. 2007b	+
Isoeugenol	97-54-1	30	6.73	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2007a	+
Isopropanol	67-63-0	50	2.22	+	-	+	+	-	-	-	-	-	-	+	+	+	+	+	Kojima et al. 2008 1	-
Isopropanol	67-63-0	50	0.98	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Kojima et al. 2008 3	-
Isopropanol	67-63-0	50	1.57	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	Kojima et al. 2008 4	-
Isopropanol	67-63-0	50	0.94	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Kojima et al. 2008 5	-
Isopropanol	67-63-0	50	2.04	+	-	+	-	-	-	-	-	-	-	+	+	+	+	+	Kojima et al. 2008 6	-
Isopropanol	67-63-0	50	1.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Kojima et al. 2008 7	-

Comparison of Multiple LLNA: BrdU-ELISA Decision Criteria and Traditional LLNA Results

Chemical Name	CASRN	Highest Conc. Tested (%)	Highest SI	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.	SI≥ 5.0	SI≥ 4.5	SI≥ 4.0	SI≥ 3.5	SI≥ 3.0	SI≥ 2.5	SI≥ 2.0	SI≥ 1.9	SI≥ 1.6	SI≥ 1.5	SI≥ 1.3	Ref. LLNA: BrdU-ELISA[1]	Trad. LLNA Result[2]
Isopropanol	67-63-0	100	0.92	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Takeyoshi et al. 2007b	-
Isopropyl myristate	110-27-0	50	4.20	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+
Lactic acid	598-82-3	50	2.53	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	Kojima et al. 2008 7	-
Lactic acid	598-82-3	50	1.89	+	-	+	+	-	-	-	-	-	-	-	-	+	+	+	Kojima et al. 2008 4	-
Lactic acid	598-82-3	50	1.80	+	-	+	-	-	-	-	-	-	-	-	-	+	+	+	Kojima et al. 2008 3	-
Linalool	78-70-6	100	1.45	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Takeyoshi unpublished 2009	+
Linalool	78-70-6	100	4.65	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Takeyoshi unpublished 2009	+
m-Aminophenol	591-27-5	25	3.06	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	Takeyoshi et al. 2007b	+
Methyl salicylate	119-36-8	50	1.43	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	Kojima et al. 2008 1	-
Methyl salicylate	119-36-8	50	1.44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Kojima et al. 2008 2	-

Comparison of Multiple LLNA: BrdU-ELISA Decision Criteria and Traditional LLNA Results

Chemical Name	CASRN	Highest Conc. Tested (%)	Highest SI	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.	SI≥ 5.0	SI≥ 4.5	SI≥ 4.0	SI≥ 3.5	SI≥ 3.0	SI≥ 2.5	SI≥ 2.0	SI≥ 1.9	SI≥ 1.6	SI≥ 1.5	SI≥ 1.3	Ref. LLNA: BrdU-ELISA[1]	Trad. LLNA Result[2]
Methyl salicylate	119-36-8	50	1.40	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Kojima et al. 2008 3	-
Nickel sulfate	7786-81-4	10	2.58	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	Kojima et al. 2008 3	+
Nickel sulfate	7786-81-4	10	4.53	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 4	+
Nickel sulfate	7786-81-4	10	2.66	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	Kojima et al. 2008 7	+
p-Benzoquinone	106-51-4	1	6.90	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2004b; Takeyoshi et al. 2007b	+
Phenyl benzoate	93-99-2	66.7	3.37	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	Takeyoshi unpublished 2009	+
Propylene glycol	57-55-6	50	1.57	+	-	+	-	-	-	-	-	-	-	-	-	-	+	+	Takeyoshi et al. 2005	-
Propylene glycol	57-55-6	10	1.20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Takeyoshi et al. 2005	-
Propylene glycol	57-55-6	50	0.91	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Takeyoshi et al. 2006; Takeyoshi et al. 2007b	-

Comparison of Multiple LLNA: BrdU-ELISA Decision Criteria and Traditional LLNA Results

Chemical Name	CASRN	Highest Conc. Tested (%)	Highest SI	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.	SI≥ 5.0	SI≥ 4.5	SI≥ 4.0	SI≥ 3.5	SI≥ 3.0	SI≥ 2.5	SI≥ 2.0	SI≥ 1.9	SI≥ 1.6	SI≥ 1.5	SI≥ 1.3	Ref. LLNA: BrdU-ELISA[1]	Trad. LLNA Result[2]
Salicylic acid	69-72-7	25	1.26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Takeyoshi unpublished 2009	-
Sodium lauryl sulfate	151-21-3	16.7	2.64	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	Takeyoshi unpublished 2009	+
Sulfanilamide	63-74-1	50	1.26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Takeyoshi unpublished 2009	-
trans-Cinnamalde-hyde	14371-10-9	10	5.90	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2005	+
trans-Cinnamalde-hyde	14371-10-9	10	4.11	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 5	+
trans-Cinnamalde-hyde	14371-10-9	10	3.50	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Kojima et al. 2008 4	+
trans-Cinnamalde-hyde	14371-10-9	10	3.37	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	Kojima et al. 2008 2	+
Trimellitic anhydride	552-30-7	10	7.85	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi unpublished 2009	+

Abbreviations: BrdU-ELISA LLNA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; CASRN = Chemical Abstract Services Registry Number; CI= confidence interval (mean absorbance of any treatment group is greater than 95% confidence interval of vehicle control group mean); Conc. = concentration; LLNA = murine local lymph node assay; Ref. = reference; SD = standard deviation (mean absorbance of any treatment group is greater than 2 or 3 SD for vehicle control group); SI = stimulation index; Stats. = statistics (analysis of variance for multiple dose groups or t-test to compare one treatment group to the vehicle control group); Trad. = traditional.

+ = sensitizer; - = nonsensitizer.

[1]Number after Kojima et al. 2008 represents the laboratory that submitted the test.

[2]References for the traditional LLNA results are provided in **Annex III-1**.

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Annex IV-1

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

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Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
VC	2003	AOO	0	1	0.07	0.97	NA	NA	NA	NA
				2	0.07	1.04				
				3	0.06	0.91				
				4	0.07	1.08				
				Mean	**0.07**	**1.00**				
HCA	2003	AOO	3.125	5	0.08	1.12	5.52	6.25	12.90	15.18
				6	0.07	0.99				
				7	0.10	1.54				
				8	0.07	1.02				
				Mean	**0.08**	**1.17**				
			6.25	9	0.06	0.81				
				10	0.17	2.54				
				11	0.12	1.73				
				12	0.09	1.33				
				Mean	**0.11**	**1.60**				
			12.5	13	0.14	2.08				
				14	0.12	1.77				
				15	0.15	2.21				
				16	0.10	1.48				
				Mean	**0.13**	**1.88**				
			25	17	0.10	1.45				
				18	0.14	2.13				
				19	0.26	3.83				
				20	0.16	2.33				
				Mean	**0.17**	**2.44**				
VC	2003	AOO	0	1	0.09	0.72	NA	NA	NA	NA
				2	0.14	1.17				
				3	0.13	1.11				
				4	0.12	1.01				
				Mean	**0.12**	**1.00**				
HCA	2003	AOO	12.5	5	0.19	1.55	11.58	12.65	17.20	18.75
				6	0.25	2.04				
				7	0.24	2.00				
				8	0.09	0.75				
				Mean	**0.19**	**1.59**				
			25	9	0.21	1.74				
				10	0.35	2.88				
				11	0.32	2.69				
				12	0.28	2.33				
				Mean	**0.29**	**2.41**				

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
HCA (continued)	2003	AOO	50	13	0.33	2.75				
				14	0.39	3.24				
				15	0.48	4.01				
				16	0.55	4.58				
				Mean	**0.44**	**3.64**				
VC	2004	AOO	0	1	0.07	0.68	NA	NA	NA	NA
				2	0.08	0.77				
				3	0.17	1.57				
				4	0.10	0.98				
				Mean	**0.11**	**1.00**				
Eugenol	2004a	AOO	1	5	0.27	2.50	5.94	6.95	10.10	11.19
				6	0.12	1.09				
				7	0.18	1.73				
				8	0.18	1.71				
				Mean	**0.19**	**1.76**				
			6	9	0.21	1.94				
				10	0.21	1.93				
				11	0.06	0.60				
				12	0.17	1.56				
				Mean	**0.16**	**1.51**				
			15	13	0.27	2.51				
				14	0.17	1.58				
				15	0.27	2.53				
				16	0.30	2.82				
				Mean	**0.25**	**2.36**				
			30	17	0.47	4.44				
				18	0.29	2.69				
				19	0.39	3.67				
				20	0.27	2.52				
				Mean	**0.36**	**3.33**				
DHEA	2004a	AOO	1	21	0.19	1.77	0.70*	0.85*	14.10	18.43
				26	0.19	1.82				
				27	0.19	1.80				
				28	0.15	1.43				
				Mean	**0.18**	**1.71**				
			6	29	0.29	2.68				
				30	0.10	0.93				
				31	0.14	1.35				
				32	0.20	1.91				
				Mean	**0.18**	**1.71**				

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
DHEA (continued)	2004a	AOO	30	33	0.12	1.14				
				34	0.32	2.98				
				35	0.31	2.90				
				36	0.22	2.06				
				Mean	**0.24**	**2.27**				
DHEB	2004a	AOO	1	37	0.19	1.75	0.60	13.95	0.78	0.83
				38	0.14	1.30				
				39	0.26	2.47				
				40	0.39	3.65				
				Mean	**0.24**	**2.29**				
			6	41	0.42	3.95				
				42	0.56	5.28				
				43	0.50	4.73				
				44	0.66	6.23				
				Mean	**0.54**	**5.05**				
			20	45	0.75	7.03				
				46	0.73	6.88				
				47	0.74	6.95				
				48	0.87	8.18				
				Mean	**0.77**	**7.26**				
p-Benzoquinone	2004b, 2007b	AOO	0	1	0.09	0.95	0.15	0.15	0.17	0.17
				2	0.08	0.79				
				3	0.09	0.95				
				4	0.13	1.31				
				Mean	**0.10**	**1.00**				
			0.25	5	0.31	3.14				
				6	0.40	4.08				
				7	0.21	2.11				
				8	0.40	4.08				
				Mean	**0.33**	**3.35**				
			0.5	9	0.38	3.90				
				10	0.68	6.93				
				11	0.89	9.09				
				12	0.32	3.21				
				Mean	**0.57**	**5.78**				
			1	13	0.74	7.58				
				14	0.72	7.28				
				15	0.60	6.09				
				16	0.67	6.84				
				Mean	**0.68**	**6.94**				

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
VC	2005	AOO	0	1	0.08	1.20	NA	NA	NA	NA
				2	0.10	1.45				
				3	0.04	0.61				
				4	0.05	0.73				
				Mean	**0.07**	**1.00**				
Isoeugenol	2005	AOO	10	5	0.97	13.93	NC	NC	NC	NC
				6	0.37	5.32				
				7	0.41	5.88				
				8	0.58	8.33				
				Mean	**0.58**	**8.36**				
p-Phenylenediamine	2005, 2007b	AOO	10	6	1.12	16.10	NC	NC	NC	NC
				7	1.03	14.90				
				8	1.02	14.70				
				9	0.92	13.20				
				Mean	**1.02**	**14.70**				
trans-Cinnamaldehyde	2005	AOO	10	13	0.55	7.93	NC	NC	NC	NC
				14	0.20	2.87				
				15	0.41	5.86				
				16	0.47	6.78				
				Mean	**0.41**	**5.86**				
Glutaraldehyde	2005, 2007b	AOO	0	1	0.08	1.20	NC	NC	NC	NC
				2	0.10	1.45				
				3	0.04	0.61				
				4	0.05	0.73				
				Mean	**0.07**	**1.00**				
			10	6	1.12	16.10				
				7	1.15	16.30				
				8	1.03	14.80				
				9	1.03	14.80				
				Mean	**1.08**	**15.50**				
Citral	2005, 2007b	AOO	0	1	0.08	1.20	NC	NC	NC	NC
				2	0.10	1.45				
				3	0.04	0.61				
				4	0.05	0.73				
				Mean	**0.07**	**1.00**				
			10	6	0.24	3.45				
				7	0.12	1.76				
				8	0.09	1.29				
				9	0.06	0.85				
				Mean	**0.13**	**1.84**				

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Citral	2005, 2007b	AOO	0	1	0.07	1.12	NC	NC	NC	NC
				2	0.06	0.87				
				3	0.08	1.26				
				4	0.05	0.76				
				Mean	**0.07**	**1.00**				
			50	6	1.08	16.40				
				7	0.99	15.00				
				8	1.32	20.10				
				9	0.91	13.90				
				Mean	**1.08**	**16.40**				
VC	2005	AOO	0	1	0.10	1.34	NA	NA	NA	NA
				2	0.11	1.40				
				3	0.04	0.57				
				4	0.05	0.69				
				Mean	**0.08**	**1.00**				
Isoeugenol	2005	AOO	10	5	0.94	12.23	NC	NC	NC	NC
				6	0.32	4.11				
				7	0.38	4.93				
				8	0.58	7.52				
				Mean	**0.55**	**7.20**				
Eugenol	2005	AOO	10	25	0.36	4.65	NC	NC	NC	NC
				26	0.15	1.89				
				27	0.14	1.84				
				28	0.33	4.33				
				Mean	**0.24**	**3.18**				
Isopropyl myristate	2005, 2007b	AOO	10	6	0.05	0.70	NC	NC	NC	NC
				7	0.07	0.95				
				8	0.13	1.71				
				9	0.07	0.94				
				Mean	**0.08**	**1.08**				
Isopropyl myristate	2005, 2007b	AOO	0	1	0.07	0.95	NC	NC	NC	NC
				2	0.06	0.91				
				3	0.08	1.11				
				4	0.07	1.03				
				Mean	**0.07**	**1.00**				
			50	6	0.22	3.15				
				7	0.41	6.03				
				8	0.24	3.55				
				9	0.28	4.02				
				Mean	**0.29**	**4.19**				

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Propylene glycol	2005	AOO	10	33	0.07	0.85	NC	NC	NC	NC
				34	0.15	1.91				
				35	0.07	0.85				
				36	0.09	1.20				
				Mean	**0.09**	**1.20**				
Hexane	2005	AOO	10	37	0.04	0.54	NC	NC	NC	NC
				38	0.09	1.12				
				39	0.05	0.61				
				40	0.05	0.67				
				Mean	**0.06**	**0.73**				
Diphenylcyclopropenone	2005, 2007b	AOO	10	41	0.52	6.84	NC	NC	NC	NC
				42	0.54	7.03				
				43	0.69	9.04				
				44	1.11	14.44				
				Mean	**0.72**	**9.34**				
VC	2005	AOO	0	1	0.09	0.94	NA	NA	NA	NA
				2	0.08	0.88				
				3	0.11	1.15				
				4	0.10	1.04				
				Mean	**0.10**	**1.00**				
DNCB	2005	AOO	2	5	1.73	18.15	NC	NC	NC	NC
				6	1.67	17.56				
				7	1.74	18.28				
				8	1.66	17.45				
				Mean	**1.70**	**17.86**				
p-Phenylenediamine	2005, 2007b	AOO	2	6	0.96	10.10	NC	NC	NC	NC
				7	1.26	13.20				
				8	1.03	10.90				
				9	1.20	12.60				
				Mean	**1.12**	**11.70**				
Glutaraldehyde	2005, 2007b	AOO	2	6	1.45	15.20	NC	NC	NC	NC
				7	1.33	14.00				
				8	1.34	14.10				
				9	1.41	14.80				
				Mean	**1.38**	**14.60**				
Diphenylcyclopropenone	2005, 2007b	AOO	2	6	1.85	19.50	NC	NC	NC	NC
				7	1.78	18.70				
				8	1.67	17.60				
				9	1.95	20.60				
				Mean	**1.81**	**19.10**				

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
VC	2005	AOO	0	1	0.07	1.12	NA	NA	NA	NA
				2	0.06	0.87				
				3	0.08	1.26				
				4	0.05	0.76				
				Mean	**0.07**	**1.00**				
HCA	2005	AOO	50	5	0.34	5.19	NC	NC	NC	NC
				6	0.37	5.57				
				7	0.45	6.91				
				8	0.39	5.95				
				Mean	**0.39**	**5.90**				
Propylene glycol	2005	AOO	50	9	0.10	1.53	NC	NC	NC	NC
				10	0.16	2.42				
				11	0.07	1.07				
				12	0.08	1.25				
				Mean	**0.10**	**1.57**				
Hexane	2005	AOO	50	13	0.12	1.86	NC	NC	NC	NC
				14	0.10	1.51				
				15	0.15	2.32				
				16	0.12	1.88				
				Mean	**0.12**	**1.89**				
Eugenol	2005	AOO	50	33	0.71	10.31	NC	NC	NC	NC
				34	0.73	10.67				
				35	1.07	15.63				
				36	0.86	12.50				
				Mean	**0.84**	**12.28**				
VC	2006	AOO	0	1	0.54	1.16	NA	NA	NA	NA
				2	0.43	0.92				
				3	0.37	0.79				
				4	0.53	1.13				
				Mean	**0.47**	**1.00**				
HCA	2006	AOO	2	5	0.49	1.04	15.87	18.67	27.10	29.86
				6	0.40	0.86				
				7	0.44	0.95				
				8	0.37	0.79				
				Mean	**0.43**	**0.91**				
			10	9	0.75	1.60				
				10	0.64	1.37				
				11	0.50	1.06				
				12	0.53	1.14				
				Mean	**0.60**	**1.29**				

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
HCA (continued)	2006	AOO	50	13	1.52	3.25				
				14	1.18	2.53				
				15	1.36	2.91				
				16	1.02	2.19				
				Mean	**1.27**	**2.72**				
Eugenol	2006	AOO	2	17	0.27	0.57	11.01	13.52	21.10	23.58
				18	0.25	0.52				
				19	0.30	0.65				
				20	0.24	0.52				
				Mean	**0.26**	**0.56**				
			10	21	0.80	1.72				
				22	0.68	1.45				
				23	0.50	1.06				
				24	0.76	1.63				
				Mean	**0.68**	**1.46**				
			50	25	1.34	2.87				
				26	1.48	3.17				
				27	1.37	2.93				
				28	1.52	3.26				
				Mean	**1.43**	**3.05**				
Isoeugenol	2006, 2007b	AOO	0	1	0.54	1.16	6.26	6.70	8.00	8.43
				2	0.43	0.92				
				3	0.37	0.79				
				4	0.53	1.13				
				Mean	**0.47**	**1.00**				
			0.4	6	0.12	0.25				
				7	0.18	0.39				
				8	0.19	0.41				
				9	0.22	0.46				
				Mean	**0.18**	**0.38**				
			2	11	0.38	0.81				
				12	0.21	0.44				
				13	0.18	0.38				
				14	0.22	0.46				
				Mean	**0.24**	**0.52**				
			10	16	1.31	2.80				
				17	1.22	2.62				
				18	0.83	1.77				
				19	1.05	2.25				
				Mean	**1.10**	**2.36**				

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
HCA	2006	AOO	0	1	0.39	1.66	18.06	19.55	24.00	25.52
				2	0.27	1.15				
				3	0.17	0.71				
				4	0.11	0.48				
				Mean	**0.24**	**1.00**				
			2	5	0.28	1.18				
				6	0.13	0.56				
				7	0.16	0.69				
				8	0.18	0.74				
				Mean	**0.19**	**0.79**				
			10	9	0.19	0.81				
				10	0.21	0.87				
				11	0.25	1.07				
				12	0.26	1.08				
				Mean	**0.23**	**0.96**				
			50	13	1.02	4.32				
				14	0.64	2.71				
				15	0.90	3.81				
				16	0.88	3.72				
				Mean	**0.86**	**3.64**				
Propylene glycol	2006, 2007b	AOO	0	1	0.39	1.54	NC	NC	NC	NC
				2	0.27	1.21				
				3	0.17	0.74				
				4	0.11	0.51				
				Mean	**0.24**	**1.00**				
			2	6	0.32	1.42				
				7	0.22	0.96				
				8	0.15	0.67				
				9	0.13	0.57				
				Mean	**0.20**	**0.91**				
			10	11	0.14	0.60				
				12	0.11	0.46				
				13	0.15	0.65				
				14	0.42	1.75				
				Mean	**0.21**	**0.87**				
			50	16	0.14	0.63				
				17	0.17	0.74				
				18	0.15	0.66				
				19	0.18	0.78				
				Mean	**0.16**	**0.70**				

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
2-Hydroxypropyl methacrylate	2007b	AOO	0	1	0.11	1.08	NC	NC	NC	NC
				2	0.09	0.87				
				3	0.10	0.90				
				4	0.12	1.15				
				Mean	**0.11**	**1.00**				
			50	6	0.10	0.96				
				7	0.14	1.35				
				8	0.14	1.32				
				9	0.09	0.88				
				Mean	**0.12**	**1.13**				
Aniline	2007b	AOO	0	1	0.08	0.86	50.00	NC	NC	NC
				2	0.09	0.90				
				3	0.09	0.92				
				4	0.13	1.33				
				Mean	**0.10**	**1.00**				
			12.5	6	0.10	1.05				
				7	0.12	1.26				
				8	0.15	1.57				
				9	0.15	1.63				
				Mean	**0.13**	**1.38**				
			25	11	0.11	1.14				
				12	0.13	1.35				
				13	0.14	1.48				
				14	0.15	1.58				
				Mean	**0.13**	**1.39**				
			50	16	0.16	1.67				
				17	0.11	1.17				
				18	0.10	1.04				
				19	0.20	2.11				
				Mean	**0.14**	**1.50**				
p-Chloroaniline	2007b	AOO	0	1	0.08	0.86	10.79	11.03	15.00	16.20
				2	0.09	0.90				
				3	0.09	0.92				
				4	0.13	1.33				
				Mean	**0.10**	**1.00**				
			12.5	6	0.15	1.60				
				7	0.14	1.47				
				8	0.15	1.59				
				9	0.20	2.07				
				Mean	**0.16**	**1.68**				

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
p-Chloroaniline (continued)	2007b	AOO	25	11	0.21	2.23				
				12	0.18	1.91				
				13	0.29	3.05				
				14	0.28	2.92				
				Mean	**0.24**	**2.53**				
Cinnamic aldehyde	2007b	AOO	0	1	0.12	0.78	6.81	4.81	8.56	9.07
				2	0.18	1.13				
				3	0.17	1.08				
				4	0.16	1.01				
				Mean	**0.16**	**1.00**				
			12.5	6	0.48	3.09				
				7	0.30	1.94				
				8	0.47	3.00				
				9	0.34	2.20				
				Mean	**0.40**	**2.56**				
			25	11	0.61	3.87				
				12	0.63	3.99				
				13	0.52	3.30				
				14	0.62	3.94				
				Mean	**0.59**	**3.77**				
			50	16	0.58	3.71				
				17	0.53	3.38				
				18	0.72	4.60				
				19	0.66	4.18				
				Mean	0.62	3.97				
Cyclamen aldehyde	2007b	AOO	0	1	0.13	0.86	69.48	75.97	93.90	NC
				2	0.17	1.20				
				3	0.13	0.90				
				4	0.15	1.04				
				Mean	**0.15**	**1.00**				
			25	6	0.13	0.91				
				7	0.13	0.90				
				8	0.20	1.39				
				9	0.13	0.86				
				Mean	**0.15**	**1.02**				
			50	11	0.16	1.11				
				12	0.20	1.35				
				13	0.15	1.04				
				14	0.19	1.32				
				Mean	**0.17**	**1.20**				

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Cyclamen aldehyde (continued)	2007b	AOO	100	16	0.24	1.65				
				17	0.20	1.35				
				18	0.39	2.69				
				19	0.32	2.20				
				Mean	**0.29**	**1.97**				
Diethyl phthalate	2007b	AOO	0	1	0.11	1.08	NC	NC	NC	NC
				2	0.09	0.87				
				3	0.10	0.90				
				4	0.12	1.15				
				Mean	**0.11**	**1.00**				
			50	6	0.09	0.87				
				7	0.07	0.71				
				8	0.08	0.77				
				9	0.12	1.16				
				Mean	**0.09**	**0.88**				
Dimethylisophthalate	2007b	AOO	0	1	0.11	1.08	NC	NC	NC	NC
				2	0.09	0.87				
				3	0.10	0.90				
				4	0.12	1.15				
				Mean	**0.11**	**1.00**				
			50	6	0.13	1.23				
				7	0.11	1.02				
				8	0.15	1.45				
				9	0.14	1.36				
				Mean	**0.13**	**1.26**				
Diphenylcyclopropenone	2007b	AOO	0	1	0.07	0.95	NC	NC	NC	NC
				2	0.06	0.91				
				3	0.08	1.11				
				4	0.07	1.03				
				Mean	**0.07**	**1.00**				
			10	6	0.93	13.50				
				7	0.82	11.90				
				8	0.50	7.23				
				9	0.95	13.80				
				Mean	**0.80**	**11.61**				
DNCB	2007b	AOO	0	1	0.44	1.76	0.07	0.07	0.10	0.11
				2	0.27	1.10				
				3	0.17	0.69				
				4	0.11	0.45				
				Mean	**0.25**	**1.00**				

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
DNCB (continued)	2007b	AOO	0.08	6	0.35	1.40				
				7	0.46	1.84				
				8	0.30	1.23				
				9	0.56	2.24				
				Mean	**0.42**	**1.68**				
			0.4	11	1.21	4.89				
				12	1.33	5.38				
				13	1.67	6.73				
				14	1.44	5.81				
				Mean	**1.41**	**5.70**				
			2	16	1.87	7.53				
				17	1.50	6.05				
				18	1.63	6.60				
				19	1.78	7.17				
				Mean	**1.69**	**6.84**				
VC	2007b	AOO	0	1	0.03	0.52	NA	NA	NA	NA
				2	0.07	1.53				
				3	0.04	0.74				
				4	0.06	1.21				
				Mean	**0.05**	**1.00**				
Eugenol	2007b	AOO	6.25	6	0.15	3.05	1.02	1.06	1.19	1.24
				7	0.35	7.48				
				8	0.25	5.26				
				9	0.42	8.85				
				Mean	**0.29**	**6.16**				
			12.5	11	0.26	5.52				
				12	0.28	5.89				
				13	0.33	6.87				
				14	0.64	13.48				
				Mean	**0.38**	**7.94**				
			25	16	0.76	16.05				
				17	0.86	18.09				
				18	0.59	12.35				
				19	0.67	14.14				
				Mean	**0.72**	**15.15**				
			50	21	0.81	17.12				
				22	0.76	15.99				
				23	0.82	17.35				
				24	0.96	20.29				
				Mean	**0.84**	**17.69**				

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Glycerol	2007b	NA	0	1	0.05	0.85	NC	NC	NC	NC
				2	0.06	0.94				
				3	0.07	1.11				
				4	0.07	1.10				
				Mean	**0.06**	**1.00**				
			10	6	0.08	1.38				
				7	0.07	1.17				
				8	0.08	1.39				
				9	0.07	1.23				
				Mean	**0.08**	**1.29**				
HCA	2007b	AOO	0	1	0.12	0.78	13.49	15.48	21.40	23.41
				2	0.18	1.13				
				3	0.17	1.08				
				4	0.16	1.01				
				Mean	**0.16**	**1.00**				
			12.5	6	0.22	1.42				
				7	0.28	1.80				
				8	0.28	1.76				
				9	0.13	0.81				
				Mean	**0.23**	**1.45**				
			25	11	0.25	1.57				
				12	0.38	2.44				
				13	0.36	2.29				
				14	0.32	2.01				
				Mean	**0.33**	**2.08**				
			50	16	0.37	2.34				
				17	0.43	2.71				
				18	0.52	3.30				
				19	0.59	3.73				
				Mean	**0.47**	**3.02**				
Hydroxycitronellal	2007b	AOO	0	1	0.13	0.86	NC	NC	NC	NC
				2	0.17	1.20				
				3	0.13	0.90				
				4	0.15	1.04				
				Mean	**0.15**	**1.00**				
			25	6	0.18	1.23				
				7	0.20	1.39				
				8	0.16	1.12				
				9	0.15	1.03				
				Mean	**0.17**	**1.19**				

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Hydroxycitronellal (continued)	2007b	AOO	50	11	0.20	1.36				
				12	0.16	1.10				
				13	0.13	0.92				
				14	0.16	1.10				
				Mean	**0.16**	**1.12**				
			100	16	0.20	1.38				
				17	0.18	1.25				
				18	0.23	1.57				
				19	0.17	1.17				
				Mean	**0.20**	**1.34**				
Isopropanol	2007b	AOO	0	1	0.13	0.85	NC	NC	NC	NC
				2	0.22	1.38				
				3	0.13	0.80				
				4	0.15	0.97				
				Mean	**0.16**	**1.00**				
			25	6	0.10	0.65				
				7	0.16	1.03				
				8	0.09	0.58				
				9	0.12	0.73				
				Mean	**0.12**	**0.75**				
			50	11	0.22	1.37				
				12	0.11	0.68				
				13	0.18	1.15				
				14	0.07	0.45				
				Mean	**0.15**	**0.92**				
			100	16	0.12	0.79				
				17	0.08	0.53				
				18	0.09	0.59				
				19	0.08	0.48				
				Mean	**0.09**	**0.60**				
m-Aminophenol	2007b	AOO	0	1	0.08	0.86	2.66	2.99	4.20	4.70
				2	0.09	0.90				
				3	0.09	0.92				
				4	0.13	1.33				
				Mean	**0.10**	**1.00**				
			6.25	6	0.28	3.00				
				7	0.18	1.86				
				8	0.21	2.22				
				9	0.18	1.90				
				Mean	**0.21**	**2.25**				

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
m-Aminophenol (continued)	2007b	AOO	12.5	11	0.15	1.61				
				12	0.47	4.97				
				13	0.21	2.17				
				14	0.25	2.67				
				Mean	**0.27**	**2.86**				
			25	16	0.27	2.81				
				17	0.35	3.71				
				18	0.25	2.67				
				19	NA	NA				
				Mean	**0.29**	**3.06**				
2-Mercaptobenzothiazole	2007b	DMF	0	1	0.17	1.11	10.08*	12.10*	NC	NC
				2	0.17	1.09				
				3	0.16	1.01				
				4	0.12	0.78				
				Mean	**0.15**	**1.00**				
			12.5	6	0.22	1.45				
				7	0.28	1.81				
				8	0.34	2.22				
				9	0.15	0.98				
				Mean	**0.25**	**1.62**				
			25	11	0.20	1.27				
				12	0.17	1.12				
				13	0.25	1.64				
				14	0.22	1.40				
				Mean	**0.21**	**1.36**				
			50	16	0.19	1.21				
				17	0.12	0.77				
				18	0.26	1.70				
				19	0.35	2.29				
				Mean	**0.23**	**1.49**				
Isoeugenol	2007a	AOO	0	1	0.15	0.83	2.92	3.62	5.92	6.69
				2	0.22	1.27				
				3	0.15	0.84				
				4	0.19	1.06				
				Mean	**0.18**	**1.00**				
			3	5	0.49	2.79				
				6	0.32	1.82				
				7	0.13	0.73				
				8	0.13	0.74				
				Mean	**0.27**	**1.52**				

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Isoeugenol (continued)	2007a	AOO	10	9	0.51	2.90				
				10	0.60	3.40				
				11	0.35	2.01				
				12	0.25	1.40				
				Mean	**0.43**	**2.43**				
			30	13	0.99	5.64				
				14	1.04	5.90				
				15	1.06	6.02				
				16	1.64	9.35				
				Mean	**1.18**	**6.73**				
Eugenol	2007a	AOO	0	1	0.15	0.83	10.68	11.18	13.70	14.56
				2	0.22	1.27				
				3	0.15	0.84				
				4	0.19	1.06				
				Mean	**0.18**	**1.00**				
			3	41	0.20	1.12				
				42	0.11	0.64				
				43	0.12	0.66				
				44	0.10	0.57				
				Mean	**0.13**	**0.75**				
			10	45	0.20	1.12				
				46	0.34	1.95				
				47	0.28	1.60				
				48	0.20	1.16				
				Mean	**0.26**	**1.46**				
			30	49	0.53	3.00				
				50	0.45	2.56				
				51	0.99	5.62				
				52	0.73	4.13				
				Mean	**0.67**	**3.83**				
Dilignol	2007a	AOO	0	1	0.15	0.85	NC	NC	NC	NC
				2	0.23	1.32				
				3	0.14	0.79				
				4	0.18	1.04				
				Mean	**0.18**	**1.00**				
			3	17	0.19	1.08				
				18	0.31	1.75				
				19	0.12	0.68				
				20	0.10	0.56				
				Mean	**0.18**	**1.02**				

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Dilignol (continued)	2007a	AOO	10	21	0.31	1.75				
				22	0.29	1.63				
				23	0.14	0.81				
				24	0.10	0.56				
				Mean	**0.21**	**1.19**				
			30	25	0.23	1.32				
				26	0.25	1.41				
				27	0.10	0.57				
				28	0.16	0.89				
				Mean	**0.19**	**1.05**				
Dehydrodiisoeugenol	2007a	AOO	0	1	0.16	0.92	1.86	1.89	2.85	3.31
				2	0.23	1.32				
				3	0.14	0.81				
				4	0.17	0.95				
				Mean	**0.18**	**1.00**				
			3	29	0.18	1.03				
				30	0.53	3.03				
				31	0.30	1.71				
				32	0.36	2.03				
				Mean	**0.34**	**1.95**				
			10	33	0.54	3.08				
				34	0.70	3.97				
				35	0.45	2.58				
				36	0.48	2.75				
				Mean	**0.54**	**3.09**				
			30	37	1.00	5.66				
				38	0.69	3.91				
				39	1.08	6.12				
				40	1.03	5.83				
				Mean	**0.95**	**5.38**				
Hexane	2009	AOO	0	1	0.07	0.91	65.79	78.95	NC	NC
				2	0.12	1.51				
				3	0.06	0.71				
				4	0.07	0.87				
				Mean	**0.08**	**1.00**				
			25	6	0.09	1.14				
				7	0.11	1.45				
				8	0.10	1.22				
				9	0.12	1.54				
				Mean	**0.11**	**1.34**				

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Hexane (continued)	2009	AOO	0	11	0.05	0.81				
				12	0.09	1.50				
				13	0.05	0.79				
				14	0.06	0.91				
				Mean	**0.06**	**1.00**				
			50	16	0.08	1.36				
				17	0.12	1.91				
				18	0.06	0.99				
				19	0.08	1.26				
				Mean	**0.08**	**1.38**				
			0	21	0.05	0.77				
				22	0.11	1.60				
				23	0.06	0.78				
				24	0.06	0.85				
				Mean	**0.07**	**1.00**				
			100	26	0.11	1.60				
				27	0.13	1.87				
				28	0.14	2.03				
				29	0.11	1.53				
				Mean	**0.12**	**1.76**				
Linalool	2009	AOO	0	1	0.11	0.81	NC	NC	NC	NC
				2	0.19	1.35				
				3	0.12	0.85				
				4	0.14	0.99				
				Mean	**0.14**	**1.00**				
			25	6	0.20	1.48				
				7	0.15	1.13				
				8	0.09	0.69				
				9	0.26	1.86				
				Mean	**0.18**	**1.29**				
			50	11	0.30	2.16				
				12	0.15	1.09				
				13	0.20	1.43				
				14	0.15	1.11				
				Mean	**0.20**	**1.45**				
			100	16	0.19	1.37				
				17	0.21	1.53				
				18	0.12	0.87				
				19	0.13	0.94				
				Mean	**0.16**	**1.18**				
Trimelittic anhydride	2009	AOO	0	1	0.07	0.96	1.76	1.81	1.97	2.03
				2	0.07	0.89				
				3	0.09	1.16				
				Mean	**0.07**	**1.00**				

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Trimelittic anhydride (continued)	2009	AOO	2.5	4	0.19	2.53				
				5	0.26	3.51				
				6	0.16	2.18				
				Mean	**0.19**	**2.74**				
			5	7	0.30	4.05				
				8	0.47	6.31				
				9	0.39	5.22				
				Mean	**0.30**	**5.19**				
			10	7	0.54	7.22				
				8	0.55	7.33				
				9	0.67	8.99				
				Mean	**0.54**	**7.85**				
VC	2009	DMSO	0	1	0.09	0.60	NA	NA	NA	NA
				2	0.18	1.13				
				3	0.19	1.18				
				4	0.18	1.10				
				Mean	**0.16**	**1.00**				
Cobalt chloride	2009	DMSO	0.5	5	0.30	1.92	0.27	0.32	0.47	0.63
				6	0.25	1.61				
				7	0.31	1.96				
				8	0.37	2.32				
				Mean	**0.31**	**1.95**				
			5	9	0.55	3.47				
				10	0.60	3.76				
				11	0.53	3.35				
				12	0.65	4.13				
				Mean	**0.58**	**3.68**				
VC	2009	DMF	0	1	0.10	0.73	NA	NA	NA	NA
				2	0.16	1.16				
				3	0.12	0.89				
				4	0.17	1.22				
				Mean	**0.14**	**1.00**				
5-Chloro-2-methyl-4-isothiazolin-3-one solution	2009	DMF	0.01	5	0.27	2.02	0.05	0.07	0.11	0.12
				6	0.10	0.77				
				7	0.09	0.66				
				8	0.18	1.33				
				Mean	**0.16**	**1.19**				
			0.5	9	0.93	6.89				
				10	0.66	4.85				
				11	0.52	3.85				
				12	0.51	3.74				
				Mean	**0.65**	**4.83**				

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Sodium lauryl sulfate	2009	DMF	10	5	0.09	0.68	13.01	13.33	14.30	14.63
				6	0.08	0.61				
				7	0.10	0.73				
				8	0.04	0.27				
				Mean	**0.08**	**0.57**				
			16.7	9	0.39	2.91				
				10	0.43	3.19				
				11	0.41	3.04				
				12	0.19	1.41				
				Mean	**0.36**	**2.64**				
Imidazolidinyl urea	2009	DMF	10	5	0.09	0.70	45.00	49.55	NC	NC
				6	0.12	0.90				
				7	0.10	0.74				
				8	0.08	0.58				
				Mean	**0.10**	**0.73**				
			50	9	0.28	2.07				
				10	0.23	1.73				
				11	0.20	1.46				
				12	0.16	1.16				
				Mean	**0.22**	**1.61**				
VC	2009	DMF	0	1	0.05	0.46	NA	NA	NA	NA
				2	0.14	1.17				
				3	0.10	0.83				
				4	0.18	1.54				
				Mean	**0.12**	**1.00**				
Sulfanlamide	2009	DMF	10	5	0.17	1.45	NC	NC	NC	NC
				6	0.08	0.68				
				7	0.08	0.73				
				8	0.13	1.09				
				Mean	**0.11**	**0.98**				
			50	9	0.20	1.70				
				10	0.11	0.93				
				11	0.12	1.06				
				12	0.16	1.36				
				Mean	**0.15**	**1.26**				
4-Methylaminophenol sulfate	2009	DMF	2	5	0.21	1.84	1.18	1.29	1.67	1.82
				6	0.16	1.34				
				7	0.21	1.77				
				8	0.41	3.50				
				Mean	**0.25**	**2.11**				

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
4-Methylaminophenol sulfate (continued)	2009	DMF	10	9	0.42	3.58				
				10	0.32	2.73				
				11	0.44	3.80				
				12	0.67	5.80				
				Mean	**0.46**	**3.98**				
Ethlene glycol dimethacrylate	2009	MEK	0	1	0.06	0.80	27.23	31.75	45.31	49.83
				2	0.06	0.83				
				3	0.11	1.42				
				4	0.07	0.94				
				Mean	**0.07**	**1.00**				
			20	5	0.10	1.41				
				6	0.12	1.63				
				7	0.09	1.28				
				8	0.08	1.05				
				Mean	**0.10**	**1.34**				
			100	9	0.18	2.46				
				10	0.23	3.14				
				11	0.29	3.93				
				12	0.21	2.89				
				Mean	**0.23**	**3.11**				
VC	2009	AOO	0	1	0.04	0.41	NA	NA	NA	NA
				2	0.20	1.87				
				3	0.10	0.91				
				4	0.09	0.82				
				Mean	**0.11**	**1.00**				
Cinnamic alcohol	2009	AOO	10	5	0.16	1.46	21.82	24.09	30.91	33.18
				6	0.10	0.94				
				7	0.06	0.54				
				8	0.10	0.99				
				Mean	**0.10**	**0.98**				
			50	9	0.28	2.61				
				10	0.31	2.97				
				11	0.27	2.51				
				12	0.31	2.89				
				Mean	**0.29**	**2.74**				
Ethyl acrylate	2009	AOO	20	5	0.11	1.07	31.34	33.33	39.30	41.29
				6	0.10	0.92				
				7	0.08	0.77				
				8	0.10	0.94				
				Mean	**0.99**	**0.93**				

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Ethyl acrylate (continued)	2009	AOO	100	9	0.56	5.31				
				10	0.67	6.37				
				11	0.38	3.65				
				12	0.47	4.47				
				Mean	**0.52**	**4.95**				
Diethyl maleate	2009	AOO	5	5	0.06	0.60	7.69	8.05	9.14	9.50
				6	0.06	0.61				
				7	0.11	1.05				
				8	0.08	0.79				
				Mean	**0.08**	**0.76**				
			25	9	0.67	6.32				
				10	0.97	9.14				
				11	0.55	5.23				
				12	0.47	4.40				
				Mean	**0.66**	**6.27**				
VC	2009	AOO	0	1	0.04	0.69	NA	NA	NA	NA
				2	0.09	1.43				
				3	0.06	0.96				
				4	0.06	0.93				
				Mean	**0.06**	**1.00**				
Phenyl benzoate	2009	AOO	10	5	0.06	0.97	NC	NC	NC	NC
				6	0.04	0.70				
				7	0.05	0.81				
				8	0.06	1.04				
				Mean	**0.05**	**0.88**				
			33	9	0.06	1.08				
				10	0.06	0.93				
				11	0.11	1.88				
				12	0.04	0.73				
				Mean	**0.07**	**1.15**				
Salicylic acid	2009	AOO	10	5	0.05	0.77	NC	NC	NC	NC
				6	0.08	1.42				
				7	0.04	0.70				
				8	0.06	1.04				
				Mean	**0.06**	**0.98**				
			25	9	0.06	0.95				
				10	0.08	1.39				
				11	0.09	1.49				
				12	0.07	1.22				
				Mean	**0,08**	**1.26**				

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Aniline	2009	AOO	0	1	0.07	0.86	67.90	73.60	90.27	95.86
				2	0.09	1.10				
				3	0.09	1.06				
				4	0.08	0.98				
				Mean	**0.08**	**1.00**				
			50	5	0.14	1.74				
				6	0.09	1.14				
				7	0.08	1.00				
				8	0.07	0.86				
				Mean	**0.10**	**1.20**				
			100	9	0.18	2.16				
				10	0.21	2.55				
				11	0.12	1.51				
				12	0.02	0.20				
				Mean	**0.17**	**2.10**				
Linalool	2009	AOO	0	1	0.08	0.75	25.22	27.60	34.72	37.09
				2	0.10	0.96				
				3	0.13	1.21				
				4	0.11	1.08				
				Mean	**0.11**	**1.00**				
			20	5	0.14	1.37				
				6	0.07	0.64				
				7	0.07	0.62				
				8	0.26	2.50				
				Mean	**0.14**	**1.30**				
			100	9	0.35	3.30				
				10	0.52	4.95				
				11	0.63	6.02				
				12	0.46	4.35				
				Mean	**0.49**	**4.70**				
VC	2009	AOO	0	1	0.05	0.70	NA	NA	NA	NA
				2	0.06	0.87				
				3	0.08	1.21				
				4	0.09	1.22				
				Mean	**0.07**	**1.00**				
Hydroxycitronellal	2009	AOO	20	5	0.19	2.69	16.20	17.12	20.55	23.31
				6	0.11	1.63				
				7	0.08	1.08				
				8	0.15	2.13				
				Mean	**0.13**	**1.88**				

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Hydroxycitronellal (continued)	2009	AOO	100	9	0.26	3.71				
				10	0.32	4.56				
				11	0.39	5.58				
				12	0.37	5.27				
				Mean	**0.33**	**4.78**				
Cyclamen aldehyde	2009	AOO	20	5	0.19	2.78	10.47	11.05	12.97	13.68
				6	0.24	3.40				
				7	0.19	2.66				
				8	0.14	2.01				
				Mean	**0.19**	**2.71**				
			100	9	0.41	5.81				
				10	0.41	5.93				
				11	0.37	5.34				
				12	0.40	5.78				
				Mean	**0.40**	**5.72**				
Phenyl benzoate	2009	DMF	0	1	0.12	1.17	14.14	16.95	25.39	28.20
				2	0.09	0.87				
				3	0.04	0.38				
				4	0.16	1.57				
				Mean	**0.10**	**1.00**				
			13.3	5	0.14	1.30				
				6	0.10	1.01				
				7	0.19	1.82				
				8	0.18	1.75				
				Mean	**0,15**	**1.47**				
			66.7	9	0.45	4.34				
				10	0.47	4.54				
				11	0.23	2.19				
				12	0.25	2.42				
				Mean	**0.35**	**3.37**				

Abbreviations: ABS = absorbance; An. No. = animal number; AOO = acetone: olive oil (4:1); Conc. = concentration; DHEA = 2,2'-dihydroxyl-3,3'-dimethoxy-5,5'-diallyl-biphenyl; DHEB = 4,5'-diallyl-2'-hydroxy-2,3'-dimethoxyphenyl ether; DMF = *N*,*N*-dimethylformamide; DMSO = dimethyl sulfoxide; DNCB = 2,4-dinitrochlorobenzene; EC = estimated concentration needed to produce a stimulation index of 1.5 (EC1.5), 1.6 (EC1.6); 1.9 (EC1.9), or 2 (EC2); HCA = hexyl cinnamic aldehyde; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; NA = not applicable; NC = not calculated because SI was not high enough to calculate EC1.5, EC1,6, EC1.9, or EC2 or because only one dose was tested; Ref. = year of Takeyoshi et al. reference for the data; SI = stimulation index; VC = vehicle control; Veh. = vehicle.

[1] mean of 3 replicates.

* EC values were calculated by linear interpolation using SI =1 and concentration = 0 as the lowest point because the dose-response was nonmonotonic.

