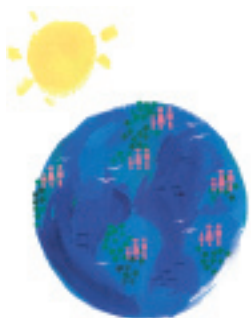


## **Recommended Performance Standards: Murine Local Lymph Node Assay**

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  
U.S. Public Health Service  
Department of Health and Human Services



**About the Interagency Coordinating Committee on the Validation of Alternative Methods  
(ICCVAM)  
and  
The National Toxicology Program Interagency Center for the Evaluation of Alternative  
Toxicological Methods (NICEATM)**

In 1997, the National Institute of Environmental Health Sciences (NIEHS), one of the National Institutes of Health, established ICCVAM to:

- Coordinate interagency technical reviews of new and revised toxicological test methods, including alternative test methods that reduce, refine, or replace the use of animals
- Coordinate cross-agency issues relating to validation, acceptance, and national and international harmonization of new, modified, and alternative toxicological test methods

On December 19, 2000, the ICCVAM Authorization Act (Public Law 106-545, 42 U.S.C. 285f-3) established ICCVAM as a permanent interagency committee of NIEHS under NICEATM.

ICCVAM consists of representatives from 15 U.S. Federal regulatory and research agencies that use, generate, or disseminate toxicological information. ICCVAM conducts technical evaluations of new, revised, and alternative methods with regulatory applicability. ICCVAM promotes the scientific validation and regulatory acceptance of toxicological test methods that more accurately assess the safety or hazards of chemicals and products and that refine (decrease or eliminate pain and distress), reduce, and/or replace animal use. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. More information about ICCVAM and NICEATM can be found on the NICEATM-ICCVAM website (<http://iccvam.niehs.nih.gov>) or obtained by contacting NICEATM (telephone: [919] 541-2384, e-mail: [niceatm@niehs.nih.gov](mailto:niceatm@niehs.nih.gov)).

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    - Agency for Toxic Substances and Disease Registry
    - National Institute of Occupational Safety and Health
  - Food and Drug Administration
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    - National Cancer Institute
    - National Institute of Environmental Health Sciences
    - National Library of Medicine
- Department of the Interior
- Department of Labor
  - Occupational Safety and Health Administration
- Department of Transportation
- Environmental Protection Agency



The NICEATM-ICCVAM graphic symbolizes the important role of new and alternative toxicological methods in protecting and advancing the health of people, animals, and our environment.

**Recommended Performance Standards:  
Murine Local Lymph Node Assay**

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

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

**National Institute of Environmental Health Sciences  
National Institutes of Health  
U.S. Public Health Service  
Department of Health and Human Services**

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## List of Abbreviations and Acronyms

ACE	Acetone
ACD	Allergic contact dermatitis
AOO	Acetone: olive oil (4:1)
BAuA	Federal Institute for Occupational Safety and Health (Germany)
BT	Buehler Test
CASRN	Chemical Abstracts Service Registry Number
CPSC	U.S. Consumer Product Safety Commission
DMF	<i>N,N</i> -dimethylformamide
DMSO	Dimethyl sulfoxide
DNCB	2,4-Dinitrochlorobenzene
dpm	Disintegrations per minute
DSA	Dose per skin area
DU	DuPont, Inc.
EC	Emulsion concentrate
EC3	Estimated concentration needed to produce a stimulation index of 3
ECPA	European Crop Protection Association
ECt	Estimated concentration needed to produce a stimulation index with a threshold other than 3, in order to distinguish between sensitizers and non-sensitizers
ECVAM	European Centre for the Validation of Alternative Methods
EFfCI	European Federation for Cosmetics Ingredients
EPA	U.S. Environmental Protection Agency
EPR	Epoxy resin
ESAC	ECVAM Scientific Advisory Committee
FCA	Freund's complete adjuvant
FDA	U.S. Food and Drug Administration
<i>FR</i>	<i>Federal Register</i>
GLP	Good Laboratory Practice
GP	Guinea pig
GPMT	Guinea Pig Maximization Test
<sup>3</sup> H	Tritiated
HCA	Hexyl cinnamic aldehyde
HMT	Human Maximization Test

HPTA	Human Patch Test Allergen
HRIPT	Human Repeat Insult Patch Test
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ILS	Integrated Laboratory Systems, Inc.
<sup>125</sup> IU	<sup>125</sup> I-iododeoxyuridine
IWG	Immunotoxicity Working Group
JaCVAM	Japanese Center for the Validation of Alternative Methods
Liq	Liquid
LLNA	Murine local lymph node assay
LNC	Lymph node cells
LOEL	Lowest observed effect level
MEK	Methyl ethyl ketone
MW	Molecular weight
NA	Not applicable
NC	Not calculated
ND	No data
NICEATM	NTP (National Toxicology Program) Interagency Center for the Evaluation of Alternative Toxicological Methods
NIH	U.S. National Institutes of Health
NOEL	No observed effect level
NTP	U.S. National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
PBS	Phosphate-buffered saline
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SC	Suspension concentrate
SD	Standard deviation
SI	Stimulation index
Sol	Solid
TCA	Trichloroacetic acid
TG	Test Guideline
U.K.	United Kingdom
U.S.	United States
Veh	Vehicle



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## Preface

Allergic contact dermatitis (ACD) is an adverse health effect that frequently develops in workers and consumers exposed to skin-sensitizing chemicals and products. ACD results in lost workdays<sup>1</sup> and can significantly diminish quality of life (Hutchings et al. 2001; Skoet et al. 2003). To minimize the occurrence of ACD, regulatory authorities require testing to identify substances that may cause ACD. Sensitizing substances must be labeled with a description of the potential hazard and the precautions necessary to avoid development of ACD.

Skin sensitization testing has typically required the use of guinea pigs (Buehler 1965; Magnusson and Kligman 1970). However, in 1999, the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended the murine (mouse) local lymph node assay (LLNA) as a valid test method to assess the skin sensitization potential of most types of substances (ICCVAM 1999). ICCVAM concluded that the LLNA (referred to herein as the “traditional LLNA”) provided several advantages compared to the guinea pig method, including elimination of potential pain and distress, use of fewer animals, less time required to perform, and availability of dose-response information. United States and international regulatory authorities subsequently accepted the traditional LLNA as an alternative test method for ACD testing. It is now commonly used around the world.

While the traditional LLNA has many advantages, it does require the use of a radioactive marker to measure cell proliferation in lymph nodes. To avoid the use of radioactive markers, scientists have recently developed several non-radioactive versions of the LLNA. In 2007, the U.S. Consumer Product Safety Commission (CPSC) asked ICCVAM and the National Toxicology Program Interagency Center for the Evaluation of Alternative Methods (NICEATM) to evaluate the scientific validity of these non-radioactive versions.<sup>2</sup>

When ICCVAM evaluated the LLNA in 1999, the concept of performance standards had not been developed. ICCVAM subsequently defined performance standards and described a process for their development (ICCVAM 2003). Performance standards provide criteria on which to evaluate the validity of functionally and mechanistically similar test methods. They are based on adequately validated and accepted test methods (e.g., the LLNA). Performance standards also specify test method components that must be included in modified versions in order to use the performance standards for their validation.

Following the CPSC nomination, ICCVAM decided to develop performance standards for the traditional LLNA that could be used to more rapidly and efficiently determine the validity of non-radioactive and other modified versions. NICEATM provided scientific and operational support for the ICCVAM Immunotoxicity Working Group (IWG), and scientists from the Japanese Center for Validation of Alternative Methods (JaCVAM) and the European Centre for the Validation of Alternative Methods (ECVAM) served as IWG liaisons. A detailed timeline of the development process for the LLNA performance standards is provided in the final Recommended Performance Standards document. Public

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<sup>1</sup> <http://www.bls.gov/IIF>

<sup>2</sup> [http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC\\_LLNA\\_nom.pdf](http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf)

comments were solicited and considered by the IWG and ICCVAM throughout the development process.

ICCVAM released initial draft LLNA Performance Standards to the public for comment on September 12, 2007 (72 FR 52130).<sup>3</sup> ICCVAM considered comments from the public and from an ECVAM workshop on non-radioactive LLNA methods and published revised draft LLNA Performance Standards in January 2008. In March 2008, ICCVAM and NICEATM convened an international independent scientific peer review panel (hereafter “Panel”) in public session to evaluate the revised draft ICCVAM LLNA Performance Standards. The Panel’s report was made available to the public and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) for comment.

Our colleagues at ECVAM and JaCVAM agreed to work with us to develop internationally harmonized LLNA performance standards. The IWG, along with the ECVAM and JaCVAM liaisons, met several times during the summer of 2008 to further revise the performance standards and to consider the comments from the Panel, the public, and SACATM. On September 23–24, 2008, ECVAM hosted a harmonization meeting to analyze and reach consensus on the remaining issues.

An important issue addressed was the number of mice used per dose group. Organisation for Economic Co-operation and Development Test Guideline 429, which describes the LLNA procedure, specifies that at least four animals per dose group must be used when lymph nodes from all animals in the dose group are pooled into one sample but requires that at least five animals per dose group be used when individual animal data are collected and analyzed. Due to animal-use regulations that require the minimum number of animals be used in studies, the Guideline has led to only pooled data being collected in many countries, a practice considered inadequate by some regulatory authorities and discouraged by ICCVAM. NICEATM analyzed LLNA data from 83 LLNA studies (275 dose groups) from six different laboratories, which showed that the use of four animals rather than five per dose group was not likely to change the hazard-classification outcome. Based on these data, ICCVAM concluded that the number of animals per dose group could be reduced from five to four when collecting individual animal data.

ICCVAM will now forward these harmonized Recommended Performance Standards for the LLNA to U.S. regulatory authorities for their approval. At its November 4-5, 2008, meeting, the ECVAM Scientific Advisory Committee endorsed their corresponding ECVAM LLNA performance standards, on which ECVAM collaborated extensively with ICCVAM to harmonize. Approved performance standards can be used by validation organizations (e.g., ICCVAM, ECVAM, and JaCVAM) and others to assess the validity of non-radioactive and other new versions and applications of the LLNA proposed for regulatory safety testing. We anticipate that these performance standards will help promote development and validation of non-radioactive LLNA methods and other innovative approaches. These new versions are expected to lead to broader use of the LLNA, which will further reduce and refine animal use for ACD testing.

We gratefully acknowledge the many organizations and scientists who provided data and information necessary to develop these LLNA performance standards. We thank the

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<sup>3</sup> [http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR\\_E7\\_18011.pdf](http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_18011.pdf)

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## Executive Summary

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended the murine (mouse) local lymph node assay (traditional LLNA<sup>4</sup>) as a valid test method to assess most types of substances for their potential to cause skin sensitization (ICCVAM 1999). United States and international regulatory authorities subsequently accepted the traditional LLNA as an alternative test method for ACD testing. It is now commonly used around the world.

Before a new test method such as the LLNA is accepted for regulatory testing, validation studies are conducted to assess its reliability (how well its results are reproduced within and across among laboratories (intra- and interlaboratory reproducibility) and its relevance (ability to correctly predict or measure the biological effect of interest) (OECD 1996, 2005; ICCVAM 1997, 2003). When a new test method is considered to have adequate relevance and reliability for regulatory testing purposes, ICCVAM develops and recommends performance standards based on the adequately validated reference test method. Such performance standards provide criteria for more efficiently evaluating the validity of test methods that are similar in function and mechanism to the reference test method.

When ICCVAM evaluated the LLNA in 1999, the concept of performance standards had not yet been developed. ICCVAM subsequently defined performance standards and described a process for their development (ICCVAM, 2003). Recognizing a need for LLNA performance standards, ICCVAM recently completed the development of performance standards for the LLNA so they can be used to evaluate the validity of modified versions of the traditional LLNA. The performance standards consist of (1) essential test method components, (2) reference substances, and (3) standards for accuracy and reliability that the proposed test method should meet or exceed.

ICCVAM recently updated its recommended LLNA test method protocol, and this was the key reference used to establish these LLNA performance standards. The updated LLNA test method protocol is appended to this document. ICCVAM revised the original ICCVAM protocol to include (1) guidance on when it may be appropriate to reduce the number of positive control animals, including statistical analysis to justify the reduction; (2) reduction in the minimum number of animals per dose group to four rather than the previous minimum of five; and (3) detailed guidance on evaluating local irritation and systemic toxicity to ensure that the appropriate highest dose is tested. The updated ICCVAM-recommended test method protocol for the LLNA is based on evaluation of extensive additional data and experience gained since the original evaluation in 1998.

### Essential Test Method Components

To be considered functionally and mechanistically similar to the traditional LLNA, a modified LLNA test method protocol must include the following components to ensure that the same biological effect is being measured:

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<sup>4</sup> “Traditional LLNA” refers to the validated ICCVAM-recommended LLNA test method protocol (ICCVAM 1999), which measures lymphocyte proliferation based on incorporation of tritiated methyl thymidine into the cells of lymph nodes draining the site of test substance application.

- The test substance must be applied topically to both ears of the mouse.
- Lymphocyte proliferation must be measured in the lymph nodes draining the site of test substance application.
- Lymphocyte proliferation must be measured during the induction phase of skin sensitization.
- The highest dose selected must be the maximum soluble concentration that does not induce systemic toxicity and/or excessive local irritation.
- A vehicle control must be included in each study, and, where appropriate, a positive control should also be used.
- A minimum of four animals per dose group must be included.
- Either individual or pooled animal data may be collected. [Note: Collection of individual animal data is recommended by ICCVAM, and also required by several regulatory authorities]

If any of these criteria are not met, then these performance standards cannot be used for validation of the modified test method.

These essential test method components have been internationally harmonized for the validation of modifications to the traditional LLNA. Test method users should be aware that certain national regulatory authorities might differ in their requirements for use of a modified LLNA test method to support regulatory submissions. For example, U.S. regulators require the following:

- As the high dose, the maximum soluble concentration that does not produce systemic toxicity and/or excessive local irritation
- Collection of individual animal data
- A concurrent positive control included in each LLNA study

The performance standards provided in this document apply to the traditional LLNA test method protocol and to LLNA test method protocols with modifications that do not affect their functional and mechanistic similarity to the traditional LLNA test method protocol. However, the modified test method protocol must incorporate the essential components listed above. Modifications must be detailed and scientifically rationalized and justified; and the modified test method must perform as well as or better than the traditional LLNA. Rationale for such changes should include descriptions of and the basis for the criteria used to distinguish between sensitizers and nonsensitizers.

### **Reference Substances**

Using established selection criteria, ICCVAM narrowed the initial database of more than 200 substances to a final list of 18 minimum reference substances for the LLNA performance standards. The criteria were: (1) the substances should be readily available from a commercial source; (2) LLNA data from guinea pig tests and (where possible) data from humans should be available for each substance; and (3) the minimum list of reference substances represent the types of substances typically tested for skin sensitization potential and the range of responses that can be assessed in the LLNA. Reference substances proposed

in draft LLNA Performance Standards by the European Centre for the Validation of Alternative Methods or included in validation studies by the Japanese Center for the Validation of Alternative Methods were also considered. To provide the opportunity to demonstrate performance equal to or better than that of the traditional LLNA, ICCVAM included four optional substances (substances that produced either false positive or false negative results in the LLNA when compared to either human or guinea pig results).

#### **Test Method Performance Standard: Accuracy**

The accuracy of a modified LLNA test method should meet or exceed that of the traditional LLNA when evaluated using the 18 minimum recommended reference substances, which have data from traditional LLNA and guinea pig tests. The proposed test method should result in the correct classification based on a “yes/no” decision. However, the modified test method might not correctly classify all of the reference substances on the minimum list. If, for example, one of the weak sensitizers were misclassified, a rationale for the misclassification and appropriate additional data (e.g., test results that provide correct classifications for other substances with physical, chemical, and sensitizing properties similar to those of the misclassified reference substance) could be considered to demonstrate equivalent performance. Under such circumstances, the validation status of the modified LLNA would be evaluated on a case-by-case basis.

#### **Test Method Performance Standard: Reliability**

Test method reliability is the degree to which a test method can be performed consistently/uniformly within (*intralaboratory reproducibility*) and among (*interlaboratory reproducibility*) laboratories over time. To determine intralaboratory reproducibility, a modified LLNA test method should be assessed using a sensitizing substance that is well characterized in the traditional LLNA. Therefore, the LLNA performance standard is based on the variability of results from repeated tests of hexyl cinnamic aldehyde (HCA).

Assessing the reliability of a modified test method requires calculating the estimated concentration needed to produce a stimulation index with a specific threshold value (an EC<sub>t</sub> value) in order to distinguish between sensitizers and non-sensitizers. To assess intralaboratory reliability, EC<sub>t</sub> values for HCA should be derived on four separate occasions with at least one week between tests. Acceptable intralaboratory reproducibility is indicated by a laboratory’s ability to obtain, in each HCA test, EC<sub>t</sub> values between 5% and 20%, which represents the range of 0.5x to 2.0x the mean EC<sub>3</sub> specified for HCA (10%) in the traditional LLNA.

Interlaboratory reproducibility of a modified LLNA test method should be assessed using two sensitizing substances that are well characterized in the traditional LLNA. The LLNA performance standard is based on the variability of results from tests of HCA and 2,4-dinitrochlorobenzene (DNCB) in different laboratories. EC<sub>t</sub> values should be derived independently from a single study conducted in at least three separate laboratories. To demonstrate acceptable interlaboratory reproducibility, each laboratory must obtain EC<sub>t</sub> values of 5% to 20% for HCA and 0.025% to 0.1% for DNCB, which represents the range of 0.5x to 2.0x the mean EC<sub>3</sub> concentrations specified for HCA (10%) and DNCB (0.05%), respectively, in the traditional LLNA.

### **Using the Performance Standards**

Test method developers are encouraged to consult directly with ICCVAM before using these performance standards to conduct a validation study of a modified LLNA test method. Developers are also encouraged to submit results of validation studies to ICCVAM for an evaluation of the validation status. Upon completing its evaluation in accordance with the ICCVAM Authorization Act (Public Law 106-545, 42 United States Code 285I-3<sup>5</sup>), ICCVAM will forward recommendations to ICCVAM agencies regarding the usefulness and limitations of the test method.

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<sup>5</sup> Available at [http://iccvam.niehs.nih.gov/docs/about\\_docs/PL106545.pdf](http://iccvam.niehs.nih.gov/docs/about_docs/PL106545.pdf)



## 1.0 Purpose and Background of Performance Standards

### 1.1 Introduction

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 interlaboratory reproducibility), and its relevance (i.e., the ability of the test method to correctly predict or measure the biological effect of interest) (OECD 1996, 2005; 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 accuracy and reliability for a specific testing purpose. These performance standards can then be used to evaluate the accuracy and reliability of other proposed test methods that are considered functionally and mechanistically similar to the accepted test method. They also allow such test methods to be evaluated with a reduced set of reference substances and tested in a minimum number of laboratories.

These test method performance standards are proposed so that modified versions of the murine local lymph node assay (LLNA) that are mechanistically and functionally similar<sup>6</sup> to the “traditional LLNA”<sup>7</sup> (ICCVAM 1999) can be effectively and efficiently evaluated for their validity 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], and the Japanese Center for Validation of Alternative Methods [JaCVAM]) or other organizations. The LLNA test method protocol recommended by ICCVAM (ICCVAM 1999) and accepted by U.S. regulatory agencies is consistent with procedures described in Test Guideline (TG) 429 of the Organisation for Economic Co-operation and Development (OECD; OECD 2002) and was used as the basis for development of the TG. However, the TG allows for much more procedural variation than the ICCVAM-recommended test method protocol. Therefore, the ICCVAM-recommended test method protocol is the key reference used for establishing these performance standards (**Appendix A**).<sup>8</sup> The test method protocol has been revised to recommend a minimum of four individual animals rather than five individual animals per group. This was based on an evaluation of data from 83 LLNA studies (275 dose groups) from six different laboratories, which indicated that a reduction in the sample size from five to four animals per group is unlikely to have a significant impact on the results of an LLNA study (**Appendix B**). This change is important since most animal-use regulations require that the minimum number of animals be used in studies. Because OECD TG 429 specifies four animals per group when pooled data are collected and five animals per group when individual animal data are collected, only pooled data have been collected in many countries.

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<sup>6</sup> Components of the traditional LLNA that a mechanistically and functionally similar modified LLNA test method protocol would need to include are summarized in **Section 2.2**.

<sup>7</sup> The “traditional LLNA” refers to the validated ICCVAM LLNA test method protocol (ICCVAM 1999), which measures lymphocyte proliferation based on incorporation of tritiated methyl thymidine into the cells of the draining lymph nodes.

<sup>8</sup> **Appendix A** includes an updated version of the validated ICCVAM-recommended LLNA test method protocol (ICCVAM 1999; Dean et al. 2001), which reflects the conclusions and recommendations of an ICCVAM Independent Scientific Peer Review Panel convened in March 2008 (see [http://iccvam.niehs.nih.gov/methods/immunotox/llna\\_PeerPanel.htm](http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel.htm)).

Modified LLNA test method protocols are expected to achieve a level of performance that is equivalent to or exceeds the accuracy and reliability of the traditional LLNA for identifying human sensitizers. All procedural modifications should be accompanied by a scientific rationale and the modified test method protocol should incorporate the essential test method components summarized in **Section 2.2** and provided in detail in **Appendix C**.

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.

Furthermore, as more experience with the LLNA is gained and additional skin sensitization data (i.e., LLNA, guinea pig, and/or human) become available these performance standards may be updated to incorporate new information on the usefulness and limitations of the LLNA for distinguishing between sensitizers and non-sensitizers.

## 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:

- **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 test method. However, since ICCVAM evaluated the LLNA (ICCVAM 1999) prior to establishing a process for developing performance standards, they were not developed for this test method. Accordingly, ICCVAM is now providing performance standards for the LLNA to support the development and validation of proposed modifications of the traditional LLNA.

A detailed timeline associated with the development of these performance standards is provided in **Appendix D**. ICCVAM released draft LLNA Performance Standards to the public for comment on September 12, 2007. After consideration of these comments, a revised version was made available on January 8, 2008, to the ICCVAM Independent Scientific Peer Review Panel (hereafter, “Panel”) for consideration at a public meeting that was convened on March 4-6, 2008, at the Consumer Product Safety Commission Headquarters in Bethesda, MD. The revised draft LLNA Performance Standards were also made available to the public for comment before the Panel meeting, and all comments were provided to the Panel for their consideration. The Panel’s conclusions and recommendations were made available to the public and to ICCVAM’s Scientific Advisory Committee on Alternative Toxicological Methods (SACATM).

The Panel’s report and all comments by the public and SACATM were considered by the ICCVAM Immunotoxicity Working Group and ICCVAM in preparing final LLNA performance standard recommendations for public release and submittal to U.S. Federal agencies. Performance standards adopted by U.S. Federal regulatory authorities can be provided or referenced in test guidelines. Availability of these performance standards and ICCVAM test method evaluation reports, which provide ICCVAM recommendations and a comprehensive evaluation of the usefulness and limitations of a test method, are announced in the *Federal Register*, in National Toxicology Program newsletters, and by email to ICCVAM email list members. Additional details regarding the chronology of updates to the ICCVAM LLNA performance standards and the specific changes that were made during this process can be found in **Appendix E**.

## **1.4 ICCVAM Development of a Performance Standard 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 depends on a substance penetrating the epidermis and subsequently binding to proteins. The antigen-presenting cells in the skin (i.e., Langerhans cells) can then process the resulting hapten complex. These cells then migrate to the draining lymph nodes, where the antigen is presented to T lymphocytes, leading to their antigen-specific clonal expansion and the production of 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).

ACD is a frequent occupational and environmental health problem. According to the U.S. Department of Labor Bureau of Labor Statistics, in 2005, 980 cases of ACD involved days

away from work.<sup>9</sup> Furthermore, ACD has also been shown to have a significant impact on quality of life in the population group affected (Hutchings et al. 2001; Skoet et al. 2003).

#### 1.4.2 Test Methods for Assessing Skin Sensitization

There are several test methods currently recognized 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 6 to 8 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 place with a dressing for 24 hours. Skin reactions (i.e., erythema, edema) are scored 24 and 48 hours after patch removal (OECD 1992).

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 6 hours over a period of 3 weeks. Two weeks after the final treatment, a patch containing the test substance is applied for 6 hours at a location different from where the initial challenges occurred. Skin reactions (i.e., erythema and edema) are then scored 24 and 48 hours after patch removal (OECD 1992).

#### 1.4.3 Intended Regulatory Uses for the LLNA

The LLNA can be used as a substitute for the traditional guinea pig tests (e.g., GPMT, BT),<sup>10</sup> where appropriate, for assessing skin sensitization. The LLNA may not be suitable for use with certain types of test materials, such as mixtures, metal compounds (particularly nickel), strong dermal irritants, and chemicals whose pharmacodynamic activity is to release dermal cytokines that cause local lymph node proliferation (e.g., certain pharmaceuticals such as imiquimod [Gaspari 2007]). Additionally, the LLNA may not be suitable for test substances that do not adhere for an acceptable length of time when applied to the dorsum of the ear during the experiment. Data to support testing of mixtures using the LLNA is currently under evaluation by ICCVAM.

#### 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. Comparatively, the guinea pig tests described in **Section 1.4.2** involve rating skin reactions evoked by the test substance, which are part of the elicitation phase of skin sensitization. The guinea pig tests therefore allow for an assessment of the entire ACD process.

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<sup>9</sup> <http://www.bls.gov/IIF>

<sup>10</sup> 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.

While the endpoints measured in the LLNA and the guinea pig 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 guinea pig test methods use subjective scoring of the skin reaction (i.e., erythema, edema) observed after test substance application.

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## 2.0 LLNA Performance Standards

### 2.1 Background

The LLNA has undergone validation studies that have demonstrated its usefulness and limitations for distinguishing between sensitizers and non-sensitizers (ICCVAM 1999). However, the 1999 evaluation determined that, while the LLNA could be used in most testing situations, certain substances might not be suitable for use with the LLNA. These include:

- Metal compounds: may produce inaccurate results and limited data are 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
- Mixtures: limited data available

ICCVAM recently obtained and is currently evaluating available LLNA data on mixtures. These performance standards may be updated to incorporate any new information on the usefulness and limitations of the LLNA for assessing the ACD potential of mixtures.

The following section briefly describes the principles of the LLNA test method, followed by the recommended performance standards that would be used to evaluate test methods that are functionally and mechanistically similar to the traditional LLNA. The performance standards consist of (1) essential test method components, (2) reference substances, and (3) the comparable accuracy and reliability that should be achieved or exceeded.

