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

Revised Draft Background Review Document

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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	*N,N*-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 two
EC3	Estimated concentration needed to produce a stimulation index of three
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	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
U.S.	United States
Unk	Unknown
Veh.	Vehicle
vs.	Versus
w/v	Weight to volume ratio

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364

Preface

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

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

NICEATM and ICCVAM provided the draft BRDs and draft recommendations to an international independent scientific peer review panel for their consideration at a public meeting on March 4-6, 2008. A report of the Panel meeting was subsequently published on the NICEATM-ICCVAM website[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

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

additional existing data that were not available to the Panel and reanalyze the performance of each non-radioactive LLNA method. NICEATM subsequently obtained additional data and prepared updated BRDs. ICCVAM also prepared revised draft test method recommendations based on the revised BRDs. This revised draft BRD addresses the validation database for the LLNA: BrdU-ELISA.

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

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

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Executive Summary

Background

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

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

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

Revisions to the LLNA: BrdU-ELISA Evaluation

NICEATM and ICCVAM convened an independent scientific peer review panel meeting on March 4-6, 2008. The Panel peer reviewed the draft BRD and commented on the extent that it supported the draft ICCVAM test method recommendations on the usefulness and limitations of the LLNA: BrdU-ELISA. Both ICCVAM and the Panel concluded that more information was needed before a recommendation on the usefulness and limitations of the LLNA: BrdU-ELISA could be made[2]. The Panel indicated that the following information was needed: a detailed protocol, individual animal data, and an evaluation of interlaboratory reproducibility. The Panel recommended that additional data be obtained by NICEATM and that a reanalysis of the performance of the LLNA: BrdU-ELISA be conducted. In response to this recommendation, NICEATM obtained additional LLNA: BrdU-ELISA data from the test sponsor, which were used to update the evaluation. These data include:

- LLNA: BrdU-ELISA data for six substances not previously provided to NICEATM. (Note: The number of substances evaluated effectively increased by seven with the location of reference data for one substance for which LLNA: BrdU-ELISA data had been previously submitted). These data were used in a reanalysis of test method accuracy, which is detailed in **Section 6.0** of this BRD.
- Individual animal data for the LLNA: BrdU-ELISA studies included in the interlaboratory validation study of 10 substances. These data were used in additional quantitative analyses of test method reproducibility, which are detailed in **Section 7.0** of this BRD.

Test Method Protocol

The protocol in this draft BRD has been revised from the January 2008 draft BRD to include the decision criterion of SI ≥ 2.0, rather than SI ≥ 3.0, to identify substances as sensitizers. The LLNA: BrdU-ELISA was originally developed by Takeyoshi et al. (2001). While the

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

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 (Dean et al. 2001; ICCVAM 1999).

Validation Database

The validation database in this draft BRD has been revised from the January 2008 draft BRD to include seven additional substances (six substances for which LLNA: BrdU-ELISA data were not previously obtained and on previously included substance for which traditional LLNA data were recently obtained). The accuracy and reliability of the LLNA: BrdU-ELISA were assessed using the individual animal data for 31 substances from six published studies (Takeyoshi et al. 2003; 2004a; 2004b; 2005; 2006; 2007a), one platform presentation (Takeyoshi 2007b), and one poster presentation (Kojima et al. 2008). The reference test data for these substances were obtained from the traditional LLNA, guinea pig (GP) skin sensitization tests, and/or human skin sensitization tests or clinical information. Of the 31 substances with traditional LLNA data, 22 were classified by the traditional LLNA as skin sensitizers and nine were classified as nonsensitizers.

Test Method Accuracy

The accuracy evaluation in this draft BRD has been revised from the January 2008 draft BRD to include the results for seven additional substances. Other revisions included the evaluation of multiple decision criteria, including the SI $\geq$ 2.0 recommended in the test method protocol, and the evaluation of two different criteria used to classify sensitizers and nonsensitizers. Based on the evaluation of multiple decision criteria, the optimal performance was achieved using SI $\geq$ 2.0 to classify sensitizers and SI < 1.3 to classify nonsensitizers. When these two criteria are used, false positive results (0/9) and false negative results (0/22) are eliminated compared with the traditional LLNA. However, using these criteria, 11

substances have an SI ≥ 1.3 to < 2.0, 6/11 substances were sensitizers and 5/11 substances were nonsensitizers when tested in the traditional LLNA. Other available information, such as peptide reactivity, could be used to interpret LLNA: BrdU-ELISA results when 1.3 ≤ SI < 2.0. Sixty-seven percent (4/6) of the sensitizers in this range had peptide reactivity data and all four had low to moderate peptide reactivity. All (5/5) of the nonsensitizers had minimal peptide reactivity.

When a single decision criterion of SI ≥ 2.0 was used to classify sensitizers vs. nonsensitizers, compared to the traditional LLNA, accuracy was 84% (26/31), with a false positive rate of 0% (0/9), and the false negative rate of 23% (5/22). Among the 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.

Test Method Reliability – Intralaboratory Reproducibility

The intralaboratory reproducibility evaluation in this draft BRD has been revised from the January 2008 draft BRD to include the results for a number of additional tests for which SI values were newly available. Intralaboratory reproducibility was assessed using a concordance analysis of sensitizer/nonsensitizer results, and a coefficient of variation (CV) analysis of SI values and EC2 values (estimated concentration needed to produce an SI of 2). The qualitative analysis shows that multiple tests of eight substances (six sensitizers and two nonsensitizers) yielded 100% concordance for sensitizer/nonsensitizer outcomes for seven of the eight substances. In the quantitative analyses, the CVs for the SI values of nine substance/concentration combinations that were tested up to five times each ranged from 1% to 79%. The CVs for the EC2 values of three substances that were tested up to five times at multiple doses ranged from 16% to 73%.

Test Method Reliability – Interlaboratory Reproducibility

The interlaboratory reproducibility evaluation is a new addition to this draft BRD because interlaboratory data were not available for evaluation in the January 2008 draft BRD. This draft BRD also includes a reproducibility analysis using separate SI criteria to identify sensitizers and nonsensitizers. When using SI ≥ 2.0 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 seven substances (six sensitizers and one nonsensitizer). There was 67% (2/3 or 4/6) agreement among the tests for the remaining sensitizer and two nonsensitizers. Interlaboratory CV values for the EC2 values of the seven sensitizers ranged from 20 to 101%.

When using SI ≥ 2.0 to classify sensitizers and SI < 1.3 to classify nonsensitizers, the concordance analysis for the 14 substances with multiple tests indicated that the SI results for 89% (8/9) of the sensitizers were 100% concordant (i.e., all yielded SI ≥ 2.0). The SI results for 40% (2/5) of the nonsensitizers were 100% (i.e., all yielded 1.3 ≤ SI < 2.0). The concordance of the other three nonsensitizers was 50% (1/2) to 57% (4/7) for SI < 1.3 and 29% (2/7) to 33% (1/3) for SI ≥ 2.0.

Animal Welfare Considerations

The animal welfare considerations in this draft BRD have not changed from the January 2008 draft BRD. 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 non-radioactive 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.

Test Method Transferability

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

ICCVAM Revised Draft Recommendations

ICCVAM developed revised draft recommendations for the LLNA: BrdU-ELISA based on the new data and analyses. Recommendations are provided for test method usefulness and

limitations, test method protocol, and future studies to further characterize its usefulness and limitations. These are provided in a separate document, *Draft ICCVAM Test Method Recommendations, Non-radioactive Murine Local Lymph Node Assay: BrdU-ELISA Test Method Protocol (LLNA: BrdU-ELISA).*

1.0 Introduction

1.1 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 presented to circulating T-lymphocytes. The T-lymphocytes are then activated, which causes release of cytokines and other inflammatory mediators. This release produces a rapid dermal immune response that can lead to ACD (ICCVAM 1999; Basketter et al. 2003; Jowsey et al. 2006).

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

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended that the LLNA is a valid substitute for currently accepted guinea pig (GP) test methods to assess the ACD potential of many, but not all, types of substances. 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

[3] Available at http://www.bls.gov/

National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)-ICCVAM website (http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf).

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

On January 10, 2007, the U.S. Consumer Product Safety Commission (CPSC) formally nominated several activities related to the LLNA for evaluation by ICCVAM and NICEATM (Available at http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf). One of the nominated activities was an assessment of the validation status of non-radioactive alternatives to the current version of the LLNA ([ICCVAM 1999, Dean et al. 2001] referred to hereafter as the "traditional LLNA"), which uses radioactivity to detect sensitizers. The information described in this background review document (BRD) was compiled by ICCVAM and NICEATM in response to this nomination. The BRD provides a comprehensive review of available data and information regarding the usefulness and limitations of one of these methods, the LLNA with detection of bromodeoxyuridine (BrdU) incorporation by enzyme-linked immunosorbent assay (ELISA) (referred to hereafter as the "LLNA: BrdU-ELISA"). ICCVAM and its IWG evaluated this method in a draft background review document (BRD) and developed draft test method recommendations based on this evaluation. An independent peer review panel (Panel) reviewed the BRD in March 2008 to evaluate the extent to which the information contained in the BRD supported the draft recommendations. The Panel concluded that additional information was needed to evaluate the method, including a detailed protocol, quantitative data for the method, and an evaluation

of interlaboratory reproducibility. After receiving the additional information, this revised draft BRD was compiled for review by the Panel.

ICCVAM will consider the conclusions and recommendations of the Panel, along with comments received from the public and the Scientific Advisory Committee for Alternative Toxicological Methods, when developing the final BRD and final recommendations on the usefulness and limitations of each non-radioactive alternative LLNA test method that is being considered.

1.3 The LLNA: BrdU-ELISA

The LLNA: BrdU-ELISA was developed by Takeyoshi et al. (2001) as a non-radioactive alternative to the traditional LLNA. While the traditional LLNA assesses cellular proliferation by measuring the incorporation of radioactivity into the deoxyribonucleic acid (DNA) of dividing lymph node cells, the LLNA: BrdU-ELISA assesses the same endpoint by measuring the incorporation of the thymidine analog BrdU, which is detected and quantified with an ELISA, which is available as a kit commercially from several sources.

This document provides:

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

2.0 LLNA: BrdU-ELISA Test Method Protocol

The protocol in this draft BRD has been revised from the January 2008 draft BRD to use the decision criterion of SI ≥ 2.0, rather than SI ≥ 3.0, to identify substances as sensitizers. The LLNA: BrdU-ELISA protocol (see **Appendix A**) 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, ^{3}H- thymidine or ^{125}I-iododeoxyuridine (in phosphate buffered saline; 250 µL/mouse) is administered via the tail vein two days after the final application of the test substance. In the LLNA: BrdU-ELISA, 5 mg BrdU in a volume of 0.5 mL physiological saline (concentration of 10 mg/mL) is administered via intraperitoneal injection two days after the final application of the test substance. Takeyoshi et al. (2001) reported that one injection of 5 mg BrdU was selected over two injections to minimize the incorporation of BrdU in the control group. Injection of BrdU two days after topical treatment with test substance yielded efficient incorporation of BrdU in comparison to injection one day or three days after topical treatment with a test substance (Takeyoshi et al. 2001). On the day following BrdU injection, lymph nodes are excised and a single cell suspension is prepared from the lymph nodes of each animal. A standard aliquot of the cell suspension is added in triplicate to the wells of a flat-bottom 96-well microplate and centrifuged. Supernatants are then removed. 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 ≥ 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 multi-laboratory validation study of the LLNA: BrdU-ELISA organized by the Japanese Society for Alternatives to Animal Experiments (JSAAE) used SI ≥ 2 to classify sensitizers (Kojima et al. 2008). The SI ≥ 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 ≥ 3 criterion was inadequate for reliably distinguishing sensitizers from nonsensitizers (Kojima H, personal communication).

3.0 LLNA: BrdU-ELISA Validation Database

The validation database in this draft BRD has been revised from the January 2008 draft BRD to include seven additional substances. To evaluate the validity of the LLNA: BrdU-ELISA, data were available for 35 substances. Twenty-seven 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 multi-laboratory validation study coordinated by JSAAE (**Table 3-1**). Most of these substances (31/35) had been previously tested in the traditional LLNA. 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-phenyoxy)-propyl]-2-methoxy-phenol and 2-methoxy-4-(7-methoxy-3-methyl-5-propenyl-2,3-dihydro-benzofuran-2yl)-phenol) (Takeyoshi et al. 2004a; 2007a). Of the 31 substances with traditional LLNA data, 22 were classified by the traditional LLNA as skin sensitizers and nine were classified as nonsensitizers. The traditional LLNA EC3 values (i.e., estimated concentration needed to produce an SI = 3) for the 22 sensitizers ranged from 0.01% to 47.5% (**Table 3-1)**.

Appendix B provides information on the physicochemical properties (e.g., physical form tested), 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's ChemID Plus database. If chemical classes were unavailable, they were assigned to each test substance using a standard classification scheme based on the National Library of Medicine Medical Subject Headings classification system (available at http://www.nlm.nih.gov/mesh/meshhome.html). A substance could be assigned to more than one chemical class; however, no substance was assigned to more than three classes. Chemical class information is presented only to provide an indication of the variety of structural elements that are present in the structures that were evaluated in this analysis. Classification of substances into chemical classes is not intended to indicate the impact of structure on biological activity with respect to sensitization potential. **Table 3-1** shows that 18 chemical classes are represented by the substances tested in the LLNA: BrdU-ELISA.

732 Five substances are classified in more than one chemical class. The classes with the highest
733 number of substances are carboxylic acids (12 substances) and aldehydes (six substances).

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

Substance Name	Chemical Class[1]	Traditional LLNA EC3 (%)[2]	N[3]
p-Benzoquinone	Quinones	0.01	1
2,4-Dinitrochlorobenzene*	Hydrocarbon, Halogenated; Nitro Compounds; Hydrocarbons, Cyclic	0.049	15
Diphenylcyclopropenone	Hydrocarbons, Cyclic	0.05	1
Glutaraldehyde	Aldehydes	0.083[4]	3
4-Phenylenediamine*	Amines	0.11	6
Formaldehyde	Aldehydes	0.50[4]	4
trans-Cinnamaldehyde	Aldehydes	1.4	1
Isoeugenol*	Carboxylic Acids	1.5	47
2-Mercaptobenzothiazole*	Heterocyclic Compounds	1.7[5]	1
Cinnamic aldehyde	Aldehydes	1.9	6
3-Aminophenol	Amines; Phenols	3.2	1
Trimellitic anhydride	Anhydrides; Carboxylic Acids	4.7	2
Nickel sulfate	Inorganic Chemicals, Metals Inorganic Chemicals, Elements	4.8[6]	1
4-Chloroaniline	Amines	6.5	1
Citral*	Hydrocarbons, Other	9.2	6
Hexyl cinnamic aldehyde*	Aldehydes	9.7	21
Eugenol*	Carboxylic Acids	10.1	11
Cyclamen aldehyde	Aldehydes	22.3	1
Hydroxycitronellal	Hydrocarbons, Other	24.0	6
Linalool	Hydrocarbons, Other	30.0	1
Isopropyl myristate	Lipids	44.0	1
Aniline	Amines	47.5	3
2-Hydroxypropyl methacrylate	Carboxylic Acids	NA	1
Diethyl phthalate	Carboxylic Acids	NA	1
Dimethyl isophthalate	Carboxylic Acids	NA	1
Glycerol	Alcohols; Carbohydrates	NA[5]	2
Hexane	Hydrocarbons, Acyclic	NA	1
Isopropanol*	Alcohols	NA	1
Lactic acid*	Carboxylic Acids	NA[6]	1
Methyl salicylate*	Carboxylic Acids	NA	9
Propylene glycol	Alcohols	NA[7]	1
2,2'-Dihydroxyl-3,3'-dimethoxy-5,5'-diallyl-biphenyl	Carboxylic Acids	NK	0

Substance Name	Chemical Class[1]	Traditional LLNA EC3 (%)[2]	N[3]
2-Methoxy-4-(7-methoxy-3-methyl-5-propenyl-2,3-dihydro-benzofuran-2yl)-phenol	Carboxylic Acids	NK	0
4,5'-Diallyl-2'-hydroxy-2,3'-dimethoxyphenyl ether	Carboxylic Acids	NK	0
4-[1-Hydroxy-2-(2-methoxy-4-propenyl-phenyoxy)-propyl]-2-methoxy-phenol (Synonym: □-O-4-Dilignol)	Carboxylic Acids	NK	0

Abbreviations: LLNA: BrdU-ELISA= Local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; EC3 = Estimated concentration needed to produce a stimulation index (SI) = 3; NA = Not applicable since maximum SI < 3.0; NK = Not known (information not found).
*Reference substance from ICCVAM (2009).
[1]Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs, developed by the National Library of Medicine (http://www.nlm.nih.gov/mesh/meshhome.html).
[2]Mean EC3 values from the NICEATM database of traditional LLNA studies. Vehicle for testing both sensitizers and nonsensitizers was acetone: olive oil (4:1) unless otherwise noted.
[3]Number of traditional LLNA studies from which the data were obtained.
[4]Vehicle = Acetone.
[5]Vehicle = *N,N*-Dimethylformamide.
[6]Vehicle = Dimethyl sulfoxide.
[6]Vehicle = Distilled water.

4.0 Reference Data

Twenty-six of the 31 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 twenty-four of these substances (**Appendix C**). 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 five substances that were not considered in the original ICCVAM evaluation (ICCVAM 1999), *trans*-cinnamaldehyde, cyclamen aldehyde, glutaraldehyde, isopropyl myristate, and linalool, were obtained from Gerberick et al. (2005), Basketter 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), Opdyke (1976), Bjorkner (1984), Gad et al. (1986), Klecak et al. (1997), ICCVAM (1999), Basketter et al. (1999, 2005), Kwon et al. (2003), Takeyoshi et al. (2004a), and Takeyoshi et al. (2007a). Although there were no traditional LLNA data available for the eugenol dimers (dihydroxyl-3,3'-dimethoxy-5,5'-diallyl-biphenyl and 4,5'-diallyl-2'-hydroxy-2,3'-dimethoxyphenyl ether) or the isoeugenol dimers (4-[1-Hydroxy-2-(2-methoxy-4-propenyl-phenyoxy)-propyl]-2-methoxy-phenol and 2-Methoxy-4-(7-methoxy-3-methyl-5-propenyl-2,3-dihydro-benzofuran-2yl)-phenol), Takeyoshi et al. (2004a and 2007a, respectively) provided results from the GPMT for these compounds.

An independent quality assurance contractor for the NTP audited the traditional LLNA data provided in ICCVAM (1999). Audit procedures and findings are presented in the quality assurance report on file at the National Institute of Environmental Health Sciences. 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 test method data in this draft BRD has been revised from the January 2008 draft BRD to include the individual animal results for all of the LLNA: BrdU-ELISA results evaluated in this BRD. 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), and one poster presentation (Kojima et al. 2008). Dr. Takeyoshi also submitted unpublished data to NICEATM in January 2009. All test results were obtained using the protocol in **Appendix A**. 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. **Appendix C** contains summary data for the LLNA: BrdU-ELISA and comparative reference data for the 35 substances tested in these studies and **Appendix D** contains the individual animal data for the LLNA: BrdU-ELISA.

6.0 Test Method Accuracy

The accuracy evaluation in this draft BRD has been revised from the January 2008 draft BRD to include the results for seven additional substances. Other revisions included the evaluation of multiple decision criteria, including the SI ≥ 2.0 recommended in the test method protocol, and the evaluation of two different criteria used simultaneously to classify sensitizers and nonsensitizers.

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

Thirty-one of the 35 substances listed in **Table 3-1** 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-phenyoxy)-propyl]-2-methoxy-phenol and 2-methoxy-4-(7-methoxy-3-methyl-5-propenyl-2,3-dihydro-

benzofuran-2yl)-phenol) were excluded from the accuracy analyses because traditional LLNA data for these substances were not identified.

Of the 31 substances tested with both LLNA: BrdU-ELISA and the traditional LLNA, 24 had 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, diphenylcyclopropenone, 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 GPMT protocol[4].

Of the 31 substances tested with both LLNA: BrdU-ELISA and the traditional LLNA, 29 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 **Appendix C**.

Multiple tests were available for 14 substances tested with the LLNA: BrdU-ELISA. For the accuracy analyses, results 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, at SI $\geq$ 2.0, isopropanol was a nonsensitizer because five of the seven tests for isopropanol were negative.

Discordant test results were noted for three of the substances with multiple test results: formaldehyde, isopropanol, and lactic acid. For all three substances, the solvents used for each test were the same. One of the three laboratories in the interlaboratory validation study reported an SI of 1.97 for formaldehyde; while the others produced SI > 2 (Kojima et al. 2008). Two of the seven tests of isopropanol yielded SI $\geq$ 2 (SI = 2.0 and SI = 2.2), while the others yielded negative results. These discordant tests were obtained by two of the six laboratories in the interlaboratory validation study. The seventh test of isopropanol yielded SI < 2 (Takeyoshi et al. 2007b). One of the three tests for lactic acid from the interlaboratory

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

validation study produced SI ≥ 2 (i.e., SI = 2.5), while the others yielded SI > 2 (Kojima et al. 2008).

6.2 Accuracy Analysis Using the SI ≥ 2.0 Decision Criterion

The performance characteristics of the LLNA: BrdU-ELISA were first evaluated using the criterion of SI ≥ 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 **Appendix A)**.

6.2.1 Accuracy vs. the Traditional LLNA

When compared to the traditional LLNA and using a decision criteria of SI ≥ 2.0 to identify sensitizers, the LLNA: BrdU-ELISA had an accuracy of 84% (26/31), a sensitivity of 77% (17/22), a specificity of 100% (9/9), a false positive rate of 0% (0/9), and a false negative rate of 23% (5/22) (**Table 6-1**).

6.2.2 Accuracy vs. Guinea Pig Data

When the accuracy of the LLNA: BrdU-ELISA (SI ≥ 2.0) and the traditional LLNA were compared based on their performance relative to the GP test, the LLNA: BrdU-ELISA had a lower accuracy (88% [21/24] vs. 100% [24/24]) and sensitivity (81% [13/16] vs. 100% [16/16]), and higher false negative rate (19% [3/16] vs. 0% [0/16]; **Table 6-1**). The specificity (100% [8/8]) and the false positive rate (0% [0/8]) 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 ≥ 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 (72% [21/29] vs. 76% [22/29]) and sensitivity (67% [14/21] vs. 81% [17/21]) and a higher false negative rate (33% [7/21] vs. 19% [4/21]) than the traditional LLNA (**Table 6-1**). The specificity for the LLNA: BrdU-ELISA was higher (88% [7/8] vs. 63% [5/8]) and the false positive rate was lower (12% [1/8) vs. 38% [3/8]) for the LLNA: BrdU-ELISA than that for the traditional LLNA.

Table 6-1 Performance of the LLNA: BrdU-ELISA in Predicting Skin Sensitizing Potential Using Decision Criteria of SI ≥ 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	31	84	26/31	77	17/22	100	9/9	0	0/9	23	5/22	100	17/17	64	9/14
Substances with LLNA: BrdU-ELISA, Traditional LLNA, and GP Data															
BrdU-ELISA vs. Traditional LLNA	24	88	21/24	81	13/16	100	8/8	0	0/8	19	3/16	100	13/13	73	8/11
LLNA: BrdU-ELISA vs. GP[3]	24	88	21/24	81	13/16	100	8/8	0	0/8	19	3/16	100	13/13	73	8/11
Traditional LLNA vs. GP[3]	24	100	24/24	100	16/16	100	8/8	0	0/8	0	0/16	100	16/16	100	8/8
Substances with LLNA: BrdU-ELISA, Traditional LLNA, and Human Data															
BrdU-ELISA vs. Traditional LLNA	29	83	24/29	75	15/20	100	9/9	0	0/9	25	5/20	100	15/15	64	9/14
LLNA: BrdU-ELISA vs. Human[4]	29	72	21/29	67	14/21	88	7/8	12	1/8	33	7/21	93	14/15	50	7/14
Traditional LLNA vs. Human[4]	29	76	22/29	81	17/21	63	5/8	38	3/8	19	4/21	85	17/20	56	5/9

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

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

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

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

[4]Human refers to outcomes obtained by studies conducting using the human maximization test, inclusion of the test substance in a human patch test allergen kit, and/or published clinical case studies/reports.

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

ICCVAM has developed recommended test method performance standards for the traditional LLNA (ICCVAM 2009)[5], 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, the LLNA: BrdU-ELISA is not being evaluated using the ICCVAM-recommended LLNA performance standards. Thus, evaluations of the LLNA: BrdU-ELISA test substances to the ICCVAM-recommended LLNA performance standards test substances are shown to provide a general comparison to a set list of reference substances (18 required reference substances and four optional reference substances) that represent a diverse substance group. As shown in **Table 6-2**, 10 of the 18 minimum reference substances included in the ICCVAM LLNA Performance Standards have been tested in the LLNA: BrdU-ELISA. Nine of the ten substances yielded the same sensitizer/nonsensitizer outcome in the LLNA: BrdU-ELISA as in the traditional LLNA.

Table 6-3 provides the range and characteristics for 31 substances tested in the LLNA: BrdU-ELISA based on traditional LLNA data. These substances are compared to the range of 18 required reference substances included on the ICCVAM-recommended LLNA performance standards reference substances list (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 range of the substances tested in the LLNA: BrdU-ELISA is similar to that included in the performance standards list. In general, there are 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 6-2 Performance of the LLNA: BrdU-ELISA (SI ≥ 2.0) Using the ICCVAM Performance Standards Reference Substances[1]

Substance	Recommended Performance Standards[1]				LLNA: BrdU-ELISA[2]			
	Vehicle	Result	EC3 (%)[1]	N[3]	Vehicle	Result	EC2 (%)	N[3]
5-Chloro-2-methyl-4-isothiazolin-3-one	DMF	+	0.009	1	NT	NT	NT	NT
2, 4-Dinitrochlorobenzene	AOO	+	0.049	15	AOO	+	0.044	8
4-Phenylenediamine	AOO	+	0.11	6	AOO	+	NC	2
Methyl methacrylate	DMF	+	90	1	NT	NT	NT	NT
Isoeugenol	AOO	+	1.5	47	AOO	+	7.6	2
2-Mercaptobenzothiazole	***DMF***	**+**	***1.7***	***1***	***DMF***	**-**	***NA (-)***	***1***
Cobalt chloride	DMSO	+	0.6	2	NT	NT	NT	NT
Citral	AOO	+	9.2	6	AOO	+	NC	1
Hexyl cinnamic aldehyde	AOO	+	9.7	21	AOO	+	17.4	11
Eugenol	AOO	+	10.1	11	AOO	+	9.8	8
Phenyl benzoate	AOO	+	13.6	3	NT	NT	NT	NT
Cinnamic alcohol	AOO	+	21	1	NT	NT	NT	NT
Imidazolidinyl urea	DMF	+	24	1	NT	NT	NT	NT
Chlorobenzene	AOO	-	NA	1	NT	NT	NT	NT
Isopropanol	AOO	-	NA	1	AOO	-	NA (-)[4]	7
Lactic acid	DMSO	-	NA	1	DMSO	+	NA (-)[5]	3
Methyl salicylate	AOO	-	NA	9	AOO	NT	NA (-)	3
Salicylic acid	AOO	-	NA	1	NT	NT	NT	NT
Ethylene glycol dimethacrylate	MEK	False +	28 (FP)	1	NT	NT	NT	NT
Sodium lauryl sulfate	DMF	False +	8.1 (FP)	5	NT	NT	NT	NT
Nickel chloride	DMSO	False -	NA (FN)	2	NT	NT	NT	NT
Xylene	AOO	False -	95.8 (FP)	1	NT	NT	NT	NT

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

Abbreviations: AOO = acetone: olive oil (4: 1); LLNA: BrdU-ELISA= murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; EC3 = estimated concentration needed to produce a stimulation index of 3; EC2 = estimated concentration needed to produce a stimulation index of 2; FN = false negative in traditional LLNA when compared to guinea pig and/or human results; FP = false positive in traditional LLNA when compared to guinea pig and/or human results; LLNA = murine local lymph node assay; MEK = methyl ethyl ketone; NA = not applicable; NC = not calculated; only one concentration tested; NT = not tested; SI = Stimulation index.

+ = Sensitizer.

- = Nonsensitizer.

[1]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). Substances for which EC2 values were not available include the outcome of the LLNA: BrdU-ELISA test (+ = sensitizer; - = nonsensitizer) in parentheses.

[3]Number of values used to derive the mean EC3 or EC2.

[4]Based on the most prevalent outcome (i.e., 5/7 tests yielded SI < 2).

[5]Based on the most prevalent outcome (i.e., 2/3 tests yielded SI < 2).

Table 6-3 Characteristics of the Substances Tested in the LLNA: BrdU-ELISA vs. the ICCVAM Performance Standards Reference Substances[1]

EC3 Range (%)	No. Chems	Solid/ Liquid	Actual EC3 Range (%)	Maximum SI Range	Human Data	Peptide Reactivity (Hi/Mod/Min/Lo/Unk)[3]
<0.1	**4**	**3/1**	**0.01 - 0.083**	**18.0 –59.0**	**4**	**4/0/0/0/0**
	2	1/1	0.009 - 0.05	22.6 - 52.3	2	2/0/0/0/0
≥ 0.1 to <1	**2**	**1/1**	**0.11 - 0.50**	**4.0 – 26.4**	**2**	**0/1/0/0/1**
	2	2/0	0.11 - 0.6	6.7 - 75.3	2	0/0/0/0/2
≥ 1 to <10	**10**	**4/6**	**1.4 - 9.7**	**3.1 – 31.0**	**8**	**2/0/1/1/6**
	4	1/3	1.5 - 9.7	8.6 - 29.5	4	1/0/1/0/2
≥ 10 to <100	**5**	**0/5**	**10.1 – 47.5**	**3.4 - 17.0**	**5**	**0/0/1/2/2**
	5	3/2	10.1 - 90	5.5 - 70.3	5	0/1/0/0/4
Negative	**10**	**2/8**	**NC**	**1.0 – 2.9**	**10**	**0/0/7/1/2**
	5	1/4	NC	0.9 - 2.8	3	0/0/2/0/3
Overall	**31**	**10/21**	**0.01 – 47.5**	**0.9 - 28.6**	**29**	**6/1/9/4/11**
	18	10/8	0.009 - 24	0.9 - 75.3	16	3/1/3/0/11

Bolded text represents characteristics of the LLNA: BrdU-ELISA database.
Abbreviations: Chems = chemicals; EC3 = Estimated concentration needed to produce SI = 3; LLNA: BrdU-ELISA= murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; NC = Not calculated because maximum SI < 3; No. = number; Lo = low; Min = minimal; Mod = moderate; 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. (2007)

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 31 substances tested in the LLNA: BrdU-ELISA (using SI ≥ 2.0) and the traditional LLNA were compared, the classifications for five substances were different. The LLNA: BrdU-ELISA classified aniline, cyclamen aldehyde, hydroxycitronellal, 2-mercaptobenzothiazole, and linalool as nonsensitizers while the traditional LLNA classified them as sensitizers (i.e., false negative outcome) (**Table 6-4**). The substances were tested in the same vehicle in both the LLNA: BrdU-ELISA and the

951 traditional LLNA tests. The only commonality noted among these four substances was their
952 molecular weights (MW), which range from 93 to 172 g/mole. No commonalities in
953 chemical class, physical form, peptide reactivity (see **Appendix B** for physical/chemical
954 information), or potential for skin irritation were noted among these substances.

955 • Aniline (MW = 93.13 g/mole) is an amine, cyclamen aldehyde is a carboxylic
956 acid (MW = 190.28 g/mole), 2-mercaptobenzothiazole (MW = 167.26 g/mole)
957 is a heterocyclic compound, and hydroxycitronellal (MW = 172.26 g/mole)
958 and linalool (MW = 154.25 g/mole) are hydrocarbons.

959 • Aniline, cyclamen aldehyde, hydroxycitronellal, and linalool are liquids, while
960 2-mercaptobenzothiazole is a solid.

961 • Of the three substances for which peptide reactivity information was
962 available, hydroxycitronellal and cyclamen aldehyde had low peptide
963 reactivity and 2-mercaptobenzothiazole had high peptide reactivity.

964 • Cyclamen aldehyde, 2-mercaptobenzothiazole and linalool are skin irritants at
965 the concentrations tested in the LLNA: BrdU-ELISA, and linalool is also a
966 skin irritant at the concentrations tested the traditional LLNA.

967 • None of the five discordant substances generated strongly positive result in
968 the traditional LLNA (EC3 = 1.7% to 47.5%).

969

Table 6-4 Discordant Results for LLNA: BrdU-ELISA (Using SI ≥ 2.0 for Sensitizers) Compared to Traditional LLNA and Guinea Pig Reference Data[1]

Substance Name[2]	Vehicle[3]	LLNA: BrdU-ELISA[4]	Traditional LLNA[4]	Guinea Pig Studies	Skin Irritant?
Aniline (47.5%)	AOO	- (1.5, 50%)	+ (3.6, 100%)[5]	+	Negative at ≤ 100%
Hydroxycitronellal (24.0%)	AOO	- (1.3, 100%)	+ (8.5, 100%)	+	Negative at ≤ 100%
Cyclamen aldehyde (22.3%)	AOO	- (1.97, 100%)	+ (5.2, 50%)	NA	Irritant at 100%
Linalool (30.0%)	AOO	- (1.45, 100%)[5]	+ (8.3, 100%)	NA	Mild irritant at 10%
2-Mercaptobenzo-thiazole (1.7%)	DMF	- (1.62, 50%)[6]	+ (8.6, 10%)	+	Negative at ≤ 10%

Abbreviations: LLNA: BrdU-ELISA= murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; GP = outcomes of guinea pig skin sensitization tests; LLNA = murine local lymph node assay; NA = not available; SI = stimulation index.
+ = Sensitizer.
- = Nonsensitizer.
[1]Data sources provided in **Appendix C-1**.
[2]Numbers in parentheses are the EC3 values for the traditional LLNA (from **Table 3-1**).
[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 50%.
[6]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 24 substances with LLNA: BrdU-ELISA, traditional LLNA, and GP test results, the results for aniline, hydroxycitronellal, and 2-mercaptobenzothiazole were also discordant with the GP test results (**Table 6-4**). The LLNA: BrdU-ELISA results for aniline, hydroxycitronellal, and 2-mercaptobenzothiazole were negative, while the traditional LLNA and GP results were positive. No guinea pig results were available for linalool or cyclamen aldehyde, which were negative in the LLNA: BrdU-ELISA and positive in the traditional LLNA. As noted in **Section 6.3.1**, there were no commonalities associated with these discordant substances.

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

When analyses were restricted to the 29 substances with LLNA: BrdU-ELISA, traditional LLNA, and human outcomes, the LLNA: BrdU-ELISA misclassified eight substances. Both the LLNA: BrdU-ELISA and the traditional LLNA misclassified four human sensitizers (diethyl phthalate, 2-hydroxypropylmethacrylate, isopropanol, and propylene glycol) as nonsensitizers (**Table 6-5**). The LLNA: BrdU-ELISA also misclassified three other sensitizers as nonsensitizers that were correctly classified by the traditional LLNA (aniline, 2-mercaptobenzothiazole, and hydroxycitronellal).

The eighth misclassified substance was isopropyl myristate, which was misclassified as a sensitizer by the LLNA: BrdU-ELISA and the traditional LLNA. Isopropyl myristate exhibited a weak response in the traditional LLNA (EC3 = 44%). It was tested in both methods at concentrations that were not irritating to skin (**Table 6-5**). Isopropyl myristate (MW = 270.46 g/mole) is a liquid lipid that exhibits low peptide reactivity.

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

There was one discordant substance (2-mercaptobenzothiazole) noted among the 10 performance standards reference substances that were tested in LLNA: BrdU-ELISA. The LLNA: BrdU-ELISA classified this substance as a nonsensitizer, while the traditional LLNA, GP, and human tests classified it as a sensitizer. The EC3 value for the traditional LLNA, 1.7%, was derived from a test of 1, 3, and 10% 2-mercaptobenzothiazole in *N,N*-dimethylformamide (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 1.6, 1.4 and 1.5, respectively.

Table 6-5 Discordant Results for LLNA: BrdU-ELISA (SI ≥ 2.0) When Compared to Traditional LLNA and Human Outcome Data[1]

Substance Name[2]	Vehicle[3]	LLNA: BrdU-ELISA[4]	Traditional LLNA[4]	Human Outcome[5]	Skin Irritant?
Diethyl phthalate	AOO	- (0.9, 50%)	- (1.5, 100%)	+ (HPTA)	Negative at ≤ 100%
2-Hydroxypro-pylmethacrylate	AOO	- (1.1, 50%)	- (1.3, 50%)	+ (case study, 0.1%)	Negative at ≤ 10%
Isopropanol	AOO	- (2.2, 50%)[6]	- (1.7, 50%)[7]	+ (case study, 0.001%)	Negative at ≤ 100%
Propylene glycol	AOO	- (1.6, 50%)	- (1.6, 100%)[8]	+ (HPTA)	Negative at ≤ 25%
Aniline (47.5%)	AOO	- (1.5, 50%)	+ (3.6, 100%)[9]	+ (7/25, 20%)	Negative at ≤ 100%
2-Mercaptoben-zothiazole (1.7%)	DMF	- (1.62, 50%)[10]	+ (8.6, 10%)	+ (5/24, 10%)	Negative at ≤ 10%
Hydroxycitro-nellal (24.0%)	AOO	- (1.3, 100%)	+ (8.5, 100%)	+ (14/73, 20%)	Negative at ≤ 100%
Isopropyl myristate (44%)	AOO	+ (4.2, 50%)	+ (3.4, 100%)	- (0/25, 20%)	Negative at ≤ 100%

Abbreviations: LLNA: BrdU-ELISA= murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; HPTA = human patch test allergen; LLNA = murine local lymph node assay.
+ = Sensitizer.
- = Nonsensitizer.
[1]Data sources listed in **Appendix C-1**.
[2]Numbers in parentheses are EC3 values for the traditional LLNA (from **Table 3-1**).
[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.
[6]Negative based on most prevalent call. Highest SI of any test is shown. Highest SIs 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 50%.
[10]Highest SI occurred at 12.5%.

