ICCVAM Test Method Evaluation Report on Using the Murine Local Lymph Node Assay for Testing Pesticide Formulations, Metals, Substances in Aqueous Solutions, and Other Products

Interagency Coordinating Committee on the Validation of Alternative Methods

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

National Institute of Environmental Health Sciences
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List of Abbreviations and Acronyms

AOO	Acetone: olive oil (4:1 by volume)
BAuA	Federal Institute for Occupational Safety and Health (Germany)
BRD	Background review document
BT	Buehler test
CPSC	U.S. Consumer Product Safety Commission
DMSO	Dimethyl sulfoxide
DNCB	Dinitrochlorobenzene
EC3	Estimated concentration needed to produce a stimulation index of 3
ECVAM	European Centre for the Validation of Alternative Methods
EPA	U.S. Environmental Protection Agency
ESAC	ECVAM Scientific Advisory Committee
FR	*Federal Register*
GP	Guinea pig
GPMT	Guinea pig maximization test
HCA	Hexyl cinnamic aldehyde
HSUS	Humane Society of the United States
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
IWG	Immunotoxicity Working Group
JaCVAM	Japanese Center for the Validation of Alternative Methods
LLNA	Murine local lymph node assay
NICEATM	National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods
OECD	Organisation for Economic Co-operation and Development
P.L.	Public Law
rLLNA	Reduced murine local lymph node assay
SACATM	Scientific Advisory Committee on Alternative Toxicological Methods
SI	Stimulation index
TG	Test Guideline
U.K.	United Kingdom
U.S.	United States
U.S.C.	United States Code

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[2] Dr. Pieters was unable to attend the public meeting on April 28-29, 2009. However, he was involved in the peer review of the documents and concurred with the conclusions and recommendations included in the *Independent Scientific Peer Review Panel Report – Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products.*

[3] Dr. Richmond was unable to attend the public meeting on March 4-6, 2008. However, he was involved in the peer review of the documents and concurred with the conclusions and recommendations included in the *Independent Scientific Peer Review Panel Report - Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products.*

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Preface

Allergic contact dermatitis (ACD) is an adverse health effect that frequently develops in workers and consumers exposed to skin-sensitizing chemicals and products. ACD results in lost workdays[1] 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 skin sensitization. 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 1998, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) evaluated and recommended an alternative known as the murine (mouse) local lymph node assay ("traditional LLNA").[2] The traditional LLNA provides several advantages compared to guinea pig test methods, including elimination of potential pain and distress, use of fewer animals, less time to perform, and availability of dose-response information. Based on the validation database and performance, ICCVAM recommended the LLNA as an alternative test method for assessing the skin sensitization potential of most types of substances (ICCVAM 1999). United States and international regulatory agencies subsequently accepted the traditional LLNA as a valid alternative test method for ACD testing.

In 2007, the U.S. Consumer Product Safety Commission requested that ICCVAM evaluate the usefulness and limitations of the LLNA for testing mixtures, metals, and substances in aqueous solutions (i.e., an evaluation of the current applicability domain of the LLNA), among other activities related to the LLNA. ICCVAM assigned this activity a high priority after considering comments from the public and ICCVAM's Scientific Advisory Committee on Alternative Toxicological Methods (SACATM). As part of their ongoing collaboration with ICCVAM, scientists from the European Centre for Validation of Alternative Methods (ECVAM) and the Japanese Center for Validation of Alternative Methods (JaCVAM) served as liaisons to the ICCVAM Immunotoxicity Working Group (IWG). A detailed timeline of the LLNA applicability domain evaluation is included with this report.

This test method evaluation report provides ICCVAM's recommendations regarding the usefulness and limitations of the LLNA for assessing the ACD potential of pesticide formulations, metals, substances tested in aqueous solutions, and other products. The report also provides the updated ICCVAM-recommended LLNA test method protocol. The database of substances used to evaluate the current applicability domain of the LLNA is discussed and summarized.

ICCVAM solicited and considered public comments and stakeholder involvement throughout the evaluation process. ICCVAM considered the SACATM comments, the Independent Scientific Peer Review Panel's report, and all public comments before finalizing this ICCVAM Test Method Evaluation Report. The ICCVAM Test Method Evaluation Report will be provided to U.S. Federal regulatory agencies for consideration and be made available to the public. The ICCVAM Authorization Act requires that Federal agencies respond to ICCVAM within 180 days after receiving the ICCVAM test method recommendations. Agency responses will be posted on the NICEATM-ICCVAM website[3] as they become available.

We gratefully acknowledge the many individuals who contributed to the preparation, review, and revision of this report. 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

[1] http://www.blf.gov/IIF

[2] The "traditional LLNA" refers to the validated ICCVAM-recommended LLNA test method protocol, which measures lymphocyte proliferation based on incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxy-uridine into the cells of the draining auricular lymph nodes (ICCVAM 1999, Dean et al. 2000).

[3] http://iccvam.niehs.nih.gov

serving as the Panel Chair and to Dr. Michael Woolhiser, Dr. Michael Olson, Dr. Stephen Ullrich, and Kim Headrick for their service as Evaluation Group Chairs. We thank the IWG for assuring a meaningful and comprehensive review. We especially thank Dr. Joanna Matheson (U.S. Consumer Product Safety Commission) and Dr. Abigail Jacobs (U.S. Food and Drug Administration Center for Drug Evaluation and Research) for serving as Co-Chairs of the IWG. We also acknowledge Integrated Laboratory Systems, Inc. (ILS), the NICEATM support contractor, for providing excellent scientific and operational support, including Dr. David Allen, Thomas Burns, Michael Paris, Dr. Eleni Salicru, Frank Stack, and Dr. Judy Strickland. Finally, we thank Dr. Silvia Casati and Dr. Hajime Kojima, the IWG liaisons from ECVAM and JaCVAM, respectively, for their participation and contributions.

This comprehensive ICCVAM evaluation of the LLNA applicability domain should facilitate regulatory agency decisions on the acceptability of the LLNA for evaluating the allergic contact dermatitis potential of pesticide formulations, metals, substances tested in aqueous solutions, and other products. Use of the method by industry can be expected to significantly reduce and refine animal use for ACD testing while continuing to support the protection of human health.

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Executive Summary

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recently evaluated the applicability domain of the murine local lymph node assay (LLNA). Applicability domain refers to defined chemicals and products for which a test method can be used to obtain accurate and reliable results. The LLNA assesses the potential of substances to cause allergic contact dermatitis (ACD). ACD is an allergic skin reaction characterized by redness, swelling, and itching that can result from contact with a sensitizing chemical or product. This Test Method Evaluation Report provides ICCVAM's recommendations regarding the usefulness and limitations of the LLNA for testing pesticide formulations, metals, substances in aqueous solutions, and other products (i.e., the current applicability domain of the LLNA). This report includes the updated ICCVAM-recommended LLNA test method protocol, the final Addendum to the ICCVAM report on the LLNA (ICCVAM 1999), and recommendations for future studies and performance standards.

The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), ICCVAM, and the ICCVAM Immunotoxicity Working Group prepared an initial draft Addendum and draft test method recommendations. The drafts were provided to an independent international scientific peer review panel (Panel) and the public for comment. The initial draft Addendum reviewed LLNA data from a database of more than 500 test substances. It built on the original ICCVAM evaluation of the LLNA, which was based on 209 substances (ICCVAM 1999). The Panel met twice in public session to review the initial and updated draft Addendums and draft ICCVAM recommendations. A detailed timeline of the evaluation of the LLNA applicability domain is included with this report.

The Panel initially met in public session on March 4–6, 2008, to discuss its peer review of the ICCVAM initial draft Addendum and to provide conclusions and recommendations regarding the LLNA applicability domain. The Panel also reviewed how well the information contained in the initial draft Addendum supported ICCVAM's draft test method recommendations. The Panel agreed with ICCVAM that the LLNA appeared useful for the testing of metal compounds, with the exception of nickel. The Panel agreed with the ICCVAM recommendations, which stated that more data were necessary before a recommendation could be made on the usefulness and limitations of the LLNA for testing mixtures and substances in aqueous solutions.

NICEATM obtained the additional data and updated the initial draft Addendum. The updated draft Addendum evaluated data derived from a database of more than 600 substances tested in the LLNA (including pesticide formulations and other products). The Panel reconvened in public session on April 28–29, 2009, to review the ICCVAM updated draft Addendum and to finalize its conclusions and recommendations on the current LLNA applicability domain. In finalizing this Test Method Evaluation Report and the Addendum, which is included as an appendix, ICCVAM considered (1) the conclusions and recommendations of the Panel, (2) comments from ICCVAM's Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), and (3) public comments.

ICCVAM Recommendations: Test Method Usefulness and Limitations

ICCVAM concludes that the accuracy performance of the LLNA supports its use for testing (1) pesticide formulations and other products; (2) metals, with the exception of nickel; (3) substances tested in aqueous solutions; and (4) other products and substances, unless these materials have unique physiochemical properties associated with them that might interfere with the LLNA's ability to detect sensitizing substances. To achieve adequate exposure, substances in aqueous solutions must be tested in an appropriate vehicle (e.g., 1% Pluronic L92 [Boverhoff et al. 2008]) that will maintain adequate contact of the test substance with the skin. The determination that a specific modification of the LLNA test method protocol is valid for evaluating new chemical classes should be relevant to other valid versions of the LLNA test method protocol (e.g., LLNA: DA and LLNA: BrdU-ELISA).

As shown in **Table 1**, the LLNA is more likely than the guinea pig test to yield a positive result for many substances. Therefore, the potential for overclassification may be a limitation of the LLNA. Federal agencies should assess how well the test materials and findings in the updated draft Addendum represent their substances of interest, particularly with respect to chemical classes and potential biological effects.

ICCVAM Recommendations: Test Method Protocol

ICCVAM recently updated the ICCVAM-recommended LLNA test method protocol (Appendix A of ICCVAM 2009a). ICCVAM recommends this revised protocol for all future LLNA studies.

Additionally, in testing situations that do not require dose-response information, the LLNA should be considered as a reduced LLNA test method protocol. The reduced LLNA tests only the high dose, further reducing animal use.

ICCVAM Recommendations: Future Studies

ICCVAM recommends several future studies to further characterize the usefulness and limitations of the LLNA. However, ICCVAM discourages formal validation of the LLNA for new classes/types of test substances unless there is a biologically-based rationale. An integrated assessment of available information, including computer-assisted structure–activity relationships, prediction/measurement of biotransformation to potential reactive species, and possibly peptide, protein, or lipid binding should be conducted for new classes of test materials. Before any animal testing is conducted, the need to test a substance for skin sensitization potential should be considered.

Table 1 Summary of LLNA Performance for Testing Pesticide Formulations and Other Products, Metal Compounds, and Substances in Aqueous Solutions

Comparison	n	Accuracy		False Positive Rate		False Negative Rate	
		%	No.	%	No.	%	No.
Pesticide Formulations							
LLNA vs. GP[1]	23	57	13/23	50	10/20	0	0/3
Dyes							
LLNA vs. GP[1]	6	33	2/6	100	1/1	60	3/5
Natural Complex Substances							
LLNA vs. Human[2]	12	42	5/12	75	6/8	25	1/4
Metal Compounds							
LLNA vs. GP[1]	6	83	5/6	100	1/1	0	0/5
LLNA vs. Human[2]	14	86	12/14	40	2/5	0	0/9
Substances Tested in Aqueous Solutions							
LLNA vs. GP[1]	25	56	14/25	48	10/21	25	1/4

Abbreviations: GP = guinea pig skin sensitization outcomes; LLNA = murine local lymph node assay; n = number of substances included in this analysis; No. = number (data on which the percentage calculation is based).
Accuracy (concordance) = the proportion of correct outcomes (positive and negative) of a test method; false positive rate = the proportion of all negative substances that are falsely identified as positive; false negative rate = the proportion of all positive substances that are falsely identified as negative.
[1] *GP* refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.
[2] *Human* refers to outcomes obtained by studies conducted using the human maximization test or a human patch test allergen kit.

ICCVAM Recommendations: Performance Standards

ICCVAM, the European Centre for the Validation of Alternative Methods, and the Japanese Center for the Validation of Alternative Methods have developed internationally harmonized test method performance standards for the LLNA (ICCVAM 2009a).[4] These performance standards can be used to evaluate the validity of LLNA test methods that incorporate specific modifications of the traditional LLNA test method.

Validation Status of the LLNA for Testing Pesticide Formulations, Metals, Substances in Aqueous Solutions, and Other Products

The Addendum summarizes information from a review of LLNA data derived from a database of more than 600 substances (including pesticide formulations and other products). It builds on the 1998-99 ICCVAM evaluation of the LLNA (ICCVAM 1999) that considered a database of 209 substances. To minimize duplication, metal formulations were not analyzed, and metal compounds were restricted to those testing single substances. The updated reference database includes (1) data for metal compounds from the original ICCVAM evaluation, (2) data published since that evaluation, and (3) data submitted in response to a *Federal Register* notice (72 FR 27815)[5] requesting LLNA, guinea pig, and/or human skin sensitization data and experience.

Pesticide Formulations: The updated LLNA database contains data for 104 pesticide formulations. Fifty-four percent of these formulations were LLNA positive, and 46% were LLNA negative.

Twenty-three pesticide formulations had associated guinea pig data for the complete formulation. An additional 46 formulations had guinea pig data for one or more of the active ingredients included in the formulation tested in the LLNA. Fourteen formulations had guinea pig data for a substance related to an active ingredient or for a related formulation.

Among the 23 formulations that had both LLNA and guinea pig data, the LLNA classified 52% (12 of 23) as sensitizers while the guinea pig tests classified 13% (3 of 23) as sensitizers. All three 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 had 57% agreement (accuracy) in 13 of 23 tests (**Table 1**). The LLNA identified as sensitizers an additional seven formulations that the guinea pig test classified as nonsensitizers, a possible overprediction (false positive) rate of 50% (10 of 20) (**Table 1**). However, 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 comparative LLNA and guinea pig data. The LLNA classified 50% of the dyes as sensitizers and 50% as nonsensitizers. By comparison, the guinea pig maximization test (GPMT) classified 83% as sensitizers and 17% as nonsensitizers. Overall, the LLNA and GPMT results had 33% accuracy (**Table 1**). The overprediction (false positive) rate for the LLNA was 100% (1 of 1), and the underprediction (false negative) rate was 60% (3 of 5) (**Table 1**).

Natural Complex Substances: The current LLNA database contains data for 12 natural complex substances (essential oils and absolutes) with comparative LLNA and human data. The LLNA classified 75% (9 of 12) of these substances as sensitizers and 25% (3 of 12) as nonsensitizers. However, human clinical studies identified only 33% (4 of 12) as sensitizers. The LLNA identified three of these four as sensitizers (75%), but six more tested positive that did not produce positive results in the human testing. Compared to human outcomes, the LLNA had an accuracy of 42% (5 of 12), a false positive rate of 75% (6 of 8), and a false negative rate of 25% (1 of 4) (**Table 1**).

[4] Available at http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm.
[5] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf.

Metal Compounds: The current LLNA database contains test results from 48 studies of 16 metal compounds. The compounds represent 13 different metals. (Formulations containing metals were excluded from this analysis.) All 16 metal compounds had comparative human data, and eight had comparative guinea pig data. Because nickel was classified as a sensitizer in three of seven studies and as a nonsensitizer in four of seven studies, nickel compounds were excluded 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 false positive rate of 40% (2 of 5), and a false negative rate of 0% (0 of 9) when compared to human results (**Table 1**). The two false positive compounds were copper chloride and zinc sulfate.

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

The performance of the LLNA and the guinea pig tests was compared to human results for the six metal compounds tested in all three species. The LLNA had accuracy of 83%, a false positive rate of 100%, and a false negative rate of 0%. By comparison, the guinea pig tests had an accuracy of 100%, a false positive rate of 0%, and a false negative rate of 0% relative to the human outcomes.

Substances Tested in Aqueous Solutions: The current LLNA database of substances tested in aqueous solutions includes results from 171 studies representing 139 substances. Ninety-one percent of these substances (123 LLNA studies) are pesticide formulations and pure compounds. Forty-eight percent (48 LLNA studies) are aqueous eluates of medical devices. The two groups were analyzed separately because of differences in the protocols for sample preparation. Of the 91 pesticide formulations and pure compounds, 63% (57 of 91) were LLNA positive, and 37% (34 of 91) were LLNA negative. The substances included in this evaluation were tested at a final concentration of at least 20% water.

Guinea pig data were available for 25 substances tested in aqueous solutions. The LLNA and the guinea pig test results disagreed for 11 (44%) of the substances. Ten of the 11 discordant substances (91%) were pesticide formulations tested in aqueous 1% Pluronic L92. These were the same 10 substances previously discussed for the pesticide formulations analysis. The LLNA overpredicted all 10 with respect to the guinea pig results (48% [10 of 21] false positive rate) (**Table 1**). The LLNA underpredicted one additional substance, neomycin sulfate, which was tested in 25% EtOH (25% [1 of 4] false negative rate) (**Table 1**). The LLNA and guinea pig results had overall agreement (accuracy) of 56% (14/25) (**Table 1**).

All 48 of the medical device eluates were negative in the LLNA. These eluates were not analyzed to determine their constituents or to determine whether any compound(s) were in fact eluted from the medical device tested.

ICCVAM Consideration of Public and SACATM Comments

The ICCVAM evaluation process provides numerous opportunities for stakeholder involvement. The public may submit written comments and provide oral comments at ICCVAM independent scientific peer review panel meetings and SACATM meetings. From May 2007 to June 2009, there were a total of 12 opportunities for public comment on the ICCVAM evaluation of the LLNA applicability domain. During this time, ICCVAM received 46 public comments, nine of which pertained directly to the LLNA applicability domain. In addition, SACATM reviewed and commented on the draft ICCVAM recommendations and associated conclusions of the Panel during their annual meetings in June 2008 and June 2009. ICCVAM considered both public and SACATM comments in finalizing the test method recommendations provided in this report.

1.0 Introduction

The murine local lymph node assay (traditional LLNA)[1] is an alternative skin sensitization test method that requires fewer animals and less time than currently accepted guinea pig (GP) tests (e.g., the guinea pig maximization test and the Buehler test). It also avoids animal discomfort that can occur in the GP tests when substances cause allergic contact dermatitis (ACD). The LLNA measures cell proliferation in the draining auricular lymph nodes of the mouse by analyzing incorporation of a radioactive marker into newly synthesized DNA. The LLNA was the first alternative test method evaluated and recommended by the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). International regulatory authorities have now recognized the traditional LLNA as an acceptable alternative to GP tests for most testing situations.

The current LLNA applicability domain was one of several LLNA-related topics nominated by the U.S. Consumer Product Safety Commission (CPSC) for evaluation by ICCVAM and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM).[2] For this evaluation, the LLNA was assessed for its ability to correctly identify the sensitization potential of pesticide formulations and other products, metals, and substances tested in aqueous solutions.

The ICCVAM Authorization Act of 2000 (Public Law 106-545, 42 United States Code 285*l*-3) charged ICCVAM with coordinating the technical evaluations of new, revised, and alternative test methods with regulatory applicability. After considering comments from the public and ICCVAM's advisory committee, the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), ICCVAM members unanimously agreed that an evaluation of the LLNA applicability domain should have a high priority for evaluation. A detailed timeline of this evaluation is provided in **Appendix A**. The updated ICCVAM-recommended LLNA test method protocol, a comparison of LLNA results for substances tested in two different mouse strains, and the final Addendum to the ICCVAM report on the LLNA (ICCVAM 1999, hereafter Addendum) are provided in **Appendices B**, **C**, and **D**, respectively.

The ICCVAM Immunotoxicity Working Group (IWG) was formed to work with NICEATM in evaluating the test methods. Dr. Silvia Casati was the European Centre for the Validation of Alternative Methods (ECVAM) liaison, and Dr. Hajime Kojima was the Japanese Center for the Validation of Alternative Methods (JaCVAM) liaison to the IWG.

To facilitate peer review of the LLNA applicability domain evaluation, the IWG and NICEATM, which administers ICCVAM and provides scientific and operational support for ICCVAM activities, prepared a comprehensive initial draft Addendum that provided information and data from validation studies and the scientific literature. A May 17, 2007, *Federal Register* (FR) notice (72 FR 27815)[3] requested data and information on these test methods and nominations of individuals to serve on an international independent scientific peer review panel (Panel). The request was also disseminated via the ICCVAM electronic mailing list and through direct requests to over 100 stakeholders. In response to this request, three individuals or organizations nominated members to the Panel (see **Section 4.0**).

In the initial draft Addendum, ICCVAM examined data derived from a database of over 500 substances (including pesticide formulations and other products) tested in the LLNA. In the original ICCVAM evaluation of the LLNA (ICCVAM 1999), the performance of the LLNA was compared to (1) results from GP tests and (2) information about sensitizers in humans (e.g., human

[1] The "traditional LLNA" refers to the validated ICCVAM-recommended LLNA protocol, which measures lymphocyte proliferation based on incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine into the cells of the draining auricular lymph nodes (ICCVAM 2009a).

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

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

maximization test results, substances used in human repeat insult patch test, clinical case reports), where available. The initial draft Addendum updated the LLNA performance analyses for pesticide formulations and other products, metals, and substances tested in aqueous solutions when compared to human and GP results. On January 8, 2008, ICCVAM announced the availability of the initial draft Addendum to the public and a public Panel meeting to review the validation status of the LLNA applicability domain (and other LLNA-related activities) (73 FR 1360).[4] All of the information provided to the Panel, including the ICCVAM initial draft Addendum, draft test method recommendations, and all public comments received prior to the Panel meeting, were made publicly available via the NICEATM-ICCVAM website.[5]

The first Panel meeting was a public session held on March 4-6, 2008, to review the ICCVAM evaluation of the LLNA for testing pesticide formulations and other products, metals, and substances in aqueous solutions and the completeness of the ICCVAM initial draft Addendum. The Panel evaluated (1) the extent to which the initial draft Addendum addressed established validation and acceptance criteria and (2) the extent to which the initial draft Addendum supported ICCVAM's draft proposed test method uses, recommended protocol, draft test method performance standards, and proposed future studies. Interested stakeholders from the public were provided opportunities to comment at the Panel meeting. The Panel considered these comments as well as those submitted prior to the meeting before concluding their deliberations. The Panel recommended that NICEATM and ICCVAM solicit more data on pesticide formulations and other products and substances tested in aqueous solutions, before making recommendations about the usefulness of the LLNA for testing such substances. On May 20, 2008, ICCVAM posted a report of the Panel's recommendations[6] (see **Appendix E**) on the NICEATM-ICCVAM website for public review and comment (announced in 73 FR 29136).[7]

ICCVAM provided SACATM with the updated draft Addendum and initial draft test method recommendations, the Panel report, and all public comments for discussion at their meeting on June 18-19, 2008, where public stakeholders were given another opportunity to comment.

NICEATM subsequently obtained a detailed test method protocol and data from an additional 140 substances and updated the initial draft Addendum to include this new information. The updated draft Addendum included an accuracy evaluation for the expanded database of over 600 substances (as compared with over 500 substances included in the January 2008 draft). Based on the analyses included in the updated draft Addendum, ICCVAM prepared updated draft test method recommendations for proposed test method uses and limitations, recommended protocol, test method performance standards, and future studies for the LLNA. ICCVAM released the updated draft documents to the public for comment on February 27, 2009, and announced a second meeting of the Panel (74 FR 8974).[8] The Panel reconvened on April 27-28, 2009, to again evaluate the LLNA applicability domain. The Panel also reviewed the completeness of the ICCVAM updated draft Addendum and the extent to which the information therein supported the ICCVAM updated draft test method recommendations. On June 1, 2009, ICCVAM posted the second report of the Panel's recommendations[9] (see **Appendix E**) on the NICEATM-ICCVAM website for public review and comment (announced in 74 FR 26242).[10]

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[4] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_25553.pdf
[5] Available at http://iccvam.niehs.nih.gov
[6] Available at http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
[7] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR-E8-11195.pdf
[8] Available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR-E9-4280.pdf
[9] Available at http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2009.pdf
[10] Announced in 74 *FR* 26242 http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR-E9-12360.pdf

ICCVAM provided SACATM with the revised draft Addendum, the second Panel report, and all public comments for discussion at their meeting on June 25-26, 2009, where public stakeholders were given another opportunity to comment.

After SACATM's meeting, ICCVAM and the IWG considered the SACATM comments, the Panel report, and all public comments before finalizing the ICCVAM Test Method Evaluation Report and the Addendum provided in this report. As required by the ICCVAM Authorization Act, ICCVAM will make this test method evaluation report and the accompanying final addendum available to the public and to U.S. Federal agencies for consideration. Federal agencies must respond to ICCVAM within 180 days after receiving ICCVAM test method recommendations. Agency responses will be made available to the public on the NICEATM-ICCVAM website as they are received.

2.0 ICCVAM Recommendations for the Updated Assessment of the Validity of the LLNA for Testing Pesticide Formulations, Metals, Substances in Aqueous Solutions, and Other Products

ICCVAM has updated the original validation report of the LLNA (ICCVAM 1999) based on a comprehensive review of available data and information regarding the current validity of the LLNA for assessing the skin-sensitizing potential of pesticide formulations and other products, metal compounds, and substances in aqueous solutions. The information is based on a retrospective review of data derived from over 600 substances, including 104 pesticide formulations, tested in the LLNA. The current evaluation builds on the previous ICCVAM evaluation of the LLNA, which was based on 209 substances (ICCVAM 1999). The Addendum updates the LLNA performance analyses for pesticide formulations and other products, metal compounds, and substances in aqueous solutions when compared to (1) the results from GP tests and (2) information about sensitizers in humans (e.g., human maximization test results, substances used in human repeat insult patch test, clinical case reports), where available (see **Section 3.0** and **Appendix D**).

2.1 ICCVAM Recommendations: Test Method Usefulness and Limitations

Pesticide Formulations: The current LLNA database contains test results on 104 pesticide formulations, 23 of which have comparative GP data. None have comparative human data. 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 10 active ingredients represent a small proportion (i.e., one insecticide, one microbioocide, six herbicides and two fungicides). Based on these 23 pesticide formulations, the concordance (accuracy) of the LLNA results compared to GP data is 57% (13/23), with an overprediction ("false positive") rate of 50% (10/20) and underprediction ("false negative") rate of 0% (0/3). Thus, there is a greater likelihood of obtaining a positive result in the LLNA (13/23; 57%) than in a GP test (3/23; 13%). All three formulations that were identified as positive in the GP tests were also identified as positive in the LLNA. Although human data are not available for these pesticide formulations to confirm their human sensitization potential, these data indicate that the LLNA is more likely to classify a pesticide formulation as a sensitizer than the GP tests. It should be noted that all 23 formulations were tested in the LLNA in the aqueous vehicle 1% Pluronic L92. Federal agencies should assess how well the test materials and findings in the Addendum represent their substances of interest, particularly with respect to chemical classes and potential biological effects. If there is any primary testing or postmarketing reports of skin sensitization, they should be used for comparison with LLNA results.

The LLNA can be used for testing pesticide formulations unless there are unique physicochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances. The potential for possible overclassification of pesticide formulations may be a limitation of the LLNA.

Natural Complex Substances: The current LLNA database also contains data for 12 natural complex substances for which there are comparative LLNA and human data. Based on LLNA results for these natural complex substances, 75% (9/12) were sensitizers and 25% (3/12) were nonsensitizers. However, based on human clinical studies, only 33% (4/12) of these substances tested as sensitizers. Based on this limited database, the concordance (accuracy) of the LLNA results compared to human sensitization data is 42% (5/12), with an overprediction ("false positive") rate of 75% (6/8) and underprediction ("false negative") rate of 25% (1/4). There are no comparative data from GP tests with these natural complex substances. Therefore, a comparison of the performance of the LLNA and the GP tests relative to the human outcome is not possible. Federal agencies should

assess how well the test materials and findings in the Addendum represent their substances of interest, particularly with respect to chemical classes and potential biological effects.

The LLNA can be used for testing natural complex substances unless there are unique physicochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances. The potential for possible overclassification of natural complex substances may be a limitation of the LLNA.

Dyes: The current LLNA database contains data for six dyes, for which there are LLNA and GP data. Compared to GPMT outcomes, the LLNA concordance (accuracy) is 33% (2/6), the overprediction ("false positive") rate is 100% (1/1) and the underprediction ("false negative") rate is 60% (3/5). Federal agencies should assess how well the test materials and findings in the Addendum represent their substances of interest, particularly with respect to chemical classes and potential biological effects.

The LLNA can be used for testing dyes unless there are unique physicochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances. The potential for possible overclassification of dyes may be a limitation of the LLNA.

Metal Compounds: The current LLNA database contains test results on 48 studies involving 16 metal compounds representing 13 different metals (formulations containing metals are excluded from this analysis). 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. Because nickel was classified as a sensitizer in three of these studies and as a nonsensitizer in the other four, nickel compounds were excluded from the LLNA metals performance analysis.

For these remaining 14 metal compounds (13 metals), the LLNA concordance (accuracy) is 86% (12/14), the overprediction ("false positive") rate is 40% (2/5) and the underprediction ("false negative") rate is 0% (0/9), when compared to human results. The two false positive compounds were copper chloride and zinc sulfate. All six of the metal compounds (six different metals with nickel compounds excluded) with comparative GP test results were predicted as sensitizers by the LLNA. For these metal compounds, the LLNA concordance (accuracy) is 83% (5/6), the overprediction ("false positive") rate is 100% (1/1), and the underprediction ("false negative") rate is 0% (0/5), when compared to GP test results. When comparing the performance of the LLNA and the GP tests for the six metal compounds tested in all three species (i.e., mice, GPs, and humans) to human results, the LLNA concordance (accuracy) is 83% (5/6), the overprediction ("false positive") rate is 100% (1/1) and the underprediction ("false negative") rate is 0% (0/5). By comparison, the GP test concordance (accuracy) is 100% (6/6), the overprediction ("false positive") rate is 0% (0/1) and the underprediction ("false negative") rate is 0% (0/5) against the human. Federal agencies should assess how well the test materials and findings in the Addendum represent their substances of interest, particularly with respect to chemical classes and potential biological effects.

The LLNA can be used for testing metal compounds, with the exception of nickel, unless there are unique physicochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances. Inconsistent results for nickel compounds obtained with the traditional LLNA suggest that the LLNA may not be suitable for testing substances containing nickel. Until the LLNA has been found to accurately identify ACD potential in substances containing nickel, further testing using a different test system is recommended when negative results are obtained for such substances.

Substances Tested in Aqueous Solutions: The current LLNA database contains test data on 44 studies that involved testing 25 substances in an aqueous solution. Pesticide formulations that were considered in the analysis discussed previously were also included in this evaluation, so this database

has the same limitations as discussed previously. The substances included in this evaluation contain at least 20% water. Most (23/25) of these substances were tested in the vehicle 1% Pluronic L92. Based on LLNA results for these substances 48% (12/25) were sensitizers and 52% (13/25) were nonsensitizers. However, based on GP results, only 20% (5/25) tested as sensitizers. Based on this limited database, the concordance (accuracy) of the LLNA compared to GP sensitization data is 56% (14/25), the overprediction ("false positive") rate is 48% (10/21) and the underprediction ("false negative") rate is 25% (1/4). Among the 11 substances for which LLNA and GP results were discordant, only one (i.e., neomycin sulfate) is negative in the LLNA and positive in the GP. These data suggest that the LLNA is more likely than the GP to classify a substance tested in an aqueous solution as a sensitizer. Human data are available for one substance that is discordant between the LLNA and the GP (i.e., neomycin sulfate). This substance is also discordant between the LLNA (i.e., negative) and the human (i.e., positive). Federal agencies should assess how well the test materials and findings in the Addendum represent their substances of interest, particularly with respect to chemical classes and potential biological effects.

The LLNA can be used for testing substances in aqueous solutions unless there are unique physicochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances. When testing substances in aqueous solutions, it is also essential to use an appropriate vehicle, to maintain the test substance in contact with the skin (e.g. 1% Pluronic L92 [Boverhoff et al. 2008]) so an adequate exposure is achieved, as demonstrated by positive control results. It should be recognized that the potential for possible overclassification of aqueous substances may be a limitation of the LLNA.

Independent Peer Review Panel Conclusions and Recommendations
The Panel concurred that the available data supported the ICCVAM updated draft test method recommendations for the LLNA with regard to testing pesticide formulations, dyes, natural complex substances, metal compounds and substances tested in aqueous solutions, in terms of the proposed test method usefulness and limitations.

On the basis of the available information, unless there are unique physicochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances, the Panel considered all of these test materials as candidates for testing in the LLNA, subject to the limitations outlined in the ICCVAM Test Method Recommendations.

2.2 ICCVAM Recommendations: Test Method Protocol

An updated version of the validated ICCVAM-recommended LLNA test method protocol has recently been developed (Appendix A of ICCVAM 2009a). This revised protocol is recommended for all future LLNA studies and includes the following key aspects:

- The high dose should be the maximum soluble concentration that does not produce systemic toxicity and/or excessive local irritation. The measurement of ear swelling is a potentially valuable adjunct for identifying local irritation.
- A minimum of four animals per dose group is recommended.
- Collection of individual animal data is recommended.
- Inclusion of a concurrent vehicle control and positive control in each study is recommended.

Additionally, ICCVAM recommends that there should be a measure of variability of the positive control response over time. Laboratories should maintain a historical database of positive control SI values such that results can be compared to the mean historical SI. There could be cause for concern when a negative test substance result is accompanied by a concurrent positive control SI value significantly lower than the mean historical SI.

In testing situations where dose-response information is not required, the LLNA should be considered for use as a reduced LLNA test method protocol in which only the high dose is tested, thus further reducing animal use.

Independent Peer Review Panel Conclusions and Recommendations
The Panel concluded that updated information on various elements in the Addendum did not suggest the need for changes to recommendations for the development of a revised standard method. Whenever discretion is permitted, the Panel recommended the inclusion of a suitable (representative) positive control from the same category of materials to be tested (e.g., for testing pesticides, select one representative positive control pesticide).

2.3 ICCVAM Recommendations: Future Studies

ICCVAM recommends the following future studies to further characterize the usefulness and limitations of the LLNA:

- To more comprehensively evaluate the ability of the LLNA to be used for testing nickel compounds, additional data from LLNA studies on such compounds with comparative human and/or GP data are needed.
- Where available, solubility data should be provided in future studies so that thermodynamic activity can be computed and compared to maximum theoretical percutaneous penetration. This information should be considered when comparing the data from LLNA studies in lipophilic delivery systems compared to that in aqueous systems. Studies done in aqueous systems should use 1% Pluronic L92 as the vehicle in order to expand the existing database for that vehicle, unless adequate scientific rationale is provided for using another aqueous vehicle.
- Revalidation of the LLNA for new classes/types of test substances should be avoided unless there is a biologically based rationale. For new classes of test materials, an integrated assessment of available information should be conducted. This should include computer-assisted structure-activity relationships, prediction/measurement of biotransformation to potential reactive species, and possibly peptide, protein, or lipid binding. Before any animal testing is conducted, consideration should be given to the necessity for a substance to be tested for skin sensitization potential.
- If any variant of the LLNA is validated for use to test novel classes, then the findings should be relevant to the family of validated LLNA tests.

Independent Peer Review Panel Conclusions and Recommendations
The Panel concurred with ICCVAM's recommendations for future studies. The Panel also suggested that, before additional animal testing is conducted, consideration should be given to the necessity for the substance to be tested for skin sensitization potential.

2.4 ICCVAM Recommendations: Performance Standards

In conjunction with ECVAM and JaCVAM, ICCVAM has developed internationally harmonized test method performance standards for the LLNA (ICCVAM 2009a)[11] to evaluate the performance of LLNA test methods that incorporate specific protocol modifications (e.g., procedures to measure lymphocyte proliferation) compared to the traditional LLNA.

[11] Available at http://iccvam.niehs.nih.gov/methods/immunotox/llna_PerfStds.htm

3.0 Evaluation of the LLNA Applicability Domain

The following is a synopsis of the information in the final Addendum to the ICCVAM report on the LLNA (ICCVAM 1999) (**Appendix D**, hereafter, Addendum), which reviews the available data and information for the LLNA applicability domain. The Addendum describes the current validation status of the LLNA for testing pesticide formulations and other products, metals, and substances in aqueous solutions, the scope of the substances tested, and standardized protocols used.

3.1 Test Method Description

The purpose of the LLNA test method is to identify potential skin sensitizers by quantifying lymphocyte proliferation in the draining auricular lymph nodes. The magnitude of lymphocyte proliferation correlates with the extent to which sensitization develops after a topical induction exposure to a potential skin-sensitizing substance.

3.1.1 General Test Method Procedures

The LLNA measures lymphocyte proliferation after topical exposure to a potential skin-sensitizing substance. The test substance is administered topically on three consecutive days to the ears of mice at a concentration that provides maximum solubility of the test substance without causing systemic toxicity and/or excessive local irritation. Two days after the final application of the test substance, ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine (in phosphate-buffered saline; 250 µL/mouse) is administered via the tail vein. Five hours later the draining auricular lymph nodes are excised, and a single-cell suspension from the lymph nodes of each animal is prepared for quantifying the incorporation of radioactivity, which correlates with lymph node cell proliferation.

The incorporation of ^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine for each mouse is expressed in disintegrations per minute (dpm). The stimulation index (SI) is calculated as the ratio of the mean dpm/mouse for each treatment group against the mean dpm/mouse for the vehicle control group. The threshold for a positive response is an SI $\geq$ 3.

3.2 LLNA Applicability Domain Database

The information summarized in the 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). To minimize duplication in this evaluation, metal formulations were 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 (**Appendix D,** Annex I), data published since that evaluation, and data submitted in response to a request in a FR notice (72 FR 27815)[12] requesting LLNA, GP, and/or human skin sensitization data and experience. An evaluation of the usefulness and limitations of the LLNA for testing pesticide formulations and other products, and substances tested in aqueous solutions was not included in the original ICCVAM validation (**Appendix D,** Annex I) because no data on these substances were available at that time. The reference database for these substances in the Addendum consists of data published since the original ICCVAM evaluation or submitted in response to the FR notice. **Table 3-1** provides information on the sources of the data and the rationale for the substances tested.

Among the LLNA studies for the pesticide formulations, 32% (29/89) used the BALB/c mouse strain rather than the CBA/J or CBA/Ca strains of mice, which are recommended in standardized LLNA

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

protocols (ICCVAM 2009a; EPA 2003; OECD 2002). One additional submitted LLNA study (from Dr. Dori Germolec at the National Institute of Environmental Health Sciences [NIEHS]) also used the BALB/c strain. The comparative performance of the LLNA using these different mouse strains relative to the GP is detailed in **Appendix C**.

Table 3-1 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 essential oils in the LLNA. RIFM and the authors submitted additional data.
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.
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 nonradioactive 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.

Continued

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

Data Source	N	Substance Selection Rationale
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 electronically 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 für 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; ECVAM = European Centre for the Validation of Alternative Methods; GP = guinea pig; LLNA = murine 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 = Netherlands Organization for Applied Scientific Research.

[1] These data were evaluated by the 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.

3.3 Reference Test Method Data

The traditional LLNA data used for evaluation of the LLNA applicability domain include the results for all tested doses of each substance. In addition to calculated SI values for each of the tested doses, the vehicles tested and EC3 values (estimated concentration needed to produce an SI value of 3) for substances classified as sensitizers were provided in Gerberick et al. (2005). If EC3 values were not included in the data source, they were calculated, where possible, using either interpolation or extrapolation (Dearman et al. 2007).

The reference data for the GP tests (guinea pig maximization test [GPMT] or Buehler test) and human data (human maximization test, human patch test allergen, or other human data) were obtained from the scientific literature or from the data submitters. The complete database (by each source) is provided in Annex II, III, and IV of the Addendum (**Appendix D**).

3.4 Test Method Accuracy

Table 3-2 presents a summary of performance statistics for the LLNA for testing pesticide formulations, dyes, natural complex substances, metal compounds, and substances tested in aqueous solutions.

Table 3-2 Evaluation of LLNA Performance for Testing Pesticide Formulations and Other Products, Metal Compounds, and Substances in Aqueous Solutions

Comparison	n[1]	Accuracy		False Positive Rate		False Negative Rate	
		%	No.[2]	%	No.[2]	%	No.[2]
Pesticide Formulations							
LLNA vs. GP[3]	23	57	13/23	50	10/20	0	0/3
Dyes							
LLNA vs. GP[3]	6	33	2/6	100	1/1	60	3/5
Natural Complex Substances							
LLNA vs. Human[4]	12	42	5/12	75	6/8	25	1/4
Metal Compounds							
LLNA vs. GP[4]	6	83	5/6	100	1/1	0	0/5
LLNA vs. Human[4]	14	86	12/14	40	2/5	0	0/9
Substances Tested in Aqueous Solutions							
LLNA vs. GP[3]	25	56	14/25	48	10/21	25	1/4

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

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

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

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

[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.

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

Seventy of the 104 pesticide formulations have LLNA and some type of associated GP 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 pesticide formulations were tested in multiple LLNA studies (25 studies total); 5/6 multiply tested pesticide formulations had LLNA results in agreement, and 1/6 pesticide formulations produced discordant results (i.e., three positive, two negative). The discordant data were for the pesticide formulation Oxyflourfen EC and were submitted to NICEATM by the European Crop Protection Association. In a five-laboratory study, SI values for the highest concentration tested (33%) ranged from 2.3 to 5.4. All lower concentrations tested showed no SI values ≥ 3.

All 70 pesticide formulations (89/89 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 GP data for the complete formulation, 46 pesticide formulations had GP data for one or more of the active ingredients included in the

complete formulation, and 14 pesticide formulations had GP data for a substance related to an active ingredient or for a related formulation.

For the 23 formulations for which there were GP data, the LLNA classified 52% (12/23) of the formulations as sensitizers while the GP tests classified only 13% (3/23) of the formulations as sensitizers. All three of the pesticide formulations identified as sensitizers in the GP test were also identified as sensitizers in the LLNA. Overall, the LLNA and the GP results were in agreement (accuracy) 57% (13/23) of the time (**Table 3-2**). The LLNA also identified an additional seven substances as sensitizers that were classified as nonsensitizers in the GP test, an overprediction (false positive) rate of 50% (10/20) (**Table 3-2**). Three of the LLNA studies for the 23 pesticide formulations were done with BALB/c mice. If these three studies are removed from the analysis, the LLNA and the GP results were in agreement 60% (12/20) of the time, and the overprediction was 47% (8/17). There were no instances of underprediction by the LLNA for these 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 for which there are LLNA and GP data. Based on LLNA results for these six dyes, 50% (3/6) were sensitizers and 50% (3/6) were nonsensitizers. By comparison, based on GP results, 83% (5/6) were sensitizers and 17% (1/6) were nonsensitizers. The LLNA and the GP results were in agreement (accuracy) 33% of the time (**Table 3-2**). The overprediction (false positive rate) for the LLNA was 100% (1/1) and the underprediction (false negative rate) was 60% (3/5) (**Table 3-2**).

Natural Complex Substances: The current LLNA database also contains data for 12 natural complex substances (essential oils and absolutes) for which there are comparative LLNA and human data. Based on LLNA results for these substances, 75% (9/12) were sensitizers and 25% (3/12) nonsensitizers. However, based on human clinical studies, only 33% (4/12) of these substances tested as sensitizers. Therefore, compared to human outcomes for these 12 substances, the LLNA was able to identify three out of four of the substances that were positive in human testing. However, an additional six substances that did not produce positive results in the human testing were positive in the LLNA. Compared to human outcomes, the LLNA had an accuracy of 42% (5/12), a false positive rate of 75% (6/8) and a false negative rate of 25% (1/4) (**Table 3-2**). There were no comparative data from GP tests with these substances. Therefore, a comparison of the performance of the LLNA and the GP tests relative to the human outcome was not possible.

Metal Compounds: The current LLNA database contains test results on 48 studies involving 16 metal compounds representing 13 different metals (formulations containing metals were excluded from this analysis). 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. Nickel was classified as a sensitizer in three of these studies and as a nonsensitizer in the other four. Two positive results occurred in aqueous vehicles, one positive result occurred in a nonaqueous vehicle, and the four negative results all occurred in nonaqueous vehicles. Because of these discordant results, a performance analysis for metals was also conducted with nickel compounds excluded.

For the remaining 14 metal compounds (13 metals), the LLNA had an accuracy of 86% (12/14), a false positive rate of 40% (2/5) and a false negative rate of 0% (0/9), when compared to human results (**Table 3-2**). The two false positive compounds were copper chloride and zinc sulfate. All six of the metal compounds (six different metals with nickel compounds excluded) with comparative GP test results were predicted as sensitizers by the LLNA. For these metal compounds, the LLNA had an accuracy of 83% (5/6), a false positive rate of 100% (1/1), and a false negative rate of 0% (0/5) (**Table 3-2**), when compared to GP test results. When comparing the performance of the LLNA and the GP tests for the six metal compounds tested in all three species to human results, the LLNA had

an accuracy of 83% (5/6), a false positive rate of 100% (1/1) and a false negative rate of 0% (0/5). By comparison, the GP tests had an accuracy of 100% (6/6), a false positive rate of 0% (0/1) and a false negative rate of 0% (0/5) relative to the human.

Substances Tested in Aqueous Solutions: The current LLNA database of substances tested in aqueous solutions includes results from 171 studies representing 139 substances; 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. 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. Of the 91 pesticide formulations and pure compounds, 63% (57/91) are LLNA positive and 37% (34/91) are LLNA negative. LLNA studies were done with either CBA (66 studies) and/or BALB/c (28 studies) mouse strains. The mouse strain was unspecified for 29 studies. The substances included in this evaluation were tested in the LLNA at a final concentration of at least 20% water.

GP data were available for 25 (four sensitizers/21 nonsensitizers in the GP) substances tested in aqueous solutions. The outcomes of 11 substances were discordant between the LLNA and the GP tests. Ten of the 11 discordant substances were pesticide formulations tested in aqueous 1% Pluronic L92; these were the same 10 substances previously discussed for the pesticide formulations analysis, and all were overpredicted by the LLNA with respect to the GP results (48% [10/21] false positive rate) (**Table 3-2**). One additional substance, neomycin sulfate, which was tested in 25% EtOH, was underpredicted by the LLNA with respect to the GP results (25% [1/4] false negative rate) (**Table 3-2**). Overall, the LLNA and the GP results were in agreement (accuracy) 56% (13/25) of the time (**Table 3-2**).

Human data were available for only four substances (three sensitizers/one nonsensitizer in humans) tested in aqueous solutions, while there were only two substances tested in aqueous solutions in the LLNA for which there was comparative GP and human data. Therefore, the database of substances tested in multiple test methods (i.e., LLNA, GP, and/or human) is too few to allow for a meaningful assessment of performance.

All 48 of the medical device eluates were negative in the LLNA. None of these eluates had associated GP or human data. 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. Therefore, the results from these eluates were not included with those from the pesticide formulations and pure substances tested in aqueous solutions.

3.5 Animal Welfare Considerations: Reduction, Refinement, and Replacement

This comprehensive evaluation of the LLNA applicability domain should facilitate regulatory agency decisions on the acceptability of submitted LLNA studies for pesticide formulations and other products, metals, and substances tested in aqueous solutions. Following regulatory acceptance, use of the method by industry may lead to further reduction in use of the GP tests, which would provide for reduced animal use and increased refinement due to the avoidance of pain and distress in the LLNA procedure. This can be expected to significantly reduce the number of animals required for ACD testing while continuing to support the protection of human health.

4.0 ICCVAM Consideration of Public and SACATM Comments

The ICCVAM evaluation process incorporates a high level of transparency. This process is designed to provide numerous opportunities for stakeholder involvement, including submitting written public comments and providing oral comments at ICCVAM independent peer review panel meetings and SACATM meetings. **Table 4-1** lists the 12 different opportunities for public comment that were provided during the ICCVAM evaluation of the validation status of new versions and applications of the LLNA. The number of public comments received in response to each of the opportunities is also indicated. A total of 49 comments were submitted. Comments received in response to or related to the *Federal Register* notices are available on the NICEATM-ICCVAM website.[13] The following sections, delineated by *Federal Register* notice, briefly discuss the public comments received.

4.1 Public Comments in Response to 72 FR 27815 (May 17, 2007): The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data

NICEATM requested the following:

1. Public comments on the appropriateness and relative priority of evaluation of the validation status of
    a. The LLNA as a stand-alone assay for determining potency (including severity) for the purpose of hazard classification
    b. The reduced LLNA approach (Kimber et al. 2006; ESAC 2007; ICCVAM 2009b)
    c. Nonradioactive LLNA methods
    d. The use of the LLNA for testing mixtures, aqueous solutions, and metals
    e. The current applicability domain
2. Nominations of expert scientists to consider as members of a possible peer review panel
3. Submission of data for the LLNA and/or modified versions of the LLNA

In response to this FR notice, NICEATM received 17 comments. Six comments included additional data and information, while two others offered data and information upon request. Three commenters nominated four potential panelists for consideration. Three commenters suggested reference publications for consideration during the Panel evaluation. The nominees were included in the database of experts from which the Panel was selected. The data and suggested references were included in the initial draft ICCVAM review documents that were provided to the Panel at the March 2008 meeting.

[13] Available at http://ntp-apps.niehs.nih.gov/iccvampb/searchPubCom.cfm

Table 4-1 Opportunities for Public Comment

Opportunities for Public Comments	Date	# of Public Comments Received
72 FR 27815: The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data	May 17, 2007	17
72 FR 52130: Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments	September 12, 2007	4
73 FR 1360: Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments	January 8, 2008	7
Independent Scientific Peer Review Panel Meeting Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay	March 4-6, 2008	16
73 FR 25754: Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)	May 7, 2008	1
73 FR 29136: Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments	May 20, 2008	0
SACATM Meeting, Radisson Hotel, RTP, NC	June 18-19, 2008	0
74 FR 8974: Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments	February 27, 2009	1
Independent Scientific Peer Review Panel Meeting Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Evaluation of the Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay	April 28-29, 2009	2
74 FR 19562: Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)	April 29, 2009	0
74 FR 26242: Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments	June 1, 2009	1
SACATM Meeting, Hilton Arlington Hotel, Arlington, VA	June 25-26, 2009	0

1. A commenter suggested rearranging the priority sequence of test method evaluation from most to least pressing: a, e, d, b, and c (see list above).

ICCVAM did not establish a relative priority for these activities because they were all considered to be high-priority activities. Accordingly, all LLNA-related activities described above were discussed at the March 2008 Panel meeting.

Two comments pertained to the LLNA applicability domain.

1. One commenter noted that the LLNA is the only method that can be used in the United Kingdom for assessment of skin sensitization potential for regulatory purposes and highlighted that in some areas of the chemical industry there is concern regarding the applicability of the LLNA for testing of preparations, mixtures and irritant substances. The commenter also noted that there is concern with regard to the view that the LLNA has not always provided results consistent with existing knowledge of the test substance or related test substances. The commenter indicated that since the LLNA offers significant scientific and animal welfare advantages over GP models for many product types, and, in the U.K., the LLNA is effectively the only available method for evaluation of skin sensitization potential for regulatory purposes, an assessment of the LLNA is welcomed.

ICCVAM initiated an assessment of the peer-reviewed literature and available data, and prepared a comprehensive background review document, to assess the LLNA applicability domain.

2. Another commenter indicated that available information should allow ICCVAM to make a rapid determination of the applicability and limitations of the LLNA for testing aqueous mixtures and metals, and, if not, then further validation efforts in this regard, should instead focus on *in vitro* methods.

In addition to *in vivo* refinement (less pain and distress) alternatives (such as the LLNA), ICCVAM is committed to identifying *in vitro* models and non-animal approaches for assessing ACD and is engaged with ECVAM and JaCVAM in the development of validation studies for such methods.

4.2 Public Comments in Response to 72 FR 52130 (September 12, 2007): Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments

NICEATM requested public comments on the September 2007 draft ICCVAM-recommended LLNA performance standards developed to facilitate evaluation of modified LLNA test method protocols with regard to the traditional LLNA. In response to this FR notice, NICEATM received four comments, two of which suggested clarifications to the text. Another comment recommended that test substances chosen for testing in the various LLNA methods should be pure, with conclusive structures, and should not be mixtures. Most comments specifically addressed the LLNA performance standards, although one comment pertained to the LLNA in general.

1. One commenter supported the development of performance standards that expedite the validation of new protocols similar to previously validated methods but was disappointed that NICEATM-ICCVAM had chosen to develop performance standards for such a narrow scope of applicability (i.e., modifications of the standard LLNA that involve incorporation of nonradioactive methods of detecting lymphocyte proliferation). The commenter suggested that limited resources available to NICEATM-ICCVAM would be better spent on activities that would have greater impact on the reduction, refinement, or replacement of animal use, such as evaluating the use of human cell lines or *in vitro* skin models as a replacement for the LLNA.

ICCVAM considered the comment and concludes that the proposed modifications to the LLNA test method protocol and expanded applications have the potential to further reduce and refine animal use. ICCVAM is committed to identifying *in vitro* models and non-animal approaches for assessing ACD and is engaged with ECVAM and JaCVAM in the development of validation studies for such methods.

There were no comments that specifically addressed the LLNA applicability domain.

4.3 Public Comments in Response to 73 FR 1360 (January 8, 2008): Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments

NICEATM requested public comments on the drafts for the January 2008 BRDs, ICCVAM test recommendations, test method protocols, and LLNA performance standards for an international independent scientific peer review panel meeting to evaluate modifications and new applications for the LLNA. NICEATM received 23 comments in response to this FR notice; seven written comments were received in advance of the meeting, and 16 oral comments were offered at the Panel meeting.

Two written comments were relevant to the LLNA applicability domain.

1. One commenter indicated that the limited data prevented a conclusive recommendation for the use of the LLNA to predict the skin sensitization potential of mixtures, metals, and aqueous solutions. Thus, the commenter viewed that the approach to expand the applicability domain of the LLNA had not been successful, and recommended that further resources be directed towards the pursuit of *in vitro* methods.

ICCVAM is committed to identifying *in vitro* models and non-animal approaches for assessing ACD and is engaged with ECVAM and JaCVAM in the development of validation studies for such methods.

