Non-Radioactive Murine Local Lymph Node Assay: Modified by Daicel Chemical Industries, Ltd. Based on ATP Content Test Method Protocol (LLNA: DA)

Revised Draft Background Review Document

March 2009

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Table of Contents

List of Tables

List of Figures

List of Abbreviations and Acronyms

ACD	Allergic contact dermatitis
ANOVA	Analysis of variance
AOO	Acetone: olive oil (4:1)
aq.	Aqueous
ATP	Adenosine triphosphate
BRD	Background review document
CASRN	Chemical Abstracts Service Registry Number
CPSC	U.S. Consumer Product Safety Commission
CI	Confidence interval
Conc.	Concentration
CV	Coefficient of variation
DMF	*N,N*-dimethylformamide
DMSO	Dimethyl sulfoxide
EC2	Estimated concentration needed to produce a stimulation index of two
EC2.5	Estimated concentration needed to produce a stimulation index of 2.5
EC3	Estimated concentration needed to produce a stimulation index of three
ECt	Estimated concentration needed to produce a stimulation index of a specified threshold
ECETOC	European Centre for Ecotoxicology and Toxicology of Chemicals
EPA	U.S. Environmental Protection Agency
FN	False negative
FP	False positive
GP	Guinea pig
HMT	Human maximization test
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ILS	Integrated Laboratory Systems
ISO	International Organization for Standardization
IWG	Immunotoxicity Working Group
JaCVAM	Japanese Center for the Validation of Alternative Methods
K_{ow}	Octanol-water partition coefficient
LLNA	Murine local lymph node assay
LLNA: DA	Murine LLNA modified by Daicel Chemical Industries, Ltd. based on ATP content
MEK	Methyl ethyl ketone
Min	Minimal
Mod	Moderate
Mol.	Molecular
NA	Not applicable
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

193	NT	Not tested
194	NTP	National Toxicology Program
195	OECD	Organisation for Economic Co-operation and Development
196	PBS	Phosphate buffered saline
197	Ref.	Reference
198	RLU	Relative luminescence units
199	SD	Standard deviation
200	SI	Stimulation index
201	SLS	Sodium lauryl sulfate
202	Stats.	Statistics
203	TG	Test guideline
204	Trad.	Traditional
205	U.S.	United States
206	Unk	Unknown
207	vs.	Versus

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Preface

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

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

NICEATM and ICCVAM provided the draft BRDs and draft test method recommendations to an international independent scientific peer review panel (referred to hereafter as "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

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

recommended that NICEATM obtain additional existing data that was not available to the Panel and reanalyze the performance of each non-radioactive LLNA test method. NICEATM subsequently obtained additional data and prepared revised draft BRDs. ICCVAM also prepared revised draft test method recommendations based on the revised draft BRDs. This revised draft BRD addresses the validation database for the LLNA developed by Daicel Chemical Industries, Ltd., based on adenosine triphosphate content (LLNA: DA).

The Panel will meet to consider the revised draft 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 on Alternative Toxicological Methods (i.e., the ICCVAM-NICEATM advisory committee), 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 revised draft 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 revised 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 (GP) 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.[2] 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.[3] One of the nominated activities was assessment of the validation status of non-radioactive modifications to the current version of the LLNA ([ICCVAM 1999; Dean et al. 2001] referred to hereafter as the "traditional LLNA"), which uses radioactivity to detect sensitizers. The information described in the original (i.e., January 2008) and 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 test methods, the LLNA based on adenosine triphosphate

[2] http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf.
[3] http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf.

(ATP) content in the draining auricular lymph nodes (referred to hereafter as the "LLNA: DA").

Revisions to the LLNA: DA 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: DA. Both ICCVAM and the Panel concluded that more information was needed before a recommendation on the usefulness and limitations of the LLNA: DA could be made.[4] 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 NICEATM obtain additional data in order to reanalyze the performance of the LLNA: DA. In response to this recommendation, NICEATM obtained additional LLNA: DA data from the test sponsor, which were used to update the evaluation. These data include:

- Individual animal data for the LLNA: DA intralaboratory validation study of 31 substances (Idehara et al. 2008). These data were used in the updated accuracy analyses represented in **Section 6.0**
- Individual animal data for 14 additional LLNA: DA substances tested in the intralaboratory validation study (Idehara unpublished). These data were used in the updated accuracy analyses represented in **Section 6.0**
- Individual animal data for the LLNA: DA two-phased interlaboratory validation study of 14 substances (Omori et al. 2008). These data were used in the updated accuracy analyses represented in **Section 6.0** and the additional quantitative analyses of test method reproducibility, which are detailed in **Section 7.0** of this BRD.

Test Method Protocol

The test method protocol in this revised draft BRD is the same as the test method protocol discussed in the January 2008 draft BRD. Daicel Chemical Industries, Ltd. developed the

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

LLNA: DA test method based on modifications to the traditional LLNA (Yamashita et al. 2005). While the traditional LLNA assesses cellular proliferation by measuring the incorporation of radioactivity into the DNA of dividing lymph node cells, the LLNA: DA assesses cellular proliferation by measuring increases in ATP content in the lymph node as an indicator of the cell number. In addition, the LLNA: DA also differs from the traditional LLNA in the timing and administration of the test substance. In the traditional LLNA, the test substance is applied on days 1, 2, and 3 and the auricular lymph nodes are excised on day 6. In the LLNA: DA, the test substance is applied on days 1, 2, 3, and 7 and the auricular lymph nodes are excised on day 8. Furthermore, one hour prior to each application of the test substance, 1% sodium lauryl sulfate is applied to increase absorption of the test substance through the skin. A stimulation index (SI) is used to identify a substance as a sensitizer (i.e., the ratio of the mean ATP content of the substance treatment group to the mean ATP content of the vehicle treatment group).

Validation Database

The validation database in this revised draft BRD has been updated from the January 2008 draft BRD to include 15 additional substances. The accuracy and reliability of the LLNA: DA was assessed using data submitted to NICEATM for 45 substances tested in one laboratory (Idehara et al. 2008; Idehara unpublished) and 14 substances, one not previously examined, tested in a two-phased interlaboratory validation study (17 laboratories). The reference test data for these substances were obtained from the traditional LLNA, GP skin sensitization tests, and/or human skin sensitization tests. One substance, benzocaine, yielded both positive and negative results in the traditional LLNA and therefore was not considered in the performance evaluation of the LLNA: DA. LLNA studies for another substance, toluene 2,4-diisocyanate, were not conducted according to the traditional LLNA test method protocol described (ICCVAM 1999; Dean et al. 2001). Of the remaining 44 substances with sufficient traditional LLNA data, 32 were classified by the traditional LLNA as skin sensitizers and 12 were classified as nonsensitizers.

Test Method Accuracy

The accuracy evaluation in this revised draft BRD has been updated from the January 2008 draft BRD to include the results for 15 additional substances. Other revisions include the

evaluation of multiple decision criteria compared to traditional LLNA results (SI ≥ 2.0 was further compared with GP and human outcomes) and the additional evaluation of two different criteria used simultaneously to classify sensitizers and nonsensitizers compared to traditional LLNA results. Based on the evaluation of multiple decision criteria, the optimal performance was achieved using SI ≥ 2.5 to classify sensitizers and SI ≤ 1.7 to classify nonsensitizers. When these two criteria are used, false positive results (0/12) and false negative results (0/32) are eliminated compared with the traditional LLNA. However, using these criteria, 10 substances have an SI > 1.7 and an SI < 2.5, which includes five substances that were sensitizers and five substances that were nonsensitizers in the traditional LLNA. Other available information could be used to interpret LLNA: DA results when the SI falls between 1.7 and 2.5, such as peptide reactivity. Forty percent (2/5) of the traditional LLNA sensitizers in this range had peptide reactivity data (i.e., one substance had minimal peptide reactivity and one substance had high peptide reactivity). Eighty percent (4/5) of the traditional LLNA nonsensitizers in this range had peptide reactivity data (i.e., all four substances had minimal peptide reactivity).

When using the decision criterion of SI ≥ 2.5 to classify sensitizers versus nonsensitizers, compared to the traditional LLNA, accuracy was 91% (40/44), with a false positive rate of 0% (0/12), and a false negative rate of 13% (4/32). Among the discordant substances, no unique characteristics were identified that could be used as rationale for excluding any particular types of substances from testing in the LLNA: DA.

Test Method Reliability – Intralaboratory Reproducibility

The intralaboratory evaluation in this revised draft BRD has been updated from the January 2008 draft BRD to include, in addition to SI ≥ 3.0, an evaluation of SI ≥ 2.5 for the same substances. Intralaboratory reproducibility for the LLNA: DA was assessed using data for two substances (isoeugenol and eugenol) that were tested at varying concentrations in three different experiments. The EC3 (estimated concentration needed to produce an SI of three) coefficient of variation (CV) for the reproducibility of isoeugenol and eugenol was 21% and 11%, respectively. The EC2.5 (estimated concentration needed to produce an SI of 2.5) CV for the reproducibility of isoeugenol and eugenol was 33% and 13%, respectively.

Test Method Reliability – Interlaboratory Reproducibility

The interlaboratory reproducibility evaluation in this revised draft BRD is a new addition because interlaboratory data were not available for evaluation in the January 2008 draft BRD. This revised draft BRD also includes a reproducibility analysis using separate SI criteria to identify sensitizers and nonsensitizers. The two-phased multilaboratory validation study included 17 different laboratories in which 14 different substances were examined. In the first phase of the study, 10 laboratories each tested up to 12 substances, while in the second phase of the study seven laboratories (different from the 10 laboratories in the first phase of the interlaboratory validation study) each tested up to five substances. In both studies, each substance was tested once at three different doses, which were provided to the participating laboratories by the validation study management team.

When using SI $\geq$ 2.5 as the decision criterion, the qualitative (positive/negative) interlaboratory concordance analysis for the 12 substances that were tested in up to 10 laboratories during the first phase of the LLNA: DA interlaboratory validation study resulted in 100% (3/3 or 10/10) concordance for 10 substances (i.e., seven sensitizers and three nonsensitizers in the traditional LLNA) and 67% (2/3) concordance for two substances (i.e., two sensitizers in the traditional LLNA). The CV values for the EC2.5 ranged from 26% (i.e., hexyl cinnamic aldehyde) to 133% (i.e., cobalt chloride) and the mean CV was 79%. The qualitative interlaboratory concordance analysis for the five substances tested in up to seven laboratories during the second phase of the validation study resulted in 100% (4/4 or 7/7) concordance for four substances (i.e., three sensitizers and one nonsensitizer in the traditional LLNA) and 75% (3/4) concordance for one substance (i.e., a sensitizer in the traditional LLNA). The CV values for the EC2.5 ranged from 20% (i.e., hexyl cinnamic aldehyde) to 92% (i.e., cobalt chloride) and the mean CV was 62%.

When using SI $\geq$ 2.5 to classify sensitizers and SI $\leq$ 1.7 to classify nonsensitizers, the concordance analysis for the 14 substances with multiple tests indicated that the SI results for 87% (27/31) of the tests that yielded SI $\leq$ 1.7 were for substances that were classified as nonsensitizers by the traditional LLNA; 13% (4/31) of the tests that yielded SI $\leq$ 1.7 were for substances that were classified as sensitizers by the traditional LLNA. Fifty-eight percent (7/12) of the tests that yielded 1.7 < SI < 2.5 were for substances that were classified as sensitizers by the traditional LLNA.

Animal Welfare Considerations

The animal welfare considerations in this revised draft BRD have not changed from the January 2008 draft BRD. The LLNA: DA will use the same number of animals when compared to the updated ICCVAM-recommended LLNA protocol (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: DA 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 revised draft BRD have not changed from the January 2008 draft BRD. The transferability of the LLNA: DA is expected to be similar to the traditional LLNA. Notably, the test method developer indicates that when the LLNA: DA test method is conducted, all the procedural steps from lymph node excision to the determination of ATP content should be performed without delay since ATP content decreases over time (Idehara et al. 2008; Omori et al. 2008). Compared to the traditional LLNA, the LLNA: DA will not require laboratories, equipment, and licensing permits for handling radioactive materials. The level of training and expertise needed to conduct the LLNA: DA should be similar to the traditional LLNA except that the understanding and practice of luciferase methodology is required.

ICCVAM Revised Draft Test Method Recommendations

ICCVAM developed revised draft test method recommendations for the LLNA: DA based on the new data and analyses. Test method recommendations are provided for test method usefulness and limitations, test method protocol, and future studies, in order 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: Modified by Daicel Chemical Industries, Ltd. Based on ATP Content Test Method Protocol.*

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.[5] ACD develops in two phases, induction and elicitation. The induction phase occurs when a susceptible individual is exposed topically to a skin-sensitizing substance. 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 antigen-specific T-lymphocytes are then activated, which causes release of cytokines and other inflammatory mediators. This release produces a rapid dermal immune response that can lead to ACD (ICCVAM 1999; Basketter et al. 2003; Jowsey et al. 2006).

1.2 Historical Background for the Murine Local Lymph Node Assay

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended that the murine local lymph node assay (LLNA) is a valid substitute for currently accepted guinea pig (GP) test methods to assess the ACD potential of many, but not all, types of substances. The recommendation was based on a comprehensive evaluation that included an independent scientific peer review panel (Panel) assessment of the validation status of the LLNA. The Panel report and the ICCVAM recommendations (ICCVAM 1999) are available at the National Toxicology Program (NTP) Interagency

[5] http://www.bls.gov/.

Center for the Evaluation of Alternative Toxicological Methods (NICEATM)-ICCVAM website.[6] 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 [TG] 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.[7] One of the nominated activities was an assessment of the validation status of non-radioactive modifications to the current version of the LLNA ([ICCVAM 1999; Dean et al. 2001] referred to hereafter as the "traditional LLNA"), which uses radioactivity to detect sensitizers. The information described in this draft background review document (BRD) was compiled by ICCVAM and NICEATM in response to this nomination. The draft BRD provides a comprehensive review of available data and information regarding the usefulness and limitations of one of these test methods, the LLNA based on adenosine triphosphate (ATP) content in the draining auricular lymph nodes (referred to hereafter as the "LLNA: DA"). Further, ICCVAM and its IWG developed draft test method recommendations based on this evaluation.

A Panel reviewed the original draft BRD in March 2008 to evaluate the extent to which the information contained in the draft BRD supported the draft test method recommendations. The Panel concluded that additional information was needed to evaluate the test method, including a detailed test method protocol, quantitative data for the test method, and an evaluation of interlaboratory reproducibility. In response to this recommendation, NICEATM

[6] http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf.
[7] http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf.

obtained additional LLNA: DA data and information, which were used in this revised draft BRD for review by the Panel. These data and information include:

- A detailed description of the standard operating procedure of the LLNA: DA test method used for the two-phased interlaboratory validation study (see **Appendix A**)
- Individual animal data for the LLNA: DA intralaboratory validation study of 31 substances (Idehara et al. 2008). These data were used in the updated accuracy analyses represented in **Section 6.0**
- Data for 14 additional LLNA: DA intralaboratory substances (Idehara unpublished). These data were used in the updated accuracy analyses represented in **Section 6.0**
- Individual animal data for the LLNA: DA two-phased interlaboratory validation study of 14 substances (Omori et al. 2008). These data were used in the updated accuracy analyses represented in **Section 6.0** and the additional quantitative analyses of test method reproducibility, which are detailed in **Section 7.0** of this BRD.

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

1.3 The LLNA: DA

The LLNA: DA was developed by Daicel Chemical Industries, Ltd. as a non-radioactive modification (Yamashita et al. 2005) to the current version of the LLNA. The traditional LLNA assesses cellular proliferation by measuring the incorporation of radioactive thymidine or iodine into the DNA of dividing lymph node cells. In contrast, the LLNA: DA assesses ATP content in the lymph node by employing a luciferin-luciferase assay to measure bioluminescence. Since ATP content is linearly related to living cell number, this measurement serves as a surrogate for cell number at the time of sampling.

This document provides:

- A comprehensive summary of the LLNA: DA 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: DA Test Method Protocol

The test method protocol in this revised draft BRD is the same as the test method protocol discussed in the January 2008 draft BRD. Notably, this revised draft BRD now includes a detailed standard operating procedure for the LLNA: DA test method and supplemental data evaluating the effect of 1% sodium lauryl sulfate (SLS) pre-treatment on lymph node proliferation that was not available for inclusion in the January 2008 draft BRD (**Appendix A**). The LLNA: DA test method protocol (**Appendix A**) differs from the ICCVAM-recommended test method protocol for the traditional LLNA (ICCVAM 2009) in the method used to assess lymphocyte proliferation in the auricular lymph nodes (**Table 2-1**). In addition, there are substantive differences between the two test method protocols regarding test substance application and timing for the collection of the lymph nodes. In the traditional LLNA, the test substance is administered on three consecutive days (days 1, 2, and 3). On day 6, radiolabeled thymidine or iodine is administered via the tail vein and the lymph nodes are excised five hours later. A lymph node cell suspension is then prepared and radioactive thymidine or iodine incorporation is determined by β-scintillation or γ-scintillation counting, respectively. In the LLNA: DA, the test substance is applied on days 1, 2, 3, and additionally on day 7. During the initial development of the LLNA: DA, the study group (Yamashita et al. 2005) determined the optimal dosing schedule by evaluating whether the addition of a fourth application (day 7) was useful for increasing lymph node proliferation. Based on a statistically significant increase in lymph node weight-based stimulation indexes (SIs) for mice that received a fourth application (day 7) of the test substance, this test method protocol was chosen. Furthermore, one hour prior to each application of the test substance, a solution of 1% SLS is applied to the dorsum of the treated ears to increase absorption of the test

substance across the skin (van Och et al. 2000). Various researchers have shown that a solution of 1% SLS does not elicit a positive response in the traditional LLNA but when applied prior to test substance administration there is generally an increased response compared to the test substance alone (van Och et al. 2000; De Jong et al. 2002). Similar results were observed by Idehara et al. (2008) (see also **Appendix A**). Lastly, twenty-four to 30 hours after the last test substance application (day 7), the auricular lymph nodes are excised and a lymph node cell suspension is prepared, and the ATP content is measured by luciferin-luciferase assay.

538 **Table 2-1 Comparison of the LLNA: DA and Traditional LLNA Experimental**
539 **Procedure**

	Days 1, 2, & 3	Days 4 & 5	Day 6	Day 7	Day 8
LLNA: DA	• Pretreat with 1% SLS solution • After one hour, apply 25 µL of test substance or vehicle to dorsum of each ear	No Treatment	No Treatment	• Pretreat with 1% SLS solution • After one hour, apply 25 µL of test substance or vehicle to dorsum of each ear	• Excision of auricular lymph nodes • Measurement of ATP content in lymph node cells
Traditional LLNA	• Apply 25 µL of test substance or vehicle to dorsum of each ear	No Treatment	• Administer ^{3}H-thymidine or ^{125}I via tail vein • Excision of auricular lymph nodes • Measurement of radioactivity incorporated into lymph node cells	No Treatment	No Treatment

540 Abbreviations: ATP = adenosine triphosphate; ^{3}H = tritiated; ^{125}I = iodine-125; LLNA = murine local lymph node assay;
541 LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content; SLS =
542 sodium lauryl sulfate.
543

544 ## 2.1. Decision Criteria

545 Similar to the traditional LLNA, an SI is used in the LLNA: DA to distinguish skin
546 sensitizers from nonsensitizers. The formula for calculating the SI in the LLNA: DA is the
547 ratio of the mean ATP content of the auricular lymph nodes collected from the test substance
548 treatment group to the mean ATP content of the auricular lymph nodes collected from the
549 vehicle treatment group (measured in relative luminescence units; RLU)

550 $$SI = \frac{mean\ ATP\ content\ of\ \ auricular\ lymph\ nodes\ in\ test\ treatment\ group\ (RLU)}{mean\ ATP\ content\ of\ \ auricular\ lymph\ nodes\ in\ vehicle\ treatment\ group\ (RLU)}$$

551 In the intra- and interlaboratory validation studies for the LLNA: DA, an SI $\geq$ 3.0 was used
552 as the threshold for labeling a substance as a sensitizer, which is the same threshold used in
553 the traditional LLNA. As noted in **Section 6.0**, alternative decision criteria are evaluated in
554 this revised draft BRD to determine the threshold that provides optimum performance.

555

3.0 LLNA: DA Validation Database

The validation database in this revised draft BRD has been updated from the January 2008 draft BRD to include 15 additional substances. To evaluate the usefulness and limitations of the LLNA: DA, Daicel Chemical Industries, Ltd., tested a total of 45 substances in one laboratory (Idehara et al. 2008; Idehara unpublished data). They further evaluated two of the 45 substances (i.e., isoeugenol and eugenol) in the LLNA: DA at varying concentrations in three different experiments in order to assess intralaboratory reproducibility. In addition, a two-phased interlaboratory validation study evaluated the reproducibility of the LLNA: DA (**Section 7.0**). In the first phase, 10 laboratories tested 12 coded substances (**Table 7-2**) and in the second phase, seven different laboratories tested five coded substances (**Table 7-3**). Between the 17 laboratories, 14 different substances were examined and one of those substances, 3-aminophenol, was not previously tested among the 45 substances in the intralaboratory validation study.

Taken together, all 46 substances tested in the LLNA: DA were previously tested in the traditional LLNA and data for 39 of the substances were considered in the original ICCVAM evaluation (ICCVAM 1999). Cinnamic alcohol, diethyl maleate, diethyl phthalate, ethyl acrylate, glutaraldehyde, methyl methacrylate, and toluene 2,4-diisocyanate were the seven substances tested in the LLNA: DA not evaluated in the ICCVAM 1999 report. Of the 46 substances tested in the LLNA: DA, 33 were classified by the traditional LLNA as skin sensitizers,[8]12 were classified as nonsensitizers, and one (i.e., benzocaine) was classified as equivocal due to highly variable results and therefore was not included in the performance analyses (ICCVAM 1999)[9] (**Table 3-1**). For the sensitizers in the traditional LLNA, the range of traditional LLNA EC3 values (estimated concentrations needed to produce a stimulation index of three) was from 0.009% to 90% (**Table 3-1**). Similar to benzocaine, traditional LLNA data for toluene 2,4-diisocyanate, not evaluated in the original ICCVAM 1999 report, were not suitable for comparison. The LLNA test method protocol followed for the study that tested toluene 2,4-diisocyanate (van Och et al. 2000) was a modified version of

[8] Resorcinol was classified as a nonsensitizer based on original LLNA data (ICCVAM 1999) but recent LLNA data have instead suggested that it is actually a sensitizer (Basketter et al. 2007) and is therefore classified as a sensitizer for this evaluation.

[9] A series of 12 tests conducted in two laboratories resulted in some positive results that were not reproducible (Basketter et al. 1995).

582 the traditional LLNA which was not performed in accordance with OECD TG 429 (OECD
583 2002) or ICCVAM 1999 and Dean et al. 2001. One variation was that the BALB/c strain of
584 mouse was used for the experiments, and not the CBA/Ca or CBA/J strains as specified by
585 ICCVAM (1999), Dean et al. (2001) or OECD TG 429 (2002). In addition, the ears of the
586 mice were pretreated with a solution of 1% SLS before treatment with the test substance. The
587 authors also stated that the auricular lymph nodes were excised and pooled for each animal.
588 Thus, of the 46 substances with LLNA: DA data and traditional LLNA data, 44 were
589 included in the accuracy analyses described in **Section 6.0**.

590 **Appendix B** provides information on the physico-chemical properties (e.g., physical form),
591 Chemical Abstracts Service Registry Number (CASRN), and chemical class for each
592 substance tested. When available, chemical classes for each substance were retrieved from
593 the National Library of Medicine's ChemID Plus database. If chemical classes were not
594 located, they were assigned for each test substance using a standard classification scheme,
595 based on the National Library of Medicine Medical Subject Headings classification system.[10]
596 A substance could be assigned to more than one chemical class; however, no substance was
597 assigned to more than three classes. Classification of substances into chemical classes is not
598 intended to indicate the impact of structure on biological activity with respect to sensitization
599 potential. Instead, chemical class information is being presented to provide an indication of
600 the variety of structural elements that are present in the substances that were evaluated in this
601 analysis.

602

[10] http://www.nlm.nih.gov/mesh/meshhome.html.

602 **Table 3-1 Traditional LLNA EC3 Values and Chemical Classifications of**
603 **Substances Tested in the LLNA: DA**

Substance Name	Chemical Class[1]	Traditional LLNA EC3 (%)[2]	No.[3]
5-Chloro-2-methyl-4-isothiazolin-3-one[b]	Sulfur Compounds; Heterocyclic Compounds	0.009	1
p-Benzoquinone[b]	Quinones	0.010	1
2,4-Dinitrochlorobenzene[a, c]	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated; Nitro Compounds	0.049	15
Benzalkonium chloride[a]	Amines; Onium Compounds	0.070[4]	1
Glutaraldehyde[a, c]	Aldehydes	0.080	3
p-Phenylenediamine[a]	Amines	0.110	6
Toluene 2,4-diisocyanate[5, a]	Hydrocarbons, Cyclic; Isocyanates	0.110	1
Potassium dichromate[a, d]	Inorganic Chemical, Chromium Compounds; Inorganic Chemical, Potassium Compounds	0.170	12
Propyl gallate[b]	Carboxylic Acids	0.320	1
Phthalic anhydride[a]	Anhydrides; Carboxylic Acids	0.360	1
Formaldehyde[a, c]	Aldehydes	0.500	4
Cobalt chloride[a, c, d]	Inorganic Chemical, Elements; Inorganic Chemical, Metals	0.600	2
Isoeugenol[a, c]	Carboxylic Acids	1.540	47
2-Mercaptobenzothiazole[a]	Heterocyclic Compounds	1.700	1
Cinnamic aldehyde[a]	Aldehydes	1.910	6
3-Aminophenol[c]	Amines; Phenols	3.200	1
Benzocaine[a]	Carboxylic Acids	3.400[6]	1
Diethyl maleate[b]	Carboxylic Acids	3.600	4
Trimellitic anhydride[a]	Anhydride; Carboxylic Acids	4.710	2
Nickel (II) sulfate hexahydrate[a, c, d]	Inorganic Chemical, Elements; Inorganic Chemical, Metals	4.800	1
Resorcinol[a]	Phenols	6.330	1
Sodium lauryl sulfate[a]	Alcohols; Sulfur Compounds; Lipids	8.080	5
Citral[a]	Hydrocarbons, Other	9.170	6
Hexyl cinnamic aldehyde[a, c, d]	Aldehydes	9.740	21
Eugenol[a]	Carboxylic Acids	10.090	11
Abietic acid[a, c]	Hydrocarbons, Cyclic; Polycyclic Compounds	11.920	5
Phenyl benzoate[b]	Carboxylic Acids	13.600	3
Cinnamic alcohol[b]	Alcohols	21.000	1
Hydroxycitronellal[a]	Hydrocarbons, Other	23.750	6
Imidazolidinyl urea[a]	Urea	24.000	1
Ethylene glycol dimethacrylate[b]	Carboxylic Acids	28.000	1
Butyl glycidyl ether[b]	Ethers	30.900	1
Ethyl acrylate[b]	Carboxylic Acids	32.800	2
Methyl methacrylate[b]	Carboxylic Acids	90.000	1
1-Bromobutane[a]	Hydrocarbons, Halogenated	NA	1
Chlorobenzene[a]	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated	NA	1
Diethyl phthalate[a]	Carboxylic Acids	NA	1
Dimethyl isophthalate[b, c]	Carboxylic Acids	NA	1
Hexane[a]	Hydrocarbons, Acyclic	NA	1
Isopropanol[a, c]	Alcohols	NA	1
Lactic acid[a, d]	Carboxylic Acids	NA	1

Substance Name	Chemical Class[1]	Traditional LLNA EC3 (%)[2]	No.[3]
Methyl salicylate[a, c]	Carboxylic Acids; Phenols	NA	9
Propylparaben[a]	Carboxylic Acids; Phenols	NA	1
Nickel (II) chloride[b]	Inorganic Chemical, Elements; Inorganic Chemical, Metals	NA	2
Salicylic acid[b]	Phenols; Carboxylic Acids	NA	1
Sulfanilamide[b]	Hydrocarbons, Cyclic; Sulfur Compounds	NA	1

Abbreviations: EC3 = estimated concentration needed to produce a stimulation index of three; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content; NA = not applicable; No. = number.

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

[2]The traditional LLNA EC3 (stimulation index needed to produce a threshold of three) listed for each substance is from traditional LLNA studies that used the same vehicle as the LLNA: DA (**Appendix D)**, except where noted.

[3]Number of traditional LLNA studies from which the data were obtained.

[4]Benzalkonium chloride was tested in the LLNA: DA using acetone: olive oil (4:1) as the vehicle (**Appendix D**) but is classified as a sensitizer in the traditional LLNA based on results using acetone as the vehicle.

[5]Not included in accuracy analyses. Comparable LLNA reference data from modified LLNA test (van Och et al. 2000).

[6]Not included in accuracy analyses. EC3 value reported in **Table 3-1** for benzocaine is based on data from the NICEATM database but variable and equivocal responses were reported by Basketter et al. (1995) and in the 1999 ICCVAM report.

[a]Substance tested in intralaboratory validation study (Idehara et al. 2008).

[b]Substance tested in intralaboratory validation study (Idehara unpublished data).

[c]Substance tested in phase one of two-phased interlaboratory validation study (Omori et al. 2008).

[d]Substance tested in phase two of two-phased interlaboratory validation study (Omori et al. 2008).

4.0 Reference Data

As mentioned in **Section 3.0**, 44 of the 46 substances tested in the LLNA: DA are included in the accuracy analyses described in **Section 6.0.** The traditional LLNA reference data used for the accuracy analyses comparisons are from ICCVAM (1999) (**Appendix C**) for 11 of those 44 substances. The traditional LLNA reference data for the remaining substances (i.e., benzalkonium chloride, cinnamic alcohol, diethyl maleate, diethyl phthalate, ethyl acrylate, formaldehyde, glutaraldehyde, imidazolidinyl urea, methyl methacrylate, and nickel [II] sulfate hexahydrate) were obtained from other sources (**Appendix C**) (Gerberick et al. 1992; Hilton et al. 1998; Ryan et al. 2002; Basketter et al. 2005; Gerberick et al. 2005; Betts et al. 2006). In addition, Basketter et al. (2007) reassessed the skin sensitization potential of resorcinol in the LLNA, in accordance with OECD TG 429 (2002), which updates information in the ICCVAM 1999 report and from Gerberick et al. (2005) that had previously stated that this substance tested negative in the LLNA.

The reference data for the GP tests (guinea pig maximization test or Buehler test) and human tests (human maximization test, human patch test allergen, or other human data) were obtained from Vandenberg and Epstein (1963), Kligman (1966), Marzulli and Maibach (1974), Jordan and King (1977), Klecak et al. (1977), Marzulli and Maibach (1980), Van der Walle et al. (1982), Gad et al. (1986), Robinson et al. (1990), Gerberick et al. (1992), ICCVAM (1999), Basketter et al. (1999, 2001, 2005, 2007), Kwon et al. (2003), Schneider and Akkan (2004), or Betts et al. (2006).

An independent quality assurance contractor for the NTP audited the traditional LLNA data provided in the ICCVAM 1999 report. 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 LLNA: DA Test Method Data and Results

The test method data in this revised draft BRD has been updated from the January 2008 draft BRD to include the individual animal data for all the LLNA: DA results evaluated in this BRD that are from published studies (Idehara et al. 2008; Omori et al. 2008). **Appendix C** represents a summary of substances for which there are LLNA: DA data. Forty-five of the substances are from an intralaboratory validation study (Idehara et al. 2008; Idehara unpublished data). In addition, 14 substances evaluated in an independent two-phased interlaboratory validation study are included (Omori et al. 2008). One of the 14 substances (3-aminophenol) was not assessed among the 45 substances evaluated in the intralaboratory validation study. Taking these studies together, **Appendix C** contains information for 46 different substances, all with available LLNA: DA and traditional LLNA data, although sufficient comparative LLNA data is only available for 44 of the 46 substances (**Section 3.0**). In addition, 42 of the 46 substances examined in the LLNA: DA have GP data and 43 of the 46 substances tested have human skin sensitization data. Based on Idehara et al. (2008, unpublished data), the 45 substances tested in the intralaboratory study were not coded prior to testing. However, the two-phased interlaboratory validation study used coded substances (Omori et al. 2008). Original data for these studies have been received.

6.0 LLNA: DA Test Method Accuracy

The accuracy evaluation in this revised draft BRD has been updated from the January 2008 draft BRD to include the results for 15 additional substances. Other revisions include the evaluation of multiple decision criteria of which SI ≥ 2.0 was chosen, based on performance in the LLNA: DA, to be further analyzed and the additional 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 test method when compared to the current reference test method (ICCVAM 2003). Additional comparisons should also be made against any available human data or experience from testing or accidental exposures. This aspect of assay performance is typically evaluated by calculating:

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

6.1 LLNA: DA Database Used for the Accuracy Analysis

An accuracy analysis for the LLNA: DA test method was conducted using data from the intralaboratory validation study and the two-phased interlaboratory validation study. Taken together, LLNA: DA test data were available for 46 different substances, 44 of which had sufficient comparative LLNA: DA and traditional LLNA data to conduct an accuracy analysis (**Section 3.0**). Thus, of the 44 substances included in the accuracy analysis, 40 had available LLNA: DA, traditional LLNA, and GP data and 41 had available LLNA: DA,

traditional LLNA, and human data. Classification of substances and data available for each substance are provided in **Appendix C**.

Multiple LLNA: DA tests were available for 14 substances tested in the intralaboratory (Idehara et al. 2008; Idehara unpublished data) and the two-phased interlaboratory LLNA: DA studies (Omori et al. 2008). For the accuracy analysis, the test results were combined so that each substance was represented by one overall result for the SI analyzed and represented the outcome that was most prevalent. For example, when using SI ≥ 3.0 as the decision criterion, cobalt chloride was positive because five of the eight LLNA: DA results were positive (**Appendix D**).

6.2 Accuracy Analysis Using the SI ≥ 3.0 Decision Criterion

The performance characteristics of the LLNA: DA test method were first evaluated using the decision criterion of SI ≥ 3.0 to identify sensitizers, which was the threshold for a positive response used in both the intralaboratory and two-phased interlaboratory validation studies (**Appendix A**).

6.2.1 Accuracy vs. the Traditional LLNA

Based on the available data (i.e., 44 substances), when compared to the traditional LLNA, the LLNA: DA had an accuracy of 91% (40/44), a sensitivity of 88% (28/32), a specificity of 100% (12/12), a false positive rate of 0% (0/12), and a false negative rate of 13% (4/32) (**Table 6-1**).

6.2.2 Accuracy vs. Guinea Pig Data

When the accuracy statistics for the LLNA: DA and the traditional LLNA were compared for substances with available LLNA: DA, traditional LLNA, and GP data, and GP results served as the reference data, the LLNA: DA had a lower accuracy (78% [31/40] vs. 85% [34/40]), sensitivity (85% [22/26] vs. 96% [25/26]), the same specificity (64% [9/14]) and false positive rate (36% [5/14]), and higher false negative rate (15% [4/26] vs. 4% [1/26]) relative to the traditional LLNA (**Table 6-1**).

6.2.3 Accuracy vs. Human Data

When substances with only comparative LLNA: DA, traditional LLNA, and human data were evaluated, and human outcomes served as the reference point, the LLNA: DA had

721 lower accuracy (78% [32/41] vs. 88% [36/41]) and sensitivity (76% [26/34] vs. 88%
722 [30/34]), the same specificity (86% [6/7]) and false positive rate (14% [1/7]), and higher false
723 negative rate (24% [8/34] vs. 12% [4/34]) relative to the traditional LLNA (**Table 6-1**).

Table 6-1 Performance of the LLNA: DA in Predicting Skin Sensitization Potential Using Decision Criterion of SI ≥ 3.0 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]
LLNA: DA vs. Traditional LLNA	44	91	40/44	88	28/32	100	12/12	0	0/12	13	4/32	100	28/28	75	12/16
Substances *with LLNA: DA, Traditional LLNA, and GP Data*															
LLNA: DA vs. Traditional LLNA	40	93	37/40	90	27/30	100	10/10	0	0/10	10	3/30	100	27/27	77	10/13
LLNA: DA vs. GP[3]	40	78	31/40	85	22/26	64	9/14	36	5/14	15	4/26	81	22/27	69	9/13
Traditional LLNA vs. GP[3]	40	85	34/40	96	25/26	64	9/14	36	5/14	4	1/26	83	25/30	90	9/10
Substances *with LLNA: DA, Traditional LLNA, and Human Data*															
LLNA: DA vs. Traditional LLNA	41	90	37/41	87	27/31	100	10/10	0	0/10	13	4/31	100	27/27	71	10/14
LLNA: DA vs. Human[4]	41	78	32/41	76	26/34	86	6/7	14	1/7	24	8/34	96	26/27	43	6/14
Traditional LLNA vs. Human[4]	41	88	36/41	88	30/34	86	6/7	14	1/7	12	4/34	97	30/31	60	6/10

Abbreviations: GP = guinea pig; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content; No. = number; vs. = versus.

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

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

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

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

6.3 Accuracy Analysis (SI ≥ 3.0) Based on ICCVAM-recommended LLNA Performance Standards Reference Substances

ICCVAM has developed recommended test method performance standards for the traditional LLNA (ICCVAM 2009),[11] which are proposed to evaluate the performance of modified LLNA test methods that are mechanistically and functionally similar to the traditional LLNA. Since the validation studies for the LLNA: DA test method were completed prior to the development of LLNA performance standards, the LLNA: DA is not being evaluated using the ICCVAM-recommended LLNA performance standards. Thus, evaluations of the LLNA: DA 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. In addition, the ICCVAM-recommended LLNA performance standards are not applicable to the LLNA: DA test method due to two main differences between the LLNA: DA and traditional LLNA test method protocols (i.e., 1% SLS pre-treatment prior to test substance application and an additional test substance application on day 7) (**Section 2.0**).

As shown in **Table 6-2**, all of the 18 required reference substances and three of the four optional reference substances included in the ICCVAM-recommended LLNA performance standards have been tested in the LLNA: DA. When compared to the traditional LLNA, the LLNA: DA at SI ≥ 3.0 predicted the same sensitization classification for 16 of the 18 required ICCVAM-recommended reference substances tested. One discordant substance, 2-mercaptobenzothiazole, was classified as a sensitizer based on traditional LLNA results (i.e., EC3 of 1.7%) but as a nonsensitizer based on LLNA: DA data. As indicated in **Table 6-2**, *N,N*-dimethylformamide (DMF) was the vehicle used in both the traditional LLNA and the LLNA: DA tests for 2-mercaptobenzothiazole. The positive result for 2-mercaptobenzothiazole reported in the ICCVAM LLNA performance standards was based on one LLNA experiment that tested the substance at 1%, 3%, and 10% (Gerberick et al. 2005). By comparison, the negative result for 2-mercaptobenzothiazole obtained with the LLNA: DA test method was based on one LLNA: DA experiment that tested the substance at 10%,

[11] http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm.

25%, and 50% (Idehara et al. 2008). The highest dose tested for 2-mercaptobenzothiazole in the traditional LLNA was the lowest dose tested in the LLNA: DA (i.e., 10%) and resulted in an SI of 8.6 versus 2.0, respectively.

Notably, a review of the original LLNA: DA laboratory records for 2-mercaptobenzothiazole indicated that the concurrent positive control (i.e., 10% eugenol in DMF) failed to yield an SI ≥ 3.0. Consequently the test method developers should have repeated the test for 2-mercaptobenzothiazole to ensure that the result obtained was correctly classified as negative and not the result of a failed experiment. This could explain the discordant result obtained between the traditional LLNA and the LLNA: DA test method for this test substance.

The second discordant substance, methyl methacrylate, was classified as a sensitizer based on traditional LLNA results (i.e., EC3 of 90%) but as a nonsensitizer based on LLNA: DA data. As indicated in **Table 6-2**, acetone: olive oil (4:1; AOO) was the vehicle used in both the traditional LLNA and the LLNA: DA tests for methyl methacrylate. The positive result for methyl methacrylate reported in the ICCVAM LLNA performance standards was based on one LLNA experiment that tested the substance at 10%, 30%, 50%, and 100% (Betts et al. 2006). By comparison, the negative result for 2-mercaptobenzothiazole obtained with the LLNA: DA test method was based on one LLNA: DA experiment that tested the substance at 25%, 50%, 75%, and 100% (Idehara, unpublished data). The highest dose tested for 2-mercaptobenzothiazole in the traditional LLNA was the same in the LLNA: DA (i.e., 100%) and resulted in an SI of 3.6 versus 1.8, respectively.

As shown in **Table 6-2**, when compared to the traditional LLNA, the LLNA: DA at SI ≥ 3.0 predicted the same sensitization for all three of the optional reference substances tested. The optional reference substances, SLS and ethylene glycol dimethacrylate, were categorized as nonsensitizers based on GP and human data but as sensitizers by the LLNA: DA. Thus, similar to the traditional LLNA, these substances were false positive in the LLNA: DA. SLS was tested in the same vehicle (i.e., DMF) in both the traditional LLNA and the LLNA: DA. In addition, the positive results for SLS reported in the ICCVAM LLNA performance standards were based on five LLNA studies that tested SLS at 1%, 2.5%, 5%, 10%, and 20% (Loveless et al. 1996). In comparison, the positive result for SLS obtained with the LLNA: DA test method was based on one LLNA: DA experiment that tested the substance at 1%,

2.5%, 5%, and 10% (Idehara et al. 2008). The EC3 values for SLS in the traditional LLNA (i.e., 8.1%) and the LLNA: DA (6.9%) were comparable. In addition, ethylene glycol dimethacrylate was tested in the same vehicle (i.e., methyl ethyl ketone) in both the traditional LLNA and the LLNA: DA. The positive result for ethylene glycol dimethacrylate reported in the ICCVAM LLNA performance standards was based on one LLNA study that tested ethylene glycol dimethacrylate at 10%, 25%, and 50% (Gerberick et al. 2005). In comparison, the positive result for ethylene glycol dimethacrylate obtained with the LLNA: DA test method was based on one LLNA: DA experiment that also tested the substance at 10%, 25%, and 50% (Idehara, unpublished data). The EC3 values for ethylene glycol dimethacrylate in the traditional LLNA (i.e., 28%) and the LLNA: DA (34%) were comparable.

Lastly, the optional reference substance, nickel (II) chloride, was categorized as a sensitizer based on GP and human data but as a nonsensitizer by the LLNA: DA. Thus, similar to the traditional LLNA, this substance was false negative in the LLNA: DA. Nickel (II) chloride was tested in the same vehicle (i.e., dimethyl sulfoxide [DMSO]) in both the traditional LLNA and the LLNA: DA. In addition, the negative results for nickel (II) chloride reported in the ICCVAM LLNA performance standards were based on two independent LLNA studies that tested the substance at 0.5%, 1%, and 2.5% (Basketter et al. 1999) and at 1%, 2.5%, and 5% (Basketter and Scholes 1992). In comparison, the negative result for nickel (II) chloride obtained with the LLNA: DA test method was based on one LLNA: DA experiment that tested the substance at 2.5%, 5%, and 10% (Idehara, unpublished data). The highest dose tested for nickel (II) chloride in the traditional LLNA was the same in the LLNA: DA (i.e., 5%) and resulted in an SI of 2.4 versus 1.3, respectively.

Table 6-2 Performance of the LLNA: DA (SI ≥ 3.0) Compared to the ICCVAM-recommended LLNA Performance Standards Reference Substances[1] (Sorted by Traditional LLNA EC3 Value)

Substance	ICCVAM-Recommended LLNA Performance Standards				LLNA: DA[2]			
	Vehicle	Result	EC3 (%)[3]	N[4]	Vehicle	Result	EC3 (%)[3]	N[4]
5-Chloro-2-methyl-4-isothiazolin-3-one	DMF	+	0.009	1	DMF	+	0.03	1
2,4-Dinitrochlorobenzene	AOO	+	0.049	15	AOO	+	0.08	11
4-Phenylenediamine	AOO	+	0.11	6	AOO	+	0.07	1
Cobalt chloride	DMSO	+	0.60	2	DMSO	+	1.27	5
Isoeugenol	AOO	+	1.5	47	AOO	+	2.94	4
2-Mercaptobenzothiazole	***DMF***	**+**	***1.7***	***1***	***DMF***	**-**	***NA***	***1***
Citral	AOO	+	9.2	6	AOO	+	15.63	1
Hexyl cinnamic aldehyde	AOO	+	9.7	21	AOO	+	11.10	18
Eugenol	AOO	+	10.1	11	AOO	+	4.50	1
Phenyl benzoate	AOO	+	13.6	3	AOO	+	2.26	1
Cinnamic alcohol	AOO	+	21.0	1	AOO	+	21.34	1
Imidazolidinyl urea	DMF	+	24.0	1	DMF	+	18.77	1
Methyl methacrylate	***AOO***	**+**	***90.0***	***1***	***AOO***	**-**	***NA***	***1***
Chlorobenzene	AOO	-	NA	1	AOO	-	NA	1
Isopropanol	AOO	-	NA	1	AOO	-	NA	11
Lactic acid	DMSO	-	NA	1	DMSO	-	NA	5
Methyl salicylate	AOO	-	NA	9	AOO	-	NA	4
Salicylic acid	AOO	**-**	NA	1	AOO	-	NA	1
Sodium lauryl sulfate	DMF	FP	8.1	5	DMF	+	6.88	1
Ethylene glycol dimethylacrylate	MEK	FP	28	1	MEK	+	34.03	1
Xylene	AOO	FP	95.8	1	NT	NT	NT	NT
Nickel chloride	DMSO	FN	NA	2	DMSO	-	NA	1

Bolded and italicized text highlights discordant LLNA: DA vs. traditional LLNA test results.

Abbreviations: AOO = acetone: olive oil (4:1); DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; EC3 = estimated concentration needed to produce a stimulation index of three; 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; ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content; MEK = methyl ethyl ketone; NA = not applicable (stimulation index < 3.0); NT = not tested; SI = stimulation index.

"+" = Sensitizer.

"-" = Nonsensitizer.

[1]From *Recommended Performance Standards: Murine Local Lymph Node Assay* (ICCVAM 2009; available at: http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm. The table lists the 18 required reference substances first (sorted from lowest to highest EC3), followed by the four optional reference substances (sorted from lowest to highest EC3).

[2]Substances tested in LLNA: DA intralaboratory validation study (Idehara et al. 2008; Idehara unpublished data) and/or two-phased interlaboratory validation study (Omori et al. 2008).

[3]Based on mean EC3 when more than one value was available.

[4]Number of LLNA studies from which data were obtained.

Table 6-3 provides the range and characteristics for 44 substances tested in the LLNA: DA 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 the range of the substances tested in the LLNA: DA is similar to that included in the performance standards list. In general, there are a proportionally increased number of substances tested in the LLNA: DA in each of the categories included in the table.

Table 6-3 Characteristics of the Substances Tested in the LLNA: DA Compared to the ICCVAM-recommended LLNA Performance Standards Reference Substances[1]

EC3 (%) Range in the Traditional LLNA	No. Substances	Solid/ Liquid	Actual EC3 Range (%)[2]	Human Data	Peptide Reactivity (High/Mod/Min/Low/Unk)[3]
<0.1	**5**	**4/2[4]**	**0.009-0.080**	**5**	**4/0/0/0/1**
	2	1/1	0.009-0.049	2	2/0/0/0/0
≥0.1 to <1	**7**	**5/2**	**0.11-0.60**	**7**	**1/2/0/0/4**
	2	2/0	0.11-0.60	2	0/0/0/0/2
≥1 to <10	**12**	**7/5**	**1.54-9.74**	**11**	**4/0/3/1/4**
	4	1/3	1.54-9.74	4	2/0/1/0/1
≥10 to <100	**10**	**4/6**	**10.09-90.00**	**10**	**2/1/0/1/6**
	5	3/2	10.09-90.00	5	0/1/0/0/4
Negative	**12**	**6/6**	**NA**	**10**	**0/0/8/1/3**
	5	1/4	NA	3	0/0/2/0/3
Overall	**46**	**26/21[4]**	**0.009-90.00**	**28**	**11/3/11/3/18**
	18	10/8	0.009-90.00	16	4/1/3/0/10

Bolded text represents characteristics of the LLNA: DA database, which includes the 44 substances tested in the intralaboratory validation study (Idehara et al. 2008; Idehara unpublished) and/or the two-phased interlaboratory validation study (Omori et al. 2008).

Abbreviations: EC3 = estimated concentration needed to produce a stimulation index of three; ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP Content; NA = not applicable because maximum SI < 3.0; No. = number; Min = minimal; Mod = moderate; SI = stimulation index; Unk = unknown.

[1]From the ICCVAM-recommended performance standards for the LLNA (ICCVAM 2009), based on the 18 required reference substances.

[2]Based on traditional LLNA studies for substances tested in the LLNA: DA (bold values) and for the 18 required reference substances in the ICCVAM-recommended LLNA performance standards (ICCVAM 2009).

[3]Data obtained from Gerberick et al. 2007.

[4]One substance tested in the LLNA: DA, benzalkonium chloride, is categorized as both a solid and a liquid.

6.4 Discordant Results for Accuracy Analysis Using the SI ≥ 3.0 Decision Criterion

6.4.1 Discordance between the LLNA: DA and the Traditional LLNA

When the outcomes for the 44 substances tested in the LLNA: DA (using SI ≥ 3.0) and the traditional LLNA were compared, the classifications for four substances were different. The LLNA: DA classified 3-aminophenol, 2-mercaptobenzothiazole, methyl methacrylate, and nickel (II) sulfate hexahydrate as nonsensitizers while the traditional LLNA classified them as sensitizers (**Tables 6-4** and **6-5**). These substances were tested in the same vehicle in both the LLNA: DA and the traditional LLNA tests. One commonality noted between three of the four discordant substances is that they are solids. Furthermore, the molecular weights for 3-aminophenol and methyl methacrylate are both about 100 g/mol and those for 2-mercaptobenzothiazole and nickel (II) sulfate hexahydrate are comparable at 160 g/mol (**Appendix B**). In addition, all four discordant substances are considered nonirritants based on GP data.

6.4.2 Discordance among the LLNA: DA, the Traditional LLNA, and/or the Guinea Pig Test

When analyses were restricted to the 40 substances with unequivocal LLNA: DA, traditional LLNA, and GP data, the LLNA: DA at SI ≥ 3.0 classified three substances differently compared with the traditional LLNA (**Table 6-4**). 2-Mercaptobenzothiazole, methyl methacrylate, and nickel (II) sulfate hexahydrate were identified as nonsensitizers by the LLNA: DA while the traditional LLNA and GP tests classified these substances as sensitizers. The discordant substances were tested at the same or higher concentrations in the LLNA: DA and in the traditional LLNA yet the substances were still classified as nonsensitizers (**Table 6-4**). There are few commonalities among these substances with regard to chemical class, physical form, molecular weight, peptide reactivity (see **Appendix B** for physico-chemical information), EC3 range (based on traditional LLNA, see **Table 3-1**) and potential for skin irritation (**Appendix C**) as follows:

- 2-Mercaptobenzothiazole is a heterocyclic compound, methyl methacrylate is carboxylic acid, and nickel (II) sulfate hexahydrate is a metal
- 2-Mercaptobenzothiazole and nickel (II) sulfate hexahydrate exist as solids and methyl methacrylate exists as a liquid

- Nickel (II) sulfate hexahydrate and methyl methacrylate are soluble in water whereas 2-mercaptobenzothizole is not
- All three discordant substances have similar molecular weights (approximately 100 to 160 g/mol)
- 2-Mercaptobenzothaizole has a high peptide reactivity, whereas the peptide reactivity for methyl methacrylate and nickel (II) sulfate hexahydrate is not known
- All three discordant substances are classified as sensitizers by the traditional LLNA (EC3 values were 90.00 for methyl methacrylate, 1.70 for 2-mercaptobenzothiazole, and 4.80 for nickel [II] sulfate hexahydrate)
- All three discordant substances are nonirritants based on data from guinea pig studies (**Table 6-4**).

In addition, benzalkonium chloride, ethyl acrylate, ethylene glycol dimethacrylate, resorcinol, and SLS were positive in both the LLNA: DA and the traditional LLNA, but were negative in the GP test (**Table 6-4**). In contrast, nickel (II) chloride was negative in both the LLNA: DA and the traditional LLNA but was positive in the GP test. There are few commonalities among these substances with regard to chemical class, physical form, molecular weight, peptide reactivity (see **Appendix B** for physico-chemical information), and potential for skin irritation (**Appendix C**) as follows:

- Benzalkonium chloride is an amine, ethyl acrylate and ethylene glycol dimethacrylate are carboxylic acids, resorcinol is a phenol, and SLS is an alcohol, sulfur, and lipid compound; nickel (II) chloride is a metal.
- Resorcinol and SLS exist as solids in their physical state and ethyl acrylate and ethylene glycol dimethacrylate exist as liquids in their physical state, whereas benzalkonium chloride can exist in both a solid and liquid physical state; nickel (II) chloride exists as a solid in its physical state.
- These five substances have varying molecular weights (100 g/mol for ethyl acrylate, 110 g/mol for resorcinol, 171 g/mol for benzalkonium chloride, 198 g/mol for ethylene glycol dimethacrylate, and 288 g/mol for SLS); the molecular weight for nickel (II) chloride is about 130 g/mol.

