# Revised Draft Assessment of the Validity of the LLNA for Mixtures, Metals, and Aqueous Solutions

**Addendum No. 1 to the ICCVAM Report:** *The Murine Local Lymph Node Assay (LLNA): A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals/Compounds (NIH Pub. No. 99-4494)*

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# List of Abbreviations and Acronyms

| | |
|---|---|
| ACD | Allergic contact dermatitis |
| AOO | Acetone: olive oil |
| BGIA | Berufsgenossenschaftliches Institut fur Arbeitsschutz (German Institute for Occupational Safety and Health) |
| BRD | Background Review Document |
| BT | Buehler Test |
| CASRN | Chemical Abstracts Service Registry Number |
| CCA | Chromated copper arsenate |
| CESIO | Comite Europeen des Agents de Surface et de Leurs Intermediaires 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 three |
| 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 ACD 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 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 provided the information, data and analyses supporting the validation status of the LLNA applicability domain. IICVAM also developed draft test method recommendations for

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

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. A report of the Panel meeting was subsequently published on the NICEATM-ICCVAM website[2]. Both ICCVAM and the Panel 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 was 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 will meet to consider the revised addendum and to evaluate the extent to which the available information supports the revised ICCVAM draft test method recommendations. ICCVAM will consider the conclusions and recommendations of the Panel, along with comments received from the public and SACATM, and finalize the addendum and test method recommendations. These will then be forwarded to Federal agencies for acceptance decisions where appropriate.

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[2] http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel.htm

# Executive Summary

***Background***

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended to U.S. Federal agencies that the murine local lymph node assay (LLNA) is a valid substitute for currently accepted guinea pig test methods to assess the allergic contact dermatitis (ACD) potential of many, but not all, types of substances. ACD is an allergic skin reaction characterized by redness, swelling, and itching that can result from contact with a sensitizing chemical or product. The recommendation was based on a comprehensive evaluation that included an independent scientific peer review panel (Panel) assessment of the validation status of the LLNA. The Panel report and the ICCVAM recommendations (ICCVAM 1999) are available at the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)-ICCVAM website (http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf). 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 Organization for Standardization [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]).

In 2007, the U.S. Consumer Product Safety Commission (CPSC) formally nominated several activities related to the LLNA for evaluation by ICCVAM and NICEATM (Available at http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf). One of the nominated activities was an assessment of the validation status of the LLNA applicability domain. The information described in the original and this revised addendum was compiled by ICCVAM and NICEATM in response to this nomination.

This addendum provides a revised comprehensive review of available data and information regarding the current usefulness and limitations of the LLNA for assessing the skin sensitizing potential of mixtures, metals, and substances tested in aqueous solutions. The information is based on a retrospective review of traditional LLNA data that were either submitted as part of the original LLNA evaluation (ICCVAM 1999), extracted from peer-reviewed publications, or submitted to the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) in response to a *Federal Register* notice requesting available data and information (Vol. 72, No. 95, pages 27815-27817, May 17, 2007[3]).

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

NICEATM and ICCVAM convened an independent scientific peer review panel meeting on March 4-6, 2008. The Panel peer reviewed the draft addendum and commented on the extent that it supports 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, 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[4]. 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 are being examined (ICCVAM 2008).

Public comments at the meeting revealed that additional relevant data from LLNA studies with pesticide formulations and other products were available that had not previously been provided in response to earlier requests for data. The Panel recommended that the additional data be obtained by NICEATM and that a reanalysis of the performance of the LLNA for testing pesticide formulations and other products be conducted. In response to this recommendation, NICEATM obtained additional LLNA data and, in some cases, corresponding reference test method data (i.e., guinea pig test and/or human data) (ICCVAM 2008). These additional data were used to revise the evaluation of the LLNA for testing pesticide formulations and other products[5](**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 evaluation remains unchanged (**Section 5.2**).

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[3] available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf
[4] http://iccvam.niehs.nih.gov/methods/immunotox/llna_PeerPanel08.htm
[5] Based on the Panel recommendation, this revised addendum does not refer to "mixtures" as a type of substance tested, but rather specifies the types of products that were tested, where possible.

The changes to the existing database that resulted from any new data received subsequent to the release of the January 2008 draft addendum are summarized as follows:

- LLNA data and corresponding *in vivo* guinea pig test method data for 52 pesticide formulations were submitted by Dow AgroSciences.
- LLNA data for 28 pesticide formulations were submitted by Dupont Chemical Company.
- Detailed LLNA study results and corresponding human data for 12 fragrance ingredients were submitted by the Research Institute for Fragrance Materials. The summary results were originally published in Lalko and Api (2006).
- LLNA data for 48 medical device eluates were submitted by AppTec Laboratory Services.

These new data sources have been added to **Table 2.1**.

***Validation Database***

This revised draft addendum considers data for 140 additional substances compared with the January 2008 draft. The information contained in this addendum is now based on a retrospective review of LLNA data derived from a current database of over 600 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) the results from guinea pig tests and 2) information about sensitizers in humans (e.g., human maximization test results, substances used in human repeat insult patch test, clinical data), where available. This addendum updates the LLNA performance analyses for pesticide formulations and other products, metals, and substances tested in aqueous solutions when compared to human and guinea pig results.

***Use of the LLNA for Testing Formulations and Other Products***

In contrast with the January 2008 draft, which used the term “mixtures” to refer to multiple component substances, this revised draft addendum categorizes substances with multiple components according to product category.

Pesticide Formulations: The revised 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 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 BALBc (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 (3 positive, 2 negative).

All of these 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-two pesticide formulations had associated guinea pig data for the complete formulation, 46 pesticide formulations had guinea pig data for one or more of the active ingredients included in the complete formulation, and 14 pesticide formulations had guinea pig data for a substance related to an active ingredient or for a related formulation.

For 22 formulations for which there were guinea pig data, the LLNA classified 54% (12/22) of the formulations as sensitizers while the guinea pig tests classified only 14% (3/22) 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 54% of the time. The LLNA also identified an additional seven substances as sensitizers that were classified as nonsensitizers in the guinea pig test, an overprediction of 53% (10/19). Three of the LLNA studies for the 22 pesticide formulations were done in BALB/c mice. If these three studies are removed from the analysis, the LLNA and the guinea pig results were in agreement 58% (11/19) of the time, and the overprediction was 50% (8/16). There were no instances of underprediction for these 22 pesticide formulations. Human data are not available for these pesticide formulations to confirm their actual sensitization potential in humans.

Dyes: The current LLNA database contains data for six dyes for which there is LLNA and guinea pig data. Based on LLNA results for these six dyes, 50% (3/6) were sensitizers and 50% (3/6) were nonsensitizers. By comparison, based on guinea pig maximization test (GPMT) results, 83% (5/6) were sensitizers (when there were multiple calls in the GPMT, a most conservative call was used) and 17% (1/6) were nonsensitizers. The LLNA and the guinea pig results were in agreement 33% of the time. The overprediction for the LLNA was 100% (1/1) and the underprediction was 60% (3/5).

Fragrance Ingredients: The current LLNA database also contains data for 12 fragrance ingredients (essential oils and absolutes) for which there are comparative LLNA and human data. 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. Based on LLNA results for these fragrance ingredients, 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 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). There are no comparative data from guinea pig tests with these fragrance ingredients. Therefore, a comparison of the performance of the LLNA and the guinea pig tests relative to the human outcome is not possible.

***Use of the LLNA for Testing Metal Compounds***

The evaluation of LLNA results for testing metal compounds has not changed from that in the January 2008 draft addendum. The NICEATM LLNA database contains test results on 48 studies involving 17 metal compounds representing 13 different metals (mixtures containing metals are excluded from this analysis). All 17 metal compounds had comparative human data and eight had comparative guinea pig data. Among the 13 metals tested multiple times, nickel was tested four times in the LLNA as nickel sulfate, three times as nickel chloride, and once as a nickel (II) salt. Because nickel was classified as a sensitizer in four of these studies and as a nonsensitizer in the other four, a decision was made to exclude nickel compounds from the LLNA metals performance analysis.

For these remaining 14 metal compounds (13 metals), the LLNA had 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. 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 guinea pig 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), when compared to guinea pig test results. When comparing the performance of the LLNA and the guinea pig 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 guinea pig test had an accuracy of 100% (6/6), a false positive rate of 0% (0/1) and a false negative rate of 0% (0/5) against the human.

***Use of the LLNA for Substances Tested in Aqueous Solutions***

The evaluation of the LLNA for substances tested in aqueous solutions includes 118 additional substances compared with that of the January 2008 draft addendum. The revised NICEATM LLNA database for aqueous solutions contains test data on 171 studies that involved testing 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 BALBc (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.

Guinea pig data were available for 24 (4 sensitizers/20 nonsensitizers in the guinea pig) substances tested in aqueous solutions. Eleven substances were discordant between the

LLNA and the guinea pig 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 guinea pig results (50% [10/20] overprediction). One additional substance, neomycin sulfate, which was tested in 25% EtOH, was underpredicted by the LLNA with respect to the guinea pig results (25% [1/4] underprediction). Overall, the LLNA and the guinea pig results were in agreement 54% (13/24) of the time.

Human data were available for only four substances (3 sensitizers/1 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 guinea pig and human data. Therefore the database of substances tested in multiple test methods (i.e., LLNA, guinea pig, and/or human) is too few to allow for a meaningful calculation.

All 48 of the medical device eluates were negative in the LLNA. None of these eluates had associated guinea pig 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.

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# 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[6]).

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.

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 **Appendix A**]).

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.

[6] 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).

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[7] 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 **Appendix A**).

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 to 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 fragrance ingredients 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.[8]

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[9]). Key words used in the online searches for this evaluation were "LLNA" OR "Local Lymph Node" OR "Local lymph node" OR "local lymph node" AND (mixture* OR formula*)" OR ("metal* OR aqueous*)". Additionally, a weekly search on SCOPUS that uses the key words (TITLE-ABS-KEY(**sensi***) AND TITLE-ABS-KEY(**skin** OR **dermal**)) is done. Since March 2008, six relevant papers were added to the database.

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[7] available at http://iccvam.niehs.nih.gov/methods/immunotox/llnadocs/CPSC_LLNA_nom.pdf
[8] available at http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
[9] 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

This section reflects substances subsequent to the release of the draft addendum. These are summarized as follows:

- LLNA data and corresponding *in vivo* guinea pig test method data for 52 pesticide formulations were submitted by Dow AgroSciences.
- LLNA data for 28 pesticide formulations were submitted by Dupont Chemical Company.
- Detailed LLNA study results and corresponding human data for 12 fragrance ingredients were submitted by the Research Institute for Fragrance Materials. The summary results were originally published in Lalko and Api (2006).
- LLNA data for 48 medical device eluates were submitted by AppTec Laboratory Services.

These new data sources have been added to **Table 2.1**.

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 (**Appendix A**), 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 (**Appendix A**), 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 2-1** provides information on the sources of the data and the rationale for the substances tested.

**Table 2-1 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. 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. |
| Bundesanstalt fur 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 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 fur Arbeitsschutz; CESIO = Comite Europeen des Agents de Surface et de Leurs Intermediaires 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 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 **Appendix C2**, 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, **Appendices B1**, **B4**, **B6**, **C1**, and **D1** provide information on the physico-chemical 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[10]. 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, Oxyflourfen EC, Quinoxyfen/cyproconazole, and Trifluralin EC) and the formulations submitted by Dow AgroSciences, using the LLNA, is included in **Appendix B3**. 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 test 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 fragrance ingredients (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.

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.

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[10] available at http://www.nlm.nih.gov/mesh/meshhome.html

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

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# 3.0 Comparative *In Vivo* Reference Data

The *in vivo* reference data in this draft addendum has been revised from the January 2008 draft addendum to include data received subsequent to the release of the draft addendum. These data are summarized in **Section 2.0**. 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 (OECD 1992, EPA 2003).

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 (OECD 1998; EPA 2006a, 2006b; FDA 2007). 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.

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# 4.0 LLNA Data and Results

The test method data in this draft addendum has been revised from the January 2008 draft addendum to include data received subsequent to the release of the draft addendum. These data are summarized in **Section 2.0**. The data used for this evaluation were obtained from 25 sources (**Table 2-1**). No new LLNA studies were conducted to generate data for this evaluation (see **Section 2.0**). Where available, specific information including name, CASRN, physico-chemical properties (e.g., molecular weight, Log $K_{ow}$), chemical class[11] and data source are indicated for each pesticide formulation, dye, fragrance ingredient, metal compound, and substance tested in an aqueous solution (**Appendices B1, B4, B6, C1,** and **D1**, 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 **Appendices B2**, **B5, B7, C2,** and **D2** for pesticide formulations, dyes, fragrance ingredients, 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 2-1** and **Appendices B1, B4, B6, C1,** and **D1,** 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 (**Appendix A**) 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; **Appendix A**) or for any of the newly obtained studies used in this evaluation.

[11] 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).

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# 5.0 Accuracy of the LLNA: Revised Applicability Domain

Since the publication of the draft addendum in 2008, NICEATM obtained additional LLNA data, which were used to revise the evaluation of the LLNA for testing pesticide formulations and other products[12](**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 evaluation remains unchanged (**Section 5.2**). The new data contained in this revised addendum are summarized in **Section 2.0.**

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 **Appendices B2**, **B5, B7, C2**, and **D2**, respectively. For comparison purposes, the performance of the LLNA database reported in the ICCVAM evaluation report (ICCVAM 1999; **Appendix A**) is included in **Tables 5-3**, **5-6**, **5-8**, **5-10**, and **5-13**. 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) (**Appendix A**) 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 physico-chemical properties of these formulations are in **Appendix B1,** and the data analyzed here are in **Appendix B2**.

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 considered. 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 BALBc (28/89) mouse strains.

Six formulations were tested in multiple LLNA studies (25 studies total [**Table 5-1]**). 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 5-2**]).

Twenty-two 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**, below. 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 **Appendix E.**

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)

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[12] Based on the Panel recommendation, this revised addendum does not refer to "mixtures" as a type of

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 inter-laboratory study (n=5 laboratories), Boverhof et al. (2008) conducted LLNA tests on three substances with known sensitization potential and four pesticide formulations for which the sensitization potential in guinea pigs and/or humans had previously been determined, along with three commonly-used positive controls in sensitization testing (hexylcinnamaldehyde, formaldehyde, and potassium dichromate), 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. **Appendix B3** contains the information on components provided by Dow AgroSciences.

substance tested, but rather specifies the types of products that were tested, where possible.

### Table 5-1 Pesticide Formulations with Multiple LLNA Studies

| Formulation | Source | No. Studies | Mouse Strain | No. Positive Studies | No. Negative Studies | No. of 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 |
| **Oxyflouren 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 5-2 LLNA Data for Pesticide Formulation with Discordant Results

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

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 22 formulations that had associated GP data for the formulation itself, 14% (3/22) were classified as sensitizers and 86% (19/22) as nonsensitizers according to the GP results (**Figure 5-1**). Twenty of these GP tests were BT and 2 were GPMT. These results are based on a positive overall GP call for formulation EXP 10810[13]. Nine out of the approximately 450 active ingredients registered with EPA were represented among these 22 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, six herbicides and two fungicides).

Nineteen 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 59% (13/22) of the formulations as sensitizers and 41% (9/22) as nonsensitizers (**Figure 5-1**). 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 5-3**).

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 19 LLNA studies, the LLNA classified 58% (11/19) of the formulations as sensitizers and 42% (8/19) as nonsensitizers (**Figure 5-1**). 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 5-3**).

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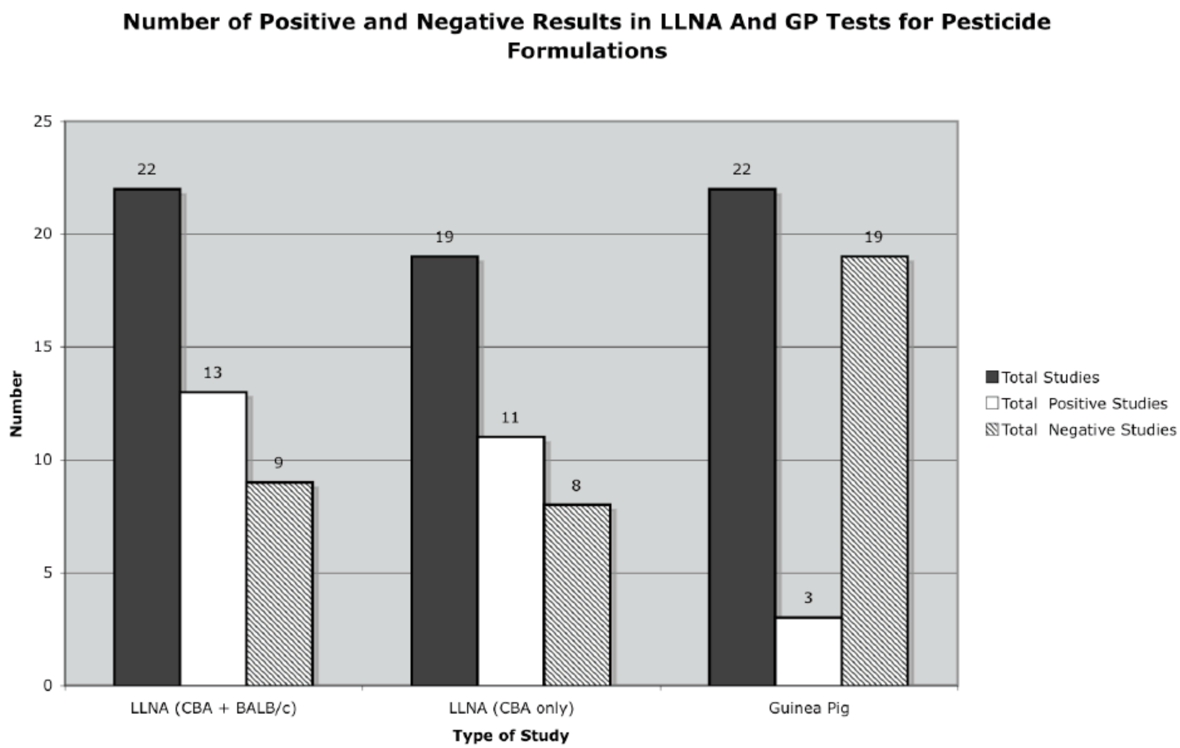
[13] Formulation EXP 10810 A (submitted by E. Debruyne, Bayer Crop Science), the only formulation for which there was data in both the GPMT and the BT, showed equivocal results in the guinea pig. This formulation tested positive in the GPMT (sensitization incidence 100%), and negative in the BT (sensitization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

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

**Figure 5-1 Numbers of Positive and Negative LLNA and GP Calls for Pesticide Formulations**




Abbreviations: BALB/c = LLNA studies conducted using the BALB/c mouse strain; CBA = LLNA studies conducted using the CBA mouse strain; GP = guinea pig; LLNA = local lymph node assay

Based on the 22 pesticide formulations tested in CBA (n=19) and BALBc (n=3) strains, the accuracy of the LLNA compared to guinea pig data was 54% (12/22), the sensitivity was 100% (3/3), the specificity was 47% (9/19), the false positive rate was 53% (10/19) 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 58% (11/19), the sensitivity was 100% (3/3), the specificity was 50% (8/16), the false positive rate was 50% (8/19) and false negative rate was 0% (0/3) (**Table 5-3**).

**Table 5-3 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] (Formulation[6])** | 22 | 54 | 12/22 | 100 | 3/3 | 47 | 9/19 | 53 | 10/19 | 0 | 0/3 |
| **LLNA[7] vs. GP[5] (Formulation[6])** | 19 | 58 | 11/19 | 100 | 3/3 | 50 | 8/16 | 50 | 8/16 | 0 | 0/3 |
| ***ICCVAM 1999 Database: Evaluation of LLNA Data vs. GP Data or Human Data[8]*** | | | | | | | | | | | |
| **LLNA[7] vs. GP[5]** | 126 | 86 | 108/126 | 87 | 81/93 | 82 | 27/33 | 18 | 6/33 | 13 | 12/93 |
| **LLNA[7] vs. Human[9]** | 74 | 72 | 53/74 | 72 | 49/68 | 67 | 4/6 | 33 | 2/6 | 28 | 19/68 |
| **GP[5] vs. Human[9]** | 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=19) and BALBc (n=3) mice.
[5] GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.
[6] Formulation refers to associated GP data for the formulation itself.
[7] LLNA studies conducted with CBA mice.
[8] 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 versus human is included here.
[9] 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 22 formulations classified as sensitizers by the LLNA that were classified as nonsensitizers in the GP (**Table 5-4**), eight were classified as nonsensitizers based on BT results and two were classified as nonsensitizers based on GPMT results.

**Table 5-4 Pesticide Formulations that are Classified as Sensitizers in the LLNA, but Classified as Nonsensitizers in the GP**

| 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; 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 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

[10]Mean from 3 positive studies

[11]Mean of 5 studies

The constituents of most of the formulations are unknown (**Appendix B3**). 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 (**Appendix B3**). Dow Agrosciences categorizes all other constituents of Formulation 2 as nonsensitizers, including the active ingredients Fluroxypyr-meptyl and Florasulam (**Appendix B3**). 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 (**Appendix B3**).

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 5-4**) 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; oxyflourfen 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 5-4**).

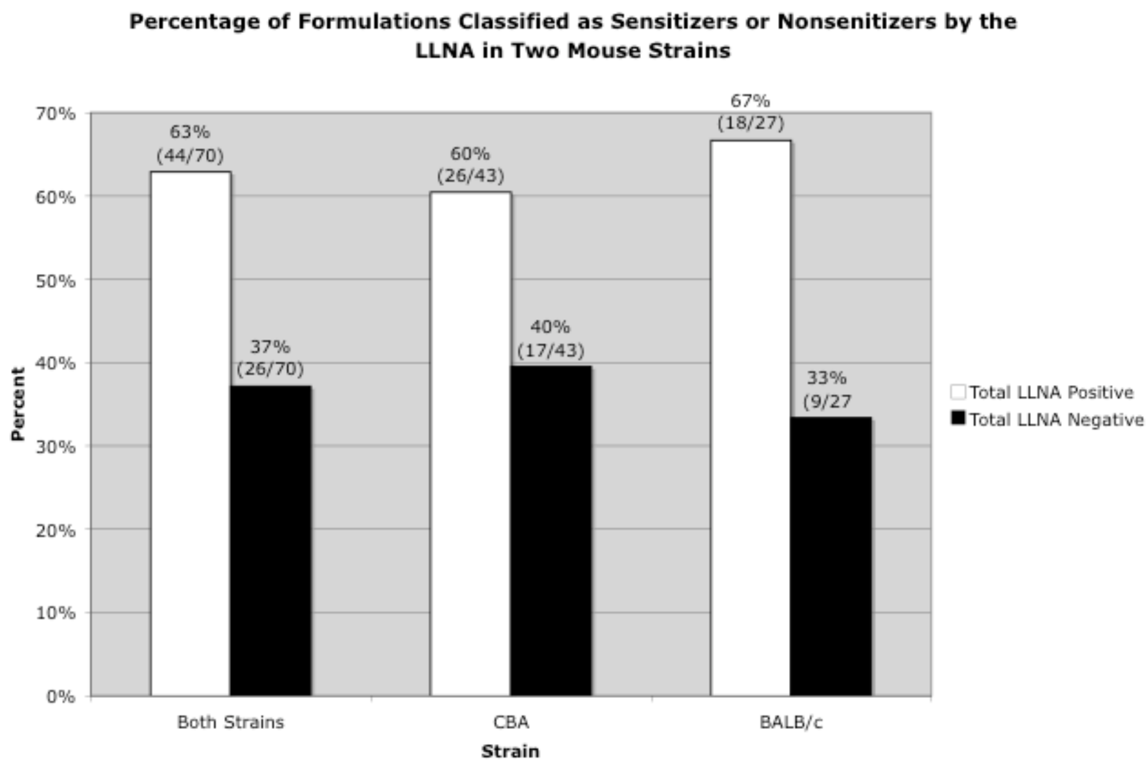
The EC3 values for most (9/10) of the formulations indicated that they produced weak to moderate responses in the LLNA (EC3 range of 5.5% to 88.5%) (**Table 5-4**). However, the EC3 value for the formulation F & Fo WG 50 + 25 (EC3 = 0.003%) is a very strong LLNA response. This could be due to the observed SI values on the LLNA dose-response curve that were used to calculate an EC3 by extrapolation (because no points fall below SI = 3) approach saturation (SI = 11.7 at 2.5%, SI = 15.2 at 25%) (**Appendix B2**). 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, Oxyflourfen 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 5-4**).

#### 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 5-2** 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 5-2 Percentage of Formulations Classified as Sensitizers or Nonsensitizers in Two Mouse Strains**




For the 22 pesticide formulations that were tested in the GP as entire formulations, the LLNA studies for 19/22 were conducted using CBA mice and 3/22 were conducted using BALBc mice. As noted in **Section 5.1.1.1, w**hen data for all 22 formulations is considered (i.e., using both CBA and BALB/c data), the overall accuracy is 54% (12/22), with false positive and false negative rates of 53% (10/19) 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 = 58% [11/19], false positive rate = 50% [8/16], 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; **Appendix A**), 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 physico-chemical properties of these dyes are in **Appendix B4,** and the data analyzed here are in **Appendix B5**. 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 5-5** provides the performance statistics for the LLNA when compared to GPMT outcomes for this limited dataset.

**Table 5-5 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; **Appendix A**) 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 5-6 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; **Appendix A**), so these data and analyses cannot be compared to previously considered data.

### 5.1.3 *Testing of Fragrance Ingredients*

The current LLNA database contains data for 12 fragrance ingredients, for which there are LLNA and human data. The physico-chemical properties of these fragrance ingredients are in **Appendix B6,** and the data analyzed here are in **Appendix B7**. For these fragrance ingredients, 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 5-7**).

### Table 5-7 Evaluation of the Performance of the LLNA for Testing Fragrance Ingredients

| 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[6]* | | | | | | | | | | | |
| 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 fragrance ingredients 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 fragrance ingredients showed discordant results between the LLNA and the HMT. These substances are shown in **Table 5-8**, 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 5-8 Fragrance Ingredients: 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]<br>5[6]<br>10[7] | 0[5]<br>0[6]<br>0[7] | - | Severe irritant at 100%[8] |
| Lemongrass oil | EtOH/DEP (1:3) | 50 | 13.1 | 6.5 | + | 4[9]<br>4[10]<br>5[10] | 0[9]<br>0[10]<br>0[10] | - | Mild irritant at 100%[4] |
| 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 fragrance ingredients 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 fragrance ingredients were evaluated in the ICCVAM evaluation report (ICCVAM 1999; **Appendix A**), so these data and analyses cannot be compared to previously considered data.

## 5.2 Testing of Metal Compounds

The evaluation of the LLNA for testing metal compounds has not changed from that in the January 2008 draft addendum. 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 (**Appendix A**). In this addendum, the original

ICCVAM analysis has been revised to include a total number of 17 metal compounds, representing 13 different metals, with corresponding human and/or GP data The physico-chemical properties of these metal compounds are in **Appendix C1,** and the data analyzed here are in **Appendix C2**. 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 17 metal compounds, 14 were tested in an aqueous vehicle, a non-aqueous vehicle, or both. The vehicle in which the three remaining metal compounds (i.e. cobalt chloride, cobalt sulfate, and nickel (II) salts) were tested in was not specified (**Appendix C2**). Similar to pesticide formulations and other products (**Section 5.1**), aqueous vehicles contained at least 20% water, while a non-aqueous vehicle contains no water.

All 17 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, three times as nickel chloride, and once as a nickel (II) salt. Because nickel was classified as a sensitizer in four of these studies and as a nonsensitizer in the other four, 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 5-9**). 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 5-9**) (**Appendix C2**).

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 5-9**). In contrast, the LLNA incorrectly identified the one human non-sensitizing 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 5-9**.

**Table 5-9 Evaluation of the Performance of the LLNA for Testing Metal Compounds**

| Comparison | n[1] | Accuracy | | Sensitivity | | Specificity | | False Positive Rate | | False Negative Rate | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | % | No.[2] | % | No.[2] | % | No.[2] | % | No.[2] | % | No.[2] |
| ***All Metal Compounds (Aqueous and Non-Aqueous Vehicles)*** | | | | | | | | | | | |
| **LLNA vs. GP[3]** | 6 | 83 | 5/6 | 100 | 5/5 | 0 | 0/1 | 100 | 1/1 | 0 | 0/5 |
| **LLNA vs. Human[4]** | 14 | 86 | 12/14 | 100 | 9/9 | 60 | 3/5 | 40 | 2/5 | 0 | 0/9 |
| **GP[3] vs. Human[4]** | 6 | 100 | 6/6 | 100 | 5/5 | 100 | 1/1 | 0 | 0/1 | 0 | 0/5 |
| **LLNA vs. Human[4] 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[5]*** | | | | | | | | | | | |
| **LLNA vs. GP[3]** | 1 | 100 | 1/1 | 100 | 1/1 | - | 0/0 | - | 0/0 | 0 | 0/1 |
| **LLNA vs. Human[4]** | 1 | 100 | 1/1 | 100 | 1/1 | - | 0/0 | - | 0/0 | 0 | 0/1 |
| **GP[3] vs. Human[4]** | 1 | 100 | 1/1 | 100 | 1/1 | - | 0/0 | - | 0/0 | 0 | 0/1 |
| ***Metal Compounds Tested in Non-Aqueous Vehicles*** | | | | | | | | | | | |
| **LLNA vs. GP[3]** | 5 | 80 | 4/5 | 100 | 4/4 | 0 | 0/1 | 100 | 1/1 | 0 | 0/4 |
| **LLNA vs. Human[4]** | 12 | 92 | 11/12 | 100 | 7/7 | 80 | 4/5 | 20 | 1/5 | 0 | 0/7 |
| **GP[3] vs. Human[4]** | 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[6]*** | | | | | | | | | | | |
| **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] All the metal compounds tested in an aqueous vehicle were also tested in a non-aqueous vehicle.
[6] 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 versus human is included here.

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 non-aqueous vehicle, while one was tested in both an aqueous and non-aqueous 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 5-9**). All of the five metal compounds with comparative GP data tested in a non-aqueous vehicle are also classified as sensitizing in the LLNA. Compared to GP data, the LLNA correctly classifies four of the five non-aqueous metal compounds. The accuracy statistics based on this limited dataset are also presented in **Table 5-9**.

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 non-aqueous vehicle, while one was tested in both an aqueous and non-aqueous 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 5-9**). In contrast, of the 12 metal compounds with comparative human data tested in a non-aqueous 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 non-aqueous 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 5-9**).

Potassium dichromate was the one metal compound with comparative GP and human data that was tested in both an aqueous and non-aqueous 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 non-aqueous 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 non-sensitizing, the most severe classification was used. For instance, for zinc sulfate, LLNA data from two studies are considered in this evaluation report (ICCVAM 1999 [**Appendix A**] 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 (**Appendix C2**).

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 (**Appendix A**). 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 (**Appendix A**). The evaluation of the LLNA for substances tested in aqueous solutions in this revised addendum includes 118 additional substances compared with that of the January 2008 draft addendum.

The revised 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 24 unique substances tested in aqueous solutions from 46 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 physico-chemical properties of these substances are in **Appendix D1,** and the data analyzed here are in **Appendix D2**. 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 2 or more studies (**Table 5-10**).

LLNA data for the two substances for which discordant LLNA study results occurred are shown in **Table 5-11**. 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 Oxyfluoren 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 5-11)**.

## Table 5-10 Substances Tested in Aqueous Solutions in Multiple LLNA Studies

| Formulation | Reference | No. Studies | Mouse Strain | Vehicle | No. Positive Studies | No. Negative Studies | No. of 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<br>$H_2O$ | 2 | 0 | 1 |
| Dinocap EC | ECPA | 5 | CBA | L92 | 5 | 0 | 5 |
| Formaldehyde | ECPA | 7 | NA | L92 | 7 | 0 | 6 |
| Formulation 7 | Dow AgroSciences | 2 | BALB/c | L92 | 2 | 0 | 1 |
| Hexyl cinnamic aldehyde | ECPA | 5 | NA | L92 | 5 | 0 | 5 |
| Methyl 2-nonynoate | Ryan et al. (2000) | 2 | NA | 80% EtOH | 2 | 0 | NA |
| Oxyflouren 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 1%; NA = not available; No. = number; SC = suspension concentrate

**Table 5-11 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 24 substances (4 sensitizers/20 nonsensitizers in the GP) tested in aqueous solutions. These substances represented a total of 43 LLNA studies. Based on these comparative data, the LLNA has an accuracy of 54% (13/24), a sensitivity of 75% (3/4), a specificity of 50% (10/20), a false positive rate of 50% (10/20), and a false negative rate of 25% (1/4) (**Table 5-12**).

**Table 5-12 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]** | 24 | 54 | 13/24 | 75 | 3/4 | 50 | 10/20 | 50 | 10/20 | 25 | 1/4 |
| **LLNA (CBA only) vs. GP[3]** | 21 | 57 | 12/21 | 75 | 3/4 | 53 | 9/17 | 47 | 8/17 | 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 |

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; **Appendix A**) showing the overall performance of the LLNA vs. GP and human, and GP versus human is included here.

Eleven substances were discordant between the LLNA and the GP tests (**Table 5-13**). 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 24 substances classified as sensitizers by the LLNA that were classified as nonsensitizers in the GP (**Table 5-13**), 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 5-13**). However, it should also be noted that neomycin sulfate also gave a negative result in the LLNA when tested at 25% in DMSO, a non-aqueous vehicle (Basketter et al. 1994).

**Table 5-13 Substances Tested in Aqueous Solution: Discordant Results Between the LLNA and GP**

| Substance Name | LLNA Results | | | | | GP Results | | | Skin Irritant? |
|---|---|---|---|---|---|---|---|---|---|
| | Vehicle | 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 three; EW = emulsion, oil in water; GP = guinea pig test; Ind. Con. = 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; 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
[10] Mean from 3 positive studies
[11] Mean of 5 studies

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 5-12**.

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 5-14**). 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 non-aqueous 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 5-14 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] | Nonirritant at 25%[7] |

Abbreviations: Conc. = concentration; EC3 = estimated concentration needed to produce a stimulation index of three; 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; Inc. 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 physico-chemical properties of any substances they contained are unknown. The submitted data are provided in **Appendix D3**.

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 (**Appendix D3**).

The sample preparation for these samples was different that 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**.

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# 6.0 LLNA Data Quality

This section has been revised to include data received subsequent to the release of the draft addendum in January 2008. These data are summarized in **Section 2.0**.

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 fur 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 was 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.

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# 7.0 Other Scientific Reports and Reviews

Six additional papers, identified since the publication of this addendum in January, 2008, have been added to this section.

A search of Medline, PubMed, and Toxline resulted in 40 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, 23 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 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.

## 7.2 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.

### 7.3 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.

### 7.4 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).

### 7.5 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 test and the LLNA, copper has a low sensitization potential. The authors then provide a diagnostic algorithm that might clarify the frequency of copper hypersensitivity.

### 7.6 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 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.

### 7.7 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.

### 7.8 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 and 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.

### 7.9 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.

### 7.10 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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# The Murine Local Lymph Node Assay:

## *A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals/Compounds*

## The Results of an Independent Peer Review Evaluation Coordinated by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

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## List of Abbreviations

| | |
|---|---|
| ACD | Allergic Contact Dermatitis |
| AOO | Acetone-Olive Oil |
| BA | Beuhler Assay |
| CAS | Chemical Abstracts Service |
| cRT-PCR | Competitive Reverse Transcriptase-Polymerase Chain Reaction |
| CV | Coefficient of Variation |
| DMF | *N*, *N*-Dimethyl formamide |
| DMSO | Dimethyl sulfoxide |
| DNCB | 2, 4 –Dinitrochlorobenzene |
| DPM | Disintegrations Per Minute |
| DTH | Delayed-Type Hypersensitivity |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| FCM | Flow Cytometric (Flow Cytometry) |
| FDA | Food and Drug Administration |
| GLP | Good Laboratory Practice Regulations |
| GPMT | Guinea Pig Maximization Test |
| GPT | Guinea Pig Tests (Nonstandard) |
| HCA | Hexylcinnamic aldehyde |
| HMT | Human Maximization Test |
| HPTA | Human Patch Test Allergen |
| ICCVAM | Interagency Coordinating Committee on the Validation of Alternative Methods |
| IgE | Immunoglobin Class E |
| IL-2 | Interleukin Type 2 |
| IL-6 | Interleukin Type 6 |
| i.v. | Intravenous |
| LLNA | Murine Local Lymph Node Assay |
| LNC | Lymph Node Cells |
| MEK | Methyl ethyl ketone |
| NICEATM | NTP Interagency Center for the Evaluation of Alternative Toxicological Methods |
| NTP | National Toxicology Program |
| PCNA | Proliferating Cell Nuclear Antigen |
| PG | Propylene glycol |
| PRP | ICCVAM Peer Review Panel Evaluating the LLNA |
| SD | Standard Deviation |
| SI | Stimulation Index |
| SLS | Sodium lauryl sulfate |
| SOP | Standard Operating Procedures |
| Th1 | T-Helper Cell Type 1 |
| Th2 | T-Helper Cell Type 2 |

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**National Institutes of Health, Office of the Director**

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**National Institute of Occupational Safety and Health**

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**Occupational Safety and Health Administration**

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## Preface

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) with support from the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) recently sponsored the independent scientific peer review of the validation status of the Murine Local Lymph Node Assay (LLNA), a new test method proposed for assessing the allergic contact dermatitis potential of chemicals. The review was one of the critical components in the ICCVAM process that culminates in achieving regulatory acceptance and implementation of scientifically validated toxicological testing methods. These methods are generally more predictive of adverse human health effects than current methods, and they may be alternative methods that provide for improved animal well-being and that reduce or eliminate the need for animals. These activities were conducted in accordance with public health directives of Public Law 103-43, which directed the National Institute of Environmental Health Sciences to develop and validate improved alternative toxicological testing methods, and to develop criteria and processes for the validation and regulatory acceptance of such methods (NIEHS, 1997).

ICCVAM was established as a collaborative effort by NIEHS and 13 other Federal regulatory and research agencies and programs. The purpose of ICCVAM is to coordinate issues within the Federal government that relate to the development, validation, acceptance, and national/international harmonization of toxicological test methods. The Committee's functions include the coordination of interagency scientific reviews of toxicological test methods and communication with outside stakeholders throughout the process of test method development and validation. The following Federal regulatory and research agencies and organizations participate in this effort:

Consumer Product Safety Commission
Department of Defense
Department of Energy
Department of Health and Human Services
- Agency for Toxic Substances and Disease Registry
- Food and Drug Administration
- National Institute for Occupational Safety and Health/CDC
- National Institutes of Health, Office of the Director
- National Cancer Institute
- National Institute of Environmental Health Sciences
- National Library of Medicine

Department of the Interior
Department of Labor
- Occupational Safety and Health Administration

Department of Transportation
- Research and Special Programs Administration

Environmental Protection Agency

The LLNA was proposed to ICCVAM in 1997 as a method that could be used as a stand alone alternative to the Guinea Pig Maximization Test (GPMT) and the Buehler Assay (BA), methods which are currently accepted by regulatory authorities for assessing the allergic contact dermatitis potential of chemicals. The LLNA was proposed by Dr. Frank Gerberick from Procter and Gamble, Dr. Ian Kimber from Zeneca (UK) and Dr. David Basketeer from Unilever (UK).

Through interactions with the sponsors, an ICCVAM Immunotoxicity Working Group

(IWG) composed of Federal employees assembled information for an independent scientific peer review of the method. The IWG reviewed and appropriately augmented the *ICCVAM Test Method Submission Guidelines* (ICCVAM, 1998) to provide useful guidance to the test method sponsors on the information needed for the review. The initial submission from the sponsors was reviewed by the IWG and additional information requested. Suggested experts for the peer review panel (PRP) were solicited from Federal agencies and national and international professional societies and organizations. The IWG recommended a PRP composition that would represent a broad range of experience and expertise, including immunotoxicology, clinical immunology, molecular biology, and biostatistics. PRP members were from industry, academia, and government, and included scientists from the US, Denmark, Japan, and Norway.

The PRP was charged with developing a scientific consensus on the usefulness and limitations of the new test method for assessing allergic contact dermatitis. In reaching this determination, the PRP was requested to evaluate all available information and data on the LLNA, and to assess the extent to which each of the ICCVAM criteria for validation and regulatory acceptance of toxicological test methods were addressed. The criteria used for the evaluation are described in the document *Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the Ad Hoc Interagency Coordinating Committee on the Validation of Alternative Methods*, NIH publication 97-3981 (ICCVAM, 1997). The PRP was provided with guidance for their evaluation (Appendix E), which included questions from the IWG to ensure that the assessment provided adequate information to facilitate ICCVAM and agency decisions on the method.

Test method submission materials were made available to the public and a request for public comments was made via a *Federal Register* Notice (Appendix G) and other announcements. Information was sought regarding the usefulness of the LLNA, including information about completed, ongoing, or planned studies, and other data or information about the LLNA All comments and information submitted in response to the request were provided to the PRP in advance of the review meeting.

The PRP met in public session on September 17, 1998, at the Gaithersburg Hilton, 620 Perry Parkway, Gaithersburg, Maryland, and opportunity for public comment was provided during the meeting. PRP members presented their evaluations and proposed conclusions and recommendations on each of the major sections and the PRP subsequently reached a consensus for each section. Following the meeting, the written evaluations, conclusions, and recommendations were consolidated as this PRP Report.

Following the peer review meeting, the IWG prepared a proposed test method protocol (Appendix J) that incorporated the recommendations of the PRP into the original test method protocol submitted by the test sponsors (Appendix D). This protocol may be helpful to regulatory authorities that find the method acceptable for their purposes. Additional data analyses prepared by NICEATM for the PRP are also included as appendices in this document, as is the original test method submission.

This entire report has been reviewed and endorsed by IWG and ICCVAM. This report along with ICCVAM recommendations on the usefulness of the method will be forwarded by ICCVAM to Federal agencies for their consideration. Federal agencies will determine the regulatory acceptability and applicability of this method according to their statutory mandates, and as deemed appropriate, issue guidelines, guidance documents, or proposed changes in regulations.

The work of the PRP was truly a team effort, and their thoughtful and unselfish contributions are gratefully acknowledged. While all members contributed to this evaluation, the exceptional efforts of Dr. Jack Dean, who served as the PRP chair, and Dr. Lorraine Twerdok, who served as executive secretary for the PRP, deserve special recognition. The efforts of the IWG, and especially the IWG Co-Chairs Ms. Denise Sailstad and Dr. David Hattan, were instrumental in assuring a meaningful and comprehensive review that would address regulatory needs. Finally, the efforts of the NICEATM staff to ensure accurate analyses and timely distribution of information for the review, particularly Dr. Raymond Tice and Ms. Karen Haneke, are acknowledged. On behalf of ICCVAM, we thank all of the many individuals who contributed to this report.

*William S. Stokes, Co-Chair, ICCVAM*
*Richard N. Hill, Co-Chair, ICCVAM*

# Executive Summary

For decades, guinea pig assays have been the standard used to assess the allergic contact dermatitis (ACD) potential of chemicals and products. These assays, in highly experienced hands, have considerable credibility, but are subject to false positive and false negative results. Interpretation of the results requires experience and expertise; follow-up testing in humans is sometimes required.

In January 1998, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) received the Local Lymph Node Assay (LLNA) Submission (Submission) from Drs. G. Frank Gerberick (Procter & Gamble, US), Ian Kimber (Zeneca, UK), and David A. Basketter (Unilever, UK) (Sponsors) for peer review. Following the receipt of this Submission, ICCVAM assembled an independent peer review panel (PRP) to evaluate the usefulness of the LLNA for hazard identification of potential human contact sensitizers. The ultimate aim of new ACD assays, such as the LLNA, is to minimize the frequency and severity of sensitization in human populations.

Evaluation of the LLNA Submission was separated into seven sections, with three to five PRP members assigned to conduct an in-depth analysis of each section. This report is organized by these sections, as follows: (1) Test Method Description; (2) Test Method Data Quality; (3) Test Method Performance; (4) Test Method Reliability (Repeatability/Reproducibility); (5) Other Scientific Reviews; (6) Other Considerations; and (7) Related Issues. The evaluations from the seven sections are then summarized in Overall Summary Conclusions. This report focuses on the performance of the LLNA, and some of the critical assumptions (i.e., the potency of the standard allergens) have only been evaluated minimally.

A public meeting of the PRP took place on September 17, 1998, in Gaithersburg, MD, to reach conclusions and make recommendations regarding the usefulness of the LLNA for hazard identification. In addition to reaching final conclusions on the analysis by section, the PRP also addressed the following two major questions:

1. *Has the LLNA been evaluated sufficiently and is its performance satisfactory to support its adoption as a stand-alone alternative to the Guinea Pig Maximization Test (GPMT)/Beuhler Assay (BA)?*

2. *Does the LLNA offer advantages with respect to animal welfare considerations (refinement [1], reduction[2], and replacement[3] alternatives)?*

In response to the first question, the consensus of the PRP was that the LLNA results, as submitted and supplemented by the Sponsors, demonstrated that the assay performed at least as well as currently accepted guinea pig methods (GPMT/BA) for the hazard identification of strong to moderate chemical sensitizing agents. The data submitted indicate that the LLNA does not accurately predict all weak sensitizers (false negative) and some strong irritants (false positive). The term weak sensitizer is somewhat arbitrary, since the terms weak, moderate, and strong apply to the percentage of animals reacting in the GPMT/BA as described in the published literature or papers submitted by the Sponsors. When comparing the LLNA with currently accepted methods (i.e., guinea pig methods), the LLNA appears to provide an equivalent prediction of the risk for human ACD. The review involved the evaluation of data on 209 chemicals, of which both LLNA and guinea pig data were available for 126 chemicals and both LLNA and human (HMT and HPTA) data were provided for 74 chemicals. An in-depth review of all the chemicals that have been defined in the published literature as human

[1] Refinement alternative: A new or revised test method that refines procedures to lessen or eliminate pain or distress to animals, or that enhances animal well-being.
[2] Reduction alternative: A new or revised test method that reduces the number of animals required.
[3] Replacement alternative: A new or revised test method that replaces animals with non-animal systems or one animal species with a phylogenetically lower one.

allergens was not conducted for this evaluation. From the analysis generated during the review process, the accuracy[1] of the LLNA vs. GPMT/BA was 89% (N=97), LLNA vs. all guinea pig tests (GPT) was 86% (N=126), the LLNA vs. human data was 72% (N=74), GPMT/BA vs. human was 72% (N=57), and all guinea pig tests (GPT) vs. human was 73% (N=62). In terms of accuracy, sensitivity[2], specificity[3], and positive[4] and negative[5] predictivity, the PRP found the performance of the LLNA to be similar to that of the GPMT/BA. Equally important, the performance of the LLNA and the GPMT/BA was similar when each were compared to human data (HMT/HPTA). Performance calculations may be found in Tables 2 and 3 of this report.

The PRP also agreed that the LLNA has several advantages over guinea pig methods for the following reasons:

(1) provides quantitative data;
(2) provides dose response assessment;
(3) reduces animal distress;
(4) potentially reduces animal numbers;
(5) potentially more cost effective;
(6) requires much less time;
(7) involves the induction phase of sensitization; and
(8) will allow for future assay improvement and mechanistic studies.

Possible assay weaknesses (e.g., false negative results with some weak sensitizing agents and metals, false positive results with some strong irritants) were identified. It was recommended that these should be evaluated in future workshops. Also, data to support the testing in the LLNA of mixtures was not provided and the evaluation of pharmaceuticals was limited.

In response to the second question, the PRP concluded that the LLNA offers several advantages with respect to animal use refinement compared to conventional guinea pig methods in that it involves less pain and distress. The method evaluates the induction phase and not the elicitation phase of the response, which significantly reduces the distress suffered by mice used in the LLNA when compared to guinea pig procedures (GPMT/BA). Furthermore, Freund's adjuvant is not used, and there is a substantial reduction in time required to perform the assay. Animal usage may also be reduced (protocol-dependent).

In summary, the PRP unanimously recommended[6] the LLNA as a stand-alone alternative for contact sensitization hazard assessment, provided that the following protocol modifications were made:

(1) Until a systematic comparison of data between (a) mouse strains, and (b) male and female mice are conducted, the protocol should specify the use of female CBA mice only;
(2) Animals should be individually identified;
(3) Body weight data should be collected at the start and end of the assay;
(4) Lymphocyte proliferation data should be collected at the level of the individual animal;
(5) Statistical analysis should be performed;
(6) A single dose of a sensitizer inducing a moderate response should be included as a concurrent positive control in each study;
(7) $^{3}$H-methyl thymidine or $^{125}$I-iododeoxyuridine may be used in the LLNA;

[1] Accuracy: (a) The closeness of agreement between a test result and an accepted reference value. (b) The proportion of correct outcomes of a method. Often used interchangeably with concordance.
[2] Sensitivity: The proportion of all positive chemicals that are correctly classified as positive in a test. A measure of test performance.
[3] Specificity: The proportion of all negative chemicals that are correctly classified as negative in a test. A measure of test performance.
[4] Positive predictivity: The proportion of correct positive responses among materials testing positive. A measure of test performance. The positive predictivity is a function of the sensitivity of the test and the prevalence of positives among the chemicals tested.
[5] Negative predictivity: The proportion of correct negative responses among materials testing negative. A measure of test performance. The negative predictivity is a function of the sensitivity of the test and the prevalence of negatives among the chemicals tested.

[6] After the peer review meeting, one absention was changed to approval.

(8) The decision process to identify a positive response should include a $SI \geq 3$, statistical significance, and dose response information;
(9) An illustration should be added to the protocol, indicating the nodes draining the exposure site that are to be harvested.

Additionally, the PRP recommended that retrospective data audits be conducted on at least three of the intra- and inter-laboratory LLNA validation studies conducted by the Sponsors. The panel commented that as additional experience is gained with the LLNA, there will be an opportunity to refine these interpretations.
Further, the PRP concluded unanimously that the LLNA is a definite improvement with respect to animal welfare (i.e., refinement and reduction) over the currently accepted GPMT.

The LLNA test as proposed measures lymphocyte proliferation using incorporation of $^{3}$H-methyl thymidine in draining lymph nodes of animals topically exposed to the test article. The measured lymphocyte proliferation response is an essential biological element in the induction phase of sensitization. In contrast, currently used guinea pig assays measure skin reactivity to a secondary challenge with the substance under investigation. It may even be argued that for hazard identification, sensitization (the primary immune response) is more relevant than the secondary response (eczematous reaction) of challenged skin. Sensitization is a prerequisite for ACD, and it is sensitization that constitutes the hazard. In a sensitized person, be it a respiratory or contact allergy, an allergic disease manifestation will not always develop upon challenge: there are individual-dependent factors, dose and mode of exposure factors, and adjuvant effects (including irritant potential and substances that increase skin penetration). All of these factors can be considered part of the risk assessment process rather than hazard identification. In the guinea pig models, hazard is combined with a set of defined risk conditions (secondary challenge conditions) and disease-analogous skin responses are measured. Thus, because of its pivotal role and obligatory presence in the process of allergic sensitization, cellular proliferative activity in the lymph node(s) draining the area of skin exposed to the substance under investigation must be considered an important and biologically relevant parameter in relation to contact allergy.

In the proposed LLNA, increased levels of radioactive thymidine or uridine incorporation, measured from lymph nodes draining the application site, results from increased proliferation of cells in the lymph node at the time of chemical exposure and of cells that migrate to the lymph node because of the chemical exposure. Thus, there are two mechanisms behind an increased stimulation index with the current protocol: a net influx of lymphoid cells/increase in cell numbers, and an increased proliferative rate. A stimulation index (SI) $\geq 3$ may predominately reflect an increase in cell numbers and/or an increased proliferative activity (per cell) of cells residing in the lymph node. This dual response probably increases the sensitivity of the test, because it measures the additive effect of two biological phenomena.

# 1. Test Method Description

## 1.1. Sufficiency of test method and protocol description

The Submission contains a thorough protocol. The scientific basis for the test is described as the measurement of the incorporation of $^3$H-methyl thymidine into lymphocytes in draining lymph nodes of animals topically exposed to the test article, as a measurement of sensitization. The endpoint of interest is stated clearly (SI $\geq$ 3). The proposed protocol provides sufficient detail such that appropriately trained personnel should be able to properly conduct independent studies. Dosing procedures, including the preparation and disposal of dosing solutions, are clear. The protocol specifies that the test article be applied to the dorsal aspect of the ear. Dosing only the dorsal aspect of the ear as opposed to splitting the dose between the dorsal and ventral aspect increases the concentration of chemical exposure per surface area. Information is provided on the appropriate choice of vehicles and the selection of doses, including the need to assess for a dose-response relationship. Problems associated with choice of vehicles and concentrations to be tested are discussed in Section III.

The range of applications of the method are described in the Submission. It is implied but not directly stated that the method is to be used for low molecular weight organic chemicals and that the assay has not been validated for all metals or larger molecular weight compounds, such as proteins. The majority of the supporting data represents the testing of simple chemicals. One publication was included in the Submission on the testing of pharmaceuticals (Kimber et. al., 1998), although the number of pharmaceuticals tested was limited. The use of the LLNA to assess the skin sensitizing potential of mixtures and extracts was also not addressed in the Submission or by the PRP.

Safety issues relating to the handling of chemicals and radioisotopes were well presented. Appropriate forms for record keeping were included as an appendix to the Submission. Acceptable variations in the protocol (e.g., the choice of animal strains, the number of mice per dose group , and the choice of vehicles) are described and prioritized. Although the use of different vehicles is described, the majority of the data presented in the Submission resulted from test articles applied in acetone-olive oil (AOO). The majority of the data was analyzed from pooled animals per group. However, the PRP strongly supports the analysis of data from individual animals.

An aspect of the protocol that could cause differences in procedure between laboratories is the description of the lymph nodes to be assayed. These nodes, referred to as the auricular lymph nodes, are a designation for nodes draining the ear. Given that this is not standard anatomical nomenclature, it is possible that different laboratories could be removing different nodes for evaluation. To the best of the reviewers' knowledge, there is no specific nomenclature for this set of lymph nodes. The anatomical location (e.g., diagram or photograph) of the auricular lymph nodes would be a beneficial addition to the protocol. Furthermore, it should be noted that locating the proper lymph nodes might be difficult when there is no induction by the test material. It is suggested that inexperienced personnel practice with a known sensitizer until competence is obtained.

### 1.1.1. Adequacy of agreement between the protocol used to generate Submission data and the proposed protocol

Much of the data presented in support of the Submission were collected by following the proposed protocol. In some cases, slight modifications were made. Variations from the protocol included the use of four days of consecutive dosing instead of three; and the use of $^{125}$I-iododeoxyuridine as compared to $^3$H-methyl thymidine. In cases where variations occurred between laboratories in inter-laboratory validation studies, similar results were obtained from modified protocols (Kimber et. al., 1995; Loveless et. al., 1996). Information on variations in the protocol used for each of the chemicals included in the provided LLNA database would have been useful in understanding the total experience with the current "standard" protocol. In most instances, there is no clear rationale for the choice of one modification over another. Having a two-day rest period prior to injecting with $^{3}$H-methyl thymidine instead of one day is more convenient in a setting where people are working five-day weeks. There has been much more experience with the use of $^{3}$H-methyl thymidine as compared to $^{125}$I-iododeoxyuridine in the LLNA. Following discussion, the PRP recommended allowing the use either of $^{3}$H-methyl thymidine or $^{125}$I -iododeoxyuridine. $^{125}$I -iododeoxyuridine has a shorter half-life which results in less cost associated with radioactive waste disposal.

### 1.1.2. Appropriateness of dose selection procedure

The dose selection process as defined by the protocol is based on previous experience in guinea pig tests, structure analysis, and solubility factors. If the LLNA is to be used as a 'stand-alone' assay on new substances, reference to guinea pig tests is inappropriate. Where no information is available, concentrations to be tested should be based on toxicity, solubility, and irritancy. The standard protocol states that three to five concentrations are selected among ten possible dose levels ranging from 0.1% to 100%. The published LLNA tests are usually performed by testing the substance of interest using a minimum of three concentrations. It is crucial to test high concentrations to avoid false negatives. An example of this potential problem is with ethylenediamine (free base) in Table 3 of Assessment of the Skin Sensitization Potential of Topical Medicaments using the Local Lymph Node Assay: An Interlaboratory Evaluation (Kimber et al., 1998). Ethylenediamine would have been classified as nonsensitizing if concentrations of 0.1 to 1.0% had been selected. Strong sensitization responses were observed at concentrations of 5.0 and 10% in AOO. Some other well known allergens require high concentrations to yield a SI $\geq$3 (i.e., eugenol, hexyl cinnmamic aldehyde, and penicillin G) (Montelius et al., 1998). For much of the data presented in the Submission, compounds were not tested at the highest possible concentrations and solubility data were not provided. The PRP recommends that a rationale for the selection of vehicle as well as for concentrations tested be included for each test article. Discussion of this issue is included in Section III.

No information was provided regarding the need for determination of dermal irritation or acute toxicity data prior to conducting the actual test. If one assumes that irritation is not a confounding issue in the LLNA as it is in the guinea pig assays where the end point is a measurement of erythema and edema, then there are benefits to being able to test higher concentrations of compounds. If one was limited to testing non-irritating concentrations of highly irritating compounds, it is possible that high enough concentrations to reach a sensitizing dose may not be tested, resulting in false negative responses. Although several reports have presented data where exposure to highly irritating concentrations of chemicals resulted in an SI $\geq$ 3, the Sponsors have addressed the issue of irritation and suggest that proliferation induced by irritation may be non-dose responsive and rarely exceeds the required three-fold increase in SI over control to predict sensitization potential. The Sponsors have stated that local or systemic toxicity may result in a suppression of the response at high doses. It is possible that, in the absence of preliminary toxicity testing, using toxic concentrations of chemicals may result in the need for repeat studies.

The protocol does not specify that animals be weighed at the beginning and end of the study. Having weight gain data available would allow for an evaluation of toxicity that may be useful in assessing data in which a decline in the dose-response relationship is seen at high doses and is recommended. To collect animal weight data, identification of individual animals is required. Individual animal identification is also a requirement for studies performed in compliance with Good Laboratory Practice (GLP) regulations.

Additional comments relating to irritation were made by PRP members. The PRP members questioned whether a grading system for dermal irritation should be developed to quantify the degree of skin irritation at the treatment sites. It is not clear as to what prevents the application of a severe irritant or a corrosive substance. Further, the PRP questions whether there is a need for a prestudy screen of the irritation potential of the test material. Although solubility and potential toxicity may influence the concentrations that will be used in a test, the protocol does not provide clear guidance on the selection of a concentration for the performance of the assay.

### 1.1.3. Appropriateness of the number of dose groups

The protocol specifies that a vehicle group and three to five test groups be assayed. Assuming that the appropriate concentrations are chosen (see No. 2 above), this study design is appropriate for a toxicology study. However, in the absence of any data on toxicity or solubility, details regarding how test concentrations should be chosen is necessary.

## 1.2. Adequacy and completeness of the test method protocol

### 1.2.1. Test method material and equipment, and animal usage

The test method protocol is detailed and provides sufficient information on materials and equipment needed and technical procedures, such that trained personnel should be able to conduct the LLNA. The appendix of the Submission provides details on reagent preparation and sample sheets for record keeping. The LLNA is analyzed based on a comparison of the mean DPM from treated animals as compared to controls. This differs from the scoring of the guinea pig assays in which a test substance is scored as positive or negative based on the percentage of animals in a group which are responders (15% in a nonadjuvant assay and at least 8% in an adjuvant test) (Marzulli and Maibach, 1996). The guinea pigs used in these assays are outbred animals with a greater genetic variability than the inbred mice chosen for use in the murine LLNA. Test results have shown that, based on using a SI≥3 as the sole criteria for determining a positive response in the LLNA, an N of four or five mice per test group provides comparable results to the guinea pig tests with 10 to 20 animals.

The specified age range of 8 to 12 weeks is appropriate for immunotoxicological studies. Mice become immune competent at approximately six to eight weeks of age (Shultz and Bailey, 1975; Tyan, 1981).

The strain chosen is a known Th1 (T-helper cell type 1) responder. However, the choice of strain has been made without a systematic comparison of alternatives. There is adequate documentation for the influence of genetic factors on contact allergy, although there is less documentation on how important a role this might have in testing. There is adequate documentation that inbred mouse strains differ in delayed-type hypersensitivity (DTH) reactions to antigens (Shultz and Bailey, 1975). Few studies have been conducted to compare the responsiveness of other inbred mouse strains to the CBA mouse in the LLNA. The documentation in the paper cited on this point (Kimber and Weisenberger, 1989) is preliminary, with only one (strong) sensitizer (2,4-dinitrochlorobenzene [DNCB]), and with a protocol different from the one submitted to ICCVAM. A range of sensitizers should be tested in parallel in a number of representative inbred strains of mice before another strain can be considered validated.

A better description of the responder properties of various mouse strains would be useful for evaluation of the robustness of the LLNA. Different lines of mice within a given strain (i.e., substrains) show genetic differences and will drift further apart genetically over time. Substrains may differ in their immune responses; one example is the DTH response to mycobacterial antigens in different substrains of C3H mice (Løvik et al., 1982). If different mouse strains are found to differ significantly in their LLNA response and genetic factors play a role, one obvious measure to help avoid false negatives would be to retest (suspicious) negative substances in a different strain of mice. Documentation provided (Kimber et al., 1998) suggests that for some CBA substrains, substrain differences have minimal effect on the LLNA response.

The Sponsor's protocol permits the use of both male and female mice, but only one sex in each experiment is proposed. Female CBA mice have been shown to develop a stronger contact dermatitis response as compared to males (Ptek et al., 1988). Furthermore, males are considered to show larger variation because of a greater tendency to fight and to be involved in 'social ranking' processes if group housed. However, this clearly is mouse strain-dependent. In the future, the use of both genders of mice might offer economic advantages, both for institutions breeding their own mice, and for users who buy their mice from commercial breeders. The documentation supplied is with female mice only. If the protocol permits the use of male mice, systematic studies on sex differences in the response should be documented.

### 1.2.2. Test method data collection procedure

The protocol adequately describes the measurement of the incorporation of $^{3}$H-methyl thymidine into proliferating lymphocytes in draining lymph nodes as a measure of sensitization. However, there appears to be two methods of performing the assay, one based on using lymph node samples pooled across mice within a treatment group (favored by the European collaborators) and another based on individual animal responses (favored by the American collaborators), which is evident in reviewing the publications from the inter-laboratory validation studies. It appears an assessment of DPM in lymph nodes from individual animals is advantageous to using lymph nodes pooled within a dose group to determine radioisotope incorporation. The pooled approach precludes statistical analysis of the data which should be used to aid in result interpretation. Thus, the draft protocol should be modified to recommend only the collection and analysis of individual animal data.

### 1.2.3. Data analysis, evaluation, and decision criteria

The protocol allows for pooling of the draining lymph nodes from multiple mice within each test group or the analysis of pooled nodes from individual animals. The mean DPM for each test group is compared to the control group and if the SI of a test group is ≥3 fold higher than the concurrent control, the test chemical is considered to be a sensitizer. The Sponsors state that the three-fold increase is an arbitrary number chosen based on the performance of the assay with a group of known sensitizers. Extensive analysis performed by NICEATM with the assay supported the three-fold increase as an adequate indicator of the sensitizing ability of chemicals. The Sponsors state that the three-fold factor takes into consideration the variability within and between groups and allow for the assumption that irritation may elicit a low level of lymphocyte proliferation.

The PRP had significant concerns about the lack of emphasis on statistical analysis in the Submission. Pooling lymph nodes from animals by dose group for radioisotope incorporation versus an evaluation of lymph nodes from individual animals to estimate the SI does not represent replicate testing and precludes any statistical analysis of the data. Statistical analysis would definitely benefit the LLNA protocol. It would confirm whether or not an apparently high SI (≥3) is due to chance variation (e.g., see Table 4, Kimber et al., 1995), thereby reducing possible false positives. It may detect whether an apparently low SI (<3) for a particular compound are statistically higher than can be explained by chance variation, and may thereby reduce the number of potential false negative responses. In both of these situations, the statistical results would at least call into question the decision based solely on SI, and thus suggest a retest. Additionally, the evaluation of individual animal data provides for trend analysis to confirm dose responsiveness. However, not all statistical differences are biologically meaningful or relevant for regulatory decision making. It is a practical question whether the qualitative statement from a statistical test is sufficient, or whether a quantitative element/magnitude of the difference also has to be considered. The SI represents one such quantitative parameter. Similar combinations of statistical and practical decision rules are used in genetic toxicology tests.

Although the statistical significance of an observed response is very important, no rigid statistical decision rule should be the sole factor in determining the biological significance of a skin sensitization response. Other factors that should be considered include the magnitude of the effect (SI ≥ 3), the strength of the dose-response relationship, chemical toxicity and solubility, and the consistency of the (positive and negative) control response with other contemporary studies.

It is the recommendation of the PRP that data be generated by analyzing lymph nodes from individual animals. This view was supported by individuals at the Public Meeting representing regulatory agencies. This would allow for the use of a SI ≥ 3 for identifying positive responses and dose-response relationship, evaluation of incidence, and statistical analysis may be used as an aid in evaluating test results. Use of individual animal data allows for a formal statistical analysis of whether or not an elevated SI is significant relative to controls. These results can be used in conjunction with the three-fold SI rule to determine the skin sensitization potential of the test chemical. The following guidelines should be considered.

The calculated measure of response (SI) will generally be simply the ratio of the mean DPM responses in the dosed and control groups. However, the investigator should be alert to possible "outlier" responses for individual animals within a group that may necessitate the use of an alternative measure of response (e.g., median rather than mean) or elimination of the outlier.

Each SI should include a measure of variability that takes into account the inter-animal variability in both the dosed and control groups. For example, dividing each dosed group animal response by the mean control response and calculating the SD of these ratios does not take into account the variability inherent in the control group. The SI is a ratio of two random variables, and the formula for the SD of this ratio is available in many standard statistical textbooks.

The statistical analysis should include an assessment of the dose-response relationship as well as pairwise dosed group vs. control comparisons. In choosing an appropriate method of statistical analysis, the investigator should maintain an awareness of possible inequality of variances and other related problems that may necessitate a data transformation or a nonparametric statistical analysis.

