Draft Background Review Document

Murine Local Lymph Node Assay (LLNA) Limit Dose Procedure

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

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List of Abbreviations and Acronyms

ACD	Allergic contact dermatitis
AOO	Acetone: olive oil
BGIA	Berufsgenossenschaftliches Institut fur Arbeitsschutz (German Institute for Occupational Safety and Health)
BRD	Background Review Document
BT	Buehler Test
CASRN	Chemical Abstracts Service Registry Number
CESIO	Comite Europeen des Agents de Surface et de Leurs Intermediaires Organiques (European Committee of Surfactants and Their Organic Intermediates)
Conc.	Concentration tested
CPSC	U.S. Consumer Product Safety Commission
DMSO	Dimethyl sulfoxide
EC3	Estimated concentration needed to produce a stimulation index of three
ECPA	European Crop Protection Association
ECVAM	European Centre for the Validation of Alternative Methods
EFfCI	European Federation for Cosmetic Ingredients
EPA	U.S. Environmental Protection Agency
ESAC	ECVAM Scientific Advisory Committee
FDA	U.S. Food and Drug Administration
FR	*Federal Register*
GHS	United Nations Globally Harmonized System for the Labelling and Classification of Chemicals
GLP	Good Laboratory Practice
GPMT	Guinea Pig Maximization Test
GSK	GlaxoSmithKline
HCA	Hexyl cinnamic aldehyde
HPTA	Human Patch Test Allergen

ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IWG	Immunotoxicity Working Group
K_{ow}	Octanol-water partition coefficient
LLNA	Local Lymph Node Assay
MTSC	Multiply tested substances combined
NC	Not calculated
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences
OECD	Organisation for Economic Co-operation and Development
OPPTS	Office of Prevention, Pesticides and Toxic Substances
rLLNA	Reduced LLNA
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SI	Stimulation index
TG	Test guideline
TNO	TNO Nutrition and Food Research
U.K.	United Kingdom
U.N.	United Nations
U.S.	United States
w/v	Weight to volume ratio

Interagency Coordinating Committee on The Validation of Alternative Methods (ICCVAM) Designated Agency Representatives[1]

Agency for Toxic Substances and Disease Registry
• Moiz Mumtaz, Ph.D.

Consumer Product Safety Commission
• Marilyn L. Wind, Ph.D. (Chair)
◊ Kristina Hatlelid, Ph.D.
* Joanna Matheson, Ph.D.

Department of Agriculture
• Jodie Kulpa-Eddy, D.V.M. (Vice-Chair)
◊ Elizabeth Goldentyer, D.V.M.

Department of Defense
• Robert E. Foster, Ph.D.
◊ Patty Decot
* Peter J. Schultheiss, D.V.M., D.A.C.L.A.M.
* Harry Salem, Ph.D.

Department of Energy
• Michael Kuperberg, Ph.D.
◊ Marvin Stodolsky, Ph.D.

Department of the Interior
• Barnett A. Rattner, Ph.D.
◊ Sarah Gerould, Ph.D.

Department of Transportation
• George Cushmac, Ph.D.
◊ Steve Hwang, Ph.D.

Environmental Protection Agency
Office of Science Coordination and Policy
• Karen Hamernik, Ph.D.
Office of Research and Development
◊ Julian Preston, Ph.D.
* Suzanne McMaster, Ph.D.
OECD Test Guidelines Program
* Jerry Smrchek, Ph.D.
Office of Pesticides Programs
* Amy Rispin, Ph.D.
* Deborah McCall

Food and Drug Administration
Office of Science
• Suzanne Fitzpatrick, Ph.D., D.A.B.T.
Center for Drug Evaluation and Research
◊ Abigail C. Jacobs, Ph.D.
Center for Devices and Radiological Health
* Melvin E. Stratmeyer, Ph.D.
Center for Biologics Evaluation and Research
* Richard McFarland, Ph.D., M.D.
* Ying Huang, Ph.D.
Center for Food Safety and Nutrition
* David G. Hattan, Ph.D.
* Robert L. Bronaugh, Ph.D.
Center for Veterinary Medicine
* Devaraya Jagannath, Ph.D.
* M. Cecilia Aguila, D.V.M.
National Center for Toxicological Research
* William T. Allaben, Ph.D.
* Paul Howard Ph.D.
Office of Regulatory Affairs
* Lawrence A. D'Hoostelaere, Ph.D.

National Cancer Institute
• Alan Poland, M.D.
◊ T. Kevin Howcroft, Ph.D.

National Institute of Environmental Health Sciences
• William S. Stokes, D.V.M., D.A.C.L.A.M.
◊ Raymond R. Tice, Ph.D.
* Rajendra S. Chhabra, Ph.D., D.A.B.T
* Jerrold J. Heindel, Ph.D.

National Institute for Occupational Safety and Health
• Paul Nicolaysen, V.M.D.
◊ K. Murali Rao, M.D., Ph.D.

National Institutes of Health
• Margaret D. Snyder, Ph.D.

National Library of Medicine
◊ Jeanne Goshorn, M.S.

Occupational Safety and Health Administration
• Surender Ahir, Ph.D.

• Principal Agency Representative
◊ Alternate Principal Agency Representative
* Other Designated Agency Representative

[1] Roster as of January 2008.

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Interagency Coordinating Committee on the Validation on Alternative Methods (ICCVAM) Immunotoxicity Working Group (IWG)

U.S. Consumer Product Safety Commission
Joanna Matheson, Ph.D. (IWG Co-Chair)
Marilyn Wind, Ph.D.

U.S. Environmental Protection Agency
Karen Hamernik, Ph.D.
Masih Hashim, Ph.D.
Marianne Lewis
Deborah McCall
Timothy McMahon, Ph.D.
Amy Rispin, Ph.D
MaryJane Selgrade, Ph.D.
Marsha Ward, Ph.D.
Ronald Ward, Ph.D.

U.S. Food and Drug Administration
Ruth Barratt, Ph.D., D.V.M.
Paul Brown, Ph.D.
Abigail Jacobs, Ph.D. (IWG Co-Chair)
Daniel Lyle, Ph.D.
Jiaqin Yao, Ph.D.

National Institute of Environmental Health Sciences
Dori Germolec, Ph.D.
William S. Stokes, D.V.M., D.A.C.L.A.M.
Raymond R. Tice, Ph.D.

National Institute for Occupational Safety and Health
Jean Meade, D.V.M., Ph.D.

ECVAM Liaison
Silvia Casati, Ph.D.

JaCVAM Liaison
Hajime Kojima, Ph.D.

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

National Institute of Environmental Health Sciences

William Stokes, D.V.M., D.A.C.L.A.M.	Director; Project Officer
Raymond Tice, Ph.D.	Deputy Director
Deborah McCarley	Special Assistant; Asst. Project Officer

NICEATM Support Contract Staff (Integrated Laboratory Systems [ILS], Inc.)

David Allen, Ph.D.	Principal Investigator
Douglas Winters, M.S.	Project Manager
Neepa Choksi, Ph.D.	Senior Staff Toxicologist
Judy Strickland, Ph.D., D.A.B.T.	Senior Staff Toxicologist
Frank Deal, M.S.	Staff Toxicologist
Elizabeth Lipscomb, Ph.D.	Staff Toxicologist
Eleni Salicru, Ph.D.	Staff Toxicologist
Thomas Burns, M.S.	Senior Project Coordinator/Technical Writer
Michael Paris	Senior Project Coordinator/Technical Writer
Patricia Ceger, M.S.	Project Coordinator/Technical Writer
James Truax, M.A.	Project Coordinator/Technical Writer
Catherine Sprankle	Communications Specialist/Web Developer
Linda Litchfield	Meeting Planner and Coordinator

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David Basketter, Ph.D.
Unilever Safety and Environmental Assurance Centre
Sharnbrook, UK

Phil Botham, Ph.D.
European Crop Protection Association
Brussels, Belgium

Eric Debruyne, Ph.D.
Bayer CropScience SA, Sophia Antipolis Cedex, France

George DeGeorge, Ph.D. and Melissa Kirk, Ph.D.
MB Research Labs
Spinnerstown, PA, USA

G. Frank Gerberick, Ph.D.
Procter and Gamble Company
Cincinnati, OH

Dori Germolec, Ph.D.
National Toxicology Program
Research Triangle Park, NC, USA

Ian Kimber, Ph.D.
Syngenta Central Toxicology Laboratory
Macclesfield, UK

Michael J. Olson, Ph.D.
GlaxoSmithKline
Research Triangle Park, NC, USA

K. Skirda
TNO Quality of Life
Delft, Netherlands

Masahiro Takeyoshi, Ph.D.
Chemicals Evaluation and Research Institute
Oita, Japan

Peter Ungeheuer, Ph.D.
European Federation for Cosmetic Ingredients
Frankfurt, Germany

Hans Werner Vohr, Ph.D.
Bayer HealthCare
Wuppertal-Elberfeld, Germany

Preface

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

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

On January 10, 2007, the U.S. Consumer Product Safety Commission (CPSC) formally nominated several activities related to the LLNA for evaluation by ICCVAM and NICEATM[4]. One of the nominated activities was an assessment of the validation status of the ‘‘cut-down’’ or ‘‘limit dose’’ LLNA procedure (also known as the reduced LLNA). After considering comments from the public and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) on this nomination, ICCVAM assigned it a high priority, and directed NICEATM and the ICCVAM Immunotoxicity Working Group (IWG) to conduct a review of the current literature and an evaluation of the available data. The information described in this background review document (BRD) was compiled by ICCVAM in response to this nomination. ICCVAM and its IWG developed draft test method

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

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

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

recommendations based on this evaluation. An independent peer review panel (Panel) is being convened to peer review the BRD and to evaluate the extent to which the information contained in the BRD support the draft recommendations. ICCVAM will consider the conclusions and recommendations of the Panel, along with comments received from the public and SACATM, when developing a final BRD and final recommendations on the usefulness and limitations of the LLNA limit dose procedure.