- These five discordant substances are soluble in water; nickel (II) chloride is slightly soluble in water.
- Peptide reactivity is identified as minimal for resorcinol, and high for ethyl acrylate and ethylene glycol dimethacrylate, but is not identified for benzalkonium chloride and SLS; peptide reactivity for nickel (II) chloride is also not identified.
- Benzalkonium chloride and SLS have been found to be skin irritants based on results in mice, rabbits, or humans, while resorcinol is considered a nonirritant based on studies in humans, and ethyl acrylate and ethylene glycol dimethacrylate are considered nonirritants based on studies in guinea pigs; nickel (II) chloride is identified as negative at ≤0.15% based on GP studies (**Table 6-4**).

Table 6-4 Discordant Results for the LLNA: DA (Using SI ≥ 3.0 for Sensitizers) Compared to Traditional LLNA and Guinea Pig Reference Data[1]

Substance Name	Vehicle[2]	LLNA: DA[3]	Traditional LLNA[3]	Guinea Pig Studies[4]	Skin Irritant?
Benzalkonium chloride	AOO ACE[5]	+ (6.7, 2.5%)	+ (11.1, 2%)[6]	-	Irritant at 2% and 1% ACE (mice)
Ethyl acrylate	AOO	+ (4.2, 50%)[7]	+ (4.0, 50%)	-	Nonirritant at 0.3 Molar (GP)
Ethylene glycol dimethacrylate	MEK	+ (4.5, 50%)	+ (7.0, 50%)	-	Nonirritant at 1% (GP)
Resorcinol	AOO	+ (4.3, 25%)[8]	+ (10.4, 50%)	-	Nonirritant at 15% (humans)
Sodium lauryl sulfate	DMF	+ (3.4, 10%)	+ (8.9, 20%)	-	Irritant at 20% aq. (rabbits); Irritant at 20% (humans)
Nickel (II) chloride	DMSO	- (1.3, 10%)	- (2.4, 5%)	+	Negative at ≤0.15% (GP)
2-Mercaptobenzothiazole	DMF	- (2.0, 50%)[8]	+ (8.6, 10%)	+	Nonirritant at 10% (GP); Nonirritant at 25% (humans)
Methyl methacrylate	AOO	- (1.8, 100%)	+ (3.6, 100%)	+	Nonirritant at 3 Molar (GP)
Nickel (II) sulfate hexahydrate	DMSO	- (11.8, 10%)	+ (3.1, 5%)	+	Irritant at 10% (humans); Nonirritant at 0.15% (GP)

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); aq. = aqueous; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; GP = guinea pig; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content; MEK = methyl ethyl ketone; SI = stimulation index.
"+" = Sensitizer.
"-" = Nonsensitizer.
[1]Data source indicated in **Appendix C.**
[2]Vehicle listed is that used in both the LLNA: DA and the traditional LLNA, unless otherwise noted.
[3]Numbers in parentheses are highest SI and maximum concentration tested; highest SI is at maximum concentration test, unless otherwise noted.
[4]Based on studies using either the guinea pig maximization test or the Buehler test.
[5]Tested in AOO in LLNA: DA and ACE in traditional LLNA.
[6]Highest SI occurred at concentration 1%.
[7]Highest SI occurred at concentration 25%.
[8]Highest SI occurred at concentration 10%.

6.4.3 Discordance among the LLNA: DA, Traditional LLNA, and/or the Human Outcome

When analyses were restricted to the 41 substances with unequivocal LLNA: DA, traditional LLNA, and human outcomes, the LLNA: DA classified four substances differently compared with the classification of the traditional LLNA (**Table 6-5**). 3-Aminophenol, 2-

mercaptobenzothiazole, methyl methacrylate, and nickel (II) sulfate hexahydrate were identified as nonsensitizers by the LLNA: DA while the traditional LLNA and human outcomes classified these substances as sensitizers. All four discordant substances were tested at similar or higher concentrations in the LLNA: DA and in the traditional LLNA yet the substances were still classified as nonsensitizers (**Table 6-5**). There are few commonalities among these substances with regard to chemical class, physical form, molecular weight, peptide reactivity (see **Appendix B** for physico-chemical information), EC3 range (based on traditional LLNA, see **Table 3-1**) and potential for skin irritation (**Appendix C**):

- 3-Aminophenol is an amine and phenol compound, 2-mercaptobenzothiazole is a heterocyclic compound, methyl methacrylate is a carboxylic acid, and nickel (II) sulfate hexahydrate is a metal.
- All four discordant substances exist as solids in their physical state except methyl methacrylate which is a liquid.
- All four discordant substances are soluble in water except 2-mercaptobenzothizole.
- Molecular weights range from 100 to 167 g/mol.
- 2-Mercaptobenzothaizole has high peptide reactivity and 3-aminophenol has minimal peptide reactivity; peptide reactivity information for methyl methacrylate and nickel (II) sulfate hexahydrate is not available.
- All four discordant substances are classified as sensitizers by the traditional LLNA (EC3 values are 1.70 for 2-mercaptobenzothiazole, 3.20 for 3-aminophenol, 4.80 for nickel [II] sulfate hexahydrate, and 90.0 for methyl methacrylate).
- All four discordant substances are classified as nonirritants based on data from guinea pig studies, although human data indicates that nickel (II) sulfate hexahydrate is an irritant at 10% (**Table 6-5**).

In addition, the LLNA: DA predicted the same outcome for SLS as the traditional LLNA (i.e., sensitizer), but was discordant when compared to the negative human test result (**Table 6-5**). Isopropanol, nickel (II) chloride, propylparaben and sulfanilamide were also predicted similarly by the LLNA: DA and the traditional LLNA (i.e., nonsensitizers), but were

discordant when compared to the positive human test result (**Table 6-5**). There are few commonalities among these substances with regard to chemical class, physical form, molecular weight, peptide reactivity (see **Appendix B** for physico-chemical information), EC3 range (based on traditional LLNA, see **Table 3-1**) and potential for skin irritation (**Appendix C**):

- SLS is an alcohol, sulfur, and lipid compound; isopropanol is an alcohol, nickel (II) chloride is a metal, propylparaben is a phenol compound, and sulfanilamide is a cyclic hydrocarbon and sulfur compound.
- SLS exists as a solid in its physical state; isopropanol is a liquid in its physical state, whereas nickel (II) chloride, propylparaben, and sulfanilamide exist as solids in their physical state.
- These substances have varying molecular weights that range from 60 to 172 g/mol for isopropanol, nickel (II) chloride, propylparaben, and sulfanilamide to 288 g/mol for SLS.
- SLS, isopropanol, nickel (II) chloride, and sulfanilamide are soluble in water and propylparaben is not.
- Isopropanol, propylparaben, and sulfanilamide have minimal peptide reactivity; peptide reactivity data for nickel (II) chloride and SLS is not available.
- SLS has been found to be a skin irritant based on results in mice, rabbits, or humans; isopropanol, nickel (II) chloride, propylparaben, and sulfanilamide are considered negative or nonirritants based on studies in rabbits or GP (**Table 6-5**).

Table 6-5 Discordant Results for the LLNA: DA (Using SI ≥ 3.0 for Sensitizers) Compared to Traditional LLNA and Human Reference Data[1]

Substance	Vehicle[2]	LLNA: DA[3]	Traditional LLNA[3]	Human Outcomes[4]	Skin Irritant?
Sodium lauryl sulfate	DMF	+ (3.4, 10%)	+ (8.9, 20%)	- (0/22 at 10%)	Irritant at 20% aq. (rabbits); Irritant at 20% (humans)
Isopropanol	AOO	- (1.97, 50%)	- (1.7, 50%)[5]	+ (case study at 0.001%)	Negative at 100% (rabbits)
Nickel (II) chloride	DMSO	- (1.3, 10%)	- (2.4, 5%)	+ (HMT, data expressed as nickel)	Negative at ≤0.15% (GP)
Propylparaben	AOO	- (1.3, 25%)	- (1.4, 25%)[6]	+ (HMT)	Nonirritant at 10% (GP)
Sulfanilamide	DMF	- (0.9, 50%)[5]	- (1.0, 50%)[7]	+ (20/25 at 25%)	Nonirritant at 25% (humans)
3-Aminophenol	AOO	- (2.8, 10%)	+ (5.7, 10%)	+	Nonirritant at 5% (GP)
2-Mercaptobenzothiazole	DMF	- (2.0, 50%)[8]	+ (8.6, 10%)	+ (24/63 at 25%)	Nonirritant at 10% (GP); Nonirritant at 25% (humans)
Methyl methacrylate	AOO	- (1.8, 100%)	+ (3.6, 100%)	+	Nonirritant at 3 M (GP)
Nickel (II) sulfate hexahydrate	DMSO	- (11.8, 10%)	+ (3.1, 5%)	+ (23/88 at 1%)	Irritant at 10% (humans); Nonirritant at 0.15% (GP)

Abbreviations: AOO = acetone: olive oil (4:1); aq. = aqueous; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; GP = guinea pig; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content; SI = stimulation index.
"+" = Sensitizer.
"-" = Nonsensitizer.
[1]Data source indicated in **Appendix C.**
[2]Vehicle listed is that used in both the LLNA: DA and the traditional LLNA, unless otherwise noted.
[3]Numbers in parentheses are highest SI and maximum concentration tested; highest SI is at maximum concentration tested, unless otherwise noted.
[4]Based on studies using either the human maximization test, inclusion of the test substance in a human patch test allergen kit, and/or published clinical case studies/reports.
[5]Highest SI occurred at concentration 25%.
[6]Highest SI occurred at concentration 5%.
[7]Highest SI occurred at concentration 10% and 25%.
[8]Highest SI occurred at concentration 10%.

6.5 Accuracy Analysis Using a Single Alternative Decision Criteria

In addition to the accuracy analysis using SI ≥ 3.0 to classify substances as sensitizers, other decision criteria were evaluated on the LLNA: DA test method performance, using the traditional LLNA (SI ≥ 3.0) as the comparative test (**Appendix C**). The performance characteristics presented in this section are for 13 decision criteria that were used to determine whether the skin sensitization potential for the substances were positive (i.e., sensitizing) or negative (i.e., nonsensitizing). The substances evaluated were the 44 substances discussed in **Section 6.1** with both LLNA: DA and sufficient comparative traditional LLNA data. The decision criteria analyzed included the following:

1. SI values ≥1.3, ≥1.5, ≥2.0, ≥2.5, ≥3.0, ≥3.5, ≥4.0, ≥4.5, or ≥5.0
2. ATP values of treated groups statistically different from control group based on analysis of variance (ANOVA) with a post-hoc Dunnett's test, when multiple treatment groups were tested, or Student's *t*-test when there was only one dosed group
3. Mean ATP values of treated groups ≥95% confidence interval (CI) of the control group mean
4. Mean ATP values of treated groups ≥2 standard deviations (SD) or ≥3 SD from the control group mean

Multiple tests were available for 14 substances tested with the LLNA: DA. The results for each of these substances were combined so that each substance was represented by one positive or negative 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 positive/negative outcome for each substance was the most prevalent outcome for each criterion. 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 using the most prevalent outcome for substances with multiple tests is presented in this section; the analyses using the highest maximum SI and the lowest maximum SI are included in **Appendix E**.

When combining multiple test results for a single substance based on the most prevalent outcome, using the decision criterion of SI ≥ 3.0 to identify sensitizers, the 44 substances analyzed yielded an accuracy of 91% (40/44), a sensitivity of 88% (28/32), a specificity of 100% (12/12), a false positive rate of 0% (0/12), and a false negative rate of 13% (4/32) (**Table 6-6)**. The decision criterion of SI ≥ 2.5 was similar to SI ≥ 3.0 in its performance characteristics. In comparison, the decision criteria using higher SI values, SI ≥ 3.5 to SI ≥ 5.0, decreased performance except for specificity, which remained at 100% (12/12), and the false positive rate, which remained at 0% (0/12) (**Figure 6-1** and **Table 6-6**). Specifically, at SI ≥ 5.0, accuracy decreased to 57% (25/44) and the false negative rate increased to 59% (19/32).

The decision criteria using lower SI values, SI ≥ 1.5 and SI ≥ 1.3, also decreased performance compared to SI ≥ 3.0 except for sensitivity, which increased to 100% (32/32), and the false negative rate, which decreased to 0% (0/32) (**Figure 6-1** and **Table 6-6**). Notably, the SI decision criterion that exhibited the best overall performance characteristics compared to SI ≥ 3.0 was the SI ≥ 2.0 (**Figure 6-1 and Table 6-6**). Compared to SI ≥ 3.0, the lower SI cutoff of 2.0 had the same accuracy (i.e., 91% [40/44]) but had an increased sensitivity of 97% (31/32), although specificity decreased to 75% (9/12) and the false positive rate increased to 25% (3/12) while the false negative rate decreased to 3% (1/32).

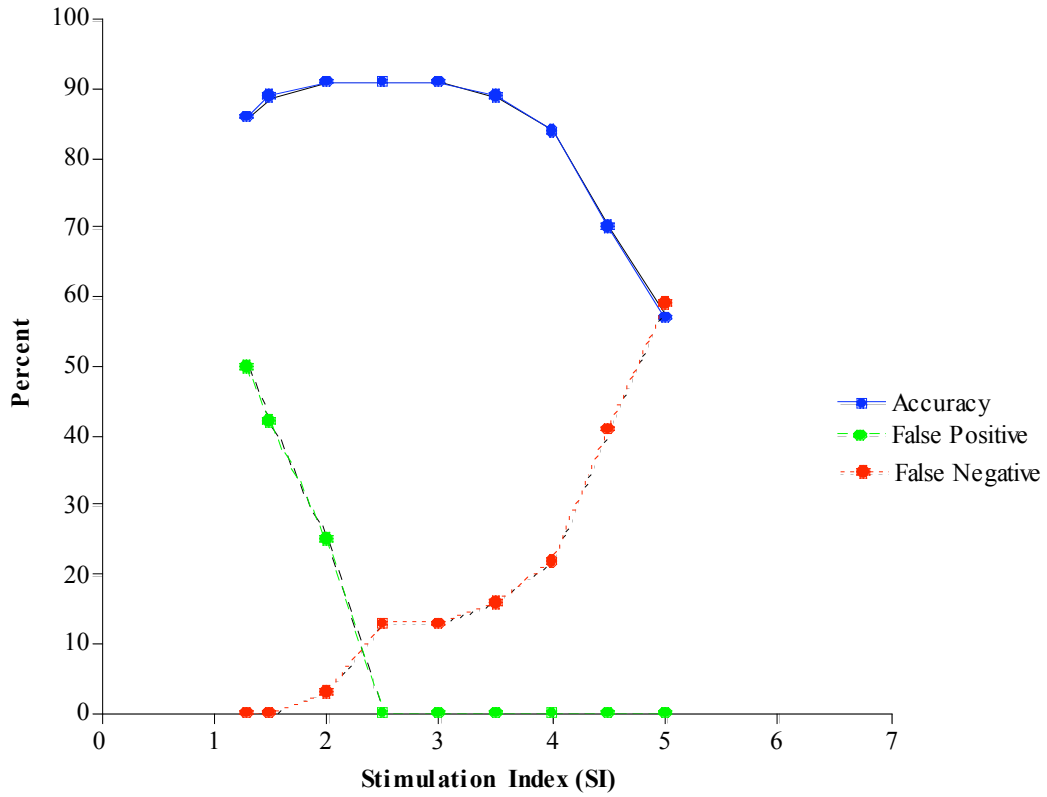
Use of ANOVA and summary statistics (i.e., mean ATP values of treated groups ≥95% confidence interval of the control group mean, or ≥2 or 3 SD from the control group mean), yielded accuracy values of 75 to 84%, with sensitivity values of 88 to 100%, and false

negative rates of 0 to 13%. The specificity for these criteria ranged from 8 to 58% and the false positive rates were 42 to 92%. None of the statistical criterion evaluated exhibited increased performance characteristics when compared to SI ≥ 3.0 (**Table 6-6**).

Since the decision criterion of SI ≥ 2.0 showed the best overall performance (i.e., similar accuracy, increased sensitivity, and decreased false negative rate compared to SI ≥ 3.0), it was further compared to SI ≥ 3.0 for accuracy against GP and human data (**Table 6-7)**. When the LLNA: DA was compared to GP outcomes for substances with available LLNA: DA, traditional LLNA, and GP data (i.e., 40 substances), SI ≥ 2.0 had the same accuracy (78% [31/40]), increased sensitivity (92% [24/26] vs. 85% [22/26]) and decreased specificity (50% [7/14] vs. 64% [9/14]) when compared with SI ≥ 3.0. Accordingly, the false positive rate was increased (50% [7/14] vs. 36% [5/14]) and the false negative rate was decreased (8% [2/26] vs. 15% [4/26]) for SI ≥ 2.0 compared to SI ≥ 3.0. The overall performance of the LLNA: DA (SI ≥ 2.0) compared to the traditional LLNA (SI ≥ 3.0) to predict GP outcomes was less (see **Table 6-7**).

When the LLNA: DA was compared to human outcomes for substances with available LLNA: DA, traditional LLNA, and human data (i.e., 41 substances), SI ≥ 2.0 increased the accuracy (80% [31/41] vs. 78% [32/41]) and sensitivity (85% [29/34] vs. 76% [26/34]) and decreased the specificity (57% [4/7] vs. 86% [6/7]) when compared with SI ≥ 3.0. Accordingly, the false positive rate was increased (43% [3/7] vs. 14% [1/7]) and the false negative rate was decreased (15% [5/34] vs. 24% [8/34]). The overall performance of the LLNA: DA (SI ≥ 2.0) compared to the traditional LLNA (SI ≥ 3.0) to predict human outcomes was less (see **Table 6-7**).

Figure 6-1 Performance of the LLNA: DA Compared to the Traditional LLNA in Predicting Skin Sensitization Potential Using Alternative SI Based on 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: DA with the SI cutoff used to identify sensitizers. This analysis used LLNA: DA and traditional LLNA results for 44 substances (32 traditional LLNA sensitizers and 12 traditional LLNA nonsensitizers). For the 14 substances with multiple test results, the results for each substance were combined by using the most prevalent outcome. The solid line shows accuracy, the dashed line shows the false positive rate, and the dotted line shows the false negative rate.

Table 6-6 Performance of the LLNA: DA Compared to the Traditional LLNA in Predicting Skin Sensitization Potential Using Alternative Decision Criteria Based on 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]	44	84	37/44	94	30/32	58	7/12	42	5/12	6	2/32	86	30/35	78	7/9
≥95% CI[4]	44	75	33/44	100	32/32	8	1/12	92	11/12	0	0/32	74	32/43	100	1/1
≥2 SD[5]	44	77	34/44	91	29/32	42	5/12	58	7/12	9	3/32	81	29/36	63	5/8
≥3 SD[6]	44	80	35/44	88	28/32	58	7/12	42	5/12	13	4/32	85	28/33	64	7/11
SI ≥ 5.0	44	57	25/44	41	13/32	100	12/12	0	0/12	59	19/32	100	13/13	39	12/31
SI ≥ 4.5	44	70	31/44	59	19/32	100	12/12	0	0/12	41	13/32	100	19/19	48	12/25
SI ≥ 4.0	44	84	37/44	78	25/32	100	12/12	0	0/12	22	7/32	100	25/25	63	12/19
SI ≥ 3.5	44	89	39/44	84	27/32	100	12/12	0	0/12	16	5/32	100	27/27	71	12/17
SI ≥ 3.0	**44**	**91**	**40/44**	**88**	**28/32**	**100**	**12/12**	**0**	**0/12**	**13**	**4/32**	**100**	**28/28**	**75**	**12/16**
SI ≥ 2.5	45	91	40/44	88	28/32	100	12/12	0	0/12	13	4/32	100	28/28	75	12/16
SI ≥ 2.0	*44*	*91*	*40/44*	*97*	*31/32*	*75*	*9/12*	*25*	*3/12*	*3*	*1/32*	*91*	*31/34*	*90*	*9/10*
SI ≥ 1.5	44	89	39/44	100	32/32	58	7/12	42	5/12	0	0/32	86	32/37	100	7/7
SI ≥ 1.3	44	86	38/44	100	32/32	50	6/12	50	6/12	0	0/32	84	32/38	100	6/6

Bolded text indicates the decision criterion chosen by the LLNA: DA validation study team; Italicized text indicates the single decision criterion that had an overall increased performance in predicting skin sensitization potential when compared to the traditional LLNA.

Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP Content; 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 ATP data were log-transformed prior to statistical analysis. For analysis of variance, significance at $p < 0.05$ was further tested by Dunnett's test.

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

[5]The mean ATP of at least one treatment group was greater than 2 SD from the mean ATP of the vehicle control group.
[6]The mean ATP of at least one treatment group was greater than 3 SD from the mean ATP of the vehicle control group.

Table 6-7 Performance of the LLNA: DA in Predicting Skin Sensitization Potential Comparing Decision Criteria of SI ≥ 3.0 versus SI ≥ 2.0 Based on the Most Prevalent Outcome for Substances with Multiple Tests

Comparison	n[1]	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate		Positive Predictivity		Negative Predictivity	
		%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]
LLNA: DA vs. Traditional LLNA	**44**	**91** *91*	**40/44** *40/44*	**88** *97*	**28/32** *31/32*	**100** *75*	**12/12** *9/12*	**0** *25*	**0/12** *3/12*	**13** *3*	**4/32** *1/32*	**100** *91*	**28/28** *31/34*	**75** *90*	**12/16** *9/10*
Substances *with LLNA: DA, Traditional LLNA, and GP Data*															
LLNA: DA vs. Traditional LLNA	**40**	**93** *93*	**37/40** *37/40*	**90** *97*	**27/30** *29/30*	**100** *80*	**10/10** *8/10*	**0** *20*	**0/10** *2/10*	**10** *3*	**3/30** *1/30*	**100** *94*	**27/27** *29/31*	**77** *89*	**10/13** *8/9*
LLNA: DA vs. GP[3]	**40**	**78** *78*	**31/40** *31/40*	**85** ***92***	**22/26** *24/26*	**64** *50*	**9/14** *7/14*	**36** *50*	**5/14** *7/14*	**15** *8*	**4/26** *2/26*	**81** *77*	**22/27** *24/31*	**69** *78*	**9/13** *7/9*
Traditional LLNA vs. GP[3]	***40***	***85***	***34/40***	***96***	***25/26***	***64***	***9/14***	***36***	***5/14***	***4***	***1/26***	***83***	***25/30***	***90***	***9/10***
Substances *with LLNA: DA, Traditional LLNA, and Human Data*															
LLNA: DA vs. Traditional LLNA	**41**	**90** *93*	**37/41** *38/41*	**87** *97*	**27/31** *30/31*	**100** *80*	**10/10** *8/10*	**0** *20*	**0/10** *2/10*	**13** *3*	**4/31** *1/31*	**100** *94*	**27/27** *30/32*	**71** *89*	**10/14** *8/9*
LLNA: DA vs. Human[4]	**41**	**78** *80*	**32/41** *31/41*	**76** *85*	**26/34** *29/34*	**86** *57*	**6/7** *4/7*	**14** *43*	**1/7** *3/7*	**24** *15*	**8/34** *5/34*	**96** *91*	**26/27** *29/32*	**43** *44*	**6/14** *4/9*
Traditional LLNA vs. Human[4]	***41***	***88***	***36/41***	***88***	***30/34***	***86***	***6/7***	***14***	***1/7***	***12***	***4/34***	***97***	***30/31***	***60***	***6/10***

Text is bolded for SI ≥ 3.0 and italicized for SI ≥ 2.0; performance for SI ≥ 3.0 is the same as SI ≥ 2.0 for traditional LLNA vs. GP and for traditional LLNA vs. human.

Abbreviations: GP = guinea pig skin sensitization outcomes; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content; No. = number; SI = stimulation index; vs. = versus.

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

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

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

[4]Human refers to outcomes obtained by studies conducted using the human maximization test, 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 a Single Alternative Decision Criteria

This section discusses the discordant results obtained for the analyses using the alternative decision criteria shown in **Tables 6-6** and **6-7,** in order to provide a comparison to the discordant substances identified when using the decision criterion of SI ≥ 3.0 to identify sensitizers. Discordant results are first discussed using the traditional LLNA as the reference test (**Section 6.6.1**) and then discordant results for SI ≥ 2.0, the single optimized alternative decision criterion, are discussed using the traditional LLNA, GP, and human outcomes as references (**Section 6.6.2**).

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

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. Using SI ≥ 2.0 as the decision criterion resulted in three nonsensitizers in the traditional LLNA (i.e., chlorobenzene, hexane, and salicylic acid) being misclassified as sensitizers in the LLNA: DA. Also, methyl methacrylate, a sensitizer in the traditional LLNA, was misclassified as a nonsensitizer in the LLNA: DA. As the SI decision criterion was further reduced to SI ≥ 1.5 and SI ≥ 1.3, two additional substances, 1-bromobutane and methyl salicylate were also misclassified as sensitizers but methyl methacrylate was no longer incorrectly classified as a nonsensitizer by the LLNA: DA when compared to traditional LLNA results. In addition, using SI ≥ 1.3 also misclassified nickel (II) chloride as a sensitizer in the LLNA: DA compared to the traditional LLNA. Increasing the SI cutoff to values greater than three increased the number of sensitizers that were misclassified as nonsensitizers. At SI ≥ 5.0, 19 substances were discordant. As **Table 6-8** shows, all 19 substances were sensitizers in the LLNA but misclassified as nonsensitizers in the LLNA: DA.

Use of a statistical test (i.e., ANOVA or *t*-test) to identify sensitizers misclassified two sensitizers in the traditional LLNA (i.e., 2-mercaptobenzothiazole and methyl methacrylate) as nonsensitizers in the LLNA: DA and five nonsensitizers (i.e., 1-bromobutane, chlorobenzene, hexane, salicylic acid, and sulfanilamide) as sensitizers. Use of summary

statistics (i.e., ≥95% CI, ≥2 SD or ≥3 SD) generally misclassified nonsensitizers in the traditional LLNA as sensitizers in the LLNA: DA. Specifically, using ≥3 SD of vehicle control mean misclassified five nonsensitizers as sensitizers: 1-bromobutane, chlorobenzene, hexane, nickel (II) chloride, and propylparaben. Using treatment group absorbance ≥2 SD of vehicle control mean misclassified the same five substances as sensitizers, as well as methyl salicylate and salicylic acid. Using the treatment group absorbance ≥95% CI of vehicle control mean misclassified all the nonsensitizers misclassified as sensitizers in the LLNA: DA when using either ≥3 SD or ≥2 SD of vehicle control mean, as well as four additional substances: diethyl phthalate, dimethyl isophthalate, isopropanol, and lactic acid. In some instances, use of summary statistics (i.e., ≥95% CI, ≥2 SD or ≥3 SD) misclassified sensitizers in the traditional LLNA as nonsensitizers in the LLNA: DA. Using ≥3 SD of vehicle control mean misclassified four traditional LLNA sensitizers as LLNA: DA nonsensitizers: butyl glycidyl ether, ethyl acrylate, methyl methacrylate, and propyl gallate. Using treatment group absorbance ≥2 SD of vehicle control mean only misclassified ethyl acrylate and propyl gallate as nonsensitizers in the LLNA; DA compared to the traditional LLNA and using the treatment group absorbance ≥95% CI did not misclassify any traditional LLNA sensitizers as LLNA: DA nonsensitizers.

6.6.2 Discordant Results for Accuracy Analysis Using a Single Optimized Alternative Decision Criteria (SI ≥ 2.0)

When analyses were restricted to the 40 substances with unequivocal LLNA: DA, traditional LLNA, and GP data based on an SI ≥ 2.0, the LLNA: DA classified three substances (i.e., chlorobenzene, salicylic acid, and methyl methacrylate) differently compared with the classification of the traditional LLNA (**Table 6-9**). Chlorobenzene and salicylic acid were classified as sensitizers in the LLNA: DA and as nonsensitizers by both the traditional LLNA and GP outcomes. Methyl methacrylate was classified as a nonsensitizer in the LLNA: DA and as a sensitizer by both the traditional LLNA and GP outcomes. In contrast, benzalkonium chloride, ethyl acrylate, ethylene glycol dimethacrylate, resorcinol, and sodium lauryl sulfate were identified as sensitizers by the LLNA: DA similar to the traditional LLNA but as nonsensitizers based on GP outcomes. Nickel (II) chloride was identified as a nonsensitizer by the LLNA: DA similar to the traditional LLNA but as a sensitizer based on GP outcomes. There are few commonalities among these substances with regard to chemical class, physical

form, molecular weight, peptide reactivity (see **Appendix B** for physico-chemical information), EC3 range (based on traditional LLNA, see **Table 3-1**) and potential for skin irritation (**Appendix C**) as follows:

- Chlorobenzene is a halogenated hydrocarbon compound and salicylic acid is a phenol and carboxylic acid; methyl methacrylate is a carboxylic acid; benzalkonium chloride is an amine (onium compound), ethyl acrylate and ethylene glycol dimethacrylate are carboxylic acids, resorcinol is a phenol, and SLS is an alcohol, sulfur, and lipid compound.
- Chlorobenzene exists as a liquid and salicylic acid exists as a solid in its physical state; methyl methacrylate is a liquid; resorcinol and SLS are solids and ethyl acrylate and ethylene glycol dimethacrylate are liquids, whereas benzalkonium chloride can exist in both a solid and liquid physical state.
- Chlorobenzene has a molecular weight of 113 g/mol and salicylic acid has a molecular weight of 138 g/mol; methyl methacrylate has a molecular weight of 100 g/mol; the other five discordant substances have varying molecular weights that range from 100 g/mol for ethyl acrylate, 110 g/mol for resorcinol, 171 g/mol for benzalkonium chloride, and 198 g/mol for ethylene glycol dimethacrylate to 288 g/mol for SLS.
- All the discordant substances are soluble in water.
- Chlorobenzene has minimal peptide reactivity; the peptide reactivity for resorcinol is identified as minimal, and that for ethyl acrylate and ethylene glycol dimethacrylate is high; peptide reactivity data for salicylic acid, methyl methacrylate, benzalkonium chloride and SLS is not available.
- Methyl methacrylate is identified as a sensitizer by the traditional LLNA (EC3 = 90%); benzalkonium chloride (EC3 = 0.1%), ethyl acrylate (EC3 = 32.8%), ethylene glycol dimethacrylate (EC3 = 28%), resorcinol (6.3%) and SLS (EC3 = 8.1%) are identified as sensitizers by the traditional LLNA.
- Chlorobenzene has low irritancy potential assumed based on clinical literature while salicylic acid is an irritant at 20% in mice; methyl methacrylate is a nonirritant in GP;

benzalkonium chloride and SLS have been found to be skin irritants based on results in mice, rabbits, or humans and ethyl acrylate, ethylene glycol dimethacrylate, and resorcinol are considered nonirritants based on studies in humans or GP (**Table 6-9**).

When analyses were restricted to the 40 substances with unequivocal LLNA: DA, traditional LLNA, and human outcomes based on an SI ≥ 2.0, the LLNA: DA classified three substances (i.e., hexane, salicylic acid, and methyl methacrylate) differently compared with the classification of the traditional LLNA (**Table 6-10**). Hexane and salicylic acid were classified as sensitizers in the LLNA: DA and as nonsensitizer by both the traditional LLNA and human outcomes. In contrast, methyl methacrylate was identified as a nonsensitizer by the LLNA: DA but as a sensitizer based on traditional LLNA and human outcomes. Isopropanol, nickel (II) chloride, propylparaben, and sulfanilamide were all classified as nonsensitizers by the LLNA: DA and the traditional LLNA but as sensitizers based on human outcomes (**Table 6-10**). In contrast, SLS was classified as a sensitizer by the LLNA: DA and traditional LLNA but as a sensitizer based on human outcomes. In instances where the substances were discordant in the LLNA: DA compared to the traditional LLNA, the discordant substances were tested at the same maximum concentration. There are few commonalities among these substances with regard to chemical class, physical form, molecular weight, peptide reactivity (see **Appendix B** for physico-chemical information), EC3 range (based on traditional LLNA, see **Table 3-1**) and potential for skin irritation (**Appendix C**):

- Hexane is an acyclic hydrocarbon compound and salicylic acid is a phenol and carboxylic acid; methyl methacrylate is a carboxylic acid; isopropanol is an alcohol, nickel (II) chloride is a metal, propylparaben is a phenol compound, and sulfanilamide is sulfur compound; SLS is an alcohol, sulfur, and lipid compound.
- Hexane is a liquid and salicylic acid is a solid; methyl methacrylate is a liquid; isopropanol is a liquid while nickel (II) chloride, propylparaben, and sulfanilamide are solids; SLS is a solid.
- Hexane has a molecular weight of 86 g/mol; methyl methacrylate has a molecular weight of 100 g/mol; the other discordant substances have varying molecular weights

that range from 60 g/mol for isopropanol, 130 g/mol for nickel (II) chloride, 172 g/mol for sulfanilamide, and 180 g/mol for propylparaben to 288 g/mol for SLS.

- Hexane, salicylic acid, isopropanol, methyl methacrylate, nickel (II) chloride, sulfanilamide, and SLS are soluble in water; propylparaben is not.
- Hexane, isopropanol, propylparaben, and sulfanilamide have minimal peptide reactivity; peptide reactivity information for salicylic acid methyl methacrylate nickel (II) chloride SLS is not available.
- Methyl methacrylate is identified as a sensitizer by the traditional LLNA (EC3 = 90%) as is SLS (EC3 = 8.1%).
- Hexane has been found to be an irritant at 100% in humans as has salicylic acid in mice; isopropanol, nickel (II) chloride, propylparaben, and sulfanilamide are considered to be nonirritants based on studies in rabbits, GP, or humans; SLS has been found to be a skin irritants based on results in mice, rabbits, or humans (**Table 6-10**).

Table 6-8 Discordant Results for the LLNA: DA Using Alternative Decision Criteria Compared to the Traditional LLNA Based on 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
3-Aminophenol (3.2%)					-	-	-	-	-	-			
p-Benzoquinone (0.01%)					-	-	-						
1-Bromobutane (-)	+	+	+	+								+	+
Butyl glycidyl ether (30.9%)				-	-								
Chlorobenzene (-)	+	+	+	+							+	+	+
Cinnamic aldehyde (1.9%)					-								
Citral (9.2%)					-	-							
Cobalt chloride (0.6%)					-	-							
Diethyl maleate (3.6%)					-	-	-						
Diethyl phthalate (-)		+											
Dimethyl isophthalate (-)		+											
Ethyl acrylate (32.8%)			-	-	-	-							
Ethylene glycol dimethacrylate (28%)					-	-							
Formaldehyde (0.5)					-								
Hexane (-)	+	+	+	+							+	+	+
Imidazolidinyl urea (24%)					-								
Isopropanol (-)		+											
Lactic acid (-)		+											

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%)	-				-	-	-	-	-	-			
Methyl methacrylate (90%)	-		-	-	-	-	-	-	-	-	-		
Methyl salicylate (-)		+	+									+	+
Nickel (II) chloride (-)		+	+	+									+
Nickel (II) sulfate hexahydrate (4.8%)					-	-	-	-	-	-			
Phenyl benzoate (13.6%)					-	-							
Propyl gallate (0.320%)			-	-	-								
Propylparaben (-)		+	+	+									
Resorcinol (6.3%)					-	-							
Salicylic acid (-)	+	+	+								+	+	+
Sulfanilamide (-)	+												
Sodium lauryl sulfate (8.1%)					-	-	-	-					
Trimellitic anhydride (4.7%)					-								

Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP Content; SD = standard deviation; SI = stimulation index.

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

[2]LLNA: DA outcomes are indicated by "+" for sensitizer results and "-" for nonsensitizer results.

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

[4]The mean ATP of at least one treatment group was outside the 95% CI for the mean ATP of the vehicle control group.

[5]The mean ATP of at least one treatment group was greater than 2 SD from the mean ATP of the vehicle control group.

[6]The mean ATP of at least one treatment group was greater than 3 SD from the mean ATP of the vehicle control group.

Table 6-9 Discordant Results for the LLNA: DA (Using SI ≥ 2.0 for Sensitizers) Compared to Traditional LLNA and GP Reference Data[1]

Substance Name	Vehicle[2]	LLNA: DA[3]	Traditional LLNA[3]	Guinea Pig Studies[4]	Skin Irritant?
Benzalkonium chloride	AOO ACE[5]	+ (6.7, 2.5%)	+ (11.1, 2%)[6]	-	Irritant at 2% and 1% ACE (mice)
Ethyl acrylate	AOO	+ (4.3, 50%)[7]	+ (4.0, 50%)	-	Nonirritant at 0.3 M (GP)
Ethylene glycol dimethacrylate	MEK	+ (4.5, 50%)	+ (7.0, 50%)	-	Nonirritant at 1% (GP)
Resorcinol	AOO	+ (4.3, 25%)[5]	+ (10.4, 50%)	-	Nonirritant at 15% (humans)
Sodium lauryl sulfate	DMF	+ (3.4, 10%)	+ (8.9, 20%)	-	Irritant at 20% aq. (rabbits); Irritant at 20% (humans)
Chlorobenzene	AOO	+ (2.4, 25%)	- (1.7, 10%)[5]	-	No data. Low irritancy potential assumed based on clinical literature.
Salicylic acid	AOO	+ (2.0, 25%)	- (2.4, 25%)	-	Irritant at 20% aq. (mice)
Methyl methacrylate	AOO	- (1.8, 100%)	+ (3.6, 100%)	+	Nonirritant at 3 M (GP)
Nickel (II) chloride	DMSO	- (1.3, 10%)	- (2.4, 5%)	+	Negative at ≤0.15% (GP)

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); aq. = aqueous ; DMF = *N,N*-dimethylformamide; GP = guinea pig; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content; SI = stimulation index.
"+" = Sensitizer.
"-" = Nonsensitizer.
[1]Data source indicated in **Appendix C.**
[2]Vehicle listed is that used in both the LLNA: DA and the traditional LLNA, unless otherwise noted.
[3]Numbers in parentheses are highest SI and maximum concentration tested; highest SI is at maximum concentration tested, unless otherwise noted.
[4]Based on studies using either the human maximization test, inclusion of the test substance in a human patch test allergen kit and/or published clinical case studies/reports.
[5]Benzalkonium chloride tested in AOO vehicle in LLNA: DA and ACE vehicle in traditional LLNA.
[6]Highest SI occurred at concentration 1%.
[7]Highest SI occurred at concentration 25%.

Table 6-10 Discordant Results for the LLNA: DA (Using SI ≥ 2.0 for Sensitizers) Compared to Traditional LLNA and Human Reference Data[1]

Substance	Vehicle[2]	LLNA: DA[3]	Traditional LLNA[3]	Human Outcomes[4]	Skin Irritant?
Hexane	AOO	+ (2.3, 100%)	- (2.2, 100%)	- (0/25 at 100%)	Irritant at 100% (humans)
Salicylic acid	AOO	+ (2.0, 25%)	- (2.4, 25%)	-	Irritant at 20% aq. (mice)
Sodium lauryl sulfate	DMF	+ (3.4, 10%)	+ (8.9, 20%)	- (0/22 at 10%)	Irritant at 20% aq. (rabbits); Irritant at 20% (humans)
Isopropanol	AOO	- (1.97, 50%)	- (1.7, 50%)[5]	+ (case study at 0.001%)	Negative at 100% (rabbits)
Nickel (II) chloride	DMSO	- (1.3, 10%)	- (2.4, 5%)	+	Negative at ≤0.15% (GP)
Propylparaben	AOO	- (1.3, 25%)	- (1.4, 25%)[6]	+ (HMT)	Nonirritant at 10% (GP)
Sulfanilamide	DMF	- (0.9, 50%)[7]	- (1.0, 50%)[8]	+	Nonirritant at 25% (humans)
Methyl methacrylate	AOO	- (1.8, 100%)	+ (3.6, 100%)	+	Nonirritant at 3 M (GP)

Abbreviations: aq. = aqueous; AOO = acetone: olive oil (4:1); DMF = *N,N*-dimethylformamide; GP = guinea pig; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content; SI = stimulation index.
"+" = Sensitizer.
"-" = Nonsensitizer.
[1]Data source indicated in **Appendix C.**
[2]Vehicle listed is that used in both the LLNA: DA and the traditional LLNA, unless otherwise noted.
[3]Numbers in parentheses are highest SI and maximum concentration tested; highest SI is at maximum concentration tested, unless otherwise noted.
[4]Based on studies using either the human maximization test, inclusion of the test substance in a human patch test allergen kit and/or published clinical case studies/reports.
[5]Highest SI occurred at concentration 10%.
[6]Highest SI occurred at concentration 5%.
[6]Highest SI occurred at concentration 25%.
[6]Highest SI occurred at concentration 10 and 25%.

6.7 Accuracy Analysis Using Multiple Alternative Decision Criteria

As detailed in **Section 6.5,** the accuracy of the LLNA: DA when using a number of alternative decision criteria was evaluated using the traditional LLNA as the reference test. Compared to the traditional LLNA (SI ≥ 3.0), the best overall performance (i.e., accuracy of 91% [40/44] and sensitivity of 97% [31/32]) was achieved using the decision criterion of SI ≥ 2.0 (**Table 6-6**). The SI ≥ 2.0 also produced a false positive rate of 25% (3/12) and a false negative rate of 3% (1/32) (**Table 6-6**). Increasing the SI decision criterion to SI ≥ 2.5

decreased the false positive rate to 0% (0/12) but increased the false negative rate to 13% (4/32). The SI ≥ 2.0 produced one false negative result for the substance methyl methacrylate (EC3 = 90%). Upon evaluating the LLNA: DA test data for methyl methacrylate, the maximum SI achieved was 1.81 at 100%. Thus, decreasing the SI decision criterion to SI ≥ 1.7 decreased the false negative rate to 0% (0/32). The 0% false positive rate using SI ≥ 2.5 and the 0% false negative rate using SI ≥ 1.7 prompted an evaluation using two decision criteria for LLNA: DA results: one criterion to classify substances as sensitizers (i.e., SI ≥ 2.5) and one criterion to classify substances as nonsensitizers (SI ≤ 1.7).

It should be noted that this analysis was based on the same strategy for combining results as that described in **Section 6.5** for the substances tested multiple times (i.e., the sensitizer/nonsensitizer outcome for each substance using the most prevalent outcome). **Section 7.3** details the reproducibility of substances tested multiple times and indicates that, there were no instances of false positive results for nonsensitizers (i.e., SI ≥ 2.5). Among the 80 tests that produced a maximum SI ≥ 2.5, 0% (0/80) were nonsensitizers (i.e., produced a false positive result). See **Section 7.3** for 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 (i.e., 1.7 < SI < 2.5) 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, molecular weight, peptide reactivity (see **Appendix B** for physico-chemical properties), traditional LLNA EC3 range (**Table 3-1**), or potential for skin irritation (**Appendix C**) were examined to identify commonalities among the substances that produced SI values between 1.7 and 2.5 in an attempt to identify similar characteristics among these substances that could be used to correctly classify such substances.

Ten substances produced SI values between 1.7 and 2.5 (**Table 6-11)**. Five of the 10 substances are nonsensitizers (i.e., chlorobenzene, hexane, isopropanol, methyl salicylate, salicylic acid) and five are sensitizers (i.e., 3-aminophenol, cobalt chloride, 2-mercaptobenzothiazole, methyl methacrylate, nickel [II] sulfate hexahydrate) based on

traditional LLNA results. Among the five nonsensitizers, six chemical classes are represented; two substances are classified as carboxylic acids (i.e., salicylic acid and methyl salicylate [also a phenol]), one substance is a halogenated and cyclic hydrocarbon (i.e., chlorobenzene), one substance is an acyclic hydrocarbon (i.e., hexane), and one substance is an alcohol (i.e., isopropanol). Other characteristics of the nonsensitizers (based on traditional LLNA data) include:

- Four substances are liquids (i.e., chlorobenzene, hexane, isopropanol, and methyl salicylate) and one substance is a solid (i.e., salicylic acid).
- Molecular weights range from 60 g/mol for isopropanol, 86 g/mol for hexane, 113 g/mol for chlorobenzene, 138 g/mol for salicylic acid to 152 g/mol for methyl salicylate.
- All five substances are soluble in water.
- The peptide reactivity for chlorobenzene, hexane, isopropanol, and methyl salicylate is minimal; peptide reactivity information for salicylic acid is not available.
- Hexane, methyl salicylate, and salicylic acid are considered irritants based on data in either mice or humans and isopropanol is considered negative based on data in rabbits; irritancy data for chlorobenzene is not available but irritancy potential is assumed to be low based on clinical literature (**Table 6-11**).

Among the five sensitizers, five chemical classes are represented; one substance is a carboxylic acid (i.e., methyl methacrylate), two substances are metals (i.e., nickel [II] sulfate hexahydrate and cobalt chloride), one substance is a phenol (i.e., 2-aminophenol [also an amine]), and one substance is a heterocyclic compound (i.e., 2-mercaptobenzothiazole). Other characteristics of the substances that are classified as sensitizers by the traditional LLNA include:

- Four substances are solids (i.e., 3-aminophenol, cobalt chloride, 2-mercaptobenzothiazole, and nickel [II] sulfate hexahydrate) and one substance is a liquid (i.e., methyl methacrylate).

- Molecular weights range from 100 g/mol for methyl methacrylate, 109 g/mol for 3-aminophenol, 130 g/mol for cobalt chloride, 155 g/mol for nickel (II) sulfate hexahydrate to 167 g/mol for 2-mercaptobenzothiazole.
- 2-Mercaptobenzothiazole is insoluble in water; the other four substances are soluble in water.
- The peptide reactivity for 2-mercaptobenzothiazole is high and that for 3-aminophenol is minimal; peptide reactivity data for the three other substances is not available.
- The EC3 values for the five substances identified as sensitizers by the traditional LLNA are: 0.6% for cobalt chloride, 1.7% for 2-mercaptobenzothiazole, 3.2% for 3-aminophenol, 4.8% for nickel [II] sulfate hexahydrate, and 90% for methyl methacrylate.
- All five substances are considered nonirritants based on available GP data (**Table 6-11**).

Table 6-11 Discordant Results for the LLNA: DA When Multiple Decision Criteria are Used[1]

Substance[2]	Vehicle[3]	LLNA: DA[4]	Traditional LLNA[4]	Skin Irritant?
Chlorobenzene	AOO	2.4, 25%	- (1.7, 25%)[5]	No data. Low irritancy potential assumed based on clinical literature.
Hexane	AOO	2.3, 100%	- (2.2, 100%)	Irritant at 100% (humans)
Isopropanol	AOO	1.97, 50%[5]	- (1.7, 50%)[5]	Negative at 100% (rabbits)
Methyl salicylate	AOO	1.77, 25%[5]	- (2.9, 20%)	Irritant at 10% AOO (mice)
Salicylic acid	AOO	2.0, 25%	- (2.4, 25%)	Irritant at 20% aq. (mice)
3-Aminophenol (3.2%) (2 LLNA: DA tests)	AOO	2.4, 10% and 1.8, 10%[6]	+ (5.7, 10%)	Nonirritant at 5% (GP)
Cobalt chloride (0.6%)	DMSO	2.0, 5%	+ (7.2, 5%)	Negative at ≤ 0.5% (GP)
2-Mercaptobenzothiazole (1.7%)	DMF	2.0, 50%[5]	+ (8.6, 10%)	Nonirritant at 10% (GP)
Methyl methacrylate (90%)	AOO	1.8, 100%	+ (3.6, 100%)	Nonirritant at 3 M (GP)
Nickel (II) sulfate hexahydrate (4.8%) (2 LLNA: DA tests)	DMSO	2.1, 10% and 2.2, 5%[7]	+ (3.1, 5%)	Nonirritant at 0.15% (GP); Irritant at 10% (humans)

Abbreviations: AOO = acetone: olive oil (4:1); aq. = aqueous; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; GP = guinea pig; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content.
"+" = Sensitizer.
"-" = Nonsensitizer.
[1]Data source indicated in **Appendix C.**
[2]Numbers in parentheses are EC3 values (concentrations needed to produce a stimulation index [SI] of three) for substances that are sensitizers in the traditional LLNA (see **Table 3-1)**.
[3]Vehicle listed is that used in both the LLNA: DA and the traditional LLNA, unless otherwise noted.
[4]Numbers indicated are highest SI and maximum concentration tested; highest SI is at maximum concentration tested, unless otherwise noted.
[5]Highest SI occurred at concentration 10%.
[6]Highest SI occurred at concentration 3%.
[7]Highest SI occurred at concentration 2.5%.

7.0 LLNA: DA Test Method Reliability

An assessment of test method reliability (intralaboratory repeatability and intra- and inter-laboratory reproducibility) is an essential element of any evaluation of the performance of an alternative test method (ICCVAM 2003). Repeatability refers to the closeness of agreement between test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions within a given time period (ICCVAM 1997, 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. With regard to the LLNA: DA test method, there are no known intralaboratory repeatability studies, which was also the situation with the traditional LLNA.

The reproducibility evaluation in this revised draft BRD has been updated from the January 2008 draft BRD to include an interlaboratory reproducibility evaluation and a reproducibility analysis using separate SI criteria to identify sensitizers and nonsensitizers. The available LLNA: DA data were amenable to both intralaboratory and interlaboratory reproducibility analyses. The evaluation of a single decision criterion in **Section 6.6** showed that SI $\geq$ 2.0 was the SI value that produced the lowest false negative rate among the alternative decision criteria evaluated (i.e., 3% [1/32]) when the traditional LLNA was the reference test (**Table 6-6)**. **Appendix F** describes the evaluation of reproducibility for the decision criterion of SI $\geq$ 2.0 to identify sensitizers, which was evaluated in **Section 6.6**.The evaluation of multiple decision criteria in **Section 6.7** evaluated SI $\geq$ 2.5 as the decision criterion for classifying substances as sensitizers when used with a decision criterion of SI $\leq$ 1.7 to identify nonsensitizers. Thus, this section provides an assessment of reproducibility for the decision criterion of SI $\geq$ 2.5 to identify sensitizers.

7.1 Intralaboratory Reproducibility

Idehara et al. (2008) evaluated intralaboratory reproducibility of EC3 values for the LLNA: DA using two substances (isoeugenol and eugenol) that were each tested in three different experiments (**Table 7-1**). The data indicate CVs of 21% and 11% for isoeugenol and

eugenol, respectively. The authors state that for both compounds the EC3 values appeared to be close and that for each test substance the SI values for the same concentration were fairly reproducible (Idehara et al. 2008). NICEATM also determined the intralaboratory reproducibility of EC2.5 values (estimated concentrations needed to produce a stimulation index of 2.5) for the same set of data. The results for EC2.5 indicate slightly larger intralaboratory variability compared to EC3 results with CVs of 33% and 13% for isoeugenol and eugenol, respectively.

Table 7-1 Intralaboratory Reproducibility of EC3 and EC2.5 Values Using the LLNA: DA[1]

Isoeugenol			
Concentration (%)	**Experiment 1[2]**	**Experiment 2[2]**	**Experiment 3[2]**
Vehicle (AOO)	1.00 ± 0.54	1.00 ± 0.54	1.00 ± 0.30
0.5	1.50 ± 0.54	-------	1.22 ± 0.13
1	2.28 ± 0.60	-------	2.77 ± 1.01
2.5	2.78 ± 0.17	3.11 ± 1.15	3.01 ± 0.98
5	3.39 ± 0.69	4.39 ± 1.25	-------
10	5.68 ± 1.19	6.77 ± 0.23	-------
EC3	**3.40%**	**2.35%**	**2.46%**
EC2.5	**0.82%**	**1.37%**	**0.75%**
Mean EC3: 2.74% ± 0.58% and 21% CV *Mean EC2.5: 1.46% ± 0.48% and 33% CV*			
Eugenol			
Concentration (%)	**Experiment 1[2]**	**Experiment 2[2]**	**Experiment 3[2]**
Vehicle (AOO)	1.00 ± 0.17	1.00 ± 0.17	1.00 ± 0.09
5	2.92 ± 1.00	2.80 ± 1.08	3.24 ± 0.70
10	7.35 ± 2.62	4.47 ± 0.98	4.79 ± 0.94
25	10.92 ± 3.63	5.62 ± 3.20	7.07 ± 0.44
EC3	**5.09%**	**5.59%**	**4.50%**
EC2.5	**4.33%**	**3.59%**	**2.87%**
Mean EC3: 5.06% ± 0.55% and 11% CV *Mean EC2.5: 4.23% ± 0.57% and 13% CV*			

Abbreviations: AOO = acetone: olive oil (4:1); CV = coefficient of variation; EC2.5 = estimated concentration needed to produce a stimulation index of 2.5; EC3 = estimated concentration needed to produce a stimulation index of three; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content.

[1]Based on results discussed in Idehara et al. 2008; the number per group was not specified.

[2]Mean stimulation index value ± standard deviation.

7.2 Interlaboratory Reproducibility

Furthermore, data were submitted to NICEATM (**Appendix D**) from a two-phased interlaboratory validation study on the LLNA: DA test method (Omori et al. 2008). In the

first phase of the interlaboratory validation study, a blinded test of 12 substances was conducted in 10 laboratories. Three substances (i.e. 2,4-dinitrochlorobenzene, hexyl cinnamic aldehyde, and isopropanol) were tested in all 10 laboratories. The remaining nine substances were randomly assigned to subsets of three of the 10 laboratories **(Table 7-2)**. In each laboratory, each substance was tested one time at three different concentrations. The dose levels for each substance were predetermined (i.e., the participating laboratories did not determine their own dose levels for testing). Nine substances are sensitizers and three substances are nonsensitizers according to the traditional LLNA. Six substances are ICCVAM-recommended LLNA performance standards reference substances: cobalt chloride, 2,4-dinitrochlorobenzene, hexyl cinnamic aldehyde, isoeugenol, isopropanol, and methyl salicylate.

