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

Draft Background Review Document

January 2008

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List of Abbreviations and Acronyms

ACD	Allergic contact dermatitis
AOO	Acetone: olive oil
ATP	Adenosine triphosphate
BRD	Background Review Document
BT	Buehler Test
CASRN	Chemical Abstracts Service Registry Number
Conc.	Concentration
CPSC	U.S. Consumer Product Safety Commission
C.V.	Coefficient of Variation
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
EC3	Estimated concentration needed to produce a stimulation index of three
ECVAM	European Centre for the Validation of Alternative Methods
EPA	U.S. Environmental Protection Agency
ESAC	ECVAM Scientific Advisory Committee
FDA	U.S. Food and Drug Administration
FHSA	Federal Hazardous Substances Act
FN	False Negative
FP	False Positive
FR	*Federal Register*
GHS	United Nations Globally Harmonized System for the Labeling and Classification of Chemicals
g/mol	Grams per mole
GLP	Good Laboratory Practice
GPMT	Guinea Pig Maximization Test
HCA	Hexyl cinnamic aldehyde
HMT	Human Maximization Test
HPTA	Human Patch Test Allergen
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IWG	Immunotoxicity Working Group
ISO	International Organization for Standardization
JaCVAM	Japanese Center for the Validation of Alternative Methods
K_{ow}	Octanol-water partition coefficient
LLNA	Local Lymph Node Assay
LLNA: DA	LLNA Modified by Daicel Chemical Industries, Ltd. Based on ATP Content
MEK	Methyl ethyl ketone
MeSH	Medical Subject Headings
Min	Minimal
Mod	Moderate
Mol.	Molecular
NA	Not applicable
NC	Not calculated

NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences
nonstd	Nonstandard
NP	Not provided
NS	Non-sensitizer
NT	Not tested
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
OPPTS	Office of Prevention, Pesticides and Toxic Substances
PBS	Phosphate buffered saline
Res	Result
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
S.D.	Standard Deviation
SI	Stimulation Index
SLS	Sodium lauryl sulfate
TG	Test Guideline
Trad.	Traditional
U.K.	United Kingdom
U.N.	United Nations
U.S.	United States
Unk	Unknown
Veh.	Vehicle
vs.	Versus
w/v	Weight to volume ratio

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Acknowledgements

The following individuals are acknowledged for their contributions to the LLNA: DA test method review process

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Other Acknowledgements

ICCVAM and NICEATM gratefully acknowledge Kenji Idehara, Ph.D. of Daicel Chemical Industries, Ltd. (Tokyo, Japan) and Takashi Omori, Ph.D. of Kyoto University School of Public Health (Kyoto, Japan) for submitting data to NICEATM used for the evaluation of the LLNA: DA.

Preface

In 1998, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) in conjunction with the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) evaluated the validation status of the murine local lymph node assay (LLNA) as an alternative to guinea pig test methods for assessing the allergic contact dermatitis (ACD) potential of substances. As described in the 1999 ICCVAM evaluation report[2], ICCVAM recommended that the LLNA could be used as a valid substitute for the accepted guinea pig test methods, in most ACD testing situations.

Based on the ICCVAM recommendations, the ICCVAM member agencies that require the regulatory submission of ACD data accepted the LLNA, with identified limitations, as an alternative to guinea pig tests for assessing ACD. In 2002, the LLNA was adopted as Test Guideline 429 by the 30-member countries of the Organisation for Economic Co-operation and Development (OECD)[3].

On January 10, 2007, the U.S. Consumer Product Safety Commission (CPSC) formally nominated several activities related to the LLNA for evaluation by ICCVAM and NICEATM[4]. One of the nominated activities was an assessment of the validation status of non-radioactive alternatives to the current version of the LLNA, which uses radioactivity. After considering comments from the public and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) on this nomination, ICCVAM assigned it a high priority, and directed NICEATM and the ICCVAM Immunotoxicity Working Group (IWG) to conduct a review of the current literature and an evaluation of the available data. The information described in this background review document (BRD) was compiled by ICCVAM in response to this nomination. ICCVAM and its IWG developed draft test method recommendations based on this evaluation. An independent peer review panel (Panel) is being convened to peer review the BRD and to evaluate the extent to which the information contained in the BRD support the draft

[2] ICCVAM 1999. The murine local lymph node assay: A test method for assessing the allergic contact dermatitis potential of chemical/compounds. NIH Publication No. 99-4494. Research Triangle Park, NC: National Toxicology Program (available at http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf

[3] OECD. 2002. Test guideline 429. Skin Sensitisation: Local Lymph Node Assay, adopted April 24, 2002. In: OECD Guidelines for Testing of Chemicals. Paris:OECD (available at http://www.oecd.org/document/55/0,2340,en_2649_34377_2349687_1_1_1_1,00.html)

[4] Available at http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf

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

We gratefully acknowledge the organizations and scientists who provided data and information for this document. We would also like to recognize the efforts of the individuals who contributed to the preparation of this BRD. These include David Allen, Ph.D., Thomas Burns, M.S., Neepa Choksi, Ph.D., Michael Paris, Eleni Salicru, Ph.D., Catherine Sprankle, Judy Strickland, Ph.D., and Douglas Winters, M.S., of Integrated Laboratory Systems, Inc., the NICEATM Support Contractor, as well as the members of the ICCVAM IWG and the ICCVAM representatives who subsequently reviewed and provided comments throughout the process leading to this final draft version. We also want to thank Raymond Tice, Ph.D., Deputy Director of NICEATM, for his contributions to this project. Finally, we want to recognize the excellent leadership of the IWG Co-chairs, Abigail Jacobs, Ph.D. (FDA) and Joanna Matheson, Ph.D. (CPSC).

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January 7, 2008

Executive Summary

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

On January 10, 2007, the U.S. Consumer Product Safety Commission (CPSC) formally nominated several activities related to the LLNA for evaluation by ICCVAM and the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) (Available at http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf). One of the nominated activities was an assessment of the validation status of non-radioactive alternatives to the current version of the LLNA ([ICCVAM 1999, Dean et al. 2001] referred to hereafter as the "traditional LLNA"), which uses radioactivity to detect sensitizers. The information described in this background review document (BRD) was compiled by ICCVAM and NICEATM in response to this nomination. The BRD provides a comprehensive review of available data and information regarding the usefulness and limitations of one of these methods, the LLNA based on adenosine triphosphate (ATP) content in the draining auricular lymph nodes (referred to hereafter as the "LLNA: DA").

The LLNA: DA was developed by Daicel Chemical Industries, Ltd. (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 (i.e., the ratio of the mean ATP content of the substance treatment group to the mean ATP content of the vehicle treatment group) equal to or greater than three is proposed as the decision criteria for identifying a substance as a sensitizer.

The accuracy and reliability of the LLNA: DA was assessed using data presented by Dr. Kenji Idehara, on behalf of Daicel Chemical Industries, Ltd., at the 6th World Congress on Alternative and Animal Use in Life Sciences (2007) and at the ECVAM Workshop on Alternative Endpoints for the LLNA (2007) and by Takashi Omori at the ECVAM Workshop on Alternative Endpoints for the LLNA (2007). These data included reports from a validation study that tested the performance of the LLNA: DA using 31 substances. The reference test data for these substances were obtained from the traditional LLNA, guinea pig (GP) skin sensitization tests, and/or human skin sensitization tests. 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. Furthermore, reference LLNA experiments with toluene 2,4-diisocyanate were not done in accordance with the traditional LLNA test method protocol described in the ICCVAM 1999 report and by Deat et al. 2001. Of the remaining 29 substances, 19 were classified by the traditional LLNA as skin sensitizers and 10 were classified as non-sensitizers. When the performance of the LLNA: DA, based on using an SI $\geq$ 3.0 to identify sensitizers, was compared to the traditional LLNA, accuracy was 93% (27/29), sensitivity was 95% (18/19), specificity was 90% (9/10), the false positive rate was 10% (1/10), and the false negative rate was 5% (1/19).

