Appendix D

Assessment of the Validity of the LLNA for Pesticide Formulations, Metals, Substances in Aqueous Solutions, and Other Products

2010 Addendum to NIH Publication Number 99-4494:
The Murine Local Lymph Node Assay (LLNA):
A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals/Compounds

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List of Abbreviations and Acronyms

ACD	Allergic contact dermatitis
AOO	Acetone: olive oil
BGIA	Berufsgenossenschaftliches Institut für Arbeitsschutz (German Institute for Occupational Safety and Health)
BRD	Background review document
BT	Buehler Test
CASRN	Chemical Abstracts Service Registry Number
CCA	Chromated copper arsenate
CESIO	Comité Européen des Agents de Surface et de leurs Intermédiaires Organiques (European Committee of Surfactants and their Organic Intermediates)
CoDEC	Cobalt diethyldithiocarbamate
Conc.	Concentration tested
CPSC	U.S. Consumer Product Safety Commission
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
EC3	Estimated concentration needed to produce a stimulation index of 3
ECPA	European Crop Protection Association
ECVAM	European Centre for the Validation of Alternative Methods
EPA	U.S. Environmental Protection Agency
EtOH	Ethanol
FDA	U.S. Food and Drug Administration
FR	*Federal Register*
GCP	Good Clinical Practice
GLP	Good Laboratory Practice
g/L	Grams per liter
GP	Guinea pig
GPMT	Guinea pig maximization test
GSK	GlaxoSmithKline
GST	Gold sodium thiosulfate
HMT	Human Maximization Test
HRIPT	Human Repeat Insult Patch Test
H_2O	Water
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ISO	International Organization for Standardization
IUD	Intrauterine device
IWG	Immunotoxicity Working Group
K_{ow}	Octanol-water partition coefficient
LLNA	Local lymph node assay

MeSH	Medical subject headings
MEST	Mouse ear swelling test
n	Number
No.	Number
NA	Not available
NC	Not calculated
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
NIEHS	National Institute of Environmental Health Sciences
NIOSH	National Institute of Occupational Safety and Health
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
OPPTS	Office of Prevention, Pesticides and Toxic Substances
QRA	Quantitative Risk Assessment
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SI	Stimulation index
TEDCD	Tetraethyldicarbamoyl disulfide
TETD	Tetraethylthiuram disulfide
TG	Test Guideline
TNO	TNO Nutrition and Food Research (Dutch - No English translation)
U.K.	United Kingdom
U.S.	United States
vs.	Versus
w/v	Weight to volume ratio
Veh.	Vehicle
ZDEC	Zinc diethyldithiocarbamate

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Preface

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

However, as described in the ICCVAM evaluation report[1], based on the lack of available data for aqueous solutions and mixtures and on discordant results for a limited number of studies with metals, ICCVAM recommended that these substances not be tested for skin sensitization potential using the LLNA.

Based on the ICCVAM recommendations, the ICCVAM member agencies that require the regulatory submission of skin sensitization data accepted the LLNA, with the identified limitations, as an alternative to the traditional guinea pig tests (Guinea Pig Maximization Test, Buehler Test).

In 2007, the U.S. Consumer Product Safety Commission (CPSC) asked ICCVAM and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to reevaluate the usefulness and limitations of the LLNA for testing mixtures, metals, and substances in aqueous solutions, among other activities related to the LLNA. ICCVAM assigned the activity a high priority, and established the ICCVAM Immunotoxicity Working Group (IWG) to work with NICEATM to review the current literature and evaluate available data to assess the status of the LLNA applicability domain. A comprehensive draft Addendum to the 1999 ICCVAM evaluation report provided the information, data and analyses supporting the validation status of the LLNA applicability domain. ICCVAM also developed draft test method recommendations for the LLNA applicability domain regarding usefulness and limitations, test method protocol, performance standards and future studies.

NICEATM and ICCVAM provided the draft Addendum and draft recommendations to an international independent scientific peer review panel for their consideration at a public meeting on March 4-6, 2008. Both the Panel and ICCVAM concluded that, due to the limitations associated with the available database for mixtures (i.e., unknown formulae, lack of human data), more data were needed before a recommendation on the usefulness and limitations of the LLNA for testing mixtures could be made. The Panel also stated that the term "mixtures" was used too broadly (i.e., can represent an infinite number of materials) and it would be more beneficial to specify types or formulations that were being examined. Public comments at the meeting revealed that additional relevant data from LLNA studies with pesticide formulations and other products were available, which had not previously been provided in response to earlier requests for data. The Panel recommended that NICEATM obtain additional existing data that were not available to the Panel, and reanalyze the performance of the LLNA for testing pesticide formulations and other products. NICEATM subsequently obtained additional data and prepared this revised Addendum. ICCVAM also prepared revised draft test method recommendations based on the revised Addendum. This revised draft Addendum addresses the validation database for the LLNA applicability domain.

The Panel reconvened on April 27-28, 2009 to assess the current validation status of the LLNA applicability domain. The Panel also reviewed the completeness and accuracy of the draft Addendum and the extent to which the information therein supported the ICCVAM draft test method

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[1] ICCVAM (1999), available at http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel98.htm

recommendations for usefulness and limitations, test method protocol, performance standards and future studies. ICCVAM considered the conclusions and recommendations of the Panel, along with comments received from the public and the Scientific Advisory Committee for Alternative Toxicological Methods, when finalizing this Addendum and test method recommendations on the LLNA applicability domain.

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 its preparation, review, and revision. We especially recognize the Panel members for their thoughtful evaluations and generous contributions of time and effort. Special thanks are extended to Dr. Michael Luster for serving as the Panel Chair and to Dr. Michael Woolhiser, Dr. Michael Olson, Kim Headrick, and Dr. Stephen Ullrich for their service as Evaluation Group Chairs. We thank the IWG for assuring a meaningful and comprehensive review. We especially thank Dr. Joanna Matheson (CPSC) and Dr. Abigail Jacobs (U.S. Food and Drug Administration Center for Drug Evaluation and Research) for serving as Co-Chairs of the IWG, as well as the IWG members and ICCVAM representatives who subsequently reviewed the Addendum and provided comments.

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Executive Summary

Background

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended the murine local lymph node assay (LLNA) to U.S. Federal agencies as a valid substitute for currently accepted guinea pig test methods. These test methods assess the potential of many types of substances to cause allergic contact dermatitis, a skin reaction characterized by redness, swelling, and itching. Allergic contact dermatitis can result from contact with a sensitizing chemical or product.

ICCVAM based its recommendation on a comprehensive evaluation that included an assessment of the LLNA's validation status by an independent international scientific peer review panel. The Panel report and the ICCVAM recommendations (ICCVAM 1999) are available at the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)–ICCVAM website (http://iccvam.niehs.nih.gov).

The LLNA was subsequently incorporated into the following national and international test guidelines for assessing skin sensitization:

- U.S. Environmental Protection Agency Health Effect Testing Guidelines on Skin Sensitization (EPA 2003)
- Organisation for Economic Co-operation and Development Test Guideline 429 (OECD 2002)
- International Organization for Standardization 10993-10: Tests for Irritation and Delayed-type Hypersensitivity (ISO 2002)

In 2007, the U.S. Consumer Product Safety Commission formally nominated several LLNA-related activities for evaluation by NICEATM and ICCVAM. The U.S. Consumer Product Safety Commission asked for an assessment of the validation status of the LLNA applicability domain. In response, NICEATM and ICCVAM compiled the information in this Addendum.

This Addendum provides a comprehensive review of available data and information about the usefulness and limitations of the LLNA for assessing the skin-sensitizing potential of pesticide formulations and other products, metals, and substances tested in aqueous solutions (i.e., its current applicability domain). The information is based on a review of traditional LLNA data that were either (1) submitted as part of the original LLNA evaluation (ICCVAM 1999), (2) extracted from peer-reviewed publications, or (3) submitted to NICEATM in response to a May 2007 *Federal Register* notice (72 FR 27815).[2]

Revisions to the NICEATM-ICCVAM Evaluation of the LLNA Applicability Domain

NICEATM and ICCVAM convened a Panel meeting on March 4–6, 2008. The Panel members reviewed the draft Addendum and commented on the extent to which it supported the draft ICCVAM test method recommendations on the usefulness and limitations of the LLNA regarding the applicability domain. Both ICCVAM and the Panel concluded that, because of insufficient information about mixtures (e.g., unknown formulas, lack of human data), more data were needed before a recommendation could be made on the usefulness and limitations of the LLNA for testing mixtures.[3] The Panel also stated that the term "mixtures" was used too broadly (i.e., it can represent an infinite number of materials). The Panel stated that it would be more beneficial to specify types or formulations that are being examined (ICCVAM 2008).

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[2] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf
[3] Available at http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel08.htm

Public comments at the meeting revealed additional relevant data from LLNA studies with pesticide formulations and other products. These data had not been provided in response to earlier requests. The Panel recommended that NICEATM obtain and analyze additional data on the performance of the LLNA for testing pesticide formulations and other products. In response, NICEATM obtained additional data and, in some cases, corresponding reference test method data (i.e., guinea pig test and/or human data) (ICCVAM 2008). NICEATM revised the evaluation of the LLNA for testing pesticide formulations and other products[4] (**Section 5.1**) and for testing substances in aqueous solutions (**Section 5.3**). No new LLNA data were received for LLNA tests with metals; therefore, this part of the evaluation remained unchanged (**Section 5.2**).

Validation Database

The information in this Addendum is based on a review of LLNA data derived from a database of more than 600 substances (including pesticide formulations and other products). In the original ICCVAM evaluation of the LLNA (ICCVAM 1999), the performance of the LLNA was compared to (1) the results from guinea pig tests and (2) information about sensitizers in humans (e.g., human maximization test results, substances used in a human repeat insult patch test, and clinical data), where available. This Addendum updates the LLNA performance analyses for (1) pesticide formulations and other products, (2) metals, and (3) substances tested in aqueous solutions when compared to human and guinea pig test results.

Use of the LLNA for Testing Formulations and Other Products

Pesticide Formulations: The revised LLNA database contains data for 104 pesticide formulations. Among these formulations, 54% (56 of 104) were LLNA positive, and 46% (48 of 104) were LLNA negative.

Seventy of the 104 pesticide formulations have LLNA data and some type of associated guinea pig reference data. Eighty-nine LLNA studies were performed using these 70 formulations. Sixty-one of the 89 LLNA studies used CBA/Ca or CBA/J strains; 28 used BALBc mice. Six pesticide formulations were tested in multiple LLNA studies (25 studies total). Five of the six had LLNA results in agreement, and one of the six produced discordant results (three positive, two negative).

All 70 pesticide formulations (89 of 89 studies) were tested in the LLNA in aqueous 1% Pluronic L92, a surfactant and wetting agent that has been evaluated as an alternative aqueous-based vehicle for use in the LLNA (Boverhof et al. 2008; Ryan et al. 2002).

Twenty-three pesticide formulations had associated guinea pig data for the complete formulation. Forty-six had guinea pig data for one or more of the active ingredients in the complete formulation. Fourteen pesticide formulations had guinea pig data for a substance related to an active ingredient or for a related formulation.

Among the 23 formulations that had guinea pig data, the LLNA classified 52% (12 of 23 formulations) as sensitizers, while the guinea pig tests classified only 13% (3 of 23 formulations) as sensitizers. All three of the pesticide formulations identified as sensitizers in the guinea pig test were also identified as sensitizers in the LLNA. Overall, the LLNA and the guinea pig results were in agreement 57% of the time. The LLNA identified as sensitizers an additional seven substances that the guinea pig test classified as nonsensitizers, an overprediction rate of 50% (10 of 20).

Three of the LLNA studies for these 23 pesticide formulations were done in BALB/c mice. The OECD Test Guideline and ICCVAM protocol use CBA/CA and CBA/J strains. If the three BALB/c studies are therefore excluded from the analysis, the LLNA and guinea pig results were in agreement 60% of the time (12 of 20), and the overprediction rate was 47% (8 of 17). There were no instances of

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[4] Based on the Panel's recommendation, this Addendum does not refer to "mixtures" as a type of substance tested but rather specifies, where possible, the types of products that were tested.

underprediction for the 23 pesticide formulations. Human data were not available for these pesticide formulations to confirm their sensitization potential in humans.

Dyes: The current LLNA database contains data for six dyes that have associated LLNA and guinea pig data. The LLNA classified 50% (3 of 6) as sensitizers and 50% (3 of 6) as nonsensitizers. By comparison, the guinea pig maximization test (GPMT) identified 83% (5 of 6) as sensitizers and 17% (1 of 6) as nonsensitizers (when there were multiple calls in the GPMT, the most conservative call was used). The LLNA and the guinea pig results were in agreement 33% of the time. The overprediction rate for the LLNA was 100% (1 of 1), and the underprediction rate was 60% (3 of 5).

Natural Complex Substances: The current LLNA database contains data for 12 natural complex substances (essential oils and absolutes) with comparative LLNA and human data. Essential oils are derived from a natural source using steam or pressure. Absolutes are purified extracts from natural products. Both essential oils and absolutes are composed of more than one component.

Of the 12 natural complex substances, the LLNA classified 75% (9 of 12) as sensitizers and 25% (3 of 12) as nonsensitizers. However, human clinical studies identified only 33% (4 of 12) of these substances as sensitizers. Therefore, among these 12 substances, the LLNA was able to identify three out of four of the substances that tested positive in human testing.

Six substances that did not produce positive results in human testing were positive in the LLNA. Compared to human outcomes, the LLNA had an accuracy of 42% (5 of 12), a sensitivity of 75% (3 of 4), a specificity of 25% (2 of 8), a false positive rate of 75% (6 of 8), and a false negative rate of 25% (1 of 4). There are no data from guinea pig tests for these natural complex substances; therefore, the performance of the LLNA and the guinea pig tests could not be compared to the human outcome.

Use of the LLNA for Testing Metal Compounds

The NICEATM LLNA database includes test results from 48 studies involving 16 metal compounds. The compounds in turn represent 13 different metals (mixtures containing metals are excluded from this analysis). All 16 metal compounds had comparative human data, and 8 had comparative guinea pig data. Among the 13 metals tested multiple times, nickel was tested four times in the LLNA as nickel sulfate, and three times as nickel chloride. Because nickel was classified as a sensitizer in three of these studies and as a nonsensitizer in four, a decision was made to exclude nickel compounds from the LLNA metals performance analysis.

