Revised Draft ICCVAM Murine Local Lymph Node Assay Performance Standards

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)

National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

National Institute of Environmental Health Sciences
National Institutes of Health
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Department of Health and Human Services

January 7, 2008

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Table of Contents

List of Abbreviations and Acronyms

ACD	Allergic contact dermatitis
AOO	Acetone: olive oil (1:4)
BT	Buehler Test
CASRN	Chemical Abstracts Service Registry Number
CV	Coefficient of variation
DMF	DMF = *N,N*-dimethylformamide
DMSO	Dimethyl sulfoxide
DNCB	2,4-dinitrochlorobenzene
EC3	Estimated concentration needed to produce a stimulation index of three
ECt	Concentration required to achieve the defined threshold stimulation index used to distinguish between sensitizers and nonsensitizers
ECVAM	European Centre for the Validation of Alternative Methods
EPA	U.S. Environmental Protection Agency
FCA	Freund's complete adjuvant
GLP	Good Laboratory Practice
GP	Guinea pig
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
JaCVAM	Japanese Center for the Validation of Alternative Methods
Liq.	Liquid
LLNA	Murine Local Lymph Node Assay
MEK	Methyl ethyl ketone
NA	Not applicable
NC	Not calculated
NICEATM	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SI	Stimulation index
Sol.	Solid
TG	Test Guideline
U.S.	United States
Veh.	Vehicle

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ACKNOWLEDGEMENTS

The following individuals are acknowledged for their contributions to the development of performance standards for the LLNA

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Other Acknowledgements

ICCVAM and NICEATM gratefully acknowledge the following individuals and institutions who submitted data to NICEATM used for the development of performance standards for the LLNA.

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Preface

The Murine Local Lymph Node Assay (LLNA) is an alternative test method used for skin sensitization testing that reduces the number of animals needed, reduces the time required for testing, and can substantially reduce the pain and distress associated with testing methods using guinea pigs. The LLNA (referred to herein as the "traditional LLNA") uses a radioactive precursor to DNA to measure cell proliferation in the draining auricular lymph nodes of the mouse. It was the first alternative test method evaluated and recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), and it has been accepted by regulatory agencies as an alternative to guinea pig tests (e.g., the Guinea Pig Maximization Test and the Buehler Test).

At the time of the ICCVAM evaluation (ICCVAM 1999), the concept of performance standards, against which test methods similar to an accepted test method can be compared, had not been developed. In January 2007, the U.S. Consumer Product Safety Commission submitted a nomination[2] to ICCVAM and the National Toxicology Program Interagency Center for the Evaluation of Alternative Methods (NICEATM) that included (among other proposed activities) an evaluation of a number of modifications to the LLNA that may eliminate the need to use radioactive materials as part of the protocol. As described in Organisation for Economic Co-operation and Development (OECD) Test Guideline 429 for the LLNA (OECD 2002), other endpoints for assessment of proliferation may be employed provided there is justification and appropriate scientific support. Accordingly, ICCVAM decided to develop performance standards to allow for a comparison of such modifications to the traditional LLNA.

In May 2007, a *Federal Register* notice[3] was published requesting comments and data relevant to the development of LLNA performance standards. An ICCVAM Immunotoxicity Working Group (IWG), which includes liaisons from the Japanese Center for Validation of Alternative Methods (JaCVAM) and the European Centre for the Validation of Alternative Methods (ECVAM), recommended with a high priority the development of performance standards for the LLNA. ICCVAM and ICCVAM's advisory committee (the Scientific Advisory Committee on Alternative Toxicological Methods [SACATM]) subsequently endorsed development of performance standards for the LLNA as a high priority activity.

The IWG with assistance from NICEATM began developing LLNA performance standards in February 2007. ICCVAM subsequently released draft performance standards to the public for comment on September 12, 2007. NICEATM and ICCVAM also interacted with ECVAM during development and during and after the public comment timeframe. These interactions included discussion of draft ICCVAM and ECVAM Performance Standards at a September 25-27, 2007 ECVAM Workshop on Alternative Endpoints for the Local Lymph Node Assay, and IWG interactions with the ECVAM Liaison.

The draft ICCVAM Performance Standards were presented to the ECVAM Scientific Advisory Committee (ESAC) at their October 30-31, 2007 semi-annual meeting, where the ESAC considered proposed ECVAM performance standards. The ESAC also considered a proposal from ICCVAM for a process aimed at achieving harmonization of the two different sets of performance standards where feasible, considering the differences in legislative mandates for

[2] http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf
[3] http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf

European Union member countries and U.S. Federal Agencies. In the interest of achieving harmonization, ICCVAM requested that the ESAC defer their final decision on the ECVAM proposed performance standards until after the March 4-6, 2008 international independent scientific peer review meeting on the LLNA, since the peer panel would be reviewing the proposed LLNA performance standards at this meeting. The ICCVAM IWG and ECVAM would then have the opportunity to jointly discuss and coordinate changes to their respective performance standards in light of the peer review panel's deliberations and recommendations, thereby increasing the likelihood of achieving greater harmonization. Revised ICCVAM-IWG performance standards would then be provided to ICCVAM for consideration, and revised ECVAM performance standards would then be provided to the ESAC for consideration.

These revised draft ICCVAM LLNA performance standards are being released to the public for comment and to members of an Independent Peer Review Panel for consideration at their public meeting on March 4-6, 2008, at the Consumer Product Safety Commission Headquarters in Bethesda, MD. This version has been modified by the IWG based on public comments received on the September 12 public draft, and in light of discussions among ICCVAM, IWG, ECVAM, ESAC and the ECVAM Task Force on Skin Sensitization. Revisions include changes to the recommended reference chemicals and the procedures for assessing test method accuracy. As a result, these draft ICCVAM LLNA performance standards and the most recent ECVAM LLNA performance standards are more similar than previously released versions. Following the Panel meeting, ECVAM, ICCVAM-IWG, and JaCVAM representatives will jointly consider the Panel's conclusions and recommendations and discuss further revisions to the Performance Standards. The Panel recommendations will also be made available for public and SACATM comment. The Panel report and all comments received will be considered by ICCVAM in preparing final test method performance standards recommendations for U.S. Federal agencies.

The goal of this transparent development and evaluation process is to produce a harmonized set of performance standards for the LLNA that can be used internationally (e.g., by ICCVAM, ECVAM, and JaCVAM) to assess the validity of non-radioactive versions and other proposed improvements to the LLNA. It is anticipated that the development and validation of non-radioactive LLNA methods will lead to broader use of the LLNA, thereby further reducing and refining animal use for allergic contact dermatitis safety assessments.

We gratefully acknowledge the organizations and scientists who provided data and information for this document. We would also like to recognize the efforts of the individuals who contributed to the preparation of this BRD. These include David Allen, Ph.D., Thomas Burns, M.S., Neepa Choksi, Ph.D., Michael Paris, Eleni Salicru, Ph.D., Catherine Sprankle, Judy Strickland, Ph.D., and Doug Winters, M.S., of Integrated Laboratory Systems, Inc., the NICEATM Support Contractor, as well as the members of the ICCVAM IWG and the ICCVAM representatives who subsequently reviewed and provided comments throughout the process leading to this revised 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, Abby Jacobs, Ph.D. (FDA) and Joanna Matheson, Ph.D. (CPSC).

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January 7, 2008

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1.0 PURPOSE AND BACKGROUND OF PERFORMANCE STANDARDS

1.1 Introduction

These test method performance standards[4] are proposed so that murine local lymph node assay (LLNA) protocols that incorporate minor modifications to the "traditional" LLNA (ICCVAM 1999, Dean et al. 2001) can be quickly and efficiently evaluated for their performance by national and international validation organizations (e.g., the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods [ICCVAM], the European Centre for the Validation of Alternative Methods [ECVAM], the Japanese Center for Validation of Alternative Methods [JaCVAM]). Because the protocol described in ICCVAM (1999) and Dean et al. (2001) is more restrictive than that described in the Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 429 (OECD 2002), the ICCVAM protocol is the key reference for establishing these performance standards. Where they occur, the differences between the ICCVAM protocol and OECD TG 429 are noted in **Appendix A**.

It is important to emphasize that the performance standards described in this document are intended for the assessment of versions of the LLNA that vary only from the traditional LLNA by using non-radioactive versus radioactive methods for assessing lymphocyte proliferation in the draining auricular lymph nodes. The modified LLNA procedure should adhere to the traditional LLNA procedures in all other aspects, such as the strain of mice, the timing of exposures, the route and sites of exposure, and the measured endpoint (lymphocyte proliferation in the draining auricular lymph nodes). All procedural modifications should be accompanied by a scientific rationale. Other, more significant changes to the traditional LLNA would necessarily be subject to a more extensive evaluation and/or validation process. New test method protocols that adhere to these performance standards would be consistent with the OECD TG 429, which states that: "other endpoints for assessment of proliferation may be employed provided there is justification and appropriate scientific support, including full citations and description of the methodology" (OECD 2002)[5].

These performance standards are not proposed for evaluating other alternative test methods for measuring skin sensitization (e.g., *in vitro* methods). Additionally, these performance standards do not imply the appropriateness of performance standards for any other *in vivo test* method. In the United States, Federal agencies will determine the regulatory acceptability and utility of the ICCVAM recommendations for their individual programs.

1.2 Elements of ICCVAM Performance Standards

Performance standards are based on an adequately validated test method and provide a basis for evaluating the comparability of a proposed test method that is mechanistically and functionally similar (ICCVAM 2003). The three elements of performance standards are:

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[4] Prior to the acceptance of a new test method for regulatory testing applications, validation studies are conducted to assess its reliability (i.e., the extent of intra- and inter-laboratory reproducibility) and its relevance (i.e., the ability of the test method to correctly predict or measure the biological effect of interest) (OECD 1996, 2002a; ICCVAM 1997, 2003). The purpose of performance standards is to communicate the basis by which new proprietary (i.e., copyrighted, trademarked, registered) and nonproprietary test methods have been determined to have sufficient relevance and reliability for specific testing purposes.

[5] Because the more restrictive ICCVAM protocol (ICCVAM 1999, Dean et al. 2001) is being used as the key reference, any modified LLNA protocols that adhere to these performance standards would therefore also adhere to OECD TG 429.

- **Essential test method components:** These consist of essential structural, functional, and procedural elements of a validated test method that should be included in the protocol of a proposed test method that is mechanistically and functionally similar to the validated method. Essential test method components include unique characteristics of the test method, critical procedural details, and quality control measures.
- **A minimum list of reference substances:** Reference substances are used to assess the accuracy and reliability of a proposed mechanistically and functionally similar test method. These substances are a representative subset of those used to demonstrate the reliability and the accuracy of the validated test method, and are the minimum number that should be used to evaluate the performance of a proposed mechanistically and functionally similar test method.
- **Accuracy and reliability values:** These are the standards for accuracy and reliability that the proposed test method should meet or exceed when evaluated using the minimum list of reference substances.

1.3 ICCVAM Process for the Development of LLNA Performance Standards

ICCVAM established and published in 2003 the process that it follows for developing performance standards (ICCVAM 2003). ICCVAM now routinely develops draft performance standards that are proposed and considered during the ICCVAM evaluation of a new alternative test method. However, since ICCVAM evaluated the LLNA (ICCVAM 1999) prior to establishment of the ICCVAM performance standards process, they were not developed at that time. Accordingly, ICCVAM is now proposing draft performance standards for the LLNA to support the validation effort of specifically identified modifications of the LLNA protocol.