2. Another commenter indicated that the dataset used to evaluate mixtures was limited due to the lack of human data for comparison (i.e., only comparative GP data were available). The commenter questioned the likelihood that GP data is representative of the human response. Thus, they did not consider using GP data as reference data to be appropriate. In addition, the usefulness of the data was limited further by the fact that information on the ingredients was known for only one of the 15 mixtures and 11 were tested in the LLNA in an aqueous vehicle (noting that the usefulness and limitations of the LLNA for testing substances in aqueous solutions was also being evaluated).

    - As indicated in the January 2008 ICCVAM draft recommendations the limitations with the database indicated that more data were needed before a recommendation on the usefulness and limitations of the LLNA for testing mixtures could be made.

The commenter further noted that Lalko and Api (2006) evaluated essential oils and included analytical data on the composition of the oils as well as LLNA data on the identified major constituents and that these data should have been included in the evaluation and not just mentioned as other available scientific reports.

    - These data are included in the ICCVAM final Addendum for the LLNA applicability domain (see **Appendix D**).

The same commenter also agreed with the January 2008 ICCVAM draft recommendation that the LLNA is useful for the testing of metal compounds but questioned the importance or need to assess the LLNA's ability to detect metal allergens since the allergenic potential in humans of most known metals has already been established. Further, whether or not the LLNA is useful for testing nickel

compounds is of limited importance as nickel is a known human contact allergen. In addition, since only one of the 14 metal compounds with LLNA and human data was tested in an aqueous vehicle, the comparison did not add much value to the assessment, especially in light of the fact that the performance of the LLNA using aqueous vehicles was being assessed in this same report.

- ICCVAM considers it important to characterize the ability of the LLNA to appropriately detect the sensitization status of metals because metals may be components of formulated products that require testing to determine their skin sensitization potential.

The commenter also agreed with the January 2008 ICCVAM draft recommendation that an assessment of the suitability of the LLNA for testing substances in aqueous solutions should not be conducted until a sufficient quantity of quality data become available.

Two oral comments were relevant to the LLNA applicability domain.

1. One commenter noted that that the LLNA could be used to test pesticide formulations and supported the efforts of the EPA and ICCVAM to confirm the validity of the LLNA for testing mixtures/formulations. If the LLNA is not accepted for testing formulations in the United States, international companies will be required to conduct both the LLNA and GP tests to satisfy the differing regulatory requirements for each formulation developed for global distribution. Such additional animal would be counter to the ICCVAM goal of reducing, refining, and replacing animal use in regulatory safety testing.

As outlined in the test method recommendations (see **Section 2.0**), ICCVAM recommends that the LLNA can be used for testing pesticide formulations, complex natural substances, dyes, metal compounds (except nickel), and substances in aqueous solutions unless there are unique physicochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances. When testing substances in aqueous solutions, it is also essential to use an appropriate vehicle to maintain the test substance in contact with the skin (e.g., 1% Pluronic L92 [Boverhoff et al, 2008] so an adequate exposure is achieved, as demonstrated by a positive control response.

2. Another commenter expressed reservations about using the LLNA to test complex mixtures and formulations because it was developed to test single substances. The commenter also stated that, since most metals have already been tested (and their sensitization potential characterized), it does not seem worthwhile to try to optimize the LLNA for hazard and potency categorization for testing metals.

    - As outlined in the test method recommendations (see **Section 2.0**), the LLNA can be used for testing pesticide formulations, complex natural substances, dyes, metal compounds (except nickel), and substances in aqueous solutions unless there are unique physicochemical properties associated with these materials that may interfere with the ability of the LLNA to detect sensitizing substances. When testing substances in aqueous solutions, it is also essential to use an appropriate vehicle, to maintain the test substance in contact with the skin (e.g. 1% Pluronic L92 [Boverhoff et al. 2008]) so an adequate exposure is achieved, as demonstrated by positive control results.
    - ICCVAM considers it important to characterize the ability of the LLNA to appropriately detect the sensitization status of metals because metals may be components of formulated products that require testing to determine their skin sensitization potential.

4.4 Public Comments in Response to 73 FR 25754 (May 7, 2008): Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

NICEATM announced the SACATM meeting and requested written and public oral comment on the agenda topics. One public comment was received in response to this FR notice. The commenter made

a general comment that the members of SACATM do not represent a cross-section of the American public.

The SACATM charter indicates that the Committee shall consist of 15 members, including the Chair. Voting members shall be appointed by the Director, NIEHS, and include representatives from an academic institution, a State government agency, an international regulatory body, or any corporation developing or marketing new or revised or alternative test methodologies, including contract laboratories. Knowledgeable representatives from public health, environmental communities, or organizations using new or alternative test methodologies may be included as appropriate. There shall be at least one knowledgeable representative having a history of expertise, development, or evaluation of new or revised or alternative test methods from each of the following categories: (1) personal care, pharmaceutical, industrial chemicals, or agricultural industry; (2) any other industry that is regulated by one of the Federal agencies on ICCVAM; and (3) a national animal protection organization established under section 501(c)(3) of the Internal Revenue Code of 1986. The Director, NIEHS, shall select the Chair from among the appointed members of SACATM.

4.5 Public Comments in Response to 73 FR 29136 (May 20, 2008): Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments

NICEATM requested submission of written public comments on the Independent Scientific Peer Review Panel Assessment. No public comments were received in response to this FR notice.

4.6 Public and SACATM Comments: SACATM Meeting on June 18-19, 2008

The June 18-19, 2008, SACATM meeting included a discussion of the ICCVAM review of the LLNA test method.

There were no public comments specific to the LLNA applicability domain.

Regarding the LLNA applicability domain, one SACATM member indicated that there was not enough data and information to offer an informed opinion.

As indicated in the January 2008 ICCVAM draft recommendations, more data and information were needed to make final recommendations for the LLNA applicability domain. NICEATM subsequently obtained additional data for pesticide formulations, dyes, and natural complex substances for inclusion in the updated draft Addendum that was evaluated by the Panel in April 2009.

4.7 Public Comments in Response to 74 FR 8974 (February 27, 2009): Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments

NICEATM requested public comments on the updated drafts for the BRDs, Addendum, ICCVAM test method recommendations, and test method protocols for the second international independent scientific peer review panel meeting to evaluate modifications and new applications for the LLNA. NICEATM received three comments in response to this FR notice; one written comment, and two oral comments offered at the Panel meeting.

1. This was a general comment expressing concern that the extensive time and resources that ICCVAM has devoted to this evaluation has detracted from focus on promising *in vitro* methods with potential to have a much greater impact on animal use.

ICCVAM considers the evaluations conducted to date have significant potential to further reduce and refine animal use, particularly where the use of the LLNA is precluded due to restrictions associated with the use of radioactivity. ICCVAM is also committed to identifying *in vitro* models and non-animal approaches for assessing ACD and is engaged with ECVAM and JaCVAM in the development of validation studies for such methods.

The commenter further made one comment relevant to the LLNA applicability domain.

1. The commenter stated that the limited availability of data or the lack of clear definition of the test substance prevented a conclusive recommendation from the previous ICCVAM review for the use of the LLNA. The commenter noted that the updated recommendations from the current review of formulation and aqueous solutions offered a potential for expanded use, if overclassification was accepted (presumably by both the manufacturer and the regulatory agency). The commenter further noted that, in the interim, little had changed in the availability of comparative human data and they supported the ICCVAM recommendation that there is a need to identify relevant human data and human experience in order to continue to evaluate the applicability of LLNA to mixtures and aqueous solutions. The commenter indicated that this approach would provide the most valuable information and would not involve further animal testing, and therefore should be a priority.

    - ICCVAM will consider this comment when prioritizing future activities.

4.8 Public Comments in Response to 74 FR 19562 (April 29, 2009): Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

NICEATM announced the SACATM meeting and requested written and public oral comment on the agenda topics. No public comments were received in response to this FR notice.

4.9 Public Comments in Response to 74 FR 26242 (June 1, 2009): Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments

NICEATM requested submission of written public comments on the Independent Scientific Peer Review Panel Assessment. One comment was received in response to this FR notice.

The commenter did not make a comment relevant to the LLNA applicability domain.

4.10 Public and SACATM Comments: SACATM Meeting on June 25-26, 2009

The June 25-26, 2009, SACATM meeting included a discussion of the ICCVAM review of the LLNA test method.

There were no public comments specific to the LLNA applicability domain.

In general, SACATM was supportive of the Panel report. However, there was general concern regarding the potential for over-labeling substances that may occur by using LLNA test results. They emphasized the need for developing non-animal test methods for identifying potential skin sensitizers.

Regarding the LLNA applicability domain, one SACATM member expressed concern about the limited additional data for the pesticide formulations. Compared to the original work on single substances, these data show that the pesticide formulations appear to produce false positives in the LLNA. The difference in sensitivity between the Buehler test and the GPMT was clarified. For the 22 substances for which there were comparative tests, 18 of the GPMTs were actually Buehler tests, so

there is a question as to whether they could have been concordant if they had been GPMTs. Strictly comparing the performance of the LLNA and the GPMT for those 22 substances, the accuracy is not great because the trend was to get a positive result more often in the LLNA.

As indicated in the ICCVAM final test method recommendations (**Section 2.1**), the potential for possible overclassification of pesticide formulations may be a limitation of the LLNA.

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Appendix A

ICCVAM Evaluation Timeline

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ICCVAM Evaluation Timeline

January 10, 2007	ICCVAM receives a letter from the Consumer Product Safety Commission (CPSC) nominating six murine local lymph node assay (LLNA) review activities for evaluation, including the LLNA applicability domain.
January 2007	The ICCVAM Immunotoxicity Working Group (IWG) is re-established to work with NICEATM to carry out LLNA evaluations.
January 24, 2007	ICCVAM endorses the six CPSC-nominated LLNA review activities, including evaluation of the LLNA applicability domain.
May 17, 2007	*Federal Register* notice (72 FR 27815) – The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data.
June 12, 2007	SACATM endorses with high priority the six CPSC-nominated LLNA review activities, including evaluation of the LLNA applicability domain.
November 12–13, 2007	ECVAM Workshop on Alternative Methods (Reduction, Refinement, Replacement).
January 8, 2008	*Federal Register* notice (73 FR 1360) – Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments.
March 4–6, 2008	Independent Scientific Peer Review Panel holds a public meeting, with opportunity for oral public comments, at CPSC Headquarters in Bethesda, MD, to discuss LLNA review activities, including the LLNA applicability domain. The Panel is charged with reviewing the current status of the LLNA applicability domain and commenting on the extent to which the information in the draft LLNA Addendum on the validity of the LLNA for mixtures, metals, and aqueous solutions supported the draft ICCVAM recommendations.
May 20, 2008	*Federal Register* notice (73 FR 29136) – Announcement of the Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments.
June 18–19, 2008	SACATM public meeting for comments on the 2008 Panel report.

February 27, 2009	*Federal Register* notice (74 FR 8974) – Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments.
April 28–29, 2009	Independent Scientific Peer Review Panel holds a public meeting with opportunity for oral public comments, at NIH Natcher Conference Center in Bethesda, MD, to discuss LLNA review activities, including the updated LLNA applicability domain. The Panel is charged with reviewing the current status of the LLNA applicability domain and commenting on the extent to which the information in the revised draft LLNA Addendum on the validity of the LLNA for mixtures, metals, and aqueous solutions supported the revised draft ICCVAM recommendations.
June 1, 2009	*Federal Register* notice (74 FR 26242) – Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments.
June 25–26, 2009	SACATM public meeting for comments on the 2009 Panel report.
October 28, 2009	ICCVAM endorses the TMER for the LLNA applicability domain, which includes the final LLNA Addendum on the validity of the LLNA for mixtures, metals, and aqueous solutions.

Appendix B

ICCVAM-Recommended Protocol
The Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products

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Preface

The murine local lymph node assay (LLNA) is a test method developed to assess whether a chemical has the potential to induce allergic contact dermatitis (ACD) in humans. In 1998, the LLNA was submitted to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) for evaluation as an alternative (i.e., stand-alone) test method to the guinea pig (GP) sensitization tests accepted by U.S. regulatory agencies. In 1999, based on a comprehensive evaluation of the LLNA by an independent scientific peer review panel (Panel),[1] ICCVAM concluded that the LLNA is an acceptable alternative to the GP test methods to assess the ACD hazard potential of most substances (Dean et al. 2001). The Panel also concluded that the LLNA offers animal welfare advantages compared to use of the traditional GP methods, in that it provides for animal use refinement (i.e., elimination of distress and pain) and reduces the total number of animals required. An ICCVAM Immunotoxicity Working Group (IWG) reviewed the 1999 Panel report and developed recommendations applicable to the regulatory use of the LLNA. The IWG then worked with the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to produce a recommended test method protocol (ICCVAM 2001)[2] that would accurately reflect the ICCVAM and Panel recommendations (ICCVAM 1999).

In March 2008, ICCVAM and NICEATM convened an independent scientific peer review panel (Panel) to evaluate new versions and applications of the LLNA. The Panel provided conclusions and recommendations in their report, many of which were applicable to the traditional LLNA test method protocol.[3] ICCVAM subsequently considered the Panel's conclusions and recommendations, as well as comments from the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) and public, and updated the 2001 ICCVAM-recommended LLNA test method protocol. The updated ICCVAM-recommended LLNA test method protocol will be forwarded with the Panel's report to agencies for their consideration.

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

We want to express our sincere appreciation to the ICCVAM IWG for their careful deliberations and efforts in updating the LLNA test method protocol, and especially appreciate the efforts of the Working Group Co-Chairs, Abigail Jacobs, Ph.D., from the U.S. Food and Drug Administration and Joanna Matheson, Ph.D., from the U.S. Consumer Products Safety Commission. We also want to acknowledge the outstanding support provided by NICEATM and the Integrated Laboratory Systems, Inc., support staff. Lastly, we appreciate the efforts of the Panel members for their diligent review, and the comments provided by SACATM and numerous stakeholders, including the public.

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[1] http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf
[2] http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/LLNAProt.pdf
[3] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

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

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

2.0 Description of the Local Lymph Node Assay

2.1 Sex and strain of animals

Young adult female mice (nulliparous and nonpregnant) of the CBA/Ca or CBA/J strain are recommended.[4] Females are used because most data in the existing database were generated using mice of this gender. At the start of the study, mice should be age 8–12 weeks. All mice should be age matched (preferably within a one-week time frame). Weight variations between the mice should not exceed 20% of the mean weight.

2.2 Preparation of animals

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

2.3 Preparation of doses

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

2.4 Test Conditions

2.4.1 Solvent/vehicle

The selected solvent/vehicle must not interfere with or bias the test result and should be selected on the basis of maximizing the test concentrations while producing a solution/suspension suitable for application of the test substance. In order of preference, recommended solvents/vehicles are acetone: olive oil (4:1 v/v), *N,N*-dimethylformamide, methyl ethyl ketone, propylene glycol, and dimethyl sulfoxide, but others may be used (Kimber and Basketter 1992). Particular care should be taken to

[4] Male mice or other strains of mice may be used if it is sufficiently demonstrated that these animals perform as well as female CBA mice in the LLNA.

ensure that hydrophilic materials are incorporated into a vehicle system that wets the skin and does not immediately run off. Thus, wholly aqueous vehicles may need to be avoided. It may be necessary for regulatory purposes to test the substance in the clinically relevant solvent or product formulation.

2.4.2 Controls

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

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

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

Inclusion of a positive control with each test is recommended to ensure that all test method protocol procedures are being conducted properly and that all aspects of the test system are working properly such that they are capable of producing a positive response. However, periodic testing (i.e., at intervals ≤6 months) of the positive control substance may be considered in laboratories that conduct the LLNA regularly (i.e., conduct the LLNA at a frequency of no less than once per month) and that have a history and a documented proficiency for obtaining consistent results with positive controls. Adequate proficiency with the LLNA can be successfully demonstrated by generating consistent results with the positive control in at least 10 independent tests conducted within a reasonable period of time (i.e., less than one year). A positive control group should always be included when there is a procedural change to the LLNA (i.e., change in trained personnel, change in test method materials and/or reagents, change in test method equipment, change in source of test animals, etc.), and such changes should be documented in laboratory reports. Consideration should be given to the impact of these changes on the adequacy of the previously established historical database in determining the necessity for establishing a new historical database to document consistency in the positive control results. Users should be aware that the decision to only include a positive control on a periodic basis instead of concurrently will have ramifications on the adequacy and acceptability of negative study results generated without a concurrent positive control during the interval between each periodic positive control study. For example, if a false negative result is obtained in the periodic positive control study, all negative test substance results obtained in the interval between the last acceptable periodic positive control study and the unacceptable periodic positive control study will be questioned. In order to demonstrate that the prior negative test substance study results are acceptable, a laboratory would be expected to repeat all negative studies, which would require additional expense and increased animal use. These implications should be carefully considered when determining whether to include concurrent positive controls or to only conduct periodic positive controls.

Consideration should also be given to using fewer animals in the concurrent positive control group when this is scientifically justified, as discussed below and in **Annex II**.

Benchmark controls may be useful to demonstrate that the test method is functioning properly for detecting the skin sensitization potential of substances of a specific chemical class or a specific range of responses, or for evaluating the relative skin sensitization potential of a test substance. Appropriate benchmark controls should have the following properties:

- Structural and functional similarity to the class of the substance being tested
- Known physical/chemical characteristics
- Supporting data on known effects in animal models
- Known potency for sensitization response

2.5 Methodology

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

As noted above, concurrent negative and positive control groups should be included, unless a laboratory can demonstrate adequate proficiency that would support the use of a periodic positive control study. The number of mice in the concurrent positive control group might be reduced compared to the vehicle and test substance groups, if the laboratory demonstrates, based on laboratory-specific historical data,[5] that fewer mice can be used without substantially increasing the frequency with which studies will need to be repeated. An example of how to reduce the number of mice in the concurrent positive control group is provided in **Annex II**.

Test substance treatment dose levels should be based on the recommendations given in Kimber and Basketter (1992) and in the ICCVAM Panel Report (ICCVAM 1999). Dose levels are selected from the concentration series 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc. The maximum concentration tested should be the highest achievable level while avoiding excessive local irritation and overt systemic toxicity (**Annex III**). Efforts should be made to identify existing information that may aid in selecting the appropriate maximum test substance dose level. In the absence of such information, an initial prescreen test, conducted under identical experimental conditions except for omission of an assessment of lymph node proliferative activity, may be necessary. In order to have adequate information from which to select a maximum dose level to use in the definitive test and to identify a dose-response relationship, data should be collected on at least three test substance dose levels with two mice per dose group, in addition to the concurrent solvent/vehicle control group.

The LLNA experimental procedure is performed as follows:

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

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

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

Days 4 and 5. No treatment.

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

For the ^{3}H-methyl thymidine method, resuspend pellets 1 mL TCA and transfer to 10 mL of scintillation fluid. Incorporation of ^{3}H-methyl thymidine is measured by β-scintillation counting as disintegrations per minute (dpm) for each mouse and expressed as dpm/mouse. For the ^{125}IU method, transfer the 1 mL TCA pellet directly into gamma-counting tubes. Incorporation of ^{125}IU is determined by gamma counting and also expressed as dpm/mouse.

2.6 Observations

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

3.0 Calculation of Results

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

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

4.0 Evaluation and Interpretation of Results

In general, when the SI for any single treatment dose group is ≥ 3, the test substance is regarded as a skin sensitizer (Kimber et al. 1994; Basketter et al. 1996; ICCVAM 1999) and a test substance not meeting this criterion is considered a non-sensitizer in this test. However, the magnitude of the observed SI should not be the sole factor used in determining the biological significance of a skin

sensitization response. Additional factors that could be considered include the outcomes of statistical analyses, the strength of the dose-response relationship, chemical toxicity, and solubility. For instance, a quantitative assessment may be performed by statistical analysis of individual mouse data and may provide a more complete evaluation of the test substance's ability to act as a sensitizer (see **Section 3.0**). Equivocal results (e.g., the SI does not reach 3, but it is near 3 and there is a positive dose-response relationship) should be clarified by performing statistical analysis, and by considering structural relationships, available toxicity information, and dose selection.

5.0 Data and Reporting

5.1 Data

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

5.2 Test Report

The test report should contain the following information:

Test Substances and Control Substances

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

Solvent/Vehicle:

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

Test Animals:

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

Test Conditions:

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

Results:

- Signs of systemic toxicity and/or local irritation

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

Discussion of the Results

Conclusion

A Quality Assurance Statement for GLP-compliant Studies

- This statement should indicate all inspections made during the study and the dates any results were reported to the Study Director. This statement should also confirm that the final report reflects the raw data.

6.0 References

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Annex I:
An Approach to Dissection and Identification of the Draining ("Auricular") Lymph Nodes

1.0 Background

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

2.0 Training and Preparation for Node Identification

2.1 Identification of the Draining Node – Dye Treatment

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

2.1.1 Evan's Blue Dye treatment:

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

2.1.2 Colloidal carbon and other dye treatments:

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

2.2 Identification of the Draining Node – Application of Strong Sensitizers

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

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

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

3.0 Dissection Approach

3.1 Lateral Dissection (Figure B-1)

Although lateral dissection is not the conventional approach used to obtain the nodes draining the ear, it may be helpful as a training procedure when used in combination with the ventral dissection. Perform this approach bilaterally (on both sides of the mouse). After euthanizing the mouse, place it in a lateral position. Wet the face and neck with 70% ethanol. Use scissors and forceps to make an initial cut from the neck area slightly below the ear. Carefully extend the incision toward the mouth and nose. Angle the tip of the scissors slightly upward during this procedure to prevent the damage of deeper tissue. Gently retract the glandular tissue in the area using the forceps. Using the masseter muscle, facial nerves, blood vessels, and the bifurcation of the jugular vein as landmarks, isolate and remove the draining node (**Figure B-1**). The draining node ("auricular") will be positioned adjacent to the masseter muscle and proximal to and slightly above the jugular bifurcation.

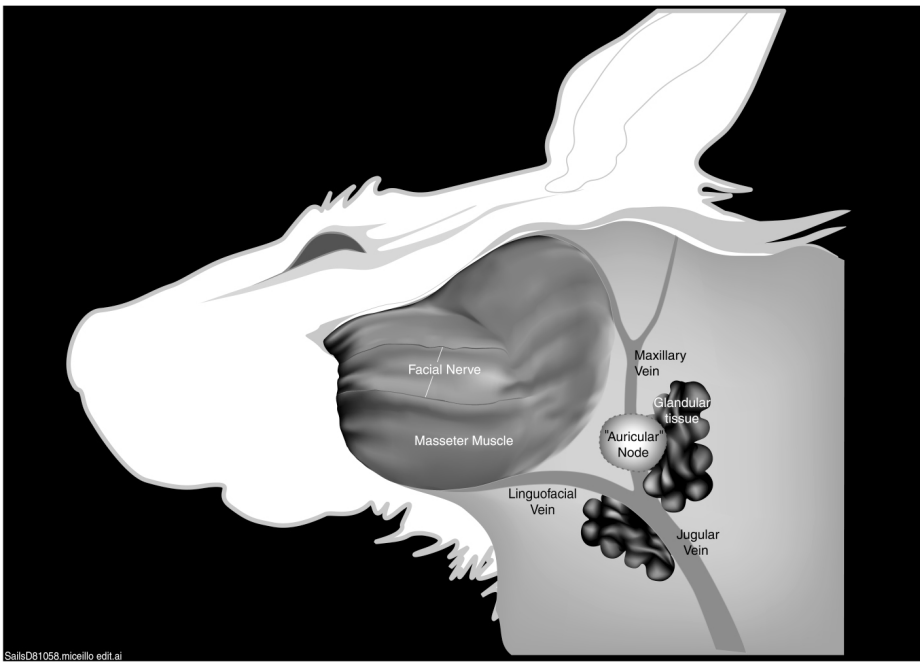
3.2 Ventral Dissection (Figure B-2)

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

4.0 Accuracy in Identification

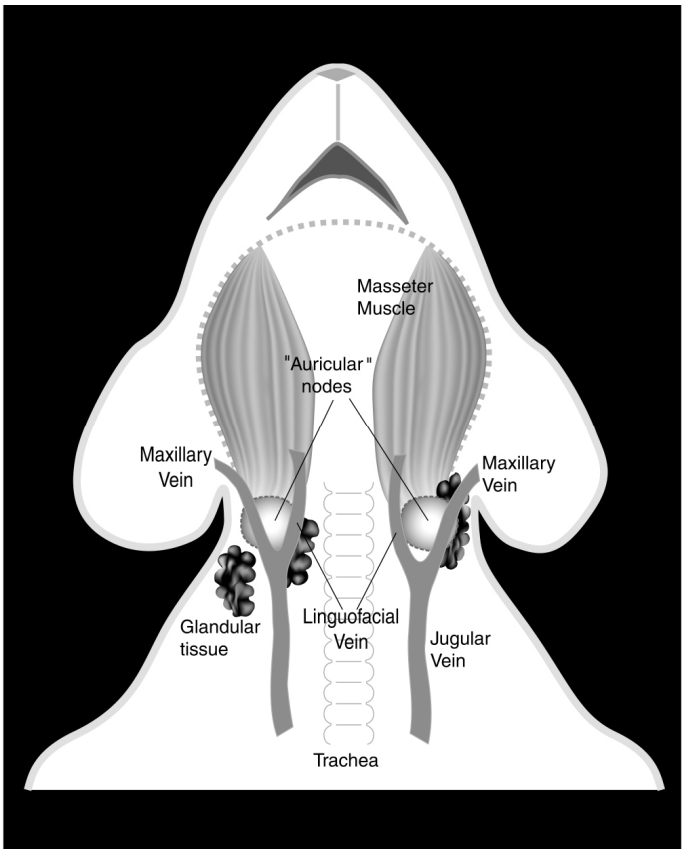
The nodes can be distinguished from glandular and connective tissue in the area by the uniformity of the nodal surface and a shiny translucent appearance. Application of sensitizing agents (especially the strong sensitizers used in training) will cause enlargement of the node size. If a dye is injected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

Figure B-1 Lateral Dissection



Credit: Dee Sailstad, U.S. EPA

Figure B-2 Ventral Dissection



Credit: Dee Sailstad, U.S. EPA

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

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

The stimulation index (SI) results for each positive control test can be used to generate mean SI values for every possible combination of SI values for as few as two animals. The mean SI values for every combination of numbers for each group size can then be used to calculate the failure rate of the positive control for each group size (i.e., the percentage of the combinations for which the mean SI < 3). **Table B-1** provides an example of positive control results from four tests in one laboratory of 30% hexyl cinnamic aldehyde (HCA) using six CBA/J mice per group. In these tests, with six animals, HCA produced "borderline" positive results (i.e., the mean SI values were marginally greater than 3). To determine whether the number of animals can be reduced, sample size reductions (i.e., N = 5, 4, 3, or 2) can be evaluated by taking all possible samples from the six values for each test given in **Table B-1**, which can occur in the following ways: N = 2 (15 samples), N = 3 (20 samples), N = 4 (15 samples), and N = 5 (6 samples).

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

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

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

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

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

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

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

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

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

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

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

Excessive local irritation is indicated by an erythema score ≥3 and/or ear swelling of ≥25%.

Table B-3 Erythema Scores

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

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

Test guidelines for assessing acute systemic toxicity recommend a number of clinical observations for assessing systemic toxicity (OECD 1987; EPA 1998). The following observations, which are based on test guidelines and current practices (ICCVAM 2009), may indicate systemic toxicity when used

as part of an integrated assessment and therefore may indicate that the maximum dose recommended for the LLNA has been exceeded:

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

Appendix C

Comparison of LLNA Responses for Substances Tested in CBA and BALB/C Mice

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1.0 Introduction

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended to U.S. Federal agencies that the LLNA is a valid substitute for currently accepted guinea pig test methods to assess the allergic contact dermatitis potential of many types of substances (Haneke, et al., 2001). The LLNA provides several advantages compared to guinea pig methods, including elimination of potential pain and distress, use of fewer animals, less time required to perform, and availability of dose-response information (Dean, et al. 2001; Sailstad et al., 2001). The recommendation was based on a comprehensive evaluation that included an independent scientific peer review panel assessment of LLNA validation status (ICCVAM 1999).

The LLNA was subsequently incorporated into national and international test guidelines for the assessment of skin sensitization (OECD 2002; ISO 2002; EPA 2003) and is now commonly used worldwide. The recently updated ICCVAM-recommended LLNA protocol states that mouse strains other than CBA may be used in the LLNA if it is sufficiently demonstrated that these animals perform as well as CBA mice in the LLNA (ICCVAM 2009).

Although CBA/J and CBA/Ca mice are currently recommended as the preferred mouse strains in national and international LLNA test guidelines (OECD 2002; EPA 2003), the LLNA was originally developed using BALB/c mice (Kimber et al. 1986). Kimber and Weisenberger (1989) observed that *in vitro* proliferation of lymph node cells in response to exposure to 2,4-dinitrochlorobenzene was stronger in CBA/Ca mice than in BALB/c, and chose to focus on using CBA/Ca mice in further development efforts for the LLNA.

Woolhiser and co-workers assessed LLNA responses in various mouse strains including CBA and BALB/c. They found essentially equal levels of lymph node proliferation (as measured by incorporation of 3H-thymidine into the draining auricular lymph nodes) in both strains following exposure to the sensitizers α-hexylcinnamaldehyde (HCA), 2,4-dinitrofluorobenzene (DNFB) and toluene diisocyanate (Woolhiser et al., 2000). Other U.S. groups have also published LLNA studies using BALB/c mice, including the National Institute for Occupational Safety and Health, the Dow Chemical Corporation, and the National Toxicology Program (Anderson et al. 2009; Boverhof et al. 2009; NTP 2005) and continue to use them today.

In order to further evaluate the impact of using different strains and substrains of mice in the LLNA, the study reported here is a retrospective evaluation of the performance of the LLNA in studies using CBA mice with studies using BALB/c mice. LLNA results are compared from studies done with CBA and BALB/c mice using the same test substances in the same vehicles.

2.0 Methodology

The information summarized here is based on LLNA data derived from a database of over 600 substances tested in the LLNA. Data were extracted from published reports or submissions 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[1]). Key words used in the online searches for this evaluation were "LLNA" OR "Local Lymph Node" OR "Local lymph node" OR "local lymph node". Papers that contained studies on BALB/c were identified by appending AND "balb/c" to this search string. Forty-one such papers identified by the AND "balb/c" search were examined for BALB/c data appropriate for inclusion in this study.

The primary consideration for inclusion of data from published studies was the identification of test substances for which LLNA studies in the same vehicle existed. In general, published studies that

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

were included in this evaluation followed the LLNA protocol in the Organisation for Economic Co-operation and Development (OECD) Test Guideline 429 (OECD 2002). However, some exceptions were made since many of the published BALB/c studies were done prior to the formal adoption of TG 429. Exceptions to the OECD protocol include studies in which lymph nodes were harvested on days 3, 4, 5, and 6 after study initiation, as well as studies that used 2 or 3 mice per treatment group. Studies that included other modifications (e.g., pretreatment of mice with sodium lauryl sulfate before application of the test substance) were excluded. The complete database is in **Annex I.**

An LLNA result was identified as positive if an SI value ≥ 3.0 occurred at any concentration tested. Overall LLNA outcomes for individual substances were made according to the most prevalent outcome, or on a most conservative basis if an equal number of positive and negative studies were found (i.e., considered positive). Since this was a retrospective study, there were substances with multiple studies using the same strain. For each such substance, LLNA outcome was based on the most prevalent study result (positive vs. negative), or considered positive if an equal number of positive and negative studies were found. EC3 values (the concentration of a test substance necessary to cause an SI value of 3) were calculated according to the methods used by Ryan and co-workers (Ryan et al., 2007). In the event that an EC3 value could not be calculated using these methods due to an inadequate dose response, the study was still designated as either positive or negative for the purpose of calculating agreement between strains, based on the decision criterion of SI> 3 as the basis for a positive.

3.0 Results

3.1 Characteristics of the Database

A summary of the responses in LLNA studies conducted with CBA and BALB/c mice is shown in **Table C-1.**

Table C-1 Summary of LLNA Responses from CBA and BALB/c

Test Substance	Vehicle	No. of Studies								
		All Strains	CBA			BALBc			Avg EC3 (%)	
		Total	Total	Pos	Neg	Total	Pos	Neg	CBA	BALBc
3-Amino-5-mercapto-1,2,4-triazole	DMSO	2	1	1	0	1	1	0	11.6	5.2
Benzocaine	AOO	5	4	1	3	1	0	1	NC	NC
Cobalt chloride	DMSO	3	2	2	0	1	0	1	0.6	NC
2,4-DNCB	AOO	14	10	10	0	4	4	0	0.052	0.116
2,4-DNFB	AOO	3	1	1	0	2	2	0	0.016	0.024
Eugenol	AOO	9	8	8	0	1	1	0	14.3	13.8
Eugenol	ACE	2	1	1	0	1	0	1	18.2	NC
Formaldehyde	DMF	2	1	1	0	1	1	0	0.27	0.11
Glutaraldehyd	DMF	2	1	1	0	1	1	0	0.07	0.09

e										
HCA	ACE	5	4	4	0	1	1	0	5.8	12.9
Isoeugenol	AOO	33	32	32	0	1	1	0	1.4	0.8

continued

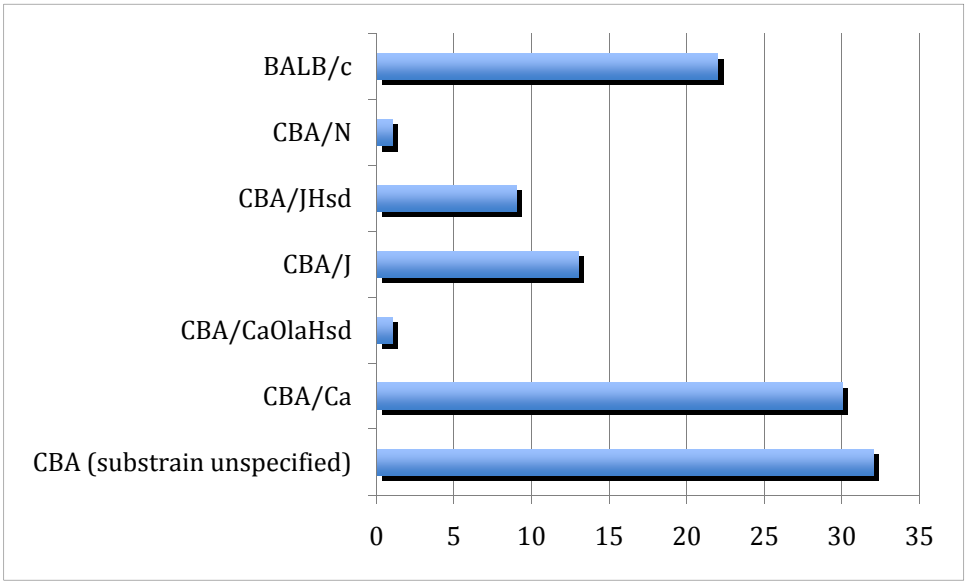
Table C-1 Summary of LLNA Responses from CBA and BALB/c (continued)

Test Substance	Vehicle	No. of Studies								
		All Strains	CBA			BALBc			Avg EC3 (%)	
		Total	Total	Pos	Neg	Total	Pos	Neg	CBA	BALBc
Methyl salicylate	AOO	7	6	0	6	1	0	1	NC	NC
Nickel sulfate	DMSO	2	1	1	0	1	0	1	1.5	NC
Oxazolone	AOO	6	5	5	0	1	1	0	0.0018	IDR
Potassium dichromate	DMSO	10	8	8	0	2	1	1	0.09	0.2
Trimellitic anhydride	AOO	3	1	1	0	2	2	0	9.2	0.15
Total No. Studies		108	86	77	9	22	16	6		

Abbreviations: ACE = acetone; AOO = acetone/olive oil; DMF = dimethylformamide; DMSO = dimethylsulfoxide; DNCB = dinitrochlorobenzene; DNFB = dinitroflurobenzene; EC3 = estimated concentration needed to produce a stimulation index of 3; HCA = α-hexylcinnamic aldehyde; IDR = Inadequate dose response to calculate an EC3 value; LLNA = local lymph node assay; N = No; NC = not calculated; Neg = negative; Pos = positive.

The database evaluated contains results from a total of 108 independent LLNA studies, representing 16 different test substances; 86 of the studies were done with CBA and 22 with BALB/c. Substrains of CBA mice used in the studies were not always specified; specified CBA substrains included CBA/Ca, CBA/CaHsd, CBA/J, CBA/JHsd and CBA/N. None of the studies using BALB/c mice specified a substrain. **Figure C-1** shows a frequency distribution of the substrains used in the studies analyzed. The substrain used in a particular study and the supplier (if known) is indicated for each study in **Annex 1**.

Figure C-1 Substrain Frequency Distribution



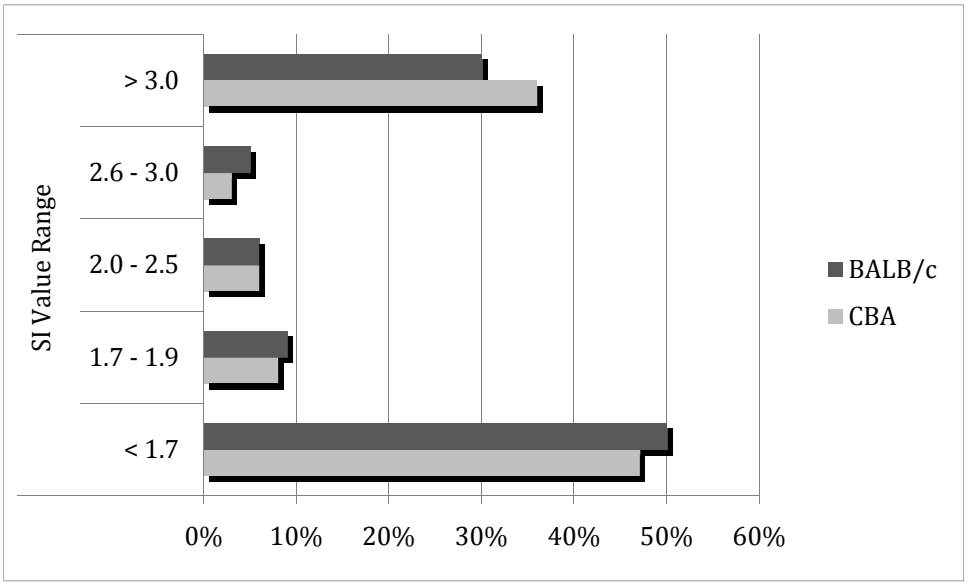
Four different vehicles were represented, with acetone-olive oil (AOO, 80 studies) being the most prevalent, followed by dimethylsulfoxide (DMSO, 17 studies), acetone (ACE, 5 studies) and dimethylformamide (DMF, 4 studies). Only one nonsensitizer (as classified by results in guinea pigs and humans), methyl salicylate, was included. The EC3 values for the 15 sensitizers (as determined from CBA LLNA data) included in the database ranged from 0.0018% (for oxazolone in AOO) to 18.2% (for eugenol in ACE) (**Table C-1**).

Current ICCVAM-recommended LLNA performance standards (ICCVAM 2009) recommend that EC3 values for HCA and DNCB determined in different laboratories should fall into a range of 0.5-2.0x of a reference value; in this study, 29% of the EC3 values for all sensitizers determined in BALB/c fall within this range, if the EC3 value determined in CBA is used as the reference. Neither the EC3 value determined in BALBc for DNCB, or for HCA, falls within this range (**Table C-1**). However, it should be noted that most of the EC3 values determined in both strains were based on a very limited number of studies; for CBA, 8/16 EC3 values were based on one or two LLNA studies, for BALB/c, 13/16 EC3 values were based on one or two LLNA studies. No EC3 value for oxazolone was determined in BALB/c because the dose response data were inadequate to do so.

3.2 Comparison of Responses in the LLNA from CBA and BALB/c Databases

Initially, results from LLNA studies using CBA mice (75 substances, 83 LLNA studies) were compared to results from LLNA studies using BALB/c mice (39 substances, 41 LLNA studies) (ICCVAM 2009). The percentage of positive LLNA studies (i.e., SI $\geq$ 3.0) using either CBA (59% [49/83]) or BALB/c (63% [26/41] mice were similar. **Figure C-2** shows the frequency distribution of LLNA responses from 277 test substance doses that fall into the indicated ranges of SI values. However, this does not include a comparison of results from the same substances tested in the same vehicles. The study described in this report was done to compare results of substances tested in the same vehicle in both CBA and BALB/c.

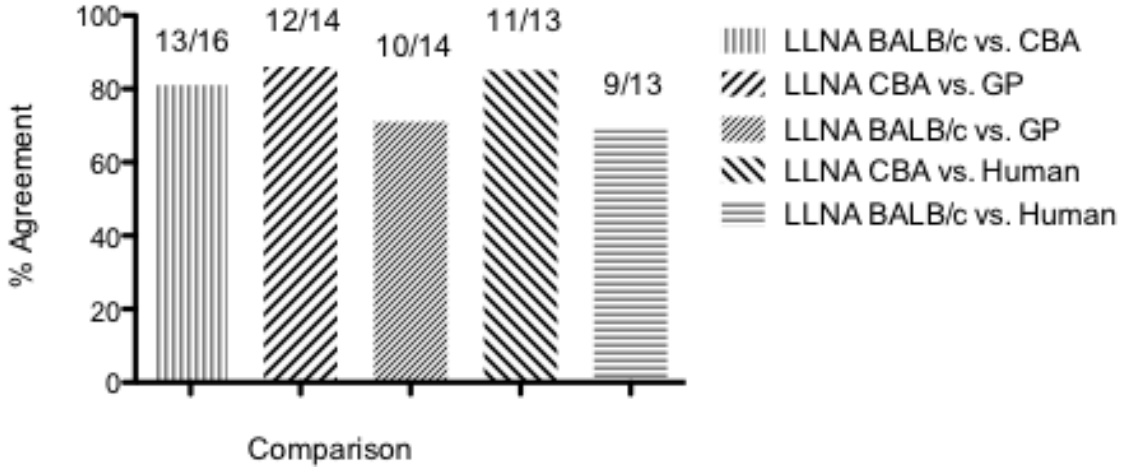
Figure C-2 Comparison of LLNA Responses from CBA and BALB/c Databases (ICCVAM 2009)



Abbreviation: No. = number; SI = stimulation index

The database analyzed here contains data for 16 substances for which there is LLNA data for both CBA and BALB/c in the same vehicle. Thirteen of these substances had GP reference data and 12 had human reference data. Two substances, 3-Amino-5-mercapto-1,2,4-triazole and 2,4-dinitrofluorobenzene, had neither GP nor human reference data; and one substance, trimellitic anhydride, had GP reference data but no human reference data. For this database, 92% (12/13) of the substances were classified as sensitizers in the GP, 92% (11/12) of the substances were classified as sensitizers in humans, 8% (1/13) were classified as nonsensitizers in the GP and 8% (1/12) were classified as nonsensitizers in humans. **Figure C-3** provides a comparison of the performance of the LLNA when the two strains are compared to each other, and to GP and human outcomes.

Figure C-3 Comparison of the Performance of the LLNA using CBA or BALB/c Mice



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

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

LLNA outcomes using BALB/c are in agreement with LLNA outcomes obtained with CBA for 81% (13/16) of the test substances. LLNA outcomes with CBA agree with GP outcomes for 86% (12/14) of the test substances and with human outcomes for 85% (11/13) of the test substances; in contrast, LLNA outcomes with BALB/c agree with GP outcomes for 71% (10/14) of the test substances and with human outcomes for 69% (9/13) of the test substances.

Table C-2 contains LLNA data for three substances (cobalt chloride, nickel sulfate, and eugenol) for which the overall LLNA results were different between CBA and BALB/c, or between one of the mouse strains and guinea pig or human reference data. In the LLNA studies for cobalt chloride and nickel sulfate considered in this investigation, the LLNA results using CBA were concordant with guinea pig and human reference tests, while those using BALB/c were discordant. However, the discordant results obtained in BALB/c were based on a single study for each metal compound. The negative study for nickel sulfate using BALB/c was a 4-day study, while the positive study in CBA was a 6-day study. Furthermore, the LLNA response was a borderline positive in CBA (maximum SI=3.1), and the maximum SI for BALB/c mice was SI=2.46; **Table C-2**). For these reasons there is insufficient information to draw conclusions about the LLNA response to metals in BALB/c. It should also be noted that metal compounds (ICCVAM 1999) are known to produce variable LLNA responses in CBA.

Table C-2 Substances Discordant Between the LLNA, GP, and Human

Chemical Name	LLNA Vehicle	Conc. (%)	SI	EC3 (%)	Mouse Strain	LLNA Call	LLNA Study Length (Days)	Overall LLNA Call[2] (CBA)	Overall LLNA Call[2] (BALB/c)	Overall GP[1] Call[2]	Overall Human[3] Call[2]	LLNA Ref	GP Ref	Human Ref
Eugenol	ACE	25, 50, 75	5.4, 10.6, 10.5	18.5	CBA/J	+	5	+	-	+	+	Gerberick et al. (1992)	Basketter et al. (1999)	Basketter et al. (1999)
		10, 20	1.07, 1.89	NC	BALB/c	-	4					Sailstad et al., (1995)		
Cobalt chloride	DMSO	0.5, 1.0, 2.5	3.2, 3.7, 2.8	0.4	CBA/Ca	+	5	+	-	+	+	Basketter and Scholes (1992)	Basketter et al. (1999)	Kligman (1966)
		0.5, 1.0, 2.5, 5.0	2.1, 3.5,3.8, 7.2	0.8	CBA/N	+	4					Ikarashi (1992b)		
		1.0, 2.5, 5.0	1.5, 1.6, 2,7	NC	BALB/c	-	4					Manderve lt et al. (1997)		
Nickel sulfate	DMSO	0.25, 0.5, 1, 2.5, 5	1.3, 1.4, 1,4, 1.8, 3.1	4.8	CBA/J	+	6	+	-	+	+	Ryan et al. (2002)	Basketter and Scholes (1992)	Kligman (1966)
		2.5, 5,	2.19, 2.46	NC	BALB/c	-	4					Ikarashi et al, (1992a)		

Abbreviations:

AOO = acetone/olive oil; Conc. = concentration; DMSO=dimethylsulfoxide; EC3 = estimated concentration needed to produce a stimulation index of 3; GP = guinea pig; Ind. Conc. = induction concentration; LLNA = local lymph node assay; NC = not calculated since SI<3.0; SI = stimulation index; Veh. = vehicle

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

[2] Human refers to outcomes obtained by studies conducted using either the human repeat insult patch test or the human maximization test, or inclusion in a human patch test allergen kit.

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

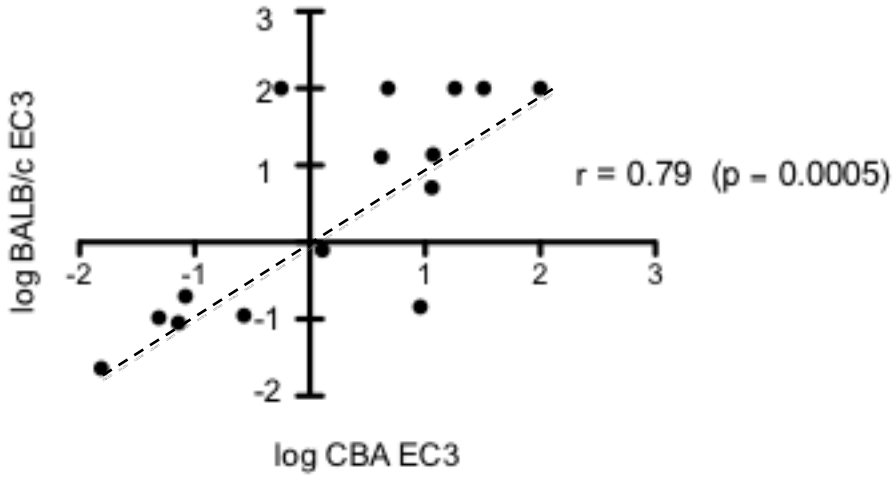
In the LLNA studies for eugenol with acetone as the vehicle, the LLNA results using CBA were concordant with guinea pig and human reference tests, while those using BALB/c were discordant. The differences between CBA and BALB/c studies may be due the large differences in the concentration ranges used, where the maximum concentration used in the CBA study was almost 4-fold higher than that used in the BALB/c study. It should also be noted that BALB/c and CBA studies for eugenol in which AOO was used as the vehicle were both positive. (**Annex 1**).

3.3 Correlation of EC3 Values Obtained with CBA and BALB/c Mice

A correlation analysis between EC3 values calculated using LLNA data from each of the two strains was done. If there were multiple LLNA studies for a strain, a geometric mean EC3 value was used in the correlation analysis. Since the EC3 values for the test substances in this analysis spanned six orders of magnitude (range = 0.0018% to 100%), the mean EC3 values were log transformed prior to analysis. Oxazolone was not included in this analysis because the dose response obtained with BALB/c mice was inadequate to allow calculation of an EC3 value (**Table C-1**).

Spearman's rank correlation is used for rating the extent of agreement with the 'true" ranking of a set of observations (Steel and Torrie, 1980). In this analysis, the CBA EC3 results were considered the "true" ranking. A highly significant ($p \leq 0.0005$) positive correlation ($r = 0.79$) was obtained between EC3 values calculated from LLNA studies in both strains (**Figure C-4**).

Figure C-4 Correlation of EC3 Values Obtained with CBA and BALB/c Mice



Log-transformed geometric mean EC3 values for 15 of the 16 substance-vehicle groups shown in **Table 2**. r = Spearman's Rank correlation coefficient.

NOTE: An EC3 value of 100% was assigned to negative LLNA results in order to exceed all positive values, so that they could be included in the correlation analysis.

Among the 10 substances for which an EC3 was calculated in both CBA and BALC/c studies, 5/10 were lower CBA and 5/10 were lower in BALB/c. (**Table C-1**).

As stated previously, it should be noted that most of the EC3 values determined in both strains were based on a very limited number of studies; for CBA, 50% (8/16) EC3 values were based on one or two LLNA studies, and for BALB/c, 81% (13/16) EC3 values were based on one or two LLNA studies (**Table C-1)**.

3.4 Conclusions

This study complements a previous study (ICCVAM 2009), which concluded that the percentage of positive LLNA responses study were the same between studies with CBA or BALB/c mice. However, there was no substance-by-substance comparison (i.e., the respective databases were compared *in toto*, regardless of test substance or vehicle). Therefore, the present study compares results from LLNA studies with CBA and BALB/c mice using the same test substances in the same vehicles.

Current testing guidelines (OECD 2002; EPA 2003) recommend using CBA mice unless it is sufficiently demonstrated that significant strain-specific differences in the LLNA response do not exist. When compared to LLNA studies using CBA mice (the strain specified in the ICCVAM-recommended LLNA protocol [ICCVAM 2009]), results of studies done on the same substances in BALB/c were in agreement most of the time (81% [13/16])
(**Figure C-3**). Also, there was a positive rank correlation ($r = 0.79$) between EC3 values ($p \leq 0.0005$) (**Figure C-4**). Where there were different outcomes (n=3) between the two mouse strains, the CBA studies were positive (which was also concordant with the human and GP outcomes) while the BALB/c studies were negative (and thereby discordant with the human and GP outcomes) (**Table C-2**).

These results suggest that further characterization of strain and substrain differences in needed. Until such additional information becomes available, caution should be used prior to selecting a mouse strain other than CBA for use in the LLNA for regulatory testing.

