Non-radioactive Murine Local Lymph Node Assay: Flow Cytometry Test Method Protocol (LLNA: BrdU-FC)

Draft Background Review Document

January 2008

[This Page Intentionally Left Blank]

Table of Contents

Page Number

List of Tables

List of Figures

Page Number

List of Abbreviations and Acronyms

ACD	Allergic contact dermatitis
AOO	Acetone: olive oil
BRD	Background Review Document
BrdU	Bromodeoxyuridine
BT	Buehler Test
CASRN	Chemical Abstracts Service Registry Number
Conc.	Concentration tested
CPSC	U.S. Consumer Product Safety Commission
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
EC3	Estimated concentration needed to produce a stimulation index of three
ECVAM	European Centre for the Validation of Alternative Methods
eLLNA: BrdU-FC	Enhanced LLNA: BrdU-FC
EPA	U.S. Environmental Protection Agency
ESAC	ECVAM Scientific Advisory Committee
FDA	U.S. Food and Drug Administration
FR	*Federal Register*
GHS	United Nations Globally Harmonized System for the Labelling and Classification of Chemicals
GLP	Good Laboratory Practice
GPMT	Guinea Pig Maximization Test
HCA	Hexyl cinnamic aldehyde
HMT	Human Maximization Test
HPTA	Human Patch Test Allergen
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IR	Information requested
ISO	International Standards Organization
IWG	Immunotoxicity Working Group
JaCVAM	Japanese Center for the Validation of Alternative Methods
K_{ow}	Octanol-water partition coefficient
LNC	Lymph node cells
LLNA	Local Lymph Node Assay
LLNA: BrdU-FC	LLNA with detection of bromodeoxyuridine incorporation by flow cytometry
MEK	Methyl ethyl ketone
MeSH	Medical Subject Headings
Min	Minimal
Mod	Moderate
NA	Not available
NC	Not calculated
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences

NT	Not tested
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
OPPTS	Office of Prevention, Pesticides and Toxic Substances
Res	Result
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
S.D.	Standard Deviation
SI	Stimulation Index
SLS	Sodium lauryl sulfate
TG	Test Guideline
U.S.	United States
Unk	Unknown
Veh.	Vehicle
vs.	Versus
w/v	Weight to volume ratio

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Designated Agency Representatives[1]

Agency for Toxic Substances and Disease Registry
• Moiz Mumtaz, Ph.D.

Consumer Product Safety Commission
• Marilyn L. Wind, Ph.D. (Chair)
◊ Kristina Hatlelid, Ph.D.
* Joanna Matheson, Ph.D.

Department of Agriculture
• Jodie Kulpa-Eddy, D.V.M.
◊ Elizabeth Goldentyer, D.V.M.

Department of Defense
• Robert E. Foster, Ph.D.
◊ Patty Decot
* Peter J. Schultheiss, D.V.M., D.A.C.L.A.M.
* Harry Salem, Ph.D.

Department of Energy
• Michael Kuperberg, Ph.D.
◊ Marvin Stodolsky, Ph.D.

Department of the Interior
• Barnett A. Rattner, Ph.D.
◊ Sarah Gerould, Ph.D.

Department of Transportation
• George Cushmac, Ph.D.
◊ Steve Hwang, Ph.D.

Environmental Protection Agency
Office of Science Coordination and Policy
• Karen Hamernik, Ph.D.
Office of Research and Development
◊ Julian Preston, Ph.D.
* Suzanne McMaster, Ph.D.
OECD Test Guidelines Program
* Jerry Smrchek, Ph.D.
Office of Pesticides Programs
* Amy Rispin, Ph.D.
* Deborah McCall

Food and Drug Administration
Office of Science
• Suzanne Fitzpatrick, Ph.D., D.A.B.T.
Center for Drug Evaluation and Research
◊ Abigail C. Jacobs, Ph.D.
Center for Devices and Radiological Health
* Melvin E. Stratmeyer, Ph.D.
Center for Biologics Evaluation and Research
* Richard McFarland, Ph.D., M.D.
* Ying Huang, Ph.D.
Center for Food Safety and Nutrition
* David G. Hattan, Ph.D.
* Robert L. Bronaugh, Ph.D.
Center for Veterinary Medicine
* Devaraya Jagannath, Ph.D.
* M. Cecilia Aguila, D.V.M.
National Center for Toxicological Research
* William T. Allaben, Ph.D.
* Paul Howard Ph.D.
Office of Regulatory Affairs
* Lawrence A. D'Hoostelaere, Ph.D.

National Cancer Institute
• Alan Poland, M.D.
◊ T. Kevin Howcroft, Ph.D.

National Institute of Environmental Health Sciences
• William S. Stokes, D.V.M., D.A.C.L.A.M.
◊ Raymond R. Tice, Ph.D.
* Rajendra S. Chhabra, Ph.D., D.A.B.T
* Jerrold J. Heindel, Ph.D.

National Institute for Occupational Safety and Health
• Paul Nicolaysen, V.M.D.
◊ K. Murali Rao, M.D., Ph.D.

National Institutes of Health
• Margaret D. Snyder, Ph.D.

National Library of Medicine
◊ Jeanne Goshorn, M.S.

Occupational Safety and Health Administration
• Surender Ahir, Ph.D.

• Principal Agency Representative
◊ Alternate Principal Agency Representative
* Other Designated Agency Representative

[1] Roster as of January 2008.

Acknowledgements

The following individuals are acknowledged for their contributions to the LLNA: BrdU-FC test method review process

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Immunotoxicity Working Group (IWG)

U.S. Consumer Product Safety Commission
Marilyn Wind, Ph.D.
Joanna Matheson, Ph.D. (IWG Co-Chair)

U.S. Environmental Protection Agency
Karen Hamernik, Ph.D.
Masih Hashim, Ph.D.
Marianne Lewis
Debbie McCall
Timothy McMahon, Ph.D.
Amy Rispin, Ph.D.
MaryJane Selgrade, Ph.D.
Marsha Ward, Ph.D.
Ronald E. Ward, Ph.D.

U.S. Food and Drug Administration
Ruth Barrett, Ph.D., D.V.M.
Paul C. Brown, Ph.D.
Abigail Jacobs, Ph.D. (IWG Co-Chair)
Dan Lyle, Ph.D.
Jiaqin Yao, Ph.D.

National Institute of Environmental Health Sciences
Dori Germolec, Ph.D.
William S. Stokes, D.V.M., D.A.C.L.A.M.
Raymond R. Tice, Ph.D.

National Institute for Occupational Health & Safety
Jean Meade, D.V.M., Ph.D.

European Centre for the Validation of Alternative Methods (ECVAM) Liaison
Silvia Casati, Ph.D.

Japanese Center for the Validation of Alternative Methods (JaCVAM) Liaison
Hajime Kojima, Ph.D.

National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

National Institute of Environmental Health Sciences

William Stokes, D.V.M., D.A.C.L.A.M.	Director; Project Officer
Raymond Tice, Ph.D.	Deputy Director
Deborah McCarley	Special Assistant; Asst. Project Officer

NICEATM Support Contract Staff (Integrated Laboratory Systems [ILS], Inc.)

David Allen, Ph.D.	Principal Investigator
Douglas Winters, M.S.	Project Manager
Neepa Choksi, Ph.D.	Senior Staff Toxicologist
Judy Strickland, Ph.D., D.A.B.T.	Senior Staff Toxicologist
Frank Deal, M.S.	Staff Toxicologist
Elizabeth Lipscomb, Ph.D.	Staff Toxicologist
Eleni Salicru, Ph.D.	Staff Toxicologist
Thomas Burns, M.S.	Senior Project Coordinator/Technical Writer
Michael Paris	Senior Project Coordinator/Technical Writer
Patricia Ceger, M.S.	Project Coordinator/Technical Writer
James Truax, M.A.	Project Coordinator/Technical Writer
Catherine Sprankle	Communications Specialist/Web Developer
Linda Litchfield	Meeting Planner and Coordinator

Other Acknowledgements

ICCVAM and NICEATM gratefully acknowledge George DeGeorge, Ph.D. and Melissa Kirk, Ph.D. of MB Research Labs (Spinnerstown, PA, USA) for submitting data to NICEATM used for the evaluation of the LLNA: BrdU-FC.

Preface

In 1998, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) in conjunction with the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) evaluated the validation status of the murine local lymph node assay (LLNA) as an alternative to guinea pig test methods for assessing the allergic contact dermatitis (ACD) potential of substances. As described in the 1999 ICCVAM evaluation report[2], ICCVAM recommended that the LLNA could be used as a valid substitute for the accepted guinea pig test methods, in most ACD testing situations.

Based on the ICCVAM recommendations, the ICCVAM member agencies that require the regulatory submission of ACD data accepted the LLNA, with identified limitations, as an alternative to guinea pig tests for assessing ACD. In 2002, the LLNA was adopted as Test Guideline 429 by the 30-member countries of the Organisation for Economic Co-operation and Development (OECD)[3].

On January 10, 2007, the U.S. Consumer Product Safety Commission (CPSC) formally nominated several activities related to the LLNA for evaluation by ICCVAM and NICEATM[4]. One of the nominated activities was an assessment of the validation status of non-radioactive alternatives to the current version of the LLNA, which uses radioactivity. After considering comments from the public and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) on this nomination, ICCVAM assigned it a high priority, and directed NICEATM and the ICCVAM Immunotoxicity Working Group (IWG) to conduct a review of the current literature and an evaluation of the available data. The information described in this background review document (BRD) was compiled by ICCVAM in response to this nomination. ICCVAM and its IWG developed draft test method recommendations based on this evaluation. An independent peer review panel (Panel) is

[2] ICCVAM 1999. The murine local lymph node assay: A test method for assessing the allergic contact dermatitis potential of chemical/compounds. NIH Publication No. 99-4494. Research Triangle Park, NC: National Toxicology Program (available at http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf

[3] OECD. 2002. Test guideline 429. Skin Sensitisation: Local Lymph Node Assay, adopted April 24, 2002. In: OECD Guidelines for Testing of Chemicals. Paris:OECD (available at http://www.oecd.org/document/55/0,2340,en_2649_34377_2349687_1_1_1_1,00.html)

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

being convened to peer review the BRD and to evaluate the extent to which the information contained in the BRD support the draft recommendations. ICCVAM will consider the conclusions and recommendations of the Panel, along with comments received from the public and SACATM, when developing a final BRD and final recommendations on the usefulness and limitations of each non-radioactive alternative LLNA test method that is being considered.