The two discordant substances in the LLNA: DA compared to the traditional LLNA were benzalkonium chloride and 2-mercaptobenzothiazole. Benzalkonium chloride was identified as a sensitizer by the LLNA: DA while the traditional LLNA classified this substance as a non-sensitizer. In contrast, 2-mercaptobenzothiazole was identified as a non-sensitizer by the LLNA:

DA while the traditional LLNA classified this substance as a sensitizer. Both of these substances exist as solids in their physical form and have similar molecular weights (about 170 g/mol). In addition, 2-mercaptobenzothaizole has high peptide reactivity but that for benzalkonium chloride was not identified for comparison. Notably, benzalkonium chloride is very soluble in water whereas 2-mercaptobenzothizole is not.

NICEATM also evaluated the effect of using decision criteria other than SI ≥ 3 to determine skin sensitization potential on test performance characteristics with the traditional LLNA (SI ≥ 3) serving as the reference test. The decision criteria analyzed included SI values ≥ 2.5, 2, and 1.5. When the SI cutoff was ≥ 2 or ≥ 1.5 the sensitivity of the LLNA: DA compared to the traditional LLNA was increased but accuracy and specificity were compromised. Furthermore, although the false negative rate was reduced completely, the false positive rate was increased to at least 40% compared to the traditional LLNA. Furthermore, although 2-mercaptobenzothiazole went from being a false negative in the LLNA: DA to being accurately predicted compared to the traditional LLNA, other substances that had been correctly predicted compared to the traditional LLNA were now predicted to be false positives in the LLNA: DA (nickel sulfate, chlorobenzene, hexane, and 1-bromobutane).

The LLNA: DA studies included 13 of the 18 minimum reference substances proposed by ICCVAM for inclusion in the draft LLNA Performance Standards. The LLNA: DA, using an SI of ≥ 3.0 to identify sensitizers, predicted the same result for 12 of the 13 ICCVAM minimum reference substances, an accuracy of 92% (12/13). When compared to the traditional LLNA, the sensitivity was 89% (8/9), and the specificity was 100% (4/4), with a false positive rate of 0% (0/4), and a false negative rate of 11% (1/9). The one false negative, 2-mercaptobenzothiazole, was tested in a 4:1 acetone: olive oil vehicle in the traditional LLNA but in dimethylformamide (DMF) in the LLNA: DA.

In addition, the LLNA: DA studies included analysis for two of the four optional reference substances proposed by ICCVAM for inclusion in the draft LLNA Performance Standards (nickel sulfate and SLS). When compared to the traditional LLNA, the LLNA: DA predicted the same sensitization for both optional substances tested. Thus, similar to the traditional LLNA, nickel sulfate was a false negative and SLS was a false positive in the LLNA: DA. While SLS

was tested in DMF in both the traditional LLNA and the LLNA: DA, nickel sulfate was tested in DMF in the traditional LLNA and in DMSO in the LLNA: DA.

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 coefficient of variation for the reproducibility of isoeugenol and eugenol was 21% and 11%, respectively.

Two multilaboratory validation studies evaluated the interlaboratory reproducibility of the LLNA: DA. In the first study, ten facilities each blindly tested 12 substances while in the second study seven facilities (different from the ten facilities in the first multilaboratory validation study) each blindly tested five substances. Hexyl cinnamic aldehyde and two metallic salts (nickel sulfate and cobalt chloride) were also tested in the first multilaboratory validation study while lactic acid and potassium dichromate were newly tested substances. Each substance was tested once in each laboratory at three different doses. In the first round, eight of the 12 substances were classified similarly in all 10 laboratories and in the second round four of the five substances were classified similarly in all 5 of the laboratories. Between the 17 different facilities, 14 different substances were examined and two of those (3-aminophenol and dimethyl isophthalate) had not been previously assessed in the LLNA: DA.

Requests for data (i.e. SI values and EC3s) were made to the study groups, but have not been made available. Thus, the conclusions made on these interlaboratory validation studies were made by the study groups and are taken from two posters presented at the 6th World Congress on Alternatives and Animal Use in the Life Sciences (Kanazawa et al. 2007, Omori et al. 2007) and a presentation given by Dr. Takashi Omori at the ECVAM workshop on Alternative Endpoints for the Local Lymph Node Assay (2007). Combining the data from both interlaboratory validation studies, “consistent results” and “small variation” in SI between laboratories were reported for 10 substances (i.e., hexyl cinnamic aldehyde, 2,4-dinitrochlorobenzene, isopropanol, 3-aminophenol, isoeugenol, dimethyl isophthalate, abietic acid, methyl salicylate, lactic acid and potassium dichromate). In contrast, “inconsistent results” were observed among laboratories for glutaraldehyde and formaldehyde although the variations in SI were “not large” thus leading to “inconclusive results”. Furthermore, both “inconsistent results” and “large interlaboratory variations” in SI values were initially observed for two metallic salts (i.e., cobalt chloride and

nickel sulfate) dissolved in dimethyl sulfoxide although further analysis of cobalt chloride revealed "inconsistencies" between laboratories but "small variations in SI". From these results, the authors concluded that there was "sufficient relevance when compared to the traditional LLNA" and "acceptable interlaboratory reproducibility was obtained for all substances based on small variation

Original data from these studies have yet to be obtained by NICEATM, but they have been requested. For this reason, a formal audit of data cannot be made at this time. However, studies performed at Daicel Chemical Industries, Ltd. during the development of the LLNA: DA were reportedly done according to the guidelines of the Japanese Association for Laboratory Animal Science (Yamashita et al. 2005). The original assessment of 31 substances at Daicel Chemical Industries, Ltd. as well as the two interlaboratory validation studies, were not conducted in compliance with Good Laboratory Practice (GLP) guidelines although all of the participating laboratories conduct GLP compliant studies. In addition, while data were not subjected to a formal audit, 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).

These experiments for the LLNA: DA were done using four animals per test group, compared to the traditional LLNA which requires five. Furthermore, the traditional LLNA uses radioactive materials and as such its use might be restricted, broader use of the non-radioactive LLNA: DA protocol in place of the GP test could further reduce the overall number of animals used to assess skin sensitization, and avoid the potential pain and distress that can occur in the GP tests.

The transferability of the LLNA: DA is expected to be similar to the traditional LLNA. Compared to the traditional LLNA, the LLNA: DA will not require facilities, 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 has developed draft recommendations for the LLNA: DA with regard to its usefulness and limitations, test method protocol, and future studies to further characterize its usefulness and limitations. These are provided in a separate document, *Draft ICCVAM Test Method Recommendations, Non-Radioactive Murine Local Lymph Node Assay: Modified by Daicel Chemical Industries, Ltd. Based on ATP Content Test Method Protocol.*

1.0 Introduction

1.1 Historical Background

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended that the murine local lymph node assay (LLNA) is a valid substitute for currently accepted guinea pig test methods to assess the allergic contact dermatitis (ACD) potential of many, but not all, types of substances. The recommendation was based on a comprehensive evaluation that included an independent scientific peer review panel (Panel) assessment of the validation status of the LLNA. The Panel report and the ICCVAM recommendations (ICCVAM 1999) are available at the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)/ICCVAM website (http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf).

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

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

1.2 The LLNA: DA

The LLNA: DA was developed by Daicel Chemical Industries, Ltd. (2005) as a non-radioactive alternative to the current version of the local lymph node assay (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 LLNA: DA protocol differs from the ICCVAM-recommended protocol for the traditional LLNA (see http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/LLNAProt.pdf) in the method used to assess lymphocyte proliferation in the auricular lymph nodes, as stated above. In addition, there are major differences between the two protocols that relate to test substance application and timing for the collection of the lymph nodes (**Table 2-1** and **Appendix A**). In the traditional LLNA, the test substance is administered on three consecutive days (days 1, 2, and 3). On day 6, tritiated thymidine or iodine-125 is administered via the tail vein and the lymph nodes are excised five hours later. A lymph node cell suspension is then prepared and tritiated thymidine or iodine-125 incorporation is determined by β-scintillation or γ-scintillation counting. In the LLNA: DA, the test substance is applied on days 1, 2, 3, and 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 of the test substance, this protocol was decided upon. Furthermore, one hour prior to each application of the test substance, 1% sodium lauryl sulfate (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 1% SDS 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). Lastly, twenty-four to 30 hours after the last test substance application, the auricular lymph nodes are excised and a lymph node cell suspension is prepared, and the ATP content is measured by luciferin-luciferase assay.