### 2.2 LLNA Essential Test Method Components and Other Validation Considerations

Certain principles are important in delineating the essential test method components that determine whether a modified test is functionally and mechanistically similar to the traditional LLNA. In the LLNA, the induction phase of contact hypersensitivity is characterized by lymphocyte proliferation and hyperplasia in the lymph nodes draining the site of topical exposure (Sikorski et al. 1996). Because test substances are applied topically to the ear, the lymphocytes in the draining auricular lymph nodes are collected for evaluation. In the traditional LLNA, the amount of incorporated radioactivity is indicative of the number of proliferating cells in the draining auricular lymph nodes. Potential skin sensitizers are identified by calculating the ratio of radioactivity incorporated into the DNA of cells of the auricular lymph nodes after topical application of a potential chemical sensitizer to that obtained after topical application of the test vehicle.

#### 2.2.1 Essential Test Method Components

The essential test method components for the validation of modifications to the traditional LLNA applicable to these performance standards, using the 18 required reference substances, are summarized as follows and are provided in detail in **Appendix C**.

1. The test substance must be applied topically to both ears of the mice.
2. Lymphocyte proliferation must be measured in the lymph nodes draining the site of test substance application.

3. Lymphocyte proliferation must be measured during the induction phase of skin sensitization.
4. For test substances, the highest dose selected must be the maximum soluble concentration that does not induce systemic toxicity and/or excessive local irritation. For positive control substances, the highest dose selected should exceed the known EC3 values (i.e., the estimated concentrations needed to produce a stimulation index [SI] of 3) of the reference substances without producing systemic toxicity and/or excessive local irritation.
5. A vehicle control must be included in each study and, where appropriate, a positive control should be used.
6. A minimum of four animals per dose group is required.
7. Either individual or pooled animal data may be collected.

In order for a modified LLNA test method protocol to be considered functionally and mechanistically similar to the traditional LLNA, the above characteristics are essential to ensure that the same biological effect is being measured accurately. If any of the criteria are not met, then these performance standards are not applicable to validation of the modified test method. For example, these performance standards would not be applicable to the popliteal lymph node assay (Pieters 2000).

These essential test method components have been internationally harmonized for the validation of modifications to the traditional LLNA. Test method users should be aware that certain national regulatory authorities might have requirements that differ from these essential test method components for the prospective use of a modified LLNA method in support of regulatory submissions. For example, U.S. regulators require the following:

1. As the high dose: the maximum soluble concentration that does not produce systemic toxicity and/or excessive local irritation
2. Collection of individual animal data
3. A concurrent positive control included in each LLNA study

### 2.2.2 Other Validation Considerations

Additional points to consider during the validation of modified LLNA test methods applicable to these performance standards, using the 18 required reference substances, are summarized as follows and are provided in detail in **Appendix C**.

1. Consideration should be given to running concurrently a mix of negative, weakly, and strongly positive substances from the reference substance list so that the strongly positive substance can act as a positive control for the weaker skin sensitizer.
2. Group housing is recommended; otherwise animal selection, preparation, housing, and feeding should be in accordance with OECD TG 429 in compliance with other relevant regulatory requirements (e.g., animal care and use).
3. Appropriate quality assurance systems (i.e., in accordance with Good Laboratory Practice guidelines, e.g., OECD 1998; EPA 2006a, 2006b; FDA 2006) are required.



4. The study should be conducted according to international validation principles (OECD Guidance Document 34 [OECD 2005]) and in compliance with other relevant regulatory requirements (e.g., animal care and use).

### 2.2.3 LLNA Test Method Protocol Modifications

These performance standards also apply to LLNA test method protocols that include modifications that do not impact on its functional and mechanistic similarity to the traditional LLNA test method protocol (**Appendix A**) provided that (1) the modified test method protocol incorporates the essential test method components described in detail in **Appendix C**, (2) such modifications are detailed and scientifically rationalized and justified, and (3) the performance of the modified test method is equal to or better than that determined for the traditional LLNA (see **Section 2.4**). Rationale for such changes should include a description of the decision criteria used to distinguish between sensitizers and non-sensitizers, and the basis for the decision criteria. In the traditional LLNA, an SI of 3 or greater is used to identify a skin-sensitizing agent (ICCVAM 1999). However, a threshold SI may be other than 3 for modified LLNA test method protocols that use a different methodology for measuring lymph node cell proliferation. In such cases, the dose of a test substance at the revised threshold limit would be other than an EC<sub>3</sub> and would therefore be defined as an EC<sub>t</sub> (i.e., the estimated concentration needed to produce an SI with a threshold other than 3).

A proposed minimum of 18 substances are selected as reference substances (i.e., required) with four optional reference substances for the LLNA performance standards. If the modified LLNA test method protocol, like the traditional LLNA (ICCVAM 1999), still uses a decision criterion of  $SI \geq 3$ , the 18 required substances could then be used to determine its validation status. If a different decision criterion (i.e.,  $SI \text{ not } \geq 3$ ) is used, additional testing will be required, the extent (i.e., number and types of substances) of which will be determined on a case-by-case basis, depending on the magnitude of the change in the decision criterion.

Test method developers are encouraged to consult directly with ICCVAM prior to conducting a validation study on modified LLNA methods in accordance with these performance standards. Following completion of a validation study using these performance standards, developers are also encouraged to submit results of studies to ICCVAM for an evaluation of the validation status. In accordance with the ICCVAM Authorization Act, upon completion of its evaluation, ICCVAM will forward recommendations on the validity of the test method to ICCVAM agencies, including adequacy of the test method with regards to these performance standards. Also in accordance with the ICCVAM Authorization Act, the regulatory agencies will determine the acceptability of the test method based on their specific regulatory needs and requirements. Before submitting it to a regulatory agency, test method developers should complete a validation review of the data using the modified test method protocol. Doing so will reduce the possibility of the regulatory agency deeming the data unacceptable or unpersuasive.

Although the SI decision criterion is the one most often used to distinguish between sensitizers and non-sensitizers, 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.

### 2.2.3.1 Calculation of EC<sub>t</sub>

The reliability assessment of a modified LLNA test method protocol requires calculation of an EC<sub>t</sub>. Acceptable reproducibility will be demonstrated by each laboratory obtaining EC<sub>t</sub> values that are generally within 0.5x to 2.0x the mean EC<sub>3</sub> concentration specified for the substance tested. The ICCVAM LLNA test method protocol (ICCVAM 1999) does not include guidance on the calculation of an EC<sub>t</sub>, which is therefore described below.

The method for determining the EC<sub>t</sub> 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 decision threshold is an SI of 3:

If the data points lying immediately above and below the SI value of 3 have the coordinates (a, b) and (c, d) respectively, then the EC<sub>3</sub> value may be calculated using the equation:  $EC_3 = c + [(3 - d)/(b - d)](a - c)$  (Basketter et al. 1999c).

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.

### 2.2.4 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. Test animals
  - Mouse strain used<sup>11</sup>
  - Number, age, and sex of animal used
  - Microbiological status of the animal, when information is available
  - Source of animal, housing conditions, diet, etc.
3. Description of the test method and protocol used to measure lymphocyte proliferation and justification for its use

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<sup>11</sup> Female CBA/Ca or CBA/J mice are currently recommended. The use of male CBA mice, or female or male mice of other strains would only be accepted if it can be adequately demonstrated that these animals perform in the LLNA as well as female CBA mice.

4. Test method conditions
  - Details on test substance preparation and application
  - Justification for dose selections, including basis for the highest dose tested (see **Appendix A**). The reason for variation away from traditional dose-selection process, if any, should be discussed.
5. Criteria for an acceptable test
  - Positive control data
  - Negative/vehicle control data
  - Laboratory-specific historical ranges of positive and negative control data. A robust historical dataset should include at least 10 independent tests conducted within a reasonable period of time (i.e., less than one year) with a minimum of four animals each per negative and positive control groups.
  - Exclusion criteria should be defined and the impact of any excluded data should be described.
6. Results
  - Weights of each animal at the start of the test and the time of lymph node collection
  - During the collection of individual animals, tabulation of data from the individual animals showing the mean and individual values for each dose (including vehicle and, where applicable, 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 EC<sub>t</sub> value, if applicable<sup>12</sup>) should be provided for all test substances and concurrent controls.
  - Statistical analysis and/or evaluation of the dose-response relationship, where appropriate
7. 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).
8. 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

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<sup>12</sup> An EC<sub>t</sub> would only be calculated where an SI greater than or equal to the defined threshold was generated.

results for classification of substances as skin sensitizers, such information should be provided.

9. Conclusions

10. If GLP-compliant studies are performed, then additional reporting requirements 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.

## 2.3 Minimum List of Reference Substances for Methods Assessing Lymphocyte Proliferation

### 2.3.1 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

### 2.3.2 Characteristics of Selected Reference Substances

The validity of the traditional LLNA was supported with test data for 211 substances. After careful consideration of the above criteria, 18 substances were selected as proposed minimum reference substances for the LLNA performance standards. An additional four “optional” substances (i.e., these substances were either false positive or false negative in the LLNA when compared to either human or guinea pig results) are also included to provide the opportunity for demonstrating equivalent or superior performance to the traditional LLNA.

The proposed substances are listed in **Appendix F**, and a detailed rationale for selection of the substances in this list is included in **Appendix E**. The selected substances have the following characteristics:

- Twenty-one of the 22 substances have data from testing in the GPMT or BT.

- Twenty of the 22 substances have human data (e.g., Human Maximization Test results, Human Repeat Insult Patch Test results, and/or clinical case studies/reports) or are used as a patch test kit allergen.
- 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.
- The selected substances include 10 solids and 12 liquids.
- The molecular weights of the substances range from 60.095 g/mol to 388.294 g/mol.
- The octanol: water partition coefficient values (Wang et al. 2000) of the substances range from -8.3 to 4.8 (from water-soluble to insoluble, respectively).
- The vehicles used for all of the substances are known. The vehicles used were acetone: olive oil (14 substances), dimethyl formamide (4 substances), dimethyl sulfoxide (3 substances), and methyl ethyl ketone (1 substance).
- Peptide reactivity information, which is available for 10 substances, ranges from minimal to high.
- The EC3 values of the positive substances range from 0.009% to 95.8%, based on results from the traditional LLNA.
- The selected substances have a wide range of SI values, from 3.1 to 43.9 for substances identified as skin sensitizers by the traditional LLNA, and 0.9 to 2.9 for substances identified as non-sensitizers by the traditional LLNA.

Studies using the proposed reference substances should be evaluated in the vehicle with which they are listed in **Appendix F**.

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 2.3.1**) may be used.

## 2.4 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 the accuracy and reliability characteristics based on the performance of the traditional LLNA (ICCVAM 1999) for the indicated reference substances; the rationale for the selection of the performance statistics is described in detail in **Appendix E**.

### 2.4.1 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 F**). Therefore, for the 18 substances with concordant traditional LLNA and guinea pig data (referred to as “required substances”), the

proposed test method should result in the correct classification based on a “yes/no” decision. However, there is a possibility that the modified test method might not correctly classify all the required substances. If, for example, one of the weak sensitizers were to be misclassified, a rationale for the discordance and appropriate additional data (e.g., test results that provide the correct classification for other substances that have similar physical, chemical, and sensitizing properties as the reference substance that was misclassified) could be considered to demonstrate equivalent performance. Therefore, an evaluation of the validation status of the modified LLNA would be on a case-by-case basis. This provision is included since the classification of three out of the five sensitizers among the required reference substances with an EC<sub>3</sub> > 10% (i.e., suggesting that they are “weak” sensitizers) is based on only one LLNA study for each of the three substances. Therefore, the likelihood of obtaining a negative result if any of these three substances were retested in the traditional LLNA is not known.

#### 2.4.2 Reliability

*Test method reliability* (intralaboratory repeatability and intra- and interlaboratory 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 indication of the inherent biological variability can be obtained by comparing the results for individual test animals administered the same dose of a test substance within the same study.

*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. *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. Intra- and interlaboratory reproducibility for the traditional LLNA are summarized in **Appendix E**.

##### 2.4.2.1 Intralaboratory Repeatability

No standard is proposed.

##### 2.4.2.2 Intralaboratory Reproducibility

Intralaboratory reproducibility can be assessed by calculating the variability resulting from testing hexyl cinnamic aldehyde (HCA). EC<sub>t</sub> 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 test, EC<sub>t</sub> values for HCA that are within 0.5x to 2.0x (5% to 20%) the mean EC<sub>3</sub> (10%) specified for HCA in **Appendix F**. Because the target EC<sub>3</sub> is provided, as few as two dose groups can be used (instead of at least three dose groups, as would be required when testing an unknown substance) since calculation of an EC<sub>3</sub> would use only doses that bracket the target EC<sub>3</sub> value (i.e., one dose above and one dose below).

#### 2.4.2.3 Interlaboratory Reproducibility

Interlaboratory reproducibility should be evaluated with at least two sensitizing chemicals with well-characterized activity in the traditional LLNA. In this regard, EC<sub>t</sub> values for 2,4-dinitrochlorobenzene (DNCB) and HCA should be derived independently from a single study conducted in at least three separate laboratories. Acceptable reproducibility will be indicated by each laboratory obtaining EC<sub>t</sub> values for HCA and DNCB that are within 0.5x to 2.0x (5% to 20% and 0.025% to 0.1%, respectively) the mean EC<sub>3</sub> concentration (10% and 0.05%, respectively) specified for these substances in **Appendix F**. As mentioned for intralaboratory reproducibility, as few as two dose groups can be used for this evaluation.

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## Appendix A

### ICCVAM-Recommended Test Method Protocol (Updated 2008): The Murine Local Lymph Node Assay:<sup>13</sup> A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products

<b>Annex I</b>	<b>An Approach to Dissection and Identification of the Draining (“Auricular”) Lymph Nodes .....</b>	<b>A-15</b>
<b>Annex II</b>	<b>An Example of How to Reduce the Number of Animals in the Concurrent Positive Control Group of the Local Lymph Node Assay .....</b>	<b>A-19</b>
<b>Annex III</b>	<b>Evaluating Local Irritation and Systemic Toxicity in the Local Lymph Node Assay .....</b>	<b>A-21</b>

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<sup>13</sup> Based on ICCVAM 1999 and Dean et al. 2001

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## Preface

The murine local lymph node assay (LLNA) is a test method developed to assess whether a chemical has the potential to induce allergic contact dermatitis (ACD) in humans. In 1998, the LLNA was submitted to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) for evaluation as an alternative (i.e., stand-alone) test method to the guinea pig (GP) sensitization tests accepted by U.S. regulatory agencies. In 1999, based on a comprehensive evaluation of the LLNA by an independent scientific peer review panel (Panel),<sup>14</sup> ICCVAM concluded that the LLNA is an acceptable alternative to the GP test methods to assess the ACD hazard potential of most substances (Dean et al. 2001). The Panel also concluded that the LLNA offers animal welfare advantages compared to use of the traditional GP methods, in that it provides for animal use refinement (i.e., elimination of distress and pain) and reduces the total number of animals required. An ICCVAM Immunotoxicity Working Group (IWG) reviewed the 1999 Panel report and developed recommendations applicable to the regulatory use of the LLNA. The IWG then worked with the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to produce a recommended test method protocol (ICCVAM 2001)<sup>15</sup> that would accurately reflect the ICCVAM and Panel recommendations (ICCVAM 1999).

In March 2008, ICCVAM and NICEATM convened an independent scientific peer review panel (Panel) to evaluate new versions and applications of the LLNA. The Panel provided conclusions and recommendations in their report, many of which were applicable to the traditional LLNA test method protocol.<sup>16</sup> ICCVAM subsequently considered the Panel's conclusions and recommendations, as well as comments from the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) and public, and updated the 2001 ICCVAM-recommended LLNA test method protocol. The updated ICCVAM-recommended LLNA test method protocol will be forwarded with the Panel's report to agencies for their consideration.

The updated ICCVAM-recommended test method protocol for the LLNA is based on evaluation of previous experience and scientific data. It is provided to Federal agencies for their consideration as a standardized test method protocol recommended for generation of data for regulatory purposes. Prior to conducting a LLNA test to meet a regulatory requirement, it is recommended that the appropriate regulatory agency be contacted for their current guidance on the conduct and interpretation of this assay. Additional information on the ICCVAM LLNA review process and deliberations of the Panel can be found at the ICCVAM website (<http://iccvam.niehs.nih.gov>) or in the Panel report (ICCVAM 2008a).

We want to express our sincere appreciation to the ICCVAM IWG for their careful deliberations and efforts in updating the LLNA test method protocol, and especially appreciate the efforts of the Working Group Co-Chairs, Abigail Jacobs, Ph.D., from the U.S. Food and Drug Administration and Joanna Matheson, Ph.D., from the U.S. Consumer Products Safety Commission. We also want to acknowledge the outstanding support

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<sup>14</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/llna/llnarep.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf)

<sup>15</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/llna/LLNAProt.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/LLNAProt.pdf)

<sup>16</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPRept2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf)

provided by NICEATM and the Integrated Laboratory Systems, Inc., support staff. Lastly, we appreciate the efforts of the Panel members for their diligent review, and the comments provided by SACATM and numerous stakeholders, including the public.

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## **1.0 General Principle of Detection of Skin Sensitization Using the Local Lymph Node Assay**

The basic principle underlying the murine local lymph node assay (LLNA) is that sensitizers induce proliferation of lymphocytes in the lymph node draining the site of substance application. Under appropriate test conditions, this proliferation is proportional to the dose applied, and provides a means of obtaining an objective, quantitative measurement of sensitization. The test measures cellular proliferation as a function of *in vivo* radioisotope incorporation into the DNA of dividing lymphocytes. The LLNA assesses this proliferation in the draining lymph nodes proximal to the application site (see **Annex I**). This effect occurs as a dose response in which the proliferation in test groups is compared to that in the concurrent vehicle-treated control group. A concurrent positive control is added to each assay to provide an indication of appropriate assay performance.

## **2.0 Description of the Local Lymph Node Assay**

### **2.1 Sex and strain of animals**

Young adult female mice (nulliparous and non-pregnant) of the CBA/Ca or CBA/J strain are recommended.<sup>17</sup> Females are used because most data in the existing database were generated using mice of this gender. At the start of the study, mice should be age 8–12 weeks. All mice should be age matched (preferably within a one-week time frame). Weight variations between the mice should not exceed 20% of the mean weight.

### **2.2 Preparation of animals**

The temperature of the experimental animal room should be 21°C (±3°C) and the relative humidity 30%–70%. When artificial lighting is used, the light cycle should be 12 hours light: 12 hours dark. For feeding, an unlimited supply of standard laboratory mouse diets and drinking water should be used. The mice should be acclimatized for at least five days prior to the start of the test (ILAR 1996). Mice should be housed in small groups unless adequate scientific rationale for housing mice individually is provided (ILAR 1996). Healthy mice are randomly assigned to the control and treatment groups. The mice are uniquely identified prior to being placed in the study. The method used to mark the mice should not involve identification via the ear (e.g., marking, clipping, or punching of the ear). All mice should be examined prior to the initiation of the test to ensure that there are no skin lesions present.

### **2.3 Preparation of doses**

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

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<sup>17</sup> Male mice or other strains of mice may be used if it is sufficiently demonstrated that these animals perform as well as female CBA mice in the LLNA.

## 2.4 Test Conditions

### 2.4.1 Solvent/vehicle

The selected solvent/vehicle must not interfere with or bias the test result and should be selected on the basis of maximizing the test concentrations while producing a solution/suspension suitable for application of the test substance. In order of preference, recommended solvents/vehicles are acetone: olive oil (4:1 v/v), *N,N*-dimethylformamide, methyl ethyl ketone, propylene glycol, and dimethyl sulfoxide, but others may be used (Kimber and Basketter 1992). Particular care should be taken to ensure that hydrophilic materials are incorporated into a vehicle system that wets the skin and does not immediately run off. Thus, wholly aqueous vehicles may need to be avoided. It may be necessary for regulatory purposes to test the substance in the clinically relevant solvent or product formulation.

### 2.4.2 Controls

Concurrent negative (solvent/vehicle) controls should be included in each test to ensure that the test system is functioning properly and that the specific test is valid. In some circumstances (e.g., when using a solvent/vehicle not recommended in **Section 2.4.1**), it may be useful to include a naïve control. Except for treatment with the test substance, the mice in the negative control groups should be handled in an identical manner to the mice of the treatment groups.

Concurrent positive controls are used to ensure the appropriate performance of the assay by demonstrating that the test method is responding with adequate and reproducible sensitivity to a sensitizing substance for which the magnitude of the response is well characterized. Inclusion of a concurrent positive control is also important since it can confirm technical competence in performing the test and can demonstrate intra- and interlaboratory reproducibility and comparability. The positive control should produce a positive LLNA response (i.e., a stimulation index [SI]  $\geq 3$  over the negative control group). In particular, for negative LLNA studies, the concurrent positive control must induce a SI  $\geq 3$  relative to its vehicle-treated control. The positive control dose should be chosen such that the induction is reproducible but not excessive (i.e., SI  $> 20$ ). Preferred positive control substances are hexyl cinnamic aldehyde or mercaptobenzothiazole. There may be circumstances where, given adequate justification, other positive control substances may be used.

Although the positive control substance should be tested in the same vehicle as the test substance, there may be certain regulatory situations where it is necessary to test the positive control substance in both a standard and a non-standard vehicle (e.g., a clinically/chemically relevant formulation) to test for possible interactions.

Inclusion of a positive control with each test is recommended to ensure that all test method protocol procedures are being conducted properly and that all aspects of the test system are working properly such that they are capable of producing a positive response. However, periodic testing (i.e., at intervals  $\leq 6$  months) of the positive control substance may be considered in laboratories that conduct the LLNA regularly (i.e., conduct the LLNA at a frequency of no less than once per month) and that have a history and a documented proficiency for obtaining consistent results with positive controls. Adequate proficiency with the LLNA can be successfully demonstrated by generating consistent results with the positive control in at least 10 independent tests conducted within a reasonable period of time (i.e., less

than one year). A positive control group should always be included when there is a procedural change to the LLNA (i.e., change in trained personnel, change in test method materials and/or reagents, change in test method equipment, change in source of test animals, etc.), and such changes should be documented in laboratory reports. Consideration should be given to the impact of these changes on the adequacy of the previously established historical database in determining the necessity for establishing a new historical database to document consistency in the positive control results. Users should be aware that the decision to only include a positive control on a periodic basis instead of concurrently will have ramifications on the adequacy and acceptability of negative study results generated without a concurrent positive control during the interval between each periodic positive control study. For example, if a false negative result is obtained in the periodic positive control study, all negative test substance results obtained in the interval between the last acceptable periodic positive control study and the unacceptable periodic positive control study will be questioned. In order to demonstrate that the prior negative test substance study results are acceptable, a laboratory would be expected to repeat all negative studies, which would require additional expense and increased animal use. These implications should be carefully considered when determining whether to include concurrent positive controls or to only conduct periodic positive controls. Consideration should also be given to using fewer animals in the concurrent positive control group when this is scientifically justified, as discussed below and in **Annex II**.

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
- Supporting data on known effects in animal models
- Known potency for sensitization response

## 2.5 Methodology

A minimum of four animals per dose group is recommended. The collection of lymph nodes from individual mice is necessary in order to identify if any of the individual animal responses are outliers (e.g., in accordance with statistical tests such as Dixon's test). This will aid in avoiding false negative results for weaker sensitizers (i.e., substances that normally would induce an SI just above 3 might be incorrectly classified as negative due to a low outlier value, because the resulting mean SI may be less than 3 if an outlier is not identified and excluded). Individual animal measurements allow for the assessment of interanimal variability, a statistical comparison of the difference between test substance and vehicle control group measurements, and the evaluation of statistical power for different group sizes. Finally, evaluating the possibility of reducing the number of mice in the positive control group is only feasible when individual animal data are collected.

As noted above, concurrent negative and positive control groups should be included, unless a laboratory can demonstrate adequate proficiency that would support the use of a periodic positive control study. The number of mice in the concurrent positive control group might be

reduced compared to the vehicle and test substance groups, if the laboratory demonstrates, based on laboratory-specific historical data,<sup>18</sup> that fewer mice can be used without substantially increasing the frequency with which studies will need to be repeated. An example of how to reduce the number of mice in the concurrent positive control group is provided in **Annex II**.

Test substance treatment dose levels should be based on the recommendations given in Kimber and Basketter (1992) and in the ICCVAM Panel Report (ICCVAM 1999). Dose levels are selected from the concentration series 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc. The maximum concentration tested should be the highest achievable level while avoiding excessive local irritation and overt systemic toxicity (**Annex III**). Efforts should be made to identify existing information that may aid in selecting the appropriate maximum test substance dose level. In the absence of such information, an initial prescreen test, conducted under identical experimental conditions except for not conducting an assessment of lymph node proliferative activity, may be necessary. In order to have adequate information on which to select a maximum dose level to use in the definitive test and to identify a dose-response relationship, data should be collected on at least three test substance dose levels with two mice per dose group, in addition to the concurrent solvent/vehicle control group.

The LLNA experimental procedure is performed as follows:

**Day 1.** Identify and record the weight of each mouse before applying the test substance. Apply 25 µL/ear of the appropriate dilution of the test substance, or the positive control, or the solvent/vehicle only, to the dorsum of both ears of each mouse.

**Days 2 and 3.** Repeat the application procedure as carried out on Day 1.

**Days 4 and 5.** No treatment.

**Day 6.** Record the weight of each mouse. Inject 250 µL of sterile phosphate-buffered saline (PBS) containing 20 µCi of tritiated (<sup>3</sup>H)-methyl thymidine or 250 µL PBS containing 2 µCi of <sup>125</sup>I-iododeoxyuridine (<sup>125</sup>IU) and 10<sup>-5</sup> M fluorodeoxyuridine into each mouse via the tail vein (Kimber et al. 1995; Loveless et al. 1996). Five hours later, each mouse is euthanized and the draining (“auricular”) lymph nodes of both ears are collected and placed in PBS (one container per mouse). Both bilateral draining lymph nodes must be collected (see diagram and description of dissection in **Annex I**). A single-cell suspension of lymph node cells (LNC) is prepared for each individual mouse. The single-cell suspension is prepared in PBS by either gentle mechanical separation through 200-mesh stainless steel gauze or another acceptable technique for generating a single-cell suspension. LNC are washed twice with an excess of PBS and the DNA precipitated with 5% trichloroacetic acid (TCA) at 4°C for approximately 18 hours.

For the <sup>3</sup>H-methyl thymidine method, pellets are resuspended in 1 mL TCA and transferred to 10 mL of scintillation fluid. Incorporation of <sup>3</sup>H-methyl thymidine is measured by β-scintillation counting as disintegrations per minute (dpm) for each mouse and expressed as dpm/mouse. For the <sup>125</sup>IU method, the 1 mL TCA pellet is transferred directly into gamma-counting tubes. Incorporation of <sup>125</sup>IU is determined by gamma counting and also expressed as dpm/mouse.

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<sup>18</sup> A robust historical dataset should include at least 10 independent tests, conducted within a reasonable period of time (i.e., less than one year), with a minimum of four mice per negative and positive control groups.

