

Appendix C

Final Background Review Document:

**Nonradioactive Murine Local Lymph Node Assay: BrdU-ELISA Test Method Protocol
(LLNA: BrdU-ELISA)**

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**Background Review Document
Nonradioactive Murine Local Lymph Node Assay: BrdU-ELISA
Test Method Protocol (LLNA: BrdU-ELISA)**

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

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

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List of Abbreviations and Acronyms

ACD	Allergic contact dermatitis
ANOVA	Analysis of variance
AOO	Acetone: olive oil
BRD	Background review document
BrdU	Bromodeoxyuridine
CI	Confidence interval
CASRN	Chemical Abstracts Service Registry Number
Conc.	Concentration tested
CPSC	U.S. Consumer Product Safety Commission
CV	Coefficient of variation
DMF	<i>N,N</i> -dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC1.5	Estimated concentration needed to produce a stimulation index of 1.5
EC2	Estimated concentration needed to produce a stimulation index of 2
EC3	Estimated concentration needed to produce a stimulation index of 3
ECt	Estimated concentration needed to produce a stimulation index equaling or greater than a specified threshold
ELISA	Enzyme-linked immunosorbent assay
EPA	U.S. Environmental Protection Agency
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
ISO	International Organization for Standardization
IWG	Immunotoxicity Working Group
JSAAE	Japanese Society for Alternatives to Animal Experiments
K _{ow}	Octanol-water partition coefficient
LLNA	Murine 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
MW	Molecular weight

NA	Not available
NC	Not calculated
NK	Not known
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NT	Not tested
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
Res	Result
SD	Standard deviation
SI	Stimulation index
TG	Test Guideline
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; Sailstad et al. 2001; Dean et al. 2001; Haneke et al. 2001). ICCVAM concluded that the LLNA (referred to herein as the “traditional LLNA”) provided several advantages compared to guinea pig test methods, 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 nonradioactive 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 Toxicological Methods (NICEATM) to evaluate the scientific validity of these nonradioactive 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. The evaluation process involved two public meetings of an international independent scientific peer review panel (referred to hereafter as “Panel”) that reviewed draft and revised draft background review documents (BRDs) and ICCVAM test method recommendations.

A comprehensive draft background review document provided the initial information, data, and analyses supporting the validation status of each of the nonradioactive 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 BRDs and draft test method recommendations to the 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.¹ 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 data that were not available to the Panel and reanalyze the performance of each nonradioactive LLNA test method. NICEATM subsequently obtained additional data and prepared revised draft BRDs. ICCVAM also prepared revised draft test method recommendations based on the revised draft BRDs. NICEATM and ICCVAM provided the revised draft BRDs and revised draft test method recommendations to the Panel for their consideration at a public meeting on April 28-29, 2009. A report of the Panel meeting was subsequently published on the NICEATM-ICCVAM website.²

Based on the revised draft ICCVAM recommendations, NICEATM submitted a proposed draft Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) for the LLNA with enzyme-linked immunosorbent assay detection of bromodeoxyuridine (referred to hereafter as the “LLNA: BrdU-ELISA”) that was circulated in July 2009 to the 30 OECD member countries for review and comment. An OECD Expert Consultation Meeting was held on October 20-22, 2009, to evaluate the comments. The expert group reviewed the draft OECD TG for the LLNA: BrdU-ELISA and proposed responses to the comments from member countries. A revised TG was again distributed to the 30 OECD member countries in December 2009 for review and comment,

¹ http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel.htm.

² http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel.htm.

and then the final draft was forwarded to the OECD Working Group of National Co-ordinators of the Test Guidelines Programme to consider for adoption at their March 23-25, 2010, meeting.

ICCVAM considered the conclusions and recommendations of the Panel and conclusions from the OECD Expert Consultation, along with comments received from the public and the Scientific Advisory Committee for Alternative Toxicological Methods (i.e., the ICCVAM-NICEATM advisory committee), and then finalized the BRDs and test method recommendations. These will be forwarded to Federal agencies for their consideration and acceptance decisions, where appropriate. This BRD addresses the validation database for the LLNA: BrdU-ELISA.

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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 (GP) test methods to assess the allergic contact dermatitis (ACD) potential of most 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 Delayed-type Hypersensitivity [ISO 2002]; U.S. Environmental Protection Agency [EPA] Health Effects Test 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 nonradioactive modifications to the current version of the LLNA ([ICCVAM 1999; Sailstad et al. 2001; Dean et al. 2001; Haneke 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 data and information regarding the usefulness and limitations of one of these test methods, the LLNA with detection of bromodeoxyuridine (BrdU) incorporation by an enzyme-linked immunosorbent assay (ELISA) (referred to hereafter as the “LLNA: BrdU-ELISA”).

Test Method Protocol

The LLNA: BrdU-ELISA was originally developed by Takeyoshi et al. (2001). 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 using an 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 control group, is used to identify 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 (ICCVAM 1999; Sailstad et al. 2001; Dean et al. 2001; Haneke et al. 2001).

Validation Database

The accuracy and reliability of the LLNA: BrdU-ELISA were assessed using the individual animal data for 43 substances from six published studies (Takeyoshi et al. 2003, 2004a, 2004b, 2005, 2006, 2007a), one platform presentation (Takeyoshi 2007b), one poster presentation (Kojima et al. 2008), and unpublished data submitted to NICEATM in 2009. The reference test data for these substances were obtained from the traditional LLNA, GP skin sensitization tests, and/or human skin sensitization tests or clinical information. Of the 43 substances with traditional LLNA data, 32 were classified by the traditional LLNA as skin sensitizers and 11 were classified as nonsensitizers.

Test Method Accuracy

The accuracy evaluation in this BRD includes the evaluation of multiple decision criteria, including the $SI \geq 2.0$ recommended in the test method protocol. Based on the evaluation of multiple decision criteria, the optimal performance was achieved using $SI \geq 1.6$ to classify sensitizers. Compared with the traditional LLNA, accuracy was 95% (41/43), with a false positive rate of 18% (2/11) and a false negative rate of 0% (0/32). The two false positive substances produced borderline positive SI values between 1.6 and 1.9 in the LLNA: BrdU-ELISA.

When the decision criterion of $SI \geq 2.0$ was used to classify sensitizers vs. nonsensitizers, compared to the traditional LLNA, accuracy was 95% (41/43), with a false positive rate of 0% (0/11) and a false negative rate of 6% (2/32). Between the two false negative substances, no unique characteristics were identified that could be used as rationale for excluding any particular types of substances from testing in the LLNA: BrdU-ELISA.

The reduced LLNA: BrdU-ELISA (rLLNA: BrdU-ELISA), which uses only the highest soluble dose of the test substance that does not produce local skin irritation or systemic toxicity, can reduce animal use by 40% for hazard classification purposes where dose-response information is not needed. Using $SI \geq 1.6$ to classify sensitizers, the accuracy of the rLLNA: BrdU-ELISA compared with the multiple-dose LLNA: BrdU-ELISA was 95% (82/85 tests), with a false positive rate of 0% (0/11 tests) and a false negative rate of 4% (3/74 tests). The three tests that were false negative in the rLLNA: BrdU-ELISA were weakly positive in the LLNA: BrdU-ELISA at a concentration lower than the highest dose ($SI = 1.62, 2.02, \text{ and } 2.22$). The highest dose tested for each of the three tests of two substances was 50%.

Test Method Reliability – Intralaboratory Reproducibility

Intralaboratory reproducibility was assessed using a concordance analysis of sensitizer/nonsensitizer results and a coefficient of variation (CV) analysis of SI values and EC1.6 values (estimated concentration needed to produce an SI of 1.6). The qualitative analysis shows that multiple tests of 12 substances (10 sensitizers and two nonsensitizers) yielded 100% concordance for the sensitizer/nonsensitizer outcomes for 10/12 substances. However, one of the nonsensitizers with 100% concordance produced false positive results in both tests that were conducted for this substance. In the quantitative analyses, the CV values for the SI values of 13 substance/concentration combinations that were tested up to five times each ranged from 1% to 80%. The CV values for the EC1.6 values of four substances that were tested up to five times at multiple doses ranged from 37% to 118%.

Test Method Reliability – Interlaboratory Reproducibility

When using $SI \geq 1.6$ to classify sensitizers, the qualitative interlaboratory reproducibility analysis of 10 substances (seven sensitizers and three nonsensitizers), that were tested in up to seven laboratories indicated 100% agreement (3/3, 6/6, or 7/7) among the laboratories for nine substances (seven sensitizers and two nonsensitizers). One of the nonsensitizers with 100% concordance, however, produced false positive results in 3/3 laboratories. There was 67% (4/6) agreement among the tests for the remaining nonsensitizer. Interlaboratory CV values for the EC1.6 values of the seven sensitizers ranged from 31% to 93%.

When using $SI \geq 1.6$ to classify sensitizers, the categorical concordance analysis for the 18 substances with multiple tests indicated that the SI results for 85% (11/13) of the LLNA sensitizers were 100% concordant (i.e., all yielded $SI \geq 1.6$ and $SI \geq 1.9$). Two of the 13 sensitizers produced one test with $SI < 1.6$ and one test with $SI > 1.6$. The SI results for 60% (3/5) of the nonsensitizers were 100% concordant. All tests for two nonsensitizers had $SI < 1.6$, and all tests of the third nonsensitizer yielded SI values between 1.6 and 1.9. The concordance of the other two nonsensitizers was 67% (2/3 tests) for SI values between 1.6 and 1.9 and 71% (5/7 tests) for $SI < 1.6$.

Animal Welfare Considerations

The LLNA: BrdU-ELISA will use the same number of animals when compared to the updated ICCVAM-recommended LLNA protocol (Appendix A of ICCVAM 2009). However, since use of the traditional LLNA is restricted in some institutions because it involves radioactivity, availability and use of the nonradioactive LLNA: BrdU-ELISA 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.

Further, the LLNA: BrdU-ELISA evaluates the induction phase of sensitization and therefore discomfort to animals associated with the elicitation phase is eliminated. Additionally, the LLNA: BrdU-ELISA protocol requires fewer mice per treatment group (a minimum of four animals per group) than either of the GP tests (10-20 animals/group for the Buehler test and 5-10 animals/group for the GPMT).

Test Method Transferability

The transferability of the LLNA: BrdU-ELISA was demonstrated by an interlaboratory validation study (Kojima et al. 2008). 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 the ELISA is required.

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, in 2005, 980 cases of ACD involved days away from work.³ ACD develops in two phases, induction and elicitation. The induction phase occurs when a susceptible individual is exposed topically to a skin-sensitizing substance. Induction depends on the substance passing 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 again topically exposed to the same substance. As in the induction phase, the substance penetrates the epidermis, is processed by the Langerhans cells, and is presented to circulating T-lymphocytes. The antigen-specific 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; Sailstad 2001; 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 the LLNA as a valid substitute for currently accepted guinea pig (GP) test methods to assess the ACD potential of most 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 nonregulatory 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 [TG] 429 [OECD 2002]; International Standards Organization [ISO] 10993-10: Tests for Irritation and Delayed-type Hypersensitivity [ISO 2002]; U.S. Environmental Protection Agency [EPA] Health Effects Test 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 nonradioactive modifications 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

³ Available at <http://www.bls.gov/>.

nomination. This 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”). ICCVAM and its Immunotoxicity Working Group (IWG) evaluated this method in a draft BRD and developed draft test method recommendations based on this initial evaluation. An independent peer review panel (Panel) reviewed the draft BRD in March 2008 to evaluate the extent to which the information contained in the BRD supported the draft test method recommendations. The Panel concluded that additional information was needed to evaluate the test method, including a detailed test method protocol, individual animal data on a larger number of reference substances that cover a wide range of physicochemical properties and sensitization potency, and an evaluation of interlaboratory reproducibility. In response to this recommendation, NICEATM obtained additional LLNA: BrdU-ELISA data and information, which were used to generate a revised draft BRD for review by the Panel in April 2009.

Based on the revised draft ICCVAM test method recommendations, NICEATM submitted a proposed draft OECD TG for the LLNA: BrdU-ELISA that was circulated in July 2009 to the 30 OECD member countries for review and comment via their National Co-ordinators, who distributed the draft TG to interested stakeholders. An OECD Expert Consultation meeting was held on October 20-22, 2009, to evaluate the comments. Scientists from the National Institute of Environmental Health Sciences (NIEHS), the Environmental Protection Agency (EPA), the Food and Drug Administration (FDA), and CPSC, as well as U.S. and international experts from industry and other stakeholder organizations, participated in the meeting, which was co-hosted by CPSC and NICEATM-ICCVAM. The expert group reviewed the draft OECD TG for the LLNA: BrdU-ELISA, proposed responses to comments from member countries, and evaluated additional LLNA: BrdU-ELISA results for 12 substances tested and submitted to NICEATM after the Panel evaluation. The OECD Expert Consultation convened a subsequent teleconference on December 1, 2009, to discuss outstanding issues identified at the October meeting. A revised TG was again distributed to the 30 OECD member countries in December 2009, via their National Co-ordinators, for review and comment by national experts and interested stakeholders. A final teleconference of the OECD Expert Consultation was convened on January 29, 2010 to discuss the member country comments received during the last round of review, and a final draft TG was developed based on these discussions. This final draft was forwarded to the OECD Working Group of National Co-ordinators of the Test Guidelines Programme to consider for adoption at their March 23-25, 2010 meeting.

ICCVAM and the IWG considered the conclusions and recommendations of the Panel, comments received from the public and its advisory committee (i.e., the Scientific Advisory Committee on Alternative Toxicological Methods [SACATM]), along with the conclusions of the OECD Expert Consultation on the LLNA, and developed this final BRD. ICCVAM provides this final BRD to regulatory agencies for consideration as part of the ICCVAM Test Method Evaluation Report.

1.3 The LLNA: BrdU-ELISA

The LLNA: BrdU-ELISA was developed by Takeyoshi et al. (2001) as a nonradioactive 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 **Annex I**) is similar to the ICCVAM-recommended protocol for the traditional LLNA (see Appendix A of ICCVAM [2009]), 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, ³H- thymidine or ¹²⁵I-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. FixDenat solution (Roche Applied Science), which fixes the cells and denatures the DNA in one step, is added to each well, and the plate is incubated at room temperature. The FixDenat 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 nonsensitizers. 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 $SI \geq 3.0$ was initially used as the threshold for labeling a substance as a sensitizer. Takeyoshi et al. (2007b) evaluated the use of other decision criteria such as specific differences in BrdU incorporation between treated and control groups (i.e., greater than the 95% confidence interval [CI] of the control group, greater than the two or three standard deviations [SD] from the control group mean, and statistically significant differences by analysis of variance [ANOVA]) and other SI values to distinguish sensitizers from nonsensitizers and found that lower cutoff values for the SI improved accuracy when compared with the results of the traditional LLNA.

A multilaboratory validation study of the LLNA: BrdU-ELISA organized by the Japanese Society for Alternatives to Animal Experiments (JSAAE) used $SI \geq 2$ to classify sensitizers (Kojima et al. 2008). The $SI \geq 2$ criterion was selected for the interlaboratory validation study because prior studies (Takeyoshi et al. 2003, 2004a, 2004b, 2005, 2006, 2007a, 2007b) indicated that the $SI \geq 3$ criterion was inadequate for reliably distinguishing sensitizers from nonsensitizers (Kojima H, personal communication).

3.0 LLNA: BrdU-ELISA Validation Database

The validation database for the LLNA: BrdU-ELISA includes data that were available for 47 substances; 43 substances had been previously tested in the traditional LLNA. Thirty-nine substances were tested in one laboratory (Takeyoshi et al. 2003, 2004a, 2004b, 2005, 2006, 2007a, 2007b, unpublished data) and four additional substances (along with six of the same substances tested by Takeyoshi et al.) were tested in the multilaboratory validation study coordinated by JSAAE (**Table C-1**). No traditional LLNA data were available for four substances, which include 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-phenoxy)-propyl]-2-methoxy-phenol and 2-methoxy-4-(7-methoxy-3-methyl-5-propenyl-2,3-dihydro-benzofuran-2yl)-phenol) (Takeyoshi et al. 2004a, 2007a). Of the 43 substances with traditional LLNA data, 32 were classified by the traditional LLNA as skin sensitizers and 11 were classified as nonsensitizers. The traditional LLNA EC₃ values (i.e., estimated concentration needed to produce an SI = 3) for the 32 sensitizers ranged from 0.009% to 47.5% (**Table C-1**).

Annex II provides information on physicochemical properties (e.g., physical form tested). For the 43 substances evaluated, the molecular weights ranged from 30.03 to 388.29 g/mole. Twenty-five substances were liquids and 18 substances are solids. Estimated log octanol-water partition coefficients, which were available for 41 substances, ranged from -3 to 3.88. Peptide reactivity, which was available for 22 substances, ranged from high to minimal (Gerberick et al. 2007a).

Annex II further provides information on the Chemical Abstracts Service Registry Number and chemical class for each substance tested. When available, chemical classes for each substance were retrieved from the National Library of Medicine Medical Subject Headings classification system (available at <http://www.nlm.nih.gov/mesh/meshhome.html>). If chemical classes were unavailable, they were assigned using a standard classification scheme based on the Medical Subject Headings classification system. 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 C-1 shows that 19 chemical classes are represented by the 47 substances tested in the LLNA: BrdU-ELISA. Eleven substances are classified in more than one chemical class. The classes with the highest number of substances are carboxylic acids (13 substances) and aldehydes (six substances). Of the 22 chemical classes represented in the NICEATM LLNA database by at least five substances (thereby providing a sufficiently large representation for further analyses), 20 classes had at least 60% of the traditional LLNA results identified as positive. For this database of more than 600 substances, these classes were identified as those most likely to be associated with skin sensitization. Fifteen of these classes were also represented in the LLNA: BrdU-ELISA database (only amides, ethers, ketones, macromolecular substances, and polycyclic compounds were not included). Among the chemical classes that have been previously identified as common skin allergens (e.g., aldehydes, ketones, quinones, and acrylates [Gerberick et al. 2004]), only ketones were not included in the LLNA: BrdU-ELISA database.