### 1.3. Positive, negative, and irritation control chemicals

The protocol does not adequately address the use of controls. The protocol specifies the inclusion of a vehicle control but not a positive or irritation control. The inclusion of a single concentration of a moderate grade sensitizer as a concurrent positive control would provide validity to the assay indicating that all procedures involved in the assay were conducted properly. In addition, a positive control will provide a standard to compare between studies and laboratories. Regulatory agency representatives present at the public meeting supported the need for a concurrent positive control with each assay. The PRP recommends the use of a positive control in the form of a sensitizer inducing a moderate response. Based on the criteria set for the evaluation of the LLNA, there is no need for an irritation control.

### 1.4. Dose response interpretation

The dose-response relationship is an advantage of this method and becomes important in the evaluation of equivocal results. The ability to evaluate multiple concentrations of the chemicals is an advantage of the LLNA because it provides added confidence that compounds that are skin sensitizers will be detected. The Sponsors have designated a SI ≥ 3 as the limit for classifying a chemical as a sensitizer. In equivocal cases where the SI does not reach three-fold, but there is a positive dose response, repeating the study to assess reproducibility may be appropriate. Also, the dose response relationship allows for the evaluation of potential systemic toxicity. In cases where a suppressed response is seen at high doses, the dose response may allow for recognition of a toxic response.

### 1.5. Strengths and/or limitations

The strengths of the LLNA are its quantitative nature, the inclusion of a dose response relationship, the ability to test colored substances, improved animal welfare, and the reduction in the time required to conduct a study. The usefulness of the method for testing mixtures and extracts was not addressed in the proposal. Some strong irritants and sensitizing metals appear to be problematic for the LLNA. A failing of the LLNA, as described, is its inability to identify some metal salts as contact allergens. Ikarashi et al. (1992a; 1992b; 1993) suggest that the use of DMSO as a vehicle results in a positive LLNA test when metal salts, including nickel and copper salts, are applied to the skin. To better evaluate interlaboratory comparisons, the PRP would like to have seen more data generated from blinded studies.

### 1.6. Editorial/technical corrections

The PRP found the protocol to be well written and easy to follow.

### 1.7. Conclusions

The PRP found the recommended protocol to be thorough. The strengths of the assay were seen as its mechanistic basis, quantitative endpoint, and the inclusion of a dose response relationship. Weakness were seen as the assay resulting in false negatives (e.g., some metals and some clinically relevant allergens) and false positives (e.g., some irritants). Furthermore, there is limited experience with pharmaceuticals and mixtures/extracts. The value of adding a concurrent positive control was seen as providing validity to the assay and giving a standard by which to compare between studies and laboratories. It is crucial to test high concentrations of test materials to avoid false negatives. The choice of the highest concentrations tested should be based on solubility and toxicity. The choice of suitable vehicles are described and prioritized. However, the majority of the data presented in the Submission resulted from exposure to test articles applied in AOO.

### 1.8. Recommendations

The following changes to the protocol were recommended:

(1) Until a systematic comparison of data between (a) mouse strains, and (b) male and female mice are conducted, the protocol should specify the use of female CBA mice only;
(2) Animals should be individually identified;
(3) Body weight data should be collected at the start and end of the assay;
(4) Lymphocyte proliferation data should be collected at the level of the individual animal;
(5) Statistical analysis should be performed;
(6) A single dose of a moderate sensitizer should be included as a concurrent positive control in each study;
(7) $^{3}$H-methyl thymidine or $^{125}$I-iododeoxyuridine may be used in the LLNA;
(8) The decision process to identify a positive response should include a SI $\geq$ 3, statistical significance, and dose response information;
(9) An illustration should be added to the protocol, indicating the nodes draining the exposure site that are to be harvested.

# 2. Test Method Data Quality

Validation studies appear to have been conducted in the "spirit" of Good Laboratory Practice (GLP) (or Good Research Practice) as determined by standard operating procedures (SOP) at the individual institutions. Formal audited reports were not prepared because the data were primarily intended for publication. By definition, without an audited final report, a study does not conform to GLP. Data record forms in the sample protocol (Appendix D) and supplemental individual animal data supplied solely for PRP review indicated that record-keeping and data collection were adequate.

## 2.1. Protocol consistency during validation

Assurance was not provided to indicate adherence to a standard protocol during the validation studies. Early validation studies were conducted before a standard protocol was available; thus, slight procedural variations occurred as described in the next section. Two protocol modifications were intentionally introduced during the later validation studies.

## 2.2. Protocol variations and modification during validation

Several variations/modifications of the standard protocol are described in the validation studies. These variations and modifications included:

(1) exposure of mice for four rather than three consecutive days;
(2) differences in the number of mice per treatment group;
(3) removal of nodes four days rather than five days after initiation of the study;
(4) use of different mouse strains;
(5) use of pooled nodes vs nodes from individual mice for each treatment group; and
(6) use of $^{125}$I-iododeoxyuridine rather than $^{3}$H-methyl thymidine.

However, data based on using a four-day treatment protocol were not included in the database and this modification is currently not considered acceptable. Procedural variations nos. 2 to 4 are difficult to identify as true changes or modifications of the standard protocol, since they appeared to have more to do with how a particular laboratory performed the LLNA, rather than being an intentional modification for assay optimization. With the available documentation, in most cases it was not possible to distinguish which studies used which of these modifications. Consequently, a rigorous evaluation of the effects of these four protocol variations on test results was not possible. Modification nos. 5 and 6 were intentional modifications and are clearly described in Kimber et al. (1998). The justification for these two modifications was to evaluate the effects of slight modification on the predictive value of the test. This justification is adequate and, overall, these variations and modifications did not significantly alter test results, indicating that the LLNA is relatively insensitive to minor variations in procedure.

## 2.3. Data audits

In the absence of formal audited reports and GLP compliance statements, it is not possible to determine if data audits were conducted by Quality Assurance Units. The Sponsors state that much of the data presented in support of the Submission were derived from audited GLP compliant studies (Appendix C), inferring that data audits were conducted. Additionally, the Sponsors state that, with retrospective audits, GLP compliance statements could be issued for the great majority of substances tested. The integrity of the validation data is also supported by the fact that all interlaboratory validation data were made available to, and scrutinized by, all participants.

## 2.4. Recommendation

Due to lack of representative quality assurance and GLP documentation in the Submission, it is recommended that data quality and adherence to protocol (in individual studies) be confirmed by retrospective auditing of at least three individual LLNA studies. The studies should be selected by NICEATM from those conducted in the later phase of the interlaboratory validation, and should include laboratories from both the US and UK.

# 3. Test Method Performance

## 3.1. Data presentation

The Sponsors' Submission applies a three-fold SI for evaluating the sensitization potential of a chemical using the LLNA. The Sponsor's initial Submission, which included only a table of "+" and "-" data, did not provide sufficient detail for the comprehensive evaluation of the LLNA. However, subsequent literature evaluation (Basketter and Scholes, 1992; Basketter et al., 1994; Basketter et al., 1996a; Basketter et al., 1998; Gerberick et al., 1992; Kimber et al., 1990; Loveless et al,. 1996) carried out by NICEATM and PRP members provided more detailed information on SI for a majority of the chemicals evaluated. This compilation permitted a more definitive evaluation of LLNA performance, in particular, the application of the SI $\geq$ 3.0 rule and the determination of sensitivity and specificity of the assay in comparison to the GPMT/BA and human sensitization data.

There were minor data inconsistencies, including double reporting under chemical synonyms for one chemical, inaccurate reporting of whether or not a standard guinea pig test method was used, and minor omissions in the Submission. Most of these inconsistencies were resolved during the review process and in discussions and teleconferences with the Sponsors. Comparison to literature citations confirmed the accuracy of almost all of the LLNA classifications provided by the Sponsors. However, the PRP could not confirm positive results (but did confirm negative results) reported for aniline, 4-chloroaniline, streptomycin sulfate, or α-trimethyl-ammonium 4-tolyoxy-4-benzenesulfonate, nor the equivocal result reported for neomycin sulfate. These chemicals were considered negative in the analysis of LLNA assay data, although it is recognized that unpublished data may exist that would support a positive call. Hydroquinone and quinol had the same CAS number and were changed to a single listing. Benzoic acid and glycerol were tested using a non-standard LLNA protocol and, in agreement with the Sponsors and consistent with other similar data, excluded from further consideration. Benzocaine yielded equivocal LLNA results among six separate studies and was excluded from subsequent performance evaluations. The revised data are compared to the Submission in Table 1.

The LLNA was validated for hazard identification of chemicals, as defined by the National Research Council (NRC, 1983) with a proclivity to produce ACD.

The LLNA assesses the induction process and does not assess the elicitation process. ACD refers to an immunologically mediated process in man or animal that is characterized by redness and swelling of the skin and is a cell mediated (type IV) process (Kawabata et al., 1996). For the purposes of this report, the LLNA assesses type IV hypersensitivity and no attempt has been made to validate this assay for immediate hypersensitivity and contact urticaria syndrome.

**Table 1. Comparison of Original and Revised Concordance Between the LLNA and Guinea Pig Tests**

| LLNA | GPT | Original | Revised |
|---|---|---|---|
| + | + | 86 | 81 |
| + | - | 6 | 6 |
| - | + | 10 | 12 |
| - | - | 28 | 27 |
| Total | | 130 | 126 |
| Concordance | | 88% (114/130) | 86% (108/126) |

## 3.2. Adequacy of the test method performance evaluation

There is a century of experience on the identification of chemicals that produce ACD in man. The definition of ACD in man is operational in nature in that several components are required for verification: this includes history, physical examination, diagnostic patch testing with appropriate controls, and natural history after removal of the contact allergen.

For this review, the PRP compared the LLNA against guinea pig data and compared both the LLNA and guinea pig test data against human data, where available. This PRP did not conduct an in-depth review of all the chemicals that have been defined in the published literature as human allergens.

The PRP, with the assistance of NICEATM, compared the LLNA to the guinea pig assays in terms of specificity, sensitivity, positive and negative predictivity, and accuracy. The purpose of this evaluation was to determine if the LLNA, as a test for hazard identification, is equivalent to or superior to the guinea pig assays. To accurately make that comparison, the guinea pig assay would have to undergo the same rigorous evaluation as the LLNA. The PRP is not aware of any such evaluation.

Although much effort was expended to compare the LLNA to the GPMT/BA, the goal of LLNA testing is for hazard identification and to prevent human sensitization. Thus, the PRP attempted to compare the performance of the LLNA to available sources of human data that were viewed as the "gold standard." Of the 209 chemicals tested in the LLNA, 97 were also tested in the GPMT/BA, an additional 29 were tested using non-standard guinea pig tests, and 39 were tested using the human maximization test (HMT). Inclusion of compounds that are included in human patch test allergen (HPTA) panels expanded the comparative human data set to 74 compounds. These human data were not further validated as that would have required an exhaustive study of the literature to determine their potency. Thus, these data should be considered with the caveat that a few of the HPTA compounds may cause human sensitization only infrequently.

Several deficiencies in the Submission materials were noted by the PRP. Since the choice of vehicle may be problematic in the LLNA, analysis of vehicle effects should have been more thoroughly evaluated. Acetone or AOO appeared to be the preferred vehicle in most studies, followed by *N*,*N*-dimethyl formamide (DMF), methyl ethyl ketone (MEK), propylene glycol (PG), dimethyl sulfoxide (DMSO), and saline or 50% acetone/saline. There are very few data available on vehicles other than AOO, DMF, and DMSO. It is desirable that predictive animal tests be performed with vehicles relevant for human exposure where possible. The choice of vehicle may be decisive for the determination of the SI. For instance, olive oil may pose problems in the LLNA since it is reported as an allergen giving an SI=16 to 23 when tested at 100%, and 2.9 to 3.6 when tested as AOO (4:1) (Montelius et al., 1996).

The choice of test concentrations is also crucial to the proper performance of the LLNA. It is given in the standard protocol that "three to five concentrations are selected among ten possibilities ranging from 0.1% – 100%." The preponderance of data is based on tests performed using three concentrations. It appears that some well known allergens require high concentrations to yield a SI $\geq 3$ (e.g., eugenol, hexylcinnamic aldehyde, ethylenediamine, and penicillin G). For some non-sensitizing irritants (e.g., nonanoic acid and methyl salicylate), it appears that high concentrations yield a SI $\geq 3$ (Montelius et al., 1998). It was not stated clearly enough in the Submission that the range of concentrations tested may be decisive for the result.

## 3.3. Adequacy of the numbers of chemicals/products evaluated

There have been a substantial number of chemicals and classes of chemicals tested using the LLNA to evaluate its performance. Few other toxicological assays have had this type of rigorous evaluation prior to use. However, the PRP noted that several classes of compounds for which the LLNA has been used were under-represented in the Submission. These include some weak sensitizers, irritants, organometals, and petroleum additives. The PRP noted that preferential testing of potent and moderate sensitizers over weak sensitizers would tend to yield better performance data for the LLNA than would be expected in general use for hazard assessment. The PRP disagrees with the statement in the Submission (Appendix C, page C-22) that a LLNA false negative for nickel sulfate is " . . . as unsurprising as it is unimportant" since ". . . new metals are not being invented." The PRP recognizes the importance of LLNA testing of new organometals, particularly in the petroleum additives industry. Data derived from the testing of coded samples in blinded studies would have allowed for a better comparison of LLNA performance to guinea pig and human data. The PRP is aware that such data exist but that it was considered proprietary and was not available for analysis.

### 3.4. Adequacy of test method performance data

There is consensus among the PRP that with the inclusion of the additional material requested of the Sponsors, plus that drawn from published sources, sufficient information was available to evaluate the LLNA. As stated above, additional data for weak sensitizers, some irritants and certain metals, plus data from blinded studies, would have added further rigor to the review.

### 3.5. Sensitivity, specificity, concordance, false positive rate, and false negative rates

The revised database described above and included in Appendix A was analyzed to determine sensitivity, specificity, false positive and false negative rates, and accuracy of the methods compared to guinea pig and human data. The results of these analyses are tabulated below in Tables 2 and 3. Table 2 is based on analysis of all available data for each comparison; Table 3 is limited to compounds for which there are LLNA, guinea pig and human sensitization data for the same compound.

#### 3.5.1. Prediction of non-sensitizers

According to a Chi square evaluation, there is a significant association between the LLNA and guinea pig test (GPMT/BA plus GPT) classification of positive and negative sensitizers ($p$ value < 0.001). Based on 126 compounds (93 guinea pig positive and 33 guinea pig negative), the LLNA exhibited a sensitivity of 87%, specificity of 82%, and accuracy of 86%. The predictive value of a positive test was 93% and the predictive value of a negative test was 69%. The latter value suggests that the LLNA is more likely than guinea pig tests to identify compounds as non-sensitizers. However, the predictive value of a negative test when compared against the GPMT/BA only was 80%. From a regulatory standpoint, false negatives are of greater concern than false positives.

In comparison to the human data, the LLNA exhibited a sensitivity of 72%, specificity of 67%, and accuracy of 72%. The predictive value of a positive test was 96% and the predictive value of a negative test was 17%. GPT gave a similar value for negative predictivity. It should be recognized that this latter value was based on only four human non-sensitizers.

These analyses were also performed applying different SI values to establish a LLNA result as positive. As shown in Table 4, no overall improvement in accuracy was demonstrated if a SI of 2.0, 2.5, 3.5 or 4.0 was chosen instead of 3.0. A higher threshold improves the specificity but reduces the sensitivity. A SI ≥ 3 provided better concordance with guinea pig tests than the other thresholds tested.

**Table 2. Comparative Evaluation of the PRP's Revised LLNA Database**[1]

| Comparison | Number of Comparisons | Sensitivity[2] | | Specificity[3] | | Positive Predictivity[4] | | Negative Predictivity[5] | | Accuracy[6] | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | % | Number | % | Number | % | Number | % | Number | % | Number |
| LLNA vs GPMT/BA | 97 | 91% | (62/68) | 83% | (24/29) | 93% | (62/67) | 80% | (24/30) | 89% | (86/97) |
| LLNA vs GPT | 126 | 87% | (81/93) | 82% | (27/33) | 93% | (81/87) | 69% | (27/39) | 86% | (108/126) |
| LLNA vs HUMAN | 74 | 72% | (49/68) | 67% | (4/6) | 96% | (49/51) | 17% | (4/23)[7] | 72% | (53/74) |
| GPMT/BA vs HUMAN | 57 | 70% | (38/54) | 100% | (3/3) | 100% | (38/38) | 16% | (3/19)[7] | 72% | (41/57) |
| GPT vs HUMAN | 62 | 71% | (42/59) | 100% | (3/3) | 100% | (42/42) | 16% | (3/20)[7] | 73% | (45/62) |

**Abbreviations: LLNA = Local Lymph Node Assay; GPMT = Guinea Pig Maximization Test; BA = Buehler Assay; GPT includes nonstandard Guinea pig tests; HUMAN = Human Maximization Test (HMT) plus Human Patch Test Allergen (HPTA)**

**Number of comparisons refers to the number of substances tested in both systems.**
**Numbers in parentheses indicate actual number of comparisons for each analysis.**

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[1] This analysis was conducted by NICEATM based on the LLNA Submission List of Chemicals provided in Appendix A of this report.
[2] Sensitivity: The proportion of all positive chemicals that are correctly classified as positive in a test. A measure of test performance.
[3] Specificity: The proportion of all negative chemicals that are correctly classified as negative in a test. A measure of test performance.
[4] Positive predictivity: The proportion of correct positive responses among materials testing positive. A measure of test performance. The positive predictivity is a function of the sensitivity of the test and the prevalence of positives among the chemicals tested.
[5] Negative predictivity: The proportion of correct negative responses among materials testing negative. A measure of test performance. The negative predictivity is a function of the sensitivity of the test method and the prevalence of negatives among the chemicals tested.
[6] Accuracy: (a) The closeness of agreement between a test result and an accepted reference value. (b) The proportion of correct outcomes of a method. Often used interchangeably with concordance.
[7] The poor but equal negative predictivity for the LLNA, GPMT/BA, and GPT test results versus human may be due to the nature of the human database used, which was biased towards substances used as HPTAs (approx. 57% when N=74; 61% when N=57; and 60% when N=62).

**Table 3. Comparative Evaluation of the PRP's LLNA Database Limited to Compounds with LLNA, Guinea Pig, and Human Data[1]**

| Comparison | Number of Comparisons | Sensitivity[2] | | Specificity[3] | | Positive Predictivity[4] | | Negative Predictivity[5] | | Accuracy[6] | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | % | Number | % | Number | % | Number | % | Number | % | Number |
| LLNA vs HUMAN | 57 | 72% | (39/54) | 67% | (2/3) | 98% | (39/40) | 12% | (2/17)[7] | 72% | (41/57) |
| GPMT/BA vs HUMAN | 57 | 70% | (38/54) | 100% | (3/3) | 100% | (38/38) | 17% | (3/19)[7] | 72% | (41/57) |
| LLNA[8] vs HUMAN | 62 | 73% | (43/59) | 67% | (2/3) | 98% | (43/44) | 11% | (2/18)[7] | 73% | (45/62) |
| GPT vs HUMAN | 62 | 71% | (42/59) | 100% | (3/3) | 100% | (42/42) | 15% | (3/20)[7] | 73% | (45/62) |

**Abbreviations: LLNA = Local Lymph Node Assay; GPMT = Guinea Pig Maximization Test; BA = Buehler Assay; GPT includes nonstandard guinea pig tests; HUMAN = Human Maximization Test (HMT) plus Human Patch Test Allergen (HPTA)**

**Numbers in parenthesis indicate actual number of comparisons for each analysis.**

[1] This analysis was conducted by NICEATM based on the LLNA Submission List of Chemicals provided in Appendix A of this report.
[2] Sensitivity: The proportion of all positive chemicals that are correctly classified as positive in a test. A measure of test performance.
[3] Specificity: The proportion of all negative chemicals that are correctly classified as negative in a test. A measure of test performance.
[4] Positive predictivity: The proportion of correct positive responses among materials testing positive. A measure of test performance. The positive predictivity is a function of the sensitivity of the test and the prevalence of positives among the chemicals tested.
[5] Negative predictivity: The proportion of correct negative responses among materials testing negative. A measure of test performance. The negative predictivity is a function of the sensitivity of the test method and the prevalence of negatives among the chemicals tested.
[6] Accuracy: (a) The closeness of agreement between a test result and an accepted reference value. (b) The proportion of correct outcomes of a method. Often used interchangeably with concordance.
[7] The poor but equal negative predictivity for the LLNA, GPMT/BA, and GPT test results versus human may be due to the nature of the human database used, which was biased towards substances used as HPTAs (approx. 61% when N=57 and 60% when N=62).
[8] This analysis includes compounds tested in nonstandard guinea pig tests. Number of comparisons refers to the number of substances tested in both systems.

**Table 4. Influence of the Threshold SI on Sensitivity and Specificity**

| Comparison | Number of Comparisons | SI Threshold | Sensitivity % | Specificity % | Accuracy % |
|---|---|---|---|---|---|
| | | 2.0 | 85% (66/78) | 59% (16/27) | 78% (82/105) |
| LLNA vs. | 105 | 2.5 | 82% (64/78) | 74% (20/27) | 80% (84/105) |
| GPT | | 3.0 | 81% (63/78) | 89% (24/27) | 83% (87/105) |
| | | 3.5 | 79% (62/78) | 89% (24/27) | 82% (86/105) |
| | | 4.0 | 78% (61/78) | 93% (25/27) | 82% (86/105) |
| | | 2.0 | 72% (39/54) | 33% (2/6) | 68% (41/60) |
| LLNA vs. | 60 | 2.5 | 72% (39/54) | 50% (3/6) | 70% (42/60) |
| Human | | 3.0 | 65% (35/54) | 67% (4/6) | 65% (39/60) |
| | | 3.5 | 65% (35/54) | 67% (4/6) | 65% (39/60) |
| | | 4.0 | 61% (33/54) | 83% (5/6) | 63% (38/60) |

Using human response data as the “gold standard”, three compounds (aniline, nickel sulfate, neomycin sulfate) were false negatives in the LLNA and one (sodium lauryl sulfate [SLS]/sodium dodecyl sulfate) was a false positive in the LLNA. The GPMT/BA registered four false negatives (musk ambrette, ammonium thioglycolate, ethylene glycol dimethacrylate, neomycin sulfate) and no false positives. While these data show one more false positive for the LLNA than the GPMT/BA, the rates of mis-classification for both are low and not significantly different.

### 3.5.2. Prediction of positive sensitizers

The LLNA shows a high concordance with human data and guinea pig test data for strong and moderate sensitizers. The Sponsors reported a 93% positive predictivity in comparison with the guinea pig assays. Improvements in the LLNA should be targeted toward enhancing the detection of weak sensitizers. It is the opinion of some of the PRP members that improved detection of weak sensitizers may be accomplished using the LLNA if the number of exposures (or dose groups) and the number of animals were increased. However, from some false negative cases, the data demonstrate that compounds negative in the LLNA are strongly so and increasing the numbers of test animals would not be likely to have any effect on the test outcome.

As stated in the previous section, three compounds yielded false negatives in the LLNA in comparison to human response data. The GPMT/BA also registered three false negatives. The analyses of sensitivity and specificity indicated the predictive value of a positive LLNA test was 93% and the predictive value of a negative test was 80% compared to GPMT/BA. When compared to human data the predictive value of a positive LLNA test was 96% and the predictive value of a negative LLNA test was 17%. Similar positive and negative predictivity values (100% and 16%, respectively) were found when the GPMT test was compared to human data.

## 3.6. Acceptability of sensitivity, specificity, concordance, and false positive and negative rates

Analysis of concordance between the LLNA and guinea pig data and the LLNA and human data give confidence that the LLNA can reasonably predict human responses to sensitizers when compared to currently accepted methods for regulatory decisionmaking. Potential problems in the

LLNA rest with certain non-sensitizing irritants mis-classified as positive for sensitization and false negatives (compared to human data) represented by compounds from several different classes.

### 3.7. Scientific validity of conclusions on assay usefulness

#### 3.7.1. Clinical relevance and human predictivity

The results of the LLNA are clinically relevant and the test is predictive except for some weak human contact allergens. The functioning of the immune systems of mice and humans are very similar as they relate to ACD. Human ACD generally arises through dermal exposure to non-abraded skin. It is a two-step process requiring first induction of specific immunity, followed by an elicitation response several weeks later. The LLNA utilizes topical application of the test compound to non-abraded skin and quantifies the induction phase (proliferation of T-lymphocytes in the draining auricular lymph nodes) as the indication of the potential of a compound to produce sensitization. One concern is that some non-sensitizing, irritant compounds may produce sufficiently profound lymphocyte proliferation to yield a false positive result. Also, some compounds that are recognized as human sensitizers do not produce a sufficiently strong proliferative response in the LLNA and are mis-classified as negative. This is also true for the guinea pig tests.

#### 3.7.2. Regulatory utility of the method

The utility of the method for regulatory use in hazard assessment of chemicals as potential human contact sensitizers has been clearly established, subject to the limitations discussed above.

## 4. Test Method Reliability (Repeatability/Reproducibility)

In general, the initial LLNA Submission presented qualitative data, which demonstrate adequate intra- and inter-laboratory repeatability and reproducibility. The Submission was deficient, however, in the presentation of quantitative data supporting the reliability of the test method.

The reproducibility of the test method results across laboratories was adequate for a biological assay. In all but one interlaboratory comparison study, all of the test chemicals were identified prior to testing. In the only blinded study, 20 of 25 test chemicals were coded and of these, six chemicals were not reproducibly identified among the four laboratories. More confidence in the intra- and inter-laboratory repeatability and reproducibility of the test method would have been achieved had more quantitative blinded studies been performed. Also, while in most cases the sensitizers and non-sensitizers were correctly identified, it is likely to be more difficult to yield repeatable data with non-sensitizing irritant compounds or weak sensitizers.

### 4.1. Adequacy of <u>intralaboratory</u> repeatability and reproducibility evaluations

The data evaluated for intralaboratory repeatability and reproducibility were limited, in that only six chemicals were evaluated. These data (i.e., Basketter et al., 1996a; Kimber et al., 1998; Loveless et al., 1996) are presented in a summarized form in Tables 1 and 2 (Appendix C, pages C-12 and C-13, respectively) of the Submission. These data, while limited, indicate sufficient agreement; however, there are some discrepancies between the tables. For example, Table 1 of the Submission indicates that three tests were carried out on DNCB and all were positive. However, Table 2 of the Submission indicates that only two tests were carried out for this chemical, not three.

Table 1 of the Submission presents qualitative intralaboratory repeatability data from one laboratory for six compounds including one potent sensitizer assayed three times, three moderate sensitizers assayed four to six times, and two non-sensitizers assayed four or six times. The data indicate that the LLNA correctly identified four known sensitizers, which occurred in three to six repeated tests on each chemical. In this same laboratory, methyl salicylate was correctly identified as a non-sensitizer in each of four tests, while benzocaine was identified as a non-sensitizer in five of six tests.

Table 2 of the Submission presents quantitative intralaboratory data (i.e., EC3 values, defined as the estimated concentration needed to produce an SI of three) from five laboratories that performed two tests each on the potent sensitizer DNCB and two laboratories that performed six tests each on the moderate sensitizer HCA. An assessment (Appendix K) of the DNCB data presented in Table 2 of the Submission indicate a lack of significant intra-laboratory variability.

The data in Table 2 of the Submission also allows for a calculation of coefficient of variation (CV) for intralaboratory variability, which is presented in Table 5.

Recognizing the limitations of such a calculation (i.e., five of the CVs were based on only two tests), overall the CVs are reasonable. In all cases, the sensitizers and non-sensitizers were correctly identified. However, it is likely to be more difficult to yield repeatable data with non-sensitizing irritant compounds or weak sensitizers.

The information provided is sufficient to show that the LLNA can be reproducibly performed in a qualitative manner. However, it would be useful if future evaluations included further statistical analysis of the data to more accurately establish responses by chemical class. Also, it would be useful if future studies include an analysis of the intralaboratory repeatability of this method with an emphasis on compounds with a maximum SI clustered around three.

**Table 5: Analysis of Intralaboratory Variability**

| Laboratory | N | Mean | SD | CV (%) |
|---|---|---|---|---|
| DNCB Laboratory 1 | 2 | 0.040 | 0.01414 | 35.4 |
| DNCB Laboratory 2 | 2 | 0.055 | 0.00707 | 12.9 |
| DNCB Laboratory 3 | 2 | 0.050 | 0.01414 | 28.3 |
| DNCB Laboratory 4 | 2 | 0.075 | 0.02121 | 28.3 |
| DNCB Laboratory 5 | 2 | 0.045 | 0.02121 | 47.1 |
| Isoeugenol | 5 | 0.420 | 0.10955 | 26.1 |
| HCA Laboratory 1 | 6 | 7.7167 | 2.0605 | 26.7 |
| HCA Laboratory 2 | 6 | 9.1667 | 1.7166 | 18.7 |
| Eugenol | 5 | 9.62 | 1.7693 | 18.4 |

### 4.2. Adequacy of interlaboratory reproducibility evaluations

The NICEATM assessment (Appendix K) of the interlaboratory reproducibility of the LLNA data presented in Table 2 of the Submission (Appendix C, page C-13) indicated a lack of significant between-laboratory variability. Interlaboratory CVs of 25.5% and 12.1% were obtained for DNCB and HCA, respectively. These CVs are adequate for a biological assay. However, these values were derived from the mean of two tests in five laboratories and six tests taken at each of two laboratories for DNCB and HCA, respectively, and thus may not be truly representative of a more general single test result at one or more laboratories. Based on EC3 values contained in Kimber et al. (1995) and Loveless et al. (1996), some calculations of inter-laboratory CVs can be made, as presented in Table 6.

**Table 6: Analysis of Interlaboratory Variability**

| Compound | Quantitative Interlaboratory Data | | | | | | Reference |
|---|---|---|---|---|---|---|---|
| | Lab 1 | Lab 2 | Lab 3 | Lab 4 | Lab 5 | CV (%) | |
| DNCB | 0.3 | 0.5 | 0.6 | 0.9 | 0.6 | 37.4 | Kimber et al. (1995)* |
| | 0.5 | 0.6 | 0.4 | 0.6 | 0.3 | 27.2 | Loveless et al. (1996)* |
| HCA | 7.9 | 7.6 | 8.4 | 7.0 | 8.1 | 6.8 | Loveless et al. (1996) |
| Isoeugenol | 1.3 | 3.3 | 1.8 | 3.1 | 1.6 | 41.2 | |
| Eugenol | 5.8 | 14.5 | 8.9 | 13.8 | 6.0 | 42.5 | |
| SLS | 13.4 | 4.4 | 1.5 | 17.1 | 4.0 | 83.7 | |

*These data are also provided in Table 2 of the Submission.

With the exception of SLS, which is a false positive irritant, these data indicate acceptable interlaboratory variability.

There were several earlier open study design interlaboratory studies performed in the UK that showed adequate concordance (72% to 100%) among methods/laboratories; however, these studies remain limited for drawing conclusions about quantitative EC3 variation. In the first study (Kimber et. al., 1991), four laboratories evaluated eight chemicals using the same protocol vehicles and test concentrations. All the laboratories appropriately identified the eight chemicals (100% concordance). In a second study, the same four laboratories tested 25 chemicals (Basketter et al., 1991). Eighteen of 25 equivalent predictions of sensitizing potential (72% concordance) were achieved. In this study, 20 of 25 test chemicals were coded and of these, six chemicals were not reproducibly identified among the four laboratories. However, in the single blinded study, there was low concordance. In a third study, four laboratories evaluated nine chemicals with a protocol deviation from the proposed protocol (i.e., the LLNA was performed on day five instead of day four after three consecutive days of topical application [Scholes et al., 1992a]). Chemicals were evaluated at three concentrations that were pre-selected and differed among the participating laboratories. Eight of nine equivalent predictions of sensitizing potential (89% concordance) were obtained, with 4-chloroaniline being the exception. In a fourth study, five laboratories (i.e., two in the UK and three in the US), in collaboration with the FDA (Kimber et al., 1998), showed five of six equivalent predictions of sensitization potential (83%), with streptomycin being the exception.

### 4.2.1. Inter- and intra-laboratory vehicle control data

There is a considerable range of values for vehicle control data; however, it is difficult to determine if the differences actually affect data quality because the endpoint (SI) in the LLNA is based on the ratio of DPM in the test lymph nodes to that in the vehicle controls. For example, the data presented in Kimber et al. (1998) indicate that the DPM for vehicle controls in the test for benzoyl peroxide ranged from a low of 262 to a high of 463, and for hydroquinone from 257 to 781. However, the SIs for these two chemicals tested at the same concentrations were comparable. Therefore, it is not apparent that the vehicle control results significantly affected data quality.

## 4.3. Reproducibility of reference chemicals or products

The studies appear to have included both clinically relevant sensitizing and non-sensitizing chemicals that represent the types of substances for which the test is proposed for use. The reproducibility of the LLNA was evaluated on a total 49 chemicals/ products (Tables 1 and 2 of the Submission, Appendix C; Kimber et al., 1991; Basketter et al., 1991; Scholes et al., 1992a; Kimber et al., 1995; Loveless et al., 1996; Kimber et al., 1998), with a concordance of 82% among laboratories for identifying the sensitization potential of these chemicals/products.

## 4.4. Repeatability and reproducibility of results

The results obtained with the LLNA appear to be sufficiently repeatable and reproducible. As indicated above (A and B) for the small data set presented in Tables 1 and 2 of the Submission, which were analyzed by NICEATM (Appendix K), sufficient intra- and inter-laboratory repeatability and reproducibility were indicated for the LLNA. However, it is not known how other LLNA data would fare in such an analysis. More confidence in the repeatability and reproducibility of the results would have been gained had an additional blinded study been performed.

## 4.5. Reproducibility and reliability of LLNA versus standard guinea pig assays

A study that directly compares the reproducibility and reliability of the LLNA with the guinea pig assay has not been performed. To the best of the reviewers' knowledge, the guinea pig data have not been evaluated for intra-and inter-laboratory reproducibility and reliability.

## 4.6. Conclusion:

The Submission presents qualitative data, which demonstrate adequate intra- and inter-laboratory repeatability and reproducibility.

## 4.7. Recommendation:

Further testing of the assay should include an additional blinded interlaboratory study with moderate and weak sensitizers.

# 5. Other Scientific Reviews

## 5.1. Literature Reviewed

A literature search was conducted on August 17, 1998 (Medline data base, 1966 to present) using "Local Lymph Node Assay" as the key phrase. A total of 69 articles were retrieved (Appendix B). Of the 69, 42 were published by one or more of the Sponsors involved in the ICCVAM Test Method Submission, or their colleagues, and 27 papers were published by others working in the field.

The PRP concentrated on papers published by investigators not directly involved with the ICCVAM Test Method Submission. Thirteen of these papers reported that the LLNA showed concordance with the GPMT or human results. Three suggested non-concordance (not including the issue of the inability of the LLNA to identify metal salts as contact allergens). The PRP did not independently verify these results. Four papers dealt with other endpoints for the LLNA, two using cytokine production *in vitro*, one using flow cytometry (FCM) to measure proliferation, and one using immunohistochemistry to measure cytokine production in vivo. Six publications covered the issue of false negatives when metal salts were used. Finally, five different papers dealt with generating LLNA data in different species (rats-four; hamsters-one).

Perhaps the most interesting were the publications suggesting that modifications in the LLNA procedure may serve to make the assay more sensitive when irritants were tested and thereby reduce the false positive rate. When common irritants are used in the LLNA, they give a false positive result, inasmuch as these irritants are not contact allergens when applied to human skin. This issue has been described in the literature by others and it is possible that a modification of the LLNA, a pre-exposure to the irritant by use of an occluded patch (Boussiquet-Leroux et al., 1995), or by intradermal injection (in Freund's complete Adjuvant) of the irritant followed by cutaneous application (Ikarashi et al., 1993), resolves this issue and renders the irritants non-sensitizers in the LLNA. As yet, these findings have not been independently verified.

A major failing of the LLNA, as described, is its inability to identify metal salts as contact allergens. This issue has also been addressed by others in the literature. In three papers, Ikarashi et al. (1992a; 1992b; 1993) suggested that the use of DMSO as a vehicle results in a positive LLNA test when metal salts, including nickel and copper salts, are applied to the skin.

Another paper describing the effect the vehicle may have on the results was published by Montelius et al. (1996). Olive oil poses problems in the LLNA as it is reported as an allergen giving SI values of at least 16 when tested at 100% concentration and at least 2.9 when tested as AOO (4: 1).

## 5.2. Conclusions

A review of the other scientific literature supports the use of the LLNA as an alternative assay to identify contact allergens. The LLNA is deficient in detecting sensitization by some weak contact sensitizers, some metals, and organometal compounds.

## 5.3. Recommendations for future workshops

1) Evaluation of whether the LLNA procedure should be modified to contain a second test, including a pre-exposure, as described by Boussiquet-Leroux et al. (1995) and/or Ikarashi et al. (1993), when positive test results are obtained in the first test, such as occurred for irritants, xylene, and pyridine. The purpose of such a modification is to avoid the number of false positive test results.

2) Consideration of whether DMSO should be required as the vehicle in order to increase the sensitivity of the assay (i.e., allow the assay to detect metal salts as contact allergens).

3) Consideration of whether the use of the differentiation index should be employed,

as described by Homey et al. (1998), to differentiate between irritant and contact allergic reactions.

4) Evaluation of the design, performance, and execution of assays for the prediction of allergic contact sensitivity. Since 1943, various agencies have attempted to minimize the frequency of ACD in man. This workshop would review the half century of experience in the hopes of refining our techniques and interpretation.

# 6. Other Considerations

### 6.1. Test method transferability

In general, the test method can be readily transferred among properly equipped and staffed laboratories. The method is tolerant of minor protocol changes, the techniques are commonly used, personnel can readily be trained, and the necessary equipment and supplies can be readily obtained. Whether the method is sensitive to more substantial changes in protocol such as varying the strain of mouse or varying the gender of the mouse is not clear. Some concern was raised regarding the availability of the CBA/Ca or CBA/J mouse worldwide. In addition, the differences in SI obtained by the Montelius group raises concern about the transfer of the method between laboratories. With the inclusion of a concurrent positive control in the protocol, the concern regarding transfer of the technique is reduced. Interlaboratory variability can be more easily determined in the future (see section III).

#### 6.1.1. Sensitivity to minor protocol changes

The LLNA appears to be insensitive to minor changes in protocol. In addition, the use of radioiodinated uridine rather than tritiated thymidine is said to produce the same assay results and conclusions.

Changing the mouse strain or gender cannot be defined as minor changes in protocol until more substantive data and comparisons are provided. No systematic comparisons of alternative mouse strains or effect of gender have been presented. Documentation provided (Kimber et al., 1998) suggests that for some CBA substrains, substrain differences do not have much effect on the LLNA response. A clear concise presentation of the effect of gender and strain of the mouse would provide evidence that any such changes in the protocol would not make a difference in the conclusion.

#### 6.1.2. Considerations regarding training and expertise

The training and expertise in biology available to perform the LLNA is substantial. Tritiated thymidine incorporation as a measure of cellular proliferation is a technique which has been used in immunology laboratories for many years. Thus, expertise in this method is widespread. Individuals skilled in animal handling, including tail vein injection and lymph node harvesting, are required. The technical skills required are significant, but not prohibitive. The test endpoint is objective and requires minimal training in judgment. The use of radioactivity adds to the training requirements of personnel and the level of expertise required.

#### 6.1.3. Ease in obtaining necessary equipment and supplies

The laboratory equipment and supplies required are standard and readily obtainable. The assay can be readily conducted in research laboratories with radioisotope facilities.

### 6.2. Cost-effectiveness

A direct comparison of the actual cost required to conduct the LLNA vs the GPMT was not provided in the Submission. It is expected that the cost of the LLNA will not exceed the current guinea pig tests and will decrease as the use of the assay is increased. The following data were obtained by NICEATM.

Animal costs: Assume that 16 to 30 mice (LLNA) or 24 to 32 guinea pigs (GPMT) are required for the testing of one chemical. Then, 16 to 30 six-week old CBA/J mice cost from \$160.80 to \$301.50. This is compared to the cost of 32 to 43 guinea pigs (400 to 450 g) from Charles River Laboratories at \$1832 to \$2462. In addition, care costs for mice are less than that for guinea pigs because of their smaller size and space requirements and shorter experimental duration.

Cost for testing of chemicals: Two US contract laboratories were contacted regarding testing using the LLNA. These labs quoted prices per chemical in the range of $4,950 (if two chemicals were tested) to $6,900 (if one chemical was tested). The only laboratory contacted regarding the cost of testing using the GPMT quoted a price of $6000 to $7000 per chemical. These estimates suggest that the dollars saved in the purchase of animals for the LLNA would be required for the technical time and expertise required to tail vein inject and harvest and process lymph nodes from the mice. However, an exact analysis of this issue is not provided in the Submission. Certainly animal costs would be reduced even if the cost for running the whole test would not necessarily be reduced. One advantage cited for the LLNA was that the amount of test chemical required is much less than for the guinea pig tests, resulting in additional cost reduction and overall safety. The actual cost of the assay will depend on how many concentrations of chemicals are tested. The cost of radio-labeled thymidine ($20 to 30/test chemical) as well as the cost of radioactive facilities and disposal of radioisotope contaminated waste must also be considered in the final analysis.

## 6.3. Considerations regarding the time needed to conduct the test

The time needed to conduct the test is reasonable. The time from the beginning treatment of animals to a final result is maximally seven days. This is a substantial improvement over the time frame required in the GPMT to obtain a result (i.e., at least 25 days).

## 6.4. Refinement, reduction, and replacement considerations

The LLNA procedure is a definite refinement in terms of reducing or eliminating distress in animals compared to the GPMT. The LLNA does not replace the use of animals for assessing the potential of compounds to cause ACD. Whether the LLNA will result in a reduction in the number of animals used will depend on the actual number of concentrations required for testing the particular compound.

### 6.4.1. Refinement

In the LLNA the induction phase of sensitization is being evaluated. Thus, discomfort to animals associated with the elicitation phase is eliminated. The ACD reaction itself is not being measured so redness and erythema are not induced unless the substance causes irritation over the three-day period of treatment of the mouse ear. Very importantly, the LLNA reduces the distress associated with administering adjuvants such as Freund's adjuvant. The animals are involved in the experiment for a considerably shorter period of time than in the GPMT (i.e., seven days compared to ≥25 days). The only manipulation of the animal is the application of the test solution to the ears on three consecutive days, and one intravenous (i.v.) injection, before the termination of the experiment. This level of manipulation is contrasted to shaving, injection into the skin, and occlusive bandaging in the guinea pig models.

### 6.4.2. Reduction

As required in the protocol, lymph nodes from individual animals are processed, five animals are used per group, and a positive control is included in each assay. Thus, for testing one chemical alone, 25 to 35 animals are required for testing three to five concentrations of a compound. Whether three or five concentrations are tested, the number of mice required will be less than or equal to the number of guinea pigs, with dose response information being obtained as well. Testing of multiple compounds in one assay will further reduce the number of animals required since the vehicle and positive controls will not need to be duplicated. In the opinion of some reviewers, testing three concentrations of each test chemical is sufficient. In this case, adoption of the LLNA would definitely result in a reduction in the number of animals used.

## 6.5. Conclusions

The test method can be readily transferred among properly equipped and staffed laboratories. The method is cost effective and the time required to conduct the assay is considerably less than the current guinea pig assays. The LLNA procedure is a refinement in terms of reducing or eliminating distress in animals compared to the GPMT.

## 6.6. Recommendation

Future submissions to ICCVAM should include quantitative cost data for determination of cost-effectiveness. This cost data should be specific with regard to the number and species/strain of animals (purchase, housing); required reagents and other equipment; and amount of labor (other than animal husbandry) reported in man-hours.

# 7. Related Issues

## 7.1. Alternative endpoints for the LLNA or test method modifications to be considered

### 7.1.1. Alternative Endpoints for the LLNA

Published results using alternative endpoints in the LLNA assays are summarized in Table 7. The concept of LLNA is based on the proliferative response of lymphocytes to allergens at the induction phase of contact dermatitis. Endpoint assays assessing cell proliferation other than $^{3}$H-methyl thymidine incorporation may be applicable to the LLNA.

One approach was published using $^{125}$I-iododeoxyuridine, which has a shorter half-life and reportedly saves on the expense for radiolabeled waste (Ladics et. al., 1995).

**Table 7. Alternative Endpoints for the LLNA**

| Assay Type | Targeted Biological Reactions | Assay Endpoint | Application Period* | Animal Strain | Test chemicals[b] | Reference |
|---|---|---|---|---|---|---|
| **Original** | LNC proliferation | $^{3}$H-methyl thymidine uptake | Day -3 to -1 | CBA/Ca | - | - |
| ***in vivo*** | LNC proliferation | $^{125}$I-iododeoxyuridine uptake | Day -5 to -3 | CBA/JHsd | P:4, N:1 | Ladics et al. (1995) |
| | LNC proliferation (Tissue) | Microscopic observation (BrdU) | Day -5 to -3 | Rat | P:1, N:1 | Arts et al. (1997) |
| | LNC proliferation (Tissue) | Microscopic observation (BrdU) | ** | CD1 | P:4, N:2 | Boussiquet-Leroux et al. (1995) |
| | LNC proliferation (PCNA) | FCM | Day -4 to -1 | BALB/c, C57/BL6 | P:3, N:2 | Kuhn et al. (1995) |
| | Cellularity & LNC phenotype | FCM | Day -4 to -2 | BALB/c, CBA/J | P:5, N:6 | Sikorski et al. (1996) |
| ***ex vivo*** | Cellularity, proliferation, & phenotype | FCM | Day -5 to -3 | BALB/c | P:1, N:1 | De Silva et al. (1993) |
| | LNC proliferation & cytokine profile | cRT-PCR, ELISA | Day -3 to -1 | BALB/c | P:1, N:0 | Ulrich et al. (1998) |
| | Cytokine production (IL-2) | ELISA | Day -3 to -1 | BALB/c | P:8, N:2 | Hatao et al. (1995) |
| | Cytokine production (IL-2) | ELISA, FCM | Day -3 to -1 | BALB/c | P:10, N:4 | Hariya et al. (1999) |
| | Cytokine production (IL-6) | ELISA | Day -3 to -1 | BALB/c | P:9, N:2 | Dearman et al. (1994) |

Abbreviations: BrdU = bromodeoxyuridine; cRT-PCR = competitive reverse transriptase-polymerase chain reaction; FCM = flow cytometry; ELISA = enzyme-linked immunosorbent assay; IL-2 = interleukin type 2; IL-6 = interleukin type 6; LNC = lymph node cell; N = negative; P = positive; PCNA = proliferating cell nuclear antigen

* Day 0=lymph node excision

** Pre-exposure with occluded patch plus three-day application

However, radioisotopes are still used. A proliferative response of lymph node cells (LNC) in rats (Arts et al., 1997) and mice (Boussiquet-Leroux et al., 1995) was assessed by a non-radioisotope method using bromodeoxyuridine (BrdU). However, these methods may not be as accurate as the original LLNA since they necessitate cell counting under microscopic observation. If the non-radioisotope method can produce a reproducible SI similar to that obtained with the standard LLNA, it may be an acceptable alternative. The proliferation of LNC was also determined by the FCM analysis of proliferating cell nuclear antigen (PCNA) (Kuhn et. al., 1995). This method could possibly be a promising alternative to the radioisotope-dependent assay but needs to be validated with a wider range of allergenic chemicals.

Other than the proliferative response, several functional approaches were reported, including phenotypic analysis of LNC subpopulations B220 positive cells which increase in number in response to allergenic chemicals (Sikorski et. al., 1996). This method does did not require the use of radioisotopes and was reportedly effective in differentiating allergens from irritants. Another non-radioisotope LLNA was based on the use of FCM (De Silva et al., 1993). The strong sensitizer DNCB induced a significant increase in CD3 positive and CD25 positive cells compared with vehicle control and SLS. This method reportedly distinguished contact allergens from irritants as well, but is unvalidated.

Cytokine production in LNC was assessed using competitive reverse transcriptase-polymerase chain reaction (cRT-PCR) or enzyme-linked immunosorbent assay (ELISA). As Thl lymphocytes are considered to play an important role in contact allergy, several efforts were attempted to detect Thl-cytokine production induced by contact allergens. Analysis of cytokine gene transcription *ex vivo* and cytokine release revealed that Thl type cytokines as well as Th2 (T-helper cell type 2) type cytokines were produced during the induction phase of contact dermatitis (Ulrich et al., 1998). Production of IL-2 (interleukin type 2), one of the important Thl-cytokines, was investigated as well (Hatao et. al., 1995). The amount of IL-2 was increased by strong allergens but was not always increased by moderate allergens. However, the inclusion of IL-2 production with lymph node weight and CD4 positive subset ratio in LNC improved the sensitivity (Hariya et al., 1999).

The CD IV positive subset ratio reportedly detected the difference between allergens and SLS although the difference is small. In addition to Thl cytokines, the production of IL-6 (interleukin type 6), an inflammatory cytokine with a co-stimulatory effect on T cell proliferation, was measured in *ex vivo* LLNA (Dearman et. al., 1994). IL-6 production was increased by strong allergens; however, the sensitivity of this method was reportedly not sufficient for routine identification of skin allergens.

Proliferation of LNC possibly includes both antigen-specific expansion by contact sensitizers and non-specific proliferation by irritants (Homey et al., 1998). Therefore, a functional analysis may have the potential to differentiate allergens from irritants in addition to the measurement of proliferative response. These approaches have not been fully validated and should be further studied using a wider range of chemicals.

### 7.1.2. Test method modifications

In addition to the *in vivo* LLNA, there have been several reports dealing with *ex vivo* LLNA. One of the major disadvantages of *in vivo* LLNA is the radioisotope-contaminated carcasses. To eliminate this disadvantage, a change from *in vivo* LLNA to *ex vivo* LLNA may be a possible alternative.

The extra work needed for *ex vivo* LLNA would be cell-counting and short-time cell culture. Nevertheless, there would be gains as follows;

(1) No need for i.v. injection;
(2) The amount of radiolabeled thymidine is reduced;
(3) Only simple precautions are necessary; and
(4) Slightly better in terms of animal welfare.

*Ex vivo* LLNA with *in vitro* thymidine uptake would offer advantages in handling but may reduce the sensitivity of the assay.

Several reports are published for the purpose of improving the sensitivity of LLNA. Vitamin A acetate enriched diet for three weeks increased the sensitivity of *ex vivo* LLNA (Sailstad et al., 1995). As a result, the allergenicity of 15% formalin and 3% glutaraldehyde (sensitizers) was detected. Also, the use of an adjuvant improved the sensitivity of the *ex vivo* LLNA (Ikarashi et. al., 1993). Mice were treated with intradermal injections of test chemical in Freund's complete adjuvant emulsion prior to sensitization. Then, the test chemicals were applied on the ears of mice for *ex vivo* LLNA. The LNC proliferation induced by allergenic chemicals was increased in this modification; however, the one by irritants was not. Another example is pre-exposure with an occluded patch, which reportedly enhanced the sensitivity of a modified LLNA (Boussiquet-Leroux et. al., 1995).

## 7.2. Potential workshops and validation efforts

### 7.2.1. General

A workshop on the evaluation process of ICCVAM would be helpful for individuals planning on making Submissions as well as for individuals who may be involved in the evaluation process.

### 7.2.2. Optimization of test conditions in LLNA

There have been several reports regarding modifications of LLNA, which are intended to improve sensitivity, specificity, or handling, and which could be considered for future research needs. The reports include the following modifications;

a. <u>Pre-exposure of test chemicals</u>: When a positive LLNA result is obtained, should the procedure be refined to include a second test including a pre-exposure, as described by Boussiquet-Leroux, et al. (1995) and/or Ikarashi et al. (1993) to avoid false positives such as is seen when the irritants, xylene and pyridine, are applied?

b. <u>Solvent used for topical application</u>: Should DMSO be considered as the vehicle to use to increase the sensitivity of the assay for metal salts?

c. <u>The administration route</u> of [$^3$H]thymidine: i.v. using the tail vein or peritoneal?

d. <u>Use of abrasion for water-soluble chemicals</u>: Should the ear skin be abraded to increase the sensitivity to water-soluble chemicals?

In addition to these future optimizations, LLNA endpoints other than $^3$H-thymidine uptake and the modified LLNA procedures cited in the section VII.A.1. of this report may be a target of research or a validation study.

### 7.2.3. Photosensitization

A photosensitization test composed of UVA irradiation and the LLNA may be a methodological target once the LLNA protocol is accepted for regulatory purposes. One methodological paper used $^3$H-thymidine uptake as an endpoint combined with UVA irradiation, which is reportedly able to detect moderate photoallergenic potential (Scholes et. al., 1991). An additional two papers are evaluated on the reactions in draining lymph node such as cytokine expression pattern (Ulrich et al., 1998), lymph node weight, LNC count, or used FCM (Vohr et al., 1994). These methods reportedly are able to differentiate photoallergenic compounds from phototoxic compounds; however, they should be further studied using a wider range of chemicals.

### 7.2.4. Immediate-type hypersensitivity

It is recommended that ICCVAM consider a workshop to identify the most predictive methods for detecting immediate-type hypersensitivity following exposure to chemicals and drugs. This is problematic in preclinical drug development as there are no robust models which have been properly evaluated or validated to predict drugs that will produce immediate-type hypersensitivity following oral exposure in humans. This continues to be a major reason for failure of new pharmaceuticals upon their introduction in clinical trials or the market.

The methods being developed use elevations in total serum immunoglobulins as well as an increase in specific IgE+ (immunoglobin class E+) lymphocyte populations as a measurement of a chemical's ability to elicit an IgE response. However, investigators have recently started to evaluate the cytokine profiles of lymphocytes following chemical exposures and examining lymphocyte phenotypes as an indication of a chemicals ability to induce irritation or type I or type IV hypersensitivity responses.

Therefore, an immediate-type hypersensitivity test utilizing LLNA could be a topic of a future workshop or validation work.

## 7.3. Summary of Related Issues

### 7.3.1 Future assay improvements should be investigated

a. Improvement for detection of weak sensitizers, strong iritants, and metals;
b. *Ex vivo* LLNA with $^{3}$H-methyl thymidine incorporation;
c. Cytokine production (ELISA or cRT-PCR); and
d. Cellularity and LNC phenotype analysis.

### 7.3.2 Future potential workshops

a. Explanation of the ICCVAM process for Sponsors and the scientific community.
b. Potential modification and research needs of LLNA.
c. Use of LLNA to assess photosensitization.
d. Models to predict immediate-type hypersensitivity

# 8. Overall Summary Conclusions

### 8.1. Compared with current methods (e.g., the GPMT), could this method be used to provide equivalent or better prediction of human ACD?

The stated objective of the ICCVAM PRP was to determine if the mouse LLNA as a test for hazard identification was equivalent to the guinea pig assays (GPMT/BA). This review involved the evaluation of data on 209 chemicals of which data on 126 chemicals were provided for both LLNA and the guinea pig, and 74 chemicals with both LLNA and human data (human maximization test and Human Patch Test Allergens). The accuracy of the LLNA vs. GPMT/BA was 89% (N = 97), LLNA vs. all guinea pig tests (GPT) was 86% (N = 126), the LLNA vs. the human data was 72% (N = 74), GMPT/BA vs. human data was 72% (N = 57), and GPT vs. human data was 73% (N = 62). The PRP found the concordance between the LLNA and the GPMT/BA to be acceptable, as was the concordance between the LLNA vs. human response, in terms of accuracy, sensitivity, specificity, and positive or negative predictive value compared to that for GPT results. Thus, the consensus of the PRP was that the LLNA results, as submitted and supplemented by the Sponsors, demonstrated that the assay performed well and gave equivalent results to guinea pig methods (GPMT/BA) for the hazard identification of strong to moderate chemical sensitizing agents. An in-depth review of all the chemicals that have been defined in the published literature as human allergens was not conducted as part of this evaluation.

The data demonstrate that the LLNA was less sensitive compared to the GPMT with certain types of agents since results were negative or equivocal in the LLNA with nickel salts, benzocaine (equivocal), 4-chloroaniline, streptomycin sulfate, and sulfanilic acid. All were positive in the GPMT. In cases where there were equivocal data, the LLNA provided more information for evaluation, often including a dose-response curve. Also, the quantitative DPM endpoint removed the subjectivity of evaluating equivocal responses as with the guinea pig assays.

The PRP determined that dose-response evaluation, individual animal data, and statistical analysis would allow one to evaluate response trends and could suggest the need to retest at higher or lower concentrations. Decision rules for the consistency of interpretation and future use of the method were recommended by the PRP, as discussed in Section I.

In evaluating the LLNA as a stand-alone method for hazard assessment, the PRP further explored discordance of chemicals between the LLNA and GPMT/BA relative to available human data. Only six chemicals were identified to be discordant after discussion between the PRP and Sponsors. For three of these chemicals, the LLNA results were discordant with human data, while the remaining three chemicals were discordant between GPMT/BA and human data.

The data submitted indicate that the LLNA does not accurately predict all weak sensitizers (false negative) and some strong irritants (false positive). The term weak sensitizer is somewhat arbitrary, since the terms weak, moderate, and strong apply to the percentage of animals reacting in the GPMT/BA as described in the published literature or papers submitted by the Sponsors. When comparing the LLNA with the current guideline guinea pig methods, the LLNA appears to provide an equivalent prediction of the risk for human ACD.

The PRP found that the test method protocol was detailed and provided sufficient information on materials and equipment needed and technical procedures such that trained personnel should have no problem in reproducing the assay. The PRP recommended a retrospective audit of at least three of the intra- and interlaboratory validation studies since these were performed in the “spirit” of GLP, but without audit.

As part of the review, the PRP also reviewed papers published by investigators not directly involved with the ICCVAM Test Method Submission. Thirteen of these papers reported that the LLNA showed concordance with the GPMT or human results while three suggested non-concordance (not including the issue of the inability of the LLNA to identify some metal salts as contact allergens). The conclusion of the PRP was that the LLNA was equivalent to the current guinea pig methods as a stand-alone method and offered several advantages including opportunities for future assay improvement and mechanistic studies.

**8.2. Does the LLNA adequately identify the <u>lack of potential</u> of chemicals to induce human ACD? If applicable, specify those circumstances (e.g., specific chemicals/chemical classes) where the LLNA, or test results from the LLNA, would be considered either (i) inadequate or (ii) equal to or better than current methods for concluding that the test article <u>is not</u> a contact sensitizer.**

Some chemicals expected to give negative results based on guinea pig data tested positive or equivocal in the LLNA. This issue was discussed in a telephone conference including PRP members and the Sponsors, and many of these discordant results were resolved to the satisfaction of the PRP.

The PRP was also concerned that some strong irritants may give false positive results in the LLNA assay although the Sponsors have evaluated these issues. In Basketter et al. (1998), a comparison of the HMT and LLNA for identifying irritants is presented. Of the eight chemical irritants tested in the HMT, the LLNA produced false positive results for SLS and false negative results for benzalkonium chloride, lactic acid and octanoic acid. This indicates that there is only a 50% chance of identifying chemicals that are irritants in humans, although irritation has also been a confounding problem with guinea pig assays. The Sponsors have suggested methods for evaluating the data that may help to distinguish the proliferative effects of irritation in the LLNA. Such improvement may be required to correctly classify irritants in the LLNA.

On a proportional basis, the LLNA appears to be better at identifying the potential of chemicals that induce contact dermatitis than in identifying a non-sensitizing chemical. Relative to GPMT/BA data, the LLNA mis-identified aniline, 4-chloroaniline, nickel chloride, nickel sulfate, streptomycin sulfate, and sulfanilic acid as non-sensitizers, and ammonium thioglycolate, copper chloride, ethylene glycol dimethacrylate, musk ambrette, and SLS as sensitizers.

The predictive value of the LLNA vs. GPMT/BA to give a positive test was 93% and the predictive value of a negative test was 80%, giving an accuracy of 89%. The negative test value suggests that the LLNA produced a slightly higher frequency of false negatives than the guinea pig methods. However, it is important to note that in some cases where there was discordance between the assays, the LLNA was a better predictor of the human response.

**8.3. Does the LLNA adequately identify <u>the potential</u> of chemicals to induce human ACD? If applicable, specify those circumstances (e.g., specific chemicals/chemical classes) where the LLNA, or test results from the LLNA, would be considered either (i) inadequate or (ii) equal to or better than current methods for concluding that the test article <u>is</u> a contact sensitizer.**

The LLNA produced negative results for 12 chemicals that tested positive in guinea pig tests, including nonstandard tests. Of the 57 chemicals tested in both the LLNA and GPMT/BA, and for which there are human data (HMT and/or HPTA), the LLNA mis-identified 16 chemicals. Similarly, the GPT misidentified 16 chemicals. It was the opinion of the PRP that detection of weak sensitizers was not a significant issue and some improvement may be accomplished if the number of treatments and the number of animals was increased. Likewise, the use of a three-fold SI to call a chemical a sensitizer along with statistical analysis should improve the decision process.

Another weakness of the LLNA, as described, was the inability to identify some metal salts as contact allergens. This issue has been addressed by others in the literature. In three different papers, Ikarashi et al. (1992a; 1992b; 1993) suggest that the use of DMSO as a vehicle results in a positive LLNA test when metal salts, including nickel and copper salts, are applied to the skin.

Circumstances where the LLNA may give discordant results would include cases where weak sensitizers require extensive exposure time or where dermal penetration does not occur or is delayed through intact skin.

As mentioned earlier, when some common irritants were used in the LLNA, they give false positive results, in as much as they were not contact allergens when applied to human skin. This issue has been described in the literature by others and it has been demonstrated that a modification of the LLNA, involving pre-exposure to the irritant by use of an occluded patch (Boussiquet-Leroux et. al., 1995), or by intradermal injection (in Freund's complete adjuvant) of the irritant followed by cutaneous application (Ikarashi et. al., 1993) renders the irritants non-reactive in the LLNA.

### 8.4. Discuss conditions/limitations/ restrictions that may affect the intended use of the LLNA, and that are justified based upon the presence or lack of scientific evidence.

Two limitations of the LLNA have been mentioned and discussed previously. Firstly, in the material provided by the Sponsor, the LLNA failed to detect certain metal salts which are sensitizers in both guinea pigs and humans. Publications by Ikarashi et al. (1992a; 1992b; 1993) may have resolved this weakness through the use of DMSO as the vehicle. Secondly, some common irritants have given false positive results in the assay. Modifications described by Boussiquet-Leroux et al. (1995) involving pre-exposure of the animal to the irritant by the occluded patch method or by Ikarashi et al. (1993) with intradermal injection (Freund's) of the irritant dissolved in Freund's adjuvant followed by cutaneous exposure improved the ability of the LLNA to discriminate irritant responses.

The protocol does not adequately address the use of a concurrent positive control. A concurrent positive control would provide validity to the assay by indicating that all procedures involved in the assay were conducted properly. In addition, a positive control will provide an internal standard to compare between studies. Guinea pig sensitization studies (e.g., BA and GMPT) usually require a reliability check every six months with substances that are known to have mild-to-moderate skin sensitization properties. The PRP recommended the inclusion of a moderate sensitizer (single dose) as a positive control in all assays.

The mouse strain chosen was a known Th1 responder although a choice based on a systematic comparison of alternative strains was not provided. The literature contains sufficient documentation for the influence of genetic factors on contact allergy, although there is less documentation on how important a role this plays in practice. Likewise, there is evidence that inbred mouse strains differ in DTH reactions to various antigens. The PRP was concerned that little had been done to compare other inbred mouse strains to the CBA mouse in the LLNA. The documentation in the paper cited on this point (Kimber and Weisenberger, 1989), is very preliminary, and with only one strong sensitizer (DNCB) evaluated, and with a protocol different from the one proposed. The PRP recommended that additional research with other strains is required before strains other than CBA are considered validated.

The majority of the data documented in the Submission was generated using female mice. Therefore, it was the opinion of the PRP that the protocol should be limited to the use of female mice until a systematic comparison of the data from male mice is made available.

The anatomical location (e.g., photograph or diagram) of the auricular lymph nodes was a highly recommended addition to the protocol.

The ability to determine and consider the dose-response relationship (three to five doses) represents an important advantage of the LLNA compared to guinea pig tests. Dose-response analysis becomes very important in the evaluation of equivocal results because the presence of a dose response provides added confidence that skin sensitizing compounds were correctly identified. The dose response also allows for the evaluation of potential toxicity.

Safety issues relating to the handling of radioisotopes were discussed and the PRP recommended that a future improvement might be a non-radioactive endpoint. The PRP saw significant advantages to the use of *ex vivo-in vitro* pulsing to assess thymidine incorporation if sensitivity was not sacrificed, and identified this method as a research need for the future.

## 8.5. Discuss advantages of the proposed LLNA, as compared to the standard guinea pig methods.

The LLNA appears to offer several advantages as compared to the standard guinea pig methods. The LLNA:

(1) evaluates the induction phase of the contact dermatitis response;
(2) has an objective and quantitative endpoint which can be analyzed to evaluate dose-response;
(3) is a relatively robust assay as indicated by test method transferability between laboratories;
(4) requires significantly shorter time to conduct;
(5) is not confounded by colored compounds; and
(6) has potential to be less costly than the guinea pig assays.

### 8.5.1. Mechanistic basis of the assay

The LLNA is based on auricular lymph node proliferation (as assessed by incorporation of radiolabeled thymidine or uridine) following topical administration of test material to the mouse ear. The results are expressed as DPM from treated animals as compared to control. This differs from the scoring of the guinea pig assays in which a test substance is scored as positive based on the percentage of animals in a group that are responders (15% in a nonadjuvant assay and at least 8% in an adjuvant test) (Marzulli and Maibach, 1996). Increased understanding of the underlying mechanisms of the induction of contact sensitization will provide many areas for future improvement of the LLNA, such as assessment of non-radioactive endpoints including cytokine production or local lymph node cell phenotyping.

### 8.5.2. Endpoint is objective and quantitative

The LLNA uses the measurement of the incorporation of $^{3}$H-methyl thymidine into proliferating lymphocytes in draining lymph nodes as a measurement of sensitization. Proliferation is directly measured by DPM count, which is an objective endpoint that requires no training in judgement. This is a distinct advantage over the subjective visual scoring of the intensity of erythema and occurrence of palpable edema used in the guinea pig tests.

### 8.5.3. Time required to conduct assay

The time from beginning the treatment of animals to a final result in is within seven days. This is a substantial improvement over the minimum 25-day time frame required to conduct the standard guinea pig tests.

### 8.5.4 Insensitivity to minor variations in protocol

The LLNA appears to be fairly insensitive to minor changes in protocol. The use of radioiodinated uridine rather than tritiated thymidine is said to produce the same assay results and conclusions.