## 2.6 Observations

Mice should be carefully observed for any clinical signs, either of local irritation at the application site or of systemic toxicity (**Annex III**). Weighing mice prior to treatment and at the time of necropsy will aid in assessing systemic toxicity. All observations are systematically recorded and records maintained for each individual mouse. Animal monitoring plans must include criteria to promptly identify mice exhibiting systemic toxicity or excessive irritation or corrosion of skin for euthanasia.

## 3.0 Calculation of Results

Results for each treatment group are expressed as the mean SI. Each SI is the ratio of the mean dpm/mouse within each test-substance treatment group or the positive control treated group against the mean dpm/mouse for the solvent/vehicle treated control group. However, the investigator should be alert to possible outlier responses for individual mice within a group that may necessitate analysis both with and without the outlier.

In addition to a formal assessment of the magnitude of the SI, a statistical analysis for presence and degree of dose response may be conducted, which is possible only with the use of individual animals. Any statistical assessment should include an assessment of the dose-response relationship as well as suitably adjusted comparisons of test groups (e.g., pair-wise dosed group versus concurrent solvent/vehicle control comparisons). Analyses may include, for instance, linear regression, William's test to assess dose-response trends, or Dunnett's test for pairwise comparisons. In choosing an appropriate method of statistical analysis, the investigator should be aware of possible inequality of variances and other related problems that may necessitate a data transformation or a non-parametric statistical analysis.

## 4.0 Evaluation and Interpretation of Results

In general, when the SI for any single treatment dose group is  $\geq 3$ , the test substance is regarded as a skin sensitizer (Kimber et al. 1994; Basketter et al. 1996; ICCVAM 1999) and a test substance not meeting this criterion is considered a non-sensitizer in this test. However, the magnitude of the observed SI should not be the sole factor used in determining the biological significance of a skin sensitization response. Additional factors that could be considered include the outcomes of statistical analyses, the strength of the dose-response relationship, chemical toxicity, and solubility. For instance, a quantitative assessment may be performed by statistical analysis of individual mouse data and may provide a more complete evaluation of the test substance's ability to act as a sensitizer (see **Section 3.0**). Equivocal results (e.g., the SI does not reach 3, but it is near 3 and there is a positive dose-response relationship) should be clarified by performing statistical analysis, and by considering structural relationships, available toxicity information, and dose selection.

## 5.0 Data and Reporting

### 5.1 Data

Individual animal dpm data should be presented in tabular form, along with the group mean dpm/mouse, its associated error term, and the mean SI (and associated error term) for each dose group compared against the concurrent solvent/vehicle control group.

### 5.2 Test Report

The test report should contain the following information:

*Test Substances and Control Substances*

- Identification data and Chemical Abstracts Service Registry Number, if known
- Physical nature and purity
- Physiochemical properties relevant to the conduct of the study
- Stability of the test substance, if known
- Lot number of the test substance

*Solvent/Vehicle:*

- Justification for choice of solvent/vehicle
- Solubility and stability of the test substance in the solvent/vehicle

*Test Animals:*

- Strain of mice used
- Number, age, and sex of mice
- Source, housing conditions, diet, etc.
- Individual weight of the mice at the start and end of the test, including body weight range, as well as mean and associated error term for each group
- Microbiological status of the mice

*Test Conditions:*

- Concurrent and historical positive and negative (solvent/vehicle) control data
- Data from range-finding study, if conducted
- Rationale for dose-level selection
- Details of test substance preparation
- Details of the administration of the test substance
- Details of food and water quality
- Detailed description of treatment and sampling schedules
- Methods for measurement of toxicity
- Criteria for considering studies as positive, negative, or equivocal



*Results:*

- Signs of systemic toxicity and/or local irritation
- Values for dpm/mouse for each mouse within each treatment group
- Mean and associated error term for dpm/mouse for each treatment group and the results of outlier analysis for each dose group should be provided
- Calculated SI and an appropriate measure of variability that takes into account the interanimal variability in both the test substance dosed and control groups
- Dose-response relationship
- Statistical analyses and method applied
- Concurrent and historical positive and negative (solvent/vehicle) control data as established in the test laboratory
- Concurrent positive control data or, if not done, the date and laboratory report for the most recent periodic positive control and a report detailing the historical positive control data for the laboratory justifying the basis for not conducting a concurrent positive control.

*Discussion of the Results*

*Conclusion*

*A Quality Assurance Statement for GLP-compliant Studies*

- This statement 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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## **Annex I: An Approach to Dissection and Identification of the Draining (“Auricular”) Lymph Nodes**

### **1.0 Background**

Although minimal technical training of the murine local lymph node assay (LLNA) is required, extreme care must be taken to ensure appropriate and consistent dissection of the lymph nodes. It is recommended that technical proficiency in the dissection and identification of the lymph nodes draining the ear be achieved by practice on mice that have been (a) injected with a colored agent (dye) and/or (b) sensitized with a strong positive sensitizer. Brief descriptions of these practice dissections are provided below. Recognizing that nodes from vehicle-treated and naïve mice are smaller, laboratories performing the LLNA must also gain proficiency in the dissection of these nodes. It may be helpful for laboratories inexperienced in this procedure to request guidance from laboratories that have successfully performed the LLNA.

### **2.0 Training and Preparation for Node Identification**

#### **2.1 Identification of the Draining Node – Dye Treatment**

There are several methods that can be used to provide color identification of the draining nodes. These techniques may be helpful for initial identification and should be performed to ensure proper isolation of the appropriate node. Examples of such treatments are listed below. It should be noted that other such protocols might be used effectively.

*Evan’s Blue Dye treatment:*

Inject approximately 0.1 mL of 2% Evan’s Blue Dye (prepared in sterile saline) intradermally into the pinnae of an ear. Euthanize the mouse after several minutes and continue with the dissection as noted below.

*Colloidal carbon and other dye treatments:*

Colloidal carbon and India ink are examples of other dye treatments that may be used (Tilney 1971).

#### **2.2 Identification of the Draining Node – Application of Strong Sensitizers**

For the purpose of node identification and training, a strong sensitizer is recommended. This agent should be applied in the standard acetone: olive oil vehicle (4:1). Suggested sensitizers for this training exercise include 0.1% oxazolone, 0.1% (w/v) 2,4-dinitrochlorobenzene, and 0.1% (v/v) dinitrofluorobenzene. After treating the ear with a strong sensitizer, the draining node will dramatically increase in size, thus aiding in identification and location of the node.

Using a procedure similar to that described in the test method protocol, apply the agent to the dorsum of both ears (25 µL/ear) for 3 consecutive days. On the fourth day, euthanize the mouse. Identification and dissection (listed below) of the node should be performed in these animals prior to practice in non-sensitized or vehicle-treated mice, where the node is significantly smaller.

Please note: Due to the exacerbated response, the suggested sensitizers are not recommended as controls for assay performance. They should only be used for training and node identification purposes.

### **3.0 Dissection Approach**

#### **3.1 Lateral Dissection (Figure A-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. Perform this approach bilaterally (on both sides of the mouse). After euthanizing the mouse, place it in a lateral position. Wet the face and neck with 70% ethanol. Use scissors and forceps to make an initial cut from the neck area slightly below the ear. Carefully extend the incision toward the mouth and nose. Angle the tip of the scissors slightly upward during this procedure to prevent the damage of deeper tissue. Gently retract the glandular tissue in the area using the forceps. Using the masseter muscle, facial nerves, blood vessels, and the bifurcation of the jugular vein as landmarks, isolate and remove the draining node (**Figure A-1**). The draining node (“auricular”) will be positioned adjacent to the masseter muscle and proximal to and slightly above the jugular bifurcation.

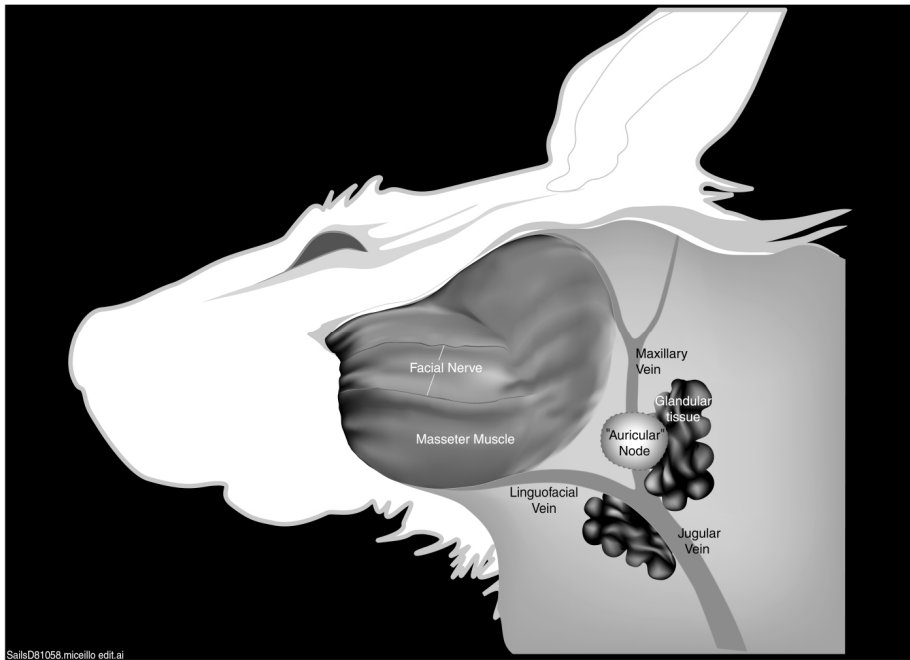
#### **3.2 Ventral Dissection (Figure A-2)**

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, wet the neck and abdomen with 70% ethanol. Use scissors and forceps to carefully make the first incision across the chest and between the arms. Make a second incision up the midline 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. Take care 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.

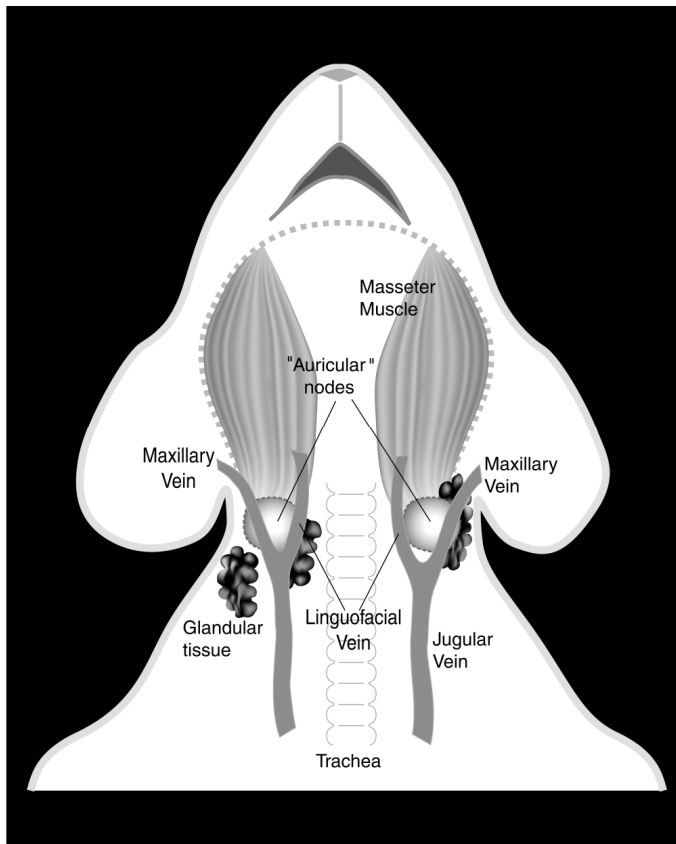
### **4.0 Accuracy in Identification**

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. Application of sensitizing agents (especially the strong sensitizers used in training) will cause enlargement of the nodes. If a dye is injected for training purposes, the node will take on the tint of the dye.

**Figure A-1 Lateral Dissection**



**Figure A-2 Ventral Dissection**



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**Annex II:**  
**An Example of How to Reduce the Number of Animals in the Concurrent Positive Control Group of the Local Lymph Node Assay**

As stated in the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Murine Local Lymph Node Assay (LLNA) test method protocol (Section 2.4.2 of Appendix A), a concurrent positive control is recommended to ensure the appropriate performance of the assay. Appropriate performance is demonstrated when the test method responds with adequate and reproducible sensitivity to a sensitizing substance for which the magnitude of the response is well characterized. The number of mice in the concurrent positive control group may possibly be reduced if the laboratory demonstrates, based on laboratory-specific historical data, that fewer mice can be used without compromising the integrity of the study (i.e., positive control results should be always be positive compared to the vehicle control results). As illustrated in the example and accompanying explanation below, reducing the number of animals in the positive control group is only feasible when individual animal data are collected.

The stimulation index (SI) results for each positive control test can be used to generate mean SI values for every possible combination of SI values for as few as two animals. The mean SI values for every combination of numbers for each group size can then be used to calculate the failure rate of the positive control for each group size (i.e., the percentage of the combinations for which the mean SI < 3). **Table A-1** provides an example of positive control results from four tests in one laboratory of 30% hexyl cinnamic aldehyde (HCA) using six CBA/J mice per group. In these tests, with six animals, HCA produced “borderline” positive results (i.e., the mean SI values were marginally greater than 3). To determine whether the number of animals can be reduced, sample size reductions (i.e., N = 5, 4, 3, or 2) can be evaluated by taking all possible samples from the six values for each test given in **Table A-1**, which can occur in the following ways: N = 2 (15 samples), N = 3 (20 samples), N = 4 (15 samples), and N = 5 (six samples).

**Table A-1 Example of SI Results from Four Local Lymph Node Assay Positive Control Studies with 30% HCA**

<b>Test</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
Animal 1	2.13	3.56	4.68	0.78
Animal 2	4.55	1.54	4.44	9.16
Animal 3	3.64	3.00	5.41	6.66
Animal 4	1.98	3.87	3.32	3.02
Animal 5	3.09	3.79	2.89	2.32
Animal 6	3.77	3.96	1.81	2.91
<b>Mean SI</b>	<b>3.19</b>	<b>3.29</b>	<b>3.76</b>	<b>4.14</b>

Abbreviations: HCA = hexyl cinnamic aldehyde; SI = stimulation index

The failure rate of the positive control was then calculated using the SI results for each group of two, three, four, or five values to determine the likelihood of obtaining a mean SI < 3. The results for these four “borderline” HCA tests were then added to the results from an additional 12 robust positive control tests included in this laboratory’s historical database to determine the overall likelihood of obtaining a mean SI < 3 for the positive control substance (**Table A-2**). The failure rate reflects the frequency with which a positive control test will fail, which would result in retesting the positive control and any concurrent test substances. Each laboratory is encouraged to determine the lowest number of animals to use in the positive control group based on the highest failure rate considered acceptable by the laboratory.

**Table A-2 Example of Positive Control Failure Rate for 30% HCA Based on Data Collected in Single Laboratory**

Number of Animals	HCA Test 1	HCA Test 2	HCA Test 3	HCA Test 4	Results from Other Tests <sup>1</sup>	Overall Likelihood of a Mean SI < 3
5	17% (1/6)	0% (0/6)	0% (0/6)	0% (0/6)	0% (0/72)	1% (1/96)
4	27% (4/15)	13% (2/15)	0% (0/15)	7% (1/15)	0% (0/180)	3% (7/240)
3	40% (8/20)	30% (6/20)	5% (1/20)	20% (4/20)	0% (0/240)	6% (19/320)
2	47% (7/15)	33% (5/15)	13% (2/15)	40% (6/15)	1% (1/180)	9% (21/240)

Abbreviations: HCA = hexyl cinnamic aldehyde; SI = stimulation index

<sup>1</sup> These represent 12 positive control studies in the same laboratory where all mice in the positive control groups treated with 30% HCA produced an SI ≥ 3.

### Annex III: Evaluating Local Irritation and Systemic Toxicity in the Local Lymph Node Assay

As noted in the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Murine Local Lymph Node Assay (LLNA) test method protocol, at least three dose levels of a test substance should be evaluated. The highest dose level tested should be a concentration of 100% (i.e., neat substance for liquid substances) or the maximum soluble concentration (for solids), unless available information suggests that this concentration induces systemic toxicity or excessive local irritation after topical application.

In the absence of such information, a prescreen test should be performed using three dose levels of the test substance, in order to define the appropriate dose level to test in the LLNA. Six mice (two per concentration) are used, and the prescreen is conducted under identical conditions as the main LLNA study, except there is no assessment of lymph node proliferation. All mice will be observed daily for any clinical signs of systemic toxicity or local irritation at the application site. For example, observations might occur before and after treatment on Days 1, 2, and 3. Body weights are recorded pre-test and prior to termination (Day 6). Both ears of each mouse are observed for erythema (and scored using **Table A-3**). Ear thickness measurements are taken using a thickness gauge (e.g., digital micrometer or Peacock Dial thickness gauge) on Day 1 (pre-dose), Day 3 (approximately 48 hours after the first dose), and Day 6.

*Excessive local irritation is indicated by an erythema score  $\geq 3$  and/or ear swelling of  $\geq 25\%$ .*

**Table A-3 Erythema Scores**

Observation	Value
No visual effect	0
Slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema (beet redness)	3
Eschar (i.e., piece of dead tissue that is cast off from the surface of the skin)	4

A 25% increase in ear swelling has been used as an initial step to identify substances that cause a skin reaction due to an irritant response rather than sensitization (Reeder et al. 2007; ICCVAM 2008b). A statistically significant difference from control animals has also been used to delineate irritants from non-irritants in the LLNA (Hayes et al. 1998; Homey et al. 1998; Woolhiser et al. 1998; Hayes and Meade 1999; Ehling et al. 2005; Vohr and Jürgen 2005; Patterson et al. 2007). While these statistical differences often occur when ear swelling is less than 25%, they have not been associated specifically with excessive irritation (Woolhiser et al. 1998; Ehling et al. 2005; Vohr and Jürgen 2005; Patterson et al. 2007). Additionally, an adequately robust statistical comparison would require that a vehicle control group be included and that more than two animals per group be tested. Both of these requirements would substantially increase the number of animals used for this prescreen test.

For this reason, a threshold increase in ear swelling above pre-dosing levels is recommended for this prescreen test.

Test guidelines for assessing acute systemic toxicity recommend a number of clinical observations for assessing systemic toxicity (OECD 1987; EPA 1998). The following observations, which are based on test guidelines and current practices (ICCVAM in press), may indicate systemic toxicity when used as part of an integrated assessment and therefore may indicate that the maximum dose recommended for the LLNA has been exceeded:

- Clinical signs
  - Changes in nervous system function (e.g., piloerection, ataxia, tremors, and convulsions)
  - Changes in behavior (e.g., aggressiveness, change in grooming activity, marked change in activity level)
  - Changes in respiratory patterns (i.e., changes in frequency and intensity of breathing such as dyspnea, gasping, and rales)
  - Changes in food and water consumption
  - Lethargy and/or unresponsiveness
  - Any clinical signs of more than slight or momentary pain and distress
- Reduction in body weight >10% from Day 1 to Day 6
- Mortality

## **Appendix B**

### **Evaluating the Impact of Reducing the Sample Size from Five to Four Animals per Group on the Performance of the Ratio Rule of $SI > 3$ in LLNA Testing**

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## 1.0 Introduction

Test Guideline 429 issued by the Organisation for Economic Co-operation and Development (OECD; OECD 2002) states that “A minimum of four animals is used per dose group, with a minimum of three concentrations of the test substance, plus a negative control group treated only with the vehicle for the test substance, and a positive control, as appropriate. *In those cases in which individual animal data are to be collected, a minimum of five animals per dose group are used.*” This analysis was undertaken to determine if the number of animals required for individual animal data collection could be harmonized with that required for pooled data without diminishing accuracy. This is important because most animal-use regulations require that the minimum number of animals be used in studies, which currently results in only pooled data being collected in many countries because it currently requires fewer animals.

Therefore, the issue under investigation in the evaluation that follows is the impact of modifying the murine local lymph node assay (LLNA) test method protocol by reducing the number of individual animals per group from 5 to 4. More specifically, the evaluation considers how often this reduction in animal usage would have an impact on the overall LLNA outcome when the decision criterion used to determine a sensitizer from a non-sensitizer is a stimulation index (SI) greater than or equal to 3 (i.e., the “Ratio Rule”). Since the “true” underlying sensitizer status for individual substances is generally not known, this investigation will focus on the degree of disagreement rather than on which observed outcome is the “correct” one. This evaluation focused primarily on the Ratio Rule, although the possible use of a formal statistical test will also be considered.

The results of the following analyses indicate that a reduction in the sample size from 5 to 4 animals per group is unlikely to have any significant impact on the results of the LLNA test when using the Ratio Rule. If using statistics, the power for detecting LLNA effects will be reduced slightly when using 4 animals per group relative to using 5 animals per group. However, the practical impact of this power difference may be minimal, in that the power difference appears to be small for detecting effects above the Ratio Rule cutoff point of SI = 3. Importantly, this analysis also indicates that a statistical test based on 4 animals per group will identify more sensitizers than using the Ratio Rule based on 5 animals per group.

## 2.0 Methods

The database evaluated includes three different strains of animals: CBA, BALB/c, and B6C3F1. This report evaluates in detail only the CBA database; the data from the other two strains are summarized (**Section 4.0** and **Table B-7**) and may be evaluated more definitively in due course. The CBA database consists of 83 individual studies, each with three or four dosed groups and a control group. There are not 83 distinct substances, because some substances are tested in multiple studies. The number of individual animals per group in these studies ranged from 2 to 9. There were a total of 277 dosed groups, two of which were excluded from the agreement-disagreement analysis since there were only 2 or 3 animals per group. Study results were evaluated on a dose-by-dose basis as well as on a study-by-study basis, recognizing that the doses within a study used a common control group. Also, for certain labs, a common control group was used for multiple substances.

For each study having 5 animals per group (i.e., N = 5), all possible random samples of size 4 (responses measured as disintegrations per minute [dpm] of a radiolabeled tracer compound) were taken from both the control and experimental groups (25 possible combinations), and the results of the Ratio Rule were compared for each of the samples with that of the full data set of 5 animals. The level of agreement was then determined.

For those studies having more than 5 animals per group, a similar procedure was applied, but in this case random samples were taken for both the N = 5 and N = 4 protocols, and there were far more combinations of samples to deal with (8100 rather than 25). Once again, the level of agreement between the N = 5 and N = 4 protocols were determined.

### 3.0 Results

Using the Ratio Rule criterion, the CBA mouse database consisted of a mix of sensitizers (49 studies) and non-sensitizers (33 studies), with one study (discussed in more detail below) producing a borderline effect. **Table B-1** shows the frequency of the various SI values in the 275 usable (for agreement-disagreement analysis) dosed groups, together with the average agreement seen between samples of N = 5 and N = 4. As can be seen in the table, the disagreement in study results is limited to SIs in the 2.1 to 4.7 range, with the disagreement increasing as the SI approaches 3. The overall average agreement between N = 4 and N = 5 studies is quite good: 97.5%. Moreover, as discussed in more detail below, the disagreement in outcome is due primarily to the inherent variability in the data (and the closeness of the SI to 3), not to the reduction in sample size.

**Table B-1 Breakdown of Individual Dosed Group SIs: CBA Strain**

SI	Frequency	Agreement between N = 5 and N = 4 samples
<2.1	154	100.00%
2.1 - 2.5	16	90.10%
2.6	2	85.00%
2.7	3	73.30%
2.8	2	64.00%
3.1	1	56.00%
3.2	2	55.50%
3.3	4	73.50%
3.4	1	88.00%
3.5	1	68.00%
3.6	1	84.00%
3.7	1	90.00%
3.8	1	100.00%
4.0 - 4.7	16	97.90%
>4.7	70	100.00%
<b>Total</b>	<b>275</b>	<b>97.50%</b>

Abbreviations: N = number of animals per dose group; SI = stimulation index

The individual study results for the CBA strain are summarized in **Annex I**.



Although the primary focus of this evaluation is on the Ratio Rule (i.e.,  $SI > 3$ ), it is possible that a formal statistical test may be used in addition to (or possibly even in place of) the Ratio Rule. For this reason, a simple Student’s *t* test (based on the logged dpm data) was also used to compare each dosed group with its concurrent control. The results of this analysis are summarized in **Table B-2**. It is clear that using a formal statistical test will identify far more “positives” than the Ratio Rule, i.e., statistical significance ( $p < 0.05$ ) was achieved for some dosed groups producing an SI well below 3. This matter is discussed in more detail below.

**Table B-2 Distribution of Statistically Significant ( $p < 0.05$ ) SIs: CBA Strain**

SI	Frequency	Percentage of statistically significant ( $p < 0.05$ ) SIs
<1.7	131	0.00%
1.7 - 1.9	23	52.20%
2.0 - 2.5	17	88.00%
2.6 - 3.0	7	85.70%
> 3.0	1	100.00%
<b>Total</b>	<b>277</b>	

Abbreviation: SI = stimulation index

#### 4.0 Discussion

It was known in advance that the reduction in sample size from  $N = 5$  to  $N = 4$  would have essentially no impact on study results for “strong sensitizers” and for “clear non-sensitizers,” and this is confirmed in **Table B-1**. What was not known was (1) how frequently such outcomes are seen in practice; (2) the specific range of SI values in which some impact on study outcome may be evident; (3) the magnitude of the impact for those studies having an SI close to 3; and (4) whether the disagreement in study outcome was due primarily to the reduction in sample size or to the inherent variability in the data (and the closeness of the SI to 3). The current investigation addresses all of these issues.

With regard to the first issue, for the CBA mouse database, only 34 of the 275 dosed groups (12%) had less than 100% agreement between  $N = 5$  and  $N = 4$  outcomes. Thus, for most dosed groups, the reduced sample size will not even be an issue when using the Ratio Rule.

Moreover, the reduced sample size becomes an issue only for a relatively narrow range of SI values. The range of SI values in this database producing less than 100% agreement was 2.1 to 4.7, but this may be somewhat misleading in that many studies in this range produced 100% agreement (see **Table B-1** and **Annex I**).

As the SI approaches 3, the disagreement between a sample of  $N = 5$  and  $N = 4$  increases notably (**Table B-1**). However, and this may be the single most important “take home” message of this entire analysis, the disagreement is far more a function of the animal-to-animal variability than it is to the reduction in sample size. That is, a second sample of 5 animals would show almost the same level of disagreement with the first sample of 5 animals, as would a sample of 4 animals. Thus, the reduction in sample size is a relatively small contributor to this difference. This important concept is illustrated below with two examples from the CBA mouse database, the first showing an SI of 2.8, just below the Ratio Rule threshold of  $SI = 3$ , the second showing an SI of 3.2, just above the Ratio Rule threshold.