Table C-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: BrdU-ELISA EC1.6 Values, and Maximum SI Values for 43 Tested Substances

Substance Name	Product Use¹	Chemical Class²	Traditional LLNA EC3 (Maximum SI)³	LLNA: BrdU-ELISA EC1.6 (Maximum SI)³
5-Chloro-2-methyl-4-isothiazolin-3-one*	Cosmetics; Manufacturing; Pesticides	Sulfur Compounds; Heterocyclic Compounds	0.009 (27.7)	0.065 (4.8)
p-Benzoquinone	Manufacturing; Pesticides; Pharmaceuticals	Quinones	0.010 (52.3)	0.150 (6.9)
2,4-Dinitrochlorobenzene *	Manufacturing; Pesticides	Hydrocarbon, Halogenated; Nitro Compounds; Hydrocarbons, Cyclic	0.049 (43.9)	0.032 (18.8)
Diphenylcyclopropenone	Pharmaceuticals	Hydrocarbons, Cyclic	0.050 (NA)	0.450 (19.1)
Glutaraldehyde	Cosmetics; Disinfectant; Manufacturing; Pesticides	Aldehydes	0.083 (18.0)	0.115 (28.6)
4-Phenylenediamine*	Intermediate in chemical synthesis; Manufacturing	Amines	0.11 (26.4)	0.285 (14.7)
Formaldehyde	Disinfectant; Manufacturing	Aldehydes	0.50 (4.0)	0.163 (16.6)
Cobalt chloride*	Manufacturing; Pesticides	Inorganic Chemical, Elements; Inorganic Chemical, Metals	0.66 (7.2)	0.316 (3.7)
4-Methylaminophenol sulfate	Manufacturing	Amines; Phenols	0.8 (6.7)	1.081 (4.0)
<i>trans</i> -Cinnamaldehyde	Food Additive; Fragrance Agent	Aldehydes	1.4 (13.1)	1.530 (5.9)
Isoeugenol*	Food Additive; Fragrance Agent	Carboxylic Acids	1.5 (31.0)	5.156 (8.4)
2-Mercaptobenzothiazole *	Manufacturing; Pesticides	Heterocyclic Compounds	1.7 (8.6)	12.097 (1.6)

continued

Table C-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: BrdU-ELISA EC1.6 Values, and Maximum SI Values for 43 Tested Substances (continued)

Substance Name	Product Use¹	Chemical Class²	Traditional LLNA EC3 (Maximum SI)³	LLNA: BrdU-ELISA EC1.6 (Maximum SI)³
Cinnamic aldehyde	Cosmetics; Food Additive; Fragrance Agent; Intermediate in chemical synthesis; Personal Care Products; Pesticides	Aldehydes	1.9 (18.4)	4.808 (4.0)
3-Aminophenol	Cosmetics; Pharmaceuticals	Amines; Phenols	3.2 (5.7)	2.990 (3.1)
Diethyl maleate	Food Additive; Intermediate in chemical synthesis	Carboxylic Acids	3.6 (22.6)	8.049 (6.3)
Trimellitic anhydride	Manufacturing	Anhydrides; Carboxylic Acids	4.7 (4.6)	0.862 (7.9)
Nickel sulfate	Manufacturing	Inorganic Chemicals, Metals; Inorganic Chemicals, Elements	4.8 (3.1)	1.027 (4.5)
4-Chloroaniline	Intermediate in chemical synthesis; Manufacturing; Pesticides; Pharmaceuticals	Amines	9.00 (3.3)	11.029 (2.5)
Sodium lauryl sulfate *	Cosmetics; Food Additive; Manufacturing; Personal Care Products; Pesticides; Pharmaceuticals	Alcohols; Sulfur Compounds; Lipids	8.1 (8.9)	13.334 (2.6)
Citral*	Fragrance Agent	Hydrocarbons, Other	9.2 (20.5)	7.143 (16.4)
Hexyl cinnamic aldehyde *	Food Additive; Fragrance Agent	Aldehydes	9.7 (20.0)	12.920 (13.5)
Eugenol*	Cosmetics; Food Additive; Intermediate in chemical synthesis; Manufacturing; Personal Care Products; Pharmaceuticals	Carboxylic Acids	10.1 (17.0)	8.851 (17.7)
Phenyl benzoate *	Manufacturing; Pesticides	Carboxylic Acids	13.6 (11.1)	16.954 (3.4)

continued

Table C-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: BrdU-ELISA EC1.6 Values, and Maximum SI Values for 43 Tested Substances (continued)

Substance Name	Product Use¹	Chemical Class²	Traditional LLNA EC3 (Maximum SI)³	LLNA: BrdU-ELISA EC1.6 (Maximum SI)³
Cinnamic alcohol*	Cosmetics; Food Additive; Fragrance Agent; Intermediate in chemical synthesis; Personal Care Products	Alcohols	21.0 (5.7)	24.091 (2.7)
Cyclamen aldehyde	Food Additive; Fragrance Agent	Aldehydes	22.3 (5.2)	41.496 (5.7)
Hydroxycitronellal	Food Additive; Fragrance Agent; Personal Care Products	Hydrocarbons, Other	24.0 (8.5)	13.636 (4.8)
Imidazolidinyl urea *	Cosmetics; Personal Care Products; Pesticides	Urea	24.0 (5.5)	49.545 (1.6)
Ethylene glycol dimethacrylate*	Manufacturing	Carboxylic Acids	28.0 (7.0)	31.751 (3.1)
Linalool	Cosmetics; Food Additive; Fragrance Agent; Personal Care Products; Pesticides	Hydrocarbons, Other	30.0 (8.3)	27.596 (4.7)
Ethyl acrylate	Manufacturing	Carboxylic Acids	32.8 (4.0)	33.333 (5.0)
Isopropyl myristate	Cosmetics; Personal Care Products; Pharmaceuticals	Lipids	44.0 (3.4)	9.404 (4.2)
Aniline	Food Additive; Manufacturing; Personal Care Products; Pesticides; Pharmaceuticals	Amines	47.5 (4.4)	73.596 (2.1)
2-Hydroxypropyl methacrylate	Intermediate in chemical synthesis; Manufacturing	Carboxylic Acids	NC (1.3)	NC (1.1)
Diethyl phthalate	Cosmetics; Manufacturing; Personal Care Products; Pesticides; Pharmaceuticals	Carboxylic Acids	NC (1.5)	NC (0.9)
Dimethyl isophthalate	Manufacturing; Fragrance Agent	Carboxylic Acids	NC (1.0)	NC (1.3)

continued

Table C-1 Product Use and Chemical Classification, Traditional LLNA EC3 Values, LLNA: BrdU-ELISA EC1.6 Values, and Maximum SI Values for 43 Tested Substances (continued)

Substance Name	Product Use ¹	Chemical Class ²	Traditional LLNA EC3 (Maximum SI) ³	LLNA: BrdU-ELISA EC1.6 (Maximum SI) ³
Glycerol	Cosmetics; Food Additive; Intermediate in chemical synthesis; Manufacturing; Personal Care Products; Pharmaceuticals; Solvent	Alcohols; Carbohydrates	NC (1.1)	NC (1.3)
Hexane	Manufacturing; Solvent	Hydrocarbons, Acyclic	NC (2.2)	56.328 (1.9)
Isopropanol*	Cosmetics; Disinfectant; Food Additive; Intermediate in chemical synthesis; Manufacturing; Personal Care Products; Pharmaceuticals; Solvent	Alcohols	NC (1.7)	5.344 (2.2) ⁴
Lactic acid*	Food Additive; Manufacturing; Pharmaceuticals	Carboxylic Acids	NC (2.2)	15.177 (2.5)
Methyl salicylate*	Cosmetics; Food Additive; Fragrance Agent; Personal Care Products; Pharmaceuticals; Solvent	Carboxylic Acids	NC (2.9)	NC (1.4)
Salicylic acid*	Food Additive; Manufacturing; Pharmaceuticals	Phenols; Carboxylic Acids	NC (2.5)	NC (1.3)
Sulfanilamide	Pharmaceuticals	Hydrocarbons, Cyclic; Sulfur Compounds	NC (1.0)	NC (1.3)
Propylene glycol	Cosmetics; Food Additive; Intermediate in chemical synthesis; Personal Care Products; Pharmaceuticals; Solvent	Alcohols	NC (1.6)	NC (1.6)

Abbreviations: EC1.6 = estimated concentration (expressed as percentage) needed to produce SI = 1.6; EC3 = estimated concentration (expressed as percentage) needed to produce SI = 3; LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; NA = not available; NC = not calculated since maximum SI < 3.0 for the traditional LLNA or maximum SI < 1.6 for the LLNA: BrdU-ELISA; SI = stimulation index.

* Reference substance from ICCVAM (2009).

¹ Information gathered from the following databases: Hazardous Substances Database (<http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB>); Haz-Map (<http://hazmap.nlm.nih.gov/>); Household Products Database (<http://hpd.nlm.nih.gov/index.htm>); International Programme on Chemical Safety INCHEM database (<http://www.inchem.org/>); and the National Toxicology Program (<http://ntp.niehs.nih.gov:8080/index.html?col=010stat>).

- ² 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>).
- ³ Mean EC3 (expressed as % concentration) and maximum SI values are from the NICEATM database of traditional LLNA studies. EC1.6 and SI values for individual LLNA: BrdU-ELISA tests are provided in Annex IV of the BRD (Appendix C).
- ⁴ Highest SI of seven tests. Because the majority (five) of the seven tests, had SI values < 1.6, isopropanol is considered to be a nonsensitizer in the LLNA: BrdU-ELISA.

4.0 Reference Data

Thirty-five of the 43 substances previously tested in the traditional LLNA were considered in the original evaluation of the LLNA by ICCVAM (ICCVAM 1999). The traditional LLNA reference data used for the accuracy evaluation described in **Section 6.0** were obtained from ICCVAM (1999) for 33 of these substances (**Annex III**). The traditional LLNA data for the two remaining substances included in the original LLNA evaluation (ICCVAM 1999), aniline and nickel sulfate, were obtained from more recent sources, Gerberick et al. (2005) and Ryan et al. (2002), respectively. The traditional LLNA results in ICCVAM (1999) for these two substances were negative, but the subsequent tests at higher concentrations produced positive results. The traditional LLNA data for the remaining eight substances that were not considered in the original ICCVAM evaluation (ICCVAM 1999), *trans*-cinnamaldehyde, cinnamic alcohol, cyclamen aldehyde, diethyl maleate, ethyl acrylate, glutaraldehyde, isopropyl myristate, and linalool, were obtained from Gerberick et al. (2005), Gerberick et al. (2005), Basketter et al. (2005), Gerberick et al. (2005), Gerberick et al. (2005), Hilton et al. (1998), Ryan et al. (2000), and Gerberick et al. (2005), respectively.

The reference data for the GP tests (guinea pig maximization test [GPMT] or Buehler test) and human tests (human maximization test, human patch test allergen, or other human data) were obtained from Marzulli and Maibach (1974), Marzulli and Maibach (1980), Opdyke (1976), Björkner (1984), Gad et al. (1986), Jordan and King (1977), Klecak et al. (1997), ICCVAM (1999), Basketter et al. (1999b, 2005), Basketter and Scholes (1992), Kwon et al. (2003), Robinson et al. (1990), Takeyoshi et al. (2004a), Van der Walle et al. (1982), and Takeyoshi et al. (2007a) (**Annex III**). 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-phenyloxy)-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.

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 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.

5.0 Test Method Data and Results

The LLNA: BrdU-ELISA data evaluated in this technical summary were obtained from individual animal data that were submitted to NICEATM. These data supported six published studies (Takeyoshi et al. 2003, 2004a, 2004b, 2005, 2006, 2007a), one platform presentation (Takeyoshi et al. 2007b), one poster presentation (Kojima et al. 2008), and unpublished data submitted by Dr. Takeyoshi in January 2009. Unpublished data submitted by Dr. Takeyoshi in May and August 2009, after the Panel review, are included in the accuracy (**Section 6**) and reproducibility analyses (**Section 7**) in this final BRD because they were evaluated by the OECD Expert Consultation on the LLNA. Unpublished data for three additional substances (xylene, chlorobenzene, and nickel chloride) and repeat tests of two previously tested substances (2-mercaptobenzothiazole and imidazolidinyl urea) using different vehicles were submitted after the OECD Expert Consultation. Because they could not be considered in an independent peer review, these data are not considered in the accuracy and reproducibility analyses; however, they are included in **Annex V**. The data for the repeat tests are discussed where relevant.

All test results were obtained using the protocol in **Annex I**. The substances tested by Takeyoshi et al. were not coded to prevent the possibility of bias in the interpretation of test results. The interlaboratory validation study reported by Kojima et al. (2008); however, used coded test substances to mask the identity of the test substances from the testing laboratories. **Annex III** contains summary data for the LLNA: BrdU-ELISA and comparative reference data for the 47 substances tested in these studies, and **Annex IV** contains the individual animal data for the LLNA: BrdU-ELISA.

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 LLNA: BrdU-ELISA Database Used for the Accuracy Analysis

Forty-three of the 47 substances listed in **Table C-1** had sufficient LLNA: BrdU-ELISA and traditional LLNA data to conduct an accuracy analysis. 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-phenyloxy)-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.

Of the 43 substances tested with both LLNA: BrdU-ELISA and the traditional LLNA, 35 had GP data for a comparison of the performance of the LLNA: BrdU-ELISA vs. GP data with that of the traditional LLNA vs. GP data. No GP data were found for *trans*-cinnamaldehyde, cyclamen aldehyde, diethyl maleate, diphenylcyclopropanone, hexane, isopropyl myristate, or linalool. Additionally, 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 GPMPT protocol.⁴

Of the 43 substances tested with both LLNA: BrdU-ELISA and the traditional LLNA, 41 had human data for a comparison of the performance of the LLNA: BrdU-ELISA vs. human data with that of the traditional LLNA vs. human data. No human data for *trans*-cinnamaldehyde or trimellitic anhydride were located. The complete set of comparative data for each substance is located in **Annex III**.

Multiple tests were available for 18 substances tested with the LLNA: BrdU-ELISA. For the accuracy analyses, results using the same vehicle for multiply tested substances were combined so that each substance was represented by one result for the accuracy analysis. In this case, the single result used for each substance represented the outcome that was most prevalent. For example, using $SI \geq 2.0$ to identify sensitizers, isopropanol was a nonsensitizer because five of the seven tests for isopropanol had $SI < 2$. If the number of positive and negative outcomes were equal, the most conservative (i.e., positive) result was used for the accuracy analyses. If there were multiple test results with multiple vehicles for a substance, the vehicle that matched that used in the traditional LLNA was used in the accuracy analysis. For example, of the five tests for glutaraldehyde, two tests used acetone: olive oil

⁴ The nonstandard GP protocol did not include the 48-hour topical patch induction that should follow induction by intradermal injection and it replaced the 24-hour skin patch challenge (usually 2 weeks after topical induction) with a 6-hour skin patch challenge (Basketter D, personal communication).

(4:1) (AOO) as the solvent (Takeyoshi et al. 2005), and three tests used acetone as the solvent (Kojima et al. 2008). The tests that used acetone for the solvent were used for the accuracy analyses because the solvent matches that used for the traditional LLNA reference data.

6.2 Accuracy Analysis Using the $SI \geq 2.0$ Decision Criterion

The performance characteristics of the LLNA: BrdU-ELISA were first evaluated using the criterion of $SI \geq 2.0$ to identify sensitizers, which was the threshold for a positive response used in the interlaboratory validation study (the complete protocol used in the validation study is included in **Annex I**).

Of the 18 substances with multiple test results, discordant test results were noted among tests for six of the substances with multiple test results: cyclamen aldehyde, hydroxycitronellal, linalool, formaldehyde, isopropanol, and lactic acid. For all six substances, the solvents used for each test were the same. Dr. Takeyoshi tested cyclamen aldehyde (2007b and unpublished), hydroxycitronellal (2007b and unpublished), and linalool (both unpublished) twice; for each substance one test produced $SI < 2$ and the other test produced $SI \geq 2$.

- Cyclamen aldehyde tests yielded $SI = 1.97$ and 5.71 .
- Hydroxycitronellal tests yielded $SI = 1.34$ and 4.78 .
- Linalool tests yielded $SI = 1.45$ and 4.65 .

Other discordances included:

- One of the three laboratories in the interlaboratory validation study reported an SI of 1.97 for formaldehyde, while the others produced $SI \geq 2$ ($SI = 4.40$ and 16.59) (Kojima et al. 2008).
- Two of the seven tests of isopropanol yielded $SI \geq 2$ ($SI = 2.04$ and $SI = 2.22$), while the others yielded $SI < 2$ ($SI = 0.92, 0.94, 0.98, 1.01, \text{ and } 1.57$). The discordant tests were obtained by two of the six laboratories in the interlaboratory validation study.
- One of the three tests for lactic acid from the interlaboratory validation study produced $SI \geq 2$ (i.e., $SI = 2.53$), while the others yielded $SI < 2$ ($SI = 1.80$ and 1.89) (Kojima et al. 2008).

6.2.1 Accuracy vs. the Traditional LLNA

When compared to the traditional LLNA and using a decision criteria of $SI \geq 2.0$ to identify sensitizers, the LLNA: BrdU-ELISA had an accuracy of 95% (41/43), a sensitivity of 94% (30/32), a specificity of 100% (11/11), a false positive rate of 0% (0/11), and a false negative rate of 6% (2/32) (**Table C-2**).

