DRAFT ICCVAM PERFORMANCE STANDARDS FOR THE MURINE LOCAL LYMPH NODE ASSAY: METHODS FOR ASSESSING LYMPHOCYTE PROLIFERATION

NATIONAL TOXICOLOGY PROGRAM (NTP) INTERAGENCY CENTER FOR THE EVALUATION OF ALTERNATIVE TOXICOLOGICAL METHODS (NICEATM)

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LIST OF ACRONYMS

ACD	Allergic contact dermatitis
BT	Buehler Test
CPSC	U.S. Consumer Product Safety Commission
CV	Coefficient of variation
DNCB	2,4-dinitrochlorobenzene
EC3	Estimated concentration needed to produce an stimulation index of three or greater
ECVAM	European Centre for the Validation of Alternative Methods
EPA	U.S. Environmental Protection Agency
FCA	Freund's complete adjuvant
GLP	Good Laboratory Practice
GPMT	Guinea Pig Maximization Test
HCA	Hexyl cinnamic aldehyde
HMT	Human Maximization Test
HPTA	Human Patch Test—Allergan
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IWG	Immunotoxicity Working Group
JaCVAM	Japanese Center for the Validation of Alternative Methods
LLNA	Murine Local Lymph Node Assay
NICEATM	NTP Interagency Center for the Evaluation of Alternative Toxicological Methods
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SI	Stimulation index

PREFACE

The Murine Local Lymph Node Assay (LLNA) is an alternative test method used for skin sensitization testing that reduces the number of animals needed, reduces the time required for testing, and can substantially reduce or minimize the pain and distress associated with testing methods using guinea pigs. The LLNA (referred to herein as the "traditional LLNA") uses a radioactive precursor to DNA to measure cell proliferation in the draining auricular lymph nodes of the mouse. It was the first alternative test method evaluated and recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), and it has been accepted by regulatory agencies. At the time of the ICCVAM evaluation (ICCVAM 1999), the concept of performance standards, against which test methods similar to an accepted test method can be compared, had not been developed. In January 2007, the U.S. Consumer Product Safety Commission (CPSC) submitted a nomination[1] to ICCVAM and the National Toxicology Program Interagency Center for the Evaluation of Alternative Methods (NICEATM) that included (among other proposed activities) an evaluation of a number of modifications to the LLNA that may eliminate the need to use radioactive materials as part of the protocol. ICCVAM endorsed the nomination and also decided to develop performance standards to allow for a comparison of such modifications to the traditional LLNA. In May 2007, a *Federal Register* notice[2] was published requesting comments and data relevant to these issues. An ICCVAM Immunotoxicity Working Group (IWG), which includes liaisons from the Japanese Center for Validation of Alternative Methods (JaCVAM) and the European Centre for the Validation of Alternative Methods (ECVAM), recommended with a high priority the development of performance standards for the LLNA. ICCVAM and ICCVAM's advisory committee (the Scientific Advisory Committee on Alternative Toxicological Methods [SACATM]) subsequently endorsed development of performance standards for the LLNA as a high priority activity. The IWG, with assistance from NICEATM, subsequently developed the draft LLNA performance standards provided in this document.

These draft test method performance standards are proposed to evaluate the performance of LLNA test methods that incorporate specific modifications to measure lymphocyte proliferation compared to the traditional LLNA. These modifications focus specifically on using non-radioactive procedures to measure lymphocyte proliferation in the draining auricular lymph nodes rather than incorporation of radioactivity (^{3}H-thymidine), which is used in the traditional LLNA.

These draft performance standards are being released to the public for comment; any comments received will be considered by ICCVAM during the development of a revised draft version of this document, which will be released in late 2007. In early March 2008, this revised version will be considered by an independent peer review panel in a public forum, in conjunction with their review of the evaluations included in the CPSC nomination[1]. The recommendations by the peer review panel will be made available for public and SACATM comment. The peer review panel report and all comments will be considered by ICCVAM in preparing final test method performance standards recommendations for Federal agencies and for publication.

[1] http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf

[2] http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf

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

1.0 PURPOSE AND BACKGROUND OF PERFORMANCE STANDARDS

1.1 Introduction

These test method performance standards[3] are proposed so that the Interagency Coordinating Committee on the Validation of Alternative Toxicological Methods (ICCVAM) can evaluate the performance of murine local lymph node assay (LLNA) protocols that incorporate specific modifications to the measurement of lymphocyte proliferation in the radioactive LLNA (referred to herein as the "traditional LLNA") and make recommendations to Federal agencies regarding these assay modifications. These modifications focus specifically on incorporating non-radioactive procedures to evaluate lymphocyte proliferation in the draining auricular lymph nodes rather than incorporation of radioactivity (i.e., ^{3}H-thymidine), which is used in the traditional LLNA.

These performance standards are not proposed for evaluating other alternative test methods for measuring skin sensitization (e.g., *in vitro* methods) and are for a narrow purpose (i.e., methods that evaluate lymphocyte proliferation). Additionally, these performance standards do not imply the appropriateness of performance standards for any other *in vivo* method. U.S. Federal agencies will determine the regulatory acceptability and utility of the ICCVAM recommendations for their individual programs.

1.2 Elements of ICCVAM Performance Standards

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

- **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, mechanistically and functionally similar test 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 accuracy and reliability characteristics that the proposed test method should be comparable to or exceed when evaluated using the minimum list of reference substances.

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

1.3 ICCVAM Process for the Development of LLNA Performance Standards

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

The proposed performance standards are being made available to the public for comment at this time. ICCVAM will consider public comments and prepare revised draft performance standards for consideration by an independent peer review panel. The ICCVAM/National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) Independent Expert Peer Review Panel will evaluate the revised draft performance standards for completeness and appropriateness at a public meeting in early March 2008. These will also be made available to the public for comment in advance of the panel meeting, and all comments will be provided to the panel for their consideration. The recommendations by the Expert Peer Review Panel will be made available for public and Scientific Advisory Committee on Alternative Toxicological Methods comment. These comments will be considered by ICCVAM in preparing final test method performance standards recommendations for Federal agencies and for publication.

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

1.4 ICCVAM Development of Performance Standards for the LLNA

1.4.1 Background on Skin Sensitization

Skin sensitization to a substance can lead to allergic contact dermatitis (ACD), a type IV hypersensitivity reaction. The development of skin sensitization occurs in two separate phases. The first phase, referred to as the induction phase, occurs when a susceptible individual is exposed topically to a skin-sensitizing substance in sufficient quantities. Induction is dependent on a substance penetrating the epidermis and subsequently binding to proteins. The resulting hapten complex can then be processed by the antigen-presenting cells in the skin (i.e., Langerhans cells). These cells then migrate to the draining lymph nodes, where the antigen is presented to T lymphocytes, leading to their clonal expansion. The lymphocytes can be divided into two subsets, memory and effector T lymphocytes. At this point, the individual has become sensitized to the exposed substance (Basketter et al. 2003; Jowsey et al. 2006).

The second phase, referred to as the elicitation phase, occurs when the individual is exposed to the same substance at the same or different skin location. As in the induction phase, the substance penetrates the epidermis where it is processed by antigen-presenting cells. The antigen is then presented to circulating effector T lymphocytes. The T lymphocytes produce a rapid

secondary immune response in the skin that can lead to ACD (Basketter et al. 2003; Jowsey et al. 2006).

1.4.2 Test Methods for Assessing Skin Sensitization

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

For the GPMT, sensitization in guinea pigs is induced by intradermal injection of the test substance and 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 placed with a dressing for 24 hours. Skin reactions (erythema and edema) are scored 24 and 48 hours after patch removal (ICCVAM 1999).

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 three weeks. Two weeks after the final treatment, a patch containing the test substance is applied for 6 hours at a location different to where the initial challenges occurred. Skin reactions (erythema and edema) are then scored 24 and 48 hours after patch removal (ICCVAM 1999).

1.4.3 Intended Regulatory Uses for the LLNA

The LLNA is an alternative method that can be used as a substitute for the traditional guinea pig tests (GPMT and BT[4]), where appropriate, for assessing skin sensitization. The LLNA may not be suitable for use with certain types of test materials, such as metallic compounds, mixtures, high molecular weight compounds that cannot penetrate the stratum corneum, strong dermal irritants, chemicals whose pharmacodynamic activity is to release dermal cytokines that cause local lymph node proliferation (e.g., certain pharmaceuticals such as imiquimod [Gaspari 2007]), and materials that do not adhere to the ear for an acceptable time during the experiment.

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 (i.e., induction phase of skin sensitization, see **Section 1.4.1**). Comparatively, the reference tests (see **Section 1.4.2**) involve rating skin reactions evoked in guinea pigs by the test substance (i.e., elicitation phase of skin sensitization; see **Section 1.4.1**), and therefore allows for an assessment of the entire process associated with allergic contact dermatitis.

While the endpoints measured in the LLNA and the reference test methods are different, the induction phase of skin sensitization is necessary for development of skin reactions (i.e., elicitation phase). Therefore, measurement of lymphocyte proliferation generally predicts whether the test substance will produce skin sensitization. Compared to the LLNA, which

[4] The GPMT and BT are widely used and are the preferred guinea pig sensitization tests as outlined in the OECD test guidelines for skin sensitization.

quantifies the amount of T lymphocyte proliferation, the reference test methods use subjective scoring of the irritation (i.e., erythema and edema) observed after test substance application.

2.0 LLNA PERFORMANCE STANDARDS FOR ASSESSING LYMPHOCYTE PROLIFERATION

2.1 Background

Validation studies have been completed to evaluate the ability of the LLNA to be used as an alternative to traditional guinea pig skin sensitization tests for "yes/no" determinations (ICCVAM 1999). Studies indicate that certain test materials, such as mixtures (limited available data), metallic compounds (which may produce unreliable results), high molecular weight compounds (which are not readily absorbed into the skin), strong dermal irritants (which may produce false positive results), and materials that do not adhere to the ear for an acceptable time during the experiment may not be suitable for use with the LLNA. This section briefly describes the principles of the LLNA test method, followed by the draft performance standards that would be used to evaluate test methods for evaluation of lymphocyte proliferation that are functionally and mechanistically similar. The performance standards consist of 1) essential test method components, 2) reference substances, and 3) the comparable accuracy and reliability that should be achieved.