The second phase of the interlaboratory validation study was designed to determine the reason for inconsistencies obtained from the two metals dissolved in DMSO (i.e., cobalt chloride and nickel (II) sulfate hexahydrate) and thus to further evaluate the reliability of the LLNA: DA for testing metallic salts using DMSO as a vehicle. Five coded substances (two of the five substances were unique to the second phase of the interlaboratory validation study) were tested in seven laboratories **(Table 7-3)**. One substance (i.e. hexyl cinnamic aldehyde) was tested in all seven laboratories. The remaining four substances (i.e., cobalt chloride, nickel (II) sulfate hexahydrate, lactic acid, and potassium dichromate) were randomly assigned to subsets of four of the seven laboratories. Each laboratory tested the substance one time at three different dose levels. Again, the dose levels for each substance were predetermined. Of the two substances not previously tested in the first phase of the interlaboratory validation study (i.e., lactic acid and potassium dichromate), one is a nonsensitizer and the other is a sensitizer according to traditional LLNA results, respectively. In addition, lactic acid is an ICCVAM-recommended LLNA performance standards reference substance.

The LLNA: DA test results from the two-phased interlaboratory validation studies are amenable to interlaboratory reproducibility analyses for three endpoints: sensitizer (positive) or nonsensitizer (negative) classification, and EC2.5 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 (EC2.5 values) (**Sections 7.2** and **7.3**).

Table 7-2 Substances and Allocation for the First Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance[1]	Vehicle	Concentration Tested (%)			Laboratory									
					1	2	3	4	5	6	7	8	9	10
2,4-Dinitro-chlorobenzene (+)	AOO	0.03	0.10	0.30	X	X	X	X	X	X	X	X	X	X
Hexyl cinnamic aldehyde (+)	AOO	5	10	25	X	X	X	X	X	X	X	X	X	X
Isopropanol (-)	AOO	10	25	50	X	X	X	X	X	X	X	X	X	X
Abietic acid (+)	AOO	5	10	25		X				X	X			
3-Aminophenol (+)	AOO	1	3	10	X		X					X		
Dimethyl isophthalate (-)	AOO	5	10	25	X		X				X			
Isoeugenol (+)	AOO	1	3	10				X	X				X	
Methyl salicylate (-)	AOO	5	10	25			X				X			X
Formaldehyde (+)	ACE	0.5	1.5	5.0	X	X			X					
Glutaraldehyde (+)	ACE	0.05	0.15	0.50	X	X			X					
Cobalt chloride[2] (+)	DMSO	0.3	1.0	3.0				X		X		X		
Nickel (II) sulfate hexahydrate (+)	DMSO	1	3	10				X		X		X		

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content.

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

[2]Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

Table 7-3 Substances and Allocation for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance[1]	Vehicle	Concentration Tested (%)			Laboratory						
					11	12	13	14	15	16	17
Hexyl cinnamic aldehyde (+)	AOO	5	10	25	X	X	X	X	X	X	X
Cobalt chloride[2] (+)	DMSO	1	3	5	X		X	X			X
Lactic acid (-)	DMSO	5	10	25	X		X		X	X	
Nickel (II) sulfate hexahydrate (+)	DMSO	1	3	10	X	X		X		X	
Potassium dichromate (+)	DMSO	0.1	0.3	1.0	X	X			X		X

Abbreviations: AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content.
[1](+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.
[2]Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

7.2.1 Interlaboratory Reproducibility – Qualitative Results

The qualitative (positive/negative) interlaboratory concordance analysis for the 12 substances that were tested during the first phase of the LLNA: DA interlaboratory validation study is shown in **Table 7-4** for SI ≥ 2.5. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), ten substances tested in either three or 10 laboratories had consistent results leading to 100% (3/3 or 10/10) interlaboratory concordance for those substances. There were two discordant substances (i.e., 3-aminophenol and nickel (II) sulfate hexahydrate) for which interlaboratory concordance was 67% (2/3). One of the three laboratories that tested 3-aminophenol reported SI ≥ 2.5, at the highest dose tested (i.e., SI = 2.83 at 10%) and two laboratories did not achieve SI ≥ 2.5 at any dose tested (**Appendix D**). One of the three laboratories that tested nickel (II) sulfate hexahydrate reported a maximum SI = 1.52, while the other two laboratories produced an SI ≥ 2.5 at all three doses tested (**Appendix D**). Notably, when analyzing the dose response curves for the 3 tests performed for nickel (II) sulfate in the first phase of the two-phased interlaboratory validation study, only one study demonstrated a sufficient dose response (i.e., a parallel increase in SI relative to increase in concentration). Since 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: DA concordance data from the first phase of the interlaboratory validation study.

Table 7-4 Qualitative Results for the First Phase of the Interlaboratory Validation Studies for the LLNA: DA (SI ≥ 2.5)

Substance[1]	Laboratory[2]										Concordance
	1	2	3	4	5	6	7	8	9	10	
2,4-Dinitrochlorobenzene (+)	+	+	+	+	+	+	+	+	+	+	10/10
Hexyl cinnamic aldehyde (+)	+	+	+	+	+	+	+	+	+	+	10/10
Isopropanol (-)	-	-	-	-	-	-	-	-	-	-	10/10
Abietic acid (+)		+				+	+				3/3
3-Aminophenol (+)	+		-					-			**2/3**
Dimethyl isophthalate (-)	-		-				-				3/3
Isoeugenol (+)				+	+				+		3/3
Methyl salicylate (-)			-				-			-	3/3
Formaldehyde (+)	+	+			+						3/3
Glutaraldehyde (+)	+	+			+						3/3
Cobalt chloride[3] (+)				+[4]		+		+			3/3
Nickel (II) sulfate hexahydrate (+)				-[5]		+		+[5]			**2/3**

Bolded substances did not achieve 100% interlaboratory concordance.
Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content; SI = stimulation index
[1](+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.
[2](+) indicates sensitizers and (-) indicates nonsensitizers according to LLNA: DA tests.
[3]Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.
[4]Data not reported for the highest dose (i.e., 3%), only for 0.3% and 1%.
[5]Insufficient dose response.

The qualitative (positive/negative) interlaboratory concordance analysis for the five substances that were tested during the second phase of the LLNA: DA interlaboratory validation study is shown in **Table 7-5**. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), four substances (i.e., hexyl cinnamic aldehyde, lactic acid, nickel [II] sulfate hexahydrate, and potassium dichromate) tested in either four or seven laboratories had consistent results leading to 100% (4/4 or 7/7) interlaboratory concordance for those substances. There was one discordant substance (i.e., cobalt chloride) for which interlaboratory concordance was 75% (3/4). One of the four laboratories that tested cobalt

chloride did not report a maximum SI ≥ 2.5 at any dose, while the other three laboratories produced an SI ≥ 2.5 at the highest dose tested. Cobalt chloride was also tested in the first phase of the interlaboratory validation study where interlaboratory concordance was 100% (3/3). Furthermore, as mentioned previously, the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), and therefore there were no traditional LLNA concordance data for comparison with the LLNA: DA concordance data from the second phase of the interlaboratory validation study.

Table 7-5 Qualitative Results for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA (SI ≥ 2.5)

Substance[1]	Laboratory[2]							Concordance
	11	12	13	14	15	16	17	
Hexyl cinnamic aldehyde (+)	+	+	+	+	+	+	+	7/7
Cobalt chloride[3] (+)	**-**		**+**	**+**			**+**	**3/4**
Lactic acid (-)	-		-		-	-		4/4
Nickel (II) sulfate hexahydrate (+)	-	-		-		-		4/4
Potassium dichromate (+)	+	+			+		+	4/4

Bolded substance did not achieve 100% interlaboratory concordance.
Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content; SI = stimulation index.
[1](+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.
[2](+) indicates sensitizers and (-) indicates nonsensitizers according to LLNA: DA tests.
[3]Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

7.2.2 Interlaboratory Reproducibility – EC2.5 Values

The available quantitative (i.e., EC2.5 value) data for interlaboratory reproducibility analysis were obtained from the LLNA: DA results for ten sensitizers that were tested during the first and second phase of the LLNA: DA interlaboratory validation study. The equation used for calculating EC2.5 values for the positive results was modified based on the method of linear interpolation reported by Gerberick et al. (2004) for the EC3:

$$EC2.5 = c + \left[\frac{(2.5 - d)}{(b - d)}\right] \times (a - c)$$

where the data points lying immediately above and below the SI = 2.5 on the dose response curve have the coordinates of (a, b) and (c, d), respectively (Gerberick et al. 2004). For substances for which the lowest concentration tested resulted in an SI > 2.5, an EC2.5 value was extrapolated according to the equation:

$$EC2.5_{ex} = 2^{\left\{\log_2(c)+\frac{(2.5-d)}{(b-d)}\times\left[\log_2(a)-\log_2(c)\right]\right\}}$$

where the point with the higher SI is denoted with the coordinates of (a, b) and the point with the lower SI is denoted (c, d) (Gerberick et al. 2004).

The EC2.5 values from each laboratory were used to calculate CV values for each substance. The resulting values for the first and second phase of the interlaboratory validation study are shown in **Tables 7-6** and **7-7**, respectively. In the first phase of the interlaboratory validation study, CV values ranged from 26% (i.e., hexyl cinnamic aldehyde) to 133% (i.e., cobalt chloride) and the mean CV was 79% (**Table 7-6**). In the second phase of the interlaboratory validation study, CV values ranged from 20% (i.e., hexyl cinnamic aldehyde) to 92% (i.e., cobalt chloride) and the mean CV was 62% (**Table 7-7**).

The ICCVAM-recommended LLNA performance standards indicate that interlaboratory reproducibility should be evaluated with at least two sensitizing chemicals with well-characterized activity in the traditional LLNA. Acceptable reproducibility is attained when each laboratory obtains ECt values (estimated concentrations needed to produce a stimulation index of a specified threshold) within 0.025% to 0.1% for 2,4-dinitrochlorobenzene and within 5% to 20% for hexyl cinnamic aldehyde (ICCVAM 2009). In the first phase of the interlaboratory validation study, five laboratories reported EC2.5 values outside the acceptance range indicated for 2,4-dinitrochlorobenzene; two of the five laboratories obtained EC2.5 values that were lower than the specified acceptance range (i.e., 0.025%) and three of the five laboratories obtained EC2.5 values that were higher than the specified acceptance range (i.e., 0.1%) (**Table 7-6**). For hexyl cinnamic aldehyde, all the laboratories obtained an EC2.5 value within the acceptance range (5% to 20%). In the second phase of the interlaboratory validation study, only hexyl cinnamic aldehyde was tested and all seven laboratories obtained EC2.5 values that were within the acceptance range indicated (**Table 7-7**).

Table 7-6 EC2.5 Values from the First Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance[1]	Laboratory										Mean EC2.5 (%)	CV (%)
	1	2	3	4	5	6	7	8	9	10		
2,4-Dinitrochlorobenzene (+)	**0.026 (11.97)**	**0.063 (9.23)**	**0.039 (9.96)**	**0.022 (8.53)**	**0.112 (7.86)**	**0.025 (15.14)**	**0.011 (13.18)**	**0.039 (12.60)**	**0.023 (10.89)**	**0.131 (4.71)**	**0.049**	**84**
Hexyl cinnamic aldehyde (+)	**8.473 (5.78)**	**9.414 (4.82)**	**11.402 (4.44)**	**7.900 (5.11)**	**14.594 (3.97)**	**10.759 (5.50)**	**6.778 (7.09)**	**7.032 (10.22)**	**12.530 (3.88)**	**9.135 (3.51)**	**9.802**	**26**
Isopropanol (-)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Abietic acid (+)		6.418				6.469	11.525				8.137	36
3-Aminophenol (+)	5.471		NA					NA			5.471	NA
Dimethyl isophthalate (-)	NA		NA				NA				NA	NA
Isoeugenol (+)				0.657	5.191				0.874		2.240	114
Methyl salicylate (-)			NA				NA			NA	NA	NA
Formaldehyde (+)	0.393	1.105			4.179						1.892	106
Glutaraldehyde (+)	0.091	0.351			0.296						0.246	56
Cobalt chloride[2] (+)				0.822[3]		0.047		0.104			0.325	133
Nickel (II) sulfate hexahydrate (+)				NA[4]		0.352		IDR			0.352	NA

Bolded text indicates substances that are ICCVAM-recommended murine local lymph node assay (LLNA) performance standards reference substances (ICCVAM 2009). Values in parentheses are highest stimulation index (SI) values achieved. For both 2,4-dinitrochlorobenzene and hexyl cinnamic aldehyde, the highest SI values achieved were from the highest dose tested (i.e., 0.30% for 2,4-dinitrochlorobenzene and 25% for hexyl cinnamic aldehyde). Shading shows EC2.5 values (estimated concentration needed to produce a stimulation index of 2.5) that are outside of the acceptable range indicated in the ICCVAM-recommended LLNA performance standards: 5 - 20% for hexyl cinnamic aldehyde and 0.025 - 0.1% for 2,4-dinitrochlorobenzene.

Abbreviations: CV = coefficient of variation; IDR = insufficient dose response; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content. NA = not applicable.

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

[2]Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

[3]Data not reported for the highest dose (i.e., 3%), only for 0.3% and 1%.

[4]Insufficient dose response.

Table 7-7 EC2.5 Values from the Second Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance[1]	Laboratory							Mean	%CV
	11	12	13	14	15	16	17		
Hexyl cinnamic aldehyde (+)	**7.737 (4.47)**	**7.374 (5.71)**	**6.772 (5.41)**	**6.361 (7.60)**	**9.902 (3.92)**	**5.366 (8.42)**	**6.783 (6.45)**	**7.185**	**20**
Cobalt chloride[2] (+)	NA		4.111	1.202			0.699	2.004	92
Lactic acid (-)	NA		NA		NA	NA		NA	NA
Nickel (II) sulfate hexahydrate (+)	NA	NA		NA		NA		NA	NA
Potassium dichromate (+)	0.372	0.269			0.087		0.063	0.198	75

Bolded text indicates substances that are ICCVAM-recommended murine local lymph node assay (LLNA) performance standards reference substances (ICCVAM 2009). Values in parentheses are highest stimulation index (SI) values achieved. For hexyl cinnamic aldehyde, the highest SI values achieved were from the highest dose tested (i.e., 25%). None of the EC2.5 values (estimated concentrations needed to produce a stimulation index of 2.5) are outside of the acceptable range indicated in the ICCVAM-recommended LLNA performance standards (i.e., 5 - 20% for hexyl cinnamic aldehyde).
Abbreviations: CV = coefficient of variation; NA = not applicable.
[1](+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.
[2]Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

The interlaboratory CV values for both the first and second phase of the interlaboratory validation study for the LLNA: DA EC2.5 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 6.8 to 83.7% 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: DA (i.e., hexyl cinnamic aldehyde, 2,4-dinitrochlorobenzene, and isoeugenol). All interlaboratory CV values for the LLNA: DA were greater than that for the traditional LLNA. The CV of 84% for 2,4-dinitrochlorobenzene was greater than the two CV values of 37.4% and 27.2% (which were calculated from five values each), reported by ICCVAM (1999). The CV of 26% and 20% for hexyl cinnamic aldehyde tested in the first and second phase of the LLNA: DA interlaboratory validation study, respectively, were both greater than the 6.8% reported by ICCVAM (1999). The CV of 114% for isoeugenol tested in the LLNA: DA was greater than the 41.2% 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	37.4
	0.5	0.6	0.4	0.6	0.3	27.2
Hexyl cinnamic aldehyde	7.9	7.6	8.4	7.0	8.1	6.8
Isoeugenol	1.3	3.3	1.8	3.1	1.6	41.2
Eugenol	5.8	14.5	8.9	13.8	6.0	42.5
SLS	13.4	4.4	1.5	17.1	4.0	83.7

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 report.

7.3 Reproducibility for the LLNA: DA Accuracy Analysis Using Multiple Alternative Decision Criteria

Section 6.7 details the accuracy analysis for the LLNA: DA (using the most prevalent outcome for substances with multiple tests) when using two decision criteria for LLNA: DA results: one criterion to classify substances as sensitizers (SI ≥ 2.5) and one criterion to classify substances as nonsensitizers (SI ≤ 1.7). SI ≥ 2.5 was evaluated for classifying sensitizers because it resulted in no false positives, and SI ≤ 1.7 was evaluated for classifying substances as nonsensitizers because it resulted in no false negatives, with respect to traditional LLNA data. 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 44 substances occurred in one of three SI categories. The three SI categories were:

- SI ≤ 1.7 for classifying nonsensitizers
- 1.7 < SI < 2.5, the range of uncertainty with respect to classification by the traditional LLNA
- SI ≥ 2.5 to classify substances as sensitizers

The validation database for the LLNA: DA consists of 123 tests of 44 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. Eighty-seven percent of the tests (27/31) that yielded SI ≤ 1.7 were for substances that were classified as nonsensitizers by the traditional LLNA; 13% of the tests (4/31) that yielded SI ≤ 1.7 were for substances that were classified as sensitizers by the traditional LLNA. Fifty-eight percent (7/12) of the tests that yielded 1.7 < SI < 2.5 were for substances that were classified as sensitizers by the traditional LLNA. Four tests produced SI values near either end of this range (i.e., SI = 1.7 or SI = 2.5). One of the 3-aminophenol studies and one of the methyl salicylate studies produced SI = 1.76 and 1.77, respectively, and the chlorobenzene test produced SI = 2.44. The remainder of the tests in this category, 42% (5/12), were classified as nonsensitizers by the traditional LLNA. One hundred percent (80/80) of the tests that yielded SI ≥ 2.5 were for substances that were classified as sensitizers by the traditional LLNA and 0% (0/80) were classified as nonsensitizers.

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

Classification Based on Traditional LLNA	Classification Concordance with Traditional LLNA[1]			
	Maximum SI ≤ 1.7	1.7 < Maximum SI < 2.5	Maximum SI ≥ 2.5	Total
Sensitizer	4 (13%)	7 (58%)	80 (100%)	91
Nonsensitizer	27 (87%)	5 (42%)	0 (0%)	32
Total	31	12	80	123

Abbreviations: LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content; 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 123 tests evaluated in **Table 7-9** include multiple tests for 14 substances. For the 14 substances, three to 18 tests were available. **Table 7-10** shows the proportion of the tests for each substance that produced SI values in each category. For the four nonsensitizers with multiple test results, there were 22 tests that produced SI ≤ 1.7 and two tests that produced an SI of between 1.7 and 2.5. For the 10 sensitizers with multiple test results, however, SI values occurred in all three SI categories. The results for nickel (II) sulfate hexahydrate were particularly variable: 50% (4/8) produced SI ≤ 1.7 (i.e., four tests with SI = 0.79, 1.24, 1.52,

and 1.56), 25% (2/8) produced 1.7 < SI < 2.5 (SI = 2.13 and 2.17), and 25% (2/8) produced SI ≥ 2.5 (SI = 3.49 and 11.78). 3-Aminophenol produced SI values in two categories: 67% (2/3) of the tests had 1.7 < SI < 2.5 (SI = 1.76 and 2.38), and 33% (1/3) of the tests had SI ≥ 2.5 (SI = 2.83). Cobalt chloride tests also produced SI values in two categories: 12.5% (1/8) of the tests had 1.7 < SI < 2.5 (SI = 2.01) and seven of eight tests (i.e., 87.5%) produced SI ≥ 2.5 (SI = 2.54, 2.66, 3.64, 4.25, 5.06, 8.07, and 20.55). The multiple test results for the remaining seven traditional LLNA sensitizers were 100% concordant (**Table 7-10**).

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

Substance	Concordance Among Multiple Tests[1]			Total
	Maximum SI ≤ 1.7	1.7 < Maximum SI < 2.5	Maximum SI ≥ 2.5	
Sensitizers[2]				
Abietic acid	0 (0%)	0 (0%)	4 (100%)	4
3-Aminophenol	0 (0%)	2 (67%)	1 (33%)	3
Cobalt chloride	0 (0%)	1 (12.5%)	7 (87.5%)	8
2,4-Dinitrochlorobenzene	0 (0%)	0 (0%)	11 (100%)	11
Formaldehyde	0 (0%)	0 (0%)	4 (100%)	4
Glutaraldehyde	0 (0%)	0 (0%)	4 (100%)	4
Hexyl cinnamic aldehyde	0 (0%)	0 (0%)	18 (100%)	18
Isoeugenol	0 (0%)	0 (0%)	4 (100%)	4
Nickel (II) sulfate hexahydrate	4 (50%)	2 (25%)	2 (25%)	8
Potassium dichromate	0 (0%)	0 (0%)	5 (100%)	5
Nonsensitizers[2]				
Dimethyl isophthalate	4 (100%)	0 (0%)	0 (0%)	4
Isopropanol	10 (91%)	1 (9%)	0 (0%)	11
Lactic acid	5 (100%)	0 (0%)	0 (0%)	5
Methyl salicylate	3 (75%)	1 (25%)	0 (0%)	4

Abbreviations: LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content; 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 LLNA: DA Data Quality

The data quality section in this revised draft BRD has been updated from the January 2008 draft BRD to indicate that all of the studies included in this performance evaluation are based on individual animal data submitted to NICEATM in the form of original data and study records. Furthermore, since the January 2008 draft BRD was made available, manuscripts detailing the results for 31 substances evaluated in the intralaboratory study and 14 substances evaluated in the two-phased interlaboratory validation have been published in the peer-reviewed literature (Idehara et al. 2008; Omori et al. 2008). Also, an independent audit has been conducted to confirm that the reported data from the intralaboratory validation study (i.e., assessment of 31 substances from Idehara et al. 2008) performed by Daicel Chemical Industries, Ltd. was the same as the data originally recorded (Idehara et al. 2008). The data from the two-phased interlaboratory validation study were not subjected to a formal audit, but the raw data were reportedly entered directly into formatted MS-Excel templates provided by the study management team prior to being used for analyses (Omori et al. 2007). In addition, data recently received for 14 substances evaluated in an intralaboratory validation study (Idehara, unpublished) were also not subjected to a formal audit. The intralaboratory assessment at Daicel Chemical Industries, Ltd. (Idehara et al. 2008; Idehara, unpublished), as well as the two-phased interlaboratory validation study (Omori et al. 2008), did not conduct their studies in compliance with Good Laboratory Practice guidelines, although all of the participating laboratories reportedly have this capability.

9.0. Other Scientific Reports and Reviews

This section has been updated to include information on the intralaboratory validation study and the two-phased interlaboratory validation based on publication of the data since the January 2008 draft BRD. In addition, information is included on the regulatory acceptance of the LLNA: DA test method by the Japanese Center for the Validation of Alternative Methods (JaCVAM).

Yamashita et al. (2005) describe the development of the LLNA: DA as an alternative non-radioisotope LLNA test method. The manuscript details the determination of an optimal dosing schedule and further compares SI values obtained from lymph node weights versus ATP content to determine an appropriate lymphocyte proliferation endpoint. The authors further assessed the intermediate precision and sensitivity/specificity of the LLNA: DA. In these experiments, four compounds (2,4-dinitrochlorbenzene, eugenol, α-hexyl cinnamic aldehyde, and methyl salicylate) were tested and no significant differences were noted in the SI levels generated from the LLNA: DA and the traditional LLNA. This study provided the basis for the expanded intralaboratory study of 31 substances analyzed by Daicel Chemical Industries, Ltd. (described in **Sections 6.0** and **7.0**) for which the data were published by Idehara et al. (2008).

Idehara et al. (2008) summarize the LLNA: DA test method in terms of test substance dosing schedule, preparation of single cell suspensions of the auricular lymph nodes, measurement of ATP content, and explanation of statistical analyses employed. The authors further describe how the results correlate between ATP content and lymph node cell number, the test results (i.e., mean SI values and EC3) obtained for the 31 substances, the concordance of the LLNA: DA versus the traditional LLNA EC3, and the reproducibility of EC3 and SI values. Based on the details included in the manuscript, the authors conclude that the SI values obtained from measuring ATP content were similar to the traditional LLNA and therefore the LLNA: DA was a promising non-radioisotope modified test method for evaluating the skin sensitization potential of substances.

Omori et al. (2008) describe the two-phased interlaboratory validation study used to evaluate the reliability and relevance of the LLNA: DA test method (see **Section 7.0**). They describe the organization and technology transfer of the test method between the laboratories, as well

as test substance selection and allocation. They further describe the development of the LLNA: DA and the resulting standard protocol for the LLNA: DA interlaboratory study. The provide the interlaboratory data for analyzing both ATP content with regard to SI values and lymph node weight and discuss assay sensitivity and interlaboratory variability. Based on the data summarized in the manuscript, the authors conclude that in the first phase of the interlaboratory validation study, a large variation was observed for two substances (i.e., cobalt chloride and nickel [II] sulfate hexahydrate) but in the second phase of the interlaboratory validation study this variation was small. The authors attributed the initial variation to application of DMSO as the solvent for the metallic salts and therefore, prior to the second phase of the interlaboratory validation study, included operation of LLNA: DA with DMSO in the technology transfer seminar. In conclusion, the authors view the LLNA: DA as a reliable test method for predicting skin sensitization potential of substances.

Regarding the LLNA: DA test method, non-commission members of JaCVAM met on August 28, 2008 at the National Institute of Health Sciences, Tokyo, Japan, and endorsed the following statement: "Following the review of the results of the Ministry of Health, Labour and Welfare (MHLW)-funded validation study on the LLNA: DA coordinated by Japanese Society for Alternative to Animal Experiments, it is concluded that the LLNA: DA can be used for distinguishing between sensitizer and nonsensitizer chemicals within the context of the OECD testing guidelines No. 429 on skin sensitization: LLNA. The JaCVAM regulatory acceptance board has been regularly kept informed of the progress of the study, and this endorsement was based on an assessment of various documents, including, in particular, the report on the results from the study, and also on the evaluation supported by MHLW of the study prepared for the JaCVAM ad hoc peer review panel." JaCVAM has informed NICEATM-ICCVAM that in January 2009 they will submit the SPSF for recommendation of the LLNA: DA from the Japanese National Coordinator to OECD secretary. They will make clear that the SPSF was produced in collaboration with NICEATM-ICCVAM.

10.0 Animal Welfare Considerations

This section of the draft BRD has not changed from the January 2008 draft BRD. The LLNA: DA will require the use of the same number of animals when compared to the updated ICCVAM LLNA protocol (Appendix A of ICCVAM 2009). However, since the traditional LLNA uses radioactive materials and as such its use might be restricted due to the complications associated with storage, use, and disposal, broader use of a non-radioactive alternative to the traditional LLNA, such as the LLNA: DA, could further reduce the number of guinea pigs that are used to assess skin sensitization.

10.1 Rationale for the Need to Use Animals

The rationale for the use of animals in the LLNA: DA is the same as the rationale for the traditional LLNA. There currently are no valid and accepted non-animal test methods 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. Additionally, 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% (15 vs. 25) could be achieved by using a reduced version of the LLNA: DA, 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 elicit 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.

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: DA

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: DA would be similar to the traditional LLNA, since their test method protocols are experimentally similar. Notably, the test method developer does indicate that when the LLNA: DA test method is conducted, all the procedural steps from lymph node excision to the determination of ATP content should be performed without delay since ATP content decreases over time (Idehara et al. 2008; Omori et al. 2008).

11.2 Laboratories and Major Fixed Equipment Required to Conduct the LLNA: DA

Compared to the traditional LLNA, the LLNA: DA will not require laboratories, equipment, and licensing permits for handling radioactive materials. However, the LLNA: DA does require access to a luminometer capable of detecting light emission by ATP for the assessment of lymphocyte proliferation. The remaining requirements (e.g., animal care laboratories) are the same between the two methods.

11.3 LLNA: DA Training Considerations

The level of training and expertise needed to conduct the LLNA: DA should be similar to the traditional LLNA, although the LLNA: DA includes an additional requirement that users operate a luminometer instead of a scintillation counter and be able process this data.

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Appendix A

Standard Operating Procedures for the LLNA: DA Test Method

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Appendix A1

Standard Operating Procedures/Protocol for the LLNA: DA Test Method

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These are the standard operating procedures performed during the two-phased interlaboratory test method validation study (Omori et al. 2008) for the murine local lymph node assay (LLNA) modified by Daicel Chemical Industries, Ltd., based on adenosine triphosphate content (ATP; referred to hereafter as the "LLNA: DA") as confirmed by the LLNA: DA Validation Committee and provided by the study director.[1] These procedures are intended for tests conducted to evaluate a single test substance. Although the standard operating procedures detailed herein are specific for the interlaboratory test method validation study, the substances tested in the intralaboratory validation study followed a technically similar LLNA: DA test method protocol (Idehara et al. 2008; Idehara unpublished data).

1.0 Preparation of Equipment and Materials

Prepare the experimental equipment, materials, and reagents given in **Table A-1**. Luminometer tubes, 15 mL test tubes, 50 mL test tubes, petri dishes, and slide glass should be disposable. The underlined items will be provided by the LLNA: DA Validation Committee but in some cases, a luminometer will be furnished by the test facilities. All other materials will be provided by the test facilities.

Table A-1 List of Required Equipment, Materials and Reagents

Name of Equipment, Material, or Reagent	Manufacturer	Comment (Trade Name, Model Number, etc.)
Luminometer	Kikkoman Corporation, Japan	LUMITESTER C-100 Detection Range: $4x10^{-12}$ – $1x10^{-6}$ M Upper Limit: 1,000,000 RLU
Luminometer tubes	Kikkoman Corporation, Japan	Polypropylene, sterilized
15 mL test tubes	IWAKI brand	Polypropylene, sterilized
50 mL test tubes	IWAKI brand	Polypropylene, sterilized
Petri dish	Corning Incorporated	Cell culture dish, sterilized
Cell scraper	Costar brand	Disposable cell scraper, sterilized
Slide glass	Matsunami	Micro slide glass
Vortex mixer		
Analytical balance		For body weight measurements (readability of at least 0.1 g)

[1] 2/6/2006: Confirmed by LLNA: DA Validation Committee; 2/17/2006: Revised by Takashi Omori; 2/19/2006: Revised by Takashi Omori; 3/27/2006: Revised by Takashi Omori; 4/2/2006: Revised by Takashi Omori; 12/2/2006: Revised by Takashi Omori.

Name of Equipment, Material, or Reagent	Manufacturer	Comment (Trade Name, Model Number, etc.)
Analytical balance		For lymph node weight measurements (readability of at least 0.1 mg)
Brush	Ikkyuen	Osho
Phosphate buffered saline	Invitrogen Gibco™	pH 7.2, sterilized
Luciferin-luciferase reagent	Kikkoman Corporation, Japan	CheckLite™ 250 Plus[1]
Cages		Capable of housing four mice, with feed and water dispensers
Micropipette		For applying test solutions (25 μL), handling phosphate buffered saline (1000 μL), tissue suspension (20 μL), cell suspension (100 μL), and dissolved Luciferin-luciferase solution (100 μL)
Micropipette tips		Sterilized
Dissecting instruments		Large and small tweezers, scissors, surgical holder, injection needle and holder
Timer		With second display
General laboratory materials		Cotton, antiseptic solution, paper towel, clean sheet, test tube rack, microtube rack

Abbreviations: etc. = et cetera; g = grams; M = molar; mg = milligrams; μL = microliter; mL = milliliter; RLU = relative luminescence units.

[1]For the substances tested in the intralaboratory validation study by Daicel Chemical Industries, Ltd. (Idehara et al. 2008), the ATP content for potassium dichromate was measured by the CheckLite™ 250 Plus Kit (Kikkoman Corporation, Japan) but that for all other substances was determined using the ViaLight® HS Kit (Lonza Rockland, Inc., USA).

2.0 Preparations Prior to Delivery of Animals

The animals to be used in the tests are young adult female mice (nulliparous and non-pregnant) of the CBA/JNCrlj strain, aged between eight to twelve weeks prior to application of test and control substances. The animals will be provided by the LLNA: DA Validation Committee. Preparations should be made according to the standards of the test facilities to begin acclimatizing the animals once they have arrived on the previously agreed upon date of delivery.

Six cages capable of holding four animals each should be prepared prior to the end of acclimatization.[2] The cages should be labeled as listed in **Table A-2**. The symbol "X" represents the code of the test substance to be provided. Mark the label using the letter indicated on the datasheets provided prior to the test. The animal test group numbers are also indicated on the datasheets. The numbers should be confirmed and the cages labeled with care. This test will be performed two or three times, so it is important to include the test number on the labels.

Table A-2 Preparation of Test Group Cages

Test Group Number	Label
Group 1	Acetone: Olive Oil (4:1)
Group 2	Positive Control
Group 3	Vehicle
Group 4	Test Substance "X" – Low Concentration
Group 5	Test Substance "X" – Medium Concentration
Group 6	Test Substance "X" – High Concentration

"X" represents the code of the test substance provided by the study management team.

3.0 Delivery, Acclimatization and Animal Assignment

On the date of delivery, 25 animals will arrive and acclimatization should begin immediately. Acclimatization should be performed according to the standards of the test facilities. The animals should be acclimatized for at least five days, but no more than 16 days.

After acclimatization healthy animals with no observable skin lesions or other abnormalities should be randomly assigned to six groups of four animals each using randomly generated numbers. After assigning the animals to groups, four animals each should be placed in the six cages prepared as described in **Section 2.0**. Any animals remaining after the assignment of 24 should be omitted from the test. Should there be fewer than 24 animals with no observed abnormalities, three animals should be assigned to each group beginning with the test group with the highest number until all of the animals are assigned.

[2] For the substances tested in the intralaboratory validation study by Daicel Chemical Industries, Ltd. (Idehara et al. 2008; Idehara unpublished data), at least three animals per dose group were used (i.e., in most cases, 4 animals per control group and three animals per test substance group).

From the delivery of the animals to the end of the test procedures the temperature of the animal housing facility should be maintained at 22ºC (±3ºC) with a relative humidity of 30-70%. The animals should be housed with a light: dark cycle of 12 hours light: 12 hours dark and should be given food and water *ad libitum*. Any deviations from the standard housing and feeding procedures should be recorded.

4.0 Confirmation of Test Materials

When the test materials sent by the LLNA: DA Validation Committee arrive, confirm that the inventory document matches the contents.

The labels for each of the treatments (acetone: olive oil [4:1], positive control, vehicle, and low, medium and high concentrations of test substances) include a test substance code and a group number. After confirming that these codes match the datasheet, arrange the treatments in a test tube rack according to group number. Sodium lauryl sulfate (SLS) solution will arrive in one tube. Apportion 3 mL of SLS solution to each of the accompanying empty test tubes, mark each tube with the group number, and arrange the tubes in order in the test tube rack.

The treatments should be refrigerated immediately and only removed when beginning the test. Refrigeration of the solutions used in these procedures should be between 0-10ºC, and preferably between 2-8ºC, except when instructed differently. Should there be specific instructions as to the handling of the solutions, the instructions will be included with the materials shipment and they should be followed. For instance:

- SLS (CASRN: 151-21-3) is a 1% solution and should be kept at room temperature
- Acetone: olive oil is 4:1 volume to volume ratio
- Positive control is a 25% acetone: olive oil (4:1) solution of hexyl cinnamic aldehyde (CASRN: 101-86-0)

5.0 Procedures on Test Days 1, 2, 3 and 7

5.1 Day 1

Mark the animals on the tail with their test group number and a number from one to four. Weigh the animals and record their weight to the nearest 0.1 g on the test forms.

Remove the test materials from the refrigerator. Should the materials arrive with instructions to heat or sonicate the treatments prior to application, perform these procedures as instructed.

5.1.1 Pre-treatment with 1% SLS Solution

Beginning with Group 1 and proceeding in order to Group 6, the SLS solution should be applied with a brush to the dorsum of both ears of the mice. The number of the SLS solution used should match the test group number. The brush should be dipped in the SLS solution and applied to the dorsum of one ear using a petting motion, covering the entire dorsum with four to five strokes. Dip the brush again in the SLS solution and apply the solution to the dorsum of the other ear in the same manner.

Record the time when beginning to apply SLS solution to Group 1 and when completing application to Group 6. The application procedure should be performed continuously without delay for Groups 1 through 6.

Six brushes should be prepared and numbered, using only one brush for each test group. When performing the same application procedure on Days 2, 3, and 7 there is the possibility of brush contamination due to residual solution on the mouse auricula. It is important to switch brushes after finishing application for one group and check the number of the next brush before proceeding to the next group. After use, the brushes should be washed thoroughly and made available for the next day.

5.1.2 Test Substance Application

One hour after starting the SLS solution application, the numbered treatments should be applied to the auriculae of the mice, beginning with Group 1 and ending with Group 6. Using a micropipette or similar device, 25 μL of the test solution should be dripped slowly on the dorsum of one of the mouse's ears, covering the dorsum entirely. Again take up 25μL of treatment solution and apply it in the same manner to the dorsum of the mouse's other ear.

When applying the treatments, micropipette tips should be changed for each test group. After completing application for one test group, remove the tip and spray the end of the micropipette with an alcohol mist and wipe to avoid contamination.

Record the time when beginning to apply the test solution to Group 1 and when completing application to Group 6. The application procedure should be performed continuously without delay for Groups 1 through 6.

Immediately after completing application the test materials should be refrigerated.

5.1.3 General Information on the 1% SLS Pre-treatment and Test Substance Application

The objective of the application procedure is to first apply SLS solution to the entirety of the dorsum of the ear and then to apply a prescribed amount of test solution to the same area. Using ether anesthesia ensures ease and accuracy of the procedure. However, special care should be taken to avoid taking the life of the animals in the course of anesthesia. If one technician immobilizes the animal and extends the ear with tweezers while the other technician applies the solution, the procedure can be performed with accuracy without using anesthesia. If this approach is used six pairs of tweezers should be prepared, one for each group, to avoid contamination. Alternatively, the tweezers should be wiped with an alcohol swab after application is completed for each test group.

5.2 Days 2 and 3

Apply SLS solution and treatments using the same procedures as for Day 1.

When performing the application procedures the animals should be observed carefully for necrosis, hardening, hyperplasia or erythema of the auricula, as well as piloerection, or a decrease in locomotor activity. Any such abnormalities observed should be recorded on the test forms.

5.3 Day 7

On Day 7 the same procedures should be performed as on Days 2 and 3.

Excision of the auricular lymph nodes will be performed from 24 to 30 hours after the start of application on Day 7. It is therefore recommended that application procedures on Day 7 begin in the morning or early afternoon.

6.0 Procedure on Test Day 8 (Excision of Auricular Lymph Nodes and ATP Assay)

6.1 Laboratory Preparation

Forty-eight 15 mL test tubes should each be filled with 1.98 mL of phosphate buffered saline (PBS). The dispensing of PBS should be conducted under aseptic manipulation. Dispense a minimum of 24 mL of PBS in a 50 mL test tube. Pipetting should be under aseptic manipulation.

Dissolve the luciferin-luciferase reagent according to the ATP assay kit instructions (at least 4.8 mL are required). The ATP assay kit provided, CheckLite™ 250 Plus, includes five bottles each of Luciferin-luciferase reagent, solvent water, and ATP releasing agent. Using one bottle of each type, create a solution according to the instructions (approximately 5.5 mL). Shield the assay solutions from light using aluminum foil and refrigerate until the time of use. Immediately before using, return to room temperature and remove the foil prior to use. Dispense 0.1 mL of the ATP releasing agent included in the ATP assay kit to each of the 48 luminometer tubes. ATP assay kit reagents should be dispensed using sterilized pipette tips under aseptic manipulation to avoid contamination with ATP and microorganisms.

6.2 Body Weight Measurement

Weigh the mice and record their body weights to the nearest 0.1 g on the test forms.

6.3 Auricular Lymph Node Excision and Weight Measurement

Perform procedures in **Sections 6.3**, **6.4** and **6.5** within 24 to 30 hours after the start of treatment application on Day 7. The necessary materials for procedures in **Sections 6.3**, **6.4** and **6.5** are given in **Annex I**.

Immediately after sacrificing the mice with ether anesthesia excise completely all auricular lymph nodes for each ear (there can be one or two auricular lymph nodes) as illustrated in **Figure A-1**. Place the excised lymph nodes for one animal in a disposable petri dish and immediately measure the wet weight to the nearest 0.1 mg with an analytical balance.

6.4 Preparation of Cell Suspension

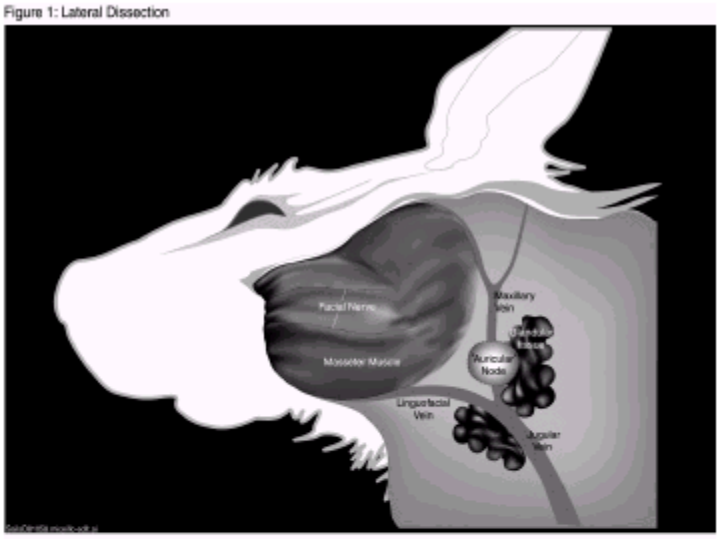
The lymph nodes from one animal should be sandwiched between two pieces of slide glass and light pressure should be applied to crush the nodes (**Figure A-2**). After confirming that the tissue has spread out thinly pull the two slides apart. Suspend the tissue on both pieces of slide glass in 1 mL of PBS. As illustrated in **Figure A-3**, each piece of slide glass should be held at an angle over the petri dish and rinsed with PBS while the tissue is scraped off of the

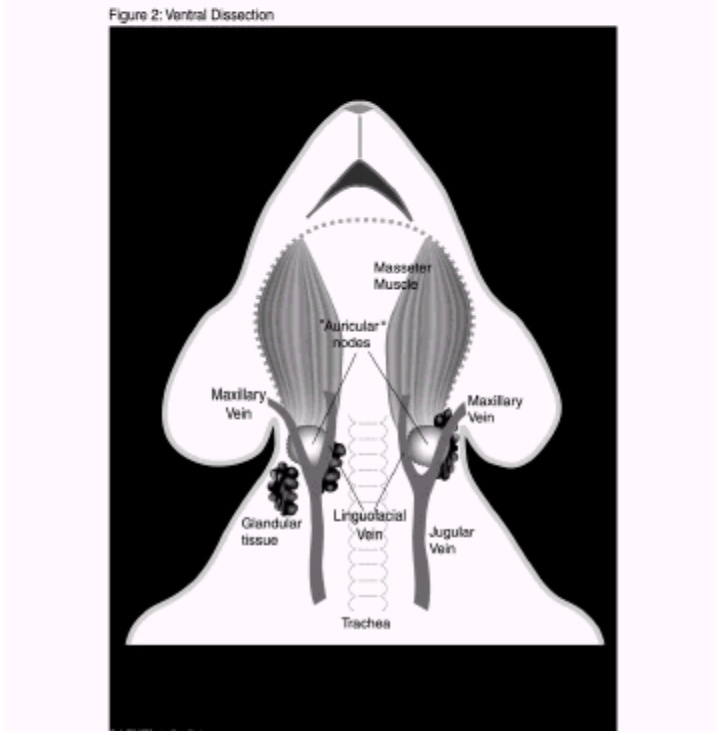
glass with repeated movements of a cell scraper. One mL of PBS should be used for rinsing both slides.

The tissue suspension in the petri dish should be homogenized lightly with the cell scraper, and 20 µL of the suspension should be taken up with a micropipette, taking care not to take up the membrane that is visible to the eye. The pipetted suspension should be added to 1.98 mL of PBS and homogenized well. This will be cell suspension No. 1. Again take up 20µL of the suspension in the petri dish, add to 1.98 mL of PBS, and homogenize well. This will be cell suspension No. 2.

These procedures should be performed while wearing gloves and a mask and micropipette tips should be sterile. Detailed step-by-step procedures are given in **Annex II**.

232 **Figure A-1 Auricular lymph nodes[3]**





233

234

[3] Taken from ICCVAM IWG LLNA Protocol (ICCVAM 2001)

Figure A-2 Preparation of cell suspension

Lymph nodes from each animal are sandwiched between two pieces of slide glass and light pressure is applied to crush the nodes.

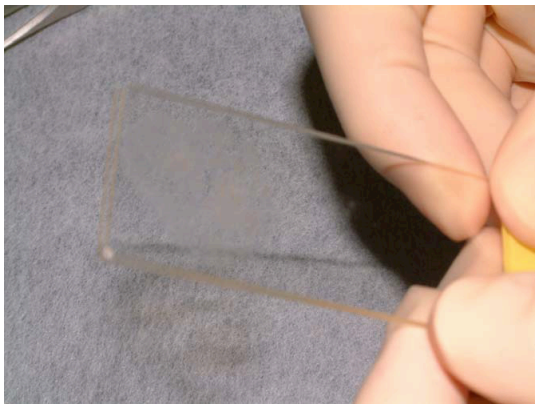
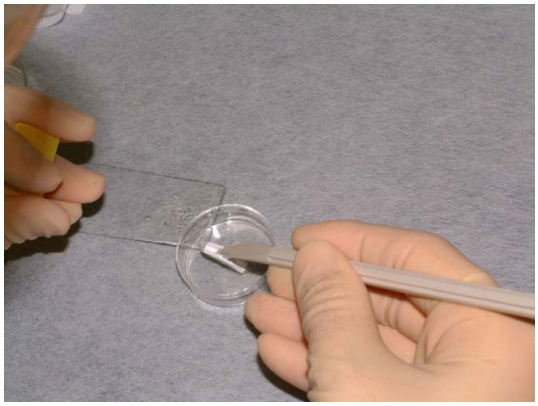


Figure A-3 Preparation of cell suspension

Rinse with PBS while scraping the tissue off of the glass with a cell scraper. Repeat the scraping motion, scooping up liquid from the petri dish as need. Use 1 mL of PBS for the nodes of each animal.



6.5 ATP Assay

Prepare 48 luminometer tubes in advance by dispensing 0.1 mL of the ATP releasing reagent provided to each tube. Add 0.1 mL of each homogenized cell suspension to the luminometer tubes and homogenize. After allowing the solution in the tube to stand for approximately 20 seconds, add 0.1 mL of the Luciferin-luciferase solution, promptly homogenize and place in the luminometer. The amount of bioluminescence (RLU; relative luminescence units) measured over 10 seconds will be displayed. Record this measurement on the test forms.

The amount of bioluminescence begins to decrease immediately after adding the Luciferin-luciferase solution. It is therefore important that the series of procedures from the addition of Luciferin-luciferase solution to switching on the luminometer are performed as quickly as possible, ideally with the same rhythm.

These procedures should be performed while wearing gloves and a mask and micropipette tips should be sterile. The detailed procedures are given in **Annex III**.

7.0 Points of Caution on Procedures from Excision to ATP Assay

The ATP content of the lymph node decreases over time after the sacrifice of the animal. It is therefore desirable that the time elapsed between sacrifice of the animal and ATP assay is uniform for each animal. The series of procedures from excision to ATP assay must be performed rapidly and without delay.

If these procedures are performed by one technician, the animals should be sacrificed one at a time. If there are multiple technicians, it is possible to divide tasks and sacrifice the animals one group at a time. If two technicians perform the procedures, steps in **Section 6.3** should be performed by one individual and steps in **Sections 6.4** and **6.5** should be performed by the other. If three technicians perform the procedures, steps in **Sections 6.3**, **6.4** and **6.5** can each be handled by one individual. If multiple technicians are involved, it is important that the timing of excision is carefully planned so that there are no delays in subsequent steps.

8.0 Data Entry

Input the body weights on Day 1 and Day 8, the lymph node weight, and the amount of ATP bioluminescence into the designated Excel file.

Annex I: Equipment and Reagents Used for the Experimental Procedures in Sections 6.3, 6.4, and 6.5

For the equipment and reagents underlined below, the items provided by the LLNA: DA Validation Committee should be used. In the event the test facility provides a luminometer, it can be used.

6.3 Auricular Lymph Node Excision and Weight Measurement

Dissecting instruments set (Tweezers, scissors, surgical holder, injection needle and holder)

Antiseptic solution

Cotton

Petri dish (24)

Analytical balance (readability of at least 0.1 mg)

6.4 Preparation of Cell Suspension

15 mL test tubes with 1.98 mL phosphate buffered saline (PBS) (48)

50 mL test tubes with at least 24 mL PBS (1)

Slide glass (48)

Tweezers (1)

Micropipette 1000 µL (1) (Volume to be measured: 1 mL)

Micropipette 100 µL (1) (Volume to be measured: 20 µL)

Cell scraper (1)

Sterilized pipette tips for 1000 µL micropipette (24) and for 100 µL micropipette (24)

Vortex mixer (1)

Paper towels

Clean sheet

Test tube rack

6.5 ATP Assay

Luminometer tubes with 0.1 mL ATP releasing agent (48)

15 mL test tube with dissolved luciferin-luciferase solution (1)

Micropipette - 100μL or 200 μL (2) (Volume to be measured: 0.1 mL)

Sterilized micropipette tips (96)

Timer (with second display) (1)

Luminometer (1)

Vortex mixer (can use same mixer listed under **Section 6.4** Preparation of Cell Suspension)

Test tube rack

Luminometer tube rack (microtube rack)

Annex II: Preparation of Cell Suspension for the Experimental Procedures in Section 6.4

1. Cover the laboratory bench with a clean sheet and place one piece of slide glass on the sheet.
2. After measuring the lymph node weights, use tweezers to move the lymph nodes from one animal from the petri dish to the center of the slide glass.
3. Place another piece of slide glass on top.
4. Pick up the two sandwiched pieces of slide glass. Squeeze the two pieces in the center to crush the lymph nodes. (Apply only light pressure. Too much pressure can break the cells.)
5. Confirm that the tissue has spread out thinly between the two slides and place the sandwiched slides on the clean sheet.
6. Fasten a tip on the 1000 μL micropipette and draw 1 mL phosphate buffered saline (PBS) from the 50 mL tube.
7. Remove the upper slide glass from the sandwiched slides and place it on the clean sheet with the side that was in contact with the lymph node tissue facing up. The other slide glass should be held at an angle in the petri dish, the side with lymph node tissue affixed facing forward, and washed with 1 mL PBS.
8. Dispose of the 1000 μL micropipette tip.
9. Scrape the tissue off of the glass with a cell scraper, scooping up PBS from the petri dish and repeating the scraping motion. Confirm that there is no tissue, or only trace amounts of tissue, left on the slide before disposing of the slide glass.
10. Pick up the slide glass laid aside at step 7; scrape the tissue off in the same manner and dispose of the slide glass. Note that it becomes difficult to scrape the tissue off of the slide glass once it has dried. Perform steps 4 through 10 without delay. The scraping should be performed while keeping the area of the slide glass to which the lymph node tissue is affixed sufficiently wet with PBS from the petri dish.

11. The tissue suspension in the petri dish should be homogenized lightly with the cell scraper. If large pieces of tissue are observed, stir with the cell scraper to break up the pieces and obtain a uniform solution.

12. Wipe the cell scraper with a paper towel. (The cell scraper will be used for the next animal.)

13. Fasten a tip to the 100 µL micropipette, tilt the petri dish at an angle and mix the suspension by pipetting in and out several times. Take up 20 µL of the suspension with the pipette, taking care not to take up any membrane that is visible to the eye.

14. Add the 20 µL of suspension to a 15 mL test tube containing 1.98 mL PBS. Pipette the solution and proceed to homogenize with the vortex mixer. (cell suspension No. 1)

15. Repeat steps 13 and 14 to prepare cell suspension No. 2.

16. Dispose of the 100 µL micropipette tip.

Annex III: ATP Assay for the Experimental Procedures in Section 6.5

1. Fasten a tip on the 100 μL (or 200 μL) micropipette and draw 0.1 mL of vortex-homogenized cell suspension No. 1.
2. To the luminometer tube filled with 0.1 mL ATP releasing reagent, add 0.1 mL of cell suspension No. 1, making sure to note the time with a timer. Dispose of the tip.
3. Homogenize with the vortex mixer and place in the luminometer tube rack.
4. Fasten a tip on a separate 100 μL (or 200 μL) micropipette and draw 0.1 mL of solution from the 15 mL tube containing dissolved Luciferin-luciferase reagent.
5. Take the luminometer tube from the rack and add 0.1 mL of Luciferin-luciferase solution to the luminometer tube 20 seconds after the time noted in step 2.
6. Promptly homogenize in the vortex mixer, place in the luminometer and turn on the switch. The amount of bioluminescence begins to decrease immediately after adding the Luciferin-luciferase solution. Step 6 should be performed as quickly as possible, ideally with the same rhythm.
7. Dispose of the tip.
8. After 10 seconds the amount of bioluminescence (RLU; relative luminescence units) will be displayed. Record this measurement on the test forms.
9. Repeat steps 1 through 8 for cell suspension No. 2, measure the bioluminescence and record.