6.5 LLNA: BrdU-ELISA Accuracy Analysis Using One Alternative Decision Criterion

In addition to the accuracy analysis using SI ≥ 2.0 to classify substances as sensitizers, other decision criteria were evaluated for test method performance with the traditional LLNA serving as the reference test. The performance characteristics for 13 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 31 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, ≥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 vehicle control group using ANOVA with a post-hoc Dunnett's test, when multiple treatment groups were tested, or Student's t-test when only there was only one treatment group.
3. Mean absorbance values of treated groups ≥ 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 14 substances tested with the LLNA: BrdU-ELISA. The results for each of these substances 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 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 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 for 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 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 are detailed in **Appendix E**.

As shown in **Section 6.1**, using the most prevalent outcome and the decision criterion of SI ≥ 2.0 resulted in an accuracy of 84% (26/31), a sensitivity of 77% (17/22), a specificity of 100% (9/9), a false positive rate of 0% (0/9), and a false negative rate of 23% (5/22) (**Tables 6-1** and **6-6)**. Although using SI ≥ 2.5 produced the same results as SI ≥ 2.0, using higher SI values (i.e., SI ≥ 3.0 to SI ≥ 5.0) resulted in reduced overall accuracy, higher false negative rates, and lower false positive rates as compared to SI ≥ 2.0 (**Figure 6-1** and **Table 6-6**). Using a lower SI value (SI ≥ 1.5) as the decision criterion produced the same accuracy as SI ≥ 2.0 (84% [26/31]), but the false positive rate increased to 33% (3/9), and the false negative rate decreased to 9% (2/22). SI ≥ 1.3 is shown for comparison as 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 ≥ 95% confidence interval of the control group, or ≥ 2 or 3 SD from the control group mean), yielded accuracy values of 81 to 87%, with false negative rates of 0 to 14%, and false positive rates were 11 to 56%.

Table 6-6 Performance of the LLNA: BrdU-ELISA in Predicting Skin Sensitizing Potential Using Alternative Decision Criteria to Identify Sensitizers and the Most Prevalent Outcome for Substances with Multiple Tests

Alternate Criterion	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]
Statistics[3]	31	81	25/31	86	19/22	67	6/9	33	3/9	14	3/22	86	19/22	67	6/9
≥ 95% CI[4]	31	84	26/31	100	22/22	44	4/9	56	5/9	0	0/22	82	22/27	100	4/4
≥ 2 SD[5]	31	84	26/31	96	21/22	56	5/9	44	4/9	5	1/22	84	21/25	83	5/6
≥ 3 SD[6]	31	87	27/31	86	19/22	89	8/9	11	1/9	14	3/22	95	19/20	73	8/11
SI ≥ 5.0	31	58	18/31	41	9/22	100	9/9	0	0/9	59	13/22	100	9/9	41	9/22
SI ≥ 4.5	31	58	18/31	41	9/22	100	9/9	0	0/9	59	13/22	100	9/9	41	9/22
SI ≥ 4.0	31	71	22/31	59	13/22	100	9/9	0	0/9	41	9/22	100	13/13	50	9/18
SI ≥ 3.5	31	74	23/31	64	14/22	100	9/9	0	0/9	36	8/22	100	14/14	53	9/17
SI ≥ 3.0	31	77	24/31	68	15/22	100	9/9	0	0/9	32	7/22	100	15/15	56	9/16
SI ≥ 2.5	31	84	26/31	77	17/22	100	9/9	0	0/9	23	5/22	100	17/17	64	9/14
SI ≥ 2.0	31	84	26/31	77	17/22	100	9/9	0	0/9	23	5/22	100	17/17	64	9/14
SI ≥ 1.5	31	84	26/31	91	20/22	67	6/9	33	3/9	9	2/22	87	20/23	75	6/8
SI ≥ 1.3	31	87	27/31	100	22/22	56	5/9	44	4/9	0	0/22	85	22/26	100	5/5

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

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

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

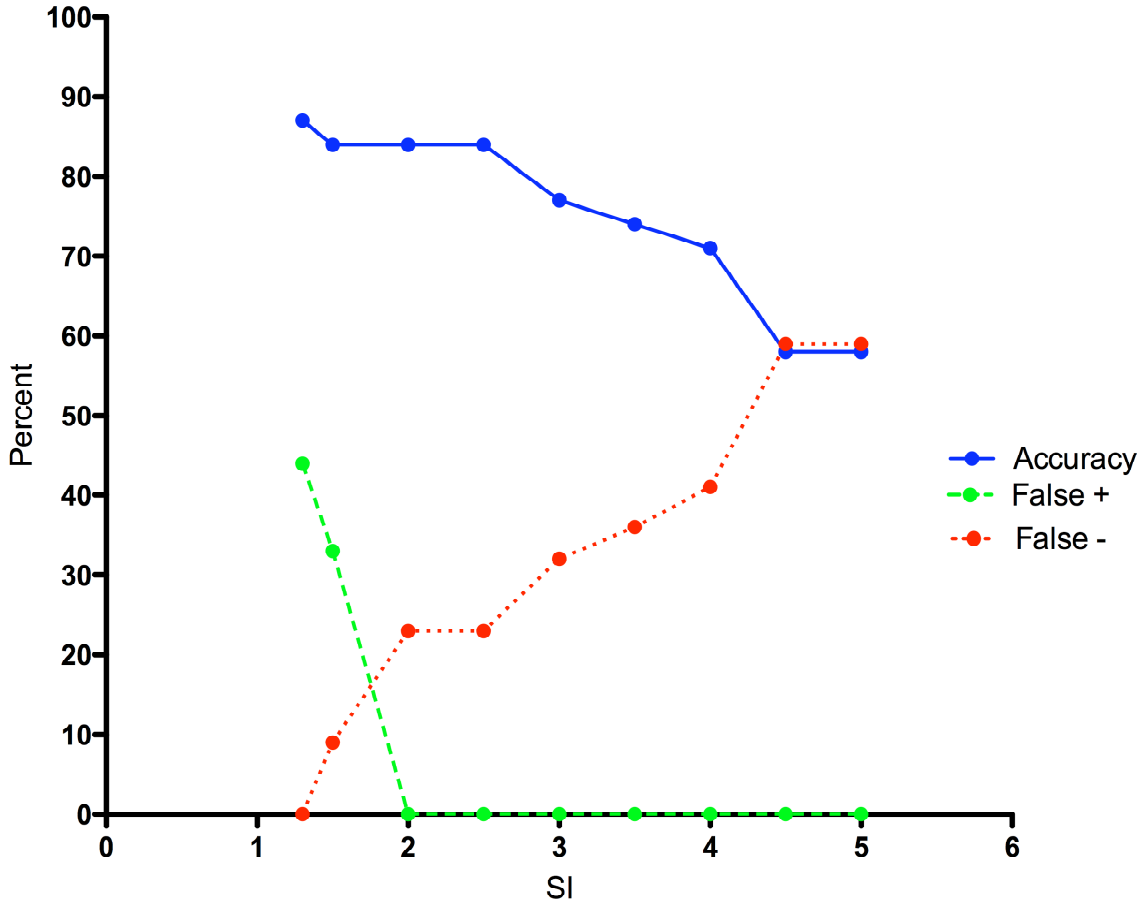
[3] Analysis of variance for difference of group means when substances were tested at multiple doses or *t*-test when substances were tested at one dose. The absorbance data were log-transformed prior to analysis of variance. Significance at $p < 0.05$ was further tested by Dunnett's test.

[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

Figure 6-1 Performance of the LLNA: BrdU-ELISA 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 29 substances (20 sensitizers and nine nonsensitizers). For the 14 substances with multiple test results, the results for each substance were combined using the most prevalent outcome. The solid line shows accuracy, the dashed green line shows the false positive rate, and the dotted red line shows the false negative rate.

The decision criteria of SI ≥ 1.5 and mean absorbance of treated group ≥ 2 SD from the vehicle control group are compared with SI ≥ 2.0 for accuracy of the LLNA: BrdU-ELISA against GP and human data in **Table 6-7**. When GP test results were used as the reference data, SI ≥ 1.5 and mean absorbance of treated group ≥ 2 SD from the vehicle control group had the same accuracy (88% [21/24]), lower false negative rates (6% [1/16] for SI ≥ 1.5 vs. 0% [0/16] for mean absorbance of treated group ≥ 2 SD from the vehicle control group vs.

19% [3/16] for SI ≥ 2.0), and increased false positive rate (25% [2/8] for SI ≥ 1.5 vs. 38% [3/8] for mean absorbance of treated group ≥ 2 SD from the vehicle control group vs. 0% [0/8] for SI ≥ 2.0) when compared with SI ≥ 2.0. Using mean absorbance of treated group ≥ 2 SD from the vehicle control group had the most impact on the false negative rate. It decreased the number of false negatives from three (for SI ≥ 2.0) to zero, but the number of false positives increased from zero (for SI ≥ 2.0) to three.

When results were compared to human data, SI ≥ 1.5 and mean absorbance of treated group ≥ 2 SD from the vehicle control group produced the same the accuracy (72% [21/29]), decreased the false negative rate (19% [4/21] for SI ≥ 1.5 vs. 14% [3/21] for mean absorbance of treated group ≥ 2 SD from the vehicle control group vs. 33% [7/21] for SI ≥ 2.0), and increased the false positive rate (50% [4/8] for SI ≥ 1.5 vs. 63% [5/8] for mean absorbance of treated group ≥ 2 SD from the vehicle control group vs. 12% [1/8] for SI ≥ 2.0) compared with SI ≥ 2.0. Using mean absorbance of treated group ≥ 2 SD from the vehicle control group had the most impact on the false negative rate. It decreased the number of false negatives from seven (for SI ≥ 2.0) to three, but the number of false positives increased from one (for SI ≥ 2.0) to five.

Table 6-7 Comparison of Performance for Decision Criteria of SI ≥ 1.5 (Bold), > 2 SD (Bold Italics), and SI ≥ 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]
BrdU-ELISA vs. Traditional LLNA	31	**84** ***84*** 84	**26/31** ***26/31*** 26/31	**91** ***96*** 77	**20/22** ***21/22*** 17/22	**67** ***56*** 100	**6/9** ***5/9*** 9/9	**33** ***44*** 0	**3/9** ***4/9*** 0/9	**9** ***5*** 23	**2/22** ***1/22*** 5/22	**87** ***84*** 100	**20/23** ***21/25*** 17/17	**75** ***83*** 64	**6/8** ***5/6*** 9/14
Substances with LLNA: BrdU-ELISA, Traditional LLNA, and GP Data															
BrdU-ELISA vs. Traditional LLNA	24	**88** ***88*** 88	**21/24** ***21/24*** 21/24	**94** ***100*** 81	**15/16** ***16/16*** 13/16	**75** ***63*** 100	**6/8** ***5/8*** 8/8	**25** ***38*** 0	**2/8** ***3/8*** 0/8	**6** ***0*** 19	**1/16** ***0/16*** 3/16	**88** ***84*** 100	**15/17** ***16/19*** 13/13	**86** ***100*** 73	**6/7** ***5/5*** 8/11
LLNA: BrdU-ELISA vs. GP[3]	24	**88** ***88*** 88	**21/24** ***21/24*** 21/24	**94** ***100*** 81	**15/16** ***16/16*** 13/16	**75** ***63*** 100	**6/8** ***5/8*** 8/8	**25** ***38*** 0	**2/8** ***3/8*** 0/8	**6** ***0*** 19	**1/16** ***0/16*** 3/16	**88** ***84*** 100	**15/17** ***16/19*** 13/13	**86** ***100*** 73	**6/7** ***5/5*** 8/11
Traditional LLNA vs. GP[3]	24	100	24/24	100	16/16	100	8/8	0	0/8	0	0/16	100	16/16	100	8/8
Substances with LLNA: BrdU-ELISA, Traditional LLNA, and Human Data															
BrdU-ELISA vs. Traditional LLNA	29	**83** ***83*** 83	**24/29** ***24/29*** 24/29	**90** ***95*** 75	**18/20** ***19/20*** 15/20	**67** ***56*** 100	**6/9** ***5/9*** 9/9	**33** ***44*** 0	**3/9** ***4/9*** 0/9	**10** ***5*** 25	**2/20** ***1/20*** 5/20	**86** ***83*** 100	**18/21** ***19/23*** 15/15	**75** ***83*** 64	**6/8** ***5/6*** 9/14
LLNA: BrdU-ELISA vs. Human[4]	29	**72** ***72*** 72	**21/29** ***21/29*** 21/29	**81** ***86*** 67	**17/21** ***18/21*** 14/21	**50** ***38*** 88	**4/8** ***3/8*** 7/8	**50** ***63*** 12	**4/8** ***5/8*** 1/8	**19** ***14*** 33	**4/21** ***3/21*** 7/21	**81** ***78*** 93	**17/21** ***18/23*** 14/15	**50** ***50*** 50	**4/8** ***3/6*** 7/14
Traditional LLNA vs. Human[4]	29	76	22/29	81	17/21	63	5/8	38	3/8	19	4/21	85	17/20	56	5/9

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.

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

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

[3]GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.

[4]Human refers to outcomes obtained by studies conducting using the human maximization test, inclusion of the test substance in a human patch test allergen kit, and/or published clinical case studies/reports.

6.6 Discordant Results for Accuracy Analysis Using One Alternative Decision Criterion

This section discusses the discordant results obtained for the analyses using the alternative decision criteria shown in **Tables 6-6** and **6-7** to provide a comparison to the discordant substances identified using the decision criteria of SI ≥ 2.0 to identify sensitizers. Discordant results are first discussed using the traditional LLNA as the reference test (**Section 6.5.1**) and then discordant results for SI ≥ 1.5, the optimized single criterion, are discussed using the traditional LLNA, GP, and human outcomes as references (**Section 6.5.2**).

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

Using decision criteria of SI ≥ 2.0 and the most prevalent outcome for the substances with multiple tests, the five discordant substances, when compared to the traditional LLNA, were aniline, cyclamen aldehyde, hydroxycitronellal, linalool, and 2-mercaptobenzothiazole (**Table 6-4**). As indicated in **Section 6.3**, all five substances were false negatives when compared to the traditional LLNA.

Table 6-8 shows how the number and identity of discordant substances changes with the alternate 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 6-6**) or summary statistics (i.e., ≥ 95% CI, or ≥ 2 or 3 SD in **Table 6-6**) did not result in substantively improved performance relative to using SI ≥ 1.5.

Table 6-8 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]	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 ≥ 1.5	SI ≥ 1.3
Formaldehyde (0.53%)					-	-							
trans-Cinnamic aldehyde (1.4%)					-	-							
2-Mercaptobenzothiazole (1.7%)	-				-	-	-	-	-	-	-		
Cinnamic aldehyde (2.4%)					-	-							
3-Aminophenol (3.2%)					-	-	-	-					
Nickel sulfate (4.8%)					-	-	-	-	-				
4-Chloroaniline (6.5%)					-	-	-	-	-				
Hexyl cinnamic aldehyde (9.7%)					-	-	-						
Cyclamen aldehyde (22.3%)					-	-	-	-	-	-	-		
Hydroxycitronellal (24%)				-	-	-	-	-	-	-	-	-	
Linalool (30%)			-	-	-	-	-	-	-	-	-	-	
Isopropyl myristate (44%)					-	-							
Aniline (63%)	-			-	-	-	-	-	-	-	-		
Glycerol (-)	+	+	+										
Hexane (-)	+	+	+	+								+	+
Lactic acid (-)	+	+	+									+	+
Methyl salicylate (-)		+											+
Propylene glycol (-)		+	+									+	+

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

[1]LLNA: BrdU-ELISA outcomes are indicated by "+" for sensitizer results and "-" for nonsensitizer results.

[2]Compared to the traditional LLNA. Traditional LLNA result in parentheses: "-" for nonsensitizers and EC3 (%) for sensitizers.

[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.

Four ICCVAM performance standards reference substances were discordant for the analysis of alternate decision criteria using the most prevalent outcome for substances with multiple tests (**Table 6-6**). Two sensitizers (2-mercaptobenzothiazole and hexyl cinnamic aldehyde) 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 2-mercaptobenzothiazole included summary statistics (i.e., ≥ 95% CI, ≥ 2 SD, or 3 ≥ SD) and SI ≥ 1.5. The criteria that yielded the correct results for hexyl cinnamic aldehyde included statistical tests (i.e., ANOVA or *t*-test), summary statistics (i.e., ≥ 95% CI, ≥ 2 SD, or 3 ≥ SD), and SI ≥ 1.5 to 3.5. The criteria that yielded the correct results for lactic acid included treatment group mean ≥ 3 SD from the vehicle control, and SI ≥ 2.0 to 5.0. All criteria yielded the correct results for methyl salicylate except for treatment group absorbance ≥ 95% CI of vehicle control mean.

6.6.2 *Discordant Results for Accuracy Analysis of the SI ≥1.5 Decision Criterion*

When the outcomes for the 31 substances tested in the LLNA: BrdU-ELISA (using SI ≥ 1.5) and the traditional LLNA were compared, the classifications for five substances were different. For the three substances with GP data, the GP tests and traditional LLNA yielded the same sensitizer/nonsensitizer results (**Table 6-9**). Two substances were misclassified in the LLNA: BrdU-ELISA as nonsensitizers (hydroxycitronellal and linalool) and three were misclassified as sensitizers (hexane, lactic acid and propylene glycol). Chemical class, physical form, MW, peptide reactivity (see **Appendix B** for physical/chemical properties), traditional LLNA EC3 range, or potential for skin irritation were examined to identify commonalities among the discordant substances. For the two substances misclassified as nonsensitizers:

- Hydroxycitronellal (MW = 172.26 g/mole) and linalool (MW = 154.25 g/mole) are hydrocarbons in a liquid form with similar molecular weights.
- Hydroxycitronellal exhibits low peptide reactivity; peptide reactivity information is not available for linalool.
- Hydroxycitronellal and linalool were not strongly positive in the traditional LLNA (EC3 = 24% and 30% with maximum SI = 8.5 and 8.3, respectively, at 100%).

- Linalool is a skin irritant at the concentrations tested in the LLNA: BrdU-ELISA and traditional LLNA, but hydroxycitronellal was not.

For the three substances misclassified as sensitizers in the LLNA: BrdU-ELISA (hexane, lactic acid and propylene glycol), although they represented different chemical classes (acyclic hydrocarbons, carboxylic acids, and alcohols, respectively) all three:

- Are liquids
- Have minimal peptide reactivity
- Have molecular weights below 100 g/mole
- Were tested at concentrations that are irritating to skin.

Table 6-9 Discordant Results for LLNA: BrdU-ELISA (Using SI ≥ 1.5 for Sensitizers) Compared to Traditional LLNA and Guinea Pig Reference Data[1]

Substance Name[2]	Vehicle[3]	LLNA: BrdU-ELISA[4]	Traditional LLNA[4]	Guinea Pig Studies	Skin Irritant?
Hydroxycitronellal (24.0%)	AOO	- (1.3, 100%)	+ (8.5, 100%)	+	Negative at ≤ 100%
Linalool (30.0%)	AOO	- (1.45, 100%)[5]	+ (8.3, 100%)	NA	Mild irritant at 10%
Hexane	AOO	+ (1.8, 100%)[6]	- (2.2, 100%)	NA	Irritant at 100%
Lactic acid	DMSO	+ (2.5, 50%)	- (2.2, 25%)	-	Slightly irritating at 10%
Propylene glycol	AOO	+ (1.6, 50%)	- (1.6, 100%)	-	Negative at ≤ 25%

Abbreviations: AOO = acetone: olive oil (4: 1); LLNA: BrdU-ELISA= murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; DMSO = dimethyl sulfoxide;; LLNA = murine local lymph node assay; NA = not available.
+ = Sensitizer.
- = Nonsensitizer.
[1]Data sources provided in **Appendix C-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 3-1**).
[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 50%.
[6]An additional test yielded an SI of 1.9 at 50%.

When the outcomes for the 29 substances with LLNA: BrdU-ELISA (using SI ≥ 1.5) and human outcome data were compared, the classifications for eight substances were different

(**Table 6-10**). The LLNA: BrdU-ELISA results for three of these substances (hydroxycitronellal, hexane, and lactic acid) were discordant with the traditional LLNA. The LLNA: BrdU-ELISA classified four human sensitizers as nonsensitizers (diethyl phthalate, 2-hydroxypropylmethacrylate, isopropanol, and hydroxycitronellal) and four human nonsensitizers as sensitizers (cyclamen aldehyde, isopropyl myristate, hexane, and lactic acid).

Table 6-10 Discordant Results for LLNA: BrdU-ELISA (SI ≥ 1.5) When Compared to Traditional LLNA and Human Outcome Data[1]

Substance Name[2]	Vehicle[3]	LLNA: BrdU-ELISA[4]	Traditional LLNA[4]	Human Outcome[5]	Skin Irritant?
Diethyl phthalate	AOO	- (0.9, 50%)	- (1.5, 100%)	+ (HPTA)	Negative at ≤ 100%
2-Hydroxypro-pylmethacrylate	AOO	- (1.1, 50%)	- (1.3, 50%)	+ (case study, 0.1%)	Negative at ≤ 10%
Isopropanol	AOO	- (2.2, 50%)[6]	- (1.7, 50%)[7]	+ (case study, 0.001%)	Negative at ≤ 100%
Hydroxycitro-nellal (24.0%)	AOO	- (1.3, 100%)	+ (8.5, 100%)	+ (14/73, 20%)	Negative at ≤ 100%
Cyclamen aldehyde (22.3%)	AOO	+ (1.97, 100%)	+ (5.2, 50%)	- (0/64, 4%)	Irritant at 100%
Isopropyl myristate (44%)	AOO	+ (4.2, 50%)	+ (3.4, 100%)	- (0/25, 20%)	Negative at ≤ 100%
Hexane	AOO	+ (1.8, 100%)[8]	- (2.2, 100%)	- (0/25, 100%)	Irritant at 100%
Lactic acid	DMSO	+ (2.5, 50%)	- (2.2, 25%)	- (no data)	Slightly irritating at 10%

Abbreviations: 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; HPTA = human patch test allergen; LLNA = murine local lymph node assay.
+ = Sensitizer.
- = Nonsensitizer.
[1]Data sources provided in **Appendix C-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 3-1**.
[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.
[6]Negative based on most prevalent call. Highest SI of any test is shown. Highest SIs for most tests occurred at < 50%.
[7]Highest SI occurred at 10%.
[8]An additional test yielded SI=1.9 at 50%.

Few commonalities in chemical class, physical form, molecular weight, peptide reactivity, traditional LLNA EC3 range, or potential for skin irritation were noted among the discordant substances. The four human sensitizers that were misclassified as nonsensitizers:

- Represented three different chemical classes: carboxylic acids (diethyl phthalate and 2-hydroxypropylmethacrylate), alcohols (isopropanol), and hydrocarbons (hydroxycitronellal) (**Tables 3-1** and **6-10**).
- Three substances were liquids (diethyl phthalate, isopropanol, and hydroxycitronellal) and one was a solid (2-hydroxypropylmethacrylate).
- Molecular weights ranged from 60.10 (isopropanol) to 222.24 g/mole (diethyl phthalate).
- All four substances exhibited low peptide reactivity.
- Three were classified as nonsensitizers by the traditional LLNA and one was classified as a sensitizer (hydroxycitronellal with EC3 = 24.0%).
- Although 2-hydroxypropylmethacrylate is a skin irritant at the concentrations tested in the LLNA: BrdU-ELISA, the other three substances were not irritating to skin at the concentrations tested (**Table 6-10**).

There were few commonalities in chemical class, physical form, molecular weight, peptide reactivity, EC3 range (based on the traditional LLNA), or potential for skin irritation noted among the four human nonsensitizers that were misclassified as sensitizers by the LLNA: BrdU-ELISA.

- The four substances represented three different chemical classes: carboxylic acids (cyclamen aldehyde and lactic acid), lipids (isopropyl myristate), and acyclic hydrocarbons (hexane) (**Tables 3-1** and **6-10**).
- While all four substances are liquids, with minimal to low peptide reactivity, molecular weights ranged from 86.15 g/mole for hexane to 270.46 g/mole for isopropyl myristate.
- Cyclamen aldehyde and isopropyl myristate were also classified as sensitizers by the traditional LLNA (EC3 values were 22.3% and 44%, respectively), but

hexane and lactic acid were classified as nonsensitizers by the traditional LLNA.

- Two of the substances (cyclamen aldehyde and lactic acid) misclassified as sensitizers were tested at concentrations that are irritating to skin, but two were not (isopropyl myristate and hexane) (**Table 6-10**).

6.7 LLNA: BrdU-ELISA 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. The lowest decision criterion with a 0% (0/9) false positive rate was SI ≥ 2.0, which was used by the JSAAE interlaboratory validation study. The accuracy at SI ≥ 2.0 was 84% (26/31) and the false negative rate was 23% (5/22) (**Table 6-6)**. Higher SI values also produced false positive rates of 0% (0/9), but the false negative rate increased as the SI increased. The lowest false negative rate was produced at SI ≥ 1.3 (0% [0/22]), but the false positive rate at SI ≥ 1.3 was 44% (4/9).

The 0% false positive rate using SI ≥ 2.0 and the 0% false negative rate using SI ≥ 1.3 prompted an evaluation of using two decision criteria for LLNA: BrdU-ELISA results: one criterion to classify substances as sensitizers (i.e., SI ≥ 2.0) and one criterion to classify substances as nonsensitizers (i.e., SI < 1.3). The SI ≥ 1.3 criterion, when used to classify sensitizers, resulted in no false negative results with respect to the traditional LLNA results. However, using SI ≤ 1.3 to classify substances as nonsensitizers resulted in one false negative result (4% [1/22]), which was for hydroxycitronellal (at 100% the LLNA: BrdU-ELISA SI = 1.30, while the traditional LLNA SI = 8.5). Thus, SI < 1.3 is proposed to classify substances as nonsensitizers because this criterion results in no false negative results.

It should be noted that this analyses was based on the same strategy for combining results from multiply tested substances described in **Section 6.5** (i.e., the sensitizer/nonsensitizer outcome for each substance was most prevalent outcome). **Section 7.3** details the reproducibility of multiply tested substances and indicates that, while there were isolated instances of false positive results for nonsensitizers (i.e., SI ≥ 2.0), there were no false negatives. Among the 78 tests that produced a maximum SI ≥ 2.0, 4% (3/78) were

nonsensitizers (i.e., produced a false positive result). These results were obtained for isopropanol and lactic acid, which produced SI = 2.0 and 2.2 in two different tests in the LLNA: BrdU-ELISA and one test of lactic acid, which produced an SI = 2.5. Isopropanol and lactic acid are classified as nonsensitizers based on maximum SI values of 1.7 and 2.2, respectively in the traditional LLNA. See **Section 7.3** from more details regarding these results.

6.8 Discordant Results for Accuracy Analysis Using Multiple Alternative Decision Criteria

While optimum false positive and false negative rates can be achieved using these two different decision criteria, a range of SI values ($1.3 \leq SI < 2.0$) now exists for which the correct classification is not definitive (i.e., there is a chance for false positives or false negatives for substances in this range). Chemical class, physical form, MW, peptide reactivity (see **Appendix B** for physical/chemical properties), traditional LLNA EC3 range, or potential for skin irritation were examined to identify commonalities among the substances that produced SI values of 1.3 to < 2.0 in an attempt to identify common characteristics among these substances that could be used to correctly classify such substances.

Eleven substances produced SI values from 1.3 to < 2.0 (see **Table 6-11**). Five of the 11 substances are nonsensitizers and six are sensitizers based on traditional LLNA results. The five substances classified by the traditional LLNA as nonsensitizers (methyl salicylate, isopropanol, propylene glycol, hexane and lactic acid), represented four chemical classes (carboxylic acids, alcohols, phenols and acyclic hydrocarbons).

- Two substances are classified as carboxylic acids (methyl salicylate [also a phenol] and lactic acid) and two were classified as alcohols (isopropanol, and propylene glycol).
- Hexane is an acyclic hydrocarbon.

Table 6-11 Discordant Results for LLNA: BrdU-ELISA When Multiple Decision Criteria Are Used [1]

Substance Name[2]	Vehicle[3]	LLNA: BrdU-ELISA[4]	Traditional LLNA[5]	Skin Irritant?
Hexane	AOO	1.76, 100%[7] 1.9, 50% (2/2 tests)	- (2.2, 100%)	Irritant at 100%
Isopropanol	AOO	1.6, 50% (1/7 tests)	- (1.7, 50%)[9]	No, up to 100%
Lactic acid	DMSO	1.8, 50% 1.9, 50% (2/2 tests)	- (2.2, 25%)	Slightly irritating at 10%
Methyl salicylate	AOO	1.4, 50% (3/3 tests at SI = 1.4)	- (2.9, 20%)	Irritant at 10%
Propylene glycol	AOO	1.6, 50% (1/2 tests)	- (1.6, 100%)	No, up to 25%
Aniline (47.5%)	AOO	1.5, 50%	+ (3.6, 100%)[7]	No, up to 100%
Hydroxycitronellal (24.0%)	AOO	1.30, 100%	+ 8.5, 100%)	No, up to 50%
Linalool (30.0%)	AOO	1.45, 100%[7]	+ (8.3, 100%)	Mild irritant at 100%
2-Mercaptobenzo-thiazole (1.7%)	DMF	1.6, 50%[10]	+ (8.6, 10%)	No, up to 10%
Cyclamen aldehyde (22.3%)	AOO	1.97, 100%	+ 5.2, 50%	Irritant at 100%
Formaldehyde (0.50%)	ACE	1.97, 10% (1/3 tests)	+ (4.0, 1.8%)	No, up to 2%

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4: 1); DMF = *N,N*-Dimethylformamide; DMSO = dimethyl sulfoxide; LLNA: BrdU-ELISA= murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; HPTA = human patch test allergen; LLNA = murine local lymph node assay; NA = not available; + = Sensitizer; - = Nonsensitizer.

[1]Data sources provided in **Appendix C-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 3-1**.

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

[4]Numbers are highest SI values achieved and maximum concentration tested.

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

[7]Highest SI occurred at 50%.

[8]The solvent for the traditional LLNA was *N,N*-dimethylformamide.
[9]Highest SI occurred at 10%.
[10]Highest SI occurred at 12.5%.

Other characteristics of the nonsensitizers (by the traditional LLNA) include:

- All of the five substances are liquids and have minimal peptide reactivity.
- Four substances have MW < 100 g/mole. The other substances, methyl salicylate, has a MW of 152.15 g/mole, respectively.
- Four of the five substances were tested at irritating concentrations in both the traditional LLNA and in the LLNA: BrdU-ELISA: methyl salicylate, propylene glycol, hexane and lactic acid. Isopropanol was tested at concentrations nonirritating to skin.
- Two of the five substances yielded SI < 2 in the traditional LLNA: isopropanol and propylene glycol. The other three substances yielded SI values between 2 and 3 (exclusive): hexane, lactic acid and methyl salicylate.

The six substances classified by the traditional LLNA as sensitizers (aniline, hydroxycitronellal, linalool, 2-mercaptobenzothiazole, formaldehyde, and cyclamen aldehyde) represent five chemical classes. Aniline is an amine, hydroxycitronellal and linalool are hydrocarbons (other), 2-mercaptobenzothiazole is a heterocyclic compound, formaldehyde is an aldehyde, and cyclamen aldehyde is a carboxylic acid. Other characteristics of the discordant substances that are classified as sensitizers by the traditional LLNA include:

- Five are liquids and one is a solid (2-mercaptobenzothiazole).
- Three substances have MW between 150 and 200 g/mole. Formaldehyde and aniline both have MW less than 100 g/mole (MW =30 g/mole and 93.13 g/mole, respectively).
- Hydroxycitronellal and cyclamen aldehyde exhibit low peptide reactivity, formaldehyde exhibits moderate peptide reactivity, 2-mercaptobenzothiazole exhibits high peptide reactivity, and peptide reactivity information is not available for the other two substances.

- Aniline, linalool, hydroxycitronellal, and cyclamen aldehyde were not strongly positive in the traditional LLNA (EC3 = 47.5%, 30%, 24%, and 22.3%) respectively, with maximum SI = 3.6, 8.3, 8.5, and 5.2, respectively, when tested at concentrations up to 100%.
- Hydroxycitronellal, linalool, and 2-mercaptobenzothiazole were tested at concentrations in the LLNA: BrdU-ELISA and traditional LLNA that were irritating to skin, but aniline was not. Formaldehyde and cyclamen aldehyde were tested at concentrations in the LLNA: BrdU-ELISA that were irritating to skin, but was not tested at irritating concentrations in the traditional LLNA.

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 reproducibility evaluation in this draft BRD has been revised from the January 2008 draft BRD to include the results for a number of additional intralaboratory tests for which SI values were newly available. The interlaboratory reproducibility evaluation is a new addition to this draft BRD because interlaboratory data were not available for evaluation in the January 2008 draft BRD. This draft BRD also includes a reproducibility analysis using separate SI criteria to identify sensitizers and nonsensitizers.

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 ≥ 2.0 to identify sensitizers. The evaluation of single decision criteria in **Section 6.6** showed that this was the lowest SI value that produced a false positive rate of 0% (0/9) when the traditional LLNA was the reference test (**Table 6-6)**. The evaluation of multiple decision criteria in **Section 6.7** evaluated SI ≥ 2.0 as the decision criterion for classifying substances as sensitizers when used with a decision criterion of <1.3 to identify nonsensitizers. In addition, the decision criterion of SI ≥ 2.0 to identify sensitizers was used in the JSAAE interlaboratory validation study. **Appendix F** describes the evaluation of reproducibility for the decision criterion of SI ≥ 1.5 to identify sensitizers, which was evaluated in **Section 6.6**.

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 EC2 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 EC3 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 eight substances that were tested multiple times and classified as sensitizers or nonsensitizers. Hexyl cinnamic aldehyde was tested six times, eugenol was tested five times, and isoeugenol was tested three times, and 2,4-dinitrochlorobenzene, glutaraldehyde, hexane, 4-phenylenendiamine, and propylene glycol were each tested twice (Takeyoshi et al. 2003, 2004a, 2005, 2006, 2007a; unpublished data) (**Table 7-1**). All substances were sensitizers in the traditional LLNA except for propylene glycol and hexane. The multiple test results for 8/8 substances were 100% concordant when SI $\geq$ 2.0 was used to classify substances as sensitizers.

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 similar for LLNA: BrdU-ELISA (7/8) and the traditional LLNA (5/6).

Table 7-1 Intralaboratory Reproducibility for the LLNA: BrdU-ELISA Outcome of Substances Tested Multiple Times

Substance	Highest Concentration Tested (%)	Highest SI	Outcome[1]	Takeyoshi et al. Reference
2,4-Dinitro-chlorobenzene	2	17.9	+	2005
	2	6.8	+	2006, 2007b
Eugenol	30	3.3	+	2004a
	30	3.8	+	2007a
	50	12.3	+	2005
	50	3.1	+	2006
	50	17.7	+	2007b
Glutaraldehyde	2	14.6	+	2005, 2007b
	10	15.5	+	2005, 2007b
Hexane	50	1.9	-	2005
	100	1.8	-	unpublished data
Hexyl cinnamic aldehyde	25	2.4	+	2003
	50	3.6	+	2003
	50	5.9	+	2005
	50	3.6	+	2006
	50	2.7	+	2006
	50	3.0	+	2007b
Isoeugenol	10	8.4	+	2005
	10	2.4	+	2006, 2007b
	30	6.7	+	
4-Phenylenediamine	2	11.7	+	2005, 2007b
	10	14.7	+	2005, 2007b
Propylene glycol	50	1.6	-	2005
	50	0.9	-	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*

There were six substances that were tested multiple times by Takeyoshi et al. (2003, 2004a, 2005, 2006, 2007a, 2007b, unpublished data). Because two substances had multiple tests for more than one concentration, there were nine 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 7-2**, the CVs ranged from 1% (25% hexyl cinnamic aldehyde) to 79% (10% isoeugenol). The intralaboratory reproducibility of the traditional LLNA was not assessed by CV analysis of SI values (ICCVAM 1999).