For the remaining 14 metal compounds (13 metals), the LLNA had an accuracy of 86% (12 of 14), a sensitivity of 100% (9 of 9), a specificity of 60% (3 of 5), a false positive rate of 40% (2 of 5), and a false negative rate of 0% (0 of 9) when compared to human results. The two false positive compounds were copper chloride and zinc sulfate.

The LLNA identified as sensitizers all six of the metal compounds (six different metals with nickel compounds excluded) with comparative guinea pig test results. The LLNA results had an accuracy of 83% (5 of 6), a false positive rate of 100% (1 of 1), and a false negative rate of 0% (0 of 5) when compared to guinea pig test results.

NICEATM compared the performance of the LLNA and the guinea pig tests to that of human tests for the six metal compounds tested in all three species. The LLNA had an accuracy of 83% (5 of 6), a false positive rate of 100% (1 of 1), and a false negative rate of 0% (0 of 5). By comparison, the guinea pig test had an accuracy of 100% (6 of 6), a false positive rate of 0% (0 of 1), and a false negative rate of 0% (0 of 5) against the human test.

Use of the LLNA for Substances Tested in Aqueous Solutions

The NICEATM LLNA database for aqueous solutions includes data from 171 studies that involved 139 substances. Ninety-one of these substances (123 LLNA studies) are pesticide formulations and pure compounds. Forty-eight substances (48 LLNA studies) are aqueous eluates of medical devices.

Because of differences in the protocols for sample preparation, NICEATM analyzed the two groups separately. Of the 91 pesticide formulations and pure compounds, 63% (57 of 91) were LLNA positive, and 37% (34 of 91) were LLNA negative. Of these 91 LLNA studies, 66 used CBA mice, and 28 used BALBc. The mouse strain was not specified for 29 studies. The substances included in this evaluation were tested in the LLNA at a final concentration of at least 20% water.

Guinea pig data were available for 25 substances tested in aqueous solutions (4 sensitizers/21 nonsensitizers in the guinea pig). Eleven substances had LLNA test results that differed from the guinea pig results. Ten of the 11 discordant substances were pesticide formulations tested in aqueous 1% Pluronic L92. These were the same 10 substances discussed for the pesticide formulations analysis. All were overpredicted by the LLNA with respect to the guinea pig results (48% overprediction [10 of 21 tests]). One additional substance, neomycin sulfate, which was tested in 25% EtOH, was underpredicted by the LLNA (25% underprediction [1 of 4]). Overall, the LLNA and the guinea pig results were in agreement 56% of the time (14 of 25).

Human data were available for only four substances tested in aqueous solutions. Three were classified as sensitizers, and one was classified as a nonsensitizer in humans. Only two substances tested in aqueous solutions in the LLNA had comparative guinea pig and human data. Thus, not enough substances were tested in multiple test methods (e.g., LLNA, guinea pig, and human) to allow for a meaningful calculation.

All 48 of the medical device eluates were negative in the LLNA. None of the eluates had associated guinea pig or human data. They were not analyzed to determine their constituents or whether any compound(s) were in fact eluted from the medical device tested. Because the LLNA results were uniformly negative and no sample preparation control was included in the studies, the effectiveness of the sample preparation could not be determined. Therefore, the results from these eluates were not included in the final analysis with those from the pesticide formulations and pure substances tested in aqueous solutions.

1.0   Introduction

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

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

In February 1998, ICCVAM received a submission from Drs. G. Frank Gerberick (Procter and Gamble, Cincinnati, United States [U.S.]), David Basketter (Unilever Safety and Environmental Assurance Centre, United Kingdom [U.K.]), and Ian Kimber (Syngenta Central Toxicology Laboratory, U.K.) requesting an evaluation of the validation status of the LLNA as an alternative to the guinea pig maximization test (GPMT) and the Buehler test (BT) for assessing skin sensitization potential. The submission summarized the performance (relevance and reliability) of the LLNA as compared to the GPMT and BT methods. An additional analysis was conducted by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to evaluate, where comparable data existed, the comparative performance of the LLNA and the guinea pig (GP) tests against sensitization results obtained in humans. An independent expert peer review panel (Panel) meeting was convened on September 17, 1998, to review the completeness of the submission, to determine whether the usefulness and limitations of the LLNA had been adequately described, and to decide whether its demonstrated performance supported recommending the LLNA as a stand-alone alternative to the GPMT and BT. The Panel also was asked to evaluate whether the LLNA offered advantages with regard to animal welfare considerations (i.e., refinement, reduction, or replacement[5]).

The Panel considered the performance of the LLNA to be similar to that of the GPMT and BT for identifying moderate to strong sensitizers. The Panel concluded that the LLNA did not accurately predict all weak sensitizers, nor did it adequately discriminate between strong skin irritants and skin sensitizers. The LLNA also produced false negative results with some metals. It was recommended that these issues be evaluated in future studies and workshops. Furthermore, data to support using the LLNA to test mixtures and substances tested in aqueous solutions were not provided and the evaluation of pharmaceuticals was limited. Still, the Panel noted that when compared with the GPMT and BT methods, the LLNA appeared to provide equivalent prediction of risk for human ACD, based on comparisons to available human data.

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[5]   *Refinement alternative* is defined as a new or revised test method that refines procedures to lessen or eliminate pain or distress to animals, or enhances animal well-being. *Reduction alternative* is defined as a new or revised test method that reduces the number of animals required. *Replacement alternative* is defined as a new or revised test method that replaces animals with non-animal systems or one animal species with a phylogenetically lower one (e.g., a mammal with an invertebrate) (ICCVAM 1997).

In addition, the Panel concluded that the LLNA could be considered a refinement alternative to the GPMT and BT, because the pain and distress due to sensitization associated with the guinea pig methods could be virtually eliminated by using the LLNA. ICCVAM agreed that the LLNA test method, when modified and used in accordance with the Panel report, can be used effectively for assessment of skin sensitization potential (ICCVAM 1999 [available in **Annex I**]).

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]).

NICEATM conducted this revised evaluation of the LLNA applicability domain in response to a nomination[6] submitted to ICCVAM in January 2007 by the U.S. Consumer Product Safety Commission. This Addendum to the ICCVAM (1999) report contains an evaluation of the current database for the LLNA when used to test pesticide formulations and other products, metals, and substances in aqueous solutions in order to fill some of the data gaps identified in the original evaluation (see **Annex I**).

An independent peer review panel (Panel) reviewed this Addendum in March 2008 to evaluate the extent to which the information contained in this Addendum supported the draft recommendations. The draft recommendations stated that more data would be needed before a recommendation on the usefulness and limitations of the traditional LLNA for testing mixtures could be made, due to the limitations associated with the available mixtures database (i.e., unknown formulae, lack of human data). The Panel agreed that the draft recommendation with respect to the traditional LLNA testing of mixtures appeared valid based on the limitations inherent in the available data set. Still, the Panel urged that the ICCVAM recommendations indicate that the approach may be viable. The Panel further recommended that the test method recommendations summary should indicate that the limitations include relatively poor concordance of traditional LLNA outcomes for mixtures with those obtained in GP tests. Routine comparisons of accuracy according to classification criteria may not be sufficient to evaluate the concordance for mixtures, and furthermore, the GP tests are not necessarily valid for mixtures. The Panel also indicated that the term *mixtures* was used too broadly (i.e., can represent an infinite number of materials) and it would be more beneficial to specify types or formulations of mixtures that are being examined. The analyses in this Addendum have been done separately on pesticide formulations, dyes, and natural complex substances in response to the Panel's comment.

The draft recommendations also stated that, based on the available data for metals, the traditional LLNA was useful for the testing of metal compounds, with the exception of nickel. Based on the available information, the Panel agreed that the draft recommendations with regard to testing metals appeared to be valid. A minority Panel opinion stated that it should not be concluded that the traditional LLNA was not suitable for testing nickel compounds, because the different vehicles used may have had a significant impact on the ability of nickel to penetrate the skin and be bioavailable.

The draft recommendations also stated that, due to the limited number of substances tested in aqueous solutions, more data would be needed before a recommendation on the usefulness and limitations of the traditional LLNA for testing substances in aqueous solutions could be made. The Panel agreed that the draft ICCVAM recommendation was appropriate and that more data were required before an adequate evaluation of the use of the traditional LLNA with aqueous solutions could be conducted.[7]

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[6] Available at http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf
[7] Available at http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

The data summarized in this Addendum are based on information obtained from the peer-reviewed scientific literature identified through online searches via PubMed and SCOPUS, through citations in publications, and in response to a *Federal Register* (*FR*) notice requesting LLNA, guinea pig, and/or human skin sensitization data and experience (Vol. 72, No. 95, pp. 27815-27817[8]). Key words used in the online searches for this evaluation were "LLNA" OR "Local Lymph Node" OR "Local lymph node" OR "local lymph node" AND (mixture* OR formula*)" OR ("metal* OR aqueous*)". Additionally, a weekly search on SCOPUS that uses the key words (TITLE-ABS-KEY(**sensi***) AND TITLE-ABS-KEY(**skin** OR **dermal**)) is done. Since March 2008, six relevant papers were added to the database.

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[8] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf

2.0 Substances Used for the Revised Evaluation of the Applicability Domain for the LLNA

The information summarized in this Addendum is based on a retrospective review of LLNA data derived from a database of over 600 substances (including pesticide formulations and other products) tested in the LLNA and builds on the previous ICCVAM evaluation of the LLNA, which was based on 209 substances (ICCVAM 1999). For this evaluation, to minimize the complexity of the analysis, metal formulations are not included in the analysis of pesticide formulations and other products, and metal compounds were restricted to those testing single substances. The reference database includes data for metal compounds from the original ICCVAM evaluation (**Annex I**), data published since that evaluation, and data submitted in response to a request in the previously cited *FR* notice. Since an evaluation of the usefulness and limitations of pesticide formulations and other products, and substances tested in aqueous solutions were not included in original ICCVAM validation (**Annex I**), because no data on these substances were available, the reference database for these substances consists of data published since the original ICCVAM evaluation or submitted in response to the *FR* notice. **Table D-1** provides information on the sources of the data and the rationale for the substances tested.

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

Data Source	N	Substance Selection Rationale
AppTec Laboratory Services	48	Aqueous eluates from medical devices
Dow AgroSciences	52	Pesticide formulations analyzed in the LLNA with associated GP data of various kinds
Dupont	28	Pesticide formulations analyzed in the LLNA
ECPA	39	Plant protection products (i.e., pesticides) were evaluated in the LLNA with a novel vehicle to assess its usefulness
Basketter et al. (1994, 1996, 1999a, 2005)	16	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
Lalko and Api (2006)	12	Original research that evaluated natural complex substances in the LLNA. Additional data were submitted by the authors and RIFM.
Ryan et al. (2000)	2	Interlaboratory study to evaluate the accuracy of the LLNA to identify human sensitizers.
Ryan et al. (2002)	11	Original research with known water soluble haptens and known skin sensitizers to assess the usefulness of a novel vehicle in the LLNA
E. Debruyne (Bayer Crop Science SA)	10	Original research on different pesticide types and formulations in the LLNA
Kimber et al. (1991, 1995, 2003)	9	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
Gerberick et al. (2005)[1]	6	Compiled from previously conducted LLNA studies (from published literature and unpublished sources) on substances of varying skin sensitization potential

Continued

Table D-1   Summary of Data Sources and Rationale for Substance Selection (Continued)

Data Source	N	Substance Selection Rationale
Bundesanstalt für Arbeitsschutz und Arbeitsmedizin	6	Original LLNA research on dye formulations
H.W. Vohr (BGIA)	4	Original LLNA research with epoxy resin components as part of a validation effort for non-radioactive versions of the LLNA
Basketter and Scholes (1992)[2]	2	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
Gerberick et al. (1992)	2	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
D. Germolec (NIEHS)	2	Substances were evaluated by NTP for skin sensitization potential in the LLNA.
Lea et al. (1999)	2	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
M.J. Olson (GlaxoSmithKline)	2	Pharmaceutical substances tested in the LLNA
Unilever (unpublished data)	2	Metal substances evaluated for skin sensitization potential in the LLNA
Basketter and Kimber (2006)	1	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
Goodwin et al. (1981)	1	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
Griem et al. (2003)	2	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
Kligman (1966)	1	Compiled from previously conducted LLNA studies on substances of varying skin sensitization potential
J. Matheson (CPSC)	1	Published LLNA data submitted to NICEATM, as a reference
K. Skirda (CESIO - TNO Report V7217)	1	Data were provided by CESIO member companies for use in paper titled "Limitations of the LLNA as preferred test for skin sensitization: concerns about false positive and false negative test result."
Total	262	

Abbreviations: BGIA = Berufsgenossenschaftliches Institut fur Arbeitsschutz; CESIO = Comité Européen des Agents de Surface et de leurs Intermédiaires Organiques; CPSC = U.S. Consumer Product Safety Commission; ECPA = European Crop Protection Association; GP = guinea pig; LLNA=local lymph node assay; NICEATM = National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods; NIEHS = National Institute of Environmental Health Sciences; NTP = National Toxicology Program; RIFM = Research Institute for Fragrance Materials: 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] These LLNA studies used both male and female mice, but single experiments were limited to one sex.

LLNA studies for 29/89 of the pesticide formulations (tested in aqueous solutions) used the BALB/c mouse strain rather than the CBA/J and CBA/Ca strains of mice, which are recommended for the LLNA by ICCVAM (ICCVAM 1999, Dean et al. 2001, EPA 2003), and the OECD (OECD 2002). The comparative performance of the LLNA using these different strains relative to the guinea pig is detailed in **Section 5.0**. Two additional submitted LLNA studies (from Dr. Dori Germolec at the National Institute of Environmental Health Sciences [NIEHS]) also used the BALB/c strain. One of these, sodium metasilicate (an aqueous solution), did not have comparative GP or human data and thus was not included in the performance analysis. The other study was for potassium dichromate (a metal), which was positive in the LLNA, GP, and human. As there are 22 LLNA studies for potassium dichromate included in **Annex III-2**, all of which are positive, excluding this study would have no impact on the performance analysis for metals. Two other studies cited in Griem et al. (2003) used both male and female mice, but single experiments were limited to one sex. These data were included in the evaluation.

To the extent possible, **Annexes II-1**, **II-4**, **II-6**, **III-1**, and **IV-1** provide information on the physicochemical properties (e.g., physical form), Chemical Abstracts Service Registry Number (CASRN), and chemical class for each pesticide formulation, dye, fragrance ingredient, metal compound, and substance tested in an aqueous solution, respectively. This information was obtained from published reports, submitted data, or through literature searches.