6.2.2 Accuracy vs. Guinea Pig Data

When the accuracy of the LLNA: BrdU-ELISA ($SI \geq 2.0$) and the traditional LLNA were compared based on their performance relative to GP tests, the LLNA: BrdU-ELISA had a lower accuracy (86% [30/35] vs. 91% [32/35]) and sensitivity (91% [20/22] vs. 100% [22/22]), and higher false negative rate (9% [2/22] vs. 0% [0/22]; **Table C-2**). The specificity (77% [10/13]) and the false positive rate (23% [3/13]) for the LLNA: BrdU-ELISA and the traditional LLNA were the same when they were compared with GP data.

6.2.3 Accuracy vs. Human Data

When the accuracy of the LLNA: BrdU-ELISA ($SI \geq 2.0$) and the traditional LLNA were compared based on their performance relative to the available human data, the LLNA: BrdU-ELISA had a lower

accuracy (73% [30/41] vs. 78% [32/41]) and sensitivity (77% [24/31] vs. 84% [26/31]) and a higher false negative rate (23% [7/31] vs. 16% [5/31]) than the traditional LLNA (**Table C-2**). The specificity (60% [6/10]) and the false positive rate (40% [4/10]) for the LLNA: BrdU-ELISA and the traditional LLNA were the same when they were compared to human data.

Table C-2 Performance of the LLNA: BrdU-ELISA in Predicting Skin-Sensitizing Potential Using the Decision Criterion of SI \geq 2.0 to Identify Sensitizers

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
BrdU-ELISA vs. Traditional LLNA	43	95	41/43	94	30/32	100	11/11	0	0/11	6	2/32	100	30/30	85	11/13
<i>Substances with LLNA: BrdU-ELISA, Traditional LLNA, and GP Data</i>															
BrdU-ELISA vs. Traditional LLNA	35	94	33/35	92	23/25	100	10/10	0	0/10	8	2/25	100	23/23	83	10/12
LLNA: BrdU-ELISA vs. GP³	35	86	30/35	91	20/22	77	10/13	23	3/13	9	2/22	87	20/23	83	10/12
Traditional LLNA vs. GP³	35	91	32/35	100	22/22	77	10/13	23	3/13	0	0/22	88	22/25	100	10/10
<i>Substances with LLNA: BrdU-ELISA, Traditional LLNA, and Human Data</i>															
BrdU-ELISA vs. Traditional LLNA	41	95	39/41	93	28/30	100	11/11	0	0/11	7	2/30	100	11/11	100	28/28
LLNA: BrdU-ELISA vs. Human⁴	41	73	30/41	77	24/31	60	6/10	40	4/10	23	7/31	86	24/28	46	6/13
Traditional LLNA vs. Human⁴	41	78	32/41	84	26/31	60	6/10	40	4/10	16	5/31	87	26/30	54	6/11

Abbreviations: LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; GP = guinea pig skin sensitization outcomes; LLNA = murine local lymph node assay; No. = number; SI = stimulation index.

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, the human repeat insult patch test, inclusion of the test substance in a human patch test allergen kit, and/or published clinical case studies/reports.

6.3 Accuracy Analysis (SI \geq 2.0) Based on the ICCVAM Performance Standards Reference Substances

ICCVAM has developed recommended test method performance standards for the traditional LLNA (ICCVAM 2009⁵), which are proposed to evaluate the performance of modified LLNA test methods that are mechanistically and functionally similar to the traditional LLNA. Because the validation studies for the LLNA: BrdU-ELISA test method were completed prior to the development of LLNA performance standards and because all of the reference substances had not been tested, the LLNA: BrdU-ELISA was not evaluated using the ICCVAM-recommended LLNA performance standards. Thus, the LLNA: BrdU-ELISA test results for the ICCVAM-recommended LLNA performance standards reference substances were evaluated to provide a general comparison of performance. As shown in **Table C-3**, 16 of the 18 required reference substances included in the ICCVAM LLNA Performance Standards have been tested in the LLNA: BrdU-ELISA. Fourteen of the 16 substances yielded the same sensitizer/nonsensitizer outcome in the LLNA: BrdU-ELISA as in the traditional LLNA.

Because all of the required ICCVAM-recommended LLNA performance standards reference substances had not been tested in the LLNA: BrdU-ELISA, selected characteristics of the substances tested in the LLNA: BrdU-ELISA were compared with those of the reference substances. **Table C-4** shows traditional LLNA and other selected characteristics of the 43 substances with traditional LLNA data that were tested in the LLNA: BrdU-ELISA. The characteristics of these substances are compared to the characteristics of the 18 required reference substances from the ICCVAM-recommended LLNA performance standards (ICCVAM 2009). The table indicates that, although not all of the 18 required reference substances from the ICCVAM-recommended performance standards reference substances have been tested, the characteristics of the substances tested in the LLNA: BrdU-ELISA is similar to that included in the performance standards list. In general, there is a proportionally increased number of substances tested in the LLNA: BrdU-ELISA in each of the categories included in the table.

⁵ Available at http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm.

Table C-3 Performance of the LLNA: BrdU-ELISA (SI ≥ 2.0) Using the ICCVAM Performance Standards Reference Substances¹

Substance Name	Recommended Performance Standards ¹				LLNA: BrdU-ELISA ²			
	Vehicle	Result	EC3 (%) (Max SI) ¹	N ³	Vehicle	Result	EC2 (%) (Max SI)	N ³
5-Chloro-2-methyl-4-isothiazolin-3-one	DMF	+	0.009 (22.7)	1	DMF	+	0.12 (4.8)	1
2, 4-Dinitrochlorobenzene	AOO	+	0.049 (43.9)	15	AOO	+	0.044 (18.8)	8
4-Phenylenediamine	AOO	+	0.11 (26.4)	6	AOO	+	NC (14.7)	2
Methyl methacrylate	DMF	+	90 (3.6)	1	NT	NT	NT	NT
Isoeugenol	AOO	+	1.5 (31.0)	47	AOO	+	7.6 (8.4)	2
<i>2-Mercaptobenzothiazole</i>	<i>DMF</i>	+	<i>1.7 (8.6)</i>	<i>1</i>	<i>DMF</i>	-	<i>NA (1.6)</i>	<i>1</i>
Cobalt chloride	DMSO	+	0.6 (7.2)	2	DMSO	+	0.63 (3.7)	1
Citral	AOO	+	9.2(20.5)	6	AOO	+	NC (16.4)	1
Hexyl cinnamic aldehyde	AOO	+	9.7 (20.0)	21	AOO	+	17.4 (13.5)	11
Eugenol	AOO	+	10.1 (17.0)	11	AOO	+	9.8 (17.7)	8
Phenyl benzoate	AOO	+	13.6 (11.1)	3	DMF	+	28.2 (3.4)	1
Cinnamic alcohol	AOO	+	21 (5.7)	1	AOO	+	33.2 (2.7)	1
<i>Imidazolidinyl urea</i>	<i>DMF</i>	+	<i>24 (5.5)</i>	<i>1</i>	<i>DMF</i>	+	<i>NA (1.6)</i>	<i>1</i>
Chlorobenzene ⁴	AOO	-	NA (1.7)	1	NT	NT	NT	NT
Isopropanol	AOO	-	NA (1.7)	1	AOO	-	NA (2.2) ⁵	7
Lactic acid	DMSO	-	NA (2.2)	1	DMSO	-	NA (2.5) ⁶	3
Methyl salicylate	AOO	-	NA (2.9)	9	AOO	-	NA (1.4)	3
Salicylic acid	AOO	-	NA (2.5)	1	AOO	-	NA (1.3)	1
Ethylene glycol dimethacrylate	MEK	False +	28 (7.0)	1	MEK	+	49.8 (3.1)	1
Sodium lauryl sulfate	DMF	False +	8.1 (8.9)	5	DMF	+	14.6 (2.6)	1
Nickel chloride ⁴	DMSO	False -	NA (2.4)	2	NT	NT	NT	NT
Xylene ⁴	AOO	False -	95.8 (3.1)	1	NT	NT	NT	NT

Boldface italic 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 = estimated concentration needed to produce a stimulation index of 3; EC2 = estimated concentration needed to produce a stimulation index of 2; LLNA = murine local lymph node assay; MEK = methyl ethyl ketone; NA = not applicable (i.e., SI outcome was less than decision criterion for sensitizers); NT = not tested; SI = stimulation index.

+ = sensitizer.

- = nonsensitizer.

- ¹ Mean EC3 values (expressed as % concentration) and maximum SI values (shown in parentheses) are from the NICEATM database of traditional LLNA studies and from *Recommended Performance Standards: Murine Local Lymph Node Assay* (ICCVAM 2009; available: http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm).
- ² Calculated from data supporting Takeyoshi et al. (2003, 2004b, 2005, 2006, 2007a, 2007b, and unpublished) and Kojima et al (2008).
- ³ Number of values used to derive the mean EC3 or EC2 values.
- ⁴ Data submitted after conclusion of the independent peer review evaluations (see **Annex V** for data).
- ⁵ Based on the most prevalent outcome (i.e., 5/7 tests yielded SI < 2).
- ⁶ Based on the most prevalent outcome (i.e., 2/3 tests yielded SI < 2).

Table C-4 Characteristics of the Substances Tested in the LLNA: BrdU-ELISA vs. the ICCVAM Performance Standards Reference Substances¹

EC3 Range (%)	No. Chems	Solid/Liquid	Actual EC3 Range (%)	Maximum SI Range	Human Data	Peptide Reactivity (Hi/Mod/Min/Lo/Unk) ³
<0.1	5	3/3	0.009 - 0.083	18.0 – 52.3	5	5/0/0/0/0
	2	1/1	0.009 - 0.05	22.6 - 52.3	2	2/0/0/0/0
≥ 0.1 to <1	4	3/1	0.11 - 0.8	4.0 – 26.4	4	0/1/0/0/3
	2	2/0	0.11 - 0.6	6.7 - 75.3	2	0/0/0/0/2
≥ 1 to <10	12	5/7	1.4 - 9.7	3.1 – 31.0	10	2/0/1/1/8
	4	1/3	1.5 - 9.7	8.6 - 29.5	4	1/0/1/0/2
≥ 10 to <100	11	3/8	10.1 – 47.5	3.4 - 17.0	11	1/0/1/2/7
	5	3/2	10.1 - 90	5.5 - 70.3	5	0/1/0/0/4
Negative	11	4/7	NC	1.0 – 2.9	11	0/0/7/1/3
	5	1/4	NC	0.9 - 2.8	3	0/0/2/0/3
Overall	43	18/25	0.009 – 47.5	0.9 - 52.3	41	8/1/9/4/21
	18	10/8	0.009 - 24	0.9 - 75.3	16	3/1/3/0/11

Boldface text represents characteristics of the LLNA: BrdU-ELISA database.

Abbreviations: Chems = chemicals; EC3 = estimated concentration needed to produce SI = 3; Hi = high; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; Lo = low; Min = minimal; Mod = moderate; NC = not calculated because maximum SI < 3; No. = number; SI = stimulation index; Unk = unknown.

¹ From *Recommended Performance Standards: Murine Local Lymph Node Assay* (ICCVAM 2009; available: http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm). Includes the 18 “required” substances for testing.

² Data obtained from Gerberick et al. (2007b)

6.4 Discordant Results for Accuracy Analysis Using the SI ≥ 2.0 Decision Criterion

6.4.1 Discordance Between the LLNA: BrdU-ELISA and the Traditional LLNA

When the outcomes for the 43 substances tested in the LLNA: BrdU-ELISA (using SI ≥ 2.0) and the traditional LLNA were compared, the classifications for two substances were different. The LLNA: BrdU-ELISA classified imidazolidinyl urea and 2-mercaptobenzothiazole as nonsensitizers, while the traditional LLNA classified them as sensitizers (i.e., false negative outcome) (**Table C-5**). Both substances were tested in *N,N*-dimethylformamide (DMF) in the LLNA: BrdU-ELISA and the traditional LLNA tests. Both substances are solids. No commonalities in chemical class, size, peptide reactivity (see **Annex II** for physicochemical information), traditional LLNA potency, or potential for skin irritation were noted in these substances.

Imidazolidinyl urea is classified as a urea compound. It has a molecular weight (MW) of 388.39 g/mole. It was originally tested in the LLNA: BrdU-ELISA at 10% and 50% (SI = 0.73 and 1.61, respectively). The EC3 value for the traditional LLNA is 24%. No peptide reactivity information is available. An additional LLNA: BrdU-ELISA test for imidazolidinyl urea that was submitted after the Panel review and OECD Expert Consultation indicated that testing at higher concentrations and in DMSO will increase the response (**Annex V**). The additional test used 50% and 75% imidazolidinyl urea in DMSO and produced SI values of 1.65 and 2.27, respectively.

2-Mercaptobenzothiazole is classified as a heterocyclic compound and has a MW of 167.26 g/mole. It was originally tested in the LLNA: BrdU-ELISA at 12.5%, 25%, and 50% (SI = 1.62, 1.36, and 1.49, respectively). The EC3 value for the traditional LLNA is 1.7%. Peptide reactivity is high. It is labeled as a skin irritant at the concentrations tested in the LLNA: BrdU-ELISA, but imidazolidinyl urea is not. An additional LLNA: BrdU-ELISA test for 2-mercaptobenzothiazole that was submitted after the Panel review and OECD Expert Consultation on the LLNA indicated that testing with dimethyl sulfoxide (DMSO) as the vehicle increases the response (**Annex V**). The additional test used 10% and 25% 2-mercaptobenzothiazole and produced SI values of 1.50 and 2.23, respectively.

Table C-5 Discordant Results for LLNA: BrdU-ELISA (SI ≥ 2.0) Compared to Traditional LLNA and Guinea Pig Reference Data¹

Substance Name ²	Vehicle ³	LLNA: BrdU-ELISA ⁴	Traditional LLNA ⁴	Guinea Pig Studies	Skin Irritant?
Imidazolidinyl urea (24.0%)	DMF	- (1.61, 50%)	+ (5.5, 50%)	+	Nonirritant at ≤75% (GP)
2-Mercaptobenzo-thiazole (1.7%)	DMF	- (1.62, 50%) ⁵	+ (8.6, 10%)	+	Nonirritant at ≤10% (GP); Nonirritant at 25% (humans)
Ethyl acrylate (32.8%)	AOO	+ (4.95, 100%)	+ (4.0, 50%)	-	Nonirritant at 3% (GP)
Ethylene glycol dimethacrylate (28.0%)	MEK	+ (3.11, 100%)	+ (7.0, 50%)	-	Nonirritant at 1% (GP)
Sodium lauryl sulfate (8.1%)	DMF	+ (2.64, 16.7%)	+ (8.9, 20%)	-	Irritant at 20% aq. (rabbits); Irritant at 20% (humans); Irritant at 10% in DMF (mice)

Abbreviations: AOO = acetone: olive oil (4:1); aq = aqueous; DMF = *N,N*-dimethylformamide; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; GP = guinea pig; LLNA = murine local lymph node assay; MEK = methyl ethyl ketone; SI = stimulation index.

+ = sensitizer.

- = nonsensitizer.

¹ Data sources provided in **Annex III-1**.

² Numbers in parentheses are the EC3 values for the traditional LLNA (from **Table C-1**).

³ Vehicles apply to tests for both the LLNA: BrdU-ELISA and the traditional LLNA.

⁴ Numbers in parentheses are highest SI values and maximum concentrations tested.

⁵ Highest SI occurred at concentration of 12.5%.

6.4.2 Discordance Among the LLNA: BrdU-ELISA, the Traditional LLNA, and/or the Guinea Pig Test

For the 35 substances with LLNA: BrdU-ELISA, traditional LLNA, and GP test results, five substances produced results that were discordant with GP test results (**Table C-5**). Two substances were negative in the LLNA: BrdU-ELISA and positive in the GP, and three substances were positive in the LLNA: BrdU-ELISA and negative in the GP. The LLNA: BrdU-ELISA results for imidazolidinyl urea and 2-mercaptobenzothiazole were negative, while the GP results were positive. As noted in **Section 6.4.1**, there were few commonalities associated with these two discordant substances.

Ethyl acrylate, ethylene glycol dimethacrylate, and sodium lauryl sulfate (SLS) were classified as sensitizers by the LLNA: BrdU-ELISA and traditional LLNA and as nonsensitizers by GP tests (**Table C-5**). There were a few commonalities among these substances with regard to chemical class, physical form, MW, peptide reactivity (see **Annex II** for physicochemical information), the range of EC3 values (based on traditional LLNA, see **Table C-1**), and potential for skin irritation (**Annex III-1**):

- Ethyl acrylate and ethylene glycol dimethacrylate are carboxylic acids; SLS is an alcohol, sulfur, and lipid compound.
- Ethylene glycol dimethacrylate and ethyl acrylate are liquids; SLS is a solid.
- MWs ranged from 100.10 to 288.38 g/mol.
- Peptide reactivity for ethyl acrylate and ethylene glycol dimethacrylate is high; peptide reactivity data for SLS is not available.
- Ethyl acrylate (EC3 = 32.8%) and ethylene glycol dimethacrylate (EC3 = 28%) are weak sensitizers in the traditional LLNA; SLS (EC3 = 8.1%) is somewhat stronger.
- Ethyl acrylate, ethylene glycol dimethacrylate, and SLS were tested at irritating concentrations in the LLNA: BrdU-ELISA, based on skin irritation data from guinea pigs, humans, or mice.