2.2 Principles of the LLNA

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

2.3 Essential Test Method Components for Methods Assessing Lymphocyte Proliferation

The essential test method components include all aspects of the Organisation for Economic Co-operation and Development (OECD) test guideline for the LLNA (OECD Test Guideline 429; OECD 2002 and **Appendix A**), with the exception of the assessment of lymphocyte proliferation and the interpretation of results. The following sections only discuss the information that should be provided to support the use of protocols that incorporate specific modifications to the measurement of lymphocyte proliferation in the traditional LLNA. These modifications focus specifically on incorporating non-radioactive procedures to assess lymphocyte proliferation. Test method reporting requirements also are discussed.

2.3.1 Assessment of Lymphocyte Proliferation and Interpretation of Results

- Lymphocyte proliferation can be assessed using a variety of methods (e.g., ^{3}H thymidine incorporation).
- The method used for assessing lymph node cell proliferation should be detailed and scientifically justified.
- Lymphocyte proliferation should be expressed in the units obtained from the method (e.g., disintegrations per minute for methods using radioactive reagents). Results should be provided for all test substances and concurrent controls.

- Raw data and calculated results (e.g., as measured or quantified by the SI) should be provided for all test substances and concurrent controls.
- A 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.
  - In the traditional LLNA, an SI of three or greater is required for a substance to be considered a skin-sensitizing agent. However, a decision criterion using an SI of three or greater may only be applicable to the measurement of ^{3}H-thymidine incorporation as conducted in the traditional LLNA (i.e, OECD TG 429). As described below, alternative decision criteria may be more appropriate for alternatives to ^{3}H-thymidine incorporation for measuring lymph node cell proliferation.
  - Although the SI is the criteria most often used, an assessment may also be performed by statistical analysis of individual animal data and may provide a more complete evaluation of the test substance. This may be particularly important in the case of equivocal results.
- If consideration is given to other properties of the test substance (e.g., structural relationship to known skin sensitizers) in addition to the calculated results in classification of substances as skin sensitizers, such information should be detailed.
- If applicable, the choice of statistical analysis described and rationale for selection provided.
- Exclusion criteria should be defined and the impact of any excluded data should be described.

2.3.2 Test Report

The test report should include information outlined below. Any deviations in essential test method components provided in **Appendix A** should be noted and justified.

1. Test Substances, Control Substances, and Vehicles
   - Name of test substance and identification data (e.g., Chemical Abstracts Service Registry Number)
   - Purity and composition of the substance or mixture
   - Physicochemical properties (e.g., physical state, water solubility) relevant to the conduct of the study
   - Treatment of the test/control substances prior to testing, if applicable (e.g., vortexing, sonication, warming; resuspension solvent)
   - Name of vehicle and identification data (e.g., purity, composition, volume used)
   - Justification for choice of vehicle
2. Justification of the Alternative Test Method and Protocol Used

3. Test Animals
   - Strain of mice used[5]
   - Microbiological status of the mice, when information is available
   - Number, age, and sex of mice used
   - Source of mice, housing conditions, diet, etc.
4. Test Method Conditions
   - Details on test substance preparation and application
   - Justification for dose selections, including basis for the highest dose tested (i.e. maximum non-irritating concentration, maximum soluble concentration, maximum concentration that does not cause systemic toxicity)
   - The basis for dose selection and reason for variation away from traditional assay dose selection process, if any, should be discussed
5. Criteria for an Acceptable Test
   - Concurrent positive control data
   - Concurrent negative control data
   - Historical ranges of positive and negative control data. Historical data can be from within the testing laboratory or provided from an external source, provided that supporting data (e.g., raw data) can be provided.
6. Results
   - Weights of each animal at the start of the test and at sacrifice
   - Tabulation of data from individual animals showing the mean and individual values for each dose (including vehicle) group
   - Statistical analysis, where appropriate
   - Time course of onset and severity of toxicity (e.g., dermal irritation)
7. Description of Animal Observations
   - Clinical signs of systemic toxicity and dermal irritation should be described (e.g., location of observed dermal irritation)
8. Discussion of the Results
9. Conclusion
10. A Quality Assurance Statement for Good Laboratory Practice (GLP)-Compliant Studies

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

- 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.

If GLP-compliant studies are performed, then additional reporting requirements provided in the relevant guidelines (e.g., OECD 1998; EPA 2006a, 2006b; FDA 2006) should be followed.

2.4 Reference Substances for Methods Assessing Lymphocyte Proliferation

2.4.1 Criteria for Choosing 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:

- Represent the range of responses that the validated test method is capable of measuring or predicting
- Reflect the accuracy of the validated test method
- 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

2.4.2 Characteristics of Chosen Reference Substances

The traditional LLNA was submitted with data from testing of over 200 substances. After careful consideration of the above criteria, 20 substances were selected as proposed minimum reference substances for the LLNA performance standards. The proposed substances are provided in **Appendix B** and a detailed rationale for selection of the substances in this list is included in **Appendix C**. The selected substances have the following characteristics:

- All of the substances have data from testing in the GPMT or BT.
- All of the substances are readily available from commercial sources.
- The substances represent the full dynamic range of responses that can be assessed in the current approved LLNA, from non-sensitizers to strong sensitizers.
- The substances approximate the overall accuracy determined for the traditional LLNA. Two LLNA false negative and two false positives, when compared to guinea pig outcomes, are included to indicate whether the modified LLNA procedure may have improved accuracy relative to the traditional LLNA.
- Nineteen of the substances have human data (e.g., Human Maximization Test results, Human Repeat Insult Patch Test results, available as a patch test kit allergen, and/or clinical case studies/reports).

- The selected substances include 9 solids and 11 liquids.
- The molecular weights of the substances range from 30.026 g/mole to 604.813 g/mole.
- The xLogP (octanol:water partition coefficient) values (Wang et al. 2000) range of the substances range from -3.1 to 4.9 (from water soluble to insoluble, respectively).
- The vehicles used for all of the substances are known. The vehicles used were acetone (1), acetone:olive oil (12), dimethyl formamide (4), dimethyl sulfoxide (2) and methyl ethyl ketone (1).
- There is peptide reactivity information for 11 substances.
- The EC3 values (the effective concentration for stimulation of a 3-fold increase in lymph node cell proliferation) of the positive substances range from 0.0099% to 28%, based on results from the traditional LLNA.
- A wide range of SI values are represented, ranging from 3.5 to 52.3 for substances identified as skin sensitizers by the traditional LLNA.

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

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

2.5 Accuracy and Reliability Performance Values

The third element of the performance standards are accuracy and reliability values that should be met or exceeded by the proposed test method when evaluated with the reference substances.

2.5.1 Accuracy

Accuracy is defined as the closeness of agreement between a test method result and an accepted reference value (ICCVAM 2003). When evaluated using the minimum list of recommended reference substances (**Appendix B**), the proposed test method should have performance characteristics that are similar to or exceed the performance of the traditional LLNA method (see **Table 2-1**).

Table 2-1 Performance Statistics for LLNA

Comparison	N[1]	Accuracy		Sensitivity		Specificity		Positive Predictivity		Negative Predictivity		False Positive		False Negative	
		%	No.[2]	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
LLNA vs. GPMT/BT[3]	20	80	16/20	85	11/13	71	5/7	85	11/13	71	5/7	29	2/7	15	2/13
LLNA vs. Human[4]	19	79	15/19	79	11/14	80	4/5	92	11/12	57	4/7	20	1/5	21	3/14
GPMT/BT vs. Human[5]	19	79	15/19	79	11/14	80	4/5	92	11/12	57	4/7	20	1/5	21	3/14

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

[1]N = Number of substances.

[2]Data used to calculate the percentage.

[3]Performance statistics for same comparison (as described in the LLNA Peer Review Panel Report (ICCVAM 1999)) were: Accuracy = 89% (86/97), Sensitivity = 91% (62/68), Specificity = 83% (24/29), Positive Predictivity = 93% (62/67), and Negative Predictivity = 80% (24/30).

[4]Performance statistics for same comparison (as described in the LLNA Peer Review Panel Report (ICCVAM 1999)) were: Accuracy = 72% (53/74), Sensitivity = 72% (49/68), Specificity = 67% (4/6), Positive Predictivity = 96% (49/51), and Negative Predictivity = 17% (4/23).

[5]Performance statistics for same comparison (as described in the LLNA Peer Review Panel Report (ICCVAM 1999)) were: Accuracy = 72% (41/57), Sensitivity = 70% (38/54), Specificity = 100% (3/3), Positive Predictivity = 100% (38/38), and Negative Predictivity = 16% (3/19).

2.5.2 Reliability

Test method reliability (intralaboratory repeatability, and intra- and inter-laboratory reproducibility) is the degree to which a test method can be performed reproducibly within and among laboratories over time (ICCVAM 2003). Repeatability refers to the closeness of agreement between test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions within a given time period. Intralaboratory 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.

The reliability of the proposed test method for the reference substances should be comparable to or better than that of traditional LLNA. The following sections provide these reference statistics for the traditional LLNA.

2.5.2.1 *Intralaboratory Repeatability*

Data was not available to assess intralaboratory repeatability for the traditional LLNA method.

2.5.2.2 *Intralaboratory Reproducibility*

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

As shown in **Table 2-2**, the agreement in identification of a sensitizer and non-sensitizer across three to six runs in an individual lab ranged from 83% to 100%. The results indicate that all four known sensitizers and one non-sensitizer were identified correctly in all the tests. One non-sensitizer, benzocaine, was identified as a non-sensitizer in five out of six tests.

Table 2-2 Intralaboratory Reproducibility Results for Six Substances Using the LLNA

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

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

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

Therefore, intralaboratory reproducibility can be assessed by calculating the variability resulting from testing of the positive control substance, such as HCA. The modified LLNA test method should have an intralaboratory reproducibility that is equivalent to or better than the intralaboratory reproducibility of HCA, or other comparable positive control substance in the traditional LLNA method (i.e., coefficient of variation [CV] <30%; see **Table 2-3**).