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

Marilyn Wind, Ph.D.

Deputy Associate Executive Director

Directorate for Health Sciences

U.S. Consumer Product Safety Commission

Chair, ICCVAM

William S. Stokes, D.V.M., D.A.C.L.A.M.

Rear Admiral, U.S. Public Heath Service

Director, NICEATM

Executive Director, ICCVAM

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Executive Summary

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

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

On January 10, 2007, the U.S. Consumer Product Safety Commission (CPSC) formally nominated several activities related to the LLNA for evaluation by ICCVAM and the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) (Available at http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf). One of the nominated activities was an assessment of the usefulness and limitations of the LLNA limit dose procedure. The information described in this background review document (BRD) was compiled by ICCVAM and NICEATM in response to this nomination. The BRD provides a comprehensive review of available data and information regarding the use of the LLNA limit dose procedure for the purpose of hazard classification.

The information summarized in this BRD is based on a retrospective review of traditional LLNA data. The data reviewed includes the data on 211 substances originally provided for review of the traditional LLNA in 1998, as well as data on an additional 255 substances from the peer-reviewed literature and from data submitted to the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) in response to a 2007 *Federal Register* (*FR*) notice.

The protocol for the LLNA limit dose procedure is identical to that for the traditional LLNA, except for the number of test substance dose levels administered. A detailed LLNA protocol can be found in the ICCVAM test method evaluation report (ICCVAM 1999) and Dean et al. (2001). The LLNA procedure is also described in the EPA Health Effects Test Guidelines (EPA 2003) and a modified procedure is described in OECD TG 429 (OECD 2002). In the traditional LLNA, three dose levels are used with the highest concentration that which does not induce systemic toxicity and/or excessive skin irritation. The LLNA limit dose procedure uses only the single highest dose tested. Like the traditional LLNA, the threshold for classifying a substance as a skin sensitizer in the LLNA limit dose procedure is a Stimulation Index (SI) ≥ 3.

The data used in the evaluation of the LLNA limit dose procedure in this BRD were obtained from 11 different sources. Three sources were published journal articles and eight were responses to a *FR* notice requesting such data. Data were obtained from a total of 471 studies representing 466 unique substances.

Chemical classes for each substance were retrieved from the National Library of Medicine's ChemID Plus database, or assigned for each test substance using a standard classification scheme, based on the National Library of Medicine Medical Subject Headings classification system (available at http://www.nlm.nih.gov/mesh/meshhome.html). Chemical class information is included to provide an indication of the variety of structural elements present in the substances that were evaluated in this analysis, but it is not intended to suggest an impact of structure on sensitization potential. Certain complex substances (n = 125) were identified simply as pharmaceutical chemicals. Ten substances included in this evaluation were formulations. Seventy substances could not be assigned to a specific chemical class due to incomplete information (e.g., CASRN, structure).

The ability of the LLNA limit dose procedure to correctly identify potential skin sensitizers was compared to traditional LLNA results. In the 471 studies, 317 detected skin sensitizers and 154 detected. When substances tested multiple times in the same vehicle were combined to yield an overall skin sensitization classification, the number of substances evaluated was 466. Of these 466 substances, 313 were classified as sensitizers and 153 were classified as non-sensitizers.

Based on the available study data, the LLNA limit dose procedure has an accuracy of 98.9% (466/471), a sensitivity of 98.4% (312/317), a specificity of 100% (154/154), a false positive rate of 0% (0/154), and a false negative rate of 1.6% (5/317) when compared to the traditional LLNA. When unique substances were evaluated, the LLNA limit dose procedure has an accuracy of 98.9% (461/466), a sensitivity of 98.4% (308/313), a specificity of 100% (153/153), a false positive rate of 0% (0/153), and a false negative rate of 1.6% (5/313).

In this analysis, five substances were false negatives in the LLNA limit dose procedure. A review of the data for these five substances indicates that the traditional LLNA classification of the substances as skin sensitizers was not based on the highest tested dose, but on a low- or mid-dose level that produced an SI >3 (i.e., the highest dose tested for these five substances resulted in an SI <3) [The basis for selecting the concentrations tested is unknown, but this information has been requested]. Since the LLNA limit dose procedure only tests substances at the highest dose level, all five substances would be incorrectly identified as non-sensitizers (i.e., false negatives). There were no patterns of consistency for these substances with regard to physicochemical properties.

There were sufficient data for five substances to assess the interlaboratory reproducibility of the LLNA limit dose procedure. Based on the available data, 100% concordance in classification of substances as sensitizers or non-sensitizers was observed for 60% (3/5) of the substances. No additional studies were available to assess the reliability of the LLNA limit dose procedure. However, since the LLNA limit dose procedure and traditional LLNA use identical protocols, and the datasets used to evaluate the accuracy of the LLNA limit dose procedure and traditional LLNA are similar, the reliability of the two methods would be expected to be similar. That is, the intra- and inter-laboratory reliability of the LLNA limit

dose procedure would be expected to be the same as the traditional LLNA (see ICCVAM [1999] for these statistics).

A review of the published literature discussing the LLNA limit dose procedure revealed only one published report in addition to Kimber et al. (2006). Ryan et al. (2007) described the impact of reducing the number of animals per group from five to two on the performance of the limit dose LLNA and concluded that the sensitivity is inadequate for hazard identification of skin sensitizers.

Compared to the traditional LLNA, the LLNA limit dose procedure will reduce the number of animals used to assess skin sensitization. Since, in the LLNA limit dose procedure, only the highest dose level of the test substance is being evaluated in addition to the concurrent control groups, the number of animals tested would be decreased by at least 40%.

This BRD provides a comprehensive summary of the current validation status of the LLNA limit dose procedure test method, including information about its reliability and relevance, and the scope of the substances evaluated. The database included in this BRD will be updated as additional information becomes available during future use of the traditional LLNA and the LLNA limit dose procedure.

1.0 Introduction And Rationale for the Proposed Use of the Murine Local Lymph Node Assay (LLNA) Limit Dose Procedure to Identify Skin Sensitizers

1.1 Introduction

1.1.1 Historical Background

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

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

1.1.2 Allergic Contact Dermatitis

ACD is a frequent occupational health problem. According to the U.S. Department of Labor Bureau of Labor Statistics, in 2005, 980 cases of allergic dermatitis involved days away from work.

ACD develops in two phases, induction and elicitation. The induction phase occurs when a susceptible individual is exposed topically to a skin-sensitizing substance. Induction depends on the substance passing through the epidermis, where it forms a hapten complex with

dermal proteins. The hapten complex is processed by the Langerhans cells, the resident antigen-presenting cells in the skin. The processed hapten complex then migrates to the draining lymph nodes. Antigen presentation to T-lymphocytes follows, which leads to the clonal expansion of these cells. At this point, the individual is sensitized to the substance (Basketter et al. 2003; Jowsey et al. 2006). Studies have shown that the magnitude of lymphocyte proliferation correlates with the extent to which sensitization develops (Kimber and Dearman 1991; Kimber and Dearman 1996).

The elicitation phase occurs when the individual is again topically exposed to the same substance. As in the induction phase, the substance penetrates the epidermis, is processed by the Langerhans cells, and presented to circulating T-lymphocytes. The T-lymphocytes are then activated, which causes release of cytokines and other inflammatory mediators. This release produces a rapid dermal immune response that can lead to ACD (ICCVAM 1999; Basketter et al. 2003; Jowsey et al. 2006).

1.1.3 U.S. Consumer Product Safety Commission (CPSC) Nomination

On January 10, 2007, the CPSC formally nominated several activities related to the LLNA for evaluation by ICCVAM and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). The nominated activities were:

- An assessment of the validation status of the LLNA as a stand-alone assay for potency determination (including severity) for classification purposes
- An assessment of the validation status of non-radioactive LLNA protocols
- The ‘‘cut-down’’ or ‘‘limit dose’’ LLNA procedure (also known as the reduced LLNA)
- An assessment of the validation status of the use of the LLNA to test mixtures, aqueous solutions, and metals

ICCVAM unanimously agreed that the nominated activities should have a high priority for evaluation. ICCVAM’s advisory committee, the Scientific Advisory Committee on Alternative Toxicological Methods, also recommended that the nominated activities be undertaken, with a high priority.