6.4.3 Discordance Among the LLNA: BrdU-ELISA, the Traditional LLNA, and/or the Human Outcome

When analyses were restricted to the 41 substances with LLNA: BrdU-ELISA, traditional LLNA, and human outcomes, the LLNA: BrdU-ELISA misclassified 11 substances. Both the LLNA: BrdU-ELISA and the traditional LLNA misclassified five human sensitizers (diethyl phthalate, 2-hydroxypropylmethacrylate, isopropanol, propylene glycol, and sulfanilamide) as nonsensitizers (**Table C-6**). The LLNA: BrdU-ELISA also misclassified two other sensitizers as nonsensitizers that were correctly classified by the traditional LLNA (2-mercaptobenzothiazole and imidazolidinyl urea). There were a few commonalities among these seven substances with regard to chemical class, physical form, MW, peptide reactivity (see **Annex II** for physicochemical information), the range of EC3 values (based on traditional LLNA, see **Table C-1**), and potential for skin irritation (**Annex III-1**):

- Diethyl phthalate and 2-hydroxypropylmethacrylate are carboxylic acids; isopropanol and propylene glycol are alcohols; sulfanilamide is a cyclic hydrocarbon and sulfur compound; 2-mercaptobenzothiazole is a heterocyclic compound; and imidazolidinyl urea is a urea.

- Diethyl phthalate, isopropanol, and propylene glycol are liquids; while the other four compounds are solids.
- MWs ranged from 60.1 to 222.2 g/mol.
- Peptide reactivity for diethyl phthalate, isopropanol, and propylene glycol is minimal; the peptide reactivity for 2-hydroxypropylmethacrylate is low; the peptide reactivity for 2-mercaptobenzothiazole is high; and peptide reactivity information for sulfanilamide and imidazolidinyl urea is unavailable.
- 2-Mercaptobenzothiazole is a strong sensitizer in the traditional LLNA (EC3 = 1.7%); imidazolidinyl urea is a weak sensitizers (EC3 = 24%); the other four substances are LLNA nonsensitizers.
- Diethyl phthalate, isopropanol, and imidazolidinyl urea were tested at nonirritating concentrations, but the other four substances were not, based on skin irritation data from guinea pigs, rabbits, and humans.

Four human nonsensitizers were classified as sensitizers by the LLNA: BrdU-ELISA and the traditional LLNA: isopropyl myristate, cyclamen aldehyde, linalool, and SLS. There were a few commonalities among these substances with regard to chemical class, physical form, MW, peptide reactivity (see **Annex II** for physicochemical information), the range of EC3 values (based on traditional LLNA, see **Table C-1**), or potential for skin irritation (**Annex III-1**):

- Isopropyl myristate and SLS are lipids; cyclamen aldehyde is an aldehyde; linalool is a hydrocarbon; and SLS is also an alcohol and sulfur compound.
- Isopropyl myristate, cyclamen aldehyde, and linalool are liquids; and SLS is a solid.
- MWs ranged from 154.2 to 288.4 g/mol.
- Peptide reactivity for isopropyl myristate is minimal; peptide reactivity for cyclamen aldehyde is low; and peptide reactivity information for linalool and SLS is unavailable.
- Isopropyl myristate (EC3 = 44.0%), cyclamen aldehyde (MW = 22.3%), and linalool (EC3 = 30.0%) are weak traditional LLNA sensitizers, while SLS (EC3 = 8.1%) is a stronger sensitizer.
- Isopropyl myristate was tested at nonirritating concentrations; cyclamen aldehyde, linalool, and SLS were tested at irritating concentrations, based on skin irritation data from rabbits, humans, or mice.

Table C-6 **Discordant Results for LLNA: BrdU-ELISA (SI ≥ 2.0) When Compared to Traditional LLNA and Human Outcome Data¹**

Substance Name ²	Vehicle ³	LLNA: BrdU-ELISA ⁴	Traditional LLNA ⁴	Human Outcome ⁵	Skin Irritant?
Diethyl phthalate	AOO	- (0.88, 50%)	- (1.5, 100%)	+ (HPTA)	Nonirritant at ≤100% (rabbits)
2-Hydroxypropylmethacrylate	AOO	- (1.13, 50%)	- (1.3, 50%)	+ (case study, 0.1%)	Nonirritant at ≤10% (GP)
Isopropanol	AOO	- (2.22, 50%) ⁶	- (1.7, 50%) ⁷	+ (case study, 0.001%)	Nonirritant at ≤100% (rabbits)

continued

Table C-6 Discordant Results for LLNA: BrdU-ELISA (SI ≥ 2.0) When Compared to Traditional LLNA and Human Outcome Data¹ (continued)

Substance Name ²	Vehicle ³	LLNA: BrdU-ELISA ⁴	Traditional LLNA ⁴	Human Outcome ⁵	Skin Irritant?
Propylene glycol	AOO ⁸	- (1.57, 50%)	- (1.6, 100%)	+ (HPTA)	Nonirritant at ≤25% (humans)
Sulfanilamide	DMF	- (1.26, 50%)	- (1.0, 50%) ⁹	+ (5/25, 25%)	Nonirritant at ≤25% (humans)
2-Mercaptoben-zothiazole (1.7%)	DMF	- (1.62, 50%) ¹⁰	+ (8.6, 10%)	+ (5/24, 10%)	Nonirritant at ≤10% (GP); Nonirritant at 25% (humans)
Imidazolidinyl urea (24.0%)	DMF	- (1.34, 100%)	+ (5.5, 50%)	+ (2/150, 2%)	Nonirritant at ≤75% (GP)
Isopropyl myristate (44.0%)	AOO	+ (4.20, 50%)	+ (3.4, 100%)	- (0/25, 20%)	Nonirritant at ≤100% (rabbits)
Cyclamen aldehyde (22.3%)	AOO	+ (1.97 and 5.71, 100%)	+ (5.2, 50%)	- (0/64, 4%)	Irritant at 100% (rabbits)
Linalool (30.0%)	AOO	+ (1.45 and 4.65, 100%)	+ (8.3, 100%)	- (0/25, 8%)	Irritant at 100% (rabbits)
Sodium lauryl sulfate (8.1%)	DMF	+ (3.4, 10%)	+ (8.9, 20%) ⁷	- (0/22 at 10%)	Irritant at 20% aq. (rabbits); Irritant at 20% (humans); Irritant at 10% in DMF (mice)

Abbreviations: AOO = acetone: olive oil (4:1); aq = aqueous; DMF = *N,N*-dimethylformamide; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; GP = guinea pig; HPTA = human patch test allergen; LLNA = murine local lymph node assay.

+ = sensitizer.

- = nonsensitizer.

¹ Data sources listed in **Annex III-1**.

² Numbers in parentheses are EC3 values for the traditional LLNA (from **Table C-1**).

³ Vehicles apply to tests for both the LLNA: BrdU-ELISA and the traditional LLNA unless otherwise noted.

⁴ Numbers in parentheses are highest SI values and maximum concentrations tested.

⁵ Information in parentheses indicates the basis for the human outcome. Numbers indicate the incidence of positive human response and the concentration tested.

⁶ Negative based on most prevalent outcome. Highest SI of any test is shown (SI = 0.92, 0.94, 0.98, 1.01, 1.57, 2.04, and 2.22). Highest SI values for most tests occurred at <50%.

⁷ Highest SI occurred at 10%.

⁸ Vehicle for the traditional LLNA was distilled water.

⁹ Highest SI occurred at 10% and 25%.

¹⁰ Highest SI occurred at 12.5%.

6.4.4 Discordance Between the LLNA: BrdU-ELISA and the Traditional LLNA When Testing the LLNA Performance Standards Substances

Using $SI \geq 2.0$, two discordant substances, 2-mercaptobenzothiazole and imidazolidinyl urea, were noted among the 16 performance standards minimum reference substances tested in the LLNA: BrdU-ELISA. The LLNA: BrdU-ELISA classified both substances as nonsensitizers, while the traditional LLNA, GP, and human tests classified them as sensitizers. The EC3 value for 2-mercaptobenzothiazole in the traditional LLNA, 1.7%, was derived from a test of 1%, 3%, and 10% 2-mercaptobenzothiazole in DMF (Gerberick et al. 2005). The maximum SI was 8.6 at 10%. The LLNA: BrdU-ELISA test used the same vehicle and tested concentrations of 12.5%, 25%, 50% 2-mercaptobenzothiazole, which yielded SI values of 1.62, 1.36, and 1.49, respectively. An additional LLNA: BrdU-ELISA test of 2-mercaptobenzothiazole in DMSO that was submitted after the Panel review and OECD Expert Consultation on the LLNA indicates that testing with DMSO as the vehicle increases the response. The additional test used 10% and 25% 2-mercaptobenzothiazole, which produced SI values of 1.50 and 2.23, respectively (**Annex V**).

The EC3 value for imidazolidinyl urea in the traditional LLNA, 24%, was derived from a test of 10%, 25%, and 50% imidazolidinyl urea in DMF (Gerberick et al. 2005). The maximum SI was 5.5 at 50%. The LLNA: BrdU-ELISA test used the same vehicle and tested concentrations of 10% and 50% imidazolidinyl urea, which yielded SI values of 0.73 and 1.61, respectively. An additional LLNA: BrdU-ELISA test for imidazolidinyl urea that was submitted after the Panel review and OECD Expert Consultation on the LLNA suggests that testing at higher concentrations and/or using DMSO as the vehicle will increase the response. This test used 50% and 75% imidazolidinyl urea in DMSO and produced SI values of 1.65 and 2.27, respectively (**Annex V**).

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

In addition to the accuracy analysis using $SI \geq 2.0$ to classify substances as sensitizers, other decision criteria were evaluated for test method performance. The traditional LLNA served as the reference test. The performance characteristics for 15 different decision criteria for determining whether the skin sensitization potential for the substances were positive or negative are reported in this section. The substances evaluated were the 43 substances with both LLNA: BrdU-ELISA and traditional LLNA data discussed in **Section 6.1**. The decision criteria included:

1. SI values ≥ 1.3 , ≥ 1.5 , ≥ 1.6 , ≥ 1.9 , ≥ 2.0 , ≥ 2.5 , ≥ 3.0 , ≥ 3.5 , ≥ 4.0 , ≥ 4.5 , or ≥ 5.0
2. Statistically significant difference between any treatment group and the vehicle control group. Absorbance values of treated groups were compared with the vehicle control group using ANOVA with a post-hoc Dunnett's test when multiple treatment groups were tested, or Student's *t*-test when there was only one treatment group
3. Mean absorbance values of treated groups $\geq 95\%$ CI of the control group
4. Mean absorbance values of treated groups ≥ 2 SD or ≥ 3 SD from the control group mean

Multiple tests were available for 18 substances tested with the LLNA: BrdU-ELISA. The results for each of these substances in the same vehicle were combined so that each substance was represented by one sensitizer or nonsensitizer result for each criterion evaluated for the accuracy analysis. The results were combined in three ways, and a separate accuracy analysis was performed for each approach.

1. The sensitizer/nonsensitizer outcome for each substance was the most prevalent outcome for each criterion. For example, for the criterion for a statistical difference between control and treatment groups, two of the three lactic acid tests exhibited statistical differences between the control and treated groups (i.e., produced sensitizer results). Thus, the single outcome for lactic acid for the accuracy analysis was a sensitizer result. If the number of positive and negative outcomes were equal, the most conservative (i.e., positive) result was used for the accuracy analyses.
2. The positive/negative outcome for each substance at each criterion was determined by the outcome of the test with the highest maximum SI of the multiple tests.
3. The positive/negative outcome for each substance at each criterion was determined by the outcome of the test with the lowest maximum SI of the multiple tests.

The analysis presented here is based on using the most prevalent outcome for substances with multiple tests, as this is representative of the most likely outcome for a given chemical. The analyses using the highest maximum SI and the lowest maximum SI of the multiple tests for each substance are detailed in **Annex VI**.

As shown in **Section 6.1**, using the most prevalent outcome and the decision criterion of $SI \geq 2.0$ resulted in an accuracy of 95% (41/43), a sensitivity of 94% (30/32), a specificity of 100% (11/11), a false positive rate of 0% (0/11), and a false negative rate of 6% (2/32) (**Tables C-2 and C-7**). Using higher SI values (i.e., $SI \geq 3.0$ to $SI \geq 5.0$) as the decision criterion resulted in reduced accuracy and higher false negative rates but the same false positive rates as compared to $SI \geq 2.0$ (**Figure C-1 and Table C-7**). Using $SI \geq 1.9$ as the decision criterion produced the same performance statistics as $SI \geq 2.0$. Using a lower SI value, down to $SI \geq 1.5$, produced the same accuracy as $SI \geq 2.0$ (95% [41/43]), but the false positive rate increased to 18% (2/11), and the false negative rate decreased to 0% (0/32). $SI \geq 1.3$ is shown for comparison because it was previously recommended by ICCVAM but was considered to be inadequate by the March 2008 Peer Review Panel (ICCVAM 2008). Use of ANOVA and summary statistics (i.e., mean absorbance values of treated groups $\geq 95\%$ confidence interval of the control group, or ≥ 2 or 3 SD from the control group mean), yielded accuracy values of 9% to 93%, with false negative rates of 0% to 6%, and false positive rates of 9% to 36%.

The optimal criterion was considered $SI \geq 1.6$ because it produced no false negatives and the accuracy (95% [41/43]) was the highest accuracy produced by any of the criteria examined. Using the most prevalent outcome, $SI \geq 1.6$ was the highest SI criterion that yielded no false negatives (0/32). The lowest SI criterion that yielded no false positives (0/11) was $SI \geq 1.9$ (**Table C-7**). Analyses to determine the robustness of the optimum SI criterion showed that the optimal SI criterion was stable (**Annex VII**). Taking different samples of the data as training/validation sets had relatively little impact on the cutoff SI criterion or on the resulting number of false positives or false negatives.

Table C-7 Performance of the LLNA: BrdU-ELISA for 43 Substances in Predicting Skin-Sensitizing Potential Using Alternative Decision Criteria to Identify Sensitizers and the Most Prevalent Outcome for Substances with Multiple Tests

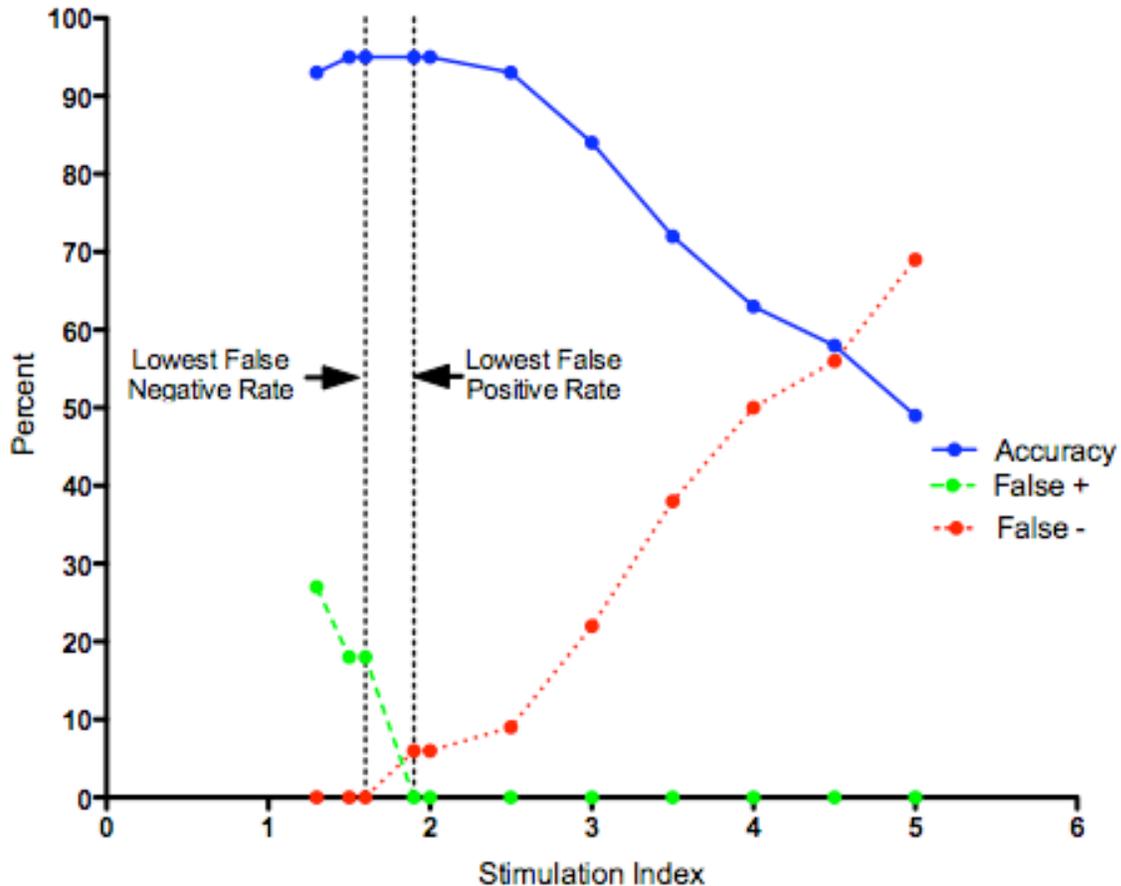
Alternative Criterion	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate		Positive Predictivity		Negative Predictivity	
	%	No. ¹	%	No. ¹	%	No. ¹	%	No. ¹	%	No. ¹	%	No. ¹	%	No. ¹
Statistics ²	91	39/43	97	31/32	73	8/11	27	3/11	3	1/32	91	31/34	89	8/9
≥95% CI ³	91	39/43	100	32/32	64	7/11	36	4/11	0	0/32	89	32/36	100	7/7
≥2 SD ⁴	93	40/43	100	32/32	73	8/11	27	3/11	0	0/32	91	32/35	100	8/8
≥3 SD ⁵	93	40/43	94	30/32	91	10/11	9	1/11	6	2/32	97	30/31	83	10/12
SI ≥ 5.0	49	21/43	31	10/32	100	11/11	0	0/11	69	22/32	100	10/10	33	11/33
SI ≥ 4.5	58	25/43	44	14/32	100	11/11	0	0/11	56	18/32	100	14/14	38	11/29
SI ≥ 4.0	63	27/43	50	16/32	100	11/11	0	0/11	50	16/32	100	16/16	41	11/27
SI ≥ 3.5	72	31/43	62	20/32	100	11/11	0	0/11	38	12/32	100	20/20	48	11/23
SI ≥ 3.0	84	36/43	78	25/32	100	11/11	0	0/11	22	7/32	100	25/25	61	11/18
SI ≥ 2.5	93	40/43	91	29/32	100	11/11	0	0/11	9	3/32	100	29/29	79	11/14
SI ≥ 2.0	95	41/43	94	30/32	100	11/11	0	0/11	6	2/32	100	30/30	85	11/13
SI ≥ 1.9	95	41/43	94	30/32	100	11/11	0	0/11	6	2/32	100	30/30	85	11/13
SI ≥ 1.6	95	41/43	100	32/32	82	9/11	18	2/11	0	0/32	94	30/32	100	9/9
SI ≥ 1.5	95	41/43	100	32/32	82	9/11	18	2/11	0	0/32	94	30/32	100	9/9
SI ≥ 1.3	93	40/43	100	32/32	73	8/11	27	3/11	0	0/32	91	32/35	100	8/8

Abbreviations: CI = confidence interval; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; No. = number; SD = standard deviation; SI = stimulation index.

