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

March 2010

NIH Publication Number 10-7551

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This document is available electronically at
http://iccvam.niehs.nih.gov/methods/immunotox/llna-DA/TMER.htm

When referencing this document, please cite as follows:
ICCVAM. 2010. 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. NIH Publication No. 10-7551. Research Triangle Park, NC: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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Other Acknowledgements

ICCVAM and NICEATM gratefully acknowledge the following individuals and institutions that submitted data to NICEATM for the evaluation of the LLNA: DA test method.

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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.

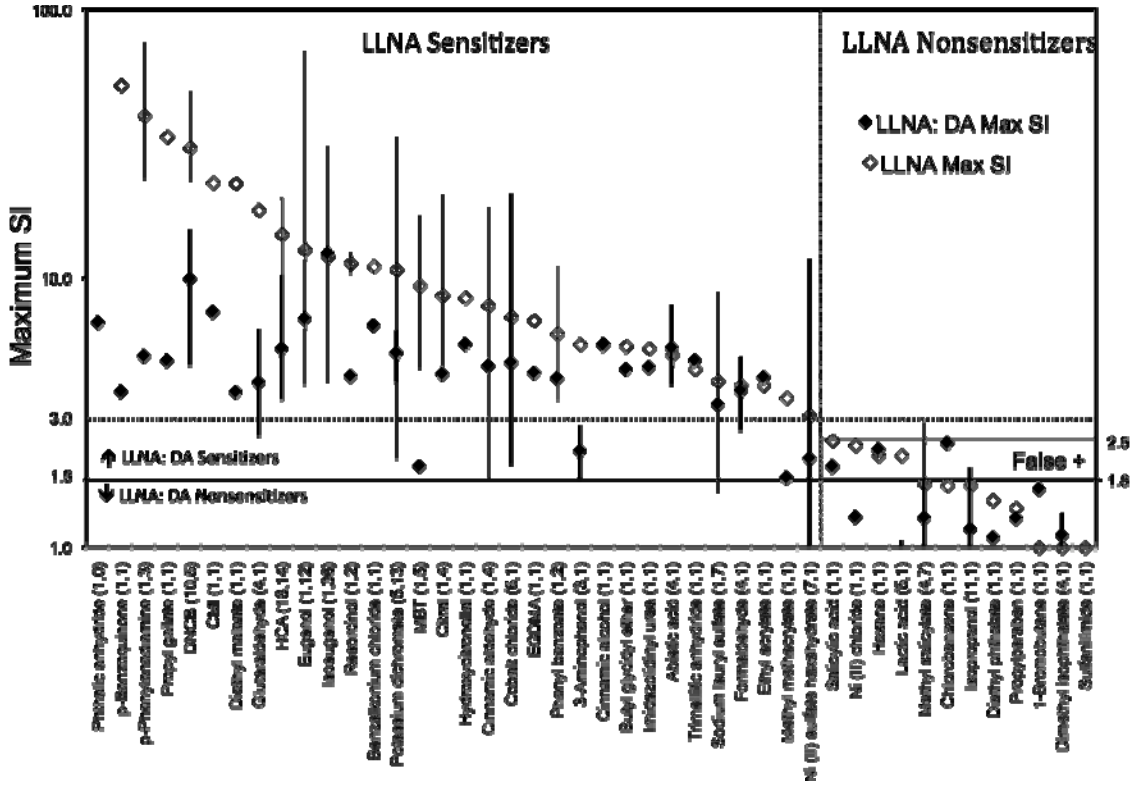
[4] The mean ATP of at least one treatment group was outside the 95% confidence interval for the mean ATP of the vehicle control group.

[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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