These revised draft performance standards are being made available to the ICCVAM Independent Expert Peer Review Panel (Panel) for consideration at a public meeting on March 4-6, 2008, to be held at the Consumer Product Safety Commission Headquarters in Bethesda, MD. These revised draft performance standards are also being made available to the public for comment in advance of the Panel meeting, and all comments received will be provided to the Panel for their consideration. The Panel recommendations will be made available to the public and to ICCVAM's Scientific Advisory Committee on Alternative Toxicological Methods for comment. The Panel report and all comments will be considered by ICCVAM in preparing final test method performance standards recommendations for United States (U.S.) Federal agencies.

Performance standards recommended by ICCVAM are incorporated into ICCVAM test method evaluation reports, which are provided to U.S. Federal agencies for consideration and made available to the public. Performance standards adopted by U.S. Federal regulatory authorities can be provided or referenced in test guidelines. Availability of ICCVAM test method evaluation reports are announced in the *Federal Register*, in NTP Newsletters, and by email to NICEATM-ICCVAM listserv groups.

1.4 ICCVAM Development of Performance Standards for the LLNA

1.4.1 Background on Skin Sensitization

Skin sensitization to a substance can lead to allergic contact dermatitis (ACD), a type IV hypersensitivity reaction. The development of skin sensitization occurs in two separate phases. The first phase, referred to as the induction phase, occurs when a susceptible individual is

exposed topically to a sufficient quantity of a skin-sensitizing substance. Induction is dependent on a substance penetrating the epidermis and subsequently binding to proteins. The resulting hapten complex can then be processed by the antigen-presenting cells in the skin (i.e., Langerhans cells). These cells then migrate to the draining lymph nodes, where the antigen is presented to T lymphocytes, leading to their clonal expansion. The lymphocytes can be divided into two subsets, memory and effector T lymphocytes. At this point, the individual has become sensitized to the exposed substance (Basketter et al. 2003; Jowsey et al. 2006).

The second phase, referred to as the elicitation phase, occurs when the individual is exposed to the same substance at the same or different skin location. As in the induction phase, the substance penetrates the epidermis where it is processed by antigen-presenting cells. The antigen is then presented to circulating effector T lymphocytes. The T lymphocytes produce a rapid secondary immune response in the skin that can lead to ACD (Basketter et al. 2003; Jowsey et al. 2006).

1.4.2 Test Methods for Assessing Skin Sensitization

There are several currently recognized test methods for evaluating skin sensitization *in vivo*. These methods are classified into two categories, adjuvant and non-adjuvant tests (see EPA 2003 for a list of acceptable test methods). Adjuvant tests use Freund's complete adjuvant (FCA) to potentiate sensitization. Examples of adjuvant tests include the Guinea Pig Maximization test (GPMT), the Maurer optimization test, the split adjuvant test, and the FCA test. Examples of non-adjuvant tests include the Buehler test (BT), the Draize sensitization test, and the Open Epicutaneous Test. All of these methods use the guinea pig as the test species.

For the GPMT, sensitization in guinea pigs is induced by intradermal injection of the test substance mixed with FCA at the start of the testing procedure. After six to eight days, an occluded patch containing the test substance is applied to the test area and held in place with a dressing for 48 hours. After 12 to 14 days, a patch containing the test substance is applied to the test area and held in placed with a dressing for 24 hours. Skin reactions (erythema and edema) are scored 24 and 48 hours after patch removal (ICCVAM 1999, Dean et al. 2001).

For the BT, a test patch containing the substance is applied to the animals. Animals are exposed once a week to the test substance for six hours over a period of three weeks. Two weeks after the final treatment, a patch containing the test substance is applied for six hours at a location different to where the initial challenges occurred. Skin reactions (erythema and edema) are then scored 24 and 48 hours after patch removal (ICCVAM 1999, Dean et al. 2001).

1.4.3 Intended Regulatory Uses for the LLNA

The LLNA is an alternative method that can be used as a substitute for the traditional guinea pig tests (GPMT and BT[6]), where appropriate, for assessing skin sensitization. The LLNA may not be suitable for use with certain types of test materials, such as metallic compounds, mixtures, high molecular weight compounds that cannot penetrate the stratum corneum, strong dermal irritants, chemicals whose pharmacodynamic activity is to release dermal cytokines that cause local lymph node proliferation (e.g., certain pharmaceuticals such as imiquimod [Gaspari 2007]), and materials that do not adhere to the ear for an acceptable length of time during the experiment.

[6]Of the methods listed in **Section 1.4.2**, the GPMT and BT are most widely used and are the preferred guinea pig sensitization tests as outlined in the OECD test guidelines for skin sensitization.

1.4.4 Similarities and Differences in the Endpoints of the LLNA and Reference Skin Sensitization Test Methods

The endpoint measured in the LLNA is induction of lymphocyte proliferation, which is part of the induction phase of skin sensitization (see **Section 1.4.1**). Comparatively, the reference tests described in **Section 1.4.2** involve rating skin reactions evoked in guinea pigs by the test substance which is part of the elicitation phase of skin sensitization (see **Section 1.4.1**). The guinea pig tests therefore allow for an assessment of the entire allergic contact dermatitis process.

While the endpoints measured in the LLNA and the reference test methods are different, the induction phase of skin sensitization is necessary for development of skin reactions (i.e., elicitation phase). Therefore, measurement of lymphocyte proliferation generally predicts whether the test substance will produce skin sensitization. Compared to the LLNA, which quantifies the amount of T lymphocyte proliferation, the reference test methods use subjective scoring of the irritation (i.e., erythema and edema) observed after test substance application.

2.0 LLNA Performance Standards for Assessing Lymphocyte Proliferation

2.1 Background

The LLNA has been adequately validated for its ability to distinguish between sensitizers and nonsensitizers (ICCVAM 1999, Dean et al. 2001). However, certain substances may not be suitable for use with the LLNA. These include:

- Mixtures: limited data available
- Metallic compounds: may produce inaccurate results and limited data available
- High molecular weight compounds: not readily absorbed into the skin
- Strong dermal irritants: may produce false positive results
- Materials that do not adhere to the ear for an acceptable time during the experiment

This section briefly describes the principles of the LLNA test method, followed by the draft performance standards that would be used to evaluate test methods for evaluation of lymphocyte proliferation that are functionally and mechanistically similar. The performance standards consist of 1) essential test method components, 2) reference substances, and 3) the comparable accuracy and reliability that should be achieved.

2.2 Principles of the LLNA

Studies have shown that chemical sensitizers induce lymphocyte proliferation in those lymph nodes that receive lymphatic drainage associated with the site of sensitizer application. Measurement of the increase in lymphocyte proliferation is used in the LLNA method to identify chemical sensitizers. The Stimulation Index (SI), defined as the ratio of lymphocyte proliferation after application of a potential chemical sensitizer to lymphocyte proliferation after application of the test vehicle, is used to assess the sensitizing potential of the test substance.

2.3 LLNA Essential Test Method Components

The essential test method components include all aspects of the traditional LLNA protocol as described by ICCVAM (1999) and Dean et al. (2001), upon which OECD TG 429 (OECD 2002)

is based, with one exception. The only exception is the method used to assess lymphocyte proliferation and the corresponding decision criteria for classifying a test substance as positive or negative. This is described in **Section 2.4**.

Appendix A provides the essential test method components associated with the ICCVAM LLNA protocol (ICCVAM 1999, Dean et al. 2001). Alternative LLNA protocols with changes to any of these essential test method components would constitute major modifications to the traditional LLNA protocol, and would therefore be subject to a more extensive evaluation and/or validation process, beyond a comparison to these performance standards.

2.4 Essential Test Method Components: Non-radioactive Alternatives to Measuring Lymphocyte Proliferation in the LLNA

This section describes the information that should be provided to support the use of LLNA protocols that incorporate modifications to the measurement of lymphocyte proliferation. These minor modifications use non-radioactive reagents to assess lymphocyte proliferation in the draining lymph nodes. As stated in **Section 2.3**, all other test method protocol components should follow the traditional LLNA protocol (see **Appendix A**).

The method used for assessing lymph node cell proliferation should be detailed and scientifically justified. It must include a description of the decision criteria for what constitutes positive and negative responses in the proposed test method, and the basis for the decision criteria, as well as the method of administration of the probe chemical (if applicable). In the traditional LLNA, an SI of three or greater is used to identify a skin-sensitizing agent. However, a decision criterion using an SI of three or greater may only be applicable to measuring the incorporation of radioactivity as conducted in the traditional LLNA (ICCVAM 1999, Dean et al. 2001). A threshold SI may be other than three for alternative LLNA protocols that are not based on the incorporation of radioactivity for measuring lymph node cell proliferation. In such cases, the concentration of test material at the revised threshold limit would be other than an EC3 (the estimated concentration needed to produce an SI of three) and would therefore be defined as ECt (the estimated concentration needed to produce an SI of a defined threshold).

Although the SI decision criteria is the one most often used to distinguish between sensitizers and nonsensitizers, a statistical analysis based on individual animal data and/or an evaluation of the dose response relationship may also be conducted in order to provide a more complete evaluation of the test substance.

2.4.1 Calculation of ECt

As described in **Sections 2.6** and **2.7**, the accuracy and reliability assessments of a modified LLNA protocol require calculation of an ECt for comparison to an acceptable range of values indicated in the list of reference substances. The ICCVAM (1999) protocol does not include guidance on the calculation of an ECt, which is therefore described below.

The method for determining the LLNA ECt is a simple linear interpolation of the points in the dose response curve that lie immediately above and below the classification threshold (e.g., SI=3 for the traditional LLNA). Consider an example where the threshold SI=3:

If the data points lying immediately above and below the SI value of 3 have the co-ordinates (a,b) and (c,d) respectively, then the EC3 value may be calculated using the equation: $EC3 = c+[(3-d)/(b-d)](a-c)$ (Basketter et al. 1999).

When there are no points below the defined threshold (e.g., SI=3), a more complex log-linear extrapolation may be applied as described in Ryan et al. (2007) in which the two lowest test concentrations from the dose response curve are used.

2.5 Data and Reporting

The test report should include information outlined below.

1. Test substances, control substances, and vehicles
   - Name of test substance and identification data (e.g., Chemical Abstracts Service Registry Number)
   - Purity and composition of the substance or mixture
   - Physicochemical properties (e.g., physical state, water solubility) relevant to the conduct of the study
   - Treatment of the test/control substances prior to testing, if applicable (e.g., vortexing, sonication, warming; resuspension solvent)
   - Name of vehicle and identification data (e.g., purity, composition, volume used)
   - Justification for choice of vehicle
2. Justification of the alternative test method and protocol used
3. Test animals
   - Strain of mouse used[7]
   - Microbiological status of the mice, when information is available
   - Number, age, and sex of mice used
   - Source of mice, housing conditions, diet, etc.
4. Description of the method used to measure lymphocyte proliferation and justification for its use
5. Test method conditions
   - Details on test substance preparation and application
   - Justification for dose selections, including basis for the highest dose tested (see **Appendix A - Test Procedure**). The reason for variation away from traditional assay dose selection process, if any, should be discussed
6. Criteria for an acceptable test
   - Concurrent positive control data
   - Concurrent negative control data

[7] Female CBA/Ca or CBA/J mice are recommended. Male mice or mice of other strains should not be used unless it is sufficiently demonstrated that significant strain- and/or gender-specific differences in the LLNA response do not exist.

- Historical ranges of positive and negative control data. Historical data can be from within the testing laboratory or provided from an external source, provided that supporting data (e.g., raw data) can be provided.
- Exclusion criteria should be defined and the impact of any excluded data should be described.

