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

Revised Draft Background Review Document

March 2009

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Table of Contents

List of Tables

List of Figures

List of Abbreviations and Acronyms

ACD	Allergic contact dermatitis
ACE	Acetone
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
GP	Guinea pig
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
MW	Molecular weight
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

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Preface

In 1999, the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended the murine (mouse) local lymph node assay (LLNA) as a valid test method to assess the skin sensitization potential of most types of substances (ICCVAM 1999). ICCVAM concluded that the LLNA (referred to herein as the "traditional LLNA") provided several advantages compared to the guinea pig method, including elimination of potential pain and distress, use of fewer animals, less time required to perform, and availability of dose-response information. United States and international regulatory authorities subsequently accepted the traditional LLNA as an alternative test method for allergic contact dermatitis testing. It is now commonly used around the world.

One disadvantage of the traditional LLNA is that it requires injection of a radioactive marker to measure cell proliferation in lymph nodes. To avoid the use of radioactive markers, scientists have recently developed several non-radioactive versions of the LLNA. In 2007, the U.S. Consumer Product Safety Commission (CPSC) asked ICCVAM and the National Toxicology Program Interagency Center for the Evaluation of Alternative Methods (NICEATM) to evaluate the scientific validity of these non-radioactive versions. ICCVAM assigned the nomination a high priority, and established the ICCVAM Immunotoxicity Working Group (IWG) to work with NICEATM to review the current literature and evaluate available data to assess the validity of three such test methods. A comprehensive draft background review document (BRD) provided the information, data, and analyses supporting the validation status of each of the non-radioactive test methods. ICCVAM also developed draft test method recommendations for each test method regarding its usefulness and limitations, test method protocol, performance standards, and future studies.

NICEATM and ICCVAM provided the draft BRD and draft recommendations to an international independent scientific peer review panel for their consideration at a public meeting on March 4-6, 2008. A report of the Panel meeting was subsequently published on the NICEATM-ICCVAM website[2]. Both the Panel and ICCVAM concluded that more information was needed before a recommendation on the usefulness and limitations of each of the three test methods could be made. The Panel recommended that NICEATM obtain additional existing data that was not available to the Panel and reanalyze the performance of

[2] http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel08.htm

each non-radioactive LLNA method. NICEATM subsequently obtained additional data and prepared updated BRDs. ICCVAM also prepared revised draft test method recommendations based on the revised BRDs. This revised draft BRD addresses the validation database for the LLNA: BrdU-FC.

The Panel will meet to consider the revised BRDs and to evaluate the extent to which the available information supports the revised ICCVAM draft test method recommendations. ICCVAM will consider the conclusions and recommendations of the Panel, along with comments received from the public and the Scientific Advisory Committee for Alternative Toxicological Methods, and then finalize the BRDs and test method recommendations. These will then be forwarded to Federal agencies for their consideration and acceptance decisions where appropriate.

We gratefully acknowledge the organizations and scientists who provided data and information for this document. We also acknowledge the efforts of those individuals contributing to the preparation of this BRD, including the following staff from the NICEATM Support Contractor, Integrated Laboratory Systems, Inc.: David Allen, Ph.D., Thomas Burns, M.S., Gregory Moyer, M.B.A., Michael Paris, Eleni Salicru, Ph.D., Catherine Sprankle, Frank Stack, and Judy Strickland, Ph.D. We also thank the members of the ICCVAM IWG, chaired by Abigail Jacobs, Ph.D. (U.S. Food and Drug Administration) and Joanna Matheson, Ph.D. (CPSC), and ICCVAM representatives who subsequently reviewed and provided comments throughout the process leading to this final draft version.

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Executive Summary

Background

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended to U.S. Federal agencies that the murine local lymph node assay (LLNA) is a valid substitute for currently accepted guinea pig test methods to assess the allergic contact dermatitis (ACD) potential of many, but not all, types of substances. ACD is an allergic skin reaction characterized by redness, swelling, and itching that can result from contact with a sensitizing chemical or product. The recommendation was based on a comprehensive evaluation that included an independent scientific peer review panel (Panel) assessment of the validation status of the LLNA. The Panel report and the ICCVAM recommendations (ICCVAM 1999) are available at the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)-ICCVAM website (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 Organization for Standardization [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]).

In 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 the original and this revised 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).

Revisions to the LLNA: BrdU-FC Evaluation

NICEATM and ICCVAM convened an independent international scientific peer review panel meeting on March 4-6, 2008. The Panel reviewed the draft BRD and commented on the extent to which it supports the draft ICCVAM test method recommendations on the usefulness and

limitations of the LLNA: BrdU-FC. Both ICCVAM and the Panel concluded that they needed more information before they could make a recommendation on the usefulness and limitations of the LLNA: BrdU-FC.[3] The Panel requested individual animal data and evaluations of both intra- and interlaboratory reproducibility. The Panel recommended that NICEATM obtain additional data and reanalyze the performance of the LLNA: BrdU-FC method. In response, NICEATM obtained additional LLNA: BrdU-FC data, which were used to update the evaluation as described below. These data include:

- LLNA: BrdU-FC data from multiple studies with 2-mercaptobenzothiazole (MBT) using different vehicles. These data were submitted in a response to a request for an explanation for the discordant results for MBT. The new data indicate a vehicle dependent response in the LLNA: BrdU-FC for identifying a positive result with MBT. Results of the retests of MBT demonstrated positive results when tested in dimethyl sulfoxide (DMSO) or dimethylformamide (DMF), but MBT gave negative results in DaAE (DMSO: acetone: ethanol; 4:3:3). Revisions for the new data are detailed in **Section 5.0** and **Appendix D**.
- Data from studies for sodium lauryl sulfate (SLS) using an enhanced LLNA: BrdU-FC protocol (eLLNA: BrdU-FC). The eLLNA: BrdU-FC includes an assessment of immunophenotypic markers to distinguish sensitizers from irritants, reportedly to reduce the incidence of false positive results. SLS was used as a positive control in DMSO tests; 2/5 animals exhibited ear swelling >25%, indicating that SLS induced an irritation response. These new data are described in **Sections 5.0** and **6.0** with details in **Appendix D**.
- New EC3 results were obtained from four tests each in LLNA: BrdU-FC for hexyl cinnamic aldehyde (HCA) and 2,4-dinitrochlorobenzene (DNCB). These new data demonstrated intralaboratory reproducibility within the range of acceptability for both substances as described in the ICCVAM LLNA Performance Standards. These data are detailed in **Section 7.0** and **Appendix D**.

Test Method Protocol

The protocol in this draft BRD has not been revised from the January 2008 draft BRD. The LLNA: BrdU-FC was developed by MB Research Labs (2001). The traditional LLNA assesses cell proliferation by measuring the incorporation of radioactivity into the deoxyribonucleic acid (DNA) of dividing lymph node cells. In contrastLLNA: BrdU-FC uses flow cytometry to assess cell proliferation by measuring the incorporation of the thymidine analog BrdU into the DNA of dividing lymphocytes. A stimulation index (SI) is the ratio of the mean BrdU incorporation into

[3] http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel.htm

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. An SI value greater than or equal to 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). As noted above, the eLLNA: BrdU-FC includes enhancements for substances with SI ≥ 3 that include an assessment of immunophenotypic markers to distinguish sensitizers from irritants.