Table 2-1 Comparison of the LLNA and Traditional LLNA Experimental 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	_____	_____	• 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
Trad. LLNA	• Apply 25 µL of test substance or vehicle to dorsum of each ear	_____	• Administer ^{3}H-thymidine or ^{125}I via tail vein • Excision of auricular lymph nodes • Measurement of radioactivity incorporated into lymph node cells	_____	_____

Abbreviations: ATP=Adenosine triphosphate; ^{3}H=Tritiated; ^{125}I=Iodine-125; LLNA=Local Lymph Node Assay; LLNA: DA=Local Lymph Node Assay Modified by Daicel Chemical Industries, Ltd. Based on ATP Content; SLS=Sodium lauryl sulfate; Trad.=Traditional

2.1. Decision Criteria

Similar to the traditional LLNA, an SI is used in the LLNA: DA to distinguish skin sensitizers from non-sensitizers. The formula for calculating the SI in the LLNA: DA is the ratio of the

mean ATP content of the auricular lymph nodes collected from the test substance treatment group to the mean ATP content of the auricular lymph nodes collected from the vehicle treatment group (measured in relative light units; RLU)

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

An SI ≥ 3 is used as the threshold for labeling a substance as a sensitizer, which is the same threshold used in the traditional LLNA.

The confidence intervals (CIs) for the SI values were calculated using the following formula:

$$\exp\left(\ln(SI) \pm 1.96\sqrt{(Var(\ln SI))}\right) \text{ where, } Var(\ln SI) \cong \frac{SE(Y)^2}{Mean(Y)^2} + \frac{SE(X)^2}{Mean(X)^2}$$

When the lower limit of the CI was greater than 1, the result was interpreted as significant.

3.0 LLNA: DA Validation Database

To evaluate the usefulness and limitations of the LLNA: DA, Daicel Chemical Industries, Ltd. tested a total of 31 substances in one laboratory (Daicel Chemical Industries, Ltd. 2007) (**Appendix B**). All of these substances were previously tested in the traditional LLNA and data for 27 out of the 31 substances were considered in the original ICCVAM evaluation (ICCVAM 1999). Diethyl phthalate, glutaraldehyde, toluene 2,4-diisocyanate, and trimellitic anhydride were the four substances tested in the LLNA: DA not evaluated in the ICCVAM 1999 report. Of the substances selected, 20 were classified by the traditional LLNA as skin sensitizers[5] and 10 were classified as non-sensitizers (**Table 3-1**). For the sensitizers, the range of traditional LLNA EC3 values was from 0.049% to 24% (**Table 3-1**). One substance (benzocaine) was classified as equivocal in the traditional LLNA (ICCVAM 1999) due to highly variable results and therefore was not included in the performance analyses[6]. In addition, traditional LLNA data for toluene 2,4-diisocyanate, not evaluated in the original ICCVAM 1999 report, was obtained from van Och et al. (2000). The LLNA protocol followed for this study was a modified version not performed

[5] Resorcinol was classified as a non-sensitizer 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.

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

in accordance with OECD TG 429 (OECD 2002) or ICCVAM 1999 and Dean et al. 2001. One variation was that the BALB/c strain of mouse was used for the experiments, and not the CBA/Ca or CBA/J strains as specified by ICCVAM (1999), Dean et al. (2001) or OECD TG 429 (2002). In addition, the ears of the mice were pretreated with 1% SDS before treatment with the test solution. The authors also stated that the auricular lymph nodes were excised and pooled for each animal.

Furthermore, two of the 31 substances (isoeugenol and eugenol) evaluated by Daicel Chemical Industries, Ltd. were tested in the LLNA: DA at varying concentrations in three different experiments in order to assess intralaboratory reproducibility. In addition, two interlaboratory validation studies evaluated the reliability and relevance of the LLNA: DA. In the first round, 10 facilities blindly tested 12 substances (**Table 3-2**) and in the second round, seven different facilities blindly tested five substances (**Table 3-3**). Between the 17 facilities, 14 different substances were examined and two of those were not previously tested among the 31 original substances assessed in the one laboratory.

Appendix B provides information on the physico-chemical properties (e.g., physical form), Chemical Abstracts Service Registry Number (CASRN), and chemical class for each substance tested. When available, chemical classes for each substance were retrieved from the National Library of Medicine's ChemID Plus database. If chemical classes were not located, they were assigned for each test substance using a standard classification scheme, based on the National Library of Medicine Medical Subject Headings (MeSH) classification system (available at http://www.nlm.nih.gov/mesh/meshhome.html). A substance could be assigned to more than one chemical class; however, no substance was assigned to more than three classes. Classification of substances into chemical classes is not intended to indicate the impact of structure on biological activity with respect to sensitization potential. Instead, chemical class information is being presented to provide an indication of the variety of structural elements that are present in the substances that were evaluated in this analysis.

Table 3-1 Traditional LLNA EC3 Values and Chemical Classification of Substances Tested in the LLNA: DA

Substance Name	Chemical Class[1]	Trad. LLNA EC3 (%)[2]	No.[3]
2,4-Dinitrochlorobenzene[a]	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated; Nitro Compounds	0.049	15
Glutaraldehyde[a]	Aldehydes	0.083	3
p-Phenylenediamine	Amines	0.11	6
Potassium dichromate[b]	Inorganic Chemical, Chromium Compounds; Inorganic Chemical, Potassium Compounds	0.11	6
Toluene 2,4-diisocyanate	Hydrocarbons, Cyclic; Isocyanates	0.11	1
Trimellitic anhydride	Anhydride; Carboxylic Acids	0.22	1
Phthalic anhydride	Anhydrides; Carboxylic Acids	0.36	1
Formaldehyde[a]	Aldehydes	0.50	4
Isoeugenol[a]	Carboxylic Acids	1.53	49
Cinnamic aldehyde	Aldehydes	2.38	5
2-Mercaptobenzothiazole	Heterocyclic Compounds	2.5	2
3-Aminophenol[c,]	Amines; Phenols	3.2	1
Cobalt chloride[a, b]	Inorganic Chemical, Elements; Inorganic Chemical, Metals	4.8	1
Resorcinol	Phenols	6.7	1
Sodium lauryl sulfate	Alcohols; Sulfur Compounds; Lipids	8.08	5
Citral	Hydrocarbons, Other	9.8	2
Hexyl cinnamic aldehyde[a, b]	Aldehydes	9.93	22
Eugenol	Carboxylic Acids	10.09	11
Abietic acid[a]	Hydrocarbons, Cyclic; Polycyclic Compounds	11.92	5
Benzocaine[4]	Carboxylic Acids	22	1
Hydroxycitronellal	Hydrocarbons, Other	23.75	6
Imidazolidinyl urea	Urea	24	1
Benzalkonium chloride	Amines; Onium Compounds	NA	NA
1-Bromobutane	Hydrocarbons, Halogenated	NA	NA
Chlorobenzene	Hydrocarbons, Cyclic; Hydrocarbons, Halogenated	NA	NA
Diethyl phthalate	Carboxylic Acids	NA	NA
Dimethyl isophthalate[c]	Carboxylic Acids	NA	NA
Hexane	Hydrocarbons, Acyclic	NA	NA
Isopropanol[a]	Alcohols	NA	NA
Lactic acid[b]	Carboxylic Acids	NA	NA
Methyl salicylate[a]	Carboxylic Acids; Phenols	NA	NA
Nickel (II) sulfate hexahydrate[a, b]	Inorganic Chemical, Elements; Inorganic Chemical, Metals	NA	NA
Propylparaben	Carboxylic Acids; Phenols	NA	NA

Abbreviations: EC3=Estimated concentration needed to produce a stimulation index of three; LLNA=Local Lymph Node Assay; LLNA: DA=Local Lymph Node Assay Modified by Daicel Chemical Industries, Ltd. Based on ATP Content; NA=Not applicable; No.=Number; Trad.=Traditional.