We gratefully acknowledge the organizations and scientists who provided data and information for this document. We would also like to recognize the efforts of the individuals who contributed to the preparation of this BRD. These include David Allen, Ph.D., Thomas Burns, M.S., Neepa Choksi, Ph.D., Michael Paris, Eleni Salicru, Ph.D., Catherine Sprankle, Judy Strickland, Ph.D., and Doug Winters, M.S., of Integrated Laboratory Systems, Inc., the NICEATM Support Contractor, as well as the members of the ICCVAM IWG and the ICCVAM representatives who subsequently reviewed and provided comments throughout the process leading to this final draft version. We also want to thank Raymond Tice, Ph.D., Deputy Director of NICEATM, for his contributions to this project. Finally, we want to recognize the excellent leadership of the IWG Co-chairs, Abigail Jacobs, Ph.D. (FDA) and Joanna Matheson, Ph.D. (CPSC).

Marilyn Wind, Ph.D.
Deputy Associate Executive Director
Directorate for Health Sciences
U.S. Consumer Product Safety Commission
Chair, ICCVAM

William S. Stokes, D.V.M., D.A.C.L.A.M.
Rear Admiral, U.S. Public Heath Service
Director, NICEATM
Executive Director, ICCVAM

January 7, 2008

Executive Summary

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended to U.S. Federal agencies that the murine local lymph node assay (LLNA) is a valid substitute for currently accepted guinea pig test methods to assess the allergic contact dermatitis (ACD) potential of many, but not all, types of substances. The recommendation was based on a comprehensive evaluation that included an independent scientific peer review panel (Panel) assessment of the validation status of the LLNA. The Panel report and the ICCVAM recommendations (ICCVAM 1999) are available at the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)/ICCVAM website (http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf). The LLNA was subsequently incorporated into national and international test guidelines for the assessment of skin sensitization (Organisation for Economic Co-operation and Development [OECD] Test Guideline 429 [OECD 2002]; International Standards Organization [ISO] 10993-10: Tests for Irritation and Sensitization [ISO 2002]; U.S. Environmental Protection Agency [EPA] Health Effect Testing Guidelines on Skin Sensitization [EPA 2003]).

On January 10, 2007, the U.S. Consumer Product Safety Commission (CPSC) formally nominated several activities related to the LLNA for evaluation by ICCVAM and NICEATM (Available at http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf). One of the nominated activities was an assessment of the validation status of non-radioactive alternatives to the current version of the LLNA ([ICCVAM 1999, Dean et al. 2001] referred to hereafter as the "traditional LLNA"), which uses radioactivity to detect sensitizers. The information described in this background review document (BRD) was compiled by ICCVAM and NICEATM in response to this nomination. The BRD provides a comprehensive review of available data and information regarding the usefulness and limitations of one of these methods, the LLNA with detection of bromodeoxyuridine (BrdU) incorporation by flow cytometry (referred to hereafter as the "LLNA: BrdU-FC").

The LLNA: BrdU-FC was developed by MB Research Labs (2001). While the traditional LLNA assesses cell proliferation by measuring the incorporation of radioactivity into the deoxyribonucleic acid (DNA) of dividing lymph node cells, the LLNA: BrdU-FC assesses cell

proliferation by measuring the incorporation of the thymidine analog bromodeoxyuridine (BrdU) into the DNA of dividing lymphocytes using flow cytometry. A Stimulation Index (SI), the ratio of the mean BrdU incorporation into the lymph nodes of mice in the test substance group to the mean BrdU incorporation into the lymph nodes of mice in the vehicle group, greater than three identifies a substance as a sensitizer. Other than the procedure for measuring lymph node cell proliferation, the protocol for the LLNA: BrdU-FC is similar to that of the traditional LLNA (Dean et al. 2001; ICCVAM 1999). The LLNA: BrdU-FC also includes enhancements (referred to hereafter as the "eLLNA: BrdU-FC"), for substances with SI $\geq$ 3 that include assessment of immunophenotypic markers to distinguish sensitizers from irritants.

The accuracy and reliability of the LLNA: BrdU-FC and the eLLNA: BrdU-FC was assessed using data for up to 45 substances that were submitted by MB Research Labs (2007). Of these 45 substances, 37 had LLNA: BrdU-FC, traditional LLNA, and GP data while 42 substances had LLNA: BrdU-FC, traditional LLNA, and human data. Three of the 45 substances produced divergent results when tested at least twice in the traditional LLNA and/or in the LLNA: BrdU-FC (referred to hereafter as "equivocal" substances). To account for the equivocal substances, two separate accuracy analyses were conducted: 1) only the substances with unequivocal LLNA: BrdU-FC results were evaluated, and 2) the three equivocal substances were included by using the more conservative result (i.e., positive) for all three substances.

When the LLNA: BrdU-FC was compared to the traditional LLNA (and excluding the three equivocal substances) the LLNA: BrdU-FC had an accuracy of 93% (39/42), a sensitivity of 100% (24/24), a specificity of 83% (15/18), a false positive rate of 17% (3/18), and a false negative rate of 0% (0/24). Including the three equivocal substances resulted in an accuracy for the LLNA: BrdU-FC of 91% (41/45), a sensitivity of 100% (26/26), a specificity of 79% (15/19), a false positive rate of 21% (4/19), and a false negative rate of 0% (0/26).

When the eLLNA: BrdU-FC was compared to the traditional LLNA, accuracy was 90% (38/42), sensitivity was 92% (22/24), specificity was 89% (16/18), the false positive rate was 11% (3/18), and false negative rate was 8% (2/24). Using the traditional LLNA as the reference classification, two nonsensitizers and two sensitizers were not identified correctly. However, the two substances identified by the eLLNA: BrdU-FC as nonsensitizers (ethylene glycol dimethacrylate and sodium lauryl sulfate) were also identified as nonsensitizers by GP skin sensitization tests.

Sodium lauryl sulfate is also considered a nonsensitizer based on human data (i.e., human maximization test), but ethylene glycol dimethacrylate is considered a sensitizer based on its inclusion as a human patch test kit allergen. Including the three equivocal substances resulted in an accuracy for the eLLNA: BrdU-FC of 89% (40/45), a sensitivity of 92% (24/26), a specificity of 84% (16/19), a false positive rate of 16% (3/19), and a false negative rate of 8% (2/26).

The LLNA: BrdU-FC and the eLLNA: BrdU-FC results included 13 of the 18 minimum substances proposed in the *Revised Draft ICCVAM Murine Local Lymph Node Assay Performance Standards* (ICCVAM 2007); there were eight sensitizers and five nonsensitizers. The sensitizer/nonsensitizer outcome of the LLNA: BrdU-FC was consistent with the outcome of the traditional LLNA with the exception of two substances (one sensitizer and one non-sensitizer) that produced equivocal results in the LLNA: BrdU-FC (i.e., produced an equal number of divergent results when tested at least twice). Three optional reference substances included in the draft ICCVAM Performance Standards (2007) were also tested in the LLNA: BrdU-FC/eLLNA: BrdU-FC. Although the LLNA: BrdU-FC classifications for two substances that yielded false positive results in the traditional LLNA were consistent with the traditional LLNA classification, the eLLNA: BrdU-FC correctly classified them as irritants rather than sensitizers. The third optional reference substance was classified by the LLNA: BrdU-FC and the eLLNA: BrdU-FC as a non-sensitizer, which is the same incorrect result produced by the traditional LLNA.

Intralaboratory reproducibility for the LLNA: BrdU-FC and the eLLNA: BrdU-FC outcomes were assessed with a coefficient of variation (CV) analysis of SI values. The CVs for the SI values of 25% hexyl cinnamic aldehyde, the positive control substance, tested in various vehicles ranged from 30.1-52.6%. Interlaboratory reproducibility was not assessed because all LLNA: BrdU-FC results were produced in one laboratory, MB Research Labs.

The LLNA: BrdU-FC and the eLLNA: BrdU-FC will use the same number of animals when compared to the traditional LLNA. However, since use of the traditional LLNA is restricted in some institutions because it involves radioactivity, availability and use of the non-radioactive LLNA: BrdU-FC and the eLLNA: BrdU-FC test methods may lead to further reduction in use of the GP tests, which would provide for reduced animal use and increased refinement due to the avoidance of pain and distress in the LLNA procedure.

The transferability of the LLNA: BrdU-FC and the eLLNA: BrdU-FC is expected to be similar to the traditional LLNA. Compared to the traditional LLNA, the LLNA: BrdU-FC and the eLLNA: BrdU-FC will not require facilities, equipment, and licensing permits for handling radioactive materials. However, these test methods require a flow cytometer. The level of training and expertise needed to conduct the LLNA: BrdU-FC and the eLLNA: BrdU-FC should be similar to the traditional LLNA except that the understanding and use of flow cytometry is required.

ICCVAM has developed draft recommendations for the LLNA: BrdU-FC with regard to its usefulness and limitations, test method protocol, and future studies to further characterize its usefulness and limitations. These are provided in a separate document, *Draft ICCVAM Test Method Recommendations, Non-radioactive Murine Local Lymph Node Assay: Flow Cytometry Test Method Protocol (LLNA: BrdU-FC).*

1.0 Introduction

1.1 Historical Background

In 1999, the Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM) recommended that the murine local lymph node assay (LLNA) is a valid substitute for currently accepted guinea pig (GP) test methods to assess the allergic contact dermatitis (ACD) potential of many, but not all, types of substances. The recommendation was based on a comprehensive evaluation that included an independent scientific peer review panel (Panel) assessment of the validation status of the LLNA. The Panel report and the ICCVAM recommendations (ICCVAM 1999) are available at the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)/ICCVAM website (http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf).

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

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

comprehensive review of available data and information regarding the usefulness and limitations of one of these methods, the LLNA with detection of bromodeoxyuridine (BrdU) (referred to hereafter as the "LLNA: BrdU-FC").

1.2 The LLNA: BrdU-FC

The LLNA: BrdU-FC was developed by MB Research Labs (2001). The flow cytometry based murine local lymph node assay was developed as a non-radioactive alternative to the current version of the traditional murine LLNA. While the traditional LLNA assesses cellular proliferation by measuring the incorporation of radioactivity into the deoxyribonucleic acid (DNA) of dividing lymph node cells, the LLNA: BrdU-FC assesses the same endpoint by measuring the incorporation of the thymidine analog BrdU. which is detected and quantified with a flow cytometer. Routine measurements of ear swelling are also included as a measure of excessive local irritation when evaluating results. Additional endpoints (e.g., immunophenotypic markers such as B220 and CD69) are incorporated into an enhanced LLNA: BrdU-FC protocol (hereafter the "eLLNA: BrdU-FC") to further distinguish irritants from sensitizers.