7. Results
   - Weights of each animal at the start of the test and the time of lymph node collection
   - Tabulation of data from individual animals showing the mean and individual values for each dose (including vehicle and positive control) group
   - Lymphocyte proliferation, which should be expressed in the units specified by the method (e.g., disintegrations per minute for methods using radioactive reagents; absorbance at a specified wavelength for methods using colorimetric reagents). Results should be provided for all test substance dose levels and concurrent controls.
   - Calculated results (e.g., as measured or quantified by the SI and the associated ECt value, if applicable[8]) should be provided for all test substances and concurrent controls.
   - Statistical analysis and/or evaluation of the dose response relationship, where appropriate
8. Description of animal observations
   - Time course of onset and severity of clinical signs of systemic toxicity and dermal irritation should be described (e.g., location of observed dermal irritation)
9. Discussion of the results
   - If consideration is given to other properties of the test substance (e.g., structural relationship to known skin sensitizers) in addition to the calculated results in classification of substances as skin sensitizers, such information should be provided.
10. Conclusion
11. If Good Laboratory Practice (GLP)-compliant studies are performed, then additional reporting requirements provided in the relevant guidelines (e.g., OECD 1998; EPA 2006a, 2006b; FDA 2006) should be followed.
    - A quality assurance statement for GLP-compliant studies should indicate all inspections made during the study and the dates any results were reported to the Study Director. This statement should also confirm that the final report reflects the raw data.

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[8] An ECt would only be calculated where an SI ≥ the defined threshold was generated.

2.6 Reference Substances for Methods Assessing Lymphocyte Proliferation

2.6.1 Criteria for Selection of Reference Substances

Reference substances are used to assess the accuracy and reliability of a proposed mechanistically and functionally similar test method and are a representative subset of those used to demonstrate the reliability and the accuracy of the validated test method (i.e., traditional LLNA). This set of reference substances should, to the extent possible:

- Represent the range of responses that the validated test method is capable of measuring or predicting
- Have well-defined chemical structures
- Have high-quality data available from the traditional test method (i.e., guinea pig tests), which is compared to the data generated by the validated test method (i.e., traditional LLNA), as well as data from the species of interest (e.g., humans), where possible
- Have produced consistent results in the validated test method
- Be readily available from commercial sources
- Not involve excessive hazard or prohibitive disposal costs

2.6.2 Characteristics of Selected Reference Substances

The traditional LLNA was submitted with data from testing of 211 substances. After careful consideration of the above criteria, 22 substances were selected as proposed minimum reference substances for the LLNA performance standards. The proposed substances are provided in **Appendix B** and a detailed rationale for selection of the substances in this list is included in **Appendix C**. The selected substances have the following characteristics:

- All of the substances have data from testing in the GPMT or BT.
- All of the substances are readily available from commercial sources.
- The substances represent the full dynamic range of responses that can be assessed in the current approved LLNA, from non-sensitizers to strong sensitizers.
- Twenty of the 22 substances have human data (e.g., Human Maximization Test results, Human Repeat Insult Patch Test results, available as a patch test kit allergen, and/or clinical case studies/reports).
- The selected substances include 13 solids and nine liquids.
- The molecular weights of the substances range from 30.026 g/mole to 604.813 g/mole.
- The xLogP (octanol:water partition coefficient) values (Wang et al. 2000) of the substances range from -3.1 to 4.9 (from water soluble to insoluble, respectively).
- The vehicles used for all of the substances are known. The vehicles used were acetone:olive oil (13), dimethyl formamide (6), dimethyl sulfoxide (2), and methyl ethyl ketone (1).
- There is peptide reactivity information for nine substances.

- The EC3 values of the positive substances range from 0.0099% to 28%, based on results from the traditional LLNA.
- The selected substances have a wide range of SI values, ranging from 5.5 to 75.3 for substances identified as skin sensitizers by the traditional LLNA, and 0.9 to 2.5 for substances identified as non-sensitizers by the traditional LLNA.

For all studies using the proposed list provided in **Appendix B**, substances should be evaluated in the vehicle with which they are listed.

In situations where a listed substance may not be available, other substances of the same class (e.g., correctly identified sensitizer, false positive) for which there are high quality *in vivo* reference data (as outlined in **Section 2.6.1**) may be used.

2.7 Accuracy and Reliability Performance Values

The final elements of performance standards are the accuracy and reliability values (i.e., test method performance) that should be met or exceeded by the proposed test method when evaluated with the reference substances. The following sections indicate these required statistics for LLNA protocols that use an endpoint other than the incorporation of radioactivity for the evaluation of lymphocyte proliferation; the rationale for their selection is described in detail in **Appendix D**.

2.7.1 Accuracy

Accuracy is defined as the closeness of agreement between a test method result and an accepted reference value (ICCVAM 2003). For these performance standards, the proposed test method should have accuracy characteristics that are equivalent to or exceed the performance of the traditional LLNA method when evaluated using the minimum list of recommended reference substances (**Appendix B**). Therefore, for the 18 substances with concordant traditional LLNA and GP data (referred to as "required substances"), the proposed test method should result in the correct classification based on a "yes/no" decision. Additionally, when tested in the relevant vehicle, the calculated ECt[9] for each of the sensitizing chemicals on the reference list should be within 0.5x to 2.0x the reference EC3 values as indicated in **Appendix B**. Instructions on properly calculating an EC3, which would apply also to the calculation of an ECt, are included in **Section 2.4.1** and **Appendix A**.

To demonstrate improved performance relative to the traditional LLNA, four "optional substances" (two LLNA false negatives and two LLNA false positives) may be tested in addition to the required set of substances described above.

2.7.2 Reliability

Test method reliability (intralaboratory repeatability, and intra- and inter-laboratory reproducibility) is the degree to which a test method can be performed reproducibly within and among laboratories over time (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. Intralaboratory repeatability for the traditional LLNA method was not assessed, although some

[9] As indicated in **Section 2.4**, a threshold SI may be other than three for alternatives to the incorporation of radioactivity for measuring lymph node cell proliferation, and in such instances the concentration of test material at the revised threshold limit would be other than an EC3. Therefore, the term ECt is used.

indication of the inherent biological variability can be obtained by comparing the results for individual test animals administered the same identical dose.

Intralaboratory reproducibility refers to the determination of the extent to which qualified personnel within the same laboratory can replicate results using a specific test protocol at different times. Intralaboratory reproducibility for the traditional LLNA is discussed in **Appendix D**.

Interlaboratory reproducibility refers to the determination of 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. Interlaboratory reproducibility for the traditional LLNA is summarized in **Appendix D**.

2.7.2.1 *Intralaboratory Repeatability*

No requirement is proposed.

2.7.2.2 *Intralaboratory Reproducibility*

Intralaboratory reproducibility can be assessed by calculating the variability resulting from testing hexyl cinnamic aldehyde (HCA). ECt values should be derived on four separate occasions with at least one week between tests. Acceptable reproducibility will be indicated by a laboratory obtaining, in each instance, ECt values for HCA that are generally within 0.5x to 2.0x (5% to 20%) the mean EC3 concentration (10%) specified for HCA in **Appendix B**.

2.7.2.3 *Interlaboratory Reproducibility*

Interlaboratory reproducibility should be evaluated with at least two sensitizing chemicals with well-characterized activity in the traditional LLNA. In this regard, ECt values for 2,4-dinitrochlorobenzene (DNCB) and HCA should be derived independently in at least three separate laboratories. Acceptable reproducibility will be indicated by each laboratory obtaining ECt values for HCA and DNCB that are generally within 0.5x to 2.0x (5% to 20% and 0.025% to 0.1%, respectively) the mean EC3 concentration (10% and 0.05%, respectively) specified for these substances in **Appendix B**.

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APPENDIX A

Essential Test Method Components for Local Lymph Node Assay[1]

and

Details of Dissection of Draining Auricular Lymph Nodes[2]

[1] Based on ICCVAM (1999) and Dean et al. (2001)

[2] From 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 (http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/LLNAProt.pdf)

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The following is a description of the essential test method components for the LLNA. These test method components are consistent with the ICCVAM recommended LLNA protocol (ICCVAM 1999, Dean et al. 2001) and the ICCVAM and ICCVAM IWG LLNA Protocol (2001).

Animal Selection and Preparation

Animal Species Selection

- Mice are the species of choice for this test method.
- Young adult female mice that are nulliparous and not pregnant (i.e., CBA/Ca or CBA/J strains) are used. Other strains and males should not be used until it is sufficiently demonstrated that significant strain- and/or gender-specific differences in the LLNA response do not exist.[10]
- At the start of the study, mice should be 8-12 weeks old. All animals should be age-matched (preferably within a one-week time frame)
- Weight variations between the mice should not exceed 20% of the mean weight.

Housing and Feeding Conditions

- Experimental animal room temperature should be 22 ± 4 °C
- Experimental animal room humidity should range between 30% and 70%. The preferred humidity for the room should range from 50% to 60%.
- Artificial lighting should be used with a cycle of 12 hours light and 12 hours dark.
- Mice may be housed individually, or caged in small groups of the same sex, and fed a conventional laboratory diet with unrestricted access to drinking water.[11]

Animal Preparation

- Mice are to be uniquely identified prior to being placed in the study. The method used to mark the mice may not involve identification via the ear (i.e., marking, clipping, or punching of the ear).
- Mice should be acclimated for at least five days prior to the start of the test.
- Healthy mice are randomly assigned to the control and treatment groups.
- All mice should be examined prior to the initiation of the test to ensure that there are no skin lesions present.

Control Substances

Negative (Solvent/Vehicle) Control

- To ensure that the test system is functioning properly and that the specific test is valid, a solvent/vehicle control should be included in each experiment.
- The solvent/vehicle control should be tested concurrently with the test substances.

[10] According to OECD TG 429, other strains and males may be used where it has been demonstrated that strain- and/or gender-specific differences are not detrimental to the performance of the test method (OECD 2002).

[11] OECD TG 429 states that mice should be individually housed (OECD 2002).

- Hydrophilic materials should be incorporated into a vehicle that does not immediately run off of the skin.
- The selected solvent/vehicle must not interfere with or bias the test result and should be selected to achieve maximum concentration/skin exposure of the test substance.
- In order of preference, recommended solvents/vehicles are acetone:olive oil (4:1 v/v), *N*,*N*-dimethylformamide, methyl ethyl ketone, propylene glycol, and dimethyl sulfoxide. Other solvents may be used if appropriate justification is provided.

Positive Control

- The purpose of a positive control substance is to demonstrate that the test method is responding with adequate sensitivity to a sensitizing substance for which the magnitude of the response is well characterized.
- The positive control should be tested concurrently[12] with the test substance, using the same vehicle, and it should elicit a response that is within 0.5x to 2.0x of the mean laboratory historical ECt value for that positive control-solvent combination.
- The positive control should be tested at a concentration that is expected to yield a positive response (e.g., for the traditional LLNA protocol, the positive control should produce an SI $\geq$ 3 over the negative control).
- The positive control dose is to be chosen such that there is a clearly positive response, but one that is not excessive (e.g., benzoquinone may be too potent to use as a positive control).
- Examples of test substances that may be used as positive controls include, but are not limited to, hexyl cinnamic aldehyde and mercaptobenzothiazole.
- Other substances may be used as a positive control, with sufficient justification. However, benzocaine should not be used as a positive control since it has been shown to produce equivocal responses in the LLNA.

Benchmark Controls

- Benchmark controls may be useful to demonstrate that the test method is functioning properly for detecting the skin sensitization potential of substances of a specific chemical class or a specific range of responses, or for evaluating the relative skin sensitization potential of a test substance.
- Appropriate benchmark controls should have the following properties:
  - Structural and functional similarity to the class of the substance being tested
  - Known physical/chemical characteristics

[12] OECD TG 429 states that there may be situations for which test laboratories will have available historic positive control data to show consistency of a satisfactory response over a 6-month or more extended period. In those situations, less frequent testing with positive controls may be appropriate at intervals no greater than six months (OECD 2002).