¹ The proportion on which the percentage calculation is based.

- ² Analysis of variance for difference of group means when substances were tested at multiple doses or *t*-test when substances were tested at one dose. The absorbance data were log-transformed prior to analysis of variance. Significance at $p < 0.05$ was further tested by Dunnett's test.
- ³ The mean absorbance of at least one treatment group was outside the 95% confidence interval for the mean absorbance of the vehicle control group.
- ⁴ The mean absorbance of at least one treatment group was greater than 3 SD from the mean absorbance of the vehicle control group.
- ⁵ The mean absorbance of at least one treatment group was greater than 2 SD from the mean absorbance of the vehicle control group.

Figure C-1 Performance of the LLNA: BrdU-ELISA for 43 Substances with SI Compared to the Traditional LLNA Using the Most Prevalent Outcome for Substances with Multiple Tests



As compared to traditional LLNA results, the lines show the change in performance characteristics for the LLNA: BrdU-ELISA with the SI cutoff used to identify sensitizers. This analysis used LLNA: BrdU-ELISA and traditional LLNA results for 32 sensitizers and 11 nonsensitizers. For the 18 substances with multiple test results, the results for each substance were combined using the most prevalent outcome. The solid line shows accuracy, the dashed line shows the false positive rate, and the dotted line shows the false negative rate.

The optimum decision criterion of $SI \geq 1.6$ is compared with $SI \geq 2.0$ for accuracy of the LLNA: BrdU-ELISA against GP and human data in **Table C-8**. When GP test results were used as the reference data, $SI \geq 1.6$ had higher accuracy (89% [31/35]) for $SI \geq 1.6$ vs. 86% [30/35], lower false negative rate (0% [0/32] for $SI \geq 1.6$ vs. 9% [2/22]), and increased false positive rate (31% [4/13] for $SI \geq 1.6$ vs. 23% [3/13]) when compared with $SI \geq 2.0$. When results were compared to human data, $SI \geq 1.6$ produced the same accuracy (73% [30/41]), decreased the false negative rate (16% [5/31] for $SI \geq 1.6$ vs. 23% [7/31]), and increased the false positive rate (60% [6/10] for $SI \geq 1.6$ vs. 40% [4/10]) compared with $SI \geq 2.0$.

Table C-8 Comparison of Performance for Decision Criteria of SI \geq 1.6 (Bold) and SI \geq 2.0 for Predicting Skin Sensitizing Potential with LLNA: BrdU-ELISA

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-ELISA vs. Traditional LLNA	43	95 95	41/43 41/43	100 94	32/32 30/32	82 100	9/11 11/11	18 0	2/11 0/11	0 6	0/32 2/32	94 100	32/34 30/30	100 85	9/9 11/13
<i>Substances with LLNA: BrdU-ELISA, Traditional LLNA, and GP Data</i>															
LLNA: BrdU-ELISA vs. Traditional LLNA	35	97 94	34/35 33/35	100 92	25/25 23/25	90 100	9/10 10/10	10 0	1/10 0/10	0 8	0/25 2/25	96 100	25/26 23/23	100 83	9/9 10/12
LLNA: BrdU-ELISA vs. GP³	35	89 86	31/35 30/35	100 91	22/22 20/22	69 77	9/13 10/13	31 23	4/13 3/13	0 9	0/22 2/22	85 87	22/26 20/23	100 83	9/9 10/12
Traditional LLNA vs. GP³	35	91	32/35	100	22/22	77	10/13	23	3/13	0	0/22	88	22/25	100	10/10
<i>Substances with LLNA: BrdU-ELISA, Traditional LLNA, and Human Data</i>															
LLNA: BrdU-ELISA vs. Traditional LLNA	41	95 95	39/41 39/41	100 93	30/30 28/30	82 100	9/11 11/11	18 0	2/11 0/11	0 7	0/30 2/30	94 100	30/32 28/28	100 85	9/9 11/13
LLNA: BrdU-ELISA vs. Human⁴	41	73 73	30/41 30/41	84 77	26/31 24/31	40 60	4/10 6/10	60 40	6/10 4/10	16 23	5/31 7/31	81 86	26/32 24/28	44 46	5/9 6/13
Traditional LLNA vs. Human⁴	41	78	32/41	84	26/31	60	6/10	40	4/10	16	5/31	87	26/30	54	6/11

Abbreviations: GP = guinea pig skin sensitization outcomes; LLNA: BrdU-ELISA= murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; 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, the human repeat insult patch test, inclusion of the test substance in a human patch test allergen kit, and/or published clinical case studies/reports.

6.6 Discordant Results for Accuracy Analysis Using Alternative Decision Criteria

This section discusses the discordant results obtained for the analyses using the alternative decision criteria shown in **Tables C-7** and **C-8** to provide a comparison to the discordant substances identified using the decision criterion of $SI \geq 2.0$ to identify sensitizers. Discordant results are first discussed for the alternative decision criteria using the traditional LLNA as the reference test (**Section 6.6.1**). Then discordant results for $SI \geq 1.6$, the optimized criterion, are discussed using the traditional LLNA, GP, and human outcomes as references (**Section 6.6.2**).

6.6.1 Discordant Results Using Alternative Decision Criteria Compared with the Traditional LLNA

Using decision criteria of $SI \geq 2.0$ and the most prevalent outcome for the substances with multiple tests, the two discordant substances, when compared to the traditional LLNA, were imidazolidinyl urea and 2-mercaptobenzothiazole (**Table C-5**). As indicated in **Section 6.4**, both substances were false negatives when compared to the traditional LLNA.

Table C-9 shows how the number and identity of discordant substances changes with the alternative decision criteria when using the most prevalent outcome for the substances with multiple tests. Use of a statistical test (i.e., ANOVA or *t*-test; “Statistics” in **Table C-7**) or summary statistics (i.e., $\geq 95\%$ CI, or ≥ 2 or 3 SD in **Table C-7**) did not result in substantively improved performance relative to using $SI \geq 1.6$. $SI \geq 1.3$ is shown for comparison because it was previously recommended by ICCVAM. It is not discussed because it was considered to be inadequate by the March 2008 Peer Review Panel (ICCVAM 2008).

Table C-9 Discordant Results for LLNA: BrdU-ELISA Using Alternative Decision Criteria Compared to the Traditional LLNA and the Most Prevalent Outcome for Substances with Multiple Tests

Discordant Substance ¹	Alternative Decision Criterion ²														
	Statistics ³	≥95% CI ⁴	≥2 SD ⁵	≥3 SD ⁶	SI ≥ 5.0	SI ≥ 4.5	SI ≥ 4.0	SI ≥ 3.5	SI ≥ 3.0	SI ≥ 2.5	SI ≥ 2.0	SI ≥ 1.9	SI ≥ 1.6	SI ≥ 1.5	SI ≥ 1.3
5-Chloro-2-methyl-4-isothiazolin-3-one solution (0.009%)					-										
Formaldehyde (0.50%)					-	-									
Cobalt chloride (0.6%)					-	-	-								
4-Methylaminophenol sulfate (0.8%)					-	-	-								
<i>trans</i> -Cinnamic aldehyde (1.4%)					-	-	-								
2-Mercaptobenzothiazole (1.7%)	-				-	-	-	-	-	-	-	-			
Cinnamic aldehyde (1.9%)					-	-	-								
3-Aminophenol (3.2%)					-	-	-	-							
Diethyl maleate (3.6%)					-	-	-	-							
Nickel sulfate (4.8%)					-	-	-	-	-						
4-Chloroaniline (6.5%)					-	-	-	-	-						
Sodium lauryl sulfate (8.1%)					-	-	-	-	-						
Hexyl cinnamic aldehyde (9.7%)					-	-	-	-							
Eugenol (10.1%)					-	-	-	-							
Phenyl benzoate (13.6%)					-	-	-	-							

continued

Table C-9 Discordant Results for LLNA: BrdU-ELISA Using Alternative Decision Criteria Compared to the Traditional LLNA and the Most Prevalent Outcome for Substances with Multiple Tests (continued)

Discordant Substance ¹	Alternate Decision Criterion ²														
	Statistics ³	≥95% CI ⁴	≥2 SD ⁵	≥3 SD ⁶	SI ≥ 5.0	SI ≥ 4.5	SI ≥ 4.0	SI ≥ 3.5	SI ≥ 3.0	SI ≥ 2.5	SI ≥ 2.0	SI ≥ 1.9	SI ≥ 1.6	SI ≥ 1.5	SI ≥ 1.3
Cinnamic alcohol (21.0%)				-	-	-	-	-	-						
Hydroxycitronellal (24.0%)					-										
Imidazolidinyl urea (24.0%)				-	-	-	-	-	-	-	-	-			
Ethylene glycol dimethacrylate (28.0%)					-	-	-	-							
Linalool (30.0%)					-										
Ethyl acrylate (32.8%)					-										
Isopropyl myristate (44.0%)					-	-									
Aniline (47.5%)					-	-	-	-	-	-					
Glycerol (-)	+	+	+												
Hexane (-)	+	+	+	+									+	+	+
Lactic acid (-)	+	+	+										+	+	+
Methyl salicylate (-)		+													+

Abbreviations: CI = confidence interval; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SD = standard deviation; SI = stimulation index.

¹ Compared to the traditional LLNA outcome. Traditional LLNA result in parentheses: “-” for nonsensitizers and EC3 values (%) for sensitizers.

² LLNA: BrdU-ELISA outcomes are indicated by “+” for sensitizer results and “-” for nonsensitizer results.

³ Analysis of variance for difference of group means when substances were tested at multiple doses or *t*-test when substances were tested at one dose. The absorbance data were log-transformed prior to analysis of variance. Significance at *p* < 0.05 was further tested by Dunnett’s test.

⁴ The mean absorbance of at least one treatment group was outside the 95% confidence interval for the mean absorbance of the vehicle control group.

⁵ The mean absorbance of at least one treatment group was greater than 3 SD from the mean absorbance of the vehicle control group.

⁶ The mean absorbance of at least one treatment group was greater than 2 SD from the mean absorbance of the vehicle control group.

Ten of the ICCVAM performance standards required reference substances were discordant for the analysis of alternative decision criteria using the most prevalent outcome for substances with multiple tests (**Table C-7**). Eight sensitizers (5-chloro-2-methyl-4-isothiazolin-3-one, cobalt chloride, 2-mercaptobenzothiazole, hexyl cinnamic aldehyde, eugenol, phenyl benzoate, cinnamic alcohol, and imidazolidinyl urea) were misclassified by some criteria as nonsensitizers, and two nonsensitizers (lactic acid and methyl salicylate) were misclassified as sensitizers by some criteria. The criteria that yielded the correct results for most of the sensitizers included summary statistics (i.e., $\geq 95\%$ CI, ≥ 2 SD, or ≥ 3 SD), statistical tests (i.e., ANOVA or *t*-test), and $SI \geq 3.0$ to ≥ 1.6 . The exceptions were:

- 2-mercaptobenzothiazole, which was incorrectly negative by a statistical test (i.e., ANOVA) and at $SI \geq 5.0$ to ≥ 1.9 .
- Cinnamic alcohol, which was incorrectly negative at $SI \geq 3.0$.
- 5-chloro-2-methyl-4-isothiazolin-3-one, which was also correctly positive at $SI \geq 4.5$ to ≥ 3.5 .
- Cobalt chloride, which was also correctly positive at $SI \geq 3.5$.

The criteria that yielded the correct results for the nonsensitizers were generally SI criterion greater than 1.9. For lactic acid, the criteria that yielded the correct results included treatment group mean ≥ 3 SD from the vehicle control, and $SI \geq 5.0$ to 1.9. All criteria yielded the correct results for methyl salicylate except for treatment group absorbance $\geq 95\%$ CI of vehicle control mean.

6.6.2 Discordant Results for Accuracy Analysis of the $SI \geq 1.6$ Decision Criterion

When the outcomes for the 43 substances tested in the LLNA: BrdU-ELISA (using $SI \geq 1.6$) and the traditional LLNA were compared, the classifications for two substances were different. Hexane and lactic acid, nonsensitizers in the traditional LLNA, were misclassified as sensitizers in the LLNA: BrdU-ELISA. In the LLNA: BrdU-ELISA and the traditional LLNA, hexane was tested in AOO and lactic acid was tested in DMSO. Chemical class, physical form, MW, peptide reactivity (see **Annex II** for physicochemical properties), and potential for skin irritation were examined to identify commonalities among the discordant substances. Hexane is a hydrocarbon, and lactic acid is a carboxylic acid. Both substances are liquids and have low MW (hexane MW = 86.18 g/mol and lactic acid MW = 90.08 g/mol) and minimal peptide reactivity. Both substances were tested in the LLNA: BrdU-ELISA at concentrations expected to produce skin irritation based on data in humans (hexane) or rabbits (lactic acid).

When the outcomes for the 35 substances tested in the LLNA: BrdU-ELISA (using $SI \geq 1.6$) and GP tests were compared, the classifications for four substances were different. Ethyl acrylate, ethylene glycol dimethacrylate, lactic acid, and SLS were classified as nonsensitizers in GP tests but were misclassified as sensitizers in the LLNA: BrdU-ELISA. The LLNA: BrdU-ELISA test result was concordant with the traditional LLNA for three of the four substances (i.e., all except lactic acid) (**Table C-10**). Chemical class, physical form, MW, peptide reactivity (see **Annex II** for physicochemical properties), and potential for skin irritation were examined to identify the following commonalities among the discordant substances:

- Ethyl acrylate, ethylene glycol dimethacrylate, and lactic acid are carboxylic acids; SLS is an alcohol, lipid, and sulfur compound.
- MWs range from 90.08 to 288.38 g/mol.
- Ethyl acrylate, ethylene glycol dimethacrylate, and lactic acid are liquids; SLS is a solid.
- Peptide reactivity for ethylene glycol dimethacrylate is high; peptide reactivity for lactic acid is minimal; peptide reactivity data for ethyl acrylate and SLS are not available.

- Ethyl acrylate (EC3 = 32.8%) and ethylene glycol dimethacrylate (EC3 = 28%) are weak sensitizers in the traditional LLNA; SLS (EC3 = 8.1%) is somewhat stronger. Lactic acid is a nonsensitizer in the traditional LLNA.
- Ethyl acrylate, ethylene glycol dimethacrylate, lactic acid, and SLS were tested at irritating concentrations in the LLNA: BrdU-ELISA, based on skin irritation data from guinea pigs, rabbits, mice, or humans.

Table C-10 Discordant Results for LLNA: BrdU-ELISA (SI ≥ 1.6) Compared to Traditional LLNA and Guinea Pig Reference Data¹

Substance Name ²	Vehicle ³	LLNA: BrdU-ELISA ⁴	Traditional LLNA ⁴	Guinea Pig Studies	Skin Irritant?
Ethyl acrylate (32.8%)	AOO	+ (4.95, 100%)	+ (4.0, 50%)	-	Nonirritant at 3% (GP)
Ethylene glycol dimethacrylate (28.0%)	MEK	+ (3.11, 100%)	+ (7.0, 50%)	-	Nonirritant at 1% (GP)
Lactic acid	DMSO	+ (1.80, 1.89, and 2.53, 50%)	- (2.2, 25%)	-	Slightly irritating at 10% (rabbits)
Sodium lauryl sulfate (8.1%)	DMF	+ (2.64, 16.7%)	+ (8.9, 20%) ⁵	-	Irritant at 20% aq. (rabbits); Irritant at 20% (humans); Irritant at 10% in DMF (mice)

Abbreviations: AOO = acetone: olive oil (4:1); aq = aqueous; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; GP = guinea pigs; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; LLNA = murine local lymph node assay; MEK = methyl ethyl ketone; NA = not available; SI = stimulation index.

+ = sensitizer.

- = nonsensitizer.

¹ Data sources provided in **Annex III-1**.

² Numbers in parentheses are the EC3 values (estimated concentration needed to produce a stimulation index [SI] of 3) for the traditional LLNA (from **Table C-1**).

