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

Draft Background Review Document

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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
ELISA	Enzyme-linked immunosorbent assay
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 Classification and Labelling 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
Java	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-ELISA	LLNA with enzyme-linked immunosorbent assay detection of bromodeoxyuridine
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

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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 being convened to peer review the BRD and to evaluate the extent to which the information contained in the BRD support the draft

[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

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).

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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 an enzyme-linked immunosorbent assay (ELISA) (referred to hereafter as the "LLNA: BrdU-ELISA").

The LLNA: BrdU-ELISA was developed by Takeyoshi et al. (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-ELISA assesses

cell proliferation by measuring the incorporation of the thymidine analog BrdU into the DNA of dividing lymphocytes using ELISA. 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-ELISA is similar to that of the traditional LLNA (Dean et al. 2001; ICCVAM 1999).

The accuracy and reliability of the LLNA: BrdU-ELISA were assessed using data for 24 substances generated from six published studies (Takeyoshi et al. 2003; 2004a; 2004b; 2005; 2006; 2007a) and one platform presentation (Takeyoshi 2007b). The reference test data for these substances were obtained from the traditional LLNA, guinea pig (GP) skin sensitization tests, and/or human skin sensitization tests. Of the 24 substances with traditional LLNA data, 16 were classified by the traditional LLNA as skin sensitizers and eight were classified as non-sensitizers.

When the LLNA: BrdU-ELISA was compared to the traditional LLNA, accuracy was 75% (18/24), sensitivity was 71% (12/17), specificity was 86% (6/7), the false positive rate was 14% (1/7), and false negative rate was 29% (5/17). Using the traditional LLNA as the reference classification, five non-sensitizers and one sensitizer were not classified correctly. No commonalities were identified among these substances.

The LLNA: BrdU-ELISA results included eight of the 18 minimum substances proposed in the *Revised Draft ICCVAM Murine Local Lymph Node Assay Performance Standards* (ICCVAM 2007); there were seven sensitizers and one non-sensitizer. The sensitizer/non-sensitizer outcome of the LLNA: BrdU-ELISA was consistent with the outcome of the traditional LLNA with the exception of one sensitizer.

When the decision criteria were altered to include SI values below three to identify a positive response, improved performance was achieved. Best overall performance was achieved using an SI $\geq$ 1.3 with an accuracy of 96% (22/23), sensitivity of 100% (17/17), specificity of 83% (5/6), a false positive rate of 17% (1/6), and false negative rate of 0% (0/17). Using an SI $\geq$ 1.3 also correctly classified all of the ICCVAM performance standards reference substances.

Intralaboratory reproducibility was assessed using a concordance analysis of sensitizer/non-sensitizer results, and a coefficient of variation (CV) analysis of SI values and EC3 values

(calculated concentration corresponding to SI = 3). Four of six substances yielded 100% concordance for sensitizer/non-sensitizer outcomes. Discordant test results were noted for two substances: a sensitizer (isoeugenol) and a non-sensitizer (propylene glycol). Isoeugenol was correctly identified as a sensitizer in 75% (3) of the four tests. Propylene glycol was correctly identified as a non-sensitizer in 50% (1) of the two tests. The CVs for the SI values of five substance/concentration combinations that were tested two times each ranged from 0.6% to 51.3%. The CVs for the EC3 values of four substances that were tested two to three times each ranged from 10.1% to 47.1%.

Interlaboratory reproducibility could not be assessed because all LLNA: BrdU-ELISA results were produced in one laboratory.

The LLNA: BrdU-ELISA 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-ELISA and the 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-ELISA is expected to be similar to the traditional LLNA. Compared to the traditional LLNA, the LLNA: BrdU-ELISA will not require facilities, equipment, and licensing permits for handling radioactive materials. The level of training and expertise needed to conduct the LLNA: BrdU-ELISA should be similar to the traditional LLNA except that the understanding and use of ELISA is required.

ICCVAM has developed draft recommendations for the LLNA: BrdU-ELISA 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: BrdU-ELISA Test Method Protocol (LLNA: BrdU-ELISA).*

1.0 Introduction

1.1 Historical Background

In 1999, the Interagency Coordinating Committee on 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) incorporation by enzyme-linked immunosorbent assay (ELISA) (referred to hereafter as the "LLNA: BrdU-ELISA").

1.2 The LLNA: BrdU-ELISA

The LLNA: BrdU-ELISA was developed by Takeyoshi et al. (2001) as a non-radioactive alternative to 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-ELISA assesses the same endpoint by measuring the incorporation of the thymidine analog BrdU, which is detected and quantified with an ELISA, which is available as a kit commercially from several sources.

This document provides:

- A comprehensive summary of the LLNA: BrdU-ELISA 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-ELISA Test Method Protocol

The LLNA: BrdU-ELISA protocol (see **Appendix A**) is similar to the ICCVAM-recommended protocol for the traditional LLNA (see http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/LLNAProt.pdf), except for the method used to assess lymphocyte proliferation. In both the LLNA: BrdU-ELISA and the traditional LLNA, the test substance is administered on three consecutive days. In the traditional LLNA, ^{3}H- thymidine or ^{125}I-iododeoxyuridine (in phosphate buffered saline; 250 µL/mouse) is administered via the tail vein two days after the final application of the test substance. In the LLNA: BrdU-ELISA, 5 mg BrdU in a volume of 0.5 mL physiological saline (concentration of 10 mg/mL) is administered via intraperitoneal injection two days after the final application of the test substance. Takeyoshi et al. (2001) reported that one injection of 5 mg BrdU was selected over two injections to minimize the incorporation of BrdU in the control group. Injection of BrdU two days after topical treatment with test substance yielded efficient incorporation of BrdU

in comparison to injection one day or three days after topical treatment with a test substance (Takeyoshi et al. 2001). On the day following BrdU injection, lymph nodes are excised and a single cell suspension is prepared from the lymph nodes of each animal. A standard aliquot of the cell suspension is added in triplicate to the wells of a flat-bottom 96-well microplate and centrifuged. Supernatants are then removed. Fix-Denat solution, which fixes the cells and denatures the DNA, is added to each well, and the plate is incubated at room temperature. The Fix-Denat solution is removed and the diluted anti-BrdU antibody solution is added to each well. After each well is washed with phosphate-buffered saline, an aliquot of substrate solution containing tetramethylbenzidine is added. After incubation at room temperature, the absorbance is measured using a microplate reader.