533 **Table 2-3 Intralaboratory Reproducibility of LLNA EC3 values, as Calculated by Coefficient of Variation**

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

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

2.5.2.3 *Interlaboratory Reproducibility*

Interlaboratory reproducibility for the traditional LLNA was evaluated based on data provided to ICCVAM and from literature searches. As shown in **Table 2-4**, the interlaboratory CVs for the EC3 values for a range of the tested sensitizers (DNCB, HCA, isoeugenol, and eugenol) ranged from 6.8% to 42.5%. Sodium lauryl sulfate, which is a false positive irritant, produced an interlaboratory CV of 83.7%.

Table 2-4 Interlaboratory Reproducibility of LLNA, as Calculated by Coefficient of Variation

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

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

Therefore, when testing DNCB and HCA, a proposed test method that is functionally and mechanistically similar to the LLNA should have an interlaboratory reproducibility that is comparable to the interlaboratory reproducibility of DNCB and HCA in the traditional LLNA method (see **Table 2-4**).

ICCVAM recognizes the limitations of this dataset with regard to the type and number of substances tested. For this reason, ICCVAM is continuing to request additional data and reliability analyses from interested stakeholders. Once additional information is received, interlaboratory reproducibility statistics will be updated.

3.0 REFERENCES

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APPENDIX A

Essential Test Method Components for Local Lymph Node Assay (based on OECD TG 429)

and

Details of Dissection of Draining Auricular Lymph Nodes from *Protocol: Murine Local Lymph Node Assay (LLNA); Recommended by ICCVAM Immunotoxicology Working Group based on an Independent Expert Peer Review Panel Evaluation of the LLNA* (http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/LLNAProt.pdf)

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The following is a description of the essential test method components for the LLNA. These test method components are consistent with the OECD test guideline for the LLNA (OECD Test Guideline 429; OECD 2002) as well as the ICCVAM recommended LLNA protocol (ICCVAM 1999).

Animal Selection and Preparation

Animal Species Selection

- Mice are the species of choice for this test method.
- Young adult female mice that are nulliparous and not pregnant (i.e., CBA/Ca or CBA/J strains) are used. Other strains and males may be used, where it has been demonstrated that strain- and/or gender-specific differences are not detrimental to the performance of the test method.
- At the start of the study, mice should be 8-12 weeks old.
- Weight variations between the mice should not exceed 20% of the mean weight.

Housing and Feeding Conditions

- Experimental animal room temperature should be 22 ± 4 °C.
- Experimental animal room humidity should range between 30% and 70%. The preferred humidity for the room should range from 50% to 60%.
- Artificial lighting should be used with a cycle of 12 hours light and 12 hours dark.
- Mice should be individually housed and fed a conventional laboratory diet. Mice should have unrestricted access to drinking water.

Animal Preparation

- Mice should be acclimated for 5 days prior to the start of the test.
- All mice should be examined prior to the initiation of the test to ensure that there are no skin lesions present.

Control Substances

Solvent/Vehicle Control

- To ensure that the test system is functioning properly and that the specific test is valid, a solvent/vehicle control should be included in each experiment.
- The solvent/vehicle control should be tested concurrently with the test substances.
- The selected solvent/vehicle must not interfere with or bias the test result and should be selected to achieve maximum concentration/skin exposure of the test substance.
- Hydrophilic materials should be incorporated into a vehicle that does not immediately run off of the skin.
- In order of preference, recommended solvents/vehicles are acetone:olive oil (4:1 v/v), *N*,*N*-dimethylformamide, methyl ethyl ketone, propylene glycol, and

dimethyl sulfoxide. Other solvents may be used if appropriate justification is provided.

Positive Control

- The purpose of a positive control substance is to demonstrate that the test method is responding with adequate sensitivity to a sensitizing substance for which the magnitude of the response is well characterized.
- The positive control should be tested concurrently with the test substances, and should be tested in the same vehicle as the test substances, if possible.
- The positive control should be tested at a concentration that is expected to yield a positive response (e.g., for the traditional LLNA protocol, the positive control should produce an SI > 3). Each test should generate a response that is comparable to the historical range generated by the laboratory.
- The positive control dose is to be chosen such that there is a clearly positive response, but that is not excessive (e.g., benzoquinone may be too potent to use as a positive control).
- Examples of test substances that may be used as positive controls include, but are not limited to, hexyl cinnamic aldehyde and mercaptobenzthiazole.
- Other substances may be used as a positive control, with sufficient justification. However, benzocaine should not be used as a positive control since it has been shown to produce equivocal responses in the LLNA.

Benchmark Controls

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

<u>Test Procedure</u>

Number of Animals per Dose

- A minimum of four mice per dose group should be used.
- A negative and positive control group should be included.

Selection of Doses

- Dose and vehicle selection should be based on the recommendations provided in Kimber et al. (1994).

- Three consecutive doses are selected (e.g., 100%, 50%, 25%).
- Higher concentration percentages (e.g., 100%) may not be applicable to mixtures.
- The highest dose tested should not induce systemic toxicity and/or excessive skin irritation.

Dosing Schedule and Collection of Lymph Node Cells

- Day 1
  - Each mouse is identified and weighed.
  - Test substance, vehicle, or positive control (25 μL) is applied to the dorsum of each ear.
- Days 2 and 3
  - Repeat the application procedure as described for Day 1.
- Days 4 and 5
  - No treatment.
- Day 6
  - Weigh each mouse.
  - Mice are euthanized.
  - The draining auricular lymph nodes from each ear are excised. The nodes are either (a) pooled in PBS for each experimental group (pooled treatment group approach) or (b) pooled in PBS for each animal (individual animal approach).

Observations

- Mice should be observed for any clinical signs of local, excessive irritation or corrosion, or systemic toxicity. Animal monitoring plans must include criteria to promptly identify animals exhibiting systemic toxicity or excessive irritation or corrosion of skin for euthanasia.
- Histopathology should be considered to evaluate questionable lesions.
- Erythema and edema formation should be noted.
- All observations should be recorded.

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

- Lymphocyte proliferation should be expressed in the units obtained from the method (i.e., disintegrations per minute). Results should be provided for all test substances and concurrent controls.
- Raw data and calculated results (i.e., as measured or quantified by the stimulation index [SI]) should be provided for all test substances and concurrent controls.

- Description of decision criteria for what constitutes positive and negative responses in the proposed test method and the basis for the decision criteria should be provided.
  - When the SI for any single treatment group is ≥ 3, the test substance is regarded as a skin sensitizer.
  - However, the magnitude of the SI should not be the sole factor used in determining the biological significance of a skin sensitization response.
  - An assessment may be performed by statistical analysis of individual animal data and may provide a more complete evaluation.
  - Factors that should be considered include the SI, statistical analyses, the strength of the dose-response relationship, chemical toxicity, solubility, and the consistency of the vehicle and positive control responses.
  - A test substance not meeting the above criteria is considered a non-sensitizer.

DISSECTION APPROACH

Lateral Dissection (Figure 1)

Although lateral dissection is not the conventional approach used to obtain the nodes draining the ear, it may be helpful as a training procedure when used in combination with the ventral dissection. This approach is performed bilaterally (on both sides of the mouse). After the mouse is euthanized, it is placed in a lateral position. The facial and neck area is wetted with 70% ethanol. Using scissors and forceps, an initial cut is made from the neck area slightly below the ear. This incision is carefully extended toward the mouth and nose. During this procedure, the tip of the scissors should be angled slightly upward to prevent the damage of deeper tissue. The glandular tissue in the area is gently retracted using the forceps. Using the masseter muscle, facial nerves, blood vessels, and the bifurcation of the jugular vein as landmarks, the draining node is isolated and removed (**Figure 1**). The draining nodes[6] ("auricular") will be positioned adjacent to the masseter muscle and proximal to and slightly above the jugular bifurcation.

Ventral Dissection (Figure 2)

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

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

Figure 1: Lateral Dissection

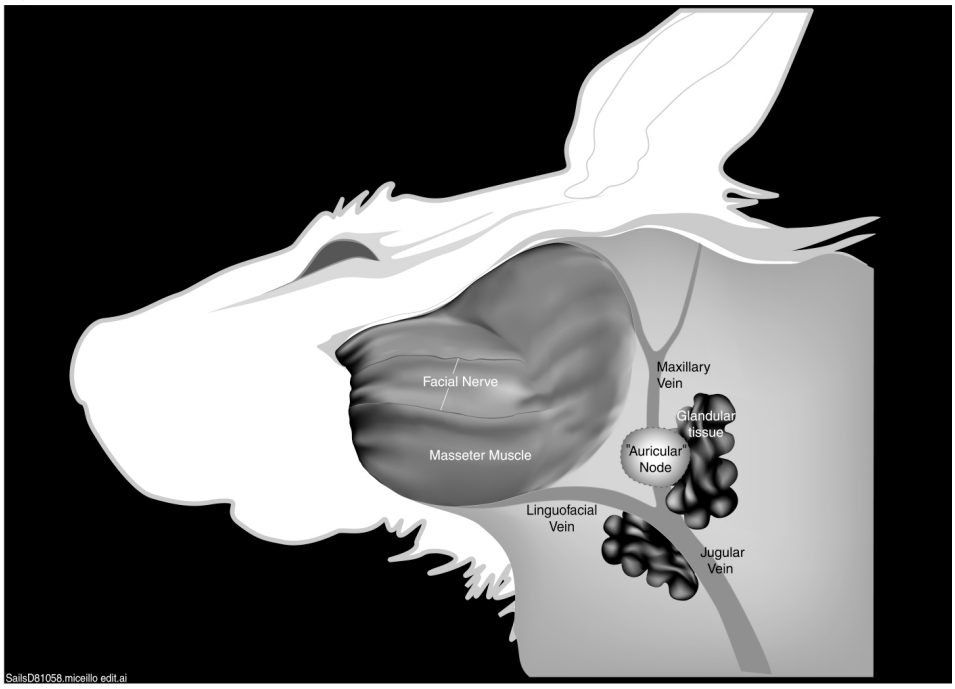
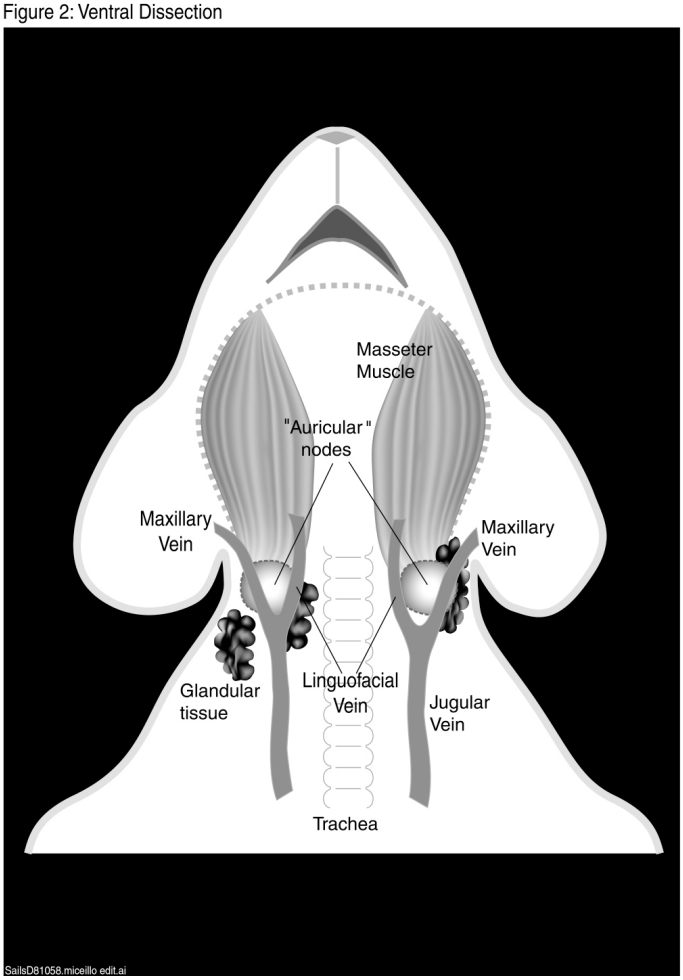