As ICCVAM and NICEATM collaborate closely with the European Centre for the Validation of Alternative Methods (ECVAM) and the Japanese Center for the Validation of Alternative Methods, both organizations identified liaisons to the ICCVAM Immunotoxicity Working Group to facilitate the evaluations requested by the CPSC.

1.1.4 Description of the LLNA Limit Dose Procedure

The LLNA limit dose procedure was initially described in a paper by Kimber and colleagues (2006). The LLNA limit dose procedure was also discussed in two posters (Basketter et al. 2007; and Chaney et al. 2007, which was subsequently published as Ryan et al. 2007) and one platform presentation (Basketter 2007) presented at the Society of Toxicology Annual Meeting in Charlotte, NC, March 25-29, 2007.

The LLNA limit dose procedure is identical to the traditional LLNA (as described in ICCVAM 1999, Dean et al. 2001), with one exception. In the traditional LLNA, three dose levels of each test substance are tested while in the LLNA limit dose procedure, only the highest test substance dose level that does not induce systemic toxicity and/or excessive skin irritation is tested for skin sensitizing activity (Kimber et al. 2006).

1.1.5 Results of Peer Reviews on the LLNA Limit Dose Procedure

The LLNA limit dose procedure was reviewed by the ECVAM Scientific Advisory Committee (ESAC) meeting on April 26-27, 2007. Prior to the meeting, ESAC established a review panel to retrospectively analyze the published LLNA data to determine if limiting the number of test substance dose levels to the highest dose level only could successfully reduce the number of animals used per test. This review was based on the evaluation published in Kimber et al. (2006).

The ESAC statement on the LLNA limit dose procedure, dated April 27, 2007 (**Appendix A**), states:

" … that the peer reviewed and published information is of a quality and nature to support the use of the rLLNA within tiered-testing strategies to reliably distinguish between substances that are skin sensitisers and non-sensitisers, and that animal use can be minimised providing:

- The concentration used to evaluate sensitisation potential is the maximum consistent with solubility and the need to avoid local and other systemic

adverse effects, and that this principle rather than strict adherence to the specific recommended absolute concentrations as in OECD TG 429 should be used.

- Negative test results associated with testing using concentrations of less than 10% should undergo further evaluation.
- Positive and negative (vehicle) control groups are used, as appropriate, per ICCVAM (1999) and Dean et al. (2001).
- The full LLNA should be performed when it is known that an assessment of sensitisation potency is required."

The ESAC statement also recommends, "that further work should be undertaken to determine if the 10% concentration threshold referenced above is optimal."

1.2 Regulatory Rationale and Applicability

Current regulatory testing needs require the assessment of the potential skin sensitization hazard of regulated substances/products. The LLNA limit dose procedure is being considered for use in the identification of skin sensitizers in a weight-of-evidence strategy, such as proposed in the United Nations (U.N.) Globally Harmonized System of Classification and Labelling of Chemicals (GHS; U.N. 2005). Unlike the traditional LLNA, the LLNA limit dose procedure evaluates the ability of a substance to be a sensitizer based on testing a single dose level and therefore dose response information is not generated. The LLNA limit dose procedure is being proposed for "yes/no" sensitization hazard identification purposes.

1.3 Scientific Basis for the Test Method

1.3.1 Purpose and Mechanistic Basis of the Test Method

The purpose of the LLNA limit dose procedure is to identify potential skin sensitizers through quantification of lymphocyte proliferation. The mechanistic basis is identical to that of the traditional LLNA (see **Section 1.1.2**).

1.3.2 Applicability Domain

The applicability domain of the LLNA limit dose procedure should be identical to that of the traditional LLNA. The traditional LLNA was not recommended for identification of skin sensitizers that were classified as metals, mixtures/extracts, pharmaceuticals, and skin irritants (ICCVAM 1999).

1.4 Validation of the LLNA Limit Dose Procedure

The ICCVAM Authorization Act (Sec. 4(c)) mandates that "[e]ach Federal Agency … shall ensure that any new or revised … test method … is determined to be valid for its proposed use prior to requiring, recommending, or encouraging [its use]." (ICCVAM 2000).

Validation is the process by which the reliability and relevance of an assay for a specific purpose are established (ICCVAM 1997). Relevance is defined as the extent to which an assay will correctly predict or measure the biological effect of interest (ICCVAM 1997). For the LLNA limit dose procedure, relevance is determined by how well the assay identifies substances that are capable of producing skin sensitization. Reliability is defined as the reproducibility of a test method within and among laboratories. Reliability should be assessed by using the test method to evaluate a diverse set of substances that are representative both of the types of chemical and product classes to be tested and of the range of responses to be identified. The validation process provides data and information that allow U.S. Federal agencies to develop guidance on the use of test methods in evaluating the skin sensitization potential of substances.

The first stage in this evaluation is the preparation of a Background Review Document (BRD) that provides a comprehensive review of the relevant data and information about a test method, including its mechanistic basis, proposed uses, reliability, and performance characteristics (ICCVAM 1997). This BRD summarizes the available information on the LLNA limit dose procedure. If the data presented are considered insufficient to support the recommendation of a standardized protocol for the LLNA limit dose procedure, this BRD will aid in identifying essential test method components that should be considered during future development and validation activities.

1.5 Selection of Citations for the BRD

The test method data summarized in this BRD are based on information obtained both from the peer-reviewed scientific literature and from responses to a published *Federal Register* (*FR*) notice requesting such data (Vol. 72, No. 95, pp. 27815-27817, available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf). A review of the literature discussing the LLNA limit dose procedure revealed two published reports (Kimber et al. 2006 and Ryan et al. 2007), two posters (Basketter et al. 2007; and Chaney et al. 2007, which was subsequently published as Ryan et al. 2007) and one platform presentation (Basketter 2007) (see **Section 1.1.4**).

2.0 Test Method Protocol Components

2.1 Overview of the LLNA Limit Dose Procedure

The technical aspects of the LLNA limit dose procedure are identical to those of the traditional LLNA; the two methods differ only in the number of test substance dose levels tested (Kimber et al. 2006). In the LLNA limit dose procedure, in addition to the concurrent vehicle and positive control groups, each test substance is tested only at the highest dose level consistent with maximum solubility while avoiding systemic toxicity and excessive local irritation. In the traditional LLNA, each test substance is tested at a minimum of three dose levels.

A detailed LLNA protocol can be found in the ICCVAM test method evaluation report (ICCVAM 1999) and Dean et al. (2001). The LLNA procedure is also described in the EPA Health Effects Test Guidelines (EPA 2003) and a modified procedure is described in OECD TG 429 (OECD 2002).

A Stimulation Index (SI) is calculated as the ratio of radioactivity incorporated into the cells of auricular lymph nodes of the treated animals to that in the vehicle control animals. In the traditional LLNA, the threshold for classifying a substance as a skin sensitizer is an $SI \geq 3$.

2.2 Basis for Selection of the LLNA Limit Dose Procedure

The LLNA limit dose procedure was proposed by Kimber et al. (2006) in an effort to further reduce the number of animals used for skin sensitization testing.

2.3 Test Method Proprietary Components

The LLNA limit dose procedure does not employ any proprietary components.

2.4 Basis for the Number of Mice Per Dose Group

The basis for the number of mice per dose group is the same as that for the traditional LLNA (ICCVAM 1999, Dean et al. 2001).

2.5 Study Acceptance Criteria

In order for an LLNA study to be considered acceptable, the concurrent positive control must yield an SI ≥ 3 (ICCVAM 1999, Dean et al. 2001).

2.6 Basis for Selection of the Limit Dose Level

Consistent with the criteria for selecting the highest dose level in the traditional LLNA, the dose level used to evaluate sensitization potential using the LLNA limit dose procedure should be the maximum soluble concentration that does not cause systemic toxicity or excessive local irritation.

3.0 Substances Used for Validation of the LLNA Limit Dose Procedure

3.1 Rationale for the Substances or Products Included in the Evaluation

Data from a total of 471 LLNA studies were obtained from 11 different sources (**Table 3-1**), including published reports and unpublished data submitted to NICEATM in response to a *FR* notice (Vol. 72, No. 95, pp. 27815-27817, available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf).

3.2 Rationale for the Number of Substances Included in the Evaluation

As indicated in **Table 3-1**, data were obtained from a total of 471 studies representing 466 unique substances; 211 of these substances were included in the original ICCVAM evaluation of the traditional LLNA (ICCVAM 1999). Among these 471 studies, there were nine substances that were evaluated two or more times in different vehicles and three substances evaluated two or more times in the same vehicle. Additionally, there were two substances (hexyl cinnamic aldehyde [HCA] and potassium dichromate) where at least two of the studies were conducted using the same vehicle and the remaining studies (one for HCA and two for potassium dichromate) were conducted using different vehicles.