Test Method Accuracy

The accuracy evaluation in this draft BRD has been revised from the January 2008 draft BRD to reduce the number of equivocal substances based on new data for MBT, and to include revisions to the reference data for the traditional LLNA and human data. The accuracy of the LLNA: BrdU-FC and the eLLNA: BrdU-FC was assessed using data submitted by MB Research Labs (2007) for up to 45 substances. Of these 45 substances, 37 had LLNA: BrdU-FC, traditional LLNA, and guinea pig data. Forty-two substances had LLNA: BrdU-FC, traditional LLNA, and human data. Two of the 45 substances (equivocal substances) produced divergent results when tested at least twice in the traditional LLNA and/or in the LLNA: BrdU-FC. To account for the equivocal substances, two separate accuracy analyses were conducted. In one, only the substances with unequivocal LLNA: BrdU-FC results were evaluated; in the other, the two equivocal substances were included by using the more conservative result (i.e., by using the positive responses) for both substances.

When the LLNA: BrdU-FC was compared to the traditional LLNA (excluding the two equivocal substances), the LLNA: BrdU-FC had an accuracy of 95% (41/43), a false positive rate of 7% (1/15), and a false negative rate of 4% (1/28).[4] Including the two equivocal substances resulted in an accuracy for the LLNA: BrdU-FC of 93% (42/45), a false positive rate of 13% (2/16), and a false negative rate of 3% (1/29).[4]

When the eLLNA: BrdU-FC was compared to the traditional LLNA, accuracy was 88% (38/43), the false positive rate was 7% (1/15), and false negative rate was 14% (4/28). Using the traditional LLNA as the reference classification, two nonsensitizers and two sensitizers were identified incorrectly. However, the two substances identified by the eLLNA: BrdU-FC as nonsensitizers (ethylene glycol dimethacrylate and sodium lauryl sulfate) were identified as nonsensitizers by guinea pig skin sensitization tests also. SLS is also considered a nonsensitizer

[4] The one false negative substance is aniline, which did not generate a strongly positive result in the traditional LLNA (EC3 = 48%, maximum SI = 3.6 at 50% in acetone: olive oil).

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 two equivocal substances resulted in an accuracy for the eLLNA: BrdU-FC of 87% (39/45), a false positive rate of 13% (2/16), and a false negative rate of 14% (4/29).

Test Method Reliability – Intralaboratory Reproducibility

The intralaboratory reproducibility has been revised to include new data for HCA and DCNB that were not available for evaluation in the January 2008 draft BRD. 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. For the SI values of 25% HCA, the positive control substance, tested in various vehicles, the CVs ranged from 30.1% to 52.6%. EC3 results were obtained from four tests each in LLNA: BrdU-FC for HCA and DNCB. These data demonstrated intralaboratory reproducibility within the range of acceptability for both substances as described in the ICCVAM LLNA Performance Standards.

Test Method Reliability – Interlaboratory Reproducibility

Nothing has been added to the interlaboratory reproducibility section since the January 2008 draft BRD. Interlaboratory reproducibility for the LLNA: BrdU-FC and the eLLNA: BrdU-FC could not be addressed because data were only available from one laboratory.

Animal Welfare Considerations

The animal welfare considerations in this draft BRD have not changed from the January 2008 draft BRD. The LLNA: BrdU-FC and the eLLNA: BrdU-FC will use the same number of animals as the traditional LLNA. However, because the traditional LLNA cannot be conducted in some institutions because it involves radioactivity, availability and use of the nonradioactive LLNA: BrdU-FC and the eLLNA: BrdU-FC test methods may further reduce use of the guinea pig test methods. Such a reduction could reduce animal use and increase refinement as pain and distress are avoided in the LLNA procedure.

Test Method Transferability

The test method transferability considerations in this draft BRD have not changed from the January 2008 draft BRD. The transferability of the LLNA: BrdU-FC and the eLLNA: BrdU-FC is expected to be similar to that of the traditional LLNA. Unlike the traditional LLNA, the LLNA: BrdU-FC and the eLLNA: BrdU-FC will not require facilities, equipment, and licensing permits for handling radioactive materials. The level of training and expertise needed to conduct the LLNA: BrdU-FC and the eLLNA: BrdU-FC should be similar to that needed for the

411 traditional LLNA except that proficiency in flow cytometry is required for the nonradioactive
412 test methods.

413 **ICCVAM Revised Draft Recommendations**

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

1.0 Introduction

1.1 Public Health Perspective

Allergic contact dermatitis (ACD) is a frequent occupational health problem. According to the U.S. Department of Labor Bureau of Labor Statistics, ACD resulted in 980 lost workdays in 2005.[5]

ACD develops in two phases, induction and elicitation. The induction phase occurs when a susceptible individual is exposed topically to a skin-sensitizing substance. During induction, the substance passes through the epidermis, where it forms a hapten complex with dermal proteins. The Langerhans cells, the resident antigen-presenting cells in the skin, process the hapten complex. The processed hapten complex then migrates to the draining lymph nodes. Antigen presentation to T-lymphocytes follows, which leads to the clonal expansion of these cells. At this point, the individual is sensitized to the substance (Basketter et al. 2003; Jowsey et al. 2006). Studies have shown that the magnitude of lymphocyte proliferation correlates with the extent to which sensitization develops (Kimber and Dearman 1991, 1996).

The elicitation phase occurs when the individual is topically exposed to the same substance again. As in the induction phase, the substance penetrates the epidermis, is processed by the Langerhans cells, and is then presented to circulating T-lymphocytes. The T-lymphocytes are then activated, which causes release of cytokines and other inflammatory mediators. This release produces a rapid dermal immune response that can lead to ACD (ICCVAM 1999; Basketter et al. 2003; Jowsey et al. 2006).

1.2 Historical Background for the Murine Local Lymph Node Assay (LLNA)

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended to U.S. Federal agencies that LLNA is a valid substitute for currently accepted guinea pig (GP) test methods to assess the ACD potential of many, but not all, types of substances. ICCVAM based its recommendation on a comprehensive evaluation that included an assessment of the validation status of the LLNA by an independent scientific peer review panel (Panel). 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).

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

ICCVAM recommended that the LLNA be considered for regulatory acceptance or other nonregulatory applications for assessing the ACD potential of substances, while acknowledging 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 for evaluation by ICCVAM and NICEATM several activities related to the LLNA (Available at http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf). The requested activities included an assessment of the validation status of nonradioactive alternatives to the current version of the LLNA (traditional LLNA) (ICCVAM 1999, Dean et al. 2001), which uses radioactivity to detect sensitizers. ICCVAM and NICEATM compiled the information in this background review document (BRD) 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) (LLNA: BrdU-FC). ICCVAM and its Immunotoxicity Working Group (IWG) evaluated this method in draft test method recommendations based on the BRD evaluation. An independent international scientific peer review panel (Panel) reviewed the BRD in March 2008 to evaluate the extent to which the information contained in the BRD supported the draft recommendations. The Panel concluded that additional information was needed to evaluate the method, including original animal data, quantitative data for the method, and an evaluation of interlaboratory reproducibility. NICEATM gathered the additional information and produced this revised draft BRD for review by the Panel.