When available, chemical classes for the test substances were retrieved from the National Library of Medicine's ChemID Plus database. If chemical classes were not located, where possible, they were assigned for each test substance using a standard classification scheme, based on the National Library of Medicine Medical Subject Headings (MeSH) classification system[9]. Some substances were assigned to more than one chemical class; however, no substance was assigned to more than three classes. One complex pharmaceutical intermediate was simply identified as a pharmaceutical substance. Material families for the active ingredients in the formulations submitted by Dow AgroSciences were provided by Dow AgroSciences.

The generic composition of some of the formulated products evaluated by the European Crop Protection Association (ECPA) (Dinocap EC, Oxyfluorfen EC, Quinoxyfen/cyproconazole, and Trifluralin EC) and the formulations submitted by Dow AgroSciences, using the LLNA, is included in **Annex II-3**. For the formulations provided by ECPA, none of the active ingredients have been tested using the LLNA but the active ingredients have been tested previously in a guinea pig test (personal communication by Dr Eric Debruyne, Bayer CropScience in France). Likewise, none of the inerts (e.g., surfactants, solvents, etc.) have been tested independently for these formulations. Dow AgroSciences provided information about LLNA and guinea pig tests on active ingredients and inerts for the formulations they submitted. The component information for the remaining pesticide formulations have been requested by NICEATM, but since some of the data is proprietary, it is not available at this time.

One hundred and four pesticide formulations (i.e., herbicides, fungicides, insecticides) were evaluated for this Addendum. All of these were liquids, though some were in the form of suspensions or emulsions, and were tested in an aqueous vehicle. Six dyes (all solids), and 12 natural complex substances (all liquids), which are a combination of essential oils and absolutes, were also evaluated. Essential oils are oils derived from a natural source using steam or pressure. Absolutes are purified extracts from natural products. Both essential oils and absolutes are substances comprised of more than one component.

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[9] Available at http://www.nlm.nih.gov/mesh/meshhome.html

Of the 13 metal compounds evaluated, one (potassium dichromate) is used in leather tanning and as an oxidizer in organic synthesis. Most of the remaining 12 metals in the analysis are used as catalysts, conductors of electricity, or for coating and plating. All of the metal compounds for which information on physical form is identified are solids.

Of the 21 substances tested in aqueous solutions included in this evaluation, six are pesticides (i.e., herbicides, fungicides, and insecticides); this is the only product class represented by more than one substance tested in an aqueous solution.

3.0 .Comparative *In Vivo* Reference Data

The reference database for this evaluation includes results using currently accepted guinea pig test methods for skin sensitization (i.e., the GPMT and the BT) and human clinical studies and experience (e.g., human repeat insult patch test [HRIPT], human maximization test [HMT], case reports). In the absence of HRIPT or HMT data, the classification of a substance as a human sensitizer was based on the classification of the authors of the report. National and international test guidelines are available for each of these standardized tests and are thus described in detail elsewhere (EPA 2003; OECD 1992).

Ongoing efforts are being made by NICEATM to obtain the original records for all of the reference data used in this evaluation. Ideally, all data supporting the validity of a test method should be obtained and reported from animal studies conducted in accordance with Good Laboratory Practice (GLP) guidelines (EPA 2006a, 2006b; FDA 2007; OECD 1998). Equally, data based on human studies should be conducted in compliance with Good Clinical Practices (GCP) guidelines (ICH 1996). Both sets of guidelines provide an internationally standardized procedure for the conduct of studies, reporting requirements, archival 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 human or guinea pig studies were compliant with GCP or GLP guidelines, respectively, is based on the information provided in published and submitted reports. The GP data obtained from E. Debruyne (Bayer CropScience SA) and P. Botham (ECPA), and Dow AgroSciences, were reportedly conducted according to GLP guidelines. None of the published references from which GP or human data were obtained include specifics on GCP or GLP compliance.

4.0   LLNA Data and Results

The data used for this evaluation were obtained from 25 sources (**Table D-1**). No new LLNA studies were conducted to generate data for this evaluation (see **Section 2.0**). Where available, specific information including name, CASRN, physicochemical properties (e.g., molecular weight, Log K_{ow}), chemical class[10] and data source are indicated for each pesticide formulation, dye, fragrance ingredient, metal compound, and substance tested in an aqueous solution (**Annexes II-1, II-4, II-6, III-1,** and **IV-1**, respectively). The concentrations tested, along with calculated stimulation index (SI) and/or EC3 (the concentration that induces an SI of 3) values, are provided in **Annexes II-2**, **II-5, B7, III-2,** and **IV-2** for pesticide formulations, dyes, natural complex substances, metal compounds, and substances tested in an aqueous solution, respectively. Individual components and concentrations of the pesticide formulations and substances tested in an aqueous solution submitted by Bayer have been requested, but due to confidential and proprietary issues, Bayer has only been able to provide the generic composition for four formulated products (see **Section 2.0**). Furthermore, provided in the submitted data or study reports, the source or purity of the test substance was not known.

LLNA classification as to whether a substance was a sensitizer or a nonsensitizer was based on study data extracted from the sources listed in **Table D-1** and **Annexes II-1, II-4, II-6, III-1,** and **IV-1,** with two exceptions. Classification of ammonium tetrachloroplatinate and gold (III) chloride (both of which are metal compounds) as sensitizers by the LLNA was based on published reference classifications (Basketter and Scholes 1992, Basketter et al. 1999a) and not on actual LLNA data.

The LLNA data included in the ICCVAM (1999) database (**Annex I**) were reviewed during the original evaluation. However, the availability of the original data for the other studies included in this evaluation has not yet been established for all data sources. Additionally, coding of substances to avoid potential scoring bias was not described in the previous evaluation of 209 substances (ICCVAM 1999; **Annex I**) or for any of the newly obtained studies used in this evaluation.

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[10] 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).

5.0   Accuracy of the LLNA: Revised Applicability Domain

The ability of the LLNA to correctly identify pesticide formulations and other products, metal compounds, and substances tested in aqueous solutions as potential skin sensitizers was evaluated when compared to human and guinea pig data. The classification of pesticide formulations, dyes, fragrance ingredients, metal compounds, and substances tested in aqueous solutions and the relevant data for each substance is located in **Annexes II-2**, **II-5, II-7, III-2**, and **IV-2**, respectively. For comparison purposes, the performance of the LLNA database reported in the ICCVAM evaluation report (ICCVAM 1999; **Annex I**) is included in **Tables D-4**, **D-6**, **D-8**, **D-11**, and **D-14**. For this addendum, substances containing multiple components were analyzed separately as pesticide formulations, dyes, and fragrance ingredients.

5.1   Testing of Pesticide Formulations and Other Products

The original ICCVAM LLNA report (ICCVAM 1999) (**Annex I**) did not include an analysis on the ability of the LLNA to predict the skin sensitizing potential of pesticide formulations and other products, because data were not available for that evaluation. Thus, all of the analyses below for pesticide formulations, dyes and fragrance ingredients are new material in this addendum.

5.1.1   Testing of Pesticide Formulations

The current LLNA database contains data for 104 pesticide formulations for which LLNA data exists. The physicochemical properties of these formulations are in **Annex II-1,** and the data analyzed here are in **Annex II-2**.

For these formulations, 54% (56/104) were classified as sensitizers in the LLNA, and 46% (48/104) were classified as nonsensitizers. For substances that were tested multiple times in the LLNA, classification as a sensitizer or nonsensitizer was made by a majority call (i.e., the most prevalent call that occurred among the studies). For example, five independent studies were considered for the formulation Oxyfluorfen EC. The highest SI values observed for the various studies were 5.4, 4.9, 3.1, 2.8, and 2.3, respectively (all of these SI values occurred with a test concentration of 33%). Since an SI value ≥ 3 occurred in three of the five studies, Oxyfluorfen EC was classified as a sensitizer in the LLNA, even though two studies (SIs = 2.8 and 2.1, respectively) would have resulted in classification as a nonsensitizer if considered alone.

Seventy of the 104 pesticide formulations have LLNA and some type of guinea pig reference data. A total of 89 LLNA studies were performed using these 70 formulations. LLNA studies were conducted with either CBA/Ca or CBA/J (61/89) and/or BALB/c (28/89) mouse strains.

Six formulations were tested in multiple LLNA studies (25 studies total [**Table D-2]**). LLNA results for 5/6 formulations were in agreement across multiple studies, and LLNA results for 1/6 formulations were discordant across multiple studies (3 positive, 2 negative [**Table D-3**]).

Twenty-three formulations had associated GP data for the formulation itself, 46 formulations had GP data for one or more of the active ingredients in the formulation, and 14 formulations had GP data for a substance related to an active ingredient, or for a related formulation. The performance of the LLNA against GP tests for pesticide formulations with GP data for the entire formulation is discussed in **Section 5.1.1.1**. The performance of the LLNA against GP tests for pesticide formulations with GP data for active ingredients or related substances and formulations is discussed in **Annex V.**

All formulations (89/89 studies) were tested in the LLNA in 1% Pluronic L92. Pluronic L92 block copolymer is a surfactant and wetting agent that has been evaluated as an alternative aqueous-based vehicle for use in the LLNA. Pluronic L92 was chosen for evaluation because it promotes test material retention on the ear by preventing run-off, and exhibits low acute toxicity and irritation potential (Boverhof et al. 2008; Ryan et al. 2002). Ryan et al. (2002) assessed the performance of Pluronic L92 relative to other solvents in the LLNA using aqueous soluble haptens. Based on their

results, they determined that, for identification of sensitization hazard of aqueous soluble materials using the LLNA, dimethylformamide (DMF), and dimethylsulfoxide (DMSO) were the preferred vehicles. However, if a test material is not soluble in DMF or DMSO, or if higher test concentrations could be achieved in an aqueous vehicle, then 1% Pluronic L92 might improve assay performance over the use of water as a vehicle.

In an interlaboratory study (n=5 laboratories), Boverhof et al. (2008) conducted LLNA tests on three substances with known sensitization potential (hexylcinnamaldehyde, formaldehyde, and potassium dichromate), and four pesticide formulations for which the sensitization potential in guinea pigs and/or humans had previously been determined, using Pluronic L92 as the vehicle. They concluded that the LLNA results for all of these substances when tested in Pluronic L92 were consistent with previous GP or human results, and that Pluronic L92 was a suitable vehicle to use when testing aqueous solutions in the LLNA.

For the 52 formulations submitted by Dow AgroSciences, a list of all of the components in the formulation (albeit some were listed generically [e.g., emulsifier, biocide, etc.]) was also provided, along with information as to whether each component was a sensitizer. For these components, the criteria for classification as a sensitizer were not specified. **Annex II-3** contains the information on components provided by Dow AgroSciences.

Table D-2   Pesticide Formulations with Multiple LLNA Studies

Formulation	Source	No. Studies	Mouse Strain	No. Positive Studies	No. Negative Studies	No. Labs
Atrazine SC	ECPA	2	CBA	2	0	2
Dinocap EC	ECPA	5	CBA	5	0	5
Formulation 7	Dow AgroSciences	2	BALB/c	2	0	1
Oxyfluorfen EC	ECPA	5	CBA	3	2	5
Quinoxyfen / cyproconazole	ECPA	6	CBA	6	0	6
Trifluralin EC	ECPA	5	CBA	5	0	5

Abbreviations:
   EC = emulsion concentrate; ECPA= European Crop Protection Association; No. = number; SC = suspension concentrate.

Table D-3   LLNA Data for Pesticide Formulation with Discordant Results

Formulation	Vehicle	Conc. (%)	SIs	Strain	EC3 (%)	Lab
Oxyfluorfen EC	L92	1, 7, 33	0.8, 1.4, 4.9	CBA/Ca	30.8	1
		1, 7, 33	0.9, 1.4, 2.8	CBA/J	NC	2
		1, 7, 33	0.3, 0.9, 2.3	CBA/J	NC	3
		1, 7, 33	1.1, 1.5, 3.1	CBA/JHsd	30.8	4
		1, 7, 33	1.2, 1.2, 5.4	CBA/CaOlaHsd	18.1	5

Abbreviations:
   Conc. = concentration; EC = emulsion concentrate; EC3 = estimated concentration needed to produce an SI of 3; L92 = 1% aqueous pluronic L92; NC = not calculated since SI<3.0; SIs = stimulation indices.

5.1.1.1 Testing of Pesticide Formulations: LLNA vs. GP with Available Reference Data for the Entire Formulation

For the 23 formulations that had associated GP data for the formulation itself, 13% (3/23) were classified as sensitizers and 87% (20/23) as nonsensitizers according to the GP results (**Figure D-1**). Twenty-one of these GP tests were BT and 2 were GPMT. These results are based on a positive overall GP call for formulation EXP 10810.[11] Ten out of the approximately 450 active ingredients registered with EPA were represented among these 23 formulations. Furthermore, approximately 40 different classes of pesticides are registered with EPA, of which these nine active ingredients represent a small proportion (i.e., one insecticide, one microbiocide, six herbicides and two fungicides).

Twenty of the LLNA studies were conducted in CBA mice (i.e., the preferred strain for use in the LLNA according to the ICCVAM recommended LLNA protocol and OECD TG 429) and three studies were conducted in BALB/c mice. The LLNA classified 57% (13/23) of the formulations as sensitizers and 43% (10/23) as nonsensitizers (**Figure D-1**). All three of the pesticide formulations identified as sensitizers in the GP test were also identified as sensitizers in the LLNA. The LLNA also identified an additional seven substances as sensitizers that were classified as nonsensitizers in the GP test (**Table D-4**).

If only LLNA studies using CBA mice are considered, three LLNA studies conducted with BALB/c mice are removed from the database, which eliminates two LLNA positive studies, and one LLNA negative study. Based on the remaining 20 LLNA studies, the LLNA classified 55% (11/20) of the formulations as sensitizers and 45% (9/20) as nonsensitizers (**Figure D-1**). This does not change the fact that all three of the pesticide formulations identified as sensitizers in the GP test were also identified as sensitizers in the LLNA, and that seven substances identified as sensitizers in the LLNA are classified as nonsensitizers in the GP test (**Table D-4**).

There were no comparative human data with which to determine the actual human sensitization potential.

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[11] Formulation EXP 10810 A (submitted by E. Debruyne, Bayer Crop Science), the only formulation for which there was data in both the GPMT and the BT, showed equivocal results in the guinea pig. This formulation tested positive in the GPMT (sensitization incidence 100%), and negative in the BT (sensitization incidence 10%). The patch concentration in the GPMT was the same as the induction concentration in the BT (50%).