- Supporting data on known effects in animal models
- Known potency in the range of response

Test Procedure

Number of Animals per Dose

- A minimum of five successfully scored mice per dose group should be used.[13]
- A negative and positive control group should be included.

Selection of Doses

- Dose and vehicle selection should be based on the recommendations provided in the ICCVAM recommended LLNA protocol (ICCVAM 1999, Dean et al. 2001).
  - The highest dose tested should be the highest soluble concentration that does not induce systemic toxicity (e.g., greater than a 10% decrease in body weight has been suggested to be an appropriate indicator of systemic toxicity in LLNA studies [Basketter et al. 2001, Cockshott et al. 2006]) and/or excessive skin irritation (e.g., increased ear swelling [Hayes et al. 1998, Manetz and Meade 1999]).
  - Animal monitoring plans must include criteria to promptly identify animals for euthanasia based on exhibiting systemic toxicity or excessive irritation or corrosion of skin.
- A minimum of three consecutive doses are selected (e.g., 100%, 50%, 25%) plus a negative (solvent/vehicle) and a positive control group.

Dosing Schedule and Collection of Lymph Node Cells

- Day 1
  - Each mouse is identified and weighed.
  - Test substance, vehicle, or positive control (25 μL) is applied to the dorsum of each ear.
- Days 2 and 3
  - Repeat the application procedure as described for Day 1.
- Days 4 and 5
  - No treatment.
- Day 6
  - Weigh each mouse.
  - Inject 250 μL of sterile phosphate-buffered saline (PBS) containing 20 μCi of ^{3}H-methyl thymidine (^{3}H-TdR) or 250 μL PBS containing 2 μCi of ^{125}I-

[13] OECD TG 429 states that in those cases in which individual animal data are to be collected, a minimum of five mice per dose group should be used. Otherwise (i.e., when pooling of lymph nodes within treatment groups is performed), a minimum of four animals per dose group should be used (OECD 2002).

iododeoxyuridine (^{125}IU) and 10^{-5} M fluorodeoxyuridine into each experimental mouse via the tail vein. Other routes of injection may be more appropriate for non-radioactive markers of lymphocyte proliferation (e.g., intraperitoneal for bromodeoxyuridine [BrdU]); the route of injection should be described in the test method protocol and the scientific rationale provided.

- Five hours later, the mice are euthanized.
- The draining auricular lymph nodes from each ear are excised. The nodes are then combined in PBS for each animal.[14]
  - Measuring cell proliferation in the lymph nodes from individual animals, rather than from lymph nodes pooled across all mice in a dose group, can highlight problems caused by technical inexperience (Cockshott et al. 2006)

Observations

- All observations should be recorded.
- Mice should be observed for any clinical signs of local, excessive irritation or corrosion, or systemic toxicity. Animal monitoring plans must include criteria to promptly identify animals exhibiting systemic toxicity or excessive irritation or corrosion of skin for euthanasia.
- Histopathology should be considered to evaluate questionable lesions.
- Evidence of local irritation (i.e., erythema/edema formation) should be noted and the method(s) used for such measurements and the criteria for what is considered excessive should be provided.

*Assessment of Lymphocyte Proliferation and Interpretation of Results (see **Section 2.3** for a description of essential test method components applicable to alternative methods for measuring lymphocyte proliferation)*

- Lymphocyte proliferation should be expressed in the units obtained from the method (e.g., disintegrations per minute). Results should be provided for all test substance dose levels and concurrent positive and vehicle controls.
- Raw data and calculated results (i.e., as measured or quantified by the stimulation index [SI]) should be provided for all test substances and concurrent controls.
- Description of decision criteria for what constitutes positive and negative responses in the proposed test method and the basis for the decision criteria should be provided.
  - For example when the threshold for a positive response is SI=3, the test substance is regarded as a skin sensitizer when the SI for any single treatment group is ≥ 3.

[14] OECD TG 429 allows pooling of lymph nodes for each animal (i.e., pooled individual animal approach) or pooling for each experimental treatment group (i.e., pooled treatment group approach) (OECD 2002).

- However, the magnitude of the SI should not be the sole factor used in determining the biological significance of a skin sensitization response.
- An assessment may be performed by statistical analysis of individual animal data and may provide a more complete evaluation. For this reason, pooling of lymph node cells from multiple test animals is discouraged.
- Factors that should be considered include the SI, statistical analyses, the strength of the dose-response relationship, chemical toxicity, solubility, and the consistency of the vehicle and positive control responses.
- A test substance not meeting the above criteria is considered a non-sensitizer.

DISSECTION APPROACH[15]

Lateral Dissection (Figure 1)

Although lateral dissection is not the conventional approach used to obtain the nodes draining the ear, it may be helpful as a training procedure when used in combination with the ventral dissection. This approach is performed bilaterally (on both sides of the mouse). After the mouse is euthanized, it is placed in a lateral position. The facial and neck area is wetted with 70% ethanol. Using scissors and forceps, an initial cut is made from the neck area slightly below the ear. This incision is carefully extended toward the mouth and nose. During this procedure, the tip of the scissors should be angled slightly upward to prevent the damage of deeper tissue. The glandular tissue in the area is gently retracted using the forceps. Using the masseter muscle, facial nerves, blood vessels, and the bifurcation of the jugular vein as landmarks, the draining node is isolated and removed (**Figure 1**). The draining nodes[16] ("auricular") will be positioned adjacent to the masseter muscle and proximal to and slightly above the jugular bifurcation.

Ventral Dissection (Figure 2)

The most commonly used dissection approach is from the ventral surface of the mouse. This approach allows both right and left draining nodes to be obtained without repositioning the mouse. With the mouse ventrally exposed, the neck and abdomen area is wetted with 70% ethanol. Using scissors and forceps, carefully make the first incision across the chest and between the arms. Make a second incision up the mid-line, perpendicular to the initial cut, and then cut up to the chin area. Reflect the skin to expose the external jugular veins in the neck area. Care should be used to avoid salivary tissue at the midline and nodes associated with this tissue. The nodes draining the ear ("auricular") are located distal to the masseter muscle, away from the midline, and near the bifurcation of the jugular veins[5].

[15] From recommeneded ICCVAM-IWG LLNA protocol (ICCVAM 2001, Available at http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/LLNAProt.pdf)

[16] It is noted while **Figures 1** and **2** represent the auricular nodes as a single entity, rodents may have more than a single node that comprises the auricular nodes.

Figure 1: Lateral Dissection

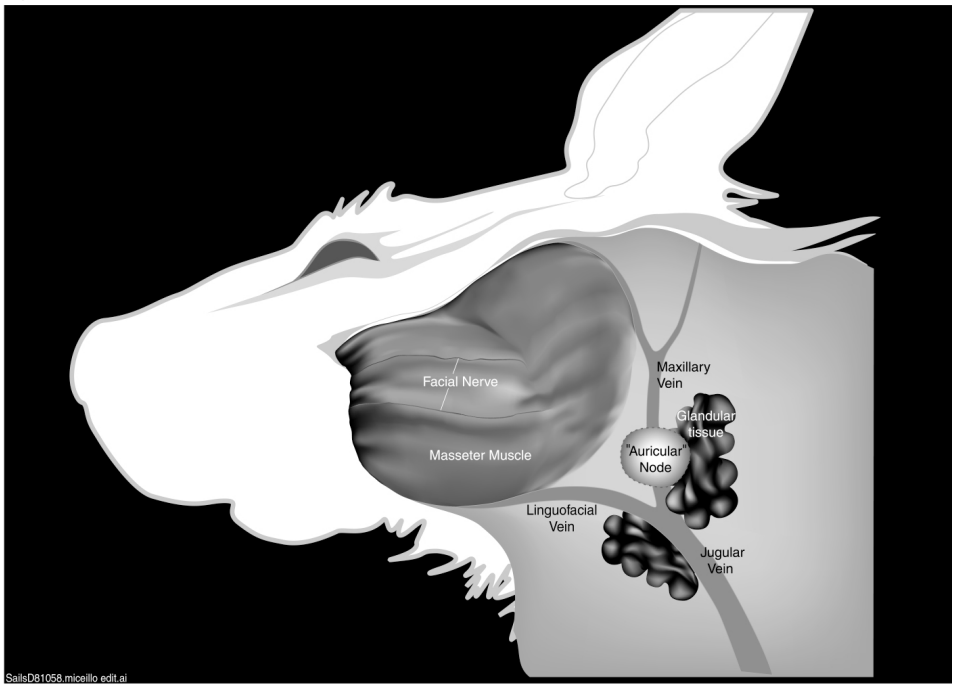
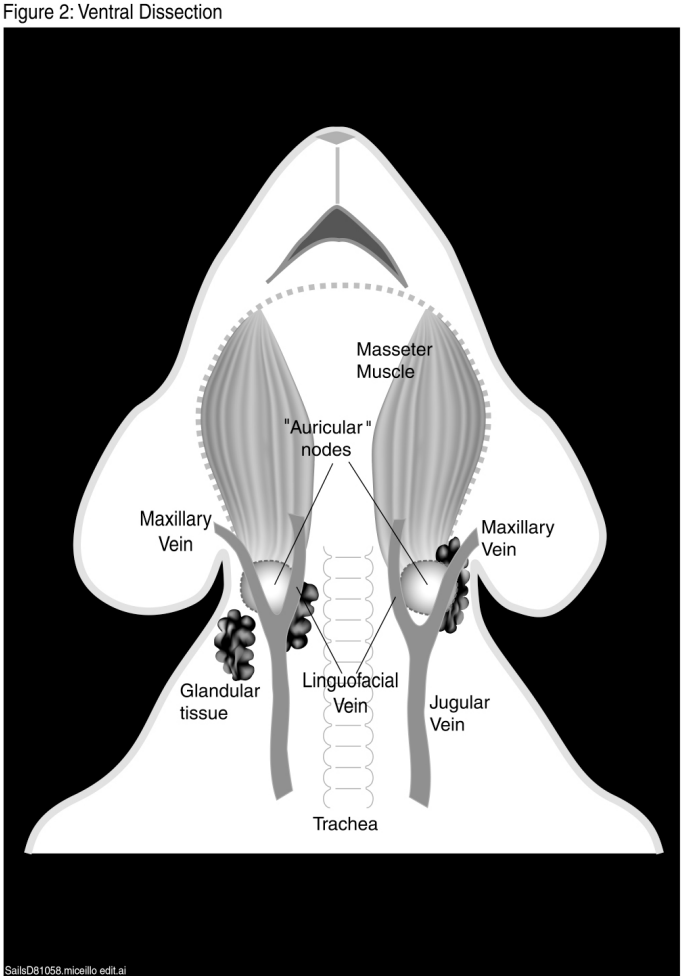


Figure 2: Ventral Dissection



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ACCURACY IN IDENTIFICATION

The nodes can be distinguished from glandular and connective tissue in the area by the uniformity of the nodal surface and a shiny translucent appearance. The application of sensitizing agents (especially the strong sensitizers used in training) will cause an enlargement of the node size. If a dye is injected for training purposes, the node will take on the tint of the dye.