ICCVAM will consider the conclusions and recommendations of the Panel, along with comments received from the public and SACATM, when developing the final BRD and final recommendations on the usefulness and limitations of each nonradioactive alternative LLNA test methods that is being considered.

1.3 The LLNA: BrdU-FC

The LLNA: BrdU-FC was developed by MB Research Labs (2001) as a nonradioactive alternative to the current version of the traditional 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 (i.e., immunophenotypic markers such as B220 and CD69) are incorporated in an enhanced LLNA: BrdU-FC protocol (eLLNA: BrdU-FC) to further distinguish irritants from sensitizers.

This document provides:

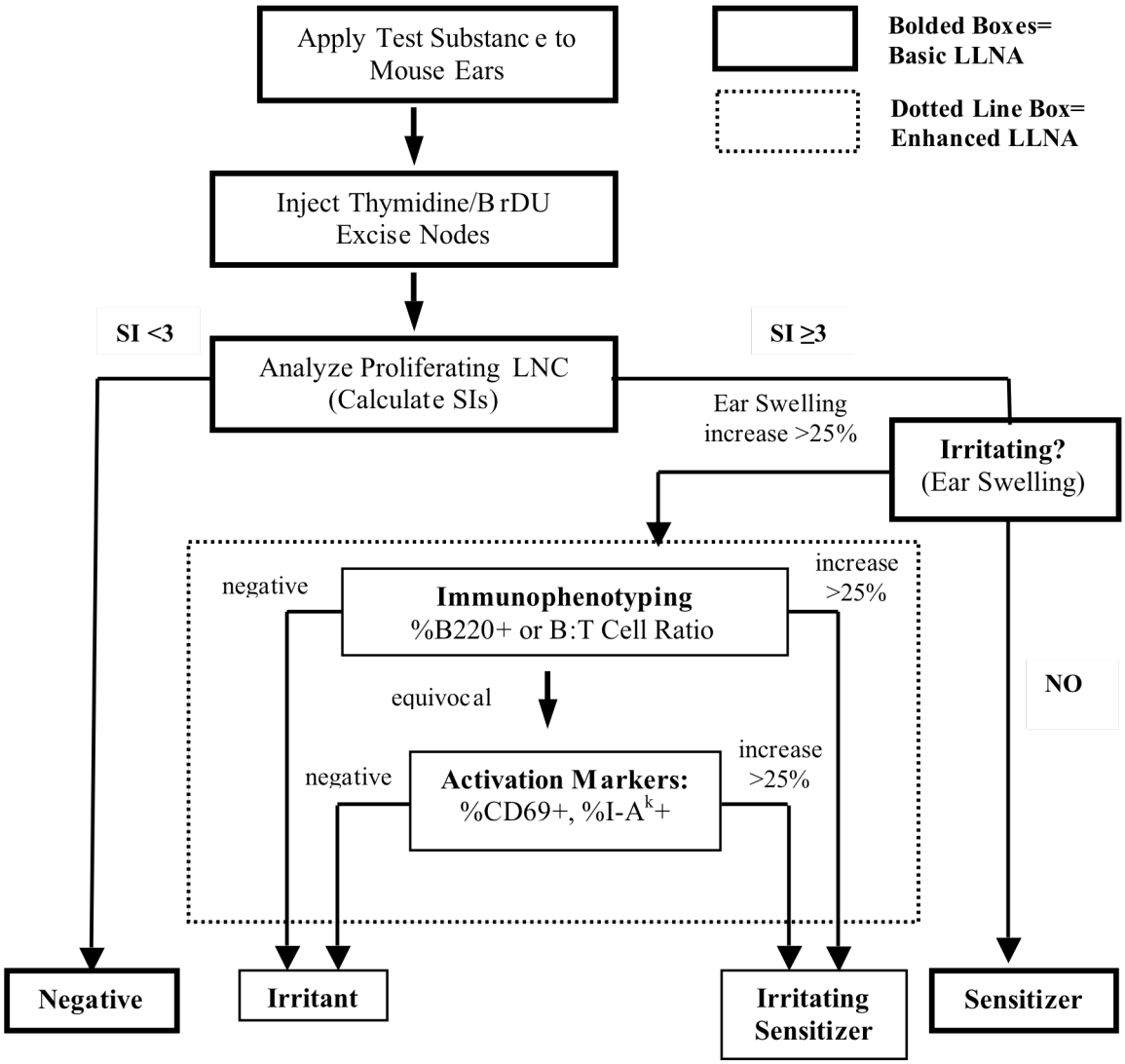
- A comprehensive summary of the LLNA: BrdU-FC test method protocol
- Identification of the substances used in the validation of the test method and the test results
- The performance characteristics (accuracy and reliability) of the test method
- Animal welfare considerations
- Other considerations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

2.0 LLNA: BrdU-FC Test Method Protocol

The protocol in this draft BRD has not been revised from the January 2008 draft BRD. 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 when determining the highest dose level, as is recommended in the ICCVAM LLNA protocol, the LLNA: BrdU-FC includes a quantitative assessment of potential dermal irritation by measuring ear thickness with a digital micrometer at three separate timepoints (once each on Days 1 [prior to dosing], 3, and 6).

In the traditional LLNA, the test substance is administered on three consecutive days. Forty-eight hours after the final application of the test substance, ^{3}H-methyl thymidine or ^{125}I-fluorodeoxyuridine (in phosphate-buffered saline; 250 μL/mouse) is injected into the tail vein. This same dosing schedule is followed in the LLNA: BrdU-FC, but 200 μL per mouse of BrdU is administered intraperitoneally rather than intravenously (see **Appendix A** for the rationale for the route of administration and amount of BrdU). 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**.

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



Abbreviations: B = B lymphocyte; BrdU = bromodeoxyuridine; LLNA = murine local lymph node assay; LNC = lymph node cells; SI = stimulation index; T = T lymphocyte

The shaded box shows that the enhancements of immunophenotyping and measurement of activation markers are used when SI ≥ 3 and mouse ear swelling ≥ 25% (i.e., the enhanced LLNA: BrdU-FC protocol [eLLNA: BrdU-FC]).

As mentioned above, the eLLNA: BrdU-FC incorporates immunophenotypic endpoints, which are evaluated sequentially using the criteria described in **Section 2.1**, to distinguish irritants from dermal sensitizers when a stimulation index (SI) ≥ 3 is recorded. For mice exhibiting ear swelling >25%, the first-tier endpoints include determination of the percentage of 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 have shown a preferential increase in the percentage of B220+ cells compared with irritant-treated mice (Gerberick et al. 2002). An increase of more than 25% for B220+ cells or a B:T cell ratio greater than 1.25 indicates that a substance is an irritating sensitizer. If the percentage of B220+ cells or the B:T cell ratio increases by less than 25%, then the substance is classified as an irritant. However, a second tier of immunophenotypic measurements can be used to reconcile outcomes in which the B220+ cells or the B:T cell ratio produce a borderline response. In those instances, an increase of greater than 25% in IA^K+ cells (B-lymphocytes) or CD69 (T-lymphocytes) indicates 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, the LLNA: BrdU-FC uses an SI value to distinguish skin sensitizers from nonsensitizers. 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:

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

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 allows further evaluation of substances that produce SI values ≥ 3 in order 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., percentage of B220+ cells or the calculation of the B:T cell ratio). If the percentage of B220+ cells 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 the

556 percentage of B220+ cells increases more than 25% above control values or the B:T cell ratio
557 is >1.25, then the substance is classified as an irritating sensitizer. If the increase in the
558 percentage of B220+ cells or the B:T cell ratio is equivocal (i.e., at least one mouse has ear
559 swelling >25% and the percentage of B220+ cells or the B:T cell ratio is significantly
560 elevated or is greater than 25% above control values), then an evaluation of the second set of
561 immunophenotypic markers is conducted (i.e., percentage of either IA^K+ cells or CD69+
562 cells). If the percentage of IA^K+ cells or CD69+ cells is >25% above control values, then the
563 substance is classified as a sensitizer. If the percentage of IA^K+ cells or CD69+ cells is <25%
564 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 in comparison to 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; therefore, they are not included in 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, 28 were classified by the traditional LLNA as skin sensitizers and 17 were classified as nonsensitizers. As shown by the EC3 values (i.e., calculated concentration that corresponds to SI=3) in **Table 3-1**, the 28 sensitizers were representative of a full range of sensitization responses (i.e., weak to strong sensitizers).