7.1.3 *Intralaboratory Reproducibility – EC2*

CV values were also calculated for the EC2 values for the three sensitizers that were tested more than once using multiple doses by Takeyoshi et al. (2003; 2004a, 2005, 2006, 2007a, 2007b). The individual animal data for eugenol, hexyl cinnamic aldehyde, and isoeugenol, were used to calculate EC2 values for the LLNA: BrdU-ELISA. The methods for calculating EC2 values for each sensitizer were modified from those used by Ryan et al. (2007) to calculate EC3 values. Linear interpolation was used to calculate EC2 values for each test with SI values higher or lower than two and extrapolation was used to calculate EC2 values for tests with no SI values below two. The equation for linear interpolation was:

$$EC2 = c + \left[\frac{(2-d)}{(b-d)}\right] \times (a-c)$$

The linear interpolation equation uses the points immediately above and below SI = 2, with the (dose, SI) coordinates of (a, b) immediately above SI = 2 and (c, d) immediately below SI = 2. The equation for extrapolation was:

$$EC2_{ex} = 2^{\left\{\log_2(c)+\frac{(2-d)}{(b-d)}\times\left[\log_2(a)-\log_2(c)\right]\right\}}$$

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

Substance	Concentration Tested (%)	SI	Mean	SD	CV (%)	Takeyoshi et al. Reference
2,4-Dinitrochlorobenzene	2	17.9	12.4	7.8	64	2005
	2	6.8				2006, 2007b
Eugenol	30	3.3	3.6	0.4	10	2004a
	30	3.8				2007a
Eugenol	50	12.3	11.0	7.4	67	2005
	50	3.1				2006
	50	17.7				2007b
Hexane	50	1.9	1.6	0.4	22	2005
	50	1.4				unpublished
Hexyl cinnamic aldehyde	12.5	1.87	1.73	0.21	12	2003
	12.5	1.58				2003
Hexyl cinnamic aldehyde	25	2.42	2.4	0.01	1	2003
	25	2.40				2003
Hexyl cinnamic aldehyde	50	3.6	3.8	1.3	34	2003
	50	5.9				2005
	50	3.6				2006
	50	2.7				2006
	50	3.0				2007b
Isoeugenol	10	8.4	5.4	4.2	79	2005
	10	2.4				2006, 2007b
Propylene glycol	50	1.6	1.1	0.6	55	2005
	50	0.7				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 = 2, with the coordinates of (a, b) for the point closest to SI = 2, and (c, d) for the higher point. As shown in **Table 7-3**, there were five EC2 values for hexyl cinnamic aldehyde, four EC2 values for eugenol, and two EC2 values for isoeugenol. The CV values were 73% for eugenol, 25% for hexyl cinnamic aldehyde, and 16% 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 ECt value (i.e., the estimated concentration needed to produce an SI of a specific threshold value; in this case, SI = 1.5) within 5% to 20% (ICCVAM 2009). Two of the five EC2 values for hexyl cinnamic aldehyde were within the acceptable range for intralaboratory reproducibility.

Table 7-3 Intralaboratory Reproducibility for the EC2 of Tested Substances in LLNA: BrdU-ELISA - Coefficient of Variation

Substance	EC2	Mean	SD	CV (%)	Takeyoshi et al. Reference
Eugenol	11.2	12.6	9.2	73	2004a
	23.6				2006
	1.2				2007b
	14.6				2007a
Hexyl cinnamic aldehyde	15.2	22.6	5.7	25	2003
	18.8				2003
	29.9				2006
	25.5				2006
	23.4				2007b
Isoeugenol	8.4	7.6	1.2	16	2006; 2007b
	6.7				2007a

Abbreviations: CV = coefficient of variation; EC2 = estimated concentration needed to produce a stimulation index of two; 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 7-4**).

Table 7-4 Intralaboratory Reproducibility for the EC3 of Tested Substances in the Traditional LLNA[1]

Substance	Number of Laboratories	Number of Tests per Laboratory	CV (%)
2, 4-Dinitrochlorobenzene	5	2	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 three; LLNA = murine local lymph node assay;
[1]From ICCVAM (1999).

For two of three substances, the intralaboratory CV values for the EC2 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 EC2 CV from the LLNA: BrdU-ELISA tests of eugenol was higher that that reported by ICCVAM (1999) (73% vs. 18%). The intralaboratory EC2 CV from the LLNA: BrdU-ELISA tests of isoeugenol was greater than that from ICCVAM (1999) (16% vs. 26%). However, the intralaboratory EC2 CV from the LLNA: BrdU-ELISA tests of hexyl cinnamic aldehyde was within the range reported by ICCVAM (1999) (25% 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 multi-laboratory 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 7-5**. All the laboratories that participated in the validation study used the same experimental protocol (**Appendix A**) and participated in a one-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 7-5 Substances and Test Allocation for the Phase II Interlaboratory Validation Study of the LLNA: BrdU-ELISA

Substance[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 EC2 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 (EC2 values) (**Sections 7.2.2** and **7.2.3**, respectively).

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 ≥ 2.0 at any dose were classified as sensitizers. 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 7-6**. The qualitative comparison of LLNA: BrdU-ELISA results (i.e., positive vs. negative) for 10 substances tested among up to 7 laboratories were consistent. The concordance results show that interlaboratory concordance was 100% (3/3, 6/6, or 7/7) for seven substances. There were three discordant substances (formaldehyde, isopropanol, and lactic acid) for which interlaboratory concordance was 67% (2/3 or 4/6). One of the three laboratories reported an SI of 1.97 for formaldehyde; while the others produced SI > 2. Two of the six tests of isopropanol yielded SI ≥ 2.0 (SI = 2.0 and SI = 2.2); while the others yielded negative results. One of the three tests for lactic acid produced SI ≥ 2.0 (i.e., SI = 2.5), while the others yielded SI < 2.0. The Validation Management Team considered the interlaboratory reproducibility to be acceptable (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 BrdU-ELISA concordance.

Table 7-6 Qualitative Results for the Phase II Interlaboratory Validation Study on the LLNA: BrdU-ELISA[1]

Substance	Laboratory							Concordance
	1	2	3	4	5	6	7	
2,4-Dinitrochlorobenzene	+	+	+	+	+	+	+	7/7
Glutaraldehyde	+				+	+		3/3
Nickel sulfate			+	+			+	3/3
trans-Cinnamic aldehyde		+		+	+			3/3
Formaldehyde	+				+	-[4]		2/3
Eugenol		+				+	+	3/3
Hexyl cinnamic aldehyde	+	-[3]	+	+	+	+[5]	+	6/6
Isopropanol	+[2]	-[3]	-	-	-	+[2,6]	-	4/6
Lactic acid			-	-			+	2/3
Methyl salicylate	-	-	-					3/3

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

[1](+) indicates sensitizer result; (-) indicates nonsensitizer result.

[2]Stimulation index [SI] ≥ 2 at lowest dose tested, but <2 at the higher doses. The Validation Management Team considered

these to be nonsensitizer results (Kojima et al. 2008).
[3]Test failed because concurrent positive control failed (i.e., SI < 2). Result not included in the concordance analysis.
[4]Maximum SI = 1.97.
[5]Three mice tested at highest dose.
[6]Three mice per dose group.

7.2.2 *Interlaboratory Reproducibility – EC2 Values*

The SI values from the interlaboratory validation study were used to calculate EC2 values for each sensitizer according to the methods reported in Section **7.1.3**. The EC2 values from each laboratory were then used to calculate CV values for each substance. The resulting values are shown in **Table 7-7**. CV values ranged from 20% (formaldehyde) to 101% (glutaraldehyde). The mean CV was 58%.

The ICCVAM LLNA performance standards indicates 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). EC2 values from two laboratories were outside these ranges for both substances. Laboratory 2 and Laboratory 5 reported EC2 values that were lower than the specified acceptance range for 2,4-dinitrochlorobenzene (0.019% and 0.0025%, respectively). For hexyl cinnamic aldehyde, Laboratory 3 obtained an EC2 value of 24.0%, which was higher than the acceptance range and Laboratory 5 obtained an EC2 value of 4.07%, which was lower than the acceptance range.

Table 7-7 EC2 Values from the Phase II Interlaboratory Validation Study on the LLNA: BrdU-ELISA[1]

Substance	Laboratory							Mean	% CV
	1	2	3	4	5	6	7		
2,4-Dinitro-chlorobenzene	0.084 (4.3 @ 1%)	***0.019 (8.37 @ 1%)***	0.029 (5.99 @ 0.3%)	0.030 (5.50 @ 1%)	***0.0025 (18.80 @ 0.3%)***	0.025 (4.83 @ 0.3%)	0.053 (12.18 @ 1%)	0.035	76
Hexyl cinnamic aldehyde	16.2 (3.4 @ 50%)	-[1] (1.83 @ 50%)	***24.0 (2.87 @ 50%)***	9.36 (3.34 @ 50%)	***4.07 (13.5 @ 50%)***	13.0[2] (3.27 @ 50%)	14.2 (3.84 @ 50%)	13.5	50
Glutaraldehyde	0.18	NT	NT	NT	0.034	0.51	NT	0.24	101
Nickel sulfate	NT	NT	3.85	0.95	NT	NT	1.31	2.0	78
trans-Cinnamic aldehyde	NT	2.59	NT	1.63	2.79	NT	NT	2.3	27
Formaldehyde	0.41	NT	NT	NT	0.31	-[3]	NT	0.36	20
Eugenol	NT	19.1	NT	NT	NT	16.4	5.06	13.5	55

Note: Bolded font indicates substances recommended for assessing interlaboratory reproducibility in *Recommended Performance Standards* (ICCVAM 2009). Bolded EC2 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; EC2 = estimated concentration needed to produce a stimulation index of two; LLNA: BrdU-ELISA = murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; NT = not tested; SI = stimulation index.

[1]Test failed because associated positive control failed (i.e., SI < 2; vehicle control absorbance was unusually high). Result not included in the mean EC2 and CV.

[2]Three mice tested at highest dose.

[3]Maximum SI = 1.97.

The interlaboratory CV values for the LLNA: BrdU-ELISA EC2 values were higher than that 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 7-8**; ICCVAM 1999). Three of the same substances were evaluated in the traditional LLNA and the LLNA: BrdU-ELISA. All interlaboratory CV values for LLNA: BrdU-ELISA were greater than that for the traditional LLNA. The CV of 76% 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 50% for hexyl cinnamic aldehyde tested in the LLNA: BrdU-ELISA was greater than the 7% reported by ICCVAM (1999). The CV of 55% for eugenol tested in the LLNA: BrdU-ELISA was greater than the 42% reported by ICCVAM (1999).

Table 7-8 Interlaboratory Reproducibility of the EC3 for Substances Tested in the Traditional LLNA[1]

Substance	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 three; LLNA = murine local lymph node assay.
[1]From ICCVAM (1999).

7.3 Reproducibility for the LLNA: BrdU-ELISA Using Multiple Alternative Decision Criteria

Section 6.7 details the accuracy analysis for the LLNA: BrdU-ELISA (using the most prevalent outcome for substances with multiple tests) when using two decision criteria for LLNA: BrdU-ELISA results: one criterion to classify substances as sensitizers (SI $\geq$ 2.0) and one criterion to classify substances as nonsensitizers (i.e., SI < 1.3). SI $\geq$ 2.0 was evaluated for classifying sensitizers because it resulted in no false positives with respect to the

traditional LLNA. SI < 1.3 was evaluated for classifying substances as nonsensitizers because it resulted in no false negatives. This section evaluates reproducibility of the concordance with the traditional LLNA results by examining the frequency with which SI values in the validation database of 31 substances occurred in one of three SI categories. The three SI categories were:

- SI < 1.3 for classifying nonsensitizers
- SI ≥ 1.3 and <2.0, the range of uncertainty with respect to classification by the traditional LLNA
- SI ≥ 2.0 to classify substances as sensitizers

The validation database for the LLNA: BrdU-ELISA consists of 102 tests of 31 substances. The maximum SI achieved by each test and the traditional LLNA outcome (sensitizer vs. nonsensitizer) were used to determine the frequency of the maximum SI. **Table 7-9** shows the proportion of sensitizers and nonsensitizers, according to the traditional LLNA for each SI category. All of the tests (9/9 [100%]) that yielded SI < 1.3 were for substances that were classified as nonsensitizers by the traditional LLNA. Forty percent (6/15) of the tests that yielded SI values of 1.3 ≤ SI < 2.0 were for substances that were classified as sensitizers by the traditional LLNA. Three tests produced SI values at either end of this range (i.e., SI = 1.3 or SI = 2.0). Hydroxycitronellal produced SI = 1.3 and the cyclamen aldehyde test and one formaldehyde test produced SI = 1.97. The remainder of the tests in this category, 60% (9/15), were classified as nonsensitizers by the traditional LLNA. Ninety-six percent (75/78) of the tests that yielded SI ≥ 2.0 were for substances that were classified as sensitizers by the traditional LLNA and only 4% (3/78) were classified as nonsensitizers. The three nonsensitizer tests were two tests of isopropanol, which yielded SI = 2.0 and 2.2 in the LLNA: BrdU-ELISA, and one test of lactic acid, which produced an SI = 2.5.

Table 7-9 Frequency of Maximum SI for LLNA: BrdU-ELISA Tests by Category and Traditional LLNA Outcome

Classification Based on Traditional LLNA	Classification Concordance with Traditional LLNA[1]			
	Maximum SI < 1.3	1.3 ≤ Maximum SI < 2.0	Maximum SI ≥ 2.0	Total
Sensitizer	0 (0%)	6 (40%)	75 (96%)	86
Nonsensitizer	9 (100%)	9 (60%)	3 (4%)	16
Total	9	15	78	102

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. Includes all tests of substances that were tested multiple times. Percentage in parentheses reflects percentage of the total number of tests for each SI category.

The 102 tests evaluated in **Table 7-9** include multiple tests for 14 substances. For the 14 substances, two to 31 tests were available. **Table 7-10** shows the proportion of the tests for each substance that produced SI values in each category. For the nine sensitizers with multiple test results, there were no tests that produced SI < 1.3 and only one test that produced an SI of 1.3 to <2. This was a formaldehyde test that produced SI = 1.97. For the five nonsensitizers with multiple test results, however, SI values occurred in all three SI categories. The results for isopropanol were particularly variable: 57% (4/7) produced SI < 1.3 (two tests with SI= 0.9 and two tests with SI = 1.0), 14% (1/7) produced 1.3 ≤ SI < 2 (SI = 1.6), and 29% (2/7) produced SI ≥ 2 (SI = 2.0 and 2.2). Lactic acid tests produced SI values in two categories: 67% (2/3) of the tests had 1.3 ≤ SI < 2 (SI = 1.8 and 1.9), and 33% (1/3) of the tests had SI ≥ 2 (SI = 2.5). Propylene glycol tests produced SI values in two categories: 50% (1/2) of the tests had SI < 1.3 (0.9) and one test produced 1.3 ≤ SI < 2 (SI = 1.9). The multiple test results for hexane and methyl salicylate were 100% concordant. The two hexane tests produced SI values in the 1.3 ≤ SI < 2 category (SI = 1.76 and 1.9) and the three methyl salicylate tests also produced SI values in that category (all three SI = 1.4).

Table 7-10 Concordance of LLNA: BrdU-ELISA Tests for Substances with Multiple Tests by Maximum SI Category

Substance	Concordance Among Multiple Tests[1]			Total
	Maximum SI < 1.3	1.3 ≤ Maximum SI < 2.0	Maximum SI ≥ 2.0	
Sensitizers[2]				
2,4-Dinitrochloro-benzene	0 (0%)	0 (0%)	9 (100%)	9
Eugenol	0 (0%)	0 (0%)	8 (100%)	8
Formaldehyde	0 (0%)	1(33%)	2 (67%)	3
Glutaraldehyde	0 (0%)	0 (0%)	5 (100%)	5
Hexyl cinnamic aldehyde	0 (0%)	0 (0%)	31 (100%)	31
Isoeugenol	0 (0%)	0 (0%)	3 (100%)	3
Nickel sulfate	0 (0%)	0 (0%)	3 (100%)	3
1,4-Phenylenediamine	0 (0%)	0 (0%)	2 (100%)	2
trans-Cinnamaldehyde	0 (0%)	0 (0%)	4 (100%)	4
Nonsensitizers[2]				
Hexane	0 (0%)	2 (100%)	0 (%)	2
Isopropanol	4 (57%)	1 (14%)	2 (29%)	7
Lactic acid	0 (0%)	2 (67%)	1 (33%)	3
Methyl salicylate	0 (0%)	3 (100%)	0 (0%)	3
Propylene glycol	1 (50%)	1 (50%)	0 (0%)	2

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 quality section in this draft BRD has been revised from the January 2008 draft BRD only to include data quality information about the interlaboratory validation study organized by the JSAAE.

The data submitted by Dr. Takeyoshi were generated at the Hita Laboratory of the Chemicals Evaluation and Research Institute, Japan. Although the Hita Laboratory is a Good Laboratory Practice (GLP)-conforming facility, the studies on the LLNA: BrdU-ELISA did not conform fully with GLP guidelines since they were not intended for regulatory purposes. However, all systems employed for these studies (i.e., test facilities, study staff, reagents, and the other study elements) were reportedly the same as those employed in the 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

This section has been revised from the January 2008 draft BRD only to include information about the interlaboratory validation study of the LLNA: BrdU-ELISA that was organized by the JSAAE. The Validation Management Team for the multi-laboratory 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 completed by the end of February 2009 (Kojima H, personal communication).

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

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

10.0 Animal Welfare Considerations

This section of the draft BRD has not changed from the January 2008 draft BRD. The LLNA: BrdU-ELISA will require the use of the same number of animals when compared to the updated ICCVAM LLNA protocol (ICCVAM 2009). However, since the traditional LLNA uses radioactivity and as such its use is restricted in some institutions, broader use of the non-radioactive LLNA: BrdU-ELISA protocol in place of the GP test could further reduce the number of guinea pigs that are still being used to assess skin sensitization.

10.1 Rationale for the Need to Use Animals

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

10.2 Basis for Determining the Number of Animals Used

The number of animals used for the experimental, vehicle, and positive control groups is based on the number of animals specified in the 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 a limit dose version of the LLNA: BrdU-ELISA in cases where dose response information is not needed for hazard identification purposes. In such an approach, only the highest soluble dose of the test article that does not produce skin irritation or systemic toxicity would be administered, and the two lower dose groups would not be used. Additional reductions could be achieved by testing more substances concurrently, so that the same vehicle and positive control group could be used for multiple substances, thus further reducing the number of animals for each additional substance by eight animals, or 40% (12 vs. 20).

11.0 Practical Considerations

This section of the draft BRD has not changed from the January 2008 draft BRD. Several issues are taken into account when assessing the practicality of using an alternative to an existing test method. In addition to performance evaluations, assessments of the laboratory equipment and supplies needed to conduct the alternative test method, level of personnel training, labor costs, and the time required to complete the test method relative to the existing test method are necessary. The time, personnel cost, and effort required to conduct the proposed test method(s) must be considered to be reasonable when compared to the existing test method it is intended to replace.

11.1 Transferability of the LLNA: BrdU-ELISA

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

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

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

11.3 LLNA: BrdU-ELISA Training Considerations

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

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Appendix A

LLNA: BrdU-ELISA Protocol

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1.0 Introduction/Principle

This document describes the recommended standard operating procedure for the non-radioisotopic modification of the LLNA, which is based on BrdU incorporation in place of tritiated thymidine to measure lymph node cell proliferation. This document is based on the protocol used in the JSAAE multi-laboratory validation study of the LLNA: BrdU-ELISA, *Recommended Standard Operating Procedure for the Non-Radioisotopic Local Lymph Node Assay using BrdU-ELISA (Non-RI LLNA), version 1.20, July 31, 2008*, by Masahiro Takeyoshi, Ph.D., Chemicals Evaluation and Research Institute, Japan. This

2.0 Description of the Method

The method is practically identical to the standard LLNA methodology excluding the use of BrdU and colorimetric detection. A single intraperitoneal injection (5 mg/mouse per injection) of BrdU is made on day 4. This administration schedule was decided as the most effective labeling protocol to yield maximum SI values based on preliminary study data with several different protocols. Approximately 24 h after the BrdU injection, the auricular lymph nodes are removed, weighed, and stored at -20˚C until analysis using an enzyme-linked immunosorbent assay to measure the level of BrdU incorporation (BrdU-ELISA).

The cell proliferation response is measured by a commercial BrdU detection kit (i.e., Roche Diagnostics GmbH, Roche Applied Science, 68298 Mannheim, Germany; Cat. No. 11 647 229 001). To perform the BrdU-ELISA, the lymph nodes are crushed, passed through a #70 nylon mesh. The lymph node cells (LNC) from individual animals are suspended in 15 ml of physiological saline. The cell suspension is added to the wells of a flatbottom microplate in triplicate. After fixation and denaturation of the LNC, anti-BrdU antibody is added to each well, and after rinsing, substrate solution containing tetramethylbenzidine (TMB) is added and allowed to produce chromogen. Absorbance at 370 nm with a reference wavelength of 492 nm is defined as the BrdU labeling index.

2.1 Animals

2.1.1 Animal source

Young adult female mice (nulliparous and non-pregnant) of the CBA/JN or other recommended mouse strains, such as CBA/Ca or CBA/J strain, should be used at age 8-12 weeks. All animals should be age matched (preferably within a one-week time frame).

2.1.2 Quarantine and Acclimation

Healthy animals in good general condition on arrival should be quarantined for more than five days. During the quarantine and acclimation period, clinical signs, body weights and excrement of the animals should be observed.

2.1.3 Grouping

Animals confirmed to be in good health with favorable body weight gains during the quarantine and acclimation period should be allocated to groups by a stratified randomization or other appropriate methods before the start of the study.

2.1.4 Identification

Animals should be identified by colored marks on the tails, ear tags, or other appropriate methods.

2.1.5 Animal Husbandry

The animals should be housed in an animal room maintained at a temperature of 22±3°C and a relative humidity of 30-70%. The rooms should be artificially lighted for 12 h daily, and the animals should be given free access to conventional laboratory diet and drinking water.

2.2 Chemicals and Vehicle

2.2.1 Vehicle

The solvent/vehicle should be selected on the basis of maximizing the test concentrations while producing a solution/suspension suitable for application of the test substance. In order of preference, recommended solvents/vehicles are acetone/olive oil (4:1 v/v), DMF, MEK, propylene glycol, and DMSO, but others may be used.

2.2.2 Test Chemicals

Solid test substances should be dissolved in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance should be prepared daily unless stability data demonstrate the acceptability of storage.

2.2.3 Controls

Concurrent negative (vehicle) and positive controls should be included in each test. Positive control (50% HCA, CAS RN. 101-86-0) should be used to ensure the appropriate performance of the assay. The positive control should produce a positive LLNA response at an exposure level expected to give an increase in the stimulation index (SI) >2 over the negative (vehicle) control group.

2.2.4 Dose selection

Doses are selected from the concentration series 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc. The maximum concentration tested should be the highest achievable level while avoiding overt systemic toxicity and excessive local irritation. All test solutions should be prepared in a day of application unless the stability is confirmed in advance.

2.2.5 Preparation of BrdU

BrdU should be accurately weighed and dissolved in physiological saline for injection) to make 10 mg/ml solution. The BrdU solution should be sterilized by a commercial filtration system (i.e. MILLEX®-HV, MILLIPORE etc.). The BrdU solution can be prepared before administration and stored in a freezer below - 20°C until use.

2.3 Animal Experiment

2.3.1 Grouping

A minimum of four successfully treated animals is used per dose group, with a minimum of three consecutive concentrations of the test substance plus a negative (vehicle) control and a positive control group.

Table 1 Structure of LLNA: BrdU-ELISA Test Groups

Group	Number of Animals
Negative (vehicle) control	4
Positive control (50% HCA)	4
Test substance-low dose	4
Test substance-middle dose	4
Test substance-high dose	4

2.3.2 Sensitization Procedure

Apply 25µl of test solution to the dorsum of both ears of the mice using micro volume pipette daily for three consecutive days.

2.3.3 BrdU Administration

A single intraperitoneal injection of 0.5 ml of BrdU solution (5 mg/mouse/injection) should be given to the mice 48 hours (h) after the final sensitization.

2.3.4 General Condition

Clinical signs should be observed at least once a day.

2.3.5 Body Weights

Body weights should be measured on the day of the first test substance application and on the lymph nodes are collected.

2.3.6 Collection of Lymph Nodes And Measurement of Lymph Node Weight

Approximately 24 h after BrdU injection, the auricular lymph nodes should be removed. The lymph nodes should be carefully dissected and trimmed of fascia and fat, weighed, and stored individually in a 1.5 ml centrifuge tube at -20°C until the ELISA is performed.

2.4 BrdU-ELISA

The incorporation of BrdU into lymph node cells should be determined using a commercial cell proliferation assay kit (Roche Diagnostics GmbH, Roche Applied Science, 68298 Mannheim, Germany; Cat. No. 11 647 229 001) after they are crushed and suspended in

physiological saline. The absorbance is defined as the BrdU labeling index. Follow the instructions in the assay kit.

2.5 Preparation of Reagents in the BrdU-ELISA Kit

The assay method should be according to the instruction manual in the assay kit excluding preparation of the BrdU labeling solution.

2.5.1 Peroxidase Conjugated Anti-BrdU Antibody (Anti-BrdU-POD) Stock Solution

Dissolve Anti-BrdU-POD (bottle 3) in 1.1 ml double distilled water for 10 minutes and mix thoroughly. This solution can be stored at 2-8°C for several months. For long-term storage it is recommended to store the solution in aliquots at -15 to -25°C.

2.5.2 Anti-BrdU-POD Working Solution

Dilute Anti-BrdU-POD stock solution 1:100 with antibody dilution solution (bottle 4). For one 96-well microtiter plate dilute 100 ml Anti-BrdU-POD stock solution in 10 ml antibody dilution solution (bottle 4). Prepare shortly before use.

2.5.3 Washing Solution

Dilute washing buffer concentrate (bottle 5) 1:10 with double distilled water. For one 96-well microtiter plate, dilute 10 ml washing buffer concentrate (bottle 5) with 90 ml double distilled water. This solution can be stored at 2-8°C for several weeks.

2.6 Preparation of Cell Suspension of Lymph Nodes

The procedure for preparing the LNC suspension is a critical step of this assay; it is most important to crush the lymph node and suspend the LNC completely. Every technician should establish the skill in advance. The lymph nodes in negative control animals are very small, so careful operation is required to avoid an artificial effect on SI values.

2.6.1 Optimizing Assay Condition

Mean absorbance of negative (vehicle) control group should be within 0.1-0.2. Because the absorbance depends on the combination of assay apparatus and the target volume of the LNC suspension, every laboratory should decide their own optimal target volume of LNC suspension in advance so that the absorbance of the negative control is within 0.1-0.2. The volume is expected to be approximately 15 ml. The volume of the LNC suspension for all

test animals should be adjusted to the optimized volume.

2.6.2 Preparation of LNC Suspension

A small amount (approximately 0.3 ml) of physiological saline should be added to the centrifuge tube that contains the collected lymph node. The lymph node should be crushed with a disposable plastic pestle to make the LNC suspension. The LNC suspension should be passed through a #70 nylon mesh and adjusted to the optimal target volume in a 50 ml Falcon tube.

[Note: Although a crushing apparatus other than a plastic pestle can be used to prepare the LNC, the target volume of the LNC suspension should be adjusted to the optimized volume.]

2.7 Assay Flow (BrdU-ELISA)

1. The cell suspension (100 µl) is added to the wells of a flat-bottom microplate (three wells per sample) after mixing thoroughly with a Vortex. Simultaneously, three blank wells should be prepared by adding 100 µl of physiological saline.
2. After filling all sample wells and blank wells, the plate should be centrifuged at 300 x *g* for 10 minutes.
3. Remove 3/4 of the supernatant volume. Great care should be taken so that the LNC are not aspirated.
4. The assay plate should be dried completely in a hot-air oven.
5. Add 200 µl of Fix-Denat solution and allow plate to stand for 30 minutes at room temperature.
6. Remove the Fix-Denat solution completely.
7. Add 100 µl of anti-BrdU-POD antibody working solution and allow it to react for 1 h.
8. Remove the anti-BrdU-POD antibody solution completely.
9. Add 200 µl of wash solution into each well, and wash the well by pippetting

10 times. Discard the wash solution completely.

10. The wash step (Step 9) should be repeated twice (three times in total).

11. Add 100 □l of TMB substrate solution and let it stand for 15 minutes at room temperature in a dark place.

12. Measure an absorbance (ABS) at 370 nm with a reference wavelength of 492 nm. When using stop solution (1M sulfuric acid, 25 µl/well), measure ABS at 450 nm with a reference wavelength of 690 nm.

3.0 Calculation of Results

BrdU labeling index and Stimulation Index (SI) are defined as follows:

3.1 Without Stop Solution

BrdU labeling index = $(ABS_{370}-ABS_{blank370}) - (ABS_{490}-ABS_{blank490})$

3.2 With Stop Solution

BrdU labeling index = $(ABS_{450}-ABS_{blank450}) - (ABS_{650}-ABS_{blank650})$

3.3 Stimulation Index

$$\text{Stimulation Index (SI)} = \frac{\text{BrdU labeling index for each test animal}}{\text{Mean BrdU labeling index for concurrent vehicle control group}}$$

4.0 Evaluation of Results

4.1 Success Criteria for Each Experiment

Employing the optimized assay condition described previously, the mean SI for the positive control group (50% HCA) should be equal to or greater than 2. If not, all data derived from the experiment should not be used for evaluation.

4.2 Evaluation of the Results

The mean BrdU labeling index for each animal should be calculated based on the results of BrdU ELISA. The SI for each animal should be calculated by dividing of the mean BrdU labeling index for each treated animal by the mean BrdU labeling index of the concurrent vehicle control group. A positive response is defined as mean SI of the test group ≥2.

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Appendix B

Physico-Chemical Properties of Substances Tested Using the LLNA: BrdU-ELISA

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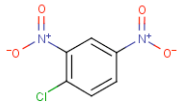
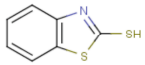
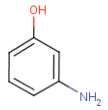
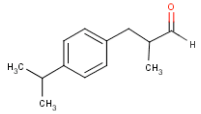
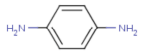
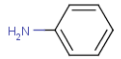
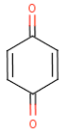
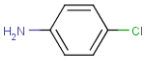
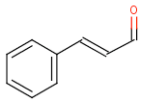
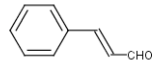
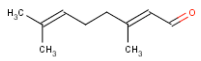
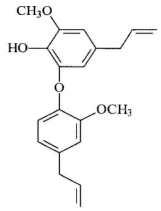
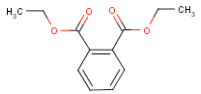
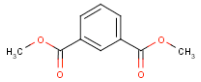
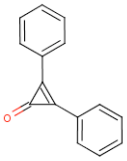
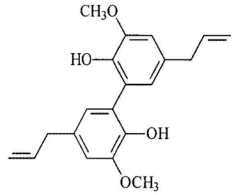
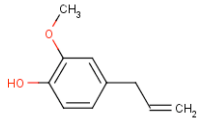
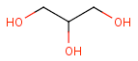
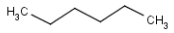
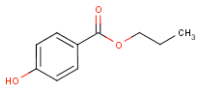
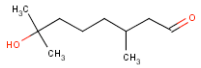
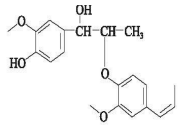
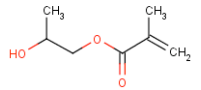
Appendix B Physicochemical Characteristics of Substances Tested in the LLNA: BrdU-ELISA (Alphanumeric Order)

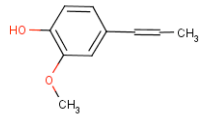
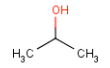
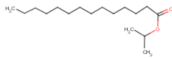
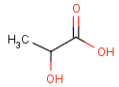
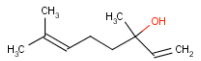
Chemical Name	Synonyms	CASRN	Mol. Weight (g/mol)	Log Kow[1,2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
1-Chloro-2-dinitrobenzene	2,4-Dinitrochloro-benzene	97-00-7	202.55	-0.057	High	Solid	Hydrocarbon, halogenated; Nitro compounds; Hydrocarbons, cyclic	
2-Mercaptobenzothiazole	Captax	149-30-4	167.253	1.8	High	Solid	Heterocyclic compounds	
3-Aminophenol	m-Aminophenol; 3-Hydroxyaniline	591-27-5	109.126	1.17	NA	Solid	Amines; Phenols	
3-(4-Isopropylphenyl) isobutyraldehyde	Cyclamen aldehyde	103-95-7	190.28	3.28	Low	Liquid	Carboxylic acids	
1,4-Phenylenediamine	p-PDA; p-Phenylenediamine	106-50-3	108.141	1.17	NA	Solid	Amines	

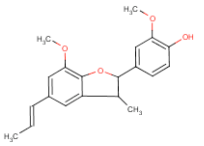
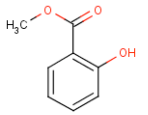
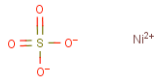
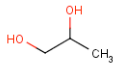
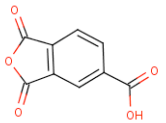
Chemical Name	Synonyms	CASRN	Mol. Weight (g/mol)	Log Kow[1,2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Aniline	Benzenamine	62-53-3	93.1265	1.56	NA	Liquid	Amines	
Benzoquinone	p-Quinone; 1,4-Cyclohexadienedione	106-51-4	108.095	1.17	High	Solid	Quinones	
4-Chloroaniline	4-Chlorobenzeneamine; Aniline, p-chloro-; Benzenamine, 4-chloro-	106-47-8	127.57	1.8	NA	Liquid	Amines	
trans-Cinnamaldehyde	3-Phenylpropenal	14371-10-9	132.6	1.82	NA	Liquid	Aldehydes	
Cinnamic aldehyde	Cinnamal; cinnamaldehyde; 3-phenyl-2-propenal	104-55-2	132.16	2.29	High	Liquid	Aldehydes	

Chemical Name	Synonyms	CASRN	Mol. Weight (g/mol)	Log Kow[1,2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Citral	3,7-Dimethyl-2,6-octadienal; Geranial-neral mixture	5392-40-5	152.233	2.54/ 3.45	NA	Liquid	Hydrocarbons, other	
4,5'-Diallyl-2'-hydroxy-2,3'-dimethoxyphenyl ether	DHEB	NA	326.39	NA	NA	NA	Carboxylic acids	
Diethyl pthalate	1,2-Benzenedicarboxylic acid, diethyl ester; Diethyl 1,2-benzenedicarboxylate	84-66-2	222.24	1.87	Minimal	Liquid	Carboxylic acids	
Dimethyl isophthalate	Dimethyl m-phthalate	1459-93-4	194.19	1.66	NA	Solid	Carboxylic acids	
Diphenylcyclopropenone	2,3-Diphenylcyclo-propenone	886-38-4	206.24	3.25	High	Solid	Hydrocarbons, cyclic	

Chemical Name	Synonyms	CASRN	Mol. Weight (g/mol)	Log Kow[1,2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
2,2'-Dihydroxyl-3,3'-dimethoxy-5,5'-diallyl-biphenyl	DHEA	NA	326.39	NA	NA	NA	Carboxylic acids	
Eugenol	2-Methoxy-4-(2-propenyl)phenol; 4-Allyl-2-methoxyphenol; 4-Allylguaiacol	97-53-0	164.201	2.15/ 2.73	NA	Liquid	Carboxylic acids	
Formaldehyde	Formalin	50-00-0	30.03	0.33	Moderate	Liquid	Aldehydes	
Glutaraldehyde	Glutaral	111-30-8	100.12	0.92	High	Liquid	Aldehydes	
Glycerol	Glycerin	56-81-5	92.09	0.05	Minimal	Liquid	Alcohols; Carbohydrates	

Chemical Name	Synonyms	CASRN	Mol. Weight (g/mol)	Log Kow[1,2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Hexane	Hexyl hydride; n-Hexane	110-54-3	86.1754	1.94	Minimal	Liquid	Hydrocarbons, acyclic	
Hexyl cinnamic aldehyde	HCA; alpha-Hexylcinnamaldehyde; 2-(Phenylmethylene)octanal	101-86-0	216.319	3.77/ 4.82	Minimal	Liquid	Aldehydes	
Hydroxycitronellal	7-Hydroxy-3,7-dimethyloctanol	107-75-5	172.26	2.15	Low	Liquid	Hydrocarbons, other	
4-[1-Hydroxy-2-(2-methoxy-4-propenyl-phenyoxy)-propyl]-2-methoxy-phenol	□-O-4-Dilignol	NA	327.39	NA	NA	NA	Carboxylic acids	
2-Hydroxypropyl methacrylate	2-HPMA	923-26-2	144.168	1.03	Low	Solid	Carboxylic acids	

Chemical Name	Synonyms	CASRN	Mol. Weight (g/mol)	Log Kow[1,2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Isoeugenol	2-Methoxy-4-propenylphenol; 4-Propenylguaiacol	97-54-1	164.201	2.15	NA	Liquid	Carboxylic acids	
Isopropanol	Isopropyl alcohol, 2-Propanol	67-63-0	60.095	0.82	Minimal	Liquid	Alcohols	
Isopropyl myristate	1-Methylethyl tetradecanoate	110-27-0	270.46	3.88	Minimal	Liquid	Lipids	
Lactic acid	2-Hydroxypropanoic acid	50-21-5	90.08	0.05	Minimal	Liquid	Carboxylic acids	
Linalool	3,7-dimethylocta-,6-dien-3-ol	78-70-6	154.25	2.54	NA	Liquid	Hydrocarbons	

Chemical Name	Synonyms	CASRN	Mol. Weight (g/mol)	Log Kow[1,2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
2-Methoxy-4-(7-methoxy-3-methyl-5-propenyl-2,3-dihydro-benzofuran-2yl)-phenol	Dehydrodiisoeugenol	2680-81-1	326.39	NA	NA	NA	Carboxylic acids	
Methyl salicylate	Oil of wintergreen; 2-Hydroxybenzoic acid methyl ester	119-36-8	152.15	1.28	Minimal	Liquid	Phenols; Carboxylic acids	
Nickel Sulfate	Nickelous sulfate	7786-81-4	154.76	NA	NA	Solid	Inorganic chemicals, metals; Inorganic chemicals, elements	
Propylene glycol	1,2-Dihydroxypropane; 1,2-Propanediol	57-55-6	76.0944	0.43	Minimal	Liquid	Alcohols	
Trimellitic anhydride	1,2,4-Benzenetricarboxylic acid, cyclic 1,2-anhydride (8CI); 1,3-Dihydro-1,3-dioxo-5-isobenzofuran-carboxylic acid; 5-Isobenzofuran-carboxylic acid; 1,3-dihydro-1,3-dioxo-Benzene-1,2,4-tricarboxylic acid 1,2-anhydride	552-30-7	192.13	1.95	Low	Solid	Anhydrides, Carboxylic acids	

Abbreviations: CASRN=Chemical Abstracts Registry Number; g/mol=grams per mole; NA = Not available.