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APPENDIX B

Draft ICCVAM LLNA Performance Standards: Recommended Reference Substances

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982 **APPENDIX B1**
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984 **Recommended Reference Substances - Alphabetically Sorted**
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Chemical Name	CASRN	MW (g/mol)	Physical Form	LLNA	Veh[1]	EC3[2]	N[3]	0.5x - 2.0x EC3	SI (Conc)	GPMT /BT[4]	HMT	HPTA	Additional Human Skin Sensitization Data/Information [5]	Peptide Reactivity[6]
5-Chloro-2 methyl-4-isothiazolin-3-one	26172-55-4	149.599	Liquid	+	DMF	0.009	1	0.0045-0.018	22.7 (0.1%)	+		+		
Chlorobenzene	108-90-7	112.557	Liquid	-	AOO	NC	1	NA	1.7 (10%)	-			No human data located*	
Cinnamic alcohol	104-54-1	134.18	Sol	+	AOO	21	1	10.5-42	5.7 (90%)	+	+		DSA05HRIPT=3474;	
Citral	5392-40-5	152.233	Liquid	+	AOO	9.8	2	4.9-19.6	6.3 (25%)	+	+		DSA05HRIPT=1266; DSA05HMT=862; DSA(NOEL)HRIPT=775	
Cobalt chloride	7646-79-9	129.84	Solid	+	DMSO	4.8	1	2.4-9.6	NA	+	+	+		
2,4-Dinitrochlorobenzene	97-00-7	202.552	Liquid	+	AOO	0.049	15	0.025-0.099		+			Results from patch test studies indicate substance produces skin sensitization[10]	
Ethylene glycol dimethacrylate	97-90-5	198.216	Liquid	+	MEK	28	1	14-56	7 (50%)	-		+		High
Eugenol	97-53-0	164.201	Liquid	+	AOO	10.1	11	5.05-20.2	14.1 (70%)	+		+		

Chemical Name	CASRN	MW (g/mol)	Physical Form	LLNA	Veh[1]	EC3[2]	N[3]	0.5x - 2.0x EC3	SI (Conc)	GPMT /BT[4]	HMT	HPTA	Additional Human Skin Sensitization Data/Information[5]	Peptide Reactivity[6]
Hexyl cinnamic aldehyde[7]	101-86-0	216.319	Liquid	+	AOO	9.9	22	5.0-19.9	17 (50%)	+			DSA(NOEL)HRIPT=23622	Minimal
Lactic acid	598-82-3	90.078	Liquid	-	DMSO	NC	2	NA	2.2 (25%)	-			No human data located*	
Imidazolidinyl urea	39236-46-9	388.294	Solid	+	DMF	24	1	12-36	5.5 (50%)	+		+	DSA05HRIPT=3846; DSA(NOEL)HRIPT=2000	Moderate
Isoeugenol	97-54-1	164.201	Liquid	+	AOO	1.5	49	0.77-3.1	12.4 (5%)	+		+	DSA05HRIPT=657; DSA(NOEL)HRIPT=250	
Isopropanol	67-63-0	60.095	Liquid	-	AOO	NC	1	NA	1.0 (50%)	-			Studies indicate substance produces skin sensitization[11]	Minimal
2-Mercaptobenzothiazole	149-30-4	167.253	Solid	+	DMF	2.5[8]	2	1.25-5.0	8.6 (10%)	+	+	+	DSA05HMT=2269	High
4-Methylaminophenol sulfate	55-55-0	344.384	Solid	+	DMF	0.8	1	0.4-0.12	6.7 (2.5%)	+		+		
Methyl salicylate	119-36-8	152.147	Liquid	-	AOO	NC	10	NA	0.9 (20%)	-	-	-		Minimal

Chemical Name	CASRN	MW (g/mol)	Physical Form	LLNA	Veh[1]	EC3[2]	N[3]	0.5x - 2.0x EC3	SI (Conc)	GPMT /BT[4]	HMT	HPTA	Additional Human Skin Sensitization Data/Information[5]	Peptide Reactivity[6]
Nickel sulfate	10101-98-1	280.864	Solid	-	DMSO[9]	NC	2	NA		+	+	+		
Phenylbenzoate	93-99-2	198.217	Solid	+	AOO[7]	13.6	3	6.8-27.2	11.1 (25%)	+			Human sensitization threshold dose = 9448 μg/cm^2	
4-Phenylenediamine	106-50-3	108.14	Solid	+	AOO	0.11	10	0.055-0.22	6.6 (1.0%)	+	+	+	DSA05HMT=111	
Salicylic acid	69-72-7	138.121	Solid	-	AOO	NC	1	NA	2.5 (25%)	-	-	-		
Sodium lauryl sulfate	151-21-3	288.38	Solid	+	DMF	8.1	5	4.05-16.2	3.5 (20%)	-	-	-		
Sulfanilamide	63-74-1	172.20	Solid	-	DMF	NC	1	NA	0.9 (50%)	-	+	+		Minimal

Abbreviations: Ac = acetone; AOO = acetone:olive oil (4:1); BT = Buehler Test; CASRN = Chemical Abstract Service Registry Number; Conc. = Maximum concentration tested; DMF = *N,N*-dimethylformamide ; DMSO = dimethyl sulfoxide; DSA = Dose per skin area; GPMT = Guinea Pig Maximization Test; HMT = Human Maximization Test; HPTA = Human Patch Test Allergen; LLNA = local lymph node assay; MEK = methyl ethyl ketone; MW = molecular weight; NC = not calculated; SI = Stimulation Index; Veh = vehicle.

* = Presumed to be a non-sensitizer in humans based on the fact that no clinical patch test results were located, it is not included as a patch test kit allergen, and no case reports of human sensitization were located

[1]Unless noted otherwise, vehicle information obtained from Gerberick et al. 2005.

[2]Unless noted otherwise, EC3 values obtained from Gerberick et al. 2005.

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

[4]Results obtained from Guinea Pig Maximization Test and Buehler Test.

[5]Human Quantitative Data obtained from literature where human data was compared to LLNA. All data are expressed as DSA (μg/cm^2). DSA05HMT and DSA05HRIPT were obtained by linear interpolation from the lowest observed effect level to a dose corresponding to the estimated sensitization incidence of 5% (Schneider and Akkan 2004). DSA (NOEL) refers to the maximum no observed effect level. In absence of negative data, the lowest observed effect level was used, provided that the percentage of people sensitized was less than 8% (Basketter et al. 2005).

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

[7]Presumed to be a strong human allergen (search for human data ongoing).

[8]EC3 values obtained from Kimber et al. 2003.

[9]Vehicle information obtained from: ICCVAM 1999.

[10]Human data based on following studies: (1) Rees et al. 1989 (2) Zina et al. 1987.

[11]Human data based on Kwon et al. 2003.

APPENDIX B2

Recommended Reference Substances - Structures and Product Uses

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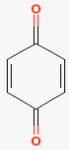
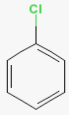
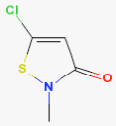
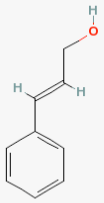
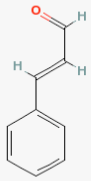
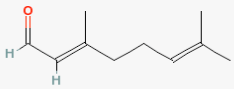
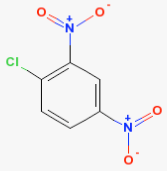
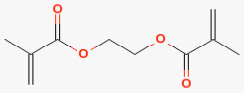
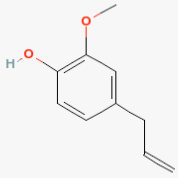
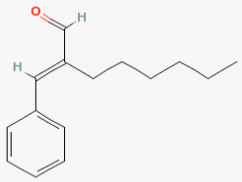
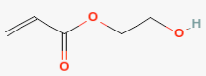
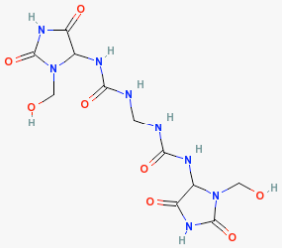
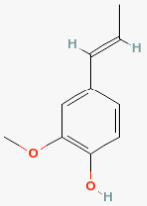
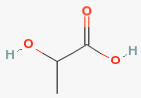
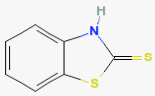
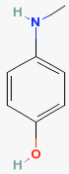
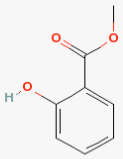
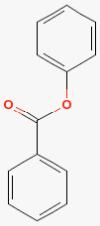
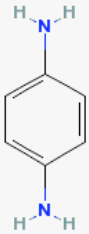
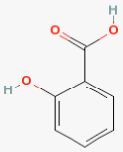
1046

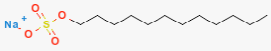
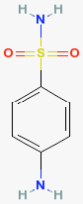
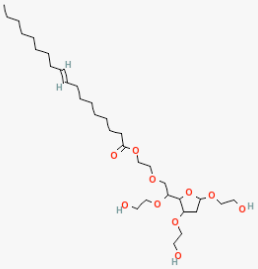
Chemical Name	CASRN	Structure	Product Uses
Benzoquinone [1]	106-51-4		Agricultural chemical Nylon manufacture Dye manufacture
Chlorobenzene [2]	108-90-7		Phenol manufacture Aniline manufacture DDT manufacture Solvent for paints
5-Chloro-2-methyl-4-isothiazolin-3-one [2]	26172-55-4		Disinfectant
Cinnamic alcohol [2]	104-54-1		Perfume manufacture
Cinnamic aldehyde [1]	104-55-2		Flavor additive Perfume manufacture Fungicide Insecticide

Chemical Name	CASRN	Structure	Product Uses
Citral [1,2]	5392-40-5		Flavor additive Perfume manufacture
Cobalt chloride [2]	7646-79-9		Humidity & water indicator Preparation of catalysts Fertilizer & feed additive Vitamin B12 manufacture
2,4-Dinitrochlorobenzene [1,2]	97-00-7		Color photo processing Explosives manufacture
Ethylene glycol dimethacrylate [1,2]	97-90-5		Polymerization agent
Eugenol [2]	97-53-0		Fragrance and flavoring agent Insect attractant

Chemical Name	CASRN	Structure	Product Uses
Formaldehyde [1]	50-00-0		Industrial chemical Embalming fluid
Hexyl cinnamic aldehyde [1,2]	101-86-0		Perfume manufacture
2-Hydroxyethyl acrylate [1]	818-61-1		Embedding resin Cosmetic
Imidazolidinyl urea [1,2]	39236-46-9		Cosmetic preservative Antimicrobial
Isoeugenol [1,2]	97-54-1		Perfume manufacture Flavoring additive Topical pharmaceutical

Chemical Name	CASRN	Structure	Product Uses
Isopropanol [1,2]	67-63-0		Topical pharmaceutical Gasoline additive Cleaning agent
Lactic Acid [2]	50-21-5		Manufacture of lactates which are used in food products, in medicine, and as solvents
2-Mercaptobenzothiazole [1,2]	149-30-4		Rubber manufacture Anticorrosive
4-Methylaminophenol [2]	150-75-4		Organic synthesis Photographic developer Developer for hair dyes
Methyl salicylate [1,2]	119-36-8		Topical pharmaceutical Flavor additive

Chemical Name	CASRN	Structure	Product Uses
Nickel chloride [1]	7718-54-9		Electroplating agent Battery manufacture
Nickel sulfate [1,2]	10101-98-1		Electroplating agent Battery manufacture Dye manufacture
Phenyl benzoate [2]	93-99-2		Production of industrial chemicals
4-Phenylenediamine [1,2]	106-50-3		Hair dye Textile dye
Salicylic acid [1,2]	69-72-7		Pharmaceutical Food preservative

Chemical Name	CASRN	Structure	Product Uses
Sodium lauryl sulfate [1,2]	151-21-3		Detergent Cosmetic
Sulfanilamide [1,2]	63-74-1		Pharmaceutical Antimicrobial
Tween 80 [1]	9005-65-6		Detergent Food additive

Abbreviations: CASRN = Chemical Abstract Service Registry Number. Shaded rows are substances that were on the original removed ICCVAM Proposed LLNA Reference Chemical List (September 12, 2007) when the list was revised (January 7, 2008).
[1] Included on the original ICCVAM Proposed LLNA Reference Chemical List (September 12, 2007).
[2] Included on the revised ICCVAM Proposed LLNA Reference Chemical List (January 7, 2008).