Table 3-1 Summary of Data Sources and Rationale for Substance Selection

Data Source	Number of Studies	Primary Data Source and Substance Selection Rationale
Gerberick et al. (2005)[1]	210	Compiled from previously conducted studies (from published literature and unpublished sources) on substances of varying skin sensitization potential
M.J. Olson/GlaxoSmithKline	124	Pharmaceuticals, pharmaceutical intermediates
Basketter, Gerberick, and Kimber[2]	31	Compiled from previously conducted studies (from published literature and unpublished sources) on substances of varying skin sensitization potential
K. Skirda/CESIO (TNO Report V7217)	18	Data were provided by CESIO member companies for use in paper titled "Limitations of the Local Lymph Node Assay (LLNA) as preferred test for skin sensitisation: concerns about false positive and false negative test result"
Lalko and Api (2006)	17	Original research conducted on essential oils, which were representative of the oils commonly used in perfumery. Each contains significant amounts of one or more known skin sensitizers.
H.W. Vohr/BGIA	16	Original research with epoxy resin components as part of a validation effort for non-radioactive versions of the Local Lymph Node Assay
Ryan et al. (2002)	15	Original research with known water soluble haptens and known skin sensitizers to assess the usefulness of a novel vehicle
D. Germolec/NIEHS	15	Substances evaluated by the National Toxicology Program for skin sensitization potential
E. Debruyne/Bayer CropScience SA	10	Original research on different pesticide types and formulations
P. Ungeheur/EFfCI	9	Data for selected unsaturated chemicals were provided in the report entitled "Comparative Experimental Study on the Skin Sensitising Potential of Selected Unsaturated Chemicals as Assessed by the Murine Local Lymph Node Assay (LLNA) and the Guinea Pig Maximisation Test (GPMT)"
P. Botham/ECPA	6	Plant protection products (i.e., pesticides) were evaluated in the Local Lymph Node Assay with a novel vehicle to assess its usefulness
Total	**471**[3]	

Abbreviations: BGIA: Berufsgenossenschaftliches Institut fur Arbeitsschutz; CESIO = Comite Europeen des Agents de Surface et de Leurs Intermediaires Organiques; ECPA = European Crop Protection Association;

EFfCI = European Federation for Cosmetic Ingredients; NIEHS = National Institute for Environmental Health Sciences: TNO = TNO Nutrition and Food Research

[1]These data were evaluated by the European Centre for the Validation of Alternative Methods (ECVAM) Scientific Advisory Committee in its evaluation of the LLNA limit dose procedure and were previously submitted to ICCVAM in 1998 for the original evaluation of the validation status of the LLNA (ICCVAM 1999, Gerberick et al. 2005).

[2]Data were included in a submission to ECVAM for the validation of traditional LLNA as a stand-alone assay for potency determination.

[3]The total number of studies does not take into account the fact that some substances were tested more than once (see **Section 3.2**)

3.3 Detailed Description of Substances Included in the Evaluation

Appendix B provides information on the physicochemical properties (e.g., physical form tested), Chemical Abstracts Service Registry Number (CASRN), and chemical class for each substance tested. This information was obtained from the published reports, submitted data, or through literature searches.

When available, chemical classes for each substance were retrieved from the National Library of Medicine's ChemID Plus database. If chemical class information was not located, chemical classes were assigned for each test substance using a standard classification scheme, based on the National Library of Medicine Medical Subject Headings classification system (available at http://www.nlm.nih.gov/mesh/meshhome.html). A substance could be assigned to more than one chemical class; however, no substance was assigned to more than three classes. Certain complex pharmaceuticals and pharmaceutical intermediates were simply identified as pharmaceutical substances.

Chemical class information is being presented only to provide an indication of the variety of structural elements that are present in the substances that were evaluated in this analysis. Classification of substances into chemical classes is not intended to make a representation regarding the impact of structure on biological activity or potency.

Table 3-2 provides the chemical class information for the test substances that were evaluated for this LLNA limit dose procedure evaluation. The table distinguishes the chemical classifications of the 211 substances included in the original evaluation of the LLNA limit dose procedure (Kimber et al. 2006; ESAC 2007) and the chemical classifications of the additional substances received in response to the *FR* notice (see **Section 3.1**). Of the 211 substances initially evaluated by Kimber et al. (2006), the chemical classes with the greatest number of substances were carboxylic acids (29) and halogenated hydrocarbons (27). Of the

additional 256 substances included in this evaluation, the chemical classes with the greatest number of substances tested were pharmaceutical chemicals (125), carboxylic acids (15), and lipids (14). Of the substances included in this evaluation, 10 were formulations. Seventy substances could not be assigned to a specific chemical class due to incomplete available information (e.g., CASRN, structure).

3.4 Coding Procedures

Coding of substances to avoid potential scoring bias was not described in the previous evaluation of 211 substances (ICCVAM 1999) or for any of the additional studies used in this evaluation.

588 **Table 3-2 Chemical Classes[1] Represented in the Current Database**

Chemical Class	Number of Substances – Original[2]	Number of Substances - Additional[2]	Chemical Class	Number of Substances - Original	Number of Substances - Additional
Alcohols	9	4	Inorganic Chemicals	0	2
Aldehydes	21	4	Isocyanates	1	0
Amides	4	0	Ketones	5	0
Amidines	1	0	Lactones	2	2
Amines	14	7	Lipids	7	14
Anhydrides	1	0	Macromolecular Substances[3]	0	5
Carbohydrates	3	2	Nitriles	1	1
Carboxylic Acids	29	15	Nitro Compounds	2	0
Esters	3	0	Nitroso Compounds	3	0
Ethers	14	2	Onium Compounds	1	0
Formulations[3]	0	10	Pharmaceutical chemicals[4]	0	125
Heterocyclic Compounds	18	4	Phenols	18	2
Hydrocarbons, Acyclic	2	1	Polycyclic Compounds	5	3
Hydrocarbons, Cyclic	14	7	Quinones	1	1
Hydrocarbons, Halogenated	27	1	Sulfur Compounds	20	2
Hydrocarbons, Other	7	8	Urea	3	0
Imines	0	1	Unknown	28	42

589 [1]Total number of chemical classes does not equal the total number of substances evaluated because some
590 substances were assigned to more than one class and some substances were not assigned to a specific chemical
591 class.

592 [2]Total Number of Substances – Original represents the substances evaluated in Kimber et al. (2006). Total
593 Number of Substances – Additional represents the substances received in response to the released *FR* notice
594 (Vol. 72, No. 95, pp. 27815-27817, available at
595 http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf)

596 [3]No chemical class could be assigned, but formulation or macromolecular substance used to identify such
597 common substances

598 [4]Chemical classification of "pharmaceutical chemicals" for the GlaxoSmithKline (GSK) substances was
599 suggested by Dr. Michael Olson of GSK which captures three types of pharmaceutical substances (actives,
600 intermediates, and starting materials).

4.0 Comparative *In Vivo* Reference Data

4.1 Protocol Used to Generate Comparative *In Vivo* Reference Data

As described in **Section 2.1**, the traditional LLNA protocol was consistent with the ICCVAM recommended protocol (ICCVAM 1999, Dean et al. 2001) and the EPA test guideline (EPA 2003) or the modified procedure that is described in OECD TG 429 (OECD 2002).

4.2 Comparative *In Vivo* Data Used

The traditional LLNA data used for this evaluation were obtained from nine sources (**Table 3-1)**. In addition to calculated SI values for each of the tested concentrations, the vehicle tested and EC3 values for substances classified as sensitizers were provided in Gerberick et al. (2005). The data received in response to the *FR* notice included calculated SI values for each of the tested concentrations and vehicle tested. Three of the submissions in response to the *FR* notice included EC3 values. The complete database (by each source) is provided in **Appendix C**.

4.3 Availability of Original Records for Comparative *In Vivo* Reference Data

An attempt was made to obtain the original records for the traditional LLNA data through the published *FR* notice and requests to specific stakeholders (Vol. 72, No. 95, pp. 27815-27817, available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf). Although the original study records were not obtained for any of the studies, compiled *in vivo* reports and/or transcribed results were obtained and/or are available for all studies included in this evaluation.

4.4 Quality of Comparative *In Vivo* Reference Data

Ideally, all data supporting the validity of a test method should be obtained and reported from studies conducted in accordance with Good Laboratory Practice (GLP) guidelines, which are nationally and internationally recognized rules designed to produce high-quality laboratory records (OECD 1998; EPA 2006a, 2006b; FDA 2007a). These guidelines provide an internationally standardized procedure for the conduct of studies, reporting requirements,

archiving of study data and records, and information about the test protocol, in order to ensure the integrity, reliability, and accountability of a study.

The extent to which the LLNA studies were compliant with GLP guidelines is based on the information provided in published and submitted reports. Based on the available information, the papers and data submissions that were identified as originating from studies that followed GLP guidelines or used data obtained according to GLP guidelines were H.W. Vohr/Berufsgenossenschaftliches Institut fur Arbeitsschutz (BGIA), P. Ungeheuer/European Federation for Cosmetic Ingredients (EFfCI), E. Debruyne/Bayer CropScience SA, P. Botham/European Crop Protection Association (ECPA), and D. Germolec/National Institute for Environmental Health Sciences (NIEHS).