[1]Physicochemical properties were obtained from PubChem (http://pubchem.ncbi.nlm.nih.gov/), ChemID (http://chem.sis.nlm.nih.gov/chemidplus/chemidheavy.jsp), or the Sigma Chemical Catalog.

[2]K_{ow} represents the octanol-water partition coefficient (expressed on log scale). When two numbers are shown, the first number is the value calculated by the method of Moriguchi et al. (1994 Chem Pharm Bull. 42:976-978) and provided in Gerberick et al. (2005 Dermatitis. 16:157-202). The second number was calculated by the method of Meylan and Howard (1995 J Pharm Science. 84:83-92) and obtained from the website: http://www.syrres.com/esc/est_kowdemo.htm.

[3]Peptide reactivity data obtained from: Gerberick et al. 2007.

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

Appendix C

Comparative LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Skin Sensitization Data

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Appendix C1

LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA (Alphanumeric Order)

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Appendix C-1 LLNA: BrdU-ELISA, Traditional LLNA, Guinea Pig, and Human Results for Substances Tested Using the LLNA: BrdU-ELISA (Alphanumeric Order)

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
m-Aminophenol	AOO	25	3.06	Takeyoshi et al. 2007b	+ (5.7, 10%)	NA	+	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999;GP was + nonstd	ICCVAM 1999	No at ≤ 5%	Basketter et al. 2007
Aniline	AOO	50	1.50	Takeyoshi et al. 2007b	+ (3.6, 100%)[7]	+	+ (7/25 at 20%)	ICCVAM 1999 (Basketter et al. 1991)	ICCVAM 1999	ICCVAM 1999 (Kligman 1966)	No at ≤ 100% (GP); Irritant at 20% in humans	Basketter et al. 2007; Kligman 1966
p-Benzoquinone	AOO	1	6.90	Takeyoshi et al. 2004b; Takeyoshi et al. 2007b	+ (52.3, 2.5%)	+	+	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 2.5%	Basketter et al. 2007
4-Chloroaniline	AOO	25	2.53	Takeyoshi et al. 2007b	+ (NA)	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999	No at 2.5%	Basketter and Scholes 1992
Cinnamic aldehyde	AOO	50	3.97	Takeyoshi et al. 2007b	+ (18.4, 25%)[8]	+	+	ICCVAM 1999 (Basketter et al. 1992)	ICCVAM 1999	ICCVAM 1999	Mild irritant at 100%	ECETOC 1995
trans-Cinnamaldehyde	AOO	10	5.90	Takeyoshi et al. 2005	+ (13.1, 25%)	NA	NA	Gerberick et al. 2005	NA	NA	NA	NA
trans-Cinnamaldehyde	AOO	10	4.11	Kojima et al. 2008 5	+ (13.1, 25%)	NA	NA	Gerberick et al. 2005	NA	NA	NA	NA
trans-Cinnamaldehyde	AOO	10	3.50	Kojima et al. 2008 4	+ (13.1, 25%)	NA	NA	Gerberick et al. 2005	NA	NA	NA	NA
trans-Cinnamaldehyde	AOO	10	3.37	Kojima et al. 2008 2	+ (13.1, 25%)	NA	NA	Gerberick et al. 2005	NA	NA	NA	NA

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
Citral	AOO	50	16.35	Takeyoshi et al. 2007b; Takeyoshi et al. 2005	+ (20.5, 20%)	+	+	ICCVAM 1999 (Basketter et al. 1991)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.5%	Basketter et al. 2007
Citral	AOO	10	1.84	Takeyoshi et al. 2007b; Takeyoshi et al. 2005	+ (20.5, 20%)	+	+	ICCVAM 1999 (Basketter et al. 1991)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.5%	Basketter et al. 2007
Cyclamen aldehyde	AOO	100	1.97	Takeyoshi et al. 2007b	+ (5.2, 50%)	NA	- (0/64, 4%)	Gerberick et al. 2005	NA	Basketter et al. 2005	Yes, at 100%	ECETOC 1995
4,5'-Diallyl-2'-hydroxy-2,3'-dimethoxyphenyl ether (DHEB)	AOO	20	7.30	Takeyoshi et al. 2004a	NA	+	NA	NA	Takeyoshi et al. 2004a	NA	NA	NA
Diethyl phthalate (Phthallic acid diethylester)	AOO	50	0.88	Takeyoshi et al. 2007b	- (1.5, 100%)	-	+ (HPTA)	ICCVAM 1999 (Gerberick et al. 2005)	Klecak et al. 1977	ICCVAM 1999	No at ≤ 100%	ECETOC 1995
2,2'-Dihydroxyl-3,3'-dimethoxy-5,5'-diallyl-biphenyl (DHEA)	AOO	30	2.30	Takeyoshi et al. 2004a	NA	-	NA	NA	Takeyoshi et al. 2004a	NA	NA	NA
Dimethyliso-phthalate	AOO	50	1.26	Takeyoshi et al. 2007b	- (1, 25%)	-	-	ICCVAM 1999 (Basketter and Scholes 1992)	ICCVAM 1999	Basketter et al. 1999	NA	NA
Diphenylcyclo-propenone	AOO	2	19.10	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+ (NA)	NA	+	ICCVAM 1999	NA	ICCVAM 1999	NA	NA
Diphenylcyclo-propenone	AOO	10	9.34	Takeyoshi et al. 2005	+ (NA)	NA	+	ICCVAM 1999	NA	ICCVAM 1999	NA	NA

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
2,4-Dinitrochloro-benzene	AOO	1	4.30	Kojima et al. 2008 1	+ (43.9, 0.25%)	+	+	ICCVAM 1999 (Kimber et al. 1995)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.1%	Basketter et al. 2007
2,4-Dinitrochlorobenzene	AOO	1	8.37	Kojima et al. 2008 2	+ (43.9, 0.25%)	+	+	ICCVAM 1999 (Kimber et al. 1995)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.1%	Basketter et al. 2007
2,4-Dinitrochlorobenzene	AOO	0.3	6.26	Kojima et al. 2008 3	+ (43.9, 0.25%)	+	+	ICCVAM 1999 (Kimber et al. 1995)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.1%	Basketter et al. 2007
2,4-Dinitrochlorobenzene	AOO	1	5.50	Kojima et al. 2008 4	+ (43.9, 0.25%)	+	+	ICCVAM 1999 (Kimber et al. 1995)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.1%	Basketter et al. 2007
2,4-Dinitrochlorobenzene	AOO	0.3	18.80	Kojima et al. 2008 5	+ (43.9, 0.25%)	+	+	ICCVAM 1999 (Kimber et al. 1995)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.1%	Basketter et al. 2007
2,4-Dinitrochlorobenzene	AOO	1	4.83	Kojima et al. 2008 6	+ (43.9, 0.25%)	+	+	ICCVAM 1999 (Kimber et al. 1995)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.1%	Basketter et al. 2007
2,4-Dinitrochlorobenzene	AOO	1	12.98	Kojima et al. 2008 7	+ (43.9, 0.25%)	+	+	ICCVAM 1999 (Kimber et al. 1995)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.1%	Basketter et al. 2007
2,4-Dinitrochlorobenzene	AOO	2	17.90	Takeyoshi et al. 2005	+ (43.9, 0.25%)	+	+	ICCVAM 1999 (Kimber et al. 1995)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.1%	Basketter et al. 2007
2,4-Dinitrochlorobenzene	AOO	2	6.84	Takeyoshi et al. 2006; Takeyoshi et al. 2007b	+ (43.9, 0.25%)	+	+	ICCVAM 1999 (Kimber et al. 1995)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.1%	Basketter et al. 2007
Eugenol	AOO	10	3.18	Takeyoshi et al. 2005	+ (17, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	ICCVAM 1999	No at ≤ 25%	Basketter et al. 2007
Eugenol	AOO	30	3.30	Takeyoshi et al. 2004a	+ (17, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	ICCVAM 1999	No at ≤ 25%	Basketter et al. 2007
Eugenol	AOO	30	3.83	Takeyoshi et al. 2007a	+ (17, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	ICCVAM 1999	No at ≤ 25%	Basketter et al. 2007

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Eugenol	AOO	50	12.30	Takeyoshi et al. 2005	+ (17, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	ICCVAM 1999	No at ≤ 25%	Basketter et al. 2007
Eugenol	AOO	50	3.10	Takeyoshi et al. 2006	+ (17, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	ICCVAM 1999	No at ≤ 25%	Basketter et al. 2007
Eugenol	AOO	50	7.09	Kojima et al. 2008 7	+ (17, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	ICCVAM 1999	No at ≤ 25%	Basketter et al. 2007
Eugenol	AOO	50	3.17	Kojima et al. 2008 2	+ (17, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	ICCVAM 1999	No at ≤ 25%	Basketter et al. 2007
Eugenol	AOO	50	3.18	Kojima et al. 2008 6	+ (17, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	ICCVAM 1999	No at ≤ 25%	Basketter et al. 2007
Eugenol	AOO	50	17.70	Takeyoshi et al. 2007b	+ (17, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	ICCVAM 1999	No at ≤ 25%	Basketter et al. 2007
Formaldehyde	ACE	10	16.59	Kojima et al. 2008 5	+ (11.9, 25%)	+	+	ICCVAM 1999 (Kimber et al. 1991)	ICCVAM 1999	ICCVAM 1999	No at ≤ 2%	Basketter et al. 2007
Formaldehyde	ACE	10	4.40	Kojima et al. 2008 1	+ (11.9, 25%)	+	+	ICCVAM 1999 (Kimber et al. 1991)	ICCVAM 1999	ICCVAM 1999	No at ≤ 2%	Basketter et al. 2007
Formaldehyde	ACE	10	1.97	Kojima et al. 2008 6	+ (11.9, 25%)	+	+	ICCVAM 1999 (Kimber et al. 1991)	ICCVAM 1999	ICCVAM 1999	No at ≤ 2%	Basketter et al. 2007
Glutaraldehyde	AOO	1	28.64	Kojima et al. 2008 5	+ (18, 2.5%)[9]	+	+	Hilton et. al 1998 (Gerberick et al. 2005)	Gad et al. 1986	Schneider and Akkan 2004	NA	NA
Glutaraldehyde	AOO	1	3.72	Kojima et al. 2008 1	+ (18, 2.5%)[9]	+	+	Hilton et. al 1998 (Gerberick et al. 2005)	Gad et al. 1986	Schneider and Akkan 2004	NA	NA
Glutaraldehyde	AOO	1	2.25	Kojima et al. 2008 6	+ (18, 2.5%)[9]	+	+	Hilton et. al 1998 (Gerberick et al. 2005)	Gad et al. 1986	Schneider and Akkan 2004	NA	NA

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Glutaraldehyde	AOO	2	14.60	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+ (18, 2.5%)[9]	+	+	Hilton et. al 1998 (Gerberick et al. 2005)	Gad et al. 1986	Schneider and Akkan 2004	NA	NA
Glutaraldehyde	AOO	10	15.50	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+ (18, 2.5%)[9]	+	+	Hilton et. al 1998 (Gerberick et al. 2005)	Gad et al. 1986	Schneider and Akkan 2004	NA	NA
Glycerol	None	50	1.29	Takeyoshi et al. 2007b	- (1.1, 100%)[10]	-	-	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	ICCVAM 1999	NA	NA
Hexane	AOO	100	1.76	Takeyoshi unpublish-ed 2009	- (2.2, 100%)	NA	- (0/25, 100%)	ICCVAM 1999 (Gerberick et al. 2005)	NA	ICCVAM 1999 (Kligman 1966)	Yes at 100%	Kligman 1966
Hexane	AOO	10	0.73	Takeyoshi et al. 2005	- (2.2, 100%)	NA	- (0/25, 100%)	ICCVAM 1999 (Gerberick et al. 2005)	NA	ICCVAM 1999 (Kligman 1966)	Yes at 100%	Kligman 1966
Hexane	AOO	50	1.89	Takeyoshi et al. 2005	- (2.2, 100%)	NA	- (0/25, 100%)	ICCVAM 1999 (Gerberick et al. 2005)	NA	ICCVAM 1999 (Kligman 1966)	Yes at 100%	Kligman 1966
Hexyl cinnamic aldehyde	AOO	50	3.60	Takeyoshi et al. 2003	+ (20, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	5.90	Takeyoshi et al. 2005	+ (20, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	3.64	Takeyoshi et al. 2006	+ (20, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	2.72	Takeyoshi et al. 2006	+ (20, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007

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Hexyl cinnamic aldehyde	AOO	50	3.02	Takeyoshi et al. 2007b	+ (20, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	3.40	Kojima et al. 2008 1	+ (20, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	2.07	Kojima et al. 2008 PC 1-1	+ (20, 50%)			ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	6.11	Kojima et al. 2008 PC 1-2	+ (20, 50%)			ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	3.43	Kojima et al. 2008 PC 1-3	+ (20, 50%)			ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	5.15	Kojima et al. 2008 PC 2-2	+ (20, 50%)			ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	2.52	Kojima et al. 2008 PC 2-3	+ (20, 50%)			ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	2.87	Kojima et al. 2008 3	+ (20, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	3.34	Kojima et al. 2008 PC 3-1	+ (20, 50%)			ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	3.54	Kojima et al. 2008 PC 3-2	+ (20, 50%)			ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	2.18	Kojima et al. 2008 PC 3-3	+ (20, 50%)			ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	3.34	Kojima et al. 2008 4	+ (20, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	2.69	Kojima et al. 2008 PC 4-1	+ (20, 50%)			ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007

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Hexyl cinnamic aldehyde	AOO	50	3.17	Kojima et al. 2008 2 PC 4-2	+ (20, 50%)			ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	6.58	Kojima et al. 2008 PC 4-3	+ (20, 50%)			ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	13.50	Kojima et al. 2008 5	+ (20, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	12.46	Kojima et al. 2008 PC 5-1	+ (20, 50%)			ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	4.24	Kojima et al. 2008 PC 5-2	+ (20, 50%)			ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	6.07	Kojima et al. 2008 PC 5-3	+ (20, 50%)			ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	3.27	Kojima et al. 2008 6	+ (20, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	5.30	Kojima et al. 2008 PC 6-1	+ (20, 50%)			ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	2.41	Kojima et al. 2008 PC 6-2	+ (20, 50%)			ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	2.52	Kojima et al. 2008 PC 6-3	+ (20, 50%)			ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	3.84	Kojima et al. 2008 7	+ (20, 50%)	+	+	ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	6.86	Kojima et al. 2008 PC 7-1	+ (20, 50%)			ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hexyl cinnamic aldehyde	AOO	50	4.39	Kojima et al. 2008 PC 7-2	+ (20, 50%)			ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007

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Hexyl cinnamic aldehyde	AOO	50	4.78	Kojima et al. 2008 PC 7-3	+ (20, 50%)			ICCVAM 1999 (Loveless et al. 1996)	ICCVAM 1999	Basketter et al. 1999	No at ≤ 10%	Basketter et al. 2007
Hydroxy-citronellal	AOO	100	1.30	Takeyoshi et al. 2007b	+ (8.5, 100%)	+	+ (14/73, 20%)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	ICCVAM 1999 (Marzulli and Maibach 1980)	No at ≤ 50%	Basketter et al. 2007
4-[1-Hydroxy-2-(2-methoxy-4-propenyl-phenyoxy)-propyl]-2-methoxy-phenol (Synonym: □-O-4-Dilignol)	AOO	30	1.19[8]	Takeyoshi et al. 2007a	NA	-	NA	NA	Takeyoshi et al. et al. 2007a	NA	NA	NA
2-Hydroxypropyl-methacrylate	AOO	50	1.13	Takeyoshi et al. 2007b	- (1.3, 50%)	-	+ (case study, 0.1%)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	Bjorkner 1984	No at ≤ 10%	Scholes et al. 1992
Isoeugenol	AOO	10	8.40	Takeyoshi et al. 2005	+ (31, 5%)	+	+	ICCVAM 1999 (Basketter and Cadby 2004)	ICCVAM 1999	ICCVAM 1999	No at ≤ 5%	Basketter et al. 2007
Isoeugenol	AOO	10	2.40	Takeyoshi et al. 2006; Takeyoshi et al. 2007b	+ (31, 5%)	+	+	ICCVAM 1999 (Basketter and Cadby 2004)	ICCVAM 1999	ICCVAM 1999	No at ≤ 5%	Basketter et al. 2007
Isoeugenol	AOO	30	6.73	Takeyoshi et al. 2007a	+ (31, 5%)	+	+	ICCVAM 1999 (Basketter and Cadby 2004)	ICCVAM 1999	ICCVAM 1999	No at ≤ 5%	Basketter et al. 2007
Isopropanol	AOO	50	2.22	Kojima et al. 2008 1	- (1.7, 50%)[8]	-	+ (case study, 0.001%)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	Kwon et al. 2003	No at ≤ 100%	ECETOC 1995

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
Isopropanol	AOO	50	0.98	Kojima et al. 2008 3	- (1.7, 50%)[8]	-	+ (case study, 0.001%)	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	No at ≤ 100%	ECETOC 1995
Isopropanol	AOO	50	1.57	Kojima et al. 2008 4	- (1.7, 50%)[8]	-	+ (case study, 0.001%)	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	No at ≤ 100%	ECETOC 1995
Isopropanol	AOO	50	0.94	Kojima et al. 2008 5	- (1.7, 50%)[8]	-	+ (case study, 0.001%)	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	No at ≤ 100%	ECETOC 1995
Isopropanol	AOO	50	2.04	Kojima et al. 2008 6	- (1.7, 50%)[8]	-	+ (case study, 0.001%)	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	No at ≤ 100%	ECETOC 1995
Isopropanol	AOO	50	1.01	Kojima et al. 2008 7	- (1.7, 50%)[8]	-	+ (case study, 0.001%)	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	No at ≤ 100%	ECETOC 1995
Isopropanol	AOO	100	0.92[7]	Takeyoshi et al. 2007b	- (1.7, 50%)[8]	-	+ (case study, 0.001%)	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	No at ≤ 100%	ECETOC 1995
Isopropyl myristate	AOO	50	4.20	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+ (3.4, 100%)	NA	- (0/25)	Ryan et al. 2000 (Gerberick et al. 2005)	NA	Opdyke 1976	No at ≤ 100%	ECETOC 1995
Isopropyl myristate	AOO	10	1.10	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+ (3.4, 100%)	NA	- (0/25)	Ryan et al. 2000 (Gerberick et al. 2005)	NA	Opdyke 1976	No at ≤ 100%	ECETOC 1995

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
Lactic acid	DMSO	50	2.53	Kojima et al. 2008 7	- (2.2, 25%)	-	- (no data)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	Basketter et al. 1999	Slightly irritating at 10%	Cosmetic Ingredient Review Panel 1998
Lactic acid	DMSO	50	1.89	Kojima et al. 2008 4	- (2.2, 25%)-	-	- (no data)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	Basketter et al. 1999	Slightly irritating at 10%	Cosmetic Ingredient Review Panel 1998
Lactic acid	DMSO	50	1.80	Kojima et al. 2008 3	- (2.2, 25%)-	-	- (no data)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	Basketter et al. 1999	Slightly irritating at 10%	Cosmetic Ingredient Review Panel 1998
Linalool	AOO	100	1.45[7]	Takeyoshi unpublish-ed 2009	+ (8.3, 100%)	NA	-	Gerberick et al. 2005	NA	Basketter et al. 2001	Mild irritant at 100%	ECETOC 1995
2-Mercaptobenzo-thiazole	DMF	50	1.62[11]	Takeyoshi et al. 2007b	+ (8.6, 10%)	+	+ (5/24, 10%)	ICCVAM 1999 (Ryan et al. 2000)	ICCVAM 1999	ICCVAM 1999 (Kligman 1966)	No at ≤ 10%	Basketter et al. 2007
Methyl salicylate	AOO	50	1.44	Kojima et al. 2008 1	- (2.9, 20%)	-	-	ICCVAM 1999 (Kimber et al. 1995)	ICCVAM 1999	ICCVAM 1999	Irritant at 10%	Gerberick et al. 2002
Methyl salicylate	AOO	50	1.44	Kojima et al. 2008 2	- (2.9, 20%)	-	-	ICCVAM 1999 (Kimber et al. 1995)	ICCVAM 1999	ICCVAM 1999	Irritant at 10%	Gerberick et al. 2002
Methyl salicylate	AOO	50	1.40	Kojima et al. 2008 3	- (2.9, 20%)	-	-	ICCVAM 1999 (Kimber et al. 1995)	ICCVAM 1999	ICCVAM 1999	Irritant at 10%	Gerberick et al. 2002

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
2-Methoxy-4-(7-methoxy-3-methyl-5-propenyl-2,3-dihydro-benzofuran-2yl)-phenol (Synonym: Dehydrodiisoeugenol)	AOO	30	5.37	Takeyoshi et al. 2007a	NA	+	NA	NA	Takeyoshi et al. et al. 2007a	NA	NA	NA
Nickel sulfate	DMSO	10	2.58	Kojima et al. 2008 3	+ (3.1, 5%)	+	+	Ryan et al 2002	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.15%	Basketter and Scholes 1992
Nickel sulfate	DMSO	10	4.53	Kojima et al. 2008 4	+ (3.1, 5%)	+	+	Ryan et al 2002	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.15%	Basketter and Scholes 1992
Nickel sulfate	DMSO	10	2.66	Kojima et al. 2008 7	+ (3.1, 5%)	+	+	Ryan et al 2002	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.15%	Basketter and Scholes 1992
1,4-Phenylene-diamine	AOO	2	11.70	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+ (26.4, 1%)	+	+	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.5%	Basketter et al. 2007
1,4-Phenylene-diamine	AOO	10	14.70	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+ (26.4, 1%)	+	+	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	ICCVAM 1999	No at ≤ 0.5%	Basketter et al. 2007
Propylene glycol	AOO	10	1.2	Takeyoshi et al. 2005	- (1.6, 100%)[12]	-	+ (HPTA)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	ICCVAM 1999	NA	NA
Propylene glycol	AOO	50	1.57	Takeyoshi et al. 2005	- (1.6, 100%)[12]	-	+ (HPTA)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	ICCVAM 1999	NA	NA

Chemical Name	Veh.[1]	Highest Conc. Tested (%)	Highest SI	LLNA: BrdU Ref.[2]	Trad. LLNA Result[3]	GP Result	Human Result[4]	Ref. Trad. LLNA[5]	Ref. GP	Ref. Human[6]	Skin Irritant?	Reference Skin Irritation
Propylene glycol	AOO	50	0.87[13]	Takeyoshi et al. 2006; Takeyoshi et al. 2007b	- (1.6, 100%)[12]	-	+ (HPTA)	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	ICCVAM 1999	NA	NA
Trimellitic anhydride	AOO	10	7.85	Takeyoshi unpublish-ed 2009	+ (4.6, 25%)	+	NA	ICCVAM 1999 (Gerberick et al. 2005)	ICCVAM 1999	NA	No at ≤ 10%	Basketter and Scholes 1992

Abbreviations: ACE = Acetone; AOO = Acetone: olive oil (4:1); DMF = *N,N*-dimethyl formamide; DMSO = Dimethyl sulfoxide; LLNA: BrdU-ELISA = Murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; CASRN = Chemical Abstract Services Registry Number; Conc.= Concentration; GP = Guinea pig; LLNA = Murine local lymph node assay; NA = Not available; nonstd = non-standard; PC = Positive control (the numbers after PC represent the laboratory that performed the test and then the order of the test) ; Ref. = Reference; SI = Stimulation index; Trad. = Traditional; Veh. = Vehicle.

+ = Sensitizer; - = Non-sensitizer

[1] Applies to both traditional LLNA and LLNA: BrdU-ELISA unless otherwise noted.

[2]Number after Kojima et al. 2008 represents the laboratory that submitted the test.

[3]Numbers in parentheses indicate the maximum SI and the highest concentration tested.

[4]Information in parentheses provides the evidence for the human result; usually as incidence of a positive human response at the challenge concentration.

[5]Reference in parentheses applies to the maximum SI and the highest concentration tested, if it is different from the reference for the traditional LLNA result.

[6]Reference in parentheses applies to the evidence for the human result if different from the sensitizer/nonsensitizer outcome.

[7]Maximum SI occurred at 50%.

[8]Maximum SI occurred at 10%.

[9]Vehicle for the traditional LLNA was acetone.

[10]Maximum SI occurred at 25%. Vehicle for the traditional LLNA was *N,N*-dimethyl formamide.

[11]Maximum SI occurred at 12.5%.

[12]Vehicle for the traditional LLNA was distilled water.

[13]Maximum SI occurred at 2% and 10%.

Appendix C2

Comparison of Multiple LLNA: BrdU-ELISA Decision Criteria and Traditional LLNA Results (Alphanumeric Order)

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Appendix C-2 Comparative Performance of Various LLNA: BrdU-ELISA SI Values and Traditional LLNA Tests (Alphanumeric Order)

Chemical Name	CASRN	Highest Conc. Tested (%)	Highest SI	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.	SI ≥ 5.0	SI ≥ 4.5	SI ≥ 4.0	SI ≥ 3.5	SI ≥ 3.0	SI ≥ 2.5	SI ≥ 2.0	SI ≥ 1.5	SI ≥ 1.3	SI ≥ 1.0	Ref. BrdU-ELISA[1]	Trad. LLNA Result	Ref. Trad. LLNA
m-Aminophenol	591-27-5	25	3.06	+	+	+	+	-	-	-	-	+	+	+	+	+	+	Takeyoshi et al. 2007b	+	ICCVAM 1999
Aniline	62-53-3	50	1.50	+	-	+	-	-	-	-	-	-	-	-	+	+	+	Takeyoshi et al. 2007b	+	ICCVAM 1999
p-Benzoquinone	106-51-4	1	6.90	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2004b; Takeyoshi et al. 2007b	+	ICCVAM 1999
4-Chloroaniline	106-47-8	25	2.53	+	+	+	+	-	-	-	-	-	+	+	+	+	+	Takeyoshi et al. 2007b	+	ICCVAM 1999
Cinnamic aldehyde	104-55-2	50	3.97	+	+	+	+	-	-	-	+	+	+	+	+	+	+	Takeyoshi et al. 2007b	+	ICCVAM 1999
trans-Cinnamaldehyde	14371-10-9	10	5.90	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2005	+	Gerberick et al. 2005
trans-Cinnamaldehyde	14371-10-9	10	4.11	+	+	+	+	-	-	+	+	+	+	+	+	+	+	Kojima et al. 2008 5	+	Gerberick et al. 2005
trans-Cinnamaldehyde	14371-10-9	10	3.50	+	+	+	+	-	-	-	+	+	+	+	+	+	+	Kojima et al. 2008 4	+	Gerberick et al. 2005
trans-Cinnamaldehyde	14371-10-9	10	3.37	+	+	+	+	-	-	-	-	+	+	+	+	+	+	Kojima et al. 2008 2	+	Gerberick et al. 2005
Citral	5392-40-5	50	16.35	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2007b; Takeyoshi et al. 2005	+	ICCVAM 1999
Cyclamen aldehyde	103-95-7	100	1.97	+	+	+	+	-	-	-	-	-	-	-	+	+	+	Takeyoshi et al. 2007b	+	Basketter et al. 2005
4,5'-Diallyl-2'-hydroxy-2,3'-dimethoxyphenyl ether (DHEB)	NA	20	7.30	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2004a	NA	NA

Chemical Name	CASRN	Highest Conc. Tested (%)	Highest SI	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.	SI ≥ 5.0	SI ≥ 4.5	SI ≥ 4.0	SI ≥ 3.5	SI ≥ 3.0	SI ≥ 2.5	SI ≥ 2.0	SI ≥ 1.5	SI ≥ 1.3	SI ≥ 1.0	Ref. BrdU-ELISA[1]	Trad. LLNA Result	Ref. Trad. LLNA
Diethyl phthalate (Phthallic acid diethylester)	84-66-2	50	0.88	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Takeyoshi et al. 2007b	-	ICCVAM 1999
2,2'-Dihydroxyl-3,3'-dimethoxy-5,5'-diallyl-biphenyl (DHEA)	NA	30	2.30	+	+	+	-	-	-	-	-	-	-	+	+	+	+	Takeyoshi et al. 2004a	NA	NA
Dimethyliso-phthalate	1454-93-4	50	1.26	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Takeyoshi et al. 2007b	-	ICCVAM 1999
Diphenylcyclo-propenone	886-38-4	2	19.10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	1	4.30	+	+	+	+	-	-	+	+	+	+	+	+	+	+	Kojima et al. 2008 1	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	1	8.37	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 2	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.3	6.26	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 3	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	1	5.50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 4	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.3	18.80	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 5	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	1	4.83	+	+	+	+	-	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 6	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	1	12.98	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 7	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	2	17.90	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2005	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	2	6.84	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2006; Takeyoshi et al. 2007b	+	ICCVAM 1999

Chemical Name	CASRN	Highest Conc. Tested (%)	Highest SI	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.	SI ≥ 5.0	SI ≥ 4.5	SI ≥ 4.0	SI ≥ 3.5	SI ≥ 3.0	SI ≥ 2.5	SI ≥ 2.0	SI ≥ 1.5	SI ≥ 1.3	SI ≥ 1.0	Ref. BrdU-ELISA[1]	Trad. LLNA Result	Ref. Trad. LLNA
Eugenol	97-53-0	30	3.30	+	+	+	+	-	-	-	-	+	+	+	+	+	+	Takeyoshi et al. 2004a	+	ICCVAM 1999
Eugenol	97-53-0	30	3.83	+	+	+	+	-	-	-	+	+	+	+	+	+	+	Takeyoshi et al. 2007a	+	ICCVAM 1999
Eugenol	97-53-0	50	12.30	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2005	+	ICCVAM 1999
Eugenol	97-53-0	50	3.10	+	+	+	+	-	-	-	-	+	+	+	+	+	+	Takeyoshi et al. 2006	+	ICCVAM 1999
Eugenol	97-53-0	50	7.09	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 7	+	ICCVAM 1999
Eugenol	97-53-0	50	3.17	+	+	+	+	-	-	-	-	+	+	+	+	+	+	Kojima et al. 2008 2	+	ICCVAM 1999
Eugenol	97-53-0	50	3.18	+	+	+	+	-	-	-	-	+	+	+	+	+	+	Kojima et al. 2008 6	+	ICCVAM 1999
Eugenol	97-53-0	50	17.70	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2007b	+	ICCVAM 1999
Formaldehyde	50-00-0	10	16.59	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 5	+	ICCVAM 1999
Formaldehyde	50-00-0	10	4.40	+	+	+	+	-	-	+	+	+	+	+	+	+	+	Kojima et al. 2008 1	+	ICCVAM 1999
Formaldehyde	50-00-0	10	1.97	+	+	+	+	-	-	-	-	-	-	-	+	+	+	Kojima et al. 2008 6	+	ICCVAM 1999
Glutaraldehyde	111-30-8	1	28.64	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 5	+	Hilton et. al 1998
Glutaraldehyde	111-30-8	1	3.72	+	+	+	+	-	-	-	+	+	+	+	+	+	+	Kojima et al. 2008 1	+	Hilton et. al 1998
Glutaraldehyde	111-30-8	1	2.25	+	+	+	+	-	-	-	-	-	-	+	+	+	+	Kojima et al. 2008 6	+	Hilton et. al 1998

Chemical Name	CASRN	Highest Conc. Tested (%)	Highest SI	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.	SI ≥ 5.0	SI ≥ 4.5	SI ≥ 4.0	SI ≥ 3.5	SI ≥ 3.0	SI ≥ 2.5	SI ≥ 2.0	SI ≥ 1.5	SI ≥ 1.3	SI ≥ 1.0	Ref. BrdU-ELISA[1]	Trad. LLNA Result	Ref. Trad. LLNA
Glutaraldehyde	111-30-8	2	14.60	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+	Hilton et. al 1998
Glutaraldehyde	111-30-8	10	15.50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+	Hilton et. al 1998
Glycerol	56-81-5	50	1.29	+	-	+	+	-	-	-	-	-	-	-	-	-	+	Takeyoshi et al. 2007b	-	ICCVAM 1999
Hexane	110-54-3	100	1.76	+	-	-	+	-	-	-	-	-	-	-	+	+	+	Takeyoshi unpub-lished 2009	-	ICCVAM 1999
Hexane	110-54-3	50	1.89	+	+	+	+	-	-	-	-	-	-	-	+	+	+	Takeyoshi et al. 2005	-	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	3.60	+	+	+	+	-	-	-	+	+	+	+	+	+	+	Takeyoshi et al. 2003	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	5.90	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2005	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	3.64	+	+	+	+	-	-	-	+	+	+	+	+	+	+	Takeyoshi et al. 2006	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	2.72	+	+	+	+	-	-	-	-	-	+	+	+	+	+	Takeyoshi et al. 2006	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	3.02	+	+	+	+	-	-	-	-	+	+	+	+	+	+	Takeyoshi et al. 2007b	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	3.40	+	+	+	+	-	-	-	-	+	+	+	+	+	+	Kojima et al. 2008 1	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	2.07	+	+	+	+	-	-	-	-	-	-	+	+	+	+	Kojima et al. 2008 PC 1-1		ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	6.11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 PC 1-2		ICCVAM 1999

Chemical Name	CASRN	Highest Conc. Tested (%)	Highest SI	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.	SI ≥ 5.0	SI ≥ 4.5	SI ≥ 4.0	SI ≥ 3.5	SI ≥ 3.0	SI ≥ 2.5	SI ≥ 2.0	SI ≥ 1.5	SI ≥ 1.3	SI ≥ 1.0	Ref. BrdU-ELISA[1]	Trad. LLNA Result	Ref. Trad. LLNA
Hexyl cinnamic aldehyde	101-86-0	50	3.43	+	+	+	+	-	-	-	-	+	+	+	+	+	+	Kojima et al. 2008 PC 1-3		ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	5.15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 PC 2-2		ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	2.52	+	+	+	+	-	-	-	-	-	+	+	+	+	+	Kojima et al. 2008 PC 2-3		ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	2.87	+	+	+	+	-	-	-	-	-	+	+	+	+	+	Kojima et al. 2008 3	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	3.34	+	+	+	+	-	-	-	-	+	+	+	+	+	+	Kojima et al. 2008 PC 3-1		ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	3.54	+	+	+	+	-	-	-	+	+	+	+	+	+	+	Kojima et al. 2008 PC 3-2		ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	2.18	+	+	+	+	-	-	-	-	-	-	+	+	+	+	Kojima et al. 2008 PC 3-3		ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	3.34	+	+	+	+	-	-	-	-	+	+	+	+	+	+	Kojima et al. 2008 4	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	2.69	+	+	+	+	-	-	-	-	-	+	+	+	+	+	Kojima et al. 2008 PC 4-1		ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	3.17	+	+	+	+	-	-	-	-	+	+	+	+	+	+	Kojima et al. 2008 PC 4-2		ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	6.58	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 PC 4-3		ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	13.50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 5	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	12.46	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 PC 5-1		ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	4.24	+	+	+	+	-	-	+	+	+	+	+	+	+	+	Kojima et al. 2008 PC 5-2		ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	6.07	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 PC 5-3		ICCVAM 1999

Chemical Name	CASRN	Highest Conc. Tested (%)	Highest SI	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.	SI ≥ 5.0	SI ≥ 4.5	SI ≥ 4.0	SI ≥ 3.5	SI ≥ 3.0	SI ≥ 2.5	SI ≥ 2.0	SI ≥ 1.5	SI ≥ 1.3	SI ≥ 1.0	Ref. BrdU-ELISA[1]	Trad. LLNA Result	Ref. Trad. LLNA
Hexyl cinnamic aldehyde	101-86-0	50	3.27	+	+	+	+	-	-	-	-	+	+	+	+	+	+	Kojima et al. 2008 6	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	5.30	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 PC 6-1		ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	2.41	+	+	+	+	-	-	-	-	-	-	+	+	+	+	Kojima et al. 2008 PC 6-2		ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	2.52	+	+	+	+	-	-	-	-	-	+	+	+	+	+	Kojima et al. 2008 PC 6-3		ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	3.84	+	+	+	+	-	-	-	+	+	+	+	+	+	+	Kojima et al. 2008 7	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	6.86	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 PC 7-1		ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	4.39	+	+	+	+	-	-	+	+	+	+	+	+	+	+	Kojima et al. 2008 PC 7-2		ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	50	4.78	+	+	+	+	-	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 PC 7-3		ICCVAM 1999
Hydroxycitronellal	107-73-5	100	1.30	+	-	+	+	-	-	-	-	-	-	-	-	+	+	Takeyoshi et al. 2007b	+	ICCVAM 1999
4-[1-Hydroxy-2-(2-methoxy-4-propenyl-phenyoxy)-propyl]-2-methoxy-phenol (Synonym: □-O-4-Dilignol)	NA	30	1.19	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Takeyoshi et al. 2007a	NA	NA
2-Hydroxypropyl-methacrylate	923-26-2	50	1.13	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Takeyoshi et al. 2007b	-	ICCVAM 1999
Isoeugenol	97-54-1	10	8.40	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2005	+	ICCVAM 1999