The first example is the high dose of the third hexyl cinnamic aldehyde study, which had an SI of 2.8 for N = 6. This is the one study noted above with a borderline effect. Since N = 6, this required selection of samples of size 5 from both the control and dosed groups, and some of these samples did not give the same result as that seen for the full six animal sample. The results are summarized below and compared with the N = 4 strategy.

**Table B-3 Example Showing Effect of Sample Size on Agreement of Results for a Test Substance with SI = 2.8**

	<b>Two N = 5 samples</b>	<b>One N = 5 sample and one N = 4 sample</b>
Agreement (SI > 3)	7.7% (10/36) (10/36)	10.5% (10/36) (85/225)
Agreement (SI < 3)	52.2% (26/36) (26/36)	44.9% (26/36) (140/225)
Disagreement (one SI > 3; one SI < 3)	40.1% (by subtraction)	44.6% (by subtraction)

Abbreviations: N = number of animals per dose group; SI = stimulation index

As can be seen from these calculations (see also **Annex I**), the agreement between N = 5 and N = 4 strategies is “only” 55%. However, the disagreement is *not* due primarily to a reduction in sample size, since the agreement is very similar to that found for two N = 5 samples (60%). In other words, only 4.5% of the observed 45% disagreement is due to the reduction in sample size. The rest is due to the inherent variability among animals (and the closeness of the SI to 3) that would be evident even if a second sample of size 5 were used.

The second example is the mid-dose of the dipropylene triamine study, which had an SI of 3.2 also for N = 6. The results are summarized below and compared with the N = 4 strategy.

**Table B-4 Example Showing Effect of Sample Size on Agreement of Results for a Test Substance with SI = 3.2**

	<b>Two N = 5 samples</b>	<b>One N = 5 sample and one N = 4 sample</b>
Agreement (SI > 3)	56.25% (27/36) (27/36)	50.67% (27/36) (152/225)
Agreement (SI < 3)	6.25% (9/36) (9/36)	8.11% (9/36) (73/225)
Disagreement (one SI > 3; one SI < 3)	37.50% (by subtraction)	41.22% (by subtraction)

Abbreviations: N = number of animals per dose group; SI = stimulation index

The results are very similar to those of the first example, in that most of the 41% disagreement between the N = 4 sample and the N = 5 sample is due to the inherent variability of the data and the closeness of the SI to 3, not to the reduction in sample size.

Another point that should be noted: in the instances in which there is disagreement, the N = 4 strategy may actually have a higher likelihood of producing an SI > 3 result than using a sample of size 5. This occurs when the underlying SI is close to but below 3. For instance, consider the first example given above in which the observed SI = 2.8. A sample of size 4 would have a 38% chance (85/225) of producing an SI > 3 compared with only 28% (10/36) when using N = 5. In that sense, N = 4 could be regarded as having greater “power” than N = 5 for these data.

However, use of the Ratio Rule implicitly assumes that an SI less than 3 is biologically unimportant and thus should not be detected. Thus, the increased likelihood of exceeding the

Ratio Rule criterion using  $N = 4$  in the example above could be regarded as an increase in the false positive rate, rather than an increase in power. Importantly, as  $N$  increases, the likelihood of detecting  $SI = 2.8$  by the Ratio Rule approaches zero, with maximum “power” occurring for  $N = 1$ .

However, some investigators may regard an  $SI$  of 2.8 as biologically important, especially if seen at the top dose, as was the case in this study. Consequently, these investigators might actually prefer the performance of  $N = 4$  rather than  $N = 5$  in this example. Of course, if  $SI < 3$  responses are considered important, it would make far more sense to carry out a formal statistical test to detect them rather than using the Ratio Rule, which will likely not detect them. Although not detected by the Ratio Rule, the  $SI = 2.8$  effect noted above in the high dose hexyl cinnamic aldehyde study is highly significant ( $p < 0.01$ ) by Student’s  $t$  test.

Moreover, it is likely that this particular  $SI = 2.8$  is a “real” effect, not only because it is highly significant statistically, but also because in four other studies with this compound, the  $SI$ s produced for this dose were 2.2, 4.1, 4.2, and 6.6, with higher doses producing even greater effects (see **Annex I**). Without these additional studies, it is possible that this effect would be “missed” since  $SI = 2.8$  does not satisfy the Ratio Rule criterion of  $SI > 3$ , and without individual animal data, it would not be possible to determine whether or not this effect was statistically significant. This is another illustration of the value of individual animal data and also the value of using a formal statistical test. It also shows that in some cases a sample of  $N = 4$  is actually more likely to produce the “correct” conclusion than  $N = 5$  when using the Ratio Rule.

As can be seen in **Table B-2**, a formal statistical test will identify as statistically significant ( $p < 0.05$ ) many responses that would not be detected by the Ratio Rule. In some cases, statistical significance is achieved for  $SI$  values as low as 1.7 (see **Annex I** and **Table B-2**). Normally, this “increased power” would be considered very desirable, but apparently it is possible that certain  $SI$ s in the 1.7 to 3.0 range, while truly different from controls, may be reflecting “irritation” rather than a true sensitizing effect, and thus may not be indicative of a meaningful human risk. Discussion of this matter is beyond the scope of this investigation, but it is logical to assume that since the Ratio Rule is widely used for LLNA data, while a formal statistical test is not, there must be concern that a formal statistical test will produce too many “significant effects” for  $SI$ s in the 2 to 3 range. That is,  $SI$ s below 3 may be statistically significant and reflect “real” dosed group effects, but responses in this range are considered biologically unimportant. As can be seen in **Table B-2**, most of the  $SI$ s in the 2 to 3 range are in fact statistically significant. Use of the Ratio Rule also implicitly assumes that false positives are more important than false negatives.

Any consideration of statistical power must take into account the variability in response among animals. To illustrate this, consider the 17 CBA mouse studies carried out at BASF (see **Table B-11** in **Annex I**). The mean control dpm response across these 17 studies was 552.3. The mean standard deviation (SD; based on the logged dpm responses) among the control animals was 0.4077. Based on this information, we can carry out a power calculation, which is summarized in **Table B-5**.

To explain further: Power is primarily a function of (1) the magnitude of the difference between the dosed and control groups, (2) the underlying variability among animals, and (3) the sample size. In the table below, “difference” is the size (on a log scale) of the “fold

increase” that is to be detected. The SD is the assumed underlying standard deviation among animals (on a log scale) as determined by the data from BASF (see **Table B-11** in **Annex I**). This SD is assumed to be the same in the dosed and control groups, an assumption consistent with the data from multiple labs obtained to date. Delta is the standardized (by SD) difference to be detected and is the key input variable into the power calculation program. The power calculations given below are based on a two-sided Student’s *t* test, and assume an underlying normal distribution for the logged data. The specific power calculations were taken from <http://www.danielsoper.com/statcalc/calc49.aspx>. In this program “Cohen’s *d*” is just the standardized difference, Delta. This is a very simple program to use, and alternative power calculations can easily be made.

**Table B-5 Post-hoc Power Calculations Based on the BASF Control Data**

	<b>Dosed Group Increase Relative to Controls</b>			
	<b>3.5-fold</b>	<b>3-fold</b>	<b>2.5-fold</b>	<b>2-fold</b>
Assumed control response	552.3	552.3	552.3	552.3
Log (Control response)	6.314	6.314	6.314	6.314
Dosed group response	1933.05	1656.90	1380.75	1104.60
Log (Dosed group response)	7.567	7.413	7.230	7.007
Difference (log scale)	1.253	1.099	0.916	0.693
Assumed SD (log scale)	0.4077	0.4077	0.4077	0.4077
Delta = Difference/SD	3.07	2.70	2.25	1.70
Power for N = 5	99.0%	96.4%	87.9%	65.8%
Power for N = 4	95.7%	89.8%	76.8%	53.0%

Abbreviations: N = number of animals per dose group; SD = standard deviation

From these calculations, the conclusion is that if the underlying variability among control animals is similar to that seen in an average BASF study, then there is an excellent chance that an underlying SI of 2.5 will be detected as statistically significant ( $p < 0.05$ ), although this likelihood is higher for N = 5 (87.9%) than for N = 4 (76.8%). This power calculation is also consistent with the empirical results summarized in **Table B-2**. An underlying SI of 2.5 would almost certainly not be detected by the Ratio Rule, nor would one want it to be detected, since use of the Ratio Rule implicitly assumes that such an effect is of no consequence, as noted earlier.

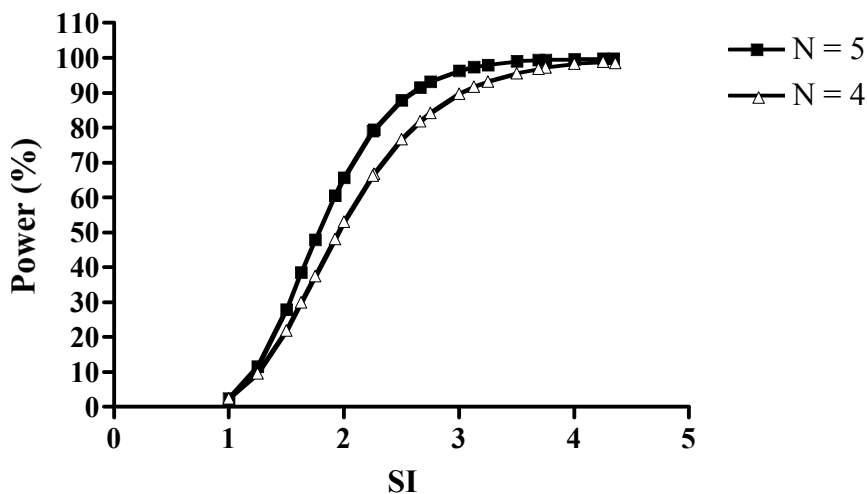
From the website given above, a general power curve can be constructed for N = 5 and N = 4 by specifying different values of Delta, which could reflect different “-fold increases (i.e., SI values),” different underlying variabilities, or a combination of these two factors. Such power comparisons are summarized below in **Table B-6** and **Figure B-1** and include the four from **Table B-5**.

**Table B-6 Selected Power Comparisons for N = 5 and N = 4 Samples Based on BASF Control Data**

<b>SI</b>	<b>Delta</b>	<b>N = 5</b>	<b>N = 4</b>
4.34	3.60	99.9%	99.1%
4.25	3.55	99.9%	98.9%
4.00	3.40	99.7%	98.3%
3.75	3.24	99.5%	97.2%
3.69	3.20	99.4%	96.9%
3.50	3.07	99.0%	95.7%
3.25	2.89	98.0%	93.3%
3.13	2.80	97.4%	91.8%
3.00	2.70	96.4%	89.8%
2.75	2.48	93.2%	84.3%
2.66	2.40	91.6%	81.9%
2.50	2.25	87.9%	76.8%
2.26	2.00	79.5%	66.8%
2.25	1.99	79.1%	66.3%
2.00	1.70	65.8%	53.0%
1.92	1.60	60.5%	48.2%
1.75	1.37	47.9%	37.4%
1.63	1.20	38.6%	30.0%
1.50	0.99	28.0%	21.9%
1.25	0.55	11.6%	9.7%
1.00	0.00	2.5%	2.5%

Abbreviations: N = number of animals per dose group; SI = stimulation index

**Figure B-1 Power Curve for N = 5 and N = 4 Samples Based on BASF Control Data**



Abbreviations: N = number of animals per dose group; SI = stimulation index

Although these particular “Deltas” could result from different combinations of -fold-increases and assumed variability, the power calculations for the BASF data indicate that the most notable differences in power between N = 5 and N = 4 occur for SIs below 3, a range for which detection of an effect is apparently viewed as a “false positive” as discussed earlier. That is, the Ratio Rule implicitly assumes that SIs less than 3 should not be detected, so the fact that samples of N = 5 are more likely than samples with N = 4 to detect significant effects for SIs below 3 could be viewed as a disadvantage rather than an advantage of a larger sample size. For SI = 3.5 (at least for the BASF data), the power is high and similar for N = 5 and N = 4 (99.0% vs. 95.7%).

Note also from **Table B-6** that there will be far more sensitizers identified by a statistical test based on 4 animals per group than would be identified by the Ratio Rule using 5 animals per group. For example, a formal statistical test with N = 4 would have approximately 90% power for detecting an SI = 3, compared with only 50% power by using the Ratio Rule (regardless of N).

Although this report focuses on the large CBA mouse database, there are two smaller LLNA databases involving BALB/c and B6C3F1 mice. Although these other databases were not evaluated in detail, the pattern of LLNA response seen in these two strains was very similar to that seen in the CBA database. This comparison is summarized in **Table B-7** below. In this table, the percentage of positive studies is the percentage of studies having SI > 3 in at least one dosed group. As can be seen in **Table B-7**, there is little evidence of a strain difference in the pattern of LLNA response, and thus there is very little likelihood that a detailed evaluation of these other two strains would change the conclusions of this report.

**Table B-7 Comparison of CBA, BALB/c, and B6C3F1 Databases**

Strain	No. of Studies	No. of Doses	% Positive Studies	Distribution of SIs				
				<1.7	1.7 – 1.9	2.0 – 2.5	2.6 – 3.0	> 3.0
CBA	83	277	59 (49/83)	131 (47%)	23 (8%)	17 (6%)	7 (3%)	99 (36%)
BALB/c	41	133	63 (26/41)	67 (50%)	12 (9%)	8 (6%)	6 (5%)	40 (30%)
B6C3F1	10	28	70 (7/10)	15 (54%)	1 (4%)	1 (4%)	2 (7%)	9 (32%)

Abbreviation: No. = number; SI = stimulation index

There is one B6C3F1 mouse study that deserves special mention: the National Toxicology Program 2,4,5-trichlorophenoxyacetic acid study, which used a sample size of 6 animals per group. The top dose in this study produced a mean SI response of 3.03, which is the weakest “Ratio Rule positive” of any study in the three databases (control dpm responses were 63-69-75-90-119-133 compared with 213-229-244-249-325-405 in the top dosed group). The impact of reducing the sample size from 6 to 5 or 4 animals per group is summarized below.

**Table B-8 Example Showing Effect of Sample Size on Agreement of Results for a Test Substance with SI = 3.03**

	Two N = 5 samples	One N = 5 sample and one N = 4 sample
Agreement (SI > 3)	25.0% (18/36) (18/36)	26.4% (18/36) (119/225)
Agreement (SI < 3)	25.0% (18/36) (18/36)	23.6% (18/36) (106/225)
Disagreement (one SI > 3; one SI < 3)	50.0% (by subtraction)	50.0% (by subtraction)

Abbreviations: N = number of animals per dose group; SI = stimulation index

For these data, there is 50% disagreement between samples of size 4 and samples of size 5, but there is also 50% disagreement between two samples of size 5. This is a somewhat extreme example of the point made earlier, namely that most of the disagreement in Ratio Rule results observed between samples of size 5 and samples of size 4 shown in **Table B-1** is not due to the reduction in sample size, but rather due to the variability in response among animals and the closeness of the SI to the cutoff point of 3.

Finally, it is important to understand that **Table B-1** is not measuring accuracy; it is measuring agreement. That is, **Table B-1** assesses the reliability of N = 5 and N = 4 samples to produce the same classification outcome using the Ratio Rule; it does not assess the ability of N = 5 and N = 4 samples to produce the correct sensitizer classification (which for most substances is not known in any case). As illustrated in this report, as SI approaches 3, different samples may produce different classifications using the Ratio Rule, regardless of sample size, because of naturally occurring variability among animals. Importantly, most of the discordance between N = 5 and N = 4 samples shown in **Table B-1** is *not* due to the reduction in sample size.

With regard to accuracy of classification using the Ratio Rule, for 90% (75/83) of the CBA studies, there is no difference in accuracy using N = 5 and N = 4, based on the top dose group SI response. For eight studies, each with a top dose SI close to 3, there are slight differences in agreement, as shown in **Table B-9**.

**Table B-9 Likelihood of SI > 3 for All CBA Studies Showing Less than Complete Agreement for the Top Dose Response Using N = 5 and N = 4 Samples**

Substance	Top Dose SI	Likelihood of SI > 3 (%)	
		N = 5	N = 4
Formulation 54	2.3	0 (0/36)	7 (16/225)
Hexyl cinnamic aldehyde	2.8	28 (10/36)	38 (85/225)
Formulation 39	3.3	92 (33/36)	78 (175/225)
Bakelite EPR 161	3.5	83 (30/36)	77 (174/225)
Formulation 55	3.7	100 (36/36)	90 (202/225)
Potassium dichromate	4.1	100 (1/1)	92 (23/25)
Formulation 51	4.5 <sup>1</sup>	100 (36/36)	96 (215/225)
1,6-(Bis(2-3-epoxypropoxy)hexane	4.7	100 (36/36)	94 (211/225)

Abbreviations: N = number of animals per dose group; SI = stimulation index

<sup>1</sup>Maximum response seen at mid-dose rather than top dose.

It is not known with certainty whether or not these eight substances are truly sensitizers. The one exception may be hexyl cinnamic aldehyde, which was confirmed in four other studies to be positive, with three showing SI > 4 at this dose. Thus, for this one compound the N = 4 sample may actually be more likely to be “accurate” than the N = 5 sample using the Ratio Rule.

If we assume that the Ratio Rule classifies all other substances correctly, and thus all six substances in **Table B-9** with SI > 3 are sensitizers, then there is a small loss in power by reducing the sample size per group from 5 to 4. However, this difference in power is small, and for all six substances, the likelihood is still quite high (77% - 96%) that the substance will be identified as a sensitizer using a sample of size 4. Recall also that these are “worst cases” and that for 90% of the CBA studies there is no difference in power at all between samples of N = 5 and N = 4. Thus, not only does the reduction in sample size from N = 5 to N = 4 have little impact on reliability using the Ratio Rule, it also appears to have little impact on the accuracy of classification.

## 5.0 Conclusion

For strong sensitizers and for obvious non-sensitizers, the reduction in sample size from 5 to 4 will have essentially no impact on the observed study outcome using the Ratio Rule. For those substances having an SI between (approximately) 2 and 4, the outcomes may be different, especially as SI approaches 3, but any such differences reflect primarily the inherent variability among animals and the closeness of the SI to 3 rather than the impact of reducing the sample size. Empirical examination of data from 83 CBA LLNA studies confirms that it is very unlikely that a reduction in sample size from 5 to 4 animals per group would have any impact on the overall interpretation of study results using the Ratio Rule.

Although the BALB/c and B6C3F1 databases were not evaluated in detail, the pattern of LLNA response seen in these strains is very similar to that seen in the larger CBA database, so a more definitive analysis of these other two strains would almost certainly not change the conclusions of this report. We conclude that a reduction in the sample size from 5 to 4 animals per group is unlikely to significantly impact the results of the LLNA test when using the Ratio Rule.



If a formal statistical test is used rather than (or in addition to) the Ratio Rule, the effect of reducing the sample size from  $N = 5$  to  $N = 4$  is to decrease the power slightly. However, for  $SI > 3$ , the power differences between samples of  $N = 5$  and  $N = 4$  are minimal. Moreover, a statistical test based on 4 animals per group will identify more sensitizers than using the Ratio Rule based on 5 animals per group. Thus, even if a formal statistical test is used rather than (or in addition to) the Ratio Rule, the practical impact of reducing the sample size from 5 to 4 animals per group on the interpretation of experimental results appears to be minimal.

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## Annex I: Summary of Study Results – CBA Mouse Database

**Table B-10 Experiments Conducted at ECPA Laboratories**

Study <sup>1</sup>	Control N	Control Mean	Control SD	Experimental N	Experimental Mean	Experimental SD	SI	Agreement (%) <sup>2</sup>
Dincocap EC 0.8	5	175	50	5	471	198	2.7 <sup>3</sup>	88 (22/25)
Dincocap EC 4.0	5	175	50	5	4007	1578	22.9 <sup>3</sup>	100
Dincocap EC 10.0	5	175	50	4	7088	1863	40.5 <sup>3</sup>	100 <sup>4</sup>
Formaldehyde-1 1.0	5	163	59	5	125	12	0.8	100
Formaldehyde-1 5.0	5	163	59	5	208	147	1.3	100
Formaldehyde-1 20.0	5	163	59	5	781	439	4.8 <sup>3</sup>	100
Formaldehyde-2 1.0	5	844	513	5	838	737	1.0	100
Formaldehyde-2 5.0	5	844	513	5	1824	1341	2.2	92 (23/25)
Formaldehyde-2 20.0	5	844	513	5	5188	2845	6.1 <sup>3</sup>	100
HCA-1 3.0	5	430	154	5	571	153	1.3	100
HCA-1 10.0	5	430	154	5	955	368	2.2 <sup>3</sup>	100
HCA-1 30.0	5	430	154	5	1870	376	4.3 <sup>3</sup>	100
HCA-2 3.0	5	708	172	5	1353	649	1.9 <sup>3</sup>	100
HCA-2 10.0	5	708	172	5	2981	1422	4.2 <sup>3</sup>	100
HCA-2 30.0	5	708	172	5	6525	4014	9.2 <sup>3</sup>	100
Oxyfluorfen EC 1	5	192	117	5	238	67	1.2	100
Oxyfluorfen EC 7	5	192	117	5	234	162	1.2	100
Oxyfluorfen EC 33	5	192	117	5	1043	311	5.4 <sup>3</sup>	100
Potassium dichromate 0.02	5	153	84	5	260	139	1.7	100
Potassium dichromate 0.10	5	153	84	5	234	135	1.5	100
Potassium dichromate 0.50	5	153	84	5	626	390	4.1 <sup>3</sup>	92 (23/25)
Quinoxifen/ cyproconazole 7	5	226	86	5	283	102	1.3	100
Quinoxifen/ cyproconazole 33	5	226	86	5	1470	276	6.5 <sup>3</sup>	100
Quinoxifen/ cyproconazole 100	5	226	86	5	3075	621	13.6 <sup>3</sup>	100
Trifluralin EC 7	5	194	46	5	357	163	1.8 <sup>3</sup>	100
Trifluralin EC 33	5	194	46	5	1585	349	8.2 <sup>3</sup>	100
Trifluralin EC 100	5	194	46	5	3965	1456	20.5 <sup>3</sup>	100

Abbreviations: EC = emulsion concentrate; ECPA = European Crop Protection Association; HCA = hexyl cinnamic aldehyde; N = number of animals per dose group; SD = standard deviation; SI = stimulation index

<sup>1</sup> Test substance and dose tested (%)

<sup>2</sup> Agreement (%) between N = 5 and N = 4 for the Ratio Rule. When agreement is less than 100%, numbers in parentheses indicate the proportion of the total number of N = 4 and N = 5 dose group combinations that agree with respect to whether SI < 3 or SI > 3. This is calculated by multiplying the proportion of N = 5 dose groups yielding SI > 3 with the proportion of N = 4 dose groups yielding SI > 3 and then adding the product of the proportion of N = 5 dose groups yielding SI < 3 with the proportion of N = 4 dose groups yielding SI < 3.

<sup>3</sup> These SIs are significantly different (p < 0.05) from 1 based on a Student's *t* test applied to the logged disintegrations per minute data.

<sup>4</sup> Although N = 4 for the experimental group, the responses in this particular group clearly would have shown 100% concordance between the outcomes for N = 5 and N = 4.

**Table B-11 Experiments Conducted at BASF Laboratories**

Study <sup>1</sup>	Control N	Control Mean	Control SD	Experimental N	Experimental Mean	Experimental SD	SI	Agreement (%) <sup>2</sup>
SC-1 3	6	626	216	6	511	124	0.8	100
SC-1 10	6	626	216	6	789	245	1.3	100
SC-1 30	6	626	216	6	1168	414	1.9 <sup>3</sup>	100
HCA-3 2.5	6	1322	465	6	1479	161	1.1	100
HCA-3 5	6	1322	465	6	1571	921	1.2	100
HCA-3 10	6	1322	465	6	3749	1791	2.8 <sup>3</sup>	55 <sup>4</sup>
HCA-4 3	6	703	197	5	3209	1479	4.6 <sup>3</sup>	100
HCA-4 10	6	703	197	6	4659	1409	6.6 <sup>3</sup>	100
HCA-4 30	6	703	197	6	6929	1187	9.9 <sup>3</sup>	100
HCA-5 10	5	176	26	5	711	240	4.1 <sup>3</sup>	100
HCA-5 30	5	176	26	5	1362	611	7.8 <sup>3</sup>	100
HCA-5 50	5	176	26	5	849	422	4.8 <sup>3</sup>	100
1,6-Bis(2,3-epoxypropoxy)hexane 0.3	6	967	454	6	913	81	0.9	100
1,6-Bis(2,3-epoxypropoxy)hexane 1.0	6	967	454	6	1611	584	1.7	100
1,6-Bis(2,3-epoxypropoxy)hexane 3.0	6	967	454	6	4500	3061	4.7 <sup>3</sup>	94 (211/225)
m-Phenylenebis (methylamine) 0.3	6	468	154	6	900	440	1.9 <sup>3</sup>	100
m-Phenylenebis (methylamine) 1.0	6	468	154	6	4256	1298	9.1 <sup>3</sup>	100
m-Phenylenebis (methylamine) 3.0	6	468	154	6	20691	6436	44.2 <sup>3</sup>	100
Oxirane, mono((C12-14-alkyloxy) methyl) derivs 0.3	6	218	96	6	512	218	2.3 <sup>3</sup>	92 (208/225)
Oxirane, mono((C12-14-alkyloxy) methyl) derivs 1.0	6	218	96	6	908	598	4.2 <sup>3</sup>	92 (206/225)
Oxirane, mono((C12-14-alkyloxy) methyl) derivs 3.0	6	218	96	6	4963	1861	22.7 <sup>3</sup>	100
1,2-Diaminocyclohexane 0.1	5	446	327	6	528	114	1.2	100
1,2-Diaminocyclohexane 0.3	5	446	327	6	810	290	1.8	100
1,2-Diaminocyclohexane 1.0	5	446	327	6	3736	1982	8.4 <sup>3</sup>	100
Trimethylhexamine diamine 1.0	6	742	448	6	1599	400	2.2 <sup>3</sup>	88 <sup>5</sup>

Study <sup>1</sup>	Control N	Control Mean	Control SD	Experimental N	Experimental Mean	Experimental SD	SI	Agreement (%) <sup>2</sup>
Trimethylhexamine diamine 3.0	6	742	448	6	2972	1191	4.0 <sup>3</sup>	93 (209/225)
Trimethylhexamine diamine 10.0	6	742	448	6	6581	1250	8.9 <sup>3</sup>	100
1-(2,3-epoxypropoxy)-2,2-bis[(2,3-epoxypropoxy) methylbutane 1.0	6	388	310	6	797	392	2.1 <sup>3</sup>	81 <sup>6</sup>
1-(2,3-epoxypropoxy)-2,2-bis[(2,3-epoxypropoxy) methylbutane 3.0	6	388	310	6	2531	1812	6.5 <sup>3</sup>	100
1-(2,3-epoxypropoxy)-2,2-bis[(2,3-epoxypropoxy) methylbutane 10.0	6	388	310	6	4644	2150	12.0 <sup>3</sup>	100
3-Aminomethyl-3,5,5-trimethylcyclohexylamine 0.3	6	309	85	6	384	134	1.2	100
3-Aminomethyl-3,5,5-trimethylcyclohexylamine 1.0	6	309	85	6	806	248	2.6 <sup>3</sup>	86 <sup>7</sup>
3-Aminomethyl-3,5,5-trimethylcyclohexylamine 3.0	6	309	85	6	6597	1867	21.4 <sup>3</sup>	100
Dipropylene triamine 0.3	6	349	101	6	753	228	2.2 <sup>3</sup>	100
Dipropylene triamine 1.0	6	349	101	6	1106	254	3.2 <sup>3</sup>	59 <sup>8</sup>
Dipropylene triamine 3.0	6	349	101	6	4344	1350	12.4 <sup>3</sup>	100
N-(2-Hydroxyethyl)-ethylendiamine 3.0	6	445	179	6	891	277	2.0 <sup>3</sup>	100
N-(2-Hydroxyethyl)-ethylendiamine 10.0	6	445	179	6	766	230	1.7 <sup>3</sup>	100
N-(2-Hydroxyethyl)-ethylendiamine 30.0	6	445	179	6	2937	626	6.6 <sup>3</sup>	100
p-tert-Butylphenyl 1-(2,3-epoxy)propyl ether 0.1	6	406	83	6	553	148	1.4	100
p-tert-Butylphenyl 1-(2,3-epoxy)propyl ether 0.3	6	406	83	6	681	230	1.7 <sup>3</sup>	100
p-tert-Butylphenyl 1-(2,3-epoxy)propyl ether 1.0	6	406	83	6	5780	3279	14.2 <sup>3</sup>	100
Bakelite EPR 161 0.1	6	770	189	6	789	108	1	100
Bakelite EPR 161 0.3	6	770	189	6	1825	733	2.4 <sup>3</sup>	99 (222/225)
Bakelite EPR 161 1.0	6	770	189	6	2694	1652	3.5 <sup>3</sup>	68 <sup>9</sup>
Bakelite EPR 162 0.3	6	591	251	6	6225	3285	10.5 <sup>3</sup>	100
Bakelite EPR 162 1.0	6	591	251	6	11790	4292	19.9 <sup>3</sup>	100
Bakelite EPR 162 3.0	6	591	251	6	23583	3469	39.9 <sup>3</sup>	100

Study <sup>1</sup>	Control N	Control Mean	Control SD	Experimental N	Experimental Mean	Experimental SD	SI	Agreement (%) <sup>2</sup>
Bakelite EPR 164 0.3	6	463	208	6	2920	1049	6.3 <sup>3</sup>	100
Bakelite EPR 164 1.0	6	463	208	6	8427	1833	18.2 <sup>3</sup>	100
Bakelite EPR 164 3.0	6	463	208	6	10387	7000	22.4 <sup>3</sup>	100

Abbreviations: EPR = epoxy resin; N = number of animals per dose group; SC = suspension concentrate; SD = standard deviation; SI = stimulation index

<sup>1</sup> Test substance and dose tested (%)

<sup>2</sup> Agreement (%) between N = 5 and N = 4 for the Ratio Rule. When agreement is less than 100%, numbers in parentheses or footnoted indicate the proportion of the total number of N = 4 and N = 5 dose group combinations that agree with respect to whether SI < 3 or SI > 3. This is calculated by multiplying the proportion of N = 5 dose groups yielding SI > 3 with the proportion of N = 4 dose groups yielding SI > 3 and then adding the product of the proportion of N = 5 dose groups yielding SI < 3 with the proportion of N = 4 dose groups yielding SI < 3.