Annex IV-2

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

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Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

Substance	Lab	Vehicle	Conc. (%)	Animal No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
VP	1	AOO	0	1	0.24	1.17	NA	NA	NA	NA
				2	0.20	0.95				
				3	0.18	0.88				
				4	0.21	1.00				
				Mean	**0.21**	**1.00**				
PC	1	AOO	0	1	0.49	2.33	NA	NA	NA	NA
				2	0.40	1.92				
				3	0.48	2.29				
				4	0.36	1.73				
				Mean	**0.43**	**2.07**				
VS	1	AOO	0	1	0.29	0.96	NA	NA	NA	NA
				2	0.30	1.00				
				3	0.37	1.22				
				4	0.25	0.82				
				Mean	**0.43**	**2.07**				
Methyl salicylate	1	AOO	10	1	0.43	1.42	NC	NC	NC	NC
				2	0.38	1.26				
				3	0.41	1.34				
				4	0.51	1.69				
				Mean	**0.43**	**1.43**				
			25	1	0.56	1.86				
				2	0.31	1.04				
				3	0.29	0.95				
				4	0.51	1.68				
				Mean	**0.42**	**1.38**				
			0.5	1	0.33	1.08				
				2	0.36	1.21				
				3	0.40	1.34				
				4	0.43	1.43				
				Mean	**0.38**	**1.26**				
2, 4-Dinitrochlorobenzene	1	AOO	0.1	1	0.68	2.24	0.06	0.06	0.08	0.08
				2	0.58	1.93				
				3	0.90	3.00				
				4	0.54	1.77				
				Mean	**0.67**	**2.23**				
			0.3	1	1.15	3.82				
				2	1.31	4.34				
				3	0.87	2.88				
				4	1.11	3.66				
				Mean	**1.11**	**3.68**				

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

Substance	Lab	Vehicle	Conc. (%)	Animal No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
2, 4-Dinitrochlorobenzene (continued)	1	AOO	1	1	1.25	4.16				
				2	1.23	4.07				
				3	1.23	4.08				
				4	1.48	4.90				
				Mean	**1.30**	**4.30**				
VP	1	AOO	0	1	0.04	0.67	NA	NA	NA	NA
				2	0.07	1.27				
				3	0.03	0.60				
				4	0.08	1.47				
				Mean	**0.06**	**1.00**				
PC	1	AOO	0	1	0.22	4.04	NA	NA	NA	NA
				2	0.37	6.73				
				3	0.38	6.88				
				4	0.38	6.79				
				Mean	**0.34**	**6.11**				
VS	1	AOO	0	1	0.15	0.93	NA	NA	NA	NA
				2	0.27	1.69				
				3	0.11	0.69				
				4	0.11	0.69				
				Mean	**0.16**	**1.00**				
Hexyl cinnamic aldehyde	1	AOO	10	1	0.23	1.48	9.40	10.40	14.80	16.20
				2	0.22	1.36				
				3	0.25	1.60				
				4	0.29	1.83				
				Mean	**0.25**	**1.57**				
			25	1	0.35	2.20				
				2	0.45	2.87				
				3	0.42	2.69				
				4	0.42	2.68				
				Mean	**0.41**	**2.61**				
			50	1	0.48	3.04				
				2	0.57	3.62				
				3	0.61	3.87				
				4	0.49	3.09				
				Mean	**0.54**	**3.40**				
Isopropanol	1	AOO	10	1	0.20	1.27	4.10*	4.92*	7.38*	8.20*
				2	0.68	4.28				
				3	0.28	1.78				
				4	0.24	1.53				
				Mean	**0.35**	**2.22**				

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

Substance	Lab	Vehicle	Conc. (%)	Animal No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Isopropanol (continued)	1	AOO	25	1	0.15	0.97				
				2	0.13	0.85				
				3	0.10	0.64				
				4	0.09	0.59				
				Mean	**0.12**	**0.76**				
			50	1	0.16	0.98				
				2	0.11	0.69				
				3	0.11	0.71				
				4	0.20	1.29				
				Mean	**0.15**	**0.92**				
VP	1	ACE	0	1	0.13	1.61	NA	NA	NA	NA
				2	0.07	0.80				
				3	0.05	0.57				
				4	0.08	1.01				
				Mean	**0.08**	**1.00**				
PC	1	ACE	0	1	0.23	2.83	NA	NA	NA	NA
				2	0.33	4.05				
				3	0.31	3.83				
				4	0.25	3.04				
				Mean	**0.28**	**3.44**				
VS	1	ACE	0	1	0.09	0.87	NA	NA	NA	NA
				2	0.09	0.84				
				3	0.17	1.57				
				4	0.08	0.73				
				Mean	**0.11**	**1.00**				
Gluteraldehyde	1	ACE	0.1	1	0.12	1.09	0.06	0.08	0.14	0.18
				2	0.21	1.96				
				3	0.25	2.31				
				4	0.18	1.66				
				Mean	**0.19**	**1.76**				
			0.3	1	0.25	2.30				
				2	0.26	2.41				
				3	0.33	3.11				
				4	0.19	1.76				
				Mean	**0.26**	**2.40**				
			1	1	0.27	2.47				
				2	0.54	5.03				
				3	0.35	3.30				
				4	0.44	4.10				
				Mean	**0.40**	**3.72**				

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

Substance	Lab	Vehicle	Conc. (%)	Animal No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Formaldehyde	1	ACE	1	1	0.28	2.59	0.27	0.29	0.37	0.41
				2	0.51	4.75				
				3	0.27	2.47				
				4	0.27	2.52				
				Mean	**0.33**	**3.08**				
			3	1	0.50	4.69				
				2	0.50	4.69				
				3	0.53	4.92				
				4	0.35	3.28				
				Mean	**0.47**	**4.40**				
			10	1	0.18	1.71				
				2	0.22	2.00				
				3	0.18	1.70				
				4	0.18	1.72				
				Mean	**0.19**	**1.78**				
VP	2	AOO	0	1	0.35	1.09	NA	NA	NA	NA
				2	0.30	0.93				
				3	0.34	1.05				
				4	0.30	0.93				
				Mean	**0.32**	**1.00**				
PC[2]	2	AOO	0	1	0.30	0.93	NA	NA	NA	NA
				2	0.38	1.17				
				3	0.50	1.53				
				4	0.49	1.53				
				Mean	**0.42**	**1.29**				
VS	2	AOO	0	1	0.21	0.70	NA	NA	NA	NA
				2	0.33	1.06				
				3	0.35	1.14				
				4	0.34	1.10				
				Mean	**0.31**	**1.00**				
Hexyl cinnamic aldehyde[2]	2	AOO	10	1	0.41	1.33	20.96	27.90	NC	NC
				2	0.43	1.39				
				3	0.36	1.17				
				4	0.42	1.37				
				Mean	**0.40**	**1.31**				
			25	1	0.45	1.47				
				2	0.57	1.84				
				3	0.54	1.77				
				4	0.37	1.19				
				Mean	**0.48**	**1.57**				

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

Substance	Lab	Vehicle	Conc. (%)	Animal No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Hexyl cinnamic aldehyde (continued)	2	AOO	50	1	0.49	1.61				
				2	0.66	2.14				
				3	0.52	1.71				
				4	0.58	1.88				
				Mean	**0.56**	**1.83**				
Isopropanol[2]	2	AOO	10	1	0.35	1.13	NC	NC	NC	NC
				2	0.39	1.27				
				3	0.27	0.87				
				4	0.33	1.07				
				Mean	**0.33**	**1.09**				
			25	1	0.31	1.02				
				2	0.24	0.77				
				3	0.11	0.36				
				4	0.38	1.23				
				Mean	**0.26**	**0.85**				
			50	1	0.24	0.78				
				2	0.25	0.81				
				3	0.36	1.16				
				4	0.17	0.55				
				Mean	**0.25**	**0.83**				
VP	2	AOO	0	1	0.14	1.06	NA	NA	NA	NA
				2	0.13	0.99				
				3	0.12	0.94				
				4	0.13	1.01				
				Mean	**0.13**	**1.00**				
PC	2	AOO	0	1	0.50	3.82	NA	NA	NA	NA
				2	0.61	4.62				
				3	0.66	5.01				
				4	0.94	7.14				
				Mean	**0.68**	**5.15**				
VS	2	AOO	0	1	0.13	0.74	NA	NA	NA	NA
				2	0.13	0.72				
				3	0.22	1.23				
				4	0.23	1.30				
				Mean	**0.18**	**1.00**				
2, 4-Dinitrochlorobenzene	2	AOO	0.1	1	1.06	5.96	0.010*	0.011*	0.017*	0.019*
				2	1.09	6.13				
				3	1.31	7.35				
				4	1.09	6.12				
				Mean	**1.14**	**6.39**				

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

Substance	Lab	Vehicle	Conc. (%)	Animal No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
2, 4-Dinitrochlorobenzene (continued)	2	AOO	0.3	1	0.94	5.26				
				2	1.10	6.18				
				3	1.30	7.28				
				4	1.32	7.39				
				Mean	**1.16**	**6.53**				
			1	1	1.32	7.42				
				2	1.49	8.38				
				3	1.53	8.60				
				4	1.62	9.07				
				Mean	**1.49**	**8.37**				
trans-Cinnamaldehyde	2	AOO	1	1	0.18	0.99	1.71	1.88	2.42	2.59
				2	0.17	0.94				
				3	0.24	1.34				
				4	0.20	1.14				
				Mean	**0.20**	**1.10**				
			3	1	0.35	1.95				
				2	0.39	2.16				
				3	0.48	2.71				
				4	0.37	2.10				
				Mean	**0.40**	**2.23**				
			10	1	0.54	3.05				
				2	0.56	3.12				
				3	0.59	3.30				
				4	0.71	4.00				
				Mean	**0.60**	**3.37**				
VP	2	AOO	0	1	0.20	1.16	NA	NA	NA	NA
				2	0.16	0.89				
				3	0.15	0.87				
				4	0.19	1.07				
				Mean	**0.17**	**1.00**				
PC	2	AOO	0	1	0.44	2.55	NA	NA	NA	NA
				2	0.49	2.83				
				3	0.40	2.32				
				4	0.41	2.37				
				Mean	**0.44**	**2.52**				
VS	2	AOO	0	1	0.22	1.26	NA	NA	NA	NA
				2	0.11	0.61				
				3	0.22	1.26				
				4	0.15	0.87				
				Mean	**0.17**	**1.00**				

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

Substance	Lab	Vehicle	Conc. (%)	Animal No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Eugenol	2	AOO	10	1	0.24	1.40	12.50	13.80	17.80	19.08
				2	0.24	1.38				
				3	0.18	1.01				
				4	0.25	1.46				
				Mean	**0.23**	**1.31**				
			25	1	0.31	1.79				
				2	0.47	2.75				
				3	0.44	2.57				
				4	0.46	2.69				
				Mean	**0.42**	**2.45**				
			50	1	0.52	3.01				
				2	0.50	2.91				
				3	0.56	3.26				
				4	0.60	3.47				
				Mean	**0.55**	**3.17**				
Methyl salicylate	2	AOO	10	1	0.20	1.13	NC	NC	NC	NC
				2	0.22	1.26				
				3	0.16	0.93				
				4	0.19	1.12				
				Mean	**0.19**	**1.11**				
			25	1	0.20	1.14				
				2	0.20	1.17				
				3	0.23	1.34				
				4	0.17	1.00				
				Mean	**0.20**	**1.16**				
			50	1	0.28	1.65				
				2	0.19	1.12				
				3	0.27	1.58				
				4	0.24	1.41				
				Mean	**0.25**	**1.44**				
VP	3	AOO	0	1	0.25	1.05	NA	NA	NA	NA
				2	0.29	1.21				
				3	0.14	0.59				
				4	0.28	1.14				
				Mean	**0.24**	**1.00**				
PC	3	AOO	0	1	0.68	2.82	NA	NA	NA	NA
				2	1.01	4.21				
				3	0.65	2.70				
				4	0.87	3.63				
				Mean	**0.80**	**3.34**				

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

Substance	Lab	Vehicle	Conc. (%)	Animal No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
VS	3	AOO	0	1	0.21	0.93	NA	NA	NA	NA
				2	0.17	0.78				
				3	0.21	0.97				
				4	0.29	1.32				
				Mean	**0.22**	**1.00**				
Methyl salicylate	3	AOO	10	1	0.23	1.06	NC	NC	NC	NC
				2	0.34	1.54				
				3	0.13	0.59				
				4	0.27	1.21				
				Mean	**0.24**	**1.10**				
			25	1	0.39	1.78				
				2	0.24	1.07				
				3	0.12	0.54				
				4	0.32	1.46				
				Mean	**0.27**	**1.21**				
			50	1	0.26	1.18				
				2	0.47	2.15				
				3	0.18	0.79				
				4	0.33	1.49				
				Mean	**0.31**	**1.40**				
2, 4-Dinitrochlorobenzene	3	AOO	0.1	1	0.77	3.50	0.022	0.023	0.027	0.029
				2	0.81	3.69				
				3	0.98	4.44				
				4	1.20	5.45				
				Mean	**0.94**	**4.27**				
			0.3	1	1.28	5.82				
				2	1.35	6.13				
				3	1.31	5.96				
				4	1.57	7.11				
				Mean	**1.38**	**6.26**				
			10	1	1.26	5.71				
				2	1.28	5.82				
				3	1.30	5.90				
				4	1.44	6.52				
				Mean	**1.32**	**5.99**				
VP	3	AOO	0	1	0.26	1.26	NA	NA	NA	NA
				2	0.20	1.00				
				3	0.15	0.73				
				4	0.21	1.01				
				Mean	**0.20**	**1.00**				

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

Substance	Lab	Vehicle	Conc. (%)	Animal No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
PC	3	AOO	0	1	0.77	3.77	NA	NA	NA	NA
				2	1.01	4.96				
				3	0.52	2.53				
				4	0.59	2.89				
				Mean	**0.72**	**3.54**				
VS	3	AOO	0	1	0.36	1.34	NA	NA	NA	NA
				2	0.18	0.67				
				3	0.32	1.20				
				4	0.21	0.79				
				Mean	**0.27**	**1.00**				
Hexyl cinnamic aldehyde	3	AOO	10	1	0.48	1.79	15.23	17.00	22.20	23.95
				2	0.35	1.33				
				3	0.23	0.85				
				4	0.22	0.84				
				Mean	**0.32**	**1.20**				
			25	1	0.55	2.06				
				2	0.72	2.72				
				3	0.30	1.14				
				4	0.62	2.32				
				Mean	**0.55**	**2.06**				
			50	1	0.57	2.13				
				2	1.01	3.82				
				3	0.64	2.42				
				4	0.83	3.14				
				Mean	**0.76**	**2.87**				
Isopropanol	3	AOO	10	1	0.30	1.14	NC	NC	NC	NC
				2	0.35	1.31				
				3	0.13	0.50				
				4	0.26	0.97				
				Mean	**0.26**	**0.98**				
			25	1	0.27	1.01				
				2	0.28	1.06				
				3	0.16	0.61				
				4	0.20	0.73				
				Mean	**0.23**	**0.85**				
			50	1	0.21	0.79				
				2	0.29	1.08				
				3	0.13	0.47				
				4	0.17	0.64				
				Mean	**0.20**	**0.75**				

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

Substance	Lab	Vehicle	Conc. (%)	Animal No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
VP	3	DMSO	0	1	0.33	1.04	NA	NA	NA	NA
				2	0.42	1.32				
				3	0.18	0.56				
				4	0.34	1.08				
				Mean	**0.32**	**1.00**				
PC	3	DMSO	0	1	0.67	2.10	NA	NA	NA	NA
				2	0.94	2.98				
				3	0.60	1.90				
				4	0.55	1.72				
				Mean	**0.69**	**2.18**				
VS	3	DMSO	0	1	0.18	0.83	NA	NA	NA	NA
				2	0.28	1.25				
				3	0.15	0.67				
				4	0.27	1.24				
				Mean	**0.22**	**1.00**				
Lactic acid	3	DMSO	10	1	0.20	0.90	20.98	23.70	NC	NC
				2	0.35	1.57				
				3	0.17	0.77				
				4	0.25	1.12				
				Mean	**0.24**	**1.09**				
			25	1	0.45	2.05				
				2	0.46	2.07				
				3	0.21	0.97				
				4	0.34	1.53				
				Mean	**0.37**	**1.65**				
			50	1	0.40	1.83				
				2	0.63	2.84				
				3	0.21	0.97				
				4	0.35	1.56				
				Mean	**0.40**	**1.80**				
Nickel sulfate	3	DMSO	1	1	0.33	1.48	1.47	1.84	2.93	3.85
				2	0.48	2.19				
				3	0.14	0.61				
				4	0.27	1.22				
				Mean	**0.30**	**1.37**				
			3	1	0.43	1.93				
				2	0.61	2.75				
				3	0.26	1.19				
				4	0.40	1.81				
				Mean	**0.42**	**1.92**				

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

Substance	Lab	Vehicle	Conc. (%)	Animal No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Nickel sulfate (continued)	3	DMSO	10	1	0.70	3.16				
				2	0.64	2.91				
				3	0.42	1.92				
				4	0.52	2.34				
				Mean	**0.57**	**2.58**				
VP	4	AOO	0	1	0.23	0.81	NA	NA	NA	NA
				2	0.21	0.74				
				3	0.33	1.19				
				4	0.35	1.26				
				Mean	**0.28**	**1.00**				
PC	4	AOO	0	1	0.83	2.95	NA	NA	NA	NA
				2	0.71	2.54				
				3	0.76	2.71				
				4	0.72	2.56				
				Mean	**0.76**	**2.69**				
VS	4	AOO	0	1	0.22	0.80	NA	NA	NA	NA
				2	0.25	0.91				
				3	0.36	1.32				
				4	0.26	0.97				
				Mean	**0.27**	**1.00**				
2, 4-Dinitrochlorobenzene	4	AOO	0.1	1	1.04	3.83	0.022	0.023	0.028	0.030
				2	1.05	3.87				
				3	0.88	3.23				
				4	1.06	3.90				
				Mean	**1.01**	**3.71**				
			0.3	1	1.43	5.27				
				2	1.38	5.10				
				3	1.44	5.30				
				4	1.49	5.50				
				Mean	**1.43**	**5.29**				
			1	1	1.43	5.27				
				2	1.54	5.68				
				3	1.47	5.43				
				4	1.52	5.62				
				Mean	**1.49**	**5.50**				
trans-Cinnamaldehyde	4	AOO	1	1	0.60	2.21	0.95	1.04	1.48	1.63
				2	0.22	0.80				
				3	0.38	1.41				
				4	0.51	1.86				
				Mean	**0.43**	**1.57**				

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

Substance	Lab	Vehicle	Conc. (%)	Animal No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
trans-Cinnamaldehyde (continued)	4	AOO	3	1	0.80	2.94				
				2	0.86	3.15				
				3	0.70	2.59				
				4	0.83	3.07				
				Mean	**0.80**	**2.94**				
			10	1	0.89	3.28				
				2	1.12	4.11				
				3	0.77	2.85				
				4	1.01	3.73				
				Mean	**0.95**	**3.50**				
VP	4	AOO	0	1	0.26	1.16	NA	NA	NA	NA
				2	0.24	1.08				
				3	0.19	0.84				
				4	0.21	0.92				
				Mean	**0.22**	**1.00**				
PC	4	AOO	0	1	0.50	2.25	NA	NA	NA	NA
				2	0.89	3.99				
				3	0.55	2.44				
				4	0.90	4.00				
				Mean	**0.71**	**3.17**				
VS	4	AOO	0	1	0.40	1.65	NA	NA	NA	NA
				2	0.22	0.92				
				3	0.17	0.72				
				4	0.17	0.71				
				Mean	**0.24**	**1.00**				
Isopropanol	4	AOO	0.1	1	0.19	0.78	45.10	NC	NC	NC
				2	0.09	0.37				
				3	0.24	0.99				
				4	0.44	1.83				
				Mean	**0.24**	**0.99**				
			0.25	1	0.58	2.41				
				2	0.11	0.46				
				3	0.22	0.92				
				4	0.26	1.06				
				Mean	**0.29**	**1.21**				
			0.5	1	0.19	0.78				
				2	0.53	2.21				
				3	0.52	2.15				
				4	0.28	1.15				
				Mean	**0.38**	**1.57**				

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

Substance	Lab	Vehicle	Conc. (%)	Animal No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Hexyl cinnamic aldehyde	4	AOO	10	1	0.58	2.39	4.07	4.80	7.92	9.36
				2	0.40	1.67				
				3	0.62	2.57				
				4	0.36	1.51				
				Mean	**0.49**	**2.04**				
			25	1	0.66	2.73				
				2	0.65	2.69				
				3	0.61	2.54				
				4	0.58	2.40				
				Mean	**0.63**	**2.59**				
			50	1	0.99	4.11				
				2	0.83	3.45				
				3	0.78	3.25				
				4	0.61	2.54				
				Mean	**0.80**	**3.34**				
VP	4	DMSO	0	1	0.05	0.32	NA	NA	NA	NA
				2	0.23	1.48				
				3	0.20	1.28				
				4	0.14	0.92				
				Mean	**0.15**	**1.00**				
PC	4	DMSO	0	1	1.04	6.75	NA	NA	NA	NA
				2	1.08	7.01				
				3	1.07	6.92				
				4	0.86	5.60				
				Mean	**1.01**	**6.57**				
VS	4	DMSO	0	1	0.16	0.75	NA	NA	NA	NA
				2	0.11	0.51				
				3	0.29	1.36				
				4	0.29	1.38				
				Mean	**0.21**	**1.00**				
Lactic acid	4	DMSO	10	1	0.24	1.13	3.43	5.71	NC	NC
				2	0.47	2.25				
				3	0.28	1.34				
				4	0.44	2.11				
				Mean	**0.36**	**1.71**				
			25	1	0.33	1.56				
				2	0.45	2.14				
				3	0.34	1.63				
				4	0.47	2.24				
				Mean	**0.40**	**1.89**				

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

Substance	Lab	Vehicle	Conc. (%)	Animal No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Lactic acid (continued)	4	DMSO	50	1	0.44	2.11				
				2	0.37	1.78				
				3	0.26	1.22				
				4	0.30	1.41				
				Mean	**0.34**	**1.63**				
Nickel sulfate	4	DMSO	1	1	0.35	1.66	0.48*	0.57*	0.86*	0.95*
				2	0.44	2.10				
				3	0.35	1.65				
				4	0.59	2.78				
				Mean	**0.43**	**2.05**				
			3	1	0.29	1.39				
				2	0.34	1.63				
				3	0.48	2.30				
				4	0.56	2.66				
				Mean	**0.42**	**2.00**				
			10	1	0.94	4.45				
				2	0.70	3.32				
				3	0.71	3.36				
				4	1.47	6.98				
				Mean	**0.95**	**4.53**				
VP	5	ACE	0	1	0.04	0.29	NA	NA	NA	NA
				2	0.15	1.17				
				3	0.07	0.52				
				4	0.25	2.01				
				Mean	**0.13**	**1.00**				
PC	5	ACE	0	1	1.40	11.10	NA	NA	NA	NA
				2	0.64	5.05				
				3	2.37	18.78				
				4	1.87	14.87				
				Mean	**1.57**	**12.45**				
VS	5	ACE	0	1	0.05	0.94	NA	NA	NA	NA
				2	0.01	0.19				
				3	0.11	2.07				
				4	0.04	0.81				
				Mean	**0.05**	**1.00**				
Formaldehyde	5	ACE	1	1	0.13	2.52	0.15*	0.19*	0.28*	0.31*
				2	0.27	5.01				
				3	0.06	1.15				
				4	0.44	8.26				
				Mean	**0.23**	**4.23**				

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

Substance	Lab	Vehicle	Conc. (%)	Animal No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Formaldehyde (continued)	5	ACE	3	1	0.07	1.26				
				2	0.12	2.18				
				3	0.08	1.56				
				4	0.09	1.63				
				Mean	**0.09**	**1.66**				
			10	1	0.54	10.22				
				2	0.30	5.60				
				3	0.85	16.02				
				4	1.84	34.52				
				Mean	**0.88**	**16.59**				
Gluteraldehyde	5	ACE	0.001	1	0.22	4.17	0.031	0.031	0.033	0.034
				2	0.29	5.37				
				3	0.20	3.66				
				4	0.88	16.47				
				Mean	**0.40**	**7.42**				
			0.003	1	0.22	4.09				
				2	0.97	18.22				
				3	0.51	9.56				
				4	1.06	19.87				
				Mean	**0.69**	**12.93**				
			0.01	1	1.65	30.93				
				2	0.98	18.40				
				3	1.77	33.31				
				4	1.70	31.91				
				Mean	**1.53**	**28.64**				
VP	5	AOO	0	1	0.05	0.30	NA	NA	NA	NA
				2	0.18	1.13				
				3	0.13	0.79				
				4	0.29	1.78				
				Mean	**0.16**	**1.00**				
PC	5	AOO	0	1	0.61	3.76	NA	NA	NA	NA
				2	0.82	5.10				
				3	0.91	5.61				
				4	0.40	2.47				
				Mean	**0.68**	**4.24**				
VS	5	AOO	0	1	0.09	0.61	NA	NA	NA	NA
				2	0.17	1.15				
				3	0.18	1.19				
				4	0.16	1.05				
				Mean	**0.15**	**1.00**				

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

Substance	Lab	Vehicle	Conc. (%)	Animal No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
2, 4-Dinitrochlorobenzene	5	AOO	0.1	1	2.56	17.07	0.0022	0.0022	0.0024	0.0025
				2	1.96	13.07				
				3	2.21	14.74				
				4	2.24	14.92				
				Mean	**2.24**	**14.95**				
			0.3	1	2.72	18.14				
				2	2.79	18.61				
				3	2.90	19.35				
				4	2.86	19.08				
				Mean	**2.82**	**18.80**				
			1	1	2.77	18.45				
				2	2.67	17.79				
				3	2.21	14.74				
				4	2.51	16.76				
				Mean	**2.54**	**16.94**				
trans-Cinnamaldehyde	5	AOO	1	1	0.12	0.78	1.75	1.96	2.58	2.79
				2	0.08	0.56				
				3	0.28	1.85				
				4	0.21	1.38				
				Mean	**0.17**	**1.14**				
			3	1	0.40	2.69				
				2	0.28	1.87				
				3	0.28	1.87				
				4	0.30	1.98				
				Mean	**0.32**	**2.10**				
			10	1	0.57	3.79				
				2	0.50	3.34				
				3	0.70	4.66				
				4	0.70	4.65				
				Mean	**0.62**	**4.11**				
VP	5	AOO	0	1	0.16	1.41	NA	NA	NA	NA
				2	0.04	0.33				
				3	0.08	0.73				
				4	0.17	1.52				
				Mean	**0.11**	**1.00**				
PC	5	AOO	0	1	0.69	6.17	NA	NA	NA	NA
				2	0.58	5.15				
				3	0.90	8.02				
				4	0.55	4.90				
				Mean	**0.68**	**6.06**				

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

Substance	Lab	Vehicle	Conc. (%)	Animal No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
VS	5	AOO	0	1	0.04	0.63	NA	NA	NA	NA
				2	0.08	1.48				
				3	0.03	0.62				
				4	0.07	1.27				
				Mean	**0.06**	**1.00**				
Isopropanol	5	AOO	10	1	0.09	1.56	NC	NA	NC	NC
				2	0.04	0.74				
				3	0.05	0.92				
				4	0.03	0.52				
				Mean	**0.05**	**0.94**				
			25	1	0.05	0.92				
				2	0.02	0.43				
				3	0.03	0.52				
				4	0.05	0.89				
				Mean	**0.04**	**0.69**				
			50	1	0.03	0.62				
				2	0.04	0.78				
				3	0.03	0.54				
				4	0.05	0.94				
				Mean	**0.04**	**0.72**				
Hexyl cinnamic aldehyde	5	AOO	10	1	0.44	7.89	3.54	3.64	3.96	4.07
				2	0.07	1.32				
				3	0.20	3.58				
				4	0.46	8.24				
				Mean	**0.29**	**5.26**				
			25	1	0.26	4.74				
				2	0.92	16.65				
				3	0.20	3.57				
				4	0.52	9.36				
				Mean	**0.47**	**8.58**				
			50	1	0.93	16.89				
				2	0.37	6.61				
				3	1.10	19.95				
				4	0.58	10.55				
				Mean	**0.75**	**13.50**				
VP	6	AOO	0	1	0.30	1.97	NA	NA	NA	NA
				2	0.08	0.55				
				3	0.15	0.99				
				4	0.07	0.49				
				Mean	**0.15**	**1.00**				

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

Substance	Lab	Vehicle	Conc. (%)	Animal No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
PC	6	AOO	0	1	0.66	4.40	NA	NA	NA	NA
				2	0.66	4.39				
				3	1.21	8.09				
				4	0.64	4.27				
				Mean	**0.79**	**5.29**				
VS	6	AOO	0	1	0.21	0.84	NA	NA	NA	NA
				2	0.13	0.53				
				3	0.37	1.47				
				4	0.29	1.16				
				Mean	**0.25**	**1.00**				
Hexyl cinnamic aldehyde	6	AOO	10	1	0.29	1.13	7.90	8.60	11.70	13.03
				2	0.48	1.91				
				3	0.33	1.32				
				4	0.70	2.76				
				Mean	**0.45**	**1.78**				
			25	1	0.67	2.66				
				2	0.57	2.25				
				3	1.04	4.12				
				4	0.62	2.47				
				Mean	**0.73**	**2.87**				
			50	1	1.37	5.41				
				2	0.59	2.31				
				3	0.53	2.10				
				4	NA	NA				
				Mean	**0.83**	**3.27**				
Isopropanol	6	AOO	10	1	0.88	3.48	4.81*	5.77*	8.65*	9.62*
				2	0.20	0.79				
				3	0.47	1.86				
				4	NA	NA				
				Mean	**0.52**	**2.04**				
			25	1	0.18	0.72				
				2	0.34	1.34				
				3	0.33	1.31				
				4	NA	NA				
				Mean	**0.28**	**1.12**				
			50	1	0.27	1.07				
				2	0.56	2.20				
				3	0.32	1.28				
				4	NA	NA				
				Mean	**0.38**	**1.52**				

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

Substance	Lab	Vehicle	Conc. (%)	Animal No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
VP	6	ACE	0	1	0.12	0.65	NA	NA	NA	NA
				2	0.25	1.36				
				3	0.16	0.90				
				4	0.20	1.09				
				Mean	**0.18**	**1.00**				
PC	6	ACE	0	1	0.37	2.04	NA	NA	NA	NA
				2	0.46	2.54				
				3	0.57	3.09				
				4	0.36	1.95				
				Mean	**0.44**	**2.40**				
VS	6	ACE	0	1	0.23	1.38	NA	NA	NA	NA
				2	0.11	0.69				
				3	0.13	0.81				
				4	0.18	1.12				
				Mean	**0.16**	**1.00**				
Gluteraldehyde	6	ACE	0.1	1	0.19	1.17	0.21	0.24	0.32	0.51
				2	0.14	0.85				
				3	0.17	1.06				
				4	0.15	0.89				
				Mean	**0.16**	**0.99**				
			0.3	1	0.40	2.42				
				2	0.31	1.88				
				3	0.31	1.88				
				4	0.22	1.37				
				Mean	**0.31**	**1.89**				
			1	1	0.36	2.20				
				2	0.42	2.58				
				3	0.30	1.87				
				4	0.38	2.34				
				Mean	**0.37**	**2.25**				
Formaldehyde	6	ACE	0.01	1	0.33	2.04	0.58	0.01	0.07	NC
				2	0.20	1.20				
				3	0.23	1.38				
				4	0.29	1.77				
				Mean	**0.26**	**1.60**				
			0.03	1	0.31	1.91				
				2	0.29	1.75				
				3	0.25	1.52				
				4	0.33	2.01				
				Mean	**0.29**	**1.80**				

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

Substance	Lab	Vehicle	Conc. (%)	Animal No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Formaldehyde (continued)	6	ACE	0.1	1	0.36	2.20				
				2	0.24	1.50				
				3	0.39	2.41				
				4	0.29	1.77				
				Mean	**0.32**	**1.97**				
VP	6	AOO	0	1	0.49	1.61	NA	NA	NA	NA
				2	0.20	0.66				
				3	0.26	0.84				
				4	0.27	0.89				
				Mean	**0.30**	**1.00**				
PC	6	AOO	0	1	0.68	2.24	NA	NA	NA	NA
				2	0.83	2.72				
				3	0.68	2.24				
				4	0.88	2.88				
				Mean	**0.77**	**2.52**				
VS	6	AOO	0	1	0.27	1.30	NA	NA	NA	NA
				2	0.14	0.69				
				3	0.24	1.15				
				4	0.18	0.86				
				Mean	**0.21**	**1.00**				
Eugenol	6	AOO	10	1	0.35	1.66	10.47	11.65	15.20	16.38
				2	0.27	1.27				
				3	0.28	1.33				
				4	0.33	1.57				
				Mean	**0.31**	**1.46**				
			25	1	0.99	4.72				
				2	0.52	2.48				
				3	0.38	1.83				
				4	0.40	1.90				
				Mean	**0.57**	**2.73**				
			50	1	0.67	3.18				
				2	0.87	4.13				
				3	0.64	3.04				
				4	0.50	2.36				
				Mean	**0.67**	**3.18**				
2, 4-Dinitrochlorobenzene	6	AOO	0.1	1	0.67	3.19	0.022	0.022	0.023	0.025
				2	0.76	3.61				
				3	0.68	3.25				
				4	0.74	3.50				
				Mean	**0.71**	**3.39**				

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

Substance	Lab	Vehicle	Conc. (%)	Animal No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
2, 4-Dinitrochlorobenzene (continued)	6	AOO	0.3	1	0.91	4.33				
				2	0.89	4.24				
				3	0.86	4.12				
				4	1.11	5.31				
				Mean	**0.94**	**4.50**				
			1	1	0.92	4.39				
				2	0.96	4.59				
				3	1.07	5.09				
				4	1.11	5.27				
				Mean	**1.01**	**4.83**				
VP	7	AOO	0	1	0.04	0.49	NA	NA	NA	NA
				2	0.15	1.71				
				3	0.08	0.93				
				4	0.08	0.87				
				Mean	**0.09**	**1.00**				
PC	7	AOO	0	1	0.69	7.71	NA	NA	NA	NA
				2	0.70	7.81				
				3	0.44	4.94				
				4	0.62	6.96				
				Mean	**0.61**	**6.85**				
VS	7	AOO	0	1	0.15	1.21	NA	NA	NA	NA
				2	0.08	0.69				
				3	0.16	1.30				
				4	0.10	0.81				
				Mean	**0.12**	**1.00**				
Hexyl cinnamic aldehyde	7	AOO	10	1	0.15	1.26	9.45	10.10	13.20	14.21
				2	0.23	1.90				
				3	0.17	1.44				
				4	0.21	1.78				
				Mean	**0.19**	**1.59**				
			25	1	0.26	2.19				
				2	0.40	3.33				
				3	0.34	2.81				
				4	0.46	3.86				
				Mean	**0.37**	**3.05**				
			50	1	0.33	2.78				
				2	0.81	6.69				
				3	0.51	4.20				
				4	0.20	1.70				
				Mean	**0.46**	**3.84**				

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

Substance	Lab	Vehicle	Conc. (%)	Animal No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Isopropanol	7	AOO	10	1	0.03	0.24	NC	NC	NC	NC
				2	0.03	0.25				
				3	0.11	0.93				
				4	0.06	0.50				
				Mean	**0.06**	**0.48**				
			25	1	0.13	1.04				
				2	0.08	0.69				
				3	0.10	0.84				
				4	0.15	1.25				
				Mean	**0.12**	**0.95**				
			50	1	0.05	0.38				
				2	0.11	0.89				
				3	0.28	2.32				
				4	0.05	0.43				
				Mean	**0.12**	**1.01**				
VP	7	AOO	0	1	0.10	1.14	NA	NA	NA	NA
				2	0.10	1.17				
				3	0.09	1.07				
				4	0.05	0.61				
				Mean	**0.09**	**1.00**				
PC	7	AOO	0	1	0.38	4.48	NA	NA	NA	NA
				2	0.35	4.15				
				3	0.32	3.80				
				4	0.43	5.12				
				Mean	**0.37**	**4.39**				
VS	7	AOO	0	1	0.15	1.21	NA	NA	NA	NA
				2	0.11	0.87				
				3	0.09	0.76				
				4	0.14	1.16				
				Mean	**0.12**	**1.00**				
Eugenol	7	AOO	10	1	0.33	2.70	3.51	3.77	4.70	5.06
				2	0.36	2.93				
				3	0.44	3.58				
				4	0.31	2.49				
				Mean	**0.36**	**2.93**				
			25	1	0.44	3.54				
				2	0.33	2.67				
				3	0.73	5.95				
				4	0.56	4.57				
				Mean	**0.51**	**4.18**				

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

Substance	Lab	Vehicle	Conc. (%)	Animal No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Eugenol (continued)	7	AOO	50	1	0.79	6.44				
				2	0.94	7.63				
				3	0.85	6.90				
				4	0.91	7.38				
				Mean	**0.87**	**7.09**				
2, 4-Dinitrochlorobenzene	7	AOO	0.1	1	0.71	5.74	0.049	0.050	0.053	0.053
				2	0.64	5.22				
				3	0.53	4.30				
				4	0.95	7.71				
				Mean	**0.71**	**5.74**				
			0.3	1	1.29	10.50				
				2	1.50	12.23				
				3	1.21	9.82				
				4	2.04	16.63				
				Mean	**1.51**	**12.29**				
			1	1	1.72	14.03				
				2	1.39	11.35				
				3	1.62	13.23				
				4	1.63	13.31				
				Mean	**1.59**	**12.98**				
VP	7	DMSO	0	1	0.08	0.67	NA	NA	NA	NA
				2	0.11	0.93				
				3	0.13	1.05				
				4	0.16	1.35				
				Mean	**0.12**	**1.00**				
PC	7	DMSO	0	1	0.45	3.68	NA	NA	NA	NA
				2	0.77	6.33				
				3	0.36	2.95				
				4	0.75	6.17				
				Mean	**0.58**	**4.78**				
VS	7	DMSO	0	1	0.08	0.54	NA	NA	NA	NA
				2	0.18	1.22				
				3	0.19	1.28				
				4	0.14	0.97				
				Mean	**0.15**	**1.00**				
Lactic acid	7	DMSO	10	1	0.19	1.30	14.58	16.16	20.90	22.47
				2	0.17	1.15				
				3	0.16	1.12				
				4	0.18	1.24				
				Mean	**0.18**	**1.21**				

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

Substance	Lab	Vehicle	Conc. (%)	Animal No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Lactic acid (continued)	7	DMSO	25	1	0.25	1.70				
				2	0.27	1.83				
				3	0.39	2.67				
				4	0.36	2.45				
				Mean	**0.31**	**2.16**				
			50	1	0.48	3.34				
				2	0.26	1.78				
				3	0.25	1.73				
				4	0.48	3.28				
				Mean	**0.37**	**2.53**				
Nickel sulfate	7	DMSO	1	1	0.22	1.52	0.59	0.67	1.05	1.31
				2	0.31	2.14				
				3	0.31	2.12				
				4	0.25	1.73				
				Mean	**0.27**	**1.88**				
			3	1	0.55	3.77				
				2	0.38	2.65				
				3	0.39	2.66				
				4	0.23	1.57				
				Mean	**0.39**	**2.66**				
			10	1	0.23	1.59				
				2	0.43	2.95				
				3	0.33	2.30				
				4	0.55	3.79				
				Mean	**0.39**	**2.66**				

Abbreviations: ABS = absorbance; ACE = acetone; AOO = acetone: olive oil; Conc. = concentration; DMSO = dimethyl sulfoxide; EC = estimated concentration needed to produce a stimulation index of 1.5 (EC1.5), 1.6 (EC1.6), 1.9 (EC1.9), or 2 (EC2); LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; NA = not applicable; NC = not calculated because the maximum SI was less than the decision criterion; No. = identification number; PC = positive control; SI = stimulation index; VP = vehicle for PC; VS = vehicle for test substance.

* Calculated using linear interpolation with SI =1, concentration =0 as the lowest point because the dose-response was nonmonotonic.

[1] Mean of 3 replicates

[2] Positive control failed because SI >2 was not achieved. Results from test substances associated with failed positive control were not considered in the accuracy and reproducibility analyses.

Annex V

Additional LLNA: BrdU-ELISA Test Results Submitted in December 2009

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Additional LLNA: BrdU-ELISA Test Results Submitted in December 2009

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Chlorobenzene	2009	AOO	0	1	0.04	0.36	18.61	21.39	29.72	32.50
				2	0.12	1.12				
				3	0.14	1.37				
				4	0.12	1.15				
				Mean	**0.10**	**1.00**				
			30	5	0.16	1.54				
				6	0.21	2.00				
				7	0.15	1.47				
				8	0.27	2.64				
				Mean	**0.20**	**1.91**				
			100	9	0.43	4.16				
				10	0.44	4.29				
				11	0.58	5.60				
				12	0.38	3.68				
				Mean	**0.46**	**4.43**				
Imidazolidinyl urea	2009	DMSO	0	1	0.10	0.70	43.95	47.98	60.08	64.11
				2	0.17	1.13				
				3	0.22	1.43				
				4	0.11	0.74				
				Mean	**0.15**	**1.00**				
			50	5	0.21	1.40				
				6	0.25	1.67				
				7	0.28	1.87				
				8	0.25	1.68				
				Mean	**0.25**	**1.65**				
			75	9	0.35	2.34				
				10	0.29	1.95				
				11	0.37	2.49				
				12	0.34	2.31				
				Mean	**0.34**	**2.27**				
VC	2009	DMSO	0	1	0.12	0.51	NA	NA	NA	NA
				2	0.57	2.36				
				3	0.12	0.49				
				4	0.16	0.65				
				Mean	**0.24**	**1.00**				
Nickel chloride	2009	DMSO	1	5	0.06	0.25	NC	NC	NC	NC
				6	0.32	1.30				
				7	0.26	1.09				
				8	0.22	0.91				
				Mean	**0.22**	**0.89**				

Additional LLNA: BrdU-ELISA Test Results Submitted in December 2009

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
Nickel chloride (continued)			5	9	0.40	1.64				
				10	0.32	1.30				
				11	0.22	0.89				
				12	0.34	1.41				
				Mean	**0.32**	**1.31**				
2-Mercaptoben-zothiazole	2009	DMSO	10	5	0.33	1.35	NC	NC	NC	NC
				6	0.16	0.65				
				7	0.23	0.93				
				8	0.09	0.37				
				Mean	**0.20**	**0.82**				
			25	9	0.27	1.11				
				10	0.25	1.01				
				11	0.20	0.81				
				12	0.47	1.95				
				Mean	**0.29**	**1.22**				
VC[2]	2009	DMSO	0	1	0.12	0.92	NA	NA	NA	NA
				3	0.12	0.89				
				4	0.16	1.18				
				Mean	**0.13**	**1.00**				
Nickel chloride[2]	2009	DMSO	1	5	0.06	0.45				
				6	0.32	2.38				
				7	0.26	1.98				
				8	0.22	1.67				
				Mean	**0.22**	**1.62**				
			5	9	0.40	3.00				
				10	0.32	2.37				
				11	0.22	1.62				
				12	0.34	2.57				
				Mean	**0.32**	**2.39**				
2-Mercaptoben-zothiazole[2]	2009	DMSO	10	5	0.33	2.46	NC	NC	NC	NC
				6	0.16	1.18				
				7	0.23	1.70				
				8	0.09	0.67				
				Mean	**0.20**	**1.50**				
			25	9	0.27	2.02				
				10	0.25	1.84				
				11	0.20	1.48				
				12	0.47	3.56				
				Mean	**0.29**	**2.23**				
2-Mercaptoben-zothiazole[3]	2009	DMSO	0	1	0.14	1.11	15.82	16.73	19.45	20.36
				2	0.12	0.96				
				3	0.06	0.48				
				4	0.18	1.45				
				Mean	**0.13**	**1.00**				

Additional LLNA: BrdU-ELISA Test Results Submitted in December 2009

Substance	Ref.	Veh.	Conc. (%)	An. No.	ABS[1]	SI	EC1.5	EC1.6	EC1.9	EC2
2-Mercaptoben-zothiazole[3] (continued)			10	5	0.11	0.84				
				6	0.14	1.10				
				7	0.17	1.34				
				8	0.02	0.17				
				Mean	**0.11**	**0.86**				
			25	9	0.22	1.73				
				10	0.37	2.90				
				11	0.40	3.16				
				12	0.28	2.23				
				Mean	**0.31**	**2.51**				
Xylene	2009	AOO	0	1	0.04	0.36	14.76	15.89	19.84	21.36
				2	0.12	1.12				
				3	0.14	1.37				
				4	0.12	1.15				
				Mean	**0.11**	**1.00**				
			30	5	0.31	2.99				
				6	0.17	1.66				
				7	0.29	2.87				
				8	0.24	2.30				
				Mean	**0.25**	**2.46**				
			100	9	0.42	4.10				
				10	0.48	4.72				
				11	0.40	3.91				
				12	0.37	3.65				
				Mean	**0.42**	**4.09**				

Abbreviations: ABS = absorbance; An. No. = animal number; AOO = acetone: olive oil (4:1); Conc. = concentration; DMSO = dimethyl sulfoxide; EC = estimated concentration needed to produce a stimulation index of 1.5 (EC1.5), 1.6 (EC1.6), 1.9 (EC1.9), or 2 (EC2); LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; NA = not available; NC = not calculated because the SI was not high enough to calculate EC1.5, EC1.6, EC1.9, or EC2; Ref. = year Dr. Takeyoshi submitted data (month was December); SI = stimulation index; Veh. = vehicle.

[1] Mean of 3 replicates

[2] Same tests as previously listed in the table for this chemical, but outlier of 0.57 in DMSO control has been excluded on the basis of the outlier test in Dixon WJ, Massey FJ. 1981. Introduction to Statistical Analysis, 4th ed. Milwaukee: Quality Press.

[3] Test used CBA/J mice, rather than CBA/JN, which were used in all other tests considered in this evaluation.

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Annex VI

Accuracy Analyses Using Additional Approaches for Combining Multiple Test Results

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1.0 LLNA: BrdU-ELISA Accuracy Analysis Using Alternative Decision Criteria and Alternate Methods for Combining Data for Substances Tested Multiple Times

This annex shows performance analyses for the LLNA: BrdU-ELISA using single alternative decision criteria and two different approaches for combining test results for the 18 substances with multiple LLNA: BrdU-ELISA tests:

1. The positive/negative outcome for each substance for each criterion was determined by the outcome of the test with the highest maximum SI of the multiple tests.
2. The positive/negative outcome for each substance for each criterion was determined by the outcome of the test with the lowest maximum SI of the multiple tests.

Appendix C, Section 6.5 provides the results for the analysis when the most prevalent outcome for each criterion was used as the result for each substance that was tested multiple times.

1.1 Results of LLNA: BrdU-ELISA Accuracy Analysis Using Single Alternative Decision Criteria and the Highest Maximum SI for the Outcome of Multiple Tests

When combining multiple test results for a single substance using the outcome of the test with the highest maximum SI, the decision criterion of SI ≥ 2.0 to identify sensitizers yielded an accuracy of 91% (39/43), a sensitivity of 91% (29/32), a specificity of 91% (10/11), a false positive rate of 9% (1/11), and a false negative rate of 9% (3/32) (**Table C-VI-1)**. SI ≥ 2.0 was the decision criterion used by the JSAAE interlaboratory validation study of the LLNA: BrdU-ELISA. The performance for the additional decision criteria is shown in **Table C-VI-1**. Over the range of SI cutoffs evaluated, the SI cutoffs of 1.5 and 1.9 to 2.5 produced the same accuracy as SI ≥ 2.0 (i.e., 91%). Accuracy at the optimum criterion of SI ≥ 1.6, identified in **Section 6.5** of the BRD, was the highest, at 93% (40/43). At SI ≥ 1.3, and at SI ≥ 3.0 and higher, the accuracy decreased to 88% (38/43) and from 86% (37/43) to 56% (24/43), respectively. SI ≥ 2.5 had the same sensitivity as SI ≥ 2.0 (91% [29/32]), but higher SI cutoffs decreased sensitivity (81% [26/32]) at SI ≥ 3.0 to 41% [13/32] at SI ≥ 5.0), increased specificity (100% [11/11] from SI ≥ 3.0 to SI ≥ 5.0), decreased the false positive rate (0% [0/11] at SI ≥ 3.0 and higher), and increased the false negative rate (19% [6/32] at SI ≥ 3.0 to 59% [19/32] at SI ≥ 5.0) (**Figure C-VI-1** and **Table C-VI-1)**. SI cutoffs lower than 2.0 increased sensitivity (94% [30/32] at SI ≥ 1.9 to 100% [32/32] at SI ≥ 1.6 to 1.3), decreased specificity (82% [9/11] at SI ≥ 1.9 to 55% [6/11] at SI ≥ 1.3), increased the false positive rate (18% [2/11] at SI ≥ 1.9 to 45% [5/11] at SI ≥ 1.3), and decreased the false negative rate (6% [2/32] at SI ≥ 1.9 to 0% [0/32] at SI ≥ 1.6 to 1.3). Use of ANOVA and summary statistics (i.e., mean absorbance values of treated groups ≥95% confidence interval [CI] of the control group mean, or ≥2 or ≥3 SD from the control group mean), yielded accuracy values of 86% (37/43) to 91% (39/43), with sensitivity values of 94% (30/32) to 100% (32/32), and false negative rates of 0% (0/32) to 6% (2/32). The specificity for these criteria ranged from 45% (5/11) to 82% (9/11) and the false positive rates were 18% (2/11) to 55% (6/11).