³ Vehicles apply to tests for both the LLNA: BrdU-ELISA and the traditional LLNA.

⁴ Numbers in parentheses are highest SI values and maximum concentrations tested.

⁵ Highest SI occurred at 10%.

When the outcomes for the 41 substances with LLNA: BrdU-ELISA (using SI ≥ 1.6) and human outcome data were compared, the classifications for 11 substances were different (**Table C-11**). The LLNA: BrdU-ELISA results for two of these substances (hexane and lactic acid) were discordant with the traditional LLNA. The LLNA: BrdU-ELISA classified five human sensitizers as nonsensitizers (diethyl phthalate, 2-hydroxypropylmethacrylate, isopropanol, propylene glycol, and sulfanilamide) and six human nonsensitizers as sensitizers (hexane, lactic acid, isopropyl myristate, cyclamen aldehyde, linalool, and SLS).

Table C-11 Discordant Results for LLNA: BrdU-ELISA (SI ≥ 1.6) When Compared to Traditional LLNA and Human Outcome Data¹

Substance Name ²	Vehicle ³	LLNA: BrdU-ELISA ⁴	Traditional LLNA ⁴	Human Outcome ⁵	Skin Irritant?
Diethyl phthalate	AOO	- (0.88, 50%)	- (1.5, 100%)	+ (HPTA)	Nonirritant at ≤ 100% (rabbits)
2-Hydroxypropylmethacrylate	AOO	- (1.13, 50%)	- (1.3, 50%)	+ (case study, 0.1%)	Nonirritant at ≤ 10% (GP)
Isopropanol	AOO	- (2.22, 50%) ⁶	- (1.7, 50%) ⁷	+ (case study, 0.001%)	Nonirritant at ≤ 100% (rabbits)
Propylene glycol	AOO ⁸	- (1.57, 50%)	- (1.6, 100%)	+ (HPTA)	Nonirritant at ≤ 25% (humans)
Sulfanilamide	DMF	- (1.26, 50%)	- (1.0, 50%) ⁹	+ (5/25, 25%)	Nonirritant at ≤ 25% (humans)
Hexane	AOO	+ (1.76, 100%) ¹⁰	- (2.2, 100%)	- (0/25, 100%)	Irritant at 100% (humans)
Lactic acid	DMSO	+ (1.80, 1.89, and 2.53, 100%)	- (2.5, 25%)	- (no data located) ¹¹	Slightly irritating at ≤ 10% (rabbits)
Isopropyl myristate (44.0%)	AOO	+ (4.20, 50%)	+ (3.4, 100%)	- (0/25, 20%)	Nonirritant at ≤ 100% (rabbits)
Cyclamen aldehyde (22.3%)	AOO	+ (1.97 and 5.71, 100%)	+ (5.2, 50%)	- (0/64, 4%)	Irritant at 100% (rabbits)
Linalool (30.0%)	AOO	+ (1.45 and 4.65, 100%) ¹²	+ (8.3, 100%)	- (0/25, 8%)	Irritant at 100% (rabbits)
Sodium lauryl sulfate (8.1%)	DMF	+ (3.4, 10%)	+ (8.9, 20%) ¹³	- (0/22 at 10%)	Irritant at 20% aq. (rabbits); Irritant at 20% (humans); Irritant at 10% in DMF (mice)

Abbreviations: AOO = acetone: olive oil (4:1); aq = aqueous; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; GP = guinea pigs; HPTA = human patch test allergen; LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SI = stimulation index.

+ = sensitizer.

- = nonsensitizer.

¹ Data sources provided in **Annex III-1**.

² Numbers in parentheses are EC3 values (estimated concentration needed to produce a stimulation index [SI] of 3) for substances that are sensitizers in the traditional LLNA; from **Table C-1**.

³ Vehicles apply to tests for both the LLNA: BrdU-ELISA and the traditional LLNA unless otherwise noted.

⁴ Numbers in parentheses are highest SI values and maximum concentrations tested.

⁵ Information in parentheses indicates the basis for the human outcome. Numbers indicate the incidence of positive human response and concentration tested.

⁶ Negative based on most prevalent call. Highest SI of any test is shown. Highest SI values for most tests occurred at <50%.

⁷ Highest SI occurred at 10%.

⁸ The vehicle for the traditional LLNA was distilled water.

⁹ Highest SI occurred at 10% and 25%.

¹⁰ An additional test yielded SI = 1.89 at 50%.

¹¹ Presumed to be a nonsensitizer in humans because no clinical patch test results were located, it is not a patch test kit allergen, and no case reports of human sensitization were located.

¹² When the number of positive and negative outcomes were equal for a substance, the most conservative result was used in the accuracy analysis (see **Section 6.5**).

¹³ Highest SI occurred at 10% and 25%.

Few commonalities in chemical class, physical form, MW, peptide reactivity, traditional LLNA range of EC3 values, and potential for skin irritation were noted among the discordant substances. For the five human sensitizers that were misclassified as nonsensitizers:

- Four different chemical classes were represented: carboxylic acids (diethyl phthalate and 2-hydroxypropylmethacrylate), alcohols (isopropanol and propylene glycol), sulfur compounds (sulfanilamide) and cyclic hydrocarbons (sulfanilamide) (**Tables C-1 and C-11**).
- Three substances were liquids (diethyl phthalate, isopropanol, and propylene glycol), and two were solids (2-hydroxypropylmethacrylate and sulfanilamide).
- MWs ranged from 60.10 (isopropanol) to 222.24 g/mole (diethyl phthalate).
- Four substances exhibited low peptide reactivity; no peptide reactivity information was available for sulfanilamide.
- All five substances were also classified as nonsensitizers by the traditional LLNA.
- Although 2-hydroxypropylmethacrylate, propylene glycol, and sulfanilamide are skin irritants at the concentrations tested in the LLNA: BrdU-ELISA (based on data from humans, rabbits, or guinea pigs), the other two substances were not irritating to skin at the concentrations tested (**Table C-11**).

There were few commonalities in chemical class, physical form, MW, peptide reactivity, range of EC3 values (based on the traditional LLNA), and potential for skin irritation noted among the six human nonsensitizers that were misclassified as sensitizers by the LLNA: BrdU-ELISA:

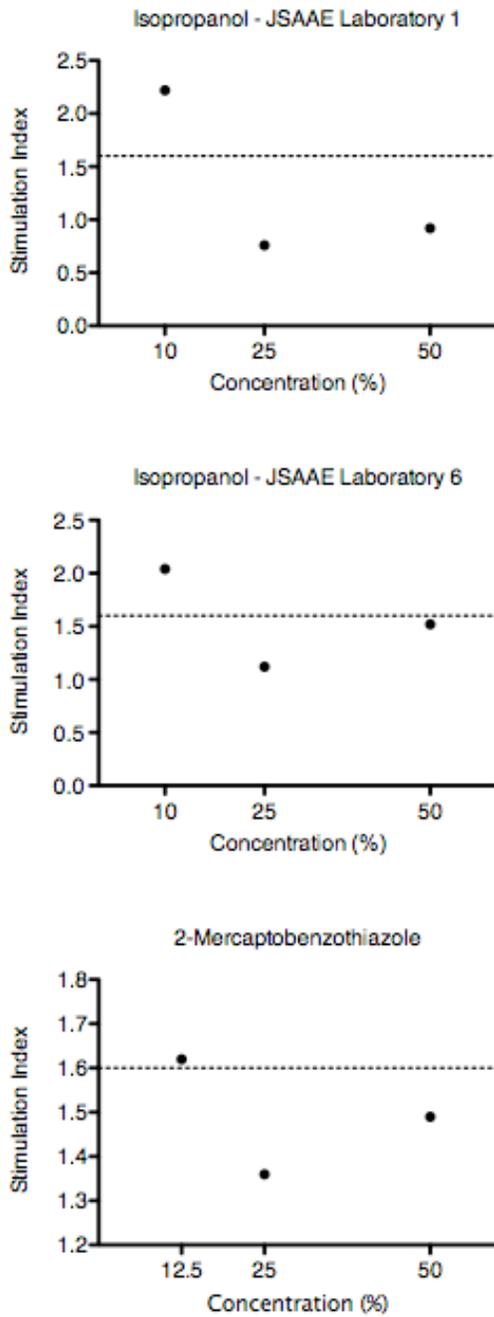
- The six substances represented six different chemical classes: carboxylic acids (cyclamen aldehyde and lactic acid), lipids (isopropyl myristate and SLS), acyclic hydrocarbons (hexane), sulfur compounds (SLS), alcohols (SLS), and hydrocarbons, other (linalool) (**Tables C-1 and C-11**).
- Five substances are liquids, and SLS is a solid.
- Four substances have minimal to low peptide reactivity, but no peptide reactivity data are available for linalool or SLS.
- MWs ranged from 86.15 g/mole for hexane to 288.38 g/mole for SLS.

- Isopropyl myristate, cyclamen aldehyde, linalool, and SLS were also classified as sensitizers by the traditional LLNA (EC3 values were 44.0%, 22.3%, 30.0%, and 8.1%, respectively); but hexane and lactic acid were classified as nonsensitizers by the traditional LLNA.
- Five of the substances misclassified as sensitizers (hexane, lactic acid, cyclamen aldehyde, linalool, and SLS) were tested at concentrations that are irritating to skin, but one was not (isopropyl myristate), based on skin irritation data from humans, mice, or rabbits (**Table C-11**).

6.7 Accuracy Analysis for the Reduced LLNA: BrdU-ELISA (rLLNA: BrdU-ELISA)

An accuracy analysis for the rLLNA: BrdU-ELISA was performed using the optimized $SI \geq 1.6$ criterion to identify sensitizers. The rLLNA: BrdU-ELISA uses only the highest soluble dose of the test substance that does not produce local skin irritation or systemic toxicity; the two lower dose groups are not used. The available validation database for the rLLNA: BrdU-ELISA analysis included 85 individual tests that used multiple doses. The performance of the rLLNA: BrdU-ELISA was evaluating by comparing the outcome of the highest dose for each test to the outcome of the same test when considering all doses tested. Using $SI \geq 1.6$ to identify sensitizers, the accuracy of the rLLNA: BrdU-ELISA was 95% (82/85), with a false positive rate of 0% (0/11) and a false negative rate of 4% (3/74). The three tests that were false negative in the rLLNA: BrdU-ELISA were weakly positive in the multiple-dose LLNA: BrdU-ELISA. Two tests of 10%, 25%, and 50% isopropanol produced maximum SI values of 2.04 and 2.22 at the lowest dose tested (**Figure C-2**). The third false negative was the test of 12.5%, 25%, and 50% 2-mercaptobenzothiazole, which produced the maximum SI of 1.62 at the lowest dose tested (**Figure C-2**).

Figure C-2 Dose-Response Curves for Substances Identified as Nonsensitizers by the rLLNA: BrdU-ELISA and Sensitizers by the LLNA: BrdU-ELISA



The horizontal dashed line shows the stimulation index of 1.6, which is the threshold for a positive response. Points above the line indicate sensitizer responses and points below the line indicate nonsensitizer responses.

Abbreviations: JSAAE = Japanese Society for Alternatives to Animal Experiments.

6.8 Accuracy Analysis Using Multiple Alternative Decision Criteria

As detailed in **Section 6.5**, the accuracy of the LLNA: BrdU-ELISA when using a number of alternative decision criteria was evaluated using the traditional LLNA as the reference test. Using the database of 31 substances that was available for the Panel review in April 2009, **Annex VII** provides an accuracy and reproducibility analysis that uses two SI decision criteria: one to identify sensitizers and another to identify nonsensitizers. The lowest SI decision criterion that produced no false positives was used to identify sensitizers, and the SI decision criterion that produced no false negatives was used to identify nonsensitizers. **Annex VII** also includes an evaluation of additional information that could be used in an integrated decision strategy for classifying indeterminate substances and an analysis of the effect of sample size on the indeterminate range of SI values.

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.

The available LLNA: BrdU-ELISA data were amenable to both intralaboratory and interlaboratory reproducibility analyses. This section provides an assessment of reproducibility for the decision criterion of $SI \geq 1.6$ to identify sensitizers. In **Section 6.5**, this criterion was identified as the optimum criterion for producing no false negatives and minimal false positives, compared with the traditional LLNA. **Annex IX** describes the evaluation of reproducibility for additional decision criteria to identify sensitizers that were evaluated in **Section 6.5**: $SI \geq 1.5$ and $SI \geq 2.0$ (used in the JSAAE interlaboratory validation study).

7.1 Intralaboratory Reproducibility

The test results for the LLNA: BrdU-ELISA were amenable to intralaboratory reproducibility analyses for three endpoints: sensitizer or nonsensitizer classification, SI values, and EC1.6 values. Analyses of intralaboratory reproducibility were performed using a concordance analysis for the qualitative results (sensitizer vs. nonsensitizer) (**Section 7.1.1**) and a coefficient of variation (CV) analysis for the quantitative results (SI values and EC1.6 values) (**Sections 7.1.2** and **7.1.3**, respectively).

7.1.1 Intralaboratory Reproducibility – Qualitative Results

The dataset available for an intralaboratory concordance analysis of the qualitative test results for the LLNA: BrdU-ELISA included 12 substances that were tested multiple times by Takeyoshi et al. (2003, 2004a, 2005, 2006, 2007a, 2007b, unpublished). Hexyl cinnamic aldehyde and eugenol were tested six times; isoeugenol, diphenylcyclopropenone, and propylene glycol were tested three times; and cyclamen aldehyde, 2,4-dinitrochlorobenzene, glutaraldehyde, hexane, hydroxycitronellal, linalool, and 4-phenylenediamine were each tested twice (Takeyoshi et al. 2003, 2004a, 2005, 2006, 2007a, unpublished) (**Table C-12**). All substances were sensitizers in the traditional LLNA except for propylene glycol and hexane. The multiple test results for 10/12 substances were 100% concordant when $SI \geq 1.6$ was used to classify substances as sensitizers. However, the concordant tests for one nonsensitizer, hexane, were incorrectly positive. The substances with discordant results were the sensitizers hydroxycitronellal and linalool, which produced one positive ($SI \geq 1.6$) and one negative ($SI < 1.6$) result in the LLNA: BrdU-ELISA.

By comparison, 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. Intralaboratory results for each substance were 100% concordant with the exception of benzocaine. One of the six benzocaine (5/6 or 83% concordance) results for the traditional LLNA was reported as equivocal because SI increased with dose but did not reach the criterion of $SI \geq 3.0$. Thus, the proportion of substances for which intralaboratory concordance of qualitative results was 100% was identical for LLNA: BrdU-ELISA (10/12) and the traditional LLNA (5/6).

Table C-12 Intralaboratory Reproducibility for the LLNA: BrdU-ELISA Outcome of Substances Tested Multiple Times

Substance Name	Highest Concentration Tested (%)	Highest SI	Outcome ¹	Takeyoshi et al. Reference
Cyclamen aldehyde	100	1.97	+	2007b
	100	5.71	+	Unpublished
2,4-Dinitrochlorobenzene	2	17.90	+	2005
	2	6.84	+	2006, 2007b
Diphenylcyclopropanone	2	19.10	+	2005; 2007b
	10	9.34	+	2005
	10	11.62	+	2007b
Eugenol	10	3.18	+	2003
	30	3.30	+	2004a
	30	3.83	+	2007a
	50	12.30	+	2005
	50	3.10	+	2006
	50	17.70	+	2007b
Glutaraldehyde	2	14.60	+	2005, 2007b
	10	15.50	+	2005, 2007b
Hexane	50	1.89	+	2005
	100	1.76	+	Unpublished
Hexyl cinnamic aldehyde	25	2.41	+	2003
	50	3.60	+	2003
	50	5.90	+	2005
	50	3.64	+	2006
	50	2.72	+	2006
	50	3.02	+	2007b
Hydroxycitronellal	100	1.34	-	2007b
	100	4.78	+	Unpublished
Isoeugenol	10	8.40	+	2005
	10	2.40	+	2006, 2007b
	30	6.73	+	2007a
Linalool	100	1.45	-	Unpublished
	100	4.65	+	Unpublished

continued

Table C-12 Intralaboratory Reproducibility for the LLNA: BrdU-ELISA Outcome of Substances Tested Multiple Times (continued)

Substance Name	Highest Concentration Tested (%)	Highest SI	Outcome ¹	Takeyoshi et al. Reference
4-Phenylenediamine	2	11.70	+	2005, 2007b
	10	14.70	+	2005, 2007b
Propylene glycol	10	1.20	-	2005
	50	1.57	-	2005
	50	0.91	-	2006, 2007b

Abbreviations: LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SI = stimulation index.

¹ (+) = sensitizer; (-) = nonsensitizer.

7.1.2 Intralaboratory Reproducibility – SI

Ten substances were tested multiple times by Takeyoshi et al. (2003, 2004a, 2005, 2006, 2007a, 2007b, unpublished data) at the same concentrations. Because two substances had multiple tests for more than one concentration, there were 13 substance/concentration combinations that were tested two to five times in separate experiments. The multiple SI values for each substance/concentration were used to calculate a CV for the assessment of intralaboratory variability. As shown by **Table C-13**, the CV values ranged from 1% (25% hexyl cinnamic aldehyde) to 80% (100% hydroxycitronellal and 10% isoeugenol). There are no data for comparison with the traditional LLNA because the intralaboratory reproducibility of the traditional LLNA was not assessed by CV analysis of SI values (ICCVAM 1999).