2.1. Decision Criteria

Like the traditional LLNA, a stimulation index (SI) is used in the LLNA: BrdU-ELISA to distinguish skin sensitizers from non-sensitizers. The SI is the ratio of the mean absorbance of the incorporated BrdU in a lymph node suspension from individual mice in the test substance group to the mean absorbance of the incorporated BrdU in a lymph node suspension from individual mice in the vehicle control group as indicated by the formula below:

$$SI = \frac{\text{Mean absorbance of the treatment group lymph nodes}}{\text{Mean absorbance of the vehicle control group lymph nodes}}$$

Consistent with the traditional LLNA, an SI ≥ 3 was initially used as the threshold for labeling a substance as a sensitizer. However, Takeyoshi et al. (2007b) also evaluated the use of statistically significant differences in BrdU incorporation between treated and control groups, and/or other SI values to distinguish sensitizers from non-sensitizers, and found that improved accuracy resulted from using lower cutoff values for the SI as the decision criteria for whether a substance was a sensitizer or non-sensitizer (see **Appendix B**).

3.0 LLNA: BrdU-ELISA Validation Database

To evaluate the validity of the LLNA: BrdU-ELISA, data were available for a total of 29 substances that had been were tested in one laboratory (**Table 3-1**). Most of these substances (24/29) had been previously tested in the traditional LLNA. No traditional LLNA data were available for the remaining five substances, which are trans-cinnamaldehyde, two dimers of eugenol (dihydroxyl-3,3'-dimethoxy-5,5'-diallyl-biphenyl and 4,5'-diallyl-2'-hydroxy-2,3'-

dimethoxyphenyl ether) and two dimers of isoeugenol (4-[1-Hydroxy-2-(2-methoxy-4-propenyl-phenyoxy)-propyl]-2-methoxy-phenol and 2-methoxy-4-(7-methoxy-3-methyl-5-propenyl-2,3-dihydro-benzofuran-2yl)-phenol).

Twenty-one of the 24 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 three remaining substances, cyclamen aldehyde, glutaraldehyde, and isopropyl myristate, and for aniline were obtained from Basketter et al. (2005), Hilton et al. (1998), Ryan et al. (2000), and Gerberick et al. (2005), respectively.

Of the 24 substances with traditional LLNA data, 16 were classified by the traditional LLNA as skin sensitizers and eight were classified as non-sensitizers. As shown in **Table 3-1**, the traditional LLNA EC3 values (i.e., estimated concentration needed to produce an SI =3) for the 16 sensitizers ranged from 0.005% to 44%.

Appendix C provides information on the physicochemical properties (e.g., physical form tested), Chemical Abstracts Service Registry Number (CASRN), and chemical class for each substance tested. When available, chemical classes for each substance were retrieved from the National Library of Medicine's ChemID Plus database. If chemical classes were unavailable, they were assigned to 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 indicate the impact of structure on biological activity with respect to sensitization potential. **Table 3-1** shows that 13 chemical classes are represented by the substances tested in the LLNA: BrdU-ELISA. Three substances are classified in more than one chemical class. The classes with the highest number of substances are carboxylic acids (9 substances) and aldehydes (5 substances).

414 **Table 3-1 Traditional LLNA EC3 Values and Chemical Classification of Substances**
415 **Tested in the LLNA: BrdU-ELISA**

Substance Name	Chemical Class[1]	Traditional LLNA EC3 (%)[2]
Diphenylcyclopropenone	Hydrocarbons, Cyclic	0.005
p-Benzoquinone	Quinones	0.01
2,4-Dinitrochlorobenzene*	Hydrocarbon, Halogenated; Nitro Compounds; Hydrocarbons, Cyclic	0.049
4-Phenylenediamine*	Amines	0.11
Glutaraldehyde	Aldehydes	0.14
Isoeugenol*	Carboxylic Acids	1.5
Cinnamic aldehyde	Aldehydes	2.4
3-Aminophenol	Amines; Phenols	3.2
4-Chloroaniline	Amines	6.5
2-Mercaptobenzothiazole*	Heterocyclic Compounds	9.8
Citral*	Hydrocarbons, Other	9.8
Hexyl cinnamic aldehyde*	Aldehydes	9.9
Eugenol*	Carboxylic Acids	10
Cyclamen aldehyde	Aldehydes	22.3
Hydroxycitronellal	Hydrocarbons, Other	23.8
Isopropyl myristate	Lipids	44
Aniline	Amines	63
Diethylpthalate	Carboxylic Acids	NA
Dimethyl isophthalate	Carboxylic Acids	NA
Glycerol	Alcohols; Carbohydrates	NA
Hexane	Hydrocarbons, Acyclic	NA
2-Hydroxypropyl methacrylate	Carboxylic Acids	NA
Isopropanol*	Alcohols	NA
Propylene glycol	Alcohols	NA
trans-Cinnamaldehyde	Aldehydes	NK
2,2'-Dihydroxyl-3,3'-dimethoxy-5,5'-diallyl-biphenyl	Carboxylic Acids	NK
2-Methoxy-4-(7-methoxy-3-methyl-5-propenyl-2,3-dihydro-benzofuran-2yl)-phenol	Carboxylic Acids	NK
4,5'-Diallyl-2'-hydroxy-2,3'-dimethoxyphenyl ether	Carboxylic Acids	NK
4-[1-Hydroxy-2-(2-methoxy-4-propenyl-phenyoxy)-propyl]-2-methoxy-phenol (Synonym: □-O-4-Dilignol)	Carboxylic Acids	NK

416 Abbreviations: LLNA: BrdU-ELISA= Local lymph node assay with enzyme-linked immunosorbent assay detection
417 of bromodeoxyuridine; EC3 = Estimated concentration needed to produce a stimulation index (SI) = 3; NA = Not
418 applicable since maximum SI < 3.0; NK = Not known (information requested but not yet obtained).
419 *Reference substance from ICCVAM (2007).

[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]Mean EC3 values from the NICEATM database of traditional LLNA studies.

4.0 Reference Data

The traditional LLNA reference data used for the accuracy evaluation described in **Section 6.0** were obtained from ICCVAM (1999), Basketter et al. (2005), Hilton et al. (1998), Ryan et al. (2000), or Gerberick et al. (2005) (**Appendix D**). An independent quality assurance contractor for the 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. No traditional LLNA data could be located for trans-cinnamaldehyde.

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 ICCVAM (1999), Basketter et al. (2000), Bjorkner (1984), Hilton et al. (1998), Marzulli et al. (1974), Opdyke (1976), Takeyoshi et al. (2004a), or Takeyoshi et al. (2007a). Although there were no traditional LLNA data available for the eugenol dimers (dihydroxyl-3,3'-dimethoxy-5,5'-diallyl-biphenyl and 4,5'-diallyl-2'-hydroxy-2,3'-dimethoxyphenyl ether) or the isoeugenol dimers (4-[1-Hydroxy-2-(2-methoxy-4-propenyl-phenyoxy)-propyl]-2-methoxy-phenol and 2-Methoxy-4-(7-methoxy-3-methyl-5-propenyl-2,3-dihydro-benzofuran-2yl)-phenol), Takeyoshi et al. (2004a and 2007a, respectively) provided results from the GPMT for these compounds.