Figure D-1   Numbers of Positive and Negative LLNA and GP Calls for Pesticide Formulations
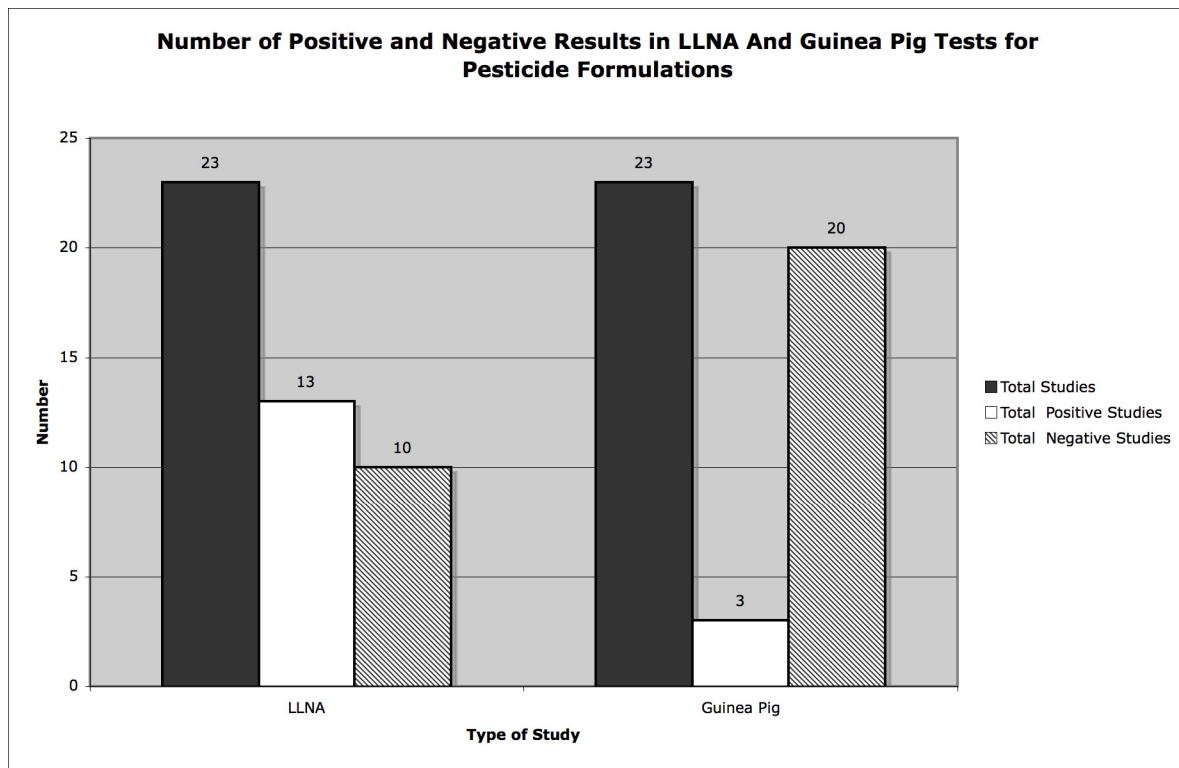
Abbreviations: LLNA = local lymph node assay.

Based on the 23 pesticide formulations tested in CBA (n=20) and BALB/c (n=3) strains, the accuracy of the LLNA compared to guinea pig data was 57% (13/23), the sensitivity was 100% (3/3), the specificity was 50% (10/20), the false positive rate was 50% (10/20) and false negative rate was 0% (0/3). If the three studies using BALB/c mice are not considered, the accuracy of the LLNA compared to guinea pig data was 60% (12/20), the sensitivity was 100% (3/3), the specificity was 53% (9/17), the false positive rate was 47% (8/17), and the false negative rate was 0% (0/3) (**Table D-4**).

Table D-4   Evaluation of the Performance of the LLNA for Testing Pesticide Formulations

Comparison[1]	n[2]	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate	
		%	No.[3]	%	No.[3]	%	No.[3]	%	No.[3]	%	No.[3]
LLNA[4] vs. GP[5]	23	57	13/23	100	3/3	50	10/20	50	10/20	0	0/3
LLNA[6] vs. GP[5]	20	60	12/20	100	3/3	53	9/17	47	8/17	0	0/3
ICCVAM 1999 Database: Evaluation of LLNA Data vs. GP Data or Human Data[7]											
LLNA[6] vs. GP[5]	126	86	108/126	87	81/93	82	27/33	18	6/33	13	12/93
LLNA[6] vs. Human[8]	74	72	53/74	72	49/68	67	4/6	33	2/6	28	19/68
GP[5] vs. Human[8]	62	73	45/62	71	42/59	100	3/3	0	0/3	29	17/59

Abbreviations:
    GP = guinea pig skin sensitization outcomes; LLNA = local Lymph Node Assay; No. = number.

Accuracy (concordance) = the proportion of correct outcomes (positive and negative) of a test method

Sensitivity = the proportion of all positive substances that are classified as positive

Specificity = the proportion of all negative substances that are classified as negative

False negative rate = the proportion of all positive substances that are falsely identified as negative

False positive rate = the proportion of all negative substances that are falsely identified as positive

[1] This accuracy analysis is only for formulations that have LLNA data and some type of associated GP data; none of the pesticide formulations analyzed had human data, so a comparison between LLNA vs. human and LLNA vs. GP is not included.

[2] n = number of substances included in this analysis

[3] The data on which the percentage calculation is based

[4] LLNA studies conducted with CBA (n=20) and BALB/c (n=3) mice

[5] P refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.

[6] LLNA studies conducted with CBA mice

[7] For comparison purposes, an excerpt from the ICCVAM evaluation report (ICCVAM 1999; Annex I) showing the overall performance of the LLNA vs. GP and human, and GP vs. human is included here.

[8] *Human* refers to outcomes obtained by studies conducted using the human maximization test or the inclusion of the test substance in a human patch test allergen kit.

Among the 10 of 23 formulations classified as sensitizers by the LLNA that were classified as nonsensitizers in the GP (**Table D-5**), eight were classified as nonsensitizers based on BT results and two were classified as nonsensitizers based on GPMT results.

Table D-5   Pesticide Formulations that are Classified as Sensitizers in the LLNA, but Classified as Nonsensitizers in the Guinea Pig

Substance Name	LLNA Results				GP Results			Skin Irritant?
	Conc. (%)[1]	SI[2]	EC3 (%)	Result[3]	Ind. Conc. (%)	Sens. Incid. (%)	Result[3]	
Atrazine SC	100	7.3	36.4[4]	+	30	0	-[5]	Nonirritant at ≤ 25%[6]
BASF SE-1	70	22.7	5.5	+	100	0	-[7]	Nonirritant at ≤ 50%[6]
EXP 11120 A	100	5.3	64.9	+	100	0	-[7]	Nonirritant at 100%[6]
F & Fo WG 50 + 25	25	15.2	0.003	+	30	0	-[7]	Nonirritant at ≤ 10%[6]
FAR01060-00	100	3.6	88.5	+	100	0	-[7]	Nonirritant at 100%[6]
Formulation 2[8]	80	15.8	15.7	+	NA	NA	-[7]	Nonirritant at 80%[9]
Formulation 7[8]	100	3.2	85	+	100	0	-[7]	Nonirritant at 80%[9]
Fx + Me EW 69	50	8.6	25.2	+	100	0	-[7]	Nonirritant at 100%[6]
Oxyfluorfen EC	33	5.4	30.8[10]	+	10	26	-[5]	Nonirritant at ≤ 25%[6]
Trifluralin EC	100	75.2	10.3[11]	+	50	10	-[7]	Nonirritant at ≤ 25%[6]

Abbreviations:

Conc. = concentration; EC = emulsion concentrate; EC3 = estimated concentration needed to produce a stimulation index of 3; EW = emulsion, oil in water; GP = guinea pig; Ind. Conc. = induction concentration; LLNA = local lymph node assay; NA = not available; SC = suspension concentrate; Sens. Incid. = sensitization incidence; SI = stimulation index; WG = water-dispersible granules

[1]   Maximum concentration tested in the LLNA

[2]   Maximum SI obtained in the LLNA

[3]   (-) = nonsensitizer, (+) = sensitizer

[4]   Mean value from two studies

[5]   Guinea pig maximization test (GPMT) result

[6]   Based on challenge concentration from a GPMT or Buehler test (BT)

[7]   BT result

[8]   LLNA conducted in BALB/c mice

[9]   Based on irritation prescreen in mice

[10]   Mean from three positive studies

[11]   Mean of five studies

The constituents of most of the formulations are unknown (**Annex II-3**). Formulation 2 contains a biocide (at a concentration of 0.54 g/L) that is a sensitizer according to constituent information provided by Dow AgroSciences (**Annex II-3**). Dow Agrosciences categorizes all other constituents of Formulation 2 as nonsensitizers, including the active ingredients fluroxypyr-meptyl and florasulam (**Annex II-3**). Formulation 7 contains the sensitizers quinoxyfen (active ingredient at a concentration of 45 g/L) and a biocide (at a concentration of 0.37 g/L); it is unknown whether this is the same biocide that is a constituent of Formulation 2. Formulation 7 also contains the active ingredient mycyclobutanil, which, when tested by Dow AgroSciences in GP sensitization tests, gave equivocal results (**Annex II-3**).

Six of the overpredicted formulations based on LLNA results compared to GP results (BASF SE-1, EXP 11120 A, F & Fo WG 50 + 25, FAR01060-00, Formulation 7, and Fx + Me EW 69; see **Table D-5**) were tested in the GP at induction concentrations equal to or greater than the highest concentration tested in the LLNA. However, atrazine tested as a sensitizer at 100% in the LLNA, but tested as a nonsensitizer at 30% induction concentration in the GPMT; oxyfluorfen tested as a sensitizer at 33% in the LLNA but tested as a nonsensitizer at 10% induction concentration in the GPMT; and trifluralin tested as a sensitizer at 100% in the LLNA, but tested as a nonsensitizer at 50% induction concentration in the BT **(Table D-5)**.

The EC3 values for most (9/10) of the formulations indicated that they produced weak to moderate responses in the LLNA (EC3 range of 5.5% to 88.5%) **(Table D-5)**. However, the EC3 value for the formulation F & Fo WG 50 + 25 (EC3 = 0.003%) is a very strong LLNA response. This could be because the LLNA dose-response curve approached saturation (i.e., SI = 11.7 at 2.5%, SI = 15.2 at 25%) and the calculation of the EC3 was performed by extrapolation because no responses were below SI = 3 (**Annex II-2**). This EC3 value is likely a poor estimate of the actual value. However, based on the concentrations test, and the resulting SI values, the LLNA data do indicate that the EC3 for formulation F & Fo WG 50 + 25 is less than 2.5% (i.e., SI = 11.7 at 2.5%, the lowest concentration tested).

Five of the overpredicted formulations (Atrazine SC, BASF SE-1, F & Fo WG 50 + 25, Oxyfluorfen EC, and Trifluralin EC) were tested in the LLNA at potentially irritating concentrations. This is based on the concentration tested in the LLNA exceeding the reported challenge concentrations used in the BT or GPMT. According to the respective protocols for these guinea pig tests, the challenge concentration should be the maximum nonirritating concentration of a test substance (**Table D-5**).

5.1.1.2 *Testing of Pesticide Formulations: Comparison Between Mouse Strains CBA and BALB/c*

For the 70 pesticide formulations that had associated GP data, 43 were tested in the LLNA in CBA mice and 27 were tested in BALB/c mice. No formulation was tested in the LLNA in both strains. **Figure D-2** shows that the percentage of formulations that were classified as sensitizers was slightly higher in BALB/c mice (67% [18/27]) than in CBA mice (60% [26/43]).

Percentage of Formulations Classified as Sensitizers or Nonsenitizers by the LLNA in Two Mouse Strains
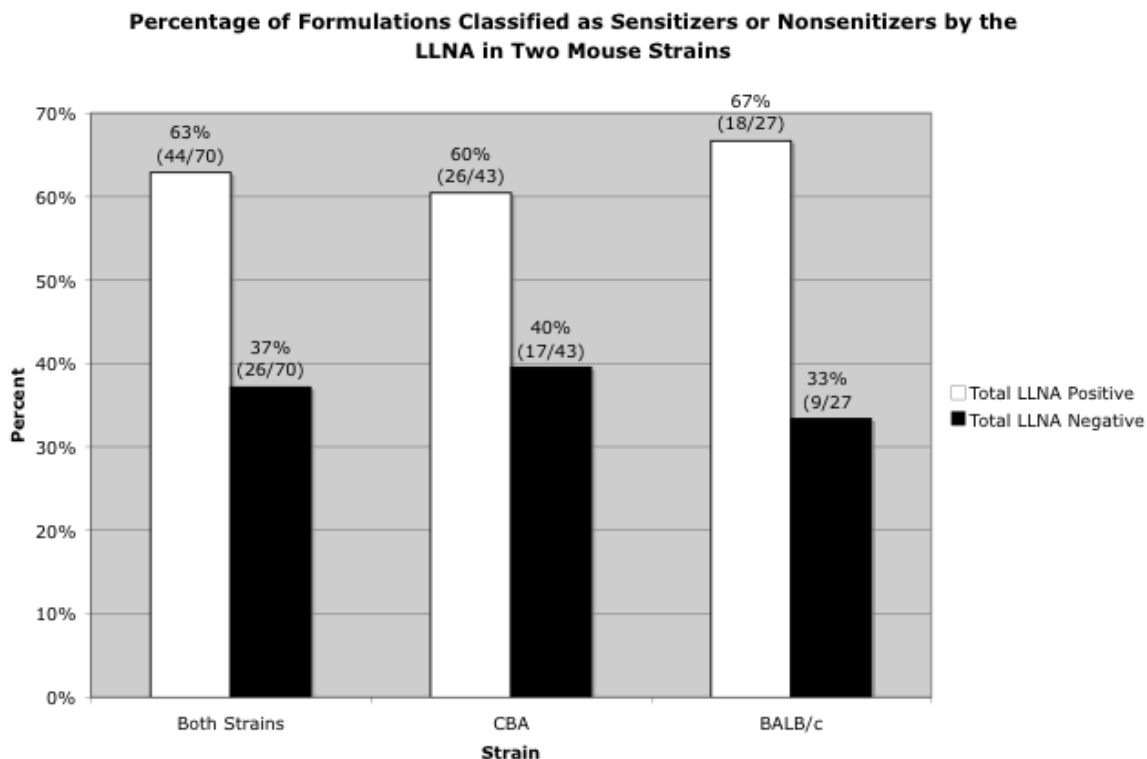
Figure D-2   Percentage of Formulations Classified as Sensitizers or Nonsensitizers in Two Mouse Strains

For the 23 pesticide formulations that were tested in both the GP and the LLNA, 20/23 were conducted using CBA mice and 3/22 were conducted using BALB/c mice. As noted in **Section 5.1.1.1**, when data for all 23 formulations is considered (i.e., using both CBA and BALB/c data), the overall accuracy is 57% (13/23), with false positive and false negative rates of 50% (10/20) and 0% (0/3), respectively. If only LLNA studies using CBA mice are considered, removing the three LLNA studies conducted with BALB/c mice from the database eliminates two LLNA positive studies, and one LLNA negative study, which only marginally impacts the overall accuracy (accuracy = 60% [12/20], false positive rate = 47% [8/17], and false negative rate = 0% [0/3]).