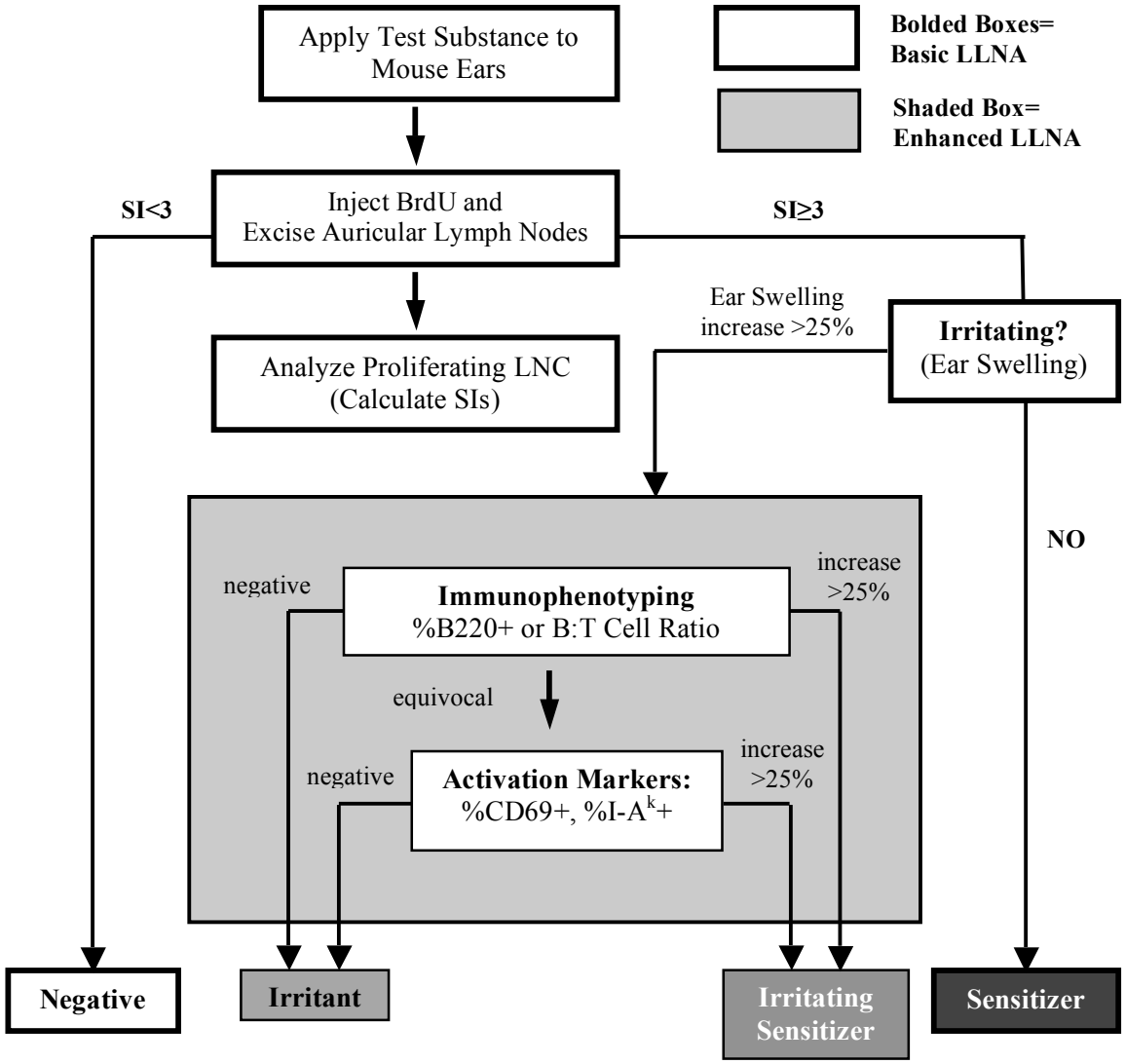
This document provides:

- A comprehensive summary of the LLNA: BrdU-FC test method protocol
- The substances used in the validation of the test method and the test results
- The performance characteristics (accuracy and reliability) of the test method
- Animal welfare considerations
- Other considerations relevant to the usefulness and limitations of this test method (e.g., transferability, cost of the test method).

2.0 LLNA: BrdU-FC Test Method Protocol

The LLNA: BrdU-FC protocol (see **Figure 2-1** and **Appendix A**) follows the ICCVAM-recommended protocol for the traditional LLNA (ICCVAM 1999; Dean et al. 2001) with the exception of the method used to assess lymphocyte proliferation. To evaluate excessive skin irritation, as recommended by the ICCVAM LLNA protocol when determining the highest dose level, the LLNA: BrdU-FC incorporates a quantitative assessment of potential dermal irritation by measuring ear thickness (i.e., with a digital micrometer) at three separate time points (on days 1 [prior to dosing], 3, and 6). The ICCVAM protocol is less specific and recommends only that mice be carefully observed daily for signs of excessive local irritation (i.e., redness and /or swelling) at the application site, and a record made of the observations.

363 **Figure 2-1 Strategy for Using the LLNA: BrdU-FC to Detect Irritants vs. Sensitizers**



364

365 Abbreviations: B = B lymphocyte; BrdU = Bromodeoxyuridine; LLNA = Murine local lymph node assay; LNC
366 = Lymph node cells; SI = Stimulation index; T = T lymphocyte
367 The shaded box shows that the enhancements of immunophenotyping and measurement of activation markers
368 are used when SI ≥ 3 and mouse ear swelling ≥ 25% (i.e., the enhanced LLNA: BrdU-FC protocol ([eLLNA:
369 BrdU-FC]).

370

In the traditional LLNA, the test substance is administered on three consecutive days. Forty-eight hour after the final application of the test substance, ^{3}H-methyl thymidine or ^{125}I-fluorodeoxyuridine (in phosphate buffered saline; 250 µL/mouse) is administered via the tail vein. This same dosing schedule is followed in the LLNA: BrdU-FC, but BrdU is administered, 200 µL per mouse, via intraperitoneal injection rather than intravenously. See **Appendix A** for the rationale for the route of administration and amount of BrdU. Again following the traditional LLNA protocol, five hours after BrdU administration, lymph nodes are excised and processed. Measurement of the total number of lymphocytes and the total number of cells with incorporated BrdU in the lymph node preparation is described in **Appendix A**.

As mentioned above, the eLLNA: BrdU-FC incorporates immunophenotypic endpoints, which are evaluated in tiers using the criteria described in **Section 2.1**, to distinguish irritants from dermal sensitizers when an SI ≥ 3 is recorded. For mice exhibiting ear swelling > 25%, the first tier endpoints include determination of the %B lymphocytes (%B220+) or the B lymphocyte to T lymphocyte ratio (B:T cell ratio) in the isolated lymph node cells of the treated mice. B220 is an isoform of a transmembrane protein expressed on B lymphocytes that assists in the activation of the cells. Allergen treated mice show a preferential increase in the percentage of B220+ cells compared with irritant treated mice (Gerberick et al. 2002). A greater than 25% increase of B220+ cells or a B:T cell ratio greater than 1.25 indicates that a substance is an irritating sensitizer. If the B220+ or B:T cell ratio increases by less than 25%, then the substance is classified as an irritant. However, if the outcome of the B220+ or B:T cell ratio produces a borderline response, a second tier of immunophenotypic measurements can be used to reconcile such cases. An increase of greater than 25% in IA^{K}+ cells (B-lymphocytes) or CD69 (T-lymphocytes) is an irritating sensitizer.

NICEATM has requested, but not obtained, a detailed protocol from MB Research Labs to describe the specific procedures used to quantify the immunophenotypic endpoints.

2.1 Decision Criteria

Like the traditional LLNA, an SI is used in the LLNA: BrdU-FC to distinguish skin sensitizers from non-sensitizers. The SI in the LLNA: BrdU-FC is the ratio of the mean number of lymph node cells with incorporated BrdU from mice in each of the test substance

dose groups to the mean number of lymph node cells with incorporated BrdU from mice in the vehicle control group. The formula is:

$$SI = \frac{\text{Mean number of BrdU - labeled cells in the treatment group}}{\text{Mean number of BrdU - labeled cells the vehicle control group}}$$

An SI ≥ 3 is the threshold for labeling a substance as a sensitizer. This same SI threshold is used in the traditional LLNA.

The eLLNA: BrdU-FC provides the opportunity for further evaluating substances producing an SI ≥ 3 to distinguish between sensitizers and irritants. As detailed in **Figure 2-1**, if mouse ear swelling exceeds 25% for substances with an SI ≥ 3, then an evaluation of the first set of immunophenotypic markers is conducted (i.e., B220+ cells or the calculation of the B:T cell ratio). If %B220+ increases less that 25% above control values or the B:T cell ratio is <1.25, then the substance is classified as an irritant. If %B220+ increases more than 25% above control values or the B:T cell ratio is >1.25, then the substance is classified as an irritating sensitizer. If the increase in %B220+ or the B:T cell ratio is equivocal (i.e., at least one mouse has ear swelling > 25% and %B220+ or B:T cell ratio is significantly elevated or is greater than 25% above control values), then an evaluation of the second set of immuophenotypic markers is conducted (i.e., %IA^K+ cells or CD69+ cells). If the %IA^K+ cells or %CD69+ cells is > 25% above control values, then the substance is classified as a sensitizer. If the %IA^K+ cells or %CD69+ cells is <25% above control values, then the substance is classified as an irritant.

3.0 LLNA: BrdU-FC Validation Database

To evaluate the performance of the LLNA: BrdU-FC and the eLLNA: BrdU-FC against the traditional LLNA, MB Research Labs tested a total of 48 substances (MB Research Labs 2007) (**Appendix B**). Traditional LLNA data were identified by NICEATM for 45 of the 48 substances (**Table 3-1**). Traditional LLNA data were not identified for 4-aminophenol HCl, chlorpromazine with ultraviolet radiation (chlorpromazine +UVR), and croton oil and therefore they were excluded from this evaluation. Forty of the 45 substances previously tested in the traditional LLNA were considered in the original evaluation of the LLNA by ICCVAM (ICCVAM 1999). The traditional LLNA data for the five remaining substances (cobalt chloride, diphenylcyclopropenone, fluorescein isothiocyanate, isopropyl myristate, and linalool) were identified from Ryan et al. (2000), Basketter et al. (2006), Gerberick et al. (2005), and Schneider and Akkan (2004). Of these 45 substances, 27 were classified by the traditional LLNA as skin sensitizers and 18 were classified as non-sensitizers. As shown by the EC3 values (i.e., calculated concentration that corresponds to SI=3) in **Table 3-1**, four sensitizers had EC3 <0.1%, six sensitizers had 0.1% ≤ EC3 <1%, nine sensitizers had 1% ≤ EC3 <10%, eight sensitizers had 10% ≤ EC3 ≤100%.