7.1.3 Intralaboratory Reproducibility – EC1.6 Values

CV values were also calculated for the EC1.6 values for the four sensitizers that were tested more than once using multiple doses by Takeyoshi et al. (2003, 2004a, 2005, 2006, 2007a, 2007b) and repeatedly yielded positive LLNA: BrdU-ELISA results. The individual animal data for cyclamen aldehyde, eugenol, hexyl cinnamic aldehyde, and isoeugenol were used to calculate EC1.6 values for the LLNA: BrdU-ELISA. The methods for calculating EC1.6 values for each sensitizer were modified from those used by Ryan et al. (2007) to calculate EC3 values. Linear interpolation was used to calculate EC1.6 values for each test with SI values higher or lower than 1.6, and extrapolation was used to calculate EC1.6 values for tests with no SI values below 1.6. The equation for linear interpolation was:

$$EC1.6 = c + \left[\frac{(1.6 - d)}{(b - d)} \right] \times (a - c)$$

The linear interpolation equation uses the points immediately above and below SI = 1.6, with the (dose, SI) coordinates of (a, b) immediately above SI = 1.6 and (c, d) immediately below SI = 1.6. The equation for extrapolation was:

$$EC1.6_{ex} = 2^{\left\{ \log_2(c) + \frac{(1.6 - d)}{(b - d)} \times [\log_2(a) - \log_2(c)] \right\}}$$

Table C-13 Intralaboratory Reproducibility for the SI of Substances Tested in LLNA: BrdU-ELISA – Coefficient of Variation

Substance Name	Concentration Tested (%)	SI	Mean	SD	CV (%)	Takeyoshi et al. Reference
Cyclamen aldehyde	100	1.97	3.84	2.64	69	2007b
		5.71				Unpublished
2,4-Dinitrochlorobenzene	2	17.86	12.35	7.79	63	2005
		6.84				2006, 2007b
Diphenylcyclopropenone	10	9.34	10.48	1.61	15	2005; 2007b
		11.62				2007b
Eugenol	30	3.33	3.58	0.35	10	2004a
		3.83				2007a
Eugenol	50	12.28	11.01	7.40	67	2005
		3.05				2006
		17.69				2007b
Hexane	50	1.89	1.64	0.36	22	2005
		1.38				Unpublished
Hexyl cinnamic aldehyde	12.5	1.88	1.74	0.21	12	2003
		1.59				2003
Hexyl cinnamic aldehyde	25	2.44	2.42	0.02	1	2003
		2.41				2003
Hexyl cinnamic aldehyde	50	3.64	3.78	1.25	33	2003
		5.90				2005
		3.64				2006
		2.72				2006
		3.02				2007b
Hydroxycitronellal	100	1.34	3.06	2.43	80	2007b
		4.78				Unpublished
Isoeugenol	10	8.36	5.09	3.15	80	2005
		7.20				2005
		2.36				2006, 2007b
		2.43				2007a
Linalool	100	1.45	3.05	2.26	74	Unpublished
		4.65				Unpublished

continued

Table C-13 Intralaboratory Reproducibility for the SI of Substances Tested in LLNA: BrdU-ELISA – Coefficient of Variation (continued)

Substance Name	Concentration Tested (%)	SI	Mean	SD	CV (%)	Takeyoshi et al. Reference
Propylene glycol	50	1.57	1.14	0.62	54	2005
		0.70				2006, 2007b

Abbreviations: CV = coefficient of variation; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SD = standard deviation; SI = stimulation index.

The extrapolation equation uses the two points immediately above SI = 1.6, with the coordinates of (a, b) for the point closest to SI = 1.6, and (c, d) for the higher point. As shown in **Table C-14**, there were five EC1.6 values for hexyl cinnamic aldehyde, four EC1.6 values for eugenol, and two EC1.6 values for cyclamen aldehyde and isoeugenol. The CV values were 118% for cyclamen aldehyde, 67% for eugenol, 37% for hexyl cinnamic aldehyde, and 42% for isoeugenol. The ICCVAM LLNA *Performance Standards* criteria for demonstrating adequate intralaboratory reproducibility is based on results from at least four independent tests of hexyl cinnamic aldehyde (ICCVAM 2009).

Intralaboratory reproducibility is considered adequate when each test yields an EC_t value (i.e., the estimated concentration needed to produce an SI of a specific threshold value; in this case, SI = 1.6) within 5% to 20% (ICCVAM 2009). All of the five EC1.6 values for hexyl cinnamic aldehyde were within the acceptable range for intralaboratory reproducibility.

Table C-14 Intralaboratory Reproducibility for the EC1.6 Values of Substances Tested in LLNA: BrdU-ELISA - Coefficient of Variation

Substance Name	EC1.6 (%)	Mean	SD	CV (%)	Takeyoshi et al. Reference
Cyclamen aldehyde	76.0	41.5	48.8	118	2007b
	7.0				Unpublished
Eugenol	7.0	8.2	5.5	67	2004a
	13.5				2006
	1.1				2007b
	11.2				2007a
Hexyl cinnamic aldehyde	6.3	14.5	5.4	37	2003
	12.7				2003
	18.7				2006
	19.6				2006
	15.5				2007b
Isoeugenol	6.7	5.2	2.2	42	2006; 2007b
	3.6				2007a

Abbreviations: CV = coefficient of variation; EC1.6 = estimated concentration needed to produce a stimulation index of 1.6; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; 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 C-15).

Table C-15 Intralaboratory Reproducibility for the EC3 Values of Substances Tested in the Traditional LLNA¹

Substance Name	Number of Laboratories	Number of Tests per Laboratory	CV (%)
2, 4-Dinitrochlorobenzene	5	2	13-47
Isoeugenol	1	5	26
Hexyl cinnamic aldehyde	2	6	19-27
Eugenol	1	5	18

Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of 3; LLNA = murine local lymph node assay.

¹From ICCVAM (1999).

The intralaboratory CV values for the EC1.6 values from LLNA: BrdU-ELISA tests were higher than EC3 values for the same substances from the traditional LLNA reported in ICCVAM (1999). The intralaboratory EC1.6 CV value from the LLNA: BrdU-ELISA tests of eugenol was higher than that reported by ICCVAM (1999) (67% vs. 18%). The intralaboratory EC1.6 CV value from the LLNA: BrdU-ELISA tests of isoeugenol was greater than that from ICCVAM (1999) (42% vs. 26%). The intralaboratory EC1.6 CV value for hexyl cinnamic aldehyde was greater than those from ICCVAM (1999) (37% vs. 19% to 27%).

7.2 Interlaboratory Reproducibility

The interlaboratory reproducibility of the LLNA: BrdU-ELISA was assessed using the individual animal data from the multilaboratory validation study organized by the JSAAE (Kojima et al. 2008). Phase I of the study evaluated the reliability and transferability of the test method protocol by testing 12 substances in three to nine laboratories. With the exception of the positive control data, neither the summary results nor the individual animal data from Phase I of the validation study have been released. Phase II of the study tested 10 substances in three to seven laboratories as shown in **Table C-16**. All the laboratories that participated in the validation study used the same experimental protocol (**Annex I**) and participated in a 1-day seminar that explained the protocol and execution of the test method. The same commercial ELISA kit, test materials, and the same doses of the test substances were used in all of the laboratories. The Validation Management Team determined the doses and vehicles for testing and coded the identity of the test substances prior to distribution to the test laboratories. Seven substances were sensitizers and three substances were nonsensitizers according to the traditional LLNA. Six substances were ICCVAM *Recommended Performance Standards* reference substances: 2,4-dinitrochlorobenzene, eugenol, hexyl cinnamic aldehyde, lactic acid, isopropanol, and methyl salicylate (ICCVAM 2009).

Table C-16 Substances and Test Allocation for the Phase II Interlaboratory Validation Study of the LLNA: BrdU-ELISA

Substance Name ¹	Vehicle	Concentrations Tested			Laboratory ²						
					1	2	3	4	5	6	7
Nickel sulfate (+)	DMSO	1%	3%	10%			X	X			X
Isopropanol (-)	AOO	10%	25%	50%	X	X	X	X	X	X	X
Eugenol (+)	AOO	10%	25%	50%		X				X	X
Cinnamic aldehyde (+)	AOO	1%	3%	10%		X		X	X		
2,4-Dinitrochlorobenzene (+)	AOO	0.1%	0.3%	1%	X	X	X	X	X	X	X
Glutaraldehyde (+)	ACE	0.1%	0.3%	1%	X				X	X	
Methyl salicylate (-)	AOO	10%	25%	50%	X	X	X				
Hexyl cinnamic aldehyde (+)	AOO	10%	25%	50%	X	X	X	X	X	X	X
Lactic acid (-)	DMSO	10%	25%	50%			X	X			X
Formaldehyde (+)	ACE	1%	3%	10%	X				X	X	

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine.

¹ (+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.

² X indicates that a substance was tested in a particular laboratory: 1 = Daicel Chemical Industries Ltd.; 2 = Food and Drug Safety Center; 3 = Otsuka Pharmaceutical Co. Ltd.; 4 = Taisho Pharmaceutical Co. Ltd.; 5 = Fuji Film Co. Ltd.; 6 = Biosafety Research Center, Foods, Drugs and Pesticides; 7 = National Institute of Health Sciences.

The LLNA: BrdU-ELISA test results from the JSAAE validation study were used for interlaboratory reproducibility analyses for three endpoints: sensitizer or nonsensitizer classification and EC1.6 values. Analyses of interlaboratory reproducibility were performed using a concordance analysis for the qualitative results (sensitizer vs. nonsensitizer) (**Section 7.2.1**) and a CV analysis for the quantitative results (EC1.6 values) (**Section 7.2.2**).

7.2.1 Interlaboratory Reproducibility – Qualitative Results

The available quantitative absorbance data for interlaboratory reproducibility analysis were used to calculate SI values for each substance and dose tested. Substances with $SI \geq 1.6$ at any dose were classified as sensitizers. Substances with $SI < 1.6$ at all doses were classified as nonsensitizers. The qualitative (sensitizer/nonsensitizer) interlaboratory concordance analysis for the 10 substances tested during Phase II of the JSAAE interlaboratory validation study is shown in **Table C-17**. The qualitative comparison evaluated the consistency of LLNA: BrdU-ELISA results (i.e., positive vs. negative) for 10 substances tested among up to 7 laboratories. The concordance results show that interlaboratory concordance was 100% (3/3, 6/6, or 7/7) for nine substances. However, one nonsensitizer, lactic acid, yielded concordant sensitizer results ($SI = 1.80, 1.89, \text{ and } 2.53$). The discordant substance was isopropanol, for which interlaboratory concordance was 67% (4/6). Two of

the six tests of isopropanol yielded $SI \geq 1.6$ ($SI = 2.04$ and $SI = 2.22$), while the others yielded negative results (i.e., $SI < 1.6$). The Validation Management Team considered the interlaboratory reproducibility to be acceptable using $SI \geq 2.0$ to identify sensitizers (Kojima et al. 2008). Because the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), there were no traditional concordance data for comparison with the LLNA: BrdU-ELISA concordance.

Table C-17 Qualitative Results for the Phase II Interlaboratory Validation Study on the LLNA: BrdU-ELISA¹

Substance Name	Laboratory							Concordance
	1	2	3	4	5	6	7	
2,4-Dinitrochlorobenzene	+	+	+	+	+	+	+	7/7
	(4.30)	(8.37)	(6.26)	(5.50)	(18.80)	(4.83)	(12.98)	
Glutaraldehyde	+				+	+		3/3
	(3.72)				(28.64)	(2.25)		
Nickel sulfate			+	+			+	3/3
			(2.58)	(4.53)			(2.66)	
<i>trans</i> -Cinnamic aldehyde		+		+	+			3/3
		(3.37)		(3.50)	(4.11)			
Formaldehyde	+				+	+		3/3
	(4.40)				(16.59)	(1.97)		
Eugenol		+				+	+	3/3
		(3.17)				(3.18)	(7.09)	
Hexyl cinnamic aldehyde	+	- ³	+	+	+	+ ⁴	+	6/6
	(3.40)		(2.87)	(3.34)	(13.50)	(3.27)	(3.84)	
Isopropanol	+ ²	- ³	-	-	-	+ ^{2,5}	-	4/6
	(2.22)		(0.98)	(1.57)	(0.94)	(2.04)	(1.01)	
Lactic acid			+	+			+	3/3
			(1.80)	(1.89)			(2.53)	
Methyl salicylate	-	-	-					3/3
	(1.43)	(1.44)	(1.40)					

Abbreviation: LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine.

¹ (+) indicates sensitizer result; (-) indicates nonsensitizer result. Highest stimulation index value for each test is shown in parentheses.

² Stimulation index (SI) ≥ 1.6 at lowest dose tested but < 1.6 at the higher doses. The Validation Management Team considered these to be nonsensitizer results using the $SI \geq 2.0$ criterion (Kojima et al. 2008).

³ Test failed because concurrent positive control failed (i.e., $SI < 1.6$). Result not included in the concordance analysis.

⁴ Three mice tested at highest dose.

⁵ Three mice per dose group.

7.2.2 Interlaboratory Reproducibility – EC1.6 Values

The SI values from the interlaboratory validation study were used to calculate EC1.6 values for each sensitizer according to the methods reported in **Section 7.1.3**. The EC1.6 values from each laboratory were then used to calculate CV values for each substance. The resulting values are shown in **Table C-18**. CV values ranged from 31% (*trans*-cinnamic aldehyde) to 93% (glutaraldehyde). The mean CV was 69%.

The ICCVAM LLNA performance standards indicate that interlaboratory reproducibility should be evaluated with at least two sensitizing chemicals with well-characterized activity in the traditional LLNA (ICCVAM 2009). Acceptable reproducibility is attained when each laboratory obtains ECt values within 0.025% to 0.1% for 2,4-dinitrochlorobenzene and within 5% to 20% for hexyl cinnamic aldehyde (ICCVAM 2009). EC1.6 values from five laboratories were outside the range for 2,4-dinitrochlorobenzene, and the EC1.6 values from two laboratories were outside the range for hexyl cinnamic aldehyde. Laboratories 2 through 6 reported EC1.6 values that were lower than the specified acceptance range for 2,4-dinitrochlorobenzene (0.011%, 0.023%, 0.023%, 0.0022%, and 0.017%, respectively). For hexyl cinnamic aldehyde, Laboratories 4 and 5 obtained EC1.6 values that were lower than the acceptance range (4.80% and 3.64%, respectively).

Table C-18 EC1.6 Values from the Phase II Interlaboratory Validation Study on the LLNA: BrdU-ELISA¹

Substance Name	Laboratory							Mean ± SD	% CV
	1	2	3	4	5	6	7		
2,4-Dinitro-chlorobenzene	0.062 (4.3 @ 1%)	<i>0.011¹</i> (8.37 @ 1%)	<i>0.023</i> (5.99 @ 0.3%)	<i>0.023</i> (5.50 @ 1%)	<i>0.0022</i> (18.80 @ 0.3%)	<i>0.017</i> (4.83 @ 0.3%)	0.050 (12.18 @ 1%)	0.027 ± 0.021	80
Hexyl cinnamic aldehyde	10.43 (3.4 @ 50%)	- ² (1.83 @ 50%)	16.98 (2.87 @ 50%)	<i>4.80</i> (3.34 @ 50%)	<i>3.64</i> (13.5 @ 50%)	8.60 ³ (3.27 @ 50%)	10.10 (3.84 @ 50%)	11.78 ± 8.33	71
Glutaraldehyde	0.079	NT	NT	NT	0.031	0.24	NT	0.12 ± 0.11	93
Nickel sulfate	NT	NT	1.84	0.57	NT	NT	0.67	1.03 ± 0.70	68
<i>trans</i> -Cinnamic aldehyde	NT	1.88	NT	1.04	1.96	NT	NT	1.63 ± 0.51	31
Formaldehyde	0.29	NT	NT	NT	0.19	0.010	NT	0.16 ± 0.14	88
Eugenol	NT	13.82	NT	NT	NT	11.65	3.77	9.75 ± 5.29	54

Note: Boldface indicates substances recommended for assessing interlaboratory reproducibility in *Recommended Performance Standards* (ICCVAM 2009). Boldface italic EC1.6 values are outside of the acceptable range from the ICCVAM LLNA performance standards: 5%-20% for hexyl cinnamic aldehyde and 0.025%-0.1% for 2,4-dinitrochlorobenzene. Values in parentheses are the highest SI values achieved.

Abbreviations: CV = coefficient of variation; EC1.6 = estimated concentration needed to produce a stimulation index of 1.6; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; NT = not tested; SI = stimulation index.

¹ EC1.6 values interpolated using lowest dose and SI = 1 at 0% concentration because the dose response at the two lowest doses (0.1% and 0.3%) was flat (SI = 6.39 and 6.53, respectively).

² Test failed because associated positive control failed (i.e., SI < 1.6; vehicle control absorbance was unusually high). Result not included in the mean EC1.6 and CV values.

³ Three mice tested at highest dose.

The interlaboratory CV values for the LLNA: BrdU-ELISA EC1.6 values were higher than those for the traditional LLNA EC3 values. The analysis of interlaboratory variation of EC3 values for the traditional LLNA reported CV values of 7% to 84% for five substances tested in five laboratories (Table C-19; ICCVAM 1999). Three of the same substances were evaluated in the traditional LLNA and the LLNA: BrdU-ELISA. All interlaboratory CV values for the LLNA: BrdU-ELISA were greater than those for the traditional LLNA. The CV of 80% for 2,4-dinitrochlorobenzene was greater than the two CV values of 37% and 27%, calculated from five values each, reported by ICCVAM (1999). The CV of 71% for hexyl cinnamic aldehyde tested in the LLNA: BrdU-ELISA was greater than the 7% reported by ICCVAM (1999). The CV of 54% for eugenol tested in the LLNA: BrdU-ELISA was greater than the 42% reported by ICCVAM (1999).

