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

Interagency Coordinating Committee on the Validation of Alternative Methods

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

National Institute of Environmental Health Sciences
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List of Abbreviations and Acronyms

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

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

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

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

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Preface

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

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

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

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

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

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

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

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

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

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

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

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

Executive Summary

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

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

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

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

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

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

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

ICCVAM Recommendations: Test Method Usefulness and Limitations

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

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

ICCVAM Recommendations: Test Method Protocol

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

ICCVAM Recommendations: Future Studies

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

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

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

ICCVAM Recommendations: Performance Standards

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

Validation Status of the LLNA: BrdU-ELISA

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

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

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

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

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

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

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

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

ICCVAM Consideration of Independent Peer Review Panel Report and Other Comments

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