Table C-VI-1 Performance of the LLNA: BrdU-ELISA for 43 Substances Compared with the Traditional LLNA Using Alternative Decision Criteria to Identify Sensitizers and the Highest Maximum SI for Substances with Multiple Tests

Alternate Criterion	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate		Positive Predictivity		Negative Predictivity	
	%	No.[1]	%	No.[1]	%	No.[1]	%	No.[1]	%	No.[1]	%	No.[1]	%	No.[1]
Statistics[2]	88	38/43	97	31/32	64	7/11	36	4/11	3	1/32	89	31/35	88	7/8
≥ 95% CI[3]	86	37/43	100	32/32	45	5/11	55	6/11	0	0/32	84	32/38	100	5/5
≥ 2 SD[4]	86	37/43	100	32/32	45	5/11	55	6/11	0	0/32	84	32/38	100	5/5
≥ 3 SD[5]	91	39/43	94	30/32	82	9/11	18	2/11	6	2/32	94	30/32	82	9/11
SI ≥ 5.0	56	24/43	41	13/32	100	11/11	0	0/11	59	19/32	100	13/13	37	11/30
SI ≥ 4.5	67	29/43	56	18/32	100	11/11	0	0/11	44	14/32	100	18/18	44	11/25
SI ≥ 4.0	72	31/43	63	20/32	100	11/11	0	0/11	38	12/32	100	20/20	48	11/23
SI ≥ 3.5	79	34/43	72	23/32	100	11/11	0	0/11	28	9/32	100	23/23	55	11/20
SI ≥ 3.0	86	37/43	81	26/32	100	11/11	0	0/11	19	6/32	100	26/26	65	11/17
SI ≥ 2.5	91	39/43	91	29/32	91	10/11	9	1/11	9	3/32	97	29/30	77	10/13
SI ≥ 2.0	91	39/43	91	29/32	91	10/11	9	1/11	9	3/32	97	29/30	77	10/13
SI ≥ 1.9	91	39/43	94	30/32	82	9/11	18	2/11	6	2/32	94	30/32	82	9/11
SI ≥ 1.6	93	40/43	100	32/32	73	8/11	27	3/11	0	0/32	91	32/35	100	8/8
SI ≥ 1.5	91	39/43	100	32/32	64	7/11	36	4/11	0	0/32	89	32/36	100	7/7
SI ≥ 1.3	88	38/43	100	32/32	55	6/11	45	5/11	0	0/32	86	32/37	100	6/6

Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; No. = number; SD = standard deviation; SI = stimulation index.

[1] The proportion on which the percentage calculation is based.

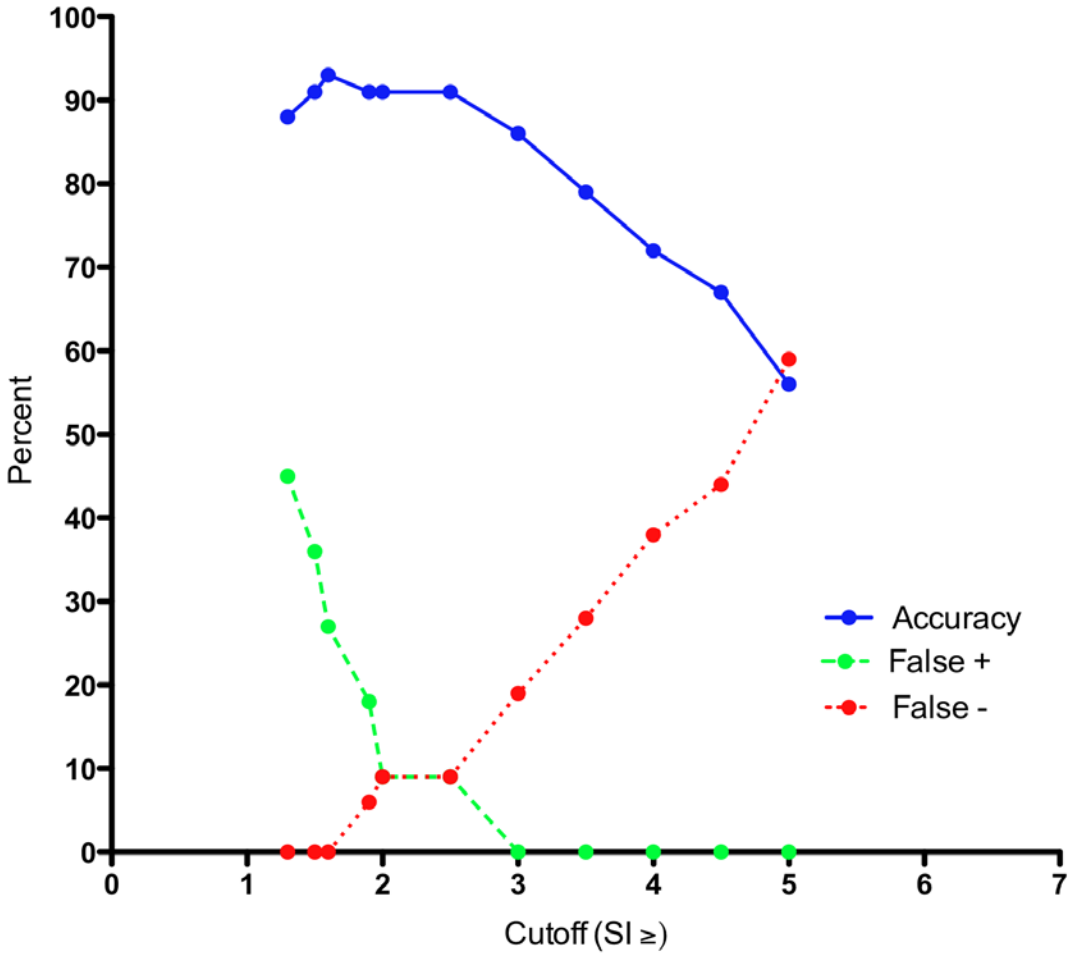
[2] Analysis of variance for difference of group means when substances were tested at multiple doses or *t*-test when substances were tested at one dose. The absorbance data were log-transformed prior to analysis of variance. Significance at $p < 0.05$ was further tested by Dunnett's test.

[3] The mean absorbance of at least one treatment group was outside the 95% confidence interval for the mean absorbance of the vehicle control group.

[4] The mean absorbance of at least one treatment group was greater than 3 SD from the mean absorbance of the vehicle control group.

[5] The mean absorbance of at least one treatment group was greater than 2 SD from the mean absorbance of the vehicle control group.

Figure C-VI-1 Performance of the LLNA: BrdU-ELISA for 43 Substances with SI Compared to the Traditional LLNA Using the Highest Maximum SI for Substances with Multiple Tests



Abbreviations: LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SI = stimulation index.

The highest accuracy and lowest false negative rate for the approach using the highest maximum SI for the substances with more than one test, was achieved using an SI ≥ 1.6, the optimum criterion identified in **Section 6.5** of the BRD. The accuracy for SI ≥ 1.6 was 93% (40/43), with sensitivity of 100% (32/32), specificity of 73% (8/11), a false positive rate of 27% (3/11), and a false negative rate of 0% (0/32). However, using an SI ≥ 1.6 incorrectly classified lactic acid and isopropanol, two of the ICCVAM performance standards reference substances, as sensitizers. Use of mean absorbance values of treated groups ≥95% CI of the control group mean, or ≥2 SD from the control group mean, to identify sensitizers also produced the false negative rates as low as the SI cutoffs of 1.6 to 1.3 (0% [0/32]), with a slightly lower accuracy of 86% (37/43) to 88% (38/43), and a higher false positive rate of 55% (6/11). These criteria also incorrectly classified lactic acid and isopropanol, as well as methyl salicylate, another ICCVAM performance standards reference substance, as sensitizers. The

lowest false positive rates (0% [0/11]) were produced by SI cutoffs of 3.0 to 5.0; however, the false negative rates at those cutoffs were 19% (6/32) to 59% (19/32).

As compared to traditional LLNA results, the lines show the change in performance characteristics for the LLNA: BrdU-ELISA with the SI cutoff used to identify sensitizers. This analysis used LLNA: BrdU-ELISA and traditional LLNA results for 43 substances (32 sensitizers and 11 nonsensitizers based on traditional LLNA results). For the 18 substances with multiple test results, the results for each substance were combined by using the outcome for the test with the highest maximum SI value. The solid line shows accuracy, the dashed line shows the false positive rate, and the dotted line shows the false negative rate.

1.2 Results of LLNA: BrdU-ELISA Accuracy Analysis Using Alternative Decision Criteria and Lowest Maximum SI for the Outcome of Multiple Tests

When combining multiple test results for a single substance using the outcome of the test with the lowest maximum SI, the decision criterion of SI ≥ 2.0 to identify sensitizers for these 43 substances yielded an accuracy of 86% (37/43), a sensitivity of 81% (26/32), a specificity of 100% (11/11), a false positive rate of 0% (0/11), and a false negative rate of 19% (6/32) (**Table C-VI-2)**. SI ≥ 2.0 was the decision criterion used by the JSAAE interlaboratory validation study of the LLNA: BrdU-ELISA. The performance for the additional decision criteria is shown in **Table C-VI-2**.

Over the range of SI cutoffs evaluated, increasing the SI cutoff compared with SI ≥ 2.0 decreased accuracy (79% [34/43] at SI ≥ 2.5 to 40% [21/43] at SI ≥ 5.0), decreased sensitivity (72% [23/32] at SI ≥ 2.5 to 19% [6/32] at SI ≥ 5.0), produced the same specificity (100% [11/11] up to SI ≥ 5.0), produced the same false positive rate (0% [0/11] up to SI ≥ 5.0), and increased the false negative rate (28% [9/32] at SI ≥ 2.5 to 81% [26/32] at SI ≥ 5.0) (**Figure C-VI-2** and **Table C-VI-2**). SI cutoffs lower than 2.0 increased sensitivity (88% [28/32] at SI ≥ 1.9 to 100% [32/32] at SI ≥ 1.3), decreased specificity (100% [11/11] at SI ≥ 1.9 to 73% [8/11] at SI ≥ 1.3), increased the false positive rate (0% [0/11] at SI ≥ 1.9 to 27% [3/11] at SI ≥ 1.3), and decreased the false negative rate (13% [4/32] at SI ≥ 1.9 to 0% [0/32] at SI ≥ 1.3). Use of ANOVA and summary statistics (i.e., mean absorbance values of treated groups ≥95% CI of the control group mean, or ≥2 or 3 SD from the control group mean), yielded accuracy of 88% (38/43) to 93% (40/43), with sensitivity values of 88% (28/32) to 97% (31/32), and false negative rates of 3% (1/32) to 13% (4/32). The specificity for these criteria ranged from 64% (7/11) to 100% (11/11) and the false positive rates were 0% (0/11) to 36% (4/11).

The highest accuracy and lowest false negative rate for the approach using the lowest maximum SI for the substances with more than one test was achieved using an SI ≥ 1.3 and mean absorbance values of treated groups ≥2 SD of the control group mean. Both criteria yielded an accuracy of 93% (40/43). The false negative rates were 0% (0/32) at SI ≥ 1.3 and 3% (1/32) at ≥2 SD. The sensitivity was 100% (32/32) at SI ≥ 1.3 and 97% (31/32) at ≥2 SD. The specificity was 73% (8/11) at SI ≥ 1.3 and 82% (9/11) at ≥2 SD. The false positive rate was 27% (3/11) at SI ≥ 1.3 and 18% (2/11) at ≥2 SD. However, SI ≥ 1.3 incorrectly classified lactic acid and methyl salicylate, two of the ICCVAM performance standards reference substances, as sensitizers. Mean absorbance values of treated groups ≥2 SD from the control group mean incorrectly classified lactic acid as a sensitizer. The lowest false positive rate (0% [0/11]) was produced by SI cutoffs of 1.9 to 5.0 and mean absorbance values of treated groups ≥3 SD; however, the false negative rates at those cutoffs were 13% (4/32) to 81% (26/32). Of those cutoffs, SI ≥ 1.9 and mean absorbance values of treated groups ≥3 SD produced the highest accuracy, 91% (39/43), and the lowest false negative rates, 13% (4/32).

Table C-VI-2 Performance of the LLNA: BrdU-ELISA for 43 Substances Compared with the Traditional LLNA Using Alternative Decision Criteria to Identify Sensitizers and the Lowest Maximum SI for Substances with Multiple Tests

Alternate Criterion	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate		Positive Predictivity		Negative Predictivity	
	%	No.[1]	%	No.[1]	%	No.[1]	%	No.[1]	%	No.[1]	%	No.[1]	%	No.[1]
Statistics[2]	88	38/43	91	29/32	82	9/11	18	2/11	9	3/32	94	29/31	75	9/12
≥ 95% CI[3]	88	38/43	97	31/32	64	7/11	36	4/11	3	1/32	89	31/35	88	7/8
≥ 2 SD[4]	93	40/43	97	31/32	82	9/11	18	2/11	3	1/32	94	31/33	90	9/10
≥ 3 SD[5]	91	39/43	88	28/32	100	11/11	0	0/11	13	4/32	100	28/28	73	11/15
SI ≥ 5.0	40	17/43	19	6/32	100	11/11	0	0/11	81	26/32	100	6/6	30	11/37
SI ≥ 4.5	44	19/43	25	8/32	100	11/11	0	0/11	75	24/32	100	8/8	31	11/35
SI ≥ 4.0	49	21/43	31	10/32	100	11/11	0	0/11	69	22/32	100	10/10	33	11/33
SI ≥ 3.5	56	24/43	41	13/32	100	11/11	0	0/11	59	19/32	100	13/13	37	11/30
SI ≥ 3.0	67	29/43	56	18/32	100	11/11	0	0/11	44	14/32	100	18/18	44	11/25
SI ≥ 2.5	79	34/43	72	23/32	100	11/11	0	0/11	28	9/32	100	23/23	55	11/20
SI ≥ 2.0	86	37/43	81	26/32	100	11/11	0	0/11	19	6/32	100	26/26	65	11/17
SI ≥ 1.9	91	39/43	88	28/32	100	11/11	0	0/11	13	4/32	100	28/28	73	11/15
SI ≥ 1.6	91	39/43	94	30/32	82	9/11	18	2/11	6	2/32	94	30/32	82	9/11
SI ≥ 1.5	91	39/43	94	30/32	82	9/11	18	2/11	6	2/32	94	30/32	82	9/11
SI ≥ 1.3	93	40/43	100	32/32	73	8/11	27	3/11	0	0/32	91	32/35	100	8/8

Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; No. = number; SD = standard deviation; SI = stimulation index.

[1] The proportion on which the percentage calculation is based.

[2] Analysis of variance for difference of group means when substances were tested at multiple doses or *t*-test when substances were tested at one dose. The absorbance data were log-transformed prior to analysis of variance. Significance at $p < 0.05$ was further tested by Dunnett's test.

[3] The mean absorbance of at least one treatment group was outside the 95% confidence interval for the mean absorbance of the vehicle control group.

[4] The mean absorbance of at least one treatment group was greater than 3 SD from the mean absorbance of the vehicle control group.

[5] The mean absorbance of at least one treatment group was greater than 2 SD from the mean absorbance of the vehicle control group.

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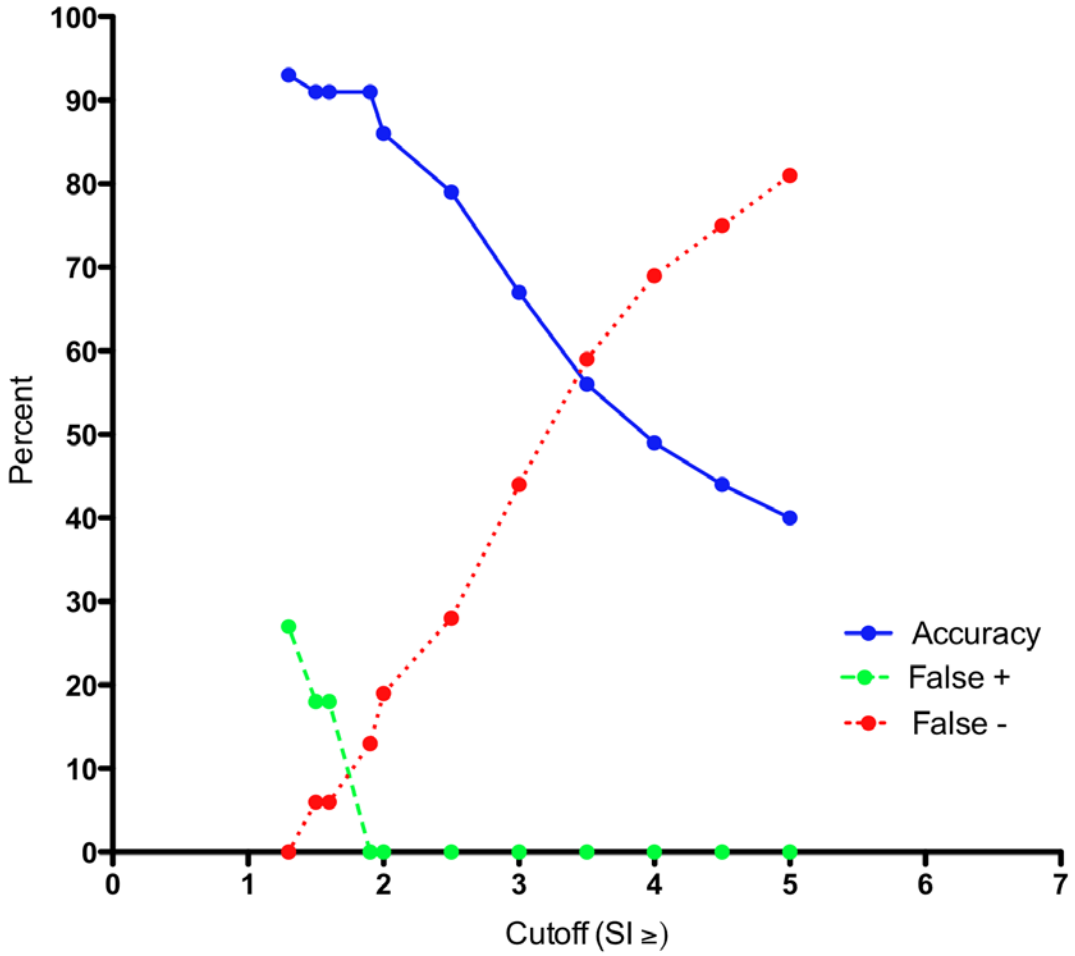
Figure C-VI-2 Performance of the LLNA: BrdU-ELISA for 43 Substances with SI Compared to the Traditional LLNA Using the Lowest Maximum SI for Substances with Multiple Tests



Abbreviations: LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SI = stimulation index.

As compared to traditional LLNA results, the lines show the change in performance characteristics for the LLNA: BrdU-ELISA with the SI cutoff used to identify sensitizers. This analysis used LLNA: BrdU-ELISA and traditional LLNA results for 43 substances (32 sensitizers and 11 nonsensitizers based on traditional LLNA results). For the 18 substances with multiple test results, the results for each substance were combined by using the outcome for the test with the lowest maximum SI value. The solid line shows accuracy, the dashed line shows the false positive rate, and the dotted line shows the false negative rate.

2.0 Discordant Results for Accuracy Analysis of Alternative Decision Criteria

Using the decision criteria of SI ≥ 2.0 to identify sensitizers and the most prevalent outcome for the substances with multiple tests, the LLNA: BrdU-ELISA outcomes yielded two discordant substances (2-mercaptobenzothiazole and imidazolidinyl urea) compared with the traditional LLNA (**Table C-5**). As indicated in **Appendix C, Section 6.4.1**, these substances were misclassified as nonsensitizers when compared to the traditional LLNA, which classified them as sensitizers.

2.1 Discordant Results Using Alternative Decision Criteria and Highest Maximum SI Outcome for Multiple Tests

Using the decision criteria of SI ≥ 2.0 to identify sensitizers and the test with the highest maximum SI as the result for substances with multiple tests, yielded two additional discordant substances: isopropanol and lactic acid, which were misclassified as sensitizers.

Table C-VI-3 shows how the number and identity of discordant substances change with the alternative decision criteria when using the test with the highest maximum SI as the result for substances with multiple tests. Using an SI cutoff less than 2.0 increased the number of traditional LLNA nonsensitizers that were misclassified as sensitizers. SI ≥ 1.3 yielded the highest number (five) of discordant substances that were misclassified as sensitizers (hexane, isopropanol, lactic acid, methyl salicylate, and propylene glycol). Increasing the SI cutoff to values greater than 2.0 increased the number of sensitizers that were misclassified as nonsensitizers. At SI ≥2.5, three sensitizers were misclassified as nonsensitizers while, at SI ≥ 5.0, 19 sensitizers were classified as nonsensitizers (**Table C-VI-3)**. At SI ≥ 2.0, two nonsensitizers were misclassified as sensitizers. Increasing the SI cutoff to values greater than 2.0 decreased the number of nonsensitizers classified as sensitizers. At SI ≥ 2.5, one nonsensitizer was misclassified as a sensitizer. At SI ≥ 3.0 and higher, no nonsensitizers were classified as sensitizers.

Use of a statistical test (i.e., ANOVA or *t*-test) or summary statistics (i.e., ≥95% CI, ≥2 SD, or ≥3 SD) tended to misclassify more nonsensitizers than sensitizers. Using ANOVA or a *t*-test to identify sensitizers misclassified one sensitizer (2-mercaptobenzothiazole) as a nonsensitizer and four nonsensitizers (glycerol, hexane, isopropanol, and lactic acid) as sensitizers. Using treatment group absorbance ≥95% CI or ≥2 SD of control group mean misclassified six nonsensitizers as sensitizers (glycerol, hexane, isopropanol, lactic acid, methyl salicylate, and propylene glycol). Using treatment group absorbance ≥3 SD of the control group mean misclassified two nonsensitizers as sensitizers (hexane and lactic acid) and two weak sensitizers as nonsensitizers (cinnamic alcohol and imidazolidinyl urea).

Eleven ICCVAM performance standards reference substances were discordant for the analysis of alternative decision criteria using the test with the highest maximum SI as the result for substances with multiple tests (**Table C-VI-3**). Eight traditional LLNA sensitizers (2-mercaptobenzothiazole, 5-chloro-2-methyl-4-isothiazolin-3-one, cinnamic alcohol, cobalt chloride, ethylene glycol dimethacrylate, imidazolidinyl urea, phenyl benzoate, and sodium lauryl sulfate) were misclassified by some criteria as nonsensitizers. Sodium lauryl sulfate, however, produces a false positive result in the traditional LLNA; it does not produce a sensitization reaction in humans or guinea pigs. Three nonsensitizers (isopropanol, lactic acid, and methyl salicylate) were misclassified as sensitizers by some criteria.

Table C-VI-3 Discordant Results for LLNA: BrdU-ELISA Using Alternative Decision Criteria Compared to the Traditional LLNA and the Highest Maximum SI for Substances with Multiple Tests

Discordant Substances[1]	Alternative Decision Criterion[2]														
	Statistics[3]	≥ 95% CI[4]	≥ 2 SD[5]	≥ 3 SD[6]	SI≥5.0	SI≥4.5	SI≥4.0	SI≥3.5	SI≥3.0	SI≥2.5	SI≥2.0	SI≥1.9	SI≥1.6	SI≥1.5	SI≥1.3
Glycerol (-)	+	+	+												
Hexane (-)	+	+	+	+									+	+	+
Isopropanol[7] (-)	+	+	+								+	+	+	+	+
Lactic acid[7] (-)	+	+	+	+						+	+	+	+	+	+
Methyl salicylate[7] (-)		+	+												+
Propylene glycol (-)		+	+											+	+
2-Mercaptobenzothiazole[7] (1.7%)	-				-	-	-	-	-	-	-	-			
3-Aminophenol (3.2%)					-	-	-	-							
4-Chloroaniline (6.5%)					-	-	-	-	-						
MAPS (0.8%)					-	-	-								
CMI solution[7] (0.009%)					-										
Aniline (48%)					-	-	-	-	-	-					
Cinnamic alcohol[7] (21%)				-	-	-	-	-	-						
Cinnamic aldehyde (1.9%)					-	-	-								
Cobalt chloride[7] (0.6%)					-	-	-								
Ethyl acrylate (33%)					-										
EGDA[7] (28%)					-	-	-	-							
Hydroxycitronellal (24%)					-										
Imidazolidinyl urea[7] (24%)				-	-	-	-	-	-	-	-	-			
Isopropyl myristate (44%)					-	-									
Linalool (30%)					-										
Nickel sulfate (4.8%)					-										

Discordant Substances[1]	Alternative Decision Criterion[2]														
	Statistics[3]	≥ 95% CI[4]	≥ 2 SD[5]	≥ 3 SD[6]	SI≥5.0	SI≥4.5	SI≥4.0	SI≥3.5	SI≥3.0	SI≥2.5	SI≥2.0	SI≥1.9	SI≥1.6	SI≥1.5	SI≥1.3
Phenyl benzoate[7] (14%)					-	-	-	-							
Sodium lauryl sulfate[7] (8.1%)					-	-	-	-	-						
trans-Cinnamaldehyde (1.4 %)					-	-									

Abbreviations: CI = confidence interval; CMI = 5-Chloro-2-methyl-4-isothiazolin-3-one; EGDA = ethylene glycol dimethacrylate; LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine (BrdU); MAPS = 4-methylaminophenol sulfate; SD = standard deviation; SI = stimulation index.

[1] Compared to the traditional LLNA. Traditional LLNA result in parentheses: "-" for nonsensitizers and EC3 (%) for sensitizers.

[2] LLNA: BrdU result shown: "+" if the decision criterion was met and "-" if the decision criterion was not met.

[3] Analysis of variance for difference of group means when substances were tested at multiple doses or *t*-test when substances were tested at one dose. The absorbance data were log-transformed prior to analysis of variance. Significance at $p < 0.05$ was further tested by Dunnett's test.

[4] The mean absorbance of at least one treatment group was outside the 95% confidence interval for the mean absorbance of the vehicle control group.

[5] The mean absorbance of at least one treatment group was greater than 3 SD from the mean absorbance of the vehicle control group.

[6] The mean absorbance of at least one treatment group was greater than 2 SD from the mean absorbance of the vehicle control group.

[7] Reference substance from *Recommended Performance Standards: Murine Local Lymph Node Assay* (ICCVAM 2009; available: http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm).

The criteria that yielded the correct results for 2-mercaptobenzothiazole included the statistics ≥95% CI, ≥2 SD, or ≥3 SD; and SI ≥ 1.6 to SI ≥ 1.3. The criteria that yielded the correct results for 5-chloro-2-methyl-4-isothiazolin-3-one included all of the summary statistics and SI ≥ 4.5 to SI ≥ 1.3. The criteria that yielded the correct results for cinnamic alcohol included the summary statistics ANOVA, ≥95% CI, and ≥2 SD; and SI ≥ 2.5 to SI ≥ 1.3. The criteria that yielded the correct results for cobalt chloride included all of the summary statistics and SI ≥ 3.5 to SI ≥ 1.3. The criteria that yielded the correct results for ethylene glycol dimethacrylate and phenyl benzoate included all of the summary statistics and SI ≥ 3.0 to SI ≥ 1.3. The criteria that yielded the correct results for imidazolidinyl urea included the summary statistics ANOVA, ≥95% CI, and ≥2 SD; and SI ≥ 1.6 to SI ≥ 1.3. The criteria that yielded the correct results for isopropanol included treatment group absorbance ≥3 SD of vehicle control group mean and SI ≥ 2.5 and higher. The criteria that yielded the correct results for lactic acid included SI ≥ 3.0 and higher. All criteria yielded the correct results for methyl salicylate except for treatment group absorbance ≥95% CI or ≥2 SD of the control group mean, and SI ≥ 1.3.

2.2 Discordant Results Using Alternative Decision Criteria and Lowest Maximum SI Outcome for Multiple Tests

Using the decision criteria of SI ≥ 2.0 to identify sensitizers and the most prevalent outcome for the substances with multiple tests, the LLNA: BrdU-ELISA outcomes yielded two discordant substances (2-mercaptobenzothiazole and imidazolidinyl urea) compared with the traditional LLNA (**Table C-5**). These substances were classified as nonsensitizers by the LLNA: BrdU-ELISA, while the traditional LLNA classified them as sensitizers. Using the test with the lowest maximum SI as the result for substances with multiple tests yielded six discordant substances at SI ≥ 2.0, including four additional sensitizers (cyclamen aldehyde, formaldehyde, hydroxycitronellal, and linalool), which were misclassified as nonsensitizers (**Table C-VI-4**). Linalool, however, is false positive in the traditional LLNA; it does not produce a sensitization reaction in humans (guinea pig data were not available).

Table C-VI-4 shows how the number and identity of discordant substances changed with the alternative decision criteria when using the test with the lowest maximum SI as the result for substances with multiple tests. Using an SI cutoff less than 2.0, SI ≥ 1.9 misclassified fewer (four) traditional LLNA sensitizers as nonsensitizers. SI ≥ 1.6 and lower increased the number of traditional LLNA nonsensitizers that were misclassified as sensitizers. SI ≥ 1.6 yielded two discordant substances that were misclassified as sensitizers (hexane and lactic acid), while SI ≥ 1.3 misclassified three nonsensitizers as sensitizers (hexane, lactic acid, and methyl salicylate). Increasing the SI cutoff to values greater than 2.0 increased the number of sensitizers that were misclassified as nonsensitizers. At SI ≥ 2.5, nine sensitizers were misclassified as nonsensitizers while, at SI ≥ 5.0, 26 sensitizers were classified as nonsensitizers (**Table C-VI-4)**. Although no nonsensitizers were misclassified as sensitizers at SI ≥ 1.9 and higher, lower SI cutoffs misclassified some nonsensitizers as sensitizers (two at SI ≥ 1.6 and SI ≥ 1.5 and three at SI ≥ 1.3).

Using the test with the lowest maximum SI as the result for substances with multiple tests caused even potent sensitizers to be misclassified as nonsensitizers at the higher SI cutoffs. At SI ≥ 4.5 and SI ≥ 5.0, 2,4-dinitrochlorobenzene, 4-methyl aminophenol sulfate, cobalt chloride, glutaraldehyde, and formaldehyde were classified as nonsensitizers. Glutaraldehyde was classified as a nonsensitizer at SI cutoffs as low as 2.5, and formaldehyde was classified as a nonsensitizer at SI cutoffs as low as 2.0.

Table C-VI-4 Discordant Results for LLNA: BrdU-ELISA Using Alternative Decision Criteria Compared to the Traditional LLNA and the Lowest Maximum SI for Substances with Multiple Tests

Discordant Substances[1]	Alternative Decision Criterion[2]														
	Statistics[3]	≥ 95% CI[4]	≥ 2 SD[5]	≥ 3 SD[6]	SI ≥5.0	SI ≥4.5	SI ≥4.0	SI ≥3.5	SI ≥3.0	SI ≥2.5	SI ≥2.0	SI ≥1.9	SI ≥1.6	SI ≥1.5	SI ≥1.3
Glycerol (-)	+	+	+												
Hexane (-)	+	+											+	+	+
Lactic acid[7] (-)		+	+										+	+	+
Methyl salicylate[7] (-)		+													+
2-Mercaptobenzothiazole[7] (1.7%)	-				-	-	-	-	-	-	-	-			
2,4-Dinitrochlorobenzene (0.05%)[7]					-	-									
3-Aminophenol (3.2%)					-	-	-	-							
4-Chloroaniline (6.5%)					-	-	-	-	-						
MAPS (0.8%)					-	-	-								
CMI solution[7] (0.009%)					-										
Aniline (48%)					-	-	-	-	-	-					
Cinnamic alcohol[7] (21%)				-	-	-	-	-	-						
Cinnamic aldehyde (1.9%)					-	-	-								
Cobalt chloride[7] (0.6%)					-	-	-								
Cyclamen aldehyde (22%)					-	-	-	-	-	-	-				
Ethyl acrylate (33%)					-										
EGDA[7] (28%)					-	-	-	-							
Eugenol[7] (10%)					-	-	-	-							
Formaldehyde (0.5%)					-	-	-	-	-	-	-				
Glutaraldehyde (0.08%)					-	-	-	-	-	-					
Hexyl cinnamic aldehyde[7] (9.7%)					-	-	-	-	-						
Hydroxycitronellal (24%)	-			-	-	-	-	-	-	-	-	-	-	-	
Imidazolidinyl urea[7] (24%)				-	-	-	-	-	-	-	-	-			
Isoeugenol[7] (1.5%)					-	-	-	-	-	-					
Isopropyl myristate (44%)					-	-									
Linalool (30%)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Nickel sulfate (4.8%)					-	-	-	-	-						

Discordant Substances[1]	Alternative Decision Criterion[2]														
	Statistics[3]	**≥ 95% CI[4]**	**≥ 2 SD[5]**	**≥ 3 SD[6]**	**SI ≥5.0**	**SI ≥4.5**	**SI ≥4.0**	**SI ≥3.5**	**SI ≥3.0**	**SI ≥2.5**	**SI ≥2.0**	**SI ≥1.9**	**SI ≥1.6**	**SI ≥1.5**	**SI ≥1.3**
Phenyl benzoate[7] (14%)					-	-	-	-							
Sodium lauryl sulfate[7] (8.1%)					-	-	-	-	-						
trans-Cinnamaldehyde (1.4%)					-	-	-	-							

Abbreviations: CI = confidence interval; CMI = 5-Chloro-2-methyl-4-isothiazolin-3-one; EGDA = ethylene glycol dimethacrylate; LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine (BrdU); MAPS = 4-methylaminophenol sulfate; SD = standard deviation; SI = stimulation index.

[1] Compared to the traditional LLNA. Traditional LLNA result in parentheses: "-" for nonsensitizers and EC3 (%) for sensitizers.

[2] LLNA: BrdU result shown: "+" if the decision criterion was met and "-" if the decision criterion was not met.

[3] Analysis of variance for difference of group means when substances were tested at multiple doses or *t*-test when substances were tested at one dose. The absorbance data were log-transformed prior to analysis of variance. Significance at $p < 0.05$ was further tested by Dunnett's test.

[4] The mean absorbance of at least one treatment group was outside the 95% confidence interval for the mean absorbance of the vehicle control group.

[5] The mean absorbance of at least one treatment group was greater than 3 SD from the mean absorbance of the vehicle control group.

[6] The mean absorbance of at least one treatment group was greater than 2 SD from the mean absorbance of the vehicle control group.

[7] Reference substance from *Recommended Performance Standards: Murine Local Lymph Node Assay* (ICCVAM 2009; available: http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm).

Use of a statistical test (i.e., ANOVA or *t*-test) or summary statistics (i.e., ≥95% CI, ≥2 SD, or 3 ≥SD) more often misclassified nonsensitizers than sensitizers (**Table C-VI-4**). Using ANOVA or *t*-tests to identify sensitizers misclassified three sensitizers (2-mercaptobenzothiazole, hydroxycitronellal, and linalool) as nonsensitizers and two nonsensitizers (glycerol and hexane) as sensitizers. Using treatment group absorbance ≥95% CI of the control group mean misclassified glycerol, hexane, lactic acid, and methyl salicylate as sensitizers. Using treatment group absorbance ≥2 SD of the control group mean misclassified glycerol and lactic acid as sensitizers. Using treatment group absorbance ≥3 SD of the control group mean misclassified three weak sensitizers as nonsensitizers (cinnamic alcohol, hydroxycitronellal, and imidazolidinyl urea). Linalool was classified as a nonsensitizer by all of the summary statistics, which is discordant with traditional LLNA results; however, linalool is false positive in the traditional LLNA. It does not produce a sensitization reaction in humans (guinea pig data were not available).

Fourteen ICCVAM performance standards reference substances were discordant for the analysis of alternative decision criteria using the test with the lowest maximum SI as the result for substances with multiple tests (**Table C-VI-4**). Three strong sensitizers, 2,4-dinitrochlorobenzene, 5-chloro-2-methyl-4-isothiazolin-3-one, and cobalt chloride were misclassified by some criteria as nonsensitizers. Nine additional sensitizers, 2-mercaptobenzothiazole, cinnamic alcohol, ethylene glycol dimethacrylate, eugenol, hexyl cinnamic aldehyde, imidazolidinyl urea, isoeugenol, phenyl benzoate, and sodium lauryl sulfated, were also misclassified as nonsensitizers by some criteria. Sodium lauryl sulfate, however, produces a false positive result in the traditional LLNA; it does not produce a sensitization reaction in humans or guinea pigs.

The criteria that yielded the correct results for 2-mercaptobenzothiazole included the summary statistics ≥95% CI, ≥2 SD, or ≥3 SD; and SI ≥ 1.6 to SI ≥ 1.3. The criteria that yielded the correct results for 2,4-dinitrochlorobenzene were all but the SI ≥ 4.5 to 5.0 criteria. The criteria that yielded the correct results for 5-chloro-2-methyl-4-isothiazolin-3-one were all but the SI ≥ 5.0 criterion. The criteria that yielded the correct results for cinnamic alcohol were all except ≥3 SD and SI ≥ 3.0 to SI ≥ 5.0. The criteria that yielded the correct results for cobalt chloride were all but the SI ≥ 5.0 to SI ≥ 4.0 criteria. The criteria that yielded the correct results for ethylene glycol dimethacrylate, eugenol, and phenyl benzoate were all but the SI ≥ 5.0 to SI ≥ 3.5 criteria. The criteria that yielded the correct results for hexyl cinnamic aldehyde were all except SI ≥ 5.0 to SI ≥ 3.0. The criteria that yielded the correct results for imidazolidinyl urea were the summary statistics ANOVA, ≥95% CI, or ≥2 SD; and SI ≥ 1.6 to SI ≥ 1.3 and SI ≥ 3.0 to SI ≥ 5.0. The criteria that yielded the correct results for isoeugenol were all except SI ≥ 5.0 to SI ≥ 2.5.

Two nonsensitizers, lactic acid and methyl salicylate, from the list of ICCVAM performance standards reference substances, were misclassified as sensitizers by some criteria. The criteria that yielded the correct results for lactic acid were all except for treatment group absorbance ≥95% CIand ≥2 SD of the control group mean, and SI ≥ 1.6 to SI ≥ 1.3. The criteria that yielded the correct results for methyl salicylate were all except for treatment group absorbance ≥95% CIof the control group mean and SI ≥ 1.3.

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Annex VII

Evaluation of the Robustness of the SI Cutoff Criteria Used for the LLNA: BrdU-ELISA and LLNA: DA Test Methods

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1.0 Evaluation of the Robustness of the SI Cutoff Criteria Used for the LLNA: BrdU-ELISA and LLNA: DA Test Methods

The analyses described in this annex aim to determine the robustness of the optimum SI criteria for the LLNA: BrdU-ELISA and LLNA: DA test methods. The analyses show that the optimal SI criteria for the LLNA: DA and the LLNA: BrdU-ELISA test methods are quite stable. Taking different samples of the data as training/validation sets has relatively little impact on the cutoff SI criteria or on the resulting number of false positives or false negatives. Both assays perform quite well for the optimized SI cutoff criteria. The proposed SI cutoff criteria should be adopted for now and re-optimized in the future after new prospective data have been collected.

1.1 Basis for Selection of the Optimized Criteria

The optimum SI criteria proposed in **Section 6.5** of the BRD were based on selecting the highest SI values that produced no false negatives, relative to traditional LLNA outcomes, in the entire databases of 43 (LLNA: BrdU-ELISA) or 44 (LLNA: DA) substances. Substances with multiple test results are represented by the most prevalent outcome for the SI criterion evaluated (e.g., if a substance had more negative than positive results at SI $\geq$ 1.6, then the substance was deemed negative). If there were an equal number of positive and negative tests for a substance at a particular SI criterion, then a conservative approach was taken where the substance was deemed positive at that criterion in order to be protective of public health. The "most prevalent outcome" approach is the same as using the median SI, or the higher of the two SI values in the middle of the data if there are an equal number of SI values.

1.2 Methods

Since there are no newly tested substances for which the optimized cutoff criteria (currently proposed to be SI $\geq$ 1.6 for the LLNA: BrdU-ELISA test method and SI $\geq$ 1.8 for the LLNA: DA test method) could be prospectively applied, a retrospective evaluation was performed. This retrospective validation involved taking various samples of the existing data as training sets, re-optimizing the SI cutoff criteria, and then applying the new criteria to the remainder of the data, which would serve as a validation set.

Such a validation exercise can be useful for situations in which the decision criteria for distinguishing between "positives" and "negatives" are quite complex and involve multiple variables. In such cases, it is quite common to discover that an apparently "successful" decision criteria based on a training set is really just an artifact unique to those substances, and cannot be generalized or extrapolated to another set of substances, such as a validation set. However, the LLNA: BrdU-ELISA and LLNA: DA criteria are extremely simple – a single SI cutoff value, which nevertheless produces an outstanding performance: no false negatives and only two false positives (<5%) for 43 LLNA: BrdU-ELISA-tested substances, and no false negatives and only three false positives (<7%) for the 44 LLNA: DA-tested substances. This excellent performance for a single SI cutoff criterion strongly argues that the criterion is robust to sampling.

When carrying out a validation exercise for the LLNA: BrdU-ELISA and LLNA: DA data, it is important to understand that only a small number of substances actually contribute to the determination and stability of the SI cutoff criterion. Thus, rather than taking various samples of the total dataset, one possible approach is a complete enumeration of all possible samples as it relates to the critical substances. Thus, one validation exercise carried out for the LLNA: BrdU-ELISA and LLNA: DA datasets was to look at all possible sample combinations of the four critical substances and examine the robustness of the optimized cutoff criterion in each case. In addition, a more traditional validation exercise for both the LLNA: BrdU-ELISA and LLNA: DA datasets was

performed. The datasets were first divided into phase I and phase II groups based on the dates that the data were submitted to NICEATM. The phase I substances were considered to be the training set, and the phase II substances were considered to be the validation set (and vice versa).

1.3 LLNA: BrdU-ELISA Results

The LLNA: BrdU-ELISA data for 43 substances are summarized and organized by test phase in **Table C-VII-1**. The decision rule applied to the data and the corresponding SI cutoff point were designed to minimize false positives while eliminating false negatives. As indicated above, the results were impressive, with a very low (<5%) false positive rate, when using SI ≥ 1.6 as the cutoff point.

It was noted that choosing SI ≥ 1.5 would produce exactly the same result as SI ≥ 1.6 for the 43 LLNA: BrdU-ELISA substances (no false negatives; two false positives). Choosing the lower critical value of 1.5 would minimize the likelihood of a false negative in the testing of future substances, while SI ≥ 1.6 minimizes the likelihood of future false positives. The calculations that follow use SI ≥ 1.6 as the critical cutoff. This same issue arises for the LLNA: DA data (see **Section 1.4** of this annex**)**. The SI ≥ 1.6 criterion was selected for the LLNA: BrdU-ELISA database because it was the highest SI value that produced no false negatives with minimal false positives.

For the first analysis, half of the LLNA: BrdU-ELISA substances were sampled to form a training set, while the remainder of the data served as the validation set. For each sample, the SI cutoff was re-optimized using the substances in the training set and then applied to the validation set. Because the criterion must be optimized to prevent false negatives and minimize the number of false positives, the SI cutoff is determined solely by the smallest positive SI response of the true positive substances in the training set. Thus, in a sample, the cutoff SI can only increase, never decrease, relative to the cutoff SI for entire database. Similarly, the false positive rate in the validation set can only go down, while the false negative rate can and does go up based on the cutoff value selected using the training set.

Table C-VII-1 SI Data for the LLNA: BrdU-ELISA[1]

Substance Name	SI for True Positives[2]	Substance Name	SI for True Negatives[3]
Phase I (N=31)			
Citral	16.35	Hexane	1.89
1, 4-Phenylenediamine	14.70	Lactic acid	1.89
Glutaraldehyde	14.60	Methyl salicylate	1.43
Diphenylcyclopropenone	11.62	Glycerol	1.29
Trimellitic anhydride	7.85	Dimethyl isophthalate	1.26
p-Benzoquinone	6.90	Propylene glycol	1.20
2, 4-Dinitrochloro-benzene	6.84	2-Hydroxypropyl-methacrylate	1.13
Isoeugenol	6.73	Isopropanol	1.01
Cyclamen aldehyde	5.71	Diethyl phthalate	0.88
Hydroxycitronellal	4.78		
Linalool	4.65		
Formaldehyde	4.40		
Isopropyl myristate	4.19		
Cinnamic aldehyde	3.97		
trans-Cinnamaldehyde	3.50		

continued

Table C-VII-1 SI Data for the LLNA: BrdU-ELISA[1] (continued)

Substance Name	SI for True Positives[2]	Substance Name	SI for True Negatives[3]
Phase I (N=31)			
Hexyl cinnamic aldehyde	3.40		
Eugenol	3.30		
3-Aminophenol	3.06		
Nickel sulfate	2.66		
4-Chloroaniline	2.53		
Aniline	2.07		
2-Mercaptobenzo-thiazole	1.62		
Phase II (N=12)			
Diethyl maleate	6.27	Salicylic acid	1.26
Ethyl acrylate	4.95	Sulfanilamide	1.26
5-Chloro-2-methyl-4-isothiazolin-3-one solution	4.83		
4-Methylaminophenol sulfate	3.98		
Cobalt chloride	3.68		
Phenyl benzoate	3.37		
Ethylene glycol dimethacrylate	3.11		
Cinnamic alcohol	2.74		
Sodium lauryl sulfate	2.64		
Imidazolidinyl urea	1.61		

Abbreviations: N = number of substances; SI = stimulation index.

[1] Substances with multiple test results are represented by the median SI, or the highest of the two SI values in the middle of the data if there are an equal number of SI values.

[2] True positive are substances that are positive in the traditional LLNA.

[3] True negatives are substances that are negative in the traditional LLNA.

The most critical substances for the LLNA: BrdU-ELISA data when evaluating the stability of the cutoff SI are the four lowest SI values for traditional LLNA positive substances. All of the 16 possible combinations of these substances are provided in **Table C-VII-2**.

Table C-VII-2 All Possible Distributions of Four Key Substances in Training (T) or Validation (V) Sets for LLNA: BrdU-ELISA

4-Chloro-aniline (SI=2.53)	Aniline (SI=2.07)	2-Mercapto-benzothiazole (SI=1.62)	Imidizolidinyl urea (SI=1.61)	Cutoff SI[1]	Validation Set	
					No. False Positives[2]	No. False Negatives
T	T	T	T	1.6	0-2	0
T	T	T	V	1.6	0-2	0
T	T	V	T	1.6	0-2	0
T	T	V	V	2.0	0	2
T	V	T	T	1.6	0-2	0
T	V	T	V	1.6	0-2	0
T	V	V	T	1.6	0-2	0
T	V	V	V	2.5	0	3
V	T	T	T	1.6	0-2	0
V	T	T	V	1.6	0-2	0
V	T	V	T	1.6	0-2	0
V	T	V	V	2.0	0	2
V	V	T	T	1.6	0-2	0
V	V	T	V	1.6	0-2	0
V	V	V	T	1.6	0-2	0
V	V	V	V	>2.5	0	≥4

Abbreviations: No. = number; SI = stimulation index; T= substance was in the training set; V = substance was in the validation set.

[1] The cutoff value is determined using the training set.

[2] The number of false positives in the validation set depend upon whether the two LLNA: BrdU-ELISA false positives with SI > 1.6, lactic acid (SI = 1.89) and hexane (SI = 1.89), are in the training set or in the validation set.

The cutoff SI values are relatively stable for the LLNA: BrdU-ELISA. The likelihood is 75% (12/16) that a validation exercise would result in an unchanged cutoff of SI ≥ 1.6, which also was the case when the phase I substances were used as the training set and the phase II substances were used as the validation set (and vice versa). The likelihood is 12.5% (2/16) that the cutoff will be elevated to SI ≥ 2, 6.25% (1/16) that it will be elevated to SI ≥ 2.5, and also 6.25% (1/16) that the re-optimized cutoff SI will exceed 2.5. The higher the cutoff SI, the greater the number of false negatives, as can be seen from **Table C-VII-2**. It is also important to recognize that most of the data are not relevant to determining the cutoff SI point. Only the "weakest positives" are critical, and the greater the variability among the SI values for these critical substances, the less stable the cutoff SI points will be.