#### 8.5.5. Evaluation is not confounded by colored compounds

Colored compounds can confound visual scoring systems for erythema and edema as used in the guinea pig sensitization tests. Measurement of incorporation of radiolabeled thymidine (or uridine) in the LLNA eliminates this confounder, making the assay more suited for testing of colored compounds.

#### 8.5.6. Cost-effectiveness

A direct comparison of the actual cost required to conduct the LLNA vs the GPMT was not provided in the Submission. It is expected that the cost of the LLNA will not exceed the current guinea pig tests and decrease as experience with the assay is obtained.

### 8.6. Has there been adequate consideration and appropriate incorporation of animal use refinement, reduction, and replacement alternatives? Will the LLNA reduce the number of animals required or refine the procedure to eliminate distress compared with the reference tests?

The LLNA procedure is a definite refinement in terms of reducing or eliminating distress in animals compared to the GPMT. The LLNA does not replace the use of animals for assessing the potential of compounds to cause ACD. Whether the LLNA will result in a reduction in the number of animals will depend on the actual number of concentrations required for testing a particular compound.

#### 8.6.1. Refinement

In the LLNA, the induction phase of sensitization is being evaluated. Thus, discomfort to animals associated with the elicitation phase is eliminated. The ACD reaction itself is not being measured so redness and erythema are not induced unless the substance causes irritation over the three-day period of treatment of the mouse ear. Very importantly, the LLNA reduces the distress associated with administering adjuvants such as Freund's adjuvant. The animals are involved in the experiment for a considerably shorter period of time than in the GPMT (i.e., seven days compared to ≥32 days) The only manipulation of the animal is the application of the test solution to the ears on three consecutive days, and one intravenous (i.v.) injection, before the termination of the experiment. This level of manipulation is contrasted to shaving, injection into the skin, and occlusive bandaging in the guinea pig models.

#### 8.6.2. Reduction

As required in the protocol, lymph nodes from individual animals are processed, five animals are used per group, and a positive control is included in each assay. Thus, for testing one chemical alone, 25 to 35 animals are required for testing three to five concentrations of a compound. Whether three or five concentrations are tested, the number of mice required will be less than or equal to the number of guinea pigs, with dose response information being obtained as well. Testing of multiple compounds in one assay will further reduce the number of animals required since the vehicle and positive controls may not need to be duplicated. In the opinion of some PRP members, testing three concentrations of each test chemical is sufficient. In this case, adoption of the LLNA would definitely result in a reduction in the number of animals used.

### 8.7. Recommendations for Future ICCVAM Workshops and Research

A workshop on the ICCVAM evaluation process would be helpful for individuals planning on making future assay Submissions as well as for individuals which may be involved in the evaluation process.

A workshop on the use of the LLNA for detecting photosensitization in conjunction with UV irradiation would be useful.

A workshop to optimize test conditions of the LLNA was recommended by the PRP.

A workshop to discuss and describe research needs for preclinical models to predict immediate type hypersensitivity to chemicals/pharmaceuticals was also recommended.

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# LLNA Submission List of Chemicals

The following list of chemicals includes information from Appendix B, Table 1 of the original submission (Appendix C), unpublished data from a laboratory participating in the validation studies, and supplemental sources.

Participating laboratories in the validation studies have provided statements indicating that the studies were conducted under Good Laboratory Practice (GLP) guidelines or within the spirit of GLP. The laboratory that submitted unpublished data also provided a representative sample of raw data for review. NICEATM concluded that the data provided supported the results given in the original submission (Appendix C).

NICEATM has included human patch test allergen information from the Contact Dermatitis web site (Truett, 1998); chemical class assignments (some of which are based on categories used by Ashby et al., 1995); product class information from *The Merck Index*, $12^{th}$ edition (Budavari, 1996), and other sources; dermal irritancy potential; sensitization incidence in a cohort of patch tested dermatitis patients (from Marzulli and Maibach, 1996) [*Dermatotoxicology*]); and other comments.

| Chemical Name | CASRN | Chemical Class | Product Class | LLNA | GPMT/BT | HMT | HPTA | Patch Concn. | References | Comment |
|---|---|---|---|---|---|---|---|---|---|---|
| Abietic acid// Sylvic acid | 514-10-3 | rosin isomer// terpene derivative// decahydrophenanthrenecarboxylic acid// pot. epoxide | cosmetics// manuf. of esters for use in lacquers and varnishes and of metal resinates for sizing// metalworking fluids | + | + | | + | 10% pet. * | Basketter and Scholes (1992); Ashby et al. (1995); Hausen et al. (1989) | Weak sensitizer in a modified FCA method. |
| 2-Acetamidofluorene// 2-AAF// 2-Acetylaminofluorene | 53-96-3 | amide// PAH | | - | | | | | Ashby et al. (1995) | |
| 2-(N-Acetoxyacetamido)fluorene// 2-AAAF | | amide// PAH// ?acetylated N-oxide// potential epoxide | | + | | | | | Ashby et al. (1995) | |
| 4-Acetylphenyl benzoate | 1523-18-8 | aromatic ester// benzoate | | - | | | | | Ashby et al. (1995) | |
| 3-Acetylphenyl benzoate | | aromatic ester// benzoate// acylating agent// benzoylating agent | | + | + | | | | Ashby et al. (1995) | |
| C16-1,3-Alkene sultone | | alkene sultone (sulfur analog of a lactone) | | + | + nonstd | | | | Unpublished Unilever data | |
| 4-Allylanisole// Estragole | 140-67-0 | aryl alkyl ether | fragrance// flavoring in foods and liqueurs | + | + | | | | Unpublished Unilever data | |
| 4-Aminobenzoic acid// p-Aminobenzoic acid// PABA | 150-13-0 | arylamine// benzoic acid derivative | UV B sunscreen (cosmetics)// manuf. esters, folic acid, and azo dyes// formerly, antirickettsial | - | - | - | + | 10% pet. * | Ashby et al. (1995); Loveless et al. (1996); Basketter et al. (1996a); Truett (1998) | Constituent of photoallergen patch test kit. |
| 3-Aminophenol// m-Aminophenol// 3-Hydroxyaniline | 591-275 | phenolic// arylamine | dye intermediate// manuf. of p-aminosalicylic acid// potential epoxide | + | + nonstd | | + | | Basketter and Scholes (1992) | |
| 2-Aminophenol// o-Aminophenol// 2-Hydroxyaniline | 95-55-6 | phenolic// arylamine// potential epoxide | manuf. azo and sulfur dyes// dyeing furs and hair | + | + nonstd | | | | Ashby et al. (1995) | |

| Chemical Name | CASRN | Chemical Class | Product Class | LLNA | GPMT/BT | HMT | HPTA | Patch Concn. | References | Comment |
|---|---|---|---|---|---|---|---|---|---|---|
| Ammonium tetrachloroplatinate// Ammonium platinous chloride | 13820-41-2 | heavy metal salt// heavy metal coordination compound | photographic chemical | + | + | | + | 0.25% pet. * | Basketter and Scholes (1992) | |
| Ammonium thioglycolate// Ammonium mercaptoacetate | 5421-46-5 | carboxylic acid salt | hairdressing (reducing agent in permanent hair waving solutions) | + | - | | + | 1% pet. * | Unpublished Unilever data | |
| Aniline// Benzenamine | 62-53-3 | arylamine | manuf. dyes, medicinals, varnishes, etc.// vulcanizing rubber// as solvent | - | + | + | | | Basketter and Scholes (1992); Basketter et al. (1994); Basketter et al. (1996a) | |
| Benzalkonium chloride | 8001-54-5 | quaternary ammonium halide | antimicrobial// cationic surfactant// Pharmaceutic aid (preservative) | - | - | | + | 0.1% water * | Basketter et al. (1996a); Basketter et al. (1998) | High human skin irritancy potential (52% of panel responded [83% to positive control]). |
| 3-(Benzenesulfonyloxymethyl)-5,5-dimethyldihydro-2(3H)-furanone | | benzenesulfonate// lactone// butyrolactone derivative | | - | | | | | Ashby et al. (1995) | |
| Benzene-1,3,4-tricarboxylic anhydride// Trimellitic anhydride | 552-30-7 | aromatic carboxylic acid anhydride// benzoylating agent// acylating agent | preparation of resins, adhesives, polymers, dyes, printing inks | + | + | | | | Ashby et al. (1995); Basketter and Scholes (1992) | |
| 1,2-Benzisothiazolin-3-one | 2634-33-5 | aromatic amide// heterocyclic | antimicrobial, preservative (sodium salt) | + | + | | + | 0.1% pet. * (Na salt) | Botham et al. (1991); Ashby et al. (1995) | |
| Benzocaine | 9/7/94 | p-aminobenzoic acid | local anesthetic | +/- | + | +/- | | | Basketter and Scholes (1992); Ashby et al. (1995); Basketter et al. (1994); Kimber and Weisenberger (1989); Kimber et al. (1989); Kimber et al. (1991); Gerberick et al. (1992) | Classified as a moderate sensitizer in the GPMT. |
| Benzo[a]pyrene | 50-32-8 | PAH// potential epoxide after metabolism? | none | + | | | | | Ashby et al. (1995) | |
| Benzoquinone// p-Quinone// 1,4-Cyclohexadienedione | 106-51-4 | quinone// potential Michael-reactive agent | oxidizing agent// manuf. hydroquinone, dyes// tanning hides, etc. | + | + | | | | Basketter and Scholes (1992); Ashby et al. (1995); | |

| Chemical Name | CASRN | Chemical Class | Product Class | LLNA | GPMT/BT | HMT | HPTA | Patch Concn. | References | Comment |
|---|---|---|---|---|---|---|---|---|---|---|
| Benzoyl chloride | 98-88-4 | aroyl halide// acylating agent// benzoylating agent | acylating agent in synthesis// manufacture of benzoyl peroxide and dyes | + | + | | | | Ashby et al. (1995); Budavari (1996) | Skin and mucous membrane irritant. |
| Benzoyloxy-3,5-benzenedicarboxylic acid// 5-Benzoyloxyisophthalic acid | | benzoate// benzoic acid derivative// isophthalic acid deriv.// acylating agent// benzoylating agent | | - | + nonstd | | | | Unpublished Unilever data | |
| Benzoyl peroxide | 94-36-0 | aromatic peroxide | pharmaceuticals// food additive (bakery series patch tests)// metalworking fluids// plastics and glues | + | + | | + | 1% pet. *** | Kimber et al. (1998); Marzulli and Maibach (1996) | 20 of 1115 dermatitis patients sensitized// 3 of 1115 showed skin irritation. |
| Benzyl bromide// .alpha.-Bromotoluene | 100-39-0 | alkyl halide | alkylating agent? | + | | | | | Unpublished Unilever data; Budavari (1996) | Strong skin irritant. |
| Beryllium sulfate | 7787-56-6 | alkaline earth metal salt | | + | + | + | | | Basketter et al. (1994); Basketter et al. (1996a) | |
| C12-13-.beta. Branched primary alcohol sulfate | | alkyl sulfate | | + | | | | | Basketter et al. (1998) | Moderate skin irritant in 4-hour human patch test (84% of panel responded// 90% in positive control). |
| 1-Bromobutane | 109-65-9 | alkyl halide | alkylating agent | - | | | | | Basketter et al. (1992); Ashby et al. (1995) | |
| 1-Bromododecane// Lauryl bromide | 143-15-7 | alkyl halide | | + | + nonstd | | | | Basketter et al. (1992) Ashby et al. (1995) | |
| 12-Bromododecanoic acid// 12-Bromolauric acid | 73367-80-3 | bromoalkanoic acid// alkyl halide// aliphatic carboxylic acid | | + | | | | | Unpublished Unilever data | |
| 12-Bromo-1-dodecanol// 12-Bromolauryl alcohol | 3344-77-2 | alkanol// bromoalkanol// alkyl halide | | + | | | | | Unpublished Unilever data | |

| Chemical Name | CASRN | Chemical Class | Product Class | LLNA | GPMT/BT | HMT | HPTA | Patch Concn. | References | Comment |
|---|---|---|---|---|---|---|---|---|---|---|
| 1-Bromoheptadecane | | | | + | | | | | Basketter et al. (1992) | |
| 1-Bromohexadecane// n-Hexadecyl bromide// Palmityl bromide// Cetyl bromide | 112-82-3 | alkyl halide | | + | + | | | | Basketter et al. (1992) Ashby et al. (1995); Basketter et al. (1996a) | |
| 1-Bromohexane// n-Hexyl bromide | 111-25-1 | alkyl halide | | + | + nonstd | | | | Basketter et al. (1992); Ashby et al. (1995) | |
| 3-Bromomethyl-3-dimethyldihydrofuranone | | lactone// butyrolactone derivative// alkyl halide | | + | + | | | | Unpublished Unilever data | |
| 1-Bromononane | | | | - | | | | | Basketter et al. (1992) | |
| 1-Bromooctadecane | | | | + | | | | | Basketter et al. (1992) | |
| 1-Bromopentadecane// n-Pentadecyl bromide | 629-72-1 | alkyl halide | | + | | | | | Basketter et al. (1992); Ashby et al. (1995) | |
| 7-Bromotetradecane// 7-Tetradecyl bromide// 7-Myristyl bromide | | alkyl halide | | + | | | | | | |
| 1-Bromotetradecane | | | | + | | | | | Basketter et al. (1992) | |
| 2-Bromotetradecanoic acid// 2-Bromomyristic acid | 10520-81-7 | aliphatic carboxylic acid// alkyl halide | | + | | | | | Unpublished Unilever data | |

| Chemical Name | CASRN | Chemical Class | Product Class | LLNA | GPMT/BT | HMT | HPTA | Patch Concn. | References | Comment |
|---|---|---|---|---|---|---|---|---|---|---|
| 1-Bromotridecane | | | | + | | | | | Basketter et al. (1992) | |
| 1-Bromoundecane | | | | + | | | | | Basketter et al. (1992) | |
| 2,3-Butanedione// Erythritol anhydride// Butadiene diepoxide | 431-03-8 | epoxide | crosslinking agent (polymers, textile fibers) | + | | | | | Unpublished Unilever data | Reasonably anticipated to be a human carcinogen |
| Butyl glycidyl ether | 2426-08-6 | epoxide// dialkyl ether | | + | + | + | | | Basketter et al. (1996a) | |
| Camphorquinone// Camphoroquinone | 465-29-2 | quinone | dental material (visible light curing of acrylic composites) | + | | | + | 1% pet. ** | Unpublished Unilever data | |
| Chloramine T | 10599-90-3 | toluenesulfonamide derivative// sulfonamide//N-chloroamide | antibacterial [antimicrobial] (pharmaceutical, veterinary topical antiseptic) | + | + | | + | | Basketter and Scholes (1992) | |
| 4-Chloroaniline | 106-47-8 | arylamine// aryl halide// aniline derivative | | - | + | | | | Basketter and Scholes (1992); Ashby et al. (1995); Basketter et al. (1996a) | |
| Chlorobenzene | 108-90-7 | aryl halide | synthetic organic intermediate// manufacture of phenol, aniline, DDT// paint solvent// heat transfer | - | - | | | | Ashby et al. (1995); Basketter et al. (1998) | Presumed to have low irritancy potential. |
| 3-(Chlorobenzenesulfonyloxymethyl)-5,5-dimethyldihydro-2(3H)-furanone | | lactone// butyrolactone derivative// benzenesulfonate | | - | | | | | Ashby et al. (1995) | |
| 2-Chloroethanol// Ethylene chlorohydrin// Glycol chlorohydrin | 107-07-3 | aliphatic alcohol// alkyl halide | solvent// insecticide manufacture | - | | | | | Ashby et al. (1995); Budavari (1996) | Skin and mucous membrane irritant. |

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|---|---|---|---|---|---|---|---|---|---|---|
| 2-Chloromethylfluorene | | alkyl halide// PAH | | + | | | | | Ashby et al. (1995) | |
| 5-Chloro-2-methyl-4-isothiazolin-3-one [no locants & different CASRN in list] | 26172-55-4 | potential Michael-reactive agent// active aryl halide | cosmetics, biocidal, antimicrobial. Major active ingredient of Kathon CG (200 ppm). | + | + | | + | 1.34% aq. ** | Botham et al. (1991); Ashby et al. (1995) | Kathon CG or MCI/MI is used in paints, hair shampoos, skin care products, and cleaning agents, typically at 35 ppm. |
| 1-Chloromethylpyrene | 1086-00-6 | alkyl halide// PAH | | + | | | | | Ashby et al. (1995) | |
| 1-Chlorononane// n-Nonyl chloride | 2473-01-0 | alkyl halide | | + | | | | | Basketter et al. (1993) | |
| 1-Chlorooctadecane// Stearyl chloride | 3386-33-2 | alkyl halide | | + | | | | | Basketter et al. (1993) | |
| 1-Chlorotetradecane// Myristyl chloride | 2425-54-9 | alkyl halide | | + | | | | | Basketter et al. (1993) | |
| Chlorpromazine | 69-09-0 | phenothiazine// tertiary amine | pharmaceutical (antiemetic// antipsychotic// veterinary tranquilizer) | + | + nonstd | + | | | Basketter et al. (1994); Basketter et al. (1996a) | |
| Cinnamic aldehyde// cinnamaldehyde | 104-55-2 | potential Michael-reactive agent | fragrance// food additive (bakery series kit) | + | + | + | + | 1 pet. *** | Basketter and Scholes (1992); Ashby et al. (1995); Basketter et al. (1994); Kligman (1990); Marzulli and Maibach (1996) | Urticariogen. Irritant (60/1048) & sensitizer (62/1048) in dermatitis patients. |
| Citral// 3,7-Dimethyl-2,6-octadienal// Geranial-Neral mixture | 5392-40-5 | terpene alcohol// potential Michael-reactive agent | fragrance// flavoring// synthesis of vitamin A, ionone, and methylionone | + | + | + | | | Basketter et al. (1994); Basketter and Scholes (1992); Ashby et al. (1995) | |
| Clotrimazole | 23593-75-1 | aryl halide//imidazole derivative | pharmaceutical (topical antifungal [antimicrobial]) | + | | | + | 5% pet. * | Scholes et al. (1994) | |

| Chemical Name | CASRN | Chemical Class | Product Class | LLNA | GPMT/BT | HMT | HPTA | Patch Concn. | References | Comment |
|---|---|---|---|---|---|---|---|---|---|---|
| Cobalt chloride | 7646-79-9 | heavy metal salt | fertilizer and feed additive// paints for glass and porcelain// vitamin B12 manufacture, etc. | + | + | + | + | 1% pet. ** | Basketter and Scholes (1992); Basketter et al. (1994); Basketter et al. (1996a) | Used in dental patch test series |
| Cocoamidopropyl betaine//CAPB | 61789-40-0 | quaternary ammonium compound// alkylaminobetaine | cosmetics// surfactant in shampoos, detergents, and cleaning agents | + | + | | + | 1% aq. *** | Ashby et al. (1995); Basketter et al. (1996a) | |
| Copper chloride// Cuprous chloride | 7758-89-6 | heavy metal salt | catalyst// condensing agent for soaps, fats, and oils// denitration of cellulose | + | - | | | | Basketter and Scholes (1992); Basketter et al. (1996a) | Cupric chloride is a skin irritant. Articles refer to copper chloride. CASRN for cupric chloride is 7447-39-4. |
| Dextran | 9004-54-0 | polysaccharide (.alpha.-D-glucopyranosyl units) | foods (soft center confections, partial substitute for barley malt)// pharmaceutical (plasma volume expander) | - | - | | | | Basketter and Scholes (1992); Basketter et al. (1996a) | |
| 1,2-Dibromo-2,4-dicyanobutane | 35691-65-7 | alkyl halide// aliphatic nitrile | antimicrobial, preservative in paints, adhesives, metalworking fluids, etc.//cosmetic and personal care products | + | + | | + | 0.1% pet. * | Unpublished Unilever data | Component of Euxyl K-400 (1:4 mixture with phenoxyethanol). Trade name for use in paints is Tektamer 38 |
| 2,4-Dichloronitrobenzene | 611-06-3 | nitroaromatic// aryl halide | | - | - | | | | Basketter et al. (1997); Basketter et al. (1996b); Basketter et al. (1996a); Gerberick et al. (1992) | |
| Diethylenetriamine | 111-40-0 | | hardener for epoxy resins// drilling muds// carbonless copy paper | + | + | + | + | | Basketter et al. (1994); Basketter et al. (1996a) | |
| Diethyl sulfate | 64-67-5 | alkyl sulfate | alkylating agent// accelerator in ethylene sulfation// used in some sulfonations | + | | | | | Ashby et al. (1995) | Reasonably anticipated to be a human carcinogen |
| Di-2-furanylethanedione// .alpha.-Furil// 2,2'-Furil | 492-94-4 | potential Michael-reactive agent | | - | | | | | | |
| 3,4-Dihydrocoumarin// Hydroxydihydrocinnamic acid lactone | 119-84-6 | lactone | fragrance | + | | | | | Ashby et al. (1995) | |

| Chemical Name | CASRN | Chemical Class | Product Class | LLNA | GPMT/BT | HMT | HPTA | Patch Concn. | References | Comment |
|---|---|---|---|---|---|---|---|---|---|---|
| Dihydroeugenol// 2-Methoxy-4-propylphenol// 4-Propylguaicol | 2785-87-7 | phenolic// alkyl aromatic ether | fragrance | + | + | | | | Unpublished Unilever data | |
| 3-Dimethylaminopropylamine//N,N-Dimethyl-1,3-propanediamine// DMAPA | 109-55-7 | alkylenediamine// tertiary amine// primary amine | chemical intermediate | + | + | | | | Basketter et al. (1996a) | Corrosive and severely irritating to skin, eyes, and respiratory tract. |
| 7,12-Dimethylbenz[a]anthracene// DMBA// 9,10-Dimethyl-1,2-benzanthracene | 57-97-6 | PAH// potential epoxide | | + | | | | | Ashby et al. (1995) | |
| Dimethyl isophthalate | 1459-93-4 | isophthalate// aromatic carboxylic acid ester | intermediate in polyester synthesis | - | - | | | | Basketter and Scholes (1992) | |
| 5,5-Dimethyl-3-(mesyloxymethyl)dihydro-2(3H)-furanone | | lactone// butyrolactone derivative | | - | + nonstd | | | | Ashby et al. (1995) | |
| 5,5-Dimethyl-3-(methoxybenzenesulfonyloxymethyl)dihydro-2(3H)-furanone | | lactone// butyrolactone derivative | | - | + nonstd | | | | Unpublished Unilever data | |
| 5,5-Dimethyl-3-methylenedihydro-2(3H)-furanone | | lactone// butyrolactone derivative// potential Michael-reactive agent | | + | - nonstd | | | | Ashby et al. (1995) | |
| 5,5-Dimethyl-3-(nitrobenzenesulfonyloxymethyl)dihydro-2(3H)-furanone | | lactone// butyrolactone derivative | | - | + nonstd | | | | Ashby et al. (1995) | |
| Dimethyl sulfate | 77-78-1 | alkyl sulfate | alkylating agent// methylating agent in organic chemical manufacture | + | | | | | Ashby et al. (1995) | Mucous membrane irritant. Reasonably anticipated to be a human carcinogen. |
| 5,5-Dimethyl-3-(thiocyanatomethyl)dihydro-2(3H)-furanone | | lactone// butyrolactone derivative// thiocyanate | | + | + nonstd | | | | Ashby et al. (1995) | |

| Chemical Name | CASRN | Chemical Class | Product Class | LLNA | GPMT/BT | HMT | HPTA | Patch Concn. | References | Comment |
|---|---|---|---|---|---|---|---|---|---|---|
| 5,5-Dimethyl-3-(tosyloxymethyl)dihydro-2(3H)-furanone | | toluenesulfonate// lactone// butyrolactone derivative | | - | - nonstd | | | | Ashby et al. (1995) | |
| 2,4-Dinitrochlorobenzene// DNCB | 97-00-7 | active aryl halide// nitroaromatic | | + | + | | | | Basketter et al. (1996a); Kimber et al. (1995) Loveless et al. (1996); Kimber and Dearman (1991) Basketter and Scholes (1992); Budavari (1996) | Used as positive control. May cause dermatitis of both primary and allergic types. |
| Dinitrofluorobenzene//DNFB | | | | + | | | | | Kimber and Weisenberger (1989); Montelius et al. (1994); Maurer and Kimber (1991) | |
| 2,4-Dinitrothiocyanobenzene// 2,4-Dinitrophenyl thiocyanate// Nirit | 1594-56-5 | aryl thiocyanate// nitroaromatic | | + | + | | | | Basketter et al. (1996a); Kimber and Dearman (1991); Kimber and Weisenberger (1989) | |
| Diphenylmethane-4,4'-diisocyanate// Methylenediphenyl diisocyanate// MDI | 101-68-8 | aryl isocyanate | monomer for polyurethane synthesis// plastics and glues | + | + | | + | 0.1% pet. * | Basketter et al. (1996a) | |
| Disodium benzoyloxy-3,5-benzenedicarboxylate | | benzoate (ester)// isophthalate (salt) | | - | - | | | | Ashby et al. (1995) | |
| Disodium 1,2-diheptanoyloxy-3,5-benzenedisulfonate | | aliphatic carboxylic acid ester// benzenesulfonate salt | | + | + nonstd | | | | Ashby et al. (1995) | |
| Ditallowdihydroxypropenetrimethylammonium | | quaternary ammonium compound | | - | - | | | | Unpublished Unilever data | |
| Dodecyl methanesulfonate// Lauryl methanesulfonate | 51323-71-8 | alkanesulfonate (ester) | | + | + nonstd | | | | Ashby et al. (1995) | |
| Dodecyl thiosulfonate// Lauryl thiosulfonate | | | | + | + | | | | Ashby et al. (1995) | |

| Chemical Name | CASRN | Chemical Class | Product Class | LLNA | GPMT/BT | HMT | HPTA | Patch Concn. | References | Comment |
|---|---|---|---|---|---|---|---|---|---|---|
| Ellipticine | 519-23-3 | | antineoplastic activity | + | | | | | Unpublished Unilever data | |
| Ethylenediamine | 107-15-3 | alkylamine// alkylenediamine | solvent, stabilizer, inhibitor, textile lubricant, pharmaceutical, cosmetics, epoxy patch test kit | + | + | | + | 1% as 2HCl pet. ** | Gerberick et al. (1992); Kimber et al. (1998); Marzulli and Maibach (1996); Prystowsky et al. (1979) | Dihydrochloride 66/1120 dermatitis patients// 0/1120 irritation// 5/1158 volunteers. |
| Ethylene glycol dimethacrylate// EGDMA | 97-90-5 | acrylate | dental materials (monomer)// plastics and glues | + | - | | + | 2% pet. * | Basketter et al. (1991) | Coded chemical results reported in this publication. |
| Ethyl methanesulfonate | 62-50-0 | alkanesulfonate (ester) | experimental mutagen, teratogen, carcinogen | - | | | | | Ashby et al. (1995) | Known human carcinogen |
| 1-Ethyl-3-nitro-1-nitrosoguanidine// ENNG | | nitrosoguanide// alkylating agent | | + | | | | | Ashby et al. (1995) | |
| N-Ethyl-N-nitrosourea// ENU | 759-73-9 | nitrosamide | | + | | | | | Ashby et al. (1995) | Reasonably anticipated to be a human carcinogen |
| Eugenol// Allylguaiacol// 4-Allyl-2-methoxyphenol | 97-53-0 | phenolic// potential epoxide after metabolism | fragrances// vanillin manufacture// dental analgesic// bakery series kit | + | + | | + | 2% pet. ** | Kimber et al. (1991); Gerberick et al. (1992); Loveless et al. (1996); Basketter and Scholes (1992); Kimber and Basketter (1997); Ashby et al. (1995); Marzulli and Maibach (1996) | Irritating to 5 of 1016 at 4% in petrolatum// 14/1016 showed sensitization in patch test. |
| Fluorescein isothiocyanate | 25168-13-2 | miscellaneous electrophile (Ashby et al., 1995)// isothiocyanate | biological stain or dye | + | | | | | Ashby et al. (1995); Krasteva et al. (1996) | Fluorescein is a skin irritant. Strong sensitizer. Product class assumption based on that of fluorescein. |
| Formaldehyde | 50-0-0 | aliphatic aldehyde | antimicrobial, disinfectant, monomer, manuf. wood products and shoes, fertilizers, plastics, textile finish | + | + | + | + | 1% aq. ** | Kimber and Weisenberger (1989); Kimber et al. (1991); Basketter and Scholes (1992); Basketter et al. (1994); Basketter et al. (1996a); Maurer and Kimber (1991); Marzulli and Maibach (1996) | Irritant to 13 of 1144 in human patch test// 70 of 1144 subjects tested were sensitized. |
| Geraniol | 106-24-1 | terpene alcohol | fragrance | - | - | - | + | 2% pet. * | Basketter et al. (1994); Basketter et al. (1996a) | |

| Chemical Name | CASRN | Chemical Class | Product Class | LLNA | GPMT/BT | HMT | HPTA | Patch Concn. | References | Comment |
|---|---|---|---|---|---|---|---|---|---|---|
| Glyoxal// Oxaldehyde// Ethanedial// Biformyl | 107-22-2 | aldehyde | biocides, antimicrobial// in textiles, organic synthesis, glues | + | + | + | | | Basketter et al. (1994); Basketter et al. (1996a); Budavari (1996) | Moderately irritating to skin and mucous membranes. |
| Gold chloride | 16903-35-8 | heavy metal salt | photography, gold-plating, gilding glass and porcelain, ruby glass manufacture, reagent for alkaloids | + | | + | | | Basketter et al. (1996a); Budavari (1996) | Caustic action (vesicant) on the skin. |
| Hexadecanoyl chloride// Palmitoyl chloride | 112-67-4 | alkanoyl chloride// acylating agent | acylating agent | + | | | | | Ashby et al. (1995) | Lacrimator |
| Hexane | 110-54-3 | alkane | solvent | - | | - | | | Basketter et al. (1996a); Basketter et al. (1998) | Presumed low irritancy potential. |
| Hexylcinnamic aldehyde// H.C.A.// .alpha.-Hexylcinnamaldehyde// 2-(Phenylmethylene)octanal | 101-86-0 | potential Michael-reactive agent | fragrance | + | + | | | | Kimber and Basketter (1997) Loveless et al. (1996) | |
| Hydrocortisone// Cortisol | 50-23-7 | steroid | pharmaceutical (anti-inflammatory) | - | | - | + | 0.1% pet. as 17-butyrate | Basketter et al. (1996a) | |
| Hydroquinone// Quinol [separate entry in submission] | 123-31-9 | quinone// potential Michael reactive agent | cosmetics// photographic developer// plastics and glues// polymn. inhibitor// antioxidant// depigmenting skin | + | + | | + | 1% pet. *** | Kimber et al. (1998); Basketter and Scholes (1992); Ashby et al. (1995) | |
| 4-Hydroxybenzoic acid | 99-96-7 | phenolic// benzoic acid derivative | chemical intermediate for dyes and fungicides | - | - | | | | Basketter and Scholes (1992); Ashby et al. (1995) | |
| Hydroxycitronellal | 107-75-5 | terpene aldehyde// potential Michael-reactive agent// potential epoxide | fragrance// food flavoring// antiseptics [antimicrobial]// insecticides | + | + | + | + | 2% pet. * | Basketter and Scholes (1992); Ashby et al. (1995); Basketter et al. (1994); Basketter et al. (1996a); Krasteva et al. (1996) | Weak human sensitizer. Two of 1049 showed irritation in human patch test at 4% in petrolatum// 16 were sensitized. |
| 2-Hydroxyethyl acrylate// HEA | 818-61-1 | potential Michael-reactive agent// acrylate ester | acrylate monomer// cosmetics (artificial nails)// adhesives, lacquers, UV-curable inks and coatings | + | + | | + | | Ashby et al. (1995); Basketter and Scholes (1992) | |

| Chemical Name | CASRN | Chemical Class | Product Class | LLNA | GPMT/BT | HMT | HPTA | Patch Concn. | References | Comment |
|---|---|---|---|---|---|---|---|---|---|---|
| 2-Hydroxypropyl methacrylate// 2-HPMA | 923-26-2 | acrylate ester// potential Michael-reactive agent | monomer used in UV-curable inks and coatings, dental composites, printing plates, sealants | - | - | | + | 0.1% pet. | Basketter and Scholes (1992); Ashby et al. (1995); Bjorkner (1984) | Reported to be a weak sensitizer in the GPMT. |
| Imidazolidinyl urea// Germall 115 | 39236-46-9 | | antimicrobial, preservative// in cosmetics | + | + | | + | 2% pet. * or aq. ** | Basketter and Scholes (1992); Marzulli and Maibach (1996) | Two of 1134 showed irritation in the human patch test// 17/1134 were sensitized. |
| 1-Iodohexadecane// Palmityl iodide// Hexadecyl iodide | 544-77-4 | alkyl halide | | + | | | | | Basketter et al. (1993) | |
| 1-Iodohexane | | | | - | | | | | Basketter et al. (1992) | |
| 1-Iodononane// n-Nonyl iodide | 4282-42-2 | alkyl halide | | + | | | | | Basketter et al. (1993) | |
| 1-Iodooctadecane | | | | - | | | | | Basketter et al. (1992) | |
| 1-Iodotetradecane// Myristyl iodide// n-Tetradecyl iodide | 192-94-1 | alkyl halide | | + | | | | | Ashby et al. (1995) | |
| Isoeugenol// 2-Methoxy-4-propenylphenol// 4-Propenylguaiacol | 97-54-1 | phenolic// alkyl aryl ether// potential epoxide | fragrance (cosmetics)// food flavor | + | + | | + | 2% pet. *** | Kimber et al. (1991); Loveless et al. (1996); Basketter and Scholes (1992); Kimber and Basketter (1997) | Isoeugenol is a mixture of cis and trans isomers. Int. Fragrance Res. Assocn. recommends up to 1% |
| Isononanoyloxybenzenesulfonate | | benzenesulfonate (ester)// aliphatic carboxylic acid ester | | + | + | | | | Basketter et al. (1996a) | |
| Isophorone diisocyanate// IPDI | 4098-71-9 | isocyanate | monomer for polyurethane plastics// biomedical polyurethane-based hydrogel | + | + | | + | | Basketter et al. (1996a) | |

| Chemical Name | CASRN | Chemical Class | Product Class | LLNA | GPMT/BT | HMT | HPTA | Patch Concn. | References | Comment |
|---|---|---|---|---|---|---|---|---|---|---|
| Isopropanol// Isopropyl alcohol// 2-Propanol | 67-63-0 | alkanol// aliphatic secondary alcohol | solvent// cosmetics// body rubs// pharmaceutic aid (solvent)// manufacture of acetone, glycerol, isopropyl acetate | - | - | | | | Basketter et al. (1996a); Basketter et al. (1998) | Low irritancy potential in human patch test. |
| Isopropylisoeugenol | 29653-00-7 | potential Michael-reactive agent | fragrance? | + | + | | | | Unpublished Unilever data | |
| Kanamycin | 25389-94-0 | glucose (glucopyranose) derivative// primary alkylamine | pharmaceutical (antibacterial [topical antimicrobial]) | - | - nonstd | + | + | 10% pet. (as sulfate) | Basketter et al. (1996a); Budavari (1996) | CASRN given in submission is for kanamycin A sulfate// that for kanamycin is 8063-07-8. |
| Lactic acid// 2-Hydroxypropanoic acid | 598-82-3 | .alpha.-hydroxy carboxylic acid// alkanoic acid | food additive, mordant, solvent, treating hides, pharmaceutical, catalyst for casting phenolaldehyde resins (polymers). | - | - | | | | Basketter et al. (1998) | CASRN is for racemic lactic acid. Highly irritant in 4-hour human patch test (81% of panel responded// 60% to pos. control). |
| Lanolin// Wool alcohols// Wool fat// Wool wax// Adeps lanae | 8006-54-0 | esters of alcohols (steroid, aliphatic, triterpenoid) and fatty acids | cosmetics// pharmaceuticals// insecticides (cancelled, e.g., flea and tick treatments for dogs and cats) | - | - | | + | | Basketter et al. (1996a); Truett (1998); Marzulli and Maibach (1996) | Lanolin allergy is most common among leg ulcer patients. In human patch test, 14/1135 were sensitized// one showed |
| Lead acetate | 15347-57-6 | heavy metal carboxylate salt | drier in paints, varnishes, and pigment inks// hair dye// manufacture of lead salts, etc. | - | | | | | Unpublished Unilever data. | Reasonably anticipated to be a human carcinogen. |
| 2-Mercaptobenzothiazole | 149-30-4 | thiazole// heterocyclic | a thiazole rubber accelerator (one of the most common classes) | + | + | + | + | 2% pet. ** | Basketter and Scholes (1992); Ashby et al. (1995); Basketter et al. (1994); Basketter et al. (1996a); Truett (1998); Marzulli and Maibach (1996) | Most commonly identified allergen in allergic contact dermatitis due to shoes. In human patch test 33/1141 were sensitized. |
| Mercuric chloride// Corrosive sublimate | 7487-94-7 | heavy metal salt | pharmaceutical ([formerly] topical antiseptic, disinfectant [antimicrobial])// preservative// numerous industrial uses | + | + | + | + | | Basketter et al. (1994); Basketter et al. (1996a); Truett (1998) | Strong sensitizer. May produce a nonspecific, pustular or irritant patch test response. |
| 2-Methoxy-4-methylphenol | 5635-98-3 | phenolic// alkyl aryl ether | | + | + | | | | | |

| Chemical Name | CASRN | Chemical Class | Product Class | LLNA | GPMT/BT | HMT | HPTA | Patch Concn. | References | Comment |
|---|---|---|---|---|---|---|---|---|---|---|
| 3-Methoxyphenyl benzoate | 5554-24-5 | benzoate// alkyl aryl ether// acylating agent// benzoylating agent | | + | | | | | Ashby et al. (1995) | |
| 4-Methylaminophenol sulfate// Metol// p-Hydroxymethylaniline sulfate | 55-55-0 | phenolic// secondary amine// potential epoxide | photographic developer// dyeing furs | + | + | | + | 1% pet. | Basketter and Scholes (1992); Ashby et al. (1995) | |
| 4-Methylcatechol | 452-86-8 | phenolic | | + | + | | | | Unpublished Unilever data | |
| 3-Methylcatechol// 3-Methyl-1,2-benzenediol// 2,3-Dihydroxytoluene | 488-17-5 | phenolic | | + | | | | | | |
| 3-Methylcholanthrene// 1,2-Dihydro-3-methylbenz[j]aceanthrylene | 56-49-5 | PAH | experimental use in cancer research | + | | | | | Unpublished Unilever data | |
| 6-Methylcoumarin// 6-MC | 92-48-8 | lactone// potential Michael-reactive agent | fragrance (synthetic)// cosmetics, soaps, toiletries | - | - | - | + | | Scholes et al. (1992);, Ashby et al. (1995);, Basketter et al. (1996a) | |
| N'-(4-Methylcyclohexyl)-N-(2-chloroethyl)-N-nitrosourea// MeCCNU | 13909-09-6 | nitrosourea// nitrosamide// alkylating agent// alkyl halide | pharmaceutical (antineoplastic agent) | - | | | | | Ashby et al. (1995) | |
| Methyl dodecanesulfonate | | | | + | + | | | | Basketter and Scholes (1992); Ashby et al. (1995) | |
| 3-Methyleugenol | | phenolic | | + | | | | | Bertrand et al. (1997) | |
| 5-Methyleugenol | | phenolic | | + | | | | | Bertrand et al. (1997) | |

| Chemical Name | CASRN | Chemical Class | Product Class | LLNA | GPMT/BT | HMT | HPTA | Patch Concn. | References | Comment |
|---|---|---|---|---|---|---|---|---|---|---|
| 6-Methyleugenol | | phenolic | | + | | | | | Bertrand et al. (1997) | |
| Methyl hexadecenesulfonate | | alkenesulfonate (ester) | | + | + nonstd | | | | Ashby et al. (1995) | |
| Methylisoeugenol | | phenolic | fragrance | + | + nonstd | | | | Bertrand et al. (1997) | Submission listed as 3-methyl isoeugenol. |
| Methyl methanesulfonate | 66-27-3 | alkanesulfonate | | + | | | | | Ashby et al. (1995) | |
| 1-Methyl-3-nitronitrosoguanidine// MNNG | 70-25-7 | nitrosoguanide | | + | | | | | Ashby et al. (1995) | Reasonably anticipated to be a human carcinogen. |
| N-Methyl-N-nitrosourea// MNU | 684-93-5 | nitrosourea | | + | | | | | Ashby et al. (1995) | Reasonably anticipated to be a human carcinogen. |
| Methyl salicylate// Oil of wintergreen// 2-Hydroxybenzoic acid methyl ester | 119-36-8 | benzoate (ester)// phenolic | fragrance// flavoring// pharmaceutical (counterirritant) | - | - | - | | | Basketter et al. (1994); Basketter et al. (1996a); Gerberick et al. (1992); Basketter et al. (1998) | Used as a negative control. Presumed to have moderate human irritancy potential. |
| Methyl(2-sulfomethyl) octadecanoate | | aliphatic carboxylic acid ester// alkanesulfonate? | | + | | | | | Ashby et al. (1995) | |
| 2-Methyl-4,5-trimethylene-4-isothiazolin-3-one | | amide// heterocyclic | | + | + | | | | Ashby et al. (1995) | Does not attribute sensitization by this substance to any structural moiety. |
| Musk ambrette | 83-66-9 | synthetic nitro musk// lactone// potential epoxide | fragrance and fixative | + | - | | + | 1% or 5% pet. *** | Scholes et al. (1992); Ashby et al. (1995); Basketter et al. (1996a); Truett (1998) | Causes photoallergy. |

| Chemical Name | CASRN | Chemical Class | Product Class | LLNA | GPMT/BT | HMT | HPTA | Patch Concn. | References | Comment |
|---|---|---|---|---|---|---|---|---|---|---|
| .alpha.-Naphthoflavone | 604-59-1 | potential Michael-reactive agent | | + | | | | | Unpublished Unilever data | |
| .beta.-Naphthoflavone | 6051-87-2 | potential Michael-reactive agent | | + | | | | | Unpublished Unilever data | |
| Neomycin sulfate | 1405-10-3 | glucose (glucopyranose & glucofuranose) derivative// primary alkylamine | pharmaceutical (antibiotic in skin creams and ointments and eye and ear drops [antimicrobial]) | - | - | | + | | Basketter et al. (1994); Basketter et al. (1996a); Gerberick et al. (1992); Truett (1998); Marzulli and Maibach (1996); Prystowsky et al. (1979) | Unusual reactions: Contact urticaria. 75/1131 allergy patients were sensitized, but only 13/1158 volunteers. |
| Nickel chloride | 7718-54-9 | heavy metal salt | metal coatings (nickel electroplating cast zinc) | - | + | | | | Basketter and Scholes (1992); Gerberick et al. (1992); Moller (1984) | May be difficult to sensitize mice to nickel salts. |
| Nickel sulfate | 10101-98-1 | heavy metal salt | metal coatings (nickel electroplating, blackening zinc and brass)// mordant in dyeing and printing fabrics | - | + | + | + | 5% pet. ** | Basketter and Scholes (1992); Basketter et al. (1994); Basketter et al. (1996a); Marzulli and Maibach (1996) | Used in dental and shoe series patch tests. 2.5% pet. in human patch test: 109/1123 sensitized// 8 showed |
| 4-Nitrobenzyl bromide// 1-(Bromomethyl)-4-nitrobenzene | 100-11-8 | nitroaromatic// alkyl halide | | + | + nonstd | | | | Unpublished Unilever data | |
| 4-Nitrobenzyl chloride// 1-(Chloromethyl)-4-nitrobenzene | 100-14-1 | nitroaromatic// alkyl halide// potential epoxide | | + | + nonstd | | | | Ashby et al. (1995) | |
| 2-Nitrofluorene// 2-Nitro-9H-fluorene | 607-57-8 | nitroaromatic// PAH | | - | | | | | Ashby et al. (1995) | |
| 4-Nitroso-N,N-dimethylaniline// N,N-Dimethyl-4-nitrosobenzenamine | 138-89-6 | arylamine// nitrosoaromatic// tertiary amine | synthetic organic intermediate// accelerator in vulcanizing rubber// printing fabrics | + | + | | | | Ashby et al. (1995) | |
| Nonanoyl chloride// Pelargonoyl chloride | 764-85-2 | alkanoyl halide// carboxylic acid halide// acylating agent | | + | | | | | Ashby et al. (1995) | |

| Chemical Name | CASRN | Chemical Class | Product Class | LLNA | GPMT/BT | HMT | HPTA | Patch Concn. | References | Comment |
|---|---|---|---|---|---|---|---|---|---|---|
| Octadecanoyl chloride// Stearoyl chloride | 112-76-5 | alkanoyl halide// carboxylic acid halide// acylating agent | | + | | | | | Ashby et al. (1995) | Lacrimator |
| Octadecyl methanesulfonate// Stearyl methanesulfonate | 31081-59-1 | alkanesulfonate (ester) | | - | + nonstd | | | | Ashby et al. (1995) | |
| Octyl gallate// Octyl 3,4,5-trihydroxybenzoic acid | 1034-01-1 | phenolic// benzoic acid derivative | antioxidant in pharmaceuticals, cosmetics, and food (e.g., in margarine and peanut butter) | + | | | + | 0.25% pet. * | Ashby et al. (1995); Truett (1998); Hausen and Beyer (1992) | Has caused dermatitis from airborne contact. Moderate to strong sensitizer in the guinea pig. |
| Oxazolone// 4-Ethoxymethylene 2-phenyloxazol-5-one | 15646-46-5 | potential Michael-reactive agent | | + | + | | | | Loveless et al. (1996); Gerberick et al. (1992); Tarayre et al. (1984) | Designated oxazolone as a weak primary irritant in the mouse. |
| Penicillin G | 61-33-6 | lactam | pharmaceutical (antibacterial [antimicrobial], antibiotic) | + | + | + | | | Kimber et al. (1998); Basketter and Scholes (1992); Ashby et al. (1995); Basketter et al. (1994); Basketter et al. (1996a) | |
| Pentachlorophenol// Penta// PCP | 87-86-5 | phenolic// aryl halide | pesticide (wood preservative, termite control [cancelled])// pre-harvest defoliant// general herbicide) | + | | + | | | Basketter et al. (1996a) | |
| Phenol// Carbolic acid | 108-95-2 | phenolic | pharmaceutical (topical anesthetic, antiseptic, and antipruritic) | - | | - | | | Basketter et al. (1996a); Basketter et al. (1998) | Skin and mucous membrane irritant// burns skin |
| Phenyl benzoate | 93-99-2 | benzoate (ester) | | + | + | | | | Ashby et al. (1995) | |
| 3-Phenylenediamine// m-Phenylenediamine | 108-45-2 | arylamine// potential epoxide | dye manufacture// rubber curing// resins and polymers// corrosion inhibitor// photography, etc. | + | + nonstd | | + | 1% pet. | Ashby et al. (1995); Marzulli and Maibach (1996) | Human patch test: 79/1138 showed sensitization, 2/1138 showed irritation. |
| 4-Phenylenediamine// p-PDA// p-Phenylenediamine | 106-50-3 | arylamine// potential epoxide | hairdressing (permanent hair dyes)// fur & leather dyes// photography// vulcanization accelerant, etc. | + | + | + | + | 1% pet. | Kimber et al. (1991); Basketter and Scholes (1992); Ashby et al. (1995); Basketter et al. (1994); Basketter et al. (1996a); Truett (1998) | Causes contact urticaria// photoallergen. |

| Chemical Name | CASRN | Chemical Class | Product Class | LLNA | GPMT/BT | HMT | HPTA | Patch Concn. | References | Comment |
|---|---|---|---|---|---|---|---|---|---|---|
| Phthalic acid diethyl ester// Diethyl phthalate | 84-66-2 | phthalate (ester) | cellulose ester plastics used in eyeglasses and hearing aids// fragrances (perfume fixative) | - | | | + | | Unpublished Unilever data | |
| Phthalic anhydride | 85-44-9 | aromatic carboxylic acid anhydride// acylating agent | chemical intermediate (manuf. of phthaleins, phthalates, benzoic acid, synthetic indigo, artificial resins (glyptal) | + | + | | | | Basketter and Scholes (1992); Ashby et al. (1995) | |
| Picryl chloride// Trinitrochlorobenzene// TNCB | 88-88-0 | nitroaromatic// aryl halide// strong electrophile | | + | + | | | | Kimber and Weisenberger (1989); Tarayre et al. (1984) | Skin and mucous membrane irritant. Designated as a primary irritant in the mouse as well as giving delayed hypersensitivity |
| Polyhexamethylene biguanide | | | | + | + | + | | | Unpublished Unilever data | |
| Potassium dichromate | 7778-50-9 | heavy metal salt// strong oxidizer | leather tanning// oxidizer in organic synthesis// pigments, etc. | + | + | + | + | 0.5% pet. *** | Kimber et al. (1991); Kimber et al. (1995); Basketter and Scholes (1992); Basketter et al. (1994); Basketter et al. (1996a); Marzulli and Maibach (1996) | Cr compds. are the most common cause of occup. dermatitis. Ulcerates skin, destroys mucous membranes. Patch test: 59/1138 sens // 34 irr |
| .beta.-Propiolactone | 57-57-8 | lactone | intermediate in organic synthesis// disinfectant [antimicrobial] | + | | | | | Ashby et al. (1995); Budavari (1996) | Skin exposure causes irritation, blistering, and burns. Reasonably anticipated to be a human carcinogen |
| Propylene glycol// 1,2-Dihydroxypropane// 1,2-Propanediol | 57-55-6 | glycol// dihydric alcohol | cosmetics and pharmaceutical vehicle// metalworking fluids// keratolytic// foods (solvent & emulsifier)// antifreeze | - | - | | + | 5% pet. *** | Basketter et al. (1998) | Contact urticaria, systemic contact dermatitis, keratolytic. Low irritancy in 4-hour human patch test (6% of panel). |
| Propyl gallate// Tenox PG// 3,4,5-Trihydroxybenzoic acid propyl ester | 121-79-9 | benzoate (ester)// phenolic | antioxidant in food (0.05 to 0.2%), cosmetics, & pharmaceuticals | + | + | | + | 1% pet. ** | Basketter and Scholes (1992); Ashby et al. (1995); Hausen and Beyer (1992) | Moderate sensitizer in the guinea pig. |
| 1-Propyl-3-nitro-1-nitrosoguanidine// PNNG | | nitrosoguanide | | + | | | | | Ashby et al. (1995) | |
| Propylparaben// Propyl 4-hydroxybenzoate | 94-13-3 | benzoate (ester)// phenolic | Parabens are the most widely used preservatives in cosmetics, foods, & topical pharmaceuticals. | - | - | +/- | + | 3% unsp. vehicle ** | Basketter and Scholes (1992); Basketter et al. (1994); Ashby et al. (1995); Basketter et al. (1996a) | |

| Chemical Name | CASRN | Chemical Class | Product Class | LLNA | GPMT/BT | HMT | HPTA | Patch Concn. | References | Comment |
|---|---|---|---|---|---|---|---|---|---|---|
| Pyridine | 110-86-1 | aromatic heterocycle | intermediate in organic synthesis// solvent for anhydrous mineral salts | + | | + | | | Basketter et al. (1996a); Budavari (1996) | Eye irritant, may cause dermatitis. |
| Resorcinol// 1,3-Dihydroxybenzene | 108-46-3 | phenolic | pharmaceutical (acne treatment, antipruritic, antiseptic, eye drops)// cosmetics// hair dyes// tanning// resins | - | - | - | + | | Basketter et al. (1996a); Basketter et al. (1994); Basketter et al. (1998) | Keratolytic agent. Skin and mucous membrane irritant. Presumed to have low irritancy potential. |
| Salicylic acid// 2-Hydroxybenzoic acid | 69-72-7 | benzoic acid derivative// phenolic | pharmaceutical (keratolytic)// food preservative// manuf. of aspirin, methyl salicylate, & other salicylates | - | - | - | | | $GEB97-97, Basketter et al. (1996a); Basketter et al. (1994); Basketter et al. (1998); Budavari (1996) | Keratolytic. May cause skin rashes in sensitive individuals (from ingestion). Presumed mod. human skin irr. |
| Sodium benzoyloxybenzenesulfonate | | benzenesulfonate (salt)// benzoate (ester) | | + | + | | | | Unpublished Unilever data | |
| Sodium benzoyloxy-2-methoxy-5-benzenesulfonate | | benzenesulfonate (salt)// benzoate (ester) | | + | + nonstd | | | | Ashby et al. (1995) | |
| Sodium 4-(2-ethylhexyloxycarboxy)benzenesulfonate | | benzenesulfonate (salt)// benzoate (ester) | | + | + nonstd | | | | Ashby et al. (1995) | |
| Sodium lauryl sulfate// Sodium dodecyl sulfate// SLS// SDS// Irium | 151-21-3 | alkyl sulfate (half ester salt) | surfactant (detergent, wetting agent, esp. textile industry)// toothpaste ingredient | + | - | - | | | Loveless et al. (1996); Basketter et al. (1996a); Basketter et al. (1998) | Moderate irritant in 4-hour human patch test (70% of panel [380/544] responded). |
| Sodium norbornanacetoxy-4-benzenesulfonate | | benzenesulfonate (salt)// aliphatic carboxylic acid ester// alkanoate ester | | + | + nonstd | | | | Ashby et al. (1995) | |
| Sodium 4-sulfophenyl acetate | | benzenesulfonate (salt?)// alkanoate (ester?)// aliphatic carboxylic acid ester?//acetylating agent? | | + | + nonstd | | | | Ashby et al. (1995) | |
| Streptomycin sulfate | 57-92-1 | glucose (glucofuranose & glucopyranose) derivative// guanidine derivative | pharmaceutical (antibacterial [antimicrobial], tuberculostatic) | - | + | | | | Kimber et al. (1998) | |

| Chemical Name | CASRN | Chemical Class | Product Class | LLNA | GPMT/BT | HMT | HPTA | Patch Concn. | References | Comment |
|---|---|---|---|---|---|---|---|---|---|---|
| Streptozotocin | 18883-66-4 | nitrosourea derivative// nitrosamide// glucose (glucopyranose) derivative | pharmaceutical (antineoplastic)// production of exptl. diabetes in lab. animals | - | | | | | Ashby et al. (1995) | Reasonably anticipated to be a human carcinogen. |
| Sulfanilamide// 4-Aminobenzenesulfonamide// p-Anilinesulfonamide// p-Sulfamidoaniline | 63-74-1 | benzenesulfonamide// arylamine | pharmaceutical (antibacterial [antimicrobial]) | - | - | + | + | 5% pet. * | Basketter et al. (1994); Basketter et al. (1996a); Truett (1998) | May cause photoallergic contact sensitivity after topical application. |
| Sulfanilic acid// p-Aminobenzenesulfonic acid// p-Anilinesulfonic acid | 121-57-3 | | pharmaceutical (antibacterial [antimicrobial])// intermediate in manuf. of dyes, other org. chem.// anal. chem. reagent | - | + | | | | Basketter and Scholes (1992); Ashby et al. (1995) | |
| Tartaric acid// [R-(R*,R*)]-2,3-Dihydroxybutanedioic acid// d-Tartaric acid// L-Tartaric acid | 87-69-4 | aliphatic carboxylic acid// glycol | food (acidulant)// photography// tanning// ceramics | - | - nonstd | | | | Unpublished Unilever data | Skin irritant |
| Tetrachlorosalicylanilide// 3,5-Dichloro-N-(3,4-dichlorophenyl)-2-hydroxybenzamide// TCS | 1154-59-2 | phenolic// benzoic acid derivative// benzamide// aryl halide | bacteriostat [antimicrobial] in surgical & laundry soaps, polishes, shampoos, deodorants// preservative in cutting oils | + | + | + | + | 0.1% pet. * | Scholes et al. (1992); Ashby et al. (1995); Basketter et al. (1994); Basketter et al. (1996a); Budavari (1996) | Photoallergen. Banned in USA from use in cosmetics. |
| Tetradecyl iodide// Iodotetradecane// Myristyl iodide | 19318-94-1 | alkyl halide | | + | | | | | Unpublished Unilever data | |
| Tetramethyl thiuram disulfide// Thiram// Bis(dimethylthiocarbamoyl) disulfide | 137-26-8 | thiourea derivative// disulfide | bacteriostat [antimicrobial] in soap, fungicide// rubber accelerator & vulcanizer// pharmaceutical | + | + nonstd | + | + | 1% pet. | Basketter et al. (1996a); Budavari (1996) | Potent skin sensitizer. Overexposure may cause dermatitis and irritation of mucous membranes. |
| 1-Thioglycerol// 3-Mercapto-1,2 propanediol | 96-27-5 | glycol | pharmaceutical (vulnerary [promotes wound healing]) | + | + | + | | | Basketter et al. (1996a); Basketter et al. (1994) | Irritates eyes, respiratory system, and skin. |
| Tixocortol pivalate// 11.beta.-11,17-Dihydroxy-21-mercaptopregn-4-ene-3,20-dione | 55560-96-8 | steroid | pharmaceutical (anti-inflammatory) | - | | | + | | Unpublished Unilever data | |
| Toluenediamine bismaleimide | | imide// potential Michael-reactive agent | hair dressing (free base) | + | + | | + | 1% pet. free base | Basketter and Scholes (1992) | |

| Chemical Name | CASRN | Chemical Class | Product Class | LLNA | GPMT/BT | HMT | HPTA | Patch Concn. | References | Comment |
|---|---|---|---|---|---|---|---|---|---|---|
| Toluenesulfonamide-formaldehyde resin | 25035-71-6 | benzenesulfonamide, 4-methyl polymer with formaldehyde | cosmetics (acrylics/ nail polish & hardeners)// plastics and glues | - | - | | + | 10% pet. *** | Unpublished Unilever data | |
| 2,4,5-Trichlorophenol | 95-95-4 | phenolic// aryl halide | fungicide [pesticide]// bactericide [antibacterial, antimicrobial] | + | | | | | Ashby et al. (1995) | |
| 2,4,6-Trichloro-1,3,5-triazine// Cyanuric chloride | 108-77-1 | active aryl halide | pharmaceutical (topical anti-infective [antimicrobial])// chlorinating agent, disinfectant | + | | | | | Ashby et al. (1995) | Submission gave CASRN 87-90-1 [trichloroisocyanuric acid]. |
| Trimethylammonium-3-tolyl-.epsilon.-caprolactimide chloride | | quaternary ammonium compound | antimicrobial? | - | | | | | Ashby et al. (1995) | |
| .alpha.-Trimethylammonium 4-tolyloxy-4-benzenesulfonate | | benzenesulfonate// benzoylating agent// acylating agent// quaternary ammonium compound | antimicrobial? | - | + nonstd | | | | Ashby et al. (1995) | Recorded as nonsensitizing. |
| 3,5,5-Trimethylhexanoyl chloride | 36727-29-4 | acylating agent | | + | + | | | | Ashby et al. (1995) | |
| Tween 80// Polysorbate 80// Polyoxyethylenesorbitan oleate | 9005-65-6 | polyoxyethylene sorbitan ester | polyol surfactant & emulsifier in cosmetics, foods, and pharmaceuticals | - | - | | + | 5% pet. * | Basketter et al. (1996a) | No dose-response data in reference, only the call. Stated to be previously unpublished. |
| Vinylpyridine | | | | + | | | | | Ashby et al. (1995); Kimber et al. (1989); Kimber and Weisenberger (1989) | CASRN of 1337-81-1 given in submission is for the 2-vinyl isomer. The references present results for 4-vinylpyridine. |
| Xylene// Dimethylbenzene (mixture of o-, m-, & p-isomers) | 1330-20-7 | aromatic hydrocarbon | solvent// intermediate in production of benzoic acid, phthalates, etc. | + | | - | | | Basketter et al. (1996a); Budavari (1996) | Causes skin irritation and dermatitis due to defatting action. Eye irritation and corneal burns. |
| Zinc sulfate | 7733-02-0 | heavy metal salt | pharmaceutical [ophthalmic astringent, zinc supplement]// zinc refining & electroplating// manuf. zinc compds.// mordant | + | | | | | Unpublished Unilever data | |

## Results of LLNA Literature Search

(August 17, 1998)

A literature search was done on August 17, 1998 (Medline data base, 1966 to present) using "Local Lymph Node Assay" as the key phrase. Following are the 69 articles retrieved.

Arts, J. H., S. C. Droge, N. Bloksma, and C. F. Kuper. 1996. Local lymph node activation in rats after dermal application of the sensitizers 2,4-dinitrochlorobenzene and trimellitic anhydride. Food Chem. Toxicol. 34:55-62.

Arts, J. H., S. C. Droge, S. Spanhaak, N. Bloksma, A. H. Penninks, and C. F. Kuper. 1997. Local lymph node activation and IgE responses in brown Norway and Wistar rats after dermal application of sensitizing and non-sensitizing chemicals. Toxicology 117:229-234.

Ashby, J., J. Hilton, R. J. Dearman, R. D. Callander, and I. Kimber. 1993. Mechanistic relationship among mutagenicity, skin sensitization, and skin carcinogenicity. Environ. Health Persp. 101:62-67.

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*The Procter & Gamble Company*
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3 April. 1998

Dr. William S. Stokes
Environmental Toxicology Program
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P.O. Box 12233
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Dear Dr. Stokes:

As promised, we have revised our ICCVAM Test Method Submission for the Local Lymph Node Assay (LLNA) based the comments we received from Ms. Sailstad (Letter dated March 16, 1998). Specifically, we revised Appendix B by giving more detailed information on areas of discordance in the LLNA data. In addition, we have provided disintegration per minute data and stimulation indices for these compounds. The submission was prepared by David Basketter, Ian Kimber and me.

As you know, the LLNA is currently accepted as a screening test in the OECD 406 guidelines as well as in the EU guidelines. In our submission, extensive data are reviewed supporting the use of the LLNA as a stand-alone method for the identification of contact allergens. Comparative studies have confirmed that the local lymph node assay is of equal predictivity to guinea pig methods used currently for the identification of skin sensitizing chemicals. Furthermore, it is clear that the local lymph node assay offers a number of important advantages, including significant animal welfare advantages.

Since the initial publication on the LLNA in 1986 by Kimber and his associates, there have been numerous publications addressing the immunological mechanisms underlying the assay as well as its use in regulatory toxicology - 61 references are listed in the submission. A list of approximately 200 chemicals which have been tested in the LLNA are listed also in the submission. Of the 130 chemicals tested in one of the reference guinea pig tests, approximately 83% gave the same result in the LLNA and the guinea pig tests.

In light of advancing knowledge and experience, and given animal welfare considerations, it is our opinion that the LLNA is now fully validated as a methodology for the identification of significant skin sensitizers and, therefore, should be adopted formally as an alternative skin sensitization test and incorporated fully into regulatory guideline documents addressing skin sensitization testing. Please note that the proposal relates to the standard LLNA. Consequently, data from modified versions of the LLNA have not been included in the submission.

Please feel free to contact us if you have any questions regarding the submission.

Sincerely yours.
/s/

G. Frank Gerberick, Ph.D.
Procter & Gamble Principal Scientist

cc: Dr. I. Kimber; Dr. D. Basketter

# The Local Lymph Node Assay

# ICCVAM Test Method Submission

**Prepared by G. Frank Gerberick, Ian Kimber and David A. Basketter**

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## The Local Lymph Node Assay

# ICCVAM Test Method Submission

## A. INTRODUCTION AND RATIONALE

Allergic contact dermatitis is a frequent occupational health problem and, in common with other forms of allergic disease, develops in two phases. The first or induction phase is initiated when a susceptible individual encounters on the skin sufficient amounts of an inducing allergen to stimulate a primary cutaneous immune response. This results in allergic sensitization. If the now sensitized individual is subsequently exposed, at the same or a different skin site, to the same allergen then an accelerated and more aggressive secondary immune response will be provoked at the site of contact. Allergen-responsive T lymphocytes are activated in the skin at the site of contact and release cytokines and other inflammatory mediators which cause the accumulation of mononuclear cells and the inflammatory reaction that is recognized clinically as allergic contact dermatitis.

For many years the species of choice for the identification of contact allergens was the guinea pig . A variety of guinea pig test methods has been described and while these vary in detail, the principles of the assays are in each case the same, sensitizing activity being measured as a function of challenge-induced erythematous and edematous reactions in previously sensitized animals. There is no doubt that some at least of these guinea pig methods have served toxicologists well. Nevertheless, it is clear that such assays are subject to some important limitations, including the fact that the endpoint is subjective and may be difficult to measure and interpret if colored or irritant chemicals are evaluated. Moreover, some of the more sensitive guinea pig methods demand the use of adjuvant. These limitations encouraged consideration of alternative approaches.

Some ten years ago the local lymph node assay was described (Kimber *et al*, 1986; Kimber *et al*, 1989; Kimber and Basketter, 1992; Kimber *et al*, 1994; Kimber, 1996). This method was founded on the belief that an increasingly sophisticated appreciation of the immune system would facilitate the design of alternative methods for the identification of chemical allergens that cause adverse effects through the stimulation of specific immune responses. The local lymph node assay employs mice, the experimental species where there is the most detailed information available about the induction and regulation of immunological responses. In contrast to guinea pig test methods, the local lymph node assay identifies potential skin sensitizing chemicals as a function of events associated with the induction, rather than elicitation, phase of skin sensitization.

The induction phase of skin sensitization is characterized by the stimulation of an allergen-specific immune response in lymph nodes draining the site of exposure. Epidermal Langerhans cells (LC) recognize, internalize and process the chemical hapten associated with protein. LC are induced to migrate to draining lymph nodes. While in transit they develop into immunostimulatory dendritic cells which in the lymph nodes are able to interact with, and present antigen to, responsive T lymphocytes (Kimber and Cumberbatch, 1992; Kimber and Dearman, 1996). Immune activation in draining lymph nodes is characterized by T lymphocyte division and differentiation, the production by activated cells of cytokines and other mediators and an increase in the size, weight and cellularity of the lymph nodes. The division of activated T cells results in an increase in the number of allergen-reactive lymphocytes; this clonal expansion being the cellular basis of immunological memory and allergic sensitization. The importance of clonal expansion is reflected by the fact that the vigor of proliferative responses induced by chemicals in draining lymph nodes correlates closely with the extent to which sensitization develops (Kimber and Dearman, 1991; Kimber and Dearman, 1996).