There is no information in the publication by Gerberick et al. (2005) regarding the GLP compliance for any of the studies discussed. Several of the substances listed in Gerberick et al. (2005) also were included in the original LLNA submission to ICCVAM (ICCVAM 1999). According to the submission, "Much of the data used to support this submission and much of the data contained within the publications cited in this document have been derived from audited Good Laboratory Practices (GLP) compliant studies. Where this is not the case all investigations have been conducted to the spirit of GLP or Good Research Practice in GLP compliant facilities." (ICCVAM 1999). Furthermore, in response to requests from ICCVAM, records indicating compliance with GLP guidelines for some of the studies conducted were provided.

4.5 Accuracy and Reliability of the *In Vivo* Reference Test Method

4.5.1 Accuracy of the Traditional LLNA

ICCVAM (1999) reviewed the performance of the traditional LLNA with comparisons to (1) the GPMT and BT (EPA 2003) and (2) human results obtained from the human maximization test[5] and human patch test allergen[6] (HPTA) panels. The evaluation concluded that the LLNA demonstrated adequate accuracy. (ICCVAM 1999).

[5] Human maximization test involves application of occluded patches on the same skin site with a rest period between each reapplication. Two weeks after the last induction patch, sensitization is evaluated using a 48-hour occluded patch test. The site is scored after 24 and 48 hours after patch removal.

4.5.2 Reliability of the Traditional LLNA

Reliability, as assessed by intra- and inter-laboratory reproducibility, of the traditional LLNA was reviewed in ICCVAM (1999). The evaluation concluded that the LLNA demonstrated adequate intra- and interlaboratory repeatability and reproducibility (ICCVAM 1999).

[6] Allergen patch tests are diagnostic tests applied to the surface of the skin to assess the cause of contact dermatitis. Chemicals and substances included in these tests (e.g., nickel, rubber, and fragrance mixes) typically cause contact dermatitis (i.e., skin sensitization) (FDA 2007b).

5.0 LLNA Limit Dose Procedure Test Method Data and Results

5.1 Description of the LLNA Limit Dose Procedure Test Method Protocol Used to Generate Data

No specific LLNA limit dose procedure studies were conducted for this evaluation; rather, data from traditional LLNA studies were retrospectively evaluated. As described in **Section 2.1**, the only difference in the test method protocols between the proposed LLNA limit dose procedure and the traditional LLNA is the number of dose levels tested for a test substance. The traditional LLNA requires at least three test substance dose levels, while the LLNA limit dose procedure requires only the highest dose level of the test substance (Kimber et al. 2006).

5.2 Availability of Copies of Original LLNA Limit Dose Procedure Data Used to Evaluate Accuracy and Reliability

As noted in **Section 4.3**, while original study records were not obtained for any of the previously conducted studies, compiled *in vivo* reports and/or transcribed results were obtained and/or available for all studies included in this evaluation[7].

5.3 Description of the Statistical Approach Used to Evaluate the Resulting Data

The performance analysis in this BRD focuses on evaluating the ability of the LLNA limit dose procedure to identify potential skin sensitizers as determined by the calculated SI for each test substance (see **Section 2.1**).

5.4 Summary of Results

The data used for this evaluation were obtained from nine sources (**Table 3-1**). Where available, the specific information extracted for each substance includes its name, CASRN, physicochemical properties (e.g., form tested, Log K_{ow}), and chemical class[8] (**Appendix B**). Dose levels tested, along with calculated SI and/or EC3 values, sensitizing hazard classification, and the data source are provided in **Appendix C**. Other than the information

[7] The LLNA data for several of the chemicals evaluated for this report were included in the database that was submitted to ICCVAM in 1998 for the initial evaluation of LLNA (ICCVAM 1999). Therefore, some of the original data for these substances were available for review.

provided in the submitted data, no additional attempt was made to identify the source or purity of the test substance.

5.5 Use of Coded Substances

Coding of substances to avoid potential scoring bias was not described in the previous evaluation of 211 substances (ICCVAM 1999) or for any of the additional studies used in this evaluation.

5.6 Lot-to-Lot Consistency of Test Substances

Ideally, a single lot of each substance is used during the validation of a test method. In situations where multiple lots of a chemical must be used, the lot-to-lot consistency of a test substance must be evaluated to ensure the consistency of the substance evaluated over the course of the study. The procedures used in evaluating lot-to-lot consistency were evaluated by what was described in the published reports. No attempt was made to review original records to assess the procedures used to evaluate different batches of tested substances.

For the data submitted by P. Botham/ECPA, P. Ungheuer/EFfCI, and D. Germolec/NIEHS, the source and the batch number of each of the tested substances were provided.

5.7 Availability of Data for External Audit

The LLNA data included in the ICCVAM (1999) database were reviewed during the original evaluation. The original data for the other studies included in this evaluation were not available.

[8] Chemical classes were assigned by NICEATM based on the classification of the National Library of Medicine's Medical Subject Heading (available at http://www.nlm.nih.gov/mesh/meshhome.html).

6.0 LLNA Limit Dose Procedure Accuracy

6.1 Performance Statistics for the LLNA Limit Dose Procedure

A critical component of a formal evaluation of the validation status of a test method is an assessment of the accuracy of the proposed tested method when compared to the current reference test method (ICCVAM 2003). This aspect of assay performance is typically evaluated by calculating:

- Accuracy (concordance): the proportion of correct outcomes (positive and negative) of a test method
- Sensitivity: the proportion of all positive substances that are classified as positive
- Specificity: the proportion of all negative substances that are classified as negative
- Positive predictivity: the proportion of correct positive responses among substances testing positive
- Negative predictivity: the proportion of correct negative responses among substances testing negative
- False positive rate: the proportion of all negative substances that are falsely identified as positive
- False negative rate: the proportion of all positive substances that are falsely identified as negative

The ability of the LLNA limit dose procedure to correctly identify potential skin sensitizers was evaluated when compared to traditional LLNA results for 471 studies[9]. In the 471 studies, 317 detected skin sensitizers and 154 detected[10]. Classification of substances and

[9] Of the 466 substances tested in the 471 studies, five were independently evaluated up to three times in the same vehicle (see **Section 7.0** for additional information). Due to the small number of repeated studies (5% of total studies), all studies were treated independently for the purpose of this accuracy evaluation.

[10] For two of the repeated studies (HCA and linalool alcohol), discordant results were obtained in the LLNA. In both cases, one study classified the substance as a non-sensitizer and the other as a sensitizer. Closer review of

724 complete data for each substance is located in **Appendix C**. When substances tested multiple
725 times in the same vehicle were combined to yield an overall skin sensitization classification,
726 the number of substances evaluated was 466. Of these 466 substances, 313 were classified as
727 sensitizers and 153 were classified as non-sensitizers.

728 Based on the available data, the LLNA limit dose procedure has an accuracy of 98.9%
729 (466/471), a sensitivity of 98.4% (312/317), a specificity of 100% (154/154), a false positive
730 rate of 0% (0/154), and a false negative rate of 1.6% (5/317) when compared to the
731 traditional LLNA. When substances tested multiple times in the same vehicle were
732 combined, the LLNA limit dose procedure has an accuracy of 98.9% (461/466), a sensitivity
733 of 98.4% (308/313), a specificity of 100% (153/153), a false positive rate of 0% (0/153), and
734 a false negative rate of 1.6% (5/313) (**Table 6-1**). For comparison purposes, the performance
735 characteristics of the LLNA limit dose procedure as discussed in Kimber et al. (2006) are
736 included in **Table 6-1**.

737

the studies indicates that the discordant results were due to differences in the highest dose levels tested. For each of the studies, the LLNA limit dose approach and the traditional LLNA classified the substance similarly.

738

739 **Table 6-1 Evaluation of the Performance of the LLNA Limit Dose Procedure in Predicting Skin Sensitizers Compared to**
740 **the Traditional LLNA**

Data	N[1]	Accuracy		Sensitivity		Specificity		Positive Predictivity		Negative Predictivity		False Positive		False Negative	
		%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
Kimber et al. (2006)	211	98.6	208/211	98.2	166/169	100	42/42	100	166/166	93.3	42/45	0	0/42	1.8	3/169
LLNA limit dose approach	471	98.9	466/471	98.4	312/317	100	154/154	100	312/312	96.9	154/159	0	0/154	1.6	5/317
LLNA limit dose approach- Multiply tested substances combined	466	98.9	461/466	98.4	308/313	100	153/153	100	308/308	96.8	153/158	0	0/153	1.6	5/313

741 Abbreviations: conc. = concentration; No. = Numbers used to calculate percentage.
742 [1]N=Number of tests

743

Kimber et al. (2006) proposed a minimum testing concentration be considered for the purpose of judging the appropriateness of a non-sensitizing classification for a test substance. For the purposes of the evaluation discussed in Kimber et al. (2006), 10% was proposed as the minimum concentration in a dose solution to test. However, lack of sensitizing potential at 10% does not necessarily indicate that a substance will not produce skin sensitization when tested at a higher concentration. In fact, 51 substances (16% [51/313]) within the current database were non-sensitizers at concentrations of ≤ 10%, but sensitizers at concentrations >10% (see **Appendix D**).