The second validation exercise considered the phase I substances as a training set and the phase II substances as a validation set (and vice versa). If the phase I data are used as the training set, the SI cutoff point remains unchanged at ≥1.6; if the phase II data are used as the training set, then the SI cutoff point also remains unchanged (≥1.6). If the phase I data cutoff point was used in the evaluation of phase II substances, then there would be no false positives or false negatives. Conversely, if the phase II cutoff point was used to evaluate the substances in phase I, then there would be no false negatives and two false positives. Once again, the results of the validation study produce quite stable results.

1.4 LLNA: DA Results

The LLNA: DA data for 44 substances are organized by test phase and summarized in **Table C-VII-3**. Again, the decision rule applied to the data and the corresponding SI cutoff point were designed to minimize false positives while totally eliminating false negatives. These data showed a low (<7%) false positive rate. The cutoff value was set at SI ≥ 1.8 based on the data from the 44 substances, although a lower cutoff point, namely SI ≥ 1.7, would have performed exactly the same for these 44 substances (no false negatives; three false positives).

For the first analysis, half of the LLNA: DA substances were sampled to form a training set, while the remainder of the data served as a validation set. For each sample, the SI cutoff is re-optimized based on the substances in the training set and then applied to the validation set. Because the criterion must be optimized to prevent false negatives and minimize the number of false positives, the SI cutoff is determined solely by the smallest SI responses of the true positive substances in the training set. Thus in a sample, the cutoff SI can only increase, never decrease, relative to the cutoff SI for entire database. Similarly, the false positive rate in the validation set can only go down, while the false negative rate can and does go up based on the cutoff value selected using the training set.

Table C-VII-3 SI Data for the LLNA: DA[1]

Phase I (N=31)			
Substance Name	**SI for True Positives[2]**	**Substance Name**	**SI for True Negatives[3]**
2, 4-Dinitrochloro-benzene	9.96	Chlorobenzene	2.44
Isoeugenol	7.09	Hexane	2.31
Eugenol	7.07	1-Bromobutane	1.65
Benzalkonium chloride	6.68	Methyl salicylate	1.55
Abietic acid	6.26	Propylparaben	1.28
Hydroxycitronellal	5.69	Dimethyl isophthalate	1.26
Hexyl cinnamic aldehyde	5.50	Isopropanol	1.21
Phthalic anhydride	5.49	Diethyl phthalate	1.09
Potassium dichromate	5.49	Lactic acid	0.97
p-Phenylenediamine	5.14		
Glutaraldehyde	5.00		
Trimellitic anhydride	4.96		
Formaldehyde	4.84		
Cinnamic aldehyde	4.73		
Imidazolidinyl urea	4.67		
Citral	4.40		
Resorcinol	4.33		
Cobalt chloride	4.25		
Sodium lauryl sulfate	3.39		
3-Aminophenol	2.38		
Nickel (II) sulfate hexahydrate	2.13		
2-Mercaptobenzo-thiazole	2.00		

continued

Table C-VII-3 SI Data for the LLNA: DA[1] (continued)

Phase II (N=13)

Substance Name	SI for True Positives[2]	Substance Name	SI for True Negatives[3]
5-Chloro-2-methyl-4-isothiazolin-3-one	7.50	Salicylic acid	2.00
Cinnamic alcohol	5.66	Nickel (II) chloride	1.30
Propyl gallate	4.95	Sulfanilamide	0.86
Butyl glycidyl ether	4.59		
Ethylene glycol dimethacrylate	4.45		
Ethyl acrylate	4.29		
Phenyl benzoate	4.24		
p-Benzoquinone	3.79		
Diethyl maleate	3.78		
Methyl methacrylate	1.81		

Abbreviations: N = number of substances; SI = stimulation index.

[1] Substances with multiple test results are represented by the median SI or the highest of the two SI values in the middle of the data if there are an equal number of SI values.

[2] True positives are substances that are positive in the traditional LLNA.

[3] True negatives are substances that are negative in the traditional LLNA.

The four most critical substances for the LLNA: DA data when evaluating the stability of the cutoff SI are the four lowest SI values for positive substances. All of the 16 possible combinations of these substances are given in **Table C-VII-4**.

Table C-VII-4 All Possible Distributions of Four Key Substances in Training (T) or Validation (V) Sets for LLNA: DA

3-Aminophenol (SI=2.38)	Nickel sulfate (SI=2.13)	2-Mercapto-benzothiazole (SI=2.00)	Methyl methacrylate (SI=1.81)	Cutoff SI[1]	Validation Set	
					No. False Positives[2]	No. False Negatives
T	T	T	T	1.8	0-3	0
T	T	T	V	2.0	0-3	1
T	T	V	T	1.8	0-3	0
T	T	V	V	2.1	0-2	2
T	V	T	T	1.8	0-3	0
T	V	T	V	2.0	0-3	1
T	V	V	T	1.8	0-3	0
T	V	V	V	2.3	0-2	3
V	T	T	T	1.8	0-3	0
V	T	T	V	2.0	0-3	1
V	T	V	T	1.8	0-3	0
V	T	V	V	2.1	0-2	2

continued

Table C-VII-4 All Possible Distributions of Four Key Substances in Training (T) or Validation (V) Sets for LLNA: DA (continued)

3-Aminophenol (SI=2.38)	Nickel sulfate (SI=2.13)	2-Mercapto-benzothiazole (SI=2.00)	Methyl methacrylate (SI=1.81)	Cutoff SI[1]	Validation Set	
					No. False Positives[2]	No. False Negatives
V	V	T	T	1.8	0-3	0
V	V	T	V	2.0	0-3	1
V	V	V	T	1.8	0-3	0
V	V	V	V	>2.3	0-2	≥4

Abbreviations: No. = number; SI = stimulation index; T= substance was in the training set; V = substance was in the validation set.

[1] The cutoff value is determined using the training set.

[2] The number of false positives in the validation set depends upon whether the three LLNA: DA false positives (salicylic acid [SI = 2.0], hexane [SI= 2.31], and chlorobenzene [SI = 2.44]) are in the training set or in the validation set.

The cutoff SI values are relatively robust for the LLNA: DA test method also. The likelihood is 50% (8/16) that a validation exercise would result in an unchanged cutoff of SI ≥ 1.8. The likelihood is 25% (4/16) that the cutoff will be increased slightly to SI ≥ 2.0. The likelihood is 12.5% (2/16) that the cutoff will be elevated to SI ≥ 2.1 and 6.25% (1/16) that it will be greater than 2.3.

This conclusion regarding the stability of the cutoff SI is supported by the phase I vs. phase II approach to validation. This approach considered the phase I substances as a training set and the phase II substances as a validation set (and vice versa). If the phase I LLNA: DA data are used as the training set, the optimized cutoff SI criterion increases slightly from 1.8 to 2.0. If the phase II data are used as the training set, then the SI cutoff criterion remains unchanged at ≥1.8. If the phase I data cutoff point was used in the evaluation of phase II substances, then there would be one false positive and one false negative (methyl methacrylate, SI ≥ 1.81). Conversely, if the phase II cutoff point was used to evaluate the substances in phase I, then there would be no false negatives and two false positives.

1.5 Conclusions

These analyses show that the SI criteria for the LLNA: DA and LLNA: BrdU-ELISA test methods are quite robust. Taking different samples of the data as training/validation sets has relatively little impact on cutoff SI criteria or on the number of false positives or false negatives. Both assays perform quite well for the optimized SI cutoff criteria. The proposed SI cutoff criteria should be adopted for now, and re-optimized in the future after new prospective data have been collected.

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Annex VIII

Analyses Using Multiple SI Decision Criteria

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1.0 Introduction

This annex provides analyses associated with using two decision criteria for classifying substances using the results from the LLNA: BrdU-ELISA: one criterion to classify substances as sensitizers and another criterion to classify substances as nonsensitizers. The data used for the analyses in this annex are the LLNA: BrdU-ELISA results for the 31 substances (22 traditional LLNA sensitizers and nine traditional LLNA nonsensitizers) that were reviewed by the Panel at the public meeting on April 28-29, 2009. **Section 2** of this annex discusses the accuracy produced by using the two decision criteria and includes an evaluation of discordant, or indeterminate, substances that produced SI values in between the sensitizer and nonsensitizer SI criteria. **Section 3** provides the reproducibility analysis using the decision criterion for sensitizers (**Sections 3.1** and **3.2**) and for tests yielding SI values in three categories: sensitizer, nonsensitizer, and indeterminate (i.e., in the range of uncertainty) (**Section 3.3**). The two SI values determined to be optimal were based on four animals per dose group and resulted in nine substances that could not be definitively classified because they produced SI values in the range of uncertainty. **Section 4** describes the impact of sample size on the range of the uncertainty between the sensitizer and nonsensitizer criteria. **Section 5** evaluates a number of physicochemical characteristics and other parameters to distinguish between traditional LLNA sensitizers and nonsensitizers in the LLNA: BrdU-ELISA when using multiple SI decision criteria for their potential use in providing additional information for use in classifying substances that produce SI values in the range of uncertainty.

2.0 Accuracy Analysis Using Multiple Stimulation Index Decision Criteria

The accuracy of the LLNA: BrdU-ELISA with respect to the traditional LLNA using a number of alternative decision criteria (and the most prevalent outcome for substances with multiple tests) was evaluated in **Section 6.5** of the BRD. This section evaluates the accuracy of the LLNA: BrdU-ELISA when two SI decision criteria are used to classify test substances: one criterion for sensitizers and another criterion for nonsensitizers. For the database of 31 substances, the lowest decision criterion with a 0% (0/9) false positive rate was SI $\geq$ 2.0, which was used by the JSAAE interlaboratory validation study. The accuracy at SI $\geq$ 2.0 was 84% (26/31) and the false negative rate was 23% (5/22). Higher SI values also produced false positive rates of 0% (0/9), but the false negative rate increased as the SI increased. The lowest false negative rate was produced at SI $\geq$ 1.3 (0% [0/22]), but the false positive rate at SI $\geq$ 1.3 was 44% (4/9).

The 0% false positive rate using SI $\geq$ 2.0 and the 0% false negative rate using SI $\geq$ 1.3 prompted the evaluation of using two decision criteria for LLNA: BrdU-ELISA results: one criterion to classify substances as sensitizers and another criterion to classify substances as nonsensitizers. Further examination of the LLNA: BrdU-ELISA results indicated that a lower SI criterion than SI $\geq$ 2.0, SI $\geq$ 1.9, also correctly identified traditional LLNA sensitizers with no false positives. Thus, SI $\geq$ 1.9 was proposed as the criterion to classify substances as sensitizers. The SI $\geq$ 1.3 criterion, when used to classify sensitizers, resulted in no false negative results with respect to the traditional LLNA results. Thus, SI $\leq$ 1.3 was proposed to classify substances as nonsensitizers because this criterion also resulted in no false negative results.

2.1 Indeterminate Results Using Multiple Alternative Decision Criteria

While optimum false positive and false negative rates can be achieved for the 31 substances evaluated in the LLNA: BrdU-ELISA accuracy analyses using these two different decision criteria, a range of SI values (i.e., 1.3 < SI < 1.9, the range of uncertainty) exists for which the correct classification is not definitive (i.e., there is a chance for false positive or false negative results for substances that produce SI values in this range). Chemical class, physical form, MW, peptide reactivity (see

Annex II for physicochemical properties), traditional LLNA EC3 range (**Table C-1**), and potential for skin irritation (**Annex III-1**) were examined to identify commonalities among the substances that produced SI values between 1.3 and 1.9 in an attempt to identify common characteristics among these substances that could be used to correctly classify such substances. **Section 5.0** of this annex provides a comprehensive evaluation of a number of physicochemical characteristics and other parameters, using the entire LLNA: BrdU-ELISA database, to distinguish between traditional LLNA sensitizers and nonsensitizers.

Of the nine substances that produced SI values in the range of uncertainty, between 1.3 and 1.9, five substances are nonsensitizers and four are sensitizers based on traditional LLNA results (**Table C-VIII-1**). The five substances classified by the traditional LLNA as nonsensitizers (hexane, isopropanol, lactic acid, methyl salicylate, and propylene glycol), represented four chemical classes (acyclic hydrocarbons, alcohols, carboxylic acids, and phenols).

- Two substances are classified as carboxylic acids (methyl salicylate, also a phenol, and lactic acid) and two were classified as alcohols (isopropanol and propylene glycol).
- Hexane is an acyclic hydrocarbon.

Other characteristics of the indeterminate substances that are traditional LLNA nonsensitizers include:

- All of the five substances are liquids and have minimal peptide reactivity.
- Four substances have MW < 100 g/mole. The other substance, methyl salicylate, has a MW of 152.15 g/mole.
- Four of the five substances were tested at irritating concentrations in the LLNA: BrdU-ELISA: hexane, lactic acid, methyl salicylate, and propylene glycol, based on skin irritation data from mice, rabbits, or humans. Isopropanol was tested at concentrations nonirritating to skin, based on skin irritation data from rabbits.
- Two of the five substances yielded SI < 2 in the traditional LLNA: isopropanol and propylene glycol. The other three substances yielded SI values between 2 and 3 (exclusive): hexane, lactic acid and methyl salicylate.

Table C-VIII-1 Indeterminate Results for LLNA: BrdU-ELISA When Multiple Decision Criteria Were Used [1]

Substance Name[2]	Vehicle[3]	LLNA: BrdU-ELISA[4]	Traditional LLNA[5]	Skin Irritant?
Hexane	AOO	1.76, 100% 1.89, 50% (2/2 tests)	- (2.2, 100%)	Irritant at 100% (humans)
Isopropanol	AOO	1.57, 50% (1/7 tests)	- (1.7, 50%)[6]	No, up to 100% (rabbits)
Lactic acid	DMSO	1.80, 50% 1.89, 50% (2/3 tests)	- (2.2, 25%)	Slightly irritating at 10% (rabbits)
Methyl salicylate	AOO	1.40, 50% 1.43, 50% 1.44, 50% (3/3 tests)	- (2.9, 20%)	Irritant at 10% (mice)

continued

Table C-VIII-1 Indeterminate Results for LLNA: BrdU-ELISA When Multiple Decision Criteria Were Used [1] (continued)

Substance Name[2]	Vehicle[3]	LLNA: BrdU-ELISA[4]	Traditional LLNA[5]	Skin Irritant?
Propylene glycol	AOO[7]	1.57, 50% (1/3 tests)	- (1.6, 100%)[8]	No, up to 25% (humans)
Aniline (47.5%)	AOO	1.50, 50%	+ (3.6, 100%)[7]	No, up to 100% (GP); Irritant at 20% (humans)
Hydroxycitronellal (24.0%)	AOO	1.34, 100%	+ 8.5, 100%)	No, up to 50% (GP)
Linalool (30.0%)	AOO	1.45, 100%[8]	+ (8.3, 100%)	Mild irritant at 100% (rabbits)
2-Mercaptobenzo-thiazole (1.7%)	DMF	1.62, 50%[9]	+ (8.6, 10%)	No, up to 10% (GP); No, up to 25% (humans)

Abbreviations: AOO = acetone: olive oil (4:1); DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; LLNA: BrdU-ELISA= murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; LLNA = murine local lymph node assay; + = sensitizer; - = nonsensitizer.

[1] Data sources provided in **Annex III-1**.

[2] Numbers in parentheses are EC3 values (estimated concentration needed to produce a stimulation index [SI] of 3) for substances that are sensitizers in the traditional LLNA; from **Table C-1**.

[3] Vehicles apply to tests for both the LLNA: BrdU-ELISA and the traditional LLNA unless otherwise noted.

[4] Numbers are highest SI values achieved and maximum concentration tested.

[5] Information in parentheses indicates the basis for the human outcome. Numbers indicate the incidence of positive human response and concentration tested.

[6] Highest SI occurred at 10%.

[7] The vehicle for the traditional LLNA was distilled water.

[8] Highest SI occurred at 50%.

[8] The solvent for the traditional LLNA was *N,N*-dimethylformamide.

[9] Highest SI occurred at 12.5%.

The four indeterminate substances classified by the traditional LLNA as sensitizers (aniline, hydroxycitronellal, linalool, and 2-mercaptobenzothiazole,) represent three chemical classes. Aniline is an amine, hydroxycitronellal and linalool are hydrocarbons (other), and 2-mercaptobenzothiazole is a heterocyclic compound. Other characteristics of the indeterminate substances that are classified as sensitizers by the traditional LLNA include:

- Three are liquids and one is a solid (2-mercaptobenzothiazole).

- All four substances have MW between 90 and 200 g/mole.
- Hydroxycitronellal exhibits low peptide reactivity, 2-mercaptobenzothiazole exhibits high peptide reactivity, and peptide reactivity information is not available for the other two substances.
- Aniline, linalool, and hydroxycitronellal were not strongly positive in the traditional LLNA (EC3 = 47.5%, 30%, and 24%, respectively), with maximum SI = 3.6, 8.3, and 8.5, respectively, when tested at concentrations up to 100%. 2-Mercaptobenzothiazole, however, was a strong positive (EC3 = 1.7%).
- All four substances were tested in the LLNA: BrdU-ELISA at concentrations that were irritating to skin, based on human, guinea pig, or rabbit data.

3.0 Test Method Reliability

An assessment of test method reliability (intra- and interlaboratory reproducibility) is an essential element of any evaluation of the performance of an alternative test method (ICCVAM 2003). Intralaboratory reproducibility refers to the extent to which qualified personnel within the same laboratory can replicate results using a specific test protocol at different times. Interlaboratory reproducibility refers to the extent to which different laboratories can replicate results using the same protocol and test substances and indicates the extent to which a test method can be transferred successfully among laboratories.

The available LLNA: BrdU-ELISA data were amenable to both intralaboratory and interlaboratory reproducibility analyses. This section provides an assessment of reproducibility for the decision criterion of SI $\geq$ 1.9 to identify sensitizers. As described in **Section 2.0** of this annex, SI $\geq$ 1.9 was evaluated as the decision criterion for classifying substances as sensitizers with SI $\leq$ 1.3 as the criterion to identify nonsensitizers.

3.1 Intralaboratory Reproducibility

The test results for the LLNA: BrdU-ELISA were amenable to intralaboratory reproducibility analyses for three endpoints: sensitizer or nonsensitizer classification, SI values, and EC1.9 values. Analyses of intralaboratory reproducibility were performed using a concordance analysis for the qualitative results (sensitizer vs. nonsensitizer) (**Section 3.1.1** of this annex) and a coefficient of variation (CV) analysis for the quantitative results (SI values and EC3 values) (**Sections 3.1.2** and **3.1.3** of this annex, respectively).

3.1.1 Intralaboratory Reproducibility – Qualitative Results

The dataset available for an intralaboratory concordance analysis of the qualitative test results for the LLNA: BrdU-ELISA included nine substances that were tested multiple times and classified as sensitizers or nonsensitizers. Hexyl cinnamic aldehyde and eugenol were tested six times; isoeugenol was tested four times; diphenycyclopropenone and propylene glycol were tested three times; and 2,4-dinitrochlorobenzene, glutaraldehyde, hexane, and 4-phenylenendiamine were each tested twice (Takeyoshi et al. 2003, 2004a, 2005, 2006, 2007a; unpublished data) (**Table C-VIII-2**). All substances were sensitizers in the traditional LLNA except for propylene glycol and hexane. The multiple test results for 9/9 substances were 100% concordant when SI $\geq$ 1.9 was used to classify substances as sensitizers.

By comparison, the qualitative intralaboratory concordance analysis for the traditional LLNA (ICCVAM 1999) was based on a dataset of six substances that included six results each for benzocaine and hexyl cinnamic aldehyde, five results for eugenol, four results each for isoeugenol and methyl salicylate, and three results for 2,4-dinitrochlorobenzene. Intralaboratory results for each substance were 100% concordant with the exception of benzocaine. One of the six benzocaine (5/6 or

83% concordance) results for the traditional LLNA was reported as equivocal because SI increased with dose, but did not reach the criterion of SI ≥ 3.0. Thus, the proportion of substances for which intralaboratory concordance of qualitative results was 100% was greater for LLNA: BrdU-ELISA (9/9) than for the traditional LLNA (5/6).

Table C-VIII-2 Intralaboratory Reproducibility for the LLNA: BrdU-ELISA Outcome of Substances Tested Multiple Times

Substance Name	Highest Concentration Tested (%)	Highest SI	Outcome	Takeyoshi et al. Reference
2,4-Dinitro-chlorobenzene	2	17.86	+	2005
	2	6.84	+	2006, 2007b
Diphenylcyclopro-penone	2	19.10	+	2005; 2007b
	10	9.34	+	2005
	10	11.62	+	2007b
Eugenol	10	3.18	+	2003
	30	3.33	+	2004a
	30	3.83	+	2007a
	50	12.28	+	2005
	50	3.05	+	2006
	50	17.69	+	2007b
Glutaraldehyde	2	14.60	+	2005, 2007b
	10	15.50	+	2005, 2007b
Hexane	50	1.89	-	2005
	100	1.76	-	unpublished data
Hexyl cinnamic aldehyde	25	2.41	+	2003
	50	3.64	+	2003
	50	5.90	+	2005
	50	3.64	+	2006
	50	2.72	+	2006
	50	3.02	+	2007b
Isoeugenol	10	8.36	+	2005
	10	2.36	+	2006, 2007b
	10	7.20	+	2005
	30	6.73	+	2007a
4-Phenylenediamine	2	11.70	+	2005, 2007b
	10	14.70	+	2005, 2007b
Propylene glycol	10	1.20	-	2005
	50	1.57	-	2005
	50	0.91	-	2006, 2007b

Abbreviations: LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SI = stimulation index.

+ = sensitizer; - = nonsensitizer.

3.1.2 Intralaboratory Reproducibility – SI

There were seven substances that were tested multiple times at the same concentrations by Takeyoshi et al. (2003, 2004a, 2005, 2006, 2007a, 2007b, unpublished data). Because two substances had multiple tests for more than one concentration, there were 10 substance/concentration combinations that were tested two to five times in separate experiments. The multiple SI values for each substance/concentration were used to calculate a CV for the assessment of intralaboratory variability. As shown by **Table C-VIII-3**, the CVs ranged from 1% (25% hexyl cinnamic aldehyde) to 80% (10% isoeugenol). The intralaboratory reproducibility of the traditional LLNA was not assessed by CV analysis of SI values (ICCVAM 1999).

3.1.3 Intralaboratory Reproducibility – EC1.9

CV values were also calculated for the EC1.9 values for the three sensitizers that were tested more than once using multiple doses by Takeyoshi et al. (2003, 2004a, 2005, 2006, 2007a, 2007b). The individual animal data for eugenol, hexyl cinnamic aldehyde, and isoeugenol were used to calculate EC1.9 values for the LLNA: BrdU-ELISA. The methods for calculating EC1.9 values for each sensitizer were modified from those used by Ryan et al. (2007) to calculate EC3 values. Linear interpolation was used to calculate EC1.9 values for each test with SI values higher or lower than 1.9, and extrapolation was used to calculate EC1.9 values for tests with no SI values below 1.9. The equation for linear interpolation was:

$$EC1.9 = c + \left[\frac{(1.9 - d)}{(b - d)}\right] \times (a - c)$$

The linear interpolation equation uses the points immediately above and below SI = 1.9, with the (dose, SI) coordinates of (a, b) immediately above SI = 1.9 and (c, d) immediately below SI = 1.9. The equation for extrapolation was:

$$EC1.9_{ex} = 2^{\left\{\log_2(c) + \frac{(1.9-d)}{(b-d)} \times \left[\log_2(a) - \log_2(c)\right]\right\}}$$

Table C-VIII-3 Intralaboratory Reproducibility for the SI of Tested Substances in LLNA: BrdU-ELISA – Coefficient of Variation

Substance Name	Concentration Tested (%)	SI	Mean	SD	CV (%)	Takeyoshi et al. Reference
2,4-Dinitrochlorobenzene	2	17.86	12.35	7.79	63	2005
		6.84				2006, 2007b
Diphenylcyclopropenone	10	9.34	10.48	1.61	15	2005; 2007b
		11.62				2007b
Eugenol	30	3.33	3.58	0.35	10	2004a
		3.83				2007a
Eugenol	50	12.28	11.01	7.40	67	2005
		3.05				2006
		17.69				2007b
Hexane	50	1.89	1.64	0.36	22	2005
		1.38				Unpublished

continued

Table C-VIII-3 Intralaboratory Reproducibility for the SI of Tested Substances in LLNA: BrdU-ELISA – Coefficient of Variation (continued)

Substance Name	Concentration Tested (%)	SI	Mean	SD	CV (%)	Takeyoshi et al. Reference
Hexyl cinnamic aldehyde	12.5	1.88	1.74	0.21	12	2003
		1.59				2003
Hexyl cinnamic aldehyde	25	2.44	2.42	0.02	1	2003
		2.41				2003
Hexyl cinnamic aldehyde	50	3.64	3.78	1.25	33	2003
		5.90				2005
		3.64				2006
		2.72				2006
		3.02				2007b
Isoeugenol	10	8.36	5.09	3.15	80	2005
		7.20				2005
		2.36				2006, 2007b
		2.43				2007a
Propylene glycol	50	1.57	1.14	0.62	54	2005
		0.70				2006, 2007b

Abbreviations: CV = coefficient of variation; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SD = standard deviation, SI = stimulation index.

The extrapolation equation uses the two points immediately above SI = 1.9, with the coordinates of (a, b) for the point closest to SI = 1.9, and (c, d) for the higher point. As shown in **Table C-VIII-4**, there were five EC1.9 values for hexyl cinnamic aldehyde, four EC1.9 values for eugenol, and two EC1.9 values for isoeugenol. The CV values were 72% for eugenol, 27% for hexyl cinnamic aldehyde, and 21% for isoeugenol. The ICCVAM LLNA *Performance Standards* criteria for demonstrating adequate intralaboratory reproducibility are based on results from at least four independent tests of hexyl cinnamic aldehyde (ICCVAM 2009). Intralaboratory reproducibility is considered adequate when each test yields an ECt value (i.e., the estimated concentration needed to produce an SI of a specific threshold value; in this case, SI = 1.9) within 5% to 20% (ICCVAM 2009). Two of the five EC1.9 values for hexyl cinnamic aldehyde were within the acceptable range for intralaboratory reproducibility.

Table C-VIII-4 Intralaboratory Reproducibility for the EC1.9 of Tested Substances in LLNA: BrdU-ELISA - Coefficient of Variation

Substance Name	EC1.9	Mean	SD	CV (%)	Takeyoshi et al. Reference
Eugenol	10.1	11.5	8.3	72	2004a
	21.1				2006
	1.2				2007b
	13.7				2007a
Hexyl cinnamic aldehyde	12.9	20.5	5.6	27	2003
	17.2				2003
	27.1				2006
	24.0				2006
	21.4				2007b
Isoeugenol	8.0	7.0	1.5	21	2006; 2007b
	5.9				2007a

Abbreviations: CV = coefficient of variation; EC1.9 = estimated concentration needed to produce a stimulation index of 1.9; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SD = standard deviation.

The intralaboratory reproducibility of the traditional LLNA was assessed by CV analysis of EC3 values using a larger dataset (ICCVAM 1999) than that available for the LLNA: BrdU-ELISA analysis. Two EC3 values were reported by each of five laboratories for 2,4-dinitrochlorobenzene, five EC3 values were reported by one laboratory for isoeugenol, six EC3 values were reported for hexyl cinnamic aldehyde by two laboratories, and five EC3 values were reported for eugenol by one laboratory (**Table C-VIII-5**).

Table C-VIII-5 Intralaboratory Reproducibility for the EC3 of Tested Substances in the Traditional LLNA[1]

Substance Name	Number of Laboratories	Number of Tests per Laboratory	CV (%)
2, 4-Dinitrochlorobenzene	5	2	13-47
Isoeugenol	1	5	26
Hexyl cinnamic aldehyde	2	6	19-27
Eugenol	1	5	18

Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of 3; LLNA = murine local lymph node assay;

[1] From ICCVAM (1999).

For one of three substances, the intralaboratory CV values for the EC1.9 values from LLNA: BrdU-ELISA tests were higher than EC3 values for the same substances from the traditional LLNA reported in ICCVAM (1999). The intralaboratory EC1.9 CV from the LLNA: BrdU-ELISA tests of eugenol was higher that that reported by ICCVAM (1999) for EC3 values (72% vs. 18%). However, the intralaboratory EC1.9 CV from the LLNA: BrdU-ELISA tests of isoeugenol was less than that for EC3 values from ICCVAM (1999) (21% vs. 26%). The intralaboratory EC1.9 CV from the

LLNA: BrdU-ELISA tests of hexyl cinnamic aldehyde was within the range reported by ICCVAM (1999) for EC3 values (27% vs. 19% to 27%).

3.2 Interlaboratory Reproducibility

The interlaboratory reproducibility of the LLNA: BrdU-ELISA was assessed using the individual animal data from the multilaboratory validation study organized by the JSAAE (Kojima et al. 2008). Phase I of the study evaluated the reliability and transferability of the test method protocol by testing 12 substances in three to nine laboratories. With the exception of the positive control data, neither the summary results nor the individual animal data from phase I of the validation study have been released. Phase II of the study tested 10 substances in three to seven laboratories as shown in **Table C-VIII-6**. All the laboratories that participated in the validation study used the same experimental protocol (**Annex I** of the BRD) and participated in a one-day seminar that explained the protocol and execution of the test method. The same commercial ELISA kit, test materials, and the same doses of the test substances were used in all of the laboratories. The Validation Management Team determined the doses and vehicles for testing and coded the identity of the test substances prior to distribution to the test laboratories. Seven substances were sensitizers and three substances were nonsensitizers according to the traditional LLNA. Six substances were ICCVAM *Recommended Performance Standards* reference substances: 2,4-dinitrochlorobenzene, eugenol, hexyl cinnamic aldehyde, lactic acid, isopropanol, and methyl salicylate (ICCVAM 2009).

Table C-VIII-6 Substances and Test Allocation for the Phase II Interlaboratory Validation Study of the LLNA: BrdU-ELISA

Substance Name[1]	Vehicle	Concentrations Tested			Laboratory[2]						
					1	2	3	4	5	6	7
Nickel sulfate (+)	DMSO	1%	3%	10%			X	X			X
Isopropanol (-)	AOO	10%	25%	50%	X	X	X	X	X	X	X
Eugenol (+)	AOO	10%	25%	50%		X				X	X
Cinnamic aldehyde (+)	AOO	1%	3%	10%		X		X	X		
2,4-Dinitrochlorobenzene (+)	AOO	0.1%	0.3%	1%	X	X	X	X	X	X	X
Glutaraldehyde (+)	ACE	0.1%	0.3%	1%	X				X	X	
Methyl salicylate (-)	AOO	10%	25%	50%	X	X	X				
Hexyl cinnamic aldehyde (+)	AOO	10%	25%	50%	X	X	X	X	X	X	X
Lactic acid (-)	DMSO	10%	25%	50%			X	X			X
Formaldehyde (+)	ACE	1%	3%	10%	X				X	X	

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.

[2] X indicates that a substance was tested in a particular laboratory. 1 = Daicel Chemical Industries Ltd.; 2 = Food and Drug Safety Center; 3 = Otsuka Pharmaceutical Co. Ltd.; 4 = Taisho Pharmaceutical Co. Ltd.;

5 = Fuji Film Co. Ltd.; 6 = Biosafety Research Center, Foods, Drugs and Pesticides; 7 = National Institute of Health Sciences.

The LLNA: BrdU-ELISA test results from the JSAAE validation study were used for interlaboratory reproducibility analyses for both qualitative and quantitative endpoints. Analyses of interlaboratory reproducibility were performed using a concordance analysis for the qualitative results (sensitizer vs. nonsensitizer) (**Section 3.2.1** of this annex) and a CV analysis for the quantitative results (EC1.9 values) (**Sections 3.2.2** of this annex).

3.2.1 Interlaboratory Reproducibility – Qualitative Results

The available quantitative absorbance data for interlaboratory reproducibility analysis were used to calculate SI values for each substance and dose tested. Substances with SI ≥ 1.9 at any dose were classified as sensitizers. The qualitative (sensitizer/nonsensitizer) interlaboratory concordance analysis for the 10 substances tested during Phase II of the JSAAE interlaboratory validation study is shown in **Table C-VIII-7**. The qualitative comparison evaluated the consistency of LLNA: BrdU-ELISA results (i.e., positive vs. negative) for 10 substances tested among up to 7 laboratories. The results show that interlaboratory concordance was 100% (3/3, 6/6, or 7/7) for eight substances. There were two discordant substances (isopropanol and lactic acid) for which interlaboratory concordance was 67% (2/3 or 4/6). Two of the six tests of isopropanol yielded SI ≥ 1.9 (SI = 2.04 and SI = 2.22), while the others yielded SI < 1.9. One of the three tests for lactic acid produced SI ≥ 1.9 (i.e., SI = 2.53), while the others yielded SI < 1.9. The Validation Management Team, which used SI ≥ 2.0 as the decision criterion, considered the interlaboratory reproducibility to be acceptable (Kojima et al. 2008). Because the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), there were no traditional concordance data for comparison with the LLNA: BrdU-ELISA concordance.

Table C-VIII-7 Qualitative Results for the Phase II Interlaboratory Validation Study on the LLNA: BrdU-ELISA[1]

Substance Name	Laboratory							Concordance
	1	2	3	4	5	6	7	
2,4-Dinitrochlorobenzene	+ (4.30)	+ (8.37)	+ (6.26)	+ (5.50)	+ (18.80)	+ (4.83)	+ (12.98)	7/7
Glutaraldehyde	+ (3.72)				+ (28.64)	+ (2.25)		3/3
Nickel sulfate			+ (2.58)	+ (4.53)			+ (2.66)	3/3
trans-Cinnamic aldehyde		+ (3.37)		+ (3.50)	+ (4.11)			3/3
Formaldehyde	+ (4.40)				+ (16.59)	+ (1.97)		3/3
Eugenol		+ (3.17)				+ (3.18)	+ (7.09)	3/3
Hexyl cinnamic aldehyde	+ (3.40)	-[3]	+ (2.87)	+ (3.34)	+ (13.50)	+[4] (3.27)	+ (3.84)	6/6
Isopropanol	+[2] (2.22)	-[3]	- (0.98)	- (1.57)	- (0.94)	+[2,5] (2.04)	- (1.01)	4/6
Lactic acid			- (1.80)	- (1.89)			+ (2.53)	2/3
Methyl salicylate	- (1.43)	- (1.44)	- (1.40)					3/3

Abbreviation: LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine.

[1] + indicates sensitizer result; - indicates nonsensitizer result.

[2] Stimulation index (SI) ≥ 1.9 at lowest dose tested, but <1.9 at the higher doses. The Validation Management Team considered these to be nonsensitizer results (Kojima et al. 2008).

[3] Test failed because concurrent positive control failed (i.e., SI < 1.9). Result not included in the concordance analysis.

[4] Three mice tested at highest dose.

[5] Three mice per dose group.

3.2.2 Interlaboratory Reproducibility – EC1.9 Values

The SI values from the interlaboratory validation study were used to calculate EC1.9 values for each sensitizer according to the methods reported in **Section 3.1.3** of this annex. The EC1.9 values from each laboratory were then used to calculate CV values for each substance. The resulting values are shown in **Table C-VIII-8**. CV values ranged from 27% (*trans*-cinnamic aldehyde) to 87% (glutaraldehyde). The mean CV was 62%.

The ICCVAM LLNA performance standards indicate that interlaboratory reproducibility should be evaluated with at least two sensitizing chemicals with well-characterized activity in the traditional LLNA (ICCVAM 2009). Acceptable reproducibility is attained when each laboratory obtains ECt values within 0.025% to 0.1% for 2,4-dinitrochlorobenzene and within 5% to 20% for hexyl cinnamic aldehyde (ICCVAM 2009). EC1.9 values from three laboratories were outside the range for 2,4-dinitrochlorobenzene, and the EC1.9 values from two laboratories were outside the range for hexyl cinnamic aldehyde. Laboratories 2, 5, and 6 reported EC1.9 values that were lower than the specified acceptance range for 2,4-dinitrochlorobenzene (0.017%, 0.0024%, and 0.023%, respectively). For hexyl cinnamic aldehyde, Laboratory 3 obtained an EC1.9 value of 22.21%, which was higher than the acceptance range. Laboratory 5 obtained an EC1.9 value of 3.96%, which was lower than the acceptance range.

The interlaboratory CV values for the LLNA: BrdU-ELISA EC1.9 values were higher than those for the traditional LLNA EC3 values. The analysis of interlaboratory variation of EC3 values for the traditional LLNA reported CV values of 7 to 84% for five substances tested in five laboratories (**Table C-VIII-9**; ICCVAM 1999). Three of the same substances were evaluated in the traditional LLNA and the LLNA: BrdU-ELISA. All interlaboratory CV values for LLNA: BrdU-ELISA were greater than those for the traditional LLNA. The CV of 77% for the EC1.9 of 2,4-dinitrochlorobenzene was greater than the two CV values of 37% and 27% calculated from five EC3 values each, reported by ICCVAM (1999). The CV of 51% for the EC1.9 of hexyl cinnamic aldehyde tested in the LLNA: BrdU-ELISA was greater than the 7% for the EC3 reported by ICCVAM (1999). The CV of 55% for the EC1.9 of eugenol tested in the LLNA: BrdU-ELISA was greater than the 42% reported by ICCVAM (1999) for the EC3.

Table C-VIII-8 EC1.9 Values from the Phase II Interlaboratory Validation Study on the LLNA: BrdU-ELISA[1]

Substance Name	Laboratory							Mean ± SD	% CV
	1	2	3	4	5	6	7		
2,4-Dinitro-chlorobenzene	0.078 (4.3 @ 1%)	***0.017 (8.37 @ 1%)***	0.027 (5.99 @ 0.3%)	0.028 (5.50 @ 1%)	***0.0024 (18.80 @ 0.3%)***	***0.023 (4.83 @ 0.3%)***	0.053 (12.18 @ 1%)	0.033 ± 0.025	77
Hexyl cinnamic aldehyde	14.76 (3.4 @ 50%)	-[1] (1.83 @ 50%)	***22.21 (2.87 @ 50%)***	7.92 (3.34 @ 50%)	***3.96 (13.5 @ 50%)***	11.65[2] (3.27 @ 50%)	13.18 (3.84 @ 50%)	12.28 ± 6.23	51
Glutaraldehyde	0.14	NT	NT	NT	0.033	0.32	NT	0.17 ± 0.14	87
Nickel sulfate	NT	NT	2.93	0.86	NT	NT	1.05	1.61 ± 1.14	71
trans-Cinnamic aldehyde	NT	2.42	NT	1.48	2.58	NT	NT	2.16 ± 0.59	27
Formaldehyde	0.37	NT	NT	NT	0.28	0.071	NT	0.24 ± 0.16	64
Eugenol	NT	17.76	NT	NT	NT	15.20	4.70	12.55 ± 6.92	55

Note: Boldface indicates substances recommended for assessing interlaboratory reproducibility in *Recommended Performance Standards* (ICCVAM 2009). Boldface italic EC1.9 values are outside of the acceptable range from the ICCVAM LLNA performance standards: 5%-20% for hexyl cinnamic aldehyde and 0.025%-0.1% for 2,4-dinitrochlorobenzene. Values in parentheses are the highest SI values achieved.

Abbreviations: CV = coefficient of variation; EC1.9 = estimated concentration needed to produce a stimulation index of 1.9; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; NT = not tested; SD = standard deviation; SI = stimulation index.

[1] Test failed because associated positive control failed (i.e., SI < 1.9; vehicle control absorbance was unusually high). Result not included in the mean EC1.9 and CV.

[2] Three mice tested at highest dose.

Table C-VIII-9 Interlaboratory Reproducibility of the EC3 for Substances Tested in the Traditional LLNA[1]

Substance Name	Laboratory					CV (%)
	1	2	3	4	5	
2, 4-Dinitrochlorobenzene	0.3	0.5	0.6	0.9	0.6	37
	0.5	0.6	0.4	0.6	0.3	27
Hexyl cinnamic aldehyde	7.9	7.6	8.4	7.0	8.1	7
Isoeugenol	1.3	3.3	1.8	3.1	1.6	41
Eugenol	5.8	14.5	8.9	13.8	6.0	42
Sodium lauryl sulfate	13.4	4.4	1.5	17.1	4.0	84

Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of 3; LLNA = murine local lymph node assay.

[1] From ICCVAM (1999).

3.3 Reproducibility for the LLNA: BrdU-ELISA Using Multiple Alternative Decision Criteria

Section 2.0 of this annex discusses the accuracy for the LLNA: BrdU-ELISA when using two decision criteria for LLNA: BrdU-ELISA results: one criterion to classify substances as sensitizers (SI ≥ 1.9) and another criterion to classify substances as nonsensitizers (i.e., SI ≤ 1.3). SI ≥ 1.9 was evaluated for classifying sensitizers because it resulted in no false positives with respect to the traditional LLNA. SI ≤ 1.3 was evaluated for classifying substances as nonsensitizers because it resulted in no false negatives. This section evaluates reproducibility of the concordance with the traditional LLNA results by examining the frequency with which SI values in the validation database of 31 substances occurred in one of three SI categories, regardless of whether the tests were performed in one or multiple laboratories (i.e., intra- and inter-laboratory data have been combined for this analysis). The three SI categories were:

- SI ≤ 1.3 for classifying nonsensitizers
- SI > 1.3 and <1.9, the range of uncertainty with respect to classification by the traditional LLNA (i.e., indeterminate results)
- SI ≥ 1.9 to classify substances as sensitizers

The database for this analysis consisted of 106 LLNA: BrdU-ELISA tests of 31 substances. The maximum SI achieved by each test and the traditional LLNA outcome (sensitizer vs. nonsensitizer) were used to determine the frequency of the maximum SI by category. **Table C-VIII-10** shows the proportion of sensitizers and nonsensitizers, according to the traditional LLNA, for each of three SI categories: SI ≤ 1.3, 1.3 < SI < 1.9, and SI ≥ 1.9. All of the tests (10/10 [100%]) that yielded SI < 1.3 were for substances that were classified as nonsensitizers by the traditional LLNA. Thirty-one percent (4/13) of the tests that yielded SI values in the range of uncertainty; 1.3 < SI < 1.9, were for substances that were classified as sensitizers by the traditional LLNA. The remainder of the tests in the 1.3 < SI < 1.9 category, 69% (9/13), were classified as nonsensitizers by the traditional LLNA. Ninety-six percent (80/83) of the tests that yielded SI ≥ 1.9 were for substances that were classified as sensitizers by the traditional LLNA, and only 4% (3/83) were classified as nonsensitizers. The three

nonsensitizer tests were two tests of isopropanol, which yielded SI = 2.02 and 2.22 in the LLNA: BrdU-ELISA, and one test of lactic acid, which produced SI = 2.53.

Table C-VIII-10 Frequency of Maximum SI for LLNA: BrdU-ELISA Tests by Category and Traditional LLNA Outcome

Classification Based on Traditional LLNA	Classification Concordance with Traditional LLNA[1]			
	Maximum SI ≤ 1.3	1.3 < Maximum SI < 1.9	Maximum SI ≥ 1.9	Total
Sensitizer	0 (0%)	4 (31%)	80 (96%)	84
Nonsensitizer	10 (100%)	9 (69%)	3 (4%)	22
Total	10	13	83	106

Abbreviations: LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SI = stimulation index.

[1] Numbers shown reflect number of tests. Includes all tests of substances that were tested multiple times. Percentage in parentheses reflects percentage of the total number of tests for each SI category.

The 106 tests evaluated in **Table C-VIII-10** include multiple tests for 15 substances. For the 15 substances, two to 12 tests were available. **Table C-VIII-11** shows the proportion of the tests for each substance that produced SI values in each category. For the 10 sensitizers with multiple test results, there were no tests that produced SI ≤ 1.3 or 1.3 < SI < 1.9. However, the LLNA: BrdU-ELISA tests for traditional LLNA nonsensitizers were more variable. The results for isopropanol were particularly variable: 57% (4/7) of the tests produced SI ≤ 1.3 (SI = 0.92, 0.94, 0.98, and 1.01), 14% (1/7) produced 1.3 < SI < 1.9 (SI = 1.57), and 29% (2/7) produced SI ≥ 1.9 (SI = 2.04 and 2.22). Lactic acid tests produced SI values in two categories: 67% (2/3) of the tests had 1.3 < SI < 1.9 (SI = 1.80 and 1.89), and 33% (1/3) of the tests had SI ≥ 1.9 (SI = 2.53). Propylene glycol tests produced SI values in two categories: 67% (2/3) of the tests had SI < 1.3 (SI = 0.91 and 1.20) and one test produced 1.3 < SI < 1.9 (SI = 1.57). The multiple test results for hexane and methyl salicylate were 100% concordant, with all results in the 1.3 < SI < 1.9 category. The two hexane tests produced SI values of 1.76 and 1.89, and the three methyl salicylate tests also produced SI values of 1.40, 1.43, and 1.44.

Table C-VIII-11 Concordance of LLNA: BrdU-ELISA Tests for Substances with Multiple Tests by Maximum SI Category

Substance Name	Concordance Among Multiple Tests[1]			Total
	Maximum SI ≤ 1.3	1.3 < Maximum SI < 1.9	Maximum SI ≥ 1.9	
Sensitizers[2]				
2,4-Dinitrochloro-benzene	0 (0%)	0 (0%)	9 (100%)	9
Diphenylcyclopro-penone	0 (0%)	0 (0%)	3 (100%)	3
Eugenol	0 (0%)	0 (0%)	9 (100%)	9
Formaldehyde	0 (0%)	0 (0%)	3 (100%)	3
Glutaraldehyde	0 (0%)	0 (0%)	5 (100%)	5
Hexyl cinnamic aldehyde	0 (0%)	0 (0%)	12 (100%)	12
Isoeugenol	0 (0%)	0 (0%)	3 (100%)	3
Nickel sulfate	0 (0%)	0 (0%)	3 (100%)	3
1,4-Phenylenediamine	0 (0%)	0 (0%)	2 (100%)	2
trans-Cinnamaldehyde	0 (0%)	0 (0%)	4 (100%)	4

continued

Table C-VIII-11 Concordance of LLNA: BrdU-ELISA Tests for Substances with Multiple Tests by Maximum SI Category (continued)

Substance Name	Concordance Among Multiple Tests[1]			Total
	Maximum SI ≤ 1.3	1.3 < Maximum SI < 1.9	Maximum SI ≥ 1.9	
Nonsensitizers[2]				
Hexane	0 (0%)	2 (100%)	0 (%)	2
Isopropanol	4 (57%)	1 (14%)	2 (29%)	7
Lactic acid	0 (0%)	2 (67%)	1 (33%)	3
Methyl salicylate	0 (0%)	3 (100%)	0 (0%)	3
Propylene glycol	2 (67%)	1 (33%)	0 (0%)	3

Abbreviations: LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SI = stimulation index.

[1] Numbers shown reflect number of tests. Percentage in parentheses reflects percentage of the total number of tests for each substance.

[2] According to traditional LLNA results.

4.0 The Impact of Increasing the LLNA: BrdU-ELISA Sample Size on the Substances in the Range of Uncertainty

This section examines the impact of increasing the number of animals used in each LLNA: BrdU-ELISA control and treatment group (i.e., sample size) on the size of the range of uncertainty (i.e., 1.3 < SI < 1.9) and on the number of substances in the range of uncertainty.

Since the LLNA: BrdU-ELISA accuracy analyses were based on studies with four animals per dose group, additional analyses were performed in order to determine if the sample size per dose group contributed to these indeterminate classifications. As detailed below, increasing the sample size for each dose group is unlikely to impact either the number of substances classified as uncertain or the SI values that define the range.

Table C-VIII-12 shows the 31 substances evaluated, along with their LLNA: BrdU-ELISA SI values and corresponding traditional LLNA results. Based on the LLNA: BrdU-ELISA SI values, 18 substances were sensitizers (SI ≥ 1.9), four were nonsensitizers (SI ≤ 1.3), and nine were in the range of uncertainty (1.3 < SI < 1.9). Of the nine substances in the range of uncertainty, four were sensitizers and five were nonsensitizers in the traditional LLNA.