Appendix B provides information on the physicochemical properties (e.g., peptide reactivity, octanol-water partition coefficient), Chemical Abstracts Service Registry Number, and chemical class for each substance tested. When available, chemical class information was retrieved from the National Library of Medicine's ChemIDplus® 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 (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 present in the structures that were evaluated in this analysis. Classification of substances into chemical classes is not intended to represent 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).

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

Substance Name	Chemical Class[1]	Traditional LLNA EC3[2]	No.[3]
Oxazalone	Heterocyclic compounds	0.003	5
Benzoyl peroxide	Carboxylic acids	0.01[5]	2
Tetrachlorosalicylanilide	Amides; Amines	0.04	1
2, 4-DNCB	Hydrocarbon, Halogenated; Nitro compounds; Hydrocarbons, Cyclic	0.05	15
Diphenylcyclopropenone	Hydrocarbons, Cyclic	0.05	1
Benzalkonium chloride	Onium compounds	0.10	1
4-Phenylenediamine	Amines	0.11	6
Potassium dichromate	Inorganic chemical, Chromium compounds, Potassium compounds	0.17	12
Copper chloride	Inorganic chemicals	0.4	1
Formaldehyde	Aldehydes	0.5	6
Cobalt chloride	Inorganic chemicals, Metals	0.6[3]	2
Isoeugenol	Carboxylic acids	1.5	47
2-Mercaptobenzothiazole	Heterocylic compounds	1.7[6]	1
Ethylenediamine	Amines	2.2	1
Diethylenetriamine	Amines	3.3	1
Benzocaine	Carboxylic acids	3.4	1
Trimellitic anhydride	Anhydrides; Carboxylic acids	4.7	2
Resorcinol	Phenols	6.3	1
Sodium lauryl sulfate	Alcohols; Sulfur compounds; Lipids	8.1[6]	5
Citral	Hydrocarbons, Other	9.2	6
Hexyl cinnamic aldehyde	Aldehydes	9.7	21
Eugenol	Carboxylic acids	10	11
Ethylene glycol dimethacrylate	Carboxylic acids	28[7]	1
Linalool	Hydrocarbons	30	1
Isopropyl myristate	Lipids	44	1
Aniline	Amines	48	3
Pyridine	Heterocyclic compounds	72	1
Xylene	Hydrocarbons, Cyclic	96[5]	1
4-Aminobenzoic acid	Carboxylic acids	NA	NA
Benzoic acid	Carboxylic acids	NA	NA
Chlorobenzene	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated	NA	NA
Glycerol	Alcohols; Carbohydrates	NA	NA
Hexane	Hydrocarbons, Acyclic	NA	NA
Hydrocortisone	Polycyclic compounds	NA	NA

Substance Name	Chemical Class[1]	Traditional LLNA EC3[2]	No.[3]
Isopropanol	Alcohols	NA	NA
Lactic acid	Carboxylic acids	NA	NA
6-Methylcoumarin	Heterocyclic compounds	NA	NA
Methyl salicylate	Phenols; Carboxylic acids	NA	NA
Nickel chloride	Inorganic chemicals	NA	NA
Propylene glycol	Alcohols	NA	NA
Propylparaben	Phenols; Carboxylic acids	NA	NA
Salicylic acid	Phenols; Carboxylic acids	NA	NA
Sulfanilimide	Amides; Sulfur compounds; Amines	NA	NA
Tween 80	Alcohols	NA	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] Number of traditional LLNA studies from which the EC3 data were obtained

[4] Vehicle= Dimethyl sulfoxide

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

[6] Vehicle not reported

[7] Vehicle = Dimethylformamide

[8] Vehicle = Methyl ethyl ketone

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. (1999, 2006), Gerberick et al. (2005), or Schneider and Akkan (2004). No traditional LLNA data were identified for three substances: 4-aminophenol HCl, chlorpromazine +UVR, and croton oil; therefore, they are 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
- fluorescein isothiocyanate

No GP data could be located for seven substances:

- diphenylcyclopropenone
- hexane
- hydrocortisone
- linalool
- pyridine
- xylene
- isopropyl myristate.

Additionally, no human data could be located for chlorobenzene or trimellitic anhydride.

5.0 Test Method Data and Results

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. Forty-two substances had LLNA: BrdU-FC, traditional LLNA, and human data. Two of the 45 substances produced discordant results when tested at least twice in the traditional LLNA and/or in the LLNA (equivocal substances): BrdU-FC (i.e., benzocaine in both tests and salicylic acid in the LLNA: BrdU-FC test). Data initially submitted for 2-mercaptobenzothiazole (MBT) indicated that it produced equivocal results in the LLNA: BrdU-FC, but results of retests that were subsequently provided to NICEATM demonstrated this variability was likely due variations in the vehicle tested. MBT produced positive results when tested in dimethyl sulfoxide (EC3 = 4.1% in DMSO; max SI = 8.0 at 25% MBT) or when tested in dimethylformamide (EC3 = 22% in DMF; max SI = 3.3 at 25% MBT); MBT (up to 25%) gave negative results in DaAE (DMSO: acetone: ethanol at a ratio of 4:3:3 parts; max SI = 1.3 at 10% MBT). Sodium lauryl sulfate (SLS) was used as a positive control in DMSO tests (SI = 3.0–4.7 at 25% SLS; 2/5 animals exhibited ear swelling >25%, indicating that SLS induced an irritation response).

All test results were obtained using the protocol in **Appendix A.** The LLNA: BrdU-FC results for 48 substances are included in **Appendix C**. 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). In order to hide their identities during testing, test substances were not coded.

6.0 Test Method Accuracy

The accuracy evaluation in this draft BRD has been revised from the January 2008 draft BRD to reduce the number of equivocal substances based on new data for MBT, and to include revisions to the reference data for the traditional LLNA and human data. 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 two equivocal substances were included by using the more conservative result (i.e., positive) for both substances. Including the two equivocal substances resulted in a net gain of one correctly identified sensitizer and one false positive result when comparing the LLNA: BrdU-FC to the traditional LLNA, guinea pig, 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 (excluding the two equivocal substances) the LLNA: BrdU-FC had an accuracy of 95% (41/43), a sensitivity of 96% (27/28), a specificity of 93% (14/15), a false positive rate of 7% (1/15), and a false negative rate of 4% (1/28) (**Table 6-1**).