<sup>3</sup> These SIs are significantly ( $p < 0.05$ ) different from 1 based on a Student's *t* test applied to the logged disintegrations per minute data.

<sup>4</sup>  $55\% = (26/36 \times 140/225) + (10/36 \times 85/225)$

<sup>5</sup>  $88\% = (35/36 \times 204/225) + (1/36 \times 21/225)$

<sup>6</sup>  $81\% = (33/36 \times 195/225) + (3/36 \times 30/225)$

<sup>7</sup>  $86\% = (35/36 \times 198/225) + (1/36 \times 27/225)$

<sup>8</sup>  $59\% = (27/36 \times 152/225) + (9/36 \times 73/225)$

<sup>9</sup>  $68\% = (30/36 \times 174/225) + (6/36 \times 51/225)$

**Table B-12 Experiments Conducted at DuPont Laboratories**

Study <sup>1</sup>	Control N	Control Mean	Control SD	Experimental N	Experimental Mean	Experimental SD	SI	Agreement (%) <sup>2</sup>
DU-1A 5	5	506	185	5	284	122	0.6	100
DU-1A 25	5	506	185	5	596	166	1.2	100
DU-1A 50	5	506	185	5	354	198	0.7	100
DU-1A 100	5	506	185	5	526	313	1.0	100
DU-1B 1	5	1067	301	5	635	202	0.6	100
DU-1B 5	5	1067	301	5	1165	386	1.1	100
DU-1B 10	5	1067	301	5	1413	1145	1.3	100
DU-1B 25	5	1067	301	5	1144	388	1.1	100
DU-1C 5	5	617	265	5	419	156	0.7	100
DU-1C 25	5	617	265	4	883	517	1.4	100 <sup>3</sup>
DU-1C 50	5	617	265	5	1075	432	1.7	100
DU-1C 100	5	617	265	4	779	262	1.3	100 <sup>3</sup>
DU-1D 5	5	1067	301	5	755	196	0.7	100
DU-1D 10	5	1067	301	5	1019	266	1.0	100
DU-1D 25	5	1067	301	5	1337	493	1.3	100
DU-1D 50	5	1067	301	4	1086	281	1.0	100 <sup>3</sup>
DU-2A 5	5	992	446	5	4132	815	4.2 <sup>4</sup>	100
DU-2A 25	5	992	446	5	5422	939	5.5 <sup>4</sup>	100
DU-2A 50	5	992	446	5	6604	1282	6.7 <sup>4</sup>	100
DU-2A 100	5	992	446	5	6482	724	6.5 <sup>4</sup>	100
DU-2E 5	5	452	219	5	433	169	1.0	100
DU-2E 25	5	452	219	5	370	142	0.8	100
DU-2E 50	5	452	219	5	509	285	1.1	100
DU-2E 100	5	452	219	5	623	200	1.4	100
DU-3 5	5	917	533	5	531	231	0.6	100
DU-3 10	5	917	533	5	720	306	0.8	100
DU-3 25	5	917	533	5	699	174	0.8	100
DU-3 50	5	917	533	5	538	179	0.6	100
DU-4 5	5	516	114	5	439	203	0.9	100
DU-4 25	5	516	114	5	505	257	1.0	100
DU-4 50	5	516	114	5	500	200	1.0	100
DU-4 100	5	516	114	5	538	65	0.9	100
DU-5A 5	5	589	317	5	1576	504	2.7 <sup>4</sup>	76 (19/25)
DU-5A 25	5	589	317	5	903	534	1.5	100
DU-5A 50	5	589	317	5	915	223	1.6	100

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Study <sup>1</sup>	Control N	Control Mean	Control SD	Experimental N	Experimental Mean	Experimental SD	SI	Agreement (%) <sup>2</sup>
DU-5A 100	5	589	317	5	499	230	0.8	100
DU-5B 5	5	1057	256	5	835	406	0.8	100
DU-5B 25	5	1057	256	5	1168	352	1.1	100
DU-5B 50	5	1057	256	5	1087	200	1.0	100
DU-5B 100	5	1057	256	5	1200	394	1.1	100
DU-5C 1	5	354	140	5	491	136	1.4	100
DU-5C 5	5	354	140	5	692	313	2.0 <sup>4</sup>	100
DU-5C 25	5	354	140	5	429	195	1.2	100
DU-5C 100	5	354	140	5	312	124	0.9	100
DU-6 5	4	468	290	5	503	300	1.1	100 <sup>3</sup>
DU-6 25	4	468	290	5	381	106	0.8	100 <sup>3</sup>
DU-6 50	4	468	290	5	400	176	0.9	100 <sup>3</sup>
DU-6 80	4	468	290	5	440	211	0.9	100 <sup>3</sup>
DU-7 5	5	721	191	5	1394	1154	1.9	100
DU-7 25	5	721	191	5	846	331	1.2	100
DU-7 50	5	721	191	5	817	286	1.1	100
DU-7 80	5	721	191	5	915	249	1.3	100
DU-8A 1	9	486	186	4	680	178	1.4	100 <sup>3</sup>
DU-8A 10	9	486	186	5	658	261	1.4	100
DU-8A 50	9	486	186	4	391	184	0.8	100 <sup>3</sup>
DU-8A 100	9	486	186	5	473	263	1.0	100
DU-8B 5	5	786	312	5	916	460	1.2	100
DU-8B 25	5	786	312	5	1515	621	1.9	100
DU-8B 50	5	786	312	5	1121	764	1.4	100
DU-8B 100	5	786	312	5	1422	921	1.8	100
DU-9A 5	5	677	307	5	2405	1569	3.6 <sup>4</sup>	84 (21/25)
DU-9A 25	5	677	307	5	3354	1463	5.0 <sup>4</sup>	100
DU-9A 50	5	677	307	5	5975	773	8.8 <sup>4</sup>	100
DU-9A 100	5	677	307	5	9118	3211	13.5 <sup>4</sup>	100
DU-9B 5	5	1049	285	5	809	362	0.8	100
DU-9B 25	5	1049	285	5	822	195	0.8	100
DU-9B 50	5	1049	285	5	622	242	0.6	100
DU-9B 100	5	1049	285	5	493	88	0.5	100
DU-10 0.5	5	177	67	5	174	25	1.0	100
DU-10 1.0	5	177	67	5	230	73	1.3	100
DU-10 2.5	5	177	67	5	265	55	1.5	100



Study <sup>1</sup>	Control N	Control Mean	Control SD	Experimental N	Experimental Mean	Experimental SD	SI	Agreement (%) <sup>2</sup>
DU-10 5.0	5	177	67	3	289	122	1.6	NC <sup>5</sup>
DU-11B 5	5	984	210	5	1362	561	1.4	100
DU-11B 25	5	984	210	5	639	449	0.6	100
DU-11B 50	5	984	210	5	651	531	0.7	100
DU-11B 100	5	984	210	5	1016	1032	1.0	100
DU-11C 5	5	769	310	5	1168	472	1.5	100
DU-11C 25	5	769	310	5	871	217	1.1	100
DU-11C 50	5	769	310	5	719	133	0.9	100
DU-11C 100	5	769	310	5	1113	300	1.4	100
DU-12 1	5	617	265	5	479	132	0.8	100
DU-12 5	5	617	265	5	749	378	1.2	100
DU-12 25	5	617	265	5	477	253	0.8	100
DU-12 50	5	617	265	5	872	497	1.4	100
DU-13A 5	5	621	455	5	284	67	0.5	100
DU-13A 25	5	621	455	5	276	93	0.4	100
DU-13A 50	5	621	455	5	322	167	0.5	100
DU-13A 100	5	621	455	5	370	56	0.6	100
DU-13B 1	5	578	161	5	703	450	1.2	100
DU-13B 10	5	578	161	5	551	179	1.0	100
DU-13B 50	5	578	161	5	413	117	0.7	100
DU-13B 100	5	578	161	5	376	201	0.7	100

Abbreviations: DU = DuPont; N = number of animals per dose group; NC = not calculated; SD = standard deviation; SI = stimulation index

<sup>1</sup> Test substance and dose tested (%)

<sup>2</sup> Agreement (%) between N = 5 and N = 4 for the Ratio Rule. When agreement is less than 100%, numbers in parentheses indicate the proportion of the total number of N = 4 and N = 5 dose group combinations that agree with respect to whether SI < 3 or SI > 3. This is calculated by multiplying the proportion of N = 5 dose groups yielding SI > 3 with the proportion of N = 4 dose groups yielding SI > 3 and then adding the product of the proportion of N = 5 dose groups yielding SI < 3 with the proportion of N = 4 dose groups yielding SI < 3.

<sup>3</sup> Although N = 4 for the experimental group, the responses in this particular group clearly would have shown 100% concordance between the outcomes for N = 5 and N = 4.

<sup>4</sup> These SIs are significantly ( $p < 0.05$ ) different from 1 based on a Student's *t* test applied to the logged disintegrations per minute data.

<sup>5</sup> Agreement could not be assessed, since N < 4.

**Table B-13 Experiments Conducted at EFfCI Laboratories**

Study <sup>1</sup>	Control N	Control Mean	Control SD	Experimental N	Experimental Mean	Experimental SD	SI	Agreement (%) <sup>2</sup>
Fumaric Acid 5	5	327	85	5	419	126	1.3	100
Fumaric Acid 10	5	327	85	5	742	284	2.3 <sup>3</sup>	100
Fumaric Acid 25	5	327	85	5	479	201	1.5	100
Linoleic Acid 10	5	223	133	5	326	176	1.5	100
Linoleic Acid 25	5	223	133	5	1567	303	7.0 <sup>3</sup>	100
Linoleic Acid 50	5	223	133	5	2025	601	9.1 <sup>3</sup>	100
Linoleic Acid 10	5	223	133	5	699	301	3.1 <sup>3</sup>	56 (14/25)
Linoleic Acid 25	5	223	133	5	2075	344	9.3 <sup>3</sup>	100
Linoleic Acid 50	5	223	133	5	2290	1174	10.3 <sup>3</sup>	100
Maleic Acid 10	5	327	85	5	2186	934	6.7 <sup>3</sup>	100
Maleic Acid 25	5	327	85	5	5262	686	16.1 <sup>3</sup>	100
Maleic Acid 50	5	327	85	5	5244	2304	16.0 <sup>3</sup>	100
Octinol 10	5	1120	512	5	6327	1446	5.6 <sup>3</sup>	100
Octinol 25	5	1120	512	5	9833	2523	8.8 <sup>3</sup>	100
Octinol 50	5	1120	512	4	12594	1250	11.2 <sup>3</sup>	100 <sup>4</sup>
Oleic Acid 10	5	223	133	5	581	408	2.6 <sup>3</sup>	84 (21/25)
Oleic Acid 25	5	223	133	5	3336	1688	14.9 <sup>3</sup>	100
Oleic Acid 50	5	223	133	5	1550	897	6.9 <sup>3</sup>	100
Squalene 10	5	223	133	5	839	245	3.8 <sup>3</sup>	100
Squalene 25	5	223	133	5	1536	209	6.9 <sup>3</sup>	100
Squalene 50	5	223	133	5	1821	327	8.2 <sup>3</sup>	100
Succinic Acid 5	5	327	85	5	376	146	1.1	100
Succinic Acid 10	5	327	85	5	407	113	1.2	100
Succinic Acid 25	5	327	85	5	420	243	1.3	100
Undecylenic Acid 10	5	223	133	5	556	140	2.5 <sup>3</sup>	80 (20/25)
Undecylenic Acid 25	5	223	133	5	736	250	3.3 <sup>3</sup>	84 (21/25)
Undecylenic Acid 50	5	223	133	5	991	149	4.4 <sup>3</sup>	100

Abbreviations: EFfCI = European Federation for Cosmetics Ingredients; N = number of animals per dose group; SD = standard deviation; SI = stimulation index

<sup>1</sup> Test substance and dose tested (%)

<sup>2</sup> Agreement (%) between N = 5 and N = 4 for the Ratio Rule. When agreement is less than 100%, numbers in parentheses indicate the proportion of the total number of N = 4 and N = 5 dose group combinations that agree with respect to whether SI < 3 or SI > 3. This is calculated by multiplying the proportion of N = 5 dose groups yielding SI > 3 with the proportion of N = 4 dose groups yielding SI > 3 and then adding the product of the proportion of N = 5 dose groups yielding SI < 3 with the proportion of N = 4 dose groups yielding SI < 3.

<sup>3</sup> These SIs are significantly (p < 0.05) different from 1 based on a Student's *t* test applied to the logged disintegrations per minute data.

<sup>4</sup> Although N = 4 for the experimental group, the responses in this particular group clearly would have shown 100% concordance between the outcomes for N = 5 and N = 4.

**Table B-14 Experiments Conducted at BAuA Laboratories**

Study <sup>1</sup>	Control N	Control Mean	Control SD	Experimental N	Experimental Mean	Experimental SD	SI	Agreement (%) <sup>2</sup>
Yellow E-JD 3442 1	5	70	21	5	70	19	1.0	100
Yellow E-JD 3442 3	5	70	21	5	52	9	0.8	100
Yellow E-JD 3442 9	5	70	21	5	60	32	0.9	100
Yellow E-JD 3442 15	5	70	21	5	61	16	0.9	100
CI Reactive Red 231 1	5	70	21	5	334	147	4.8 <sup>3</sup>	100
CI Reactive Red 231 3	5	70	21	5	234	78	3.4 <sup>3</sup>	88 (22/25)
CI Reactive Red 231 9	5	70	21	5	305	121	4.4 <sup>3</sup>	100
CI Reactive Red 231 15	5	70	21	5	317	105	4.6 <sup>3</sup>	100
P-46 1	5	70	21	5	167	86	2.4 <sup>3</sup>	100
P-46 3	5	70	21	5	175	73	2.5 <sup>3</sup>	96 (24/25)
P-46 9	5	70	21	5	135	39	1.9 <sup>3</sup>	100
P-46 15	5	70	21	5	175	45	2.5 <sup>3</sup>	100
CI Reactive Yellow 174 1	5	70	21	5	288	62	4.1 <sup>3</sup>	100
CI Reactive Yellow 174 3	5	70	21	5	231	70	3.3 <sup>3</sup>	80 (20/25)
CI Reactive Yellow 174 9	5	70	21	5	385	242	5.5 <sup>3</sup>	100
CI Reactive Yellow 174 15	5	70	21	5	539	114	7.8 <sup>3</sup>	100
Navy 14 08 723 1	5	70	21	5	353	54	5.1 <sup>3</sup>	100
Navy 14 08 723 3	5	70	21	5	335	116	4.8 <sup>3</sup>	100
Navy 14 08 723 9	5	70	21	5	398	102	5.7 <sup>3</sup>	100
Navy 14 08 723 15	5	70	21	5	361	90	5.2 <sup>3</sup>	100
Dispersionsrot 2754 1	5	70	21	5	68	27	1.0	100
Dispersionsrot 2754 3	5	70	21	5	65	19	0.9	100
Dispersionsrot 2754 9	5	70	21	5	67	40	1.0	100

Abbreviations: BAuA = Federal Institute for Occupational Safety and Health (Germany); N = number of animals per dose group; SD = standard deviation; SI = stimulation index

<sup>1</sup> Test substance and dose tested (%)

<sup>2</sup> Agreement (%) between N = 5 and N = 4 for the Ratio Rule. When agreement is less than 100%, numbers in parentheses indicate the proportion of the total number of N = 4 and N = 5 dose group combinations that agree with respect to whether SI < 3 or SI > 3. This is calculated by multiplying the proportion of N = 5 dose groups yielding SI > 3 with the proportion of N = 4 dose groups yielding SI > 3 and then adding the product of the proportion of N = 5 dose groups yielding SI < 3 with the proportion of N = 4 dose groups yielding SI < 3.

<sup>3</sup> These SIs are significantly (p < 0.05) different from 1 based on a Student's *t* test applied to the logged disintegrations per minute data.

**Table B-15 Experiments Conducted at Dow AgroSciences Laboratories**

Study <sup>1</sup>	Control N	Control Mean	Control SD	Experimental N	Experimental Mean	Experimental SD	SI	Agreement (%) <sup>2</sup>
Formulation 29 5	6	567	305	6	1036	663	1.8	100
Formulation 29 25	6	567	305	6	913	200	1.6	100
Formulation 29 100	6	567	305	6	823	373	1.5	100
Formulation 30 5	6	536	258	6	947	253	1.8 <sup>3</sup>	100
Formulation 30 25	6	536	258	6	3839	736	7.2 <sup>3</sup>	100
Formulation 30 100	6	536	258	6	7269	1014	13.6 <sup>3</sup>	100
Formulation 31 5	6	385	121	5	393	223	1.0	100
Formulation 31 25	6	385	121	5	724	215	1.9 <sup>3</sup>	100
Formulation 31 100	6	385	121	6	696	262	1.8 <sup>3</sup>	100
Formulation 32 5	6	332	346	6	2136	737	6.5 <sup>3</sup>	100
Formulation 32 25	6	332	346	6	14833	6139	44.7 <sup>3</sup>	100
Formulation 32 100	6	332	346	6	22965	5480	69.3 <sup>3</sup>	100
Formulation 33 5	6	672	249	6	479	194	0.7	100
Formulation 33 25	6	672	249	6	913	496	1.4	100
Formulation 33 100	6	672	249	6	843	303	1.3	100
Formulation 34 5	6	385	121	6	713	331	1.9	100
Formulation 34 25	6	385	121	6	528	227	1.4	100
Formulation 34 100	6	385	121	6	581	216	1.5	100
Formulation 35 5	6	332	346	6	360	294	1.1	100
Formulation 35 25	6	332	346	6	383	158	1.2	100
Formulation 35 100	6	332	346	6	412	317	1.3	100
Formulation 37 1	6	744	359	6	1008	525	1.4	100
Formulation 37 5	6	744	359	6	1999	1687	2.7	56 <sup>4</sup>
Formulation 37 15	6	744	359	6	5586	4162	7.5 <sup>3</sup>	100
Formulation 38 5	6	889	520	6	960	515	1.1	100
Formulation 38 25	6	889	520	6	4098	1541	4.6 <sup>3</sup>	100
Formulation 38 100	6	889	520	6	11232	2102	12.7 <sup>3</sup>	100
Formulation 39 1	6	627	256	6	1076	268	1.7 <sup>3</sup>	100
Formulation 39 5	6	627	256	6	1551	650	2.5 <sup>3</sup>	84 <sup>5</sup>
Formulation 39 25	6	627	256	6	2083	259	3.3 <sup>3</sup>	73 <sup>6</sup>
Formulation 40 1	5	821 <sup>7</sup>	263	6	1481	621	1.8	100
Formulation 40 5	5	821 <sup>7</sup>	263	6	2316	401	2.8 <sup>3</sup>	73 (55/75)
Formulation 40 25	5	821 <sup>7</sup>	263	6	4646	1833	5.7 <sup>3</sup>	100
Formulation 41 5	6	1017	325	6	1936	1024	1.9 <sup>3</sup>	100
Formulation 41 25	6	1017	325	6	1891	1133	1.9	100
Formulation 41 100	6	1017	325	5	5653 <sup>7</sup>	2750	5.6 <sup>3</sup>	100
Formulation 49 5	5	626 <sup>7</sup>	298	6	442	250	0.7	100

Study <sup>1</sup>	Control N	Control Mean	Control SD	Experimental N	Experimental Mean	Experimental SD	SI	Agreement (%) <sup>2</sup>
Formulation 49 25	5	626 <sup>7</sup>	298	6	880	444	1.4	100
Formulation 49 100	5	626 <sup>7</sup>	298	5	2958	489	4.7 <sup>3</sup>	100
Formulation 50 5	6	1208	882	6	796	183	0.7	100
Formulation 50 25	6	1208	882	6	786	436	0.7	100
Formulation 50 100	6	1208	882	6	9439	4239	7.8 <sup>3</sup>	100
Formulation 51 5	6	863	526	6	1346	537	1.6	100
Formulation 51 25	6	863	526	6	3893	2120	4.5 <sup>3</sup>	96 (215/225)
Formulation 51 100	6	863	526	6	2084	1725	2.4	66 <sup>8</sup>
Formulation 53 2.5	5	392 <sup>7</sup>	159	6	596	317	1.5	100
Formulation 53 7.5	5	392 <sup>7</sup>	159	6	1240	987	3.2 <sup>3</sup>	52 <sup>9</sup>
Formulation 53 15	5	392 <sup>7</sup>	159	4	2609	1494	6.7 <sup>3</sup>	100 <sup>10</sup>
Formulation 54 5	6	438	143	6	551	357	1.3	100
Formulation 54 25	6	438	143	6	502	262	1.2	100
Formulation 54 100	6	438	143	6	1016	583	2.3	93 (209/225)
Formulation 55 5	6	529	238	6	781	602	1.5	100
Formulation 55 25	6	529	238	6	1348	947	2.5 <sup>3</sup>	68 <sup>11</sup>
Formulation 55 100	6	529	238	6	1972	758	3.7 <sup>3</sup>	90 (202/225)
Formulation 56 5	6	529	238	6	1726	831	3.3 <sup>3</sup>	57 <sup>12</sup>
Formulation 56 25	6	529	238	6	3217	1996	6.1 <sup>3</sup>	100
Formulation 56 100	6	529	238	2	2064	21	3.9 <sup>3</sup>	NC <sup>13</sup>

Abbreviations: N = number of animals per dose group; NC = not calculated; SD = standard deviation; SI = stimulation index

<sup>1</sup> Test substance and dose tested (%)

<sup>2</sup> Agreement (%) between N = 5 and N = 4 for the Ratio Rule. When agreement is less than 100%, numbers in parentheses or footnoted indicate the proportion of the total number of N = 4 and N = 5 dose group combinations that agree with respect to whether SI < 3 or SI > 3. This is calculated by multiplying the proportion of N = 5 dose groups yielding SI > 3 with the proportion of N = 4 dose groups yielding SI > 3 and then adding the product of the proportion of N = 5 dose groups yielding SI < 3 with the proportion of N = 4 dose groups yielding SI < 3.

<sup>3</sup> These SIs are significantly ( $p < 0.05$ ) different from 1 based on a Student's *t* test applied to the logged disintegrations per minute data.

<sup>4</sup> 56% = (26/36 x 142/225) + (10/36 x 83/225)

<sup>5</sup> 84% = (35/36 x 194/225) + (1/36 x 31/225)

<sup>6</sup> 73% = (33/36 x 175/225) + (3/36 x 50/225)

<sup>7</sup> Data reflects elimination of one control outlier (4258) in Formulation 40, one dosed group outlier (428) in Formulation 41, one control outlier (3) and one dosed group outlier (6273) in Formulation 49, and one control outlier (3172) in Formulation 53.

<sup>8</sup> 66% = (29/36 x 172/225) + (7/36 x 53/225)

<sup>9</sup> 52% = (4/6 x 42/75) + (2/6 x 33/75)

<sup>10</sup> Although N = 4 for the experimental group, the responses in this particular group clearly would have shown 100% concordance between the outcomes for N = 5 and N = 4.

<sup>11</sup> 68% = (31/36 x 168/225) + (5/36 x 57/225)

<sup>12</sup> 57% = (26/36 x 150/225) + (10/36 x 75/225)

<sup>13</sup> Agreement could not be assessed, since N < 4.