Appendix B provides information on the physicochemical properties (e.g., peptide reactivity, octanol-water partition coefficient), Chemical Abstracts Service Registry Number (CASRN), and chemical class for each substance tested. When available, chemical class information was retrieved from the National Library of Medicine's ChemID Plus database. If chemical class information was not located, they were assigned for each test substance using a standard classification scheme, based on the National Library of Medicine Medical Subject Headings (MeSH) classification system (available at http://www.nlm.nih.gov/mesh/meshhome.html). A substance could be assigned to more than one chemical class; however, no substance was assigned to more than three classes. Chemical class information is presented only to provide an indication of the variety of structural elements that are present in the structures that were evaluated in this analysis. Classification of substances into chemical classes is not intended to make a representation regarding the impact of structure on biological activity with respect to sensitization potential. **Table 3-1** shows that 23 chemical classes are represented by the 45 substances included in this evaluation. Fifteen substances are classified in more than one chemical class. The classes

with the highest number of substances are carboxylic acids (12 substances) and amines (seven substances).

4.0 Reference Data

The reference data for the traditional LLNA used for the accuracy evaluation described in **Section 6.0** were obtained from ICCVAM (1999), Ryan et al. (2000), Basketter et al. (2006), Gerberick et al. (2005), or Schneider and Akkan (2004). As stated in **Section 3.0**, no traditional LLNA data were identified for three substances: 4-aminophenol HCl, chlorpromazine +UVR, and croton oil. Therefore they were not included in this evaluation. An independent quality assurance contractor for the National Toxicology Program (NTP) audited the traditional LLNA data provided in ICCVAM (1999). Audit procedures and findings are presented in the quality assurance report on file at the National Institute of Environmental Health Sciences (NIEHS). The audit supports the conclusion that the transcribed test data in the submission were accurate, consistent, and complete as compared to the original study records. A similar audit of the traditional LLNA data in Ryan et al. (200l), Schneider and Akkan (2004), Gerberick et al. (2005), and Basketter et al. (2006) has not been possible, but copies of original data have been requested.

The reference data for the GP tests (Guinea Pig Maximization Test [GPMT] or Buehler Test [BT]) and human tests (Human Maximization Test [HMT], Human Patch Test Allergen [HPTA], or other human data) were obtained from Poole et al. (1970), Opdyke (1976a, 1976b), Gad et al. (1986), Gerberick et al. (1992, 2005), Kimber and Basketter (1997), ICCVAM (1999), Rasanen et al. (1999), Basketter et al. (2000, 2003), Kwon et al. (2003), and Schneider and Akkan (2004). Neither GP nor human data could be located for four substances: croton oil, chlorpromazine +UVR, 4-aminophenol HCl, and fluorescein isothiocyanate. No GP data could be located for seven substances: diphenylcyclopropenone, hexane, hydrocortisone, linalool, pyridine, xylene, and isopropyl myristate. Additionally, no human data could be located for copper chloride and lactic acid.

476 **Table 3-1 Traditional LLNA EC3 Values and Chemical Classification of Substances**
477 **Tested in the LLNA: BrdU-FC (Sorted by EC3 Value)**

Substance Name	Chemical Class[1]	Traditional LLNA EC3[2]
Oxazalone	Heterocyclic compounds	0.0034
Tetrachlorosalicylanilide	Amides; Amines	0.04
2, 4-Dinitrochlorobenzene	Hydrocarbon, Halogenated; Nitro compounds; Hydrocarbons, Cyclic	0.049
Diphenylcyclopropenone	Hydrocarbons, Cyclic	0.05
4-Phenylenediamine	Amines	0.11
Potassium dichromate	Inorganic chemical, Chromium compounds; Potassium compounds	0.11[3]
Fluorescein isothiocyanate	Polycyclic compounds; Isocyanates; Sulfur compounds	0.143[4]
Benzoyl peroxide	Carboxylic acids	0.3[5]
Copper chloride	Inorganic chemicals	0.4
Formaldehyde	Aldehydes	0.53
Isoeugenol	Carboxylic acids	1.53
Ethylenediamine	Amines	2.2
Trimellitic anhydride	Anhydrides; Carboxylic acids	4.71
Cobalt chloride	Inorganic chemicals, Metals	4.8[3]
Diethylenetriamine	Amines	5.8
Sodium lauryl sulfate	Alcohols; Sulfur compounds; Lipids	8.08[6]
2-Mercaptobenzothiazole	Heterocylic compounds	9.8
Citral	Hydrocarbons, Other	9.8
Hexyl cinnamic aldehyde	Aldehydes	9.9
Eugenol	Carboxylic acids	10.1
Benzocaine	Carboxylic acids	22
Ethylene glycol dimethacrylate	Carboxylic acids	28[7]
Linalool	Hydrocarbons	30
Isopropyl myristate	Lipids	44
Aniline	Amines	63
Pyridine	Heterocyclic compounds	72
Xylene	Hydrocarbons, Cyclic	95.8[5]
4-Aminobenzoic acid	Carboxylic acids	NA
6-Methylcoumarin	Heterocyclic compounds	NA
Benzalkonium chloride	Onium compounds	NA
Benzoic acid	Carboxylic acids	NA
Chlorobenzene	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated	NA
Glycerol	Alcohols; Carbohydrates	NA

Substance Name	Chemical Class[1]	Traditional LLNA EC3[2]
Hexane	Hydrocarbons, Acyclic	NA
Hydrocortisone	Polycyclic compounds	NA
Isopropanol	Alcohols	NA
Lactic acid	Carboxylic acids	NA
Methyl salicylate	Phenols; Carboxylic acids	NA
Nickel chloride	Inorganic chemicals	NA
Propylene glycol	Alcohols	NA
Propylparaben	Phenols; Carboxylic acids	NA
Resorcinol	Phenols	NA
Salicylic acid	Phenols; Carboxylic acids	NA
Sulfanilimide	Amides; Sulfur compounds; Amines	NA
Tween 80	Alcohols	NA

Abbreviations: LLNA: BrdU-FC = Murine local lymph node assay with flow cytometry measurement of bromodeoxyuridine incorporation; EC3 = Estimated concentration needed to produce a stimulation index (SI) = 3; NA = Not applicable, since maximum SI < 3.

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

[2]Average EC3 values from the NICEATM LLNA database. All tests use acetone:olive oil (4:1) as the vehicle unless otherwise noted.

[3]Vehicle= Dimethyl sulfoxide.

[4]Vehicle = acetone/dibutyl phthalate (50:50).

[5]Vehicle not reported.

[6]Vehicle = Dimethylformamide.

[7]Vehicle = Methyl ethyl ketone.

5.0 Test Method Data and Results

See **Appendix C** for the LLNA: BrdU-FC data for the 48 substances tested in this study. All substances were also evaluated in the eLLNA: BrdU-FC protocol (only substances with SI ≥ 3 and mouse ear swelling ≥ 25% were evaluated with the additional immunophenotypic markers included in the eLLNA: FC-BrdU). Test substances were not coded to hide their identities during testing. All test results were obtained using the protocol in **Appendix A.**

As indicated in **Section 3.0**, traditional LLNA data were identified by NICEATM for 45 of the 48 substances. Of these 45 substances, 37 had LLNA: BrdU-FC, traditional LLNA, and GP data while 42 substances had LLNA: BrdU-FC, traditional LLNA, and human data. Three of the 45 substances produced divergent results when tested at least twice in the traditional LLNA and/or in the LLNA: BrdU-FC (i.e., benzocaine in both tests, and 2-mercaptobenzothiazole and salicylic acid in the LLNA: BrdU-FC test). These three substances are hereafter referred to as producing "equivocal" results in the LLNA: BrdU-FC.

6.0 Test Method Accuracy

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

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

An accuracy analysis for the LLNA: BrdU-FC was conducted using data on 45 substances tested by MB Research Labs (2007); these substances had also been tested in the traditional LLNA. Thirty-seven of these substances had LLNA: BRDU-FC, traditional LLNA, and GP data while 42 substances had LLNA: BRDU-FC, traditional LLNA, and human data. To account for the substances that produced equivocal results in the LLNA: BrdU-FC (see **Section 5.0**) two separate analyses were conducted: 1) only the substances with unequivocal LLNA: BrdU-FC results were evaluated, and 2) the three equivocal substances were included by using the more conservative result (i.e., positive) for all three substances. Including the three equivocal substances resulted in a net gain of two correctly identified sensitizers and one false positive result when comparing the LLNA: BrdU-FC to the traditional LLNA, guinea, and human results.

6.1 LLNA: BrdU-FC Database Analysis

6.1.1 Accuracy vs. the Traditional LLNA

Based on the available data, when compared to the traditional LLNA (and excluding the three equivocal substances) the LLNA: BrdU-FC had an accuracy of 93% (39/42), a sensitivity of 100% (24/24), a specificity of 83% (15/18), a false positive rate of 17% (3/18), and a false negative rate of 0% (0/24) (**Table 6-1**).

Including the three equivocal substances resulted in an accuracy for the LLNA: BrdU-FC of 91% (41/45), a sensitivity of 100% (26/26), a specificity of 79% (15/19), a false positive rate of 21% (4/19), and a false negative rate of 0% (0/26) (**Table 6-1**).

6.1.2 Accuracy vs. Guinea Pig Data

When the accuracy statistics for the LLNA: BrdU-FC and the traditional LLNA were compared when GP results served as the reference data, the LLNA: BrdU-FC had a lower accuracy rate (79% [27/34] vs. 85% [29/34]), higher sensitivity (94% [15/16] vs. 88% [15/17]), and lower specificity (67% [12/18] vs. 82% [14/17]) compared with the traditional LLNA. The LLNA: BrdU-FC also had a higher false positive rate (33% [6/18] vs. 18% [3/17]) and a lower false negative rate of (6% [1/16] vs. 12% [2/17]) than the traditional LLNA (**Table 6-1**).