Figure 2: Ventral Dissection



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ACCURACY IN IDENTIFICATION

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

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APPENDIX B

Recommended Reference Substances for Methods Assessing Lymphocyte Proliferation

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APPENDIX B1

Recommended Reference Substances for Methods Assessing Lymphocyte Proliferation - Alphabetically Sorted

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Chemical Name	CASRN	MW (g/mol)	Physical Form	LLNA	Veh[1]	EC3[2]	SI (Conc)	GPMT/BT[3]	HMT	HPTA	Additional Human Skin Sensitization Data/Information[4]	Peptide Reactivity[5]
Benzoquinone[6]	106-51-4	108.095	Solid	+	AOO	0.0099	52.3 (2.5%)	+				High
Cinnamic aldehyde	104-55-2	132.159	Liquid	+	AOO	3	15.8 (10%)	+	+	+	DSA05HRIPT=639; DSA05HMT=216	High
Citral	5392-40-5	152.233	Liquid	+	AOO	13	6.3 (25%)	+	+		DSA05HRIPT=1266; DSA05HMT=862; DSA(NOEL)HRIPT=775	
2,4-Dinitrochlorobenzene	97-00-7	202.552	Liquid	+	AOO	0.05		+			Results from patch test studies indicate substance produces skin sensitization[11]	
Ethylene glycol dimethacrylate	97-90-5	198.216	Liquid	+	MEK	28	7 (50%)	-		+		High
Formaldehyde	50-00-0	30.026	Liquid	+	Ac	0.61	4.0 (1.85%)	+	+	+	DSA05HRIPT=411; DSA05HMT=89; DSA(NOEL)HRIPT=37	Moderate
Hexyl cinnamic aldehyde[6]	101-86-0	216.319	Liquid	+	AOO	11	17 (50%)	+			DSA(NOEL)HRIPT=23622	Minimal
2-Hydroxyethyl acrylate	818-61-1	116.115	Liquid	+	AOO	1.4	18.1 (25%)	+		+		High

Chemical Name	CASRN	MW (g/mol)	Physical Form	LLNA	Veh[1]	EC3[2]	SI (Conc)	GPMT/BT[3]	HMT	HPTA	Additional Human Skin Sensitization Data/Information[4]	Peptide Reactivity[5]
Imidazolidinyl urea	39236-46-9	388.294	Solid	+	DMF	24	5.5 (50%)	+		+	DSA05HRIPT=3846; DSA(NOEL)HRIPT=2000	Moderate
Isoeugenol	97-54-1	164.201	Liquid	+	AOO	1.2	12.4 (5%)	+		+	DSA05HRIPT=657; DSA(NOEL)HRIPT=250	
Isopropanol	67-63-0	60.095	Liquid	-	AOO	NC	1.0 (50%)	-			Studies indicate substance produces skin sensitization[12]	Minimal
2-Mercaptobenzothiazole	149-30-4	167.253	Solid	+	DMF	1.7[7]	8.6 (10%)	+	+	+	DSA05HMT=2269	High
Methyl salicylate	119-36-8	152.147	Liquid	-	AOO	NC	0.9 (20%)	-	-	-		Minimal
Nickel chloride	7718-54-9	129.599	Solid	-	DMSO[8]	NC	2.4 (5%)	+	-	-	Results from patch test studies indicate substance produces skin sensitization[13]	
Nickel sulfate	10101-98-1	280.864	Solid	-	DMSO[8]	NC		+	+	+		
4-Phenylenediamine	106-50-3	108.14	Solid	+	AOO	0.16	6.6 (1.0%)	+	+	+	DSA05HMT=111	

Chemical Name	CASRN	MW (g/mol)	Physical Form	LLNA	Veh[1]	EC3[2]	SI (Conc)	GPMT/BT[3]	HMT	HPTA	Additional Human Skin Sensitization Data/Information[4]	Peptide Reactivity[5]
Salicylic acid	69-72-7	138.121	Solid	-	AOO	NC	2.5 (25%)	-	-	-		
Sodium lauryl sulfate	151-21-3	288.38	Solid	+	DMF	14	3.5 (20%)	-	-	-		
Sulfanilamide	63-74-1	172.20	Solid	-	DMF	NC	0.9 (50%)	-	+	+		Minimal
Tween 80	9005-65-6	604.81	Liquid	-	AOO[9]	NC[10]		-	-	+		

Abbreviations: Ac = Acetone; AOO = Acetone:OliveOil; BT = Buehler Test; CASRN = Chemical Abstract Service Registry Number; DMF = Dimethylformamide; DMSO = Dimethyl sulfoxide; GPMT = Guinea Pig Maximization Test; HMT = Human Maximization Test; HPTA = Human Patch Test Allergan; LLNA = Local Lymph Node Assay; MEK = methyl ethyl ketone; MW = Molecular Weight; NC = Not Calculated SI = Stimulation Index; Veh = Vehicle.

[1]Unless noted otherwise, vehicle information obtained from: Gerberick et al, 2005. Compilation of historical Local Lymph Node data for evaluation of skin sensitization alternative methods. Dermatitis. 16:157-202.

[2]Unless noted otherwise, EC3 values obtained from: Gerberick et al, 2005. Compilation of historical Local Lymph Node data for evaluation of skin sensitization alternative methods. Dermatitis. 16:157-202,

[3]Results obtained from guinea pig maximization test and Buehler test.

[4]Human Quantitative Data obtained from literature where human data was compared to LLNA. All data are expressed as dose per skin area (μg/cm^2). DSA05HMT and DSA05HRIPT were obtained by linear interpolation from the lowest observed effect level to a dose corresponding to the estimated sensitization incidence of 5% (Schneider K, Akkan Z. 2004. Quantitative relationship between the local lymph node assay and human skin sensitization assays. Reg Toxicol Pharmacol. 39:245-255). 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 D, Clapp C, Jeffries D, Safford B, Ryan C, Gerberick F, Dearman R, Kimber I. 2005. Predictive identification of human skin sensitization thresholds. Contact Dermatitis. 53:260-267).

[5]Peptide reactivity data obtained from: Gerberick et al. Quantification of chemical peptide reactivity for screening contact allergens: A classification tree model approach. Tox Sci Advance Access (March 30, 2007).

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

[7]EC3 values obtained from Kimber et al. 2003. Classification of contact allergens according to potency: proposals. Food Chem Toxicol. 41:1799-1809.

871 [8]Vehicle information obtained from: ICCVAM 1999. The murine local lymph node assay: A test method for assessing the allergic contact dermatitis potential of
872 chemical/compounds. NIH Publication No. 99-4494. Research Triangle Park, NC: National Toxicology Program.
873 [9]Vehicle information obtained from: Basketter et al. 2000. Use of the local lymph node assay for the estimation of relative contact allergenic potency. Contact Dermatitis
874 42(6):344-348.
875 [10]EC3 values obtained from: Basketter and Kimber, 2006. Predictive test for irritants and allergens and their use in quantitative risk assessment. In "Contact Dermatitis", 4th
876 Edition. Eds, Frosch P J, Menné T and Lepoittevin J-P, Springer Verlag, Heidelberg, pp 179 – 188.
877 [11]Human data based on following studies: (1) Rees JL, Friedmann PS, Matthews JN. 1989. Sex differences in susceptibility to development of contact hypersensitivity to
878 dinitrochlorobenzene (DNCB). Br J Dermatol. 120:371-374. (2) Zina AM, Bedello PG, Cane D, Bundinio S, Benedetto A. 1987. Dermatitis in a rubber tyre factory. Contact
879 Dermatitis. 17:17-20.
880 [12]Human data based on following study: Kwon JA, Lee MS, Kim MI, Park YM, Kim HO, Kim CW. 2003. Allergic contact dermatitis from dodecyldiaminoethylglycine and
881 isopropyl alcohol in a commercial disinfectant swab. Contact Dermatitis. 48:339-340.
882 [13]Human data based on following study: Rasanen L, Mattila U, Kalimo K. 1999. Patch testing with nickel sulfate versus nickel chloride. Contact Dermatitis. 40:287-288.
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892 **APPENDIX B2**
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894 **Recommended Reference Substances for Methods Assessing Lymphocyte Proliferation -**
895 **Structures and Product Uses**