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## **Appendix C**

### **Essential Test Method Components and Other Validation Considerations for the Murine Local Lymph Node Assay<sup>19</sup>**

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<sup>19</sup> Based on the updated ICCVAM-recommended LLNA test method protocol in **Appendix A**.

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## 1.0 Essential Test Method Components

The following is a detailed description of the essential test method components for the validation of modifications to the murine local lymph node assay (LLNA) using the Interagency Committee on the Validation of Alternative Methods (ICCVAM) performance standards and the 18 required reference substances. Adherence to these essential test method components ensures that a modified test is functionally and mechanistically similar to the traditional LLNA. The essential test method components are provided as bolded text and are accompanied by additional guidance information in the bulleted text.

### 1. The test substance must be applied topically to both ears of the mice.

- On treatment days, an appropriate volume (e.g., 25 µL) of the test substance, vehicle control, and positive control (where appropriate) should be applied to each ear.
- Since the ear is the site of test substance application, any unique identification of the animals prior to placement in the study should not involve identification via the ear (i.e., marking, clipping, or punching of the ear).
- The ears of all animals should be examined prior to initiation of the test to ensure there are no skin lesions present.

### 2. Lymphocyte proliferation must be measured in the lymph nodes draining the site of test substance application.

- The basic principle underlying the LLNA is that sensitizers induce proliferation of lymphocytes during the induction phase of skin sensitization in the lymph nodes that drain the site of substance application. Test method endpoints may include cell turnover and/or cell number.
- Under appropriate test conditions, this proliferation is proportional to the dose applied, and provides a means of obtaining an objective, quantitative measurement of sensitization.
- Since topical application of the test substance must be to the ear, the LLNA essential test method components state that measurement of lymphocyte proliferation should be from lymph nodes that drain the auricular site of test substance application.
- **Annex I in Appendix A** of the ICCVAM Recommended LLNA Performance Standards describes an approach to dissection and identification of the draining auricular lymph nodes.

### 3. Lymphocyte proliferation must be measured during the induction phase of skin sensitization.

- The LLNA measures events during the induction phase, rather than in the elicitation phase, of allergic contact dermatitis (ACD).
- In order for a modified LLNA test method protocol to remain mechanistically and functionally similar to the LLNA, the dosing schedule should ensure that lymphocyte proliferation is only measured during the induction phase of ACD.

- Usually, the induction phase lasts eight to 15 days in humans, and five to seven days in the mouse (Saint-Mezard et al. 2003)
- Raw data and calculated results (i.e., as measured or quantified by the stimulation index [SI]) should be provided for all test substance dose levels 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  $\geq 3$ .
  - However, the magnitude of the SI should not be the sole factor used in determining the biological significance of a skin sensitization response. Factors that could be considered in addition to the SI include: statistical analyses of individual animal data (if available), the nature of the dose-response relationship, test substance toxicity, and test substance solubility.
  - Statistical analysis of individual animal data may provide a more complete evaluation.

**4. For test substances, the highest dose selected must be the maximum soluble concentration that does not induce systemic toxicity and/or excessive local irritation. For positive control substances, the highest dose selected should exceed the known EC3 values (i.e., the estimated concentrations needed to produce an SI of 3) of the reference substances without producing systemic toxicity and/or excessive local irritation.**

- If dose-response information is desired, then a minimum of three dose levels should be tested plus concurrent vehicle control and, where appropriate positive control. Test substance treatment dose levels should be based on the recommendations given in Kimber and Basketter (1992) and in the ICCVAM Panel Report (ICCVAM 1999). Dose levels are normally selected from the concentration series 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc.
- Efforts should be made to identify existing information that may aid in selecting the appropriate maximum test substance dose level.
  - Guidance for determining the appropriate maximum dose based on the avoidance of excessive local irritation (indicated by erythema and/or ear swelling) and/or systemic toxicity (indicated by clinical observations) is detailed in the updated ICCVAM-recommended LLNA test method protocol (**Appendix A** of the ICCVAM Recommended LLNA Performance Standards).

**5. A vehicle control must be included in each study and, where appropriate, a positive control should be used.**

*Vehicle control*

- The response of the vehicle control group is used as the reference value against which the SI is calculated and therefore, a vehicle control must be included in each experiment.
- The choice of vehicle should be informed by the relevant literature.
- Other vehicles may be used if appropriate justification is provided. This may necessitate the use of additional controls in order to demonstrate that the alternative vehicle does not adversely impact the outcome of a test substance. Recommended vehicles are acetone: olive oil (4:1 v/v), N,N-dimethylformamide, methyl ethyl ketone, propylene glycol, and dimethyl sulfoxide.

*Positive control*

- The purpose of the 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.
- If sensitizer(s) are run with non-sensitizers, no positive control is required (i.e., for any test, a known sensitizer from the reference substance list may serve as a positive control). If non-sensitizers are run by themselves, a positive control is required.

**6. A minimum of four animals per dose group is required.**

**7. Either individual or pooled animal data may be collected.**

*Individual animal data*

The updated ICCVAM-recommended LLNA test method protocol recommends the collection of lymph nodes from individual animals. This approach allows for:

- Detection of problems caused by technical inexperience (Cockshott et al. 2006)
- Identification of potential outlier responses that may aid in avoiding false negative results for weaker sensitizers (i.e., substances that normally would induce an SI just above 3 in the traditional LLNA might be incorrectly classified as negative due to an outlier value because the resulting mean SI may be less than 3 if the outlier is not identified and excluded)
- The assessment of interanimal variability
- Statistical comparison of the difference between test substance and vehicle control group measurements and an assessment of statistical power associated with the number of animals per group
- Evaluation of the possibility of reducing the number of animals in the positive control group, which is only feasible when individual animal data are collected

- Recognition that certain regulatory authorities (e.g., U.S. Environmental Protection Agency [EPA], U.S. Food and Drug Administration [FDA]) require data from single animals

#### *Pooled animal data*

- The use of pooled nodes has the advantage of technical simplicity. It is the view of those who favor this approach that pooling of nodes serves to minimize variability and also serves to minimize the inevitable loss of material associated with the handling and processing of very small amounts of tissue. Although this may be of little impact generally, it may be of importance in relation to the detection of the weakest skin-sensitizing substances.
- In addition, it is worth recognizing that the great majority of the data employed in the original validation of the assay was drawn from experiments using pooled nodes from four mice, and that data generated in this manner still represents the greater part of the published data.

#### *Assessment of lymphocyte proliferation and interpretation of results*

- Lymphocyte proliferation should be expressed in the units obtained from 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 positive and vehicle controls.

## **2.0 Other Validation Considerations**

The following should also be considered during the validation of a modified LLNA test method using the ICCVAM LLNA performance standards and the 18 required reference substances.

### **1. Use of the positive control**

- Consideration should be given to concurrently running a mix of negative, weakly, and strongly positive substances from the reference substance list so that the strongly positive substance can act as a positive control for the weaker skin sensitizer.

### **2. Group housing is recommended; otherwise animal selection, preparation, housing, and feeding should be in accordance with Organisation for Economic Co-operation and Development (OECD) Test Guideline 429 (OECD 2002) in compliance with other relevant regulatory requirements (e.g., animal care and use).**

### **3. Appropriate quality assurance systems (e.g., Good Laboratory Practice guidelines e.g., OECD 1998; EPA 2006a, 2006b; FDA 2006) are required.**

- Collection, recording and retention of raw and processed data
- Data available upon request

- 4. The study should be conducted according to international validation principles (e.g., OECD Guidance Document 34; OECD 2005) and in compliance with other relevant regulatory requirements (e.g., animal care and use).**

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## **Appendix D**

### **Timeline for Development of the ICCVAM Murine Local Lymph Node Assay Performance Standards**

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<b>January 10, 2007</b>	ICCVAM receives a letter from the CPSC nominating six LLNA review activities. <sup>20</sup>
<b>January 2007</b>	The ICCVAM IWG is re-established to work with NICEATM to carry out LLNA evaluations.
<b>January 24, 2007</b>	ICCVAM endorses the six CPSC-nominated LLNA review activities and the development of ICCVAM LLNA performance standards.
<b>May 17, 2007</b>	<i>Federal Register</i> notice (72 FR 27815) – <i>The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data.</i>
<b>June 12, 2007</b>	SACATM endorses with high priority the six CPSC-nominated LLNA review activities and the development of ICCVAM LLNA performance standards.
<b>August 14, 2007</b>	IWG endorses release of the draft ICCVAM LLNA Performance Standards for public comment.
<b>September 12, 2007</b>	<i>Federal Register</i> notice (72 FR 52130) – <i>Announcement of Draft ICCVAM Performance Standards for the Murine Local Lymph Node Assay: Request for Comments.</i>
<b>November 12-13, 2007</b>	ECVAM Workshop on Alternative Methods (Reduction, Refinement, Replacement).
<b>January 8, 2008</b>	<i>Federal Register</i> notice (73 FR 1360) – <i>Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments.</i>
<b>March 4-6, 2008</b>	Independent Peer Review Panel Meeting on seven LLNA review activities, which includes the ICCVAM LLNA performance standards, CPSC Headquarters, Bethesda, MD; public meeting with opportunity for oral public comments. The Panel was charged with reviewing the draft ICCVAM LLNA Performance Standards and considering if they were adequate for assessing the accuracy and reliability of test method protocols that are based on similar scientific principles and that measure the same biologic effect as the traditional LLNA.

<sup>20</sup>[http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC\\_LLNA\\_nom.pdf](http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf)

<b>May 7-8, 2008</b>	Meeting of the ECVAM Scientific Advisory Committee to discuss ECVAM LLNA performance standards and to consider ICCVAM’s request for ECVAM and ICCVAM to develop harmonized LLNA performance standards.
<b>May 20, 2008</b>	<i>Federal Register notice (73 FR 29136) – Announcement of the Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments.</i> <sup>21</sup>
<b>June 18-19, 2008</b>	SACATM public meeting for comments on the Panel report.
<b>October 29, 2008</b>	ICCVAM endorses the ICCVAM LLNA performance standards.

Abbreviations: CPSC = U.S. Consumer Product Safety Commission; ECVAM = European Centre for the Validation of Alternative Methods; ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; IWG = ICCVAM Immunotoxicity Working Group; LLNA = murine local lymph node assay; NICEATM = National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods; SACATM = Scientific Advisory Committee on Alternative Toxicological Methods

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<sup>21</sup> [http://iccvam.niehs.nih.gov/docs/immunotox\\_docs/LLNAPRPRept2008.pdf](http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf)

## Appendix E

### Supporting Documents: Development of the ICCVAM LLNA Performance Standards

<b>E1</b>	<b>Methods Applicable to the ICCVAM LLNA Performance Standards and Essential Test Method Components .....</b>	<b>E-3</b>
<b>E2</b>	<b>Selection of Proposed Performance Standards Reference Substances .....</b>	<b>E-9</b>
<b>E3</b>	<b>Rationale for the Required Accuracy and Reliability Statistics Included in the Test Method Performance Evaluation .....</b>	<b>E-25</b>

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## **Appendix E1**

### **Methods Applicable to the ICCVAM LLNA Performance Standards and Essential Test Method Components**

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## 1.0 Introduction

In 2007, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) developed draft murine local lymph node assay (LLNA) Performance Standards, which were announced and released to the public for comment in a *Federal Register (FR)* notice on September 12, 2007 (72 FR 52130).<sup>22</sup> The European Centre for the Validation of Alternative Methods (ECVAM) also independently drafted LLNA performance standards in 2007, and the Japanese Center for the Validation of Alternative Methods (JaCVAM) initiated two validation studies of modified LLNA test methods using a list of proposed reference substances to evaluate their validity. With obvious international interest in developing LLNA performance standards, ICCVAM, JaCVAM, and ECVAM agreed that it would be useful to work together to attempt to develop internationally harmonized LLNA performance standards that could be proposed for inclusion in the Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 429, which describes the use of the LLNA for determining allergic contact dermatitis potential of chemicals and other substances.

The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and ICCVAM interacted closely with ECVAM and JaCVAM through the ECVAM and JaCVAM liaisons to the ICCVAM Immunotoxicity Working Group (IWG) and representatives of the ECVAM Task Force on Skin Sensitization. Additionally, at their October 2007 meeting, the ECVAM Scientific Advisory Committee considered both drafts of the LLNA performance standards (i.e., ICCVAM and ECVAM versions), along with the ICCVAM recommendations for a process to achieve harmonization of the two documents and subsequently deferred their evaluation of LLNA performance standards until their November 2008 meeting. They encouraged ECVAM and ICCVAM to continue working together to reach agreement on any outstanding differences.

After considering these comments, ICCVAM announced in an *FR* notice on January 8, 2008 (73 FR 1360),<sup>23</sup> the availability of a revised draft version of the LLNA Performance Standards. The ICCVAM Independent Scientific Peer Review Panel (Panel) considered the revised draft Performance Standards at a public meeting convened on March 4-6, 2008, at the Consumer Product Safety Commission Headquarters in Bethesda, MD. All comments received in response to the *FR* notice were provided to the Panel for their consideration. Subsequently, the Panel's conclusions and recommendations were announced in a May 2008 *FR* notice (73 FR 29136),<sup>24</sup> released to the public and to ICCVAM's Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) for comment at their public meeting convened on June 18-19, 2008. The Panel Report and all comments by the public and SACATM were considered by the ICCVAM IWG and ICCVAM in preparing final LLNA performance standard recommendations for submittal to U.S. Federal agencies and for release to the public. Performance standards adopted by U.S. Federal regulatory authorities can be provided or referenced in test guidelines. Availability of these performance standards and ICCVAM test method evaluation reports, which provide ICCVAM recommendations

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<sup>22</sup> [http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR\\_E7\\_18011.pdf](http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_18011.pdf)

<sup>23</sup> [http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR\\_E7\\_25553.pdf](http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_25553.pdf)

<sup>24</sup> <http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR-E8-11195.pdf>

and a comprehensive evaluation of the usefulness and limitations of a test method, are announced in the *FR*, NTP newsletters, and by email to NICEATM-ICCVAM email list subscribers.

## **2.0 Revisions to the Methods Applicable to the ICCVAM LLNA Performance Standards**

The original draft ICCVAM LLNA Performance Standards (i.e., the version released on September 12, 2007) stated that they were intended for LLNA test method protocols that incorporate modifications that use non-radioactive procedures (rather than radioactivity, which is used in the traditional LLNA) to evaluate lymphocyte proliferation in the draining auricular lymph nodes. After discussions with members of the ECVAM Skin Sensitization Task Force, the draft document was updated to reflect increased specificity with respect to the allowable modifications to the LLNA test method protocol. The performance standards released on January 8, 2008, were applicable only to LLNA test method protocols with “minor” modifications that vary only by using non-radioactive methods for assessing lymphocyte proliferation in the draining auricular lymph nodes. All other test method protocol modifications 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) were considered “major” modifications. The performance standards stated that LLNA test method protocols with “major” modifications would be subjected to a more extensive validation effort.

However, the Panel considered the draft LLNA Performance Standards to be appropriate for evaluating modifications other than those defined as “minor.” The Panel recommended that, instead of defining “minor” and “major” modifications, the performance standards should define criteria to ensure that a modified test method is mechanistically and functionally similar to the traditional LLNA. Thus, taking into consideration the Panel’s comments, along with those of SACATM and the public, and relevant IWG discussions, the final ICCVAM LLNA Performance Standards indicate that they are to be applied to modified methods that are mechanistically and functionally similar to the traditional LLNA (see **Section 2.2** of the ICCVAM Recommended LLNA Performance Standards).

## **3.0 Revisions to the Essential Test Method Components of the ICCVAM LLNA Performance Standards**

The original draft ICCVAM LLNA Performance Standards, released on September 12, 2007, stated that the essential test method components included all aspects of the traditional LLNA test method protocol as described by ICCVAM (1999) and Dean et al. (2001), upon which OECD TG 429 (OECD 2002) was based, with the exceptions being the method used to assess lymphocyte proliferation and the corresponding decision criteria for classifying a test substance as positive or negative. The original draft Performance Standards then described the information that should be provided to support the use of test method protocols that incorporate specific modifications, which were to focus specifically on incorporating non-radioactive procedures to assess to the measurement of lymphocyte proliferation. The essential test method components included as appendix to the document provided a list of the test method protocol elements such as animal species and housing, number of doses to test, selection of doses, etc.



The January 8, 2008, draft ICCVAM LLNA Performance Standards elaborated by noting that modified LLNA test method protocols with changes to any of the essential test method components were defined as “major” modifications to the traditional LLNA test method protocol and would therefore be subject to a more extensive evaluation and/or validation process than a comparison to the LLNA performance standards.

As noted above, the Panel recommended that, instead of defining “minor” and “major” modifications, the performance standards should define criteria to ensure that a modified test method is mechanistically and functionally similar to the traditional LLNA. In this regard, the final ICCVAM LLNA Performance Standards document now describes all of the essential test method components for the LLNA, detailed in **Appendix C**. This document indicates that modified LLNA test method protocols could include modifications that do not impact the functional and mechanistic basis of the method. Seven essential test method components are identified as the elements that determine whether a modified LLNA test method protocol is functionally and mechanistically similar to the traditional LLNA. If any of the criteria are not met, then these performance standards are not applicable to validation of the modified test method.

1. The test substance must be applied topically to both ears of the mice.
2. Lymphocyte proliferation must be measured in the lymph nodes draining the site of test substance application.
3. Lymphocyte proliferation must be measured during the induction phase of skin sensitization.
4. For test substances, the highest dose selected for testing must be the maximum soluble concentration that does not induce systemic toxicity and/or excessive local irritation. For positive control substances, the highest dose selected should exceed the known EC3 values (i.e., the estimated concentration needed to produce a stimulation index of 3) of the reference substances without producing systemic toxicity and/or excessive local irritation.
5. A vehicle control must be included in each study and, where appropriate, a positive control should be used.
6. A minimum of four animals per dose group is required.
7. Either individual or pooled animal data may be collected.

Following are additional points to consider during the validation of modified LLNA test methods applicable to these performance standards, using the 18 required reference substances:

1. Consideration should be given to running concurrently a mix of negative, weakly, and strongly positive substances from the reference substance list so that the strongly positive substances can act as a positive control for the weaker skin sensitizer.
2. Group housing is recommended; otherwise animal selection, preparation, housing, and feeding should be in accordance with OECD TG 429 in compliance with other relevant regulatory requirements (e.g., animal care and use).

3. Appropriate quality assurance systems (i.e., in accordance with Good Laboratory Practice guidelines e.g., OECD 1998; EPA 2006a, 2006b; FDA 2006) are required.
4. The study should be conducted according to international validation principles (OECD Guidance Document 34 [OECD 2005]) and in compliance with other relevant regulatory requirements (e.g., animal care and use).

Thus, the final ICCVAM LLNA Performance Standards can be applied to a modified LLNA test method protocol provided that (1) the modified test method protocol incorporates the essential test method components, (2) test method protocol modifications are detailed and scientifically justified, and (3) the performance of the modified test method is equal to or better than that determined for the traditional LLNA.

## **Appendix E2**

### **Selection of Proposed Performance Standards Reference Substances**

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## 1.0 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 murine local lymph node assay (LLNA) performance standards. The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) subsequently reviewed the original proposed list of minimum reference substances, and prepared revised draft LLNA Performance Standards and a revised draft proposed reference substances list (i.e., released to the public on January 8, 2008; 73 FR 1360<sup>25</sup>). As in the original draft ICCVAM LLNA Performance Standards (released to the public on September 12, 2007; announced in 72 FR 52130<sup>26</sup>), the criteria for consideration on any subsequent revisions to the reference substances list was that the substances:

- Are readily available commercially
- Have available LLNA data (including stimulation index [SI] and EC3, i.e., the estimated concentration needed to produce an SI of 3)
- Have available guinea pig data (i.e., Guinea Pig Maximization Test [GPMT] or Buehler Test [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 substances on 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 European Centre for the Validation of Alternative Methods (ECVAM) LLNA Performance Standards and/or included in Japanese Center for the Validation of Alternative Methods (JaCVAM) validation studies

The final list of reference substances 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 with the provision that a weak sensitizer may be missed. In addition, there are four optional substances that may be tested to demonstrate improved performance relative to the traditional LLNA. The revisions to the draft ICCVAM performance standards reference substance list for the LLNA were based on all comments received and on comparison to the proposed substances in the ECVAM draft LLNA

<sup>25</sup> [http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR\\_E7\\_25553.pdf](http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_25553.pdf)

<sup>26</sup> [http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR\\_E7\\_18011.pdf](http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_18011.pdf)

Performance Standards. Since during this period ECVAM also revised their draft LLNA Performance Standards and changed their list of reference substances, all 22 substances are included in both the final ICCVAM and ECVAM reference substances lists. In addition there are six substances in common between the final ICCVAM list and the list of substances used by JaCVAM in their recent validation efforts. **Table E-1** provides the final list of proposed ICCVAM LLNA performance standards reference substances.

**Table E-1 ICCVAM-Recommended Performance Standards Reference Substances for the LLNA**

Number	Substance	CASRN	Form	Veh	EC3 (%) <sup>1</sup>	N <sup>2</sup>	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	106-50-3	Sol	AOO	0.11	6	0.055-0.22	0.07-0.16	+/+	+/+
4	Cobalt chloride	7646-79-9	Sol	DMSO	0.6	2	0.3-1.2	0.4-0.8	+/+	+/+
5	Isoeugenol	97-54-1	Liq	AOO	1.5	47	0.77-3.1	0.5-3.3	+/+	+/+
6	2-Mercaptobenzothiazole	149-30-4	Sol	DMF	1.7	1	0.85-3.4	NC	+/+	+/+
7	Citral	5392-40-5	Liq	AOO	9.2	6	4.6-18.3	5.1-13	+/+	+/+
8	HCA	101-86-0	Liq	AOO	9.7	21	4.8-19.5	4.4-14.7	+/+	+/+
9	Eugenol	97-53-0	Liq	AOO	10.1	11	5.05-20.2	4.9-15	+/+	+/+
10	Phenyl benzoate	93-99-2	Sol	AOO	13.6	3	6.8-27.2	1.2-20	+/+	+/+
11	Cinnamic alcohol	104-54-1	Sol	AOO	21	1	10.5-42	NC	+/+	+/+
12	Imidazolidinyl urea	39236-46-9	Sol	DMF	24	1	12-48	NC	+/+	+/+
13	Methyl methacrylate	80-62-6	Liq	AOO	90	1	45-100	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	50-21-5	Liq	DMSO	NA	1	NA	NA	-/-	-/*
17	Methyl salicylate	119-36-8	Liq	AOO	NA	9	NA	NA	-/-	-/-
18	Salicylic acid	69-72-7	Sol	AOO	NA	1	NA	NA	-/-	-/-

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Number	Substance	CASRN	Form	Veh	EC3 (%) <sup>1</sup>	N <sup>2</sup>	0.5x - 2.0x EC3	Actual Range	LLNA vs. GP	LLNA vs. Human
<b>Optional Substances to Demonstrate Improved Performance Relative to the Traditional LLNA</b>										
19	Sodium lauryl sulfate	151-21-3	Sol	DMF	8.1	5	4.05-16.2	1.5-17.1	+/-	+/-
20	Ethylene glycol dimethacrylate	97-90-5	Liq	MEK	28	1	14-56	NC	+/-	+/+
21	Xylene	1330-20-7	Liq	AOO	95.8	1	47.9-100	NC	+/**	+/-
22	Nickel chloride	7718-54-9	Sol	DMSO	NA	2	NA	NA	-/+	-/+

Abbreviations: AOO = acetone: olive oil (4:1); CASRN = Chemical Abstracts Service Registry Number; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; DNCB = 2,4-dinitrochlorobenzene; EC3 = estimated concentration needed to produce a stimulation index of 3; 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 data was obtained from a single study; Sol = solid; Veh = vehicle

<sup>1</sup> Mean value where more than one EC3 value was available

<sup>2</sup> 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.

\*\* = GP data not available



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

**Table E-2** details the current revisions to the draft ICCVAM-recommended performance standards reference substances for the LLNA based on the LLNA Peer Review Panel meeting, public comments, and comparison with the revised draft ECVAM LLNA Performance Standards. The original ICCVAM list represents the draft version released for public comment on September 12, 2007, and initial revisions to the original ICCVAM list were provided to the Peer Review Panel and released to the public on January 8, 2008. The revised ECVAM list represents the version distributed to the ECVAM Scientific Advisory Committee (ESAC) members for discussion at its 28<sup>th</sup> ESAC meeting on May 7-8, 2008.

Initially, based on comments received from ECVAM and additional searches by NICEATM for reference data, six substances from the original ICCVAM list (i.e., the September 12, 2007 version) were not included on the revised list of ICCVAM reference substances (i.e., the January 8, 2008 draft). These substances and the rationale for their exclusion are as follows:

- Benzoquinone was removed because no human data were located. Another substance, 5-chloro-2-methyl-4-isothiazolin-3-one, was identified as an adequate replacement 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.
- Cinnamic aldehyde was removed in response to an ECVAM comment noting that another aldehyde (hexyl cinnamic aldehyde [HCA]) was already on the list, which is also a positive control substance used in the traditional LLNA.
- Formaldehyde was 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 was removed in response to an ECVAM comment that suggested this substance is unstable and is therefore susceptible to variable results.
- Nickel sulfate was removed in response to the ECVAM comment that inclusion of two nickel salts is unnecessary.
- Tween 80 was 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.

One substance (i.e., ethyl acrylate) included on the revised draft ECVAM reference substances list but not on the original draft ICCVAM list (i.e., the September 12, 2007 draft) is still not included in the final ICCVAM LLNA Performance Standards because no guinea pig test reference data were located.