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Annex I

Data for Substances Tested in the LLNA in s CBA and BALB/c Mice

List of Abbreviations and Acronyms

ACE	acetone
AOO	acetone: olive oil (4:1)
CASRN	Chemical Abstract Services Registry Number
Conc.	concentration
DMF	N, N-dimethyl formamide
DMSO	dimethyl sulfoxide
EC3	estimated concentration needed to produce a stimulation index of 3
GP	guinea pig
LLNA	murine local lymph node assay
MEK	methyl ethyl ketone
NA	not available
Veh.	Vehicle
SI	Stimulation index
+	Sensitizer
-	Non-sensitizer

Chemical Name	CASRN	Veh.	Conc. (%)	SI	EC3 (%)	LLNA Mouse Strain	Mouse Supplier	LLNA Outcome	LLNA Reference	Guinea Pig Reference	Human Reference	Notes
3-Amino-5-mercapto-1,2,4-triazole	16691-43-3	DMSO	5, 15, 25	2.95, 6.2, 8.66	5.2	BALB/c	Taconic Laboratories (Germantown, NY)	+	Klink & Meade (2003)	NA	NA	
3-Amino-5-mercapto-1,2,4-triazole	16691-43-3	DMSO	1, 5, 15, 25	1.23, 2.13, 3.45, 4.08	11.6	CBA	Taconic Laboratories (Germantown, NY)	+	Klink & Meade (2003)			
Benzocaine	94-09-7	AOO	2.5, 5, 10, 25, 50	2.1, 1.8, 2.7, 1.8, 1.2	NC	CBA	Harlan Olac, Bicester, Oxfordshire, UK	-	Gerberick et al. (2005)	Basketter and Scholes (1992)	Kligman (1966c)	
Benzocaine	94-09-7	AOO	1, 5, 25	1.3, 1.8, 2.9	NC	CBA/Ca	B&K Universal AB, Sollentuna, Sweden	-	Montelius et al. (1994)			
Benzocaine	94-09-7	AOO	10, 25, 50	1.7, 2.0, 0.9	NC	CBA/Ca	Harlan Olac, Bicester, Oxfordshire, UK	-	Basketter et al. (1995)			
Benzocaine	94-09-7	AOO	5, 10, 20	4.5, 7.2, 7.6	3.4	CBA/Ca	Harlan Olac, Bicester, Oxfordshire, UK	+	Kimber et al (1989b)			
Benzocaine	94-09-7	AOO	10, 25	0.95, 1.05	NC	BALB/c	Japan SLC Inc, Shizuoka, Japan	-	Ikarashi et al, (1993a)			
Cobalt chloride	1332-82-7	DMSO	0.5, 1, 2.5	3.2, 3.7, 2.8	0.4	CBA/Ca	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter and Scholes (1992)	Basketter et al. (1999b)	Kligman (1966c)	
Cobalt chloride	1332-82-7	DMSO	0.5, 1, 2.5, 5	2.1, 3.5, 3.8, 7.2	0.8	CBA/N	Japan SLC Inc, Shizuoka, Japan	+	Ikarashi et al. (1992b)			
Cobalt chloride	1332-82-7	DMSO	1, 2.5, 5	1.5, 1.6, 2.7	NC	BALB/c	Charles River, Germany	-	Mandervelt et al. (1997)			
2,4-Dinitrochloro benzene	97-00-7	AOO	0.01, 0.025, 0.05, 0.1, 0.25	1.5, 1.8, 2.4, 8.9, 38.0	0.055	CBA/JHsd	Harlan Sprague Dawley, Inc., Frederick, MD	+	Gerberick et al. (2005)	Basketter et al. (1999b)	Kligman (1996b)	
2,4 Dinitrochloro benzene	97-00-7	AOO	0.01, 0.025, 0.05, 0.1, 0.25	1.4. 2.2, 4.0, 9.8, 16.2	0.036	CBA/Ca	Harlan Olac, Bicester, Oxfordshire, UK	+	Kimber et al. (1995)			
2,4 Dinitrochloro benzene	97-00-7	AOO	0.01, 0.025, 0.05, 0.1, 0.25	2.0, 2.3, 5.3, 10.5, 35.5	0.027	CBA/Ca	Harlan Olac, Bicester, Oxfordshire, UK	+	Kimber et al. (1995)			
2,4 Dinitrochloro benzene	97-00-7	AOO	0.01, 0.025, 0.05, 0.1, 0.25	0.8, 1.8, 3.3, 8.7, 40.9	0.046	CBA/Ca	Harlan Olac, Bicester, Oxfordshire, UK	+	Kimber et al. (1995)			

Chemical Name	CASRN	Veh.	Conc. (%)	SI	EC3 (%)	LLNA Mouse Strain	Mouse Supplier	LLNA Outcome	LLNA Reference	Guinea Pig Reference	Human Reference	Notes
2,4 Dinitrochloro benzene	97-00-7	AOO	0.01, 0.025, 0.05, 0.1, 0.25	1.1, 1.4, 2.5, 4.6, 11.5	0.062	CBA/J	Harlan Sprague Dawley Inc, Indianapolis, IN	+	Kimber et al. (1995)			
2,4 Dinitrochloro benzene	97-00-7	AOO	0.01, 0.025, 0.05, 0.1, 0.25	0.8, 1.2, 1.7, 3.1, 22.5	0.094	CBA/J	Harlan Sprague Dawley Inc, Indianapolis, IN	+	Kimber et al. (1995)			
2,4 Dinitrochloro benzene	97-00-7	AOO	0.01, 0.025, 0.05, 0.1, 0.25	1.3, 1.5, 2.1, 7.7, 43.9	0.057	CBA/J	Harlan Sprague Dawley Inc, Indianapolis, IN	+	Kimber et al. (1995)			
2,4- Dinitrochloro benzene	97-00-7	AOO	0.01, 0.025, 0.05, 0.1, 0.25	1.5, 1.9, 3.1, 6.5, 25.0	0.05	CBA/Ca	Harlan Olac, Bicester, Oxfordshire, UK	+	Loveless et al. (1996)			
2,4- Dinitrochloro benzene	97-00-7	AOO	0.01, 0.025, 0.05, 0.1, 0.25	1.2, 0.9, 2.9, 4.5, 13.0	0.06	CBA/Ca	Harlan Olac, Bicester, Oxfordshire, UK	+	Loveless et al. (1996)			
2,4- Dinitrochloro benzene	97-00-7	AOO	0.01, 0.025, 0.05, 0.1, 0.25	2.5, 2.9, 3.2, 7.1, 25.0	0.033	CBA/JHsd	Harlan Sprague Dawley, Inc., Frederick, MD	+	Loveless et al. (1996)			
2,4- Dinitrochloro benzene	97-00-7	AOO	0.01, 0.025, 0.05, 0.1, 0.25	1.2, 1.1, 1.9, 2.0, 7.1	0.13	BALB/c	Charles River Laboratories (location unspecified)	+	NTP Study Submitted by: Dori Germolec			
2,4- Dinitrochloro benzene	97-00-7	AOO	0.03, 0.1, 0.3, 1.0	1.6, 5.0, 15.8, 24.6	0.06	BALB/c	Charles River Japan Laboratories, Atugi, Kanagawa, Japan	+	Fukuyama et al. (2008b)			
2,4- Dinitrochloro benzene	97-00-7	AOO	0.5, 1.0	8.7, 12.9	0.19	BALB/c	Japan SLC Inc, Shizuoka, Japan	+	Ikarashi et al, (1993a)			
2,4- Dinitrochloro benzene	97-00-7	AOO	0.1, 0.5, 1.0	3.5, 7.4, 12.3	0.083	BALB/c	Japan SLC Inc, Shizuoka, Japan	+	Ikarashi et al, (1993a)			
2,4- Dinitrochloro benzene	70-34-8	AOO	0.02, 0.1, 0.5	6.4, 28.0, 39.9	0.016	CBA/Ca	B&K Universal AB, Sollentuna, Sweden	+	Montelius et al. (1994)	NA	NA	
2,4- Dinitrochloro benzene	70-34-8	AOO	NA	NA	0.032	BALB/c	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter et al. (1997a)			

Chemical Name	CASRN	Veh.	Conc. (%)	SI	EC3 (%)	LLNA Mouse Strain	Mouse Supplier	LLNA Outcome	LLNA Reference	Guinea Pig Reference	Human Reference	Notes
2,4-D initrochloro benzene	70-34-8	AOO	0.01, 0.025, 0.05	2, 4.5, 6.5	0.016	BALB/c	Taconic Laboratories, Rockville, MD	+	Pattterson et al. (2004)			
Eugenol	97-53-0	AOO	2.5, 5, 10, 25, 50	1.6, 1.5, 2.4, 5.5, 16.1	11.9	CBA/Ca	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter and Scholes (1992)	Basketter et al. (1999d)	Basketter et al. (1999d)	SI values were estimated from a graph of dpm v s conc in LLNA Ref
Eugenol	97-53-0	AOO	25, 50	1.2, 4.0	40.9	CBA/Ca	Barriered Animal Breeding Unit, Adderly Park, UK	+	Kimber & Weisenberger (1991)			Mice were exposed to AOO under an occluded patech 5 days before exposure to eugenol in AOO on the ears.
Eugenol	97-53-0	AOO	2.5, 5, 10, 25, 50	2.0, 2.8, 3.2, 13.0, 17.0	5.8	CBA/Ca	Harlan Olac, Bicester, Oxfordshire, UK	+	Loveless et al. (1996)			
Eugenol	97-53-0	AOO	2.5, 5, 10, 25, 50	1.6, 1.5, 2.4, 5.5, 16.0	14.5	CBA/Ca	Harlan Olac, Bicester, Oxfordshire, UK	+	Loveless et al. (1996)			
Eugenol	97-53-0	AOO	2.5, 5, 10, 25, 50	1.1, 1.7, 1.8, 9.1, 12.4	8.9	CBA/JHsd	Harlan Sprague Dawley, Inc., Frederick, MD	+	Loveless et al. (1996)			
Eugenol	97-53-0	AOO	2.5, 5, 10, 25, 50	2.4, 2.1, 1.2, 5.3, 9.6	13.8	CBA/JHsd	Harlan Sprague Dawley, Inc., Frederick, MD	+	Loveless et al. (1996)			
Eugenol	97-53-0	AOO	2.5, 5, 10, 25, 50	1.5, 4.3, 4.6, 14.0, 6.1	6	CBA/JHsd	Harlan Sprague Dawley, Inc., Frederick, MD	+	Loveless et al. (1996)			
Eugenol	97-53-0	AOO	10, 25, 50	2.4, 5.5, 16.1	12.9	CBA/Ca	Harlan Olac, Bicester, Oxfordshire, UK	+	Bertrand et al. (1997)			
Eugenol	97-53-0	ACE	25, 50, 75	5.4, 10.6, 10.5	18.2	CBA/J	Harlan Olac, Bicester, Oxfordshire, UK	+	Gerberick et al. (1992)			Mice were treated with the test substance for 4 consective days instaed of 3 days as per the ICCVAM protocol
Eugenol	97-53-0	AOO	5, 10, 25	1, 2, 6	13.8	BALB/c	Harlan Olac, Bicester, Oxfordshire, UK	+	Hilton et al. (1996a)			SI values were estimated from a graph of dpm x 103 vs conc in LLNA Ref
Eugenol	97-53-0	ACE	10, 20	1.1, 1.9	NC	BALB/c	Charles River, Raleigh, NC	-	Sailstad et al. (1995)			
Formaldehyde	50-00-0	DMF	1, 10, 20	6.7, 13.2, 17.7	0.27	CBA/J	Jackson Laboratories, Bar Harbor, ME	+	Ryan et al. (2002)	Basketter et al. (1999b)	Kligman (1966c)	

Chemical Name	CASRN	Veh.	Conc. (%)	SI	EC3 (%)	LLNA Mouse Strain	Mouse Supplier	LLNA Outcome	LLNA Reference	Guinea Pig Reference	Human Reference	Notes
Formaldehyde	50-00-0	DMF	10, 25, 50	8.6, 9.7, 9.0	0.11	BALB/c	Harlan Olac, Bicester, Oxfordshire, UK	+	Hilton et al. (1996b)			
Glutaraldehyde	111-30-8	DMF	0.1, 0.75, 2.5	4.9, 16.4, 31.5	0.07	CBA	Taconic Laboratories, Germantown, NY	+	Azadi et al. (2004)	Gad et al. (1986)	Marzulli & Maibach (1974)	
Glutaraldehyde	111-30-8	DMF	0.1, 0.75, 2.5	3.5, 12.7, 25.5	0.09	BALB/c	Taconic Laboratories, Germantown, NY	+	Azadi et al. (2004)			
Hexyl cinnamic aldehyde	101-86-0	ACE	3, 10, 30	4.6, 6.6, 9.9	1.2	CBA/CaOlaHsd	Charles River Laboratories, Inc., Kingston, NY	+	Report; Project No.: BGIA Project FP251, submitted by Bayer	Basketter et al. (1999b)	Basketter et al. (1999b)	
Hexyl cinnamic aldehyde	101-86-0	ACE	1, 3, 10	1.8, 3.2, 3.7	2.7	CBA/J	Charles River, Germany	+	BASF, submitted by C. Hastings			
Hexyl cinnamic aldehyde	101-86-0	ACE	1, 3, 10	1.8, 2.4, 3.3	8	CBA/J	Charles River, Germany	+	BASF, submitted by C. Hastings			
Hexyl cinnamic aldehyde	101-86-0	ACE	5, 25, 50	2.5, 4.1, 9.4	11.3	CBA	Jackson Laboratories, Bar Harbor, ME	+	Woolhiser et al. (2000)			
Hexyl cinnamic aldehyde	101-86-0	ACE	5, 25, 50	1.7, 5, 10.9	12.9	BALB/c	Jackson Laboratories, Bar Harbor, ME	+	Woolhiser et al. (2000)			
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	0.7, 2.3, 13.8	1	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)	Wahlberg & Boman (1985)	Basketter et al. (1999b)	
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	0.8, 1.6, 14.1	1.1	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	0.8, 2.8, 5.6	2.1	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	0.9, 6.3, 31	0.5	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	0.9, 1, 7.2	1.9	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1, 1.1, 12.4	1.2	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Gerberick et al. (2005)			

Chemical Name	CASRN	Veh.	Conc. (%)	SI	EC3 (%)	LLNA Mouse Strain	Mouse Supplier	LLNA Outcome	LLNA Reference	Guinea Pig Reference	Human Reference	Notes
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1, 1.3, 7.5	1.8	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.1, 1.8, 23.2	0.8	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.1, 1.9, 15.3	1.3	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.2, 4.2, 18.4	0.7	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.2, 1.4, 19.3	1.8	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.2, 3.2, 8.7	1.3	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.3, 2.2, 13.1	1	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.3, 3.3, 14.7	1.5	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.4, 1.5, 4.9	2.6	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.4, 1.2, 6.7	2	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.5, 2.6, 19.2	0.8	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.5, 2.5, 29.8	0.6	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.6, 1.6, 14.7	1.4	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.6, 2.2, 7.5	1.6	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			

Chemical Name	CASRN	Veh.	Conc. (%)	SI	EC3 (%)	LLNA Mouse Strain	Mouse Supplier	LLNA Outcome	LLNA Reference	Guinea Pig Reference	Human Reference	Notes
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.6, 2.2, 19	0.8	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.6, 4.3, 24.4	0.6	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.7, 1.2, 5	2.6	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	1.8, 2.9, 23.2	0.6	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	2, 1.4, 7.6	1.6	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Isoeugenol	97-54-1	AOO	0.5, 1.0, 5.0	2.3, 1.6, 23.6	0.6	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter & Cadby (2004)			
Isoeugenol	97-54-1	AOO	NA	NA	1.3	CBA/Ca	Harlan Olac, Bicester, Oxfordshire, UK	+	Loveless et al. (1996)			
Isoeugenol	97-54-1	AOO	0.25, 0.5, 1.0, 2.5, 5.0	1.5, 2.2, 2.5, 4.9, 10	1.3	CBA/Ca	Harlan Olac, Bicester, Oxfordshire, UK	+	Loveless et al. (1996)			
Isoeugenol	97-54-1	AOO	0.25, 0.5, 1.0, 2.5, 5.0	1, 1.3, 2.1, 2.3, 4.1	3.3	CBA/Ca	Harlan Sprague Dawley, Inc., Frederick, MD	+	Loveless et al. (1996)			
Isoeugenol	97-54-1	AOO	0.25, 0.5, 1.0, 2.5, 5.0	2.9, 1.7, 2.3, 3.8, 6.8	1.8	CBA/Ca	Harlan Sprague Dawley, Inc., Frederick, MD	+	Loveless et al. (1996)			
Isoeugenol	97-54-1	AOO	0.25, 0.5, 1.0, 2.5, 5.0	0.7, 0.7, 0.9, 2.1, 7.2	3.1	CBA/Ca	Harlan Sprague Dawley, Inc., Frederick, MD	+	Loveless et al. (1996)			
Isoeugenol	97-54-1	AOO	0.25, 0.5, 1.0, 2.5, 5.0	1.2, 1.7, 2.6, 4.3, 11	1.6	CBA/Ca	Harlan Sprague Dawley, Inc., Frederick, MD	+	Loveless et al. (1996)			
Isoeugenol	97-54-1	AOO	5, 10, 25	7, 8.5, 26	0.8	BALB/c	Harlan Olac, Bicester, Oxfordshire, UK	+	Hilton et al. (1996a)			SI values were estimated from a graph of dpm x 103 vs conc in Ref1

Chemical Name	CASRN	Veh.	Conc. (%)	SI	EC3 (%)	LLNA Mouse Strain	Mouse Supplier	LLNA Outcome	LLNA Reference	Guinea Pig Reference	Human Reference	Notes
Methyl salicylate	119-36-8	AOO	1, 2.5, 5, 10, 20	1.1, 1, 1.1, 1.6, 1.9	NC	CBA/J	Harlan Sprague Dawley Inc, Indianapolis, IN	-	Kimber et al. (1995)	Basketter et al. (1999b)	Basketter et al. (1999b)	
Methyl salicylate	119-36-8	AOO	1, 2.5, 5, 10, 20	1.2, 1.5, 1.2, 1.8, 2.9	NC	CBA/Ca	Harlan Olac, Bicester, Oxfordshire, UK	-	Kimber et al. (1995)			
Methyl salicylate	119-36-8	AOO	1, 2.5, 5, 10, 20	2.1, 1.4, 1.5, 1.9, 2.1	NC	CBA/J	Harlan Sprague Dawley Inc, Indianapolis, IN	-	Kimber et al. (1995)			
Methyl salicylate	119-36-8	AOO	1, 2.5, 5, 10, 20	0.7, 0.9, 0.8, 0.5, 1.1	NC	CBA/J	Harlan Sprague Dawley Inc, Indianapolis, IN	-	Kimber et al. (1995)			
Methyl salicylate	119-36-8	AOO	1, 2.5, 5, 10, 20	0.9, 1.2, 1.8, 1.6, 2.3	NC	CBA/Ca	Harlan Olac, Bicester, Oxfordshire, UK	-	Kimber et al. (1995)			
Methyl salicylate	119-36-8	AOO	1, 2.5, 5, 10, 20	1, 1.1, 1.6, 1.4, 0.9	NC	CBA/JHsd	Harlan Sprague Dawley, Inc., Frederick, MD	-	Gerberick et al. (2005)			
Methyl salicylate	119-36-8	AOO	1, 2.5, 5, 10, 20	0.9, 1.2, 1.2, 1.4, 1.7	NC	BALB/c	Charles River Laboratories (location unspecified)	-	NTP Study Submitted by: Dori Germolec			
Nickel sulfate	7786-81-4	DMSO	0.25, 0.5, 1.0, 2.5, 5.0	1.3, 1.4, 1.4, 1.8, 3.1	1.5	CBA/J	Jackson Laboratories, Bar Harbor, ME	+	Ryan et al. (2002)	Basketter and Scholes (1992)	Kligman (1966c)	
Nickel sulfate	7786-81-4	DMSO	2.5, 5.0	2.2, 2.5	NC	BALBc	Japan SLC Inc, Shizuoka, Japan	-	Ikarashi et al, (1993a)			
Oxazolone	15646-46-5	AOO	0.0025, 0.005, 0.01, 0.025, 0.05	2.9, 4.9, 12, 22, 33	0.0026	CBA/JHsd	Harlan Sprague Dawley, Inc., Frederick, MD	+	Loveless et al. (1996)	Basketter et al. (1999b)	Basketter et al. (1999b)	
Oxazolone	15646-46-5	AOO	0.0025, 0.005, 0.01, 0.025, 0.05	3.4, 4.4, 4, 5.9, 8.9	0.002	CBA/Ca	Harlan Olac, Bicester, Oxfordshire, UK	+	Loveless et al. (1996)			
Oxazolone	15646-46-5	AOO	0.0025, 0.005, 0.01, 0.025, 0.05	3.9, 4.8, 6, 12, 13	0.0014	CBACa	Harlan Olac, Bicester, Oxfordshire, UK	+	Loveless et al. (1996)			

Chemical Name	CASRN	Veh.	Conc. (%)	SI	EC3 (%)	LLNA Mouse Strain	Mouse Supplier	LLNA Outcome	LLNA Reference	Guinea Pig Reference	Human Reference	Notes
Oxazolone	15646-46-5	AOO	0.0025, 0.005, 0.01, 0.025, 0.05	4, 6.9, 16, 40, 59	0.0025	CBA/JHsd	Harlan Sprague Dawley, Inc., Frederick, MD	+	Loveless et al. (1996)			
Oxazolone	15646-46-5	AOO	0.0025, 0.005, 0.01, 0.025, 0.05	3.8, 6.2, 7.7, 15, 23	0.0007	CBA/JHsd	Harlan Sprague Dawley, Inc., Frederick, MD	+	Loveless et al. (1996)			
Oxazolone	15646-46-5	AOO	1, 2, 4	25.2, 25.5, 19	IDR	BALB/c	Charles River, Germany	+	Mandervelt et al. (1997)			
Potassium dichromate	7778-50-9	DMSO	0.025, 0.05, 0.1, 0.25, 0.5	1.6, 1.4, 3.8, 5.3, 16.1	0.08	CBA/J	Harlan Olac, Bicester, Oxfordshire, UK	+	Gerberick et al. (2005)	Basketter et al. (1999b)	Kligman (1966c)	
Potassium dichromate	7778-50-9	DMSO	0.025, 0.05, 0.1, 0.25, 0.5	1.4, 2.5, 9.5, 25.9, 10.1	0.05	CBA/J	Jackson Laboratories, Bar Harbor, ME	+	Ryan et al. (2002)			
Potassium dichromate	7778-50-9	DMSO	0.025, 0.05, 0.1, 0.25, 0.5	1.1, 1.3, 2.3, 5.1, 13.1	0.15	CBA/Ca	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter et al. (1999a)			
Potassium dichromate	7778-50-9	DMSO	0.1, 0.25, 0.5	3.5, 10.2, 10.4	0.03	CBA/Ca	Harlan Olac, Bicester, Oxfordshire, UK	+	Basketter and Scholes (1992)			
Potassium dichromate	7778-50-9	DMSO	0.025, 0.05, 0.1, 0.25, 0.5	1.7, 2.9, 4.5, 10.4, 19.1	0.058	CBA/Ca	Harlan Olac, Bicester, Oxfordshire, UK	+	Kimber et al. (1995)			
Potassium dichromate	7778-50-9	DMSO	0.025, 0.05, 0.1, 0.25, 0.5	1.2, 2.1, 3.4, 4.5, 11.2	0.132	CBA/J	Harlan Sprague Dawley Inc, Indianapolis, IN	+	Kimber et al. (1995)			
Potassium dichromate	7778-50-9	DMSO	0.025, 0.05, 0.1, 0.25, 0.5	1.9, 1.7, 2.2, 5.9, 13	0.122	CBA/J	Harlan Sprague Dawley Inc, Indianapolis, IN	+	Kimber et al. (1995)			
Potassium dichromate	7778-50-9	DMSO	0.025, 0.05, 0.1, 0.25, 0.5	1.6, 1.4, 3.8, 5.3, 16.1	0.126	CBA/J	Harlan Sprague Dawley Inc, Indianapolis, IN	+	Kimber et al. (1995)			
Potassium dichromate	7778-50-9	DMSO	0.5, 1, 2	1.8, 1.4, 1.5	NC	BALB/c	Charles River, Germany	-	Mandervelt et al. (1997)			
Potassium dichromate	7778-50-9	DMSO	0.025, 0.05, 0.1, 0.25	1.2, 1.8, 2.2, 3.4	0.2	BALB/c	Charles River Laboratories (location unspecified)	+	NTP Study Submitted by: Dori Germolec			

Chemical Name	CASRN	Veh.	Conc. (%)	SI	EC3 (%)	LLNA Mouse Strain	Mouse Supplier	LLNA Outcome	LLNA Reference	Guinea Pig Reference	Human Reference	Notes
Trimellitic anhydride	552-30-7	AOO	1, 2.5, 5, 10, 25	1.1, 2.0, 2.0, 3.2, 4.6	9.2	CBA	Harlan Olac, Bicester, Oxfordshire, UK	+	Gerberick et al. (2005)	Basketter and Scholes (1992)	NA	
Trimellitic anhydride	552-30-7	AOO	0.5, 1.0, 2.5, 5.0, 10	2.6, 2.7, 3.7, 7.5, 11.6	0.11	BALB/c	Charles River Laboratories, Inc., Kingston, NY	+	Boverhof et al. (2009)			
Trimellitic anhydride	552-30-7	AOO	5, 10, 25	7, 8.5, 26	0.19	BALB/c	Charles River Japan Laboratories, Atugi, Kanagawa, Japan	+	Fukuyama et al. (2008b)			

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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Final 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

[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.

Integrated Laboratory Systems, Inc., the NICEATM support contractor, provided excellent scientific and operational support for which we thank Dr. David Allen, Thomas Burns, Linda Litchfield, Dr. Steven Morefield, Michael Paris, Dr. Eleni Salicru, Catherine Sprankle, Frank Stack, and Dr. Judy Strickland. Finally, we want to thank Dr. Silvia Casati and Dr. Hajime Kojima, the IWG liaisons from the European Centre for the Validation of Alternative Methods and the Japanese Center for the Validation of Alternative Methods, respectively for their participation.

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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

[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.

[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.

[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).

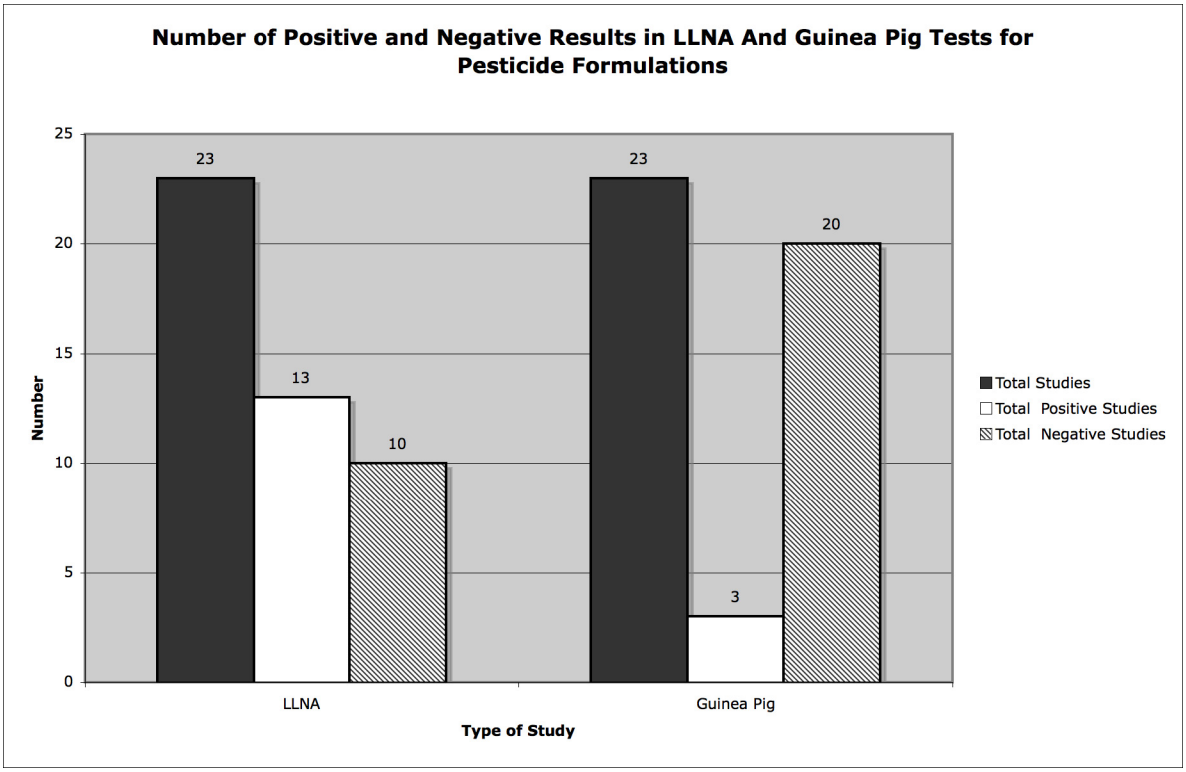
Twenty of the LLNA studies were conducted in CBA mice (i.e., the preferred strain for use in the LLNA according to the ICCVAM recommended LLNA protocol and OECD TG 429) and three studies were conducted in BALB/c mice. The LLNA classified 57% (13/23) of the formulations as sensitizers and 43% (10/23) 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.

[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]).

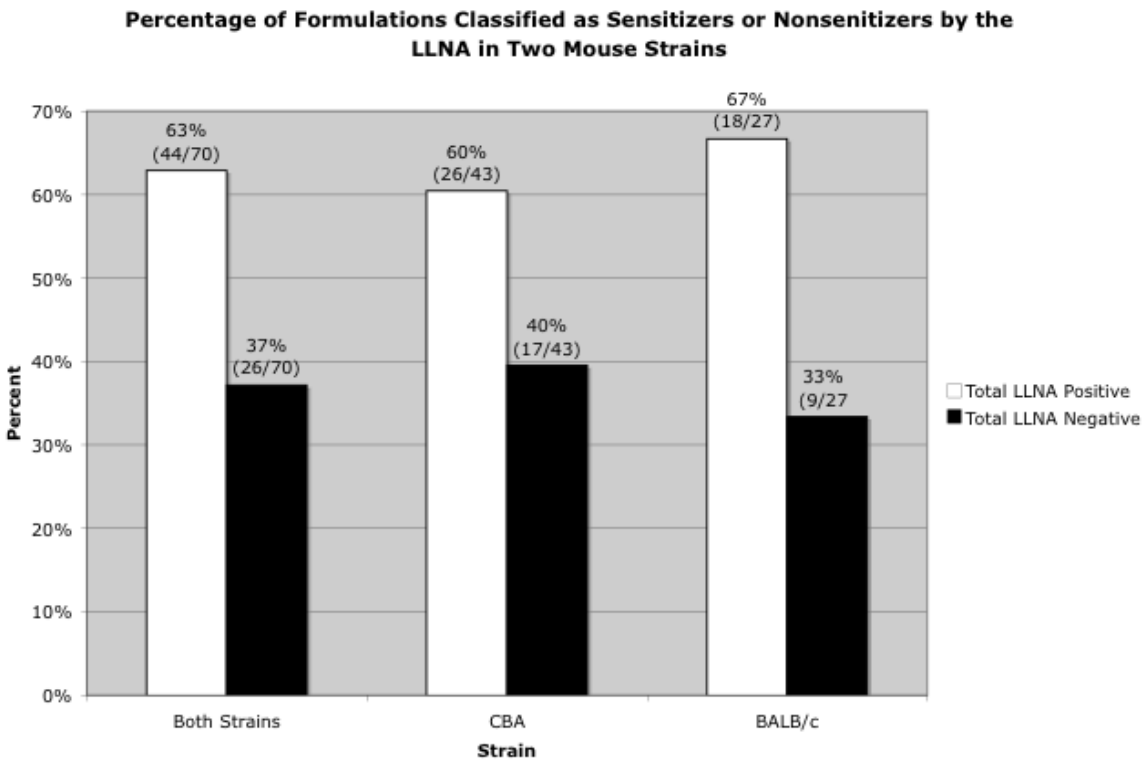


Figure D-2 Percentage of Formulations Classified as Sensitizers or Nonsensitizers in Two Mouse Strains

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	2	0	1
				H_2O			
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

[2] Maximum SI obtained in the LLNA

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

[4] Mean value from 2 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

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_2O	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_2O	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.

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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.

Annex I

The Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals/Compounds
(NIH Publication No. 99-4494)

This document is available electronically at:
https://ntp.niehs.nih.gov/iccvam/docs/immunotox_docs/llna/llnarep.pdf

This document is also available on request from NICEATM:

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Annex II

Available Data and Information for Pesticide Formulations and Other Products Tested in the LLNA

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Annex II-1

Physicochemical Properties and Chemical Classes of Pesticide Formulations Tested in the LLNA

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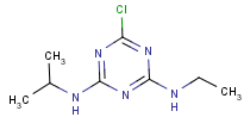
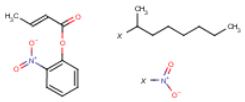
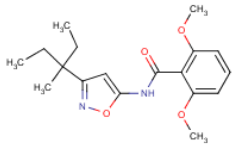
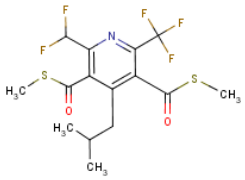
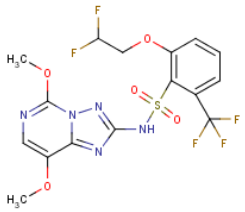
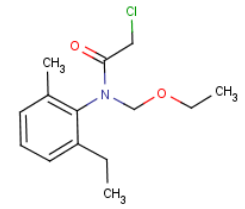
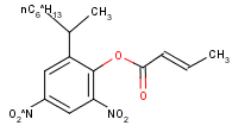
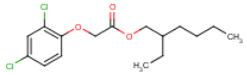
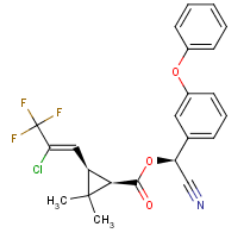
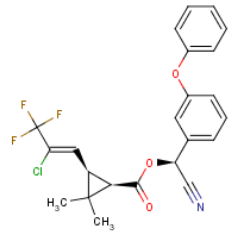
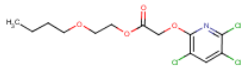
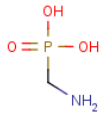
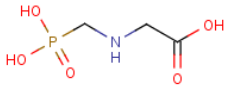
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
AE F016382 00 TK71 A101	NA	NA	NA	NA	NA	Formulation	NA
A SC600	NA	NA	NA	NA	NA	Formulation	NA
Atrazine	Atrazine SC 1-chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine	1912-24-9	215.68	2.82	Solid	Heterocyclic Compounds	
BASF #1	NA	NA	NA	NA	Emulsion	NA	NA
BASF #2	NA	NA	NA	NA	Emulsion	NA	NA
BASF #3	NA	NA	NA	NA	Liquid	NA	NA
BASF #4	NA	NA	NA	NA	Emulsion	NA	NA
BASF #5	NA	NA	NA	NA	Suspension	NA	NA
BASF #6	BAS 493 05 F	NA	NA	NA	Dispersion	NA	NA
BASF SC-1	NA	NA	NA	NA	Emulsion	NA	NA
BASF SE-1	NA	NA	NA	NA	Emulsion	NA	NA
D EC25	NA	NA	NA	NA	NA	Formulation	NA
D EW 15	NA	NA	NA	NA	NA	Formulation	NA

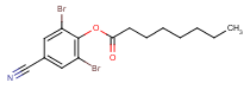
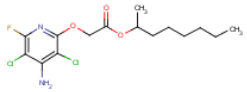
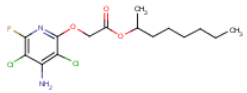
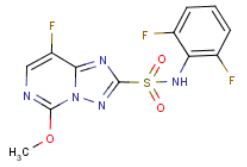
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Dinocap	Butenoic acid, 2-(or 4)-isooctyl-4,6(or 2,6)-dinitrophenyl ester (9CI); Crotonic acid, 2(or 4)-(1-methylheptyl)-4,6(or 2,6)-dinitrophenylester	39300-45-3	364.39	5.76	Liquid	Nitro Compounds; Hydrocarbons, Cyclic	
DU-10	NA	NA	NA	NA	NA	Formulation	NA
DU-11A	NA	NA	NA	NA	NA	Formulation	NA
DU-11B	NA	NA	NA	NA	NA	Formulation	NA
DU-11C	NA	NA	NA	NA	NA	Formulation	NA
DU-12	NA	NA	NA	NA	NA	Formulation	NA
DU-13A	NA	NA	NA	NA	NA	Formulation	NA
DU-13B	NA	NA	NA	NA	NA	Formulation	NA
DU-1A	NA	NA	NA	NA	NA	Formulation	NA
DU-1B	NA	NA	NA	NA	NA	Formulation	NA
DU-1C	NA	NA	NA	NA	NA	Formulation	NA
DU-1D	NA	NA	NA	NA	NA	Formulation	NA
DU-2A	NA	NA	NA	NA	NA	Formulation	NA
DU-2B	NA	NA	NA	NA	NA	Formulation	NA
DU-2C	NA	NA	NA	NA	NA	Formulation	NA
DU-2D	NA	NA	NA	NA	NA	Formulation	NA

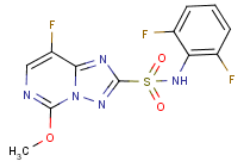
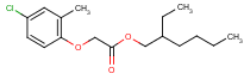
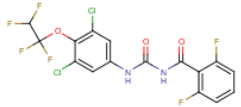
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
DU-2E	NA	NA	NA	NA	NA	Formulation	NA
DU-2F	NA	NA	NA	NA	NA	Formulation	NA
DU-3	NA	NA	NA	NA	NA	Formulation	NA
DU-4	NA	NA	NA	NA	NA	Formulation	NA
DU-5A	NA	NA	NA	NA	NA	Formulation	NA
DU-5B	NA	NA	NA	NA	NA	Formulation	NA
DU-5C	NA	NA	NA	NA	NA	Formulation	NA
DU-6	NA	NA	NA	NA	NA	Formulation	NA
DU-7	NA	NA	NA	NA	NA	Formulation	NA
DU-8A	NA	NA	NA	NA	NA	Formulation	NA
DU-8B	NA	NA	NA	NA	NA	Formulation	NA
DU-9A	NA	NA	NA	NA	NA	Formulation	NA
DU-9B	NA	NA	NA	NA	NA	Formulation	NA
EXP 10810 A	NA	NA	NA	NA	NA	Formulation	NA
EXP 11120 A	NA	NA	NA	NA	NA	Formulation	NA
FAR01042-00	NA	NA	NA	NA	NA	Formulation	NA
FAR01060-00	NA	NA	NA	NA	NA	Formulation	NA

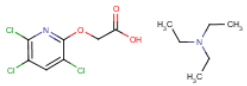
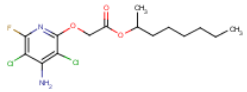
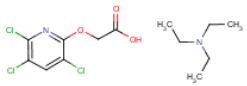
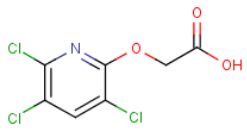
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 1	Isoxaben	82558-50-7	332.40	NA	Liquid	Formulation	
Formulation 10	22.9% w/w dithiopyr	97886-45-8	401.42	NA	Liquid	Formulation	
Formulation 11	0.31 wt % penoxsulam 84.2 wt % acetochlor	219714-96-2 34256-82-1	483.37 269.77	NA	Liquid	Formulation	 

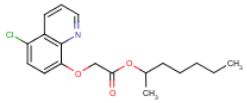
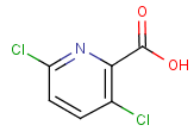
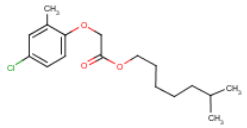
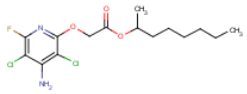
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 12	34.7% w/w 2,4-dinitro-6-(1-methylheptyl)phenyl crotonate DE-126	6119-92-2	364.40	NA	Liquid	Formulation	
Formulation 13	87.6% w/w 2,4-dichlorophenoxyacetic acid 2-ethylhexyl ester 2,4-D-2-ethylhexyl	1928-43-4	333.25	NA	Liquid	Formulation	
Formulation 14	1.5 wt. % gamma-cyhalothrin Nexide Fentrol	76703-62-3	449.85	NA	Liquid	Formulation	
Formulation 15	5.8 wt.% gamma-cyhalothrin Nexide Fentrol	76703-62-3	449.85	NA	Liquid	Formulation	

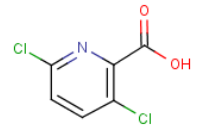
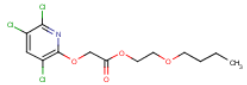
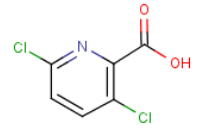
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 16	85.3% w/w triclopyr butoxyethyl ester	64470-88-8	356.63	NA	Liquid	Formulation	
Formulation 17	50.8% wt/wt glyphosate dimethylammonium salt (active ingredient) 40.1% wt/wt glyphosate (acid equivalent) 8.3% w/w Geronol CF/AS 30 (ammonium adjuvant)	1066-51-9 1071-83-6	111.04 169.02	NA	Liquid	Formulation	 

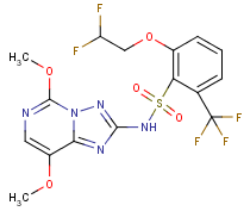
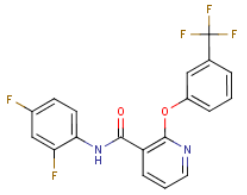
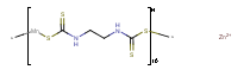
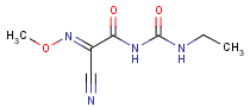
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 19	37.1 wt% bromoxynil octanoate 9.23 wt% fluroxypyr-1-methylheptyl	1689-99-2 81406-37-3	403.11 367.25	NA	Liquid	Formulation	 
Formulation 2	14.2% w/w fluroxypyr-1-methylheptyl 0.22% w/w florasulam	81406-37-3 145701-23-1	367.25 359.29	NA	Liquid	Formulation	 

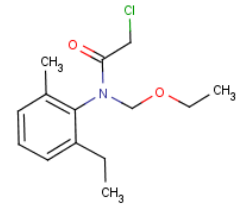
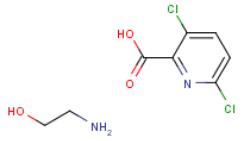
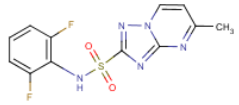
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 20	0.39 wt% Florasulam 41.9 wt% 2-methyl-4-chlorophenoxyacetic acid 2-ethylhexyl ester (MCPA, 2-ethyl hexyl ester)	145701-23-1 29450-45-1	359.29 312.84	NA	Liquid	Formulation	 
Formulation 21	50.4% Hexaflumuron N-(((3,5-dichloro-4-(1,1,2,2-tetrafluoroethoxy)phenyl)amino)carbonyl)-2,6-difluoro benzamide	86479-06-3	461.14	NA	Liquid	Formulation	

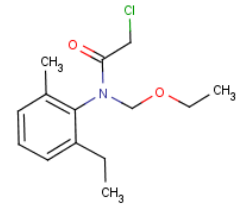
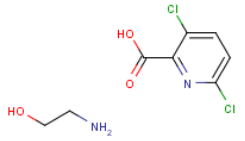
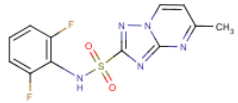
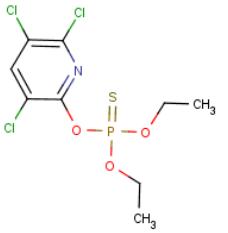
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 22	8.3 wt. % Triclopyr triethylammonium 2.8 wt. % fluroxypyr-methyl heptyl ester	57213-69-1 81406-37-3	357.66 367.25	NA	Liquid	Formulation	 
Formulation 23	16.1 wt% Triclopyr triethylammonium 11.6 wt% triclopyr acid	57213-69-1 55335-06-3	357.66	NA	Liquid	Formulation	 

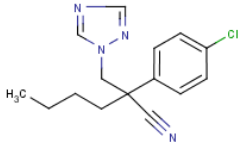
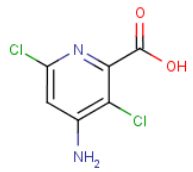
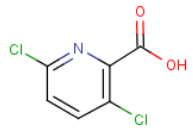
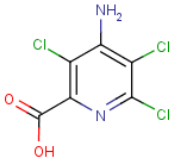
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 24	8.8 wt% Cloquintocet-mexyl	99607-70-2	335.83	NA	Liquid	Formulation	
Formulation 25	2.2 wt.% Clopyralid 37.7 wt.% MCPA-2-ethylhexyl ester 8.2 wt.% fluroxypyr -meptyl	1702-17-6 26544-20-7 81406-37-3	192.00 312.84/ 367.25	NA	Liquid	Formulation	  

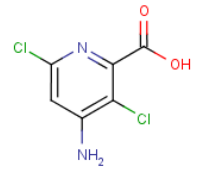
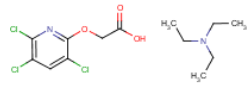
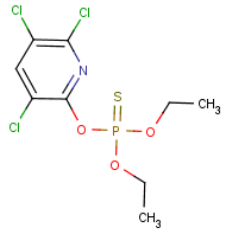
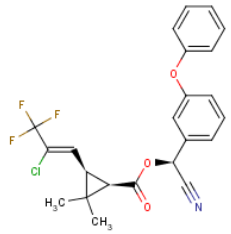
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 26	5.9 wt. % Clopyralid 32.9 wt. % Triclopyr-butotyl	1702-17-6 64700-56-7	192.00 356.63	NA	Liquid	Formulation	 
Formulation 27	45.2 wt. % Fluroxypyr-meptyl	81406-37-3	192.00	NA	Liquid	Formulation	

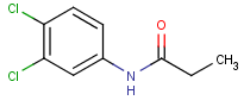
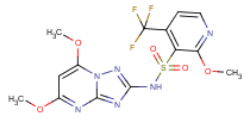
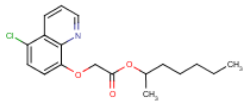
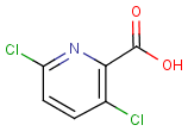
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 28	1.4 wt. % Penoxsulam 9.37 wt. % Diflufenican	219714-96-2 83164-33-4	483.37 394.30	NA	Liquid	Formulation	 
Formulation 29	35.6% Mancozeb 4.92% Cymoxanil	8018-01-7 57966-95-7	541.1 198.18	NA	Liquid	Formulation	 

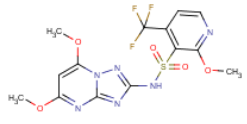
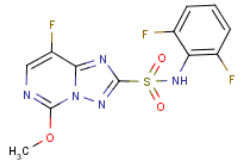
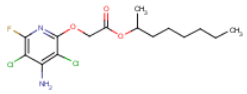
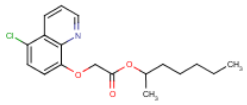
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 3	455 g/L Acetochlor 47 g/L Clopyralid-olamine 14 g/L Flumetsulam	34256-82-1 57754-85-5 98967-40-9	269.77 253.08 325.30	NA	Liquid	Formulation	  

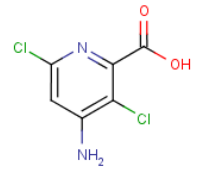
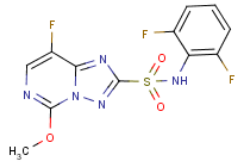
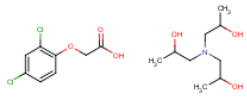
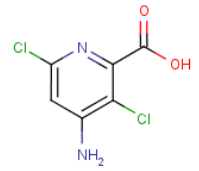
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 30	455 g/L Acetochlor 47 g/L Clopyralid-olamine 14 g/L Flumetsulam	34256-82-1 57754-85-5 98967-40-9	269.77 253.08 325.30	NA	Liquid	Formulation	  
Formulation 31	18.7 wt. % Chlorpyrifos	2921-88-2	350.59	NA	Liquid	Formulation	

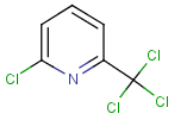
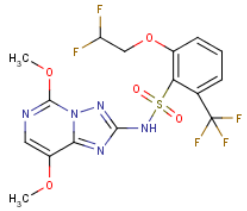
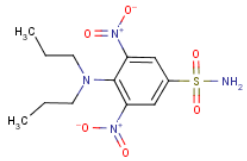
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 32	11.2 wt. % ((E)-2-(1-methylheptyl) -4,6-dinitrophenyl ester-2-butenoic acid 4.68% wt/wt Myclobutanil	88671-89-0	288.78	NA	Liquid/ Solid	Formulation	
Formulation 33	4.5 wt. % Aminopyralid-olamine 27.1 wt. % Clopyralid-olamine 8.7 wt. % Picloram-olamine 3.5 wt. % Aminopyralid 20.6 wt. % Clopyralid 7.0 wt. % Picloram	150114-71-9 1702-17-6 1918-02-1	207.02 192.00 241.46	NA	Liquid	Formulation	  

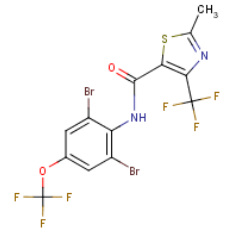
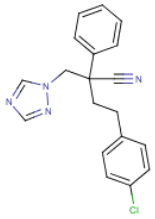
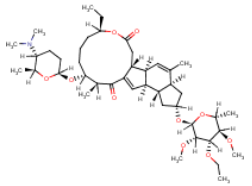
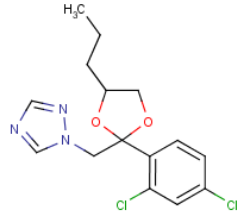
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 34	3.0 wt. % Aminopyralid	150114-71-9		NA	Liquid	Formulation	
Formulation 35	2.15 wt. % Aminopyralid-triisopropanolammonium 16.0 wt. % triclopyr-triethylammonium	566191-89-7 57213-69-1	NA 357.66	NA	Liquid	Formulation	NA 
Formulation 37	30.6 wt. % Chlorpyrifos 0.54 wt. % Gamma-cyhalothrin	2921-88-2 76703-62-3	350.60 449.85	NA	Liquid	Formulation	 

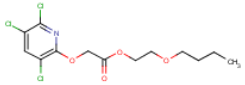
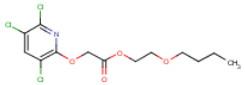
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 38	44.4 wt. % Propanil	709-98-8	218.08	NA	Liquid	Formulation	
Formulation 39	4.2 wt. % Pyroxsulam 8.7 wt. % Cloquintocet mexyl	422556-08-9 99607-70-2	434.35 335.83	NA	Liquid	Formulation	 
Formulation 4	100 g/L Clopyralid mono-ethanolamine salt)	1702-17-6	192.00	NA	Liquid	Formulation	

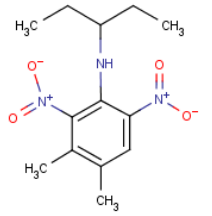
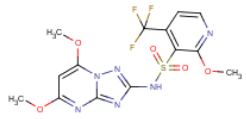
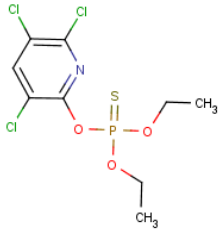
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 40	1.2 wt. % Pyroxsulam 0.21 wt. % Florasulam 11.8 wt. % Fluroxypyr-meptyl 3.6 wt. % Cloquintocet-mexyl	422556-08-9 145701-23-1 81406-37-3 99607-70-2	434.35 359.29 367.25 335.83	NA	Liquid	Formulation	   

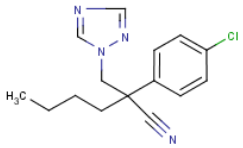
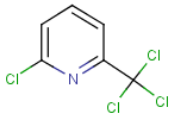
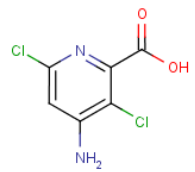
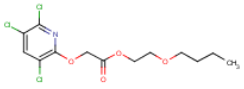
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 41	1.10 wt. % Aminopyralid potassium salt 0.47 wt. % Florasulam	150114-71-9 145701-23-1	207.02 359.29	NA	Liquid	Formulation	 
Formulation 42	31 wt. % 2,4-D-triisoproanolamine 1.52 wt. % Aminopyralid triisopropanolammonium	18584-79-7 150114-71-9	412.31 207.2	NA	NA	Formulation	 

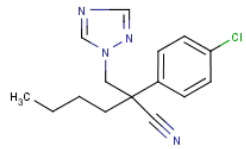
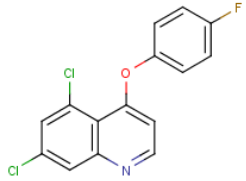
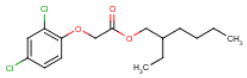
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 43	17.9 wt. % Nitrapyrin	1929-82-4	230.91	NA	NA	Formulation	
Formulation 44	0.12 wt. % Penoxsulam 40.38 wt. % Oryzalin	219714-96-2 19044-88-3	483.37 346.36	NA	NA	Formulation	 

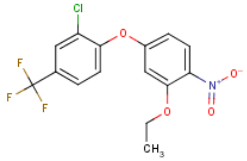
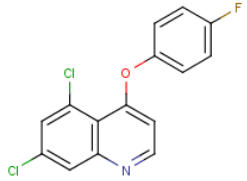
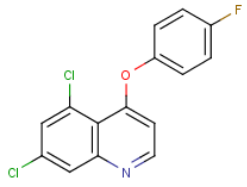
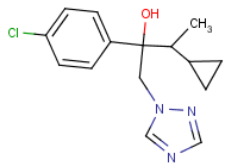
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 45	7.53 wt. % Thifluzamide 9.42 wt. % Fenbuconazole	130000-40-7 114369-43-6	528.06 336.82	NA	NA	Formulation	 
Formulation 46	5.87 wt. % Spinetoram	187166-15-0	760.02	NA	NA	Formulation	
Formulation 47	14.56 wt. % Propiconazole	60207-90-1	342.22	NA	NA	Formulation	

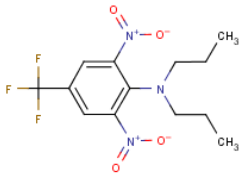
Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 49	23.7 wt. % Triclopyr BEE	64700-56-7	356.63	NA	Liquid	Formulation	
Formulation 5	3,5,6-trichloro-2-pyridyloxyacetic acid, butoxy ethyl ester Triclopyr-butotyl triclopyr BEE	64700-56-7	356.63	NA	Liquid	Formulation	
Formulation 50	Glyphosate dimethylamine salt Glyphosate dimethylammonium salt	34494-04-7 NA	NA	NA	Liquid	Formulation	NA

Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 51	29.6 wt. % Pendimethalin 0.51 wt. % Pyroxsulam	40487-42-1 422556-08-9	281.31 434.35	NA	Liquid	Formulation	 
Formulation 53	41.1 wt. % Chlorpyrifos	2921-88-2	350.60	NA	Liquid	Formulation	
Formulation 54	49.9 wt. % Glyphosate dimethylammonium salt	NA	NA	NA	Liquid	Formulation	NA

Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 55	4.6 wt. % Myclobutanil	88671-89-0	288.78	NA	Liquid	Formulation	
Formulation 56	20.5 wt. % Nitrapyrin	1929-82-4	230.91	NA	Liquid	Formulation	
Formulation 6	Aminopyralid potassium + Triclopyr-butotyl form Aminopyralid herbicide	150114-71-9 64700-56-7	207.02	NA	Liquid	Formulation	 

Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 7	45 g/L Myclobutanil + 45 g/L quinoxyfen	88671-89-0 124495-18-7	288.78 308.14	NA	Liquid	Formulation	 
Formulation 8	81.8% w/w 2,4-dichlorophenoxyacetic acid 2-ethylhexyl ester 2,4-D EHE	1928-43-4	333.25	NA	Liquid	Formulation	
Formulation 9	NA	NA	NA	NA	Liquid	Formulation	NA
F & Fo WG 50 + 25	NA	NA	NA	NA	NA	Formulation	NA
Fx + Me EW 69	NA	NA	NA	NA	NA	Formulation	NA

Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Oxyfluorfen	Oxirane, mono; ((C12-14-alkyloxy) methyl) derivatives	42874-03-3	361.70	5.21	Solid	Ethers	
Quinoxyfen	5,7-dichloro-4-(4-fluorophenoxy)quinoline	124495-18-7	308.14	5.69	Liquid	Heterocyclic Compounds	
Quinoxyfen / Cyproconazole	5,7-dichloro-4-(4-fluorophenoxy)quinoline/ H-1,2,4-triazole-1-ethanol, alpha-(4-chlorophenyl)-alpha-(1-cyclopropylethyl)-	124495-18-7 113096-99-4	308.14 291.78	5.69 3.25	Liquid	Heterocyclic Compounds	 

Substance Name	Active Ingredient(s)	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Trifluralin	2,6-dinitro-4-trifluormethyl-N,N-dipropylanilin	1582-09-8	335.28	5.31	NA	Hydrocarbons, Cyclic; Amine	

Abbreviations: CASRN = Chemical Abstract Services Registry Number; g/mol = grams per mole; Kow = octanol-water partition coefficient; NA = not available.