Increasing the sample size could effectively move any of the borderline substances into or out of the range of uncertainty. Also, changing the sample size could widen or narrow the range of the uncertainty interval and thus either increase or decrease the number of substances in the range of uncertainty.

Table C-VIII-12 Distribution of LLNA: BrdU-ELISA Maximum SI Data for 31 Substances

Substance Name	LLNA: BrdU-ELISA Maximum SI Values[1]	Traditional LLNA Result
LLNA: BrdU-ELISA Positive; SI ≥ 1.9 (N = 18)		
2,4-Dinitrochlorobenzene	4.30, 4.83, 5.50, 6.26, 6.84, 8.37, 12.98, 17.90, 18.80	+
3-Aminophenol	3.06	+
4-Chloroaniline	2.53	+
Benzoquinone	6.90	+
Cinnamic aldehyde	3.97	+
Citral	1.84, 16.35	+
Cyclamen aldehyde	1.97	+
Diphenylcyclopropenone	9.34, 19.10	+
Eugenol	3.10, 3.17, 3.18, 3.18, 3.30, 3.83, 7.09, 12.30, 17.70	+
Formaldehyde	1.97, 4.40, 16.59	+
Glutaraldehyde	2.25, 3.72, 14.60, 15.50, 28.64	+
Hexyl cinnamic aldehyde	2.44, 2.72, 2.87, 3.02, 3.27, 3.34, 3.40, 3.64, 3.64, 3.84, 5.90, 13.50	+
Isoeugenol	2.40, 6.73, 8.40	+
Isopropyl myristate	1.10, 4.20	+
Nickel sulfate	2.58, 2.66, 4.53	+
4-Phenylenediamine	11.70, 14.70	+
trans-Cinnamaldehyde	3.37, 3.50, 4.11	+
Trimellitic anhydride	7.85	+
LLNA: BrdU-ELISA Negative; SI ≤ 1.3 (N = 4)		
2-Hydroxypropyl methacrylate	1.13	-
Diethyl phthalate	0.88	-
Dimethyl isophthalate	1.26	-
Glycerol	1.29	-
LLNA: BrdU-ELISA Range of Uncertainty; 1.3 < SI < 1.9 (N = 9)		
2-Mercaptobenzothiazole	**1.62**	+
Aniline	**1.50**	+
Hexane	0.73, **1.76, 1.89**	-
Hydroxycitronellal	**1.34**	+
Isopropanol	0.92, 0.94, 0.98, 1.01, **1.57**, 2.04, 2.22	-
Lactic acid	**1.80, 1.89,** 2.53	-
Linalool	**1.45**	+
Methyl salicylate	**1.40, 1.43, 1.44**	-
Propylene glycol	0.87, 1.20, **1.57**	-

Abbreviations: LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; N = number of substances; SI = stimulation index; + = sensitizer; - = nonsensitizer.

[1] Multiple values indicate multiple test results. The bold text indicates LLNA: BrdU-ELISA tests with maximum SI values between 1.3 and 1.9.

4.1 Impact of Sample Size on the Size of the Range of Uncertainty

There are two substances that determine the limits of the range of uncertainty: hydroxycitronellal (the sensitizer, based on traditional LLNA data, with the lowest SI value in the range of uncertainty, 1.34) and lactic acid (the nonsensitizer, based on traditional LLNA data, with the highest SI value in the range of uncertainty, 1.89).

To illustrate the impact of additional animals, consider hydroxycitronellal. Based on the individual animal data, the four animals had SI values of 1.38, 1.25, 1.57, and 1.17 (Annex IV-1). The mean SI value for these four animals is 1.34, which is effectively the lower limit of the range of uncertainty. The standard deviation (SD) is 0.18. Assume an underlying normal distribution with a mean of 1.34 and an SD of 0.18 (range of 1.16 to 1.52) and consider how the range might change if, for example, 10 animals are used rather than four. A mean will be contained in a range of the mean plus or minus 1.28 times the standard error (SE) for 80% of the time. For a sample of size 10, the SE is 0.055. There is 80% confidence that if a mean SI had been calculated based on 10 animals, it would fall between 1.27 and 1.41, which does not have any appreciable impact on the size of the range of uncertainty.

4.2 Impact of Sample Size on the Number of Substances in the Range of Uncertainty

Regarding the number of substances within the range, if the revised mean SI were as low as 1.27, then it is possible that glycerol (which had an overall mean SI of 1.29) could be added to the range of uncertainty. The most likely outcome is no change at all and only a minor shift in the lower end of the range (either slightly upward or slightly downward).

The upper limit is somewhat different, since the SI data for lactic acid are more variable, and, importantly, there are three tests rather than one. The individual animal SI values for one test were 1.83, 2.84, 0.97, 1.56 (Annex IV-2), producing a mean SI of 1.80 and an SE (for N = 10) of 0.25. Thus, the upper limit (with 80% confidence) could shift as low as SI = 1.48 or as high as SI = 2.12. If this were the only study, then raising the upper limit would potentially add three substances to the range of uncertainty.

However, the lower limit for the range of uncertainty could not be reduced to SI = 1.48, because of hexane (negative, despite SI = 1.76 and SI = 1.89). Reducing the lower limit below an SI of 1.76 would make hexane a false positive. Lactic acid had three studies, not one, and in order to lower the range of uncertainty, two of the three would have to be revised downward. The likelihood of both the SI = 1.80 and the SI = 1.89 lactic acid studies being revised downward to SI = 1.48 based on additional animals is quite small (less than 5%). So, because of the multiple studies for lactic acid (and the results for hexane) additional animals would have little appreciable effect on the upper limit of the range of uncertainty.

There is not a single SI value that would produce accurate classifications for all the substances in the range of uncertainty. For example, if the range of uncertainty is eliminated, and an SI = 1.50 is proposed as the cutoff point, even with more animals, there is a strong likelihood that lactic acid (traditional LLNA negative, despite LLNA: BrdU-ELISA SI of 1.80, 1.89, and 2.53) and hexane (traditional LLNA negative, despite LLNA: BrdU-ELISA SI of 1.76 and 1.89) would be still be misclassified, as likely would hydroxycitronellal (traditional LLNA positive, despite LLNA: BrdU-ELISA SI of 1.34). Different proposed SI cutoff points (higher or lower than 1.50) would still produce misclassifications. As the number of animals approaches infinity, the means converge to the observed mean, so in the limit, there would be no change at all in the range of uncertainty by increasing the sample size (assuming that the means observed are essentially correct).

The SI values determined for these 31 substances were based on four animals per dose. The analyses described above indicates that additional animals would likely not have had an appreciable impact on either the number of substances in the range of uncertainty or on the range of the uncertainty interval.

5.0 Analysis of Physicochemical Characteristics of Substances in the Range of Uncertainty

5.1 Introduction

The following information is presented to evaluate the use of physicochemical characteristics and other parameters to distinguish between traditional LLNA sensitizers and nonsensitizers in the LLNA: BrdU-ELISA when using multiple SI decision criteria to identify sensitizers and nonsensitizers (SI ≥ 1.9 and SI ≤1.3 for sensitizers and nonsensitizers, respectively). Characteristics that distinguish between sensitizers and nonsensitizers may aid in the interpretation of LLNA: BrdU-ELISA SI values that fall within the range of uncertainty, 1.3 < SI < 1.9.

The physicochemical information evaluated included peptide reactivity, molecular weight, octanol/water partition coefficient, physical form, and chemical class. The other parameters evaluated were vehicle control substance and potential local skin irritation at the highest concentration tested. The "potentially irritating" concentration is based on either (1) skin irritation at the concentration tested based on hazard identification (e.g., ECETOC skin irritation database; published traditional LLNA studies that provided skin irritation data), (2) the concentration tested in the LLNA exceeded the challenge concentration used in the GPMT (i.e., the maximum nonirritating concentration is used in the GPMT), (3) human skin irritation data from predictive skin sensitization patch testing, or (4) mouse skin irritation data. The information used for this analysis is provided in **Annexes II** and **III** unless otherwise noted.

The nine substances in the range of uncertainty, 1.3 < SI < 1.9, for the LLNA: BrdU-ELISA test method along with the LLNA: BrdU-ELISA SI values and the traditional LLNA SI values are listed in **Table C-VIII-13**. Based on the traditional LLNA, four substances were sensitizers and five substances were nonsensitizers.

Table C-VIII-13 Substances with Tests in the Range of Uncertainty: 1.3 < SI < 1.9

Substance	Maximum SI LLNA: BrdU-ELISA[1]	Maximum SI Traditional LLNA	Traditional LLNA Result
Aniline	1.50	3.6	+
Hexane	1.76, 0.73, 1.89	2.2	-
Hydroxycitronellal	1.34	8.5	+
Isopropanol	2.22, 0.98, 1.57, 0.94, 2.04, 1.01, 0.92	1.7	-
Lactic acid	2.53, 1.89, 1.80	2.2	-
Linalool	1.45	8.3	+
2-Mercaptobenzothiazole	1.62	8.6	+
Methyl salicylate	1.44, 1.43, 1.40	2.9	-
Propylene glycol	1.2, 1.57, 0.91	1.6	-

Abbreviations: + = sensitizer; - = nonsensitizer; LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay with ELISA detection of bromodeoxyuridine; SI = stimulation index.

[1] Multiple values indicate multiple test results.

5.2 Peptide Reactivity

Because the ability to form stable conjugates with protein is a key requirement for a substance to produce skin sensitization, peptide reactivity information may assist in determining skin sensitization potential (Jowsey et al. 2006).

5.2.1 Categorical Analysis

Gerberick et al. (2007) classified peptide reactivity as high, moderate, low, and minimal based on a classification tree model used to relate the depletion of cysteine- and lysine-containing peptides to relative skin sensitization potency categories from Kimber et al. (2003) that were based on LLNA EC3 values. The preferred model, which was based on the average of two peptide depletion measurements (i.e., one using a cysteine-containing peptide at a 1:10 molar ratio with the test substance and one using a lysine-containing peptide at a 1:50 molar ratio with the test substance), accurately predicted the sensitizer or nonsensitizer outcomes of 89% (72/81) of the substances evaluated (Gerberick et al. 2007). The peptide reactivity categories for 20/31 substances tested in the LLNA: BrdU-ELISA were available from Gerberick et al. (2007). These data were used to analyze the association of the high, moderate, low, and minimal peptide reactivity categories with the traditional LLNA sensitizer and nonsensitizer status of the 20 test substances (12 traditional LLNA sensitizers and eight traditional LLNA nonsensitizers).

Table C-VIII-14 lists the nine substances in the range of uncertainty and the corresponding peptide reactivity categories available from Gerberick et al. (2007). Peptide reactivity categories were available for 7/9 substances. **Annex VIIIa** shows the peptide reactivity information for all 20 substances available from Gerberick et al. (2007).

Table C-VIII-14 Peptide Reactivity Data for Substances in the Range of Uncertainty

Substance	Traditional LLNA Result	Peptide Reactivity Category[1]	% Cysteine Depletion[2]
Aniline	+	NA	NA
Hexane	-	Minimal	-0.4
Hydroxycitronellal	+	Low	46.7
Isopropanol	-	Minimal	0.3
Lactic acid	-	Minimal	2.5
Linalool	+	NA	2.0
2-Mercaptobenzothiazole	+	High	100
Methyl salicylate	-	Minimal	0.3
Propylene glycol	-	Minimal	-0.9

Abbreviations: LLNA = murine local lymph node assay.

+ = sensitizer; - = nonsensitizer.

[1] Categories from Gerberick et al. (2007).

[2] Values from Natsch et al. (2009).

Table C-VIII-15 shows the proportions of the 12 sensitizers and eight nonsensitizers in each category of peptide reactivity. Traditional LLNA nonsensitizers, across all relevant LLNA: BrdU-ELISA SI categories (i.e., whether SI ≤ 1.3 or 1.3 < SI < 1.9) were associated with minimal to low peptide reactivity; 100% (8/8) of the nonsensitizers with peptide reactivity data had low or moderate peptide reactivity. The 12 traditional LLNA sensitizers with peptide reactivity data, across both relevant LLNA: BrdU-ELISA SI categories (i.e., whether 1.3 < SI < 1.9 or SI ≥ 1.9), were generally associated with moderate to high peptide reactivity (58% [7/12]); however, 25% (3/12) of the sensitizers were associated with low peptide reactivity, and 17% (2/12) of the sensitizers were associated with minimal peptide reactivity.

Table C-VIII-15 Peptide Reactivity for Sensitizers vs. Nonsensitizers[1]

Peptide Reactivity Category[2]	Sensitizer[3]/ LLNA: BrdU-ELISA SI ≥ 1.9	Nonsensitizer[3]/ LLNA: BrdU-ELISA SI ≤ 1.3	Sensitizer[3]/ 1.3 < LLNA: BrdU-ELISA SI < 1.9	Nonsensitizer[3]/ 1.3 < LLNA: BrdU-ELISA SI < 1.9
High	50% (5/10)	0% (0/3)	50% (1/2)	0% (0/5)
Moderate	10% (1/10)	0% (0/3)	0% (0/2)	0% (0/5)
Low	20% (2/10)	33% (1/3)	50% (1/2)	0% (0/5)
Minimal	20% (2/10)	67% (2/3)	0% (0/2)	100% (5/5)
NA	8	1	2	0
Total Substances	18	4	4	5

Abbreviations: LLNA: BrdU-ELISA = murine local lymph node assay with ELISA detection of bromodeoxyuridine; NA = peptide reactivity information was not available; SI = stimulation index.

[1] Number of substances shown. Proportion in parentheses based on number of substances with peptide reactivity data.

[2] Determined using data in Gerberick et al. (2007).

[3] Based on traditional LLNA.

There are insufficient data to definitively choose a single "breakpoint" for using peptide reactivity to predict sensitizers. However, a range of reactivity (i.e., low to high vs. minimal) could be useful since Fisher's exact test shows that peptide reactivity is highly associated ($p < 0.001$) with the traditional LLNA result using the low to high vs. minimal breakpoint (**Table C-VIII-16**).

Table C-VIII-16 Fisher's Exact Test for Association of Peptide Reactivity with Sensitizers and Nonsensitizers[1]

Peptide Reactivity Category	Sensitizer	Nonsensitizer	Peptide Reactivity Category	Sensitizer	Nonsensitizer
Low to High	10	1	Moderate to High	7	0
Minimal	2	7	Minimal to Low	5	8
p = 0.0045 (Fisher's Exact Test)			p = 0.0147 (Fisher's Exact Test)		

[1] Number of substances with peptide reactivity in each category shown.

Low to high vs. minimal would correctly classify 100% (7/7) of the substances in the range of uncertainty that have peptide reactivity data (**Table C-VIII-5**). Moderate to high vs. minimal to low would correctly classify 86% (6/7) substances in the range of uncertainty. The association is highly

significant, and peptide reactivity could be used as a "tiebreaker" for those substances for which the LLNA: BrdU-ELISA assay produces SI values in the range of uncertainty.

5.2.2 Numerical Analysis

Peptide reactivity data as percent cysteine depletion were available for 27/31 substances tested in the LLNA: BrdU-ELISA. Most of the cysteine depletion data were obtained from Natsch et al. (2009). Natsch et al. (2009) measured peptide depletion with methods similar to Gerberick et al. (2007) using a cysteine-containing peptide at a 1:10 molar ratio with the test substance. Thus, cysteine depletion data was obtained from Gerberick et al. (2007) for substances that were not included in Natsch et al. (2009). Natsch et al. (2009) demonstrated that using >15% cysteine-containing peptide depletion to classify sensitizers yielded an overall accuracy of 80% (93/116). The cysteine depletion data were used to analyze sensitizer/nonsensitizer classification using various peptide depletion cutoff values. Cysteine depletion data were available for 8/9 substances in the range of uncertainty (see **Table C-VIII-14**).

The analysis evaluated the performance of several different % cysteine depletion values by determining the accuracy, false negative rate, and false positive rate for classifying substances as sensitizers and nonsensitizers. The results indicated that the highest accuracy (81% [22/27]) occurred for three different cysteine depletion cutoffs, >0.55%, >1.40, or >4.75%, that were used to classify substances as sensitizers. The associated false positive rates were 38% (3/8), 25% (2/8), and 13% (1/8), respectively. False negative rates were 11% (2/19), 16% (3/19), and 22% (4/19), respectively. Thus, the cutoff with the lowest false negative rate was >0.55%. See **Annex VIIIb** for the performance of other cysteine depletion cutoffs.

Table C-VIII-17 shows that the percentages of sensitizers with LLNA: BrdU-ELISA SI ≥ 1.9 with cysteine depletion values of >0.55%, >1.40, or >4.75% were 88% (14/16), 81% (13/16), and 81% (13/16), respectively. The percentages of nonsensitizers with LLNA: BrdU-ELISA SI ≤ 1.3 for cysteine depletion values ≤0.55%, ≤1.40 or ≤4.75% were 0% (0/3), 67% (2/3), and 67% (2/3), respectively. For the substances with 1.3 < SI < 1.9, 100% (3/3) of the sensitizers had cysteine depletion values >0.55% or >1.40%, and 100% (5/5) of the nonsensitizers had cysteine depletion ≤4.75.

Table C-VIII-17 Correct Classification Rate of Sensitizers vs. Nonsensitizers by Cysteine Depletion[1]

Cysteine Depletion Cutoff	Sensitizer[2]/ LLNA: BrdU-ELISA SI ≥ 1.9	Nonsensitizer[2]/ LLNA: BrdU-ELISA SI ≤ 1.3	Sensitizer[2]/ 1.3 < LLNA: BrdU-ELISA SI < 1.9	Nonsensitizer[2]/ 1.3 < LLNA: BrdU-ELISA SI < 1.9
≤0.55%	12% (2/16)	0% (0/3)	0% (0/3)	80% (4/5)
>0.55%	88% (14/16)	100% (3/3)	100% (3/3)	20% (1/5)
≤1.40%	19% (3/16)	67% (2/3)	0% (0/3)	80% (4/5)
>1.40%	81% (13/16)	33% (1/3)	100% (3/3)	20% (1/5)
≤4.75%	19% (3/16)	67% (2/3)	33% (1/3)	100% (5/5)
>4.75%	81% (13/16)	33% (1/3)	67% (2/3)	0% (0/5)
≤ 15%	25% (4/16)	67% (2/3)	33% (1/3)	100% (5/5)
> 15%	75% (12/16)	33% (1/3)	67% (2/3)	0% (0/5)
NA	3	1	1	0
Total Substances	18	4	4	5

Abbreviations: LLNA: BrdU-ELISA = murine local lymph node assay with ELISA detection of bromodeoxyuridine; NA = peptide reactivity information was not available; SI = stimulation index.

[1] Number of substances shown. Proportion in parentheses based on the total number of substances with peptide reactivity data.

[2] Based on traditional LLNA.

Natsch et al. (2009) indicated that at least 15% peptide depletion is needed for significant results. The percentage of sensitizers and nonsensitizers associated with peptide depletion ≤15% and >15% are also shown in **Table C-VIII-17**. The results were similar to the cutoff value of 4.75% cysteine depletion. Of the sensitizers with SI ≥ 1.9, 75% (12/16) had cysteine depletion values >15%, and 67% (2/3) of the nonsensitizers with SI ≤ 1.3 had cysteine depletion values ≤15%. For the substances with 1.3 < SI < 1.9, 67% (2/3) of the sensitizers had cysteine depletion >15%, and 100% (5/5) of the nonsensitizers had cysteine depletion ≤15%.

The cysteine depletion cutoffs of 4.75% and 15% (evaluated in **Table C-VIII-17)** would have accurately classified 88% (7/8) of the substances in the range of uncertainty that had cysteine depletion data. This is similar to the result yielded by the categorical analysis when using low to high peptide reactivity to classify sensitizers and minimal peptide reactivity to classify nonsensitizers, which classified 100% (7/7) of the substances (with categorical peptide reactivity data) in the range of uncertainty.

5.3 Molecular Weight

The molecular weights of the 22 sensitizers and nine nonsensitizers were not different, as shown by the means and standard deviations in **Table C-VIII-18**. The standard deviations for sensitizers and nonsensitizers have a large range of overlap.

Table C-VIII-18 Molecular Weight (g/mol) for Sensitizers vs. Nonsensitizers

	Sensitizer[1]/ LLNA: BrdU-ELISA SI ≥ 1.9	**Nonsensitizer[1]/ LLNA: BrdU-ELISA SI ≤ 1.3**	**Sensitizer[1]/ 1.3 < LLNA: BrdU-ELISA SI < 1.9**	**Nonsensitizer[1]/ 1.3 < LLNA: BrdU-ELISA SI < 1.9**
Mean	153.4	163.2	146.7	92.9
Standard Deviation	55.1	57.3	36.5	35.1
Total	18	4	4	5

Abbreviation: LLNA: BrdU-ELISA = murine local lymph node assay with ELISA detection of bromodeoxyuridine; SI = stimulation index.

[1] Based on traditional LLNA.

5.4 Octanol-Water Partition Coefficient (log K_{ow})

The octanol-water partition coefficients (log K_{ow}) of the sensitizers and nonsensitizers were not different, as shown by the means and overlapping standard deviations in **Table C-VIII-19**. The log K_{ow} value was unavailable for one substance.

Table C-VIII-19 Log K_{ow} for Sensitizers vs. Nonsensitizers

	Sensitizer[1]/ LLNA: BrdU-ELISA SI ≥ 1.9	Nonsensitizer[1]/ LLNA: BrdU-ELISA SI ≤ 1.3	Sensitizer[1]/ 1.3 < LLNA: BrdU-ELISA SI < 1.9	Nonsensitizer[1]/ 1.3 < LLNA: BrdU-ELISA SI < 1.9
Mean	1.98	1.15	2.01	0.90
Standard Deviation	1.13	0.82	0.43	0.74
Total	17[2]	4	4	5

Abbreviation: LLNA: BrdU-ELISA = murine local lymph node assay with ELISA detection of bromodeoxyuridine; SI = stimulation index.

[1] Based on traditional LLNA.

[2] No log K_{ow} available for nickel sulfate.

5.5 Physical Form

Table C-VIII-20 shows the association of physical form with traditional LLNA sensitizer/ nonsensitizer outcome. The sensitizers with SI ≥ 1.9 and the nonsensitizers with SI ≤ 1.3 were divided approximately equally into solids and liquids. The majority of the substances (89% [8/9]) with 1.3 < SI < 1.9 were liquids regardless of whether they were sensitizers or nonsensitizers.

Table C-VIII-20 Physical Form for Sensitizers vs. Nonsensitizers[1]

Physical Form	Sensitizer[2]/ LLNA: BrdU-ELISA SI ≥1.9	Nonsensitizer[2]/ LLNA: BrdU-ELISA SI ≤ 1.3	Sensitizer[2]/ 1.3 < LLNA: BrdU-ELISA SI < 1.9	Nonsensitizer/[2] 1.3 < LLNA: BrdU-ELISA SI < 1.9
Solid	7 (39%)	2 (50%)	1 (25%)	0 (0%)
Liquid	11 (61%)	2 (50%)	3 (75%)	5 (100%)
Total	18	4	4	5

Abbreviation: LLNA: BrdU-ELISA = murine local lymph node assay with ELISA detection of bromodeoxyuridine; SI = stimulation index.

[1] Number of substances shown. Proportion in parentheses is based on the total number of substances.

[2] Based on traditional LLNA.

5.6 Vehicle Control Substances

Table C-VIII-21 shows the proportions of sensitizers and nonsensitizers for each vehicle control substance used for traditional LLNA and LLNA: BrdU-ELISA testing. Because there were too many vehicles with few substances to make an adequate comparison, the substances tested in AOO were compared with all other vehicles combined. The proportions of sensitizers and nonsensitizers tested in AOO vs. all other vehicles were similar.

Table C-VIII-21 Vehicle Control for Sensitizers vs. Nonsensitizers[1]

Vehicle	Sensitizer[2]/ LLNA: BrdU-ELISA SI ≥ 1.9	Nonsensitizer[2]/ LLNA: BrdU-ELISA SI ≤ 1.3	Sensitizer[2]/ 1.3 < LLNA: BrdU-ELISA SI < 1.9	Nonsensitizer[2]/ 1.3 < LLNA: BrdU-ELISA SI < 1.9
Acetone: olive oil (4:1)	15 (83%)	3 (75%)	3 (75%)	3 (60%)
Dimethylformamide	0 (0%)	1 (25%)	1 (25%)	0 (0%)
Acetone	2 (11%)	0 (0%)	0 (0%)	0 (0%)
Dimethyl sulfoxide	1 (6%)	0 (0%)	0 (0%)	1 (20%)
Water	0 (0%)	0 (0%)	0 (0%)	1 (20%)
Total	18	4	4	5
Acetone: Olive Oil vs. Other Vehicles				
Acetone: olive oil (4:1)	15 (83%)	3 (75%)	3 (75%)	3 (60%)
Other	3 (17%)	1 (25%)	1 (25%)	2 (40%)
Total	18	4	4	5

Abbreviations: LLNA: BrdU-ELISA = murine local lymph node assay with ELISA detection of bromodeoxyuridine; SI = stimulation index.

[1] Number of substances shown. Proportion of total is shown in parentheses.

[2] Based on traditional LLNA.

5.7 Skin Irritation Data

The maximum concentrations tested in the traditional LLNA were compared with concentrations known to produce skin irritation to determine whether there was a relationship between skin irritation and sensitizer or nonsensitizer results in the traditional LLNA. For the sensitizers, 73% (16/22) were tested at potentially irritating concentrations while 56% (5/9) of the nonsensitizers were tested at irritating concentrations. For the entire group of substances tested, 68% (21/31) were tested at irritating concentrations.

5.8 Conclusion

Based on the available data, peptide reactivity is the only promising characteristic for a positive association with LLNA sensitizer/nonsensitizer results that could be used to assist in classifying substances that produce LLNA: BrdU-ELISA SI values in the range of uncertainty. While there are insufficient data to definitively choose a single "breakpoint" for using peptide reactivity to predict sensitizers, ranges of peptide reactivity were highly associated ($p < 0.001$) with the traditional LLNA results using the low to high vs. minimal breakpoints. Thus, peptide reactivity could be used as a "tiebreaker" for those substances for which the LLNA: BrdU-ELISA produces SI values in the range of uncertainty. The numerical analysis using different cysteine depletion cutoffs also supports the conclusion that peptide reactivity is associated with sensitization outcomes.

5.9 References

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Annex VIIIa Data for 31 Substances Tested Using the LLNA: BrdU-ELISA Method

Substance	Vehicle	Trad. LLNA SI	LLNA: BrdU-ELISA SI and Highest Conc. Tested (%)[1]	MW (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Cys Depletion (%)[3]	Physical Form	Chemical Class[4]	Skin Irritant[5]	Highest Conc. Tested (%)[6]	Maximum Non-Irritating Conc. (%) (unless noted)[7]
1,4-Phenylene-diamine	AOO	26.4	11.70, 14.70; (2, 10)	108.141	1.17	NA	95.2	Solid	Amines	YES	1	0.5
2,4-Dinitrochloro-benzene	AOO	43.9	4.30, 8.37, 6.26, 5.50, 18.80, 4.83, 12.98, 17.90, 6.84; (1, 1, 0.3, 1, 0.3, 1, 1, 2, 2)	202.55	-0.057	High	100	Solid	Hydrocarbon, halogenated; Nitro compounds; Hydrocarbons, cyclic	YES	0.25	0.1
3-Aminophenol	AOO	5.7	3.06; (25)	109.126	1.17	NA	7	Solid	Amines; Phenols	YES	10	5
4-Chloroaniline	AOO	+NA	2.53; (25)	127.57	1.8	NA	NA	Liquid	Amines	NA	NA	2.5
Benzoquinone	AOO	52.3	6.90; (1)	108.095	1.17	High	91.8	Solid	Quinones	YES	2.5	2.5
Cinnamic aldehyde	AOO	18.4	3.97; (50)	132.16	2.29	High	90.5	Liquid	Aldehydes	NO	25	100
Citral	AOO	20.5	16.35, 1.84; (50, 10)	152.233	2.54	NA	34.7	Liquid	Hydrocarbons, other	YES	20	0.5
Cyclamen aldehyde	AOO	5.2	1.97; (100)	190.28	3.28	Low	59.9	Liquid	Carboxylic acids	NO	50	100
Diphenylcyclo-propenone	AOO	+NA	19.10, 9.34; (2, 10)	206.24	3.25	High	98.8	Solid	Hydrocarbons, cyclic	NA	NA	NA
Eugenol	AOO	17	3.18, 3.30, 3.83, 12.30, 3.10, 7.09, 3.17, 3.18, 17.70; (10, 30, 30, 50, 50, 50, 50, 50, 50)	164.201	2.15	NA	54	Liquid	Carboxylic acids	YES	50	25
Formaldehyde	ACE	11.9	16.59, 4.40, 1.97; (10)	30.03	0.33	Moderate	56.5	Liquid	Aldehydes	YES	25	2
Glutaraldehyde	ACE	18	28.64, 3.72, 2.25, 14.60, 15.50; (1, 1, 1, 2, 10)	100.12	0.92	High	30	Liquid	Aldehydes	NA	2.5	NA

Substance	Vehicle	Trad. LLNA SI	LLNA: BrdU-ELISA SI and Highest Conc. Tested (%)[1]	MW (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Cys Depletion (%)[3]	Physical Form	Chemical Class[4]	Skin Irritant[5]	Highest Conc. Tested (%)[6]	Maximum Non-Irritating Conc. (%) (unless noted)[7]
Hexyl cinnamic aldehyde	AOO	20	3.60, 5.90, 3.64, 2.72, 3.02, 3.40, 2.07, 6.11, 3.43, 5.15, 2.52, 2.87, 3.34, 3.54, 2.18, 3.34, 2.69, 3.17, 6.58, 13.50, 12.46, 4.24, 6.07, 3.27, 5.30, 2.41, 2.52, 3.84, 6.86, 4.39, 4.78; (50)	216.319	3.77	Minimal	-0.3	Liquid	Aldehydes	YES	50	10
Isoeugenol	AOO	31	8.40, 2.40, 6.73; (10, 10, 30)	164.201	2.15	NA	98.4	Liquid	Carboxylic acids	YES	5	5
Isopropyl myristate	AOO	3.4	4.20, 1.10 (50, 100)	270.46	3.88	Minimal	0.8	Liquid	Lipids	YES	100	100
Nickel Sulfate	DMSO	3.1	2.58, 4.53, 2.66; (10)	154.76	NA	NA	35.5	Solid	Inorganic chemicals, metals; Inorganic chemicals, elements	YES	5	0.15
trans-Cinnamaldehyde	AOO	13.1	4.11, 3.50, 3.37; (10)	132.6	1.82	NA	NA	Liquid	Aldehydes	NA	25	NA
Trimellitic anhydride	AOO	4.6	7.85; (10)	192.13	1.95	Low	-1.1	Solid	Anhydrides, Carboxylic acids	YES	25	10
2-Mercaptobenzo-thiazole	DMF	8.6	1.62; (50)	167.253	1.8	High	100	Solid	Heterocyclic compounds	YES	10	10
Aniline	AOO	3.6	1.50; (50)	93.1265	1.56	NA	NA	Liquid	Amines	YES	100	100
Hexane	AOO	2.2	1.76, 0.73, 1.89; (100, 10, 50)	86.1754	1.94	Minimal	-0.4[8]	Liquid	Hydrocarbons, acyclic	YES	100	100
Hydroxycitronellal	AOO	8.5	1.30; (100)	172.26	2.15	Low	46.7	Liquid	Hydrocarbons, other	YES	100	50
Isopropanol	AOO	1.7	2.22, 0.98, 1.57, 0.94, 2.04, 1.01, 0.92; (50, 50, 50, 50, 50, 50, 100)	60.095	0.82	Minimal	0.3	Liquid	Alcohols	NO	50	100
Lactic acid	DMSO	2.2	2.53, 1.89, 1.80; (50)	90.08	0.05	Minimal	2.5	Liquid	Carboxylic acids	YES	25	10
Linalool	AOO	8.3	1.45; (100)	154.25	2.54	NA	2	Liquid	Hydrocarbons	YES	100	100
Methyl salicylate	AOO	2.9	1.44, 1.44, 1.40; (50)	152.15	1.28	Minimal	0.3	Liquid	Phenols; Carboxylic acids	YES	20	10
Propylene glycol	H2O	1.6	1.2, 1.57, 0.87; (10, 50, 50)	76.0944	0.43	Minimal	-0.9	Liquid	Alcohols	NA	100	NA

Substance	Vehicle	Trad. LLNA SI	LLNA: BrdU-ELISA SI and Highest Conc. Tested (%)[1]	MW (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Cys Depletion (%)[3]	Physical Form	Chemical Class[4]	Skin Irritant[5]	Highest Conc. Tested (%)[6]	Maximum Non-Irritating Conc. (%) (unless noted)[7]
2-Hydroxypropyl methacrylate	AOO	1.3	1.13; (50)	144.168	1.03	Low	58.4[8]	Solid	Carboxylic acids	YES	50	10
Diethyl phthalate	AOO	1.5	0.88; (50)	222.24	1.87	Minimal	0.8	Liquid	Carboxylic acids	YES	100	100
Dimethyl isophthalate	AOO	1	1.26; (50)	194.19	1.66	NA	NA	Solid	Carboxylic acids	NA	25	NA
Glycerol	DMF	1.1	1.29; (50)	92.09	0.05	Minimal	-3.8	Liquid	Alcohols; Carbohydrates	NA	100	NA

Note: Shaded cells contain substances in the range of certainty.

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); Cys = cysteine-containing peptide; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; GP = guinea pig; K_{ow} = octanol/water partition coefficient; LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; MEK = methyl ethyl ketone; MW = molecular weight; NA = not available; Trad. = traditional;+ = sensitizer; - = nonsensitizer.

[1] Highest SI value from LLNA: DA test(s); respective highest concentration tested for each SI value in parentheses.

2 Kow represents the estimated octanol-water partition coefficient (expressed on log scale) calculated by an interactive demo at the SRC website: http://www.srcinc.com/what-we-do/databaseforms.aspx?id=385.

[3] Peptide reactivity categories based on cysteine and lysine depletion as reported in Gerberick et al. (2007). Cysteine depletion values are primarily from Natsch et al. (2009) unless otherwise noted.

4 Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs, as developed by the National Library of Medicine: http://www.nlm.nih.gov/mesh/meshhome.html.

[5] Highest concentration tested compared to the maximum nonirritating concentration.

[6] Highest concentration tested in the traditional LLNA.

[7] Guinea pig data unless noted.

[8] Data from Gerberick et al. (2007).

Annex VIIIb Performance of Cysteine Depletion Cutoffs for Prediction of 19 Sensitizers and Eight Nonsensitizers Tested in the LLNA: BrdU-ELISA

Cys Depletion (%)	Accuracy (%)	Sensitivity (%)	Specificity (%)	False Positive (%)	False Negative (%)	Number of Substances Çorrect +	Number of Substances False +	Number of Substances False -	Number of Substances Çorrect -
> -2.450	74	100	13	88	0	19	7	0	1
> -1.000	70	95	13	88	5	18	7	1	1
> -0.6500	74	95	25	75	5	18	6	1	2
> -0.3500	78	95	38	63	5	18	5	1	3
> 0.0	74	89	38	63	11	17	5	2	3
> 0.5500	81	89	63	38	11	17	3	2	5
> 1.400	81	84	75	25	16	16	2	3	6
> 2.250	78	79	75	25	21	15	2	4	6
> 4.750	81	79	88	13	21	15	1	4	7
> 18.50	78	74	88	13	26	14	1	5	7
> 32.35	74	68	88	13	32	13	1	6	7
> 35.10	70	63	88	13	37	12	1	7	7
> 41.10	67	58	88	13	42	11	1	8	7
> 50.35	63	53	88	13	47	10	1	9	7
> 55.25	59	47	88	13	53	9	1	10	7
> 57.45	56	42	88	13	58	8	1	11	7
> 59.15	59	42	100	0	58	8	0	11	8
> 75.20	56	37	100	0	63	7	0	12	8
> 91.15	52	32	100	0	68	6	0	13	8
> 93.50	48	26	100	0	74	5	0	14	8
> 96.80	44	21	100	0	79	4	0	15	8
> 98.60	41	16	100	0	84	3	0	16	8
> 99.40	37	11	100	0	89	2	0	17	8

Abbreviations: Cys = cysteine-containing peptide; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; + = sensitizer; - = nonsensitizer.

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Annex IX

Reproducibility Analyses for LLNA: BrdU-ELISA with Decision Criterion of SI ≥ 1.5 or SI ≥ 2.0

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1.0 Test Method Reliability

Appendix C, Section 7 provides the reproducibility analyses for the LLNA: BrdU-ELISA using SI ≥ 1.6 to classify substances as sensitizers. This annex provides the reproducibility analyses using SI ≥ 1.5 or SI ≥ 2.0 to classify substances as sensitizers. The data used for the analyses in this annex are the LLNA: BrdU-ELISA results for the 31 substances (22 traditional LLNA sensitizers and nine traditional LLNA nonsensitizers) that were reviewed by the Panel at the public meeting on April 28-29, 2009. The decision criterion of SI ≥ 2.0 was used in the JSAAE interlaboratory validation study. The SI ≥ 2.0 criterion produced an accuracy of 87% (27/31), a false positive rate of 0% (0/9), and a false negative rate of 18% (4/22) when LLNA: BrdU-ELISA results were compared to the results of the traditional LLNA. The SI ≥ 1.5 criterion, which was one of the alternative SI criterion evaluated, produced an accuracy of 84% (26/31), a false positive rate of 33% (3/9), and a false negative rate of 9% (2/22) when LLNA: BrdU-ELISA results were compared to the results of the traditional LLNA.

1.1 Intralaboratory Reproducibility for SI ≥ 1.5

The test results for the LLNA: BrdU-ELISA were amenable to intralaboratory reproducibility analyses for three endpoints: sensitizer or nonsensitizer classification, SI values, and EC1.5 values. Analyses of intralaboratory reproducibility were performed using a concordance analysis for the qualitative results (sensitizer vs. nonsensitizer in **Section 1.1.1** of this annex) and a CV analysis for the quantitative results (SI values and EC1.5 in **Sections 1.1.2** and **1.1.3** of this annex, respectively).

1.1.1 Intralaboratory Reproducibility – Qualitative Results

The dataset available for an intralaboratory concordance analysis of the qualitative test results for the LLNA: BrdU-ELISA included nine substances that were tested multiple times and classified as sensitizers or nonsensitizers. Hexyl cinnamic aldehyde and eugenol were tested six times; isoeugenol was tested four times; diphenycyclopropenone and propylene glycol were tested three times; and 2,4-dinitrochlorobenzene, glutaraldehyde, hexane, and 4-phenylenendiamine were each tested twice (Takeyoshi et al. 2003, 2004a, 2005, 2006, 2007a; unpublished data) (**Table C-IX-1**). All substances were sensitizers in the traditional LLNA except for propylene glycol and hexane. The multiple test results for 8/9 substances were 100% concordant when SI ≥ 1.5 was used to classify substances as sensitizers; however, the concordant results for hexane were false positive with respect to the traditional LLNA. Discordant test results were noted for propylene glycol. The test results from Takeyoshi et al. (2005), which were tested at maximum concentrations of 10% and 50% were negative (SI = 1.20) and positive (SI = 1.57), respectively. The result from Takeyoshi et al. (2006) produced a negative result (SI = 0.91). All tests used AOO as the vehicle.

By comparison, the qualitative intralaboratory concordance analysis for the traditional LLNA (ICCVAM 1999) was based on a dataset of six substances that included six results each for benzocaine and hexyl cinnamic aldehyde, five results for eugenol, four results each for isoeugenol and methyl salicylate, and three results for 2,4-dinitrochlorobenzene. Intralaboratory results for each substance were 100% concordant with the exception of benzocaine. One of the six benzocaine (5/6 or 83% concordance) results in the traditional LLNA was reported as equivocal because SI increased with dose, but did not reach the criterion of SI ≥ 3.0. Thus, the proportion of substances for which intralaboratory concordance of qualitative results was 100% was similar for LLNA: BrdU-ELISA (8/9) and the traditional LLNA (5/6).

Table C-IX-1 Intralaboratory Reproducibility for the LLNA: BrdU-ELISA Outcome of Substances Tested Multiple Times for SI ≥ 1.5

Substance	Highest Concentration Tested (%)	Highest SI	Outcome[1]	Takeyoshi et al. Reference
2,4-Dinitro-chlorobenzene	2	17.86	+	2005
	2	6.84	+	2006, 2007b
Diphenylcyclopro-penone	2	19.10	+	2005; 2007b
	10	9.34	+	2005
	10	11.62	+	2007b
Eugenol	10	3.18	+	2003
	30	3.33	+	2004a
	30	3.83	+	2007a
	50	12.28	+	2005
	50	3.05	+	2006
	50	17.69	+	2007b
Glutaraldehyde	2	14.60	+	2005, 2007b
	10	15.50	+	2005, 2007b
Hexane	50	1.89	+	2005
	100	1.76	+	unpublished data
Hexyl cinnamic aldehyde	25	2.41	+	2003
	50	3.64	+	2003
	50	5.90	+	2005
	50	3.64	+	2006
	50	2.72	+	2006
	50	3.02	+	2007b
Isoeugenol	10	8.36	+	2005
	10	2.36	+	2006, 2007b
	10	7.20	+	2005
	30	6.73	+	2007a
4-Phenylenediamine	2	11.70	+	2005, 2007b
	10	14.70	+	2005, 2007b
Propylene glycol	10	1.20	-	2005
	50	1.57	+	2005
	50	0.91	-	2006, 2007b

Abbreviations: LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine (BrdU); SI = stimulation index.

[1] + = sensitizer; - = nonsensitizer.

1.1.2 Intralaboratory Reproducibility – SI ≥ 1.5

There were seven substances that were tested multiple times using the same concentrations by Takeyoshi et al. (2003, 2004a, 2005, 2006, 2007a, 2007b, unpublished data). Because two substances had multiple tests for more than one concentration, there were 10 substance/concentration combinations that were tested two to five times in separate experiments. The multiple SI values for each substance/concentration were used to calculate a CV for the assessment of intralaboratory variability. As shown by **Table C-IX-2**, the CVs ranged from 1% (25% hexyl cinnamic aldehyde) to 80% (10% isoeugenol). The intralaboratory reproducibility of the traditional LLNA was not assessed by CV analysis of SI values (ICCVAM 1999).

1.1.3 Intralaboratory Reproducibility – EC1.5

CV values were also calculated for the EC1.5 values for the three sensitizers that were tested more than once using multiple doses by Takeyoshi et al. (2003; 2004a, 2005, 2006, 2007a, 2007b). The individual animal data for eugenol, hexyl cinnamic aldehyde, and isoeugenol were used to calculated EC1.5 values for the LLNA: BrdU-ELISA. The methods for calculating EC1.5 values for each sensitizer were modified from those used by Ryan et al. (2007) to calculate EC3 values. Linear interpolation was used to calculate EC1.5 values for each test with SI values higher or lower than 2 and extrapolation was used to calculate EC1.5 values for tests with no SI values below 2. The equation for linear interpolation was:

$$EC1.5 = c + \left[\frac{(1.5 - d)}{(b - d)}\right] \times (a - c)$$

The linear interpolation equation uses the points immediately above and below SI = 2, with the (dose, SI) coordinates of (a, b) immediately above SI = 2 and (c, d) immediately below SI = 2. The equation for extrapolation was:

$$EC1.5_{ex} = 2^{\left\{\log_2(c) + \frac{(1.5-d)}{(b-d)} \times \left[\log_2(a) - \log_2(c)\right]\right\}}$$

The extrapolation equation uses the two points immediately above SI = 2, with the coordinates of (a, b) for the point closest to SI = 2 and (c, d) for the higher point.

Table C-IX-2 Intralaboratory Reproducibility for the SI of Tested Substances in LLNA: BrdU-ELISA - Coefficient of Variation

Substance	Concentration Tested (%)	SI	Mean	SD	CV (%)	Takeyoshi et al. Reference
2,4-Dinitrochlorobenzene	2	17.86	12.35	7.79	63	2005
		6.84				2006, 2007b
Diphenylcyclopropenone	10	9.34	10.48	1.61	15	2005; 2007b
		11.62				2007b
Eugenol	30	3.33	3.58	0.35	10	2004a
		3.83				2007a

continued

Table C-IX-2 Intralaboratory Reproducibility for the SI of Tested Substances in LLNA: BrdU-ELISA - Coefficient of Variation (continued)

Substance	Concentration Tested (%)	SI	Mean	SD	CV (%)	Takeyoshi et al. Reference
Eugenol	50	12.28	11.01	7.40	67	2005
		3.05				2006
		17.69				2007b
Hexane	50	1.89	1.64	0.36	22	2005
		1.38				Unpublished
Hexyl cinnamic aldehyde	12.5	1.88	1.74	0.21	12	2003
		1.59				2003
	25	2.44	2.42	0.02	1	2003
		2.41				2003
	50	3.64	3.78	1.25	33	2003
		5.90				2005
		3.64				2006
		2.72				2006
		3.02				2007b
Isoeugenol	10	8.36	5.09	3.15	80	2005
		7.20				2005
		2.36				2006, 2007b
		2.43				2007a
Propylene glycol	50	1.57	1.14	0.62	54	2005
		0.70				2006, 2007b

Abbreviations: CV = coefficient of variation; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SD = standard deviation; SI = stimulation index.

As shown in **Table C-IX-3**, there were five EC1.5 values for hexyl cinnamic aldehyde, four EC1.5 values for eugenol, and two EC1.5 values for isoeugenol. The CV values were 37% for hexyl cinnamic aldehyde, 66% for eugenol, and 52% for isoeugenol. The ICCVAM LLNA *Performance Standards* criteria for demonstrating adequate intralaboratory reproducibility is based on results from at least four independent tests of hexyl cinnamic aldehyde (ICCVAM 2008a). Intralaboratory reproducibility is considered adequate when each test yields an ECt value (i.e., the estimated concentration needed to produce an SI of a specific threshold value, 1.5, in this case) within 5% to 20% (ICCVAM 2008a). All five EC1.5 values for hexyl cinnamic aldehyde were within the acceptable range for intralaboratory reproducibility.

Table C-IX-3 Intralaboratory Reproducibility for the EC1.5 of Tested Substances in LLNA: BrdU-ELISA - Coefficient of Variation

Substance	EC1.5	Mean	SD	CV (%)	Takeyoshi et al. Reference
Eugenol	5.9	7.2	4.7	66	2004a
	11.0				2006
	10.7				2007a
	1.0				2007b
Hexyl cinnamic aldehyde	11.6	12.9	4.8	37	2003
	5.5				2003
	15.9				2006
	18.1				2006
	13.5				2007b
Isoeugenol	6.3	4.6	2.4	52	2006, 2007b
	2.9				2007a

Abbreviations: CV = coefficient of variation; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; EC1.5 = estimated concentration needed to produce a stimulation index of 1.5; SD = standard deviation.

The intralaboratory reproducibility of the traditional LLNA was assessed by CV analysis of EC3 values using a larger dataset (ICCVAM 1999) than that available for the LLNA: BrdU-ELISA analysis. Two EC3 values were reported by each of five laboratories for 2, 4-dinitro-chlorobenzene, five EC3 values were reported by one laboratory for isoeugenol, six EC3 values were reported for hexyl cinnamic aldehyde by two laboratories, and five EC3 values were reported for eugenol by one laboratory (**Table C-IX-4**).

Table C-IX-4 Intralaboratory Reproducibility for the EC3 of Tested Substances in the Traditional LLNA[1]

Substance	Number of Laboratories	Number of Tests per Laboratory	CV (%)
2, 4-Dinitrochlorobenzene	5	2	13 – 47
Isoeugenol	1	5	26
Hexyl cinnamic aldehyde	2	6	19-27
Eugenol	1	5	18

Abbreviations: CV = coefficient of variation; LLNA = murine local lymph node assay); EC3 = estimated concentration needed to produce a stimulation index of 3.