Appendix A2

Results in the LLNA: DA Test Method for 1% Sodium Lauryl Sulfate (SLS) Pretreatment versus without 1% SLS Pretreatment

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Appendix A2 Summary of Results in the LLNA: DA Test Method with 1% SLS Pretreatment versus without 1% SLS Pretreatment

Substance Name	Vehicle	Concentration (%)	SI[1] (+ SLS)	SI[1] (- SLS)	Calculated EC3[2] (%) (+ SLS)	Calculated EC3[2] (%) (- SLS)
2, 4-Dinitrochloro-benzene	AOO	0.03	2.10	1.88	0.05	0.06
		0.10	5.02	4.46		
		0.30	9.74	14.61		
Potassium dichromate	DMSO	0.1	2.61	2.54	0.15	0.22
		0.3	4.24	3.34		
		1.0	5.51	5.66		
Isoeugenol	AOO	1.0	2.05	1.32	2.46	4.24
		2.5	3.02	2.21		
		5.0	2.85	3.35		
Citral	AOO	5	1.93	1.88	7.4	10.4
		10	4.15	2.91		
		25	6.97	5.90		
Hexyl cinnamic aldehyde	AOO	5	1.51	0.99	7.5	8.8
		10	4.52	3.64		
		25	4.84	3.79		
Cinnamic alcohol	AOO	10	2.46	2.44	14.1	18.5
		25	4.40	3.43		
		50	6.36	4.01		
Hydroxycitronellal	AOO	10	1.98	1.49	15.8	19.8
		25	4.61	3.81		
		50	6.59	6.74		
Imidazolidinyl urea	DMF	10	2.36	2.54	20.3	33.0
		25	3.29	2.38		
		50	6.02	4.31		
Methyl methacrylate	AOO	25	0.73	1.11	NA	NA
		50	0.68	0.92		

Substance Name	Vehicle	Concentration (%)	SI[1] (+ SLS)	SI[1] (- SLS)	Calculated EC3[2] (%) (+ SLS)	Calculated EC3[2] (%) (- SLS)
		100	1.31	1.83		
Nickel (II) chloride	DMSO	2.5	1.53	0.98	NA	NA
		5.0	1.57	1.16		
		10.0	2.24	1.87		
Methyl salicylate	AOO	5	0.89	0.83	NA	NA
		10	1.59	1.32		
		25	1.69	2.34		
Salicylic acid	AOO	5	1.21	1.13	NA	NA
		10	2.05	1.29		
		25	2.48	2.44		
Sulfanilamide	DMF	10	1.08	0.92	NA	NA
		25	1.03	0.90		
		50	0.94	0.84		

Abbreviations: AOO = acetone: olive oil (4:1); DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; EC3 = estimated concentration required to produce a stimulation index of three; NA = not applicable; RLU = relative luminescence units; SI = stimulation index; SLS = sodium lauryl sulfate.

[1]SI determined from mean ATP content (RLU).

[2]EC3 value was calculated based on interpolation or extrapolation formulas discussed in Gerberick et al. 2004.

+ SLS = with pretreatment of 1% SLS prior to test substance application

- SLS = without pretreatment of 1% SLS prior to test substance application

Appendix B

Physico-Chemical Properties and Chemical Classes of Substances Tested in the LLNA: DA

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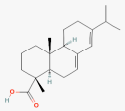
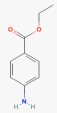
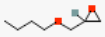
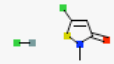
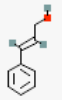
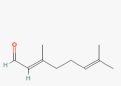
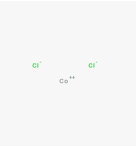
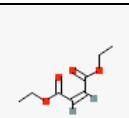
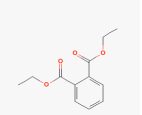
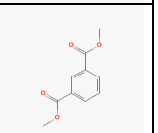
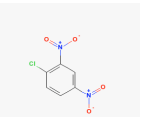
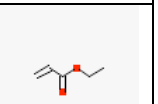
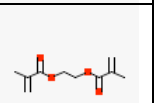
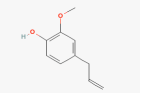
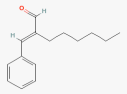
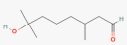
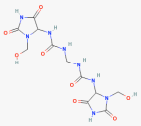
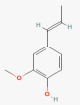
30 **Physico-Chemical Properties and Chemical Classes of Substances Tested in the LLNA: DA**

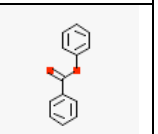
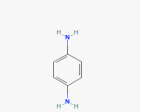
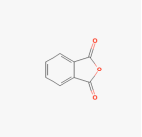
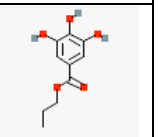
Substance Name[1]	Synonyms	CASRN	Mol. Weight (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Abietic acid[a, c]	Sylvic acid	514-10-3	302.46	6.46	NA	Solid	Hydrocarbons, Cyclic; Polycyclic Compounds	
3-Aminophenol[c]	m-Aminophenol	591-27-5	109.13	0.24	Minimal	Solid	Amines; Phenols	
Benzalkonium chloride[a]	Alkylbenzyldimethyl-ammonium chloride; Germitol; Zephiral	8001-54-5	170.66	NA	NA	Solid/ Liquid	Amines; Onium Compounds	
Benzocaine[a]	Ethyl 4-aminobenzoate	94-09-7	165.19	1.80	NA	Solid	Carboxylic Acids	
Benzoquinone[b]	p-Quinone 1,4-benzoquinone Cyclohexadienedione	106-51-4	108.10	1.17	High	Solid	Quinones	
1-Bromobutane[a]	Butyl bromide	109-65-9	137.02	2.65	Low	Liquid	Hydrocarbons, Halogenated	
Butyl glycidyl ether[b]	n- Butyl glycidyl ether	2426-08-6	130.19	1.42	NA	Liquid	Ethers	

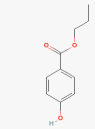
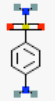
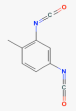
Substance Name[1]	Synonyms	CASRN	Mol. Weight (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
5-Chloro-2-methyl-4-isothiazolin-3-one[b]	Chloromethyliso-thiazolinone CMI	26172-55-4	132.30	0.92	High	Liquid	Sulfur Compounds Heterocyclic Compounds	
Chlorobenzene[a]	Phenyl chloride	108-90-7	112.56	2.64	Minimal	Liquid	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated	
Cinnamic alcohol[b]	3-Phenyl-2-propen-1-ol Cinnamyl alcohol	104-54-1	134.18	2.29	NA	Solid	Alcohols	
Cinnamic aldehyde[a]	Cinnamaldehyde	104-55-2	132.16	1.82	High	Liquid	Aldehydes	
Citral[a]	2,6-Octadienal, 3,7-dimethyl-	5392-40-5	152.24	3.45	High	Liquid	Hydrocarbons, Other	
Cobalt chloride[a, c, d]	Cobaltous chloride	7646-79-9	129.84	0.85	NA	Solid	Inorganic Chemical, Elements; Inorganic Chemical, Metals	
Diethyl maleate[b]	Ethyl maleate	141-05-9	172.18	0.89	High	Liquid	Carboxylic Acids	

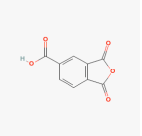
Substance Name[1]	Synonyms	CASRN	Mol. Weight (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Diethyl phthalate[a]	Ethyl phthalate	84-66-2	222.24	2.65	Minimal	Liquid	Carboxylic Acids	
Dimethyl isophthalate[b,c]	1,3-Benzenedicarboxylic acid, dimethyl ester	1459-93-4	194.19	1.66	NA	Solid	Carboxylic Acids	
2,4-Dinitrochloro-benzene[a, c]	Dinitrochlorobenzene; DNCB	97-00-7	202.55	2.27	High	Solid	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated; Nitro Compounds	
Ethyl acrylate[b]	2-Propenoic acid, ethyl ester	140-88-5	100.10	NA	High	Liquid	Carboxylic Acids	
Ethylene glycol dimethacrylate[b]	EGDMA	97-90-5	198.22	1.38	High	Liquid	Carboxylic Acids	
Eugenol[a]	2-Methoxy-4-(2-propenyl)phenol; Allylguaiacol	97-53-0	164.20	2.73	NA	Liquid	Carboxylic Acids	

Substance Name[1]	Synonyms	CASRN	Mol. Weight (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Formaldehyde[a, c]	Formalin	50-00-0	30.03	0.35	Moderate	Liquid	Aldehydes	
Glutaraldehyde[a, c]	Glutaral; Pentanedial	111-30-8	100.12	-0.18	High	Liquid	Aldehydes	
Hexane[a]	Hexyl hydride; n-Hexane	110-54-3	86.18	3.29	Minimal	Liquid	Hydrocarbons, Acyclic	
Hexyl cinnamic aldehyde[a, c, d]	alpha-Hexylcinnamaldehyde; HCA	101-86-0	216.32	4.82	Minimal	Liquid	Aldehydes	
Hydroxycitronellal[a]	Citronellal hydrate	107-75-5	172.26	2.11	Low	Liquid	Hydrocarbons, Other	
Imidazolidinyl urea[a]	Germall 115; Imidurea	39236-46-9	388.30	-8.28	Moderate	Solid	Urea	
Isoeugenol[a, c]	2-Methoxy-4-propenylphenol; 4-Propenylguaiacol	97-54-1	164.20	2.65	NA	Liquid	Carboxylic Acids	

Substance Name[1]	Synonyms	CASRN	Mol. Weight (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Isopropanol[a, c]	Isopropyl alcohol, 2-Propanol	67-63-0	60.10	0.28	Minimal	Liquid	Alcohols	
Lactic acid[a, d]	2-Hydroxypropanoic acid	50-21-5	90.08	-0.65	Minimal	Solid	Carboxylic Acids	
2-Mercaptobenzo-thiazole[a]	Captax	149-30-4	167.26	2.86	High	Solid	Heterocyclic Compounds	
Methyl methacrylate[b]	MMA	80-62-6	100.12	NA	NA	Liquid	Carboxylic Acids	
Methyl salicylate[a, c]	Oil of wintergreen; Methyl 2-hydroxybenzoate	119-36-8	152.15	2.60	Minimal	Liquid	Carboxylic Acids; Phenols	
Nickel (II) chloride[b]	Nickel chloride	7718-54-9	129.60	NA	NA	Solid	Inorganic Chemical, Elements; Inorganic Chemical, Metals	

Substance Name[1]	Synonyms	CASRN	Mol. Weight (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Nickel (II) sulfate hexahydrate[a, c, d]	Nickel sulfate hexahydrate	10101-97-0	154.76	NA	NA	Solid	Inorganic Chemical, Elements; Inorganic Chemical, Metals	
Phenyl benzoate[b]	Diphenylcarboxylate	93-99-2	198.22	2.89	NA	Solid	Carboxylic Acids	
p-Phenylenediamine[a]	4-Phenylenediamine	106-50-3	108.14	-0.39	NA	Solid	Amines	
Phthalic anhydride[a]	1,2-Benzenedicarboxylic anhydride; 1,3-Dioxophthalan	85-44-9	148.12	2.07	Moderate	Solid	Anhydrides; Carboxylic Acids	
Potassium dichromate[a,d]	PDC; Dipotassium bichromate	7778-50-9	294.18	-3.59	NA	Solid	Inorganic Chemical, Chromium Compounds; Inorganic Chemical, Potassium Compounds	
Propyl gallate[b]	Benzoic acid, 3,4,5-trihydroxy-, propyl ester; Gallic acid, propyl ester;	121-79-9	212.20	NA	High	Solid	Carboxylic Acids	

Substance Name[1]	Synonyms	CASRN	Mol. Weight (g/mol)	K_{ow}[2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
	Propyl 3,4,5-trihydroxybenzoate							
Propylparaben[a]	4-Hydroxybenzoic acid, propyl ester; Propyl p-hydroxybenzoate	94-13-3	180.20	2.98	Minimal	Solid	Carboxylic Acids; Phenols	
Resorcinol[a]	1,3-Dihydroxybenzene	108-46-3	110.11	1.03	Minimal	Solid	Phenols	
Salicylic acid[b]	2-Hydroxybenzoic acid	69-72-7	138.12	1.03	NA	Solid	Phenols; Carboxylic Acids	
Sodium lauryl sulfate[a]	Sodium dodecyl sulfate, SLS, SDS, Irium	151-21-3	288.38	1.69	NA	Solid	Alcohols; Sulfur Compounds; Lipids	
Sulfanilamide[b]	4-Aminobenzene-sulfonamide, p-Anilinesulfonamide, p-Sulfamidoaniline	63-74-1	172.21	0.40	Minimal	Solid	Hydrocarbons, Cyclic; Sulfur Compounds	
Toluene 2,4-diisocyanate[a]	2,4-TDI	584-54-9	174.16	3.74	NA	Liquid	Hydrocarbons, Cyclic; Isocyanates	

Substance Name[1]	Synonyms	CASRN	Mol. Weight (g/mol)	K_{ow} [2]	Peptide Reactivity[3]	Physical Form	Chemical Class[4]	Structure
Trimellitic anhydride[a]	4-Carboxyphthalic anhydride	552-30-7	192.13	1.95	Low	Solid	Anhydride; Carboxylic Acids	

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; K_{ow} = octanol-water partition coefficient; Mol. = molecular; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content; NA = not available.

[1]Total of 46 substances: intralaboratory validation study tested 45 substances (Idehara et al. 2008; Idehara unpublished data) and the two-phased interlaboratory validation study tested 13 of the 45 substances from the intralaboratory validation study and one substance not previously evaluated.

[2]K_{ow} represents the estimated octanol-water partition coefficient (expressed on log scale) calculated by the Syracuse Research Corporation from the website: http://www.syrres.com/esc/est_kowdemo.htm.

[3]Peptide reactivity based on Cys (1:10) and Lys (1:50) data as reported in Gerberick et al. 2004 and/or Gerberick et al. 2007.

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

[a]Substance tested in intralaboratory validation study (Idehara et al. 2008).

[b]Substance tested in intralaboratory validation study (Idehara unpublished data).

[c]Substance tested in phase one of two-phased interlaboratory validation study (Omori et al. 2008).

[d]Substance tested in phase two of two-phased interlaboratory validation study (Omori et al. 2008).

Appendix C

Comparative LLNA: DA, Traditional LLNA, Guinea Pig, and Human Skin Sensitization Data

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Appendix C1

Comparison of LLNA: DA, Traditional LLNA, Guinea Pig, and Human Results (Alphanumeric Order)

Appendix C-1 Comparative Performance of the LLNA: DA, Traditional LLNA, Guinea Pig, and Human Tests (Alphanumeric Order)

Substance Name	CASRN	LLNA: DA Highest Conc. Tested (%)	LLNA: DA Highest SI[1]	LLNA: DA Reference	Trad. LLNA Result	GP Result[2]	Human Result[3]	Trad. LLNA Reference	GP Reference	Human Reference	Skin Irritant?	Skin Irritation Data Reference
Abietic acid	514-10-3	25	6.26	Idehara et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	No at ≤ 2.5% (GP)	Basketter et al. 2007
Abietic acid	514-10-3	25	4.64	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	No at ≤ 2.5% (GP)	Basketter et al. 2007
Abietic acid	514-10-3	25	7.96	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	No at ≤ 2.5% (GP)	Basketter et al. 2007
Abietic acid	514-10-3	25	3.98 at 10%	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	No at ≤ 2.5% (GP)	Basketter et al. 2007
3-Aminophenol	591-27-5	10	2.83	Omori et al. 2008	+	NA	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 5% (GP)	Basketter et al. 2007b
3-Aminophenol	591-27-5	10	1.76 at 3%	Omori et al. 2008	+	NA	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 5% (GP)	Basketter et al. 2007b
3-Aminophenol	591-27-5	10	2.38	Omori et al. 2008	+	NA	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 5% (GP)	Basketter et al. 2007b
Benzalkonium chloride	8001-54-5	2.5	6.68	Idehara et al. 2008	+	-	+	Gerberick 1992	ICCVAM 1999	ICCVAM 1999	Irritant at 2% ACE (mice)	Gerberick et al 2002 Manetz et al. 1999
Benzocaine	94-09-7	25	4.84	Idehara et al. 2008	+/-[4]	+	+/-	ICCVAM 1999	ICCVAM 1999	Poole et al. 1970; ICCVAM 1999 (Equivocal data)	Negative at ≤ 10% (GP)	Basketter and Scholes
p-Benzoquinone	106-51-4	0.100	3.79	Idehara unpublished	+	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Nonirritant at 2.5% (GP)	Basketter et al. 2007b
1-Bromobutane	109-65-9	25	1.65	Idehara et al. 2008	-	NA	NA	ICCVAM 1999	NA	NA	NA	NA
Butyl glycidyl ether	2426-08-6	50	4.59	Idehara unpublished	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.1% (GP)	Wahlberg and Boman 1985
Chlorobenzene	108-90-7	25	2.44	Idehara et al. 2008	-	-	NA	ICCVAM 1999	ICCVAM 1999	NA	No data. Low irritancy	Basketter et al. 1998

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											potential assumed based on clinical literature.	
5-Chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	0.100	7.50	Idehara unpublished	+	+	+	ICCVAM 1999; Gerberick et al. 2005	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
Cinnamic alcohol	104-54-1	90	5.66 at 50%	Idehara unpublished	+	+	+	Gerberick et al. 2005	Robinson et al. 1990	Jordan and King 1977	Nonirritant at 1% (GP)	Robinson et al. 1990
Cinnamic aldehyde	104-55-2	15	4.73	Idehara et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.75% (GP); Mild Irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC #66, 1995
Citral	5392-40-5	25	4.40	Idehara et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.5% (GP)	Basketter et al. 2007b
Cobalt chloride	7646-79-9	5	3.64	Idehara et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes
Cobalt chloride	7646-79-9	1[5]	2.66	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes
Cobalt chloride	7646-79-9	3	20.55	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes
Cobalt chloride	7646-79-9	3	8.07	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes
Cobalt chloride	7646-79-9	5	2.01	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes
Cobalt chloride	7646-79-9	5	2.54	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes
Cobalt chloride	7646-79-9	5	4.25	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes
Cobalt chloride	7646-79-9	5	5.06	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 0.5% (GP)	Basketter and Scholes

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Diethyl maleate	141-05-9	10.0	3.78	Idehara unpublished				Gerberick et al. 2005	NA	Marzulli and Maibach 1980	Nonirritant at 100% (GP)	Basketter et al. 2007b
Diethyl phthalate	84-66-2	100	1.09	Idehara et al. 2008	-	-	-	Gerberick et al. 2005	Klecak et al. 1977	Schneider and Akkan 2004	Negative at 100% (rabbits)	ECETOC #66, 1995
Dimethyl isophthalate	1459-93-4	25	0.89 at 5%	Idehara unpublished	-	-	-	ICCVAM 1999; Basketter et al. 1999b	ICCVAM 1999; Basketter et al. 1999b	Basketter et al. 1999b	Negative at ≤ 10% (GP)	Basketter and Scholes
Dimethyl isophthalate	1459-93-4	25	1.34 at 5%	Omori et al. 2008	-	-	-	ICCVAM 1999; Basketter et al. 1999b	ICCVAM 1999; Basketter et al. 1999b	Basketter et al. 1999b	Negative at ≤ 10% (GP)	Basketter and Scholes
Dimethyl isophthalate	1459-93-4	25	1.00 at 5%	Omori et al. 2008	-	-	-	ICCVAM 1999; Basketter et al. 1999b	ICCVAM 1999; Basketter et al. 1999b	Basketter et al. 1999b	Negative at ≤ 10% (GP)	Basketter and Scholes
Dimethyl isophthalate	1459-93-4	25	1.26 at 5%	Omori et al. 2008	-	-	-	ICCVAM 1999; Basketter et al. 1999b	ICCVAM 1999; Basketter et al. 1999b	Basketter et al. 1999b	Negative at ≤ 10% (GP)	Basketter and Scholes
2,4-Dinitrochloro-benzene	97-00-7	1	7.10	Idehara et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	97-00-7	0.30	11.97	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	97-00-7	0.30	9.23	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	97-00-7	0.30	9.96	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	97-00-7	0.30	8.53	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b

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2,4-Dinitrochloro-benzene	97-00-7	0.30	7.86	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	97-00-7	0.30	15.14	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	97-00-7	0.30	13.18	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	97-00-7	0.30	12.60	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	97-00-7	0.30	10.89	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	97-00-7	0.30	4.71	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
2,4-Dinitrochloro-benzene	97-00-7	1	7.10	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Schneider and Akkan 2004	Nonirritant at 0.1% (GP)	Basketter et al. 2007b
Ethyl acrylate	140-88-5	50	4.29 at 25%	Idehara unpublished	+	-	+	Gerberick et al. 2005	Van der Walle et al. 1982	Marzulli and Maibach 1974	Nonirritant at 0.3 M (GP)	Van der Walle et al. 1982
Ethylene glycol dimethacrylate	97-90-5	50	4.45	Idehara unpublished	+	-	+	ICCVAM 1999	ICCVAM 1999; Gerberick 1992	ICCVAM 1999; Basketter et al. 1999b	Nonirritant at 1% (GP)	Wahlberg and Boman 1985
Eugenol	97-53-0	25	7.07	Idehara et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 25% (GP); Mild irritant at 100% (rabbits)	Basketter et al. 2007b; ECETOC #66, 1995
Formaldehyde	50-00-0	2.50	5.10	Idehara et al. 2008	+	+	+	Gerberick et al. 2005;	ICCVAM 1999	ICCVAM 1999; Kwon et	2% (GP)	Basketter et al.

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								Hilton et al. 1998		al. 2003		2007b
Formaldehyde	50-00-0	5.0	4.84	Omori et al. 2008	+	+	+	Gerberick et al. 2005; Hilton et al. 1998	ICCVAM 1999	ICCVAM 1999; Kwon et al. 2003	Nonirritant at 2% (GP)	Basketter et al. 2007b
Formaldehyde	50-00-0	5.0	3.18	Omori et al. 2008	+	+	+	Gerberick et al. 2005; Hilton et al. 1998	ICCVAM 1999	ICCVAM 1999; Kwon et al. 2003	Nonirritant at 2% (GP)	Basketter et al. 2007b
Formaldehyde	50-00-0	5.0	2.69	Omori et al. 2008	+	+	+	Gerberick et al. 2005; Hilton et al. 1998	ICCVAM 1999	ICCVAM 1999; Kwon et al. 2003	Nonirritant at 2% (GP)	Basketter et al. 2007b
Glutaraldehyde	111-30-8	0.25	6.45	Idehara et al. 2008	+	+	+	Basketter et al. 2005; Hilton et al. 1998	Gad et al. 1986	Marzulli et al. 1974; Schneider and Akkan 2004	NA	NA
Glutaraldehyde	111-30-8	0.50	5.00	Omori et al. 2008	+	+	+	Basketter et al. 2005; Hilton et al. 1998	Gad et al. 1986	Marzulli et al. 1974; Schneider and Akkan 2004	NA	NA
Glutaraldehyde	111-30-8	0.50	3.39	Omori et al. 2008	+	+	+	Basketter et al. 2005; Hilton et al. 1998	Gad et al. 1986	Marzulli et al. 1974; Schneider and Akkan 2004	NA	NA
Glutaraldehyde	111-30-8	0.50	2.57	Omori et al. 2008	+	+	+	Basketter et al. 2005; Hilton et al. 1998	Gad et al. 1986	Marzulli et al. 1974; Schneider and Akkan 2004	NA	NA
Hexane	110-54-3	100	2.31	Idehara et al. 2008	-	NA	-	ICCVAM 1999	NA	ICCVAM 1999	Irritant at 100% (humans)	Kligman 1966c
Hexyl cinnamic aldehyde	101-86-0	25	6.47	Idehara et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Mild irritant at 100% (rabbits)	ECETOC #66, 1995
Hexyl cinnamic aldehyde	101-86-0	25	5.78	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Mild irritant at 100% (rabbits)	ECETOC #66, 1995
Hexyl cinnamic aldehyde	101-86-0	25	4.82	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Mild irritant at 100% (rabbits)	ECETOC #66, 1995
Hexyl cinnamic aldehyde	101-86-0	25	4.44	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Mild irritant at 100% (rabbits)	ECETOC #66, 1995

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Hexyl cinnamic aldehyde	101-86-0	25	5.11	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Mild irritant at 100% (rabbits)	ECETOC #66, 1995
Hexyl cinnamic aldehyde	101-86-0	25	3.97	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Mild irritant at 100% (rabbits)	ECETOC #66, 1995
Hexyl cinnamic aldehyde	101-86-0	25	5.50	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Mild irritant at 100% (rabbits)	ECETOC #66, 1995
Hexyl cinnamic aldehyde	101-86-0	25	7.09	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Mild irritant at 100% (rabbits)	ECETOC #66, 1995
Hexyl cinnamic aldehyde	101-86-0	25	10.22	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Mild irritant at 100% (rabbits)	ECETOC #66, 1995
Hexyl cinnamic aldehyde	101-86-0	25	3.88	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Mild irritant at 100% (rabbits)	ECETOC #66, 1995
Hexyl cinnamic aldehyde	101-86-0	25	3.51	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Mild irritant at 100% (rabbits)	ECETOC #66, 1995
Hexyl cinnamic aldehyde	101-86-0	25	4.47	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Mild irritant at 100% (rabbits)	ECETOC #66, 1995
Hexyl cinnamic aldehyde	101-86-0	25	5.71	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Mild irritant at 100% (rabbits)	ECETOC #66, 1995
Hexyl cinnamic aldehyde	101-86-0	25	5.41	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Mild irritant at 100% (rabbits)	ECETOC #66, 1995
Hexyl cinnamic aldehyde	101-86-0	25	7.60	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Mild irritant at 100% (rabbits)	ECETOC #66, 1995
Hexyl cinnamic aldehyde	101-86-0	25	3.92	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Mild irritant at 100% (rabbits)	ECETOC #66, 1995
Hexyl cinnamic aldehyde	101-86-0	25	8.42	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Mild irritant at 100% (rabbits)	ECETOC #66, 1995
Hexyl cinnamic aldehyde	101-86-0	25	6.45	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Mild irritant at 100% (rabbits)	ECETOC #66, 1995
Hydroxycitronellal	107-75-5	50	5.69	Idehara et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 50% (GP); Negative at 100% (rabbits)	Basketter et al. 2007b; ECETOC #66, 1995
Imidazolidinyl urea	39236-46-9	50	4.67	Idehara et al. 2008	+	+	+	Gerberick et al. 2005	ICCVAM 1999	ICCVAM 1999	Negative at ≤ 75% (GP)	Basketter and Scholes
Isoeugenol	97-54-1	50	12.36 at 25%	Idehara et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 5% (GP)	Basketter et al. 2007b
Isoeugenol	97-54-1	10	6.11	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 5% (GP)	Basketter et al.

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												2007b
Isoeugenol	97-54-1	10	5.54	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 5% (GP)	Basketter et al. 2007b
Isoeugenol	97-54-1	10	7.09	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 5% (GP)	Basketter et al. 2007b
Isopropanol	67-63-0	50	1.08 at 25%	Idehara et al. 2008	-	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC #66, 1995
Isopropanol	67-63-0	50	1.54 at 10%	Omori et al. 2008	-	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC #66, 1995
Isopropanol	67-63-0	50	0.91 at 10%	Omori et al. 2008	-	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC #66, 1995
Isopropanol	67-63-0	50	1.01 at 10%	Omori et al. 2008	-	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC #66, 1995
Isopropanol	67-63-0	50	1.57 at 10%	Omori et al. 2008	-	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC #66, 1995
Isopropanol	67-63-0	50	0.76 at 25%	Omori et al. 2008	-	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC #66, 1995
Isopropanol	67-63-0	50	1.97 at 10%	Omori et al. 2008	-	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC #66, 1995
Isopropanol	67-63-0	50	1.45 at 10%	Omori et al. 2008	-	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC #66, 1995
Isopropanol	67-63-0	50	1.21 at 10%	Omori et al. 2008	-	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC #66, 1995
Isopropanol	67-63-0	50	0.70 at 25%	Omori et al. 2008	-	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC #66, 1995
Isopropanol	67-63-0	50	1.25	Omori et al. 2008	-	-	+	ICCVAM 1999	ICCVAM 1999	Kwon et al. 2003	Negative at 100% (rabbits)	ECETOC #66, 1995
Lactic acid	50-21-5	50	1.06 at 10%	Idehara et al. 2008	-	-	-	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Slightly irritating at 10% aq. (rabbits)	Cosmetic Ingredient Review Expert Panel 1998
Lactic acid	50-21-5	25	0.93 at 5%	Omori et al. 2008	-	-	-	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Slightly irritating at 10% aq. (rabbits)	Cosmetic Ingredient Review Expert Panel

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												1998
Lactic acid	50-21-5	25	0.99 at 5%	Omori et al. 2008	-	-	-	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Slightly irritating at 10% aq. (rabbits)	Cosmetic Ingredient Review Expert Panel 1998
Lactic acid	50-21-5	25	0.97 at 10%	Omori et al. 2008	-	-	-	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Slightly irritating at 10% aq. (rabbits)	Cosmetic Ingredient Review Expert Panel 1998
Lactic acid	50-21-5	25	0.91	Omori et al. 2008	-	-	-	ICCVAM 1999	ICCVAM 1999	Basketter et al. 1999b	Slightly irritating at 10% aq. (rabbits)	Cosmetic Ingredient Review Expert Panel 1998
2-Mercaptobenzo-thiazole	149-30-4	50	2.00	Idehara et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 10% (GP)	Basketter et al. 2007b
Methyl methacrylate	80-62-6	100	1.81	Idehara unpublished	+	+	+ (case studies, no exposure concen-tration)	Betts et al. 2006	Van der Walle et al. 1982	Betts et al. 2006	Nonirritant at 3 M (GP)	Van der Walle et al. 1982
Methyl salicylate	119-36-8	25	1.20	Idehara et al. 2008	-	-	-	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Irritant at 10% AOO (mice)	Gerberick et al 2002
Methyl salicylate	119-36-8	25	1.55	Omori et al. 2008	-	-	-	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Irritant at 10% AOO (mice)	Gerberick et al 2002
Methyl salicylate	119-36-8	25	1.77 at 10%	Omori et al. 2008	-	-	-	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Irritant at 10% AOO (mice)	Gerberick et al 2002
Methyl salicylate	119-36-8	25	0.83	Idehara et al. 2008	-	-	-	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Irritant at 10% AOO (mice)	Gerberick et al 2002
Nickel (II) chloride	7718-54-9	10	1.30	Idehara unpublished	-	+	+	ICCVAM 1999	ICCVAM 1999	Vandenberg and Epstein 1963	Negative at ≤ 0.15% (GP)	Basketter and Scholes
Nickel (II) sulfate hexahydrate	10101-97-0	5.0	2.17 at 2.5%	Omori et al. 2008	+	+	+	Ryan et al. 2002	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (humans);	Kligman 1966c;

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											Nonirritant at 0.15% (GP)	Scholes et al. 1992
Nickel (II) sulfate hexahydrate	10101-97-0	10	1.52 at 3%	Omori et al. 2008	+	+	+	Ryan et al. 2002	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (humans); Nonirritant at 0.15% (GP)	Kligman 1966c; Scholes et al. 1992
Nickel (II) sulfate hexahydrate	10101-97-0	10	11.78	Omori et al. 2008	+	+	+	Ryan et al. 2002	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (humans); Nonirritant at 0.15% (GP)	Kligman 1966c; Scholes et al. 1992
Nickel (II) sulfate hexahydrate	10101-97-0	10	3.49 at 1%	Omori et al. 2008	+	+	+	Ryan et al. 2002	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (humans); Nonirritant at 0.15% (GP)	Kligman 1966c; Scholes et al. 1992
Nickel (II) sulfate hexahydrate	10101-97-0	10	0.79 at 3%	Omori et al. 2008	+	+	+	Ryan et al. 2002	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (humans); Nonirritant at 0.15% (GP)	Kligman 1966c; Scholes et al. 1992
Nickel (II) sulfate hexahydrate	10101-97-0	10	1.24 at 3%	Omori et al. 2008	+	+	+	Ryan et al. 2002	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (humans); Nonirritant at 0.15% (GP)	Kligman 1966c; Scholes et al. 1992
Nickel (II) sulfate hexahydrate	10101-97-0	10	2.13	Omori et al. 2008	+	+	+	Ryan et al. 2002	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (humans); Nonirritant at 0.15% (GP)	Kligman 1966c; Scholes et al. 1992
Nickel (II) sulfate hexahydrate	10101-97-0	10	1.56 at 3%	Omori et al. 2008	+	+	+	Ryan et al. 2002	ICCVAM 1999	ICCVAM 1999	Irritant at 10% (humans); Nonirritant at 0.15% (GP)	Kligman 1966c; Scholes et al. 1992
Phenyl benzoate	93-99-2	10	4.24 at 5%	Idehara unpublished	+	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al 2005a	NA	NA
p-Phenylenediamine	106-50-3	1	5.14 at 0.25%	Idehara et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.5% (GP)	Basketter et al. 2007b
Phthalic anhydride	85-44-9	1.0	5.49	Idehara et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	Basketter et al. 2001	Negative at ≤ 10% (GP)	Basketter and Scholes
Potassium dichromate	7778-50-9	1.0	4.78	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.15% (GP)	Basketter et al. 2007b

Substance Name	CASRN	LLNA: DA Highest Conc. Tested (%)	LLNA: DA Highest SI[1]	LLNA: DA Reference	Trad. LLNA Result	GP Result[2]	Human Result[3]	Trad. LLNA Reference	GP Reference	Human Reference	Skin Irritant?	Skin Irritation Data Reference
Potassium dichromate	7778-50-9	1.0	4.08	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.15% (GP)	Basketter et al. 2007b
Potassium dichromate	7778-50-9	1.0	6.01	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.15% (GP)	Basketter et al. 2007b
Potassium dichromate	7778-50-9	1.0	6.37	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.15% (GP)	Basketter et al. 2007b
Potassium dichromate	7778-50-9	1.0	5.49	Omori et al. 2008	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 0.15% (GP)	Basketter et al. 2007b
Propyl gallate	121-79-9	2.5	4.95	Idehara unpublished	+	+	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 5% (GP)	Basketter and Scholes
Propylparaben	94-13-3	25	1.28	Idehara et al. 2008	-	-	+	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Nonirritant at 10% (GP)	Basketter and Scholes
Resorcinol	108-46-3	25	4.33	Idehara et al. 2008	+	-	+	Basketter et al. 2007	ICCVAM 1999	ICCVAM 1999; Basketter et al. 2007	Nonirritant at 15% (humans)	Kligman 1966c
Salicylic acid	69-72-7	25	2.00	Idehara unpublished	-	-	+ (6/23 at 1%)	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999 (Kligman 1966b for nickel sulfate)	Irritant at 20% aq. (mice)	Gerberick et al. 2002
Sodium lauryl sulfate	151-21-3	10	3.39	Idehara et al. 2008	+	-	-	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999	Irritant at 20% (rabbits)	ECETOC #66, 1995
Sulfanilamide	63-74-1	50	0.86 at 25%	Idehara unpublished	-	-	-	ICCVAM 1999	ICCVAM 1999	ICCVAM 1999; Kligman 1966c	Nonirritant at 25% (humans)	Kligman 1966c
Toluene 2,4-diisocyanate	584-84-9	0.25	9.43	Idehara et al. 2008	+	+	+	van Och et al. 2001	NA	Basketter et al. 2001	NA	NA
Trimellitic anhydride	552-30-7	0.50	4.96	Idehara et al. 2008	+	+	NA	ICCVAM 1999; Basketter and Scholes 1992	ICCVAM 1999; Gad et al. 1986	ICCVAM 1999; Basketter et al. 2001	Negative at ≤ 10% (GP)	Basketter and Scholes

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); aq. = aqueous; CASRN = Chemical Abstracts Service Registry Number; Conc. = concentration; ECETOC = European Centre for Ecotoxicology and Toxicology of Chemicals; GP = guinea pig; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content; NA = not available; SI = stimulation index; Trad. = traditional;
"+" = Sensitizer.
"-" = Nonsensitizer.

[1]Highest SI achieved at highest concentration tested, unless otherwise noted.
[2]GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.
[3]Human refers to outcomes obtained by studies conducted using the human maximization test, inclusion of the test substance in a human patch test allergen kit, and/or published clinical case studies/reports.
[4]Equivocal traditional LLNA data (ICCVAM 1999); substance not included in accuracy analyses.
[5]Data not reported for the highest dose (i.e., 3%), only for 0.3% and 1%.

Appendix C2

Comparison of Alternative LLNA: DA Decision Criteria and Traditional LLNA Results (Alphanumeric Order)

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

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.[2]	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	LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
Abietic acid	514-10-3	25	6.26	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Abietic acid	514-10-3	25	4.64	+	+	+	+	-	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Abietic acid	514-10-3	25	7.96	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Abietic acid	514-10-3	25	3.98 at 10%	+	+	+	+	-	-	-	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
3-Aminophenol	591-27-5	10	2.83	+	+	+	+	-	-	-	-	-	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
3-Aminophenol	591-27-5	10	1.76 at 3%	+	+	+	+	-	-	-	-	-	-	-	+	+	+	Omori et al. 2008	+	ICCVAM 1999
3-Aminophenol	591-27-5	10	2.38	+	+	+	+	-	-	-	-	-	-	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Benzalkonium chloride	8001-54-5	2.5	6.68	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	Gerberick 1992
Benzocaine	94-09-7	25	4.84	+	+	+	+	-	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+/-[3]	ICCVAM 1999
p-Benzoquinone	106-51-4	0.100	3.79	+	+	+	+	-	-	-	+	+	+	+	+	+	+	Idehara unpublished	+	ICCVAM 1999
1-Bromobutane	109-65-9	25	1.65	+	+	+	+	-	-	-	-	-	-	-	+	+	+	Idehara et al. 2008	-	ICCVAM 1999
Butyl glycidyl ether	2426-08-6	50	4.59	+	-	+	+	-	+	+	+	+	+	+	+	+	+	Idehara unpublished	+	ICCVAM 1999
Chlorobenzene	108-90-7	25	2.44	+	+	+	+	-	-	-	-	-	-	+	+	+	+	Idehara et al. 2008	-	ICCVAM 1999

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.[2]	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	LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
5-Chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	0.100	7.50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara unpublished	+	ICCVAM 1999; Gerberick et al. 2005
Cinnamic alcohol	104-54-1	90	5.66 at 50%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara unpublished	+	Gerberick et al. 2005
Cinnamic aldehyde	104-55-2	15	4.73	+	+	+	+	-	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Citral	5392-40-5	25	4.40	+	+	+	+	-	-	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Cobalt chloride	7646-79-9	5	3.64	+	+	+	+	-	-	-	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Cobalt chloride	7646-79-9	1[4]	2.66	+	+	+	+	-	-	-	-	-	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Cobalt chloride	7646-79-9	3	20.55	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Cobalt chloride	7646-79-9	3	8.07	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Cobalt chloride	7646-79-9	5	2.01	+	+	+	+	-	-	-	-	-	-	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Cobalt chloride	7646-79-9	5	2.54	+	+	+	+	-	-	-	-	-	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Cobalt chloride	7646-79-9	5	4.25	+	+	+	+	-	-	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Cobalt chloride	7646-79-9	5	5.06	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Diethyl maleate	141-05-9	10.0	3.78	+	+	+	+	-	-	-	+	+	+	+	+	+	+	Idehara unpublished	+	Gerberick et al. 2005

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.[2]	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	LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
Diethyl phthalate	84-66-2	100	1.09	+	-	-	-	-	-	-	-	-	-	-	-	-	+	Idehara et al. 2008	-	Gerberick et al. 2005
Dimethyl isophthalate	1459-93-4	25	0.89 at 5%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Idehara unpublished	-	ICCVAM 1999; Basketter et al. 1999b
Dimethyl isophthalate	1459-93-4	25	1.34 at 5%	+	-	-	-	-	-	-	-	-	-	-	-	+	+	Omori et al. 2008	-	ICCVAM 1999; Basketter et al. 1999b
Dimethyl isophthalate	1459-93-4	25	1.00 at 5%	+	-	-	-	-	-	-	-	-	-	-	-	-	+	Omori et al. 2008	-	ICCVAM 1999; Basketter et al. 1999b
Dimethyl isophthalate	1459-93-4	25	1.26 at 5%	+	-	-	-	-	-	-	-	-	-	-	-	-	+	Omori et al. 2008	-	ICCVAM 1999; Basketter et al. 1999b
2,4-Dinitrochloro-benzene	97-00-7	1	7.10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	11.97	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	9.23	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	9.96	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	8.53	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.[2]	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	LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
2,4-Dinitrochloro-benzene	97-00-7	0.30	7.86	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	15.14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	13.18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	12.60	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	10.89	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	0.30	4.71	+	+	+	+	-	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
2,4-Dinitrochloro-benzene	97-00-7	1	7.10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Ethyl acrylate	140-88-5	50	4.29 at 25%	+	-	-	+	-	-	+	+	+	+	+	+	+	+	Idehara unpublished	+	Gerberick et al. 2005
Ethylene glycol dimethacrylate	97-90-5	50	4.45	+	+	+	+	-	-	+	+	+	+	+	+	+	+	Idehara unpublished	+	ICCVAM 1999
Eugenol	97-53-0	25	7.07	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Formaldehyde	50-00-0	2.50	5.10	+	+	+	+	-	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	Gerberick et al. 2005; Hilton et al. 1998
Formaldehyde	50-00-0	5.0	4.84	+	+	+	+	-	-	-	-	+	+	+	+	+	+	Omori et al. 2008	+	Gerberick et al. 2005; Hilton et al. 1998
Formaldehyde	50-00-0	5.0	3.18	+	+	+	+	-	-	-	-	-	+	+	+	+	+	Omori et al. 2008	+	Gerberick et al. 2005; Hilton et al. 1998

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.[2]	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	LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
Formaldehyde	50-00-0	5.0	2.69	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	Gerberick et al. 2005; Hilton et al. 1998
Glutaraldehyde	111-30-8	0.25	6.45	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	Basketter et al. 2005; Hilton et al. 1998
Glutaraldehyde	111-30-8	0.50	5.00	+	+	+	+	-	-	-	-	+	+	+	+	+	+	Omori et al. 2008	+	Basketter et al. 2005; Hilton et al. 1998
Glutaraldehyde	111-30-8	0.50	3.39	+	+	+	+	-	-	-	-	-	+	+	+	+	+	Omori et al. 2008	+	Basketter et al. 2005; Hilton et al. 1998
Glutaraldehyde	111-30-8	0.50	2.57	+	+	+	+	-	-	-	-	-	-	+	+	+	+	Omori et al. 2008	+	Basketter et al. 2005; Hilton et al. 1998
Hexane	110-54-3	100	2.31	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	-	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	6.47	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	5.78	+	+	+	+	-	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	4.82	+	+	+	+	-	-	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	4.44	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	5.11	+	+	+	+	-	-	-	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.[2]	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	LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
Hexyl cinnamic aldehyde	101-86-0	25	3.97	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	5.50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	7.09	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	10.22	+	+	+	+	-	-	-	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	3.88	+	+	+	+	-	-	-	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	3.51	+	+	+	+	-	-	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	4.47	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	5.71	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	5.41	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	7.60	+	+	+	+	-	-	-	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	3.92	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	8.42	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hexyl cinnamic aldehyde	101-86-0	25	6.45	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Hydroxycitronellal	107-75-5	50	5.69	+	+	+	+	-	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Imidazolidinyl urea	39236-46-9	50	4.67	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	Gerberick et al. 2005

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.[2]	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	LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
Isoeugenol	97-54-1	50	12.36 at 25%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Isoeugenol	97-54-1	10	6.11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Isoeugenol	97-54-1	10	5.54	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Isoeugenol	97-54-1	10	7.09	+	-	-	-	-	-	-	-	-	-	-	-	-	+	Omori et al. 2008	+	ICCVAM 1999
Isopropanol	67-63-0	50	1.08 at 25%	+	-	+	-	-	-	-	-	-	-	-	+	+	+	Idehara et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	1.54 at 10%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Omori et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	0.91 at 10%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Omori et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	1.01 at 10%	+	-	+	+	-	-	-	-	-	-	-	+	+	+	Omori et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	1.57 at 10%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Omori et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	0.76 at 25%	+	+	+	-	-	-	-	-	-	-	-	+	+	+	Omori et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	1.97 at 10%	+	-	+	-	-	-	-	-	-	-	-	-	+	+	Omori et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	1.45 at 10%	+	-	-	-	-	-	-	-	-	-	-	-	-	+	Omori et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	1.21 at 10%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Omori et al. 2008	-	ICCVAM 1999
Isopropanol	67-63-0	50	0.70 at 25%	+	-	+	-	-	-	-	-	-	-	-	-	-	+	Omori et al. 2008	-	ICCVAM 1999

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.[2]	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	LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
Isopropanol	67-63-0	50	1.25	+	-	-	-	-	-	-	-	-	-	-	-	-	+	Omori et al. 2008	-	ICCVAM 1999
Lactic acid	50-21-5	50	1.06 at 10%	+	-	-	-	-	-	-	-	-	-	-	-	-	+	Idehara et al. 2008	-	ICCVAM 1999
Lactic acid	50-21-5	25	0.93 at 5%	+	-	-	+	-	-	-	-	-	-	-	-	-	+	Omori et al. 2008	-	ICCVAM 1999
Lactic acid	50-21-5	25	0.99 at 5%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Omori et al. 2008	-	ICCVAM 1999
Lactic acid	50-21-5	25	0.97 at 10%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Omori et al. 2008	-	ICCVAM 1999
Lactic acid	50-21-5	25	0.91	+	+	+	-	-	-	-	-	-	-	+	+	+	+	Omori et al. 2008	-	ICCVAM 1999
2-Mercaptobenzo-thiazole	149-30-4	50	2.00	+	-	+	-	-	-	-	-	-	-	-	-	-	+	Idehara et al. 2008	+	ICCVAM 1999
Methyl methacrylate	80-62-6	100	1.81	+	-	-	+	-	-	-	-	-	-	-	+	+	+	Idehara unpublished	+	Betts et al. 2006
Methyl salicylate	119-36-8	25	1.20	+	+	+	-	-	-	-	-	-	-	-	+	+	+	Idehara et al. 2008	-	ICCVAM 1999
Methyl salicylate	119-36-8	25	1.55	+	-	-	-	-	-	-	-	-	-	-	+	+	+	Omori et al. 2008	-	ICCVAM 1999
Methyl salicylate	119-36-8	25	1.77 at 10%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Omori et al. 2008	-	ICCVAM 1999
Methyl salicylate	119-36-8	25	0.83	+	+	+	+	-	-	-	-	-	-	+	+	+	+	Idehara et al. 2008	-	ICCVAM 1999
Nickel (II) chloride	7718-54-9	10	1.30	+	+	+	-	-	-	-	-	-	-	-	-	+	+	Idehara unpublished	-	ICCVAM 1999
Nickel (II) sulfate hexahydrate	10101-97-0	5.0	2.17 at 2.5%	+	-	-	-	-	-	-	-	-	-	-	+	+	+	Omori et al. 2008	+	Ryan et al. 2002
Nickel (II) sulfate hexahydrate	10101-97-0	10	1.52 at 3%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	Ryan et al. 2002

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.[2]	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	LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
Nickel (II) sulfate hexahydrate	10101-97-0	10	11.78	+	+	+	+	-	-	-	-	+	+	+	+	+	+	Omori et al. 2008	+	Ryan et al. 2002
Nickel (II) sulfate hexahydrate	10101-97-0	10	3.49 at 1%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Omori et al. 2008	+	Ryan et al. 2002
Nickel (II) sulfate hexahydrate	10101-97-0	10	0.79 at 3%	+	+	+	-	-	-	-	-	-	-	-	-	-	+	Omori et al. 2008	+	Ryan et al. 2002
Nickel (II) sulfate hexahydrate	10101-97-0	10	1.24 at 3%	+	+	+	+	-	-	-	-	-	-	+	+	+	+	Omori et al. 2008	+	Ryan et al. 2002
Nickel (II) sulfate hexahydrate	10101-97-0	10	2.13	+	-	-	-	-	-	-	-	-	-	-	+	+	+	Omori et al. 2008	+	Ryan et al. 2002
Nickel (II) sulfate hexahydrate	10101-97-0	10	1.56 at 3%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	Ryan et al. 2002
Phenyl benzoate	93-99-2	10.0	4.24 at 5%	+	+	+	+	-	-	+	+	+	+	+	+	+	+	Idehara unpublished	+	ICCVAM 1999
p-Phenylenediamine	106-50-3	1	5.14 at 0.25%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Phthalic anhydride	85-44-9	1.0	5.49	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Potassium dichromate	7778-50-9	1.0	4.78	+	+	+	+	-	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Potassium dichromate	7778-50-9	1.0	4.08	+	+	+	+	-	-	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Potassium dichromate	7778-50-9	1.0	6.01	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Potassium dichromate	7778-50-9	1.0	6.37	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Omori et al. 2008	+	ICCVAM 1999
Potassium dichromate	7778-50-9	1.0	5.49	+	+	+	-	-	-	-	-	-	-	-	-	-	+	Omori et al. 2008	+	ICCVAM 1999

Substance Name	CASRN	Highest Conc. Tested (%)	Highest SI[1]	≥95 % CI	≥ 3 SD	≥ 2 SD	Stats.[2]	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	LLNA: DA Ref.	Trad. LLNA Result	Trad. LLNA Ref.
Propyl gallate	121-79-9	2.5	4.95	+	-	-	+	-	+	+	+	+	+	+	+	+	+	Idehara unpublished	+	ICCVAM 1999
Propylparaben	94-13-3	25	1.28	+	+	+	+	-	-	+	+	+	+	+	+	+	+	Idehara et al. 2008	-	ICCVAM 1999
Resorcinol	108-46-3	25	4.33	+	+	+	+	-	-	-	-	+	+	+	+	+	+	Idehara et al. 2008	+	Basketter et al. 2007
Salicylic acid	69-72-7	25	2.00	+	-	+	+	-	-	-	-	-	-	+	+	+	+	Idehara unpublished	-	ICCVAM 1999
Sodium lauryl sulfate	151-21-3	10	3.39	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999
Sulfanilamide	63-74-1	50	0.86 at 25%	-	-	-	+	-	-	-	-	-	-	-	-	-	-	Idehara unpublished	-	ICCVAM 1999
Toluene 2,4-diisocyanate	584-84-9	0.25	9.43	+	+	+	+	-	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	Van Och et al. 2001
Trimellitic anhydride	552-30-7	0.50	4.96	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Idehara et al. 2008	+	ICCVAM 1999; Basketter and Scholes 1992

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; CI = confidence interval (mean ATP measurement of any treatment group is greater than 95% CI of mean ATP measurement for vehicle control group); Conc. = concentration; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content; Ref. = reference; SD = standard deviation (mean ATP measurement of any treatment group is greater than two or three 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.

"-" = Nonsensitizer.

[1]Highest SI achieved at highest concentration tested, unless otherwise noted.

[2]The ATP data were log-transformed prior to statistical analyses. For analysis of variance, significance at $p < 0.05$ was further tested by Dunnett's test.

[3]Equivocal traditional LLNA data (ICCVAM 1999). Substance not included in accuracy analyses.

[4]Data not reported for the highest dose (i.e., 3%), only for 0.3% and 1%.