Including the three equivocal substances resulted in only a slight reduction in overall performance for the LLNA: BrdU-FC (e.g., accuracy reduced to 78% [29/37] from 79% [27/34]) when compared to GP results (**Table 6-1**).

6.1.3 Accuracy vs. Human Data

When substances with only comparative LLNA: BrdU-FC data, traditional LLNA data, and human outcomes were evaluated, the LLNA: BrdU-FC had a higher accuracy rate (69% [27/39] vs. 62% [24/39]), higher sensitivity (72% [22/31] vs. 61% [19/31]), and the same specificity (63% [5/8]) compared with the traditional LLNA. The LLNA: BrdU-FC had a false positive rate (38% [3/8]) equal to that of the traditional LLNA and a lower false negative rate (29% [9/31] vs. 39% [12/31]) than the traditional LLNA, when each was compared to human sensitization outcomes.

Including the three equivocal substances resulted in no change in overall performance for the LLNA: BrdU-FC (e.g., accuracy remained 69% [29/42]) when compared to human sensitization outcomes (**Table 6-1**).

562 **Table 6-1 Evaluation of the Performance of the LLNA: BrdU-FC In Predicting Skin Sensitizing Potential**

Comparison	N[1]	Accuracy		Sensitivity		Specificity		Positive Predictivity		Negative Predictivity		False Positive Rate		False Negative Rate	
		%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]
LLNA: BrdU-FC vs. Traditional LLNA	42	93	39/42	100	24/24	83	15/18	89	24/27	100	15/15	17	3/18	0	0/24
LLNA: BrdU-FC vs. Traditional LLNA*	45	91	41/45	100	26/26	79	15/19	87	26/30	100	15/15	21	4/19	0	0/26
Substances with LLNA: BrdU-FC, Traditional LLNA, and GP Data															
LLNA: BrdU-FC vs. Traditional LLNA	34	91	31/34	100	18/18	81	13/16	86	18/21	100	13/13	19	3/16	0	0/18
LLNA: BrdU-FC vs. Traditional LLNA*	37	89	33/37	100	20/20	76	13/17	83	20/24	100	13/13	24	4/17	0	0/20
LLNA: BrdU-FC vs. GP[3]	34	79	27/34	94	15/16	67	12/18	71	15/21	92	12/13	33	6/18	6	1/16
LLNA: BrdU-FC vs. GP[3]*	37	78	29/37	94	17/18	63	12/19	71	17/24	92	12/13	37	7/19	5	1/18
Traditional LLNA vs. GP[3]	34	85	29/34	88	15/17	82	14/17	83	15/18	88	14/16	18	3/17	12	2/17
Traditional LLNA vs. GP[3]*	37	86	32/37	89	17/19	83	15/18	85	17/20	88	15/17	17	3/18	11	2/19
Substances with LLNA: BrdU-FC, Traditional LLNA, and Human Data															
LLNA: BrdU-FC vs. Traditional LLNA	39	92	36/39	100	22/22	82	14/17	88	22/25	100	14/14	18	3/17	0	0/22
LLNA: BrdU-FC vs. Traditional LLNA*	42	90	38/42	100	24/24	78	14/18	86	24/28	100	14/14	22	4/18	0	0/24

Comparison	N[1]	Accuracy		Sensitivity		Specificity		Positive Predictivity		Negative Predictivity		False Positive Rate		False Negative Rate	
		%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]
LLNA: BrdU-FC vs. Human[4]	39	69	27/39	71	22/31	63	5/8	88	22/25	36	5/14	38	3/8	29	9/31
LLNA: BrdU-FC vs. Human[4]*	42	69	29/42	73	24/33	56	5/9	86	24/28	36	5/14	44	4/9	27	9/33
Traditional LLNA vs. Human[4]	39	62	24/39	61	19/31	63	5/8	86	19/22	29	5/17	38	3/8	39	12/31
Traditional LLNA vs. Human[4]*	42	64	27/42	64	21/33	67	6/9	88	21/24	33	6/18	33	3/9	36	12/33

Abbreviations: LLNA: BrdU-FC = Murine local lymph node assay with flow cytometry measurement of bromodeoxyuridine incorporation; GP = Guinea pig skin sensitization outcomes; LLNA = Local Lymph Node Assay; No. = Number.

* Includes 3 additional substances that produced divergent results when tested in the LLNA: BrdU-FC. In order to include these substances in the analysis, they were assigned the more conservative classification (i.e., sensitizer).

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

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

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

[4] Human refers to outcomes obtained by studies conducting using the Human Maximization Test or the inclusion of the test substance in a Human Patch Test Allergen Kit.

6. 2 eLLNA: BrdU-FC Database Analysis

6.2.1 Accuracy vs. the Traditional LLNA

A separate accuracy analysis was conducted for the eLLNA: BrdU-FC. As noted in **Section 2.0**, only substances with SI ≥ 3 and mouse ear swelling ≥ 25% are evaluated with the additional immunophenotypic markers included in the eLLNA: FC-BrdU. The results of the eLLNA: BrdU-FC were the same as those for the LLNA: BrdU-FC with the exception of ethylene glycol dimethacrylate, benzalkonium chloride, and sodium lauryl sulfate. These substances, which were classified as sensitizers by the LLNA: BrdU-FC, were identified as irritants (i.e., non-sensitizers) by the eLLNA: BrdU-FC. Since the traditional LLNA incorrectly identified two of these substances (ethylene glycol dimethacrylate and sodium lauryl sulfate) as sensitizers, the concordance of the eLLNA: BrdU-FC with the traditional LLNA was decreased (compared to the LLNA: BrdU-FC without the immunophenotypic endpoints). Thus, based on the 42 substances with unequivocal eLLNA: BrdU-FC and traditional LLNA results, the eLLNA: BrdU-FC decreased the accuracy (90% [38/42] vs. 93% [39/42]) and sensitivity (92% [22/24] vs. 100% [24/24]) and increased the false negative rate (8% [2/24] vs. 0% [0/24]) relative to the LLNA: BrdU-FC (compare **Table 6-2** with **Table 6-1**). The specificity rates (89% [16/18] vs. 83% [15/18]) were higher and the false positive rates (11% [2/18] vs. 17% [3/18]) were lower for the eLLNA: BrdU-FC vs. the traditional LLNA compared to the LLNA: BrdU-FC vs. the traditional LLNA.

Including the three equivocal substances resulted in an accuracy for the eLLNA: BrdU-FC of 89% (40/45), a sensitivity of 92% (24/26), a specificity of 84% (16/19), a false positive rate of 16% (3/19), and a false negative rate of 8% (2/26) (**Table 6-2**).

596 **Table 6-2 Evaluation of the Performance of the eLLNA: BrdU-FC[1] In Predicting Skin Sensitizing Potential**

Comparison	N[2]	Accuracy		Sensitivity		Specificity		Positive Predictivity		Negative Predictivity		False Positive Rate		False Negative Rate	
		%	No.[3]	%	No.[3]	%	No.[3]	%	No.[3]	%	No.[3]	%	No.[3]	%	No.[3]
eLLNA: BrdU-FC vs. Traditional LLNA	42	90	38/42	92	22/24	89	16/18	92	22/24	89	16/18	11	2/18	8	2/24
eLLNA: BrdU-FC vs. Traditional LLNA*	45	89	40/45	92	24/26	84	16/19	89	24/27	89	16/18	16	3/19	8	2/26
Substances with eLLNA: BrdU-FC, Traditional LLNA, and GP Data															
eLLNA: BrdU-FC vs. Traditional LLNA	34	88	30/34	89	16/18	88	14/16	89	16/18	88	14/16	13	2/16	11	2/18
eLLNA: BrdU-FC vs. Traditional LLNA*	37	86	32/37	90	18/20	82	14/17	86	18/21	88	14/16	18	3/17	10	2/20
eLLNA: BrdU-FC vs. GP[4]	34	88	30/34	94	15/16	83	15/18	83	15/18	94	15/16	17	3/18	6	1/16
eLLNA: BrdU-FC vs. GP[4]*	37	86	32/37	94	17/18	79	15/19	81	17/21	94	15/16	21	4/19	6	1/18
Traditional LLNA vs. GP[4]	34	85	29/34	88	15/17	82	14/17	83	15/18	88	14/16	18	3/17	12	2/17
Traditional LLNA vs. GP[4]*	37	86	32/37	89	17/19	83	15/18	85	17/20	88	15/17	17	3/18	11	2/19
Substances with eLLNA: BrdU-FC, Traditional LLNA, and Human Data															
eLLNA: BrdU-FC vs. Traditional LLNA	39	90	35/39	91	20/22	88	15/17	91	20/22	88	15/17	12	2/17	9	2/22
eLLNA: BrdU-FC vs. Traditional LLNA*	42	88	37/42	92	22/24	83	15/18	88	22/25	88	15/17	17	3/18	8	2/24

Comparison	N[2]	Accuracy		Sensitivity		Specificity		Positive Predictivity		Negative Predictivity		False Positive Rate		False Negative Rate	
		%	No.[3]	%	No.[3]	%	No.[3]	%	No.[3]	%	No.[3]	%	No.[3]	%	No.[3]
eLLNA: BrdU-FC vs. Human[5]	39	67	26/39	67	20/30	67	6/9	87	20/23	38	6/16	33	3/9	33	10/30
eLLNA: BrdU-FC vs. Human[5]*	42	67	28/42	69	22/32	60	6/10	85	22/26	38	6/16	40	4/10	31	10/32
Traditional LLNA vs. Human[5]	39	62	24/39	61	19/31	63	5/8	86	19/22	29	5/17	38	3/8	39	12/31
Traditional LLNA vs. Human[5]*	42	64	27/42	64	21/33	67	6/9	88	21/24	33	6/18	33	3/9	36	12/33

Abbreviations: eLLNA: BrdU-FC = Murine local lymph node assay with flow cytometry measurement of bromodeoxyuridine incorporation enhanced with immunophenotypic endpoints; GP = Guinea pig skin sensitization outcomes; LLNA = Murine local lymph node assay; No. = Number.

* Includes 3 additional substances that produced divergent results when tested in the LLNA: BrdU-FC. In order to include these substances in the analysis, they were assigned the more conservative classification (i.e., sensitizer).

[1] The results of the eLLNA: BrdU-FC were the same as those for the LLNA: BrdU-FC with the exception of ethylene glycol dimethacrylate and sodium lauryl sulfate, which were classified as irritants rather than sensitizers.