In initial investigations several parameters of draining lymph node activation were measured following topical exposure of mice to contact allergens and to non-sensitizing chemicals. These comprised changes in lymph node weight and cellularity and lymphocyte proliferation measured as a function of radiolabelled thymidine incorporation during culture of lymph node cells (Kimber *et al*, 1986; Kimber and Weisenberger, 1989a; Kimber, 1989). The marker that proved to be the most sensitive and selective correlate of skin sensitizing activity was the induction of lymph node cell proliferation and subsequent investigations focused upon this. Another change introduced following these preliminary experiments was to measure the proliferative activity *in situ,* by intravenous injection of tritiated thymidine, rather than following culture of isolated lymph node cells ( Kimber and Weisenberger 1989b; Kimber *et al*, 1989). It is this version of the method that has been evaluated extensively in the context of national and international collaborative trials and which has been the subject of detailed comparisons with guinea pig tests and with human data. The results of these evaluations and comparisons will be discussed later.

A criterion of positivity was required to facilitate decisions regarding the sensitizing potential of chemicals based on activity in the local lymph node assay. The decision was made, based on extensive experience gained with the method, that a chemical should be classified as a skin sensitizer if, at one or more test concentrations, proliferative activity three-fold or greater than that measured in concurrent vehicle treated controls was induced. The validity of the use of a stimulation index of 3 for the identification of contact allergens is discussed later in this submission.

In summary, the local lymph node assay provides a novel approach to the identification of skin allergens where immunobiological events stimulated during the induction phase of skin sensitization are measured. Decisions are based upon assessment of draining lymph node cell proliferative responses - responses that are known to be essential for, and to correlate with, the induction of skin sensitization.

For practical purposes the following recommendations are made for use of the local lymph node assay:

- A chemical which, at one or more test concentrations, elicits a three-fold or greater increase in proliferative activity compared with concurrent vehicle treated controls should be classified as being a contact allergen and handled and labeled accordingly.
- Chemicals that fail at all test concentrations to elicit a positive response in the local lymph node should be classified as lacking significant skin sensitizing potential and should be handled and labeled accordingly. No further confirmation of negative results is required.

There is currently some interest in comparing and contrasting the nature of immune responses induced in mice by different types of chemical allergens. It is very important to emphasize here, however, that the proposal is that the local lymph node assay can be used to identify those chemicals that are able to cause skin sensitization. A case is not being made here for use of the local lymph node assay in the identification of any other classes of chemical allergen. Moreover, this submission is focused on the standard LLNA. Consequently, papers describing modified versions of the assay are not reviewed in this document.

The proposal is that the local lymph node assay provides an alternative method for use in the identification of skin sensitizing chemicals and for confirming that chemicals lack a significant potential to cause skin sensitization. This does not necessarily imply that in all instances the local lymph node assay should be used in place of guinea pig tests, but rather that the assay is of equal merit and may be employed as a full alternative in which positive and negative results require no further confirmation.

The local lymph node assay is not an *in vitro* method and as a consequence will not eliminate the use of animals in the assessment of contact sensitizing activity. It will, however, permit a **reduction** in the number of animals required for this purpose. It has been estimated that, in practice, on average half the number of animals required for a standard guinea pig test is needed for conduct of a local lymph node assay. Moreover, the local lymph node assay does offer a substantial **refinement** of the way in which animals are used for contact sensitization testing. One important point is that, unlike some of the guinea pig methods, such as the guinea pig maximization test, the local lymph node assay does not require the use of adjuvant. Furthermore, the local lymph node assay is based upon consideration of immunobiological events stimulated by chemicals during the induction phase of sensitization. Unlike guinea pig tests the local lymph node assay does not require that challenged-induced dermal hypersensitivity reactions are elicited.

Due to the fact that the local lymph node assay requires far fewer animals than needed for standard guinea pig tests, it can be conducted for approximately half the cost. The time taken for conduct of a local lymph node assay is some eight times less than that needed for a standard guinea pig method.

It is estimated currently that in excess of 25 separate laboratories world-wide are conducting the local lymph node assay.

## B. TEST METHOD PROTOCOL

The contact allergenic potential of a test substance, under the conditions of this protocol, is evaluated by its ability to cause proliferation of draining lymph node cells in mice treated topically compared to appropriate concurrent vehicle treated controls. Direct epicutaneous application of a test substance to the ears is an appropriate route of administration for assessing the contact allergic potential of a test substance. Incorporation of $^3$H-thymidine into DNA of lymphocytes results from the stimulation of S-phase prior to proliferation of the cells after receipt of antigenic stimulation. Measurement of $^3$H-thymidine uptake by the cells is an objective and quantifiable correlate of immune activation.

<u>Protocol</u> The standard protocol described previously (Kimber and Basketter, 1992) utilizes young adult (6-16 week old) female CBA/Ca stain mice. In strain comparisons, CBA/Ca mice were found to exhibit a more marked

response to contact allergens than did the other strains examined (Kimber and Weisenberger, 1989a). However, female CBA/J and CBA/JHsd strain mice are also acceptable for use in the assay as, in several interlaboratory validation studies, they display responses comparable with those of CBA/Ca strain mice (Kimber *et al*, 1995; Loveless *et al*, 1996). Mice are housed under standard conditions, individually or by treatment group, in plastic shoe box type cages for the duration of the study. Food and tap water are provided *ad libitum*. Control of bias is addressed by randomization of mice prior to initiation of the study.

Groups of mice (n=4 or 5) are treated by topical application, on the dorsum of both ears, of 25 µl of one of several concentrations of test material, or with an equal volume of the relevant vehicle alone. Treatments are performed daily for three consecutive days and the mice are then rested for 2 days prior to analysis. On the sixth day (five days after initiation of treatment), the mice are injected intravenously via the tail vein with 250 µl of sterile phosphate buffered saline (PBS) containing 20 µCi of [$^3$H] methyl thymidine ($^3$H-TdR; specific activity between 2 and 7 Ci/mmol). Five hours later, the mice are killed and the draining auricular lymph nodes excised and pooled for each experimental group or for each individual animal. Single cell suspensions of lymph node cells (LNC) are prepared by gentle mechanical disaggregation through 200-mesh nylon or stainless steel gauze. LNC are washed twice with an excess of PBS and precipitated with 5% trichloroacetic acid (TCA) at 4$^o$C. Twelve-18 hours later the samples, pelleted by centrifugation, are resuspended in 1 ml 5% TCA and transferred to 10 ml of scintillation cocktail. Incorporation of $^3$H-TdR is measured by _-scintillation counting and expressed as disintegrations per minute (dpm). The use of $^{125}$IUdR rather than $^3$H-TdR as the isotope has been shown to be comparably robust in the LLNA (Kimber *et al*, 1995; Loveless *et al*, 1996).

A sample protocol is provided in Appendix D.

<u>Dose selection</u> No additional animals are used for dose range finding. The current practice is to select at least three consecutive concentrations from the following range: 100, 50, 25, 10, 5, 2.5, 1, 0.5, 0.25 and 0.1% (w/v). The selection is made to provide the highest possible test concentration, limited by compatibility with the vehicle chosen (and the suitability of the resultant preparation for unoccluded dermal application ), while avoiding dermal trauma or

systemic toxicity. The test chemical is dissolved in an appropriate vehicle. Vehicle selection is important and a variety of organic solvents is suitable. The following are recommended, in order of preference: acetone-olive oil (4:1) (AOO), acetone, dimethylformamide, methyl ethyl ketone, propylene glycol and dimethylsulfoxide (Kimber and Basketter, 1992). While aqueous vehicles are not recommended, aqueous and aqueous-organic mixtures such as 3:1 acetone:water have been used successfully.

Control Materials The current OECD positive control sensitizers hexyl cinnamic aldehyde, 2-mercaptobenzothiazole and benzocaine have each been evaluated in the local lymph node assay. Results with these positive controls in the local lymph node assay met or exceeded the minimum acceptable standard set forth by the OECD (Basketter *et al*, 1993). The strong sensitizer 2,4-dinitrochlorobenzene (DNCB) may be used as a positive control as it has produced consistent responses in the LLNA, including when tested in two recent international interlaboratory trials (Kimber *et al*, 1995; Loveless *et al*, 1996). Currently, there are no recommended negative controls for the LLNA as is the case with the reference guinea pig methods. However, methyl salicylate, tested at 1, 2.5, 5, 10 and 20% (w/v) in acetone:olive oil (4:1) (Kimber *et al*, 1995; Kimber *et al*, 1998) and *para*-aminobenzoic acid tested at 0.5, 1, 2.5, 5 and 10% (w/v) in acetone:olive oil (Loveless *et al*, 1996) have been used successfully as negative control chemicals in interlaboratory validation studies. In common with other skin sensitization tests, a control substance for irritation has not been defined for the LLNA.

Data collection and analysis *In vivo* $^{3}$H-thymidine incorporation into lymph node cell DNA associated with proliferation induced by application of a contact sensitizer (measured by liquid scintillation counting) is an objective and quantifiable response. Data are collected as disintegrations per minute (dpm).

The data are expressed as mean dpm for each experimental group and the stimulation indices (SI) for each experimental group are determined as the increase in $^{3}$H-TdR incorporation relative to concurrent vehicle-treated controls (test/control ratio). A test material which at one or more concentrations causes a stimulation index of 3 or greater is considered to have skin sensitizing activity. Thus, whether the draining auricular lymph nodes are excised and pooled for each experimental group or for each individual animal, the three-fold or greater increase in proliferative activity compared with concurrent vehicle treated control animals is the sole criterion for a classification of skin sensitizing activity.

In cases where individual mice are being used for determining the mean dpm value for an experimental group, statistical analysis may be performed. The value of statistical analyses, either alone or in conjunction with the three-fold stimulation index, has not yet been established and is still the subject of investigations. Where isotope incorporation is determined for individual mice, a mean dpm value ± standard error of the mean (SEM) is calculated for each experimental group. A stimulation index is derived for each experimental group by dividing the mean dpm of that group by the mean dpm of the vehicle-control group.

One approach to the development of statistical methods that may prove of value in the local lymph node assay is as follows. For statistical analyses, the mean dpm values for each treatment group and the vehicle control group are initially normalized by obtaining their log value. Bartlett's test (Bartlett, 1937) is then used to examine the data for homogeneity of the within-chemical treatment variance. When analysis of variance reveals significant differences in parametric data, experimental groups are compared with vehicle-treated controls using Dunnett's *t* test (Dunnett, 1955). For non-parametric data, a Kurskal-Wallis test (Kruskal and Wallis, 1952) followed by Dunn's multiple comparison procedure (Dunn, 1964) is used. Groups differing from vehicle-treated controls at the level of $P \leq 0.05$ are considered significantly different. Alternately, if Bartlett's test for homogeneity of variance is not significant, comparisons with the control group (and other specific, pairwise comparisons of groups) are based on the least significant difference criterion. If Bartlett's test is significant, these comparisons are based on Wilcoxon's rank sum test.

In addition, an estimate of the test material concentration required to produce a stimulation index of 3 ($EC_3$) can be calculated using fitted quadratic regression analyses. An advantage of the $EC_3$ calculation is that data from the entire dose response curve are used to produce a single value of intrinsic potency (Loveless *et al*, 1996). The $EC_3$ value can then be used to rank order the skin sensitizing potential of chemicals. Stronger sensitizers such as DNCB and oxazolone have lower $EC_3$ values than more moderate sensitizers such as hexyl cinnamic aldehyde and eugenol

(Loveless *et al*, 1996). Dose response analyses in the local lymph node assay, combined with the mathematical derivation of the lowest test concentration of a chemical required for a defined stimulation index, such as the $EC_3$, provides a convenient, reliable and realistic approach to evaluation of relative potency (Kimber and Basketer, 1997).

An examination of the application of statistical analyses to the local lymph node assay is continuing. At present, it is not clear whether, or in what way, an evaluation of statistical significance would add value to the interpretation of the local lymph node assay. This, together with consideration of $EC_3$ values for measurement of relative potency are areas of investigation that may pay dividends in the future, but which are not currently part of the standard protocol.

Summary of control data The recommended positive control material, hexyl cinnamic aldehyde (HCA), was tested independently by five laboratories over a dose range of 2.5, 5.0, 10.0, 25.0, and 50% (w/v) in AOO (Loveless et al., 1996). All five correctly identified HCA as a contact allergen. Four of the five laboratories found the lowest concentration to produce an SI of 3 or greater was 10%. The fifth laboratory reported an SI of 2.5 for this concentration. Calculations of the $EC_3$ for HCA ranged from 7.0 to 8.4%. DNCB was tested in two separate trials by the same five laboratories at concentrations of 0.01, 0.025, 0.05,0.1, and 0.25% (w/v) in AOO. $EC_3$ calculations for DNCB from both trials ranged from 0.03 to 0.09%.

Recently the stability with time of responses induced in the local lymph node assay by HCA has been evaluated in a single laboratory. Over a ten month period HCA elicited very similar $EC_3$ values in the local lymph node assay (Dearman *et al*, 1998). These issues are discussed further in Section D below.

## C. CHARACTERIZATION OF MATERIALS TESTED

Two of the interlaboratory evaluations of the LLNA were carried out under conditions where all details of the test materials and test conditions were not known to the participating laboratories. In the first of these studies, 20 substances were coded and supplied to each of 4 laboratories (Basketter *et al*, 1991). In a subsequent study, the chemical names were given, but no advice on dose/vehicle selection was provided (Scholes *et al*, 1992). The results from both of these investigations demonstrated a high degree of interlaboratory agreement. It is interesting to compare these results with those from unblinded interlaboratory studies of the GPMT and the Buehler test (Robinson *et al*, 1990; Andersen *et al*, 1985). In these instances, relatively poor interlaboratory reproducibility was achieved, which is in sharp contrast to experience with LLNA.

## D. ASSESSMENT OF RELIABILITY (REPEATABILITY AND REPRODUCIBILITY)

There are considerable data on intralaboratory reproducibility of the LLNA, some of which has been published (Basketter *et al*, 1996; Kimber *et al*, 1998) and some of which is based on unpublished individual laboratory experience. Table 1 summarizes the information on this topic.

Although it is not the aim within the current validation to examine assessment of relative skin sensitizing potency, it is possible to derive such information from the LLNA (Basketter *et al*, 1996; Kimber and Basketter, 1997). For this, the estimated concentration of the test chemical which is sufficient to cause a 3-fold stimulation ($EC_3$) is determined by interpolation of the dose response data. What precise value this may have for risk assessment is currently the subject of various pieces of work (eg Basketter *et al*, 1996; Kimber and Basketter, 1997; Basketter, 1998). However, the approach taken also allows better comparison of individual LLNA results. Examples of this type of data are contained in Table 2.

**Table 1: Intralaboratory reproducibility of the LLNA**

| **Chemical** | **Test 1** | **Test 2** | **Test 3** | **Test 4** | **Test 5** | **Test 6** |
|---|---|---|---|---|---|---|
| DNCB | + | + | + | ND | ND | ND |
| Hexyl cinnamic aldehyde | + | + | + | + | + | + |
| Isoeugenol | + | + | + | + | ND | ND |
| Eugenol | + | + | + | + | + | ND |
| Methyl salicylate | - | - | - | - | ND | ND |
| Benzocaine | - | - | +/- | - | - | - |

ND = No data

The first collaborative LLNA validation trial involved four independent laboratories in the UK which evaluated the same batch of eight chemicals, using the same protocol, vehicles and test concentrations. Each laboratory identified 2,4-dinitrochlorobenzene (DNCB), formalin, eugenol, isoeugenol, paraphenylenediamine (p-PDA), and potassium dichromate as positive with benzocaine and methyl salicylate as negatives. With the exception of isoeugenol, no significant differences between the laboratories were found with respect to the characteristics of dose-response curves (Kimber *et al*, 1991).

The same four laboratories participated in a more extensive evaluation involving 25 chemicals (Basketter *et al*, 1991). Of the 25 chemicals, equivalent predictions of sensitizing potential were made for 18 chemicals by all laboratories. An additional five chemicals were identified as potential sensitizers in the LLNA by two or three laboratories. Three of these subsequently gave a positive response in laboratories which initially failed to detect them when retested under identical or altered conditions (e.g. higher concentration, different vehicle). It should be noted that these investigations were conducted prior to publication of the definitive LLNA protocol.

**Table 2 Reproducibility of LLNA quantitative data**

| Chemical | Test 1 | Test 2 | Test 3 | Test 4 | Test 5 | Test 6 |
|---|---|---|---|---|---|---|
| DNCB - Laboratory 1 | 0.05[1] | 0.03 | ND[2] | ND | ND | ND |
| DNCB - Laboratory 2 | 0.06 | 0.05 | ND | ND | ND | ND |
| DNCB - Laboratory 3 | 0.04 | 0.06 | ND | ND | ND | ND |
| DNCB - Laboratory 4 | 0.06 | 0.09 | ND | ND | ND | ND |
| DNCB - Laboratory 5 | 0.03 | 0.06 | ND | ND | ND | ND |
| Isoeugenol | 0.3 | 0.4 | 0.4 | 0.4 | 0.6 | ND |
| Hexyl cinnamic aldehyde | 7.9 | 6.9 | 9.6 | 8.7 | 4.0 | 9.2 |
| Hexyl cinnamic aldehyde | 7.6 | 7.2 | 8.8 | 9.5 | 10.0 | 11.9 |
| Eugenol | 5.1 | 6.1 | 10.5 | 11.9 | 14.5 | ND |
| Methyl salicylate | NS[3] | NS | NS | NS | ND | ND |
| Benzocaine | NS | NS | ?[4] | NS | NS | NS |

[1]% concentration required to give a stimulation index of 3
[2]ND = Not done
[3]NS = Not a sensitizer
[4]Not possible to determine an EC3 value from the dose response data.

For the final phase of this national collaboration, nine chemicals were evaluated and each laboratory independently selected the test concentrations and vehicles (Scholes *et al*, 1992). One modification that all laboratories employed was applying chemicals topically for three consecutive days and then terminating the experiment five days after the initiation of exposure, rather than four days. Chemicals were evaluated at three concentrations which were chosen independently by each laboratory with regard to potential toxicity. The choice of vehicle was based upon solubility and viscosity. For eight chemicals, equivalent predictions were made by all laboratories and by three of the four laboratories for the remaining chemical. Identical vehicles and concentrations were selected independently by all laboratories for two chemicals and by three laboratories for six chemicals. In those cases where different concentrations or vehicles were chosen, equivalent predictions (positive or negative LLNA results) were still made.

To determine what effect minor protocol modifications would have on the predictive value of the test, the LLNA was evaluated in an international study by five independent laboratories, two of which had participated in the UK national validation exercise. Modifications to the standard protocol included exposure of mice for four, rather than three, consecutive days, removal of auricular lymph nodes four rather than five days after study initiation, the use of an alternative isotope and analysis of lymph nodes from individual mice to allow for statistical evaluation proposed (reviewed in Gerberick *et al*, 1992; Ladics *et al*, 1995).

In the first phase of this international validation, two skin sensitizers, DNCB and potassium dichromate, and one non-sensitizer, methyl salicylate, were evaluated (Kimber *et al*, 1995). In the LLNA, the criteria for a positive result is a three-fold or greater stimulation of proliferative activity relative to vehicle controls. In the laboratories analyzing nodes from individual mice, a positive result was also defined, for the purpose of this investigation, as treatment groups differing from vehicle treated controls at a predetermined level of statistical significance ($p<0.05$ or $p<0.01$ depending upon the statistical method employed). By either criterion, and regardless of the protocol utilized, all five laboratories identified the two known sensitizers as being positive in the LLNA. Estimates of the test concentration required to yield a stimulation index of three ($EC_3$) were very similar for all laboratories for both chemicals. Using the stimulation index criteria, all laboratories reported a negative finding for methyl salicylate at all concentrations tested. Two of the three laboratories evaluating nodes from individual mice did detect a statistically significant increase in radioisotope incorporation at the highest of the five concentrations tested (20%).

In the second phase of the international collaborative trial, the sensitivity and selectivity of the assay were examined further by analysis of six additional chemicals: hexylcinnamic aldehyde (HCA), oxazolone, isoeugenol, eugenol, sodium lauryl sulphate (SLS), and para-aminobenzoic acid (pABA) (Loveless *et al*, 1996). The last two are considered to be non-sensitizing chemicals, while the remainder exhibit skin sensitizing potential to varying extents, with HCA being one of three chemicals recommended by the OECD for use as positive controls in skin sensitization studies (OECD, 1993). All laboratories retested DNCB under the conditions employed in phase I of the trial (Kimber *et al*, 1995) to provide information on the temporal stability of assay data. All five laboratories identified as positive the five moderate to strong sensitizers (DNCB, HCA, oxazolone, isoeugenol and eugenol). SLS, considered to be a non-sensitizing skin irritant, also induced a positive response in the assay. pABA, a non-sensitizing chemical, was negative in each laboratory.

Oxazolone was clearly the most potent sensitizer evaluated in Phase II, with predicted $EC_3$ values ranging from 0.0007 to 0.0026%. This chemical highlights the benefit of utilizing the entire dose response curve for predicting the concentration required for a SI of 3, since four of the five laboratories recorded stimulation indices of above three at the lowest concentration tested. It also demonstrates that determination of an $EC_3$ maybe useful in assessing the relative sensitizing potency of a class of chemicals. Results with HCA, eugenol, isoeugenol and pABA were similar to published LLNA results (Basketter *et al*, 1993; Basketter and Scholes, 1992; Basketter *et al*, 1994 ).

The results of Phase I and II provide strong support that the incorporation of minor procedural modifications did not affect the performance of the LLNA. In that regard applying a test chemical for either three or four consecutive days, with removal of lymph nodes five or four days, respectively, after the initiation of treatment, did not change the ability of the assay to detect skin allergens. Three consecutive daily exposures to a chemical is therefore considered sufficient for the purpose of the identification of potential skin sensitization hazard.

Concerning the choice of isotope utilized for detection of proliferation, there was no difference in the ability of $^{3}$HTdR or $^{125}$IUdR to identify correctly the chemicals evaluated in this study. Either isotope can be used in the LLNA (Ladics *et al*, 1995; Kimber *et al*, 1995; Loveless *et al*, 1996).

An important modification assessed during Phase I and II of this international validation study was the analysis of proliferation within lymph nodes of individual mice as opposed to lymph nodes pooled for each experimental group.

In the majority of cases, the lowest concentration yielding a positive response was identical by either method of analysis.

One objective of Phase II was to examine inter-experimental variability by evaluating DNCB twice. Three of the five laboratories obtained identical results to the first study (Kimber *et al*, 1995). Depending upon which of the criteria were used, the other two participating laboratories had either identical inter-experimental results or were within one adjacent concentration level. Therefore, the intralaboratory inter-experimental variability was very low.

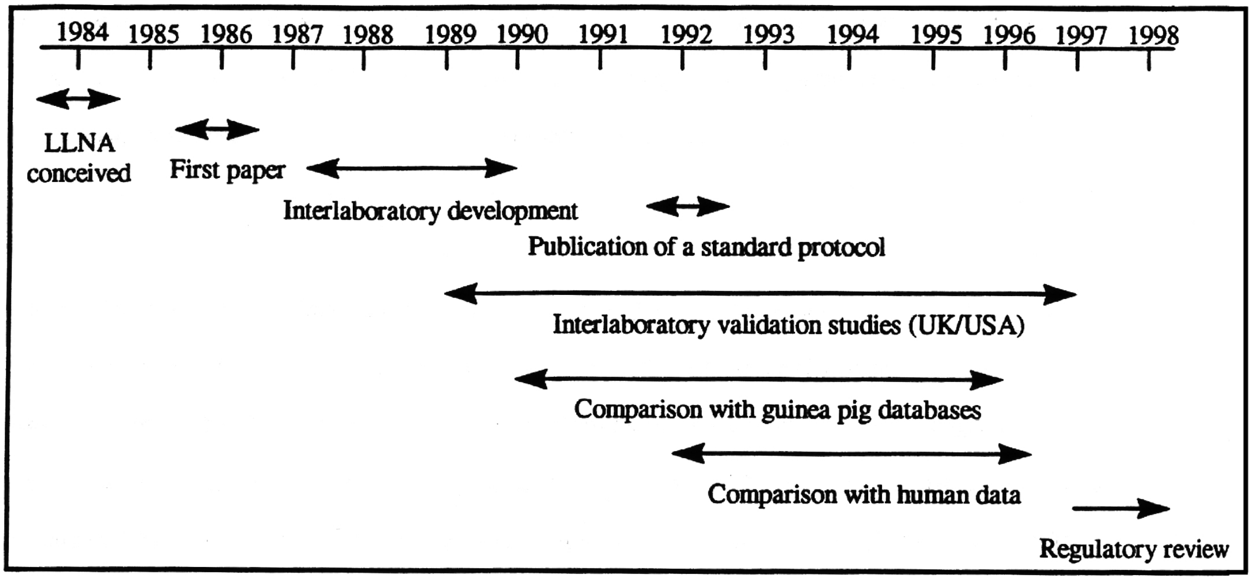
The overall conclusion from this and the previous phase of the validation study (Kimber *et al*, 1995) is that five independent laboratories, despite the use of procedural modifications and different methods for data analysis, successfully and consistently employed the LLNA to reach identical conclusions on the sensitizing potential of nine chemicals.

The most recent interlaboratory validation study involved the same five laboratories working in collaboration with the US FDA. In this study (Kimber *et al*, 1998), a small series of chemicals used in topical drug products was examined. Again there was very close agreement between laboratories, with all five identifying correctly benzoyl peroxide, hydroquinone, penicillin G and methyl salicylate. Streptomycin sulfate induced equivocal responses, insofar as this material provoked a positive LLNA response in only one of the five laboratories, and then only at the highest concentration tested. Ethylenediamine dihydrochloride was uniformly negative. Collectively these data serve to confirm that the LLNA is sufficiently robust to yield equivalent results when performed independently in separate laboratories. The data indicate also that the LLNA is of value in assessing the skin sensitization potential of topical medicaments.

A total of 7 laboratories have been involved in interlaboratory validations of the LLNA. The results of the work have appeared in the several associated publications (Kimber *et al*, 1991; Basketter *et al*, 1991; Scholes *et al*, 1992; Kimber *et al*, 1995; Loveless *et al*, 1996; Kimber *et al*, 1998). This work has involved investigation of more than 40 different chemicals.

An overview of the time frame for the development and validation of the LLNA is displayed in Figure 1 (adapted from Chamberlain and Basketter, 1996). Information on consistency/performance over time has been given earlier in this section.

**Figure 1. LLNA Timeline**




**E. REFERENCE DATA**

A variety of guinea pig tests has been developed for evaluation of the skin sensitizing potential of chemicals. Among those most widely applied are the guinea pig maximization test (GPMT) (Magnusson and Kligman, 1969,1970) and the occluded patch test of Buehler (Buehler, 1965, 1985; Robinson et al, 1990). These two assays are the preferred guinea pig sensitization tests outlined in the OECD 406 guideline for skin sensitization.

Figure 1. LLNA Timeline

The GPMT used for comparisons with LLNA results is based on and similar to that described by Magnusson and Kligman (1970) which uses Freund's adjuvant. Albino Dunkin-Hartley guinea pigs, weighing approximately 350g at the start of each study, are used. Preliminary irritation tests are carried out to determine the concentrations of the test substances suitable for induction of sensitization and for challenge. Guinea pigs are then treated by a series of six intradermal injections in the shoulder region to induce sensitization. After 6-8 days, sensitization is boosted by a 48 hr occluded patch placed over the injection site. Twelve to fourteen days later, the animals are challenged on one

flank by a 24 hr occluded patch at the maximum non-irritant concentration. Challenge sites are scored for erythema (scale 0-3) and edema 24 and 48 hr after removal of the patches. The EC guidelines state that a material is positive if the incidence is ≥30% (European Communities, 1993).

The standard Buehler test (BT) protocol uses an occluded topical patch technique for the induction and elicitation of contact sensitization (Buehler, 1965, 1985; Robinson *et al*, 1990). The procedure calls for 20 animals in the test (sensitized) group, 10 naive (control) animals for challenge, and 10 separate naive control animals for rechallenge. For induction, a single dorsal site is used for three 6 hour induction patches (applied occluded once per week to the same pre-shaven induction site on the dorsal surface of the test animals). Following a two week rest period, the test and non-induced control animals receive 6 hour challenge patches at a naive skin site for the primary challenge. The same test animals and additional new control animals can be rechallenged by this procedure 7-15 days after primary challenge at any remaining naive skin sites. Reactions are graded for erythema 24 and 48 hours after patch removal, according to a 5 point grading scale. The grades "1", "2" and "3" denote increasing severity of erythema with grades ≥"1" considered positive. The EC guidelines state that a material is positive if the incidence is ≥15% (European Communities, 1993).

In addition to comparison of the LLNA with guinea pig sensitization test data, the LLNA has also been compared with human data (Basketter *et al*, 1994; Basketter *et al*, 1996). Specifically, the LLNA has been compared with the human maximization test (HMT) (Kligman, 1966a,b,c). This method was specifically designed to provide a rigorous assessment of the skin sensitization potential of chemicals in humans. In principle, a group of 25 subjects is subjected to 48 hour occlusive patch treatments with as high a concentration of test chemical as possible. This treatment is repeated five times over a two week period. If the substance is not sufficiently irritating, the irritancy is enhanced by prior treatment of the site for 24 hours with sodium lauryl sulfate prior to each 48 hour patch. The extent of sensitization in the panel is assessed by 48 hour treatments on a slightly irritated skin site using the maximum non-irritant concentration of the test substance. The challenge sites are scored at 48 hours and 96 hours post-application. In essence, this procedure can provide a stringent assessment of intrinsic sensitization hazard and its relative potency.

To define the role of the LLNA in predictive testing, results from the assay have been compared with predictions from guinea pig and human tests. In some instances, the LLNA results and the reference results (guinea pig or human) are presented together. In other cases, LLNA studies have been conducted with chemicals whose sensitization potential, or lack thereof, are well known. Basketter and Scholes (1992) investigated the correlation between results in the LLNA and those derived from the GPMT for materials that covered a range of chemical types and levels of skin sensitization potency. Kimber *et al* (1990) reported comparative analyses in which 24 chemicals, of previously unknown contact sensitizing potential, were evaluated in both the local lymph node assay and the occluded patch test of Buehler. The data reported demonstrate that the local lymph node assay identified successfully those chemicals that were classified as moderate or strong skin sensitizers in the Buehler test. Basketter *et al* (1991) evaluated the performance of the LLNA with 25 chemicals for which guinea pig maximization test or Buehler occluded patch test data were available. The 25 chemicals included preservatives, perfume ingredients, surfactants, plastics/resin chemicals and oil additives. A high level of agreement between the results of local lymph node assays and guinea pig test data was found.

As stated above, an essential point of comparison for the LLNA is with human data. Basketter *et al* (1994 and 1996) compared human maximization tests results with those obtained with the LLNA for the same 38 chemicals. The former being a rigorous assessment of the sensitization potential of chemicals in humans. The authors reported that the LLNA identifies those chemicals that are significant human contact allergens and that the specificity of the assay is good. A comprehensive review of published and unpublished LLNA data is given in Appendix A.

## F. TEST METHOD RESULTS AND PERFORMANCE ASSESSMENT

The predictive power of the LLNA in comparison to standard guinea pig methods is given in Appendix B. This type of information has been reviewed in detail in a recent paper (Basketter *et al*, 1996). While it is clear that the LLNA is not quite as sensitive as the GPMT, it is of similar or greater sensitivity than the Buehler test. It is important to note that this comparison is only true where the guinea pig tests have been conducted to the very highest standards. In terms of predictive identification of important skin sensitizers, the LLNA is at least as sensitive as, and much more reliable than, current guinea pig tests. Of the 130 chemicals tested in one of the reference guinea pig tests, approximately 88% gave the same result in the LLNA and the guinea pig tests. An overview of this information is contained in the 2 X 2 contingency table (Table 3).

**Table 3: Comparison of LLNA and guinea pig classifications**

**Guinea Pig Classification**[a]

| LLNA Classification | | Guinea Pig Positive | Guinea Pig Negative | unclear | total |
|---|---|---|---|---|---|
| | LLNA Positive | **86** | **6** | **0** | **92** |
| | LLNA Negative | **10** | **28** | **0** | **38** |
| | total | **96** | **34** | **0** | **130** |

**table statistics for the shadowed 2 x 2 table**

| | | | |
|---|---|---|---|
| *sensitivity:* | 90% | *prevalence:* | 2.82 |
| *specificity:* | 82% | | |
| *positive predictivity:* | 93% | | |
| *negative predictivity:* | 74% | | |
| ***accuracy:*** | **88%** | | |
| ***x_:*** | **59.38** | **(p<0.001)** | |

[a]Guinea pig classifications are based on GPMT or Buehler results - some of the results are derived from non-standard GPMT guinea pig tests.

The 2 x 2 contingency table is a means to compare the *in vivo* classifications of skin sensitization of the guinea pig test with the *in vivo* predictions obtained in the LLNA. This procedure is recommended as a standard way of assessing data from validation studies (Balls *et al*, 1990). However, it is critical to point out that not all the guinea pig results are based on data generated by a standard protocol. Moreover, the guinea pig classifications are derived from both GPMT and Buehler studies. With these limitations in mind, the accuracy of the prediction of the LLNA amounts to 88%, with a sensitivity of 90% and a specificity of 82%. The test is characterized by a high positive predictivity of 93% and by a negative predicitivity of 74%. Obviously, the LLNA does an excellent job of correctly identifying chemicals that are classified as skin sensitizers in the guinea pig tests. The high $X^2$ value confirms that the classification of test chemicals by the LLNA is significant ($p<0.001$). Overall, the results given in Appendix B, Table 1, and Table 3 above, reveal a high level of concordance between the LLNA and guinea pig data in the determination of skin sensitization potential of a wide range of chemicals.

Appendix B-Table 2 lists those chemicals for which there is discord in results between the LLNA and guinea pig or human test methods. It is important to emphasize, however, that comparisons between LLNA data and the results of guinea pig tests should be viewed with caution. Guinea pig test data cannot be regarded as representing the gold standard in skin sensitization testing. Thus, for instance, it should not be concluded that the failure of the LLNA to identify as a contact allergen a chemical that is know to elicit a positive response in a guinea pig test necessarily suggests a false negative in the former method. A case in point is sulfanilic acid, a chemical that is positive in the GPMT but which fails to provoke a response in the LLNA. There is compelling evidence that sulfanilic acid fails to induce allergic contact dermatitis in humans despite extensive occupational exposure (Basketter *et al*, 1992). In contrast to the case of sulphanilic acid, ammonium thioglycolate, a well described, important, occupational contact allergen, notably among hairdressers, was positive in the LLNA, but was found not to give a significant response in the GPMT of Magnusson and Kligman. This particular chemical would be expected to test positive in a predictive assay. Thus, the LLNA result is the correct one. Ethylene glycol dimethacrylate (EGDMA) produced a positive LLNA response but was negative in guinea pig testing. Acrylate allergy is a complex subject, with many acrylate derivatives being suspected of giving rise to at least some degree of clinical disease. In the case of EGDMA, the LLNA result may be the more accurate reflection of the true importance of this substance as a potential human contact allergen, however, the clinical evidence is lacking.

Guinea pig or mouse data may not always mirror precisely and quantitatively the extent of the hazard to humans. Benzocaine, a substance selected as an OECD positive control for skin sensitization (OECD, 1993), has proven notoriously difficult to obtain reliable/reproducible positive results in either the LLNA or the GPMT (Basketter *et al*, 1993). Although it is well known as a skin sensitizer, one of its most common presentations arises from its use in puritis ani. In this situation, it is the repeated semi-occlusive exposure to inflamed mucosal tissue that renders a rather weak allergen positive. At the opposite end of the spectrum from ammonium thioglycollate, is the preservative propyl paraben. It is negative in both the LLNA and GPMT (Basketter and Scholes, 1992). This is not altogether suprising as except for behaving as a medicament allergen, notably in stasis ulcers, it is a very rare skin sensitizer, despite extensive skin exposure, e.g. from cosmetics. The consequence, is that it is unreasonable to expect a normal predictive skin sensitization test to identify this substance as an allergen. Neither nickel chloride nor nickel sulphate produced clear positive results in the standard LLNA. In contrast, and although nickel has been documented as a difficult allergen in predictive tests (Wahlberg, 1989), positive results can be obtained in the GPMT. While nickel is a common allergen, it is not a strong allergen, since it is the extensive and intimate exposure (e.g. pierced ears) which results in the high incidence of allergy. Thus, the conclusion is that the failure of the LLNA to identify nickel salts as allergens is as unsuprising as it is unimportant.

Comparison of skin sensitization data from predictive tests such as the GPMT and the LLNA with human clinical information is far from simple. Clinical data are complicated by the varying nature and extent of exposure to which individuals may have been subjected together with their individual sensitivities. Thus, it is easy to confuse a strong allergen with a common one (e.g. nickel) or to expect that the parabens esters or lanolin should be positive in predictive tests because clinicians often refer to these as allergens. In this latter case, skin allergies do arise, but most commonly in a special group of patients (stasis eczema/medicament allergy) which cause dermatologists particular problems. However, it is evident from the large list of chemicals in Appendix B, Table 1, that the LLNA is quite capable of detecting essentially all of the major human contact allergens. It is worth repeating here what has been said elsewhere about metals - that the precise mechanisms of metal allergy are probably rather different than those for organic chemical; since it is known which metals are allergens and which are not, and given that new metals are not being invented, the ability of the LLNA, or indeed any other predictive sensitization assay to detect metal allergens is rather irrelevant to the main need - the identification of new organic chemical skin sensitizers.

The data for the discordant results are reported in Appendix B-Table 3. Specifically, the disintegrations per minute (dpm) and stimulation indices (SI) are given for each concentration of test material tested. For comparison, a positive control (hexyl cinnamic aldehyde) and negative control (para-aminobenzoic acid) are listed to illustrate typical results obtained in the LLNA. For the allergen, benzocaine, one can see that the SI increase with increasing concentrations tested, but the 3-fold level is not reached and the material is classified as negative in the LLNA. In contrast, the irritant, sodium lauryl sulphate, leads to SI above the 3-fold level leading to its positive classification in the LLNA.

In relation to the mouse ear swelling test (MEST) (Gad *et al*, 1986), the LLNA offers several important animal welfare advantages, not least that unlike the MEST it does not use adjuvant. In addition, the state of validation of the MEST is quite preliminary. The data which does exist suggests that results are not wholly reliable, but clearly a great deal more work would be required to establish in detail its merits as a full replacement for the current guinea pig methods.

It is not expected, from our current knowledge of the mechanism of skin sensitization to organic chemicals, and what is known of the immunology of guinea pigs, mice and man, that the LLNA will face special problems. Little is known of the impact of interspecies differences in skin metabolism of prohaptens and its importance in predictive testing. What limited information exists has suggested that there may be species differences (Bertrand *et al*, 1997) but examination of the concordance in the identification of skin sensitizers implies that these may not be of major practical importance.

One question commonly asked about skin sensitization tests concerns their ability to discriminate allergens from irritants. This question has been posed for the LLNA (Montelius *et al*, 1995), as it has for the guinea pig maximization test (Kligman and Basketter, 1995; Buehler, 1996). In practice, all guinea pig skin sensitization tests may have such difficulties and strategies for dealing with them are available (Kligman and Basketter, 1995; Frankild *et al*, 1996). The LLNA deals well with irritancy - it is not a confounding factor for dose selection and the majority of irritants are negative in the assay. Strategies for dealing with potential false positives in the LLNA and other predictive skin sensitization tests have been reviewed recently (Basketter *et al*, 1998).

If the LLNA is determined to be an acceptable alternative, then it will enhance further what is already happening, that this assay begins to be used ever more widely as the first choice method when it is necessary to assess skin sensitization potential of an unknown chemical. The limitations of the assay are minor compared with its advantages. They comprise the inability to evaluate the elicitation response and to test for cross challenge reactions. This latter item is of some use in research, but rarely forms part of testing for regulatory purposes, which is the reason for this assay validation.

## G. DATA INTERPRETATION

In the local lymph node assay skin sensitizing activity is measured as a function of proliferative activity induced in draining lymph nodes by repeated topical exposure of mice to a test chemical. For the purposes of developing a criterion for identification of contact allergens a stimulation index of 3, relative to background cell turnover measured in concurrent vehicle treated controls, was proposed as an empirical arbiter. This value was chosen on the basis of previous experience with the local lymph node assay and an apparent high level of discrimination between contact allergens and non-sensitizing chemicals. Since that proposal was first adopted in 1990 a number of independent laboratories has gained considerably greater experience with the method and in excess of one hundred additional chemicals have been tested. The accumulated evidence reveals that the use of a stimulation index of 3 continues to provide an accurate and reliable criterion for the identification of skin sensitizing chemicals. However, as discussed in a review article published in 1992 (Kimber and Basketter, 1992), while the three-fold stimulation index provides a very useful criterion for judging sensitizing activity, in practice a dose-related increase in proliferative activity that approaches, but does not reach, a stimulation index of 3 might trigger a repeat analysis using higher concentrations and/or an alternative application vehicle (Robinson and Cruze, 1996). In this context the potential utility of a higher or lower stimulation index for the identification of sensitizing activity has been considered, but there is no evidence that this would enhance further the specificity or selectivity of the method.

Whether the draining auricular lymph nodes are excised and pooled for each experimental group or for each individual animal, a stimulation index of 3 is used as the sole criterion against which to judge skin sensitizing activity. The use of statistical analysis for classifying the skin sensitization potential of chemicals is still under investigation. This is also the case for using $EC_3$ values for determining the potency of a sensitizing chemical. Further research will be required to determine the usefulness of these approaches in LLNA testing. In the meantime, the approach is the use of the three-fold stimulation index.

In the standard local lymph node assay protocol test chemicals are evaluated using 3 application concentrations. In the vast majority of assays conventional dose responses are recorded with sensitizing chemicals such that increasing concentrations of the allergen provoke increasingly more vigorous proliferative responses. In some instances the dose response profile may be relatively flat which suggests either that saturation kinetics for absorption have been achieved or that maximal immune stimulation has been induced. In such instances where a repeat analysis is performed using lower concentrations of the test chemical then invariably a conventional dose response profile is achieved. Very rarely there may be some indication at the top concentration of an inversed dose response. In these cases the cause is either local or systemic toxicity. Again, repeat studies conducted with reduced application concentrations yield normal dose responses. The local lymph node assay is not associated normally, and certainly no more frequently than any other biological analytical system, with ambiguous dose responses.

In conclusion, the view is that the local lymph node assay should be employed as a 'stand alone' method for reaching decisions about the skin sensitizing potential of chemicals. There would be no added value in using instead a battery of methods that included, with the local lymph node assay for instance, analyses of skin penetration or identification of structural alerts using structure-activity relationships. The local lymph node assay provides a holistic mechanistically-based assessment of the ability of a test chemical to provoke the cutaneous immune response necessary for the induction of contact sensitization. If the chemical tested fails to gain access through the skin, or is unable to interact with protein to form an immunogenic hapten-macromolecular complex, then immune activation will not be initiated and sensitization will fail to develop. The current status of the LLNA and its application in regulatory toxicology has been reviewed in detail elsewhere (Basketter *et al*, 1996).

## H. DATA QUALITY

Much of the data used here to support this submission and much of the data contained within the publications cited in this document have been derived from audited Good Laboratory Practices (GLP) compliant studies. Where this is not the case all investigations have been conducted to the spirit of GLP or Good Research Practice in GLP compliant facilities. Data quality audits when conducted have been satisfactory.

It is worth emphasizing that in all collaborative studies, both national and international, all data from each of the participating units have been made available to, and have been scrutinized by, all laboratories.

There is now a long history of the local lymph node assay being used successfully in many independent laboratories for conduct of GLP compliant studies.

## I. SUPPORTING MATERIALS

The LLNA is already mentioned in detail in the main internationally accepted regulatory guideline describing test methods, namely, by the OECD (1993), where it is presented as a screening method. It is also similarly represented in EU guidelines (EC, 1996). If the result is positive, then the chemical can be defined as a contact allergen. On the basis of this OECD update to the skin sensitization test guideline, the European Commission adopted the LLNA as a screening method acceptable for the identification of skin sensitizers which in its view should be formally classified and labeled as such (European Communities, 1993). Chemicals classified would carry the R43 risk phase May cause sensitization by skin contact . However, both the OECD and EC tests state that, when the result of the LLNA is negative, it is necessary to conduct a confirmatory guinea pig test according to the standard protocol. It is important to point out that these guidelines were crafted before most of the LLNA validation work had been completed. In fact, the references cited in the OECD 406 guidelines dated from 1989 and 1990.

Recently, Dr. Peter Evans (UK-Health and Safety Executive) stated that the LLNA has been extensively and rigorously validated against both animal and human data and that the assay should be adopted by the OECD and accepted by the EU as suitable method for classification purposes for skin sensitization (Evans, 1998). In light of advancing knowledge and experience, and given animal welfare considerations, it is our opinion that the LLNA is now fully validated as a methodology for the identification of significant skin sensitizers and, therefore, should be adopted formally as an alternative skin sensitization test and incorporated fully into OECD Guideline 406.

Since the initial publication on the LLNA in 1986 by Kimber and his associates, there have been numerous publications addressing the immunological mechanisms underlying the assay as well as its use in regulatory toxicology. In Appendix A, a bibliography of 61 relevant publications is provided. These papers are related directly to the development of the LLNA for its use in assessing the skin sensitization potential of chemicals. Copies of ten selected manuscripts are included in Appendix C to permit reference to specific information supporting the validation of this assay for regulatory toxicology.

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APPENDIX B (of Original Submission): Table 1. Chemicals Tested in Local Lymph Node Asay

| Chemical | CAS number | LLNA | GPMT /BT# | HMT | MEST |
|---|---|---|---|---|---|
| Abietic acid | 514-10-3 | + | + | | |
| 2-(N-acetoxy-acetamido)fluorene | | + | | | |
| 3-Acetylphenylbenzoate | | + | + | | |
| 4-Allylanisole | 140-67-0 | + | + | | |
| 2-Aminophenol | 95-55-6 | + | +* | | |
| 3-Aminophenol | 591-275 | + | +* | | |
| Ammonium tetrachloroplatinate | 13820-41-2 | + | + | | |
| Ammonium thioglycollate | 5421-46-5 | + | - | | |
| Aniline | 62-53-3 | + | + | + | |
| Benzene-1,3,4-tricarboxylic anhydride | | + | + | | |
| 1,2-Benzisothiazolin-3-one | | + | + | | |
| Benzo[a]pyrene | 50-32-8 | + | | | |
| Benzocaine | 94-09-7 | +/-** | +/-** | + | |
| Benzoquinone | 106-51-4 | + | + | | |
| Benzoyl chloride | 98-88-4 | + | + | | |
| Benzoyl peroxide | 94-36-0 | + | + | | + |
| Benzyl bromide | 100-39-0 | + | | | |
| Beryllium sulphate | 7787-56-6 | + | + | + | |
| 1-Bromododecane | 143-15-7 | + | +* | | |
| 12-Bromododecanoic acid | 73367-80-3 | + | | | |
| 12-Bromo-1-dodecanol | 3344-77-2 | + | | | |
| 1-Bromohexadecane | 112-82-3 | + | + | | |
| 1-Bromohexane | 111-25-1 | + | +* | | |
| 3-Bromomethyl-3-dimethyldihydrofuranone | | + | + | | |
| 1-Bromopentadecane | 629-72-1 | + | | | |
| 7-Bromotetradecane | | + | | | |
| 2-Bromotetradecanoic acid | 10520-81-7 | + | | | |
| 2,3-Butanedione | 431-03-8 | + | | | |
| Butylglycidyl ether | 2426-08-6 | + | + | + | |
| $C_{12-13}$-β branched primary alcohol sulphate | | + | | | |
| $C_{16}$-1,3-alkene sultone | | + | +* | | |
| Camphorquinone | 465-29-2 | + | | | |
| Chloramine T | 10599-90-3 | + | + | | |
| 4-Chloroaniline | 106-47-8 | + | + | | |
| 2-Chloromethylfluorene | | + | | | |
| (Chloro)methylisothiazolinone | 55965-84-9 | + | + | | |
| 1-Chloromethylpyrene | 1086-00-6 | + | | | |
| 1-Chlorononane | 2473-01-0 | + | | | |
| 1-Chlorooctadecane | 3386-33-2 | + | | | |
| 1-Chlorotetradecane | 2425-54-9 | + | | | |
| Chlorpromazine | 69-09-0 | + | +* | + | |
| Cinnamic aldehyde | 104-55-2 | + | + | + | + |
| Citral | 5392-40-5 | + | + | + | |
| Clotrimazole | 23593-75-1 | + | | | |
| Cobalt chloride | 7646-79-9 | + | + | + | |
| Cocoamidopropyl betaine | 59141-98-9 | + | + | | |

**APPENDIX B (of Original Submission): Table 1. Chemicals Tested in Local Lymph Node Asay**

| Chemical | CAS number | LLNA | GPMT /BT# | HMT | MEST |
|---|---|---|---|---|---|
| Copper chloride | 7758-89-6 | + | - | | |
| Dibromodicyanobutane | | + | + | | |
| Diethyl sulphate | 64-67-5 | + | | | |
| Diethylenetriamine | 111-40-0 | + | + | + | |
| 3,4-Dihydrocoumarin | 119-84-6 | + | | | |
| Dihydroeugenol | 2785-87-7 | + | + | | |
| 3-Dimethylaminopropylamine | 109-55-7 | + | + | | |
| 7,12-Dimethylbenz[a]anthracene | 57-97-6 | + | | | |
| 5,5-Dimethyl-3-methylenedihydro-2(3H)-furanone | | + | -* | | |
| 5,5-Dimethyl-3-(thiocyanatomethyl)dihydro-2(3H) -furanone | | + | +* | | |
| Dimethyl sulphate | 77-78-1 | + | | | |
| 2,4-Dinitrochlorobenzene | 97-00-7 | + | + | | + |
| 2,4-Dinitrothiocyanobenzene | 1594-56-5 | + | + | | |
| Diphenylmethane-4-4'diisocyanate | 101-68-8 | + | + | | |
| Disodium 1,2-diheptanoyloxy-3,5-benzenedisulphonate | | + | +* | | |
| Dodecylmethanesulphonate | 51323-71-8 | + | +* | | |
| Dodecylthiosulphonate | | + | + | | |
| Ellipticine | 519-23-3 | + | | | |
| Ethylene diamine | 107-15-3 | + | + | | + |
| Ethylene glycol dimethacrylate | 97-90-5 | + | - | | |
| 1-Ethyl-3-nitro-1-nitrosoguanidine | | + | | | |
| Eugenol | 97-53-0 | + | + | | + |
| Fluorescein isothiocyanate | 25168-13-2 | + | | | |
| Formaldehyde | 50-0-0 | + | + | + | + |
| Glyoxal | 107-22-2 | + | + | + | |
| Gold chloride | 16903-35-8 | + | | + | |
| Hexadecanoyl chloride | 112-67-4 | + | | | |
| Hexyl cinnamic aldehyde | 101-86-0 | + | + | | |
| Hydroquinone | 123-31-9 | + | + | | |
| Hydroxycitronellal | 107-75-5 | + | + | + | |
| 2-Hydroxyethyl acrylate | 818-61-1 | + | + | | |
| Imidazolidinyl urea | 39236-46-9 | + | + | | |
| 1-Iodohexadecane | 544-77-4 | + | | | |
| 1-Iodononane | 4282-42-2 | + | | | |
| 1-Iodotetradecane | 192-94-1 | + | | | |
| Isoeugenol | 97-54-1 | + | + | | |
| Isopropylisoeugenol | 29653-00-7 | + | + | | |
| Isononanoyloxybenzene sulphonate | | + | + | | |
| Isophorone diisocyanate | 4098-71-9 | + | + | | |
| 2-Mercaptobenzothiazole | 149-30-4 | + | + | + | |
| Mercuric chloride | 7487-94-7 | + | + | + | |
| 2 Methoxy-4-methyl phenol | 5635-98-3 | + | + | | |
| 3-Methoxyphenylbenzoate | 5554-24-5 | + | | | |
| 4-Methylaminophenol sulphate | 55-55-0 | + | + | | |
| 3-Methylcatechol | 488-17-5 | + | | | |
| 4-Methylcatechol | 452-86-8 | + | + | | |

**APPENDIX B (of Original Submission): Table 1. Chemicals Tested in Local Lymph Node Asay**

| Chemical | CAS number | LLNA | GPMT /BT# | HMT | MEST |
|---|---|---|---|---|---|
| 3-Methylcholanthrene | 56-49-5 | + | | | |
| Methyl dodecane sulphonate | | + | + | | |
| 3-Methyleugenol | | + | | | |
| 5-Methyleugenol | | + | | | |
| 6-Methyleugenol | | + | | | |
| Methyl hexadecane sulphonate | | + | +* | | |
| 3-Methyl isoeugenol | | + | +* | | |
| Methyl methane sulphonate | 66-27-3 | + | | | |
| 1-Methyl-3-nitro-1-nitrosoguanidine | 70-25-7 | + | | | |
| Methyl(2-sulphomethyl)octadecanoate | | + | | | |
| 2-Methyl-4,5-trimethylene-4-isothiazolin-3-one | | + | + | | |
| Musk ambrette | 83-66-9 | + | - | | |
| N-Ethyl-N-nitrosourea | | + | | | |
| N-Methyl-N-nitrosourea | 684-93-5 | + | | | |
| α-Naphthoflavone | 604-59-1 | + | | | |
| β-Naphthoflavone | 6051-87-2 | + | | | |
| Neomycin sulphate | 1405-10-3 | +/- | + | | |
| 4-Nitrobenzyl bromide | 100-11-8 | + | +* | | |
| 4-Nitrobenzyl chloride | 100-14-1 | + | +* | | |
| 4-Nitroso-N,N-dimethylaniline | 138-89-6 | + | + | | |
| Nonanoyl chloride | 764-85-2 | + | | | |
| Octadecanoyl chloride | 112-76-5 | + | | | |
| Octyl gallate | 1034-01-1 | + | | | |
| Oxazolone | 15646-46-5 | + | + | | + |
| Penicillin G | 61-33-6 | + | + | + | |
| Pentachlorophenol | 87-86-5 | + | | + | |
| Phenyl benzoate | 93-99-2 | + | + | | |
| 3-Phenylenediamine | 108-45-2 | + | +* | | |
| 4-Phenylenediamine | 106-50-3 | + | + | + | + |
| Phthalic anhydride | 85-44-9 | + | + | | - |
| Picryl chloride | 88-88-0 | + | + | | - |
| Polyhexamethylene biguanide | | + | + | + | - |
| Potassium dichromate | 7778-50-9 | + | + | + | - |
| β-Propiolactone | 57-57-8 | + | | | |
| Propylgallate | 121-79-9 | + | + | | |
| 1-Propyl-3-nitro-1-nitrosoguanidine | | + | | | |
| Pyridine | 110-86-1 | + | | +/- | |
| Quinol | 123-31-9 | + | +* | | |
| Sodium benzoyloxybenzene sulphonate | | + | + | | |
| Sodium 4-(2-ethylhexyloxycarboxy)benzene sulphonate | | + | +* | | |
| Sodium 4-sulphophenyl acetate | | + | +* | | |
| Sodium benzoyloxy-2-methoxy-5-benzene sulphonate | | + | +* | | |
| Sodium lauryl sulphate | 151-21-3 | + | - | - | - |
| Sodium norbornanacetoxy-4-benzene sulphonate | | + | +* | | |
| Streptomycin | 57-92-1 | + | + | | |
| Tetrachlorosalicylanilide | 7426-07-5 | + | + | + | |
| Tetradecyl iodide | 19218-94-1 | + | | | |

APPENDIX B (of Original Submission): Table 1. Chemicals Tested in Local Lymph Node Asay

| Chemical | CAS number | LLNA | GPMT /BT# | HMT | MEST |
|---|---|---|---|---|---|
| Tetramethyl thiuram disulphide | 137-26-8 | + | +* | + | |
| 1-Thioglycerol | 96-27-5 | + | + | + | |
| Toluene diamine bismaleimide | | + | + | | |
| 2,4,5-Trichlorophenol | 95-95-4 | + | | | |
| 2,4,6-Trichloro-1,3,5-triazine | 87-90-1 | + | | | |
| α-Trimethylammonium-4-tolyloxy-4-benzene sulphonate | | + | +* | | |
| 3,5,5-Trimethylhexanoyl chloride | 36727-29-4 | + | + | | |
| Vinyl pyridine | 1337-81-1 | + | | | |
| Xylene | 1330-20-7 | + | | - | |
| Zinc sulphate | 7733-02-0 | + | | | |
| 2-Acetamidofluorene | 53-96-3 | - | | | |
| 4-Acetylphenylbenzoate | 1523-18-8 | - | | | |
| 4-Aminobenzoic acid | 150-13-0 | - | - | - | + |
| Benzalkonium chloride | 8001-54-5 | - | - | | + |
| 3-(Benzenesulphonyloxymethyl)-5,5-dimethyldihydro -2(3H)-furanone | | - | | | |
| Benzoic acid | 65-85-0 | - | - | | - |
| Benzoyloxy-3,5 benzene dicarboxylic acid | | - | +* | | |
| 1-Bromobutane | 109-65-9 | - | | | |
| Chlorobenzene | 108-90-7 | - | - | | |
| 3-(Chlorobenzenesulphonyloxymethyl)-5,5-dimethyl dihydro-2(3H)-furanone | | - | | | |
| 2-Chloroethanol | 107-07-3 | - | | | |
| Dextran | 9004-54-0 | - | - | | |
| 2,4-Dichloronitrobenzene | 611-06-3 | - | - | | |
| Di-2-furanylethanedione | 492-94-4 | - | | | |
| 5,5-Dimethyl-3-(mesyloxymethyl)dihydro-2(3H)-furanone | | - | +* | | |
| 5,5-Dimethyl-3-(methoxybenzenesulphonyloxymethyl) dihydro-2(3H)-furanone | | - | +* | | |
| 5,5-Dimethyl-3-(nitrobenzenesulphonyloxymethyl) dihydro-2(3H)-furanone | | - | +* | | |
| Dimethylisophthalate | 1459-93-4 | - | - | | |
| 5,5-Dimethyl-3-(tosyloxymethyl)dihydro-2(3H) -furanone | | - | -* | | |
| Disodium benzoyloxy-3,5-benzenedicarboxylate | | - | - | | |
| Ditallowdihydroxypropenetrimethyl ammonium | | - | - | | |
| Ethylmethanesulphonate | 62-50-0 | - | | | |
| Geraniol | 106-24-1 | - | - | - | |
| Glycerol | 56-81-5 | - | - | | - |
| Hexane | 110-54-3 | - | | - | |
| Hydrocortisone | 50-23-7 | - | | - | |
| 4-Hydroxybenzoic acid | 99-96-7 | - | - | | |
| 2-Hydroxypropylmethacrylate | 923-26-2 | - | - | | |
| Isopropanol | 67-63-0 | - | - | | |
| Kanamycin | 25389-94-0 | - | -* | + | |
| Lactic acid | 50-21-5 | - | - | | |

**APPENDIX B (of Original Submission): Table 1. Chemicals Tested in Local Lymph Node Asay**

| Chemical | CAS number | LLNA | GPMT /BT# | HMT | MEST |
|---|---|---|---|---|---|
| Lanolin | 8006-54-0 | - | - | | |
| Lead acetate | 15347-57-6 | - | | | |
| 6-Methylcoumarin | 92-48-8 | - | - | - | |
| Methyl salicylate | 119-36-8 | - | - | - | |
| N'-(4-Methylcyclohexyl)-N-(2-chloroethyl)-N-nitrosourea | | - | | | |
| Nickel chloride | 7718-54-9 | - | + | | |
| Nickel sulphate | 10101-98-1 | - | + | + | + |
| 2-Nitrofluorene | 607-57-8 | - | | | |
| Octadecylmethane sulphonate | 31081-59-1 | - | +* | | |
| Phenol | 108-95-2 | - | | - | - |
| Phthalic acid diethyl ester | | - | | | |
| Propylparaben | 94-13-3 | - | - | +/- | |
| Propylene glycol | 57-55-6 | - | - | | - |
| Resorcinol | 108-46-3 | - | - | - | |
| Salicylic acid | 69-72-7 | - | - | - | - |
| Streptozotocin | 18883-66-4 | - | | | |
| Sulphanilamide | 63-74-1 | - | - | + | |
| Sulphanilic acid | 121-57-3 | - | + | | + |
| Tartaric acid | 87-69-4 | - | -* | | |
| Tixocortol pivalate | 55560-96-8 | - | | | |
| Toluene sulphonamide formaldehyde resin | | - | - | | |
| Trimethylammonium-3-tolyl-ε-caprolactimide chloride | | - | | | |
| Tween 80 | 9005-65-6 | - | - | | - |

# Positive results based on EC classification threshold
* result obtained in a non-standard guinea pig test
** ref Benzocaine paper

**APPENDIX B (of Original Submission): Table 2. Discordant Results Between the Local Lymph Node Assay and Guinea Pig or Human Test Methods**

| Chemical | CAS number | LLNA | GPMT/ BT# | HMT |
|---|---|---|---|---|
| Ammonium thioglycollate[1] | 5421-46-5 | + | - | |
| Benzocaine[2] | 94-09-7 | +/-** | +/-** | + |
| Copper chloride[3] | 7758-89-6 | + | - | |
| 5,5-Dimethyl-3-methylenedihydro-2(3H)-furanone[4] | | + | -* | |
| Ethylene glycol dimethacrylate[5] | 97-90-5 | + | - | |
| Musk ambrette | 83-66-9 | + | - | |
| Neomycin sulphate[6] | 1405-10-3 | +/- | + | |
| Pyridine[7] | 110-86-1 | + | | +/- |
| Sodium lauryl sulphate[3] | 151-21-3 | + | - | - |
| Xylene | 1330-20-7 | + | | - |
| Benzoyloxy-3,5 benzene dicarboxylic acid[8] | | - | +* | |
| 5,5-Dimethyl-3-(mesyloxymethyl)dihydro-2(3H)-furanone[9] | | - | +* | |
| 5,5-Dimethyl-3-(methoxybenzenesulphonyloxymethyl) dihydro-2(3H)-furanone[9] | | - | +* | |
| 5,5-Dimethyl-3-(nitrobenzenesulphonyloxymethyl) dihydro-2(3H)-furanone[9] | | - | +* | |
| Kanamycin[6] | 25389-94-0 | - | -* | + |
| Nickel chloride[9] | 7718-54-9 | - | + | |
| Nickel sulphate[9] | 10101-98-1 | - | + | + |
| Octadecylmethane sulphonate[10] | 31081-59-1 | - | +* | |
| Propylparaben[11] | 94-13-3 | - | - | +/- |
| Sulphanilamide[12] | 63-74-1 | - | - | + |
| Sulphanilic acid[13] | 121-57-3 | - | + | |

# Positive results based on EC classification threshold
* result obtained in a non-standard guinea pig test
** ref Benzocaine paper

[1] Significant human contact allergen that should be positive in a predictive test.
[2] Very weak, difficult sensitiser in predictive tests that is only a human allergen under forcing exposure conditions.
[3] A false positive in the LLNA.
[4] Likely to be a true positive based on both the LLNA and structure activity considerations; the guinea pig data are from a non-standard version of the GPMT that omits the patch induction phase.
[5] Acrylate allergy is a complex subject, with many acrylate derivatives being suspected of giving rise at least to some degree of clinical disease.
[6] A well described contact allergen in medicaments, but which was much weaker than Kanamycin in a human predictive test.
[7] A very weak allergen in human predictive test (equivalent to paraben) and which is thus an unexpected positive in the LLNA.
[8] Whilst this substance was positive in the GPMT (which involves injection), its size and charge will result in extremely poor skin penetration, such that it is unlikely to cause allergic contact dermatitis. Thus, the LLNA result is likely to be the most meaningful.
[9] False negative in the LLNA.
[10] A false negative probably due to poor skin penetration engendered by the size of the compound, its very high log P and the presence of a charged group.
[11] This substance is a rare allergen except in specific disease states; it is not positive in predictive assays except the human maximization test.
[12] Unexpected negative in both the LLNA and guinea pig tests.
[13] Although a clear positive in the GPMT, this substance was negative in both the LLNA and on the basis of substantial human exposure experience, suggesting it is the LLNA result which is correct.

