ICCVAM Test Method Evaluation Report on the Murine Local Lymph Node Assay: DA
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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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	2,4-Dinitrochlorobenzene
EC1.8	Estimated concentration needed to produce a stimulation index of 1.8
EC2.5	Estimated concentration needed to produce a stimulation index of 2.5
EC3	Estimated concentration needed to produce a stimulation index of 3.0
ECVAM	European Centre for the Validation of Alternative Methods
EGDMA	Ethylene glycol dimethacrylate
ELISA	Enzyme-linked immunosorbent assay
EPA	U.S. Environmental Protection Agency
ESAC	ECVAM Scientific Advisory Committee
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
K_{ow}	Estimated log octanol-water partition coefficient
LLNA	Murine local lymph node assay
LLNA: DA	Murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content
LNC	Lymph node cells
Max.	Maximum
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
PBS	Phosphate buffered saline
rLLNA: DA	Reduced murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content
RLU	Relative luminescence units
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SD	Standard deviation
SEM	Standard error of the mean
SI	Stimulation index
SLS	Sodium lauryl sulfate
TCA	Trichloroacetic acid
TG	Test Guideline
U.K.	United Kingdom
U.S.	United States
U.S.C.	United States Code

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[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 developed by Dr. Kenji Idehara at Daicel Chemical Industries, Ltd. in Hyogo, Japan. This version (referred to as the "LLNA: DA") measures increases in ATP content instead of using a radioactive marker to measure lymphocyte proliferation. The 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 Validation of Alternative Methods (ECVAM) and JaCVAM served as liaisons to the ICCVAM Immunotoxicity Working Group (IWG). A detailed timeline of the LLNA: DA evaluation is included with this report.

This Test Method Evaluation Report provides ICCVAM's recommendations regarding the LLNA: DA for assessing the ACD hazard potential of chemicals and products. Since the LLNA: DA does not require the use of 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 severely limit or discourage the use of radioactive materials required by the traditional LLNA. The report also summarizes the validation status of the LLNA: DA and provides the ICCVAM-recommended LLNA: DA 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: DA that 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. 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

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

[2] The "traditional LLNA" refers to the 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).

OECD Working Group of National Co-ordinators of the Test Guidelines Programme, which was approved as TG 442A at their March 23-25, 2010 meeting.

ICCVAM solicited and considered public comments and stakeholder involvement throughout the LLNA: DA 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: DA. The recommendations and the Background Review Document, 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 (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 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 (CPSC) 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: DA 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 required for ACD testing while continuing to support the protection of human health.

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[3] http://iccvam.niehs.nih.gov/methods/immunotox/llna-DA/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 modified by Daicel Chemical Industries, Ltd., based on ATP content (LLNA: DA). 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: DA measures increases in ATP content by luciferin-luciferase assay as an indicator of increases in lymphocyte cell number 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: DA as a variation of the traditional LLNA. The report includes the ICCVAM-recommended LLNA: DA test method protocol, the final LLNA: DA 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: DA 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 the public for comment. The Panel met twice in public session to review the initial and revised draft BRDs and draft ICCVAM recommendations. The initial draft BRD evaluated data for 29 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: DA 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 44 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: DA 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: DA. 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: DA and proposed responses to comments from member countries. 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 approved the LLNA: DA as TG 442A 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.

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[4] *Traditional LLNA* refers to the 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).

ICCVAM Recommendations: Test Method Usefulness and Limitations

ICCVAM concludes that the accuracy and reliability of the LLNA: DA support use of the test method to identify substances as potential skin sensitizers and nonsensitizers. For the validation database of 44 substances, the LLNA: DA correctly identified all 32 LLNA sensitizers (0% [0/32] false negatives), and nine of the 12 LLNA nonsensitizers (25% [3/12] false positives).[5] ICCVAM recommends that a stimulation index (SI) ≥ 1.8 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 ≥ 1.8 is used.

A limitation of the LLNA: DA is the potential for false positive results when borderline positive responses between an SI of 1.8 and 2.5 are obtained. Further, the use of the LLNA: DA might not be appropriate for testing substances that affect ATP levels (e.g., substances that function as ATP inhibitors) or those that affect the accurate measurement of intracellular ATP (e.g., presence of ATP degrading enzymes, presence of extracellular ATP in the lymph node).

ICCVAM Recommendations: Test Method Protocol

The ICCVAM-recommended LLNA: DA test method protocol, which is based on the protocol developed by Yamashita et al. (2005) and Idehara et al. (2008), incorporates all aspects of the ICCVAM-recommended traditional LLNA test method protocol except for those procedures unique to the conduct of the LLNA: DA. In testing situations that do not require dose-response information, or negative results are anticipated, the LLNA: DA should be considered for use as a reduced test method protocol. The reduced LLNA: DA tests only the high dose, thus further reducing animal use.

ICCVAM Recommendations: Future Studies

To further characterize the LLNA: DA test method, ICCVAM recommends that efforts 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 postmarketing surveillance of consumers for allergic reactions and occupational surveillance of potentially exposed workers. Additional nonsensitizing skin irritants should be tested to determine the impact of such substances on the false positive rate of the LLNA: DA.

ICCVAM also recommends that efforts be made to further characterize the sensitization potential of borderline positive substances that produce SI values between 1.8 and 2.5 to determine if such results might be false positives. This could include (1) evaluations of peptide reactivity; (2) determination of molecular weight; (3) identification of results from related chemicals; (4) human studies where ethically and scientifically justified; and (5) review of occupational exposures, postmarketing experience or monitoring, and/or *in vitro* testing data. All decision criteria should be reassessed as additional discriminators and data become available.

ICCVAM Recommendations: Performance Standards

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

Validation Status of the LLNA: DA

The mechanistic basis of the LLNA: DA 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 three-fold or more higher

[5] These results used the most prevalent outcome for substances that were tested multiple times.

than the vehicle control is considered a positive response indicative of a skin sensitizing substance. The LLNA: DA assesses cell proliferation by measuring increases in ATP content in the draining auricular lymph nodes as an indicator of cell number. The LLNA: DA also differs from the traditional LLNA in the test substance treatment and sampling schedule. In addition, the LLNA: DA includes pretreatment of the application site with an aqueous solution of 1% sodium lauryl sulfate (SLS).

The accuracy of the LLNA: DA was compared to that of the traditional LLNA. Optimal LLNA: DA performance was achieved using SI ≥ 1.8 to classify sensitizers versus nonsensitizers. Compared to the traditional LLNA, accuracy was 93% (41/44), with a false positive rate of 25% (3/12) and a false negative rate of 0% (0/32). The three false positive substances using SI ≥ 1.8 produced SI values between 1.8 and 2.5 in the LLNA: DA. 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 optimum SI ≥ 1.8 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 criterion or on the resulting number of false or false negative results.

ICCVAM concludes that the reproducibility of the LLNA: DA 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. A two-phased study was conducted to assess interlaboratory reproducibility.

Intralaboratory reproducibility was assessed using a coefficient of variation (CV) analysis of EC3 (estimated concentration needed to produce an SI of 3.0) and EC1.8 values (estimated concentration needed to produce an SI of 1.8) for isoeugenol and eugenol. (Each substance was tested in three different experiments.) The mean EC3 value for isoeugenol was 2.74% ± 0.58%, with a corresponding CV of 21%. Eugenol had an EC3 of 5.06% ± 0.55% and a CV of 11%. The mean EC1.8 value and corresponding CV for isoeugenol and eugenol were 0.87% ± 0.31% (36% CV) and 3.38% ± 0.79% (23% CV), respectively.

Both phases of an interlaboratory validation study included qualitative analyses of LLNA: DA reproducibility. An SI ≥ 1.8 was used as the threshold to distinguish sensitizers from nonsensitizers. In the first phase, 12 substances (nine sensitizers and three nonsensitizers based on traditional LLNA test results) were tested in either three or 10 laboratories. There was 100% agreement among the laboratories for 10 substances (seven sensitizers and three nonsensitizers based on traditional LLNA results). There was 67% (2/3) agreement among the tests for the remaining two traditional LLNA sensitizers. Interlaboratory CV values for the EC1.8 values of the nine sensitizers ranged from 15% to 140%.

The second phase included five substances (four sensitizers and one nonsensitizer based on traditional LLNA test results) tested in either four or seven laboratories. There was 100% agreement among the laboratories for four substances (three sensitizers and one nonsensitizer based on traditional LLNA results). There was 75% (3/4) agreement among the tests for the remaining traditional LLNA sensitizer. Interlaboratory CV values for the EC1.8 values of the four traditional LLNA sensitizers ranged from 14% to 93%.

Reproducibility of results for the 14 substances (10 traditional LLNA sensitizers and four traditional LLNA nonsensitizers) that had three to 18 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 ≥ 1.8 decision criterion was used to classify sensitizers versus nonsensitizers the SI results for 80%

(8/10) of the sensitizers (based on traditional LLNA results) were 100% concordant (i.e., all tests for that substance yielded maximum SI ≥ 1.8) in the LLNA: DA for three to 18 tests. The SI results for 75% (3/4) of the nonsensitizers (based on traditional LLNA results) were 100% concordant in the LLNA: DA (i.e., all tests for that substance yielded SI < 1.8) for four to 11 tests. The other nonsensitizer had 91% concordance (10/11). This test for the nonsensitizer yielded SI values between 1.8 and 2.5, the narrow region in which false positive results occurred.

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: DA included two public review meetings by an independent scientific peer review panel, multiple opportunities for public comments, consideration of reports from an OECD Consultation, 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: DA.

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 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 guinea pig tests for most testing situations.

The LLNA modified by Daicel Chemical Industries, Ltd., based on ATP content (referred to hereafter as the "LLNA: DA") 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 by detecting increases in ATP content as an indicator of cell number at the end of cell proliferation rather than by quantifying the incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine. The increase in ATP content in lymph nodes from test animals compared to vehicle control animals is then quantified using a luciferin-luciferase assay. The LLNA: DA can reduce the use of animals for skin sensitization testing when it is used in place of guinea pig 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 evaluations 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: DA should have a high priority for evaluation. A detailed timeline of the LLNA: DA evaluation is provided in **Appendix A**. The ICCVAM-recommended LLNA: DA test method protocol and the final LLNA: DA 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: DA 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 to the IWG.

To facilitate peer review of the LLNA: DA 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: DA 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 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 29 substances with adequate traditional LLNA data (19 sensitizers and 10 nonsensitizers, as classified by the traditional LLNA) that were tested in a single laboratory (Idehara et al. 2008). 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: DA (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: DA and the completeness of the ICCVAM draft BRD (see **Appendix D**). The Panel evaluated (1) the extent to which the draft BRD addressed established validation and acceptance criteria and (2) the extent to which the draft 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: DA 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: DA 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 for the validation database; and (3) an evaluation of interlaboratory reproducibility. On May 20, 2008, ICCVAM posted a report of the Panel's recommendations[6] (see **Appendix D**) 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 44 substances with adequate traditional LLNA data (32 sensitizers and 12 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: DA.

On November 4, 2008, JaCVAM released a statement that at a meeting concerning the LLNA: DA at the National Institute of Health Sciences, Tokyo, Japan, on August 28, 2008, the noncommissioned members of the JaCVAM Regulatory Acceptance Board unanimously endorsed the following statement (see **Appendix E**): "Following the review of the results of the Ministry of Health, Labour and Welfare-funded validation study of the LLNA: DA coordinated by the Japanese Society for Alternative to Animal Experimentation, it is concluded that the LLNA: DA can be used for distinguishing between sensitizer and nonsensitizer chemicals within the context of the Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 429 on skin sensitization: LLNA."

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[4] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_25553.pdf
[5] Available at 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

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: DA (see **Appendix D**). 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 **Appendix D**) 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 OECD TG for the LLNA: DA 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, the Food and Drug Administration, and the 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: DA and proposed responses to comments from member countries. 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 in December 2009 for review and comment to national experts and interested stakeholders of the 30 OECD member countries. 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 SACATM comments, the Panel report, conclusions of the OECD Expert Consultation, and all public comments before finalizing ICCVAM test method recommendations for the LLNA: DA. The recommendations (**Section 2.0**) and the final BRD (**Appendix C**) are incorporated in this ICCVAM Test Method Evaluation Report. As required by the ICCVAM Authorization Act of 2000 (Public Law 106-545, 42 United States Code 285l-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 will also be made available on the website as they are received.

[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
[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: DA Test Method

ICCVAM evaluated the validation status of the LLNA: DA as a nonradioactive modification of the traditional LLNA (ICCVAM 1999; Dean et al. 2001; Haneke et al. 2001; Sailstad et al. 2001) to identify substances that may cause ACD for regulatory hazard classification and labeling purposes. While the traditional LLNA assesses cell proliferation by measuring the incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine into the DNA of dividing cells in the draining auricular lymph nodes, the LLNA: DA assesses cell proliferation by measuring increases in ATP content in the draining auricular lymph nodes as an indicator of the cell number at the end of cell proliferation. The LLNA: DA also differs from the traditional LLNA in the test substance treatment and sampling schedule, as well as pretreatment at the application site with an aqueous solution of 1% sodium lauryl sulfate (SLS) (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: DA support use of the test method to identify substances as potential skin sensitizers and nonsensitizers. For the validation database of 44 substances,[11] the LLNA: DA correctly identified all 32 LLNA sensitizers (0% [0/32] false negatives), and nine of the 12 LLNA nonsensitizers (25% [3/12] false positives). ICCVAM recommends that a stimulation index (SI) $\geq$ 1.8 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 $\geq$ 1.8 is used.

A limitation of the LLNA: DA is the potential for false positive results when borderline positive responses between an SI of 1.8 and 2.5 are obtained (see **Section 3.4**). ICCVAM considers the applicability domain for the LLNA: DA 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: DA. For instance, the use of the LLNA: DA might not be appropriate for testing substances that affect ATP levels (e.g., substances that function as ATP inhibitors) or those that affect the accurate measurement of intracellular ATP (e.g., presence of ATP degrading enzymes, presence of extracellular ATP in the lymph node). In contrast, the LLNA: DA can be used for testing metal compounds, with the exception of nickel. Inconsistent results for nickel sulfate in the interlaboratory validation study suggest that the LLNA: DA may not be suitable for testing substances containing nickel and therefore further testing using a different test system is recommended when negative results are obtained for such substances.

2.2 ICCVAM Recommendations: Test Method Protocol

ICCVAM recommends a LLNA: DA test method protocol (**Appendix B**) that is based on the test method protocol developed by Yamashita et al. (2005) and Idehara et al. (2008). The ICCVAM-recommended LLNA: DA test method 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: DA (**Appendix B**). Key aspects from the ICCVAM-recommended LLNA test method protocol (Appendix A of ICCVAM 2009a) included in the ICCVAM-recommended LLNA: DA test method protocol (**Appendix B**) are the following:

[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 14 substances tested with the LLNA: DA.

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

Additionally, ICCVAM recommends that 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: DA should be considered and used where determined appropriate. The reduced LLNA: DA test method 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: DA 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 postmarketing 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: DA.
- Inconsistent results for nickel sulfate suggest that the LLNA: DA may not be suitable for testing nickel compounds. Therefore, the accrual of additional data from LLNA: DA studies on such compounds with comparative human and/or guinea pig data is needed in order to more comprehensively evaluate the suitability of the LLNA: DA for testing nickel compounds.
- Efforts should be made to further characterize the sensitization potential of borderline positive substances (i.e., those that produce SI values between 1.8 and 2.5) in the LLNA: DA 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: DA. The ICCVAM-recommended performance standards for the traditional LLNA apply to the LLNA: DA 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: DA are not proposed at this time.

3.0 Validation Status of the LLNA: DA Test Method

The ICCVAM BRD for the LLNA: DA test method (**Appendix C**) provides a comprehensive review of the current validation status of the LLNA: DA test method, including its accuracy and reliability, the substances tested, the rationale for the standardized test method 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: DA test method.

3.1 Test Method Description

Originally developed by Yamashita et al. (2005) and Idehara et al. (2008), the purpose of the LLNA: DA 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: DA 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 days one, two, three, and seven to the dorsum of the ears of mice at a concentration that provides maximum solubility of the test substance without producing systemic toxicity and/or excessive local skin irritation. One hour prior to each test substance application, an aqueous solution of 1% SLS is applied to the dorsum of the mouse ears to increase absorption of the test substance across the skin (van Och et al. 2000). Approximately 24 hours after the last test substance administration, the draining auricular lymph nodes are excised, and a single-cell suspension from the lymph nodes of each animal is prepared for quantifying the increase in ATP content, which serves as an indicator of cell number at the end of cell proliferation.

The increase in ATP content for each mouse is measured by luciferin-luciferase assay and is expressed in relative luminescence units (RLU). The SI is calculated as the ratio of the mean RLU/mouse for each treatment group against the mean RLU/mouse for the vehicle control group. Substances producing an SI greater than a specified threshold are considered to be potential skin sensitizers. Based on the accuracy evaluation described in **Section 3.4**, the optimum accuracy was at SI $\geq$ 1.8.

3.1.2 Similarities and Differences Between the Test Method Protocols for the Traditional LLNA and the LLNA: DA

While the traditional LLNA assesses cell proliferation by measuring the incorporation of radioactive thymidine or iodine into the DNA of dividing cells in the draining auricular lymph nodes (ICCVAM 1999; Dean et al. 2001), the LLNA: DA assesses cell proliferation by measuring increases in ATP content in the draining auricular lymph nodes as an indicator of cell number at the end of cell proliferation. The LLNA: DA also differs from the traditional LLNA in the test substance treatment and sampling schedule, as well as pretreatment at the application site with an aqueous solution of 1% SLS (see **Appendix B**).

In the traditional LLNA, the test substance is topically applied on three consecutive days. Two days after the last treatment, 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. By comparison, in the LLNA: DA, the test substance is administered topically on days one, two, three, and seven, with each treatment preceded by application of an aqueous solution of 1% SLS. The draining auricular lymph nodes are excised 24 hrs after the last test substance application

and prepared for quantifying the increase in ATP content, which does not require injection of a marker chemical.

3.2 Validation Database

The current validation database for the LLNA: DA includes results from studies for 46 substances that had previously been tested in the traditional LLNA. The LLNA: DA results were obtained from either the intralaboratory (Idehara et al. 2008; unpublished data) and/or the two-phased interlaboratory (Omori et al. 2008) validation study. These data were available and reviewed by the Panel in April 2009.

The reference test data for the 46 substances were obtained from traditional LLNA tests. Of the 46 substances, 33 were classified by the traditional LLNA as skin sensitizers, 12 were classified as nonsensitizers, and one (benzocaine) was classified as equivocal due to highly variable results (Basketter et al. 1995; ICCVAM 1999) and was not included in the performance analyses. Similar to benzocaine, traditional LLNA data for toluene 2,4-diisocyanate (van Och et al. 2000) were not suitable for comparison (i.e., a modified version of the traditional LLNA test method protocol was used that was not in accordance with OECD TG 429 [OECD 2002] or ICCVAM 1999 and Dean et al. 2001) and results for this test substance were not included in the performance analysis. Thus, the validation database is comprised of 44 substances tested in the LLNA: DA that have adequate traditional LLNA reference data for use in the performance analyses. Results from guinea pig skin sensitization testing and human skin sensitization testing and/or published clinical case report information are also provided where they were available (see **Appendix C, Annex III**). Of the 46 substances, 42 had guinea pig skin sensitization testing data and 43 had human skin sensitization testing data and/or published clinical case report information. Similar to LLNA: DA comparisons with the traditional LLNA, benzocaine and toluene 2,4-diisocyanate were not included in comparisons between the LLNA: DA and guinea pig or human outcomes.

Table 3-1 lists the chemical classifications, traditional LLNA EC3 values with maximum SI values, and LLNA: DA EC1.8 values with maximum SI values for the 44 substances with adequate comparative LLNA data that were evaluated in the LLNA: DA performance analyses. Twenty chemical classes were represented by the 44 substances evaluated in the LLNA: DA performance analyses; 13 substances were classified in more than one chemical class. The classes with the highest number of substances were carboxylic acids (16 substances) and phenols (5 substances). Further, 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. Seventeen of these classes were also represented in the LLNA: DA database (only amides, ketones, and macromolecular substances 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: DA database. Nevertheless, the Panel considered the database of substances tested in the LLNA: DA 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 an SI = 3) for the 32 sensitizers ranged from 0.009% to 90%.

Table 3-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: DA EC1.8 Values, and Maximum SI Values for 44 Substances Evaluated in the LLNA: DA Performance Analyses

Substance Name	Product Use[1]	Chemical Class[2]	Trad. LLNA EC3 (%) (Max. SI)[3]	LLNA: DA EC1.8 (%) (Max. SI)[3]
5-Chloro-2-methyl-4-isothiazolin-3-one[4]	Cosmetics; Manufacturing; Pesticides	Sulfur Compounds; Heterocyclic Compounds	0.009 (27.7)	0.009 (7.5)
p-Benzoquinone[4]	Manufacturing; Pesticides; Pharmaceuticals	Quinones	0.010 (52.3)	0.003 (3.8)
2,4-Dinitrochlorobenzene[5, 6]	Manufacturing; Pesticides	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated; Nitro Compounds	0.049 (43.9)	0.032 (15.1)
Benzalkonium chloride[5]	Cosmetics; Disinfectant; Manufacturing; Personal care products; Pesticides	Amines; Onium Compounds	0.070[7] (11.1)	0.402 (6.7)
Glutaraldehyde[5, 6]	Cosmetics; Disinfectant; Manufacturing; Pesticides	Aldehydes	0.083 (18.0)	0.118 (6.5)
p-Phenylenediamine[5]	Intermediate in chemical synthesis; Manufacturing	Amines	0.110 (26.4)	0.036 (5.1)
Potassium dichromate[5, 8]	Manufacturing; Pharmaceuticals	Inorganic Chemical, Chromium Compounds; Inorganic Chemical, Potassium Compounds	0.170 (33.6)	0.062 (6.4)
Propyl gallate[4]	Cosmetics; Food additive	Carboxylic Acids	0.320 (33.6)	0.225 (5.0)
Phthalic anhydride[5]	Intermediate in chemical synthesis; Manufacturing; Pharmaceuticals	Anhydrides; Carboxylic Acids	0.360 (26.0)	0.030 (6.9)
Formaldehyde[5, 6]	Disinfectant; Manufacturing	Aldehydes	0.495 (4.0)	0.699 (5.1)
Cobalt chloride[5, 6, 8]	Manufacturing; Pesticides	Inorganic Chemical, Elements; Inorganic Chemical, Metals	0.600 (7.2)	0.859 (20.6)
Isoeugenol[5, 6]	Food additive; Fragrance agent	Carboxylic Acids	1.540 (31.0)	1.477 (12.4)

continued

Table 3-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: DA EC1.8 Values, and Maximum SI Values for 44 Substances Evaluated in the LLNA: DA Performance Analyses (continued)

Substance Name	Product Use[1]	Chemical Class[2]	Trad. LLNA EC3 (%) (Max. SI)[3]	LLNA: DA EC1.8 (%) (Max. SI)[3]
2-Mercaptobenzothiazole[5]	Manufacturing; Pesticides	Heterocyclic Compounds	1.700 (8.6)	7.992 (2.0)
Cinnamic aldehyde[5]	Cosmetics; Food additive; Fragrance agent; Intermediate in chemical synthesis; Personal care products; Pesticides	Aldehydes	1.910 (18.4)	0.635 (4.7)
3-Aminophenol[6]	Cosmetics; Pharmaceuticals	Amines; Phenols	3.200 (5.7)	1.841 (2.8)
Diethyl maleate[4]	Food additive; Intermediate in chemical synthesis	Carboxylic Acids	3.600 (22.6)	0.442 (3.8)
Trimellitic anhydride[5]	Manufacturing	Anhydride; Carboxylic Acids	4.710 (4.6)	0.058 (5.0)
Nickel (II) sulfate hexahydrate[5, 6, 8]	Manufacturing	Inorganic Chemical, Elements; Inorganic Chemical, Metals	4.800 (3.1)	2.606 (11.8)
Resorcinol[5]	Cosmetics; Manufacturing; Personal care products; Pesticides; Pharmaceuticals	Phenols	6.330 (10.4)	3.902 (4.3)
Sodium lauryl sulfate[5]	Cosmetics; Food additive; Manufacturing; Personal care products; Pesticides; Pharmaceuticals	Alcohols; Sulfur Compounds; Lipids	8.080 (8.9)	1.640 (3.4)
Citral[5]	Fragrance agent	Hydrocarbons, Other	9.170 (20.5)	2.053 (4.4)
Hexyl cinnamic aldehyde[5, 6, 8]	Food additive; Fragrance agent	Aldehydes	9.740 (20.0)	6.275 (10.2)

continued

Table 3-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: DA EC1.8 Values, and Maximum SI Values for 44 Substances Evaluated in the LLNA: DA Performance Analyses (continued)

Substance Name	Product Use[1]	Chemical Class[2]	Trad. LLNA EC3 (%) (Max. SI)[3]	LLNA: DA EC1.8 (%) (Max. SI)[3]
Eugenol[5]	Cosmetics; Food additive; Intermediate in chemical synthesis; Manufacturing; Personal care products; Pharmaceuticals	Carboxylic Acids	10.090 (17.0)	2.629 (7.1)
Abietic acid[5, 6]	Manufacturing	Hydrocarbons, Cyclic; Polycyclic Compounds	11.920 (5.2)	4.530 (8.0)
Phenyl benzoate[4]	Manufacturing; Pesticides	Carboxylic Acids	13.600 (11.1)	0.653 (4.2)
Cinnamic alcohol[4]	Cosmetics; Food additive; Fragrance agent; Intermediate in chemical synthesis; Personal care products	Alcohols	21.000 (5.7)	5.218 (5.7)
Hydroxycitronellal[5]	Food additive; Fragrance agent; Personal care products	Hydrocarbons, Other	23.750 (8.5)	8.674 (5.7)
Imidazolidinyl urea[5]	Cosmetics; Personal care products; Pesticides	Urea	24.000 (5.5)	6.275 (4.7)
Ethylene glycol dimethacrylate[4]	Manufacturing	Carboxylic Acids	28.000 (7.0)	19.236 (4.5)
Butyl glycidyl ether[4]	Intermediate in chemical synthesis; Manufacturing	Ethers	30.900 (5.6)	17.507 (4.6)
Ethyl acrylate[4]	Manufacturing	Carboxylic Acids	32.800 (4.0)	6.790 (4.3)
Methyl methacrylate[4]	Manufacturing	Carboxylic Acids	90.000 (3.6)	99.347 (1.8)
1-Bromobutane[5]	Intermediate in chemical synthesis; Pharmaceuticals; Solvent	Hydrocarbons, Halogenated	NA (1.2)	NA (1.7)

continued

Table 3-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: DA EC1.8 Values, and Maximum SI Values for 44 Substances Evaluated in the LLNA: DA Performance Analyses (continued)

Substance Name	Product Use[1]	Chemical Class[2]	Trad. LLNA EC3 (%) (Max. SI)[3]	LLNA: DA EC1.8 (%) (Max. SI)[3]
Chlorobenzene[5]	Manufacturing; Solvent	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated	NA (1.7)	17.877 (2.4)
Diethyl phthalate[5]	Cosmetics; Manufacturing; Personal care products; Pesticides; Pharmaceuticals	Carboxylic Acids	NA (1.5)	NA (1.1)
Dimethyl isophthalate[4, 6]	Manufacturing; Fragrance agent	Carboxylic Acids	NA (1.0)	NA (1.3)
Hexane[5]	Manufacturing; Solvent	Hydrocarbons, Acyclic	NA (2.2)	82.232 (2.3)
Isopropanol[5, 6]	Cosmetics; Disinfectant; Food additive; Intermediate in chemical synthesis; Manufacturing; Personal care products; Pharmaceuticals; Solvent	Alcohols	NA (1.7)	NA (2.0)
Lactic acid[5, 8]	Food additive; Manufacturing; Pharmaceuticals	Carboxylic Acids	NA (2.2)	NA (1.1)
Methyl salicylate[5, 6]	Cosmetics; Food additive; Fragrance agent; Personal care products; Pharmaceuticals; Solvent	Carboxylic Acids; Phenols	NA (2.9)	NA (1.8)
Propylparaben[5]	Food additive; Pesticides; Pharmaceuticals	Carboxylic Acids; Phenols	NA (1.4)	NA (1.3)
Nickel (II) chloride[4]	Manufacturing; Pesticides	Inorganic Chemical, Elements; Inorganic Chemical, Metals	NA (2.4)	NA (1.3)
Salicylic acid[4]	Food additive; Manufacturing; Pharmaceuticals	Phenols; Carboxylic Acids	NA (2.5)	17.768 (2.0)

continued

Table 3-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: DA EC1.8 Values, and Maximum SI Values for 44 Substances Evaluated in the LLNA: DA Performance Analyses (continued)

Substance Name	Product Use[1]	Chemical Class[2]	Trad. LLNA EC3 (%) (Max. SI)[3]	LLNA: DA EC1.8 (%) (Max. SI)[3]
Sulfanilamide[4]	Pharmaceuticals	Hydrocarbons, Cyclic; Sulfur Compounds	NA (1.0)	NA (0.9)

Abbreviations: EC3 = estimated concentration needed to produce a stimulation index of three; EC1.8 = estimated concentration needed to produce a stimulation index of 1.8; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; Max. = maximum; NA = not available; SI = stimulation index.

[1] Information for product use was gathered from the following databases:
Hazardous Substances Database - National Library of Medicine – TOXNET: http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB.
Haz-Map: National Library of Medicine-Toxicology and Environmental Health Information Program: http://hazmap.nlm.nih.gov/.
Household Products Database - National Library of Medicine: http://hpd.nlm.nih.gov/index.htm.
International Programme on Chemical Safety INCHEM database in partnership with Canadian Centre for Occupational Health and Safety: 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, as developed by the National Library of Medicine: http://www.nlm.nih.gov/mesh/meshhome.html.

[3] The traditional LLNA EC3 or LLNA: DA EC1.8 values listed for each substance is averaged from respective studies. The substance was tested in the same vehicle in both the traditional LLNA and the LLNA: DA, except where noted. Numbers in parentheses indicate the maximum SI.

[4] Substance tested in the intralaboratory validation study (Idehara unpublished).

[5] Substance tested in the intralaboratory validation study (Idehara et al. 2008).

[6] Substance tested in phase one of the two-phased interlaboratory validation study (Omori et al. 2008).

[7] Benzalkonium chloride was tested in the LLNA: DA using acetone: olive oil (4:1) as the vehicle but the traditional LLNA EC3 value reported is based on results using acetone as the vehicle.

[8] Substance tested in phase two of a two-phased interlaboratory validation study (Omori et al. 2008).

Annex II of the BRD (**Appendix C**) lists various physicochemical properties for the substances tested in the LLNA: DA. For the 44 substances that were evaluated in the LLNA: DA performance analyses, the molecular weights ranged from 30 to 388 g/mol. Twenty-two of the 44 substances were solids, 21 were liquids, and one substance (benzalkonium chloride) exists as either a solid or a liquid. The estimated log octanol-water partition coefficients (K_{ow}) were available for 38 substances and ranged from -8.28 to 6.46. Peptide reactivity, which was available for 28 substances, ranged from high to minimal (Gerberick et al. 2004, 2007).

3.3 Reference Test Method Data

The traditional LLNA reference data used for the accuracy analyses were from ICCVAM (1999) for 34 of the 44 substances that were evaluated. The traditional LLNA reference data for the remaining 10 substances were obtained from the scientific literature (Gerberick et al. 1992; Hilton et al. 1998; Ryan et al. 2002; Basketter et al. 2005; Gerberick et al. 2005; Betts et al. 2006; Basketter et al. 2007). The reference data for the guinea pig tests (GPMT or Buehler test) and human tests (human maximization test, human patch test allergen, or other human data) were also obtained from the scientific literature. The LLNA, guinea pig, and human reference data and their sources for each of the 44 substances evaluated are provided in Annex III of the BRD (**Appendix C**).

3.4 Test Method Accuracy

The ICCVAM evaluation of the LLNA: DA included an assessment of multiple decision criteria (see **Table 3-2**) including SI ≥ 3.0, the threshold for distinguishing sensitizers and nonsensitizers that is recommended in the LLNA: DA developer's test method protocol. When the optimal decision criterion of SI ≥ 1.8 was used to identify sensitizers vs. nonsensitizers, compared to the traditional LLNA, accuracy was 93% (41/44), with a false positive rate of 25% (3/12), and a false negative rate of 0% (0/32). All three false positive substances were tested once in the LLNA: DA and had resulting maximum SI values between 1.8 and 2.5 (chlorobenzene maximum SI = 2.44; hexane maximum SI = 2.31; salicylic acid maximum SI = 2.00). 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. For example, peptide reactivity (Gerberick et al. 2007), could be used to interpret LLNA: DA results when borderline positive results (e.g., SI values between 1.8 and 2.5) are produced to confirm that such results are not false positive. Two of the three traditional LLNA nonsensitizers with positive LLNA: DA SI values in this range had minimal peptide reactivity and one did not have peptide reactivity data available. No unique characteristics were identified that could be used as rationale for excluding any particular types of substances from testing in the LLNA: DA.

An evaluation to determine the robustness of the optimum SI ≥ 1.8 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 criterion or on the resulting number of false or false negative results.

Table 3-2 Performance of the LLNA: DA for 44 Substances Compared to the Traditional LLNA in Predicting Skin Sensitization Potential Using Alternative Decision Criteria Based on the Most Prevalent Outcome for Substances with Multiple Tests

Alternate Criterion	N[1]	Accuracy % (No.[2])	Sensitivity % (No.[2])	Specificity % (No.[2])	False Positive Rate % (No.[2])	False Negative Rate % (No.[2])	Positive Predictivity % (No.[2])	Negative Predictivity % (No.[2])
Statistics[3]	44	84 (37/44)	94 (30/32)	58 (7/12)	42 (5/12)	6 (2/32)	86 (30/35)	78 (7/9)
≥95% CI[4]	44	75 (33/44)	100 (32/32)	8 (1/12)	92 (11/12)	0 (0/32)	74 (32/43)	100 (1/1)
≥2 SD[5]	44	77 (34/44)	91 (29/32)	42 (5/12)	58 (7/12)	9 (3/32)	81 (29/36)	63 (5/8)
≥3 SD[6]	44	80 (35/44)	88 (28/32)	58 (7/12)	42 (5/12)	13 (4/32)	85 (28/33)	64 (7/11)
SI ≥ 5.0	44	57 (25/44)	41 (13/32)	100 (12/12)	0 (0/12)	59 (19/32)	100 (13/13)	39 (12/31)
SI ≥ 4.5	44	70 (31/44)	59 (19/32)	100 (12/12)	0 (0/12)	41 (13/32)	100 (19/19)	48 (12/25)
SI ≥ 4.0	44	84 (37/44)	78 (25/32)	100 (12/12)	0 (0/12)	22 (7/32)	100 (25/25)	63 (12/19)
SI ≥ 3.5	44	89 (39/44)	84 (27/32)	100 (12/12)	0 (0/12)	16 (5/32)	100 (27/27)	71 (12/17)
SI ≥ 3.0	*44*	*91 (40/44)*	*88 (28/32)*	*100 (12/12)*	*0 (0/12)*	*13 (4/32)*	*100 (28/28)*	*75 (12/16)*
SI ≥ 2.5	44	91 (40/44)	88 (28/32)	100 (12/12)	0 (0/12)	13 (4/32)	100 (28/28)	75 (12/16)
SI ≥ 2.0	44	91 (40/44)	97 (31/32)	75 (9/12)	25 (3/12)	3 (1/32)	91 (31/34)	90 (9/10)
SI ≥ 1.8	**44**	**93 (41/44)**	**100 (32/32)**	**75 (9/12)**	**25 (3/12)**	**0 (0/32)**	**91 (32/35)**	**100 (9/9)**
SI ≥ 1.5	44	89 (39/44)	100 (32/32)	58 (7/12)	42 (5/12)	0 (0/32)	86 (32/37)	100 (7/7)
SI ≥ 1.3	44	86 (38/44)	100 (32/32)	50 (6/12)	50 (6/12)	0 (0/32)	84 (32/38)	100 (6/6)

Italicized text indicates the decision criterion chosen by the LLNA: DA validation study team; Bolded text indicates the single decision criterion that had an overall increased performance in predicting skin sensitization potential when compared to the traditional LLNA.

Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; No. = number; SD = standard deviation; SI = stimulation index.

[1] N = Number of substances included in this analysis.

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

[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 ATP data were log-transformed prior to statistical analysis. For analysis of variance, significance at $p < 0.05$ was further tested by Dunnett's test.

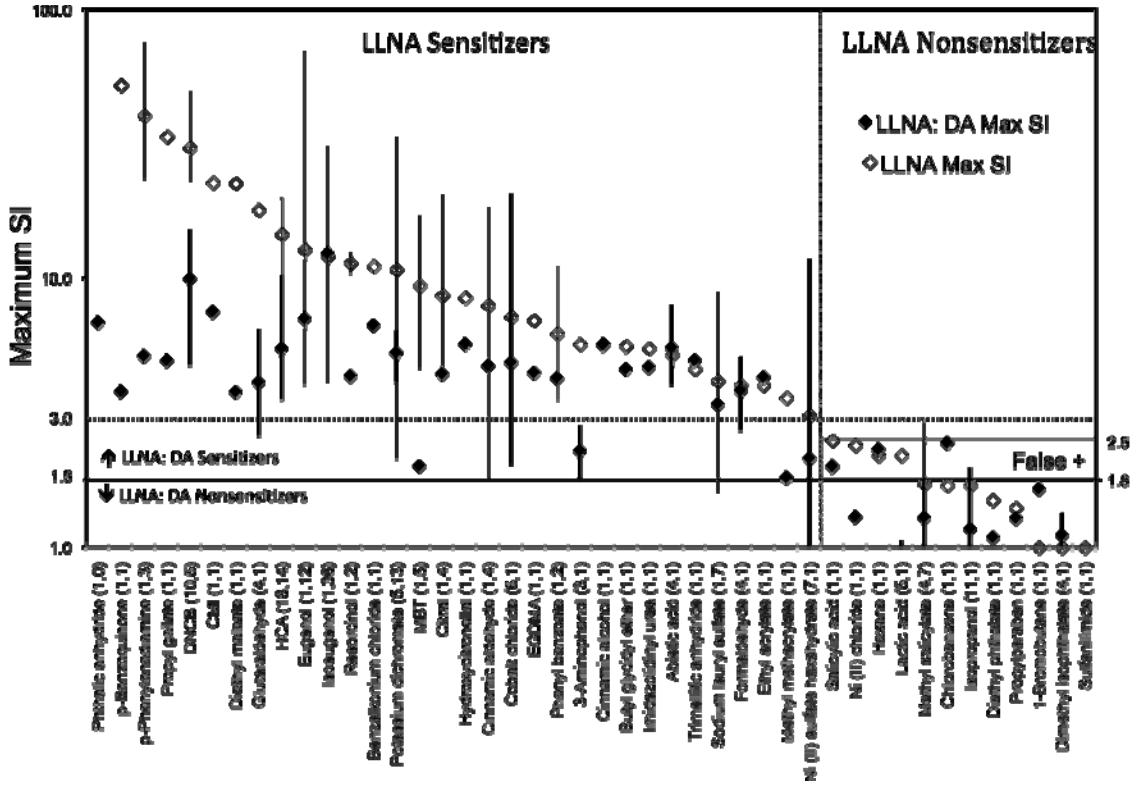
[4] The mean ATP of at least one treatment group was outside the 95% confidence interval for the mean ATP of the vehicle control group.

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

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

Figure 3-1 shows that SI values for the LLNA: DA are generally lower than those for traditional LLNA tests at similar 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: DA results. However, the accuracy analyses reported in the BRD are based on individual test results and not on a geometric mean. **Table 3-3** lists the maximum SI values for the substances included in **Figure 3-1**.

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



Abbreviations: CMI = 5-chloro-2-methyl-4-isothiazolin-3-one; DNCB = 2,4-dinitrochlorobenzene; EGDMA = ethylene glycol dimethacrylate; HCA = hexyl cinnamic aldehyde; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; MBT = 2-mercaptobenzothiazole; Ni = nickel; False + = false positive results in the LLNA: DA based on majority call were in the SI range between 1.8 and 2.5; SI = stimulation index.

[1] LLNA: DA and traditional LLNA tests at similar doses are shown. Symbols show the maximum SI for substances with one test result or geometric mean maximum SI for substances with more than one test result. Bars show the range of values reported for multiple test results (heavy bars for LLNA: DA and light bars for traditional LLNA). Numbers in parentheses beside the substance names indicate the number of tests for the LLNA: DA followed by the traditional LLNA, which may differ from the total number of tests available since only tests with similar maximum doses 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 at least one positive LLNA: DA test result in the SI range between 1.8 and 2.5 include salicylic acid, hexane, chlorobenzene, and isopropanol.

Table 3-3 Maximum SI Values of 44 Substances Evaluated in the LLNA: DA Compared to Traditional LLNA Tests with Similar Doses[1]

Substance Name[2]	Test Vehicle[3]	LLNA: DA Maximum SI Values[4]	Traditional LLNA Maximum SI Values
Sensitizers (LLNA: DA SI ≥ 1.8 and Traditional LLNA SI ≥ 3.0)			
Phthalic anhydride (1, 0)	AOO	6.85	NA
p-Benzoquinone (1, 1)	AOO	3.79	52.30
p-Phenylenediamine (1, 3)	AOO	5.14	23.30, 37.40, 75.30
Propyl gallate (1, 1)	AOO	4.95	33.60
DNCB (10, 5)	AOO	4.71, 7.86, 8.53, 9.23, 9.96, 10.89, 11.97, 12.60, 13.18, 15.14	23.00, 24.00, 26.80, 36.70, 49.60
CMI (1, 1)	DMF	7.50	22.70
Diethyl maleate (1, 1)	AOO	3.78	22.60
Glutaraldehyde (4, 1)	ACE	2.57, 3.39, 5.00, 6.45	18.00
HCA (18, 14)	AOO	3.51, 3.88, 3.92, 3.97, 4.44, 4.47, 4.82, 5.11, 5.41, 5.50, 5.71, 5.78, 6.45, 6.47, 7.09, 7.60, 8.42, 10.22	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
Eugenol (1, 12)	AOO	7.07	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 (1, 36)	AOO	12.36	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
Resorcinol (1, 2)	AOO	4.33	10.40, 12.50
Benzalkonium chloride (1, 1)	AOO / ACE	6.68	11.10
Potassium dichromate (5, 13)	DMSO	4.08, 4.78, 5.49, 6.01, 6.37	2.12, 5.40, 6.90, 10.10, 10.10, 10.40, 11.20, 13.00, 13.10, 16.10, 16.10, 19.10, 33.60
Citral (1, 4)	AOO	4.40	4.70, 6.20, 9.30, 20.50
Hydroxycitronellal (1, 1)	AOO	5.69	8.50
Cinnamic aldehyde (1, 4)	AOO	4.73	1.80, 7.60, 15.80, 18.40
EGDMA (1, 1)	MEK	4.45	7.00
Phenyl benzoate (1, 2)	AOO	4.24	3.50, 11.10

continued

Table 3-3 Maximum SI Values of 44 Substances Evaluated in the LLNA: DA Compared to Traditional LLNA Tests with Similar Doses[1] (continued)

Substance Name[2]	Test Vehicle[3]	LLNA: DA Maximum SI Values[4]	Traditional LLNA Maximum SI Values
Sensitizers (LLNA: DA SI ≥ 1.8 and Traditional LLNA SI ≥ 3.0)			
Cinnamic alcohol (1, 1)	AOO	5.66	5.70
Butyl glycidyl ether (1, 1)	AOO	4.59	5.60
Imidazolidinyl urea (1, 1)	DMF	4.67	5.50
Abietic acid (4, 1)	AOO	3.98, 4.64, 6.26, 7.96	5.20
Trimellitic anhydride (1, 1)	AOO	4.96	4.60
Sodium lauryl sulfate (1, 7)	DMF	3.39	1.60, 2.60, 4.10, 5.10, 5.10, 5.40, 8.90
Formaldehyde (4, 1)	ACE	2.69, 3.18, 4.84, 5.10	4.00
Ethyl acrylate (1, 1)	AOO	4.29	3.98
MBT (1, 5)	DMF	**2.00**	4.60, 9.10, 9.50, 10.80, 17.10
Cobalt chloride (6, 1)	DMSO	**2.01**, 2.54, 3.64, 4.25, 8.07, 20.55	7.21
3-Aminophenol (3, 1)	AOO	1.76, **2.38, 2.83**	5.70
Methyl methacrylate (1, 1)	AOO	**1.81**	3.60
Ni (II) sulfate hexahydrate (7, 1)	DMSO	0.79, 1.24, 1.52, 1.56, **2.13**, 3.49, 11.78	3.10
Traditional LLNA Nonsensitizers (SI < 3.0) with Borderline Positive SI Values in LLNA: DA (1.8 < SI <2.5; see bold text)			
Salicylic acid (1, 1)	AOO	**2.00**	2.50
Hexane (1, 1)	AOO	**2.31**	2.20
Chlorobenzene (1, 1)	AOO	**2.44**	1.70
Nonsensitizers (LLNA: DA SI < 1.8 and Traditional LLNA SI < 3.0)			
Ni (II) chloride (1, 1)	DMSO	1.30	2.40
Lactic acid (5, 1)	DMSO	0.91, 0.93, 0.97, 0.99, 1.06	2.20
Methyl salicylate (4, 7)	AOO	0.83, 1.20, 1.55, 1.77	0.90, 1.10, 1.72, 1.90, 2.10, 2.30, 2.90
Isopropanol (11, 1)	AOO	0.70, 0.76, 0.91, 1.01, 1.08, 1.21, 1.25, 1.45, 1.54, 1.57, **1.97**	1.70
Diethylphthalate (1, 1)	AOO	1.09	1.50
Propylparaben (1, 1)	AOO	1.28	1.40
1-Bromobutane (1, 1)	AOO	1.65	1.00

continued

Table 3-3 Maximum SI Values of 44 Substances Evaluated in the LLNA: DA Compared to Traditional LLNA Tests with Similar Doses[1] (continued)

Substance Name[2]	Test Vehicle[3]	LLNA: DA Maximum SI Values[4]	Traditional LLNA Maximum SI Values
Nonsensitizers (LLNA: DA SI < 1.8 and Traditional LLNA SI < 3.0)			
Dimethyl isophthalate (4, 1)	AOO	0.89, 1.00, 1.26, 1.34	1.00
Sulfanilimide (1, 1)	DMF	0.86	1.00

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); CMI = 5-Chloro-2-methyl-4-isothiazolin-3-one; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; DNCB = 2,4-dinitrochlorobenzene; EGDMA = ethylene glycol dimethacrylate; HCA = hexyl cinnamic aldehyde; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; MBT = 2-mercaptobenzothiazole; MEK = methyl ethyl ketone; NA = not available; Ni = nickel; SI = stimulation index.

[1] LLNA: DA and traditional LLNA tests at similar doses are shown and correspond to the same data depicted in **Figure 3-1**.

[2] Numbers in parentheses beside the substance names indicate the number of tests for the LLNA: DA followed by the traditional LLNA, which may differ from the total number of tests available since only tests with similar doses were included.

[3] The vehicle used was the same in LLNA: DA and traditional LLNA tests except for one substance, and in this case (for benzalkonium chloride) the first entry is the vehicle used for the LLNA: DA, and the second entry is for the traditional LLNA.

[4] The bold text indicates LLNA: DA tests with maximum SI values between 1.8 and 2.5.

3.5 Test Method Reliability (Intra- and Interlaboratory Reproducibility)

The BRD details the evaluation of intra- and interlaboratory reproducibility of the LLNA: DA test method (see **Section 7.0** of **Appendix C**). Intralaboratory reproducibility was assessed using a coefficient of variation (CV) analysis of EC3 (estimated concentration needed to produce an SI of 3.0) and EC1.8 values (estimated concentration needed to produce an SI of 1.8) for isoeugenol and eugenol (each substance was tested in three different experiments). The mean EC3 values and corresponding CVs for isoeugenol and eugenol were 2.74% ± 0.58% with a 21% CV, and 5.06% ± 0.55%, with an 11% CV, respectively. The mean EC1.8 values and corresponding CVs for isoeugenol and eugenol were 0.87% ± 0.31% (36% CV), and 3.38% ± 0.79% (23% CV), respectively.

Qualitative analyses of LLNA: DA reproducibility were conducted in both phases of an interlaboratory validation study, using SI ≥ 1.8 as the threshold to distinguish sensitizers from nonsensitizers. In the first phase (n = 12 substances [nine sensitizers and three nonsensitizers based on traditional LLNA test results] tested in three or 10 laboratories) there was 100% agreement among the laboratories for 10 substances (seven sensitizers and three nonsensitizers based on traditional LLNA test results). There was 67% (2/3) agreement among the tests for the remaining two traditional LLNA sensitizers. The interlaboratory CV values for the EC1.8 values for eight of the nine traditional LLNA sensitizers ranged from 15% to 140%. The interlaboratory CV value for the EC1.8 values for the traditional LLNA sensitizer nickel (II) sulfate hexahydrate could not be calculated since an EC1.8 value was only available from one of the three laboratories that tested it.

In the second phase (n = 5 substances [four sensitizers and one nonsensitizer based on traditional LLNA test results] tested in four or seven laboratories) there was 100% agreement among the

laboratories for four substances (three sensitizers and one nonsensitizer based on traditional LLNA results). There was 75% (3/4) agreement among the tests for the remaining traditional LLNA sensitizer. Interlaboratory CV values for the EC1.8 values of the four traditional LLNA sensitizers ranged from 14% to 93%.

There were 14 substances with multiple tests across the two phases of the interlaboratory validation study that could be used for analyses of reproducibility when using SI ≥ 1.8 to identify potential sensitizers. The SI results for 80% (8/10) of the sensitizers (based on traditional LLNA results) were 100% concordant in the LLNA: DA (i.e., all tests for that substance yielded maximum SI ≥ 1.8) (**Table 3-4**). The two traditional LLNA sensitizers with LLNA: DA tests that yielded maximum SI values less than 1.8 were 3-aminophenol and nickel (II) sulfate hexahydrate. The SI results for 75% (3/4) of the nonsensitizers (based on traditional LLNA results) were 100% concordant in the LLNA: DA (i.e., all tests for that substance yielded SI < 1.8). The concordance of the other nonsensitizer, isopropanol, was 91% (10/11).

Table 3-4 Concordance of LLNA: DA Tests for Substances with Multiple Tests Based on Maximum SI Category

Substance Name	LLNA: DA Nonsensitizers (Maximum SI < 1.8)[1]	LLNA: DA Sensitizers (SI ≥ 1.8)		Total Tests
		1.8 < Maximum SI < 2.5[1]	Maximum SI ≥ 2.5[1]	
Sensitizers[2]				
Abietic acid	0 (0%)	0 (0%)	4 (100%)	4
3-Aminophenol	1 (33.3%)	1 (33.3%)	1 (33.3%)	3
Cobalt chloride	0 (0%)	1 (12.5%)	7 (87.5%)	8
2,4-Dinitrochlorobenzene	0 (0%)	0 (0%)	11 (100%)	11
Formaldehyde	0 (0%)	0 (0%)	4 (100%)	4
Glutaraldehyde	0 (0%)	0 (0%)	4 (100%)	4
Hexyl cinnamic aldehyde	0 (0%)	0 (0%)	18 (100%)	18
Isoeugenol	0 (0%)	0 (0%)	4 (100%)	4
Nickel (II) sulfate hexahydrate	4 (50%)	2 (25%)	2 (25%)	8
Potassium dichromate	0 (0%)	0 (0%)	5 (100%)	5
Nonsensitizers[2]				
Dimethyl isophthalate	4 (100%)	0 (0%)	0 (0%)	4
Isopropanol	10 (91%)	1 (9%)	0 (0%)	11
Lactic acid	5 (100%)	0 (0%)	0 (0%)	5
Methyl salicylate	4 (100%)	0 (0%)	0 (0%)	4

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; 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] Based on traditional LLNA test results.

3.6 Animal Welfare Considerations: Reduction, Refinement, and Replacement

The LLNA: DA will use the same number of animals as the updated ICCVAM-recommended traditional LLNA test method 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: DA may lead to further reduction in use of the guinea pig tests, which would provide for reduced animal use and increased refinement by avoiding the discomfort that can occur in the guinea pig tests when substances cause ACD. Additionally, the LLNA: DA test method protocol requires fewer mice per treatment group (a minimum of four animals per group) than either of the guinea pig 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: DA included two public review meetings by an independent scientific peer review panel, multiple opportunities for public comments (see **Section 1.0**), consideration of reports from an OECD Expert Consultation, 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: DA. This section summarizes the ICCVAM consideration of these reports and comments. The Panel reports and public comments are provided in **Appendices D** and **F**.

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: DA 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 Expert Consultation viewed that despite certain limitations, the LLNA: DA 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. Like the Panel, OECD member country experts questioned the regulatory utility of the LLNA: DA since specific guidance on how to classify substances with SI values in the range of uncertainty has yet to be 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 $\geq$ 1.8 provided 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 1.8 and 2.5) as potential skin sensitizers. Additionally, the OECD Expert Consultation agreed that the use of the LLNA: DA might not be appropriate for testing substances that affect ATP levels (e.g., substances that function as ATP inhibitors) or those that affect the accurate measurement of intracellular ATP (e.g., presence of ATP degrading enzymes, presence of extracellular ATP in the lymph node).

ICCVAM considered the Panel report and the OECD Expert Consultation recommendations, and concluded that the single SI decision criterion of SI ≥ 1.8 to classify sensitizers would avoid false negative results as well as indeterminate results, which are not useful for regulatory purposes. Borderline positive results that may occur between 1.8 and 2.5 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 (see **Appendix D2**). 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 (ICCVAM 2009a), a prescreen test should be performed in order to define the appropriate dose level to test in the LLNA: DA. The Panel and the OECD Expert Consultation agreed in principle with ICCVAM that use of a reduced LLNA: DA test method protocol instead of the multi-dose LLNA: DA 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 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 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 ≥ 1.8, the OECD Expert Consultation recommended that borderline positive results (i.e., SI values between 1.8 and 2.5) 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 1.8 and 2.5 in the LLNA: DA 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 that the LLNA: DA 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: DA 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
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

continued

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

Table 4-1 Opportunities for Public Comments (continued)

Opportunities for Public Comments	Date	Number of Public Comments Received
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 ICCVAM draft 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: DA.

1. One commenter indicated that several nonradioactive detection methods for the LLNA (e.g., bromodeoxyuridine [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: DA. 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: DA 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: DA.

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 revised 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.

One written comment was relevant to the LLNA: DA.

1. The commenter indicated that beyond the method to assess lymph node cell proliferation, the test method protocol for the LLNA: DA contained several key deviations from the OECD TG 429 recommended protocol and the essential test method components as described in the January 2008 draft ICCVAM-recommended LLNA performance standards (i.e., major modifications from the traditional LLNA in both the test substance treatment and sampling schedule). The commenter viewed that the LLNA: DA should not be considered for validation as an alternative to the traditional LLNA since the modifications extended beyond the specifications in the January 2008 draft ICCVAM-recommended LLNA performance standards.

- The validation studies for the LLNA: DA test method were completed prior to the development of LLNA performance standards and thus, the ICCVAM-recommended LLNA performance standards were not used to evaluate the LLNA: DA. Further, despite the differences between the LLNA: DA test method protocol and the traditional LLNA test method protocol, ICCVAM concurs with the Panel that the LLNA: DA is mechanistically and functionally similar to the traditional LLNA and therefore the LLNA performance standards would otherwise be applicable.

Two oral comments were relevant to the LLNA: DA.

1. One commenter agreed with ICCVAM that the LLNA: DA (and also the LLNA: BrdU by enzyme-linked immunosorbent assay [ELISA]) should be evaluated separately because of different treatment schedules. The commenter also questioned whether the extra topical dose in the LLNA: DA was necessary, and expressed concern that additional doses may cause skin irritation. For this reason, the commenter suggested that the SI should be evaluated at earlier sample times and without SLS pretreatment.

- Yamashita et al. (2005) examined the effect of various dosing regimens on the SI value produced in the LLNA: DA. The fourth topical application of test substance was required for sensitizers to produce SI $\geq$ 3.0.
- The effect of SLS pretreatment on the SI values of selected substances is presented in the final BRD (**Annex I** of **Appendix C**) and Idehara et al. (2008). Briefly, the data indicated that the calculated EC3 values were lower for substances pretreated with an aqueous solution of 1% SLS than for substances not pretreated with an aqueous solution of 1% SLS. This included some weak sensitizers for which an enhanced response would be important to detect.
- The SLS pretreatment constitutes application of a 1% aqueous solution, which does not induce excessive local skin irritation. SLS is an irritant in mice at 10% in *N,N,*-dimethylformamide (Antonopoulos et al. 2008).

2. Another commenter cited data from Ullmann (2002) that indicates differences in 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 showed that CBA/JNCrj mice had markedly lower responses compared to the other strains tested, which may explain the negative result for 2-mercaptobenzothiazole produced by the LLNA: DA test method.

- Validation studies for the LLNA: DA were conducted exclusively with the CBA/JNCrlj strain, which is therefore considered the preferred strain. There were insufficient LLNA: DA data in multiple strains to allow for an evaluation of potential strain differences.

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 comment 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, 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 (**Appendix F3**).

There were no public comments specific to the LLNA: DA.

Regarding the LLNA: DA, one SACATM member indicated that it was uncertain whether the test method would perform well for mixtures, metals, or aqueous solutions.

- As outlined in the test method recommendations, ICCVAM considers the applicability domain for the LLNA: DA 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: DA. However, inconsistent results for nickel sulfate in the LLNA: DA suggest that the LLNA: DA may not be suitable for testing nickel compounds. Therefore, ICCVAM recommends the accrual of additional data from LLNA: DA studies on such nickel compounds with comparative human and/or guinea pig data in order to more comprehensively evaluate the suitability of the LLNA: DA for testing nickel compounds.

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, and revised draft test method protocols 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: DA.

1. The commenter supported the revised draft ICCVAM recommendation that the LLNA: DA can be used for ACD testing with specific defined limitations in the decision criteria. The commenter viewed that substances falling within the intermediate SI (i.e., when maximum SI results were between the SI decision criteria for sensitizers and nonsensitizers) 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 $\geq$ 1.8, to classify potential sensitizers. However, ICCVAM recommends that borderline positive results (i.e., SI values between 1.8 and 2.5) should be evaluated with other available 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, other testing data) to confirm that such results are positive.

The commenter further noted that the Panel recommended that the LLNA: DA 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.

- As outlined in the test method recommendations, ICCVAM considers the applicability domain for the LLNA: DA 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: DA. However, inconsistent results for nickel sulfate in the LLNA: DA suggest that the LLNA: DA may not be suitable for testing nickel compounds. Therefore, ICCVAM recommends the accrual of additional data from LLNA: DA studies on such nickel compounds with comparative human and/or guinea pig data in order to more comprehensively evaluate the suitability of the LLNA: DA for testing nickel compounds.

One oral comment was relevant to the LLNA: DA.

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: DA 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 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: DA.

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 guinea pig test methods where radioactivity is prohibited.

The commenter also indicated that for the LLNA: DA an explanation of the use of SLS was needed.

- As indicated in Section 2.0 of the final ICCVAM BRD (**Appendix C**), 1% SLS pretreatment is used in the LLNA: DA because various researchers have shown that an aqueous solution of 1% SLS does not elicit a positive response in the traditional LLNA but when applied prior to test substance administration there is generally an increased response compared to the test substance alone (van Och et al. 2000; De Jong et al. 2002).

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 (**Appendix F4**).

There were no public comments specific to the LLNA: DA.

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

Regarding the LLNA: DA, one SACATM member did not consider ATP content to be an accurate measure of lymphocyte proliferation and therefore considered methods that use BrdU incorporation (i.e., LLNA: BrdU-ELISA and LLNA: BrdU by flow cytometry) to be higher priority for moving forward.

- Measuring ATP content by bioluminescence, as is done in the LLNA: DA by the luciferin-luciferase assay, is known to correlate with living cell number (Crouch et al. 1993) and therefore indicates an increased number of proliferating cells in the draining auricular lymph nodes (Ishizaka et al. 1984; Dexter et al. 2003). As indicated in Section 2.0 of the final ICCVAM BRD (**Appendix C**), the emitted light intensity (measured using a luminometer) is linearly related to the ATP concentration and the luciferin-luciferase assay is a sensitive method for ATP quantitation used in a wide variety of applications (Lundin 2000).

Another SACATM member asked if the SLS pretreatment had ever been validated.

- Annex I of the final ICCVAM BRD (**Appendix C**) and Idehara et al. (2008) provide comparative results in the LLNA: DA for a number of substances tested both with and without SLS pretreatment. Briefly, the data indicate that the calculated EC3 values were lower for substances pretreated with SLS than for substances not pretreated with SLS. This included some weak sensitizers for which an enhanced response would be important to detect.

Another SACATM member indicated that the use of two SI decision criteria in the LLNA: DA (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 $\geq$ 1.8 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 $\geq$ 1.8 as the decision criterion results in a false positive rate of 25% (3/12) compared to the traditional LLNA. Since the three false positive substances in the LLNA: DA produced SI values between 1.8 and 2.5, 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, other testing data) to confirm that results in this SI range are positive.