Table C-19 Interlaboratory Reproducibility of the EC3 Values for Substances Tested in the Traditional LLNA¹

Substance Name	Laboratory					CV (%)
	1	2	3	4	5	
2, 4-Dinitrochlorobenzene	0.3	0.5	0.6	0.9	0.6	37
	0.5	0.6	0.4	0.6	0.3	27
Hexyl cinnamic aldehyde	7.9	7.6	8.4	7.0	8.1	7
Isoeugenol	1.3	3.3	1.8	3.1	1.6	41
Eugenol	5.8	14.5	8.9	13.8	6.0	42
Sodium lauryl sulfate	13.4	4.4	1.5	17.1	4.0	84

Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of 3; LLNA = murine local lymph node assay.

¹ From ICCVAM (1999).

7.3 Reproducibility Analysis for Substances With Multiple Tests

This section examines the reproducibility of the tests for the 18 substances that had multiple test results, regardless of whether the tests were performed in one laboratory or multiple laboratories. For the 18 substances, two to 12 tests were available. The frequency with which SI values for the 18 substances occurred in one of three SI categories was considered. The three SI categories were:

- LLNA: BrdU-ELISA nonsensitizers with SI < 1.6
- LLNA: BrdU-ELISA sensitizers with SI between 1.6 and 1.9 (borderline positive results with potential to be false positives with respect to classification by the traditional LLNA)
- LLNA: BrdU-ELISA sensitizers with SI ≥ 1.9

Table C-20 shows the proportion of the tests for each substance that produced SI values in each category. When using SI ≥ 1.6 to classify sensitizers, the categorical concordance analysis for the 18 substances with multiple tests indicated that the SI results for 85% (11/13) of the LLNA sensitizers were 100% concordant (i.e., all yielded SI ≥ 1.6 and SI ≥ 1.9). For the 13 traditional LLNA sensitizers with multiple test results, there were two tests that produced SI < 1.6: one test of linalool (SI = 1.45) and one test of hydroxycitronellal (SI = 1.34). The other tests of linalool and hydroxycitronellal produced SI ≥ 1.6 and SI ≥ 1.9. Both tests of these substances were performed in the same laboratory. None of the tests for the 13 sensitizers produced 1.6 < SI < 1.9.

The LLNA: BrdU-ELISA tests for traditional LLNA nonsensitizers were more variable than those for traditional LLNA sensitizers. The results for isopropanol were particularly variable: 71% (5/7) of the tests produced $SI \leq 1.6$ ($SI = 0.92, 0.94, 0.98, 1.01, \text{ and } 1.57$) and 29% (2/7) produced $SI \geq 1.9$ ($SI = 2.04 \text{ and } 2.22$). All isopropanol tests were performed in different laboratories. Lactic acid tests produced SI values in two categories: 67% (2/3) of the tests had $1.6 \leq SI < 1.9$ ($SI = 1.80 \text{ and } 1.89$), and 33% (1/3) of the tests had $SI \geq 1.9$ ($SI = 2.53$). All isopropanol tests were performed in different laboratories. The multiple test results for hexane, methyl salicylate, and propylene glycol were 100% concordant. However, the two hexane tests produced SI values in the $1.6 \leq SI < 1.9$ category ($SI = 1.76 \text{ and } 1.89$) (i.e., sensitizer). Both tests were performed in the same laboratory. The three methyl salicylate ($SI = 1.40, 1.43, \text{ and } 1.44$) tests performed in different laboratories and the three propylene glycol ($SI = 1.20, 1.57, \text{ and } 0.91$) tests performed in the same laboratory produced SI values in the $SI < 1.6$ category (i.e., nonsensitizer).

Table C-20 Concordance of LLNA: BrdU-ELISA Tests for Substances with Multiple Tests by Maximum SI Category

Substance	LLNA: BrdU-ELISA Nonsensitizers (Maximum $SI < 1.6^1$)	LLNA: BrdU-ELISA Sensitizers (Maximum $SI \geq 1.6$)		Total Tests
		$1.6 \leq \text{Maximum } SI < 1.9^1$	Maximum $SI \geq 1.9^1$	
<i>Sensitizers²</i>				
Cyclamen aldehyde	0 (0%)	0 (0%)	0 (100%)	2
2,4-Dinitrochlorobenzene	0 (0%)	0 (0%)	9 (100%)	9
Diphenylcyclopro-penone	0 (0%)	0 (0%)	3 (100%)	3
Eugenol	0 (0%)	0 (0%)	9 (100%)	9
Formaldehyde	0 (0%)	0 (0%)	3 (100%)	3
Glutaraldehyde	0 (0%)	0 (0%)	5 (100%)	5
Hexyl cinnamic aldehyde	0 (0%)	0 (0%)	12 (100%)	12
Hydroxycitronellal	1 (50%)	0 (0%)	1 (50%)	2
Isoeugenol	0 (0%)	0 (0%)	3 (100%)	3
Linalool	1 (50%)	0 (0%)	1 (50%)	2
Nickel sulfate	0 (0%)	0 (0%)	3 (100%)	3
1,4-Phenylenediamine	0 (0%)	0 (0%)	2 (100%)	2
<i>trans</i> -Cinnamaldehyde	0 (0%)	0 (0%)	4 (100%)	4
<i>Nonsensitizers²</i>				
Hexane	0 (0%)	2 (100%)	0 (%)	2
Isopropanol	5 (71%)	0 (0%)	2 (29%)	7
Lactic acid	0 (0%)	2 (67%)	1 (33%)	3
Methyl salicylate	3 (100%)	0 (0%)	0 (0%)	3
Propylene glycol	3 (100%)	0 (0%)	0 (0%)	3

Abbreviations: LLNA = murine local lymph node assay; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; SI = stimulation index.

¹ Numbers shown reflect number of tests. Percentage in parentheses reflects percentage of the total number of tests for each substance.

² According to traditional LLNA results.

8.0 Data Quality

The data submitted by Dr. Takeyoshi were generated at the Hita Laboratory and the Tokyo Laboratory of the Chemicals Evaluation and Research Institute, Japan (Takeyoshi M, personal communication). Although the laboratories conduct studies routinely that conform to Good Laboratory Practices (GLP), 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 fully 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.

The data from the interlaboratory validation study (Kojima et al. 2008) were generated in GLP laboratories, but the LLNA: BrdU-ELISA studies were not fully GLP compliant. The data from each laboratory were reviewed by the chief of the Validation Management Team and the biostatistician.

9.0 Other Scientific Reports and Reviews

The Validation Management Team for the multilaboratory validation study concluded that the LLNA: BrdU-ELISA, using the $SI \geq 2$ criterion to identify sensitizers, had sufficient relevance compared with the traditional LLNA and acceptable interlaboratory reproducibility (Kojima et al. 2008). The validation study has been peer reviewed in Japan. The peer review report is expected to be released in 2010 (Kojima H, personal communication).

A set of studies was 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 in this BRD. Compared to the method of Takeyoshi et al., which administered 5 mg BrdU/mouse, the concentration of BrdU administered (via intraperitoneal injection) by Yamano et al. 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 naphthenic 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 tested. Therefore, a comparison of the accuracy of the LLNA: BrdU-ELISA of Yamano et al. with the traditional LLNA could not be conducted.

10.0 Animal Welfare Considerations

The LLNA: BrdU-ELISA evaluates only the induction phase of skin sensitization; therefore, the discomfort to animals that can occur in the guinea pig tests with the elicitation phase of ACD is eliminated. Additionally, the LLNA: BrdU-ELISA test method protocol requires fewer mice per treatment group (a minimum of four animals per group) than either of the GP tests (10-20 animals/group for Buehler and 5-10 animals/group for GPMT).

The LLNA: BrdU-ELISA will require the use of the same number of animals as the updated ICCVAM LLNA protocol (ICCVAM 2009). However, since the traditional LLNA uses radioactivity, which is restricted in some countries and institutions, broader use of the nonradioactive 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 that 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 used in the development (Takeyoshi et al. 2003, 2004a, 2004b, 2005, 2006, 2007a, 2007b, unpublished data) and interlaboratory validation (Kojima et al. 2008) of the LLNA: BrdU-ELISA test method, which is the same as that specified in the updated ICCVAM LLNA protocol (Appendix A of ICCVAM 2009).

10.3 Reduction Considerations

A further reduction of 40% (12 vs. 20) could be achieved by using the rLLNA: 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 eight animals, or 40% (12 vs. 20).

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. The transferability of the LLNA: BrdU-ELISA was demonstrated by the interlaboratory validation study (Kojima et al. 2008) (**Section 7.2**).

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, or licensing permits for handling radioactive materials. The remaining facilities (e.g., animal care facilities) are the same for 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 know how to perform ELISAs.

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13.0 Glossary

Accuracy⁶: (a) The closeness of agreement between a test method result and an accepted reference value. (b) The proportion of correct outcomes of a test method. It is a measure of test method performance and one aspect of *relevance*. The term is often used interchangeably with concordance (see also *two-by-two table*). Accuracy is highly dependent on the prevalence of positives in the population being examined.

Allergic contact dermatitis (ACD): A Type IV allergic reaction of the skin that results from repeated skin contact with a skin sensitizer. Clinical signs of ACD include the development of erythema (redness) and edema (swelling), blistering, and itching. Also referred to as skin sensitization.

Assay⁶: The experimental system used. Often used interchangeably with *test* and *test method*.

Coded substances: Substances labeled by code rather than name so that they can be tested and evaluated without knowledge of their identity or anticipation of test results. Coded substances are used to avoid intentional or unintentional bias when evaluating laboratory or test method performance.

Concordance⁶: The proportion of all substances tested that are correctly classified as positive or negative. It is a measure of test method performance and one aspect of *relevance*. The term is often used interchangeably with *accuracy* (see also *two-by-two table*). Concordance is highly dependent on the prevalence of positives in the population being examined.

EC1.6: The estimated concentration needed to produce a stimulation index of 1.6, as compared to the concurrent vehicle control.

EC3: The estimated concentration needed to produce a stimulation index of 3, as compared to the concurrent vehicle control.

ECt: The estimated concentration needed to produce a stimulation index of a specific threshold.

False negative⁶: A substance incorrectly identified as negative by a test method.

False negative rate⁶: The proportion of all positive substances falsely identified by a test method as negative (see *two-by-two table*). It is one indicator of test method accuracy.

False positive⁶: A substance incorrectly identified as positive by a test method.

False positive rate⁶: The proportion of all negative substances that are falsely identified by a test method as positive (see *two-by-two table*). It is one indicator of test method accuracy.

Good Laboratory Practices (GLP)⁶: Regulations promulgated by the U.S. Food and Drug Administration and the U.S. Environmental Protection Agency, and principles and procedures adopted by the Organization for Economic Cooperation and Development and Japanese authorities, that describe record keeping and quality assurance procedures for laboratory records that will be the basis for data submissions to national regulatory agencies.

Hazard⁶: The potential for an adverse health or ecological effect. A hazard potential results only if an exposure occurs that leads to the possibility of an adverse effect being manifested.

Interlaboratory reproducibility⁶: A measure of whether different qualified laboratories using the same protocol and test substances can produce qualitatively and quantitatively similar results.

⁶ Definition used by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM 2003).

Interlaboratory reproducibility is determined during the prevalidation and validation processes and indicates the extent to which a test method can be transferred successfully among laboratories.

Intralaboratory repeatability⁶: The closeness of agreement between test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions within a given time period.

Intralaboratory reproducibility⁶: The first stage of validation; a determination of whether qualified people within the same laboratory can successfully replicate results using a specific test protocol at different times.

Immunological: Relating to the immune system and immune responses.

In vivo: In the living organism. Refers to assays performed in multicellular organisms.

Lymphocyte: A white blood cell found in the blood, lymph, and lymphoid tissues, which regulates and plays a role in acquired immunity.

Murine local lymph node assay (LLNA): An *in vivo* test method used to assess the skin sensitization potential of a substance by measuring the proliferation of lymphocytes in the lymph nodes draining the ears (i.e., auricular lymph nodes) of mice, subsequent to topical exposure of the ear to the substance. The traditional LLNA measures lymphocyte proliferation by quantifying the amount of ³H-thymidine or ¹²⁵I-iododeoxyuridine incorporated into the cells of the draining lymph nodes.

Murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine (LLNA: BrdU-ELISA): An *in vivo* test method used to assess the skin sensitization potential of a substance by measuring the proliferation of lymphocytes in the lymph nodes draining the ears (i.e., auricular lymph nodes) of mice, subsequent to topical exposure of the ear to the substance. The LLNA: BrdU-ELISA is a nonradioactive modification of the traditional LLNA and assesses lymphocyte proliferation by quantifying the amount of bromodeoxyuridine (BrdU) incorporated into the cells of the draining lymph nodes using an enzyme-linked immunosorbent assay (ELISA).

Negative predictivity⁶: The proportion of correct negative responses among substances testing negative by a test method (see *two-by-two table*). It is one indicator of test method accuracy. Negative predictivity is a function of the sensitivity of the test method and the prevalence of negatives among the substances tested.

Nonsensitizer: A substance that does not cause skin sensitization following repeated skin contact.

Performance⁶: The accuracy and reliability characteristics of a test method (see *accuracy, reliability*).

Positive control: A substance known to induce a positive response, which is used to demonstrate the sensitivity of the test method and to allow for an assessment of variability in the conduct of the assay over time. For most test methods, the positive control substance is tested concurrently with the test substance and the vehicle/solvent control. However, for some *in vivo* test methods, periodic studies using a positive control substance is considered adequate by the OECD.

Positive predictivity⁶: The proportion of correct positive responses among substances testing positive by a test method (see *two-by-two table*). It is one indicator of test method accuracy. Positive predictivity is a function of the sensitivity of the test method and the prevalence of positives among the substances tested.

Prevalence⁶: The proportion of positives in the population of substances tested (see *two-by-two table*).

Protocol⁶: The precise, step-by-step description of a test, including the listing of all necessary reagents, criteria, and procedures for the evaluation of the test data.

Quality assurance⁶: A management process by which adherence to laboratory testing standards, requirements, and record keeping procedures is assessed independently by individuals other than those performing the testing.

Reduction alternative⁶: A new or modified test method that reduces the number of animals required.

Reference test method⁶: The accepted *in vivo* test method used for regulatory purposes to evaluate the potential of a test substance to be hazardous to the species of interest.

Refinement alternative⁶: A new or modified test method that refines procedures to lessen or eliminate pain or distress in animals or enhances animal well-being.

Relevance⁶: The extent to which a test method correctly predicts or measures the biological effect of interest in humans or another species of interest. Relevance incorporates consideration of the *accuracy* or *concordance* of a test method.

Reliability⁶: A measure of the degree to which a test method can be performed reproducibly within and among laboratories over time. It is assessed by calculating intra- and interlaboratory reproducibility and intralaboratory repeatability.

Replacement alternative⁶: A new or modified test method that replaces animals with non-animal systems or one animal species with a phylogenetically lower one (e.g., a mammal with an invertebrate).

Reproducibility⁶: The consistency of individual test results obtained in a single laboratory (intralaboratory reproducibility) or in different laboratories (interlaboratory reproducibility) using the same protocol and test substances (see intra- and inter-laboratory reproducibility).

rLLNA: BrdU-ELISA (reduced LLNA: BrdU-ELISA): A variant of the LLNA: BrdU-ELISA that employs a single, high dose of the test substance rather than multiple doses to determine its skin sensitization potential, thus using fewer animals.

Sensitivity⁶: The proportion of all positive substances that are classified correctly as positive in a test method. It is a measure of test method accuracy (see *two-by-two table*).

Skin sensitizer: A substance that induces an allergic response following skin contact (UN 2005).

Specificity⁶: The proportion of all negative substances that are classified correctly as negative in a test method. It is a measure of test method accuracy (see *two-by-two table*).

Stimulation index (SI): A value calculated for the LLNA, to assess the skin sensitization potential of a test substance. The value is calculated as the ratio of the bromodeoxyuridine incorporated into the auricular lymph nodes of a group of treated mice to the bromodeoxyuridine incorporated into the corresponding lymph nodes of a group of vehicle control mice. For the traditional LLNA: BrdU-ELISA and the rLLNA: BrdU-ELISA, an $SI \geq 1.6$ classifies a substance as a skin sensitizer.

Test⁶: The experimental system used; used interchangeably with *test method* and *assay*.

Test method⁶: A process or procedure used to obtain information on the characteristics of a substance or agent. Toxicological test methods generate information regarding the ability of a substance or agent to produce a specified biological effect under specified conditions. Used interchangeably with *test* and *assay*. See also *validated test method* and *reference test*.

Transferability⁶: The ability of a test method or procedure to be accurately and reliably performed in different, competent laboratories.

Two-by-two table⁶: The two-by-two table can be used for calculating accuracy (concordance) ($(a+d)/(a+b+c+d)$), negative predictivity ($d/(c+d)$), positive predictivity ($a/(a+b)$), prevalence ($(a+c)/(a+b+c+d)$), sensitivity ($a/(a+c)$), specificity ($d/(b+d)$), false positive rate ($b/(b+d)$), and false negative rate ($c/(a+c)$).

		New Test Outcome		
		Positive	Negative	Total
Reference Test Outcome	Positive	a	c	a + c
	Negative	b	d	b + d
	Total	a + b	c + d	a + b + c + d

Validated test method⁶: An accepted test method for which validation studies have been completed to determine the relevance and reliability of this method for a specific proposed use.

Validation⁶: The process by which the reliability and relevance of a procedure are established for a specific purpose.

Vehicle control: An untreated sample containing all components of a test system, including the vehicle that is processed with the test substance-treated and other control samples to establish the baseline response for the samples treated with the test substance dissolved in the same vehicle.

Weight-of-evidence (process): The strengths and weaknesses of a collection of information are used as the basis for a conclusion that may not be evident from the individual data.

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