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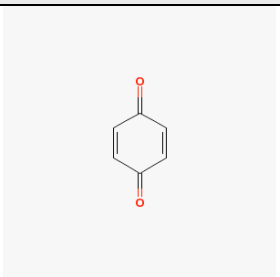
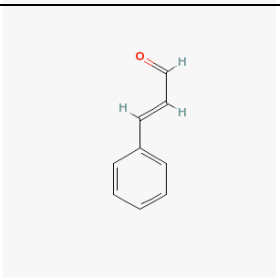
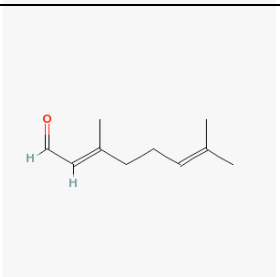
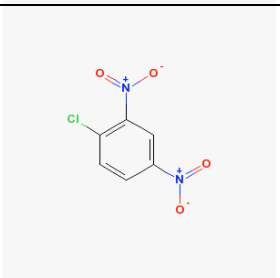
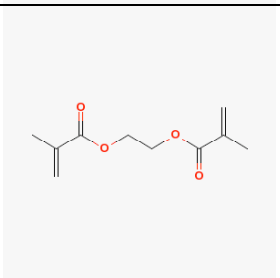
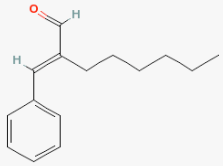
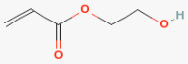
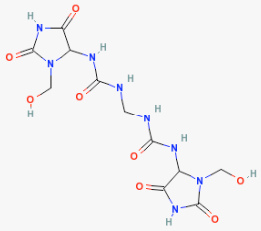
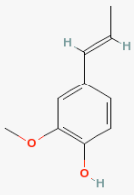
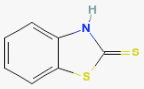
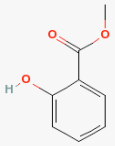
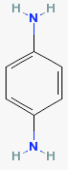
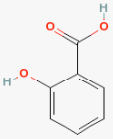
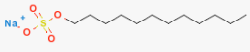
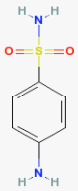
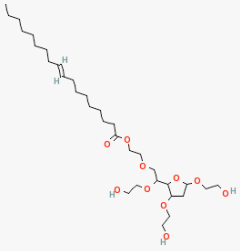
Chemical Name	CASRN	Structure	Product Uses
Benzoquinone	106-51-4		Agricultural chemical Nylon manufacture Dye manufacture
Cinnamic aldehyde	104-55-2		Flavor additive Perfume manufacture Fungicide Insecticide
Citral	5392-40-5		Flavor additive Perfume manufacture
2,4-Dinitrochlorobenzene	97-00-7		Color photo processing Explosives manufacture
Ethylene glycol dimethacrylate	97-90-5		Polymerization agent

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

Chemical Name	CASRN	Structure	Product Uses
Isopropanol	67-63-0		Topical pharmaceutical Gasoline additive Cleaning agent
2-Mercaptobenzothiazole	149-30-4		Rubber manufacture Anticorrosive
Methyl salicylate	119-36-8		Topical pharmaceutical Flavor additive
Nickel chloride	7718-54-9		Electroplating agent Battery manufacture
Nickel sulfate	10101-98-1		Electroplating agent Battery manufacture Dye manufacture
4-Phenylenediamine	106-50-3		Hair dye Textile dye

Chemical Name	CASRN	Structure	Product Uses
Salicylic acid	69-72-7		Pharmaceutical Food preservative
Sodium lauryl sulfate	151-21-3		Detergent Cosmetic
Sulfanilamide	63-74-1		Pharmaceutical Antimicrobial
Tween 80	9005-65-6		Detergent Food additive

912 Abbreviations: CASRN = Chemical Abstract Service Registry Number.

APPENDIX C

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

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The candidate list used to select proposed minimum reference substances ("reference list") for the draft proposed local lymph node assay (LLNA) performance standards was initially generated from the database originally submitted to ICCVAM for the 1998 evaluation of the LLNA. This database of 209 substances was reduced to 97 candidate substances by identifying those substances for which unequivocal comparative guinea pig maximization test (GPMT) or Buehler test (BT) data that were collected using a standard protocol (e.g., EPA Health Effects Test Guideline OPPTS 870.2600) were available. The availability of such data is important because any accuracy comparisons of new or revised methods must include the currently accepted regulatory test methods (i.e., in this case, the LLNA, and the GPMT and/or BT), as well as comparison to available human data and/or experience. Substances must also be readily available from commercial sources. Further limiting the list of substances to those that are readily available commercially reduced the list from 97 to 81 candidate substances. **Table 1** provides a breakdown of the impact that specific criteria had the list of candidate substances.

Table 1. Impact of Selection Criteria on Candidate List

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

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

The candidate list was then reduced to a candidate list of 40 substances taking into consideration, where feasible, the following criteria:

- Maintainance of similar performance statistics to those achieved in the original validation report
- Availability of human data
- Approximately equal distribution of solids and liquids
- An adequate range of responses in the LLNA based on EC3[7] and Stimulation Index (SI) values.
- Consideration of substances used in the Japanese Center for the Validation of Alternative Methods (JaCVAM) validation studies (12 substances) and in the draft performance standards proposed by the European Centre for the Validation of Alternative Methods (ECVAM) LLNA (14 substances).

The candidate list and characteristics of this candidate list are provided in **Appendix C1**[8].

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

[8] Comparative GPMT or BT data generated using a standardized protocol or human data were unavailable for six of the 14 substances proposed in the draft ECVAM performance standards and two of the 12 substances used in the

A list of 20 proposed reference substances was then selected from the list of 40 candidate substances (see **Appendix C2**). This list was based on the same criteria for selection listed above. **Table 1** provides the distribution of responses for the substances in the proposed reference list. The number of substances that have concurrent human data (i.e., human maximization test (HMT) data; included as part of a human patch test allergen (HPTA) kit; clinical case studies) also is provided. While the selection criteria included the availability of human data whenever possible, one substance without such data was included in order to maintain the desired performance statistics, dynamic range of responses, and range of physical and chemical characteristics.

JaCVAM validation study. Therefore, these substances were not included on the candidate list. All of the remaining substances (eight from the ECVAM list and 10 from the JaCVAM list) were included in the candidate list.

Table 1. Distribution of Substances and Available Human Data for the 20 Proposed Reference Substances

LLNA	GPMT/BT	No.	No. w/ HMT, HPTA, or Other Human Data[1]	HMT only	HPTA only	Both HMT and HPTA	Other Human Data[1]
+	+	11	10	1	3	4	1
+	-	2	2	1	1	0	0
-	+	2	2	0	0	1	1
-	-	5	5	2	1	1	1
Totals		20	19	4	5	6	3

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

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

Table 2 provides a breakdown of the various characteristics of the proposed list of 20 substances, including EC3 ranges, physical form information, and peptide reactivity.

985 **Table 2. Characteristics of the Proposed List of Reference Chemicals**

Potency Category[1] (EC3 range)	No. Chems	Solid/ Liquid	EC3 Range	SI Range	Human Data	Peptide Reactivity (High/Mod/Min/Unk)[2]	ECVAM/JaCVAM/ Both?
Extreme (<0.1)	2	1/1	0.0099-.05	52.3	1	1/0/0/1	0/1/0
Strong (≥0.1 to <1)	2	1/1	0.16-0.61	4-6.6	2	1/0/0/1	0/1/0
Moderate (≥1 to <10)	4	1/3	1.2-3	8.6-18.1	4	3/0/0/1	1/0/1
Weak (≥10 to <100)	5	2/3	11-28	3.5-17	5	1/1/1/2	2/0/1
Negative	7	4/3	-	0.9-2.5	7	0/0/3/4	0/2/1
Overall	**20**	**9/11**	**0.0099-28**	**0.9-52.3**	**19**	**6/1/4/9**	**3/4/3**

986 Abbreviations: Chems = Chemicals; ECVAM = European Centre for the Validation of Alternative Methods; JaCVAM = Japanese Center for the Validation of
987 Alternative Methods; No. = Number; Min = Minimal; Mod = Moderate; SI = Stimulation Index; Unk = Unknown.
988 [1]Proposed potency categories based on EC3 values as proposed by Gerberick et al. (2004)
989 [2]Data obtained from: Gerberick et al. Tox Sci Advance Access. March 2007.

A comparison of the chemicals on the ECVAM and JaCVAM proposed lists with those included on the ICCVAM candidate list, and the proposed reference chemicals list is provided in **Table 3**.

Table 3. Comparison of Chemicals on the Proposed ECVAM, JaCVAM, ICCVAM Candidate List (40 Chemicals), and Draft ICCVAM Reference Chemicals List (20 Chemicals)

Chemical	ECVAM (N=14)	JaCVAM (N=12)	ICCVAM – Candidate List (N=40)	ICCVAM – Reference Substances List (N=20)
Abietic acid		X	X	
4-Aminobenzoic acid			X	
3-Aminophenol		X		
Benzoquinone			X	X
Benzoyl peroxide			X	
Chloramine T			X	
Cinnamic alcohol	X			
Cinnamic aldehyde			X	X
Citral	X		X	X
Cobalt chloride		X	X	
Copper chloride			X	
Diethyl maleate	X		X	
Diethylenetriamine			X	
Dihydroeugenol			X	
Dimethyl isophthalate		X	X	
DNCB		X	X	X
Ethyl acrylate	X			
Ethylene glycol dimethacrylate			X	X
Eugenol	X		X	
Formaldehyde		X	X	X
Glutaraldehyde		X		
Glycerol	X			
Glyoxal			X	
HCA	X	X	X	X
2-Hydroxyethyl acrylate			X	X
Hexane	X			
Imidazolidinyl urea	X		X	X
Isoeugenol	X	X	X	X
Isophorone diisocyanate				
Isopropanol		X	X	X
Lactic acid	X			
2-Mercaptobenzothialzole	X		X	X
Mercuric chloride			X	
4-Methylaminophenol			X	

Chemical	ECVAM (N=14)	JaCVAM (N=12)	ICCVAM – Candidate List (N=40)	ICCVAM – Reference Substances List (N=20)
sulfate				
Methyl salicylate	X	X	X	X
Nickel chloride			X	X
Nickel sulfate		X	X	X
Phenyl benzoate	X		X	
4-Phenylenediamine			X	X
Potassium dichromate			X	
Propylene glycol			X	
Propylparaben			X	
Salicylic acid			X	X
Sodium lauryl sulfate			X	X
Sulfanilamide			X	X
Sulfanilic acid			X	
Tetrachlorosalicylanilide			X	
Tween 80			X	X
Total Number of Chemicals	**14**	**12**	**40**	**20**

Abbreviations: DNCB = 2, 4-Dinitrochlorobenzene; ECVAM = European Centre for the Validation of Alternative Methods; HCA = Hexyl Cinnamic Aldehyde; ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods; JaCVAM = Japanese Center for the Validation of Alternative Methods.