Another 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.

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Appendix A

Timeline for ICCVAM Evaluation of the LLNA: DA

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January 10, 2007	ICCVAM receives nomination from CPSC for seven LLNA review activities,[1] including evaluation of the LLNA: DA test method.
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) – *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*.
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: DA).

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

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: DA.
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: DA.
March 2010	ICCVAM endorses the TMER for the LLNA: DA, 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: DA.

Abbreviations: BRD = background review document; CPSC = U.S. Consumer Product Safety Commission; ECVAM = European Centre for the Validation of Alternative Methods; FR = *Federal Register*; ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; IWG = Immunotoxicity Working Group; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; 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.

Appendix B

ICCVAM-Recommended Test Method Protocol: The Murine Local Lymph Node Assay: DA, a Nonradioactive Alternative Test Method to Assess 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: Modified by Daicel Chemical Industries, Ltd., Based on ATP Content (LLNA: DA)

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: DA[1] was therefore developed as a nonradioactive modification to the LLNA that measures increases in ATP content in the lymph node as an indicator of the cell number at the end of cell proliferation (Yamashita et al. 2005; Idehara et al. 2008). 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: DA provides quantitative data suitable for dose-response assessment. 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: DA 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 ≥1.8 before a test substance can be considered as a skin sensitizer with specific limitations for borderline positive results (i.e., SI between 1.8 and 2.5) as described in Section 3 of this Test Method Evaluation Report.

The methods described here are based on the use of measuring ATP content by luciferin-luciferase assay to indicate an increased number of proliferating cells in the draining auricular lymph nodes. The luciferin-luciferase assay is a sensitive method for ATP quantitation used in a wide variety of applications (Lundin 2000). It utilizes the luciferase enzyme to catalyze the formation of light from ATP and luciferin according to the following reaction:

$$ATP + Luciferin + O_2 \xrightarrow{Luciferase} Oxyluciferin + AMP + PP_i + CO_2 + Light$$

The emitted light intensity is linearly related to the ATP concentration and is measured using a luminometer. A concurrent positive control is added to each assay to provide an indication of appropriate assay performance.

2.0 Description of the LLNA: DA

2.1 Sex and Strain of Animals

The mouse is the species of choice for the LLNA: DA. Validation studies for the LLNA: DA were conducted exclusively with young adult female mice (nulliparous and non-pregnant) of the CBA/JNCrlj strain, and therefore these are the recommended sex and mouse strain.[2] At the start of the

[1] Daicel Chemical Industries, Ltd., Japan.

[2] Male mice and other substrains of CBA mice (e.g., CBA/Ca or CBA/J) may be used if it is sufficiently demonstrated that these animals perform as well as female CBA/JNCrlj mice in the LLNA: DA.

study, mice should be 8-12 weeks of age. All 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 reveal 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), *N*,*N*-dimethyl-formamide (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 wets the skin and does not immediately run off by incorporation of appropriate solubilizers (e.g., 1% Pluronic® L92). 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: DA response resulting in an SI that is at least 1.8 over that observed in 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 25% hexyl cinnamic aldehyde (HCA; Chemical Abstracts Service Registry Number [CASRN] 101-86-0) or 10% eugenol (CASRN 97-53-0) in acetone: olive oil (4:1 v/v). 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., acetone: olive oil), there may be certain regulatory situations in which testing in a nonstandard 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: DA regularly (i.e., conduct the LLNA: DA 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: DA 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: DA (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 < 1.8 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: DA
- 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: DA experimental procedure is performed as follows:

Day 1. Individually identify and record the weight of each animal and any clinical observations. Apply 1% sodium lauryl sulfate (SLS) aqueous solution to the dorsum of each ear by using a brush dipped in the SLS solution to cover the entire dorsum of each ear with four to five strokes. One hour after the SLS treatment, 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, 3, and 7. Repeat the 1% SLS aqueous solution pretreatment and test substance application procedure carried out on Day 1.

Days 4, 5, and 6. No treatment.

Day 8. Record the weight of each animal and any clinical observations. Approximately 24 to 30 hours after the start of application on Day 7, 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 the draining auricular lymph nodes from each mouse ear and process separately in phosphate buffered saline for each animal. Details and diagrams of the node identification and dissection can be found in **Annex I**.

A single-cell suspension of lymph node cells (LNC) excised bilaterally from each mouse is prepared by sandwiching the lymph nodes between two glass slides and applying light pressure to crush the nodes. After confirming that the tissue has spread out thinly pull the two slides apart. Suspend the tissue on both slides in phosphate buffered saline (PBS) by holding each slide at an angle over the petri dish and rinsing with PBS while concurrently scraping the tissue off of the slide with a cell scraper. A total volume of 1 mL PBS should be used for rinsing both slides. The tissue suspension in the petri dish should be homogenized lightly with the cell scraper. A 20 µL aliquot of the

homogenized suspension is then collected with a micropipette and mixed with 1.98 mL PBS to yield a 2 mL sample. This procedure is repeated so that two samples per animal are collected for immediate ATP measurement.

ATP is measured by the luciferin/luciferase method using a commercially available ATP measurement kit that measures bioluminescence in relative luminescence units (RLU). Follow the instructions in the assay kit. The assay timeframe from animal sacrifice to measurement of ATP content for each individual animal should be uniform, within approximately 30 minutes, because the ATP content is considered to gradually decrease with time after animal sacrifice (Idehara et al. 2008). Thus, the series of procedures from excision of auricular lymph nodes to ATP measurement should be completed within 20 minutes by the predetermined time schedule that is the same for each animal. ATP luminescence should be measured in each 2 mL sample so that a total of two ATP measurements are collected for each animal. The mean ATP luminescence is then determined and used in subsequent calculations.

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.

2.6 Reduced LLNA

Using this test method protocol, there is also the opportunity to perform a reduced LLNA: DA (rLLNA: DA). Use of the rLLNA: DA has the potential to reduce the number of animals by omitting the middle and low dose groups from the LLNA: DA (Kimber 2006; ESAC 2007; ICCVAM 2009b). This is the only difference between the LLNA: DA and the rLLNA: DA. Thus, the test substance concentration evaluated in the rLLNA: DA should be the maximum concentration that does not induce overt systemic toxicity and/or excessive local irritation in the mouse (**Annex II**). The rLLNA: DA 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: DA 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 for euthanasia those mice exhibiting systemic toxicity, excessive irritation, or corrosion of skin (OECD 2000).

3.0 Calculation of Results

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

The decision process regards a result as positive when SI $\geq$ 1.8 (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., pairwise

dosed group versus concurrent solvent/vehicle control comparisons). Statistical analyses may include, for instance, linear regression or Williams' 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 1.8 and 2.5 are obtained. This is based on the validation database of 44 substances using an SI ≥ 1.8 for which the LLNA: DA correctly identified all 32 LLNA sensitizers, but incorrectly identified three of 12 LLNA nonsensitizers with SI values between 1.8 and 2.5 (i.e. borderline positive) (see Section 3.0 of this Test Method Evaluation Report). If an SI value between 1.8 and 2.5 is obtained, other available information such as 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 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 (25% HCA or 10% eugenol) should be equal to or greater than 1.8. 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 RLU values, the group mean RLU/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 (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 RLU values and SI values for each treatment group
- Mean and associated error term (e.g., SD, SEM) for RLU/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

6.0 References

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Annex I

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

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1.0 Background

Although minimal technical training of the LLNA: DA 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: DA 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: DA.

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 acetone: olive oil vehicle (4:1). 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.

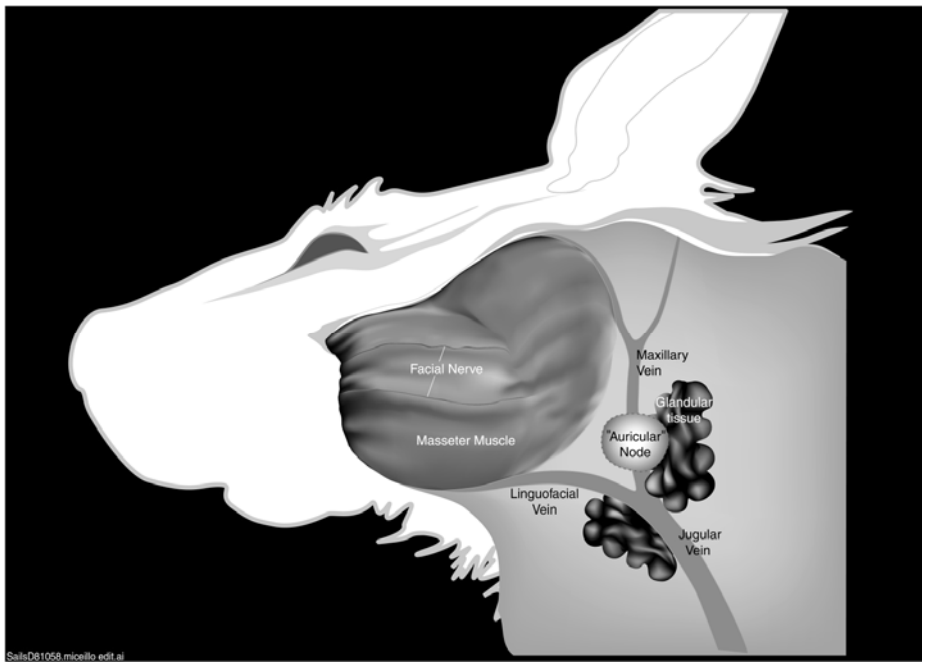
3.2 Ventral Dissection (Figure B-I-2)

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

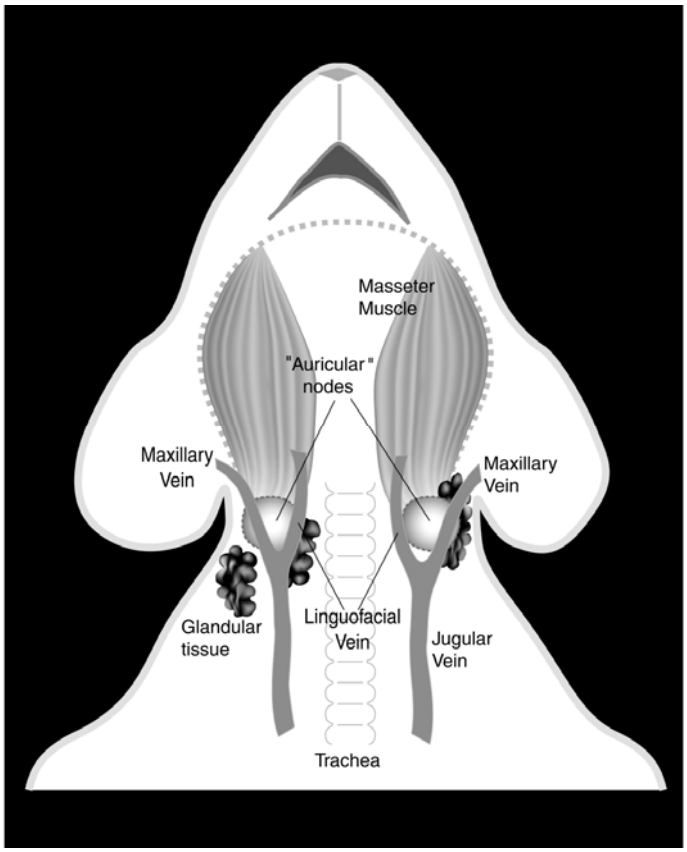
The nodes can be distinguished from glandular and connective tissue in the area by the uniformity of the nodal surface and a shiny translucent appearance. Application of sensitizing agents (especially the strong sensitizers used in training) will cause enlargement of the node size. If a dye is injected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

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Evaluating Local Irritation and Systemic Toxicity in the LLNA: DA

As noted in the ICCVAM-recommended LLNA: DA test method protocol, the maximum dose tested should be the maximum possible concentration that does not produce systemic toxicity and/or excessive local skin 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: DA.

The prescreen test is conducted under identical conditions as the main LLNA: DA study, except there is no assessment of lymph node 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 and/or local skin irritation at the application site. Body weights are recorded pretest and prior to termination (Day 8). 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), Day 7 (24 hours prior to termination), and Day 8 (termination). Additionally on Day 8, 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: DA 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	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: DA:

- 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 8
- Mortality

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

Appendix C

Final Background Review Document:

The Nonradioactive Murine Local Lymph Node Assay: DA

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Background Review Document
Nonradioactive Murine Local Lymph Node Assay: DA

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
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March 2010

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List of Abbreviations and Acronyms

ACD	Allergic contact dermatitis
ACE	Acetone
Anim.	Animal
ANOVA	Analysis of variance
AOO	Acetone: olive oil (4:1)
aq.	Aqueous
BRD	Background review document
Calc.	Calculated
CASRN	Chemical Abstracts Service Registry Number
CPSC	U.S. Consumer Product Safety Commission
CI	Confidence interval
Conc.	Concentration
CV	Coefficient of variation
Cys	Cysteine-containing peptide
DMF	*N,N*-dimethylformamide
DMSO	Dimethyl sulfoxide
EC1.8	Estimated concentration needed to produce a stimulation index of 1.8
EC2	Estimated concentration needed to produce a stimulation index of two
EC2.5	Estimated concentration needed to produce a stimulation index of 2.5
EC3	Estimated concentration needed to produce a stimulation index of three
ECt	Estimated concentration needed to produce a stimulation index of a specified threshold
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
ECVAM	European Centre for the Validation of Alternative Methods
EPA	U.S. Environmental Protection Agency
FN	False negative
FP	False positive
GP	Guinea pig
GPMT	Guinea pig maximization test
HMT	Human maximization test
HPTA	Human patch test antigen
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IDR	Insufficient dose response
ILS	Integrated Laboratory Systems
ISO	International Organization for Standardization
IWG	Immunotoxicity Working Group
JaCVAM	Japanese Center for the Validation of Alternative Methods

K_{ow}	Estimated log octanol-water partition coefficient
LLNA	Murine local lymph node assay
LLNA: DA	Murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content
LLNA: BrdU-ELISA	Murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine
MEK	Methyl ethyl ketone
MHLW	Ministry of Health, Labour and Welfare (Japan)
Min	Minimal
Mod	Moderate
Mol.	Molecular
NA	Not applicable
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NR	Not reported
NT	Not tested
OECD	Organisation for Economic Co-operation and Development
PBS	Phosphate buffered saline
PC	Positive control
Ref.	Reference
rLLNA: DA	Reduced murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content
RLU	Relative luminescence units
SD	Standard deviation
SI	Stimulation index
SLS	Sodium lauryl sulfate
Stats.	Statistics
TG	Test guideline
Trad.	Traditional
U.S.	United States
Unk	Unknown
VC	Vehicle control
Veh.	Vehicle
vs.	Versus

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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 and ICCVAM test method recommendations.

A comprehensive draft background review document (BRD) 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 existing 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 modified by Daicel Chemical Industries, Ltd., based on ATP content (referred to hereafter as the "LLNA: DA") 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: DA 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 and then the final draft was

[1] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf.
[2] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2009.pdf.

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 on Alternative Toxicological Methods (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: DA.

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 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 many, but not all, 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 international 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.[3] 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.[4] 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 modified by Daicel Chemical Industries, Ltd., based on ATP content in the draining auricular lymph nodes (referred to hereafter as the "LLNA: DA").

Test Method Protocol

Daicel Chemical Industries, Ltd. developed the LLNA: DA test method based on modifications to the traditional LLNA (Yamashita et al. 2005). While the traditional LLNA assesses cell proliferation by measuring the incorporation of radioactivity into the DNA of dividing lymph node cells, the LLNA: DA assesses cell proliferation by measuring increases in ATP content in the lymph node as an indicator of the cell number at the end of cell proliferation. The LLNA: DA also differs from the traditional LLNA in the timing and administration of the test substance. In the traditional LLNA, the test substance is applied on days 1, 2, and 3 and the auricular lymph nodes are excised on day 6. In the LLNA: DA, the test substance is applied on days 1, 2, 3, and 7 and the auricular lymph nodes are excised on day 8. Furthermore, one hour prior to each application of the test substance, 1% aqueous solution of sodium lauryl sulfate is applied to increase absorption of the test substance through the skin. A stimulation index (SI) is used to identify a substance as a sensitizer (the ratio of the mean ATP content of the substance treatment group to the mean ATP content of the vehicle treatment group).

Validation Database

The accuracy and reliability of the LLNA: DA were assessed using data submitted to NICEATM for 45 substances tested in one laboratory (Idehara et al. 2008; Idehara unpublished) and 14 substances

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[3] Hhttp://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdfH.

[4] Hhttp://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdfH.

tested in a two-phased interlaboratory validation study (17 laboratories) (Omori et al. 2008). Of the 14 substances tested in the two-phased interlaboratory study (Omori et al. 2008) only one was different from the 45 substances tested initially (Idehara et al. 2008; Idehara unpublished). Thus, data were available for 46 unique substances tested in the LLNA: DA. The reference test data for these substances were obtained from the traditional LLNA, GP skin sensitization tests, and/or human skin sensitization tests. One substance, benzocaine, yielded both positive and negative results in the traditional LLNA (ICCVAM 1999) and therefore was not considered in the performance evaluation of the LLNA: DA. LLNA studies for another substance, toluene 2,4-diisocyanate (van Och et al. 2000), were not conducted according to the traditional LLNA test method protocol described (ICCVAM 1999; Dean et al. 2001). Thus of the 46 substances with LLNA: DA data, 44 substances had adequate traditional LLNA data (32 were classified by the traditional LLNA as skin sensitizers and 12 were classified as nonsensitizers).

Test Method Accuracy

The accuracy evaluation in this BRD includes the evaluation of multiple decision criteria, including the SI ≥ 3.0 recommended by the test method developer. Based on the evaluation of multiple decision criteria, the optimal performance was achieved using SI ≥ 1.8 to classify potential skin sensitizers. Compared to the traditional LLNA, accuracy was 93% (41/44), with a false positive rate of 25% (3/12), and a false negative rate of 0% (0/32). The three false positive substances produced SI values between 1.8 and 2.5 in the LLNA: DA.

When the decision criterion of SI ≥ 3.0 was used to classify sensitizers versus nonsensitizers, compared to the traditional LLNA, accuracy was 91% (40/44), with a false positive rate of 0% (0/12), and a false negative rate of 13% (4/32). Among the four discordant substances, no unique characteristics were identified that could be used as rationale for excluding any particular types of substances from testing in the LLNA: DA.

The reduced LLNA: DA (rLLNA: DA), which uses only the highest dose of the test substance that does not elicit excessive skin irritation and/or systemic toxicity, has the potential to reduce animal use by up to 40% for hazard classification purposes when dose-response information is not needed. Using SI ≥ 1.8 to classify potential sensitizers for 123 individual tests which used multiple doses, overall accuracy of the rLLNA: DA compared to the multi-dose LLNA: DA was 98% (121/123), with a false positive rate of 0% (0/33) and a false negative rate of 2% (2/90). The two tests that were false negative in the rLLNA: DA were borderline positive in the LLNA: DA at a concentration lower than the highest dose (maximum SI = 1.97 and 2.00). The highest dose tested for each of the two tests of the two substances was 50%.

Test Method Reliability – Intralaboratory Reproducibility

Intralaboratory reproducibility for the LLNA: DA was assessed using data for two substances (isoeugenol and eugenol) that were tested at varying concentrations in three different experiments. The coefficient of variation (CV) for the reproducibility of the EC3 values (estimated concentration needed to produce an SI of three) for isoeugenol and eugenol was 21% and 11%, respectively. The CV for the reproducibility of the EC1.8 values (estimated concentration needed to produce an SI of 1.8) for isoeugenol and eugenol was 36% and 23%, respectively.

Test Method Reliability – Interlaboratory Reproducibility

This BRD includes a reproducibility analysis using SI ≥ 1.8 to identify potential sensitizers. The two-phased multilaboratory validation study included 17 different laboratories in which 14 different substances were examined. In the first phase of the study, 10 laboratories each tested up to 12 substances, while in the second phase of the study seven laboratories (different from the 10 laboratories in the first phase of the interlaboratory validation study) each tested up to five substances (2/5 substances unique compared to the first phase). In both studies, each substance was tested once at

three different doses, which were provided to the participating laboratories by the validation study management team.

When using SI ≥ 1.8 as the decision criterion, the qualitative (positive/negative) interlaboratory concordance analysis for the 12 substances that were tested in up to 10 laboratories during the first phase of the LLNA: DA interlaboratory validation study resulted in 100% (3/3 or 10/10) concordance for 9 substances (seven sensitizers and two nonsensitizers in the traditional LLNA), 90% (9/10) concordance for one substance (one nonsensitizer in the traditional LLNA), and 67% (2/3) concordance for two substances (two sensitizers in the traditional LLNA). The coefficient of variation (CV) values for the estimated concentration needed to produce a stimulation index of 1.8 (EC1.8) values ranged from 15% (abietic acid) to 140% (isoeugenol) and the mean CV was 71%. The qualitative interlaboratory concordance analysis for the five substances tested in up to seven laboratories during the second phase of the validation study resulted in 100% (4/4 or 7/7) concordance for four substances (three sensitizers and one nonsensitizer in the traditional LLNA) and 75% (3/4) concordance for one substance (a sensitizer in the traditional LLNA). The CV values for the EC1.8 values ranged from 14% (hexyl cinnamic aldehyde) to 93% (cobalt chloride) and the mean CV was 49%.

When using SI ≥ 1.8 to classify potential sensitizers, the tally of concordant tests for the 14 substances with multiple LLNA: DA tests indicated that the SI results for 80% (8/10) of the sensitizers (based on traditional LLNA results) were 100% concordant in the LLNA: DA (i.e., all tests for that substance yielded maximum SI ≥ 1.8). The concordance of the other two sensitizers (based on traditional LLNA results) was 50% (4/8) to 67% (2/3) for SI ≥ 1.8. The SI results for 75% (3/4) of the nonsensitizers (based on traditional LLNA results) were 100% concordant in the LLNA: DA (i.e., all tests for that substance yielded maximum SI ≤ 1.8). The concordance of the other nonsensitizer (based on traditional LLNA results) was 91% (10/11) for SI ≤ 1.8.

Animal Welfare Considerations

The LLNA: DA will use the same number of animals when compared to the updated ICCVAM-recommended LLNA protocol (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: DA 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: DA evaluates the induction phase of sensitization and therefore discomfort to animals associated with the elicitation phase is eliminated. Additionally, the LLNA: DA protocol requires fewer mice per treatment group (a minimum of four animals per group) than either of the guinea pig tests (10-20 animals/group for the Buehler test and 5-10 animals/group for the guinea pig maximization test [GPMT]).

Test Method Transferability

The transferability of the LLNA: DA was demonstrated by a two-phased interlaboratory validation study (Omori et al. 2008). Notably, the test method developer indicates that when the LLNA: DA test method is conducted, all the procedural steps from lymph node excision to the determination of ATP content should be performed without delay since ATP content decreases over time (Idehara et al. 2008; Omori et al. 2008). Compared to the traditional LLNA, the LLNA: DA will not require facilities, equipment, and licensing permits for handling radioactive materials. The level of training and expertise needed to conduct the LLNA: DA should be similar to the traditional LLNA except that the understanding and practice of luciferase methodology is required.

1.0 Introduction

1.1 Public Health Perspective

Allergic contact dermatitis (ACD) is a frequent occupational health problem that often results in lost workdays[5] and can significantly diminish quality of life (Hutchings et al. 2001; Skoet et al. 2003). 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 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 et al. 2001; Basketter et al. 2003; Jowsey et al. 2006).

1.2 Historical Background for the Murine Local Lymph Node Assay

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended that the murine local lymph node assay (LLNA) is a valid substitute for currently accepted guinea pig (GP) test methods to assess the ACD potential of many, but not all, 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 Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)-ICCVAM website.[6] 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.[7] 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 nomination. This BRD provides a comprehensive review of available data and information regarding the usefulness and limitations of one of these test methods, the LLNA modified by Daicel Chemical

[5] http://www.bls.gov/IIF

[6] http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf.

[7] Hhttp://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdfH.

Industries, Ltd., based on ATP content (referred to hereafter as the "LLNA: DA") in the draining auricular lymph nodes. 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.

A Panel reviewed the draft BRD in March 2008 to evaluate the extent to which the information contained in the draft 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, quantitative data for the test method, and an evaluation of interlaboratory reproducibility. In response to this recommendation, NICEATM obtained additional LLNA: DA 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: DA 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 this meeting, which was co-hosted by CPSC and NICEATM-ICCVAM. The expert group reviewed the draft OECD TG for the LLNA: DA and proposed responses to comments from member countries. 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 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 (the Scientific Advisory Committee on Alternative Toxicological Methods), 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: DA

Daicel Chemical Industries, Ltd. developed the LLNA: DA as a nonradioactive modification (Yamashita et al. 2005; Idehara et al. 2008) to the traditional LLNA. The traditional LLNA assesses cell proliferation by measuring the incorporation of radioactive thymidine or iodine into the DNA of dividing lymph node cells. In contrast, the LLNA: DA assesses increases in ATP content in the draining auricular lymph nodes by employing a luciferin-luciferase assay to measure bioluminescence. Since ATP content is linearly related to living cell number, this measurement serves as a surrogate for cell number at the time of sampling (Crouch et al. 1993).

This document provides:

- A comprehensive summary of the LLNA: DA 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: DA Test Method Protocol

This BRD includes the detailed standard operating procedure for the LLNA: DA test method that was used in the validation studies (**Annex I**). The LLNA: DA test method protocol (**Annex I**) differs from the ICCVAM-recommended test method protocol for the traditional LLNA (ICCVAM 2009) in the method used to assess lymphocyte proliferation in the auricular lymph nodes (**Table C-1**). In addition, there are substantive differences between the two test method protocols regarding test substance application and timing for the collection of the lymph nodes. In the traditional LLNA, the test substance is administered on three consecutive days (days 1, 2, and 3). On day 6, radiolabeled thymidine or iodine is administered via the tail vein and the lymph nodes are excised five hours later. A lymph node cell suspension is then prepared and radioactive thymidine or iodine incorporation is determined by β-scintillation or γ-scintillation counting, respectively. In the LLNA: DA, the test substance is applied on days 1, 2, 3, and additionally on day 7. During the initial development of the LLNA: DA, the study group (Yamashita et al. 2005) determined the optimal dosing schedule by evaluating whether the addition of a fourth application (day 7) was useful for increasing lymph node proliferation. Based on a statistically significant increase in lymph node weight-based stimulation index (SI) values for mice that received a fourth application (day 7) of the test substance, this test method protocol was chosen. Furthermore, one hour prior to each application of the test substance, an aqueous solution of 1% sodium lauryl sulfate (SLS) is applied to the dorsum of the treated ears to increase absorption of the test substance across the skin (van Och et al. 2000). Various researchers have shown that an aqueous solution of 1% SLS does not elicit a positive response in the traditional LLNA but when applied prior to test substance administration there is generally an increased response compared to the test substance alone (van Och et al. 2000; De Jong et al. 2002). Idehara et al. (2008) observed similar results (see also **Annex I** for supplemental data submitted to NICEATM evaluating the effect of 1% SLS pretreatment on lymph node cell proliferation [Idehara unpublished]). Lastly, 24 to 30 hours after the last test substance application on day 7, the auricular lymph nodes are excised and a lymph node cell suspension is prepared, and the ATP content is measured by luciferin-luciferase assay (day 8). The luciferin-luciferase assay is a sensitive method for ATP quantitation used in a wide variety of applications (Lundin 2000). It utilizes the luciferase enzyme to catalyze the formation of light from ATP and luciferin according to the following reaction:

The emitted light intensity is linearly related to the ATP concentration and is measured using a luminometer.

Table C-1 Comparison of the LLNA: DA and Traditional LLNA Experimental Procedure

Day	LLNA: DA	Traditional LLNA
1, 2, & 3	• Pretreat with 1% SLS aqueous solution • After one hour, apply 25 µL of test substance or vehicle to dorsum of each ear	• Apply 25 µL of test substance or vehicle to dorsum of each ear
4 & 5	• No treatment	• No treatment
6	• No treatment	• Administer ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine via tail vein • Excision of auricular lymph nodes • Measurement of radioactivity incorporated into lymph node cells
7	• Pretreat with 1% SLS aqueous solution • After one hour, apply 25 µL of test substance or vehicle to dorsum of each ear	• No treatment
8	• Excision of auricular lymph nodes • Measurement of ATP content in lymph node cells	• No treatment

Abbreviations: ^{3}H = tritiated; ^{125}I = iodine-125; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SLS = sodium lauryl sulfate.

2.1 Decision Criteria

Similar to the traditional LLNA, an SI is used in the LLNA: DA to distinguish skin sensitizers from nonsensitizers. The formula for calculating the SI in the LLNA: DA is the ratio of the mean ATP content of the auricular lymph nodes collected from the test substance treatment group to the mean ATP content of the auricular lymph nodes collected from the vehicle treatment group (measured in relative luminescence units; RLU):

$$SI = \frac{\text{mean ATP content of auricular lymph nodes in test treatment group (RLU)}}{\text{mean ATP content of auricular lymph nodes in vehicle treatment group (RLU)}}$$

In the intra- and interlaboratory validation studies for the LLNA: DA, an SI ≥ 3.0 was used as the threshold for identifying a substance as a sensitizer, which is the same threshold used in the traditional LLNA. As noted in **Section 6.0**, alternative decision criteria are evaluated in this BRD to determine the threshold that provides optimum performance.

3.0 LLNA: DA Validation Database

To evaluate the usefulness and limitations of the LLNA: DA, Daicel Chemical Industries, Ltd. tested a total of 45 substances in one laboratory (Idehara et al. 2008; Idehara unpublished). They further evaluated two of the 45 substances (isoeugenol and eugenol) in the LLNA: DA at varying concentrations in three different experiments in order to assess intralaboratory reproducibility. In addition, a two-phased interlaboratory validation study evaluated the reproducibility of the LLNA: DA (**Section 7.0**). In the first phase 10 laboratories tested 12 coded substances and in the second phase seven different laboratories tested five coded substances. Between the 17 laboratories, 14 different substances were examined and one of those substances, 3-aminophenol, was not previously tested among the 45 substances in the intralaboratory validation study, yielding a total of 46 substances tested in the LLNA: DA.

All 46 substances tested in the LLNA: DA were previously tested in the traditional LLNA, including 40 substances that were considered in the original ICCVAM evaluation of the traditional LLNA (ICCVAM 1999). Cinnamic alcohol, diethyl maleate, ethyl acrylate, glutaraldehyde, methyl methacrylate, and toluene 2,4-diisocyanate were the six substances tested in the LLNA: DA not evaluated in the ICCVAM 1999 report.

Of the 46 substances tested in the LLNA: DA, 33 were classified by the LLNA as skin sensitizers,[8] 12 were classified as nonsensitizers, and one (benzocaine) was classified as equivocal due to highly variable results and therefore was not included in the performance analyses (ICCVAM 1999)[9] (**Table C-2**). For the sensitizers in the LLNA, the range of traditional LLNA EC3 values (estimated concentrations needed to produce an SI of three) was from 0.009% to 90% (**Table C-2**). Similar to benzocaine, LLNA data for toluene 2,4-diisocyanate, not evaluated in the original ICCVAM 1999 report, were not suitable for comparison. The LLNA test method protocol followed for the study that tested toluene 2,4-diisocyanate (van Och et al. 2000) was a modified version of the traditional LLNA which was not performed in accordance with OECD TG 429 (OECD 2002) or ICCVAM 1999 and Dean et al. (2001). One variation included use of the BALB/c strain of mouse for the experiments, and not the CBA/Ca or CBA/J strains as specified by ICCVAM (1999), Dean et al. (2001) or OECD TG 429 (2002). In addition, the ears of the mice were pretreated with an aqueous solution of 1% SLS before treatment with the test substance. The authors also stated that the auricular lymph nodes were excised and pooled for each animal. Thus, of the 46 substances with LLNA: DA and LLNA data, 44 had adequate traditional LLNA data and were included in the accuracy analyses described in **Section 6.0**.

Annex II provides information on physicochemical properties (e.g., physical form tested). For the 44 substances that were evaluated in the LLNA: DA performance analyses, the molecular weights ranged from 30 to 388 g/mol. Twenty-two of the 44 substances were solids, 21 were liquids, and one substance (benzalkonium chloride) exists as either a solid or a liquid. The estimated log octanol-water partition coefficients (K_{ow}) were available for 38 substances and ranged from -8.28 to 6.46. Peptide reactivity, which was available for 28 substances, ranged from high to minimal (Gerberick et al. 2004, 2007).

Annex II further provides information on the Chemical Abstracts Service Registry Number (CASRN) 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. If

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[8] Resorcinol was classified as a nonsensitizer based on original LLNA data (ICCVAM 1999) but recent LLNA data have instead suggested that it is actually a sensitizer (Basketter et al. 2007a) and is therefore classified as a sensitizer for this evaluation.

[9] A series of 12 tests conducted in two laboratories resulted in some positive results that were not reproducible (Basketter et al. 1995).

chemical classes were not located, they were assigned for each test substance using a standard classification scheme, based on the National Library of Medicine Medical Subject Headings classification system.[10] A substance could be assigned to more than one chemical class; however, no substance was assigned to more than three classes. Classification of substances into chemical classes is not intended to indicate the impact of structure on biological activity with respect to sensitization potential. Instead, chemical class information is being presented to provide an indication of the variety of structural elements that are present in the substances that were evaluated in this analysis.

Table C-2 shows that 20 chemical classes are represented by the 44 substances tested in the LLNA: DA with adequate traditional LLNA data; 13 substances were classified in more than one chemical class. The classes with the highest number of substances were carboxylic acids (16 substances) and phenols (five substances). Further, 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. Seventeen of these classes were also represented in the LLNA: DA database (only amides, ketones, and macromolecular substances 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: DA database.

[10] Hhttp://www.nlm.nih.gov/mesh/meshhome.htmlH.

Table C-2 Product Use, Chemical Classification, and Traditional LLNA EC3 Values of 46 Substances Tested in the LLNA: DA

Substance Name	Product Use[1]	Chemical Class[2]	Traditional LLNA EC3 (%) (Max. SI)[3]	N[4]
5-Chloro-2-methyl-4-isothiazolin-3-one[5]	Cosmetics; Manufacturing; Pesticides	Sulfur Compounds; Heterocyclic Compounds	0.009 (27.7)	1
p-Benzoquinone[5]	Manufacturing; Pesticides; Pharmaceuticals	Quinones	0.010 (52.3)	1
2,4-Dinitrochlorobenzene[6, 7]	Manufacturing; Pesticides	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated; Nitro Compounds	0.049 (43.9)	15
Benzalkonium chloride[6]	Cosmetics; Disinfectant; Manufacturing; Personal care products; Pesticides	Amines; Onium Compounds	0.070[8] (11.1)	1
Glutaraldehyde[6, 7]	Cosmetics; Disinfectant; Manufacturing; Pesticides	Aldehydes	0.083 (18.0)	3
p-Phenylenediamine[6]	Intermediate in chemical synthesis; Manufacturing	Amines	0.110 (26.4)	6
Toluene 2,4-diisocyanate[6, 9]	Intermediate in chemical synthesis	Hydrocarbons, Cyclic; Isocyanates	0.110 (NR)	1
Potassium dichromate[6, 10]	Manufacturing; Pharmaceuticals	Inorganic Chemical, Chromium Compounds; Inorganic Chemical, Potassium Compounds	0.170 (33.6)	12
Propyl gallate[5]	Cosmetics; Food additive	Carboxylic Acids	0.320 (33.6)	1
Phthalic anhydride[6]	Intermediate in chemical synthesis; Manufacturing; Pharmaceuticals	Anhydrides; Carboxylic Acids	0.360 (26.0)	1
Formaldehyde[6, 7]	Disinfectant; Manufacturing	Aldehydes	0.495 (4.0)	4
Cobalt chloride[6, 7, 10]	Manufacturing; Pesticides	Inorganic Chemical, Elements; Inorganic Chemical, Metals	0.600 (7.2)	2

Substance Name	Product Use[1]	Chemical Class[2]	Traditional LLNA EC3 (%) (Max. SI)[3]	N[4]
Isoeugenol[6, 7]	Food additive; Fragrance agent	Carboxylic Acids	1.540 (31.0)	47
2-Mercaptobenzothiazole[6]	Manufacturing; Pesticides	Heterocyclic Compounds	1.700 (8.6)	1
Cinnamic aldehyde[6]	Cosmetics; Food additive; Fragrance agent; Intermediate in chemical synthesis; Personal care products; Pesticides	Aldehydes	1.910 (18.4)	6
3-Aminophenol[7]	Cosmetics; Pharmaceuticals	Amines; Phenols	3.200 (5.7)	1
Benzocaine[6]	Medication	Carboxylic Acids	3.400[11] (7.6)	1
Diethyl maleate[5]	Food additive; Intermediate in chemical synthesis	Carboxylic Acids	3.600 (22.6)	4
Trimellitic anhydride[6]	Manufacturing	Anhydride; Carboxylic Acids	4.710 (4.6)	2
Nickel (II) sulfate hexahydrate[6, 7, 10]	Manufacturing	Inorganic Chemical, Elements; Inorganic Chemical, Metals	4.800 (3.1)	1
Resorcinol[6]	Cosmetics; Manufacturing; Personal care products; Pesticides; Pharmaceuticals	Phenols	6.330 (10.4)	1
Sodium lauryl sulfate[6]	Cosmetics; Food additive; Manufacturing; Personal care products; Pesticides; Pharmaceuticals	Alcohols; Sulfur Compounds; Lipids	8.080 (8.9)	5
Citral[6]	Fragrance agent	Hydrocarbons, Other	9.170 (20.5)	6
Hexyl cinnamic aldehyde[6, 7, 10]	Food additive; Fragrance agent	Aldehydes	9.740 (20.0)	21

Substance Name	Product Use[1]	Chemical Class[2]	Traditional LLNA EC3 (%) (Max. SI)[3]	N[4]
Eugenol[6]	Cosmetics; Food additive; Intermediate in chemical synthesis; Manufacturing; Personal care products; Pharmaceuticals	Carboxylic Acids	10.090 (17.0)	11
Abietic acid[6, 7]	Manufacturing	Hydrocarbons, Cyclic; Polycyclic Compounds	11.920 (5.2)	5
Phenyl benzoate[5]	Manufacturing; Pesticides	Carboxylic Acids	13.600 (11.1)	3
Cinnamic alcohol[5]	Cosmetics; Food additive; Fragrance agent; Intermediate in chemical synthesis; Personal care products	Alcohols	21.000 (5.7)	1
Hydroxycitronellal[6]	Food additive; Fragrance agent; Personal care products	Hydrocarbons, Other	23.750 (8.5)	6
Imidazolidinyl urea[6]	Cosmetics; Personal care products; Pesticides	Urea	24.000 (5.5)	1
Ethylene glycol dimethacrylate[5]	Manufacturing	Carboxylic Acids	28.000 (7.0)	1
Butyl glycidyl ether[5]	Intermediate in chemical synthesis; Manufacturing	Ethers	30.900 (5.6)	1
Ethyl acrylate[5]	Manufacturing	Carboxylic Acids	32.800 (4.0)	2
Methyl methacrylate[5]	Manufacturing	Carboxylic Acids	90.000 (3.6)	1
1-Bromobutane[6]	Intermediate in chemical synthesis; Pharmaceuticals; Solvent	Hydrocarbons, Halogenated	NA (1.2)	1
Chlorobenzene[6]	Manufacturing; Solvent	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated	NA (1.7)	1

Substance Name	Product Use[1]	Chemical Class[2]	Traditional LLNA EC3 (%) (Max. SI)[3]	N[4]
Diethyl phthalate[6]	Cosmetics; Manufacturing; Personal care products; Pesticides; Pharmaceuticals	Carboxylic Acids	NA (1.5)	1
Dimethyl isophthalate[5, 7]	Manufacturing; Fragrance agent	Carboxylic Acids	NA (1.0)	1
Hexane[6]	Manufacturing; Solvent	Hydrocarbons, Acyclic	NA (2.2)	1
Isopropanol[6, 7]	Cosmetics; Disinfectant; Food additive; Intermediate in chemical synthesis; Manufacturing; Personal care products; Pharmaceuticals; Solvent	Alcohols	NA (1.7)	1
Lactic acid[6, 10]	Food additive; Manufacturing; Pharmaceuticals	Carboxylic Acids	NA (2.2)	1
Methyl salicylate[6, 7]	Cosmetics; Food additive; Fragrance agent; Personal care products; Pharmaceuticals; Solvent	Carboxylic Acids; Phenols	NA (2.9)	9
Propylparaben[6]	Food additive; Pesticides; Pharmaceuticals	Carboxylic Acids; Phenols	NA (1.4)	1
Nickel (II) chloride[5]	Manufacturing; Pesticides	Inorganic Chemical, Elements; Inorganic Chemical, Metals	NA (2.4)	2
Salicylic acid[5]	Food additive; Manufacturing; Pharmaceuticals	Phenols; Carboxylic Acids	NA (2.5)	1
Sulfanilamide[5]	Pharmaceuticals	Hydrocarbons, Cyclic; Sulfur Compounds	NA (1.0)	1

Abbreviations: EC3 = estimated concentration needed to produce a stimulation index of three; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; Max. = maximum; NA = not applicable; NR = not reported; SI = stimulation index.

[1] Information for product use was gathered from the following databases:
Hazardous Substances Database (HSDB)-National Library of Medicine-TOXNET .http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB.
Haz-Map: National Library of Medicine-Toxicology and Environmental Health Information Program .http://hazmap.nlm.nih.gov/.
Household Products Database-National Library of Medicine .http://hpd.nlm.nih.gov/index.htm
International Programme on Chemical Safety (IPCS) INCHEM database in partnership with Canadian Centre for Occupational Health and Safety (CCOHS) .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, as developed by the National Library of Medicine: .http://www.nlm.nih.gov/mesh/meshhome.html.

[3] The traditional LLNA EC3 value (estimated concentration needed to produce a stimulation index of three) listed for each substance is averaged from respective studies. The substance was tested in the same vehicle in both the traditional LLNA and the LLNA: DA (**Annex IV)**, except where noted. Numbers in parentheses indicate the maximum stimulation index, where reported.

[4] Number of traditional LLNA studies from which the data were obtained.

[5] Substance tested in intralaboratory validation study (Idehara unpublished).

[6] Substance tested in intralaboratory validation study (Idehara et al. 2008).

[7] Substance tested in first phase of a two-phased interlaboratory validation study (Omori et al. 2008).

[8] Benzalkonium chloride was tested in the LLNA: DA using acetone: olive oil (4:1) as the vehicle (**Annex IV**) but the traditional LLNA EC3 value reported is based on results using acetone as the vehicle.

[9] Not included in accuracy analyses. Comparable LLNA reference data from modified LLNA test (van Och et al. 2000).

[10] Substance tested in second phase of a two-phased interlaboratory validation study (Omori et al. 2008).

[11] Not included in accuracy analyses. EC3 value reported in **Table C-2** for benzocaine is based on data from the NICEATM database but variable and equivocal (i.e., results that were not reproducible) responses were reported by Basketter et al. (1995) and in the 1999 ICCVAM report.

4.0 Reference Data

As mentioned in **Section 3.0**, 44 of the 46 substances tested in the LLNA: DA have adequate traditional LLNA data and are included in the accuracy analyses described in **Section 6.0.** The traditional LLNA reference data used for the accuracy analyses comparisons are from ICCVAM (1999) (**Annex III**) for 34 of those 44 substances. The traditional LLNA reference data for the remaining 10 substances (benzalkonium chloride, cinnamic alcohol, diethyl maleate, diethyl phthalate, ethyl acrylate, formaldehyde, glutaraldehyde, imidazolidinyl urea, methyl methacrylate, and nickel [II] sulfate hexahydrate) were obtained from other sources (**Annex III**) (Gerberick et al. 1992; Hilton et al. 1998; Ryan et al. 2002; Basketter et al. 2005; Gerberick et al. 2005; Betts et al. 2006). In addition, Basketter et al. (2007a) reassessed the skin sensitization potential of resorcinol in the LLNA, in accordance with OECD TG 429 (2002), which updates information in the ICCVAM 1999 report and from Gerberick et al. (2005) that had previously stated that this substance tested negative in the LLNA.

The reference data for the GP tests (guinea pig maximization test or Buehler test) and human tests (human maximization test, human patch test allergen, or other human data) were obtained from Vandenberg and Epstein (1963), Kligman (1966a, 1966b, 1966c), Marzulli and Maibach (1974), Jordan and King (1977), Klecak et al. (1977), Marzulli and Maibach (1980), Van der Walle et al. (1982), Gad et al. (1986), Robinson et al. (1990), Gerberick et al. (1992), ICCVAM (1999), Basketter et al. (1999a, 1999b, 2001, 2005, 2007a), Kwon et al. (2003), Schneider and Akkan (2004), and Betts et al. (2006).

An independent quality assurance contractor for the National Toxicology Program audited the traditional LLNA data provided in the ICCVAM 1999 report. Audit procedures and findings are presented in the quality assurance report on file at the National Institute of Environmental Health Sciences. 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 LLNA: DA Test Method Data and Results

The test method data in this BRD include the individual animal data for the LLNA: DA results from the validation studies by Idehara et al. (2008) and Omori et al. (2008). In addition, individual animal data for 14 unpublished studies (Idehara unpublished) were submitted to NICEATM and were included in the evaluation (although the individual animal data were submitted to NICEATM they are not included in the BRD at the request of the test method developer since they are not yet published). **Annex III** represents a summary of data for the 46 different substances tested in the LLNA: DA, and includes the comparative traditional LLNA data that were available for 44 of the 46 substances (see also **Section 3.0**). In addition, 42 of the 46 substances examined in the LLNA: DA have GP data and 43 of the 46 substances tested have human skin sensitization data. Based on Idehara et al. (2008; unpublished), the 45 substances tested in the intralaboratory study were not coded prior to testing. However, the two-phased interlaboratory validation study used coded substances (Omori et al. 2008). Original data for these studies are included in **Annex IV**.

6.0 LLNA: DA 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 test method when compared to the current reference test method (ICCVAM 2003). Additional comparisons should also be made against any available human data or 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: DA Database Used for the Accuracy Analysis

An accuracy analysis for the LLNA: DA test method was conducted using data from the intralaboratory validation study (Idehara et al. 2008; Idehara unpublished) and the two-phased interlaboratory validation study (Omori et al. 2008). Taken together, LLNA: DA test data were available for 46 different substances, 44 of which had adequate comparative traditional LLNA data to conduct an accuracy analysis (**Section 3.0**). Thus, of the 44 substances included in the accuracy analysis, 40 had LLNA: DA, traditional LLNA, and GP data and 41 had LLNA: DA, traditional LLNA, and human data. Classification of substances and data available for each substance are provided in **Annex III**.

Multiple LLNA: DA tests were available for 14 substances tested in the intralaboratory (Idehara et al. 2008; Idehara unpublished) and the two-phased interlaboratory LLNA: DA studies (Omori et al. 2008). For the accuracy analyses, the test results were combined so that each substance was represented by one overall result for the SI analyzed and represented the outcome that was most prevalent. For example, when using SI $\geq$ 3.0 as the decision criterion, cobalt chloride was positive because five of the eight LLNA: DA results were positive (**Annex IV**). Also, using SI $\geq$ 3.0 as the decision criterion, inconsistent test results were noted for two of the 14 substances with multiple test results: cobalt chloride and nickel (II) sulfate hexahydrate. Three of the validation laboratories that tested cobalt chloride reported SI < 3.0 and five laboratories yielded SI $\geq$ 3.0. For nickel (II) sulfate hexahydrate, six validation laboratories reported SI < 3.0 and two laboratories yielded SI $\geq$ 3.0.

6.2 Accuracy Analysis Using the SI $\geq$ 3.0 Decision Criterion

The performance characteristics of the LLNA: DA test method were first evaluated using the decision criterion of SI $\geq$ 3.0 to identify sensitizers, which was the threshold for a positive response used in both the intralaboratory and two-phased interlaboratory validation studies (**Annex I**).

6.2.1 Accuracy vs. the Traditional LLNA

Based on the data (44 substances), when compared to the traditional LLNA, the LLNA: DA had an accuracy of 91% (40/44), a sensitivity of 88% (28/32), a specificity of 100% (12/12), a false positive rate of 0% (0/12), and a false negative rate of 13% (4/32) (**Table C-3**).

6.2.2 Accuracy vs. Guinea Pig Data

When the accuracy statistics for the LLNA: DA and the traditional LLNA were compared for substances with LLNA: DA, traditional LLNA, and GP data, and GP results served as the reference data, the LLNA: DA had a lower accuracy (78% [31/40] vs. 85% [34/40]), sensitivity (85% [22/26]

vs. 96% [25/26]), the same specificity (64% [9/14]) and false positive rate (36% [5/14]), and higher false negative rate (15% [4/26] vs. 4% [1/26]) relative to the traditional LLNA (**Table C-3**).

6.2.3 Accuracy vs. Human Data

When substances with only comparative LLNA: DA, traditional LLNA, and human data were evaluated, and human outcomes served as the reference point, the LLNA: DA had lower accuracy (76% [31/41] vs. 85% [35/41]) and sensitivity (74% [26/35] vs. 86% [30/35]), the same specificity (83% [5/6]) and false positive rate (17% [1/6]), and higher false negative rate (26% [9/35] vs. 14% [5/35]) relative to the traditional LLNA (**Table C-3**).

Table C-3 Performance of the LLNA: DA in Predicting Skin Sensitization Potential Using Decision Criterion of SI ≥ 3.0 to Identify Sensitizers

Comparison	n[1]	Accuracy % (No.[2])	Sensitivity % (No.[2])	Specificity % (No.[2])	False Positive Rate % (No.[2])	False Negative Rate % (No.[2])	Positive Predictivity % (No.[2])	Negative Predictivity % (No.[2])
LLNA: DA vs. Traditional LLNA	44	91 (40/44)	88 (28/32)	100 (12/12)	0 (0/12)	13 (4/32	100 (28/28)	75 (12/16)
Substances with LLNA: DA, Traditional LLNA, and GP Data								
LLNA: DA vs. Traditional LLNA	40	93 (37/40)	90 (27/30)	100 (10/10)	0 (0/10)	10 (3/30)	100 (27/27)	77 (10/13)
LLNA: DA vs. GP[3]	40	78 (31/40)	85 (22/26)	64 (9/14)	36 (5/14)	15 (4/26)	81 (22/27)	69 (9/13)
Traditional LLNA vs. GP[3]	40	85 (34/40)	96 (25/26)	64 (9/14)	36 (5/14)	4 (1/26)	83 (25/30)	90 (9/10)
Substances with LLNA: DA, Traditional LLNA, and Human Data								
LLNA: DA vs. Traditional LLNA	41	90 (37/41)	87 (27/31)	100 (10/10)	0 (0/10)	13 (4/31)	100 (27/27)	71 (10/14)
LLNA: DA vs. Human[4]	41	76 (31/41)	74 (26/35)	83 (5/6)	17 (1/6)	26 (9/35)	96 (26/27)	36 (5/14)
Traditional LLNA vs. Human[4]	41	85 (35/41)	86 (30/35)	83 (5/6)	17 (1/6)	14 (5/35)	97 (30/31)	50 (5/10)

Abbreviations: GP = guinea pig; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; No. = number; SI = stimulation index; vs. = versus.

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

[2] The proportion 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, inclusion of the test substance in a human patch test allergen kit, and/or published clinical case studies/reports.

6.3 Accuracy Analysis (SI ≥ 3.0) Based on ICCVAM-recommended LLNA Performance Standards Reference Substances

In conjunction with the European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Center for the Validation of Alternative Methods (JaCVAM), ICCVAM has developed internationally harmonized test method performance standards for the traditional LLNA (ICCVAM 2009),[11] which are proposed to evaluate the performance of modified LLNA test methods that are mechanistically and functionally similar to the traditional LLNA. Since the validation studies for the LLNA: DA test method were completed prior to the development of LLNA performance standards, the LLNA: DA is not being evaluated using the ICCVAM-recommended LLNA performance standards. Thus, evaluations of the LLNA: DA test substances to the ICCVAM-recommended LLNA performance standards test substances are shown to provide a general comparison to a set list of reference substances (18 required reference substances and four optional reference substances) that represent a diverse substance group.

As shown in **Table C-4**, all of the 18 required reference substances and three of the four optional reference substances included in the ICCVAM-recommended LLNA performance standards have been tested in the LLNA: DA. When compared to the traditional LLNA, the LLNA: DA at SI ≥ 3.0 (SI decision criterion used in the intralaboratory and the interlaboratory validation studies) predicted the same sensitization classification for 16 of the 18 required ICCVAM-recommended reference substances tested. One discordant substance, 2-mercaptobenzothiazole, was classified as a sensitizer based on traditional LLNA results (EC3 = 1.7%) but as a nonsensitizer based on LLNA: DA data. As indicated in **Table C-4**, *N,N*-dimethylformamide (DMF) was the vehicle used in both the traditional LLNA and the LLNA: DA tests for 2-mercaptobenzothiazole. The positive result for 2-mercaptobenzothiazole reported in the ICCVAM-recommended LLNA performance standards was based on one LLNA experiment that tested the substance at 1%, 3%, and 10% (Gerberick et al. 2005). By comparison, the negative result for 2-mercaptobenzothiazole obtained with the LLNA: DA test method was based on one LLNA: DA experiment that tested the substance at 10%, 25%, and 50% (Idehara et al. 2008). The highest dose tested for 2-mercaptobenzothiazole in the traditional LLNA was the lowest dose tested in the LLNA: DA (10%) and resulted in an SI of 8.6 versus 2.0, respectively.

Notably, a review of the original LLNA: DA laboratory records for 2-mercaptobenzothiazole indicated that the concurrent positive control (10% eugenol in DMF) failed to yield an SI ≥ 3.0. Consequently the test method developers should have repeated the test for 2-mercaptobenzothiazole to ensure that the result obtained was correctly classified as negative and not the result of a failed experiment. This could explain the discordant result obtained between the traditional LLNA and the LLNA: DA test method for this test substance.

The second discordant substance, methyl methacrylate, was classified as a sensitizer based on traditional LLNA results (EC3 = 90%) but as a nonsensitizer based on LLNA: DA data. As indicated in **Table C-4**, acetone: olive oil (AOO; 4:1) was the vehicle used in both the traditional LLNA and the LLNA: DA tests for methyl methacrylate. The positive result for methyl methacrylate reported in the ICCVAM-recommended LLNA performance standards was based on one LLNA experiment that tested the substance at 10%, 30%, 50%, and 100% (Betts et al. 2006). By comparison, the negative result for methyl methacrylate obtained with the LLNA: DA test method was based on one LLNA: DA experiment that tested the substance at 25%, 50%, 75%, and 100% (Idehara unpublished). The highest dose tested for methyl methacrylate in the traditional LLNA was the same in the LLNA: DA (100%) and resulted in an SI of 3.6 versus 1.8, respectively.

[11] Hhttp://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htmH.

As shown in **Table C-4**, when compared to the traditional LLNA, the LLNA: DA at SI ≥ 3.0 predicted the same sensitization for all three of the optional reference substances tested. The optional reference substances, SLS and ethylene glycol dimethacrylate, were categorized as nonsensitizers based on GP and human data but as sensitizers by the LLNA: DA. Thus, similar to the traditional LLNA, these substances were false positive in the LLNA: DA. SLS was tested in the same vehicle (DMF) in both the traditional LLNA and the LLNA: DA. In addition, the positive results for SLS reported in the ICCVAM-recommended LLNA performance standards were based on five LLNA studies that tested SLS at 1%, 2.5%, 5%, 10%, and 20% (Loveless et al. 1996). In comparison, the positive result for SLS obtained with the LLNA: DA test method was based on one LLNA: DA experiment that tested the substance at 1%, 2.5%, 5%, and 10% (Idehara et al. 2008). The EC3 values for SLS in the traditional LLNA (8.1%) and the LLNA: DA (6.9%) were comparable. In addition, ethylene glycol dimethacrylate was tested in the same vehicle (methyl ethyl ketone) in both the traditional LLNA and the LLNA: DA. The positive result for ethylene glycol dimethacrylate reported in the ICCVAM-recommended LLNA performance standards was based on one LLNA study that tested the substance at 10%, 25%, and 50% (Gerberick et al. 2005). In comparison, the positive result for ethylene glycol dimethacrylate obtained with the LLNA: DA test method was based on one LLNA: DA experiment that also tested the substance at 10%, 25%, and 50% (Idehara unpublished). The EC3 values for ethylene glycol dimethacrylate in the traditional LLNA (28%) and the LLNA: DA (34%) were comparable.

Lastly, the optional reference substance, nickel (II) chloride, was categorized as a sensitizer based on GP and human data but as a nonsensitizer by the LLNA: DA. Thus, similar to the traditional LLNA, this substance was false negative in the LLNA: DA. Nickel (II) chloride was tested in the same vehicle (dimethyl sulfoxide [DMSO]) in both the traditional LLNA and the LLNA: DA. In addition, the negative results for nickel (II) chloride reported in the ICCVAM-recommended LLNA performance standards were based on two independent LLNA studies that tested the substance at 0.5%, 1%, and 2.5% (Basketter et al. 1999a) and at 1%, 2.5%, and 5% (Basketter and Scholes 1992). In comparison, the negative result for nickel (II) chloride obtained with the LLNA: DA test method was based on one LLNA: DA experiment that tested the substance at 2.5%, 5%, and 10% (Idehara unpublished). The highest dose tested for nickel (II) chloride in the traditional LLNA was the same in the LLNA: DA (5%) and resulted in an SI of 2.4 versus 1.3, respectively.

Table C-4 Performance of the LLNA: DA (SI ≥ 3.0) Compared to the ICCVAM-recommended LLNA Performance Standards Reference Substances[1] (Sorted by Traditional LLNA EC3 Value)

Substance Name	ICCVAM-recommended LLNA Performance Standards				LLNA: DA[2]			
	Vehicle	Result	EC3 (%) (Max. SI)[3]	N[4]	Vehicle	Result	EC3 (%) (Max. SI)[3]	N[4]
5-Chloro-2-methyl-4-isothiazolin-3-one	DMF	+	0.009 (27.7)	1	DMF	+	0.03 (7.5)	1
2,4-Dinitrochlorobenzene	AOO	+	0.049 (43.9)	15	AOO	+	0.08 (15.1)	11
4-Phenylenediamine	AOO	+	0.110 (26.4)	6	AOO	+	0.07 (5.1)	1
Cobalt chloride	DMSO	+	0.600 (7.2)	2	DMSO	+	1.27 (20.6)	5

continued

Table C-4 Performance of the LLNA: DA (SI ≥ 3.0) Compared to the ICCVAM-recommended LLNA Performance Standards Reference Substances[1] (Sorted by Traditional LLNA EC3 Value) (continued)

Substance Name	ICCVAM-recommended LLNA Performance Standards				LLNA: DA[2]			
	Vehicle	Result	EC3 (%) (Max. SI)[3]	N[4]	Vehicle	Result	EC3 (%) (Max. SI)[3]	N[4]
Isoeugenol	AOO	+	1.540 (31.0)	47	AOO	+	2.94 (12.4)	4
2-Mercaptobenzothiazole	***DMF***	**+**	***1.700 (8.6)***	***1***	***DMF***	**-**	***NA (2.0)***	***1***
Citral	AOO	+	9.170 (20.5)	6	AOO	+	15.63 (4.4)	1
Hexyl cinnamic aldehyde	AOO	+	9.740 (20.0)	21	AOO	+	11.10 (10.2)	18
Eugenol	AOO	+	10.090 (17.0)	11	AOO	+	4.50 (7.1)	1
Phenyl benzoate	AOO	+	13.600 (11.1)	3	AOO	+	2.26 (4.2)	1
Cinnamic alcohol	AOO	+	21.000 (5.7)	1	AOO	+	21.34 (5.7)	1
Imidazolidinyl urea	DMF	+	24.000 (5.5)	1	DMF	+	18.77 (4.7)	1
Methyl methacrylate	***AOO***	**+**	***90.000 (3.6)***	***1***	***AOO***	**-**	***NA (1.8)***	***1***
Chlorobenzene	AOO	-	NA (1.7)	1	AOO	-	NA (2.4)	1
Isopropanol	AOO	-	NA (1.7)	1	AOO	-	NA (2.0)	11
Lactic acid	DMSO	-	NA (2.2)	1	DMSO	-	NA (1.1)	5
Methyl salicylate	AOO	-	NA (2.9)	9	AOO	-	NA (1.8)	4
Salicylic acid	AOO	-	NA (2.5)	1	AOO	-	NA (2.0)	1
Sodium lauryl sulfate	DMF	FP	8.1 (8.9)	5	DMF	+	6.88 (3.4)	1
Ethylene glycol dimethylacrylate	MEK	FP	28.000 (7.0)	1	MEK	+	34.03 (4.5)	1
Xylene	AOO	FP	95.800 (3.1)	1	NT	NT	NT	NT
Nickel (II) chloride	DMSO	FN	NA (2.4)	2	DMSO	-	NA (1.3)	1

Bolded and italicized text highlights discordant LLNA: DA vs. traditional LLNA test results.