Appendix D

Data for the LLNA: DA Intralaboratory and Interlaboratory Validation Studies

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Appendix D1

Individual Animal Data for the LLNA: DA (Intralaboratory)

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Appendix D1 Individual Animal Data for the LLNA: DA Intralaboratory Validation Study[1]

Substance[2]	Veh.	An. #	1 Conc. (%)	1 Mean ATP[3]	1 SI	An. #	2 Conc. (%)	2 Mean ATP[3]	2 SI	An. #	3 Conc. (%)	3 Mean ATP[3]	3 SI	An. #	4 Conc. (%)	4 Mean ATP[3]	4 SI	An. #	5 Conc. (%)	5 Mean ATP[3]	5 SI	An. #	6 Conc. (%)	6 Mean ATP[3]	6 SI	Calc. EC3[4]	Calc. EC2[5]
Vehicle Control	AOO	1-1	0	4927	1.12																						
Vehicle Control	AOO	1-2	0	3547	0.80																						
Vehicle Control	AOO	1-3	0	4758	1.08																						
Vehicle Control	**AOO**	**Mean**	**0**	**4411**	**1.0**																						
Positive Control - Eugenol	AOO	2-1	10	17020	3.86																						
Positive Control - Eugenol	AOO	2-2	10	14029	3.18																						
Positive Control - Eugenol	AOO	2-3	10	12117	2.75																						
Positive Control - Eugenol	**AOO**	**Mean**	**10**	**14388**	**3.26**																						
Citral	AOO	3-1	5	9191	2.08	4-1	10	9937	2.25	5-1	15	12297	2.79	6-1	25	18200	4.13										
Citral	AOO	3-2	5	12120	2.75	4-2	10	7447	1.69	5-2	15	11863	2.69	6-2	25	22609	5.13										
Citral	AOO	3-3	5	4808	1.09	4-3	10	10528	2.39	5-3	15	14283	3.24	6-3	25	17469	3.96										
Citral	**AOO**	**Mean**	**5**	**8706**	**1.97**	**Mean**	**10**	**9304**	**2.11**	**Mean**	**15**	**12814**	**2.91**	**Mean**	**25**	**19426**	**4.40**									**15.63**	**5.96**
Cinnamic aldehyde	AOO	7-1	1.0	6780	1.54	8-1	2.5	13624	3.09	9-1	5.0	21945	4.98	10-1	15	20037	4.54										
Cinnamic aldehyde	AOO	7-2	1.0	13271	3.01	8-2	2.5	8924	2.02	9-2	5.0	17313	3.93	10-2	15	18085	4.10										
Cinnamic aldehyde	AOO	7-3	1.0	7545	1.71	8-3	2.5	12681	2.88	9-3	5.0	19218	4.36	10-3	15	24421	5.54										
Cinnamic aldehyde	**AOO**	**Mean**	**1.0**	**9199**	**2.09**	**Mean**	**2.5**	**11743**	**2.66**	**Mean**	**5.0**	**19492**	**4.42**	**Mean**	**15**	**20848**	**4.73**									**2.98**	**0.92**
Vehicle Control	AOO	1-1	0	3759	0.97																						
Vehicle Control	AOO	1-2	0	3995	1.03																						
Vehicle Control	AOO	1-3	0	3461	0.89																						
Vehicle Control	AOO	1-4	0	4269	1.10																						
Vehicle Control	**AOO**	**Mean**	**0**	**3871**	**1.00**																						

Substance[2]	Veh.	An. #	1 Conc. (%)	1 Mean ATP[3]	1 SI	An. #	2 Conc. (%)	2 Mean ATP[3]	2 SI	An. #	3 Conc. (%)	3 Mean ATP[3]	3 SI	An. #	4 Conc. (%)	4 Mean ATP[3]	4 SI	An. #	5 Conc. (%)	5 Mean ATP[3]	5 SI	An. #	6 Conc. (%)	6 Mean ATP[3]	6 SI	Calc. EC3[4]	Calc. EC2[5]
Positive Control - Eugenol	AOO	3-1	10	16624	4.30																						
Positive Control - Eugenol	AOO	3-2	10	23785	6.15																						
Positive Control - Eugenol	AOO	3-3	10	15667	4.05																						
Positive Control - Eugenol	AOO	3-4	10	18066	4.67																						
Positive Control - Eugenol	**AOO**	**Mean**	**10**	**18535**	**4.79**																						
Eugenol	AOO	2-1	5	12594	3.25	3-1	10	16624	4.30	4-1	25	26107	6.75														
Eugenol	AOO	2-2	5	15216	3.93	3-2	10	23785	6.15	4-2	25	26713	6.90														
Eugenol	AOO	2-3	5	9790	2.53	3-3	10	15667	4.05	4-3	25	29297	7.57														
Eugenol	NT	NT	NT	NT	NT	3-4	10	18066	4.67	NT	NT	NT	NT														
Eugenol	**AOO**	**Mean**	**5**	**12533**	**3.24**	**Mean**	**10**	**18535**	**4.79**	**Mean**	**25**	**27372**	**7.07**													**4.50**	**2.88**
Propylparaben	AOO	5-1	5	5058	1.31	6-1	10	5539	1.43	7-1	25	6385	1.65														
Propylparaben	AOO	5-2	5	4773	1.233	6-2	10	3919	1.012	7-2	25	5813	1.50														
Propylparaben	AOO	5-3	5	3034	0.784	6-3	10	3713	0.959	7-3	25	2679	0.69														
Propylparaben	**AOO**	**Mean**	**5**	**4288**	**1.11**	**Mean**	**10**	**4390**	**1.13**	**Mean**	**25**	**4959**	**1.28**													**NA**	**NA**
HCA	AOO	8-1	5	7375	1.91	9-1	10	9217	2.38	10-1	25	30420	7.86														
HCA	AOO	8-2	5	3858	1.00	9-2	10	12654	3.27	10-2	25	27682	7.15														
HCA	AOO	8-3	5	3782	1.00	9-3	10	8072	2.09	10-3	25	17014	4.40														
HCA	**AOO**	**Mean**	**5**	**5005**	**1.29**	**Mean**	**10**	**9981**	**2.58**	**Mean**	**25**	**25038**	**6.47**													**11.62**	**7.75**
Methyl salicylate	AOO	11-1	5	3250	0.84	12-1	10	4499	1.16	13-1	25	4542	1.17														
Methyl salicylate	AOO	11-2	5	3310	0.86	12-2	10	4637	1.20	13-2	25	5445	1.41														
Methyl salicylate	AOO	11-3	5	1760	0.46	12-3	10	2035	0.53	13-3	25	3996	1.03														
Methyl salicylate	**AOO**	**Mean**	**5**	**2773**	**0.72**	**Mean**	**10**	**3723**	**0.96**	**Mean**	**25**	**4661**	**1.20**													**NA**	**NA**

Substance[2]	Veh.	An. #	1 Conc. (%)	1 Mean ATP[3]	1 SI	An. #	2 Conc. (%)	2 Mean ATP[3]	2 SI	An. #	3 Conc. (%)	3 Mean ATP[3]	3 SI	An. #	4 Conc. (%)	4 Mean ATP[3]	4 SI	An. #	5 Conc. (%)	5 Mean ATP[3]	5 SI	An. #	6 Conc. (%)	6 Mean ATP[3]	6 SI	Calc. EC3[4]	Calc. EC2[5]
Vehicle Control 1	AOO	1-1	0	3529	1.17																						
Vehicle Control 1	AOO	1-2	0	3106	1.03																						
Vehicle Control 1	AOO	1-3	0	2949	0.98																						
Vehicle Control 1	AOO	1-4	0	2473	0.82																						
Vehicle Control 1	**AOO**	**Mean**	**0**	**3014**	**1.00**																						
Positive Control 1 - Eugenol	AOO	2-1	10	20105	6.67																						
Positive Control 1 - Eugenol	AOO	2-2	10	14663	4.87																						
Positive Control 1 - Eugenol	AOO	2-3	10	14233	4.72																						
Positive Control 1 - Eugenol	AOO	2-4	10	13137	4.36																						
Positive Control 1 - Eugenol	**AOO**	**Mean**	**10**	**15535**	**5.15**																						
Vehicle Control 2	DMSO	3-1	0	4770	0.72																						
Vehicle Control 2	DMSO	3-2	0	6914	1.04																						
Vehicle Control 2	DMSO	3-3	0	8487	1.27																						
Vehicle Control 2	DMSO	3-4	0	6527	0.98																						
Vehicle Control 2	**DMSO**	**Mean**	**0**	**6674**	**1.00**																						
Positive Control 2 - Eugenol	DMSO	4-1	10	10887	1.63																						
Positive Control 2 - Eugenol	DMSO	4-2	10	16454	2.47																						
Positive Control 2 - Eugenol	DMSO	4-3	10	9982	1.50																						
Positive Control 2 - Eugenol	DMSO	4-4	10	12245	1.84																						
Positive Control 2 - Eugenol	**DMSO**	**Mean**	**10**	**12392**	**1.86**	**Failed PC**																					
Abietic acid	AOO	5-1	5	4143	1.38	6-1	10	13190	4.3	7-1	25	20693	6.87														
Abietic acid	AOO	5-2	5	9059	3.01	6-2	10	8354	2.772	7-2	25	17109	5.68														
Abietic acid	AOO	5-3	5	7056	2.34	6-3	10	10561	3.50	7-3	25	18770	6.23														

Substance[2]	Veh.	An. #	1 Conc. (%)	1 Mean ATP[3]	1 SI	An. #	2 Conc. (%)	2 Mean ATP[3]	2 SI	An. #	3 Conc. (%)	3 Mean ATP[3]	3 SI	An. #	4 Conc. (%)	4 Mean ATP[3]	4 SI	An. #	5 Conc. (%)	5 Mean ATP[3]	5 SI	An. #	6 Conc. (%)	6 Mean ATP[3]	6 SI	Calc. EC3[4]	Calc. EC2[5]
Abietic acid	**AOO**	**Mean**	**5**	**6752**	**2.24**	**Mean**	**10**	**10701**	**3.55**	**Mean**	**25**	**18857**	**6.26**													**7.90**	**4.40**
Cobalt II chloride	DMSO	8-1	1.0	17709	2.65	9-1	2.5	17680	2.65	10-1	5.0	28248	4.23														
Cobalt II chloride	DMSO	8-2	1.0	12673	1.90	9-2	2.5	17863	2.68	10-2	5.0	27268	4.09														
Cobalt II chloride	DMSO	8-3	1.0	12428	1.86	9-3	2.5	18809	2.82	10-3	5.0	17378	2.60														
Cobalt II chloride	**DMSO**	**Mean**	**1.0**	**14270**	**2.14**	**Mean**	**2.5**	**18117**	**2.71**	**Mean**	**5.0**	**24298**	**3.64**													**3.27**	**0.88**
Nickel (II) sulfate hexahydrate	DMSO	11-1	1.0	7672	1.15	12-1	2.5	10829	1.62	13-1	5.0	15969	2.39														
Nickel (II) sulfate hexahydrate	DMSO	11-2	1.0	11041	1.65	12-2	2.5	10925	1.64	13-2	5.0	9433	1.41														
Nickel (II) sulfate hexahydrate	DMSO	11-3	1.0	8581	1.29	12-3	2.5	21735	3.26	13-3	5.0	11636	1.74														
Nickel (II) sulfate hexahydrate	**DMSO**	**Mean**	**1.0**	**9098**	**1.36**	**Mean**	**2.5**	**14496**	**2.17**	**Mean**	**5.0**	**12346**	**1.85**													**NA**	**2.18**
Vehicle Control 1	AOO	1-1	0	2660	1.03																						
Vehicle Control 1	AOO	1-2	0	2856	1.11																						
Vehicle Control 1	AOO	1-3	0	1828	0.71																						
Vehicle Control 1	AOO	1-4	0	2975	1.15																						
Vehicle Control 1	**AOO**	**Mean**	**0**	**2580**	**1.00**																						
Positive Control 1 - Eugenol	AOO	2-1	10	19298	7.48																						
Positive Control 1 - Eugenol	AOO	2-2	10	17360	6.73																						
Positive Control 1 - Eugenol	AOO	2-3	10	14953	5.80																						
Positive Control 1 - Eugenol	AOO	2-4	10	11827	4.59																						
Positive Control 1 - Eugenol	**AOO**	**Mean**	**10**	**15859**	**6.15**																						
Vehicle Control 2	DMF	3-1	0	4424	1.29																						
Vehicle Control 2	DMF	3-2	0	3087	0.90																						
Vehicle Control 2	DMF	3-3	0	2348	0.69																						
Vehicle Control 2	DMF	3-4	0	3854	1.12																						

Substance[2]	Veh.	An. #	1 Conc. (%)	1 Mean ATP[3]	1 SI	An. #	2 Conc. (%)	2 Mean ATP[3]	2 SI	An. #	3 Conc. (%)	3 Mean ATP[3]	3 SI	An. #	4 Conc. (%)	4 Mean ATP[3]	4 SI	An. #	5 Conc. (%)	5 Mean ATP[3]	5 SI	An. #	6 Conc. (%)	6 Mean ATP[3]	6 SI	Calc. EC3[4]	Calc. EC2[5]
Vehicle Control 2	**DMF**	**Mean**	**0**	**3428**	**1.00**																						
Positive Control 2 - Eugenol	DMF	4-1	10	5738	1.67																						
Positive Control 2 - Eugenol	DMF	4-2	10	5644	1.65																						
Positive Control 2 - Eugenol	DMF	4-3	10	3688	1.08																						
Positive Control 2 - Eugenol	DMF	4-4	10	8185	2.39																						
Positive Control 2 - Eugenol	**DMF**	**Mean**	**10**	**5813**	**1.70**	**Failed PC**																					
Benzocaine	AOO	5-1	5	10495	4.07	6-1	10	10314	4.00	7-1	25	10512	4.08														
Benzocaine	AOO	5-2	5	3052	1.18	6-2	10	10880	4.22	7-2	25	14366	5.57														
Benzocaine	AOO	5-3	5	6751	2.62	6-3	10	8378	3.25	7-3	25	12564	4.87														
Benzocaine	**AOO**	**Mean**	**5**	**6766**	**2.62**	**Mean**	**10**	**9857**	**3.82**	**Mean**	**25**	**12480**	**4.84**													**6.57**	**3.49**
Imidazolidinyl urea	DMF	8-1	10	7333	2.14	9-1	25	9854	2.88	10-1	50	14760	4.31														
Imidazolidinyl urea	DMF	8-2	10	6777	1.98	9-2	25	13907	4.06	10-2	50	15299	4.46														
Imidazolidinyl urea	DMF	8-3	10	10143	2.96	9-3	25	11783	3.44	10-3	50	17971	5.24														
Imidazolidinyl urea	**DMF**	**Mean**	**10**	**8084**	**2.36**	**Mean**	**25**	**11848**	**3.46**	**Mean**	**50**	**16010**	**4.67**													**18.77**	**7.42**
2-Mercaptbenzo-thiazole	DMF	11-1	10	7829	2.28	12-1	25	6978	2.04	13-1	50	3976	1.16														
2-Mercaptbenzo-thiazole	DMF	11-2	10	7102	2.07	12-2	25	2425	0.71	13-2	50	4375	1.28														
2-Mercaptbenzo-thiazole	DMF	11-3	10	5647	1.65	12-3	25	4401	1.28	13-3	50	2675	0.78														
2-Mercaptbenzo-thiazole	**DMF**	**Mean**	**10**	**6859**	**2.00**	**Mean**	**25**	**4601**	**1.34**	**Mean**	**50**	**3675**	**1.07**													**NA**	**9.99**
Vehicle Control	AOO	1-1	0	1453	0.28																						
Vehicle Control	AOO	1-2	0	11748	2.27																						
Vehicle Control	AOO	1-3	0	4663	0.90																						
Vehicle Control	AOO	1-4	0	2810	0.54																						
Vehicle Control	**AOO**	**Mean**	**0**	**5168**	**1.00**																						

Substance[2]	Veh.	An. #	1 Conc. (%)	1 Mean ATP[3]	1 SI	An. #	2 Conc. (%)	2 Mean ATP[3]	2 SI	An. #	3 Conc. (%)	3 Mean ATP[3]	3 SI	An. #	4 Conc. (%)	4 Mean ATP[3]	4 SI	An. #	5 Conc. (%)	5 Mean ATP[3]	5 SI	An. #	6 Conc. (%)	6 Mean ATP[3]	6 SI	Calc. EC3[4]	Calc. EC2[5]
Positive Control - Eugenol	AOO	2-1	10	13351	2.58																						
Positive Control - Eugenol	AOO	2-2	10	27023	5.229																						
Positive Control - Eugenol	AOO	2-3	10	12875	2.49																						
Positive Control - Eugenol	AOO	2-4	10	15921	3.08																						
Positive Control - Eugenol	**AOO**	**Mean**	**10**	**17292**	**3.35**																						
2-4-Dinitro-chlorobenzene	AOO	10-1	0.025	11884	2.30	11-1	0.05	10848	2.10	12-1	0.10	13205	2.56	13-1	0.25	34300	6.64	14-1	0.5	33092	6.40	15-1	1.0	40795	7.89		
2-4-Dinitro-chlorobenzene	AOO	10-2	0.025	11146	2.16	11-2	0.05	7394	1.43	12-2	0.10	8679	1.68	13-2	0.25	26924	5.21	14-2	0.5	46685	9.03	15-2	1.0	36807	7.12		
2-4-Dinitro-chlorobenzene	AOO	10-3	0.025	5799	1.12	11-3	0.05	8468	1.64	12-3	0.10	6740	1.30	13-3	0.25	15631	3.03	14-3	0.5	30241	5.85	15-3	1.0	32445	6.29		
2-4-Dinitro-chlorobenzene	**AOO**	**Mean**	**0.025**	**9610**	**1.86**	**Mean**	**0.05**	**8903**	**1.72**	**Mean**	**0.10**	**9541**	**1.85**	**Mean**	**0.25**	**25618**	**4.96**	**Mean**	**0.5**	**36673**	**7.10**	**Mean**	**1.0**	**36682**	**7.10**	**0.16**	**0.11**
Vehicle Control	AOO	1-1	0	1460	0.41																						
Vehicle Control	AOO	1-2	0	5137	1.46																						
Vehicle Control	AOO	1-3	0	3988	1.13																						
Vehicle Control	**AOO**	**Mean**	**0**	**3528**	**1.00**																						
Positive Control - Eugenol	AOO	2-1	10	22813	6.47																						
Positive Control - Eugenol	AOO	2-2	10	21142	5.99																						
Positive Control - Eugenol	AOO	2-3	10	30985	8.78																						
Positive Control - Eugenol	**AOO**	**Mean**	**10**	**24980**	**7.08**																						
Isoeugenol	AOO	3-1	2.5	15638	4.43	4-1	5.0	15773	4.47	5-1	10	24776	7.02	6-1	25	40328	11.43	7-1	50	43389	12.30						
Isoeugenol	AOO	3-2	2.5	9113	2.58	4-2	5.0	19726	5.59	5-2	10	23236	6.59	6-2	25	50432	14.30	7-2	50	28424	8.06						
Isoeugenol	AOO	3-3	2.5	8197	2.32	4-3	5.0	10920	3.10	5-3	10	23595	6.69	6-3	25	40035	11.35	7-3	50	40263	11.41						
Isoeugenol	**AOO**	**Mean**	**2.5**	**10982**	**3.11**	**Mean**	**5.0**	**15473**	**4.39**	**Mean**	**10**	**23869**	**6.77**	**Mean**	**25**	**43598**	**12.36**	**Mean**	**50**	**37359**	**10.59**					**2.35**	**1.36**
Vehicle Control	AOO	1-1	0	836	0.55																						
Vehicle Control	AOO	1-2	0	1815	1.20																						

Substance[2]	Veh.	An. #	1 Conc. (%)	1 Mean ATP[3]	1 SI	An. #	2 Conc. (%)	2 Mean ATP[3]	2 SI	An. #	3 Conc. (%)	3 Mean ATP[3]	3 SI	An. #	4 Conc. (%)	4 Mean ATP[3]	4 SI	An. #	5 Conc. (%)	5 Mean ATP[3]	5 SI	An. #	6 Conc. (%)	6 Mean ATP[3]	6 SI	Calc. EC3[4]	Calc. EC2[5]
Vehicle Control	AOO	1-3	0	1752	1.16																						
Vehicle Control	AOO	1-4	0	1631	1.08																						
Vehicle Control	**AOO**	**Mean**	**0**	**1508**	**1.00**																						
Positive Control - Eugenol	AOO	2-1	10	13707	9.09																						
Positive Control - Eugenol	AOO	2-2	10	6746	4.47																						
Positive Control - Eugenol	AOO	2-3	10	10475	6.95																						
Positive Control - Eugenol	AOO	2-4	10	6855	4.54																						
Positive Control - Eugenol	**AOO**	**Mean**	**10**	**9446**	**6.26**																						
Benzalkonium chloride	AOO	12-1	0.5	3027	2.01	13-1	1.0	9672	6.41	14-1	2.5	10292	6.82														
Benzalkonium chloride	AOO	12-2	0.5	5780	3.83	13-2	1.0	7809	5.18	14-2	2.5	11879	7.88														
Benzalkonium chloride	AOO	12-3	0.5	4183	2.77	13-3	1.0	10868	7.21	14-3	2.5	8070	5.35														
Benzalkonium chloride	**AOO**	**Mean**	**0.5**	**4330**	**2.87**	**Mean**	**1.0**	**9449**	**6.26**	**Mean**	**2.5**	**10080**	**6.68**													**0.52**	**0.42**
Vehicle Control	DMF	1-1	0	2926	1.10																						
Vehicle Control	DMF	1-2	0	1674	0.63																						
Vehicle Control	DMF	1-3	0	3984	1.49																						
Vehicle Control	DMF	1-4	0	2091	0.78																						
Vehicle Control	**DMF**	**Mean**	**0**	**2668**	**1.00**																						
Positive Control - Cinnamic aldehyde	DMF	2-1	5	17595	6.59																						
Positive Control - Cinnamic aldehyde	DMF	2-2	5	12322	4.62																						
Positive Control - Cinnamic aldehyde	DMF	2-3	5	10331	3.87																						
Positive Control - Cinnamic aldehyde	DMF	2-4	5	12297	4.61																						

Substance[2]	Veh.	An. #	1 Conc. (%)	1 Mean ATP[3]	1 SI	An. #	2 Conc. (%)	2 Mean ATP[3]	2 SI	An. #	3 Conc. (%)	3 Mean ATP[3]	3 SI	An. #	4 Conc. (%)	4 Mean ATP[3]	4 SI	An. #	5 Conc. (%)	5 Mean ATP[3]	5 SI	An. #	6 Conc. (%)	6 Mean ATP[3]	6 SI	Calc. EC3[4]	Calc. EC2[5]
Positive Control - Cinnamic aldehyde	**DMF**	**Mean**	**5**	**13136**	**4.92**																						
Sodium lauryl sulfate	DMF	3-1	1.0	3870	1.45	4-1	2.5	7965	2.99	5-1	5.0	2945	1.10	6-1	10	10337	3.87										
Sodium lauryl sulfate	DMF	3-2	1.0	2899	1.09	4-2	2.5	4802	1.80	5-2	5.0	7161	2.68	6-2	10	6881	2.58										
Sodium lauryl sulfate	DMF	3-3	1.0	3777	1.42	4-3	2.5	6838	2.56	5-3	5.0	7913	2.97	6-3	10	9932	3.72										
Sodium lauryl sulfate	**DMF**	**Mean**	**1.0**	**3515**	**1.32**	**Mean**	**2.5**	**6535**	**2.45**	**Mean**	**5.0**	**6006**	**2.25**	**Mean**	**10**	**9050**	**3.39**									**6.88**	**1.91**
Vehicle Control	AOO	1-1	0	2045	0.97																						
Vehicle Control	AOO	1-2	0	1990	0.94																						
Vehicle Control	AOO	1-3	0	2212	1.05																						
Vehicle Control	AOO	1-4	0	2212	1.05																						
Vehicle Control	**AOO**	**Mean**	**0**	**2115**	**1.00**																						
Positive Control - HCA	AOO	2-1	15	14020	6.63																						
Positive Control - HCA	AOO	2-2	15	9078	4.29																						
Positive Control - HCA	AOO	2-3	15	8912	4.21																						
Positive Control - HCA	**AOO**	**Mean**	**15**	**10670**	**5.05**																						
Isopropanol	AOO	6-1	10	1364	0.65	7-1	25	3820	1.81	8-1	50	2249	1.06														
Isopropanol	AOO	6-2	10	2872	1.36	7-2	25	1746	0.83	8-2	50	700	0.33														
Isopropanol	AOO	6-3	10	2417	1.14	7-3	25	1298	0.61	8-3	50	2454	1.16														
Isopropanol	**AOO**	**Mean**	**10**	**2218**	**1.05**	**Mean**	**25**	**2288**	**1.08**	**Mean**	**50**	**1801**	**0.85**													**NA**	**NA**
Vehicle Control	AOO	1-1	0	2386	0.76																						
Vehicle Control	AOO	1-2	0	2967	0.95																						
Vehicle Control	AOO	1-3	0	4347	1.39																						
Vehicle Control	AOO	1-4	0	2816	0.90																						
Vehicle Control	**AOO**	**Mean**	**0**	**3129**	**1.00**																						
Positive Control - HCA	AOO	2-1	15	9352	2.99																						

Substance[2]	Veh.	An. #	1 Conc. (%)	1 Mean ATP[3]	1 SI	An. #	2 Conc. (%)	2 Mean ATP[3]	2 SI	An. #	3 Conc. (%)	3 Mean ATP[3]	3 SI	An. #	4 Conc. (%)	4 Mean ATP[3]	4 SI	An. #	5 Conc. (%)	5 Mean ATP[3]	5 SI	An. #	6 Conc. (%)	6 Mean ATP[3]	6 SI	Calc. EC3[4]	Calc. EC2[5]
Positive Control - HCA	AOO	2-2	15	16201	5.18																						
Positive Control - HCA	AOO	2-3	15	10538	3.37																						
Positive Control - HCA	AOO	2-4	15	9135	2.92																						
Positive Control - HCA	**AOO**	**Mean**	**15**	**11306**	**3.61**																						
Hexane	AOO	12-1	25	3755	1.20	13-1	50	3070	0.98	14-1	100	9027	2.89														
Hexane	AOO	12-2	25	3240	1.04	13-2	50	2491	0.80	14-2	100	6802	2.17														
Hexane	AOO	12-3	25	3136	1.00	13-3	50	2658	0.85	14-3	100	5850	1.87														
Hexane	**AOO**	**Mean**	**25**	**3377**	**1.08**	**Mean**	**50**	**2740**	**0.88**	**Mean**	**100**	**7226**	**2.31**													**NA**	**89.19**
Vehicle Control	AOO	1-1	0	2370	0.84																						
Vehicle Control	AOO	1-2	0	3124	1.11																						
Vehicle Control	AOO	1-3	0	2314	0.82																						
Vehicle Control	AOO	1-4	0	3464	1.23																						
Vehicle Control	**AOO**	**Mean**	**0**	**2818**	**1.00**																						
Positive Control - HCA	AOO	2-1	15	7739	2.75																						
Positive Control - HCA	AOO	2-2	15	10867	3.86																						
Positive Control - HCA	AOO	2-3	15	5290	1.88																						
Positive Control - HCA	AOO	2-4	15	8570	3.04																						
Positive Control - HCA	**AOO**	**Mean**	**15**	**8116**	**2.88**	**Failed PC when SI ≥ 3.0**																					
Toluene-2,4-diisocyanate	AOO	12-1	0.05	9445	3.35	13-1	0.10	12732	4.52	14-1	0.25	25104	8.91														
Toluene-2,4-diisocyanate	AOO	12-2	0.05	11471	4.07	13-2	0.10	17962	6.38	14-2	0.25	27791	9.86														
Toluene-2,4-diisocyanate	AOO	12-3	0.05	5999	2.13	13-3	0.10	16204	5.75	14-3	0.25	26785	9.51														
Toluene-2,4-diisocyanate	**AOO**	**Mean**	**0.05**	**8972**	**3.18**	**Mean**	**0.10**	**15632**	**5.55**	**Mean**	**0.25**	**26560**	**9.43**													**0.05**	**0.04**

Substance[2]	Veh.	An. #	1 Conc. (%)	1 Mean ATP[3]	1 SI	An. #	2 Conc. (%)	2 Mean ATP[3]	2 SI	An. #	3 Conc. (%)	3 Mean ATP[3]	3 SI	An. #	4 Conc. (%)	4 Mean ATP[3]	4 SI	An. #	5 Conc. (%)	5 Mean ATP[3]	5 SI	An. #	6 Conc. (%)	6 Mean ATP[3]	6 SI	Calc. EC3[4]	Calc. EC2[5]
Vehicle Control	AOO	1-1	0	1727	0.80																						
Vehicle Control	AOO	1-2	0	2122	0.99																						
Vehicle Control	AOO	1-3	0	2111	0.98																						
Vehicle Control	AOO	1-4	0	2645	1.23																						
Vehicle Control	**AOO**	**Mean**	**0**	**2151**	**1.00**																						
Positive Control - HCA	AOO	2-1	15	14931	6.94																						
Positive Control - HCA	AOO	2-2	15	15575	7.24																						
Positive Control - HCA	AOO	2-3	15	13043	6.06																						
Positive Control - HCA	AOO	2-4	15	11199	5.21																						
Positive Control - HCA	**AOO**	**Mean**	**15**	**13687**	**6.36**																						
1-Bromobutane	AOO	3-1	5	2701	1.26	4-1	10	1810	0.84	5-1	25	3483	1.62														
1-Bromobutane	AOO	3-2	5	2491	1.16	4-2	10	2130	0.99	5-2	25	2916	1.36														
1-Bromobutane	AOO	3-3	5	4272	1.99	4-3	10	878	0.41	5-3	25	4220	1.96														
1-Bromobutane	**AOO**	**Mean**	**5**	**3154**	**1.47**	**Mean**	**10**	**1606**	**0.75**	**Mean**	**25**	**3539**	**1.65**													**NA**	**NA**
Chlorobenzene	AOO	6-1	5	1875	0.87	7-1	10	2505	1.16	8-1	25	2848	1.32														
Chlorobenzene	AOO	6-2	5	2180	1.01	7-2	10	1840	0.86	8-2	25	5302	2.47														
Chlorobenzene	AOO	6-3	5	1088	0.51	7-3	10	2682	1.25	8-3	25	7615	3.54														
Chlorobenzene	**AOO**	**Mean**	**5**	**1714**	**0.80**	**Mean**	**10**	**2342**	**1.09**	**Mean**	**25**	**5255**	**2.44**													**NA**	**20.09**
Diethyl-phthalate	AOO	9-1	25	1543	0.72	10-1	50	1781	0.83	11-1	100	1808	0.84														
Diethyl-phthalate	AOO	9-2	25	2561	1.19	10-2	50	1371	0.64	11-2	100	1288	0.60														
Diethyl-phthalate	AOO	9-3	25	2906	1.35	10-3	50	2477	1.15	11-3	100	2139	0.99														
Diethyl-phthalate	**AOO**	**Mean**	**25**	**2336**	**1.09**	**Mean**	**50**	**1876**	**0.87**	**Mean**	**100**	**1745**	**0.81**													**NA**	**NA**
Hydroxycitro-nellal	AOO	12-1	10	5201	2.42	13-1	25	9519	4.43	14-1	50	14400	6.70														
Hydroxycitro-nellal	AOO	12-2	10	4094	1.90	13-2	25	13562	6.31	14-2	50	8741	4.06														
Hydroxycitro-nellal	AOO	12-3	10	5293	2.46	13-3	25	10656	4.95	14-3	50	13563	6.31														

Substance[2]	Veh.	An. #	1 Conc. (%)	1 Mean ATP[3]	1 SI	An. #	2 Conc. (%)	2 Mean ATP[3]	2 SI	An. #	3 Conc. (%)	3 Mean ATP[3]	3 SI	An. #	4 Conc. (%)	4 Mean ATP[3]	4 SI	An. #	5 Conc. (%)	5 Mean ATP[3]	5 SI	An. #	6 Conc. (%)	6 Mean ATP[3]	6 SI	Calc. EC3[4]	Calc. EC2[5]
Hydroxycitro-nellal	**AOO**	**Mean**	**10**	**4862**	**2.26**	**Mean**	**25**	**11246**	**5.23**	**Mean**	**50**	**12234**	**5.69**													**13.74**	**9.23**
Vehicle Control	ACE	9-1	0	2232	1.39																						
Vehicle Control	ACE	9-2	0	1509	0.94																						
Vehicle Control	ACE	9-3	0	1287	0.80																						
Vehicle Control	ACE	9-4	0	1419	0.88																						
Vehicle Control	**ACE**	**Mean**	**0**	**1611**	**1.00**																						
Positive Control - HCA	ACE	10-1	15	13901	8.63																						
Positive Control - HCA	ACE	10-2	15	16265	10.09																						
Positive Control - HCA	ACE	10-3	15	15531	9.64																						
Positive Control - HCA	ACE	10-4	15	15749	9.77																						
Positive Control - HCA	**ACE**	**Mean**	**15**	**15361**	**9.53**																						
Glutaraldehyde	ACE	11-1	0.05	1821	1.13	12-1	0.10	5389	3.34	13-1	0.25	16484	10.23														
Glutaraldehyde	ACE	11-2	0.05	2181	1.35	12-2	0.10	2496	1.55	13-2	0.25	6814	4.23														
Glutaraldehyde	ACE	11-3	0.05	1931	1.12	12-3	0.10	6344	3.94	13-3	0.25	7889	4.90														
Glutaraldehyde	**ACE**	**Mean**	**0.05**	**1978**	**1.23**	**Mean**	**0.10**	**4743**	**2.94**	**Mean**	**0.25**	**10396**	**6.45**													**0.10**	**0.07**
Vehicle Control	AOO	1-1	0	3101	0.92																						
Vehicle Control	AOO	1-2	0	3253	0.97																						
Vehicle Control	AOO	1-3	0	2687	0.80																						
Vehicle Control	AOO	1-4	0	4407	1.31																						
Vehicle Control	**AOO**	**Mean**	**0**	**3362**	**1.00**																						
Positive Control - HCA	AOO	2-1	15	22800	6.78																						
Positive Control - HCA	AOO	2-2	15	16696	4.97																						
Positive Control - HCA	AOO	2-3	15	17973	5.35																						
Positive Control -	AOO	2-4	15	18757	5.58																						

Substance[2]	Veh.	An. #	1 Conc. (%)	1 Mean ATP[3]	1 SI	An. #	2 Conc. (%)	2 Mean ATP[3]	2 SI	An. #	3 Conc. (%)	3 Mean ATP[3]	3 SI	An. #	4 Conc. (%)	4 Mean ATP[3]	4 SI	An. #	5 Conc. (%)	5 Mean ATP[3]	5 SI	An. #	6 Conc. (%)	6 Mean ATP[3]	6 SI	Calc. EC3[4]	Calc. EC2[5]
HCA																											
Positive Control - HCA	**AOO**	**Mean**	**15**	**19056**	**5.67**																						
Trimellitic anhydride	AOO	7-1	0.10	5681	1.69	8-1	0.25	13902	4.14	9-1	0.50	14361	4.27														
Trimellitic anhydride	AOO	7-2	0.10	7841	2.33	8-2	0.25	11270	3.35	9-2	0.50	18976	5.64														
Trimellitic anhydride	AOO	7-3	0.10	11293	3.36	8-3	0.25	10963	3.26	9-3	0.50	16673	4.96														
Trimellitic anhydride	**AOO**	**Mean**	**0.10**	**8272**	**2.46**	**Mean**	**0.25**	**12045**	**3.58**	**Mean**	**0.50**	**16670**	**4.96**													**0.17**	**0.07**
Phthalic anhydride	AOO	10-1	0.10	11304	3.36	11-1	0.25	8332	2.48	12-1	0.50	22051	6.56	13-1	1.0	19987	5.95										
Phthalic anhydride	AOO	10-2	0.10	13066	3.89	11-2	0.25	15717	4.68	12-2	0.50	12828	3.82	13-2	1.0	32118	9.55										
Phthalic anhydride	AOO	10-3	0.10	12448	3.70	11-3	0.25	9833	2.93	12-3	0.50	24315	7.23	13-3	1.0	17006	5.09										
Phthalic anhydride	**AOO**	**Mean**	**0.10**	**12272**	**3.65**	**Mean**	**0.25**	**11294**	**3.36**	**Mean**	**0.50**	**19731**	**5.87**	**Mean**	**1.0**	**23037**	**6.85**									**0.08**	**0.04**
Vehicle Control 1	DMSO	1-1	0	13832	1.36																						
Vehicle Control 1	DMSO	1-2	0	9930	0.97																						
Vehicle Control 1	DMSO	1-3	0	9958	0.98																						
Vehicle Control 1	DMSO	1-4	0	7097	0.70																						
Vehicle Control 1	**DMSO**	**Mean**	**0**	**10204**	**1.00**																						
Positive Control 1 - HCA	DMSO	2-1	15	17741	1.74																						
Positive Control 1 - HCA	DMSO	2-2	15	18810	1.84																						
Positive Control 1 - HCA	DMSO	2-3	15	18045	1.77																						
Positive Control 1 - HCA	DMSO	2-4	15	12293	1.21																						
Positive Control 1 - HCA	**DMSO**	**Mean**	**15**	**16722**	**1.64**	**Failed PC**																					
Lactic acid	DMSO	3-1	5	6741	0.66	4-1	10	11054	1.08	5-1	25	7025	0.69	6-1	50	8623	0.85										
Lactic acid	DMSO	3-2	5	12789	1.25	4-2	10	11929	1.17	5-2	25	13796	1.35	6-2	50	10101	0.99										
Lactic acid	DMSO	3-3	5	12217	1.12	4-3	10	9542	0.94	5-3	25	8677	0.85	6-3	50	11594	1.14										
Lactic acid	**DMSO**	**Mean**	**5**	**10582**	**1.04**	**Mean**	**10**	**10841**	**1.06**	**Mean**	**25**	**9832**	**0.96**	**Mean**	**50**	**10106**	**0.99**									**NA**	**NA**

Substance[2]	Veh.	An. #	1 Conc. (%)	1 Mean ATP[3]	1 SI	An. #	2 Conc. (%)	2 Mean ATP[3]	2 SI	An. #	3 Conc. (%)	3 Mean ATP[3]	3 SI	An. #	4 Conc. (%)	4 Mean ATP[3]	4 SI	An. #	5 Conc. (%)	5 Mean ATP[3]	5 SI	An. #	6 Conc. (%)	6 Mean ATP[3]	6 SI	Calc. EC3[4]	Calc. EC2[5]
Vehicle Control 2	AOO	7-1	0	5263	1.07																						
Vehicle Control 2	AOO	7-2	0	4970	1.01																						
Vehicle Control 2	AOO	7-3	0	5431	1.11																						
Vehicle Control 2	AOO	7-4	0	3965	0.81																						
Vehicle Control 2	**AOO**	**Mean**	**0**	**4907**	**1.00**																						
Positive Control 2 - HCA	AOO	8-1	15	25796	5.26																						
Positive Control 2 - HCA	AOO	8-2	15	24279	4.95																						
Positive Control 2 - HCA	AOO	8-3	15	13979	2.85																						
Positive Control 2 - HCA	AOO	8-4	15	23991	4.89																						
Positive Control 2 - HCA	**AOO**	**Mean**	**15**	**22011**	**4.49**																						
Resorcinol	AOO	9-1	5	12461	2.54	10-1	10	25798	5.26	11-1	25	20760	4.23														
Resorcinol	AOO	9-2	5	11743	2.39	10-2	10	16771	3.42	11-2	25	21215	4.32														
Resorcinol	AOO	9-3	5	12095	2.47	10-3	10	21121	4.30	11-3	25	9659	1.97														
Resorcinol	**AOO**	**Mean**	**5**	**12099**	**2.47**	**Mean**	**10**	**21230**	**4.33**	**Mean**	**25**	**17211**	**3.51**													**6.44**	**4.20**
Vehicle Control	ACE	1-1	0	3937	1.45																						
Vehicle Control	ACE	1-2	0	2374	0.88																						
Vehicle Control	ACE	1-3	0	2360	0.87																						
Vehicle Control	ACE	1-4	0	2173	0.80																						
Vehicle Control	**ACE**	**Mean**	**0**	**2711**	**1.00**																						
Positive Control - HCA	ACE	2-1	15	21117	7.79																						
Positive Control - HCA	ACE	2-2	15	19843	7.32																						
Positive Control - HCA	ACE	2-3	15	12203	4.50																						
Positive Control - HCA	ACE	2-4	15	13734	5.07																						

Substance[2]	Veh.	An. #	1 Conc. (%)	1 Mean ATP[3]	1 SI	An. #	2 Conc. (%)	2 Mean ATP[3]	2 SI	An. #	3 Conc. (%)	3 Mean ATP[3]	3 SI	An. #	4 Conc. (%)	4 Mean ATP[3]	4 SI	An. #	5 Conc. (%)	5 Mean ATP[3]	5 SI	An. #	6 Conc. (%)	6 Mean ATP[3]	6 SI	Calc. EC3[4]	Calc. EC2[5]
Positive Control - HCA	**ACE**	**Mean**	**15**	**16724**	**6.17**																						
Formaldehyde	ACE	3-1	0.10	5222	1.93	4-1	0.25	6167	2.28	5-1	0.50	2317	0.86	6-1	1.0	7846	2.90	7-1	2.5	17242	6.36						
Formaldehyde	ACE	3-2	0.10	3045	1.12	4-2	0.25	2933	1.08	5-2	0.50	4479	1.65	6-2	1.0	10628	3.92	7-2	2.5	14355	5.30						
Formaldehyde	ACE	3-3	0.10	2923	1.08	4-3	0.25	5093	1.88	5-3	0.50	5263	1.94	6-3	1.0	3894	1.44	7-3	2.5	9904	3.65						
Formaldehyde	**ACE**	**Mean**	**0.10**	**3730**	**1.38**	**Mean**	**0.25**	**4731**	**1.75**	**Mean**	**0.50**	**4019**	**1.48**	**Mean**	**1.0**	**7456**	**2.75**	**Mean**	**2.5**	**13833**	**5.10**					**1.16**	**0.44**
Vehicle Control	DMSO	1-1	0	82453	1.27																						
Vehicle Control	DMSO	1-2	0	78192	1.21																						
Vehicle Control	DMSO	1-3	0	42838	0.66																						
Vehicle Control	DMSO	1-4	0	56114	0.87																						
Vehicle Control	**DMSO**	**Mean**	**0**	**64899**	**1.00**																						
Positive Control	NT	NT	NT	NT	NT																						
Positive Control	NT	NT	NT	NT	NT																						
Positive Control	NT	NT	NT	NT	NT																						
Positive Control	NT	NT	NT	NT	NT																						
Positive Control	**NT**	**Mean**	**NT**	**NT**	**NT**	**No PC**																					
Potassium dichromate	DMSO	4-1	0.1	193231	2.98	5-1	0.3	209189	3.22	6-1	1.0	286418	4.41														
Potassium dichromate	DMSO	4-2	0.1	140171	2.16	5-2	0.3	274466	4.23	6-2	1.0	304081	4.69														
Potassium dichromate	DMSO	4-3	0.1	186039	2.87	5-3	0.3	421230	6.49	6-3	1.0	440493	6.79														
Potassium dichromate	DMSO	4-4	0.1	152378	2.35	5-4	0.3	253302	3.90	6-4	1.0	394755	6.08														
Potassium dichromate	**DMSO**	**Mean**	**0.1**	**167954**	**2.59**	**Mean**	**0.3**	**289546**	**4.46**	**Mean**	**1.0**	**356437**	**5.49**													**0.14**	**0.07**
Vehicle Control	AOO	1-1	0	4172	1.44																						
Vehicle Control	AOO	1-2	0	3078	1.06																						
Vehicle Control	AOO	1-3	0	2136	0.74																						
Vehicle Control	AOO	1-4	0	2192	0.76																						
Vehicle Control	**AOO**	**Mean**	**0**	**2894**	**1.00**																						
Positive Control - HCA	AOO	2-1	15	10569	3.65																						

Substance[2]	Veh.	An. #	1 Conc. (%)	1 Mean ATP[3]	1 SI	An. #	2 Conc. (%)	2 Mean ATP[3]	2 SI	An. #	3 Conc. (%)	3 Mean ATP[3]	3 SI	An. #	4 Conc. (%)	4 Mean ATP[3]	4 SI	An. #	5 Conc. (%)	5 Mean ATP[3]	5 SI	An. #	6 Conc. (%)	6 Mean ATP[3]	6 SI	Calc. EC3[4]	Calc. EC2[5]
Positive Control - HCA	AOO	2-2	15	11027	3.81																						
Positive Control - HCA	AOO	2-3	15	12928	4.47																						
Positive Control - HCA	AOO	2-4	15	12520	4.33																						
Positive Control - HCA	**AOO**	**Mean**	**15**	**11761**	**4.06**																						
p-Phenylene-diamine	AOO	3-1	0.10	8259	2.85	4-1	0.25	12197	4.21	5-1	0.50	16392	5.66	6-1	1.0	10644	3.68										
p-Phenylene-diamine	AOO	3-2	0.10	11194	3.87	4-2	0.25	15785	5.45	5-2	0.50	9781	3.38	6-2	1.0	10669	3.69										
p-Phenylene-diamine	AOO	3-3	0.10	11454	3.96	4-3	0.25	16610	5.74	5-3	0.50	10173	3.52	6-3	1.0	5942	2.05										
p-Phenylene-diamine	**AOO**	**Mean**	**0.10**	**10302**	**3.56**	**Mean**	**0.25**	**14864**	**5.14**	**Mean**	**0.50**	**12115**	**4.19**	**Mean**	**1.0**	**9085**	**3.14**									**0.07**	**0.04**

Abbreviations: ACE = acetone; An. # = animal number; AOO = acetone: olive oil (4:1); ATP = adenosine triphosphate; Calc. = calculated; conc. = Concentration; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; HCA = hexyl cinnamic aldehyde; NA = not applicable; No. = number; NT = not tested; PC = positive control; SI = stimulation Index; Veh = vehicle.

[1]Original laboratory records with individual animal data for the 31 substances tested in the LLNA: DA intralaboratory validation study (Idehara et al. 2008) provided by Kenji Idehara, Ph.D., Daicel Chemical Industries, Ltd.

[2]The 31 substances in the intralaboratory validation study were evaluated during one of 18 LLNA: DA tests that were conducted between July 2003 through September 2007 and are listed in order based on the date that they were tested.

[3]Two ATP measurements were taken for each animal and the mean ATP is indicated.

[4]EC3 value was calculated based on interpolation or extrapolation formulas discussed in Gerberick et al. 2004.

[5]EC2 value was calculated based on modified interpolation or extrapolation formulas for EC3 discussed in Gerberick et al. 2004.

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Appendix D2

Summary Data for 14 Additional Substances Tested in the LLNA: DA (Intralaboratory)

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116 **Appendix D2 Summary of the Results for 14 Additional Substances Tested in the LLNA: DA (Intralaboratory)**

Substance Name	Vehicle	Concentration (%)	SI[1]	Calculated EC3[2] (%)	Calculated EC2[3] (%)
5-Chloro-2-methyl-4-isothiazolin-3-one (CMI)	DMF	0.005	1.2	0.03	0.01
		0.010	1.9		
		0.025	2.7		
		0.050	4.0		
		0.100	7.5		
p-Benzoquinone	AOO	0.005	2.6	0.06	0.003
		0.010	2.6		
		0.025	2.5		
		0.050	2.7		
		0.100	3.8		
Propyl gallate	AOO	0.5	2.8	1.09	0.28
		1.0	2.9		
		2.5	4.9		
Phenyl benzoate	AOO	1.0	2.2	2.26	0.80
		2.5	3.2		
		5.0	4.2		
		10.0	3.7		
Diethyl maleate	AOO	0.5	1.9	3.71	1.18

Substance Name	Vehicle	Concentration (%)	SI[1]	Calculated EC3[2] (%)	Calculated EC2[3] (%)
		1.0	1.9		
		2.5	2.7		
		5.0	3.3		
		10.0	3.8		
Ethyl acrylate	AOO	10	2.5	13.94	7.54
		25	4.3		
		50	3.4		
Cinnamic alcohol	AOO	10	2.4	21.34	6.54
		25	3.2		
		50	5.7		
		90	4.4		
Ethylene glycol dimethacrylate	MEK	10	1.2	34.03	22.27
		25	2.2		
		50	4.4		
Butyl glycidyl ether	AOO	10	1.2	31.68	19.92
		25	2.4		
		50	4.6		
Nickel (II) chloride	DMSO	2.5	0.9	NA	NA

Substance Name	Vehicle	Concentration (%)	SI[1]	Calculated EC3[2] (%)	Calculated EC2[3] (%)
		5.0	1.1		
		10.0	1.3		
Salicylic acid	AOO	5	1.5	NA	25.00
		10	1.6		
		25	2.0		
Sulfanilamide	DMF	10	0.8	NA	NA
		25	0.9		
		50	0.6		
Methyl methacrylate	AOO	25	1.0	NA	NA
		50	1.2		
		75	1.3		
		100	1.8		
Dimethyl isophthalate[4]	AOO	5	0.9	NA	NA
		10	0.9		
		25	0.8		

Abbreviations: AOO = acetone: olive oil (4:1); DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; EC3 = estimated concentration needed to produce a stimulation index of three; MEK = methyl ethyl ketone; NA = not applicable; RLU = relative luminescence units; SI = stimulation index.

[1]SI determined from mean ATP content (RLU).

[2]EC3 value was calculated based on interpolation or extrapolation formulas discussed in Gerberick et al. 2004.

[3]EC2 value was calculated based on modified interpolation or extrapolation formulas for EC3 discussed in Gerberick et al. 2004

[4]This substance was also tested in the first phase of the interlaboratory validation study (Omori et al. 2008).