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

Substance <sup>1</sup>	CASRN	Form	Veh	EC3 (%) <sup>2</sup>	N <sup>3</sup>	Orig I	Rev I	Curr I	E	J	Rationale for Exclusion/Inclusion or Current Data Gap
5-Chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	Liq	DMF	0.009	1		X	X	X		Concordant GP and human data
Benzoquinone	106-51-4	Sol	AOO	0.01	1	X					No available human data
DNCB	97-00-7	Sol	AOO	0.049	15	X	X	X	X	X	
4-Phenylenediamine	106-50-3	Sol	AOO	0.11	6	X	X	X	X		
Cobalt chloride	7646-79-9	Sol	DMSO	0.6	2		X	X	X	X	Concordant GP and human data and also on JaCVAM list
Formaldehyde	50-00-0	Liq	ACE	0.61	1	X				X	Another aldehyde (HCA) already on the list
4-Methylaminophenol sulfate	55-55-0	Sol	DMF	0.8	1		X				Replaced with an acrylate that is a "weak" sensitizer with available GP and human data (methyl methacrylate)
2-Hydroxyethyl acrylate	818-61-1	Liq	AOO	1.4	1	X					Unstable compound
Isoeugenol	97-54-1	Liq	AOO	1.5	47	X	X	X	X	X	
2-Mercaptobenzothiazole	149-30-4	Sol	DMF	1.7	1	X	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)
Citral	5392-40-5	Liq	AOO	9.2	6	X	X	X	X		
HCA	101-86-0	Liq	AOO	9.7	21	X	X	X	X	X	
Eugenol	97-53-0	Liq	AOO	10.1	11		X	X	X		
Phenyl benzoate	93-99-2	Sol	AOO	13.6	3		X	X	X		
Cinnamic alcohol	104-54-1	Sol	AOO	21	1		X	X	X		
Imidazolidinyl urea	39236-45-9	Sol	DMF	24	1	X	X	X	X		
Ethyl acrylate	140-88-5	Liq	AOO	32.4	2						No available GP data. ECVAM agreed to replace with methyl methacrylate in September 2008.
Methyl methacrylate	80-62-6	Liq	AOO	90	1			X	X		Acrylate with concordant GP and human data
Chlorobenzene	108-90-7	Liq	AOO	NA	1		X	X	X		Concordant GP data*
Isopropanol	67-63-0	Liq	AOO	NA	1	X	X	X	X	X	Case report of human sensitizer
Lactic acid	50-21-5	Liq	DMSO	NA	1		X	X	X		Concordant GP data*
Methyl salicylate	119-36-8	Liq	AOO	NA	9	X	X	X	X	X	
Salicylic acid	69-72-7	Sol	AOO	NA	1	X	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
<b>Optional Substances to Demonstrate Improved Performance Relative to the Traditional LLNA</b>											
Sodium lauryl sulfate	151-21-3	Sol	DMF	8.1	5	X	X	X	X		Included as a false positive

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Substance <sup>1</sup>	CASRN	Form	Veh	EC3 (%) <sup>2</sup>	N <sup>3</sup>	Orig I	Rev I	Curr I	E	J	Rationale for Exclusion/Inclusion or Current Data Gap
Ethylene glycol dimethacrylate	97-90-5	Liq	MEK	28	1	X	X	X	X		Included as 1 of 3 false positives (with respect to GP only) on ICCVAM list
Xylene	1330-20-7	Liq	AOO	95.8	1				X		Substituted for sulfanilamide as a false positive (with respect to human only)
Nickel chloride	7718-54-9	Sol	DMSO	NA	2	X			X		Included as a false negative
Nickel sulfate	7786-81-4	Sol	DMF	NA	2	X	X	X		X	Don't need two nickel salts
Sulfanilamide	63-74-1	Sol	DMF	NA	1	X	X	X			Excluded as a false negative because the human results were equivocal (i.e., usually negative rather than positive)

ACE = acetone; AOO = acetone: olive oil (4:1); CASRN = Chemical Abstracts Service Registry Number; Curr I = final ICCVAM LLNA Performance Standards list; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; DNCB = 2,4-dinitrochlorobenzene; E = draft ECVAM LLNA Performance Standards list; EC3 = estimated concentration needed to produce a stimulation index of 3; 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 data was obtained from a single study; NP = not provided in draft ECVAM LLNA Performance Standards; Orig I = September 12, 2007, ICCVAM LLNA Performance Standards list; Rev I = January 8, 2008, ICCVAM LLNA Performance Standards list; Sol = solid; Veh = vehicle

<sup>1</sup> Substances are listed by EC3 value in ascending order. Substances for which no EC3 value was available are listed after those with the highest EC3 values. Substances that are on the final ICCVAM list are indicated in boldface (see also **Table E-1**).

<sup>2</sup> Mean value where more than one EC3 value was available

<sup>3</sup> 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.

### **3.0 Rationale for Inclusion of Substances on the Revised Draft ICCVAM List**

Four of the substances included in the draft ECVAM reference substances list but not on the original draft ICCVAM list (i.e., the September 12, 2007, draft) were included in the revised draft ICCVAM list (i.e., the January 8, 2008, draft):

- Cinnamic alcohol was 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. It also has available concordant reference data for the guinea pig and human.
- Eugenol was 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. It also has available concordant reference data for the guinea pig and human, and it has been extensively evaluated in the traditional LLNA.
- Lactic acid was included in the revised list as a non-sensitizer based on available concordant guinea pig data, although human data were not located. 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 was 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. It also has available concordant reference data for the guinea pig and human.

At the time, there were also six substances that were included on the revised draft ICCVAM list (i.e., the January 8, 2008, draft) 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 was identified, as indicated above, as an adequate replacement for benzoquinone based on the availability of concordant guinea pig and human data. It has a history of demonstrated results in the guinea pig and human as an extreme sensitizer.
- Chlorobenzene was included as a non-sensitizer based on available concordant guinea pig data, although no human data were located. 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 was 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 was not included by ECVAM, as their list only includes one false positive substance. The revised ICCVAM list included 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 was included as a strong sensitizer based on LLNA results with available concordant guinea pig and human data.
- Sulfanilamide was not included by ECVAM, as their list only included one false negative substance. The revised ICCVAM list included two false negative substances that may be tested if improved performance relative to the traditional LLNA is the goal of a validation study.

For the May 7-8, 2008, ESAC meeting, ECVAM revised their list and cited the rationale for their revisions as follows:

- Benzoquinone was replaced with 5-chloro-2-methyl-4-isothiazolin-3-one for reasons mentioned above.
- Diethyl maleate was replaced with cobalt chloride to aid the process of harmonization, despite it being unnecessary to have another metal on the list.
- Hexane was replaced with chlorobenzene as there are no guinea pig data for hexane.
- A proposal to substitute ethyl acrylate with 4-methylaminophenol sulfate was rejected. Ethyl acrylate represents the acrylates and is a weak sensitizer, and therefore substituting that compound with 4-methylaminophenol, which is not an acrylate and a strong sensitizer, is not acceptable. ECVAM would consider substituting ethyl acrylate with another weak sensitizer for which guinea pig and human data are available.

Subsequently, ICCVAM replaced 4-methylaminosulfate with methyl methacrylate, to represent an acrylate and a weak sensitizer with available guinea pig and human data.

Finally, at the September 23-24, 2008 meeting for the Harmonization of Performance Standards for the LLNA, ECVAM and ICCVAM agreed upon a list of 18 required reference substances and four optional substances. At this meeting, there was agreement to:

- Accept methyl methacrylate as a replacement for ethyl acrylate as a weak sensitizer
- Replace the nickel sulfate with nickel chloride as an optional test substance because the available LLNA results for nickel sulfate were equivocal (i.e., both positive and negative), while the results for nickel chloride were consistently negative
- Include a total of four optional test substances. This included replacement of sulfanilamide with xylene because the reliability of the positive human result with sulfanilamide was questioned. Thus, the four optional substances are ethylene glycol dimethacrylate, sodium lauryl sulfate, nickel chloride, and xylene.

#### 4.0 Database Used to Select Reference Substances

The candidate list used to select proposed minimum reference substances (“reference list”) for the draft proposed 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 GPMT or BT data that were collected using a standard test method protocol (e.g., U.S. Environmental Protection Agency [EPA] Health Effects Test Guideline OPPTS 870.2600 [EPA 2003]) 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 E-3** provides a breakdown of the impact that specific criteria had on the list of candidate substances.

**Table E-3 Impact of Selection Criteria on Candidate List**

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

Abbreviations: BT = Buehler Test; GPMT = Guinea Pig Maximization Test; LLNA = murine 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 and SI values
- Consideration of substances used in the JaCVAM validation studies (6 substances) and in the draft LLNA Performance Standards proposed by ECVAM (22 substances)

**Table E-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 data; included as part of a human patch test allergen kit; clinical case studies) also is provided. While the selection criteria included the availability of human data whenever possible, two substances without such data was included in order to maintain the desired dynamic range of responses, and range of physical and chemical characteristics.

**Table E-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 <sup>1</sup>	HMT only	HPTA only	Both HMT and HPTA	Other Human Data <sup>1</sup>
+	+	13	13	2	4	3	4
+	-	2	2	0	1	1	0
-	+	1	1	0	0	0	1
-	-	5	3 <sup>2</sup>	0	0	2	1
+	NA	1	1	1	0	0	0
<b>Totals</b>		<b>22</b>	<b>20</b>	<b>3</b>	<b>5</b>	<b>6</b>	<b>6</b>

Abbreviations: BT = Buehler Test; GPMT = Guinea Pig Maximization Test; HMT = Human Maximization Test; HPTA = Human Patch Test Allergen; LLNA = murine local lymph node assay; NA = not available; No. = number

<sup>1</sup> Other human data include published reports of patch tests or case studies with the substance in question.

<sup>2</sup> 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 E-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 E-5 Characteristics of the Proposed List of Reference Chemicals**

<b>No. Chems</b>	<b>Solid/Liquid</b>	<b>EC3 Range</b>	<b>Maximum SI Range</b>	<b>Human Data</b>	<b>Peptide Reactivity (High/Mod/Min/Unk)<sup>1</sup></b>	<b>Included on lists: ECVAM/JaCVAM/Both</b>
2	1/1	0.009 - 0.05	22.7 - 43.9	2	2/0/0/0	2/1/1
2	2/0	0.11 - 0.6	7.2 - 26.4	2	0/0/0/2	2/1/1
4	1/3	1.5 - 9.7	8.6 - 25.3	4	1/0/1/2	4/2/2
5	3/2	10.1 - 90.1	3.6 - 17.0	5	0/1/0/4	5/0/0
5	1/4	NA	1.7 - 2.7	3	0/0/4/1	5/2/2
<b>Optional Substances to Demonstrate Improved Performance Relative to the Traditional LLNA</b>						
3	1/2	8.1 - 95.8	3.1 - 8.9	3	1/0/0/2	3/0/0
1	1/0	NA	2.4	1	0/0/0/1	1/0/0
<b>Totals</b>						
<b>22</b>	<b>10/12</b>	<b>0.009 - 95.8</b>	<b>1.7 - 43.9</b>	<b>20</b>	<b>4/1/5/12</b>	<b>22/6/6</b>

Abbreviations: Chems = chemicals; EC3 = estimated concentration needed to produce a stimulation index of 3; ECVAM = European Centre for the Validation of Alternative Methods; JaCVAM = Japanese Center for the Validation of Alternative Methods; LLNA = murine local lymph node assay; NA = not applicable; No. = number; Min = minimal; mod = Moderate; SI = stimulation index; Unk = unknown

<sup>1</sup> Data obtained from: Gerberick et al. 2007.



The proposed list of substances includes an adequate number of correctly identified sensitizers, non-sensitizers, 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 chloride is included on the reduced list of 22 chemicals 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.

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## **Appendix E3**

### **Rationale for the Required Accuracy and Reliability Statistics Included in the Test Method Performance Evaluation**

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## 1.0 Introduction

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Murine Local Lymph Node Assay (LLNA) performance standards describe performance statistics (**Section 2.4**) to be used in the development of new functionally and mechanistically similar test methods. The following text provides an overview of how the performance statistics (i.e., accuracy and reliability values) were selected. Similar to the list of reference substances (**Appendix F**), these recommended statistics represent the culmination of interactions between the ICCVAM Immunotoxicity Working Group (IWG) and 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 of the ECVAM Sensitization Task Force.

## 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 (announced in *Federal Register* [FR] notice 72 FR 52130),<sup>27</sup> the accuracy evaluation was based on meeting or exceeding the performance to the traditional LLNA based on calculated accuracy, sensitivity, specificity, and false negative and false positive rates when using the minimum list of recommended reference substances.

After consideration and discussions with ECVAM, an FR notice released on January 8, 2008 (73 FR 1360),<sup>28</sup> announced the availability of a draft version that required a "chemical by chemical" match which required 100% concordance with the traditional LLNA results for the 18 required substances. An optional list of four substances (two false positive/two false negative with respect to guinea pig data) was provided to allow for a modified LLNA test method protocol to demonstrate that its performance exceeded that of the traditional LLNA.

As an additional measure of test method accuracy, the January 8, 2008, draft included a range of EC<sub>t</sub> values (i.e., the concentration required to achieve the defined threshold stimulation index used to distinguish between sensitizers and non-sensitizers) for the sensitizing substances on the reference list (these values are based on the EC<sub>3</sub> values, i.e., the estimated concentrations needed to produce a stimulation index of 3, for each sensitizer). This provided assurance that, not only does a modified LLNA test method 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. This range was originally proposed by ECVAM based on the personal experience of members of the ECVAM Sensitization Task Force.

In their review of the January 8, 2008, draft ICCVAM LLNA Performance Standards, an international independent scientific peer review panel (hereafter, "Panel") concluded that the acceptability range of 0.5x to 2.0x was too restrictive. They also emphasized that it was not appropriate to define an acceptability range for which there was only one or two EC<sub>3</sub> values available to calculate the range. The Panel also recommended that modified LLNA test

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<sup>27</sup> [http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR\\_E7\\_18011.pdf](http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_18011.pdf)

<sup>28</sup> [http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR\\_E7\\_25553.pdf](http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_25553.pdf)

methods should be evaluated with all 22 substances (including false negatives and false positives) and accuracy statistics calculated. To the extent possible, rationale for discordant results should be provided. However, the most potent sensitizers (e.g., 2,4-dinitrochlorobenzene [DNCB]) should always be identified correctly.

Considering comments from the Panel, the Scientific Advisory Committee on Alternative Toxicological Methods and the public, IWG discussions, and discussions with ECVAM, the final ICCVAM LLNA Performance Standards state that the proposed test method should result in the correct sensitizer/non-sensitizer classification for each of the 18 required reference substances, but that a misclassification of one weak sensitizer could be allowed. The rationale for the discrepancy must be provided and would be assessed on a case-by-case basis to determine acceptability. In addition, to demonstrate equivalent or improved performance relative to the traditional LLNA, any of the four optional substances may be tested in addition to the required 18 substances.

### **3.0 Test Method Reliability**

The original draft ICCVAM LLNA Performance Standards (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 hexyl cinnamic aldehyde (HCA), or other comparable positive control substance in the traditional LLNA. EC<sub>t</sub> 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 producing an EC<sub>t</sub> that is within an acceptable range of the historical EC<sub>3</sub> concentration for HCA, based on traditional LLNA studies. Instead, the test method could achieve an acceptable coefficient of variation that is based on EC<sub>3</sub> concentrations that differ significantly from the historical range (i.e., the method could produce reproducible, but inaccurate results).

For this reason, the January 8, 2008, draft of the ICCVAM LLNA Performance Standards criteria for intralaboratory reproducibility was revised to reflect that acceptable reproducibility is indicated when each of at least three laboratories obtain EC<sub>t</sub> values for HCA and DNCB that are generally within 0.5x to 2.0x the historical mean EC<sub>3</sub> concentration (5% to 20% and 0.025 to 0.1%, respectively) for these substances when tested in the traditional LLNA. The Panel agreed with the proposed intralaboratory reproducibility standard. This section remains unchanged from the January 8, 2008, draft.

#### **3.1 Interlaboratory Reproducibility**

The original draft ICCVAM LLNA Performance Standards (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, which would be based on coefficients of variations. However, similar to the assessment of intralaboratory reproducibility, this evaluation also did not take into account the acceptable range of the historical EC<sub>3</sub> 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 EC<sub>3</sub> values that is being applied the assessment of test method accuracy (i.e., 0.5x to 2.0x EC<sub>t</sub>). Acceptable reproducibility will now be indicated by each of at least three laboratories obtaining EC<sub>t</sub> values for HCA and DNCB that are generally within 0.5x to 2.0x the EC<sub>3</sub> concentration (5% to 20%

and 0.025 to 0.1%, respectively) as specified for these substances when tested in the traditional LLNA. The Panel agreed with the proposed interlaboratory reproducibility standard. This section remains unchanged from the January 8, 2008, draft.

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## **Appendix F**

### **ICCVAM LLNA Performance Standards: Recommended Reference Substances**

<b>F1</b>	<b>Recommended Reference Substances – Alphabetically Sorted .....</b>	<b>F-3</b>
<b>F2</b>	<b>Recommended Reference Substances – Structures and Product Uses .....</b>	<b>F-7</b>
<b>F3</b>	<b>Recommended Reference Substances – Murine Local Lymph Node Assay Data .....</b>	<b>F-15</b>

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## **Appendix F1**

### **Recommended Reference Substances – Alphabetically Sorted**

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Substance Name	CASRN	MW (g/mol)	Physical Form	LLNA	Veh <sup>1</sup>	EC3 <sup>2</sup>	N <sup>3</sup>	0.5x - 2.0x EC3	Max SI (Conc)	GPMT/BT <sup>4</sup>	HMT	HPTA	Additional Human Skin Sensitization Data/Information <sup>5</sup>	Peptide Reactivity <sup>6</sup>
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%)	+		+	DSA05HRIPT=4.3 (Cardin et al. 1986) Human data is for mixture (Kathon CG) – no human data for single substance	High
Chlorobenzene	108-90-7	112.557	Liquid	-	AOO	NC	1	NA	1.7 (10%)	-			No human data located <sup>7</sup>	Minimal
Cinnamic alcohol	104-54-1	134.18	Solid	+	AOO	21	1	10.5-42	5.7 (90%)	+	+		DSA05HRIPT=3704; DSA05HMT=625 (Jordan and King 1977)	
Citral	5392-40-5	152.233	Liquid	+	AOO	9.2	6	4.6-18.3	20.5 (20%)	+	+		DSA05HRIPT=840; DSA05HMT=862 (Steltenkamp et al. 1980)	
Cobalt chloride	7646-79-9	129.84	Solid	+	DMSO <sup>8</sup>	0.6	2	0.3-1.2	7.2 (5%)	+	+	+	DSA05HMT=172 (Kligman 1966b) human data is for cobalt sulfate	
2,4-Dinitrochlorobenzene	97-00-7	202.552	Solid	+	AOO	0.049	15	0.025-0.099	43.9 (0.025%)	+			DSA05HRIPT=5.5 (Friedmann et al. 1983)	High
Ethylene glycol dimethacrylate	97-90-5	198.216	Liquid	+	MEK	28	1	14-56	7 (50%)	-		+	+ human from Basketter et al. 1999a	High
Eugenol	97-53-0	164.201	Liquid	+	AOO	10.1	11	5.05-20.2	17 (50%)	+		+	DSA05HRIPT=5926 (Marzulli and Maibach 1980)	
Hexyl cinnamic aldehyde <sup>9</sup>	101-86-0	216.319	Liquid	+	AOO <sup>10</sup>	9.7	21	4.8-19.5	20 (50%)	+			DSA(NOEL)HRIPT=23622 (RIFM submission and Basketter et al. 2005b)	Minimal
Lactic acid	50-21-5	90.078	Liquid	-	DMSO	NC	1	NA	2.2 (25%)	-			No human data located <sup>7</sup>	Minimal
Imidazolidinyl urea	39236-46-9	388.294	Solid	+	DMF	24	1	12-48	5.5 (50%)	+		+	DSA05HRIPT=3846 (Jordan and King 1977)	Moderate
Isoeugenol	97-54-1	164.201	Liquid	+	AOO <sup>11</sup>	1.5	47	0.77-3.1	31 (5%)	+		+	DSA(LOEL) HRIPT=69 (Griem et al. 2003); DSA(LOEL)HMT=5217	
Isopropanol	67-63-0	60.095	Liquid	-	AOO	NC	1	NA	1.7 (10%)	-			Studies indicate substance produces skin sensitization <sup>12</sup>	Minimal
2-Mercaptobenzo-thiazole	149-30-4	167.253	Solid	+	DMF	1.7	1	0.85-3.4	8.6 (10%)	+	+	+	DSA05HMT=1642 (Kligman 1966a)	High
Methyl methacrylate	80-62-6	100.12	Liquid	+	AOO <sup>13</sup>	90 <sup>13</sup>	1	45-100	3.6 (100%)	+			Information derived from clinical experience <sup>13</sup>	
Methyl salicylate	119-36-8	152.147	Liquid	-	AOO	NC	9	NA	2.7 (20%)	-	-	-	DSA05HMT=5517 (Schneider and Akkan 2004)	Minimal

ICCVAM LLNA Performance Standards – Appendix F1

Substance Name	CASRN	MW (g/mol)	Physical Form	LLNA	Veh <sup>1</sup>	EC3 <sup>2</sup>	N <sup>3</sup>	0.5x - 2.0x EC3	Max SI (Conc)	GPMT/BT <sup>4</sup>	HMT	HPTA	Additional Human Skin Sensitization Data/Information <sup>5</sup>	Peptide Reactivity <sup>6</sup>
Nickel chloride	7718-54-9	129.599	Solid	-	DMSO <sup>14</sup>	NC	2	NA	2.4 (5%)	+			DSA05HMT=28 (Kligman 1966a for nickel sulfate – but data expressed as nickel)	
Phenyl benzoate <sup>9</sup>	93-99-2	198.217	Solid	+	AOO	13.6	3	6.8-27.2	11.1 (25%)	+			DSA05HRIPT=52489 (Basketter et al. 2005b)	
4-Phenylenediamine	106-50-3	108.14	Solid	+	AOO	0.11	6	0.055-0.22	26.4 (1%)	+	+	+	DSA05HMT=16.4 (Kligman 1966a); DSA05HRIPT=6.9 (Marzulli and Maibach 1974)	
Salicylic acid	69-72-7	138.121	Solid	-	AOO	NC	1	NA	2.5 (25%)	-	-	-	DSA(NOEL) HMT=13793 (Kligman 1966b)	
Sodium lauryl sulfate	151-21-3	288.38	Solid	+	DMF	8.1	5	4.05-16.2	8.9 (10%)	-	-	-	DSA(NOEL) HMT=6897 (Kligman 1966b)	
Xylene	1330-20-7	106.17	Liquid	+	AOO	95.8 <sup>15</sup>	1	47.9-100	3.1 (100%)	NA <sup>16</sup>	-		DSA (NOEL)=68966 (Kligman 1966b)	

Abbreviations: AOO = acetone: olive oil (4:1); BT = Buehler Test; CASRN = Chemical Abstracts Service Registry Number; Conc. = concentration tested; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; DSA = dose per skin area; DSA05 = dose per skin area leading to a sensitization incidence of 5%; EC3 = estimated concentration needed to produce a stimulation index of 3; GPMT = Guinea Pig Maximization Test; HMT = Human Maximization Test; HPTA = Human Patch Test Allergen; HRIPT = Human Repeat Insult Patch Test; LLNA = murine local lymph node assay; LOEL = lowest observed effect level; Max = maximum; MEK = methyl ethyl ketone; MW = molecular weight; NA = not available; NC = not calculated; NOEL = no observed effect level; RIFM = Research Institute for Fragrance Materials; SI = stimulation index; Veh = vehicle

<sup>1</sup> Unless noted otherwise, vehicle information obtained from Gerberick et al. 2005.

<sup>2</sup> Unless noted otherwise, where the number of LLNA studies equals one, EC3 values obtained from Gerberick et al. 2005.

<sup>3</sup> Number of LLNA studies from which data were obtained.

<sup>4</sup> Results obtained from Guinea Pig Maximization Test and/or Buehler Test.

<sup>5</sup> Human Quantitative Data obtained from literature where human data was compared to LLNA. All data are expressed as DSA ( $\mu\text{g}/\text{cm}^2$ ). 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. 2005b).

<sup>6</sup> Peptide reactivity data obtained from Gerberick et al. 2007.

<sup>7</sup> 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.

<sup>8</sup> Basketter and Scholes 1992; Ikarashi et al. 1992

<sup>8</sup> Human data based on following studies: (1) Rees et al. 1989 (2) Zina et al. 1987.