Abbreviations: AOO = acetone: olive oil (4:1); DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; EC3 = estimated concentration needed to produce a stimulation index of three; FN = false negative in traditional LLNA when compared to guinea pig and/or human results; FP = false positive in traditional LLNA when compared to guinea pig and/or human results; ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; Max. = maximum; MEK = methyl ethyl ketone; NA = not applicable (stimulation index < 3.0); NT = not tested; SI = stimulation index.

+ = sensitizer.

- = nonsensitizer.

[1] From *Recommended Performance Standards: Murine Local Lymph Node Assay* (ICCVAM 2009; available at: http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm). The table lists the 18 required reference substances first (sorted from lowest to highest EC3 value), followed by the four optional reference substances (sorted from lowest to highest EC3 value).

[2] Substances tested in LLNA: DA intralaboratory validation study (Idehara et al. 2008; Idehara unpublished) and/or two-phased interlaboratory validation study (Omori et al. 2008).

[3] Based on mean EC3 value when more than one value was available. Numbers in parentheses indicate the maximum SI.

[4] Number of LLNA studies from which data were obtained.

Table C-5 provides the range and characteristics for 44 substances tested in the LLNA: DA based on sufficient traditional LLNA data. These substances are compared to the range of 18 required reference substances included on the ICCVAM-recommended LLNA performance standards reference substances list (ICCVAM 2009). The table indicates that the range of the substances tested in the LLNA: DA is similar to that included in the performance standards list. In general, there is a proportionally increased number of substances tested in the LLNA: DA in each of the categories included in the table.

Table C-5 Characteristics of the Substances Tested in the LLNA: DA Compared to the ICCVAM-recommended LLNA Performance Standards Reference Substances[1]

EC3 Range in the Traditional LLNA (%)	No. Substances	Solid/ Liquid	Actual EC3 Range (%)[2]	Human Data	Peptide Reactivity (High/Mod/Min/Low/Unk)[3]
<0.1	**5**	**3/3[4]**	**0.009-0.083**	**5**	**4/0/0/0/1**
	2	1/1	0.009-0.049	2	2/0/0/0/0
≥0.1 to <1	**6**	**5/1**	**0.110-0.600**	**6**	**1/2/0/0/3**
	2	2/0	0.110-0.600	2	0/0/0/0/2
≥1 to <10	**11**	**6/5**	**1.540-9.740**	**10**	**4/0/3/1/3**
	4	1/3	1.540-9.740	4	2/0/1/0/1
≥10 to <100	**10**	**4/6**	**10.090-90.000**	**10**	**2/1/0/1/6**
	5	3/2	10.090-90.000	5	0/1/0/0/4
Negative	**12**	**7/5**	**NA**	**10**	**0/0/8/1/3**
	5	1/4	NA	3	0/0/2/0/3
Overall	**44**	**25/20[4]**	**0.009-90.000**	**41**	**11/3/11/3/16**
	18	8/10	0.009-90.000	16	4/1/3/0/10

Boldface represents characteristics of the LLNA: DA database, which includes the 44 substances with adequate traditional LLNA data, tested in the intralaboratory validation study (Idehara et al. 2008; Idehara unpublished) and/or the two-phased interlaboratory validation study (Omori et al. 2008).

Abbreviations: EC3 = estimated concentration needed to produce a stimulation index of three; ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP Content; NA = not applicable because maximum stimulation index < 3.0; No. = number; Min = minimal; Mod = moderate; Unk = unknown.

[1] From *Recommended Performance Standards: Murine Local Lymph Node Assay* (ICCVAM 2009; available at: http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm), based on the 18 required reference substances.

[2] Based on traditional LLNA studies for substances tested in the LLNA: DA (bold values) and for the 18 required reference substances in the ICCVAM-recommended LLNA performance standards (ICCVAM 2009).

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

[4] One substance tested in the LLNA: DA, benzalkonium chloride, is categorized as both a solid and a liquid.

6.4 Discordant Results for Accuracy Analysis Using the SI ≥ 3.0 Decision Criterion

6.4.1 Discordance Between the LLNA: DA and the Traditional LLNA

When the outcomes for the 44 substances tested in the LLNA: DA (using SI ≥ 3.0) and the traditional LLNA were compared, the classifications for four substances were different. The LLNA: DA classified 3-aminophenol, 2-mercaptobenzothiazole, methyl methacrylate, and nickel (II) sulfate hexahydrate as nonsensitizers while the traditional LLNA classified them as sensitizers (**Tables C-6** and **C-7**). These substances were tested in the same vehicle in both the LLNA: DA and the traditional LLNA tests. One commonality noted between three of the four discordant substances is that they are solids. Furthermore, the molecular weights for 3-aminophenol and methyl methacrylate are both about 100 g/mol and those for 2-mercaptobenzothiazole and nickel (II) sulfate hexahydrate are comparable at 160 g/mol (**Annex II**). In addition, all four discordant substances are considered nonirritants based on GP data (**Table C-6**).

6.4.2 Discordance Among the LLNA: DA, the Traditional LLNA, and/or the Guinea Pig Test

When analyses were restricted to the 40 substances with unequivocal LLNA: DA, traditional LLNA, and GP data, the LLNA: DA at SI ≥ 3.0 classified three substances differently compared with the traditional LLNA (**Table C-6**). 2-Mercaptobenzothiazole, methyl methacrylate, and nickel (II) sulfate hexahydrate were identified as nonsensitizers by the LLNA: DA while the traditional LLNA and GP tests classified these substances as sensitizers. The discordant substances were tested at the same or higher concentrations in the LLNA: DA and in the traditional LLNA yet the substances were still classified as nonsensitizers (**Table C-6**). There are few commonalities among these substances with regard to chemical class, physical form, molecular weight, peptide reactivity (see **Annex II** for physicochemical information), range of EC3 values (based on traditional LLNA, see **Table C-2**), and potential for skin irritation (**Annex III**) as follows:

- 2-Mercaptobenzothiazole is a heterocyclic compound, methyl methacrylate is carboxylic acid, and nickel (II) sulfate hexahydrate is a metal.
- 2-Mercaptobenzothiazole and nickel (II) sulfate hexahydrate exist as solids and methyl methacrylate exists as a liquid.
- Nickel (II) sulfate hexahydrate and methyl methacrylate are soluble in water whereas 2-mercaptobenzothizole is not.

- All three discordant substances have similar molecular weights (approximately 100 to 160 g/mol).
- 2-Mercaptobenzothaizole has high peptide reactivity, whereas the peptide reactivity for methyl methacrylate and nickel (II) sulfate hexahydrate is not known.
- All three discordant substances are classified as sensitizers by the traditional LLNA (EC3 values were 90% for methyl methacrylate, 1.7% for 2-mercaptobenzothiazole, and 4.8% for nickel [II] sulfate hexahydrate).
- All three discordant substances are nonirritants based on data from GP studies (**Table C-6**).

In addition, benzalkonium chloride, ethyl acrylate, ethylene glycol dimethacrylate, resorcinol, and SLS were positive in both the LLNA: DA and the traditional LLNA, but were negative in GP tests (**Table C-6**). In contrast, nickel (II) chloride was negative in both the LLNA: DA and the traditional LLNA but was positive in GP tests. There are few commonalities among these substances with regard to chemical class, physical form, molecular weight, peptide reactivity (see **Annex II** for physicochemical information), and potential for skin irritation (**Annex III**) as follows:

- Benzalkonium chloride is an amine, ethyl acrylate and ethylene glycol dimethacrylate are carboxylic acids, resorcinol is a phenol, and SLS is an alcohol, sulfur, and lipid compound; nickel (II) chloride is a metal.
- Resorcinol and SLS exist as solids in their physical state and ethyl acrylate and ethylene glycol dimethacrylate exist as liquids in their physical state, whereas benzalkonium chloride can exist in both a solid and liquid physical state; nickel (II) chloride exists as a solid in its physical state.
- These five substances have varying molecular weights (100 g/mol for ethyl acrylate, 110 g/mol for resorcinol, 171 g/mol for benzalkonium chloride, 198 g/mol for ethylene glycol dimethacrylate, and 288 g/mol for SLS); the molecular weight for nickel (II) chloride is about 130 g/mol.
- These five discordant substances are soluble in water; nickel (II) chloride is slightly soluble in water.
- Peptide reactivity is identified as minimal for resorcinol, and high for ethyl acrylate and ethylene glycol dimethacrylate, but is not identified for benzalkonium chloride and SLS; peptide reactivity for nickel (II) chloride is also not identified.
- Benzalkonium chloride and SLS have been found to be skin irritants based on results in mice, rabbits, or humans, while resorcinol is considered a nonirritant based on studies in humans, and ethyl acrylate and ethylene glycol dimethacrylate are considered nonirritants based on studies in GPs; nickel (II) chloride is identified as negative at ≤0.15% based on GP studies (**Table C-6**).

Table C-6 Discordant Results for the LLNA: DA (Using SI ≥ 3.0 for Sensitizers) Compared to Traditional LLNA and Guinea Pig Reference Data[1]

Substance Name[2]	Vehicle[3]	LLNA: DA[4]	Traditional LLNA[4]	Guinea Pig Studies[5]	Skin Irritant?
Benzalkonium chloride (0.07%)	AOO ACE[6]	+ (6.7, 2.5%)	+ (11.1, 2%)[7]	-	Irritant at 2% and 1% ACE (mice)
Ethyl acrylate (32.8%)	AOO	+ (4.2, 50%)[8]	+ (4.0, 50%)	-	Nonirritant at 0.3 Molar (GP)
Ethylene glycol dimethacrylate (28%)	MEK	+ (4.5, 50%)	+ (7.0, 50%)	-	Nonirritant at 1% (GP)

continued

Table C-6 Discordant Results for the LLNA: DA (Using SI ≥ 3.0 for Sensitizers) Compared to Traditional LLNA and Guinea Pig Reference Data[1] (continued)

Substance Name[2]	Vehicle[3]	LLNA: DA[4]	Traditional LLNA[4]	Guinea Pig Studies[5]	Skin Irritant?
Resorcinol (6.33%)	AOO	+ (4.3, 25%)[9]	+ (10.4, 50%)	-	Nonirritant at 15% (humans)
Sodium lauryl sulfate (8.08%)	DMF	+ (3.4, 10%)	+ (8.9, 20%)	-	Irritant at 20% aq. (rabbits); Irritant at 20% (humans)
Nickel (II) chloride	DMSO	- (1.3, 10%)	- (2.4, 5%)	+	Negative at ≤0.15% (GP)
2-Mercapto-benzothiazole (1.7%)	DMF	- (2.0, 50%)[9]	+ (8.6, 10%)	+	Nonirritant at 10% (GP); Nonirritant at 25% (humans)
Methyl methacrylate (90%)	AOO	- (1.8, 100%)	+ (3.6, 100%)	+	Nonirritant at 3 Molar (GP)
Nickel (II) sulfate hexahydrate (4.8%)	DMSO	- (11.8, 10%)	+ (3.1, 5%)	+	Irritant at 10% (humans); Nonirritant at 0.15% (GP)

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); aq. = aqueous; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; GP = guinea pig; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; MEK = methyl ethyl ketone; SI = stimulation index.

+ = sensitizer.

- = nonsensitizer.

[1] References for traditional LLNA, guinea pig, and skin irritant data are indicated in **Annex III-1.**

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

[3] Vehicle listed is that used in both the LLNA: DA and the traditional LLNA, unless otherwise noted.

[4] Numbers in parentheses are highest SI and maximum concentration tested; highest SI is at maximum concentration test, unless otherwise noted.

[5] Based on studies using either the guinea pig maximization test or the Buehler test.

[6] Tested in AOO in LLNA: DA and ACE in traditional LLNA.

[7] Highest SI occurred at concentration 1%.

[8] Highest SI occurred at concentration 25%.

[9] Highest SI occurred at concentration 10%.

6.4.3 Discordance Among the LLNA: DA, Traditional LLNA, and/or the Human Outcome

When analyses were restricted to the 41 substances with unequivocal LLNA: DA, traditional LLNA, and human outcomes, the LLNA: DA classified four substances differently compared with the classification of the traditional LLNA (**Table C-7**). 3-Aminophenol, 2-mercaptobenzothiazole, methyl methacrylate, and nickel (II) sulfate hexahydrate were identified as nonsensitizers by the LLNA: DA while the traditional LLNA and human outcomes classified these substances as

sensitizers. All four discordant substances were tested at similar or higher concentrations in the LLNA: DA and in the traditional LLNA yet the substances were still classified as nonsensitizers (**Table C-7**). There are few commonalities among these substances with regard to chemical class, physical form, molecular weight, peptide reactivity (see **Annex II** for physicochemical information), range of EC3 values (based on traditional LLNA, see **Table C-2**), and potential for skin irritation (**Annex III**):

- 3-Aminophenol is an amine and phenol compound, 2-mercaptobenzothiazole is a heterocyclic compound, methyl methacrylate is a carboxylic acid, and nickel (II) sulfate hexahydrate is a metal.
- All four discordant substances exist as solids in their physical state except methyl methacrylate, which is a liquid.
- All four discordant substances are soluble in water except 2-mercaptobenzothizole.
- Molecular weights range from 100 to 167 g/mol.
- 2-Mercaptobenzothaizole has high peptide reactivity and 3-aminophenol has minimal peptide reactivity; peptide reactivity information for methyl methacrylate and nickel (II) sulfate hexahydrate is not available.
- All four discordant substances are classified as sensitizers by the traditional LLNA (EC3 values are 1.7% for 2-mercaptobenzothiazole, 3.2% for 3-aminophenol, 4.8% for nickel [II] sulfate hexahydrate, and 90% for methyl methacrylate).
- All four discordant substances are classified as nonirritants based on data from GP studies, although human data indicate that nickel (II) sulfate hexahydrate is an irritant at 10% (**Table C-7**).

In addition, the LLNA: DA predicted the same outcome for SLS as the traditional LLNA (i.e., sensitizer), but was discordant when compared to the negative human test result (**Table C-7**). Diethyl phthalate, isopropanol, nickel (II) chloride, propylparaben and sulfanilamide were also predicted similarly by the LLNA: DA and the traditional LLNA (i.e., nonsensitizers) but were discordant when compared to the positive human test result (**Table C-7**). There are few commonalities among these substances with regard to chemical class, physical form, molecular weight, peptide reactivity (see **Annex II** for physicochemical information), range of EC3 values (based on traditional LLNA, see **Table C-2**), and potential for skin irritation (**Annex III**):

- SLS is an alcohol, sulfur, and lipid compound; diethyl phthalate is a carboxylic acid, isopropanol is an alcohol, nickel (II) chloride is a metal, propylparaben is a phenol compound, and sulfanilamide is a cyclic hydrocarbon and sulfur compound.
- SLS exists as a solid in its physical state; diethyl phthalate and isopropanol are liquids in their physical state, whereas nickel (II) chloride, propylparaben, and sulfanilamide exist as solids in their physical state.
- These substances have varying molecular weights that range from 60 to 222 g/mol for diethyl phthalate, isopropanol, nickel (II) chloride, propylparaben, and sulfanilamide to 288 g/mol for SLS.
- SLS, diethyl phthalate, isopropanol, nickel (II) chloride, and sulfanilamide are soluble in water and propylparaben is not.
- Diethyl phthalate, isopropanol, propylparaben, and sulfanilamide have minimal peptide reactivity; peptide reactivity data for nickel (II) chloride and SLS are not available.
- SLS has been found to be a skin irritant based on results in mice, rabbits, or humans; diethyl phthalate, isopropanol, nickel (II) chloride, propylparaben, and sulfanilamide are considered negative or nonirritants based on studies in rabbits or GP (**Table C-7**).

Table C-7 Discordant Results for the LLNA: DA (Using SI ≥ 3.0 for Sensitizers) Compared to Traditional LLNA and Human Reference Data[1]

Substance Name[2]	Vehicle[3]	LLNA: DA[4]	Traditional LLNA[4]	Human Outcomes[5]	Skin Irritant?
Sodium lauryl sulfate (8.08%)	DMF	+ (3.4, 10%)	+ (8.9, 20%)	- (0/22 at 10%)	Irritant at 20% aq. (rabbits); Irritant at 20% (humans)
Diethyl phthalate	AOO	- (1.09, 100%)[6]	- (1.5, 100%)	+ (HPTA)	Negative at 100% (rabbits)
Isopropanol	AOO	- (1.97, 50%)	- (1.7, 50%)[6]	+ (case study at 0.001%)	Negative at 100% (rabbits)
Nickel (II) chloride	DMSO	- (1.3, 10%)	- (2.4, 5%)	+ (HMT, data expressed as nickel)	Negative at ≤0.15% (GP)
Propylparaben	AOO	- (1.3, 25%)	- (1.4, 25%)[7]	+ (HMT)	Nonirritant at 10% (GP)
Sulfanilamide	DMF	- (0.9, 50%)[6]	- (1.0, 50%)[8]	+ (20/25 at 25%)	Nonirritant at 25% (humans)
3-Aminophenol (3.2%)	AOO	- (2.8, 10%)	+ (5.7, 10%)	+	Nonirritant at 5% (GP)
2-Mercapto-benzothiazole (1.7%)	DMF	- (2.0, 50%)[9]	+ (8.6, 10%)	+ (24/63 at 25%)	Nonirritant at 10% (GP); Nonirritant at 25% (humans)
Methyl methacrylate (90%)	AOO	- (1.8, 100%)	+ (3.6, 100%)	+	Nonirritant at 3 M (GP)
Nickel (II) sulfate hexahydrate (4.8%)	DMSO	- (11.8, 10%)	+ (3.1, 5%)	+ (23/88 at 1%)	Irritant at 10% (humans); Nonirritant at 0.15% (GP)

Abbreviations: AOO = acetone: olive oil (4:1); aq. = aqueous; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; GP = guinea pig; HMT = human maximization test; HPTA = human patch test allergen; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

+ = sensitizer.

- = nonsensitizer.

[1] References for traditional LLNA, human, and skin irritant data are indicated in **Annex III-1.**

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

[3] Vehicle listed is that used in both the LLNA: DA and the traditional LLNA, unless otherwise noted.

[4] Numbers in parentheses are highest SI and maximum concentration tested; highest SI is at maximum concentration tested, unless otherwise noted.

[5] Based on studies using either the human maximization test, inclusion of the test substance in a human patch test allergen kit, and/or published clinical case studies/reports.

[6] Highest SI occurred at concentration 25%.

[7] Highest SI occurred at concentration 5%.

[8] Highest SI occurred at concentration 10% and 25%.

[9] Highest SI occurred at concentration 10%.

6.5 Accuracy Analysis Using Single Alternative Decision Criteria

In addition to the accuracy analysis using SI ≥ 3.0 to classify substances as sensitizers, other decision criteria were evaluated on the LLNA: DA test method performance, using the traditional LLNA (SI ≥ 3.0) as the comparative test (**Annex III**). The performance characteristics presented in this section are for 14 decision criteria that were used to determine whether the skin sensitization potential for the substances were positive (i.e., sensitizing) or negative (i.e., nonsensitizing). The substances evaluated were the 44 substances discussed in **Section 6.1** with both LLNA: DA and adequate comparative traditional LLNA data. The decision criteria analyzed included the following:

1. SI values ≥1.3, ≥1.5, ≥1.8, ≥2.0, ≥2.5, ≥3.0, ≥3.5, ≥4.0, ≥4.5, or ≥5.0
2. Log-transformed ATP values of treated groups statistically different from control group based on analysis of variance (ANOVA) with a post-hoc Dunnett's test, when multiple treatment groups were tested, or Student's *t*-test when there was only one dosed group
3. Mean ATP values of treated groups ≥95% confidence interval (CI) of the control group mean
4. Mean ATP values of treated groups ≥2 standard deviations (SD) or ≥3 SD from the control group mean

Multiple tests were available for 14 substances tested with the LLNA: DA. The results for each of these substances were combined so that each substance was represented by one positive or negative result for each criterion evaluated for the accuracy analyses. The results were combined in three ways and a separate accuracy analysis was performed for each approach.

1. The positive/negative outcome for each substance was the most prevalent outcome for each criterion. 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 for 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 was determined by the outcome of the test with the lowest maximum SI of the multiple tests.

The analysis using the most prevalent outcome for substances with multiple tests is presented in this section; the analyses using the highest maximum SI and the lowest maximum SI are included in **Annex V**.

When combining multiple test results for a single substance based on the most prevalent outcome, using the decision criterion of SI ≥ 3.0 to identify sensitizers, the 44 substances analyzed yielded an accuracy of 91% (40/44), a sensitivity of 88% (28/32), a specificity of 100% (12/12), a false positive rate of 0% (0/12), and a false negative rate of 13% (4/32) (**Table C-8)**. The decision criterion of SI ≥ 2.5 was similar to SI ≥ 3.0 in its performance characteristics. In comparison, the decision criteria using higher SI values, SI ≥ 3.5 to SI ≥ 5.0, decreased performance except for specificity, which remained at 100% (12/12), and the false positive rate, which remained at 0% (0/12) (**Figure C-1** and **Table C-8**). Specifically, at SI ≥ 5.0, accuracy decreased to 57% (25/44) and the false negative rate increased to 59% (19/32).

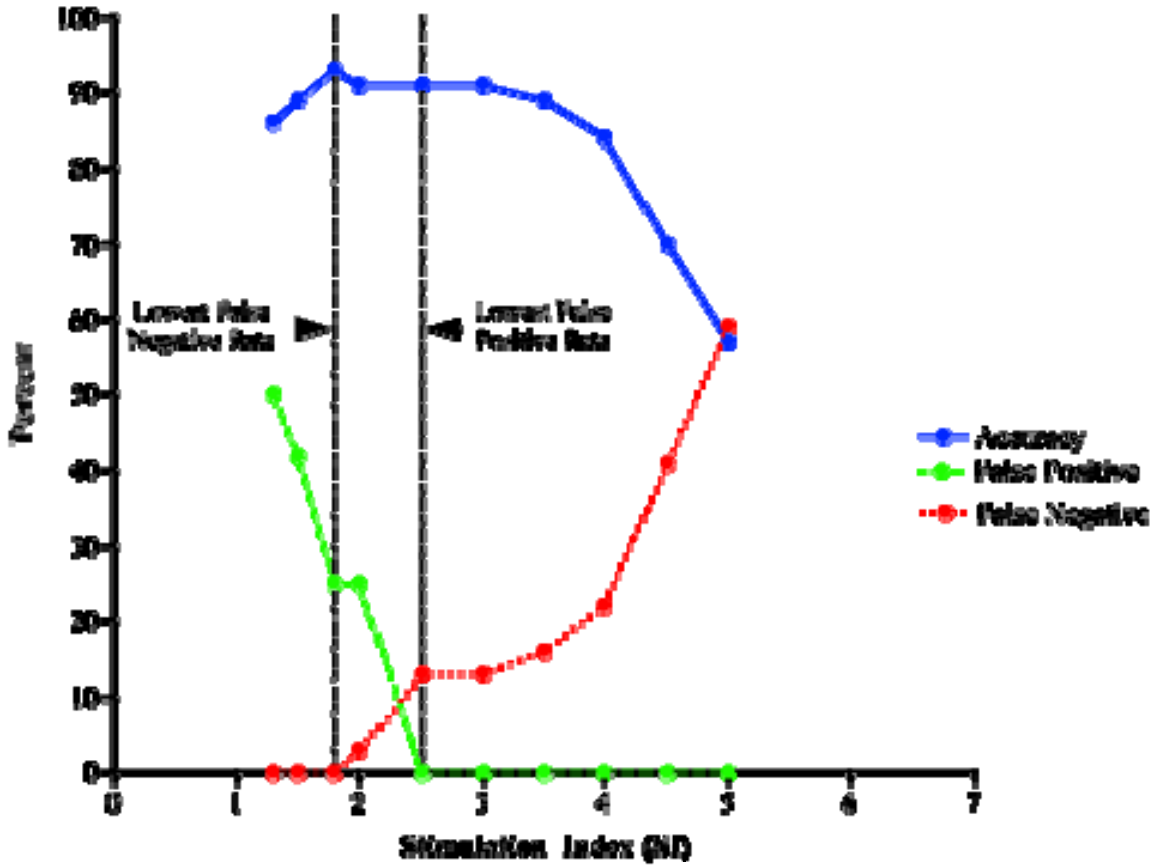
The decision criteria using lower SI values, SI ≥ 1.5 and SI ≥ 1.3, also decreased performance compared to SI ≥ 3.0 except for sensitivity, which increased to 100% (32/32), and the false negative rate, which decreased to 0% (0/32) (**Figure C-1** and **Table C-8**). Further, compared to SI ≥ 3.0, the lower SI cutoff of 2.0 had the same accuracy (91% [40/44]) but had an increased sensitivity of 97% (31/32), although specificity decreased to 75% (9/12) and the false positive rate increased to 25% (3/12) while the false negative rate decreased to 3% (1/32) (**Figure C-1** and **Table C-8**). Notably, the SI decision criterion that exhibited optimum performance characteristics compared to SI ≥ 3.0 was SI ≥ 1.8 (**Figure C-1** and **Table C-8**). Compared to SI ≥ 3.0, the lower SI cutoff of 1.8 had increased accuracy (93% [41/44]) and sensitivity (100% [32/32]), although specificity decreased to 75% (9/12) and the false positive rate increased to 25% (3/12) while the false negative rate decreased to 0% (0/32) (**Figure C-1** and **Table C-8**).

Use of ANOVA and summary statistics (i.e., mean ATP values of treated groups ≥95% confidence interval of the control group mean, or ≥2 or 3 SD from the control group mean), yielded accuracy values of 75 to 84%, with sensitivity values of 88 to 100%, and false negative rates of 0 to 13%. The specificity for these criteria ranged from 8 to 58% and the false positive rates were 42 to 92%. None of the statistical criterion evaluated exhibited increased performance characteristics when compared to SI ≥ 3.0 (**Table C-8**).

An evaluation to determine the robustness of the optimum SI ≥ 1.8 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 criterion or on the resulting number of false or false negative results (see **Annex VI**). Since the decision criterion of SI ≥ 1.8 showed optimum performance (i.e., increased accuracy and sensitivity, and decreased false negative rate compared to SI ≥ 3.0), it was further compared to SI ≥ 3.0 for accuracy against GP and human data (**Table C-9)**. When the LLNA: DA was compared to GP outcomes for substances with LLNA: DA, traditional LLNA, and GP data (40 substances), SI ≥ 1.8 had increased accuracy (80% [32/40] vs. 78% [31/40]), increased sensitivity (96% [25/26] vs. 85% [22/26]) and decreased specificity (50% [7/14] vs. 64% [9/14]) when compared with SI ≥ 3.0. Accordingly, the false positive rate was increased (50% [7/14] vs. 36% [5/14]) and the false negative rate was decreased (4% [1/26] vs. 15% [4/26]) for SI ≥ 1.8 compared to SI ≥ 3.0. The overall performance of the LLNA: DA (SI ≥ 1.8 or SI ≥ 3.0) compared to the traditional LLNA (SI ≥ 3.0) to predict GP outcomes was less (see **Table C-9**).

When the LLNA: DA was compared to human outcomes for substances with LLNA: DA, traditional LLNA, and human data (41 substances), SI ≥ 1.8 increased the accuracy (80% [33/41] vs. 76% [31/41]) and sensitivity (86% [30/35] vs. 74% [26/35]) and decreased the specificity (50% [3/6] vs. 83% [5/6]) when compared with SI ≥ 3.0. Accordingly, the false positive rate was increased (50% [3/6] vs. 17% [1/6]) and the false negative rate was decreased (14% [5/35] vs. 26% [9/35]). The overall performance of the LLNA: DA (SI ≥ 1.8 or SI ≥ 3.0) compared to the traditional LLNA (SI ≥ 3.0) to predict human outcomes was less (see **Table C-9**).

Figure C-1 Performance of the LLNA: DA for 44 Substances Compared to the Traditional LLNA in Predicting Skin Sensitization Potential Using Alternative SI Based on 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: DA with the SI cutoff used to identify sensitizers. This analysis used LLNA: DA and traditional LLNA results for 44 substances (32 traditional LLNA sensitizers and 12 traditional LLNA nonsensitizers). For the 14 substances with multiple test results in the LLNA: DA, the results for each substance were combined by 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.

Abbreviations: LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

Table C-8 Performance of the LLNA: DA for 44 Substances Compared to the Traditional LLNA in Predicting Skin Sensitization Potential Using Alternative Decision Criteria Based on the Most Prevalent Outcome for Substances with Multiple Tests

Alternate Criterion	N[1]	Accuracy % (No.[2])	Sensitivity % (No.[2])	Specificity % (No.[2])	False Positive Rate % (No.[2])	False Negative Rate % (No.[2])	Positive Predictivity % (No.[2])	Negative Predictivity % (No.[2])
Statistics[3]	44	84 (37/44)	94 (30/32)	58 (7/12)	42 (5/12)	6 (2/32)	86 (30/35)	78 (7/9)
≥95% CI[4]	44	75 (33/44)	100 (32/32)	8 (1/12)	92 (11/12)	0 (0/32)	74 (32/43)	100 (1/1)
≥2 SD[5]	44	77 (34/44)	91 (29/32)	42 (5/12)	58 (7/12)	9 (3/32)	81 (29/36)	63 (5/8)
≥3 SD[6]	44	80 (35/44)	88 (28/32)	58 (7/12)	42 (5/12)	13 (4/32)	85 (28/33)	64 (7/11)
SI ≥ 5.0	44	57 (25/44)	41 (13/32)	100 (12/12)	0 (0/12)	59 (19/32)	100 (13/13)	39 (12/31)
SI ≥ 4.5	44	70 (31/44)	59 (19/32)	100 (12/12)	0 (0/12)	41 (13/32)	100 (19/19)	48 (12/25)
SI ≥ 4.0	44	84 (37/44)	78 (25/32)	100 (12/12)	0 (0/12)	22 (7/32)	100 (25/25)	63 (12/19)
SI ≥ 3.5	44	89 (39/44)	84 (27/32)	100 (12/12)	0 (0/12)	16 (5/32)	100 (27/27)	71 (12/17)
SI ≥ 3.0	*44*	*91 (40/44)*	*88 (28/32)*	*100 (12/12)*	*0 (0/12)*	*13 (4/32)*	*100 (28/28)*	*75 (12/16)*
SI ≥ 2.5	44	91 (40/44)	88 (28/32)	100 (12/12)	0 (0/12)	13 (4/32)	100 (28/28)	75 (12/16)
SI ≥ 2.0	44	91 (40/44)	97 (31/32)	75 (9/12)	25 (3/12)	3 (1/32)	91 (31/34)	90 (9/10)
SI ≥ 1.8	**44**	**93 (41/44)**	**100 (32/32)**	**75 (9/12)**	**25 (3/12)**	**0 (0/32)**	**91 (32/35)**	**100 (9/9)**
SI ≥ 1.5	44	89 (39/44)	100 (32/32)	58 (7/12)	42 (5/12)	0 (0/32)	86 (32/37)	100 (7/7)
SI ≥ 1.3	44	86 (38/44)	100 (32/32)	50 (6/12)	50 (6/12)	0 (0/32)	84 (32/38)	100 (6/6)

Italicized text indicates the decision criterion chosen by the LLNA: DA validation study team; Bold text indicates the single decision criterion that had an overall increased performance in predicting skin sensitization potential when compared to the traditional LLNA.

Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP Content; No. = number; SD = standard deviation; SI = stimulation index.

[1] N = Number of substances included in this analysis.

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

[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 ATP data were log-transformed prior to statistical analysis. For analysis of variance, significance at $p < 0.05$ was further tested by Dunnett's test.

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

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

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

Table C-9 Performance of the LLNA: DA in Predicting Skin Sensitization Potential Comparing Decision Criteria of SI ≥ 3.0 versus SI ≥ 1.8 Based on the Most Prevalent Outcome for Substances with Multiple Tests

Comparison	n[1]	Decision Criterion	Accuracy % (No.[2])	Sensitivity % (No.[2])	Specificity % (No.[2])	False Positive Rate % (No.[2])	False Negative Rate % (No.[2])	Positive Predictivity % (No.[2])	Negative Predictivity % (No.[2])
LLNA: DA vs. Traditional LLNA	44	SI ≥ 3.0	91 (40/44)	88 (28/32)	100 (12/12)	0 (0/12)	13 (4/32)	100 (28/28)	75 (12/16)
		SI ≥ 1.8	93 (41/44)	100 (32/32)	75 (9/12)	25 (3/12)	0 (0/32)	91 (32/35)	100 (9/9)
Substances with LLNA: DA, Traditional LLNA, and GP Data									
LLNA: DA vs. Traditional LLNA	40	SI ≥ 3.0	93 (37/40)	90 (27/30)	100 (10/10)	0 (0/10)	10 (3/30)	100 (27/27)	77 (10/13)
		SI ≥ 1.8	95 (38/40)	100 (30/30)	80 (8/10)	20 (2/10)	0 (0/30)	94 (30/32)	100 (8/8)
LLNA: DA vs. GP[3]	40	SI ≥ 3.0	78 (31/40)	85 (22/26)	64 (9/14)	36 (5/14)	15 (4/26)	81 (22/27)	69 (9/13)
		SI ≥ 1.8	80 (32/40)	96 (25/26)	50 (7/14)	50 (7/14)	4 (1/26)	78 (25/32)	88 (7/8)
Traditional LLNA vs. GP[3]	40	SI ≥ 3.0	85 (34/40)	96 (25/26)	64 (9/14)	36 (5/14)	4 (1/26)	83 (25/30)	90 (9/10)
Substances with LLNA: DA, Traditional LLNA, and Human Data									
LLNA: DA vs. Traditional LLNA	41	SI ≥ 3.0	90 (37/41)	87 (27/31)	100 (10/10)	0 (0/10)	13 (4/31)	100 (27/27)	71 (10/14)
		SI ≥ 1.8	95 (39/41)	100 (31/31)	80 (8/10)	20 (2/10)	0 (0/31)	94 (31/33)	100 (8/8)
LLNA: DA vs. Human[4]	41	SI ≥ 3.0	76 (31/41)	74 (26/35)	83 (5/6)	17 (1/6)	26 (9/35)	96 (26/27)	36 (5/14)
		SI ≥ 1.8	80 (33/41)	86 (30/35)	50 (3/6)	50 (3/6)	14 (5/35)	91 (30/33)	38 (3/8)
Traditional LLNA vs. Human[4]	41	SI ≥ 3.0	85 (35/41)	86 (30/35)	83 (5/6)	17 (1/6)	14 (5/35)	97 (30/31)	50 (5/10)

Abbreviations: GP = guinea pig skin sensitization outcomes; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; No. = number; SI = stimulation index; vs. = versus.

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

[2] The proportion 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, 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 Single Alternative Decision Criteria

This section discusses the discordant results obtained for the analyses using the alternative decision criteria shown in **Tables C-8** and **C-9,** in order to provide a comparison to the discordant substances identified when using the decision criterion of SI ≥ 3.0 to identify sensitizers. Discordant results for the alternative decision criteria are first discussed in general using the traditional LLNA as the reference test (**Section 6.6.1**) and then discordant results for SI ≥ 1.8, the single optimized alternative decision criterion, are discussed using the traditional LLNA, GP, and human outcomes as references (**Section 6.6.2**).

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

Table C-10 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. Using SI ≥ 2.0 as the decision criterion resulted in three nonsensitizers in the traditional LLNA (chlorobenzene, hexane, and salicylic acid) being misclassified as sensitizers in the LLNA: DA. Also, methyl methacrylate, a sensitizer in the traditional LLNA, was misclassified as a nonsensitizer in the LLNA: DA. Using SI ≥ 1.8 as the decision criterion still resulted in chlorobenzene, hexane, and salicylic acid being misclassified as sensitizers in the LLNA: DA compared to the traditional LLNA, although methyl methacrylate was no longer misclassified as a nonsensitizer in the LLNA: DA compared to SI ≥ 2.0. As the SI decision criterion was further reduced to SI ≥ 1.5 and SI ≥ 1.3, two additional substances, 1-bromobutane and methyl salicylate, were also misclassified as sensitizers when compared to traditional LLNA results. In addition, using SI ≥ 1.3 also misclassified nickel (II) chloride as a sensitizer in the LLNA: DA compared to the traditional LLNA. Increasing the SI cutoff to values greater than three increased the number of sensitizers that were misclassified as nonsensitizers. At SI ≥ 5.0, 19 substances were discordant. As **Table C-10** shows, all 19 substances were sensitizers in the LLNA but misclassified as nonsensitizers in the LLNA: DA.

Use of a statistical test (i.e., ANOVA or *t*-test) to identify sensitizers misclassified two sensitizers in the traditional LLNA (2-mercaptobenzothiazole and methyl methacrylate) as nonsensitizers in the LLNA: DA and five nonsensitizers (1-bromobutane, chlorobenzene, hexane, salicylic acid, and sulfanilamide) as sensitizers. Use of summary statistics (i.e., ≥95% CI, ≥2 SD or ≥3 SD) generally misclassified nonsensitizers in the traditional LLNA as sensitizers in the LLNA: DA. Specifically, using ≥3 SD of vehicle control mean misclassified five nonsensitizers as sensitizers: 1-bromobutane, chlorobenzene, hexane, nickel (II) chloride, and propylparaben. Using treatment group absorbance ≥2 SD of vehicle control mean misclassified the same five substances as sensitizers, as well as methyl salicylate and salicylic acid. Using the treatment group absorbance ≥95% CI of vehicle control mean misclassified all the nonsensitizers misclassified as sensitizers in the LLNA: DA when using either ≥3 SD or ≥2 SD of vehicle control mean, as well as four additional substances: diethyl phthalate, dimethyl isophthalate, isopropanol, and lactic acid. In some instances, use of summary statistics (i.e., ≥95% CI, ≥2 SD or ≥3 SD) misclassified sensitizers in the traditional LLNA as nonsensitizers in the LLNA: DA. Using ≥3 SD of vehicle control mean misclassified four traditional LLNA sensitizers as LLNA: DA nonsensitizers: butyl glycidyl ether, ethyl acrylate, methyl methacrylate, and propyl gallate. Using treatment group absorbance ≥2 SD of vehicle control mean only misclassified ethyl acrylate and propyl gallate as nonsensitizers in the LLNA: DA compared to the traditional LLNA and using the treatment group absorbance ≥95% CI did not misclassify any traditional LLNA sensitizers as LLNA: DA nonsensitizers.

6.6.2 Discordant Results for Accuracy Analysis Using a Single Optimized Alternative Decision Criterion (SI ≥ 1.8)

When analyses were restricted to the 40 substances with unequivocal LLNA: DA, traditional LLNA, and GP data based on an SI ≥ 1.8, the LLNA: DA classified two substances (chlorobenzene and salicylic acid) differently compared with the classification of the traditional LLNA (**Table C-11**). Chlorobenzene and salicylic acid were classified as sensitizers in the LLNA: DA and as nonsensitizers by both the traditional LLNA and GP outcomes. In contrast, benzalkonium chloride, ethyl acrylate, ethylene glycol dimethacrylate, resorcinol, and sodium lauryl sulfate were identified as sensitizers by the LLNA: DA similar to the traditional LLNA but as nonsensitizers based on GP outcomes. Further, nickel (II) chloride was identified as a nonsensitizer by the LLNA: DA similar to the traditional LLNA but as a sensitizer based on GP outcomes. There are few commonalities among these substances with regard to chemical class, physical form, molecular weight, peptide reactivity (see **Annex II** for physicochemical information), range of EC3 values (based on traditional LLNA, see **Table C-2**), and potential for skin irritation (**Annex III**) as follows:

- Chlorobenzene is a halogenated hydrocarbon compound and salicylic acid is a phenol and carboxylic acid; benzalkonium chloride is an amine (also an onium compound), ethyl acrylate and ethylene glycol dimethacrylate are carboxylic acids, resorcinol is a phenol, and SLS is an alcohol, sulfur, and lipid compound; nickel (II) chloride is a metal.
- Chlorobenzene exists as a liquid and salicylic acid exists as a solid in its physical state; benzalkonium chloride can exist in both a solid and liquid physical state, whereas ethyl acrylate and ethylene glycol dimethacrylate are liquids, and resorcinol and SLS are solids; nickel (II) chloride is a solid.
- Chlorobenzene has a molecular weight of 113 g/mol and salicylic acid has a molecular weight of 138 g/mol; the five substances that are concordant with the traditional LLNA but discordant with GP outcomes have varying molecular weights that range from 100 g/mol for ethyl acrylate, 110 g/mol for resorcinol, 171 g/mol for benzalkonium chloride, and 198 g/mol for ethylene glycol dimethacrylate to 288 g/mol for SLS; the molecular weight for nickel (II) chloride is 130 g/mol.
- All the discordant substances are soluble in water.
- Chlorobenzene has minimal peptide reactivity while peptide reactivity data for salicylic acid are not available; the peptide reactivity for resorcinol is identified as minimal, and that for ethyl acrylate and ethylene glycol dimethacrylate is high while peptide reactivity data for benzalkonium chloride and SLS are not available; peptide reactivity data for nickel (II) chloride are not available.
- Benzalkonium chloride (EC3 = 0.07%), ethyl acrylate (EC3 = 32.8%), ethylene glycol dimethacrylate (EC3 = 28%), resorcinol (EC3 = 6.33%), and SLS (EC3 = 8.08%) are identified as sensitizers by the traditional LLNA.
- Chlorobenzene has low irritancy potential assumed based on clinical literature while salicylic acid is an irritant at 20% in mice; benzalkonium chloride and SLS have been found to be skin irritants based on results in mice, rabbits, or humans and ethyl acrylate, ethylene glycol dimethacrylate, and resorcinol are considered nonirritants based on studies in humans or GP; nickel (II) chloride is considered a negative at ≤0.15% based on GP data (**Table C-11**).

When analyses were restricted to the 40 substances with unequivocal LLNA: DA, traditional LLNA, and human outcomes based on an SI ≥ 1.8, the LLNA: DA classified two substances (hexane and salicylic acid) differently compared with the classification of the traditional LLNA (**Table C-12**). Hexane and salicylic acid were classified as sensitizers in the LLNA: DA and as nonsensitizers by both the traditional LLNA and human outcomes. Further, SLS was classified as a sensitizer by the LLNA: DA and traditional LLNA but as a nonsensitizer based on human outcomes. In contrast,

diethyl phthalate, isopropanol, nickel (II) chloride, propylparaben, and sulfanilamide were all classified as nonsensitizers by the LLNA: DA and the traditional LLNA but as sensitizers based on human outcomes (**Table C-12**). In instances where the substances were discordant in the LLNA: DA compared to the traditional LLNA, the discordant substances were tested at the same maximum concentration. There are few commonalities among these substances with regard to chemical class, physical form, molecular weight, peptide reactivity (see **Annex II** for physicochemical information), range of EC3 values (based on traditional LLNA, see **Table C-2**), and potential for skin irritation (**Annex III**):

- Hexane is an acyclic hydrocarbon compound and salicylic acid is a phenol and carboxylic acid; SLS is an alcohol, sulfur, and lipid compound; diethyl phthalate is a carboxylic acid, isopropanol is an alcohol, nickel (II) chloride is a metal, propylparaben is a phenol compound, and sulfanilamide is sulfur compound.
- Hexane is a liquid and salicylic acid is a solid; SLS is a solid; diethyl phthalate and isopropanol are liquids while nickel (II) chloride, propylparaben, and sulfanilamide are solids.
- Hexane and salicylic acid have molecular weights of 86 g/mol and 138 g/mol, respectively; the molecular weight for SLS is 288 g/mol; the other discordant substances have varying molecular weights that range from 60 g/mol for isopropanol, 130 g/mol for nickel (II) chloride, 172 g/mol for sulfanilamide, and 180 g/mol for propylparaben to 222 g/mol for diethyl phthalate.
- Hexane, salicylic acid, SLS, diethyl phthalate, isopropanol, nickel (II) chloride, and sulfanilamide are soluble in water; propylparaben is not.
- Hexane, diethyl phthalate, isopropanol, propylparaben, and sulfanilamide have minimal peptide reactivity; peptide reactivity information for salicylic acid, nickel (II) chloride, and SLS is not available.
- SLS is identified as a sensitizer by the traditional LLNA (EC3 = 8.08%).
- Hexane has been found to be an irritant at 100% in humans as has salicylic acid at 20% in mice; SLS has been found to be a skin irritant based on results in mice, rabbits, or humans; diethyl phthalate, isopropanol, nickel (II) chloride, propylparaben, and sulfanilamide are considered to be nonirritants based on studies in rabbits, GP, or humans (**Table C-12**).

Table C-10 Discordant Results for the LLNA: DA Using Alternative Decision Criteria Compared to the Traditional LLNA Based on 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 ≥ 1.8	SI ≥ 1.5	SI ≥ 1.3
3-Aminophenol (3.2%)					-	-	-	-	-	-				
p-Benzoquinone (0.01%)					-	-	-							
1-Bromobutane (-)	+	+	+	+									+	+
Butyl glycidyl ether (30.9%)				-	-									
Chlorobenzene (-)	+	+	+	+							+	+	+	+
Cinnamic aldehyde (1.91%)					-									
Citral (9.17%)					-	-								
Cobalt chloride (0.6%)					-	-								
Diethyl maleate (3.6%)					-	-	-							
Diethyl phthalate (-)		+												
Dimethyl isophthalate (-)		+												
Ethyl acrylate (32.8%)			-	-	-	-								
Ethylene glycol dimethacrylate (28%)					-	-								
Formaldehyde (0.5%)					-									
Hexane (-)	+	+	+	+							+	+	+	+
Imidazolidinyl urea (24%)					-									
Isopropanol (-)		+												
Lactic acid (-)		+												
2-Mercaptobenzothiazole (1.7%)	-				-	-	-	-	-	-				

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 ≥ 1.8	SI ≥ 1.5	SI ≥ 1.3
Methyl methacrylate (90%)	-		-	-	-	-	-	-	-	-	-			
Methyl salicylate (-)		+	+										+	+
Nickel (II) chloride (-)		+	+	+										+
Nickel (II) sulfate hexahydrate (4.8%)					-	-	-	-	-	-				
Phenyl benzoate (13.6%)					-	-								
Propyl gallate (0.32%)			-	-	-									
Propylparaben (-)		+	+	+										
Resorcinol (6.33%)					-	-								
Salicylic acid (-)	+	+	+								+	+	+	+
Sulfanilamide (-)	+													
Sodium lauryl sulfate (8.08%)					-	-	-	-						
Trimellitic anhydride (4.71%)					-									

Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP Content; SD = standard deviation; SI = stimulation index.

[1] Compared to the traditional LLNA; traditional LLNA result in parentheses are "-" for nonsensitizers and EC3 value for sensitizers.

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

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

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

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

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

Table C-11 Discordant Results for the LLNA: DA (Using SI ≥ 1.8 for Sensitizers) Compared to Traditional LLNA and GP Reference Data[1]

Substance Name[2]	Vehicle[3]	LLNA: DA[4]	Traditional LLNA[4]	Guinea Pig Studies[5]	Skin Irritant?
Chlorobenzene (-)	AOO	+ (2.4, 25%)	- (1.7, 10%)[6]	-	No data. Low irritancy potential assumed based on clinical literature.
Salicylic acid (-)	AOO	+ (2.0, 25%)	- (2.4, 25%)	-	Irritant at 20% aq. (mice)
Benzalkonium chloride (0.07%)	AOO ACE[7]	+ (6.7, 2.5%)	+ (11.1, 2%)[8]	-	Irritant at 2% and 1% ACE (mice)
Ethyl acrylate (32.8%)	AOO	+ (4.3, 50%)[6]	+ (4.0, 50%)	-	Nonirritant at 0.3 M (GP)
Ethylene glycol dimethacrylate (28%)	MEK	+ (4.5, 50%)	+ (7.0, 50%)	-	Nonirritant at 1% (GP)
Resorcinol (6.33%)	AOO	+ (4.3, 25%)[9]	+ (10.4, 50%)	-	Nonirritant at 15% (humans)
Sodium lauryl sulfate (8.08%)	DMF	+ (3.4, 10%)	+ (8.9, 20%)	-	Irritant at 20% aq. (rabbits); irritant at 20% (humans)
Nickel (II) chloride (-)	DMSO	- (1.3, 10%)	- (2.4, 5%)	+	Negative at ≤0.15% (GP)

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); aq. = aqueous ; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; GP = guinea pig; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; MEK = methyl ethyl ketone; SI = stimulation index.

+ = sensitizer.

- = nonsensitizer.

[1] References for traditional LLNA, guinea pig, and skin irritant data are indicated in **Annex III-1.**

[2] Numbers in parentheses are EC3 values (estimated concentration needed to produce a stimulation index [SI] of three) for substances that are sensitizers in the traditional LLNA (see **Table C-2**). Minus signs (-) indicate substances that were negative in the traditional LLNA.

[3] Vehicle listed is that used in both the LLNA: DA and the traditional LLNA, unless otherwise noted.

[4] Numbers in parentheses are highest SI and maximum concentration tested; highest SI is at maximum concentration tested, unless otherwise noted.

[5] Based on studies using either the guinea pig maximization test or the Buehler test.

[6] Highest SI occurred at concentration 25%.

[7] Benzalkonium chloride tested in AOO vehicle in LLNA: DA and ACE vehicle in traditional LLNA.

[8] Highest SI occurred at concentration 1%.

[9] Highest SI occurred at concentration 10%.

Table C-12 Discordant Results for the LLNA: DA (Using SI ≥ 1.8 for Sensitizers) Compared to Traditional LLNA and Human Reference Data[1]

Substance Name[2]	Vehicle[3]	LLNA: DA[4]	Traditional LLNA[4]	Human Outcomes[5]	Skin Irritant?
Hexane (-)	AOO	+ (2.3, 100%)	- (2.2, 100%)	- (0/25 at 100%)	Irritant at 100% (humans)
Salicylic acid (-)	AOO	+ (2.0, 25%)	- (2.4, 25%)	-	Irritant at 20% aq. (mice)
Sodium lauryl sulfate (8.08%)	DMF	+ (3.4, 10%)	+ (8.9, 20%)	- (0/22 at 10%)	Irritant at 20% aq. (rabbits); irritant at 20% (humans)
Diethyl phthalate (-)	AOO	- (1.09, 100%)[6]	- (1.5, 100%)	+ (HPTA)	Negative at 100% (rabbits)
Isopropanol (-)	AOO	- (1.97, 50%)	- (1.7, 50%)[7]	+ (case study at 0.001%)	Negative at 100% (rabbits)
Nickel (II) chloride (-)	DMSO	- (1.3, 10%)	- (2.4, 5%)	+	Negative at ≤0.15% (GP)
Propylparaben (-)	AOO	- (1.3, 25%)	- (1.4, 25%)[8]	+ (HMT)	Nonirritant at 10% (GP)
Sulfanilamide (-)	DMF	- (0.9, 50%)[6]	- (1.0, 50%)[9]	+	Nonirritant at 25% (humans)

Abbreviations: aq. = aqueous; AOO = acetone: olive oil (4:1); DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; GP = guinea pig; HMT = human maximization test; HPTA = human patch test allergen; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

+ = sensitizer.

- = nonsensitizer.

[1] References for traditional LLNA, human, and skin irritant data are indicated in **Annex III-1.**

[2] Numbers in parentheses are EC3 values (estimated concentration needed to produce a stimulation index [SI] of three) for substances that are sensitizers in the traditional LLNA (see **Table C-2**). Minus signs (-) indicate substances that were negative in the traditional LLNA.

[3] Vehicle listed is that used in both the LLNA: DA and the traditional LLNA, unless otherwise noted.

[4] Numbers in parentheses are highest SI and maximum concentration tested; highest SI is at maximum concentration tested, unless otherwise noted.

[5] Based on studies using either the human maximization test, inclusion of the test substance in a human patch test allergen kit, and/or published clinical case studies/reports.

[6] Highest SI occurred at concentration 25%.

[7] Highest SI occurred at concentration 10%.

[8] Highest SI occurred at concentration 5%.

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

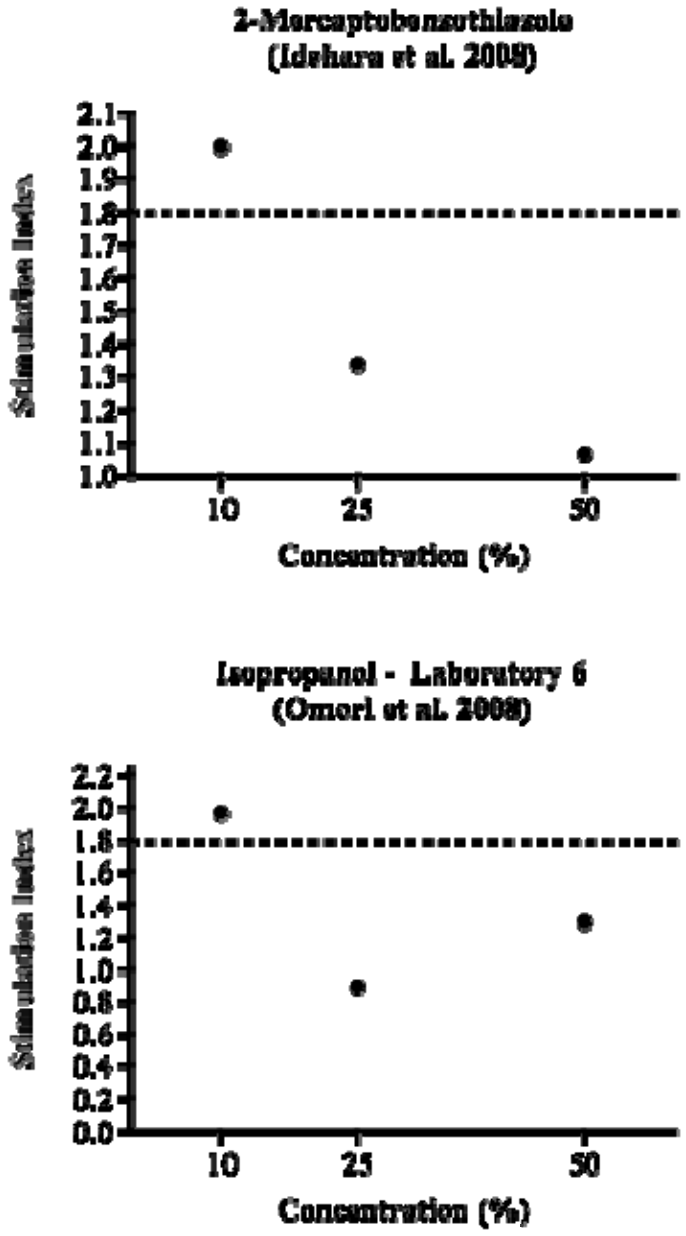
6.7 Accuracy Analysis for the Reduced LLNA: DA Using the SI ≥ 1.8 Decision Criterion

An accuracy analysis for the rLLNA: DA was performed using the optimized SI ≥ 1.8 criterion to identify sensitizers. The rLLNA: DA uses only the highest dose of the test substance that does not produce excessive skin irritation and/or systemic toxicity; the two lower dose groups are not used. The available validation database for the rLLNA: DA analysis included 123 individual tests that used multiple doses. The performance of the rLLNA: DA was evaluated 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 ≥ 1.8 to identify sensitizers, the accuracy of the rLLNA: DA was 98% (121/123), with a false positive rate of 0% (0/33) and a false negative rate of 2% (2/90). The two tests that were false negative in the rLLNA: DA were borderline positive in the multiple-dose LLNA: DA. One study that tested 2-mercaptobenzothiazole at 10%, 25%, and 50% produced a maximum SI value of 2.00 at the lowest dose tested (**Figure C-2**). The second false negative test was for isopropanol at 10%, 25%, and 50%, which produced the maximum SI of 1.97 at the lowest dose tested (**Figure C-2**).

6.8 Analyses Using Multiple Alternative Decision Criteria

As detailed in **Section 6.5,** the accuracy of the LLNA: DA when using various single alternative decision criteria was evaluated using the traditional LLNA as the reference test. Compared to the traditional LLNA (SI ≥ 3.0), the optimum performance (i.e., accuracy of 93% [41/44] and sensitivity of 100% [32/32]) was achieved using the decision criterion of SI ≥ 1.8 (**Table C-8**). Although the SI ≥ 1.8 produced a false positive rate of 25% (3/12) it yielded a false negative rate of 0% (0/32) (**Table C-8**). Increasing the SI decision criterion to SI ≥ 2.5 decreased the false positive rate to 0% (0/12) but increased the false negative rate to 13% (4/32). The 0% false positive rate using SI ≥ 2.5 and the 0% false negative rate using SI ≥ 1.8 prompted an evaluation using two SI decision criteria for determining LLNA: DA results: one criterion to classify substances as sensitizers (SI ≥ 2.5) and one criterion to classify substances as nonsensitizers (SI ≤ 1.8). This evaluation is described in detail in **Annex VII**.

Figure C-2 Dose Response Curves for Tests Identified as Sensitizers by the LLNA: DA but as Nonsensitizers by the Reduced LLNA: DA



Note: The horizontal line in each figure indicates an SI ≥ 1.8, which is the threshold that is considered optimum for providing a positive response in the LLNA: DA. Points on or above this line would indicate a positive (sensitizer) response, while points below this line would indicate a negative (nonsensitizer) response.

7.0 LLNA: DA Test Method Reliability

An assessment of test method reliability (intralaboratory repeatability and intra- and interlaboratory reproducibility) is an essential element of any evaluation of the performance of an alternative test method (ICCVAM 2003). Repeatability refers to 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 (ICCVAM 1997, 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. With regard to the LLNA: DA test method, there are no known intralaboratory repeatability studies, which was also the situation with the traditional LLNA.

The LLNA: DA data were amenable to both intralaboratory and interlaboratory reproducibility analyses. The evaluation of a single decision criterion in **Section 6.5** showed that SI ≥ 1.8 was the SI value that produced the most optimum results (i.e., accuracy of 93% [41/44], sensitivity of 100% [32/32], and false negative rate of 0% [0/32]) among the alternative decision criteria evaluated when the traditional LLNA was the reference test (**Table C-8)**. Thus, this section provides an assessment of reproducibility for the decision criterion of SI ≥ 1.8 to identify sensitizers. For additional reproducibility analyses using a single decision criterion see **Annex VIII,** which describes the evaluation of reproducibility for the decision criterion of SI ≥ 3.0 (SI decision criterion used in the intralaboratory and the interlaboratory validation studies) and SI ≥ 2.0 (previously evaluated as an optimum decision criterion in the March 2009 revised draft BRD evaluated by the Panel) to identify sensitizers. Further, the reproducibility analyses based on the evaluation of multiple decision criteria briefly mentioned in **Section 6.8** (i.e., SI ≥ 2.5 as the decision criterion for classifying substances as sensitizers when used with a decision criterion of SI ≤ 1.8 to identify nonsensitizers) is detailed in **Annex VII**.

7.1 Intralaboratory Reproducibility

Idehara et al. (2008) evaluated intralaboratory reproducibility of EC3 values for the LLNA: DA using two substances (isoeugenol and eugenol) that were each tested in three different experiments (**Table C-13**). The data indicate CV values of 21% and 11% for isoeugenol and eugenol, respectively. The authors state that for both compounds the EC3 values appeared to be close and that for each test substance the SI values for the same concentration were fairly reproducible (Idehara et al. 2008). NICEATM also determined the intralaboratory reproducibility of EC1.8 values (estimated concentration needed to produce an SI of 1.8) for the same set of data. This resulted in CV values of 36% and 23% for isoeugenol and eugenol indicating larger intralaboratory variability compared to EC3 values with CV values of 21% and 11% for isoeugenol and eugenol, respectively.

Table C-13 Intralaboratory Reproducibility of EC3 and EC1.8 Values Using the LLNA: DA[1]

Isoeugenol			
Concentration (%)	**Experiment 1[2]**	**Experiment 2[2]**	**Experiment 3[2]**
Vehicle (AOO)	1.00 ± 0.54	1.00 ± 0.54	1.00 ± 0.30
0.5	1.50 ± 0.54	-------	1.22 ± 0.13
1	2.28 ± 0.60	-------	2.77 ± 1.01
2.5	2.78 ± 0.17	3.11 ± 1.15	3.01 ± 0.98

continued

Table C-13 Intralaboratory Reproducibility of EC3 and EC1.8 Values Using the LLNA: DA[1] (continued)

Isoeugenol			
Concentration (%)	**Experiment 1[2]**	**Experiment 2[2]**	**Experiment 3[2]**
5	3.39 ± 0.69	4.39 ± 1.25	-------
10	5.68 ± 1.19	6.77 ± 0.23	-------
EC3	**3.40%**	**2.35%**	**2.46%**
EC1.8	**0.69%**	**1.23%**	**0.69%**
Mean EC3: 2.74% ± 0.58% and 21% CV *Mean EC1.8: 0.87% ± 0.31% and 36% CV*			
Eugenol			
Concentration (%)	**Experiment 1[2]**	**Experiment 2[2]**	**Experiment 3[2]**
Vehicle (AOO)	1.00 ± 0.17	1.00 ± 0.17	1.00 ± 0.09
5	2.92 ± 1.00	2.80 ± 1.08	3.24 ± 0.70
10	7.35 ± 2.62	4.47 ± 0.98	4.79 ± 0.94
25	10.92 ± 3.63	5.62 ± 3.20	7.07 ± 0.44
EC3	**5.09%**	**5.59%**	**4.50%**
EC1.8	**4.20%**	**3.30%**	**2.63%**
Mean EC3: 5.06% ± 0.55% and 11% CV *Mean EC1.8: 3.38% ± 0.79% and 23% CV*			

Abbreviations: AOO = acetone: olive oil (4:1); CV = coefficient of variation; EC1.8 = estimated concentration needed to produce a stimulation index of 1.8; EC3 = estimated concentration needed to produce a stimulation index of three; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

[1] Based on results discussed in Idehara et al. 2008; the number per group was not specified.