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

[2]Traditional LLNA EC3% for the LLNA: DA test substance vehicle listed in **Appendix C**.

[3]Number of LLNA studies from which the EC3 data were obtained.

[4]EC3 value is reported for benzocaine, but variable and equivocal responses were reported in the ICCVAM 1999 report.

[a]Tested among the 31 substances used to assess the performance of the LLNA: DA (Daicel Chemical Industries, Ltd. 2007) and in the first interlaboratory validation study on the LLNA: DA (Ikarashi et al. 2007).

[b]Tested among the 31 substances used to assess the performance of the LLNA: DA (Daicel Chemical Industries, Ltd. 2007) and in the second interlaboratory validation study on the LLNA: DA (Kanazawa et al. 2007).

[c]Not tested among the 31 substances used to assess the performance of the LLNA: DA (Daicel Chemical Industries, Ltd. 2007) but in the first interlaboratory validation study on the LLNA: DA (Ikarashi et al. 2007).

Table 3-2 Substances and Allocation for the First Interlaboratory Validation Study on the LLNA: DA[1]

Substance	Vehicle	Laboratory									
		1	2	3	4	5	6	7	8	9	10
2,4-Dinitrochlorobenzene	AOO	o	o	o	o	o	o	o	o	o	o
Hexyl cinnamic aldehyde	AOO	o	o	o	o	o	o	o	o	o	o
3-Aminophenol	AOO	o		o					o		
Glutaraldehyde	ACE	o	o			o					
Cobalt chloride	DMSO				o		o		o		
Isoeugenol	AOO				o	o				o	
Formaldehyde	ACE	o	o			o					
Dimethyl isophthalate	AOO	o		o				o			
Isopropanol	AOO	o	o	o	o	o	o	o	o	o	o
Nickel sulfate	DMSO				o		o		o		
Abietic acid	AOO		o				o	o			
Methyl salicylate	AOO			o				o			o

Abbreviations: ACE=Acetone; AOO=4:1 Acetone: olive oil; DMSO=Dimethyl sulfoxide; LLNA: DA=Local Lymph Node Assay Modified by Daicel Chemical Industries, Ltd. Based on ATP content.
[1]Ikarashi et al. 2007.

Table 3-3 Substances and Allocation for the Second Interlaboratory Validation Study on the LLNA: DA[1]

Substance	Vehicle	Laboratory						
		11	12	13	14	15	16	17
Hexyl cinnamic aldehyde	AOO	o	o	o	o	o	o	o
Cobalt chloride	DMSO	o		o	o			o
Nickel sulfate	DMSO	o	o		o		o	
Lactic acid	DMSO	o		o		o	o	
Potassium dichromate	DMSO	o	o			o		o

Abbreviations: AOO=4:1 Acetone: olive oil; DMSO=Dimethyl sulfoxide; LLNA: DA=Local Lymph Node Assay Modified by Daicel Chemical Industries, Ltd. Based on ATP Content.
[1]Kanazawa et al. 2007.

4.0 Reference Data

The reference data for the traditional LLNA used for the accuracy evaluation described in **Section 6.0** were obtained from Basketter and Scholes (1992), ICCVAM (1999), Gerberick et al. (2005), or Basketter et al. 2007 (**Appendix B**). An independent quality assurance contractor for the National Toxicology Program (NTP) audited the traditional LLNA data provided in ICCVAM (1999). Audit procedures and findings are presented in the quality assurance report on file at the National Institute of Environmental Health Sciences (NIEHS). The audit supports the conclusion that the transcribed test data in the submission were accurate, consistent, and complete as compared to the original study records. Two of the three substances not evaluated in the original ICCVAM 1999 report (diethyl phthalate and gluataraldehyde) were obtained from Gerberick et al. (2005). This report compiled historical LLNA data from numerous laboratories and each of the substances was listed in a table and referenced. The authors state that the data were derived from previous studies that used LLNA methodology as described in OECD Test Guideline (TG) 429 (OECD 2002). A brief summary of the LLNA protocol indicates that the draining auricular lymph nodes were excised and pooled for each experimental group or each individual animal, without specifying which method was used for each substance. In addition, Basketter et al. (2007) reassessed the skin sensitizing potential of resorcinol in the LLNA, in accordance with OECD TG 429 (2002), to update information in ICCVAM 1999 and Gerberick et al. (2005) that had previously stated this substance tested negative in the LLNA. For these experiments, the auricular lymph nodes were drained and pooled within each dose group. Lastly, traditional LLNA data for the remaining substance (trimellitic anhydride) not evaluated in the original ICCVAM 1999 report was obtained from Basketter and Scholes (1992).

5.0 LLNA: DA Test Method Data and Results

Appendix C represents a summary of the LLNA: DA data, which includes the 31 substances originally assessed. In addition, the 14 different substances evaluated in the two independent interlaboratory validation studies are included. Two of the 14 substances (3-aminophenol and dimethyl isophthalate) were not included among the 31 substances originally assessed. Taking these studies together, **Appendix C** contains 33 different substances and there are comparative LLNA: DA and traditional LLNA data listed for all but toluene 2,4-diisocyanate. In addition, there is GP skin sensitization data available for 28 of the 33 substances and human sensitization

data for 29 of the 33 substances examined. According to the presentation given at the 2007 6th World Congress on Alternatives and Animal Use in the Life Sciences from which the data were evaluated (see **Appendix D**), there is no indication of whether the 31 original substances were coded prior to testing (Daicel 2007). In contrast, the two interlaboratory validation studies reportedly used coded substances (Ikarashi et al. 2007; Kanazawa et al. 2007). Original data for these studies have been requested but not yet received.

6.0 LLNA:DA Test Method Accuracy

A critical component of a formal evaluation of the validation status of a test method is an assessment of the accuracy of the proposed 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 Analysis

An accuracy analysis for the LLNA: DA was conducted using data from the validation study conducted by Daicel Chemical Industries, Ltd. and presented at the 6th World Congress on Alternatives and Animal Use in Life Sciences in 2007. In this study, test data were provided for 31 substances, 29 of which had sufficient comparative LLNA: DA and traditional LLNA data to conduct an accuracy analysis. The one substance that yielded an equivocal result in the traditional LLNA (i.e., benzocaine) was excluded from the accuracy analysis (see **Section 3.0**). Furthermore, available LLNA data for toluene 2,4-diisocyanate was not included in the accuracy

analysis because the experiments were not performed in accordance with ICCVAM 1999 and Dean et al. 2001 (see **Section 3.0**). Of the substances analyzed, 25 had available LLNA: DA, traditional LLNA, and GP data while 26 substances had available LLNA: DA, traditional LLNA, and human data. Classification of substances and data available for each substance are provided in **Appendix C**.

6.1.1 Accuracy vs. the Traditional LLNA

Based on the available data, when compared to the traditional LLNA, the LLNA: DA had an accuracy of 93% (27/29), a sensitivity of 95% (18/19), a specificity of 90% (9/10), a false positive rate of 10% (1/10), and a false negative rate of 5% (1/19) (**Table 6-1**).

6.1.2 Accuracy vs. Guinea Pig Data

When the accuracy statistics for the LLNA: DA and the traditional LLNA were compared, when GP results served as the reference data, the LLNA: DA had a lower accuracy rate (80% [20/25] vs. 88% [22/25]), a lower sensitivity rate (88% [15/17] vs. 94% [16/17]) and a lower specificity rate ((62% [5/8] vs. 75% [6/8]) leading to a higher false positive rate (38% [3/8] vs. 25% [2/8]), and a higher false negative rate (12% [2/17] vs. 6% [1/17]) relative to the traditional LLNA (**Table 6-1**).