**APPENDIX B (of Original Submission): Table 3. Disintegrations Per Minute (DPM) Data and Stimulation Indices (SI) for Discordant Results**

| Chemical | Concentration[1] (%) | DPM | SI |
|---|---|---|---|
| **Hexyl cinnamic aldehyde** | Vehicle (AOO) | 495 | 1.0 |
| **Example of positive LLNA response** | 2.5 | 691 | 1.4 |
| | 5.0 | 1056 | 2.1 |
| | 10.0 | 1615 | 3.3 |
| | 25.0 | 4107 | 8.3 |
| | 50.0 | 6857 | 14.0 |
| | | | |
| **para-Aminobenzoic acid** | Vehicle (AOO) | 453 | 1.0 |
| **Example of negative LLNA response** | 0.5 | 399 | 0.9 |
| | 1.0 | 457 | 1.0 |
| | 2.5 | 626 | 1.4 |
| | 5.0 | 519 | 1.1 |
| | 10.0 | 452 | 1.0 |
| | | | |
| Ammonium thioglycollate | Vehicle (DMSO) | 807 | 1.0 |
| | 10.0 | 2389 | 3.0 |
| | 25.0 | 2490 | 3.1 |
| | 50.0 | 3250 | 4.0 |
| | | | |
| Benzocaine | Vehicle (DMF) | 325 | 1.0 |
| | 2.5 | 562 | 1.7 |
| | 5.0 | 574 | 1.8 |
| | 10.0 | 698 | 2.1 |
| | 25.0 | 794 | 2.4 |
| | | | |
| Copper chloride | Vehicle (DMSO) | 605 | 1.0 |
| | 1.0 | 4920 | 8.1 |
| | 2.5 | 8341 | 13.8 |
| | 5.0 | 8225 | 13.6 |
| | | | |
| 5,5-Dimethyl-3-methylenedihydro | Vehicle (AOO) | 672 | 1.0 |
| -2(3H)-furanone | 1.77 | 2022 | 3.0 |
| | 3.53 | 5002 | 7.4 |
| | 7.06 | 6213 | 9.2 |
| | | | |
| Ethylene glycol dimethacrylate | Vehicle (Acetone) | 365 | 1.0 |
| | 10.0 | 675 | 1.8 |
| | 25.0 | 1312 | 3.6 |
| | 50.0 | 4046 | 11.1 |

| | | | |
|---|---|---|---|
| Neomycin sulphate | Vehicle (DMSO) | 355 | 1.0 |
| | 25.0 | 379 | 1.1 |
| | | | |

**APPENDIX B (of Original Submission): Table 3. Disintegrations Per Minute (DPM) Data and Stimulation Indices (SI) for Discordant Results**

| Chemical | Concentration[1] (%) | DPM | SI |
|---|---|---|---|
| Pyridine | Vehicle (AOO) | 250 | 1.0 |
| | 25.0 | 274 | 1.1 |
| | 50.0 | 578 | 2.3 |
| | 100.0 | 978 | 3.9 |
| | | | |
| Sodium lauryl sulphate | Vehicle (DMF) | 369 | 1.0 |
| | 1.0 | 747 | 2.0 |
| | 2.5 | 954 | 2.6 |
| | 5.0 | 1301 | 3.5 |
| | 10.0 | 1814 | 4.9 |
| | 20.0 | 1628 | 4.4 |
| | | | |
| Xylene | Vehicle (AOO) | 382 | 1.0 |
| | 25.0 | 487 | 1.3 |
| | 50.0 | 1138 | 3.0 |
| | 100.0 | 1182 | 3.1 |
| | | | |
| Benzoyloxy-3,5 benzene dicarboxylic | Veh (Ace/Sal, 1:1) | 382 | 1.0 |
| acid | 2.5 | 346 | 0.9 |
| | 5.0 | 315 | 0.8 |
| | 10.0 | 419 | 1.1 |
| | | | |
| 5,5-Dimethyl-3-(mesyloxymethyl) | Vehicle (AOO) | 526 | 1.0 |
| -dihydro-2(3H)-furanone | 3.42 | 494 | 0.9 |
| | 6.83 | 791 | 1.5 |
| | 13.66 | 702 | 1.3 |
| | | | |
| 5,5-Dimethyl-3-(methoxybenzenesulpho | Vehicle (AOO) | 672 | 1.0 |
| -nyloxymethyl)dihydro-2(3H)-furanone | 4.84 | 802 | 1.2 |
| | 9.67 | 612 | 0.9 |
| | 19.34 | 690 | 1.0 |
| | | | |
| 5,5-Dimethyl-3-(nitrobenzenesulphonyl | Vehicle (AOO) | 657 | 1.0 |
| -oxymethyl)dihydro-2(3H)-furanone | 5.07 | 493 | 0.8 |
| | 10.13 | 490 | 0.7 |
| | 20.26 | 585 | 0.9 |
| Hydrocortisone | Vehicle (AOO) | 250 | 1.0 |
| | 2.5 | 74 | 0.3 |
| | 5.0 | 29 | 0.1 |
| | 10.0 | 16 | 0.06 |

**APPENDIX B (of Original Submission): Table 3. Disintegrations Per Minute (DPM) Data and Stimulation Indices (SI) for Discordant Results**

| Chemical | Concentration[1] (%) | DPM | SI |
|---|---|---|---|
| | | | |
| Kanamycin | Vehicle (AOO) | 382 | 1.0 |
| | 5.0 | 842 | 2.2 |
| | 10.0 | 301 | 0.8 |
| | 25.0 | 391 | 1.0 |
| | | | |
| Nickel chloride | Vehicle (DMSO) | 898 | 1.0 |
| | 1.0 | 1363 | 1.5 |
| | 2.5 | 1940 | 2.2 |
| | 5.0 | 2133 | 2.4 |
| | | | |
| Nickel sulphate | Vehicle (DMSO) | 898 | 1.0 |
| | 0.5 | 986 | 1.1 |
| | 1.0 | 1315 | 1.5 |
| | 2.5 | 1376 | 1.5 |
| | | | |
| Octadecylmethane sulphonate | Vehicle (AOO) | 510 | 1.0 |
| | 2.5 | 594 | 1.2 |
| | 5.0 | 374 | 0.7 |
| | 10.0 | 444 | 0.9 |
| | | | |
| Propylparaben | Vehicle (AOO) | 433 | 1.0 |
| | 10.0 | 595 | 1.4 |
| | 25.0 | 445 | 1.0 |
| | 50.0 | 575 | 1.3 |
| | | | |
| Sulphanilamide | Vehicle (DMF) | 416 | 1.0 |
| | 10.0 | 429 | 1.0 |
| | 25.0 | 415 | 1.0 |
| | 50.0 | 393 | 0.9 |
| | | | |
| Sulphanilic acid | Vehicle (DMSO) | 436 | 1.0 |
| | 2.5 | 667 | 1.5 |
| | 5.0 | 827 | 1.9 |
| | 10.0 | 967 | 2.2 |

[1] Abbreviations Used: DMSO = dimethylsulphoxide; DMF = dimethylformamide; AOO = acetone:olive oil (4:1); Ace/Sal = acetone:saline (1:1)

## Local Lymph Node Assay References Included in ICCVAM Submission


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9. Kimber I., Hilton J., Dearman R.J., Gerberick G.F., Ryan C.A., Basketter D.A., Lea L., House R.V., Ladics G.S., Loveless S.E. and Hastings K.L. (1998) Assessment of the skin sensitization potential of topical medicaments using the local lymph node assay: an inter-laboratory exercise. *J Toxicol. Environ. Health*. 53(7): 563-579.

10. Loveless S.E., Ladics G.S., Gerberick G.F., Ryan C.A., Basketter D.A., Scholes E.W., House R.V., Hilton J., Dearman R.J. and Kimber I. (1996) Further evaluation of the local lymph node assay in the final phase of an international collaborative trial. *Toxicology* **108**, 141-152.

## APPENDIX D

## SAMPLE PROTOCOL

## STANDARD OPERATING PROCEDURE

## THE LOCAL LYMPH NODE ASSAY (LLNA)

**STANDARD OPERATING PROCEDURE**
**METHOD:**

# THE LOCAL LYMPH NODE ASSAY (LLNA)

## 1. PRE-TEST PREPARATION

The Local Lymph Node Assay (LLNA) has been developed as an alternative method for the identification of skin sensitizing substances and measures the proliferation of lymphocytes isolated from lymph nodes draining the site of exposure in mice.

Each test is defined by a Protocol. The Protocol states the purpose of the test, test substance and concentrations to be assayed, and other details necessary to ensure that the test is conducted properly in compliance with the principles of Good Laboratory Practice (GLP).

Upon receipt of the protocol, the Test Operator plans the test, prepares test documents and requests test samples.

## 2. THE LOCAL LYMPH NODE ASSAY - TEST METHOD

### 2.1 Introduction

The LLNA determines the extent to which sensitization to a test substance has developed by measuring the proliferation of lymphocytes in the auricular lymph nodes draining the site of exposure (ears). Lymphocyte proliferation is measured by determining the incorporation of $^{3}$H-methyl thymidine ($^{3}$HTdR).

The LLNA involves treatment of laboratory mice which is performed by experienced, trained and qualified personnel. Such persons have been granted a Home Office License which permits them to carry out experiments on animals listed in this section.

This Standard Operating Procedure fully describes the LLNA. The completion of each treatment/task outlined must be recorded immediately on the appropriate sheet by signature and date (APPENDIX 1).

### 2.2 Summary of experimental design

| LLNA PROTOCOL | DAY 0 | DAY 1 | DAY 2 | DAY 3 | DAY 4 | DAY 5 | DAY 6 |
|---|---|---|---|---|---|---|---|
| 5 DAYS | T | T | T | - | - | 3H | C |

T - Topical application of test substance/vehicle.

$^{3}$H - 0hrs - Administration of 20µCi $^{3}$HTdR.
+5hrs - Excision and processing of pooled lymph nodes. Incubation of pooled LNC with TCA overnight.

C - $^{3}$HTdR incorporation into pooled LNC determined.

The method is based upon the assay developed some years ago at the Central Toxicology Laboratory, Zeneca (see References, section 2.15).

Mice, housed in groups of four, are treated by topical application of the test substance to the dorsum of each ear one time per day over three consecutive days to induce sensitization. Control mice are treated with the vehicle alone. Five days after the first topical application, the mice are injected with radiolabelled thymidine ($^{3}$H-methyl thymidine). Approximately five hours after injection, the mice are sacrificed and the draining auricular lymph nodes excised and pooled per group. Single cell suspensions of lymph node cells (LNC) are prepared from pooled lymph nodes which are subsequently washed and incubated with trichloroacetic acid (TCA) overnight. The proliferative capacity of pooled LNC is then determined by the incorporation of $^{3}$H-methyl thymidine measured on a β-scintillation counter. Each stage of the method is described below:

## 2.3 Animals

CBA/Ca strain mice, purchased from Harlan UK Ltd or Charles River UK Ltd, are housed in groups of four in cages lined with `Lignocel' animal bedding RS Grade 3/4. Diets consists of SDS PCD 3/8" SQC pellets and water ad libitum. The mice are acclimated for at least six days before initiation of a study. At the start of a study, 4 young female adults (approximately 8-12 weeks) per test group are housed according to treatment.

All clinical signs, especially at the treatment sites (ie. skin reactions), should be recorded for the animals during a study. Details concerning the care and maintenance of mice can be found in the testing facility's SOPs. Cage and bottle washing procedures can be found in the testing facility's SOPs.

More information concerning animal maintenance (including diet batch numbers) are detailed on the 'Animal Log' sheet archived separately from the Study Report.

## 2.4 Test substance

Handling and characterisation of test substances must comply with the principles established in the testing facility's GLP policy documents and SOPs. Subsample archiving is conducted by the sample processing unit of the testing facility.

The amount of sample used is recorded on the Sample Accountability form (APPENDIX 1). Details of the subsample (including date received, appearance and the subsample identification) are filled in when the sample is received from the sample processing unit. The amount of sample actually weighed out and the weight of sample + container before and after removing a sample is recorded. Significant deviations of amount of sample used from the difference in weight of sample container before and after must be noted and commented upon on the back of the form. If a sample is sent for analysis, this should also be recorded on the form. At the end of the test, record the amount of material returned to the sample processing unit and the date returned. The Sample Accountability form must be signed by the test operator and archived with the final Study Report.

## 2.5 Solvent vehicle selection and preparation

When preparing solutions, a suitable solvent vehicle is selected from the following list or according to instructions from the Study Director:

4:1 v/v Acetone/Olive oil (AOO)
Acetone
Methyl ethyl ketone (MEK)
4:1 v/v Methyl ethyl ketone/paraffin oil (MEKPO)
Dimethyl sulfoxide (DMSO)
N,N-Dimethylformamide (DMF)
Propylene glycol (PG)
Physiological saline (0.9%)
50% v/v acetone saline

The vehicles AOO, MEKPO and acetone saline are prepared as follows:

AOO - add 160ml of acetone to 40ml of olive oil.

MEKPO - add 160ml of MEK to 40ml of paraffin oil.
Acetone saline - add 100ml of acetone to 100ml of physiological saline (0.9%).

All vehicles are labelled with "Name" of contents, date of preparation, expiry date/condition, storage/handling and the name/initials of the operator who prepared it.

Where possible the following vehicles should be used (in order of preference): A00 > DMF > MEK > PG > DMSO.

## 2.6 Test solution preparation

Safety glasses and gloves must be worn during solution preparation and all procedures must be carried out in a fume cupboard where the test substance and/or vehicle is known to present an inhalation hazard.

The test substance is normally assayed at three to five consecutive concentrations from within the following range:

100%, 50%, 25%, 10%, 5%, 2.5%, 1.0%, 0.5%, 0.25%, 0.1%,

using a suitable vehicle. Test concentrations are primarily based upon previous experience in guinea pig tests, structure analysis and solubility factors. In the event of no such support data optimal test concentrations will be prepared based upon the solubility of the test substance in the vehicle.

Solids and liquids are weighed and solutions prepared on a weight upto (-->) a volume basis (this must be specified in the record of solution preparation as w/v). 0.2ml graduated stoppered 10ml measuring cylinders, stoppered 5ml/10ml volumetric flasks and disposable 1.0ml syringes are used in the preparation of solutions. Such measuring cylinders/volumetric flasks are deemed sufficiently accurate for solution preparation . 1.0ml syringes are also sufficiently accurate for solution preparation. Details of solution preparation are recorded in the data sheets for the particular study and archived with the Study Report (APPENDIX 1).

Substances of low solubility can be mixed using a mechanical agitator or using a magnetic stirrer. Heat above $38^{o}$C is not used unless the substance is known to be heat stable.

## 2.7 Topical application

Gloves must be worn during this operation.

Each group of mice are treated by topical application to the dorsal surface of each ear with a different selected concentration of the test substance. A further group of mice is treated with the vehicle alone. The application volume, 25µl, is administered using a 0-50µl positive displacement pipette and is spread over the entire dorsal surface of the ear. For treatment, one mouse is removed from the home cage, treated and placed in an empty cage. When all mice from that group have been treated they are returned to the home cage. Topical application is performed once daily over three consecutive days. After the final topical application each group of mice are transferred into plastic disposable cages.

After treatments excess sample or the empty container is returned to the sample processing unit. Excess solutions, in small quantities, can normally be emptied down the drains using plenty of cold water. Hazardous solutions, however, must be returned to the sample processing unit for correct disposal.

## 2.8 Working with radiation

All work with radionuclides is conducted in a room which is a designated area approved by the test facility’s Radiation Safety Office. The workstation has a 'Designated Workstation Log' in which details of the work undertaken and monitoring data is recorded.

Only suitably trained and approved staff will be allowed to work with unsealed radioactive sources.

Bench surfaces where radionuclides are handled are lined with absorbent plastic-lined paper, such as 'Benchkote' and plastic 'lipped' trays are used to confine contamination in the event of spills. Personal protection must be used when handling radionuclides, these include a labcoat, plastic gloves and safety glasses.

2.9 Preparation of $^{3}$H-methyl thymidine

The radionuclide $^{3}$H-methyl thymidine ($^{3}$HTdR) is used in the LLNA. $^{3}$HTdR is purchased from Amersham International, catalogue Code No. TRA.310 (specific activity, 2.0Ci/mMol; concentration 1.0mCi/ml). 'Radiochemical Batch Analysis' sheets received with each batch of 3HTdR are recorded separately from the Study Report.

The $^{3}$HTdR is diluted to a working concentration of 80µCi/ml on a volume to volume basis using sterile phosphate buffered saline (PBS). $^{3}$HTdR is prepared in sterile 30ml disposable `Universal' containers and is prepared fresh prior to the study. A disposable B-D plastipak 1ml syringe + 26G $^{3}$/8" hypodermic needle and disposable B-D plastipak 1ml/10ml/30ml syringes + 0.2mm micropore filter are used for the measurement of volumes of $^{3}$HTdR and PBS respectively.

The concentration of 80mCi/ml of $^{3}$HTdR is confirmed by removing a 80µl aliquot, diluting to 200ml with tap water and 'counting' two 1ml aliquots of this dilution in a β-Scintillation Counter after adding 10mls of 'Optiphase-mp' scintillant.

Details of $^{3}$HTdR preparation and confirmation of the concentration are recorded in the data sheets for the particular Study and archived with the Study Report (APPENDIX 1, Section 3). Further details concerning $^{3}$HTdR preparation and use are also detailed on `Radioactive Log' sheets archived separately from the Study Report.

2.10 Incorporation of $^{3}$H-methyl thymidine in vivo

Five days after the first topical application treatment, all mice are administered $^{3}$H-methyl thymidine ($^{3}$HTdR). Several minutes prior to $^{3}$HTdR administration mouse tail veins are visualised by placing the mice in a warm air environment. This is achieved using a 'Thermacage' (Beta medical and Scientific; Datesand Ltd) which consists of four separate compartments each fitted with a lid, catch and vent control enabling temperature adjustment of each chamber. 20µCi $^{3}$HTdR is administered per mouse by injecting intravenously via tail vein with 250µl of 80µCi/ml $^{3}$HTdR using B-D Plastipak 1.0ml disposable syringes + 26G $^{3}/_{8}$" hypodermic needles. 1.0ml disposable syringes are deemed sufficiently accurate for the measurement of volumes in the range 0.2-1.0ml.

2.11 Preparation of single cell suspensions

Approximately five hours after $^{3}$HTdR injection all mice are sacrificed by carbon dioxide asphyxiation, the draining auricular lymph nodes rapidly excised and pooled for each experimental group (8 nodes per group). Pooled lymph nodes are collected into 7ml disposable bijou bottles containing 1.0ml of phosphate buffered saline (PBS). A single cell suspension (SCS) of pooled lymph node cells (LNC) is prepared and collected into the base of a 90mm plastic Petri dish by gentle mechanical disaggregation of pooled lymph nodes through stainless steel gauze (200 mesh size) using the plunger of a B-D `Discardit' 5.0ml disposable syringe (catalogue code no. 309050). The gauze is washed with 4-5mls of PBS into the base of the Petri dish, and the SCS transferred into a 10ml graduated plastic round-bottomed Sarstedt centrifuge tube. The SCS is finally made up to 10 mls with 4-5mls of PBS used to rinse the Petri dish. This procedure is repeated for each group of pooled lymph nodes.

Pooled LNC are pelleted with a relative centrifugal force (RCF) of 190 x g (RCF calculated to bottom of centrifuge tube) for 10 minutes in a centrifuge set at 4$^{o}$C. After centrifugation each supernatant is removed by aspiration using disposable plastic pipettes leaving 1-2mls of supernatant above each pellet. Each pellet is gently agitated before making up to 10mls with PBS and resuspending the LNC. This washing procedure is repeated twice.

2.12 Determination of incorporated $^{3}$H-methyl thymidine

Safety glasses and gloves must be worn when handling TCA and 'Optiphase mp' scintillation fluid.

After the final wash each supernatant is removed leaving just a small volume (<0.5ml) of supernatant above each pellet. Each pellet is gently agitated before resuspending the LNC in 3mls of 5% TCA for precipitation of macromolecules. After incubation with 5% TCA at +4$^{o}$C overnight, each precipitate is recovered by centrifugation at 190 x g for 10 minutes, removing each supernatant and resuspending in 1ml of 5% TCA. Each precipitate is transferred to a 25ml glass scintillation vial with 10mls of 'Optiphase mp' scintillation liquid and thoroughly mixed. The vials are loaded into a β-scintillation counter, and after approximately 30 minutes $^{3}$HTdR incorporation is measured. The β-counter expresses $^{3}$HTdR incorporation as the number of radioactive disintegrations per minute (DPM), the results of which are produced on a printout. Similarly, background $^{3}$HTdR levels are also measured in two 1ml aliquots of 5% TCA.

## 2.13 Radioactive contamination monitoring

After completing an otherwise uneventful work routine the workplace must be thoroughly monitored. Such monitoring must be carried out regardless of the level of activity at which the work is done. Monitoring data is recorded in the 'Designated Workstation Log' and on 'Radioactive Monitoring Swabs' sheets which are archived separately from the Study Report. If necessary these will be made available to the Radiation Safety Officer. If contamination has been detected then the area contaminated must be decontaminated immediately using a suitable detergent such as 'Decon 90'.
In addition personal exposure to $^{3}$HTdR is monitored by monthly urine analysis.
Prompt whole body examination will be compulsory for staff who have been exposed to radionuclides as a result of accidents and major spillages.

Accidental contamination of personnel and equipment must be immediately reported to the local Radiation Safety Officer and medical department. Decontamination measures must be undertaken without delay. Contaminated protective clothing may be laundered in a 'Hot Lab' and personal contamination must be reduced by washing and scrubbing. Success of decontamination measures must be assessed by monitoring.

## 2.14 Disposal of radioactive waste

All contaminated solid waste from each experiment including animal carcasses is placed in biohazard plastic bags lined with plastic bin liners, sealed, labeled `Radioactive material' and sent for incineration. If radioactive carcasses cannot be incinerated immediately then they must be placed in double plastic bags and frozen until it is convenient to do so.

Contaminated liquid waste is temporarily stored in a 2.5 litre impact resistant bottle and the contents sent for incineration when full.

Contaminated waste should not be allowed to accumulate and should be sent for incineration as soon as practically possible.

The quantity of radioactivity present within the waste is recorded on the 'Radioactive Log' sheet and archived separately from the Study Report. The quantity of radioactivity incinerated each week is submitted to the Radiation Safety Officer.

## 2.15 References

Kimber, I. and Weisenberger, C. 1989. A modified murine local lymph node assay for the identification of contact allergens. In "Current Topics in Contact Dermatitis". pp 592-595. Eds. Frosch, P.J. et al., Springer-Verlag Berlin Heidelberg.

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## 3. RESULTS

### 3.1 Interpretation/treatment of results

The proliferative response of lymph node cells (LNC) is expressed as the number of radioactive disintegrations per minute per lymph node (DPM/NODE) and as the ratio of $^3$HTdR incorporation into LNC of test lymph nodes relative to that recorded for control lymph nodes (TEST/CONTROL RATIO). Before DPM/NODE values are determined, background $^3$HTdR is subtracted from test and control raw DPM data.

A substance is regarded as a sensitizer in the LLNA if at least one concentration of the test substance results in a 3-fold or greater increase in $^3$HTdR incorporation into LNC of test lymph nodes relative to that recorded for control lymph nodes, as indicated by the TEST/CONTROL RATIO. The data should also not be incompatible with a biological dose response, although allowance must be made, especially at high topical application concentrations, for either local toxicity or immunological suppression.

3.2 Example

Raw data: Background $^3$HTdR in two 1ml TCA samples - 90 DPM
100 DPM

$^3$HTdR incorporation into LNC of 8 control lymph nodes - 3,000 DPM
$^3$HTdR incorporation into LNC of 8 test lymph nodes - 21,000 DPM

Derived data: Mean background $^3$HTdR $= \dfrac{90\ \text{DPM} + 100\ \text{DPM}}{2}$

= **95 DPM**

Control DPM/NODE $= \dfrac{3000\ \text{DPM} - 95\ \text{DPM}}{8\ \text{NODES}}$

= **363 DPM/NODE**

Test DPM/NODE $= \dfrac{21{,}000\ \text{DPM} - 95\ \text{DPM}}{8\ \text{NODES}}$

= **2613 DPM/NODE**

TEST/CONTROL RATIO $= \dfrac{2613\ \text{DPM/NODE}}{363\ \text{DPM/NODE}}$

= **7.2**

Since the TEST/CONTROL RATIO is greater than 3, the test substance fulfils the criteria to be classified as a sensitizer in the LLNA. If the TEST/CONTROL RATIO is less than 3, the test substance fails to fulfil the criteria to be classified as a sensitizer in the LLNA.

## 4. EQUIPMENT DETAILS

Refer to the appropriate test facility SOPs; for instruction guides, calibration and maintenance care for the equipment. Calibration and Service records associated with the studies are archived independent to studies annually. Refer to the SOP for instruction guides, calibration and maintenance care for use of β-scintillation counters.

## 5. DATA HANDLING

The recording and handling of data must comply with the principles established in the GLP policy document of the testing facility and any applicable SOPs.

Data is transferred from the data sheets to produce a Study Report. All original data, Protocols and data sheets must be retained and archived with the Study Report as a Study Package.

Archiving procedures are described in the testing facility's SOPs. Study Packages should be archived within 6 months of completion of the Study. Other supporting data which is not included in the Study Package (calibration/maintenance and animal room day books, animal and radioactive logs) are archived annually.

APPENDIX 1

Local Lymph Node Assay Data Test Sheets

Sample Accountability

Solution Preparation

Reagent Preparation

Background and Control Raw Data

Test Raw Data

Expression and Interpretation of Results

Mouse Maintenance, Treatment and Task Record

THE LOCAL LYMPH NODE ASSAY - STUDY NUMBER .

Sample Accountability

Test substance :
Sub-sample ref. no. :
Appearance of sub-sample :
Active ingredient level :

Date sample received:

| Procedure | Wt. Sample + Container | | Amount Used | Operator | Date |
|---|---|---|---|---|---|
| | Before | After | | | |
| Initial Weight | | | | | |
| To Archive | | | | | |
| To Analytical | | | | | |
| Solvent Determination | | | | | |
| Topical Application | | | | | |
| Returned to Sample Processing | | | | | |

Comments:

THE LOCAL LYMPH NODE ASSAY - STUDY NUMBER        .

Table

Test substance :
Sample ref. no. :
Active ingredient level :
Storage :
Handling :

1. Description of test solutions and preparation

Solvent vehicle:

| Test Conc. (%) | Preparation | Description | Operator | Date |
|---|---|---|---|---|
| | | | | |
| | | | | |
| | | | | |
| | | | | |
| | | | | |

2. Method of test solution preparation

| Test Conc. (%) | Method of Preparation | Storage Conditions | Other Comments |
|---|---|---|---|
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |

THE LOCAL LYMPH NODE ASSAY - STUDY NUMBER .

Reagent Preparation

(i) Phosphate buffered saline (PBS) - 1 sachet of PBS powder ------> 1000ml distilled water. Stored at + 4$^{o}$C. Prepared

(ii) Trichloroacetic acid (TCA) - 7.5g TCA ------> 150ml tap water. Stored at + 4$^{o}$C. Prepared

(iii) $^{3}$H-methyl thymidine ($^{3}$HTdR), specific activity 2.0Ci/mMol (Concentration 1.0mCi/ml). Stored at + 4$^{o}$C. `**Radiochemical Batch Analysis'** sheets received with each batch of $^{3}$HTdR are recorded separately from this study. 80µCi/ml activity $^{3}$HTdR was prepared as follows:

| $^{3}$HTdR Code No. | In-Use Activity | Preparation | Operator | Date |
|---|---|---|---|---|
| | 80µCi/ml* | ml of 1mCi/ml $^{3}$HTdR + ml of sterile PBS. | | |

* Dilution activity of $^{3}$HTdR confirmed by removing a 80µl aliquot, diluting to 200ml with tap water and removing two 1ml aliquots (0.032 µCi) and counting these on the β-scintillation counter:

β-Counter printout inserted here

Mean Count : DPM

Since 1.0µCi = 2220000 DPM (37000 Bq)
then 0.032µCi = 71040 DPM.

Therefore DPM = $\frac{\text{\_\_\_\_ DPM}}{\text{71040 DPM}}$ x 80µCi/ml

= ____**µCi/ml**

More information concerning 3HTdR preparation, use, disposal and monitoring during this study are detailed on the `**Radioactive Log'** and `**Radioactive Monitoring Swabs'** sheets recorded separately from this study.

Signed : Date:

THE LOCAL LYMPH NODE ASSAY - STUDY NUMBER     .

Table

Background and control raw data retrieved from the β-scintillation counter

Results:

(i) Background and Control results

Background $^3$HTdR in two 1ml TCA samples was determined and $^3$HTdR incorporation into Control LNC determined  days after the first vehicle topical application.

β-Counter printout inserted here

| Rack/Sample Position | Sample Description | No. Lymph Nodes | Sample DPM |
|---|---|---|---|
| | | | |
| | | | |
| | | | |
| | | | |

Mean background count: DPM

Signed : Date :

THE LOCAL LYMPH NODE ASSAY - STUDY NUMBER ______
INDIVIDUAL ANIMAL LYMPH NODES

Table

Control raw data retrieved from the β-scintillation counter

Results:

(i) Control results

$^{3}$HTdR incorporation into Control LNC determined days after the first vehicle topical application.

β-Counter printout inserted here

| Rack/Sample Position | Sample Description | Control Group Animal No. | Sample DPM |
|---|---|---|---|
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |

Mean background count: DPM

Signed : Date :

THE LOCAL LYMPH NODE ASSAY - STUDY NUMBER .

Table

Test Raw data retrieved from b-Scintillation Counter

Test substance :
Sample ref. no. :

$^3$HTdR incorporation into test LNC determined days after the first test substance topical application.

Results

(ii) Test results

β-Counter printout inserted here

| Rack/Sample Position | Sample Description | No. Lymph Nodes | Sample DPM |
|---|---|---|---|
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Signed : Date :

THE LOCAL LYMPH NODE ASSAY - STUDY NUMBER .

Table

Test Raw data retrieved from b-Scintillation Counter

Test substance :
Sample ref. no. :

$^3$HTdR incorporation into test LNC determined days after the first test substance topical application.

Results

(ii) Test results

β-Counter printout inserted here

| Rack/Sample Position | Sample Description | No. Lymph Nodes | Sample DPM |
|---|---|---|---|
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Signed : Date :

THE LOCAL LYMPH NODE ASSAY - STUDY NUMBER .

Table

Expression and Interpretation of results

Test Substance :
Sample ref. no. :
Exposure period (days) :

The proliferative response of lymph node cells (LNC) is expressed as the number of radioactive disintegrations per minute per lymph node (DPM/NODE) and as the ratio of $^{3}$HTdR incorporation into LNC of test nodes relative to that recorded for control nodes (TEST/CONTROL RATIO). The test substance can be regarded as `a sensitizer' if at least one test concentration produces a test/control ratio equal to or greater than 3.0. The data must also be compatible with a biological dose response, although allowance must be made, especially at high topical application concentrations, for local toxicity and/or immunological suppression. Where the data does not fulfill these criteria, the test substance can be regarded as `unlikely to be a strong sensitizer'.

Background count : DPM

| Sample Description | Sample DPM - B'grd DPM | No. Lymph Nodes | DPM/NODE | TEST/CONTROL RATIO | +/- |
|---|---|---|---|---|---|
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Biological dose response - Yes/No.

Comments:

Signed : Date :

## THE LOCAL LYMPH NODE ASSAY - STUDY NUMBER .

Mouse maintenance, treatment record and task sheet

Strain : CBA/Ca.
Sex : Female.
Age :
Source :.
Diet :
Water : Ad libitum.
Housing : Experimental groups of 4 mice housed in plastic disposable cages.

Test substance :
Sample ref. no. :

| Animal Group | Topical Application | | | Admin. of $^3$HTdR | | Mice Killed | | Processing of Nodes | Samples Counted |
|---|---|---|---|---|---|---|---|---|---|
| | Day 0 | Day 1 | Day 2 | | No. Mice Inj. | | No. Nodes Excised | | |
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| | | | | | | | | | |
| Operator | | | | | | | | | |
| Date | | | | | | | | | |

More information concerning animal maintenance (including diet batch numbers) are detailed on the `**Animal Log'** sheet recorded separately from this study.

Comments:

THE LOCAL LYMPH NODE ASSAY - STUDY NUMBER
INDIVIDUAL ANIMAL LYMPH NODES

Table

Expression and Interpretation of results

Test Substance
Sample ref. no.
Exposure period (days)

The proliferative response of lymph node cells (LNC) is expressed as the number of radioactive disintegrations per minute per individual animal (DPM), the test or control group mean DPM and as the ratio of $^3$HTdR incorporation into LNC of test nodes relative to that recorded for control nodes (TEST/CONTROL RATIO). The test substance can be regarded as 'a sensitizer' if at least one test concentration produces a test/control ratio equal to or greater than 3.0. The data must also be compatible with a biological dose response, although allowance must be made, especially at high topical application concentrations, for local toxicity and/or immunological suppression. Where the data does not fulfill these criteria, the test substance can be regarded as 'unlikely to be a strong sensitizer'.

| Sample Description<br>Test or control Group | Group Mean<br>DPM | Group Mean<br>Standard Error | TEST/CONTROL<br>RATIO | +/- |
|---|---|---|---|---|
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Biological dose response - Yes/No.

Comments:

Signed :

Date :

# Evaluation Guidance to the Peer Review Panel

## A. Instructions for Peer Review Panel Members

The Peer Review Panel was charged with developing a consensus on the usefulness of the proposed LLNA test method (appendix D) as an alternative for the currently accepted guinea pig assay. In reaching this determination, the panel was asked to evaluate all of the available information in the submission in accordance with the published criteria for validation and acceptance of toxicological test methods (NIEHS, 1997). The Peer Review Panel was charged with preparing a written report that summarized the extent to which each of these criteria were addressed, and that addressed the acceptability of this method as a substitute for the guinea pig assay.

An outline of the major items addressed in the Peer Review Panel report is provided below in "B. Points for Evaluation." Specific questions and considerations were added by the Interagency Immunotoxicity Working Group to ensure that the assessment provided adequate information to facilitate agency decisions on the regulatory acceptability of the method.

One primary and at least two secondary reviewers were designated for each section by the NIEHS Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) in consultation with the Peer Review Panel Chair. These individuals were requested to prepare draft written responses for their assigned sections. All reviewers were encouraged to familiarize themselves with the entire set of questions and to comment on any or all sections. All reviewers were asked to complete the summary conclusions section.

In conducting this review, the primary focus of the Peer Review Panel was to evaluate the information supporting the usefulness of the proposed LLNA Test Method Protocol (LLNA ICCVAM Submission). Based on the information provided in the Submission, the panel was asked to determine if the LLNA is an acceptable alternative to standard guinea pig assays for identifying human contact allergens. Two overall questions that they were asked to address were:

***Has the LLNA been evaluated sufficiently and is its performance satisfactory to support its adoption as a stand-alone alternative?***

***Does the LLNA offer advantages with respect to animal welfare considerations (refinement, reduction, and replacement)?***

The focus of the Peer Review Panel evaluation was on the utility of the LLNA, as described in the proposed Test Method Protocol, for detecting possible human contact allergens. The Panel was made aware that modifications to the proposed LLNA protocol have been made or were under development (*e.g.*, ex-vivo use of radiolabeled thymidine, use of nonradioactive methods) which were outside the scope of this evaluation. However, the Panel was asked to submit suggestions for future evaluations or workshops to review proposed test method revisions.

## B. Points for Evaluation

### 1. Summary Conclusions

Based on the information provided:

a. Compared with current methods [e.g., the guinea pig maximization test (GPMT)], could this method be used to provide equivalent or better prediction of human allergic contact dermatitis?

b. Does the LLNA adequately identify the <u>lack of potential</u> of chemicals to induce human allergic contact dermatitis? If applicable, specify those circumstances (e.g., specific chemicals/chemical classes) where the LLNA, or test results from the LLNA, would be considered either (i) inadequate or (ii) equal to or better than current methods for concluding that the test article <u>is not</u> a contact sensitizer.

c. Does the LLNA adequately identify <u>the potential</u> of chemicals to induce human allergic contact dermatitis? If applicable, specify those circumstances (e.g., specific chemicals/chemical classes) where the LLNA, or test results from the LLNA, would be considered either (i) inadequate or (ii) equal to or better than current methods for concluding that the test article <u>is</u> a contact sensitizer.

d. Discuss conditions/limitations/restrictions that may affect the intended use of the LLNA, and that are justified based upon the presence or lack of scientific evidence.

e. Discuss advantages of the proposed LLNA, as compared to the standard guinea pig methods.

f. Has there been adequate consideration and appropriate incorporation of animal use refinement, reduction, and replacement alternatives? Will the LLNA reduce the number of animals required or refine the procedure to eliminate pain or distress compared with the reference tests?

### 2. Test Method Description (see Appendix D, LLNA Protocol)

a. Are the test method and protocol described in sufficient detail, including the scientific and mechanistic basis of the test, range of applications, endpoints, numbers of replicates, need for dose-response curves, and acceptable variations in the protocol?

   1) Is the protocol used to generate the supporting submission data in agreement with the proposed protocol (Section II. D.)? If not, discuss the adequacy of the rationale provided for changes incorporated in the proposed protocol.

   2) Evaluate the appropriateness of the dose selection procedure. Discuss the need for determination of dermal irritation (e.g., as done for the guinea pig test) or acute toxicity data prior to conducting the actual test.

3) Evaluate the appropriateness of the number of dose groups recommended as necessary for an adequate study.

b. Comment on the adequacy and completeness of the test method protocol, including:

1) Description of the material and equipment needed to conduct the test. Is the number of mice per dose group appropriate? Is the age range appropriate? Is the designated gender and strain appropriate?

2) Description of what is measured and how it is used.

3) Description of data analysis, evaluation, and decision criteria (*i.e.*, a >3-fold stimulation factor) used to identify substances as: 1) a positive skin sensitizer, and 2) a negative skin sensitizer.

c. Are there appropriate provisions for the use of positive, negative, and irritation control chemicals?

d. Discuss the role of a dose response relationship in interpreting the results of this assay.

e. What are the strengths and/or limitations of the LLNA and are they described adequately, including the usefulness for testing mixtures, extracts, and metals?

f. Are there editorial/technical corrections necessary for the proposed protocol?

## 3. Test Method Data Quality

Is there evidence of sufficient quality assurance/quality control [i.e., were experiments conducted and data collected and maintained in accordance with Good Laboratory Practice (GLP) standards and procedures; in the “spirit” of GLPs (e.g., GLP standards without audits)]? If not, is there clear indication from the technical data that there was adequate record-keeping or data collection.

a. Is there an assurance provided that indicates there was adherence to the protocol during the validation studies? Are deviations from the standard protocol clearly described and justified?

b. If changes were made to the test method protocol during the validation studies, is the rationale for the changes provided, are data clearly identified to indicate which protocol was used, and are the potential impact of these changes on evaluation of the test method presented?

c. Was a data audit conducted by a Quality Assurance Unit? If so, is the data quality satisfactory based on the audit results (e.g., adequate adherence to protocols, record-keeping following GLPs)?

## 4. Test Method Performance

a. Are the data provided in sufficient detail for you to evaluate the results and conclusions obtained with the LLNA?

b. Comment on the adequacy of the methods used to evaluate the performance of the test method. Are results of the LLNA and the reference test(s) compared and evaluated appropriately?

c. Comment on the adequacy of the numbers of chemicals/products selected to evaluate the performance (end result) of the method for each chemical/product class. Are there limitations in application of this assay to specific chemical/product classes?

d. Are sufficient data provided to adequately evaluate the performance of the method for its proposed use?

e. Comment on the sensitivity, specificity, concordance, false positive rate, and false negative rates for the chemical/product classes that the method is proposed to be used for.

   1) To what extent does the method correctly predict negative effects for some or all chemicals/products?

   2) To what extent does the method correctly predict positive effects correctly for some or all classes? Does it consistently over or under predict toxicity compared with the current test method?

f. Are the sensitivity, specificity, concordance, and false positive and negative rates acceptable for the chemical/product classes tested?

g. Are the conclusions on the usefulness of this method scientifically sound?

   1) Are results of the LLNA clinically relevant and is the test predictive for human contact allergens?

   2) Is the utility of the method clearly established for regulatory use in hazard assessment of chemicals as potential contact sensitizers?

## 5. Determination of Test Method Reliability (Repeatability/Reproducibility)

Are intra- and inter-laboratory reproducibility adequately evaluated?

a. Comment on the adequacy of the evaluation of <u>intralaboratory</u> repeatability and reproducibility of the test method, and the data used to define and describe the level of intralaboratory variability.

b. Comment on the adequacy of the evaluation of <u>interlaboratory</u> reproducibility of the test method, and the data used to define and describe the level of interlaboratory variation.

   1) Consider the range of vehicle control data within and across laboratories in the validation studies. Do these differences affect data quality

(reproducibility, sensitivity, etc)?

c. Was the reproducibility of the test method evaluated on a series of appropriate reference chemicals or products, and do these adequately represent the types of substances for which the test method is proposed to be used?

d. Are the results obtained with the LLNA sufficiently repeatable and reproducible?

e. Comment on the reproducibility and reliability of the LLNA as compared to standard guinea pig assays.

### 6. Other Scientific Reviews

Comment on and compare the conclusions published in independent peer-reviewed reports or other independent scientific reviews of the test method, compared to the conclusions reached in this report, and comment on any other ongoing evaluations of this method.

### 7. Other Considerations

a. Can the test method be readily transferred among properly equipped and staffed laboratories; that is:

1) Is it relatively insensitive to minor changes in protocol (e.g., the acceptable temperature range for reagents and for the location where the test will be conducted)?

2) Are the level of training and expertise required to conduct the test reasonable?

3) Are the necessary equipment and supplies relatively easy to obtain?

b. Is the method cost-effective, relative to the cost of conducting the currently accepted test methods for hypersensitivity?

c. Is the time needed to conduct the test reasonable?

d. Is there any other information that should be added to the report, published or unpublished?

e. Has there been adequate consideration and appropriate incorporation of animal use refinement, reduction, and replacement alternatives? Will the LLNA reduce the number of animals required or refine the procedure to reduce or eliminate pain or distress compared with the reference tests?

## C. Related Issues

1. Although this evaluation is for a specific LLNA protocol proposed as an alternative for currently used guinea pig tests, what other endpoints or test methods would you like to see evaluated by ICCVAM in the future?

2. Are there ideas for potential workshops and validation efforts that you think that ICCVAM or others should support in this area of contact hypersensitivity?

## Reference:

NIEHS (National Institute of Environmental Health Sciences). 1997. Validation and regulatory acceptence of toxicological test methods: A report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods. NIH Publication No. 97-3981. NIEHS, Research Triangle Park, NC.

# ICCVAM Validation and Regulatory Acceptance Criteria

## Validation Criteria[1]

For a new or revised test method to be considered validated for regulatory risk assessment purposes, it should generally meet the following criteria (the extent to which these criteria are met will vary with the method and its proposed use). However, there needs to be flexibility in assessing a method given its purpose and the supporting database. Because tests can be designed and used for different purposes by different organizations and for different categories of substances, the determination of whether a specific test method is considered by an agency to be useful for a specific purpose must be made on a case-by-case basis. Validation of a test method is a prerequisite for it to be considered for regulatory acceptance.

- The scientific and regulatory rationale for the test method, including a clear statement of its proposed use, should be available.

- The relationship of the test method's endpoint(s) to the biologic effect of interest must be described. Although the relationship may be mechanistic or correlative, tests with biologic relevance to the toxic process being evaluated are preferred.

- A detailed protocol for the test method must be available and should include a description of the materials needed, a description of what is measured and how it is measured, acceptable test performance criteria (e.g., positive and negative control responses), a description of how data will be analyzed, a list of the species for which the test results are applicable, and a description of the known limitations of the test including a description of the classes of materials that the test can and cannot accurately assess.

- The extent of within-test variability, and the reproducibility of the test within and among laboratories must have been demonstrated. Data must be provided describing the level of intra- and interlaboratory reproducibility and how it varies over time. The degree to which biological variability affects this test reproducibility should be addressed.

- The test method's performance must have been demonstrated using reference chemicals or test agents representative of the types of substances to which the test method will be applied, and should include both known positive and known negative agents. Unless it is hazardous to do so, chemicals or test agents should be tested under code to exclude bias.

- Sufficient data should be provided to permit a comparison of the performance of a proposed substitute test with that of the test it is designed to replace. Performance should be evaluated in relation to existing relevant toxicity testing data, and relevant toxicity information from the species of concern. Reference data from the comparable traditional test method should be available and of acceptable quality.

- The limitations of the method must be described; for example, in vitro or other non-animal test methods may not replicate all of the metabolic processes relevant to chemical toxicity that occur in vivo.

[1]From: National Institute of Environmental Health Sciences (NIEHS). Validation and Regulatory Acceptance of Toxicological Test Methods: A report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). NIH Publication No. 97-3981, NIEHS, Research Triangle Park, NC, USA; 1997

- Ideally, all data supporting the validity of a test method should be obtained and reported in accordance with Good Laboratory Practices (GLPs). Aspects of data collection not performed according to GLPs must be fully described, along with their potential impact.

- All data supporting the assessment of the validity of the test method must be available for review.

- Detailed protocols should be readily available and in the public domain.

- The method(s) and results should be published or submitted for publication in an independent, peer-reviewed publication.

- The methodology and results should have been subjected to independent scientific review

**Regulatory Acceptance Criteria**[1]

Validated methods are not automatically accepted by regulatory agencies; they need to fit into the regulatory structure. Flexibility is essential in determining the acceptability of methods to ensure that appropriate scientific information is considered in regulatory risk assessment. A test method proposed for regulatory acceptance generally should be supported by the following attributes:

- The method should have undergone independent scientific peer review by disinterested persons who are experts in the field, knowledgeable in the method, and financially unencumbered by the outcome of the evaluation.

- There should be a detailed protocol with standard operating procedures (SOPs), a list of operating characteristics, and criteria for judging test performance and results.

- Data generated by the method should adequately measure or predict the endpoint of interest and demonstrate a linkage between either the new test and an existing test, or the new test and effects in the target species.

- There should be adequate test data for chemicals and products representative of those administered by the regulatory program or agency and for which the test is proposed.

- The method should generate data useful for risk assessment purposes, i.e., for hazard identification, dose-response assessment, and/or exposure assessment. Such methods may be useful alone or as part of a battery or tiered approach.

- The specific strengths and limitations of the test must be clearly identified and described.

- The test method must be robust (relatively insensitive to minor changes in protocol) and transferable among properly equipped and staffed laboratories.

- The method should be time and cost effective.

- The method should be one that can be harmonized with similar testing requirements of other agencies and international groups.

- The method should be suitable for international acceptance.

- The method must provide adequate consideration for the reduction, refinement, and replacement of animal use.

[1]From: National Institute of Environmental Health Sciences (NIEHS). Validation and Regulatory Acceptance of Toxicological Test Methods: A report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). NIH Publication No. 97-3981, NIEHS, Research Triangle Park, NC, USA; 1997.

## DEPARTMENT OF HEALTH AND HUMAN SERVICES

### Public Health Service

### National Institute of Environmental Health Sciences (NIEHS); Notice of Meeting to Review the Murine Local Lymph Node Assay (LLNA) as an Alternative Test Method for Contact Hypersensitivity; Request for Comments

**SUMMARY:** Pursuant to Public Law 103–43, notice is hereby given of a public meeting sponsored by the NIEHS and the National Toxicology Program (NTP), and coordinated by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NTP Center). The agenda topic is the scientific peer review of the murine local lymph node assay (LLNA), which is proposed as an alternative toxicological test method for assessing contact hypersensitivity (allergic contact dermatitis) potential of chemicals and products. The meeting will be held on September 17, 1998, at the Gaithersburg Hilton, 620 Perry Parkway, Gaithersburg, Maryland. The meeting will take place from 8:30 a.m. to 4:30 p.m. and is open to the public.

**Background**

Public Law 103–43 directed the NIEHS to develop and validate alternative methods that can reduce or eliminate the use of animals in acute or chronic toxicity testing, establish criteria for the validation and regulatory acceptance of alternative testing methods, and recommend a process through which scientifically validated alternative methods can be accepted for regulatory use. Criteria and processes for validation and regulatory acceptance were developed in conjunction with 13 other Federal agencies and programs with broad input from the public. These are described in the document "Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the Ad Hoc Interagency Coordinating Committee on the Validation of Alternative Methods" NIH publication 97–3981, March 1997, which is available on the internet at http://ntp-server.niehs.nih.gov/htdocs/ICCVAM/ICCVAM htm. An Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) was subsequently established in a collaborative effort by NIEHS and 13 other Federal regulatory and research agencies and programs. The Committee's functions include the coordination of interagency reviews of toxicological test methods and communication with stakeholders throughout the process of test method development and validation. The following Federal regulatory and research agencies and organizations are participating in this effort:

Consumer Product Safety Commission
Department of Defense
Department of Energy
Department of Health and Human Services
- Agency for Toxic Substances and Disease Registry
- Food and Drug Administration
- National Institute for Occupational Safety and Health/CDC
- National Institutes of Health
- National Cancer Institute
- National Institute of Environmental Health Sciences
- National Library of Medicine

Department of the Interior
Department of Labor
- Occupational Safety and Health Administration

Department of Transportation
- Research and Special Programs Administration

Environmental Protection Agency

The LLNA was proposed to the ICCVAM for consideration as a stand-alone test to identify chemicals that have a potential to cause contact hypersensitivity (allergic contact dermatitis). An ICCVAM Immunotoxicity Working Group composed of Federal employees determined that there was sufficient information available to merit an independent scientific peer review of the LLNA test method. Peer review has been determined to be an essential prerequisite for consideration of a method for regulatory acceptance. The peer review panel will be charged with developing a scientific consensus on the usefulness of the test method to generate information for various human health risk assessment purposes. Following evaluation at this peer review meeting, the proposed test method and results of the peer review will be forwarded by ICCVAM to Federal agencies for consideration. Federal agencies will determine the regulatory acceptability of a method according to their mandates.

**Agenda**

There will be a brief orientation on the ICCVAM and the ICCVAM review process, followed by peer review of the proposed LLNA test method and supporting information. The peer review panel will discuss the usefulness of the LLNA as an alternative to test methods currently accepted by government regulatory authorities for the assessment of the contact hypersensitivity potential of chemicals and products. Copies of the proposed LLNA Test Method Protocol and supporting documentation may be obtained from the NTP Center for the Evaluation of Alternative Toxicological Methods, MD EC–17, P.O. Box 12233, Research Triangle Park, NC, 27709 (919–541–3398), FAX (919–541–0947), e-mail: *ICCVAM@niehs.nih.gov*. The LLNA test method documents and copies of written public comments can also be viewed at the Documents Management Branch, Food and Drug Administration, 5630 Fishers Lane, Room 1061, Rockville, MD, 20852 on Monday through Friday from 9:00 a.m. to 4:00 p.m.

**Public Comment**

The NTP Center invites the submission of written comments on the proposed LLNA test method, and other available information regarding the usefulness of the LLNA, including information about completed, ongoing, or planned studies. Written comments and additional information should be sent by mail, fax, or e-mail to the NTP Center at the address listed above by August 14th. Written comments will be made available to the peer review panel members, ICCVAM agency representatives and experts, and will be made available for attendees at the meeting. Members of the public who wish to present oral statements at the meeting should also contact the NTP Center as soon as possible, but not later than September 11, 1998. Speakers will be assigned on a first-come, first-serve basis and will be limited to a maximum of five minutes in presentation length. Written comments accompanying the oral statement should be submitted in advance so that copies can be made and distributed to the peer panel members.

The NTP Center will furnish an agenda and a roster of peer review panel members just prior to the meeting. Summary minutes and a final report of the LLNA peer review meeting will be available subsequent to the meeting upon request to the Center. Persons needing special assistance, such as sign language interpretation or other special accommodations should contact the NTP Center as described above.

Dated: June 30, 1998.

Kenneth Olden,

*Director, National Toxicology Program.*

[FR Doc. 98–18320 Filed 7–9–98; 8:45 am]

BILLING CODE 4140-01-M

# LLNA Peer Review Meeting Agenda

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

*September 17, 1998, 8:30 a.m. to 5:30 p.m.*

Ballroom A
Gaithersburg Hilton, 620 Perry Parkway
Gaithersburg, Maryland

| | | |
|---|---|---|
| 8:30 a.m. | Introductions | Dr. Jack Dean |
| | Welcome from the National Toxicology Program | Dr. George Lucier |
| | Introduction to ICCVAM and NICEATM | Dr. William Stokes |
| | Overview of the LLNA Peer Review Process | Ms. Denise Sailstad |
| | Summary of Current Agency Requirements | Dr. David Hattan |
| | Overview of the Proposed LLNA Test Method Protocol | Drs. G. Frank Gerberick, Ian Kimber, and David Basketter |
| | Questions Regarding the Test Method Protocol | |
| 9:55 a.m. | Peer Review Panel Discussion | |
| | Test Method Description | Dr. Jean Meade, Coordinator<br>Drs. Paul Bailey, Martinus Lovik, Howard Maibach, and Jean Regal |
| | Break | |
| 10:50 a.m. | Peer Review Panel Discussion (continued) | |
| | Test Method Data Quality | Dr. Lorraine Twerdok, Coordinator<br>Drs. Martinus Lovik, Ralph Smialowicz, and Stephen Ullrich |
| | Test Method Performance | Dr. Peter Thorne, Coordinator<br>Drs. Klaus Andersen, Paul Bailey, Jean Meade, and Joe Haseman |
| 12:30 p.m. | Public Comment | |
| 1:00 p.m. | Lunch Break | |

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|---|---|---|
| 2:00 p.m. | Peer Review Panel Discussion (continued) | |
| | Test Method Performance (cont.) | Dr. Peter Thorne, Coordinator<br>Drs. Klaus Andersen, Paul Bailey, Jean Meade, and Joe Haseman |
| | Test Method Reliability | Dr. Ralph Smialowicz, Coordinator<br>Drs. Robert Hamilton, Masato Hatao, Joe Haseman, and Peter Thorne |
| | Other Literature and Scientific Reviews | Dr. StephenUllrich, Coordinator<br>Drs. Klaus Andersen, Howard Maibach, and Jean Regal |
| | Other Considerations | Dr. Jean Regal, Coordinator<br>Drs. Robert Hamilton and Masato Hatao |
| 3:30 p.m. | Break | |
| 3:50 p.m. | Peer Review Panel Discussion (continued) | |
| | Related Issues | Dr. Masato Hatao, Coordinator<br>Drs. Howard Maibach, Jean Meade, and Stephen Ullrich |
| 4:10 p.m. | Public Comments | |
| 4:30 p.m. | Peer Review Panel Conclusions | Drs. Jack Dean and Lorraine Twerdok |
| 5:30 p.m. | Adjourn | |

# LLNA Peer Review Meeting Summary Minutes

## Introduction

A public meeting of an independent peer review panel was convened on September 17, 1998, in Gaithersburg, Maryland to review the murine local lymph node assay (LLNA), which was proposed as an alternative toxicological test method for assessing contact hypersensitivity (allergic contact dermatitis) potential of chemicals and products. The meeting was coordinated by ICCVAM and the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and was sponsored by the National Institute of Environmental Health Sciences (NIEHS) and the National Toxicology Program (NTP).

The following expert scientists served on the peer review panel:

- Jack Dean, Ph.D., Sanofi Pharmaceuticals, Inc., Malvern, Pennsylvania (Panel Chair)
- Klaus Andersen, M.D., Ph.D., Odense University Hospital, Odense, Denmark
- Paul Bailey, Ph.D., Mobil Oil Corporation, Paulsboro, New Jersey
- Robert G. Hamilton, Ph.D., Johns Hopkins University, Baltimore, Maryland
- Joseph Haseman, Ph.D., National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina
- Masato Hatao, Ph.D., Shiseido Research Center, Yokohama, Japan
- Martinus Lovik, M.D., Ph.D., National Institute of Public Health, Oslo, Norway
- Howard Maibach, M.D., University of California/SF, San Francisco, California
- B. Jean Meade, D.V.M., Ph.D., National Institute of Occupational Safety and Health, Morgantown, West Virginia
- Jean Regal, Ph.D., University of Minnesota, Duluth, Minnesota
- Ralph Smialowicz, Ph.D., US Environmental Protection Agency, Research Triangle Park, North Carolina
- Peter Thorne, Ph.D., University of Iowa, Iowa City, Iowa
- Lorraine E. Twerdok, Ph.D., American Petroleum Institute, Washington, District of Columbia
- Stephen E. Ullrich, Ph.D., MD Anderson Cancer Center, Houston, Texas

## Introductions

Dr. Jack Dean, chair, called the meeting to order at 8:30 a.m., and asked each person in attendance to state their name and affiliation.

## Welcome from the National Toxicology Program

Dr. George Lucier, Director of the National Toxicology Program, thanked the ICCVAM participating agencies and stakeholders, the LLNA sponsors, and the peer review panel for their efforts. Dr. Lucier also provided a brief overview of the history of ICCVAM and NICEATM.

## Introduction to ICCVAM and NICEATM

Dr. William Stokes, ICCVAM Co-Chair and Director of NICEATM, explained the ICCVAM review process, and the steps that had been undertaken in the review of LLNA. He discussed the role of the ICCVAM committee, its expert subgroup

(Immunotoxicology Working Group) and the peer review panel, and the process by which test methods are reviewed and forwarded to agencies for action.

Public Law 103-43 directed the NIEHS to develop and validate alternative methods that can reduce or eliminate the use of animals in acute or chronic toxicity testing, to establish criteria for the validation and regulatory acceptance of alternative testing methods, and to recommend a process through which scientifically validated alternative methods can be accepted for regulatory use. Criteria and processes for validation and regulatory acceptance were developed in conjunction with 14 other Federal agencies and programs with broad input from the public. These are described in the document "Validation and Regulatory Acceptance of Toxicological Test Methods: A Report of the Ad Hoc Interagency Coordinating Committee on the Validation of Alternative Methods," NIH Publication 97-3981, March, 1997. This document is available via the internet at http://ntp-server.niehs.nih.gov/htdocs /ICCVAM.htm.

ICCVAM was subsequently established in a collaborative effort by NIEHS and 13 other Federal regulatory and research agencies and programs. The Committee's functions include the coordination of interagency reviews of toxicological test methods and communication with stakeholders throughout the process of test method development and validation. The following Federal regulatory and research agencies and organizations are participating in this effort:

- Consumer Product Safety Commission
- Department of Defense
- Department of Energy
- Department of Health and Human Services
  - Agency for Toxic Substances and Disease Registry
  - Food and Drug Administration
  - National Institutes of Health
    - Office of the Director
    - National Cancer Institute
    - National Institute of Environmental Health Sciences
    - National Library of Medicine
- National Institute for Occupational Safety and Health/CDC
- Department of the Interior
- Department of Labor
  - Occupational Safety and Health Administration
- Department of Transportation
  - Research and Special Programs Administration
- Environmental Protection Agency

The LLNA was proposed to ICCVAM for consideration as a stand-alone test to identify chemicals that have the potential to cause contact hypersensitivity (allergic contact dermatitis). The test method submission was prepared by three co-sponsors: Drs. G. Frank Gerberick (Procter & Gamble, US); Ian Kimber (Zeneca, UK); and David A. Basketter (Unilever, UK). Independent peer review is an essential prerequisite for consideration of a method for regulatory acceptance (NIEHS, 1997). The peer review panel (PRP) was charged with developing a scientific consensus on the usefulness of the test method to generate information for human health risk assessment purposes. The proposed test method and results of the peer review will be forwarded by ICCVAM to Federal agencies for consideration. Federal agencies will determine the regulatory acceptability of the method according to their mandates.

**Overview of the LLNA Peer Review Process**

Ms. Denise Sailstad, IWG Co-Chair, provided an overview of the role of the IWG in the review of the LLNA, outlining the specific accomplishments of the IWG. She reiterated the two main questions that the working group had drafted as the focus of the review. The questions were as follows:

1. Has the LLNA been evaluated sufficiently and is it performance satisfactory to support its adoption as a stand-alone alternative?
2. Does the LLNA offer advantages with respect to animal welfare considerations (refinement, reduction, and replacement)?

**Summary of Current Agency Requirements**

Dr. David Hattan, IWG Co-Chair, summarized Federal agency and international regulations and recommendations for dermal contact hypersensitivity testing. Several test methods are currently accepted by the EPA. EPA OPPTS and the OECD (Guideline Number 405) both currently accept the LLNA as a screening test for dermal hypersensitivity. If the test results are positive, no further testing is required. However, if the LLNA test is negative, then one of the guinea pig tests must be conducted; FDA currently recommends the use of the Guinea Pig Maximization Test (GPMT) or the Buehler Assay (BA).

**Overview of the Proposed LLNA Test Method Protocol**

Each of the test method sponsors (Drs. G. Frank Gerberick, David Basketter, and Ian Kimber) gave a brief introduction to the LLNA. Allergic contact dermatitis results from two separate but related sequential immunological events caused by a chemical substance. First, an initial exposure(s) causes a primary immune response known as sensitization. If there is additional exposure following sensitization, then a secondary immune-mediated response occurs, which is characterized by skin erythema, swelling, and pruritis. The scientific basis for the proposed LLNA test is that lymphocytes in draining lymph nodes of ears of mice proliferate as the primary response to topical exposure with chemicals that cause dermal sensitization. This proliferation is detected by measuring the amount of $^{3}$H-methyl thymidine incorporated into dividing lymphocytes. Radioactive thymidine incorporation results from increased proliferation of resident or migratory lymphocytes in the lymph node in response to the chemical challenge. The resulting data are measured on an individual lymph node basis and presented as a stimulation index (SI) after comparing the level of radioactive incorporation in treated versus the control mice. The measured lymphocyte response is an essential element in the process of sensitization. In contrast, currently accepted guinea pig assays measure skin reactivity to a secondary challenge with the test substance. Their presentations were followed by assay-related questions from the PRP.

**Review of the LLNA submission**

The PRP then proceeded to present and discuss the various sections that they were asked to evaluate. The conclusions for each of the sections are summarized below.

**Test Method Description**

Dr. J. Meade, the section coordinator, presented the analysis and conclusions reached by the test method description section reviewers, which included Drs. P. Bailey, M. Lovik, H. Maibach, and J. Regal

The panel concluded that the proposed test method protocol (Local Lymph Node Assay ICCVAM Submission, April, 1998) was generally adequate, but recommended the following additions and/or changes:

1. Until a systematic comparison of data between (a) mouse strains, and (b) male and female mice are conducted, the protocol should specify the use of female CBA mice only.
2. Animals should be individually identified.
3. Body weight data should be collected at the start and end of the assay.
4. Lymphocyte proliferation data should be collected at the level of the individual animal.
5. Statistical analysis should be performed.
6. A single dose of a moderate sensitizer should be included as a concurrent positive control in each study.
7. $^{3}$H-methyl thymidine or $^{125}$I-iododeoxyuridine may be used in the LLNA.
8. The decision process to identify a positive response should include an SI ≥ 3, statistical significance, and dose response information.
9. An illustration should be added to the protocol, indicating the nodes draining the exposure site that are to be harvested.

**Test Method Data Quality**

Dr. L. Twerdok, the section coordinator, presented the analysis and conclusions reached by the test method data quality section reviewers, which included Drs. M. Lovik, R. Smialowicz, and S. Ullrich. The PRP recommended that retrospective data audits be conducted on at least three of the intra- and inter-laboratory LLNA validation studies conducted by the Sponsors.

**Test Method Performance**

Dr. P. Thorne, the section coordinator, presented the analysis and conclusions reached by the test method performance section reviewers, which included Drs. K. Andersen, P. Bailey, J. Meade, and J. Haseman. The panel concluded that the LLNA performed at least as well as the currently accepted guinea pig methods (GPMT/BA) for the hazard identification of chemical sensitizing agents. The review involved the evaluation of LLNA data on 203 chemicals, of which both LLNA and guinea pig data were provided for 126 chemicals. Both LLNA and human (Human Maximization Test [HMT]/ Human Patch Test Allergen [HPTA]) data were provided for 74 of the 203 chemicals. From the analysis generated during the review process, the accuracy[1] of the LLNA when compared to the GPMT/BA was 89% (N = 97), and when compared to all guinea pig tests (GPT) was 86% (N = 126). The accuracy of the LLNA when compared to human tests was 72% (N = 74). The accuracy of the GPMT/BA when compared to human tests was 72% (N = 57), and the accuracy of the GPT when compared to human tests was 73% (N = 62).

Additionally, when the analysis was limited to only those compounds for which there was LLNA, guinea pig, and human data, the accuracy of the LLNA when compared to human tests and the accuracy of theGPMT/BA when compared to human tests was 72% (N = 57) in both comparisons. In terms of accuracy, sensitivity, specificity, and positive and negative predictivity, the PRP found the performance of the LLNA to be similar to that of the GPMT/BA. Equally important, the performance of the LLNA and GPMT/BA were similar in regard to human data (HMT/HPMT)

**Test Method Reliability**

Dr. R. Smialowicz, the section coordinator, presented the analysis and conclusions reached by the test method reliability section reviewers, which included Drs. R. Hamilton, M. Hatao, J. Haseman, and P. Thorne.

The panel concluded that the data submitted for review demonstrated that the LLNA has adequate repeatability and reproducibility, and that the qualitative data demonstrated good inter- and intra-laboratory reliability.

**Other Literature and Scientific Reviews**

Dr. S. Ullrich, the section coordinator, presented the analysis and conclusions reached by the reviewers for the other literature and scientific reviews section, which included Drs. K. Andersen, H. Maibach, and J. Regal.

This section evaluated the published literature on the LLNA that was not generated by the test sponsors. The results presented in the literature support the use of the LLNA for testing the sensitization potential of chemicals. Future protocol modifications may allow for the assay to more accurately predict the sensitizing potential of metal salts and irritants; these groups of chemicals appear to have high false positive and false negative rates, respectively, when evaluated using the submitted protocol.

**Other Considerations**

Dr. J. Regal, the section coordinator, presented the analysis and conclusions reached by the other considerations section reviewers, which included Drs. R. Hamilton and M. Hatao.

The panel discussed the transferability of the test method, and issues relating to cost and time effectiveness. It was concluded that the test method was transferable among labs and that there is potential for the method to be more cost effective than the guinea pig assays.

**Related Issues**

Dr. M. Hatao, the section coordinator, presented the analysis and conclusions reached by the related issues section reviewers, which included Drs. H. Maibach, J. Meade, and S. Ullrich.
This section reviewed other potential endpoints and modifications that could be considered in the future. The following workshops were recommended:

1. A workshop on the ICCVAM evaluation process focusing on providing guidance for individuals planning on making future assay submissions as well as for individuals that may be involved in the evaluation process;
2. A workshop on the use of the LLNA for detecting the photosensitization potential in conjunction with UVA irradiation;
3. A workshop to identify the most predictive methods for detecting immediate-type hypersensitivity following oral exposure to chemicals and drugs;
4. A workshop to explore alternative endpoints of the LLNA; and
5. A workshop to consider the potential of the *ex vivo* LLNA as well as other

[2]One abstaining member of the panel expressed agreement with the PRP conclusion after the public meeting.

possible refinements. It was concluded by the PRP that more research is needed before such a workshop should be planned.

**Public Comments**

Several individuals from Federal regulatory agencies made comments at the meeting with respect to issues that would be important from a regulatory standpoint. Dr. Ken Hastings, FDA/CDER, stated that their agency would want individual animal data collected in order to consider the data.

Dr. John Langone, FDA/CDRH, stated that the dataset definitely supports the use of the LLNA for detecting the sensitization potential of moderate and potent sensitizers, but that the data was not as conclusive for weak sensitizers. Because of this point, Dr. Langone recommended using statistics as part of the criteria for identifying sensitization hazard potential. He further stated that established reference statistical data would help in future refinements to the assay.

Dr. Al Munson, NIOSH, encouraged the PRP to accept the 3-fold index as the method for determining contact hypersensitivity potential. He added that this method of determination came about as a judgement factor, and that to this point, the use of this index has been adequate. Further, Dr. Munson felt that as further knowledge of the assay is collected, it may be appropriate to consider other factors, such as statistical analysis. He reiterated that the test was designed and validated using the 3-fold index, and that there was no data to support the use of a different measurement as the predictive endpoint.

Dr. Lynnda Reid, FDA/CDER, stated that her agency would like to see the use of concurrent positive controls when testing using the LLNA. Dr. Reid stated that without such controls, it would be difficult for her agency to accept negative results.

Other public comments were also offered. A representative from the Institute for In Vitro Sciences requested caution in adding items to the existing validation model. He stated that to adequately address the use of statistics instead of the 3-fold index, the data would need to be entirely reevaluated.