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

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

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

[5] Human refers to outcomes obtained by studies conducting using the Human Maximization Test or the inclusion of the test substance in a Human Patch Test Allergen Kit.

6.2.2 *Accuracy vs. Guinea Pig Data*

However, the concordance of the eLLNA: BrdU-FC with GP data was increased (compared with the concordance of LLNA: BrdU-FC data to GP data), since ethylene glycol dimethacrylate and sodium lauryl sulfate were classified as non-sensitizers in both eLLNA: BrdU-FC and GP tests. These substances were classified as sensitizers by the LLNA: BrdU-FC. For the 34 substances with eLLNA: BrdU-FC, GP, and traditional LLNA data, the eLLNA: BrdU-FC protocol improved the performance (in reference to the GP tests) of the LLNA: BrdU-FC (compare **Table 6-2** with **Table 6-1**). Accuracy increased to 88% (30/34) from 79% (27/34), specificity increased to 83% (15/18) from 67% (12/18), and the false positive rate decreased from 33% (6/18) to 17% (3/18). The sensitivity (94% [15/16]) and the false negative rates (6% [1/16]) were the same for the LLNA: BrdU-FC and the eLLNA: BrdU-FC (in reference to GP test results).

Like the LLNA: BrdU-FC, including the three equivocal substances resulted in only a slight reduction in overall performance for the eLLNA: BrdU-FC (e.g., accuracy reduced to 86% [32/37] from 88% [30/34]) when compared to GP results (**Table 6-2**).

6.2.3 *Accuracy vs. Human Data*

When the substances with comparative eLLNA: BrdU-FC data, traditional LLNA data, and human outcomes were evaluated, the eLLNA: BrdU-FC had similar accuracy, sensitivity, and false negative rates to the LLNA: BrdU-FC. The accuracy for the eLLNA: BrdU-FC (in reference to human data) was slightly decreased to 67% (26/39) from 69% (27/39), the sensitivity slightly decreased to 67% (20/31) from 71% (22/31), and the false negative rate slightly increased to 33% (10/30) from 29% (9/31). The specificity for the eLLNA: BrdU-FC increased to 67% (6/9) from 63% (5/8), the false positive rate decreased to 33% (3/9) from 38% (3/8), and the sensitivity was similar to that of the LLNA: BrdU-FC (68% [21/31] for eLLNA: BrdU-FC vs. 71% [22/31] for LLNA: BrdU-FC).

Including the three equivocal substances resulted in no change in overall performance for the LLNA: BrdU-FC (e.g., accuracy remained 67% [28/42]) when compared to human sensitization outcomes (**Table 6-2**).

6.3 Accuracy Analysis Based on Revised Draft ICCVAM Draft Performance Standards

ICCVAM is currently developing draft performance standards for the traditional LLNA (http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm). These draft test method performance standards are proposed to evaluate the performance of LLNA test methods that incorporate specific modifications to measure lymphocyte proliferation compared to the traditional LLNA. As shown in **Table 6-3**, 13 of the 18 minimum reference substances have been tested in the LLNA: BrdU-FC and the eLLNA: BrdU-FC. Eight substances were sensitizers and five substances were non-sensitizers. Two substances, 2-mercaptobenzothiazole (sensitizer, mean EC3 = 2.5%) and salicylic acid (non-sensitizer), produced equivocal results in the LLNA: BrdU-FC and the eLLNA: BrdU-FC. The LLNA: BrdU-FC and the eLLNA: BrdU-FC results for the remaining 11 substances were consistent with those of the traditional LLNA.

Three of the four optional reference substances included in ICCVAM (2007) were also tested in the LLNA: BrdU-FC and produced false positive responses for ethylene glycol dimethacrylate and sodium lauryl sulfate (which were also false positive in the traditional LLNA). However, when tested in the eLLNA: BrdU-FC, they were identified as irritants rather than sensitizers. The third optional reference substance, sulfanilamide (false negative in the traditional LLNA), also produced a false negative result when tested in either the LLNA: BrdU-FC or the eLLNA: BrdU-FC.

EC3 values based on LLNA: BrdU-FC and eLLNA: BrdU-FC results were reported for 10 substances (**Table 6-3**). EC3 values were available for eight of the list of required substances included on the draft ICCVAM performance standards substances list. However, since EC3 ranges are not reported for sulfanilamide or isopropanol (because they are classified as nonsensitizers in the traditional LLNA), the LLNA: BrdU-FC and the eLLNA: BrdU-FC EC3 values for six substances could be compared with the ICCVAM criteria. The EC3 values for two substances (hexyl cinnamic aldehyde and eugenol) were within the proposed acceptability range of 0.5x to 2.0x the mean historical EC3 value. The other four substances had EC3 values that were either above (4-phenylenediamine) or below (2,4-dinitrochlorobenzene, citral, and cobalt chloride) the proposed acceptability range.

Table 6-4 provides the range of substances tested in the LLNA: BrdU-FC based on the overall database of 45 substances in comparison to the range of substances included on the revised draft

ICCVAM LLNA performance standards substances list. The table indicates that although not all of the draft ICCVAM performance standards reference substances have been tested, the range of the substances tested in the LLNA: BrdU-FC is similar to that included in the draft performance standards list. In general, there is a proportionally increased number of substances tested in the LLNA: BrdU-FC in each of the categories included in the table.

6.4 Discordant Results

Substances that yielded different sensitizer/non-sensitizer classifications when tested by the LLNA: BrdU-FC and the reference methods (i.e., GP tests, human tests) were evaluated to compare numbers of discordant substances with those for the traditional LLNA and to identify commonalities among the discordant substances. The effect of testing with different vehicles could not be evaluated because the MB Research Lab submission did not identify the vehicle used for each test substance. To date, this information has not been received, but a request has been made by NICEATM, and MB Research Labs has agreed to supply this information at their earliest convenience.

When analyses were restricted to the 34 substances with unequivocal LLNA: BrdU-FC, traditional LLNA, and GP data, the LLNA: BrdU-FC classified three substances differently compared with the traditional LLNA (**Table 6-5**). Benzalkonium chloride, resorcinol, and Tween 80 were identified as sensitizers by the LLNA: BrdU-FC while the traditional LLNA classified these substances as non-sensitizers. No commonalities were identified for these three substances. They represent three different chemical classes: onium compounds, alcohols, and, phenols. Information on peptide reactivity and lipid solubility (octanol-water partition coefficient) was available only for resorcinol. Resorcinol is a solid, Tween 80 is a liquid, and benzalkonium chloride could be either liquid or solid. The eLLNA: BrdU-FC correctly identified benzalkonium chloride as a non-sensitizer (based on GP results). The eLLNA: BrdU-FC also correctly identified sodium lauryl sulfate and ethylene glycol dimethacrylate as non-sensitizers, unlike both the LLNA: BrdU-FC and traditional LLNA.

Table 6-3 Evaluation of the Performance of the LLNA: BrdU-FC When Compared to the ICCVAM Draft Performance Standards Reference Substances (Sorted by Ascending Traditional LLNA EC3 Value)[1]

Name	ICCVAM Draft LLNA Performance Standards[1]					LLNA: BrdU-FC[2]		
	Res	EC3 (%)	N	0.5x – 2.0x EC3 (%)	Veh	Result	EC3 (%)	Vehicle
5-Chloro-2-methyl-4-isothiazolin-3-one	+	0.009	1	0.0045 – 0.018	DMF	NT	NT	IR
2,4-Dinitrochlorobenzene	+	0.049	15	***0.025 – 0.099***	***AOO***	+	***0.01-*** *0.09*	***IR***
4-Phenylenediamine	+	0.11	10	***0.055 – 0.22***	***AOO***	+	***0.45***	***IR***
4-Methylaminophenol sulfate	+	0.8	1	0.4 – 0.12	DMF	NT	NT	IR
Isoeugenol	+	1.5	49	0.77 – 3.1	AOO	+	NR	IR
2-Mercaptobenzothiazole	+	2.5	2	***1.25 – 5.0***	***AOO***	***+/-***	***NR***	***IR***
Cobalt chloride	+	4.8	1	***2.4 – 9.6***	***DMSO***	+	***1***	***IR***
Citral	+	9.8	2	4.9 – 19.6	AOO	+	***2***	IR
Hexyl cinnamic aldehyde	+	9.9	22	5.0 – 19.9	AOO	+	6.3	IR
Eugenol	+	10.1	11	5.05 – 20.2	AOO	+	13.2	IR
Phenyl benzoate	+	13.6	3	6.8 – 27.2	AOO	NT	NT	IR
Cinnamic alcohol	+	21	1	10.5 - 42	AOO	NT	NT	IR
Imidazolidinyl urea	+	24	1	12 - 36	DMF	NT	NT	IR
Chlorobenzene	-	NA	1	NA	AOO	-	NA	IR
Isopropanol	-	NA	1	NA	AOO	-	>50%	IR
Lactic acid	-	NA	2	NA	DMSO	-	NA	IR
Methyl salicylate	-	NA	10	NA	AOO	-	NA	IR
Salicylic acid	***-***	NA	1	***NA***	***AOO***	***+/-***	***NA***	***IR***
Ethylene glycol dimethylacrylate	FP	28	1	14 - 56	MEK	+[3]	40.0	IR
Sodium lauryl sulfate	FP	8.1	5	4.05 – 16.2	DMF	+[3]	4.84	IR
Nickel sulfate	FN	NA	2	NA	DMF	NT	NA	IR
Sulfanilamide	FN	NA	1	NA	DMF	-	>50%	IR

Bolded italics text highlights discordant LLNA: BrdU-FC vs. traditional LLNA test results.

Abbreviations: FN = False negative; FP = False positive; LLNA: BrdU-FC = Murine local lymph node assay with flow cytometry measurement of bromodeoxyuridine incorporation; IR = Information requested; NA = Not applicable (Stimulation Index < 3); NR = Not reported; NT = Not tested; + = Sensitizer; - = Nonsensitizer; +/- = equivocal compounds that were not included in contingency table evaluations. Some tests of this substance were positive, while others were negative

[1]From Revised Draft ICCVAM Performance Standards for the LLNA (available: http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm)

[2]From MB Research Labs (2007).

[3]Classified by the LLNA: BrdU-FC as an irritant, but not a sensitizer, using an enhanced LLNA: BrdU-FC with immunophenotypic endpoints (i.e., the eLLNA: BrdU-FC).