6.1.3 Accuracy vs. Human Data

When substances with only comparative LLNA: DA data, traditional LLNA data, and human outcomes were evaluated, and human data was the reference point, the LLNA: DA and the traditional LLNA had the same accuracy rate (85% [22/26]), the same sensitivity (86% [18/21]) and the same specificity (80% [4/5]) resulting in the same false positive rate (20% [1/5]) and false negative rate (14% [3/22]) (**Table 6-1**).

602 **Table 6-1 Evaluation of the Performance of the LLNA: DA in Predicting Skin Sensitizing Potential**

Comparison	n[1]	Accuracy		Sensitivity		Specificity		Positive Predictivity		Negative Predictivity		False Positive Rate		False Negative Rate	
		%	No.[2]	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
LLNA: DA vs. LLNA	29	93	27/29	95	18/19	90	9/10	95	18/19	90	9/10	10	1/10	5	1/19
Substances *with LLNA: DA, Traditional LLNA, and GP Data*															
LLNA: DA vs. LLNA	25	92	23/25	94	17/18	86	6/7	94	17/18	86	6/7	14	1/7	6	1/18
LLNA: DA vs. GP[3]	25	80	20/25	88	15/17	62	5/8	83	15/18	71	5/7	38	3/8	12	2/17
LLNA vs. GP	25	88	22/25	94	16/17	75	6/8	89	16/18	86	6/7	25	2/8	6	1/17
Substances *with LLNA: DA, Traditional LLNA, and Human Data*															
LLNA: DA vs. LLNA	26	92	24/26	95	18/19	86	6/7	95	18/19	86	6/7	14	1/7	5	1/19
LLNA: DA vs. Human[4]	26	85	22/26	86	18/21	80	4/5	95	18/19	57	4/7	20	1/5	14	3/21
LLNA vs. Human	26	85	22/26	86	18/21	80	4/5	95	18/19	57	4/7	20	1/5	14	3/21

603 Abbreviations: GP=Guinea Pig Skin Sensitization Outcomes; LLNA=Local Lymph Node Assay; LLNA: DA=Local Lymph Node Assay Modified by Daicel Chemical Industries,
604 Ltd. Based on ATP Content; No.=Number; vs.=Versus.
605 [1]n = Number of substances included in this analysis.
606 [2]The data on which the percentage calculation is based.
607 [3]GP refers to outcomes obtained by studies conducted using either the Guinea Pig Maximization Test or the Buehler Test.
608 [4]Human refers to outcomes obtained by studies conducting using the Human Maximization Test or the inclusion of the test substance in a Human Patch Test Allergen Kit.

6.2 Accuracy Analysis Based on ICCVAM Draft Performance Standards

ICCVAM is currently developing draft performance standards for the traditional LLNA (http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm). These draft test method performance standards are proposed to evaluate the performance of LLNA test methods that incorporate specific modifications to measure lymphocyte proliferation compared to the traditional LLNA. However, the major changes to the traditional LLNA protocol reflected in the LLNA: DA (**Section 2.0**) prevents a direct comparison to the draft ICCVAM performance standards. Thus, in the evaluation of the LLNA: DA results for the draft ICCVAM recommended test substances that follows below is performed to provide a general comparison to a set list of reference substances that represent a diverse substance group.

As shown in **Table 6-2**, 13 of the list of 18 minimum reference substances and two of the four optional substances included in the draft ICCVAM performance standards have been tested in the LLNA: DA. When compared to the traditional LLNA, the LLNA: DA predicted the same sensitization classification for 12 of the 13 proposed ICCVAM minimum reference substances tested. Thus, when compared with the traditional LLNA, the accuracy of the LLNA: DA was 92% (12/13), the sensitivity was 89% (8/9), and the specificity was 100% (4/4), with a false positive rate of 0% (0/4), and a false negative rate of 11% (1/9) (**Table 6-1**). The discordant substance, 2-mercaptobenzothiazole, was classified as a moderate sensitizer (EC3 of 2.5%) based on traditional LLNA results but as a non-sensitizer based on LLNA: DA data. One difference in the testing of this substance was that in the traditional LLNA the vehicle was 4:1 acetone: olive oil (AOO) (Appendix C, Table 1 of Revised Draft ICCVAM LLNA Performance Standards and **Table 6-2**) while in the LLNA: DA, the vehicle used was dimethylformamide (DMF) (**Table 6-2**). This variation might account for the discordance between the assays.

As shown in **Table 6-2**, when compared to the traditional LLNA, the LLNA: DA predicted the same sensitization for both optional substances tested. One discordant optional substance, nickel sulfate, was categorized as a sensitizer based on GP and human data but as a non-sensitizer by the LLNA: DA. Thus, as occurred with the traditional LLNA, nickel sulfate was a false negative in the LLNA: DA. The other discordant optional substance, sodium lauryl sulfate (SLS), was categorized as a nonsensitizer based on GP and human data but as a sensitizer by the LLNA: DA. Thus, similar to the traditional LLNA, SLS was a false positive in the LLNA: DA. While SLS

was tested in the same vehicle (DMF) in both the traditional LLNA and the LLNA: DA, nickel sulfate was tested in DMF in the traditional LLNA and in DMSO in the LLNA: DA.

Table 6-2 Evaluation of the Performance of the LLNA: DA when Compared to the ICCVAM Draft Performance Standards Reference Substances (Sorted by Traditional LLNA EC3 Value)[1]

Name	ICCVAM Draft LLNA Performance Standards[1]					LLNA: DA		
	Res	EC3 (%)[3]	N	0.5x – 2.0x EC3 (%)	Veh.	Veh.	Res	EC3 (%)[5]
5-Chloro-2-methyl-4-isothiazolin-3-one	+	0.009	1	0.0045 – 0.018	DMF	NT	NT	NT
2,4-Dinitrochlorobenzene	+	0.049	15	0.025 – 0.099	AOO	AOO	+	0.05
4-Phenylenediamine	+	0.11	10	0.055 – 0.22	AOO	AOO	+	0.07
4-Methylaminophenol sulfate	+	0.8	1	0.4 – 0.12	DMF	NT	NT	NT
Isoeugenol	+	1.5	49	0.77 – 3.1	AOO	AOO	+	2.35
2-Mercaptobenzothiazole	+	***2.5***	2	***1.25 – 5.0***	***AOO***	***DMF***	-	***NC (SI = 1.00, 2.00, 1.34, 1.07)***
Cobalt chloride	+	4.8	1	2.4 – 9.6	DMSO	DMSO	+	3.27
Citral	+	9.8	2	4.9 – 19.6	AOO	AOO	+	15.63
Hexyl cinnamic aldehyde	+	9.9	22	5.0 – 19.9	AOO	AOO	+	11.62
Eugenol	+	***10.1***	11	***5.05 – 20.2***	***AOO***	***AOO***	+	***4.50***
Phenyl benzoate	+	13.6	3	6.8 – 27.2	AOO	NT	NT	NT
Cinnamic alcohol	+	21	1	10.5 - 42	AOO	NT	NT	NT
Imidazolidinyl urea	+	24	1	12 - 36	DMF	DMF	+	18.77
Chlorobenzene	-	NS	1	NC	AOO	AOO	-	NC
Isopropanol	-	NS	1	NC	AOO	AOO	-	NC
Lactic acid	-	NS	2	NC	DMSO	DMSO	-	NC
Methyl salicylate	-	NS	10	NC	AOO	AOO	-	NC
Salicylic acid	-	NS	1	NC	AOO	NT	NT	NT
Ethylene glycol dimethylacrylate	FP	28 (FP)	1	14 - 56	MEK	NT	NT	NT
Sodium lauryl sulfate	FP	8.1 (FP)	5	4.05 – 16.2	DMF	DMF	+	8.28
Nickel sulfate	FN	NS (FN)	2	NC	DMF	DMSO	-	NC (1.00, 1.36, 2.17, 1.85)
Sulfanilamide	FN	NS (FN)	1	NC	DMF	NT	NT	NT