<sup>9</sup> Presumed to be a strong human allergen (search for human data ongoing)

<sup>10</sup> Dearman et al. 2001

<sup>11</sup> Basketter and Cadby 2004

<sup>12</sup> Human data based on Kwon et al. 2003

<sup>13</sup> Betts et al. 2006

<sup>14</sup> Basketter and Scholes 1992

<sup>15</sup> Estrada et al. 2003

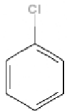
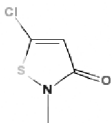
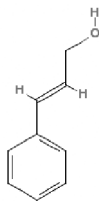
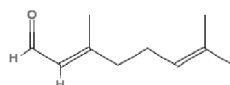
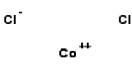
<sup>16</sup> Personal Communication (D. Basketter 2008)

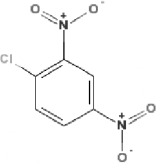
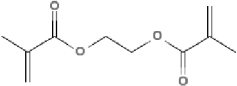
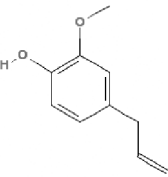
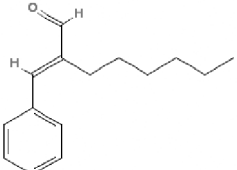
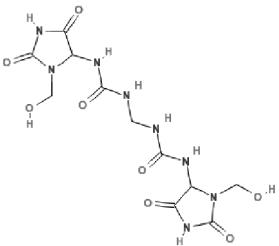
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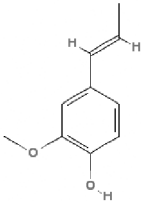

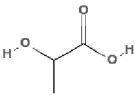
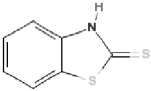
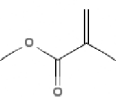
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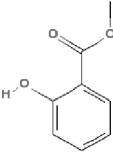

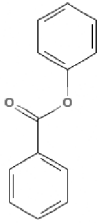
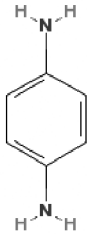
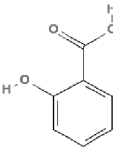
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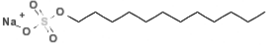
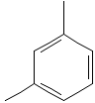


Substance Name	CASRN	Structure	Product Uses
Chlorobenzene	108-90-7		Phenol manufacture Aniline manufacture DDT manufacture Solvent for paints
5-Chloro-2-methyl-4-isothiazolin-3-one	26172-55-4		Disinfectant
Cinnamic alcohol	104-54-1		Perfume manufacture
Citral	5392-40-5		Flavor additive Perfume manufacture
Cobalt chloride	7646-79-9		Humidity and water indicator Preparation of catalysts Fertilizer and feed additive Vitamin B12 manufacture

Substance Name	CASRN	Structure	Product Uses
2,4-Dinitrochlorobenzene	97-00-7		Color photo processing Explosives manufacture
Ethylene glycol dimethacrylate	97-90-5		Polymerization agent
Eugenol	97-53-0		Fragrance and flavoring agent Insect attractant
Hexyl cinnamic aldehyde	101-86-0		Perfume manufacture
Imidazolidinyl urea	39236-46-9		Cosmetic preservative Antimicrobial

Substance Name	CASRN	Structure	Product Uses
Isoeugenol	97-54-1		<p>Perfume manufacture Flavoring additive Topical pharmaceutical</p>
Isopropanol	67-63-0		<p>Topical pharmaceutical Gasoline additive Cleaning agent</p>
Lactic Acid	50-21-5		<p>Manufacture of lactates which are used in food products, in medicine, and as solvents</p>
2-Mercaptobenzothiazole	149-30-4		<p>Rubber manufacture Anticorrosive</p>
Methyl methacrylate	80-62-6		<p>Used in the production of polymers such as surface coating resins, plastics (Plexiglas and Lucite), ion exchange resins and plastic dentures.</p>

Substance Name	CASRN	Structure	Product Uses
Methyl salicylate	119-36-8		Topical pharmaceutical Flavor additive
Nickel chloride	7718-54-9		Electroplating agent Battery manufacture
Phenyl benzoate	93-99-2		Production of industrial chemicals
4-Phenylenediamine	106-50-3		Hair dye Textile dye
Salicylic acid	69-72-7		Pharmaceutical Food preservative

Substance Name	CASRN	Structure	Product Uses
Sodium lauryl sulfate	151-21-3		<p>Detergent Cosmetic</p>
Xylene	1330-20-7		<p>Solvent Production of industrial chemicals</p>

Abbreviations: CASRN = Chemical Abstracts Service Registry Number

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## **Appendix F3**

### **Recommended Reference Substances – Murine Local Lymph Node Assay Data**

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Substance Name	CASRN	Veh	Dose 1 Conc. (%)	Dose 1 SI	Dose 2 Conc. (%)	Dose 2 SI	Dose 3 Conc. (%)	Dose 3 SI	Dose 4 Conc. (%)	Dose 4 SI	Dose 5 Conc. (%)	Dose 5 SI	EC3 (%)	Rationale for High Dose <sup>1</sup>	LLNA Result	Reference
Chlorobenzene	108-90-7	AOO	5	1.1	10	1.7	25	1.6	ND	ND	ND	ND	NC	A, C	-	Gerberick et al. 2005
5-Chloro-2-methyl-4-isothiazolin-3-one	26172-55-4	DMF	0.01	3.5	0.03	12.3	0.10	22.7	ND	ND	ND	ND	0.009	A, C	+	Gerberick et al. 2005
Cinnamic alcohol	104-54-1	AOO	10	1.8	25	3.5	50	3.9	90	5.7	ND	ND	21	C	+	Gerberick et al. 2005
Citral	5392-40-5	AOO	5	1.2	10	2.1	25	6.3	ND	ND	ND	ND	13	C	+	Gerberick et al. 2005
Citral	5392-40-5	AOO	5	2.1	10	5.0	20	9.3	ND	ND	ND	ND	6.6	B	+	Basketter et al. 1991
Citral	5392-40-5	AOO	5	0.9	10	2.2	20	6.2	ND	ND	ND	ND	12.0	B	+	Basketter et al. 1991
Citral	5392-40-5	AOO	5	2.2	10	8.1	20	20.5	ND	ND	ND	ND	5.7	B	+	Basketter et al. 1991
Citral	5392-40-5	AOO	5	0.9	10	2.4	20	4.7	ND	ND	ND	ND	12.6	B	+	Basketter et al. 1991
Citral PQ extra	5392-40-5	AOO	5	2.9	10	6.4	25	12.9	ND	ND	ND	ND	5.1	A, C	+	Ashby et al. 1995
Cobalt chloride	7646-79-9	DMSO	0.5	3.2	1	3.7	2.5	2.8	ND	ND	ND	ND	0.4	B	+	Basketter and Scholes 1992
Cobalt chloride	7646-79-9	DMSO	0.5	2.1	1	3.5	2.5	3.8	5	7.2	ND	ND	0.8	A	+	Ikarashi et al. 1992
DNCB	97-00-7	AOO	0.010	1.5	0.025	1.8	0.050	2.4	0.100	8.9	0.250	38.0	0.048	E	+	Gerberick et al. 2005
DNCB	97-00-7	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.08	N	+	Basketter et al. 2005a
DNCB	97-00-7	AOO	0.010	1.4	0.025	2.2	0.050	4.0	0.100	9.8	0.250	16.2	0.036	C	+	Betts et al. 2006
DNCB	97-00-7	AOO	0.010	2.0	0.025	2.3	0.050	5.3	0.100	10.5	0.250	35.5	0.027	C	+	Kimber et al. 1995
DNCB	97-00-7	AOO	0.010	0.8	0.025	1.8	0.050	3.3	0.100	8.7	0.250	40.9	0.046	C	+	Kimber et al. 1995
DNCB	97-00-7	AOO	0.010	1.1	0.025	1.4	0.050	2.5	0.100	4.6	0.250	11.5	0.062	C	+	Kimber et al. 1995
DNCB	97-00-7	AOO	0.010	0.8	0.025	1.2	0.050	1.7	0.100	3.1	0.250	22.5	0.094	C	+	Kimber et al. 1995
DNCB	97-00-7	AOO	0.010	1.3	0.025	1.5	0.050	2.1	0.100	7.7	0.250	43.9	0.057	C	+	Kimber et al. 1995
DNCB	97-00-7	AOO	0.010	1.5	0.025	1.9	0.050	3.1	0.100	6.5	0.250	25.0	0.05	E	+	Loveless et al. 1996
DNCB	97-00-7	AOO	0.010	1.2	0.025	0.9	0.050	2.9	0.100	4.5	0.250	13.0	0.06	E	+	Loveless et al. 1996
DNCB	97-00-7	AOO	0.010	2.5	0.025	2.9	0.050	3.2	0.100	7.1	0.250	25.0	0.033	E	+	Loveless et al. 1996
DNCB	97-00-7	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.02	A, C, D	+	Basketter et al. 2007
DNCB	97-00-7	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.05	A, C, D	+	Basketter et al. 2007
DNCB	97-00-7	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.03	A, C, D	+	Basketter et al. 2007
DNCB	97-00-7	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.05	A, C, D	+	Basketter et al. 2007

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Substance Name	CASRN	Veh	Dose 1 Conc. (%)	Dose 1 SI	Dose 2 Conc. (%)	Dose 2 SI	Dose 3 Conc. (%)	Dose 3 SI	Dose 4 Conc. (%)	Dose 4 SI	Dose 5 Conc. (%)	Dose 5 SI	EC3 (%)	Rationale for High Dose <sup>1</sup>	LLNA Result	Reference
Ethylene glycol dimethacrylate	97-90-5	MEK	10	1.2	25	2.4	50	7.0	ND	ND	ND	ND	28	N	+	Gerberick et al. 2005
Eugenol	97-53-0	AOO	2.5	1.6	5	1.5	10	2.4	25	5.5	50	16.1	11.9	E	+	Gerberick et al. 2005
Eugenol	97-53-0	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	9.80	E	+	Loveless et al. 1996
Eugenol	97-53-0	AOO	2.5	2.0	5	2.8	10	3.2	25	13.0	50	17.0	5.80	E	+	Loveless et al. 1996
Eugenol	97-53-0	AOO	2.5	1.6	5	1.5	10	2.4	25	5.5	50	16.0	14.50	E	+	Loveless et al. 1996
Eugenol	97-53-0	AOO	2.5	1.1	5	1.7	10	1.8	25	9.1	50	12.4	8.90	E	+	Loveless et al. 1996
Eugenol	97-53-0	AOO	2.5	2.4	5	2.1	10	1.2	25	5.3	50	9.6	13.80	E	+	Loveless et al. 1996
Eugenol	97-53-0	AOO	2.5	1.5	5	4.3	10	4.6	25	14.0	50	6.1	6.00	E	+	Loveless et al. 1996
Eugenol	97-53-0	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	15	E	+	Basketter et al. 2007
Eugenol	97-53-0	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	12.9	E	+	Basketter et al. 2007
Eugenol	97-53-0	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	4.9	E	+	Basketter et al. 2007
Eugenol	97-53-0	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	7.5	E	+	Basketter et al. 2007
HCA	101-86-0	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	8	N	+	Basketter and Kimber 2001
HCA	101-86-0	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	11	C	+	Dearman et al. 2001
HCA	101-86-0	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	12.02	C	+	Patelwicz et al. 2001
HCA	101-86-0	AOO	10	3.2	25	6.0	50	10.0	ND	ND	ND	ND	9.40	A, C	+	Ashby et al. 1995
HCA	101-86-0	AOO	2.5	1.3	5	1.1	10	2.5	25	10.0	50	17.0	11	E	+	Loveless et al. 1996
HCA	101-86-0	AOO	2.5	1.3	5	1.1	10	2.5	25	10.0	50	17.0	8.40	E	+	Loveless et al. 1996
HCA	101-86-0	AOO	2.5	1.3	5	2.1	10	2.7	25	7.8	50	13.4	10.6	C	+	Dearman et al. 2001
HCA	101-86-0	AOO	2.5	1.7	5	2.1	10	4.4	25	8.1	50	14.5	6.6	C	+	Dearman et al. 2001
HCA	101-86-0	AOO	2.5	1.7	5	2.1	10	2.4	25	7.2	50	14.1	11.3	C	+	Dearman et al. 2001
HCA	101-86-0	AOO	2.5	2.2	5	3.2	10	7.1	25	13.9	50	17.6	4.4	C	+	Basketter et al. 1999c
HCA	101-86-0	AOO	2.5	1.0	5	1.4	10	2.0	25	8.7	50	11.6	11.5	C	+	Basketter et al. 1999c
HCA	101-86-0	AOO	2.5	1.3	5	1.5	10	4.4	25	8.8	50	16.0	7.60	E	+	Loveless et al. 1996
HCA	101-86-0	AOO	2.5	1.4	5	2.1	10	3.3	25	8.3	50	14.0	7.90	E	+	Loveless et al. 1996
HCA	101-86-0	AOO	2.5	1.1	5	2.2	10	4.4	25	9.8	50	20.0	7.00	E	+	Loveless et al. 1996
HCA	101-86-0	AOO	5	1.6	10	2.5	25	6.8	ND	ND	ND	ND	11.70	C	+	Dearman et al. 2001

Substance Name	CASRN	Veh	Dose 1 Conc. (%)	Dose 1 SI	Dose 2 Conc. (%)	Dose 2 SI	Dose 3 Conc. (%)	Dose 3 SI	Dose 4 Conc. (%)	Dose 4 SI	Dose 5 Conc. (%)	Dose 5 SI	EC3 (%)	Rationale for High Dose <sup>1</sup>	LLNA Result	Reference
HCA	101-86-0	AOO	5	1.4	10	2.7	25	5.3	ND	ND	ND	ND	11.70	C	+	Dearman et al. 2001
HCA	101-86-0	AOO	2.5	1.7	5	2.2	10	2.8	25	8.2	ND	ND	10.60	C	+	Dearman et al. 2001
HCA	101-86-0	AOO	2.5	1.4	5	2.1	10	3.3	25	8.4	50	14.0	8.8	C	+	Dearman et al. 2001
HCA	101-86-0	AOO	2.5	1.0	5	1.4	10	2.0	25	8.7	50	11.6	12.20	C	+	Dearman et al. 2001
HCA	101-86-0	AOO	2.5	1.3	5	1.3	10	4.2	25	8.8	50	17.0	8.10	E	+	Loveless et al. 1996
HCA	101-86-0	AOO	1	1.0	2.5	1.0	5	1.5	10	1.8	25	5.7	14.7	N	+	Basketter et al. 2001
Imidazolidinyl urea	39236-46-9	DMF	10	1.7	25	3.1	50	5.5	ND	ND	ND	ND	24	B	+	Gerberick et al. 2005
Isoeugenol	97-54-1	AOO	0.5	0.7	1	2.3	5	13.8	ND	ND	ND	ND	1	G	+	Basketter and Cadby 2004
Isoeugenol	97-54-1	AOO	0.5	0.8	1	1.6	5	14.1	ND	ND	ND	ND	1.1	G	+	Basketter and Cadby 2004
Isoeugenol	97-54-1	AOO	0.5	0.8	1	2.8	5	5.6	ND	ND	ND	ND	2.1	G	+	Basketter and Cadby 2004
Isoeugenol	97-54-1	AOO	0.5	0.9	1	6.3	5	31.0	ND	ND	ND	ND	0.5	G	+	Basketter and Cadby 2004
Isoeugenol	97-54-1	AOO	0.5	0.9	1	1.0	5	7.2	ND	ND	ND	ND	1.9	G	+	Basketter and Cadby 2004
Isoeugenol	97-54-1	AOO	0.5	1.0	1	1.1	5	12.4	ND	ND	ND	ND	1.2	G	+	Gerberick et al. 2005
Isoeugenol	97-54-1	AOO	0.5	1.0	1	1.3	5	7.5	ND	ND	ND	ND	1.8	G	+	Basketter and Cadby 2004
Isoeugenol	97-54-1	AOO	0.5	1.1	1	1.8	5	23.2	ND	ND	ND	ND	0.8	G	+	Basketter and Cadby 2004
Isoeugenol	97-54-1	AOO	0.5	1.2	1	4.2	5	18.4	ND	ND	ND	ND	0.7	G	+	Basketter and Cadby 2004
Isoeugenol	97-54-1	AOO	0.5	1.2	1	1.4	5	19.3	ND	ND	ND	ND	1.8	G	+	Basketter and Cadby 2004
Isoeugenol	97-54-1	AOO	0.5	1.3	1	2.2	5	13.1	ND	ND	ND	ND	1	G	+	Basketter and Cadby 2004
Isoeugenol	97-54-1	AOO	0.5	1.3	1	3.3	5	14.7	ND	ND	ND	ND	1.5	G	+	Basketter and Cadby 2004
Isoeugenol	97-54-1	AOO	0.5	1.4	1	1.5	5	4.9	ND	ND	ND	ND	2.6	G	+	Basketter and Cadby 2004

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Substance Name	CASRN	Veh	Dose 1 Conc. (%)	Dose 1 SI	Dose 2 Conc. (%)	Dose 2 SI	Dose 3 Conc. (%)	Dose 3 SI	Dose 4 Conc. (%)	Dose 4 SI	Dose 5 Conc. (%)	Dose 5 SI	EC3 (%)	Rationale for High Dose <sup>1</sup>	LLNA Result	Reference
Isoeugenol	97-54-1	AOO	0.5	1.4	1	1.2	5	6.7	ND	ND	ND	ND	2	G	+	Basketter and Cadby 2004
Isoeugenol	97-54-1	AOO	0.5	1.5	1	2.6	5	19.2	ND	ND	ND	ND	0.8	G	+	Basketter and Cadby 2004
Isoeugenol	97-54-1	AOO	0.5	1.5	1	2.5	5	29.8	ND	ND	ND	ND	0.6	G	+	Basketter and Cadby 2004
Isoeugenol	97-54-1	AOO	0.5	1.6	1	2.2	5	7.5	ND	ND	ND	ND	1.6	G	+	Basketter and Cadby 2004
Isoeugenol	97-54-1	AOO	0.5	1.6	1	2.2	5	19.0	ND	ND	ND	ND	0.8	G	+	Basketter and Cadby 2004
Isoeugenol	97-54-1	AOO	0.5	1.6	1	4.3	5	24.4	ND	ND	ND	ND	0.6	G	+	Basketter and Cadby 2004
Isoeugenol	97-54-1	AOO	0.5	1.7	1	1.2	5	5.0	ND	ND	ND	ND	2.6	G	+	Basketter and Cadby 2004
Isoeugenol	97-54-1	AOO	0.5	1.8	1	2.9	5	23.2	ND	ND	ND	ND	0.6	G	+	Basketter and Cadby 2004
Isoeugenol	97-54-1	AOO	0.5	2.0	1	1.4	5	7.6	ND	ND	ND	ND	1.6	G	+	Basketter and Cadby 2004
Isoeugenol	97-54-1	AOO	0.5	2.3	1	1.6	5	23.6	ND	ND	ND	ND	0.6	G	+	Basketter and Cadby 2004
Isoeugenol	97-54-1	AOO	2.5	7.8	5	13.1	10	14.6	ND	ND	ND	ND	1.3	A, B	+	Kimber et al. 1991
Isoeugenol	97-54-1	AOO	2.5	9.9	5	17.0	10	29.5	ND	ND	ND	ND	1.3	A, B	+	Kimber et al. 1991
Isoeugenol	97-54-1	AOO	2.5	4.2	5	11.8	10	21.3	ND	ND	ND	ND	2.2	A	+	Kimber et al. 1991
Isoeugenol	97-54-1	AOO	2.5	7.5	5	13.1	10	25.3	ND	ND	ND	ND	1.4	A, B	+	Kimber et al. 1991
Isoeugenol	97-54-1	AOO	0.25	1.5	0.50	2.2	1	2.5	2.5	4.9	5	10.0	1.3	E	+	Loveless et al. 1996
Isoeugenol	97-54-1	AOO	0.25	1.0	0.50	1.3	1	2.1	2.5	2.3	5	4.1	3.3	E	+	Loveless et al. 1996
Isoeugenol	97-54-1	AOO	0.25	2.9	0.50	1.7	1	2.3	2.5	3.8	5	6.8	1.8	E	+	Loveless et al. 1996
Isoeugenol	97-54-1	AOO	0.25	0.7	0.50	0.7	1	0.9	2.5	2.1	5	7.2	3.1	E	+	Loveless et al. 1996
Isoeugenol	97-54-1	AOO	0.25	1.2	0.50	1.7	1	2.6	2.5	4.3	5	11.0	1.6	E	+	Loveless et al. 1996
Isoeugenol	97-54-1	AOO	0.5	1.8	1	2.9	2.5	7.7	5	11.1	10	11.7	1		+	No reference
Isoeugenol	97-54-1	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.9	C, E	+	Basketter et al. 2007
Isoeugenol	97-54-1	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.3	C, E	+	Basketter et al. 2007
Isoeugenol	97-54-1	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.8	C, E	+	Basketter et al. 2007

Substance Name	CASRN	Veh	Dose 1 Conc. (%)	Dose 1 SI	Dose 2 Conc. (%)	Dose 2 SI	Dose 3 Conc. (%)	Dose 3 SI	Dose 4 Conc. (%)	Dose 4 SI	Dose 5 Conc. (%)	Dose 5 SI	EC3 (%)	Rationale for High Dose <sup>1</sup>	LLNA Result	Reference
Isoeugenol	97-54-1	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.9	C, E	+	Basketter et al. 2007
Isoeugenol	97-54-1	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.7	C, E	+	Basketter et al. 2007
Isoeugenol	97-54-1	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.2	C, E	+	Basketter et al. 2007
Isoeugenol	97-54-1	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.4	C, E	+	Basketter et al. 2007
Isoeugenol	97-54-1	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.3	C, E	+	Basketter et al. 2007
Isoeugenol	97-54-1	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.2	C, E	+	Basketter et al. 2007
Isoeugenol	97-54-1	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.7	C, E	+	Basketter et al. 2007
Isoeugenol	97-54-1	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.3	C, E	+	Basketter et al. 2007
Isoeugenol	97-54-1	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.3	C, E	+	Basketter et al. 2007
Isoeugenol	97-54-1	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.7	C, E	+	Basketter et al. 2007
Isoeugenol	97-54-1	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.7	C, E	+	Basketter et al. 2007
Isopropanol	67-63-0	AOO	10	1.7	25	1.1	50	1.0	ND	ND	ND	ND	NC	C	-	Gerberick et al. 2005
Lactic acid	598-82-3	DMSO	5	1.0	10	1.4	25	2.2	ND	ND	ND	ND	NC	C	-	Gerberick et al. 2005
2-Mercapto-benzothiazole	149-30-4	DMF	1	2.3	3	4.4	10	8.6	ND	ND	ND	ND	1.7	A, C	+	Gerberick et al. 2005
Methyl methacrylate	80-62-6	AOO	10	1.4	30	1.5	50	1.5	75	2.1	100	3.6	90	C	+	Betts et al. 2006
Methyl salicylate	119-36-8	AOO	1	1.0	2.5	1.1	5	1.6	10	1.4	20	0.9	NC	C	-	Gerberick et al. 2005
Methyl salicylate	119-36-8	AOO	1	1.1	2.5	1.0	5	1.1	10	1.6	20	1.9	NC	C	-	Kimber et al. 1995
Methyl salicylate	119-36-8	AOO	1	1.8	2.5	2.7	5	2.6	ND	ND	ND	ND	NC	A, B	-	Kimber et al. 1991
Methyl salicylate	119-36-8	AOO	1	1.0	2.5	0.7	5	1.2	ND	ND	ND	ND	NC	C	-	Kimber et al. 1991
Methyl salicylate	119-36-8	AOO	1	1.2	2.5	1.5	5	1.2	10	1.8	20	2.9	NC	C	-	Kimber et al. 1995
Methyl salicylate	119-36-8	AOO	1	2.1	2.5	1.4	5	1.5	10	1.9	20	2.1	NC	C	-	Kimber et al. 1995
Methyl salicylate	119-36-8	AOO	1	0.7	2.5	0.9	5	0.8	10	0.5	20	1.1	NC	C	-	Kimber et al. 1995
Methyl salicylate	119-36-8	AOO	1	1.3	2.5	1.0	5	0.8	ND	ND	ND	ND	NC	C	-	Kimber et al. 1991

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Substance Name	CASRN	Veh	Dose 1 Conc. (%)	Dose 1 SI	Dose 2 Conc. (%)	Dose 2 SI	Dose 3 Conc. (%)	Dose 3 SI	Dose 4 Conc. (%)	Dose 4 SI	Dose 5 Conc. (%)	Dose 5 SI	EC3 (%)	Rationale for High Dose <sup>1</sup>	LLNA Result	Reference
Methyl salicylate	119-36-8	AOO	1	0.9	2.5	1.2	5	1.8	10	1.6	20	2.3	NC	C	-	Kimber et al. 1995
Nickel chloride	7718-54-9	DMSO	0.5	1.0	1	1.7	2.5	2.2	ND	ND	ND	ND	NC	C	-	Basketter et al. 1999b
Nickel chloride	7718-54-9	DMSO	1	1.5	2.5	2.2	5	2.4	ND	ND	ND	ND	NC	B	-	Basketter and Scholes 1992
Phenyl benzoate	93-99-2	AOO	5	2.3	10	2.1	25	3.5	ND	ND	ND	ND	20	N	+	Gerberick et al. 2005
Phenyl benzoate	93-99-2	AOO	1	2.0	2.5	6.4	5	9.3	10	8.7	25	11.1	1.2	C	+	Basketter et al. 1999c
Phenyl benzoate	93-99-2	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	19.60	F	+	Basketter et al. 2005b
4-Phenylene-diamine	106-50-3	AOO	0.05	2.0	0.10	3.3	0.25	10.2	0.50	20.5	1	26.4	0.10	B, C	+	Gerberick et al. 2004
4-Phenylene-diamine	106-50-3	AOO	0.05	1.9	0.10	2.3	0.25	4.0	0.50	5.7	1	6.6	0.16	C	+	Gerberick et al. 2005
4-Phenylene-diamine	106-50-3	AOO	0.05	2.2	0.10	4.2	0.25	13.7	0.50	20.8	1	25.3	0.07	C	+	Warbrick et al. 1999
4-Phenylene-diamine	106-50-3	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.09	F	+	Basketter et al. 2005b
4-Phenylene-diamine	106-50-3	AOO	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.07	C	+	Warbrick et al. 1999
4-Phenylene-diamine	106-50-3	AOO	0.05	1.6	0.10	2.6	0.25	5.6	0.50	9.5	1	9.4	0.15	C	+	Warbrick et al. 1999
Salicylic acid	69-72-7	AOO	5	0.8	10	1.5	25	2.5	ND	ND	ND	ND	NC	C	-	Gerberick et al. 2005
Sodium lauryl sulfate	151-21-3	DMF	1	0.9	2.5	1.1	5	1.7	10	2.6	20	3.5	13.40	E	+	Loveless et al. 1996
Sodium lauryl sulfate	151-21-3	DMF	1	1.5	2.5	2.3	5	3.8	10	4.1	20	5.3	4.40	E	+	Loveless et al. 1996
Sodium lauryl sulfate	151-21-3	DMF	1	2.7	2.5	4.2	5	4.6	10	8.9	20	8.6	1.50	E	+	Loveless et al. 1996
Sodium lauryl sulfate	151-21-3	DMF	1	1.6	2.5	2.1	5	2.8	10	1.6	20	3.6	17.10	E	+	Loveless et al. 1996
Sodium lauryl sulfate	151-21-3	DMF	1	1.2	2.5	1.7	5	4.3	10	5.4	20	8.0	4.00	E	+	Loveless et al. 1996

Substance Name	CASRN	Veh	Dose 1 Conc. (%)	Dose 1 SI	Dose 2 Conc. (%)	Dose 2 SI	Dose 3 Conc. (%)	Dose 3 SI	Dose 4 Conc. (%)	Dose 4 SI	Dose 5 Conc. (%)	Dose 5 SI	EC3 (%)	Rationale for High Dose <sup>1</sup>	LLNA Result	Reference
Xylene	1330-20-7	AOO	25	1.3	50	3.0	100	3.1	ND	ND	ND	ND	95.8	C	+	Basketter et al. 1996; Personal Communication (Basketter 2008); Estrada et al. 2003

Abbreviations: AOO = acetone: olive oil (4:1); CASRN = Chemical Abstracts Service Registry Number; Conc. = concentration; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; DNCB = 2,4-dinitrochlorobenzene; EC3 = estimated concentration needed to produce a stimulation index of 3; HCA = hexyl cinnamic aldehyde; LLNA = murine local lymph node assay; MEK = methyl ethyl ketone; NC = not calculated; ND = no data; SI = stimulation index

<sup>1</sup> Rationale for High Dose Key:

A: Kimber and Weisenberger 1989. Test concentrations were determined from data available in the literature and previous experience in this laboratory.

B: Basketter et al. 1991. The choice of test concentrations was based primarily upon previous experience in guinea pig tests and the physical properties of the test material (e.g., solubility and viscosity).

C: Kimber and Basketter 1992. Selected three consecutive concentrations from the following range: 50, 25, 10, 5, 2.5, 1, 0.5, 0.25 and 0.1% (w/v). The selection was made to provide the highest possible test concentration, while avoiding unacceptable dermal trauma or systemic toxicity.

D: Kimber et al. 1995. Test concentrations were selected on the basis of previous experience in these and in other laboratories.

E: Loveless et al. 1996. Test concentrations were selected on the basis of previous experience in these or in other laboratories.

F: Ryan et al. 2000. Test concentrations were chosen to provide the highest possible concentration based on either solubility in the selected vehicle or lack of systemic toxicity of the chemical. For those chemicals that were deemed to be non-sensitizing in man, the highest possible dose, up to 100% in some cases, was used.

G: OECD TG 429 (OECD 2002). Doses were selected from the concentration series 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5% etc. Three consecutive concentrations were selected so that the highest concentration maximizes exposure whilst avoiding systemic toxicity and excessive local skin irritation.

N: No information; reference did not provide specific information for test chemical preparations.





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