As mentioned previously, since comparative human data are not available for any of the formulations analyzed, an evaluation of these formulations in the LLNA compared to human performance could not be assessed. For the same reason, an evaluation of GP versus human outcomes is also not possible. Also, no formulations were evaluated in the ICCVAM evaluation report (ICCVAM 1999; **Annex I**), so these data and analyses cannot be compared to previously considered data.

5.1.2 Testing of Dyes

The current LLNA database contains data for six dyes, for which there is LLNA and GP data. The physicochemical properties of these dyes are in **Annex II-4,** and the data analyzed here are in **Annex II-5**. For these dyes, 50% (3/6) were classified as sensitizers in the LLNA, and 50% (3/6) were classified as nonsensitizers in the LLNA. In the GPMT, 83% (5/6) dyes tested as sensitizers. **Table D-6** provides the performance statistics for the LLNA when compared to GPMT outcomes for this limited dataset.

Table D-6 Evaluation of the Performance of the LLNA for Testing Dyes

Comparison[1]	n[2]	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate	
		%	No.[3]	%	No.[3]	%	No.[3]	%	No.[3]	%	No.[3]
LLNA vs. GPMT	6	33	2/6	40	2/5	0	0/1	100	1/1	60	3/5
ICCVAM 1999 Database: Evaluation of LLNA Data vs. GP Data or Human Data[4]											
LLNA vs. GP[5]	126	86	108/126	87	81/93	82	27/33	18	6/33	13	12/93
LLNA vs. Human[6]	74	72	53/74	72	49/68	67	4/6	33	2/6	28	19/68
GP[5] vs. Human[6]	62	73	45/62	71	42/59	100	3/3	0	0/3	29	17/59

Abbreviations:

  GP = guinea pig; GPMT = guinea pig maximization test; LLNA = local lymph node assay; No. = number.

Accuracy (concordance) = the proportion of correct outcomes (positive and negative) of a test method

Sensitivity = the proportion of all positive substances that are classified as positive

Specificity = the proportion of all negative substances that are classified as negative

False negative rate: the proportion of all positive substances that are falsely identified as negative

False positive rate = the proportion of all negative substances that are falsely identified as positive

[1] This accuracy analysis is only for dyes that have LLNA data and some type of associated GP data; none of the dyes analyzed had human data, so a comparison between LLNA vs. human and LLNA vs. GP is not included.

[2] n = number of substances included in this analysis

[3] The data on which the percentage calculation is based

[4] For comparison purposes, an excerpt from the ICCVAM evaluation report (ICCVAM 1999; Annex I) showing the overall performance of the LLNA vs. GP and human, and GP versus human is included here.

[5] *GP* refers to outcomes obtained by studies conducted using either the guinea pig maximization test, the Buehler test, or the McGuire test.

[6] *Human* refers to outcomes obtained by studies conducted using the human maximization test or the inclusion of the test substance in a human patch test allergen kit.

Four of the six dyes showed discordant results between the LLNA and the GPMT. These substances are shown in **Table 5-6**, including the maximum concentration tested in the LLNA and the maximum SI value attained, as well as the induction concentration and sensitization incidence in the GPMT. These results indicate that the discordant outcomes between the LLNA and the GPMT cannot be explained based on the concentrations tested (i.e., the maximum concentration tested in the LLNA was higher than the GPMT induction concentration in all four cases).

Table D-7   Dyes Discordant Between the LLNA and GPMT

Substance Name	LLNA Results					GPMT Results			Skin Irritant?
	Veh.	Conc. (%)[1]	SI[2]	EC3 (%)	Result[3]	Ind. Conc. (%)	Sens. Incid. (%)	Result[3]	
C.I. Reactive Yellow 174	AOO	15	7.8	7.8	+	5	11	-	NA
Dispersionsrot 2754	AOO	9	1	NC	-	5	100	+	NA
Produkt P-4G	AOO	15	2.5	NC	-	5	90	+	NA
Yellow E-JD 3442	AOO	15	0.9	NC	-	5	90	+	NA

Abbreviations:
  AOO = acetone/olive oil; Conc. = concentration; EC3 = estimated concentration needed to produce a stimulation index of three; GPMT = guinea pig maximization test; Ind. Conc. = induction concentration; LLNA = local lymph node assay; NA = not available; NC = not calculated since SI<3.0; ND = not done; Sens. Incid. = sensitization incidence; SI = stimulation index; Veh. = vehicle.

[1] Maximum concentration tested in the LLNA

[2] Maximum SI obtained in the LLNA

[3] (-) = nonsensitizer, (+) = sensitizer

As mentioned previously, since comparative human data are not available for any of the dyes analyzed, an evaluation of these substances in the LLNA or the GP compared to human performance could not be assessed. Also, no dyes were evaluated in the ICCVAM evaluation report (ICCVAM 1999; **Annex I**), so these data and analyses cannot be compared to previously considered data.

5.1.3   *Testing of Natural Complex Substances*

The current LLNA database contains data for 12 natural complex substances, for which there are LLNA and human data. The physicochemical properties of these substances are in **Annex II-6,** and the data analyzed here are in **Annex II-7**. For these substances, 75% (9/12) were classified as sensitizers in the LLNA, and 25% (3/12) were classified as nonsensitizers in the LLNA. In the human, 33% (4/12) of these substances tested as sensitizers. One of these human sensitizers (treemoss) was underpredicted by the LLNA. Compared to human outcomes, the LLNA had an accuracy of 42% (5/12), a sensitivity of 75% (3/4), a specificity of 25% (2/8), a false positive rate of 75% (6/8), and a false negative rate of 25% (1/4) (**Table D-8**).

Table D-8   **Evaluation of the Performance of the LLNA for Testing Natural Complex Substances**

Comparison[1]	n[2]	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate	
		%	No.[3]	%	No.[3]	%	No.[3]	%	No.[3]	%	No.[3]
LLNA vs. Human[4]	12	42	5/12	75	3/4	25	2/8	75	6/8	25	1/4
ICCVAM 1999 Database: Evaluation of LLNA Data vs. GP Data or Human Data											
LLNA vs. GP[5]	126	86	108/126	87	81/93	82	27/33	18	6/33	13	12/93
LLNA vs. Human[4]	74	72	53/74	72	49/68	67	4/6	33	2/6	28	19/68
GP[3] vs. Human[4]	62	73	45/62	71	42/59	100	3/3	0	0/3	29	17/59

Abbreviations:
   GP = guinea pig; LLNA = local lymph node assay; No. = number.

Accuracy (concordance) = the proportion of correct outcomes (positive and negative) of a test method

Sensitivity = the proportion of all positive substances that are classified as positive

Specificity = the proportion of all negative substances that are classified as negative

False negative rate: the proportion of all positive substances that are falsely identified as negative

False positive rate = the proportion of all negative substances that are falsely identified as positive

[1] This accuracy analysis is only for substances that have LLNA data and associated human data; none of the natural complex substances analyzed had GP data, so a comparison between LLNA vs. human and LLNA vs. GP is not included.

[2] n = Number of substances included in this analysis

[3] The data on which the percentage calculation is based

[4] Human refers to outcomes obtained by studies conducted using the human maximization test or the inclusion of the test substance in a human patch test allergen kit.

[5] GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test, the Buehler test, or the McGuire test.

Seven of 12 natural complex substances showed discordant results between the LLNA and the HMT. These substances are shown in **Table D-9**, along with the maximum concentration tested in the LLNA and the maximum SI value attained, and the test concentration and sensitization incidence from the HMT. Most (6/7) of the discordant substances were LLNA positive/human negative. All substances for which concentration information was available for both the LLNA and HMT (5/7) were tested at higher concentrations in the LLNA than the induction concentration in the HMT. All false positives in the LLNA produced maximum SI values greater than 6.0, with the exception of spearmint oil, which produced an SI of 3.6 at a test concentration of 10%. All of the discordant LLNA positive fragrance ingredients had EC3 values in a narrow range (3.6% to 9.6%). All false positives were clearly nonsensitizers in the HMT with a sensitization index of 0%. The one human sensitizer underpredicted by the LLNA (treemoss) is classified as a sensitizer based on a sensitization incidence of 2% (3/145) in humans. The concentrations tested in the LLNA and the human were not available.

Table D-9   Natural Complex Substances: Discordant Results Between the LLNA and Human

Substance Name	LLNA Results					HMT Results			Skin Irritant?
	Veh.	Conc. (%)[1]	SI[2]	EC3 (%)	Result[3]	Test Conc. (%)	Sens. Incid. (%)	Result[3]	
Basil oil	EtOH/DEP (1:3)	50	25.2	6.2	+	4	0	-	Mild irritant at 100%[4]
Clove oil	EtOH/DEP (1:3)	50	11.4	7.1	+	5[5]	0[5]	-	Severe irritant at 100%[8]
						5[6]	0[6]		
						10[7]	0[7]		
Lemongrass oil	EtOH/DEP (1:3)	50	13.1	6.5	+	4[9]	0[9]	-	Mild irritant at 100%[4]
						4[10]	0[10]		
						5[10]	0[10]		
Litsea cubeb oil	EtOH/DEP (1:3)	50	16.0	8.4	+	8	0	-	Strong irritant at 100%[4]
Palmarosa oil	EtOH/DEP (1:3)	50	5.0	9.6	+	NA	0	-	NA
Spearmint oil	EtOH/DEP (1:3)	10	3.6	3.6	+	4	0	-	Nonirritant at 100%[4]
Treemoss	EtOH/DEP (1:3)	NA	NA	NC	-	NA	2[11]	+	Nonirritant at 100%[4]

Abbreviations:

Conc. = concentration; DEP = diethyl phthalate: EtOH = ethanol: HMT = human maximization test; LLNA = local lymph node assay; NA = Not available; NC = not calculated since SI<3.0; Sens. Incid. = sensitization incidence; SI = stimulation index; Veh. = vehicle.

[1] Maximum concentration tested in the LLNA

[2] Maximum SI obtained in the LLNA

[3] (-) = nonsensitizer, (+) = sensitizer

[4] Test in mice

[5] Test substance was clove bud oil (Opdyke 1975a)

[6] Test substance was clove stem oil (Opdyke 1975b)

[7] Test substance was clove leaf oil Madagascar (Opdyke 1978)

[8] Test in mice with clove stem oil (Opdyke 1976a)

[9] Test substance was lemongrass oil, East Indian (Opdyke 1976a)

[10] Test substance was lemongrass oil, East Indian (Opdyke 1976b)

[11] HMT or human repeat insult patch test data, submitted by the Research Institute for Fragrance Materials

As mentioned previously, since comparative GP data are not available for any of the natural complex substances analyzed, an evaluation of these substances in the LLNA compared to GP performance could not be assessed. For the same reason, an evaluation of GP versus human outcomes is also not

possible. Also, no natural complex substances were evaluated in the ICCVAM evaluation report (ICCVAM 1999; **Annex I**), so these data and analyses cannot be compared to previously considered data.

5.2   Testing of Metal Compounds

The ICCVAM LLNA report (ICCVAM 1999) includes a summary on the ability of the LLNA to predict the skin-sensitizing potential of 11 metal compounds, representing 10 different metals (**Annex I**). In this addendum, the original ICCVAM analysis has been revised to include a total number of 16 metal compounds, representing 13 different metals, with corresponding human and/or GP data. The physicochemical properties of these metal compounds are in **Annex III-1,** and the data analyzed here are in **Annex III-2**. To reduce the complexity of the analysis, pesticide formulations and other products containing metals were not classified as metal compounds in this evaluation. Among these 16 metal compounds, 14 were tested in an aqueous vehicle, a nonaqueous vehicle, or both. The vehicle in which the two remaining metal compounds (i.e. cobalt chloride and cobalt sulfate) were tested in was not specified (**Annex III-2**). Similar to pesticide formulations and other products (**Section 5.1**), aqueous vehicles contained at least 20% water, while a nonaqueous vehicle contains no water.

All 16 metal compounds had comparative human data and eight had comparative GP data. Among the 13 metals tested multiple times, nickel was tested four times in the LLNA as nickel sulfate, and three times as nickel chloride. The LLNA results for these studies with nickel-containing compounds are shown in **Table D-10**.

Table D-10   Behavior of Nickel-containing Compounds in the LLNA

Substance	LLNA Vehicle	LLNA Call	Max. SI (Conc. [%])	Max. Conc. Tested (%)	Mouse Strain	Reference
Nickel chloride	30% ETOH	+	6.6 (10)	10	CBA/J	Gerberick et al. (1992)
Nickel chloride	DMSO	-	2.2 (2.5)	2.5	CBA/Ca	Basketter et al. (1999d)
Nickel chloride	DMSO	-	2.4 (5)	5	CBA/Ca	Basketter and Scholes (1992)
Nickel sulfate	DMSO	+	3.1 (5)	5	CBA/J	Ryan et al. (2002)
Nickel sulfate	DMSO	-	1.5 (2.5	2.5	CBA/Ca	Basketter and Scholes (1992)
Nickel sulfate	DMF	-	2.2 (5)	5	CBA/J	Ryan et al. (2002)
Nickel sulfate	Pluronic L92 (1%)	+	3 (2,5)	5	CBA/J	Ryan et al. (2002)

Nickel was classified as a sensitizer in three of these studies and as a nonsensitizer in the other four. Two of the three positive results occurred in aqueous vehicles (30% ethanol and 1% Pluronic L92), one of the positive results occurred in a nonaqueous vehicle (DMSO), and all four of the negative results occurred in a nonaqueous vehicle (three in DMSO and one in DMF). Because of these discordant results, a decision was made to exclude nickel compounds from the LLNA metals performance analysis.