APPENDIX C

Rationale for Selection of Proposed Performance Standards Reference Substances for the Local Lymph Node Assay

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Revisions to the Draft ICCVAM List of Reference Substances for LLNA Performance Standards

Twenty substances were originally selected as proposed minimum reference substances for the LLNA performance standards. These draft LLNA performance standards were released to the public for comment on September 12, 2007 (*Federal Register* Vol. 72, No. 176, pages 52130-52131). NICEATM and ICCVAM also interacted closely with ECVAM during this period through the ICCVAM Immunotoxicity Working Group liaison, as well as the ECVAM Scientific Advisory Committee (ESAC), at their October 30-31, 2007 biannual meeting. During this meeting, the ESAC considered draft performance standards for the LLNA developed separately by ECVAM and ICCVAM, and ICCVAM recommendations for a process to achieve harmonization of the two documents. The ESAC deferred approval of the ECVAM peformance standards, and encouraged ECVAM and ICCVAM to work together to achieve harmonized performance standards. NICEATM and the IWG also sought the input of the ECVAM task force on the LLNA for additional comments and suggestions for achieving a harmonized list of reference substances.

NICEATM and ICCVAM subsequently revised the draft LLNA performance standards, including the proposed list of minimum reference substances that are provided below. As in the original draft ICCVAM performance standards, the criteria for consideration on the reference substances list was that the substances:

- Are readily available commercially
- Have available LLNA data (including SI and EC3)
- Have available guinea pig data (i.e., GPMT or BT)
- Where possible, have available human data/experience (e.g., Human Maximization Test results, Human Repeat Insult Patch Test results, available as a patch test kit allergen, and/or clinical case studies/reports).

The criteria used to narrow this list to the draft reference substances were that the list also:

- Represent the full range of responses in the LLNA, from negative to highly positive/extreme sensitizer, based on EC3 and SI ranges
- Represent a relevant range of chemistry and chemical classes
- Have an approximately equal distribution of solids and liquids
- Include consideration of substances that were proposed in draft ECVAM LLNA performance standards and/or included in JaCVAM validation studies.

The revised draft list now includes 22 substances based on the revised design of the performance analysis, where 18 required substances must be tested and produce the same response as the traditional LLNA, and four optional substances (two LLNA false negatives and two LLNA false positives) may be tested to demonstrate improved performance relative to the traditional LLNA. The revisions to the ICCVAM draft recommended performance standards reference substances for the LLNA were based on

all comments received and comparison to the ECVAM draft performance standards proposed substances. There are now 16 substances in common between the ICCVAM and ECVAM draft reference substances lists, and seven substances in common between the draft ICCVAM list and the list of substances used by JaCVAM in their recent validation efforts. **Table 1** provides the revised list of proposed ICCVAM performance standards substances.

Rationale for Exclusion of Substances from the ECVAM List or Removal of Substances from the Original ICCVAM Draft List

Table 2 details the revisions to the ICCVAM draft recommended performance standards reference substances for the LLNA based on public comments and comparison with the ECVAM draft performance standards. The original ICCVAM list represents the draft version released for public comment on September 12, 2007, and the ECVAM list represents the version discussed at the October 30-31, 2007 ESAC meeting. Based on comments received from ECVAM and additional searches by NICEATM for reference data, six substances from the original ICCVAM list were not included on the revised ICCVAM list of reference substances. These substances and the rationale for their exclusion are as follows:

- Benzoquinone: removed because no human data were located, and another substance, 5-chloro-2-methyl-4-isothiazolin-3-one was identified as an adequate replacement based the availability of concordant guinea pig and human data for this substance, and its associated history of demonstrated results in the guinea pig and human as an extreme sensitizer.
- Cinnamic aldehyde: removed in response to an ECVAM comment noting that another aldehyde (hexylcinnamic aldehyde [HCA]) was already on the list, which is also a positive control substance used in the traditional LLNA.
- Formaldehyde: removed in response to an ECVAM comment noting that another aldehyde (HCA) was already on the list. HCA has also been extensively studied as a sensitizing substance and is a positive control substance used in the traditional LLNA.
- 2-Hydroxyethyl acrylate: removed in response to an ECVAM comment that suggested this substance is unstable and is therefore susceptible to variable results.
- Nickel chloride: removed in response to the ECVAM comment that inclusion of two nickel salts is unnecessary. Nickel sulfate was favored because of the available LLNA, GP, and human data (both HMT and HPTA data), as well as the fact that the ECVAM draft list includes nickel sulfate.
- Tween 80: removed in response to an ECVAM comment that commercially available batches of Tween 80 may vary and the substance is therefore susceptible to variable results.

Three of the substances included on the ECVAM draft reference substances list but not on the original ICCVAM draft list (diethyl maleate, ethyl acrylate, and hexane) were not

included on the revised ICCVAM draft because no guinea pig test reference data were located.

Rationale for Inclusion of Substances on the Revised ICCVAM Draft List

Four of the substances included on the ECVAM draft reference substances list but not on the original ICCVAM draft list were included on the revised ICCVAM draft list. These substances are:

- Cinnamic alcohol: Included in the revised list to help achieve the goal of a reference list with a range of sensitizing potency and a variety of different chemical classes. Also has available concordant reference data for the guinea pig and human.
- Eugenol: Included in the revised list to help achieve the goal of a reference list with a range of sensitizing potency and a variety of different chemical classes. Also has available concordant reference data for the guinea pig and human, and this substance has been extensively evaluated in the traditional LLNA.
- Lactic acid: Although human data were not located for this substance, it was included in the revised list as a non-sensitizer based on available concordant guinea pig data. It was presumed to be a non-sensitizer in humans based on the fact that no clinical patch test results were located, it is not included as a patch test kit allergen, and no case reports of human sensitization were located.
- Phenyl benzoate: Included in the revised list to help achieve the goal of a reference list with a range of sensitizing potency and a variety of different chemical classes. Also has available concordant reference data for the guinea pig and human.

There were also six substances that were included on the revised draft ICCVAM list that were not included on the ECVAM list. These substances and their rationale for inclusion are as follows:

- 5-Chloro-2-methyl-4-isothiazolin-3-one: As indicated above, this substance was identified as an adequate replacement for benzoquinone based on the availability of concordant guinea pig and human data for this substance and its associated history of demonstrated results in the guinea pig and human as an extreme sensitizer.
- Chlorobenzene: Although no human data were located, it is included as a non-sensitizer based on available concordant guinea pig data. It was also presumed to be a non-sensitizer in humans based on the fact that no clinical patch test results were located, it is not included as a patch test kit allergen, and no case reports of human sensitization were located.
- Cobalt chloride: Included as a moderate sensitizer based on LLNA results with concordant guinea pig and human data. It was also included on the JaCVAM list of substances used for validation.

- Ethylene glycol dimethacrylate: Not included by ECVAM, as their list only includes one false positive substance. The revised ICCVAM list includes two false positive substances that may be tested if improved performance relative to the traditional LLNA is the goal of a validation study.
- 4-Methylaminosulfate: Included as a strong sensitizer based on LLNA results with available concordant guinea pig and human data.
- Sulfanilimide: Not included by ECVAM, as their list only includes one false negative substance. The revised ICCVAM list includes two false negative substances that may be tested if improved performance relative to the traditional LLNA is the goal of a validation study.

Table 1 ICCVAM Draft Recommended Performance Standards Reference Substances for the LLNA

Number	Chemical	CASRN	Form	Veh	EC3 (%)[1]	N[2]	0.5x - 2.0x EC3	Actual Range	LLNA vs GP	LLNA vs Human
1	5-Chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	Liq	DMF	0.009	1	0.0045-0.018	NC	+/+	+/+
2	DNCB	97-00-7	Sol	AOO	0.049	15	0.025-0.099	0.02-0.094	+/+	+/+
3	4-Phenylenediamine	160-50-3	Sol	AOO	0.11	10	0.055-0.22	0.07-0.16	+/+	+/+
4	4-Methylaminophenol sulfate	55-55-0	Sol	DMF	0.8	1	0.4-0.12	NC	+/+	+/+
5	Isoeugenol	97-54-1	Liq	AOO	1.5	49	0.77-3.1	0.5-3.3	+/+	+/+
6	2-Mercaptobenzothiazole	149-30-4	Sol	AOO	2.5	2	1.25-5.0	1.7-3.3	+/+	+/+
7	Cobalt chloride	7646-79-9	Sol	DMSO	4.8	1	2.4-9.6	NC	+/+	+/+
8	Citral	5392-40-5	Liq	AOO	9.8	2	4.9-19.6	6.6-13.0	+/+	+/+
9	HCA	101-86-0	Liq	AOO	9.9	22	5.0-19.9	4.4-14.7	+/+	+/+
10	Eugenol	97-53-0	Liq	AOO	10.1	11	5.05-20.2	4.9-15	+/+	+/+
11	Phenyl benzoate	93-99-2	Sol	AOO	13.6	3	6.8-27.2	1.2-20	+/+	+/+
12	Cinnamic alcohol	104-54-1	Sol	AOO	21	1	10.5-42	NC	+/+	+/+
13	Imidazolidinyl urea	39236-45-9	Sol	DMF	24	1	12-36	NC	+/+	+/+
14	Chlorobenzene	108-90-7	Liq	AOO	NA	1	NA	NA	-/-	-/*
15	Isopropanol	67-63-0	Liq	AOO	NA	1	NA	NA	-/-	-/+
16	Lactic acid	598-82-3	Liq	DMSO	NA	2	NA	NA	-/-	-/*
17	Methyl salicylate	119-36-8	Liq	AOO	NA	10	NA	NA	-/-	-/-
18	Salicylic acid	69-72-7	Sol	AOO	NA	1	NA	NA	-/-	-/-
Optional Substances to Demonstrate Improved Performance Relative to the Traditional LLNA										
19	Ethylene glycol dimethacrylate	97-90-5	Liq	MEK	28 (FP)	1	14-56	NC	+/-	+/+
20	Sodium lauryl sulfate	151-21-3	Sol	DMF	8.1 (FP)	5	4.05-16.2	1.5-17.1	+/-	+/-
21	Nickel sulfate	7786-81-4	Sol	DMF	NA (FN)	2	NA	NA	-/+	-/+
22	Sulfanilamide	63-74-1	Sol	DMF	NA (FN)	1	NA	NA	-/-	-/+

Abbreviations: AOO = acetone:olive oil (4:1); CASRN = Chemical Abstract Services Registry Number; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; DNCB = 2,4-dinitrochlorobenzene; FN= false negative; FP = false positive; GP = guinea pig test result; HCA = hexyl cinnamic aldehyde; Liq = liquid; LLNA = murine local lymph node assay result; MEK = methyl ethyl ketone; NA = not applicable since stimulation index < 3; NC = not calculated since n = 1; Sol = solid; Veh = vehicle

[1]Mean value where EC3 > 1 available

[2]Number of LLNA studies from which data were obtained

* = Presumed to be a non-sensitizer in humans based on the fact that no clinical patch test results were located, it is not included as a patch test kit allergen, and no case reports of human sensitization were located.