5.0 Test Method Data and Results

The LLNA: BrdU-ELISA data evaluated in this technical summary were obtained from six published studies (Takeyoshi et al. 2003; 2004a; 2004b; 2005; 2006; 2007a) and one platform presentation (Takeyoshi 2007b). All test results were obtained using the protocol in **Appendix A**. **Appendix D** contains the LLNA: BrdU-ELISA data for the 29 substances tested in these studies. The test substances were not coded to prevent the possibility of bias.

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 available human data, including 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.

6.1 Total LLNA: BrdU-ELISA Database Analysis Using SI ≥ 3 Decision Criteria

The performance characteristics of the LLNA: BrdU-ELISA were first evaluated using the criterion of SI ≥ 3 to identify sensitizers. Twenty-four of the 29 substances listed in **Table 3-1** had sufficient LLNA: BrdU-ELISA and traditional LLNA data to conduct an accuracy analysis. Of the remaining substances tested with the LLNA: BrdU-ELISA, 17 had LLNA: BrdU-ELISA, traditional LLNA, and GP data; and 21 had LLNA: BrdU-ELISA, traditional LLNA, and human data. 3-Aminophenol was excluded from the accuracy analyses for the dataset with LLNA: BrdU-ELISA, traditional LLNA, and GP data since the available GP data were generated with a nonstandard GPMT protocol. The nonstandard protocol did not include the 48-hour topical patch induction that should follow induction by intradermal injections and it replaced the 24-hour skin patch challenge (usually two weeks after topical induction) with a 6-hour skin patch challenge (Basketter D, personal communication). Trans-cinnamaldehyde, the eugenol dimers (dihydroxyl-3,3'-dimethoxy-5,5'-diallyl-biphenyl and 4,5'-diallyl-2'-hydroxy-2,3'-dimethoxyphenyl ether), and the isoeugenol dimers (4-[1-Hydroxy-2-(2-methoxy-4-propenyl-phenyoxy)-propyl]-2-

methoxy-phenol and 2-methoxy-4-(7-methoxy-3-methyl-5-propenyl-2,3-dihydro-benzofuran-2yl)-phenol) were excluded from the accuracy analyses because traditional LLNA data for these substances were not identified. The complete set of data for each substance is located in **Appendix D**.

Discordant test results were noted for two substances. Four LLNA: BrdU-ELISA test results were available for isoeugenol; three tests were positive for skin sensitization potential (Takeyoshi et al. 2005; 2007a) and one was negative (Takeyoshi et al. 2006). Based on a weight of evidence, a positive result was used for analysis of performance characteristics. Two discordant test results were noted for propylene glycol. The most conservative result with respect to prediction of adverse health effects, positive for skin sensitization, was used for the accuracy analyses. The test result in Takeyoshi et al. (2005) produced a positive result as indicated by individual animal data submitted by Dr. Takeyoshi to support the graphical data shown in the paper. The test result in Takeyoshi et al. (2006) was negative.

6.1.1 *Accuracy vs. the Traditional LLNA*

When compared to the traditional LLNA and using a decision criteria of SI $\geq$ 3.0 to identify sensitizers, the LLNA: BrdU-ELISA had an accuracy of 75% (18/24), a sensitivity of 71% (12/17), a specificity of 86% (6/7), a false positive rate of 14% (1/7), and a false negative rate of 29% (5/17) (**Table 6-1**).

Table 6-1 Evaluation of the Performance of the LLNA: BrdU-ELISA In Predicting Skin Sensitizing Potential Using Decision Criteria of SI ≥ 3.0 to Identify Sensitizers

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]
BrdU-ELISA vs. Traditional LLNA	24	75	18/24	71	12/17	86	6/7	92	12/13	55	6/11	14	1/7	29	5/17
Substances with LLNA: BrdU-ELISA, Traditional LLNA, and GP Data															
BrdU-ELISA vs. Traditional LLNA	17	71	12/17	67	8/12	80	4/5	89	8/9	50	4/8	20	1/5	33	4/12
LLNA: BrdU-ELISA vs. GP[3]	17	71	12/17	67	8/12	80	4/5	89	8/9	50	4/8	20	1/5	33	4/12
Traditional LLNA vs. GP[3]	17	100	17/17	100	12/12	100	5/5	100	12/12	100	5/5	0	0/5	0	0/12
Substances with LLNA: BrdU-ELISA, Traditional LLNA, and Human Data															
BrdU-ELISA vs. Traditional LLNA	21	76	16/21	73	11/15	83	5/6	92	11/12	56	5/9	17	1/6	27	4/15
LLNA: BrdU-ELISA vs. Human[4]	21	62	13/21	61	11/18	67	2/3	92	11/12	22	2/9	33	1/3	39	7/18
Traditional LLNA vs. Human[4]	21	76	16/21	78	14/18	67	2/3	93	13/14	33	2/6	33	1/3	22	4/18

Abbreviations: LLNA: BrdU-ELISA= Murine local lymph node assay (LLNA) with enzyme-linked immunosorbent assay (ELISA) detection of bromodeoxyuridine (BrdU); GP = Guinea pig skin sensitization outcomes; ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; LLNA = Murine local lymph node assay; No. = Number.

[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, inclusion of the test substance in a Human Patch Test Allergen Kit, and/or published clinical case studies/reports.

6.1.2 *Accuracy vs. Guinea Pig Data*

When the accuracy of the LLNA: BrdU-ELISA (SI ≥ 3.0) and the traditional LLNA were compared based on their performance relative to the GP test, the LLNA: BrdU-ELISA had a lower accuracy rate (71% [12/17] vs. 100% [17/17]) and sensitivity (67% [8/12] vs. 100% [12/12]), and higher false negative rate (33% [4/12] vs. 0% [0/12] (**Table 6-1**). The specificity was lower (80% [4/5] vs. 100% [5/5]) and the false positive rate was higher (20%, [1/5] vs. 0% [0/5]) for the LLNA: BrdU-ELISA than that for the traditional LLNA.