[1] Kow represents the octanol-water partition coefficient (expressed on log scale) obtained from the website: http://www.syrres.com/esc/est_kowdemo.htm.

[2] Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs, as developed by the National Library of Medicine at: http://www.nlm.nih.gov/mesh/meshhome.html.

[3] Chemical structures of active ingredients, based on CASRN, were obtained from ChemID available at: http://chem.sis.nlm.gov/chemidplus/chemidheavy.jsp.

Annex II-2

Pesticide Formulations Tested in the LLNA – Comparative Data

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Substance Name	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC_3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	Overall Call						GP Reference
										GP (F)	GP (any)[3]	GP (AI)[4]	BT (AI)[4]	GPMT (AI)[4]	GP (RC/RF)[5]	
A SC600	NA	10, 25, 50, 100	1.4, 1.8, 2.3, 1.6	NC	1% L92	CBA/J	-	Bayer Crop Science, submitted by E. Debruyne	-	-	-	NA	NA	NA	NA	Bayer Crop Science, submitted by E. Debruyne
AE F016382 00 TK71 A101	NA	3.6, 7.1, 17.9, 35.7	1.0, 0.8, 1.0, 1.1	NC	1% L92	CBA/J	-	Bayer Crop Science, submitted by E. Debruyne	-	-	-	NA	NA	NA	NA	Bayer Crop Science, submitted by E. Debruyne
Atrazine	SC	12.5, 25, 50, 75, 100	1.8, 2.8, 3.6, 7.1, 7.3	31.3	1% L92	CBA/J	+	ECPA LLNA Project Report submitted by Dow Chemical	+	-	-	NA	NA	NA	+	NA
		7, 33, 100	0.8, 2.9, 3.7	41.4	1% L92	CBA/J	+	ECPA LLNA Project Report submitted by Dow Chemical								
BASF #1	NA	10, 30, 70	2.0, 2.9, 4.9	31.2	1% L92	CBA/Ca	+	BASF, submitted by C. Hastings	+	NA	NA	NA	NA	NA	NA	NA
BASF #2	NA	3, 10, 30	0.8, 1.0, 3.0	29.7	1% L92	CBA/J	+	BASF, submitted by C. Hastings	+	NA	NA	NA	NA	NA	NA	NA
BASF #3	NA	3, 10, 30	6.9, 14.6, 16.1	1.6	ACE	CBA/J	+	BASF, submitted by C. Hastings	+	NA	NA	NA	NA	NA	NA	NA
BASF #4	NA	3, 10, 50	2.4, 2.7, 5.4	14.1	1% L92	CBA/Ca	+	BASF, submitted by C. Hastings	+	NA	NA	NA	NA	NA	NA	NA

Substance Name	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC_3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	Overall Call						GP Reference
										GP (F)	GP (any)[3]	GP (AI)[4]	BT (AI)[4]	GPMT (AI)[4]	GP (RC/RF)[5]	
BASF #5	NA	3, 10, 50	1.6, 1.2, 3.9	36.9	1% L92	CBA/Ca	+	BASF, submitted by C. Hastings	+	NA	NA	NA	NA	NA	NA	NA
BASF #6	NA	3, 10, 30	2.7, 9.9, 23.1	3.3	1% L92	CBA/Ca	+	BASF, submitted by C. Hastings	+	NA	NA	NA	NA	NA	NA	NA
BASF SC-1	SC	3, 10, 30	0.8, 1.3, 1.9	NC	1% L92	CBA/Ca	-	BASF, submitted by C. Hastings	-	-	-	NA	NA	NA	NA	NA
BASF SE-1	SE	10, 30, 70	8.0, 17.3, 22.7	5.5	1% L92	CBA/Ca	+	BASF, submitted by C. Hastings	+	-	-	NA	NA	NA	NA	NA
D EC25®	EC	0.5, 1.0, 2.5	0.6, 0.6, 0.6	NC	1% L92	CBA/Ca	-	Bayer Crop Science, submitted by E. Debruyne	-	-	-	NA	NA	NA	NA	NA
D EW 15	EW	2.5, 5.0, 10.0, 25.0	1.9, 1.5, 2.5, 2.5	NC	1% L92	CBA/J	-	Bayer Crop Science, submitted by E. Debruyne	-	-	-	NA	NA	NA	NA	NA

Substance Name	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC_3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	Overall Call						GP Reference
										GP (F)	GP (any)[3]	GP (AI)[4]	BT (AI)[4]	GPMT (AI)[4]	GP (RC/RF)[5]	
Dinocap	EC	0.8, 4, 21	2.2, 25.8, 14.4	0.9	1% L92	CBA/Ca	+	ECPA LLNA Project Report submitted by BASF	+	+	+	NA	NA	NA	NA	NA
		0.8, 4, 20	1.3, 11.5, 15.6	1.3	1% L92	CBA/J	+									
		0.8, 4, 21	2.0, 4.0, 26.7	1.1	1% L92	CBA/J	+									
		0.8, 4, 10	1.3, 4.1, 10.9	2.8	1% L92	CBA/JHsd	+									
		0.8, 4, 10	2.7, 22.9, 40.5	0.8	1% L92	CBA/Ca OlaHsd	+									
DU-10	NA	0.5, 1, 2.5, 5	1.0, 1.3, 1.5, 1.6	NC	PG	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA
DU-11A	NA	5, 25, 50, 100	3.2, 1.6, 0.7, 0.5	NC	AOO	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA
DU-11B	NA	5, 25, 50,100	1.4, 0.7, 0.7, 1.0	NC	DMF	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA
DU-11C	NA	5, 25, 50,100	1.5, 1.1, 0.9, 1.5	NC	DMF	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA
DU-12	NA	1, 5, 25, 50	0.8, 1.2, 0.8, 1.4	NC	DMF	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA
DU-13A	NA	5, 25, 50, 100	0.5, 0.4, 0.5, 0.6	NC	DMF	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA
DU-13B	NA	1, 10, 50, 100	1.2, 1.0, 0.7, 0.6	NC	AOO	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA

Substance Name	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC_3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	Overall Call						GP Reference
										GP (F)	GP (any)[3]	GP (AI)[4]	BT (AI)[4]	GPMT (AI)[4]	GP (RC/RF)[5]	
DU-1A	NA	5, 25, 50, 100	0.6, 1.2, 0.7, 1.0	NC	PG	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA
DU-1B	NA	1, 5, 10, 25	0.6, 1.1, 1.3, 1.1	NC	DMSO	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA
DU-1C	NA	5, 25, 50, 100	0.7, 1.4, 1.7, 1.3	NC	DMF	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA
DU-1D	NA	5, 10, 25, 50	0.7, 1.0, 1.3, 1.0	NC	DMF	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA
DU-2A	NA	5, 25, 50, 100	4.1, 5.4, 6.7, 6.5	1.2	AOO	CBA/JHsd	+	Submitted by Dupont	+	NA	NA	NA	NA	NA	NA	NA
DU-2B	NA	5, 25, 50, 100	2.1, 4.5, 7.3, 9.3	12.4	DMF	CBA/JHsd	+	Submitted by Dupont	+	NA	NA	NA	NA	NA	NA	NA
DU-2C	NA	10, 50, 100	2.1, 2.7, 3.7	62.9	DMF	CBA/JHsd	+	Submitted by Dupont	+	NA	NA	NA	NA	NA	NA	NA
DU-2D	NA	5, 25, 50, 100	4.5, 8.1, 14.8, 14.5	2.5	DMF	CBA/JHsd	+	Submitted by Dupont	+	NA	NA	NA	NA	NA	NA	NA
DU-2E	NA	5, 25, 50, 100	1.0, 0.8, 1.1, 1.4	NC	PG	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA
DU-2F	NA	5, 25, 50, 100	2.0, 3.8, 7.5, 5.8	15.6	DMF	CBA/JHsd	+	Submitted by Dupont	+	NA	NA	NA	NA	NA	NA	NA
DU-3	NA	5, 10, 25, 50	0.6, 0.8, 0.8, 0.6	NC	DMSO	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA
DU-4	NA	5, 25, 50, 100	0.9, 1.0, 1.0, 0.9	NC	DMF	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA
DU-5A	NA	5, 25, 50, 100	2.7, 1.5, 1.6, 0.9	NC	DMSO	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA

Substance Name	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC_3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	Overall Call						GP Reference
										GP (F)	GP (any)[3]	GP (AI)[4]	BT (AI)[4]	GPMT (AI)[4]	GP (RC/RF)[5]	
DU-5B	NA	5, 25, 50, 100	0.8, 1.1, 1.0, 1.1	NC	DMSO	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA
DU-5C	NA	1, 5, 25, 100	1.4, 2.0, 1.2, 0.9	NC	DMSO	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA
DU-6	NA	5, 25, 50, 80	1.1, 0.8, 0.9, 0.9	NC	DMF	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA
DU-7	NA	5, 25, 50, 80	1.9, 1.2, 1.1, 1.3	NC	DMF	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA
DU-8A	NA	1, 10, 50, 100	1.4, 1.4, 0.8, 1.0	NC	AOO	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA
DU-8B	NA	5, 25, 50, 100	1.2, 1.9, 1.4, 1.8	NC	DMF	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA
DU-9A	NA	5, 25, 50, 100	3.6, 5.0, 8.8, 13.5	2.7	AOO	CBA/JHsd	+	Submitted by Dupont	+	NA	NA	NA	NA	NA	NA	NA
DU-9B	NA	5, 25, 50, 100	0.8, 0.8, 0.6, 0.5	NC	AOO	CBA/JHsd	-	Submitted by Dupont	-	NA	NA	NA	NA	NA	NA	NA
EXP 10810 A	NA	10, 25, 50	6.4, 8.4, 9.2	2.1	1% L92	CBA/J	+	Bayer Crop Science, submitted by E. Debruyne	+	+	+	NA	NA	NA	NA	Bayer Crop Science, submitted by E. Debruyne
EXP 11120 A	NA	10, 25, 50, 100	1.0, 0.7, 1.6, 6.3	64.9	1% L92	CBA/J	+	Bayer Crop Science, submitted by E. Debruyne	+	-	-	NA	NA	NA	NA	Bayer Crop Science, submitted by E. Debruyne

Substance Name	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC_3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	Overall Call						GP Reference
										GP (F)	GP (any)[3]	GP (AI)[4]	BT (AI)[4]	GPMT (AI)[4]	GP (RC/RF)[5]	
F & Fo WG 50 + 25	WG	2.5, 5.0, 10.0, 25.0	11.7, 12.6, 14.4, 15.2	0.003	1% L92	CBA/J	+	Bayer Crop Science, submitted by E. Debruyne	+	-	-	NA	NA	NA	NA	Bayer Crop Science, submitted by E. Debruyne
FAR01042-00	NA	10, 25, 50, 100	1.4, 2.1, 1.4, 2.5	NC	1% L92	CBA/J	-	Bayer Crop Science, submitted by E. Debruyne	-	-	-	NA	NA	NA	NA	Bayer Crop Science, submitted by E. Debruyne
FAR01060-00	NA	10, 25, 50, 100	0.4, 0.8, 1.0, 3.6	88.5	1% L92	CBA/J	+	Bayer Crop Science, submitted by E. Debruyne	+	-	-	NA	NA	NA	NA	Bayer Crop Science, submitted by E. Debruyne
Formulation 1	SC	5, 20, 80	1.1, 1.3, 1.3	NC	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	NA	+	+	-	+	NA	Submitted by Dow AgroSciences
Formulation 10	EW	2, 10, 50	1, 1, 5.2	29.0	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	-	-	-	NA	NA	Submitted by Dow AgroSciences
Formulation 11	OD	0.4, 2, 10	1.2, 1.2, 3.2	9.2	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	+	+	+	NA	Submitted by Dow AgroSciences
Formulation 12	EC	0.2, 1, 5	1.2, 3, 11.6	1.00	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	NA	NA	NA	NA	Submitted by Dow AgroSciences
Formulation 13	EC	1, 5, 25	1.2, 1.3, 10.4	8.7	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	+	NA	NA	+	Submitted by Dow AgroSciences

Substance Name	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC_3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	Overall Call						GP Reference
										GP (F)	GP (any)[3]	GP (AI)[4]	BT (AI)[4]	GPMT (AI)[4]	GP (RC/RF)[5]	
Formulation 14	CS	0.1, 1, 10	0.7, 0.7, 1.3	NC	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	NA	+	+	NA	+	NA	Submitted by Dow AgroSciences
Formulation 15	CS	0.2, 1, 5	0.8, 1.4, 3.2	4.6	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	+	NA	+	NA	Submitted by Dow AgroSciences
Formulation 16	EC	1, 5, 25	1.3, 2.2, 12.3	6.6	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	+	+	NA	NA	Submitted by Dow AgroSciences
Formulation 17	SL	5, 25, 75	1.7, 9.3, 18.5	8.4	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	NA	NA	NA	-	Submitted by Dow AgroSciences
Formulation 19	EC	1, 10, 25, 50	4.9, 7.9, 20, 50.5	0.23	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	+	-	-	-	Submitted by Dow AgroSciences
Formulation 2	SE	5, 20, 80	2, 3.4, 15.8	15.7	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	-	+	-	-	-	NA	Submitted by Dow AgroSciences
Formulation 20	SE	2, 10, 50	1.1, 1.4, 3.3	43.7	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	+	-	NA	NA	Submitted by Dow AgroSciences
Formulation 21	TK	5, 25, 100	1.3, 1.2, 1.9	NC	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	NA	+	-	-	NA	NA	Submitted by Dow AgroSciences
Formulation 22	ME	5, 25, 100	1.2, 1.4, 5.8	52.3	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	+	+	-	NA	Submitted by Dow AgroSciences

Substance Name	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC_3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	Overall Call						GP Reference
										GP (F)	GP (any)[3]	GP (AI)[4]	BT (AI)[4]	GPMT (AI)[4]	GP (RC/RF)[5]	
Formulation 23	SL	5, 25, 100	0.8, 1, 1	NC	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	NA	+	+	+	NA	NA	Submitted by Dow AgroSciences
Formulation 24	OD	2, 10, 50	1.4, 4.1, 11.7	6.7	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	NA	NA	NA	+	Submitted by Dow AgroSciences
Formulation 25	EC	1, 5, 25	1.8, 2.6, 14.7	5.6	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	+	-	+	NA	Submitted by Dow AgroSciences
Formulation 26	EC	1, 5, 25	1, 1, 4	18	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	+	+	NA	NA	Submitted by Dow AgroSciences
Formulation 27	EC	1, 5, 25	2.3, 2.5, 11.2	6.1	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	-	-	-	-	NA	Submitted by Dow AgroSciences
Formulation 28	SC	5, 25, 100	1, 1, 1.1	NC	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	NA	+	-	NA	NA	NA	Submitted by Dow AgroSciences
Formulation 29	SC	5, 25, 100	1.8, 1.6, 1.5	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	+	+	NA	+	+	Submitted by Dow AgroSciences
Formulation 3	SC	5, 20, 80	1, 1.2, 1.7	NC	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	-	+	-	-	-	NA	Submitted by Dow AgroSciences
Formulation 30	EW	5, 25, 100	1.8, 7.2, 13.6	9.4	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	+	+	+	+	NA	Submitted by Dow AgroSciences

Substance Name	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC_3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	Overall Call						GP Reference
										GP (F)	GP (any)[3]	GP (AI)[4]	BT (AI)[4]	GPMT (AI)[4]	GP (RC/RF)[5]	
Formulation 31	CS	5, 25, 100	1, 1.9, 1.8	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	+	+	+	-	NA	Submitted by Dow AgroSciences
Formulation 32	EC	5, 25, 100	6.5, 44.7, 69.3	4.3	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	+	NA	NA	NA	+	Submitted by Dow AgroSciences
Formulation 33	SL	5, 25, 100	0.7, 1.4, 1.3	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	+	NA	NA	NA	+	Submitted by Dow AgroSciences
Formulation 34	SL	5, 25, 100	1.9, 1.4, 1.5	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	-	-	NA	-		Submitted by Dow AgroSciences
Formulation 35	SL	5, 25, 100	1.1, 1.2, 1.3	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	+	+	+	NA	NA	Submitted by Dow AgroSciences
Formulation 37	EC	1, 5, 15	1.4, 2.7, 7.5	5.6	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	+	+	+	NA	NA	Submitted by Dow AgroSciences
Formulation 38	EC	5, 25, 100	1.1, 4.6, 12.7	15.9	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	-	-	-	NA	NA	Submitted by Dow AgroSciences
Formulation 39	OD	1, 5, 25	1.7, 2.5, 3.3	17.5	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	+	+	NA	NA	+	Submitted by Dow AgroSciences
Formulation 4	SL	5, 20, 80	1.4, 1.1, 1.2	NC	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	NA	-	-	-	NA	+	Submitted by Dow AgroSciences

Substance Name	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC_3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	Overall Call						GP Reference
										GP (F)	GP (any)[3]	GP (AI)[4]	BT (AI)[4]	GPMT (AI)[4]	GP (RC/RF)[5]	
Formulation 40	OD	1, 5, 25	1.8, 2.8, 5.7	6.4	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	+	+	NA	NA	+	Submitted by Dow AgroSciences
Formulation 41	SE	5, 25, 100	1.9, 1.9, 4.7	54.5	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	+	+	NA	NA	+	Submitted by Dow AgroSciences
Formulation 42	SL	10, 50, 100	1.2, 2.0, 3.1	95.5	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	-	-	NA	NA	NA	Submitted by Dow AgroSciences
Formulation 43	CS	5, 25, 75	NA	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	+	+	+	NA	NA	Submitted by Dow AgroSciences
Formulation 44	SC	5, 25, 100	NA	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	+	-	NA	NA	NA	Submitted by Dow AgroSciences
Formulation 45	SC	5, 25, 100	NA	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	+	-	NA	NA	NA	Submitted by Dow AgroSciences
Formulation 46	SC	5, 25, 100	NA	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	-	+	NA	NA	NA	NA	Submitted by Dow AgroSciences
Formulation 47	EW	5, 25, 100	2.1, 2.1, 6.0	42.3	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	+	+	NA	NA	NA	Submitted by Dow AgroSciences
Formulation 49	AL	5, 25, 100	0.7, 1.4, 4.7	61.4	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	+	+	+	NA	NA	Submitted by Dow AgroSciences

Substance Name	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC_3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	Overall Call						GP Reference
										GP (F)	GP (any)[3]	GP (AI)[4]	BT (AI)[4]	GPMT (AI)[4]	GP (RC/RF)[5]	
Formulation 5	EC	3, 10, 30	1.4, 4, 11.5	7.3	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	+	+	NA	NA	Submitted by Dow AgroSciences
Formulation 50	SL	5, 25, 100	1.2, 1.2, 14.7	35	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	-	-	NA	NA	NA	Submitted by Dow AgroSciences
Formulation 51	OD	5, 25, 100	1.6, 4.5, 2.9	14.7	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	+	+	NA	+	+	Submitted by Dow AgroSciences
Formulation 53	EW	2.5, 7.5, 15	1.5, 3.2, 6.7	6.9	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	+	+	+	-	NA	Submitted by Dow AgroSciences
Formulation 54	SL	5, 25, 100	1.3, 1.2, 2.3	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	-	NA	NA	NA	NA	Submitted by Dow AgroSciences
Formulation 55	EW	5, 25, 100	1.5, 2.5, 3.7	56.3	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	+	+	+	-	NA	Submitted by Dow AgroSciences
Formulation 56	SL	5, 25, 100	3.3, 6.1, 3.9	4.2	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	+	+	NA	NA	NA	Submitted by Dow AgroSciences
Formulation 6	EW	5, 20, 80	1.3, 2.7, 11.6	23.7	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	+	+	NA	NA	Submitted by Dow AgroSciences
Formulation 7	SC	20, 80, 100	1, 1.9, 3,2	96.9	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	-	+	+	-	+	NA	Submitted by Dow AgroSciences
		5, 20, 80	2.6, 1.4, 3.2	73.3	1% L92	BALB/c	+									

Substance Name	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC_3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	Overall Call						GP Reference
										GP (F)	GP (any)[3]	GP (AI)[4]	BT (AI)[4]	GPMT (AI)[4]	GP (RC/RF)[5]	
Formulation 8	EC	1, 5, 25	0.9, 1.1, 7.3	11.1	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	+	+	NA	NA	+	Submitted by Dow AgroSciences
Formulation 9	SC	4, 20, 80	1.1, 1.7, 1.3	NC	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	NA	+	NA	NA	NA	NA	Submitted by Dow AgroSciences
Fx + Me EW 69	EW	5.0, 10.0, 25.0, 50.0	0.8, 1.6, 3.0, 8.6	25.2	1% L92	CBA/J	+	Bayer Crop Science, submitted by E. Debruyne	+	-	-	NA	NA	NA	NA	Bayer Crop Science, submitted by E. Debruyne
Oxyfluorfen	EC	1, 7, 33	0.81, 1.4, 4.9	18.8	1% L92	CBA/Ca	+	ECPA LLNA Project Report submitted by BASF	+	-	-	NA	NA	NA	NA	ECPA LLNA Project Report submitted by Dow Chemical
		1, 7, 33	0.9, 1.4, 2.8	NC	1% L92	CBA/J	-	ECPA LLNA Project Report submitted by Bayer								
		1, 7, 33	0.3, 0.9, 2.3	NC	1% L92	CBA/J	-	ECPA LLNA Project Report submitted by Dow Chemical								
		1, 7, 33	1.1, 1.5, 3.1	30.8	1% L92	CBA/JHsd	+	ECPA LLNA Project Report submitted by Dupont								
		1, 7, 33	1.2, 1.2, 5.4	18.1	1% L92	CBA/CaOlaHsd	+	ECPA LLNA Project Report submitted by								

Substance Name	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC_3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	Overall Call						GP Reference
										GP (F)	GP (any)[3]	GP (AI)[4]	BT (AI)[4]	GPMT (AI)[4]	GP (RC/RF)[5]	
								Syngenta/RCC								
Quinoxyfen	SC	7, 33, 100	1.1, 0.7, 0.8	NC	1% L92	CBA/J	-	ECPA LLNA Project Report submitted by Dow Chemical	-	-	+	+	-	+	NA	ECPA LLNA Project Report submitted by Dow Chemical
Quinoxyfen / cyproconazole	NA	7, 33, 100	2.1, 10.7, 20.3	9.8	1% L92	CBA/Ca	+	ECPA LLNA Project Report submitted by BASF	+	+	+	+	-	+	NA	ECPA LLNA Project Report submitted by Dow Chemical
		7, 33, 100	1.2, 7.2, 12.4	14.8	1% L92	CBA/J	+	ECPA LLNA Project Report submitted by Bayer								
		7, 33, 100	0.4, 3.8, 2.0	26.9	1% L92	CBA/J	+	ECPA LLNA Project Report submitted by Dow Chemical								
		7, 33, 100	1.4, 2.0, 6.2	49.8	1% L92	CBA/JHsd	+	ECPA LLNA Project Report submitted by Dow Chemical								
		7, 33, 100	1.3, 6.5, 13.6	15.5	1% L92	CBA/CaOlaHsd	+	ECPA LLNA Project Report submitted by Dupont								
		12.5, 25. 50, 75, 100	2, 2.3, 8.6, 15.8, 30.1	27.8	1% L92	CBA/J	+	ECPA LLNA Project Report submitted by Syngenta/RCC								

Substance Name	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC_3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	Overall Call						GP Reference
										GP (F)	GP (any)[3]	GP (AI)[4]	BT (AI)[4]	GPMT (AI)[4]	GP (RC/RF)[5]	
Trifluralin	EC	7, 33, 100	6.0, 30.0, 75.2	5.8	1% L92	CBA/Ca	+	ECPA LLNA Project Report submitted by BASF	+	-	-	NA	NA	NA	NA	ECPA LLNA Project Report submitted by Dow Chemical
		7, 33, 100	1.9, 8.7, 25.7	11.2	1% L92	CBA/J	+	ECPA LLNA Project Report submitted by Bayer								
		7, 33, 100	3.1, 26.3, 61.5	7.0	1% L92	CBA/J	+	ECPA LLNA Project Report submitted by Dow Chemical								
		7, 33, 100	1.0, 7.0, 16.1	15.6	1% L92	CBA/ JHsd	+	ECPA LLNA Project Report submitted by Dupont								
		7, 33, 100	1.8, 8.2, 20.5	11.9	1% L92	CBA/ CaOlaHsd	+	ECPA LLNA Project Report submitted by Syngenta/RCC								

Abbreviations: AL = any other liquid; AOO = acetone olive-oil (4:1); ACE = acetone; BT = Buehler Test; Conc. = concentration; CS = capsule suspension; DMF = dimethyl formamide; DMSO = dimethyl sulfoxide; EC = emulsion concentrate; ECPA = European Crop Protection Association; EW = emulsion, oil in water; GPMT = Guinea Pig Maximization Test; LLNA = Local Lymph Node Assay; OD = oil dispersion; ME = micro-emulsion; NA = not available; NC = not calculated since SI>3; PG = propylene glycol; SC = suspension concentrate; SE = suspo-emulsion; SI = stimulation index; SL = soluble concentrate; TK = technical concentrate.

[1] "+" = sensitizer; "-" = nonsensitizer

[2] Overall GP call made on the basis of a test on the entire formulation

[3] Overall GP call made with priority entire formulation > active ingredient > related compound or formulation

[4] Overall GP call made on the basis of a test on an active ingredient

[5] Overall GP call made on the basis of a test on a related compound or formulation

Annex II-3

Composition of Pesticide Formulations Tested in the LLNA

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Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/ w)	Existing Sensitization Information[1]
Dinocap	EC	ECPA	NA	Dinocap	350	NA	NA
			NA	Solvent	542	NA	NA
			NA	Surfactant	78	NA	NA
Formulation 1	SC	Dow AgroSciences	Benzamide	Isoxaben	125	12.14%	- (Dow Data)
			NA	Water	735.2	NA	-
			NA	Thickener	4	NA	- (MSDS)
			NA	Antifoam	2	NA	- (MSDS)
			NA	Surfactant	30	NA	- (MSDS)
			NA	Surfactant	20	NA	- (MSDS)
			NA	Performance Aid	8.5	NA	- (MSDS)
			NA	pH Buffer	1.3	NA	- (MSDS)
			NA	Surfactant	100	NA	- (MSDS)
			NA	Biocide	4	< 0.1 %	+ (MSDS)

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/ w)	Existing Sensitization Information[1]
Formulation 2	SE	Dow AgroSciences	Pyridinyloxy acetic acid	Fluroxypyr-meptyl	144.09	14.53%	- (Dow Data)
			Sulfonamides	Florasulam	2.5	0.25 %	- (Dow Data)
			NA	Emulsifier	58.92	NA	- (MSDS)
			NA	Emulsifier	31.84	NA	- (MSDS)
			NA	Solvent	326.8	NA	- (MSDS)
			NA	Suspending Aid	3.24	NA	- (MSDS)
			NA	Suspending Aid	0.91	NA	- (MSDS)
			NA	Emulsifier	1.81	NA	- (MSDS)
			NA	Emulsifier	1.81	NA	- (MSDS)
			NA	Biocide	0.54	0.05 %	+ (MSDS)
			NA	Antifoam	1.06	NA	- (MSDS)
			NA	Antifreeze	34.62	NA	- (MSDS)
			NA	Suspending Aid	0.05	NA	- (MSDS)
			NA	Dispersant	0.1	NA	- (MSDS)
			NA	pH Buffer	0.003	NA	- (MSDS)
			NA	Dispersant	0.2	NA	- (MSDS)
			NA	Water	383.66	NA	-

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information[1]
Formulation 3	SC	Dow AgroSciences	Sulfonamides	Florasulam	50	4.84 %	- (Dow Data)
			NA	Water	869.12	NA	-
			NA	Biocide	0.93	0.09 %	+ (MSDS)
			NA	Dispersant	10.03	NA	- (MSDS)
			NA	Thickener	10.03	NA	- (MSDS)
			NA	Dispersant	1.96	NA	- (MSDS)
			NA	Antifoam	0.21	NA	- (MSDS)
			NA	Thickener	1.76	NA	- (MSDS)
			NA	Antifreeze	89.96	NA	- (MSDS)
			NA	pH Buffer	0.1	NA	- (MSDS)
Formulation 4	SL	Dow AgroSciences	Pyridine carboxylic acids	Clopyralid-olamine (MEA salt)	131.75	12.5 2%	- (Dow Data) (Clopyralid)
			NA	Water	920.25	NA	-
Formulation 5	EC	Dow AgroSciences	Pyridinyloxy acetic acid	Triclopyr-butotyl	670.39	60.4 5%	+ (Dow Data)
			NA	Emulsifier	55.45	NA	- (MSDS)
			NA	Solvent	383.16	NA	- (MSDS)

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/ w)	Existing Sensitization Information[1]
Formulation 6	EW	Dow AgroSciences	Pyridinyloxy acetic acid	Triclopyr-butotyl	333.567	29.44%	+ (Dow Data)
			Pyridine carboxylic acids	Aminopyralid potassium	35.507	3.13 %	- (Dow Data) (Aminopyralid)
			NA	Antifreeze	50	NA	- (MSDS)
			NA	Emulsifier	32.5	NA	- (MSDS)
			NA	Emulsifier	32.5	NA	- (MSDS)
			NA	Biocide	1	0.09 %	+ (MSDS)
			NA	Thickener	7.5	NA	- (MSDS)
			NA	Thickener	1.875	NA	- (MSDS)
			NA	pH Buffer	27.33	NA	- (MSDS)
			NA	pH Buffer	2.67	NA	- (MSDS)
			NA	Antifoam	2	NA	- (MSDS)
			NA	Water	606.831	NA	-

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information[1]
Formulation 7	SC	Dow AgroSciences	Triazole	Myclobutanil	45	4.12 %	Equivocal (Dow Data)
			Phenoxyquinoline	Quinoxyfen	45	4.12 %	+ (Dow Data)
			NA	Antifreeze	74.89	NA	- (MSDS)
			NA	Dispersant	31.81	NA	- (MSDS)
			NA	Wetter	14.96	NA	- (MSDS)
			NA	Suspending Aid	7.45	NA	- (MSDS)
			NA	Carrier	57.12	NA	-
			NA	Antifoam	1.09	NA	- (MSDS)
			NA	Biocide	0.37	0.03 %	+ (MSDS)
			NA	Water	785.84	NA	-
			NA	Filler	26.5	NA	- (MSDS)
			NA	Thickener	1.97	NA	- (WHO)
Formulation 8	EC	Dow AgroSciences	Phenoxyacetic acids	2,4-D-ethylhexyl	905	81.68%	+ (Dow Data)
			NA	Emulsifier	37	3.34 %	- (MSDS)
			NA	Emulsifier	43	3.88 %	- (MSDS)
			NA	Solvent	123	NA	- (MSDS)

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/ w)	Existing Sensitization Information[1]
Formulation 9	SC	Dow AgroSciences	Spinosoids	DE-175	120	11.71%	Equivocal (+/- LLNA)
			Nicotinoates	Wetter	20.5	NA	- (MSDS)
			NA	Antifreeze	61.5	NA	- (MSDS)
			NA	Biocide	2	0.20 %	+ (MSDS)
			NA	Thickener	1.8	NA	- (WHO)
			NA	Thickener	4.1	NA	- (MSDS)
			NA	Antifoam	3.6	NA	- (MSDS)
			NA	Dispersant	46.1	NA	- (MSDS)
			NA	Water	765.4	NA	-
Formulation 10	EW	Dow AgroSciences	NA	Dithiopyr	240	24 %	- (Dow Data)
			NA	Solvent	130	13 %	- (MSDS)
			NA	Emulsifier	470	47 %	- (MSDS)
			NA	Water	160	16 %	-

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/ w)	Existing Sensitization Information[1]
Formulation 11	OD	Dow AgroSciences	Chloroacetamides	Acetochlor	950	84.15%	+ (Dow Data)
			Sulfonamides	Penoxsulam	3.5	0.31 %	- (Dow Data)
			NA	Suspending Aid	28.5	NA	- (MSDS)
			NA	Antifoam	0.035	NA	- (MSDS)
			NA	Thickener	0.035	NA	- (MSDS)
			NA	pH Buffer	0.014	NA	- (MSDS)
			NA	Dispersant	0.28	NA	- (MSDS)
			NA	Wetter	0.07	NA	- (MSDS)
			NA	Antifreeze	0.21	NA	- (MSDS)
			NA	Water, Deionized	2.84	NA	-
			NA	Nutrient	4.75	0.42 %	- (Human Data from IUCLID)
			NA	Related Process Inert Impurities	45.98	NA	- (MSDS)
			NA	Anticaking Agent	0.007	NA	- (MSDS)
			NA	Biocide	0.007	0% (0.007)	+ (MSDS)
			NA	Emulsifier	92.94	NA	- (MSDS)
Formulation 12	EC	Dow AgroSciences	Dinitrophenol	Meptyldinocap	350	35.71%	+ (Dow Data)
			NA	Emulsifier	41.7	NA	- (MSDS)
			NA	Emulsifier	25.76	NA	- (MSDS)
			NA	Solvent	562.54	NA	- (MSDS)
Formulation 13	EC	Dow AgroSciences	Phenoxyacetic acids	2,4-D-ethylhexyl	995.5	87.17%	+ (Dow Data)
			NA	Emulsifier	48	NA	- (MSDS)

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/ w)	Existing Sensitization Information[1]
			NA	Emulsifier	48	NA	-
			NA	Unspecified Inert	50.5	NA	-
Formulation 14	CS	Dow AgroSciences	Pyrethroids	Gamma-cyhalothrin	15	1.5 %	+ (Dow Data)
			NA	Solvent	10.02	NA	- (MSDS)
			NA	Emulsifier	1.25	NA	- (MSDS)
			NA	Emulsifier	1.25	NA	- (MSDS)
			NA	Encapsulating Agent	1.63	NA	-
			NA	pH Buffer	1	NA	- (MSDS)
			NA	Thickener	0.02	NA	- (MSDS)
			NA	Biocide	1.5	0.15 %	+ (MSDS)
			NA	Thickener	1.5	NA	- (MSDS)
			NA	Thickener	0.02	NA	- (MSDS)
			NA	Thickener	15.03	NA	-
			NA	Water	953.8	NA	-

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information[1]
Formulation 15	CS	Dow AgroSciences	Pyrethroids	Gamma-cyhalothrin	60	5.9 %	+ (Dow Data)
			NA	Solvent	48.82	NA	- (MSDS)
			NA	Emulsifier	5.09	NA	- (MSDS)
			NA	Emulsifier	5.09	NA	- (MSDS)
			NA	Encapsulating Agent	6.81	NA	-
			NA	Thickener	0.09	NA	- (MSDS)
			NA	Biocide	1.53	0.15 %	+ (MSDS)
			NA	Thickener	1.53	NA	- (MSDS)
			NA	Thickener	0.09	NA	- (MSDS)
			NA	pH Buffer	4.07	NA	- (MSDS)
			NA	Thickener	10.68	NA	-
			NA	Water	873.4	NA	-
Formulation 16	EC	Dow AgroSciences	Pyridinyloxy acetic acid	Triclopyr-butotyl	1050.07	83.94%	+ (Dow Data)
			NA	Emulsifier	200.93	NA	- (MSDS)
Formulation 17	SL	Dow AgroSciences	Glycines	Glyphosate dimethyl-ammonium salt	1.1	1.2	1.3 - (EPA Tolerance)
			NA	Adjuvant	50	7.0	No Data
			NA	Adjuvant	100	NA	- (MSDS)
			NA	Water	453	NA	-

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/ w)	Existing Sensitization Information[1]
Formulation 19	EC	Dow AgroSciences	Pyridinyloxy acetic acid	Fluroxypyr-meptyl	100.865	9.23 %	- (Dow Data)
			Benzonitriles	Bromoxynil-octanoate	407.569	37.29%	+ (Dow Data)
			NA	Emulsifier	44	4.03 %	- (MSDS)
			NA	Emulsifier	44	NA	- (MSDS)
			NA	Solvent	496.566	45.43%	- (IUCLID Datasheet)
Formulation 20	SE	Dow AgroSciences	Sulfonamides	Florasulam	4	0.39 %	- (Dow Data)
			NA	MCPA-2-ethylhexyl	436.817	42.25%	- (Dow Data); + (EPA RED)
			NA	Emulsifier	12	NA	- (MSDS)
			NA	Thickener	4.34	NA	- (MSDS)
			NA	Dispersant	0.17	NA	- (MSDS)
			NA	Antifoam	1	NA	- (MSDS)
			NA	Stabilizer	1.5	NA	- (MSDS)
			NA	Thickener	0.54	NA	- (MSDS)
			NA	Stabilizer	45.14	NA	- (MSDS)
			NA	pH Buffer	0.01	NA	- (MSDS)
			NA	Stabilizer	0.34	NA	- (MSDS)
			NA	Antifreeze	49.75	NA	- (MSDS)
			NA	Biocide	0.93	0.09 %	+ (MSDS)
			NA	pH Buffer	1.03	NA	- (MSDS)
			NA	Water	476.443	NA	-

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information[1]
Formulation 21	TK	Dow AgroSciences	Acyl Ureas	Hexaflumuron	645	50 %	- (Dow Data)
			NA	Water	497.42	NA	-
			NA	Biocide	9.68	0.75 %	+ (MSDS)
			NA	Surfactant	64.5	NA	- (MSDS)
			NA	Antifoam	3.48	NA	- (MSDS)
			NA	Surfactant	69.92	5.42 %	- (MSDS)
Formulation 22	ME	Dow AgroSciences	Pyridinyloxy acetic acid	Fluroxypyr-meptyl	28.8	2.83 %	- (Dow Data)
			NA	Triclopyr-triethyl-ammonium	83.67	8.23 %	+ (EPA RED)
			NA	Surfactant	29.59	NA	- (MSDS)
			NA	Carrier	29.59	NA	- (MSDS)
			NA	Surfactant	84	NA	- (MSDS)
			NA	Emulsifier	48	NA	- (MSDS)
			NA	Solvent	86.34	NA	- (MSDS)
			NA	Unspecified Inert	104.98	NA	-
			NA	Water	522.03	NA	-
Formulation 23	SL	Dow AgroSciences	Pyridinyloxy acetic acid	Triclopyr-triethyl-ammonium	167.36	16 %	+ (EPA RED)
			NA	Water	837	NA	-
			NA	Antifoam	0.02	NA	- (MSDS)
			NA	Wetter	3.77	NA	- (MSDS)
			NA	Chelating Agent	8.68	NA	- (MSDS)
			NA	Surfactant	10.04	NA	- (MSDS)
			NA	Neutralizer	11.3	NA	- (67/548/EEC

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/ w)	Existing Sensitization Information[1]
							)
			NA	Carrier	7.85	NA	- (67/548/EEC)
Formulation 24	OD	Dow AgroSciences	Sulfonamides	Pyroxsulam	30	2.87 %	+ (Dow Data)
			NA	Safener	90	8.6 %	+ (EPA Tolerance Petition)
			NA	Emulsifier	40	NA	- (MSDS)
			NA	Emulsifier	50	NA	- (MSDS)
			NA	Emulsifier	20	NA	- (MSDS)
			NA	Stabilizer	10	NA	-
			NA	Suspending Aid	40	NA	- (MSDS)
			NA	Diluent	767	NA	- (MSDS)
Formulation 25	EC	Dow AgroSciences	Pyridine carboxylic acids	Clopyralid	23.34	2.21 %	- (Dow Data)
			Pyridinyloxy acetic acid	Fluroxypyr-meptyl	86.455	8.19 %	- (Dow Data)
			NA	MCPA-2-ethylhexyl	416.1	39.4 %	- (Dow Data); + (EPA RED)
			NA	Solvent	38.54	NA	- (MSDS)
			NA	Emulsifier	52.27	NA	- (MSDS)
			NA	Emulsifier	428.205	NA	- (MSDS)
			NA	Solvent	11.09	NA	- (MSDS)
Formulation 26	EC	Dow AgroSciences	Pyridine carboxylic acids	Clopyralid	60	5.83 %	- (Dow Data)
			Pyridinyloxy acetic acid	Triclopyr-butotyl	333.797	32.41%	+ (Dow Data)
			NA	Emulsifier	43.7	NA	- (MSDS)
			NA	Emulsifier	29.2	NA	- (MSDS)
			NA	Solvent	88.9	NA	- (MSDS)

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information[1]
			NA	Solvent	474.403	NA	- (IUCLID Datasheet)
Formulation 27	EC	Dow AgroSciences	Pyridinyloxy acetic acid	Fluroxypyr-meptyl	479.827	45.52%	- (Dow Data)
			NA	Emulsifier	78.46	NA	- (MSDS)
			NA	Solvent	417.253	NA	- (MSDS)
			NA	Emulsifier	78.46	NA	-(MSDS)
Formulation 28	SC	Dow AgroSciences	Unclassified Herbicide	Diflufenican	100	9.48 %	- (MSDS)
			Sulfonamides	Penoxsulam	15	1.42 %	- (Dow Data)
			NA	Wetter	15	NA	- (MSDS)
			NA	Dispersant	10	NA	- (MSDS)
			NA	Thickener	10	NA	- (MSDS)
			NA	Thickener	2	NA	- (MSDS)
			NA	Biocide	1.5	0.14 %	+ (MSDS)
			NA	Antifreeze	50	NA	- (MSDS)
			NA	pH Buffer	0.462	NA	- (MSDS)
			NA	Antifoam	5	NA	- (MSDS)
			NA	Water	846.038	NA	-

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/ w)	Existing Sensitization Information[1]
Formulation 29	SC	Dow AgroSciences	Dithiocarbamate	Mancozeb	462	35.95%	Equivocal (EPA RED)
			Unspecified	Cymoxanil	70.03	5.45 %	- (EPA Fact Sheet)
			NA	Anticaking Agent	29.81	NA	- (MSDS)
			NA	Stabilizer	25.7	NA	-
			NA	Stabilizer	12.85	NA	-
			NA	Emulsifier	12.85	NA	- (MSDS)
			NA	Dispersant	2.57	NA	- (MSDS)
			NA	Thickener	1.29	NA	- (MSDS)
			NA	Adjuvant	131.58	NA	- (MSDS)
			NA	Water	536.32	NA	-
Formulation 30	EW	Dow AgroSciences	Chloroacetamides	Acetochlor	450	41.82%	+(Dow Data)
			Pyridine carboxylic acids	Clopyralid-olamine	46.11	4.29 %	- (Dow Data)
			Sulfonamides	Flumetsulam	14.0	1.3 %	- (MSDS)
			NA	pH Buffer	2.37	0.22 %	- (67/548/EEC)
			NA	Emulsifier	21.52	2%	- (IUCLID Datasheet)
			NA	Solvent	10.76	1%	- (IUCLID Datasheet)
			NA	Biocide	1.076	0.10 %	+ (MSDS)
			NA	Thickener	1.076	0.10 %	- (WHO)
			NA	Antifoam	1.61	NA	- (MSDS)
			NA	Dispersant	5.38	NA	- (MSDS)
			NA	Wetter	2.69	NA	- (MSDS)

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/ w)	Existing Sensitization Information[1]
			NA	Water	519.408	NA	-
Formulation 31	CS	Dow AgroSciences	Organophosphates	Chlorpyrifos	200	18.96%	Equivocal (Dow Data)
			NA	Encapsulating Agent	6.49	NA	-
			NA	Dispersant	29.59	NA	- (MSDS)
			NA	Biocide	1.055	0.10 %	+ (MSDS)
			NA	Thickener	5.92	NA	- (MSDS)
			NA	Thickener	0.738	NA	- (MSDS)
			NA	Dispersant	16.47	NA	- (MSDS)
			NA	Solvent	120	NA	- (IUCLID Datasheet)
			NA	Water	674.737	NA	-
Formulation 32	EC	Dow AgroSciences	Dinitrophenol	Meptyldinocap	105	11.27%	+ (Dow Data)
			Triazole	Myclobutanil	45	4.83 %	Equivocal (Dow Data)
			NA	pH Buffer	15	NA	- (67/548/EEC)
			NA	Emulsifier	23	NA	- (MSDS)
			NA	Emulsifier	68	NA	- (MSDS)
			NA	Solvent	676	NA	- (MSDS)
Formulation 33	SL	Dow AgroSciences	Pyridine carboxylic acids	Clopyralid-olamine	316.206	26.66%	- (Dow Data)
			Pyridine carboxylic acids	Picloram-olamine	100.251	8.45 %	- (EPA RED)
			Pyridine carboxylic acids	Aminopyralid-olamine	51.8	4.37 %	- (Dow Data)
			NA	Neutralizer	22	NA	- (67/548/EEC

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/ w)	Existing Sensitization Information[1]
							)
			NA	Water, Deionized	695.743	NA	-
Formulation 34	SL	Dow AgroSciences	Pyridine carboxylic acids	Aminopyralid	30	2.95 %	- (Dow Data)
			NA	Neutralizer	8.1	NA	- (67/548/EEC)
			NA	Water	978.9	NA	-
Formulation 35	SL	Dow AgroSciences	Pyridine carboxylic acids	Aminopyralid triisopropanol-ammonium	23.08	2.22 %	- (Dow Data) (Aminopyralid)
			Pyridinyloxy acetic acid	Triclopyr-triethyl-ammonium	167.36	16.09 %	+ (EPA RED)
			NA	Neutralizer	1.14	NA	-
			NA	Wetter	38	NA	- (MSDS)
			NA	Antifoam	0.19	NA	- (MSDS)
			NA	Neutralizer	14.82	NA	- (67/548/EEC)
			NA	Sequestrant	8.74	NA	- (MSDS)
			NA	Water	786.67	NA	-
Formulation 37	EC	Dow AgroSciences	Organophosphates	Chlorpyrifos	300	30 %	Equivocal (Dow Data)
			Pyrethroids	Gamma-cyhalothrin	5.4	0.54 %	+ (DOW Data)
			NA	Emulsifier	55	5.50 %	- (MSDS)
			NA	Emulsifier	4.4	0.44 %	- (MSDS)
			NA	Solvent	635.2	63.52%	- (IUCLID Datasheet)

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/ w)	Existing Sensitization Information[1]
Formulation 38	EC	Dow AgroSciences	Acetamides	Propanil	479.81	44.80%	- (EPA RED)
			NA	Solvent	362	NA	- (MSDS)
			NA	Solvent	122.09	NA	- (IUCLID Datasheet)
			NA	Emulsifier	107.1	10 %	- (IUCLID Datasheet)
Formulation 39	OD	Dow AgroSciences	Sulfonamides	Pyroxsulam	45	4.31 %	+ (DOW Data)
			NA	Safener	90	8.61 %	+ (EPA Tolerance Petition)
			NA	Dispersant	6	0.57 %	- (MSDS)
			NA	Dispersant	10	NA	- (MSDS)
			NA	Emulsifier	80	NA	- (MSDS)
			NA	Stabilizer	10	0.96 %	- (MSDS)
			NA	Suspending Aid	27	NA	- (MSDS)
			NA	Solvent	777	NA	- (MSDS)

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/ w)	Existing Sensitization Information[1]
Formulation 40	OD	Dow AgroSciences	Sulfonamides	Pyroxsulam	12.8	1.20 %	+ (Dow Data)
			NA	Safener	38.5	3.62 %	+ (EPA Tolerance Petition)
			NA	Active Ingredient	2.14	0.20 %	- (EPA Fact Sheet)
			NA	Active Ingredient	123.199	11.57%	- (Dow Data)
			NA	Dispersant	4	0.38 %	- (MSDS)
			NA	Dispersant	10	NA	- (MSDS)
			NA	Emulsifier	80	NA	- (MSDS)
			NA	Stabilizer	10	NA	- (MSDS)
			NA	Thickener	30	NA	- (MSDS)
			NA	Solvent	754.361	NA	- (MSDS)

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information[1]
Formulation 41	SE	Dow AgroSciences	Phenoxyacetic acids	2,4-D-ethylhexyl	271.493	25.61%	+ (Dow Data)
			Pyridine carboxylic acids	Aminopyralid	11.834	1.12 %	- (Dow Data)
			Sulfonamides	Florasulam	5	0.47 %	- (Dow Data)
			NA	Solvent	73.2	NA	- (MSDS)
			NA	Emulsifier	60.4	NA	- (MSDS)
			NA	Thickener	0.1	NA	- (MSDS)
			NA	Biocide	0.9	0.08 %	+ (MSDS)
			NA	Antifoam	2	NA	- (MSDS)
			NA	Dispersant	0.2	NA	- (MSDS)
			NA	Antifoam	0.02	NA	- (MSDS)
			NA	Antifreeze	50.5	NA	- (MSDS)
			NA	Suspending Aid	1.6	NA	- (MSDS)
			NA	pH Buffer	0.1	NA	- (MSDS)
			NA	Water	582.873	NA	-
Formulation 42	SL	Dow AgroSciences	Phenoxyacetic acids	2,4-D-triisoproanolamine	339	31.00%	- (EPA RED)
			Pyridine carboxylic acids	Aminopyralid triisopropanol-ammonium	17	1.52 %	- (Dow Data) (Aminopyralid)
			NA	Neutralizer	4.962	NA	- (MSDS)
			NA	Sequestrant	2.19	NA	- (MSDS)
			NA	Antifreeze	38.26	NA	- (MSDS)
			NA	Water	694.48	NA	-
Formulation 43	CS	Dow AgroSciences	Unspecified nitrification inhibitor	Nitrapyrin	200	17.90%	+ (Dow Data)

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/ w)	Existing Sensitization Information[1]
			NA	Solvent	234.79	0.12 %	+ (R43)
			NA	Solvent	99.65	NA	- (MSDS)
			NA	Thickener	22.31	NA	- (MSDS)
			NA	Dispersant	13.36	NA	+ (MSDS)
			NA	Emulsifier	13.36	0.24 %	- (MSDS)
			NA	Dispersant	2.67	1.19 %	- (MSDS)
			NA	Thickener	2.14	8.87 %	+ (DOW Data)
			NA	Biocide	1.34	NA	- (MSDS)
			NA	Water	534.38	NA	-

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information[1]
Formulation 44	SC	Dow AgroSciences	Sulfonamides	Penoxsulam	1.4	0.12 %	- (Dow Data)
			Dinitroanilines	Oryzalin	478.9	40.38%	Equivocal (Dow Data)
			NA	Antifoam	5.92	NA	- (MSDS)
			NA	Dispersant	71	5.99 %	- (MSDS)
			NA	Antifreeze	47.3	NA	- (MSDS)
			NA	Dispersant	17.7	1.49 %	- (MSDS)
			NA	Antifreeze	71.1	5.99 %	- (MSDS)
			NA	Biocide	0.59	0.05 %	+ (MSDS)
			NA	Suspending Aid	1.78	0.15 %	- (WHO)
			NA	Carrier	8.88	NA	- (MSDS)
			NA	Antifoam	0.01	NA	- (MSDS)
			NA	Suspending Aid	0.01	0%	- (MSDS)
			NA	pH Buffer	0.01	NA	- (MSDS)
			NA	Dispersant	0.11	0.01 %	- (MSDS)
			NA	Wetter	0.03	0%	- (MSDS)
			NA	Water	481.32	40.58%	-

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/ w)	Existing Sensitization Information[1]
Formulation 45	SC	Dow AgroSciences	Carboxanilide	Thifluzamide	80	7.53 %	- (Dow Data) (25%)
			Triazole	Fenbuconazole	100	9.42 %	- (Dow Data)
			NA	Adjuvant	51.4008	NA	- (MSDS)
			NA	Wetter	12.8502	NA	- (MSDS)
			NA	Biocide	1.062	0.10 %	+ (MSDS)
			NA	Suspending Aid	4.248	NA	- (MSDS)
			NA	Antifoam	5.32	NA	- (MSDS)
			NA	Emulsifier	11.682	NA	- (MSDS)
			NA	Dispersant	40.887	NA	- (MSDS)
			NA	Water	754.55	71.05%	-
Formulation 46	SC	Dow AgroSciences	Spinosoids	Spinetoram	60	5.87 %	Equivocal (+/- LLNA)
			NA	Dispersant	30.75	NA	- (MSDS)
			NA	Wetter	20.5	2%	- (MSDS)
			NA	Antifreeze	61.4	NA	- (MSDS)
			NA	Biocide	2	0.20 %	+ (MSDS)
			NA	Thickener	2	0.20 %	- (WHO)
			NA	Thickener	4.1	NA	- (MSDS)
			NA	Antifoam	10	0.98 %	- (MSDS)
			NA	Water	832.25	81.35%	-

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/ w)	Existing Sensitization Information[1]
Formulation 47	EW	Dow AgroSciences	Triazole	Propiconazole	150	14.56%	+ (EPA RED)
			NA	Solvent	5.15	NA	- (MSDS)
			NA	Emulsifier	20.6	2.00 %	- (MSDS)
			NA	Emulsifier	15.45	0.50 %	- (MSDS)
			NA	Antifreeze	51.5	5.00 %	- (MSDS)
			NA	Emulsifier	51.5	1.50 %	- (MSDS)
			NA	Water		66.44%	-
			NA	Solvent	735.8	5.00 %	- (IUCLID Datasheet)
Formulation 49	AL	Dow AgroSciences	Pyridinyloxy acetic acid	Triclopyr-butotyl	200.3	23.16%	+ (Dow Data)
			NA	Diluent	664.7	76.84%	- (IUCLID Datasheet)
Formulation 50	SL	Dow AgroSciences	Glycines	Glyphosate dimethyl-ammonium salt	608	50.54%	- (EPA Tolerance)
			NA	Adjuvant	90	7.48 %	- (MSDS for Similar)
			NA	Water	505	41.98%	-

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/ w)	Existing Sensitization Information[1]
Formulation 51	OD	Dow AgroSciences	Dinitroanilines	Pendimethalin	314	29.76%	- (EPA RED)
			Sulfonamides	Pyroxsulam	5.4	0.51 %	+ (Dow Data)
			NA	Safener	5.4	0.51 %	+ (EPA Tolerance Petition)
			NA	Stabilizer	5	NA	- (MSDS)
			NA	Suspending Aid	20	NA	- (MSDS)
			NA	Emulsifier	60	NA	- (MSDS)
			NA	Emulsifier	10	0.95 %	- (MSDS)
			NA	Emulsifier	30	NA	- (MSDS)
			NA	Antifoam	1	0.09 %	- (MSDS)
			NA	Solvent	604.2	NA	- (MSDS)
Formulation 53	EW	Dow AgroSciences	Organophosphates	Chlorpyrifos	450	40.18%	Equivocal (Dow Data)
			NA	Emulsifier	56	5%	No Data
			NA	Antifreeze	28	NA	- (MSDS)
			NA	Dispersant	134.5	12.01%	- (MSDS)
			NA	Biocide	1.12	0.10 %	+ (MSDS)
			NA	Antifoam	4.5	NA	- (MSDS)
			NA	Solvent	224	20 %	- (IUCLID Datasheet)
			NA	Water	221.88	19.81%	-

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/w)	Existing Sensitization Information[1]
Formulation 54	SL	Dow AgroSciences	Glycines	Glyphosate dimethyl-ammonium salt	608	49.88%	- (EPA Tolerance)
			NA	Adjuvant	100	NA	- (MSDS)
			NA	Adjuvant	50	NA	- (MSDS)
			NA	Water	461	37.82%	-
Formulation 55	EW	Dow AgroSciences	Triazole	Myclobutanil	45	4.5 %	Equivocal (Dow Data)
			NA	Emulsifier	26.5	2.65 %	- (MSDS)
			NA	Emulsifier	18.5	1.85 %	8.0 - (MSDS)
			NA	Antifreeze	100	NA	- (MSDS)
			NA	Solvent	200	20 %	- (IUCLID Datasheet)
			NA	Diluent	40.5	NA	- (MSDS)
			NA	Emulsifier	5	0.50 %	No Data
			NA	Water	561.5	56.15%	-
			NA	Biocide	3	0.30 %	+ (MSDS)
Formulation 56	SL	Dow AgroSciences	Unspecified nitrification inhibitor	Nitrapyrin	216	19.89%	+ (Dow Data)
			NA	Impurities	24	2.21 %	No Data
			NA	Stabilizer	14.4	1.33 %	- (MSDS)
			NA	Solvent	831.6	76.57%	- (IUCLID Datasheet)

Substance Name	Formu-lation Type	Source	Material Family	Active Ingredient/ Inert Function	Conc. (g/L)	Amount (% w/ w)	Existing Sensitization Information[1]
Oxyfluorfen	EC	ECPA	NA	Oxyfluorfen	240	NA	NA
			NA	Solvent	732	NA	NA
			NA	Surfactant	108	NA	NA
Quinoxyfen / Cyproconazole	NA	ECPA	NA	Cyproconazole	80	NA	NA
			NA	Quinoxyfen	75	NA	NA
			NA	Antifreeze	75	NA	NA
			NA	Thickener	10	NA	NA
			NA	Water/Other Components	842	NA	NA
Trifluralin	EC	ECPA	NA	Triflualin	480	NA	NA
			NA	Solvent	500	NA	NA
			NA	Surfactant	60	NA	NA

Abbreviations: AL = any other liquid; AOO = acetone olive oil (4:1); ACE = acetone; Conc. = concentration; CS = capsule suspension; EC = emulsion concentrate; ECPA = European Crop Protection Association; EEC = European Economic Community; EPA = U.S. Environmental Protection Agency; EW = emulsion, oil in water; IUCLID = International Uniform Chemical Information Database; LLNA = Local Lymph Node Assay; OD = oil dispersion; ME = micro-emulsion; MSDS = Material Safety Data Sheet; NA = not available; RED = reregistration eligibility decision; SC = suspension concentrate; SE = suspo-emulsion; SI = stimulation index; SL = soluble concentrate; TK = technical concentrate; WHO = World Health Organization.