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Appendix D3

Individual Animal Data for the LLNA: DA (Interlaboratory)

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Appendix D3 Individual Animal Data for the LLNA: DA Two-Phased Interlaboratory Validation Study

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Vehicle - Positive Control	1		1	0	27373	1.09									
Vehicle - Positive Control	1		2	0	23473	0.93									
Vehicle - Positive Control	1		3	0	30778	1.22									
Vehicle - Positive Control	1		4	0	19231	0.76									
Vehicle - Positive Control	**1**		**MEAN**	**0**	**25214**	**1.00**									
Positive Control	1		1	NA	163662	6.49									
Positive Control	1		2	NA	118724	4.71									
Positive Control	1		3	NA	120098	4.76									
Positive Control	1		4	NA	172911	6.86									
Positive Control	**1**		**MEAN**	**NA**	**143849**	**5.71**									
Vehicle - Substance	1	AOO	1	0	30365	1.24									
Vehicle - Substance	1	AOO	2	0	26124	1.06									
Vehicle - Substance	1	AOO	3	0	25218	1.03									
Vehicle - Substance	1	AOO	4	0	16624	0.68									
Vehicle - Substance	**1**	**AOO**	**MEAN**	**0**	**24583**	**1.00**									
Hexyl cinnamic aldehyde	1	AOO	1	5	39462	1.61	10	94155	3.83	25	174255	7.09			
Hexyl cinnamic aldehyde	1	AOO	2	5	29952	1.22	10	60720	2.47	25	140034	5.70			
Hexyl cinnamic aldehyde	1	AOO	3	5	37759	1.54	10	70595	2.87	25	103168	4.20			
Hexyl cinnamic aldehyde	1	AOO	4	5	25613	1.04	10	70068	2.85	25	151064	6.15			
Hexyl cinnamic aldehyde	**1**	**AOO**	**MEAN**	**5**	**33196**	**1.35**	**10**	**73884**	**3.01**	**25**	**142130**	**5.78**	**9.98**	**8.47**	**6.96**
Isopropanol	1	AOO	1	10	49049	2.00	25	28917	1.18	50	32979	1.34			
Isopropanol	1	AOO	2	10	46692	1.90	25	28183	1.15	50	28219	1.15			
Isopropanol	1	AOO	3	10	22501	0.92	25	28099	1.14	50	28788	1.17			
Isopropanol	1	AOO	4	10	32783	1.33	25	23206	0.94	50	24907	1.01			
Isopropanol	**1**	**AOO**	**MEAN**	**10**	**37756**	**1.54**	**25**	**27101**	**1.10**	**50**	**28723**	**1.17**	**NA**	**NA**	**NA**
Vehicle - Positive Control	1		1	0	27603	1.19									
Vehicle - Positive Control	1		2	0	29165	1.26									
Vehicle - Positive Control	1		3	0	13867	0.60									
Vehicle - Positive Control	1		4	0	21857	0.95									
Vehicle - Positive Control	**1**		**MEAN**	**0**	**23123**	**1.00**									
Positive Control	1		1	NA	187061	8.09									
Positive Control	1		2	NA	192723	8.33									

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Positive Control	1		3	NA	152209	6.58									
Positive Control	1		4	NA	120141	5.20									
Positive Control	**1**		**MEAN**	**NA**	**163033**	**7.05**									
Vehicle - Substance	1	ACE	1	0	23522	1.31									
Vehicle - Substance	1	ACE	2	0	17328	0.97									
Vehicle - Substance	1	ACE	3	0	19286	1.07									
Vehicle - Substance	1	ACE	4	0	11653	0.65									
Vehicle - Substance	**1**	**ACE**	**MEAN**	**0**	**17947**	**1.00**									
Glutaraldehyde	1	ACE	1	0.05	39029	2.17	0.15	86407	4.81	0.50	117767	6.56			
Glutaraldehyde	1	ACE	2	0.05	21473	1.20	0.15	69645	3.88	0.50	91139	5.08			
Glutaraldehyde	1	ACE	3	0.05	17442	0.97	0.15	44897	2.50	0.50	85284	4.75			
Glutaraldehyde	1	ACE	4	0.05	24434	1.36	0.15	90044	5.02	0.50	64878	3.62			
Glutaraldehyde	**1**	**ACE**	**MEAN**	**0.05**	**25594**	**1.43**	**0.15**	**72748**	**4.05**	**0.50**	**89767**	**5.00**	**0.11**	**0.09**	**0.07**
Formaldehyde	1	ACE	1	0.5	54229	3.02	1.5	65799	3.67	5.0	92516	5.16			
Formaldehyde	1	ACE	2	0.5	65863	3.67	1.5	35118	1.96	5.0	131184	7.31			
Formaldehyde	1	ACE	3	0.5	49268	2.75	1.5	48274	2.69	5.0	52728	2.94			
Formaldehyde	1	ACE	4	0.5	39499	2.20	1.5	56430	3.14	5.0	71309	3.97			
Formaldehyde	**1**	**ACE**	**MEAN**	**0.5**	**52214**	**2.91**	**1.5**	**51405**	**2.86**	**5.0**	**86934**	**4.84**	**1.75**	**0.39**	**0.26**
Vehicle - Positive Control	1		1	0	25568	1.13									
Vehicle - Positive Control	1		2	0	30989	1.37									
Vehicle - Positive Control	1		3	0	15244	0.68									
Vehicle - Positive Control	1		4	0	18525	0.82									
Vehicle - Positive Control	**1**		**MEAN**	**0**	**22582**	**1.00**									
Positive Control	1		1	NA	160326	7.10									
Positive Control	1		2	NA	97979	4.34									
Positive Control	1		3	NA	126572	5.61									
Positive Control	1		4	NA	151977	6.73									
Positive Control	**1**		**MEAN**	**NA**	**134213**	**5.94**									
Vehicle - Substance	1	AOO	1	0	36866	1.36									
Vehicle - Substance	1	AOO	2	0	33905	1.25									
Vehicle - Substance	1	AOO	3	0	15218	0.56									
Vehicle - Substance	1	AOO	4	0	22764	0.84									
Vehicle - Substance	**1**	**AOO**	**MEAN**	**0**	**27188**	**1.00**									

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
2,4-Dinitrochlorobenzene	1	AOO	1	0.03	108431	3.99	0.10	185139	6.81	0.30	334363	12.30			
2,4-Dinitrochlorobenzene	1	AOO	2	0.03	83821	3.08	0.10	159188	5.86	0.30	258002	9.49			
2,4-Dinitrochlorobenzene	1	AOO	3	0.03	68037	2.50	0.10	133437	4.91	0.30	366438	13.48			
2,4-Dinitrochlorobenzene	1	AOO	4	0.03	48931	1.80	0.10	110880	4.08	0.30	343140	12.62			
2,4-Dinitrochlorobenzene	**1**	**AOO**	**MEAN**	**0.03**	**77305**	**2.84**	**0.10**	**147161**	**5.41**	**0.30**	**325485**	**11.97**	**0.03**	**0.03**	**0.02**
Dimethyl isophthalate	1	AOO	1	5	41322	1.52	10	46499	1.71	25	39741	1.46			
Dimethyl isophthalate	1	AOO	2	5	32753	1.20	10	27887	1.03	25	21245	0.78			
Dimethyl isophthalate	1	AOO	3	5	24319	0.89	10	29565	1.09	25	38401	1.41			
Dimethyl isophthalate	1	AOO	4	5	47742	1.76	10	20851	0.77	25	20734	0.76			
Dimethyl isophthalate	**1**	**AOO**	**MEAN**	**5**	**36534**	**1.34**	**10**	**31200**	**1.15**	**25**	**30030**	**1.10**	**NA**	**NA**	**NA**
3-Aminophenol	1	AOO	1	1	48998	1.80	3	65491	2.41	10	93723	3.45			
3-Aminophenol	1	AOO	2	1	50122	1.84	3	55831	2.05	10	57142	2.10			
3-Aminophenol	1	AOO	3	1	47237	1.74	3	55478	2.04	10	82054	3.02			
3-Aminophenol	1	AOO	4	1	44007	1.62	3	75285	2.77	10	74792	2.75			
3-Aminophenol	**1**	**AOO**	**MEAN**	**1**	**47591**	**1.75**	**3**	**63021**	**2.32**	**10**	**76927**	**2.83**	**NA**	**5.49**	**1.88**
Vehicle - Positive Control	2		1	0	29854	0.94									
Vehicle - Positive Control	2		2	0	36425	1.15									
Vehicle - Positive Control	2		3	0	42387	1.34									
Vehicle - Positive Control	2		4	0	18060	0.57									
Vehicle - Positive Control	**2**		**MEAN**	**0**	**31681**	**1.00**									
Positive Control	2		1	NA	194745	6.15									
Positive Control	2		2	NA	196510	6.20									
Positive Control	2		3	NA	202311	6.39									
Positive Control	2		4	NA	171703	5.42									
Positive Control	**2**		**MEAN**	**P**	**191317**	**6.04**									
Vehicle - Substance	2	AOO	1	0	26727	0.65									
Vehicle - Substance	2	AOO	2	0	62370	1.51									
Vehicle - Substance	2	AOO	3	0	48632	1.18									
Vehicle - Substance	2	AOO	4	0	27029	0.66									
Vehicle - Substance	**2**	**AOO**	**MEAN**	**0**	**41189**	**1.00**									
Hexyl cinnamic aldehyde	2	AOO	1	5	49355	1.20	10	129128	3.13	25	259210	6.29			
Hexyl cinnamic aldehyde	2	AOO	2	5	57775	1.40	10	98419	2.39	25	185538	4.50			

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Hexyl cinnamic aldehyde	2	AOO	3	5	62556	1.52	10	96062	2.33	25	176096	4.28			
Hexyl cinnamic aldehyde	2	AOO	4	5	55479	1.35	10	113209	2.75	25	173235	4.21			
Hexyl cinnamic aldehyde	**2**	**AOO**	**MEAN**	**5**	**56291**	**1.37**	**10**	**109204**	**2.65**	**25**	**198520**	**4.82**	**12.41**	**9.41**	**7.46**
Isopropanol	2	AOO	1	10	48933	1.19	25	40741	0.99	50	31132	0.76			
Isopropanol	2	AOO	2	10	26716	0.65	25	33529	0.81	50	44432	1.08			
Isopropanol	2	AOO	3	10	38147	0.93	25	36625	0.89	50	30372	0.74			
Isopropanol	2	AOO	4	10	35351	0.86	25	29201	0.71	50	27101	0.66			
Isopropanol	**2**	**AOO**	**MEAN**	**10**	**37286**	**0.91**	**25**	**35024**	**0.85**	**50**	**33259**	**0.81**	**NA**	**NA**	**NA**
Vehicle - Positive Control	2		1	0	16450	0.51									
Vehicle - Positive Control	2		2	0	56211	1.74									
Vehicle - Positive Control	2		3	0	29690	0.92									
Vehicle - Positive Control	2		4	0	26911	0.83									
Vehicle - Positive Control	**2**		**MEAN**	**0**	**32315**	**1.00**									
Positive Control	2		1	NA	100365	3.11									
Positive Control	2		2	NA	144864	4.48									
Positive Control	2		3	NA	121515	3.76									
Positive Control	2		4	NA	131149	4.06									
Positive Control	**2**		**MEAN**	**NA**	**124473**	**3.85**									
Vehicle - Substance	2	AOO	1	0	26982	1.03									
Vehicle - Substance	2	AOO	2	0	26503	1.01									
Vehicle - Substance	2	AOO	3	0	23078	0.88									
Vehicle - Substance	2	AOO	4	0	28074	1.07									
Vehicle - Substance	**2**	**AOO**	**MEAN**	**0**	**26159**	**1.00**									
2,4-Dinitrochlorobenzene	2	AOO	1	0.03	46482	1.78	0.10	54947	2.10	0.30	154655	5.91			
2,4-Dinitrochlorobenzene	2	AOO	2	0.03	45109	1.72	0.10	79087	3.02	0.30	244903	9.36			
2,4-Dinitrochlorobenzene	2	AOO	3	0.03	64419	2.46	0.10	103400	3.95	0.30	231793	8.86			
2,4-Dinitrochlorobenzene	2	AOO	4	0.03	87361	3.34	0.10	44369	1.70	0.30	334511	12.79			
2,4-Dinitrochlorobenzene	**2**	**AOO**	**MEAN**	**0.03**	**60843**	**2.33**	**0.10**	**70451**	**2.69**	**0.30**	**241465**	**9.23**	**0.11**	**0.06**	**0.02**
Abietic acid	2	AOO	1	5	53429	2.04	10	76437	2.92	25	109226	4.18			
Abietic acid	2	AOO	2	5	44953	1.72	10	106616	4.08	25	165358	6.32			
Abietic acid	2	AOO	3	5	55417	2.12	10	106351	4.07	25	78960	3.02			
Abietic acid	2	AOO	4	5	66359	2.54	10	77421	2.96	25	131863	5.04			

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Abietic acid	**2**	**AOO**	**MEAN**	**5**	**55039**	**2.10**	**10**	**91706**	**3.51**	**25**	**121351**	**4.64**	**8.20**	**6.41**	**4.76**
Vehicle - Positive Control	2		1	0	15977	0.59									
Vehicle - Positive Control	2		2	0	29941	1.11									
Vehicle - Positive Control	2		3	0	25288	0.94									
Vehicle - Positive Control	2		4	0	36217	1.35									
Vehicle - Positive Control	**2**		**MEAN**	**0**	**26856**	**1.00**									
Positive Control	2		1	NA	105933	3.94									
Positive Control	2		2	NA	170707	6.36									
Positive Control	2		3	NA	134656	5.01									
Positive Control	2		4	NA	173488	6.46									
Positive Control	**2**		**MEAN**	**NA**	**146196**	**5.44**									
Vehicle - Substance	2	ACE	1	0	56525	1.49									
Vehicle - Substance	2	ACE	2	0	38645	1.02									
Vehicle - Substance	2	ACE	3	0	28667	0.75									
Vehicle - Substance	2	ACE	4	0	28339	0.74									
Vehicle - Substance	**2**	**ACE**	**MEAN**	**0**	**38044**	**1.00**									
Glutaraldehyde	2	ACE	1	0.05	34115	0.90	0.15	50405	1.32	0.50	172747	4.54			
Glutaraldehyde	2	ACE	2	0.05	37388	0.98	0.15	36212	0.95	0.50	104608	2.75			
Glutaraldehyde	2	ACE	3	0.05	17955	0.47	0.15	54707	1.44	0.50	105731	2.78			
Glutaraldehyde	2	ACE	4	0.05	22926	0.60	0.15	54598	1.44	0.50	133355	3.51			
Glutaraldehyde	**2**	**ACE**	**MEAN**	**0.05**	**28096**	**0.74**	**0.15**	**48980**	**1.29**	**0.50**	**129110**	**3.39**	**0.44**	**0.35**	**0.27**
Formaldehyde	2	ACE	1	0.5	71257	1.87	1.5	120557	3.17	5.0	148089	3.89			
Formaldehyde	2	ACE	2	0.5	61368	1.61	1.5	110027	2.89	5.0	111959	2.94			
Formaldehyde	2	ACE	3	0.5	74954	1.97	1.5	139716	3.67	5.0	97241	2.56			
Formaldehyde	2	ACE	4	0.5	50290	1.32	1.5	90274	2.37	5.0	126577	3.33			
Formaldehyde	**2**	**ACE**	**MEAN**	**0.5**	**64467**	**1.69**	**1.5**	**115143**	**3.03**	**5.0**	**120966**	**3.18**	**1.48**	**1.11**	**0.73**
Vehicle - Positive Control	3		1	0	14012	0.68									
Vehicle - Positive Control	3		2	0	25742	1.25									
Vehicle - Positive Control	3		3	0	18482	0.90									
Vehicle - Positive Control	3		4	0	24206	1.17									
Vehicle - Positive Control	**3**		**MEAN**	**0**	**20610**	**1.00**									
Positive Control	3		1	NA	147051	7.13									
Positive Control	3		2	NA	129657	6.29									

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Positive Control	3		3	NA	119376	5.79									
Positive Control	3		4	NA	132756	6.44									
Positive Control	**3**		**MEAN**	**NA**	**132210**	**6.41**									
Vehicle - Substance	3	AOO	1	0	22801	0.95									
Vehicle - Substance	3	AOO	2	0	28208	1.17									
Vehicle - Substance	3	AOO	3	0	19180	0.80									
Vehicle - Substance	3	AOO	4	0	26000	1.08									
Vehicle - Substance	**3**	**AOO**	**MEAN**	**0**	**24047**	**1.00**									
Methyl salicylate	3	AOO	1	5	22109	0.92	10	35176	1.46	25	53142	2.21			
Methyl salicylate	3	AOO	2	5	22812	0.95	10	22115	0.92	25	31027	1.29			
Methyl salicylate	3	AOO	3	5	21410	0.89	10	21251	0.88	25	31120	1.29			
Methyl salicylate	3	AOO	4	5	36725	1.53	10	26904	1.12	25	34146	1.42			
Methyl salicylate	**3**	**AOO**	**MEAN**	**5**	**25764**	**1.07**	**10**	**26361**	**1.10**	**25**	**37359**	**1.55**	**NA**	**NA**	**NA**
3-Aminophenol	3	AOO	1	1	40069	1.67	3	51109	2.13	10	39746	1.65			
3-Aminophenol	3	AOO	2	1	31036	1.29	3	34706	1.44	10	38143	1.59			
3-Aminophenol	3	AOO	3	1	28933	1.20	3	53201	2.21	10	35330	1.47			
3-Aminophenol	3	AOO	4	1	35464	1.47	3	30394	1.26	10	53816	2.24			
3-Aminophenol	**3**	**AOO**	**MEAN**	**1**	**33875**	**1.41**	**3**	**42352**	**1.76**	**10**	**41759**	**1.74**	**NA**	**NA**	**NA**
Vehicle - Positive Control	3		1	0	32037	1.14									
Vehicle - Positive Control	3		2	0	27673	0.98									
Vehicle - Positive Control	3		3	0	25512	0.91									
Vehicle - Positive Control	3		4	0	27174	0.97									
Vehicle - Positive Control	**3**		**MEAN**	**0**	**28099**	**1.00**									
Positive Control	3		1	NA	133836	4.76									
Positive Control	3		2	NA	122152	4.35									
Positive Control	3		3	NA	164019	5.84									
Positive Control	3		4	NA	133810	4.76									
Positive Control	**3**		**MEAN**	**NA**	**138454**	**4.93**									
Vehicle - Substance	3	AOO	1	0	52047	1.46									
Vehicle - Substance	3	AOO	2	0	31377	0.88									
Vehicle - Substance	3	AOO	3	0	36296	1.02									
Vehicle - Substance	3	AOO	4	0	22887	0.64									
Vehicle - Substance	**3**	**AOO**	**MEAN**	**0**	**35652**	**1.00**									

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Hexyl cinnamic aldehyde	3	AOO	1	5	38213	1.07	10	69749	1.96	25	124915	3.50			
Hexyl cinnamic aldehyde	3	AOO	2	5	35942	1.01	10	85956	2.41	25	168780	4.73			
Hexyl cinnamic aldehyde	3	AOO	3	5	68561	1.92	10	97018	2.72	25	188378	5.28			
Hexyl cinnamic aldehyde	3	AOO	4	5	50818	1.43	10	75438	2.12	25	151145	4.24			
Hexyl cinnamic aldehyde	**3**	**AOO**	**MEAN**	**5**	**48383**	**1.36**	**10**	**82040**	**2.30**	**25**	**158304**	**4.44**	**14.90**	**11.39**	**8.40**
Isopropanol	3	AOO	1	10	32440	0.91	25	30325	0.85	50	29038	0.81			
Isopropanol	3	AOO	2	10	45395	1.27	25	27645	0.78	50	28736	0.81			
Isopropanol	3	AOO	3	10	38482	1.08	25	23613	0.66	50	37489	1.05			
Isopropanol	3	AOO	4	10	28304	0.79	25	12277	0.34	50	28026	0.79			
Isopropanol	**3**	**AOO**	**MEAN**	**10**	**36155**	**1.01**	**25**	**23465**	**0.66**	**50**	**30822**	**0.86**	**NA**	**NA**	**NA**
Vehicle - Positive Control	3		1	0	19428	0.70									
Vehicle - Positive Control	3		2	0	34843	1.26									
Vehicle - Positive Control	3		3	0	30475	1.11									
Vehicle - Positive Control	3		4	0	25568	0.93									
Vehicle - Positive Control	**3**		**MEAN**	**0**	**27578**	**1.00**									
Positive Control	3		1	NA	152890	5.54									
Positive Control	3		2	NA	150397	5.45									
Positive Control	3		3	NA	179030	6.49									
Positive Control	3		4	NA	164124	5.95									
Positive Control	**3**		**MEAN**	**NA**	**161610**	**5.86**									
Vehicle - Substance	3	AOO	1	0	27832	0.78									
Vehicle - Substance	3	AOO	2	0	43858	1.23									
Vehicle - Substance	3	AOO	3	0	39077	1.10									
Vehicle - Substance	3	AOO	4	0	31673	0.89									
Vehicle - Substance	**3**	**AOO**	**MEAN**	**0**	**35610**	**1.00**									
2,4-Dinitrochlorobenzene	3	AOO	1	0.03	78157	2.19	0.10	121518	3.41	0.30	333041	9.35			
2,4-Dinitrochlorobenzene	3	AOO	2	0.03	124013	3.48	0.10	178885	5.02	0.30	332166	9.33			
2,4-Dinitrochlorobenzene	3	AOO	3	0.03	79811	2.24	0.10	152199	4.27	0.30	364546	10.24			
2,4-Dinitrochlorobenzene	3	AOO	4	0.03	40213	1.13	0.10	149717	4.20	0.30	388959	10.92			
2,4-Dinitrochlorobenzene	**3**	**AOO**	**MEAN**	**0.03**	**80548**	**2.26**	**0.10**	**150579**	**4.23**	**0.30**	**354678**	**9.96**	**0.06**	**0.04**	**0.03**
Dimethyl isophthalate	3	AOO	1	5	31045	0.87	10	42990	1.21	25	21801	0.61			
Dimethyl isophthalate	3	AOO	2	5	35735	1.00	10	26663	0.75	25	20892	0.59			

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Dimethyl isophthalate	3	AOO	3	5	28933	0.81	10	27736	0.78	25	29220	0.82			
Dimethyl isophthalate	3	AOO	4	5	47129	1.32	10	40039	1.12	25	23687	0.67			
Dimethyl isophthalate	**3**	**AOO**	**MEAN**	**5**	**35710**	**1.00**	**10**	**34357**	**0.96**	**25**	**23900**	**0.67**	**NA**	**NA**	**NA**
Vehicle - Positive Control	4		1	0	48083	1.06									
Vehicle - Positive Control	4		2	0	39428	0.87									
Vehicle - Positive Control	4		3	0	55411	1.22									
Vehicle - Positive Control	4		4	0	38284	0.85									
Vehicle - Positive Control	**4**		**MEAN**	**0**	**45301**	**1.00**									
Positive Control	4		1	NA	211896	4.68									
Positive Control	4		2	NA	262733	5.80									
Positive Control	4		3	NA	242739	5.36									
Positive Control	4		4	NA	275773	6.09									
Positive Control	**4**		**MEAN**	**NA**	**248285**	**5.48**									
Vehicle - Substance	4	DMSO	1	0	132462	1.32									
Vehicle - Substance	4	DMSO	2	0	79967	0.80									
Vehicle - Substance	4	DMSO	3	0	82192	0.82									
Vehicle - Substance	4	DMSO	4	0	106964	1.07									
Vehicle - Substance	**4**	**DMSO**	**MEAN**	**0**	**100396**	**1.00**									
Cobalt chloride	4	DMSO	1	0.3	175468	1.75	1.0	272071	2.71	NA	NA	NA			
Cobalt chloride	4	DMSO	2	0.3	192922	1.92	1.0	206730	2.06	NA	NA	NA			
Cobalt chloride	4	DMSO	3	0.3	230415	2.30	1.0	333152	3.32	NA	NA	NA			
Cobalt chloride	4	DMSO	4	0.3	216774	2.16	1.0	256734	2.56	NA	NA	NA			
Cobalt chloride	**4**	**DMSO**	**MEAN**	**0.3**	**203895**	**2.03**	**1.0**	**267172**	**2.66**	**NA**	**NA**	**NA**	**NA**	**0.82**	**0.28**
Nickel (II) sulfate hexahydrate	4	DMSO	1	1	136287	1.36	3	152054	1.51	10	129555	1.29			
Nickel (II) sulfate hexahydrate	4	DMSO	2	1	84335	0.84	3	166405	1.66	10	89825	0.89			
Nickel (II) sulfate hexahydrate	4	DMSO	3	1	125617	1.25	3	188337	1.88	10	85180	0.85			
Nickel (II) sulfate hexahydrate	4	DMSO	4	1	118828	1.18	3	105499	1.05	10	109822	1.09			
Nickel (II) sulfate hexahydrate	**4**	**DMSO**	**MEAN**	**1**	**116266**	**1.16**	**3**	**153074**	**1.52**	**10**	**103595**	**1.03**	**NA**	**NA**	**NA**

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Vehicle - Positive Control	4		1	0	42028	0.90									
Vehicle - Positive Control	4		2	0	49964	1.07									
Vehicle - Positive Control	4		3	0	44351	0.95									
Vehicle - Positive Control	4		4	0	50162	1.08									
Vehicle - Positive Control	**4**		**MEAN**	**0**	**46626**	**1.00**									
Positive Control	4		1	NA	266538	5.72									
Positive Control	4		2	NA	297022	6.37									
Positive Control	4		3	NA	208438	4.47									
Positive Control	4		4	NA	238300	5.11									
Positive Control	**4**		**MEAN**	**NA**	**252574**	**5.42**									
Vehicle - Substance	4	AOO	1	0	38814	0.90									
Vehicle - Substance	4	AOO	2	0	40081	0.93									
Vehicle - Substance	4	AOO	3	0	36876	0.86									
Vehicle - Substance	4	AOO	4	0	56256	1.31									
Vehicle - Substance	**4**	**AOO**	**MEAN**	**0**	**43007**	**1.00**									
Hexyl cinnamic aldehyde	4	AOO	1	5	66346	1.54	10	92375	2.15	25	183245	4.26			
Hexyl cinnamic aldehyde	4	AOO	2	5	63590	1.48	10	128592	2.99	25	237260	5.52			
Hexyl cinnamic aldehyde	4	AOO	3	5	71486	1.66	10	121376	2.82	25	208440	4.85			
Hexyl cinnamic aldehyde	4	AOO	4	5	55427	1.29	10	213148	4.96	25	249803	5.81			
Hexyl cinnamic aldehyde	**4**	**AOO**	**MEAN**	**5**	**64212**	**1.49**	**10**	**138873**	**3.23**	**25**	**219687**	**5.11**	**9.34**	**7.90**	**6.46**
Isopropanol	4	AOO	1	10	62566	1.45	25	29136	0.68	50	33511	0.78			
Isopropanol	4	AOO	2	10	86226	2.00	25	45518	1.06	50	41282	0.96			
Isopropanol	4	AOO	3	10	63529	1.48	25	42708	0.99	50	36712	0.85			
Isopropanol	4	AOO	4	10	56908	1.32	25	38074	0.89	50	26023	0.61			
Isopropanol	**4**	**AOO**	**MEAN**	**10**	**67307**	**1.57**	**25**	**38859**	**0.90**	**50**	**34382**	**0.80**	**NA**	**NA**	**NA**
Vehicle - Positive Control	4		1	0	61301	1.49									
Vehicle - Positive Control	4		2	0	42018	1.02									
Vehicle - Positive Control	4		3	0	31933	0.78									
Vehicle - Positive Control	4		4	0	29486	0.72									
Vehicle - Positive Control	**4**		**MEAN**	**0**	**41184**	**1.00**									
Positive Control	4		1	NA	188993	4.59									
Positive Control	4		2	NA	168896	4.10									
Positive Control	4		3	NA	258012	6.26									

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Positive Control	4		4	NA	307187	7.46									
Positive Control	**4**		**MEAN**	**NA**	**230772**	**5.60**									
Vehicle - Substance	4	AOO	1	0	55245	1.29									
Vehicle - Substance	4	AOO	2	0	32859	0.77									
Vehicle - Substance	4	AOO	3	0	37143	0.87									
Vehicle - Substance	4	AOO	4	0	46219	1.08									
Vehicle - Substance	**4**	**AOO**	**MEAN**	**0**	**42866**	**1.00**									
Isoeugenol	4	AOO	1	1	117220	2.73	3	167018	3.90	10	278270	6.49			
Isoeugenol	4	AOO	2	1	159050	3.71	3	172577	4.03	10	266047	6.21			
Isoeugenol	4	AOO	3	1	114887	2.68	3	190296	4.44	10	212878	4.97			
Isoeugenol	4	AOO	4	1	112197	2.62	3	171216	3.99	10	291279	6.80			
Isoeugenol	**4**	**AOO**	**MEAN**	**1**	**125838**	**2.94**	**3**	**175277**	**4.09**	**10**	**262118**	**6.11**	**1.11**	**0.66**	**0.41**
2,4-Dinitrochlorobenzene	4	AOO	1	0.03	99433	2.32	0.10	239929	5.60	0.30	351048	8.19			
2,4-Dinitrochlorobenzene	4	AOO	2	0.03	124385	2.90	0.10	248752	5.80	0.30	304028	7.09			
2,4-Dinitrochlorobenzene	4	AOO	3	0.03	156964	3.66	0.10	226511	5.28	0.30	426667	9.95			
2,4-Dinitrochlorobenzene	4	AOO	4	0.03	131177	3.06	0.10	125633	2.93	0.30	381330	8.90			
2,4-Dinitrochlorobenzene	**4**	**AOO**	**MEAN**	**0.03**	**127990**	**2.99**	**0.10**	**210206**	**4.90**	**0.30**	**365768**	**8.53**	**0.03**	**0.02**	**0.02**
Vehicle - Positive Control	5		1	0	7783	0.65									
Vehicle - Positive Control	5		2	0	7273	0.61									
Vehicle - Positive Control	5		3	0	22835	1.92									
Vehicle - Positive Control	5		4	0	9704	0.82									
Vehicle - Positive Control	**5**		**MEAN**	**0**	**11899**	**1.00**									
Positive Control	5		1	NA	60519	5.09									
Positive Control	5		2	NA	57983	4.87									
Positive Control	5		3	NA	48159	4.05									
Positive Control	5		4	NA	72951	6.13									
Positive Control	**5**		**MEAN**	**NA**	**59903**	**5.03**									
Vehicle - Substance	5	AOO	1	0	31442	1.49									
Vehicle - Substance	5	AOO	2	0	12103	0.57									
Vehicle - Substance	5	AOO	3	0	20941	0.99									
Vehicle - Substance	5	AOO	4	0	20115	0.95									
Vehicle - Substance	**5**	**AOO**	**MEAN**	**0**	**21150**	**1.00**									

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
2,4-Dinitrochlorobenzene	5	AOO	1	0.03	19491	0.92	0.10	40351	1.91	0.30	199476	9.43			
2,4-Dinitrochlorobenzene	5	AOO	2	0.03	14102	0.67	0.10	76157	3.60	0.30	109134	5.16			
2,4-Dinitrochlorobenzene	5	AOO	3	0.03	17254	0.82	0.10	39813	1.88	0.30	155961	7.37			
2,4-Dinitrochlorobenzene	5	AOO	4	0.03	21584	1.02	0.10	26445	1.25	0.30	200326	9.47			
2,4-Dinitrochlorobenzene	**5**	**AOO**	**MEAN**	**0.03**	**18107**	**0.86**	**0.10**	**45691**	**2.16**	**0.30**	**166224**	**7.86**	**0.13**	**0.11**	**0.09**
Isoeugenol	5	AOO	1	1	20321	0.96	3	12620	0.60	10	123238	5.83			
Isoeugenol	5	AOO	2	1	19512	0.92	3	28001	1.32	10	110582	5.23			
Isoeugenol	5	AOO	3	1	33957	1.61	3	20937	0.99	10	118049	5.58			
Isoeugenol	5	AOO	4	1	17792	0.84	3	32921	1.56	10	116524	5.51			
Isoeugenol	**5**	**AOO**	**MEAN**	**1**	**22896**	**1.08**	**3**	**23619**	**1.12**	**10**	**117098**	**5.54**	**5.98**	**5.19**	**4.40**
Vehicle - Positive Control	5		1	0	22681	1.23									
Vehicle - Positive Control	5		2	0	15429	0.84									
Vehicle - Positive Control	5		3	0	20405	1.11									
Vehicle - Positive Control	5		4	0	15143	0.82									
Vehicle - Positive Control	**5**		**MEAN**	**0**	**18414**	**1.00**									
Positive Control	5		1	NA	97304	5.28									
Positive Control	5		2	NA	83132	4.51									
Positive Control	5		3	NA	67441	3.66									
Positive Control	5		4	NA	117794	6.40									
Positive Control	**5**		**MEAN**	**NA**	**91418**	**4.96**									
Vehicle - Substance	5	AOO	1	0	16435	0.86									
Vehicle - Substance	5	AOO	2	0	22909	1.20									
Vehicle - Substance	5	AOO	3	0	25965	1.36									
Vehicle - Substance	5	AOO	4	0	11275	0.59									
Vehicle - Substance	**5**	**AOO**	**MEAN**	**0**	**19146**	**1.00**									
Hexyl cinnamic aldehyde	5	AOO	1	5	17037	0.89	10	32966	1.72	25	73109	3.82			
Hexyl cinnamic aldehyde	5	AOO	2	5	30640	1.60	10	38027	1.99	25	83266	4.35			
Hexyl cinnamic aldehyde	5	AOO	3	5	26481	1.38	10	17968	0.94	25	77637	4.05			
Hexyl cinnamic aldehyde	5	AOO	4	5	19509	1.02	10	52769	2.76	25	70103	3.66			
Hexyl cinnamic aldehyde	**5**	**AOO**	**MEAN**	**5**	**23417**	**1.22**	**10**	**35432**	**1.85**	**25**	**76029**	**3.97**	**18.13**	**14.59**	**11.06**
Isopropanol	5	AOO	1	10	9967	0.52	25	15066	0.79	50	18749	0.98			
Isopropanol	5	AOO	2	10	5679	0.30	25	15418	0.81	50	13502	0.71			

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Isopropanol	5	AOO	3	10	12157	0.63	25	12221	0.64	50	10223	0.53			
Isopropanol	5	AOO	4	10	12621	0.66	25	15418	0.81	50	11851	0.62			
Isopropanol	**5**	**AOO**	**MEAN**	**10**	**10106**	**0.53**	**25**	**14531**	**0.76**	**50**	**13581**	**0.71**	**NA**	**NA**	**NA**
Vehicle - Positive Control	5		1	0	15918	1.04									
Vehicle - Positive Control	5		2	0	13724	0.90									
Vehicle - Positive Control	5		3	0	10819	0.71									
Vehicle - Positive Control	5		4	0	20489	1.34									
Vehicle - Positive Control	**5**		**MEAN**	**0**	**15237**	**1.00**									
Positive Control	5		1	NA	67799	4.45									
Positive Control	5		2	NA	56834	3.73									
Positive Control	5		3	NA	60000	3.94									
Positive Control	5		4	NA	84607	5.55									
Positive Control	**5**		**MEAN**	**NA**	**67310**	**4.42**									
Vehicle - Substance	5	ACE	1	0	8265	0.50									
Vehicle - Substance	5	ACE	2	0	23012	1.40									
Vehicle - Substance	5	ACE	3	0	14503	0.88									
Vehicle - Substance	5	ACE	4	0	19975	1.22									
Vehicle - Substance	**5**	**ACE**	**MEAN**	**0**	**16439**	**1.00**									
Glutaraldehyde	5	ACE	1	0.05	23621	1.44	0.15	38622	2.35	0.50	34431	2.09			
Glutaraldehyde	5	ACE	2	0.05	11837	0.72	0.15	64431	3.92	0.50	42955	2.61			
Glutaraldehyde	5	ACE	3	0.05	14251	0.87	0.15	24666	1.50	0.50	42380	2.58			
Glutaraldehyde	5	ACE	4	0.05	18389	1.12	0.15	33558	2.04	0.50	49184	2.99			
Glutaraldehyde	**5**	**ACE**	**MEAN**	**0.05**	**17024**	**1.04**	**0.15**	**40319**	**2.45**	**0.50**	**42237**	**2.57**	**NA**	**0.29**	**0.12**
Formaldehyde	5	ACE	1	0.5	24898	1.51	1.5	36696	2.23	5.0	44219	2.69			
Formaldehyde	5	ACE	2	0.5	18454	1.12	1.5	29172	1.77	5.0	47739	2.90			
Formaldehyde	5	ACE	3	0.5	21972	1.34	1.5	43949	2.67	5.0	33377	2.03			
Formaldehyde	5	ACE	4	0.5	12719	0.77	1.5	14018	0.85	5.0	51542	3.14			
Formaldehyde	**5**	**ACE**	**MEAN**	**0.5**	**19510**	**1.19**	**1.5**	**30959**	**1.88**	**5.0**	**44219**	**2.69**	**NA**	**4.18**	**2.02**
Vehicle - Positive Control	6		1	0	16022	1.79									
Vehicle - Positive Control	6		2	0	9436	1.05									
Vehicle - Positive Control	6		3	0	3788	0.42									
Vehicle - Positive Control	6		4	0	6561	0.73									
Vehicle - Positive Control	**6**		**MEAN**	**0**	**8952**	**1.00**									

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Positive Control	6		1	NA	80444	8.99									
Positive Control	6		2	NA	92491	10.33									
Positive Control	6		3	NA	73767	8.24									
Positive Control	6		4	NA	101082	11.29									
Positive Control	**6**		**MEAN**	**NA**	**86946**	**9.71**									
Vehicle - Substance	6	DMSO	1	0	7575	1.81									
Vehicle - Substance	6	DMSO	2	0	4135	0.99									
Vehicle - Substance	6	DMSO	3	0	2759	0.66									
Vehicle - Substance	6	DMSO	4	0	2267	0.54									
Vehicle - Substance	**6**	**DMSO**	**MEAN**	**0**	**4184**	**1.00**									
Nickel (II) sulfate hexahydrate	6	DMSO	1	1	30363	7.26	3	32830	7.85	10	46902	11.21			
Nickel (II) sulfate hexahydrate	6	DMSO	2	1	12902	3.08	3	28614	6.84	10	64448	15.40			
Nickel (II) sulfate hexahydrate	6	DMSO	3	1	22353	5.34	3	31319	7.49	10	56156	13.42			
Nickel (II) sulfate hexahydrate	6	DMSO	4	1	22343	5.34	3	19101	4.57	10	29707	7.10			
Nickel (II) sulfate hexahydrate	**6**	**DMSO**	**MEAN**	**1**	**21990**	**5.26**	**3**	**27966**	**6.68**	**10**	**49303**	**11.78**	**0.47**	**0.35**	**0.24**
Cobalt chloride	6	DMSO	1	0.3	88782	21.22	1.0	59079	14.12	3.0	108860	26.02			
Cobalt chloride	6	DMSO	2	0.3	40452	9.67	1.0	24246	5.80	3.0	62637	14.97			
Cobalt chloride	6	DMSO	3	0.3	22788	5.45	1.0	69511	16.61	3.0	106164	25.38			
Cobalt chloride	6	DMSO	4	0.3	23988	5.73	1.0	25023	5.98	3.0	66252	15.84			
Cobalt chloride	**6**	**DMSO**	**MEAN**	**0.3**	**44002**	**10.52**	**1.0**	**44465**	**10.63**	**3.0**	**85978**	**20.55**	**0.06**	**0.05**	**0.03**
Vehicle - Positive Control	6		1	0	7997	0.75									
Vehicle - Positive Control	6		2	0	10763	1.01									
Vehicle - Positive Control	6		3	0	13602	1.27									
Vehicle - Positive Control	6		4	0	10360	0.97									
Vehicle - Positive Control	**6**		**MEAN**	**0**	**10680**	**1.00**									
Positive Control	6		1	NA	52468	4.91									
Positive Control	6		2	NA	66048	6.18									
Positive Control	6		3	NA	81979	7.68									

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Positive Control	6		4	NA	76135	7.13									
Positive Control	**6**		**MEAN**	**NA**	**69157**	**6.48**									
Vehicle - Substance	6	AOO	1	0	8621	0.62									
Vehicle - Substance	6	AOO	2	0	14670	1.05									
Vehicle - Substance	6	AOO	3	0	18086	1.30									
Vehicle - Substance	6	AOO	4	0	14263	1.03									
Vehicle - Substance	**6**	**AOO**	**MEAN**	**0**	**13910**	**1.00**									
Abietic acid	6	AOO	1	5	38117	2.74	10	57039	4.10	25	98752	7.10			
Abietic acid	6	AOO	2	5	18850	1.36	10	73842	5.31	25	129426	9.30			
Abietic acid	6	AOO	3	5	25525	1.83	10	56561	4.07	25	139343	10.02			
Abietic acid	6	AOO	4	5	18617	1.34	10	43018	3.09	25	75268	5.41			
Abietic acid	**6**	**AOO**	**MEAN**	**5**	**25277**	**1.82**	**10**	**57615**	**4.14**	**25**	**110697**	**7.96**	**7.54**	**6.47**	**5.39**
2,4-Dinitrochlorobenzene	6	AOO	1	0.03	29344	2.11	0.10	32064	2.31	0.30	170451	12.25			
2,4-Dinitrochlorobenzene	6	AOO	2	0.03	53129	3.82	0.10	78273	5.63	0.30	258700	18.60			
2,4-Dinitrochlorobenzene	6	AOO	3	0.03	39348	2.83	0.10	66285	4.77	0.30	241703	17.38			
2,4-Dinitrochlorobenzene	6	AOO	4	0.03	31167	2.24	0.10	60587	4.36	0.30	171691	12.34			
2,4-Dinitrochlorobenzene	**6**	**AOO**	**MEAN**	**0.03**	**38247**	**2.75**	**0.10**	**59302**	**4.26**	**0.30**	**210636**	**15.14**	**0.04**	**0.03**	**0.02**
Vehicle - Positive Control	6		1	0	18240	1.56									
Vehicle - Positive Control	6		2	0	4174	0.36									
Vehicle - Positive Control	6		3	0	11817	1.01									
Vehicle - Positive Control	6		4	0	12605	1.08									
Vehicle - Positive Control	**6**		**MEAN**	**0**	**11709**	**1.00**									
Positive Control	6		1	NA	105716	9.03									
Positive Control	6		2	NA	92508	7.90									
Positive Control	6		3	NA	86410	7.38									
Positive Control	6		4	NA	107936	9.22									
Positive Control	**6**		**MEAN**	**NA**	**98142**	**8.38**									
Vehicle - Substance	6	AOO	1	0	13188	0.81									
Vehicle - Substance	6	AOO	2	0	16677	1.02									
Vehicle - Substance	6	AOO	3	0	13789	0.84									
Vehicle - Substance	6	AOO	4	0	21847	1.33									
Vehicle - Substance	**6**	**AOO**	**MEAN**	**0**	**16375**	**1.00**									

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Hexyl cinnamic aldehyde	6	AOO	1	5	34939	2.13	10	50225	3.07	25	61340	3.75			
Hexyl cinnamic aldehyde	6	AOO	2	5	34548	2.11	10	38763	2.37	25	71280	4.35			
Hexyl cinnamic aldehyde	6	AOO	3	5	18582	1.13	10	26933	1.64	25	110980	6.78			
Hexyl cinnamic aldehyde	6	AOO	4	5	21408	1.31	10	37387	2.28	25	116668	7.12			
Hexyl cinnamic aldehyde	**6**	**AOO**	**MEAN**	**5**	**27369**	**1.67**	**10**	**38327**	**2.34**	**25**	**90067**	**5.50**	**13.13**	**10.76**	**7.46**
Isopropanol	6	AOO	1	10	71570	4.37	25	14610	0.89	50	16623	1.02			
Isopropanol	6	AOO	2	10	20763	1.27	25	19836	1.21	50	19168	1.17			
Isopropanol	6	AOO	3	10	19846	1.21	25	17188	1.05	50	28176	1.72			
Isopropanol	6	AOO	4	10	16753	1.02	25	7416	0.45	50	21474	1.31			
Isopropanol	**6**	**AOO**	**MEAN**	**10**	**32233**	**1.97**	**25**	**14762**	**0.90**	**50**	**21360**	**1.30**	**NA**	**NA**	**NA**
Vehicle - Positive Control	7		1	0	10954	0.47									
Vehicle - Positive Control	7		2	0	14547	0.62									
Vehicle - Positive Control	7		3	0	33870	1.44									
Vehicle - Positive Control	7		4	0	34460	1.47									
Vehicle - Positive Control	**7**		**MEAN**	**0**	**23458**	**1.00**									
Positive Control	7		1	NA	93512	3.99									
Positive Control	7		2	NA	104433	4.45									
Positive Control	7		3	NA	114003	4.86									
Positive Control	7		4	NA	180482	7.69									
Positive Control	**7**		**MEAN**	**NA**	**123107**	**5.25**									
Vehicle - Substance	7	AOO	1	0	15339	0.71									
Vehicle - Substance	7	AOO	2	0	11627	0.54									
Vehicle - Substance	7	AOO	3	0	17793	0.83									
Vehicle - Substance	7	AOO	4	0	41425	1.92									
Vehicle - Substance	**7**	**AOO**	**MEAN**	**0**	**21546**	**1.00**									
Methyl salicylate	7	AOO	1	5	26796	1.24	10	30066	1.40	25	14218	0.66			
Methyl salicylate	7	AOO	2	5	23023	1.07	10	45494	2.11	25	31612	1.47			
Methyl salicylate	7	AOO	3	5	12934	0.60	10	41639	1.93	25	31551	1.46			
Methyl salicylate	7	AOO	4	5	31083	1.44	10	35433	1.64	25	42145	1.96			
Methyl salicylate	**7**	**AOO**	**MEAN**	**5**	**23459**	**1.09**	**10**	**38158**	**1.77**	**25**	**29881**	**1.39**	**NA**	**NA**	**NA**
Abietic acid	7	AOO	1	5	28706	1.33	10	50807	2.36	25	45895	2.13			
Abietic acid	7	AOO	2	5	46411	2.15	10	92597	4.30	25	102739	4.77			
Abietic acid	7	AOO	3	5	46541	2.16	10	105497	4.90	25	87409	4.06			

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Abietic acid	7	AOO	4	5	39654	1.84	10	94381	4.38	25	91230	4.23			
Abietic acid	**7**	**AOO**	**MEAN**	**5**	**40328**	**1.87**	**10**	**85821**	**3.98**	**25**	**81818**	**3.80**	**7.68**	**11.53**	**6.33**
Vehicle - Positive Control	7		1	0	17271	0.75									
Vehicle - Positive Control	7		2	0	23663	1.03									
Vehicle - Positive Control	7		3	0	24070	1.04									
Vehicle - Positive Control	7		4	0	27154	1.18									
Vehicle - Positive Control	**7**		**MEAN**	**0**	**23039**	**1.00**									
Positive Control	7		1	NA	127080	5.52									
Positive Control	7		2	NA	150247	6.52									
Positive Control	7		3	NA	122132	5.30									
Positive Control	7		4	NA	128311	5.57									
Positive Control	**7**		**MEAN**	**NA**	**131942**	**5.73**									
Vehicle - Substance	7	AOO	1	0	36823	1.23									
Vehicle - Substance	7	AOO	2	0	31245	1.04									
Vehicle - Substance	7	AOO	3	0	21937	0.73									
Vehicle - Substance	7	AOO	4	0	29694	0.99									
Vehicle - Substance	**7**	**AOO**	**MEAN**	**0**	**29925**	**1.00**									
Hexyl cinnamic aldehyde	7	AOO	1	5	42392	1.42	10	106569	3.56	25	170985	5.71			
Hexyl cinnamic aldehyde	7	AOO	2	5	33988	1.14	10	151880	5.08	25	193134	6.45			
Hexyl cinnamic aldehyde	7	AOO	3	5	66350	2.22	10	161431	5.39	25	198620	6.64			
Hexyl cinnamic aldehyde	7	AOO	4	5	41865	1.40	10	87141	2.91	25	286402	9.57			
Hexyl cinnamic aldehyde	**7**	**AOO**	**MEAN**	**5**	**46148**	**1.54**	**10**	**126755**	**4.24**	**25**	**212285**	**7.09**	**7.71**	**6.78**	**5.85**
Isopropanol	7	AOO	1	10	30442	1.02	25	15392	0.51	50	26039	0.87			
Isopropanol	7	AOO	2	10	32600	1.09	25	39028	1.30	50	25885	0.87			
Isopropanol	7	AOO	3	10	41239	1.38	25	22387	0.75	50	27685	0.93			
Isopropanol	7	AOO	4	10	69502	2.32	25	32333	1.08	50	19497	0.65			
Isopropanol	**7**	**AOO**	**MEAN**	**10**	**43446**	**1.45**	**25**	**27285**	**0.91**	**50**	**24776**	**0.83**	**NA**	**NA**	**NA**
Vehicle - Positive Control	7		1	0	20353	0.71									
Vehicle - Positive Control	7		2	0	31709	1.10									
Vehicle - Positive Control	7		3	0	34254	1.19									
Vehicle - Positive Control	7		4	0	29038	1.01									
Vehicle - Positive Control	**7**		**MEAN**	**0**	**28838**	**1.00**									
Positive Control	7		1	NA	170163	5.90									

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Positive Control	7		2	NA	142824	4.95									
Positive Control	7		3	NA	167113	5.79									
Positive Control	7		4	NA	135621	4.70									
Positive Control	**7**		**MEAN**	**NA**	**153930**	**5.34**									
Vehicle - Substance	7	AOO	1	0	25299	1.13									
Vehicle - Substance	7	AOO	2	0	25685	1.14									
Vehicle - Substance	7	AOO	3	0	19870	0.88									
Vehicle - Substance	7	AOO	4	0	19010	0.85									
Vehicle - Substance	**7**	**AOO**	**MEAN**	**0**	**22466**	**1.00**									
Dimethyl isophthalate	7	AOO	1	5	30872	1.37	10	28765	1.28	25	24457	1.09			
Dimethyl isophthalate	7	AOO	2	5	23829	1.06	10	27567	1.23	25	25583	1.14			
Dimethyl isophthalate	7	AOO	3	5	26046	1.16	10	22517	1.00	25	18065	0.80			
Dimethyl isophthalate	7	AOO	4	5	32477	1.45	10	23373	1.04	25	26228	1.17			
Dimethyl isophthalate	**7**	**AOO**	**MEAN**	**5**	**28306**	**1.26**	**10**	**25555**	**1.14**	**25**	**23583**	**1.05**	**NA**	**NA**	**NA**
2,4-Dinitrochlorobenzene	7	AOO	1	0.03	54379	2.42	0.10	142045	6.32	0.30	282805	12.59			
2,4-Dinitrochlorobenzene	7	AOO	2	0.03	95575	4.25	0.10	139187	6.20	0.30	336813	14.99			
2,4-Dinitrochlorobenzene	7	AOO	3	0.03	95094	4.23	0.10	108882	4.85	0.30	258764	11.52			
2,4-Dinitrochlorobenzene	7	AOO	4	0.03	99284	4.42	0.10	93969	4.18	0.30	305713	13.61			
2,4-Dinitrochlorobenzene	**7**	**AOO**	**MEAN**	**0.03**	**86083**	**3.83**	**0.10**	**121021**	**5.39**	**0.30**	**296024**	**13.18**	**0.02**	**0.01**	**0.01**
Vehicle - Positive Control	8		1	0	18303	0.95									
Vehicle - Positive Control	8		2	0	25980	1.34									
Vehicle - Positive Control	8		3	0	17493	0.90									
Vehicle - Positive Control	8		4	0	15606	0.81									
Vehicle - Positive Control	**8**		**MEAN**	**0**	**19345**	**1.00**									
Positive Control	8		1	NA	98761	5.11									
Positive Control	8		2	NA	72937	3.77									
Positive Control	8		3	NA	86236	4.46									
Positive Control	8		4	NA	76278	3.94									
Positive Control	**8**		**MEAN**	**NA**	**83553**	**4.32**									
Vehicle - Substance	8	AOO	1	0	9463	0.78									
Vehicle - Substance	8	AOO	2	0	13874	1.14									
Vehicle - Substance	8	AOO	3	0	17229	1.41									

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Vehicle - Substance	8	AOO	4	0	8262	0.68									
Vehicle - Substance	**8**	**AOO**	**MEAN**	**0**	**12207**	**1.00**									
Isopropanol	8	AOO	1	10	12562	1.03	25	17249	1.41	50	14510	1.19			
Isopropanol	8	AOO	2	10	17330	1.42	25	9264	0.76	50	14113	1.16			
Isopropanol	8	AOO	3	10	11886	0.97	25	11845	0.97	50	12238	1.00			
Isopropanol	8	AOO	4	10	17410	1.43	25	11193	0.92	50	13342	1.09			
Isopropanol	**8**	**AOO**	**MEAN**	**10**	**14797**	**1.21**	**25**	**12387**	**1.01**	**50**	**13551**	**1.11**	**NA**	**NA**	**NA**
Hexyl cinnamic aldehyde	8	AOO	1	5	16997	1.39	10	40975	3.36	25	155208	12.71			
Hexyl cinnamic aldehyde	8	AOO	2	5	15777	1.29	10	56754	4.65	25	133055	10.90			
Hexyl cinnamic aldehyde	8	AOO	3	5	22473	1.84	10	58346	4.78	25	75582	6.19			
Hexyl cinnamic aldehyde	8	AOO	4	5	11217	0.92	10	47242	3.87	25	135369	11.09			
Hexyl cinnamic aldehyde	**8**	**AOO**	**MEAN**	**5**	**16616**	**1.36**	**10**	**50829**	**4.16**	**25**	**124803**	**10.22**	**7.92**	**7.03**	**6.14**
Vehicle - Positive Control	8		1	0	11818	0.62									
Vehicle - Positive Control	8		2	0	22893	1.19									
Vehicle - Positive Control	8		3	0	21441	1.12									
Vehicle - Positive Control	8		4	0	20608	1.07									
Vehicle - Positive Control	**8**		**MEAN**	**0**	**19190**	**1.00**									
Positive Control	8		1	NA	117067	6.10									
Positive Control	8		2	NA	100222	5.22									
Positive Control	8		3	NA	91462	4.77									
Positive Control	8		4	NA	80907	4.22									
Positive Control	**8**		**MEAN**	**NA**	**97414**	**5.08**									
Vehicle - Substance	8	DMSO	1	0	15322	0.77									
Vehicle - Substance	8	DMSO	2	0	24630	1.24									
Vehicle - Substance	8	DMSO	3	0	16802	0.85									
Vehicle - Substance	8	DMSO	4	0	22460	1.13									
Vehicle - Substance	**8**	**DMSO**	**MEAN**	**0**	**19803**	**1.00**									
Nickel (II) sulfate hexahydrate	8	DMSO	1	1	64139	3.24	3	64301	3.25	10	40447	2.04			
Nickel (II) sulfate hexahydrate	8	DMSO	2	1	59705	3.01	3	70343	3.55	10	45033	2.27			
Nickel (II) sulfate hexahydrate	8	DMSO	3	1	61654	3.11	3	55459	2.80	10	62589	3.16			

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Nickel (II) sulfate hexahydrate	8	DMSO	4	1	90810	4.59	3	53420	2.70	10	54206	2.74			
Nickel (II) sulfate hexahydrate	**8**	**DMSO**	**MEAN**	**1**	**69077**	**3.49**	**3**	**60881**	**3.07**	**10**	**50568**	**2.55**	**IDR**	**IDR**	**IDR**
Cobalt chloride	8	DMSO	1	0.3	68800	3.47	1.0	123857	6.25	3.0	175242	8.85			
Cobalt chloride	8	DMSO	2	0.3	98124	4.95	1.0	178916	9.03	3.0	143477	7.25			
Cobalt chloride	8	DMSO	3	0.3	95925	4.84	1.0	96477	4.87	3.0	155827	7.87			
Cobalt chloride	8	DMSO	4	0.3	87399	4.41	1.0	124765	6.30	3.0	164687	8.32			
Cobalt chloride	**8**	**DMSO**	**MEAN**	**0.3**	**87562**	**4.42**	**1.0**	**131004**	**6.62**	**3.0**	**159808**	**8.07**	**0.14**	**0.10**	**0.08**
Vehicle - Positive Control	8		1	0	17139	1.02									
Vehicle - Positive Control	8		2	0	23311	1.39									
Vehicle - Positive Control	8		3	0	14001	0.84									
Vehicle - Positive Control	8		4	0	12548	0.75									
Vehicle - Positive Control	**8**		**MEAN**	**0**	**16749**	**1.00**									
Positive Control	8		1	NA	133873	7.99									
Positive Control	8		2	NA	147108	8.78									
Positive Control	8		3	NA	114171	6.82									
Positive Control	8		4	NA	97568	5.83									
Positive Control	**8**		**MEAN**	**NA**	**123180**	**7.35**									
Vehicle - Substance	8	AOO	1	0	18744	0.91									
Vehicle - Substance	8	AOO	2	0	20074	0.98									
Vehicle - Substance	8	AOO	3	0	15187	0.74									
Vehicle - Substance	8	AOO	4	0	28298	1.38									
Vehicle - Substance	**8**	**AOO**	**MEAN**	**0**	**20576**	**1.00**									
2,4-Dinitrochlorobenzene	8	AOO	1	0.03	40777	1.98	0.10	41930	2.04	0.30	228871	11.12			
2,4-Dinitrochlorobenzene	8	AOO	2	0.03	45024	2.19	0.10	50135	2.44	0.30	393845	19.14			
2,4-Dinitrochlorobenzene	8	AOO	3	0.03	30526	1.48	0.10	107465	5.22	0.30	273309	13.28			
2,4-Dinitrochlorobenzene	8	AOO	4	0.03	82593	4.01	0.10	50754	2.47	0.30	140789	6.84			
2,4-Dinitrochlorobenzene	**8**	**AOO**	**MEAN**	**0.03**	**49730**	**2.42**	**0.10**	**62571**	**3.04**	**0.30**	**259203**	**12.60**	**0.10**	**0.04**	**0.01**
3-Aminophenol	8	AOO	1	1	25653	1.25	3	51618	2.51	10	57296	2.78			
3-Aminophenol	8	AOO	2	1	27127	1.32	3	47941	2.33	10	52938	2.57			
3-Aminophenol	8	AOO	3	1	28861	1.40	3	36281	1.76	10	38134	1.85			