6.1.3 *Accuracy vs. Human Data*

When the accuracy of the LLNA: BrdU-ELISA (SI ≥ 3.0) and the traditional LLNA were compared based on their performance relative to the available human data, the LLNA: BrdU-ELISA had a lower accuracy (62% [13/21] vs. 76% [16/21]) and sensitivity (61% [11/18] vs. 78% [14/18]) and a higher false negative rate (39% [7/18] vs. 22% [4/18]) than the traditional LLNA (**Table 6-1**). The specificity (67% [2/3]) and the false positive rate (33%, [1/3]) for the LLNA: BrdU-ELISA was not different from that of the traditional LLNA.

6.2 Accuracy Analysis (SI ≥ 3.0) Based on Revised Draft ICCVAM Performance Standards Reference Substances

As shown in **Table 6-2**, eight of the 18 minimum reference substances included in the *Revised Draft ICCVAM Performance Standards for the Local Lymph Node Assay* (ICCVAM 2007) have been tested in the LLNA: BrdU-ELISA. Seven of the eight substances yielded the same sensitizer/non-sensitizer outcome in the LLNA: BrdU-ELISA as in the traditional LLNA. 2-Mercaptobenzothiazole, a sensitizer in the LLNA, tested negative in the LLNA: BrdU-ELISA. One could suspect that testing in different vehicles produced the difference between the ICCVAM (2007) EC3 values and those for the LLNA: BrdU-ELISA. The test results used by ICCVAM (2007) used acetone:olive oil (4:1; AOO) for the vehicle while those for the LLNA: BrdU-ELISA used dimethylformamide (DMF). However, the NICEATM database of traditional LLNA studies indicates that 2-mercaptobenzothiazole has a higher EC3 when tested in AOO (mean EC3 =9.8%) compared with DMF (mean EC3 =2.5%). Thus, the use of DMF as the vehicle should have made 2-mercaptobenzothiazole more likely to be positive in the LLNA: BrdU-ELISA.

Table 6-2 Evaluation of the Performance of the LLNA: BrdU-ELISA (SI ≥ 3.0) Using the Revised Draft ICCVAM Performance Standards Reference Substances[1]

Substance	ICCVAM Draft LLNA Performance Standards[1]					LLNA: BrdU-ELISA[2]		
	Vehicle	Result	EC3 (%)[1]	N[2]	0.5x – 2.0x EC3 (%)	Vehicle	Result	EC3 (%)
5-Chloro-2-methyl-4-isothiazolin-3-one	DMF	+	0.009	1	0.0045 – 0.018	NA	NT	NT
2, 4-Dinitrochlorobenzene	AOO	+	0.049	15	0.025 – 0.099	AOO	+	***0.15 (n=2)***[3]
4-Phenylenediamine	AOO	+	0.11	10	0.055 – 0.22	AOO	+	NR (+)
4-Methylaminophenol sulfate	DMF	+	0.8	1	0.4 – 0.12	NA	NT	NT
Isoeugenol	AOO	+	1.5	49	0.77 – 3.1	AOO	+	**9.3 (n=3)**[3]
2-Mercaptobenzothiazole	***AOO***	+	2.5	2	***1.25 – 5.0***	***DMF***	**-**	***NA (-)***
Cobalt chloride	DMSO	+	4.8	1	2.4 – 9.6	NA	NT	NT
Citral	AOO	+	9.8	2	4.9 – 19.6	AOO	+	NR ***(+)***
Hexyl cinnamic aldehyde	AOO	+	9.9	22	5.0 – 19.9	AOO	+	***41.2 (n=3)***[3]
Eugenol	AOO	+	10.1	11	5.05 – 20.2	AOO	+	***29.5 (n=3)***[3]
Phenyl benzoate	AOO	+	13.6	3	6.8 – 27.2	NA	NT	NT
Cinnamic alcohol	AOO	+	21	1	10.5 - 42	NA	NT	NT
Imidazolidinyl urea	DMF	+	24	1	12 - 36	NA	NT	NT
Chlorobenzene	AOO	-	NA	1	NA	NA	NT	NT
Isopropanol	AOO	-	NA	1	NA	AOO	-	NA (-)
Lactic acid	DMSO	-	NA	2	NA	NA	NT	NT
Methyl salicylate	AOO	-	NA	10	NA	NA	NT	NT
Salicylic acid	AOO	-	NA	1	***NA***	NA	NT	NT
Ethylene glycol dimethacrylate	MEK	False +	28 (FP)	1	14-56	NA	NT	NT
Sodium lauryl sulfate	DMF	False +	8.1 (FP)	5	4.05 – 16.2	NA	NT	NT
Nickel sulfate	DMF	False -	NA (FN)	2	NA	NA	NT	NT
Sulfanilamide	DMF	False -	NA (FN)	1	NA	NA	NT	NT

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

Abbreviations: AOO = acetone:olive oil (4:1); LLNA: BrdU-ELISA= Murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; DMF = *N,N*-dimethylformamide; DMSO = Dimethyl sulfoxide; EC3 = Calculated concentration that corresponds to SI=3; FN = False negative in traditional LLNA when compared to guinea pig and/or human results; FP = False positive in traditional LLNA when compared to guinea pig and/or human results; LLNA = Murine local lymph node assay; MEK = Methyl ethyl ketone; NA = Not applicable (Stimulation Index < 3); NR = Not reported (information requested by NICEATM); NT = Not tested.

+ = Sensitizer.

- = Non-sensitizer.

[1]Mean EC3 when more than one value was available. From Revised Draft ICCVAM Performance Standards for the LLNA (ICCVAM 2007; available: http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm.

[2]From Takeyoshi et al. (2003, 2004b, 2005, 2006, 2007a, 2007b). Substances for which EC3 values were not available include in parentheses the outcome of the BrdU-ELISA test (+ = sensitizer; - = nonsensitizer>

[3]Number of values used to derive the mean EC3.

553 The *Revised Draft ICCVAM Performance Standards for the Local Lymph Node Assay* (ICCVAM
554 2007) recommend a range of 0.5X to 2.0X the historical mean EC3 traditional LLNA as the
555 criteria for accuracy for nontraditional LLNA methods. EC3 values from the LLNA: BrdU-
556 ELISA were reported for four of the seven ICCVAM reference sensitizers tested. The EC3
557 values for all four substances were outside of the proposed acceptability range.

558 Selected characteristics of the substances tested using the LLNA: BrdU-ELISA were compared
559 with the characteristics of the 18 minimum reference substances included in the *Revised Draft*
560 *ICCVAM Performance Standards for the Local Lymph Node Assay* (ICCVAM 2007). **Table 6-3**
561 provides the range of substances tested in the LLNA: BrdU-ELISA based on the overall database
562 of 24 substances in comparison to the range of substances included on the revised draft
563 ICCVAM LLNA performance standards substances list. The table indicates that although not all
564 of the draft ICCVAM performance standards reference substances have been tested, the range of
565 the substances tested in the LLNA: BrdU-ELISA based on traditional LLNA EC3 values,
566 physical form of the test substance, and availability of reference data is similar to that included in
567 the draft performance standards list. In general, there are a proportionally increased number of
568 substances tested in the LLNA: BrdU-ELISA in each of the categories included in the table.