Including the two equivocal substances resulted in an accuracy for the LLNA: BrdU-FC of 93% (42/45), a sensitivity of 97% (28/29), a specificity of 88% (14/16), a false positive rate of 12% (2/16), and a false negative rate of 3% (1/29) (**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 (74% [26/35] vs. 81% [29/36]), lower sensitivity (84% [16/19] vs. 90% [17/19]), and lower specificity (63% [10/16] vs. 71% [12/17]) compared with the traditional LLNA. The LLNA: BrdU-FC also had a higher false positive rate (38% [6/16] vs. 29% [5/17]) and a higher false negative rate of (16% [3/19] vs. 11% [2/19]) than the traditional LLNA (**Table 6-1**).

Including the two equivocal substances resulted in only a slight reduction in overall performance for the LLNA: BrdU-FC (e.g., accuracy reduced to 73% [27/37] from 74% [26/35]) 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 similar accuracy (72% [29/40] vs. 73% [30/41]), similar specificity (61% [8/13] vs. 64% [9/14]), and the same sensitivity (78% [21/27]) as the traditional LLNA when using human sensitization outcomes as the reference data. Similarly, the LLNA: BrdU-FC had a false positive rate (39% [5/13] vs. 36% [5/14]) that was similar to the traditional LLNA, and the same false negative rate (22% [6/27]) as the traditional LLNA, when each was compared to human sensitization outcomes.

Including the two equivocal substances resulted in a slight reduction in test method accuracy for the LLNA: BrdU-FC (accuracy was reduced from 72% [29/40] to 71% [30/42]) when compared to human sensitization outcomes (**Table 6-1**).

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

Comparison	N[1]	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate		Positive Predictivity		Negative Predictivity	
		%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]
LLNA: BrdU-FC vs. Traditional LLNA	43	95	41/43	96	27/28	93	14/15	7	1/15	4	1/28	96	27/28	93	14/15
LLNA: BrdU-FC vs. Traditional LLNA*	45	93	42/45	97	28/29	88	14/16	13	2/16	3	1/29	93	28/30	93	14/15
Substances with LLNA: BrdU-FC, Traditional LLNA, and GP Data															
LLNA: BrdU-FC vs. Traditional LLNA	35	94	33/35	96	21/22	92	12/13	8	1/13	4	1/22	95	21/22	92	12/13
LLNA: BrdU-FC vs. Traditional LLNA*	37	92	34/37	96	22/23	86	12/14	14	2/14	4	1/23	92	22/24	92	12/13
LLNA: BrdU-FC vs. GP[3]	35	74	26/35	84	16/19	63	10/16	37	6/16	16	3/19	73	16/22	77	10/13
LLNA: BrdU-FC vs. GP[3]*	37	73	27/37	85	17/20	59	10/17	41	7/17	15	3/20	71	17/24	77	10/13
Traditional LLNA vs. GP[3]	35	80	28/35	90	17/19	69	11/16	31	5/16	10	2/19	77	17/22	85	11/13
Traditional LLNA vs. GP[3]*	37	81	30/37	90	18/20	71	12/17	29	5/17	10	2/20	78	18/23	86	12/14
Substances with LLNA: BrdU-FC, Traditional LLNA, and Human Data															
LLNA: BrdU-FC vs. Traditional LLNA	40	95	38/40	96	25/26	93	13/14	7	1/14	4	1/26	96	25/26	93	13/14
LLNA: BrdU-FC vs. Traditional LLNA*	42	93	39/42	96	26/27	87	13/15	13	2/15	4	1/27	93	26/28	93	13/14
LLNA: BrdU-FC vs. Human[4]	40	72	29/40	78	21/27	61	8/13	39	5/13	22	6/27	81	21/26	57	8/14
LLNA: BrdU-FC vs. Human[4]*	42	71	30/42	79	22/28	57	8/14	43	6/14	21	6/28	79	22/28	57	8/14
Traditional LLNA vs. Human[4]	41	73	30/41	78	21/27	64	9/14	36	5/14	22	6/27	81	21/26	60	9/15
Traditional LLNA vs. Human[4]*	42	74	31/42	79	22/28	64	9/14	36	5/14	21	6/28	81	22/27	60	9/15

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 2 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 conducted 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., nonsensitizers) 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 43 substances with unequivocal eLLNA: BrdU-FC and traditional LLNA results, the eLLNA: BrdU-FC decreased the accuracy (88% [38/43] vs. 95% [41/43]) and sensitivity (86% [24/28] vs. 96% [27/28]) and increased the false negative rate (14% [4/28] vs. 4% [1/28]) relative to the LLNA: BrdU-FC (compare **Table 6-2** with **Table 6-1**). The specificity rates (93% [14/15]) and the false positive rates (7% [1/15]) were the same for the eLLNA: BrdU-FC vs. the traditional LLNA compared to the LLNA: BrdU-FC vs. the traditional LLNA.

Including the two equivocal substances resulted in an accuracy for the eLLNA: BrdU-FC of 87% (39/45), a sensitivity of 86% (25/29), a specificity of 88% (14/16), a false positive rate of 13% (2/16), and a false negative rate of 14% (4/29) (**Table 6-2**).

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

Comparison	N	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate		Positive Predictivity		Negative Predictivity	
		%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]
eLLNA: BrdU-FC vs. Traditional LLNA	43	88	38/43	86	24/28	93	14/15	7	1/15	14	4/28	96	24/25	78	14/18
eLLNA: BrdU-FC vs. Traditional LLNA*	45	87	39/45	86	25/29	88	14/16	13	2/16	14	4/29	93	25/27	78	14/18
Substances with eLLNA: BrdU-FC, Traditional LLNA, and GP Data															
eLLNA: BrdU-FC vs. Traditional LLNA	35	86	30/35	82	18/22	92	12/13	8	1/13	18	4/22	95	18/19	75	12/16
eLLNA: BrdU-FC vs. Traditional LLNA*	37	84	31/37	83	19/23	86	12/14	14	2/14	17	4/23	91	19/21	75	12/16
eLLNA: BrdU-FC vs. GP	35	83	29/35	84	16/19	81	13/16	19	3/16	16	3/19	84	16/19	81	13/16
eLLNA: BrdU-FC vs. GP*	37	81	30/37	85	17/20	77	13/17	23	4/17	15	1/18	81	17/21	81	13/16
Traditional LLNA vs. GP	35	80	28/35	90	17/19	69	11/16	31	5/16	10	2/19	77	17/22	85	11/13
Traditional LLNA vs. GP*	37	81	30/37	90	18/20	71	12/17	29	5/17	10	2/20	78	18/23	86	12/14
Substances with eLLNA: BrdU-FC, Traditional LLNA, and Human Data															
eLLNA: BrdU-FC vs. Traditional LLNA	40	88	35/40	85	22/26	93	13/14	7	1/14	15	4/26	96	22/23	77	13/17
eLLNA: BrdU-FC vs. Traditional LLNA*	42	86	36/42	85	23/27	87	13/15	13	2/15	15	4/27	92	23/25	77	13/17
eLLNA: BrdU-FC vs. Human[3]	40	70	28/40	70	19/27	69	9/13	31	4/13	30	8/27	83	19/23	53	9/17
eLLNA: BrdU-FC vs. Human[3]*	42	69	29/42	71	20/28	64	9/14	36	5/14	29	8/28	80	20/25	53	9/17
Traditional LLNA vs. Human[3]	41	73	30/41	78	21/27	64	9/14	36	5/14	22	6/27	81	21/26	60	9/15
Traditional LLNA vs. Human[3]*	42	74	31/42	79	22/28	64	9/14	36	5/14	21	6/28	82	22/27	60	9/15

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 obtained using either the Guinea Pig Maximization Test or the Buehler Test; LLNA = Murine local lymph node assay; N = Number of substances included in this analysis; 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] The data on which the percentage calculation is based.