Chemical Name	CASRN	Highest Conc. Tested (%)	Highest SI	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.	SI ≥ 5.0	SI ≥ 4.5	SI ≥ 4.0	SI ≥ 3.5	SI ≥ 3.0	SI ≥ 2.5	SI ≥ 2.0	SI ≥ 1.5	SI ≥ 1.3	SI ≥ 1.0	Ref. BrdU-ELISA[1]	Trad. LLNA Result	Ref. Trad. LLNA
Isoeugenol	97-54-1	10	2.40	+	+	+	+	-	-	-	-	-	-	+	+	+	+	Takeyoshi et al. 2006; Takeyoshi et al. 2007b	+	ICCVAM 1999
Isoeugenol	97-54-1	30	6.73	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2007a	+	ICCVAM 1999
Isopropanol	67-63-0	50	2.22	+	-	+	+	-	-	-	-	-	-	+	+	+	+	Kojima et al. 2008 1	-	ICCVAM 1999
Isopropanol	67-63-0	50	0.98	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Kojima et al. 2008 3	-	ICCVAM 1999
Isopropanol	67-63-0	50	1.57	-	-	-	-	-	-	-	-	-	-	-	+	+	+	Kojima et al. 2008 4	-	ICCVAM 1999
Isopropanol	67-63-0	50	0.94	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Kojima et al. 2008 5	-	ICCVAM 1999
Isopropanol	67-63-0	50	2.04	+	-	+	-	-	-	-	-	-	-	+	+	+	+	Kojima et al. 2008 6	-	ICCVAM 1999
Isopropanol	67-63-0	50	1.01	-	-	-	-	-	-	-	-	-	-	-	-	-	+	Kojima et al. 2008 7	-	ICCVAM 1999
Isopropanol	67-63-0	100	0.92	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Takeyoshi et al. 2007b	-	ICCVAM 1999
Isopropyl myristate	110-27-0	50	4.20	+	+	+	+	-	-	+	+	+	+	+	+	+	+	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+	Ryan et al. 2000
Lactic acid	598-82-3	50	2.53	+	+	+	+	-	-	-	-	-	+	+	+	+	+	Kojima et al. 2008 7	-	ICCVAM 1999
Lactic acid	598-82-3	50	1.89	+	-	+	+	-	-	-	-	-	-	-	+	+	+	Kojima et al. 2008 4	-	ICCVAM 1999
Lactic acid	598-82-3	50	1.80	+	-	+	-	-	-	-	-	-	-	-	+	+	+	Kojima et al. 2008 3	-	ICCVAM 1999

Chemical Name	CASRN	Highest Conc. Tested (%)	Highest SI	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.	SI ≥ 5.0	SI ≥ 4.5	SI ≥ 4.0	SI ≥ 3.5	SI ≥ 3.0	SI ≥ 2.5	SI ≥ 2.0	SI ≥ 1.5	SI ≥ 1.3	SI ≥ 1.0	Ref. BrdU-ELISA[1]	Trad. LLNA Result	Ref. Trad. LLNA
Linalool	78-70-6	100	1.45	+	-	-	-	-	-	-	-	-	-	-	-	+	+	Takeyoshi unpub-lished 2009	+	Gerberick et al. 2005
2-Mercaptobenzo-thiazole	149-30-4	50	1.62	+	+	+	-	-	-	-	-	-	-	-	+	+	+	Takeyoshi et al. 2007b	+	ICCVAM 1999
Methyl salicylate	119-36-8	50	1.44	+	-	+	-	-	-	-	-	-	-	-	-	+	+	Kojima et al. 2008 1	-	ICCVAM 1999
Methyl salicylate	119-36-8	50	1.44	-	-	-	-	-	-	-	-	-	-	-	-	+	+	Kojima et al. 2008 2	-	ICCVAM 1999
Methyl salicylate	119-36-8	50	1.40	+	-	-	-	-	-	-	-	-	-	-	-	+	+	Kojima et al. 2008 3	-	ICCVAM 1999
2-Methoxy-4-(7-methoxy-3-methyl-5-propenyl-2,3-dihydro-benzofuran-2yl)-phenol (Syn: Dehydrodi-isoeugenol)		30	5.37	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2007a	NA	NA
Nickel sulfate	10101-97-0	10	2.58	+	+	+	+	-	-	-	-	-	+	+	+	+	+	Kojima et al. 2008 3	+	Ryan et al 2002
Nickel sulfate	10101-97-0	10	4.53	+	+	+	+	-	+	+	+	+	+	+	+	+	+	Kojima et al. 2008 4	+	Ryan et al 2002
Nickel sulfate	10101-97-0	10	2.66	+	+	+	+	-	-	-	-	-	+	+	+	+	+	Kojima et al. 2008 7	+	Ryan et al 2002
1,4-Phenylene-diamine	106-50-3	2	11.70	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+	ICCVAM 1999
1,4-Phenylene-diamine	106-50-3	10	14.70	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi et al. 2005; Takeyoshi et al. 2007b	+	ICCVAM 1999

Chemical Name	CASRN	Highest Conc. Tested (%)	Highest SI	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.	SI ≥ 5.0	SI ≥ 4.5	SI ≥ 4.0	SI ≥ 3.5	SI ≥ 3.0	SI ≥ 2.5	SI ≥ 2.0	SI ≥ 1.5	SI ≥ 1.3	SI ≥ 1.0	Ref. BrdU-ELISA[1]	Trad. LLNA Result	Ref. Trad. LLNA
Propylene glycol	57-55-6	50	1.57	+	-	+	-	-	-	-	-	-	-	-	+	+	+	Takeyoshi et al. 2005	-	ICCVAM 1999
Propylene glycol	57-55-6	50	0.87	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Takeyoshi et al. 2006; Takeyoshi et al. 2007b	-	ICCVAM 1999
Trimellitic anhydride	552-30-7	10	7.85	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Takeyoshi unpub-lished 2009	+	ICCVAM 1999

Abbreviations: BrdU-ELISA LLNA = Murine local lymph node assay with enzyme-linked immunosorbent assay detection of bromodeoxyuridine; BrdU = Bromodeoxyuridine CASRN = Chemical Abstract Services Registry Number; CI= Confidence interval (mean absorbance of any treatment group is greater than 95% confidence interval of vehicle control group mean); Conc. = Concentration; LLNA = Murine local lymph node assay; NA = Not available; PC = Positive control (numbers after PC designates laboratory and test number); Ref. = Reference; SD = Standard deviation (mean absorbance of any treatment group is greater than 2 or 3 SD for vehicle control group); SI = Stimulation index; Stats. = Statistics (analysis of variance for multiple dose groups or t-test to compare one treatment group to the vehicle control group); Trad. = Traditional.
+ = Sensitizer; - = Non-sensitizer
[1] Number after Kojima et al. 2008 represents the laboratory that submitted the test.

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Appendix D

Individual Animal Data for the LLNA: BrdU-ELISA

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Appendix D1

Individual Animal Data for the LLNA: BrdU-ELISA - Takeyoshi et al.

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57 **Appendix D-1 Absorbance Data for Individual Animals for LLNA: BrdU-ELISA Tests Submitted by Dr. Takeyoshi**

Substance	Veh.	An. No.	1 Conc. (%)	1 ABS	1 SI	An. No.	2 Conc. (%)	2 ABS	2 SI	An. No.	3 Conc. (%)	3 ABS	3 SI	An. No.	4 Conc. (%)	4 ABS	4 SI	EC 1.5	EC 2	Ref.
VC	AOO	1	0	0.065	0.97	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2003
VC	AOO	2	0	0.070	1.04	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2003
VC	AOO	3	0	0.062	0.91	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2003
VC	AOO	4	0	0.073	1.08	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2003
VC	AOO	Mean	0	0.068	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2003
HCA	AOO	5	3.125	0.076	1.12	9	6.25	0.055	0.81	13	12.5	0.140	2.08	17	25	0.098	1.45	NA	NA	2003
HCA	AOO	6	3.125	0.067	0.99	10	6.25	0.171	2.54	14	12.5	0.120	1.77	18	25	0.144	2.13	NA	NA	2003
HCA	AOO	7	3.125	0.104	1.54	11	6.25	0.117	1.73	15	12.5	0.149	2.21	19	25	0.259	3.83	NA	NA	2003
HCA	AOO	8	3.125	0.069	1.02	12	6.25	0.090	1.33	16	12.5	0.100	1.48	20	25	0.158	2.33	NA	NA	2003
HCA	AOO	Mean	3.125	0.079	1.17	Mean	6.25	0.108	1.60	Mean	12.5	0.127	1.88	Mean	25	0.165	2.44	5.52	15.18	2003
VC	AOO	1	0	0.086	0.72	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2003
VC	AOO	2	0	0.140	1.17	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2003
VC	AOO	3	0	0.133	1.11	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2003
VC	AOO	4	0	0.121	1.01	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2003
VC	AOO	Mean	0	0.120	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2003
HCA	AOO	5	12.5	0.186	1.55	9	25	0.209	1.74	13	50	0.329	2.75	NA	NA	NA	NA	NA	NA	2003
HCA	AOO	6	12.5	0.245	2.04	10	25	0.345	2.88	14	50	0.388	3.24	NA	NA	NA	NA	NA	NA	2003
HCA	AOO	7	12.5	0.239	2.00	11	25	0.322	2.69	15	50	0.480	4.01	NA	NA	NA	NA	NA	NA	2003
HCA	AOO	8	12.5	0.090	0.75	12	25	0.279	2.33	16	50	0.548	4.58	NA	NA	NA	NA	NA	NA	2003
HCA	AOO	Mean	12.5	0.190	1.59	Mean	25	0.289	2.41	Mean	50	0.436	3.64	NA	NA	NA	NA	11.58	18.75	2003
VC	AOO	1	0	0.073	0.68	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2004
VC	AOO	2	0	0.082	0.77	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2004
VC	AOO	3	0	0.167	1.57	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2004
VC	AOO	4	0	0.104	0.98	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2004
VC	AOO	Mean	0	0.107	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2004
Eugenol	AOO	5	1	0.267	2.50	9	6	0.206	1.94	13	15	0.267	2.51	17	30	0.473	4.44	NA	NA	2004a
Eugenol	AOO	6	1	0.116	1.09	10	6	0.206	1.93	14	15	0.168	1.58	18	30	0.287	2.69	NA	NA	2004a
Eugenol	AOO	7	1	0.184	1.73	11	6	0.064	0.60	15	15	0.269	2.53	19	30	0.391	3.67	NA	NA	2004a
Eugenol	AOO	8	1	0.182	1.71	12	6	0.166	1.56	16	15	0.300	2.82	20	30	0.268	2.52	NA	NA	2004a
Eugenol	AOO	Mean	1	0.187	1.76	Mean	6	0.161	1.51	Mean	15	0.251	2.36	Mean	30	0.355	3.33	5.94	11.19	2004a
DHEA	AOO	21	1	0.189	1.77	29	6	0.285	2.68	33	30	0.121	1.14	NA	NA	NA	NA	NA	NA	2004a
DHEA	AOO	26	1	0.194	1.82	30	6	0.099	0.93	34	30	0.317	2.98	NA	NA	NA	NA	NA	NA	2004a
DHEA	AOO	27	1	0.191	1.80	31	6	0.144	1.35	35	30	0.309	2.90	NA	NA	NA	NA	NA	NA	2004a
DHEA	AOO	28	1	0.153	1.43	32	6	0.203	1.91	36	30	0.219	2.06	NA	NA	NA	NA	NA	NA	2004a
DHEA	AOO	Mean	1	0.182	1.71	Mean	6	0.183	1.71	Mean	30	0.242	2.27	NA	NA	NA	NA	0.70*	18.43	2004a
DHEB	AOO	37	1	0.187	1.75	41	6	0.421	3.95	45	20	0.749	7.03	NA	NA	NA	NA	NA	NA	2004a
DHEB	AOO	38	1	0.138	1.30	42	6	0.563	5.28	46	20	0.733	6.88	NA	NA	NA	NA	NA	NA	2004a
DHEB	AOO	39	1	0.263	2.47	43	6	0.504	4.73	47	20	0.741	6.95	NA	NA	NA	NA	NA	NA	2004a
DHEB	AOO	40	1	0.389	3.65	44	6	0.664	6.23	48	20	0.872	8.18	NA	NA	NA	NA	NA	NA	2004a

Substance	Veh.	An. No.	1 Conc. (%)	1 ABS	1 SI	An. No.	2 Conc. (%)	2 ABS	2 SI	An. No.	3 Conc. (%)	3 ABS	3 SI	An. No.	4 Conc. (%)	4 ABS	4 SI	EC 1.5	EC 2	Ref.
DHEB	AOO	Mean	1	0.244	2.29	Mean	6	0.538	5.05	Mean	20	0.774	7.26	NA	NA	NA	NA	0.60	0.83	2004a
p-Benzoquinone	AOO	1	0	0.093	0.95	5	0.25	0.308	3.14	9	0.50	0.383	3.90	13	1	0.744	7.58	NA	NA	2004b, 2007b
p-Benzoquinone	AOO	2	0	0.078	0.79	6	0.25	0.401	4.08	10	0.50	0.681	6.93	14	1	0.715	7.28	NA	NA	2004b, 2007b
p-Benzoquinone	AOO	3	0	0.093	0.95	7	0.25	0.207	2.11	11	0.50	0.893	9.09	15	1	0.598	6.09	NA	NA	2004b, 2007b
p-Benzoquinone	AOO	4	0	0.129	1.31	8	0.25	0.401	4.08	12	0.50	0.315	3.21	16	1	0.672	6.84	NA	NA	2004b, 2007b
p-Benzoquinone	AOO	Mean	0	0.098	1.00	Mean	0.25	0.329	3.35	Mean	0.50	0.568	5.78	Mean	1	0.682	6.94	0.15	0.17	2004b, 2007b
VC	AOO	1	0	0.084	1.20	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
VC	AOO	2	0	0.101	1.45	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
VC	AOO	3	0	0.042	0.61	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
VC	AOO	4	0	0.051	0.73	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
VC	AOO	Mean	0	0.070	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Isoeugenol	AOO	5	10	0.968	13.93	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Isoeugenol	AOO	6	10	0.370	5.32	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Isoeugenol	AOO	7	10	0.408	5.88	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Isoeugenol	AOO	8	10	0.579	8.33	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Isoeugenol	AOO	Mean	10	0.581	8.36	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
p-Phenylene diamine	AOO	6	10	1.115	16.1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
p-Phenylene diamine	AOO	7	10	1.034	14.9	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
p-Phenylene diamine	AOO	8	10	1.018	14.7	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
p-Phenylene diamine	AOO	9	10	0.919	13.2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
p-Phenylene diamine	AOO	Mean	10	1.022	14.7	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
trans-Cinnamaldehyde	AOO	13	10	0.551	7.93	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
trans-Cinnamaldehyde	AOO	14	10	0.200	2.87	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
trans-Cinnamaldehyde	AOO	15	10	0.407	5.86	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
trans-Cinnamaldehyde	AOO	16	10	0.471	6.78	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
trans-Cinnamaldehyde	AOO	Mean	10	0.407	5.86	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Glutaraldehyde	AOO	1	0	0.084	1.20	6	10	1.116	16.1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Glutaraldehyde	AOO	2	0	0.101	1.45	7	10	1.146	16.3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Glutaraldehyde	AOO	3	0	0.042	0.61	8	10	1.028	14.8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Glutaraldehyde	AOO	4	0	0.051	0.73	9	10	1.028	14.8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b

Substance	Veh.	An. No.	1 Conc. (%)	1 ABS	1 SI	An. No.	2 Conc. (%)	2 ABS	2 SI	An. No.	3 Conc. (%)	3 ABS	3 SI	An. No.	4 Conc. (%)	4 ABS	4 SI	EC 1.5	EC 2	Ref.
Glutaraldehyde	AOO	Mean	0	0.070	1.00	Mean	10	1.080	15.5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Citral	AOO	1	0	0.084	1.20	6	10	0.240	3.45	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005; 2007b
Citral	AOO	2	0	0.101	1.45	7	10	0.123	1.76	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005; 2007b
Citral	AOO	3	0	0.042	0.61	8	10	0.089	1.29	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005; 2007b
Citral	AOO	4	0	0.051	0.73	9	10	0.059	0.85	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005; 2007b
Citral	AOO	Mean	0	0.070	1.00	Mean	10	0.128	1.84	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005; 2007b
Citral	AOO	1	0	0.074	1.12	6	50	1.080	16.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005; 2007b
Citral	AOO	2	0	0.057	0.87	7	50	0.985	15.0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005; 2007b
Citral	AOO	3	0	0.083	1.26	8	50	1.323	20.1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005; 2007b
Citral	AOO	4	0	0.050	0.76	9	50	0.911	13.9	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005; 2007b
Citral	AOO	Mean	0	0.066	1.00	Mean	50	1.075	16.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005; 2007b
VC	AOO	1	0	0.102	1.34	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
VC	AOO	2	0	0.107	1.40	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
VC	AOO	3	0	0.044	0.57	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
VC	AOO	4	0	0.053	0.69	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
VC	AOO	Mean	0	0.077	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Isoeugenol	AOO	5	10	0.937	12.23	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Isoeugenol	AOO	6	10	0.315	4.11	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Isoeugenol	AOO	7	10	0.377	4.93	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Isoeugenol	AOO	8	10	0.576	7.52	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Isoeugenol	AOO	Mean	10	0.551	7.20	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Eugenol	AOO	25	10	0.356	4.65	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Eugenol	AOO	26	10	0.145	1.89	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Eugenol	AOO	27	10	0.141	1.84	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Eugenol	AOO	28	10	0.332	4.33	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Eugenol	AOO	Mean	10	0.243	3.18	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Isopropyl myristate	AOO	6	10	0.054	0.70	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Isopropyl myristate	AOO	7	10	0.073	0.95	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Isopropyl myristate	AOO	8	10	0.131	1.71	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Isopropyl myristate	AOO	9	10	0.072	0.94	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Isopropyl myristate	AOO	Mean	10	0.083	1.08	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Propylene glycol	AOO	33	10	0.065	0.85	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005

Substance	Veh.	An. No.	1 Conc. (%)	1 ABS	1 SI	An. No.	2 Conc. (%)	2 ABS	2 SI	An. No.	3 Conc. (%)	3 ABS	3 SI	An. No.	4 Conc. (%)	4 ABS	4 SI	EC 1.5	EC 2	Ref.
Propylene glycol	AOO	34	10	0.146	1.91	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Propylene glycol	AOO	35	10	0.065	0.85	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Propylene glycol	AOO	36	10	0.092	1.20	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Propylene glycol	AOO	Mean	10	0.092	1.20	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Hexane	AOO	37	10	0.041	0.54	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Hexane	AOO	38	10	0.086	1.12	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Hexane	AOO	39	10	0.047	0.61	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Hexane	AOO	40	10	0.051	0.67	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Hexane	AOO	Mean	10	0.056	0.73	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Diphenylcyclopropenone	AOO	41	10	0.524	6.84	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Diphenylcyclopropenone	AOO	42	10	0.538	7.03	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Diphenylcyclopropenone	AOO	43	10	0.693	9.04	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Diphenylcyclopropenone	AOO	44	10	1.106	14.44	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Diphenylcyclopropenone	AOO	Mean	10	0.715	9.34	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
VC	AOO	1	0	0.089	0.94	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
VC	AOO	2	0	0.083	0.88	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
VC	AOO	3	0	0.109	1.15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
VC	AOO	4	0	0.099	1.04	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
VC	AOO	Mean	0	0.095	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
DNCB	AOO	5	2	1.725	18.15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
DNCB	AOO	6	2	1.668	17.56	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
DNCB	AOO	7	2	1.736	18.28	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
DNCB	AOO	8	2	1.658	17.45	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
DNCB	AOO	Mean	2	1.697	17.86	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
p-Phenylene diamine	AOO	6	2	0.960	10.1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
p-Phenylene diamine	AOO	7	2	1.257	13.2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
p-Phenylene diamine	AOO	8	2	1.031	10.9	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
p-Phenylene diamine	AOO	9	2	1.198	12.6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
p-Phenylene diamine	AOO	Mean	2	1.115	11.7	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Glutaraldehyde	AOO	6	2	1.447	15.2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Glutaraldehyde	AOO	7	2	1.331	14.0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Glutaraldehyde	AOO	8	2	1.344	14.1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Glutaraldehyde	AOO	9	2	1.410	14.8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b

Substance	Veh.	An. No.	1 Conc. (%)	1 ABS	1 SI	An. No.	2 Conc. (%)	2 ABS	2 SI	An. No.	3 Conc. (%)	3 ABS	3 SI	An. No.	4 Conc. (%)	4 ABS	4 SI	EC 1.5	EC 2	Ref.
Glutaraldehyde	AOO	Mean	2	1.383	14.6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Diphencyclopro penone	AOO	6	2	1.850	19.5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Diphencyclopro penone	AOO	7	2	1.775	18.7	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Diphencyclopro penone	AOO	8	2	1.672	17.6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Diphencyclopro penone	AOO	9	2	1.952	20.6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Diphencyclopro penone	AOO	Mean	2	1.812	19.1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
VC	AOO	1	0	0.074	1.12	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
VC	AOO	2	0	0.057	0.87	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
VC	AOO	3	0	0.083	1.26	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
VC	AOO	4	0	0.050	0.76	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
VC	AOO	Mean	0	0.066	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
HCA	AOO	5	50	0.341	5.19	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
HCA	AOO	6	50	0.366	5.57	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
HCA	AOO	7	50	0.454	6.91	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
HCA	AOO	8	50	0.391	5.95	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
HCA	AOO	Mean	50	0.388	5.90	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Propylene glycol	AOO	9	50	0.100	1.53	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Propylene glycol	AOO	10	50	0.159	2.42	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Propylene glycol	AOO	11	50	0.071	1.07	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Propylene glycol	AOO	12	50	0.082	1.25	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Propylene glycol	AOO	Mean	50	0.103	1.57	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Hexane	AOO	13	50	0.122	1.86	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Hexane	AOO	14	50	0.099	1.51	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Hexane	AOO	15	50	0.153	2.32	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Hexane	AOO	16	50	0.124	1.88	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Hexane	AOO	Mean	50	0.124	1.89	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Eugenol	AOO	33	50	0.708	10.31	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Eugenol	AOO	34	50	0.733	10.67	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Eugenol	AOO	35	50	1.073	15.63	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Eugenol	AOO	36	50	0.859	12.50	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Eugenol	AOO	Mean	50	0.843	12.28	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005
Isopropyl myristate	AOO	1	0	0.065	0.95	6	50	0.216	3.15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Isopropyl myristate	AOO	2	0	0.062	0.91	7	50	0.414	6.03	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Isopropyl myristate	AOO	3	0	0.076	1.11	8	50	0.244	3.55	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
Isopropyl myristate	AOO	4	0	0.071	1.03	9	50	0.276	4.02	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b

Substance	Veh.	An. No.	1 Conc. (%)	1 ABS	1 SI	An. No.	2 Conc. (%)	2 ABS	2 SI	An. No.	3 Conc. (%)	3 ABS	3 SI	An. No.	4 Conc. (%)	4 ABS	4 SI	EC 1.5	EC 2	Ref.
Isopropyl myristate	AOO	Mean	0	0.069	1.00	Mean	50	0.288	4.19	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2005, 2007b
VC	AOO	1	0	0.543	1.16	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2006
VC	AOO	2	0	0.430	0.92	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2006
VC	AOO	3	0	0.367	0.79	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2006
VC	AOO	4	0	0.529	1.13	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2006
VC	AOO	Mean	0	0.467	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2006
HCA	AOO	5	2	0.486	1.04	9	10	0.745	1.60	13	50	1.517	3.25	NA	NA	NA	NA	NA	NA	2006
HCA	AOO	6	2	0.402	0.86	10	10	0.639	1.37	14	50	1.184	2.53	NA	NA	NA	NA	NA	NA	2006
HCA	AOO	7	2	0.442	0.95	11	10	0.495	1.06	15	50	1.362	2.91	NA	NA	NA	NA	NA	NA	2006
HCA	AOO	8	2	0.370	0.79	12	10	0.531	1.14	16	50	1.021	2.19	NA	NA	NA	NA	NA	NA	2006
HCA	AOO	Mean	2	0.425	0.91	Mean	10	0.603	1.29	Mean	50	1.271	2.72	NA	NA	NA	NA	15.87	29.86	2006
Eugenol	AOO	17	2	0.265	0.57	21	10	0.803	1.72	25	50	1.341	2.87	NA	NA	NA	NA	NA	NA	2006
Eugenol	AOO	18	2	0.245	0.52	22	10	0.676	1.45	26	50	1.479	3.17	NA	NA	NA	NA	NA	NA	2006
Eugenol	AOO	19	2	0.302	0.65	23	10	0.495	1.06	27	50	1.367	2.93	NA	NA	NA	NA	NA	NA	2006
Eugenol	AOO	20	2	0.242	0.52	24	10	0.761	1.63	28	50	1.523	3.26	NA	NA	NA	NA	NA	NA	2006
Eugenol	AOO	Mean	2	0.263	0.56	Mean	10	0.684	1.46	Mean	50	1.428	3.05	NA	NA	NA	NA	11.01	23.58	2006
Isoeugenol	AOO	1	0	0.543	1.16	6	0.40	0.116	0.25	11	2	0.379	0.81	16	10	1.308	2.80	NA	NA	2006, 2007b
Isoeugenol	AOO	2	0	0.430	0.92	7	0.40	0.180	0.39	12	2	0.205	0.44	17	10	1.224	2.62	NA	NA	2006, 2007b
Isoeugenol	AOO	3	0	0.367	0.79	8	0.40	0.191	0.41	13	2	0.176	0.38	18	10	0.826	1.77	NA	NA	2006, 2007b
Isoeugenol	AOO	4	0	0.529	1.13	9	0.40	0.217	0.46	14	2	0.216	0.46	19	10	1.053	2.25	NA	NA	2006, 2007b
Isoeugenol	AOO	Mean	0	0.467	1.00	Mean	0.40	0.176	0.38	Mean	2	0.244	0.52	Mean	10	1.102	2.36	6.26	8.43	2006, 2007b
HCA	AOO	1	0	0.392	1.66	5	2	0.280	1.18	9	10	0.191	0.81	13	50	1.023	4.32	NA	NA	2006
HCA	AOO	2	0	0.273	1.15	6	2	0.132	0.56	10	10	0.207	0.87	14	50	0.642	2.71	NA	NA	2006
HCA	AOO	3	0	0.169	0.71	7	2	0.163	0.69	11	10	0.252	1.07	15	50	0.902	3.81	NA	NA	2006
HCA	AOO	4	0	0.113	0.48	8	2	0.175	0.74	12	10	0.255	1.08	16	50	0.880	3.72	NA	NA	2006
HCA	AOO	Mean	0	0.236	1.00	Mean	2	0.188	0.79	Mean	10	0.226	0.96	Mean	50	0.862	3.64	18.06	25.52	2006
Propylene glycol	AOO	1	0	0.347	1.54	6	2	0.321	1.42	11	10	0.143	0.63	16	50	0.143	0.63	NA	NA	2006, 2007b
Propylene glycol	AOO	2	0	0.273	1.21	7	2	0.216	0.96	12	10	0.108	0.48	17	50	0.167	0.74	NA	NA	2006, 2007b
Propylene glycol	AOO	3	0	0.166	0.74	8	2	0.150	0.67	13	10	0.154	0.68	18	50	0.149	0.66	NA	NA	2006, 2007b
Propylene glycol	AOO	4	0	0.116	0.51	9	2	0.129	0.57	14	10	0.415	1.84	19	50	0.176	0.78	NA	NA	2006, 2007b
Propylene glycol	AOO	Mean	0	0.225	1.00	Mean	2	0.204	0.91	Mean	10	0.205	0.91	Mean	50	0.159	0.70	NA	NA	2006, 2007b
2-Hydroxypropyl methacrylate	AOO	1	0	0.114	1.08	6	50	0.101	0.96	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
2-Hydroxypropyl methacrylate	AOO	2	0	0.091	0.87	7	50	0.143	1.35	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b

Substance	Veh.	An. No.	1 Conc. (%)	1 ABS	1 SI	An. No.	2 Conc. (%)	2 ABS	2 SI	An. No.	3 Conc. (%)	3 ABS	3 SI	An. No.	4 Conc. (%)	4 ABS	4 SI	EC 1.5	EC 2	Ref.
2-Hydroxypropyl methacrylate	AOO	3	0	0.095	0.90	8	50	0.139	1.32	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
2-Hydroxypropyl methacrylate	AOO	4	0	0.122	1.15	9	50	0.093	0.88	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
2-Hydroxypropyl methacrylate	AOO	Mean	0	0.105	1.00	Mean	50	0.119	1.13	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
Aniline	AOO	1	0	0.081	0.86	6	12.5	0.099	1.05	11	25	0.107	1.14	16	50	0.158	1.67	NA	NA	2007b
Aniline	AOO	2	0	0.085	0.90	7	12.5	0.119	1.26	12	25	0.128	1.35	17	50	0.111	1.17	NA	NA	2007b
Aniline	AOO	3	0	0.087	0.92	8	12.5	0.148	1.57	13	25	0.140	1.48	18	50	0.099	1.04	NA	NA	2007b
Aniline	AOO	4	0	0.125	1.33	9	12.5	0.154	1.63	14	25	0.149	1.58	19	50	0.199	2.11	NA	NA	2007b
Aniline	AOO	Mean	0	0.095	1.00	Mean	12.5	0.130	1.38	Mean	25	0.131	1.39	Mean	50	0.142	1.50	50.0	NC	2007b
p-Chloroaniline	AOO	1	0	0.081	0.86	6	12.5	0.151	1.60	11	25	0.211	2.23	NA	NA	NA	NA	NA	NA	2007b
p-Chloroaniline	AOO	2	0	0.085	0.90	7	12.5	0.139	1.47	12	25	0.181	1.91	NA	NA	NA	NA	NA	NA	2007b
p-Chloroaniline	AOO	3	0	0.087	0.92	8	12.5	0.150	1.59	13	25	0.289	3.05	NA	NA	NA	NA	NA	NA	2007b
p-Chloroaniline	AOO	4	0	0.125	1.33	9	12.5	0.196	2.07	14	25	0.276	2.92	NA	NA	NA	NA	NA	NA	2007b
p-Chloroaniline	AOO	Mean	0	0.095	1.00	Mean	12.5	0.159	1.68	Mean	25	0.239	2.53	NA	NA	NA	NA	10.79	17.21	2007b
Cinnamic aldehyde	AOO	1	0	0.123	0.78	6	12.5	0.484	3.09	11	25	0.606	3.87	16	50	0.581	3.71	NA	NA	2007b
Cinnamic aldehyde	AOO	2	0	0.177	1.13	7	12.5	0.304	1.94	12	25	0.625	3.99	17	50	0.529	3.38	NA	NA	2007b
Cinnamic aldehyde	AOO	3	0	0.170	1.08	8	12.5	0.470	3.00	13	25	0.517	3.30	18	50	0.721	4.60	NA	NA	2007b
Cinnamic aldehyde	AOO	4	0	0.158	1.01	9	12.5	0.344	2.20	14	25	0.617	3.94	19	50	0.655	4.18	NA	NA	2007b
Cinnamic aldehyde	AOO	Mean	0	0.157	1.00	Mean	12.5	0.401	2.56	Mean	25	0.591	3.77	Mean	50	0.621	3.97	6.81	9.07	2007b
Cyclamen aldehyde	AOO	1	0	0.125	0.86	6	25	0.132	0.91	11	50	0.161	1.11	16	100	0.239	1.65	NA	NA	2007b
Cyclamen aldehyde	AOO	2	0	0.173	1.20	7	25	0.130	0.90	12	50	0.195	1.35	17	100	0.195	1.35	NA	NA	2007b
Cyclamen aldehyde	AOO	3	0	0.130	0.90	8	25	0.201	1.39	13	50	0.150	1.04	18	100	0.389	2.69	NA	NA	2007b
Cyclamen aldehyde	AOO	4	0	0.151	1.04	9	25	0.125	0.86	14	50	0.191	1.32	19	100	0.319	2.20	NA	NA	2007b
Cyclamen aldehyde	AOO	Mean	0	0.145	1.00	Mean	25	0.147	1.02	Mean	50	0.174	1.20	Mean	100	0.285	1.97	69.48	NC	2007b
Diethyl phthalate	AOO	1	0	0.114	1.08	6	50	0.091	0.87	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
Diethyl phthalate	AOO	2	0	0.091	0.87	7	50	0.074	0.71	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
Diethyl phthalate	AOO	3	0	0.095	0.90	8	50	0.081	0.77	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
Diethyl phthalate	AOO	4	0	0.122	1.15	9	50	0.122	1.16	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
Diethyl phthalate	AOO	Mean	0	0.106	1.00	Mean	50	0.092	0.88	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
Dimethyliso-phthalate	AOO	1	0	0.114	1.08	6	50	0.129	1.23	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
Dimethyliso-phthalate	AOO	2	0	0.091	0.87	7	50	0.108	1.02	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
Dimethyliso-phthalate	AOO	3	0	0.095	0.90	8	50	0.152	1.45	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
Dimethyliso-phthalate	AOO	4	0	0.122	1.15	9	50	0.143	1.36	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
Dimethyliso-phthalate	AOO	Mean	0	0.106	1.00	Mean	50	0.133	1.26	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
Diphencyclopro-penone	AOO	1	0	0.065	0.95	6	10	0.929	13.5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b

Substance	Veh.	An. No.	1 Conc. (%)	1 ABS	1 SI	An. No.	2 Conc. (%)	2 ABS	2 SI	An. No.	3 Conc. (%)	3 ABS	3 SI	An. No.	4 Conc. (%)	4 ABS	4 SI	EC 1.5	EC 2	Ref.
Diphencyclopro-penone	AOO	2	0	0.062	0.91	7	10	0.816	11.9	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
Diphencyclopro-penone	AOO	3	0	0.076	1.11	8	10	0.497	7.23	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
Diphencyclopro-penone	AOO	4	0	0.071	1.03	9	10	0.949	13.8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
Diphencyclopro-penone	AOO	Mean	0	0.069	1.00	Mean	10	0.798	11.6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
DNCB	AOO	1	0	0.436	1.76	6	0.08	0.348	1.40	11	0.40	1.210	4.89	16	2	1.865	7.53	NA	NA	2007b
DNCB	AOO	2	0	0.272	1.10	7	0.08	0.456	1.84	12	0.40	1.332	5.38	17	2	1.497	6.05	NA	NA	2007b
DNCB	AOO	3	0	0.171	0.69	8	0.08	0.304	1.23	13	0.40	1.666	6.73	18	2	1.634	6.60	NA	NA	2007b
DNCB	AOO	4	0	0.110	0.45	9	0.08	0.556	2.24	14	0.40	1.439	5.81	19	2	1.775	7.17	NA	NA	2007b
DNCB	AOO	Mean	0	0.248	1.00	Mean	0.08	0.416	1.68	Mean	0.40	1.412	5.70	Mean	2	1.693	6.84	0.11	0.072	2007b
VC	AOO	1	0	0.025	0.52	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
VC	AOO	2	0	0.073	1.53	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
VC	AOO	3	0	0.035	0.74	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
VC	AOO	4	0	0.058	1.21	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
VC	AOO	Mean	0	0.048	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
Eugenol	AOO	6	6.25	0.145	3.05	11	12.5	0.261	5.52	16	25	0.760	16.05	21	50	0.81	17.12	NA	NA	2007b
Eugenol	AOO	7	6.25	0.354	7.48	12	12.5	0.279	5.89	17	25	0.856	18.09	22	50	0.76	15.99	NA	NA	2007b
Eugenol	AOO	8	6.25	0.249	5.26	13	12.5	0.325	6.87	18	25	0.585	12.35	23	50	0.82	17.35	NA	NA	2007b
Eugenol	AOO	9	6.25	0.419	8.85	14	12.5	0.638	13.48	19	25	0.669	14.14	24	50	0.96	20.29	NA	NA	2007b
Eugenol	AOO	Mean	6.25	0.292	6.16	Mean	12.5	0.376	7.94	Mean	25	0.717	15.15	Mean	50	0.84	17.69	1.02	1.2	2007b
Glycerol	NA	1	0	0.050	0.85	6	10	0.082	1.38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
Glycerol	NA	2	0	0.056	0.94	7	10	0.069	1.17	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
Glycerol	NA	3	0	0.066	1.11	8	10	0.083	1.39	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
Glycerol	NA	4	0	0.065	1.10	9	10	0.073	1.23	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
Glycerol	NA	Mean	0	0.059	1.00	Mean	10	0.077	1.29	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2007b
HCA	AOO	1	0	0.123	0.78	6	12.5	0.223	1.42	11	25	0.246	1.57	16	50	0.366	2.34	NA	NA	2007b
HCA	AOO	2	0	0.177	1.13	7	12.5	0.282	1.80	12	25	0.382	2.44	17	50	0.425	2.71	NA	NA	2007b
HCA	AOO	3	0	0.170	1.08	8	12.5	0.276	1.76	13	25	0.359	2.29	18	50	0.517	3.30	NA	NA	2007b
HCA	AOO	4	0	0.158	1.01	9	12.5	0.127	0.81	14	25	0.316	2.01	19	50	0.585	3.73	NA	NA	2007b
HCA	AOO	Mean	0	0.157	1.00	Mean	12.5	0.227	1.45	Mean	25	0.326	2.08	Mean	50	0.473	3.02	13.49	23.41	2007b
Hydroxycitronellal	AOO	1	0	0.125	0.86	6	25	0.178	1.23	11	50	0.197	1.36	16	100	0.200	1.38	NA	NA	2007b
Hydroxycitronellal	AOO	2	0	0.173	1.20	7	25	0.202	1.39	12	50	0.159	1.10	17	100	0.181	1.25	NA	NA	2007b
Hydroxycitronellal	AOO	3	0	0.130	0.90	8	25	0.162	1.12	13	50	0.133	0.92	18	100	0.228	1.57	NA	NA	2007b
Hydroxycitronellal	AOO	4	0	0.151	1.04	9	25	0.149	1.03	14	50	0.159	1.10	19	100	0.169	1.17	NA	NA	2007b
Hydroxycitronellal	AOO	Mean	0	0.145	1.00	Mean	25	0.172	1.19	Mean	50	0.162	1.12	Mean	100	0.195	1.34	NA	NA	2007b
Isopropanol	AOO	1	0	0.134	0.85	6	25	0.103	0.65	11	50	0.217	1.37	16	100	0.124	0.79	NA	NA	2007b
Isopropanol	AOO	2	0	0.218	1.38	7	25	0.162	1.03	12	50	0.108	0.68	17	100	0.084	0.53	NA	NA	2007b
Isopropanol	AOO	3	0	0.127	0.80	8	25	0.092	0.58	13	50	0.182	1.15	18	100	0.093	0.59	NA	NA	2007b