569

Table 6-3 Characteristics of the Substances Tested in the LLNA: BrdU-ELISA 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	**3**	**3/0**	**0.005 - 0.049**	**2**	**3/0/0/0**
	2	1/1	0.009-0.05	2	0/1/0/1
≥ 0.1 to <1	**2**	**1/1**	**0.11 - 0.14**	**2**	**1/0/0/1**
	2	2/0	0.11-0.8	2	1/0/0/1
≥ 1 to <10	**7**	**2/5**	**1.5 - 9.9**	**6**	**1/0/1/5**
	5	2/3	1.6-9.9	5	1/0/1/3
≥ 10 to <100	**5**	**0/5**	**10 - 63**	**4**	**0/0/3/2**
	4	3/1	10.1-24	4	0/1/0/3
Negative	**7**	**2/5**	**NC**	**4**	**0/0/6/1**
	5	2/3	NC	3	0/0/2/3
Overall	**24**	**8/16**	**0.005 - 63**	**18**	**5/0/10/9**
	18	10/8	0.009-24	16	2/2/3/11

Bolded text represents characteristics of the LLNA: BrdU-ELISA 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- ELISA database (bold values) and the draft ICCVAM LLNA performance standards substances.
[5]Data obtained from: Gerberick et al. (2007)

6.3 Discordant Results for Accuracy Analysis of SI ≥ 3.0 Decision Criterion

When the outcomes for the 24 substances tested in the LLNA: BrdU-ELISA (using SI ≥ 3.0) and the traditional LLNA were compared, the classifications for six substances were different. The LLNA: BrdU-ELISA classified aniline, 4-chloroaniline, cyclamen aldehyde, hydroxycitronellal, and 2-mercaptobenzothiazole as non-sensitizers while the traditional LLNA classified them as sensitizers (**Table 6-4**). No commonalities in chemical class, physical form, or EC3 value (based on the traditional LLNA) were noted among these substances. The LLNA: BrdU-ELISA classified propylene glycol as a sensitizer while the traditional LLNA classified it as a non-sensitizer. Two discordant LLNA: BrdU-ELISA results for propylene glycol were obtained. The most conservative result was used for the accuracy analyses to be conservative with respect to

prediction of adverse health effects. Additionally, the substances were tested in the same vehicle in both the LLNA: BrdU-ELISA and the traditional LLNA tests. Aniline, 4-chloroaniline, cyclamen aldehyde, propylene glycol, and hydroxycitronellal were tested in AOO, while 2-mercaptobenzothiazole was tested in DMF.

Table 6-4 Discordant Results for LLNA: BrdU-ELISA (Using SI ≥ 3.0 for Sensitizers) Compared to Traditional LLNA and Guinea Pig Reference Data

Substance Name	LLNA: BrdU-ELISA[1]	Traditional LLNA[2]	Guinea Pig Studies[2]
Aniline	-	+[3]	+
4-Chloroaniline	-	+	+
Cyclamen aldehyde	-	+[5]	NA
Hydroxycitronellal	-	+	+
2-Mercaptobenzothiazole	-	+	+
Propylene glycol	+[4]	-	-

Abbreviations: LLNA: BrdU-ELISA= Murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; GP = Outcomes of guinea pig skin sensitization tests; LLNA = Murine local lymph node assay; NA = Not available.
+ = Sensitizer.
- = Non-sensitizer.
[1]From Takeyoshi et al. (2005, 2006, 2007b).
[2]From ICCVAM (1999) unless otherwise noted.
[3]From Gerberick et al. (2005)
[4]The test result in Takeyoshi et al. (2005) produced a positive result as indicated by individual animal data submitted by Dr. Takeyoshi to support the graphical data shown in the paper. The test result in Takeyoshi et al. (2006) produced a negative result. Both tests used a maximum concentration of 50%. The overall result was deemed to be positive (i.e., a conservative approach was used).
[5]From Basketter et al. (2005).

For the 17 substances with LLNA: BrdU-ELISA, traditional LLNA, and GP test results, The results for aniline, 4-chloroaniline, hydroxycitronellal, 2-mercaptobenzothiazole, and propylene glycol were discordant with the GP test results. The LLNA: BrdU-ELISA results for aniline, 4-chloroaniline, hydroxycitronellal, and 2-mercaptobenzothiazole were negative, while the traditional LLNA and GP results were positive. The LLNA: BrdU-ELISA result for propylene glycol was positive, while the traditional LLNA and GP results were negative.

When analyses were restricted to the 21 substances with LLNA: BrdU-ELISA, traditional LLNA, and human outcomes, both LLNA methods misclassified three sensitizers (2-

hydroxypropylmethacrylate, isopropanol, and diethyl phthalate) as non-sensitizers (**Table 6-5**). The LLNA: BrdU-ELISA also misclassified four more sensitizers as non-sensitizers that were correctly classified by the traditional LLNA: aniline, hydroxycitronellal, cyclamen aldehyde, and 2-mercaptobenzothiazole. No commonalities in chemical class, physical form, or EC3 range (based on the traditional LLNA) were noted among these substances. Both the LLNA: BrdU-ELISA and the traditional LLNA misclassified isopropyl myristate as a sensitizer.

Table 6-5 Discordant Results for LLNA: BrdU-ELISA (SI ≥ 3.0) When Compared to Traditional LLNA and Human Outcome Data

Substance Name	LLNA: BrdU-ELISA[1]	Traditional LLNA[2]	Human Outcome[2]
Aniline	-	+[7]	+
Cyclamen aldehyde	-	+[8]	+[8]
Hydroxycitronellal	-	+	+
2-Hydroxypropylmethacrylate	-	-	+[3]
Isopropanol	-	-	+[4]
Isopropyl myristate	+	+[5]	-[6]
2-Mercaptobenzothiazole	-	+	+
Diethyl phthalate	-	-	+

Abbreviations: LLNA: BrdU-ELISA= Murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; GP = outcomes of guinea pig skin sensitization tests; LLNA = Murine local lymph node assay.
+ = Sensitizer.
- = Nonsensitizer.
[1]From Takeyoshi et al. (2005, 2006, 2007b).
[2]From ICCVAM (1999) unless otherwise noted.
[3]From Bjorkner (1984).
[4]From Kwon et al. (2003).
[5]From Ryan et al. (2000).
[6]From Opdyke (1976).
[7]From Gerberick et al. (2005).
[8]From Basketter et al. (2005).