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
3-Aminophenol	8	AOO	4	1	19026	0.92	3	27846	1.35	10	47782	2.32			
3-Aminophenol	**8**	**AOO**	**MEAN**	**1**	**25167**	**1.22**	**3**	**40921**	**1.99**	**10**	**49037**	**2.38**	**NA**	**NA**	**3.18**
Vehicle - Positive Control	9		1	0	25729	0.98									
Vehicle - Positive Control	9		2	0	31786	1.22									
Vehicle - Positive Control	9		3	0	24343	0.93									
Vehicle - Positive Control	9		4	0	22785	0.87									
Vehicle - Positive Control	**9**		**MEAN**	**0**	**26161**	**1.00**									
Positive Control	9		1	NA	155962	5.96									
Positive Control	9		2	NA	112682	4.31									
Positive Control	9		3	NA	124334	4.75									
Positive Control	9		4	NA	122066	4.67									
Positive Control	**9**		**MEAN**	**NA**	**128761**	**4.92**									
Vehicle - Substance	9	AOO	1	0	21600	0.73									
Vehicle - Substance	9	AOO	2	0	38136	1.29									
Vehicle - Substance	9	AOO	3	0	34690	1.17									
Vehicle - Substance	9	AOO	4	0	23981	0.81									
Vehicle - Substance	**9**	**AOO**	**MEAN**	**0**	**29602**	**1.00**									
Hexyl cinnamic aldehyde	9	AOO	1	5	35263	1.19	10	32104	1.08	25	109826	3.71			
Hexyl cinnamic aldehyde	9	AOO	2	5	34558	1.17	10	68901	2.33	25	114755	3.88			
Hexyl cinnamic aldehyde	9	AOO	3	5	20309	0.69	10	61583	2.08	25	101116	3.42			
Hexyl cinnamic aldehyde	9	AOO	4	5	12277	0.41	10	99972	3.38	25	133469	4.51			
Hexyl cinnamic aldehyde	**9**	**AOO**	**MEAN**	**5**	**25602**	**0.86**	**10**	**65640**	**2.22**	**25**	**114791**	**3.88**	**17.07**	**12.55**	**9.19**
Isopropanol	9	AOO	1	10	16071	0.54	25	18605	0.63	50	11350	0.38			
Isopropanol	9	AOO	2	10	29909	1.01	25	12916	0.44	50	14836	0.50			
Isopropanol	9	AOO	3	10	16721	0.56	25	26806	0.91	50	13840	0.47			
Isopropanol	9	AOO	4	10	12462	0.42	25	24183	0.82	50	20129	0.68			
Isopropanol	**9**	**AOO**	**MEAN**	**10**	**18791**	**0.63**	**25**	**20627**	**0.70**	**50**	**15039**	**0.51**	**NA**	**NA**	**NA**
Vehicle - Positive Control	9		1	0	21626	0.82									
Vehicle - Positive Control	9		2	0	28191	1.06									
Vehicle - Positive Control	9		3	0	36208	1.37									
Vehicle - Positive Control	9		4	0	19953	0.75									
Vehicle - Positive Control	**9**		**MEAN**	**0**	**26494**	**1.00**									
Positive Control	9		1	NA	152153	5.74									

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Positive Control	9		2	NA	173639	6.55									
Positive Control	9		3	NA	117177	4.42									
Positive Control	9		4	NA	165097	6.23									
Positive Control	**9**		**MEAN**	**NA**	**152016**	**5.74**									
Vehicle - Substance	9	AOO	1	0	37188	1.39									
Vehicle - Substance	9	AOO	2	0	20177	0.75									
Vehicle - Substance	9	AOO	3	0	17473	0.65									
Vehicle - Substance	9	AOO	4	0	32530	1.21									
Vehicle - Substance	**9**	**AOO**	**MEAN**	**0**	**26842**	**1.00**									
Isoeugenol	9	AOO	1	1	43063	1.60	3	82412	3.07	10	241256	8.99			
Isoeugenol	9	AOO	2	1	92318	3.44	3	114677	4.27	10	169293	6.31			
Isoeugenol	9	AOO	3	1	73315	2.73	3	83819	3.12	10	153506	5.72			
Isoeugenol	9	AOO	4	1	68329	2.55	3	65486	2.44	10	197513	7.36			
Isoeugenol	**9**	**AOO**	**MEAN**	**1**	**69256**	**2.58**	**3**	**86598**	**3.23**	**10**	**190392**	**7.09**	**2.30**	**0.87**	**0.38**
2,4-Dinitrochlorobenzene	9	AOO	1	0.03	80731	3.01	0.10	81426	3.03	0.30	294486	10.97			
2,4-Dinitrochlorobenzene	9	AOO	2	0.03	46072	1.72	0.10	105837	3.94	0.30	287848	10.72			
2,4-Dinitrochlorobenzene	9	AOO	3	0.03	82472	3.07	0.10	164718	6.14	0.30	287739	10.72			
2,4-Dinitrochlorobenzene	9	AOO	4	0.03	91886	3.42	0.10	97148	3.62	0.30	298846	11.13			
2,4-Dinitrochlorobenzene	**9**	**AOO**	**MEAN**	**0.03**	**75290**	**2.80**	**0.10**	**112282**	**4.18**	**0.30**	**292230**	**10.89**	**0.04**	**0.02**	**0.02**
Vehicle - Positive Control	10		1	0	20162	0.95									
Vehicle - Positive Control	10		2	0	15285	0.72									
Vehicle - Positive Control	10		3	0	30517	1.43									
Vehicle - Positive Control	10		4	0	19166	0.90									
Vehicle - Positive Control	**10**		**MEAN**	**0**	**21282**	**1.00**									
Positive Control	10		1	NA	116157	5.46									
Positive Control	10		2	NA	142905	6.71									
Positive Control	10		3	NA	135316	6.36									
Positive Control	10		4	NA	117862	5.54									
Positive Control	**10**		**MEAN**	**NA**	**128060**	**6.02**									
Vehicle - Substance	10	AOO	1	0	45394	0.85									
Vehicle - Substance	10	AOO	2	0	67917	1.27									
Vehicle - Substance	10	AOO	3	0	36479	0.68									

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Vehicle - Substance	10	AOO	4	0	63610	1.19									
Vehicle - Substance	**10**	**AOO**	**MEAN**	**0**	**53350**	**1.00**									
2,4-Dinitrochlorobenzene	10	AOO	1	0.03	52123	0.98	0.10	113324	2.12	0.30	202245	3.79			
2,4-Dinitrochlorobenzene	10	AOO	2	0.03	66363	1.24	0.10	80089	1.50	0.30	264292	4.95			
2,4-Dinitrochlorobenzene	10	AOO	3	0.03	36583	0.69	0.10	127648	2.39	0.30	298490	5.59			
2,4-Dinitrochlorobenzene	10	AOO	4	0.03	92933	1.74	0.10	127592	2.39	0.30	239662	4.49			
2,4-Dinitrochlorobenzene	**10**	**AOO**	**MEAN**	**0.03**	**62000**	**1.16**	**0.10**	**112163**	**2.10**	**0.30**	**251172**	**4.71**	**0.17**	**0.13**	**0.09**
Methyl salicylate	10	AOO	1	5	36446	0.68	10	47420	0.89	25	53941	1.01			
Methyl salicylate	10	AOO	2	5	34905	0.65	10	47616	0.89	25	54989	1.03			
Methyl salicylate	10	AOO	3	5	37286	0.70	10	40117	0.75	25	43082	0.81			
Methyl salicylate	10	AOO	4	5	26017	0.49	10	31641	0.59	25	25692	0.48			
Methyl salicylate	**10**	**AOO**	**MEAN**	**5**	**33663**	**0.63**	**10**	**41698**	**0.78**	**25**	**44426**	**0.83**	**NA**	**NA**	**NA**
Vehicle - Positive Control	10		1	0	20445	0.88									
Vehicle - Positive Control	10		2	0	15079	0.65									
Vehicle - Positive Control	10		3	0	26464	1.13									
Vehicle - Positive Control	10		4	0	31358	1.34									
Vehicle - Positive Control	**10**		**MEAN**	**0**	**23336**	**1.00**									
Positive Control	10		1	NA	89914	3.85									
Positive Control	10		2	NA	107768	4.62									
Positive Control	10		3	NA	93418	4.00									
Positive Control	10		4	NA	102331	4.39									
Positive Control	**10**		**MEAN**	**NA**	**98357**	**4.21**									
Vehicle - Substance	10	AOO	1	0	28181	0.97									
Vehicle - Substance	10	AOO	2	0	33325	1.15									
Vehicle - Substance	10	AOO	3	0	27821	0.96									
Vehicle - Substance	10	AOO	4	0	26981	0.93									
Vehicle - Substance	**10**	**AOO**	**MEAN**	**0**	**29077**	**1.00**									
Hexyl cinnamic aldehyde	10	AOO	1	5	35684	1.23	10	86735	2.98	25	78538	2.70			
Hexyl cinnamic aldehyde	10	AOO	2	5	30080	1.03	10	88833	3.06	25	107305	3.69			
Hexyl cinnamic aldehyde	10	AOO	3	5	62393	2.15	10	75607	2.60	25	129081	4.44			
Hexyl cinnamic aldehyde	10	AOO	4	5	34584	1.19	10	66109	2.27	25	93013	3.20			
Hexyl cinnamic aldehyde	**10**	**AOO**	**MEAN**	**5**	**40685**	**1.40**	**10**	**79321**	**2.73**	**25**	**101984**	**3.51**	**15.24**	**9.14**	**7.26**

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Isopropanol	10	AOO	1	10	19691	0.68	25	30241	1.04	50	42188	1.45			
Isopropanol	10	AOO	2	10	28293	0.97	25	24774	0.85	50	37228	1.28			
Isopropanol	10	AOO	3	10	29845	1.03	25	29230	1.01	50	35247	1.21			
Isopropanol	10	AOO	4	10	28091	0.97	25	38461	1.32	50	30201	1.04			
Isopropanol	**10**	**AOO**	**MEAN**	**10**	**26480**	**0.91**	**25**	**30676**	**1.06**	**50**	**36216**	**1.25**	**NA**	**NA**	**NA**
Vehicle - Positive Control	11		1	0	13452	0.45									
Vehicle - Positive Control	11		2	0	32469	1.09									
Vehicle - Positive Control	11		3	0	37235	1.25									
Vehicle - Positive Control	11		4	0	35940	1.21									
Vehicle - Positive Control	**11**		**MEAN**	**0**	**29774**	**1.00**									
Positive Control	11		1	NA	113708	3.82									
Positive Control	11		2	NA	108755	3.65									
Positive Control	11		3	NA	57560	1.93									
Positive Control	11		4	NA	97736	3.28									
Positive Control	**11**		**MEAN**	**NA**	**94440**	**3.17**									
Vehicle - Substance	11	AOO	1	0	16175	0.76									
Vehicle - Substance	11	AOO	2	0	31955	1.50									
Vehicle - Substance	11	AOO	3	0	24257	1.14									
Vehicle - Substance	11	AOO	4	0	12926	0.61									
Vehicle - Substance	**11**	**AOO**	**MEAN**	**0**	**21328**	**1.00**									
Hexyl cinnamic aldehyde	11	AOO	1	5	24541	1.15	10	73959	3.47	25	56324	2.64			
Hexyl cinnamic aldehyde	11	AOO	2	5	31920	1.50	10	73920	3.47	25	81323	3.81			
Hexyl cinnamic aldehyde	11	AOO	3	5	42454	1.99	10	74762	3.51	25	117271	5.50			
Hexyl cinnamic aldehyde	11	AOO	4	5	30308	1.42	10	60117	2.82	25	126476	5.93			
Hexyl cinnamic aldehyde	**11**	**AOO**	**MEAN**	**5**	**32306**	**1.51**	**10**	**70689**	**3.31**	**25**	**95348**	**4.47**	**9.13**	**7.74**	**6.35**
Vehicle - Positive Control	11		1	0	6855	0.32									
Vehicle - Positive Control	11		2	0	23315	1.10									
Vehicle - Positive Control	11		3	0	27767	1.30									
Vehicle - Positive Control	11		4	0	27187	1.28									
Vehicle - Positive Control	**11**		**MEAN**	**0**	**21281**	**1.00**									
Positive Control	11		1	NA	118741	5.58									
Positive Control	11		2	NA	114600	5.39									
Positive Control	11		3	NA	86525	4.07									

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Positive Control	11		4	NA	115969	5.45									
Positive Control	**11**		**MEAN**	**NA**	**108959**	**5.12**									
Vehicle - Substance	11	DMSO	1	0	67859	1.04									
Vehicle - Substance	11	DMSO	2	0	76567	1.18									
Vehicle - Substance	11	DMSO	3	0	60349	0.93									
Vehicle - Substance	11	DMSO	4	0	55465	0.85									
Vehicle - Substance	**11**	**DMSO**	**MEAN**	**0**	**65060**	**1.00**									
Potassium dichromate	11	DMSO	1	0.1	134992	2.07	0.3	194686	2.99	1.0	283541	4.36			
Potassium dichromate	11	DMSO	2	0.1	133187	2.05	0.3	104933	1.61	1.0	340279	5.23			
Potassium dichromate	11	DMSO	3	0.1	130433	2.00	0.3	166086	2.55	1.0	318543	4.90			
Potassium dichromate	11	DMSO	4	0.1	97134	1.49	0.3	117627	1.81	1.0	301673	4.64			
Potassium dichromate	**11**	**DMSO**	**MEAN**	**0.1**	**123936**	**1.90**	**0.3**	**145833**	**2.24**	**1.0**	**311009**	**4.78**	**0.51**	**0.37**	**0.16**
Lactic acid	11	DMSO	1	5	34889	0.54	10	57810	0.89	25	73850	1.14			
Lactic acid	11	DMSO	2	5	70275	1.08	10	60103	0.92	25	38479	0.59			
Lactic acid	11	DMSO	3	5	81876	1.26	10	42148	0.65	25	54647	0.84			
Lactic acid	11	DMSO	4	5	55263	0.85	10	36073	0.55	25	41547	0.64			
Lactic acid	**11**	**DMSO**	**MEAN**	**5**	**60576**	**0.93**	**10**	**49033**	**0.75**	**25**	**52131**	**0.80**	**NA**	**NA**	**NA**
Vehicle - Positive Control	11		1	0	25338	0.96									
Vehicle - Positive Control	11		2	0	29261	1.11									
Vehicle - Positive Control	11		3	0	21131	0.80									
Vehicle - Positive Control	11		4	0	29732	1.13									
Vehicle - Positive Control	**11**		**MEAN**	**0**	**26365**	**1.00**									
Positive Control	11		1	NA	136936	5.19									
Positive Control	11		2	NA	81100	3.08									
Positive Control	11		3	NA	114598	4.35									
Positive Control	11		4	NA	79191	3.00									
Positive Control	**11**		**MEAN**	**NA**	**102956**	**3.90**									
Vehicle - Substance	11	DMSO	1	0	86043	1.05									
Vehicle - Substance	11	DMSO	2	0	65589	0.80									
Vehicle - Substance	11	DMSO	3	0	117592	1.43									
Vehicle - Substance	11	DMSO	4	0	59151	0.72									
Vehicle - Substance	**11**	**DMSO**	**MEAN**	**0**	**82093**	**1.00**									
Cobalt chloride	11	DMSO	1	1	113621	1.38	3	123437	1.50	5	167985	2.05			

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Cobalt chloride	11	DMSO	2	1	130468	1.59	3	115859	1.41	5	167593	2.04			
Cobalt chloride	11	DMSO	3	1	97082	1.18	3	189281	2.31	5	174922	2.13			
Cobalt chloride	11	DMSO	4	1	147603	1.80	3	139101	1.69	5	150902	1.84			
Cobalt chloride	**11**	**DMSO**	**MEAN**	**1**	**122193**	**1.49**	**3**	**141919**	**1.73**	**5**	**165350**	**2.01**	**NA**	**NA**	**4.93**
Nickel (II) sulfate hexahydrate	11	DMSO	1	1	65339	0.80	3	89247	1.09	10	80662	0.98			
Nickel (II) sulfate hexahydrate	11	DMSO	2	1	51981	0.63	3	49391	0.60	10	49864	0.61			
Nickel (II) sulfate hexahydrate	11	DMSO	3	1	46829	0.57	3	83879	1.02	10	41820	0.51			
Nickel (II) sulfate hexahydrate	11	DMSO	4	1	50461	0.61	3	37620	0.46	10	69460	0.85			
Nickel (II) sulfate hexahydrate	**11**	**DMSO**	**MEAN**	**1**	**53652**	**0.65**	**3**	**65034**	**0.79**	**10**	**60451**	**0.74**	**NA**	**NA**	**NA**
Vehicle - Positive Control	12		1	0	31062	1.15									
Vehicle - Positive Control	12		2	0	34769	1.28									
Vehicle - Positive Control	12		3	0	19233	0.71									
Vehicle - Positive Control	12		4	0	23272	0.86									
Vehicle - Positive Control	**12**		**MEAN**	**0**	**27084**	**1.00**									
Positive Control	12		1	NA	32499	1.20									
Positive Control	12		2	NA	149284	5.51									
Positive Control	12		3	NA	138062	5.10									
Positive Control	12		4	NA	155617	5.75									
Positive Control	**12**		**MEAN**	**NA**	**118865**	**4.39**									
Vehicle - Substance	12	AOO	1	0	34707	1.27									
Vehicle - Substance	12	AOO	2	0	19823	0.72									
Vehicle - Substance	12	AOO	3	0	21963	0.80									
Vehicle - Substance	12	AOO	4	0	33252	1.21									
Vehicle - Substance	**12**	**AOO**	**MEAN**	**0**	**27436**	**1.00**									
Hexyl cinnamic aldehyde	12	AOO	1	5	45866	1.67	10	96208	3.51	25	146684	5.35			
Hexyl cinnamic aldehyde	12	AOO	2	5	32444	1.18	10	70432	2.57	25	176112	6.42			
Hexyl cinnamic aldehyde	12	AOO	3	5	52964	1.93	10	121167	4.42	25	135063	4.92			
Hexyl cinnamic aldehyde	12	AOO	4	5	49440	1.80	10	90169	3.29	25	168604	6.15			

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Hexyl cinnamic aldehyde	**12**	**AOO**	**MEAN**	**5**	**45178**	**1.65**	**10**	**94494**	**3.44**	**25**	**156615**	**5.71**	**8.76**	**7.37**	**5.98**
Vehicle - Positive Control	12		1	0	26207	0.79									
Vehicle - Positive Control	12		2	0	39177	1.18									
Vehicle - Positive Control	12		3	0	37398	1.13									
Vehicle - Positive Control	12		4	0	30062	0.91									
Vehicle - Positive Control	**12**		**MEAN**	**0**	**33211**	**1.00**									
Positive Control	12		1	NA	151987	4.58									
Positive Control	12		2	NA	169589	5.11									
Positive Control	12		3	NA	209928	6.32									
Positive Control	12		4	NA	134469	4.05									
Positive Control	**12**		**MEAN**	**NA**	**166493**	**5.01**									
Vehicle - Substance	12	DMSO	1	0	78629	0.95									
Vehicle - Substance	12	DMSO	2	0	88765	1.07									
Vehicle - Substance	12	DMSO	3	0	76637	0.92									
Vehicle - Substance	12	DMSO	4	0	88155	1.06									
Vehicle - Substance	**12**	**DMSO**	**MEAN**	**0**	**83046**	**1.00**									
Nickel (II) sulfate hexahydrate	12	DMSO	1	1	98797	1.19	3	84327	1.02	10	105221	1.27			
Nickel (II) sulfate hexahydrate	12	DMSO	2	1	80665	0.97	3	86877	1.05	10	71971	0.87			
Nickel (II) sulfate hexahydrate	12	DMSO	3	1	86949	1.05	3	137747	1.66	10	55567	0.67			
Nickel (II) sulfate hexahydrate	12	DMSO	4	1	65175	0.78	3	104430	1.26	10	89624	1.08			
Nickel (II) sulfate hexahydrate	**12**	**DMSO**	**MEAN**	**1**	**82896**	**1.00**	**3**	**103345**	**1.24**	**10**	**80596**	**0.97**	**NA**	**NA**	**NA**
Potassium dichromate	12	DMSO	1	0.1	170554	2.05	0.3	198199	2.39	1.0	301077	3.63			
Potassium dichromate	12	DMSO	2	0.1	113710	1.37	0.3	205018	2.47	1.0	323900	3.90			
Potassium dichromate	12	DMSO	3	0.1	166200	2.00	0.3	273194	3.29	1.0	378405	4.56			
Potassium dichromate	12	DMSO	4	0.1	179394	2.16	0.3	191835	2.31	1.0	351057	4.23			
Potassium dichromate	**12**	**DMSO**	**MEAN**	**0.1**	**157464**	**1.90**	**0.3**	**217061**	**2.61**	**1.0**	**338610**	**4.08**	**0.49**	**0.27**	**0.13**
Vehicle - Positive Control	13		1	0	21808	0.80									
Vehicle - Positive Control	13		2	0	23919	0.87									

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Vehicle - Positive Control	13		3	0	24606	0.90									
Vehicle - Positive Control	13		4	0	39312	1.43									
Vehicle - Positive Control	**13**		**MEAN**	**0**	**27411**	**1.00**									
Positive Control	13		1	NA	138513	5.05									
Positive Control	13		2	NA	94225	3.44									
Positive Control	13		3	NA	118316	4.32									
Positive Control	13		4	NA	161413	5.89									
Positive Control	**13**		**MEAN**	**NA**	**128117**	**4.67**									
Vehicle - Substance	13	AOO	1	0	33895	1.37									
Vehicle - Substance	13	AOO	2	0	20013	0.81									
Vehicle - Substance	13	AOO	3	0	20945	0.85									
Vehicle - Substance	13	AOO	4	0	24103	0.97									
Vehicle - Substance	**13**	**AOO**	**MEAN**	**0**	**24739**	**1.00**									
Hexyl cinnamic aldehyde	13	AOO	1	5	28705	1.16	10	106862	4.32	25	164960	6.67			
Hexyl cinnamic aldehyde	13	AOO	2	5	19630	0.79	10	92835	3.75	25	116945	4.73			
Hexyl cinnamic aldehyde	13	AOO	3	5	45958	1.86	10	83026	3.36	25	118296	4.78			
Hexyl cinnamic aldehyde	13	AOO	4	5	45943	1.86	10	159832	6.46	25	135132	5.46			
Hexyl cinnamic aldehyde	**13**	**AOO**	**MEAN**	**5**	**35059**	**1.42**	**10**	**110638**	**4.47**	**25**	**133833**	**5.41**	**7.59**	**6.77**	**5.95**
Vehicle - Positive Control	13		1	0	16810	0.75									
Vehicle - Positive Control	13		2	0	25921	1.15									
Vehicle - Positive Control	13		3	0	21544	0.96									
Vehicle - Positive Control	13		4	0	25627	1.14									
Vehicle - Positive Control	**13**		**MEAN**	**0**	**22475**	**1.00**									
Positive Control	13		1	NA	156378	6.96									
Positive Control	13		2	NA	133906	5.96									
Positive Control	13		3	NA	140685	6.26									
Positive Control	13		4	NA	152161	6.77									
Positive Control	**13**		**MEAN**	**NA**	**145782**	**6.49**									
Vehicle - Substance	13	DMSO	1	0	93878	1.15									
Vehicle - Substance	13	DMSO	2	0	70631	0.87									
Vehicle - Substance	13	DMSO	3	0	91822	1.13									
Vehicle - Substance	13	DMSO	4	0	68974	0.85									
Vehicle - Substance	**13**	**DMSO**	**MEAN**	**0**	**81326**	**1.00**									

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Cobalt chloride	13	DMSO	1	1	120105	1.48	3	199869	2.46	5	192357	2.37			
Cobalt chloride	13	DMSO	2	1	148835	1.83	3	195046	2.40	5	215391	2.65			
Cobalt chloride	13	DMSO	3	1	93820	1.15	3	207281	2.55	5	224902	2.77			
Cobalt chloride	13	DMSO	4	1	172802	2.12	3	195145	2.40	5	192928	2.37			
Cobalt chloride	**13**	**DMSO**	**MEAN**	**1**	**133890**	**1.65**	**3**	**199335**	**2.45**	**5**	**206394**	**2.54**	**NA**	**4.13**	**1.88**
Lactic acid	13	DMSO	1	5	71011	0.87	10	58052	0.71	25	61451	0.76			
Lactic acid	13	DMSO	2	5	58742	0.72	10	44480	0.55	25	47962	0.59			
Lactic acid	13	DMSO	3	5	95883	1.18	10	56725	0.70	25	79235	0.97			
Lactic acid	13	DMSO	4	5	96922	1.19	10	62219	0.77	25	51848	0.64			
Lactic acid	**13**	**DMSO**	**MEAN**	**5**	**80639**	**0.99**	**10**	**55369**	**0.68**	**25**	**60124**	**0.74**	**NA**	**NA**	**NA**
Vehicle - Positive Control	14		1	0	25953	0.86									
Vehicle - Positive Control	14		2	0	42071	1.39									
Vehicle - Positive Control	14		3	0	22870	0.76									
Vehicle - Positive Control	14		4	0	30199	1.00									
Vehicle - Positive Control	**14**		**MEAN**	**0**	**30273**	**1.00**									
Positive Control	14		1	NA	198381	6.55									
Positive Control	14		2	NA	164826	5.44									
Positive Control	14		3	NA	205542	6.79									
Positive Control	14		4	NA	198361	6.55									
Positive Control	**14**		**MEAN**	**NA**	**191777**	**6.33**									
Vehicle - Substance	14	AOO	1	0	21623	0.89									
Vehicle - Substance	14	AOO	2	0	27737	1.14									
Vehicle - Substance	14	AOO	3	0	33618	1.38									
Vehicle - Substance	14	AOO	4	0	14415	0.59									
Vehicle - Substance	**14**	**AOO**	**MEAN**	**0**	**24348**	**1.00**									
Hexyl cinnamic aldehyde	14	AOO	1	5	45466	1.87	10	100580	4.13	25	164791	6.77			
Hexyl cinnamic aldehyde	14	AOO	2	5	40112	1.65	10	134453	5.52	25	155059	6.37			
Hexyl cinnamic aldehyde	14	AOO	3	5	72779	2.99	10	18994	0.78	25	249145	10.23			
Hexyl cinnamic aldehyde	14	AOO	4	5	43275	1.78	10	101713	4.18	25	171572	7.05			
Hexyl cinnamic aldehyde	**14**	**AOO**	**MEAN**	**5**	**50408**	**2.07**	**10**	**88935**	**3.65**	**25**	**185142**	**7.60**	**7.94**	**6.36**	**4.85**
Vehicle - Positive Control	14		1	0	18024	0.74									
Vehicle - Positive Control	14		2	0	24615	1.02									
Vehicle - Positive Control	14		3	0	28493	1.18									

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Vehicle - Positive Control	14		4	0	25735	1.06									
Vehicle - Positive Control	**14**		**MEAN**	**0**	**24216**	**1.00**									
Positive Control	14		1	NA	116341	4.80									
Positive Control	14		2	NA	213773	8.83									
Positive Control	14		3	NA	182037	7.52									
Positive Control	14		4	NA	192821	7.96									
Positive Control	**14**		**MEAN**	**NA**	**176243**	**7.28**									
Vehicle - Substance	14	DMSO	1	0	33858	0.81									
Vehicle - Substance	14	DMSO	2	0	31373	0.75									
Vehicle - Substance	14	DMSO	3	0	60046	1.44									
Vehicle - Substance	14	DMSO	4	0	41804	1.00									
Vehicle - Substance	**14**	**DMSO**	**MEAN**	**0**	**41770**	**1.00**									
Cobalt chloride	14	DMSO	1	1	104955	2.51	3	193202	4.63	5	239096	5.72			
Cobalt chloride	14	DMSO	2	1	83477	2.00	3	147696	3.54	5	128719	3.08			
Cobalt chloride	14	DMSO	3	1	85107	2.04	3	165128	3.95	5	160037	3.83			
Cobalt chloride	14	DMSO	4	1	114867	2.75	3	179062	4.29	5	182970	4.38			
Cobalt chloride	**14**	**DMSO**	**MEAN**	**1**	**97101**	**2.32**	**3**	**171272**	**4.10**	**5**	**177705**	**4.25**	**1.76**	**1.20**	**0.82**
Nickel (II) sulfate hexahydrate	14	DMSO	1	1	104492	2.50	3	72152	1.73	10	71690	1.72			
Nickel (II) sulfate hexahydrate	14	DMSO	2	1	58854	1.41	3	48034	1.15	10	NA	NA			
Nickel (II) sulfate hexahydrate	14	DMSO	3	1	94853	2.27	3	68084	1.63	10	97605	2.34			
Nickel (II) sulfate hexahydrate	14	DMSO	4	1	53019	1.27	3	72530	1.74	10	97675	2.34			
Nickel (II) sulfate hexahydrate	**14**	**DMSO**	**MEAN**	**1**	**77804**	**1.86**	**3**	**65200**	**1.56**	**10**	**88990**	**2.13**	**NA**	**NA**	**8.40**
Vehicle - Positive Control	15		1	0	39487	1.12									
Vehicle - Positive Control	15		2	0	45663	1.30									
Vehicle - Positive Control	15		3	0	28492	0.81									
Vehicle - Positive Control	15		4	0	26819	0.76									
Vehicle - Positive Control	**15**		**MEAN**	**0**	**35115**	**1.00**									
Positive Control	15		1	NA	157090	4.47									

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Positive Control	15		2	NA	164583	4.69									
Positive Control	15		3	NA	77120	2.20									
Positive Control	15		4	NA	157960	4.50									
Positive Control	**15**		**MEAN**	**NA**	**139188**	**3.96**									
Vehicle - Substance	15	AOO	1	0	26758	0.86									
Vehicle - Substance	15	AOO	2	0	46603	1.49									
Vehicle - Substance	15	AOO	3	0	23061	0.74									
Vehicle - Substance	15	AOO	4	0	28334	0.91									
Vehicle - Substance	**15**	**AOO**	**MEAN**	**0**	**31189**	**1.00**									
Hexyl cinnamic aldehyde	15	AOO	1	5	38890	1.25	10	71984	2.31	25	124344	3.99			
Hexyl cinnamic aldehyde	15	AOO	2	5	55784	1.79	10	66130	2.12	25	85306	2.74			
Hexyl cinnamic aldehyde	15	AOO	3	5	43619	1.40	10	84295	2.70	25	142287	4.56			
Hexyl cinnamic aldehyde	15	AOO	4	5	49120	1.57	10	91478	2.93	25	136649	4.38			
Hexyl cinnamic aldehyde	**15**	**AOO**	**MEAN**	**5**	**46853**	**1.50**	**10**	**78471**	**2.52**	**25**	**122146**	**3.92**	**15.18**	**9.92**	**7.45**
Vehicle - Positive Control	15		1	0	43807	1.36									
Vehicle - Positive Control	15		2	0	26515	0.82									
Vehicle - Positive Control	15		3	0	29210	0.90									
Vehicle - Positive Control	15		4	0	29709	0.92									
Vehicle - Positive Control	**15**		**MEAN**	**0**	**32310**	**1.00**									
Positive Control	15		1	NA	118146	3.66									
Positive Control	15		2	NA	172004	5.32									
Positive Control	15		3	NA	135989	4.21									
Positive Control	15		4	NA	163682	5.07									
Positive Control	**15**		**MEAN**	**NA**	**147455**	**4.56**									
Vehicle - Substance	15	DMSO	1	0	35762	0.72									
Vehicle - Substance	15	DMSO	2	0	32858	0.67									
Vehicle - Substance	15	DMSO	3	0	49385	1.00									
Vehicle - Substance	15	DMSO	4	0	79406	1.61									
Vehicle - Substance	**15**	**DMSO**	**MEAN**	**0**	**49353**	**1.00**									
Lactic acid	15	DMSO	1	5	35838	0.73	10	40908	0.83	25	31906	0.65			
Lactic acid	15	DMSO	2	5	46572	0.94	10	44335	0.90	25	37990	0.77			
Lactic acid	15	DMSO	3	5	43793	0.89	10	70146	1.42	25	33696	0.68			
Lactic acid	15	DMSO	4	5	56717	1.15	10	36323	0.74	25	37444	0.76			

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Lactic acid	**15**	**DMSO**	**MEAN**	**5**	**45730**	**0.93**	**10**	**47928**	**0.97**	**25**	**35259**	**0.71**	**NA**	**NA**	**NA**
Potassium dichromate	15	DMSO	1	0.1	121714	2.47	0.3	215997	4.38	1.0	360162	7.30			
Potassium dichromate	15	DMSO	2	0.1	177882	3.60	0.3	210129	4.26	1.0	191584	3.88			
Potassium dichromate	15	DMSO	3	0.1	132281	2.68	0.3	226134	4.58	1.0	340917	6.91			
Potassium dichromate	15	DMSO	4	0.1	93102	1.89	0.3	115017	2.33	1.0	293061	5.94			
Potassium dichromate	**15**	**DMSO**	**MEAN**	**0.1**	**131244**	**2.66**	**0.3**	**191819**	**3.89**	**1.0**	**296431**	**6.01**	**0.16**	**0.09**	**0.06**
Vehicle - Positive Control	16		1	0	40980	1.14									
Vehicle - Positive Control	16		2	0	29750	0.83									
Vehicle - Positive Control	16		3	0	37809	1.05									
Vehicle - Positive Control	16		4	0	35687	0.99									
Vehicle - Positive Control	**16**		**MEAN**	**0**	**36056**	**1.00**									
Positive Control	16		1	NA	166596	4.62									
Positive Control	16		2	NA	324494	9.00									
Positive Control	16		3	NA	309550	8.59									
Positive Control	16		4	NA	255550	7.09									
Positive Control	**17**		**MEAN**	**NA**	**264047**	**7.32**									
Vehicle - Substance	16	AOO	1	0	28428	1.00									
Vehicle - Substance	16	AOO	2	0	25378	0.89									
Vehicle - Substance	16	AOO	3	0	40570	1.43									
Vehicle - Substance	16	AOO	4	0	19307	0.68									
Vehicle - Substance	**16**	**AOO**	**MEAN**	**0**	**28421**	**1.00**									
Hexyl cinnamic aldehyde	16	AOO	1	5	68037	2.39	10	134273	4.72	25	255545	8.99			
Hexyl cinnamic aldehyde	16	AOO	2	5	75307	2.65	10	132074	4.65	25	274377	9.65			
Hexyl cinnamic aldehyde	16	AOO	3	5	70208	2.47	10	192936	6.79	25	235997	8.30			
Hexyl cinnamic aldehyde	16	AOO	4	5	47285	1.66	10	127598	4.49	25	190963	6.72			
Hexyl cinnamic aldehyde	**16**	**AOO**	**MEAN**	**5**	**65209**	**2.29**	**10**	**146720**	**5.16**	**25**	**239220**	**8.42**	**6.23**	**5.36**	**4.66**
Vehicle - Positive Control	16		1	0	45989	1.19									
Vehicle - Positive Control	16		2	0	31080	0.80									
Vehicle - Positive Control	16		3	0	40234	1.04									
Vehicle - Positive Control	16		4	0	37535	0.97									
Vehicle - Positive Control	**16**		**MEAN**	**0**	**38709**	**1.00**									
Positive Control	16		1	NA	266865	6.89									
Positive Control	16		2	NA	266443	6.88									

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Positive Control	16		3	NA	291111	7.52									
Positive Control	16		4	NA	264989	6.85									
Positive Control	**16**		**MEAN**	**NA**	**272352**	**7.04**									
Vehicle - Substance	16	DMSO	1	0	78052	1.02									
Vehicle - Substance	16	DMSO	2	0	111835	1.47									
Vehicle - Substance	16	DMSO	3	0	43088	0.57									
Vehicle - Substance	16	DMSO	4	0	71636	0.94									
Vehicle - Substance	**16**	**DMSO**	**MEAN**	**0**	**76153**	**1.00**									
Nickel (II) sulfate hexahydrate	16	DMSO	1	1	104880	1.38	3	109460	1.44	10	78555	1.03			
Nickel (II) sulfate hexahydrate	16	DMSO	2	1	80888	1.06	3	116987	1.54	10	115405	1.52			
Nickel (II) sulfate hexahydrate	16	DMSO	3	1	92663	1.22	3	110261	1.45	10	88420	1.16			
Nickel (II) sulfate hexahydrate	16	DMSO	4	1	81686	1.07	3	139021	1.83	10	71548	0.94			
Nickel (II) sulfate hexahydrate	**16**	**DMSO**	**MEAN**	**1**	**90029**	**1.18**	**3**	**118932**	**1.56**	**10**	**88482**	**1.16**	**NA**	**NA**	**NA**
Lactic acid	16	DMSO	1	5	56025	0.74	10	44029	0.58	25	72313	0.95			
Lactic acid	16	DMSO	2	5	72079	0.95	10	67039	0.88	25	47618	0.63			
Lactic acid	16	DMSO	3	5	58768	0.77	10	63161	0.83	25	75699	0.99			
Lactic acid	16	DMSO	4	5	90115	1.18	10	68256	0.90	25	80804	1.06			
Lactic acid	**16**	**DMSO**	**MEAN**	**5**	**69247**	**0.91**	**10**	**60621**	**0.80**	**25**	**69108**	**0.91**	**NA**	**NA**	**NA**
Vehicle - Positive Control	17		1	0	16598	1.00									
Vehicle - Positive Control	17		2	0	21167	1.28									
Vehicle - Positive Control	17		3	0	20244	1.22									
Vehicle - Positive Control	17		4	0	8376	0.50									
Vehicle - Positive Control	**17**		**MEAN**	**0**	**16596**	**1.00**									
Positive Control	17		1	NA	130759	7.88									
Positive Control	17		2	NA	159307	9.60									
Positive Control	17		3	NA	101692	6.13									
Positive Control	17		4	NA	105306	6.35									
Positive Control	**17**		**MEAN**	**NA**	**124266**	**7.49**									

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Vehicle - Substance	17	AOO	1	0	22001	0.92									
Vehicle - Substance	17	AOO	2	0	17205	0.72									
Vehicle - Substance	17	AOO	3	0	38937	1.63									
Vehicle - Substance	17	AOO	4	0	17407	0.73									
Vehicle - Substance	**17**	**AOO**	**MEAN**	**0**	**23888**	**1.00**									
Hexyl cinnamic aldehyde	17	AOO	1	5	37307	1.56	10	96209	4.03	25	123470	5.17			
Hexyl cinnamic aldehyde	17	AOO	2	5	23097	0.97	10	106660	4.47	25	144993	6.07			
Hexyl cinnamic aldehyde	17	AOO	3	5	33287	1.39	10	109225	4.57	25	191859	8.03			
Hexyl cinnamic aldehyde	17	AOO	4	5	32984	1.38	10	129230	5.41	25	156101	6.53			
Hexyl cinnamic aldehyde	**17**	**AOO**	**MEAN**	**5**	**31668**	**1.33**	**10**	**110331**	**4.62**	**25**	**154106**	**6.45**	**7.54**	**6.78**	**6.02**
Vehicle - Positive Control	17		1	0	11526	0.63									
Vehicle - Positive Control	17		2	0	12942	0.71									
Vehicle - Positive Control	17		3	0	16830	0.92									
Vehicle - Positive Control	17		4	0	31658	1.74									
Vehicle - Positive Control	**17**		**MEAN**	**0**	**18239**	**1.00**									
Positive Control	17		1	NA	152686	8.37									
Positive Control	17		2	NA	167020	9.16									
Positive Control	17		3	NA	133016	7.29									
Positive Control	17		4	NA	160607	8.81									
Positive Control	**17**		**MEAN**	**NA**	**153332**	**8.41**									
Vehicle - Substance	17	DMSO	1	0	47192	0.93									
Vehicle - Substance	17	DMSO	2	0	45146	0.89									
Vehicle - Substance	17	DMSO	3	0	57466	1.13									
Vehicle - Substance	17	DMSO	4	0	53459	1.05									
Vehicle - Substance	**17**	**DMSO**	**MEAN**	**0**	**50815**	**1.00**									
Cobalt chloride	17	DMSO	1	1	134969	2.66	3	206718	4.07	5	297901	5.86			
Cobalt chloride	17	DMSO	2	1	249468	4.91	3	243849	4.80	5	231316	4.55			
Cobalt chloride	17	DMSO	3	1	104002	2.05	3	212124	4.17	5	192465	3.79			
Cobalt chloride	17	DMSO	4	1	106668	2.10	3	201772	3.97	5	306231	6.03			
Cobalt chloride	**17**	**DMSO**	**MEAN**	**1**	**148776**	**2.93**	**3**	**216116**	**4.25**	**5**	**256978**	**5.06**	**1.11**	**0.70**	**0.46**
Potassium dichromate	17	DMSO	1	0.1	212537	4.18	0.3	281536	5.54	1.0	349431	6.88			
Potassium dichromate	17	DMSO	2	0.1	192220	3.78	0.3	284296	5.59	1.0	269795	5.31			
Potassium dichromate	17	DMSO	3	0.1	110195	2.17	0.3	229749	4.52	1.0	278313	5.48			

Substance	Lab No.[1]	Vehicle	Animal No.	1 Conc. (%)	1 Mean ATP[2]	1 SI	2 Conc. (%)	2 Mean ATP[2]	2 SI	3 Conc. (%)	3 Mean ATP[2]	3 SI	Calc. EC3[3]	Calc. EC2.5[4]	Calc. EC2[5]
Potassium dichromate	17	DMSO	4	0.1	146041	2.87	0.3	232971	4.58	1.0	397799	7.83			
Potassium dichromate	**17**	**DMSO**	**MEAN**	**0.1**	**165248**	**3.25**	**0.3**	**257138**	**5.06**	**1.0**	**323834**	**6.37**	**0.09**	**0.06**	**0.05**

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); ATP = adenosine triphosphate; Calc. = calculated; Conc. = concentration; DMSO = dimethyl sulfoxide; IDR = insufficient dose response; NA = not applicable; No. = number; NT = not tested; SI = stimulation Index.

[1]Laboratories 1 – 10 participated in the first phase, and laboratories 11 – 17 participated in the second phase of the two-phased interlaboratory validation study.

[2]Two ATP measurements were taken for each animal and the mean ATP is indicated.

[3]EC3 value was calculated based on interpolation or extrapolation formulas discussed in Gerberick et al. 2004.

[4]EC2.5 value was calculated based on modified interpolation or extrapolation formulas for EC3 discussed in Gerberick et al. 2004.

[5]EC2 value was calculated based on modified interpolation or extrapolation formulas for EC3 discussed in Gerberick et al. 2004.

Appendix E

LLNA: DA Accuracy Analysis Using Additional Approaches for Combining Multiple Test Results

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1.0 Accuracy Analysis Using Alternative Decision Criteria and Alternate Methods for Combining Data for Substances Tested Multiple Times

This appendix shows performance analyses for the murine local lymph node assay (LLNA) modified by Daicel Chemical Industries, Ltd., based on adenosine triphosphate content (ATP; referred to hereafter as the "LLNA: DA") for alternative decision criteria when using two different approaches for combining test results for the 14 substances with multiple LLNA: DA tests.

1. The positive/negative outcome for each substance for each criterion was determined by the outcome of the test with the highest maximum stimulation index (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.

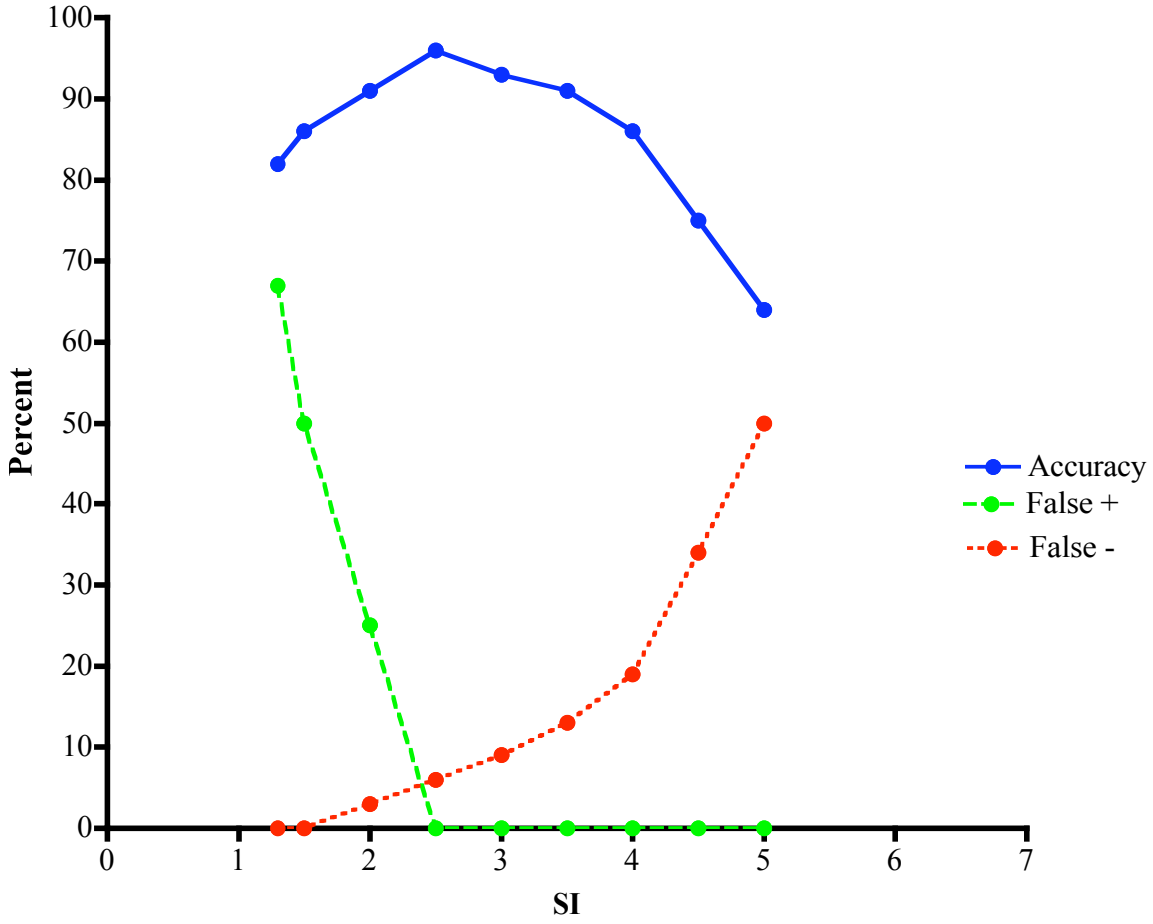
Section 6.0 of this background review document provides the results for the analysis when the most prevalent outcome was used to represent the result for each substance tested multiple times (for each criterion).

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

When combining multiple test results for a single substance by using the outcome of the test with the highest maximum SI, the decision criterion of SI ≥ 3.0 (used by the LLNA: DA validation study team) to identify sensitizers yielded an accuracy of 93% (41/44), a sensitivity of 91% (29/32), a specificity of 100% (12/12), a false positive rate of 0% (0/12), and a false negative rate of 9% (3/32) (**Table E-1)**. The decision criteria using higher SI values, SI ≥ 3.5 to SI ≥ 5.0, decreased performance except for the specificity and the false positive rate, which remained at 100% (12/12) and 0% (0/12), respectively (**Figure E-1** and **Table E-1)**. The lower SI criterion, SI ≥ 2.5, increased accuracy to 96% (42/44) and sensitivity to 94% (30/32), while the specificity and the false positive rate remained the same at 100% (12/12) and 0% (0/12), respectively. Further, the false negative rate decreased to 6%

(2/32) at SI ≥ 2.5. At an even lower SI criterion, SI ≥ 1.3, accuracy was 82% (36/44) and sensitivity was 100% (32/32), while the specificity was low (33% [4/12]) and the false positive rate was high (67% [8/12]). Further, the false negative rate decreased to 0% (0/32) at SI ≥ 1.3. The use of analysis of variance (ANOVA) and summary statistics (i.e., mean ATP measurement of treated groups ≥95% confidence interval (CI) of the control group, or ≥2 or ≥3 standard deviation [SD] from the control group mean), yielded accuracy values of 75% to 84%, with sensitivity values of 88% to 100%, and false negative rates of 0 to 13%. The specificity for these criteria ranged from 8% to 58% and the false positive rates were 42% to 92%. Of these alternative decision criteria, the best overall performance for the approach using the highest maximum SI for the substances with more than one test was achieved using an SI ≥ 2.5, as summarized above. Using an SI ≥ 2.5, however, incorrectly classified 2-mercaptobenzothiazole, a commonly know skin sensitizer.

Figure E-1 Performance of the LLNA: DA 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: DA with the SI cutoff used to identify sensitizers. This analysis used LLNA: DA and traditional LLNA results for 44 substances (32 traditional LLNA sensitizers and 12 traditional LLNA nonsensitizers). 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.

Table E-1 Performance of the LLNA: DA Compared with the Traditional LLNA Using Alternative Decision Criteria to Identify Sensitizers Based on 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]	44	84	37/44	94	30/32	58	7/12	42	5/12	6	2/32	86	30/35	78	7/9
≥ 95% CI[4]	44	75	33/44	100	32/32	8	1/12	92	11/12	0	0/32	74	32/43	100	1/1
≥ 2 SD[5]	44	77	34/44	91	29/32	42	5/12	58	7/12	9	3/32	81	29/36	63	5/8
≥ 3 SD[6]	44	77	34/44	88	28/32	50	6/12	50	6/12	13	4/32	82	28/34	60	6/10
SI ≥ 5.0	44	64	28/44	50	16/32	100	12/12	0	0/12	50	16/32	100	16/16	43	12/28
SI ≥ 4.5	44	75	33/44	66	21/32	100	12/12	0	0/12	34	11/32	100	21/21	52	12/23
SI ≥ 4.0	44	86	38/44	81	26/32	100	12/12	0	0/12	19	6/32	100	26/26	67	12/18
SI ≥ 3.5	44	91	40/44	88	28/32	100	12/12	0	0/12	13	4/32	100	28/28	75	12/16
SI ≥ 3.0	***44***	***93***	***41/44***	***91***	***29/32***	***100***	***12/12***	***0***	***0/12***	***9***	***3/32***	***100***	***29/29***	***80***	***12/15***
SI ≥ 2.5	*44*	*96*	*42/44*	*94*	*30/32*	*100*	*12/12*	*0*	*0/12*	*6*	*2/32*	*100*	*30/30*	*86*	*12/14*
SI ≥ 2.0	44	91	40/44	97	31/32	75	9/12	25	3/12	3	1/32	91	31/34	90	9/10
SI ≥ 1.5	44	86	38/44	100	32/32	50	6/12	50	6/12	0	0/32	84	32/38	100	6/6
SI ≥ 1.3	44	82	36/44	100	32/32	33	4/12	67	8/12	0	0/32	80	32/40	100	4/4

Bolded text indicates the decision criterion chosen by the LLNA: DA validation study team; Italicized text indicates the single decision criterion that had an overall increased performance in predicting skin sensitization potential when compared to the traditional LLNA.

Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP Content; 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 ATP data were log-transformed prior to statistical analyses. For analysis of variance, significance at $p < 0.05$ was further tested by Dunnett's test.

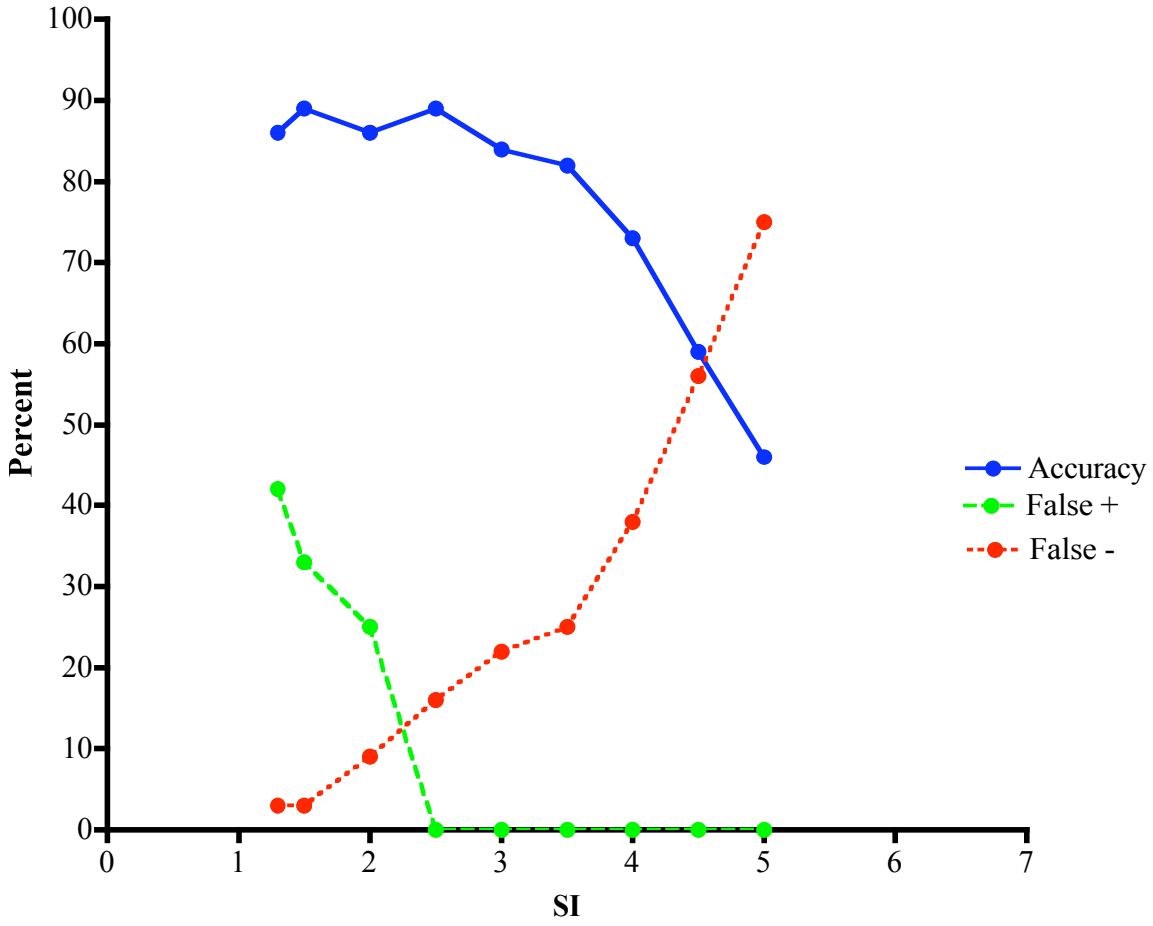
[4]The mean ATP of at least one treatment group was outside the 95% CI for the mean ATP of the vehicle control group.