The proposed list of substances includes an adequate number of correctly identified sensitizers, nonsensitizers, false positives, and false negatives, as well as a range of physicochemical properties (e.g., distribution of solids and liquids) to provide meaningful data relevant to the wide range of substances associated with this type of testing. Some of the 20 substances in the proposed reference list lacked data on peptide reactivity and/or from human testing in order to satisfy other criteria for selection or meet specific goals. For example, nickel sulfate is included on the reduced list of 20 chemicals, despite the lack of SI data, because it belongs to a chemical class (metal salts) that is not correctly identified by the traditional LLNA. This provides the opportunity for superior performance to be demonstrated by a modified LLNA.

In development of the reference chemical list, two additional chemicals on the proposed ECVAM reference substances list and three additional chemicals on the proposed JaCVAM validation chemicals list were excluded. The ECVAM chemicals and one JaCVAM chemical (see **Table 4**) that were not included were moderate sensitizers (EC3 at least 1% and lower than 10%) that were correctly identified by LLNA. These chemicals were excluded from the reference chemicals list since inclusion of these chemicals would have altered the performance characteristics (addition of 3 correctly identified positives) significantly compared to the LLNA. Replacing chemicals on the list with the ECVAM and JaCVAM chemicals would have excluded chemicals identified as false positives, thus altering the performance characteristics. One correctly identified positive was excluded because EC3 and SI data were unavailable.

The remaining JaCVAM chemical (a correctly identified negative) was not included since comparative human data was not available. In the current reference chemicals list, all but one of

the correctly identified negative substances has comparative human data. The only correctly identified negative chemical without human data included on the reference chemicals list was isopropanol, which was included because it was part of the JaCVAM validation study.

Table 4. Rationale for Exclusion of ECVAM and JaCVAM Substances from Reduced List

Chemical	ECVAM	JaCVAM	LLNA Identification	Rationale for Exclusion
Abietic Acid		X	Correctly identified moderate sensitizer	Sufficient number of correctly identified moderate sensitizers currently included on list
Cobalt Chloride		X	Correctly identified sensitizer	No EC3/SI data
Dimethyl isophthalate		X	Correctly identified non-sensitizer	No human data
Eugenol	X		Correctly identified moderate sensitizer	Sufficient number of correctly identified moderate sensitizers currently included on list
Phenyl Benzoate	X		Correctly identified moderate sensitizer	Sufficient number of correctly identified moderate sensitizers currently included on list

Abbreviations: ECVAM = European Centre for the Validation of Alternative Methods; JaCVAM = Japanese Center for the Validation of Alternative Methods.

Searches and requests for BT, GPMT, and/or human data for those substances from the JaCVAM and ECVAM chemical lists that were excluded are ongoing.

References

Gerberick GF, Ryan CA, Kern PS, Dearman RJ, Kimber I, Patlewicz GY, Basketter DA. 2004. A chemical dataset for evaluation of alternative approaches to skin-sensitization testing. Contact Dermatitis. 50:274-288.

APPENDIX C1

Candidate List

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CANDIDATE LIST

Chemical Name	CASRN	MW (g/mol)	Physical Form	LLNA	Veh[1]	EC3[2]	SI (Conc)	GPMT/ BT[3]	HMT	HPTA	Additional Human Skin Sensitization Data/Information	Peptide Reactivity[4]
Abietic acid	514-10-3	302.45	Solid	+	AOO	15	5.2 (25%)	+		+		
4-Aminobenzoic acid	150-13-0	137.136	Solid	-				-	-	+		
Benzoquinone	106-51-4	108.095	Solid	+	AOO	0.0099	52.3 (2.5%)	+				High
Benzoyl peroxide	94-36-0	242.227	Solid	+		0.30[5]		+		+	DSA05HRIPT= 895; DSA05HMT=987	High
Chloramine T	149358-73-6	227.644	Solid	+		0.40[5]		+		+		
Cinnamic aldehyde	104-55-2	132.159	Liquid	+	AOO	3	15.8 (10%)	+	+	+	DSA05HRIPT=639; DSA05HMT=216	High
Citral	5392-40-5	152.233	Liquid	+	AOO	13	6.3 (25%)	+	+		DSA05HRIPT= 1266; DSA05HMT=862; DSA(NOEL)HRIPT =775	
Cobalt chloride	7646-79-9	129.839	Solid	+				+	+	+		
Copper chloride	7758-89-6	98.9987	Solid	+				-				

CANDIDATE LIST

Chemical Name	CASRN	MW (g/mol)	Physical Form	LLNA	Veh[1]	EC3[2]	SI (Conc)	GPMT/ BT[3]	HMT	HPTA	Additional Human Skin Sensitization Data/Information	Peptide Reactivity[4]
Diethylenetriamine	111-40-0	103.166	Liquid	+	AOO	5.8	12.1 (25%)	+	+	+	DSA05HMT=411	
Dihydroeugenol	2785-87-7	166.217	Liquid	+	AOO	6.8	7.8 (25.3%)	+				
Dimethyl isophthalate	1459-93-4	194.184	Solid	-				-				
2,4-Dinitrochlorobenzene	97-00-7	202.552	Liquid	+	AOO	0.05		+			Results from patch test studies indicate substance produces skin sensitization[7]	
Ethylene glycol dimethacrylate	97-90-5	198.216	Liquid	+	MEK	28	7 (50%)	-		+		High
Eugenol	97-53-0	164.201	Liquid	+	AOO	13	5.5 (25%)	+		+	DSA05HRIPT= 5926; DSA(NOEL)HRIPT =5905	
Formaldehyde	50-00-0	30.026g	Liquid	+	Ac	0.61	4.0 (1.85%)	+	+	+	DSA05HRIPT=411; DSA05HMT=89; DSA(NOEL)HRIPT =37	Moderate
Glyoxal	107-22-2	58.0361	Liquid	+	AOO	1.4	15.8 (25%)	+	+		DSA05HMT=345	High

CANDIDATE LIST

Chemical Name	CASRN	MW (g/mol)	Physical Form	LLNA	Veh[1]	EC3[2]	SI (Conc)	GPMT/ BT[3]	HMT	HPTA	Additional Human Skin Sensitization Data/Information	Peptide Reactivity[4]
Hexyl cinnamic aldehyde	101-86-0	216.319	Liquid	+	AOO	11	17 (50%)	+			DSA(NOEL)HRIPT =23622	Minimal
2-Hydroxyethyl acrylate	818-61-1	116.115	Liquid	+	AOO	1.4	18.1 (25%)	+		+		High
Imidazolidinyl urea	39236-46-9	388.294	Solid	+	DMF	24	5.5 (50%)	+		+	DSA05HRIPT= 3846; DSA(NOEL)HRIPT =2000	Moderate
Isoeugenol	97-54-1	164.201	Liquid	+	AOO	1.2	12.4 (5%)	+		+	DSA05HRIPT=657; DSA(NOEL)HRIPT =250	
Isophorone diisocyanate	4098-71-9	222.284	Liquid	+				+		+		
Isopropanol	67-63-0	60.095	Liquid	-	AOO	NC	1.0 (50%)	-			Studies indicate substance produces skin sensitization[8]	Minimal
2-Mercaptobenzothiazole	149-30-4	167.253	Solid	+	DMF	1.7[6]	8.6 (10%)	+	+	+	DSA05HMT=2269	High
Mercuric chloride	7487-94-7	271.495	Solid	+				+	+	+		

CANDIDATE LIST

Chemical Name	CASRN	MW (g/mol)	Physical Form	LLNA	Veh[1]	EC3[2]	SI (Conc)	GPMT/ BT[3]	HMT	HPTA	Additional Human Skin Sensitization Data/Information	Peptide Reactivity[4]
4-Methylaminophenol sulfate	55-55-0	344.384	Solid	+	DMF	0.8	6.7 (2.5%)	+		+		High
Methyl salicylate	119-36-8	152.147	Liquid	-	AOO	NC	0.9 (20%)	-	-			Minimal
Nickel chloride	7718-54-9	129.599	Solid	-	DMSO	NC	2.4 (5%)	+			Results from patch test studies indicate substance produces skin sensitization[9]	
Nickel sulfate	10101-98-1	280.864	Solid	-	DMSO	NC		+	+	+		
Phenyl benzoate	93-99-2	198.217	Solid	+	AOO	20	3.5 (25%)	+			DSA(NOEL)HRIPT =9448	
4-Phenylenediamine	106-50-3	108.141	Solid	+	AOO	0.16	6.6 (1%)	+	+	+	DSA05HRIPT=6.9; DSA05HMT=16.4; DSA(NOEL)HRIPT =10	