The accuracy analyses for the eight reference substances from the Revised Draft ICCVAM Performance Standards (ICCVAM 2007) tested in LLNA: BrdU-ELISA yielded one discordant substance, 2-mercaptobenzothiazole. The LLNA: BrdU-ELISA classified this substance as a non-sensitizer, while the traditional LLNA, GP, and human tests classified it as a sensitizer. While the vehicles for the historical results reported in ICCVAM (2007) were AOO and that

used by Takeyoshi et al. (2007b) was DMF, the different vehicles were not responsible for the discordant results. Other reports of traditional LLNA tests using DMF have also classified 2-mercaptobenzothiazole as a sensitizer (e.g., Ashby et al. 1995; Gerberick et al. 2005).

6.4 LLNA: BrdU-ELISA Accuracy Analysis Using Alternative Decision Criteria

Takeyoshi et al (2007b) evaluated the effect of decision criteria other than SI ≥ 3 to determine skin sensitization potential on test performance characteristics with the traditional LLNA serving as the reference test. The performance characteristics for nine alternate decision criteria for determining whether the skin sensitization potential for the substances were positive or negative are reported in this section. **Appendix B** also reports results for intermediate SI cutoff values. The substances evaluated were the same as those evaluated in **Sections 6.1** through **6.3** except that hexane was not included. The decision criteria included:

1. SI values ≥ 1.3, ≥ 1.5, ≥ 2, ≥ 2.5, or ≥ 3
2. Mean BrdU labeling index is statistically different from control group
3. Mean BrdU labeling index ≥ 95% confidence interval of the control group
4. Mean BrdU labeling index is ≥ 2 standard deviations (SD) or ≥ 3 SD from the control group mean

Using a decision criteria of SI ≥ 3.0 to identify sensitizers for these 23 substances, the LLNA: BrdU-ELISA had an accuracy of 74% (17/23), a sensitivity of 71% (12/17), a specificity of 83% (5/6), a false positive rate of 17% (1/6), and a false negative rate of 29% (5/17) (**Table 6-6)**. However, when the decision criteria are altered to include lower SI values, improved performance was achieved. When the mean labeling index for the treatment group was outside the 95% confidence interval of the control group or ≥ 2 SD from the index for the vehicle control group, or when SI ≥ 1.5, the LLNA: BrdU-ELISA accuracy improved to 91% (21/23), with a sensitivity of 94% (16/17), and a false negative rate of 6% (1/17). The specificity (83% [5/6]) and false positive rate (17% [1/6]) were the same as that for SI ≥ 3.

The best overall performance was achieved using an SI ≥ 1.3 with an accuracy of 96% (22/23), sensitivity of 100% (17/17), specificity of 83% (5/6), a false positive rate of 17% (1/6), and false

672 negative rate of 0% (0/17). Using an SI ≥ 1.3 also correctly classified all of the ICCVAM
673 performance standards reference substances.

674 6.5 Discordant Results for Accuracy Analysis of Alternative Decision Criterion

675 Using the decision criteria of SI ≥ 3.0 to identify sensitizers for the 23 substances used in the
676 analysis of alternative decision criteria, the six discordant substances (when compared to the
677 traditional LLNA) were propylene glycol, 4-chloroaniline, hydroxycitronellal, aniline, cyclamen
678 aldehyde, and 2-mercaptobenzothiazole (**Table 6-7**). As indicated in **Section 6.3**, 4-
679 chloroaniline, aniline, hydroxycitronellal, cyclamen aldehyde, and 2-mercaptobenzothiazole
680 were misclassified as non-sensitizers, and propylene glycol was misclassified as a sensitizer
681 when compared to the traditional LLNA.

682 **Table 6-7** shows how the number and identity of discordant substances changes with the
683 alternate decision criteria. Using SI ≥ 2.0 or SI ≥ 2.5 yielded the same five discordant substances:
684 propylene glycol, hydroxycitronellal, aniline, cyclamen aldehyde, and 2-mercaptobenzothiazole.
685 Three discordant substances, propylene glycol, aniline, and hydroxycitronellal, were noted when
686 a statistical test was used to determine a difference between the treatment and vehicle control
687 group means and when the treatment group mean >3 SD from the control group mean. Using SI
688 ≥ 1.5, ≥ 95% CI, or ≥ 2 SD yielded two discordant substances, propylene glycol and
689 hydroxycitronellal. Using SI ≥ 1.3 to classify substances as sensitizers yielded only one
690 discordant substance (propylene glycol). As noted in **Section 6.4**, using the decision criterion of
691 SI ≥ 1.3 would correctly classify the ICCVAM reference substance 2-mercaptobenzothiazole as
692 a sensitizer, which was incorrectly classified as a non-sensitizer using SI ≥ 3.0 as the criterion
693 (see **Section 6.2**).

694

Table 6-6 Evaluation of the Performance of the LLNA: BrdU-ELISA In Predicting Skin Sensitizing Potential Using Alternative Decision Criteria to Identify Sensitizers

Alternate Criterion	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]
Statistics[3]	23	87	20/23	88	15/17	83	5/6	94	15/16	71	5/7	17	1/6	12	2/17
≥ 95% CI	23	91	21/23	94	16/17	83	5/6	94	16/17	83	5/6	17	1/6	6	1/17
≥ 2 SD	23	91	21/23	94	16/17	83	5/6	94	16/17	83	5/6	17	1/6	6	1/17
≥ 3 SD	23	87	20/23	88	15/17	83	5/6	94	15/16	71	5/7	17	1/6	12	2/17
SI ≥ 3.0	23	74	17/23	71	12/17	83	5/6	92	12/13	50	5/10	17	1/6	29	5/17
SI ≥ 2.5	23	78	18/23	77	13/17	83	5/6	93	13/14	56	5/9	17	1/6	24	4/17
SI ≥ 2.0	23	78	18/23	77	13/17	83	5/6	93	13/14	56	5/9	17	1/6	24	4/17
SI ≥ 1.5	23	91	21/23	94	16/17	83	5/6	94	16/17	83	5/6	17	1/6	6	1/17
SI ≥ 1.3	23	96	22/23	100	17/17	83	5/6	94	17/18	100	5/5	17	1/6	0	0/17

Abbreviations: LLNA: BrdU-ELISA = Murine local lymph node assay (LLNA) with enzyme-linked immunosorbent assay (ELISA) detection of bromodeoxyuridine (BrdU); CI = Confidence interval; No. = Number; SD = Standard deviation; SI = Stimulation index

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

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

[3] Statistical test for difference of group means.