According to the ICCVAM-recommended LLNA protocol, the maximum concentration tested should be "the highest achievable level while avoiding overt systemic toxicity and excessive local irritation" (ICCVAM 1999, Dean et al. 2001). Similar text is included in OECD TG 429 (OECD 2002).

6.2 Discordant Results

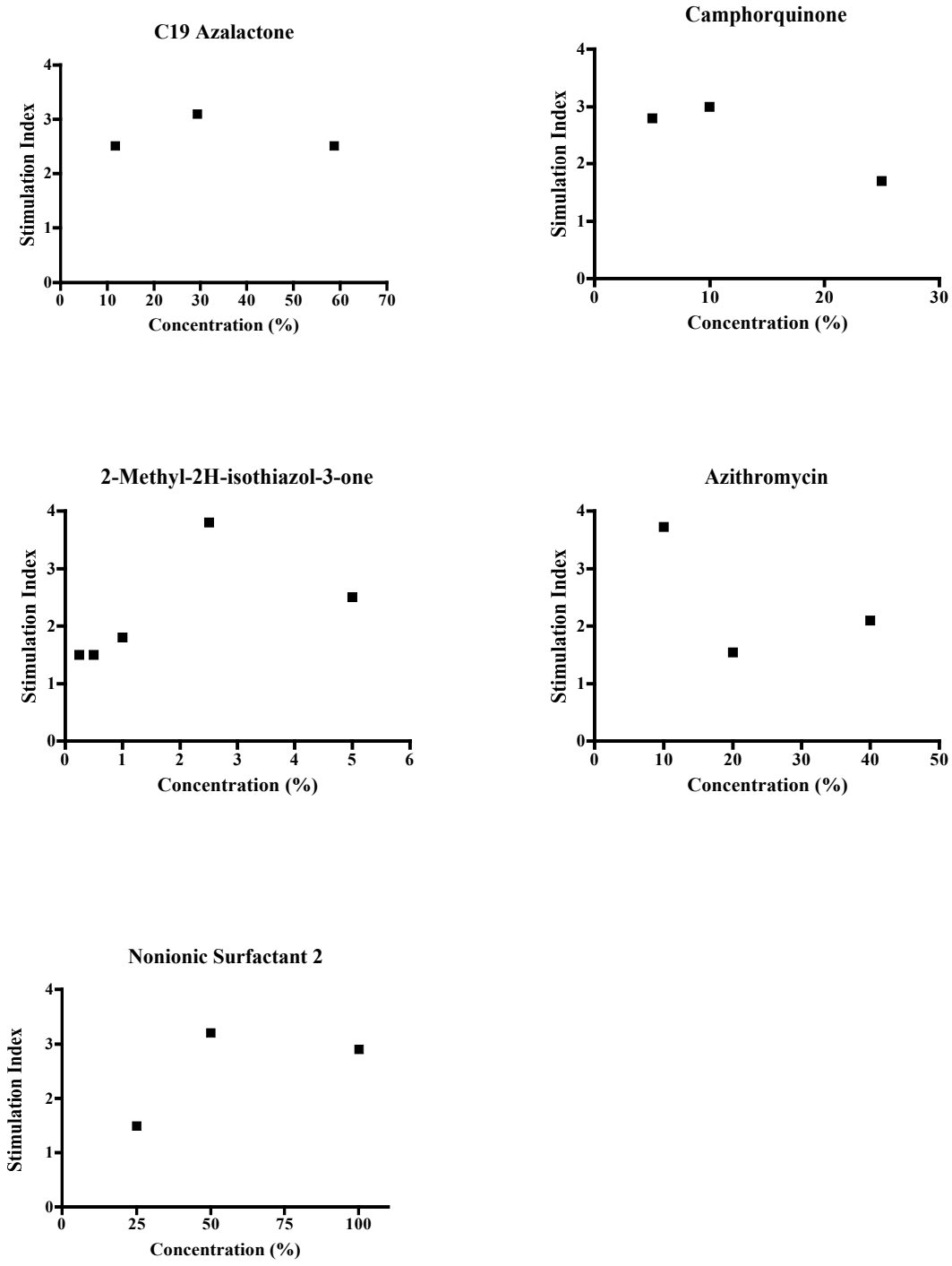
In this analysis, five substances were false negatives in the LLNA limit dose procedure. The misclassified substances were 2-methyl-2H-isothiazol-3-one, C19-azlactone, camphorquinone, azithromycin, and a substance designated as non-ionic surfactant 2. A review of the data for the false negatives indicates that the traditional LLNA classification of the substances as skin sensitizers was not based on the highest tested dose level producing an SI greater than three, but on a low- or mid-dose level that produced an SI greater than three (see **Table 6-2**). Since the LLNA limit dose procedure only evaluates the highest dose level tested, all of which produced an SI value below three, all five substances were incorrectly identified as non-sensitizers (i.e., false negatives). Graphs of the dose-response curves for the five substances incorrectly identified are provided in **Figure 6-1**.

767 **Table 6-2 LLNA Data for Substances Incorrectly Identified as Negative by the**
768 **LLNA Limit Dose Procedure**

Chemical	EC3	LLNA Data (Low- to Mid-Dose Group)		LLNA Data (Highest Dose Group)	
		Concentration (%)	SI	Concentration (%)	SI
C19-azlactone	26	29.33	3.1	58.67	2.5
Camphorquinone	10	10	3.0	25	1.7
2-Methyl-2H-isothiazol-3-one	1.9	2.5	3.8	5.0	2.5
Azithromycin	NC[1]	10	3.72	40	2.1
Non-ionic surfactant 2	47.1	50	3.2	100	2.9

769 Abbreviation: NC = Not Calculated; SI = Stimulation Index.
770 [1] Data was not calculated because a concentration that produced an SI less than 3 was not evaluated. Therefore
771 extrapolation between points that bracket an SI of 3 could not be done.

772 **Figure 6-1 Dose-Response Graphs for False Negatives, as Identified by the LLNA**
773 **Limit Dose Procedure**



774

775 **Table 6-3** provides a summary of the available physicochemical properties of these
776 substances and the test vehicle.

777 **Table 6-3 Summary of Available Physicochemical Properties for False Negatives, as**
778 **Identified by the LLNA Limit Dose Procedure**

Chemical	CASRN	Vehicle	Molecular Weight (g/mol)	K_{OW}[1]
C19-azlactone	--	Acetone:Olive Oil	379.63	5.21[2]
Camphorquinone	465-29-2	Acetone:Olive Oil	166.217	2.15[2]
2-Methyl-2H-isothiazol-3-one	2682-20-4	Acetone:Olive Oil	115.15	0.68[2]
Azithromycin	83905-01-5	Acetone	748.985	3.243[3]
Non-ionic surfactant 2	--	Acetone:Olive Oil	---	--

779 Abbreviations: CASRN = Chemical Abstracts Service Registry Number.
780 [1] K_{OW} represents the octanol-water partition coefficient (expressed on log scale).
781 [2] K_{OW} calculated by the method of Moriguchi et al. (1994) and provided in Gerberick et al. (2005 Dermatitis.
782 16:157-2002).
783 [3] K_{OW} calculated by the method of Meylan and Howard (1995) and obtained from the website:
784 http://www.syrres.com/esc/est_kowdemo.htm.
785
786

7.0 LLNA Limit Dose Procedure Reliability

An assessment of test method reliability (intralaboratory repeatability and intra- and inter-laboratory reproducibility) is an essential element of any evaluation of the performance of an alternative test method (ICCVAM 2003). Repeatability refers to the closeness of agreement between test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions within a given time period (ICCVAM 1997, 2003). Intralaboratory reproducibility refers to the 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.

Based on a review of the data (**Appendix C**), there were only five substances with sufficient traditional LLNA data to assess the interlaboratory reproducibility of the LLNA limit dose procedure. These are linalool alcohol, DCNB, HCA, methyl salicylate, and potassium dichromate. **Table 7-1** provides a summary of the responses obtained by the LLNA limit dose procedure. However, since the LLNA limit dose procedure and traditional LLNA use identical protocols, and the datasets used to evaluate the accuracy of the LLNA limit dose procedure and traditional LLNA are similar, the reliability of the two methods would be expected to be similar. That is, the intra- and inter-laboratory reliability of the LLNA limit dose procedure would be expected to be equal to the traditional LLNA (see ICCVAM [1999] for these statistics).