CANDIDATE LIST

Chemical Name	CASRN	MW (g/mol)	Physical Form	LLNA	Veh[1]	EC3[2]	SI (Conc)	GPMT/ BT[3]	HMT	HPTA	Additional Human Skin Sensitization Data/Information	Peptide Reactivity[4]
Potassium dichromate	7778-50-9	294.185	Solid	+	DMSO	0.08	16.1 (0.5%)	+	+	+	DSA05HMT=111	
Propylene glycol	57-55-6	76.0944	Liquid	-	dH2O	NC	16 (100%)	-		+		Minimal
Propylparaben	94-13-3	180.2	Solid	-	AOO	NC	1.3 (25%)	-	+/-	+		Minimal
Salicylic acid	69-72-7	138.121	Solid	-	AOO	NC	2.5 (25%)	-	-			
Sodium lauryl sulfate	151-21-3	288.38	Solid	+	DMF	14	3.5 (20%)	-	-			
Sulfanilamide	63-74-1	172.206	Solid	-	DMF	NC	0.9 (50%)	-	+	+		Minimal
Sulfanilic acid	121-57-3	173.191	Solid	-	DMF	NC	2.2 (25%)	+				Minimal

CANDIDATE LIST

Chemical Name	CASRN	MW (g/mol)	Physical Form	LLNA	Veh[1]	EC3[2]	SI (Conc)	GPMT/ BT[3]	HMT	HPTA	Additional Human Skin Sensitization Data/Information	Peptide Reactivity[4]
Tetrachlorosalicylanilide	1154-59-2	351.011	Solid	+	Ac	0.04	18.0 (1%)	+	+	+	DSA05HMT=14.4	Moderate
Tween 80	9005-65-6	604.813	Liquid	-	AOO	NC[5]		-		+		

Abbreviations: Ac = Acetone; AOO = Acetone:OliveOil; BT = Buehler Test; CASRN = Chemical Abstract Service Registry Number; DMF = Dimethylformamide; DMSO = Dimethyl sulfoxide; GPMT = Guinea Pig Maximization Test; HMT = Human Maximization Test; HPTA = Human Patch Test Allergan; LLNA = Local Lymph Node Assay; MEK = methyl ethyl ketone; MW = Molecular Weight; SI = Stimulation Index; Veh = Vehicle.

[1]Vehicle information obtained from: Gerberick et al, 2005. Compilation of historical Local Lymph Node data for evaluation of skin sensitization alternative methods. Dermatitis. 16:157-202.

[2] Unless noted otherwise, EC3 values obtained from: Gerberick et al, 2005. Compilation of historical Local Lymph Node data for evaluation of skin sensitization alternative methods. Dermatitis. 16:157-202,

[3]Results obtained from guinea pig maximization test and Buehler test.

[4]Peptide reactivity data obtained from: Gerberick et al. Quantification of chemical peptide reactivity for screening contact allergens: A classification tree model approach. Tox Sci Advance Access (March 30, 2007).

[5]EC3 values obtained from: Basketter and Kimber, 2006. Predictive test for irritants and allergens and their use in quantitative risk assessment. In "Contact Dermatitis", 4th Edition. Eds, Frosch P J, Menné T and Lepoittevin J-P, Springer Verlag, Heidelberg, pp 179 – 188.

[6]EC3 values obtained from: Kimber et al. 2003. Classification of contact allergens according to potency: proposals. Food Chem Toxicol. 41:1799-1809. 3

[7]Human data based on following studies: (1) Rees JL, Friedmann PS, Matthews JN. 1989. Sex differences in susceptibility to development of contact hypersensitivity to dinitrochlorobenzene (DNCB). Br J Dermatol. 120:371-374. (2) Zina AM, Bedello PG, Cane D, Bundinio S, Benedetto A. 1987. Dermatitis in a rubber tyre factory. Contact Dermatitis. 17:17-20.

[8]Human data based on following study: Kwon JA, Lee MS, Kim MI, Park YM, Kim HO, Kim CW. 2003. Allergic contact dermatitis from dodecyldiaminoethylglycine and isopropyl alcohol in a commercial disinfectant swab. Contact Dermatitis. 48:339-340.

[9]Human data based on following study: Rasanen L, Mattila U, Kalimo K. 1999. Patch testing with nickel sulfate versus nickel chloride. Contact Dermatitis. 40:287-288.

APPENDIX C2

Proposed List of 20 Reference Substances

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TABLE 1. PROPOSED LIST OF 20 REFERENCE SUBSTANCES (Alphabetical Sort)

Chemical Name	CASRN	MW (g/mol)	Physical Form	LLNA	Veh[1]	EC3[2]	SI (Conc)	GPMT/BT[3]	HMT	HPTA	Additional Human Skin Sensitization Data/Information[4]	Peptide Reactivity[5]
Benzoquinone[6]	106-51-4	108.095	Solid	+	AOO	0.0099	52.3 (2.5%)	+				High
Cinnamic aldehyde	104-55-2	132.159	Liquid	+	AOO	3	15.8 (10%)	+	+	+	DSA05HRIPT=639; DSA05HMT=216	High
Citral	5392-40-5	152.233	Liquid	+	AOO	13	6.3 (25%)	+	+		DSA05HRIPT=1266; DSA05HMT=862; DSA(NOEL)HRIPT=775	
2,4-Dinitrochlorobenzene[6]	97-00-7	202.552	Liquid	+	AOO	0.05		+			Results from patch test studies indicate substance produces skin sensitization[11]	
Ethylene glycol dimethacrylate	97-90-5	198.216	Liquid	+	MEK	28	7 (50%)	-		+		High
Formaldehyde	50-00-0	30.026	Liquid	+	Ac	0.61	4.0 (1.85%)	+	+	+	DSA05HRIPT=411; DSA05HMT=89; DSA(NOEL)HRIPT=37	Moderate
Hexyl cinnamic aldehyde[6]	101-86-0	216.319	Liquid	+	AOO	11	17 (50%)	+			DSA(NOEL)HRIPT=23622	Minimal
2-Hydroxyethyl acrylate	818-61-1	116.115	Liquid	+	AOO	1.4	18.1 (25%)	+		+		High

TABLE 1. PROPOSED LIST OF 20 REFERENCE SUBSTANCES (Alphabetical Sort)

Chemical Name	CASRN	MW (g/mol)	Physical Form	LLNA	Veh[1]	EC3[2]	SI (Conc)	GPMT/BT[3]	HMT	HPTA	Additional Human Skin Sensitization Data/Information[4]	Peptide Reactivity[5]
Imidazolidinyl urea	39236-46-9	388.294	Solid	+	DMF	24	5.5 (50%)	+		+	DSA05HRIPT=3846; DSA(NOEL)HRIPT=2000	Moderate
Isoeugenol	97-54-1	164.201	Liquid	+	AOO	1.2	12.4 (5%)	+		+	DSA05HRIPT=657; DSA(NOEL)HRIPT=250	
Isopropanol	67-63-0	60.095	Liquid	-	AOO	NC	1.0 (50%)	-			Studies indicate substance produces skin sensitization[12]	Minimal
2-Mercaptobenzothiazole	149-30-4	167.253	Solid	+	DMF	1.7[7]	8.6 (10%)	+	+	+	DSA05HMT=2269	High
Methyl salicylate	119-36-8	152.147	Liquid	-	AOO	NC	0.9 (20%)	-	-			Minimal
Nickel chloride	7718-54-9	129.599	Solid	-	DMSO[8]	NC	2.4 (5%)	+			Results from patch test studies indicate substance produces skin sensitization[13]	
Nickel sulfate	10101-98-1	280.864	Solid	-	DMSO[8]	NC		+	+	+		

TABLE 1. PROPOSED LIST OF 20 REFERENCE SUBSTANCES (Alphabetical Sort)

Chemical Name	CASRN	MW (g/mol)	Physical Form	LLNA	Veh[1]	EC3[2]	SI (Conc)	GPMT/BT[3]	HMT	HPTA	Additional Human Skin Sensitization Data/Information[4]	Peptide Reactivity[5]
4-Phenylenediamine	106-50-3	108.14	Solid	+	AOO	0.16	6.6 (1.0%)	+	+	+	DSA05HMT=111	
Salicylic acid	69-72-7	138.121	Solid	-	AOO	NC	2.5 (25%)	-	-			
Sodium lauryl sulfate	151-21-3	288.38	Solid	+	DMF	14	3.5 (20%)	-	-			
Sulfanilamide	63-74-1	172.20	Solid	-	DMF	NC	0.9 (50%)	-	+	+		Minimal
Tween 80	9005-65-6	604.81	Liquid	-	AOO[9]	NC[10]		-		+		

Abbreviations: Ac = Acetone; AOO = Acetone:OliveOil; BT = Buehler Test; CASRN = Chemical Abstract Service Registry Number; DMF = Dimethylformamide; DMSO = Dimethyl sulfoxide; GPMT = Guinea Pig Maximization Test; HMT = Human Maximization Test; HPTA = Human Patch Test Allergan; LLNA = Local Lymph Node Assay; MEK = methyl ethyl ketone; MW = Molecular Weight; NC = Not Calculated SI = Stimulation Index; Veh = Vehicle.

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[4]Human Quantitative Data obtained from literature where human data was compared to LLNA. All data are expressed as dose per skin area (μg/cm^2). DSA05HMT and DSA05HRIPT were obtained by linear interpolation from the lowest observed effect level to a dose corresponding to the estimated sensitization incidence of 5% (Schneider K, Akkan Z. 2004. Quantitative relationship between the local lymph node assay and human skin sensitization assays. Reg Toxicol Pharmacol. 39:245-255). DSA (NOEL) refers to

TABLE 1. PROPOSED LIST OF 20 REFERENCE SUBSTANCES (Alphabetical Sort)

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 D, Clapp C, Jeffries D, Safford B, Ryan C, Gerberick F, Dearman R, Kimber I. 2005. Predictive identification of human skin sensitization thresholds. Contact Dermatitis. 53:260-267.)

[5]Peptide reactivity data obtained from: Gerberick et al. Quantification of chemical peptide reactivity for screening contact allergens: A classification tree model approach. Tox Sci Advance Access (March 30, 2007).

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[12]Human data based on following study: Kwon JA, Lee MS, Kim MI, Park YM, Kim HO, Kim CW. 2003. Allergic contact dermatitis from dodecyldiaminoethylglycine and isopropyl alcohol in a commercial disinfectant swab. Contact Dermatitis. 48:339-340.