Table 6-7 Discordant Results for LLNA: BrdU-ELISA Using Alternative Decision Criteria Compared to the Traditional LLNA

Discordant Substance	Alternate Decision Criterion								
	Statistics[1]	≥ 95% CI	≥ 2 SD	≥ 3 SD	SI ≥ 3.0	SI ≥ 2.5	SI ≥ 2.0	SI ≥ 1.5	SI ≥ 1.3
Propylene glycol	X	X	X	X	X	X	X	X	X
4-Chloroaniline					X				
Hydroxycitronellal	X	X	X	X	X	X	X	X	
Aniline	X			X	X	X	X		
Cyclamen aldehyde					X	X	X		
2-Mercaptobenzothiazole					X	X	X		

Abbreviations: LLNA: BrdU-ELISA = Murine local lymph node assay (LLNA) with enzyme-linked immunosorbent assay (ELISA) detection of bromodeoxyuridine (BrdU); CI = Confidence interval; SD = Standard deviation; SI = Stimulation index; X = Discordant result obtained in the LLNA: BrdU-ELISA when compared to the traditional LLNA

[1] Statistical test for difference of group means.

7.0 Test Method 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.

Since several substances were tested in the LLNA: BrdU-ELISA multiple times**,** 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-ELISA are amenable to intralaboratory reproducibility analyses for three endpoints: sensitizer (positive) or non-sensitizer (negative) classification, SI values, and EC3 values. Analyses of intralaboratory reproducibility were performed using a concordance analysis for the qualitative results (sensitizer vs. non-sensitizer) (**Section 7.1**) and a coefficient of variation (CV) analysis for the quantitative results (SI values and EC3 values) (**Sections 7.2** and **7.3**).

7.1 Intralaboratory Reproducibility – Qualitative Results

The dataset available for an intralaboratory concordance analysis of the qualitative test results for the LLNA: BrdU-ELISA included six substances that were tested multiple times and classified as sensitizers or non-sensitizers. Eugenol and isoeugenol were each tested four times, hexyl cinnamic aldehyde and isoeugenol were each tested three times, and 2,4-dinitrochlorobenzene and propylene glycol were each tested twice (Takeyoshi et al. 2003, 2004a, 2005, 2006, 2007a). All substances were sensitizers in the traditional LLNA except for propylene glycol. The multiple test results for eugenol, hexyl cinnamic aldehyde, and 2,4-dinitrochlorobenzene were 100% concordant.

Discordant test results were noted for isoeugenol and propylene glycol. Three of the four LLNA: BrdU-ELISA results for isoeugenol were positive for skin sensitization potential (Takeyoshi et al. 2005; 2007a) and one was negative (Takeyoshi et al. 2006), which yields a 75% intralaboratory concordance. Two of the positive results (Takeyoshi et al. 2006) and one negative

result (Takeyoshi et al. 2006) were obtained at a maximum concentration of 10% isoeugenol. The remaining positive result was obtained using a maximum concentration of 30% isoeugenol (Takeyoshi et al. 2007a). Two discordant test results were noted for propylene glycol. A positive result was indicated by individual animal data submitted by Dr. Takeyoshi to support the graphical data shown in Takeyoshi et al. (2005) (although the graphical display indicated a negative result). The test result from Takeyoshi et al. (2006) produced a negative result. Both tests used a maximum concentration of 50%.

The qualitative intralaboratory concordance analysis for the traditional LLNA (ICCVAM 1999) was based on a dataset of six substances that included six results each for benzocaine and hexyl cinnamic aldehyde, five results for eugenol, four results each for isoeugenol and methyl salicylate, and three results for 2,4-dinitrochlorobenzene. All intralaboratory results for each substance were 100% concordant with the exception of one of the six benzocaine (5/6 or 83% concordance) results that was reported as +/- (i.e., equivocal). An equivocal result was described as one in which SI increases with dose, but does not reach the criterion of three for classification as a sensitizer.

Thus, the intralaboratory concordance of qualitative results for the LLNA: BrdU-ELISA was lower than that of the traditional LLNA, but it was based on a smaller dataset.

7.2 Intralaboratory Reproducibility – SI

Three of the Takeyoshi et al. (2003, 2005, 2007a) studies reported numerical SI values (i.e., values were reported in the text or tables rather than plotted on graphs) that allowed for an assessment of intralaboratory reproducibility. The SI values reported for five substance/concentration combinations that were tested twice (in separate experiments) were used to calculate a CV for the assessment of intralaboratory variability. Hexyl cinnamic aldehyde was tested twice at three different concentrations. Eugenol and isoeugenol were each tested twice at one concentration. As shown by **Table 7-1**, the CVs ranged from 0.6% (hexyl cinnamic aldehyde) to 51.3% (isoeugenol). The intralaboratory reproducibility of the traditional LLNA was not assessed by CV analysis of SI values (ICCVAM 1999).

Table 7-1 Intralaboratory Reproducibility for the SI of Tested Substances in LLNA: BrdU-ELISA - Coefficient of Variation

Substance	Concentration Tested (%)	SI	Mean	SD	CV (%)	Takeyoshi et al. Reference
Eugenol	30	3.30	3.57	0.37	10.5%	2004a
Eugenol	30	3.83				2007
Hexyl cinnamic aldehyde	12.5	1.87	1.73	0.21	11.9%	2003
Hexyl cinnamic aldehyde	12.5	1.58				2003
Hexyl cinnamic aldehyde	25	2.42	2.41	0.01	0.6%	2003
Hexyl cinnamic aldehyde	25	2.40				2003
Hexyl cinnamic aldehyde	50	3.63	3.62	0.02	0.6%	2003
Hexyl cinnamic aldehyde	50	3.60				2005
Isoeugenol	10	5.20	3.82	1.96	51.3%	2005
Isoeugenol	10	2.43				2007

Abbreviations: CV = Coefficient of variation; SD = Standard deviation, SI = Stimulation index

7.3 Intralaboratory Reproducibility – EC3

CV values were also calculated for the EC3 values of substances that were tested multiple times. Five Takeyoshi et al. (2003, 2004a, 2005, 2006, 2007a) studies reported multiple EC3 values, or SI values that could be used to interpolate EC3 values, for multiple tests of the same substances. Multiple EC3 values were available for four substances. Two EC3 values were reported for 2-dinitrochlorobenzene and three EC3 values each were reported for isoeugenol, hexyl cinnamic aldehyde, and isoeugenol. As shown by **Table 7-2**, the CVs ranged from 10.1% (hexyl cinnamic aldehyde) to 47.1% (2, 4-dinitrochlorobenzene).