[2] Mean stimulation index value ± standard deviation.

7.2 Interlaboratory Reproducibility

Furthermore, data were submitted to NICEATM (**Annex IV**) from a two-phased interlaboratory validation study on the LLNA: DA test method (Omori et al. 2008). In the first phase of the interlaboratory validation study, a blinded test of 12 substances was conducted in 10 laboratories. Three substances (2,4-dinitrochlorobenzene, hexyl cinnamic aldehyde, and isopropanol) were tested in all 10 laboratories. The remaining nine substances were randomly assigned to subsets of three of the 10 laboratories **(Table C-14)**. In each laboratory, each substance was tested one time at three different concentrations. The dose levels for each substance were predetermined (i.e., the participating laboratories did not determine their own dose levels for testing). Nine substances are sensitizers and three substances are nonsensitizers according to traditional LLNA results. Six substances are ICCVAM-recommended LLNA performance standards reference substances: cobalt chloride, 2,4-dinitrochlorobenzene, hexyl cinnamic aldehyde, isoeugenol, isopropanol, and methyl salicylate.

The second phase of the interlaboratory validation study was designed to evaluate the reliability of the LLNA: DA for testing metallic salts using DMSO as a vehicle since two metals dissolved in DMSO (cobalt chloride and nickel (II) sulfate hexahydrate) from the first phase of the interlaboratory validation study yielded inconsistent results. Five coded substances (two of the five substances were unique to the second phase of the interlaboratory validation study) were tested in seven laboratories

(Table C-15). One substance (i.e. hexyl cinnamic aldehyde) was tested in all seven laboratories. The remaining four substances (cobalt chloride, nickel (II) sulfate hexahydrate, lactic acid, and potassium dichromate) were randomly assigned to subsets of four of the seven laboratories. Each laboratory tested the substance one time at three different dose levels. Again, the dose levels for each substance were predetermined. Of the two substances not previously tested in the first phase of the interlaboratory validation study (lactic acid and potassium dichromate), one is a nonsensitizer and the other is a sensitizer according to traditional LLNA results, respectively. In addition, lactic acid is an ICCVAM-recommended LLNA performance standards reference substance.

The LLNA: DA test results from the two-phased interlaboratory validation study are amenable to interlaboratory reproducibility analyses for three endpoints: sensitizer (positive) or nonsensitizer (negative) classification, and EC1.8 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 (EC1.8 values) (**Sections 7.2** and **7.3**).

Table C-14 Substances and Allocation for the First Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	Vehicle	Concentration Tested (%)			Laboratory									
					1	2	3	4	5	6	7	8	9	10
2,4-Dinitro-chlorobenzene (+)	AOO	0.03	0.10	0.30	X	X	X	X	X	X	X	X	X	X
Hexyl cinnamic aldehyde (+)	AOO	5	10	25	X	X	X	X	X	X	X	X	X	X
Isopropanol (-)	AOO	10	25	50	X	X	X	X	X	X	X	X	X	X
Abietic acid (+)	AOO	5	10	25		X				X	X			
3-Aminophenol (+)	AOO	1	3	10	X		X					X		
Dimethyl isophthalate (-)	AOO	5	10	25	X		X				X			
Isoeugenol (+)	AOO	1	3	10				X	X				X	
Methyl salicylate (-)	AOO	5	10	25			X				X			X
Formaldehyde (+)	ACE	0.5	1.5	5.0	X	X			X					
Glutaraldehyde (+)	ACE	0.05	0.15	0.50	X	X			X					
Cobalt chloride[2] (+)	DMSO	0.3	1.0	3.0				X		X		X		
Nickel (II) sulfate hexahydrate (+)	DMSO	1	3	10				X		X		X		

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

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

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

Table C-15 Substances and Allocation for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	Vehicle	Concentration Tested (%)			Laboratory						
					11	12	13	14	15	16	17
Hexyl cinnamic aldehyde (+)	AOO	5	10	25	X	X	X	X	X	X	X
Cobalt chloride[2] (+)	DMSO	1	3	5	X		X	X			X
Lactic acid (-)	DMSO	5	10	25	X		X		X	X	
Nickel (II) sulfate hexahydrate (+)	DMSO	1	3	10	X	X		X		X	
Potassium dichromate (+)	DMSO	0.1	0.3	1.0	X	X			X		X

Abbreviations: AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

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

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

7.2.1 Interlaboratory Reproducibility – Qualitative Results

The qualitative (positive/negative) interlaboratory concordance analysis for the 12 substances that were tested during the first phase of the LLNA: DA interlaboratory validation study is shown in **Table C-16** for SI ≥ 1.8. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), nine substances tested in either three or 10 laboratories had consistent results leading to 100% (3/3 or 10/10) interlaboratory concordance for those substances. There were three substances with discordant results between the labs (isopropanol, 3-aminophenol and nickel [II] sulfate hexahydrate). The interlaboratory concordance for isopropanol was 90% (9/10) and the one discordant lab reported a maximum SI = 1.97 at the lowest dose tested. The interlaboratory concordance for 3-aminophenol and nickel (II) sulfate hexahydrate was 67% (2/3). Two of the three laboratories that tested 3-aminophenol reported SI ≥ 1.8 at the middle dose tested (SI = 2.32 and SI = 1.99 at 10%) and one laboratory did not achieve SI ≥ 1.8 at any dose tested (**Annex IV**). One of the three laboratories that tested nickel (II) sulfate hexahydrate reported a maximum SI = 1.52, while the other two laboratories produced an SI ≥ 1.8 at all three doses tested (**Annex IV**). Notably, when analyzing the dose response curves for the three tests performed for nickel (II) sulfate in the first phase of the two-phased interlaboratory validation study, only one study demonstrated a sufficient dose response (i.e., a parallel increase in SI relative to increase in concentration). Since 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: DA concordance data from the first phase of the interlaboratory validation study.

Table C-16 Qualitative Results for the First Phase of the Interlaboratory Validation Study for the LLNA: DA (SI ≥ 1.8)

Substance Name[1]	Qualitative Results (Maximum SI)[2]										Concordance
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	
2,4-Dinitro-chlorobenzene (+)	+ (11.97)	+ (9.23)	+ (9.96)	+ (8.53)	+ (7.86)	+ (15.14)	+ (13.18)	+ (12.60)	+ (10.89)	+ (4.71)	10/10
Hexyl cinnamic aldehyde (+)	+ (5.78)	+ (4.82)	+ (4.44)	+ (5.11)	+ (3.97)	+ (5.50)	+ (7.09)	+ (10.22)	+ (3.88)	+ (3.51)	10/10
Isopropanol (-)	**- (1.54)**	**- (0.91)**	**- (1.01)**	**- (1.57)**	**- (0.76)**	**+ (1.97)**	**- (1.45)**	**- (1.21)**	**- (0.70)**	**- (1.25)**	**9/10**
Abietic acid (+)		+ (4.64)				+ (7.96)	+ (3.98)				3/3
3-Aminophenol (+)	**+ (2.83)**		**- (1.76)**					**+ (2.38)**			**2/3**
Dimethyl isophthalate (-)	- (1.34)		- (1.29)				- (1.26)				3/3
Isoeugenol (+)				+ (6.11)	+ (5.54)				+ (7.09)		3/3
Methyl salicylate (-)			- (1.55)				- (1.77)			- (0.83)	3/3
Formaldehyde (+)	+ (4.84)	+ (3.18)			+ (2.69)						3/3
Glutaraldehyde (+)	+ (5.00)	+ (3.39)			+ (2.57)						3/3
Cobalt chloride[3] (+)				+[4] (2.66)		+ (20.55)		+ (8.07)			3/3
Nickel (II) sulfate hexahydrate (+)				**-[5] (1.52)**		**+ (11.78)**		**+[5] (3.49)**			**2/3**

Bolded substances did not achieve 100% interlaboratory concordance.

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index

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

[2] (+) indicates sensitizers and (-) indicates nonsensitizers according to LLNA: DA tests. Highest stimulation index value for each test is shown in parentheses.

[3] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

[4] Data not reported for the highest dose (3%), only for 0.3% and 1%.

[5] Insufficient dose response.

The qualitative (positive/negative) interlaboratory concordance analysis for the five substances that were tested during the second phase of the LLNA: DA interlaboratory validation study is shown in **Table C-17**. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), four substances (hexyl cinnamic aldehyde, cobalt chloride, lactic acid, and potassium dichromate) tested in either four or seven laboratories had consistent results leading to 100% (4/4 or 7/7) interlaboratory concordance for those substances. There was one discordant substance (nickel [II] sulfate hexahydrate) for which interlaboratory concordance was 75% (3/4). Three of the four laboratories that tested nickel (II) sulfate hexahydrate did not report a maximum SI ≥ 1.8 at any dose, while one laboratory produced an SI ≥ 1.8 at the lowest dose tested. Nickel (II) sulfate hexahydrate was also tested in the first phase of the interlaboratory validation study where interlaboratory concordance was 67% (2/3). Furthermore, as mentioned previously, the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), and therefore there were no traditional LLNA concordance data for comparison with the LLNA: DA concordance data from the second phase of the interlaboratory validation study.

Table C-17 Qualitative Results for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA (SI ≥ 1.8)

Substance Name[1]	Qualitative Results (Maximum SI)[2]							Concordance
	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17	
Hexyl cinnamic aldehyde (+)	+ (4.47)	+ (5.71)	+ (5.41)	+ (7.60)	+ (3.92)	+ (8.42)	+ (6.45)	7/7
Cobalt chloride[3] (+)	+ (2.01)		+ (2.54)	+ (4.25)			+ (5.06)	4/4
Lactic acid (-)	- (0.93)		- (0.99)		- (0.97)	- (0.91)		4/4
Nickel (II) sulfate hexahydrate (+)	**- (0.79)**	**- (1.24)**		**+ (2.13)**		**- (1.56)**		**3/4**
Potassium dichromate (+)	+ (4.78)	+ (4.08)			+ (6.01)		+ (6.37)	4/4

Bolded substance did not achieve 100% interlaboratory concordance.

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

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

[2] (+) indicates sensitizers and (-) indicates nonsensitizers according to LLNA: DA tests. Highest stimulation index value for each test is shown in parentheses,

[3] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

7.2.2 Interlaboratory Reproducibility – EC1.8 Values

The quantitative (i.e., EC1.8 value) data for interlaboratory reproducibility analysis were obtained from the LLNA: DA results that yielded positive results (SI ≥ 1.8) during the first and second phases of the LLNA: DA interlaboratory validation study. The equation used for calculating EC1.8 values for the positive results was modified based on the method of linear interpolation reported by Gerberick et al. (2004) for the EC3 value:

$$EC1.8 = c + \left[\frac{(1.8 - d)}{(b - d)}\right] \times (a - c)$$

where the data points lying immediately above and below the SI = 1.8 on the dose response curve have the coordinates of (a, b) and (c, d), respectively (Gerberick et al. 2004). For substances for which the lowest concentration tested resulted in an SI ≥ 1.8, an EC1.8 value was extrapolated according to the equation:

$$EC1.8_{ex} = 2^{\left\{\log_2(c) + \frac{(1.8 - d)}{(b - d)} \times [\log_2(a) - \log_2(c)]\right\}}$$

where the point with the higher SI is denoted with the coordinates of (a, b) and the point with the lower SI is denoted (c, d) (Gerberick et al. 2004).

The EC1.8 values from each laboratory were used to calculate CV values for each substance. The resulting values for the first and second phases of the interlaboratory validation study are shown in **Tables C-19** and **C-20**, respectively. In the first phase of the interlaboratory validation study, CV values ranged from 15% (abietic acid) to 140% (isoeugenol) and the mean CV was 71% (**Table C-18**). In the second phase of the interlaboratory validation study, CV values ranged from 14% (hexyl cinnamic aldehyde) to 93% (cobalt chloride) and the mean CV was 49% (**Table C-19**).

The ICCVAM-recommended LLNA performance standards indicate that interlaboratory reproducibility should be evaluated with at least two sensitizing chemicals with well-characterized activity in the traditional LLNA. Acceptable reproducibility is attained when each laboratory obtains ECt values (estimated concentrations needed to produce an SI of a specified threshold) within 0.025% to 0.1% for 2,4-dinitrochlorobenzene and within 5% to 20% for hexyl cinnamic aldehyde (ICCVAM 2009). In the first phase of the interlaboratory validation study, eight laboratories reported EC1.8 values outside the acceptance range indicated for 2,4-dinitrochlorobenzene; all of the eight laboratories obtained EC1.8 values that were lower than the specified acceptance range (<0.025%) (**Table C-18**). For hexyl cinnamic aldehyde, all the laboratories participating in the first phase of the interlaboratory validation study obtained an EC1.8 value within the acceptance range (5% to 20%). In the second phase of the interlaboratory validation study, only hexyl cinnamic aldehyde was tested and five of the seven laboratories obtained EC1.8 values that were within the acceptance range indicated (**Table C-19**).

Table C-18 EC1.8 Values from the First Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name	EC1.8 (%)										Mean EC1.8 (%) ± SD	CV (%)
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10		
2,4-Dinitrochlorobenzene (+)	**0.018 (11.97)**	**0.018 (9.23)**	**0.023 (9.96)**	**0.014 (8.53)**	**0.081 (7.86)**	**0.014 (15.14)**	**0.006 (13.18)**	**0.017 (12.60)**	**0.012 (10.89)**	**0.077 (4.71)**	**0.028 ± 0.027**	**97**
Hexyl cinnamic aldehyde (+)	**6.358 (5.78)**	**6.687 (4.82)**	**7.346 (4.44)**	**5.884 (5.11)**	**9.597 (3.97)**	**5.961 (5.50)**	**5.479 (7.09)**	**5.783 (10.22)**	**8.457 (3.88)**	**6.508 (3.51)**	**6.806 ± 1.312**	**19**
Isopropanol (-)	NA	NA	NA	NA	NA	IDR	NA	NA	NA	NA	NA	NA
Abietic acid (+)		3.636				4.878	4.598				4.371 ± 0.651	15
3-Aminophenol (+)	1.175		NA					2.507			1.841 ± 0.942	51
Dimethyl isophthalate (-)	NA		NA				NA				NA	NA
Isoeugenol (+)				0.337	4.082				0.265		1.561 ± 2.183	140
Methyl salicylate (-)			NA				NA			NA	NA	NA
Formaldehyde (+)	0.209	0.579			1.380						0.723 ± 0.599	83
Glutaraldehyde (+)	0.064	0.235			0.104						0.134 ± 0.089	67
Cobalt chloride[2] (+)				0.233[3]		0.025		0.071			0.110 ± 0.109	99
Nickel (II) sulfate hexahydrate (+)				NA		0.188		IDR			0.188 ± NA	NA

Bolded text indicates substances that are ICCVAM-recommended murine local lymph node assay (LLNA) performance standards reference substances for evaluating interlaboratory reproducibility (ICCVAM 2009). Values in parentheses are highest stimulation index (SI) values achieved. For both 2,4-dinitrochlorobenzene and hexyl cinnamic aldehyde, the highest SI values achieved were from the highest dose tested (0.3% for 2,4-dinitrochlorobenzene and 25% for hexyl cinnamic aldehyde). Shading shows EC1.8 values that are outside of the acceptable range indicated in the ICCVAM-recommended LLNA performance standards: 5-20% for hexyl cinnamic aldehyde and 0.025-0.1% for 2,4-dinitrochlorobenzene.

Abbreviations: CV = coefficient of variation; EC1.8 = estimated concentration needed to produce a stimulation index of 1.8; IDR = insufficient dose response for calculation of EC1.8; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; NA = not applicable; SD = standard deviation.

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

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

[3] Data not reported for the highest dose (3%), only for 0.3% and 1%.

Table C-19 EC1.8 Values from the Second Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	EC1.8 (%)							Mean EC1.8 (%) ± SD	CV (%)
	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17		
Hexyl cinnamic aldehyde (+)	**5.793 (4.47)**	**5.426 (5.71)**	**5.627 (5.41)**	**4.442 (7.60)**	**6.469 (3.92)**	**4.437 (8.42)**	**5.720 (6.45)**	**5.416 ± 0.741**	**14**
Cobalt chloride[2] (+)	3.499		1.382	0.723			0.393	1.499 ± 1.395	93
Lactic acid (-)	NA		NA		NA	NA		NA	NA
Nickel (II) sulfate hexahydrate (+)	NA	NA		5.938		NA		5.938 ± NA	NA
Potassium dichromate (+)	0.089	0.089			0.046		0.041	0.066 ± 0.026	39

Bolded text indicates a substance that is an ICCVAM-recommended murine local lymph node assay (LLNA) performance standards reference substance for evaluating interlaboratory reproducibility (ICCVAM 2009). Values in parentheses are highest stimulation index (SI) values achieved. For hexyl cinnamic aldehyde, the highest SI values achieved were from the highest dose tested (25%). Two of the EC1.8 values (shaded cells) are outside of the acceptable range indicated in the ICCVAM-recommended LLNA performance standards (5-20% for hexyl cinnamic aldehyde).

Abbreviations: CV = coefficient of variation; EC1.8 = estimated concentrations needed to produce a stimulation index of 1.8; NA = not applicable; SD = standard deviation.

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

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

The interlaboratory CV values for both the first and second phases of the interlaboratory validation study for the LLNA: DA EC1.8 values were higher than that for the traditional LLNA EC3 values. The analysis of interlaboratory variation of EC3 values for the traditional LLNA reported CV values of 6.8% to 83.7% for five substances tested in five laboratories (**Table C-20**; ICCVAM 1999). Three of the same substances were evaluated in the traditional LLNA and the LLNA: DA (hexyl cinnamic aldehyde, 2,4-dinitrochlorobenzene, and isoeugenol). All interlaboratory CV values for the LLNA: DA were greater than that for the traditional LLNA. The CV of 97% for 2,4-dinitrochlorobenzene was greater than the two CV values of 37.4% and 27.2% (which were calculated from five values each), reported by ICCVAM (1999). The CV of 19% and 14% for hexyl cinnamic aldehyde tested in the first and second phases of the LLNA: DA interlaboratory validation study, respectively, were both greater than the 6.8% reported by ICCVAM (1999). The CV of 140% for isoeugenol tested in the LLNA: DA was greater than the 41.2% reported by ICCVAM (1999).

Table C-20 Interlaboratory Reproducibility of the EC3 Values for Substances Tested in the Traditional LLNA[1]

Substance Name	EC3 (%)					CV (%)
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	
2, 4-Dinitrochlorobenzene	0.3	0.5	0.6	0.9	0.6	37.4
	0.5	0.6	0.4	0.6	0.3	27.2
Hexyl cinnamic aldehyde	7.9	7.6	8.4	7.0	8.1	6.8
Isoeugenol	1.3	3.3	1.8	3.1	1.6	41.2
Eugenol	5.8	14.5	8.9	13.8	6.0	42.5
SLS	13.4	4.4	1.5	17.1	4.0	83.7

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

[1] From ICCVAM 1999 report.

7.3 Reproducibility Analysis for Substances with Multiple Tests

Section 6.5 details the accuracy analysis for the LLNA: DA (using the most prevalent outcome for substances with multiple tests) when using one optimized criterion to classify substances as potential sensitizers (SI ≥ 1.8). SI ≥ 1.8 was evaluated for classifying substances as potential sensitizers because it resulted in no false negative results, with respect to traditional LLNA data. This section examines the reproducibility of the tests for the 14 substances that had multiple LLNA: DA test results, regardless of whether the tests were performed in one laboratory or multiple laboratories. The frequency with which SI values for the 14 substances occurred in one of three SI categories was considered. The three SI categories were:

- LLNA: DA nonsensitizers with SI < 1.8
- LLNA: DA sensitizers with SI between 1.8 and 2.5 (borderline positive results with potential to be false positives with respect to classification by the traditional LLNA)
- LLNA: DA sensitizers with SI ≥ 2.5

For the 14 substances, three to 18 tests were available. **Table C-21** shows the proportion of the tests for each substance that produced SI values in each category. For the four traditional LLNA nonsensitizers with multiple test results, there were 23 LLNA: DA tests that produced SI < 1.8 and one LLNA: DA test that produced an SI between 1.8 and 2.5. For the 10 traditional LLNA sensitizers with multiple LLNA: DA test results, however, SI values occurred in all three SI categories. The results for nickel (II) sulfate hexahydrate were particularly variable: 50% (4/8) produced SI < 1.8 (four tests with SI = 0.79, 1.24, 1.52, and 1.56), 25% (2/8) produced 1.8 < SI < 2.5 (SI = 2.13 and 2.17), and 25% (2/8) produced SI ≥ 2.5 (SI = 3.49 and 11.78). 3-Aminophenol also produced SI values in all three categories: 33% (1/3) of the tests had SI < 1.8 (SI = 1.76), 33% (1/3) of the tests had 1.8 < SI < 2.5 (SI = 2.38), and 33% (1/3) of the tests had SI ≥ 2.5 (SI = 2.83). Cobalt chloride tests produced SI values in two categories: 12.5% (1/8) of the tests had 1.8 < SI < 2.5 (SI = 2.01) and seven of eight tests (87.5%) produced SI ≥ 2.5 (SI = 2.54, 2.66, 3.64, 4.25, 5.06, 8.07, and 20.55). The multiple test results for the remaining seven traditional LLNA sensitizers were 100% concordant (**Table C-21**).

Table C-21 Concordance of LLNA: DA Tests for Substances with Multiple Tests by Maximum SI Category

Substance Name	LLNA: DA Nonsensitizers (Maximum SI < 1.8)[1]	LLNA: DA Sensitizers (SI ≥ 1.8)		
		1.8 < Maximum SI < 2.5[1]	Maximum SI ≥ 2.5[1]	Total Tests
Sensitizers[2]				
Abietic acid	0 (0%)	0 (0%)	4 (100%)	4
3-Aminophenol	1 (33.3%)	1 (33.3%)	1 (33.3%)	3
Cobalt chloride	0 (0%)	1 (12.5%)	7 (87.5%)	8
2,4-Dinitrochlorobenzene	0 (0%)	0 (0%)	11 (100%)	11
Formaldehyde	0 (0%)	0 (0%)	4 (100%)	4
Glutaraldehyde	0 (0%)	0 (0%)	4 (100%)	4
Hexyl cinnamic aldehyde	0 (0%)	0 (0%)	18 (100%)	18
Isoeugenol	0 (0%)	0 (0%)	4 (100%)	4
Nickel (II) sulfate hexahydrate	4 (50%)	2 (25%)	2 (25%)	8
Potassium dichromate	0 (0%)	0 (0%)	5 (100%)	5
Nonsensitizers[2]				
Dimethyl isophthalate	4 (100%)	0 (0%)	0 (0%)	4
Isopropanol	10 (91%)	1 (9%)	0 (0%)	11
Lactic acid	5 (100%)	0 (0%)	0 (0%)	5
Methyl salicylate	4 (100%)	0 (0%)	0 (0%)	4

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; 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 LLNA: DA Data Quality

All of the studies included in this performance evaluation are based on individual animal data submitted to NICEATM in the form of original data and study records. Furthermore, manuscripts detailing the results for 31 substances evaluated in the intralaboratory study and 14 substances evaluated in the two-phased interlaboratory validation have been published in the peer-reviewed literature (Idehara et al. 2008; Omori et al. 2008). An independent audit has been conducted to confirm that the reported data from the intralaboratory validation study (assessment of 31 substances from Idehara et al. 2008) performed by Daicel Chemical Industries, Ltd. were the same as the data originally recorded (Idehara et al. 2008). The data from the two-phased interlaboratory validation study were not subjected to a formal audit, but the raw data were reportedly entered directly into formatted MS-Excel templates provided by the study management team prior to being used for analyses (Omori et al. 2007). Data recently received for 14 substances evaluated in an intralaboratory validation study (Idehara unpublished) were also not subjected to a formal audit. The intralaboratory assessment at Daicel Chemical Industries, Ltd. (Idehara et al. 2008; Idehara unpublished), as well as the two-phased interlaboratory validation study (Omori et al. 2008), did not conduct their studies in compliance with Good Laboratory Practice guidelines, although all of the participating laboratories reportedly have this capability.

9.0 Other Scientific Reports and Reviews

Yamashita et al. (2005) describe the development of the LLNA: DA as an alternative nonradioisotope LLNA test method. The manuscript details the determination of an optimal dosing schedule and further compares SI values obtained from lymph node weights versus ATP content to determine an appropriate lymphocyte proliferation endpoint. The authors further assess the intermediate precision and sensitivity/specificity of the LLNA: DA. In those experiments, four compounds (2,4-dinitrochlorobenzene, eugenol, α-hexyl cinnamic aldehyde, and methyl salicylate) were tested and no significant differences were noted in the SI levels generated from the LLNA: DA and the traditional LLNA. The studies by Yamashita et al. provided the basis for the expanded intralaboratory study of 31 substances performed by Daicel Chemical Industries, Ltd. and published by Idehara et al. (2008) (described in **Sections 6.0** and **7.0**).

Idehara et al. (2008) summarize the LLNA: DA test method in terms of test substance dosing schedule, preparation of single cell suspensions of the auricular lymph nodes, measurement of ATP content, and explanation of statistical analyses employed. The authors further describe how the results correlate between ATP content and lymph node cell number, the test results (i.e., mean SI values and EC3 values) obtained for the 31 substances, the concordance of the LLNA: DA versus the traditional LLNA EC3 values, and the reproducibility of EC3 and SI values. Based on the details included in the manuscript, the authors conclude that the SI values obtained from measuring ATP content were similar to the traditional LLNA and therefore the LLNA: DA was a promising nonradioisotope modified test method for evaluating the skin sensitization potential of substances.

Omori et al. (2008) describe the two-phased interlaboratory validation study used to evaluate the reliability and relevance of the LLNA: DA test method (see **Section 7.0**). They describe the organization and technology transfer of the test method between the laboratories, as well as test substance selection and allocation. They further describe the development of the LLNA: DA and the resulting standard protocol for the LLNA: DA interlaboratory study. They provide the interlaboratory data for analyzing both ATP content with regard to SI values and lymph node weight and discuss assay sensitivity and interlaboratory variability. Based on the data summarized in the manuscript, the authors conclude that in the first phase of the interlaboratory validation study, a large variation was observed for two substances (cobalt chloride and nickel [II] sulfate hexahydrate) but in the second phase of the interlaboratory validation study this variation was small. The authors attribute the initial variation to application of DMSO as the solvent for the metallic salts and therefore, prior to the second phase of the interlaboratory validation study, include operation of LLNA: DA with DMSO in the technology transfer seminar. In conclusion, the authors view the LLNA: DA as a reliable test method for predicting skin sensitization potential of substances.

Regarding the LLNA: DA test method, noncommission members of JaCVAM met on August 28, 2008 at the National Institute of Health Sciences, Tokyo, Japan, and endorsed the following statement: “Following the review of the results of the Ministry of Health, Labour and Welfare (MHLW)-funded validation study on the LLNA: DA coordinated by Japanese Society for Alternative to Animal Experiments, it is concluded that the LLNA: DA can be used for distinguishing between sensitizer and nonsensitizer chemicals within the context of the OECD testing guidelines No. 429 on skin sensitization: LLNA. The JaCVAM regulatory acceptance board has been regularly kept informed of the progress of the study, and this endorsement was based on an assessment of various documents, including, in particular, the report on the results from the study, and also on the evaluation supported by MHLW of the study prepared for the JaCVAM ad hoc peer review panel.”

10.0 Animal Welfare Considerations

The LLNA: DA will require the use of the same number of animals when compared to the updated ICCVAM-recommended LLNA protocol (Appendix A of ICCVAM 2009). However, since the traditional LLNA uses radioactive materials and as such its use might be restricted in some countries and institutions due to the complications associated with storage, use, and disposal, broader use of a nonradioactive alternative to the traditional LLNA, such as the LLNA: DA, could further reduce the number of GPs that are used to assess skin sensitization.

Further, the LLNA: DA offers increased refinement by avoiding the discomfort that can occur in the guinea pig tests when substances cause ACD. Additionally, the LLNA: DA test method protocol requires fewer mice per treatment group (a minimum of four animals per group) than either of the guinea pig tests (10-20 animals/group for the Buehler test and 5-10 animals/group for the GPMT).

10.1 Rationale for the Need to Use Animals

The rationale for the use of animals in the LLNA: DA is the same as the rationale for the traditional LLNA. There currently are no valid and accepted non-animal test methods 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. Additionally, 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 (Yamashita et al. 2005) and validation of the test method (Idehara et al. 2008; Omori et al. 2008), which is the same as that specified in the updated ICCVAM-recommended LLNA protocol (Appendix A of ICCVAM 2009).

10.3 Reduction Considerations

A further reduction of up to 40% (15 vs. 25) could be achieved by using a reduced version of the LLNA: DA, in cases where dose-response information is not needed for hazard identification purposes. In such an approach, only the highest dose of the test article that does not elicit excessive 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.

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: DA

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. It would be expected that the transferability of the LLNA: DA would be similar to the traditional LLNA, since their test method protocols are experimentally similar. Notably, the test method developer does indicate that when the LLNA: DA test method is conducted, all the procedural steps from lymph node excision to the determination of ATP content should be performed without delay since ATP content decreases over time (Idehara et al. 2008; Omori et al. 2008). The first and second phases of the interlaboratory validation study have demonstrated that this test method is transferable (see **Section 7.0**).

11.2 Laboratories and Major Fixed Equipment Required to Conduct the LLNA: DA

Compared to the traditional LLNA, the LLNA: DA will not require laboratories, equipment, and licensing permits for handling radioactive materials. However, the LLNA: DA does require access to a luminometer capable of detecting light emission by ATP for the assessment of lymphocyte proliferation. The remaining requirements (e.g., animal care laboratories) are the same between the two methods.

11.3 LLNA: DA Training Considerations

The level of training and expertise needed to conduct the LLNA: DA should be similar to the traditional LLNA, although the LLNA: DA includes an additional requirement that users operate a luminometer instead of a scintillation counter and be able to process this data.

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13.0 Glossary

Accuracy:[12] (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:[12] 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:[12] 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.

EC1.8: The estimated concentration needed to produce a stimulation index of 1.8, as compared to the concurrent vehicle control.

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

ECt: The estimated concentration needed to produce a stimulation index of a specific threshold, as compared to the concurrent vehicle control.

False negative:[12] A substance incorrectly identified as negative by a test method.

False negative rate:[12] 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:[12] A substance incorrectly identified as positive by a test method.

False positive rate:[12] 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):[12] Regulations promulgated by the U.S. Food and Drug Administration and the U.S. Environmental Protection Agency, and principles and procedures adopted by the Organisation for Economic Co-operation and Development (OECD) 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[12]**:** 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:[12] 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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[12] 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:[12] 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:[12] 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 on 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 modified by Daicel Chemical Industries, Ltd., based on ATP content (LLNA: DA): 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 on the ear to the substance. The LLNA: DA is a nonradioactive modification of the traditional LLNA and assesses lymphocyte cell proliferation by measuring increases in ATP content in the lymph node as an indicator of the cell number at the end of cell proliferation.

Negative predictivity:[12] 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:[12] 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 are considered adequate by the OECD.

Positive predictivity:[12] 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:[12] The proportion of positives in the population of substances tested (see *two-by-two table*).

Protocol:[12] 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:[12] 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:[12] A new or modified test method that reduces the number of animals required.

Reference test method:[12] 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:[12] A new or modified test method that refines procedures to lessen or eliminate pain or distress in animals or enhances animal well-being.

Relevance:[12] 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:[12] 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:[12] 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:[12] 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 interlaboratory reproducibility).

rLLNA: DA (reduced LLNA: DA): A variant of the LLNA: DA 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:[12] 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.

Specificity:[12] 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: DA to assess the skin sensitization potential of a test substance. The value is calculated as the ratio of the mean ATP content of the auricular lymph nodes from a group of treated mice to the mean ATP content of the auricular lymph nodes from a group of vehicle control mice. The mean ATP content is measured in relative luminescence units. For the LLNA: DA and the rLLNA: DA, an SI $\geq$ 1.8 classifies a substance as a potential skin sensitizer.

Test:[12] The experimental system used; used interchangeably with *test method* and *assay*.

Test method:[12] 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:[12] The ability of a test method or procedure to be accurately and reliably performed in different, competent laboratories.

Two-by-two table:[12] 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:[12] 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:[12] 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: DA Test Method Protocol

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Annex I-1

Standard Operating Procedures Used for the LLNA: DA Test Method Validation Studies

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1.0 Introduction

These are the standard operating procedures for the two-phased interlaboratory test method validation study (Omori et al. 2008) for the murine local lymph node assay (LLNA) modified by Daicel Chemical Industries, Ltd., based on ATP content (referred to hereafter as the "LLNA: DA") as confirmed by the LLNA: DA Validation Committee and provided by the study director.[1] These procedures are intended for tests conducted to evaluate a single test substance. Although the standard operating procedures detailed herein are specific for the two-phased interlaboratory test method validation study (Omori et al. 2008), the substances tested in the intralaboratory validation study followed a technically similar LLNA: DA test method protocol (Idehara et al. 2008; Idehara unpublished).

2.0 Preparation of Equipment and Materials

Prepare the experimental equipment, materials, and reagents given in **Table C-I-1**. Luminometer tubes, 15 mL test tubes, 50 mL test tubes, petri dishes, and slide glass should be disposable. The underlined items will be provided by the LLNA: DA Validation Committee but in some cases, a luminometer will be furnished by the test facilities. All other materials will be provided by the test facilities.

Table C-I-1 List of Required Equipment, Materials and Reagents

Name of Equipment, Material, or Reagent	Manufacturer	Comment (Trade Name, Model Number, etc.)
Luminometer	Kikkoman Corporation, Japan	LUMITESTER C-100 Detection Range: $4x10^{-12}$ – $1x10^{-6}$ M Upper Limit: 1,000,000 RLU
Luminometer tubes	Kikkoman Corporation, Japan	Polypropylene, sterilized
15 mL test tubes	IWAKI brand	Polypropylene, sterilized
50 mL test tubes	IWAKI brand	Polypropylene, sterilized
Petri dish	Corning Incorporated	Cell culture dish, sterilized
Cell scraper	Costar brand	Disposable cell scraper, sterilized
Slide glass	Matsunami	Micro slide glass
Vortex mixer		
Analytical balance		For body weight measurements (readability of at least 0.1 g)
Analytical balance		For lymph node weight measurements (readability of at least 0.1 mg)
Brush	Ikkyuen	Osho
Phosphate buffered saline	Invitrogen Gibco™	pH 7.2, sterilized
Luciferin-luciferase reagent	Kikkoman Corporation, Japan	CheckLite™ 250 Plus[1]

continued

[1] Confirmed by LLNA: DA Validation Committee on 2/6/2006; Revised by Takashi Omori, Study Director on: 2/17/2006, 2/19/2006, 3/27/2006, 4/2/2006, and 12/2/2006.

Table C-I-1 List of Required Equipment, Materials and Reagents (continued)

Name of Equipment, Material, or Reagent	Manufacturer	Comment (Trade Name, Model Number, etc.)
Cages		Capable of housing four mice, with feed and water dispensers
Micropipette		For applying test solutions (25 µL), handling phosphate buffered saline (1000 µL), tissue suspension (20 µL), cell suspension (100 µL), and dissolved luciferin-luciferase solution (100 µL)
Micropipette tips		Sterilized
Dissecting instruments		Large and small tweezers, scissors, surgical holder, injection needle and holder
Timer		With second display
General laboratory materials		Cotton, antiseptic solution, paper towel, clean sheet, test tube rack, microtube rack

Abbreviations: RLU = relative luminescence units.

[1] For the intralaboratory validation study by Daicel Chemical Industries, Ltd. (Idehara et al. 2008; Idehara unpublished), only the ATP content for potassium dichromate was measured by the CheckLite™ 250 Plus Kit (Kikkoman Corporation, Japan) and the ViaLight® HS Kit (Lonza Rockland, Inc., USA) was used for determining the ATP content of all the other substances in the intralaboratory validation.

3.0 Preparations Prior to Delivery of Animals

The animals to be used in the tests are young adult female mice (nulliparous and non-pregnant) of the CBA/JNCrlj strain, aged between 8-12 weeks prior to application of test and control substances. The animals will be provided by the LLNA: DA Validation Committee. Preparations should be made according to the standards of the test facilities to begin acclimatizing the animals once they have arrived on the previously agreed upon date of delivery.

Six cages capable of holding four animals each should be prepared prior to the end of acclimatization. The cages should be labeled as listed in **Table C-I-2**. The symbol "X" represents the code of the test substance to be provided. Mark the label using the letter indicated on the datasheets provided prior to the test. The animal test group numbers are also indicated on the datasheets. The numbers should be confirmed and the cages labeled with care. This test will be performed two or three times, so it is important to include the test number on the labels.

Table C-I-2 Preparation of Test Group Cages

Test Group Number	Label
Group 1	Acetone: Olive Oil (4:1)
Group 2	Positive Control
Group 3	Vehicle
Group 4	Test Substance "X" – Low Concentration
Group 5	Test Substance "X" – Medium Concentration
Group 6	Test Substance "X" – High Concentration

"X" represents the code of the test substance provided by the study management team.

4.0 Delivery, Acclimatization and Animal Assignment

On the date of delivery, 25 animals will arrive and acclimatization should begin immediately. Acclimatization should be performed according to the standards of the test facilities. The animals should be acclimatized for at least five days, but no more than 16 days.

After acclimatization healthy animals with no observable skin lesions or other abnormalities should be randomly assigned to six groups of four[2] animals each using randomly generated numbers. After assigning the animals to groups, four animals each should be placed in the six cages prepared as described in **Section 3.0**. Any animals remaining after the assignment of 24 should be omitted from the test. Should there be fewer than 24 animals with no observed abnormalities, three animals should be assigned to each group beginning with the test group with the highest number until all of the animals are assigned.

From the delivery of the animals to the end of the test procedures the temperature of the animal housing facility should be maintained at 22ºC (±3ºC) with a relative humidity of 30-70%. The animals should be housed with a light: dark cycle of 12 hours light: 12 hours dark and should be given food and water *ad libitum*. Any deviations from the standard housing and feeding procedures should be recorded.

5.0 Confirmation of Test Materials

Upon arrival of the test materials, sent by the LLNA: DA Validation Committee, confirm that the inventory document matches the contents.

The labels for each of the treatments (acetone: olive oil [4:1], positive control, vehicle, and low, medium and high concentrations of test substances) include a test substance code and a group number. After confirming that these codes match the datasheet, arrange the treatments in a test tube rack according to group number. Sodium lauryl sulfate (SLS) solution will arrive in one tube. Apportion 3 mL of SLS solution to each of the accompanying empty test tubes, mark each tube with the group number, and arrange the tubes in order in the test tube rack.

The treatments should be refrigerated immediately and only removed when beginning the test. Refrigeration of the solutions used in these procedures should be between 0-10ºC, and preferably between 2-8ºC, except when instructed differently. Should there be specific instructions as to the handling of the solutions, the instructions will be included with the materials shipment and they should be followed. For instance:

- SLS (CASRN: 151-21-3) is a 1% aqueous solution and should be kept at room temperature
- Acetone: olive oil is 4:1 volume to volume ratio
- Positive control is a 25% acetone: olive oil (4:1) solution of hexyl cinnamic aldehyde (CASRN: 101-86-0)[3]

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[2] For the tests conducted as part of the intralaboratory validation study by Daicel Chemical Industries, Ltd. (Idehara et al. 2008; Idehara unpublished), at least three animals per dose group were used (i.e., in most cases, four animals per control group and three animals per treatment group).

[3] For the tests conducted as part of the intralaboratory validation study by Daicel Chemical Industries, Ltd., either 15% hexyl cinnamic aldehyde (CASRN: 101-86-0), 10% eugenol (CASRN: 101-86-0), or 5% cinnamic aldehyde (CASRN: 104-55-2) were used as positive controls (Idehara et al. 2008).

6.0 Procedures on Test Days 1, 2, 3 and 7

6.1 Day 1

Mark the animals on the tail with their test group number and a number from 1-4. Weigh the animals and record their weight to the nearest 0.1 g on the test forms.

Remove the test materials from the refrigerator. Should the materials arrive with instructions to heat or sonicate the treatments prior to application, perform these procedures as instructed.

6.1.1 Pre-treatment with 1% SLS Aqueous Solution

Beginning with Group 1 and proceeding in order to Group 6, the SLS solution should be applied with a brush to the dorsum of both ears of the mice. The number of the SLS solution used should match the test group number. The brush should be dipped in the SLS solution and applied to the dorsum of one ear using a petting motion, covering the entire dorsum with four to five strokes. Dip the brush again in the SLS solution and apply the solution to the dorsum of the other ear in the same manner.

Record the time when beginning to apply SLS solution to Group 1 and when completing application to Group 6. The application procedure should be performed continuously without delay for Groups 1-6.

Six brushes should be prepared and numbered, using only one brush for each test group. When performing the same application procedure on Days 2, 3, and 7 there is the possibility of brush contamination due to residual solution on the mouse auricula. It is important to switch brushes after finishing application for one group and check the number of the next brush before proceeding to the next group. After use, the brushes should be washed thoroughly and made available for the next day.

6.1.2 Test Substance Application

One hour after starting the SLS solution application, the numbered treatments should be applied to the auriculae of the mice, beginning with Group 1 and ending with Group 6. Using a micropipette or similar device, 25 µL of the test solution should be dripped slowly on the dorsum of one of the mouse's ears, covering the dorsum entirely. Again take up 25 µL of treatment solution and apply it in the same manner to the dorsum of the mouse's other ear.

When applying the treatments, micropipette tips should be changed for each test group. After completing application for one test group, remove the tip and spray the end of the micropipette with an alcohol mist and wipe to avoid contamination.

Record the time when beginning to apply the test solution to Group 1 and when completing application to Group 6. The application procedure should be performed continuously without delay for Groups 1-6.

Immediately after completing application the test materials should be refrigerated.

6.1.3 General Information on the 1% SLS Pre-treatment and Test Substance Application

The objective of the application procedure is to first apply SLS solution to the entirety of the dorsum of the ear and then to apply a prescribed amount of test solution to the same area. Using ether anesthesia ensures ease and accuracy of the procedure. However, special care should be taken to avoid taking the life of the animals in the course of anesthesia. If one technician immobilizes the animal and extends the ear with tweezers while the other technician applies the solution, the procedure can be performed with accuracy without using anesthesia. If this approach is used six pairs of tweezers should be prepared, one for each group, to avoid contamination. Alternatively, the tweezers should be wiped with an alcohol swab after application is completed for each test group.

6.2 Days 2 and 3

Apply SLS solution and treatments using the same procedures as for Day 1.

When performing the application procedures the animals should be observed carefully for necrosis, hardening, hyperplasia or erythema of the auricula, as well as piloerection, or a decrease in locomotor activity. Any such abnormalities observed should be recorded on the test forms.

6.3 Day 7

On Day 7 the same procedures should be performed as on Days 1, 2, and 3.

Excision of the auricular lymph nodes will be performed from 24-30 hours after the start of application on Day 7. It is therefore recommended that application procedures on Day 7 begin in the morning or early afternoon.

7.0 Procedure on Test Day 8 (Excision of Auricular Lymph Nodes and ATP Assay)

7.1 Laboratory Preparation

Forty-eight 15 mL test tubes should each be filled with 1.98 mL of phosphate buffered saline (PBS). The dispensing of PBS should be conducted under aseptic manipulation. Dispense a minimum of 24 mL of PBS in a 50 mL test tube. Pipetting should be under aseptic manipulation.

Dissolve the luciferin-luciferase reagent according to the ATP assay kit instructions (at least 4.8 mL are required). The ATP assay kit provided, CheckLite™ 250 Plus,[4] includes five bottles each of luciferin-luciferase reagent, solvent water, and ATP releasing agent. Using one bottle of each type, create a solution according to the instructions (approximately 5.5 mL). Shield the assay solutions from light using aluminum foil and refrigerate until the time of use. Immediately before using, return to room temperature and remove the foil prior to use. Dispense 0.1 mL of the ATP releasing agent included in the ATP assay kit to each of the 48 luminometer tubes. ATP assay kit reagents should be dispensed using sterilized pipette tips under aseptic manipulation to avoid contamination with ATP and microorganisms.

7.2 Body Weight Measurement

Weigh the mice and record their body weights to the nearest 0.1 g on the test forms.

7.3 Auricular Lymph Node Excision and Weight Measurement

Perform procedures in **Sections 7.3**, **7.4** and **7.5** within 24 to 30 hours after the start of treatment application on Day 7. The necessary materials for procedures in **Sections 7.3**, **7.4** and **7.5** are given in **Annex Ia**.

Immediately after sacrificing the mice with ether anesthesia excise completely all auricular lymph nodes for each ear (there can be one or two auricular lymph nodes) as illustrated in **Figure C-I-1**. Place the excised lymph nodes for one animal in a disposable petri dish and immediately measure the wet weight to the nearest 0.1 mg with an analytical balance.

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[4] For the intralaboratory validation study by Daicel Chemical Industries, Ltd. (Idehara et al. 2008; Idehara unpublished), only the ATP content for potassium dichromate was measured by the CheckLite™ 250 Plus Kit (Kikkoman Corporation, Japan) and the ViaLight® HS Kit (Lonza Rockland, Inc., USA) was used for determining the ATP content of all the other substances in the intralaboratory validation.

7.4 Preparation of Cell Suspension

The lymph nodes from one animal should be sandwiched between two pieces of slide glass and light pressure should be applied to crush the nodes (**Figure C-I-2**). After confirming that the tissue has spread out thinly pull the two slides apart. Suspend the tissue on both pieces of slide glass in 1 mL of PBS. As illustrated in **Figure C-I-3**, each piece of slide glass should be held at an angle over the petri dish and rinsed with PBS while the tissue is scraped off of the glass with repeated movements of a cell scraper. One mL of PBS should be used for rinsing both slides.

The tissue suspension in the petri dish should be homogenized lightly with the cell scraper, and 20 μL of the suspension should be taken up with a micropipette, taking care not to take up the membrane that is visible to the eye. The pipetted suspension should be added to 1.98 mL of PBS and homogenized well. This will be cell suspension No. 1. Again take up 20 μL of the suspension in the petri dish, add to 1.98 mL of PBS, and homogenize well. This will be cell suspension No. 2.

These procedures should be performed while wearing gloves and a mask, and micropipette tips should be sterile. Detailed step-by-step procedures are given in **Annex Ib**.

Figure C-I-1 Auricular lymph nodes[5]

[5] Taken from ICCVAM IWG LLNA Protocol (ICCVAM 2001).

Figure 1: Lateral Dissection

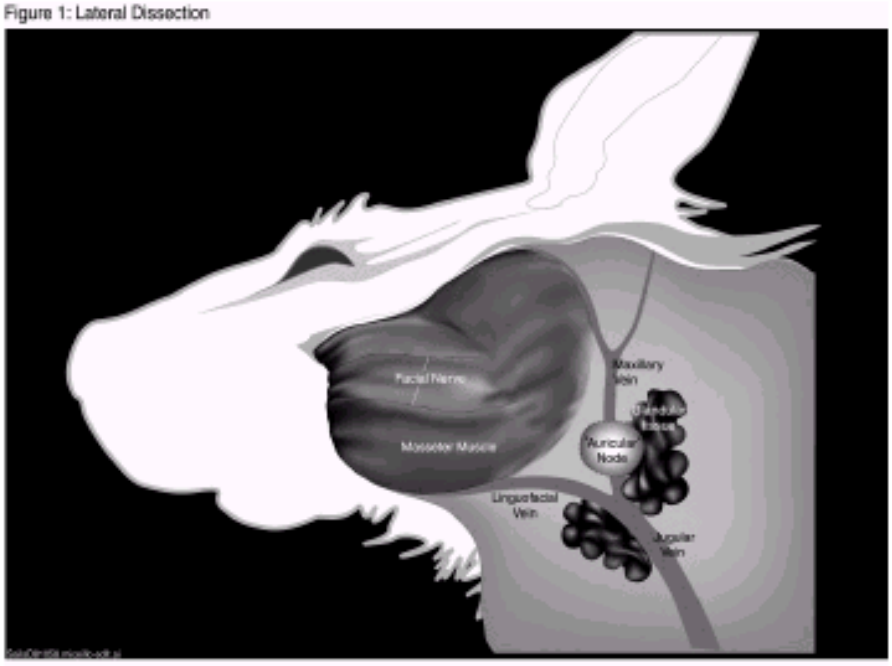


Figure 2: Ventral Dissection

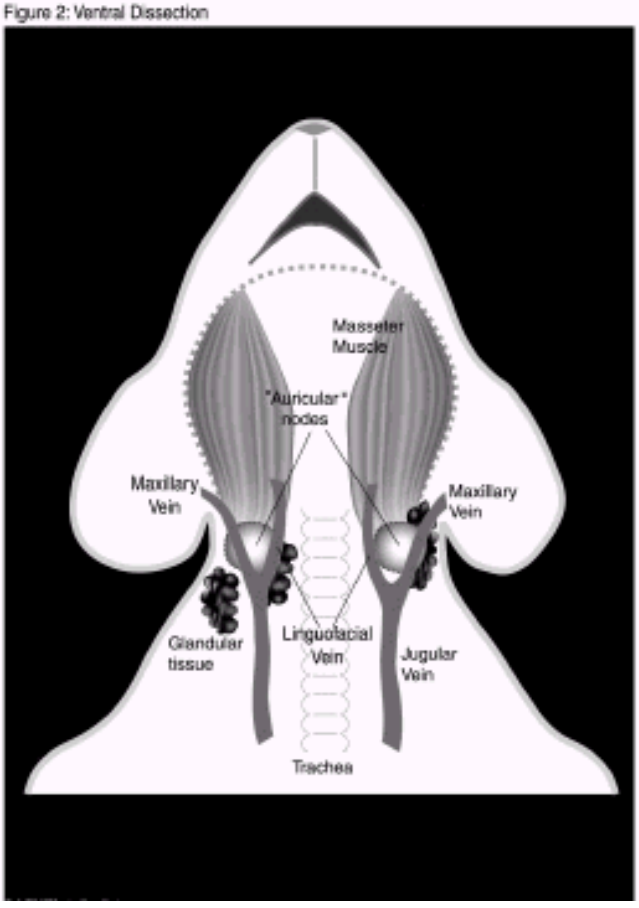


Figure C-I-2 Preparation of cell suspension

Lymph nodes from each animal are sandwiched between two pieces of slide glass and light pressure is applied to crush the nodes.

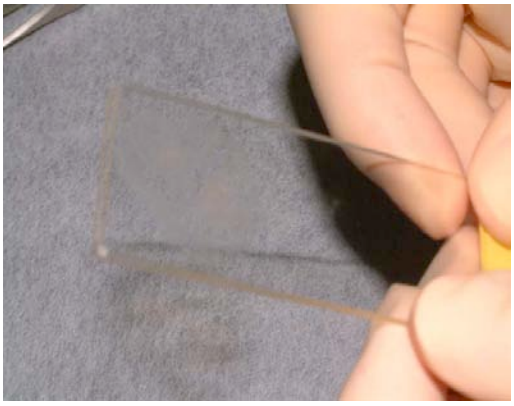
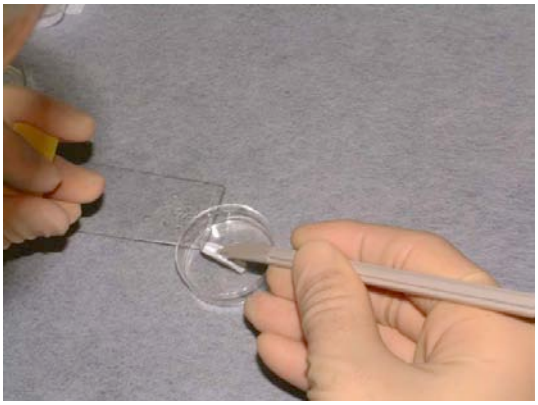


Figure C-I-3 Preparation of cell suspension

Rinse with PBS while scraping the tissue off of the glass with a cell scraper. Repeat the scraping motion, scooping up liquid from the petri dish as needed. Use 1 mL of PBS for the nodes of each animal.



7.5 ATP Assay

Prepare 48 luminometer tubes in advance by dispensing 0.1 mL of the ATP releasing reagent provided to each tube. Add 0.1 mL of each homogenized cell suspension to the luminometer tubes and homogenize. After allowing the solution in the tube to stand for approximately 20 seconds, add 0.1 mL of the luciferin-luciferase solution, promptly homogenize and place in the luminometer. The amount of bioluminescence (RLU; relative luminescence units) measured over 10 seconds will be displayed. Record this measurement on the test forms.

The amount of bioluminescence begins to decrease immediately after adding the luciferin-luciferase solution. It is therefore important that the series of procedures from the addition of luciferin-luciferase solution to switching on the luminometer are performed as quickly as possible, ideally with the same rhythm.

These procedures should be performed while wearing gloves and a mask, and micropipette tips should be sterile. The detailed procedures are given in **Annex Ic**.

8.0 Points of Caution on Procedures from Excision to ATP Assay

The ATP content of the lymph node decreases over time after the sacrifice of the animal. It is therefore desirable that the time elapsed between sacrifice of the animal and ATP assay is uniform for each animal. The series of procedures from excision to ATP assay must be performed rapidly and without delay.

If one technician performs these procedures, the animals should be sacrificed one at a time. If there are multiple technicians, it is possible to divide tasks and sacrifice the animals one group at a time. If two technicians perform the procedures, one individual should perform steps in **Section 7.3**, and the other individual should perform steps in **Sections 7.4** and **7.5**. If three technicians perform the procedures, one individual can handle steps in **Sections 7.3**, **7.4** and **7.5**. If multiple technicians are involved, it is important that the timing of excision is carefully planned so that there are no delays in subsequent steps.

9.0 Data Entry

Input the body weights on Day 1 and Day 8, the lymph node weight, and the amount of ATP bioluminescence into the designated Excel file.

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Annex Ia: Equipment and Reagents Used for the Experimental Procedures in Sections 7.3, 7.4, and 7.5

For the equipment and reagents underlined below, the items provided by the LLNA: DA Validation Committee should be used. In the event the test facility provides a luminometer, it can be used. Numbers in parentheses indicate the number of equipment or reagents required.

7.3 Auricular Lymph Node Excision and Weight Measurement

Dissecting instruments set (tweezers, scissors, surgical holder, injection needle and holder)

Antiseptic solution

Cotton

Petri dish (24)

Analytical balance (readability of at least 0.1 mg)

7.4 Preparation of Cell Suspension

15 mL test tubes with 1.98 mL PBS (48)

50 mL test tubes with at least 24 mL PBS (1)

Slide glass (48)

Tweezers (1)

Micropipette 1000 µL (1) (volume to be measured: 1 mL)

Micropipette 100 µL (1) (volume to be measured: 20 µL)

Cell scraper (1)

Sterilized pipette tips for 1000 µL micropipette (24) and for 100 µL micropipette (24)

Vortex mixer (1)

Paper towels

Clean sheet

Test tube rack

7.5 ATP Assay

Luminometer tubes with 0.1 mL ATP releasing agent (48)

15 mL test tube with dissolved luciferin-luciferase solution (1)

Micropipette – 100 µL or 200 µL (2) (volume to be measured: 0.1 mL)

Sterilized micropipette tips (96)

Timer (with second display) (1)

Luminometer (1)

Vortex mixer (can use same mixer listed under **Section 7.4** Preparation of Cell Suspension)

Test tube rack and luminometer tube rack (microtube rack)

Annex Ib: Preparation of Cell Suspension for the Experimental Procedures in Section 7.4

1. Cover the laboratory bench with a clean sheet and place one piece of slide glass on the sheet.
2. After measuring the lymph node weights, use tweezers to move the lymph nodes from one animal from the petri dish to the center of the slide glass.
3. Place another piece of slide glass on top.
4. Pick up the two sandwiched pieces of slide glass. Squeeze the two pieces in the center to crush the lymph nodes. (Apply only light pressure. Too much pressure can break the cells.)
5. Confirm that the tissue has spread out thinly between the two slides and place the sandwiched slides on the clean sheet.
6. Fasten a tip on the 1000 μL micropipette and draw 1 mL phosphate buffered saline (PBS) from the 50 mL tube.
7. Remove the upper slide glass from the sandwiched slides and place it on the clean sheet with the side that was in contact with the lymph node tissue facing up. The other slide glass should be held at an angle in the petri dish, the side with lymph node tissue affixed facing forward, and washed with 1 mL PBS.
8. Dispose of the 1000 μL micropipette tip.
9. Scrape the tissue off of the glass with a cell scraper, scooping up PBS from the petri dish and repeating the scraping motion. Confirm that there is no tissue, or only trace amounts of tissue, left on the slide before disposing of the slide glass.
10. Pick up the slide glass laid aside at step 7; scrape the tissue off in the same manner and dispose of the slide glass. Note that it becomes difficult to scrape the tissue off of the slide glass once it has dried. Perform steps 4-10 without delay. The scraping should be performed while keeping the area of the slide glass to which the lymph node tissue is affixed sufficiently wet with PBS from the petri dish.
11. The tissue suspension in the petri dish should be homogenized lightly with the cell scraper. If large pieces of tissue are observed, stir with the cell scraper to break up the pieces and obtain a uniform solution.
12. Wipe the cell scraper with a paper towel. (The cell scraper will be used for the next animal.)
13. Fasten a tip to the 100 μL micropipette, tilt the petri dish at an angle and mix the suspension by pipetting in and out several times. Take up 20 μL of the suspension with the pipette, taking care not to take up any membrane that is visible to the eye.
14. Add the 20 μL of suspension to a 15 mL test tube containing 1.98 mL PBS. Pipette the solution and proceed to homogenize with the vortex mixer. (cell suspension No. 1)
15. Repeat steps 13 and 14 to prepare cell suspension No. 2.
16. Dispose of the 100 μL micropipette tip.

Annex Ic: ATP Assay for the Experimental Procedures in Section 7.5

1. Fasten a tip on the 100 μL (or 200 μL) micropipette and draw 0.1 mL of vortex-homogenized cell suspension No. 1.
2. To the luminometer tube filled with 0.1 mL ATP releasing reagent, add 0.1 mL of cell suspension No. 1, making sure to note the time with a timer. Dispose of the tip.
3. Homogenize with the vortex mixer and place in the luminometer tube rack.
4. Fasten a tip on a separate 100 μL (or 200 μL) micropipette and draw 0.1 mL of solution from the 15 mL tube containing dissolved luciferin-luciferase reagent.
5. Take the luminometer tube from the rack and add 0.1 mL of luciferin-luciferase solution to the luminometer tube 20 seconds after the time noted in step 2.
6. Promptly homogenize in the vortex mixer, place in the luminometer and turn on the switch. The amount of bioluminescence begins to decrease immediately after adding the luciferin-luciferase solution. Step 6 should be performed as quickly as possible, ideally with the same rhythm.
7. Dispose of the tip.
8. After 10 seconds the amount of bioluminescence (RLU) will be displayed. Record this measurement on the test forms.
9. Repeat steps 1-8 for cell suspension No. 2, measure the bioluminescence and record.

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Annex I-2

LLNA: DA Test Method Data Comparing With and Without 1% SLS Pretreatment

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Table C-I-2-1 Summary of LLNA: DA Test Method Results Comparing With and Without 1% SLS Pretreatment[1]

Substance Name	Vehicle	Concentration (%)	SI[2] (+ SLS)	SI[2] (- SLS)	Calculated EC3[3] (+ SLS)	Calculated EC3[3] (- SLS)
2, 4-Dinitrochloro-benzene	AOO	0.03	2.10	1.88	0.05%	0.06%
		0.10	5.02	4.46		
		0.30	9.74	14.61		
Potassium dichromate	DMSO	0.1	2.61	2.54	0.15%	0.22%
		0.3	4.24	3.34		
		1.0	5.51	5.66		
Isoeugenol	AOO	1.0	2.05	1.32	2.46%	4.24%
		2.5	3.02	2.21		
		5.0	2.85	3.35		
Citral	AOO	5	1.93	1.88	7.4%	10.4%
		10	4.15	2.91		
		25	6.97	5.90		
Hexyl cinnamic aldehyde	AOO	5	1.51	0.99	7.5%	8.8%
		10	4.52	3.64		
		25	4.84	3.79		
Cinnamic alcohol	AOO	10	2.46	2.44	14.1%	18.5%
		25	4.40	3.43		
		50	6.36	4.01		
Hydroxycitronellal	AOO	10	1.98	1.49	15.8%	19.8%
		25	4.61	3.81		
		50	6.59	6.74		
Imidazolidinyl urea	DMF	10	2.36	2.54	20.3%	33.0%
		25	3.29	2.38		
		50	6.02	4.31		

Substance Name	Vehicle	Concentration (%)	SI[2] (+ SLS)	SI[2] (- SLS)	Calculated EC3[3] (+ SLS)	Calculated EC3[3] (- SLS)
Methyl methacrylate	AOO	25	0.73	1.11	NA	NA
		50	0.68	0.92		
		100	1.31	1.83		
Nickel (II) chloride	DMSO	2.5	1.53	0.98	NA	NA
		5.0	1.57	1.16		
		10.0	2.24	1.87		
Methyl salicylate	AOO	5	0.89	0.83	NA	NA
		10	1.59	1.32		
		25	1.69	2.34		
Salicylic acid	AOO	5	1.21	1.13	NA	NA
		10	2.05	1.29		
		25	2.48	2.44		
Sulfanilamide	DMF	10	1.08	0.92	NA	NA
		25	1.03	0.90		
		50	0.94	0.84		

Abbreviations: AOO = acetone: olive oil (4:1); DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; EC3 = estimated concentration required to produce a stimulation index of three; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; NA = not applicable; SI = stimulation index; SLS = sodium lauryl sulfate; + SLS = with pretreatment of 1% aqueous solution of SLS prior to test substance application; - SLS = without pretreatment of 1% aqueous solution of SLS prior to test substance application.