Bolded italics text highlights discordant LLNA: DA vs. traditional LLNA test results.
Abbreviations: AOO=4:1 Acetone: olive oil; DMF=Dimethylformamide; DMSO=Dimethyl sulfoxide; EC3=Estimated concentration needed to produce a stimulation index of three; FN=False negative; FP=False positive; ICCVAM=Interagency Coordinating Committee on the Validation of Alternative Methods; LLNA=Local Lymph Node Assay; LLNA: DA=Local Lymph Node Assay Modified by Daicel Chemical Industries, Ltd. Based on ATP content; MEK=Methyl ethyl ketone; NA=Not applicable; NC=Not calculated (Stimulation Index < 3); NS=Non-sensitizer; NT=Not tested; Res = Result; SI = Stimulation Index; Veh.=Vehicle.
[1]From ICCVAM Draft Performance Standards for the LLNA. The table lists the 18 minimum reference substances first, sorted from lowest to highest. The four optional reference substances are listed last (available: http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm)

[2]From Daicel Chemical Industries, Ltd..
[3]Based on mean EC3 value.
[4]Number of LLNA studies from which data were obtained.
[5]Based on EC3 values calculated by Daicel Chemical Industries, Ltd. (2007); For substances predicted as non-sensitizers by the LLNA: DA, the mean SI for each dose tested is provided in parenthesis.

Table 6-3 provides the range of substances tested in the LLNA: DA based on the overall database of the 29 substances evaluated in the accuracy analysis of the LLNA: DA versus the traditional LLNA. These substances are compared to the range of substances included on the revised draft ICCVAM LLNA performance standards substances list. The table indicates that although not all of the draft ICCVAM performance standards reference substances have been tested, the range of the substances tested in the LLNA: DA is similar to that included in the draft 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 vs. the Revised Draft ICCVAM Performance Standards Substances List[1]

EC3 range (%)	No. Chems	Solid/ Liquid	Actual EC3 Range (%)[2]	Human Data	Peptide Reactivity (High/Mod/ Min/Unk)[3]
<0.1	**3**	**3/0**	**0.05-<0.1[4]**	**3**	**1/1/0/1**
	2	1/1	0.009-0.05	2	0/1/0/1
≥0.1 to <1	**4**	**3/2[5]**	**0.1-0.58**	**4**	**1/0/1/2**
	2	2/0	0.11-0.8	2	1/0/0/1
≥1 to <10	**8**	**4/4**	**1.16-8.28**	**8**	**1/1/1/5**
	5	2/3	1.6-9.9	5	1/0/1/3
≥10 to <100	**4**	**1/3**	**11.62-18.77**	**4**	**0/1/2/1**
	4	3/1	10.1-24	4	0/1/0/3
Negative	**10**	**4/6**	**NC**	**7**	**1/0/8/1**
	5	2/3	NC	3	0/0/2/3
Overall	**29**	**15/15[5]**	**0.099-18.77**	**26**	**4/3/12/10**
	18	10/8	0.009-24	16	2/2/3/11

Bolded text represents characteristics of the LLNA: DA database, which includes the 31 substances tested in the original validation study on the LLNA: DA.
Abbreviations: Chems=Chemicals; EC3=Estimated concentration needed to produce a stimulation index of three; ICCVAM=Interagency Coordinating Committee on the Validation of Alternative Methods; LLNA=Local Lymph Node Assay; LLNA: DA=Local Lymph Node Assay Modified by Daicel Chemical Industries, Ltd. Based on ATP Content; NC = Not calculated because maximum SI < 3.0; No.=Number; Min=Minimal; Mod=Moderate; SI=Stimulation Index; Unk=Unknown; vs.=Versus.
[1]From Revised Draft ICCVAM Performance Standards for the LLNA (http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm). Includes the 18 "required" substances for testing.
[2] Based on traditional LLNA studies for substances in the LLNA: DA database (bold values) and the draft ICCVAM LLNA performance standards substances.
[3]Data obtained from: Gerberick et al. (2007).

682 [4]For one substance tested in the LLNA: DA, phthalic anhydride, the EC3 was reported as <0.1 by the study group.
683 [5]One substance tested in the LLNA: DA, benzalkonium chloride, is categorized as both a solid and a liquid.

684

6.3 Discordant Results

When analyses were restricted to the 25 substances with unequivocal LLNA: DA, traditional LLNA, and GP data, the LLNA: DA classified two substances differently compared with the traditional LLNA (**Table 6-4**). Benzalkonium chloride was identified as a sensitizer by the LLNA: DA while the traditional LLNA and GP studies classified this substance as a non-sensitizer. In contrast, 2-mercaptobenzothiazole was identified as a non-sensitizer by the LLNA: DA while the traditional LLNA and GP tests classified this substance as a sensitizer. Both of these substances exist as solids in their physical form and have similar molecular weights (about 170 g/mol) (**Appendix B**). In addition, 2-mercaptobenzothaizole has a high peptide reactivity but that for benzalkonium chloride was not identified for comparison (**Appendix B**). Notably, benzalkonium chloride is very soluble in water whereas 2-mercaptobenzothizole is not.

In addition, resorcinol, SLS, and nickel sulfate predicted the same outcome in the LLNA: DA as in the traditional LLNA but were discordant when compared to the GP test results (**Table 6-4**). All three of these substances exist as solids in their physical state, have varying molecular weights (**Appendix B**) and are soluble in water. Resorcinol also has minimal peptide reactivity but that for SLS and nickel sulfate was not identified (**Appendix B**).

Table 6-4 Discordant Results with Respect to Traditional LLNA and Guinea Pig Reference Data

Substance Name	Classification			
	LLNA: DA[1]	Traditional LLNA[2]	Guinea Pig Studies[3]	Human Outcome[4]
Benzalkonium chloride	+	-	-	+
Resorcinol	+	+[5]	-	+
Sodium lauryl sulfate	+	+	-	-
2-Mercaptobenzothiazole	-	+	+	+
Nickel sulfate	-	-	+	+

Abbreviations: LLNA=Local Lymph Node Assay; LLNA: DA=Local Lymph Node Assay Modified by Daicel Chemical Industries, Ltd. Based on ATP Content.

[1]From Daicel Chemical Industries, Ltd. presented at 6th World Congress on Alternatives and Animal Use in the Life Sciences (2007).

[2]From ICCVAM (1999) unless otherwise noted.

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

[4]Basketter et al. 2007.

[5]From ICCVAM (1999) and based on studies using either the Human Maximization Test or the inclusion of the test substance in a Human Patch Test Allergen Kit.

When analyses were restricted to the 26 substances with unequivocal LLNA: DA, traditional LLNA, and human outcomes, the LLNA: DA classified two substances differently compared with the classification of the traditional LLNA. Again, benzalkonium chloride was identified as a sensitizer by the LLNA: DA while the traditional LLNA classified this substance as a non-sensitizer. In contrast, 2-mercaptobenzothiazole was identified as a non-sensitizer by the LLNA: DA while the traditional LLNA classified this substance as a sensitizer. Notable physico-chemical similarities and differences between these two substances are mentioned above.

In addition, SLS, nickel sulfate, and propyl paraben predicted the same outcome in the LLNA: DA as in the traditional LLNA but were discordant when compared to the human test results (**Table 6-5**). All three of these substances exist as solids in their physical state, have diverse molecular weights (**Appendix B**), and are soluble in water. Propyl paraben also has minimal peptide reactivity but that for SLS and nickel sulfate was not identified (**Appendix B**).