A representative from Eli Lilly stated that for determining if a compound is immunotoxic, a review of incidences would be important. Thus, he stated that he would want the lymph nodes to be collected at the level of the individual animal, and statistics to be used in decisionmaking.

Dr. Martin Stephens, Humane Society of the United States (HSUS), stated that HSUS is pleased with the ICCVAM process since it allows for consideration of animal welfare in new assay development. Dr. Andrew Rowan, HSUS, further stated that the HSUS would like to see alternative tests approved when they are at least as good as current animal tests; he felt that it is unnecessary (and inappropriate from an animal welfare perspective) to wait until enough data is gathered to show that the alternative method is better than the animal test.

**Peer Review Panel Conclusions**

The peer review panel conclusions were summarized by Drs. J. Dean and L. Twerdok.

The PRP unanimously[1] concluded to recommend the LLNA as a stand-alone alternative for contact sensitization hazard

[1] After the peer review meeting, one abstention was changed to approval

assessment, provided that the protocol modifications discussed under the test method description (above) were made.

The PRP also agreed that the LLNA had several advantages over guinea pig methods in that it provided quantitative data, allowed dose-response assessment, reduced animal distress, potentially reduced animal numbers, was potentially more cost effective, required much less time, involved the induction phase of sensitization, and will allow future refinement and mechanistic studies. Possible assay weaknesses (e.g., false negative results with some metals and weak sensitizing agents, false positive results with some strong irritants) were identified; it was concluded that these should be addressed in future workshops. Also, data to support the testing in the LLNA of mixtures was not provided and the evaluation of pharmaceuticals was limited.

**Adjournment**

The meeting was adjourned by Dr. Jack Dean at 5:30 p.m.

[2]One abstaining member of the panel expressed agreement with the PRP conclusion after the public meeting.

# SAMPLE PROTOCOL[1]:

## TESTING OF CHEMICALS FOR CONTACT SENSITIZING (ALLERGIC CONTACT DERMATITIS) POTENTIAL: LOCAL LYMPH NODE ASSAY (LLNA)

### INTRODUCTION

1. OECD Guidelines for Testing of Chemicals are reviewed periodically in light of scientific progress and animal welfare considerations. Guideline 406 (1992) describes methods for assessing skin sensitization potential of chemicals in animals (1). While this Guideline mentions certain alternative screening tests, it relies on guinea pigs tests, notably the Guinea Pig Maximization Test and the Buehler Assay, for the hazard identification of skin sensitizers and non-sensitizers.

2. The details that follow in this Guideline describe the Local Lymph Node Assay (LLNA), an alternative procedure using the mouse (2-4). The LLNA provides advantages with regard to animal welfare (both reduction and refinement) and scientific aspects (specifically, the objective and quantitative nature of the endpoint measured). This method was mentioned in Guideline 406 (1) as a screening test, but has now undergone sufficient validation that it should be considered as a stand-alone method. The details of this validation and a review of the associated work have been published (5-8). In addition, it should be noted that the mild/moderate sensitizers recommended as suitable positive control substances for guinea pig test methods are also appropriate for use with the LLNA (6, 8, 9, 10).

3. Prior to modification of this protocol, changes should be adequately validated and determined to be acceptable (11).

### GENERAL PRINCIPLE OF DETECTION OF SKIN SENSITIZATION USING THE LOCAL LYMPH NODE ASSAY

4. The basic principle underlying the LLNA is that sensitizers induce proliferation of lymphocytes in the lymph node draining the site of chemical application. Generally, 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 Appendix 1). This effect occurs as a dose-response in which the proliferation in test groups is compared to that in concurrent vehicle-treated controls. A positive control is added to each assay to provide an indication of appropriate assay performance.

[1] This protocol is a modification of the "Draft OECD Guideline for Testing of Chemicals. Skin Sensitisation: Local Lymph Node Assay," and was provided to ICCVAM by R. J. Fielder, Department of Health (UK), on August 6, 1998 as background information for the peer review. The protocol was modified by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) to reflect the conclusions and recommendations of the ICCVAM-coordinated LLNA peer review meeting that took place on September 17, 1998 in Gaithersburg, MD.

## DESCRIPTION OF THE LOCAL LYMPH NODE ASSAY

### Sex and strain of animals

5. Young adult female mice (nulliparous and non-pregnant) of the CBA/Ca or CBA/J strain should be used at age 8-12 weeks. All animals should be age-matched (preferably within a one-week time frame). Females are used because the existing database is predominantly based on this gender. Other strains and males should not be used until it is sufficiently demonstrated that significant strain- and/or gender-specific differences in the LLNA response do not exist.

### Preparation of animals

6. The temperature of the experimental animal room should be 21ºC (± 3ºC) and the relative humidity 30-70%. When artificial lighting is used, the light cycle should be 12 hours light:12 hours dark. For feeding, standard laboratory mouse diets should be used with an unlimited supply of drinking water. The mice should be acclimatised for at least 5 days prior to the start of the test. Animals may be housed individually, or caged in small groups of the same sex. Healthy animals are randomly assigned to the control and treatment groups. The animals are uniquely identified prior to being placed on study. Although a variety of techniques exist to uniquely mark mice, any method that involves identification via ear marking (e.g., ear tags) should not be used.

### Preparation of doses

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

### Test conditions

<u>Solvent/vehicle</u>

8. The solvent/vehicle 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 (DMF), methyl ethyl ketone (MEK), propylene glycol (PG), and dimethyl sulfoxide (DMSO), but others may be used (2). Particular care should be taken to ensure that hydrophilic materials are incorporated into a vehicle system that wets the skin and does not immediately run off. Thus, wholly aqueous vehicles are to be avoided. It may be necessary for regulatory purposes to test the chemical in the clinically relevant solvent or product formulation.

<u>Controls</u>

9. Concurrent negative (solvent/vehicle) and positive controls should be included in each test. In some circumstances, it may be useful to include a naïve control. Except for treatment with the test substance, animals in the control groups should be handled in an identical manner to animals of the treatment groups.

10. Positive controls are used to ensure the appropriate performance of the assay. The positive control should produce a positive LLNA response at an exposure level expected to give an increase in the stimulation index (SI) >3 over the negative control group. The positive control dose should be chosen such that the induction is clear but not excessive. Preferred positive control substances are hexyl cinnamic aldehyde (HCA) and 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 vehicle that is known to elicit a consistent response (i.e., acetone:olive oil), there may be certain regulatory situations where a non-standard vehicle (clinically/chemically relevant formulation) is necessary to test the effect (interaction) of a positive control with this unconventional vehicle.

**Methodology**

11. A minimum of five successfully treated animals are used per dose group, with a minimum of three consecutive concentrations of the test substance plus a solvent/vehicle control and a positive control group. Test substance treatment doses should be based on the recommendations given in Kimber and Basketter (1992) (2) and in the ICCVAM Peer Review Panel Report (8). Doses 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 overt systemic toxicity and excessive local irritation. To identify the appropriate maximum test substance dose, an initial toxicity test, conducted under identical experimental conditions except for an assessment of lymph node proliferative activity, may be necessary. To support an ability to identify a dose-response relationship, data must be collected on at least three test substance treatment doses, in addition to the concurrent solvent/vehicle control group. For negative LLNA studies, the concurrent positive control must induce a SI >3 relative to its vehicle-treated control (see Section 10.).

12. The LLNA experimental procedure is performed as follows:

    *Day 1* – Individually identify and record the weight of each mouse prior to dermal applications. Apply 25 µL/ear of the appropriate dilution of the test substance, or the positive control, or the vehicle alone to the dorsum of both ears.

    *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 $^{3}$H-methyl thymidine ($^{3}$H – TdR) or 250 µL PBS containing 2 µCi of $^{125}$I-iododeoxyuridine ($^{125}$IU) and $10^{-5}$ M fluorodeoxyuride into each experimental mouse via the tail vein (12, 13). Five hours later, the draining (auricular) lymph node of each ear (8) is excised and pooled in PBS for each animal. Both bilateral draining lymph nodes must be collected (see diagram and description of dissection in Appendix 1). A single cell suspension of lymph node cells (LNC) is prepared for each mouse. The single cell suspension is prepared in PBS by either gentle mechanical separation through 200-mesh stainless steel gauze or another acceptable technique for generating a single cell suspension. LNC are washed twice with an excess of PBS and the DNA precipitated with 5% trichloroacetic acid (TCA) at 4$^{o}$C for approximately 18h.

For $^{3}$H – TdR method, pellets are resuspended in 1 mL TCA and transferred to 10 mL of scintillation fluid. Incorporation of tritiated thymidine is measured by $\beta$-scintillation counting as disintegrations per minute (dpm) for each mouse and expressed as dpm/mouse. For the $^{125}$IU method,the 1 mL TCA pellet is transferred directly into gamma counting tubes. Incorporation of $^{125}$IU is determined by gamma counting and also expressed as dpm/mouse.

<u>Observations</u>: Mice should be carefully observed for any clinical signs, either of local irritation at the application site or of systemic toxicity. Weighing mice prior to treatment and at the time of necropsy will aid in assessing systemic toxicity. All observations are systematically recorded, with records being maintained for each individual mouse.

13. Results for each treatment group are expressed as the mean SI. The SI is the ratio of the mean dpm/mouse within each test substance treatment group and the positive control treated group against the mean dpm/mouse for the solvent/vehicle treated control group. However, the investigator should be alert to possible "outlier" responses for individual animals within a group that may necessitate the use of an alternative measure of response (e.g., median rather than mean) or elimination of the outlier. Each SI should include an appropriate measure of variability that takes into account the inter-animal variability in both the dosed and control groups (8).

    In addition to an assessment of the magnitude of the SI, a statistical analysis should be conducted which includes an assessment of the dose-response relationship as well as pairwise dosed group versus concurrent solvent/vehicle concurrent control comparisons (e.g., linear regression analysis to assess dose-response trends; Dunnett's test to make 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 nonparametric statistical analysis.

## DATA AND REPORTING

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

### Evaluation and interpretation of results

15. In general, when the SI for any single treatment dose group is ≥3, the test substance is regarded as a skin sensitizer (3, 6, 8). However, the magnitude of the SI should not be the sole factor used in determining the biological significance of a skin sensitization response. A quantitative assessment may be performed by statistical analysis of individual animal data and may provide a more complete evaluation of the test agents (see Section 13). Factors that should be considered

include the results of the SI, statistical analyses, the strength of the dose-response relationship, chemical toxicity, solubility, and the consistency of the vehicle and positive control responses. Equivocal results should be clarified by considering statistical analysis, structural relationships, available toxicity information, and dose selection.

16. A test substance not meeting the above criteria is considered a non-sensitizer in this test.

17. The test report must contain the following information:

<u>Test substance, controls, and solvent/vehicles</u>

- identification data and CAS no., if known;
- physical nature and purity;
- physiochemical properties relevant to the conduct of the study;
- stability of the test substance, if known; and
- lot number of the test substance.

<u>Solvent/vehicle</u>:

- use of the regulatory relevant vehicle;
- justification for choice of solvent/vehicle; and
- solubility and stability of the test substance in the solvent/vehicle.

<u>Test animals</u>:

- strain of mice used;
- number, age, and sex of mice;
- source, housing conditions, diet, etc.;
- individual weight of the animals at the start and end of the test, including body weight range, mean and associated error term for each group; and
- microbiological status of the mouse

<u>Test conditions</u>:

- positive and negative (vehicle/solvent) 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.

<u>Results</u>:

- signs of toxicity;
- dpm/mouse values for each mouse within each treatment group;
- mean and associated error term for dpm/mouse for each treatment group;
- calculated SI and associated error term for each test substance treatment dose group and concurrent positive control group;
- dose-response relationship;
- statistical analyses and method applied;
- concurrent and historical negative control data as established in the testers laboratory;
- concurrent positive control data

<u>Discussion of the results</u>

<u>Conclusion</u>

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# APPENDIX 1: DISSECTION AND IDENTIFICATION OF THE DRAINING LYMPH NODES

## Background

Although minimal technical training of the LLNA is required, extreme care must be taken to obtain appropriate and consistent dissection of the lymph nodes. It is recommended that technical proficiency be achieved by the dissection and identification of the lymph nodes draining the ear by:a) practice dissection on mice that have been injected with a colored agent (dye); and/or b) practice dissection with mice 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.

## Training and preparation for node identification

### *Identification of the draining node – colored 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 may be used effectively.

**A.** ***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.

**B.** ***Colloidal carbon and other dye treatments:***

Colloidal carbon and India ink are examples of other dye treatments that may be used (14).

### *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 used 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 the identification and location of the node.

Using a procedure similar to that listed in the protocol, the agent is applied to the dorsum of both ears (25 µL/ear) for three consecutive days. On the fourth day, the mouse is euthanized. 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 the assay performance. They should only be used for training and node identification purposes.

**Dissection Approach**
*Lateral Dissection (Figure 1):*

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

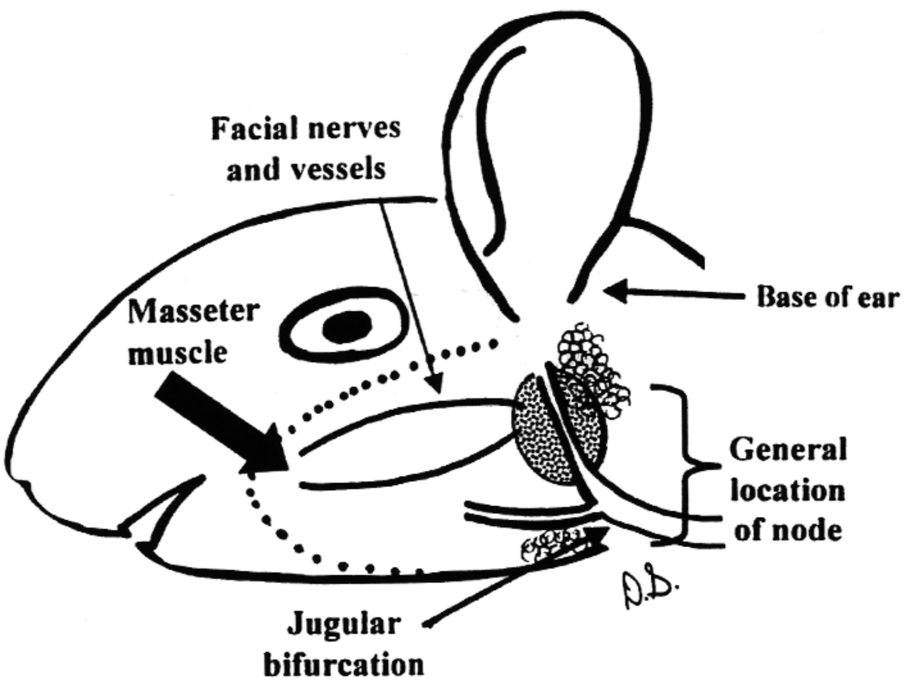



**Figure 1. Lateral view**

Illustration by Denise Sailstad, EPA, RTP, NC, USA. May be reprinted with permission.

**Ventral Dissection (Figure 2):**

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

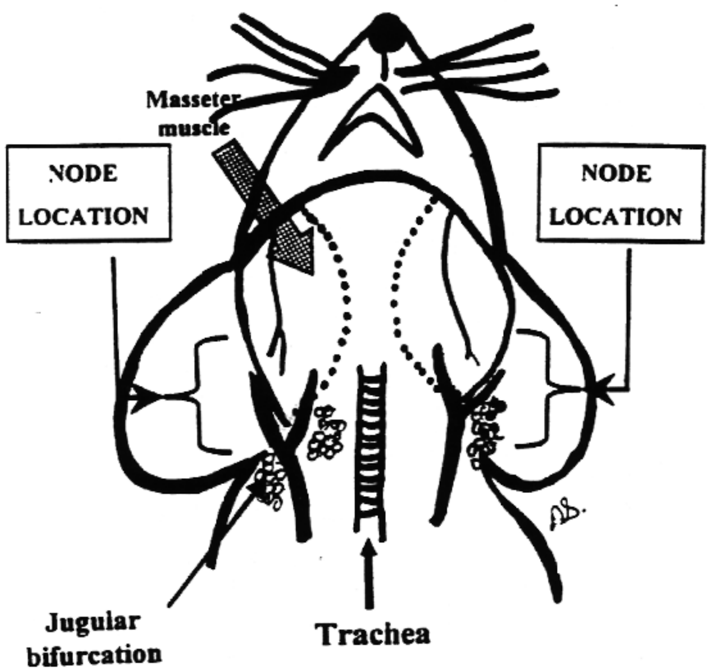



**Figure 2. Ventral view**

Illustration by Denise Sailstad. EPA. RTP. NC. USA. May be reprinted with permission.

**Accuracy in identification:**

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

# NICEATM Assessment of Intra/Inter-Laboratory Variability in the LLNA

(July 11, 1998)

This assessment of the extent of intra- and inter- laboratory variability was based on the data provided in Table 2, page 12, of the LLNA Submission (Tab B). These are the only data located which are amenable to the type of analysis described in ASTM E691-92 A Standard Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method. Two data sets were analyzed. The first one consisted of $EC_3$ (dose calculated to induce a stimulation index of 3) data for DNCB tested twice in each of 5 laboratories. The second consisted of $EC_3$ data for HCA tested six times in each of two laboratories. This analysis calculates h, the within laboratory consistency statistic, where h = d (the difference between each laboratory mean value and the mean for all laboratories)/the standard deviation of test averages, and k, the between laboratory consistency statistic, where k = the standard deviation for individual laboratories/ repeatability standard deviation. Once calculated, 95% confidence limits can be derived from a table provided in the ASTM Guideline. It should be appreciated that (i) the analysis is based on $EC_3$ data, the calculation of which is not a part of the submitted protocol, and (ii) a corresponding analysis of guinea pig test data may not be feasible given the nature of the assay.

1. DNCB Data.

The original data and calculations are provided in the attached table, the individual h and k values for each laboratory ate presented graphically in the accompanying figures. The 95% confidence limits for h and k were 1.74 and 2.11, respectively. None of the h and k values for the individual laboratories exceeded these confidence limits, indicating the lack of significant within and between laboratory variability.

2. HCA Data.

The original data and calculations are provided in the attached table. The 95% confidence limits for h could not be calculated due to the fact that only two laboratories were involved; k was 1.52. The k values for the two laboratories did not exceed this confidence limit, indicating the lack of significant between laboratory variability.

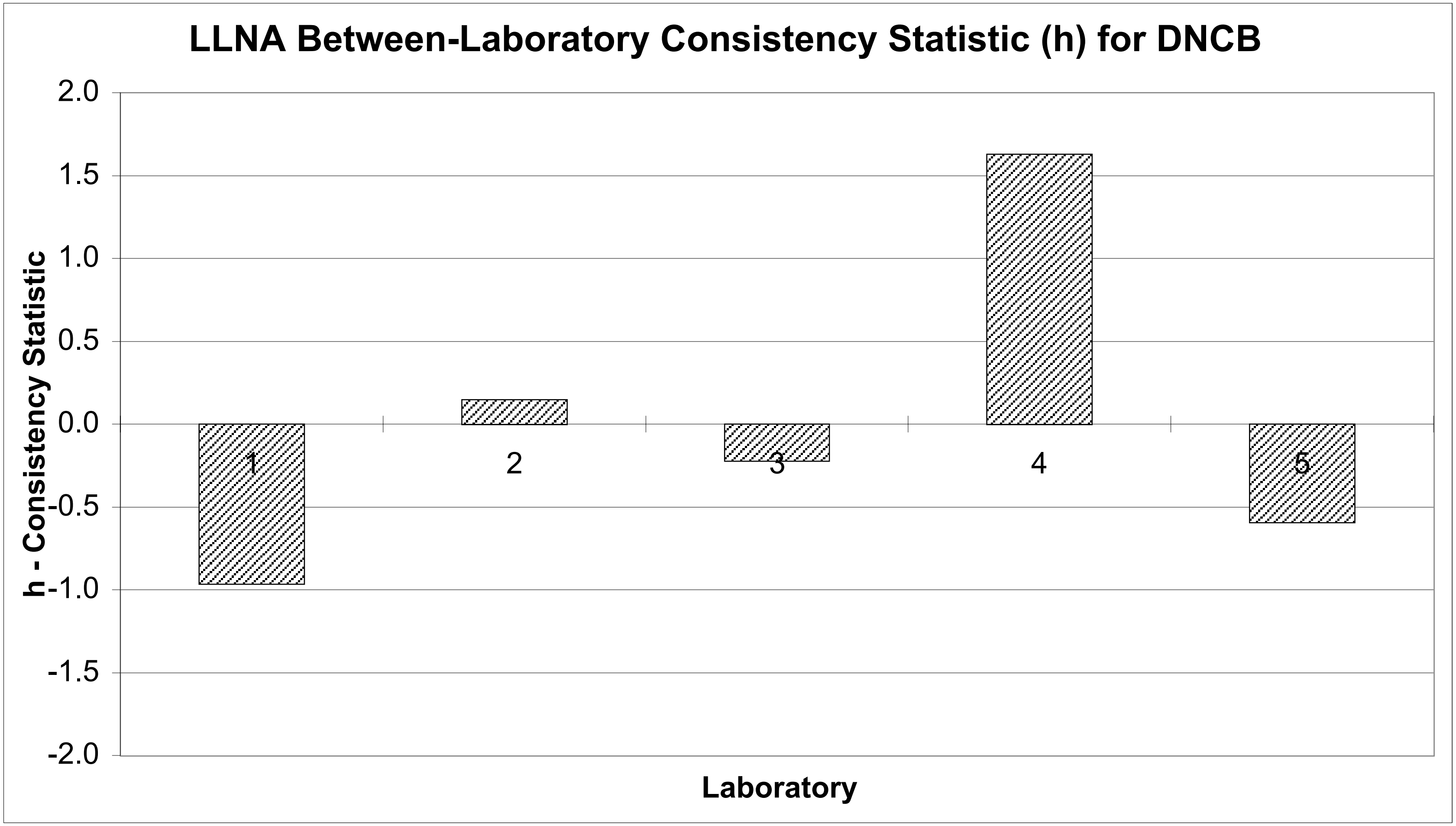
## Interlaboratory Comparison for LLNA[a]
## Assessment of DNCB Data from Five Laboratories[b]

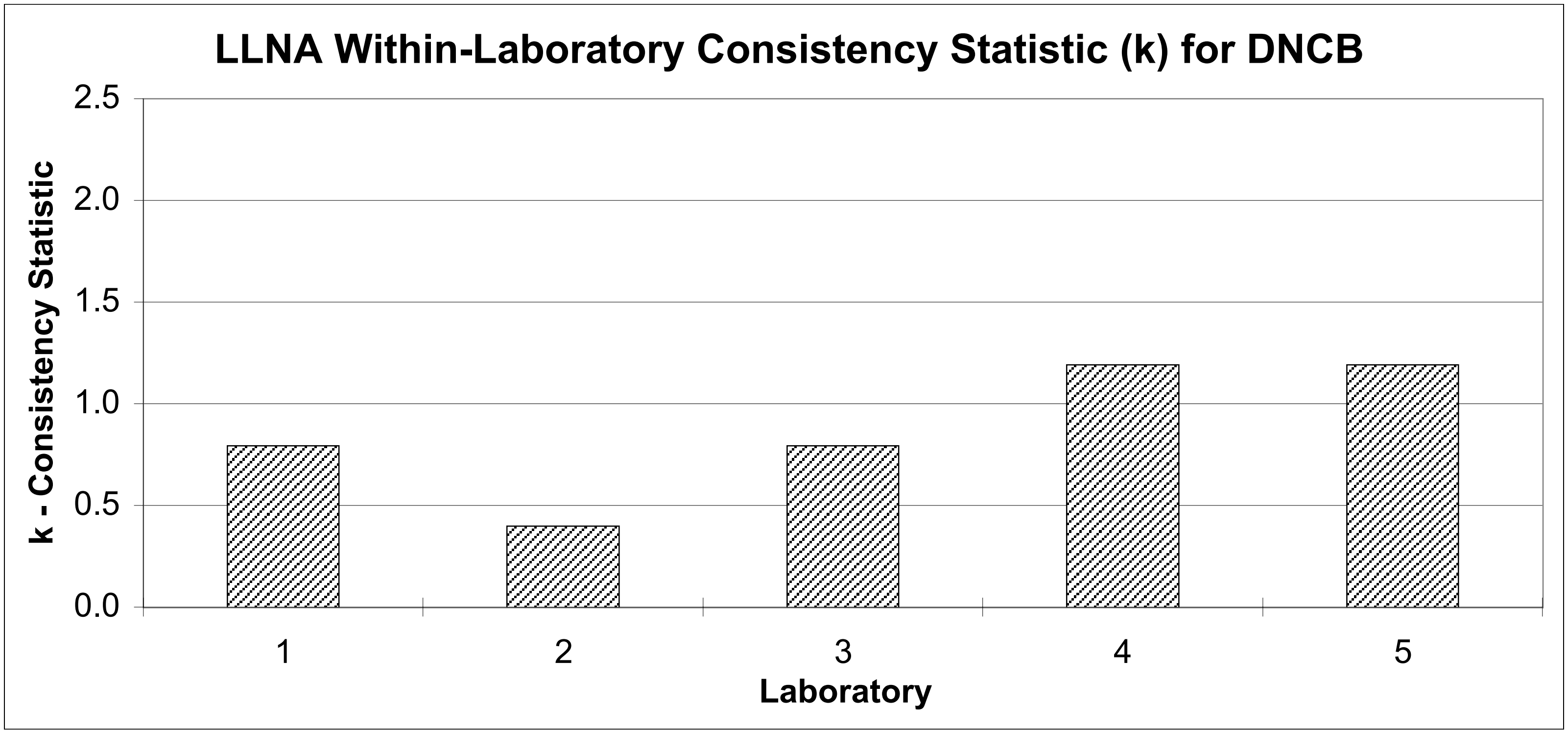
| Laboratory | Test Results 1 | Test Results 2 | Average | Standard Deviation | d | h | k |
|---|---|---|---|---|---|---|---|
| 1 | 0.05 | 0.03 | 0.0400 | 0.0141 | -0.0130 | -0.96 | 0.79 |
| 2 | 0.06 | 0.05 | 0.0550 | 00.0071 | 0.0020 | 0.15 | 0.40 |
| 3 | 0.04 | 0.06 | 0.0500 | 0.0141 | -0.0030 | -0.22 | 0.79 |
| 4 | 0.06 | 0.09 | 0.0750 | 0.0212 | 0.0220 | 1.63 | 1.19 |
| 5 | 0.03 | 0.06 | 0.0450 | 0.0212 | -0.0080 | -0.59 | 1.19 |
| | Average of test averages | | 0.0530 | 95% confidence limits | | ±1.74 | 2.11 |
| | Standard deviation of test averages | | 0.0135 | | | | |
| | Repeatability standard deviation | | 0.0164 | | | | |
| | Reproducibility standard deviation | | 0.0178 | | | | |

[a] Analysis as described in ASTM E691-92 Standard Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method.

[b] $EC_3$ (dose calculated to induce a stimulation index of 3) data from LLNA Submission, Tab B, page 12, Table 2 -Reproducibility of LLNA Quantitative Data.

Abbreviations: d = difference between individual laboratory mean and mean for all laboratories; h = within laboratory consistency statistic = d / standard deviation of test averages; k = between laboratory consistency statistic = standard deviation for individual laboratories / repeatability standard deviation.


LLNA Between-Laboratory Consistency Statistic (h) for DNCB
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
h - Consistency Statistic
1
2
3
4
5
Laboratory


LLNA Within-Laboratory Consistency Statistic (k) for DNCB
k - Consistency Statistic
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
4
5
Laboratory

**Interlaboratory Comparison for LLNA[a]**
**Preliminary Assessment of HCA Data from Two Laboratories[b]**

| Laboratory | Test Results 1 | 2 | 3 | 4 | 5 | 6 | Average | Standard deviation | d | h | k |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 7.9 | 6.9 | 9.6 | 8.7 | 4.0 | 9.2 | 7.7167 | 2.0605 | -0.7250 | -0.71 | 1.02 |
| 2 | 7.6 | 7.2 | 8.8 | 9.5 | 10.0 | 11.9 | 9.1667 | 1.7166 | 0.7250 | 0.71 | 0.85 |
| | | Average of test averages | | | | | 8.4417 | 95% confidence limits | | --- | 1.52 |
| | | Standard deviation of test averages | | | | | 1.0253 | | | | |
| | | Repeatability standard deviation | | | | | 1.8964 | | | | |
| | | Reproducibility standard deviation | | | | | 2.0120 | | | | |

[a] Analysis as described in ASTM E691-92 Standard Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method.
[b] $EC_3$ (dose calculated to induce a stimulation index of 3) data from LLNA Submission, Tab B, page 12, Table 2 –Reproducibility of LLNA Quantitative Data.
Abbreviations: d = difference between individual laboratory mean and mean for all laboratories; h = within laboratory consistency statistic = d / standard deviation of test averages; k = between laboratory consistency statistic = standard deviation for individual laboratories / repeatability standard deviation.

# NICEATM Assessment of the Performance of Irritants in the LLNA

(July 11, 1998)

In Basketter et al. (1998), information is provided on the performance of human irritants in the LLNA. The irritants are classified as low, moderate, or high, while the LLNA data are classified as negative, equivocal, or positive. These data are summarized in the attached 3 by 3 table, showing that 2 of 14 known irritants tested positive in the LLNA.

Performance of a Range of Irritants in the LLNA

| **Chemical name** | **Irritancy potential** | | **LLNA result** | | |
|---|---|---|---|---|---|
| *Test substance* | *Human 4 hour patch test data*[1] | *Conclusion* | *Concentration (%)* | *Stimulation indices*[3] | *Conclusion*[4] |
| Chlorbenzene | Not done | Low[5] | 5.0/10/25 | 1.1/1.7/1.6 | Negative |
| Hexane | Not done | Low[5] | 25/50/100 | 0.8/0.8/2.2 | Negative |
| Isopropanol | 0% / 53% | Low | 10/25/50 | 1.7/1.1/1.0 | Negative |
| Propylene glycol | 6% / 72% | Low | 50/100 | 1.2/1.6 | Negative |
| Resorcinol | Not done | Low[5] | 5.0/10/25 | 2.2/2.2/2.7 | Negative |
| Cetyltrimethyl ammonium chloride | 25% / 75% | Moderate | 3.5/8.8/17.5 | 3.0/3.0/1.1 | Equivocal |
| $C_{12-13}$ ß-branched primary alcohol sulphate | 84% / 90% | Moderate | 7.7/15.4/38.5 | 2.1/3.1/4.3 | Positive |
| Methyl salicylate | Not done | Moderate[5] | 25/50/100 | 0.9/1.0/2.6 | Negative |
| Salicylic acid | Not done | Moderate[5] | 5.0/10/25 | 0.8/1.5/2.5 | Negative |
| Sodium lauryl sulphate | 70%[6] | Moderate | 2.5/5.0/10/25 | 2.3/3.8/4.1/5.3 | Positive |
| Benzalkonium chloride | 52% / 83% | High | 1/2.5 | 2/5/2.4 | Negative |
| Lactic acid | 81% / 60% | High | 5.0/10/25 | 1.0/1.4/2.2 | Negative |
| Octanoic acid | 68% / 58% | High | 10/25/50 | 0.7/1.0/1.6 | Negative |
| Phenol | Not done | High[5] | 1/2.5/5.0 | 0.7/1.5/1.6 | Negative |

[1]Results taken from human 4 hour patch tests carried out according to the standard protocol (York et al, 1996); most of the data is reported elsewhere (Basketter et al, 1997). The first figure is the % of the panel responding to the test material, the second the % reacting to the 25% SLS positive control.
[2]Overall judgement on the irritation potential of the substance based on human 4 hour patch test data together with other information available in the general literature, including standard patch test concentrations used in diagnostic testing (de Groot, 1994).
[3]Proliferation in test animals was compared with that in sham treated controls.
[4]Overall judgement on sensitization potential from data generated in the standard LLNA and using the criteria previously described (Kimber and Basketter, 1992).
[5]As there is no data from a human 4 hour patch test, the judgement on irritation potential has been based on information available in the general clinical literature.
[6]Average derived from 18 experiments, representing 380 positives amongst the 544 individuals tested.

PERFORMANCE OF A RANGE OF IRRITANTS IN THE LLNA

| | | LLNA RESULTS Negative | Equivocal | Positive | TOTAL # CHEMICALS |
|---|---|---|---|---|---|
| IRRITANCY | Low | 5 | | | 5 |
| | Moderate | 2 | 1 | 2 | 5 |
| | High | 4 | | | 4 |
| TOTAL # CHEMICALS | | 11 | 1 | 2 | 14 |

Data from Basketter et al. (Submitted key paper - Tab 5, Table 2).

# NICEATM Assessment of Cost and Time Differences Between the LLNA and the Guinea Pig Maximization Test (GPMT)

Table 1 provides a summary of information gathered regarding the number of animals used in the LLNA and the GPMT and the time involved in conducting the test. The revised protocol supplied by Gerberick et al. (1998) states that groups of four or five mice per dose group are used, depending on whether the lymph nodes will be pooled by treatment group or whether individual animal nodes will be scored. A control group and three to five testing groups are evaluated. Therefore, the total number of animals used in the LLNA for testing one chemical ranges from 16 to 30. The revised protocol indicates that the LLNA takes 7 days to conduct, as calculated from the time of initial treatment to the time that $^{3}$HTdR incorporation into lymph nodes is determined. Based on information provided in Klecak (1996) regarding procedures for conducting the GPMT, 20 test and 10 to 20 control guinea pigs are used. A pilot study using two to three animals is recommended to determine appropriate concentrations. Therefore, the total number of animals used in the GPMT ranges from 32 to 43. The time to conduct the GPMT is 25 days, as calculated from the time of the initial induction to the observation time 48 hours after removal of the challenge patch. Adding a one week period for the pilot study increases the length to a total of 32 days.

**Table 1**

| Test Method | Total Number of Animals | Time to Conduct Test (days) | Reference |
|---|---|---|---|
| LLNA | 16-30 mice | 7 | Gerberick et al. (1998) |
| GPMT * | 32-43 guinea pigs | 32 | Klecak (1996) |

* Includes 7-day toxicity test

Table 2 presents a comparison of the animal cost associated with conducting the LLNA and GPMT. Costs per animal are presented based the 1998 price lists for the laboratories supplying the animals. For the LLNA, Jackson Laboratories, Bar Harbor, ME quoted the cost of a 6-week-old CBA/J mouse as \$10.05. Using the number of animals as specified in Table 1, the animal cost associated with conducting the LLNA ranges from \$160.80 to \$301.50. For the GPMT, the cost of one 400 to 450 gram outbred Crl:(Ha)BR Hartley guinea pig, as quoted by Charles River Laboratories, MA, is \$57.25. When the number of animals necessary to conduct the test is factored in, the animal cost associated with conducting the GPMT ranges from \$1,832.00 to \$2,461.75.

**Table 2**

| Test Method | Species, Strain, and Age or Weight | Cost per Animal | Total Animal Cost | Source of Animal |
|---|---|---|---|---|
| LLNA | Mice (CBA/J, 6 weeks old) | $10.05 | $160.80-$301.50 | Jackson Laboratories, Bar Harbor, ME |
| GPMT | Guinea Pigs (Outbred Crl:(Ha)BR Hartley, 400-450 g) | $57.25 | $1,832.00-$2,461.75 | Charles River Laboratories, MA |

Table 3 outlines cost estimates for conducting the LLNA and the GPMT. Illinois Institute of Technology Research Institute, IL (IITRI, 1998) quoted the costs of conducting the LLNA as $6,900 if one chemical is tested and $4,950 each if two chemicals are tested. WIL Research Laboratories, Inc., Ashland, OH (1998) provided a written estimate of $6,000 for conducting the LLNA regardless of the number of chemicals tested. IITRI stated that, in their particular situation, disposal costs were not increased due to the need to dispose of radioactive carcasses.

IITRI's estimate of the cost for conducting the GPMT was $6,000 to $7,000 regardless of the number of chemicals tested (IITRI, 1998). No other estimates were collected for the GPMT.

These cost estimates do not appear to reflect the actual cost to conduct each of the assays, however, judging by the differences in time to conduct each of the tests (Table 1) and the differences in animal costs (Table 2).

**Table 3**

| Test Method | IITRI Estimate (single chemical) | IITRI Estimate (two chemicals) | WIL Research Labs Estimate |
|---|---|---|---|
| LLNA | $6,900 | $4,950 each | $6,000 |
| GPMT | $6,000-7,000 | $6,000-7,000 | not provided |

References

Charles River Laboratories, Inc. 1998. CRL Product Catalogue.

Gerberick, G. F., I. Kimber, and D. A. Basketter. 1998. Sample Protocol: Standard Operating Procedure, the Local Lymph Node Assay (LLNA). (Supplied as a replacement for the protocol provided in the Local Lymph Node Assay ICCVAM Submission).

IITRI. 1998. Phone conversation between Robert House, IITRI, and Bonnie Carson, ILS, Inc. (NICEATM), on June 1, 1998 regarding a comparison of prices between the LLNA and the GPMT.

Jackson Laboratories. 1998. Phone conversation between the customer service representative, Jackson Laboratories, and Karen Haneke, ILS, Inc. (NICEATM), on July 15, 1998 regarding the cost of CBA/J mice.

Klecak, G. 1996. Chapter 34: Test methods for allergic contact dermatitis in animals. In: F. N. Marzulli and H. I. Maibach (Eds.), Dermatotoxicology, 5th ed. Taylor and Francis, Washington, DC. pp. 437-459.

WIL Research Laboratories, Inc. 1998. Written cost proposal for conducting the LLNA prepared by Tom Kern. Received by Karen Haneke, ILS, Inc. (NICEATM) on July 13, 1998, by fax.

# NICEATM Assessment of the Effect of Different Stimulation Index (SI) Levels on Performance of the LLNA

Data on maximal dose tested and maximal SI response for each test substance included in Appendix A were obtained, when available, and used to generate a database capable of being analyzed for the effect of different SI criteria on sensitivity, specificity, positive predictivity, negative predictivity, and accuracy for the LLNA. The revised list, containing only chemicals were SI data were located, and for which guinea pig and/or human data were available, is attached. Multiple entries (highlighted in the list) for the same test substance were included where multiple tests had been conducted. Where the same data were present in multiple citations, only the earliest citation is provided. Arbitrary fold-increase SI criteria for a positive call (i.e., 4.0, 3.5, 2.4, 2.0) in addition to the standard increase SI criteria of 3.0 were used to distinguish a positive response from a negative one. The resulting calls were used to compare the sensitivity, specificity, positive predictivity, negative predictivity, and accuracy of the LLNA versus:

- The Guinea Pig Maximization Test (GPMT)/Buehler Assay (BA)

- Guinea Pig Tests (GPT) (i.e., GPMT/BA plus nonstandard guinea pig tests)

- Human Data, which included Human Maximization Test (HMT) results plus substances used as Human Patch Test Allergens.

The results of these analyses are presented in the accompanying table.

In making these comparisons and to be consistent with the previous evaluation, (1) discordant LLNA results (i.e., where multiple tests were conducted, with some positive and some negative calls) which could not be reconciled by inspection, were classified as negative; (2) equivocal HMT results were classified as positive; and (3) in cases where a negative result was recorded for the HMT but the substance was used as a HPTA, the chemical was classified as positive for human senistization. In regard to item (1), one data set was omitted from each of 3 chemicals (cinnamic aldehyde, formaldehyde, sodium lauryl sulfate) as indicated in accompanying data list, because the low response was associated with a maximum dose considerable lower than that used in the other tests.

The resulting analyses indicates that an SI of 3.0 is a reasonable criteria for classifying an LLNA response as positive.

Effect of Different Stimulation Index (SI) Levels on Sensitivity, Specificity, Positive Predictivity, Negative Predictivity, and Accuracy of LLNA

| Comparison | # of Comparisons | SI Level | Sensitivity % | Sensitivity Ratio | Specificity % | Specificity Ratio | Positive Predictivity % | Positive Predictivity Ratio | Negative Predictivity % | Negative Predictivity Ratio | Accuracy % | Accuracy Ratio |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LLNA vs GPT | 105 | >4.0 | 77% | (59/77) | 82% | (23/28) | 92% | (61/64) | 56% | (25/41) | 78% | (82/105) |
| | | >3.5 | 78% | (60/77) | 79% | (22/28) | 91% | (62/66) | 56% | (24/39) | 78% | (82/105) |
| | | >3.0 | 79% | (61/77) | 79% | (22/28) | 91% | (63/67) | 58% | (24/38) | 79% | (83/105) |
| | | >2.5 | 81% | (62/77) | 68% | (19/28) | 87% | (64/71) | 56% | (20/34) | 77% | (81/105) |
| | | >2.0 | 83% | (64/77) | 64% | (18/28) | 86% | (66/74) | 58% | (16/31) | 78% | (82/105) |
| LLNA vs Human | 60 | >4.0 | 64% | (38/59) | 80% | (4/5) | 97% | (38/39) | 16% | (4/25) | 66% | (42/64) |
| | | >3.5 | 68% | (40/59) | 60% | (3/5) | 95% | (40/42) | 14% | (3/22) | 67% | (43/64) |
| | | >3.0 | 69% | (41/59) | 60% | (3/5) | 95% | (41/43) | 14% | (3/21) | 69% | (44/64) |
| | | >2.5 | 76% | (45/59) | 40% | (2/5) | 94% | (45/48) | 13% | (2/16) | 73% | (47/64) |
| | | >2.0 | 78% | (46/59) | 20% | (1/5) | 92% | (46/50) | 7% | (1/14) | 63% | (47/64) |

LLNA = Local Lymph Node Assay; GPMT = Guinea Pig Maximization Test; BA = Buehler Assay; GPT includes GPMT/BT plus nonstandard Guinea pig tests; Human includes Human Maximization Test results and substances used as Human Patch Test Allergens.

| Chemical Name | >4 | >3.5 | >3 | >2.5 | >2 | Max Dose (%) | Max. Increase | GPMT/BT | HMT | HPTA | LLNA References |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Abietic acid// Sylvic acid | + | + | + | + | + | 25 | 6.4 | + | | + | $BAS92-65 |
| Abietic acid// Sylvic acid | + | + | + | + | + | 25 | 5.9 | + | | + | $BAS91-30 |
| Abietic acid// Sylvic acid | + | + | + | + | + | 25 | 5.2 | + | | + | $ASH95-177 |
| Abietic acid// Sylvic acid | + | + | + | + | + | 25 | 4.2 | + | | + | $BAS91-30 |
| Abietic acid// Sylvic acid | - | - | - | + | + | 25 | 2.9 | + | | + | $BAS91-30 |
| 3-Acetylphenyl benzoate | + | + | + | + | + | 25 | 7.1 | + | | | $ASH95-177 |
| 4-Aminobenzoic acid// p-Aminobenzoic acid// PABA | - | - | - | - | - | 10 | 1.6 | - | - | + | $LOV96-141 |
| 4-Aminobenzoic acid// p-Aminobenzoic acid// PABA | - | - | - | - | - | 10 | 1.6 | - | - | + | $LOV96-141 |
| 4-Aminobenzoic acid// p-Aminobenzoic acid// PABA | - | - | - | - | - | 10 | 1.4 | - | - | + | $LOV96-141 |
| 4-Aminobenzoic acid// p-Aminobenzoic acid// PABA | - | - | - | - | - | 10 | 1.2 | - | - | + | $LOV96-141 |
| 4-Aminobenzoic acid// p-Aminobenzoic acid// PABA | - | - | - | - | - | 10 | 1.1 | - | - | + | $BAS94-543 |
| 4-Aminobenzoic acid// p-Aminobenzoic acid// PABA | - | - | - | - | - | 10 | 1.1 | - | - | + | $LOV96-141 |
| 3-Aminophenol// m-Aminophenol// 3-Hydroxyaniline | + | + | + | + | + | 10 | 9.7 | + nonstd | | + | $BAS91-30 |
| 3-Aminophenol// m-Aminophenol// 3-Hydroxyaniline | + | + | + | + | + | 10 | 8.1 | + nonstd | | + | $BAS91-30 |
| 2-Aminophenol// o-Aminophenol// 2-Hydroxyaniline | + | + | + | + | + | 2.5 | 7.4 | + nonstd | | | $ASH95-177 |
| 3-Aminophenol// m-Aminophenol// 3-Hydroxyaniline | + | + | + | + | + | 10 | 7.1 | + nonstd | | + | $BAS91-30 |
| 3-Aminophenol// m-Aminophenol// 3-Hydroxyaniline | + | + | + | + | + | 10 | 5.7 | + nonstd | | + | $BAS91-30 |
| Ammonium tetrachloroplatinate// Ammonium platinous chloride | + | + | + | + | + | 10 | 18.1 | + | | + | $BAS92-65 |
| Ammonium thioglycolate// Ammonium mercaptoacetate | + | + | + | + | + | 50 | 4.0 | - | | + | Appen B |
| Aniline// Benzenamine | - | - | - | + | + | 50 | 2.9 | + | + | | $BAS92-65 |
| Aniline// Benzenamine | - | - | - | + | + | 50 | 2.6 | + | + | | $BAS91-30 |
| Aniline// Benzenamine | - | - | - | + | + | 50 | 2.5 | + | + | | $BAS91-30 |
| Aniline// Benzenamine | - | - | - | - | - | 50 | 1.0 | + | + | | $BAS91-30 |
| Benzalkonium chloride | - | - | - | + | + | 2.5 | 2.5 | - | | + | $GER97-97 |
| Benzene-1,3,4-tricarboxylic anhydride// Trimellitic anhydride | + | + | + | + | + | 10 | 50.5 | + | | | $BAS92-65 |
| 1,2-Benzisothiazolin-3-one | + | + | + | + | + | 50 | 4.9 | + | | + | $BOT91-172 |
| Benzocaine | + | + | + | + | + | 20 | 7.7 | + | + | + | $KIM89-215 |
| Benzocaine | - | - | - | + | + | 25 | 2.9 | + | + | + | $MON94-22 |
| Benzocaine | - | - | - | - | + | 25 | 2.4 | + | + | + | Append B |
| Benzocaine | - | - | - | - | + | 50 | 2.3 | + | + | + | $KIM89-203 |
| Benzocaine | - | - | - | - | - | 50 | 1.8 | + | + | + | $KIM91-203 |
| Benzocaine | - | - | - | - | - | 50 | 1.5 | + | + | + | $KIM89-203 |
| Benzocaine | - | - | - | - | - | 50 | 1.4 | + | + | + | $KIM91-203 |
| Benzoquinone// p-Quinone// 1,4-Cyclohexadienedione | + | + | + | + | + | 2.5 | 52.3 | + | | | $BAS92-65 |
| Benzoyl chloride | + | + | + | + | + | 5 | 25.9 | + | | + | $ASH95-177 |
| Benzoyloxy-3,5-benzenedicarboxylic acid// 5-Benzoyloxyisophthalic acid | - | - | - | - | - | 10 | 1.1 | -nonstd | | | Append B |
| Benzoyl peroxide | + | + | + | + | + | 10 | 26.5 | + | | + | $KIM98-563 |
| Benzoyl peroxide | + | + | + | + | + | 10 | 21.8 | + | | + | $KIM98-563 |
| Benzoyl peroxide | + | + | + | + | + | 10 | 18.6 | + | | + | $KIM98-563 |
| Benzoyl peroxide | + | + | + | + | + | 10 | 17.3 | + | | + | $KIM98-563 |
| Benzoyl peroxide | + | + | + | + | + | 10 | 16.1 | + | | + | $KIM98-563 |
| Beryllium sulfate | + | + | + | + | + | 10 | 9.4 | + | + | | $BAS94-543 |
| 1-Bromododecane// Lauryl bromide | + | + | + | + | + | 25 | 17.6 | + nonstd | | | $ASH95-177 |
| 1-Bromododecane// Lauryl bromide | + | + | + | + | + | 25 | 4.5 | + nonstd | | | $BAS92-137 |
| 1-Bromohexadecane// n-Hexadecyl bromide// Palmityl bromide// Cetyl bromide | + | + | + | + | + | 50 | 16.8 | + | | | $BAS92-137 |
| 1-Bromohexadecane// n-Hexadecyl bromide// Palmityl bromide// Cetyl bromide | + | + | + | + | + | 25 | 15.6 | + | | | $BAS92-137 |
| 1-Bromohexane// n-Hexyl bromide | + | + | + | + | + | 50 | 18.6 | + nonstd | | | Data supplied by sponsor |
| 1-Bromohexane// n-Hexyl bromide | - | - | - | - | + | 25 | 2.1 | + nonstd | | | $BAS92-137 |
| 1-Bromohexane// n-Hexyl bromide | - | - | - | - | - | 25 | 1.4 | + nonstd | | | Data supplied by sponsor |
| Butyl glycidyl ether | + | + | + | + | + | 50 | 5.6 | + | + | | $BAS94-542 |
| Chloramine T | + | + | + | + | + | 25 | 10.7 | + | | + | $BAS92-65 |

| Chemical Name | >4 | >3.5 | >3 | >2.5 | >2 | Max Dose (%) | Max. Increase | GPMT/BT | HMT | HPTA | LLNA References |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 4-Chloroaniline | + | + | + | + | + | 25 | 4.5 | + | | | Data supplied by sponsor |
| 4-Chloroaniline | - | - | + | + | + | 10 | 3.3 | + | | | $SCH92-217 |
| 4-Chloroaniline | - | - | - | + | + | 10 | 2.5 | + | | | $SCH92-217 |
| 4-Chloroaniline | - | - | - | - | - | 10 | 1.8 | + | | | $SCH92-217 |
| 4-Chloroaniline | - | - | - | - | - | 10 | 1.8 | + | | | $BAS92-65 |
| 4-Chloroaniline | - | - | - | - | - | 25 | 1.4 | + | | | Data supplied by sponsor |
| Chlorobenzene | - | - | - | - | - | 25 | 1.7 | - | | | $ASH95-177 |
| 5-Chloro-2-methyl-4-isothiazolin-3-one [no locants & different CASRN in list] | + | + | + | + | + | 0.1 | 27.7 | + | | + | BOT91-172 |
| Chlorpromazine | + | + | + | + | + | 50 | 8.9 | + nonstd | + | | $BAS94-543 |
| Cinnamic aldehyde// cinnamaldehyde | + | + | + | + | + | 25 | 15.4 | + | + | + | $BAS92-65 |
| Cinnamic aldehyde// cinnamaldehyde | + | + | + | + | + | 25 | 12.8 | + | + | + | $MON94-22 |
| Cinnamic aldehyde// cinnamaldehyde | + | + | + | + | + | 5 | 9.8 | + | + | + | $KIM89-215 |
| Cinnamic aldehyde// cinnamaldehyde | - | - | + | + | + | 2 | 3.3 | + | + | + | $MAU91-209 |
| Citral// 3,7-Dimethyl-2,6-octadienal// Geranial-Neral mixture | + | + | + | + | + | 25 | 20.5 | + | + | | $BAS91-30 |
| Citral// 3,7-Dimethyl-2,6-octadienal// Geranial-Neral mixture | + | + | + | + | + | 25 | 9.3 | + | + | | $BAS91-30 |
| Citral// 3,7-Dimethyl-2,6-octadienal// Geranial-Neral mixture | + | + | + | + | + | 50 | 9.3 | + | + | | $BAS94-543 |
| Citral// 3,7-Dimethyl-2,6-octadienal// Geranial-Neral mixture | + | + | + | + | + | 25 | 6.2 | + | + | | $BAS91-30 |
| Citral// 3,7-Dimethyl-2,6-octadienal// Geranial-Neral mixture | + | + | + | + | + | 25 | 4.7 | + | + | | $BAS91-30 |
| Cobalt chloride | + | + | + | + | + | 5 | 13.6 | + | + | + | Append B |
| Cobalt chloride | - | + | + | + | + | 2.5 | 3.7 | + | + | + | $BAS92-65 |
| Cocoamidopropyl betaine//CAPB | + | + | + | + | + | 25 | 11.3 | + | | + | $ASH95-177 |
| Copper chloride// Cuprous chloride | + | + | + | + | + | 5 | 13.8 | - | | | $BAS92-65 |
| Dextran | - | - | - | - | - | 10 | 1.5 | - | | | $BAS92-65 |
| 2,4-Dichloronitrobenzene | - | - | - | - | + | 1 | 2.2 | - | | | $BAS96-55 |
| Diethylenetriamine | + | + | + | + | + | 10 | 12.1 | + | + | + | $BAS94-543 |
| Dimethyl isophthalate | - | - | - | - | - | 25 | 1.8 | - | | | $SCH92-217 |
| Dimethyl isophthalate | - | - | - | - | - | 50 | 1.6 | - | | | $SCH92-217 |
| Dimethyl isophthalate | - | - | - | - | - | 25 | 1.5 | - | | | $SCH92-217 |
| Dimethyl isophthalate | - | - | - | - | - | 25 | 1.0 | - | | | $BAS92-65 |
| 5,5-Dimethyl-3-(mesyloxymethyl)dihydro-2(3H)-furanone | - | - | - | - | - | 13.66 | 1.5 | + nonstd | | | Append B |
| 5,5-Dimethyl-3-(mesyloxymethyl)dihydro-2(3H)-furanone | - | - | - | - | - | 20 | 1.2 | + nonstd | | | $ASH95-177 |
| 5,5-Dimethyl-3-(methoxybenzenesulfonyloxymethyl)dihydro-2(3H)-furanone | - | - | - | - | - | 20 | 1.2 | + nonstd | | | Unpublished Unilever data |
| 5,5-Dimethyl-3-methylenedihydro-2(3H)-furanone | + | + | + | + | + | 8 | 9.2 | - nonstd | | | $ASH95-177 |
| 5,5-Dimethyl-3-(nitrobenzenesulfonyloxymethyl)dihydro-2(3H)-furanone | - | - | - | - | - | 20 | 0.9 | + nonstd | | | $ASH95-177 |
| 5,5-Dimethyl-3-(thiocyanatomethyl)dihydro-2(3H)-furanone | + | + | + | + | + | 13 | 8.6 | + nonstd | | | $ASH95-177 |
| 5,5-Dimethyl-3-(tosyloxymethyl)dihydro-2(3H)-furanone | - | - | - | - | - | 18 | 1.4 | - nonstd | | | $ASH95-177 |
| 2,4-Dinitrochlorobenzene// DNCB | + | + | + | + | + | 0.25 | 78.0 | + | | | $LOV96-141 |
| 2,4-Dinitrochlorobenzene// DNCB | + | + | + | + | + | 0.25 | 43.9 | + | | | $KIM95-63 |
| 2,4-Dinitrochlorobenzene// DNCB | + | + | + | + | + | 2 | 41.5 | + | | | $KIM89-215 |
| 2,4-Dinitrochlorobenzene// DNCB | + | + | + | + | + | 0.25 | 40.9 | + | | | $KIM95-63 |
| 2,4-Dinitrochlorobenzene// DNCB | + | + | + | + | + | 0.25 | 38.0 | + | | | $LOV96-141 |
| 2,4-Dinitrochlorobenzene// DNCB | + | + | + | + | + | 0.25 | 35.5 | + | | | $KIM95-63 |
| 2,4-Dinitrochlorobenzene// DNCB | + | + | + | + | + | 1 | 29.5 | + | | | $HIL96-571 |
| 2,4-Dinitrochlorobenzene// DNCB | + | + | + | + | + | 0.25 | 25.0 | + | | | $LOV96-141 |
| 2,4-Dinitrochlorobenzene// DNCB | + | + | + | + | + | 0.25 | 25.0 | + | | | $LOV96-141 |
| 2,4-Dinitrochlorobenzene// DNCB | + | + | + | + | + | 0.1 | 24.0 | + | | | $BAS92-65 |
| 2,4-Dinitrochlorobenzene// DNCB | + | + | + | + | + | 0.5 | 23.0 | + | | | $MON94-22 |
| 2,4-Dinitrochlorobenzene// DNCB | + | + | + | + | + | 0.25 | 22.5 | + | | | $KIM95-63 |
| 2,4-Dinitrochlorobenzene// DNCB | + | + | + | + | + | 0.1 | 21.1 | + | | | $GER92-438 |
| 2,4-Dinitrochlorobenzene// DNCB | + | + | + | + | + | 0.1 | 15.0 | + | | | $ASH95-177 |
| 2,4-Dinitrochlorobenzene// DNCB | + | + | + | + | + | 0.25 | 13.0 | + | | | $LOV96-141 |
| 2,4-Dinitrochlorobenzene// DNCB | + | + | + | + | + | 0.25 | 11.5 | + | | | $KIM95-63 |
| 2,4-Dinitrothiocyanobenzene// 2,4-Dinitrophenyl thiocyanate// Nirit | + | + | + | + | + | 2 | 10.3 | + | | | $KIM89-274 |

| Chemical Name | >4 | >3.5 | >3 | >2.5 | >2 | Max Dose (%) | Max. Increase | GPMT/BT | HMT | HPTA | LLNA References |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Disodium benzoyloxy-3,5-benzenedicarboxylate | - | - | - | - | + | 25 | 2.1 | - | | | $ASH95-177 |
| Disodium 1,2-diheptanoyloxy-3,5-benzenedisulfonate | + | + | + | + | + | 25 | 15.4 | + nonstd | | | $ASH95-177 |
| Dodecyl methanesulfonate// Lauryl methanesulfonate | + | + | + | + | + | 25 | 9.0 | + nonstd | | | $ASH95-177 |
| Ethylenediamine | - | - | - | - | - | 2.5 | 1.7 | + | | + | $KIM98-563 |
| Ethylenediamine | - | - | - | - | - | 2.5 | 1.6 | + | | + | $KIM98-563 |
| Ethylenediamine | - | - | - | - | - | 2.5 | 1.5 | + | | + | $KIM98-563 |
| Ethylenediamine | - | - | - | - | - | 2.5 | 0.9 | + | | + | $KIM98-563 |
| Ethylenediamine | - | - | - | - | - | 2.5 | 0.7 | + | | + | $KIM98-563 |
| Ethylene glycol dimethacrylate// EGDMA | + | + | + | + | + | 50 | 9.2 | - | | + | Append. B |
| Eugenol// Allylguaiacol// 4-Allyl-2-methoxyphenol | + | + | + | + | + | 100 | 70.3 | + | | + | $KIM91-203 |
| Eugenol// Allylguaiacol// 4-Allyl-2-methoxyphenol | + | + | + | + | + | 50 | 17.0 | + | | + | $LOV96-141 |
| Eugenol// Allylguaiacol// 4-Allyl-2-methoxyphenol | + | + | + | + | + | 50 | 16.0 | + | | + | $LOV96-141 |
| Eugenol// Allylguaiacol// 4-Allyl-2-methoxyphenol | + | + | + | + | + | 50 | 14.1 | + | | + | $KIM91-203 |
| Eugenol// Allylguaiacol// 4-Allyl-2-methoxyphenol | + | + | + | + | + | 50 | 12.4 | + | | + | $LOV96-141 |
| Eugenol// Allylguaiacol// 4-Allyl-2-methoxyphenol | + | + | + | + | + | 75 | 10.6 | + | | + | $GER92-438 |
| Eugenol// Allylguaiacol// 4-Allyl-2-methoxyphenol | + | + | + | + | + | 100 | 10.2 | + | | + | $KIM91-203 |
| Eugenol// Allylguaiacol// 4-Allyl-2-methoxyphenol | + | + | + | + | + | 50 | 9.6 | + | | + | $LOV96-141 |
| Eugenol// Allylguaiacol// 4-Allyl-2-methoxyphenol | + | + | + | + | + | 100 | 9.3 | + | | + | $KIM91-203 |
| Eugenol// Allylguaiacol// 4-Allyl-2-methoxyphenol | + | + | + | + | + | 50 | 6.1 | + | | + | $LOV96-141 |
| Formaldehyde | + | + | + | + | + | 25 | 11.9 | + | + | + | $KIM89-274 |
| Formaldehyde | + | + | + | + | + | 50 | 9.0 | + | + | + | $HIL96-571 |
| Formaldehyde | + | + | + | + | + | 25 | 6.6 | + | + | + | $KIM91-203 |
| Formaldehyde | + | + | + | + | + | 25 | 5.8 | + | + | + | $KIM91-203 |
| Formaldehyde | + | + | + | + | + | 25 | 4.2 | + | + | + | $KIM91-203 |
| Formaldehyde | - | - | - | - | + | 2 | 2.3 | + | + | + | $MAU91-209 |
| Geraniol | - | - | - | + | + | 50 | 2.6 | - | - | + | $BAS94-543 |
| Glyoxal// Oxaldehyde// Ethanedial// Biformyl | + | + | + | + | + | 25 | 18.1 | + | + | | $BAS94-543 |
| Gold chloride | | + | + | + | + | | 17.2 | | + | | $BAS96-985 |
| Hexane | - | - | - | - | + | 100 | 2.2 | | - | | $BAS96-985 |
| Hexylcinnamic aldehyde// H.C.A.// .alpha.-Hexylcinnamaldehyde// 2-(Phenylmethylene)octana | + | + | + | + | + | 50 | 20.0 | + | | | $LOV96-141 |
| Hexylcinnamic aldehyde// H.C.A.// .alpha.-Hexylcinnamaldehyde// 2-(Phenylmethylene)octana | + | + | + | + | + | 50 | 17.0 | + | | | $LOV96-141 |
| Hexylcinnamic aldehyde// H.C.A.// .alpha.-Hexylcinnamaldehyde// 2-(Phenylmethylene)octana | + | + | + | + | + | 50 | 17.0 | + | | | $LOV96-141 |
| Hexylcinnamic aldehyde// H.C.A.// .alpha.-Hexylcinnamaldehyde// 2-(Phenylmethylene)octana | + | + | + | + | + | 50 | 16.0 | + | | | $LOV96-141 |
| Hexylcinnamic aldehyde// H.C.A.// .alpha.-Hexylcinnamaldehyde// 2-(Phenylmethylene)octana | + | + | + | + | + | 50 | 14.0 | + | | | $LOV96-141 |
| Hexylcinnamic aldehyde// H.C.A.// .alpha.-Hexylcinnamaldehyde// 2-(Phenylmethylene)octana | + | + | + | + | + | 50 | 10.0 | + | | | $BAS93-63 |
| Hexylcinnamic aldehyde// H.C.A.// .alpha.-Hexylcinnamaldehyde// 2-(Phenylmethylene)octana | + | + | + | + | + | 50 | 4.6 | + | | | $BAS93-63 |
| Hydrocortisone// Cortisol | - | - | - | - | - | 10 | 0.3 | | - | + | $BAS96-985 |
| Hydroquinone// Quinol [separate entry in submission] | + | + | + | + | + | 2.5 | 33.4 | + nonstd | | + | $KIM98-563 |
| Hydroquinone// Quinol [separate entry in submission] | + | + | + | + | + | 2.5 | 23.2 | + nonstd | | + | $KIM98-563 |
| Hydroquinone// Quinol [separate entry in submission] | + | + | + | + | + | 2.5 | 16.4 | + nonstd | | + | $BAS92-65 |
| Hydroquinone// Quinol [separate entry in submission] | + | + | + | + | + | 2.5 | 15.0 | + nonstd | | + | $KIM98-563 |
| Hydroquinone// Quinol [separate entry in submission] | + | + | + | + | + | 2.5 | 13.1 | + nonstd | | + | $KIM98-563 |
| Hydroquinone// Quinol [separate entry in submission] | + | + | + | + | + | 2.5 | 12.2 | + nonstd | | + | $KIM98-563 |
| 4-Hydroxybenzoic acid | - | - | - | - | - | 25 | 1.5 | + | | | $BAS92-65$ |
| 4-Hydroxybenzoic acid | - | - | - | - | - | 25 | 1.5 | + | | | $SCH92-217 |
| 4-Hydroxybenzoic acid | - | - | - | - | - | 25 | 1.0 | + | | | $SCH92-217 |
| 4-Hydroxybenzoic acid | - | - | - | - | - | 10 | 0.8 | + | | | $SCH92-217 |
| Hydroxycitronellal | + | + | + | + | + | 100 | 8.5 | + | + | + | $BAS92-65 |
| Hydroxycitronellal | + | + | + | + | + | 50 | 6.7 | + | + | + | $BAS94-543 |
| Hydroxycitronellal | + | + | + | + | + | 25 | 3.4 | + | + | + | $MON94-22 |
| 2-Hydroxyethyl acrylate// HEA | + | + | + | + | + | 25 | 18.1 | + | | + | $SCH92-217 |
| 2-Hydroxyethyl acrylate// HEA | + | + | + | + | + | 50 | 11.7 | + | | + | $SCH92-217 |
| 2-Hydroxyethyl acrylate// HEA | + | + | + | + | + | 50 | 9.9 | + | | + | $SCH92-217 |