Table 6-4 Characteristics of the Substances Tested in the LLNA: BrdU-FC vs. the Revised Draft ICCVAM Performance Standards Substances List[1]

EC3 range (%)	No. Chems	Solid/ Liquid	Actual EC3 Range (%)[2]	Human Data	Peptide Reactivity (High/Mod/ Min/Unk)[3]
<0.1	**4**	**4/0**	**0.0034-0.05**	**4**	**3/1/0/0**
	2	1/1	0.009-0.05	2	0/1/0/1
≥0.1 to <1	**5**	**4/1**	**0.1-0.53**	**4**	**2/1/0/2**
	2	2/0	0.11-0.8	2	1/0/0/1
≥1 to <10	**9**	**4/5**	**1.53-9.9**	**9**	**1/0/2/6**
	5	2/3	1.6-9.9	5	1/0/1/3
≥10 to <100	**8**	**1/7**	**10.1-95.8**	**8**	**1/0/1/6**
	4	3/1	10.1-24	4	0/1/0/3
Negative	**19**	**12/7**	**NC**	**18**	**0/0/0/19**
	5	2/3	NC	3	0/0/2/3
Overall	**45**	**25/20**	**0.0034-95.8**	**43**	**7/2/3/33**
	18	10/8	0.009-24	16	2/2/3/11

Bolded text represents characteristics of the LLNA: BrdU-FC database.

Abbreviations: Chems = Chemicals; EC3 = Estimated concentration needed to produce a stimulation index of three; NC = Not calculated because maximum SI < 3.0; No. = Number; Min = Minimal; Mod = Moderate; SI = Stimulation Index; Unk = Unknown.

[1]From Revised Draft ICCVAM Performance Standards for the LLNA (available: http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm). Includes the 18 "required" substances for testing.

[2] Based on traditional LLNA studies for substances in the LLNA: BrdU-FC database (bold values) and the draft ICCVAM LLNA performance standards substances.

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

Table 6-5 Discordant Results with Respect to Traditional LLNA and Guinea Pig Reference Data

Substance Name	Classification				
	LLNA: BrdU-FC	eLLNA: BrdU-FC[1]	Traditional LLNA	Guinea Pig Tests[2]	Human Outcome
Benzalkonium chloride	+	-	-	-	+
Resorcinol	+	+	-	-	+
Copper chloride	+	+	+	-	NA
Ethylene glycol methacrylate	+	-	+	-	+
Sodium lauryl sulfate	+	-	+	-	-
Tween 80	+	+	-	-	-
Aniline	-	-	-	+	+
Nickel chloride	-	-	-	+	+

Abbreviations: LLNA: BrdU-FC = Murine local lymph node assay with flow cytometry measurements of bromodeoxyuridine; eLLNA: BrdU-FC = enhanced LLNA: BrdU-FC that includes immunophenotypic measurements to distinguish irritants from sensitizers; NA = not available.
+ = Sensitizer.
- = Nonsensitizer.
[1]The results of the eLLNA: BrdU-FC were the same as those for the LLNA: BrdU-FC with the exception of ethylene glycol dimethacrylate and sodium lauryl sulfate, which were classified as irritants rather than sensitizers.
[2]From ICCVAM (1999) and based on studies using either the Guinea Pig Maximization Test or the Buehler Test.

When compared to the outcomes of GP tests, the LLNA: BrdU-FC misclassified eight substances, the eLLNA: BrdU-FC misclassified five substances, and the traditional LLNA misclassified five substances. The LLNA: BrdU-FC and the traditional LLNA had four discordant substances in common. Copper chloride, ethylene glycol dimethacrylate, and sodium lauryl sulfate were incorrectly classified as sensitizers (compared with the GP results) by the LLNA: BrdU-FC and the traditional LLNA. No commonalities were identified for these three substances. They represent five different chemical classes: inorganics, alcohols, carboxylic acids, organic sulfur compounds, and lipids. There are two solids and one liquid, ranging in molecular weight from 99 to 288, with octanol-water partition coefficients ranging from 1.4 to 1.7. One substance, ethylene glycol dimethacrylate, is considered to be highly peptide reactive.

Aniline (a liquid, MW = 93) and nickel chloride (a solid, MW = 130) were incorrectly classified as non-sensitizers by the LLNA: BrdU-FC and the traditional LLNA. The eLLNA: BrdU-FC protocol correctly classified benzalkonium chloride, ethylene glycol dimethacrylate, and sodium lauryl sulfate as non-sensitizers.

When analyses were restricted to the 39 substances with unequivocal LLNA: BrdU-FC, traditional LLNA, and human outcomes, the discordant substances for the LLNA: BrdU-FC and the eLLNA: BrdU-FC and traditional LLNA were the same as that for the set of 34 substances with unequivocal LLNA: BrdU-FC, traditional LLNA, and GP outcomes (**Table 6-4**). The LLNA: BrdU-FC classified three substances differently compared with the classification of the traditional LLNA. Resorcinol, benzalkonium chloride, and Tween 80 were identified as sensitizers by the LLNA: BrdU-FC while the traditional LLNA classified these substances as non-sensitizers. The eLLNA: BrdU-FC protocol, however, identified benzalkonium chloride as a non-sensitizer, which was consistent with the traditional LLNA classification. The eLLNA: BrdU-FC protocol identified ethylene glycol dimethacrylate and sodium lauryl sulfate as non-sensitizers while the traditional LLNA classified them as sensitizers.

When comparing to the outcomes of human tests, the LLNA: BrdU-FC misclassified 12 substances, the eLLNA: BrdU-FC misclassified 14 substances, and the traditional LLNA misclassified 14 substances (**Table 6-6**). All 12 discordant substances misclassified by the LLNA: BrdU-FC were also misclassified by the traditional LLNA. Of these 12 substances, three were misclassified as sensitizers (sodium lauryl sulfate, xylene, and isopropyl myristate) and nine (6-methylcoumarin, aniline, hydrocortisone, 4-aminobenzoic acid, propylene glycol, propylparaben, sulfanilamide, nickel chloride, and isopropanol) were misclassified as non-sensitizers by both methods. Among the three false positives, two are liquids and one is a solid; they range in molecular weight from 107 to 288, with octanol-water partition coefficients that range from 1.7 to 3.9. One substance, isopropyl myristate, is considered to be minimally peptide reactive. Peptide reactivity data on the other substances could not be located.

There were no commonalities noted among the nine human sensitizers that were misclassified as non-sensitizers by both LLNA: BrdU-FC and traditional methods. The nine substances represent alcohols, amides, amines, carboxylic acids, heterocylic compounds, phenols, organic sulfur compounds, lipids, polycylic compounds, and inorganic chemicals. Six are solids and three are liquids, with molecular weights ranging from 60 to 362, with octanol-water partition coefficients ranging from 0.3 to 2.2. Four of the false negative substances are considered to be minimally peptide reactive. The eLLNA: BrdU-FC protocol also misclassified these same nine sensitizing substances as non-sensitizers. In addition, the eLLNA: BrdU-FC misclassified benzalkonium

chloride and ethylene glycol dimethacrylate as non-sensitizers, but correctly classified sodium lauryl sulfate as a non-sensitizer when compared with human outcomes.

Table 6-6 Discordant Results with Respect to Human Outcomes[1]

Substance Name	Classification			
	LLNA: BrdU-FC	eLLNA: BrdU-FC	LLNA	Human Outcomes
Benzalkonium chloride	+	-	-	+
Ethylene glycol dimethacrylate	+	-	+	+
Isopropyl myristate	+	+	+	-
Resorcinol	+	+	-	+
Sodium lauryl sulfate	+	-	+	-
Tween 80	+	+	-	+
Xylene	+	+	+	-
4-Aminobenzoic acid	-	-	-	+
Aniline	-	-	-	+
Hydrocortisone	-	-	-	+
Isopropanol	-	-	-	+
6-Methylcoumarin	-	-	-	+
Nickel chloride	-	-	-	+
Propylene glycol	-	-	-	+
Propylparaben	-	-	-	+
Sulfanilamide	-	-	-	+

Abbreviations: LLNA: BrdU-FC = Murine local lymph node assay with flow cytometry measurements of bromodeoxyuridine; eLLNA: BrdU-FC = enhanced LLNA: BrdU-FC that includes immunophenotypic measurements to distinguish irritants from sensitizers.
+ = Sensitizer.
- = Nonsensitizer.
[1]Outcomes obtained by studies conducted with the Human Maximization Test or the inclusion of the test substance in a Human Patch Test Allergen Kit.

7.0 LLNA: BrdU-FC Reliability

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

The only available data on multiply tested substances in the LLNA: BrdU-FC is for hexyl cinnamic aldehyde. Thus, data were available for an evaluation of intralaboratory reproducibility. However, interlaboratory reproducibility could not be assessed because the test results were generated in one laboratory. The test results for the LLNA: BrdU-FC are amenable to intralaboratory reproducibility analyses only for the SI values for hexyl cinnamic aldehyde since only one concentration was tested multiple times. The data submission did not include EC3 values for hexyl cinnamic aldehyde.

Presumably, there are additional data that could be used in an intralaboratory reproducibility analysis from multiply tested substances in the LLNA: BrdU-FC based on the equivocal classifications assigned to benzocaine, and 2-mercaptobenzothiazole, and salicylic acid (see **Section 5.0**). These data have been requested, but have not been obtained.

7.1 Intralaboratory Reproducibility – SI

MB Research Labs provided SI data for multiple tests of hexyl cinnamic aldehyde in different vehicles. The SI values reported for 2 to 26 tests of 25% hexyl cinnamic aldehyde in each of six vehicles were used to calculate a coefficient of variation (CV) for the assessment of intralaboratory variability. As shown by **Table 7-1**, the CVs ranged from 30.1% to 52.6%. The intralaboratory reproducibility of the traditional LLNA was not assessed by CV analysis of SI values (ICCVAM 1999).