Table 6-5 Discordant Results with Respect to Traditional LLNA and Human Reference Data

Substance Name	Classification		
	LLNA: DA[1]	Traditional LLNA[2]	Human Outcomes[3]
Benzalkonium chloride	+	-	+
Sodium lauryl sulfate	+	+	-
2-Mercaptobenzothiazole	-	+	+
Nickel sulfate	-	-	+
Propyl paraben	-	-	+

Abbreviations: LLNA=Local Lymph Node Assay; LLNA: DA=Local Lymph Node Assay Modified by Daicel Chemical Industries, Ltd. Based on ATP Content.
[1]From Daicel Chemical Industries, Ltd. presented at 6th World Congress on Alternatives and Animal Use in the Life Sciences (2007).
[2]From ICCVAM (1999).
[3]From ICCVAM (1999) and based on studies using either the Human Maximization Test or the inclusion of the test substance in a Human Patch Test Allergen Kit.

6.4 Accuracy Analysis Using Alternative Decision Criteria

NICEATM evaluated the effect of using decision criteria other than SI ≥ 3 to determine skin sensitization potential on test performance characteristics with the traditional LLNA (SI ≥ 3) serving as the reference test (**Appendix E**). The decision criteria analyzed included SI values ≥ 2.5, 2, and 1.5. As **Table 6-6** shows, changing the SI cutoff value to 1.5 increased the sensitivity

of the LLNA: DA compared to the traditional LLNA but compromised accuracy and specificity (i.e. the false negative rate was reduced completely (0%) but the false positive rate was increased at least 40% compared to the traditional LLNA).

Table 6-6 Evaluation of the Performance of the LLNA: DA in Predicting Skin Sensitizing Potential Using Alternative Decision Criteria

Comparison	n[1]	Accuracy		Sensitivity		Specificity		Positive Predictivity		Negative Predictivity		False Positive Rate		False Negative Rate	
		%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]
SI ≥ 3	29	93	27/29	95	18/19	90	9/10	95	18/19	90	9/10	10	1/10	5	1/19
SI ≥ 2.5	29	93	27/29	95	18/19	90	9/10	95	18/19	90	9/10	10	1/10	5	1/19
SI ≥ 2	29	86	25/29	100	19/19	60	6/10	83	19/23	100	6/6	40	4/10	0	0/19
SI ≥ 1.5	29	83	24/29	100	19/19	50	5/10	79	19/24	100	5/5	50	5/10	0	0/19

Abbreviations: LLNA: DA=Local Lymph Node Assay Modified by Daicel Chemical Industries, Ltd. Based on ATP Content; No.=Number; SI=Stimulation Index.

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

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

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 method, there are no known intralaboratory repeatability studies, which was also the situation with the traditional LLNA.

Dr. Kenji Idehara of Daicel Chemical Industries, Ltd. presented data at the ECVAM Workshop on Alternative Endpoints for the LLNA (**Appendix D-3**) that showed the 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 study group reported CVs of 22% and 14% for isoeugenol and eugenol, respectively. For both compounds, the study group stated that the variation between experiments was "small" and that the EC3 values obtained by the LLNA: DA were similar to historical values reported by the traditional LLNA for those same compounds.

Table 7-1 Intralaboratory Reproducibility of EC3 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%**
Mean: 2.74% ± 0.58% and 21% 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%**
Mean: 5.06% ± 0.55% and 11% CV			

Abbreviations: AOO=4:1 Acetone: olive oil; CV=Coefficient of variation; EC3=Estimated concentration needed to produce a stimulation index of three; LLNA: DA=Local Lymph Node Assay Modified by Daicel Chemical Industries, Ltd. Based on ATP Content.

[1]From Daicel Chemical Industries, Ltd. presented at 6th World Congress on Alternatives and Animal Use in the Life Sciences (2007); The number per group was not specified.

[2]Mean SI Value ± S.D.

Furthermore, there are data (**Appendices C and D**) from two rounds of interlaboratory validation studies on the LLNA: DA method that were presented as posters at the 6th World Congress on Alternatives and Animal Use in the Life Sciences (Ikarashi et al. 2007, Kanazawa et al. 2007) and as a presentation by Dr. Takashi Omori at the ECVAM Workshop on Alternative Endpoints for the Local Lymph Node Assay (**Appendix D-2**). Since requests for this data have been made to the study group but have not yet been provided, the conclusions made are based on the above-mentioned abstracts and presentation. In the first 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 3-2)**. 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). For the three substances tested in all 10 laboratories, the study group reported that "consistent results" and "small variations" in

SI values were obtained for each of them. In addition, "consistent results" and "small variation" in SI between laboratories were also reported for five additional substances (i.e., 3-aminophenol, isoeugenol, dimethyl isophthalate, abietic acid and methyl salicylate). In contrast, "inconsistent results" were observed among the three laboratories for glutaraldehyde and formaldehyde although the variations in SI were "not large" thus leading to "inconclusive results". Furthermore, both "inconsistent results" and "large" interlaboratory variations in SI values were reported for two metallic salts (i.e., cobalt chloride and nickel sulfate) dissolved in DMSO (**Appendix D-2**). In general, 67% (8/12) of the substances tested were classified the same by all three participating laboratories. Among the substances tested, seven of the substances categorized as sensitizing by the traditional LLNA were also found to be sensitizing by the LLNA: DA. Four of these seven substances (57%) were correctly identified as sensitizing in all participating laboratories tested while the remaining three substances (43%) were not. From these results, the study group concluded that acceptable interlaboratory reproducibility was obtained for 10 of the 12 substances examined while "large variations" were observed for the two metallic salts dissolved in DMSO. Thus, the study group concluded that performance for the LLNA: DA was similar to that of the traditional LLNA.

Based on results from the first interlaboratory validation study, a second interlaboratory validation study was designed to determine the reason for the inconsistency in SI values for the two metals dissolved in DMSO and to evaluate the reliability of the LLNA: DA for metallic salts using DMSO as a vehicle. A blinded test of five substances was conducted in seven laboratories (different from the 10 laboratories that performed the first interlaboratory validation study) **(Table 3-3)**. One substance (i.e. hexyl cinnamic aldehyde) was tested in all seven laboratories. The remaining four substances (i.e., cobalt chloride, nickel sulfate, 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. The results indicate that four of the five substances in the study showed "consistent results" between laboratories and "small SI variations". In contrast, cobalt chloride showed "inconsistent results" among laboratories, but the variations in SI were "not large" (**Appendix D-2**). In general, 80% (4/5) of the substances tested were classified the same by all participating laboratories. Among the substances tested, all three substances categorized as sensitizing by the LLNA: DA were also classified as sensitizing by the traditional LLNA. Two

of the three substances were correctly identified as sensitizing in all the laboratories tested while the remaining one substance (cobalt chloride) was correctly identified by two of the four (50%) participating laboratories. Furthermore, two substances classified as nonsensitizing by the traditional LLNA were also classified as nonsensitizing by the LLNA: DA and participating laboratories that tested it were in agreement). Based on these findings, and that the two metals dissolved in DMSO (i.e. cobalt chloride and nickel sulfate) showed "small variations in SI", the study group concluded that the LLNA: DA was an "acceptable method to assess the sensitization potential of metals". Still, both metals tested yielded variable interlaboratory results in the first validation study and cobalt chloride yielded inconsistent results in the second study (**Appendix D-2**). Furthermore, the study group did not evaluate the reliability of the LLNA: DA for the metallic salts dissolved in a vehicle other than DMSO. Thus, results obtained when DMSO is used as a solvent should be carefully assessed and the applicability of the LLNA: DA for testing metals should be further characterized.

8.0 LLNA: DA Data Quality

All of the studies included in this performance evaluation are based on data obtained from poster or platform presentations. Manuscripts detailing these results are reported to be currently undergoing peer review for publication. For this reason, original data and records from these studies have been requested by NICEATM but have not yet been obtained. As a result, an independent audit could not be conducted to confirm that the reported data is the same as the data originally recorded. However, studies performed at Daicel Chemical Industries, Ltd. during the development of the LLNA: DA were reportedly done according to the guidelines of the Japanese Association for Laboratory Animal Science (Yamashita et al. 2005). The original assessment of 31 substances at Daicel Chemical Industries, Ltd., as well as the two interlaboratory validation studies, did not conduct their studies in compliance with GLP guidelines, although all of the participating laboratories reportedly have this capability. In addition, while data were not subjected to a formal audit, 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).