| Chemical Name | >4 | >3.5 | >3 | >2.5 | >2 | Max Dose (%) | Max. Increase | GPMT/BT | HMT | HPTA | LLNA References |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2-Hydroxyethyl acrylate// HEA | + | + | + | + | + | 25 | 8.2 | + | | + | $BAS92-65 |
| 2-Hydroxypropyl methacrylate// 2-HPMA | - | - | - | - | - | 50 | 1.9 | - | | + | $SCH92-217 |
| 2-Hydroxypropyl methacrylate// 2-HPMA | - | - | - | - | - | 50 | 1.4 | - | | + | $SCH92-217 |
| 2-Hydroxypropyl methacrylate// 2-HPMA | - | - | - | - | - | 50 | 1.3 | - | | + | $BAS92-65 |
| 2-Hydroxypropyl methacrylate// 2-HPMA | - | - | - | - | - | 50 | 1.0 | - | | + | $SCH92-217 |
| Imidazolidinyl urea// Germall 115 | + | + | + | + | + | 50 | 5.5 | + | | + | $BAS92-65 |
| Isoeugenol// 2-Methoxy-4-propenylphenol// 4-Propenylguaiacol | + | + | + | + | + | 10 | 29.5 | + | | + | $KIM91-203 |
| Isoeugenol// 2-Methoxy-4-propenylphenol// 4-Propenylguaiacol | + | + | + | + | + | 10 | 25.3 | + | | + | $KIM91-203 |
| Isoeugenol// 2-Methoxy-4-propenylphenol// 4-Propenylguaiacol | + | + | + | + | + | 10 | 21.3 | + | | + | $KIM91-203 |
| Isoeugenol// 2-Methoxy-4-propenylphenol// 4-Propenylguaiacol | + | + | + | + | + | 10 | 14.6 | + | | + | $KIM91-203 |
| Isoeugenol// 2-Methoxy-4-propenylphenol// 4-Propenylguaiacol | + | + | + | + | + | 10 | 12.9 | + | | + | $ASH95-177 |
| Isoeugenol// 2-Methoxy-4-propenylphenol// 4-Propenylguaiacol | + | + | + | + | + | 10 | 11.0 | + | | + | $LOV96-141 |
| Isoeugenol// 2-Methoxy-4-propenylphenol// 4-Propenylguaiacol | + | + | + | + | + | 10 | 10.0 | + | | + | $LOV96-141 |
| Isoeugenol// 2-Methoxy-4-propenylphenol// 4-Propenylguaiacol | + | + | + | + | + | 10 | 7.2 | + | | + | $LOV96-141 |
| Isoeugenol// 2-Methoxy-4-propenylphenol// 4-Propenylguaiacol | + | + | + | + | + | 10 | 6.8 | + | | + | $LOV96-141 |
| Isoeugenol// 2-Methoxy-4-propenylphenol// 4-Propenylguaiacol | + | + | + | + | + | 10 | 4.1 | + | | + | $LOV96-141 |
| Isopropanol// Isopropyl alcohol// 2-Propanol | - | - | - | - | - | 50 | 1.7 | - | | | $BAS96-985 |
| Kanamycin | - | - | - | - | + | 25 | 2.2 | - nonstd | + | + | $BAS96-985 |
| Lactic acid// 2-Hydroxypropanoic acid | - | - | - | - | + | 25 | 2.2 | - | | | BAS98-327 |
| 2-Mercaptobenzothiazole | + | + | + | + | + | 25 | 17.1 | + | + | + | $MON94-22 |
| 2-Mercaptobenzothiazole | + | + | + | + | + | 50 | 8.9 | + | + | + | $SCH92-217 |
| 2-Mercaptobenzothiazole | + | + | + | + | + | | 8.6 | + | + | + | $BAS93-63 |
| 2-Mercaptobenzothiazole | + | + | + | + | + | 50 | 8.1 | + | + | + | $SCH92-217 |
| 2-Mercaptobenzothiazole | + | + | + | + | + | 50 | 5.5 | + | + | + | $BAS92-65 |
| 2-Mercaptobenzothiazole | + | + | + | + | + | | 5.0 | + | + | + | $BAS93-63 |
| 2-Mercaptobenzothiazole | + | + | + | + | + | 50 | 4.8 | + | + | + | $SCH92-217 |
| Mercuric chloride// Corrosive sublimate | + | + | + | + | + | 10 | 19.9 | + | + | + | $BAS94-543 |
| 4-Methylaminophenol sulfate// Metol// p-Hydroxymethylaniline sulfate | + | + | + | + | + | 2.5 | 6.7 | + | | + | $BAS92-65 |
| 6-Methylcoumarin// 6-MC | - | - | - | - | - | 25 | 1.2 | - | - | + | $SCH92-249 |
| 6-Methylcoumarin// 6-MC | - | - | - | - | - | 25 | 1.1 | - | - | + | $ASH95-177 |
| Methyl dodecanesulfonate | + | + | + | + | + | 5 | 48.6 | + | | | $BAS92-65 |
| Methyl dodecanesulfonate | + | + | + | + | + | 25 | 46.3 | + | | | $ASH95-177 |
| Methyl hexadecenesulfonate | + | + | + | + | + | 25 | 35.4 | + nonstd | | | $ASH95-177 |
| Methyl salicylate// Oil of wintergreen// 2-Hydroxybenzoic acid methyl ester | - | - | - | + | + | 20 | 2.9 | - | - | | $KIM95-63 |
| Methyl salicylate// Oil of wintergreen// 2-Hydroxybenzoic acid methyl ester | - | - | - | + | + | 5 | 2.7 | - | - | | $KIM91-203 |
| Methyl salicylate// Oil of wintergreen// 2-Hydroxybenzoic acid methyl ester | - | - | - | + | + | 100 | 2.6 | - | - | | $BAS98-327 |
| Methyl salicylate// Oil of wintergreen// 2-Hydroxybenzoic acid methyl ester | - | - | - | - | + | 20 | 2.3 | - | - | | $KIM95-63 |
| Methyl salicylate// Oil of wintergreen// 2-Hydroxybenzoic acid methyl ester | - | - | - | - | + | 25 | 2.2 | - | - | | $ASH95-177 |
| Methyl salicylate// Oil of wintergreen// 2-Hydroxybenzoic acid methyl ester | - | - | - | - | + | 20 | 2.1 | - | - | | $KIM95-63 |
| Methyl salicylate// Oil of wintergreen// 2-Hydroxybenzoic acid methyl ester | - | - | - | - | + | 20 | 2.0 | - | - | | $KIM98-563 |
| Methyl salicylate// Oil of wintergreen// 2-Hydroxybenzoic acid methyl ester | - | - | - | - | + | 20 | 2.0 | - | - | | $KIM98-563 |
| Methyl salicylate// Oil of wintergreen// 2-Hydroxybenzoic acid methyl ester | - | - | - | - | - | 20 | 1.9 | - | - | | $KIM95-63 |
| Methyl salicylate// Oil of wintergreen// 2-Hydroxybenzoic acid methyl ester | - | - | - | - | - | 20 | 1.9 | - | - | | $KIM98-563 |
| Methyl salicylate// Oil of wintergreen// 2-Hydroxybenzoic acid methyl ester | - | - | - | - | - | 20 | 1.6 | - | - | | $KIM98-563 |
| Methyl salicylate// Oil of wintergreen// 2-Hydroxybenzoic acid methyl ester | - | - | - | - | - | 20 | 1.4 | - | - | | $KIM98-563 |
| Methyl salicylate// Oil of wintergreen// 2-Hydroxybenzoic acid methyl ester | - | - | - | - | - | 5 | 1.3 | - | - | | $KIM91-203 |
| Methyl salicylate// Oil of wintergreen// 2-Hydroxybenzoic acid methyl ester | - | - | - | - | - | 5 | 1.2 | - | - | | $KIM91-203 |
| Methyl salicylate// Oil of wintergreen// 2-Hydroxybenzoic acid methyl ester | - | - | - | - | - | 5 | 1.1 | - | - | | $KIM91-203 |
| Methyl salicylate// Oil of wintergreen// 2-Hydroxybenzoic acid methyl ester | - | - | - | - | - | 20 | 1.1 | - | - | | $KIM95-63 |
| Methyl salicylate// Oil of wintergreen// 2-Hydroxybenzoic acid methyl ester | - | - | - | - | - | 5 | 0.8 | - | - | | $GER92-438 |
| Methyl salicylate// Oil of wintergreen// 2-Hydroxybenzoic acid methyl ester | - | - | - | - | - | 25 | 0.5 | - | - | | $KIM91-203 |
| 2-Methyl-4,5-trimethylene-4-isothiazolin-3-one | + | + | + | + | + | 30 | 7.0 | + | | | $ASH95-177 |
| Musk ambrette | + | + | + | + | + | 25 | 8.2 | - | | + | $SCH92-249 |

| Chemical Name | >4 | >3.5 | >3 | >2.5 | >2 | Max Dose (%) | Max. Increase | GPMT/BT | HMT | HPTA | LLNA References |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Musk ambrette | + | + | + | + | + | 25 | 6.5 | - | | + | $SCH92-249 |
| Neomycin sulfate | - | - | - | - | - | 25 | 1.1 | - | + | + | Append B |
| Neomycin sulfate | - | - | - | - | - | 25 | 1.0 | - | + | + | $BAS94-543 |
| Nickel chloride | - | - | - | - | + | 5 | 2.4 | + | | | $BAS92-65 |
| Nickel sulfate | - | - | - | - | + | 10 | 2.0 | + | + | + | $SCH92-217 |
| Nickel sulfate | - | - | - | - | - | 2.5 | 1.5 | + | + | + | $BAS92-65 |
| Nickel sulfate | - | - | - | - | - | 10 | 1.4 | + | + | + | $SCH92-217 |
| Nickel sulfate | - | - | - | - | - | 25 | 0.8 | + | + | + | $SCH92-217 |
| Nickel sulfate | - | - | - | - | - | 10 | 0.7 | + | + | + | $SCH92-217 |
| 4-Nitrobenzyl chloride// 1-(Chloromethyl)-4-nitrobenzene | + | + | + | + | + | 5 | 40.0 | + nonstd | | | $ASH95-177 |
| 4-Nitroso-N,N-dimethylaniline// N,N-Dimethyl-4-nitrosobenzenamine | + | + | + | + | + | 10 | 60.4 | + | | | $KIM89-215 |
| 4-Nitroso-N,N-dimethylaniline// N,N-Dimethyl-4-nitrosobenzenamine | + | + | + | + | + | 2 | 19.7 | + | | | $MAU91-209 |
| Octadecyl methanesulfonate// Stearyl methanesulfonate | - | - | - | - | - | 10 | 1.2 | + nonstd | | | $ASH95-177 |
| Oxazolone// 4-Ethoxymethylene-2-phenyloxazol-5-one | + | + | + | + | + | 2 | 93.0 | + | | | $MAU91-209 |
| Oxazolone// 4-Ethoxymethylene-2-phenyloxazol-5-one | + | + | + | + | + | 1 | 63.0 | + | | | $KIM89-215 |
| Oxazolone// 4-Ethoxymethylene-2-phenyloxazol-5-one | + | + | + | + | + | 0.05 | 59.0 | + | | | $LOV96-141 |
| Oxazolone// 4-Ethoxymethylene-2-phenyloxazol-5-one | + | + | + | + | + | 0.05 | 55.2 | + | | | $GER92-438 |
| Oxazolone// 4-Ethoxymethylene-2-phenyloxazol-5-one | + | + | + | + | + | 0.5 | 44.6 | + | | | $ASH95-177 |
| Oxazolone// 4-Ethoxymethylene-2-phenyloxazol-5-one | + | + | + | + | + | 0.05 | 33.0 | + | | | $LOV96-141 |
| Oxazolone// 4-Ethoxymethylene-2-phenyloxazol-5-one | + | + | + | + | + | 0.5 | 32.0 | + | | | $MON94-22 |
| Oxazolone// 4-Ethoxymethylene-2-phenyloxazol-5-one | + | + | + | + | + | 0.05 | 23.0 | + | | | $LOV96-141 |
| Oxazolone// 4-Ethoxymethylene-2-phenyloxazol-5-one | + | + | + | + | + | 0.05 | 13.0 | + | | | $LOV96-141 |
| Oxazolone// 4-Ethoxymethylene-2-phenyloxazol-5-one | + | + | + | + | + | 0.05 | 8.9 | + | | | $LOV96-141 |
| Penicillin G | + | + | + | + | + | 50 | 17.0 | + | + | | $SCH92-217 |
| Penicillin G | + | + | + | + | + | 50 | 8.9 | + | + | | $BAS92-65 |
| Penicillin G | + | + | + | + | + | 25 | 8.9 | + | + | | $SCH92-217 |
| Penicillin G | + | + | + | + | + | 50 | 6.6 | + | + | | $KIM98-563 |
| Penicillin G | + | + | + | + | + | 50 | 6.5 | + | + | | $SCH92-217 |
| Penicillin G | + | + | + | + | + | 50 | 4.6 | + | + | | $KIM98-563 |
| Penicillin G | - | + | + | + | + | 50 | 3.6 | + | + | | $KIM98-563 |
| Penicillin G | - | - | + | + | + | 50 | 3.4 | + | + | | $KIM98-563 |
| Penicillin G | - | - | + | + | + | 50 | 3.4 | + | + | | $KIM98-563 |
| Pentachlorophenol// Penta// PCP | + | + | + | + | + | | 5.4 | | + | | $BAS96-985 |
| Phenol// Carbolic acid | - | - | - | - | - | | 1.6 | | - | | $BAS96-985 |
| 4-Phenylenediamine// p-PDA// p-Phenylenediamine | + | + | + | + | + | 10 | 75.3 | + | + | + | $KIM91-203 |
| 4-Phenylenediamine// p-PDA// p-Phenylenediamine | + | + | + | + | + | 10 | 37.4 | + | + | + | $KIM91-203 |
| 4-Phenylenediamine// p-PDA// p-Phenylenediamine | + | + | + | + | + | 10 | 26.5 | + | + | + | $KIM89.215 |
| 4-Phenylenediamine// p-PDA// p-Phenylenediamine | + | + | + | + | + | 5 | 23.7 | + | + | + | $KIM91-203 |
| 4-Phenylenediamine// p-PDA// p-Phenylenediamine | + | + | + | + | + | 10 | 23.3 | + | + | + | $KIM91-203 |
| 4-Phenylenediamine// p-PDA// p-Phenylenediamine | + | + | + | + | + | 10 | 20.4 | + | + | + | $ASH95-177 |
| 3-Phenylenediamine// m-Phenylenediamine | + | + | + | + | + | 10 | 19.2 | + nonstd | | | $ASH95-177 |
| 4-Phenylenediamine// p-PDA// p-Phenylenediamine | + | + | + | + | + | 2 | 16.3 | + | + | + | $MON94-22 |
| 4-Phenylenediamine// p-PDA// p-Phenylenediamine | + | + | + | + | + | 2 | 5.3 | + | + | + | $MAU91-209 |
| Phthalic anhydride | + | + | + | + | + | 10 | 73.1 | + | | | $KIM89-215 |
| Phthalic anhydride | + | + | + | + | + | 10 | 26.0 | + | | | $BAS92-65 |
| Picryl chloride// Trinitrochlorobenzene// TNCB | + | + | + | + | + | 0.1 | 103.3 | + | | | $GER92-438 |
| Picryl chloride// Trinitrochlorobenzene// TNCB | + | + | + | + | + | 2 | 55.8 | + | | | $MAU91-209 |
| Potassium dichromate | + | + | + | + | + | 0.5 | 33.6 | + | + | + | $KIM91-203 |
| Potassium dichromate | + | + | + | + | + | 0.5 | 19.1 | + | + | + | $KIM95-63 |
| Potassium dichromate | + | + | + | + | + | 0.5 | 16.1 | + | + | + | $KIM95-63 |
| Potassium dichromate | + | + | + | + | + | 0.5 | 13.1 | + | + | + | $KIM95-63 |
| Potassium dichromate | + | + | + | + | + | 0.5 | 13.0 | + | + | + | $KIM95-63 |
| Potassium dichromate | + | + | + | + | + | 0.5 | 11.2 | + | + | + | $KIM95-63 |

| Chemical Name | >4 | >3.5 | >3 | >2.5 | >2 | Max Dose (%) | Max. Increase | GPMT/BT | HMT | HPTA | LLNA References |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Potassium dichromate | + | + | + | + | + | 0.5 | 10.4 | + | + | + | $BAS92-65 |
| Potassium dichromate | + | + | + | + | + | 0.5 | 10.1 | + | + | + | $KIM91-203 |
| Potassium dichromate | + | + | + | + | + | 0.5 | 6.9 | + | + | + | $KIM91-203 |
| Potassium dichromate | + | + | + | + | + | 0.5 | 5.4 | + | + | + | $KIM91-203 |
| Propylene glycol// 1,2-Dihydroxypropane// 1,2-Propanediol | - | - | - | - | - | 50 | 1.6 | - | | + | BAS98-327 |
| Propyl gallate// Tenox PG// 3,4,5-Trihydroxybenzoic acid propyl ester | + | + | + | + | + | 25 | 33.6 | + | | + | $BAS92-65 |
| Propylparaben// Propyl 4-hydroxybenzoate | - | - | - | - | + | 25 | 2.1 | - | +/- | + | $BAS91-30 |
| Propylparaben// Propyl 4-hydroxybenzoate | - | - | - | - | + | 25 | 2.0 | - | +/- | + | $BAS91-30 |
| Propylparaben// Propyl 4-hydroxybenzoate | - | - | - | - | - | 25 | 1.6 | - | +/- | + | $BAS91-30 |
| Propylparaben// Propyl 4-hydroxybenzoate | - | - | - | - | - | 25 | 1.5 | - | +/- | + | $BAS91-30 |
| Pyridine | - | + | + | + | + | | 3.9 | | + | | $BAS96-985 |
| Resorcinol// 1,3-Dihydroxybenzene | - | - | - | + | + | 25 | 2.7 | - | - | + | $BAS94-543 |
| Salicylic acid// 2-Hydroxybenzoic acid | - | - | - | + | + | 25 | 2.5 | - | - | | $BAS94-543 |
| Sodium benzoyloxy-2-methoxy-5-benzenesulfonate | + | + | + | + | + | 25 | 7.2 | + nonstd | | | $ASH95-177 |
| Sodium 4-(2-ethylhexyloxycarboxy)benzenesulfonate | + | + | + | + | + | 25 | 24.0 | + nonstd | | | $ASH95-177 |
| Sodium lauryl sulfate// Sodium dodecyl sulfate// SLS// SDS// Irium | + | + | + | + | + | 20 | 8.6 | - | - | | $LOV96-141 |
| Sodium lauryl sulfate// Sodium dodecyl sulfate// SLS// SDS// Irium | + | + | + | + | + | 20 | 8.0 | - | - | | $LOV96-141 |
| Sodium lauryl sulfate// Sodium dodecyl sulfate// SLS// SDS// Irium | + | + | + | + | + | 25 | 7.6 | - | - | | $MON94-22 |
| Sodium lauryl sulfate// Sodium dodecyl sulfate// SLS// SDS// Irium | + | + | + | + | + | 25 | 6.7 | - | - | | $MON94-22 |
| Sodium lauryl sulfate// Sodium dodecyl sulfate// SLS// SDS// Irium | + | + | + | + | + | 20 | 5.3 | - | - | | $LOV96-141 |
| Sodium lauryl sulfate// Sodium dodecyl sulfate// SLS// SDS// Irium | + | + | + | + | + | 25 | 4.2 | - | - | | $BAS94-543 |
| Sodium lauryl sulfate// Sodium dodecyl sulfate// SLS// SDS// Irium | - | + | + | + | + | 20 | 3.6 | - | - | | $LOV96-141 |
| Sodium lauryl sulfate// Sodium dodecyl sulfate// SLS// SDS// Irium | - | + | + | + | + | 20 | 3.5 | - | - | | $LOV96-141 |
| Sodium lauryl sulfate// Sodium dodecyl sulfate// SLS// SDS// Irium | - | - | - | - | - | 2 | 1.0 | - | - | | $MAU91-209 |
| Sodium norbornanacetoxy-4-benzenesulfonate | + | + | + | + | + | 25 | 13.6 | + nonstd | | | $ASH95-177 |
| Sodium 4-sulfophenyl acetate | + | + | + | + | + | 25 | 10.1 | + nonstd | | | $ASH95-177 |
| Streptomycin sulfate | - | - | + | + | + | 50 | 3.2 | + | | | $KIM98-563 |
| Streptomycin sulfate | - | - | - | - | - | 50 | 1.9 | + | | | $KIM98-563 |
| Streptomycin sulfate | - | - | - | - | - | 50 | 1.3 | + | | | $KIM98-563 |
| Streptomycin sulfate | - | - | - | - | - | 50 | 1.3 | + | | | $KIM98-563 |
| Streptomycin sulfate | - | - | - | - | - | 50 | 1.2 | + | | | $KIM98-563 |
| Sulfanilamide// 4-Aminobenzenesulfonamide// p-Anilinesulfonamide// p-Sulfamidoaniline | - | - | - | - | - | 50 | 0.9 | - | + | + | $BAS94-543 |
| Sulfanilic acid// p-Aminobenzenesulfonic acid// p-Anilinesulfonic acid | - | - | - | - | - | 25 | 2.2 | + | | | $BAS92-209 |
| Sulfanilic acid// p-Aminobenzenesulfonic acid// p-Anilinesulfonic acid | - | - | - | - | - | 10 | 2.2 | + | | | Append B |
| Sulfanilic acid// p-Aminobenzenesulfonic acid// p-Anilinesulfonic acid | - | - | - | - | - | 25 | 1.8 | + | | | $BAS92-209 |
| Sulfanilic acid// p-Aminobenzenesulfonic acid// p-Anilinesulfonic acid | - | - | - | - | - | 10 | 1.5 | + | | | $BAS92-65 |
| Sulfanilic acid// p-Aminobenzenesulfonic acid// p-Anilinesulfonic acid | - | - | - | - | - | 25 | 1.3 | + | | | $BAS92-209 |
| Tetrachlorosalicylanilide// 3,5-Dichloro-N-(3,4-dichlorophenyl)-2-hydroxybenzamide// TCS | + | + | + | + | + | 0.5 | 40.5 | + | + | + | $SCH92-249 |
| Tetrachlorosalicylanilide// 3,5-Dichloro-N-(3,4-dichlorophenyl)-2-hydroxybenzamide// TCS | + | + | + | + | + | 1 | 18.0 | + | + | + | $BAS94-543 |
| Tetramethyl thiuram disulfide// Thiram// Bis(dimethylthiocarbamoyl) disulfide | + | + | + | + | + | | 5.1 | + nonstd | + | + | $BAS96-985 |
| 1-Thioglycerol// 3-Mercapto-1,2-propanediol | + | + | + | + | + | 50 | 10.0 | + | + | | $BAS94-543 |
| Toluenediamine bismaleimide | + | + | + | + | + | 10 | 35.3 | + | | +? | $SCH92-217 |
| Toluenediamine bismaleimide | + | + | + | + | + | 25 | 25.7 | + | | +? | $SCH92-217 |
| Toluenediamine bismaleimide | + | + | + | + | + | 25 | 19.1 | + | | +? | $SCH92-217 |
| Toluenediamine bismaleimide | + | + | + | + | + | 25 | 12.2 | + | | +? | $BAS92-65 |
| .alpha.-Trimethylammonium 4-tolyloxy-4-benzenesulfonate | - | - | - | - | + | 25 | 2.2 | + nonstd | | | $ASH95-177 |
| 3,5,5-Trimethylhexanoyl chloride | + | + | + | + | + | 25 | 19.0 | + | | | $ASH95-177 |
| Xylene// Dimethylbenzene (mixture of o-, m-, & p-isomers) | + | + | + | + | + | | 4.2 | | - | | $BAS96-985 |

## NICEATM Assessment of LLNA vs. GPMT/BA Discordant Compounds

As requested, NICEATM has reviewed the LLNA data base in regard to compounds which have tested discordant in the LLNA vs GPMT/BA and for which there is human response information. Information on the six discordant and one potentially discordant (benzalkonium chloride) compounds located are provided in the accompanying table. Notes attached to the table explain the information provided. The results can be summarized as follows:

| Number of Compounds | LLNA Response | GPMT/BT Response | Human Response |
|---|---|---|---|
| 4 | Negative | Positive | Positive |
| 0 | Negative | Positive | Negative |
| 2 | Positive | Negative | Positive |
| 1 | Positive | Negative | Negative |

Discordant Compounds for LLNA vs GPMT/BA Which Have Human Response Information

| Compound | LLNA[1] | SI Values[2] | # of Tests | GPMT/BA | Max. Incidence[3] | HMT | HPTA |
|---|---|---|---|---|---|---|---|
| Aniline// Benzenamine | - | 2.9,2.6,2.5,1.0 | 4 | + | 95% | + | |
| Benzocaine[4] | - | 7.7,2.9,2.3,1.8,1.5,1.4 | 6 | + | 50% | + | + |
| Nickel sulfate[5] | - | 2.0,1.5,1.4,0.8,0.7 | 5 | + | 33% | + | + |
| Sodium lauryl sulfate// Sodium dodecyl sulfate[6] | + | 8.6,8.0,7.6,6.7,5.3,4.2,3.6,2.5 | 8 | - | | - | |
| Benzalkonium chloride[7] | +? | 11.1,2.5 | 2 | - | | | + |
| Ethylenediamine[8] | - | 2.2,1.7,1.6,1.5,0.9,0.7 | 6 | + | | | + |
| Musk ambrette[9] | + | 8.2,6.5 | 2 | - | | | + |

Abbreviations: - = negative call; + = positive call; LLNA = Local Lymph Node Assay; GPMT/BA = Guinea Pig Maximization Test/ Buehler Assay; HMT = Human Maximization Test; HPTA = Human Patch Test Allergen

Notes:

1. LLNA: the call provided is based on the SI data presented for these compounds.
2. SI Values: these are the maximum SI values obtained (ranked from high to low) for the number of LLNA tests conducted.
3. The maximum incidence of responding animals in the GPMT/BA
4. Benzocaine: Sponsor states that GPMT/BA results are +/-. Some have classified as a moderate sensitizer.
   Nonirritant// 40/1135 dermatitis patients (Marzulli & Maibach, 1996), 2/1158 volunteers (Prystowsky et al., 1979)
5. Nickel Sulfate: 2.5% pet. in human patch test: 109/1123 sensitized// 8 showed irritation (Marzulli & Maibach, 1996).
6. SDS: Moderate irritant in 4-hour human patch test (70% of panel [380/544] responded) (Basketter et al., 1998)
7. Benzalkonium chloride was classified as negative for the LLNA submission but the SI for one test was 11.1 and for another 2.5.
   High human skin irritancy potential (52% of panel responded) (Basketter et al., 1998)
8. Ethylenediamine was classified as positive in the submission but the SI was not above 3.0.
   Human response: 66/1120 dermatitis patients (Marzulli & Maibach, 1996)// 5/1158 volunteers (Prystowsky et al., 1979)
9. Musk ambrette: causes photoallergy (Truitt, 1998)

# Comparison of LLNA versus GPMT/BA and Human Data, by Chemical and Product Class

The tabulated LLNA data provided in Appendix A was used to compare, by chemical and product class, the sensitivity, specificity, positive predictivity, negative predictivity, and accuracy of the:

- LLNA versus the Guinea Pig Maximization Test (GPMT)/Buehler Assay (BA);

- LLNA versus Guinea Pig Tests (GPT) (i.e., GPMT/BA plus nonstandard Guinea pig tests);

- LLNA versus human results, which includes Human Maximization Test (HMT) data and substances used as Human Patch Test Allergens (HPTA); and

- GPT versus the human results.

The results of these analysis are presented in the accompanying four tables. Tables 1 - 4 are based on a comparison by chemical class; Tables 5 - 8 by product class. The accuracy of each comparison are presented graphically in Figures 1 through 4.

Center staff member Bonnie Carson, M.S. Organic Chemistry, assigned the chemical classes based on subsituent groups when a graphic molecular structure was readily available or could be drawn based on the chemical name. Some chemical class assignments, such as potential Michael-reactive agent, were based on assignments by Ashby et al. (1995). Chemical classes selected for the Center's analysis were generally those that possessed electrophilic moieties. The sources for the product classes were Budavari (1996), Truett (1998) and Chemfinder (1997). A chemical or product may be present in more than one chemical or product class and not all chemicals listed could be placed in one of the classes used.

A number of these class/product comparisons are of very limited value considering the small number of chemicals tested in common among the various assays, and especially in terms of human sensitization results. To increase the number of possible comparisons to human data, all guinea pig test data were considered and human patch test allergens were included in the analyses. Their inclusion was based on an assumption that the substance would not be in use in a commercial test kit if it did not test positive in at least some individuals. In making these comparisons, unpublished data (as indicated in the Appendix) were included.

Although several chemical or product classes are clearly underrepresented in these analyses, the correlation between the LLNA and guinea pig tests appeared to be disparate, by chemical class, only for lactones and salts. However, when compared against human sensitization results, the LLNA and GPT appear to be equal in accuracy.

Table 1. Comparison of LLNA versus GPMT/BA by Chemical Class

| Chemical Class | # Tested | # of Comparisons | Sensitivity | Specificity | Positive Predictivity | Negative Predictivity | Accuracy |
|---|---|---|---|---|---|---|---|
| Acylating Agents | 9 | 7 | 100% (2/2) | 100% (5/5) | 100% (2/2) | 100% (5/5) | 100% (7/7) |
| Alcohols/Glycols | 8 | 6 | 100% (2/2) | 100% (4/4) | 100% (2/2) | 100% (4/4) | 100% (6/6) |
| Alkyl Halides | 25 | 3 | 100% (3/3) | (0/0) | 100% (3/3) | (0/0) | 100% (3/3) |
| Amides | 11 | 6 | 100% (4/4) | 100% (2/2) | 100% (4/4) | 100% (2/2) | 100% (6/6) |
| Aromatic Amines | 9 | 6 | 50% (2/4) | 100% (2/2) | 100% (2/2) | 50% (2/4) | 67% (4/6) |
| Aryl Halides | 11 | 7 | 80% (4/5) | 100% (2/2) | 100% (4/4) | 67% (2/3) | 86% (6/7) |
| Epoxides (Actual/Potential) | 15 | 8 | 100% (7/7) | 0% (0/1) | 88% (7/8) | (0/0) | 88% (7/8) |
| Esters | 26 | 14 | 100% (6/6) | 88% (7/8) | 86% (6/7) | 100% (7/7) | 93% (13/14) |
| Lactones | 14 | 3 | 100% (1/1) | 50% (1/2) | 50% (1/2) | 100% (1/1) | 67% (2/3) |
| Michael-reactive Agents | 17 | 13 | 100% (11/11) | 100% (2/2) | 100% (11/11) | 100% (2/2) | 100% (13/13) |
| Nitroso Compounds | 8 | 1 | 100% (1/1) | (0/0) | 100% (1/1) | (0/0) | 100% (1/1) |
| Nitroaromatics | 7 | 4 | 100% (3/3) | 100% (1/1) | 100% (3/3) | 100% (1/1) | 100% (4/4) |
| Phenolic Compounds | 24 | 13 | 100% (8/8) | 100% (5/5) | 100% (8/8) | 100% (5/5) | 100% (13/13) |
| Salts | 20 | 12 | 75% (6/8) | 25% (1/4) | 67% (6/9) | 33% (1/3) | 58% (7/12) |

LLNA = Local Lymph Node Assay; GPMT = Guinea Pig Maximization Test; BA = Buehler Assayt.
Number tested refers to the number of substances tested in the LLNA.
Number of comparisons refers to the number of substances tested in both LLNA and GPMT/BA.
Numbers in parenthesis indicate actual number of resulting comparisons for each analysis.

Table 2. Comparison of LLNA versus Guinea Pig Test (GPT) by Chemical Class

| Chemical Class | # Tested | # of Comparisons | Sensitivity | Specificity | Positive Predictivity | Negative Predictivity | Accuracy |
|---|---|---|---|---|---|---|---|
| Acylating Agents | 9 | 8 | 100% (2/2) | 100% (6/6) | 100% (2/2) | 100% (6/6) | 100% (8/8) |
| Alcohols/Glycols | 8 | 7 | 100% (2/2) | 100% (5/5) | 100% (2/2) | 100% (5/5) | 100% (7/7) |
| Alkyl Halides | 25 | 7 | 100% (7/7) | (0/0) | 100% (7/7) | (0/0) | 100% (7/7) |
| Amides | 25 | 6 | 100% (4/4) | 100% (2/2) | 100% (4/4) | 100% (2/2) | 100% (6/6) |
| Aromatic Amines | 9 | 9 | 71% (5/7) | 100% (2/2) | 100% (5/5) | 50% (2/4) | 78% (7/9) |
| Aryl Halides | 11 | 7 | 80% (4/5) | 100% (2/2) | 100% (4/4) | 67% (2/3) | 86% (6/7) |
| Epoxides (Actual/Potential) | 15 | 11 | 100% (10/10) | 0% (0/1) | 91% (10/11) | (0/0) | 91% (10/11) |
| Esters | 26 | 22 | 93% (13/14) | 88% (7/8) | 93% (13/14) | 88% (7/8) | 91% (20/22) |
| Lactones | 14 | 10 | 50% (3/6) | 50% (2/4) | 60% (3/5) | 40% (2/5) | 50% (5/10) |
| Michael-reactive Agents | 17 | 14 | 100% (11/11) | 67% (2/3) | 92% (11/12) | 100% (2/2) | 93% (13/14) |
| Nitroso Compounds | 8 | 1 | 100% (1/1) | (0/0) | 100% (1/1) | (0/0) | 100% (1/1) |
| Nitroaromatics | 7 | 6 | 100% (5/5) | 100% (1/1) | 100% (5/5) | 100% (1/1) | 100% (6/6) |
| Phenolic Compounds | 24 | 16 | 100% (11/11) | 100% (5/5) | 100% (11/11) | 100% (5/5) | 100% (16/16) |
| Salts | 20 | 17 | 85% (11/13) | 25% (1/4) | 79% (11/14) | 33% (1/3) | 71% (12/17) |

LLNA = Local Lymph Node Assay; GPT includes Guinea Pig Maximization Test, Buehler Assay, and nonstandard Guinea pig tests.
Number tested refers to the number of substances tested in the LLNA.
Number of comparisons refers to the number of substances tested in both LLNA and GPT.
Numbers in parenthesis indicate actual number of resulting comparisons for each analysis.

Table 3. Comparison of LLNA versus Human Data by Chemical Class

| Chemical Class | # Tested | # of Comparisons | Sensitivity | Specificity | Positive Predictivity | Negative Predictivity | Accuracy |
|---|---|---|---|---|---|---|---|
| Acylating Agents | 11 | 0 | | | | | |
| Alcohols/Glycols | 8 | 5 | 40% (2/5) | (0/0) | 100% (2/2) | 0% (0/3) | 40% (2/5) |
| Alkyl Halides | 25 | 1 | 100% (1/1) | (0/0) | 100% (1/1) | (0/0) | 100% (1/1) |
| Amides | 11 | 5 | 60% (3/5) | (0/0) | 100% (3/3) | 0% (0/2) | 60% (3/5) |
| Aromatic Amines | 7 | 7 | 57% (4/7) | (0/0) | 100% (4/4) | 0% (0/3) | 57% (4/7) |
| Aryl Halides | 11 | 4 | 100% (4/4) | (0/0) | 100% (4/4) | (0/0) | 100% (4/4) |
| Epoxides (Actual/Potential) | 15 | 9 | 100% (9/9) | (0/0) | 100% (9/9) | (0/0) | 100% (9/9) |
| Esters | 26 | 8 | 29% (2/7) | 0% (0/1) | 67% (2/3) | 0% (0/5) | 25% (2/8) |
| Lactones | 14 | 2 | 50% (1/2) | (0/0) | 100% (1/1) | 0% (0/1) | 50% (1/2) |
| Michael-reactive Agents | 17 | 8 | 75% (6/8) | (0/0) | 100% (6/6) | 0% (0/2) | 75% (6/8) |
| Nitroso Compounds | 8 | 0 | | | | | |
| Nitroaromatics | 7 | 0 | | | | | |
| Phenolic Compounds | 24 | 14 | 80% (8/10) | 100% (4/4) | 100% (8/8) | 67% (4/6) | 86% (12/14) |
| Salts | 20 | 7 | 83% (5/6) | 0% (0/1) | 83% (5/6) | 0% (0/1) | 71% (5/7) |

LLNA = Local Lymph Node Assay; Human data includes results from Human Maximization Test and Human Patch Test Allergens.
Number tested refers to the number of substances tested in the LLNA.
Number of comparisons refers to the number of substances tested in both LLNA and GPT.
Numbers in parenthesis indicate actual number of resulting comparisons for each analysis.

Table 4. Comparison of Guinea Pig Tests (GPT) versus Human Data by Chemical Class

| Chemical Class | # Tested | # of Comparisons | Sensitivity | Specificity | Positive Predictivity | Negative Predictivity | Accuracy |
|---|---|---|---|---|---|---|---|
| Acylating Agents | 7 | 0 | | | | | |
| Alcohols/Glycols | 7 | 5 | 40% (2/5) | (0/0) | 100% (2/2) | 0% (0/3) | 40% (2/5) |
| Alkyl Halides | 7 | 1 | 100% (1/1) | (0/0) | 100% (1/1) | (0/0) | 100% (1/1) |
| Amides | 6 | 5 | 60% (3/5) | (0/0) | 100% (3/3) | 0% (0/2) | 60% (3/5) |
| Aromatic Amines | 10 | 7 | 57% (4/7) | (0/0) | 100% (4/4) | 0% (0/3) | 57% (4/7) |
| Aryl Halides | 7 | 2 | 100% (2/2) | (0/0) | 100% (2/2) | (0/0) | 100% (2/2) |
| Epoxides (Actual/Potential) | 11 | 9 | 89% (8/9) | (0/0) | 100% (8/8) | 0% (0/1) | 89% (8/9) |
| Esters | 22 | 9 | 33% (2/6) | 33% (1/3) | 50% (2/4) | 20% (1/5) | 33% (3/9) |
| Lactones | 10 | 2 | 0% (0/2) | (0/0) | (0/0) | 0% (0/2) | 0% (0/2) |
| Michael-reactive Agents | 14 | 8 | 75% (6/8) | (0/0) | 100% (6/6) | 0% (0/2) | 75% (6/8) |
| Nitroso Compounds | 1 | 0 | | | | | |
| Nitroaromatics | 6 | 0 | | | | | |
| Phenolic Compounds | 16 | 11 | 75% (6/8) | 100% (3/3) | 100% (6/6) | 60% (3/5) | 82% (9/11) |
| Salts | 17 | 8 | 86% (6/7) | 100% (1/1) | 100% (6/6) | 50% (1/2) | 88% (7/8) |

GPT includes Guinea Pig Maximization Test, Buehler Assay, and nonstandard Guinea pig tests; Human data includes results from Human Maximization Test and Human Patch Test Allergens.

Number tested refers to the number of substances tested in the LLNA.

Number of comparisons refers to the number of substances tested in both LLNA and GPT.

Numbers in parenthesis indicate actual number of resulting comparisons for each analysis.

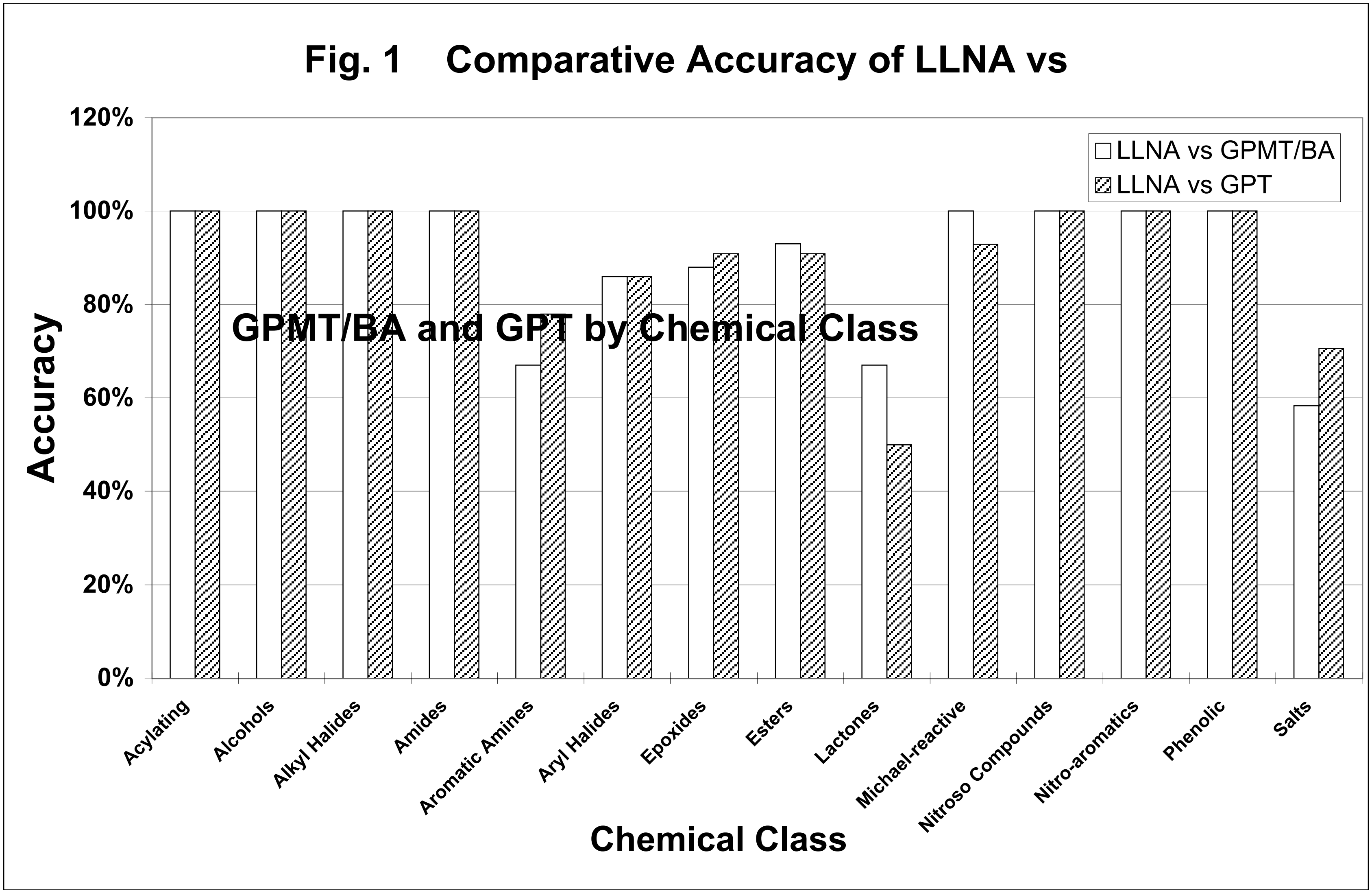


Fig. 1 Comparative Accuracy of LLNA vs
GPMT/BA and GPT by Chemical Class
120%
100%
80%
60%
40%
20%
0%
Accuracy
LLNA vs GPMT/BA
LLNA vs GPT
Acylating
Alcohols
Alkyl Halides
Amides
Aromatic Amines
Aryl Halides
Epoxides
Esters
Lactones
Michael-reactive
Nitroso Compounds
Nitro-aromatics
Phenolic
Salts
Chemical Class

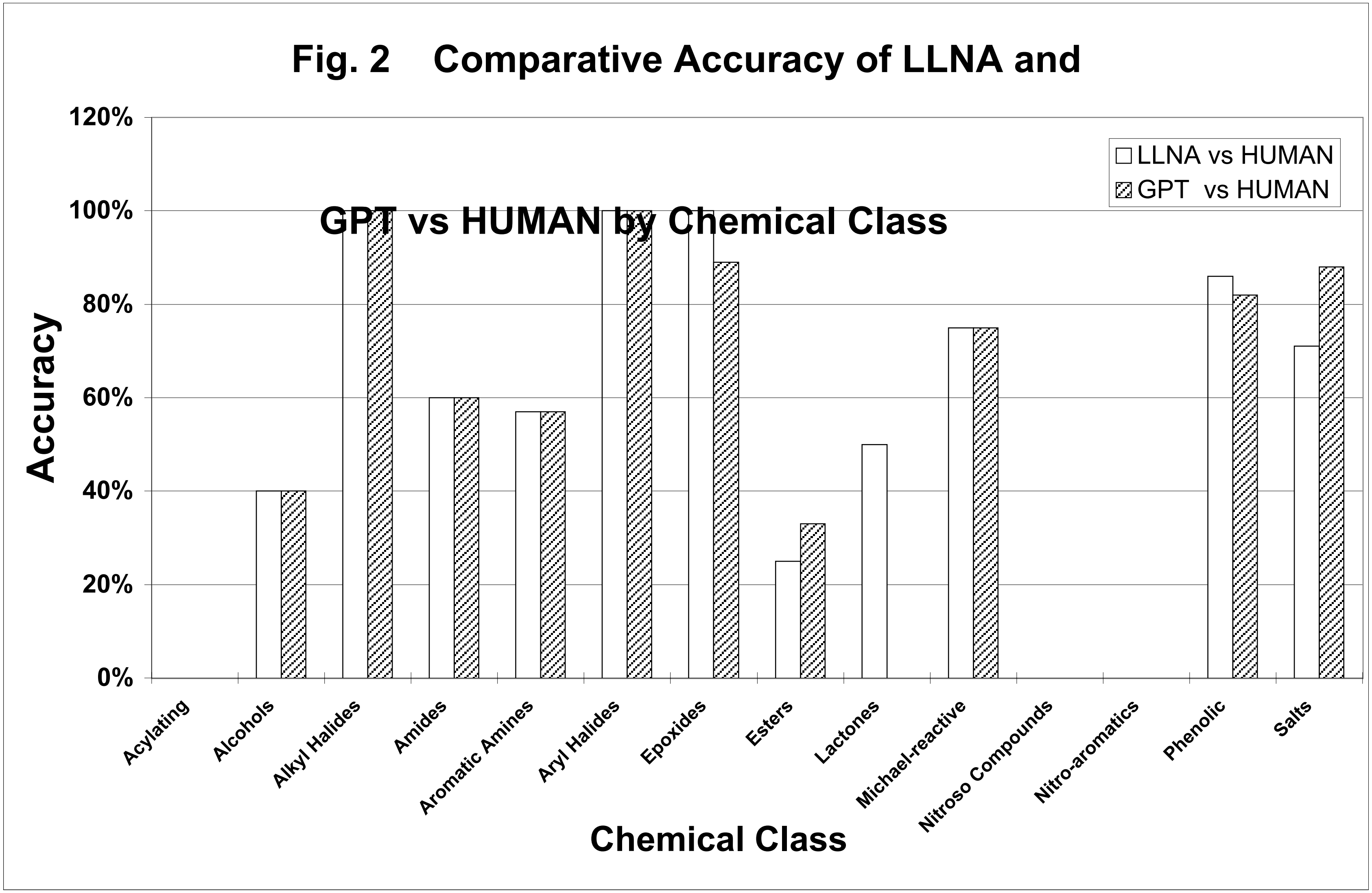


Fig. 2 Comparative Accuracy of LLNA and
GPT vs HUMAN by Chemical Class
120%
100%
80%
60%
40%
20%
0%
Accuracy
LLNA vs HUMAN
GPT vs HUMAN
Acylating
Alcohols
Alkyl Halides
Amides
Aromatic Amines
Aryl Halides
Epoxides
Esters
Lactones
Michael-reactive
Nitroso Compounds
Nitro-aromatics
Phenolic
Salts
Chemical Class

Table 5. Comparison of LLNA versus GPMT/BA by Product Class

| Product Class | # Tested | # of Comparisons | Sensitivity | Specificity | Positive Predictivity | Negative Predictivity | Accuracy |
|---|---|---|---|---|---|---|---|
| Antimicrobial | 24 | 16 | 85% (11/13) | 100% (3/3) | 100% (11/11) | 60% (3/5) | 88% (14/16) |
| Chemical Intermediates | 38 | 25 | 88% (15/17) | 100% (8/8) | 100% (15/15) | 80% (8/10) | 92% (23/25) |
| Cosmetics (including hair and fragrances) | 38 | 32 | 100% (20/20) | 92% (11/12) | 95% (20/21) | 100% (11/11) | 97% (31/32) |
| Dyes (or Dye Intermediates) | 16 | 11 | 63% (5/8) | 100% (3/3) | 100% (5/5) | 50% (3/6) | 73% (8/11) |
| Food Additives | 14 | 12 | 100% (6/6) | 100% (6/6) | 100% (6/6) | 100% (6/6) | 100% (12/12) |
| Pesticides | 6 | 2 | 100% (1/1) | 100% (1/1) | 100% (1/1) | 100% (1/1) | 100% (2/2) |
| Pharmaceuticals | 34 | 22 | 78% (7/9) | 100% (13/13) | 100% (7/7) | 87% (13/15) | 91% (20/22) |
| Photographic Chemicals | 7 | 4 | 100% (4/4) | (0/0) | 100% (4/4) | (0/0) | 100% (4/4) |
| Polymers (including monomers, resins plastics, but not rubber) | 16 | 12 | 100% (7/7) | 80% (4/5) | 88% (7/8) | 100% (4/4) | 92% (11/12) |

LLNA = Local Lymph Node Assay; GPMT = Guinea Pig Maximization Test; BA = Buehler Assayt.
Number tested refers to the number of substances tested in the LLNA.
Number of comparisons refers to the number of substances tested in both LLNA and GPMT/BA.
Numbers in parenthesis indicate actual number of resulting comparisons for each analysis.

Table 6. Comparison of LLNA versus Guinea Pig Test (GPT) by Product Class

| Product Class | # Tested | # of Comparisons | Sensitivity | Specificity | Positive Predictivity | Negative Predictivity | Accuracy |
|---|---|---|---|---|---|---|---|
| Antimicrobial | 24 | 19 | 80% (12/15) | 100% (4/4) | 100% (12/12) | 57% (4/7) | 84% (16/19) |
| Chemical Intermediates | 38 | 28 | 95% (18/19) | 100% (9/9) | 100% (18/18) | 90% (9/10) | 96% (27/28) |
| Cosmetics (including hair and fragrances) | 38 | 34 | 100% (22/22) | 92% (11/12) | 96% (22/23) | 100% (11/11) | 97% (33/34) |
| Dyes (or Dye Intermediates) | 16 | 14 | 73% (8/11) | 100% (3/3) | 100% (8/8) | 50% (3/6) | 79% (11/14) |
| Food Additives | 14 | 13 | 100% (6/6) | 100% (7/7) | 100% (6/6) | 100% (7/7) | 100% (13/13) |
| Pesticides | 6 | 3 | 100% (2/2) | 100% (1/1) | 100% (2/2) | 100% (1/1) | 100% (3/3) |
| Pharmaceuticals | 34 | 25 | 82% (9/11) | 100% (14/14) | 100% (9/9) | 87% (14/16) | 92% (23/25) |
| Photographic Chemicals | 7 | 6 | 100% (5/5) | 100% (1/1) | 100% (5/5) | 100% (1/1) | 100% (6/6) |
| Polymers (including monomers, resins plastics, but not rubber) | 16 | 14 | 100% (9/9) | 80% (4/5) | 90% (9/10) | 100% (4/4) | 93% (13/14) |

LLNA = Local Lymph Node Assay; GPT includes Guinea Pig Maximization Test, Buehler Assay, and nonstandard Guinea pig tests.
Number tested refers to the number of substances tested in the LLNA.
Number of comparisons refers to the number of substances tested in both LLNA and GPT.
Numbers in parenthesis indicate actual number of resulting comparisons for each analysis.

Table 7. Comparison of LLNA versus Human Data by Product Class

| Product Class | # Tested | # of Comparisons | Sensitivity | Specificity | Positive Predictivity | Negative Predictivity | Accuracy |
|---|---|---|---|---|---|---|---|
| Antimicrobial | 24 | 17 | 76% (13/17) | (0/0) | 100% (13/13) | 0% (0/4) | 76% (13/17) |
| Chemical Intermediates | 35 | 19 | 76% (13/17) | 50% (1/2) | 93% (13/14) | 20% (1/5) | 74% (14/19) |
| Cosmetics (including hair and fragrances) | 38 | 28 | 63% (17/27) | 100% (1/1) | 100% (17/17) | 9% (1/11) | 64% (18/28) |
| Dyes (or Dye Intermediates) | 16 | 8 | 100% (4/4) | (0/0) | 100% (4/4) | 0% (0/4) | 50% (4/8) |
| Food Additives | 14 | 8 | 57% (4/7) | 0% (0/1) | 80% (4/5) | 0% (0/3) | 50% (4/8) |
| Pesticides | 6 | 4 | 75% (3/4) | (0/0) | 100% (3/3) | 0% (0/1) | 75% (3/4) |
| Pharmaceuticals | 34 | 26 | 50% (11/22) | 100% (4/4) | 100% (11/11) | 27% (4/15) | 58% (15/26) |
| Photographic Chemicals | 7 | 6 | 100% (6/6) | (0/0) | 100% (6/6) | (0/0) | 100% (6/6) |
| Polymers (including monomers, resins plastics, but not rubber) | 16 | 11 | 100% (7/7) | 100% (4/4) | 100% (7/7) | 100% (4/4) | 100% (11/11) |

LLNA = Local Lymph Node Assay; Human data includes results from Human Maximization Test and Human Patch Test Allergens.
Number tested refers to the number of substances tested in the LLNA.
Number of comparisons refers to the number of substances tested in both LLNA and GPT.
Numbers in parenthesis indicate actual number of resulting comparisons for each analysis.

Table 8. Comparison of Guinea Pig Tests (GPT) versus Human Data by Product Class

| Product Class | # Tested | # of Comparisons | Sensitivity | Specificity | Positive Predictivity | Negative Predictivity | Accuracy |
|---|---|---|---|---|---|---|---|
| Antimicrobial | 19 | 17 | 81% (13/17) | (0/0) | 100% (13/13) | 0% (0/3) | 81% (13/17) |
| Chemical Intermediates | 28 | 16 | 80% (12/15) | 100% (1/1) | 100% (12/12) | 25% (1/4) | 81% (13/16) |
| Cosmetics (including hair and fragrances) | 34 | 26 | 64% (16/25) | 100% (1/1) | 100% (16/16) | 10% (1/10) | 65% (17/26) |
| Dyes (or Dye Intermediates) | 14 | 9 | 67% (6/9) | (0/0) | 100% (6/6) | 0% (0/3) | 67% (6/9) |
| Food Additives | 13 | 8 | 57% (4/7) | 0% (0/1) | 80% (4/5) | 0% (0/3) | 50% (4/8) |
| Pesticides | 3 | 3 | 67% (2/3) | (0/0) | 100% (2/2) | 0% (0/1) | 67% (2/3) |
| Pharmaceuticals | 25 | 20 | 50% (9/18) | 100% (2/2) | 100% (9/9) | 18% (2/11) | 55% (11/20) |
| Photographic Chemicals | 6 | 5 | 100% (5/5) | (0/0) | 100% (5/5) | (0/0) | 100% (5/5) |
| Polymers (including monomers, resins plastics, but not rubber) | 14 | 12 | 100% (7/7) | 100% (5/5) | 100% (7/7) | 100% (5/5) | 100% (12/12) |

GPT includes Guinea Pig Maximization Test, Buehler Assay, and nonstandard Guinea pig tests; Human data includes results from Human Maximization Test and Human Patch Test Allergens.

Number tested refers to the number of substances tested in the LLNA.

Number of comparisons refers to the number of substances tested in both LLNA and GPT.

Numbers in parenthesis indicate actual number of resulting comparisons for each analysis.

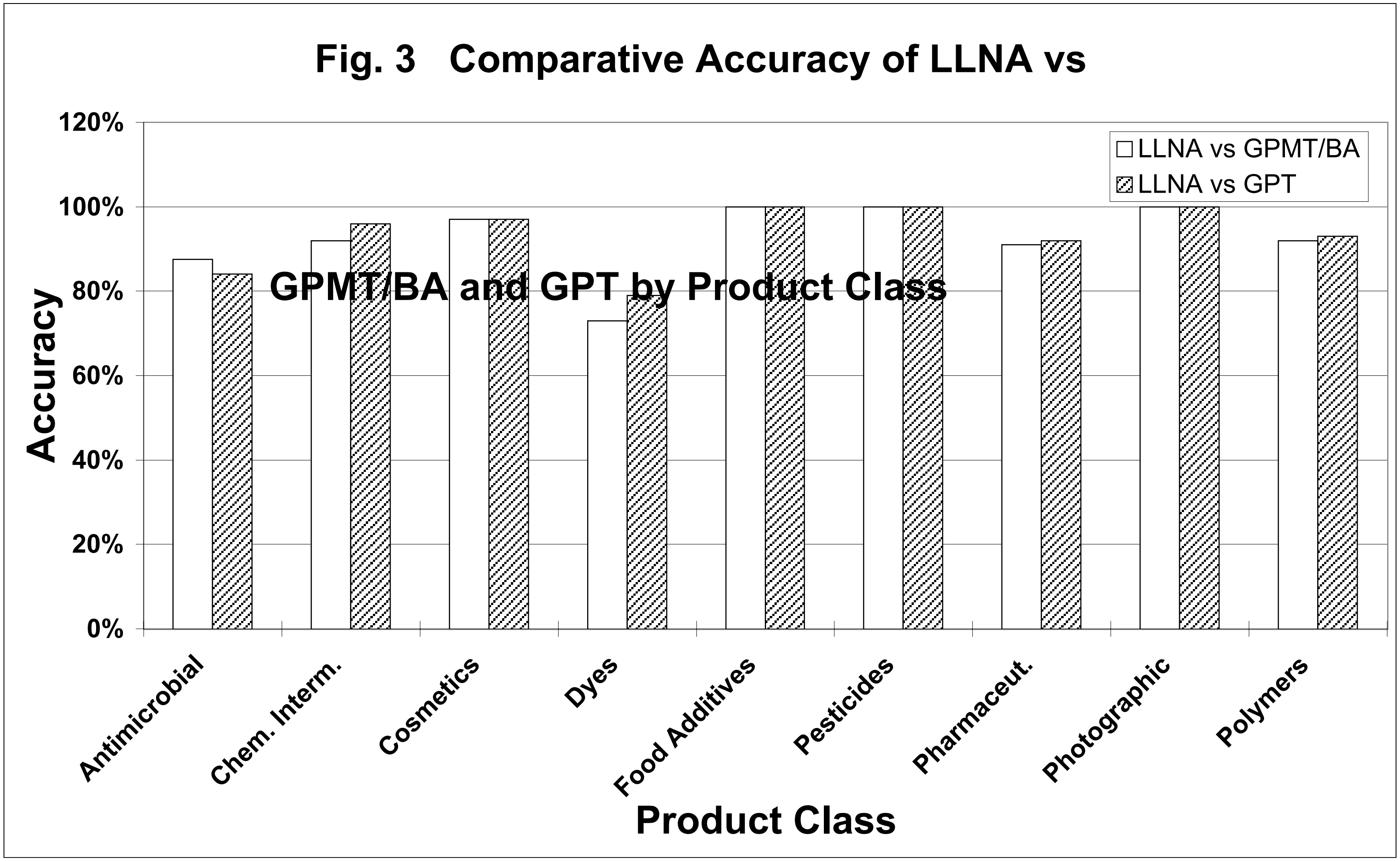


Fig. 3 Comparative Accuracy of LLNA vs
GPMT/BA and GPT by Product Class
Accuracy
120%
100%
80%
60%
40%
20%
0%
LLNA vs GPMT/BA
LLNA vs GPT
Antimicrobial
Chem. Interm.
Cosmetics
Dyes
Food Additives
Pesticides
Pharmaceut.
Photographic
Polymers
Product Class

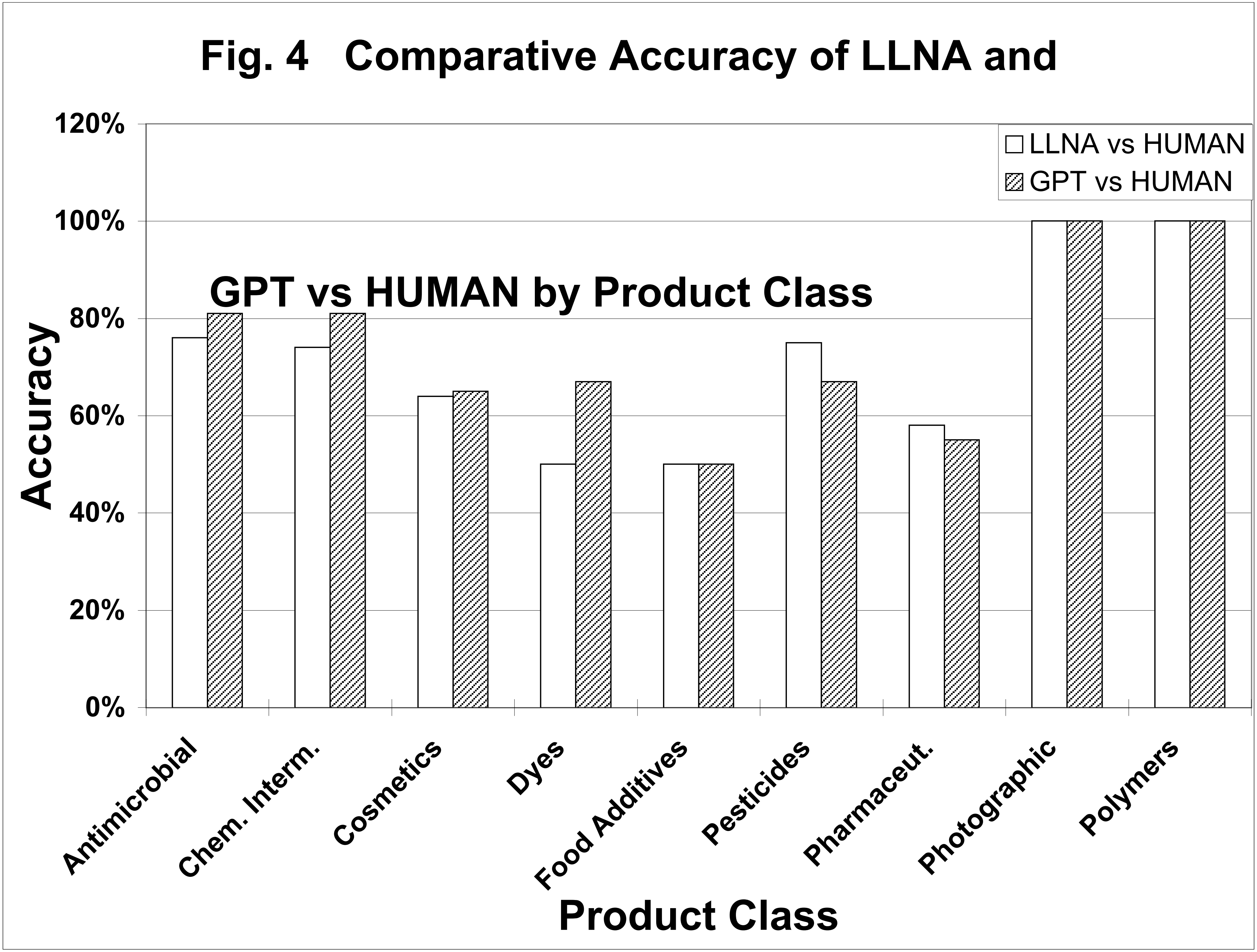


Fig. 4 Comparative Accuracy of LLNA and
GPT vs HUMAN by Product Class
LLNA vs HUMAN
GPT vs HUMAN
Accuracy
120%
100%
80%
60%
40%
20%
0%
Antimicrobial
Chem. Interm.
Cosmetics
Dyes
Food Additives
Pesticides
Pharmaceut.
Photographic
Polymers
Product Class

# NICEATM Quality Assurance Audit Summary

As recommended by the LLNA Peer Review Panel, a retrospective data audit was conducted by a National Toxicology Program (NTP) independent quality assurance contractor on the intra- and inter-laboratory LLNA validation studies submitted by the Sponsors. The purpose of the audit was to provide an independent assessment of published test data provided in the submission for accuracy, consistency, and completeness as compared to the original study records.

The published results on individual chemicals were compared against the original laboratory records from the following participating laboratories:

- Zeneca Central Toxicology Laboratory, Cheshire, UK;
- Unilever Safety and Environmental Assurance Center, Bedforshire, UK;
- Procter & Gamble Company, Cincinnati, OH;
- ITT Research Institute (IITRI), Chicago, IL[1]; and
- E. I. du Pont de Nemours, Inc., Newark, DE.

The pertinent data from each laboratory for one chemical from each of the three published papers provided below were reviewed for completeness and accuracy. The chemical evaluated is provided in parentheses.

- Kimber, I., J. Hilton, R. J. Dearman, G. F. Gerberick, C. A. Ryan, D. A. Basketter, E. W. Scholes, G. S. Ladics, S. E. Loveless, R. V. House, and A. Guy. 1995. An international evaluation of the murine local lymph node assay and comparison of modified procedures. Toxicology 103:63-73. (2,4-dinitrochlorobenzene [DNCB])

- Kimber, I., J. Hilton, R. J. Dearman, G. F. Gerberick, C. A. Ryan, D. A. Basketter, L. Lea, R. V. House, G. S. Ladics, S. E. Loveless, and K. L. Hastings. 1998. Assessment of the skin sensitization potential of topical medicaments using the local lymph node assay: An interlaboratory evaluation. J. Toxicol. Environ. Health 53:563-579. (penicillin-G)

- Loveless, S. E., G. S. Ladics, G. F. Gerberick, C. A. Ryan, D. A. Basketter, E. W. Scholes, R. V. House, J. Hiltong, R. J. Dearman, and I. Kimber. 1996. Further evaluation of the local lymph node assay in the final phase of an international collaborative trial. Toxicology 108:141-152. (sodium lauryl sulfate [SLS])

Minimal findings were identified in the audit report. Audit procedures and findings are presented in the quality assurance report on file at the National Institute of Environmental Health Sciences (NIEHS). The audit supports the conclusion that the transcribed test data in the submission were accurate, consistent, and complete as compared to the original study records.

[1] Records from IITRI were not received prior to the publication of this report, thus the findings discussed here do not include audit findings from IITRI.