Table 7-1 Reproducibility of Hexyl Cinnamic Aldehyde (25% w/v) Tested by LLNA: BrdU-FC in Different Vehicles

Vehicle	N	Mean SI	SD	CV (%)
Dimethylacetamide:Acetone: Ethanol (DAE 433)	5	13.4	6.2	45.9
Acetone:Olive Oil (4:1) (AOO)	19	10.7	5.5	51.0
Dimethyl sulfoxide (DMSO)	26	6.7	3.4	51.6
N,N-Dimethylformamide	4	8.7	4.6	52.6
Ethanol:Water (50%/50%)	4	15.2	6.3	41.4
Acetone	2	21.3	6.4	30.1

Abbreviations: CV = Coefficient of variation; N = number of tests conducted; SD = Standard deviation; SI = Stimulation Index; w/v = weight to volume ratio

8.0 Data Quality

MB Research Labs stated that, while most of the LLNA: BrdU-FC and the eLLNA: BrdU-FC data evaluated were not generated in complete compliance with Good Laboratory Practice (GLP) guidelines, their facilities routinely conduct GLP-compliant studies and they have an accredited quality assurance unit. In response to a request for the original data, MB Research Labs indicated that resources were not available to extract these data, or to determine which of the individual tests were conducted in compliance with GLPs. MB Research Labs staff members did check the reported data for consistency with the raw data, but there has not been an independent audit of the data.

9.0 Other Scientific Reports and Reviews

All available data for the LLNA: BrdU-FC and the eLLNA: BrdU-FC test methods provided by MB Research has been presented and discussed in the above sections. No other relevant data or scientific reviews of the LLNA: BrdU-FC and the eLLNA: BrdU-FC were identified based on an online literature search of entries in MEDLINE and SCOPUS (last updated December 10, 2007).

10.0 Animal Welfare Considerations

The LLNA: BrdU-FC and the eLLNA: BrdU-FC test methods will require the use of the same number of animals as the traditional LLNA. However, since the traditional LLNA uses radioactivity and as such its use might be restricted due to the complications associated with handling radioactive materials (e.g., storage, disposal) use of a non-radioactive alternative to the traditional LLNA, such as the LLNA: BrdU-FC and the eLLNA: BrdU-FC could further reduce the number of guinea pigs that are used to assess skin sensitization.

10.1 Rationale for the Need to Use Animals

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

10.2 Basis for Determining the Number of Animals Used

The number of animals used for the experimental, vehicle, and positive control groups is based on the number of animals specified in the ICCVAM recommended traditional LLNA protocol (ICCVAM 1999; Dean et al. 2001).

10.3 Reduction Considerations

A further reduction of 40% (15 vs. 25) could be achieved by using a limit dose version of the LLNA: BrdU-FC, in cases where dose response information is not needed for hazard identification purposes. In such an approach, only the highest soluble dose of test substances that does not induce systemic toxicity or excessive local irritation would be administered, and the two lower dose groups would not be used. Additional reductions could be achieved by testing more substances concurrently, so that the same vehicle and positive control group could be used for multiple substances, thus reducing the number of animals for each additional substance by 10 animals, or 40% (15 vs. 25).

11.0 Practical Considerations

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

11.1 Transferability of the LLNA: BrdU-FC and the eLLNA: BrdU-FC

Test method transferability addresses the ability of a method to be accurately and reliably performed by multiple laboratories (ICCVAM 2003), including those experienced in the particular type of procedure as well as laboratories with less or no experience in the particular procedure. It would be expected that the transferability of the LLNA: BrdU-FC and the eLLNA: BrdU-FC would be similar to the traditional LLNA, since the protocols of the two methods (except for the detection of lymphocyte proliferation and immunophenotypic measurements) are identical. However, a definitive assessment of the extent of transferability of the LLNA: BrdU-

FC and the eLLNA: BrdU-FC cannot be made in the absence of interlaboratory reproducibility data.

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

Compared to the traditional LLNA, the LLNA: BrdU-FC and the eLLNA: BrdU-FC will not require facilities, equipment, and licensing permits for handling radioactive materials. However, the LLNA: BrdU-FC does require access to a flow cytometer for the assessment of lymphocyte proliferation. A flow cytometer is not routinely included in many laboratories and a new flow cytometer can cost as much as $100,000 or more. The remaining requirements (e.g., animal care facilities) are the same between the two methods.

11.3 LLNA: BrdU-FC Training Considerations

The level of training and expertise needed to conduct the LLNA: BrdU-FC and the eLLNA: BrdU-FC should be similar to the traditional LLNA, although the LLNA: BrdU-FC and the eLLNA: BrdU-FC includes an additional requirement that users operate a flow cytometer instead of a scintillation counter and be able process flow cytometric data.

12.0 References

Basketter DA, Balikie L, Dearman RJ, Kimber I, Ryan CA, Gerberick GF, Harvey P, Evans P, White IR, Rycroft RJ. 2000. Use of the local lymph node assay for the estimation of relative contact allergenic potency. Contact Dermatitis. 42(6):344-8.

Basketter DA, Smith Pease CK, Patlewicz GY. 2003. Contact allergy: The local lymph node assay for the prediction of hazard and risk. Clinical and Experimental Dermatology 28(2):218-221.

Basketter DA, Kimber I. 2006. Predictive tests for irritants and allergens and their use in quantitative risk assessment. In "Contact Dermatitis", 4th Edition. Eds, Frosch P J, Menné T, Lepoittevin J-P, Springer Verlag, Heidelberg, pp 179 – 188.

Dean J, Twerdok L, Tice R, Sailstad D, Hattan D, Stokes WS. 2001. Evaluation of the murine local lymph node assay (LLNA) II: conclusions and recommendations of an independent scientific peer review panel. Regul Toxicol Pharmacol 34:258-273.

EPA. 2003. Health Effects Test Guideline, OPPTS 870.2600. Skin Sensitization EPA 712–C–03–197. Washington, DC: U.S. Environmental Protection Agency.

Gad SC, Dunn BJ, Dobbs DW, Reilly C, Walsh RD. 1986. Development and validation of an alternative dermal sensitization test: the mouse ear swelling test (MEST). Toxicol Appl Pharmacol. 15;84(1):93-114.

Gerberick GF, House RV, Fletcher ER, Ryan CA. 1992. Examination of the local lymph node assay for use in contact sensitization risk assessment Fundamental and Applied Toxicology 19:438-445

Gerberick GF, Cruse LW, Ryan CA, Hulette BC, Chaney JG, Skinner RA, Dearman RJ, Kimber I. 2002. Use of a B cell marker (B220) to discriminate between allergens and irritants in the local lymph node assay. Toxicol Sci 68:420-428.

Gerberick GF, Ryan CA, Kern PS, Schlatter H, Dearman RJ, Kimber I, Patlewicz GY, Basketter DA. 2005. Compilation of historical local lymph node data for evaluation of skin sensitization alternative methods. Dermatitis 16:157-202.

912 Gerberick GF, Vassallo JD, Foertsch LM, Price BB, Chaney JG, Lepoittevin JP. 2007.
913 Quantification of chemical peptide reactivity for screening contact allergens: a classification tree
914 model approach. Toxicol Sci. 97(2):417-27.

915 ICCVAM. 1997. Validation and Regulatory Acceptance of Toxicological Test Methods: A
916 Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative
917 Methods. NIH Publication No.: 97-3981. Research Triangle Park:National Toxicology Program.

918 ICCVAM 1999. The murine local lymph node assay: A test method for assessing the allergic
919 contact dermatitis potential of chemical/compounds. NIH Publication No. 99-4494. Research
920 Triangle Park, NC: National Toxicology Program.

921 ICCVAM. 2003. ICCVAM Guidelines for the Nomination and Submission of New, Revised, and
922 Alternative Test Methods. NIH Publication No: 03-4508. Research Triangle Park:National
923 Toxicology Program.

924 ICCVAM. 2007. Revised Draft ICCVAM Murine Local Lymph Node Assay Performance
925 Standards. Interagency Coordinating Committee on the Validation of Alternative Methods.
926 Research Triangle Park:National Toxicology Program. Available at
927 http://iccvam.niehs.nih.gov/methods/immunotox/.

928 ISO. 2002. Biological evaluation of medical devices -- Part 10: Tests for irritation and delayed-
929 type hypersensitivity. Available for purchase at: http://www.iso.org/iso/home.htm.

930 Jowsey IR, Basketter DA, Westmoreland C, Kimber I. 2006. A future approach to measuring
931 relative skin sensitising potency: A proposal. Journal of Applied Toxicology 26:341-350.

932 Kimber I, Basketter DA 1997. Contact sensitization: A new approach to risk assessment. Human
933 and Ecological Risk Assessment (HERA)3:385-395.

934 Kimber I, Basketter DA, Butler M, Gamer A, Garrigue J-L, Gerberick GF, Newsome C, Steiling
935 W, Vohr H-W. 2003. Classification of contact allergens according to potency: proposals. Food
936 Chem Toxicol 41:1799-1809.

937 Kwon JA, Lee MS, Kim MI, Park YM, Kim HO, Kim CW. 2003. Allergic contact dermatitis
938 from dodecyldiaminoethylglycine and isopropyl alcohol in a commercial disinfectant swab.
939 Contact Dermatitis. 48:339-340.

MB Research Labs. 2007. Data Submission in response to FR. Accessed at: http://iccvam.niehs.nih.gov/pubcomment/LLNA07/MBresLabs.pdf [last accessed on 28 September 2007].

OECD. 2002. Test guideline 429. Skin Sensitisation: Local Lymph Node Assay, adopted April 24, 2002. In: OECD Guidelines for Testing of Chemicals. Paris:OECD.

Opdyke DLJ. 1976a. Fragrance raw materials monographs. Isopropyl myristate. Food Cosmet Toxicol 14:323-325.

Opdyke DLJ. 1976b. Fragrance raw materials monographs. 6-Methylcoumarin. Food Cosmet Toxicol 14:605.

Poole, R. L., Griffith, J. F., and Macmillan, F. S. K. 1970. Experimental contact sensitization with benzoyl peroxide. Arch. Dermatol. 102:635–641

Rasanen L, Mattila U, Kalimo K. 1999. Patch testing with nickel sulfate versus nickel chloride. Contact Dermatitis. 40:287-288.

Ryan CA, Gerberick G F, Cruse LW, Basketter DA, Lea L, Blaikie L, Dearman RJ, Warbrick EV, Kimber I. 2000. Activity of human contact allergens in the murine local lymph node assay Contact Dermatitis 43:95-102.

Russell WMS, Burch RL. 1992. The Principles of Humane Experimental Technique. 14th ed. South Mimms, Potters Bar, Herts, England:Universities Federation for Animal Welfare.

Sailstad DM, Hattan D, Hill RN, Stokes WS. 2001. ICCVAM Evaluation of the Murine Local Lymph Node Assay (LLNA) I: The ICCVAM Review Process. Regulatory Toxicology and Pharmacology 34:249-257.

Schneider K, Akkan Z. 2004. Quantitative relationship between the local lymph node assay and human skin sensitization assays. Regul Toxicol Pharmacol. Jun;39(3):245-55.