[1] Data submitted to NICEATM in February 2009 (Idehara unpublished).

[2] SI determined from mean ATP content (relative luminescence units).

[3] EC3 value was calculated based on interpolation or extrapolation formulas discussed in Gerberick et al. 2004.

Annex II

Physicochemical Properties and Chemical Classes of Substances Tested in the LLNA: DA

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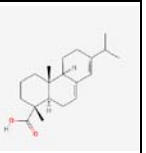
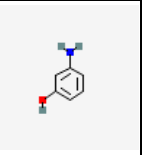
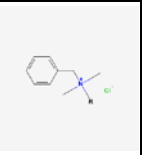
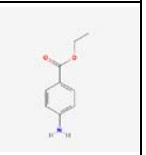
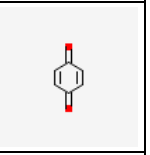
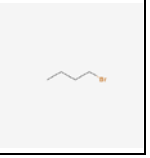
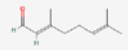
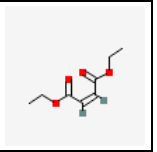
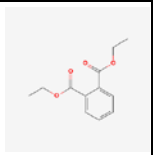
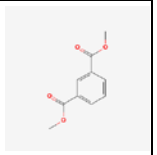
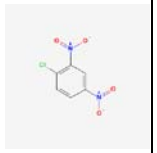
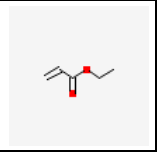
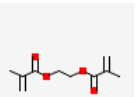
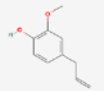
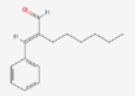
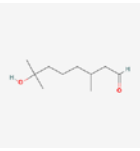
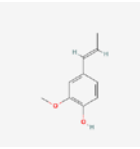
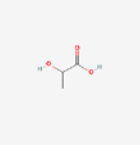
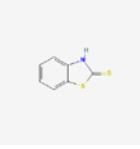
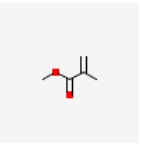
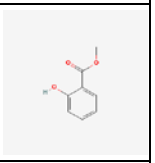
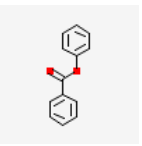
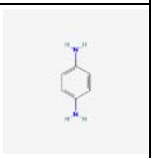
Substance Name[1]	Synonyms	CASRN	Mol. Weight (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Abietic acid[5, 6]	Sylvic acid	514-10-3	302.46	11	NA	Solid	Hydrocarbons, cyclic; Polycyclic compounds	
3-Aminophenol[6]	*m*-Aminophenol	591-27-5	109.13	0.24	Minimal	Solid	Amines; Phenols	
Benzalkonium chloride[5]	Alkylbenzyldimethyl-ammonium chloride; Germitol; Zephiral	8001-54-5	170.66	NA	NA	Solid/ Liquid	Amines; Onium compounds	
Benzocaine[5]	Ethyl 4-aminobenzoate	94-09-7	165.19	1.80	NA	Solid	Carboxylic acids	
Benzoquinone[7]	*p*-Quinone; 1,4-benzoquinone; Cyclohexadienedione	106-51-4	108.10	1.17	High	Solid	Quinones	
1-Bromobutane[5]	Butyl bromide	109-65-9	137.02	2.65	Low	Liquid	Hydrocarbons, halogenated	

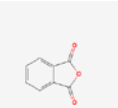
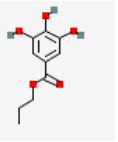
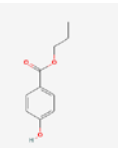
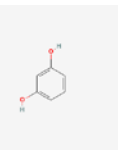
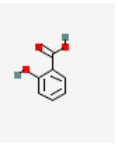
Substance Name[1]	Synonyms	CASRN	Mol. Weight (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Butyl glycidyl ether[7]	*n*-Butyl glycidyl ether	2426-08-6	130.19	1.42	NA	Liquid	Ethers	
5-Chloro-2-methyl-4-isothiazolin-3-one[7]	Chloromethyliso-thiazolinone; CMI	26172-55-4	132.30	0.92	High	Liquid	Sulfur compounds; Heterocyclic compounds	
Chlorobenzene[5]	Phenyl chloride	108-90-7	112.56	2.64	Minimal	Liquid	Hydrocarbons, cyclic; Hydrocarbons, halogenated	
Cinnamic alcohol[7]	3-Phenyl-2-propen-1-ol; Cinnamyl alcohol	104-54-1	134.18	2.29	NA	Solid	Alcohols	
Cinnamic aldehyde[5]	Cinnamaldehyde	104-55-2	132.16	1.82	High	Liquid	Aldehydes	
Citral[5]	2,6-Octadienal, 3,7-dimethyl-	5392-40-5	152.24	3.45	High	Liquid	Hydrocarbons, other	

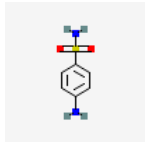
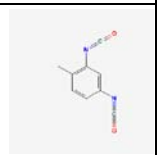
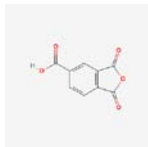
Substance Name[1]	Synonyms	CASRN	Mol. Weight (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Cobalt chloride[5, 6, 8]	Cobaltous chloride	7646-79-9	129.84	0.85	NA	Solid	Inorganic chemical, elements; Inorganic chemical, metals	
Diethyl maleate[7]	Ethyl maleate	141-05-9	172.18	0.89	High	Liquid	Carboxylic acids	
Diethyl phthalate[5]	Ethyl phthalate; Phthalic acid, diethyl ester	84-66-2	222.24	2.65	Minimal	Liquid	Carboxylic acids	
Dimethyl isophthalate[6, 7]	1,3-Benzenedicarboxylic acid, dimethyl ester	1459-93-4	194.19	1.66	NA	Solid	Carboxylic acids	
2,4-Dinitrochloro-benzene[5, 6]	Dinitrochlorobenzene; DNCB	97-00-7	202.55	2.27	High	Solid	Hydrocarbons, cyclic; Hydrocarbons, halogenated; Nitro compounds	
Ethyl acrylate[7]	2-Propenoic acid, ethyl ester	140-88-5	100.10	NA	High	Liquid	Carboxylic acids	

Substance Name[1]	Synonyms	CASRN	Mol. Weight (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Ethylene glycol dimethacrylate[7]	EGDMA	97-90-5	198.22	1.38	High	Liquid	Carboxylic acids	
Eugenol[5]	2-Methoxy-4-(2-propenyl)phenol; Allylguaiacol	97-53-0	164.20	2.73	NA	Liquid	Carboxylic acids	
Formaldehyde[5, 6]	Formalin	50-00-0	30.03	0.35	Moderate	Liquid	Aldehydes	
Glutaraldehyde[5, 6]	Glutaral; Pentanedial	111-30-8	100.12	-0.18	High	Liquid	Aldehydes	
Hexane[5]	Hexyl hydride; *n*-Hexane	110-54-3	86.18	3.29	Minimal	Liquid	Hydrocarbons, acyclic	
Hexyl cinnamic aldehyde[5, 6, 8]	alpha-Hexylcinnamaldehyde; HCA	101-86-0	216.32	4.82	Minimal	Liquid	Aldehydes	

Substance Name[1]	Synonyms	CASRN	Mol. Weight (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Hydroxycitronellal[5]	Citronellal hydrate	107-75-5	172.26	2.11	Low	Liquid	Hydrocarbons, other	
Imidazolidinyl urea[5]	Germall 115; Imidurea	39236-46-9	388.30	-8.28	Moderate	Solid	Urea	
Isoeugenol[5, 6]	2-Methoxy-4-propenylphenol; 4-Propenylguaiacol	97-54-1	164.20	2.65	NA	Liquid	Carboxylic acids	
Isopropanol[5, 6]	Isopropyl alcohol; 2-Propanol	67-63-0	60.10	0.28	Minimal	Liquid	Alcohols	
Lactic acid[5, 8]	2-Hydroxypropanoic acid	50-21-5	90.08	-0.65	Minimal	Liquid	Carboxylic acids	
2-Mercaptobenzo-thiazole[5]	Captax	149-30-4	167.26	2.86	High	Solid	Heterocyclic compounds	

Substance Name[1]	Synonyms	CASRN	Mol. Weight (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Methyl methacrylate[7]	MMA	80-62-6	100.12	NA	NA	Liquid	Carboxylic acids	
Methyl salicylate[5, 6]	Oil of wintergreen; Methyl 2-hydroxybenzoate	119-36-8	152.15	2.60	Minimal	Liquid	Carboxylic acids; Phenols	
Nickel (II) chloride[7]	Nickel chloride	7718-54-9	129.60	NA	NA	Solid	Inorganic chemical, elements; Inorganic chemical, metals	
Nickel (II) sulfate hexahydrate[5, 6, 8]	Nickel sulfate hexahydrate	10101-97-0	154.76	NA	NA	Solid	Inorganic chemical, elements; Inorganic chemical, metals	
Phenyl benzoate[7]	Diphenylcarboxylate	93-99-2	198.22	2.89	NA	Solid	Carboxylic acids	
p-Phenylenediamine[5]	4-Phenylenediamine	106-50-3	108.14	-0.39	NA	Solid	Amines	

Substance Name[1]	Synonyms	CASRN	Mol. Weight (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Phthalic anhydride[5]	1,2-Benzenedicarboxylic anhydride; 1,3-Dioxophthalan	85-44-9	148.12	2.07	Moderate	Solid	Anhydrides; Carboxylic acids	
Potassium dichromate[5, 8]	PDC; Dipotassium bichromate	7778-50-9	294.18	-3.59	NA	Solid	Inorganic chemical, chromium compounds; Inorganic chemical, potassium compounds	
Propyl gallate[7]	Benzoic acid, 3,4,5-trihydroxy-, propyl ester; Gallic acid, propyl ester; Propyl 3,4,5-trihydroxybenzoate	121-79-9	212.20	NA	High	Solid	Carboxylic acids	
Propylparaben[5]	4-Hydroxybenzoic acid, propyl ester; Propyl p-hydroxybenzoate	94-13-3	180.20	2.98	Minimal	Solid	Carboxylic acids; Phenols	
Resorcinol[5]	1,3-Dihydroxybenzene	108-46-3	110.11	1.03	Minimal	Solid	Phenols	
Salicylic acid[7]	2-Hydroxybenzoic acid	69-72-7	138.12	1.03	NA	Solid	Phenols; Carboxylic acids	

Substance Name[1]	Synonyms	CASRN	Mol. Weight (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Sodium lauryl sulfate[5]	Sodium dodecyl sulfate; SLS; SDS; Irium	151-21-3	288.38	1.69	NA	Solid	Alcohols; Sulfur compounds; Lipids	
Sulfanilamide[7]	4-Aminobenzene-sulfonamide; *p*-Anilinesulfonamide; *p*-Sulfamidoaniline	63-74-1	172.21	0.40	Minimal	Solid	Hydrocarbons, cyclic; Sulfur compounds	
Toluene 2,4-diisocyanate[5]	2,4-TDI	584-54-9	174.16	3.74	NA	Liquid	Hydrocarbons, cyclic; Isocyanates	
Trimellitic anhydride[5]	4-Carboxyphthalic anhydride	552-30-7	192.13	1.95	Low	Solid	Anhydrides; Carboxylic acids	

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; K_{ow} = octanol-water partition coefficient; Mol. = molecular; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; NA = not available.

[1] Total of 46 substances: intralaboratory validation study (Idehara et al. 2008; Idehara unpublished) tested 45 substances and the two-phased interlaboratory validation study (Omori et al. 2008) tested 14 substances (i.e., 13 of the 45 substances from the intralaboratory validation study and one unique substance not tested in the intralaboratory validation study).

[2] K_{ow} represents the estimated octanol-water partition coefficient (expressed on log scale) calculated by the Syracuse Research Corporation from the website: http://www.srcinc.com/what-we-do/databaseforms.aspx?id=385.

[3] Peptide reactivity based on Cys (1:10) and Lys (1:50) data as reported in Gerberick et al. 2004 and/or Gerberick et al. 2007.

[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] Substance tested in intralaboratory validation study (Idehara et al. 2008).

[6] Substance tested in phase one of two-phased interlaboratory validation study (Omori et al. 2008).

[7] Substance tested in intralaboratory validation study (Idehara unpublished).

[8] Substance tested in phase two of two-phased interlaboratory validation study (Omori et al. 2008).

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Annex III

Comparative LLNA: DA, Traditional LLNA, Guinea Pig, and Human Skin Sensitization Data

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Annex III-1

Comparison of LLNA: DA, Traditional LLNA, Guinea Pig, and Human Results (Alphanumeric Order)

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Table C-III-1-1 Comparative Performance of the LLNA: DA, Traditional LLNA, Guinea Pig, and Human Tests (Alphanumeric Order)

Substance Name	Veh.[1]	LLNA: DA Highest Conc. Tested (%)	LLNA: DA Highest SI[2]	LLNA: DA Reference	Trad. LLNA Result[3]	GP Result[4]	Human Result[5]	Trad. LLNA Reference	GP Reference	Human Reference	Skin Irritation Data	Skin Irritation Reference
Abietic acid	AOO	25	6.26	Idehara et al. 2008	+ (5.2, 25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 25% (GP)	Basketter et al. 2007b
Abietic acid	AOO	25	4.64	Omori et al. 2008	+ (5.2, 25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 25% (GP)	Basketter et al. 2007b
Abietic acid	AOO	25	7.96	Omori et al. 2008	+ (5.2, 25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 25% (GP)	Basketter et al. 2007b
Abietic acid	AOO	25	3.98 at 10%	Omori et al. 2008	+ (5.2, 25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 25% (GP)	Basketter et al. 2007b
3-Aminophenol	AOO	10	2.83	Omori et al. 2008	+ (5.7, 10%)	NA (+ nonstd)	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 5% (GP)	Basketter et al. 2007b
3-Aminophenol	AOO	10	1.76 at 3%	Omori et al. 2008	+ (5.7, 10%)	NA (+ nonstd)	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 5% (GP)	Basketter et al. 2007b
3-Aminophenol	AOO	10	2.38	Omori et al. 2008	+ (5.7, 10%)	NA (+ nonstd)	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 5% (GP)	Basketter et al. 2007b
Benzalkonium chloride	AOO[6]	2.5	6.68	Idehara et al. 2008	+ (11.1, 2%)[7]	-	+	Gerberick 1992	ICCVAM 1999	ICCVAM 1999	Irritant at 2% in ACE (mice); irritant at 1% in ACE (mice)	Gerberick et al 2002; Manetz and Meade 1999
Benzocaine	**AOO**	**25**	**4.84**	**Idehara et al. 2008**	**+/- (7.6, 20%)[8]**	**+**	**+/-**	**ICCVAM 1999**	**ICCVAM 1999**	**Poole et al. 1970; ICCVAM 1999 (Equivocal data)**	**Negative at ≤ 10% (GP)**	**Basketter and Scholes 1992**

Substance Name	Veh.[1]	LLNA: DA Highest Conc. Tested (%)	LLNA: DA Highest SI[2]	LLNA: DA Reference	Trad. LLNA Result[3]	GP Result[4]	Human Result[5]	Trad. LLNA Reference	GP Reference	Human Reference	Skin Irritation Data	Skin Irritation Reference
p-Benzo-quinone	AOO	0.100	3.79	Idehara unpublished	+ (52.3, 2.5%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at 2.5% (GP)	Basketter et al. 2007b
1-Bromo-butane	AOO	25	1.65	Idehara et al. 2008	- (1.2, 25%)[9]	NA	NA	ICCVAM 1999	NA	NA	NA	NA
Butyl glycidyl ether	AOO	50	4.59	Idehara unpublished	+ (5.6, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.1% (GP)	Wahlberg and Boman 1985
Chlorobenzene	AOO	25	2.44	Idehara et al. 2008	- (1.7, 25%)[9]	-	NA	ICCVAM 1999	ICCVAM 1999	NA	No data. Low irritancy potential assumed based on clinical literature.	Basketter et al. 1998
5-Chloro-2-methyl-4-isothiazolin-3-one	DMF	0.100	7.50	Idehara unpublished	+ (27.7, 0.1%)	+	+	ICCVAM 1999; Gerberick et al. 2005	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
Cinnamic alcohol	AOO	90	5.66 at 50%	Idehara unpublished	+ (5.7, 90%)	+	+	Gerberick et al. 2005	Robinson et al. 1990	Jordan and King 1977	Nonirritant at 1% (GP)	Robinson et al. 1990
Cinnamic aldehyde	AOO	15	4.73	Idehara et al. 2008	+ (18.4, 25%)[9]	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.75% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Citral	AOO	25	4.40	Idehara et al. 2008	+ (20.5, 20%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.5% (GP)	Basketter et al. 2007b
Cobalt chloride	DMSO	5	3.64	Idehara et al. 2008	+ (7.2, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes 1992

Substance Name	Veh.[1]	LLNA: DA Highest Conc. Tested (%)	LLNA: DA Highest SI[2]	LLNA: DA Reference	Trad. LLNA Result[3]	GP Result[4]	Human Result[5]	Trad. LLNA Reference	GP Reference	Human Reference	Skin Irritation Data	Skin Irritation Reference
Cobalt chloride	DMSO	1[10]	2.66	Omori et al. 2008	+ (7.2, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes 1992
Cobalt chloride	DMSO	3	20.55	Omori et al. 2008	+ (7.2, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes 1992
Cobalt chloride	DMSO	3	8.07	Omori et al. 2008	+ (7.2, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes 1992
Cobalt chloride	DMSO	5	2.01	Omori et al. 2008	+ (7.2, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes 1992
Cobalt chloride	DMSO	5	2.54	Omori et al. 2008	+ (7.2, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes 1992
Cobalt chloride	DMSO	5	4.25	Omori et al. 2008	+ (7.2, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes 1992
Cobalt chloride	DMSO	5	5.06	Omori et al. 2008	+ (7.2, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes 1992
Diethyl maleate	AOO	10.0	3.78	Idehara unpublished	+ (22.6, 100%)[11]	NA	+	Gerberick et al. 2005	NA	Marzulli and Maibach 1980	Nonirritant at 100% (GP)	Basketter et al. 2007b
Diethyl phthalate	AOO	100	1.09	Idehara et al. 2008	- (1.5, 100%)	-	+	Gerberick et al. 2005	Klecak et al. 1977	ICCVAM 1999	Negative at 100% (rabbits)	ECETOC 1995
Dimethyl isophthalate	AOO	25	0.89 at 5%	Idehara unpublished	- (1.0, 25%)	-	-	ICCVAM 1999; Basketter et al. 1999b	ICCVAM 1999; Basketter et al. 1999b	Basketter et al. 1999b	Negative at ≤ 10% (GP)	Basketter and Scholes 1992

Substance Name	Veh.[1]	LLNA: DA Highest Conc. Tested (%)	LLNA: DA Highest SI[2]	LLNA: DA Reference	Trad. LLNA Result[3]	GP Result[4]	Human Result[5]	Trad. LLNA Reference	GP Reference	Human Reference	Skin Irritation Data	Skin Irritation Reference
Dimethyl isophthalate	AOO	25	1.34 at 5%	Omori et al. 2008	- (1.0, 25%)	-	-	ICCVAM 1999; Basketter et al. 1999b	ICCVAM 1999; Basketter et al. 1999b	Basketter et al. 1999b	Negative at ≤ 10% (GP)	Basketter and Scholes 1992
Dimethyl isophthalate	AOO	25	1.00 at 5%	Omori et al. 2008	- (1.0, 25%)	-	-	ICCVAM 1999; Basketter et al. 1999b	ICCVAM 1999; Basketter et al. 1999b	Basketter et al. 1999b	Negative at ≤ 10% (GP)	Basketter and Scholes 1992
Dimethyl isophthalate	AOO	25	1.26 at 5%	Omori et al. 2008	- (1.0, 25%)	-	-	ICCVAM 1999; Basketter et al. 1999b	ICCVAM 1999; Basketter et al. 1999b	Basketter et al. 1999b	Negative at ≤ 10% (GP)	Basketter and Scholes 1992
2,4-Dinitrochloro-benzene	AOO	1	7.10	Idehara et al. 2008	+ (43.9, 0.25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	AOO	0.30	11.97	Omori et al. 2008	+ (43.9, 0.25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	AOO	0.30	9.23	Omori et al. 2008	+ (43.9, 0.25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	AOO	0.30	9.96	Omori et al. 2008	+ (43.9, 0.25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	AOO	0.30	8.53	Omori et al. 2008	+ (43.9, 0.25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b

Substance Name	Veh.[1]	LLNA: DA Highest Conc. Tested (%)	LLNA: DA Highest SI[2]	LLNA: DA Reference	Trad. LLNA Result[3]	GP Result[4]	Human Result[5]	Trad. LLNA Reference	GP Reference	Human Reference	Skin Irritation Data	Skin Irritation Reference
2,4-Dinitrochloro-benzene	AOO	0.30	7.86	Omori et al. 2008	+ (43.9, 0.25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	AOO	0.30	15.14	Omori et al. 2008	+ (43.9, 0.25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	AOO	0.30	13.18	Omori et al. 2008	+ (43.9, 0.25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	AOO	0.30	12.60	Omori et al. 2008	+ (43.9, 0.25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	AOO	0.30	10.89	Omori et al. 2008	+ (43.9, 0.25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	AOO	0.30	4.71	Omori et al. 2008	+ (43.9, 0.25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
Ethyl acrylate	AOO	50	4.29 at 25%	Idehara unpublished	+ (4, 50%)	-	+	Gerberick et al. 2005	Van der Walle et al. 1982	Marzulli and Maibach 1974	Nonirritant at 0.3 M (GP)	Van der Walle et al. 1982
Ethylene glycol dimethacrylate	MEK	50	4.45	Idehara unpublished	+ (7, 50%)	-	+	ICCVAM 1999	ICCVAM 1999; Gerberick et al.1992	ICCVAM 1999; Basketter et al. 1999b	Nonirritant at 1% (GP)	Wahlberg and Boman 1985
Eugenol	AOO	25	7.07	Idehara et al. 2008	+ (17, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 25% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995

Substance Name	Veh.[1]	LLNA: DA Highest Conc. Tested (%)	LLNA: DA Highest SI[2]	LLNA: DA Reference	Trad. LLNA Result[3]	GP Result[4]	Human Result[5]	Trad. LLNA Reference	GP Reference	Human Reference	Skin Irritation Data	Skin Irritation Reference
Formaldehyde	ACE	2.50	5.10	Idehara et al. 2008	+ (4, 1.85%)	+	+	Gerberick et al. 2005; Hilton et al. 1998	ICCVAM 1999	ICCVAM 1999; Kwon et al. 2003	Nonirritant at 2% (GP)	Basketter et al. 2007b
Formaldehyde	ACE	5.0	4.84	Omori et al. 2008	+ (4, 1.85%)	+	+	Gerberick et al. 2005; Hilton et al. 1998	ICCVAM 1999	ICCVAM 1999; Kwon et al. 2003	Nonirritant at 2% (GP)	Basketter et al. 2007b
Formaldehyde	ACE	5.0	3.18	Omori et al. 2008	+ (4, 1.85%)	+	+	Gerberick et al. 2005; Hilton et al. 1998	ICCVAM 1999	ICCVAM 1999; Kwon et al. 2003	Nonirritant at 2% (GP)	Basketter et al. 2007b
Formaldehyde	ACE	5.0	2.69	Omori et al. 2008	+ (4, 1.85%)	+	+	Gerberick et al. 2005; Hilton et al. 1998	ICCVAM 1999	ICCVAM 1999; Kwon et al. 2003	Nonirritant at 2% (GP)	Basketter et al. 2007b
Glutaraldehyde	ACE	0.25	6.45	Idehara et al. 2008	+ (18, 2.5%)	+	+	Basketter et al. 2005; Hilton et al. 1998	Gad et al. 1986	Marzulli and Maibach 1974; Schneider and Akkan 2004	NA	NA
Glutaraldehyde	ACE	0.50	5.00	Omori et al. 2008	+ (18, 2.5%)	+	+	Basketter et al. 2005; Hilton et al. 1998	Gad et al. 1986	Marzulli and Maibach 1974; Schneider and Akkan 2004	NA	NA
Glutaraldehyde	ACE	0.50	3.39	Omori et al. 2008	+ (18, 2.5%)	+	+	Basketter et al. 2005; Hilton et al. 1998	Gad et al. 1986	Marzulli and Maibach 1974; Schneider and Akkan 2004	NA	NA
Glutaraldehyde	ACE	0.50	2.57	Omori et al. 2008	+ (18, 2.5%)	+	+	Basketter et al. 2005; Hilton et al. 1998	Gad et al. 1986	Marzulli and Maibach 1974; Schneider and Akkan 2004	NA	NA

Substance Name	Veh.[1]	LLNA: DA Highest Conc. Tested (%)	LLNA: DA Highest SI[2]	LLNA: DA Reference	Trad. LLNA Result[3]	GP Result[4]	Human Result[5]	Trad. LLNA Reference	GP Reference	Human Reference	Skin Irritation Data	Skin Irritation Reference
Hexane	AOO	100	2.31	Idehara et al. 2008	- (2.2, 100%)	NA	-	ICCVAM 1999	NA	ICCVAM 1999	Irritant at 100% (humans)	Kligman 1966c
Hexyl cinnamic aldehyde	AOO	25	6.47	Idehara et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	5.78	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	4.82	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	4.44	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	5.11	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	3.97	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	5.50	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	7.09	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995

Substance Name	Veh.[1]	LLNA: DA Highest Conc. Tested (%)	LLNA: DA Highest SI[2]	LLNA: DA Reference	Trad. LLNA Result[3]	GP Result[4]	Human Result[5]	Trad. LLNA Reference	GP Reference	Human Reference	Skin Irritation Data	Skin Irritation Reference
Hexyl cinnamic aldehyde	AOO	25	10.22	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	3.88	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	3.51	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	4.47	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	5.71	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	5.41	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	7.60	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	3.92	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hexyl cinnamic aldehyde	AOO	25	8.42	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995

Substance Name	Veh.[1]	LLNA: DA Highest Conc. Tested (%)	LLNA: DA Highest SI[2]	LLNA: DA Reference	Trad. LLNA Result[3]	GP Result[4]	Human Result[5]	Trad. LLNA Reference	GP Reference	Human Reference	Skin Irritation Data	Skin Irritation Reference
Hexyl cinnamic aldehyde	AOO	25	6.45	Omori et al. 2008	+ (20, 50%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at ≤10% (GP); mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Hydroxy-citronellal	AOO	50	5.69	Idehara et al. 2008	+ (8.5, 100%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 50% (GP); negative at 100% (rabbits)	Basketter et al. 2007b; ECETOC 1995
Imidazolidinyl urea	DMF	50	4.67	Idehara et al. 2008	+ (5.5, 50%)	+	+	Gerberick et al. 2005	ICCVAM 1999	ICCVAM 1999	Negative at ≤75% (GP)	Basketter and Scholes 1992
Isoeugenol	AOO	50	12.36 at 25%	Idehara et al. 2008	+ (31, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 5% (GP)	Basketter et al. 2007b
Isoeugenol	AOO	10	6.11	Omori et al. 2008	+ (31, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 5% (GP)	Basketter et al. 2007b
Isoeugenol	AOO	10	5.54	Omori et al. 2008	+ (31, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 5% (GP)	Basketter et al. 2007b
Isoeugenol	AOO	10	7.09	Omori et al. 2008	+ (31, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 5% (GP)	Basketter et al. 2007b
Isopropanol	AOO	50	1.08 at 25%	Idehara et al. 2008	- (1.7, 50%)[9]	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC 1995
Isopropanol	AOO	50	1.54 at 10%	Omori et al. 2008	- (1.7, 50%)[9]	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC 1995
Isopropanol	AOO	50	0.91 at 10%	Omori et al. 2008	- (1.7, 50%)[9]	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC 1995
Isopropanol	AOO	50	1.01 at 10%	Omori et al. 2008	- (1.7, 50%)[9]	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC 1995

Substance Name	Veh.[1]	LLNA: DA Highest Conc. Tested (%)	LLNA: DA Highest SI[2]	LLNA: DA Reference	Trad. LLNA Result[3]	GP Result[4]	Human Result[5]	Trad. LLNA Reference	GP Reference	Human Reference	Skin Irritation Data	Skin Irritation Reference
Isopropanol	AOO	50	1.57 at 10%	Omori et al. 2008	- (1.7, 50%)[9]	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC 1995
Isopropanol	AOO	50	0.76 at 25%	Omori et al. 2008	- (1.7, 50%)[9]	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC 1995
Isopropanol	AOO	50	1.97 at 10%	Omori et al. 2008	- (1.7, 50%)[9]	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC 1995
Isopropanol	AOO	50	1.45 at 10%	Omori et al. 2008	- (1.7, 50%)[9]	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC 1995
Isopropanol	AOO	50	1.21 at 10%	Omori et al. 2008	- (1.7, 50%)[9]	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC 1995
Isopropanol	AOO	50	0.70 at 25%	Omori et al. 2008	- (1.7, 50%)[9]	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC 1995
Isopropanol	AOO	50	1.25	Omori et al. 2008	- (1.7, 50%)[9]	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC 1995
Lactic acid	DMSO	50	1.06 at 10%	Idehara et al. 2008	- (2.2, 25%)	-	-	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Slightly irritating at 10% aq. (rabbits)	Cosmetic Ingredient Review Expert Panel 1998
Lactic acid	DMSO	25	0.93 at 5%	Omori et al. 2008	- (2.2, 25%)	-	-	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Slightly irritating at 10% aq. (rabbits)	Cosmetic Ingredient Review Expert Panel 1998
Lactic acid	DMSO	25	0.99 at 5%	Omori et al. 2008	- (2.2, 25%)	-	-	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Slightly irritating at 10% aq. (rabbits)	Cosmetic Ingredient Review Expert Panel 1998

Substance Name	Veh.[1]	LLNA: DA Highest Conc. Tested (%)	LLNA: DA Highest SI[2]	LLNA: DA Reference	Trad. LLNA Result[3]	GP Result[4]	Human Result[5]	Trad. LLNA Reference	GP Reference	Human Reference	Skin Irritation Data	Skin Irritation Reference
Lactic acid	DMSO	25	0.97 at 10%	Omori et al. 2008	- (2.2, 25%)	-	-	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Slightly irritating at 10% aq. (rabbits)	Cosmetic Ingredient Review Expert Panel 1998
Lactic acid	DMSO	25	0.91	Omori et al. 2008	- (2.2, 25%)	-	-	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Slightly irritating at 10% aq. (rabbits)	Cosmetic Ingredient Review Expert Panel 1998
2-Mercaptobenzo-thiazole	DMF	50	2.00	Idehara et al. 2008	+ (8.6, 10%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 10% (GP); nonirritant at 25% (humans)	Basketter et al. 2007b; Kligman 1966c
Methyl methacrylate	AOO	100	1.81	Idehara unpublished	+ (3.6, 100%)	+	+ (case studies, no exposure concen-tration)	Betts et al. 2006	Van der Walle et al. 1982	Betts et al. 2006	Nonirritant at 3 M (GP)	Van der Walle et al. 1982
Methyl salicylate	AOO	25	1.20	Idehara et al. 2008	- (2.9, 20%)	-	-	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Irritant at 10% AOO (mice)	Gerberick et al 2002
Methyl salicylate	AOO	25	1.55	Omori et al. 2008	- (2.9, 20%)	-	-	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Irritant at 10% AOO (mice)	Gerberick et al 2002
Methyl salicylate	AOO	25	1.77 at 10%	Omori et al. 2008	- (2.9, 20%)	-	-	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Irritant at 10% AOO (mice)	Gerberick et al 2002
Methyl salicylate	AOO	25	0.83	Omori et al. 2008	- (2.9, 20%)	-	-	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Irritant at 10% AOO (mice)	Gerberick et al 2002
Nickel (II) chloride	DMSO	10	1.30	Idehara unpublished	- (2.4, 5%)	+	+	ICCVAM 1999	ICCVAM 1999	Vandenberg and Epstein 1963	Negative at ≤ 0.15% (GP)	Basketter and Scholes 1992

Substance Name	Veh.[1]	LLNA: DA Highest Conc. Tested (%)	LLNA: DA Highest SI[2]	LLNA: DA Reference	Trad. LLNA Result[3]	GP Result[4]	Human Result[5]	Trad. LLNA Reference	GP Reference	Human Reference	Skin Irritation Data	Skin Irritation Reference
Nickel (II) sulfate hexahydrate	DMSO	5.0	2.17 at 2.5%	Idehara et al. 2008	+ (3.1, 5%)	+	+	Ryan et al. 2002	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (humans); nonirritant at 0.15% (GP)	Kligman 1966c; Scholes et al. 1992
Nickel (II) sulfate hexahydrate	DMSO	10	1.52 at 3%	Omori et al. 2008	+ (3.1, 5%)	+	+	Ryan et al. 2002	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (humans); nonirritant at 0.15% (GP)	Kligman 1966c; Scholes et al. 1992
Nickel (II) sulfate hexahydrate	DMSO	10	11.78	Omori et al. 2008	+ (3.1, 5%)	+	+	Ryan et al. 2002	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (humans); nonirritant at 0.15% (GP)	Kligman 1966c; Scholes et al. 1992
Nickel (II) sulfate hexahydrate	DMSO	10	3.49 at 1%	Omori et al. 2008	+ (3.1, 5%)	+	+	Ryan et al. 2002	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (humans); nonirritant at 0.15% (GP)	Kligman 1966c; Scholes et al. 1992
Nickel (II) sulfate hexahydrate	DMSO	10	0.79 at 3%	Omori et al. 2008	+ (3.1, 5%)	+	+	Ryan et al. 2002	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (humans); nonirritant at 0.15% (GP)	Kligman 1966c; Scholes et al. 1992
Nickel (II) sulfate hexahydrate	DMSO	10	1.24 at 3%	Omori et al. 2008	+ (3.1, 5%)	+	+	Ryan et al. 2002	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (humans); nonirritant at 0.15% (GP)	Kligman 1966c; Scholes et al. 1992
Nickel (II) sulfate hexahydrate	DMSO	10	2.13	Omori et al. 2008	+ (3.1, 5%)	+	+	Ryan et al. 2002	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (humans); nonirritant at 0.15% (GP)	Kligman 1966c; Scholes et al. 1992
Nickel (II) sulfate hexahydrate	DMSO	10	1.56 at 3%	Omori et al. 2008	+ (3.1, 5%)	+	+	Ryan et al. 2002	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (humans); nonirritant at 0.15% (GP)	Kligman 1966c; Scholes et al. 1992
Phenyl benzoate	AOO	10	4.24 at 5%	Idehara unpublished	+ (11.1, 25%)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al 2005	NA	NA

Substance Name	Veh.[1]	LLNA: DA Highest Conc. Tested (%)	LLNA: DA Highest SI[2]	LLNA: DA Reference	Trad. LLNA Result[3]	GP Result[4]	Human Result[5]	Trad. LLNA Reference	GP Reference	Human Reference	Skin Irritation Data	Skin Irritation Reference
p-Phenylenediamine	AOO	1	5.14 at 0.25%	Idehara et al. 2008	+ (26, 1%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.5% (GP)	Basketter et al. 2007b
Phthalic anhydride	AOO	1.0	6.85	Idehara et al. 2008	+ (26.0, 10%)[12]	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 2001	Negative at ≤ 10% (GP)	Basketter and Scholes 1992
Potassium dichromate	DMSO	1.0	5.49	Idehara et al. 2008	+ (33.6, 0.5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.15% (GP)	Basketter et al. 2007b
Potassium dichromate	DMSO	1.0	4.78	Omori et al. 2008	+ (33.6, 0.5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.15% (GP)	Basketter et al. 2007b
Potassium dichromate	DMSO	1.0	4.08	Omori et al. 2008	+ (33.6, 0.5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.15% (GP)	Basketter et al. 2007b
Potassium dichromate	DMSO	1.0	6.01	Omori et al. 2008	+ (33.6, 0.5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.15% (GP)	Basketter et al. 2007b
Potassium dichromate	DMSO	1.0	6.37	Omori et al. 2008	+ (33.6, 0.5%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.15% (GP)	Basketter et al. 2007b
Propyl gallate	AOO	2.5	4.95	Idehara unpublished	+ (33.6, 25%)	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 5% (GP)	Basketter and Scholes 1992
Propylparaben	AOO	25	1.28	Idehara et al. 2008	- (1.4, 25%)[13]	-	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 10% (GP)	Basketter and Scholes 1992
Resorcinol	AOO	25	4.33	Idehara et al. 2008	+ (10.4, 50%)	-	+	Basketter et al. 2007a	ICCVAM 1999	ICCVAM 1999; Basketter et al. 2007a	Nonirritant at 15% (humans)	Kligman 1966c
Salicylic acid	AOO	25	2.00	Idehara unpublished	- (2.5, 25%)	-	-	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Irritant at 20% aq. (mice)	Gerberick et al. 2002

Substance Name	Veh.[1]	LLNA: DA Highest Conc. Tested (%)	LLNA: DA Highest SI[2]	LLNA: DA Reference	Trad. LLNA Result[3]	GP Result[4]	Human Result[5]	Trad. LLNA Reference	GP Reference	Human Reference	Skin Irritation Data	Skin Irritation Reference
Sodium lauryl sulfate	DMF	10	3.39	Idehara et al. 2008	+ (8.9, 20%)[9]	-	-	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Irritant at 20% (humans); Irritant at 20% (rabbits); irritant at 10% in DMF (mice)	Kligman 1966c; ECETOC 1995; Antonopoulos et al. 2008
Sulfanilamide	DMF	50	0.86 at 25%	Idehara unpublished	- (1, 50%)[14]	-	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Kligman 1966c	Nonirritant at 25% (humans)	Kligman 1966c
Toluene 2,4-diisocyanate	**AOO**	**0.25**	**9.43**	**Idehara et al. 2008**	**+[15] (NA)**	**+**	**+**	**van Och et al. 2001**	**NA**	**Basketter et al. 2001**	**NA**	**NA**
Trimellitic anhydride	AOO	0.50	4.96	Idehara et al. 2008	+ (4.6, 25%)	+	NA	ICCVAM 1999; Basketter and Scholes 1992	ICCVAM 1999; Gad et al. 1986	ICCVAM 1999; Basketter et al. 2001	Negative at ≤ 10% (GP)	Basketter and Scholes 1992

Bold Substances not included in accuracy analyses.

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); aq. = aqueous; CASRN = Chemical Abstracts Service Registry Number; Conc. = concentration; DMF = *N,N*-dimethylformamide; DMSO = dimethylsulfoxide; ECETOC = European Centre for Ecotoxicology and Toxicology of Chemicals; GP = guinea pig; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; MEK = methyl ethyl ketone; NA = not available; nonstd = nonstandard; SI = stimulation index; Trad. = traditional; "+" = Sensitizer.

"-" = Nonsensitizer.

[1] Applies to both traditional LLNA and LLNA: DA, unless otherwise noted.

[2] Highest SI occurred at highest concentration tested, unless otherwise noted.

[3] Numbers in parentheses indicate the highest SI and the highest concentration tested. Highest SI occurred at highest concentration tested, unless otherwise footnoted.

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

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

[6] Vehicle for traditional LLNA was acetone.

[7] Highest SI occurred at 1%.

[8] Equivocal traditional LLNA data (ICCVAM 1999); substance not included in accuracy analyses.

[9] Highest SI occurred at 10%.

[10] Data not reported for the highest dose (i.e., 3%), only for 0.3% and 1%.

[11] Highest SI occurred at 50%.

[12] Highest SI occurred at 2.5%.

[13] Highest SI occurred at 5%.

[14] Highest SI occurred at both 10% and 25%.

[15] Comparable LLNA reference data from modified LLNA test (van Och et al. 2000).

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Annex III-2

Comparison of Alternative LLNA: DA Decision Criteria and Traditional LLNA Results

(Alphanumeric Order)

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Table C-III-2-1 Comparative Performance of Various LLNA: DA SI Values and Traditional LLNA Tests (Alphanumeric Order)

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	Stats.[2]	≥95 % CI	≥3 SD	≥2 SD	SI ≥											LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
								5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.8	1.5	1.3	1.0			
Abietic acid	514-10-3	25	6.26	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Abietic acid	514-10-3	25	4.64	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Abietic acid	514-10-3	25	7.96	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Abietic acid	514-10-3	25	3.98 at 10%	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
3-Amino-phenol	591-27-5	10	2.83	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
3-Amino-phenol	591-27-5	10	1.76 at 3%	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	Omori et al. 2008	+	ICCVAM 1999
3-Amino-phenol	591-27-5	10	2.38	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Benzalkonium chloride	8001-54-5	2.5	6.68	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	Gerberick et al. 1992
Benzocaine	**94-09-7**	**25**	**4.84**	**+**	**+**	**+**	**+**	**-**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**Idehara et al. 2008**	**+/-[3]**	**ICCVAM 1999**
p-Benzo-quinone	106-51-4	0.100	3.79	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Idehara unpublished	+	ICCVAM 1999
1-Bromo-butane	109-65-9	25	1.65	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	Idehara et al. 2008	-	ICCVAM 1999
Butyl glycidyl ether	2426-08-6	50	4.59	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	Idehara unpublished	+	ICCVAM 1999
Chlorobenzene	108-90-7	25	2.44	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	Idehara et al. 2008	-	ICCVAM 1999
5-Chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	0.100	7.50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara unpublished	+	ICCVAM 1999; Gerberick et al. 2005

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	Stats.[2]	≥95 % CI	≥3 SD	≥2 SD	SI ≥											LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
								5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.8	1.5	1.3	1.0			
Cinnamic alcohol	104-54-1	90	5.66 at 50%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara unpublished	+	Gerberick et al. 2005
Cinnamic aldehyde	104-55-2	15	4.73	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Citral	5392-40-5	25	4.40	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Cobalt chloride	7646-79-9	5	3.64	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Cobalt chloride	7646-79-9	1[4]	2.66	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Cobalt chloride	7646-79-9	3	20.55	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Cobalt chloride	7646-79-9	3	8.07	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Cobalt chloride	7646-79-9	5	2.01	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Cobalt chloride	7646-79-9	5	2.54	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Cobalt chloride	7646-79-9	5	4.25	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Cobalt chloride	7646-79-9	5	5.06	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Diethyl maleate	141-05-9	10.0	3.78	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Idehara unpublished	+	Gerberick et al. 2005
Diethyl phthalate	84-66-2	100	1.09	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	Idehara et al. 2008	-	Gerberick et al. 2005
Dimethyl isophthalate	1459-93-4	25	0.89 at 5%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Idehara unpublished	-	ICCVAM 1999; Basketter et al. 1999b

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	Stats.[2]	≥95 % CI	≥3 SD	≥2 SD	SI ≥											LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
								5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.8	1.5	1.3	1.0			
Dimethyl isophthalate	1459-93-4	25	1.34 at 5%	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	Omori et al. 2008	-	ICCVAM 1999; Basketter et al. 1999b
Dimethyl isophthalate	1459-93-4	25	1.00 at 5%	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	Omori et al. 2008	-	ICCVAM 1999; Basketter et al. 1999b
Dimethyl isophthalate	1459-93-4	25	1.26 at 5%	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	Omori et al. 2008	-	ICCVAM 1999; Basketter et al. 1999b
2,4-Dinitrochloro-benzene	97-00-7	1	7.10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	11.97	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	9.23	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	9.96	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	8.53	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	7.86	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	15.14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	13.18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	12.60	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	10.89	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	Stats.[2]	≥95 % CI	≥3 SD	≥2 SD	SI ≥											LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
								5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.8	1.5	1.3	1.0			
2,4-Dinitrochloro-benzene	97-00-7	0.30	4.71	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Ethyl acrylate	140-88-5	50	4.29 at 25%	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	Idehara unpublished	+	Gerberick et al. 2005
Ethylene glycol dimethacrylate	97-90-5	50	4.45	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	Idehara unpublished	+	ICCVAM 1999
Eugenol	97-53-0	25	7.07	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Formaldehyde	50-00-0	2.50	5.10	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	Idehara et al. 2008	+	Gerberick et al. 2005; Hilton et al. 1998
Formaldehyde	50-00-0	5.0	4.84	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	Gerberick et al. 2005; Hilton et al. 1998
Formaldehyde	50-00-0	5.0	3.18	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	Omori et al. 2008	+	Gerberick et al. 2005; Hilton et al. 1998
Formaldehyde	50-00-0	5.0	2.69	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	Omori et al. 2008	+	Gerberick et al. 2005; Hilton et al. 1998
Glutaraldehyde	111-30-8	0.25	6.45	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	Basketter et al. 2005; Hilton et al. 1998
Glutaraldehyde	111-30-8	0.50	5.00	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	Basketter et al. 2005; Hilton et al. 1998
Glutaraldehyde	111-30-8	0.50	3.39	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	Omori et al. 2008	+	Basketter et al. 2005; Hilton et al. 1998
Glutaraldehyde	111-30-8	0.50	2.57	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	Omori et al. 2008	+	Basketter et al. 2005; Hilton et al. 1998

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	Stats.[2]	≥95 % CI	≥3 SD	≥2 SD	SI ≥											LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
								5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.8	1.5	1.3	1.0			
Hexane	110-54-3	100	2.31	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	Idehara et al. 2008	-	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	6.47	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	5.78	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	4.82	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	4.44	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	5.11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	3.97	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	5.50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	7.09	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	10.22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	3.88	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	3.51	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	4.47	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	5.71	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	5.41	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	Stats.[2]	≥95 % CI	≥3 SD	≥2 SD	SI ≥											LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
								5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.8	1.5	1.3	1.0			
Hexyl cinnamic aldehyde	101-86-0	25	7.60	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	3.92	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	8.42	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	6.45	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hydroxycitronellal	107-75-5	50	5.69	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Imidazolidinyl urea	39236-46-9	50	4.67	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	Gerberick et al. 2005
Isoeugenol	97-54-1	50	12.36 at 25%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Isoeugenol	97-54-1	10	6.11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Isoeugenol	97-54-1	10	5.54	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Isoeugenol	97-54-1	10	7.09	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Isopropanol	67-63-0	50	1.08 at 25%	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	Idehara et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	1.54 at 10%	-	+	-	+	-	-	-	-	-	-	-	-	+	+	+	Omori et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	0.91 at 10%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Omori et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	1.01 at 10%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Omori et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	1.57 at 10%	+	+	-	+	-	-	-	-	-	-	-	-	+	+	+	Omori et al. 2008	-	ICCVAM 1999

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	Stats.[2]	≥95 % CI	≥3 SD	≥2 SD	SI ≥											LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
								5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.8	1.5	1.3	1.0			
Isopropanol	67-63-0	50	0.76 at 25%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Omori et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	1.97 at 10%	-	+	+	+	-	-	-	-	-	-	-	+	+	+	+	Omori et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	1.45 at 10%	-	+	-	+	-	-	-	-	-	-	-	-	-	+	+	Omori et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	1.21 at 10%	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	Omori et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	0.70 at 25%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Omori et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	1.25	-	+	-	+	-	-	-	-	-	-	-	-	-	-	+	Omori et al. 2008	-	ICCVAM 1999
Lactic acid	50-21-5	50	1.06 at 10%	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	Idehara et al. 2008	-	ICCVAM 1999
Lactic acid	50-21-5	25	0.93 at 5%	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Omori et al. 2008	-	ICCVAM 1999
Lactic acid	50-21-5	25	0.99 at 5%	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Omori et al. 2008	-	ICCVAM 1999
Lactic acid	50-21-5	25	0.97 at 10%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Omori et al. 2008	-	ICCVAM 1999
Lactic acid	50-21-5	25	0.91	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Omori et al. 2008	-	ICCVAM 1999
2-Mercapto-benzothiazole	149-30-4	50	2.00	-	+	+	+	-	-	-	-	-	-	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Methyl methacrylate	80-62-6	100	1.81	-	+	-	-	-	-	-	-	-	-	-	+	+	+	+	Idehara unpublished	+	Betts et al. 2006
Methyl salicylate	119-36-8	25	1.20	-	+	-	+	-	-	-	-	-	-	-	-	-	-	+	Idehara et al. 2008	-	ICCVAM 1999
Methyl salicylate	119-36-8	25	1.55	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+	Omori et al. 2008	-	ICCVAM 1999

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	Stats.[2]	≥95 % CI	≥3 SD	≥2 SD	SI ≥											LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
								5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.8	1.5	1.3	1.0			
Methyl salicylate	119-36-8	25	1.77 at 10%	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	Omori et al. 2008	-	ICCVAM 1999
Methyl salicylate	119-36-8	25	0.83	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	Omori et al. 2008	-	ICCVAM 1999
Nickel (II) chloride	7718-54-9	10	1.30	-	+	+	+	-	-	-	-	-	-	-	-	-	+	+	Idehara unpublished	-	ICCVAM 1999
Nickel (II) sulfate hexahydrate	10101-97-0	5.0	2.17 at 2.5%	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	Idehara et al. 2008	+	Ryan et al. 2002
Nickel (II) sulfate hexahydrate	10101-97-0	10	1.52 at 3%	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	Omori et al. 2008	+	Ryan et al. 2002
Nickel (II) sulfate hexahydrate	10101-97-0	10	11.78	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	Ryan et al. 2002
Nickel (II) sulfate hexahydrate	10101-97-0	10	3.49 at 1%	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	Omori et al. 2008	+	Ryan et al. 2002
Nickel (II) sulfate hexahydrate	10101-97-0	10	0.79 at 3%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Omori et al. 2008	+	Ryan et al. 2002
Nickel (II) sulfate hexahydrate	10101-97-0	10	1.24 at 3%	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	Omori et al. 2008	+	Ryan et al. 2002
Nickel (II) sulfate hexahydrate	10101-97-0	10	2.13	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	Omori et al. 2008	+	Ryan et al. 2002
Nickel (II) sulfate hexahydrate	10101-97-0	10	1.56 at 3%	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	Omori et al. 2008	+	Ryan et al. 2002
Phenyl benzoate	93-99-2	10.0	4.24 at 5%	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Idehara unpublished	+	ICCVAM 1999
p-Phenylene-diamine	106-50-3	1	5.14 at 0.25%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Phthalic anhydride	85-44-9	1.0	6.85	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	Stats.[2]	≥95 % CI	≥3 SD	≥2 SD	SI ≥											LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
								5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.8	1.5	1.3	1.0			
Potassium dichromate	7778-50-9	1.0	5.49	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Potassium dichromate	7778-50-9	1.0	4.78	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Potassium dichromate	7778-50-9	1.0	4.08	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Potassium dichromate	7778-50-9	1.0	6.01	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Potassium dichromate	7778-50-9	1.0	6.37	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Propyl gallate	121-79-9	2.5	4.95	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	Idehara unpublished	+	ICCVAM 1999
Propylparaben	94-13-3	25	1.28	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	Idehara et al. 2008	-	ICCVAM 1999
Resorcinol	108-46-3	25	4.33	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	Basketter et al. 2007a
Salicylic acid	69-72-7	25	2.00	+	+	-	+	-	-	-	-	-	-	+	+	+	+	+	Idehara unpublished	-	ICCVAM 1999
Sodium lauryl sulfate	151-21-3	10	3.39	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Sulfanilamide	63-74-1	50	0.86 at 25%	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Idehara unpublished	-	ICCVAM 1999
Toluene 2,4-diisocyanate	**584-84-9**	**0.25**	**9.43**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**Idehara et al. 2008**	**+[5]**	**Van Och et al. 2001**
Trimellitic anhydride	552-30-7	0.50	4.96	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999; Basketter and Scholes 1992

Entries in boldface indicate substances not included in accuracy analyses.

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; CI = confidence interval (mean ATP measurement of any treatment group is greater than 95% CI of mean ATP measurement for vehicle control group); Conc. = concentration; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP

content; Ref. = reference; SD = standard deviation (mean ATP measurement of any treatment group is greater than two or three 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] Highest SI occurred at highest concentration tested, unless otherwise noted.

[2] The ATP data were log-transformed prior to statistical analyses. For analysis of variance, significance at $p < 0.05$ was further tested by Dunnett's test.

[3] Equivocal (i.e., results that were not reproducible) traditional LLNA data (ICCVAM 1999). Substance not included in accuracy analyses.

[4] Data not reported for the highest dose (i.e., 3%), only for 0.3% and 1%.

[5] LLNA reference data from modified LLNA test (van Och et al. 2000). Substance not included in accuracy analyses.