[1] From ICCVAM (1999).

For all three substances in common, the intralaboratory CV values for the EC1.5 values from LLNA: BrdU-ELISA tests were higher than those reported in ICCVAM (1999) for EC3 values from the traditional LLNA. The intralaboratory EC1.5 CV for the LLNA: BrdU-ELISA tests of eugenol was 66% vs. 18% for the CV of EC3 values reported by ICCVAM (1999). The intralaboratory EC1.5 CV for isoeugenol was 52% vs. 26% for the CV of EC3 values from ICCVAM (1999), and the intralaboratory EC1.5 CV for hexyl cinnamic aldehyde was 37% vs. 19% to 27% for the CV reported by ICCVAM (1999) for EC3 values.

1.2 Interlaboratory Reproducibility for SI ≥ 1.5

The interlaboratory reproducibility of the LLNA: BrdU-ELISA was assessed using the individual animal data from the multilaboratory validation study organized by the JSAAE (Kojima et al. 2008). The study design is described in **Appendix C, Section 7.2**. The LLNA: BrdU-ELISA test results from the study are amenable to interlaboratory reproducibility analyses for two endpoints: sensitizer or nonsensitizer classification and EC2 values. Analyses of interlaboratory reproducibility were performed using a concordance analysis for the qualitative results (sensitizer vs. nonsensitizer based on SI ≥ 1.5 in **Section 1.2.1** of this annex) and a CV analysis for the quantitative results (EC1.5 in **Section 1.2.2** of this annex).

1.2.1 Interlaboratory Reproducibility – Qualitative Results (SI ≥ 1.5)

The available quantitative absorbance data for interlaboratory reproducibility analysis were used to calculate SI values for each substance and dose tested. Substances with SI ≥ 1.5 at any dose were classified as sensitizers. The qualitative (i.e., sensitizer vs. nonsensitizer) interlaboratory concordance analysis for the 10 substances tested during phase II of the JSAAE interlaboratory validation study is shown in **Table C-IX-5**. The qualitative comparison of LLNA: BrdU-ELISA results for nine substances tested in up to seven laboratories show that interlaboratory concordance was 100% (3/3, 6/6, or 7/7). However, one of these substances, lactic acid, was misclassified as a nonsensitizer in all three laboratories. The concordance for isopropanol, the substance that produced discordant results among the laboratories, was 50% (3/6). The test of isopropanol at Laboratory 2 failed (SI = 1.09) because the concurrent positive control (SI = 1.29) failed the acceptance criterion of SI ≥ 2.The other six laboratories reported maximum SI values of 2.22, 0.98, 1.57, 0.94, 2.04, and 1.01. Thus, three tests were positive (SI ≥ 1.5) and three were negative (SI < 1.5). Isopropanol produces a nonsensitizer result in the traditional LLNA.

The Validation Management Team, which evaluated the reproducibility using SI ≥ 2 to identify sensitizers, considered the interlaboratory reproducibility to be acceptable (Kojima et al. 2008). Because the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), there were no traditional LLNA concordance data for comparison with the LLNA: BrdU-ELISA concordance.

Table C-IX-5 Qualitative Results for the Phase II Interlaboratory Validation Study on the LLNA: BrdU-ELISA[1]

Substance	Laboratory							Concordance
	1	2	3	4	5	6	7	
2,4-Dinitrochloro-benzene	+ (4.30)	+ (8.37)	+ (6.26)	+ (5.50)	+ (18.80)	+ (4.83)	+ (12.98)	7/7
Glutaraldehyde	+ (3.72)				+ (28.64)	+ (2.25)		3/3
Nickel sulfate			+ (2.58)	+ (4.53)			+ (2.66)	3/3
trans-Cinnamic aldehyde		+ (3.37)		+ (3.50)	+ (4.11)			3/3
Formaldehyde	+ (4.40)				+ (16.59)	+ (1.97)		3/3
Eugenol		+ (3.17)				+ (3.18)	+ (7.09)	3/3

continued

Table C-IX-5 Qualitative Results for the Phase II Interlaboratory Validation Study on the LLNA: BrdU-ELISA[1] (continued)

Substance	Laboratory							Concordance
	1	2	3	4	5	6	7	
Hexyl cinnamic aldehyde	+ (3.40)	-[3]	+ (2.87)	+ (3.34)	+ (13.50)	+[4] (3.27)	+ (3.84)	6/6
Isopropanol	+[2] (2.22)	-[3]	- (0.98)	+ (1.57)	- (0.94)	+[2,5] (2.04)	- (1.01)	3/6
Lactic acid			+ (1.80)	+ (1.89)			+ (2.53)	3/3
Methyl salicylate	- (1.43)	- (1.44)	- (1.40)					3/3

Abbreviation: LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine.

[1] + indicates sensitizer result; - indicates nonsensitizer result using SI ≥ 1.5 to classify sensitizers. Maximum stimulation index values for each test are shown in parentheses.

[2] Test failed because concurrent positive control (SI = 1.29) failed the acceptance criterion (i.e., SI < 2). The positive control would have also failed if the acceptance criterion was SI ≥ 1.5. This isopropanol result was not included in the concordance analysis.

[3] Three mice tested at highest dose.

[4] Three mice per dose group.

1.2.2 Interlaboratory Reproducibility – EC1.5 Values

The SI values for each test were used to calculate EC1.5 values for each sensitizer according to the methods reported in **Section 1.1.3** of this annex. The EC1.5 values from each laboratory were used to calculate CV values for each substance. The resulting values are shown in **Table C-IX-6**. CV values ranged from 31% (*trans*-cinnamic aldehyde) to 95% (glutaraldehyde). The mean CV was 63%.

The ICCVAM LLNA *Performance Standards* indicate that interlaboratory reproducibility should be evaluated with at least two sensitizing chemicals with well-characterized activity in the traditional LLNA (ICCVAM 2008a). Acceptable reproducibility is attained when each laboratory obtains ECt values within 0.025% to 0.1% for 2,4-dinitrochlorobenzene and within 5% to 20% for hexyl cinnamic aldehyde (ICCVAM 2008a). For 2,4-dinitrochloro-benzene, the EC1.5 values from four laboratories were outside the acceptable range. For hexyl cinnamic aldehyde, the EC1.5 values from two laboratories were outside the acceptable range. All values outside the acceptable ranges were below the low end of the range. This indicates that the discordance was due to the LLNA: BrdU-ELISA producing a more sensitive result.

Table C-IX-6 EC1.5 Values from the Phase II Interlaboratory Validation Study of the LLNA: BrdU-ELISA

Substance	Laboratory							Mean	% CV
	1	2	3	4	5	6	7		
2,4-Dinitro-chlorobenzene	0.058 (4.3 @ 1%)	***0.010 (8.37 @ 1%)***	***0.022 (5.99 @ 0.3%)***	***0.022 (5.50 @ 1%)***	***0.0022 (18.80 @ 0.3%)***	***0.015 (4.83 @ 0.3%)***	0.049 (12.18 @ 1%)	0.025	81
Hexyl cinnamic aldehyde	9.4 (3.4 @ 50%)	-[1] (1.83 @ 50%)	15.2 (2.87 @ 50%)	***4.1 (3.34 @ 50%)***	***3.5 (13.5 @ 50%)***	7.9[2] (3.27 @ 50%)	9.5 (3.84 @ 50%)	8.3	52
Glutaraldehyde	0.064	NT	NT	NT	0.031	0.21	NT	0.10	95
Nickel sulfate	NT	NT	1.5	0.5	NT	NT	0.6	0.8	65
trans-Cinnamic aldehyde	NT	1.7	NT	1.0	1.8	NT	NT	1.5	31
Formaldehyde	0.3	NT	NT	NT	0.2	0.6	NT	0.3	66
Eugenol	NT	12.5	NT	NT	NT	10.5	3.5	8.8	54

Note: Boldface indicates substances recommended for assessing interlaboratory reproducibility in *Recommended Performance Standards* (ICCVAM 2008a). Boldface italics show EC1.5 values that are outside of the acceptable range from the ICCVAM *LLNA Performance Standards*: 5% - 20% for hexyl cinnamic aldehyde and 0.025% - 0.1% for 2,4-dinitrochlorobenzene. Values in parentheses are highest SI values achieved.

Abbreviations: CV =coefficient of variation; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; NT = not tested; SI = stimulation index.

[1] Test failed because associated positive control failed acceptance criterion (i.e., SI < 2; vehicle control absorbance was unusually high). At SI = 1.29, the positive control would have failed even if the acceptance criterion was SI≥ 1.5. Result not included in the mean EC1.5 and CV.

[2] Three mice tested at highest dose.

The interlaboratory CV values for the LLNA: BrdU-ELISA EC1.5 values were higher than those for the traditional LLNA EC3 values. The analysis of interlaboratory variation of EC3 values for the traditional LLNA reported CV values of 7% to 84% for five substances tested in five laboratories (**Table C-IX-7**; ICCVAM 1999). Three of the same substances were evaluated in the traditional LLNA and the LLNA: BrdU-ELISA. All interlaboratory CV values for the EC1.5 from LLNA: BrdU-ELISA tests were greater than that for EC3 values from the traditional LLNA. The CV of 81% for EC1.5 values for 2,4-dinitrochlorobenzene was greater than the two CV values of 37% and 27%, calculated from five EC3 values each, reported by ICCVAM (1999). The CV of 52% for EC1.5 values for hexyl cinnamic aldehyde tested in the LLNA: BrdU-ELISA was greater than the 7% CV for EC3 values reported by ICCVAM (1999). The CV of 54% for EC1.5 values for eugenol tested in the LLNA: BrdU-ELISA was greater than the CV of 42% for EC3 values reported by ICCVAM (1999).

Table C-IX-7 Interlaboratory Reproducibility of the EC3 for Substances Tested in the Traditional LLNA[1]

Substance	Laboratory					CV (%)
	1	2	3	4	5	
2, 4-Dinitrochlorobenzene	0.3	0.5	0.6	0.9	0.6	37
	0.5	0.6	0.4	0.6	0.3	27
Hexyl cinnamic aldehyde	7.9	7.6	8.4	7.0	8.1	7
Isoeugenol	1.3	3.3	1.8	3.1	1.6	41
Eugenol	5.8	14.5	8.9	13.8	6.0	42
SLS	13.4	4.4	1.5	17.1	4.0	84

Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of 3; LLNA = murine local lymph node assay; SLS = sodium lauryl sulfate.

[1] From ICCVAM (1999).

1.3 Intralaboratory Reproducibility for SI ≥ 2.0

The dataset available for an intralaboratory concordance analysis of the qualitative test results for the LLNA: BrdU-ELISA included nine substances that were tested multiple times and classified as sensitizers or nonsensitizers. Hexyl cinnamic aldehyde and eugenol were tested six times; isoeugenol was tested four times; diphenylcyclopropenone and propylene glycol were tested three times; and 2,4-dinitrochlorobenzene, glutaraldehyde, hexane, and 4-phenylenendiamine were each tested twice (Takeyoshi et al. 2003, 2004a, 2005, 2006, 2007a; unpublished data) (**Table C-IX-8**). All substances were sensitizers in the traditional LLNA except for propylene glycol and hexane. The multiple test results for 9/9 substances were 100% concordant when SI ≥ 2.0 was used to classify substances as sensitizers.

By comparison, the qualitative intralaboratory concordance analysis for the traditional LLNA (ICCVAM 1999) was based on a dataset of six substances that included six results each for benzocaine and hexyl cinnamic aldehyde, five results for eugenol, four results each for isoeugenol and methyl salicylate, and three results for 2,4-dinitrochlorobenzene. Intralaboratory results for each substance were 100% concordant with the exception of benzocaine. One of the six benzocaine (5/6 or 83% concordance) results for the traditional LLNA was reported as equivocal because SI increased

with dose, but did not reach the criterion of SI ≥ 3.0. Thus, the proportion of substances for which intralaboratory concordance of qualitative results was 100% was greater for the LLNA: BrdU-ELISA (9/9) than that for the traditional LLNA (5/6).

Table C-IX-8 Intralaboratory Reproducibility for the LLNA: BrdU-ELISA Outcome of Substances Tested Multiple Times

Substance	Highest Concentration Tested (%)	Highest SI	Outcome[1]	Takeyoshi et al. Reference
2,4-Dinitro-chlorobenzene	2	17.86	+	2005
	2	6.84	+	2006, 2007b
Diphenylcyclopro-penone	2	19.10	+	2005; 2007b
	10	9.34	+	2005
	10	11.62	+	2007b
Eugenol	10	3.18	+	2003
	30	3.33	+	2004a
	30	3.83	+	2007a
	50	12.28	+	2005
	50	3.05	+	2006
	50	17.69	+	2007b
Glutaraldehyde	2	14.60	+	2005, 2007b
	10	15.50	+	2005, 2007b
Hexane	50	1.89	-	2005
	100	1.76	-	unpublished data
Hexyl cinnamic aldehyde	25	2.41	+	2003
	50	3.64	+	2003
	50	5.90	+	2005
	50	3.64	+	2006
	50	2.72	+	2006
	50	3.02	+	2007b
Isoeugenol	10	7.20	+	2005
	10	8.36	+	2005
	10	2.36	+	2006, 2007b
	30	6.73	+	2007a
4-Phenylenediamine	2	11.70	+	2005, 2007b
	10	14.70	+	2005, 2007b
Propylene glycol	10	1.20	-	2005
	50	1.57	-	2005
	50	0.91	-	2006, 2007b

Abbreviations: LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SI = stimulation index.

[1] + = sensitizer; - = nonsensitizer.

1.3.1 Intralaboratory Reproducibility – SI ≥ 2.0

There were seven substances that were tested multiple times by Takeyoshi et al. (2003, 2004a, 2005, 2006, 2007a, 2007b, unpublished data). Because two substances had multiple tests for more than one concentration, there were 10 substance/concentration combinations that were tested two to five times in separate experiments. The multiple SI values for each substance/concentration were used to calculate a CV for the assessment of intralaboratory variability. As shown by **Table C-IX-2**, the CVs ranged from 1% (25% hexyl cinnamic aldehyde) to 80% (10% isoeugenol). The intralaboratory reproducibility of the traditional LLNA was not assessed by CV analysis of SI values (ICCVAM 1999).

1.3.2 Intralaboratory Reproducibility – EC2

CV values were also calculated for the EC2 values for the three sensitizers that were tested more than once using multiple doses by Takeyoshi et al. (2003; 2004a, 2005, 2006, 2007a, 2007b). The individual animal data for eugenol, hexyl cinnamic aldehyde, and isoeugenol were used to calculate EC2 values for the LLNA: BrdU-ELISA. The methods for calculating EC2 values for each sensitizer were modified from those used by Ryan et al. (2007) to calculate EC3 values. Linear interpolation was used to calculate EC2 values for each test with SI values higher or lower than 2 and extrapolation was used to calculate EC2 values for tests with no SI values below 2. The equation for linear interpolation was:

$$EC2 = c + \left[\frac{(2-d)}{(b-d)}\right] \times (a-c)$$

The linear interpolation equation uses the points immediately above and below SI = 2, with the (dose, SI) coordinates of (a, b) immediately above SI = 2 and (c, d) immediately below SI = 2. The equation for extrapolation was:

$$EC2_{ex} = 2^{\left\{\log_2(c) + \frac{(2-d)}{(b-d)} \times \left[\log_2(a) - \log_2(c)\right]\right\}}$$

The extrapolation equation uses the two points immediately above SI = 2, with the coordinates of (a, b) for the point closest to SI = 2, and (c, d) for the higher point. As shown in **Table C-IX-9**, there were five EC2 values for hexyl cinnamic aldehyde, four EC2 values for eugenol, and two EC2 values for isoeugenol. The CV values were 73% for eugenol, 25% for hexyl cinnamic aldehyde, and 16% for isoeugenol. The ICCVAM LLNA *Performance Standards* criteria for demonstrating adequate intralaboratory reproducibility is based on results from at least four independent tests of hexyl cinnamic aldehyde (ICCVAM 2009). Intralaboratory reproducibility is considered adequate when each test yields an ECt value (i.e., the estimated concentration needed to produce an SI of a specific threshold value; in this case, SI = 1.5) within 5% to 20% (ICCVAM 2009). Two of the five EC2 values for hexyl cinnamic aldehyde were within the acceptable range for intralaboratory reproducibility.

Table C-IX-9 Intralaboratory Reproducibility for the EC2 of Tested Substances in LLNA: BrdU-ELISA - Coefficient of Variation

Substance	EC2	Mean	SD	CV (%)	Takeyoshi et al. Reference
Eugenol	11.2	12.6	9.2	73	2004a
	23.6				2006
	1.2				2007b
	14.6				2007a
Hexyl cinnamic aldehyde	15.2	22.6	5.7	25	2003
	18.8				2003
	29.9				2006
	25.5				2006
	23.4				2007b
Isoeugenol	8.4	7.6	1.2	16	2006; 2007b
	6.7				2007a

Abbreviations: CV = coefficient of variation; EC2 = estimated concentration needed to produce a stimulation index of 2; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SD = standard deviation.

The intralaboratory reproducibility of the traditional LLNA was assessed by CV analysis of EC3 values using a larger dataset (ICCVAM 1999) than that available for the LLNA: BrdU-ELISA analysis. Two EC3 values were reported by each of five laboratories for 2, 4-dinitrochlorobenzene, five EC3 values were reported by one laboratory for isoeugenol, six EC3 values were reported for hexyl cinnamic aldehyde by two laboratories, and five EC3 values were reported for eugenol by one laboratory (**Table C-IX-4**).

For two of three substances, the intralaboratory CV values for the EC2 values from LLNA: BrdU-ELISA tests were higher than EC3 values for the same substances from the traditional LLNA reported in ICCVAM (1999). The intralaboratory EC2 CV from the LLNA: BrdU-ELISA tests of eugenol was higher that that reported by ICCVAM (1999) for the EC3 (73% vs. 18%). The intralaboratory EC2 CV from the LLNA: BrdU-ELISA tests of isoeugenol was greater than the EC3 CV from ICCVAM (1999) (26% vs. 16%). However, the intralaboratory EC2 CV from the LLNA: BrdU-ELISA tests of hexyl cinnamic aldehyde was within the EC3 CV range reported by ICCVAM (1999) (25% vs. 19% to 27%).

1.4 Interlaboratory Reproducibility for SI ≥ 2.0

The interlaboratory reproducibility of the LLNA: BrdU-ELISA was assessed using the individual animal data from the multi-laboratory validation study organized by the JSAAE (Kojima et al. 2008). The study design is described in **Appendix C, Section 7.2**. The LLNA: BrdU-ELISA test results from the study are amenable to interlaboratory reproducibility analyses for two endpoints: sensitizer or nonsensitizer classification and EC2 values. Analyses of interlaboratory reproducibility were performed using a concordance analysis for the qualitative results (sensitizer vs. nonsensitizer in **Section 1.4.1** of this annex) and a CV analysis for the quantitative results (EC2 values in **Section 1.4.2** of this annex).

1.4.1 Interlaboratory Reproducibility – Qualitative Results

The available quantitative absorbance data for interlaboratory reproducibility analysis were used to calculate SI values for each substance and dose tested. Substances with SI ≥ 2.0 at any dose were

classified as sensitizers. The qualitative (sensitizer/nonsensitizer) interlaboratory concordance analysis for the 10 substances tested during Phase II of the JSAAE interlaboratory validation study is shown in **Table C-IX-10**. The concordance results show that interlaboratory concordance was 100% (3/3, 6/6, or 7/7) for seven substances. There were three discordant substances (formaldehyde, isopropanol, and lactic acid) for which interlaboratory concordance was 67% (2/3 or 4/6). One of the three laboratories reported an SI of 1.97 for formaldehyde, while the others produced SI > 2. Two of the six tests of isopropanol yielded SI ≥ 2.0 (SI = 2.0 and SI = 2.2); while the others yielded SI < 2. One of the three tests for lactic acid produced SI ≥ 2.0 (i.e., SI = 2.5), while the others yielded SI < 2.0. The Validation Management Team considered the interlaboratory reproducibility to be acceptable (Kojima et al. 2008). There were no traditional LLNA concordance data for comparison with the LLNA: BrdU-ELISA concordance because the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999).

Table C-IX-10 Qualitative Results for the Phase II Interlaboratory Validation Study on the LLNA: BrdU-ELISA[1]

Substance	Laboratory							Concordance
	1	2	3	4	5	6	7	
2,4-Dinitrochloro-benzene	+ (4.30)	+ (8.37)	+ (6.26)	+ (5.50)	+ (18.80)	+ (4.83)	+ (12.98)	7/7
Glutaraldehyde	+ (3.72)				+ (28.64)	+ (2.25)		3/3
Nickel sulfate			+ (2.58)	+ (4.53)			+ (2.66)	3/3
trans-Cinnamic aldehyde		+ (3.37)		+ (3.50)	+ (4.11)			3/3
Formaldehyde	+ (4.40)				+ (16.59)	- (1.97)		2/3
Eugenol		+ (3.17)				+ (3.18)	+ (7.09)	3/3
Hexyl cinnamic aldehyde	+ (3.40)	-[3]	+ (2.87)	+ (3.34)	+ (13.50)	+3,[4] (3.27)	+ (3.84)	6/6
Isopropanol	+[2] (2.22)	-[3]	- (0.98)	- (1.57)	- (0.94)	+[2,3,5] (2.04)	- (1.01)	4/6
Lactic acid			- (1.80)	- (1.89)			+ (2.53)	2/3
Methyl salicylate	- (1.43)	- (1.44)	- (1.40)					3/3

Abbreviation: LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine.

[1] + indicates sensitizer result; - indicates nonsensitizer result. Maximum stimulation index values for each test are shown in parentheses.

[2] Stimulation index (SI) ≥ 2 at lowest dose tested, but <2 at the higher doses. The Validation Management Team considered these to be nonsensitizer results (Kojima et al. 2008).

[3] Test failed because concurrent positive control failed (i.e., SI < 2). Result not included in the concordance analysis.

[4] Maximum SI = 1.97.

[5] Three mice tested at highest dose.

[6] Three mice per dose group.

1.4.2 Interlaboratory Reproducibility – EC2 Values

The SI values from the interlaboratory validation study were used to calculate EC2 values for each sensitizer according to the methods reported in **Section 1.3.3** of this annex. The EC2 values from each laboratory were then used to calculate CV values for each substance. The resulting values are shown in **Table C-IX-11**. CV values ranged from 20% (formaldehyde) to 101% (glutaraldehyde). The mean CV was 58%.

The ICCVAM LLNA performance standards indicate that interlaboratory reproducibility should be evaluated with at least two sensitizing chemicals with well-characterized activity in the traditional LLNA (ICCVAM 2009). Acceptable reproducibility is attained when each laboratory obtains ECt values within 0.025% to 0.1% for 2,4-dinitrochlorobenzene and within 5% to 20% for hexyl cinnamic aldehyde (ICCVAM 2009). EC2 values from two laboratories were outside these ranges for both substances. Laboratory 2 and Laboratory 5 reported EC2 values that were lower than the specified acceptance range for 2,4-dinitrochlorobenzene (0.019% and 0.0025%, respectively). For hexyl cinnamic aldehyde, Laboratory 3 obtained an EC2 value of 24.0%, which was higher than the acceptance range, and Laboratory 5 obtained an EC2 value of 4.07%, which was lower than the acceptance range.

Table C-IX-11 EC2 Values from the Phase II Interlaboratory Validation Study on the LLNA: BrdU-ELISA[1]

Substance	Laboratory							Mean	% CV
	1	2	3	4	5	6	7		
2,4-Dinitro-chlorobenzene	0.084 (4.3 @ 1%)	***0.019 (8.37 @ 1%)***	0.029 (5.99 @ 0.3%)	0.030 (5.50 @ 1%)	***0.0025 (18.80 @ 0.3%)***	0.025 (4.83 @ 0.3%)	0.053 (12.18 @ 1%)	0.035	76
Hexyl cinnamic aldehyde	16.2 (3.4 @ 50%)	-[1] (1.83 @ 50%)	***24.0 (2.87 @ 50%)***	9.36 (3.34 @ 50%)	***4.07 (13.5 @ 50%)***	13.0[2] (3.27 @ 50%)	14.2 (3.84 @ 50%)	13.5	50
Glutaraldehyde	0.18	NT	NT	NT	0.034	0.51	NT	0.24	101
Nickel sulfate	NT	NT	3.85	0.95	NT	NT	1.31	2.0	78
trans-Cinnamic aldehyde	NT	2.59	NT	1.63	2.79	NT	NT	2.3	27
Formaldehyde	0.41	NT	NT	NT	0.31	-[3]	NT	0.36	20
Eugenol	NT	19.1	NT	NT	NT	16.4	5.06	13.5	55

Note: Boldface indicates substances recommended for assessing interlaboratory reproducibility in *Recommended Performance Standards* (ICCVAM 2009). Boldface italic EC2 values are outside of the acceptable range from the ICCVAM LLNA performance standards: 5% - 20% for hexyl cinnamic aldehyde and 0.025% - 0.1% for 2,4-dinitrochlorobenzene. Values in parentheses are the highest SI values achieved.

Abbreviations: CV = coefficient of variation; EC2 = estimated concentration needed to produce a stimulation index of 2; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; NT = not tested; SI = stimulation index.

[1] Test failed because associated positive control failed (i.e., SI < 2; vehicle control absorbance was unusually high). Result not included in the mean EC2 and CV.

[2] Three mice tested at highest dose.

[3] Maximum SI = 1.97.

The interlaboratory CV values for the LLNA: BrdU-ELISA EC2 values were higher than those for the traditional LLNA EC3 values. The analysis of interlaboratory variation of EC3 values for the traditional LLNA reported CV values of 7% to 84% for five substances tested in five laboratories (**Table C-IX-12**; ICCVAM 1999). Three of the same substances were evaluated in the traditional LLNA and the LLNA: BrdU-ELISA. All interlaboratory CV values for LLNA: BrdU-ELISA were greater than those for the traditional LLNA. The CV of 76% for 2,4-dinitrochlorobenzene was greater than the two CV values of 37% and 27%, calculated from five values each, reported by ICCVAM (1999). The CV of 50% for hexyl cinnamic aldehyde tested in the LLNA: BrdU-ELISA was greater than the 7% reported by ICCVAM (1999). The CV of 55% for eugenol tested in the LLNA: BrdU-ELISA was greater than the 42% reported by ICCVAM (1999).

Table C-IX-12 Interlaboratory Reproducibility of the EC3 for Substances Tested in the Traditional LLNA[1]

Substance	Laboratory					CV (%)
	1	2	3	4	5	
2,4-Dinitrochlorobenzene	0.3	0.5	0.6	0.9	0.6	37
	0.5	0.6	0.4	0.6	0.3	27
Hexyl cinnamic aldehyde	7.9	7.6	8.4	7.0	8.1	7
Isoeugenol	1.3	3.3	1.8	3.1	1.6	41
Eugenol	5.8	14.5	8.9	13.8	6.0	42
Sodium lauryl sulfate	13.4	4.4	1.5	17.1	4.0	84

Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of 3; LLNA = murine local lymph node assay.

[1] From ICCVAM (1999).

Appendix D

Independent Scientific Peer Review Panel Assessment

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Appendix D1

Summary Minutes from the Independent Scientific Peer Review Panel Meeting on March 4-6, 2008

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Summary Minutes

Independent Scientific Peer Review Panel Meeting

Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products

Consumer Product Safety Commission (CPSC), Headquarters
Bethesda, MD
March 4 – 6, 2008
8:30 a.m. – 5:30 p.m.

Peer Review Panel Members:

Michael Luster, Ph.D. (Peer Review Panel Chair)	Senior Consultant to the NIOSH Health Effects Laboratory, Morgantown, WV, U.S.
Nathalie Alépée, Ph.D.	Associate Research Fellow, Pfizer PDRD MCT Laboratory, France
Anne Marie Api, Ph.D.	Vice President, Human Health Sciences, Research Institute for Fragrance Materials, Woodcliff Lake, NJ, U.S.
Nancy Flournoy, M.S., Ph.D.	Professor and Chair, Dept. of Mathematics and Statistics, University of Missouri-Columbia, Columbia, MO, U.S.
Thomas Gebel, Ph.D.	Regulatory Toxicologist, Federal Institute for Occupational Safety and Health, Dortmund, Germany
Kim Headrick, B. Admin., B.Sc.	International Harmonization Senior Policy Advisor, Health Canada, Ottawa, Ontario, Canada
Dagmar Jírová, M.D., Ph.D.	Toxicologist, Research Manager, Head of Reference Center for Cosmetics, Head of Reference Laboratory for Experimental Immunotoxicology, National Institute of Public Health, Czech Republic
David Lovell, Ph.D	Reader in Medical Statistics, Postgraduate Medical School, University of Surrey, Guildford, Surrey, U.K.
Howard Maibach, M.D.	Professor, Dept. of Dermatology, University of California-San Francisco, San Francisco, CA, U.S.

Peer Review Panel Members:

James McDougal, Ph.D.	Professor and Director of Toxicology Research, Dept. of Pharmacology and Toxicology, Boonshoft School of Medicine, Wright State University, Dayton, OH, U.S.
Michael Olson, Ph.D.	Director of Occupational Toxicology, Corporate Environment Health and Safety, GlaxoSmithKline, RTP, NC, U.S.
Raymond Pieters, Ph.D.	Associate Professor, Immunotoxicology Group Leader, Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands
Jean Regal, Ph.D.	Professor, Dept. of Pharmacology, University of Minnesota Medical School, Duluth, MN, U.S.
Peter Theran, V.M.D.	Massachusetts Society for the Prevention of Cruelty to Animals, Novato, CA, U.S.
Stephen Ullrich, Ph.D.	Dallas/Ft. Worth Living Legends Professor & Professor of Immunology, Graduate School of Biomedical Science, University of Texas M.D. Anderson Cancer Center, Houston, TX, U.S.
Michael Woolhiser, Ph.D.	Technical Leader - Immunotoxicology, Toxicology and Environmental Research and Consulting Immunology, Dow Chemical, Midland, MI, U.S.
Takahiko Yoshida, M.D., Ph.D.	Professor, Dept. of Health Science, Asahikawa Medical College, Hokkaido, Japan

ICCVAM and ICCVAM IWG Members:

Paul Brown, Ph.D.	FDA, Silver Spring, MD, U.S.
Ruth Barratt, Ph.D., D.V.M.	FDA, Rockville, MD, U.S.
Karen Hamernik, Ph.D.	EPA, Washington, DC, U.S.
Masih Hashim, Ph.D.	EPA, Washington, DC, U.S.
Abigail Jacobs, Ph.D. (IWG Co-Chair)	FDA, Silver Spring, MD, U.S.
Kristina Hatlelid, Ph.D.	CPSC, Bethesda, MD, U.S.
Joanna Matheson, Ph.D. (IWG Co-Chair)	CPSC, Bethesda, MD, U.S.
Tim McMahon, Ph.D.	EPA, Washington, DC, U.S.

ICCVAM and ICCVAM IWG Members:

Amy Rispin, Ph.D.	EPA, Washington, DC, U.S.
William Stokes, D.V.M., DACLAM	NIEHS, RTP, NC, U.S.
Raymond Tice, Ph.D.	NIEHS, RTP, NC, U.S.
Ron Ward, Ph.D.	EPA, Washington, DC, U.S.
Marilyn Wind, Ph.D. (ICCVAM Chair)	CPSC, Bethesda, MD, U.S.
Jiaqin Yao, Ph.D.	FDA, Silver Spring, MD, U.S.

ECVAM Observer:

David Basketter, Ph.D.	DABMEB Consultancy Ltd., Bedfordshire, U.K.

Invited Experts:

George DeGeorge, Ph.D., DABT	MB Research Laboratories, Spinnerstown, PA, U.S.
Kenji Idehara, Ph.D.	Daicel Chemical Industries, Hyogo, Japan
Masahiro Takeyoshi, Ph.D.	Chemicals Evaluation and Research Institute, Saitama, Japan

Public Attendees:

Odette Alexander	Syngenta Crop Protection, Inc., Greensboro, NC, U.S.
Nancy Beck, Ph.D.	PCRM, Washington, DC, U.S.
Ann Blacker, Ph.D.	Bayer CropScience, RTP, NC, U.S.
Stuart Cagan, Ph.D.	Shell Oil Company, Houston, TX, U.S.
Joan Chapdelaine, Ph.D.	Calvert Laboratories, Inc., Olyphant, PA, U.S.
Adriana Doi, Ph.D.	BASF Corporation, RTP, NC, U.S.
Carol Eisenmann, Ph.D.	Personal Care Products Council, Washington, DC, U.S.
Charles Hastings, Ph.D.	BASF Corporation, RTP, NC, U.S.
Kailash Gupta, D.V.M., Ph.D.	Retired CPSC, Bethesda, MD, U.S.
John Lyssikatos	Hill Top Research, Miamiville, OH, U.S.
Laurence Musset, Ph.D.	OECD, Paris, France
Carol O'Neil	NuPathe, Conshohocken, PA, U.S.

Public Attendees:

Kui Lea Park, Ph.D.	National Institute of Toxicological Research, KFDA, Seoul, Korea
Rafael Rivas	AFRRI/USHUS, Bethesda, MD, U.S.
Terri Sebree	NuPathe, Conshohocken, PA, U.S.
Libby Sommer	EPA, Washington, DC, U.S.
Merrill Tisdel	Syngenta Crop Protection Inc., Greensboro, NC, U.S.
Jeffrey Toy, Ph.D.	FDA, Rockville, MD, U.S.

NICEATM:

William Stokes, D.V.M., DACLAM	Director
Raymond Tice, Ph.D.	Deputy Director
Debbie McCarley	Special Assistant to the Director

Support Contract Staff— Integrated Laboratory Systems, Inc. (ILS)

David Allen, Ph.D.	Michael Paris
Thomas Burns, M.S.	Eleni Salicru, Ph.D.
Linda Litchfield	Judy Strickland, Ph.D., DABT
Douglas Winters, M.S.	

Abbreviations:

AFFRI = Armed Forces Radiobiology Research Institute

CPSC = U.S. Consumer Product Safety Commission

ECVAM = European Centre for the Validation of Alternative Methods

EPA = U.S. Environmental Protection Agency

FDA = U.S. Food and Drug Administration

ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods

ILS = Integrated Laboratory Systems

IWG = Immunotoxicology Working Group

KFDA = Korea Food and Drug Administration

NICEATM = National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

NIEHS = National Institute of Environmental Health Sciences

NIOSH = National Institute of Occupational Safety and Health

OECD = Organisation for Economic Co-operation and Development

PCRM = Physicians Committee for Responsible Medicine

USDA = U.S. Department of Agriculture

USHUS = Uniformed Services University of the Health Sciences

TUESDAY, MARCH 4, 2008

Call to Order and Introductions—

Dr. Michael Luster (Peer Review Panel Chair) called the meeting to order at 8:30 a.m. and introduced himself. He then asked all Peer Review Panel (hereafter Panel) members to introduce themselves and to state their name and affiliation for the record. He then asked all the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) staff, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) members, the ICCVAM Immunotoxicity Working Group (IWG) members, the European Centre for the Validation of Alternative Methods (ECVAM) observer, and members of the public to also introduce themselves. Dr. Luster stated that there would be opportunity for public comments during each of the seven local lymph node assay (LLNA)-related topics. He asked that all those interested in making a comment register at the registration table and provide a written copy of their comments, if available, to NICEATM staff. Dr. Luster emphasized that the comments would be limited to seven minutes per individual and that, while an individual would be welcome to make comments during each commenting period, repeating the same comments at each comment period would be inappropriate. He further stated that the meeting was being recorded and that Panel members should speak directly their microphone. Finally, Dr. Luster noted that if the Panel finished early with the assigned topics on the agenda for that day, they would proceed to the next day's topics if time permitted.

Welcome from the ICCVAM Chair—

Dr. Marilyn Wind, U.S. Consumer Product Safety Commission (CPSC) and Chair of ICCVAM, welcomed everyone to CPSC and to the Panel meeting. Dr. Wind stressed the importance of this Panel's efforts especially considering recent reports that allergies and asthma have increased markedly over the past number of years and that contact dermatitis is the most common occupational illness in the United States. Dr. Wind thanked the Panel members for giving their expertise, time, and effort and acknowledged their important role to the ICCVAM test method evaluation process. Dr. Wind also emphasized the important role of the public and their comments in this process.

Welcome from the Director of NICEATM, and Conflict of Interest Statements—

Dr. William Stokes, Director of NICEATM, stated the Panel meeting was being convened as a National Institutes of Health (NIH) special emphasis panel and was being held in accordance with the Federal Advisory Committee Act regulations. As such, Dr. Stokes indicated that he would serve as the Designated Federal Official for this public meeting. He reminded the Panel that they had signed a conflict-of-interest statement when they were selected for the Panel, in which they identified any potential conflicts of interest. He then read this statement to provide another opportunity for members of the Panel to identify any conflicts not previously declared. Dr. Luster asked the Panel members to declare any direct or indirect conflicts based on Dr. Stokes statements and to recuse themselves from discussion and voting on any aspect of the meeting where there might be a conflict. None of the Panel members declared a conflict of interest.

Overview of the ICCVAM Test Method Evaluation Process

Dr. Stokes provided an overview of the ICCVAM test method evaluation process. He stated that the Panel was made up of 19 different scientists from eight different countries (Canada, Czech Republic, France, Germany, Japan, The Netherlands, United Kingdom, and the United States). Dr. Stokes thanked the Panel members for the significant amount of time and effort that they had devoted to prepare for and attend the meeting. He explained that the purpose of the Panel was to assist ICCVAM by carrying out an independent scientific peer review of the information provided on a series of proposed new versions of the LLNA and some expanded applications of the assay. Dr. Stokes

mentioned that the original LLNA peer review panel in 1998 considered the LLNA a valid substitute for the guinea pig-based test in most testing situations, but not all. He mentioned that three Panel members from the 1998 review are also on the current Panel (i.e., Drs. Howard Maibach, Jean Regal, and Stephen Ullrich). Dr. Stokes also reviewed the nomination that was received from CPSC in January 2007,[1] which provides the basis for the current evaluation.

Dr. Stokes then identified the 15 Federal agencies that comprise ICCVAM and summarized ICCVAM's mission. He noted that ICCVAM, as an interagency committee, does not carry out research and development or validation studies. Instead, ICCVAM, in conjunction with NICEATM, carries out the critical scientific evaluation of proposed test methods with regard to their usefulness and limitations for regulatory testing and then makes formal recommendations to ICCVAM agencies.

Dr. Stokes provided a brief review of ICCVAM's history and summarized the ICCVAM Authorization Act of 2000,[2] detailing the purpose and duties of ICCVAM. He noted that one of ICCVAM's duties is to review and evaluate new, revised, and alternative test methods applicable to regulatory testing. He stated that all of the reports produced by NICEATM are available on the NICEATM-ICCVAM website or can be obtained upon request from NICEATM. He also mentioned that ICCVAM provides guidance on test method development, validation criteria, and processes, and helps to facilitate not only the acceptance of scientifically valid alternative methods, but also encourages international harmonization.

Dr. Stokes then described the ICCVAM test method evaluation process, which begins with a test method nomination or submission. NICEATM conducts a prescreen evaluation to summarize the extent to which the proposed submission or nomination addresses the ICCVAM prioritization criteria. A report of this evaluation is then provided to ICCVAM, which in turn develops recommendations regarding the priority for evaluation. ICCVAM then seeks input on their recommendations from the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) and the public. Given sufficient regulatory applicability, sufficient data, resources, and priority, a test method will move forward into a formal evaluation. A draft background review document (BRD), which provides a comprehensive review of all available data and information, is prepared by NICEATM, in conjunction with an ICCVAM working group designated for the relevant toxicity testing area (e.g., the IWG). In addition, ICCVAM considers all of the available information and makes draft test method recommendations on the proposed usefulness and limitations of the test methods, test method protocol, performance standards, and future studies. The BRD and the draft ICCVAM test method recommendations are made available to the Panel and the public for review and comment. The Panel peer reviews the BRD and evaluates the extent to which it supports the draft ICCVAM test method recommendations. A Panel report is published, which is then considered along with public and SACATM comments by ICCVAM in making final recommendations. These final recommendations are forwarded to the ICCVAM member agencies for their consideration and possible incorporation into relevant testing guidelines.

Dr. Stokes reviewed the ICCVAM criteria for adequate validation. He stated that validation is defined by ICCVAM as the process by which the reliability and relevance of a procedure are established for a specific purpose, and that adequate validation is a prerequisite for consideration of a test method by U.S. Federal regulatory agencies. Dr. Stokes listed the ICCVAM acceptance criteria for test method validation and acceptance. He concluded by summarizing the timeline of the review activities beginning with CPSC's nomination in January 2007 and ending with the present Panel meeting.

[1] http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf
[2] http://iccvam.niehs.nih.gov/docs/about_docs/PL106545.pdf

ICCVAM Charge to the Panel

Dr. Stokes reviewed the charge to the Panel, which was to: (1) review the draft BRDs, the draft Addendum to the traditional[3] LLNA, and the draft performance standards for completeness and identify any errors or omissions; (2) determine the extent to which each of the applicable criteria for validation and regulatory acceptance had been addressed for the proposed revised or modified versions of the LLNA; and (3) consider and provide comment on the extent to which the ICCVAM draft test method recommendations including the proposed use, standardized protocols, performance standards, and additional studies are supported by the information provided in the draft BRDs and draft Addendum.

Dr. Stokes thanked the IWG and ICCVAM for their contributions to this project, and acknowledged the contributions from the participating liaisons from ECVAM and JaCVAM (Japanese Center for the Validation of Alternative Methods). He also acknowledged the NICEATM staff for their support and assistance in organizing the Panel meeting and preparing the materials being reviewed.

Current Regulatory Testing Requirements and Hazard Classification Schemes for Allergic Contact Dermatitis and the Traditional LLNA Procedure

Dr. Joanna Matheson, Chair of the IWG, briefly reviewed the regulatory testing requirements of U.S. Federal agencies for skin-sensitization hazard identification and provided a brief description of the LLNA protocol.

Overview of the Agenda

Dr. Luster provided a brief synopsis of the agenda. He stated that there were six test methods and applications along with the draft LLNA performance standards for review and that the same agenda would be followed for each: (1) introductory summary of the draft ICCVAM recommendations from one of the NICEATM staff members; in addition, test method developers would provide a brief description of the methodology for each of the three nonradioactive tests, (2) presentation of the Evaluation Group draft comments by the Evaluation Group leader, (3) Panel discussion, (4) public comments, (5) recommendations and conclusions by the Panel.

Overview of the Draft LLNA Limit Dose Procedure[4] BRD and Draft ICCVAM Test Method Recommendations

Dr. David Allen, Integrated Laboratory Systems, Inc., the NICEATM support contractor, presented an overview of the draft ICCVAM BRD for the LLNA limit dose procedure. He mentioned that the draft ICCVAM BRD provided a comprehensive review of the available data and information regarding the usefulness and limitations of the LLNA limit dose procedure. The method was reviewed for its accuracy in correctly identifying sensitizers and non-sensitizers, when compared to the traditional LLNA.

NICEATM published a series of *Federal Register* (FR) notices, including an FR notice (72 FR 27815, May 17, 2007) requesting original data from the LLNA. This FR notice was also sent to over 100 potentially interested stakeholders for their input and comment. As a result, data on 255 substances tested in the LLNA were received. The resulting LLNA database consisted of 471 studies of 466 unique substances, 211 of which were included in the original ICCVAM 1999 evaluation. Dr. Allen briefly summarized the performance characteristics of the LLNA limit dose procedure test

[3] For the purposes of this document, the radioactive LLNA test method, which was first evaluated by ICCVAM in 1999, and subsequently recommended to U.S. Federal agencies as a valid substitute for currently accepted guinea pig test methods to assess the allergic contact dermatitis potential of many, but not all, types of substances, is referred to as the traditional LLNA.

[4] Also known as the reduced LLNA (rLLNA).

method, which is detailed in the draft ICCVAM BRD,[5] and briefly summarized the draft ICCVAM test method recommendations for the LLNA limit dose procedure.[6]

Panel Evaluation:

Dr. Michael Olson led the Panel discussion on the LLNA limit dose procedure and specifically thanked the members of his Evaluation Group (i.e., Drs. James McDougal, Raymond Pieters, Jonathan Richmond [not present], and Takahiko Yoshida) for their collegial review of the information presented in the draft ICCVAM LLNA Limit Dose Procedure BRD. Dr. Olson also thanked the NICEATM staff for their technical support during the BRD review process. He then presented the draft responses to ICCVAM's questions to the Panel for consideration by the entire Panel. The focus was on review of the BRD for errors and omissions, assessment of the validation status of the test method, and review of draft ICCVAM test method recommendations. The Panel discussion and their recommended revisions to each section of the draft ICCVAM BRD and recommendations are reflected in the *Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products*, published in May 2008 (hereafter, the Panel report[7]).

During the Panel's evaluation, discussion arose regarding what might have resulted in the inverted-U-shaped dose response that was seen with the false-negative substances in the LLNA limit dose procedure. Dr. Olson responded that although it was difficult to understand what the cause might have been, he speculated that the top dose was either toxic at a systemic-effect level or that those substances were immunosuppressive at the highest dose level. He also stated that there did not seem to be any structural features of the substances that could be attributed for the false negative response in the LLNA limit dose procedure.

The Panel also discussed the use of concurrent versus intermittent positive controls in the LLNA limit dose procedure. Dr. Olson indicated that the Evaluation Group had discussed the possibility to allow intermittent positive controls for laboratories that exhibited repeatable and adequate performance with the LLNA but he indicated that it would be important to describe a set of performance criteria that would determine when this practice would be acceptable. Clearly, if the laboratory was not performing the assay routinely or if there were other reasons to suspect variability in response with any substance, the positive control would be necessary. Dr. Stokes indicated that this discussion was pertinent and indicated that the Panel's suggestions for what the performance criteria might be for intermittent positive control testing would be of interest to the IWG. Dr. Stokes also wanted to clarify that the OECD TG is consistent with the EPA TG and the ICCVAM-recommended test method protocol for the LLNA although the OECD TG allows additional latitude in how tests are run (i.e., four animals per dose group, use of pooled data, and the option to not run a positive concurrent positive).

Public Comments:

Dr. Amy Rispin, EPA

Dr. Rispin stated that the ICCVAM LLNA report (1999[8]) and standardized protocol (2001[9]) recommends the use of a concurrent positive control in addition to the concurrent negative control required for each study. Subsequently, the OECD (Organisation for Economic Co-operation and Development) Test Guideline (TG) 429 (Skin Sensitisation: Local Lymph Node Assay) was finalized (2002). She said that originally, OECD TG 429 was drafted without a concurrent positive control but that language was added to include the recommended use of a concurrent positive control until

[5] http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-LD/LLNAldBRD07Jan08FD.pdf
[6] http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-LD/IWGrecLLNA-LD07Jan08FD.pdf
[7] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
[8] http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf
[9] http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/LLNAProt.pdf

laboratories demonstrate competence. Subsequent to that, EPA put forth its LLNA guideline for sensitization,[10] which states that concurrent positive and negative controls are to be included in each study. Dr. Rispin then added that U.S. Federal regulatory agencies, most notably the EPA and FDA, received LLNA data from studies in which the positive control did not achieve the appropriate limits of performance (i.e., the control values were not in the appropriate range) and therefore the studies were deemed unacceptable, underscoring the importance of a concurrent positive control for regulatory acceptance in the United States.

In response to Dr. Rispin's public comment, Drs. Ullrich and Theran asked how competence is determined and if laboratories have difficulties reaching a level of competence, respectively. Dr. Abby Jacobs responded by stating that the FDA has seen large data variations in laboratories that conduct the LLNA. It is often difficult to determine what the variations might be due to (e.g., new technicians, tail vein injection, lymph node removal) and these variations have been seen both in laboratories that are established and those that are not.