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

Annex II-4

Physicochemical Properties and Chemical Classes of Dye Formulations Tested in the LLNA

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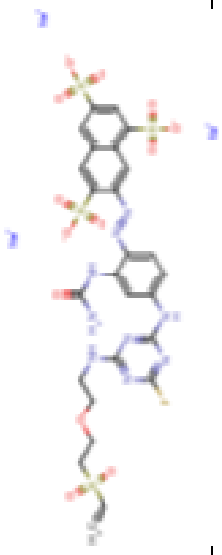
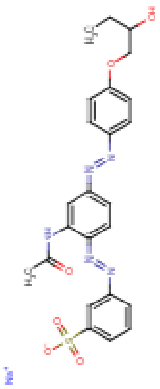
Substance Name	Synonyms	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Phys. Form	Chemical Class[2]	Structure[3]
C.I. Reactive Red 231	NA	NA	NA	NA	Solid	Formulation	NA
C.I. Reactive Yellow 174	1,3,6-Naphthalene-trisulfonic acid, 7-(2-(2-((aminocarbonyl) amino)-4-((4-((2-(2-(ethenylsulfonyl) ethoxy) ethyl)amino)-6-fluoro-1,3,5-triazin-2-yl) amino)phenyl) diazenyl)-, sodium salt (1:3)	106359-91-5	885.72	NA	Solid	Formulation	
Dispersionsrot 2754	NA	NA	NA	NA	Solid	Formulation	NA
Navy 14 08 723	NA	NA	NA	NA	Solid	Formulation	NA
Produkt P-4G	NA	185461-17-0	NA	NA	Solid	Formulation	NA
Yellow E-JD 3442	Benzenesulfonic acid, 3-(2-(2-(acetylamino)-4-(2-(4-(2-hydroxybutoxy) phenyl)diazenyl)phenyl) diazenyl)-, sodium salt (1:1)	147703-65-9	533.54	NA	Solid	Formulation	

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; g/mol = grams per mole; Kow = octanol-water partition coefficient; NA = not available.

[1]Kow represents the octanol-water partition coefficient (expressed on log scale) obtained from the website: http://www.syrres.com/esc/est_kowdemo.htm.

[2]Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs, as developed by the National Library of Medicine at: http://www.nlm.nih.gov/mesh/meshhome.html.

[3]Chemical structures, based on CASRN, were obtained from ChemID available at: http://chem.sis.nlm.gov/chemidplus/chemidheavy.jsp.

Annex II-5

Dye Formulations Tested in the LLNA - Comparative Data

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Substance Name	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC_3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	GPMT i.d. Induction Conc. (%)	GPMT Patch Conc. (%)	GPMT Challenge Conc. (%)	GPMT No. Animals with + rxn After Challenge & Rechallenge	GPMT % Sens. Incidence	GPMT Result[1]	Reference
C.I. Reactive Red 231	Dye	1, 3, 9, 15	4.8, 3.4, 4.4, 4.6	0.6	AOO	CBA/Ca	+	1	75	75	NA	~50	+	Forschung Projekt F 1877 submitted by Bundesanstalt für Arbeitsschutz und Arbeitsmedizin
C.I. Reactive Yellow 174	Dye	1, 3, 9, 15	4.2, 5.3, 5.5, 7.8	0.3	AOO	CBA/Ca	+	5	25	25	2	11	-	Forschung Projekt F 1877 submitted by Bundesanstalt für Arbeitsschutz und Arbeitsmedizin
Dispersionsrot 2754	Dye	1, 3, 9	1.0, 0.9, 1.0	NC	AOO	CBA/Ca	-	5	25	25	8	100	+	Forschung Projekt F 1877 submitted by Bundesanstalt für Arbeitsschutz und Arbeitsmedizin

Substance Name	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC_3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	GPMT i.d. Induction Conc. (%)	GPMT Patch Conc. (%)	GPMT Challenge Conc. (%)	GPMT No. Animals with + rxn After Challenge & Rechallenge	GPMT % Sens. Incidence	GPMT Result[1]	Reference
Navy 14 08 723	Dye	1, 3, 9, 15	5.1, 4.8, 5.7, 5.2	IDR	AOO	CBA/Ca	+	5	25	10	20	100	+	Forschung Projekt F 1877 submitted by Bundesanstalt für Arbeitsschutz und Arbeitsmedizin
Produkt P-4G	Dye	1, 3, 9, 15	2.4, 2.5, 1.9, 2.5	NC	AOO	CBA/Ca	-	5	25	25	9	90	+	Forschung Projekt F 1877 submitted by Bundesanstalt für Arbeitsschutz und Arbeitsmedizin
Yellow E-JD 3442	Dye	1, 3, 9, 15	1.0, 0.8, 0.9, 0.9	NC	AOO	CBA/Ca	-	5	50	50	2	10	-	Forschung Projekt F 1877 submitted by Bundesanstalt für Arbeitsschutz und Arbeitsmedizin

Abbreviations: AOO = acetone olive-oil (4:1); Conc. = concentration; GPMT = Guinea Pig Maximization Test; i.d. = intradermal; IDR = inadequate dose response; LLNA = Local Lymph Node Assay; NA = not available; NC = not calculated since SI<3; rxn = reaction; sens. = sensitization; SI = stimulation index.

[1] "+" = sensitizer; "-" = nonsensitizer

Annex II-6

Physicochemical Properties and Chemical Classes of Natural Complex Substances Tested in the LLNA

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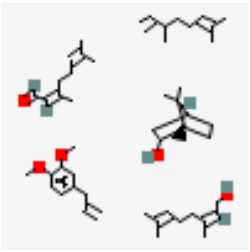
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow [1]	Physical Form	Chemical Class[2]	Structure[3]
Basil oil	Ocimum basilicum oil	8015-73-4	NA	NA	Liquid	Lipids	NA
Citronella oil	Cymbopogon nardus oil	8000-29-1	NA	3.53	Liquid	Lipids	NA
Clove Oil	Clove leaf oil Clove stem oil	8000-34-8	NA	NA	Liquid	Lipids	NA
Geranium oil	Geranium maculatum oil	8000-46-2	NA	NA	Liquid	NA	NA
Jasmine absolute	Gardenia jasminoides, ext.	92457-01-7	NA	NA	NA	NA	NA
Lemongrass oil	Citral terpenes; 1,2-dimethoxy-4-prop-2-enylbenzene	8007-02-1	777.21	NA	Liquid	NA	
Litsea cubeb oil	Litsea cubeba	68855-99-2	NA	NA	Liquid	NA	NA
Oakmoss	Oak moss extract, absolute	68917-10-2	NA	NA	NA	NA	NA
Palmarosa oil	Cymbopogon martini oil	8014-19-5	NA	NA	NA	NA	NA
Spearmint oil	Mentha spicata oil	8008-79-5	NA	NA	Liquid	NA	NA
Treemoss	Cedar moss extract	68648-41-9	NA	NA	NA	NA	NA
Ylang Ylang oil	Cananga oil	68606-83-7 8006-81-3	NA	NA	NA	NA	NA

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; g/mol = grams per mole; Kow = octanol-water partition coefficient; NA = not available.

[1]Kow represents the octanol-water partition coefficient (expressed on log scale) obtained from the website: http://www.syrres.com/esc/est_kowdemo.htm.

[2]Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs, as developed by the National Library of Medicine at: http://www.nlm.nih.gov/mesh/meshhome.html

[3]Chemical structures, based on CASRN, were obtained from ChemID available at: http://chem.sis.nlm.gov/chemidplus/chemidheavy.jsp.

Annex II-7

Natural Complex Substances Tested in the LLNA – Comparative Data

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Substance Name	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	Overall LLNA Result[1]	LLNA Reference	Test Conc. (%)	% Sens. Incidence	Result[1]	Overall Human Result[1]	Human Reference
Basil Oil	Fragrance Ingredient	2.5, 5, 10, 25, 50	3.0, 3.0, 8.0, 17.6, 25.2	6.2	1:3 EtOH/DEP	CBA/Ca	+	+	Lalko & Api (2006) submitted by RIFM	4	0	-	-	Opdyke (1973a)
Citronella Oil	Fragrance Ingredient	2.5, 5, 10, 25, 50	1.4, 0.9, 1.2, 1.2, 2.7	NC	1:3 EtOH/DEP	CBA/Ca	-	-	Lalko & Api (2006) submitted by RIFM	8	0	-	-	Opdyke (1973b)
										8	0	-		
										8	0	-		
Clove Oil	Fragrance Ingredient	1.0, 2.5, 5, 10, 25	1.1, 1.8, 2.5, 3.7, 5.9	7.1	1:3 EtOH/DEP	CBA/Ca	+	+	Lalko & Api (2006) submitted by RIFM	5	0	-	-	Opdyke (1975a)
		2.5, 5, 10, 25, 50	1.6, 1.5, 4.0, 9.5, 11.4	7.1	1:3 EtOH/DEP	CBA/Ca	+		Lalko & Api (2006) submitted by RIFM	5	0	-		Opdyke (1978a)
		1.0, 2.5, 5, 10, 25	1.6, 1.7, 2.2, 4.2, 8.9	7.0	1:3 EtOH/DEP	CBA/Ca	+		Lalko & Api (2006) submitted by RIFM	10	0	-		Opdyke (1975b)
Geranium Oil	Fragrance Ingredien	2.5, 5, 10,	1.2, 0.7, 1.7,	NC	1:3 EtOH/DEP	CBA/Ca	-	-	Lalko & Api (2006) submitted by	10	0	-	-	Opdyke (1975c)

Substance Name	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	Overall LLNA Result[1]	LLNA Reference	Test Conc. (%)	% Sens. Incidence	Result[1]	Overall Human Result[1]	Human Reference
	t	25, 50	1.8, 2.8						RIFM					
Jasmine Absolute	Fragrance Ingredient	1.0, 2.5, 5, 10, 25	1.2, 1.8, 2.0, 7.4, 11.8	5.9	1:3 EtOH/DEP	CBA/Ca	+	+	Lalko & Api (2006) submitted by RIFM	3	8	+[2]	+	Opdyke (1976c)
		10, 25, 50, 75, 100	1.7, 2.5, 3.6, 1.8, 16.2	36.4	1:3 EtOH/DEP	CBA/Ca	+		Lalko & Api (2006) submitted by RIFM	3	0	-		
Lemongrass Oil	Fragrance Ingredient	2.5, 5, 10, 25, 50	0.9, 2.1, 5.1, 10.3, 13.1	6.5	1:3 EtOH/DEP	CBA/Ca	+	+	Lalko & Api (2006) submitted by RIFM	4	0	-	-	Opdyke (1976e)
										4	0	-		Opdyke (1976d)
Litsea cubeb Oil	Fragrance Ingredient	2.5, 5, 10, 25, 50	2.0, 2.3, 3.3, 7.9, 16.0	8.4	1:3 EtOH/DEP	CBA/Ca	+	+	Lalko & Api (2006) submitted by RIFM	8	0	-	-	Opdyke (1982)
Oakmoss	Fragrance Ingredient	NA	NA	3.9	1:3 EtOH/DEP	CBA/Ca	+	+	Lalko & Api (2006) submitted by RIFM	10	0	-	+	Opdyke (1976a)

Substance Name	Formulation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	Overall LLNA Result[1]	LLNA Reference	Test Conc. (%)	% Sens. Incidence	Result[1]	Overall Human Result[1]	Human Reference
Palmarosa Oil	Fragrance Ingredient	2.5, 5, 10, 25, 50	1.1, 2.1, 3.1, 3.6, 5.0	9.6	1:3 EtOH/DEP	CBA/Ca	+	+	Lalko & Api (2006) submitted by RIFM	NA	NA	NA	-	Lalko & Api (2006) submitted by RIFM
Spearmint Oil	Fragrance Ingredient	0.5, 1.0, 2.5, 5, 10	1.2, 1.1, 1.2, 1.9, 3.6	8.2	1:3 EtOH/DEP	CBA/Ca	+	+	Lalko & Api (2006) submitted by RIFM	4	0	-	-	Opdyke (1978b)
Treemoss	Fragrance Ingredient	NA	NA	NC	1:3 EtOH/DEP	CBA/Ca	-	-	Lalko & Api (2006) submitted by RIFM	NA	NA	NA	+	RIFM, submitted by AM Api
Ylang Ylang Oil	Fragrance Ingredient	0.5, 1.0, 2.5, 5, 10	1.5. 1.7, 2.1, 2.6, 2.6	NC	1:3 EtOH/DEP	CBA/Ca	-	+	Lalko & Api (2006) submitted by RIFM	10	0	-	+	Opdyke (1974)
										10	0	-		
		0.5, 1.0, 2.5, 5, 10	1.5, 1.4, 2.1, 2.5, 3.9	6.8	1:3 EtOH/DEP	CBA/Ca	+			10	5	+		
										10	0	-		
										10	0	-		

Abbreviations: Conc. = concentration; DEP = diethyl phthalate; EtOH = ethanol; HMT = Human Maximization Test; HRIPT = Human Repeat Insult Patch Test; LLNA = Local Lymph Node Assay; NA = not available; NC = not calculated since SI< 3; RIFM = Research Institute for Fragrance Materials; Sens. = sensitization; SI = stimulation index.

[1] "+" = sensitizer; "-" = nonsensitizer

[2] Positive result possibly due to "Spillover effect." "In maximization testing, four unrelated materials are tested on each of 25 human subjects. In the event that one of the four test materials turns out to be a potent sensitizer (in this case it was Costus oil, which sensitized 25/25 subjects), false weak positive results may occur with the other three materials. When these three materials are subsequently retested out of the context of the serious allergen, and in the same or different groups of subjects, they prove to be negative. We refer to this as the ‘spillover effect’" (Opdyke 1976c).

Annex III

Available Data and Information for Metals Tested in the LLNA

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Annex III-1

Physicochemical Properties and Chemical Classes of Metals Tested in the LLNA

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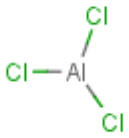
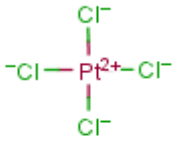
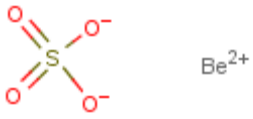
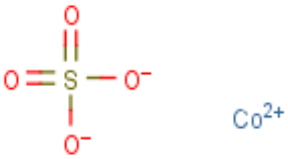
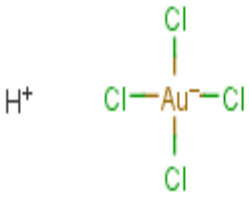
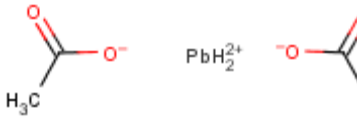
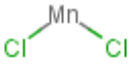
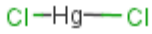
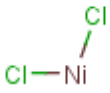
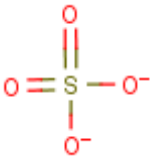
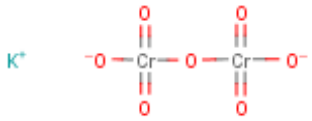
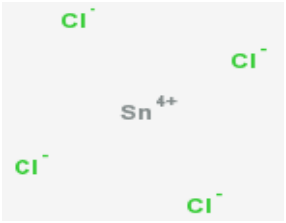
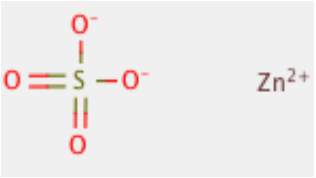
Substance Name	Synonyms	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Aluminum chloride	Aluminum chloride, anhydrous	7446-70-0	NA	NA	Solid	Inorganic Chemicals, Aluminum Compounds, Inorganic Chemicals, Chlorine Compounds	
Ammonium tetrachloro-platinate	***Ammonium platinous chloride; Ammonium chloroplatinate***	***13820-41-2***	***372.97***	***0.47***	***Solid***	***Inorganic Chemicals, Platinum Compounds***	 1.1
Beryllium sulfate	***Beryllium sulfate tetrahydrate***	***7787-56-6***	***177.14***	***NA***	***Solid***	***Inorganic Chemicals, Metals, Salts***	
Cobalt chloride	***Cobaltous chloride***	***7646-79-9***	***129.84***	***0.85***	***Solid***	***Inorganic Chemicals, Metals, Salts***	
Cobalt (II) salts	NA	NA	NA	NA	Solid	Inorganic Chemicals, Metals, 1.2 Salts	1.3 NA
Cobalt sulfate	Cobaltous sulfate	10124-43-3	154.99	0.63	Solid	Inorganic Chemicals, 1.4 Metals, Salts	

Substance Name	Synonyms	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Copper chloride	*Cuprous chloride*	*7758-89-6*	*98.99*	*-0.26*	*NA*	*Inorganic Chemicals, Metals, Salts*	
Gold chloride	*Gold tetrachloride*	*16903-35-8*	*339.79*	*0.16*	*Solid*	*Inorganic Chemicals, Gold Compounds, Salts*	
Lead acetate	*Acetic acid, lead salt*	*15347-57-6*	*325.29*	*-0.08*	*Solid*	*Inorganic Chemicals, 1.5 Metals, Salts*	
Manganese chloride	Manganese chloride, anhydrous	7773-01-5	125.84	0.85	Solid	Inorganic Chemicals, Manganese Compounds, Salts	
Mercuric chloride	*Mercuric (II) chloride*	*7487-94-7*	*271.5*	*0.15*	*Solid*	*Inorganic Chemicals, Mercury Compounds, Salts*	
Nickel chloride	*Nickelous chloride*	*7718-54-9*	*129.6*	*0.05*	*Solid*	*Inorganic Chemicals, Metals, Salts*	
Nickel sulfate	*Nickel (II) sulfate*	*7786-81-4*	*154.76*	*-0.17*	*Solid*	*Inorganic Chemicals, Metals, 1.6 Salts*	 *1.7*

Substance Name	Synonyms	CASRN	Mol. Wt. (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Potassium dichromate	***PDC***	***7778-50-9***	***294.18***	***-2.24***	***Solid***	***Inorganic Chemicals, Chromium Compounds, Inorganic Chemicals Potassium Compounds***	
Tin chloride	NA	1344-13-14	260.52	NA	Solid	Inorganic Chemicals, Tin Compounds, Salts	
Zinc sulfate	***Sulfuric acid, zinc salt; Zinc sulphate***	***7733-02-0***	***NA***	***NA***	***Solid***	Inorganic Chemicals, Zinc Compounds, Salts	

Bold, italicized text represents the 11 metals reported in the original LLNA Evaluation Report (ICCVAM 1999).

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; g/mol = grams per mole; Kow = octanol-water partition coefficient; NA = not available.

[1] Kow represents the octanol-water partition coefficient (expressed on log scale) obtained from the website: http://www.syrres.com/esc/est_kowdemo.htm..

[2] Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs, as developed by the National Library of Medicine at: http://www.nlm.nih.gov/mesh/meshhome.html.

[3] Chemical structures, based on CASRN, were obtained from ChemID available at: http://chem.sis.nlm.nih.gov/chemidplus/chemidheavy.jsp.

Annex III-2

Metals Tested in the LLNA – Comparative Data

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Substance Name	CASRN	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	Vehicle	LLNA[1] Result	Overall LLNA Result[1,2]	Overall LLNA Result[1,2,3] (Aqueous Metals)	Overall LLNA Result[1,2,3] (Nonaqueous Metals)	LLNA References	Guinea Pig Studies Outcome[1] (GPMT/ BT)	Guinea Pig References	Human Outcome[1]	Human References
Aluminum $_c$ hloride	7446-70-0	5, 10, 25	0.8, 0.8, 0.7	NC	Petrolatum	-	-	NA	-	Basketter et al. (1999a)	NA	NT	-	Basketter et al. (1999a)
Ammonium tetrachloroplatinate [4]	13820-41-2	2.5, 5, 10	16, 15.4, 18.1	IDR	DMSO	+	+	NA	+	Basketter and Scholes (1992); Basketter et al. (1999a,b)	+	Basketter and Scholes (1992); Basketter et al. (1999a)	+[7]	Basketter et al. (1999a,b)
Beryllium sulfate	7787-56-6	NA	NA	0.03	NA	+	+	NA	+	Basketter et al. (1994); Mandervelt et al. (1997); Basketter et al.	+	Basketter et al. (1999a)	+[8,9]	Basketter et al. (1994); Kligman (1966); Basketter et
		2.5, 5, 10	8.4, 7.1, 9.4	IDR	DMF	+								
Cobalt chloride	7646-79-9	0.5, 1.0, 2.5	3.2, 2.7, 2.8	0.4	NA	+	+	NA	NA	Basketter and Scholes (1992); Basketter et al. (1994); Basketter et al. (1999b)	+	Basketter and Scholes (1992)	+[7,8]	Basketter et al. (1999a,b)
Cobalt (II) salts	7440-48-4	NA	NA	NA	DMSO	+	+	NA	+	Ikarashi et al. (1992); Griem et al. (2003); Mandervelt et al. (1997); Schneider and Akkan (2004)	NA	NT	+[8]	Kligman (1966); Griem et al. (2003); Schneider and Akkan (2004)
Cobalt sulfate	10124-43-3	NA	NA	NA	NA	+	+	NA	NA	NP	NA	NT	+[9]	Kligman (1966)
Copper chloride	7758-89-6	1, 2.5, 5	8.1, 13.8, 13.6	0.4	DMSO	+	+	NA	+	Basketter and Scholes (1992); Basketter et al. (1999a); ICCVAM (1999)	-	Basketter and Scholes (1992); ICCVAM (1999)	-	Basketter et al. (1999a,b)
		NA	NA	NA	DMSO	+								
Gold chloride	16903-35-8	NA	NA	0.31	DMSO	+	+	NA	+	Basketter et al. (1999a); Schneider and Akkan (2004)	NA	NT	+[8,9]	Kligman (1966); Basketter et al. (1999a,b); Schneider and Akkan (2004)
		5, 10, 25	21.8, 10.9, 17.9	IDR	DMSO	+								
Lead acetate	15347-57-6	2.5, 5, 10	0.7, 0.8, 1	NC	DMSO	-	-	NA	-	Basketter et al. (1999b); ICCVAM (1999)	NA	NT	-	Basketter et al. (1999a,b)
		NA	NA	NA	NA	-								
Manganese chloride	1/5/7773	5, 10, 25	1.10, 0.60, 1.00	NC	Petrolatum	-	-	NA	-	Basketter et al. (1999a)	NA	NT	-	Basketter et al. (1999a,b)
Mercuric (II) chloride	7484-94-7	5, 10	19.9, 11.8	0.39	AOO	+	+	NA	+	Basketter et al. (1994); Basketter et al. (1999a); Schneider and Akkan (2004)	+	Magnusson and Kligman (1969); Basketter et al. (1999a)	+[7,8,9]	Kligman (1966); Marzulli and Maibach (1974); Magnusson and Kligman (1969); Basketter et al. (1994); Basketter et al. (1999a,b)
Nickel chloride	7718-54-9	2.5, 5, 10	1.3, 2.6, 6.6	5.5	30% ETOH	+	+	+	-	Basketter and Scholes (1992); Gerberick et al. (1992); Basketter et al. (1999a,b); ICCVAM (1999); Griem et al. (2003)	+	Hicks et al. (1979); Goodwin et al. (1981); Möller (1984); Wahlberg and Boman (1985); Basketter and	+	Vandenberg and Epstein (1963); Goodwin et al. (1981); Menne (1994); Basketter et al. (1999a,b); Griem et al. (2003)
		0.5, 1.0, 2.5	1, 1.7, 2.2	NC	DMSO	-								
		1, 2.5, 5	1.5, 2.2, 2.4	NC	DMSO	-								

Substance Name	CASRN	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	Vehicle	LLNA[1] Result	Overall LLNA Result[1,2]	Overall LLNA Result[1,2,3] (Aqueous Metals)	Overall LLNA Result[1,2,3] (Nonaqueous Metals)	LLNA References	Guinea Pig Studies Outcome[1] (GPMT/ BT)	Guinea Pig References	Human Outcome[1]	Human References
Nickel sulfate	7786-81-4	0.25, 0.5, 1, 2.5	2, 2.4, 2.8, 3	2.5	1% Pluronic L92	+	+	+	-	Basketter and Scholes (1992); Basketter et al. (1994); Basketter et al. (1999a); Ryan et al. (2000, 2002); Griem et al. (2003)	+	Magnusson and Kligman (1969); Bourrinet et al. (1979); Maurer et al. (1979); Wahlberg and Boman (1985); Gad et al. (1986); Basketter and Scholes (1992)	+[7,8]	Magnusson and Kligman (1969); Marzulli and Maibach (1976); Bourrinet et al. (1979); Gad et al. (1986); Basketter et al. (1994); Uter et al. (1995); Basketter et al. (1999a,b); Griem et al. (2003)
		0.25, 0.5, 1, 2.5	0.9, 1.1, 1.6, 1.6	NC	DMF	-								
		0.25, 0.5, 1, 2.5	1.3, 1.4, 1.4, 1.8	4.8	DMSO	+								
		0.5, 1.0, 2.5	1.1, 1.5, 1.5	NC	DMSO	-								
Potassium dichromate	7778-50-9	0.025, 0.05, 0.1, 0.25, 0.5	1.6, 1.4, 3.8, 5.3, 16.1	0.08	DMSO	+	+	+	+	ECPA LLNA Project Report[5]; NTP Study[6]; Kimber et al. (1991); Basketter and Scholes (1992); Basketter et al. (1994); Kimber et al. (1995); Basketter et al. (1999a,b); Ryan et al. (2002); Schneider and Akkan (2004); Basketter and Kimber (2006)	+	Magnusson and Kligman (1969); Goodwin et al. (1981); Gad et al. (1986); Kimber et al. (1991); Basketter and Scholes 1992); Kimber et al. (2003)	+[7,8,9]	Kligman (1966); Magnusson and Kligman (1969); Marzulli and Maibach (1976); Goodwin et al. (1981); Basketter et al. (1994); Basketter et al. (1999a,b); Schneider and Akkan (2004); Basketter and Kimber (2006)
		0.025, 0.05, 0.1, 0.25, 0.5	1.4, 2.5, 9.5, 25.9, 10.1	0.05	DMSO	+								
		0.025, 0.05, 0.1, 0.25	1.21, 1.84, 2.22, 3.39	0.2	DMSO	+								
		0.025, 0.05, 0.1, 0.25, 0.5	1.1, 1.1, 1.4, 4.9, 5.4	0.17	1% Pluronic L92	+								
		0.025, 0.05, 0.1, 0.25, 0.5	2.9, 4.3, 9.1, 15.1, 22.6	0.33	DMF	+								
		0.02, 0.1, 0.5	1.5, 4.5, 15.2	0.06	1% Pluronic L92	+								

Substance Name	CASRN	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	Vehicle	LLNA[1] Result	Overall LLNA Result[1,2]	Overall LLNA Result[1,2,3] (Aqueous Metals)	Overall LLNA Result[1,2,3] (Nonaqueous Metals)	LLNA References	Guinea Pig Studies Outcome[1] (GPMT/ BT)	Guinea Pig References	Human Outcome[1]	Human References
Potassium dichromate (continued)	7778-50-9	0.02, 0.1, 0.5	1.06, 1.04, 5.55	0.3	1% Pluronic L92	+	+	+	+	ECPA LLNA Project Report[5]; NTP Study[6]; Kimber et al. (1991); Basketter and Scholes (1992); Basketter et al. (1994); Kimber et al. (1995); Basketter et al. (1999a,b); Ryan et al. (2002); Schneider and Akkan (2004); Basketter and Kimber (2006)	+	Magnusson and Kligman (1969); Goodwin et al. (1981); Gad et al. (1986); Kimber et al. (1991); Basketter and Scholes 1992); Kimber et al. (2003)	+[7,8,9]	Kligman (1966); Magnusson and Kligman (1969); Marzulli and Maibach (1976); Goodwin et al. (1981); Basketter et al. (1994); Basketter et al. (1999a,b); Schneider and Akkan (2004); Basketter and Kimber (2006)
		0.02, 0.1, 0.5	2.4, 2.9, 7.9	0.11	1% Pluronic L92	+								
		0.02, 0.1, 0.5	1.4, 1.8, 7.8	0.18	1% Pluronic L92	+								
		0.02, 0.1, 0.5	1.7, 1.5, 4.1	0.33	1% Pluronic L92	+								
		0.025, 0.05, 0.1, 0.25, 0.5	1.1, 1.3, 2.3, 5.1, 13.1	0.15	DMSO	+								
		0.1, 0.25, 0.5	3.5, 10.2, 10.4	0.03	DMSO	+								
		NA	NA	0.46	NA	+								
		0.1, 0.25, 0.5	7.9, 22.6, 33.6	0.07	DMSO	+								
		0.1, 0.25, 0.5	1.8, 5.1, 6.9	0.15	DMSO	+								
		0.1, 0.25, 0.5	NA, 8.8, 10.1	0.01	DMSO	+								
		0.1, 0.25, 0.5	2.0, 4.4, 5.4	0.17	DMSO	+								
		0.025, 0.05, 0.1, 0.25, 0.5	1.7, 2.9, 4.5, 10.4, 19.1	0.058	DMSO	+								
		0.025, 0.05, 0.1, 0.25, 0.5	1.2, 2.1, 3.4, 4.5, 11.2	0.132	DMSO	+								
		0.025, 0.05, 0.1, 0.25, 0.5	1.9, 1.7, 2.2, 5.9, 13.0	0.122	DMSO	+								

Substance Name	CASRN	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	Vehicle	LLNA[1] Result	Overall LLNA Result[1,2]	Overall LLNA Result[1,2,3] (Aqueous Metals)	Overall LLNA Result[1,2,3] (Nonaqueous Metals)	LLNA References	Guinea Pig Studies Outcome[1] (GPMT/ BT)	Guinea Pig References	Human Outcome[1]	Human References
		0.025, 0.05, 0.1, 0.25, 0.5	1.6, 1.4, 3.8, 5.3, 16.1	0.126	DMSO	+								
		0.025, 0.05, 0.1, 0.25, 0.5	NA	0.08	NA	+								
Tin chloride	NA	5, 10, 25	4.1, 6.5, 6.3	3.6	AOO	+	+	NA	+	Basketter et al. (1999b)	NA	NT	+	Basketter et al. (1999a,b)
Zinc sulfate	7730-02-0	5, 10, 25	1.3, 2, 2.3	NC	DMSO	-	+	NA	-	Basketter et al. (1999a); ICCVAM (1999)	NA	NT	-	Basketter et al. (1999a,b)
		NA	NA	NA	NA	+								

[1] (+) = sensitizer; (-) = nonsensitizer

Annex IV

Available Data and Information for Substances in Aqueous Solutions Tested in the LLNA

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Annex IV-1

Physicochemical Properties and Chemical Classes of Substances Tested in Aqueous Solutions in the LLNA

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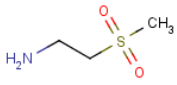
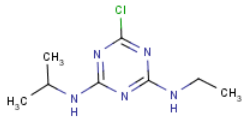
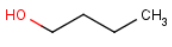
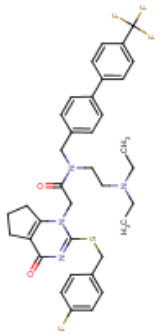
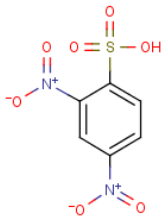
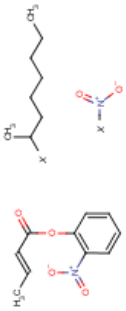
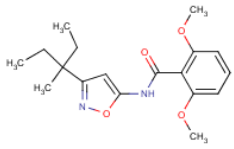
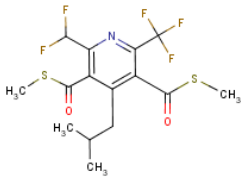
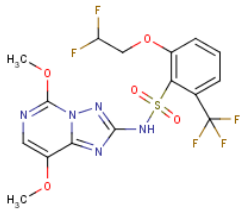
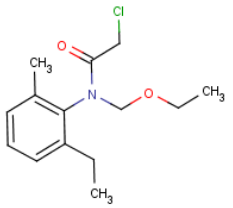
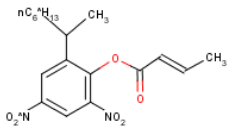
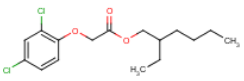
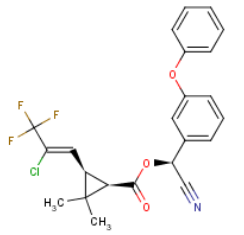
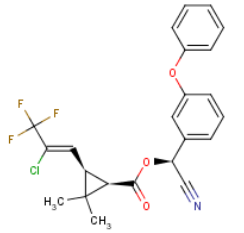
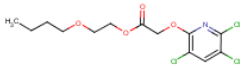
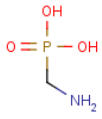
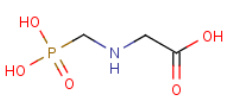
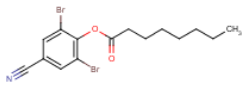
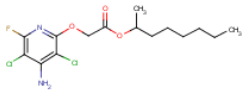
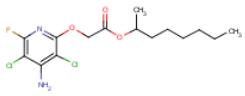
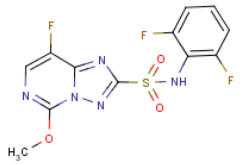
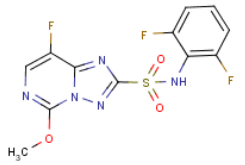
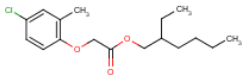
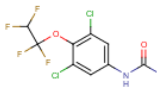
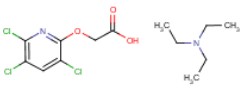
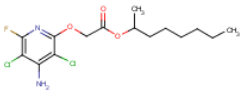
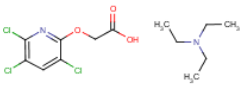
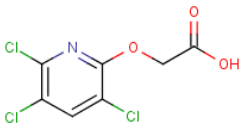
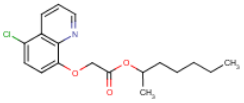
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
AE F016382 00 TK71 A101	NA	NA	NA	NA	NA	Formulation	NA
A SC600	NA	NA	NA	NA	NA	Formulation	NA
2-Aminoethyl-methylsulfone	Ethanamine, 2-(methylsulfonyl)-	49773-20-8	159.63	NA	Solid	Sulfur Compounds	
Atrazine	Atrizine SC 1-Chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine	1912-24-9	215.68	2.82	Solid	Heterocyclic Compounds	
BASF #1	NA	NA	NA	NA	Emulsion	NA	NA
BASF #2	NA	NA	NA	NA	Emulsion	NA	NA
BASF #4	NA	NA	NA	NA	Emulsion	NA	NA
6.0 BASF #5	NA	7.0 NA	8.0	9.0	Suspension	NA	10.0 NA
11.0 BASF #6	BAS 493 05 F	12.0 NA	13.0	14.0	Dispersion	NA	15.0 NA
16.0 BASF SC-1	Suspension concentrate 1	NA	NA	NA	Emulsion	NA	NA
BASF SE-1	Suspo-emulsion 1	NA	NA	NA	Emulsion	NA	NA
1-Butanol	n-Butyl alcohol	71-36-3	74.12	1.06	Liquid	Alcohols; Lipids	
D EC25	NA	NA	NA	NA	NA	Formulation	NA
D EW 15	NA	NA	NA	NA	NA	Formulation	NA

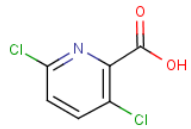
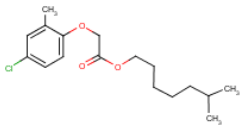
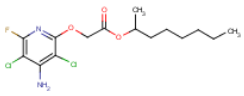
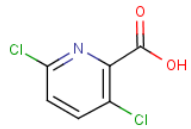
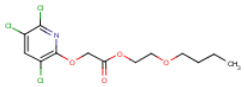
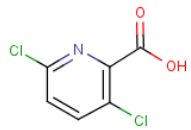
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
n-[2-(diethylamino)ethyl]-2-[[(4-fluorophenyl)-methyl]thio]-4,5,6,7-tetrahydro-4-oxo-n-[[4'-(trifluoromethyl)-[1,1'-biphenyl]-4-yl]methyl]-1h-cyclopentapyrim-idine-1-acetamide	Darapladib	356057-34-6	666.78	NA	Solid	Pharmaceutical Intermediate	
1,4-Dihydroquinone	Hydroquinone p-hydroquinone	123-31-9	110.11	1.17	Solid	Phenols	
2,4-Dinitrobenzene sulfonic acid	2,4-Dinitrophenyl-sulfonic acid	89-02-1	248.17	-1.53	Solid	Hydrocarbons, Cyclic	
Dinocap	Butenoic acid, 2-(or 4)-isooctyl-4,6(or 2,6)-dinitrophenyl ester(9CI) Crotonic acid, 2(or 4)-(1-methylheptyl)-4,6(or 2,6)-dinitrophenylester	39300-45-3	364.39	5.76	Liquid	Nitro Compounds; Hydrocarbons, Cyclic	
EXP 10810 A	NA	NA	NA	NA	NA	Formulation	NA
EXP 11120 A	NA	NA	NA	NA	NA	Formulation	NA
FAR01042-00	NA	NA	NA	NA	NA	Formulation	NA
FAR01060-00	NA	NA	NA	NA	NA	Formulation	NA
F & Fo WG 50 + 25	NA	NA	NA	NA	NA	Formulation	NA

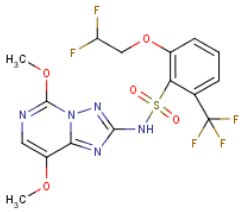
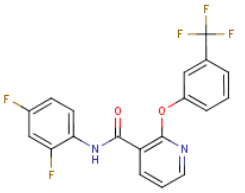
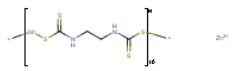
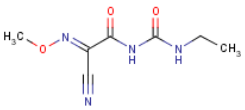
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formaldehyde	Formalin	50-00-0	30.03	0.33	Liquid	Aldehydes	
Formulation 1	Isoxaben	82558-50-7	332.40	NA	Liquid	Formulation	
Formulation 10	22.9% w/w dithiopyr	97886-45-8	401.42	NA	Liquid	Formulation	
Formulation 11	0.31 wt.% penoxsulam, 84.2 wt.% acetochlor	219714-96-2 34256-82-1	483.37 269.77	NA	Liquid	Formulation	 
Formulation 12	34.7% w/w 2,4-dinitro-6-(1-methylheptyl)-phenyl crotonate DE-126	6119-92-2	364.40	NA	Liquid	Formulation	

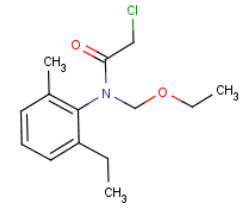
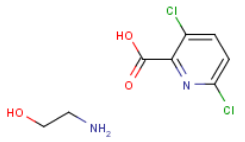
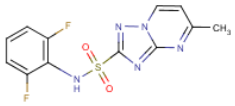
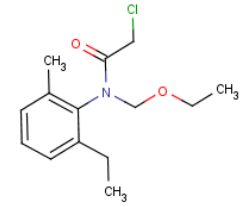
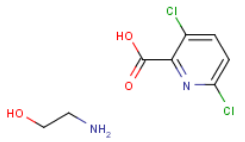
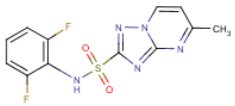
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 13	87.6% w/w 2,4-dichlorophenoxy-acetic acid 2-ethylhexyl ester 2,4-D-2-ethylhexyl	1928-43-4	333.25	NA	Liquid	Formulation	
Formulation 14	1.5 wt.% gamma-cyhalothrin Nexide Fentrol	76703-62-3	449.85	NA	Liquid	Formulation	
Formulation 15	5.8 wt.% gamma-cyhalothrin Nexide Fentrol	76703-62-3	449.85	NA	Liquid	Formulation	
Formulation 16	85.3% w/w triclopyr butoxyethyl ester	64470-88-8	356.63	NA	Liquid	Formulation	
Formulation 17	50.8% wt/wt glyphosate dimethyl-ammonium salt (active ingredient) 40.1% wt/wt glyphosate (acid equivalent) 8.3% w/w Geronol CF/AS 30 (ammonium adjuvant)	1066-51-9 1071-83-6	111.04 169.02	NA	Liquid	Formulation	 

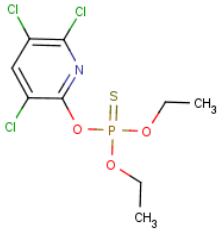
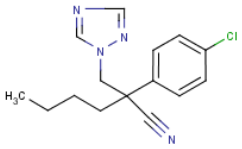
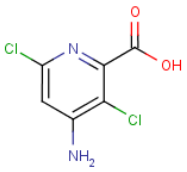
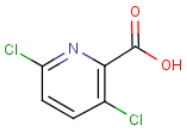
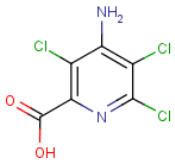
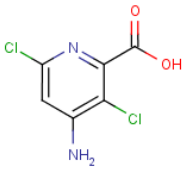
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 19	37.1 wt.% bromoxynil octanoate 9.23 wt.% fluroxypyr-1-methylheptyl	1689-99-2 81406-37-3	403.11 367.25	NA	Liquid	Formulation	 
Formulation 2	14.2% w/w fluroxypyr - meptyl 0.22% w/w florasulam	81406-37-3 145701-23-1	367.25 359.29	NA	Liquid	Formulation	 
Formulation 20	0.39 wt.% Florasulam 41.9 wt.% 2-methyl-4-chlorophenoxy-acetic acid 2-ethylhexyl ester (MCPA, 2-ethyl hexyl ester)	145701-23-1 29450-45-1	359.29 312.84	NA	Liquid	Formulation	 

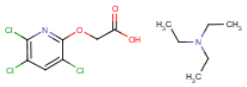
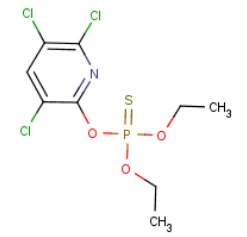
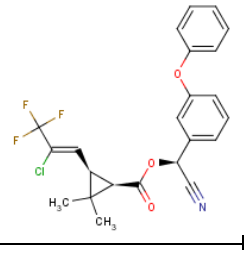
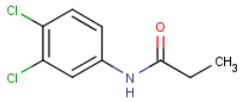
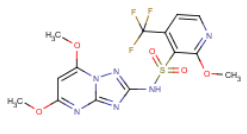
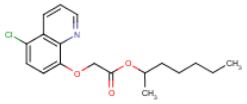
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 21	50.4% hexaflumuron N-(((3,5-dichloro-4-(1,1,2,2-tetrafluoroethoxy)-phenyl)amino)-carbonyl)-2,6-difluoro benzamide	86479-06-3	461.14	NA	Liquid	Formulation	 16.1
Formulation 22	8.3 wt.% triclopyr triethyl-ammonium 2.8 wt.% fluroxypyr-methyl heptyl ester	57213-69-1 81406-37-3	357.66 367.25	NA	Liquid	Formulation	 
Formulation 23	16.1 wt.% triclopyr - triethylammonium 11.6 wt.% triclopyr acid	57213-69-1 55335-06-3	357.66	NA	Liquid	Formulation	 
Formulation 24	8.8 wt.% cloquintocet-mexyl	99607-70-2	335.83	NA	Liquid	Formulation	

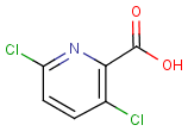
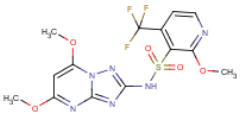
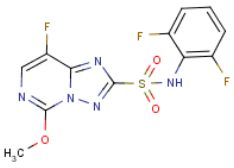
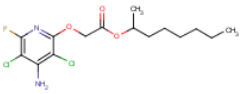
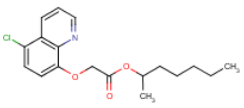
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 25	2.2 wt.% clopyralid 37.7 wt.% MCPA-2-ethyl-hexyl ester 8.2 wt.% fluroxypyr - meptyl	1702-17-62 6544-20-7 81406-37-3	192.00 312.84 367.25	NA	Liquid	Formulation	  
Formulation 26	5.9 wt.% clopyralid 32.9 wt.% triclopyr-butotyl	1702-17-6 64700-56-7	192.00 356.63	NA	Liquid	Formulation	 
Formulation 27	45.2 wt.% fluroxypyr-meptyl	81406-37-3	192.00	NA	Liquid	Formulation	

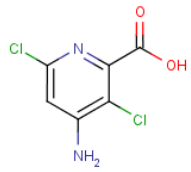
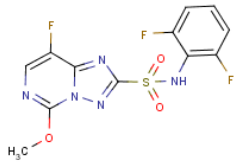
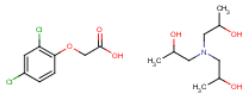
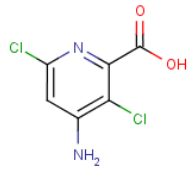
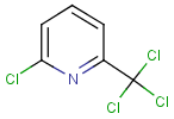
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 28	1.4 wt.% penoxsulam 9.37 wt.% diflufenican	219714-96-2 83164-33-4	483.37 394.30	NA	Liquid	Formulation	 
Formulation 29	35.6% mancozeb, 4.92% cymoxanil	8018-01-7 57966-95-7	541.1 198.18	NA	Liquid	Formulation	 

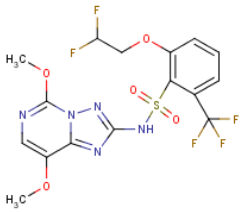
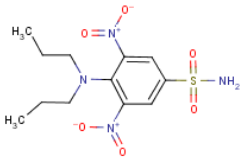
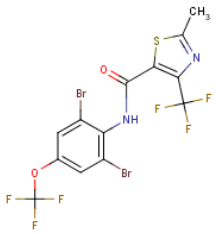
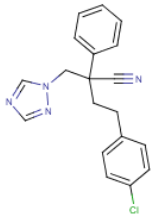
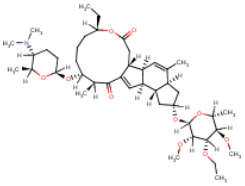
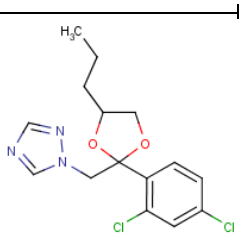
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 3	455 g/L acetochlor 47 g/L clopyralid-olamine 14 g/L flumetsulam	34256-82-1 57754-85-5 98967-40-9	269.77 253.08 325.30	NA	Liquid	Formulation	  
Formulation 30	455 g/L acetochlor 47 g/L clopyralid-olamine 14 g/L flumetsulam	34256-82-1 57754-85-5 98967-40-9	269.77 253.08 325.30	NA	Liquid	Formulation	  

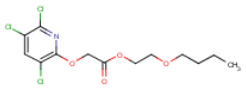
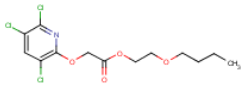
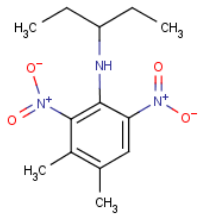
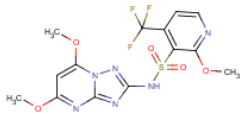
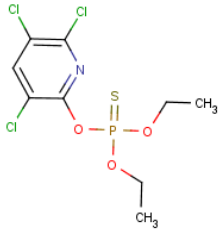
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 31	18.7 wt.% chlorpyrifos	2921-88-2	350.59	NA	Liquid	Formulation	
Formulation 32	11.2 wt.% ((E)-2-(1-methylheptyl) -4,6-dinitrophenyl ester-2-butenoic acid 4.68% wt/wt myclobutanil	88671-89-0	288.78	NA	Liquid/ Solid	Formulation	
Formulation 33	4.5 wt.% aminopyralid-olamine 27.1 wt.% clopyralid-olamine 8.7 wt.% picloram-olamine 3.5 wt.% aminopyralid 20.6 wt.% clopyralid 7.0 wt.% picloram	150114-71-9 1702-17-6 1918-02-1	207.02 192.00 241.46	NA	Liquid	Formulation	  
Formulation 34	3.0 wt.% aminopyralid	150114-71-9		NA	Liquid	Formulation	

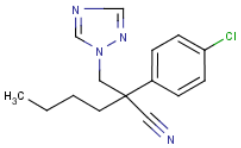
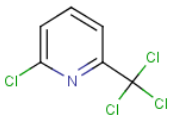
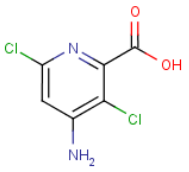
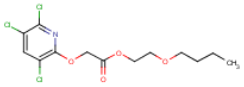
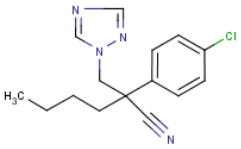
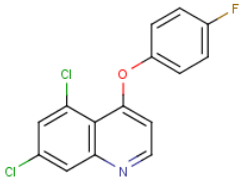
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 35	2.15 wt.% aminopyralid-triisopropanol-ammonium 16.0 wt.% triclopyr-triethylammonium	566191-89-7 57213-69-1	NA 357.66	NA	Liquid	Formulation	NA 
Formulation 37	30.6 wt.% chlorpyrifos 0.54 wt.% gamma-cyhalothrin	2921-88-2 76703-62-3	350.60 449.85	NA	Liquid	Formulation	 
Formulation 38	44.4 wt.% propanil	709-98-8	218.08	NA	Liquid	Formulation	
Formulation 39	4.2 wt.% pyroxsulam 8.7 wt.% cloquintocetmexyl	422556-08-9 99607-70-2	434.35 335.83	NA	Liquid	Formulation	 

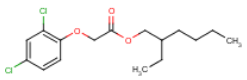
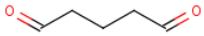
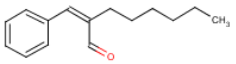
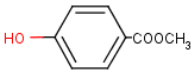
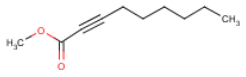
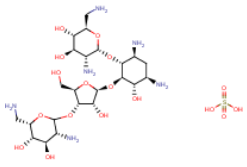
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 4	100 g/L clopyralid mono-ethanolamine salt)	1702-17-6	192.00	NA	Liquid	Formulation	
Formulation 40	1.2 wt.% pyroxsulam 0.21 wt.% florasulam 11.8 wt.% fluroxypyr-meptyl 3.6 wt.% cloquintocetmexyl	422556-08-9 145701-23-1 81406-37-3 99607-70-2	434.35 359.29 367.25 335.83	NA	Liquid	Formulation	   

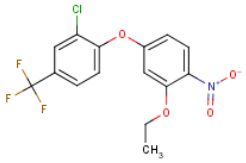
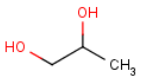
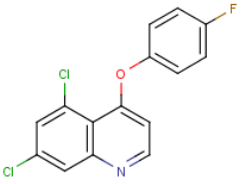
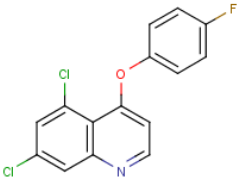
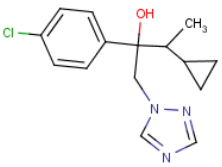
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 41	1.10 wt.% aminopyralid potassium salt 0.47 wt.% florasulam	150114-71-9 145701-23-1	207.02 359.29	NA	Liquid	Formulation	 
Formulation 42	31 wt.% 2,4-D-triisoproanolamine 1.52 wt.% aminopyralid triisopropanol-ammonium	18584-79-7 150114-71-9	412.31 207.2	NA	NA	Formulation	 
Formulation 43	17.9 wt.% nitrapyrin	1929-82-4	230.91	NA	NA	Formulation	

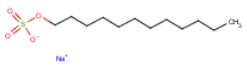
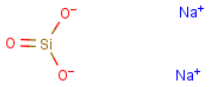
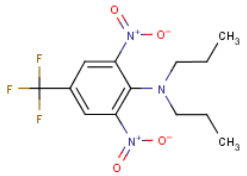
Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 44	0.12 wt.% penoxsulam 40.38 wt.% oryzalin	219714-96-2 19044-88-3	483.37 346.36	NA	NA	Formulation	 
Formulation 45	7.53 wt.% thifluzamide 9.42 wt.% fenbuconazole	130000-40-7 114369-43-6	528.06 336.82	NA	NA	Formulation	 
Formulation 46	5.87 wt.% spinetoram	187166-15-0	760.02	NA	NA	Formulation	
Formulation 47	14.56 wt.% propiconazole	60207-90-1	342.22	NA	NA	Formulation	

Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 49	23.7 wt.% triclopyr BEE	64700-56-7	356.63		Liquid	Formulation	
Formulation 5	3,5,6-trichloro-2-pyridyloxyacetic acid, butoxy ethyl ester Triclopyr-butotyl triclopyr BEE	64700-56-7	356.63		Liquid	Formulation	
Formulation 50	Glyphosate dimethylamine salt Glyphosate dimethyl-ammonium salt	34494-04-7 NA	NA	NA	Liquid	Formulation	NA
Formulation 51	29.6 wt.% pendimethalin 0.51 wt.% pyroxsulam	40487-42-1 422556-08-9	281.31 434.35		Liquid	Formulation	 
Formulation 53	41.1 wt.% chlorpyrifos	2921-88-2	350.60	NA	Liquid	Formulation	

Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 54	t.% glyphosate l-ammonium salt	NA	NA	NA	Liquid	Formulation	NA
Formulation 55	4.6 wt.% myclobutanil	88671-89-0	288.78	NA	Liquid	Formulation	
Formulation 56	20.5 wt.% nitrapyrin	1929-82-4	230.91	NA	Liquid	Formulation	
Formulation 6	Aminopyralid potassium + triclopyr-butotyl form Aminopyralid herbicide	150114-71-9 64700-56-7	207.02	NA	Liquid	Formulation	 
Formulation 7	45 g/L myclobutanil + 45 g/L quinoxyfen)	88671-89-0 124495-18-7	288.78 308.14	NA	Liquid	Formulation	 

Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Formulation 8	81.8% w/w 2,4-dichlorophenoxy-acetic acid 2-ethylhexyl ester 2,4-D EHE	1928-43-4	333.25	NA	Liquid	Formulation	
Formulation 9	NA	NA	NA	NA	Liquid	Formulation	NA
Fx + Me EW 69	NA	NA	NA	NA	NA	Formulation	NA
Glutaraldehyde	Glutaral	111-30-8	100.12	NA		Aldehydes	
Hexyl cinnamic aldehyde	HCA, alpha-hexylcinnamic aldehyde, alpha-hexyl cinnamaldehyde	101-86-0	216.32	3.77	Liquid	Aldehydes	
Methyl 4-hydroxybenzoate	Methylparaben	99-76-3	152.15	1.28	Solid	Carboxylic Acids	
Methyl 2-nonynoate	Methyl octine carbonate	111-80-8	168.24	2.15	Liquid	Lipids	
Neomycin sulfate	Neomycin, sulfate (salt)	1405-10-3	908.88	NA	Solid	Carbohydrates	

Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Oxyfluorfen	Oxirane, mono; ((C12-14-alkyloxy) methyl) derivatives	42874-03-3	361.70	5.21	Solid	Ethers	
Pluronic L92	NA	NA	NA	NA	NA	NA	NA
Propylene glycol	1,2- dihydroxypropane 1,2-propanediol	57-55-6	76.10	0.43	Liquid	Alcohols	
Quinoxyfen	5,7-dichloro-4-(4-fluorophenoxy)-quinoline	124495-18-7	308.14	5.69	Liquid	Heterocyclic Compounds	
Quinoxyfen/ Cyproconazole	5,7-dichloro-4-(4-fluorophenoxy) quinoline/ H-1,2,4-triazole-1-ethanol, alpha-(4-chlorophenyl)-alpha-(1-cyclopropylethyl)-	124495-18-7 113096-99-4	308.14 291.78	5.69 3.25	Liquid	Heterocyclic Compounds	 
Saturated diglycerin	NA	NA	NA	NA	NA	NA	NA

Substance Name	Synonyms	CASRN	Molecular Weight (g/mol)	Log Kow[1]	Physical Form	Chemical Class[2]	Structure[3]
Sodium lauryl sulfate	Sodium dodecyl sulfate, SLS, SDS, irium	151-21-3	288.38	1.87	Solid	Alcohols, Sulfur Compounds, Lipids	
Sodium metasilicate	Silicic acid, disodium salt	6834-92-0	122.063	NA	Solid	Inorganic Chemical, Sodium Compounds, Inorganic Chemical, Silicon Compounds	
Trifluralin	2,6-dinitro-4-trifluormethyl-N,N-dipropylanilin	16.2 1582-09-8	16.3	16.4	NA	Hydrocarbons, Cyclic, Amine	

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; g/mol = grams per mole; Kow = octanol-water partition coefficient; NA = not available.