Annex IV-1

Individual Animal Data for the LLNA: DA (Intralaboratory)

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Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
VC	AOO	0	1	4927	1.12				
			2	3547	0.80				
			3	4758	1.08				
			Mean	**4411**	**1.00**				
PC - Eugenol	AOO	10	1	17020	3.86				
			2	14029	3.18				
			3	12117	2.75				
			Mean	**14388**	**3.26**				
Citral	AOO	5	1	9191	2.08	**15.63**	**12.46**	**5.96**	**4.11**
			2	12120	2.75				
			3	4808	1.09				
			Mean	**8706**	**1.97**				
		10	1	9937	2.25				
			2	7447	1.69				
			3	10528	2.39				
			Mean	**9304**	**2.11**				
		15	1	12297	2.79				
			2	11863	2.69				
			3	14283	3.24				
			Mean	**12814**	**2.91**				
		25	1	18200	4.13				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
			2	22609	5.13				
			3	17469	3.96				
			Mean	**19426**	**4.40**				
Cinnamic aldehyde	AOO	1	1	6780	1.54	**2.98**	**2.08**	**0.92**	**0.63**
			2	13271	3.01				
			3	7545	1.71				
			Mean	**9199**	**2.09**				
		2.5	1	13624	3.09				
			2	8924	2.02				
			3	12681	2.88				
			Mean	**11743**	**2.66**				
		5	1	21945	4.98				
			2	17313	3.93				
			3	19218	4.36				
			Mean	**19492**	**4.42**				
		15	1	20037	4.54				
			2	18085	4.10				
			3	24421	5.54				
			Mean	**20848**	**4.73**				
VC	AOO	0	1	3759	0.97				
			2	3995	1.03				
			3	3461	0.89				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
			4	4269	1.10				
			Mean	**3871**	**1.00**				
PC - Eugenol	AOO	10	1	16624	4.30				
			2	23785	6.15				
			3	15667	4.05				
			4	18066	4.67				
			Mean	**18535**	**4.79**				
Eugenol	AOO	5	1	12594	3.25	**4.50**	**3.60**	**2.88**	**2.63**
			2	15216	3.93				
			3	9790	2.53				
			4	NT	NT				
			Mean	**12533**	**3.24**				
		10	1	16624	4.30				
			2	23785	6.15				
			3	15667	4.05				
			4	18066	4.67				
			Mean	**18535**	**4.79**				
		25	1	26107	6.75				
			2	26713	6.90				
			3	29297	7.57				
			4	NT	NT				
			Mean	**27372**	**7.07**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
Propylparaben	AOO	5	1	5058	1.31	**NA**	**NA**	**NA**	**NA**
			2	4773	1.23				
			3	3034	0.78				
			Mean	**4288**	**1.11**				
		10	1	5539	1.43				
			2	3919	1.01				
			3	3713	0.96				
			Mean	**4390**	**1.13**				
		25	1	6385	1.65				
			2	5813	1.50				
			3	2679	0.69				
			Mean	**4959**	**1.28**				
Hexyl cinnamic aldehyde	AOO	5	1	7375	1.91	**11.62**	**9.69**	**7.75**	**6.97**
			2	3858	1.00				
			3	3782	1.00				
			Mean	**5005**	**1.29**				
		10	1	9217	2.38				
			2	12654	3.27				
			3	8072	2.09				
			Mean	**9981**	**2.58**				
		25	1	30420	7.86				
			2	27682	7.15				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
			3	17014	4.40				
			Mean	**25038**	**6.47**				
Methyl salicylate	AOO	5	1	3250	0.84	**NA**	**NA**	**NA**	**NA**
			2	3310	0.86				
			3	1760	0.46				
			Mean	**2773**	**0.72**				
		10	1	4499	1.16				
			2	4637	1.20				
			3	2035	0.53				
			Mean	**3723**	**0.96**				
		25	1	4542	1.17				
			2	5445	1.41				
			3	3996	1.03				
			Mean	**4661**	**1.20**				
VC 1	AOO	0	1	3529	1.17				
			2	3106	1.03				
			3	2949	0.98				
			4	2473	0.82				
			Mean	**3014**	**1.00**				
PC 1 - Eugenol	AOO	10	1	20105	6.67				
			2	14663	4.87				
			3	14233	4.72				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
			4	13137	4.36				
			Mean	**15535**	**5.15**				
VC 2	DMSO	0	1	4770	0.72				
			2	6914	1.04				
			3	8487	1.27				
			4	6527	0.98				
			Mean	**6674**	**1.00**				
PC 2 - Eugenol	DMSO	10	1	10887	1.63				
			2	16454	2.47				
			3	9982	1.50				
			4	12245	1.84				
			Mean	**12392**	**1.86**				
Abietic acid	AOO	5	1	4143	1.38	**7.90**	**5.99**	**4.40**	**3.96**
			2	9059	3.01				
			3	7056	2.34				
			Mean	**6752**	**2.24**				
		10	1	13190	4.30				
			2	8354	2.77				
			3	10561	3.50				
			Mean	**10701**	**3.55**				
		25	1	20693	6.87				
			2	17109	5.68				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
			3	18770	6.23				
			Mean	**18857**	**6.26**				
Cobalt II chloride	DMSO	1	1	17709	2.65	**3.27**	**1.94**	**0.88**	**0.70**
			2	12673	1.90				
			3	12428	1.86				
			Mean	**14270**	**2.14**				
		2.5	1	17680	2.65				
			2	17863	2.68				
			3	18809	2.82				
			Mean	**18117**	**2.71**				
		5	1	28248	4.23				
			2	27268	4.09				
			3	17378	2.60				
			Mean	**24298**	**3.64**				
Nickel (II) sulfate hexahydrate	DMSO	1	1	7672	1.15	**NA**	**NA**	**2.18**	**1.81**
			2	11041	1.65				
			3	8581	1.29				
			Mean	**9098**	**1.36**				
		2.5	1	10829	1.62				
			2	10925	1.64				
			3	21735	3.26				
			Mean	**14496**	**2.17**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
		5	1	15969	2.39				
			2	9433	1.41				
			3	11636	1.74				
			Mean	**12346**	**1.85**				
VC 1	AOO	0	1	2660	1.03				
			2	2856	1.11				
			3	1828	0.71				
			4	2975	1.15				
			Mean	**2580**	**1.00**				
PC 1 - Eugenol	AOO	10	1	19298	7.48				
			2	17360	6.73				
			3	14953	5.80				
			4	11827	4.59				
			Mean	**15859**	**6.15**				
VC 2	DMF	0	1	4424	1.29				
			2	3087	0.90				
			3	2348	0.69				
			4	3854	1.12				
			Mean	**3428**	**1.00**				
PC 2 - Eugenol	DMF	10	1	5738	1.67				
			2	5644	1.65				
			3	3688	1.08				
			4	8185	2.39				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
			Mean	**5813**	**1.70**				
Benzocaine	AOO	5	1	10495	4.07	**6.57**	**4.66**	**3.49**	**3.11**
			2	3052	1.18				
			3	6751	2.62				
			Mean	**6766**	**2.62**				
		10	1	10314	4.00				
			2	10880	4.22				
			3	8378	3.25				
			Mean	**9857**	**3.82**				
		25	1	10512	4.08				
			2	14366	5.57				
			3	12564	4.87				
			Mean	**12480**	**4.84**				
Imidazolidinyl urea	DMF	10	1	7333	2.14	**18.77**	**11.94**	**7.42**	**6.28**
			2	6777	1.98				
			3	10143	2.96				
			Mean	**8084**	**2.36**				
		25	1	9854	2.88				
			2	13907	4.06				
			3	11783	3.44				
			Mean	**11848**	**3.46**				
		50	1	14760	4.31				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
			2	15299	4.46				
			3	17971	5.24				
			Mean	**16010**	**4.67**				
2-Mercaptobenzothiazole	DMF	10	1	7829	2.28	**NA**	**NA**	**9.99**	**7.99**
			2	7102	2.07				
			3	5647	1.65				
			Mean	**6859**	**2.00**				
		25	1	6978	2.04				
			2	2425	0.71				
			3	4401	1.28				
			Mean	**4601**	**1.34**				
		50	1	3976	1.16				
			2	4375	1.28				
			3	2675	0.78				
			Mean	**3675**	**1.07**				
VC	AOO	0	1	1453	0.28				
			2	11748	2.27				
			3	4663	0.90				
			4	2810	0.54				
			Mean	**5168**	**1.00**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
PC - Eugenol	AOO	10	1	13351	2.58				
			2	27023	5.23				
			3	12875	2.49				
			4	15921	3.08				
			Mean	**17292**	**3.35**				
2-4-Dinitrochlorobenzene	AOO	0.03	1	11884	2.30	**0.16**	**0.13**	**0.11**	**0.08**
			2	11146	2.16				
			3	5799	1.12				
			Mean	**9610**	**1.86**				
		0.05	1	10848	2.10				
			2	7394	1.43				
			3	8468	1.64				
			Mean	**8903**	**1.72**				
		0.1	1	13205	2.56				
			2	8679	1.68				
			3	6740	1.30				
			Mean	**9541**	**1.85**				
		0.25	1	34300	6.64				
			2	26924	5.21				
			3	15631	3.03				
			Mean	**25618**	**4.96**				
		0.5	1	33092	6.40				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
			2	46685	9.03				
			3	30241	5.85				
			Mean	**36673**	**7.10**				
		1	1	40795	7.89				
			2	36807	7.12				
			3	32445	6.29				
			Mean	**36682**	**7.10**				
VC	AOO	0	1	1460	0.41				
			2	5137	1.46				
			3	3988	1.13				
			Mean	**3528**	**1.00**				
PC - Eugenol	AOO	10	1	22813	6.47				
			2	21142	5.99				
			3	30985	8.78				
			Mean	**24980**	**7.08**				
Isoeugenol	AOO	2.5	1	15638	4.43	**2.35**	**1.79**	**1.36**	**1.22**
			2	9113	2.58				
			3	8197	2.32				
			Mean	**10982**	**3.11**				
		5	1	15773	4.47				
			2	19726	5.59				
			3	10920	3.10				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
			Mean	**15473**	**4.39**				
		10	1	24776	7.02				
			2	23236	6.59				
			3	23595	6.69				
			Mean	**23869**	**6.77**				
Isoeugenol (continued)		25	1	40328	11.43				
			2	50432	14.30				
			3	40035	11.35				
			Mean	**43598**	**12.36**				
		50	1	43389	12.30				
			2	28424	8.06				
			3	40263	11.41				
			Mean	**37359**	**10.59**				
VC	AOO	0	1	836	0.55				
			2	1815	1.20				
			3	1752	1.16				
			4	1631	1.08				
			Mean	**1508**	**1.00**				
PC - Eugenol	AOO	10	1	13707	9.09				
			2	6746	4.47				
			3	10475	6.95				
			4	6855	4.54				
			Mean	**9446**	**6.26**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
Benzalkonium chloride	AOO	0.5	1	3027	2.01	**0.52**	**0.46**	**0.42**	**0.40**
			2	5780	3.83				
			3	4183	2.77				
			Mean	**4330**	**2.87**				
		1	1	9672	6.41				
			2	7809	5.18				
			3	10868	7.21				
			Mean	**9449**	**6.26**				
		2.5	1	10292	6.82				
			2	11879	7.88				
			3	8070	5.35				
			Mean	**10080**	**6.68**				
VC	DMF	0	1	2926	1.10				
			2	1674	0.63				
			3	3984	1.49				
			4	2091	0.78				
			Mean	**2668**	**1.00**				
PC - Cinnamic aldehyde	DMF	5	1	17595	6.59				
			2	12322	4.62				
			3	10331	3.87				
			4	12297	4.61				
			Mean	**13136**	**4.92**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
Sodium lauryl sulfate	DMF	1	1	3870	1.45	**6.88**	**2.91**	**1.91**	**1.64**
			2	2899	1.09				
			3	3777	1.42				
			Mean	**3515**	**1.32**				
Sodium lauryl sulfate		2.5	1	7965	2.99				
(continued)			2	4802	1.80				
			3	6838	2.56				
			Mean	**6535**	**2.45**				
		5	1	2945	1.10				
			2	7161	2.68				
			3	7913	2.97				
			Mean	**6006**	**2.25**				
		10	1	10337	3.87				
			2	6881	2.58				
			3	9932	3.72				
			Mean	**9050**	**3.39**				
VC	AOO	0	1	2045	0.97				
			2	1990	0.94				
			3	2212	1.05				
			4	2212	1.05				
			Mean	**2115**	**1.00**				
PC - Hexyl cinnamic	AOO	15	1	14020	6.63				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
aldehyde			2	9078	4.29				
			3	8912	4.21				
			Mean	**10670**	**5.05**				
Isopropanol	AOO	10	1	1364	0.65	**NA**	**NA**	**NA**	**NA**
			2	2872	1.36				
			3	2417	1.14				
			Mean	**2218**	**1.05**				
		25	1	3820	1.81				
			2	1746	0.83				
			3	1298	0.61				
			Mean	**2288**	**1.08**				
		50	1	2249	1.06				
			2	700	0.33				
			3	2454	1.16				
			Mean	**1801**	**0.85**				
VC	AOO	0	1	2386	0.76				
			2	2967	0.95				
			3	4347	1.39				
			4	2816	0.90				
			Mean	**3129**	**1.00**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
PC - Hexyl cinnamic aldehyde	AOO	15	1	9352	2.99				
			2	16201	5.18				
			3	10538	3.37				
			4	9135	2.92				
			Mean	**11306**	**3.61**				
Hexane	AOO	25	1	3755	1.20	**NA**	**NA**	**89.19**	**82.22**
			2	3240	1.04				
			3	3136	1.00				
			Mean	**3377**	**1.08**				
		50	1	3070	0.98				
			2	2491	0.80				
			3	2658	0.85				
			Mean	**2740**	**0.88**				
		100	1	9027	2.89				
			2	6802	2.17				
			3	5850	1.87				
			Mean	**7226**	**2.31**				
VC	AOO	0	1	2370	0.84				
			2	3124	1.11				
			3	2314	0.82				
			4	3464	1.23				
			Mean	**2818**	**1.00**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
PC - Hexyl cinnamic aldehyde	AOO	15	1	7739	2.75				
			2	10867	3.86				
			3	5290	1.88				
			4	8570	3.04				
			Mean	**8116**	**2.88**				
Toluene-2,4-diisocyanate	AOO	0.05	1	9445	3.35	**0.05**	**0.04**	**0.04**	**0.03**
			2	11471	4.07				
			3	5999	2.13				
			Mean	**8972**	**3.18**				
		0.1	1	12732	4.52				
			2	17962	6.38				
			3	16204	5.75				
			Mean	**15632**	**5.55**				
		0.25	1	25104	8.91				
			2	27791	9.86				
			3	26785	9.51				
			Mean	**26560**	**9.43**				
VC	AOO	0	1	1727	0.80				
			2	2122	0.99				
			3	2111	0.98				
			4	2645	1.23				
			Mean	**2151**	**1.00**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
PC - Hexyl cinnamic aldehyde	AOO	15	1	14931	6.94				
			2	15575	7.24				
			3	13043	6.06				
			4	11199	5.21				
			Mean	**13687**	**6.36**				
1-Bromobutane	AOO	5	1	2701	1.26	**NA**	**NA**	**NA**	**NA**
			2	2491	1.16				
			3	4272	1.99				
			Mean	**3154**	**1.47**				
		10	1	1810	0.84				
			2	2130	0.99				
			3	878	0.41				
			Mean	**1606**	**0.75**				
		25	1	3483	1.62				
			2	2916	1.36				
			3	4220	1.96				
			Mean	**3539**	**1.65**				
Chlorobenzene	AOO	5	1	1875	0.87	**NA**	**NA**	**20.09**	**17.88**
			2	2180	1.01				
			3	1088	0.51				
			Mean	**1714**	**0.80**				
		10	1	2505	1.16				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
			2	1840	0.86				
			3	2682	1.25				
			Mean	**2342**	**1.09**				
		25	1	2848	1.32				
			2	5302	2.47				
			3	7615	3.54				
			Mean	**5255**	**2.44**				
Diethyl phthalate	AOO	25	1	1543	0.72	**NA**	**NA**	**NA**	**NA**
			2	2561	1.19				
			3	2906	1.35				
			Mean	**2336**	**1.09**				
		50	1	1781	0.83				
			2	1371	0.64				
			3	2477	1.15				
			Mean	**1876**	**0.87**				
		100	1	1808	0.84				
			2	1288	0.60				
			3	2139	0.99				
			Mean	**1745**	**0.81**				
Hydroxycitronellal	AOO	10	1	5201	2.42	**13.74**	**11.21**	**9.23**	**8.67**
			2	4094	1.90				
			3	5293	2.46				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
			Mean	**4862**	**2.26**				
		25	1	9519	4.43				
			2	13562	6.31				
			3	10656	4.95				
			Mean	**11246**	**5.23**				
Hydroxycitronellal (continued)		50	1	14400	6.70				
			2	8741	4.06				
			3	13563	6.31				
			Mean	**12234**	**5.69**				
VC	ACE	0	1	2232	1.39				
			2	1509	0.94				
			3	1287	0.80				
			4	1419	0.88				
			Mean	**1611**	**1.00**				
PC - Hexyl cinnamic aldehyde	ACE	15	1	13901	8.63				
			2	16265	10.09				
			3	15531	9.64				
			4	15749	9.77				
			Mean	**15361**	**9.53**				
Glutaraldehyde	ACE	0.05	1	1821	1.13	**0.10**	**0.09**	**0.07**	**0.07**
			2	2181	1.35				
			3	1931	1.12				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
			Mean	**1978**	**1.23**				
		0.1	1	5389	3.34				
			2	2496	1.55				
			3	6344	3.94				
			Mean	**4743**	**2.94**				
		0.25	1	16484	10.20				
			2	6814	4.23				
			3	7889	4.90				
			Mean	**10396**	**6.45**				
VC	AOO	0	1	3101	0.92				
			2	3253	0.97				
			3	2687	0.80				
			4	4407	1.31				
			Mean	**3362**	**1.00**				
PC - Hexyl cinnamic aldehyde	AOO	15	1	22800	6.78				
			2	16696	4.97				
			3	17973	5.35				
			4	18757	5.58				
			Mean	**19056**	**5.67**				
Trimellitic anhydride	AOO	0.1	1	5681	1.69	**0.17**	**0.11**	**0.07**	**0.06**
			2	7841	2.33				
			3	11293	3.36				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
			Mean	**8272**	**2.46**				
		0.25	1	13902	4.14				
			2	11270	3.35				
			3	10963	3.26				
			Mean	**12045**	**3.58**				
Trimellitic anhydride (continued)		0.5	1	14361	4.27				
			2	18976	5.64				
			3	16673	4.96				
			Mean	**16670**	**4.96**				
Phthalic anhydride	AOO	0.1	1	11304	3.36	**0.08**	**0.06**	**0.04**	**0.03**
			2	13066	3.89				
			3	12448	3.70				
			Mean	**12272**	**3.65**				
		0.25	1	8332	2.48				
			2	15717	4.68				
			3	9833	2.93				
			Mean	**11294**	**3.36**				
		0.5	1	22051	6.56				
			2	12828	3.82				
			3	24315	7.23				
			Mean	**19731**	**5.87**				
		1	1	19987	5.95				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
			2	32118	9.55				
			3	17006	5.09				
			Mean	**23037**	**6.85**				
VC 1	DMSO	0	1	13832	1.36				
			2	9930	0.97				
			3	9958	0.98				
			4	7097	0.70				
			Mean	**10204**	**1.00**				
PC 1 - Hexyl cinnamic aldehyde	DMSO	15	1	17741	1.74				
			2	18810	1.84				
			3	18045	1.77				
			4	12293	1.21				
			Mean	**16722**	**1.64**				
Lactic acid	DMSO	5	1	6741	0.66	**NA**	**NA**	**NA**	**NA**
			2	12789	1.25				
			3	12217	1.12				
			Mean	**10582**	**1.04**				
		10	1	11054	1.08				
			2	11929	1.17				
			3	9542	0.94				
			Mean	**10841**	**1.06**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
		25	1	7025	0.69				
			2	13796	1.35				
			3	8677	0.85				
			Mean	**9832**	**0.96**				
Lactic acid (continued)		50	1	8623	0.85				
			2	10101	0.99				
			3	11594	1.14				
			Mean	**10106**	**0.99**				
VC 2	AOO	0	1	5263	1.07				
			2	4970	1.01				
			3	5431	1.11				
			4	3965	0.81				
			Mean	**4907**	**1.00**				
PC 2 - Hexyl cinnamic aldehyde	AOO	15	1	25796	5.26				
			2	24279	4.95				
			3	13979	2.85				
			4	23991	4.89				
			Mean	**22011**	**4.49**				
Resorcinol	AOO	5	1	12461	2.54	**6.44**	**5.09**	**4.20**	**3.90**
			2	11743	2.39				
			3	12095	2.47				
			Mean	**12099**	**2.47**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
		10	1	25798	5.26				
			2	16771	3.42				
			3	21121	4.30				
			Mean	**21230**	**4.33**				
		25	1	20760	4.23				
			2	21215	4.32				
			3	9659	1.97				
			Mean	**17211**	**3.51**				
VC	ACE	0	1	3937	1.45				
			2	2374	0.88				
			3	2360	0.87				
			4	2173	0.80				
			Mean	**2711**	**1.00**				
PC - Hexyl cinnamic aldhedye	ACE	15	1	21117	7.79				
			2	19843	7.32				
			3	12203	4.50				
			4	13734	5.07				
			Mean	**16724**	**6.17**				
Formaldehyde	ACE	0.1	1	5222	1.93	**1.16**	**0.81**	**0.44**	**0.29**
			2	3045	1.12				
			3	2923	1.08				
			Mean	**3730**	**1.38**				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
		0.25	1	6167	2.28				
			2	2933	1.08				
			3	5093	1.88				
			Mean	**4731**	**1.75**				
Formaldehyde (continued)		0.5	1	2317	0.86				
			2	4479	1.65				
			3	5263	1.94				
			Mean	**4019**	**1.48**				
		1	1	7846	2.90				
			2	10628	3.92				
			3	3894	1.44				
			Mean	**7456**	**2.75**				
		2.5	1	17242	6.36				
			2	14355	5.30				
			3	9904	3.65				
			Mean	**13833**	**5.10**				
VC	DMSO	0	1	82453	1.27				
			2	78192	1.21				
			3	42838	0.66				
			4	56114	0.87				
			Mean	**64899**	**1.00**				
PC	NT	NT	1	NT	NT				
			2	NT	NT				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
			3	NT	NT				
			4	NT	NT				
			Mean	**NT**	**NT**				
Potassium dichromate	DMSO	0.1	1	193231	2.98	**0.14**	**0.09**	**0.07**	**0.06**
			2	140171	2.16				
			3	186039	2.87				
			4	152378	2.35				
			Mean	**167954**	**2.59**				
		0.3	1	209189	3.22				
			2	274466	4.23				
			3	421230	6.49				
			Mean	253302	3.90				
		0.3	1	**289546**	**4.46**				
			2	286418	4.41				
			3	304081	4.69				
			Mean	440493	6.79				
		1	1	394755	6.08				
			2	**356437**	**5.49**				
VC	AOO	0	1	4172	1.44				
			2	3078	1.06				
			3	2136	0.74				
			4	2192	0.76				

Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance Name[2]	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3 (%)[4]	Calc. EC2.5 (%)[5]	Calc. EC2 (%)[5]	Calc. EC1.8 (%)[5]
			Mean	**2894**	**1.00**				
PC - Hexyl cinnamic aldehyde	AOO	15	1	10569	3.65				
			2	11027	3.81				
			3	12928	4.47				
			4	12520	4.33				
			Mean	**11761**	**4.06**				
p-Phenylenediamine	AOO	0.1	1	8259	2.85	**0.07**	**0.05**	**0.04**	**0.04**
			2	11194	3.87				
			3	11454	3.96				
			Mean	**10302**	**3.56**				
		0.25	1	12197	4.21				
			2	15785	5.45				
			3	16610	5.74				
			Mean	**14864**	**5.14**				
		0.5	1	16392	5.66				
			2	9781	3.38				
			3	10173	3.52				
			Mean	**12115**	**4.19**				
		1	1	10644	3.68				
			2	10669	3.69				
			3	5942	2.05				
			Mean	**9085**	**3.14**				

Abbreviations: ACE = acetone; Anim. = Animal; AOO = acetone: olive oil (4:1); ATP = adenosine triphosphate; Calc. = calculated; Conc. = concentration; DMF = *N*,*N*-dimethylformamide; DMSO = dimethyl sulfoxide; EC3 = estimated concentration needed to produce a stimulation index of three; EC2.5 = estimated concentration needed to produce a stimulation index of 2.5; EC2 = estimated concentration needed to produce a stimulation index of two; EC1.8 = estimated concentration needed to produce a stimulation index of 1.8; NA = not applicable; No. = number; NT = not tested; PC = positive control; SI = stimulation index; VC = vehicle control; Veh. = vehicle.

[1] Original laboratory records with individual animal data for the 31 substances tested in the LLNA: DA intralaboratory validation study (Idehara et al. 2008) provided by Kenji Idehara, Ph.D., Daicel Chemical Industries, Ltd.

[2] The 31 substances in the intralaboratory validation study were evaluated during one of 18 LLNA: DA tests that were conducted between July 2003 through September 2007 and are listed in order based on the date that they were tested.

[3] Two ATP measurements were taken for each animal and the mean ATP is indicated.

[4] EC3 value was calculated based on interpolation or extrapolation formulas discussed in Gerberick et al. 2004.

[5] EC value (i.e., EC1.8, EC2, or EC2.5) was calculated based on modified interpolation or extrapolation formulas for EC3 values discussed in Gerberick et al. 2004.

Table C-IV-2-1 Summary of the Results for 14 Additional Substances Tested in the LLNA: DA (Intralaboratory)[1]

Substance Name	Vehicle	Concentration (%)	SI[2]	Calculated EC3 (%)[3]	Calculated EC2.5 (%)[4]	Calculated EC2 (%)[4]	Calculated EC1.8 (%)[4]
5-Chloro-2-methyl-4-isothiazolin-3-one (CMI)	DMF	0.005	1.2	0.031	0.021	0.011	0.008
		0.010	1.9				
		0.025	2.7				
		0.050	4.0				
		0.100	7.5				
p-Benzoquinone	AOO	0.005	2.6	0.063	0.005	0.003	0.003
		0.010	2.6				
		0.025	2.5				
		0.050	2.7				
		0.100	3.8				
Propyl gallate	AOO	0.5	2.8	1.094	0.421	0.281	0.225
		1.0	2.9				
		2.5	4.9				
Phenyl benzoate	AOO	1.0	2.2	2.255	1.440	0.795	0.652
		2.5	3.2				
		5.0	4.2				
		10.0	3.7				
Diethyl maleate	AOO	0.5	1.9	3.705	2.084	1.181	0.889
		1.0	1.9				
		2.5	2.7				
		5.0	3.3				
		10.0	3.8				
Ethyl acrylate	AOO	10	2.5	13.943	9.793	7.537	6.788
		25	4.3				
		50	3.4				

Substance Name	Vehicle	Concentration (%)	SI[2]	Calculated EC3 (%)[3]	Calculated EC2.5 (%)[4]	Calculated EC2 (%)[4]	Calculated EC1.8 (%)[4]
Cinnamic alcohol	AOO	10	2.4	21.341	12.195	6.540	5.230
		25	3.2				
		50	5.7				
		90	4.4				
Ethylene glycol dimethacrylate	MEK	10	1.2	34.031	28.524	22.273	19.242
		25	2.2				
		50	4.4				
Butyl glycidyl ether	AOO	10	1.2	31.682	25.922	19.919	17.500
		25	2.4				
		50	4.6				
Nickel (II) chloride	DMSO	2.5	0.9	NA	NA	NA	NA
		5.0	1.1				
		10.0	1.3				
Salicylic acid	AOO	5	1.5	NA	NA	25.000	17.683
		10	1.6				
		25	2.0				
Sulfanilamide	DMF	10	0.8	NA	NA	NA	NA
		25	0.9				
		50	0.6				
Methyl methacrylate	AOO	25	1.0	NA	NA	NA	NA
		50	1.2				
		75	1.3				
		100	1.8				
Dimethyl isophthalate[5]	AOO	5	0.9	NA	NA	NA	NA
		10	0.9				
		25	0.8				

Abbreviations: AOO = acetone: olive oil (4:1); DMF = *N*,*N*-dimethylformamide; DMSO = dimethyl sulfoxide; EC3 = estimated concentration needed to produce a stimulation index of three; EC2.5 = estimated

concentration needed to produce a stimulation index of 2.5; EC2 = estimated concentration needed to produce a stimulation index of two; EC1.8 = estimated concentration needed to produce a stimulation index of 1.8; MEK = methyl ethyl ketone; NA = not applicable; SI = stimulation index.

[1] Original laboratory records with individual animal data for the 14 additional substances tested in the LLNA: DA intralaboratory validation study (Idehara unpublished) provided by Kenji Idehara, Ph.D., Daicel Chemical Industries, Ltd.

[2] SI determined from mean ATP content (relative luminescence units).

[3] EC3 value was calculated based on interpolation or extrapolation formulas discussed in Gerberick et al. 2004.

[4] EC value (i.e., EC2.5, EC2, or EC1.8) was calculated based on modified interpolation or extrapolation formulas for EC3 value discussed in Gerberick et al. 2004.

[5] This substance was also tested in the first phase of the interlaboratory validation study (Omori et al. 2008).

Annex IV-3

Individual Animal Data for the LLNA: DA (Interlaboratory)

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Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
1	Vehicle - Positive Control		0	1	27373	1.09				
				2	23473	0.93				
				3	30778	1.22				
				4	19231	0.76				
				Mean	**25214**	**1.00**				
1	Positive Control		NA	1	163662	6.49				
				2	118724	4.71				
				3	120098	4.76				
				4	172911	6.86				
				Mean	**143849**	**5.71**				
1	Vehicle – Substance	AOO	0	1	30365	1.24				
				2	26124	1.06				
				3	25218	1.03				
				4	16624	0.68				
				Mean	**24583**	**1.00**				
1	Hexyl cinnamic aldehyde	AOO	5	1	39462	1.61	**9.98**	**8.47**	**6.96**	**6.36**
				2	29952	1.22				
				3	37759	1.54				
				4	25613	1.04				
				Mean	**33196**	**1.35**				
			10	1	94155	3.83				
				2	60720	2.47				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				3	70595	2.87				
				4	70068	2.85				
				Mean	**73884**	**3.01**				
			25	1	174255	7.09				
				2	140034	5.70				
				3	103168	4.20				
				4	151064	6.15				
				Mean	**142130**	**5.78**				
1	Isopropanol	AOO	10	1	49049	2.00	**NA**	**NA**	**NA**	**NA**
				2	46692	1.90				
				3	22501	0.92				
				4	32783	1.33				
				Mean	**37756**	**1.54**				
			25	1	28917	1.18				
				2	28183	1.15				
				3	28099	1.14				
				4	23206	0.94				
				Mean	**27101**	**1.10**				
			50	1	32979	1.34				
				2	28219	1.15				
				3	28788	1.17				
				4	24907	1.01				
				Mean	**28723**	**1.17**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
1	Vehicle - Positive Control		0	1	27603	1.19				
				2	29165	1.26				
				3	13867	0.60				
				4	21857	0.95				
				Mean	**23123**	**1.00**				
1	Positive Control		NA	1	187061	8.09				
				2	192723	8.33				
				3	152209	6.58				
				4	120141	5.20				
				Mean	**163033**	**7.05**				
1	Vehicle - Substance	ACE	0	1	23522	1.31				
				2	17328	0.97				
				3	19286	1.07				
				4	11653	0.65				
				Mean	**17947**	**1.00**				
1	Glutaraldehyde	ACE	0.05	1	39029	2.17	**0.11**	**0.09**	**0.07**	**0.06**
				2	21473	1.20				
				3	17442	0.97				
				4	24434	1.36				
				Mean	**25594**	**1.43**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
			0.15	1	86407	4.81				
				2	69645	3.88				
				3	44897	2.50				
				4	90044	5.02				
				Mean	**72748**	**4.05**				
			0.50	1	117767	6.56				
				2	91139	5.08				
				3	85284	4.75				
				4	64878	3.62				
				Mean	**89767**	**5.00**				
1	Formaldehyde	ACE	0.5	1	54229	3.02	**1.75**	**0.39**	**0.26**	**0.21**
				2	65863	3.67				
				3	49268	2.75				
				4	39499	2.20				
				Mean	**52214**	**2.91**				
			1.5	1	65799	3.67				
				2	35118	1.96				
				3	48274	2.69				
				4	56430	3.14				
				Mean	**51405**	**2.86**				
			5.0	1	92516	5.16				
				2	131184	7.31				
				3	52728	2.94				
				4	71309	3.97				
				Mean	**86934**	**4.84**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
1	Vehicle - Positive Control		0	1	25568	1.13				
				2	30989	1.37				
				3	15244	0.68				
				4	18525	0.82				
				Mean	**22582**	**1.00**				
1	Positive Control		NA	1	160326	7.10				
				2	97979	4.34				
				3	126572	5.61				
				4	151977	6.73				
				Mean	**134213**	**5.94**				
1	Vehicle - Substance	AOO	0	1	36866	1.36				
				2	33905	1.25				
				3	15218	0.56				
				4	22764	0.84				
				Mean	**27188**	**1.00**				
1	2,4-Dinitrochloro-benzene	AOO	0.03	1	108431	3.99	**0.03**	**0.03**	**0.02**	**0.02**
				2	83821	3.08				
				3	68037	2.50				
				4	48931	1.80				
				Mean	**77305**	**2.84**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
1	2,4-Dinitrochloro-benzene (continued)		0.10	1	185139	6.81				
				2	159188	5.86				
				3	133437	4.91				
				4	110880	4.08				
				Mean	**147161**	**5.41**				
			0.30	1	334363	12.30				
				2	258002	9.49				
				3	366438	13.48				
				4	343140	12.62				
				Mean	**325485**	**11.97**				
1	Dimethyl isophthalate	AOO	5	1	41322	1.52	**NA**	**NA**	**NA**	**NA**
				2	32753	1.20				
				3	24319	0.89				
				4	47742	1.76				
				Mean	**36534**	**1.34**				
			10	1	46499	1.71				
				2	27887	1.03				
				3	29565	1.09				
				4	20851	0.77				
				Mean	**31200**	**1.15**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
			25	1	39741	1.46				
				2	21245	0.78				
				3	38401	1.41				
				4	20734	0.76				
				Mean	**30030**	**1.10**				
1	3-Aminophenol	AOO	1	1	48998	1.80	**NA**	**5.49**	**1.88**	**1.17**
				2	50122	1.84				
				3	47237	1.74				
				4	44007	1.62				
				Mean	**47591**	**1.75**				
			3	1	65491	2.41				
				2	55831	2.05				
				3	55478	2.04				
				4	75285	2.77				
				Mean	**63021**	**2.32**				
			10	1	93723	3.45				
				2	57142	2.10				
				3	82054	3.02				
				4	74792	2.75				
				Mean	**76927**	**2.83**				
2	Vehicle - Positive Control		0	1	29854	0.94				
				2	36425	1.15				
				3	42387	1.34				
				4	18060	0.57				
				Mean	**31681**	**1.00**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
2	Positive Control		NA	1	194745	6.15				
				2	196510	6.20				
				3	202311	6.39				
				4	171703	5.42				
				Mean	**191317**	**6.04**				
2	Vehicle - Substance	AOO	0	1	26727	0.65				
				2	62370	1.51				
				3	48632	1.18				
				4	27029	0.66				
				Mean	**41189**	**1.00**				
2	Hexyl cinnamic aldehyde	AOO	5	1	49355	1.20	**12.41**	**9.41**	**7.46**	**6.69**
				2	57775	1.40				
				3	62556	1.52				
				4	55479	1.35				
				Mean	**56291**	**1.37**				
			10	1	129128	3.13				
				2	98419	2.39				
				3	96062	2.33				
				4	113209	2.75				
				Mean	**109204**	**2.65**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
			25	1	259210	6.29				
				2	185538	4.50				
				3	176096	4.28				
				4	173235	4.21				
				Mean	**198520**	**4.82**				
2	Isopropanol	AOO	10	1	48933	1.19	**NA**	**NA**	**NA**	**NA**
				2	26716	0.65				
				3	38147	0.93				
				4	35351	0.86				
				Mean	**37286**	**0.91**				
			25	1	40741	0.99				
				2	33529	0.81				
				3	36625	0.89				
				4	29201	0.71				
				Mean	**35024**	**0.85**				
			50	1	31132	0.76				
				2	44432	1.08				
				3	30372	0.74				
				4	27101	0.66				
				Mean	**33259**	**0.81**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
2	Vehicle - Positive Control		0	1	16450	0.51				
				2	56211	1.74				
				3	29690	0.92				
				4	26911	0.83				
				Mean	**32315**	**1.00**				
2	Positive Control		NA	1	100365	3.11				
				2	144864	4.48				
				3	121515	3.76				
				4	131149	4.06				
				Mean	**124473**	**3.85**				
2	Vehicle - Substance	AOO	0	1	26982	1.03				
				2	26503	1.01				
				3	23078	0.88				
				4	28074	1.07				
				Mean	**26159**	**1.00**				
2	2,4-Dinitrochloro-benzene	AOO	0.03	1	46482	1.78	**0.11**	**0.06**	**0.02**	**0.02**
				2	45109	1.72				
				3	64419	2.46				
				4	87361	3.34				
				Mean	**60843**	**2.33**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
			0.10	1	54947	2.10				
				2	79087	3.02				
				3	103400	3.95				
				4	44369	1.70				
				Mean	**70451**	**2.69**				
2	2,4-Dinitrochloro-benzene		0.30	1	154655	5.91				
				2	244903	9.36				
				3	231793	8.86				
				4	334511	12.79				
				Mean	**241465**	**9.23**				
2	Abietic acid	AOO	5	1	53429	2.04	**8.20**	**6.41**	**4.76**	**3.64**
				2	44953	1.72				
				3	55417	2.12				
				4	66359	2.54				
				Mean	**55039**	**2.10**				
			10	1	76437	2.92				
				2	106616	4.08				
				3	106351	4.07				
				4	77421	2.96				
				Mean	**91706**	**3.51**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
			25	1	109226	4.18				
				2	165358	6.32				
				3	78960	3.02				
				4	131863	5.04				
				Mean	**121351**	**4.64**				
2	Vehicle - Positive Control		0	1	15977	0.59				
				2	29941	1.11				
				3	25288	0.94				
				4	36217	1.35				
				Mean	**26856**	**1.00**				
2	Positive Control		NA	1	105933	3.94				
				2	170707	6.36				
				3	134656	5.01				
				4	173488	6.46				
				Mean	**146196**	**5.44**				
2	Vehicle - Substance	ACE	0	1	56525	1.49				
				2	38645	1.02				
				3	28667	0.75				
				4	28339	0.74				
				Mean	**38044**	**1.00**				
2	Glutaraldehyde	ACE	0.05	1	34115	0.90	**0.44**	**0.35**	**0.27**	**0.24**

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				2	37388	0.98				
				3	17955	0.47				
				4	22926	0.60				
				Mean	**28096**	**0.74**				
			0.15	1	50405	1.32				
				2	36212	0.95				
				3	54707	1.44				
				4	54598	1.44				
				Mean	**48980**	**1.29**				
			0.50	1	172747	4.54				
				2	104608	2.75				
				3	105731	2.78				
				4	133355	3.51				
				Mean	**129110**	**3.39**				
2	Formaldehyde	ACE	0.5	1	71257	1.87	**1.48**	**1.11**	**0.73**	**0.58**
				2	61368	1.61				
				3	74954	1.97				
				4	50290	1.32				
				Mean	**64467**	**1.69**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
2	Formaldehyde (continued)		1.5	1	120557	3.17				
				2	110027	2.89				
				3	139716	3.67				
				4	90274	2.37				
				Mean	**115143**	**3.03**				
			5.0	1	148089	3.89				
				2	111959	2.94				
				3	97241	2.56				
				4	126577	3.33				
				Mean	**120966**	**3.18**				
3	Vehicle - Positive Control		0	1	14012	0.68				
				2	25742	1.25				
				3	18482	0.90				
				4	24206	1.17				
				Mean	**20610**	**1.00**				
3	Positive Control		NA	1	147051	7.13				
				2	129657	6.29				
				3	119376	5.79				
				4	132756	6.44				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				Mean	**132210**	**6.41**				
3	Vehicle - Substance	AOO	0	1	22801	0.95				
				2	28208	1.17				
				3	19180	0.80				
				4	26000	1.08				
				Mean	**24047**	**1.00**				
3	Methyl salicylate	AOO	5	1	22109	0.92	**NA**	**NA**	**NA**	**NA**
				2	22812	0.95				
				3	21410	0.89				
				4	36725	1.53				
				Mean	**25764**	**1.07**				
			10	1	35176	1.46				
				2	22115	0.92				
				3	21251	0.88				
				4	26904	1.12				
				Mean	**26361**	**1.10**				
			25	1	53142	2.21				
				2	31027	1.29				
				3	31120	1.29				
				4	34146	1.42				
				Mean	**37359**	**1.55**				
3	3-Aminophenol	AOO	1	1	40069	1.67	**NA**	**NA**	**NA**	**NA**
				2	31036	1.29				
				3	28933	1.20				
				4	35464	1.47				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				Mean	**33875**	**1.41**				
			3	1	51109	2.13				
				2	34706	1.44				
				3	53201	2.21				
				4	30394	1.26				
				Mean	**42352**	**1.76**				
			10	1	39746	1.65				
				2	38143	1.59				
				3	35330	1.47				
				4	53816	2.24				
				Mean	**41759**	**1.74**				
3	Vehicle - Positive Control		0	1	32037	1.14				
				2	27673	0.98				
				3	25512	0.91				
				4	27174	0.97				
				Mean	**28099**	**1.00**				
3	Positive Control		NA	1	133836	4.76				
				2	122152	4.35				
				3	164019	5.84				
				4	133810	4.76				
				Mean	**138454**	**4.93**				
3	Vehicle - Substance	AOO	0	1	52047	1.46				
				2	31377	0.88				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				3	36296	1.02				
				4	22887	0.64				
				Mean	**35652**	**1.00**				
3	Hexyl cinnamic aldehyde	AOO	5	1	38213	1.07	**14.90**	**11.39**	**8.40**	**7.35**
				2	35942	1.01				
				3	68561	1.92				
				4	50818	1.43				
				Mean	**48383**	**1.36**				
			10	1	69749	1.96				
				2	85956	2.41				
				3	97018	2.72				
				4	75438	2.12				
				Mean	**82040**	**2.30**				
			25	1	124915	3.50				
				2	168780	4.73				
				3	188378	5.28				
				4	151145	4.24				
				Mean	**158304**	**4.44**				
3	Isopropanol	AOO	10	1	32440	0.91	**NA**	**NA**	**NA**	**NA**
				2	45395	1.27				
				3	38482	1.08				
				4	28304	0.79				
				Mean	**36155**	**1.01**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
			25	1	30325	0.85				
				2	27645	0.78				
				3	23613	0.66				
				4	12277	0.34				
				Mean	**23465**	**0.66**				
			50	1	29038	0.81				
				2	28736	0.81				
				3	37489	1.05				
				4	28026	0.79				
				Mean	**30822**	**0.86**				
3	Vehicle - Positive Control		0	1	19428	0.70				
				2	34843	1.26				
				3	30475	1.11				
				4	25568	0.93				
				Mean	**27578**	**1.00**				
3	Positive Control		NA	1	152890	5.54				
				2	150397	5.45				
				3	179030	6.49				
				4	164124	5.95				
				Mean	**161610**	**5.86**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
3	Vehicle - Substance	AOO	0	1	27832	0.78				
				2	43858	1.23				
				3	39077	1.10				
				4	31673	0.89				
				Mean	**35610**	**1.00**				
3	2,4-Dinitrochloro-benzene	AOO	0.03	1	78157	2.19	**0.06**	**0.04**	**0.03**	**0.02**
				2	124013	3.48				
				3	79811	2.24				
				4	40213	1.13				
				Mean	**80548**	**2.26**				
			0.10	1	121518	3.41				
				2	178885	5.02				
				3	152199	4.27				
				4	149717	4.20				
				Mean	**150579**	**4.23**				
			0.30	1	333041	9.35				
				2	332166	9.33				
				3	364546	10.24				
				4	388959	10.92				
				Mean	**354678**	**9.96**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
3	Dimethyl isophthalate	AOO	5	1	31045	0.87	**NA**	**NA**	**NA**	**NA**
				2	35735	1.00				
				3	28933	0.81				
				4	47129	1.32				
				Mean	**35710**	**1.00**				
			10	1	42990	1.21				
				2	26663	0.75				
				3	27736	0.78				
				4	40039	1.12				
				Mean	**34357**	**0.96**				
			25	1	21801	0.61				
				2	20892	0.59				
				3	29220	0.82				
				4	23687	0.67				
				Mean	**23900**	**0.67**				
4	Vehicle - Positive Control		0	1	48083	1.06				
				2	39428	0.87				
				3	55411	1.22				
				4	38284	0.85				
				Mean	**45301**	**1.00**				
4	Positive Control		NA	1	211896	4.68				
				2	262733	5.80				
				3	242739	5.36				
				4	275773	6.09				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				Mean	**248285**	**5.48**				
4	Vehicle - Substance	DMSO	0	1	132462	1.32				
				2	79967	0.80				
				3	82192	0.82				
				4	106964	1.07				
				Mean	**100396**	**1.00**				
4	Cobalt chloride	DMSO	0.3	1	175468	1.75	**NA**	**0.82**	**0.28**	**0.23**
				2	192922	1.92				
				3	230415	2.30				
				4	216774	2.16				
				Mean	**203895**	**2.03**				
4	Cobalt chloride (continued)		1.0	1	272071	2.71				
				2	206730	2.06				
				3	333152	3.32				
				4	256734	2.56				
				Mean	**267172**	**2.66**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
			NA	1	NA	NA				
				2	NA	NA				
				3	NA	NA				
				4	NA	NA				
				Mean	**NA**	**NA**				
4	Nickel (II) sulfate hexahydrate	DMSO	1	1	136287	1.36	**NA**	**NA**	**NA**	**NA**
				2	84335	0.84				
				3	125617	1.25				
				4	118828	1.18				
				Mean	**116266**	**1.16**				
			3	1	152054	1.51				
				2	166405	1.66				
				3	188337	1.88				
				4	105499	1.05				
				Mean	**153074**	**1.52**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
			10	1	129555	1.29				
				2	89825	0.89				
				3	85180	0.85				
				4	109822	1.09				
				Mean	**103595**	**1.03**				
4	Vehicle - Positive Control		0	1	42028	0.90				
				2	49964	1.07				
				3	44351	0.95				
				4	50162	1.08				
				Mean	**46626**	**1.00**				
4	Positive Control		NA	1	266538	5.72				
				2	297022	6.37				
				3	208438	4.47				
				4	238300	5.11				
				Mean	**252574**	**5.42**				
4	Vehicle - Substance	AOO	0	1	38814	0.90				
				2	40081	0.93				
				3	36876	0.86				
				4	56256	1.31				
				Mean	**43007**	**1.00**				
4	Hexyl cinnamic aldehyde	AOO	5	1	66346	1.54	**9.34**	**7.90**	**6.46**	**5.8**
				2	63590	1.48				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				3	71486	1.66				
				4	55427	1.29				
				Mean	**64212**	**1.49**				
			10	1	92375	2.15				
				2	128592	2.99				
				3	121376	2.82				
				4	213148	4.96				
				Mean	**138873**	**3.23**				
			25	1	183245	4.26				
				2	237260	5.52				
				3	208440	4.85				
				4	249803	5.81				
				Mean	**219687**	**5.11**				
4	Isopropanol	AOO	10	1	62566	1.45	**NA**	**NA**	**NA**	**NA**
				2	86226	2.00				
				3	63529	1.48				
				4	56908	1.32				
				Mean	**67307**	**1.57**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
			25	1	29136	0.68				
				2	45518	1.06				
				3	42708	0.99				
				4	38074	0.89				
				Mean	**38859**	**0.90**				
			50	1	33511	0.78				
				2	41282	0.96				
				3	36712	0.85				
				4	26023	0.61				
				Mean	**34382**	**0.80**				
4	Vehicle - Positive Control		0	1	61301	1.49				
				2	42018	1.02				
				3	31933	0.78				
				4	29486	0.72				
				Mean	**41184**	**1.00**				
4	Positive Control		NA	1	188993	4.59				
				2	168896	4.10				
				3	258012	6.26				
				4	307187	7.46				
				Mean	**230772**	**5.60**				
4	Vehicle - Substance	AOO	0	1	55245	1.29				
				2	32859	0.77				
				3	37143	0.87				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				4	46219	1.08				
				Mean	**42866**	**1.00**				
4	Isoeugenol	AOO	1	1	117220	2.73	**1.11**	**0.66**	**0.41**	**0.34**
				2	159050	3.71				
				3	114887	2.68				
				4	112197	2.62				
				Mean	**125838**	**2.94**				
			3	1	167018	3.90				
				2	172577	4.03				
				3	190296	4.44				
				4	171216	3.99				
				Mean	**175277**	**4.09**				
			10	1	278270	6.49				
				2	266047	6.21				
				3	212878	4.97				
				4	291279	6.80				
				Mean	**262118**	**6.11**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
4	2,4-Dinitrochloro-benzene	AOO	0.03	1	99433	2.32	**0.03**	**0.02**	**0.02**	**0.01**
				2	124385	2.90				
				3	156964	3.66				
				4	131177	3.06				
				Mean	**127990**	**2.99**				
			0.10	1	239929	5.60				
				2	248752	5.80				
				3	226511	5.28				
				4	125633	2.93				
				Mean	**210206**	**4.90**				
4	2,4-Dinitrochloro-benzene (continued)		0.30	1	351048	8.19				
				2	304028	7.09				
				3	426667	9.95				
				4	381330	8.90				
				Mean	**365768**	**8.53**				
5	Vehicle - Positive Control		0	1	7783	0.65				
				2	7273	0.61				
				3	22835	1.92				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				4	9704	0.82				
				Mean	**11899**	**1.00**				
5	Positive Control		NA	1	60519	5.09				
				2	57983	4.87				
				3	48159	4.05				
				4	72951	6.13				
				Mean	**59903**	**5.03**				
5	Vehicle - Substance	AOO	0	1	31442	1.49				
				2	12103	0.57				
				3	20941	0.99				
				4	20115	0.95				
				Mean	**21150**	**1.00**				
5	2,4-Dinitrochloro-benzene	AOO	0.03	1	19491	0.92	**0.13**	**0.11**	**0.09**	**0.08**
				2	14102	0.67				
				3	17254	0.82				
				4	21584	1.02				
				Mean	**18107**	**0.86**				
			0.10	1	40351	1.91				
				2	76157	3.60				
				3	39813	1.88				
				4	26445	1.25				
				Mean	**45691**	**2.16**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
			0.30	1	199476	9.43				
				2	109134	5.16				
				3	155961	7.37				
				4	200326	9.47				
				Mean	**166224**	**7.86**				
5	Isoeugenol	AOO	1	1	20321	0.96	**5.98**	**5.19**	**4.40**	**4.08**
				2	19512	0.92				
				3	33957	1.61				
				4	17792	0.84				
				Mean	**22896**	**1.08**				
			3	1	12620	0.60				
				2	28001	1.32				
				3	20937	0.99				
				4	32921	1.56				
				Mean	**23619**	**1.12**				
			10	1	123238	5.83				
				2	110582	5.23				
				3	118049	5.58				
				4	116524	5.51				
				Mean	**117098**	**5.54**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
5	Vehicle - Positive Control		0	1	22681	1.23				
				2	15429	0.84				
				3	20405	1.11				
				4	15143	0.82				
				Mean	**18414**	**1.00**				
5	Positive Control		NA	1	97304	5.28				
				2	83132	4.51				
				3	67441	3.66				
				4	117794	6.40				
				Mean	**91418**	**4.96**				
5	Vehicle - Substance	AOO	0	1	16435	0.86				
				2	22909	1.20				
				3	25965	1.36				
				4	11275	0.59				
				Mean	**19146**	**1.00**				
5	Hexyl cinnamic aldehyde	AOO	5	1	17037	0.89	**18.13**	**14.59**	**11.06**	**9.60**
				2	30640	1.60				
				3	26481	1.38				
				4	19509	1.02				
				Mean	**23417**	**1.22**				
			10	1	32966	1.72				
				2	38027	1.99				
				3	17968	0.94				
				4	52769	2.76				
				Mean	**35432**	**1.85**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
			25	1	73109	3.82				
				2	83266	4.35				
				3	77637	4.05				
				4	70103	3.66				
				Mean	**76029**	**3.97**				
5	Isopropanol	AOO	10	1	9967	0.52	**NA**	**NA**	**NA**	**NA**
				2	5679	0.30				
				3	12157	0.63				
				4	12621	0.66				
				Mean	**10106**	**0.53**				
			25	1	15066	0.79				
				2	15418	0.81				
				3	12221	0.64				
				4	15418	0.81				
				Mean	**14531**	**0.76**				
			50	1	18749	0.98				
				2	13502	0.71				
				3	10223	0.53				
				4	11851	0.62				
				Mean	**13581**	**0.71**				
5	Vehicle - Positive Control		0	1	15918	1.04				
				2	13724	0.90				
				3	10819	0.71				
				4	20489	1.34				
				Mean	**15237**	**1.00**				
5	Positive Control		NA	1	67799	4.45				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				2	56834	3.73				
				3	60000	3.94				
				4	84607	5.55				
				Mean	**67310**	**4.42**				
5	Vehicle - Substance	ACE	0	1	8265	0.50				
				2	23012	1.40				
				3	14503	0.88				
				4	19975	1.22				
				Mean	**16439**	**1.00**				
5	Glutaraldehyde	ACE	0.05	1	23621	1.44	**NA**	**0.29**	**0.12**	**0.10**
				2	11837	0.72				
				3	14251	0.87				
				4	18389	1.12				
				Mean	**17024**	**1.04**				
			0.15	1	38622	2.35				
				2	64431	3.92				
				3	24666	1.50				
				4	33558	2.04				
				Mean	**40319**	**2.45**				
			0.50	1	34431	2.09				
				2	42955	2.61				
				3	42380	2.58				
				4	49184	2.99				
				Mean	**42237**	**2.57**				
5	Formaldehyde	ACE	0.5	1	24898	1.51	**NA**	**4.18**	**2.02**	**1.38**
				2	18454	1.12				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				3	21972	1.34				
				4	12719	0.77				
				Mean	**19510**	**1.19**				
			1.5	1	36696	2.23				
				2	29172	1.77				
				3	43949	2.67				
				4	14018	0.85				
				Mean	**30959**	**1.88**				
			5.0	1	44219	2.69				
				2	47739	2.90				
				3	33377	2.03				
				4	51542	3.14				
				Mean	**44219**	**2.69**				
6	Vehicle - Positive Control		0	1	16022	1.79				
				2	9436	1.05				
				3	3788	0.42				
				4	6561	0.73				
				Mean	**8952**	**1.00**				
6	Positive Control		NA	1	80444	8.99				
				2	92491	10.33				
				3	73767	8.24				
				4	101082	11.29				
				Mean	**86946**	**9.71**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
6	Vehicle - Substance	DMSO	0	1	7575	1.81				
				2	4135	0.99				
				3	2759	0.66				
				4	2267	0.54				
				Mean	**4184**	**1.00**				
6	Nickel (II) sulfate hexahydrate	DMSO	1	1	30363	7.26	**0.47**	**0.35**	**0.24**	**0.19**
				2	12902	3.08				
				3	22353	5.34				
				4	22343	5.34				
				Mean	**21990**	**5.26**				
			3	1	32830	7.85				
				2	28614	6.84				
				3	31319	7.49				
				4	19101	4.57				
				Mean	**27966**	**6.68**				
6	Nickel (II) sulfate hexahydrate (continued)		10	1	46902	11.21				
				2	64448	15.40				
				3	56156	13.42				
				4	29707	7.10				
				Mean	**49303**	**11.78**				
6	Cobalt chloride	DMSO	0.3	1	88782	21.22	**0.06**	**0.05**	**0.03**	**0.03**
				2	40452	9.67				
				3	22788	5.45				
				4	23988	5.73				
				Mean	**44002**	**10.52**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
			1.0	1	59079	14.12				
				2	24246	5.80				
				3	69511	16.61				
				4	25023	5.98				
				Mean	**44465**	**10.63**				
			3.0	1	108860	26.02				
				2	62637	14.97				
				3	106164	25.38				
				4	66252	15.84				
				Mean	**85978**	**20.55**				
6	Vehicle - Positive Control		0	1	7997	0.75				
				2	10763	1.01				
				3	13602	1.27				
				4	10360	0.97				
				Mean	**10680**	**1.00**				
6	Positive Control		NA	1	52468	4.91				
				2	66048	6.18				
				3	81979	7.68				
				4	76135	7.13				
				Mean	**69157**	**6.48**				
6	Vehicle - Substance	AOO	0	1	8621	0.62				
				2	14670	1.05				
				3	18086	1.30				
				4	14263	1.03				
				Mean	**13910**	**1.00**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
6	Abietic acid	AOO	5	1	38117	2.74	**7.54**	**6.47**	**5.39**	**4.88**
				2	18850	1.36				
				3	25525	1.83				
				4	18617	1.34				
				Mean	**25277**	**1.82**				
			10	1	57039	4.10				
				2	73842	5.31				
				3	56561	4.07				
				4	43018	3.09				
				Mean	**57615**	**4.14**				
			25	1	98752	7.10				
				2	129426	9.30				
				3	139343	10.02				
				4	75268	5.41				
				Mean	**110697**	**7.96**				
6	2,4-Dinitrochloro-benzene	AOO	0.03	1	29344	2.11	**0.04**	**0.03**	**0.02**	**0.01**
				2	53129	3.82				
				3	39348	2.83				
				4	31167	2.24				
				Mean	**38247**	**2.75**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
6	2,4-Dinitrochloro-benzene (continued)		0.10	1	32064	2.31				
				2	78273	5.63				
				3	66285	4.77				
				4	60587	4.36				
				Mean	**59302**	**4.26**				
			0.30	1	170451	12.25				
				2	258700	18.60				
				3	241703	17.38				
				4	171691	12.34				
				Mean	**210636**	**15.14**				
6	Vehicle - Positive Control		0	1	18240	1.56				
				2	4174	0.36				
				3	11817	1.01				
				4	12605	1.08				
				Mean	**11709**	**1.00**				
6	Positive Control		NA	1	105716	9.03				
				2	92508	7.90				
				3	86410	7.38				
				4	107936	9.22				
				Mean	**98142**	**8.38**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
6	Vehicle - Substance	AOO	0	1	13188	0.81				
				2	16677	1.02				
				3	13789	0.84				
				4	21847	1.33				
				Mean	**16375**	**1.00**				
6	Hexyl cinnamic aldehyde	AOO	5	1	34939	2.13	**13.13**	**10.76**	**7.46**	**5.96**
				2	34548	2.11				
				3	18582	1.13				
				4	21408	1.31				
				Mean	**27369**	**1.67**				
			10	1	50225	3.07				
				2	38763	2.37				
				3	26933	1.64				
				4	37387	2.28				
				Mean	**38327**	**2.34**				
			25	1	61340	3.75				
				2	71280	4.35				
				3	110980	6.78				
				4	116668	7.12				
				Mean	**90067**	**5.50**				
6	Isopropanol	AOO	10	1	71570	4.37	**NA**	**NA**	**NA**	**IDR**
				2	20763	1.27				
				3	19846	1.21				
				4	16753	1.02				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				Mean	**32233**	**1.97**				
			25	1	14610	0.89				
				2	19836	1.21				
				3	17188	1.05				
				4	7416	0.45				
				Mean	**14762**	**0.90**				
			50	1	16623	1.02				
				2	19168	1.17				
				3	28176	1.72				
				4	21474	1.31				
				Mean	**21360**	**1.30**				
7	Vehicle - Positive Control		0	1	10954	0.47				
				2	14547	0.62				
				3	33870	1.44				
				4	34460	1.47				
				Mean	**23458**	**1.00**				
7	Positive Control		NA	1	93512	3.99				
				2	104433	4.45				
				3	114003	4.86				
				4	180482	7.69				
				Mean	**123107**	**5.25**				
7	Vehicle - Substance	AOO	0	1	15339	0.71				
				2	11627	0.54				
				3	17793	0.83				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				4	41425	1.92				
				Mean	**21546**	**1.00**				
7	Methyl salicylate	AOO	5	1	26796	1.24	**NA**	**NA**	**NA**	**NA**
				2	23023	1.07				
				3	12934	0.60				
				4	31083	1.44				
				Mean	**23459**	**1.09**				
			10	1	30066	1.40				
				2	45494	2.11				
				3	41639	1.93				
				4	35433	1.64				
				Mean	**38158**	**1.77**				
			25	1	14218	0.66				
				2	31612	1.47				
				3	31551	1.46				
				4	42145	1.96				
				Mean	**29881**	**1.39**				
7	Abietic acid	AOO	5	1	28706	1.33	**7.68**	**11.53**	**6.33**	**4.60**
				2	46411	2.15				
				3	46541	2.16				
				4	39654	1.84				
				Mean	**40328**	**1.87**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
			10	1	50807	2.36				
				2	92597	4.30				
				3	105497	4.90				
				4	94381	4.38				
				Mean	**85821**	**3.98**				
			25	1	45895	2.13				
				2	102739	4.77				
				3	87409	4.06				
				4	91230	4.23				
				Mean	**81818**	**3.80**				
7	Vehicle - Positive Control		0	1	17271	0.75				
				2	23663	1.03				
				3	24070	1.04				
				4	27154	1.18				
				Mean	**23039**	**1.00**				
7	Positive Control		NA	1	127080	5.52				
				2	150247	6.52				
				3	122132	5.30				
				4	128311	5.57				
				Mean	**131942**	**5.73**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
7	Vehicle - Substance	AOO	0	1	36823	1.23				
				2	31245	1.04				
				3	21937	0.73				
				4	29694	0.99				
				Mean	**29925**	**1.00**				
7	Hexyl cinnamic aldehyde	AOO	5	1	42392	1.42	**7.71**	**6.78**	**5.85**	**5.48**
				2	33988	1.14				
				3	66350	2.22				
				4	41865	1.40				
				Mean	**46148**	**1.54**				
			10	1	106569	3.56				
				2	151880	5.08				
				3	161431	5.39				
				4	87141	2.91				
				Mean	**126755**	**4.24**				
			25	1	170985	5.71				
				2	193134	6.45				
				3	198620	6.64				
				4	286402	9.57				
				Mean	**212285**	**7.09**				
7	Isopropanol	AOO	10	1	30442	1.02	**NA**	**NA**	**NA**	**NA**

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				2	32600	1.09				
				3	41239	1.38				
				4	69502	2.32				
				Mean	**43446**	**1.45**				
			25	1	15392	0.51				
				2	39028	1.30				
				3	22387	0.75				
				4	32333	1.08				
				Mean	**27285**	**0.91**				
			50	1	26039	0.87				
				2	25885	0.87				
				3	27685	0.93				
				4	19497	0.65				
				Mean	**24776**	**0.83**				
7	Vehicle - Positive Control		0	1	20353	0.71				
				2	31709	1.10				
				3	34254	1.19				
				4	29038	1.01				
				Mean	**28838**	**1.00**				
7	Positive Control		NA	1	170163	5.90				
				2	142824	4.95				
				3	167113	5.79				
				4	135621	4.70				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				Mean	**153930**	**5.34**				
7	Vehicle - Substance	AOO	0	1	25299	1.13				
				2	25685	1.14				
				3	19870	0.88				
				4	19010	0.85				
				Mean	**22466**	**1.00**				
7	Dimethyl isophthalate	AOO	5	1	30872	1.37	**NA**	**NA**	**NA**	**NA**
				2	23829	1.06				
				3	26046	1.16				
				4	32477	1.45				
				Mean	**28306**	**1.26**				
7	Dimethyl isophthalate (continued)		10	1	28765	1.28				
				2	27567	1.23				
				3	22517	1.00				
				4	23373	1.04				
				Mean	**25555**	**1.14**				
			25	1	24457	1.09				
				2	25583	1.14				
				3	18065	0.80				
				4	26228	1.17				
				Mean	**23583**	**1.05**				
7	2,4-Dinitrochloro-benzene	AOO	0.03	1	54379	2.42	**0.02**	**0.01**	**0.01**	**0.01**
				2	95575	4.25				
				3	95094	4.23				
				4	99284	4.42				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				Mean	**86083**	**3.83**				
			0.10	1	142045	6.32				
				2	139187	6.20				
				3	108882	4.85				
				4	93969	4.18				
				Mean	**121021**	**5.39**				
			0.30	1	282805	12.59				
				2	336813	14.99				
				3	258764	11.52				
				4	305713	13.61				
				Mean	**296024**	**13.18**				
8	Vehicle - Positive Control		0	1	18303	0.95				
				2	25980	1.34				
				3	17493	0.90				
				4	15606	0.81				
				Mean	**19345**	**1.00**				
8	Positive Control		NA	1	98761	5.11				
				2	72937	3.77				
				3	86236	4.46				
				4	76278	3.94				
				Mean	**83553**	**4.32**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
8	Vehicle - Substance	AOO	0	1	9463	0.78				
				2	13874	1.14				
				3	17229	1.41				
				4	8262	0.68				
				Mean	**12207**	**1.00**				
8	Isopropanol	AOO	10	1	12562	1.03	**NA**	**NA**	**NA**	**NA**
				2	17330	1.42				
				3	11886	0.97				
				4	17410	1.43				
				Mean	**14797**	**1.21**				
			25	1	17249	1.41				
				2	9264	0.76				
				3	11845	0.97				
				4	11193	0.92				
				Mean	**12387**	**1.01**				
			50	1	14510	1.19				
				2	14113	1.16				
				3	12238	1.00				
				4	13342	1.09				
				Mean	**13551**	**1.11**				
8	Hexyl cinnamic aldehyde	AOO	5	1	16997	1.39	**7.92**	**7.03**	**6.14**	**5.78**
				2	15777	1.29				
				3	22473	1.84				
				4	11217	0.92				
				Mean	**16616**	**1.36**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
			10	1	40975	3.36				
				2	56754	4.65				
				3	58346	4.78				
				4	47242	3.87				
				Mean	**50829**	**4.16**				
			25	1	155208	12.71				
				2	133055	10.90				
				3	75582	6.19				
				4	135369	11.09				
				Mean	**124803**	**10.22**				
8	Vehicle - Positive Control		0	1	11818	0.62				
				2	22893	1.19				
				3	21441	1.12				
				4	20608	1.07				
				Mean	**19190**	**1.00**				
8	Positive Control		NA	1	117067	6.10				
				2	100222	5.22				
				3	91462	4.77				
				4	80907	4.22				
				Mean	**97414**	**5.08**				
8	Vehicle - Substance	DMSO	0	1	15322	0.77				
				2	24630	1.24				
				3	16802	0.85				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				4	22460	1.13				
				Mean	**19803**	**1.00**				
8	Nickel (II) sulfate hexahydrate	DMSO	1	1	64139	3.24	**IDR**	**IDR**	**IDR**	**IDR**
				2	59705	3.01				
				3	61654	3.11				
				4	90810	4.59				
				Mean	**69077**	**3.49**				
			3	1	64301	3.25				
				2	70343	3.55				
				3	55459	2.80				
				4	53420	2.70				
				Mean	**60881**	**3.07**				
			10	1	40447	2.04				
				2	45033	2.27				
				3	62589	3.16				
				4	54206	2.74				
				Mean	**50568**	**2.55**				
8	Cobalt chloride	DMSO	0.3	1	68800	3.47	**0.14**	**0.10**	**0.08**	**0.07**
				2	98124	4.95				
				3	95925	4.84				
				4	87399	4.41				
				Mean	**87562**	**4.42**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
			1.0	1	123857	6.25				
				2	178916	9.03				
				3	96477	4.87				
				4	124765	6.30				
				Mean	**131004**	**6.62**				
8	Cobalt chloride (continued)		3.0	1	175242	8.85				
				2	143477	7.25				
				3	155827	7.87				
				4	164687	8.32				
				Mean	**159808**	**8.07**				
8	Vehicle - Positive Control		0	1	17139	1.02				
				2	23311	1.39				
				3	14001	0.84				
				4	12548	0.75				
				Mean	**16749**	**1.00**				
8	Positive Control		NA	1	133873	7.99				
				2	147108	8.78				
				3	114171	6.82				
				4	97568	5.83				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				Mean	**123180**	**7.35**				
8	Vehicle - Substance	AOO	0	1	18744	0.91				
				2	20074	0.98				
				3	15187	0.74				
				4	28298	1.38				
				Mean	**20576**	**1.00**				
8	2,4-Dinitrochloro-benzene	AOO	0.03	1	40777	1.98	**0.10**	**0.04**	**0.02**	**0.02**
				2	45024	2.19				
				3	30526	1.48				
				4	82593	4.01				
				Mean	**49730**	**2.42**				
			0.10	1	41930	2.04				
				2	50135	2.44				
				3	107465	5.22				
				4	50754	2.47				
				Mean	**62571**	**3.04**				
			0.30	1	228871	11.12				
				2	393845	19.14				
				3	273309	13.28				
				4	140789	6.84				
				Mean	**259203**	**12.60**				
8	3-Aminophenol	AOO	1	1	25653	1.25	**NA**	**NA**	**3.18**	**2.51**

