

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

**Interagency Coordinating Committee on the
Validation of Alternative Methods**

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

**National Institute of Environmental Health Sciences
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List of Abbreviations and Acronyms

ACD	Allergic contact dermatitis
ACE	Acetone
AOO	Acetone: olive oil (4:1 by volume)
BRD	Background review document
BrdU	Bromodeoxyuridine
CASRN	Chemical Abstracts Service Registry Number
CI	Confidence interval
CMI	5-Chloro-2-methyl-4-isothiazolin-3-one
CPSC	U.S. Consumer Product Safety Commission
CV	Coefficient of variation
DMF	<i>N,N</i> -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	<i>Federal Register</i>
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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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 workdays¹ 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](http://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 ³H-methyl thymidine or ¹²⁵I-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 2851-3), 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 website³ 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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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 ³H-methyl thymidine or ¹²⁵I-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.

⁴ *Traditional LLNA* refers to the ICCVAM-recommended LLNA protocol, which measures lymphocyte proliferation based on incorporation of ³H-methyl thymidine or ¹²⁵I-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 $SI \geq 1.8$ 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 $SI \geq 1.8$ 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 $SI \geq 1.8$ 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 $2.74\% \pm 0.58\%$, with a corresponding CV of 21%. Eugenol had an EC3 of $5.06\% \pm 0.55\%$ and a CV of 11%. The mean EC1.8 value and corresponding CV for isoeugenol and eugenol were $0.87\% \pm 0.31\%$ (36% CV) and $3.38\% \pm 0.79\%$ (23% CV), respectively.

Both phases of an interlaboratory validation study included qualitative analyses of LLNA: DA reproducibility. An $SI \geq 1.8$ 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 $SI \geq 1.8$ 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 $SI \geq 1.8$) 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.