[1] Kow represents the octanol-water partition coefficient (expressed on log scale) obtained from the website: http://www.syrres.com/esc/est_kowdemo.htm.

[2] Chemical classifications based on the Medical Subject Headings classification for chemicals and drugs, as developed by the National Library of Medicine at: http://www.nlm.nih.gov/mesh/meshhome.html.

[3] Chemical structures, based on CASRN, were obtained from ChemID, available at: http://chem.sis.nlm.gov/chemidplus/chemidheavy.jsp.

Annex IV-2

Substances in Aqueous Solutions Tested in the LLNA – Comparative Data

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Abbreviations

ACE	Acetone
AL	Any other liquid
AOO	Acetone olive-oil (4:1)
BT	Buehler Test
Conc.	Concentration
CS	Capsule suspension
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
EC	Emulsion concentrate
ECPA	European Crop Protection Association
EW	Emulsion, oil in water
GPMT	Guinea Pig Maximization Test
LLNA	Local Lymph Node Assay
ME	Micro-emulsion
NA	Not available
NC	Not calculated
NT	Not tested
OD	Oil dispersion
PG	Propylene glycol
SC	Suspension concentrate
SE	Suspo-emulsion
SI	Stimulation index
SL	Soluble concentrate
TK	Technical concentrate
WG	Water dispersible granules

Substance Name	CASRN	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	GP Call[2]	GP Test	GP Reference	Human Call	Human References
A SC600		NA	10, 25, 50, 100	1.4, 1.8, 2.3, 1.6	NC	1% L92	CBA/J	-	Bayer Crop Science, submitted by E. Debruyne	-	-	BT	Bayer Crop Science, submitted by E. Debruyne	NA	NT
AE F016382 00 TK71 A101		NA	3.6, 7.1, 17.9, 35.7	1.0, 0.8, 1.0, 1.1	NC	1% L92	CBA/J	-	Bayer Crop Science, submitted by E. Debruyne	-	-	BT	Bayer Crop Science, submitted by E. Debruyne	NA	NT
2-aminoethyl-methylsulfone	49773-20-8		10, 25, 50	0.4, 0.3, 0.3	NC	0.5% Tween 80/ H2O		-	GSK[3]	-	NA		NT	NA	NT
Atrazine	1912-24-9	SC	12.5, 25, 50, 75, 100	1.8, 2.8, 3.6, 7.1, 7.3	31.3	1% L92	CBA/J	+	ECPA LLNA Project Report submitted by Dow Chemical	+	-	GPMT	NA	NA	NT
			7, 33, 100	0.8, 2.9, 3.7	41.4	1% L92	CBA/J	+	ECPA LLNA Project Report submitted by Dow Chemical						
BASF #1		NA	10, 30, 70	2.0, 2.9, 4.9	31.2	1% L92	CBA/Ca	+	BASF, submitted by C. Hastings	+	NA	NA	NA	NA	NT
BASF #2		NA	3, 10, 30	0.8, 1.0, 3.0	29.7	1% L92	CBA/J	+	BASF, submitted by C. Hastings	+	NA	NA	NA	NA	NT
BASF #4		NA	3, 10, 50	2.4, 2.7, 5.4	14.1	1% L92	CBA/Ca	+	BASF, submitted by C. Hastings	+	NA	NA	NA	NA	NT
BASF #5		NA	3, 10, 50	1.6, 1.2, 3.9	36.9	1% L92	CBA/Ca	+	BASF, submitted by C. Hastings	+	NA	NA	NA	NA	NT
BASF #6		NA	3, 10, 30	2.7, 9.9, 23.1	3.3	1% L92	CBA/Ca	+	BASF, submitted by C. Hastings	+	NA	NA	NA	NA	NT
BASF SC-1		SC	3, 10, 30	0.8, 1.3, 1.9	NC	1% L92	CBA/Ca	-	BASF, submitted by C. Hastings	-	-	BT	NA	NA	NT
BASF SE-1		SE	10, 30, 70	8.0, 17.3, 22.7	5.5	1% L92	CBA/Ca	+	BASF, submitted by C. Hastings	+	-	BT	NA	NA	NT

Substance Name	CASRN	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	GP Call[2]	GP Test	GP Reference	Human Call	Human References
1-butanol	71-36-3		5, 10, 20	1.6, 1.2, 1.4	NC	H2O		-	Ryan et al. (2000); Gerberick et al. (2005)	-	NA	NA	NT	-	Ryan et al. (2000)
D EC25®		EC	0.5, 1.0, 2.5	0.6, 0.6, 0.6	NC	1% L92	CBA/Ca	-	Bayer Crop Science, submitted by E. Debruyne	-	-	BT	NA	NA	NT
D EW 15		EW	2.5, 5.0, 10.0, 25.0	1.9, 1.5, 2.5, 2.5	NC	1% L92	CBA/J	-	Bayer Crop Science, submitted by E. Debruyne	-	-	BT	NA	NA	NT
n-[2-(diethylamino)ethyl]-2-[[(4-fluorophenyl)-methyl]thio]-4,5,6,7-tetrahydro-4-oxo-n-[[4'-(trifluoromethyl)-[1,1'-biphenyl]-4-yl]methyl]-1h-cyclopentapyrim-idine-1-acetamide	356057-34-6		5, 10, 25	1.1, 2.4, 12.7	10.8	80% ETOH		+	GSK	+	NA	NA	NT	NA	NT
1,4-dihydroquinone	123-31-9		0.05, 0.1, 0.25, 0.5, 1.0	0.7, 1.0, 0.9, 1.9, 1.9	NC	ACE/saline (1:1)		-	Lea et al. (1999)	+	NA	NA	NT	NA	NT
			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, 11.1	1.3	ACE/saline (1:1)		+							

Substance Name	CASRN	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	GP Call[2]	GP Test	GP Reference	Human Call	Human References
2.4-dinitrobenzene sulfonic acid	89-02-1		1, 10, 20	1.7, 1.5, 4.4	15.2	H2O		+	Ryan et al. (2002)	+	NA	NA	NT	NA	NT
			1, 10, 20	0.9, 4.4, 11.6	6.4	1% Pluronic L92/H2O		+							
Dinocap	39300-45-3	EC	0.8, 4, 21	2.2, 25.8, 14.4	0.9	1% L92	CBA/Ca	+	ECPA LLNA Project Report submitted by BASF	+	+	BT	NA	NA	NT
			0.8, 4, 20	1.3, 11.5, 15.6	1.3	1% L92	CBA/J	+	ECPA LLNA Project Report submitted by Bayer						
			0.8, 4, 21	2.0, 4.0, 26.7	1.1	1% L92	CBA/J	+	ECPA LLNA Project Report submitted by Dow Chemical						
			0.8, 4, 10	1.3, 4.1, 10.9	2.8	1% L92	CBA/JHsd	+	ECPA LLNA Project Report submitted by Dupont						
			0.8, 4, 10	2.7, 22.9, 40.5	0.8	1% L92	CBA/ CaOlaHsd	+	ECPA LLNA Project Report submitted by Syngenta/RCC						
EXP 10810 A		NA	10, 25, 50	6.4, 8.4, 9.2	2.1	1% L92	CBA/J	+	Bayer Crop Science, submitted by E. Debruyne	+	+	BT	Bayer Crop Science, submitted by E. Debruyne	NA	NT
EXP 11120 A		NA	10, 25, 50, 100	1.0, 0.7, 1.6, 6.3	64.9	1% L92	CBA/J	+	Bayer Crop Science, submitted by E. Debruyne	+	-	BT	Bayer Crop Science, submitted by E. Debruyne	NA	NT

Substance Name	CASRN	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	GP Call[2]	GP Test	GP Reference	Human Call	Human References
F & Fo WG 50 + 25		WG	2.5, 5.0, 10.0, 25.0	11.7, 12.6, 14.4, 15.2	0.003	1% L92	CBA/J	+	Bayer Crop Science, submitted by E. Debruyne	+	-	BT	Bayer Crop Science, submitted by E. Debruyne	NA	NT
FAR01042-00		NA	10, 25, 50, 100	1.4, 2.1, 1.4, 2.5	NC	1% L92	CBA/J	-	Bayer Crop Science, submitted by E. Debruyne	-	-	BT	Bayer Crop Science, submitted by E. Debruyne	NA	NT
FAR01060-00		NA	10, 25, 50, 100	0.4, 0.8, 1.0, 3.6	88.5	1% L92	CBA/J	+	Bayer Crop Science, submitted by E. Debruyne	+	-	BT	Bayer Crop Science, submitted by E. Debruyne	NA	NT

Substance Name	CASRN	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	GP Call[2]	GP Test	GP Reference	Human Call	Human References
Formaldehyde	50-00-0		1, 10, 20	1.2, 2.5, 3.6	14.5	H2O		+	Ryan et al. (2002)	+	+	GPMT	ECPA LLNA Project Report; Andersen et al. (1984); Wahlberg and Boman (1985)	+	Kligman (1966); Marzulli and Maibach (1974)
			1, 10, 20	2, 4.8, 8.8	4.2	1% Pluronic L92/H2O		+							
			1, 5, 20	1.1, 3.8, 10.6	3.8	1% Pluronic L92/H2O		+	ECPA LLNA Project Report submitted by BASF						
			1, 5, 20	1, 2.2, 6.2	8.2	1% Pluronic L92/H2O		+	ECPA LLNA Project Report submitted by Bayer						
			1, 5, 20	1.6, 2.6, 12	5.6	1% Pluronic L92/H2O		+	ECPA LLNA Project Report submitted by Dow Chemical						
			1, 5, 20	1.1, 2.5, 4.8	8.3	1% Pluronic L92/H2O		+	ECPA LLNA Project Report submitted by Dupont						
			1, 5, 20	0.8, 1.3, 4.8	12.3	1% Pluronic L92/H2O		+	ECPA LLNA Project Report submitted by Syngenta/RCC						
Formulation 1		SC	5, 20, 80	1.1, 1.3, 1.3	NC	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
Formulation 10		EW	2, 10, 50	1, 1, 5.2	29	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 11		OD	0.4, 2, 10	1.2, 1.2, 3.2	9.2	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT

Substance Name	CASRN	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	GP Call[2]	GP Test	GP Reference	Human Call	Human References
Formulation 12		EC	0.2, 1, 5	1.2, 3, 11.6	1	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 13		EC	1, 5, 25	1.2, 1.3, 10.4	8.7	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 14		CS	0.1, 1, 10	0.7, 0.7, 1.3	NC	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
Formulation 15		CS	0.2, 1, 5	0.8, 1.4, 3.2	4.6	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 16		EC	1, 5, 25	1.3, 2.2, 12.3	6.6	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 17		SL	5, 25, 75	1.7, 9.3, 18.5	8.4	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 19		EC	1, 10, 25, 50	4.9, 7.9, 20, 50.5	0.23	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 2		SE	5, 20, 80	2, 3.4, 15.8	15.7	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	-	NA	Submitted by Dow AgroSciences	NA	NT
Formulation 20		SE	2, 10, 50	1.1, 1.4, 3.3	43.7	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 21		TK	5, 25, 100	1.3, 1.2, 1.9	NC	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT

Substance Name	CASRN	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	GP Call[2]	GP Test	GP Reference	Human Call	Human References
Formulation 22		ME	5, 25, 100	1.2, 1.4, 5.8	52.3	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 23		SL	5, 25, 100	0.8, 1, 1	NC	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
Formulation 24		OD	2, 10, 50	1.4, 4.1, 11.7	6.7	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 25		EC	1, 5, 25	1.8, 2.6, 14.7	5.6	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 26		EC	1, 5, 25	1, 1, 4	18	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 27		EC	1, 5, 25	2.3, 2.5, 11.2	6.1	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 28		SC	5, 25, 100	1, 1, 1.1	NC	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
Formulation 29		SC	5, 25, 100	1.8, 1.6, 1.5	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
Formulation 3		SC	5, 20, 80	1, 1.2, 1.7	NC	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	-	NA	Submitted by Dow AgroSciences	NA	NT
Formulation 30		EW	5, 25, 100	1.8, 7.2, 13.6	9.4	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 31		CS	5, 25, 100	1, 1.9, 1.8	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT

Substance Name	CASRN	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	GP Call[2]	GP Test	GP Reference	Human Call	Human References
Formulation 32		EC	5, 25, 100	6.5, 44.7, 69.3	4.3	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 33		SL	5, 25, 100	0.7, 1.4, 1.3	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
Formulation 34		SL	5, 25, 100	1.9, 1.4, 1.5	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
Formulation 35		SL	5, 25, 100	1.1, 1.2, 1.3	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
Formulation 37		EC	1, 5, 15	1.4, 2.7, 7.5	5.6	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 38		EC	5, 25, 100	1.1, 4.6, 12.7	15.9	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 39		OD	1, 5, 25	1.7, 2.5, 3.3	17.5	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 4		SL	5, 20, 80	1.4, 1.1, 1.2	NC	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
Formulation 40		OD	1, 5, 25	1.8, 2.8, 5.7	6.4	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 41		SE	5, 25, 100	1.9, 1.9, 4.7	54.5	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 42		SL	10, 50, 100	1.2, 2.0, 3.1	95.5	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT

Substance Name	CASRN	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	GP Call[2]	GP Test	GP Reference	Human Call	Human References
Formulation 43		CS	5, 25, 75	NA	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
Formulation 44		SC	5, 25, 100	NA	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
Formulation 45		SC	5, 25, 100	NA	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
Formulation 46		SC	5, 25, 100	NA	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	-	NA	Submitted by Dow AgroSciences	NA	NT
Formulation 47		EW	5, 25, 100	2.1, 2.1, 6.0	42.3	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 49		AL	5, 25, 100	0.7, 1.4, 4.7	61.4	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 5		EC	3, 10, 30	1.4, 4, 11.5	7.3	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 50		SL	5, 25, 100	1.2, 1.2, 14.7	35	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 51		OD	5, 25, 100	1.6, 4.5, 2.9	14.7	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 53		EW	2.5, 7.5, 15	1.5, 3.2, 6.7	6.9	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 54		SL	5, 25, 100	1.3, 1.2, 2.3	NC	1% L92	CBA/J	-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT

Substance Name	CASRN	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	GP Call[2]	GP Test	GP Reference	Human Call	Human References
Formulation 55		EW	5, 25, 100	1.5, 2.5, 3.7	56.3	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 56		SL	5, 25, 100	3.3, 6.1, 3.9	4.2	1% L92	CBA/J	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 6		EW	5, 20, 80	1.3, 2.7, 11.6	23.7	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 7		SC	20, 80, 100	1, 1.9, 3.2	96.9	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	-	BT	Submitted by Dow AgroSciences	NA	NT
		SC	5, 20, 80	2.6, 1.4, 3.2	73.3	1% L92	BALB/c	+						NA	NT
Formulation 8		EC	1, 5, 25	0.9, 1.1, 7.3	11.1	1% L92	BALB/c	+	Submitted by Dow AgroSciences	+	NA	NA	NA	NA	NT
Formulation 9		SC	4, 20, 80	1.1, 1.7, 1.3	NC	1% L92	BALB/c	-	Submitted by Dow AgroSciences	-	NA	NA	NA	NA	NT
Fx + Me EW 69		EW	5.0, 10.0, 25.0, 50.0	0.8, 1.6, 3.0, 8.6	25.2	1% L92	CBA/J	+	Bayer Crop Science, submitted by E. Debruyne	+	-	BT	Bayer Crop Science, submitted by E. Debruyne	NA	NT
Glutaraldehyde	111-30-8		3.1, 6.2, 12.5	9.8, 21.4, 22.9	2.1	DMF/H2O (l/l)		+	Gerberick et al. (1992)	+	NA	NA	NT	NA	NT

Substance Name	CASRN	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	GP Call[2]	GP Test	GP Reference	Human Call	Human References
Hexyl cinnamic aldehyde	101-86-0		3, 10, 30	1.2, 4.6, 18	6.7	1% Pluronic L92/H2O		+	ECPA LLNA Project Report submitted by BASF	+	NA	NA	NT	NA	NT
			3, 10, 30	1.9, 4.2, 9.2	6.3	1% Pluronic L92/H2O		+	ECPA LLNA Project Report submitted by Bayer						
			3, 10, 30	1.9, 2.2, 10.3	12	1% Pluronic L92/H2O		+	ECPA LLNA Project Report submitted by Dow Chemical						
			3, 10, 30	1.1, 2.5, 15.6	10.8	1% Pluronic L92/H2O		+	ECPA LLNA Project Report submitted by Dupont						
			3, 10, 30	1.3, 2.2, 4.3	17.6	1% Pluronic L92/H2O		+	ECPA LLNA Project Report submitted by Syngenta/RCC						
Methyl 4-hydroxybenzoate	99-76-3		10, 25, 50	0.8, 0.9, 0.8	NC	80% ETOH		-	Ryan et al. (2000)	-	NA	NA	NT	NA	Ryan et al. (2000)
Methyl 2-nonynoate	111-80-8		5, 10, 20	10.4, 17.7, 24.4	2.5	80% ETOH		+	Ryan et al. (2000); Basketter et al. (2005); Gerberick et al. (2005)	+	NA	NA	NT	+[7]	Ryan et al. (2000); Basketter et al. (2005)
			NA	NA	2.5	80% ETOH		+							

Substance Name	CASRN	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	GP Call[2]	GP Test	GP Reference	Human Call	Human References
Neomycin sulfate	1405-10-3		0.5, 1, 2	0.9, 0.9, 0.9	NC	25% ETOH		-	Basketter et al. (1994); Basketter et al. (1999a); Gerberick et al. (1992); Schneider and Akkan (2004)	-	+	BT	Gad et al. (1986); Basketter et al. (1999a)	+[7,8]	Basketter et al. (1994); Kligman (1966); Magnusson and Kligman (1969); Marzulli and Maibach (1974); Schneider and Akkan (2004)
Oxyfluorfen	42874-03-3	EC	1, 7, 33	0.81, 1.4, 4.9	18.8	1% L92	CBA/Ca	+	ECPA LLNA Project Report submitted by BASF	+	-	GPMT	ECPA LLNA Project Report submitted by: Dow Chemical	NA	NT
			1, 7, 33	0.9, 1.4, 2.8	NC	1% L92	CBA/J	-	ECPA LLNA Project Report submitted by Bayer						
			1, 7, 33	0.3, 0.9, 2.3	NC	1% L92	CBA/J	-	ECPA LLNA Project Report submitted by Dow Chemical						
			1, 7, 33	1.1, 1.5, 3.1	30.8	1% L92	CBA/JHsd	+	ECPA LLNA Project Report submitted by Dupont						
			1, 7, 33	1.2, 1.2, 5.4	18.1	1% L92	CBA/ CaOlaHsd	+	ECPA LLNA Project Report submitted by Syngenta/RCC						
Pluronic L92®	NA		1, 2.5, 5, 10, 25, 50	1.3, 1.0, 1.0, 0.8, 0.8, 2.0	NC	H2O		-	Ryan et al. (2002)	-	NA	NA	NT	NA	NT

Substance Name	CASRN	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	GP Call[2]	GP Test	GP Reference	Human Call	Human References
Propylene glycol	57-55-6		50, 100	1.2, 1.6	NC	H2O		-	Basketter et al. (1998); Basketter et al. (1999a); Gerberick et al. (2005)	-	-	GPMT	Guillot et al. (1983); Wahlberg and Boman (1985); Gad et al. (1986); Basketter et al. (1999a)	+[8]	Kligman (1966); Basketter et al. (1998); Basketter et al. (1999a)
Quinoxyfen	124495-18-7	SC	7, 33, 100	1.1, 0.7, 0.8	NC	1% L92	CBA/J	-	ECPA LLNA Project Report submitted by Dow Chemical	-	-	BT	ECPA LLNA Project Report submitted by: Dow Chemical	NA	NT
Quinoxyfen/ Cyproconazole	124495-18-7 / 113096-99-4	NA	7, 33, 100	2.1, 10.7, 20.3	9.8	1% L92	CBA/Ca	+	ECPA LLNA Project Report submitted by BASF	+	+	BT	ECPA LLNA Project Report submitted by Dow Chemical	NA	NT
			7, 33, 100	1.2, 7.2, 12.4	14.8	1% L92	CBA/J	+	ECPA LLNA Project Report submitted by Bayer						
			7, 33, 100	0.4, 3.8, 2.0	26.9	1% L92	CBA/J	+	ECPA LLNA Project Report submitted by Dow Chemical						
			7, 33, 100	1.4, 2.0, 6.2	49.8	1% L92	CBA/JHsd	+	ECPA LLNA Project Report submitted by Dupont						
			7, 33, 100	1.3, 6.5, 13.6	15.5	1% L92	CBA/ CaOlaHsd	+	ECPA LLNA Project Report submitted by Syngenta/RCC						
			12.5, 25, 50, 75, 100	2, 2.3, 8.6, 15.8, 30.1	27.8	1% L92	CBA/J	+	ECPA LLNA Project Report submitted by Dow Chemical						
Saturated diglycerin	NA		25, 50, 100	1.4, 2.1, 1.9	NC	ETOH/H2O		-	TNO Report[4]	-	NA	NA	NT	NA	NT

Substance Name	CASRN	Formu-lation Type	LLNA Conc. Tested (%)	LLNA SIs	LLNA EC3 (%)	LLNA Vehicle	LLNA Mouse Strain	LLNA Result[1]	LLNA Reference	Overall LLNA Result[1] (Majority)	GP Call[2]	GP Test	GP Reference	Human Call	Human References
Sodium lauryl sulfate	151-21-3		5, 10, 25	3.0, 4.8, 8.5	4.9	1% Pluronic L92/H2O		+	BGIA Project FP251[5]	+	NA	NA	NT	NA	Kligman (1966)
Sodium metasilicate	6834-92-0		2, 4, 6	0.9, 1.4, 1.3	NC	15% ETOH		-	NTP Study[6]	-	NA	NA	NT	NA	NT
Trifluralin	1582-09-8	EC	7, 33, 100	6.0, 30.0, 75.2	5.8	1% L92	CBA/Ca	+	ECPA LLNA Project Report submitted by BASF	+	-	BT	ECPA LLNA Project Report submitted by Dow Chemical	NA	NT
			7, 33, 100	1.9, 8.7, 25.7	11.2	1% L92	CBA/J	+	ECPA LLNA Project Report submitted by Bayer						
			7, 33, 100	3.1, 26.3, 61.5	7	1% L92	CBA/J	+	ECPA LLNA Project Report submitted by Dow Chemical						
			7, 33, 100	1.0, 7.0, 16.1	15.6	1% L92	CBA/JHsd	+	ECPA LLNA Project Report submitted by Dupont						
			7, 33, 100	1.8, 8.2, 20.5	11.9	1% L92	CBA/ CaOlaHsd	+	ECPA LLNA Project Report submitted by Syngenta/RCC						

[1] Overall LLNA result based on the majority and/or most severe result: "+" = sensitizer; "-" = nonsensitizer.

Annex IV-3

Medical Device Eluates Tested in Aqueous Solutions in the LLNA – Comparative Data

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Project #	NS Negative Control (dpm)[1]	NS Extract[2] (dpm)[1]	SI	LLNA Result[4]	NS Extract (spiked)[3] (dpm)[1]	SI	LLNA Result[4]	NS Positive Control[5] (dpm)[1]	SI	LLNA Result[4]
1	133.3	221.6	1.7	-	1,704.1	12.8	+	20,206.3	151.6	+
2	165.2	236.3	1.4	-	2,209.5	13.4	+	5,703.7	34.5	+
3	331.7	376.7	1.1	-	895.1	2.7	+	4,101.7	12.4	+
4	197.8	186.9	0.9	-	1,056.8	5.3	+	2,664.1	13.5	+
5	244.3	195.1	0.8	-	1,311.0	5.4	+	1,851.8	7.6	+
6	381.3	375.0	1.0	-	1,125.5	3.0	+	3,920.6	10.3	+
7	233.7	234.6	1.0	-	456.7	2.0	+	2,396.6	10.3	+
8	314.5	329.4	1.0	-	1,515.1	4.8	+	3,397.2	10.8	+
9	420.6	191.9	0.5	-	1,261.8	3.0	+	2,479.5	5.9	+
10	215.3	194.3	0.9	-	1,822.0	8.5	+	3,736.4	17.4	+
11	175.6	170.9	1.0	-	1,259.9	7.2	+	2,124.1	12.1	+
12	726.6	424.6	0.6	-	1,940.8	2.7	+	8,907.2	12.3	+
13	285.6	377.3	1.3	-	1,586.3	5.6	+	2,819.0	9.9	+
14	390.9	329.7	0.8	-	3,296.0	8.4	+	8,521.3	21.8	+
15	789.2	304.5	0.4	-	1,577.9	2.0	+	4,331.8	5.5	+
16	379.3	849.0	2.2	-	3,824.0	10.1	+	10,466.7	27.6	+
17	461.9	603.9	1.3	-	1,075.3	2.3	+	4,774.0	10.3	+
18	871.9	945.0	1.1	-	8,875.3	10.2	+	10,247.9	11.8	+
19	332.8	316.4	1.0	-	2,719.8	8.2	+	4,534.5	13.6	+
20	198.5	224.4	1.1	-	790.1	4.0	+	3,101.7	15.6	+
21	759.2	902.9	1.2	-	2,323.1	3.1	+	5,725.8	7.5	+
22	261.7	276.9	1.1	-	3,604.0	13.8	+	4,531.7	17.3	+
23	1,513.3	992.2	0.7	-	3,788.0	2.5	+	11,505.5	7.6	+
24	1,453.9	865.9	0.6	-	7,543.1	5.2	+	9,564.9	6.6	+
25	825.3	438.1	0.5	-	5,262.8	6.4	+	9,808.9	11.9	+

Project #	NS Negative Control (dpm)[1]	NS Extract[2] (dpm)[1]	SI	LLNA Result[4]	NS Extract (spiked)[3] (dpm)[1]	SI	LLNA Result[4]	NS Positive Control[5] (dpm)[1]	SI	LLNA Result[4]
26	777.5	893.8	1.1	-	5,173.9	6.7	+	11,150.1	14.3	+
27	595.5	503.9	0.8	-	5,840.9	9.8	+	7,727.1	13.0	+
28	370.4	601.3	1.6	-	7,842.8	21.2	+	13,347.0	36.0	+
29	1,318.8	1,475.9	1.1	-	5,706.1	4.3	+	12,477.5	9.5	+
30	1,177.9	2,268.3	1.9	-	7,555.7	6.4	+	9,089.1	7.7	+
31	558.6	784.5	1.4	-	4,850.6	8.7	+	6,124.0	11.0	+
32	944.5	1,018.5	1.1	-	6,922.7	7.3	+	10,209.2	10.8	+
33	1,243.8	691.6	0.6	-	3,475.9	2.8	+	8,882.2	7.1	+
34	872.1	867.8	1.0	-	11,532.6	13.2	+	10,109.2	11.6	+
35	1,009.6	525.4	0.5	-	4,753.8	4.7	+	7,112.1	7.0	+
36	684.3	1,224.8	1.8	-	6,559.5	9.6	+	9,624.1	14.1	+
37	1,282.0	1,258.5	1.0	-	16,400.3	12.8	+	19,533.0	15.2	+
38	529.0	1,003.9	1.9	-	3,588.5	6.8	+	8,043.5	15.2	+
39	207.7	443.4	2.1	-	2,016.1	9.7	+	4,094.1	19.7	+
40	518.5	904.9	1.7	-	2,755.1	5.3	+	4,874.7	9.4	+
41	862.9	877.3	1.0	-	4,171.6	4.8	+	7,437.7	8.6	+
42	599.8	808.0	1.3	-	3,174.3	5.3	+	7,399.7	12.3	+
43	1,134.8	852.4	0.8	-	8,424.8	7.4	+	10,621.8	9.4	+
44	769.5	636.2	0.8	-	4,422.1	5.7	+	10,450.4	13.6	+
45	389.2	600.8	1.5	-	3,677.9	9.4	+	9,347.1	24.0	+
46	674.1	662.3	1.0	-	2,292.3	3.4	+	3,332.9	4.9	+
47	269.1	584.0	2.2	-	1,557.4	5.8	+	5,865.7	21.8	+
48	602.8	930.0	1.5	-	4,184.8	6.9	+	10,186.1	16.9	+

Abbreviations: dpm = disintegrations per minute; NS = normal saline; SI = stimulation index.

[1] Values are an average of dpms from 5 individual animals.

[2] Eluate mixed 5:1 with Pluronic L92

[3] Eluate spiked with 20% dinitrobenzenesulfonic acid (DNBS) (1:1)

[4] (+) = sensitizer; (-) + nonsensitizer

[5] Positive control is 20% DNBS.

Annex V

Supplementary Analysis of Pesticide Formulations in the LLNA

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

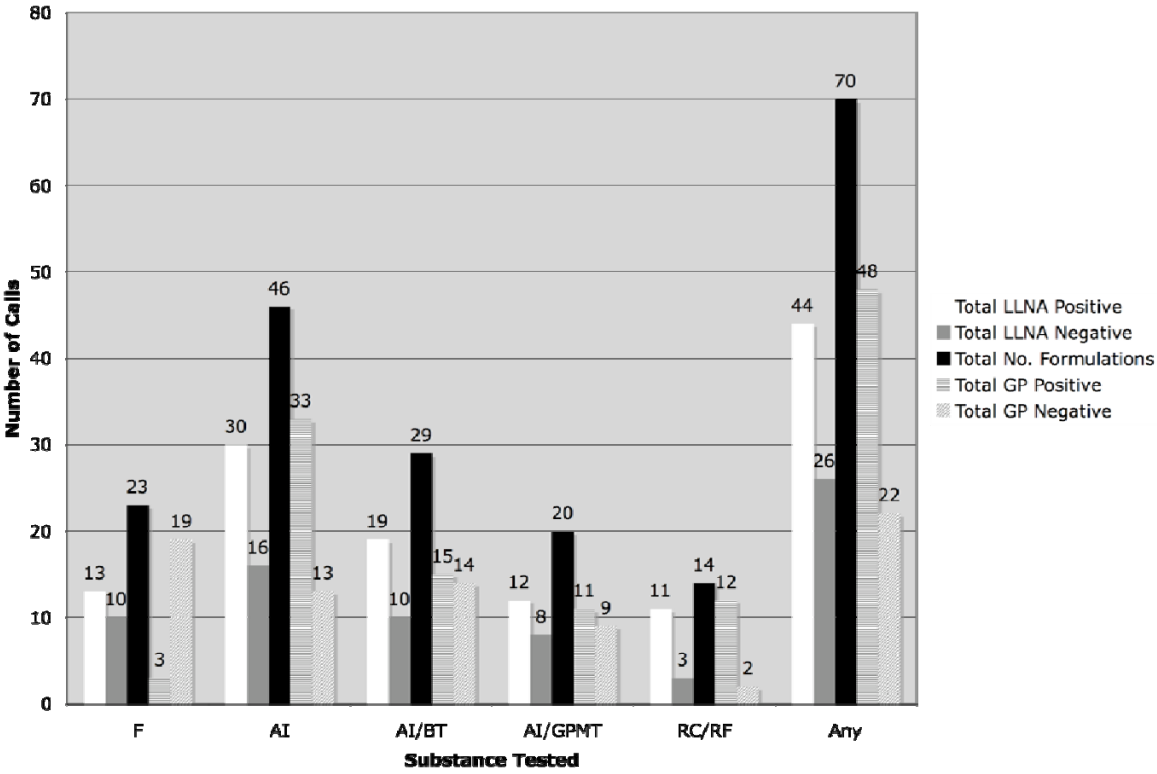
For the 23 formulations that had associated GP data for the formulation itself, 13% (3/23) were classified as sensitizers and 87% (20/23) as nonsensitizers according to the GP results (**Figure D-V-1-1**). These results are based on a positive overall GP call for formulation EXP 10810.[1] The LLNA classified 59% (13/22) of the formulations as sensitizers and 41% (9/22) as nonsensitizers (**Figure D-V-1-1**). 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 six substances as sensitizers that were classified as nonsensitizers in the GP test (**Table D-V-1-1**). There were no comparative human data with which to determine the actual human sensitization potential.

Testing of Pesticide Formulations: LLNA vs. GP with Any Available Reference Data for Relevant Substances

Of the 70 formulations, 69% (48/70) were classified as sensitizers and 31% (22/70) as nonsensitizers on the basis of various types of GP data (**Figure D-V-1-1**). To assign these classifications, a most conservative approach was used. That is, if a GP result for the formulation, any active ingredient, a substance related to an active ingredient, or a related formulation indicated sensitization, the formulation was classified as a sensitizer. Additionally, a GP result for the formulation itself was given priority over a result for an active ingredient. A result for an active ingredient was given priority over results for a substance related to an active ingredient, or a related formulation. Based on the LLNA result with the entire formulation for these same 70 pesticide formulations, 63% (44/70) were classified as sensitizers and 37% (26/70) as nonsensitizers (**Figure D-V-1-1**). Sixty-five percent (31/48) of the pesticide formulations classified as sensitizers by a GP test, based on the criteria given above, would also have been classified as sensitizers in the LLNA (**Table D-V-1-1**). The LLNA also identified an additional 14 formulations as sensitizers that would have been classified as nonsensitizers by a GP test based on these criteria. However, the LLNA failed to identify as sensitizers an additional 36% (17/48) of formulations that would have been classified as such by a GP test, based on the criteria given above.

[1] 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-V-1-1Numbers of Positive and Negative LLNA (All Mouse Strains) and GP Calls for Pesticide Formulations



Abbreviations: AI = Active Ingredient Test: BT = Buehler Test; F = Formulation Test; GP = guinea pig; GPMT = Guinea Pig Maximization Test; RC/RF = Related Substance or Related Formulation Test

Table D-V-1-1 Evaluation of the Performance of the LLNA in 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 vs. GP[4] (Formulation[5])	23	57	12/23	100	3/3	50	10/20	50	10/20	0	0/3
LLNA vs. GP[4] (Any[6])	70	56	39/70	65	31/48	36	8/22	64	14/22	35	17/48
LLNA vs. GP[4] (Active Ingredient[7])	46	72	33/46	76	25/33	62	8/13	38	5/13	24	8/33
LLNA vs. BT (Active Ingredient[7])	29	59	17/29	73	11/15	43	6/14	57	8/14	27	4/15
LLNA vs. GPMT (Active Ingredient[7])	20	55	11/20	64	7/11	44	4/9	56	5/9	36	4/11
LLNA vs. GP[4] (Related Substance or Formulation[8])	14	64	9/14	75	9/12	0	0/2	100	2/2	25	3/12
ICCVAM 1999 Database: Evaluation of LLNA Data vs. GP Data or Human Data[9]											
LLNA vs. GP[4]	126	86	108/126	87	81/93	82	27/33	18	6/33	13	12/93
LLNA vs. Human[10]	74	72	53/74	72	49/68	67	4/6	33	2/6	28	19/68
GP[4] vs. Human[10]	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] *GP* refers to outcomes obtained by studies conducted using either the Guinea Pig Maximization Test, the Buehler Test, or the McGuire Test.

[5] *Formulation* refers to associated GP data for the formulation itself.

[6] *Any* refers to associated GP data for the formulation itself, any active ingredient in the formulation, a substance related to an active ingredient, or a related formulation.

[7] *Active ingredient* refers to associated GP data for any active ingredient in the formulation.

[8] *Related substance or formulation* refers to associated GP data for a substance related to an active ingredient, or a related formulation.

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

[10] *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.

Testing of Pesticide Formulations: LLNA vs. GP with Available Reference Data for Active Ingredients

Of the 46 formulations that had associated GP data for one or more of the active ingredients, 72% (33/46) were classified as sensitizers and 28% (13/46) as nonsensitizers on the basis of an active ingredient in a GP test. Based on the LLNA result with the entire formulation for these same 46 pesticide formulations, 65% (30/46) were classified as sensitizers and 35% (16/46) as nonsensitizers (**Figure D-V-1-1**). Seventy-six percent (25/33) of the pesticide formulations identified as sensitizers based on a GP test on an active ingredient were identified as sensitizers in the LLNA (**Table D-V-1-1**). The LLNA also identified as sensitizers an additional five substances that were classified as nonsensitizers in the GP test. However, the LLNA failed to identify 24% (8/33) of the formulations as sensitizers that would have been classified as such by a GP test on an active ingredient (**Table D-V-1-1**).

Among these same 46 formulations with available GP data for one or more of the active ingredients, 29 had BT data and 20 had GPMT data (**Figure D-V-1-1**).

Of the 29 pesticide formulations with BT data for the active ingredient, 52% (15/29) were classified as sensitizers and 48% (14/29) as nonsensitizers. By comparison, LLNA results with the complete formulation for each of these products identified 66% (19/29) as sensitizers and 34% (10/29) as nonsensitizers (**Figure D-V-1-1**). Eleven of the pesticide formulations identified as sensitizers based on a BT of an active ingredient were identified as sensitizers in the LLNA (**Table D-V-1-1**). The LLNA also identified as sensitizers an additional eight substances that would have been classified as nonsensitizers in a BT on an active ingredient. However, the LLNA failed to identify 27% (4/15) formulations as sensitizers that would have been classified as such by a BT on an active ingredient.

Similarly, of the 20 pesticide formulations with GPMT data for the active ingredient, 55% (11/20) were classified as sensitizers and 45% (9/20) as nonsensitizers. The proportion of formulations classified as sensitizers was similar to the proportion classified as sensitizers by the BT on an active ingredient. By comparison, LLNA results with the complete formulation for each of these products identified 60% (12/20) as sensitizers and 40% (8/20) as nonsensitizers. Sixty-four percent (7/11) of the pesticide formulations identified as sensitizers based on a GPMT of an active ingredient were identified as sensitizers in the LLNA (**Table D-V-1-1**). The LLNA also identified as sensitizers an additional five formulations that would have been classified as nonsensitizers by a GPMT on an active ingredient. However, the LLNA failed to identify as sensitizers 36% (4/11) of formulations that would have been classified as such by a GPMT based on an active ingredient (**Table D-V-1-1**).

Testing of Pesticide Formulations: LLNA vs. GP with Available Reference Data for a Related Substance

Of the 14 formulations that had associated GP data for a substance related to an active ingredient, or a related formulation, 86% (12/14) were classified as sensitizers and 14% (2/14) as nonsensitizers on the basis of the related substance or formulation in a GP test. By comparison, LLNA results with the complete formulation identified 79% (11/14) as sensitizers and 21% (3/14) as nonsensitizers (**Figure D-V-1-1**). Nine of the pesticide formulations identified as sensitizers based on a GP test on a substance related to an active ingredient, or a related formulation, were identified as sensitizers in the LLNA (**Table D-V-1-1**). The LLNA also identified as sensitizers an additional two formulations that would have been classified as nonsensitizers by a GP test on a substance related to an active

ingredient, or a related formulation. However, the LLNA failed to identify as sensitizers an additional three formulations that would have been classified as such by a GP test on a substance related to an active ingredient, or a related formulation (**Table D-V-1-1**).

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Appendix E

Independent Scientific Peer Review Panel Assessment

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Appendix E1

Summary Minutes from the Independent Scientific Peer Review Panel Meeting on March 4-6, 2008

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Summary Minutes

Independent Scientific Peer Review Panel Meeting

Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products

Consumer Product Safety Commission (CPSC), Headquarters
Bethesda, MD
March 4 – 6, 2008
8:30 a.m. – 5:30 p.m.

Peer Review Panel Members:

Michael Luster, Ph.D. (Peer Review Panel Chair)	Senior Consultant to the NIOSH Health Effects Laboratory, Morgantown, WV, U.S.
Nathalie Alépée, Ph.D.	Associate Research Fellow, Pfizer PDRD MCT Laboratory, France
Anne Marie Api, Ph.D.	Vice President, Human Health Sciences, Research Institute for Fragrance Materials, Woodcliff Lake, NJ, U.S.
Nancy Flournoy, M.S., Ph.D.	Professor and Chair, Dept. of Mathematics and Statistics, University of Missouri-Columbia, Columbia, MO, U.S.
Thomas Gebel, Ph.D.	Regulatory Toxicologist, Federal Institute for Occupational Safety and Health, Dortmund, Germany
Kim Headrick, B. Admin., B.Sc.	International Harmonization Senior Policy Advisor, Health Canada, Ottawa, Ontario, Canada
Dagmar Jírová, M.D., Ph.D.	Toxicologist, Research Manager, Head of Reference Center for Cosmetics, Head of Reference Laboratory for Experimental Immunotoxicology, National Institute of Public Health, Czech Republic
David Lovell, Ph.D	Reader in Medical Statistics, Postgraduate Medical School, University of Surrey, Guildford, Surrey, U.K.
Howard Maibach, M.D.	Professor, Dept. of Dermatology, University of California-San Francisco, San Francisco, CA, U.S.

Peer Review Panel Members:

James McDougal, Ph.D.	Professor and Director of Toxicology Research, Dept. of Pharmacology and Toxicology, Boonshoft School of Medicine, Wright State University, Dayton, OH, U.S.
Michael Olson, Ph.D.	Director of Occupational Toxicology, Corporate Environment Health and Safety, GlaxoSmithKline, RTP, NC, U.S.
Raymond Pieters, Ph.D.	Associate Professor, Immunotoxicology Group Leader, Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands
Jean Regal, Ph.D.	Professor, Dept. of Pharmacology, University of Minnesota Medical School, Duluth, MN, U.S.
Peter Theran, V.M.D.	Massachusetts Society for the Prevention of Cruelty to Animals, Novato, CA, U.S.
Stephen Ullrich, Ph.D.	Dallas/Ft. Worth Living Legends Professor & Professor of Immunology, Graduate School of Biomedical Science, University of Texas M.D. Anderson Cancer Center, Houston, TX, U.S.
Michael Woolhiser, Ph.D.	Technical Leader - Immunotoxicology, Toxicology and Environmental Research and Consulting Immunology, Dow Chemical, Midland, MI, U.S.
Takahiko Yoshida, M.D., Ph.D.	Professor, Dept. of Health Science, Asahikawa Medical College, Hokkaido, Japan

ICCVAM and ICCVAM IWG Members:

Paul Brown, Ph.D.	FDA, Silver Spring, MD, U.S.
Ruth Barratt, Ph.D., D.V.M.	FDA, Rockville, MD, U.S.
Karen Hamernik, Ph.D.	EPA, Washington, DC, U.S.
Masih Hashim, Ph.D.	EPA, Washington, DC, U.S.
Abigail Jacobs, Ph.D. (IWG Co-Chair)	FDA, Silver Spring, MD, U.S.
Kristina Hatlelid, Ph.D.	CPSC, Bethesda, MD, U.S.
Joanna Matheson, Ph.D. (IWG Co-Chair)	CPSC, Bethesda, MD, U.S.
Tim McMahon, Ph.D.	EPA, Washington, DC, U.S.

ICCVAM and ICCVAM IWG Members:

Amy Rispin, Ph.D.	EPA, Washington, DC, U.S.
William Stokes, D.V.M., DACLAM	NIEHS, RTP, NC, U.S.
Raymond Tice, Ph.D.	NIEHS, RTP, NC, U.S.
Ron Ward, Ph.D.	EPA, Washington, DC, U.S.
Marilyn Wind, Ph.D. (ICCVAM Chair)	CPSC, Bethesda, MD, U.S.
Jiaqin Yao, Ph.D.	FDA, Silver Spring, MD, U.S.

ECVAM Observer:

David Basketter, Ph.D.	DABMEB Consultancy Ltd., Bedfordshire, U.K.

Invited Experts:

George DeGeorge, Ph.D., DABT	MB Research Laboratories, Spinnerstown, PA, U.S.
Kenji Idehara, Ph.D.	Daicel Chemical Industries, Hyogo, Japan
Masahiro Takeyoshi, Ph.D.	Chemicals Evaluation and Research Institute, Saitama, Japan

Public Attendees:

Odette Alexander	Syngenta Crop Protection, Inc., Greensboro, NC, U.S.
Nancy Beck, Ph.D.	PCRM, Washington, DC, U.S.
Ann Blacker, Ph.D.	Bayer CropScience, RTP, NC, U.S.
Stuart Cagan, Ph.D.	Shell Oil Company, Houston, TX, U.S.
Joan Chapdelaine, Ph.D.	Calvert Laboratories, Inc., Olyphant, PA, U.S.
Adriana Doi, Ph.D.	BASF Corporation, RTP, NC, U.S.
Carol Eisenmann, Ph.D.	Personal Care Products Council, Washington, DC, U.S.
Charles Hastings, Ph.D.	BASF Corporation, RTP, NC, U.S.
Kailash Gupta, D.V.M., Ph.D.	Retired CPSC, Bethesda, MD, U.S.
John Lyssikatos	Hill Top Research, Miamiville, OH, U.S.
Laurence Musset, Ph.D.	OECD, Paris, France
Carol O'Neil	NuPathe, Conshohocken, PA, U.S.

Public Attendees:

Kui Lea Park, Ph.D.	National Institute of Toxicological Research, KFDA, Seoul, Korea
Rafael Rivas	AFRRI/USHUS, Bethesda, MD, U.S.
Terri Sebree	NuPathe, Conshohocken, PA, U.S.
Libby Sommer	EPA, Washington, DC, U.S.
Merrill Tisdel	Syngenta Crop Protection Inc., Greensboro, NC, U.S.
Jeffrey Toy, Ph.D.	FDA, Rockville, MD, U.S.

NICEATM:

William Stokes, D.V.M., DACLAM	Director
Raymond Tice, Ph.D.	Deputy Director
Debbie McCarley	Special Assistant to the Director

Support Contract Staff— Integrated Laboratory Systems, Inc. (ILS)

David Allen, Ph.D.	Michael Paris
Thomas Burns, M.S.	Eleni Salicru, Ph.D.
Linda Litchfield	Judy Strickland, Ph.D., DABT
Douglas Winters, M.S.	

Abbreviations:

AFFRI = Armed Forces Radiobiology Research Institute

CPSC = U.S. Consumer Product Safety Commission

ECVAM = European Centre for the Validation of Alternative Methods

EPA = U.S. Environmental Protection Agency

FDA = U.S. Food and Drug Administration

ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods

ILS = Integrated Laboratory Systems

IWG = Immunotoxicology Working Group

KFDA = Korea Food and Drug Administration

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

OECD = Organisation for Economic Co-operation and Development

PCRM = Physicians Committee for Responsible Medicine

USDA = U.S. Department of Agriculture

USHUS = Uniformed Services University of the Health Sciences

TUESDAY, MARCH 4, 2008

Call to Order and Introductions—

Dr. Michael Luster (Peer Review Panel Chair) called the meeting to order at 8:30 a.m. and introduced himself. He then asked all Peer Review Panel (hereafter Panel) members to introduce themselves and to state their name and affiliation for the record. He then asked all the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) staff, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) members, the ICCVAM Immunotoxicity Working Group (IWG) members, the European Centre for the Validation of Alternative Methods (ECVAM) observer, and members of the public to also introduce themselves. Dr. Luster stated that there would be opportunity for public comments during each of the seven local lymph node assay (LLNA)-related topics. He asked that all those interested in making a comment register at the registration table and provide a written copy of their comments, if available, to NICEATM staff. Dr. Luster emphasized that the comments would be limited to seven minutes per individual and that, while an individual would be welcome to make comments during each commenting period, repeating the same comments at each comment period would be inappropriate. He further stated that the meeting was being recorded and that Panel members should speak directly their microphone. Finally, Dr. Luster noted that if the Panel finished early with the assigned topics on the agenda for that day, they would proceed to the next day's topics if time permitted.

Welcome from the ICCVAM Chair—

Dr. Marilyn Wind, U.S. Consumer Product Safety Commission (CPSC) and Chair of ICCVAM, welcomed everyone to CPSC and to the Panel meeting. Dr. Wind stressed the importance of this Panel's efforts especially considering recent reports that allergies and asthma have increased markedly over the past number of years and that contact dermatitis is the most common occupational illness in the United States. Dr. Wind thanked the Panel members for giving their expertise, time, and effort and acknowledged their important role to the ICCVAM test method evaluation process. Dr. Wind also emphasized the important role of the public and their comments in this process.