809 **Table 7-1 LLNA Limit Dose Procedure Responses for Repeated Studies**

Chemical	Data Source	Vehicle	LLNA Limit Dose Procedure Response					LLNA Limit Dose Procedure Classification [1]
			Conc (%)/SI	Conc (%)/SI	Conc (%)/SI	Conc (%)/SI	Conc (%)/SI	
Hexyl cinnamic aldehyde	Data Submitted by H.W. Vohr	AOO	2.5/1.1	5/1.2	10/2.84	NA	NA	-
	Gerberick et al. (2005)		2.5/1.3	5/1.1	10/2.5	25/10	50/17	+
Linalool alcohol	Gerberick et al. (2005)	AOO	25/2.5	50/4.8	100/8.3	NA	NA	+
	Data Submitted by D. Basketter, I. Kimber, and F. Gerberick		1/1.0	10/1.3	30/1.3	NA	NA	-
1-Chloro-2-dinitrobenzene	Gerberick et al. (2005)	AOO	0.01/1.5	0.025/1.8	0.05/2.4	0.1/8.9	0.25/38	+
	Data submitted by D. Germolec		0.01/1.17	0.03/1.12	0.05/1.93	0.1/1.95	0.25/7.10	+
Methyl salicylate	Gerberick et al. (2005)	AOO	1.0/1.0	2.5/1.1	5.0/1.6	10/1.4	20/0.9	-
	Data submitted by D. Germolec		1/0.86	2.5/1.19	5/1.16	10/1.41	20/1.72	-
Potassium dichromate	Gerberick et al. (2005)	DMSO	0.025/1.6	0.05/1.4	0.1/3.8	0.25/5.3	0.5/16.1	+
	Data submitted by D. Germolec		0.025/1.21	0.05/1.84	0.1/2.22	0.25/3.39	NA	+
	Ryan et al. (2002)		0.025/1.4	0.05/2.5	0.1/9.5	0.25/25.9	0.5/10.1	+

810 Abbreviations: AOO = Acetone:Olive Oil; Conc = Concentration tested; DMSO = Dimethylsulfoxide; NA = Not applicable since only three concentrations were
811 tested; SI = Stimulation Index.
812 [1] - = non-sensitizer, + = sensitizer

8.0 LLNA Limit Dose Procedure Data Quality

8.1 Adherence to National and International GLP Guidelines

The extent to which the LLNA studies were compliant with GLP guidelines is based on the information provided in published and submitted reports. Based on the available information, the papers and data submissions that were identified as originating from studies that followed GLP guidelines or used data obtained according to GLP guidelines were H.W. Vohr/Berufsgenossenschaftliches Institut fur Arbeitsschutz (BGIA), P. Ungeheuer/European Federation for Cosmetic Ingredients (EFfCI), E. Debruyne/Bayer CropScience SA, P. Botham/European Crop Protection Association (ECPA), and D. Germolec/National Institute for Environmental Health Sciences (NIEHS).

8.2 Data Quality Audits

Formal assessments of data quality, such as a quality assurance audit, generally involve a systematic and critical comparison of the data provided in a study report to the laboratory records generated for a study.

Much of the data published by Gerberick et al. (2005) was conducted following GLP guidelines or were conducted in GLP-compliant facilities. Therefore, it was previously inferred that data audits were conducted on the data (ICCVAM 1999).

A formal assessment of the quality of the remainder of the LLNA data included in this BRD was not feasible. The published data on the LLNA were limited to tested concentrations and calculated SI and EC3 values. Auditing the reported values would require obtaining the original individual animal data for each LLNA experiment, which were not obtained. However, as stated in **Section 8.1**, many of the studies were conducted according to GLP guidelines, which implies that an independent quality assurance audit was conducted.

8.3 Impact of Deviations from GLP Guidelines

The impact of deviations from GLP guidelines cannot be evaluated for the data reviewed in this BRD, since no information on data quality audits was obtained.

8.4 Availability of Laboratory Notebooks or Other Records

As noted in **Section 5.2,** the original records were not obtained for the studies included in this evaluation. Data were available for several of the substances included in the ICCVAM (1999) evaluation and thus some of the raw data for these substances were available for review.

9.0 OTHER SCIENTIFIC REPORTS AND REVIEWS

9.1 Reports in the Peer-Reviewed Literature

A search of MEDLINE, TOXLINE, and Web of Science revealed one published report, in addition to that of Kimber et al. (2006), that was relevant to the LLNA limit dose procedure. Additionally, three presentations (two posters and one platform) were included in the Society of Toxicology 2007 Annual Meeting program. One of the posters (Basketter et al. 2007) and the platform presentation (Basketter 2007) detailed the evaluation that resulted in the Kimber et al. (2006) publication and are therefore not discussed below. The information in the second poster, Chaney et al. (2007), described the impact of reducing the number of animals per dose group on the performance of the LLNA limit dose procedure and is summarized below from the subsequent publication (Ryan et al. 2007).

9.1.1 Ryan et al. (2007)

Ryan et al. (2007) evaluated the impact of reducing the number of mice (from five animals to two) on the performance characteristics using the LLNA limit dose procedure. For the evaluation, 41 datasets on 24 substances were evaluated. The 19 sensitizers and five non-sensitizers were represented by 33 sensitizer datasets and eight non-sensitizer datasets.

SI values were determined for all possible two-animal combinations for the control and high dose groups; there were 10 possible data combinations per experimental group. Thus, there were a total of 100 possible results (two control animals and two high dose animals) for each dataset. The 100 possible SI values, which were each based on a unique set of four values, were plotted for each chemical and the percentage of the combinations that resulted in SI $\geq$ 3 was calculated. Of the sensitizers evaluated, SI $\geq$ 3 was obtained for at least 96% of the combinations for 76% (25/33) of the datasets. The non-sensitizers (excluding three datasets for sodium lauryl sulfate) had $\leq$ 13% of the possible combinations yielding SI $\geq$ 3. For the datasets with threshold SI values (2-4.9), however, greater than or equal to 90% of the combinations resulted in SI $\geq$ 3 for 20% (4/20) of the sensitizers. Thirteen of the 20 (65%) sensitizer datasets had less than 75% of the combinations producing SI $\geq$ 3. The authors concluded that the decreased sensitivity produced by using two mice per group was

872 inappropriate for hazard identification of skin sensitization using the LLNA limit dose
873 procedure.

10.0 Animal Welfare Considerations

10.1 How the LLNA Limit Dose Procedure Will Refine, Reduce, or Replace Animal Use

Compared to the traditional LLNA, the LLNA limit dose procedure will reduce the number of animals used to assess skin sensitization. In addition to concurrent vehicle and positive control groups, the traditional LLNA requires testing from four to five mice for each of at least three test substance dose levels (ICCVAM 1999, Dean et al. 2001, OECD 2002, EPA 2003). Since, in the LLNA limit dose procedure, only the highest dose level of the test substance is being evaluated in addition to the concurrent control groups, the number of animals tested would be decreased by at least 40%.

10.2 Requirements for the Use of Animals

The rationale for the use of animals, and the basis for determining the number of animals used in the LLNA limit dose procedure, is the same as the rationale for the traditional LLNA (ICCVAM 1999, Dean et al. 2001).

11.0 Practical Considerations

Several issues are taken into account when assessing the practicality of using an alternative to an existing test method. In addition to performance evaluations, assessments of the laboratory equipment and supplies needed to conduct the alternative test method, level of personnel training, labor costs, and the time required to complete the test method relative to the existing test method are necessary. The time, personnel cost, and effort required to conduct the proposed test method(s) must be considered to be reasonable when compared to the test method it is intended to replace.

11.1 Transferability of the LLNA Limit Dose Procedure

Test method transferability addresses the ability of a method to be accurately and reliably performed by multiple laboratories (ICCVAM 2003), including those experienced in the particular type of procedure as well as laboratories with less or no experience in the particular procedure. The degree of transferability of a test method can be evaluated by its interlaboratory reproducibility. The results presented in **Section 7.0** provide a discussion of the minimum variability to be expected. The transferability of the LLNA limit dose procedure is equal to that of the traditional LLNA (ICCVAM 1999, Dean et al. 2001), which includes considerations for the required facilities, major fixed equipment, and any other necessary supplies.

11.2 LLNA Limit Dose Procedure Training Considerations

The level of training and expertise needed to conduct the LLNA limit dose procedure, and the training requirements needed to demonstrate proficiency, are identical to that for the traditional LLNA (ICCVAM 1999, Dean et al. 2001).

11.3 Cost Considerations

The LLNA limit dose procedure uses the same basic protocol as the traditional LLNA. However, as described in **Section 1.2.2**, since fewer animals are tested, the costs related to conducting the test (e.g., animal care, radioactivity, scintillation fluid, etc.) would be expected to be proportionally lower than the traditional LLNA.

11.4 Time Considerations

Since at least 40% fewer animals are tested in the LLNA limit dose procedure relative to the traditional LLNA, the overall time required to conduct the method (e.g., dosing mice, removing the auricular lymph nodes from the animals) would be expected to be proportionally decreased.

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13.0 Glossary[11]

Accuracy[12]: (a) The closeness of agreement between a test method result and an accepted reference value. (b) The proportion of correct outcomes of a test method. It is a measure of test method performance and one aspect of *relevance*. The term is often used interchangeably with "concordance" (see also *two-by-two table*). Accuracy is highly dependent on the prevalence of positives in the population being examined.