Substance	Veh.	An. No.	1 Conc. (%)	1 ABS	1 SI	An. No.	2 Conc. (%)	2 ABS	2 SI	An. No.	3 Conc. (%)	3 ABS	3 SI	An. No.	4 Conc. (%)	4 ABS	4 SI	EC 1.5	EC 2	Ref.
Isopropanol	AOO	4	0	0.153	0.97	9	25	0.116	0.73	14	50	0.071	0.45	19	100	0.076	0.48	NA	NA	2007b
Isopropanol	AOO	Mean	0	0.158	1.00	Mean	25	0.118	0.75	Mean	50	0.145	0.92	Mean	100	0.094	0.60	NA	NA	2007b
m-Aminophenol	AOO	1	0	0.081	0.86	6	6.25	0.284	3.00	11	12.5	0.152	1.61	16	25	0.266	2.81	NA	NA	2007b
m-Aminophenol	AOO	2	0	0.085	0.90	7	6.25	0.175	1.86	12	12.5	0.470	4.97	17	25	0.351	3.71	NA	NA	2007b
m-Aminophenol	AOO	3	0	0.087	0.92	8	6.25	0.210	2.22	13	12.5	0.205	2.17	18	25	0.252	2.67	NA	NA	2007b
m-Aminophenol	AOO	4	0	0.125	1.33	9	6.25	0.180	1.90	14	12.5	0.252	2.67	19	25	NA	NA	NA	NA	2007b
m-Aminophenol	AOO	Mean	0	0.095	1.00	Mean	6.25	0.212	2.25	Mean	12.5	0.270	2.86	Mean	25	0.290	3.06	2.66	4.70	2007b
2-Mercaptobenzo-thiazole	DMF	1	0	0.170	1.11	6	12.5	0.222	1.45	11	25	0.195	1.27	16	50	0.185	1.21	NA	NA	2007b
2-Mercaptobenzo-thiazole	DMF	2	0	0.167	1.09	7	12.5	0.277	1.81	12	25	0.172	1.12	17	50	0.118	0.77	NA	NA	2007b
2-Mercaptobenzo-thiazole	DMF	3	0	0.155	1.01	8	12.5	0.340	2.22	13	25	0.251	1.64	18	50	0.260	1.70	NA	NA	2007b
2-Mercaptobenzo-thiazole	DMF	4	0	0.120	0.78	9	12.5	0.151	0.98	14	25	0.215	1.40	19	50	0.350	2.29	NA	NA	2007b
2-Mercaptobenzo-thiazole	DMF	Mean	0	0.153	1.00	Mean	12.5	0.248	1.62	Mean	25	0.208	1.36	Mean	50	0.229	1.49	10.08 *	NC	2007b
Isoeugenol	AOO	1	0	0.146	0.83	5	3	0.490	2.79	9	10	0.510	2.90	13	30	0.993	5.64	NA	NA	2007a
Isoeugenol	AOO	2	0	0.223	1.27	6	3	0.320	1.82	10	10	0.597	3.40	14	30	1.038	5.90	NA	NA	2007a
Isoeugenol	AOO	3	0	0.148	0.84	7	3	0.128	0.73	11	10	0.354	2.01	15	30	1.060	6.02	NA	NA	2007a
Isoeugenol	AOO	4	0	0.186	1.06	8	3	0.130	0.74	12	10	0.246	1.40	16	30	1.644	9.35	NA	NA	2007a
Isoeugenol	AOO	Mean	0	0.176	1.00	Mean	3	0.267	1.52	Mean	10	0.427	2.43	Mean	30	1.184	6.73	2.92	6.69	2007a
Eugenol	AOO	1	0	0.146	0.83	41	3	0.198	1.12	45	10	0.196	1.12	49	30	0.528	3.00	NA	NA	2007a
Eugenol	AOO	2	0	0.223	1.27	42	3	0.113	0.64	46	10	0.343	1.95	50	30	0.451	2.56	NA	NA	2007a
Eugenol	AOO	3	0	0.148	0.84	43	3	0.116	0.66	47	10	0.282	1.60	51	30	0.989	5.62	NA	NA	2007a
Eugenol	AOO	4	0	0.186	1.06	44	3	0.101	0.57	48	10	0.204	1.16	52	30	0.727	4.13	NA	NA	2007a
Eugenol	AOO	Mean	0	0.176	1.00	Mean	3	0.132	0.75	Mean	10	0.256	1.46	Mean	30	0.674	3.83	10.68	14.56	2007a
Dilignol	AOO	1	0	0.149	0.85	17	3	0.189	1.08	21	10	0.309	1.75	25	30	0.233	1.32	NA	NA	2007a
Dilignol	AOO	2	0	0.232	1.32	18	3	0.308	1.75	22	10	0.287	1.63	26	30	0.249	1.41	NA	NA	2007a
Dilignol	AOO	3	0	0.140	0.79	19	3	0.120	0.68	23	10	0.142	0.81	27	30	0.100	0.57	NA	NA	2007a
Dilignol	AOO	4	0	0.184	1.04	20	3	0.099	0.56	24	10	0.098	0.56	28	30	0.157	0.89	NA	NA	2007a
Dilignol	AOO	Mean	0	0.176	1.00	Mean	3	0.179	1.02	Mean	10	0.209	1.19	Mean	30	0.185	1.05	NC	NC	2007a
Dehydrodiiso-eugenol	AOO	1	0	0.162	0.92	29	3	0.182	1.03	33	10	0.541	3.08	37	30	0.995	5.66	NA	NA	2007a
Dehydrodiiso-eugenol	AOO	2	0	0.232	1.32	30	3	0.534	3.03	34	10	0.699	3.97	38	30	0.688	3.91	NA	NA	2007a
Dehydrodiiso-eugenol	AOO	3	0	0.143	0.81	31	3	0.300	1.71	35	10	0.454	2.58	39	30	1.077	6.12	NA	NA	2007a
Dehydrodiiso-eugenol	AOO	4	0	0.168	0.95	32	3	0.358	2.03	36	10	0.483	2.75	40	30	1.025	5.83	NA	NA	2007a
Dehydrodiiso-eugenol	AOO	Mean	0	0.176	1.00	Mean	3	0.343	1.95	Mean	10	0.544	3.09	Mean	30	0.946	5.38	1.86	3.31	2007a
Hexane	AOO	1	0	0.072	0.91	6	25	0.090	1.14	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2009
Hexane	AOO	2	0	0.119	1.51	7	25	0.114	1.45	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2009

Substance	Veh.	An. No.	1 Conc. (%)	1 ABS	1 SI	An. No.	2 Conc. (%)	2 ABS	2 SI	An. No.	3 Conc. (%)	3 ABS	3 SI	An. No.	4 Conc. (%)	4 ABS	4 SI	EC 1.5	EC 2	Ref.
Hexane	AOO	3	0	0.056	0.71	8	25	0.096	1.22	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2009
Hexane	AOO	4	0	0.068	0.87	9	25	0.121	1.54	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2009
Hexane	AOO	Mean	0	0.079	1.00	Mean	25	0.105	1.34	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2009
Hexane	AOO	11	0	0.049	0.81	16	50	0.082	1.36	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2009
Hexane	AOO	12	0	0.090	1.50	17	50	0.115	1.91	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2009
Hexane	AOO	13	0	0.048	0.79	18	50	0.060	0.99	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2009
Hexane	AOO	14	0	0.055	0.91	19	50	0.076	1.26	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2009
Hexane	AOO	Mean	0	0.060	1.00	Mean	50	0.083	1.38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2009
Hexane	AOO	21	0	0.054	0.77	26	100	0.113	1.60	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2009
Hexane	AOO	22	0	0.113	1.60	27	100	0.132	1.87	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2009
Hexane	AOO	23	0	0.055	0.78	28	100	0.143	2.03	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2009
Hexane	AOO	24	0	0.060	0.85	29	100	0.108	1.53	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2009
Hexane	AOO	Mean	0	0.071	1.00	Mean	100	0.124	1.76	NA	NA	NA	NA	NA	NA	NA	NA	65.79	NC	2009
Linalool	AOO	1	0	0.111	0.81	6	25	0.203	1.48	11	50	0.295	2.16	16	100	0.187	1.37	NA	NA	2009
Linalool	AOO	2	0	0.185	1.35	7	25	0.154	1.13	12	50	0.149	1.09	17	100	0.209	1.53	NA	NA	2009
Linalool	AOO	3	0	0.116	0.85	8	25	0.094	0.69	13	50	0.196	1.43	18	100	0.119	0.87	NA	NA	2009
Linalool	AOO	4	0	0.136	0.99	9	25	0.255	1.86	14	50	0.152	1.11	19	100	0.129	0.94	NA	NA	2009
Linalool	AOO	Mean	0	0.137	1.00	Mean	25	0.176	1.29	Mean	50	0.198	1.45	Mean	100	0.161	1.18	NC	NC	2009
Timelittic anhydride	AOO	1	0	0.071	0.96	4	2.5	0.188	2.53	7	5	0.301	4.05	7	10	0.537	7.22	NA	NA	2009
Timelittic anhydride	AOO	2	0	0.066	0.89	5	2.5	0.261	3.51	8	5	0.469	6.31	8	10	0.545	7.33	NA	NA	2009
Timelittic anhydride	AOO	3	0	0.086	1.16	6	2.5	0.162	2.18	9	5	0.388	5.22	9	10	0.668	8.99	NA	NA	2009
Timelittic anhydride	AOO	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	2009
Timelittic anhydride	AOO	Mean	0	0.071	1.00	Mean	2.5	0.188	2.74	Mean	5	0.301	5.19	Mean	10	0.537	7.85	1.76	2.03	2009

Abbreviations: ABS = Absorbance; An. No. = Animal number; AOO = Acetone: olive oil (4:1); Conc. = Concentration; DHEA = 2,2'-Dihydroxyl-3,3'-dimethoxy-5,5'-diallyl-biphenyl; DHEB = 4,5'-Diallyl-2'-hydroxy-2,3'-dimethoxyphenyl ether; DMF = *N,N*-Dimethylfluoride; DNCB = 2,4-Dinitrochlorobenzene; EC1.5 = Estimated concentration needed to produce a stimulation index of 1.5; EC2 = Estimated concentration needed to produce a stimulation index of two; HCA = Hexyl cinnamic aldehyde; LLNA: BrdU-ELISA = Murine local lymph node assay (LLNA) with enzyme-linked immunosorbent assay (ELISA) detection of bromodeoxyuridine (BrdU); NA = Not available; NC = Not calculated (i.e., SI was not high enough to calculated EC1.5 or EC2); Ref. = Year of Takeyoshi et al. reference for the data; SI = Stimulation index; Veh. = Vehicle.

* EC1.5 (or EC2) was calculated by linear interpolation using SI =1 and concentration = 0 as the lowest point because the dose-response was nonmonotonic.

Appendix D2

Individual Animal Data for the LLNA: BrdU-ELISA – Kojima et al. 2008

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Appendix D2 Individual Animal Data for the LLNA: BrdU-ELISA Submitted by Dr. Kojima[1]

Substance[1]	Lab No.	Vehicle	Animal No.	1 Conc. (%)	1 ABS	1 SI	Animal No.	2 Conc. (%)	2 ABS	2 SI	Animal No.	3 Conc. (%)	3 ABS	3 SI	EC1.5	EC2
VP	1	AOO	1	0	0.244	1.17	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	1	AOO	2	0	0.198	0.95	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	1	AOO	3	0	0.184	0.88	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	1	AOO	4	0	0.21	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	1	AOO	MEAN	0	0.209	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	1	AOO	1	0	0.487	2.33	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	1	AOO	2	0	0.401	1.92	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	1	AOO	3	0	0.479	2.29	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	1	AOO	4	0	0.362	1.73	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	1	AOO	MEAN	0	0.432	2.07	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	1	AOO	1	0	0.291	0.96	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	1	AOO	2	0	0.301	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	1	AOO	3	0	0.367	1.22	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	1	AOO	4	0	0.248	0.82	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	1	AOO	MEAN	0	0.432	2.07	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Methyl salicylate	1	AOO	1	10	0.428	1.42	1	25	0.561	1.86	1	0.5	0.326	1.08	NA	NA
Methyl salicylate	1	AOO	2	10	0.381	1.26	2	25	0.314	1.04	2	0.5	0.364	1.21	NA	NA
Methyl salicylate	1	AOO	3	10	0.405	1.34	3	25	0.287	0.95	3	0.5	0.403	1.34	NA	NA
Methyl salicylate	1	AOO	4	10	0.511	1.69	4	25	0.508	1.68	4	0.5	0.430	1.43	NA	NA
Methyl salicylate	1	AOO	MEAN	10	0.431	1.43	MEAN	25	0.418	1.38	MEAN	0.5	0.381	1.26	NC	NC
2, 4-Dinitrochlorobenzene	1	AOO	1	0.1	0.676	2.24	1	0.3	1.153	3.82	1	1	1.254	4.16	NA	NA
2, 4-Dinitrochlorobenzene	1	AOO	2	0.1	0.581	1.93	2	0.3	1.311	4.34	2	1	1.229	4.07	NA	NA
2, 4-Dinitrochlorobenzene	1	AOO	3	0.1	0.904	3.00	3	0.3	0.870	2.88	3	1	1.231	4.08	NA	NA
2, 4-Dinitrochlorobenzene	1	AOO	4	0.1	0.535	1.77	4	0.3	1.105	3.66	4	1	1.478	4.90	NA	NA
2, 4-Dinitrochlorobenzene	1	AOO	MEAN	0.1	0.674	2.23	MEAN	0.3	1.110	3.68	MEAN	1	1.298	4.30	0.058	0.084
VP	1	AOO	1	0	0.037	0.67	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	1	AOO	2	0	0.070	1.27	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	1	AOO	3	0	0.033	0.60	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	1	AOO	4	0	0.081	1.47	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	1	AOO	MEAN	0	0.055	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	1	AOO	1	0	0.223	4.04	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	1	AOO	2	0	0.372	6.73	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	1	AOO	3	0	0.380	6.88	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	1	AOO	4	0	0.375	6.79	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	1	AOO	MEAN	0	0.338	6.11	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Substance[1]	Lab No.	Vehicle	Animal No.	1 Conc. (%)	1 ABS	1 SI	Animal No.	2 Conc. (%)	2 ABS	2 SI	Animal No.	3 Conc. (%)	3 ABS	3 SI	EC1.5	EC2
VS	1	AOO	1	0	0.147	0.93	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	1	AOO	2	0	0.267	1.69	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	1	AOO	3	0	0.109	0.69	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	1	AOO	4	0	0.108	0.69	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	1	AOO	MEAN	0	0.158	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Hexyl cinnamic aldehyde	1	AOO	1	10	0.234	1.48	1	25	0.347	2.20	1	50	0.479	3.04	NA	NA
Hexyl cinnamic aldehyde	1	AOO	2	10	0.215	1.36	2	25	0.453	2.87	2	50	0.571	3.62	NA	NA
Hexyl cinnamic aldehyde	1	AOO	3	10	0.252	1.60	3	25	0.424	2.69	3	50	0.611	3.87	NA	NA
Hexyl cinnamic aldehyde	1	AOO	4	10	0.289	1.83	4	25	0.422	2.68	4	50	0.487	3.09	NA	NA
Hexyl cinnamic aldehyde	1	AOO	MEAN	10	0.248	1.57	MEAN	25	0.412	2.61	MEAN	50	0.537	3.40	9.40	16.20
Isopropanol	1	AOO	1	10	0.201	1.27	1	25	0.153	0.97	1	50	0.155	0.98	NA	NA
Isopropanol	1	AOO	2	10	0.675	4.28	2	25	0.134	0.85	2	50	0.109	0.69	NA	NA
Isopropanol	1	AOO	3	10	0.281	1.78	3	25	0.101	0.64	3	50	0.112	0.71	NA	NA
Isopropanol	1	AOO	4	10	0.241	1.53	4	25	0.093	0.59	4	50	0.203	1.29	NA	NA
Isopropanol	1	AOO	MEAN	10	0.350	2.22	MEAN	25	0.120	0.76	MEAN	50	0.145	0.92	4.10*	8.20*
VP	1	ACE	1	0	0.132	1.61	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	1	ACE	2	0	0.066	0.80	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	1	ACE	3	0	0.047	0.57	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	1	ACE	4	0	0.083	1.01	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	1	ACE	MEAN	0	0.082	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	1	ACE	1	0	0.232	2.83	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	1	ACE	2	0	0.332	4.05	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	1	ACE	3	0	0.314	3.83	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	1	ACE	4	0	0.249	3.04	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	1	ACE	MEAN	0	0.282	3.44	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	1	ACE	1	0	0.093	0.87	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	1	ACE	2	0	0.090	0.84	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	1	ACE	3	0	0.168	1.57	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	1	ACE	4	0	0.078	0.73	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	1	ACE	MEAN	0	0.107	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Glutaradehyde	1	ACE	1	0.1	0.117	1.09	1	0.3	0.247	2.30	1	1	0.265	2.47	NA	NA
Glutaradehyde	1	ACE	2	0.1	0.210	1.96	2	0.3	0.258	2.41	2	1	0.539	5.03	NA	NA
Glutaradehyde	1	ACE	3	0.1	0.248	2.31	3	0.3	0.334	3.11	3	1	0.354	3.30	NA	NA
Glutaradehyde	1	ACE	4	0.1	0.178	1.66	4	0.3	0.189	1.76	4	1	0.440	4.10	NA	NA
Glutaradehyde	1	ACE	MEAN	0.1	0.188	1.76	MEAN	0.3	0.257	2.40	MEAN	1	0.400	3.72	0.064	0.18
Formaldehyde	1	ACE	1	1	0.278	2.59	1	3	0.503	4.69	1	10	0.183	1.71	NA	NA
Formaldehyde	1	ACE	2	1	0.509	4.75	2	3	0.503	4.69	2	10	0.215	2.00	NA	NA

Substance[1]	Lab No.	Vehicle	Animal No.	1 Conc. (%)	1 ABS	1 SI	Animal No.	2 Conc. (%)	2 ABS	2 SI	Animal No.	3 Conc. (%)	3 ABS	3 SI	EC1.5	EC2
Formaldehyde	1	ACE	3	1	0.265	2.47	3	3	0.528	4.92	3	10	0.182	1.70	NA	NA
Formaldehyde	1	ACE	4	1	0.270	2.52	4	3	0.352	3.28	4	10	0.184	1.72	NA	NA
Formaldehyde	1	ACE	MEAN	1	0.331	3.08	MEAN	3	0.472	4.40	MEAN	10	0.191	1.78	0.27	0.41
VP	2	AOO	1	0	0.353	1.09	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	2	AOO	2	0	0.300	0.93	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	2	AOO	3	0	0.341	1.05	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	2	AOO	4	0	0.300	0.93	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	2	AOO	MEAN	0	0.324	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	2	AOO	1	0	0.301	0.93	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	2	AOO	2	0	0.380	1.17	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	2	AOO	3	0	0.495	1.53	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	2	AOO	4	0	0.494	1.53	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	2	AOO	MEAN	0	0.418	1.29[2]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	2	AOO	1	0	0.214	0.70	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	2	AOO	2	0	0.326	1.06	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	2	AOO	3	0	0.351	1.14	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	2	AOO	4	0	0.338	1.10	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	2	AOO	MEAN	0	0.307	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Hexyl cinnamic aldehyde	2	AOO	1	10	0.410	1.33	1	25	0.451	1.47	1	50	0.494	1.61	NA	NA
Hexyl cinnamic aldehyde	2	AOO	2	10	0.426	1.39	2	25	0.566	1.84	2	50	0.658	2.14	NA	NA
Hexyl cinnamic aldehyde	2	AOO	3	10	0.359	1.17	3	25	0.544	1.77	3	50	0.524	1.71	NA	NA
Hexyl cinnamic aldehyde	2	AOO	4	10	0.421	1.37	4	25	0.366	1.19	4	50	0.577	1.88	NA	NA
Hexyl cinnamic aldehyde	2	AOO	MEAN	10	0.404	1.31	MEAN	25	0.482	1.57	MEAN	50	0.563	1.83	20.96[2]	NC[2]
Isopropanol	2	AOO	1	10	0.346	1.13	1	25	0.312	1.02	1	50	0.240	0.78	NA	NA
Isopropanol	2	AOO	2	10	0.391	1.27	2	25	0.236	0.77	2	50	0.249	0.81	NA	NA
Isopropanol	2	AOO	3	10	0.268	0.87	3	25	0.112	0.36	3	50	0.356	1.16	NA	NA
Isopropanol	2	AOO	4	10	0.329	1.07	4	25	0.379	1.23	4	50	0.169	0.55	NA	NA
Isopropanol	2	AOO	MEAN	10	0.334	1.09	MEAN	25	0.260	0.85	MEAN	50	0.254	0.83	NC[2]	NC[2]
VP	2	AOO	1	0	0.140	1.06	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	2	AOO	2	0	0.130	0.99	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	2	AOO	3	0	0.123	0.94	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	2	AOO	4	0	0.133	1.01	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	2	AOO	MEAN	0	0.132	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	2	AOO	1	0	0.502	3.82	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	2	AOO	2	0	0.608	4.62	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	2	AOO	3	0	0.659	5.01	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	2	AOO	4	0	0.939	7.14	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Substance[1]	Lab No.	Vehicle	Animal No.	1 Conc. (%)	1 ABS	1 SI	Animal No.	2 Conc. (%)	2 ABS	2 SI	Animal No.	3 Conc. (%)	3 ABS	3 SI	EC1.5	EC2
PC	2	AOO	MEAN	0	0.677	5.15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	2	AOO	1	0	0.132	0.74	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	2	AOO	2	0	0.129	0.72	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	2	AOO	3	0	0.219	1.23	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	2	AOO	4	0	0.232	1.30	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	2	AOO	MEAN	0	0.178	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2, 4-Dinitrochlorobenzene	2	AOO	1	0.1	1.060	5.96	1	0.3	0.936	5.26	1	1	1.321	7.42	NA	NA
2, 4-Dinitrochlorobenzene	2	AOO	2	0.1	1.091	6.13	2	0.3	1.100	6.18	2	1	1.491	8.38	NA	NA
2, 4-Dinitrochlorobenzene	2	AOO	3	0.1	1.308	7.35	3	0.3	1.296	7.28	3	1	1.531	8.60	NA	NA
2, 4-Dinitrochlorobenzene	2	AOO	4	0.1	1.090	6.12	4	0.3	1.315	7.39	4	1	1.615	9.07	NA	NA
2, 4-Dinitrochlorobenzene	2	AOO	MEAN	0.1	1.137	6.39	MEAN	0.3	1.162	6.53	MEAN	1	1.490	8.37	0.010*	0.019*
trans-Cinnamaldehyde	2	AOO	1	1	0.176	0.99	1	3	0.347	1.95	1	10	0.543	3.05	NA	NA
trans-Cinnamaldehyde	2	AOO	2	1	0.167	0.94	2	3	0.385	2.16	2	10	0.555	3.12	NA	NA
trans-Cinnamaldehyde	2	AOO	3	1	0.238	1.34	3	3	0.483	2.71	3	10	0.587	3.30	NA	NA
trans-Cinnamaldehyde	2	AOO	4	1	0.203	1.14	4	3	0.373	2.10	4	10	0.712	4.00	NA	NA
trans-Cinnamaldehyde	2	AOO	MEAN	1	0.196	1.10	MEAN	3	0.397	2.23	MEAN	10	0.599	3.37	1.71	2.59
VP	2	AOO	1	0	0.202	1.16	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	2	AOO	2	0	0.155	0.89	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	2	AOO	3	0	0.152	0.87	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	2	AOO	4	0	0.187	1.07	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	2	AOO	MEAN	0	0.174	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	2	AOO	1	0	0.444	2.55	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	2	AOO	2	0	0.492	2.83	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	2	AOO	3	0	0.403	2.32	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	2	AOO	4	0	0.413	2.37	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	2	AOO	MEAN	0	0.438	2.52	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	2	AOO	1	0	0.217	1.26	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	2	AOO	2	0	0.106	0.61	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	2	AOO	3	0	0.217	1.26	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	2	AOO	4	0	0.150	0.87	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	2	AOO	MEAN	0	0.173	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Eugenol	2	AOO	1	10	0.242	1.40	1	25	0.308	1.79	1	50	0.520	3.01	NA	NA
Eugenol	2	AOO	2	10	0.238	1.38	2	25	0.474	2.75	2	50	0.502	2.91	NA	NA
Eugenol	2	AOO	3	10	0.175	1.01	3	25	0.443	2.57	3	50	0.563	3.26	NA	NA
Eugenol	2	AOO	4	10	0.251	1.46	4	25	0.464	2.69	4	50	0.599	3.47	NA	NA
Eugenol	2	AOO	MEAN	10	0.227	1.31	MEAN	25	0.422	2.45	MEAN	50	0.546	3.17	12.50	19.08
Methyl salicylate	2	AOO	1	10	0.195	1.13	1	25	0.196	1.14	1	50	0.284	1.65	NA	NA

Substance[1]	Lab No.	Vehicle	Animal No.	1 Conc. (%)	1 ABS	1 SI	Animal No.	2 Conc. (%)	2 ABS	2 SI	Animal No.	3 Conc. (%)	3 ABS	3 SI	EC1.5	EC2
Methyl salicylate	2	AOO	2	10	0.218	1.26	2	25	0.202	1.17	2	50	0.194	1.12	NA	NA
Methyl salicylate	2	AOO	3	10	0.161	0.93	3	25	0.231	1.34	3	50	0.272	1.58	NA	NA
Methyl salicylate	2	AOO	4	10	0.194	1.12	4	25	0.173	1.00	4	50	0.244	1.41	NA	NA
Methyl salicylate	2	AOO	MEAN	10	0.192	1.11	MEAN	25	0.201	1.16	MEAN	50	0.249	1.44	NC	NC
VP	3	AOO	1	0	0.253	1.05	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	3	AOO	2	0	0.291	1.21	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	3	AOO	3	0	0.143	0.59	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	3	AOO	4	0	0.275	1.14	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	3	AOO	MEAN	0	0.241	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	3	AOO	1	0	0.678	2.82	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	3	AOO	2	0	1.013	4.21	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	3	AOO	3	0	0.650	2.70	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	3	AOO	4	0	0.874	3.63	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	3	AOO	MEAN	0	0.804	3.34	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	3	AOO	1	0	0.205	0.93	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	3	AOO	2	0	0.172	0.78	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	3	AOO	3	0	0.214	0.97	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	3	AOO	4	0	0.290	1.32	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	3	AOO	MEAN	0	0.220	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Methyl salicylate	3	AOO	1	10	0.234	1.06	1	25	0.391	1.78	1	50	0.260	1.18	NA	NA
Methyl salicylate	3	AOO	2	10	0.339	1.54	2	25	0.235	1.07	2	50	0.473	2.15	NA	NA
Methyl salicylate	3	AOO	3	10	0.130	0.59	3	25	0.120	0.54	3	50	0.175	0.79	NA	NA
Methyl salicylate	3	AOO	4	10	0.266	1.21	4	25	0.321	1.46	4	50	0.328	1.49	NA	NA
Methyl salicylate	3	AOO	MEAN	10	0.242	1.10	MEAN	25	0.267	1.21	MEAN	50	0.309	1.40	NC	NC
2, 4-Dinitrochlorobenzene	3	AOO	1	0.1	0.771	3.50	1	0.3	1.281	5.82	1	10	1.257	5.71	NA	NA
2, 4-Dinitrochlorobenzene	3	AOO	2	0.1	0.813	3.69	2	0.3	1.350	6.13	2	10	1.282	5.82	NA	NA
2, 4-Dinitrochlorobenzene	3	AOO	3	0.1	0.979	4.44	3	0.3	1.313	5.96	3	10	1.299	5.90	NA	NA
2, 4-Dinitrochlorobenzene	3	AOO	4	0.1	1.200	5.45	4	0.3	1.567	7.11	4	10	1.437	6.52	NA	NA
2, 4-Dinitrochlorobenzene	3	AOO	MEAN	0.1	0.941	4.27	MEAN	0.3	1.378	6.26	MEAN	10	1.319	5.99	0.022	0.029
VP	3	AOO	1	0	0.257	1.26	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	3	AOO	2	0	0.203	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	3	AOO	3	0	0.148	0.73	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	3	AOO	4	0	0.206	1.01	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	3	AOO	MEAN	0	0.204	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	3	AOO	1	0	0.767	3.77	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	3	AOO	2	0	1.009	4.96	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	3	AOO	3	0	0.515	2.53	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Substance[1]	Lab No.	Vehicle	Animal No.	1 Conc. (%)	1 ABS	1 SI	Animal No.	2 Conc. (%)	2 ABS	2 SI	Animal No.	3 Conc. (%)	3 ABS	3 SI	EC1.5	EC2
PC	3	AOO	4	0	0.588	2.89	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	3	AOO	MEAN	0	0.720	3.54	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	3	AOO	1	0	0.356	1.34	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	3	AOO	2	0	0.178	0.67	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	3	AOO	3	0	0.320	1.20	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	3	AOO	4	0	0.209	0.79	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	3	AOO	MEAN	0	0.266	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Hexyl cinnamic aldehyde	3	AOO	1	10	0.477	1.79	1	25	0.547	2.06	1	50	0.565	2.13	NA	NA
Hexyl cinnamic aldehyde	3	AOO	2	10	0.354	1.33	2	25	0.724	2.72	2	50	1.014	3.82	NA	NA
Hexyl cinnamic aldehyde	3	AOO	3	10	0.225	0.85	3	25	0.303	1.14	3	50	0.642	2.42	NA	NA
Hexyl cinnamic aldehyde	3	AOO	4	10	0.222	0.84	4	25	0.616	2.32	4	50	0.834	3.14	NA	NA
Hexyl cinnamic aldehyde	3	AOO	MEAN	10	0.320	1.20	MEAN	25	0.548	2.06	MEAN	50	0.764	2.87	15.23	23.95
Isopropanol	3	AOO	1	10	0.302	1.14	1	25	0.268	1.01	1	50	0.209	0.79	NA	NA
Isopropanol	3	AOO	2	10	0.349	1.31	2	25	0.282	1.06	2	50	0.288	1.08	NA	NA
Isopropanol	3	AOO	3	10	0.133	0.50	3	25	0.162	0.61	3	50	0.125	0.47	NA	NA
Isopropanol	3	AOO	4	10	0.259	0.97	4	25	0.195	0.73	4	50	0.171	0.64	NA	NA
Isopropanol	3	AOO	MEAN	10	0.261	0.98	MEAN	25	0.227	0.85	MEAN	50	0.198	0.75	NC	NC
VP	3	DMSO	1	0	0.328	1.04	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	3	DMSO	2	0	0.417	1.32	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	3	DMSO	3	0	0.177	0.56	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	3	DMSO	4	0	0.342	1.08	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	3	DMSO	MEAN	0	0.316	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	3	DMSO	1	0	0.665	2.10	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	3	DMSO	2	0	0.943	2.98	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	3	DMSO	3	0	0.601	1.90	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	3	DMSO	4	0	0.545	1.72	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	3	DMSO	MEAN	0	0.689	2.18	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	3	DMSO	1	0	0.184	0.83	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	3	DMSO	2	0	0.277	1.25	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	3	DMSO	3	0	0.149	0.67	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	3	DMSO	4	0	0.273	1.24	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	3	DMSO	MEAN	0	0.221	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Lactic acid	3	DMSO	1	10	0.199	0.90	1	25	0.453	2.05	1	50	0.404	1.83	NA	NA
Lactic acid	3	DMSO	2	10	0.347	1.57	2	25	0.456	2.07	2	50	0.626	2.84	NA	NA
Lactic acid	3	DMSO	3	10	0.170	0.77	3	25	0.214	0.97	3	50	0.214	0.97	NA	NA
Lactic acid	3	DMSO	4	10	0.247	1.12	4	25	0.338	1.53	4	50	0.345	1.56	NA	NA
Lactic acid	3	DMSO	MEAN	10	0.241	1.09	MEAN	25	0.365	1.65	MEAN	50	0.397	1.80	20.98	NC