Welcome from the Director of NICEATM, and Conflict of Interest Statements—

Dr. William Stokes, Director of NICEATM, stated the Panel meeting was being convened as a National Institutes of Health (NIH) special emphasis panel and was being held in accordance with the Federal Advisory Committee Act regulations. As such, Dr. Stokes indicated that he would serve as the Designated Federal Official for this public meeting. He reminded the Panel that they had signed a conflict-of-interest statement when they were selected for the Panel, in which they identified any potential conflicts of interest. He then read this statement to provide another opportunity for members of the Panel to identify any conflicts not previously declared. Dr. Luster asked the Panel members to declare any direct or indirect conflicts based on Dr. Stokes statements and to recuse themselves from discussion and voting on any aspect of the meeting where there might be a conflict. None of the Panel members declared a conflict of interest.

Overview of the ICCVAM Test Method Evaluation Process

Dr. Stokes provided an overview of the ICCVAM test method evaluation process. He stated that the Panel was made up of 19 different scientists from eight different countries (Canada, Czech Republic, France, Germany, Japan, The Netherlands, United Kingdom, and the United States). Dr. Stokes thanked the Panel members for the significant amount of time and effort that they had devoted to prepare for and attend the meeting. He explained that the purpose of the Panel was to assist ICCVAM by carrying out an independent scientific peer review of the information provided on a series of proposed new versions of the LLNA and some expanded applications of the assay. Dr. Stokes

mentioned that the original LLNA peer review panel in 1998 considered the LLNA a valid substitute for the guinea pig-based test in most testing situations, but not all. He mentioned that three Panel members from the 1998 review are also on the current Panel (i.e., Drs. Howard Maibach, Jean Regal, and Stephen Ullrich). Dr. Stokes also reviewed the nomination that was received from CPSC in January 2007,[1] which provides the basis for the current evaluation.

Dr. Stokes then identified the 15 Federal agencies that comprise ICCVAM and summarized ICCVAM's mission. He noted that ICCVAM, as an interagency committee, does not carry out research and development or validation studies. Instead, ICCVAM, in conjunction with NICEATM, carries out the critical scientific evaluation of proposed test methods with regard to their usefulness and limitations for regulatory testing and then makes formal recommendations to ICCVAM agencies.

Dr. Stokes provided a brief review of ICCVAM's history and summarized the ICCVAM Authorization Act of 2000,[2] detailing the purpose and duties of ICCVAM. He noted that one of ICCVAM's duties is to review and evaluate new, revised, and alternative test methods applicable to regulatory testing. He stated that all of the reports produced by NICEATM are available on the NICEATM-ICCVAM website or can be obtained upon request from NICEATM. He also mentioned that ICCVAM provides guidance on test method development, validation criteria, and processes, and helps to facilitate not only the acceptance of scientifically valid alternative methods, but also encourages international harmonization.

Dr. Stokes then described the ICCVAM test method evaluation process, which begins with a test method nomination or submission. NICEATM conducts a prescreen evaluation to summarize the extent to which the proposed submission or nomination addresses the ICCVAM prioritization criteria. A report of this evaluation is then provided to ICCVAM, which in turn develops recommendations regarding the priority for evaluation. ICCVAM then seeks input on their recommendations from the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) and the public. Given sufficient regulatory applicability, sufficient data, resources, and priority, a test method will move forward into a formal evaluation. A draft background review document (BRD), which provides a comprehensive review of all available data and information, is prepared by NICEATM, in conjunction with an ICCVAM working group designated for the relevant toxicity testing area (e.g., the IWG). In addition, ICCVAM considers all of the available information and makes draft test method recommendations on the proposed usefulness and limitations of the test methods, test method protocol, performance standards, and future studies. The BRD and the draft ICCVAM test method recommendations are made available to the Panel and the public for review and comment. The Panel peer reviews the BRD and evaluates the extent to which it supports the draft ICCVAM test method recommendations. A Panel report is published, which is then considered along with public and SACATM comments by ICCVAM in making final recommendations. These final recommendations are forwarded to the ICCVAM member agencies for their consideration and possible incorporation into relevant testing guidelines.

Dr. Stokes reviewed the ICCVAM criteria for adequate validation. He stated that validation is defined by ICCVAM as the process by which the reliability and relevance of a procedure are established for a specific purpose, and that adequate validation is a prerequisite for consideration of a test method by U.S. Federal regulatory agencies. Dr. Stokes listed the ICCVAM acceptance criteria for test method validation and acceptance. He concluded by summarizing the timeline of the review activities beginning with CPSC's nomination in January 2007 and ending with the present Panel meeting.

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

[2] http://iccvam.niehs.nih.gov/docs/about_docs/PL106545.pdf

ICCVAM Charge to the Panel

Dr. Stokes reviewed the charge to the Panel, which was to: (1) review the draft BRDs, the draft Addendum to the traditional[3] LLNA, and the draft performance standards for completeness and identify any errors or omissions; (2) determine the extent to which each of the applicable criteria for validation and regulatory acceptance had been addressed for the proposed revised or modified versions of the LLNA; and (3) consider and provide comment on the extent to which the ICCVAM draft test method recommendations including the proposed use, standardized protocols, performance standards, and additional studies are supported by the information provided in the draft BRDs and draft Addendum.

Dr. Stokes thanked the IWG and ICCVAM for their contributions to this project, and acknowledged the contributions from the participating liaisons from ECVAM and JaCVAM (Japanese Center for the Validation of Alternative Methods). He also acknowledged the NICEATM staff for their support and assistance in organizing the Panel meeting and preparing the materials being reviewed.

Current Regulatory Testing Requirements and Hazard Classification Schemes for Allergic Contact Dermatitis and the Traditional LLNA Procedure

Dr. Joanna Matheson, Chair of the IWG, briefly reviewed the regulatory testing requirements of U.S. Federal agencies for skin-sensitization hazard identification and provided a brief description of the LLNA protocol.

Overview of the Agenda

Dr. Luster provided a brief synopsis of the agenda. He stated that there were six test methods and applications along with the draft LLNA performance standards for review and that the same agenda would be followed for each: (1) introductory summary of the draft ICCVAM recommendations from one of the NICEATM staff members; in addition, test method developers would provide a brief description of the methodology for each of the three nonradioactive tests, (2) presentation of the Evaluation Group draft comments by the Evaluation Group leader, (3) Panel discussion, (4) public comments, (5) recommendations and conclusions by the Panel.

Overview of the Draft LLNA Limit Dose Procedure[4] BRD and Draft ICCVAM Test Method Recommendations

Dr. David Allen, Integrated Laboratory Systems, Inc., the NICEATM support contractor, presented an overview of the draft ICCVAM BRD for the LLNA limit dose procedure. He mentioned that the draft ICCVAM BRD provided a comprehensive review of the available data and information regarding the usefulness and limitations of the LLNA limit dose procedure. The method was reviewed for its accuracy in correctly identifying sensitizers and non-sensitizers, when compared to the traditional LLNA.

NICEATM published a series of *Federal Register* (FR) notices, including an FR notice (72 FR 27815, May 17, 2007) requesting original data from the LLNA. This FR notice was also sent to over 100 potentially interested stakeholders for their input and comment. As a result, data on 255 substances tested in the LLNA were received. The resulting LLNA database consisted of 471 studies of 466 unique substances, 211 of which were included in the original ICCVAM 1999 evaluation. Dr.

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[3] For the purposes of this document, the radioactive LLNA test method, which was first evaluated by ICCVAM in 1999, and subsequently recommended to U.S. Federal agencies as a valid substitute for currently accepted guinea pig test methods to assess the allergic contact dermatitis potential of many, but not all, types of substances, is referred to as the traditional LLNA.

[4] Also known as the reduced LLNA (rLLNA).

Allen briefly summarized the performance characteristics of the LLNA limit dose procedure test method, which is detailed in the draft ICCVAM BRD,[5] and briefly summarized the draft ICCVAM test method recommendations for the LLNA limit dose procedure.[6]

Panel Evaluation:

Dr. Michael Olson led the Panel discussion on the LLNA limit dose procedure and specifically thanked the members of his Evaluation Group (i.e., Drs. James McDougal, Raymond Pieters, Jonathan Richmond [not present], and Takahiko Yoshida) for their collegial review of the information presented in the draft ICCVAM LLNA Limit Dose Procedure BRD. Dr. Olson also thanked the NICEATM staff for their technical support during the BRD review process. He then presented the draft responses to ICCVAM's questions to the Panel for consideration by the entire Panel. The focus was on review of the BRD for errors and omissions, assessment of the validation status of the test method, and review of draft ICCVAM test method recommendations. The Panel discussion and their recommended revisions to each section of the draft ICCVAM BRD and recommendations are reflected in the *Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products*, published in May 2008 (hereafter, the Panel report[7]).

During the Panel's evaluation, discussion arose regarding what might have resulted in the inverted-U-shaped dose response that was seen with the false-negative substances in the LLNA limit dose procedure. Dr. Olson responded that although it was difficult to understand what the cause might have been, he speculated that the top dose was either toxic at a systemic-effect level or that those substances were immunosuppressive at the highest dose level. He also stated that there did not seem to be any structural features of the substances that could be attributed for the false negative response in the LLNA limit dose procedure.

The Panel also discussed the use of concurrent versus intermittent positive controls in the LLNA limit dose procedure. Dr. Olson indicated that the Evaluation Group had discussed the possibility to allow intermittent positive controls for laboratories that exhibited repeatable and adequate performance with the LLNA but he indicated that it would be important to describe a set of performance criteria that would determine when this practice would be acceptable. Clearly, if the laboratory was not performing the assay routinely or if there were other reasons to suspect variability in response with any substance, the positive control would be necessary. Dr. Stokes indicated that this discussion was pertinent and indicated that the Panel's suggestions for what the performance criteria might be for intermittent positive control testing would be of interest to the IWG. Dr. Stokes also wanted to clarify that the OECD TG is consistent with the EPA TG and the ICCVAM-recommended test method protocol for the LLNA although the OECD TG allows additional latitude in how tests are run (i.e., four animals per dose group, use of pooled data, and the option to not run a positive concurrent positive).

Public Comments:

Dr. Amy Rispin, EPA

Dr. Rispin stated that the ICCVAM LLNA report (1999[8]) and standardized protocol (2001[9]) recommends the use of a concurrent positive control in addition to the concurrent negative control required for each study. Subsequently, the OECD (Organisation for Economic Co-operation and Development) Test Guideline (TG) 429 (Skin Sensitisation: Local Lymph Node Assay) was finalized

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[5] http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-LD/LLNAldBRD07Jan08FD.pdf
[6] http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-LD/IWGrecLLNA-LD07Jan08FD.pdf
[7] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
[8] http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf
[9] http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/LLNAProt.pdf

(2002). She said that originally, OECD TG 429 was drafted without a concurrent positive control but that language was added to include the recommended use of a concurrent positive control until laboratories demonstrate competence. Subsequent to that, EPA put forth its LLNA guideline for sensitization,[10] which states that concurrent positive and negative controls are to be included in each study. Dr. Rispin then added that U.S. Federal regulatory agencies, most notably the EPA and FDA, received LLNA data from studies in which the positive control did not achieve the appropriate limits of performance (i.e., the control values were not in the appropriate range) and therefore the studies were deemed unacceptable, underscoring the importance of a concurrent positive control for regulatory acceptance in the United States.

In response to Dr. Rispin's public comment, Drs. Ullrich and Theran asked how competence is determined and if laboratories have difficulties reaching a level of competence, respectively. Dr. Abby Jacobs responded by stating that the FDA has seen large data variations in laboratories that conduct the LLNA. It is often difficult to determine what the variations might be due to (e.g., new technicians, tail vein injection, lymph node removal) and these variations have been seen both in laboratories that are established and those that are not.

Dr. David Basketter, ECVAM Observer

Dr. Basketter said that the main point he wanted to address is that efforts should be made to harmonize the LLNA protocol with that described in OECD TG 429. He stated that although there is referral to the "ICCVAM protocol" throughout the BRDs under consideration, OECD TG 429 is more globally recognized for regulatory use of the LLNA and therefore should be the referenced protocol. Dr. Basketter further stated that if the LLNA limit dose procedure followed the ICCVAM protocol using five animals per group instead of following OECD TG 429, which allows using four animals per group, there would only be a savings of one animal for substances that were negative. He stated that the goal of ECVAM was actually to halve the number of animals by omitting the mid- and low-dose groups and that this would achieve significant animal savings since the likely prevalence of non-sensitizers is approximately two-thirds of chemicals tested and non-sensitizers would not require further testing even if dose response information for sensitizers was needed.

Dr. Basketter also mentioned that the retrospective evaluation of the LLNA being presented to the Panel analyzed whether the top dose could identify a substance as a sensitizer and how that compares to the traditional LLNA's performance. Since the traditional LLNA assay was determined to be positive or negative based on a stimulation index (SI) of three, it is problematic if the focus is on statistics when using the five-animal model as this would require also going back and re-evaluating all the preceding data using the statistical approach.

Dr. McDougal responded to Dr. Basketter's comment by stating that one wouldn't have to go back and retrospectively re-evaluate previous data but that new data generated could be analyzed statistically. This approach would include determining if the treatment group was statistically different from the vehicle control group and then determining the biological relevance. This might help to eliminate irritants.

Panel Conclusions and Recommendations:

Dr. Luster asked the Panel to review the conclusions and recommendations for the LLNA limit dose procedure they had discussed earlier and to make any revisions, if necessary. One particular question that was asked during the Panel's conclusions and recommendations was whether an OECD TG existed for the LLNA limit dose procedure. Dr. Stokes indicated that the OECD TG would need to be updated to allow for the provision of a limit dose procedure and that's why the Panel's conclusions

[10] http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Revised/870r-2600.pdf

and recommendations are even more relevant. Dr. Stokes indicated that ICCVAM has already submitted a proposal to update the OECD TG based on the outcome of these deliberations and recommendations from the IWG.

The Panel agreed to use the term weight-of-evidence to refer to existing information that would aid the LLNA limit dose procedure in identifying a substance as a sensitizer or a non-sensitizer. The Panel also discussed the use of concurrent positive controls and recommended that a laboratory that is proficient at conducting the limit dose procedure can test a positive control at routine intervals rather than concurrently (although the Panel did not identify what constituted routine intervals). The Panel also discussed the use of individual versus pooled data and agreed with the ICCVAM-recommended protocol that individual animal data should always be collected. The Panel concluded that individual animal response data are necessary in order to allow for statistical analyses of any differences between treated and control data. In addition, having data from individual animals also allows for identification of technical problems and outlier animals within a dose group. Dr. Luster asked the Panel if they agreed with the changes and revisions made at this point and with the Panel conclusions and recommendations as presented and revised. The Panel unanimously agreed. The Panel's detailed recommendations and conclusions on the LLNA limit dose procedure are included in their final Panel report.[11]

Overview of the Draft Addendum for the Applicability Domain of the LLNA and Draft ICCVAM Test Method Recommendations

Dr. Eleni Salicru, Integrated Laboratory Systems, Inc. (the NICEATM support contractor), summarized the information provided in the draft ICCVAM Addendum to the ICCVAM LLNA report (1999). This Addendum provided an updated assessment of the validity of the LLNA for testing the sensitizing potential of mixtures, metals, and aqueous solutions. The database used for this evaluation contained traditional LLNA data submitted as part of the original LLNA evaluation (ICCVAM 1999), data extracted from peer-reviewed articles published after the original evaluation, and data submitted to NICEATM in response to the FR notice (72 FR 27815, May 17, 2007) requesting such data. Dr. Salicru then summarized the performance characteristics of the LLNA when used to test mixtures, metals, and aqueous solutions,[12] as well as the draft ICCVAM test method recommendations for each of the three categories of test substances.[13]

Panel Evaluation:

Dr. McDougal, on behalf of his Evaluation Group, presented for consideration by the entire Panel the draft responses to the questions asked of the Panel by ICCVAM. The Panel then discussed the completeness of the draft ICCVAM Addendum, identified any errors and omissions, and reviewed the draft ICCVAM test method recommendations with regard to the ability of the LLNA to be used to test the sensitizing potential of mixtures, metals, and aqueous solutions. The Panel discussion and their recommended revisions to each section of the draft ICCVAM Addendum are reflected in the Panel report, published in May 2008.[14] During the Panel's evaluation of the LLNA's applicability domain, the difficulty of testing metals in the LLNA was discussed and Dr. Woolhiser asked if testing metals was also problematic in the guinea pig. Dr. Api indicated that with the metals, most of the data has come from the clinical experience because animal studies are not predicting accurately what is happening in the clinic. Dr. Maibach indicated that metals have been tested in the guinea pig and that they are sensitized easily. Dr. Maibach further commented that metals in man need to be patch-tested for clinical relevance at a level close to the irritant dose and that a thoughtful series of algorithms is

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[11] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
[12] http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-app/LLNAappADD19Jan08FD.pdf
[13] http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-app/LLNAappRecs19Jan08FD.pdf
[14] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

necessary to determine this. He also pointed out that patch test results to some metals (e.g., nickel, palladium) may indicate that a cell mediated reaction is occurring (i.e., contact allergy) but it needs to be sorted out if this cell mediated reaction actually results in a disease (i.e., allergic contact dermatitis) and this is where the LLNA could prove useful.

With regard to mixtures, Dr Api commented that based on her experience, when the mixture tested in the LLNA contains a predominant material (loosely defined that as greater than 70 percent) then the LLNA for the mixture mirrors what occurs for that one material. When evidence indicates that the substance is a true mixture, some times the LLNA does what is expected and other times the results are unexpected. In those cases, a weight-of-evidence approach (e.g., structure-activity relationships, clinical evidence) is employed.

Public Comments:

Dr. Charles Hastings, BASF Corporation

Dr. Hastings, representing CropLife America (an industry association of companies in the crop protection business), provided an overview of current activities in industry related to the use of the LLNA to detect dermal sensitizers and the global issues that are of importance. Dr. Hastings mentioned that CropLife America's primary concern is the testing of pesticide mixtures and formulations. He stated that they support the use of the LLNA for testing the dermal sensitization of mixtures and formulations as well as single ingredients.

Dr. Hastings mentioned that in the United States, EPA OPPTS (Office of Prevention, Pesticides and Toxic Substances) Guideline 870.2600[15] allows for the use of the LLNA as the preferred alternative to the standard guinea pig test. Based on this recommendation, member companies of CropLife America conducted a large number of LLNA studies for both active ingredients and formulations in the European Union (E.U.) and were at the point of submitting data in the United States, as well. Then, in early 2007, they were informed that EPA had concerns about the validity of using the LLNA to test mixtures and formulations, and were advised to discontinue using this test method for that purpose until it had been adequately validated. Dr. Hastings stated that, in contrast to the EPA, E.U. regulators consider the LLNA acceptable for testing pesticide formulations and actually prefer it to a guinea pig test.

Dr. Pieters asked if the E.U. has conducted any evaluations of the validity of the LLNA for testing mixtures and formulations. Dr. Hastings replied that he was not certain if they had performed an extensive evaluation or not but that the E.U. considered the LLNA a validated method and therefore likely considered it appropriate to test not only the active ingredient but also the formulation or mixture.

Dr. Hastings mentioned that one concern in terms of using the LLNA for testing mixtures or formulations, particularly in the E.U., is the testing of aqueous substances. Many of the industry formulations are aqueous-based and may be incompatible with traditional LLNA vehicles. The European Crop Protection Association sponsored a study that evaluated the use of an aqueous vehicle known as Pluronic L92, which helps adhere the test material to the mouse ear. In the study, they tested three aqueous pesticide formulations that contained known sensitizers, using Pluronic L92 as the vehicle. As expected, the test results demonstrated sensitizing activity. Regarding global considerations, Dr. Hastings mentioned that if the LLNA is not accepted for mixture/formulation testing in the United States, industry will have no choice but to conduct both the LLNA, with 18 to 24 animals, and a guinea pig test, with 20 to 30 animals, for each formulation they may develop for

[15] http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Revised/870r-2600.pdf

global distribution. This scenario counters the ICCVAM goal of "reducing, refining, and replacing" animal use in regulatory safety testing.

Dr. Hastings ended with the following conclusions:

- CropLife America believes the LLNA test can be used for pesticide formulations.
- CropLife America supports the efforts of EPA and ICCVAM to confirm the validity of the LLNA for testing mixtures/formulations and encourages a quick evaluation.
- CropLife America is willing to help, as needed.
- If and, when, it is determined that the LLNA is acceptable, CropLife America requests that EPA notify them so they can then begin conducting the LLNA again for the United States.

Dr. Api asked if CropLife America has data comparing pesticides that have been evaluated in the LLNA and in guinea pigs and/or humans. Dr. Hastings replied that they do and that generally there is not much discrepancy with guinea pig test results. Occasionally they might see a false positive compared to a guinea pig test, but he did not recall ever seeing a false negative. In most cases, they would feel comfortable accepting an occasional false positive because human health is still protected.

Dr. David Basketter, ECVAM Observer

Dr. Basketter stated that he had personal reservations about testing complex mixtures and formulations in assays that were designed for testing substances (e.g., the LLNA) since no single test has ever been validated for testing mixtures. On another point, he stated that most of the metals of importance have been tested in both the guinea pig and the LLNA and the "right" answers have been generated. Thus, it does not seem worthwhile to produce new tests with revised protocols for hazard and potency categorization for testing metals.

Panel Conclusions and Recommendations:

Dr. Luster asked the Panel if they agreed with the comments and recommendations that were made earlier during the Panel discussion. The Panel agreed with the draft ICCVAM recommendation for continued collection of information from traditional LLNA evaluations of mixtures, metals, and aqueous solutions with comparative data for guinea pig (i.e., guinea pig maximization test [GPMT] or Buehler test [BT]) and human (i.e., human maximization test [HMT] or human repeat insult patch test [HRIPT]) tests. However, the Panel suggested that, given resource limitations, it would be important to organize the recommendations based on relative priority. Dr. Luster asked the Panel if they agreed with this suggestion about prioritization of activities; all members of the Panel agreed with one abstention. Dr. Howard Maibach abstained from voting stating that he hoped this public meeting and the subsequent Panel report would emphasize to industry the need for them to submit more data on mixtures, metals, and aqueous substances in order to provide a clearer evidence of the validity of the LLNA in testing these types of substances. The Panel's detailed recommendations and conclusions on the applicability domain of the LLNA are included in their final Panel report.[16]

Method Description and Overview of the LLNA: Daicel Adenosine Triphosphate (LLNA: DA) Test Method

Dr. Kenji Idehara, Daicel Chemical Industries, Ltd. (private limited company), summarized the technical aspects of the LLNA: DA test method. He described the LLNA: DA as a non-radioisotopic version of the LLNA method in which lymph node adenosine triphosphate (ATP) content is used as a measure of cell proliferation instead of radiolabeled thymidine incorporation. Dr. Idehara indicated

[16] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

that the LLNA: DA was developed six years ago at Daicel Chemical Industries, Ltd., and that they use the test method regularly for in-house assessments of the skin-sensitization potential of chemical materials, intermediates, or products. He summarized the protocol differences between the LLNA: DA and the traditional LLNA. In the LLNA: DA, the application site is treated with 1% sodium lauryl sulfate (SLS) one hour before each test substance (or vehicle control) application, and the test substance is applied to the test site on day 7 as well as on days 1, 2, and 3. The auricular lymph nodes are excised from individual animals on day 8 rather than on day 6 and the amount of ATP in the lymph nodes is measured with a luciferin-luciferase assay. Dr. Idehara mentioned that these modifications (i.e., 1% SLS pretreatment and additional application on day 7) enhance lymph node cell proliferation in order to achieve an SI = 3 in the LLNA: DA, which allows for a more direct comparison to the traditional LLNA.

Dr. Idehara mentioned that after excision, ATP content gradually decreased with time. Therefore, the overall assay time for measuring ATP content needs to be similar (i.e., within approximately 30 minutes) among all test animals. He noted that this was an important point for this method and recommended that the LLNA: DA be conducted by at least two persons. Dr. Idehara mentioned that ATP content assays are conducted using commercially available kits, and his laboratory has experience with two different commercial sources in Japan, Kikkoman and Lonzar.

Overview of the Draft LLNA: DA BRD and Draft ICCVAM Test Method Recommendations

Dr. Allen then presented an overview of the draft ICCVAM BRD for the LLNA: DA test method. He mentioned that the draft ICCVAM BRD provided a comprehensive review of the available data and information regarding the usefulness and limitations of the LLNA: DA to distinguish between sensitizers and non-sensitizers, compared to the traditional LLNA. The objective of the BRD was to describe the current validation status of the LLNA: DA test method, including its relevance and reliability, scope of substances tested, and the availability of a standardized protocol.

Dr. Allen mentioned that the data analyzed in the BRD included data provided by Daicel Chemical Industries, Ltd., on 31 substances tested at their laboratories. In addition, data for 14 different coded substances were generated from a two-phased interlaboratory validation study that included 17 total labs. Taken together, the total database represented in the LLNA: DA BRD included 33 different substances. Dr. Allen briefly summarized the performance characteristics of the LLNA: DA test method, which is detailed in the draft ICCVAM BRD.[17] Dr. Allen concluded by briefly summarizing the draft ICCVAM test method recommendations for the LLNA: DA test method.[18]

Panel Evaluation:

Dr. Michael Woolhiser thanked the Panel members of his Evaluation Group (i.e., Drs. Nathalie Alépeé, Thomas Gebel, Sidney Green [not present], and Jean Regal) for their tireless efforts in reviewing their Evaluation Group's assigned documents. He also thanked the NICEATM staff for their technical support during the review process. Dr. Woolhiser then presented the draft responses to ICCVAM's questions about this test method for consideration by the entire Panel. This included their review of the draft BRD for errors and omissions, their overall assessment of the validation status of the test method, and their comments on the draft ICCVAM test method recommendations. The Panel discussion and their recommended revisions to each section of the draft ICCVAM BRD are reflected in the Panel report, published in May 2008.[19]

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[17] http://iccvam.niehs.nih.gov/methods/immunotox/llna-DA/LLNA-DAbrd07Jan08FD.pdf
[18] http://iccvam.niehs.nih.gov/methods/immunotox/llna-DA/LLNA-DARecs07Jan08FD.pdf
[19] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

Adjournment—

The meeting was adjourned for the day at 5:03 p.m., to reconvene at 8:30 a.m., Wednesday, March 5, 2008.

WEDNESDAY, MARCH 5, 2008

Reconvening of the Panel Meeting

Dr. Luster reconvened the Panel Meeting at 8:30 a.m. He introduced himself and then asked that all Panel members, followed by all others in attendance, introduce themselves as well.

Overview of the Draft LLNA: DA BRD and Draft ICCVAM Test Method Recommendations

Panel Evaluation:

Dr. Woolhiser continued his presentation from the previous day of the draft responses to ICCVAM's questions to the Panel, for consideration by the entire Panel. The Panel discussion and their recommended revisions to each section of the draft ICCVAM BRD are reflected in the Panel report, published in May 2008.[20] Dr. Woolhiser indicated that the Evaluation Group had two main concerns with the LLNA: DA test method. The first concern related to pretreatment with 1% SLS and understanding how this impacted the biology of the response. Second, the time course of the study was different than the traditional LLNA because it extended the study by one day and included an additional challenge. This brought forth a question about the immunology of the response as it relates to the potential for elicitation and whether or not that is a significant change from the traditional LLNA, which is purely an induction model.

Public Comments:

Dr. George DeGeorge, MB Research Laboratories
In response to a question raised during the Panel discussion, Dr. DeGeorge commented that using lymph node weight as the readout to differentiate between sensitizers and non-sensitizers in the LLNA is problematic because although there are more lymph node cells packed into a node, each cell has less cytoplasm. The lymph nodes swell to a point, and then excrete water and become smaller lymphocytes that are countable. He cited examples from his laboratory with several different sensitizers, which demonstrate that lymphocytes in the node are smaller when a large SI (e.g., SI = 25) is obtained relative to when a smaller SI (e.g., SI = 3) is obtained.

Dr. DeGeorge also commented that he agreed with a point made during the Panel discussion that the LLNA: DA method and the LLNA: Bromodeoxyuridine Detected by ELISA (LLNA: BrdU-ELISA) method should be considered separately, because they are so dissimilar.

In his final comment, Dr. DeGeorge stated that in the traditional LLNA, in the LLNA: Bromodeoxyuridine Detected by Flow Cytometry (LLNA: BrdU-FC), and probably also in the LLNA: DA, strong sensitizing substances do not need to be administered three times. For instance, if one administers a single, moderately high dose of dinitrochlorobenzene (DNCB) (i.e., one that would induce an SI of 20 to 40) and then measures lymph node cell proliferation on day 1, 2, 3, or 4, an increase in the number of cells in the node and the number of cells that are positive for BrdU would likely be observed. Thus, administrations of additional applications have the potential to cause cumulative irritation. Dr. DeGeorge stated that the LLNA: DA method, which extends the assay to eight days instead of six days, should evaluate what happens to lymph node cell number at earlier

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[20] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

sample times. In addition, if the animals receive just one application using a high dose, with or without the SLS, is there an increase in the SI? If so, that would lead to the possibility that the extra applications are not necessary and might lead to cumulative irritation.

Dr. David Basketter, ECVAM Observer
Dr. Basketter made a statement that from a clinical perspective, substances are typically described as significant sensitizers or not significant sensitizers, and within that latter group some of the substances may indeed be non-sensitizing. Thus, just because a substance has been shown in an isolated case report to be a human sensitizer does not mean that there is sufficient evidence to consider it as positive for comparison with outcomes of predictive assays. It has to be of sufficient importance (i.e., potency) to trigger a positive classification. Dr. Basketter mentioned SLS, methyl salicylate, and isopropanol, as substances which will always be positive in some human cases although they shouldn't be positive in a predictive assay.

Dr. Basketter also commented that caution should be given to making sensitization assumptions based on chemical class references. As an example, eugenol and isoeugenol are structurally similar and have similar physical properties, but they act by different chemical reaction mechanisms and could fit into distinctly different chemical classes.

Dr. Basketter's last comment acknowledged that much work has been done in terms of validating the traditional LLNA. If one makes minor changes to the LLNA in terms of a different readout for proliferation, then they benefit from all the experience generated in validating the traditional LLNA and less effort is needed to prove that the minor modification is valid. In contrast, if more significant modifications are made, one cannot rely on that same experience. Dr. Basketter cautioned that more importance should be placed on distinguishing whether something has changed substantially enough such that you can no longer rely on the traditional LLNA as a reference.

Dr. Masahiro Takeyoshi, Chemicals Evaluation and Research Institute
Dr. Takeyoshi made a short presentation about differences in LLNA sensitization responsiveness among different strains of mice. He mentioned that this was an important issue when evaluating the modified LLNA methods being developed in Japan. He showed differences in responsiveness among three different mouse strains commonly used in Japan (i.e., BALB/cAnN, CBA/JN, and CD-1) tested with parabenzoquinone in his group's non-radioactive LLNA (i.e., LLNA: BrdU-ELISA). The data indicated that the CBA/JN mouse strain exhibited a higher responsiveness, as indicated by an increased SI, to parabenzoquinone than the other two mouse strains tested. Based on these results, CBA/JN mice were chosen for testing substances in the LLNA: BrdU-ELISA test method.
Dr. Takeyoshi also indicated that based on evaluating different SI cutoffs in the LLNA: BrdU-ELISA, 2-mercaptobenzothiazole, 3-(4-isopropylphenyl)isobutyraldehyde, and hydroxycitronellal had low responsiveness (i.e., SI values). He noted that 2-mercaptobenzothiazole is an OECD TG 429 recommended positive control for the LLNA; however, repeat tests could not detect this substance as positive when using an SI value of 1.7 or more. Dr. Takeyoshi suggested that a substance-specific lower response might exist in the test system. Dr. Takeyoshi also summarized LLNA data by Dr. Ullmann and coworkers with the contract lab RCC, Ltd. in which they investigated the responsiveness of six different mouse strains (CBA/CaOlaHsd, CBA/Ca (CruBR), CBA/Jlbm (SPF), CBA/JNCrj, BALB/c and NMRI) to 25% 2-mercaptobenzothiazole. The data indicated that CBA/JNCrj mice showed markedly lower responsiveness compared to the other strains tested. These studies indicate that strain related differences would not be negligible with regard to measuring different endpoints of cellular proliferation in the LLNA because depending on the chemicals tested, responsiveness might be potentially impacted. For instance, some of the discordance seen in the LLNA: DA test method (e.g., 2-mercaptobenzothiazole) could be a strain specific effect.

Panel Conclusions and Recommendations:

Dr. Luster asked the Panel to review their conclusions and recommendations and discuss any

revisions, if necessary. The Panel viewed the difference in treatment schedule between the LLNA: DA and the traditional LLNA to potentially be significant if the treatment schedule for the LLNA: DA corresponds to entering the elicitation phase of skin sensitization. The Panel was concerned that the 1% SLS pretreatment step in the LLNA: DA might modify the inherent sensitivity of the LLNA. They recommended that the test method developer (Daicel Chemical Industries, Ltd.) justify the use of 1% SLS or consider an alternative decision criterion (i.e., an SI threshold other than three) such that the 1% SLS pretreatment is no longer necessary. Dr. Luster asked the Panel if they agreed with the recommendations and conclusions that the Panel made along with the revisions; unanimously, the Panel agreed. The Panel's detailed recommendations and conclusions on the LLNA: DA test method are included in their final Panel report.[21]

Method Description and Overview of the LLNA: BrdU-FC Test Method

Dr. George DeGeorge, MB Research Laboratories, presented an overview of the LLNA: BrdU-FC test method. He stated that mice are dosed topically on the ears once daily for three consecutive days (i.e., days 1, 2, and 3), just like the traditional LLNA protocol. On day 6, the mice receive an intraperitoneal injection with bromodeoxyuridine (BrdU), and five hours later, the auricular lymph nodes are removed. The lymph nodes from individual animals are processed and, using flow cytometry, the number of BrdU-positive cells are counted from treated animals and compared to control animals as a measure of lymph node cell proliferation.

Dr. DeGeorge described in detail how the cells are processed and gated for flow cytometric analysis. He mentioned that the cells are also permeabilized and treated with propidium iodide which allows gates to be drawn around the G_0, G_1, S, and G_2M phases of the cell cycle. Dr. DeGeorge projected specific examples of flow cytometry plots and histograms for DNCB, hexyl cinnamic aldehyde (HCA), and positive and negative control data.

Dr. DeGeorge also described the tiered protocol for the assessment of sensitization potential using the LLNA: BrdU-FC and how ear swelling measurements and additional immunophenotypic endpoints (i.e., the enhanced LLNA: BrdU-FC) aid in distinguishing skin irritants from an irritating sensitizer.

Overview of the Draft LLNA: BrdU-FC BRD and Draft ICCVAM Test Method Recommendations

Dr. Judy Strickland, Integrated Laboratory Systems, Inc. (the NICEATM support contractor), presented an overview of the draft ICCVAM BRD for the LLNA: BrdU-FC test method. She stated that the draft ICCVAM BRD provided a comprehensive review of the available data and information regarding the usefulness and limitations of the LLNA: BrdU-FC test method. Specifically, the test method was reviewed for its ability to distinguish between sensitizers and non-sensitizers compared with the traditional LLNA. The objective of the BRD was to describe the current validation status of the LLNA: BrdU-FC test method, including its relevance and reliability, scope of substances tested, and the availability of a standardized protocol.

Dr. Strickland indicated that MB Research Laboratories submitted data to NICEATM for the 48 substances analyzed in the BRD in response to an FR notice (72 FR 27815, May 17, 2007) that requested such data. Dr. Strickland briefly summarized the performance characteristics of the LLNA: BrdU-FC test method, which is detailed in the draft ICCVAM BRD,[22] and the draft ICCVAM test method recommendations for the LLNA: BrdU-FC test method.[23]

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[21] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
[22] http://iccvam.niehs.nih.gov/methods/immunotox/fcLLNA/FC-LLNAbrd07Jan08FD.pdf
[23] http://iccvam.niehs.nih.gov/methods/immunotox/fcLLNA/FCLLNARecs07Jan08FD.pdf

Panel Evaluation:

Dr. Raymond Pieters, on behalf of his Evaluation Group, presented the Evaluation Group's review of the draft BRD and the draft test method recommendations for the LLNA: BrdU-FC test method. Specifically, he presented the draft responses to ICCVAM's questions to the Panel for consideration by the entire Panel. This included their review of the draft BRD for errors and omissions, their overall assessment of the validation status of this test method, and their comments on the draft ICCVAM test method recommendations. The Panel discussion and their recommended revisions to each section of the draft ICCVAM BRD are reflected in the Panel report, published in May 2008.[24] The applicability of the draft ICCVAM-recommended LLNA performance standards to the LLNA: BrdU-FC test method was discussed, particularly with regard to the number of substances tested in the LLNA: BrdU-FC method and whether more data would be necessary for review before the validation status of the assay could be determined. Dr. Stokes reminded the Panel that the proposed LLNA performance standards didn't exist when the studies for the LLNA: BrdU-FC test method were performed. The questions should be whether the adequacy of the substances that have been tested is sufficient or if more studies need to be done to cover any gaps that might exist (e.g., range of potencies or activity, chemical classes).

Public Comments

Dr. David Basketter, ECVAM Observer

Dr. Basketter commented on the statement that Dr. DeGeorge made during his overview of the LLNA: BrdU-FC test method that HCA is irritating. He said that he is not convinced it is a significant irritant. Based on previous data, they had to use 50% HCA in a 48 hour occlusive application in the guinea pig in order to produce a mildly irritating response. Dr. Api added to Dr. Basketter's comment by stating that RIFM has also not found HCA to be an irritant when tested up to 20% in humans.

Dr. Basketter also commented that in the draft BRD for the LLNA: BrdU-FC, resorcinol was noted to be negative in the traditional LLNA and this is not correct. Dr. Basketter's group published results in 2007 in the journal Contact Dermatitis that resorcinol is clearly positive in the traditional LLNA when tested at higher concentrations and therefore this should be corrected for the record.

Dr. George DeGeorge, MB Research Laboratories

Dr. DeGeorge wanted to clarify that the LLNA: BrdU-FC test method was compared to the traditional LLNA to determine if the LLNA: BrdU-FC was more predictive of skin-sensitization potential. He stated that in some cases it was better while in others it wasn't, but overall, using human data as the gold standard reference, the LLNA: BrdU-FC exceeded the traditional LLNA predictivity values and accuracy. He also noted that the additional endpoints included in the LLNA: BrdU-FC allow for them to distinguish irritating substances that typically are considered false positives in the LLNA.

Dr. DeGeorge also noted that since the LLNA: BrdU-FC is so similar to the traditional LLNA the issue of refinement and reduction in animal use is not immediately apparent but if the assay is done in as few as four mice per group with a periodic positive control (e.g., every six months) this represents a significant decrease in animal numbers compared to guinea pig tests. Furthermore, there is a refinement since mice are phylogenetically lower than guinea pigs, and undergo less pain and distress during the assay than guinea pigs undergo.

With regard to the discussion of coefficients of variation (CVs) and the 0.5x to 2.0x EC3 (i.e., the estimated concentration needed to produce a stimulation index of three) range, Dr. DeGeorge suggested that a larger range might be more reasonable because the current range is likely too restrictive.

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[24] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

Dr. George also noted that ICCVAM requires interlaboratory validation if a test method is to be transferred to other laboratories. With regard to the LLNA: BrdU-FC, it is a "me-too" assay and only has "minor" changes from the traditional LLNA and is currently only used in one laboratory. Therefore, the current dataset should suffice for determining the validity of the LLNA: BrdU-FC. In response to Dr. DeGeorge's comment, Dr. Stokes stated that if a method is only proposed to be used by one laboratory, having only intralaboratory data certainly would suffice but if it was proposed for broader use (e.g., adopted or endorsed by regulatory authorities), then other laboratories would have to demonstrate interlaboratory reproducibility. Dr. Luster asked if there was any mechanism available so that a company or small laboratory could apply for funding to help support an interlaboratory validation. Dr. Stokes indicated that they could nominate the test method for additional validation studies to ICCVAM. It would go through a nomination review process and a prioritization would be given to that. The nomination would then be considered by the member agencies as to whether funding would be provided.

Panel Conclusions and Recommendations:

Dr. Luster asked the Panel to review their conclusions and recommendations and discuss any revisions, if necessary. The Panel suggested that the utility of ear swelling or other methods to detect inflammation appeared warranted for inclusion in every variation of the LLNA (including the traditional LLNA), but should be further investigated before routine inclusion in the protocol is recommended. The Panel further agreed that the draft ICCVAM test method recommendations for future studies highlighted the unanswered questions raised by the available data set. Specifically, conducting interlaboratory studies as a part of the validation process is important.

The Panel considered the immunological markers suggested for the LLNA: BrdU-FC to be appropriate, but noted that other immunological markers for discrimination of irritant versus sensitization phenomena were also available. In general, for any future work, efforts should be made to decrease the variability and to thereby increase the power of the test in order to ensure that more animals were not needed relative to the traditional LLNA or other modified LLNA protocols.

Dr. Luster asked the Panel to indicate if they agreed with the recommendations and conclusions that the Panel made along with the revisions; the Panel unanimously agreed. The Panel's detailed recommendations and conclusions on the LLNA: BrdU-FC test method are included in their final Panel report.[25]

Method Description and Overview of the LLNA: BrdU-ELISA Test Method

Dr. Masahiro Takeyoshi, Chemicals Evaluation and Research Institute, presented an overview of the LLNA: BrdU-ELISA test method. He stated that the LLNA: BrdU-ELISA test method is very similar to the traditional LLNA test method. Unique to the LLNA: BrdU-ELISA test method, after test substance applications on days 1, 2, and 3, BrdU is injected interperitoneally on day 5. Approximately 24 hours after the BrdU injection, lymph nodes are collected, and detection of the amount of BrdU incorporated into the DNA of lymph node cells is conducted with an ELISA.

In the development process of this method, experiments were conducted to detect the most efficient injection schedule of BrdU. Based on the various injection schedules tested, a single injection protocol on day four was identified as the optimal injection schedule for BrdU administration.

Dr. Takeyoshi then showed a video of laboratory personnel preparing the lymph node cells for BrdU detection by ELISA. He went on to describe data for the LLNA: BrdU-ELISA compared to the traditional LLNA and how performance could be improved using alternative decision criteria (i.e., an SI other than three as the threshold for a positive response).

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[25] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

Overview of the Draft LLNA: BrdU-ELISA BRD and Draft ICCVAM Test Method Recommendations

Dr. Salicru presented an overview of the draft ICCVAM BRD for the LLNA: BrdU-ELISA test method. She noted that the draft ICCVAM BRD provided a comprehensive review of the available data and information regarding the usefulness and limitations of the LLNA: BrdU-ELISA test method. Specifically, the test method was reviewed for its ability to distinguish between sensitizers and non-sensitizers compared with the traditional LLNA and guinea pig test methods. The objective of the BRD was to describe the current validation status of the LLNA: BrdU-ELISA test method, including its relevance and reliability, scope of substances tested, and the availability of a standardized protocol.

Dr. Salicru stated that data from a total of 29 substances were considered in the accuracy analysis for the LLNA: BrdU-ELISA, and they were all tested in one laboratory. Dr. Salicru briefly summarized the performance characteristics of the LLNA: BrdU-ELISA test method, which are detailed in the draft ICCVAM BRD,[26] and the draft ICCVAM test method recommendations for the LLNA: BrdU-ELISA test method.[27]

Panel Evaluation:

Ms. Kim Headrick presented her Evaluation Group's (Drs. Anne Marie Api, Howard Maibach, Peter Theran, and Stephen Ullrich) review of the draft BRD and draft ICCVAM test method recommendations for the LLNA: BrdU-ELISA test method. Specifically, she presented the draft responses to ICCVAM's questions to the Panel for consideration by the entire Panel. This included their review of the draft BRD for errors and omissions, their overall assessment of the validation status of the test method, and their comments on the draft ICCVAM test method recommendations. The Panel discussion and their recommended revisions to each section of the draft ICCVAM BRD are reflected in the Panel report, published in May 2008.[28]

Public Comments:

Dr. David Basketter, ECVAM Observer

Dr. Basketter noted that when the traditional LLNA was first suggested as an alternative to the guinea pig tests, it went through a comprehensive validation process, and one of the concerns was that it should perform reliably and distinctly better than the guinea pig assays. He emphasized that this point should be kept in mind when thinking about the modified LLNA protocols with alternative endpoints that are currently being reviewed. He stated that the current rigor of examination for the modified LLNA protocols being reviewed for validation is higher than that for the traditional LLNA. He speculated that in the not-too-distant future, *in vitro* alternatives are likely to be going through a similar review process and it is going to become ever more difficult to put these alternatives in place, not because there is ill-will against the selections but because of the high standard of being good scientists. Thus, it is important that pragmatic decisions are made using the tools that are available.

Dr. George DeGeorge, MB Research Laboratories

Dr. DeGeorge commented that he agreed with Dr. Basketter's statements. He said that based on his experience in this peer review process, it is unlikely that he would bring any of the three *in vitro* test methods that MB Research Laboratories is developing for consideration by ICCVAM, given the many high hurdles that have to be negotiated.

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[26] http://iccvam.niehs.nih.gov/methods/immunotox/llna-ELISA/BrdUELISAbrd07Jan08.pdf
[27] http://iccvam.niehs.nih.gov/methods/immunotox/llna-ELISA/BrdUELISARecs07Jan08FD.pdf
[28] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

In response to the comments by Drs. Basketter and DeGeorge, Dr. McDougal commented that it does not seem unreasonable to raise the bar for what is expected of new or modified tests. Dr. Luster added that understandably, the focus on animal refinement and reduction is paramount, but that as scientists we have to ensure that the bar is maintained sufficiently high so that as the years go by scientific quality is not compromised.

Panel Conclusions and Recommendations:

Dr. Luster asked the Panel to review their conclusions and recommendations and discuss any revisions, if necessary. The Panel concluded that the available data and test method performance for the LLNA: BrdU-ELISA support the draft ICCVAM test method recommendations that it may be useful for identifying substances as potential skin sensitizers and non-sensitizers, but that more information and existing data must be made available before the LLNA: BrdU-ELISA can be recommended for use. The Panel also stated that a detailed protocol was needed, in addition to sufficient quantitative data for broader analysis on a larger set of balanced reference substances that take into account physicochemical properties and sensitization potency, as well as an appropriate evaluation of interlaboratory reproducibility.

The Panel's main concern with this test method was that the accuracy of the LLNA: BrdU-ELISA at SI $\geq$ 3 was inadequate and not equivalent to the traditional LLNA. Furthermore, although using a decision criterion of SI $\geq$ 1.3 improved the test's performance in identifying sensitizers from non-sensitizers, it did not resolve concerns about the test method, particularly considering that power calculations suggest a much larger number of animals per group would be required to identify a positive response. Thus, the Panel also concluded that it might be more appropriate to use a statistically based decision criterion rather than a stimulation index to classify substances as sensitizers, and that this should be further investigated. Dr. Luster asked the Panel to indicate if they agreed with the recommendations and conclusions that the Panel made along with the revisions; unanimously, the Panel agreed. The Panel's detailed recommendations and conclusions on the LLNA: BrdU-ELISA test method are included in their final Panel report.[29]

Overview of the Draft ICCVAM Performance Standards for the LLNA

Dr. Allen presented an overview of the draft ICCVAM Performance Standards for the LLNA. He briefly summarized the overall purpose of performance standards (i.e., to provide a basis for evaluating the performance of a proposed test method that is mechanistically and functionally similar to the validated test method) and the three elements encompassed within such performance standards (i.e., essential test method components, a minimum list of reference substances, and accuracy/reliability values). He noted that the proposed applicability of these draft ICCVAM LLNA performance standards is for the evaluation of LLNA protocols that deviate from the ICCVAM-recommended LLNA protocol only with respect to the method for assessing lymphocyte proliferation (e.g., using non-radioactive instead of radioactive reagents). Dr. Allen then provided an overview of the essential test method components, the minimum list of reference substances, and the accuracy/reliability values as detailed in the draft ICCVAM LLNA Performance Standards.[30]

Panel Evaluation:

Dr. Woolhiser, on behalf of his Evaluation Group, presented the Evaluation Group's responses to the ICCVAM questions asked about the draft ICCVAM LLNA Performance Standards for the entire Panel to consider. The overall question for the Panel was whether these performance standards were considered adequate for assessing the accuracy and reliability of test method protocols that were based on similar scientific principles and that measured the same biological effect as the traditional

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[29] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
[30] http://iccvam.niehs.nih.gov/methods/immunotox/PerfStds/LLNAPerfStd07Jan08FD.pdf

LLNA. The Panel discussion and their recommended revisions to the draft ICCVAM LLNA Performance Standards are reflected in the Panel report published in May 2008.[31]

Adjournment—

The meeting was adjourned at 5:42 p.m., to reconvene at 8:30 a.m., Thursday, March 6, 2008.

THURSDAY, MARCH 6, 2008

Reconvening of the Panel Meeting

Dr. Luster reconvened the Panel Meeting at 8:30 a.m. He introduced himself and then asked that all Panel members and all others in attendance introduce themselves as well.

Overview of the Draft ICCVAM LLNA Performance Standards

Panel Evaluation:

Dr. Woolhiser reviewed some of the important points highlighted during the previous day's discussion on this topic, and then continued to summarize the remaining comments of his Evaluation Group on the questions asked by ICCVAM on the draft ICCVAM LLNA Performance Standards for consideration by the entire Panel. As mentioned above, the Panel discussion and their recommended revisions to the draft ICCVAM LLNA Performance Standards are reflected in the Panel report published in May 2008.[32]

Dr. Woolhiser noted that there were general comments on the topic order for the Panel's review. He asked if Dr. Stokes would comment on the rationale for the topic order. Dr. Stokes indicated that as the IWG deliberated the order of topics for this review, consideration was given to the fact that the three non-radioactive methods had undergone validation studies prior to the creation of LLNA performance standards. Thus, the non-radioactive test methods were reviewed before the performance standards, so as to not bias the Panel's assessment of each test method's performance. The performance standards could then be considered for their application to future test methods.

Public Comments:

Dr. Amy Rispin, EPA

Dr. Rispin stated that her intent was to provide some additional regulatory perspective on some of the points that have been discussed. When Federal agencies evaluate the validation status of a test method under ICCVAM, they conduct a comprehensive analysis of overall performance (i.e., accuracy and reliability) in the context of making regulatory decisions with data from the test method. Thus, in a regulatory situation, equal or greater accuracy compared to the reference test method is the expectation. If the number of animals can be decreased only at the expense of accuracy, the acceptability of such a test method for the particular regulatory purpose would need to be carefully considered. Certain methods, instead of being complete replacements, might have to be relegated to the role of screens, where positives would be accepted, but negatives would require further testing - a less than ideal situation.

Dr. Rispin commented that performance standards are the regulating agencies' basis for the acceptability of variations of accepted test methods. If an agency receives data from a modified LLNA method that has not been reviewed and validated in the ICCVAM process, there is unlikely to be a comprehensive peer review of it within the agency, given resource limitations. Therefore, the question of major versus minor departures from the functional criteria is important to ICCVAM and

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[31] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
[32] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

its member agencies. One cannot anticipate that there will be anything other than these performance standards to adequately evaluate the usefulness and limitations of a new method.

Dr. David Basketter, ECVAM Observer
Dr. Basketter first commented on a point that Dr. Thomas Gebel alluded to during the Panel's discussion of the draft ICCVAM LLNA Performance Standards, which was that if a new laboratory performed the traditional LLNA to assess 18 or 22 chemicals, they probably wouldn't get a complete match. Dr. Basketter disagreed with Dr. Gebel's statement and viewed that a competent laboratory performing the LLNA would get it 100% correct.

Dr. Basketter then provided some comments that he stated were "from the ECVAM perspective." He stated that the ECVAM performance standards tried to address adhering to a standard protocol and that any change to the protocol other than the method for evaluating lymph node proliferation (e.g., strain, species, number of applications, time) was considered not to be minor, and therefore such a protocol would not be applied to these performance standards. By restricting the performance standards to minor changes, ECVAM was trying to minimize the number of chemicals required to evaluate sensitivity. Furthermore, the EC3 value could be used to see if the test method could classify substances in the appropriate range of sensitization potency.

ECVAM initially chose their reference substances in order to determine whether a modified method (differing only in the method for measuring cell proliferation) would give the same answer as the traditional LLNA. Thus, there was no intent to compare to the guinea pig or human data.

Dr. Basketter speculated that it is doubtful that data from multiple LLNA studies on the same substance are available and therefore it is unlikely that much larger sample sizes from which to calculate mean EC3 values and associated ranges will be obtained.

Dr. Basketter concluded by stating that ECVAM will not include more false positives and false negatives in its list. It has included one false positive and false negative in order to harmonize with ICCVAM but they don't see an added statistical value of just having one more false positive and false negative.

Karen Hamernik, EPA
Dr. Hamernik concurred with the comments that Dr. Rispin made previously, that performance standards, if developed such that they are too generalized with respect to minor versus major changes, would be problematic for regulatory agencies when they are reviewing submissions that include data from a modified LLNA protocol. Dr. Hamernik also asked for clarification from the Panel on a statement made during their discussions that a test for concordance for measuring the accuracy of classification (i.e., yes/no answer) should be done and that a chemical-for-chemical match is not necessary. Dr. Flournoy responded that concordance is not absolute but a continuum. Dr. Luster further clarified that the Panel discussion was based on the fact that the traditional LLNA is not a perfect match when compared to the guinea pig tests. Because there are false negatives and false positives compared to the guinea pig, there should be some flexibility so that an absolute chemical-by-chemical match is not required. In addition, a scientifically valid explanation can be provided for any discordance. Dr. Stokes emphasized that this was an important point and that additional clarity on the differences between a chemical-by-chemical match and overall accuracy need to be carefully considered before the final test method accuracy requirements are defined.

Panel Conclusions and Recommendations:

Dr. Luster asked the Panel to review the conclusions and recommendations for the ICCVAM LLNA performance standards they had discussed earlier and to make any revisions, if necessary. The Panel indicated that modified LLNA protocols that are undergoing validation should contain essential test

method components that follow the ICCVAM-recommended protocol,[33] unless adequate scientific rationale for deviating from this protocol was provided. The Panel also identified aspects of the LLNA that should be required as part of the test method validation process, if more extensive changes to the protocol are being considered: (1) application of the test substance to the skin with sampling of the lymph nodes draining that site, (2) measurement of cell proliferation in the draining lymph node, (3) absence of a skin reaction that could be indicative of the onset of the elicitation phase of skin sensitization, (4) data collected at the level of the individual animal to allow for an estimate of the variance within control and treatment groups,[34] and (5) if dose response information is needed, there are an adequate number of dose groups ($n \geq 3$) with which to accurately characterize the dose response for a given test substance.