Dr. David Basketter, ECVAM Observer

Dr. Basketter said that the main point he wanted to address is that efforts should be made to harmonize the LLNA protocol with that described in OECD TG 429. He stated that although there is referral to the "ICCVAM protocol" throughout the BRDs under consideration, OECD TG 429 is more globally recognized for regulatory use of the LLNA and therefore should be the referenced protocol. Dr. Basketter further stated that if the LLNA limit dose procedure followed the ICCVAM protocol using five animals per group instead of following OECD TG 429, which allows using four animals per group, there would only be a savings of one animal for substances that were negative. He stated that the goal of ECVAM was actually to halve the number of animals by omitting the mid- and low-dose groups and that this would achieve significant animal savings since the likely prevalence of non-sensitizers is approximately two-thirds of chemicals tested and non-sensitizers would not require further testing even if dose response information for sensitizers was needed.

Dr. Basketter also mentioned that the retrospective evaluation of the LLNA being presented to the Panel analyzed whether the top dose could identify a substance as a sensitizer and how that compares to the traditional LLNA's performance. Since the traditional LLNA assay was determined to be positive or negative based on a stimulation index (SI) of three, it is problematic if the focus is on statistics when using the five-animal model as this would require also going back and re-evaluating all the preceding data using the statistical approach.

Dr. McDougal responded to Dr. Basketter's comment by stating that one wouldn't have to go back and retrospectively re-evaluate previous data but that new data generated could be analyzed statistically. This approach would include determining if the treatment group was statistically different from the vehicle control group and then determining the biological relevance. This might help to eliminate irritants.

Panel Conclusions and Recommendations:

Dr. Luster asked the Panel to review the conclusions and recommendations for the LLNA limit dose procedure they had discussed earlier and to make any revisions, if necessary. One particular question that was asked during the Panel's conclusions and recommendations was whether an OECD TG existed for the LLNA limit dose procedure. Dr. Stokes indicated that the OECD TG would need to be updated to allow for the provision of a limit dose procedure and that's why the Panel's conclusions and recommendations are even more relevant. Dr. Stokes indicated that ICCVAM has already submitted a proposal to update the OECD TG based on the outcome of these deliberations and recommendations from the IWG.

[10]http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Revised/870r-2600.pdf

The Panel agreed to use the term weight-of-evidence to refer to existing information that would aid the LLNA limit dose procedure in identifying a substance as a sensitizer or a non-sensitizer. The Panel also discussed the use of concurrent positive controls and recommended that a laboratory that is proficient at conducting the limit dose procedure can test a positive control at routine intervals rather than concurrently (although the Panel did not identify what constituted routine intervals). The Panel also discussed the use of individual versus pooled data and agreed with the ICCVAM-recommended protocol that individual animal data should always be collected. The Panel concluded that individual animal response data are necessary in order to allow for statistical analyses of any differences between treated and control data. In addition, having data from individual animals also allows for identification of technical problems and outlier animals within a dose group. Dr. Luster asked the Panel if they agreed with the changes and revisions made at this point and with the Panel conclusions and recommendations as presented and revised. The Panel unanimously agreed. The Panel's detailed recommendations and conclusions on the LLNA limit dose procedure are included in their final Panel report.[11]

Overview of the Draft Addendum for the Applicability Domain of the LLNA and Draft ICCVAM Test Method Recommendations

Dr. Eleni Salicru, Integrated Laboratory Systems, Inc. (the NICEATM support contractor), summarized the information provided in the draft ICCVAM Addendum to the ICCVAM LLNA report (1999). This Addendum provided an updated assessment of the validity of the LLNA for testing the sensitizing potential of mixtures, metals, and aqueous solutions. The database used for this evaluation contained traditional LLNA data submitted as part of the original LLNA evaluation (ICCVAM 1999), data extracted from peer-reviewed articles published after the original evaluation, and data submitted to NICEATM in response to the FR notice (72 FR 27815, May 17, 2007) requesting such data. Dr. Salicru then summarized the performance characteristics of the LLNA when used to test mixtures, metals, and aqueous solutions,[12] as well as the draft ICCVAM test method recommendations for each of the three categories of test substances.[13]

Panel Evaluation:

Dr. McDougal, on behalf of his Evaluation Group, presented for consideration by the entire Panel the draft responses to the questions asked of the Panel by ICCVAM. The Panel then discussed the completeness of the draft ICCVAM Addendum, identified any errors and omissions, and reviewed the draft ICCVAM test method recommendations with regard to the ability of the LLNA to be used to test the sensitizing potential of mixtures, metals, and aqueous solutions. The Panel discussion and their recommended revisions to each section of the draft ICCVAM Addendum are reflected in the Panel report, published in May 2008.[14] During the Panel's evaluation of the LLNA's applicability domain, the difficulty of testing metals in the LLNA was discussed and Dr. Woolhiser asked if testing metals was also problematic in the guinea pig. Dr. Api indicated that with the metals, most of the data has come from the clinical experience because animal studies are not predicting accurately what is happening in the clinic. Dr. Maibach indicated that metals have been tested in the guinea pig and that they are sensitized easily. Dr. Maibach further commented that metals in man need to be patch-tested for clinical relevance at a level close to the irritant dose and that a thoughtful series of algorithms is necessary to determine this. He also pointed out that patch test results to some metals (e.g., nickel, palladium) may indicate that a cell mediated reaction is occurring (i.e., contact allergy) but it needs to be sorted out if this cell mediated reaction actually results in a disease (i.e., allergic contact dermatitis) and this is where the LLNA could prove useful.

[11] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
[12] http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-app/LLNAappADD19Jan08FD.pdf
[13] http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-app/LLNAappRecs19Jan08FD.pdf
[14] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

With regard to mixtures, Dr Api commented that based on her experience, when the mixture tested in the LLNA contains a predominant material (loosely defined that as greater than 70 percent) then the LLNA for the mixture mirrors what occurs for that one material. When evidence indicates that the substance is a true mixture, some times the LLNA does what is expected and other times the results are unexpected. In those cases, a weight-of-evidence approach (e.g., structure-activity relationships, clinical evidence) is employed.

Public Comments:

Dr. Charles Hastings, BASF Corporation

Dr. Hastings, representing CropLife America (an industry association of companies in the crop protection business), provided an overview of current activities in industry related to the use of the LLNA to detect dermal sensitizers and the global issues that are of importance. Dr. Hastings mentioned that CropLife America's primary concern is the testing of pesticide mixtures and formulations. He stated that they support the use of the LLNA for testing the dermal sensitization of mixtures and formulations as well as single ingredients.

Dr. Hastings mentioned that in the United States, EPA OPPTS (Office of Prevention, Pesticides and Toxic Substances) Guideline 870.2600[15] allows for the use of the LLNA as the preferred alternative to the standard guinea pig test. Based on this recommendation, member companies of CropLife America conducted a large number of LLNA studies for both active ingredients and formulations in the European Union (E.U.) and were at the point of submitting data in the United States, as well. Then, in early 2007, they were informed that EPA had concerns about the validity of using the LLNA to test mixtures and formulations, and were advised to discontinue using this test method for that purpose until it had been adequately validated. Dr. Hastings stated that, in contrast to the EPA, E.U. regulators consider the LLNA acceptable for testing pesticide formulations and actually prefer it to a guinea pig test.

Dr. Pieters asked if the E.U. has conducted any evaluations of the validity of the LLNA for testing mixtures and formulations. Dr. Hastings replied that he was not certain if they had performed an extensive evaluation or not but that the E.U. considered the LLNA a validated method and therefore likely considered it appropriate to test not only the active ingredient but also the formulation or mixture.

Dr. Hastings mentioned that one concern in terms of using the LLNA for testing mixtures or formulations, particularly in the E.U., is the testing of aqueous substances. Many of the industry formulations are aqueous-based and may be incompatible with traditional LLNA vehicles. The European Crop Protection Association sponsored a study that evaluated the use of an aqueous vehicle known as Pluronic L92, which helps adhere the test material to the mouse ear. In the study, they tested three aqueous pesticide formulations that contained known sensitizers, using Pluronic L92 as the vehicle. As expected, the test results demonstrated sensitizing activity. Regarding global considerations, Dr. Hastings mentioned that if the LLNA is not accepted for mixture/formulation testing in the United States, industry will have no choice but to conduct both the LLNA, with 18 to 24 animals, and a guinea pig test, with 20 to 30 animals, for each formulation they may develop for global distribution. This scenario counters the ICCVAM goal of "reducing, refining, and replacing" animal use in regulatory safety testing.

Dr. Hastings ended with the following conclusions:

- CropLife America believes the LLNA test can be used for pesticide formulations.

[15]http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Revised/870r-2600.pdf

- CropLife America supports the efforts of EPA and ICCVAM to confirm the validity of the LLNA for testing mixtures/formulations and encourages a quick evaluation.
- CropLife America is willing to help, as needed.
- If and, when, it is determined that the LLNA is acceptable, CropLife America requests that EPA notify them so they can then begin conducting the LLNA again for the United States.

Dr. Api asked if CropLife America has data comparing pesticides that have been evaluated in the LLNA and in guinea pigs and/or humans. Dr. Hastings replied that they do and that generally there is not much discrepancy with guinea pig test results. Occasionally they might see a false positive compared to a guinea pig test, but he did not recall ever seeing a false negative. In most cases, they would feel comfortable accepting an occasional false positive because human health is still protected.

Dr. David Basketter, ECVAM Observer
Dr. Basketter stated that he had personal reservations about testing complex mixtures and formulations in assays that were designed for testing substances (e.g., the LLNA) since no single test has ever been validated for testing mixtures. On another point, he stated that most of the metals of importance have been tested in both the guinea pig and the LLNA and the "right" answers have been generated. Thus, it does not seem worthwhile to produce new tests with revised protocols for hazard and potency categorization for testing metals.

Panel Conclusions and Recommendations:

Dr. Luster asked the Panel if they agreed with the comments and recommendations that were made earlier during the Panel discussion. The Panel agreed with the draft ICCVAM recommendation for continued collection of information from traditional LLNA evaluations of mixtures, metals, and aqueous solutions with comparative data for guinea pig (i.e., guinea pig maximization test [GPMT] or Buehler test [BT]) and human (i.e., human maximization test [HMT] or human repeat insult patch test [HRIPT]) tests. However, the Panel suggested that, given resource limitations, it would be important to organize the recommendations based on relative priority. Dr. Luster asked the Panel if they agreed with this suggestion about prioritization of activities; all members of the Panel agreed with one abstention. Dr. Howard Maibach abstained from voting stating that he hoped this public meeting and the subsequent Panel report would emphasize to industry the need for them to submit more data on mixtures, metals, and aqueous substances in order to provide a clearer evidence of the validity of the LLNA in testing these types of substances. The Panel's detailed recommendations and conclusions on the applicability domain of the LLNA are included in their final Panel report.[16]

Method Description and Overview of the LLNA: Daicel Adenosine Triphosphate (LLNA: DA) Test Method

Dr. Kenji Idehara, Daicel Chemical Industries, Ltd. (private limited company), summarized the technical aspects of the LLNA: DA test method. He described the LLNA: DA as a non-radioisotopic version of the LLNA method in which lymph node adenosine triphosphate (ATP) content is used as a measure of cell proliferation instead of radiolabeled thymidine incorporation. Dr. Idehara indicated that the LLNA: DA was developed six years ago at Daicel Chemical Industries, Ltd., and that they use the test method regularly for in-house assessments of the skin-sensitization potential of chemical materials, intermediates, or products. He summarized the protocol differences between the LLNA: DA and the traditional LLNA. In the LLNA: DA, the application site is treated with 1% sodium lauryl sulfate (SLS) one hour before each test substance (or vehicle control) application, and the test substance is applied to the test site on day 7 as well as on days 1, 2, and 3. The auricular lymph nodes are excised from individual animals on day 8 rather than on day 6 and the amount of ATP in the

[16] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

lymph nodes is measured with a luciferin-luciferase assay. Dr. Idehara mentioned that these modifications (i.e., 1% SLS pretreatment and additional application on day 7) enhance lymph node cell proliferation in order to achieve an SI = 3 in the LLNA: DA, which allows for a more direct comparison to the traditional LLNA.

Dr. Idehara mentioned that after excision, ATP content gradually decreased with time. Therefore, the overall assay time for measuring ATP content needs to be similar (i.e., within approximately 30 minutes) among all test animals. He noted that this was an important point for this method and recommended that the LLNA: DA be conducted by at least two persons. Dr. Idehara mentioned that ATP content assays are conducted using commercially available kits, and his laboratory has experience with two different commercial sources in Japan, Kikkoman and Lonzar.

Overview of the Draft LLNA: DA BRD and Draft ICCVAM Test Method Recommendations

Dr. Allen then presented an overview of the draft ICCVAM BRD for the LLNA: DA test method. He mentioned that the draft ICCVAM BRD provided a comprehensive review of the available data and information regarding the usefulness and limitations of the LLNA: DA to distinguish between sensitizers and non-sensitizers, compared to the traditional LLNA. The objective of the BRD was to describe the current validation status of the LLNA: DA test method, including its relevance and reliability, scope of substances tested, and the availability of a standardized protocol.

Dr. Allen mentioned that the data analyzed in the BRD included data provided by Daicel Chemical Industries, Ltd., on 31 substances tested at their laboratories. In addition, data for 14 different coded substances were generated from a two-phased interlaboratory validation study that included 17 total labs. Taken together, the total database represented in the LLNA: DA BRD included 33 different substances. Dr. Allen briefly summarized the performance characteristics of the LLNA: DA test method, which is detailed in the draft ICCVAM BRD.[17] Dr. Allen concluded by briefly summarizing the draft ICCVAM test method recommendations for the LLNA: DA test method.[18]

Panel Evaluation:

Dr. Michael Woolhiser thanked the Panel members of his Evaluation Group (i.e., Drs. Nathalie Alépeé, Thomas Gebel, Sidney Green [not present], and Jean Regal) for their tireless efforts in reviewing their Evaluation Group's assigned documents. He also thanked the NICEATM staff for their technical support during the review process. Dr. Woolhiser then presented the draft responses to ICCVAM's questions about this test method for consideration by the entire Panel. This included their review of the draft BRD for errors and omissions, their overall assessment of the validation status of the test method, and their comments on the draft ICCVAM test method recommendations. The Panel discussion and their recommended revisions to each section of the draft ICCVAM BRD are reflected in the Panel report, published in May 2008.[19]

Adjournment—

The meeting was adjourned for the day at 5:03 p.m., to reconvene at 8:30 a.m., Wednesday, March 5, 2008.

[17] http://iccvam.niehs.nih.gov/methods/immunotox/llna-DA/LLNA-DAbrd07Jan08FD.pdf
[18] http://iccvam.niehs.nih.gov/methods/immunotox/llna-DA/LLNA-DARecs07Jan08FD.pdf
[19] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

WEDNESDAY, MARCH 5, 2008

Reconvening of the Panel Meeting

Dr. Luster reconvened the Panel Meeting at 8:30 a.m. He introduced himself and then asked that all Panel members, followed by all others in attendance, introduce themselves as well.

Overview of the Draft LLNA: DA BRD and Draft ICCVAM Test Method Recommendations

Panel Evaluation:

Dr. Woolhiser continued his presentation from the previous day of the draft responses to ICCVAM's questions to the Panel, for consideration by the entire Panel. The Panel discussion and their recommended revisions to each section of the draft ICCVAM BRD are reflected in the Panel report, published in May 2008.[20] Dr. Woolhiser indicated that the Evaluation Group had two main concerns with the LLNA: DA test method. The first concern related to pretreatment with 1% SLS and understanding how this impacted the biology of the response. Second, the time course of the study was different than the traditional LLNA because it extended the study by one day and included an additional challenge. This brought forth a question about the immunology of the response as it relates to the potential for elicitation and whether or not that is a significant change from the traditional LLNA, which is purely an induction model.

Public Comments:

Dr. George DeGeorge, MB Research Laboratories
In response to a question raised during the Panel discussion, Dr. DeGeorge commented that using lymph node weight as the readout to differentiate between sensitizers and non-sensitizers in the LLNA is problematic because although there are more lymph node cells packed into a node, each cell has less cytoplasm. The lymph nodes swell to a point, and then excrete water and become smaller lymphocytes that are countable. He cited examples from his laboratory with several different sensitizers, which demonstrate that lymphocytes in the node are smaller when a large SI (e.g., SI = 25) is obtained relative to when a smaller SI (e.g., SI = 3) is obtained.

Dr. DeGeorge also commented that he agreed with a point made during the Panel discussion that the LLNA: DA method and the LLNA: Bromodeoxyuridine Detected by ELISA (LLNA: BrdU-ELISA) method should be considered separately, because they are so dissimilar.

In his final comment, Dr. DeGeorge stated that in the traditional LLNA, in the LLNA: Bromodeoxyuridine Detected by Flow Cytometry (LLNA: BrdU-FC), and probably also in the LLNA: DA, strong sensitizing substances do not need to be administered three times. For instance, if one administers a single, moderately high dose of dinitrochlorobenzene (DNCB) (i.e., one that would induce an SI of 20 to 40) and then measures lymph node cell proliferation on day 1, 2, 3, or 4, an increase in the number of cells in the node and the number of cells that are positive for BrdU would likely be observed. Thus, administrations of additional applications have the potential to cause cumulative irritation. Dr. DeGeorge stated that the LLNA: DA method, which extends the assay to eight days instead of six days, should evaluate what happens to lymph node cell number at earlier sample times. In addition, if the animals receive just one application using a high dose, with or without the SLS, is there an increase in the SI? If so, that would lead to the possibility that the extra applications are not necessary and might lead to cumulative irritation.

Dr. David Basketter, ECVAM Observer
Dr. Basketter made a statement that from a clinical perspective, substances are typically described as

[20] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

significant sensitizers or not significant sensitizers, and within that latter group some of the substances may indeed be non-sensitizing. Thus, just because a substance has been shown in an isolated case report to be a human sensitizer does not mean that there is sufficient evidence to consider it as positive for comparison with outcomes of predictive assays. It has to be of sufficient importance (i.e., potency) to trigger a positive classification. Dr. Basketter mentioned SLS, methyl salicylate, and isopropanol, as substances which will always be positive in some human cases although they shouldn't be positive in a predictive assay.

Dr. Basketter also commented that caution should be given to making sensitization assumptions based on chemical class references. As an example, eugenol and isoeugenol are structurally similar and have similar physical properties, but they act by different chemical reaction mechanisms and could fit into distinctly different chemical classes.

Dr. Basketter's last comment acknowledged that much work has been done in terms of validating the traditional LLNA. If one makes minor changes to the LLNA in terms of a different readout for proliferation, then they benefit from all the experience generated in validating the traditional LLNA and less effort is needed to prove that the minor modification is valid. In contrast, if more significant modifications are made, one cannot rely on that same experience. Dr. Basketter cautioned that more importance should be placed on distinguishing whether something has changed substantially enough such that you can no longer rely on the traditional LLNA as a reference.

Dr. Masahiro Takeyoshi, Chemicals Evaluation and Research Institute

Dr. Takeyoshi made a short presentation about differences in LLNA sensitization responsiveness among different strains of mice. He mentioned that this was an important issue when evaluating the modified LLNA methods being developed in Japan. He showed differences in responsiveness among three different mouse strains commonly used in Japan (i.e., BALB/cAnN, CBA/JN, and CD-1) tested with parabenzoquinone in his group's non-radioactive LLNA (i.e., LLNA: BrdU-ELISA). The data indicated that the CBA/JN mouse strain exhibited a higher responsiveness, as indicated by an increased SI, to parabenzoquinone than the other two mouse strains tested. Based on these results, CBA/JN mice were chosen for testing substances in the LLNA: BrdU-ELISA test method.
Dr. Takeyoshi also indicated that based on evaluating different SI cutoffs in the LLNA: BrdU-ELISA, 2-mercaptobenzothiazole, 3-(4-isopropylphenyl)isobutyraldehyde, and hydroxycitronellal had low responsiveness (i.e., SI values). He noted that 2-mercaptobenzothiazole is an OECD TG 429 recommended positive control for the LLNA; however, repeat tests could not detect this substance as positive when using an SI value of 1.7 or more. Dr. Takeyoshi suggested that a substance-specific lower response might exist in the test system. Dr. Takeyoshi also summarized LLNA data by Dr. Ullmann and coworkers with the contract lab RCC, Ltd. in which they investigated the responsiveness of six different mouse strains (CBA/CaOlaHsd, CBA/Ca (CruBR), CBA/Jlbm (SPF), CBA/JNCrj, BALB/c and NMRI) to 25% 2-mercaptobenzothiazole. The data indicated that CBA/JNCrj mice showed markedly lower responsiveness compared to the other strains tested. These studies indicate that strain related differences would not be negligible with regard to measuring different endpoints of cellular proliferation in the LLNA because depending on the chemicals tested, responsiveness might be potentially impacted. For instance, some of the discordance seen in the LLNA: DA test method (e.g., 2-mercaptobenzothiazole) could be a strain specific effect.

Panel Conclusions and Recommendations:

Dr. Luster asked the Panel to review their conclusions and recommendations and discuss any revisions, if necessary. The Panel viewed the difference in treatment schedule between the LLNA: DA and the traditional LLNA to potentially be significant if the treatment schedule for the LLNA: DA corresponds to entering the elicitation phase of skin sensitization. The Panel was concerned that the 1% SLS pretreatment step in the LLNA: DA might modify the inherent sensitivity of the LLNA. They recommended that the test method developer (Daicel Chemical Industries, Ltd.) justify the use of 1% SLS or consider an alternative decision criterion (i.e., an SI threshold other than

three) such that the 1% SLS pretreatment is no longer necessary. Dr. Luster asked the Panel if they agreed with the recommendations and conclusions that the Panel made along with the revisions; unanimously, the Panel agreed. The Panel's detailed recommendations and conclusions on the LLNA: DA test method are included in their final Panel report.[21]

Method Description and Overview of the LLNA: BrdU-FC Test Method

Dr. George DeGeorge, MB Research Laboratories, presented an overview of the LLNA: BrdU-FC test method. He stated that mice are dosed topically on the ears once daily for three consecutive days (i.e., days 1, 2, and 3), just like the traditional LLNA protocol. On day 6, the mice receive an intraperitoneal injection with bromodeoxyuridine (BrdU), and five hours later, the auricular lymph nodes are removed. The lymph nodes from individual animals are processed and, using flow cytometry, the number of BrdU-positive cells are counted from treated animals and compared to control animals as a measure of lymph node cell proliferation.

Dr. DeGeorge described in detail how the cells are processed and gated for flow cytometric analysis. He mentioned that the cells are also permeabilized and treated with propidium iodide which allows gates to be drawn around the G_0, G_1, S, and G_2M phases of the cell cycle. Dr. DeGeorge projected specific examples of flow cytometry plots and histograms for DNCB, hexyl cinnamic aldehyde (HCA), and positive and negative control data.

Dr. DeGeorge also described the tiered protocol for the assessment of sensitization potential using the LLNA: BrdU-FC and how ear swelling measurements and additional immunophenotypic endpoints (i.e., the enhanced LLNA: BrdU-FC) aid in distinguishing skin irritants from an irritating sensitizer.

Overview of the Draft LLNA: BrdU-FC BRD and Draft ICCVAM Test Method Recommendations

Dr. Judy Strickland, Integrated Laboratory Systems, Inc. (the NICEATM support contractor), presented an overview of the draft ICCVAM BRD for the LLNA: BrdU-FC test method. She stated that the draft ICCVAM BRD provided a comprehensive review of the available data and information regarding the usefulness and limitations of the LLNA: BrdU-FC test method. Specifically, the test method was reviewed for its ability to distinguish between sensitizers and non-sensitizers compared with the traditional LLNA. The objective of the BRD was to describe the current validation status of the LLNA: BrdU-FC test method, including its relevance and reliability, scope of substances tested, and the availability of a standardized protocol.

Dr. Strickland indicated that MB Research Laboratories submitted data to NICEATM for the 48 substances analyzed in the BRD in response to an FR notice (72 FR 27815, May 17, 2007) that requested such data. Dr. Strickland briefly summarized the performance characteristics of the LLNA: BrdU-FC test method, which is detailed in the draft ICCVAM BRD,[22] and the draft ICCVAM test method recommendations for the LLNA: BrdU-FC test method.[23]

Panel Evaluation:

Dr. Raymond Pieters, on behalf of his Evaluation Group, presented the Evaluation Group's review of the draft BRD and the draft test method recommendations for the LLNA: BrdU-FC test method. Specifically, he presented the draft responses to ICCVAM's questions to the Panel for consideration by the entire Panel. This included their review of the draft BRD for errors and omissions, their overall assessment of the validation status of this test method, and their comments on the draft ICCVAM test

[21] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
[22] http://iccvam.niehs.nih.gov/methods/immunotox/fcLLNA/FC-LLNAbrd07Jan08FD.pdf
[23] http://iccvam.niehs.nih.gov/methods/immunotox/fcLLNA/FCLLNARecs07Jan08FD.pdf

method recommendations. The Panel discussion and their recommended revisions to each section of the draft ICCVAM BRD are reflected in the Panel report, published in May 2008.[24] The applicability of the draft ICCVAM-recommended LLNA performance standards to the LLNA: BrdU-FC test method was discussed, particularly with regard to the number of substances tested in the LLNA: BrdU-FC method and whether more data would be necessary for review before the validation status of the assay could be determined. Dr. Stokes reminded the Panel that the proposed LLNA performance standards didn't exist when the studies for the LLNA: BrdU-FC test method were performed. The questions should be whether the adequacy of the substances that have been tested is sufficient or if more studies need to be done to cover any gaps that might exist (e.g., range of potencies or activity, chemical classes).

Public Comments

Dr. David Basketter, ECVAM Observer

Dr. Basketter commented on the statement that Dr. DeGeorge made during his overview of the LLNA: BrdU-FC test method that HCA is irritating. He said that he is not convinced it is a significant irritant. Based on previous data, they had to use 50% HCA in a 48 hour occlusive application in the guinea pig in order to produce a mildly irritating response. Dr. Api added to Dr. Basketter's comment by stating that RIFM has also not found HCA to be an irritant when tested up to 20% in humans.

Dr. Basketter also commented that in the draft BRD for the LLNA: BrdU-FC, resorcinol was noted to be negative in the traditional LLNA and this is not correct. Dr. Basketter's group published results in 2007 in the journal Contact Dermatitis that resorcinol is clearly positive in the traditional LLNA when tested at higher concentrations and therefore this should be corrected for the record.

Dr. George DeGeorge, MB Research Laboratories

Dr. DeGeorge wanted to clarify that the LLNA: BrdU-FC test method was compared to the traditional LLNA to determine if the LLNA: BrdU-FC was more predictive of skin-sensitization potential. He stated that in some cases it was better while in others it wasn't, but overall, using human data as the gold standard reference, the LLNA: BrdU-FC exceeded the traditional LLNA predictivity values and accuracy. He also noted that the additional endpoints included in the LLNA: BrdU-FC allow for them to distinguish irritating substances that typically are considered false positives in the LLNA.

Dr. DeGeorge also noted that since the LLNA: BrdU-FC is so similar to the traditional LLNA the issue of refinement and reduction in animal use is not immediately apparent but if the assay is done in as few as four mice per group with a periodic positive control (e.g., every six months) this represents a significant decrease in animal numbers compared to guinea pig tests. Furthermore, there is a refinement since mice are phylogenetically lower than guinea pigs, and undergo less pain and distress during the assay than guinea pigs undergo.

With regard to the discussion of coefficients of variation (CVs) and the 0.5x to 2.0x EC3 (i.e., the estimated concentration needed to produce a stimulation index of three) range, Dr. DeGeorge suggested that a larger range might be more reasonable because the current range is likely too restrictive.

Dr. George also noted that ICCVAM requires interlaboratory validation if a test method is to be transferred to other laboratories. With regard to the LLNA: BrdU-FC, it is a "me-too" assay and only has "minor" changes from the traditional LLNA and is currently only used in one laboratory. Therefore, the current dataset should suffice for determining the validity of the LLNA: BrdU-FC. In response to Dr. DeGeorge's comment, Dr. Stokes stated that if a method is only proposed to be used by one laboratory, having only intralaboratory data certainly would suffice but if it was proposed for broader use (e.g., adopted or endorsed by regulatory authorities), then other laboratories would have to demonstrate

[24] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

interlaboratory reproducibility. Dr. Luster asked if there was any mechanism available so that a company or small laboratory could apply for funding to help support an interlaboratory validation. Dr. Stokes indicated that they could nominate the test method for additional validation studies to ICCVAM. It would go through a nomination review process and a prioritization would be given to that. The nomination would then be considered by the member agencies as to whether funding would be provided.

Panel Conclusions and Recommendations:

Dr. Luster asked the Panel to review their conclusions and recommendations and discuss any revisions, if necessary. The Panel suggested that the utility of ear swelling or other methods to detect inflammation appeared warranted for inclusion in every variation of the LLNA (including the traditional LLNA), but should be further investigated before routine inclusion in the protocol is recommended. The Panel further agreed that the draft ICCVAM test method recommendations for future studies highlighted the unanswered questions raised by the available data set. Specifically, conducting interlaboratory studies as a part of the validation process is important.

The Panel considered the immunological markers suggested for the LLNA: BrdU-FC to be appropriate, but noted that other immunological markers for discrimination of irritant versus sensitization phenomena were also available. In general, for any future work, efforts should be made to decrease the variability and to thereby increase the power of the test in order to ensure that more animals were not needed relative to the traditional LLNA or other modified LLNA protocols.

Dr. Luster asked the Panel to indicate if they agreed with the recommendations and conclusions that the Panel made along with the revisions; the Panel unanimously agreed. The Panel's detailed recommendations and conclusions on the LLNA: BrdU-FC test method are included in their final Panel report.[25]

Method Description and Overview of the LLNA: BrdU-ELISA Test Method

Dr. Masahiro Takeyoshi, Chemicals Evaluation and Research Institute, presented an overview of the LLNA: BrdU-ELISA test method. He stated that the LLNA: BrdU-ELISA test method is very similar to the traditional LLNA test method. Unique to the LLNA: BrdU-ELISA test method, after test substance applications on days 1, 2, and 3, BrdU is injected interperitoneally on day 5. Approximately 24 hours after the BrdU injection, lymph nodes are collected, and detection of the amount of BrdU incorporated into the DNA of lymph node cells is conducted with an ELISA.

In the development process of this method, experiments were conducted to detect the most efficient injection schedule of BrdU. Based on the various injection schedules tested, a single injection protocol on day four was identified as the optimal injection schedule for BrdU administration.

Dr. Takeyoshi then showed a video of laboratory personnel preparing the lymph node cells for BrdU detection by ELISA. He went on to describe data for the LLNA: BrdU-ELISA compared to the traditional LLNA and how performance could be improved using alternative decision criteria (i.e., an SI other than three as the threshold for a positive response).

Overview of the Draft LLNA: BrdU-ELISA BRD and Draft ICCVAM Test Method Recommendations

Dr. Salicru presented an overview of the draft ICCVAM BRD for the LLNA: BrdU-ELISA test method. She noted that the draft ICCVAM BRD provided a comprehensive review of the available data and information regarding the usefulness and limitations of the LLNA: BrdU-ELISA test method. Specifically, the test method was reviewed for its ability to distinguish between sensitizers

[25] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

and non-sensitizers compared with the traditional LLNA and guinea pig test methods. The objective of the BRD was to describe the current validation status of the LLNA: BrdU-ELISA test method, including its relevance and reliability, scope of substances tested, and the availability of a standardized protocol.

Dr. Salicru stated that data from a total of 29 substances were considered in the accuracy analysis for the LLNA: BrdU-ELISA, and they were all tested in one laboratory. Dr. Salicru briefly summarized the performance characteristics of the LLNA: BrdU-ELISA test method, which are detailed in the draft ICCVAM BRD,[26] and the draft ICCVAM test method recommendations for the LLNA: BrdU-ELISA test method.[27]

Panel Evaluation:

Ms. Kim Headrick presented her Evaluation Group's (Drs. Anne Marie Api, Howard Maibach, Peter Theran, and Stephen Ullrich) review of the draft BRD and draft ICCVAM test method recommendations for the LLNA: BrdU-ELISA test method. Specifically, she presented the draft responses to ICCVAM's questions to the Panel for consideration by the entire Panel. This included their review of the draft BRD for errors and omissions, their overall assessment of the validation status of the test method, and their comments on the draft ICCVAM test method recommendations. The Panel discussion and their recommended revisions to each section of the draft ICCVAM BRD are reflected in the Panel report, published in May 2008.[28]

Public Comments:

Dr. David Basketter, ECVAM Observer
Dr. Basketter noted that when the traditional LLNA was first suggested as an alternative to the guinea pig tests, it went through a comprehensive validation process, and one of the concerns was that it should perform reliably and distinctly better than the guinea pig assays. He emphasized that this point should be kept in mind when thinking about the modified LLNA protocols with alternative endpoints that are currently being reviewed. He stated that the current rigor of examination for the modified LLNA protocols being reviewed for validation is higher than that for the traditional LLNA. He speculated that in the not-too-distant future, *in vitro* alternatives are likely to be going through a similar review process and it is going to become ever more difficult to put these alternatives in place, not because there is ill-will against the selections but because of the high standard of being good scientists. Thus, it is important that pragmatic decisions are made using the tools that are available.

Dr. George DeGeorge, MB Research Laboratories
Dr. DeGeorge commented that he agreed with Dr. Basketter's statements. He said that based on his experience in this peer review process, it is unlikely that he would bring any of the three *in vitro* test methods that MB Research Laboratories is developing for consideration by ICCVAM, given the many high hurdles that have to be negotiated.

In response to the comments by Drs. Basketter and DeGeorge, Dr. McDougal commented that it does not seem unreasonable to raise the bar for what is expected of new or modified tests. Dr. Luster added that understandably, the focus on animal refinement and reduction is paramount, but that as scientists we have to ensure that the bar is maintained sufficiently high so that as the years go by scientific quality is not compromised.

Panel Conclusions and Recommendations:

Dr. Luster asked the Panel to review their conclusions and recommendations and discuss any revisions, if necessary. The Panel concluded that the available data and test method performance for

[26] http://iccvam.niehs.nih.gov/methods/immunotox/llna-ELISA/BrdUELISAbrd07Jan08.pdf
[27] http://iccvam.niehs.nih.gov/methods/immunotox/llna-ELISA/BrdUELISARecs07Jan08FD.pdf
[28] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

the LLNA: BrdU-ELISA support the draft ICCVAM test method recommendations that it may be useful for identifying substances as potential skin sensitizers and non-sensitizers, but that more information and existing data must be made available before the LLNA: BrdU-ELISA can be recommended for use. The Panel also stated that a detailed protocol was needed, in addition to sufficient quantitative data for broader analysis on a larger set of balanced reference substances that take into account physicochemical properties and sensitization potency, as well as an appropriate evaluation of interlaboratory reproducibility.

The Panel's main concern with this test method was that the accuracy of the LLNA: BrdU-ELISA at SI ≥ 3 was inadequate and not equivalent to the traditional LLNA. Furthermore, although using a decision criterion of SI ≥ 1.3 improved the test's performance in identifying sensitizers from non-sensitizers, it did not resolve concerns about the test method, particularly considering that power calculations suggest a much larger number of animals per group would be required to identify a positive response. Thus, the Panel also concluded that it might be more appropriate to use a statistically based decision criterion rather than a stimulation index to classify substances as sensitizers, and that this should be further investigated. Dr. Luster asked the Panel to indicate if they agreed with the recommendations and conclusions that the Panel made along with the revisions; unanimously, the Panel agreed. The Panel's detailed recommendations and conclusions on the LLNA: BrdU-ELISA test method are included in their final Panel report.[29]

Overview of the Draft ICCVAM Performance Standards for the LLNA

Dr. Allen presented an overview of the draft ICCVAM Performance Standards for the LLNA. He briefly summarized the overall purpose of performance standards (i.e., to provide a basis for evaluating the performance of a proposed test method that is mechanistically and functionally similar to the validated test method) and the three elements encompassed within such performance standards (i.e., essential test method components, a minimum list of reference substances, and accuracy/reliability values). He noted that the proposed applicability of these draft ICCVAM LLNA performance standards is for the evaluation of LLNA protocols that deviate from the ICCVAM-recommended LLNA protocol only with respect to the method for assessing lymphocyte proliferation (e.g., using non-radioactive instead of radioactive reagents). Dr. Allen then provided an overview of the essential test method components, the minimum list of reference substances, and the accuracy/reliability values as detailed in the draft ICCVAM LLNA Performance Standards.[30]

Panel Evaluation:

Dr. Woolhiser, on behalf of his Evaluation Group, presented the Evaluation Group's responses to the ICCVAM questions asked about the draft ICCVAM LLNA Performance Standards for the entire Panel to consider. The overall question for the Panel was whether these performance standards were considered adequate for assessing the accuracy and reliability of test method protocols that were based on similar scientific principles and that measured the same biological effect as the traditional LLNA. The Panel discussion and their recommended revisions to the draft ICCVAM LLNA Performance Standards are reflected in the Panel report published in May 2008.[31]

Adjournment—

The meeting was adjourned at 5:42 p.m., to reconvene at 8:30 a.m., Thursday, March 6, 2008.

[29] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
[30] http://iccvam.niehs.nih.gov/methods/immunotox/PerfStds/LLNAPerfStd07Jan08FD.pdf
[31] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

THURSDAY, MARCH 6, 2008

Reconvening of the Panel Meeting

Dr. Luster reconvened the Panel Meeting at 8:30 a.m. He introduced himself and then asked that all Panel members and all others in attendance introduce themselves as well.

Overview of the Draft ICCVAM LLNA Performance Standards

Panel Evaluation:

Dr. Woolhiser reviewed some of the important points highlighted during the previous day's discussion on this topic, and then continued to summarize the remaining comments of his Evaluation Group on the questions asked by ICCVAM on the draft ICCVAM LLNA Performance Standards for consideration by the entire Panel. As mentioned above, the Panel discussion and their recommended revisions to the draft ICCVAM LLNA Performance Standards are reflected in the Panel report published in May 2008.[32]

Dr. Woolhiser noted that there were general comments on the topic order for the Panel's review. He asked if Dr. Stokes would comment on the rationale for the topic order. Dr. Stokes indicated that as the IWG deliberated the order of topics for this review, consideration was given to the fact that the three non-radioactive methods had undergone validation studies prior to the creation of LLNA performance standards. Thus, the non-radioactive test methods were reviewed before the performance standards, so as to not bias the Panel's assessment of each test method's performance. The performance standards could then be considered for their application to future test methods.

Public Comments:

Dr. Amy Rispin, EPA

Dr. Rispin stated that her intent was to provide some additional regulatory perspective on some of the points that have been discussed. When Federal agencies evaluate the validation status of a test method under ICCVAM, they conduct a comprehensive analysis of overall performance (i.e., accuracy and reliability) in the context of making regulatory decisions with data from the test method. Thus, in a regulatory situation, equal or greater accuracy compared to the reference test method is the expectation. If the number of animals can be decreased only at the expense of accuracy, the acceptability of such a test method for the particular regulatory purpose would need to be carefully considered. Certain methods, instead of being complete replacements, might have to be relegated to the role of screens, where positives would be accepted, but negatives would require further testing - a less than ideal situation.

Dr. Rispin commented that performance standards are the regulating agencies' basis for the acceptability of variations of accepted test methods. If an agency receives data from a modified LLNA method that has not been reviewed and validated in the ICCVAM process, there is unlikely to be a comprehensive peer review of it within the agency, given resource limitations. Therefore, the question of major versus minor departures from the functional criteria is important to ICCVAM and its member agencies. One cannot anticipate that there will be anything other than these performance standards to adequately evaluate the usefulness and limitations of a new method.

Dr. David Basketter, ECVAM Observer

Dr. Basketter first commented on a point that Dr. Thomas Gebel alluded to during the Panel's discussion of the draft ICCVAM LLNA Performance Standards, which was that if a new laboratory performed the traditional LLNA to assess 18 or 22 chemicals, they probably wouldn't get a complete match. Dr. Basketter disagreed with Dr. Gebel's statement and viewed that a competent laboratory performing the LLNA would get it 100% correct.

[32] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

Dr. Basketter then provided some comments that he stated were "from the ECVAM perspective." He stated that the ECVAM performance standards tried to address adhering to a standard protocol and that any change to the protocol other than the method for evaluating lymph node proliferation (e.g., strain, species, number of applications, time) was considered not to be minor, and therefore such a protocol would not be applied to these performance standards. By restricting the performance standards to minor changes, ECVAM was trying to minimize the number of chemicals required to evaluate sensitivity. Furthermore, the EC3 value could be used to see if the test method could classify substances in the appropriate range of sensitization potency.

ECVAM initially chose their reference substances in order to determine whether a modified method (differing only in the method for measuring cell proliferation) would give the same answer as the traditional LLNA. Thus, there was no intent to compare to the guinea pig or human data.

Dr. Basketter speculated that it is doubtful that data from multiple LLNA studies on the same substance are available and therefore it is unlikely that much larger sample sizes from which to calculate mean EC3 values and associated ranges will be obtained.

Dr. Basketter concluded by stating that ECVAM will not include more false positives and false negatives in its list. It has included one false positive and false negative in order to harmonize with ICCVAM but they don't see an added statistical value of just having one more false positive and false negative.

Karen Hamernik, EPA

Dr. Hamernik concurred with the comments that Dr. Rispin made previously, that performance standards, if developed such that they are too generalized with respect to minor versus major changes, would be problematic for regulatory agencies when they are reviewing submissions that include data from a modified LLNA protocol. Dr. Hamernik also asked for clarification from the Panel on a statement made during their discussions that a test for concordance for measuring the accuracy of classification (i.e., yes/no answer) should be done and that a chemical-for-chemical match is not necessary. Dr. Flournoy responded that concordance is not absolute but a continuum. Dr. Luster further clarified that the Panel discussion was based on the fact that the traditional LLNA is not a perfect match when compared to the guinea pig tests. Because there are false negatives and false positives compared to the guinea pig, there should be some flexibility so that an absolute chemical-by-chemical match is not required. In addition, a scientifically valid explanation can be provided for any discordance. Dr. Stokes emphasized that this was an important point and that additional clarity on the differences between a chemical-by-chemical match and overall accuracy need to be carefully considered before the final test method accuracy requirements are defined.

Panel Conclusions and Recommendations:

Dr. Luster asked the Panel to review the conclusions and recommendations for the ICCVAM LLNA performance standards they had discussed earlier and to make any revisions, if necessary. The Panel indicated that modified LLNA protocols that are undergoing validation should contain essential test method components that follow the ICCVAM-recommended protocol,[33] unless adequate scientific rationale for deviating from this protocol was provided. The Panel also identified aspects of the LLNA that should be required as part of the test method validation process, if more extensive changes to the protocol are being considered: (1) application of the test substance to the skin with sampling of the lymph nodes draining that site, (2) measurement of cell proliferation in the draining lymph node, (3) absence of a skin reaction that could be indicative of the onset of the elicitation phase of skin sensitization, (4) data collected at the level of the individual animal to allow for an estimate of the

[33] http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/LLNAProt.pdf

variance within control and treatment groups,[34] and (5) if dose response information is needed, there are an adequate number of dose groups ($n \geq 3$) with which to accurately characterize the dose response for a given test substance.

The Panel also recommended that statistical tests to analyze the data might allow for a more accurate interpretation. They recommended that a suitable variance-stabilizing transformation (e.g., log transformation, square root transformation) be applied in all statistical analyses and in reporting summary standard deviations. The Panel also recommended that a more rigorous evaluation be conducted of what would be considered an appropriate range of ECt values (i.e., estimated concentration needed to produce a stimulation index that is indicative of a positive response) to include as a requirement. This would be a statistical evaluation that considers the variability of ECt values generated among the sensitizers included on the performance standards reference substances list and the statistical multiple comparisons problem.

Dr. Luster asked the Panel if they agreed with the changes and revisions made at this point and with the Panel conclusions and recommendations as presented and revised. The members of the Panel agreed with one abstention; Dr. McDougal abstained from voting stating that he still had a concern about what constitutes a "major/minor" change. The Panel's detailed recommendations and conclusions on the ICCVAM LLNA performance standards are included in their final Panel report.[35]

Overview of the Draft LLNA Potency Determinations BRD and Draft ICCVAM Test Method Recommendations

Dr. Strickland presented an overview of the draft ICCVAM BRD for the use of the LLNA to determine skin-sensitization potency. She mentioned that the draft ICCVAM BRD provided a comprehensive review of the available data and information regarding the usefulness and limitations of the LLNA as a stand-alone assay for hazard categorization of skin-sensitization potency. In the BRD, the LLNA was evaluated for its ability to categorize substances for skin-sensitization potency using EC3 values.

Dr. Strickland noted that the analyses conducted in the BRD were based on LLNA studies obtained from ICCVAM (1999), the published literature, and data received in response to an FR notice (72 FR 27815, May 17, 2007) requesting original data from the LLNA. As a result, the analyzed data included 170 substances with LLNA, human, and/or guinea pig data. Dr. Strickland noted that three sets of data were analyzed and briefly summarized the results which are detailed in the draft ICCVAM BRD.[36] Dr. Strickland also briefly summarized the draft ICCVAM test method recommendations for potency determinations.[37]

Panel Evaluation:

Ms. Headrick presented her Evaluation Group's draft responses to ICCVAM's questions to the Panel for consideration by the entire Panel. These included their review of the draft BRD for errors and omissions, their overall assessment of the validation status of the test method, and their comments on the draft ICCVAM test method recommendations. The Panel discussion and their recommended revisions to each section of the draft ICCVAM BRD and recommendations are reflected in the Panel report published in May 2008.[38]

During the course of the discussion on the potency applicability of the LLNA, Dr. Woolhiser asked what the basis for the human threshold concentration cutoff values of 250 and 500 $\mu g/cm^2$ were. Dr.

[34] Individual animal data will allow the application of a formal statistical test, if deemed necessary, and will also allow power calculations associated with the modified LLNA test.
[35] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
[36] http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-pot/LLNApotency18Jan08FD.pdf
[37] http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-pot/LLNAPotencyRecs18Jan08FD.pdf
[38] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

Wind replied that a number of experts and clinicians from throughout the world went back and looked at what, in their countries, they demarcated as strong sensitizers. The proposed Globally Harmonized System of Classification and Labeling of Chemicals (GHS) subcategory guidance values for the LLNA, guinea pig tests (GPMT, BT) and human data (HMT and HRIPT) were made on the basis of an impact analysis of 175 chemicals. In addition, the two proposed cut-offs were evaluated by the GHS Expert Group on Sensitization based upon chemicals already regulated as strong sensitizers to ensure their inclusion within the GHS categorization scheme. Clinical members of the Expert Group also confirmed relevance of the cut-off values such that clinically important skin sensitizers fell into the appropriate subcategory. The proposed guidance values were also in line with the European Commission's Expert Working Group recommendations.

Public Comments:

Dr. David Basketter, ECVAM Observer
Dr. Basketter commented that reviewing the potency data by splitting it into pooled and unpooled groups could be interesting but might be difficult since the majority of available data likely comes from pooled groups. Furthermore, much of the deliberation concluding that individual animal data must be used was derived from analyses based only or largely on pooled data from four animals.

Dr. Basketter further stated that he viewed the analyses, which make the assumption that the human threshold data is the gold standard, as fundamentally flawed. Human data comes from studies conducted at different times, with different protocols, according to varying quality standards, and by different people. Therefore, there is no definitive knowledge of the reproducibility of the data. However, he considers the analyses adequate for recommending the LLNA as a part of a weight-of-evidence decision on human sensitization potency categorizations.

Dr. Amy Rispin, EPA
Dr. Rispin noted that there has been much discussion about various ways of handling the potency data. The OECD expert task force on skin sensitization needs to see an analytical comparison of what is considered to be the most appropriate approach for evaluating the data. The question for categorization purposes is, *What is the ideal testing modality for separating strong versus weak sensitizers for potency categorization*? A regulator who must assign a categorization is going to be confronted with all available test data and must know which data should be given the greatest weight in their evaluation.

Dr. Rispin noted that the OECD task force also reviewed the draft BRD on potency determinations and sent a list of several questions to the Panel, some of which have been answered, many of which have not been. One of the questions is, can the LLNA protocols be refined (e.g., by selection of solvents or choice of other test parameters) to improve correlation? She concluded by noting that she hopes that the additional analyses that the Panel has suggested will bring some clarity to the matter.