Allergic Contact Dermatitis (ACD): A Type IV allergic reaction of the skin that results from skin contact with an allergen. Symptoms of ACD include, but are not limited to, development of erythema (redness) and edema (swelling).

Assay[14]: The experimental system used. Often used interchangeably with *test* and *test method*.

Coded substances: Substances labeled by code rather than name so that they can be tested and evaluated without knowledge of their identity or anticipation of test results. Coded substances are used to avoid intentional or unintentional bias when evaluating laboratory or test method performance.

Concordance[14]: The proportion of all substances tested that are correctly classified as positive or negative. It is a measure of test method performance and one aspect of *relevance*. The term is often used interchangeably with *accuracy* (see also *two-by-two table*). Concordance is highly dependent on the prevalence of positives in the population being examined.

EC3: The estimated concentration needed to produce a stimulation index of three, as compared to the concurrent vehicle control.

Essential test method component[14]: Structural, functional, and procedural elements of a test method that are used to develop the test method protocol. These components include unique characteristics of the test method, critical procedural details, and quality control measures. Adherence to essential test method components is necessary when the acceptability of a

[11] The definitions in this Glossary are restricted to their uses with respect to the LLNA limit dose approach and the traditional LLNA.

proposed test method is being evaluated based on performance standards derived from mechanistically and functionally similar validated test method. [Note: Previously referred to as *minimum procedural standards*]

False negative[14]: A substance incorrectly identified as negative by a test method.

False negative rate[14]: The proportion of all positive substances falsely identified by a test method as negative (see *two-by-two table*). It is one indicator of test method accuracy.

False positive[14]: A substance incorrectly identified as positive by a test method.

False positive rate[14]: The proportion of all negative substances that are falsely identified by a test method as positive (see *two-by-two table*). It is one indicator of test method accuracy.

Good Laboratory Practices (GLP)[14]: Regulations promulgated by the U.S. Food and Drug Administration and the U.S. Environmental Protection Agency, and principles and procedures adopted by the Organization for Economic Cooperation and Development and Japanese authorities, that describe record keeping and quality assurance procedures for laboratory records that will be the basis for data submissions to national regulatory agencies.

Hazard[14]: The potential for an adverse health or ecological effect. A hazard potential results only if an exposure occurs that leads to the possibility of an adverse effect being manifested.

Interlaboratory reproducibility[14]: A measure of whether different qualified laboratories using the same protocol and test substances can produce qualitatively and quantitatively similar results. Interlaboratory reproducibility is determined during the prevalidation and validation processes and indicates the extent to which a test method can be transferred successfully among laboratories.

Intralaboratory repeatability[14]: 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.

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[12] Definition used by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM 2003).

Intralaboratory reproducibility[14]: The first stage of validation; a determination of whether qualified people within the same laboratory can successfully replicate results using a specific test protocol at different times.

Immunological: Relating to the immune system and immune responses.

In vivo: In the living organism. Refers to assays performed in multicellular organisms.

Local Lymph Node Assay (LLNA): An *in vivo* test method used to assess the skin sensitization potential of a substance by measuring the proliferation of lymphocytes in the lymph nodes draining the ears (i.e., auricular lymph nodes) of mice, subsequent to topical exposure on the ear to the substance. The traditional LLNA relates lymphocyte proliferation to the incorporation of tritiated thymidine (^{3}H) into the cells of the draining lymph nodes.

Lymphocyte: A white blood cell found in the blood, lymph, and lymphoid tissues, which regulates and plays a role in acquired immunity.

Negative predictivity[14]: The proportion of correct negative responses among substances testing negative by a test method (see *two-by-two table*). It is one indicator of test method accuracy. Negative predictivity is a function of the sensitivity of the test method and the prevalence of negatives among the substances tested.

Non-sensitizer: A substance that does not cause skin sensitization following skin contact.

Performance[14]: The accuracy and reliability characteristics of a test method (see *accuracy*, *reliability*).

Positive control: A substance known to induce a positive response, which is used to demonstrate the sensitivity of the test method and to allow for an assessment of variability in the conduct of the assay over time. For most test methods, the positive control substance is tested concurrently with the test substance and the vehicle/solvent control. However, for some *in vivo* test methods, periodic studies using a positive control substance is considered adequate by the OECD.

Positive predictivity[14]: The proportion of correct positive responses among substances testing positive by a test method (see *two-by-two table*). It is one indicator of test method

accuracy. Positive predictivity is a function of the sensitivity of the test method and the prevalence of positives among the substances tested.

Prevalence[14]: The proportion of positives in the population of substances tested (see *two-by-two table*).

Protocol[14]: The precise, step-by-step description of a test, including the listing of all necessary reagents, criteria and procedures for the evaluation of the test data.

Quality assurance[14]: A management process by which adherence to laboratory testing standards, requirements, and record keeping procedures is assessed independently by individuals other than those performing the testing.

Reduction alternative[14]: A new or modified test method that reduces the number of animals required.

Reference test method[14]: The accepted *in vivo* test method used for regulatory purposes to evaluate the potential of a test substance to be hazardous to the species of interest.

Refinement alternative[14]: A new or modified test method that refines procedures to lessen or eliminate pain or distress in animals or enhances animal well-being.

Relevance[14]: The extent to which a test method correctly predicts or measures the biological effect of interest in humans or another species of interest. Relevance incorporates consideration of the *accuracy* or *concordance* of a test method.

Reliability[14]: A measure of the degree to which a test method can be performed reproducibly within and among laboratories over time. It is assessed by calculating intra- and inter-laboratory reproducibility and intralaboratory repeatability.

Replacement alternative[14]: A new or modified test method that replaces animals with nonanimal systems or one animal species with a phylogenetically lower one (e.g., a mammal with an invertebrate).

Reproducibility[14]: The consistency of individual test results obtained in a single laboratory (intralaboratory reproducibility) or in different laboratories (interlaboratory reproducibility) using the same protocol and test substances (see intra- and inter-laboratory reproducibility).

rLLNA (reduced LLNA): Also called the cut-down LLNA, limit test LLNA, or LLNA limit dose procedure. A variant of the traditional LLNA that employs a single, high dose level of the test substance rather than multiple dose levels to determine its skin sensitization potential.

Sensitivity[14]: The proportion of all positive substances that are classified correctly as positive in a test method. It is a measure of test method accuracy (see *two-by-two table*).

Skin sensitizer: A substance that induces an allergic response following skin contact. (U.N. 2005)

Specificity[14]: The proportion of all negative substances that are classified correctly as negative in a test method. It is a measure of test method accuracy (see *two-by-two table*).

Stimulation Index (SI): A value calculated for the Local Lymph Node Assay, to assess the skin sensitization potential of a test substance. The value is calculated as the ratio of radioactivity incorporated into the auricular lymph nodes of a group of treated mice to the radioactivity incorporated into the corresponding lymph nodes of a group of vehicle control mice. For the traditional LLNA and the LLNA limit dose procedure, an SI equal to or greater than 3 classifies a substance as a skin sensitizer.

Test[14]: The experimental system used; used interchangeably with *test method* and *assay*.

Test method[14]: A process or procedure used to obtain information on the characteristics of a substance or agent. Toxicological test methods generate information regarding the ability of a substance or agent to produce a specified biological effect under specified conditions. Used interchangeably with *test* and *assay*. See also *validated test method* and *reference test*.

Transferability[14]: The ability of a test method or procedure to be accurately and reliably performed in different, competent laboratories.

Two-by-two table[14]: The two-by-two table can be used for calculating accuracy (concordance) ([a+d]/[a+b+c+d]), negative predictivity (d/[c+d]), positive predictivity (a/[a+b]), prevalence ([a+c]/[a+b+c+d]), sensitivity (a/[a+c]), specificity (d/[b+d]), false positive rate (b/[b+d]), and false negative rate (c/[a+c]).

		New Test Outcome		
		Positive	**Negative**	**Total**
Reference Test Outcome	Positive	a	c	a + c
	Negative	b	d	b + d
	Total	a + b	a + d	a + b + c + d

Validated test method[14]: An accepted test method for which validation studies have been completed to determine the relevance and reliability of this method for a specific proposed use.

Validation[14]: The process by which the reliability and relevance of a procedure are established for a specific purpose.

Vehicle control: An untreated sample containing all components of a test system, including the vehicle that is processed with the test substance-treated and other control samples to establish the baseline response for the samples treated with the test substance dissolved in the same vehicle.

Weight-of-evidence (process): The strengths and weaknesses of a collection of information are used as the basis for a conclusion that may not be evident from the individual data.

APPENDIX A

ECVAM Scientific Advisory Committee (ESAC) Statement on the rLLNA

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APPENDIX B

Physico-chemical Properties for Substances Evaluated in the LLNA Limit Dose Procedure

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APPENDIX C

LLNA Limit Dose Procedure Data

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APPENDIX D

Substances in the NICEATM LLNA Database for Which a Concentration of ≥10% Elicited a Negative Result, but an Increased Concentration Elicited a Positive Response

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