The Panel also recommended that statistical tests to analyze the data might allow for a more accurate interpretation. They recommended that a suitable variance-stabilizing transformation (e.g., log transformation, square root transformation) be applied in all statistical analyses and in reporting summary standard deviations. The Panel also recommended that a more rigorous evaluation be conducted of what would be considered an appropriate range of ECt values (i.e., estimated concentration needed to produce a stimulation index that is indicative of a positive response) to include as a requirement. This would be a statistical evaluation that considers the variability of ECt values generated among the sensitizers included on the performance standards reference substances list and the statistical multiple comparisons problem.

Dr. Luster asked the Panel if they agreed with the changes and revisions made at this point and with the Panel conclusions and recommendations as presented and revised. The members of the Panel agreed with one abstention; Dr. McDougal abstained from voting stating that he still had a concern about what constitutes a "major/minor" change. The Panel's detailed recommendations and conclusions on the ICCVAM LLNA performance standards are included in their final Panel report.[35]

Overview of the Draft LLNA Potency Determinations BRD and Draft ICCVAM Test Method Recommendations

Dr. Strickland presented an overview of the draft ICCVAM BRD for the use of the LLNA to determine skin-sensitization potency. She mentioned that the draft ICCVAM BRD provided a comprehensive review of the available data and information regarding the usefulness and limitations of the LLNA as a stand-alone assay for hazard categorization of skin-sensitization potency. In the BRD, the LLNA was evaluated for its ability to categorize substances for skin-sensitization potency using EC3 values.

Dr. Strickland noted that the analyses conducted in the BRD were based on LLNA studies obtained from ICCVAM (1999), the published literature, and data received in response to an FR notice (72 FR 27815, May 17, 2007) requesting original data from the LLNA. As a result, the analyzed data included 170 substances with LLNA, human, and/or guinea pig data. Dr. Strickland noted that three sets of data were analyzed and briefly summarized the results which are detailed in the draft ICCVAM BRD.[36] Dr. Strickland also briefly summarized the draft ICCVAM test method recommendations for potency determinations.[37]

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[33] http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/LLNAProt.pdf

[34] Individual animal data will allow the application of a formal statistical test, if deemed necessary, and will also allow power calculations associated with the modified LLNA test.

[35] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

[36] http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-pot/LLNApotency18Jan08FD.pdf

[37] http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-pot/LLNAPotencyRecs18Jan08FD.pdf

Panel Evaluation:

Ms. Headrick presented her Evaluation Group's draft responses to ICCVAM's questions to the Panel for consideration by the entire Panel. These included their review of the draft BRD for errors and omissions, their overall assessment of the validation status of the test method, and their comments on the draft ICCVAM test method recommendations. The Panel discussion and their recommended revisions to each section of the draft ICCVAM BRD and recommendations are reflected in the Panel report published in May 2008.[38]

During the course of the discussion on the potency applicability of the LLNA, Dr. Woolhiser asked what the basis for the human threshold concentration cutoff values of 250 and 500 $\mu g/cm^2$ were. Dr. Wind replied that a number of experts and clinicians from throughout the world went back and looked at what, in their countries, they demarcated as strong sensitizers. The proposed Globally Harmonized System of Classification and Labeling of Chemicals (GHS) subcategory guidance values for the LLNA, guinea pig tests (GPMT, BT) and human data (HMT and HRIPT) were made on the basis of an impact analysis of 175 chemicals. In addition, the two proposed cut-offs were evaluated by the GHS Expert Group on Sensitization based upon chemicals already regulated as strong sensitizers to ensure their inclusion within the GHS categorization scheme. Clinical members of the Expert Group also confirmed relevance of the cut-off values such that clinically important skin sensitizers fell into the appropriate subcategory. The proposed guidance values were also in line with the European Commission's Expert Working Group recommendations.

Public Comments:

Dr. David Basketter, ECVAM Observer

Dr. Basketter commented that reviewing the potency data by splitting it into pooled and unpooled groups could be interesting but might be difficult since the majority of available data likely comes from pooled groups. Furthermore, much of the deliberation concluding that individual animal data must be used was derived from analyses based only or largely on pooled data from four animals.

Dr. Basketter further stated that he viewed the analyses, which make the assumption that the human threshold data is the gold standard, as fundamentally flawed. Human data comes from studies conducted at different times, with different protocols, according to varying quality standards, and by different people. Therefore, there is no definitive knowledge of the reproducibility of the data. However, he considers the analyses adequate for recommending the LLNA as a part of a weight-of-evidence decision on human sensitization potency categorizations.

Dr. Amy Rispin, EPA

Dr. Rispin noted that there has been much discussion about various ways of handling the potency data. The OECD expert task force on skin sensitization needs to see an analytical comparison of what is considered to be the most appropriate approach for evaluating the data. The question for categorization purposes is, *What is the ideal testing modality for separating strong versus weak sensitizers for potency categorization*? A regulator who must assign a categorization is going to be confronted with all available test data and must know which data should be given the greatest weight in their evaluation.

Dr. Rispin noted that the OECD task force also reviewed the draft BRD on potency determinations and sent a list of several questions to the Panel, some of which have been answered, many of which have not been. One of the questions is, can the LLNA protocols be refined (e.g., by selection of solvents or choice of other test parameters) to improve correlation? She concluded by noting that she hopes that the additional analyses that the Panel has suggested will bring some clarity to the matter.

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[38] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

Panel Conclusions and Recommendations:

Dr. Luster asked the Panel to review the conclusions and recommendations for the LLNA potency determinations they had discussed earlier and to make any revisions, if necessary. The Panel agreed with the draft ICCVAM recommendation that the LLNA should not be used as a stand-alone assay for categorizing skin sensitizers as strong versus weak, but that it could be used as part of a weight-of-evidence evaluation (e.g., along with quantitative structure-activity relationships, peptide reactivity, human evidence, historical data from other experimental animal studies) for this purpose. The Panel also agreed with ICCVAM's recommendation that any LLNA studies conducted for the purpose of evaluating skin-sensitization potency should use the ICCVAM-recommended LLNA protocol. In addition, the Panel stated that the relevant testing guidelines for the traditional LLNA should be revised to include the procedure for calculating an EC3 value. Dr. Luster asked the Panel if they agreed with the changes and revisions made at this point and with the Panel conclusions and recommendations as presented and revised; the Panel unanimously agreed. The Panel's detailed recommendations and conclusions on the LLNA potency determinations are included in their final Panel report.[39]

Concluding Remarks—

Dr. Luster, on behalf of the Panel, thanked the NICEATM-ICCVAM staff for their continued assistance during the review process and the Panel meeting. He also thanked Drs. Joanna Matheson and Abby Jacobs, the IWG co-chairs, and Dr. Marilyn Wind, ICCVAM Chair and IWG member, for the hard work they put into the project. Dr. Luster also thanked the Panel and the Panel Chairs for their involvement in the huge task of reviewing seven topics. He commented that, for future reference for ICCVAM, the Panel in their individual groups were able to do a good job in reviewing the materials, but because they were so focused on their particular topics due to serious time constraints, there may not have been the full benefit of their expertise for other topics in all cases.

Drs. Wind and Stokes thanked the Panel again for their hard work, thoughtful and objective deliberations, and advice. Dr. Stokes further thanked the invited test method developers for their excellent summaries of their method for the benefit of the Panel, and CPSC for hosting the Panel meeting. He mentioned that there has been discussion about obtaining additional existing data (i.e., on mixtures, on one or more of the non-radiolabeled test methods), and that should these data become available in a timely manner and if NICEATM is able to assimilate and analyze the data, the Panel might be reconvened by teleconference to review the data. Dr. Stokes concluded by saying he looked forward to further working with the Panel members to complete their Panel report.

Adjournment—

The meeting was adjourned and concluded at 3:20 p.m.

[39] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

William S. Stokes, D.V.M.
NIEHS
P.O. Box 12233
MD-EC17
Research Triangle Park, NC 27709

Dear Dr. Stokes,

The Meeting Summary Minutes, Independent Scientific Peer Review Panel Meeting, Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products, accurately summarizes the Peer Review Panel meeting of March 4-6, 2008, in Bethesda, MD.

Sincerely,

Signature	Printed Name	Date
	MICHAEL I LUSTER	11-18-08

Appendix E2

Peer Review Panel Report: Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products

This document is available at:
https://ntp.niehs.nih.gov/iccvam/docs/immunotox_docs/llnaprprept2008.pdf

The document is also available on request from NICEATM:

NICEATM
National Institute of Environmental Health Sciences
P.O. Box 1233, MD K2-16
Research Triangle Park, NC 27709 USA
Telephone: 919-541-2384 Fax: 919-541-0947
E-mail: niceatm@niehs.nih.gov

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Appendix E3

Summary Minutes of Independent Scientific Peer Review Panel Meeting on April 28-29, 2009

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Summary Minutes

Independent Scientific Peer Review Panel Meeting

Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Evaluation of the Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA)

William H. Natcher Conference Center
National Institutes of Health
Bethesda, MD
April 28 - 29, 2009
8:30 a.m. - 5:30 p.m.

Peer Review Panel Members:

Michael Luster, Ph.D. (Peer Review Panel Chair)	Senior Consultant to the NIOSH Health Effects Laboratory, Morgantown, WV
Nathalie Alépée, Ph.D.	Scientific Coordinator on Alternatives Methods in Life Science, L'Oréal Research and Development, Aulnay sous Bois, France
Anne Marie Api, Ph.D.	Vice President, Human Health Sciences, Research Institute for Fragrance Materials, Woodcliff Lake, NJ
Nancy Flournoy, M.S., Ph.D.	Professor and Chair, Dept. of Mathematics and Statistics, University of Missouri – Columbia, Columbia, MO
Dagmar Jírová, M.D., Ph.D.	Toxicologist, Research Manager, Head of Reference Center for Cosmetics, Head of Reference Laboratory for Experimental Immunotoxicology, National Institute of Public Health, Czech Republic
David Lovell, Ph.D.	Reader in Medical Statistics, Postgraduate Medical School, University of Surrey, Guildford, Surrey, U.K.
Howard Maibach, M.D.	Professor, Dept. of Dermatology, University of California – San Francisco, San Francisco, CA
Michael Olson, Ph.D.	Director of Occupational Toxicology, Corporate Environment Health and Safety, GlaxoSmithKline, Research Triangle Park, NC

Peer Review Panel Members:

Raymond Pieters, Ph.D.[40]	Associate Professor, Immunotoxicology Group Leader, Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands
Jean Regal, Ph.D.	Professor, Dept. of Pharmacology, University of Minnesota Medical School, Duluth, MN
Jonathan Richmond, MB ChB, FRCSEd	Head, Animals Scientific Procedures Division, Home Office, London, U.K.
Peter Theran, V.M.D.	Massachusetts Society for the Prevention of Cruelty to Animals, Novato, CA
Stephen Ullrich, Ph.D.	Dallas/Ft. Worth Living Legends Professor and Professor of Immunology, Postgraduate School of Biomedical Science, University of Texas M.D. Anderson Cancer Center, Houston, TX
Michael Woolhiser, Ph.D.	Science and Technology Leader – Toxicology and Environmental Research and Consulting, The Dow Chemical Company, Midland, MI
Takahiko Yoshida, M.D., Ph.D.	Professor, Dept. of Health Science, Asahikawa Medical College, Hokkaido, Japan

ICCVAM and ICCVAM Immunotoxicity Working Group Members:

Paul Brown, Ph.D.	FDA, Center for Drug Evaluation and Research, Silver Spring, MD
Masih Hashim, Ph.D.	EPA, Office of Pesticide Programs, Washington, DC
Ying Huang, Ph.D.	FDA, Center for Biologics Evaluation and Research, Silver Spring, MD
Abigail Jacobs, Ph.D. (IWG Co-Chair)	FDA, Center for Drug Evaluation and Research, Silver Spring, MD
Jodie Kulpa-Eddy, D.V.M.	USDA, Animal and Plant Health Inspection Service, Riverdale, MD
Elizabeth Margosches, Ph.D.	EPA, Office of Pollution Prevention and Toxics, Washington, DC
Joanna Matheson, Ph.D. (IWG Co-Chair)	CPSC, Bethesda, MD

[40] Dr. Pieters was unable to attend the public meeting on April 28-29, 2009. However, he was involved in the review of the revised draft background review documents and the revised draft LLNA applicability domain Addendum.

ICCVAM and ICCVAM Immunotoxicity Working Group Members:

Deborah McCall	EPA, Office of Pesticide Programs, Washington, DC
Tim McMahon, Ph.D.	EPA, Office of Pesticide Programs, Washington, DC
John Redden, M.S.	EPA, Office of Pesticide Programs, Washington, DC
R. Adm. William Stokes, D.V.M., DACLAM	NIEHS, Research Triangle Park, NC
Ron Ward, Ph.D.	EPA, Office of Pollution Prevention and Toxics, Washington, DC
Marilyn Wind, Ph.D. (ICCVAM Chair)	CPSC, Bethesda, MD

Invited Experts:

George DeGeorge, Ph.D., DABT	MB Research Labs, Spinnerstown, PA
Kenji Idehara, Ph.D.	Daicel Chemical Industries, Ltd., Hyogo, Japan
Masahiro Takeyoshi, Ph.D.	Chemicals Evaluation and Research Institute, Saitama, Japan

JaCVAM Observer:

Hajime Kojima, Ph.D.	National Institute of Health Sciences, Tokyo, Japan

Public Attendees:

Joan Chapdelaine, Ph.D.	Calvert Laboratories, Inc., Olyphant, PA
Merrill Tisdel	Syngenta Crop Protection Inc., Greensboro, NC
Gary Wnorowski, M.B.A, L.A.T.	Eurofins Product Safety Labs

NICEATM:

R. Adm. William Stokes, D.V.M., DACLAM	Director
Debbie McCarley	Special Assistant to the Director

Contract Support Staff – Integrated Laboratory Systems, Inc. (ILS)

David Allen, Ph.D.	Eleni Salicru, Ph.D.
Thomas Burns, M.S.	Frank Stack

NICEATM:

Linda Litchfield Judy Strickland, Ph.D., DABT

Greg Moyer, M.B.A.

Abbreviations:

CPSC = U.S. Consumer Product Safety Commission

EPA = U.S. Environmental Protection Agency

FDA = U.S. Food and Drug Administration

ICCVAM = Interagency Coordinating Committee on the Validation of Alternative Methods

ILS = Integrated Laboratory Systems

IWG = Immunotoxicity Working Group

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

USDA = U.S. Department of Agriculture

Tuesday, April 28, 2009

Call to Order and Introductions

Dr. Michael Luster (Peer Review Panel Chair) called the meeting to order at 8:30 a.m. and introduced himself. He then asked all Peer Review Panel (hereafter Panel) members to introduce themselves and to state their name and affiliation for the record. He then asked all the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) staff, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) members, the ICCVAM Immunotoxicity Working Group (IWG) members, and members of the public to also introduce themselves. Dr. Luster stated that there would be opportunity for public comments during each of the four murine local lymph node assay (LLNA)-related topics. He asked that all those interested in making a comment register at the registration table and provide a written copy of their comments, if available, to NICEATM staff. Dr. Luster emphasized that the comments would be limited to seven minutes per individual and that, while comments from one individual would be welcomed during each commenting period, repeating the same comments at each comment period would be inappropriate.

Welcome from the ICCVAM Chair

Dr. Marilyn Wind, U.S. Consumer Product Safety Commission (CPSC) and Chair of ICCVAM, welcomed everyone to the National Institutes of Health and to the Panel meeting. Dr. Wind thanked the ICCVAM IWG and NICEATM staff for their efforts in preparing the draft documents being reviewed and for arranging the logistics of the meeting. Dr. Wind thanked the Panel members for dedicating their time, effort, and expertise to this review and acknowledged their important role to the ICCVAM test method evaluation process. Dr. Wind also emphasized the important role of the public and their comments in this process.

Welcome from the Director of NICEATM, and Conflict of Interest Statements

Dr. William Stokes, Director of NICEATM, stated the Panel meeting was being convened as an NIH Special Emphasis Panel and was being held in accordance with applicable U.S. Federal Advisory Committee Act regulations. As such, Dr. Stokes indicated that he would be serving as the Designated Federal Official for this public meeting. He reminded the Panel that they signed a conflict of interest (COI) statement during the Panel selection process, in which they identified any potential real or perceived COI. He read the COI statement and then Dr. Luster asked that panelists again declare any potential direct or indirect COI and to recuse themselves from discussion and voting on any aspect of the meeting where there might be a conflict.

Dr. Michael Woolhiser declared a COI regarding the Panel's review of the LLNA Applicability Domain, because The Dow Chemical Company, Dr. Woolhiser's employer, submitted much of the data that were being considered. He indicated that he would recuse himself from the Panel's evaluation of the applicability domain, but would remain available to answer any questions that the Panel might have about the test substances or the data.

Overview of the ICCVAM Test Method Evaluation Process

Dr. Stokes began by thanking the 15 Panel scientists from six different countries (Czech Republic, France, Japan, The Netherlands, United Kingdom, and the United States) for their significant commitment of time and effort preparing for and attending the meeting. He explained that the purpose of the Panel was to conduct an independent scientific peer review of the information provided on a series of proposed new versions of the LLNA and proposed expanded applications of the assay. The Panel is then asked to comment on the extent that the available information supports the draft ICCVAM recommendations. Dr. Stokes indicated that the original LLNA peer review panel in 1998 considered the LLNA a valid substitute for the guinea pig-based test in most but not all testing

situations. He noted that three Panel members from the 1998 review are also on the current Panel (i.e., Drs. Howard Maibach, Jean Regal, and Stephen Ullrich). Dr. Stokes also reviewed the nomination that was received from CPSC in January 2007,[41] which provides the basis for the current evaluation.

Dr. Stokes then identified the 15 Federal agencies that comprise ICCVAM and summarized ICCVAM's mission. He noted that ICCVAM, as an interagency committee, does not carry out research and development or validation studies. Instead, ICCVAM, in conjunction with NICEATM, carries out the critical scientific evaluation of the results of validation studies for proposed test methods to assess their usefulness and limitations for regulatory testing, and then makes formal recommendations to ICCVAM agencies.

Dr. Stokes provided a brief review of ICCVAM's history and summarized the ICCVAM Authorization Act of 2000,[42] including the purpose and duties of ICCVAM. He noted that one of ICCVAM's primary duties is to review and evaluate new, revised, and alternative test methods applicable to regulatory testing. He stated that all of the reports produced by NICEATM are available on the NICEATM-ICCVAM website or can be obtained upon request from NICEATM. He also mentioned that ICCVAM provides guidance on test method development, validation processes, and helps to facilitate not only the acceptance of scientifically valid alternative test methods, but also encourages internationally harmonized recommendations on the usefulness and limitations of alternative test methods.

Dr. Stokes then described the ICCVAM test method evaluation process, which begins with a test method nomination or submission. NICEATM conducts a prescreen evaluation to summarize the extent to which the proposed submission or nomination addresses the ICCVAM prioritization criteria. A report of this evaluation is then provided to ICCVAM, which in turn develops recommendations regarding the priority for evaluation. ICCVAM then seeks input on their recommendations from the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) and the public and determines whether the test method should move forward into a formal evaluation. If so, a draft background review document (BRD), which provides a comprehensive review of all available data and information, is prepared by NICEATM in conjunction with an ICCVAM working group designated for the relevant toxicity testing area (e.g., the IWG). In addition, ICCVAM considers all available information and develops draft test method recommendations on the proposed usefulness and limitations of the test methods, test method protocol, performance standards, and future optimization/validation studies. The draft BRD and the draft ICCVAM test method recommendations are made available to the Panel and the public for review and comment. The Panel peer reviews the draft BRD and evaluates the extent to which it supports the draft ICCVAM test method recommendations. A Panel report is published, which is then considered along with public and SACATM comments by ICCVAM in developing final recommendations. These final recommendations are forwarded to the ICCVAM member agencies for their consideration and possible incorporation into relevant testing guidelines. Agencies have 180 days to respond to the ICCVAM recommendations.

Dr. Stokes reviewed the ICCVAM criteria for adequate validation. He stated that validation is defined by ICCVAM as the process by which the reliability and relevance of a procedure are established for a specific purpose, and that adequate validation is a prerequisite for consideration of a test method by U.S. Federal regulatory agencies. Dr. Stokes listed the ICCVAM acceptance criteria for test method validation and acceptance. He concluded by summarizing the timeline of the review activities beginning with CPSC's nomination in January 2007 and ending with the present Panel meeting.

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[41] http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf
[42] http://iccvam.niehs.nih.gov/docs/about_docs/PL106545.pdf

ICCVAM Charges to the Panel

Dr. Stokes reviewed the charges to the Panel: (1) review the draft BRDs and the draft Addendum to the traditional[43] LLNA for completeness and identify any errors or omissions; (2) determine the extent to which each of the applicable criteria for validation and regulatory acceptance had been appropriately addressed for the proposed revised or modified versions of the LLNA; and (3) comment on the extent to which the ICCVAM draft test method recommendations including the proposed usefulness and limitations, standardized test method protocols, performance standards, and additional studies are supported by the information provided in the draft BRDs and draft Addendum.

Overview of the Agenda

Dr. Luster then reviewed the agenda and the order of presentations. He stated that for each review topic, the test method developer would present an overview of the test method protocol, followed by a presentation by NICEATM staff summarizing each revised draft BRD, and lastly a member of the IWG would present the draft ICCVAM recommendations. Following presentations, the Panel Evaluation Group Leader for the topic under consideration would present the group's draft recommendations, followed by Panel discussion. Public comments would then be presented, followed by the opportunity for additional Panel discussion in consideration of the public comments. The Panel would then vote to accept the Panel consensus, with any minority opinions being so noted with the rationale provided for the minority opinion.

Current Regulatory Testing Requirements and Hazard Classification Schemes for Allergic Contact Dermatitis (ACD) and the Traditional LLNA Procedure

Dr. Matheson presented an overview of ACD and relevant regulatory requirements. She briefly discussed the ICCVAM final recommendations for the LLNA Performance Standards, the updated ICCVAM LLNA test method protocol, and the reduced LLNA (rLLNA), all of which were reviewed by the Panel at their meeting in March 2008.

The Panel questioned who was responsible for conducting the future studies referred to in the revised draft ICCVAM test method recommendations. Dr. Stokes replied that these recommendations are provided for consideration by the stakeholder community. Those organizations with appropriate resources can use this information to guide their research, development, and validation activities.

A question arose from the Panel as to why pooled data (as opposed to individual animal data) are collected for the LLNA.

Dr. Matheson replied that, pooled data are often collected since OECD Test Guideline 429 allows the use of a minimum of four animals per treatment group when collecting pooled data, but requires a minimum of five animals per treatment group when collecting individual animal data. Legislation in some countries, and many Animal Care and Use Committees, require that the test method to be used is the one requiring the fewest animals. Dr. Matheson also noted that the ICCVAM LLNA test method protocol has recently been revised to allow the use of a minimum of four animals per treatment group when collecting individual animal data, so there is now no reason not to collect individual animal data. At the Panel meeting in March 2008, the Panel stated that all future LLNA studies should require that lymph nodes be collected from individual animals instead of pooling them

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[43] For the purposes of this document, the radioactive LLNA test method, which was first evaluated by ICCVAM in 1999, and subsequently recommended to U.S. Federal agencies as a valid substitute for currently accepted guinea pig test methods to assess the allergic contact dermatitis potential of many, but not all, types of substances, is referred to as the traditional LLNA.

with other animals in a treatment group since individual animal response data allows for identification of technical problems and outlier animals within a dose group.[44]

A question arose as to whether the U.S. Environmental Protection Agency (EPA) prefers LLNA or guinea pig data for submission. Dr. Matheson ceded the floor to Ms. Debbie McCall of EPA Office of Pesticide Programs, who was in attendance. Ms. McCall said that EPA prefers LLNA data, but will accept either guinea pig maximization test (GPMT) or Buehler test (BT) data.

Overview of the Revised Draft LLNA: DA Test Method Procedure BRD and Revised Draft ICCVAM Test Method Recommendations

The first test method reviewed was the LLNA: DA test method. This test method measures the ATP content of lymph node cells by the luciferin/luciferase method, as an index of lymphocyte proliferation, after exposure to a test substance.

Dr. Kenji Idehara of Daicel Chemical Industries, Ltd., Japan (the test method developer) presented a synopsis of the test method to the Panel.

A Panelist asked about the half-life of ATP in the lymph node cells after the mouse is sacrificed. Dr. Idehara replied that the ATP concentration declines 20 to 30% in an hour, with a half-life of about 2 to 2.5 hours. The assay time from animal sacrifice to complete measurement of ATP content for each individual animal is maintained as similar as possible, within approximately 30 min. He also said that the time between sacrifice and ATP assay is not a problem when collecting individual animal data, if the time between the excision of the lymph nodes, the preparation of the cell suspensions, and the measurement of the ATP concentrations is kept relatively constant between animals.

A Panelist asked if the lymph node samples were randomized before the ATP assays were conducted. Dr. Idehara replied that the samples were not randomized.

On behalf of NICEATM, Dr. Salicru presented an overview of the revised draft LLNA: DA BRD to the Panel.

A question arose about NICEATM's use of different decision criteria for the accuracy analysis, and the reproducibility analyses in the revised draft BRD. Dr. Salicru noted that a decision criterion of SI ≥ 2.5 was used for the reproducibility analyses because it was found to be the optimal decision criterion for identifying sensitizers (i.e., it resulted in a 0% false positive rate).

Dr. Wind presented the revised draft ICCVAM test method recommendations for the LLNA: DA test method to the Panel. She noted that ICCVAM favored the multiple decision criteria to eliminate any false positives or false negatives. A Panelist commented that, as more data are accumulated using the test method, false positives and false negatives might appear.

A Panelist asked, if the true stimulation index (SI) value for a compound was 2.0, if that compound would be classified as a sensitizer or a nonsensitizer. Dr. Wind replied that, as described in the revised draft ICCVAM recommendations, other information would be necessary to definitively answer that question.

Dr. Kojima presented the results of the Japanese Society for Alternatives to Animal Experiments (JSAAE) interlaboratory validation studies of the LLNA: DA and the LLNA: BrdU-ELISA test methods to the Panel. In the presentation, he noted that the JaCVAM Regulatory Acceptance Board has examined the results of the studies for both test methods and accepted the LLNA: DA as a replacement for the traditional LLNA. The JaCVAM Regulatory Acceptance Board has requested additional data for the LLNA: BrdU-ELISA.

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[44] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf

Panel Evaluation:

Dr. Woolhiser presented the draft position developed by Evaluation Group B, which was charged with primary review of the LLNA: DA test method. The Panel agreed that the available data and test method performance support the use of the LLNA: DA to identify substances as potential skin sensitizers and nonsensitizers, with certain limitations. They concurred with ICCVAM's proposal that, based on the current validation database, the multiple SI decision criteria should be used to identify sensitizers and nonsensitizers (i.e., SI $\geq$ 2.5 for sensitizers, SI $\leq$ 1.7 for nonsensitizers). The Panel also noted that the limitation of these test methods when using the proposed multiple decision criteria is the indeterminate classification of substances that fall in the range of SI values for which a classification is uncertain (i.e., 1.7 < SI < 2.5). The Panel recommended that when such results are obtained, users should carefully interpret the results using an integrated decision strategy in conjunction with all other available information (e.g., dose response and quantitative structure-activity relationship [QSAR] information, peptide-binding activity, molecular weight, results from related chemicals, other testing data) to determine if there is adequate information for an accurate sensitization hazard classification or if additional testing is necessary. The Panel emphasized that, from an animal welfare perspective, retesting should not be undertaken until all other available information is evaluated, and a determination is made that such testing is required to fill a data gap. The Panel also recommended that more detailed guidance be developed for regulatory agencies on how the multiple decision criteria could be used in practice.

Subsequent Panel discussions focused on ICCVAM's recommendation to use multiple decision criteria to identify sensitizers and nonsensitizers. In general, the Panel preferred the multiple decision criteria to a single decision criterion for identifying sensitizers and nonsensitizers. A Panelist recommended that graphs showing the maximum SI obtained with the modified test method (the LLNA: DA, in this case) plotted against the maximum SI obtained with the traditional LLNA, for each test substance, be included in the final BRD. This was a general recommendation for both test methods that use multiple decision criteria (i.e., the LLNA: DA and LLNA: BrdU-ELISA). It was also pointed out that, as more data are accumulated for these test methods, the cut-off SI values for sensitizers and nonsensitizers would likely change.

Bootstrapping analysis was mentioned as a means to provide some measure of variability of the chosen cut-off values. It was also mentioned that the tables in Section 7.0 of the revised draft BRD provide no measurement of variation for the data. It was suggested that all of these tables include treatment means, standard deviations, and the mean squares, so that F-values can be calculated for between and among laboratory means. However, the Panel agreed that, while this information would be useful for inclusion in the final BRD, it would not impact the Panel's overall conclusions about the test method.

Some discussion followed about variations in the LLNA: DA test method protocol from the updated ICCVAM-recommended traditional LLNA test method protocol (i.e., sodium lauryl sulfate pretreatment prior to test substance application and an additional test substance application on day 7). The Panel agreed that despite these variations, the LLNA: DA was still mechanistically and functionally similar to the traditional LLNA.

Public Comments:

At the conclusion of the Panel discussion, Dr. Luster called for public comments. None were presented.

Panel Conclusions and Recommendations:

Dr. Luster asked if the Panel was in agreement with the conclusions in the draft Panel Report as reflected in the updated Evaluation Group presentation as modified during the discussions. The Panel approved unanimously.

Applicability Domain of the LLNA and Revised Draft ICCVAM Test Method Recommendations

NICEATM provided an overview of the revised draft Addendum on the LLNA applicability domain. Subsequent to the 2008 Panel consideration of this topic, new data were obtained for pesticide formulations, dyes, essential oils, and substances tested in aqueous solution, but none were obtained for metals. Since the Panel previously considered the use of the term *mixtures* too broad, data were separately evaluated by product subgroups in the revised draft Addendum, and they were identified in general terms as pesticide formulations and other products. Dr. Wind presented the revised draft ICCVAM test method recommendations for the LLNA applicability domain to the Panel.

Subsequent to Dr. Wind's presentation, Dr. Luster asked Ms. McCall of EPA to clarify EPA's position on the use of LLNA data for pesticide formulations. Ms. McCall replied that EPA accepted positive or negative LLNA data on single substance technical grade additives. Between 2003 and 2007, EPA received few LLNA studies on pesticide formulations. Positive LLNA results were accepted, but for negative results, EPA required a confirmatory test. The majority of sensitization data submitted to EPA for pesticide formulations are from the guinea pig BT. There are limited human data available on pesticides due to the ethics limitations for conducting human studies, and applicants provide all of EPA's data.

A Panelist commented that the GPMT is more sensitive that the BT; he said that, in his experience, the GPMT showed roughly 60% positive results versus 20% positive results for the BT, for the same group of formulations. He said that the LLNA is more concordant with the GPMT than it is with the BT. He said that the GPMT is the preferred test in Europe. The Panel agreed that this should be reflected in the comparisons of LLNA and guinea pig results.

Panel Evaluation:

Dr. Olson presented the draft position developed by Evaluation Group A, which was charged with primary review of the LLNA applicability domain, to the Panel. While the Panel agreed that there were too few data in the revised draft Addendum for some of the test substance classes (e.g., dyes, essential oils) to make a firm statement about concordance of the LLNA with other test methods for these classes, the Panel stated that any material should be suitable for testing in the LLNA unless there is a biologically-based rationale for exclusion, such as unique physicochemical properties that might affect their ability to interact with immune processes. The Panel therefore agreed that the LLNA should be considered appropriate for testing pesticide formulations and other products, unless there is a biologically-based rationale for exclusion.

The Panel also concurred that, while studies done with BALB/c mice should not be excluded from the evaluations in the revised draft Addendum, CBA should remain the preferred strain for the updated ICCVAM-recommended LLNA test method protocol, and that the use of any other strain, or of male rather than female mice, should be justified by the investigator.

The Panel did not agree that Pluronic L92 should be added to the list of preferred vehicles for the LLNA, but it did agree that studies done with Pluronic L92 should not be excluded from the evaluations in the revised draft Addendum.

While the concordance of LLNA results for essential oils was properly compared with human results, the Panel noted that the revised draft Addendum neglected to consider information that showed LLNA results were more concordant with human results when the major component was ≥70%, compared to the concordance for the essential oil itself. The Panel also commented that the term *natural complex substances* was more appropriate for these types of substances than *essential oils*, because this is the terminology used for the Registration, Evaluation, Authorisation and Restriction of Chemical substances program now in force in the European Union (EU).

In reference to the data for the medical device eluates in the revised draft Addendum, the Panel commented that ISO Standard 1099 requires the chemical analysis of such materials before skin sensitization testing is undertaken, and therefore agreed that the data provided were of little use for evaluating the performance of the LLNA for testing these types of substances.

Public Comments:

At the conclusion of the Panel discussion, Dr. Luster called for public comments.

Mr. Gary Wnorowski, Eurofins Product Safety Labs
Mr. Gary Wnorowski said he had registered to make a public comment, but that Ms. McCall of EPA had already addressed his question by her answer to Dr. Luster's question regarding acceptability of pesticide formulation data.

Panel Conclusions and Recommendations:

Dr. Luster asked if the Panel was in agreement with the conclusions in the draft Panel Report as reflected in the updated presentation. The Panel approved unanimously.

Adjournment

At the conclusion of the discussion on the applicability domain, Dr. Luster adjourned the Panel for the day at 5:30 p.m., to reconvene at 8:30 a.m. on Wednesday, April 29, 2009.

Wednesday, April 29, 2009
Overview of the Draft LLNA: BrdU-ELISA Test Method Revised Draft BRD and Revised Draft ICCVAM Test Method Recommendations

Dr. Luster called for Panel consideration of the LLNA: BrdU-ELISA test method. This test method measures bromodeoxyuridine (BrdU), a thymidine analog, instead of radioactive thymidine, incorporated into the DNA of proliferating lymphocytes, via an enzyme-linked immunosorbent assay (ELISA).

Dr. Masahiro Takeyoshi of Chemicals Evaluation and Research Institute, Japan (the test method developer) presented a synopsis of the test method to the Panel.

On behalf of NICEATM, Dr. Strickland presented an overview of the revised draft ICCVAM LLNA: BrdU-ELISA BRD to the Panel.

A Panelist asked why ICCVAM proposes an SI value of 2.0 as the cutoff value for a sensitizer instead of a value of 2.5, since the data indicated that no false positives would result if either value were used. Dr. Strickland replied that the value of 2.0 was chosen because this was the lowest value that resulted in a 0% false positive rate, thus minimizing the range of uncertainty.

Dr. Jacobs presented the revised draft ICCVAM test method recommendations for the LLNA: BrdU-ELISA test method to the Panel.

Panel Evaluation:

Dr. Ullrich presented the draft position developed by Evaluation Group B, which was charged with primary review of the LLNA: BrdU-ELISA test method, to the Panel.

The Panel agreed that the LLNA: BrdU-ELISA test method was mechanistically and functionally similar to the traditional LLNA, and the ICCVAM LLNA Performance Standards could be used to evaluate it. The Panel also concurred that the available data and test method performance support the use of the LLNA: BrdU-ELISA to identify substances as potential skin sensitizers and nonsensitizers, with certain limitations. They agreed with ICCVAM's proposal that, based on the current validation database, the multiple SI decision criteria should be used to identify sensitizers and nonsensitizers

(i.e., SI ≥ 2.0 for sensitizers, SI > 1.3 for nonsensitizers). The Panel also noted that the limitation of these test methods when using the proposed multiple decision criteria is the indeterminate classification of substances that fall in the range of SI values for which a classification is uncertain (i.e., 2.0 > SI ≥ 1.3). The Panel recommended that when such results are obtained, users should carefully interpret the results in an integrated decision strategy in conjunction with all other available information (e.g., dose-response and QSAR information, peptide-binding activity, molecular weight, results from related chemicals, other testing data) to determine if there is adequate information for an accurate sensitization hazard classification or if additional testing is necessary. The Panel emphasized that, from an animal welfare perspective, retesting should not be undertaken until all other available information is evaluated, and a determination is made that such testing is required to fill a data gap. The Panel also recommended that more detailed guidance be developed for regulatory agencies on how the multiple decision criteria could be used in practice.

Subsequent Panel discussions focused on ICCVAM's recommendation to use multiple decision criteria to identify sensitizers and nonsensitizers. In general, the Panel preferred the multiple decision criteria to a single decision criterion for identifying sensitizers and nonsensitizers. The Panel agreed that all of the comments for the LLNA: DA test method regarding the graphs and tables in the revised draft BRD, and the provision of measures of variation for interlaboratory reproducibility data, apply to the BrdU-ELISA also.

A Panelist commented that the use of interpolation for determining ECt values presupposed a monotonic increase in SI values and that isotonic regression might be more appropriate in cases in which a monotonic increase does not occur. More Panel discussion occurred regarding the practical usefulness of the multiple decision criteria. It was agreed that the term *integrated assessment* was more appropriate than *weight-of-evidence* to describe the approach taken to classify substances that fell into the uncertainty range.

The Panel discussed when it was appropriate to rely on hypothesis testing (as opposed to decision criteria based on a cutoff SI value) to classify substances. The Panel commented that, in some cases, statistical significance might not indicate a biological effect. The Panel agreed with the language regarding hypothesis testing in the current ICCVAM LLNA Performance Standards (Appendix A - Section 3.0).

Public Comments:

At the conclusion of the Panel discussion, Dr. Luster called for public comments.

Dr. George De George, MB Research Labs
Dr. De George raised the following points:

- The data evaluated for the 1999 ICCVAM evaluation of the LLNA were statistically analyzed.
- As a result of that analysis, the optimum SI cutoff for a sensitizer was determined as 3.16.
- The Panel for the 1999 evaluation chose 3.0 as the SI cutoff to provide an added level of confidence.
- Routine statistical analysis of LLNA data to classify test substances was not recommended in the 1999 evaluation. In Dr. DeGeorge's opinion, the best reason to collect individual animal data was so that, in the future, studies could be done to determine an optimum method for hypothesis testing of LLNA data.
- Newer variant LLNA tests should be subjected to the same level (and not held to a higher level) of requirements for validation as the traditional LLNA.

Panel Conclusions and Recommendations:

At the conclusion of the public comments, Dr. Luster asked if the Panel was in agreement with the conclusions in the draft Panel Report as reflected in the updated presentation. The Panel approved unanimously.

Overview of the Revised Draft LLNA: BrdU-FC Test Method BRD and Revised Draft ICCVAM Test Method Recommendations

Dr. Luster called for Panel consideration of the LLNA: BrdU-FC test method. This test method measures bromodeoxyuridine (BrdU), a thymidine analog, instead of radioactive thymidine, incorporated into the DNA of proliferating lymphocytes, via flow cytometric analysis. The test method also allows for the measurement of immunophenotypic markers in the lymphocyte population, ostensibly aiding in discrimination between irritants and sensitizers.

Dr. George DeGeorge of MB Research Labs, Spinnerstown, PA (the test method developer) presented a synopsis of the test method to the Panel. In addition to a brief description of the test method protocol, Dr. DeGeorge made the following points:

- The test method protocol was based on the ICCVAM-recommended LLNA test method protocol, using SI $\geq$ 3.0 as the decision criterion for a sensitizer.
- Test substances were chosen to include those tested in the traditional LLNA.
- Guinea pig data and human results are considered less reliable.
- The LLNA: BrdU-FC uses lower doses of test substances than the traditional LLNA to avoid irritating concentrations.
- The LLNA: BrdU-FC makes correct calls for some substances for which the traditional LLNA does not.
- All of the data generated by MB Research Labs using the LLNA: BrdU-FC are available for review at the laboratory (although not all data are available electronically).
- MB Research Labs is currently attempting to find other laboratories interested in participating in an interlaboratory validation study.

Following Dr. De George's presentation, a Panelist asked the following questions:

- Does MB Research Labs conduct LLNA: BrdU-FC studies according to GLP? Dr. De George said yes.
- What is the treatment group size? Dr. DeGeorge responded that five animals per treatment group were used.
- Can measurement of ear swelling be added to any LLNA variant test method as an additional endpoint? Dr. DeGeorge replied that it could, and that it could help resolve which doses to test.

On behalf of NICEATM, Dr. Allen presented a summary of the revised draft LLNA: BrdU-FC BRD to the Panel. At the conclusion of Dr. Allen's presentation, Dr. DeGeorge pointed out that an in-house flow cytometer and trained operators weren't necessary to conduct the test method, because the lymphocytes were fixed as part of the test method protocol, and the flow cytometry analysis could be outsourced.

Dr. Jacobs then presented the revised draft ICCVAM test method recommendations for the LLNA: BrdU-FC test method to the Panel.

Panel Evaluation:

Dr. Richmond presented the draft position developed by Evaluation Group B, which was charged with primary review of the LLNA: BrdU-FC test method, to the Panel.

The Panel agreed that the LLNA: BrdU-FC test method was mechanistically and functionally similar to the traditional LLNA, and the ICCVAM LLNA Performance Standards could be used to evaluate it. The Panel also concurred that the database of more than 45 representative test substances yielded adequate accuracy based on results from one laboratory, and that intralaboratory reproducibility also had been adequately demonstrated. However, the Panel agreed with the ICCVAM proposal to defer a formal recommendation on the validity of the LLNA: BrdU-FC until an independent audit of all data supporting the analysis has been conducted and until transferability has been demonstrated in an interlaboratory validation study. The Panel recommended that ICCVAM should work with NICEATM to support and facilitate the independent audit and interlaboratory validation study. The Panel recommended that upon completion of these tasks and determination of satisfactory data quality, power, and interlaboratory reproducibility, that the LLNA: BrdU-FC could be considered to have adequate validation and performance to support its consideration for regulatory use.

Much Panel discussion about the necessary statistical power of the test method occurred. Power is defined as the probability that the test method would determine that a test group showing a positive result is different from the negative control (i.e., that a sensitizer would be detected as such). Data presented to the Panel during their 2008 evaluation indicated that the test method would require nine animals per treatment group to achieve 95% power; the power with five animals per group was estimated at 80% in that evaluation. The Panel agreed that, before an interlaboratory validation study was begun, it should be verified that the LLNA: BrdU-FC test method has power at least equal to that of the traditional LLNA using five animals per treatment group.

Public Comments:

At the conclusion of the Panel discussion, Dr. Luster called for public comments.

Dr. George De George, MB Research Labs

Dr. De George raised the following points:

- Power calculations on a subset of the data are not as reliable as accuracy statistics calculated from the entire dataset for 45 chemicals.
- Power calculations are a new requirement for validation, and not contained in the ICCVAM LLNA Performance standards.
- It was Dr. De George's opinion that it would be difficult, if not impossible, to get three qualified testing laboratories to participate in an interlaboratory validation study.

Panel Conclusions and Recommendations:

Subsequent to the public comments, the Panel commented that the flow cytometric analysis for samples from all three laboratories in an interlaboratory study could be done at MB Research Labs. Power calculations could be done by NICEATM on the most recent data generated by the LLNA: BrdU-FC test method.

The Panel decided to make a nomination to ICCVAM, with high priority, that NICEATM organize and supervise an interlaboratory validation study for the LLNA: BrdU-FC test method.

Dr. Luster asked if the Panel was in agreement with the conclusions in the draft Panel Report. The Panel approved unanimously.

Concluding Remarks

Dr. Luster, on behalf of the Panel, thanked the NICEATM-ICCVAM staff for their continued assistance during the review process and the Panel meeting. He also thanked Drs. Joanna Matheson and Abby Jacobs, the IWG co-chairs, and Dr. Marilyn Wind, ICCVAM Chair and IWG member, for the hard work they put into the project. Dr. Luster also thanked the Panel, the Evaluation Group Chairs, and the experts on the test methods, who presented them to the Panel.

Drs. Wind and Stokes thanked the Panel again for their hard work, thoughtful and objective deliberations, and advice. Dr. Stokes further thanked the invited test method developers for their excellent summaries of their test method for the benefit of the Panel. Dr. Stokes concluded by saying he looked forward to further working with the Panel members to complete their Panel report.

Adjournment

Dr. Luster adjourned the Panel at 11:30 a.m., concluding the meeting.

William S. Stokes, D.V.M., D.A.C.L.A.M.
NIEHS
P.O. Box 12233
Mail Stop: K2-16
Research Triangle Park, NC 27709

Dear Dr. Stokes,

The Meeting Summary Minutes, Independent Scientific Peer Review Panel Meeting, Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Updated Evaluation of the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA), accurately summarizes the Peer Review Panel meeting of April 28-29, 2009, in Bethesda, MD.

Sincerely,

Signature	Printed Name	Date
	MICHAEL LUSTER	8/21/09

Appendix E4

Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products

This document is available at:
https://ntp.niehs.nih.gov/iccvam/docs/immunotox_docs/llnaprprept2009.pdf

The document is also available on request from NICEATM:

NICEATM
National Institute of Environmental Health Sciences
P.O. Box 1233, MD K2-16
Research Triangle Park, NC 27709 USA
Telephone: 919-541-2384 Fax: 919-541-0947
E-mail: niceatm@niehs.nih.gov

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Appendix F

Federal Register Notices and Public Comments

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Appendix F1

Federal Register Notices

All *Federal Register* notices are available at https://www.federalregister.gov/

72 FR 27815 (May 17, 2007)
The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data

72 FR 52130 (September 12, 2007)
Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments

73 FR 1360 (January 8, 2008)
Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments

73 FR 25754 (May 7, 2008)
Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

73 FR 29136 (May 20, 2008)
Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments

74 FR 8974 (February 27, 2009)
Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments

74 FR 19562 (April 29, 2009)
Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

74 FR 26242 (June 1, 2009)
Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments

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Appendix F2

Public Comments Received in Response to *Federal Register* Notices

Public comments are available upon request from NICEATM

72 FR 27815 (May 17, 2007)
The Murine Local Lymph Node Assay: Request for Comments, Nominations of Scientific Experts, and Submission of Data

- Dr. Eric Debruyne (BAYER CropScience)
- Dr. H.-W. Vohr (Bayer HealthCare AG)
- Dr. H.-W. Vohr (Bayer HealthCare AG)
- Dr. H.-W. Vohr (Bayer HealthCare AG)
- Dr. Kirill Skirda (CESIO)
- Mark S. Maier, Ph.D., DABT (CropLife America)
- Dr. Phil Botham (European Crop Protection Association)
- Peter Ungeheuer (European Federation for Cosmetic Ingredients)
- Dori Germolec (NIEHS)
- Dori Germolec (NIEHS)
- Robert L. Guest (Safepharm Laboratories Ltd)
- Daniel R. Cerven, M.S. and Melissa K. Kirk, Ph.D. (MB Research Laboratories)
- Daniel Marsman, D.V.M., Ph.D. (Procter & Ganble)
- Michael J. Olson, Ph.D. (GlaxoSmithKline)
- Anne Marie Api, Ph.D. (Research Institute for Fragrance Manufacturers)
- Peter S. Thorne, Ph.D. (The University of Iowa)
- Catherine Willett, Ph.D. (People for the Ethical Treatment of Animals), Sara Amundson (Humane Society Legislative Fund), Dr. Martin Stephens (Humane Society of the United States), Kristie Stoick, M.P.H. (Physicians Committtee for Responsible Medicine), Sue A. Leary (Alternatives Research & Development Foundation), and Tracie Letterman, Esq. (American Anti-Vivisection Society)

72 FR 52130 (September 12, 2007)
Draft Performance Standards for the Murine Local Lymph Node Assay: Request for Comments

- Ann-Therese Karlberg (Goteborg University)
- Dr. Jon Richmond
- Prof. dr. Henk Van Loveren (National Institute of Public Health and the Environment, the Netherlands)
- Catherine Willett, Ph.D. (People for the Ethical Treatment of Animals), Sara Amundson (Humane Society Legislative Fund), Dr. Martin Stephens (Humane

Society of the United States), Kristie Stoick, M.P.H. (Physicians Committee for Responsible Medicine), Sue A. Leary (Alternatives Research & Development Foundation), and Tracie Letterman, Esq. (American Anti-Vivisection Society)

73 FR 1360 (January 8, 2008)
Announcement of an Independent Scientific Peer Review Panel Meeting on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents; Request for Comments

- Dr. David Basketter
- Dr. David Basketter
- Kenneth T. Bogen, Dr.P.H., DABT (Exponent)
- G. Frank Gerberick, Ph.D. (The Procter & Gamble Company)
- Laurence Musset (OECD)
- B. Schau
- Catherine Willett, Ph.D. (People for the Ethical Treatment of Animals) and Kristie Stoick, M.P.H. (Physicians Committee for Responsible Medicine)

73 FR 25754 (May 7, 2008)
Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

- B. Sachau

73 FR 29136 (May 20, 2008)
Peer Review Panel Report on the Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments

- No responses received

74 FR 8974 (February 27, 2009)
Announcement of a Second Meeting of the Independent Scientific Peer Review Panel on the Murine Local Lymph Node Assay; Availability of Draft Background Review Documents (BRD); Request for Comments

- Nancy Douglas, Ph.D. and Catherine Willett, Ph.D. (People for the Ethical Treatment of Animals), Kristie Stoick, M.P.H. (Physicians Committee for Responsible Medicine), Martin Stephens, Ph.D. (The Humane Society of the United States), Sara Amundson (Humane Society Legal Fund, Doris Day Animal League), Sue Leary (Alternatives Research & Development Foundation), and Tracie Letterman, Esq. (American Anti-Vivisection Society)

74 FR 19562 (April 29, 2009)
Meeting of the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM)

- No responses received

74 FR 26242 (June 1, 2009)
Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for

Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products: Notice of Availability and Request for Public Comments

- Brian E. Harvey, M.D., Ph.D. (Sanofi Aventis)

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Appendix F3

Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) Comments

SACATM Meeting on June 18-19, 2008

SACATM meeting minutes are available online at:
https://ntp.niehs.nih.gov/events/past/index.html?type=SACATM

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Appendix F4

Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) Comments

SACATM Meeting on June 25-26, 2009

SACATM meeting minutes are available online at:
https://ntp.niehs.nih.gov/events/past/index.html?type=SACATM

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Appendix G

Relevant Skin Sensitization Regulations and Testing Guidelines

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Appendix G1

Table of Relevant Skin Sensitization Test Regulations

Note to the Reader:
Regulations may be updated in the future. It is recommended that users review the most current version of all regulations identified.

Electronic versions of United States Code (U.S.C.) can be obtained at:
http://www.gpoaccess.gov/uscode/index.html

Electronic versions of the Code of Federal Regulations (CFR) can be obtained at:
http://www.gpoaccess.gov/cfr/index.html

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Skin Sensitization Testing: Relevant US Federal Laws, Regulations, Guidelines, and Recommendations				
Agency, Center, or Office	**Regulated Products**	**Statutory Requirements**	**Regulations**	**Guidelines and Recommendations**
FDA/CDER	Pharmaceuticals	Federal Food, Drug, and Cosmetic Act (U.S.C. Title 21, Chapter 9) Public Health Service Act (U.S.C. Title 42, Chapter 6A)	21 CFR 312 21 CFR 314	Guidance for Industry Immunotoxicology Evaluation of Investigational New Drugs (2002)
EPA/OPPTS	Chemicals as defined by Section 5 of the Act Pesticides	Toxic Substances Control Act (U.S.C. Title 15, Chapter 53) Federal Insecticide, Fungicide, and Rodenticide Act (U.S.C. Title 7, Chapter 6)	40 CFR 158.50 40 CFR 158.100 40 CFR 158.340 40 CFR 700-799	OPPTS 870.2600 (2003) (see **Appendix G2**)
CPSC	Consumer Products	Federal Hazardous Substances Act (U.S.C. Title 15, Chapters 1261-1278)	16 CFR 1500.3	No Specific Guidelines, Guidances, or Recommendations
OSHA	Chemicals	Occupational Safety and Health Act of 1970 (U.S.C. Title 29, Chapter 15)	29 CFR 1910.1200	No Specific Guidelines, Guidances, or Recommendations

Relevant Skin Sensitization Regulations and Guidelines Europe			
Agency, Center, or Office	**Regulated Products**	**Regulations and Directives**	
EU	Dangerous Preparations (Chemicals and Chemical Mixtures)	Directive 1999/45/EC of the European Parliament and of the Council of 31 May 1999 Annex V to Directive 67/548/EEC of 27 June 1967	
	Pesticides	Directive 91/414/EEC of the European Parliament and of the Council of 15 July 1991	
Relevant Skin Sensitization Regulations and Guidelines International			
Organizations	**Regulated Products**	**Legal Instruments and Recommendations**	**Guidelines, Guidance, and Recmmendations**
GHS	Chemicals	GHS Part 3, Chapter 3.4	No Specific Guidelines, Guidances, or Recommendations
ISO	Medical Devices	NA	ISO 10993-10 (2002) (see **Appendix G3**)
OECD	Chemicals	NA	OECD Test Guideline 429 (2002) (see **Appendix G4**) OECD Test Guideline 406 (1992) (see **Appendix G5**)
ICH	NA	NA	No Specific Guidelines, Guidances, or Recommendations

Appendix G2

EPA Health Effects Test Guidelines OPPTS 870.2600: Skin Sensitization (March 2003)

EPA Health Effects Test Guidelines are available at:
https://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-870-health-effects-test-guidelines

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Appendix G3

International Organization for Standardization - ISO 10993-10: Biological Evaluation of Medical Devices Part 10: Tests for Irritation and Delayed-type Hypersensitivity (2002)

Document available from the ISO website:

http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=33364

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Appendix G4

OECD Test Guideline 429: Skin Sensitisation – Local Lymph Node Assay (Adopted April 2002)

Available at:
https://www.oecd-ilibrary.org/environment/test-no-429-skin-sensitisation_9789264071100-en

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Appendix G5

OECD Test Guideline 406: Skin Sensitisation
(Adopted July 1992)

Available at:
https://www.oecd-ilibrary.org/environment/test-no-406-skin-sensitisation_9789264070660-en

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