[3] Human refers to outcomes obtained by studies conducted 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

The concordance of the eLLNA: BrdU-FC with GP data was greater than the concordance of LLNA: BrdU-FC data to GP data) because ethylene glycol dimethacrylate and sodium lauryl sulfate were classified as nonsensitizers in both eLLNA: BrdU-FC and GP tests. These substances were classified as sensitizers by the LLNA: BrdU-FC. For the 35 substances with eLLNA: BrdU-FC, GP, and traditional LLNA data, the eLLNA: BrdU-FC protocol improved the performance of the LLNA: BrdU-FC (compare **Table 6-2** with **Table 6-1**). Accuracy increased to 83% (29/35) from 74% (26/35); specificity increased to 81% (13/16) from 63% (10/16); and the false positive rate decreased from 38% (6/16) to 19% (3/16). The sensitivity (84% [16/19]) and the false negative rates (16% [3/19]) were the same for the LLNA: BrdU-FC and the eLLNA: BrdU-FC.

As in the LLNA: BrdU-FC, including the two equivocal substances resulted in only a slight reduction in overall performance for the eLLNA: BrdU-FC (accuracy reduced from 83% [29/35] to 81% [30/37]) 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 70% (28/40) from 72% (29/40) for LLNA: BrdU-FC; the sensitivity decreased to 70% (19/27) from 78% (21/27); and the false negative rate increased from 22% (6/27) to 30% (8/27). The specificity for the eLLNA: BrdU-FC increased to 69% (9/13) from 61% (8/13); and the false positive rate decreased to 31% (4/13) from 39% (5/13) for LLNA: BrdU-FC.

Including the two equivocal substances did not change overall performance for the eLLNA: BrdU-FC (e.g., accuracy remained 69% [29/42]) when compared to human sensitization outcomes (**Table 6-2**).

6.3 Accuracy Analysis Based on ICCVAM Draft Performance Standards

ICCVAM has proposed test method performance standards for the LLNA (ICCVAM 2009) These test method performance standards are proposed to evaluate the performance of LLNA test methods that incorporate specific protocol 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 nonsensitizers. Two substances, 2-mercaptobenzothiazole (sensitizer, mean EC3 = 2.5%) and salicylic acid (nonsensitizer), 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 the ICCVAM LLNA performance standards were also tested in the LLNA: BrdU-FC. Ethylene glycol dimethacrylate and sodium lauryl sulfate, two nonsensitizers, were both false positives in the LLNA: BrdU-FC. They were also false positives in the traditional LLNA. However, when tested in the eLLNA: BrdU-FC, ethylene glycol dimethacrylate and sodium lauryl sulfate 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.

Table 6-4 shows the EC3 range of substances tested in the LLNA: BrdU-FC based on the overall database of 45 substances in comparison to that of substances from list of minimum reference standards in the revised draft ICCVAM LLNA performance standards substances list. The table reveals that, although not all of the draft ICCVAM performance standards reference substances have been tested in the LLNA: BrdU-FC, the EC3 range of those tested is similar to that for substances on 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.

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

Name	ICCVAM Draft LLNA Performance Standards[1]				LLNA: BrdU-FC[2]		
	Result	EC3 (%)	N	Vehicle	Result	EC3 (%)	Vehicle
5-Chloro-2-methyl-4-isothiazolin-3-one	+	0.009	1	DMF	NT	NT	IR
2,4-Dinitrochlorobenzene	+	0.049	15	AOO	+	0.01-*0.09*	AOO
4-Phenylenediamine	+	0.11	10	AOO	+	0.45	IR
4-Methylaminophenol sulfate	+	0.8	1	DMF	NT	NT	IR
Isoeugenol	+	1.5	49	AOO	+	NR	IR
2-Mercaptobenzothiazole	+	2.5	2	AOO	+	4.1	DMSO
Cobalt chloride	+	0.6	2	DMSO	+	1	L92
Citral	+	9.8	6	AOO	+	2	DaAE
Hexyl cinnamic aldehyde	+	9.7	22	AOO	+	6-16	AOO
Eugenol	+	10.1	11	AOO	+	13.2	IR
Phenyl benzoate	+	13.6	3	AOO	NT	NT	IR
Cinnamic alcohol	+	21	1	AOO	NT	NT	IR
Imidazolidinyl urea	+	24	1	DMF	NT	NT	IR
Chlorobenzene	-	NA	1	AOO	-	NA	IR
Isopropanol	-	NA	1	AOO	-	>50%	IR
Lactic acid	-	NA	2	DMSO	-	NA	IR
Methyl salicylate	-	NA	10	AOO	-	NA	IR
Salicylic acid	**-**	NA	1	***AOO***	**+/-**	***NA***	***IR***
Ethylene glycol dimethylacrylate	FP	28	1	MEK	+[3]	40.0	IR
Sodium lauryl sulfate	FP	8.1	5	DMF	+[3]	4.8	DMSO
Nickel sulfate	FN	NA	2	DMF	NT	NT	IR
Sulfanilamide	FN	NA	1	DMF	-	>50%	IR

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

Abbreviations: AOO = acetone and olive oil; DaAE = DMSO, acetone, and ethanol; DMF = dimethylformamide; DMSO = dimethyl sulfoxide; FN = false negative; FP = false positive; LLNA: BrdU-FC = Murine local lymph node assay with flow cytometry measurement of bromodeoxyuridine incorporation; IR = Information requested; L92 = 1% pluronic acid L92 surfactant in water; NA = Not applicable (stimulation index < 3); NR = Not reported; NT = Not tested; + = Sensitizer; - = Nonsensitizer; +/- = equivocal compounds that were not included in contingency table evaluations.

[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).

838 **Table 6-4 Characteristics of the Substances Tested in the LLNA: BrdU-FC vs. the**
839 **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

840 **Bolded text** represents characteristics of the LLNA: BrdU-FC database.
841 Abbreviations: Chems = Chemicals; EC3 = Estimated concentration needed to produce a stimulation index of 3; NC
842 = Not calculated because maximum stimulation index < 3.0; No. = Number; Min = Minimal; Mod = Moderate;
843 SI = Stimulation index; Unk = Unknown
844 [1] From Revised Draft ICCVAM Performance Standards for the LLNA (available:
845 http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm). Includes the 18 "required" substances for
846 testing
847 [2] Based on traditional LLNA studies for substances in the LLNA: BrdU-FC database (bold values) and the draft
848 ICCVAM LLNA performance standards substances
849 [3] Data obtained from Gerberick et al. (2007)

6.4 Discordant Results

The number of substances that yielded different sensitizer/nonsensitizer classifications in the LLNA: BrdU-FC and the reference methods (i.e., GP tests, human tests) were compared to the number of discordant results in the traditional LLNA. Substances were evaluated to identify commonalities among the discordant substances. The effect of testing with different vehicles could not be evaluated because the submission from MB Research Labs did not identify the vehicle used for each test substance. NICEATM has requested this information, and MB Research Labs has agreed to supply it as soon as possible.