Table 7-2 Intralaboratory Reproducibility for the EC3 of Tested Substances in LLNA: BrdU-ELISA - Coefficient of Variation

Substance	EC3	Mean	SD	CV (%)	Takeyoshi et al. Reference
2, 4-Dinitrochlorobenzene	0.2	0.15	0.07	47.1%	2005
2, 4-Dinitrochlorobenzene	0.1				2006
Isoeugenol	5.6	9.3	3.6	38.3%	2005
Isoeugenol	9.6				2006
Isoeugenol	12.7				2007b
Hexyl cinnamic aldehyde	40.8	41.2	4.2	10.1%	2005
Hexyl cinnamic aldehyde	45.5				2006
Hexyl cinnamic aldehyde	37.2				2003
Eugenol	25.1	29.5	9.7	32.8%	2004a
Eugenol	40.6				2006
Eugenol	22.8				2007b

Abbreviations: CV = Coefficient of variation; EC3 = Estimated concentration needed to produce a stimulation index of three; SD = Standard deviation.

The intralaboratory reproducibility of the traditional LLNA was assessed by CV analysis of EC3 values using a larger dataset (ICCVAM 1999) than that available for the LLNA: BrdU-ELISA analysis. Two EC3 values were reported by each of five laboratories for 2, 4-dinitrochlorobenzene, five EC3 values were reported by one laboratory for isoeugenol, six EC3 values were reported for hexyl cinnamic aldehyde by two laboratories, and five EC3 values were reported for eugenol by one laboratory (**Table 7-3**).

Most intralaboratory CV values for the EC3 values from LLNA: BrdU-ELISA tests were higher than those reported in ICCVAM (1999) for the traditional LLNA. At 47.1%, the intralaboratory EC3 CV values from the LLNA: BrdU-ELISA tests of 2, 4-dinitrochlorobenzene (**Table 7-2**)

were at the top of the range cited in ICCVAM (1999) (**Table 7-3**). The intralaboratory EC3 CV from the LLNA: BrdU-ELISA tests of isoeugenol was greater than that from ICCVAM (1999) (38.3% vs. 26.1%). The intralaboratory EC3 CV from the LLNA: BrdU-ELISA tests of hexyl cinnamic aldehyde was lower than that reported by ICCVAM (1999) (10.1% vs. 18.7 to 26.7%). The intralaboratory EC3 CV from the LLNA: BrdU-ELISA tests of eugenol were higher that that reported by ICCVAM (1999) (32.8% vs. 18.4%).

Table 7-3 Intralaboratory Reproducibility for the EC3 of Tested Substances in the Traditional LLNA[1]

Substance	Number of Laboratories	Number of Tests per Laboratory	CV (%)
2, 4-Dinitrochlorobenzene	5	2	12.9 – 47.1
Isoeugenol	1	5	26.1
Hexyl cinnamic aldehyde	2	6	18.7-26.7
Eugenol	1	5	18.4

Abbreviations: CV = Coefficient of variation; EC3 = Estimated concentration needed to produce a stimulation index of three.
[1]From ICCVAM (1999).

8.0 Data Quality

All of data were generated at the Hita Laboratory of the Chemicals Evaluation and Research Institute, Japan. Although the Hita Laboratory is a GLP-conforming facility, the studies on the LLNA: BrdU-ELISA did not conform fully with GLP guidelines since they were not intended for regulatory purposes. However, all systems employed for these studies (i.e., test facilities, study staff, reagents, and the other study elements) were reportedly the same as those employed in the fullly GLP-compliant studies conducted in the laboratory. Although multiple staff members checked the reported data for consistency with the raw data, no audit report is available (Takeyoshi M, personal communication). The raw data are also not available for audit.

9.0. Other Scientific Reports and Reviews

A multi-laboratory validation study of the LLNA: BrdU-ELISA is underway in Japan (Kojima H, personal communication). Seven laboratories are testing 10 substances (different from those evaluated by Dr. Takeyoshi) using a revised version of Dr. Takeyoshi's protocol. The final tests were scheduled for completion by the end of December 2007. The validation study management team is scheduled to meet on February 15, 2008, to discuss the results. More information about the validation study, including the protocol, will be added as it is received. NICEATM has requested the identities of the substances tested, the number of laboratories participating, and the number of times each substance was tested in each laboratory.

A set of studies were conducted by Yamano et al. using a similar LLNA: BrdU-ELISA based method (Yamano et al. 2003, 2004, 2005, 2006, 2007). The test method protocol (e.g., application of test substance to ear of mouse) was similar to what was described in the Takeyoshi et al. studies discussed above. Compared to the method Takeyoshi et al., which administered 5 mg BrdU/mouse, the concentration of BrdU administered (via intraperitoneal injection) was 150 mg/kg/15 mL saline, which would be approximately 3 mg BrdU/mouse (based on a 20 g mouse). The studies discussed the use of a BrdU-ELISA based method to assess the skin sensitization potential of a variety of substances including metal salts of napthenic acid, methylated phenols, industrial biocides, and preservatives.

The outcomes of these studies were not included in this evaluation since comparative traditional LLNA data were not available for the substances evaluated. Therefore, a comparison of the accuracy of the LLNA: BrdU-ELISA versus the traditional LLNA, when outcomes were compared to guinea pig or human results, could not be conducted.

10.0 Animal Welfare Considerations

The LLNA: BrdU-ELISA will require the use of the same number of animals when compared to the traditional LLNA. However, since the traditional LLNA uses radioactivity and as such its use is restricted in some institutions, broader use of the non-radioactive LLNA: BrdU-ELISA protocol in place of the GP test could further reduce the number of guinea pigs that are still being 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-ELISA is the same as the rationale for the traditional LLNA; there are no valid and accepted non-animal ways to determine the ACD potential of substances and products, 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 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-ELISA in cases where dose response information is not needed for hazard identification purposes. In such an approach, only the highest soluble dose of the test article that does not produce skin irritation or systemic toxicity 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 further 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-ELISA

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-ELISA would similar to the traditional LLNA, since the protocols of the two methods (except for the detection of lymphocyte proliferation) are similar.

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

Compared to the traditional LLNA, the LLNA: BrdU-ELISA will not require facilities, equipment, and licensing permits for handling radioactive materials. The remaining facilities (e.g., animal care facilities) are the same between the two methods.

11.3 LLNA: BrdU-ELISA Training Considerations

The level of training and expertise needed to conduct the LLNA: BrdU-ELISA should be similar to the traditional LLNA. Additionally, individuals will need to understand and perform ELISAs.

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