Table 2 Revisions to the ICCVAM Draft Recommended Performance Standards Reference Substances for the LLNA Based on Public Comments and Comparison the ECVAM Draft Performance Standards

Chemical	CASRN	Form	Veh	EC3 (%)[1]	N[2]	Orig I	Rev I	E	J	Rationale for Exclusion/Inclusion or Current Data Gap
Benzoquinone	106-51-4	Sol	AOO	0.01	1	X		X		No available human data
5-Chloro-2-methyl-4-isothiazolin-3-one	**26172-55-4**	**Liq**	**DMF**	**0.009**	**1**		**X**			**Concordant GP and human data**
Formaldehyde	50-00-0	Liq	Ac	0.61	1	X			X	Another aldehyde (HCA) already on the list
DNCB	**97-00-7**	**Sol**	**AOO**	**0.049**	**15**	**X**	**X**	**X**	**X**	
4-Phenylenediamine	**160-50-3**	**Sol**	**AOO**	**0.11**	**10**	**X**	**X**	**X**		
4-Methylaminophenol sulfate	**55-55-0**	**Sol**	**DMF**	**0.8**	**1**		**X**			**Concordant GP and human data**
Isoeugenol	**97-54-1**	**Liq**	**AOO**	**1.5**	**49**	**X**	**X**	**X**	**X**	
2-Mercaptobenzothiazole	**149-30-4**	**Sol**	**AOO**	**2.5**	**2**	**X**	**X**	**X**		
Cinnamic aldehyde	104-55-2	Liq	AOO	3.0	1	X				Only need HCA (since it is an OECD positive control, and also because it has been tested extensively in the standard LLNA)
Cobalt chloride	**7646-79-9**	**Sol**	**DMSO**	**4.8**	**1**		**X**		**X**	**Concordant GP and human data and also on JaCVAM list**
Diethyl maleate	141-05-9	Liq	AOO	3.9	2			X		No available GP data
Citral	**5392-40-5**	**Liq**	**AOO**	**9.8**	**2**	**X**	**X**	**X**		
HCA	**101-86-0**	**Liq**	**AOO**	**9.9**	**22**	**X**	**X**	**X**	**X**	
2-Hydroxyethyl acrylate	818-61-1	Liq	AOO	1.4	1	X				Unstable compound
Eugenol	**97-53-0**	**Liq**	**AOO**	**10.1**	**11**		**X**	**X**		
Phenyl benzoate	**93-99-2**	**Sol**	**AOO**	**13.6**	**3**		**X**	**X**		
Cinnamic alcohol	**104-54-1**	**Sol**	**AOO**	**21**	**1**		**X**	**X**		
Ethyl acrylate	140-88-5	Liq	AOO	32.4	2			X		No available GP data
Imidazolidinyl urea	**39236-45-9**	**Sol**	**DMF**	**24**	**1**	**X**	**X**	**X**		
Chlorobenzene	**108-90-7**	**Liq**	**AOO**	**NA**	**1**		**X**			**Concordant GP data***
Hexane	110-54-3	Liq	NP	NA	NP			X		No available GP data
Isopropanol	**67-63-0**	**Liq**	**AOO**	**NA**	**1**	**X**	**X**	**X**	**X**	**Case report of human sensitizer**
Lactic acid	**598-82-3**	**Liq**	**DMSO**	**NA**	**2**		**X**	**X**		**Concordant GP data***
Methyl salicylate	**119-36-8**	**Liq**	**AOO**	**NA**	**10**	**X**	**X**	**X**	**X**	
Salicylic acid	**69-72-7**	**Sol**	**AOO**	**NA**	**1**	**X**	**X**	**X**		**Concordant human and GP data**
Tween 80	9005-65-6	Liq	AOO	NA	1	X				This is a mixture and commercially available batches may vary
Ethylene glycol dimethacrylate	**97-90-5**	**Liq**	**MEK**	**28**	**1**	**X**	**X**			**ECVAM excluded to have only 1 false positive and 1 false negative in their final list. Included as 1 of 2 false positives on ICCVAM list**
Sodium lauryl sulfate	**151-21-3**	**Sol**	**DMF**	**8.1**	**5**	**X**	**X**	**X**		**Included as 1 of 2 false positives**
Nickel chloride	7718-54-9	Sol	DMSO	NA	1	X				Don't need two nickel salts
Nickel sulfate	**7786-81-4**	**Sol**	**DMF**	**NA**	**2**	**X**	**X**	**X**	**X**	**Included as 1 of 2 false negatives**
Sulfanilamide	**63-74-1**	**Sol**	**DMF**	**NA**	**1**	**X**	**X**			**Included as 1 of 2 false negatives**

Ac = acetone; AOO = acetone:olive oil (4:1); CASRN = Chemical Abstract Services Registry Number; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; DNCB = 2,4-dinitrochlorobenzene; E = Draft ECVAM Performance Standards List; GP = guinea pig test result; HCA = hexyl cinnamic aldehyde; J = JaCVAM List of substances used in non-radiolabeled LLNA validation studies; Liq = liquid; LLNA = murine local lymph node assay results; MEK = methyl ethyl ketone; NA = not applicable since stimulation index < 3; NC = not calculated since n = 1; NP = not provided in ECVAM draft performance standards; Orig I = Sep 12, 2007 ICCVAM List; Rev I = Nov 13, 2007 ICCVAM List; Sol = solid; Veh = vehicle

[1]Mean value where EC3 > 1 available

[2]Number of LLNA studies from which data were obtained

* = Presumed to be a non-sensitizer in humans based on the fact that no clinical patch test results were located, it is not included as a patch test kit allergen, and no case reports of human sensitization were located..; Bolded text = Revised ICCVAM draft performance standards reference substances (see also **Table 1**)

The candidate list used to select proposed minimum reference substances ("reference list") for the draft proposed local lymph node assay (LLNA) performance standards was initially generated from the database originally submitted to ICCVAM for the 1998 evaluation of the LLNA. This database of 209 substances was reduced to 97 candidate substances by identifying those substances for which comparative guinea pig maximization test (GPMT) or Buehler test (BT) data that were collected using a standard protocol (e.g., EPA Health Effects Test Guideline OPPTS 870.2600) were available. The availability of such data is important because any accuracy comparisons of new or revised methods must include the currently accepted regulatory test methods (i.e., in this case, the LLNA, and the GPMT and/or BT), as well as comparison to available human data and/or experience. Substances must also be readily available from commercial sources. Further limiting the list of substances to those that are readily available commercially reduced the list from 97 to 81 candidate substances. **Table 3** provides a breakdown of the impact that specific criteria had the list of candidate substances.

Table 3 Impact of Selection Criteria on Candidate List

Criteria for Substance Selection	Number of Substances
Original 1998 LLNA Database	209
Substances with LLNA and GPMT/BT data	127
Substances where GPMT/BT data collected using standard protocol	98
Substances where LLNA result was not equivocal	97
Commercially available substances	**81**

Abbreviations: BT = Buehler Test; GPMT = Guinea Pig Maximization Test; LLNA = Local Lymph Node Assay.

The candidate list was then reduced to a draft list of 22 reference substances taking into consideration, where feasible, the following criteria:

- Availability of human data
- Approximately equal distribution of solids and liquids
- Have produced consistent results and an adequate range of responses in the LLNA based on EC3[17] and Stimulation Index (SI) values.
- Consideration of substances used in the Japanese Center for the Validation of Alternative Methods (JaCVAM) validation studies (12 substances) and in the draft performance standards proposed by the European Centre for the Validation of Alternative Methods (ECVAM) LLNA (20 substances).

Table 4 provides the distribution of responses for the substances in the proposed reference list. The number of substances that have concurrent human data (i.e., human maximization test (HMT) data; included as part of a human patch test allergen (HPTA) kit; clinical case studies) also is provided. While the selection criteria included the availability of human data whenever

[17] Concentration required to induce a three-fold increase over the negative control in lymphocyte proliferation in the traditional LLNA.

possible, one substance without such data was included in order to maintain the desired dynamic range of responses, and range of physical and chemical characteristics.

Table 4 Distribution of Substances and Available Human Data for the 22 Proposed Reference Substances

LLNA	GPMT/BT	No.	No. w/ HMT, HPTA, or Other Human Data[1]	HMT only	HPTA only	Both HMT and HPTA	Other Human Data[1]
+	+	13	13	2	5	3	5
+	-	2	2	0	1	1	0
-	+	2	2	0	0	2	0
-	-	5	3*	0	1	2	0
Totals		22	20	2	7	8	5

Abbreviations: BT = Buehler Test; GPMT = Guinea Pig Maximization Test; HMT = Human Maximization Test; HPTA = Human Patch Test Allergen; LLNA = Local Lymph Node Assay; No. = Number.

* = Presumed to be a non-sensitizer in humans based on the fact that no clinical patch test results were located, it is not included as a patch test kit allergen, and no case reports of human sensitization were located;.

[1]Other human data include published reports of patch tests or case studies with the substance in question.

Table 5 provides a breakdown of the various characteristics of the proposed list of 22 substances, including EC3 ranges, physical form information, and peptide reactivity.

Table 5 Characteristics of the Proposed List of Reference Chemicals

No. Chems	Solid/ Liquid	EC3 Range	SI Range	Human Data	Peptide Reactivity (High/Mod/Min/Unk)[2]	ECVAM/JaCVAM/ Both?
2	1/1	0.009 - 0.05	22.6 - 52.3	2	0/1/0/1	1/1/1
2	2/0	0.11 - 0.8	6.7 - 75.3	2	1/0/0/1	1/0/0
5	2/3	1.5 - 9.9	8.6 - 29.5	5	1/0/1/3	3/2/1
4	3/1	10.1 – 24	5.5 -70.3	4	0/1/0/3	4/0/1
5	2/3	-	0.9 - 2.8	3	0/0/2/3	5/3/3
2	1/1	8.1 – 28	3.5 – 7	2	1/0/0/1	1/0/0
2	2/0	-	0.9	2	0/0/1/1	1/1/1
22	**13/9**	**0.009 - 28**	**0.9 - 75.3**	**20**	**3/2/4/13**	**16/7/7**

Abbreviations: Chems = Chemicals; ECVAM = European Centre for the Validation of Alternative Methods; JaCVAM = Japanese Center for the Validation of Alternative Methods; No. = Number; Min = Minimal; Mod = Moderate; SI = Stimulation Index; Unk = Unknown.

[1]Proposed potency categories based on EC3 values as proposed by Gerberick et al. (2004)

[2]Data obtained from: Gerberick et al. (2007).

The proposed list of substances includes an adequate number of correctly identified sensitizers, nonsensitizers, false positives, and false negatives, as well as a range of physicochemical properties (e.g., distribution of solids and liquids) to provide meaningful data relevant to the wide range of substances associated with this type of testing. Some of the 22 substances in the proposed reference list lacked data on peptide reactivity and/or from human testing in order to satisfy other criteria for selection or meet specific goals. For example, nickel sulfate is included on the reduced list of 22 chemicals, despite the lack of SI data, because it belongs to a chemical class (metal salts) that is not correctly identified by the traditional LLNA. This provides the opportunity for superior performance to be demonstrated by a modified LLNA.

APPENDIX D

Rationale for the Required Accuracy and Reliability Statistics Included in the Test Method Performance Evaluation (Sections 2.6 and 2.7)

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1.0 Introduction

The following text provides an overview of how the performance statistics (i.e., accuracy and reliability values) included in **Sections 2.6** and **2.7** were selected for these draft Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Murine Local Lymph Node Assay (LLNA) Performance Standards. Similar to the list of reference substances (**Appendix B**), these recommended statistics represent the culmination of interactions between ICCVAM, and the ICCVAM Immunotoxicity Working Group (IWG), which includes liaisons from the Japanese Center for Validation of Alternative Methods (JaCVAM) and the European Centre for the Validation of Alternative Methods (ECVAM), and with members the ECVAM Task Force on Skin Sensitizaton.