When analyses were restricted to the 35 substances with unequivocal LLNA: BrdU-FC, traditional LLNA, and GP data, the LLNA: BrdU-FC classified two substances differently compared with the traditional LLNA (**Table 6-5**). The LLNA: BrdU-FC identified Tween® 80 (a liquid surfactant, MW = 1310 g/mol) as a sensitizer, while the traditional LLNA classified it as a nonsensitizer. Conversely, in the LLNA: BrdU-FC, aniline (a liquid, MW = 93 g/mol) was negative (SI value, concentrations tested, and vehicle used were not available), but it was positive in the traditional LLNA (SI=3.6@ 50% aniline in AOO). Note that Tween® 80 is a sensitizer in humans, indicating that the traditional LLNA underpredicted the sensitization potential in humans, and that the positive response in the LLNA: BrdU-FC agrees with the human outcome.

Table 6-5 Discordant Results with Respect to Traditional LLNA and Guinea Pig Reference Data[1]

Substance Name	Vehicle[2]	LLNA: BrdU-FC[3]	Traditional LLNA[3]	Guinea Pig Studies[4]	Skin Irritant?
Benzalkonium chloride	ACE	+	+ 11.1, 2%[5]	-	Irritant at 2% (mice)
Copper chloride	DMSO	+	+ 13.8, 5%[6]	-	Nonirritant at 0.25% (GP)
Resorcinol	AOO	+	+ 10.4, 50%	-	Nonirritant at 15% (humans)
Sodium lauryl sulfate	DMF	+ 3.0, 25%	+ 8.9, 20%	-	Irritant at 20% (rabbits)
Ethylene glycol dimethacrylate	MEK	+	+ 7, 50%	-	Nonirritant at 1% (GP)
Tween 80	AOO	+	- NR	-	Nonirritant at 25% (humans)
Aniline	AOO	-	+ 3.6, 100%[7]	+	Negative at 100% (GP)
4-Aminobenzoic acid	AOO	-	- 1.6, 10%[8]	+	Irritant at 25% (humans)
Nickel chloride	DMSO	-	- 2.4, 5%	+	Negative at ≤ 0.15% (GP)

Abbreviations: LLNA: BrdU-FC = Murine local lymph node assay with flow cytometry measurements of bromodeoxyuridine; Traditional LLNA = Murine local lymph node assay using radioactivity to detect sensitizers; GP = Guinea pig; NA = Not available; SI = Stimulation index; + = Sensitizer; - = Nonsensitizer

[1] Data sources are listed in **Appendix C1**.

[2] Vehicles apply to tests for the traditional LLNA; ACE = acetone; AOO = acetone: olive oil; DMF = dimethylformamide; DMSO = dimethyl sulfoxide; MEK = methyl ethyl ketone. Vehicle information was generally not reported for LLNA: BrdU-FC, except for sodium lauryl sulfate, for which the vehicle was DMSO.

[3] The numbers under the + or - calls are the highest SI and the maximum concentration tested. The results of the eLLNA: BrdU-FC were the same as those for the LLNA: BrdU-FC with the exception of benzalkonium chloride, ethylene glycol dimethacrylate, and sodium lauryl sulfate, which were classified as irritants rather than sensitizers.

[4] From ICCVAM (1999) and based on studies using either the Guinea Pig Maximization Test or the Buehler Test.

[5] Highest SI occurred at a concentration of 1%.

[6] Highest SI occurred at a concentration of 2.5%.

[7] Highest SI occurred at a concentration of 50%.

[8] Highest SI occurred at a concentration of 5%.

When compared to the outcomes of GP tests, the LLNA: BrdU-FC misclassified nine substances; the eLLNA: BrdU-FC misclassified six substances; and the traditional LLNA misclassified seven substances. The LLNA: BrdU-FC and the traditional LLNA had six discordant substances in common.

Benzalkonium chloride, copper chloride, resorcinol, 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 five substances. They represent seven different chemical classes: onium compounds, phenols, inorganics, alcohols, carboxylic acids, organic sulfur compounds, and lipids. There are four solids and one liquid, ranging in molecular weight from 99 to 288, with octanol-water partition coefficients ranging from 1.0 to 1.7. One substance, ethylene glycol dimethacrylate, is considered highly peptide reactive.

Nickel chloride (a solid, MW = 130 g/mol) and 4-Aminobenzoic acid (a solid carboxylic acid, MW = 137 g/mol) were incorrectly classified as nonsensitizers by the LLNA: BrdU-FC and the traditional LLNA. Both of the BrdU-FC tests misclassified aniline (a liquid amine, MW = 93 g/mol) as a nonsensitizer, but the traditional LLNA did not. The eLLNA: BrdU-FC protocol classified benzalkonium chloride, ethylene glycol dimethacrylate, and sodium lauryl sulfate as irritants.

When analyses were restricted to the 40 substances with unequivocal LLNA: BrdU-FC, traditional LLNA, and human outcomes, the discordant substances for the LLNA: BrdU-FC, 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**). As described earlier in this section, the LLNA: BrdU-FC and the traditional LLNA classified two substances differently (Tween® 80 and aniline).

When comparing to the outcomes of human tests, both the LLNA: BrdU-FC and the traditional LLNA misclassified 11 substances (**Table 6-6**). Ten of the 11 discordant substances misclassified by the LLNA: BrdU-FC were also misclassified by the traditional LLNA. Of these 10 substances, five were misclassified as sensitizers (copper chloride, isopropyl myristate, linalool, sodium lauryl sulfate, and xylene) and the other five (isopropanol, nickel chloride, propylene glycol, propylparaben, and sulfanilamide) were misclassified as nonsensitizers by both methods. Among the five false positives, three are liquids and two are solids; they range in molecular weight from 99 to 288 g/mol, with octanol-water partition coefficients that range from 1.7 to 3.9. One substance, isopropyl myristate, is considered minimally peptide reactive. Peptide reactivity data on the other substances could not be located.

No commonalities were noted among the five human sensitizers that were misclassified as nonsensitizers by both LLNA: BrdU-FC and traditional methods. The five substances represent alcohols, amides, amines, carboxylic acids, phenols, sulfur compounds, and inorganic chemicals (some of the substances could fit in more than one chemical class). Three are solids and two are liquids, with molecular weights ranging from 60 to 180, and octanol-water partition coefficients ranging from 0.3 to 3.0. Four of the false negative substances are considered minimally peptide

926 reactive. The eLLNA: BrdU-FC protocol also misclassified these same five sensitizing
927 substances as nonsensitizers. Both of the BrdU-FC tests misclassified aniline, but the traditional
928 LLNA did not.