Of the 14 remaining metal compounds (13 metals) tested in the LLNA and with human data, nine are sensitizers and five are nonsensitizers in humans. For these 14 metal compounds, the LLNA has an accuracy of 86% (12/14), a sensitivity of 100% (9/9), a specificity of 60% (3/5), a false positive rate

of 40% (2/5), and a false negative rate of 0% (0/9), when compared to human results (**Table D-11**). For the six metal compounds (after excluding nickel compounds) with GP data (five sensitizers and one nonsensitizer in the GP), the LLNA has an accuracy of 83% (5/6), a sensitivity of 100% (5/5), a specificity of 0% (0/1), a false positive rate of 100% (1/1), and a false negative rate of 0% (0/5), when compared to GP test results (**Table D-11**) (**Annex III-2**).

Furthermore, all six of the 14 metal compounds with GP data have human data for comparison and there is a chemical-by-chemical match in classification between the GP and human outcomes (**Table D-11**). In contrast, the LLNA incorrectly identified the one human nonsensitizing metal compound as a sensitizer. For comparative purposes, the corresponding performance of the LLNA in predicting the human response for these same six metal compounds is also provided in **Table D-11**.

Table D-11   Evaluation of the Performance of the LLNA for Testing Metal Compounds[1]

Comparison	n[2]	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate	
		%	No.[3]	%	No.[3]	%	No.[3]	%	No.[3]	%	No.[3]
All Metal Compounds (Aqueous and Nonaqueous Vehicles)											
LLNA vs. GP[4]	6	83	5/6	100	5/5	0	0/1	100	1/1	0	0/5
LLNA vs. Human[5]	14	86	12/14	100	9/9	60	3/5	40	2/5	0	0/9
GP[3] vs. Human[5]	6	100	6/6	100	5/5	100	1/1	0	0/1	0	0/5
LLNA vs. Human[5] for the same GP metal compounds	6	83	5/6	100	5/5	0	0/1	100	1/1	0	0/5
Metal Compounds Tested in Aqueous Vehicles[6]											
LLNA vs. GP[4]	1	100	1/1	100	1/1	-	0/0	-	0/0	0	0/1
LLNA vs. Human[5]	1	100	1/1	100	1/1	-	0/0	-	0/0	0	0/1
GP[3] vs. Human[5]	1	100	1/1	100	1/1	-	0/0	-	0/0	0	0/1
Metal Compounds Tested in Nonaqueous Vehicles											
LLNA vs. GP[4]	5	80	4/5	100	4/4	0	0/1	100	1/1	0	0/4
LLNA vs. Human[5]	12	92	11/12	100	7/7	80	4/5	20	1/5	0	0/7
GP[3] vs. Human[5]	5	100	5/5	100	4/4	100	1/1	0	0/1	0	0/4
ICCVAM 1999 Database: Evaluation of LLNA Data vs. GP Data or Human Data[7]											
LLNA vs. GP[4]	126	86	108/126	87	81/93	82	27/33	18	6/33	13	12/93
LLNA vs. Human[5]	74	72	53/74	72	49/68	67	4/6	33	2/6	28	19/68
GP[3] vs. Human[5]	62	73	45/62	71	42/59	100	3/3	0	0/3	29	17/59

Abbreviations:
  GP = Guinea pig skin sensitization outcomes; LLNA = local lymph node assay; No. = number.

Accuracy (concordance) = the proportion of correct outcomes (positive and negative) of a test method

Sensitivity = the proportion of all positive substances that are classified as positive

Specificity = the proportion of all negative substances that are classified as negative

False negative rate = the proportion of all positive substances that are falsely identified as negative

continued

False positive rate = the proportion of all negative substances that are falsely identified as positive

[1] Because of discordant results obtained with nickel-containing compound in multiple studies, nickel-containing compounds were omitted from this analysis.

[2] n = Number of substances included in this analysis

[3] The data on which the percentage calculation is based

[4] *GP* refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.

[5] *Human* refers to outcomes obtained by studies conducted using the human maximization test or the inclusion of the test substance in a human patch test allergen kit.

[6] All the metal compounds tested in an aqueous vehicle were also tested in a nonaqueous vehicle.

[7] For comparison purposes, an excerpt from the ICCVAM evaluation report (ICCVAM 1999; Annex I

Of the six metal compounds with GP data, the vehicle is known for five of the six compounds. Four of these metal compounds were tested only in a nonaqueous vehicle, while one was tested in both an aqueous and nonaqueous vehicle. Thus, when considering only the metal compound with GP data that was tested in an aqueous vehicle, it was a sensitizer in the LLNA and the LLNA correctly classified it compared to the GP data (**Table D-11**). All five of the metal compounds with comparative GP data tested in a nonaqueous vehicle are also classified as sensitizing in the LLNA. Compared to GP data, the LLNA correctly classifies four of the five nonaqueous metal compounds. The accuracy statistics based on this limited dataset are also presented in **Table D-11**.

Of the 14 metal compounds with human data, the vehicle is known for 12 of the 14 compounds. Eleven of these metal compounds were tested only in a nonaqueous vehicle, while one was tested in both an aqueous and nonaqueous vehicle. Thus, when considering only the metal compound with human data that was tested in an aqueous vehicle, the LLNA correctly classified it as a sensitizer compared to the human data (**Table D-11**). In contrast, of the 12 metal compounds with comparative human data tested in a nonaqueous vehicle, eight are classified as sensitizers and the remaining four are nonsensitizers in the LLNA. Compared to human data, the LLNA correctly classifies 11 of the 12 nonaqueous metal compounds. This results in an accuracy of 92% (11/12), a sensitivity of 100% (7/7), a specificity of 80% (4/5), a false positive rate of 20% (1/5) and a false negative rate of 0% (0/7) (**Table D-11**).

Potassium dichromate was the one metal compound with comparative GP and human data that was tested in both an aqueous and nonaqueous vehicle. Vehicle information was available for 20 of the 22 LLNA studies included in this analysis on potassium dichromate, indicating that it was tested six times in an aqueous vehicle (i.e., 1% Pluronic L92) and 14 times in a nonaqueous vehicle (DMF or DMSO). In all cases, it was found to be sensitizing by the LLNA regardless of the vehicle used.

For the purpose of this addendum, a case-by-case analysis was carried out to determine whether the overall LLNA classification for each metal compound is as a sensitizer or a nonsensitizer. In most cases, the majority result determined the overall LLNA skin sensitizing classification for each metal compound. In instances where there were an equal number of reports classifying the metal compound as sensitizing or nonsensitizing, the most severe classification was used. For instance, for zinc sulfate, LLNA data from two studies are considered in this evaluation report (ICCVAM 1999 [**Annex I**] and Basketter et al. 1999a). Zinc sulfate is classified as a sensitizer in ICCVAM 1999 (neither the vehicle nor the raw data were included) whereas Basketter et al. (1999a) classified zinc sulfate as a nonsensitizer when using DMSO as the vehicle (SI = 2.3 at 25%). For the purposes of this evaluation, to be conservative, zinc sulfate is classified as a sensitizer (**Annex III-2**).

Based on the data compiled for this evaluation, the LLNA classification for nine of the 11 metal compounds evaluated in the 1999 ICCVAM report remained the same in this evaluation because either no new data were available or classifications based on new data were consistent with the original classification (**Annex I**). For the remaining two metal compounds (nickel chloride and nickel sulfate), additional LLNA data were available, but as described above, discordant results with nickel compounds in eight different LLNA studies precluded a definitive classification and it was therefore excluded from this analysis.

5.3   Testing of Substances in Aqueous Solutions

The ICCVAM report (ICCVAM 1999) did not include an analysis of the ability of the LLNA to predict the skin sensitizing potential of substances tested in aqueous solutions, because data were not available for that evaluation (**Annex I**). The current database contains LLNA data for 139 substances tested in aqueous solutions, representing 171 LLNA studies; 91 (123 LLNA studies) of these substances are pesticide formulations and pure compounds and 48 of these substances (48 LLNA studies) are aqueous eluates of medical devices. As mentioned previously in **Section 5.1.1**, all pesticide formulations were tested in the LLNA in 1% Pluronic L92. Because of differences in the protocols for sample preparation between the 91 pesticide formulations and pure compounds and the 48 medical device eluates, these groups were analyzed separately.

In this addendum, the ICCVAM 1999 report has been revised to include a total of 25 unique substances tested in aqueous solutions from 47 LLNA studies with corresponding human and/or GP data. The substances included in this evaluation were tested in the LLNA at a final concentration of at least 20% water. The group of substances analyzed for this section of the addendum does not include metal compounds tested in aqueous vehicles, which have instead been included in the analyses discussed in **Section 5.2**.

5.3.1 Pesticide Formulations and Pure Compounds Tested in Aqueous Solutions

Of the 91 pesticide formulations and pure compounds considered in this analysis, 63% (57/91) are LLNA positive and 37% (34/91) are LLNA negative. Where available, the physicochemical properties of these substances are in **Annex IV-1,** and the data analyzed here are in **Annex IV-2**. If there were multiple LLNA studies for a substance, a majority call was used, so there was one LLNA call for each substance. Eleven substances were tested in multiple LLNA studies (43 total studies); 9/11 of these substances had concordant LLNA results among all studies, and 2/11 substances had discordant results among two or more studies (**Table D-12**).

LLNA data for the two substances for which discordant LLNA study results occurred are shown in **Table D-13**. The discordance for 1,4 dihydroquinone is likely due to differing concentration ranges between the two LLNA studies (i.e., only one study tested up to at least 5%, where a positive result was first noted). For Oxyfluorfen EC, the range of EC3 values for the positive LLNA studies (> 20%) is associated with a weak response in the LLNA, where the greatest variability would be expected. Similarly, the SI values for the negative LLNA studies (2.3 and 2.8) are near the threshold for a positive response (i.e., SI=3), again where the greatest variability would be expected **(Table D-13)**.

Table D-12   Substances Tested in Aqueous Solutions in Multiple LLNA Studies

Formulation	Reference	No. Studies	Mouse Strain	Vehicle	No. Positive Studies	No. Negative Studies	No. Labs
Atrazine SC	ECPA	2	CBA	L92	2	0	2
1,4 Dihydroquinone	Lea et al. (1999)	2	NA	ACE/saline (1:1)	1	1	2
2,4 Dinitrobenzene sulfonic acid	Ryan et al. (2002)	2	NA	L92 / H_2O	2	0	1
Dinocap EC	ECPA	5	CBA	L92	5	0	5
Formaldehyde	ECPA	7	NA	L92	7	0	6
Formulation 7	Dow AgroSciences	2	BALB/c	L92	2	0	1
Hexyl cinnamic aldehyde	ECPA	5	NA	L92	5	0	5
Methyl 2-nonynoate	Ryan et al. (2000)	2	NA	80% EtOH	2	0	NA
Oxyfluorfen EC	ECPA	5	CBA	L92	3	2	2
Quinoxyfen / cyproconazole	ECPA	6	CBA	L92	6	0	6
Trifluralin EC	ECPA	5	CBA	L92	5	0	6

Abbreviations:
 ACE = acetone; EC = emulsion concentrate; ECPA= European Crop Protection Association; EtOH = ethanol (diluent not specified); L92 = 1% aqueous Pluronic L92; NA = not available; No. = number; SC = suspension concentrate.

Table D-13   Substances Tested in Multiple LLNA Studies in Aqueous Solutions with Discordant Results

Substance	Vehicle	Conc. (%)	SIs	Strain	EC3	Lab
1,4 Dihydroquinone	ACE/saline (1:1)	0.05, 0.1, 0.25, 0.5, 1.0	0.7, 1.0, 0.9, 1.9, 1.9	NA	NC	1
	ACE/saline (1:1)	0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5, 10	1.4, 0.8, 1.2, 1.3, 1.9, 6.8, 10.9	NA	1.3	2
Oxyfluorfen EC	L92	1, 7, 33	0.81, 1.4, 4.9	CBA/Ca	30.8	1
	L92	1, 7, 33	0.9, 1.4, 2.8	CBA/J	NC	2
	L92	1, 7, 33	0.3, 0.9, 2.3	CBA/J	NC	3
	L92	1, 7, 33	1.1, 1.5, 3.1	CBA/JHsd	30.8	4
	L92	1, 7, 33	1.2, 1.2, 5.4	CBA/CaOlaHsd	18.1	5

Abbreviations:
 ACE = acetone; Conc. = concentration; EC = emulsion concentrate; EC3 = estimated concentration needed to produce a stimulation index of 3; L92 = 1% aqueous Pluronic L92; LLNA = local lymph node assay; NA = Not available; NC = not calculated since SI<3.0; SIs = stimulation indices.

GP data were available for 25 substances (4 sensitizers/21 nonsensitizers in the GP) tested in aqueous solutions. These substances represented a total of 44 LLNA studies. Based on these comparative data, the LLNA has an accuracy of 56% (14/25), a sensitivity of 75% (3/4), a specificity of 52% (11/21), a false positive rate of 48% (10/21), and a false negative rate of 25% (1/4) (**Table D-14**).

Table D-14   Evaluation of the Performance of the LLNA for Testing Aqueous Solutions

Comparison	n[1]	Accuracy		Sensitivity		Specificity		False Positive Rate		False Negative Rate	
		%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]	%	No.[2]
Pesticide Formulations and Pure Compounds Tested in Aqueous Solutions											
LLNA (CBA & BALB/c) vs. GP[3]	25	56	14/25	75	3/4	52	11/21	48	10/21	25	1/4
LLNA (CBA only) vs. GP[3]	22	57	13/22	75	3/4	56	10/18	44	8/18	25	1/4
LLNA (CBA only) vs. Human[4]	4	50	2/4	33	1/3	100	1/1	0	0/1	67	2/3
GP[3] vs. Human[4]	2	100	2/2	100	1/1	100	1/1	0	0/1	0	0/1
ICCVAM 1999 Database: Evaluation of LLNA Data vs. GP Data or Human Data[5]											
LLNA vs. GP[3]	126	86	108/126	87	81/93	82	27/33	18	6/33	13	12/93
LLNA vs. Human[4]	74	72	53/74	72	49/68	67	4/6	33	2/6	28	19/68
GP[3] vs. Human[4]	62	73	45/62	71	42/59	100	3/3	0	0/3	29	17/59
ICCVAM 1999 Database: Evaluation of LLNA Data vs. GP Data or Human Data[5]											
LLNA vs. GP[3]	126	86	108/126	87	81/93	82	27/33	18	6/33	13	12/93
LLNA vs. Human[4]	74	72	53/74	72	49/68	67	4/6	33	2/6	28	19/68
GP[3] vs. Human[4]	62	73	45/62	71	42/59	100	3/3	0	0/3	29	17/59

Abbreviations:
  GP = guinea pig skin sensitization outcomes; LLNA = local lymph node assay; No. = number.