Panel Conclusions and Recommendations:

Dr. Luster asked the Panel to review the conclusions and recommendations for the LLNA potency determinations they had discussed earlier and to make any revisions, if necessary. The Panel agreed with the draft ICCVAM recommendation that the LLNA should not be used as a stand-alone assay for categorizing skin sensitizers as strong versus weak, but that it could be used as part of a weight-of-evidence evaluation (e.g., along with quantitative structure-activity relationships, peptide reactivity, human evidence, historical data from other experimental animal studies) for this purpose. The Panel also agreed with ICCVAM's recommendation that any LLNA studies conducted for the purpose of evaluating skin-sensitization potency should use the ICCVAM-recommended LLNA protocol. In addition, the Panel stated that the relevant testing guidelines for the traditional LLNA should be revised to include the procedure for calculating an EC3 value. Dr. Luster asked the Panel if they agreed with the changes and revisions made at this point and with the Panel conclusions and recommendations as presented and revised; the Panel unanimously agreed. The Panel's detailed

recommendations and conclusions on the LLNA potency determinations are included in their final Panel report.[39]

Concluding Remarks—

Dr. Luster, on behalf of the Panel, thanked the NICEATM-ICCVAM staff for their continued assistance during the review process and the Panel meeting. He also thanked Drs. Joanna Matheson and Abby Jacobs, the IWG co-chairs, and Dr. Marilyn Wind, ICCVAM Chair and IWG member, for the hard work they put into the project. Dr. Luster also thanked the Panel and the Panel Chairs for their involvement in the huge task of reviewing seven topics. He commented that, for future reference for ICCVAM, the Panel in their individual groups were able to do a good job in reviewing the materials, but because they were so focused on their particular topics due to serious time constraints, there may not have been the full benefit of their expertise for other topics in all cases.
Drs. Wind and Stokes thanked the Panel again for their hard work, thoughtful and objective deliberations, and advice. Dr. Stokes further thanked the invited test method developers for their excellent summaries of their method for the benefit of the Panel, and CPSC for hosting the Panel meeting. He mentioned that there has been discussion about obtaining additional existing data (i.e., on mixtures, on one or more of the non-radiolabeled test methods), and that should these data become available in a timely manner and if NICEATM is able to assimilate and analyze the data, the Panel might be reconvened by teleconference to review the data. Dr. Stokes concluded by saying he looked forward to further working with the Panel members to complete their Panel report.

Adjournment—

The meeting was adjourned and concluded at 3:20 p.m.

[39] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

William S. Stokes, D.V.M.
NIEHS
P.O. Box 12233
MD-EC17
Research Triangle Park, NC 27709

Dear Dr. Stokes,

The Meeting Summary Minutes, Independent Scientific Peer Review Panel Meeting, Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products, accurately summarizes the Peer Review Panel meeting of March 4-6, 2008, in Bethesda, MD.

Sincerely,

Signature	Printed Name	Date
	MICHAEL I LUSTER	11-18-08

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Appendix D2

Peer Review Panel Report: Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products

This document is available at:
https://ntp.niehs.nih.gov/iccvam/docs/immunotox_docs/llnaprprept2008.pdf

The document is also available on request from NICEATM:

NICEATM
National Institute of Environmental Health Sciences
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Appendix D3

Summary Minutes of Independent Scientific Peer Review Panel Meeting on April 28-29, 2009

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Summary Minutes

Independent Scientific Peer Review Panel Meeting

Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Evaluation of the Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA)

William H. Natcher Conference Center
National Institutes of Health
Bethesda, MD
April 28 - 29, 2009
8:30 a.m. - 5:30 p.m.

Peer Review Panel Members:

Michael Luster, Ph.D. (Peer Review Panel Chair)	Senior Consultant to the NIOSH Health Effects Laboratory, Morgantown, WV
Nathalie Alépée, Ph.D.	Scientific Coordinator on Alternatives Methods in Life Science, L'Oréal Research and Development, Aulnay sous Bois, France
Anne Marie Api, Ph.D.	Vice President, Human Health Sciences, Research Institute for Fragrance Materials, Woodcliff Lake, NJ
Nancy Flournoy, M.S., Ph.D.	Professor and Chair, Dept. of Mathematics and Statistics, University of Missouri – Columbia, Columbia, MO
Dagmar Jírová, M.D., Ph.D.	Toxicologist, Research Manager, Head of Reference Center for Cosmetics, Head of Reference Laboratory for Experimental Immunotoxicology, National Institute of Public Health, Czech Republic
David Lovell, Ph.D.	Reader in Medical Statistics, Postgraduate Medical School, University of Surrey, Guildford, Surrey, U.K.
Howard Maibach, M.D.	Professor, Dept. of Dermatology, University of California – San Francisco, San Francisco, CA
Michael Olson, Ph.D.	Director of Occupational Toxicology, Corporate Environment Health and Safety, GlaxoSmithKline, Research Triangle Park, NC

Peer Review Panel Members:

Raymond Pieters, Ph.D.[1]	Associate Professor, Immunotoxicology Group Leader, Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands
Jean Regal, Ph.D.	Professor, Dept. of Pharmacology, University of Minnesota Medical School, Duluth, MN
Jonathan Richmond, MB ChB, FRCSEd	Head, Animals Scientific Procedures Division, Home Office, London, U.K.
Peter Theran, V.M.D.	Massachusetts Society for the Prevention of Cruelty to Animals, Novato, CA
Stephen Ullrich, Ph.D.	Dallas/Ft. Worth Living Legends Professor and Professor of Immunology, Postgraduate School of Biomedical Science, University of Texas M.D. Anderson Cancer Center, Houston, TX
Michael Woolhiser, Ph.D.	Science and Technology Leader – Toxicology and Environmental Research and Consulting, The Dow Chemical Company, Midland, MI
Takahiko Yoshida, M.D., Ph.D.	Professor, Dept. of Health Science, Asahikawa Medical College, Hokkaido, Japan

ICCVAM and ICCVAM Immunotoxicity Working Group Members:

Paul Brown, Ph.D.	FDA, Center for Drug Evaluation and Research, Silver Spring, MD
Masih Hashim, Ph.D.	EPA, Office of Pesticide Programs, Washington, DC
Ying Huang, Ph.D.	FDA, Center for Biologics Evaluation and Research, Silver Spring, MD
Abigail Jacobs, Ph.D. (IWG Co-Chair)	FDA, Center for Drug Evaluation and Research, Silver Spring, MD
Jodie Kulpa-Eddy, D.V.M.	USDA, Animal and Plant Health Inspection Service, Riverdale, MD
Elizabeth Margosches, Ph.D.	EPA, Office of Pollution Prevention and Toxics, Washington, DC
Joanna Matheson, Ph.D. (IWG Co-Chair)	CPSC, Bethesda, MD

[1] Dr. Pieters was unable to attend the public meeting on April 28-29, 2009. However, he was involved in the review of the revised draft background review documents and the revised draft LLNA applicability domain Addendum.

ICCVAM and ICCVAM Immunotoxicity Working Group Members:

Deborah McCall	EPA, Office of Pesticide Programs, Washington, DC
Tim McMahon, Ph.D.	EPA, Office of Pesticide Programs, Washington, DC
John Redden, M.S.	EPA, Office of Pesticide Programs, Washington, DC
R. Adm. William Stokes, D.V.M., DACLAM	NIEHS, Research Triangle Park, NC
Ron Ward, Ph.D.	EPA, Office of Pollution Prevention and Toxics, Washington, DC
Marilyn Wind, Ph.D. (ICCVAM Chair)	CPSC, Bethesda, MD

Invited Experts:

George DeGeorge, Ph.D., DABT	MB Research Labs, Spinnerstown, PA
Kenji Idehara, Ph.D.	Daicel Chemical Industries, Ltd., Hyogo, Japan
Masahiro Takeyoshi, Ph.D.	Chemicals Evaluation and Research Institute, Saitama, Japan

JaCVAM Observer:

Hajime Kojima, Ph.D.	National Institute of Health Sciences, Tokyo, Japan

Public Attendees:

Joan Chapdelaine, Ph.D.	Calvert Laboratories, Inc., Olyphant, PA
Merrill Tisdel	Syngenta Crop Protection Inc., Greensboro, NC
Gary Wnorowski, M.B.A, L.A.T.	Eurofins Product Safety Labs

NICEATM:

R. Adm. William Stokes, D.V.M., DACLAM	Director
Debbie McCarley	Special Assistant to the Director

Contract Support Staff – Integrated Laboratory Systems, Inc. (ILS)

David Allen, Ph.D.	Eleni Salicru, Ph.D.
Thomas Burns, M.S.	Frank Stack

NICEATM:

Linda Litchfield

Judy Strickland, Ph.D., DABT

Greg Moyer, M.B.A.

Abbreviations:

CPSC = U.S. Consumer Product Safety Commission

EPA = U.S. Environmental Protection Agency

FDA = U.S. Food and Drug Administration

ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods

ILS = Integrated Laboratory Systems

IWG = Immunotoxicity Working Group

NICEATM = National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

NIEHS = National Institute of Environmental Health Sciences

NIOSH = National Institute of Occupational Safety and Health

USDA = U.S. Department of Agriculture

Tuesday, April 28, 2009
Call to Order and Introductions

Dr. Michael Luster (Peer Review Panel Chair) called the meeting to order at 8:30 a.m. and introduced himself. He then asked all Peer Review Panel (hereafter Panel) members to introduce themselves and to state their name and affiliation for the record. He then asked all the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) staff, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) members, the ICCVAM Immunotoxicity Working Group (IWG) members, and members of the public to also introduce themselves. Dr. Luster stated that there would be opportunity for public comments during each of the four murine local lymph node assay (LLNA)-related topics. He asked that all those interested in making a comment register at the registration table and provide a written copy of their comments, if available, to NICEATM staff. Dr. Luster emphasized that the comments would be limited to seven minutes per individual and that, while comments from one individual would be welcomed during each commenting period, repeating the same comments at each comment period would be inappropriate.

Welcome from the ICCVAM Chair

Dr. Marilyn Wind, U.S. Consumer Product Safety Commission (CPSC) and Chair of ICCVAM, welcomed everyone to the National Institutes of Health and to the Panel meeting. Dr. Wind thanked the ICCVAM IWG and NICEATM staff for their efforts in preparing the draft documents being reviewed and for arranging the logistics of the meeting. Dr. Wind thanked the Panel members for dedicating their time, effort, and expertise to this review and acknowledged their important role to the ICCVAM test method evaluation process. Dr. Wind also emphasized the important role of the public and their comments in this process.

Welcome from the Director of NICEATM, and Conflict of Interest Statements

Dr. William Stokes, Director of NICEATM, stated the Panel meeting was being convened as an NIH Special Emphasis Panel and was being held in accordance with applicable U.S. Federal Advisory Committee Act regulations. As such, Dr. Stokes indicated that he would be serving as the Designated Federal Official for this public meeting. He reminded the Panel that they signed a conflict of interest (COI) statement during the Panel selection process, in which they identified any potential real or perceived COI. He read the COI statement and then Dr. Luster asked that panelists again declare any potential direct or indirect COI and to recuse themselves from discussion and voting on any aspect of the meeting where there might be a conflict.

Dr. Michael Woolhiser declared a COI regarding the Panel's review of the LLNA Applicability Domain, because The Dow Chemical Company, Dr. Woolhiser's employer, submitted much of the data that were being considered. He indicated that he would recuse himself from the Panel's evaluation of the applicability domain, but would remain available to answer any questions that the Panel might have about the test substances or the data.

Overview of the ICCVAM Test Method Evaluation Process

Dr. Stokes began by thanking the 15 Panel scientists from six different countries (Czech Republic, France, Japan, The Netherlands, United Kingdom, and the United States) for their significant commitment of time and effort preparing for and attending the meeting. He explained that the purpose of the Panel was to conduct an independent scientific peer review of the information provided on a series of proposed new versions of the LLNA and proposed expanded applications of the assay. The Panel is then asked to comment on the extent that the available information supports the draft ICCVAM recommendations. Dr. Stokes indicated that the original LLNA peer review panel in 1998 considered the LLNA a valid substitute for the guinea pig-based test in most but not all testing

situations. He noted that three Panel members from the 1998 review are also on the current Panel (i.e., Drs. Howard Maibach, Jean Regal, and Stephen Ullrich). Dr. Stokes also reviewed the nomination that was received from CPSC in January 2007,[2] which provides the basis for the current evaluation.

Dr. Stokes then identified the 15 Federal agencies that comprise ICCVAM and summarized ICCVAM's mission. He noted that ICCVAM, as an interagency committee, does not carry out research and development or validation studies. Instead, ICCVAM, in conjunction with NICEATM, carries out the critical scientific evaluation of the results of validation studies for proposed test methods to assess their usefulness and limitations for regulatory testing, and then makes formal recommendations to ICCVAM agencies.

Dr. Stokes provided a brief review of ICCVAM's history and summarized the ICCVAM Authorization Act of 2000,[3] including the purpose and duties of ICCVAM. He noted that one of ICCVAM's primary duties is to review and evaluate new, revised, and alternative test methods applicable to regulatory testing. He stated that all of the reports produced by NICEATM are available on the NICEATM-ICCVAM website or can be obtained upon request from NICEATM. He also mentioned that ICCVAM provides guidance on test method development, validation processes, and helps to facilitate not only the acceptance of scientifically valid alternative test methods, but also encourages internationally harmonized recommendations on the usefulness and limitations of alternative test methods.

Dr. Stokes then described the ICCVAM test method evaluation process, which begins with a test method nomination or submission. NICEATM conducts a prescreen evaluation to summarize the extent to which the proposed submission or nomination addresses the ICCVAM prioritization criteria. A report of this evaluation is then provided to ICCVAM, which in turn develops recommendations regarding the priority for evaluation. ICCVAM then seeks input on their recommendations from the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) and the public and determines whether the test method should move forward into a formal evaluation. If so, a draft background review document (BRD), which provides a comprehensive review of all available data and information, is prepared by NICEATM in conjunction with an ICCVAM working group designated for the relevant toxicity testing area (e.g., the IWG). In addition, ICCVAM considers all available information and develops draft test method recommendations on the proposed usefulness and limitations of the test methods, test method protocol, performance standards, and future optimization/validation studies. The draft BRD and the draft ICCVAM test method recommendations are made available to the Panel and the public for review and comment. The Panel peer reviews the draft BRD and evaluates the extent to which it supports the draft ICCVAM test method recommendations. A Panel report is published, which is then considered along with public and SACATM comments by ICCVAM in developing final recommendations. These final recommendations are forwarded to the ICCVAM member agencies for their consideration and possible incorporation into relevant testing guidelines. Agencies have 180 days to respond to the ICCVAM recommendations.

Dr. Stokes reviewed the ICCVAM criteria for adequate validation. He stated that validation is defined by ICCVAM as the process by which the reliability and relevance of a procedure are established for a specific purpose, and that adequate validation is a prerequisite for consideration of a test method by U.S. Federal regulatory agencies. Dr. Stokes listed the ICCVAM acceptance criteria for test method validation and acceptance. He concluded by summarizing the timeline of the review activities beginning with CPSC's nomination in January 2007 and ending with the present Panel meeting.

[2] http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf
[3] http://iccvam.niehs.nih.gov/docs/about_docs/PL106545.pdf

ICCVAM Charges to the Panel

Dr. Stokes reviewed the charges to the Panel: (1) review the draft BRDs and the draft Addendum to the traditional[4] LLNA for completeness and identify any errors or omissions; (2) determine the extent to which each of the applicable criteria for validation and regulatory acceptance had been appropriately addressed for the proposed revised or modified versions of the LLNA; and (3) comment on the extent to which the ICCVAM draft test method recommendations including the proposed usefulness and limitations, standardized test method protocols, performance standards, and additional studies are supported by the information provided in the draft BRDs and draft Addendum.

Overview of the Agenda

Dr. Luster then reviewed the agenda and the order of presentations. He stated that for each review topic, the test method developer would present an overview of the test method protocol, followed by a presentation by NICEATM staff summarizing each revised draft BRD, and lastly a member of the IWG would present the draft ICCVAM recommendations. Following presentations, the Panel Evaluation Group Leader for the topic under consideration would present the group's draft recommendations, followed by Panel discussion. Public comments would then be presented, followed by the opportunity for additional Panel discussion in consideration of the public comments. The Panel would then vote to accept the Panel consensus, with any minority opinions being so noted with the rationale provided for the minority opinion.

Current Regulatory Testing Requirements and Hazard Classification Schemes for Allergic Contact Dermatitis (ACD) and the Traditional LLNA Procedure

Dr. Matheson presented an overview of ACD and relevant regulatory requirements. She briefly discussed the ICCVAM final recommendations for the LLNA Performance Standards, the updated ICCVAM LLNA test method protocol, and the reduced LLNA (rLLNA), all of which were reviewed by the Panel at their meeting in March 2008.

The Panel questioned who was responsible for conducting the future studies referred to in the revised draft ICCVAM test method recommendations. Dr. Stokes replied that these recommendations are provided for consideration by the stakeholder community. Those organizations with appropriate resources can use this information to guide their research, development, and validation activities.

A question arose from the Panel as to why pooled data (as opposed to individual animal data) are collected for the LLNA.

Dr. Matheson replied that, pooled data are often collected since OECD Test Guideline 429 allows the use of a minimum of four animals per treatment group when collecting pooled data, but requires a minimum of five animals per treatment group when collecting individual animal data. Legislation in some countries, and many Animal Care and Use Committees, require that the test method to be used is the one requiring the fewest animals. Dr. Matheson also noted that the ICCVAM LLNA test method protocol has recently been revised to allow the use of a minimum of four animals per treatment group when collecting individual animal data, so there is now no reason not to collect individual animal data. At the Panel meeting in March 2008, the Panel stated that all future LLNA studies should require that lymph nodes be collected from individual animals instead of pooling them

[4] For the purposes of this document, the radioactive LLNA test method, which was first evaluated by ICCVAM in 1999, and subsequently recommended to U.S. Federal agencies as a valid substitute for currently accepted guinea pig test methods to assess the allergic contact dermatitis potential of many, but not all, types of substances, is referred to as the traditional LLNA.

with other animals in a treatment group since individual animal response data allows for identification of technical problems and outlier animals within a dose group.[5]

A question arose as to whether the U.S. Environmental Protection Agency (EPA) prefers LLNA or guinea pig data for submission. Dr. Matheson ceded the floor to Ms. Debbie McCall of EPA Office of Pesticide Programs, who was in attendance. Ms. McCall said that EPA prefers LLNA data, but will accept either guinea pig maximization test (GPMT) or Buehler test (BT) data.

Overview of the Revised Draft LLNA: DA Test Method Procedure BRD and Revised Draft ICCVAM Test Method Recommendations

The first test method reviewed was the LLNA: DA test method. This test method measures the ATP content of lymph node cells by the luciferin/luciferase method, as an index of lymphocyte proliferation, after exposure to a test substance.

Dr. Kenji Idehara of Daicel Chemical Industries, Ltd., Japan (the test method developer) presented a synopsis of the test method to the Panel.

A Panelist asked about the half-life of ATP in the lymph node cells after the mouse is sacrificed. Dr. Idehara replied that the ATP concentration declines 20 to 30% in an hour, with a half-life of about 2 to 2.5 hours. The assay time from animal sacrifice to complete measurement of ATP content for each individual animal is maintained as similar as possible, within approximately 30 min. He also said that the time between sacrifice and ATP assay is not a problem when collecting individual animal data, if the time between the excision of the lymph nodes, the preparation of the cell suspensions, and the measurement of the ATP concentrations is kept relatively constant between animals.

A Panelist asked if the lymph node samples were randomized before the ATP assays were conducted. Dr. Idehara replied that the samples were not randomized.

On behalf of NICEATM, Dr. Salicru presented an overview of the revised draft LLNA: DA BRD to the Panel.

A question arose about NICEATM's use of different decision criteria for the accuracy analysis, and the reproducibility analyses in the revised draft BRD. Dr. Salicru noted that a decision criterion of SI ≥ 2.5 was used for the reproducibility analyses because it was found to be the optimal decision criterion for identifying sensitizers (i.e., it resulted in a 0% false positive rate).

Dr. Wind presented the revised draft ICCVAM test method recommendations for the LLNA: DA test method to the Panel. She noted that ICCVAM favored the multiple decision criteria to eliminate any false positives or false negatives. A Panelist commented that, as more data are accumulated using the test method, false positives and false negatives might appear.

A Panelist asked, if the true stimulation index (SI) value for a compound was 2.0, if that compound would be classified as a sensitizer or a nonsensitizer. Dr. Wind replied that, as described in the revised draft ICCVAM recommendations, other information would be necessary to definitively answer that question.

Dr. Kojima presented the results of the Japanese Society for Alternatives to Animal Experiments (JSAAE) interlaboratory validation studies of the LLNA: DA and the LLNA: BrdU-ELISA test methods to the Panel. In the presentation, he noted that the JaCVAM Regulatory Acceptance Board has examined the results of the studies for both test methods and accepted the LLNA: DA as a replacement for the traditional LLNA. The JaCVAM Regulatory Acceptance Board has requested additional data for the LLNA: BrdU-ELISA.

[5] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

Panel Evaluation:

Dr. Woolhiser presented the draft position developed by Evaluation Group B, which was charged with primary review of the LLNA: DA test method. The Panel agreed that the available data and test method performance support the use of the LLNA: DA to identify substances as potential skin sensitizers and nonsensitizers, with certain limitations. They concurred with ICCVAM's proposal that, based on the current validation database, the multiple SI decision criteria should be used to identify sensitizers and nonsensitizers (i.e., SI ≥ 2.5 for sensitizers, SI ≤ 1.7 for nonsensitizers). The Panel also noted that the limitation of these test methods when using the proposed multiple decision criteria is the indeterminate classification of substances that fall in the range of SI values for which a classification is uncertain (i.e., 1.7 < SI < 2.5). The Panel recommended that when such results are obtained, users should carefully interpret the results using an integrated decision strategy in conjunction with all other available information (e.g., dose response and quantitative structure-activity relationship [QSAR] information, peptide-binding activity, molecular weight, results from related chemicals, other testing data) to determine if there is adequate information for an accurate sensitization hazard classification or if additional testing is necessary. The Panel emphasized that, from an animal welfare perspective, retesting should not be undertaken until all other available information is evaluated, and a determination is made that such testing is required to fill a data gap. The Panel also recommended that more detailed guidance be developed for regulatory agencies on how the multiple decision criteria could be used in practice.

Subsequent Panel discussions focused on ICCVAM's recommendation to use multiple decision criteria to identify sensitizers and nonsensitizers. In general, the Panel preferred the multiple decision criteria to a single decision criterion for identifying sensitizers and nonsensitizers. A Panelist recommended that graphs showing the maximum SI obtained with the modified test method (the LLNA: DA, in this case) plotted against the maximum SI obtained with the traditional LLNA, for each test substance, be included in the final BRD. This was a general recommendation for both test methods that use multiple decision criteria (i.e., the LLNA: DA and LLNA: BrdU-ELISA). It was also pointed out that, as more data are accumulated for these test methods, the cut-off SI values for sensitizers and nonsensitizers would likely change.

Bootstrapping analysis was mentioned as a means to provide some measure of variability of the chosen cut-off values. It was also mentioned that the tables in Section 7.0 of the revised draft BRD provide no measurement of variation for the data. It was suggested that all of these tables include treatment means, standard deviations, and the mean squares, so that F-values can be calculated for between and among laboratory means. However, the Panel agreed that, while this information would be useful for inclusion in the final BRD, it would not impact the Panel's overall conclusions about the test method.

Some discussion followed about variations in the LLNA: DA test method protocol from the updated ICCVAM-recommended traditional LLNA test method protocol (i.e., sodium lauryl sulfate pretreatment prior to test substance application and an additional test substance application on day 7). The Panel agreed that despite these variations, the LLNA: DA was still mechanistically and functionally similar to the traditional LLNA.

Public Comments:

At the conclusion of the Panel discussion, Dr. Luster called for public comments. None were presented.

Panel Conclusions and Recommendations:

Dr. Luster asked if the Panel was in agreement with the conclusions in the draft Panel Report as reflected in the updated Evaluation Group presentation as modified during the discussions. The Panel approved unanimously.

Applicability Domain of the LLNA and Revised Draft ICCVAM Test Method Recommendations

NICEATM provided an overview of the revised draft Addendum on the LLNA applicability domain. Subsequent to the 2008 Panel consideration of this topic, new data were obtained for pesticide formulations, dyes, essential oils, and substances tested in aqueous solution, but none were obtained for metals. Since the Panel previously considered the use of the term *mixtures* too broad, data were separately evaluated by product subgroups in the revised draft Addendum, and they were identified in general terms as pesticide formulations and other products. Dr. Wind presented the revised draft ICCVAM test method recommendations for the LLNA applicability domain to the Panel.

Subsequent to Dr. Wind's presentation, Dr. Luster asked Ms. McCall of EPA to clarify EPA's position on the use of LLNA data for pesticide formulations. Ms. McCall replied that EPA accepted positive or negative LLNA data on single substance technical grade additives. Between 2003 and 2007, EPA received few LLNA studies on pesticide formulations. Positive LLNA results were accepted, but for negative results, EPA required a confirmatory test. The majority of sensitization data submitted to EPA for pesticide formulations are from the guinea pig BT. There are limited human data available on pesticides due to the ethics limitations for conducting human studies, and applicants provide all of EPA's data.

A Panelist commented that the GPMT is more sensitive that the BT; he said that, in his experience, the GPMT showed roughly 60% positive results versus 20% positive results for the BT, for the same group of formulations. He said that the LLNA is more concordant with the GPMT than it is with the BT. He said that the GPMT is the preferred test in Europe. The Panel agreed that this should be reflected in the comparisons of LLNA and guinea pig results.

Panel Evaluation:

Dr. Olson presented the draft position developed by Evaluation Group A, which was charged with primary review of the LLNA applicability domain, to the Panel. While the Panel agreed that there were too few data in the revised draft Addendum for some of the test substance classes (e.g., dyes, essential oils) to make a firm statement about concordance of the LLNA with other test methods for these classes, the Panel stated that any material should be suitable for testing in the LLNA unless there is a biologically-based rationale for exclusion, such as unique physicochemical properties that might affect their ability to interact with immune processes. The Panel therefore agreed that the LLNA should be considered appropriate for testing pesticide formulations and other products, unless there is a biologically-based rationale for exclusion.

The Panel also concurred that, while studies done with BALB/c mice should not be excluded from the evaluations in the revised draft Addendum, CBA should remain the preferred strain for the updated ICCVAM-recommended LLNA test method protocol, and that the use of any other strain, or of male rather than female mice, should be justified by the investigator.

The Panel did not agree that Pluronic L92 should be added to the list of preferred vehicles for the LLNA, but it did agree that studies done with Pluronic L92 should not be excluded from the evaluations in the revised draft Addendum.

While the concordance of LLNA results for essential oils was properly compared with human results, the Panel noted that the revised draft Addendum neglected to consider information that showed LLNA results were more concordant with human results when the major component was ≥70%, compared to the concordance for the essential oil itself. The Panel also commented that the term *natural complex substances* was more appropriate for these types of substances than *essential oils*, because this is the terminology used for the Registration, Evaluation, Authorisation and Restriction of Chemical substances program now in force in the European Union (EU).

In reference to the data for the medical device eluates in the revised draft Addendum, the Panel commented that ISO Standard 1099 requires the chemical analysis of such materials before skin sensitization testing is undertaken, and therefore agreed that the data provided were of little use for evaluating the performance of the LLNA for testing these types of substances.

Public Comments:

At the conclusion of the Panel discussion, Dr. Luster called for public comments.

Mr. Gary Wnorowski, Eurofins Product Safety Labs
Mr. Gary Wnorowski said he had registered to make a public comment, but that Ms. McCall of EPA had already addressed his question by her answer to Dr. Luster's question regarding acceptability of pesticide formulation data.

Panel Conclusions and Recommendations:

Dr. Luster asked if the Panel was in agreement with the conclusions in the draft Panel Report as reflected in the updated presentation. The Panel approved unanimously.

Adjournment

At the conclusion of the discussion on the applicability domain, Dr. Luster adjourned the Panel for the day at 5:30 p.m., to reconvene at 8:30 a.m. on Wednesday, April 29, 2009.

Wednesday, April 29, 2009
Overview of the Draft LLNA: BrdU-ELISA Test Method Revised Draft BRD and Revised Draft ICCVAM Test Method Recommendations

Dr. Luster called for Panel consideration of the LLNA: BrdU-ELISA test method. This test method measures bromodeoxyuridine (BrdU), a thymidine analog, instead of radioactive thymidine, incorporated into the DNA of proliferating lymphocytes, via an enzyme-linked immunosorbent assay (ELISA).

Dr. Masahiro Takeyoshi of Chemicals Evaluation and Research Institute, Japan (the test method developer) presented a synopsis of the test method to the Panel.

On behalf of NICEATM, Dr. Strickland presented an overview of the revised draft ICCVAM LLNA: BrdU-ELISA BRD to the Panel.

A Panelist asked why ICCVAM proposes an SI value of 2.0 as the cutoff value for a sensitizer instead of a value of 2.5, since the data indicated that no false positives would result if either value were used. Dr. Strickland replied that the value of 2.0 was chosen because this was the lowest value that resulted in a 0% false positive rate, thus minimizing the range of uncertainty.

Dr. Jacobs presented the revised draft ICCVAM test method recommendations for the LLNA: BrdU-ELISA test method to the Panel.

Panel Evaluation:

Dr. Ullrich presented the draft position developed by Evaluation Group B, which was charged with primary review of the LLNA: BrdU-ELISA test method, to the Panel.

The Panel agreed that the LLNA: BrdU-ELISA test method was mechanistically and functionally similar to the traditional LLNA, and the ICCVAM LLNA Performance Standards could be used to evaluate it. The Panel also concurred that the available data and test method performance support the use of the LLNA: BrdU-ELISA to identify substances as potential skin sensitizers and nonsensitizers, with certain limitations. They agreed with ICCVAM's proposal that, based on the current validation database, the multiple SI decision criteria should be used to identify sensitizers and nonsensitizers

(i.e., SI ≥ 2.0 for sensitizers, SI > 1.3 for nonsensitizers). The Panel also noted that the limitation of these test methods when using the proposed multiple decision criteria is the indeterminate classification of substances that fall in the range of SI values for which a classification is uncertain (i.e., 2.0 > SI ≥ 1.3). The Panel recommended that when such results are obtained, users should carefully interpret the results in an integrated decision strategy in conjunction with all other available information (e.g., dose-response and QSAR information, peptide-binding activity, molecular weight, results from related chemicals, other testing data) to determine if there is adequate information for an accurate sensitization hazard classification or if additional testing is necessary. The Panel emphasized that, from an animal welfare perspective, retesting should not be undertaken until all other available information is evaluated, and a determination is made that such testing is required to fill a data gap. The Panel also recommended that more detailed guidance be developed for regulatory agencies on how the multiple decision criteria could be used in practice.

Subsequent Panel discussions focused on ICCVAM's recommendation to use multiple decision criteria to identify sensitizers and nonsensitizers. In general, the Panel preferred the multiple decision criteria to a single decision criterion for identifying sensitizers and nonsensitizers. The Panel agreed that all of the comments for the LLNA: DA test method regarding the graphs and tables in the revised draft BRD, and the provision of measures of variation for interlaboratory reproducibility data, apply to the BrdU-ELISA also.

A Panelist commented that the use of interpolation for determining ECt values presupposed a monotonic increase in SI values and that isotonic regression might be more appropriate in cases in which a monotonic increase does not occur. More Panel discussion occurred regarding the practical usefulness of the multiple decision criteria. It was agreed that the term *integrated assessment* was more appropriate than *weight-of-evidence* to describe the approach taken to classify substances that fell into the uncertainty range.

The Panel discussed when it was appropriate to rely on hypothesis testing (as opposed to decision criteria based on a cutoff SI value) to classify substances. The Panel commented that, in some cases, statistical significance might not indicate a biological effect. The Panel agreed with the language regarding hypothesis testing in the current ICCVAM LLNA Performance Standards (Appendix A - Section 3.0).

Public Comments:

At the conclusion of the Panel discussion, Dr. Luster called for public comments.

Dr. George De George, MB Research Labs
Dr. De George raised the following points:

- The data evaluated for the 1999 ICCVAM evaluation of the LLNA were statistically analyzed.
- As a result of that analysis, the optimum SI cutoff for a sensitizer was determined as 3.16.
- The Panel for the 1999 evaluation chose 3.0 as the SI cutoff to provide an added level of confidence.
- Routine statistical analysis of LLNA data to classify test substances was not recommended in the 1999 evaluation. In Dr. DeGeorge's opinion, the best reason to collect individual animal data was so that, in the future, studies could be done to determine an optimum method for hypothesis testing of LLNA data.
- Newer variant LLNA tests should be subjected to the same level (and not held to a higher level) of requirements for validation as the traditional LLNA.

Panel Conclusions and Recommendations:

At the conclusion of the public comments, Dr. Luster asked if the Panel was in agreement with the conclusions in the draft Panel Report as reflected in the updated presentation. The Panel approved unanimously.

Overview of the Revised Draft LLNA: BrdU-FC Test Method BRD and Revised Draft ICCVAM Test Method Recommendations

Dr. Luster called for Panel consideration of the LLNA: BrdU-FC test method. This test method measures bromodeoxyuridine (BrdU), a thymidine analog, instead of radioactive thymidine, incorporated into the DNA of proliferating lymphocytes, via flow cytometric analysis. The test method also allows for the measurement of immunophenotypic markers in the lymphocyte population, ostensibly aiding in discrimination between irritants and sensitizers.

Dr. George DeGeorge of MB Research Labs, Spinnerstown, PA (the test method developer) presented a synopsis of the test method to the Panel. In addition to a brief description of the test method protocol, Dr. DeGeorge made the following points:

- The test method protocol was based on the ICCVAM-recommended LLNA test method protocol, using SI $\geq$ 3.0 as the decision criterion for a sensitizer.
- Test substances were chosen to include those tested in the traditional LLNA.
- Guinea pig data and human results are considered less reliable.
- The LLNA: BrdU-FC uses lower doses of test substances than the traditional LLNA to avoid irritating concentrations.
- The LLNA: BrdU-FC makes correct calls for some substances for which the traditional LLNA does not.
- All of the data generated by MB Research Labs using the LLNA: BrdU-FC are available for review at the laboratory (although not all data are available electronically).
- MB Research Labs is currently attempting to find other laboratories interested in participating in an interlaboratory validation study.

Following Dr. De George's presentation, a Panelist asked the following questions:

- Does MB Research Labs conduct LLNA: BrdU-FC studies according to GLP? Dr. De George said yes.
- What is the treatment group size? Dr. DeGeorge responded that five animals per treatment group were used.
- Can measurement of ear swelling be added to any LLNA variant test method as an additional endpoint? Dr. DeGeorge replied that it could, and that it could help resolve which doses to test.

On behalf of NICEATM, Dr. Allen presented a summary of the revised draft LLNA: BrdU-FC BRD to the Panel. At the conclusion of Dr. Allen's presentation, Dr. DeGeorge pointed out that an in-house flow cytometer and trained operators weren't necessary to conduct the test method, because the lymphocytes were fixed as part of the test method protocol, and the flow cytometry analysis could be outsourced.

Dr. Jacobs then presented the revised draft ICCVAM test method recommendations for the LLNA: BrdU-FC test method to the Panel.

Panel Evaluation:

Dr. Richmond presented the draft position developed by Evaluation Group B, which was charged with primary review of the LLNA: BrdU-FC test method, to the Panel.

The Panel agreed that the LLNA: BrdU-FC test method was mechanistically and functionally similar to the traditional LLNA, and the ICCVAM LLNA Performance Standards could be used to evaluate it. The Panel also concurred that the database of more than 45 representative test substances yielded adequate accuracy based on results from one laboratory, and that intralaboratory reproducibility also had been adequately demonstrated. However, the Panel agreed with the ICCVAM proposal to defer a formal recommendation on the validity of the LLNA: BrdU-FC until an independent audit of all data supporting the analysis has been conducted and until transferability has been demonstrated in an interlaboratory validation study. The Panel recommended that ICCVAM should work with NICEATM to support and facilitate the independent audit and interlaboratory validation study. The Panel recommended that upon completion of these tasks and determination of satisfactory data quality, power, and interlaboratory reproducibility, that the LLNA: BrdU-FC could be considered to have adequate validation and performance to support its consideration for regulatory use.

Much Panel discussion about the necessary statistical power of the test method occurred. Power is defined as the probability that the test method would determine that a test group showing a positive result is different from the negative control (i.e., that a sensitizer would be detected as such). Data presented to the Panel during their 2008 evaluation indicated that the test method would require nine animals per treatment group to achieve 95% power; the power with five animals per group was estimated at 80% in that evaluation. The Panel agreed that, before an interlaboratory validation study was begun, it should be verified that the LLNA: BrdU-FC test method has power at least equal to that of the traditional LLNA using five animals per treatment group.

Public Comments:

At the conclusion of the Panel discussion, Dr. Luster called for public comments.

Dr. George De George, MB Research Labs

Dr. De George raised the following points:

- Power calculations on a subset of the data are not as reliable as accuracy statistics calculated from the entire dataset for 45 chemicals.
- Power calculations are a new requirement for validation, and not contained in the ICCVAM LLNA Performance standards.
- It was Dr. De George's opinion that it would be difficult, if not impossible, to get three qualified testing laboratories to participate in an interlaboratory validation study.

Panel Conclusions and Recommendations:

Subsequent to the public comments, the Panel commented that the flow cytometric analysis for samples from all three laboratories in an interlaboratory study could be done at MB Research Labs. Power calculations could be done by NICEATM on the most recent data generated by the LLNA: BrdU-FC test method.

The Panel decided to make a nomination to ICCVAM, with high priority, that NICEATM organize and supervise an interlaboratory validation study for the LLNA: BrdU-FC test method.

Dr. Luster asked if the Panel was in agreement with the conclusions in the draft Panel Report. The Panel approved unanimously.

Concluding Remarks

Dr. Luster, on behalf of the Panel, thanked the NICEATM-ICCVAM staff for their continued assistance during the review process and the Panel meeting. He also thanked Drs. Joanna Matheson and Abby Jacobs, the IWG co-chairs, and Dr. Marilyn Wind, ICCVAM Chair and IWG member, for the hard work they put into the project. Dr. Luster also thanked the Panel, the Evaluation Group Chairs, and the experts on the test methods, who presented them to the Panel.

Drs. Wind and Stokes thanked the Panel again for their hard work, thoughtful and objective deliberations, and advice. Dr. Stokes further thanked the invited test method developers for their excellent summaries of their test method for the benefit of the Panel. Dr. Stokes concluded by saying he looked forward to further working with the Panel members to complete their Panel report.

Adjournment

Dr. Luster adjourned the Panel at 11:30 a.m., concluding the meeting.

William S. Stokes, D.V.M., D.A.C.L.A.M.
NIEHS
P.O. Box 12233
Mail Stop: K2-16
Research Triangle Park, NC 27709

Dear Dr. Stokes,

The Meeting Summary Minutes, Independent Scientific Peer Review Panel Meeting, Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Updated Evaluation of the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA), accurately summarizes the Peer Review Panel meeting of April 28-29, 2009, in Bethesda, MD.

Sincerely,

Signature	Printed Name	Date
	MICHAEL LUSTER	8/21/09

Appendix D4

Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products

This document is available at:
https://ntp.niehs.nih.gov/iccvam/docs/immunotox_docs/llnaprprept2009.pdf

The document is also available on request from NICEATM:

NICEATM
National Institute of Environmental Health Sciences
P.O. Box 1233, MD K2-16
Research Triangle Park, NC 27709 USA
Telephone: 919-541-2384 Fax: 919-541-0947
E-mail: niceatm@niehs.nih.gov

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Appendix E

Federal Register Notices and Public Comments

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Appendix E1

Federal Register Notices

All Federal Register notices are available at https://www.federalregister.gov/

72 FR 27815 (May 17, 2007)
The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data

72 FR 52130 (September 12, 2007)
Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments

73 FR 1360 (January 8, 2008)
Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments

73 FR 25754 (May 7, 2008)
Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

73 FR 29136 (May 20, 2008)
Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments

74 FR 8974 (February 27, 2009)
Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments

74 FR 19562 (April 29, 2009)
Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

74 FR 26242 (June 1, 2009)
Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for

Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments

Appendix E2

Public Comments Received in Response to *Federal Register* Notices

72 FR 27815 (May 17, 2007)
The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data: Comments available on request from NICEATM

- Dr. Eric Debruyne (BAYER CropScience)
- Dr. H.-W. Vohr (Bayer HealthCare AG)
- Dr. H.-W. Vohr (Bayer HealthCare AG)
- Dr. H.-W. Vohr (Bayer HealthCare AG)
- Dr. Kirill Skirda (CESIO)
- Mark S. Maier, Ph.D., DABT (CropLife America)
- Dr. Phil Botham (European Crop Protection Association)
- Peter Ungeheuer (European Federation for Cosmetic Ingredients)
- Dori Germolec (NIEHS)
- Dori Germolec (NIEHS)
- Robert L. Guest (Safepharm Laboratories Ltd)
- Daniel R. Cerven, M.S. and Melissa K. Kirk, Ph.D. (MB Research Laboratories)
- Daniel Marsman, D.V.M., Ph.D. (Procter & Ganble)
- Michael J. Olson, Ph.D. (GlaxoSmithKline)
- Anne Marie Api, Ph.D. (Research Institute for Fragrance Manufacturers)
- Peter S. Thorne, Ph.D. (The University of Iowa)
- Catherine Willett, Ph.D. (People for the Ethical Treatment of Animals), Sara Amundson (Humane Society Legislative Fund), Dr. Martin Stephens (Humane Society of the United States), Kristie Stoick, M.P.H. (Physicians Committtee for

Responsible Medicine), Sue A. Leary (Alternatives Research & Development Foundation), and Tracie Letterman, Esq. (American Anti-Vivisection Society)

72 FR 52130 (September 12, 2007)
Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments: Comments available on request from NICEATM

- Ann-Therese Karlberg (Goteborg University)
- Dr. Jon Richmond
- Prof. dr. Henk Van Loveren (National Institute of Public Health and the Environment, the Netherlands)
- Catherine Willett, Ph.D. (People for the Ethical Treatment of Animals), Sara Amundson (Humane Society Legislative Fund), Dr. Martin Stephens (Humane Society of the United States), Kristie Stoick, M.P.H. (Physicians Committee for Responsible Medicine), Sue A. Leary (Alternatives Research & Development Foundation), and Tracie Letterman, Esq. (American Anti-Vivisection Society

73 FR 1360 (January 8, 2008)
Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments: Comments available on request from NICEATM

- Dr. David Basketter
- Dr. David Basketter
- Kenneth T. Bogen, Dr.P.H., DABT (Exponent)
- G. Frank Gerberick, Ph.D. (The Procter & Gamble Company)
- Laurence Musset (OECD)
- B. Schau
- Catherine Willett, Ph.D. (People for the Ethical Treatment of Animals) and Kristie Stoick, M.P.H. (Physicians Committee for Responsible Medicine)

73 FR 25754 (May 7, 2008)
Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM): Comments available on request from NICEATM

- B. Sachau

73 FR 29136 (May 20, 2008)
Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments

- No responses received

74 FR 8974 (February 27, 2009)
Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments: Comments available on request from NICEATM

- Nancy Douglas, Ph.D. and Catherine Willett, Ph.D. (People for the Ethical Treatment of Animals), Kristie Stoick, M.P.H. (Physicians Committee for Responsible

Medicine), Martin Stephens, Ph.D. (The Humane Society of the United States), Sara Amundson (Humane Society Legal Fund, Doris Day Animal League), Sue Leary (Alternatives Research & Development Foundation), and Tracie Letterman, Esq. (American Anti-Vivisection Society)

74 FR 19562 (April 29, 2009)
Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

- No responses received

74 FR 26242 (June 1, 2009)
Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments: Comments available on request from NICEATM

- Brian E. Harvey, M.D., Ph.D. (Sanofi Aventis)

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Appendix E3

Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) Comments

SACATM Meeting on June 18-19, 2008

The full meeting minutes are available online at:
https://ntp.niehs.nih.gov/events/past/index.html

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Appendix E4

Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) Comments

SACATM Meeting on June 25-26, 2009

The full meeting minutes are available online at:
https://ntp.niehs.nih.gov/events/past/index.html

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Appendix F

Relevant Skin Sensitization Regulations and Testing Guidelines

The documents below are available at
https://ntp.niehs.nih.gov/pubhealth/evalatm/regs-guidelines/index.html

F2 EPA Health Effects Test Guidelines OPPTS 870.2600: Skin Sensitization (March 2003)

F3 ISO 10993-10: Biological Evaluation of Medical Devices Part 10: Tests for Irritation and Delayed-type Hypersensitivity (2002)

F4 OECD Test Guideline 429: Skin Sensitisation – Local Lymph Node Assay (Adopted April 2002)

F5 OECD Test Guideline 406: Skin Sensitisation (Adopted July 1992)

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Appendix F1

Table of Relevant Skin Sensitization Test Regulations

Note to the Reader:
Regulations may be updated in the future. It is recommended that users review the most current version of all regulations identified.

Electronic versions of United States Code (U.S.C.) can be obtained at:
http://www.gpoaccess.gov/uscode/index.html

Electronic versions of the Code of Federal Regulations (CFR) can be obtained at:
http://www.gpoaccess.gov/cfr/index.html

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Skin Sensitization Testing: Relevant US Federal Laws, Regulations, Guidelines, and Recommendations				
Agency, Center, or Office	**Regulated Products**	**Statutory Requirements**	**Regulations**	**Guidelines and Recommendations**
FDA/CDER	Pharmaceuticals	Federal Food, Drug, and Cosmetic Act (U.S.C. Title 21, Chapter 9) Public Health Service Act (U.S.C. Title 42, Chapter 6A)	21 CFR 312 21 CFR 314	Guidance for Industry Immunotoxicology Evaluation of Investigational New Drugs (2002)
EPA/OPPTS	Chemicals as defined by Section 5 of the Act Pesticides	Toxic Substances Control Act (U.S.C. Title 15, Chapter 53) Federal Insecticide, Fungicide, and Rodenticide Act (U.S.C. Title 7, Chapter 6)	40 CFR 158.50 40 CFR 158.100 40 CFR 158.340 40 CFR 700-799	OPPTS 870.2600 (2003) (see **Appendix F2**)
CPSC	Consumer Products	Federal Hazardous Substances Act (U.S.C. Title 15, Chapters 1261-1278)	16 CFR 1500.3	No Specific Guidelines, Guidances, or Recommendations
OSHA	Chemicals	Occupational Safety and Health Act of 1970 (U.S.C. Title 29, Chapter 15)	29 CFR 1910.1200	No Specific Guidelines, Guidances, or Recommendations

Relevant Skin Sensitization Regulations and Guidelines Europe		
Agency, Center, or Office	**Regulated Products**	**Regulations and Directives**
EU	Dangerous Preparations (Chemicals and Chemical Mixtures)	Directive 1999/45/EC of the European Parliament and of the Council of 31 May 1999 Annex V to Directive 67/548/EEC of 27 June 1967
	Pesticides	Directive 91/414/EEC of the European Parliament and of the Council of 15 July 1991

Relevant Skin Sensitization Regulations and Guidelines International			
Organizations	**Regulated Products**	**Legal Instruments and Recommendations**	**Guidelines, Guidance, and Recmmendations**
GHS	Chemicals	GHS Part 3, Chapter 3.4	No Specific Guidelines, Guidances, or Recommendations
ISO	Medical Devices	NA	ISO 10993-10 (2002) (see **Appendix F3**)
OECD	Chemicals	NA	OECD Test Guideline 429 (2002) (see **Appendix F4**) OECD Test Guideline 406 (1992) (see **Appendix F5**)
ICH	NA	NA	No Specific Guidelines, Guidances, or Recommendations