[13]Human data based on following study: Rasanen L, Mattila U, Kalimo K. 1999. Patch testing with nickel sulfate versus nickel chloride. Contact Dermatitis. 40:287-288.

TABLE 2. PROPOSED LIST OF 20 REFERENCE SUBSTANCES (EC3 Sort)

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Chemical Name	CASRN	MW (g/mol)	Physical Form	LLNA	Veh[1]	EC3[2]	SI (Conc)	GPMT/BT[3]	HMT	HPTA	Additional Human Skin Sensitization Data/Information[4]	Peptide Reactivity[5]
Benzoquinone[6]	106-51-4	108.095	Solid	+	AOO	0.0099	52.3 (2.5%)	+				High
2,4-Dinitrochlorobenzene[6]	97-00-7	202.552	Liquid	+	AOO	0.05		+			Results from patch test studies indicate substance produces skin sensitization[11]	
4-Phenylenediamine	106-50-3	108.14	Solid	+	AOO	0.16	6.6 (1.0%)	+	+	+	DSA05HMT=111	
Formaldehyde	50-00-0	30.026	Liquid	+	Ac	0.61	4.0 (1.85%)	+	+	+	DSA05HRIPT=411; DSA05HMT=89; DSA(NOEL)HRIPT=37	Moderate
Isoeugenol	97-54-1	164.201	Liquid	+	AOO	1.2	12.4 (5%)	+		+	DSA05HRIPT=657; DSA(NOEL)HRIPT=250	
2-Hydroxyethyl acrylate	818-61-1	116.115	Liquid	+	AOO	1.4	18.1 (25%)	+		+		High
2-Mercaptobenzothiazole	149-30-4	167.253	Solid	+	DMF	1.7[7]	8.6 (10%)	+	+	+	DSA05HMT=2269	High
Cinnamic aldehyde	104-55-2	132.159	Liquid	+	AOO	3	15.8 (10%)	+	+	+	DSA05HRIPT=639; DSA05HMT=216	High

TABLE 2. PROPOSED LIST OF 20 REFERENCE SUBSTANCES (EC3 Sort)

Chemical Name	CASRN	MW (g/mol)	Physical Form	LLNA	Veh[1]	EC3[2]	SI (Conc)	GPMT/BT[3]	HMT	HPTA	Additional Human Skin Sensitization Data/Information[4]	Peptide Reactivity[5]
Hexyl cinnamic aldehyde[6]	101-86-0	216.319	Liquid	+	AOO	11	17 (50%)	+			DSA(NOEL)HRIPT=23622	Minimal
Citral	5392-40-5	152.233	Liquid	+	AOO	13	6.3 (25%)	+	+		DSA05HRIPT=1266; DSA05HMT=862; DSA(NOEL)HRIPT=775	
Sodium lauryl sulfate	151-21-3	288.38	Solid	+	DMF	14	3.5 (20%)	-	-			
Imidazolidinyl urea	39236-46-9	388.294	Solid	+	DMF	24	5.5 (50%)	+		+	DSA05HRIPT=3846; DSA(NOEL)HRIPT=2000	Moderate
Ethylene glycol dimethacrylate	97-90-5	198.216	Liquid	+	MEK	28	7 (50%)	-		+		High
Nickel chloride	7718-54-9	129.599	Solid	-	DMSO[8]	NC	2.4 (5%)	+			Results from patch test studies indicate substance produces skin sensitization[13]	
Nickel sulfate	10101-98-1	280.864	Solid	-	DMSO[8]	NC		+	+	+		

TABLE 2. PROPOSED LIST OF 20 REFERENCE SUBSTANCES (EC3 Sort)

Chemical Name	CASRN	MW (g/mol)	Physical Form	LLNA	Veh[1]	EC3[2]	SI (Conc)	GPMT/BT[3]	HMT	HPTA	Additional Human Skin Sensitization Data/Information[4]	Peptide Reactivity[5]
Isopropanol	67-63-0	60.095	Liquid	-	AOO	NC	1.0 (50%)	-			Studies indicate substance produces skin sensitization[12]	Minimal
Methyl salicylate	119-36-8	152.147	Liquid	-	AOO	NC	0.9 (20%)	-	-			Minimal
Salicylic acid	69-72-7	138.121	Solid	-	AOO	NC	2.5 (25%)	-	-			
Sulfanilamide	63-74-1	172.20	Solid	-	DMF	NC	0.9 (50%)	-	+	+		Minimal
Tween 80	9005-65-6	604.81	Liquid	-	AOO[9]	NC[10]		-		+		

Abbreviations: Ac = Acetone; AOO = Acetone:OliveOil; BT = Buehler Test; CASRN = Chemical Abstract Service Registry Number; DMF = Dimethylformamide; DMSO = Dimethyl sulfoxide; GPMT = Guinea Pig Maximization Test; HMT = Human Maximization Test; HPTA = Human Patch Test Allergan; LLNA = Local Lymph Node Assay; MEK = methyl ethyl ketone; MW = Molecular Weight; NC = Not Calculated SI = Stimulation Index; Veh = Vehicle.

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[5]Peptide reactivity data obtained from: Gerberick et al. Quantification of chemical peptide reactivity for screening contact allergens: A classification tree model approach. Tox Sci Advance Access (March 30, 2007).

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

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

[9]Vehicle information obtained from: Basketter et al. 2000. Use of the local lymph node assay for the estimation of relative contact allergenic potency. Contact Dermatitis 42(6):344-348.

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[12]Human data based on following study: Kwon JA, Lee MS, Kim MI, Park YM, Kim HO, Kim CW. 2003. Allergic contact dermatitis from dodecyldiaminoethylglycine and isopropyl alcohol in a commercial disinfectant swab. Contact Dermatitis. 48:339-340.

[13]Human data based on following study: Rasanen L, Mattila U, Kalimo K. 1999. Patch testing with nickel sulfate versus nickel chloride. Contact Dermatitis. 40:287-288.

TABLE 3. PROPOSED LIST OF 20 REFERENCE SUBSTANCES (SI Sort)

Chemical Name	CASRN	MW (g/mol)	Physical Form	LLNA	Veh[1]	EC3[2]	SI (Conc)	GPMT/BT[3]	HMT	HPTA	Additional Human Skin Sensitization Data/Information[4]	Peptide Reactivity[5]
Methyl salicylate	119-36-8	152.147	Liquid	-	AOO	NC	0.9 (20%)	-	-			Minimal
Sulfanilamide	63-74-1	172.20	Solid	-	DMF	NC	0.9 (50%)	-	+	+		Minimal
Isopropanol	67-63-0	60.095	Liquid	-	AOO	NC	1.0 (50%)	-			Studies indicate substance produces skin sensitization[12]	Minimal
Nickel chloride	7718-54-9	129.599	Solid	-	DMSO[8]	NC	2.4 (5%)	+			Results from patch test studies indicate substance produces skin sensitization[13]	
Salicylic acid	69-72-7	138.121	Solid	-	AOO	NC	2.5 (25%)	-	-			
Sodium lauryl sulfate	151-21-3	288.38	Solid	+	DMF	14	3.5 (20%)	-	-			
Formaldehyde	50-00-0	30.026	Liquid	+	Ac	0.61	4.0 (1.85%)	+	+	+	DSA05HRIPT=411; DSA05HMT=89; DSA(NOEL)HRIPT=37	Moderate

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Chemical Name	CASRN	MW (g/mol)	Physical Form	LLNA	Veh[1]	EC3[2]	SI (Conc)	GPMT/BT[3]	HMT	HPTA	Additional Human Skin Sensitization Data/Information[4]	Peptide Reactivity[5]
Imidazolidinyl urea	39236-46-9	388.294	Solid	+	DMF	24	5.5 (50%)	+		+	DSA05HRIPT=3846; DSA(NOEL)HRIPT=2000	Moderate
Citral	5392-40-5	152.233	Liquid	+	AOO	13	6.3 (25%)	+	+		DSA05HRIPT=1266; DSA05HMT=862; DSA(NOEL)HRIPT=775	
4-Phenylenediamine	106-50-3	108.14	Solid	+	AOO	0.16	6.6 (1.0%)	+	+	+	DSA05HMT=111	
Ethylene glycol dimethacrylate	97-90-5	198.216	Liquid	+	MEK	28	7 (50%)	-		+		High
2-Mercaptobenzothiazole	149-30-4	167.253	Solid	+	DMF	1.7[6]	8.6 (10%)	+	+	+	DSA05HMT=2269	High
Isoeugenol	97-54-1	164.201	Liquid	+	AOO	1.2	12.4 (5%)	+		+	DSA05HRIPT=657; DSA(NOEL)HRIPT=250	
Cinnamic aldehyde	104-55-2	132.159	Liquid	+	AOO	3	15.8 (10%)	+	+	+	DSA05HRIPT=639; DSA05HMT=216	High
Hexyl cinnamic aldehyde[7]	101-86-0	216.319	Liquid	+	AOO	11	17 (50%)	+			DSA(NOEL)HRIPT=23622	Minimal

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2-Hydroxyethyl acrylate	818-61-1	116.115	Liquid	+	AOO	1.4	18.1 (25%)	+		+		High
Benzoquinone[7]	106-51-4	108.095	Solid	+	AOO	0.0099	52.3 (2.5%)	+				High
2,4-Dinitrochlorobenzene[7]	97-00-7	202.552	Liquid	+	AOO	0.05		+			Results from patch test studies indicate substance produces skin sensitization[11]	
Nickel sulfate	10101-98-1	280.864	Solid	-	DMSO[8]	NC		+	+	+		
Tween 80	9005-65-6	604.81	Liquid	-	AOO[9]	NC[10]		-		+		

Abbreviations: Ac = Acetone; AOO = Acetone:OliveOil; BT = Buehler Test; CASRN = Chemical Abstract Service Registry Number; DMF = Dimethylformamide; DMSO = Dimethyl sulfoxide; GPMT = Guinea Pig Maximization Test; HMT = Human Maximization Test; HPTA = Human Patch Test Allergan; LLNA = Local Lymph Node Assay; MEK = methyl ethyl ketone; MW = Molecular Weight; NC = Not Calculated SI = Stimulation Index; Veh = Vehicle.

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