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				2	27127	1.32				
				3	28861	1.40				
				4	19026	0.92				
				Mean	**25167**	**1.22**				
			3	1	51618	2.51				
				2	47941	2.33				
				3	36281	1.76				
				4	27846	1.35				
				Mean	**40921**	**1.99**				
			10	1	57296	2.78				
				2	52938	2.57				
				3	38134	1.85				
				4	47782	2.32				
				Mean	**49037**	**2.38**				
9	Vehicle - Positive Control		0	1	25729	0.98				
				2	31786	1.22				
				3	24343	0.93				
				4	22785	0.87				
				Mean	**26161**	**1.00**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
9	Positive Control		NA	1	155962	5.96				
				2	112682	4.31				
				3	124334	4.75				
				4	122066	4.67				
				Mean	**128761**	**4.92**				
9	Vehicle - Substance	AOO	0	1	21600	0.73				
				2	38136	1.29				
				3	34690	1.17				
				4	23981	0.81				
				Mean	**29602**	**1.00**				
9	Hexyl cinnamic aldehyde	AOO	5	1	35263	1.19	**17.07**	**12.55**	**9.19**	**8.46**
				2	34558	1.17				
				3	20309	0.69				
				4	12277	0.41				
				Mean	**25602**	**0.86**				
			10	1	32104	1.08				
				2	68901	2.33				
				3	61583	2.08				
				4	99972	3.38				
				Mean	**65640**	**2.22**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
			25	1	109826	3.71				
				2	114755	3.88				
				3	101116	3.42				
				4	133469	4.51				
				Mean	**114791**	**3.88**				
9	Isopropanol	AOO	10	1	16071	0.54	**NA**	**NA**	**NA**	**NA**
				2	29909	1.01				
				3	16721	0.56				
				4	12462	0.42				
				Mean	**18791**	**0.63**				
			25	1	18605	0.63				
				2	12916	0.44				
				3	26806	0.91				
				4	24183	0.82				
				Mean	**20627**	**0.70**				
			50	1	11350	0.38				
				2	14836	0.50				
				3	13840	0.47				
				4	20129	0.68				
				Mean	**15039**	**0.51**				
9	Vehicle - Positive Control		0	1	21626	0.82				
				2	28191	1.06				
				3	36208	1.37				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				4	19953	0.75				
				Mean	**26494**	**1.00**				
9	Positive Control		NA	1	152153	5.74				
				2	173639	6.55				
				3	117177	4.42				
				4	165097	6.23				
				Mean	**152016**	**5.74**				
9	Vehicle - Substance	AOO	0	1	37188	1.39				
				2	20177	0.75				
				3	17473	0.65				
				4	32530	1.21				
				Mean	**26842**	**1.00**				
9	Isoeugenol	AOO	1	1	43063	1.60	**2.30**	**0.87**	**0.38**	**0.27**
				2	92318	3.44				
				3	73315	2.73				
				4	68329	2.55				
				Mean	**69256**	**2.58**				
			3	1	82412	3.07				
				2	114677	4.27				
				3	83819	3.12				
				4	65486	2.44				
				Mean	**86598**	**3.23**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
			10	1	241256	8.99				
				2	169293	6.31				
				3	153506	5.72				
				4	197513	7.36				
				Mean	**190392**	**7.09**				
9	2,4-Dinitrochloro-benzene	AOO	0.03	1	80731	3.01	**0.04**	**0.02**	**0.02**	**0.01**
				2	46072	1.72				
				3	82472	3.07				
				4	91886	3.42				
				Mean	**75290**	**2.80**				
			0.10	1	81426	3.03				
				2	105837	3.94				
				3	164718	6.14				
				4	97148	3.62				
				Mean	**112282**	**4.18**				
			0.30	1	294486	10.97				
				2	287848	10.72				
				3	287739	10.72				
				4	298846	11.13				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				Mean	**292230**	**10.89**				
10	Vehicle - Positive Control		0	1	20162	0.95				
				2	15285	0.72				
				3	30517	1.43				
				4	19166	0.90				
				Mean	**21282**	**1.00**				
10	Positive Control		NA	1	116157	5.46				
				2	142905	6.71				
				3	135316	6.36				
				4	117862	5.54				
				Mean	**128060**	**6.02**				
10	Vehicle - Substance	AOO	0	1	45394	0.85				
				2	67917	1.27				
				3	36479	0.68				
				4	63610	1.19				
				Mean	**53350**	**1.00**				
10	2,4-Dinitrochloro-benzene	AOO	0.03	1	52123	0.98	**0.17**	**0.13**	**0.09**	**0.08**
				2	66363	1.24				
				3	36583	0.69				
				4	92933	1.74				
				Mean	**62000**	**1.16**				
			0.10	1	113324	2.12				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				2	80089	1.50				
				3	127648	2.39				
				4	127592	2.39				
				Mean	**111163**	**2.10**				
10	2,4-Dinitrochloro-benzene (continued)		0.30	1	202245	3.79				
				2	264292	4.95				
				3	298490	5.59				
				4	239662	4.49				
				Mean	**251172**	**4.71**				
10	Methyl salicylate	AOO	5	1	36446	0.68	**NA**	**NA**	**NA**	**NA**
				2	34905	0.65				
				3	37286	0.70				
				4	26017	0.49				
				Mean	**33663**	**0.63**				
			10	1	47420	0.89				
				2	47616	0.89				
				3	40117	0.75				
				4	31641	0.59				
				Mean	**41698**	**0.78**				
			25	1	53941	1.01				
				2	54989	1.03				
				3	43082	0.81				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				4	25692	0.48				
				Mean	**44426**	**0.83**				
10	Vehicle - Positive Control		0	1	20445	0.88				
				2	15079	0.65				
				3	26464	1.13				
				4	31358	1.34				
				Mean	**23336**	**1.00**				
10	Positive Control		NA	1	89914	3.85				
				2	107768	4.62				
				3	93418	4.00				
				4	102331	4.39				
				Mean	**98357**	**4.21**				
10	Vehicle - Substance	AOO	0	1	28181	0.97				
				2	33325	1.15				
				3	27821	0.96				
				4	26981	0.93				
				Mean	**29077**	**1.00**				
10	Hexyl cinnamic aldehyde	AOO	5	1	35684	1.23	**15.24**	**9.14**	**7.26**	**6.51**
				2	30080	1.03				
				3	62393	2.15				
				4	34584	1.19				
				Mean	**40685**	**1.40**				
			10	1	86735	2.98				
				2	88833	3.06				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				3	75607	2.60				
				4	66109	2.27				
				Mean	**79321**	**2.73**				
			25	1	78538	2.70				
				2	107305	3.69				
				3	129081	4.44				
				4	93013	3.20				
				Mean	**101984**	**3.51**				
10	Isopropanol	AOO	10	1	19691	0.68	**NA**	**NA**	**NA**	**NA**
				2	28293	0.97				
				3	29845	1.03				
				4	28091	0.97				
				Mean	**26480**	**0.91**				
10	Isopropanol (continued)		25	1	30241	1.04				
				2	24774	0.85				
				3	29230	1.01				
				4	38461	1.32				
				Mean	**30676**	**1.06**				
			50	1	42188	1.45				
				2	37228	1.28				
				3	35247	1.21				
				4	30201	1.04				
				Mean	**36216**	**1.25**				
11	Vehicle - Positive		0	1	13452	0.45				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
	Control			2	32469	1.09				
				3	37235	1.25				
				4	35940	1.21				
				Mean	**29774**	**1.00**				
11	Positive Control		NA	1	113708	3.82				
				2	108755	3.65				
				3	57560	1.93				
				4	97736	3.28				
				Mean	**94440**	**3.17**				
11	Vehicle - Substance	AOO	0	1	16175	0.76				
				2	31955	1.50				
				3	24257	1.14				
				4	12926	0.61				
				Mean	**21328**	**1.00**				
11	Hexyl cinnamic aldehyde	AOO	5	1	24541	1.15	**9.13**	**7.74**	**6.35**	**5.79**
				2	31920	1.50				
				3	42454	1.99				
				4	30308	1.42				
				Mean	**32306**	**1.51**				
			10	1	73959	3.47				
				2	73920	3.47				
				3	74762	3.51				
				4	60117	2.82				
				Mean	**70689**	**3.31**				
			25	1	56324	2.64				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				2	81323	3.81				
				3	117271	5.50				
				4	126476	5.93				
				Mean	**95348**	**4.47**				
11	Vehicle - Positive Control		0	1	6855	0.32				
				2	23315	1.10				
				3	27767	1.30				
				4	27187	1.28				
				Mean	**21281**	**1.00**				
11	Positive Control		NA	1	118741	5.58				
				2	114600	5.39				
				3	86525	4.07				
				4	115969	5.45				
				Mean	**108959**	**5.12**				
11	Vehicle - Substance	DMSO	0	1	67859	1.04				
				2	76567	1.18				
				3	60349	0.93				
				4	55465	0.85				
				Mean	**65060**	**1.00**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
11	Potassium dichromate	DMSO	0.1	1	134992	2.07	**0.51**	**0.37**	**0.16**	**0.09**
				2	133187	2.05				
				3	130433	2.00				
				4	97134	1.49				
				Mean	**123936**	**1.90**				
			0.3	1	194686	2.99				
				2	104933	1.61				
				3	166086	2.55				
				4	117627	1.81				
				Mean	**145833**	**2.24**				
			1.0	1	283541	4.36				
				2	340279	5.23				
				3	318543	4.90				
				4	301673	4.64				
				Mean	**311009**	**4.78**				
11	Lactic acid	DMSO	5	1	34889	0.54	**NA**	**NA**	**NA**	**NA**
				2	70275	1.08				
				3	81876	1.26				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				4	55263	0.85				
				Mean	**60576**	**0.93**				
			10	1	57810	0.89				
				2	60103	0.92				
				3	42148	0.65				
				4	36073	0.55				
				Mean	**49033**	**0.75**				
			25	1	73850	1.14				
				2	38479	0.59				
				3	54647	0.84				
				4	41547	0.64				
				Mean	**52131**	**0.80**				
11	Vehicle - Positive Control		0	1	25338	0.96				
				2	29261	1.11				
				3	21131	0.80				
				4	29732	1.13				
				Mean	**26365**	**1.00**				
11	Positive Control		NA	1	136936	5.19				
				2	81100	3.08				
				3	114598	4.35				
				4	79191	3.00				
				Mean	**102956**	**3.90**				
11	Vehicle - Substance	DMSO	0	1	86043	1.05				
				2	65589	0.80				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				3	117592	1.43				
				4	59151	0.72				
				Mean	**82093**	**1.00**				
11	Cobalt chloride	DMSO	1	1	113621	1.38	**NA**	**NA**	**4.93**	**3.5**
				2	130468	1.59				
				3	97082	1.18				
				4	147603	1.80				
				Mean	**122193**	**1.49**				
			3	1	123437	1.50				
				2	115859	1.41				
				3	189281	2.31				
				4	139101	1.69				
				Mean	**141919**	**1.73**				
11	Cobalt chloride (continued)		5	1	167985	2.05				
				2	167593	2.04				
				3	174922	2.13				
				4	150902	1.84				
				Mean	**165350**	**2.01**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
11	Nickel (II) sulfate hexahydrate	DMSO	1	1	65339	0.80	**NA**	**NA**	**NA**	**NA**
				2	51981	0.63				
				3	46829	0.57				
				4	50461	0.61				
				Mean	**53652**	**0.65**				
			3	1	89247	1.09				
				2	49391	0.60				
				3	83879	1.02				
				4	37620	0.46				
				Mean	**65034**	**0.79**				
			10	1	80662	0.98				
				2	49864	0.61				
				3	41820	0.51				
				4	69460	0.85				
				Mean	**60451**	**0.74**				
12	Vehicle - Positive Control		0	1	31062	1.15				
				2	34769	1.28				
				3	19233	0.71				
				4	23272	0.86				
				Mean	**27084**	**1.00**				
12	Positive Control		NA	1	32499	1.20				
				2	149284	5.51				
				3	138062	5.10				
				4	155617	5.75				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				Mean	**118865**	**4.39**				
12	Vehicle - Substance	AOO	0	1	34707	1.27				
				2	19823	0.72				
				3	21963	0.80				
				4	33252	1.21				
				Mean	**27436**	**1.00**				
12	Hexyl cinnamic aldehyde	AOO	5	1	45866	1.67	**8.76**	**7.37**	**5.98**	**5.43**
				2	32444	1.18				
				3	52964	1.93				
				4	49440	1.80				
				Mean	**45178**	**1.65**				
			10	1	96208	3.51				
				2	70432	2.57				
				3	121167	4.42				
				4	90169	3.29				
				Mean	**94494**	**3.44**				
			25	1	146684	5.35				
				2	176112	6.42				
				3	135063	4.92				
				4	168604	6.15				
				Mean	**156615**	**5.71**				
12	Vehicle - Positive		0	1	26207	0.79				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
	Control			2	39177	1.18				
				3	37398	1.13				
				4	30062	0.91				
				Mean	**33211**	**1.00**				
12	Positive Control		NA	1	151987	4.58				
				2	169589	5.11				
				3	209928	6.32				
				4	134469	4.05				
				Mean	**166493**	**5.01**				
12	Vehicle - Substance	DMSO	0	1	78629	0.95				
				2	88765	1.07				
				3	76637	0.92				
				4	88155	1.06				
				Mean	**83046**	**1.00**				
12	Nickel (II) sulfate hexahydrate	DMSO	1	1	98797	1.19	**NA**	**NA**	**NA**	**NA**
				2	80665	0.97				
				3	86949	1.05				
				4	65175	0.78				
				Mean	**82896**	**1.00**				
			3	1	84327	1.02				
				2	86877	1.05				
				3	137747	1.66				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				4	104430	1.26				
				Mean	**103345**	**1.24**				
			10	1	105221	1.27				
				2	71971	0.87				
				3	55567	0.67				
				4	89624	1.08				
				Mean	**80596**	**0.97**				
12	Potassium dichromate	DMSO	0.1	1	170554	2.05	**0.49**	**0.27**	**0.13**	**0.09**
				2	113710	1.37				
				3	166200	2.00				
				4	179394	2.16				
				Mean	**157464**	**1.90**				
			0.3	1	198199	2.39				
				2	205018	2.47				
				3	273194	3.29				
				4	191835	2.31				
				Mean	**217061**	**2.61**				
			1.0	1	301077	3.63				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				2	323900	3.90				
				3	378405	4.56				
				4	351057	4.23				
				Mean	**338610**	**4.08**				
13	Vehicle - Positive Control		0	1	21808	0.80				
				2	23919	0.87				
				3	24606	0.90				
				4	39312	1.43				
				Mean	**27411**	**1.00**				
13	Positive Control		NA	1	138513	5.05				
				2	94225	3.44				
				3	118316	4.32				
				4	161413	5.89				
				Mean	**128117**	**4.67**				
13	Vehicle - Substance	AOO	0	1	33895	1.37				
				2	20013	0.81				
				3	20945	0.85				
				4	24103	0.97				
				Mean	**24739**	**1.00**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
13	Hexyl cinnamic aldehyde	AOO	5	1	28705	1.16	**7.59**	**6.77**	**5.95**	**5.63**
				2	19630	0.79				
				3	45958	1.86				
				4	45943	1.86				
				Mean	**35059**	**1.42**				
			10	1	106862	4.32				
				2	92835	3.75				
				3	83026	3.36				
				4	159832	6.46				
				Mean	**110638**	**4.47**				
			25	1	164960	6.67				
				2	116945	4.73				
				3	118296	4.78				
				4	135132	5.46				
				Mean	**133833**	**5.41**				
13	Vehicle - Positive Control		0	1	16810	0.75				
				2	25921	1.15				
				3	21544	0.96				
				4	25627	1.14				
				Mean	**22475**	**1.00**				
13	Positive Control		NA	1	156378	6.96				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				2	133906	5.96				
				3	140685	6.26				
				4	152161	6.77				
				Mean	**145782**	**6.49**				
13	Vehicle - Substance	DMSO	0	1	93878	1.15				
				2	70631	0.87				
				3	91822	1.13				
				4	68974	0.85				
				Mean	**81326**	**1.00**				
13	Cobalt chloride	DMSO	1	1	120105	1.48	**NA**	**4.13**	**1.88**	**1.38**
				2	148835	1.83				
				3	93820	1.15				
				4	172802	2.12				
				Mean	**133890**	**1.65**				
			3	1	199869	2.46				
				2	195046	2.40				
				3	207281	2.55				
				4	195145	2.40				
				Mean	**199335**	**2.45**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
			5	1	192357	2.37				
				2	215391	2.65				
				3	224902	2.77				
				4	192928	2.37				
				Mean	**206394**	**2.54**				
13	Lactic acid	DMSO	5	1	71011	0.87	**NA**	**NA**	**NA**	**NA**
				2	58742	0.72				
				3	95883	1.18				
				4	96922	1.19				
				Mean	**80639**	**0.99**				
			10	1	58052	0.71				
				2	44480	0.55				
				3	56725	0.70				
				4	62219	0.77				
				Mean	**55369**	**0.68**				
13	Lactic acid (continued)		25	1	61451	0.76				
				2	47962	0.59				
				3	79235	0.97				
				4	51848	0.64				
				Mean	**60124**	**0.74**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
14	Vehicle - Positive Control		0	1	25953	0.86				
				2	42071	1.39				
				3	22870	0.76				
				4	30199	1.00				
				Mean	**30273**	**1.00**				
14	Positive Control		NA	1	198381	6.55				
				2	164826	5.44				
				3	205542	6.79				
				4	198361	6.55				
				Mean	**191777**	**6.33**				
14	Vehicle - Substance	AOO	0	1	21623	0.89				
				2	27737	1.14				
				3	33618	1.38				
				4	14415	0.59				
				Mean	**24348**	**1.00**				
14	Hexyl cinnamic aldehyde	AOO	5	1	45466	1.87	**7.94**	**6.36**	**4.85**	**4.44**
				2	40112	1.65				
				3	72779	2.99				
				4	43275	1.78				
				Mean	**50408**	**2.07**				
			10	1	100580	4.13				
				2	134453	5.52				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				3	18994	0.78				
				4	101713	4.18				
				Mean	**88935**	**3.65**				
			25	1	164791	6.77				
				2	155059	6.37				
				3	249145	10.23				
				4	171572	7.05				
				Mean	**185142**	**7.60**				
14	Vehicle - Positive Control		0	1	18024	0.74				
				2	24615	1.02				
				3	28493	1.18				
				4	25735	1.06				
				Mean	**24216**	**1.00**				
14	Positive Control		NA	1	116341	4.80				
				2	213773	8.83				
				3	182037	7.52				
				4	192821	7.96				
				Mean	**176243**	**7.28**				
14	Vehicle - Substance	DMSO	0	1	33858	0.81				
				2	31373	0.75				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				3	60046	1.44				
				4	41804	1.00				
				Mean	**41770**	**1.00**				
14	Cobalt chloride	DMSO	1	1	104955	2.51	**1.76**	**1.20**	**0.82**	**0.72**
				2	83477	2.00				
				3	85107	2.04				
				4	114867	2.75				
				Mean	**97101**	**2.32**				
14	Cobalt chloride (continued)		3	1	193202	4.63				
				2	147696	3.54				
				3	165128	3.95				
				4	179062	4.29				
				Mean	**171272**	**4.10**				
			5	1	239096	5.72				
				2	128719	3.08				
				3	160037	3.83				
				4	182970	4.38				
				Mean	**177705**	**4.25**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
14	Nickel (II) sulfate hexahydrate	DMSO	1	1	104492	2.50	**NA**	**NA**	**8.40**	**5.94**
				2	58854	1.41				
				3	94853	2.27				
				4	53019	1.27				
				Mean	**77804**	**1.86**				
			3	1	72152	1.73				
				2	48034	1.15				
				3	68084	1.63				
				4	72530	1.74				
				Mean	**65200**	**1.56**				
			10	1	71690	1.72				
				2	NA	NA				
				3	97605	2.34				
				4	97675	2.34				
				Mean	**88990**	**2.13**				
15	Vehicle - Positive Control		0	1	39487	1.12				
				2	45663	1.30				
				3	28492	0.81				
				4	26819	0.76				
				Mean	**35115**	**1.00**				
15	Positive Control		NA	1	157090	4.47				
				2	164583	4.69				
				3	77120	2.20				
				4	157960	4.50				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				Mean	**139188**	**3.96**				
15	Vehicle - Substance	AOO	0	1	26758	0.86				
				2	46603	1.49				
				3	23061	0.74				
				4	28334	0.91				
				Mean	**31189**	**1.00**				
15	Hexyl cinnamic aldehyde	AOO	5	1	38890	1.25	**15.18**	**9.92**	**7.45**	**6.47**
				2	55784	1.79				
				3	43619	1.40				
				4	49120	1.57				
				Mean	**46853**	**1.50**				
			10	1	71984	2.31				
				2	66130	2.12				
				3	84295	2.70				
				4	91478	2.93				
				Mean	**78471**	**2.52**				
			25	1	124344	3.99				
				2	85306	2.74				
				3	142287	4.56				
				4	136649	4.38				
				Mean	**122146**	**3.92**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
15	Vehicle - Positive Control		0	1	43807	1.36				
				2	26515	0.82				
				3	29210	0.90				
				4	29709	0.92				
				Mean	**32310**	**1.00**				
15	Positive Control		NA	1	118146	3.66				
				2	172004	5.32				
				3	135989	4.21				
				4	163682	5.07				
				Mean	**147455**	**4.56**				
15	Vehicle - Substance	DMSO	0	1	35762	0.72				
				2	32858	0.67				
				3	49385	1.00				
				4	79406	1.61				
				Mean	**49353**	**1.00**				
15	Lactic acid	DMSO	5	1	35838	0.73	**NA**	**NA**	**NA**	**NA**
				2	46572	0.94				
				3	43793	0.89				
				4	56717	1.15				
				Mean	**45730**	**0.93**				
			10	1	40908	0.83				
				2	44335	0.90				
				3	70146	1.42				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				4	36323	0.74				
				Mean	**47928**	**0.97**				
			25	1	31906	0.65				
				2	37990	0.77				
				3	33696	0.68				
				4	37444	0.76				
				Mean	**35259**	**0.71**				
15	Potassium dichromate	DMSO	0.1	1	121714	2.47	**0.16**	**0.09**	**0.06**	**0.05**
				2	177882	3.60				
				3	132281	2.68				
				4	93102	1.89				
				Mean	**131244**	**2.66**				
			0.3	1	215997	4.38				
				2	210129	4.26				
				3	226134	4.58				
				4	115017	2.33				
				Mean	**191819**	**3.89**				
			1.0	1	360162	7.30				
				2	191584	3.88				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				3	340917	6.91				
				4	293061	5.94				
				Mean	**296431**	**6.01**				
16	Vehicle - Positive Control		0	1	40980	1.14				
				2	29750	0.83				
				3	37809	1.05				
				4	35687	0.99				
				Mean	**36056**	**1.00**				
16	Positive Control		NA	1	166596	4.62				
				2	324494	9.00				
				3	309550	8.59				
				4	255550	7.09				
				Mean	**264047**	**7.32**				
16	Vehicle - Substance	AOO	0	1	28428	1.00				
				2	25378	0.89				
				3	40570	1.43				
				4	19307	0.68				
				Mean	**28421**	**1.00**				
16	Hexyl cinnamic aldehyde	AOO	5	1	68037	2.39	**6.23**	**5.36**	**4.66**	**4.44**
				2	75307	2.65				
				3	70208	2.47				
				4	47285	1.66				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				Mean	**65209**	**2.29**				
			10	1	134273	4.72				
				2	132074	4.65				
				3	192936	6.79				
				4	127598	4.49				
				Mean	**146720**	**5.16**				
			25	1	255545	8.99				
				2	274377	9.65				
				3	235997	8.30				
				4	190963	6.72				
				Mean	**239220**	**8.42**				
16	Vehicle - Positive Control		0	1	45989	1.19				
				2	31080	0.80				
				3	40234	1.04				
				4	37535	0.97				
				Mean	**38709**	**1.00**				
16	Positive Control		NA	1	266865	6.89				
				2	266443	6.88				
				3	291111	7.52				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				4	264989	6.85				
				Mean	**272352**	**7.04**				
16	Vehicle - Substance	DMSO	0	1	78052	1.02				
				2	111835	1.47				
				3	43088	0.57				
				4	71636	0.94				
				Mean	**76153**	**1.00**				
16	Nickel (II) sulfate hexahydrate	DMSO	1	1	104880	1.38	**NA**	**NA**	**NA**	**NA**
				2	80888	1.06				
				3	92663	1.22				
				4	81686	1.07				
				Mean	**90029**	**1.18**				
			3	1	109460	1.44				
				2	116987	1.54				
				3	110261	1.45				
				4	139021	1.83				
				Mean	**118932**	**1.56**				
			10	1	78555	1.03				
				2	115405	1.52				
				3	88420	1.16				
				4	71548	0.94				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				Mean	**88482**	**1.16**				
16	Lactic acid	DMSO	5	1	56025	0.74	**NA**	**NA**	**NA**	**NA**
				2	72079	0.95				
				3	58768	0.77				
				4	90115	1.18				
				Mean	**69247**	**0.91**				
16	Lactic acid (continued)		10	1	44029	0.58				
				2	67039	0.88				
				3	63161	0.83				
				4	68256	0.90				
				Mean	**60621**	**0.80**				
			25	1	72313	0.95				
				2	47618	0.63				
				3	75699	0.99				
				4	80804	1.06				
				Mean	**69108**	**0.91**				
17	Vehicle - Positive Control		0	1	16598	1.00				
				2	21167	1.28				
				3	20244	1.22				
				4	8376	0.50				
				Mean	**16596**	**1.00**				
17	Positive Control		NA	1	130759	7.88				
				2	159307	9.60				
				3	101692	6.13				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				4	105306	6.35				
				Mean	**124266**	**7.49**				
17	Vehicle - Substance	AOO	0	1	22001	0.92				
				2	17205	0.72				
				3	38937	1.63				
				4	17407	0.73				
				Mean	**23888**	**1.00**				
17	Hexyl cinnamic aldehyde	AOO	5	1	37307	1.56	**7.54**	**6.78**	**6.02**	**5.72**
				2	23097	0.97				
				3	33287	1.39				
				4	32984	1.38				
				Mean	**31668**	**1.33**				
			10	1	96209	4.03				
				2	106660	4.47				
				3	109225	4.57				
				4	129230	5.41				
				Mean	**110331**	**4.62**				
			25	1	123470	5.17				
				2	144993	6.07				
				3	191859	8.03				
				4	156101	6.53				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				Mean	**154106**	**6.45**				
17	Vehicle - Positive Control		0	1	11526	0.63				
				2	12942	0.71				
				3	16830	0.92				
				4	31658	1.74				
				Mean	**18239**	**1.00**				
17	Positive Control		NA	1	152686	8.37				
				2	167020	9.16				
				3	133016	7.29				
				4	160607	8.81				
				Mean	**153332**	**8.41**				
17	Vehicle - Substance	DMSO	0	1	47192	0.93				
				2	45146	0.89				
				3	57466	1.13				
				4	53459	1.05				
				Mean	**50815**	**1.00**				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
17	Cobalt chloride	DMSO	1	1	134969	2.66	**1.11**	**0.70**	**0.46**	**0.39**
				2	249468	4.91				
				3	104002	2.05				
				4	106668	2.10				
				Mean	**148776**	**2.93**				
			3	1	206718	4.07				
				2	243849	4.80				
				3	212124	4.17				
				4	201772	3.97				
				Mean	**216116**	**4.25**				
			5	1	297901	5.86				
				2	231316	4.55				
				3	192465	3.79				
				4	306231	6.03				
				Mean	**256978**	**5.06**				
17	Potassium dichromate	DMSO	0.1	1	212537	4.18	**0.09**	**0.06**	**0.05**	**0.04**
				2	192220	3.78				

Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study[1]

Lab No.[2]	Substance Name	Veh.	Conc. (%)	Anim. No.	Mean ATP[3]	SI	Calc. EC3[4]	Calc. EC2.5[5]	Calc. EC2[5]	Calc. EC1.8[5]
				3	110195	2.17				
				4	146041	2.87				
				Mean	**165248**	**3.25**				
			0.3	1	281536	5.54				
				2	284296	5.59				
				3	229749	4.52				
				4	232971	4.58				
				Mean	**257138**	**5.06**				
			1.0	1	349431	6.88				
				2	269795	5.31				
				3	278313	5.48				
				4	397799	7.83				
				Mean	**323834**	**6.37**				

Abbreviations: ACE = acetone; Anim. = animal; AOO = acetone: olive oil (4:1); ATP = adenosine triphosphate; Calc. = calculated; Conc. = concentration; DMSO = dimethyl sulfoxide; EC3 = estimated concentration needed to produce a stimulation index of 3; EC2.5 = estimated concentration needed to produce a stimulation index of 2.5; EC2 = estimated concentration needed to produce a stimulation index of 2; EC1.8 = estimated concentration needed to produce a stimulation index of 1.8; IDR = insufficient dose response; NA = not applicable; No. = number; SI = stimulation index; Veh. = vehicle.

[1] Original laboratory records with individual animal data from the LLNA: DA two-phased interlaboratory validation study (Omori et al. 2008) were provided by Study Director Takashi Omori from the Kyoto University School of Public Health in Kyoto, Japan.

[2] Laboratories 1 – 10 participated in the first phase, and laboratories 11 – 17 participated in the second phase of the two-phased interlaboratory validation study.

[3] Two ATP measurements were taken for each animal and the mean ATP is indicated.

[4] EC3 value was calculated based on interpolation or extrapolation formulas discussed in Gerberick et al. 2004.

[5] EC value (i.e., EC2.5, EC2, or EC1.8) was calculated based on modified interpolation or extrapolation formulas for EC3 discussed in Gerberick et al. 2004.

Annex V

Accuracy Analyses Using Additional Approaches for Combining Multiple Test Results

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1.0 Accuracy Analyses Using Alternative Decision Criteria and Alternate Methods for Combining Data for Substances Tested Multiple Times

This annex shows performance analyses for the murine local lymph node assay (LLNA) modified by Daicel Chemical Industries, Ltd., based on ATP content (referred to hereafter as the "LLNA: DA") for alternative decision criteria when using two different approaches for combining test results for the 14 substances with multiple LLNA: DA tests.

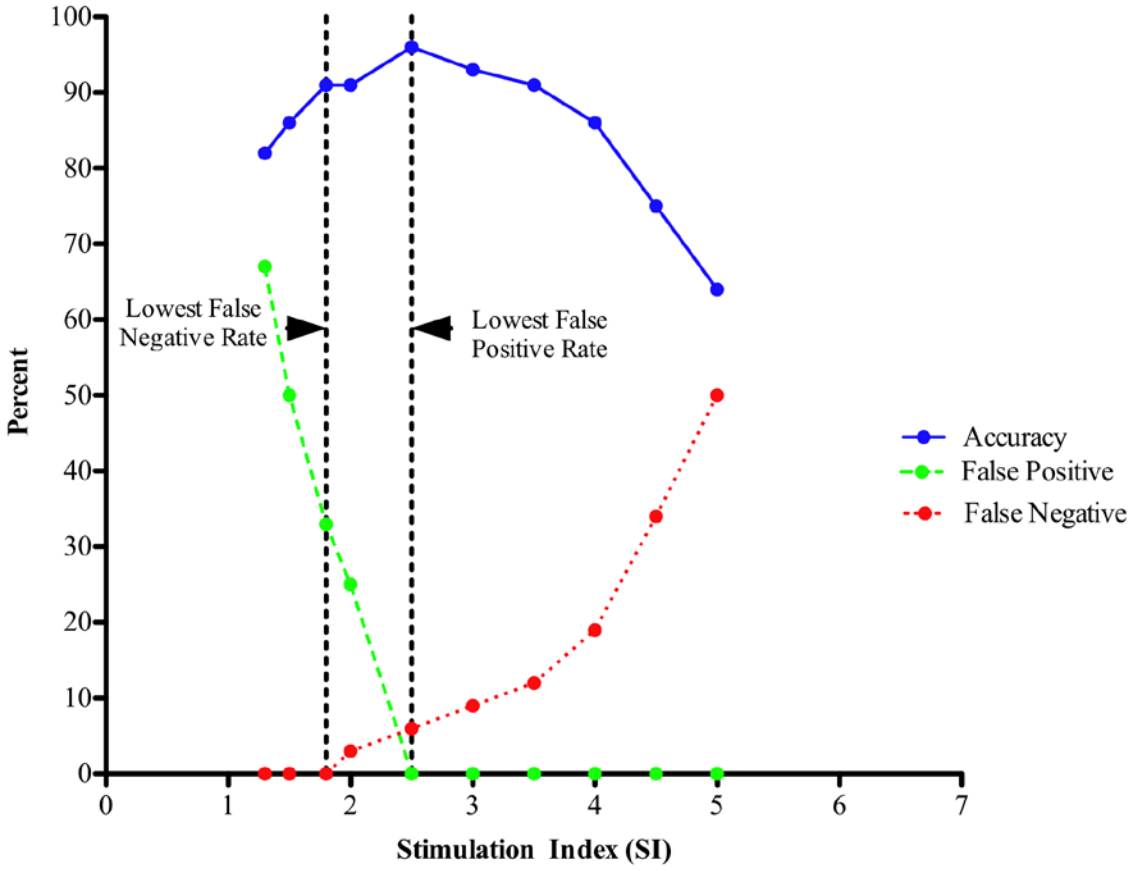
1. The positive/negative outcome for each substance for each criterion was determined by the outcome of the test with the highest maximum stimulation index (SI) of the multiple tests.
2. The positive/negative outcome for each substance for each criterion was determined by the outcome of the test with the lowest maximum SI of the multiple tests.

Section 6.0 of this background review document provides the results for the analysis when the most prevalent outcome was used to represent the result for each substance tested multiple times (for each criterion).

1.1 Results of LLNA: DA Accuracy Analysis Using Alternative Decision Criteria and Highest Maximum SI for the Outcome of Multiple Tests

When combining multiple test results for a single substance by using the outcome of the test with the highest maximum SI to identify potential sensitizers, the decision criterion of SI ≥ 3.0 (used by the LLNA: DA validation study team) yielded an accuracy of 93% (41/44), a sensitivity of 91% (29/32), a specificity of 100% (12/12), a false positive rate of 0% (0/12), and a false negative rate of 9% (3/32) (**Table C-V-1)**. The decision criteria using higher SI values, SI ≥ 3.5 to SI ≥ 5.0, decreased performance except for the specificity and the false positive rate, which remained at 100% (12/12) and 0% (0/12), respectively (**Figure C-V-1** and **Table C-V-1)**. The lower SI criterion, SI ≥ 1.8, decreased accuracy to 91% (40/44) but increased sensitivity to 100% (32/32), while the specificity and the false positive rate decreased to 67% (8/12) and 33% (4/12), respectively. Further, the false negative rate decreased to 0% (0/32) at SI ≥ 1.8. The use of analysis of variance (ANOVA) and summary statistics (i.e., mean ATP measurement of treated groups ≥95% confidence interval [CI] of the control group, or ≥2 or ≥3 standard deviation [SD] from the control group mean), yielded accuracy values of 75% to 84%, with sensitivity values of 88% to 100%, and false negative rates of 0 to 13%. The specificity for these criteria ranged from 8% to 58% and the false positive rates were 42% to 92%. As summarized above, the best overall performance of these alternative decision criteria (based on the highest SI cutoff that yielded no false positives) was achieved using an SI ≥ 1.8 and using the highest maximum SI for substances with more than one test. Using a cutoff at SI ≥ 1.8, however, misclassified four nonsensitizers in the traditional LLNA (including isopropanol based on its highest maximum SI of 1.97).

Figure C-V-1 Performance of the LLNA: DA for 44 Substances Compared to the Traditional LLNA in Predicting Skin Sensitization Potential Using the Highest Maximum SI for Substances with Multiple Tests



As compared to traditional LLNA results, the lines show the change in performance characteristics for the LLNA: DA with the SI cutoff used to identify sensitizers. This analysis used LLNA: DA and traditional LLNA results for 44 substances (32 traditional LLNA sensitizers and 12 traditional LLNA nonsensitizers). For the 14 substances with multiple test results, the result for each substance was based on 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.

Abbreviations: LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content; SI = stimulation index.

Table C-V-1 Performance of the LLNA: DA for 44 Substances Compared to the Traditional LLNA in Predicting Skin Sensitization Potential Using Alternative Decision Criteria Based on the Highest Maximum SI for Substances with Multiple Tests

Alternate Criterion	N[1]	Accuracy % (No.[2])	Sensitivity % (No.[2])	Specificity % (No.[2])	False Positive Rate % (No.[2])	False Negative Rate % (No.[2])	Positive Predictivity % (No.[2])	Negative Predictivity % (No.[2])
Statistics[3]	44	84 (37/44)	94 (30/32)	58 (7/12)	42 (5/12)	6 (2/32)	86 (30/35)	78 (7/9)
≥95% CI[4]	44	75 (33/44)	100 (32/32)	8 (1/12)	92 (11/12)	0 (0/32)	74 (32/43)	100 (1/1)
≥2 SD[5]	44	77 (34/44)	91 (29/32)	42 (5/12)	58 (7/12)	9 (3/32)	81 (29/36)	63 (5/8)
≥3 SD[6]	44	77 (34/44)	88 (28/32)	50 (6/12)	50 (6/12)	13 (4/32)	82 (28/34)	60 (6/10)
SI ≥ 5.0	44	64 (28/44)	50 (16/32)	100 (12/12)	0 (0/12)	50 (16/32)	100 (16/16)	43 (12/28)
SI ≥ 4.5	44	75 (33/44)	66 (21/32)	100 (12/12)	0 (0/12)	34 (11/32)	100 (21/21)	52 (12/23)
SI ≥ 4.0	44	86 (38/44)	81 (26/32)	100 (12/12)	0 (0/12)	19 (6/32)	100 (26/26)	67 (12/18)
SI ≥ 3.5	44	91 (40/44)	88 (28/32)	100 (12/12)	0 (0/12)	13 (4/32)	100 (28/28)	75 (12/16)
SI ≥ 3.0	*44*	*93 (41/44)*	*91 (29/32)*	*100 (12/12)*	*0 (0/12)*	*9 (3/32)*	*100 (29/29)*	*80 (12/15)*
SI ≥ 2.5	*44*	*96 (42/44)*	*94 (30/32)*	*100 (12/12)*	*0 (0/12)*	*6 (2/32)*	*100 (30/30)*	*86 (12/14)*
SI ≥ 2.0	44	91 (40/44)	97 (31/32)	75 (9/12)	25 (3/12)	3 (1/32)	91 (31/34)	90 (9/10)
SI ≥ 1.8	**44**	**91 (40/44)**	**100 (32/32)**	**67 (8/12)**	**33 (4/12)**	**0 (0/32)**	**89 (32/36)**	**100 (8/8)**
SI ≥ 1.5	44	86 (38/44)	100 (32/32)	50 (6/12)	50 (6/12)	0 (0/32)	84 (32/38)	100 (6/6)
SI ≥ 1.3	44	82 (36/44)	100 (32/32)	33 (4/12)	67 (8/12)	0 (0/32)	80 (32/40)	100 (4/4)

Italicized text indicates the decision criterion chosen by the LLNA: DA validation study team; boldface indicates the single decision criterion that had an overall increased performance in predicting skin sensitization potential when compared to the traditional LLNA (i.e., no false negatives).

Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP Content; No. = number; SD = standard deviation; SI = stimulation index

[1] N = Number of substances included in this analysis.

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

[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 ATP data were log-transformed prior to statistical analyses. For analysis of variance, significance at $p < 0.05$ was further tested by Dunnett's test.

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

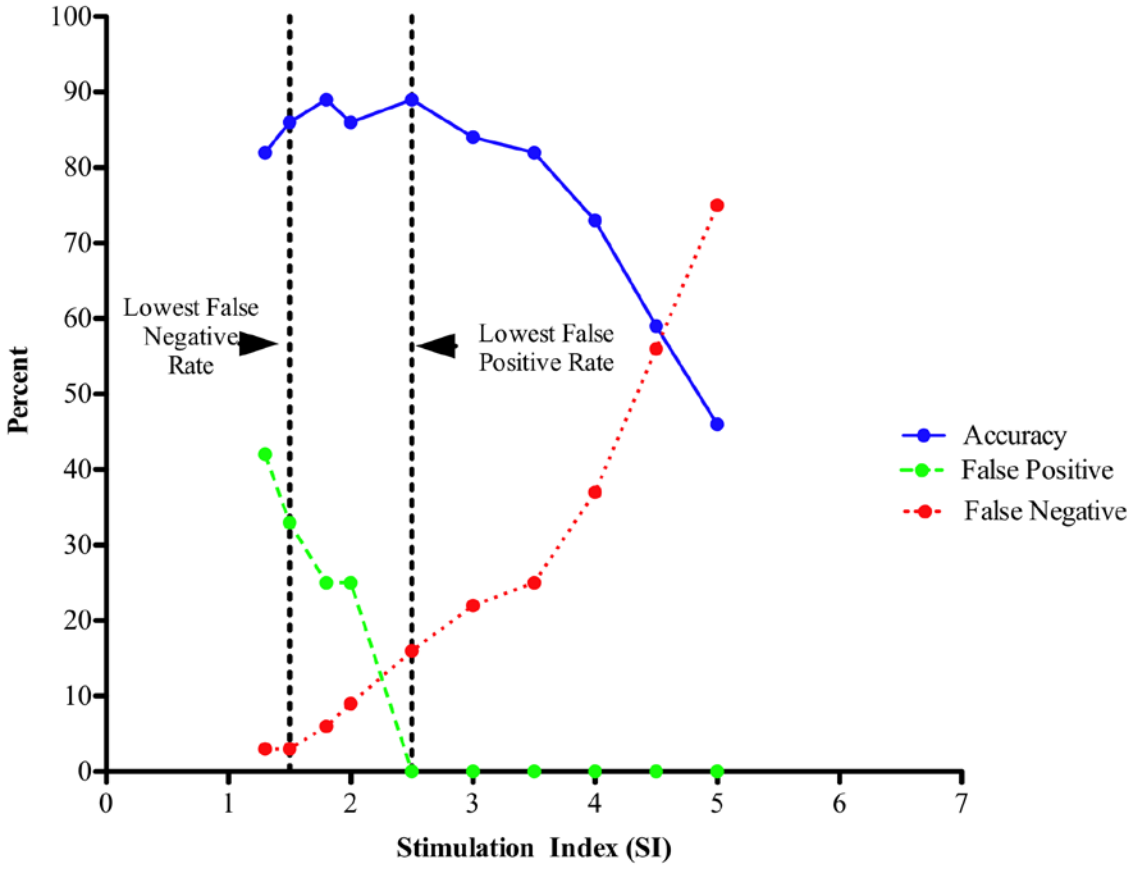
[5] The mean ATP of at least one treatment group was greater than 2 SD from the mean ATP of the vehicle control group.

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

1.2 Results of LLNA: DA 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 to identify potential sensitizers, the decision criterion of SI ≥ 3.0 (used by the LLNA: DA validation study team) yielded an accuracy of 84% (37/44), a sensitivity of 78% (25/32), a specificity of 100% (12/12), a false positive rate of 0% (0/12), and a false negative rate of 22% (7/32) (**Table C-V-2)**. The decision criteria using higher SI values, SI ≥ 3.5 to SI ≥ 5.0, decreased performance except for the specificity and the false positive rate, which remained at 100% (12/12) and 0% (0/12), respectively (**Figure C-V-2** and **Table C-V-2**). At SI ≥ 5.0, accuracy decreased to 46% (20/44) and the false negative rate increased to 75% (24/32). Use of a lower SI cutoff at SI ≥ 2.5 increased accuracy to 89% (39/44) and sensitivity to 84% (27/32), while the specificity and false positive rate remained the same at 100% (12/12) and 0% (0/12), respectively. Further, the false negative rate decreased to 16% (5/32) at SI ≥ 2.5. At SI ≥ 1.8, accuracy was unchanged at 89% (39/44) with an increased sensitivity of 94% (30/32) and decreased false negative rate of 6% (2/32), while specificity was 75% (9/12) and the false positive rate was 25% (3/12). At an even lower SI criterion, SI ≥ 1.3, accuracy was decreased to 86% (38/44) but the sensitivity increased to 97% (31/32), while the specificity was 58% (7/12) and the false positive rate was 42% (5/12). Further, the false negative rate decreased to 3% (1/32) at SI ≥ 1.3. Use of a statistical test (i.e., ANOVA or *t*-test) and summary statistics (i.e., mean ATP measurements of treated groups ≥95% CI of the control group, or ≥2 or ≥3 SD from the control group mean), yielded accuracy values of 77 to 82%, with sensitivity values of 84 to 97%, and false negative rates of 3 to 16%. Both the specificity and false positive rate for these criteria ranged from 42 to 58%. Of these alternative decision criteria, the best overall performance (i.e., lowest combined false positive and false negative rate) for the approach using the lowest maximum SI for substances with more than one test was achieved using SI ≥ 1.8, as summarized above.

Figure C-V-2 Performance of the LLNA: DA for 44 Substances Compared to the Traditional LLNA in Predicting Skin Sensitization Potential Using the Lowest Maximum SI for Substances with Multiple Tests



As compared to traditional LLNA results, the lines show the change in performance characteristics for the LLNA: DA with the SI cutoff used to identify sensitizers. This analysis used LLNA: DA and traditional LLNA results for 44 substances (32 traditional LLNA sensitizers and 12 traditional LLNA nonsensitizers). For the 14 substances with multiple test results, the result for each substance was based on 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.

Abbreviations: LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content; SI = stimulation index.

Table C-V-2 Performance of the LLNA: DA for 44 Substances Compared to the Traditional LLNA in Predicting Skin Sensitization Potential Using Alternative Decision Criteria Based on the Lowest Maximum SI for Substances with Multiple Tests

Alternate Criterion	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]
Statistics[3]	44	82	36/44	91	29/32	58	7/12	42	5/12	9	3/32	85	29/34	70	7/10
≥95% CI[4]	44	82	36/44	97	31/32	42	5/12	58	7/12	3	1/32	82	31/38	83	5/6
≥2 SD[5]	44	77	34/44	88	28/32	50	6/12	50	6/12	13	4/32	82	28/34	60	6/10
≥3 SD[6]	44	77	34/44	84	27/32	58	7/12	42	5/12	16	5/32	84	27/32	58	7/12
SI ≥ 5.0	44	46	20/44	25	8/32	100	12/12	0	0/12	75	24/32	100	8/8	33	12/36
SI ≥ 4.5	44	59	26/44	44	14/32	100	12/12	0	0/12	56	18/32	100	14/14	40	12/30
SI ≥ 4.0	44	73	32/44	63	20/32	100	12/12	0	0/12	38	12/32	100	20/20	50	12/24
SI ≥ 3.5	44	82	36/44	75	24/32	100	12/12	0	0/12	25	8/32	100	24/24	60	12/20
SI ≥ 3.0	*44*	*84*	*37/44*	*78*	*25/32*	*100*	*12/12*	*0*	*0/12*	*22*	*7/32*	*100*	*25/25*	*63*	*12/19*
SI ≥ 2.5	*44*	*89*	*39/44*	*84*	*27/32*	*100*	*12/12*	*0*	*0/12*	*16*	*5/32*	*100*	*27/27*	*71*	*12/17*
SI ≥ 2.0	44	86	38/44	91	29/32	75	9/12	25	3/12	9	3/32	91	29/32	75	9/12
SI ≥ 1.8	**44**	**89**	**39/44**	**94**	**30/32**	**75**	**9/12**	**25**	**3/12**	**6**	**2/32**	**91**	**30/33**	**82**	**9/11**
SI ≥ 1.5	44	89	39/44	97	31/32	67	8/12	33	4/12	3	1/32	89	31/35	89	8/9
SI ≥ 1.3	44	86	38/44	97	31/32	58	7/12	42	5/12	3	1/32	86	31/36	88	7/8

Italicized text indicates the decision criterion chosen by the LLNA: DA validation study team; boldface indicates the single decision criterion that had an overall increased performance in predicting skin sensitization potential when compared to the traditional LLNA (i.e., lowest combined false positive and false negative rate).

Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP Content; No. = number; SD = standard deviation; SI = stimulation index.

[1] N = Number of substances included in this analysis.

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

[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 ATP data were log-transformed prior to statistical analyses. For analysis of variance, significance at $p < 0.05$ was further tested by Dunnett's test.

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

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

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

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2.0 Discordant Results for Accuracy Analyses Using Alternative Decision Criteria

This section discusses the discordant results obtained for the analyses using the alternative decision criteria shown in **Tables C-V-1** and **C-V-2**. Discordant results using alternative decision criteria and the highest maximum SI outcome for multiple tests are discussed first (**Section 2.1**), followed by discussion of discordant results using alternative decision criteria and lowest maximum SI outcome for multiple tests (**Section 2.2**). In all cases, discordant results for the alternative decision criteria are discussed using the traditional LLNA as the reference test.

2.1 Discordant Results Using Alternative Decision Criteria and Highest Maximum SI Outcome for Multiple Tests

Table C-V-3 shows how the number and identity of discordant substances changes with the alternative decision criteria when using the test with the highest maximum SI to represent the outcome for substances with multiple tests. Using the decision criterion of SI ≥ 3.0 to identify sensitizers and the test with the highest maximum SI as the representative result for substances with multiple tests yielded three discordant substances (i.e., 3-aminophenol, 2-mercaptobenzothiazole, and methyl methacrylate), all misclassified as nonsensitizers by the LLNA: DA. Using an SI cutoff lower than three to identify sensitizers, such as SI ≥ 2.0, yielded four discordant substances: chlorobenzene, hexane, and salicylic acid were misclassified as sensitizers and methyl methacrylate was misclassified as a nonsensitizer. As mentioned in **Section 1.1**, using the decision criterion of SI ≥ 1.8 to identify sensitizers (based on the test with the highest maximum SI for substances with multiple test results) yielded the highest SI cutoff with no false negatives among the alternative decision criteria evaluated. Yet, when compared to the traditional LLNA, four substances (chlorobenzene, hexane, isopropanol, and salicylic acid) were misclassified as sensitizers by the LLNA: DA. Using a lower SI cutoff of SI ≥ 1.3 to identify sensitizers, yielded eight discordant substances that were all misclassified as sensitizers (i.e., 1-bromobutane, dimethyl isophthalate, methyl salicylate, and nickel [II] chloride plus the four substances misclassified at SI ≥ 1.8). Increasing the SI cutoff to values greater than three increased the number of sensitizers that were misclassified as nonsensitizers. At SI ≥ 4.0, six traditional LLNA sensitizers were misclassified as nonsensitizers by the LLNA: DA while at SI ≥ 5.0, 16 sensitizers were misclassified as nonsensitizers (**Table C-V-3)**.

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 nonsensitizers in the traditional LLNA as sensitizers in the LLNA: DA. Using ANOVA or *t*-test to identify sensitizers misclassified five nonsensitizers (i.e., 1-bromobutane, chlorobenzene, hexane, salicylic acid, and sulfanilamide) as sensitizers and two sensitizers (i.e., 2-mercaptobenzothiazole and methyl methacrylate) as nonsensitizers. Using treatment group ATP measurement with ≥2 SD or ≥3 SD of the vehicle control mean or a ≥95% CI of the vehicle control mean, all misclassified the following six traditional LLNA nonsensitizers as sensitizers: 1-bromobutane, chlorobenzene, hexane, isopropanol, nickel (II) chloride, and propylparaben. The ≥95% CI of the vehicle control mean misclassified four additional nonsensitizers (i.e., diethyl phthalate, dimethyl isophthalate, lactic acid, and methyl salicylate) as sensitizers. In addition, ≥2 SD or ≥3 SD of the vehicle control mean commonly misclassified three sensitizers as nonsensitizers (i.e., ethyl acrylate, methyl methacrylate, and propyl gallate).

Thirteen of the 22 ICCVAM-recommended LLNA performance standards reference substances (ICCVAM 2009) tested in the LLNA: DA were discordant for the analysis of alternative decision criteria using the test with the highest maximum SI to represent substances with multiple tests (**Table C-V-3**) when compared to the traditional LLNA. Six nonsensitizers in the traditional LLNA (i.e., chlorobenzene, isopropanol, lactic acid, methyl salicylate, nickel [II] chloride, and salicylic acid) were misclassified by some criteria in the LLNA: DA as a sensitizers, and seven sensitizers in the

traditional LLNA (i.e., citral, ethylene glycol dimethacrylate, imidazolidinyl urea, 2-mercaptobenzothiazole, methyl methacrylate, phenyl benzoate, and sodium lauryl sulfate) were misclassified as nonsensitizers by some criteria when tested in the LLNA: DA.

2.2 Discordant Results Using Alternative Decision Criteria and Lowest Maximum SI Outcome for Multiple Tests

Table C-V-4 shows how the number and identity of discordant substances changes with the alternative decision criteria when using the test with the lowest maximum SI as the representative result for substances with multiple tests. Using an SI cutoff less than three, SI ≥ 2.0, to identify sensitizers yielded six discordant substances. Three of the six discordant substances (i.e., 3-aminophenol, methyl methacrylate, and nickel [II] sulfate hexahydrate) were misclassified as nonsensitizers by the LLNA: DA compared to the traditional LLNA and the remaining three (i.e., chlorobenzene, hexane, and salicylic acid) were misclassified as sensitizers. As mentioned in **Section 1.2**, using the decision criterion of SI ≥ 1.8 to identify sensitizers (based on the test with the lowest maximum SI for substances with multiple tests) yielded optimum performance (i.e., lowest combined false positive and false negative rate) for the LLNA: DA when compared to the traditional LLNA. This decision criterion yielded five discordant substances; two were sensitizers in the traditional LLNA but were misclassified as nonsensitizers in the LLNA: DA (i.e., 3-aminophenol and nickel [II] sulfate hexahydrate) and three were nonsensitizers in the traditional LLNA but were misclassified as sensitizers in the LLNA: DA (i.e., chlorobenzene, hexane, and salicylic acid) (**Table C-V-4**).

Using an even lower SI to identify sensitizers, SI ≥ 1.3, also yielded six discordant substances. Chlorobenzene, hexane, and salicylic acid were still misclassified as sensitizers and nickel (II) sulfate hexahydrate was still misclassified as a nonsensitizer by the LLNA: DA compared to the traditional LLNA. In addition, 1-bromobutane and nickel (II) chloride were also misclassified as sensitizers. Increasing the SI cutoff to values greater than three increased the number of sensitizers that were misclassified as nonsensitizers. At SI ≥ 4.0, 12 sensitizers were misclassified as nonsensitizers while at SI ≥ 5.0, 24 sensitizers were misclassified as nonsensitizers. 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. For instance, at SI ≥ 5.0, 2,4-dinitrochlorobenzene and glutaraldehyde were classified as nonsensitizers (**Table C-V-4)**.

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 traditional LLNA nonsensitizers than sensitizers (**Table C-V-4**). Using ANOVA or *t*-test to identify sensitizers misclassified three sensitizers in the traditional LLNA (i.e., 2-mercaptobenzothiazole, methyl methacrylate, and nickel [II] sulfate hexahydrate) as nonsensitizers in the LLNA: DA. Further, five nonsensitizers in the traditional LLNA (i.e., 1-bromobutane, chlorobenzene, hexane, salicylic acid, and sulfanilamide) were misclassified as sensitizers in the LLNA: DA. Using treatment group ATP measurement ≥95% CI, ≥2 SD or ≥3 SD of vehicle control mean commonly misclassified 1-bromobutane, chlorobenzene, hexane, nickel (II) chloride, and propylparaben as sensitizers and nickel (II) sulfate hexahydrate as a nonsensitizer compared to traditional LLNA results. In addition each summary statistic misclassified from two to four additional substances when compared to traditional LLNA results (see **Table C-V-4**).

Table C-V-3 Discordant Results for the LLNA: DA Using Alternative Decision Criteria Compared to the Traditional LLNA Based on the Highest Maximum SI 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 ≥ 1.8	SI ≥ 1.5	SI ≥ 1.3
3-Aminophenol (3.2%)					-	-	-	-	-					
p-Benzoquinone (0.01%)					-	-	-							
1-Bromobutane (-)	+	+	+	+									+	+
Butyl glycidyl ether (30.9%)				-	-									
Chlorobenzene (-)	+	+	+	+							+	+	+	+
Cinnamic aldehyde (1.9%)					-									
Citral (9.2%)					-	-								
Diethyl maleate (3.6%)					-	-	-							
Diethyl phthalate (-)		+												
Dimethyl isophthalate (-)		+												+
Ethyl acrylate (32.8%)			-	-	-	-								
Ethylene glycol dimethacrylate (28.0%)					-	-								
Hexane (-)	+	+	+	+							+	+	+	+
Imidazolidinyl urea (24.0%)					-									
Isopropanol (-)		+	+	+								+	+	+
Lactic acid (-)		+												
2-Mercaptobenzothiazole (1.7%)	-				-	-	-	-	-	-				

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 ≥ 1.8	SI ≥ 1.5	SI ≥ 1.3
Methyl methacrylate (90.0%)	-		-	-	-	-	-	-	-	-	-			
Methyl salicylate (-)		+											+	+
Nickel (II) chloride (-)		+	+	+										+
Phenyl benzoate (13.6%)					-	-								
Propyl gallate (0.32%)			-	-	-									
Propylparaben (-)		+	+	+										
Resorcinol (6.3%)					-	-								
Salicylic acid (-)	+	+	+								+	+	+	+
Sodium lauryl sulfate (8.1%)					-	-	-	-						
Sulfanilamide (-)	+													
Trimellitic anhydride (4.7%)					-									

Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP Content; SD = standard deviation; SI = stimulation index.

[1] Compared to the traditional LLNA; traditional LLNA result in parentheses are "-" for nonsensitizers and EC3 values for sensitizers.

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

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

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

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

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

Table C-V-4 Discordant Results for the LLNA: DA Using Alternative Decision Criteria Compared to the Traditional LLNA Based on the Lowest Maximum SI 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 ≥ 1.8	SI ≥ 1.5	SI ≥ 1.3
Abietic Acid (11.9%)					-	-	-							
3-Aminophenol (3.2%)					-	-	-	-	-	-	-	-		
p-Benzoquinone (0.01%)					-	-	-							
1-Bromobutane (-)	+	+	+	+									+	+
Butyl glycidyl ether (30.9%)				-	-									
Chlorobenzene (-)	+	+	+	+							+	+	+	+
Cinnamic aldehyde (1.9%)					-									
Citral (9.2%)					-	-								
Cobalt chloride (0.60%)					-	-	-	-	-	-				
Diethyl phthalate (-)		+												
Dimethyl isophthalate (-)														
Diethyl maleate (3.6%)					-	-	-							
2,4-Dinitrochlorobenzene (0.05%)					-									
Ethyl acrylate (32.8%)			-	-	-	-								
Ethylene glycol dimethacrylate (28.0)					-	-								
Formaldehyde (0.50%)					-	-	-	-	-					
Glutaraldehyde (0.08%)					-	-	-	-	-					

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 ≥ 1.8	SI ≥ 1.5	SI ≥ 1.3
Hexane (-)	+	+	+	+							+	+	+	+
Hexyl cinnamic aldehyde (9.7%)					-	-	-							
Imidazolidinyl urea (24.0%)					-									
2-Mercaptobenzothiazole (1.7%)	-				-	-	-	-	-	-				
Methyl methacrylate (90.0%)	-		-	-	-	-	-	-	-	-	-			
Nickel (II) chloride (-)		+	+	+										+
Nickel (II) sulfate hexahydrate (4.8%)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phenyl benzoate (13.6%)					-	-								
Potassium dichromate (0.17%)					-	-								
Propyl gallate (0.32%)			-	-	-									
Propylparaben (-)		+	+	+										
Resorcinol (6.3%)					-	-								
Salicylic acid (-)	+	+	+								+	+	+	+
Sulfanilamide (-)	+													
Sodium lauryl sulfate (8.1%)					-	-	-	-						
Trimellitic anhydride (4.7%)					-									

Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP Content; SD = Standard deviation; SI = Stimulation index.

[1] Compared to the traditional LLNA; traditional LLNA result in parentheses are "-" for nonsensitizers and EC3 values for sensitizers.

[2] LLNA: DA 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 ATP data were log-transformed prior to statistical analyses. Significance by analysis of variance at $p < 0.05$ was further tested by Dunnett's test.