93 [5] The mean ATP of at least one treatment group was greater than 2 SD from the mean ATP of the vehicle control group.
94 [6] The mean ATP of at least one treatment group was greater than 3 SD from the mean ATP of the vehicle control group.

1.2 Results of LLNA: DA 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 to identify sensitizers, the decision criterion of SI ≥ 3.0 (used by the LLNA: DA validation study team) yielded an accuracy of 84% (37/44), a sensitivity of 78% (25/32), a specificity of 100% (12/12), a false positive rate of 0% (0/12), and a false negative rate of 22% (7/32) (**Table E-2)**. The decision criteria using higher SI values, SI ≥ 3.5 to SI ≥ 5.0, decreased performance except for the specificity and the false positive rate, which remained at 100% (12/12) and 0% (0/12), respectively (**Figure E-2** and **Table E-2**). At SI ≥ 5.0, accuracy decreased to 46% (20/44) and the false negative rate increased to 75% (24/32). Use of a lower SI at SI ≥ 2.5 increased accuracy to 89% (39/44) and sensitivity to 84% (27/32), while the specificity and false positive rate remained the same at 100% (12/12) and 0% (0/12), respectively. Further, the false negative rate decreased to 16% (5/32) at SI ≥ 2.5. At an even lower SI criterion, SI ≥ 1.3, accuracy was decreased to 86% (38/44) but the sensitivity increased to 97% (31/32), while the specificity was 58% (7/12) and the false positive rate was 42% (5/12). Further, the false negative rate decreased to 3% (1/32) at SI ≥ 1.3. Use of a statistical test (i.e., ANOVA or *t*-test) and summary statistics (i.e., mean ATP measurements of treated groups ≥95% CI of the control group, or ≥2 or ≥3 SD from the control group mean), yielded accuracy values of 77 to 82%, with sensitivity values of 84 to 97%, and false negative rates of 3 to 16%. Both the specificity and false positive rate for these criteria ranged from 42 to 58%. Of these alternative decision criteria, the best overall performance for the approach using the lowest maximum SI for the substances with more than one test was achieved using SI ≥ 2.5, as summarized above.

Figure E-2 Performance of the LLNA: DA 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: DA with the SI cutoff used to identify sensitizers. This analysis used LLNA: DA and traditional LLNA results for 44 substances (32 traditional LLNA sensitizers and 12 traditional LLNA nonsensitizers). 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.

Table E-2 Performance of the LLNA: DA Compared with the Traditional LLNA Using Alternative Decision Criteria to Identify Sensitizers Based on 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]	44	82	36/44	91	29/32	58	7/12	42	5/12	9	3/32	85	29/34	70	7/10
≥ 95% CI[4]	44	82	36/44	97	31/32	42	5/12	58	7/12	3	1/32	82	31/38	83	5/6
≥ 2 SD[5]	44	77	34/44	88	28/32	50	6/12	50	6/12	13	4/32	82	28/34	60	6/10
≥ 3 SD[6]	44	77	34/44	84	27/32	58	7/12	42	5/12	16	5/32	84	27/32	58	7/12
SI ≥ 5.0	44	46	20/44	25	8/32	100	12/12	0	0/12	75	24/32	100	8/8	33	12/36
SI ≥ 4.5	44	59	26/44	44	14/32	100	12/12	0	0/12	56	18/32	100	14/14	40	12/30
SI ≥ 4.0	44	73	32/44	63	20/32	100	12/12	0	0/12	38	12/32	100	20/20	50	12/24
SI ≥ 3.5	44	82	36/44	75	24/32	100	12/12	0	0/12	25	8/32	100	24/24	60	12/20
SI ≥ 3.0	**44**	**84**	**37/44**	**78**	**25/32**	**100**	**12/12**	**0**	**0/12**	**22**	**7/32**	**100**	**25/25**	**63**	**12/19**
SI ≥ 2.5	*44*	*89*	*39/44*	*84*	*27/32*	*100*	*12/12*	*0*	*0/12*	*16*	*5/32*	*100*	*27/27*	*71*	*12/17*
SI ≥ 2.0	44	86	38/44	91	29/32	75	9/12	25	3/12	9	3/32	91	29/32	75	9/12
SI ≥ 1.5	44	89	39/44	97	31/32	67	8/12	33	4/12	3	1/32	89	31/35	89	8/9
SI ≥ 1.3	44	86	38/44	97	31/32	58	7/12	42	5/12	3	1/32	86	31/36	88	7/8

Bolded text indicates the decision criterion chosen by the LLNA: DA validation study team; Italicized text indicates the single decision criterion that had an overall increased performance in predicting skin sensitization potential when compared to the traditional LLNA.

Abbreviations: CI = confidence interval; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP Content; 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 ATP data were log-transformed prior to statistical analyses. For analysis of variance, significance at $p < 0.05$ was further tested by Dunnett's test.

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

[5] The mean ATP of at least one treatment group was greater than 2 SD from the mean ATP of the vehicle control group.
[6] The mean ATP of at least one treatment group was greater than 3 SD from the mean ATP of the vehicle control group.
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2.0 Discordant Results for Accuracy Analysis of Alternative Decision Criteria

As mentioned above, for the 14 substances with multiple test results using the decision criteria of SI ≥ 2.5 to identify sensitizers (based on the test with the highest maximum SI) yielded the best overall performance among the alternative decision criteria evaluated. When compared to the traditional LLNA, 2-mercaptobenzothiazole, a well-known skin sensitizer was misclassified as a nonsensitizer (**Table E-3**).

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

Using the decision criterion of SI ≥ 3.0 to identify sensitizers and the test with the highest maximum SI as the representative result for substances with multiple tests yielded three discordant substances (i.e., 3-aminophenol, 2-mercaptobenzothiazole, and methyl methacrylate), all misclassified as nonsensitizers by the LLNA: DA.

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 to represent the outcome for substances with multiple tests. Using an SI cutoff lower than three to identify sensitizers, such as SI ≥ 2.0, yielded four discordant substances: chlorobenzene, hexane, and salicylic acid were misclassified as sensitizers and methyl methacrylate was misclassified as a nonsensitizer. Using an even lower SI cutoff of SI ≥ 1.3 to identify sensitizers, yielded five additional discordant substances that were all misclassified as sensitizers (i.e., 1-bromobutane, dimethyl isophthalate, isopropanol, methyl salicylate, and nickel [II] chloride). Increasing the SI cutoff to values greater than three, increased the number of sensitizers that were misclassified as nonsensitizers. At SI ≥ 4.0, six traditional LLNA sensitizers were misclassified as nonsensitizers by the LLNA: DA while at SI ≥ 5.0, 17 sensitizers were classified as nonsensitizers (**Table E-3)**.

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 nonsensitizers in the traditional LLNA as sensitizers in the LLNA: DA. Using ANOVA or *t*-test to identify sensitizers misclassified five nonsensitizers (i.e., 1-bromobutane, chlorobenzene, hexane, salicylic acid, and sulfanilamide) as sensitizers

and two sensitizers (i.e., 2-mercaptobenzothiazole and methyl methacrylate) as nonsensitizers. Using treatment group ATP measurement with ≥2 SD or ≥3 SD of the vehicle control mean or a ≥95% CI of the vehicle control mean, all misclassified the following six traditional LLNA nonsensitizers as sensitizers: 1-bromobutane, chlorobenzene, hexane, isopropanol, nickel (II) chloride, and propylparaben. The ≥95% CI of the vehicle control mean misclassified four additional nonsensitizers (i.e., diethyl phthalate, dimethyl isophthalate, lactic acid, and methyl salicylate) as sensitizers. In addition, ≥2 SD or ≥3 SD of the vehicle control mean commonly misclassified three sensitizers as nonsensitizers (i.e., ethyl acrylate, methyl methacrylate, and propyl gallate).

Thirteen of the 22 ICCVAM-recommended LLNA performance standards reference substances (ICCVAM 2009) tested in the LLNA: DA were discordant for the analysis of alternate decision criteria using the test with the highest maximum SI to represent substances with multiple tests (**Table E-3**) when compared to the traditional LLNA. Six nonsensitizers in the traditional LLNA (i.e., chlorobenzene, isopropanol, lactic acid, methyl salicylate, nickel [II] chloride, and salicylic acid) were misclassified by some criteria in the LLNA: DA as a sensitizers, and seven sensitizers in the traditional LLNA (i.e., citral, ethylene glycol dimethacrylate, imidazolidinyl urea, 2-mercaptobenzothiazole, methyl methacrylate, phenyl benzoate, and sodium lauryl sulfate) were misclassified as nonsensitizers by some criteria when tested in the LLNA: DA.

Table E-3 Discordant Results for the LLNA: DA Using Alternative Decision Criteria Compared to the Traditional LLNA Based on 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
3-Aminophenol (3.2%)					-	-	-	-	-				
p-Benzoquinone (0.01%)					-	-	-						
1-Bromobutane (-)	+	+	+	+								+	+
Butyl glycidyl ether (30.9%)				-	-								
Chlorobenzene (-)	+	+	+	+							+	+	+
Cinnamic aldehyde (1.9%)					-								
Citral (9.2%)					-	-							
Diethyl maleate (3.6%)					-	-	-						
Diethyl phthalate (-)		+											
Dimethyl isophthalate (-)													+
Ethyl acrylate (32.8%)			-	-	-	-							
Ethylene glycol dimethacrylate (28.0%)					-	-							
Hexane (-)	+	+	+	+							+	+	+
Imidazolidinyl urea (24.0%)					-								
Isopropanol (-)		+	+	+								+	+
Lactic acid (-)		+											
2-Mercaptobenzothiazole	-				-	-	-	-	-	-			

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**
(1.7%)													
Methyl methacrylate (90.0%)	-		-	-	-	-	-	-	-	-	-		
Methyl salicylate (-)		+										+	+
Nickel (II) chloride (-)		+	+	+									+
Phenyl benzoate (13.6%)					-	-							
Propyl gallate (0.32%)			-	-	-								
Propylparaben (-)		+	+	+									
Resorcinol (6.3%)					-	-							
Salicylic acid (-)	+	+	+								+	+	+
Sodium lauryl sulfate (8.1%)					-	-	-	-					
Sulfanilamide (-)	+												
Trimellitic anhydride (4.7%)					-								

Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP Content; SD = standard deviation; SI = stimulation index.

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

[2]LLNA: DA outcomes are indicated by "+" for sensitizer results and "-" for nonsensitizer results.

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

[4]The mean ATP of at least one treatment group was outside the 95% CI for the mean ATP of the vehicle control group.

[5]The mean ATP of at least one treatment group was greater than 2 SD from the mean ATP of the vehicle control group.

[6]The mean ATP of at least one treatment group was greater than 3 SD from the mean ATP of the vehicle control group.

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

As mentioned above, for the substances with multiple tests, using the decision criterion of SI ≥ 2.5 to identify sensitizers (based on the test with the lowest maximum SI) yielded the best overall performance for the LLNA: DA when compared to the traditional LLNA. This decision criterion yielded five discordant substances; all five were sensitizers in the traditional LLNA but were misclassified as nonsensitizers in the LLNA: DA (i.e., 3-aminophenol, cobalt chloride, 2-mercaptobenzothiazole, methyl methacrylate, and nickel [II] sulfate hexahydrate) (**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 representative result for substances with multiple tests. Using an SI cutoff less than three, SI ≥ 2.0, to identify sensitizers yielded six discordant substances. Three of the six discordant substances (i.e., 3-aminophenol, methyl methacrylate, and nickel [II] sulfate hexahydrate] were misclassified as nonsensitizers by the LLNA: DA compared to the traditional LLNA and the remaining three (i.e., chlorobenzene, hexane, and salicylic acid) were misclassified as sensitizers. Using an even lower SI to identify sensitizers, SI ≥1.3, also yielded six discordant substances. Chlorobenzene, hexane, and salicylic acid were still misclassified as sensitizers and nickel (II) sulfate hexahydrate was still misclassified as a nonsensitizer by the LLNA: DA compared to the traditional LLNA. In addition, 1-bromobutane and nickel (II) chloride were also misclassified as sensitizers. Increasing the SI cutoff to values greater than three, increased the number of sensitizers that were misclassified as nonsensitizers. At SI ≥ 4.0, 12 sensitizers were misclassified as nonsensitizers while at SI ≥ 5.0, 24 sensitizers were misclassified as nonsensitizers (**Table E-4)**. 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. For instance, at SI ≥ 5.0, 2,4-dinitrochlorobenzene and glutaraldehyde were classified as nonsensitizers.

Table E-4 Discordant Results for the LLNA: DA Using Alternative Decision Criteria Compared to the Traditional LLNA Based on 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
Abietic Acid (11.9%)					-	-	-						
3-Aminophenol (3.2%)					-	-	-	-	-	-	-		
p-Benzoquinone (0.01%)					-	-	-						
1-Bromobutane (-)	+	+	+	+	-							+	+
Butyl glycidyl ether (30.9%)				-	-								
Chlorobenzene (-)	+	+	+	+							+	+	+
Cinnamic aldehyde (1.9%)					-								
Citral (9.2%)					-	-							
Cobalt chloride (0.60%)					-	-	-	-	-	-			
Diethyl phthalate (-)		+											
Dimethyl isophthalate (-)													
Diethyl maleate (3.6%)					-	-	-						
2,4-Dinitrochlorobenzene (0.05%)					-								
Ethyl acrylate (32.8%)			-	-	-	-							
Ethylene glycol dimethacrylate (28.0)					-	-							
Formaldehyde (0.50%)					-	-	-	-	-				
Glutaraldehyde (0.08%)					-	-	-	-	-				

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
Hexane (-)	+	+	+	+							+	+	+
Hexyl cinnamic aldehyde (9.7%)					-	-	-						
Imidazolidinyl urea (24.0%)					-								
2-Mercaptobenzothiazole (1.7%)	-				-	-	-	-	-	-			
Methyl methacrylate (90.0%)	-		-	-		-	-	-	-	-	-		
Nickel (II) chloride (-)		+	+	+									+
Nickel (II) sulfate hexahydrate (4.8%)	-	-	-	-	-	-	-	-	-	-	-	-	-
Phenyl benzoate (13.6%)					-	-							
Potassium dichromate (0.17%)					-	-							
Propyl gallate (0.32%)			-	-	-								
Propylparaben (-)		+	+	+									
Resorcinol (6.3%)					-	-							
Salicylic acid (-)	+	+	+								+	+	+
Sulfanilamide (-)	+												
Sodium lauryl sulfate (8.1%)					-	-	-	-					
Trimellitic anhydride (4.7%)					-								

Abbreviations: CI = confidence interval; LLNA = murine local lymph node assay; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP Content; SD = Standard deviation; SI = Stimulation index.

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

[2]LLNA: DA outcomes are indicated by "+" for sensitizer results and "-" for nonsensitizer results.

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

[4]The mean ATP of at least one treatment group was outside the 95% CI for the mean ATP of the vehicle control group.

[5]The mean ATP of at least one treatment group was greater than 2 SD from the mean ATP of the vehicle control group.

[6]The mean ATP of at least one treatment group was greater than 3 SD from the mean ATP 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 traditional LLNA nonsensitizers than sensitizers (**Table E-4**). Using ANOVA or *t*-test to identify sensitizers misclassified three sensitizers in the traditional LLNA (i.e., 2-mercaptobenzothiazole, methyl methacrylate, and nickel [II] sulfate hexahydrate) as nonsensitizers in the LLNA: DA. Further, five nonsensitizers in the traditional LLNA (i.e., 1-bromobutane, chlorobenzene, hexane, salicylic acid, and sulfanilamide) were misclassified as sensitizers in the LLNA: DA. Using treatment group ATP measurement ≥95% CI, ≥2 SD or ≥3 SD of vehicle control mean commonly misclassified 1-bromobutane, chlorobenzene, hexane, nickel (II) chloride, and propylparaben as sensitizers and nickel (II) sulfate hexahydrate as a nonsensitizer compared to traditional LLNA results. In addition each summary statistic misclassified from two to four additional substances when compared to traditional LLNA results (see Table E-4.

Thirteen of the 22 ICCVAM-recommended LLNA performance standards reference substances (ICCVAM 2009) were discordant for the analysis of alternate decision criteria using the test with the lowest maximum SI as the representative result for substances with multiple tests (**Table E-4**). One strong sensitizer in the traditional LLNA, 2,4-dinitrochlorobenzene, was misclassified by SI ≥ 5.0 as a nonsensitizer in the LLNA: DA. Nine additional sensitizers (i.e., citral, cobalt chloride, ethylene glycol dimethacrylate, hexyl cinnamic aldehyde, imidazolidinyl urea, 2-mercaptobenzothiazole, methyl methacrylate, phenyl benzoate, and sodium lauryl sulfate) were also misclassified as nonsensitizers by some criteria in the LLNA: DA. Three nonsensitizers in the traditional LLNA (i.e., chlorobenzene, nickel [II] chloride, and salicylic acid) were misclassified as sensitizers by some criteria in the LLNA: DA.

Appendix F

Reproducibility Analyses for the LLNA: DA Using a Decision Criterion of SI ≥ 3.0 or SI ≥ 2.0

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1.0 LLNA: DA Test Method Reliability

An assessment of test method reliability (intralaboratory repeatability and intra- and inter-laboratory reproducibility) is an essential element of any evaluation of the performance of an alternative test method (ICCVAM 2003). Repeatability refers to the closeness of agreement between test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions within a given time period (ICCVAM 1997, 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. With regard to the murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content (referred to hereafter as the "LLNA: DA") test method, there are no known intralaboratory repeatability studies, which was also the situation with the traditional murine local lymph node assay (LLNA).

The reproducibility evaluation in this revised draft background review document (BRD) has been updated from the January 2008 draft BRD to include an interlaboratory reproducibility evaluation and a reproducibility analysis using separate stimulation index (SI) criteria to identify sensitizers and nonsensitizers (see **Section 7.0**). The available LLNA: DA data were amenable to both intralaboratory and interlaboratory reproducibility analyses. The evaluation of a single decision criterion in **Section 6.6** showed that SI $\geq$ 2.0 was the SI value that produced the lowest false negative rate among the alternative decision criteria evaluated (i.e., 3% [1/32]) when the traditional LLNA was the reference test (**Table 6-6)**. Thus, this appendix describes the evaluation of reproducibility for the decision criterion of SI $\geq$ 2.0 to identify sensitizers, which was evaluated in **Section 6.6**. In addition the reproducibility for SI $\geq$ 3.0, the SI cut-off used in the LLNA: DA validation studies, is also evaluated in this appendix.

1.1 Intralaboratory Reproducibility (SI $\geq$ 3.0 and SI $\geq$ 2.0)

Idehara et al. (2008) evaluated the intralaboratory reproducibility of EC3 (i.e., estimated concentration needed to produce a stimulation index of three) values for the LLNA: DA

using two substances (i.e., isoeugenol and eugenol) that were each tested in three different experiments (**Table F-1**). The data indicate coefficient of variations (CVs) of 21% and 11% for isoeugenol and eugenol, respectively. The authors state that for both compounds the EC3 values appeared to be close and that for each test substance the SI values for the same concentration were fairly reproducible (Idehara et al. 2008). The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) also determined the intralaboratory reproducibility of EC2 (i.e., estimated concentration needed to produce a stimulation index of two) values for the same set of data. The EC2 results indicate slightly larger intralaboratory variability compared to EC3 results with CVs of 35% and 20% for isoeugenol and eugenol, respectively.

Table F-1 Intralaboratory Reproducibility of EC3 and EC2 Values Using the LLNA: DA[1]

Isoeugenol			
Concentration (%)	**Experiment 1[2]**	**Experiment 2[2]**	**Experiment 3[2]**
Vehicle (AOO)	1.00 ± 0.54	1.00 ± 0.54	1.00 ± 0.30
0.5	1.50 ± 0.54	-------	1.22 ± 0.13
1	2.28 ± 0.60	-------	2.77 ± 1.01
2.5	2.78 ± 0.17	3.11 ± 1.15	3.01 ± 0.98
5	3.39 ± 0.69	4.39 ± 1.25	-------
10	5.68 ± 1.19	6.77 ± 0.23	-------
EC3	**3.40%**	**2.35%**	**2.46%**
EC2	**0.82%**	**1.37%**	**0.75%**
Mean EC3: 2.74% ± 0.58% and 21% CV *Mean EC2: 0.98% ± 0.34% and 35% CV*			
Eugenol			
Concentration (%)	**Experiment 1[2]**	**Experiment 2[2]**	**Experiment 3[2]**
Vehicle (AOO)	1.00 ± 0.17	1.00 ± 0.17	1.00 ± 0.09
5	2.92 ± 1.00	2.80 ± 1.08	3.24 ± 0.70
10	7.35 ± 2.62	4.47 ± 0.98	4.79 ± 0.94
25	10.92 ± 3.63	5.62 ± 3.20	7.07 ± 0.44
EC3	**5.09%**	**5.59%**	**4.50%**
EC2	**4.33%**	**3.59%**	**2.87%**
Mean EC3: 5.06% ± 0.55% and 11% CV *Mean EC2: 3.60% ± 0.73% and 20% CV*			

Abbreviations: AOO = acetone: olive oil (4:1); CV = coefficient of variation; EC2 = estimated concentration needed to produce a stimulation index of two; EC3 = estimated concentration needed to produce a stimulation index of three; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content.

[1]Based on results discussed in Idehara et al. 2008; the number per group was not specified.

[2]Mean stimulation index value ± standard deviation.

1.2 Interlaboratory Reproducibility

Furthermore, data were submitted to NICEATM (**Appendix D**) from a two-phased interlaboratory validation study on the LLNA: DA test method (Omori et al. 2008). In the first phase of the interlaboratory validation study, a blinded test of 12 substances was conducted in 10 laboratories. Three substances (i.e. 2,4-dinitrochlorobenzene, hexyl cinnamic aldehyde, and isopropanol) were tested in all 10 laboratories. The remaining nine substances were randomly assigned to subsets of three of the 10 laboratories **(Table F-2)**. In each laboratory, each substance was tested one time at three different concentrations. The dose levels for each substance were pre-determined (i.e., the participating laboratories did not determine their own dose levels for testing). Nine substances are sensitizers and three substances are nonsensitizers according to the traditional LLNA. Six substances are recommended LLNA performance standards reference substances: cobalt chloride, 2,4-dinitrochlorobenzene, hexyl cinnamic aldehyde, isoeugenol, isopropanol, and methyl salicylate (ICCVAM 2009).

The second phase of the interlaboratory validation study was designed to determine the reason for inconsistencies obtained from the two metals dissolved in dimethyl sulfoxide (DMSO) (i.e., cobalt chloride and nickel [II] sulfate hexahydrate) and thus to further evaluate the reliability of the LLNA: DA for testing metallic salts using DMSO as a vehicle. A blinded test of five substances (two of the five substances were unique to the second phase of the interlaboratory validation study) was conducted in seven laboratories (different from the 10 laboratories that performed the first interlaboratory validation study) **(Table F-3)**. One substance (i.e. hexyl cinnamic aldehyde) was tested in all seven laboratories. The remaining four substances (i.e., cobalt chloride, nickel [II] sulfate hexahydrate, lactic acid, and potassium dichromate) were randomly assigned to subsets of four of the seven laboratories. Each laboratory tested the substance one time at three different dose levels. Again, the dose levels for each substance were pre-determined. Of the two substances not previously tested in the first phase of the interlaboratory validation study (i.e., lactic acid and potassium dichromate), one is a nonsensitizer and the other is a sensitizer according to traditional LLNA results, respectively. In addition, lactic acid is a recommended LLNA performance standards reference substance (ICCVAM 2009).

The LLNA: DA test results from the two-phased interlaboratory validation study are amenable to interlaboratory reproducibility analyses for three endpoints: sensitizer (positive) or nonsensitizer (negative) classification (based on SI ≥ 3.0 and SI ≥ 2.0), and EC3 and EC2 values. Analyses of interlaboratory reproducibility were performed using a concordance analysis for the qualitative results (sensitizer vs. nonsensitizer based on SI ≥ 3.0 and SI ≥ 2.0) (**Sections 1.2.1 and 1.2.3, respectively**) and a CV analysis for the quantitative results (EC3 and EC2values) (**Sections 1.2.2** and **1.2.4, respectively**).

Table F-2 Substances and Allocation for the First Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance[1]	Vehicle	Concentration Tested (%)			Laboratory									
					1	2	3	4	5	6	7	8	9	10
2,4-Dinitrochlorobenzene (+)	AOO	0.03	0.10	0.30	X	X	X	X	X	X	X	X	X	X
Hexyl cinnamic aldehyde (+)	AOO	5	10	25	X	X	X	X	X	X	X	X	X	X
Isopropanol (-)	AOO	10	25	50	X	X	X	X	X	X	X	X	X	X
Abietic acid (+)	AOO	5	10	25		X				X	X			
3-Aminophenol (+)	AOO	1	3	10	X		X					X		
Dimethyl isophthalate (-)	AOO	5	10	25	X		X				X			
Isoeugenol (+)	AOO	1	3	10				X	X				X	
Methyl salicylate (-)	AOO	5	10	25			X				X			X
Formaldehyde (+)	ACE	0.5	1.5	5.0	X	X			X					
Glutaraldehyde (+)	ACE	0.05	0.15	0.50	X	X			X					
Cobalt chloride[2] (+)	DMSO	0.3	1.0	3.0				X		X		X		
Nickel (II) sulfate hexahydrate (+)	DMSO	1	3	10				X		X		X		

Abbreviations: ACE = acetone; AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content.

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

[2]Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

Table F-3 Substances and Allocation for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance[1]	Vehicle	Concentration Tested (%)			Laboratory						
					11	12	13	14	15	16	17
Hexyl cinnamic aldehyde (+)	AOO	5	10	25	X	X	X	X	X	X	X
Cobalt chloride[2] (+)	DMSO	1	3	5	X		X	X			X
Lactic acid (-)	DMSO	5	10	25	X		X		X	X	
Nickel (II) sulfate hexahydrate (+)	DMSO	1	3	10	X	X		X		X	
Potassium dichromate (+)	DMSO	0.1	0.3	1.0	X	X			X		X

Abbreviations: AOO = acetone: olive oil (4:1); DMSO = dimethyl sulfoxide; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content.
[1](+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.
[2]Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

1.2.1 Interlaboratory Reproducibility – Qualitative Results (SI ≥ 3.0)

The qualitative (i.e., positive/negative) interlaboratory concordance analysis for the 12 substances that were tested during the first phase of the LLNA: DA interlaboratory validation study is shown in **Table F-4** using SI ≥ 3.0 as the decision criterion to distinguish sensitizers from nonsensitizers. In a qualitative comparison of LLNA: DA calls (i.e., positive/negative), eight substances tested in either three or 10 laboratories had consistent results leading to 100% (3/3 or 10/10) interlaboratory concordance for those substances. There were four discordant substances (i.e., formaldehyde, glutaraldehyde, cobalt chloride, and nickel [II] sulfate hexahydrate) for which interlaboratory concordance was 67% (2/3). One of the three laboratories that tested formaldehyde reported a maximum SI = 2.69 while the other two laboratories produced at least one SI ≥ 3.0. Similarly, one of the three laboratories that tested glutaraldehyde reported a maximum SI = 2.57 while the other two laboratories had at least one SI ≥ 3.0. Two of the three laboratories that tested cobalt chloride yielded an SI ≥ 3.0 at all three doses tested (0.3%, 1.0%, and 3.0%) and therefore classified the substance as a sensitizer similar to the traditional LLNA test method. Notably, the laboratory that did not generate an SI ≥ 3.0 did not test cobalt chloride at the highest dose and the middle dose yielded an SI = 2.66. One of the three laboratories that tested nickel (II) sulfate hexahydrate reported a maximum SI = 1.52, while the other two laboratories had at least two doses that

yielded an SI ≥ 3.0. Since 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: DA concordance data from the first phase of the interlaboratory validation study.

Table F-4 Qualitative Results for the First Phase of the Interlaboratory Validation Study for the LLNA: DA (SI ≥ 3.0)

Substance[1]	Laboratory[2]										Concordance
	1	2	3	4	5	6	7	8	9	10	
2,4-Dinitrochlorobenzene (+)	+	+	+	+	+	+	+	+	+	+	10/10
Hexyl cinnamic aldehyde (+)	+	+	+	+	+	+	+	+	+	+	10/10
Isopropanol (-)	-	-	-	-	-	-	-	-	-	-	10/10
Abietic acid (+)		+				+	+				3/3
3-Aminophenol (+)	-		-					-			3/3
Dimethyl isophthalate (-)	-		-				-				3/3
Isoeugenol (+)				+	+				+		3/3
Methyl salicylate (-)			-				-			-	3/3
Formaldehyde (+)	+	+			-						**2/3**
Glutaraldehyde (+)	+	+			-						**2/3**
Cobalt chloride[3] (+)				-[4]		+		+			**2/3**
Nickel (II) sulfate hexahydrate (+)				-[5]		+		+[5]			**2/3**

Bolded substances did not achieve 100% interlaboratory concordance.
Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP content; SI = stimulation index.
[1](+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.
[2](+) indicates sensitizers and (-) indicates nonsensitizers according to LLNA: DA tests.
[3]Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.
[4]Data not reported for the highest dose (i.e., 3%), only for 0.3% and 1%.
[5]Insufficient dose response.

The qualitative (positive/negative) interlaboratory concordance analysis for the five substances that were tested during the second phase of the LLNA: DA interlaboratory validation study is shown in **Table F-5** using SI ≥ 3.0 as the decision criterion to distinguish sensitizers from nonsensitizers. In a qualitative comparison of LLNA: DA calls (i.e., positive/negative), four substances (i.e., hexyl cinnamic aldehyde, lactic acid, nickel [II] sulfate hexahydrate, and potassium dichromate) tested in either four or seven laboratories had

consistent results leading to 100% (4/4 or 7/7) interlaboratory concordance for those substances. There was one discordant substance (i.e., cobalt chloride) for which interlaboratory concordance was 50% (2/4). Two of the four laboratories that tested cobalt chloride reported a maximum SI = 2.01 and 2.54, respectively, while the other two laboratories had at least two doses that yielded an SI ≥ 3.0. As was discussed previously, cobalt chloride was also discordant among the laboratories that tested the substance in the first phase of the interlaboratory validation study and interlaboratory concordance was 67% (2/3). Notably, different doses of cobalt chloride were tested in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study. Furthermore, as mentioned previously, the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), and therefore there were no traditional LLNA concordance data for comparison with the LLNA: DA concordance data from the second phase of the interlaboratory validation study.

Table F-5 Qualitative Results for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA (SI ≥ 3.0)

Substance[1]	Laboratory[2]							Concordance
	11	12	13	14	15	16	17	
Hexyl cinnamic aldehyde (+)	+	+	+	+	+	+	+	7/7
Cobalt chloride[3] (+)	**-**		**-**	**+**			**+**	**2/4**
Lactic acid (-)	-		-		-	-		4/4
Nickel (II) sulfate hexahydrate (+)	-	-		-		-		4/4
Potassium dichromate (+)	+	+			+		+	4/4

Bolded substances did not achieve 100% interlaboratory concordance.
Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd. based on ATP Content; SI = stimulation index.
[1](+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.
[2](+) indicates sensitizers and (-) indicates nonsensitizers according to LLNA: DA tests.
[3]Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

1.2.2 Interlaboratory Reproducibility – EC3 Values

The available quantitative (i.e., EC3 value) data for interlaboratory reproducibility analysis were obtained from the LLNA: DA results for the nine sensitizers that were tested during the first and second phase of the LLNA: DA interlaboratory validation study. The method for

calculating EC3 values for the positive results was based on the method of linear interpolation reported by Gerberick et al. (2004) according to the equation:

$$EC3 = c + \left[\frac{(3-d)}{(b-d)}\right] \times (a-c)$$

where the data points lying immediately above and below the SI = 3.0 on the dose response curve have the coordinates of (a, b) and (c, d), respectively (Gerberick et al. 2004). For substances for which the lowest concentration tested resulted in an SI ≥ 3.0, an EC3 value was extrapolated according to the equation:

$$EC3_{ex} = 2^{\left\{\log_2(c)+\frac{(3-d)}{(b-d)}\times[\log_2(a)-\log_2(c)]\right\}}$$

where the point with the higher SI is denoted with the coordinates of (a, b) and the point with the lower SI is denoted (c, d) (Gerberick et al. 2004).

The EC3 values from each laboratory were used to calculate CV values for each substance. The resulting values for the first and second phase of the interlaboratory validation study are shown in **Tables F-6** and **F-7**, respectively. In the first phase of the interlaboratory validation study, CV values ranged from 4% (i.e., abietic acid) to 84% (i.e., glutaraldehyde) and the mean CV was 48% (**Table F-6**). Notably, although nickel (II) sulfate hexahydrate was a sensitizer in two of three laboratories, a CV could not be determined because one of the two laboratories that yielded a positive test demonstrated an insufficient dose response from which to calculate an EC3 (i.e., an inverse dose response curve). In the second phase of the interlaboratory validation study, CV values ranged from 32% (i.e., cobalt chloride) to 71% (i.e., potassium dichromate) and the mean CV was 45% (**Table F-7**).

Recommended Performance Standards: Murine Local Lymph Node Assay (ICCVAM 2009) indicates that interlaboratory reproducibility should be evaluated with at least two sensitizing chemicals with well-characterized activity in the traditional LLNA. Acceptable reproducibility is attained when each laboratory obtains ECt values (i.e., estimated concentration needed to produce a stimulation index of a specified threshold) within 0.025% to 0.1% for 2,4-dinitrochlorobenzene and within 5% to 20% for hexyl cinnamic aldehyde (ICCVAM 2009). In the first phase of the interlaboratory validation study, four laboratories reported EC3 values outside the range indicated for 2,4-dinitrochlorobenzene; one laboratory

obtained an EC3 value that was lower than the specified acceptance range (i.e., 0.025%) and three laboratories obtained EC3 values that were higher than the specified acceptance range (i.e., 0.1%) (**Table F-6**). For hexyl cinnamic aldehyde, all the laboratories obtained an EC3 value within the acceptance range (5% to 20%). In the second phase of the interlaboratory validation study, only hexyl cinnamic aldehyde was tested and all seven laboratories obtained EC3 values that were within the acceptance range indicated (**Table F-7**).

231 **Table F-6 EC3 Values from the First Phase of the Interlaboratory Validation Study for the LLNA: DA**

Substance[1]	Laboratory										Mean EC3 (%)	CV (%)
	1	2	3	4	5	6	7	8	9	10		
2,4-Dinitrochlorobenzene (+)	**0.034 (11.97)**	**0.109 (9.23)**	**0.056 (9.96)**	**0.031 (8.53)**	**0.129 (7.86)**	**0.042 (15.14)**	**0.016 (13.18)**	**0.095 (12.60)**	**0.040 (10.89)**	**0.169 (4.71)**	**0.072**	**70**
Hexyl cinnamic aldehyde (+)	**9.983 (5.78)**	**12.412 (4.82)**	**14.90 (4.44)**	**9.340 (5.11)**	**18.131 (3.97)**	**13.130 (5.50)**	**7.706 (7.09)**	**7.924 (10.22)**	**17.070 (3.88)**	**15.235 (3.51)**	**12.583**	**30**
Isopropanol (-)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Abietic acid (+)		8.196				7.544	7.676				7.805	4
3-Aminophenol (+)	NA		NA					NA			NA	NA
Dimethyl isophthalate (-)	NA		NA				NA				NA	NA
Isoeugenol (+)				1.112	5.983				2.300		3.131	81
Methyl salicylate (-)			NA				NA			NA	NA	NA
Formaldehyde (+)	1.747	1.480			NA						1.614	12
Glutaraldehyde (+)	0.110	0.435			NA						0.272	84
Cobalt chloride[2] (+)				NA[3]		0.063		0.137			0.100	53
Nickel (II) sulfate hexahydrate (+)				NA[4]		0.469		IDR			0.469	NA

232 Note: Bolded text indicates recommended LLNA performance standards reference substances (ICCVAM 2009). Values in parentheses are highest SI values achieved. For both 2,4-
233 dinitrochlorobenzene and hexyl cinnamic aldehyde, the highest SI values achieved are from the highest dose tested (i.e., 0.30% for 2,4-dinitrochlorobenzene and 25% for hexyl
234 cinnamic aldehyde). Shading shows EC3 values that are outside of the acceptable range indicated by the recommended LLNA performance standards: 5 - 20% for hexyl cinnamic
235 aldehyde and 0.025 - 0.1% for 2,4-dinitrochlorobenzene.
236 Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of three; LLNA: DA = murine local lymph node assay
237 modified by Daicel Chemical Industries, Ltd., based on ATP content; IDR = insufficient dose response; NA = not available.
238 [1](+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.
239 [2]Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.
240 [3]Data not reported for the highest dose (i.e., 3%), only for 0.3% and 1%.
241 [4]Insufficient dose response.

Table F-7 EC3 Values from the Second Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance[1]	Laboratory							Mean EC3 (%)	CV (%)
	11	12	13	14	15	16	17		
Hexyl cinnamic aldehyde (+)	**9.127 (4.47)**	**8.764 (5.71)**	**7.590 (5.41)**	**7.938 (7.60)**	**15.184 (3.92)**	**6.230 (8.42)**	**7.542 (6.45)**	**8.911**	**33**
Cobalt chloride[2] (+)	NA		NA	1.761			1.109	1.435	32
Lactic acid (-)	NA		NA		NA	NA		NA	NA
Nickel (II) sulfate hexahydrate (+)	NA	NA		NA		NA		NA	NA
Potassium dichromate (+)	0.509	0.485			0.156		0.086	0.309	71

Bolded text indicates a recommended LLNA performance standards reference substance (ICCVAM 2009). Values in parentheses are highest SI values achieved. For hexyl cinnamic aldehyde, the highest SI values achieved are from the highest dose tested (i.e., 25%). None of the EC3 values are outside of the acceptable range indicated by the recommended LLNA performance standards (i.e., 5 - 20% for hexyl cinnamic aldehyde).
Abbreviations: CV = coefficient of variation; EC3 = estimated concentration needed to produce a stimulation index of three; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; NA = not available.
[1](+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.
[2]Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

The interlaboratory CV values for both the first and second phases of the interlaboratory validation study for the LLNA: DA EC3 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 6.8 to 83.7% 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: DA (i.e., hexyl cinnamic aldehyde, 2,4-dinitrochlorobenzene, and isoeugenol). All interlaboratory CV values for the LLNA: DA were greater than that for the traditional LLNA. The CV of 70% for 2,4-dinitrochlorobenzene was greater than the two CV values of 37.4% and 27.2%, calculated from five values each, reported by ICCVAM (1999). The CV values of 30% and 33% for hexyl cinnamic aldehyde tested in the first and second phase of the LLNA: DA interlaboratory validation study, respectively, were both greater than the 6.8% reported by ICCVAM (1999). The CV of 81% for isoeugenol tested in the LLNA: DA was greater than the 41.2% 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	37.4
	0.5	0.6	0.4	0.6	0.3	27.2
Hexyl cinnamic aldehyde	7.9	7.6	8.4	7.0	8.1	6.8
Isoeugenol	1.3	3.3	1.8	3.1	1.6	41.2
Eugenol	5.8	14.5	8.9	13.8	6.0	42.5
Sodium lauryl sulfate	13.4	4.4	1.5	17.1	4.0	83.7

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 report.

1.2.3 Interlaboratory Reproducibility – Qualitative Results (SI ≥ 2.0)

The qualitative (positive/negative) interlaboratory concordance analysis for the 12 substances that were tested during the first phase of the LLNA: DA interlaboratory validation study is shown in **Table F-9** for SI ≥ 2.0. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), ten substances tested in either three or 10 laboratories had consistent results leading to 100% (3/3 or 10/10) interlaboratory concordance for those substances. There were two discordant substances (i.e., 3-aminophenol and nickel [II] sulfate hexahydrate) for which interlaboratory concordance was 67% (2/3). Two of the three laboratories that tested 3-aminophenol reported SI ≥ 2.0, at least at the highest dose tested (i.e., SI = 2.83 and 2.38, respectively) but one lab did not achieve SI ≥ 2.0 at any dose tested (**Appendix D**). One of the three laboratories that tested nickel (II) sulfate hexahydrate reported a maximum SI = 1.52, while the other two laboratories produced SI ≥ 2.0 at all three doses tested (**Appendix D**). Since 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: DA concordance data from the first phase of the interlaboratory validation study.

Table F-9 Qualitative Results for the First Phase of the Interlaboratory Validation Studies for the LLNA: DA (SI ≥ 2.0)

Substance[1]	Laboratory[2]										Concordance
	1	2	3	4	5	6	7	8	9	10	
2,4-Dinitrochlorobenzene (+)	+	+	+	+	+	+	+	+	+	+	10/10
Hexyl cinnamic aldehyde (+)	+	+	+	+	+	+	+	+	+	+	10/10
Isopropanol (-)	-	-	-	-	-	-	-	-	-	-	10/10
Abietic acid (+)		+				+	+				3/3
3-Aminophenol (+)	+		-					+			**2/3**
Dimethyl isophthalate (-)	-		-				-				3/3
Isoeugenol (+)				+	+				+		3/3
Methyl salicylate (-)			-				-			-	3/3
Formaldehyde (+)	+	+			+						3/3
Glutaraldehyde (+)	+	+			+						3/3
Cobalt chloride[3] (+)				+[4]		+		+			3/3
Nickel (II) sulfate hexahydrate (+)				-[5]		+		+[5]			**2/3**

Bolded substances did not achieve 100% interlaboratory concordance.
Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.
[1](+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.
[2](+) indicates sensitizer result and (-) indicates nonsensitizer result in the LLNA: DA test.
[3]Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.
[4]Data not reported for the highest dose (i.e., 3%), only for 0.3% and 1%.
[5]Insufficient dose response.

The qualitative (positive/negative) interlaboratory concordance analysis for the five substances that were tested during the second phase of the LLNA: DA interlaboratory validation study is shown in **Table F-10**. In a qualitative comparison of LLNA: DA calls (i.e., sensitizer/nonsensitizer), four substances (i.e., hexyl cinnamic aldehyde, cobalt chloride, lactic acid, and potassium dichromate) tested in either four or seven laboratories had consistent results leading to 100% (4/4 or 7/7) interlaboratory concordance for those substances. There was one discordant substance (i.e., nickel [II] sulfate hexahydrate) for which interlaboratory concordance was 75% (3/4). Three of the four laboratories that tested nickel (II) sulfate hexahydrate did not report a maximum SI ≥ 2.0, while the other laboratory produced an SI ≥ 2.0 at the highest dose tested. As was discussed previously, nickel (II)

sulfate hexahydrate was also discordant among the laboratories that tested the substance in the first phase of the interlaboratory validation study and interlaboratory concordance was 67% (2/3). Notably, when analyzing the dose response curves for the seven tests performed for nickel (II) sulfate hexahydrate in the two-phased interlaboratory validation study, only one study demonstrated a sufficient dose response (i.e., a parallel increase in SI relative to increase in concentration). Furthermore, as mentioned previously, the evaluation of interlaboratory reproducibility for the traditional LLNA did not include an evaluation of qualitative results (ICCVAM 1999), and therefore there were no traditional LLNA concordance data for comparison with the LLNA: DA concordance data from the second phase of the interlaboratory validation study.

Table F-10 Qualitative Results for the Second Phase of the Interlaboratory Validation Study for the LLNA: DA (SI ≥ 2.0)

Substance[1]	Laboratory[2]							Concordance
	11	12	13	14	15	16	17	
Hexyl cinnamic aldehyde (+)	+	+	+	+	+	+	+	7/7
Cobalt chloride[3] (+)	+		+	+			+	4/4
Lactic acid (-)	-		-		-	-		4/4
Nickel (II) sulfate hexahydrate (+)	**-**	**-**		**+**		**-**		**3/4**
Potassium dichromate (+)	+	+			+		+	4/4

Bolded substance did not achieve 100% interlaboratory concordance.
Abbreviations: LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; SI = stimulation index.
[1](+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.
[2](+) indicates sensitizer result and (-) indicates nonsensitizer result in the LLNA: DA test.
[3]Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) interlaboratory validation studies.

1.2.4 Interlaboratory Reproducibility – EC2 Values

The available quantitative (i.e., EC2 value) data for interlaboratory reproducibility analysis were obtained from the LLNA: DA results for the ten sensitizers that were tested during the first and second phase of the LLNA: DA interlaboratory validation study. The equation used for calculating EC2 values for the positive results was modified based on the method of linear interpolation reported by Gerberick et al. (2004) for the EC3:

339 $$EC2 = c + \left[\frac{(2-d)}{(b-d)}\right] \times (a-c)$$

340 where the data points lying immediately above and below the SI = 2.0 on the dose response
341 curve have the coordinates of (a, b) and (c, d), respectively (Gerberick et al. 2004). For
342 substances for which the lowest concentration tested resulted in an SI ≥ 2.0, an EC2 value
343 was extrapolated according to the equation:

344 $$EC2_{ex} = 2^{\left\{\log_2(c) + \frac{(2-d)}{(b-d)} \times \left[\log_2(a) - \log_2(c)\right]\right\}}$$

345 where the point with the higher SI is denoted with the coordinates of (a, b) and the point with
346 the lower SI is denoted (c, d) (Gerberick et al. 2004).

347 The EC2 values from each laboratory were used to calculate CV values for each substance.
348 The resulting values for the first and second phase of the interlaboratory validation study are
349 shown in **Tables F-11** and **F-12**, respectively. In the first phase of the interlaboratory
350 validation study, CV values ranged from 14% (i.e., abietic acid) to 134% (isoeugenol) and
351 the mean CV was 70% (**Table F-11**). In the second phase of the interlaboratory validation
352 study, CV values ranged from 16% (i.e., hexyl cinnamic aldehyde) to 100% (i.e., cobalt
353 chloride) and the mean CV was 57% (**Table F-12**).

354 The recommended LLNA performance standards indicate that interlaboratory reproducibility
355 should be evaluated with at least two sensitizing chemicals with well-characterized activity in
356 the traditional LLNA (ICCVAM 2009). Acceptable reproducibility is attained when each
357 laboratory obtains ECt (i.e., estimated concentration needed to produce a stimulation index
358 threshold) values within 0.025% to 0.1% for 2,4-dinitrochlorobenzene and within 5% to 20%
359 for hexyl cinnamic aldehyde (ICCVAM 2009). In the first phase of the interlaboratory
360 validation study, seven laboratories reported EC2 values outside the range indicated for 2,4-
361 dinitrochlorobenzene; all seven laboratories obtained EC2 values that were lower than the
362 specified acceptance range (i.e., 0.025%) (**Table F-11**). For hexyl cinnamic aldehyde, all the
363 laboratories obtained an EC2 value within the acceptance range (5% to 20%). In the second
364 phase of the interlaboratory validation study, only hexyl cinnamic aldehyde was tested and
365 two of the seven laboratories obtained EC2 values that were below the acceptance range
366 indicated (**Table F-12**).

Table F-11 EC2 Values from the First Phase Interlaboratory Validation Study for the LLNA: DA

Substance[1]	Laboratory										Mean EC2 (%)	CV (%)
	1	2	3	4	5	6	7	8	9	10		
2,4-Dinitrochlorobenzene (+)	**0.020 (11.97)**	**0.023 (9.23)**	**0.026 (9.96)**	**0.016 (8.53)**	**0.091 (7.86)**	**0.016 (15.14)**	**0.007 (13.18)**	**0.013 (12.60)**	**0.019 (10.89)**	**0.093 (4.71)**	**0.032**	**98**
Hexyl cinnamic aldehyde (+)	**6.962 (5.78)**	**7.461 (4.82)**	**8.404 (4.44)**	**6.460 (5.11)**	**11.057 (3.97)**	**7.463 (5.50)**	**5.850 (7.09)**	**6.140 (10.22)**	**9.191 (3.88)**	**7.256 (3.51)**	**7.624**	**21**
Isopropanol (-)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Abietic acid (+)		4.760				5.393	6.333				5.495	14
3-Aminophenol (+)	1.877		NA					3.179			2.528	36
Dimethyl isophthalate (-)	NA		NA				NA				NA	NA
Isoeugenol (+)				0.407	4.399				0.375		1.727	134
Methyl salicylate (-)			NA				NA			NA	NA	NA
Formaldehyde (+)	0.262	0.729			2.019						1.003	91
Glutaraldehyde (+)	0.072	0.268			0.118						0.153	67
Cobalt chloride[2] (+)				0.283[3]		0.032		0.079			0.131	102
Nickel (II) sulfate hexahydrate (+)				NA[4]		0.235		IDR			0.235	NA

Bolded text indicates substances that are recommended LLNA performance standards reference substances (ICCVAM 2009). Values in parentheses are highest SI values achieved. For both 2,4-dinitrochlorobenzene and hexyl cinnamic aldehyde, the highest SI values achieved were from the highest dose tested (i.e., 0.30% for 2,4-dinitrochlorobenzene and 25% for hexyl cinnamic aldehyde). Shading shows EC2 values that are outside of the acceptable range indicated by the recommended LLNA performance standards: 5 - 20% for hexyl cinnamic aldehyde and 0.025 - 0.1% for 2,4-dinitrochlorobenzene.

Abbreviations: CV = coefficient of variation; EC2 = estimated concentration needed to produce a stimulation index of two; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; IDR = insufficient dose response; NA = not available.

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

[2]Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) interlaboratory validation studies.

[3]Data not reported for the highest dose (i.e., 3%), only for 0.3% and 1%.

[4]Insufficient dose response.

Table F-12 EC2 Values from the Second Phase of the Interlaboratory Validation Study for the LLNA: DA

Substance[1]	Laboratory							Mean EC2 (%)	CV (%)
	11	12	13	14	15	16	17		
Hexyl cinnamic aldehyde (+)	**6.348 (4.47)**	**5.983 (5.71)**	**5.954 (5.41)**	**4.849 (7.60)**	**7.451 (3.92)**	**4.662 (8.42)**	**6.024 (6.45)**	**5.896**	**16**
Cobalt chloride[2] (+)	4.929		1.875	0.821			0.461	2.021	100
Lactic acid (-)	NA		NA		NA	NA		NA	NA
Nickel (II) sulfate hexahydrate (+)	NA	NA		NA		8.404		8.404	
Potassium dichromate (+)	0.159	0.128			0.055		0.047	0.097	56

Bolded text indicates substances that are recommended LLNA performance standards reference substances. Values in parentheses are highest SI values achieved. For hexyl cinnamic aldehyde, the highest SI values achieved were from the highest dose tested (i.e., 25%). Two of the EC2 values are outside of the acceptable range indicated by the recommended LLNA performance standards (i.e., 5 - 20% for hexyl cinnamic aldehyde), indicated by shading.
Abbreviations: CV = coefficient of variation; EC2 = estimated concentration needed to produce a stimulation index of two; LLNA: DA = murine local lymph node assay modified by Daicel Chemical Industries, Ltd., based on ATP content; NA = not available.
[1](+) indicates sensitizers and (-) indicates nonsensitizers according to traditional LLNA tests.
[2]Different doses tested for cobalt chloride in the first phase (0.3%, 1%, and 3%) and in the second phase (1%, 3%, and 10%) of the interlaboratory validation study.

The interlaboratory CV values for both the first and second phases of the interlaboratory validation study for the LLNA: DA 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 6.8 to 83.7% 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: DA (i.e., hexyl cinnamic aldehyde, 2,4-dinitrochlorobenzene, and isoeugenol). All interlaboratory CV values for LLNA: DA EC2 were greater than that for the traditional LLNA. The CV of 98% for 2,4-dinitrochlorobenzene was greater than the two CV values of 37.4% and 27.2% (which were calculated from five values each), reported by ICCVAM (1999). The CV of 21% and 16% for hexyl cinnamic aldehyde tested in the first and second phase of the LLNA: DA interlaboratory validation study, respectively, were both greater than the 6.8% reported by ICCVAM (1999). The CV of 134% for isoeugenol tested in the LLNA: DA was greater than the 41.2% reported by ICCVAM (1999).