Substance[1]	Lab No.	Vehicle	Animal No.	1 Conc. (%)	1 ABS	1 SI	Animal No.	2 Conc. (%)	2 ABS	2 SI	Animal No.	3 Conc. (%)	3 ABS	3 SI	EC1.5	EC2
Nickel sulfate	3	DMSO	1	1	0.326	1.48	1	3	0.426	1.93	1	10	0.697	3.16	NA	NA
Nickel sulfate	3	DMSO	2	1	0.484	2.19	2	3	0.607	2.75	2	10	0.642	2.91	NA	NA
Nickel sulfate	3	DMSO	3	1	0.135	0.61	3	3	0.263	1.19	3	10	0.424	1.92	NA	NA
Nickel sulfate	3	DMSO	4	1	0.269	1.22	4	3	0.399	1.81	4	10	0.517	2.34	NA	NA
Nickel sulfate	3	DMSO	MEAN	1	0.304	1.37	MEAN	3	0.424	1.92	MEAN	10	0.570	2.58	1.47	3.85
VP	4	AOO	1	0	0.228	0.81	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	4	AOO	2	0	0.208	0.74	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	4	AOO	3	0	0.333	1.19	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	4	AOO	4	0	0.354	1.26	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	4	AOO	MEAN	0	0.281	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	4	AOO	1	0	0.827	2.95	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	4	AOO	2	0	0.714	2.54	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	4	AOO	3	0	0.762	2.71	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	4	AOO	4	0	0.719	2.56	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	4	AOO	MEAN	0	0.756	2.69	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	4	AOO	1	0	0.217	0.80	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	4	AOO	2	0	0.246	0.91	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	4	AOO	3	0	0.357	1.32	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	4	AOO	4	0	0.264	0.97	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	4	AOO	MEAN	0	0.271	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2, 4-Dinitrochlorobenzene	4	AOO	1	0.1	1.039	3.83	1	0.3	1.427	5.27	1	1	1.427	5.27	NA	NA
2, 4-Dinitrochlorobenzene	4	AOO	2	0.1	1.050	3.87	2	0.3	1.382	5.10	2	1	1.539	5.68	NA	NA
2, 4-Dinitrochlorobenzene	4	AOO	3	0.1	0.875	3.23	3	0.3	1.435	5.30	3	1	1.471	5.43	NA	NA
2, 4-Dinitrochlorobenzene	4	AOO	4	0.1	1.056	3.90	4	0.3	1.491	5.50	4	1	1.524	5.62	NA	NA
2, 4-Dinitrochlorobenzene	4	AOO	MEAN	0.1	1.005	3.71	MEAN	0.3	1.434	5.29	MEAN	1	1.490	5.50	0.022	0.030
trans-Cinnamaldehyde	4	AOO	1	1	0.599	2.21	1	3	0.797	2.94	1	10	0.889	3.28	NA	NA
trans-Cinnamaldehyde	4	AOO	2	1	0.216	0.80	2	3	0.855	3.15	2	10	1.115	4.11	NA	NA
trans-Cinnamaldehyde	4	AOO	3	1	0.383	1.41	3	3	0.702	2.59	3	10	0.773	2.85	NA	NA
trans-Cinnamaldehyde	4	AOO	4	1	0.505	1.86	4	3	0.832	3.07	4	10	1.012	3.73	NA	NA
trans-Cinnamaldehyde	4	AOO	MEAN	1	0.426	1.57	MEAN	3	0.797	2.94	MEAN	10	0.947	3.50	0.95	1.63
VP	4	AOO	1	0	0.261	1.16	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	4	AOO	2	0	0.242	1.08	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	4	AOO	3	0	0.188	0.84	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	4	AOO	4	0	0.206	0.92	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	4	AOO	MEAN	0	0.224	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	4	AOO	1	0	0.504	2.25	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	4	AOO	2	0	0.894	3.99	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Substance[1]	Lab No.	Vehicle	Animal No.	1 Conc. (%)	1 ABS	1 SI	Animal No.	2 Conc. (%)	2 ABS	2 SI	Animal No.	3 Conc. (%)	3 ABS	3 SI	EC1.5	EC2
PC	4	AOO	3	0	0.547	2.44	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	4	AOO	4	0	0.897	4.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	4	AOO	MEAN	0	0.711	3.17	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	4	AOO	1	0	0.397	1.65	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	4	AOO	2	0	0.221	0.92	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	4	AOO	3	0	0.174	0.72	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	4	AOO	4	0	0.172	0.71	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	4	AOO	MEAN	0	0.241	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Isopropanol	4	AOO	1	0.1	0.189	0.78	1	0.25	0.581	2.41	1	0.5	0.188	0.78	NA	NA
Isopropanol	4	AOO	2	0.1	0.090	0.37	2	0.25	0.111	0.46	2	0.5	0.533	2.21	NA	NA
Isopropanol	4	AOO	3	0.1	0.239	0.99	3	0.25	0.221	0.92	3	0.5	0.519	2.15	NA	NA
Isopropanol	4	AOO	4	0.1	0.441	1.83	4	0.25	0.255	1.06	4	0.5	0.278	1.15	NA	NA
Isopropanol	4	AOO	MEAN	0.1	0.240	0.99	MEAN	0.25	0.292	1.21	MEAN	0.5	0.380	1.57	45.14	NC
Hexyl cinnamic aldehyde	4	AOO	1	10	0.576	2.39	1	25	0.657	2.73	1	50	0.990	4.11	NA	NA
Hexyl cinnamic aldehyde	4	AOO	2	10	0.403	1.67	2	25	0.649	2.69	2	50	0.832	3.45	NA	NA
Hexyl cinnamic aldehyde	4	AOO	3	10	0.619	2.57	3	25	0.613	2.54	3	50	0.783	3.25	NA	NA
Hexyl cinnamic aldehyde	4	AOO	4	10	0.364	1.51	4	25	0.579	2.40	4	50	0.612	2.54	NA	NA
Hexyl cinnamic aldehyde	4	AOO	MEAN	10	0.491	2.04	MEAN	25	0.625	2.59	MEAN	50	0.804	3.34	4.07	9.36
VP	4	DMSO	1	0	0.050	0.32	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	4	DMSO	2	0	0.228	1.48	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	4	DMSO	3	0	0.197	1.28	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	4	DMSO	4	0	0.141	0.92	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	4	DMSO	MEAN	0	0.154	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	4	DMSO	1	0	1.040	6.75	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	4	DMSO	2	0	1.080	7.01	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	4	DMSO	3	0	1.066	6.92	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	4	DMSO	4	0	0.863	5.60	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	4	DMSO	MEAN	0	1.01	6.57	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	4	DMSO	1	0	0.157	0.75	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	4	DMSO	2	0	0.107	0.51	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	4	DMSO	3	0	0.286	1.36	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	4	DMSO	4	0	0.291	1.38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	4	DMSO	MEAN	0	0.210	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Lactic acid	4	DMSO	1	10	0.237	1.13	1	25	0.327	1.56	1	50	0.444	2.11	NA	NA
Lactic acid	4	DMSO	2	10	0.474	2.25	2	25	0.449	2.14	2	50	0.374	1.78	NA	NA
Lactic acid	4	DMSO	3	10	0.282	1.34	3	25	0.342	1.63	3	50	0.257	1.22	NA	NA
Lactic acid	4	DMSO	4	10	0.444	2.11	4	25	0.472	2.24	4	50	0.297	1.41	NA	NA

Substance[1]	Lab No.	Vehicle	Animal No.	1 Conc. (%)	1 ABS	1 SI	Animal No.	2 Conc. (%)	2 ABS	2 SI	Animal No.	3 Conc. (%)	3 ABS	3 SI	EC1.5	EC2
Lactic acid	4	DMSO	MEAN	10	0.359	1.71	MEAN	25	0.398	1.89	MEAN	50	0.343	1.63	3.43	NC
Nickel sulfate	4	DMSO	1	1	0.349	1.66	1	3	0.293	1.39	1	10	0.935	4.45	NA	NA
Nickel sulfate	4	DMSO	2	1	0.441	2.10	2	3	0.343	1.63	2	10	0.699	3.32	NA	NA
Nickel sulfate	4	DMSO	3	1	0.347	1.65	3	3	0.484	2.30	3	10	0.706	3.36	NA	NA
Nickel sulfate	4	DMSO	4	1	0.585	2.78	4	3	0.560	2.66	4	10	1.467	6.98	NA	NA
Nickel sulfate	4	DMSO	MEAN	1	0.431	2.05	MEAN	3	0.420	2.00	MEAN	10	0.952	4.53	0.48	0.95*
VP	5	ACE	1	0	0.037	0.29	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	5	ACE	2	0	0.148	1.17	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	5	ACE	3	0	0.066	0.52	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	5	ACE	4	0	0.253	2.01	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	5	ACE	MEAN	0	0.126	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	5	ACE	1	0	1.398	11.10	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	5	ACE	2	0	0.636	5.05	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	5	ACE	3	0	2.366	18.78	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	5	ACE	4	0	1.874	14.87	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	5	ACE	MEAN	0	1.569	12.45	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	5	ACE	1	0	0.050	0.94	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	5	ACE	2	0	0.01	0.19	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	5	ACE	3	0	0.110	2.07	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	5	ACE	4	0	0.043	0.81	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	5	ACE	MEAN	0	0.053	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Formaldehyde	5	ACE	1	1	0.134	2.52	1	3	0.067	1.26	1	10	0.544	10.22	NA	NA
Formaldehyde	5	ACE	2	1	0.267	5.01	2	3	0.116	2.18	2	10	0.298	5.60	NA	NA
Formaldehyde	5	ACE	3	1	0.061	1.15	3	3	0.083	1.56	3	10	0.853	16.02	NA	NA
Formaldehyde	5	ACE	4	1	0.440	8.26	4	3	0.087	1.63	4	10	1.838	34.52	NA	NA
Formaldehyde	5	ACE	MEAN	1	0.226	4.23	MEAN	3	0.088	1.66	MEAN	10	0.883	16.59	0.15*	0.31*
Glutaradehyde	5	ACE	1	0.001	0.222	4.17	1	0.003	0.218	4.09	1	0.01	1.647	30.93	NA	NA
Glutaradehyde	5	ACE	2	0.001	0.286	5.37	2	0.003	0.970	18.22	2	0.01	0.980	18.40	NA	NA
Glutaradehyde	5	ACE	3	0.001	0.195	3.66	3	0.003	0.509	9.56	3	0.01	1.774	33.31	NA	NA
Glutaradehyde	5	ACE	4	0.001	0.877	16.47	4	0.003	1.058	19.87	4	0.01	1.699	31.91	NA	NA
Glutaradehyde	5	ACE	MEAN	0.001	0.395	7.42	MEAN	0.003	0.689	12.93	MEAN	0.01	1.525	28.64	0.031	0.034
VP	5	AOO	1	0	0.048	0.30	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	5	AOO	2	0	0.182	1.13	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	5	AOO	3	0	0.128	0.79	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	5	AOO	4	0	0.287	1.78	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	5	AOO	MEAN	0	0.161	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	5	AOO	1	0	0.607	3.76	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Substance[1]	Lab No.	Vehicle	Animal No.	1 Conc. (%)	1 ABS	1 SI	Animal No.	2 Conc. (%)	2 ABS	2 SI	Animal No.	3 Conc. (%)	3 ABS	3 SI	EC1.5	EC2
PC	5	AOO	2	0	0.822	5.10	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	5	AOO	3	0	0.905	5.61	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	5	AOO	4	0	0.399	2.47	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	5	AOO	MEAN	0	0.683	4.24	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	5	AOO	1	0	0.091	0.61	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	5	AOO	2	0	0.172	1.15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	5	AOO	3	0	0.179	1.19	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	5	AOO	4	0	0.158	1.05	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	5	AOO	MEAN	0	0.150	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2, 4-Dinitrochlorobenzene	5	AOO	1	0.1	2.561	17.07	1	0.3	2.721	18.14	1	1	2.767	18.45	NA	NA
2, 4-Dinitrochlorobenzene	5	AOO	2	0.1	1.960	13.07	2	0.3	2.792	18.61	2	1	2.669	17.79	NA	NA
2, 4-Dinitrochlorobenzene	5	AOO	3	0.1	2.211	14.74	3	0.3	2.902	19.35	3	1	2.211	14.74	NA	NA
2, 4-Dinitrochlorobenzene	5	AOO	4	0.1	2.238	14.92	4	0.3	2.862	19.08	4	1	2.514	16.76	NA	NA
2, 4-Dinitrochlorobenzene	5	AOO	MEAN	0.1	2.243	14.95	MEAN	0.3	2.819	18.80	MEAN	1	2.540	16.94	0.0022	0.0025
trans-Cinnamaldehyde	5	AOO	1	1	0.117	0.78	1	3	0.403	2.69	1	10	0.569	3.79	NA	NA
trans-Cinnamaldehyde	5	AOO	2	1	0.084	0.56	2	3	0.280	1.87	2	10	0.501	3.34	NA	NA
trans-Cinnamaldehyde	5	AOO	3	1	0.277	1.85	3	3	0.280	1.87	3	10	0.699	4.66	NA	NA
trans-Cinnamaldehyde	5	AOO	4	1	0.207	1.38	4	3	0.297	1.98	4	10	0.697	4.65	NA	NA
trans-Cinnamaldehyde	5	AOO	MEAN	1	0.171	1.14	MEAN	3	0.315	2.10	MEAN	10	0.617	4.11	1.75	2.79
VP	5	AOO	1	0	0.158	1.41	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	5	AOO	2	0	0.037	0.33	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	5	AOO	3	0	0.082	0.73	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	5	AOO	4	0	0.170	1.52	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	5	AOO	MEAN	0	0.112	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	5	AOO	1	0	0.689	6.17	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	5	AOO	2	0	0.576	5.15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	5	AOO	3	0	0.896	8.02	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	5	AOO	4	0	0.548	4.90	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	5	AOO	MEAN	0	0.677	6.06	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	5	AOO	1	0	0.035	0.63	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	5	AOO	2	0	0.082	1.48	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	5	AOO	3	0	0.034	0.62	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	5	AOO	4	0	0.070	1.27	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	5	AOO	MEAN	0	0.055	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Isopropanol	5	AOO	1	10	0.086	1.56	1	25	0.051	0.92	1	50	0.034	0.62	NC	NC
Isopropanol	5	AOO	2	10	0.041	0.74	2	25	0.024	0.43	2	50	0.043	0.78	NA	NA
Isopropanol	5	AOO	3	10	0.051	0.92	3	25	0.029	0.52	3	50	0.030	0.54	NA	NA

Substance[1]	Lab No.	Vehicle	Animal No.	1 Conc. (%)	1 ABS	1 SI	Animal No.	2 Conc. (%)	2 ABS	2 SI	Animal No.	3 Conc. (%)	3 ABS	3 SI	EC1.5	EC2
Isopropanol	5	AOO	4	10	0.029	0.52	4	25	0.049	0.89	4	50	0.052	0.94	NA	NA
Isopropanol	5	AOO	MEAN	10	0.052	0.94	MEAN	25	0.038	0.69	MEAN	50	0.040	0.72	NC	NC
Hexyl cinnamic aldehyde	5	AOO	1	10	0.436	7.89	1	25	0.262	4.74	1	50	0.933	16.89	NA	NA
Hexyl cinnamic aldehyde	5	AOO	2	10	0.073	1.32	2	25	0.920	16.65	2	50	0.365	6.61	NA	NA
Hexyl cinnamic aldehyde	5	AOO	3	10	0.198	3.58	3	25	0.197	3.57	3	50	1.102	19.95	NA	NA
Hexyl cinnamic aldehyde	5	AOO	4	10	0.455	8.24	4	25	0.517	9.36	4	50	0.583	10.55	NA	NA
Hexyl cinnamic aldehyde	5	AOO	MEAN	10	0.291	5.26	MEAN	25	0.474	8.58	MEAN	50	0.746	13.50	3.54	4.07
VP	6	AOO	1	0	0.296	1.97	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	6	AOO	2	0	0.082	0.55	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	6	AOO	3	0	0.149	0.99	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	6	AOO	4	0	0.073	0.49	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	6	AOO	MEAN	0	0.150	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	6	AOO	1	0	0.660	4.40	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	6	AOO	2	0	0.659	4.39	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	6	AOO	3	0	1.214	8.09	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	6	AOO	4	0	0.641	4.27	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	6	AOO	MEAN	0	0.794	5.29	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	6	AOO	1	0	0.213	0.84	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	6	AOO	2	0	0.134	0.53	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	6	AOO	3	0	0.372	1.47	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	6	AOO	4	0	0.292	1.16	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	6	AOO	MEAN	0	0.253	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Hexyl cinnamic aldehyde	6	AOO	1	10	0.286	1.13	1	25	0.673	2.66	1	50	1.367	5.41	NA	NA
Hexyl cinnamic aldehyde	6	AOO	2	10	0.484	1.91	2	25	0.568	2.25	2	50	0.585	2.31	NA	NA
Hexyl cinnamic aldehyde	6	AOO	3	10	0.334	1.32	3	25	1.041	4.12	3	50	0.531	2.10	NA	NA
Hexyl cinnamic aldehyde	6	AOO	4	10	0.697	2.76	4	25	0.624	2.47	4	50	NA	NA	NA	NA
Hexyl cinnamic aldehyde	6	AOO	MEAN	10	0.450	1.78	MEAN	25	0.727	2.87	MEAN	50	0.828	3.27	7.90	13.03
Isopropanol	6	AOO	1	10	0.880	3.48	1	25	0.181	0.72	1	50	0.270	1.07	NA	NA
Isopropanol	6	AOO	2	10	0.200	0.79	2	25	0.339	1.34	2	50	0.556	2.20	NA	NA
Isopropanol	6	AOO	3	10	0.469	1.86	3	25	0.330	1.31	3	50	0.324	1.28	NA	NA
Isopropanol	6	AOO	4	10	NA	NA	4	25	NA	NA	4	50	NA	NA	NA	NA
Isopropanol	6	AOO	MEAN	10	0.516	2.04	MEAN	25	0.283	1.12	MEAN	50	0.383	1.52	4.81*	9.62*
VP	6	ACE	1	0	0.118	0.65	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	6	ACE	2	0	0.249	1.36	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	6	ACE	3	0	0.164	0.90	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	6	ACE	4	0	0.200	1.09	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	6	ACE	MEAN	0	0.183	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Substance[1]	Lab No.	Vehicle	Animal No.	1 Conc. (%)	1 ABS	1 SI	Animal No.	2 Conc. (%)	2 ABS	2 SI	Animal No.	3 Conc. (%)	3 ABS	3 SI	EC1.5	EC2
PC	6	ACE	1	0	0.372	2.04	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	6	ACE	2	0	0.464	2.54	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	6	ACE	3	0	0.565	3.09	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	6	ACE	4	0	0.357	1.95	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	6	ACE	MEAN	0	0.440	2.40	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	6	ACE	1	0	0.225	1.38	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	6	ACE	2	0	0.113	0.69	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	6	ACE	3	0	0.132	0.81	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	6	ACE	4	0	0.182	1.12	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	6	ACE	MEAN	0	0.163	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Glutaradehyde	6	ACE	1	0.1	0.191	1.17	1	0.3	0.395	2.42	1	1	0.358	2.20	NA	NA
Glutaradehyde	6	ACE	2	0.1	0.138	0.85	2	0.3	0.306	1.88	2	1	0.421	2.58	NA	NA
Glutaradehyde	6	ACE	3	0.1	0.173	1.06	3	0.3	0.306	1.88	3	1	0.304	1.87	NA	NA
Glutaradehyde	6	ACE	4	0.1	0.145	0.89	4	0.3	0.223	1.37	4	1	0.382	2.34	NA	NA
Glutaradehyde	6	ACE	MEAN	0.1	0.162	0.99	MEAN	0.3	0.308	1.89	MEAN	1	0.366	2.25	0.21	0.51
Formaldehyde	6	ACE	1	0.01	0.333	2.04	1	0.03	0.312	1.91	1	0.1	0.359	2.20	NA	NA
Formaldehyde	6	ACE	2	0.01	0.196	1.20	2	0.03	0.285	1.75	2	0.1	0.244	1.50	NA	NA
Formaldehyde	6	ACE	3	0.01	0.225	1.38	3	0.03	0.248	1.52	3	0.1	0.393	2.41	NA	NA
Formaldehyde	6	ACE	4	0.01	0.288	1.77	4	0.03	0.328	2.01	4	0.1	0.289	1.77	NA	NA
Formaldehyde	6	ACE	MEAN	0.01	0.261	1.60	MEAN	0.03	0.293	1.80	MEAN	0.1	0.321	1.97	0.58	NC
VP	6	AOO	1	0	0.489	1.61	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	6	AOO	2	0	0.200	0.66	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	6	AOO	3	0	0.256	0.84	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	6	AOO	4	0	0.270	0.89	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	6	AOO	MEAN	0	0.304	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	6	AOO	1	0	0.679	2.24	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	6	AOO	2	0	0.825	2.72	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	6	AOO	3	0	0.679	2.24	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	6	AOO	4	0	0.875	2.88	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	6	AOO	MEAN	0	0.765	2.52	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	6	AOO	1	0	0.273	1.30	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	6	AOO	2	0	0.144	0.69	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	6	AOO	3	0	0.241	1.15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	6	AOO	4	0	0.181	0.86	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	6	AOO	MEAN	0	0.210	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Eugenol	6	AOO	1	10	0.349	1.66	1	25	0.991	4.72	1	50	0.668	3.18	NA	NA
Eugenol	6	AOO	2	10	0.266	1.27	2	25	0.521	2.48	2	50	0.867	4.13	NA	NA

Substance[1]	Lab No.	Vehicle	Animal No.	1 Conc. (%)	1 ABS	1 SI	Animal No.	2 Conc. (%)	2 ABS	2 SI	Animal No.	3 Conc. (%)	3 ABS	3 SI	EC1.5	EC2
Eugenol	6	AOO	3	10	0.280	1.33	3	25	0.383	1.83	3	50	0.637	3.04	NA	NA
Eugenol	6	AOO	4	10	0.329	1.57	4	25	0.399	1.90	4	50	0.496	2.36	NA	NA
Eugenol	6	AOO	MEAN	10	0.306	1.46	MEAN	25	0.574	2.73	MEAN	50	0.667	3.18	10.47	16.38
2, 4-Dinitrochlorobenzene	6	AOO	1	0.1	0.669	3.19	1	0.3	0.909	4.33	1	1	0.920	4.39	NA	NA
2, 4-Dinitrochlorobenzene	6	AOO	2	0.1	0.757	3.61	2	0.3	0.890	4.24	2	1	0.963	4.59	NA	NA
2, 4-Dinitrochlorobenzene	6	AOO	3	0.1	0.682	3.25	3	0.3	0.864	4.12	3	1	1.067	5.09	NA	NA
2, 4-Dinitrochlorobenzene	6	AOO	4	0.1	0.735	3.50	4	0.3	1.113	5.31	4	1	1.105	5.27	NA	NA
2, 4-Dinitrochlorobenzene	6	AOO	MEAN	0.1	0.711	3.39	MEAN	0.3	0.944	4.50	MEAN	1	1.014	4.83	0.015	0.025
VP	7	AOO	1	0	0.044	0.49	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	7	AOO	2	0	0.153	1.71	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	7	AOO	3	0	0.083	0.93	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	7	AOO	4	0	0.078	0.87	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	7	AOO	MEAN	0	0.090	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	7	AOO	1	0	0.690	7.71	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	7	AOO	2	0	0.699	7.81	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	7	AOO	3	0	0.442	4.94	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	7	AOO	4	0	0.623	6.96	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	7	AOO	MEAN	0	0.614	6.85	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	7	AOO	1	0	0.145	1.21	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	7	AOO	2	0	0.083	0.69	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	7	AOO	3	0	0.156	1.30	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	7	AOO	4	0	0.097	0.81	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	7	AOO	MEAN	0	0.120	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Hexyl cinnamic aldehyde	7	AOO	1	10	0.152	1.26	1	25	0.263	2.19	1	50	0.334	2.78	NA	NA
Hexyl cinnamic aldehyde	7	AOO	2	10	0.228	1.90	2	25	0.400	3.33	2	50	0.805	6.69	NA	NA
Hexyl cinnamic aldehyde	7	AOO	3	10	0.173	1.44	3	25	0.338	2.81	3	50	0.505	4.20	NA	NA
Hexyl cinnamic aldehyde	7	AOO	4	10	0.214	1.78	4	25	0.464	3.86	4	50	0.204	1.70	NA	NA
Hexyl cinnamic aldehyde	7	AOO	MEAN	10	0.192	1.59	MEAN	25	0.366	3.05	MEAN	50	0.462	3.84	9.45	14.21
Isopropanol	7	AOO	1	10	0.029	0.24	1	25	0.125	1.04	1	50	0.046	0.38	NA	NA
Isopropanol	7	AOO	2	10	0.030	0.25	2	25	0.083	0.69	2	50	0.107	0.89	NA	NA
Isopropanol	7	AOO	3	10	0.112	0.93	3	25	0.101	0.84	3	50	0.279	2.32	NA	NA
Isopropanol	7	AOO	4	10	0.060	0.50	4	25	0.150	1.25	4	50	0.052	0.43	NA	NA
Isopropanol	7	AOO	MEAN	10	0.058	0.48	MEAN	25	0.115	0.95	MEAN	50	0.121	1.01	NC	NC
VP	7	AOO	1	0	0.097	1.14	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	7	AOO	2	0	0.099	1.17	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	7	AOO	3	0	0.091	1.07	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	7	AOO	4	0	0.052	0.61	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Substance[1]	Lab No.	Vehicle	Animal No.	1 Conc. (%)	1 ABS	1 SI	Animal No.	2 Conc. (%)	2 ABS	2 SI	Animal No.	3 Conc. (%)	3 ABS	3 SI	EC1.5	EC2
VP	7	AOO	MEAN	0	0.085	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	7	AOO	1	0	0.380	4.48	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	7	AOO	2	0	0.352	4.15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	7	AOO	3	0	0.322	3.80	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	7	AOO	4	0	0.434	5.12	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	7	AOO	MEAN	0	0.372	4.39	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	7	AOO	1	0	0.148	1.21	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	7	AOO	2	0	0.107	0.87	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	7	AOO	3	0	0.093	0.76	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	7	AOO	4	0	0.143	1.16	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	7	AOO	MEAN	0	0.123	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Eugenol	7	AOO	1	10	0.331	2.70	1	25	0.435	3.54	1	50	0.790	6.44	NA	NA
Eugenol	7	AOO	2	10	0.360	2.93	2	25	0.328	2.67	2	50	0.937	7.63	NA	NA
Eugenol	7	AOO	3	10	0.440	3.58	3	25	0.730	5.95	3	50	0.847	6.90	NA	NA
Eugenol	7	AOO	4	10	0.306	2.49	4	25	0.561	4.57	4	50	0.906	7.38	NA	NA
Eugenol	7	AOO	MEAN	10	0.359	2.93	MEAN	25	0.514	4.18	MEAN	50	0.870	7.09	3.51	5.06
2, 4-Dinitrochlorobenzene	7	AOO	1	0.1	0.705	5.74	1	0.3	1.289	10.50	1	1	1.722	14.03	NA	NA
2, 4-Dinitrochlorobenzene	7	AOO	2	0.1	0.641	5.22	2	0.3	1.501	12.23	2	1	1.393	11.35	NA	NA
2, 4-Dinitrochlorobenzene	7	AOO	3	0.1	0.528	4.30	3	0.3	1.205	9.82	3	1	1.624	13.23	NA	NA
2, 4-Dinitrochlorobenzene	7	AOO	4	0.1	0.946	7.71	4	0.3	2.041	16.63	4	1	1.634	13.31	NA	NA
2, 4-Dinitrochlorobenzene	7	AOO	MEAN	0.1	0.705	5.74	MEAN	0.3	1.509	12.29	MEAN	1	1.593	12.98	0.049	0.053
VP	7	DMSO	1	0	0.081	0.67	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	7	DMSO	2	0	0.113	0.93	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	7	DMSO	3	0	0.128	1.05	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	7	DMSO	4	0	0.164	1.35	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VP	7	DMSO	MEAN	0	0.122	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	7	DMSO	1	0	0.447	3.68	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	7	DMSO	2	0	0.769	6.33	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	7	DMSO	3	0	0.358	2.95	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	7	DMSO	4	0	0.750	6.17	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	7	DMSO	MEAN	0	0.581	4.78	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	7	DMSO	1	0	0.078	0.54	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	7	DMSO	2	0	0.177	1.22	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	7	DMSO	3	0	0.185	1.28	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	7	DMSO	4	0	0.140	0.97	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VS	7	DMSO	MEAN	0	0.145	1.00	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Lactic acid	7	DMSO	1	10	0.189	1.30	1	25	0.246	1.70	1	50	0.484	3.34	NA	NA

Substance[1]	Lab No.	Vehicle	Animal No.	1 Conc. (%)	1 ABS	1 SI	Animal No.	2 Conc. (%)	2 ABS	2 SI	Animal No.	3 Conc. (%)	3 ABS	3 SI	EC1.5	EC2
Lactic acid	7	DMSO	2	10	0.167	1.15	2	25	0.266	1.83	2	50	0.258	1.78	NA	NA
Lactic acid	7	DMSO	3	10	0.163	1.12	3	25	0.387	2.67	3	50	0.251	1.73	NA	NA
Lactic acid	7	DMSO	4	10	0.180	1.24	4	25	0.355	2.45	4	50	0.475	3.28	NA	NA
Lactic acid	7	DMSO	MEAN	10	0.175	1.21	MEAN	25	0.314	2.16	MEAN	50	0.367	2.53	14.58	22.47
Nickel sulfate	7	DMSO	1	1	0.220	1.52	1	3	0.546	3.77	1	10	0.230	1.59	NA	NA
Nickel sulfate	7	DMSO	2	1	0.311	2.14	2	3	0.384	2.65	2	10	0.428	2.95	NA	NA
Nickel sulfate	7	DMSO	3	1	0.307	2.12	3	3	0.385	2.66	3	10	0.334	2.30	NA	NA
Nickel sulfate	7	DMSO	4	1	0.251	1.73	4	3	0.227	1.57	4	10	0.549	3.79	NA	NA
Nickel sulfate	7	DMSO	MEAN	1	0.272	1.88	MEAN	3	0.386	2.66	MEAN	10	0.385	2.66	0.59	1.31

Abbreviations: ABS = Absorbance; ACE = Acetone; AOO = Acetone: olive oil; Conc. = Concentration; DMSO = Dimethyl sulfoxide; EC1.5 = Estimated concentration needed to produce a stimulation index of 1.5; EC2 = Estimated concentration needed to produce a stimulation index of two; LLNA: BrdU-ELISA = Murine local lymph node assay (LLNA) with enzyme-linked immunosorbent assay (ELISA) detection of bromodeoxyuridine (BrdU); NA = Not applicable; NC = Not calculated (i.e., maximum SI was less than decision criterion); No. = Identification number; PC = Positive control; SI = Stimulation index; VP = Vehicle for PC; VS = Vehicle for test substance.

*Calculated using linear interpolation with SI =1, concentration =0 as the lowest point because the dose-response was nonmonotonic.

[1]Results of the Japanese Society for Alternatives to Animal Experiements interlaboratory validation study.

[2]Positive control failed because SI >2 was not achieved. Results from test substances associated with failed positive control were not considered in the accuracy and reproducibility analyses.

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Appendix E

Accuracy Analyses Using Additional Approaches for Combining Multiple Test Results

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1.0 LLNA: BrdU-ELISA Accuracy Analysis Using Alternative Decision Criteria and Alternate Methods for Combining Data for Substances Tested Multiple Times

This appendix shows performance analyses for the LLNA: BrdU-ELISA using single alternative decision criteria and two different approaches for combining test results for the 14 substances with multiple LLNA: BrdU-ELISA tests:

1. The positive/negative outcome for each substance for each criterion was determined by the outcome of the test with the highest maximum SI of the multiple tests.
2. The positive/negative outcome for each substance for each criterion was determined by the outcome of the test with the lowest maximum SI of the multiple tests.

Section 6.4 provides the results for the analysis when the most prevalent outcome was used as the result for each substance tested multiple times (for each criterion).

1.1 Results of LLNA: BrdU-ELISA Accuracy Analysis Using Single Alternative Decision Criteria and the Highest Maximum SI for the Outcome of Multiple Tests

When combining multiple test results for a single substance using the outcome of the test with the highest maximum SI, the decision criterion of SI ≥ 2.0 to identify sensitizers yielded an accuracy of 77% (24/31), a sensitivity of 77% (17/22), a specificity of 78% (7/9), a false positive rate of 22% (2/9), and a false negative rate of 23% (5/22) (**Table E-1)**. SI ≥ 2.0 was the decision criterion used by the JSAAE interlaboratory validation study of the LLNA: BrdU-ELISA. The performance for the additional decision criteria described in **Section 6.4**, are also shown in **Table E-1**. Over the range of SI cutoffs evaluated, increasing the SI cutoff decreased accuracy (84% at SI ≥ 1.3 to 68% at SI ≥ 5.0), decreased sensitivity (100% at SI ≥ 1.3 to 54% at SI ≥ 5.0), increased specificity (44% at SI ≥ 1.3 to 100% at SI ≥ 5.0), decreased the false positive rate (56% at SI ≥ 1.3 to 0% at SI ≥ 5.0), and increased the false negative rate (0% at SI ≥ 1.3 to 46% at SI ≥ 5.0) (**Figure E-1** and **Table E-1)**. Use of ANOVA and summary statistics (i.e., mean absorbance values of treated groups ≥95%

confidence interval of the control group, or ≥2 or ≥3 SD from the control group mean), yielded accuracy values of 74 to 81%, with sensitivity values of 86 to 100%, and false negative rates of 0 to 14%. The specificity for these criteria ranged from 33 to 56% and the false positive rates were 44 to 67%.

The highest accuracy and lowest false negative rate, for the approach using the highest maximum SI for the substances with more than one test, was achieved using an SI ≥ 1.3. The accuracy for SI ≥ 1.3 was 84% (26/31), with sensitivity of 100% (22/22), specificity of 44% (5/9), a false positive rate of 56% (5/9), and false negative rate of 0% (0/22). However, using an SI ≥ 1.3 incorrectly classified lactic acid, isopropanol, and methyl salicylate, three of the ICCVAM performance standards reference substances, as sensitizers. Use of mean absorbance values of treated groups ≥95% confidence interval of the control group to identify sensitizers also produced the lowest false negative rate 0% (0/9), with a slightly lower accuracy of 81% (25/31), and a higher false positive rate of 67% (6/9). This criterion also incorrectly classified lactic acid and isopropanol as sensitizers. The lowest false positive rates (0% [0/9]) were produced by SI cutoffs of 3.0 to 5.0, however the false negative rates at those cutoffs were 27% (6/22) to 46% (10/22).

Table E-1 Performance of the LLNA: BrdU-ELISA Compared with the Traditional LLNA Using Alternative Decision Criteria to Identify Sensitizers and the Highest Maximum SI for Substances with Multiple Tests

Alternate Criterion	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]
Statistics[3]	31	74	23/31	86	19/22	44	4/9	56	5/9	14	3/22	79	19/24	57	4/7
≥ 95% CI[4]	31	81	25/31	100	22/22	33	3/9	67	6/9	0	0/22	79	22/28	100	3/3
≥ 2 SD[5]	31	77	24/31	96	21/22	33	3/9	67	6/9	5	1/22	78	21/27	75	3/4
≥ 3 SD[6]	31	81	25/31	91	20/22	56	5/9	44	4/9	9	2/22	83	20/24	71	5/7
SI ≥ 5.0	31	68	21/31	54	12/22	100	9/9	0	0/9	46	10/22	100	12/12	47	9/19
SI ≥ 4.5	31	71	22/31	59	13/22	100	9/9	0	0/9	41	9/22	100	13/13	50	9/18
SI ≥ 4.0	31	77	24/31	68	15/22	100	9/9	0	0/9	32	7/22	100	15/15	56	9/16
SI ≥ 3.5	31	77	24/31	68	15/22	100	9/9	0	0/9	32	7/22	100	15/15	56	9/16
SI ≥ 3.0	31	81	25/31	73	16/22	100	9/9	0	0/9	27	6/22	100	16/16	60	9/15
SI ≥ 2.5	31	81	25/31	77	17/22	89	8/9	11	1/9	23	5/22	94	17/18	62	8/13
SI ≥ 2.0	31	77	24/31	77	17/22	78	7/9	22	2/9	23	5/22	90	17/19	58	7/12
SI ≥ 1.5	31	81	25/31	91	20/22	56	5/9	44	4/9	9	2/22	83	20/24	71	5/7
SI ≥ 1.3	31	84	26/31	100	22/22	44	4/9	56	5/9	0	0/22	82	22/27	100	4/4

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

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

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

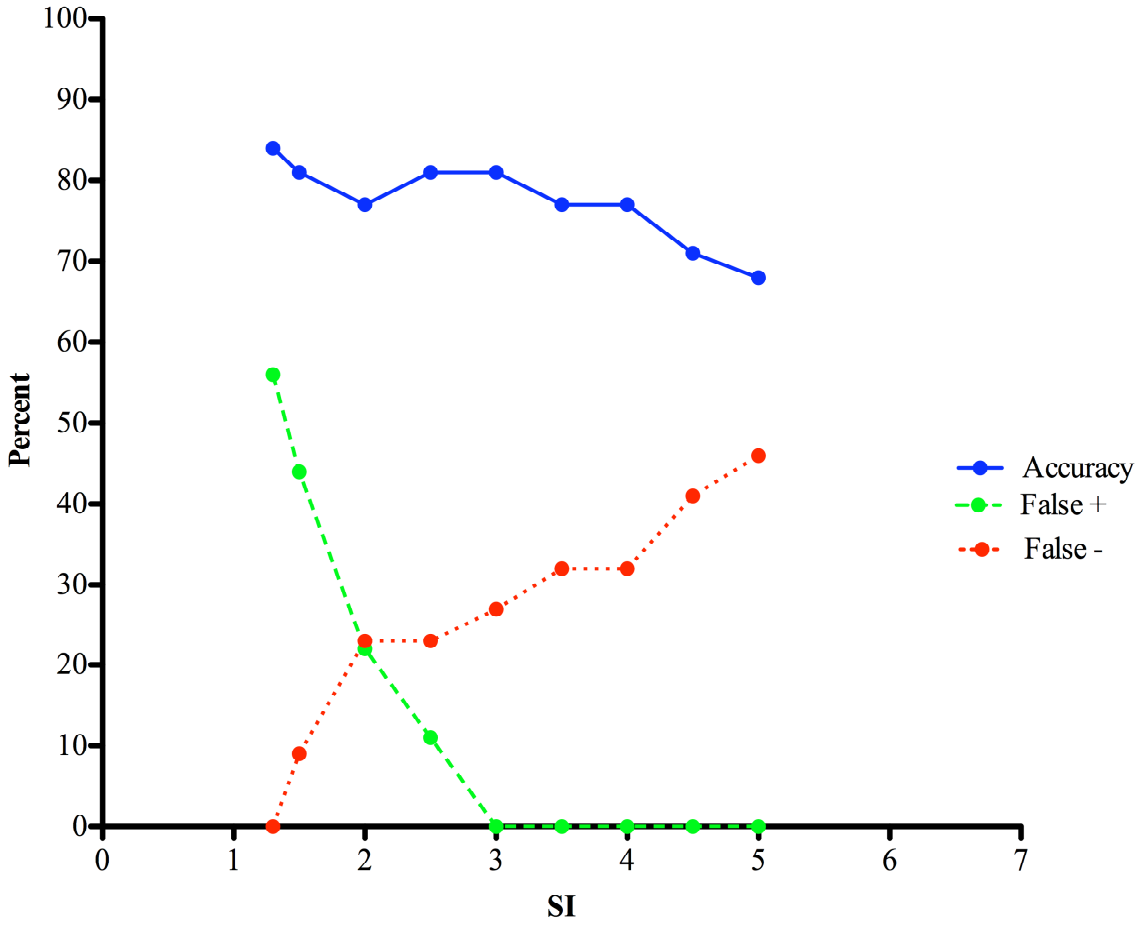
[3] Analysis of variance for difference of group means when substances were tested at multiple doses or *t*-test when substances were tested at one dose. The absorbance data were log-transformed prior to analysis of variance. Significance at $p < 0.05$ was further tested by Dunnett's test.

[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.

Figure E-1 Performance of the LLNA: BrdU-ELISA with SI Compared to the Traditional LLNA Using the Highest Maximum SI 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 31 substances (22 sensitizers and nine nonsensitizers based on traditional LLNA results). For the 14 substances with multiple test results, the results for each substance were combined by using the outcome for the test with the highest maximum SI value. The solid line shows accuracy, the dashed line shows the false positive rate, and the dotted line shows the false negative rate.