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

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

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

Thirteen of the 22 ICCVAM-recommended LLNA performance standards reference substances (ICCVAM 2009) were discordant for the analysis of alternative decision criteria using the test with the lowest maximum SI as the representative result for substances with multiple tests (**Table C-V-4**). One strong sensitizer in the traditional LLNA, 2,4-dinitrochlorobenzene, was misclassified by SI ≥ 5.0 as a nonsensitizer in the LLNA: DA. Nine additional sensitizers (i.e., citral, cobalt chloride, ethylene glycol dimethacrylate, hexyl cinnamic aldehyde, imidazolidinyl urea, 2-mercaptobenzothiazole, methyl methacrylate, phenyl benzoate, and sodium lauryl sulfate) were also misclassified as nonsensitizers by some criteria in the LLNA: DA. Three nonsensitizers in the traditional LLNA (i.e., chlorobenzene, nickel [II] chloride, and salicylic acid) were misclassified as sensitizers by some criteria in the LLNA: DA.

Annex VI

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 stimulation index (SI) criteria for the murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine (LLNA: BrdU-ELISA) and murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content (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 reoptimized 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 background review document (BRD) were based on selecting the highest SI values that produced no false negatives, relative to traditional murine local lymph node assay (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 were 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, reoptimizing 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-VI-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-VI-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-Dinitrochlorobenzene	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		

continued

Table C-VI-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)			
Isopropyl myristate	4.19		
Cinnamic aldehyde	3.97		
trans-Cinnamaldehyde	3.50		
Hexyl cinnamic aldehyde	3.40		
Eugenol	3.30		
3-Aminophenol	3.06		
Nickel sulfate	2.66		
4-Chloroaniline	2.53		
Aniline	2.07		
2-Mercaptobenzothiazole	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: LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; 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 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-VI-2**.

Table C-VI-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: LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; 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 reoptimized 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-VI-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-VI-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 reoptimized 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-VI-3 SI Data for the LLNA: DA[1]

Substance Name	SI for True Positives[2]	Substance Name	SI for True Negatives[3]
Phase I (N = 31)			
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		

continued

Table C-VI-3 SI Data for the LLNA: DA[1] (continued)

Substance Name	SI for True Positives[1]	Substance Name	SI for True Negatives[2]
Phase I (N = 31)			
Sodium lauryl sulfate	3.39		
3-Aminophenol	2.38		
Nickel (II) sulfate hexahydrate	2.13		
2-Mercaptobenzothiazole	2.00		
Phase II (N = 13)			
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: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; 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-VI-4**.

Table C-VI-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
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: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; 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, 6.25% (1/16) that it will be SI ≥ 2.3, 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 reoptimized in the future after new prospective data have been collected.

Annex VII

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 murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content (LLNA: DA); 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: DA results for the 44 substances (32 traditional murine local lymph node assay [LLNA] sensitizers and 12 traditional LLNA nonsensitizers) that were reviewed by the independent peer review 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 stimulation index (SI) values 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: DA, when using multiple SI decision criteria, for potential use in providing additional information for classifying substances that produce SI values in the range of uncertainty.

2.0 Accuracy Analysis Using Multiple Stimulation Index Decision Criteria

As detailed in Section 6.5 of the background review document (BRD), the accuracy of the LLNA: DA when using various single alternative decision criteria was evaluated using the traditional LLNA as the reference test. Compared to the traditional LLNA ($SI \geq 3.0$), the optimum performance (accuracy of 93% [41/44] and sensitivity of 100% [32/32]) was achieved using the decision criterion of $SI \geq 1.8$ (Table C-8 of the BRD). Although the $SI \geq 1.8$ produced a false positive rate of 25% (3/12) it yielded a false negative rate of 0% (0/32) (Table C-8 of the BRD). Increasing the SI decision criterion to $SI \geq 2.5$ decreased the false positive rate to 0% (0/12) but increased the false negative rate to 13% (4/32). The 0% false positive rate using $SI \geq 2.5$ and the 0% false negative rate using $SI \geq 1.8$ prompted an evaluation using two SI decision criteria for determining LLNA: DA results: one criterion to classify substances as sensitizers ($SI \geq 2.5$) and one criterion to classify substances as nonsensitizers ($SI \leq 1.8$). The evaluation of this accuracy analysis is described below.

It should be noted that this analysis was based on the same strategy for combining results as that described in Section 6.5 of the BRD for the substances tested multiple times (i.e., the sensitizer/nonsensitizer outcome for each substance using the most prevalent outcome). **Section 3.0** details the reproducibility of substances tested multiple times and indicates that, there were no instances of false positive results for nonsensitizers (0% [0/80] of the substances classified as traditional LLNA nonsensitizers had an $SI \geq 2.5$ in the LLNA: DA). See **Section 3.0** for more details regarding these results.

2.1 Indeterminate Results Using Multiple Stimulation Index Decision Criteria

While optimum false positive and false negative rates can be achieved for the 44 substances evaluated in the LLNA: DA accuracy analyses using these two different decision criteria, a range of SI values (i.e., between 1.8 and 2.5) 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, molecular weight, peptide reactivity (see Annex II of the BRD for physicochemical properties), traditional LLNA EC3 range (estimated concentration needed to

produce a stimulation index of 3) (Table C-2 of the BRD), or potential for skin irritation (Annex III of the BRD) were examined to identify commonalities among the substances that produced SI values between 1.8 and 2.5 in an attempt to identify similar 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 LLNA: DA database to distinguish between traditional LLNA sensitizers and nonsensitizers.

Of the nine substances that produced SI values between 1.8 and 2.5 (**Table C-VII-1)**, four are nonsensitizers (chlorobenzene, hexane, isopropanol, salicylic acid) and five are sensitizers (3-aminophenol, cobalt chloride, 2-mercaptobenzothiazole, methyl methacrylate, and nickel [II] sulfate hexahydrate) based on traditional LLNA results. Among the four traditional LLNA nonsensitizers, six chemical classes are represented; one substance is classified as both a carboxylic acid and phenol (salicylic acid), one substance is both a halogenated and a cyclic hydrocarbon (chlorobenzene), one substance is an acyclic hydrocarbon (hexane), and one substance is an alcohol (isopropanol). Other characteristics of the nonsensitizers (based on traditional LLNA data) include:

- Three substances are liquids (chlorobenzene, hexane, and isopropanol) and one substance is a solid (salicylic acid).
- Molecular weights range from 60 g/mol for isopropanol, 86 g/mol for hexane, 113 g/mol for chlorobenzene, to 138 g/mol for salicylic acid.
- All four substances are soluble in water.
- The peptide reactivity for chlorobenzene, hexane, and isopropanol is minimal; peptide reactivity information for salicylic acid is not available.
- Hexane and salicylic acid are considered irritants based on data in either mice or humans and isopropanol is considered negative based on data in rabbits; irritancy data for chlorobenzene are not available but irritancy potential is assumed as low based on clinical literature (**Table C-VII-1**).
- Among the five traditional LLNA sensitizers, five chemical classes are represented; one substance is a carboxylic acid (methyl methacrylate), two substances are metals (nickel [II] sulfate hexahydrate and cobalt chloride), one substance is both an amine and a phenol (3-aminophenol), and one substance is a heterocyclic compound (2-mercaptobenzothiazole).

Other characteristics of the substances that are classified as sensitizers by the traditional LLNA include:

- Four substances are solids (3-aminophenol, cobalt chloride, 2-mercaptobenzothiazole, and nickel [II] sulfate hexahydrate) and one substance is a liquid (methyl methacrylate).
- Molecular weights range from 100 g/mol for methyl methacrylate, 109 g/mol for 3-aminophenol, 130 g/mol for cobalt chloride, 155 g/mol for nickel (II) sulfate hexahydrate to 167 g/mol for 2-mercaptobenzothiazole.
- 2-Mercaptobenzothiazole is insoluble in water; the other four substances are soluble in water.
- The peptide reactivity for 2-mercaptobenzothiazole is high and that for 3-aminophenol is minimal; peptide reactivity data for the three other substances are not available.
- The EC3 values for the five substances identified as sensitizers by the traditional LLNA are: 0.6% for cobalt chloride, 1.7% for 2-mercaptobenzothiazole, 3.2% for 3-aminophenol, 4.8% for nickel [II] sulfate hexahydrate, and 90% for methyl methacrylate.
- All five substances are considered nonirritants based on GP data (**Table C-VII-1**).

Table C-VII-1 Indeterminate Results for the LLNA: DA When Multiple Stimulation Index Decision Criteria are Used[1]

Substance Name[2]	Vehicle[3]	LLNA: DA[4]	Traditional LLNA[4]	Skin Irritant?
Chlorobenzene	AOO	2.44, 25% (1/1 tests)	- (1.7, 25%)[5]	No data. Low irritancy potential assumed based on clinical literature.
Hexane	AOO	2.31, 100% (1/1 tests)	- (2.2, 100%)	Irritant at 100% (humans)
Isopropanol	AOO	1.97, 50%[5] (1/11 tests)	- (1.7, 50%)[5]	Negative at 100% (rabbits)
Salicylic acid	AOO	2.00, 25% (1/1 tests)	- (2.4, 25%)	Irritant at 20% aq. (mice)
3-Aminophenol (3.2%)	AOO	2.38, 10%[6] (1/3 tests)	+ (5.7, 10%)	Nonirritant at 5% (GP)
Cobalt chloride (0.6%)	DMSO	2.0, 5% (1/8 tests)	+ (7.2, 5%)	Negative at ≤ 0.5% (GP)
2-Mercaptobenzothiazole (1.7%)	DMF	2.01, 50%[5] (1/1 tests)	+ (8.6, 10%)	Nonirritant at 10% (GP)
Methyl methacrylate (90%)	AOO	1.81, 100% (1/1 tests)	+ (3.6, 100%)	Nonirritant at 3 M (GP)
Nickel (II) sulfate hexahydrate (4.8%)	DMSO	2.13, 10% 2.17, 5%[7] (2/8 tests)	+ (3.1, 5%)	Nonirritant at 0.15% (GP); irritant at 10% (humans)

Abbreviations: AOO = acetone: olive oil (4:1); aq. = aqueous; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; GP = guinea pig; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

"+" = Sensitizer.

"-" = Nonsensitizer.

[1] Data source(s) indicated in Annex III of the BRD.

[2] Numbers in parentheses are EC3 values (concentrations needed to produce a stimulation index [SI] of three) for substances that are sensitizers in the traditional LLNA (see Table C-2 of the BRD).

[3] Vehicle listed is that used in both the LLNA: DA and the traditional LLNA, unless otherwise noted.

[4] Numbers indicated are highest SI and maximum concentration tested; highest SI is at maximum concentration tested, unless otherwise noted.

[5] Highest SI occurred at concentration 10%.

[6] Highest SI occurred at concentration 3%.

[7] Highest SI occurred at concentration 2.5%.

3.0 Test Method Reliability

An assessment of test method reliability (intralaboratory repeatability and intra- and interlaboratory reproducibility) is an essential element of any evaluation of the performance of an alternative test method (ICCVAM 2003). Repeatability refers to 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 (ICCVAM 1997, 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. With regard to the LLNA: DA test method, there are no known intralaboratory repeatability studies, which was also the situation with the traditional LLNA.

The LLNA: DA data were amenable to both intralaboratory and interlaboratory reproducibility analyses. The evaluation of multiple SI decision criteria in **Section 2.0** of this Annex evaluated SI ≥ 2.5 as the decision criterion for classifying substances as sensitizers when used with a decision criterion of SI ≤ 1.8 to identify nonsensitizers. Thus, this section provides an assessment of reproducibility for the decision criterion of SI ≥ 2.5 to identify sensitizers.

3.1 Intralaboratory Reproducibility

Idehara et al. (2008) evaluated intralaboratory reproducibility of EC3 values for the LLNA: DA using two substances (isoeugenol and eugenol) that were each tested in three different experiments (**Table C-VII-2**). The data indicate coefficients of variation (CVs) of 21% and 11% for isoeugenol and eugenol, respectively. The authors state that for both compounds the EC3 values appeared to be close and that for each test substance the SI values for the same concentration were fairly reproducible (Idehara et al. 2008). The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) also determined the intralaboratory reproducibility of EC2.5 values (estimated concentrations needed to produce an SI of 2.5) for the same set of data. The results for EC2.5 values indicate slightly larger intralaboratory variability compared to EC3 values with CVs of 33% and 13% for isoeugenol and eugenol, respectively.

Table C-VII-2 Intralaboratory Reproducibility of EC3 and EC2.5 Values Using the LLNA: DA[1]

Isoeugenol			
Concentration (%)	**Experiment 1[2]**	**Experiment 2[2]**	**Experiment 3[2]**
Vehicle (AOO)	1.00 ± 0.54	1.00 ± 0.54	1.00 ± 0.30
0.5	1.50 ± 0.54	-------	1.22 ± 0.13
1	2.28 ± 0.60	-------	2.77 ± 1.01
2.5	2.78 ± 0.17	3.11 ± 1.15	3.01 ± 0.98
5	3.39 ± 0.69	4.39 ± 1.25	-------
10	5.68 ± 1.19	6.77 ± 0.23	-------
EC3	3.40%	2.35%	2.46%
EC2.5	0.82%	1.37%	0.75%
Mean EC3: 2.74% ± 0.58% and 21% CV *Mean EC2.5: 1.46% ± 0.48% and 33% CV*			

continued

Table C-VII-2 Intralaboratory Reproducibility of EC3 and EC2.5 Values Using the LLNA: DA[1] (continued)

Eugenol			
Concentration (%)	**Experiment 1[2]**	**Experiment 2[2]**	**Experiment 3[2]**
Vehicle (AOO)	1.00 ± 0.17	1.00 ± 0.17	1.00 ± 0.09
5	2.92 ± 1.00	2.80 ± 1.08	3.24 ± 0.70
10	7.35 ± 2.62	4.47 ± 0.98	4.79 ± 0.94
25	10.92 ± 3.63	5.62 ± 3.20	7.07 ± 0.44
EC3	5.09%	5.59%	4.50%
EC2.5	4.33%	3.59%	2.87%
Mean EC3: 5.06% ± 0.55% and 11% CV *Mean EC2.5: 4.23% ± 0.57% and 13% CV*			

Abbreviations: AOO = acetone: olive oil (4:1); CV = coefficient of variation; EC2.5 = estimated concentration needed to produce a stimulation index of 2.5; EC3 = estimated concentration needed to produce a stimulation index of three; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

[1] Based on results discussed in Idehara et al. 2008; the number per group was not specified.

[2] Mean stimulation index value ± standard deviation.

3.2 Interlaboratory Reproducibility

Furthermore, data were submitted to NICEATM (Annex IV of the BRD) from a two-phased interlaboratory validation study on the LLNA: DA test method (Omori et al. 2008). In the first phase of the interlaboratory validation study, a blinded test of 12 substances was conducted in 10 laboratories. Three substances (2,4-dinitrochlorobenzene, hexyl cinnamic aldehyde, and isopropanol) were tested in all 10 laboratories. The remaining nine substances were randomly assigned to subsets of three of the 10 laboratories **(Table C-VII-3)**. In each laboratory, each substance was tested one time at three different concentrations. The dose levels for each substance were predetermined (i.e., the participating laboratories did not determine their own dose levels for testing). Nine substances are sensitizers and three substances are nonsensitizers according to traditional LLNA results. Six substances are reference substances included in LLNA performance standards recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM): cobalt chloride, 2,4-dinitrochlorobenzene, hexyl cinnamic aldehyde, isoeugenol, isopropanol, and methyl salicylate.

The second phase of the interlaboratory validation study was designed to evaluate the reliability of the LLNA: DA for testing metallic salts using dimethylsulfoxide (DMSO) as a vehicle since two metals dissolved in DMSO (cobalt chloride and nickel [II] sulfate hexahydrate) from the first phase of the interlaboratory validation study yielded inconsistent results. Five coded substances (two of the five substances were unique to the second phase of the interlaboratory validation study) were tested in seven laboratories **(Table C-VII-4)**. One substance (i.e. hexyl cinnamic aldehyde) was tested in all seven laboratories. The remaining four substances (cobalt chloride, nickel (II) sulfate hexahydrate, lactic acid, and potassium dichromate) were randomly assigned to subsets of four of the seven laboratories. Each laboratory tested the substance one time at three different dose levels. Again, the dose levels for each substance were predetermined. Of the two substances not previously tested in the first phase of the interlaboratory validation study (lactic acid and potassium dichromate), one is a nonsensitizer and the other is a sensitizer according to traditional LLNA results, respectively. In addition, lactic acid is an ICCVAM-recommended LLNA performance standards reference substance.

The LLNA: DA test results from the two-phased interlaboratory validation studies are amenable to interlaboratory reproducibility analyses for three endpoints: sensitizer (positive) or nonsensitizer (negative) classification, and EC2.5 values. Analyses of interlaboratory reproducibility were performed using a concordance analysis for the qualitative results (sensitizer vs. nonsensitizer) (**Section 3.2.1**) and a CV analysis for the quantitative results (EC2.5 values) (**Sections 3.2** and **3.3**).

Table C-VII-3 Substances and Allocation for the First Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	Vehicle	Concentration Tested (%)			Laboratory									
					1	2	3	4	5	6	7	8	9	10
2,4-Dinitro-chlorobenzene (+)	AOO	0.03	0.10	0.30	X	X	X	X	X	X	X	X	X	X
Hexyl cinnamic aldehyde (+)	AOO	5	10	25	X	X	X	X	X	X	X	X	X	X
Isopropanol (-)	AOO	10	25	50	X	X	X	X	X	X	X	X	X	X
Abietic acid (+)	AOO	5	10	25		X				X	X			
3-Aminophenol (+)	AOO	1	3	10	X		X					X		
Dimethyl isophthalate (-)	AOO	5	10	25	X		X				X			
Isoeugenol (+)	AOO	1	3	10				X	X				X	
Methyl salicylate (-)	AOO	5	10	25			X				X			X
Formaldehyde (+)	ACE	0.5	1.5	5.0	X	X			X					
Glutaraldehyde (+)	ACE	0.05	0.15	0.50	X	X			X					
Cobalt chloride[2] (+)	DMSO	0.3	1.0	3.0				X		X		X		
Nickel (II) sulfate hexahydrate (+)	DMSO	1	3	10				X		X		X		

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

Table C-VII-4 Substances and Allocation for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	Vehicle	Concentration Tested (%)			Laboratory						
					11	12	13	14	15	16	17
Hexyl cinnamic aldehyde (+)	AOO	5	10	25	X	X	X	X	X	X	X
Cobalt chloride[2] (+)	DMSO	1	3	5	X		X	X			X
Lactic acid (-)	DMSO	5	10	25	X		X		X	X	
Nickel (II) sulfate hexahydrate (+)	DMSO	1	3	10	X	X		X		X	
Potassium dichromate (+)	DMSO	0.1	0.3	1.0	X	X			X		X

Abbreviations: AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

3.2.1 Interlaboratory Reproducibility – Qualitative Results

The qualitative (positive/negative) interlaboratory concordance analysis for the 12 substances that were tested during the first phase of the LLNA: DA interlaboratory validation study is shown in **Table C-VII-5** for SI ≥ 2.5. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), ten substances tested in either three or 10 laboratories had consistent results leading to 100% (3/3 or 10/10) interlaboratory concordance for those substances. There were two discordant substances (3-aminophenol and nickel [II] sulfate hexahydrate) for which interlaboratory concordance was 67% (2/3). One of the three laboratories that tested 3-aminophenol reported SI ≥ 2.5 at the highest dose tested (SI = 2.83 at 10%) and two laboratories did not achieve SI ≥ 2.5 at any dose tested (Annex IV of the BRD). One of the three laboratories that tested nickel (II) sulfate hexahydrate reported a maximum SI = 1.52, while the other two laboratories produced an SI ≥ 2.5 at all three doses tested (Annex IV of the BRD). Notably, when analyzing the dose response curves for the three tests performed for nickel (II) sulfate in the first phase of the two-phased interlaboratory validation study, only one study demonstrated a sufficient dose response (i.e., a parallel increase in SI relative to increase in concentration). Since 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: DA concordance data from the first phase of the interlaboratory validation study.

Annex VIII

Reproducibility Analyses for the LLNA: DA
Using a Single Decision Criterion of SI ≥ 3.0 or SI ≥ 2.0

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1.0 LLNA: DA Test Method Reliability

An assessment of test method reliability (intralaboratory repeatability and intra- and interlaboratory reproducibility) is an essential element of any evaluation of the performance of an alternative test method (ICCVAM 2003). Repeatability refers to 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 (ICCVAM 1997, 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. With regard to the murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content (referred to hereafter as the "LLNA: DA") test method, there are no known intralaboratory repeatability studies, which was also the situation with the traditional murine local lymph node assay (LLNA).

The LLNA: DA data were amenable to both intralaboratory and interlaboratory reproducibility analyses. The evaluation of a single decision criterion in Section 6.5 of this background review document (BRD) showed that stimulation index (SI) ≥ 1.8 produced the most optimum results (i.e., 93% accuracy and 0% false negative rate) among the alternate decision criteria evaluated. Thus Section 7.0 of this BRD provides an assessment of reproducibility for the decision criterion of SI ≥ 1.8 to identify potential sensitizers. Further, since SI ≥ 3.0 was used by the validation management team in the intralaboratory and interlaboratory validation studies, and SI ≥ 2.0 was previously evaluated as an optimum decision criterion in the March 2009 draft BRD reviewed by the independent scientific peer review Panel, this annex details additional reproducibility analyses for SI ≥ 3.0 and SI ≥ 2.0.

1.1 Intralaboratory Reproducibility (SI ≥ 3.0 or SI ≥ 2.0)

Idehara et al. (2008) evaluated the intralaboratory reproducibility of EC3 values (estimated concentration needed to produce an SI of three) for the LLNA: DA using two substances (isoeugenol and eugenol) that were each tested in three different experiments (**Table C-VIII-1**). The data indicate coefficients of variation (CVs) of 21% and 11% for isoeugenol and eugenol, respectively. The authors state that for both compounds the EC3 values appeared to be close and that for each test substance the SI values for the same concentration were fairly reproducible (Idehara et al. 2008). The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) also determined the intralaboratory reproducibility of EC2 values (estimated concentration needed to produce an SI of two) for the same set of data. The results for EC2 values with CV values of 35% and 20% for isoeugenol and eugenol, respectively, indicate slightly larger intralaboratory variability compared to EC3 value results.

1.2 Interlaboratory Reproducibility

Furthermore, data were submitted to NICEATM (Annex IV of this BRD) from a two-phased interlaboratory validation study on the LLNA: DA test method (Omori et al. 2008). In the first phase of the interlaboratory validation study, a blinded test of 12 substances was conducted in 10 laboratories. Three substances (i.e. 2,4-dinitrochlorobenzene, hexyl cinnamic aldehyde, and isopropanol) were tested in all 10 laboratories. The remaining nine substances were randomly assigned to subsets of three of the 10 laboratories **(Table C-VIII-2)**. In each laboratory, each substance was tested one time at three different concentrations. The dose levels for each substance were predetermined (i.e., the participating laboratories did not determine their own dose levels for testing). Nine substances are sensitizers and three substances are nonsensitizers according to traditional LLNA results. Six substances are ICCVAM-recommended LLNA performance standards

reference substances: cobalt chloride, 2,4-dinitrochlorobenzene, hexyl cinnamic aldehyde, isoeugenol, isopropanol, and methyl salicylate (ICCVAM 2009).

Table C-VIII-1Intralaboratory Reproducibility of EC3 and EC2 Values Using the LLNA: DA[1]

Isoeugenol			
Concentration (%)	**Experiment 1[2]**	**Experiment 2[2]**	**Experiment 3[2]**
Vehicle (AOO)	1.00 ± 0.54	1.00 ± 0.54	1.00 ± 0.30
0.5	1.50 ± 0.54	-------	1.22 ± 0.13
1	2.28 ± 0.60	-------	2.77 ± 1.01
2.5	2.78 ± 0.17	3.11 ± 1.15	3.01 ± 0.98
5	3.39 ± 0.69	4.39 ± 1.25	-------
10	5.68 ± 1.19	6.77 ± 0.23	-------
EC3	3.40%	2.35%	2.46%
EC2	0.82%	1.37%	0.75%
Mean EC3: 2.74% ± 0.58% and 21% CV *Mean EC2: 0.98% ± 0.34% and 35% CV*			
Eugenol			
Concentration (%)	**Experiment 1[2]**	**Experiment 2[2]**	**Experiment 3[2]**
Vehicle (AOO)	1.00 ± 0.17	1.00 ± 0.17	1.00 ± 0.09
5	2.92 ± 1.00	2.80 ± 1.08	3.24 ± 0.70
10	7.35 ± 2.62	4.47 ± 0.98	4.79 ± 0.94
25	10.92 ± 3.63	5.62 ± 3.20	7.07 ± 0.44
EC3	5.09%	5.59%	4.50%
EC2	4.33%	3.59%	2.87%
Mean EC3: 5.06% ± 0.55% and 11% CV *Mean EC2: 3.60% ± 0.73% and 20% CV*			

Abbreviations: AOO = acetone: olive oil (4:1); CV = coefficient of variation; EC2 = estimated concentration needed to produce a stimulation index of two; EC3 = estimated concentration needed to produce a stimulation index of three; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

[1] Based on results discussed in Idehara et al. 2008; the number per group was not specified.

[2] Mean stimulation index value ± standard deviation.

Table C-VIII-2 Substances and Allocation for the First Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	Vehicle	Concentration Tested (%)			Laboratory									
					1	2	3	4	5	6	7	8	9	10
2,4-Dinitrochloro-benzene (+)	AOO	0.03	0.10	0.30	X	X	X	X	X	X	X	X	X	X
Hexyl cinnamic aldehyde (+)	AOO	5	10	25	X	X	X	X	X	X	X	X	X	X
Isopropanol (-)	AOO	10	25	50	X	X	X	X	X	X	X	X	X	X
Abietic acid (+)	AOO	5	10	25		X				X	X			
3-Aminophenol (+)	AOO	1	3	10	X		X					X		
Dimethyl isophthalate (-)	AOO	5	10	25	X		X				X			
Isoeugenol (+)	AOO	1	3	10				X	X				X	
Methyl salicylate (-)	AOO	5	10	25			X				X			X
Formaldehyde (+)	ACE	0.5	1.5	5.0	X	X			X					
Glutaraldehyde (+)	ACE	0.05	0.15	0.50	X	X			X					
Cobalt chloride[2] (+)	DMSO	0.3	1.0	3.0				X		X		X		
Nickel (II) sulfate hexahydrate (+)	DMSO	1	3	10				X		X		X		

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

The second phase of the interlaboratory validation study was designed to evaluate the reliability of the LLNA: DA for testing metallic salts using dimethyl sulfoxide (DMSO) as a vehicle since two metal salts dissolved in DMSO (cobalt chloride and nickel [II] sulfate hexahydrate) from the first phase of the interlaboratory validation study yielded inconsistent results. Five coded substances (two of the five substances were unique to the second phase of the interlaboratory validation study) were tested in seven laboratories (different from the 10 laboratories that performed the first interlaboratory validation study) **(Table C-VIII-3)**. One substance (i.e. hexyl cinnamic aldehyde) was tested in all seven laboratories. The remaining four substances (cobalt chloride, nickel [II] sulfate hexahydrate, lactic acid, and potassium dichromate) were randomly assigned to subsets of four of the seven laboratories. Each laboratory tested the substance one time at three different dose levels. Again, the dose levels for each substance were predetermined. Of the two substances not previously tested in the first phase of the interlaboratory validation study (lactic acid and potassium dichromate), one is a nonsensitizer and the other is a sensitizer according to traditional LLNA results, respectively. In addition, lactic acid is a reference substance included in performance standards recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM; ICCVAM 2009).

The LLNA: DA test results from the two-phased interlaboratory validation study are amenable to interlaboratory reproducibility analyses for three endpoints: sensitizer (positive) or nonsensitizer

(negative) classification (based on SI ≥ 3.0 and SI ≥ 2.0), and EC3 and EC2 values. Analyses of interlaboratory reproducibility were performed using a concordance analysis for the qualitative results (sensitizer vs. nonsensitizer based on SI ≥ 3.0 and SI ≥ 2.0) (**Sections 1.2.1** and **1.2.3,** respectively) and a CV analysis for the quantitative results (EC3 and EC2 values) (**Sections 1.2.2** and **1.2.4,** respectively).

Table C-VIII-3 Substances and Allocation for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	Vehicle	Concentration Tested (%)			Laboratory						
					11	12	13	14	15	16	17
Hexyl cinnamic aldehyde (+)	AOO	5	10	25	X	X	X	X	X	X	X
Cobalt chloride[2] (+)	DMSO	1	3	5	X		X	X			X
Lactic acid (-)	DMSO	5	10	25	X		X		X	X	
Nickel (II) sulfate hexahydrate (+)	DMSO	1	3	10	X	X		X		X	
Potassium dichromate (+)	DMSO	0.1	0.3	1.0	X	X			X		X

Abbreviations: AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional Murine local lymph node assay results.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

1.2.1 Interlaboratory Reproducibility – Qualitative Results (SI ≥ 3.0)

The qualitative (i.e., positive/negative) interlaboratory concordance analysis for the 12 substances that were tested during the first phase of the LLNA: DA interlaboratory validation study is shown in **Table C-VIII-4** using SI ≥ 3.0 as the decision criterion to distinguish sensitizers from nonsensitizers. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), eight substances tested in either three or 10 laboratories had consistent results leading to 100% (3/3 or 10/10) interlaboratory concordance for those substances. There were four discordant substances (formaldehyde, glutaraldehyde, cobalt chloride, and nickel [II] sulfate hexahydrate) for which interlaboratory concordance was 67% (2/3). One of the three laboratories that tested formaldehyde reported a maximum SI = 2.69 while the other two laboratories produced at least one SI ≥ 3.0. Similarly, one of the three laboratories that tested glutaraldehyde reported a maximum SI = 2.57 while the other two laboratories had at least one SI ≥ 3.0. Two of the three laboratories that tested cobalt chloride yielded an SI ≥ 3.0 at all three doses tested (0.3%, 1.0%, and 3.0%) and therefore classified the substance as a sensitizer similar to the traditional LLNA test method. Notably, the laboratory that did not generate an SI ≥ 3.0 did not test cobalt chloride at the highest dose and the middle dose yielded an SI = 2.66. One of the three laboratories that tested nickel (II) sulfate hexahydrate reported a maximum SI = 1.52, while the other two laboratories had at least two doses that yielded an SI ≥ 3.0. Since 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: DA concordance data from the first phase of the interlaboratory validation study.

Table C-VIII-4Qualitative Results for the First Phase of the Interlaboratory Validation Study for the LLNA: DA (SI ≥ 3.0)

Substance Name[1]	Qualitative Results (Maximum SI)[2]										Concordance
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	
2,4-Dinitrochlorobenzene (+)	+ (11.97)	+ (9.23)	+ (9.96)	+ (8.53)	+ (7.86)	+ (15.14)	+ (13.18)	+ (12.60)	+ (10.89)	+ (4.71)	10/10
Hexyl cinnamic aldehyde (+)	+ (5.78)	+ (4.82)	+ (4.44)	+ (5.11)	+ (3.97)	+ (5.50)	+ (7.09)	+ (10.22)	+ (3.88)	+ (3.51)	10/10
Isopropanol (-)	- (1.54)	- (0.91)	- (1.01)	- (1.57)	- (0.76)	- (1.97)	- (1.45)	- (1.21)	- (0.70)	- (1.25)	10/10
Abietic acid (+)		+ (4.64)				+ (7.96)	+ (3.98)				3/3
3-Aminophenol (+)	- (2.83)		- (1.76)					- (2.38)			3/3
Dimethyl isophthalate (-)	- (1.34)		- (1.29)				- (1.26)				3/3
Isoeugenol (+)				+ (6.11)	+ (5.54)				+ (7.09)		3/3
Methyl salicylate (-)			- (1.55)				- (1.77)			- (0.83)	3/3
Formaldehyde (+)	**+ (4.84)**	**+ (3.18)**			**- (2.69)**						**2/3**
Glutaraldehyde (+)	**+ (5.00)**	**+ (3.39)**			**- (2.57)**						**2/3**
Cobalt chloride[3] (+)				**-[4] (2.66)**		**+ (20.55)**		**+ (8.07)**			**2/3**
Nickel (II) sulfate hexahydrate (+)				**-[5] (1.52)**		**+ (11.78)**		**+[5] (3.49)**			**2/3**

Bolded substances did not achieve 100% interlaboratory concordance.

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] (+) indicates sensitizers and (-) indicates nonsensitizers according to LLNA: DA tests. Highest stimulation index value for each test is shown in parentheses.

[3] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

[4] Data not reported for the highest dose (3%), only for 0.3% and 1%.

[5] Insufficient dose response.

The qualitative (positive/negative) interlaboratory concordance analysis for the five substances that were tested during the second phase of the LLNA: DA interlaboratory validation study is shown in **Table C-VIII-5** using SI ≥ 3.0 as the decision criterion to distinguish sensitizers from nonsensitizers. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), four substances (hexyl cinnamic aldehyde, lactic acid, nickel [II] sulfate hexahydrate, and potassium dichromate) tested in either four or seven laboratories had consistent results leading to 100% (4/4 or 7/7) interlaboratory concordance for those substances. There was one discordant substance (cobalt chloride) for which interlaboratory concordance was 50% (2/4). Two of the four laboratories that tested cobalt chloride reported a maximum SI = 2.01 and 2.54, respectively, while the other two laboratories had at least two doses that yielded an SI ≥ 3.0. As was discussed previously, cobalt chloride was also discordant among the laboratories that tested the substance in the first phase of the interlaboratory validation study and interlaboratory concordance was 67% (2/3). Notably, different doses of cobalt chloride were tested in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study. Furthermore, as mentioned previously, the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), and therefore there were no traditional LLNA concordance data for comparison with the LLNA: DA concordance data from the second phase of the interlaboratory validation study.

Table C-VIII-5 Qualitative Results for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA (SI ≥ 3.0)

Substance Name[1]	Qualitative Results (Maximum SI)[2]							Concordance
	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17	
Hexyl cinnamic aldehyde (+)	+ (4.47)	+ (5.71)	+ (5.41)	+ (7.60)	+ (3.92)	+ (8.42)	+ (6.45)	7/7
Cobalt chloride[3] (+)	**- (2.01)**		**- (2.54)**	**+ (4.25)**			**+ (5.06)**	**2/4**
Lactic acid (-)	- (0.93)		- (0.99)		- (0.97)	- (0.91)		4/4
Nickel (II) sulfate hexahydrate (+)	- (0.79)	- (1.24)		- (2.13)		- (1.56)		4/4
Potassium dichromate (+)	+ (4.78)	+ (4.08)			+ (6.01)		+ (6.37)	4/4

Boldface type indicates substances that did not achieve 100% interlaboratory concordance.

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP Content; SI = stimulation index.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] (+) indicates sensitizers and (-) indicates nonsensitizers according to LLNA: DA tests. Highest stimulation index value for each test is shown in parentheses.

[3] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

1.2.2 Interlaboratory Reproducibility – EC3 Values

The available quantitative (i.e., EC3 value) data for interlaboratory reproducibility analysis were obtained from the LLNA: DA tests that yielded positive results (SI ≥ 3.0) during the first and second phase of the LLNA: DA interlaboratory validation study. The method for calculating EC3 values for the positive results was based on the method of linear interpolation reported by Gerberick et al. (2004) according to the equation:

$$EC3 = c + \left[\frac{(3-d)}{(b-d)}\right] \times (a-c)$$

where the data points lying immediately above and below the SI = 3.0 on the dose response curve have the coordinates of (a, b) and (c, d), respectively (Gerberick et al. 2004). For substances for which the lowest concentration tested resulted in an SI ≥ 3.0, an EC3 value was extrapolated according to the equation:

$$EC3_{ex} = 2^{\left\{\log_2(c) + \frac{(3-d)}{(b-d)} \times [\log_2(a) - \log_2(c)]\right\}}$$

where the point with the higher SI is denoted with the coordinates of (a, b) and the point with the lower SI is denoted (c, d) (Gerberick et al. 2004).

The EC3 values from each laboratory were used to calculate CV values for each substance. The resulting values for the first and second phase of the interlaboratory validation study are shown in **Tables C-VIII-6** and **C-VIII-7**, respectively. In the first phase of the interlaboratory validation study, CV values ranged from 4% (abietic acid) to 84% (glutaraldehyde) and the mean CV was 48% (**Table C-VIII-6**). Notably, although nickel (II) sulfate hexahydrate was a sensitizer in two of three laboratories, a CV could not be determined because one of the two laboratories that yielded a positive test demonstrated an insufficient dose response (i.e., an inverse dose response curve) from which to calculate an EC3 value. In the second phase of the interlaboratory validation study, CV values ranged from 32% (cobalt chloride) to 71% (potassium dichromate) and the mean CV was 45% (**Table C-VIII-7**).

The ICCVAM-recommended LLNA performance standards (ICCVAM 2009) indicate that interlaboratory reproducibility should be evaluated with at least two sensitizing chemicals with well-characterized activity in the traditional LLNA. Acceptable reproducibility is attained when each laboratory obtains ECt values (estimated concentration needed to produce an SI of a specified threshold) within 0.025% to 0.1% for 2,4-dinitrochlorobenzene and within 5% to 20% for hexyl cinnamic aldehyde (ICCVAM 2009). In the first phase of the interlaboratory validation study, four laboratories reported EC3 values outside the range indicated for 2,4-dinitrochlorobenzene; one laboratory obtained an EC3 value that was lower than the specified acceptance range (0.025%) and three laboratories obtained EC3 values that were higher than the specified acceptance range (0.1%) (**Table C-VIII-6**). For hexyl cinnamic aldehyde, all the laboratories obtained an EC3 value within the acceptance range (5% to 20%). In the second phase of the interlaboratory validation study, only hexyl cinnamic aldehyde was tested and all seven laboratories obtained EC3 values that were within the acceptance range indicated (**Table C-VIII-7**).

Table C-VIII-6EC3 Values from the First Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	EC3 (%)										Mean EC3 (%) ± SD	CV (%)
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10		
2,4-Dinitrochlorobenzene (+)	**0.034 (11.97)**	**0.109 (9.23)**	**0.056 (9.96)**	**0.031 (8.53)**	**0.129 (7.86)**	**0.042 (15.14)**	**0.016 (13.18)**	**0.095 (12.60)**	**0.040 (10.89)**	**0.169 (4.71)**	**0.072 ± 0.051**	**70**
Hexyl cinnamic aldehyde (+)	**9.983 (5.78)**	**12.412 (4.82)**	**14.90 (4.44)**	**9.340 (5.11)**	**18.131 (3.97)**	**13.130 (5.50)**	**7.706 (7.09)**	**7.924 (10.22)**	**17.070 (3.88)**	**15.235 (3.51)**	**12.583 ± 3.748**	**30**
Isopropanol (-)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Abietic acid (+)		8.196				7.544	7.676				7.805 ± 0.345	4
3-Aminophenol (+)	NA		NA					NA			NA	NA
Dimethyl isophthalate (-)	NA		NA				NA				NA	NA
Isoeugenol (+)				1.112	5.983				2.300		3.131 ± 2.540	81
Methyl salicylate (-)			NA				NA			NA	NA	NA
Formaldehyde (+)	1.747	1.480			NA						1.614 ± 0.189	12
Glutaraldehyde (+)	0.110	0.435			NA						0.272 ± 0.230	84
Cobalt chloride[2] (+)				NA[3]		0.063		0.137			0.100 ± 0.053	53
Nickel (II) sulfate hexahydrate (+)				NA		0.469		IDR			0.469 ± NA	NA

Note: Bolded text indicates substances that are ICCVAM-recommended murine local lymph node assay (LLNA) performance standards reference substances for evaluating interlaboratory reproducibility (ICCVAM 2009). Values in parentheses are highest stimulation index (SI) values achieved. For both 2,4-dinitrochlorobenzene and hexyl cinnamic aldehyde, the highest SI values achieved are from the highest dose tested (0.30% for 2,4-dinitrochlorobenzene and 25% for hexyl cinnamic aldehyde). Shading shows EC3 values (estimated concentration needed to produce an SI of three) that are outside of the acceptable range indicated in the ICCVAM-recommended LLNA performance standards: 5 - 20% for hexyl cinnamic aldehyde and 0.025 - 0.1% for 2,4-dinitrochlorobenzene.

Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of three; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; IDR = insufficient dose response; NA = not applicable; SD = standard deviation.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and second phase (1%, 3%, and 10%) of the interlaboratory validation study.

[3] Data not reported for the highest dose (3%), only for 0.3% and 1%.

Table C-VIII-7EC3 Values from the Second Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	EC3 (%)							Mean EC3 (%) ± SD	CV (%)
	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17		
Hexyl cinnamic aldehyde (+)	**9.127 (4.47)**	**8.764 (5.71)**	**7.590 (5.41)**	**7.938 (7.60)**	**15.184 (3.92)**	**6.230 (8.42)**	**7.542 (6.45)**	**8.911 ± 2.920**	**33**
Cobalt chloride[2] (+)	NA		NA	1.761			1.109	1.435 ± 0.461	32
Lactic acid (-)	NA		NA		NA	NA		NA	NA
Nickel (II) sulfate hexahydrate (+)	NA	NA		NA		NA		NA	NA
Potassium dichromate (+)	0.509	0.485			0.156		0.086	0.309 ± 0.219	71

Bolded text indicates a substance that is an ICCVAM-recommended murine local lymph node assay performance standards reference substance for evaluating interlaboratory reproducibility (ICCVAM 2009). Values in parentheses are highest stimulation index (SI) values achieved. For hexyl cinnamic aldehyde, the highest SI values achieved are from the highest dose tested (25%). None of the EC3 values are outside of the acceptable range indicated in the ICCVAM-recommended LLNA performance standards (5 - 20% for hexyl cinnamic aldehyde).

Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of three; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; NA = not applicable; SD = standard deviation.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

The interlaboratory CV values for both the first and second phases of the interlaboratory validation study for the LLNA: DA EC3 values were higher than that for the traditional LLNA EC3 values. The analysis of interlaboratory variation of EC3 values for the traditional LLNA reported CV values of 6.8 to 83.7% for five substances tested in five laboratories (**Table C-VIII-8**; ICCVAM 1999). Three of the same substances were evaluated in the traditional LLNA and the LLNA: DA (hexyl cinnamic aldehyde, 2,4-dinitrochlorobenzene, and isoeugenol). All interlaboratory CV values for the LLNA: DA were greater than that for the traditional LLNA. The CV of 70% for 2,4-dinitrochlorobenzene was greater than the two CV values of 37.4% and 27.2%, calculated from five values each, reported by ICCVAM (1999). The CV values of 30% and 33% for hexyl cinnamic aldehyde tested in the first and second phase of the LLNA: DA interlaboratory validation study, respectively, were both greater than the 6.8% reported by ICCVAM (1999). The CV of 81% for isoeugenol tested in the LLNA: DA was greater than the 41.2% reported by ICCVAM (1999).

Table C-VIII-8 Interlaboratory Reproducibility of the EC3 Values for Substances Tested in the Traditional LLNA[1]

Substance Name	EC3 (%)					CV (%)
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	
2,4-Dinitrochlorobenzene	0.3	0.5	0.6	0.9	0.6	37.4
	0.5	0.6	0.4	0.6	0.3	27.2
Hexyl cinnamic aldehyde	7.9	7.6	8.4	7.0	8.1	6.8
Isoeugenol	1.3	3.3	1.8	3.1	1.6	41.2
Eugenol	5.8	14.5	8.9	13.8	6.0	42.5
Sodium lauryl sulfate	13.4	4.4	1.5	17.1	4.0	83.7

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

[1] From ICCVAM 1999 report.

1.2.3 Interlaboratory Reproducibility – Qualitative Results (SI ≥ 2.0)

The qualitative (positive/negative) interlaboratory concordance analysis for the 12 substances that were tested during the first phase of the LLNA: DA interlaboratory validation study is shown in **Table C-VIII-9** for SI ≥ 2.0. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), ten substances tested in either three or 10 laboratories had consistent results leading to 100% (3/3 or 10/10) interlaboratory concordance for those substances. There were two discordant substances (3-aminophenol and nickel [II] sulfate hexahydrate) for which interlaboratory concordance was 67% (2/3). Two of the three laboratories that tested 3-aminophenol reported SI ≥ 2.0, at least at the highest dose tested (SI = 2.83 and 2.38, respectively) but one lab did not achieve SI ≥ 2.0 at any dose tested (Annex IV of this BRD). One of the three laboratories that tested nickel (II) sulfate hexahydrate reported a maximum SI = 1.52, while the other two laboratories produced SI ≥ 2.0 at all three doses tested (Annex IV of this BRD). Since 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: DA concordance data from the first phase of the interlaboratory validation study.

Table C-VIII-9 Qualitative Results for the First Phase of the Interlaboratory Validation Studies for the LLNA: DA (SI ≥ 2.0)

Substance Name[1]	Qualitative Results (Maximum SI)[2]										Concordance
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	
2,4-Dinitrochlorobenzene (+)	+ (11.97)	+ (9.23)	+ (9.96)	+ (8.53)	+ (7.86)	+ (15.14)	+ (13.18)	+ (12.60)	+ (10.89)	+ (4.71)	10/10
Hexyl cinnamic aldehyde (+)	+ (5.78)	+ (4.82)	+ (4.44)	+ (5.11)	+ (3.97)	+ (5.50)	+ (7.09)	+ (10.22)	+ (3.88)	+ (3.51)	10/10
Isopropanol (-)	- (1.54)	- (0.91)	- (1.01)	- (1.57)	- (0.76)	- (1.97)	- (1.45)	- (1.21)	- (0.70)	- (1.25)	10/10
Abietic acid (+)		+ (4.64)				+ (7.96)	+ (3.98)				3/3
3-Aminophenol (+)	**+ (2.83)**		**- (1.76)**					**+ (2.38)**			**2/3**
Dimethyl isophthalate (-)	- (1.34)		- (1.29)				- (1.26)				3/3
Isoeugenol (+)				+ (6.11)	+ (5.54)				+ (7.09)		3/3
Methyl salicylate (-)			- (1.55)				- (1.77)			- (0.83)	3/3
Formaldehyde (+)	+ (4.84)	+ (3.18)			+ (2.69)						3/3
Glutaraldehyde (+)	+ (5.00)	+ (3.39)			+ (2.57)						3/3
Cobalt chloride[3] (+)				+[4] (2.66)		+ (20.55)		+ (8.07)			3/3
Nickel (II) sulfate hexahydrate (+)				**-[5] (1.52)**		**+ (11.78)**		**+[5] (3.49)**			**2/3**

Boldface text indicates substances did not achieve 100% interlaboratory concordance.

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] (+) indicates sensitizer result and (-) indicates nonsensitizer result in the LLNA: DA test. Highest stimulation index value for each test is shown in parentheses.

[3] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

[4] Data not reported for the highest dose (3%), only for 0.3% and 1%.

[5] Insufficient dose response.

The qualitative (positive/negative) interlaboratory concordance analysis for the five substances that were tested during the second phase of the LLNA: DA interlaboratory validation study is shown in **Table C-VIII-10**. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), four substances (hexyl cinnamic aldehyde, cobalt chloride, lactic acid, and potassium dichromate) tested in either four or seven laboratories had consistent results leading to 100% (4/4 or 7/7) interlaboratory concordance for those substances. There was one discordant substance (nickel [II] sulfate hexahydrate) for which interlaboratory concordance was 75% (3/4). Three of the four laboratories that tested nickel (II) sulfate hexahydrate did not report a maximum SI ≥ 2.0, while the other laboratory produced an SI ≥ 2.0 at the highest dose tested. As was discussed previously, nickel (II) sulfate hexahydrate was also discordant among the laboratories that tested the substance in the first phase of the interlaboratory validation study and interlaboratory concordance was 67% (2/3). Notably, when analyzing the dose response curves for the seven tests performed for nickel (II) sulfate hexahydrate in the two-phased interlaboratory validation study, only one study demonstrated a sufficient dose response (i.e., a parallel increase in SI relative to increase in concentration). Furthermore, as mentioned previously, the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), and therefore there were no traditional LLNA concordance data for comparison with the LLNA: DA concordance data from the second phase of the interlaboratory validation study.

Table C-VIII-10 Qualitative Results for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA (SI ≥ 2.0)

Substance Name[1]	Qualitative Results (Maximum SI)[2]							Concordance
	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17	
Hexyl cinnamic aldehyde (+)	+ (4.47)	+ (5.71)	+ (5.41)	+ (7.60)	+ (3.92)	+ (8.42)	+ (6.45)	7/7
Cobalt chloride[3] (+)	+ (2.01)		+ (2.54)	+ (4.25)			+ (5.06)	4/4
Lactic acid (-)	- (0.93)		- (0.99)		- (0.97)	- (0.91)		4/4
Nickel (II) sulfate hexahydrate (+)	**- (0.79)**	**- (1.24)**		**+ (2.13)**		**- (1.56)**		**3/4**
Potassium dichromate (+)	+ (4.78)	+ (4.08)			+ (6.01)		+ (6.37)	4/4

Boldface text indicates substance that did not achieve 100% interlaboratory concordance.

Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] (+) indicates sensitizer result and (-) indicates nonsensitizer result in the LLNA: DA test. Highest stimulation index value for each test is shown in parentheses.

[3] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) interlaboratory validation studies.

1.2.4 Interlaboratory Reproducibility – EC2 Values

The available quantitative (i.e., EC2 value) data for interlaboratory reproducibility analysis were obtained from the LLNA: DA tests that yielded positive results (i.e., SI ≥ 2.0) during the first and second phase of the LLNA: DA interlaboratory validation study. The equation used for calculating EC2 values for the positive results was modified based on the method of linear interpolation reported by Gerberick et al. (2004) for the EC3 value:

$$EC2 = c + \left[\frac{(2-d)}{(b-d)}\right] \times (a-c)$$

where the data points lying immediately above and below the SI = 2.0 on the dose response curve have the coordinates of (a, b) and (c, d), respectively (Gerberick et al. 2004). For substances for which the lowest concentration tested resulted in an SI ≥ 2.0, an EC2 value was extrapolated according to the equation:

$$EC2_{ex} = 2^{\left\{\log_2(c) + \frac{(2-d)}{(b-d)} \times [\log_2(a) - \log_2(c)]\right\}}$$

where the point with the higher SI is denoted with the coordinates of (a, b) and the point with the lower SI is denoted (c, d) (Gerberick et al. 2004).

The EC2 values from each laboratory were used to calculate CV values for each substance. The resulting values for the first and second phase of the interlaboratory validation study are shown in **Tables C-VIII-11** and **C-VIII-12**, respectively. In the first phase of the interlaboratory validation study, CV values ranged from 14% (abietic acid) to 134% (isoeugenol) and the mean CV was 70% (**Table C-VIII-11**). In the second phase of the interlaboratory validation study, CV values ranged from 16% (hexyl cinnamic aldehyde) to 100% (cobalt chloride) and the mean CV was 57% (**Table C-VIII-12**).

The ICCVAM-recommended 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 (estimated concentration needed to produce an SI of a specific threshold) within 0.025% to 0.1% for 2,4-dinitrochlorobenzene and within 5% to 20% for hexyl cinnamic aldehyde (ICCVAM 2009). In the first phase of the interlaboratory validation study, seven laboratories reported EC2 values outside the range indicated for 2,4-dinitrochlorobenzene; all seven laboratories obtained EC2 values that were lower than the specified acceptance range (0.025%) (**Table C-VIII-11**). For hexyl cinnamic aldehyde, all the laboratories obtained an EC2 value within the acceptance range (5% to 20%). In the second phase of the interlaboratory validation study, only hexyl cinnamic aldehyde was tested and two of the seven laboratories obtained EC2 values that were below the acceptance range indicated (**Table C-VIII-12**).

Table C-VIII-11 EC2 Values from the First Phase Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	EC2 (%)										Mean EC2 (%) ± SD	CV (%)
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10		
2,4-Dinitrochlorobenzene (+)	**0.020 (11.97)**	**0.023 (9.23)**	**0.026 (9.96)**	**0.016 (8.53)**	**0.091 (7.86)**	**0.016 (15.14)**	**0.007 (13.18)**	**0.013 (12.60)**	**0.019 (10.89)**	**0.093 (4.71)**	**0.032 ± 0.032**	**98**
Hexyl cinnamic aldehyde (+)	**6.962 (5.78)**	**7.461 (4.82)**	**8.404 (4.44)**	**6.460 (5.11)**	**11.057 (3.97)**	**7.463 (5.50)**	**5.850 (7.09)**	**6.140 (10.22)**	**9.191 (3.88)**	**7.256 (3.51)**	**7.624 ± 1.570**	**21**
Isopropanol (-)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Abietic acid (+)		4.760				5.393	6.333				5.495 ± 0.791	14
3-Aminophenol (+)	1.877		NA					3.179			2.528 ± 0.921	36
Dimethyl isophthalate (-)	NA		NA				NA				NA	NA
Isoeugenol (+)				0.407	4.399				0.375		1.727 ± 2.314	134
Methyl salicylate (-)			NA				NA			NA	NA	NA
Formaldehyde (+)	0.262	0.729			2.019						1.003 ± 0.910	91
Glutaraldehyde (+)	0.072	0.268			0.118						0.153 ± 0.103	67
Cobalt chloride[2] (+)				0.283[3]		0.032		0.079			0.131 ± 0.134	102
Nickel (II) sulfate hexahydrate (+)				IDR		0.235		IDR			0.235 ± NA	NA

Bolded text indicates substances that are ICCVAM-recommended murine local lymph node assay (LLNA) performance standards reference substances for evaluating interlaboratory reproducibility (ICCVAM 2009). Values in parentheses are highest stimulation index (SI) values achieved. For both 2,4-dinitrochlorobenzene and hexyl cinnamic aldehyde, the highest SI values achieved were from the highest dose tested (0.30% for 2,4-dinitrochlorobenzene and 25% for hexyl cinnamic aldehyde). Shading shows EC2 values that are outside of the acceptable range indicated by the ICCVAM-recommended LLNA performance standards: 5 - 20% for hexyl cinnamic aldehyde and 0.025 - 0.1% for 2,4-dinitrochlorobenzene.

Abbreviations: CV = coefficient of variation; EC2 = estimated concentration needed to produce a stimulation index of two; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; IDR = insufficient dose response; NA = not applicable; SD = standard deviation.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) interlaboratory validation studies.

[3] Data not reported for the highest dose (3%), only for 0.3% and 1%.

Table C-VIII-12 EC2 Values from the Second Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance Name[1]	EC2 (%)							Mean EC2 (%) ± SD	CV (%)
	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17		
Hexyl cinnamic aldehyde (+)	**6.348 (4.47)**	**5.983 (5.71)**	**5.954 (5.41)**	**4.849 (7.60)**	**7.451 (3.92)**	**4.662 (8.42)**	**6.024 (6.45)**	**5.896 ± 0.937**	**16**
Cobalt chloride[2] (+)	4.929		1.875	0.821			0.461	2.021 ± 2.029	100
Lactic acid (-)	NA		NA		NA	NA		NA	NA
Nickel (II) sulfate hexahydrate (+)	NA	NA		NA		8.404		8.404 ± NA	NA
Potassium dichromate (+)	0.159	0.128			0.055		0.047	0.097 ± 0.055	56

Bolded text indicates a substance that is an ICCVAM-recommended murine local lymph node assay (LLNA) performance standards reference substance for evaluating interlaboratory reproducibility (ICCVAM 2009). Values in parentheses are highest stimulation index (SI) values achieved. For hexyl cinnamic aldehyde, the highest SI values achieved were from the highest dose tested (25%). Two of the EC2 values are outside of the acceptable range indicated by the ICCVAM-recommended LLNA performance standards (5 - 20% for hexyl cinnamic aldehyde), indicated by shading.

Abbreviations: CV = coefficient of variation; EC2 = estimated concentration needed to produce a stimulation index of two; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; NA = not applicable; SD = standard deviation.

[1] (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional murine local lymph node assay results.

[2] Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

The interlaboratory CV values for both the first and second phases of the interlaboratory validation study for the LLNA: DA EC2 values were higher than that for the traditional LLNA EC3 values. The analysis of interlaboratory variation of EC3 values for the traditional LLNA reported CV values of 6.8 to 83.7% for five substances tested in five laboratories (**Table C-VIII-8**; ICCVAM 1999). Three of the same substances were evaluated in the traditional LLNA and the LLNA: DA (hexyl cinnamic aldehyde, 2,4-dinitrochlorobenzene, and isoeugenol). All interlaboratory CV values for LLNA: DA EC2 values were greater than that for the traditional LLNA. The CV of 98% for 2,4-dinitrochlorobenzene was greater than the two CV values of 37.4% and 27.2% (which were calculated from five values each), reported by ICCVAM (1999). The CV of 21% and 16% for hexyl cinnamic aldehyde tested in the first and second phase of the LLNA: DA interlaboratory validation study, respectively, were both greater than the 6.8% reported by ICCVAM (1999). The CV of 134% for isoeugenol tested in the LLNA: DA was greater than the 41.2% reported by ICCVAM (1999).

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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

The 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
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 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

The 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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JaCVAM statement

on the LLNA-DA for skin sensitization testing

At the meeting concerning the above method, held on 28 August 2008 at the National Institute of Health Sciences (NIHS), Tokyo, Japan, the noncommission members of the Japanese Center for the Validation of Alternative Methods (JaCVAM) Regulatory Acceptance Board [1] unanimously endorsed the following statement:

Following the review of the results of the Ministry of Health, Labour and Welfare (MHLW)-funded validation study on the LLNA (Local Lymph Node Assay) -DA coordinated by Japanese Society for Alternative to Animal Experiments (JSAAE), it is concluded that the LLNA-DA can be used for distinguishing between sensitizer and non-sensitizer chemicals within the context of the OECD testing guideline No. 429 on Skin sensitization: LLNA.

The JaCVAM Regulatory Acceptance Board has been regularly kept informed of the progress of the study, and this endorsement is based on an assessment of various documents, including, in particular, the report on the results from the study, and also on the evaluation supported by JSAAE of the study prepared for the JaCVAM ad hoc peer review panel.

[redacted]

Hajime Kojima,
Director,
JaCVAM,
National Centre for Biological Safety and Research (NCBSR)
NIHS,
Tokyo

Tohru Inoue,
Director,
NCBSR,
NIHS,
Tokyo

4 November 2008

1. The JaCVAM Regulatory Acceptance Board was established by the JaCVAM Steering Committee, and is composed of nominees from the industry and academia.

This statement was endorsed by the following members of the JaCVAM Regulatory Acceptance Board:

Mr. Tohru Inoue (NIHS)
Mr. Makoto Hayashi (An-pyo Center*)
Mr. Noriho Tanaka (FDSC*)
Mr. Takemi Yoshida (Showa Univ.)
Ms Masako Mizoguchi (St. Marianna Univ. School of Medicine)
Mr. Fumio Sagami (Eisai Co., Ltd./JPMA*)
Ms Yuko Okamoto (KOSÉ Corporation/JCIA*)
Mr. Hiroshi Onodera (PMDA*)
Mr. Yoshiaki Ikarashi (NIHS)

The following members of the JaCVAM Steering Committee were involved as observers in the consultation process, but not in the endorsement process itself.

Mr. Yasuo Ohno (NIHS)
Mr. Kenichi Nakazawa (NIHS)
Mr. Hiroshi Itagaki (JSAAE)
Mr. Mitsuteru Masuda (JaCVAM)
Mr. Hajime Kojima (JaCVAM)

* An-pyo Cneter: Biosafety Research Center Food, Drugs and Pesticides
FDSC: Food and Drug Safety Center
PMDA: Pharmaceuticals and Medical Devices Agency
JPMA: Japan Pharmaceutical Manufacturers Association
JCIA: Japan Cosmetic Industry Association

Appendix F

Federal Register Notices and Public Comments

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Appendix F1

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 F2

Public Comments Received in Response to *Federal Register* Notices

Public comments are available on request from NICEATM

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

- 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

- 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

- 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)

- 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

- 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

- Brian E. Harvey, M.D., Ph.D. (Sanofi Aventis)

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Appendix F3

Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) Comments

SACATM Meeting on June 18-19, 2008

The fmeeting minutes are available online at:
https://ntp.niehs.nih.gov/events/past/index.html?type=SACATM

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Appendix F4

Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) Comments

SACATM Meeting on June 25-26, 2009

The meeting minutes are available online at:
https://ntp.niehs.nih.gov/events/past/index.html?type=SACATM

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Appendix G

Relevant Skin Sensitization Regulations and Testing Guidelines

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Appendix G1

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 G2**)
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 G3**)
OECD	Chemicals	NA	OECD Test Guideline 429 (2002) (see **Appendix G4**) OECD Test Guideline 406 (1992) (see **Appendix G5**)
ICH	NA	NA	No Specific Guidelines, Guidances, or Recommendations

Appendix G2

EPA Health Effects Test Guidelines OPPTS 870.2600: Skin Sensitization (March 2003)

EPA Health Effects Test Guidelines are available at:
https://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-870-health-effects-test-guidelines

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Appendix G3

International Organization for Standardization - ISO 10993-10: Biological Evaluation of Medical Devices Part 10: Tests for Irritation and Delayed-type Hypersensitivity (2002)

Document available from the ISO website:

http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=33364

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Appendix G4

OECD Test Guideline 429: Skin Sensitisation – Local Lymph Node Assay

Available at:
https://www.oecd-ilibrary.org/environment/test-no-429-skin-sensitisation_9789264071100-en

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Appendix G5

OECD Test Guideline 406: Skin Sensitisation

Available at:

https://www.oecd-ilibrary.org/environment/test-no-406-skin-sensitisation_9789264070660-en

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