Accuracy (concordance) = the proportion of correct outcomes (positive and negative) of a test method

Sensitivity = the proportion of all positive substances that are classified as positive

Specificity = the proportion of all negative substances that are classified as negative

False negative rate = the proportion of all positive substances that are falsely identified as negative

False positive rate = the proportion of all negative substances that are falsely identified as positive

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

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

[3] *GP* refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.

[4] *Human* refers to outcomes obtained by studies conducted using the human maximization test or the inclusion of the test substance in a human patch test allergen kit.

[5] For comparison purposes, an excerpt from the ICCVAM evaluation report (ICCVAM 1999; Annex I) showing the overall performance of the LLNA vs. GP and human, and GP vs. human is included here.

Eleven substances were discordant between the LLNA and the GP tests (**Table D-15**). Ten of the 11 discordant substances (all overpredicted by the LLNA) were pesticide formulations tested in aqueous 1% Pluronic L92. These were the same 10 formulations noted in **Section 5.1.1.1**, where a detailed discussion of the discordant results is also detailed. The other discordant substance was neomycin sulfate, which was tested in 25% EtOH. Among the 11 of 25 substances classified as sensitizers by the LLNA that were classified as nonsensitizers in the GP (**Table D-15**), 9/11 were based on BT results and 2/11 were based on GPMT results.

The one false negative substance based on LLNA results as compared to GP results, neomycin sulfate, was tested in the LLNA at a maximum concentration 12.5-fold lower than the induction concentration used in the guinea pig (**Table D-15**). However, it should also be noted that neomycin sulfate also gave a negative result in the LLNA when tested at 25% in DMSO, a nonaqueous vehicle (Basketter et al. 1994).

Table D-15   Substances Tested in Aqueous Solution: Discordant Results Between the LLNA and GP

Substance Name	LLNA Results					GP Results			Skin Irritant?
	Veh.	Conc. (%)[1]	SI[2]	EC3 (%)	Result[3]	Ind. Conc. (%)	Sens. Incid. (%)	Result[3]	
Atrazine SC	L92	100	7.3	36.4[4]	+	30	0	-[5]	Nonirritant at ≤ 25%[6]
BASF SE-1	L92	70	22.7	5.5	+	100	0	-[7]	Nonirritant at ≤ 50%[6]
EXP 11120 A	L92	100	5.3	64.9	+	100	0	-[7]	Nonirritant at 100%[6]
F & Fo WG 50 + 25	L92	25	15.2	0.003	+	30	0	-[7]	Nonirritant at ≤ 10%[6]
FAR01060-00	L92	100	3.6	88.5	+	100	0	-[7]	Nonirritant at 100%[6]
Formulation 2[8]	L92	80	15.8	15.7	+	NA	NA	-[7]	Nonirritant at 80%[9]
Formulation 7[8]	L92	100	3.2	85	+	100	0	-[7]	Nonirritant at 80%[9]
Fx + Me EW 69	L92	50	8.6	25.2	+	100	0	-[7]	Nonirritant at 100%[6]
Neomycin sulfate	25% EtOH	2	0.9	NC	-	25	76	+	Nonirritant at ≤ 25%[6]
Oxyfluorfen EC	L92	33	5.4	30.8[7]	+	10	26	-[5]	Nonirritant at ≤ 25%[6]
Trifluralin EC	L92	100	75.2	10.3[8]	+	50	10	-[7]	Nonirritant at ≤ 25%[6]

Abbreviations:
Conc. = concentration; EC = emulsion concentrate; EC3 = estimated concentration needed to produce a stimulation index of 3; EW = emulsion, oil in water; GP = guinea pig test; Ind. Conc. = induction concentration; L92 = 1% aqueous Pluronic L92; LLNA = local lymph node assay; NA = not available; NC = not calculated since SI<3.0; SC = suspension concentrate; Sens. Incid. = sensitization incidence; SI = stimulation index; Veh. = vehicle; WG = water-dispersible granules.

[1] Maximum concentration tested in the LLNA

Among the substances tested in aqueous solutions, human data were available for only four (3 sensitizers/1 nonsensitizer in humans). Of these four, two were correctly identified by the LLNA when compared to human data. The accuracy statistics for the LLNA for this limited database are presented in **Table D-14**.

Two substances, which had comparative human and GP data, were tested in aqueous solutions. Of these, one (neomycin sulfate) was correctly identified in the GP as a sensitizer, compared to human results (Magnusson and Kligman 1969) (**Table D-16**). Neomycin sulfate, when tested in aqueous solution (25% EtOH) in the LLNA (Gerberick et al. 1992) is false negative in the LLNA when compared to human results. As noted above, the maximum concentration of neomycin sulfate tested in the LLNA in aqueous solution (2%), is 12.5-fold less than the induction concentration (25%) used in both the GPMT and the HMT tests that gave positive results (Kligman 1966), but again, neomycin sulfate was also negative in the LLNA when tested at 25% in DMSO, a nonaqueous vehicle (Basketter et al.1994). The other substance for which there was both GP and human data, propylene glycol, was false negative in both the LLNA and the GPMT. It was classified as a sensitizer for this study based on its inclusion in a human patch test allergen test kit (ICCVAM 1999), along with the fact that Guillot et al. (1983) note anecdotal evidence of sensitization reactions in humans. However, there is published HMT data for propylene glycol that indicates it is a nonsensitizer (Kligman 1966; Guillot et al. 1983) and a weak human irritant (Basketter et al. 1997). The maximum concentration of propylene glycol that has been tested in humans is 25% (Kligman 1966). Given these uncertainties, this false negative result could be considered equivocal.

Table D-16　　Substances with Human Data Tested in Aqueous Solution

Substance Name	LLNA Results					GP Results				Human Results				Skin Irritant?
	Veh.	Conc. (%)[1]	SI[2]	EC3 (%)	Result[3]	Test	Ind. Conc. (%)	Sens. Incid. (%)	Result [3]	Test	Ind. Conc. (%)	Sens. Incid. (%)	Result [3]	
Butanol	H$_2$O	20	1.6[4]	NC	-	NA	NA	NA	NA	NA	NA	NA	-	NA
Methyl 2-nonynoate	80% EtOH	20	24.4	2.5	+	NA	NA	NA	NA	HRIPT	0.2	0	+	NA
Neomycin sulfate	25% EtOH	2	0.9	NC	-	GPMT	25	76	+	HMT	25	28	+	NA
Propylene glycol	H$_2$O	100	1.6	NC	-	GPMT[5]	1	0	-	--	--	--	+[6]	Non-irritant at 25%[7]

Abbreviations:

Conc. = concentration; EC3 = estimated concentration needed to produce a stimulation index of 3; EtOH = ethanol; GP = guinea pig; GPMT = guinea pig maximization test; HMT = human maximization test; HRIPT = human repeat insult patch test; Ind. = incidence; Conc. = induction concentration; LLNA = local lymph node assay; NA = not available; NC = not calculated since SI<3.0; Sens. Incid. = sensitization incidence; SI = stimulation index; Veh. = vehicle.

[1] Maximum concentration tested in the LLNA

[2] Maximum SI obtained in the LLNA

[3] (-) = nonsensitizer, (+) = sensitizer

[4] Test concentration that produced this SI was 5%.

[5] Also tested in Buehler test: Ind. Conc. = 0.2, Sens. Ind. = 0%

[6] Positive call on the basis that propylene glycol is included as a human patch test allergen (ICCVAM 1999)

[7] Test in humans

5.3.2 Medical Device Eluates Tested in Aqueous Solutions

Of the 48 medical device eluates considered in this analysis, 100% (48/48) are LLNA negative. The constituents of these eluates were not provided by the submitter, so physicochemical properties of any substances they contained are unknown. The submitted data are provided in **Annex IV-3**.

None of these eluates had associated GP data or human data. All of the LLNA studies were reportedly done according to the ICCVAM-recommended protocol (ICCVAM 1999). The LLNA data provided by the submitter were average dpm for each treatment group (n = 5 animals); the individual animal data were not submitted (although the study report indicates that individual animal data were collected). SI values were calculated by NICEATM based on the submitted average values (**Annex IV-3**).

The sample preparation for these samples was different from that for the pesticide formulations and pure substances discussed in **Section 5.3.1**. The test substances for the LLNA were eluates of medical devices prepared according to standard procedures (ASTM 2008, ISO 2002), rather than dilutions of specific substances. A concurrent positive control was included in each LLNA study. Another treatment group treated with an eluate sample spiked with a known sensitizer, 2,4-dinitrobenzenesulfonic acid, was also included in each LLNA study. The purpose of the spiked samples was reportedly to demonstrate that there was nothing present in the eluate that would attenuate a positive LLNA response.

These eluates were not analyzed to determine their constituents, or whether in fact any compound(s) were eluted from the medical device tested. Since the LLNA results were uniformly negative and no sample preparation control was included in the studies, the effectiveness of the sample preparation could not be determined, so the results from these eluates were not included with those from the pesticide formulations and pure substances discussed in **Section 5.3.1**.

6.0   LLNA Data Quality

Based on the available information, the published papers, and data submissions, information on compliance with GLP guidelines was available for data obtained from Dow AgroSciences, Dupont, Gerberick et al. (2005), H.W. Vohr (BGIA), E. Debruyne (Bayer CropScience SA), P. Botham (ECPA), Bundesanstalt für Arbeitsschutz und Arbeitsmedizin, and D. Germolec (NIEHS).

A formal assessment of the quality of the remainder of the LLNA data considered here 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 have been requested, but not yet obtained. However, many of the studies were conducted according to GLP guidelines, which implies that an independent quality assurance audit was conducted. The impact of any deviations from GLP guidelines cannot be evaluated for the data reviewed here, since no data quality audits were obtained.

As noted in **Section 5.0,** the original records were not obtained for all of 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.

7.0   Other Scientific Reports and Reviews

A search of Medline, PubMed, and Toxline resulted in 46 published reports relevant to the applicability domain of the LLNA and the use of the LLNA for testing pesticide formulations and other products, metals and aqueous solutions for skin sensitizing potential. Of these reports, 26 have been published since the 1999 ICCVAM report on the LLNA. Included below are the reports most relevant to the evaluation included in this Addendum, with the most salient points summarized for each.

7.1   Maibach (1986)

The author evaluated the herbicide glyphosate, an active ingredient of a formulation considered in this Addendum (see **Annex II-3**), for acute and cumulative irritation, photoirritation, and allergic and photoallergic contact sensitization potential in 346 volunteers. The skin sensitization study used a modified Draize protocol in 204 adults with 0.2 mg of a commercial glyphosate formulation applied on patches. It was concluded that glyphosate is a nonsensitizer. A 10% concentration was suggested for a diagnostic patch test series.

7.2   Sharma and Kaur (1990)

The authors prepared a patch test series of 37 most prevalent pesticides used in the Chandigarh, India region, including insecticides, fungicides and herbicides. They tested 30 farmers with dermatoses and 20 controls. The only pesticide with active ingredients considered in this Addendum (see **Annex II-3**) that showed a positive patch test reaction was 1% 2,4-D (3/20, incidence = 15%). The only pesticide with active ingredients considered in this Addendum (see **Annex II-3**) that showed a negative patch test reaction was 1% atrazine.

7.3   Lisi (1992)

This is a review article that is primarily focused on pesticides sold and used in Italy at the time it was published. It covers both irritants and allergens and a broad array of pesticides (fungicides, herbicides, insecticides, soil fumigants, and contaminants in formulations). It contains a list of pesticides and active ingredients that caused positive reactions, with concentrations tested, for patch tests done by the International Contact Dermatitis Group and the Italian Group for the Study of Contact and Environmental Dermatitis. Pesticides with active ingredients considered in this Addendum (see **Annex II-3**) included in patch test series of 10% glyphosate and 1% dinocap.

7.4   Basketter et al. (1999a)

Basketter et al. (1999a) used the LLNA to evaluate the skin sensitization potential of 13 metal salts. For the purposes of their evaluation, eight of the 13 metals were considered to be human sensitizers. Their results show that the LLNA had an accuracy of 85% (11/13), sensitivity 88% (7/8), specificity of 80% (4/5), false negative rate of 12% (1/8), and false positive rate of 20% (1/5). Nickel chloride (tested up to 5% in DMSO) was false negative in the LLNA based on an SI $\leq$ 2.4. Copper chloride (tested up to 5% in DMSO) was false positive in the LLNA based on an SI $\geq$ 8.1. The authors concluded that these data support the potential utility of the LLNA for testing metal contact allergens.

7.5   Wright et al. (2001)

The authors investigate the influence of application vehicle on sensitizing potency, using the LLNA to examine the activity of four recognized human contact allergens: isoeugenol and cinnamic aldehyde and two fragrance chemicals; 3-dimethylaminopropylamine (a sensitizing impurity of cocamidopropyl betaine, a surfactant used in shower gel) and dibromodicyanobutane (the sensitizing component of Euxyl K 400, a preservative used in cosmetics). The four chemicals were applied in each of seven different vehicles (acetone: olive oil [4:1; AOO]; DMSO: methyl ethyl ketone; dimethylformamide; propylene glycol; and both 50:50 and 90:10 mixtures of ethanol and water). It was found that the vehicle in which a chemical is presented to the epidermis can have a marked effect

on sensitizing activity. EC3 values ranged from 0.9 to 4.9% for isoeugenol, from 0.5 to 1.7% for cinnamic aldehyde, from 1.7 to > 10% for dimethylaminopropylamine and from 0.4 to 6.4% for dibromodicyanobutane. These authors confirm that the vehicle in which a chemical is encountered on the skin has an important influence on the relative skin sensitizing potency of chemicals and may have a significant impact on the acquisition of allergic contact dermatitis. The data also demonstrate the utility of the LLNA as a method for the prediction of these effects and thus for the development of more accurate risk assessments.

7.6   Ikarashi et al. (2002)

The authors examined the sensitization potential of gold sodium thiosulfate (GST) in the GP and the mouse. GST has been included in a standard human patch test series, and the incidence of patients showing positive reactions to gold is increasing (contact allergy rates to gold were reported to be in the range 1–23% from various countries). GST was tested in the GPMT and in several *in vivo* assays in the mouse, including the mouse ear swelling test (MEST) (Gad et al. 1986), an ex-vivo variant of the LLNA, the sensitive LLNA (Ikarashi et al. 1993), and the mouse IgE test (Hilton et al. 1995, Dearman et al. 1992). GST was identified as a sensitizer in the GPMT (GST intradermal induction concentration, 1%; sensitization index 60% [6/10]. However, only 2/6 mice showed a positive response (ear swelling ≥ 20%) in the MEST, and GST did not induce an SI ≥ 3 in either variant of the LLNA. There was a significant difference in total serum IgE concentrations between vehicle- and GST-treated groups (p < 0.05). The authors concluded that GST was a weak sensitizer.