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

Substance Name	Vehicle[2]	LLNA: BrdU-FC[3]	Traditional LLNA[3]	Human Call[4]	Skin Irritant?
Copper chloride	DMSO	+	+ 13.8, 2.5%	-	Nonirritant at 0.25% (GP)
Isopropyl myristate	AOO	+	+ 3.4, 100%	-	Negative at 100% (rabbits)
Linalool	AOO	+	+ 8.3, 100%	-	Mild Irritant at 100% (rabbits)
Sodium lauryl sulfate	DMF	+ 4.7, 25%	+ 8.9, 20%	-	Irritant at 20% (rabbits)
Xylene	AOO	+	+ 3.1, 100%	-	Irritant at 100% (humans)
Tween 80	AOO	+	- NR	+	Nonirritant at 25% (humans)
Aniline	AOO	-	+ 3.6, 100%[5]	+	Negative at 100% (GP)
Isopropanol	AOO	-	- 1.7, 50%[6]	+	Negative at 100% (rabbits)
Nickel chloride	DMSO	-	- 2.4, 5%	+	Negative at ≤ 0.15% (GP)
Propylene glycol	Water	-	- 1.6, 100%	+	Nonirritant at 25% (humans)
Propylparaben	AOO	-	- 1.4, 25%[7]	+	Nonirritant at 10% (GP)
Sulfanilimide	DMF	-	- 1, 50%[6]	+	Nonirritant at 25% (humans)

930 Abbreviations: LLNA: BrdU-FC = Murine local lymph node assay with flow cytometry measurements of
931 bromodeoxyuridine; Traditional LLNA = Murine local lymph node assay using radioactivity to detect sensitizers;
932 + = Sensitizer; - = Nonsensitizer; NR = Not reported
933 [1] Data sources are listed in **Appendix C1**.
934 [2] Vehicles apply to tests for the traditional LLNA; AOO = acetone: olive oil; DMF = dimethylformamide; DMSO =
935 dimethyl sulfoxide. Vehicle information was generally not reported for LLNA: BrdU-FC, except for sodium
936 lauryl sulfate, for which the vehicle was DMSO.
937 [3] The numbers under the + or - calls are the highest SI and the maximum concentration tested.
938 [4] Outcomes obtained by studies conducted with the human maximization test or the inclusion of the test substance
939 in a human patch test allergen kit
940 [5] Highest SI occurred at a concentration of 50%.
941 [6] Highest SI occurred at a concentration of 10%.
942 [7] Highest SI occurred at a concentration of 5%.

7.0 LLNA: BrdU-FC Reliability

An assessment of test method reliability (intra- and interlaboratory reproducibility) is essential to 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. Interlaboratory reproducibility indicates the extent to which a test method can be transferred successfully among laboratories.

For an evaluation of intralaboratory reproducibility, the only available data on multiply tested substances in the LLNA: BrdU-FC is for hexyl cinnamic aldehyde (HCA). However, interlaboratory reproducibility could not be assessed because the test results were generated in one laboratory. The HCA test results for the LLNA: BrdU-FC are amenable to intralaboratory reproducibility analyses only for the SI values for HCA because only one concentration was tested multiple times. The initial data submission did not include EC3 values for HCA; however, data were submitted later that included EC3 results for two positive controls, HCA and 2,4-dintrochlorobenzene.

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

7.1 Intralaboratory Reproducibility – SI

The intralaboratory reproducibility has been revised to include new data for HCA and 2,4-DCNB that were not available for evaluation in the January 2008 draft BRD. MB Research Labs provided SI data for multiple tests of HCA in different vehicles. The SI values reported for 2 to 26 tests of 25% HCA in each of six vehicles were used to calculate a coefficient of variation (CV) for the assessment of intralaboratory variability. As shown in **Table 7-1**, the CVs ranged from 30% to 53%. 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 (%)	N for SI<3
Dimethylacetamide:Acetone: Ethanol (DAE 433)	5	13	6.2	46	0
Acetone:Olive Oil (4:1) (AOO)	19	11	5.5	51	0
Dimethyl sulfoxide (DMSO)	26	6.7	3.4	52	2
N,N-Dimethylformamide	4	8.7	4.6	53	0
Ethanol:Water (50%/50%)	4	15	6.3	41	0
Acetone	2	21	6.4	30	0

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

MB Research Labs subsequently provided EC3 results from four tests each in LLNA: BrdU-FC for HCA and 2,4-DNCB. As shown in **Table 7-2** the intralaboratory reproducibility of the EC3 values ranged from 8-16% for HCA and from 0.03-0.06% for 2,4-DNCB. It should be noted that these values are within the range of acceptability for reproducibility as described in the ICCVAM LLNA Performance Standards.

Table 7-2 Intralaboratory Reproducibility – EC3 Results for Positive Controls in the LLNA: BrdU-FC

Test Substance (Vehicle)	Test 1	Test 2	Test 3	Test 4	Acceptable Range[1]
HCA (AOO)	15%	16%	13%	8.4%	5-20%
DNCB (AOO)	0.06%	0.03%	0.05%	0.03%	0.03-0.10%

Abbreviations: AOO = Acetone:olive oil (4:1); DNCB = 2,4-Dinitrochlorobenzene; HCA = Hexyl cinnamic aldehyde; EC3 = Estimated concentration necessary to produce a stimulation index of 3

[1] ICCVAM LLNA Performance Standards (http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm)

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 the data has not been independently audited.

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 have 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 in online literature search of entries in MEDLINE and SCOPUS (last updated December 10, 2007).

10.0 Animal Welfare Considerations

The animal welfare considerations in this draft BRD have not changed from the January 2008 draft BRD. The LLNA: BrdU-FC and the eLLNA: BrdU-FC test methods will require the same number of animals as the traditional LLNA. However, because the traditional LLNA uses radioactivity and, accordingly, its use might be restricted due to the complications associated with handling radioactive materials (e.g., storage, disposal) use of a nonradioactive alternative to the traditional LLNA, such as the LLNA: BrdU-FC or the eLLNA: BrdU-FC could further reduce the number of guinea pigs used to assess skin sensitization.

10.1 Rationale for the Use of Animals

The rationale for the use of animals in the LLNA: BrdU-FC and the eLLNA: BrdU-FC is the same as that for the traditional LLNA: there are no valid and accepted nonanimal ways to determine the potential of substances and products to produce skin sensitization, except for situations in which human studies could be conducted ethically and 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, when dose-response information is not needed for hazard identification. 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, thereby reducing the number of animals by 10, or 40%, for each additional substance (15 vs. 25).

11.0 Practical Considerations

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

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

The test method transferability considerations in this draft BRD have not changed from the January 2008 draft BRD. Test method transferability addresses the ability of a method to be accurately and reliably performed by multiple laboratories (ICCVAM 2003), including both those experienced in the particular type of procedure and those with less or no experience in the procedure. It would be expected that the transferability of the LLNA: BrdU-FC and the eLLNA: BrdU-FC would be similar to that of the traditional LLNA because the protocols of the two methods (except for the detection of lymphocyte proliferation and immunophenotypic measurements) are identical. However, without interlaboratory reproducibility data, the extent of transferability of the LLNA: BrdU-FC and the eLLNA: BrdU-FC cannot be definitively assessed.

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

Unlike 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 to assess lymphocyte proliferation. A flow cytometer is not routinely included in many laboratories, and a new flow cytometer can cost $100,000 or more. The remaining requirements (e.g., animal care facilities) are the same for 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 require that users operate a flow cytometer instead of a scintillation counter and be able process flow cytometric data.

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