2.0 Test Method Accuracy

Accuracy is defined as the closeness of agreement between a test method result and an accepted reference value (ICCVAM 2003). In the draft LLNA Performance Standards released to the public for comment on September 12, 2007 (*Federal Register* Vol. 72, No. 176, pages 52130-52131), the accuracy evaluation was based on meeting or exceeding the performance to the traditional LLNA based on calculated accuracy, sensitivity, specificity, false negative and false positive rates when using the minimum list of recommended reference substances.

However, after further consideration, the ICCVAM IWG determined that a "chemical by chemical" match would be a more appropriate assessment of test method accuracy for modified LLNA protocols. Considering that the modified LLNA protocols for which the performance standards are intended would have only minor modifications to the ICCVAM (1999) LLNA protocol (as defined in **Section 2.3**), it was considered appropriate to require 100% concordance with the traditional LLNA results for a list of 18 substances. An optional list of four discordant chemicals is provided to allow for a modified LLNA protocol to demonstrate that its performance exceeds that of the traditional LLNA.

2.1 Defining ECt Ranges

As an additional measure of test method accuracy, a range of ECt values (the concentration required to achieve the defined threshold stimulation index used to distinguish between sensitizers and nonsensitizers) was included for the sensitizing substances on the reference list (these values are based on the EC3 values for each sensitizer). This provides assurance that, not only does a modified LLNA protocol achieve the correct call (i.e., sensitizer versus non-sensitizer), but that it does so at a substance dose level similar to that observed in the traditional LLNA. These performance standards include an acceptability range of 0.5x to 2.0x ECt. This range was originally proposed by ECVAM based on the personal experience of members of the ECVAM Skin Sensitization Task Force.

Prior to establishing this acceptability range, NICEATM performed several analyses in an attempt to identify a statistically derived acceptability range. These included calculating the 95% confidence intervals around the mean EC3 value and calculating logEC3 ± 2 standard deviations. These ranges take into account the number and the variability of EC3 values for each individual substance. However, a problem with the 95% confidence interval as a criterion for defining acceptable variability is that the range becomes increasingly narrower as the number of values increases, and the number of studies per compound varies widely. For the substances with a large number of available EC3 values, the resulting ranges were unacceptably narrow (e.g., isoeugenol

= 1.3 to 1.7% [n=49], see **Table 2-1**). The logEC3 ± 2 standard deviations approach accounts for the skewness associated with the actual data, and thus potentially is more appropriate. However, because of large variability coupled with a small number of EC3 values for certain substances, their calculated EC3 ranges were unacceptably large (e.g., phenylbenzoate = 0.3 to 198 [n=3], see "**Table 2-1**).

Therefore, the range of 0.5x to 2.0x EC3 was selected based on the NICEATM database of LLNA studies that includes a wide range of skin sensitizers demonstrating that EC3 values from replicate tests for a sensitizing chemical were rarely outside of this range, which agrees with the experience of the ECVAM Skin Sensitization Task Force.

Table 2-1 EC3 Values for the Proposed List of Reference Substances and Their Acceptable Ranges Based on Different Approaches[1]

Chemical	EC3 (%)[2]	N[3]	0.5x - 2.0x EC3	EC3 ± 2SD[4]	Actual Range
DNCB	0.049	15	0.025-0.099	0-2.4	0.02-0.094
4-Phenylenediamine	0.11	10	0.055-0.22	0.05-0.2	0.07-0.16
Isoeugenol	1.5	49	0.77-3.1	0.5-3.7	0.5-3.3
2-Mercaptobenzothiazole	2.5	2	1.25-5.0	0.9-6.0	1.7-3.3
Citral	9.8	2	4.9-19.6	3.6-24.2	6.6-13.0
HCA	9.9	22	5.0-19.9	5.5-16.8	4.4-14.7
Eugenol	10.1	11	5.05-20.2	4.2-21.1	4.9-15
Phenyl benzoate	13.6	3	6.8-27.2	0.3-198	1.2-20

Abbreviations: DNCB = 2,4-dinitrocholorbenzene; HCA = hexyl cinnamic aldehyde

[1]5/13 sensitizers on the ICCVAM list have only one EC3 value (i.e., only one LLNA study available) and therefore were not included in this evaluation. By comparison, 6/13 of the ECVAM sensitizers have only one EC3 value

[2]Mean EC3 value

[3]N = number of EC3 values used to calculate the EC3

[4]Log(EC3) used to generate mean and standard deviation (SD); 2SD = 2 x standard deviation

3.0 Test Method Reliability

The reliability (intralaboratory repeatability, and intra- and inter-laboratory reproducibility) of a modified LLNA protocol should meet or exceed that of traditional LLNA. In the original draft ICCVAM LLNA Performance Standards (*Federal Register* Vol. 72, No. 176, pages 52130-52131, September 12, 2007), the assessment of reliability focused on the statistics calculated for the traditional LLNA during its validation (ICCVAM 1999) as discussed in the following sections. The following sections provide these reference statistics for the traditional LLNA.

3.1 *Intralaboratory Repeatability*

Data were not available to assess intralaboratory repeatability for the traditional LLNA method and therefore comparative repeatability of a modified LLNA protocol cannot be evaluated.

3.2 *Intralaboratory Reproducibility*

During the validation of the traditional LLNA, intralaboratory reproducibility was assessed with six substances. The substances included four sensitizers (2,4-dinitrochlorobenzene [DNCB], hexyl cinnamic aldehyde [HCA], isoeugenol, and eugenol) and two non-sensitizers (methyl salicylate and benzocaine). Results are presented qualitatively and quantitatively.

As shown in **Table 3-1**, the agreement in identification of a sensitizer and non-sensitizer across three to six runs in an individual lab ranged from 83% to 100%. The results indicate that all four

1393 known sensitizers and one non-sensitizer were identified correctly in all the tests. One non-
1394 sensitizer, benzocaine, was identified as a non-sensitizer in five out of six tests.

1395

Table 3-1 Intralaboratory Reproducibility Results for Six Substances Using the Traditional LLNA

Substance	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6	Percent Agreement
2,4-Dinitrochlorobenzene	+	+	+	ND	ND	ND	100% (3/3)
Hexyl cinnamic aldehyde	+	+	+	+	+	+	100% (6/6)
Isoeugenol	+	+	+	+	ND	ND	100% (4/4)
Eugenol	+	+	+	+	+	ND	100% (5/5)
Methyl salicylate	-	-	-	-	ND	ND	100% (4/4)
Benzocaine	-	-	+/-	-	-	-	83% (5/6)

ND = Not Determined.
+ indicates a positive response, - indicates a negative response, +/- indicates an equivocal response.

Table 3-2 shows quantitative results (EC3 values; estimated concentration needed to produce an SI=3) for LLNA studies. **Table 3-2** shows that the intralaboratory reproducibility coefficient of variation (CV) for the tested substances, which ranged from 12.9% to 47.1%. In all cases, the sensitizers and non-sensitizers were correctly identified.

The original draft ICCVAM LLNA Performance Standards (*Federal Register* Vol. 72, No. 176, pages 52130-52131, September 12, 2007) stated that the modified LLNA test method should have an intralaboratory reproducibility that is equivalent to or better than the intralaboratory reproducibility of HCA, or other comparable positive control substance in the traditional LLNA (e.g., CV < 30% for HCA; see **Table 3-2**). ECt values should be derived on four separate occasions with at least one week between tests to ensure that there is no overlap between tests. However, this evaluation did not take into consideration the importance of calculating an ECt that is within an acceptable range of the historical EC3 concentration for HCA, based on traditional LLNA studies. Instead, the test method could achieve an acceptable CV that is based on EC3 concentrations that differ significantly from the historical range (i.e., the method could produce reproducible, but inaccurate results).

For this reason, the evaluation of intralaboratory reproducibility was revised to reflect the same range of acceptable EC3 concentrations that is being applied the assessment of test method accuracy (i.e., 0.5x to 2.0x ECt). An individual laboratory must now calculate ECt values for HCA with a modified LLNA protocol on four separate occasions that are within the specified range.

Table 3-2 Intralaboratory Reproducibility of EC3 Concentrations in the Traditional LLNA, as Calculated by Coefficient of Variation

Substance	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6	Mean	Standard Deviation	CV (%)
2,4-Dinitrochlorobenzene– Laboratory 1	0.05	0.03	ND	ND	ND	ND	0.040	0.01414	35.4
2,4-Dinitrochlorobenzene– Laboratory 2	0.06	0.05	ND	ND	ND	ND	0.055	0.00707	12.9
2,4-Dinitrochlorobenzene– Laboratory 3	0.04	0.06	ND	ND	ND	ND	0.050	0.01414	28.3
2,4-Dinitrochlorobenzene– Laboratory 4	0.06	0.09	ND	ND	ND	ND	0.075	0.2121	28.3
2,4-Dinitrochlorobenzene– Laboratory 5	0.03	0.06	ND	ND	ND	ND	0.045	0.02121	47.1
Hexyl cinnamic aldehyde– Laboratory 1	7.9	6.9	9.6	8.7	4.0	9.2	7.7167	2.0605	26.7
Hexyl cinnamic aldehyde– Laboratory 2	7.6	7.2	8.8	9.5	10.0	11.9	9.1667	1.7166	18.7
Isoeugenol	0.3	0.4	0.4	0.4	0.6	ND	0.420	0.10955	26.1
Eugenol	5.1	6.1	10.5	11.9	14.5	ND	9.62	1.7693	18.4
Methyl salicylate	NS	NS	NS	NS	NS	ND	-	-	-
Benzocaine	NS	NS	-	NS	NS	NS	-	-	-

Abbreviations: CV = coefficient of variation; ND = Not Determined; NS = Non-sensitizer.

3.3 *Interlaboratory Reproducibility*

The original draft ICCVAM LLNA Performance Standards (*Federal Register* Vol. 72, No. 176, pages 52130-52131, September 12, 2007) stated that a modified LLNA test method should be equally (or more) reproducible than the traditional LLNA, based on DNCB and HCA test results in the traditional LLNA (see **Table 3-3**). As shown in **Table 3-3**, the interlaboratory CVs for a range of the tested sensitizers (DNCB, HCA, isoeugenol, and eugenol) based on EC values ranged from 6.8% to 42.5%. Sodium lauryl sulfate, which is a false positive irritant, produced an interlaboratory CV of 83.7%.

Table 3-3 Interlaboratory Reproducibility of EC3 Concentrations in the Traditional LLNA, as Calculated by Coefficient of Variation

Substance	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Mean	SD	CV (%)
2,4-Dinitrochlorobenzene–Test 1	0.05	0.06	0.04	0.06	0.03	0.048	0.013	37.4
2,4-Dinitrochlorobenzene–Test 2	0.03	0.05	0.06	0.09	0.06	0.058	0.0217	27.2
Hexyl cinnamic aldehyde	7.9	7.6	8.4	7.0	8.1	7.8	0.5339	6.8
Isoeugenol	1.3	3.3	1.8	3.1	1.6	2.22	0.9149	41.2
Eugenol	5.8	14.5	8.9	13.8	6.0	9.8	4.1635	42.5
Sodium Lauryl Sulfate	13.4	4.4	1.5	17.1	4.0	8.08	6.7666	83.7

Abbreviations: CV = coefficient of variation, SD = standard deviation.

However, similar to the assessment of intralaboratory reproducibility, this evaluation also did not take into account the acceptable range of the historical EC3 values for HCA and DNCB, based on traditional LLNA studies. For this reason, the evaluation of interlaboratory reproducibility was revised to reflect the same range of acceptable EC3 values that is being applied the assessment of test method accuracy (i.e., 0.5x to 2.0x ECt). Acceptable reproducibility will now be indicated by each of at least three laboratories obtaining ECt values for HCA and DNCB that are generally within 0.5x to 2.0x the EC3 concentration (5% to 20% and 0.025 to 0.1%, respectively) as specified for these substances when tested in the traditional LLNA.