7.7   Griem et al. (2003)

The authors propose a quantitative risk assessment methodology for skin sensitization aimed at deriving "safe" exposure levels for sensitizing substances. In their analysis they used cinnamic aldehyde and nickel as examples of how they apply their risk assessment proposal to sensitizing substances. In their discussion of nickel, they reference data supporting that nickel is an allergen with a relatively low sensitizing potency but a high prevalence in the general population (Kligman 1966; Vandenberg and Epstein 1963). Consequently, as in humans, nickel salts (i.e. nickel chloride and nickel sulfate) are weak sensitizers in animals and often give negative results in standardized tests (e.g., LLNA). Clinical experience in humans indicates that nickel allergy preferentially develops after nickel exposure on irritated or inflamed, but not on healthy skin (Kligman 1966; Vandenberg and Epstein 1963). Similarly, previously false negative results with nickel salts in the mouse LLNA could recently be overcome by the addition of a detergent (1% surfactant in water) to the nickel test solution (Ryan et al. 2002).

7.8   Hostynek and Maibach (2003 and 2004)

In these two review papers, the authors consider reports of immediate and delayed type immune reactions to cutaneous or systemic exposure to copper in humans. They mention that the electropositive copper ion is potentially immunogenic due to its ability to diffuse through biological membranes to form complexes in contact with tissue protein. Reports of immune reactions to copper include ACD, immunologic contact urticaria, systemic allergic reactions and contact stomatitis. They state that considering the widespread use of copper intrauterine devices (IUDs) and the importance of copper in coinage, items of personal adornment and industry, unambiguous reports of sensitization to the metal are extremely rare, and even fewer are the cases, which appear clinically relevant. Reports of immune reactions to copper mainly describe systemic exposure from IUDs and prosthetic materials in dentistry, implicitly excluding induction of the hypersensitivity from contact with the skin as a risk factor. Based on predictive GP testing and the LLNA, copper has a low sensitization potential. The authors then provide a diagnostic algorithm that might clarify the frequency of copper hypersensitivity.

7.9   Penagos et al. (2004)

The authors prepared a pesticide patch test series specific to the most prevalent pesticides used on banana plantations in Panama. They examined 366 plantation workers from four different plantations for dermatoses, and tested 37 workers with dermatoses that they judged most likely to be pesticide-related. Twenty-three control workers, without dermatoses, were also patch-tested. Twenty-four workers showed a positive reaction to one or more of the pesticides tested; these positive reactions included 15 ACD cases (20 positive reactions) in 37 workers diagnosed with dermatoses and three control workers who had allergic reactions to pesticides (4 positive reactions). Pesticides with active ingredients considered in this Addendum (see **Annex II-3**) that showed positive patch test reactions were 10% glyphosate (2/60, incidence = 3.3%), 0.02% oxyfluorfen (1/60, incidence = 1.6%), 1% chlorpyrifos (1/60, incidence = 1.6%), and 0.44% propiconazole (1/60, incidence = 1.6%).

7.10   Tinkle et al. (2004)

The authors investigated the skin sensitization potential of beryllium, the cause of chronic beryllium disease, an incurable occupational lung disease that begins as a cell-mediated immune response to beryllium. Since occupational respiratory beryllium exposures have been decreasing and the rate of beryllium sensitization has not declined, the authors hypothesized that skin exposure to beryllium particles might be an alternative route for sensitization. Optical scanning laser confocal microscopy and size-selected fluorospheres were used to demonstrate that ultrafine beryllium particles penetrate the stratum corneum of human skin, reaching the epidermis and, occasionally, the dermis. Skin sensitization in mice was suggested by peripheral blood and LN beryllium lymphocyte proliferation tests (BeLPT), and by changes in LN T-cell activation markers, increased expression of CD44, and decreased CD62L following topical application of beryllium. Topically applied beryllium also increased ear thickness in mice following challenge. The authors believe that these observations are consistent with development of a cell-mediated immune response following topical application of beryllium, and hypothesize a link between the persistent rate of occupational beryllium sensitization and skin exposure to ultrafine particles.

7.11   Lalko and Api (2006)

The authors tested seven essential oils (basil, citronella, clove leaf, geranium, litsea cubeba, lemongrass, and palmarosa oils) as well as three of the major components (citral, eugenol, and geraniol) in the LLNA. Each of these essential oils contains one or more known sensitizers. If the concentration of a major component that was a sensitizer was approximately 70% or more, the potency of an essential oil (as indicated by an EC3 value adjusted for the concentration of the major component as measured by GC/MS or HPLC) showed less than a 2-fold difference from the EC3 value calculated for that individual component. *Quenching*, a phenomenon that occurs when some component in a mixture inhibits the sensitization potential of a known sensitizer that is present in the mixture at a sensitizing concentration, was not observed for any of the essential oils tested in this study.

7.12   Shelnutt et al. (2007)

This is a review of the literature on the skin sensitization potential of hexavalent chromium. Hexavalent chromium is both a dermal irritant and a dermal sensitizer, causing ulceration of the skin and ACD. While the trivalent form of chromium is the naturally occurring valence, hexavalent chromium is one of the more prevalent sensitizers in the environment, present in detergents, cement, cosmetics, and foods. Research indicates that the hexavalent form exhibits greater skin-penetration properties than the trivalent form, although it is hypothesized that hexavalent chromium is transformed to trivalent chromium in the body and it is the trivalent form that induces sensitization. Repeated exposure to 4–25 ppm of hexavalent chromium can both cause sensitization and elicit ACD. Exposure to 20 ppm hexavalent chromium can cause skin ulcers in nonsensitized people. Chromium

ACD can be persistent and debilitating, perhaps because of the high prevalence and ubiquity of hexavalent chromium.

7.13   Chipinda et al. (2008)

Zinc diethyldithiocarbamate (ZDEC) and its disulfide, tetraethylthiuram disulfide (TETD) occur in rubber products, and are well-documented contact sensitizers in animals and humans. They are cross-reactive, as sensitization to one often confers sensitization to the other. This paper explored haptenation mechanisms of ZDEC by using high-performance liquid chromatography and mass spectrometry to identify ZDEC oxidation/reduction products and sites of protein binding. The LLNA was employed to test ZDEC and its oxidation products for sensitization potential and to examine possible mechanisms of hapten formation via elimination of oxidation and chelation mechanisms by substituting cobalt for zinc in ZDEC, to produce CoDEC. Oxidation of ZDEC produced TETD, tetraethylthiocarbamoyl disulfide, and tetraethyldicarbamoyl disulfide (TEDCD). The LLNA identified ZDEC, sodium diethyldithiocarbamate, TEDCD, and TETD as sensitizers, and CoDEC, as a nonsensitizer. While ZDEC bound to the copper-containing active site of superoxide dismutase, CoDec did not, suggesting chelation of metal-containing proteins as a possible mechanism of hapten formation.

7.14   Fukuyama et al. (2008)

The authors used the LLNA to test the sensitization potential of chromated copper arsenate (CCA), a commonly used wood preservative, and its components, for sensitization potential. LLNA studies were done using both AOO and DMSO as vehicles. CCA components tested included As_2O_5, CrO_3, and CuO_2. Trimellitic anhydride in AOO was used as a positive control. All metal compounds were detected as sensitizers by the LLNA. EC3 values for metal compounds tested in AOO and DMSO were different (CCA: EC3 in AOO = 1.86%, EC3 in DMSO < 0.3%; As_2O_5: EC3 in AOO = 0.8%, EC3 in DMSO < 0.3%). CuO_2 (EC3 = 1.69%) and CrO_3 (EC3 < 0.3%) were tested in DMSO only. ATP was also measured in an aliquot of the lymph node suspension via a luciferin-luciferase assay and found to increase with increasing dose of the metal compounds.

7.15   Horiuchi et al. (2008)

This paper describes case reports tabulated by the Division of Dermatology, Sake Central Hospital, Saku, Japan from 1975 to 2000. Of pesticides with active ingredients considered in this Addendum (see **Annex II-3**), three cases in which trifluralin was implicated as the causative agent, and two cases in which glyphosate was implicated as the causative agent were documented. These causative agents were identified by either anecdotal evidence related to exposure or by patch testing.

7.16   Jowsey et al. (2008)

The authors conducted a retrospective examination of LLNA data in AOO for 18 substances that had been tested multiple times in AOO (2 - 15 studies per substance) to determine the inherent variability in the calculated EC3 values. The highest observed variability was for isoeugenol (31 studies) at 4.1-fold. A second retrospective analysis of data from the literature and previously unpublished studies for 18 substances that had been tested in the LLNA using at least two of 15 different vehicles was conducted. For 6/18 substances (ethylene glycol dimethacrylate, eugenol, geraniol, imidazolidinyl urea, hydroxycitronellal, and nickel sulfate), the variability was less than 5-fold. For 6/18 chemicals (3-dimethylaminopropylamine, cinnamic aldehyde, isoeugenol, p-tert-butyl-a-ethyl hydrocinnamal, methylchloroisothiazolinone/methylisothiazolinone, and potassium dichromate), the variability was greater than 5-fold but less than 10-fold. For 6/18 chemicals (dinitrobenzene sulfonate, 1,4-hydroquinone, 1,4-phenylenediamine, methyldibromoglutaronitrile, formaldehyde, and glutaraldehyde), the observed range was greater than 10-fold. Further examination of the data for the substances in the highest-variability group suggested that the high variability might be due to an underestimation of potency in the LLNA associated with the use of predominantly aqueous vehicles

or propylene glycol. In contrast, use of AOO, DMF, methyl ethyl ketone, DMSO, and 9:1 ethanol:water resulted in less variable potency estimates for most substances.

8.0   References

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9.0   Glossary

Absolute: A natural complex substance prepared from plant material by chemical extraction.

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 repeated skin contact with a skin sensitizer. Clinical signs of ACD include the development of erythema (redness) and edema (swelling), blistering, and itching. Also referred to as skin sensitization.

Assay[12]: The experimental system used. Often used interchangeably with *test* and *test method*.

Buehler test (BT): An *in vivo* test method used to assess the skin sensitization potential of a substance. A sensitization phase uses topical application of the test substance using an occluded patch. The sensitization phase is followed by a challenge with the test substance, also with an occluded patch, to elicit an ACD reaction, which occurs if the animal has become sensitized (Buehler 1965).

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[12]: 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.

Dye: A chemical compound that can impart color when applied to a substance. Various dyes are used as tissue stains, test reagents, therapeutic agents, and coloring agents.

EC3: The estimated concentration needed to produce a stimulation index of 3, as compared to the concurrent vehicle control.

Essential oil: A natural complex substance, in the form of a concentrated hydrophobic liquid, which contains volatile compounds. Prepared commercially from plants by distillation.

False negative[12]: A substance incorrectly identified as negative by a test method.

False negative rate[12]: 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[12]: A substance incorrectly identified as positive by a test method.

False positive rate[12]: 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.

Formulation: A particular mixture of base chemicals and additives required for a product. Formulations typically contain one or more active ingredients and inert ingredients to facilitate mixing, application, penetration, etc.

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

Good Laboratory Practices (GLP)[12]: 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.

Guinea pig maximization test (GPMT): An *in vivo* test method used to assess the skin sensitization potential of a substance. A sensitization phase combines intradermal induction using the test substance and Freund's complete adjuvant, followed by topical application using an occluded patch. The sensitization phase is followed by a challenge with the test substance, also with an occluded patch, to elicit an ACD reaction, which occurs if the animal has become sensitized (Magnusson and Kligman 1969).

Hazard[12]: 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.

Human maximization test (HMT): An *in vivo* test method used to assess the skin sensitization potential of a substance. Skin is pretreated with sodium lauryl sulfate, an anionic surfactant, to cause irritation and facilitate dermal penetration of the test substance. A sensitization phase via topical application of the test substance using an occluded patch follows. The sensitization phase is followed by a challenge with the test substance, also with an occluded patch, to elicit an ACD reaction, which occurs if the person has become sensitized (Kligman 1966c).

Human repeat insult patch test (HRIPT): An *in vivo* test method used to assess the skin sensitization potential of a substance. A number of 24-hour or 48-hour exposures to test substances are delivered by occluded patch over a 3-week period to 100–200 volunteers. Two weeks later, a challenge exposure is made at the induction site and a unexposed site, again using a 24-/48-hour patch to elicit an ACD reaction, which occurs if the person has become sensitized (Stots 1980).

Interlaboratory reproducibility[12]: 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[12]: 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[12]: 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.

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

Murine 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 of the ear to the substance. The traditional LLNA measures lymphocyte proliferation by quantifying the amount of ^{3}H-thymidine or ^{125}I-iododeoxyuridine incorporated into the cells of the draining lymph nodes.

Natural complex substance: A substance that occurs in nature that is a mixture of several individual chemical constituents. Examples are essential oils and absolutes.

Negative predictivity[12]: The proportion of correct negative responses among substances testing negative in 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.

Nonsensitizer: A substance that does not cause skin sensitization following repeated skin contact.

Performance[12]: 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 are considered adequate by the OECD.

Positive predictivity[12]: 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[12]: The proportion of positives in the population of substances tested (see *two-by-two table*).

Protocol[12]: 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[12]: 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[12]: A new or modified test method that reduces the number of animals required.

Reference test method[12]: 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[12]: A new or modified test method that refines procedures to lessen or eliminate pain or distress in animals or enhance animal wellbeing.

Relevance[12]: 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[12]: 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 interlaboratory reproducibility and intralaboratory repeatability.

Replacement alternative[12]: A new or modified test method that replaces animals with non-animal systems or one animal species with a phylogenetically lower one (e.g., a mammal with an invertebrate).

Reproducibility[12]: 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 *interlaboratory reproducibility*).

rLLNA: A variant of the LLNA that employs a single high dose of the test substance rather than multiple doses to determine its skin sensitization potential, thus using fewer animals.

Sensitivity[12]: 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 (UN 2005).

Specificity[12]: 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 LLNA to assess the skin sensitization potential of a test substance. The value is calculated as the ratio of radioacrivity 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 rLLNA, an SI $\geq$ 3.0 classifies a substance as a skin sensitizer.

Test[12]: The experimental system used; used interchangeably with *test method* and *assay*.

Test method[12]: 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[12]: The ability of a test method or procedure to be accurately and reliably performed in different competent laboratories.

Two-by-two table[12]: 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	c + d	a + b + c + d

Validated test method[12]: 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[12]: 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): In the 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.