9.0. Other Scientific Reports and Reviews

Yamashita et al. (2005) describe the development of the LLNA: DA as an alternative non-radioisotope LLNA 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 provides the basis for the expanded study of 31 substances described in **Sections 6.0** and **7.0**. No other scientific reviews of the LLNA: DA have been located.

10.0 Animal Welfare Considerations

The LLNA: DA will require the use of the same number of animals when compared to the traditional LLNA. 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

Four animals per experimental, vehicle, or positive control groups were used in the LLNA: DA test method protocol compared to five per group specified in the validated traditional LLNA protocol (ICCVAM 1999, Dean et al. 2001).

10.3 Reduction considerations

A further reduction of 40% (15 vs. 25) could be achieved by using a limit dose version of the LLNA: 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

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 protocols are experimentally similar. Furthermore, as stated above, results from two interlaboratory validation studies indicated that interlaboratory variability is small.

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

Compared to the traditional LLNA, the LLNA: DA will not require facilities, 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 facilities) 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.

12.0 References

Basketter DA, Scholes EW. 1992. Comparison of the local lymph node assay with the guinea-pig maximization test for the detection of a range of contact allergens. Food Chem Toxicol 30(1):65-9.

Basketter DA, Scholes EW, Wahlkyist H, Montelius J. 1995. An evaluation of the suitability of benzocaine as a positive control skin sensitizer. Contact Dermatitis 33(1):28-32.

Basketter DA, Wright ZM, Warbrick EV, Dearman RJ, Kimber I, Ryan CA, Gerberick GF, White IR. 2001. Human potency predictions for aldehydes using the local lymph node assay. Contact Dermatitis. 45(2):89-94.

Basketter DA, Clapp C, Jefferies D, Safford B, Ryan CA, Gerberick F, Dearman RJ, Kimber I. Contact Dermatitis. 2005. Predictive identification of human skin sensitization thresholds. 53(5):260-7.

Basketter DA, Sanders D, Jowsey IR. 2007. The skin sensitization potential of resorcinol: experience with the local lymph node assay. Contact Dermatitis. 56(4):196-200.

Dean, J., Twerdok, L., Tice, R., Sailstad, D., Hattan, D. and Stokes, W.S., Evaluation of the Murine Local Lymph Node Assay (LLNA) II: Conclusions and Recommendations of an Independent Scientific Peer Review Panel. Regul. Toxicol. Pharmacol. 34(3):258-273, 2001.

De Jong WH, Tentij M, Spiekstra SW, Vandebriel RJ, Van Loveren H. 2002. Determination of the sensitising activity of the rubber contact sensitisers TMTD, ZDMC, MBT and DEA in a modified local lymph node assay and the effect of sodium dodecyl sulfate pretreatment on local lymph node responses. Toxicology. 176(1-2):123-34.

EPA. 2003. Health Effects Test Guideline, OPPTS 870.2600. Skin Sensitization EPA 712–C–03–197. Washington, DC: U.S. Environmental Protection Agency.

Gad SC, Dunn BJ, Dobbs DW, Reilly C, Walsh RD. 1986. Development and validation of an alternative dermal sensitization test: the mouse ear swelling test (MEST). Toxicol Appl Pharmacol. 15;84(1):93-114.

Gerberick GF, Vassallo JD, Foertsch LM, Price BB, Chaney JG, Lepoittevin JP. 2007. Quantification of chemical peptide reactivity for screening contact allergens: a classification tree model approach. Toxicol Sci. 97(2):417-27.

Gerberick GF, Ryan CA, Kern PS, Schlatter H, Dearman RJ, Kimber I, Patlewicz GY, Basketter DA. 2005. Compilation of historical local lymph node data for evaluation of skin sensitization alternative methods. Dermatitis 16(4):157-202.

ICCVAM. 1997. Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods. NIH Publication No.: 97-3981. Research Triangle Park: National Toxicology Program.

ICCVAM. 1999. The murine local lymph node assay: A test method for assessing the allergic contact dermatitis potential of chemical/compounds. NIH Publication No. 99-4494. Research Triangle Park: National Toxicology Program.

ICCVAM. 2001. Protocol: murine local lymph node assay (LLNA); Recommended by ICCVAM Immunotoxicology Working Group based on an Independent Expert Peer Review Panel Evaluation of the LLNA. Research Triangle Park: National Toxicology Program.

ICCVAM. 2003. ICCVAM Guidelines for the Nomination and Submission of New, Revised, and Alternative Test Methods. NIH Publication No: 03-4508. Research Triangle Park:National Toxicology Program.

Ikarashi Y, Omori T, Idehara K, Kojima H, Sozu T, Arima K, Goto H, Hanada T, Inoda T, Kanazawa Y, Kosaka T, Maki E, Morimoto T, Shinoda S, Shinoda N, Takeyoshi M, Tanaka M, Uratani M, Usami M, Yamanaka A, Yoneda T, Yoshimura I, Yuasa A. 2007. First inter-laboratory validation study on LLNA: DA [Abstract]. Sixth World Congress on Alternatives and Animal Use in the Life Sciences. Tokyo, Japan.

ISO. 2002. Biological evaluation of medical devices -- Part 10: Tests for irritation and delayed-type hypersensitivity. Available for purchase at: http://www.iso.org/iso/home.htm.

Jowsey IR, Basketter DA, Westmoreland C, Kimber I. 2006. A future approach to measuring relative skin sensitising potency: A proposal. Journal of Applied Toxicology 26(4):341-350.

Kanazawa Y, Omori T, Idehara K, Kojima H, Sozu T, Arima K, Goto H, Hanada T, Ikarashi Y, Inoda T, Kanazawa Y, Kosaka T, Maki E, Morimoto T, Shinoda S, Shinoda N, Takeyoshi M,

967 Tanaka M, Tanaka M, Uratani M, Usami M, Yamanaka A, Yoneda T, Yoshimura I, Yuasa A.
968 2007. Second inter-laboratory validation study on LLNA: DA [Abstract]. Sixth World Congress
969 on Alternatives and Animal Use in the Life Sciences. Tokyo, Japan.

970 OECD. 2002. Test guideline 429. Skin Sensitisation: Local Lymph Node Assay, adopted April
971 24, 2002. In: OECD Guidelines for Testing of Chemicals. Paris:OECD.

972 Omori T, Idehara K, Kojima H, Sozu T, Arima K, Goto H, Hanada T, Ikarashi Y, Inoda T,
973 Kanazawa Y, Kosaka T, Maki E, Morimoto T, Shinoda S, Shinoda N, Takeyoshi M, Tanaka M,
974 Tanaka M, Uratani M, Usami M, Yamanaka A, Yoneda T, Yoshimura I, Yuasa A. 2007.
975 Validation studies on LLNA: DA: importance of study management [Abstract]. Sixth World
976 Congress on Alternatives and Animal Use in the Life Sciences. Tokyo, Japan.

977 Sailstad DM, Hattan D, Hill RN, Stokes WS. 2001. ICCVAM Evaluation of the Murine Local
978 Lymph Node Assay (LLNA) I: The ICCVAM Review Process. Regulatory Toxicology and
979 Pharmacology 34:249-257.

980 Schneider K, Akkan Z. 2004. Quantitative relationship between the local lymph node assay and
981 human skin sensitization assays. Regul Toxicol Pharmacol. Jun;39(3):245-55.

982 van Och FM, Slob W, de Jong WH, Vandebriel RJ, van Loveren H. 2000. A quantitative
983 method for assessing the sensitizing potency of low molecular weight chemicals using a local
984 lymph node assay: employment of a regression method that includes determination of the
985 uncertainty margins. Toxicology 146(1):49-59.

986 Yamashita K, Idehara K, Fukuda N, Yamagishi G, Kawada N. 2005. Development of a Modified
987 Local Lymph Node Assay using ATP Measurement as an Endpoint. AATX 11:136-144.

988