1.2 Results of LLNA: BrdU-ELISA Accuracy Analysis Using Alternative Decision Criteria and Lowest Maximum SI for the Outcome of Multiple Tests

When combining multiple test results for a single substance using the outcome of the test with the lowest maximum SI, the decision criterion of SI ≥ 2.0 to identify sensitizers for these 31 substances yielded an accuracy of 81% (25/31), a sensitivity of 73% (16/22), a specificity of 100% (9/9), a false positive rate of 0% (0/9), and a false negative rate of 27% (6/22) (**Table E-2)**. SI ≥ 2.0 was the decision criterion used by the JSAAE interlaboratory validation study of the LLNA: BrdU-ELISA. The performance for the additional decision criteria described in **Section 6.4**, are shown in **Table E-2**.

Over the range of SI cutoffs evaluated, increasing the SI cutoff decreased accuracy (90% at SI ≥ 1.3 to 45% at SI ≥ 5.0), decreased sensitivity (100% at SI ≥ 1.3 to 23% at SI ≥ 5.0), increased specificity (67% at SI ≥ 1.3 to 100% at SI ≥ 5.0), decreased the false positive rate (33% at SI ≥ 1.3 to 0% at SI ≥ 5.0), and increased the false negative rate (0% at SI ≥ 1.3 to 77% at SI ≥ 5.0) (**Figure E-2** and **Table E-2**). Use of ANOVA and summary statistics (i.e., mean absorbance values of treated groups ≥95% confidence interval of the control group, or ≥2 or 3 SD from the control group mean), yielded accuracy of 84 to 90%, with sensitivity values of 86 to 100%, and false negative rates of 0 to 14%. The specificity for these criteria ranged from 67 to 89% and the false positive rates were 11 to 33%.

The highest accuracy and lowest false negative rate, for the approach using the lowest maximum SI for the substances with more than one test, was achieved using an SI ≥ 1.3 and mean absorbance values of treated groups ≥95% confidence interval of the control group. Both criteria yielded an accuracy of 90% (26/31) and a false negative rate of 0% (0/22). Both criteria also yielded sensitivity =100% (22/22), specificity = 67% (6/9), and false positive rate = 33% (3/9). However, these criteria incorrectly classified lactic acid, isopropanol, and methyl salicylate, three of the ICCVAM performance standards reference substances, as sensitizers. The lowest false positive rate (0% [0/9]) was produced by SI cutoffs of 2.0 to 5.0, however the false negative rates at those cutoffs were 27% to 77%. Of those cutoffs, SI ≥ 2.0 produced the highest accuracy, 81% (25/31), and the lowest false negative rate, 27% (6/22).

Table E-2 Performance of the LLNA: BrdU-ELISA Compared with the Traditional LLNA Using Alternative Decision Criteria to Identify Sensitizers and the Lowest Maximum SI for Substances with Multiple Tests

Alternate Criterion	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]
Statistics[3]	31	84	26/31	86	19/22	78	7/9	22	2/9	14	3/22	91	19/21	70	7/10
≥ 95% CI[4]	31	90	28/31	100	22/22	67	6/9	33	3/9	0	0/22	88	22/25	100	6/6
≥ 2 SD[5]	31	87	27/31	96	21/22	67	6/9	33	3/9	5	1/22	88	21/24	86	6/7
≥ 3 SD[6]	31	87	27/31	86	19/22	89	8/9	11	1/9	14	3/22	95	19/20	73	8/11
SI ≥ 5.0	31	45	14/31	23	5/22	100	9/9	0	0/9	77	17/22	100	5/5	35	9/26
SI ≥ 4.5	31	45	14/31	23	5/22	100	9/9	0	0/9	77	17/22	100	5/5	35	9/26
SI ≥ 4.0	31	55	17/31	36	8/22	100	9/9	0	0/9	64	14/22	100	8/8	39	9/23
SI ≥ 3.5	31	55	17/31	36	8/22	100	9/9	0	0/9	64	14/22	100	8/8	39	9/23
SI ≥ 3.0	31	64	20/31	50	11/22	100	9/9	0	0/9	50	11/22	100	11/11	45	9/20
SI ≥ 2.5	31	71	22/31	59	13/22	100	9/9	0	0/9	41	9/22	100	13/13	50	9/18
SI ≥ 2.0	31	81	25/31	73	16/22	100	9/9	0	0/9	27	6/22	100	16/16	60	9/15
SI ≥ 1.5	31	87	27/31	91	20/22	78	7/9	22	2/9	9	2/22	91	20/22	78	7/9
SI ≥ 1.3	31	90	28/31	100	22/22	67	6/9	33	3/9	0	0/22	88	22/25	100	6/6

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

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

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

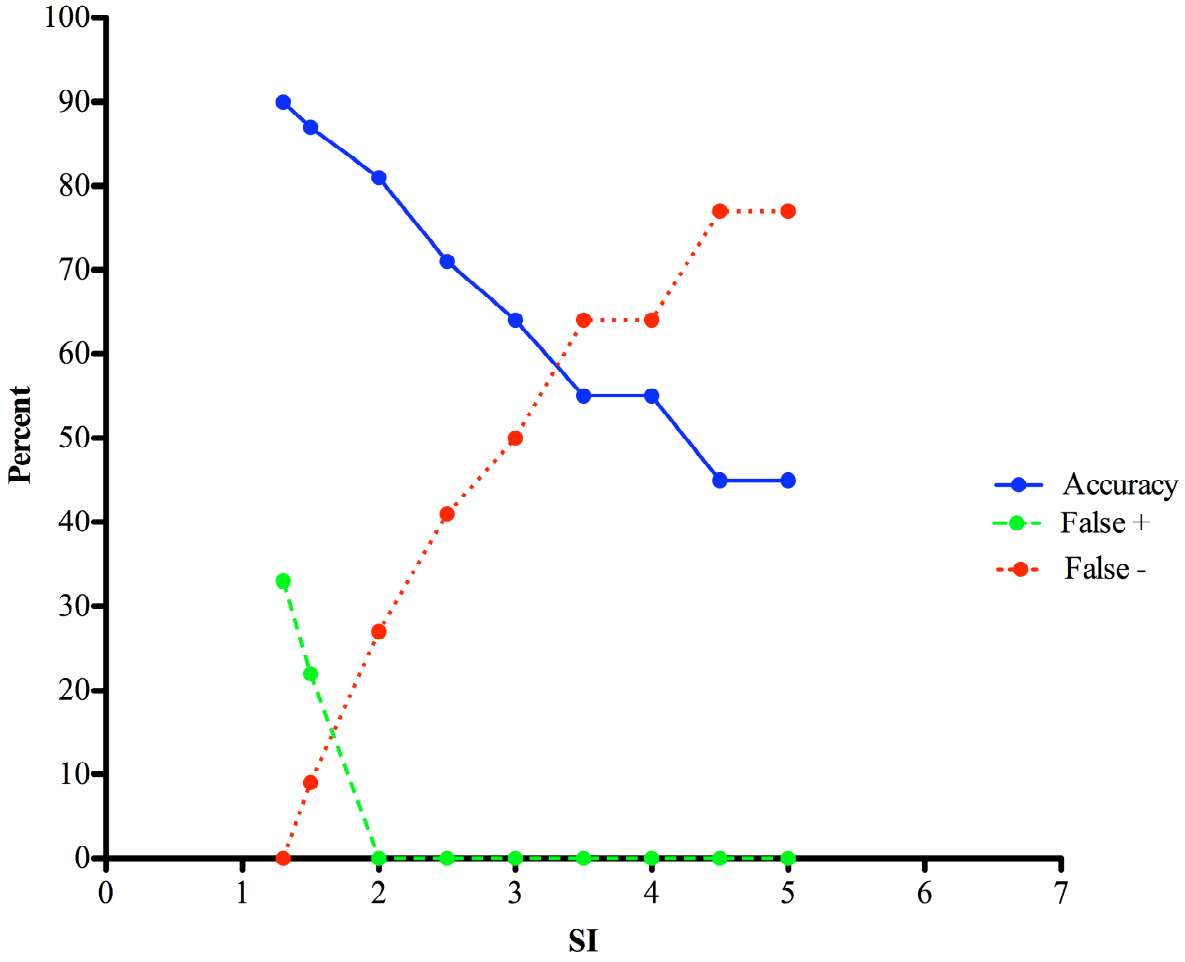
[3]Analysis of variance for difference of group means when substances were tested at multiple doses or t-test when substances were tested at one dose. The absorbance data were log-transformed prior to analysis of variance. Significance at $p < 0.05$ was further tested by Dunnett's test.

[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.

Figure E-2 Performance of the LLNA: BrdU-ELISA with SI Compared to the Traditional LLNA Using the Lowest Maximum SI 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 31 substances (22 sensitizers and nine nonsensitizers based on traditional LLNA results). For the 14 substances with multiple test results, the results for each substance were combined by using the outcome for the test with the lowest maximum SI value. The solid line shows accuracy, the dashed line shows the false positive rate, and the dotted line shows the false negative rate.

2.0 Discordant Results For Accuracy Analysis of Alternative Decision Criteria

Using the decision criteria of SI ≥ 2.0 to identify sensitizers and the most prevalent outcome for the substances with multiple tests for the analysis of alternative decision criteria, the five discordant substances (when compared to the traditional LLNA) were aniline, cyclamen aldehyde, hydroxycitronellal, 2-mercaptobenzothiazole, and linalool (**Table 6-4**). As indicated in **Section 6.4.1**, all five substances were misclassified as nonsensitizers when compared to the traditional LLNA, which classified them as sensitizers.

2.1 Discordant Results Using Alternative Decision Criteria and Highest Maximum SI Outcome for Multiple Tests

Using the decision criteria of SI ≥ 2.0 to identify sensitizers and the test with the highest maximum SI as the result for substances with multiple tests, yielded two additional discordant substances: isopropanol and lactic acid, which were misclassified as sensitizers.

Table E-3 shows how the number and identity of discordant substances changes with the alternate decision criteria when using the test with the highest maximum SI as the result for substances with multiple tests. Using an SI cutoff less than 2.0, SI ≥ 1.5, to identify sensitizers yielded six discordant substances. Two substances, hydroxycitronellal and linalool, were misclassified as nonsensitizers, and four substances, hexane, isopropanol, lactic acid and propylene glycol, were misclassified as sensitizers. Using an even lower SI to identify sensitizers, SI ≥ 1.3, yielded five discordant substances that were all misclassified as sensitizers (hexane, isopropanol, lactic acid, methyl salicylate, and propylene glycol). Increasing the SI cutoff to values greater than 2.0, increased the number of sensitizers that were misclassified as nonsensitizers. At SI ≥ 2.0, five sensitizers were misclassified as nonsensitizers while at SI ≥ 5.0, 10 sensitizers were classified as nonsensitizers (**Table E-3)**. At SI ≥ 2.0, two nonsensitizers were misclassified as sensitizers while, at SI ≥ 5.0, no nonsensitizers were classified as sensitizers.

Use of a statistical test (i.e., ANOVA or *t*-test) or summary statistics (i.e., ≥95% CI, ≥2 SD, or ≥3 SD) tended to misclassify more nonsensitizers than sensitizers. Using ANOVA or a *t*-test to identify sensitizers misclassified three sensitizers (linalool, 2-mercaptobenzothiazole, and

aniline) as nonsensitizers and four nonsensitizers (glycerol, hexane, isopropanol, and lactic acid) as sensitizers. Using treatment group absorbance ≥95% CI or ≥2 SD of vehicle control mean misclassified six nonsensitizers as sensitizers (glycerol, hexane, isopropanol, lactic acid, methyl salicylate, and propylene glycol). Treatment group absorbance ≥2 SD of vehicle control mean also misclassified one weak sensitizer as a nonsensitizer (linalool). Using treatment group absorbance ≥3 SD of vehicle control mean misclassified three nonsensitizers as sensitizers (hexane, isopropanol, and lactic acid) and three weak sensitizers as nonsensitizers (hydroxycitronellal, linalool, and aniline).

Four ICCVAM performance standards reference substances were discordant for the analysis of alternate decision criteria using the test with the highest maximum SI as the result for substances with multiple tests (**Table E-3**). One sensitizer, 2-mercaptobenzothiazole, was misclassified by some criteria as a nonsensitizer, and three nonsensitizers, isopropanol, lactic acid, and methyl salicylate, were misclassified as sensitizers by some criteria. The criteria that yielded the correct results for 2-mercaptobenzothiazole included summary statistics (i.e., ≥95% CI, ≥2 SD, or ≥3 SD), SI ≥ 1.5, and SI ≥ 1.3. The criteria that yielded the correct results for isopropanol included SI ≥ 2.5 to 5.0. The criteria that yielded the correct results for lactic acid included SI ≥ 3.0 to 5.0. All criteria yielded the correct results for methyl salicylate except for treatment group absorbance ≥95% CI or ≥2 SD of vehicle control mean, and SI ≥ 1.3.

Table E-3 Discordant Results for LLNA: BrdU-ELISA Using Alternative Decision Criteria Compared to the Traditional LLNA and the Highest Maximum SI for Substances with Multiple Tests

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 ≥ 1.5	SI ≥ 1.3
2-Mercaptobenzothiazole (1.7%)	-				-	-	-	-	-	-	-		
Cinnamic aldehyde (2.4%)					-	-							
3-Aminophenol (3.2%)					-	-	-	-					
Nickel sulfate (4.8%)					-								
4-Chloroaniline (6.5%)					-	-	-	-	-				
Cyclamen aldehyde (22.3%)					-	-	-	-	-	-	-		
Hydroxycitronellal (24%)				-	-	-	-	-	-	-	-	-	
Linalool (30%)	-		-	-	-	-	-	-	-	-	-	-	
Isopropyl myristate (44%)					-	-							
Aniline (63%)	-			-	-	-	-	-	-	-	-		
Glycerol (-)	+	+	+										
Hexane (-)	+	+	+	+								+	+
Isopropanol (-)	+	+	+	+							+	+	+
Lactic acid (-)	+	+	+	+						+	+	+	+
Methyl salicylate (-)		+	+										+
Propylene glycol (-)		+	+									+	+

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

[1]Compared to the traditional LLNA. Traditional LLNA result in parentheses: "-" for nonsensitizers and EC3 (%) for sensitizers.

[2]LLNA: BrdU result shown: "+" if the decision criterion was met and "-" if the decision criterion was not met.

[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.

2.2 Discordant Results Using Alternative Decision Criteria and Lowest Maximum SI Outcome for Multiple Tests

Using the decision criteria of SI ≥ 2.0 to identify sensitizers and the most prevalent outcome for the substances with multiple tests for the analysis of alternative decision criteria yielded five discordant substances (when compared to the traditional LLNA). Aniline, cyclamen aldehyde, hydroxycitronellal, 2-mercaptobenzothiazole, and linalool were misclassified as nonsensitizers (**Table 6-4**), while the traditional LLNA classified them as sensitizers. Using the test with the lowest maximum SI as the result for substances with multiple tests yielded six discordant substances at SI ≥ 2.0. One additional sensitizer, formaldehyde, was misclassified as a nonsensitizer (**Table E-4**).

Table E-4 shows how the number and identity of discordant substances changes with the alternate decision criteria when using the test with the lowest maximum SI as the result for substances with multiple tests. Using an SI cutoff less than 2.0, SI ≥ 1.5, to identify sensitizers yielded four discordant substances. Two substances, hydroxycitronellal and linalool, were misclassified as nonsensitizers, and two substances, hexane and lactic acid, were misclassified as sensitizers. Using an even lower SI to identify sensitizers, SI ≥ 1.3, yielded three discordant substances that were all misclassified as sensitizers (hexane, lactic acid, and methyl salicylate). Increasing the SI cutoff to values greater than 2.0, increased the number of sensitizers that were misclassified as nonsensitizers. At SI ≥ 2.0, six sensitizers were misclassified as nonsensitizers while at SI ≥ 4.5 and SI ≥ 5.0, 17 sensitizers were classified as nonsensitizers (**Table E-4)**. From SI ≥ 2.0 to SI ≥ 5.0 no nonsensitizers were misclassified as sensitizers.

Using the test with the lowest maximum SI as the result for substances with multiple tests caused even potent sensitizers to be misclassified as nonsensitizers at the higher SI cutoffs. At SI ≥ 4.5 and SI ≥ 5.0, 2,4-dinitrochlorobenzene, glutaraldehyde, and formaldehyde were classified as nonsensitizers. Glutaraldehyde was classified as a nonsensitizer at SI cutoffs as low as 2.5 and formaldehyde was classified as a nonsensitizer at SI cutoffs as low as 2.0.

Table E-4 Discordant Results for LLNA: BrdU-ELISA Using Alternative Decision Criteria Compared to the Traditional LLNA and the Lowest Maximum SI for Substances with Multiple Tests

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 ≥ 1.5	SI ≥ 1.3
2,4-Dinitrochlorobenzene (0.049%)					-	-							
Glutaraldehyde (0.14%)					-	-	-	-	-	-			
Formaldehyde (0.53%)					-	-	-	-	-	-	-		
trans-Cinnamic aldehyde (1.4%)					-	-	-	-					
Isoeugenol (1.5%)					-	-	-	-	-	-			
2-Mercaptobenzothiazole (1.7%)	-				-	-	-	-	-	-	-		
Cinnamic aldehyde (2.4%)					-	-							
3-Aminophenol (3.2%)					-	-	-	-					
Nickel sulfate (4.8%)					-	-	-	-	-				
4-Chloroaniline (6.5%)					-	-	-	-	-				
Hexyl cinnamic aldehyde (9.7%)					-	-	-	-	-	-			
Eugenol (10.1%)					-	-	-	-					
Cyclamen aldehyde (22.3%)					-	-	-	-	-	-	-		
Hydroxycitronellal (24%)				-	-	-	-	-	-	-	-	-	
Linalool (30%)	-		-	-	-	-	-	-	-	-	-	-	
Isopropyl myristate (44%)					-	-							
Aniline (63%)	-			-	-	-	-	-	-	-	-		
Glycerol (-)	+	+	+										
Hexane (-)	+	+										+	+
Lactic acid (-)		+	+									+	+
Methyl salicylate (-)													+

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

[1]Compared to the traditional LLNA. Traditional LLNA result in parentheses: "-" for nonsensitizers and EC3 (%) for sensitizers.

[2]LLNA: BrdU result shown: "+" if the decision criterion was met and "-" if the decision criterion was not met.
[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.

Use of a statistical test (i.e., ANOVA or *t*-test) or summary statistics (i.e., ≥95% CI, ≥2 SD, or 3 ≥SD) more often misclassified nonsensitizers than sensitizers (**Table E-4**). Using ANOVA or *t*-tests to identify sensitizers misclassified three sensitizers (2-mercaptobenzothiazole, linalool, and aniline) as nonsensitizers and two nonsensitizers (glycerol and hexane) as sensitizers. Using treatment group absorbance ≥95% CI or ≥2 SD of vehicle control mean misclassified glycerol and lactic acid as sensitizers. Using treatment group absorbance ≥95% CI also misclassified hexane as a sensitizer. Additionally, treatment group absorbance ≥2 SD of vehicle control mean misclassified one weak sensitizer (linalool) as a nonsensitizer. Using treatment group absorbance ≥3 SD of vehicle control mean, however, misclassified three weak sensitizers as nonsensitizers (hydroxycitronellal, linalool, and aniline).

Seven ICCVAM performance standards reference substances were discordant for the analysis of alternate decision criteria using the test with the lowest maximum SI as the result for substances with multiple tests (**Table E-4**). One strong sensitizer, 2,4-dinitrochlorobenzene, was misclassified by some criteria as a nonsensitizer. Four additional sensitizers, isoeugenol, 2-mercaptobenzothiazole, hexyl cinnamic aldehyde, and eugenol, were also misclassified as nonsensitizers by some criteria. The criteria that yielded the correct results for 2,4-dinitrochlorobenzene were all but the SI ≥ 4.5 to 5.0 criteria. The criteria that yielded the correct results for 2-mercaptobenzothiazole included summary statistics (i.e., ≥95% CI, ≥2 SD, or ≥3 SD), SI ≥ 1.5 and SI ≥ 1.3. The criteria that yielded the correct results for isoeugenol and hexyl cinnamic aldehyde were SI ≥ 1.5 to 2.0. The criteria that yielded the correct results for eugenol were SI ≥ 1.3 to 3.0.

Two nonsensitizers, lactic acid and methyl salicylate, from the list of ICCVAM performance standards reference substances, were misclassified as sensitizers by some criteria. The criteria that yielded the correct results for lactic acid were all except for treatment group absorbance ≥95% CI or ≥2 SD of vehicle control mean, SI ≥ 1.5, and SI ≥ 1.3. The criteria that yielded the correct results for methyl salicylate were all except for SI ≥ 1.3.

Appendix F

Reproducibility Analyses for LLNA: BrdU-ELISA with Decision Criterion of SI ≥ 1.5

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1.0 Test Method Reliability

Section 7 provides the reproducibility analyses for the LLNA: BrdU-ELISA using SI ≥ 2.0 to classify substances as sensitizers. The decision criterion of SI ≥ 2.0 was used in the JSAAE interlaboratory validation study. The SI ≥ 2.0 criterion produced an accuracy of 87% (27/31), a false positive rate of 0% (0/9), and a false negative rate of 18% (4/22) when LLNA: BrdU-ELISA results were compared to the results of the traditional LLNA (**Table 6-6**). This appendix provides the reproducibility analyses using SI ≥ 1.5 to classify substances as sensitizers. This was one of the alternate SI criterion evaluated in **Section 6.5**. The SI ≥ 1.5 criterion produced an accuracy of 84% (26/31), a false positive rate of 33% (3/9), and a false negative rate of 9% (2/22) when LLNA: BrdU-ELISA results were compared to the results of the traditional LLNA (**Table 6-6**).

1.1 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 EC1.5 values. Analyses of intralaboratory reproducibility were performed using a concordance analysis for the qualitative results (sensitizer vs. nonsensitizer) (**Section 1.1.1**) and a CV analysis for the quantitative results (SI values and EC3 values) (**Sections 1.1.2** and **1.1.3**, respectively).

1.1.1 *Intralaboratory Reproducibility – Qualitative Results*

The dataset available for an intralaboratory concordance analysis of the qualitative test results for the LLNA: BrdU-ELISA included eight substances that were tested multiple times and classified as sensitizers or nonsensitizers. Hexyl cinnamic aldehyde was tested six times, eugenol was tested five times, and isoeugenol was tested three times, and 2,4-dinitrochlorobenzene, glutaraldehyde, hexane, 4-phenylenendiamine, and propylene glycol were each tested twice (Takeyoshi et al. 2003, 2004a, 2005, 2006, 2007a; unpublished data) (**Table F-1**). All substances were sensitizers in the traditional LLNA except for propylene glycol and hexane. The multiple test results for 7/8 substances were 100% concordant when SI ≥ 1.5 was used to classify substances as sensitizers. Discordant test results were noted for propylene glycol tested at a maximum concentration of 50%. The test result from Takeyoshi

et al. (2005) was positive (SI = 1.6) while the result from Takeyoshi et al. (2006) produced a negative result (SI = 0.9). Both tests used AOO as the vehicle.

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 in 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 similar for LLNA: BrdU-ELISA (7/8) and the traditional LLNA (5/6).

Table F-1 Intralaboratory Reproducibility for the LLNA: BrdU-ELISA Outcome of Substances Tested Multiple Times

Substance	Highest Concentration Tested (%)	Highest SI	Outcome[1]	Takeyoshi et al. Reference
2,4-Dinitro-chlorobenzene	2	17.9	+	2005
	2	6.8	+	2006, 2007b
Eugenol	30	3.3	+	2004a
	30	3.8	+	2007a
	50	12.3	+	2005
	50	3.1	+	2006
	50	17.7	+	2007b
Glutaraldehyde	2	14.6	+	2005, 2007b
	10	15.5	+	2005, 2007b
Hexane	50	1.9	+	2005
	100	1.8	+	Unpublished data
Hexyl cinnamic aldehyde	25	2.4	+	2003
	50	3.6	+	2003
	50	5.9	+	2005
	50	3.6	+	2006
	50	2.7	+	2006
	50	3.0	+	2007b
Isoeugenol	10	8.4	+	2005
	10	2.4	+	2006, 2007b
	30	6.7	+	
4-Phenylenediamine	2	11.7	+	2005, 2007b
	10	14.7	+	2005, 2007b
Propylene glycol	50	1.6	+	2005
	50	0.9	-	2006, 2007b

Abbreviations: LLNA: BrdU-ELISA = Murine local lymph node assay (LLNA) with enzyme-linked immunosorbent assay (ELISA) detection of bromodeoxyuridine (BrdU); SI = Stimulation index.
[1](+) = Sensitizer; (-) = nonsensitizer

1.1.2 *Intralaboratory Reproducibility – SI*

There were six substances that were tested multiple times by Takeyoshi et al. (2003, 2004a, 2005, 2006, 2007a, 2007b, unpublished data). Because two substances had multiple tests for more than one concentration, there were nine 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 F-2**, the CVs ranged from 1% (25% hexyl cinnamic aldehyde) to 79% (10% isoeugenol). The intralaboratory reproducibility of the traditional LLNA was not assessed by CV analysis of SI values (ICCVAM 1999).

1.1.3 *Intralaboratory Reproducibility – EC1.5*

CV values were also calculated for the EC1.5 values for the three sensitizers that were tested more than once using multiple doses by Takeyoshi et al. (2003; 2004a, 2005, 2006, 2007a, 2007b). The individual animal data for eugenol, hexyl cinnamic aldehyde, and isoeugenol, were used to calculated EC1.5 values for the LLNA: BrdU-ELISA. The methods for calculating EC1.5 values for each sensitizer were modified from those used by Ryan et al. (2007) to calculate EC3 values. Linear interpolation was used to calculate EC1.5 values for each test with SI values higher or lower than two and extrapolation was used to calculate EC1.5 values for tests with no SI values below two. The equation for linear interpolation was:

$$EC1.5 = c + \left[\frac{(1.5 - d)}{(b - d)}\right] \times (a - c)$$

The linear interpolation equation uses the points immediately above and below SI = 2, with the (dose, SI) coordinates of (a, b) immediately above SI = 2 and (c, d) immediately below SI = 2. The equation for extrapolation was:

$$EC1.5_{ex} = 2^{\left\{\log_2(c) + \frac{(1.5-d)}{(b-d)} \times \left[\log_2(a) - \log_2(c)\right]\right\}}$$

The extrapolation equation uses the two points immediately above SI = 2, with the coordinates of (a, b) for the point closest to SI = 2, and (c, d) for the higher point.

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

Substance	Concentration Tested (%)	SI	Mean	SD	CV (%)	Takeyoshi et al. Reference
2,4-Dinitrochlorobenzene	2	17.9	12.4	7.8	64	2005
	2	6.8				2006, 2007b
Eugenol	30	3.3	3.6	0.4	10	2004a
	30	3.8				2007a
Eugenol	50	12.3	11.0	7.4	67	2005
	50	3.1				2006
	50	17.7				2007b
Hexane	50	1.9	1.8	0.07	4	2005
	50	1.8				Unpublished
Hexyl cinnamic aldehyde	12.5	1.87	1.73	0.21	12	2003
	12.5	1.58				2003
Hexyl cinnamic aldehyde	25	2.42	2.4	0.01	1	2003
	25	2.40				2003
Hexyl cinnamic aldehyde	50	3.6	3.8	1.3	34	2003
	50	5.9				2005
	50	3.6				2006
	50	2.7				2006
	50	3.0				2007b
Isoeugenol	10	8.4	5.4	4.2	79	2005
	10	2.4				2006, 2007b
Propylene glycol	50	1.6	1.1	0.6	55	2005
	50	0.7				2006, 2007b

Abbreviations: LLNA: BrdU-ELISA = Murine local lymph node assay (LLNA) with enzyme-linked immunosorbent assay (ELISA) detection of bromodeoxyuridine (BrdU); CV = Coefficient of variation; SD = Standard deviation; SI = Stimulation index.

As shown in **Table F-3**, there were five EC1.5 values for hexyl cinnamic aldehyde, four EC1.5 values for eugenol, and two EC1.5 values for isoeugenol. The CV values were 37% for hexyl cinnamic aldehyde, 66% for eugenol, and 52% 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 ECt value (i.e., the estimated concentration needed to produce an SI of a specific threshold value, 1.5, in this case) within 5% to 20% (ICCVAM 2009). All five EC1.5 values for hexyl cinnamic aldehyde were within the acceptable range for intralaboratory reproducibility.

Table F-3 Intralaboratory Reproducibility for the EC1.5 of Tested Substances in LLNA: BrdU-ELISA - Coefficient of Variation

Substance	EC1.5	Mean	SD	CV (%)	Takeyoshi et al. Reference
Eugenol	5.9	7.2	4.7	66	2004a
	11.0				2006
	10.7				2007a
	1.0				2007b
Hexyl cinnamic aldehyde	11.6	12.9	4.8	37	2003
	5.5				2003
	15.9				2006
	18.1				2006
	13.5				2007b
Isoeugenol	6.3	4.6	2.4	52	2006, 2007b
	2.9				2007a

Abbreviations: LLNA: BrdU-ELISA = Murine local lymph node assay (LLNA) with enzyme-linked immunosorbent assay (ELISA) detection of bromodeoxyuridine (BrdU); CV = Coefficient of variation; EC1.5 = Estimated concentration needed to produce a stimulation index of two; 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-dinitro-chlorobenzene, 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 F-4**).

Table F-4 Intralaboratory Reproducibility for the EC3 of Tested Substances in the Traditional LLNA[1]

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

Abbreviations: LLNA = Murine local lymph node assay (LLNA) with enzyme-linked immunosorbent assay (ELISA) detection of bromodeoxyuridine (BrdU); CV = Coefficient of variation; EC3 = Estimated concentration needed to produce a stimulation index of three.
[1]From ICCVAM (1999).

For all three substances in common, the intralaboratory CV values for the EC1.5 values from LLNA: BrdU-ELISA tests were higher than those reported in ICCVAM (1999) for EC3 values from the traditional LLNA. The intralaboratory EC1.5 CV for the LLNA: BrdU-ELISA tests of eugenol was 66% vs. 18% for the CV of EC3 values reported by ICCVAM (1999). The intralaboratory EC1.5 CV for isoeugenol was 52% vs. 26% for the CV of EC3 values from ICCVAM (1999), and the intralaboratory EC1.5 CV for hexyl cinnamic aldehyde was 37% vs. 19 to 27% for the CV reported by ICCVAM (1999) for EC3 values.

1.2 Interlaboratory Reproducibility

The interlaboratory reproducibility of the LLNA: BrdU-ELISA was assessed using the individual animal data from the multi-laboratory validation study organized by the JSAAE (Kojima et al. 2008). The study design is described in **Section 7.2**. The LLNA: BrdU-ELISA test results from the study are amenable to interlaboratory reproducibility analyses for two endpoints: sensitizer or nonsensitizer classification and EC2 values. Analyses of interlaboratory reproducibility were performed using a concordance analysis for the

qualitative results (sensitizer vs. nonsensitizer) (**Section 1.2.1**) and a CV analysis for the quantitative results (EC1.5 values) (**Section 1.2.2**).

1.2.1 *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 ≥ 1.5 at any dose were classified as sensitizers. The qualitative (i.e., sensitizer vs. nonsensitizer) interlaboratory concordance analysis for the 10 substances tested during Phase II of the JSAAE interlaboratory validation study is shown in **Table F-6**. The qualitative comparison of LLNA: BrdU-ELISA results for nine substances tested in up to seven laboratories show that interlaboratory concordance was 100% (3/3, 6/6, or 7/7). However, one of these substances, lactic acid, was misclassified as a nonsensitizer in all three laboratories. The concordance for isopropanol, the substance that produced discordant results among laboratories, the concordance was 50% (3/6). The test of isopropanol at Laboratory 2 failed (SI = 1.09) because the concurrent positive control (SI = 1.29) failed the acceptance criterion of SI ≥ 2. The other six laboratories reported maximum SI values of 2.22, 0.98, 1.57, 0.94, 2.04, and 1.01 for isopropanol. Isopropanol produces a nonsensitizer result in the traditional LLNA.

The Validation Management Team, which evaluated the reproducibility using SI ≥ 2 to identify sensitizers, considered the interlaboratory reproducibility to be acceptable (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 LLNA concordance data for comparison with the LLNA: BrdU-ELISA concordance.

Table F-6 Qualitative Results for the Phase II Interlaboratory Validation Study on the LLNA: BrdU-ELISA[1]

Substance	Laboratory							Concordance
	1	2	3	4	5	6	7	
2,4-Dinitrochlorobenzene	+	+	+	+	+	+	+	7/7
Glutaraldehyde	+				+	+		3/3
Nickel sulfate			+	+			+	3/3
trans-Cinnamic aldehyde		+		+	+			3/3
Formaldehyde	+				+	+		3/3
Eugenol		+				+	+	3/3
Hexyl cinnamic aldehyde	+	-[3]	+	+	+	+[5]	+	6/6
Isopropanol	+	-[3]	-	+	-	+[4]	-	3/6
Lactic acid			+	+			+	3/3
Methyl salicylate	-	-	-					3/3

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

[1](+) indicates sensitizer result; (-) indicates nonsensitizer result using SI ≥ 1.5 to classify sensitizers.

[2]Test failed because concurrent positive control (SI = 1.29) failed the acceptance criterion (i.e., SI < 2). The positive control would have also failed if the acceptance criterion was SI ≥ 1.5. This isopropanol result was not included in the concordance analysis.

[3]Three mice tested at highest dose.

[4]Three mice per dose group.

1.2.2 *Interlaboratory Reproducibility – EC1.5 Values*

The SI values for each test used to calculate EC1.5 values for each sensitizer according to the methods reported in Section **1.1.3**. The EC1.5 values from each laboratory were used to calculate CV values for each substance. The resulting values are shown in **Table F-7**. CV values ranged from 31% (*trans*-cinnamic aldehyde) to 95% (glutaraldehyde). The mean CV was 63%.

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). For 2,4-dinitrochloro-benzene, the EC1.5 values from four laboratories were outside the acceptable range, and for hexyl cinnamic aldehyde, the EC1.5 values from four laboratories were outside the acceptable range. All values outside the acceptable ranges were below the low end of the range. This indicates that the discordance was due to the LLNA: BrdU-ELISA producing a more sensitive result.

198 **Table F-7 EC1.5 Values from the Phase II Interlaboratory Validation Study of the LLNA: BrdU-ELISA[1]**

Substance	Laboratory							Mean	% CV
	1	2	3	4	5	6	7		
Glutaraldehyde	0.064	NT	NT	NT	0.031	0.21	NT	0.10	95
Nickel sulfate	NT	NT	1.5	0.5	NT	NT	0.6	0.8	65
trans-Cinnamic aldehyde	NT	1.7	NT	1.0	1.8	NT	NT	1.5	31
Formaldehyde	0.3	NT	NT	NT	0.2	0.6	NT	0.3	66
Eugenol	NT	12.5	NT	NT	NT	10.5	3.5	8.8	54
2,4-Dinitro-chlorobenzene	0.058 (4.3 @ 1%)	0.010 (8.37 @1%)	0.022 (5.99 @0.3%)	0.022 (5.50 @1%)	0.0022 (18.80 @ 0.3%)	0.015 (4.83 @ 0.3%)	0.049 (12.18 @ 1%)	0.025	81
Hexyl cinnamic aldehyde	9.4 (3.4 @ 50%)	-[1] (1.83 @ 50%)	15.2 (2.87 @ 50%)	4.1 (3.34 @ 50%)	3.5 (13.5 @ 50%)	7.9[2] (3.27 @ 50%)	9.5 (3.84 @ 50%)	8.3	52

199 Note: Bolded font indicates substances recommended for assessing interlaboratory reproducibility in *Recommended Performance Standards* (ICCVAM 2009). Shading shows
200 EC1.5 values that 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-
201 dinitrochlorobenzene. Values in parentheses are highest SI values achieved.
202 Abbreviations: CV =Coefficient of variation; LLNA: BrdU-ELISA = Murine local lymph node assay (LLNA) with enzyme-linked immunosorbent assay (ELISA) detection
203 of bromodeoxyuridine (BrdU); NT = Not tested; SI = Stimulation index.
204 [1]Test failed because associated positive control failed acceptance criterion (i.e., SI < 2; vehicle control absorbance was unusually high). At SI= 1.29, the positive control
205 would have failed even if the acceptance criterion was SI ≥ 1.5. Result not included in the mean EC1.5 and CV.
206 [2]Three mice tested at highest dose.
207

The interlaboratory CV values for the LLNA: BrdU-ELISA EC1.5 values were higher than that 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 F-8**; ICCVAM 1999). Three of the same substances were evaluated in the traditional LLNA and the LLNA: BrdU-ELISA. All interlaboratory CV values for the EC1.5 from LLNA: BrdU-ELISA tests were greater than that for EC3 values from the traditional LLNA. The CV of 81% for EC1.5 values for 2,4-dinitrochlorobenzene was greater than the two CV values of 37% and 27%, calculated from five EC3 values each, reported by ICCVAM (1999). The CV of 52% for EC1.5 values for hexyl cinnamic aldehyde tested in the LLNA: BrdU-ELISA was greater than the CV for EC3 values reported by ICCVAM (1999). The CV of 54% for EC1.5 values for eugenol tested in the LLNA: BrdU-ELISA was greater than the CV of 42% for EC3 values reported by ICCVAM (1999).

Table F-8 Interlaboratory Reproducibility of the EC3 for Substances Tested in the Traditional LLNA[1]

Substance	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
SLS	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 three; LLNA = Murine local lymph node assay; SLS = Sodium lauryl sulfate.
[1]From ICCVAM (1999).

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