# Appendix B1

## Physico-Chemical Properties and Chemical Classes of Pesticide Formulations Tested in the LLNA (Sorted Alphabetically)

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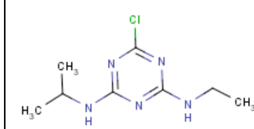
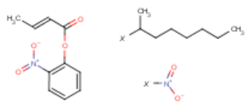
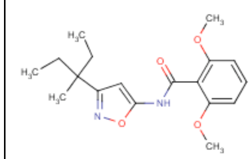
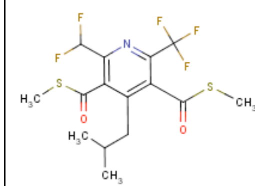
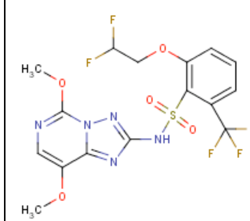
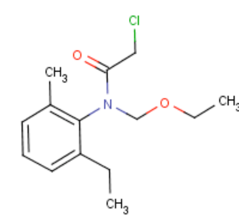
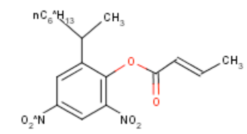
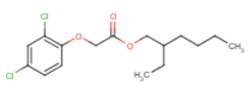
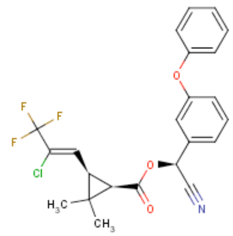
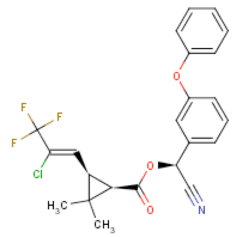
## Physico-Chemical Properties and Chemical Classes of Pesticide Formulations Tested in the LLNA (Sorted Alphabetically)

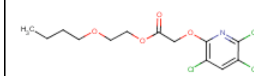
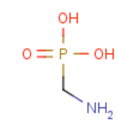
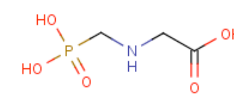
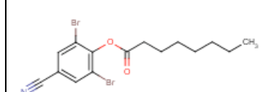
| Substance Name | Active Ingredient(s) | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| AE F016382 00 TK71 A101 | NA | NA | NA | NA | NA | Formulation | NA |
| A SC600 | NA | NA | NA | NA | NA | Formulation | NA |
| 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 #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 |

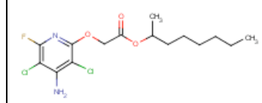
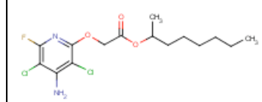
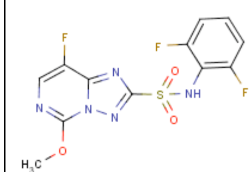
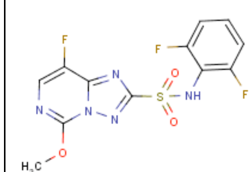
| Substance Name | Active Ingredient(s) | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| Dinocap | Butenoic acid, 2-(or 4)-isooctyl-4,6(or 2,6)-dinitrophenyl ester (9CI) Crotonic acid, 2(or 4)-(1-methylheptyl)-4,6(or 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-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 |

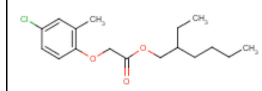
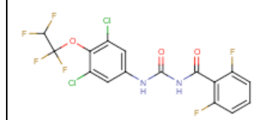
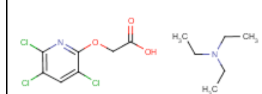
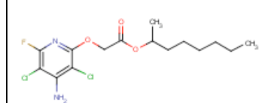
| Substance Name | Active Ingredient(s) | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| DU-2E | NA | NA | NA | NA | NA | Formulation | NA |
| DU-2F | NA | NA | NA | NA | NA | Formulation | NA |
| DU-3 | NA | NA | 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 |

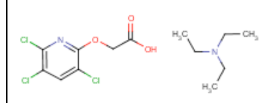
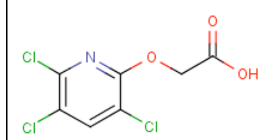
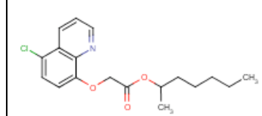
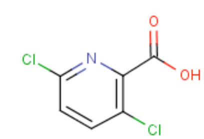
| Substance Name | Active Ingredient(s) | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| Formulation 1 | Isoxaben | 82558-50-7 | 332.40 | NA | Liquid | Formulation |  |
| Formulation 10 | 22.9% w/w dithiopyr | 97886-45-8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

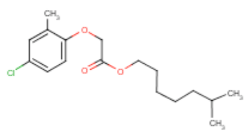
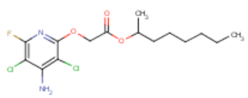
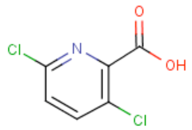
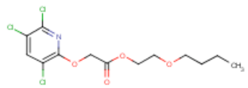
| Substance Name | Active Ingredient(s) | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| Formulation 12 | 34.7% w/w 2,4-dinitro-6-(1-methylheptyl)phenyl crotonate<br>DE-126 | 6119-92-2 | 364.40 | NA | Liquid | Formulation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

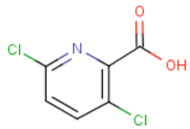
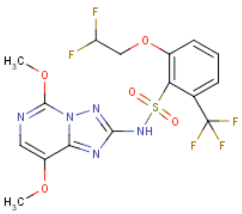
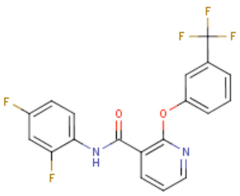
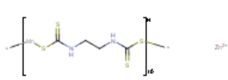
| Substance Name | Active Ingredient(s) | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| Formulation 16 | 85.3% w/w triclopyr butoxyethyl ester | 64470-88-8 | 356.63 | NA | Liquid | Formulation |  |
| Formulation 17 | 50.8% wt/wt glyphosate dimethylammonium salt (active ingredient)<br>40.1% wt/wt glyphosate (acid equivalent)<br>8.3% w/w Geronol CF/AS 30 (ammonium adjuvant) | 1066-51-9<br>1071-83-6 | 111.04<br>169.02 | NA | Liquid | Formulation | <br> |
| Formulation 19 | 37.1 wt% Bromoxynil octanoate<br>9.23 wt% fluroxypyr-1-methylheptyl | 1689-99-2<br>81406-37-3 | 403.11<br>367.25 | NA | Liquid | Formulation |  |

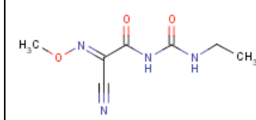
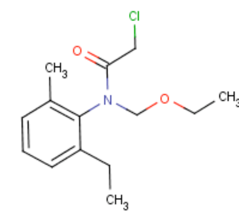
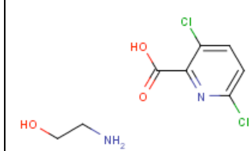
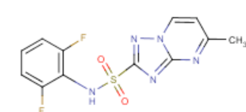
| Substance Name | Active Ingredient(s) | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| | | | | | | |  |
| Formulation 2 | 14.2% w/w fluroxypyr -meptyl 0.22% w/w florasulam | 81406-37-3 145701-23-1 | 367.25 359.29 | NA | Liquid | Formulation |   |
| Formulation 20 | 0.39 wt% Florasulam 41.9 wt% 2-methyl-4-chlorophenoxyacetic acid 2-ethylhexyl ester (MCPA, 2- | 145701-23-1 29450-45-1 | 359.29 312.84 | NA | Liquid | Formulation |  |

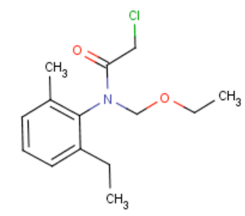
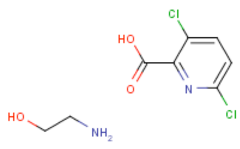
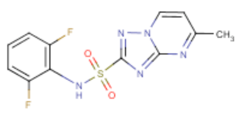
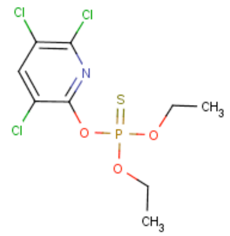
| Substance Name | Active Ingredient(s) | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| | ethyl hexyl ester) | | | | | |  |
| Formulation 21 | 50.4% Hexaflumuron N-(((3,5-dichloro-4-(1,1,2,2-tetrafluoroethoxy)phenyl)amino)carbonyl)-2,6-difluoro benzamide | 86479-06-3 | 461.14 | NA | Liquid | Formulation |  |
| Formulation 22 | 8.3 wt. % triclopyr triethylammonium 2.8 wt, % fluroxypyr-methyl heptyl ester | 57213-69-1<br>81406-37-3 | 357.66<br>367.25 | NA | Liquid | Formulation | <br> |

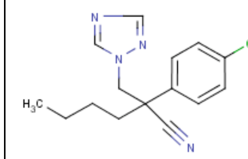
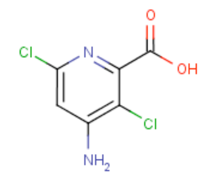
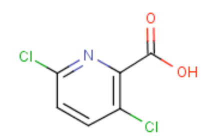
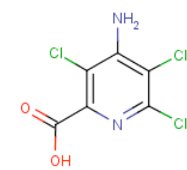
| Substance Name | Active Ingredient(s) | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| Formulation 23 | 16.1 wt% Triclopyr -triethylammonium 11.6 wt% triclopyr acid | 57213-69-1 55335-06-3 | 357.66 | NA | Liquid | Formulation |   |
| Formulation 24 | 8.8 wt% Cloquintocet-mexyl | 99607-70-2 | 335.83 | NA | Liquid | Formulation |  |
| 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 |  |

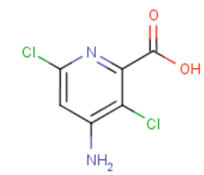
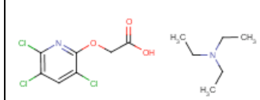
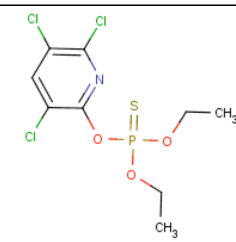
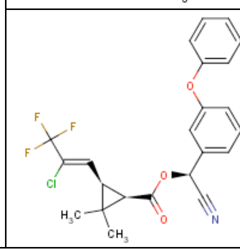
| Substance Name | Active Ingredient(s) | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| | | | | | | |   |
| Formulation 26 | 5.9 wt.% clopyralid<br>32.9 wt.% triclopyr-butotyl | 1702-17-6<br>64700-56-7 | 192.00<br>356.63 | NA | Liquid | Formulation |   |

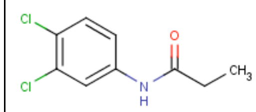
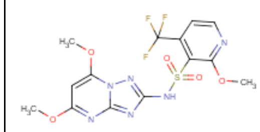
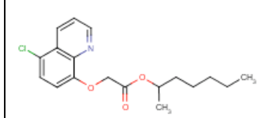
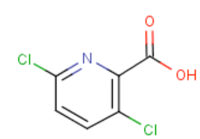
| Substance Name | Active Ingredient(s) | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| Formulation 27 | 45.2 wt% fluroxypyr-meptyl | 81406-37-3 | 192.00 | NA | Liquid | Formulation |  |
| Formulation 28 | 1.4 wt% penoxsulam<br>9.37 wt% diflufenican | 219714-96-2<br>83164-33-4 | 483.37<br>394.30 | NA | Liquid | Formulation | <br> |
| Formulation 29 | 35.6% mancozeb,<br>4.92% cymoxanil | 8018-01-7<br>57966-95-7 | 541.1<br>198.18 | NA | Liquid | Formulation |  |

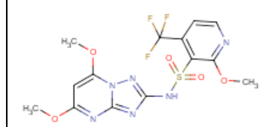
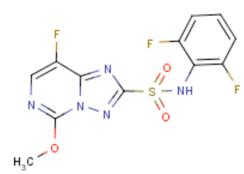
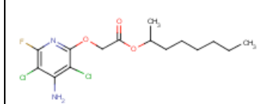
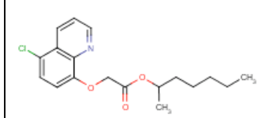
| Substance Name | Active Ingredient(s) | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| | | | | | | |  |
| Formulation 3 | 455 g/L acetochlor<br>47 g/L clopyralid-olamine<br>14 g/L flumetsulam | 34256-82-1<br>57754-85-5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

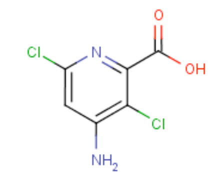
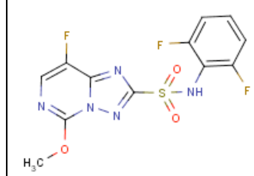
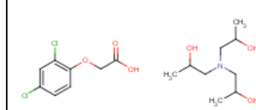
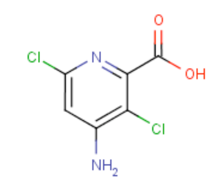
| Substance Name | Active Ingredient(s) | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| Formulation 30 | 455 g/L acetochlor<br>47 g/L clopyralid-olamine<br>14 g/L flumetsulam | 34256-82-1<br>57754-85-5<br>98967-40-9 | 269.77<br>253.08<br>325.30 | NA | Liquid | Formulation | <br><br> |
| Formulation 31 | 18.7 wt.% chlorpyrifos | 2921-88-2 | 350.59 | NA | Liquid | Formulation |  |

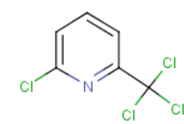
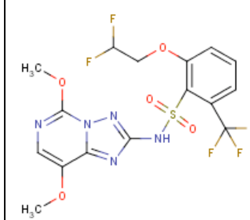
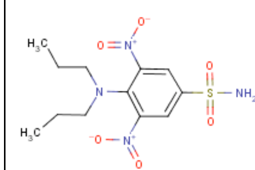
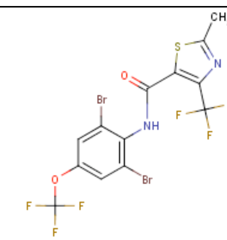
| Substance Name | Active Ingredient(s) | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| Formulation 32 | 11.2 wt % ((E)-2-(1-methylheptyl) -4,6-dinitrophenyl ester-2-butenoic acid<br>4.68% wt/wt myclobutanil | 88671-89-0 | 288.78 | NA | Liquid/ Solid | Formulation |  |
| Formulation 33 | 4.5 wt% aminopyralid-olamine<br>27.1 wt% clopyralid-olamine<br>8.7 wt% picloram-olamine<br>3.5 wt% aminopyralid<br>20.6 wt% clopyralid<br>7.0 wt% picloram | 150114-71-9<br>1702-17-6<br>1918-02-1 | 207.02<br>192.00<br>241.46 | NA | Liquid | Formulation | <br><br> |

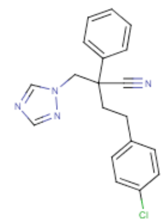
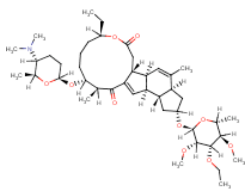
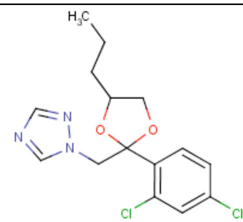
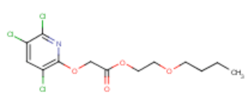
| Substance Name | Active Ingredient(s) | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| Formulation 34 | 3.0 wt% aminopyralid | 150114-71-9 | | NA | Liquid | Formulation |  |
| Formulation 35 | 2.15 wt% aminopyralid-triisopropanolammonium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

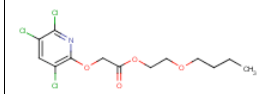
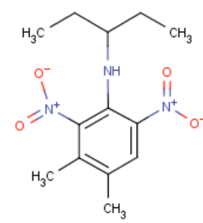
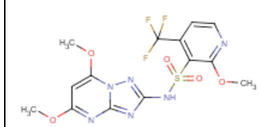
| Substance Name | Active Ingredient(s) | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| Formulation 38 | 44.4 wt.% propanil | 709-98-8 | 218.08 | NA | Liquid | Formulation |  |
| Formulation 39 | 4.2 wt% Pyroxsulam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

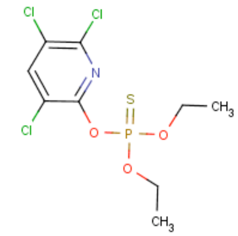
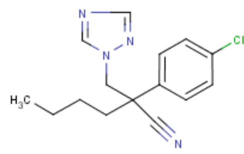
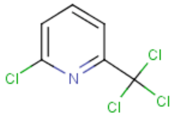
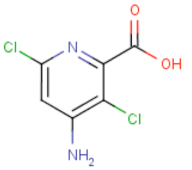
| Substance Name | Active Ingredient(s) | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| Formulation 40 | 1.2 wt% Pyroxsulam<br>0.21 wt% Florasulam<br>11.8 wt% Fluroxypyr-meptyl<br>3.6 wt% Cloquintocet-mexyl | 422556-08-9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

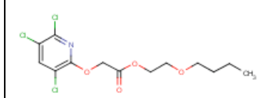
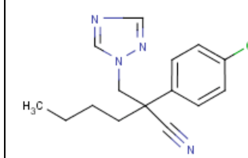
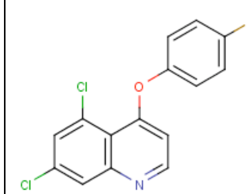
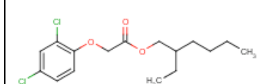
| Substance Name | Active Ingredient(s) | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| Formulation 41 | 1.10 wt% Aminopyralid potassium salt 0.47 wt% Florasulam | 150114-71-9 145701-23-1 | 207.02 359.29 | NA | Liquid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

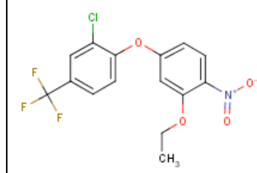
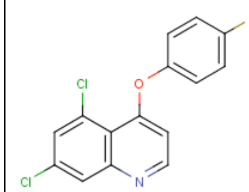
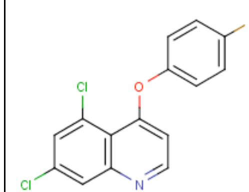
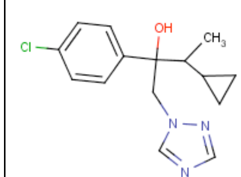
| Substance Name | Active Ingredient(s) | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| Formulation 43 | 17.9 wt % Nitrapyrin | 1929-82-4 | 230.91 | NA | NA | Formulation |  |
| Formulation 44 | 0.12 wt % Penoxsulam<br><br>40.38 wt % Oryzalin | 219714-96-2<br>19044-88-3 | 483.37<br>346.36 | NA | NA | Formulation | <br> |
| Formulation 45 | 7.53 wt % Thifluzamide<br><br>9.42 wt % Fenbuconazole | 130000-40-7<br>114369-43-6 | 528.06<br>336.82 | NA | NA | Formulation |  |

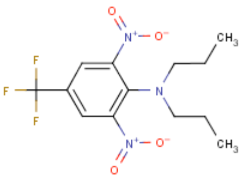
| Substance Name | Active Ingredient(s) | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| | | | | | | |  |
| Formulation 46 | 5.87 wt % Spinetoram | 187166-15-0 | 760.02 | NA | NA | Formulation |  |
| Formulation 47 | 14.56 wt % propiconazole | 60207-90-1 | 342.22 | NA | NA | Formulation |  |
| Formulation 49 | 23.7 WT% Triclopyr BEE | 64700-56-7 | 356.63 | NA | Liquid | Formulation |  |

| Substance Name | Active Ingredient(s) | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| 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 |
| Formulation 51 | 29.6 wt% Pendimethalin 0.51 wt% Pyroxsulam | 40487-42-1 422556-08-9 | 281.31 434.35 | NA | Liquid | Formulation |   |

| Substance Name | Active Ingredient(s) | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| 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 |
| 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 | Molecular Weight (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<br>124495-18-7 | 288.78<br>308.14 | NA | Liquid | Formulation | <br> |
| 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 |

| Substance Name | Active Ingredient(s) | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| Fx + Me EW 69 | NA | NA | NA | NA | NA | Formulation | NA |
| 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/<br><br>H-1,2,4-Triazole-1-ethanol, alpha-(4-chlorophenyl)-alpha-(1-cyclopropylethyl)- | 124495-18-7<br>113096-99-4 | 308.14<br>291.78 | 5.69<br>3.25 | Liquid | Heterocyclic Compounds | <br> |

| Substance Name | Active Ingredient(s) | CASRN | Molecular Weight (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.

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# Appendix B2

## Pesticide Formulations Tested in the LLNA - Comparative Data (Sorted Alphabetically)

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## Pesticide Formulations Tested in the LLNA - Comparative Data (Sorted Alphabetically)

| Substance Name | Formulation 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 GP Call (F) | Overall GP Call (Any)[3] | Overall GP Call (AI)[4] | Overall BT Call (AI)[4] | Overall GPMT Call (AI)[4] | Overall GP Call (RC/RF)[5] | GP Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 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 | Submitted by: E. Debruyne, Bayer Crop Science |
| 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 | Submitted by: E. Debruyne, Bayer Crop Science |
| 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 |
| 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 | 0.3 | 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 |
| 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/CaOlaHsd | + | | | | | | | | | |
| 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 |
| 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-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 |
| 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, 5.3 | 64.9 | 1% L92 | CBA/J | + | Bayer Crop Science, submitted by: E. Debruyne, | + | - | - | NA | NA | NA | NA | Bayer Crop Science, submitted by: E. Debruyne, |
| F & Fo WG 50 + 25 | WG | 2.5, 5.0, 10.0, 25.0 | 11.7, 12.6, 14.4, 15.2 | 0.0 | 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 |
| 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 |

| Substance Name | Formulation 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 GP Call (F) | Overall GP Call (Any)[3] | Overall GP Call (AI)[4] | Overall BT Call (AI)[4] | Overall GPMT Call (AI)[4] | Overall GP Call (RC/RF)[5] | GP Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Formulation 19 | EC | 1, 10, 25, 50 | 4.9,7.9,20,50.5 | 0.0 | 1% L92 | BALB/c | + | Submitted by Dow AgroSciences | + | NA | + | + | - | - | - | Submitted by Dow AgroSciences |
| Formulation 2 | SE | 5, 20, 80 | 2, 3.4, 15.8 | NC | 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 | 0.4 | 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 | 0.5 | 1% L92 | BALB/c | + | Submitted by Dow AgroSciences | + | NA | + | + | + | - | NA | Submitted by Dow AgroSciences |
| 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 | 0.1 | 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 | 0.1 | 1% L92 | BALB/c | + | Submitted by Dow AgroSciences | + | NA | + | + | - | + | NA | Submitted by Dow AgroSciences |
| Formulation 26 | EC | 1, 5, 25 | 1, 1, 4 | 0.2 | 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 | 0.1 | 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 | 0.1 | 1% L92 | CBA/J | + | Submitted by Dow AgroSciences | + | NA | + | + | + | + | NA | Submitted by Dow AgroSciences |
| 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 | 0.0 | 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 | 0.1 | 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 | 0.2 | 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 | 0.2 | 1% L92 | CBA/J | + | Submitted by Dow AgroSciences | + | NA | + | + | NA | NA | + | Submitted by Dow AgroSciences |
| Formulation 4 | SL | 5, 25, 100 | 1.4, 1.1, 1.2 | NC | 1% L92 | BALB/c | - | Submitted by Dow AgroSciences | - | NA | - | - | - | NA | + | Submitted by Dow AgroSciences |
| Formulation 40 | OD | 1, 5, 25 | 1.8, 2.8, 5.7 | 0.1 | 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 | 0.5 | 1% L92 | CBA/J | + | Submitted by Dow AgroSciences | + | NA | + | + | NA | NA | + | Submitted by Dow AgroSciences |
| Formulation 42 | SL | 10, 50, 100 | NA | 1.0 | 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 | NA | 0.4 | 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 | 0.6 | 1% L92 | CBA/J | + | Submitted by Dow AgroSciences | + | NA | + | + | + | NA | NA | Submitted by Dow AgroSciences |
| Formulation 5 | EC | 3, 10, 30 | 1.4, 4, 11.5 | 0.1 | 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 | 0.4 | 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 | 0.1 | 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 | 0.1 | 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 | 0.6 | 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 | 0.0 | 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 | 0.2 | 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 | 1.0 | 1% L92 | BALB/c | + | Submitted by Dow AgroSciences | + | - | + | + | - | + | NA | Submitted by Dow AgroSciences |
| Formulation 7 | | 5, 20, 80 | 2.6, 1.4, 3.2 | 0.7 | 1% L92 | BALB/c | + | | | | | | | | | |
| Formulation 8 | EC | 1, 5, 25 | 0.9, 1.1, 7.3 | 0.1 | 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 | 30.8 | 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: 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 |

| Substance Name | Formulation 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 GP Call (F) | Overall GP Call (Any)[3] | Overall GP Call (AI)[4] | Overall BT Call (AI)[4] | Overall GPMT Call (AI)[4] | Overall GP Call (RC/RF)[5] | GP Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 7, 33, 100 | 1.2, 7.2, 12.4 | 14.8 | 1% L92 | CBA/J | + | ECPA LLNA Project Report submitted by: Bayer | | | | | | | | Chemical |
| | | 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 | | | | | | | | |
| | | 7, 33, 100 | 6.0, 30.0, 75.2 | 5.8 | 1% L92 | CBA/Ca | + | ECPA LLNA Project Report submitted by: BASF | | | | | | | | |
| | | 7, 33, 100 | 1.9, 8.7, 25.7 | 11.2 | 1% L92 | CBA/J | + | ECPA LLNA Project Report submitted by: Bayer | | | | | | | | |
| Trifluralin | EC | 7, 33, 100 | 3.1, 26.3, 61.5 | 7.0 | 1% L92 | CBA/J | + | ECPA LLNA Project Report submitted by: Dow Chemical | + | - | - | NA | NA | NA | NA | 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; "-" = Non-sensitizer

[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.

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# Appendix B3

## Composition of Pesticide Formulations Tested in the LLNA

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**Composition of Pesticide Formulations Tested in the LLNA**

| Substance Name | Formulation Type | Scource | Material Family | Active Ingredient/Inert function | Conc. | Amount (%w/w) | Existilng Sensitization Information[1] |
|---|---|---|---|---|---|---|---|
| Dinocap | EC | ECPA | NA | Dinocap | 350 G/L | NA | NA |
| | | | NA | Solvent | 542 G/l | NA | NA |
| | | | NA | Surfactant | 78 G/L | NA | NA |
| Formulation 1 | SC | Dow AgroSciences | Benzamide | Isoxaben | 125 G/L | 12.14% | - (Dow Data) |
| | | | NA | Water | 735.2 G/L | NA | - |
| | | | NA | Thickener | 4 G/L | NA | - (MSDS) |
| | | | NA | Antifoam | 2 G/L | NA | - (MSDS) |
| | | | NA | Surfactant | 30 G/L | NA | - (MSDS) |
| | | | NA | Surfactant | 20 G/L | NA | - (MSDS) |
| | | | NA | Performance aid | 8.5 G/L | NA | - (MSDS) |
| | | | NA | pH Buffer | 1.3 G/L | NA | - (MSDS) |
| | | | NA | Surfactant | 100 G/L | NA | - (MSDS) |
| | | | NA | Biocide | 4 G/L | < 0.1% | + (MSDS) |
| Formulation 2 | SE | Dow AgroSciences | Pyridinyloxy acetic acid | Fluroxypyr-meptyl | 144.09 G/L | 14.53% | - (Dow Data) |
| | | | Sulfonamides | Florasulam | 2.5 G/L | 0.25% | - (Dow Data) |
| | | | NA | Emulsifier | 58.92 G/L | NA | - (MSDS) |
| | | | NA | Emulsifier | 31.84 G/L | NA | - (MSDS) |
| | | | NA | Solvent | 326.8 G/L | NA | - (MSDS) |
| | | | NA | Suspending Aid | 3.24 G/L | NA | - (MSDS) |
| | | | NA | Suspending Aid | 0.91 G/L | NA | - (MSDS) |
| | | | NA | Emulsifier | 1.81 G/L | NA | - (MSDS) |
| | | | NA | Emulsifier | 1.81 G/L | NA | - (MSDS) |
| | | | NA | Biocide | 0.54 G/L | 0.05% | + (MSDS) |
| | | | NA | Antifoam | 1.06 G/L | NA | - (MSDS) |
| | | | NA | Antifreeze | 34.62 G/L | NA | - (MSDS) |
| | | | NA | Suspending Aid | 0.05 G/L | NA | - (MSDS) |
| | | | NA | Dispersant | 0.1 G/L | NA | - (MSDS) |
| | | | NA | pH Buffer | 0.003 G/L | NA | - (MSDS) |
| | | | NA | Dispersant | 0.2 G/L | NA | - (MSDS) |
| | | | NA | Water | 383.66 G/L | NA | - |
| Formulation 3 | SC | Dow AgroSciences | Sulfonamides | Florasulam | 50 G/L | 4.84% | - (Dow Data) |
| | | | NA | Water | 869.12 G/L | NA | - |
| | | | NA | Biocide | 0.93 G/L | 0.09% | + (MSDS) |
| | | | NA | Dispersant | 10.03 G/L | NA | - (MSDS) |
| | | | NA | Thickener | 10.03 G/L | NA | - (MSDS) |
| | | | NA | Dispersant | 1.96 G/L | NA | - (MSDS) |
| | | | NA | Antifoam | 0.21 G/L | NA | - (MSDS) |
| | | | NA | Thickener | 1.76 G/L | NA | - (MSDS) |
| | | | NA | Antifreeze | 89.96 G/L | NA | - (MSDS) |
| | | | NA | pH Buffer | 0.1 G/L | NA | - (MSDS) |
| Formulation 4 | SL | Dow AgroSciences | Pyridine carboxylic acids | Clopyralid-olamine (MEA salt) | 131.75 G/L | 12.52% | - (Dow Data) (clopyralid) |
| | | | NA | Water | 920.25 G/L | NA | - |
| Formulation 5 | EC | Dow AgroSciences | Pyridinyloxy acetic acid | Triclopyr-butotyl | 670.39 G/L | 60.45% | + (Dow Data) |
| | | | NA | Emulsifier | 55.45 G/L | NA | - (MSDS) |
| | | | NA | Solvent | 383.16 G/L | NA | - (MSDS) |
| Formulation 6 | EW | Dow AgroSciences | Pyridinyloxy acetic acid | Triclopyr-butotyl | 333.567 G/L | 29.44% | + (Dow Data) |
| | | | Pyridine carboxylic acids | Aminopyralid potassium | 35.507 G/L | 3.13% | - (Dow Data) (Aminopyralid) |
| | | | NA | Antifreeze | 50 G/L | NA | - (MSDS) |
| | | | NA | Emulsifier | 32.5 G/L | NA | - (MSDS) |
| | | | NA | Emulsifier | 32.5 G/L | NA | - (MSDS) |
| | | | NA | Biocide | 1 G/L | 0.09% | + (MSDS) |
| | | | NA | Thickener | 7.5 G/L | NA | - (MSDS) |
| | | | NA | Thickener | 1.875 G/L | NA | - (MSDS) |
| | | | NA | pH Buffer | 27.33 G/L | NA | - (MSDS) |
| | | | NA | pH Buffer | 2.67 G/L | NA | - (MSDS) |
| | | | NA | Antifoam | 2 G/L | NA | - (MSDS) |
| | | | NA | Water | 606.831 G/L | NA | - |
| Formulation 7 | SC | Dow AgroSciences | Triazole | Myclobutanil | 45 G/L | 4.12% | Equivocal Dow Data) |
| | | | Phenoxyquinoline | Quinoxyfen | 45 G/L | 4.12% | + (Dow Data) |
| | | | NA | Antifreeze | 74.89 G/L | NA | - (MSDS) |
| | | | NA | Dispersant | 31.81 G/L | NA | - (MSDS) |
| | | | NA | Wetter | 14.96 G/L | NA | - (MSDS) |
| | | | NA | Suspending Aid | 7.45 G/L | NA | - (MSDS) |
| | | | NA | Carrier | 57.12 G/L | NA | - |
| | | | NA | Antifoam | 1.09 G/L | NA | - (MSDS) |
| | | | NA | Biocide | .37 G/L | 0.03% | + (MSDS) |
| | | | NA | Water | 785.84 G/L | NA | - |
| | | | NA | Filler | 26.5 G/L | NA | - (MSDS) |
| | | | NA | Thickener | 1.97 G/L | NA | - (WHO) |
| Formulation 8 | EC | Dow AgroSciences | Phenoxyacetic acids | 2,4-D-ethylhexyl | 905 G/L | 81.68% | + (Dow Data) |
| | | | NA | Emulsifier | 37 G/L | 3.34% | - (MSDS) |
| | | | NA | Emulsifier | 43 G/L | 3.88% | - (MSDS) |
| | | | NA | Solvent | 123 G/L | NA | - (MSDS) |
| Formulation 9 | SC | Dow AgroSciences | Spinosoids | DE-175 | 120 G/L | 11.71% | equivocal (+/- LLNA) |
| | | | Nicotinoates | Wetter | 20.5 G/L | NA | - (MSDS) |
| | | | NA | Antifreeze | 61.5 G/L | NA | - (MSDS) |
| | | | NA | Biocide | 2 G/L | 0.20% | + (MSDS) |
| | | | NA | Thickener | 1.8 G/L | NA | - (WHO) |
| | | | NA | Thickener | 4.1 G/L | NA | - (MSDS) |
| | | | NA | Antifoam | 3.6 G/L | NA | - (MSDS) |
| | | | NA | Dispersant | 46.1 G/L | NA | - (MSDS) |
| | | | NA | Water | 765.4 G/L | NA | - |

| Substance Name | Formulation Type | Scource | Material Family | Active Ingredient/Inert function | Conc. | Amount (%w/w) | Existilng Sensitization Information[1] |
|---|---|---|---|---|---|---|---|
| Formulation 10 | EW | Dow AgroSciences | NA | Dithiopyr | 240 G/L | 24% | - (Dow Data) |
| | | | NA | Solvent | 130 G/L | 13% | - (MSDS) |
| | | | NA | Emulsifier | 470 G/L | 47% | - (MSDS) |
| | | | NA | Water | 160 G/L | 16% | - |
| Formulation 11 | OD | Dow AgroSciences | Chloroacetamides | Acetochlor | 950 G/L | 84.15% | + (Dow Data) |
| | | | Sulfonamides | Penoxsulam | 3.5 G/L | 0.31% | - (Dow Data) |
| | | | NA | Suspending Aid | 28.5 G/L | NA | - (MSDS) |
| | | | NA | Antifoam | 0.035 G/L | NA | - (MSDS) |
| | | | NA | Thickener | 0.035 G/L | NA | - (MSDS) |
| | | | NA | pH Buffer | 0.014 G/L | NA | - (MSDS) |
| | | | NA | Dispersant | 0.28 G/L | NA | - (MSDS) |
| | | | NA | Wetter | 0.07 G/L | NA | - (MSDS) |
| | | | NA | Antifreeze | 0.21 G/L | NA | - (MSDS) |
| | | | NA | Water, deionized | 2.84 G/L | NA | - |
| | | | NA | Nutrient | 4.75 G/L | 0.42% | - (human Data from IUCLID) |
| | | | NA | Related Process Inert Impurities | 45.98 G/L | NA | - (MSDS) |
| | | | NA | Anti-Caking Agent | 0.007 G/L | NA | - (MSDS) |
| | | | NA | Biocide | 0.007 G/L | 0% (0.007 g/L) | + (MSDS) |
| | | | NA | Emulsifier | 92.94 G/L | NA | - (MSDS) |
| Formulation 12 | EC | Dow AgroSciences | Dinitrophenol | Meptyldinocap | 350 G/L | 35.71% | + (Dow Data) |
| | | | NA | Emulsifier | 41.7 G/L | NA | - (MSDS) |
| | | | NA | Emulsifier | 25.76 G/L | NA | - (MSDS) |
| | | | NA | Solvent | 562.54 G/L | NA | - (MSDS) |
| Formulation 13 | EC | Dow AgroSciences | Phenoxyacetic acids | 2,4-D-ethylhexyl | 995.5 G/L | 87.17% | + (Dow Data) |
| | | | NA | Emulsifier | 48 G/L | NA | - (MSDS) |
| | | | NA | Emulsifier | 48 G/L | NA | - |
| | | | NA | Unspecified Inert | 50.5 G/L | NA | - |
| Formulation 14 | CS | Dow AgroSciences | Pyrethroids | Gamma-cyhalothrin | 15 G/L | 1.5% | + (Dow Data) |
| | | | NA | Solvent | 10.02 G/L | NA | - (MSDS) |
| | | | NA | Emulsifier | 1.25 G/L | NA | - (MSDS) |
| | | | NA | Emulsifier | 1.25 G/L | NA | - (MSDS) |
| | | | NA | Encapsulating Agent | 1.63 G/L | NA | - |
| | | | NA | pH Buffer | 1 G/L | NA | - (MSDS) |
| | | | NA | Thickener | 0.02 G/L | NA | - (MSDS) |
| | | | NA | Biocide | 1.5 G/L | 0.15% | + (MSDS) |
| | | | NA | Thickener | 1.5 G/L | NA | - (MSDS) |
| | | | NA | Thickener | 0.02 G/L | NA | - (MSDS) |
| | | | NA | Thickener | 15.03 G/L | NA | - |
| | | | NA | Water | 953.8 G/L | NA | - |
| Formulation 15 | CS | Dow AgroSciences | Pyrethroids | Gamma-cyhalothrin | 60 G/L | 5.9% | + (Dow Data) |
| | | | NA | Solvent | 48.82 G/L | | - (MSDS) |
| | | | NA | Emulsifier | 5.09 G/L | | - (MSDS) |
| | | | NA | Emulsifier | 5.09 G/L | | - (MSDS) |
| | | | NA | Encapsulating Agent | 6.81 G/L | | - |
| | | | NA | Thickener | 0.09 G/L | | - (MSDS) |
| | | | NA | Biocide | 1.53 G/L | 0.15% | + (MSDS) |
| | | | NA | Thickener | 1.53 G/L | | - (MSDS) |
| | | | NA | Thickener | 0.09 G/L | | - (MSDS) |
| | | | NA | pH Buffer | 4.07 G/L | | - (MSDS) |
| | | | NA | Thickener | 10.68 G/L | | - |
| | | | NA | Water | 873.4 G/L | | - |
| Formulation 16 | EC | Dow AgroSciences | Pyridinyloxy acetic acid | Triclopyr-butotyl | 1050.07 G/L | 83.94% | + (Dow Data) |
| | | | NA | Emulsifier | 200.93 G/L | | - (MSDS) |
| Formulation 17 | SL | Dow AgroSciences | Glycines | Glyphosate dimethylammonium salt | 608 G/L | 50.21% | - (EPA tolerance) |
| | | | NA | Adjuvant | 50 G/L | 4.13% | no Data |
| | | | NA | Adjuvant | 100 G/L | | - (MSDS) |
| | | | NA | Water | 453 G/L | | - |
| Formulation 19 | EC | Dow AgroSciences | Pyridinyloxy acetic acid | Fluroxypyr-meptyl | 100.865 G/L | 9.23% | - (Dow Data) |
| | | | Benzonitriles | Bromoxynil-octanoate | 407.569 G/L | 37.29% | + (Dow Data) |
| | | | NA | Emulsifier | 44 G/L | 4.03% | - (MSDS) |
| | | | NA | Emulsifier | 44 G/L | | - (MSDS) |
| | | | NA | Solvent | 496.566 G/L | 45.43% | - (IUCLID Datasheet) |
| Formulation 20 | SE | Dow AgroSciences | Sulfonamides | Florasulam | 4 G/L | 0.39% | - (Dow Data) |
| | | | NA | MCPA-2-ethylhexyl | 436.817 G/L | 42.25% | - (Dow Data);+ (EPA RED) |
| | | | NA | Emulsifier | 12 G/L | | - (MSDS) |
| | | | NA | Thickener | 4.34 G/L | | - (MSDS) |
| | | | NA | Dispersant | 0.17 G/L | | - (MSDS) |
| | | | NA | Antifoam | 1 G/L | | - (MSDS) |
| | | | NA | Stabilizer | 1.5 G/L | | - (MSDS) |
| | | | NA | Thickener | 0.54 G/L | | - (MSDS) |
| | | | NA | Stabilizer | 45.14 G/L | | - (MSDS) |
| | | | NA | pH Buffer | 0.01 G/L | | - (MSDS) |
| | | | NA | Stabilizer | 0.34 G/L | | - (MSDS) |
| | | | NA | Antifreeze | 49.75 G/L | | - (MSDS) |
| | | | NA | Biocide | 0.93 G/L | 0.09% | + (MSDS) |
| | | | NA | pH Buffer | 1.03 G/L | | - (MSDS) |
| | | | NA | Water | 476.443 G/L | | - |
| Formulation 21 | TK | Dow AgroSciences | Acyl Ureas | Hexaflumuron | 645 G/L | 50% | - (Dow Data) |
| | | | NA | Water | 497.42 G/L | | - |
| | | | NA | Biocide | 9.68 G/L | 0.75% | + (MSDS) |
| | | | NA | Surfactant | 64.5 G/L | | - (MSDS) |
| | | | NA | Antifoam | 3.48 G/L | | - (MSDS) |
| | | | NA | Surfactant | 69.92 G/L | 5.42% | - (MSDS) |

| Substance Name | Formulation Type | Scource | Material Family | Active Ingredient/Inert function | Conc. | Amount (%w/w) | Existilng Sensitization Information[1] |
|---|---|---|---|---|---|---|---|
| Formulation 22 | ME | Dow AgroSciences | Pyridinyloxy acetic acid | Fluroxypyr-meptyl | 28.8 G/L | 2.83% | - (Dow Data) |
| | | | NA | Triclopyr-triethylammonium | 83.67 G/L | 8.23% | + (EPA RED) |
| | | | NA | Surfactant | 29.59 G/L | | - (MSDS) |
| | | | NA | Carrier | 29.59 G/L | | - (MSDS) |
| | | | NA | Surfactant | 84 G/L | | - (MSDS) |
| | | | NA | Emulsifier | 48 G/L | | - (MSDS) |
| | | | NA | Solvent | 86.34 G/L | | - (MSDS) |
| | | | NA | Unspecified Inert | 104.98 G/L | | - |
| | | | NA | Water | 522.03 G/L | | - |
| Formulation 23 | SL | Dow AgroSciences | Pyridinyloxy acetic acid | Triclopyr-triethylammonium | 167.36 G/L | 16% | + (EPA RED) |
| | | | NA | Water | 837 G/L | | - |
| | | | NA | Antifoam | 0.02 G/L | | - (MSDS) |
| | | | NA | Wetter | 3.77 G/L | | - (MSDS) |
| | | | NA | Chelating agent | 8.68 G/L | | - (MSDS) |
| | | | NA | Surfactant | 10.04 G/L | | - (MSDS) |
| | | | NA | Neutralizer | 11.3 G/L | | - (67/548/EEC) |
| | | | NA | Carrier | 7.85 G/L | | - (67/548/EEC) |
| Formulation 24 | OD | Dow AgroSciences | Sulfonamides | Pyroxsulam | 30 G/L | 2.87% | + (Dow Data) |
| | | | NA | Safener | 90 G/L | 8.6% | + (EPA tolerance petition) |
| | | | NA | Emulsifier | 40 G/L | | - (MSDS) |
| | | | NA | Emulsifier | 50 G/L | | - (MSDS) |
| | | | NA | Emulsifier | 20 G/L | | - (MSDS) |
| | | | NA | Stabilizer | 10 G/L | | - |
| | | | NA | Suspending Aid | 40 G/L | | - (MSDS) |
| | | | NA | Diluent | 767 G/L | | - (MSDS) |
| Formulation 25 | EC | Dow AgroSciences | Pyridine carboxylic acids | Clopyralid | 23.34 G/L | 2.21% | - (Dow Data) |
| | | | Pyridinyloxy acetic acid | Fluroxypyr-meptyl | 86.455 G/L | 8.19% | - (Dow Data) |
| | | | NA | MCPA-2-ethylhexyl | 416.1 G/L | 39.4% | - (Dow Data); + (EPA RED) |
| | | | NA | Solvent | 38.54 G/L | | - (MSDS) |
| | | | NA | Emulsifier | 52.27 G/L | | - (MSDS) |
| | | | NA | Emulsifier | 428.205 G/L | | - (MSDS) |
| | | | NA | Solvent | 11.09 G/L | | - (MSDS) |
| Formulation 26 | EC | Dow AgroSciences | Pyridine carboxylic acids | Clopyralid | 60 G/L | 5.83% | - (Dow Data) |
| | | | Pyridinyloxy acetic acid | Triclopyr-butotyl | 333.797 G/L | 32.41% | + (Dow Data) |
| | | | NA | Emulsifier | 43.7 G/L | | - (MSDS) |
| | | | NA | Emulsifier | 29.2 G/L | | - (MSDS) |
| | | | NA | Solvent | 88.9 G/L | | - (MSDS) |
| | | | NA | Solvent | 474.403 G/L | | - (IUCLID Datasheet) |
| Formulation 27 | EC | Dow AgroSciences | Pyridinyloxy acetic acid | Fluroxypyr-meptyl | 479.827 G/L | 45.52% | - (Dow Data) |
| | | | NA | Emulsifier | 78.46 G/L | | - (MSDS) |
| | | | NA | Solvent | 417.253 G/L | | - (MSDS) |
| | | | NA | Emulsifier | 78.46 G/L | | -(MSDS) |
| Formulation 28 | SC | Dow AgroSciences | Unclassified Herbicide | Diflufenican | 100 G/L | 9.48% | - (MSDS) |
| | | | Sulfonamides | Penoxsulam | 15 G/L | 1.42% | - (Dow Data) |
| | | | NA | Wetter | 15 G/L | | - (MSDS) |
| | | | NA | Dispersant | 10 G/L | | - (MSDS) |
| | | | NA | Thickener | 10 G/L | | - (MSDS) |
| | | | NA | Thickener | 2 G/L | | - (MSDS) |
| | | | NA | Biocide | 1.5 G/L | 0.14% | + (MSDS) |
| | | | NA | Antifreeze | 50 G/L | | - (MSDS) |
| | | | NA | pH Buffer | 0.462 G/L | | - (MSDS) |
| | | | NA | Antifoam | 5 G/L | | - (MSDS) |
| | | | NA | Water | 846.038 G/L | | - |
| Formulation 29 | SC | Dow AgroSciences | Dithiocarbamate | Mancozeb | 462 G/L | 35.95% | equivocal (EPA RED) |
| | | | Unspecified | Cymoxanil | 70.03 G/L | 5.45% | - (EPA fact sheet) |
| | | | NA | Anti-Caking Agent | 29.81 G/L | | - (MSDS) |
| | | | NA | Stabilizer | 25.7 G/L | | - |
| | | | NA | Stabilizer | 12.85 G/L | | - |
| | | | NA | Emulsifier | 12.85 G/L | | - (MSDS) |
| | | | NA | Dispersant | 2.57 G/L | | - (MSDS) |
| | | | NA | Thickener | 1.29 G/L | | - (MSDS) |
| | | | NA | Adjuvant | 131.58 G/L | | - (MSDS) |
| | | | NA | Water | 536.32 G/L | | - |
| Formulation 30 | EW | Dow AgroSciences | Chloroacetamides | Acetochlor | 450 G/L | 41.82% | +(Dow Data) |
| | | | Pyridine Carboxylic Acids | Clopyralid-olamine | 46.11 G/L | 4.29% | - (Dow Data) |
| | | | Sulfonamides | Flumetsulam | 14.0 G/L | 1.3% | - (MSDS) |
| | | | NA | pH Buffer | 2.37 G/L | 0.22% | - (67/548/EEC) |
| | | | NA | Emulsifier | 21.52 G/L | 2% | - (IUCLID Datasheet) |
| | | | NA | Solvent | 10.76 G/L | 1% | - (IUCLID Datasheet) |
| | | | NA | Biocide | 1.076 G/L | 0.10% | + (MSDS) |
| | | | NA | Thickener | 1.076 G/L | 0.10% | - (WHO) |
| | | | NA | Antifoam | 1.61 G/L | | - (MSDS) |
| | | | NA | Dispersant | 5.38 G/L | | - (MSDS) |
| | | | NA | Wetter | 2.69 G/L | | - (MSDS) |
| | | | NA | Water | 519.408 G/L | | - |
| Formulation 31 | CS | Dow AgroSciences | Organophosphates | Chlorpyrifos | 200 G/L | 18.96% | Equivocal (Dow Data) |
| | | | NA | Encapsulating Agent | 6.49 G/L | | - |
| | | | NA | Dispersant | 29.59 G/L | | - (MSDS) |
| | | | NA | Biocide | 1.055 G/L | 0.10% | + (MSDS) |
| | | | NA | Thickener | 5.92 G/L | | - (MSDS) |
| | | | NA | Thickener | .738 G/L | | - (MSDS) |
| | | | NA | Dispersant | 16.47 G/L | | - (MSDS) |
| | | | NA | Solvent | 120 G/L | | - (IUCLID Datasheet) |
| | | | NA | Water | 674.737 G/L | | - |

| Substance Name | Formulation Type | Scource | Material Family | Active Ingredient/Inert function | Conc. | Amount (%w/w) | Existilng Sensitization Information[1] |
|---|---|---|---|---|---|---|---|
| Formulation 32 | EC | Dow AgroSciences | Dinitrophenol | Meptyldinocap | 105 G/L | 11.27% | + (Dow Data) |
| | | | Triazole | Myclobutanil | 45 G/L | 4.83% | Equivocal (Dow Data) |
| | | | NA | pH Buffer | 15 G/L | | - (67/548/EEC) |
| | | | NA | Emulsifier | 23 G/L | | - (MSDS) |
| | | | NA | Emulsifier | 68 G/L | | - (MSDS) |
| | | | NA | Solvent | 676 G/L | | - (MSDS) |
| Formulation 33 | SL | Dow AgroSciences | Pyridine carboxylic acids | Clopyralid-olamine | 316.206 G/L | 26.66% | - (Dow Data) |
| | | | Pyridine Carboxylic Acids | Picloram-olamine | 100.251 G/L | 8.45% | - (EPA RED) |
| | | | Pyridine Carboxylic Acids | Aminopyralid-olamine | 51.8 G/L | 4.37% | - (Dow Data) |
| | | | NA | Neutralizer | 22 G/L | | - (67/548/EEC) |
| | | | NA | Water, deionized | 695.743 G/L | | - |
| Formulation 34 | SL | Dow AgroSciences | Pyridine Carboxylic Acids | Aminopyralid | 30 G/L | 2.95% | - (Dow Data) |
| | | | NA | Neutralizer | 8.1 G/L | | - (67/548/EEC) |
| | | | NA | Water | 978.9 G/L | | - |
| Formulation 35 | SL | Dow AgroSciences | Pyridine Carboxylic Acids | Aminopyralid triisopropanolammonium | 23.08 G/L | 2.22 % | - (Dow Data) (Aminopyralid) |
| | | | Pyridinyloxy Acetic Acid | Triclopyr-triethylammonium | 167.36 G/L | 16.09 % | + (EPA RED) |
| | | | NA | Neutralizer | 1.14 G/L | | - |
| | | | NA | Wetter | 38 G/L | | - (MSDS) |
| | | | NA | Antifoam | .19 G/L | | - (MSDS) |
| | | | NA | Neutralizer | 14.82 G/L | | - (67/548/EEC) |
| | | | NA | Sequestrant | 8.74 G/L | | - (MSDS) |
| | | | NA | Water | 786.67 G/L | | - |
| Formulation 37 | EC | Dow AgroSciences | Organophosphates | Chlorpyrifos | 300 G/L | 30% | Equivocal (Dow Data) |
| | | | Pyrethroids | Gamma-cyhalothrin | 5.4 G/L | 0.54% | + (DOW Data) |
| | | | NA | Emulsifier | 55 G/L | 5.50% | - (MSDS) |
| | | | NA | Emulsifier | 4.4 G/L | 0.44% | - (MSDS) |
| | | | NA | Solvent | 635.2 G/L | 63.52% | - (IUCLID Datasheet) |
| Formulation 38 | EC | Dow AgroSciences | Acetamides | Propanil | 479.81 G/L | 44.80% | - (EPA RED) |
| | | | NA | Solvent | 362 G/L | | - (MSDS) |
| | | | NA | Solvent | 122.09 G/L | | - (IUCLID Datasheet) |
| | | | NA | Emulsifier | 107.1 G/L | 10% | - (IUCLID Datasheet) |
| Formulation 39 | OD | Dow AgroSciences | Sulfonamides | Pyroxsulam | 45 G/L | 4.31% | + (DOW Data) |
| | | | NA | Safener | 90 G/L | 8.61% | + (EPA tolerance petition) |
| | | | NA | Dispersant | 6 G/L | 0.57% | - (MSDS) |
| | | | NA | Dispersant | 10 G/L | | - (MSDS) |
| | | | NA | Emulsifier | 80 G/L | | - (MSDS) |
| | | | NA | Stabilizer | 10 G/L | 0.96% | - (MSDS) |
| | | | NA | Suspending Aid | 27 G/L | | - (MSDS) |
| | | | NA | Solvent | 777 G/L | | - (MSDS) |
| Formulation 40 | OD | Dow AgroSciences | Sulfonamides | Pyroxsulam | 12.8 G/L | 1.20% | + (DOW Data) |
| | | | NA | Safener | 38.5 G/L | 3.62% | + (EPA tolerance petition) |
| | | | NA | Active Ingredient | 2.14 G/L | 0.20% | - (EPA Fact Sheet) |
| | | | NA | Active Ingredient | 123.199 G/L | 11.57% | - (Dow Data) |
| | | | NA | Dispersant | 4 G/L | 0.38% | - (MSDS) |
| | | | NA | Dispersant | 10 G/L | | - (MSDS) |
| | | | NA | Emulsifier | 80 G/L | | - (MSDS) |
| | | | NA | Stabilizer | 10 G/L | | - (MSDS) |
| | | | NA | Thickener | 30 G/L | | - (MSDS) |
| | | | NA | Solvent | 754.361 G/L | | - (MSDS) |
| Formulation 41 | SE | Dow AgroSciences | Phenoxyacetic acids | 2,4-D-ethylhexyl | 271.493 G/L | 25.61% | + (DOW Data) |
| | | | Pyridine Carboxylic Acids | Aminopyralid | 11.834 G/L | 1.12% | - (Dow Data) |
| | | | Sulfonamides | Florasulam | 5 G/L | 0.47% | - (Dow Data) |
| | | | NA | Solvent | 73.2 G/L | | - (MSDS) |
| | | | NA | Emulsifier | 60.4 G/L | | - (MSDS) |
| | | | NA | Thickener | 0.1 G/L | | - (MSDS) |
| | | | NA | Biocide | 0.9 G/L | 0.08% | + (MSDS) |
| | | | NA | Antifoam | 2 G/L | | - (MSDS) |
| | | | NA | Dispersant | 0.2 G/L | | - (MSDS) |
| | | | NA | Antifoam | 0.02 G/L | | - (MSDS) |
| | | | NA | Antifreeze | 50.5 G/L | | - (MSDS) |
| | | | NA | Suspending Aid | 1.6 G/L | | - (MSDS) |
| | | | NA | pH Buffer | 0.1 G/L | | - (MSDS) |
| | | | NA | Water | 582.873 G/L | | - |
| Formulation 42 | SL | Dow AgroSciences | Phenoxyacetic Acids | 2,4-D-triisoproanolamine | 339 G/L | 31.00% | - (EPA RED) |
| | | | Pyridine Carboxylic Acids | Aminopyralid triisopropanolammonium | 17 G/L | 1.52% | - (Dow Data) (Aminopyralid) |
| | | | NA | Neutralizer | 4.962 G/L | | - (MSDS) |
| | | | NA | Sequestrant | 2.19 G/L | | - (MSDS) |
| | | | NA | Antifreeze | 38.26 G/L | | - (MSDS) |
| | | | NA | Water | 694.48 G/L | | - |
| Formulation 43 | CS | Dow AgroSciences | Unspecified Nitrification Inhibitor | Nitrapyrin | 200 G/L | 17.90% | + (Dow Data) |
| | | | NA | Solvent | 234.79 G/L | 0.12% | + (R43) |
| | | | NA | Solvent | 99.65 G/L | | - (MSDS) |
| | | | NA | Thickener | 22.31 G/L | | - (MSDS) |
| | | | NA | Dispersant | 13.36 G/L | | + (MSDS) |
| | | | NA | Emulsifier | 13.36 G/L | 0.24% | - (MSDS) |
| | | | NA | Dispersant | 2.67 G/L | 1.19% | - (MSDS) |
| | | | NA | Thickener | 2.14 G/L | 8.87% | + (DOW Data) |
| | | | NA | Biocide | 1.34 G/L | | - (MSDS) |
| | | | NA | Water | 534.38 G/L | | - |

| Substance Name | Formulation Type | Scource | Material Family | Active Ingredient/Inert function | Conc. | Amount (%w/w) | Existilng Sensitization Information[1] |
|---|---|---|---|---|---|---|---|
| Formulation 44 | SC | Dow AgroSciences | Sulfonamides | Penoxsulam | 1.4 G/L | 0.12% | - (Dow Data) |
| | | | Dinitroanilines | Oryzalin | 478.9 G/L | 40.38% | Equivocal (Dow Data) |
| | | | NA | Antifoam | 5.92 G/L | | - (MSDS) |
| | | | NA | Dispersant | 71 G/L | 5.99% | - (MSDS) |
| | | | NA | Antifreeze | 47.3 G/L | | - (MSDS) |
| | | | NA | Dispersant | 17.7 G/L | 1.49% | - (MSDS) |
| | | | NA | Antifreeze | 71.1 G/L | 5.99% | - (MSDS) |
| | | | NA | Biocide | .59 G/L | 0.05% | + (MSDS) |
| | | | NA | Suspending Aid | 1.78 G/L | 0.15% | - (WHO) |
| | | | NA | Carrier | 8.88 G/L | | - (MSDS) |
| | | | NA | Antifoam | .01 G/L | | - (MSDS) |
| | | | NA | Suspending Aid | .01 G/L | 0% | - (MSDS) |
| | | | NA | pH Buffer | .01 G/L | | - (MSDS) |
| | | | NA | Dispersant | .11 G/L | 0.01% | - (MSDS) |
| | | | NA | Wetter | .03 G/L | 0% | -(MSDS) |
| | | | NA | Water | 481.32 G/L | 40.58% | - |
| Formulation 45 | SC | Dow AgroSciences | Carboxanilide | Thifluzamide | 80 G/L | 7.53 % | - (Dow Data) (25%) |
| | | | Triazole | Fenbuconazole | 100 G/L | 9.42 % | - (Dow Data) |
| | | | NA | Adjuvant | 51.4008 G/L | | - (MSDS) |
| | | | NA | Wetter | 12.8502 G/L | | - (MSDS) |
| | | | NA | Biocide | 1.062 G/L | 0.10% | + (MSDS) |
| | | | NA | Suspending Aid | 4.248 G/L | | - (MSDS) |
| | | | NA | Antifoam | 5.32 G/L | | - (MSDS) |
| | | | NA | Emulsifier | 11.682 G/L | | - (MSDS) |
| | | | NA | Dispersant | 40.887 G/L | | - (MSDS) |
| | | | NA | Water | 754.55 G/L | 71.05 % | - |
| Formulation 46 | SC | Dow AgroSciences | Spinosoids | Spinetoram | 60 G/L | 5.87% | equivocal (+/- LLNA) |
| | | | NA | Dispersant | 30.75 G/L | | - (MSDS) |
| | | | NA | Wetter | 20.5 G/L | 2% | - (MSDS) |
| | | | NA | Antifreeze | 61.4 G/L | | - (MSDS) |
| | | | NA | Biocide | 2 G/L | 0.20% | + (MSDS) |
| | | | NA | Thickener | 2 G/L | 0.20% | - (WHO) |
| | | | NA | Thickener | 4.1 G/L | | - (MSDS) |
| | | | NA | Antifoam | 10 G/L | 0.98% | - (MSDS) |
| | | | NA | Water | 832.25 G/L | 81.35% | - |
| Formulation 47 | EW | Dow AgroSciences | Triazole | propiconazole | 150 G/L | 14.56% | + (EPA RED) |
| | | | NA | Solvent | 5.15 G/L | | - (MSDS) |
| | | | NA | Emulsifier | 20.6 G/L | 2.00% | - (MSDS) |
| | | | NA | Emulsifier | 15.45 G/L | 0.50% | - (MSDS) |
| | | | NA | Antifreeze | 51.5 G/L | 5.00% | - (MSDS) |
| | | | NA | Emulsifier | 51.5 G/L | 1.50% | - (MSDS) |
| | | | NA | water | | 66.44% | - |
| | | | NA | Solvent | 735.8 G/L | 5.00% | -( IUCLID Datasheet) |
| Formulation 49 | AL | Dow AgroSciences | Pyridinyloxy acetic acid | Triclopyr-butotyl | 200.3 G/L | 23.16% | + (Dow Data) |
| | | | NA | Diluent | 664.7 G/L | 76.84 % | - (IUCLID Datasheet) |
| Formulation 50 | SL | Dow AgroSciences | Glycines | Glyphosate dimethylammonium salt | 608 G/L | 50.54 % | - (EPA tolerance) |
| | | | NA | Adjuvant | 90 G/L | 7.48 % | - (MSDS for similar) |
| | | | NA | Water | 505 G/L | 41.98 % | - |
| Formulation 51 | OD | Dow AgroSciences | Dinitroanilines | Pendimethalin | 314 G/L | 29.76 % | - (EPA RED) |
| | | | Sulfonamides | Pyroxsulam | 5.4 G/L | 0.51% | + (Dow Data) |
| | | | NA | Safener | 5.4 G/L | 0.51% | + (EPA tolerance petition) |
| | | | NA | Stabilizer | 5 G/L | | - (MSDS) |
| | | | NA | Suspending Aid | 20 G/L | | - (MSDS) |
| | | | NA | Emulsifier | 60 G/L | | - (MSDS) |
| | | | NA | Emulsifier | 10 G/L | 0.95% | -(MSDS) |
| | | | NA | Emulsifier | 30 G/L | | - (MSDS) |
| | | | NA | Antifoam | 1 G/L | 0.09% | - (MSDS) |
| | | | NA | Solvent | 604.2 G/L | | - (MSDS) |
| Formulation 53 | EW | Dow AgroSciences | Organophosphates | Chlorpyrifos | 450 G/L | 40.18 % | Equivocal (Dow Data) |
| | | | NA | Emulsifier | 56 G/L | 5 % | no Data |
| | | | NA | Antifreeze | 28 G/L | | - (MSDS) |
| | | | NA | Dispersant | 134.5 G/L | 12.01 % | - (MSDS) |
| | | | NA | Biocide | 1.12 G/L | 0.10% | + (MSDS) |
| | | | NA | Antifoam | 4.5 G/L | | - (MSDS) |
| | | | NA | Solvent | 224 G/L | 20% | - (IUCLID Datasheet) |
| | | | NA | Water | 221.88 G/L | 19.81% | - |
| Formulation 54 | SL | Dow AgroSciences | Glycines | Glyphosate dimethylammonium salt | 608 G/L | 49.88 % | - (EPA tolerance) |
| | | | NA | Adjuvant | 100 G/L | | - (MSDS) |
| | | | NA | Adjuvant | 50 G/L | | - (MSDS) |
| | | | NA | Water | 461 G/L | 37.82 % | - |
| Formulation 55 | EW | Dow AgroSciences | Triazole | Myclobutanil | 45 G/L | 4.5 % | Equivocal (Dow Data) |
| | | | NA | Emulsifier | 26.5 G/L | 2.65 % | - (MSDS) |
| | | | NA | Emulsifier | 18.5 G/L | 1.85 % | -(MSDS) |
| | | | NA | Antifreeze | 100 G/L | | - (MSDS) |
| | | | NA | Solvent | 200 G/L | 20 % | -( IUCLID Datasheet) |
| | | | NA | Diluent | 40.5 G/L | | - (MSDS) |
| | | | NA | Emulsifier | 5 G/L | 0.50% | no Data |
| | | | NA | Water | 561.5 G/L | 56.15 % | - |
| | | | NA | Biocide | 3 G/L | 0.30% | + (MSDS) |
| Formulation 56 | SL | Dow AgroSciences | Unspecified Nitrification Inhibitor | Nitrapyrin | 216 G/L | 19.89% | + (Dow Data) |
| | | | NA | impurities | 24 G/L | 2.21% | no Data |
| | | | NA | Stabilizer | 14.4 G/L | 1.33% | - (MSDS) |
| | | | NA | Solvent | 831.6 G/L | 76.57% | - (IUCLID Datasheet) |
| Oxyflourfen | EC | ECPA | NA | Oxyflourfen | 240 G/L | NA | NA |
| | | | NA | Solvent | 732 G/L | NA | NA |

| Substance Name | Formulation Type | Scource | Material Family | Active Ingredient/Inert function | Conc. | Amount (%w/w) | Existilng Sensitization Information[1] |
|---|---|---|---|---|---|---|---|
| | | | NA | Surfactant | 108 G/L | NA | NA |
| Quinoxyfen/ cyproconazole | NA | ECPA | NA | Cyproconazole | 80 G/L | NA | NA |
| | | | NA | Quinoxyfen | 75 G/L | NA | NA |
| | | | NA | Antifreeze | 75 G/L | NA | NA |
| | | | NA | Thickener | 10 G/L | NA | NA |
| | | | NA | Water/Other Components | 842 G/L | NA | NA |
| Trifluralin | EC | ECPA | NA | Triflualin | 480 G/L | NA | NA |
| | | | NA | Solvent | 500 G/L | NA | NA |
| | | | NA | Surfactant | 60 G/L | 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. Environmantal 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

# Appendix B4

## Physico-Chemical Properties and Chemical Classes of Dye Formulations Tested in the LLNA

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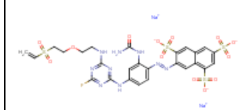
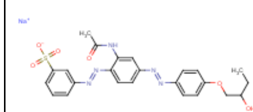
## Dye Formulations Tested in the LLNA – Comparative Data (Sorted Alphabetically)

| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical 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-Naphthalenetrisulfonic 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 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, based on CASRN, were obtained from ChemID available at: http://chem.sis.nlm.gov/chemidplus/chemidheavy.jsp.

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# Appendix B5

## Dye Formulations Tested in the LLNA - Comparative Data (Sorted Alphabetically)

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**Dye Formulations Tested in the LLNA - Comparative Data (Sorted Alphabetically**

| 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 fur 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 fur 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 fur Arbeitsschutz und Arbeitsmedizin |
| 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 fur 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 fur 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 fur 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; "-" = Non-sensitizer

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# Appendix B6

## Physico-Chemical Properties and Chemical Classes of Fragrance Ingredients Tested in the LLNA

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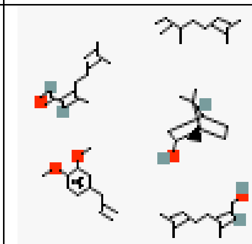
## Physico-Chemical Properties and Chemical Classes of Fragrance Ingredients Tested in the LLNA (Sorted Alphabetically)

| 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 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, based on CASRN, were obtained from ChemID available at: http://chem.sis.nlm.gov/chemidplus/chemidheavy.jsp.

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# Appendix B7

## Fragrance Ingredients Tested in the LLNA - Comparative Data (Sorted Alphabetically)

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**Fragrance Ingredients Tested in the LLNA - Comparative Data (Sorted Alphabetically)**

| Substance Name | Formulation Type | LLNA Conc. tested (%) | LLNA Sis | LLNA $EC_3$ (%) | 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 ingredient | 2.5, 5. 10, 25, 50 | 1.2, 0.7, 1.7, 1.8, 2,8 | NC | 1:3 EtOH/DEP | CBA/Ca | - | - | Lalko & Api (2006). submitted by RIFM | 10 | 0 | - | - | Opdyke 1975c |
| 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 |
| 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; "-" = Non-sensitizer

[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).

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# APPENDIX C1

## Physico-Chemical Properties and Chemical Classes of Metals Analyzed in the Applicability Domain of the LLNA

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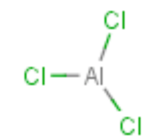
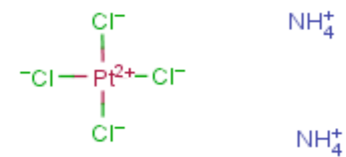
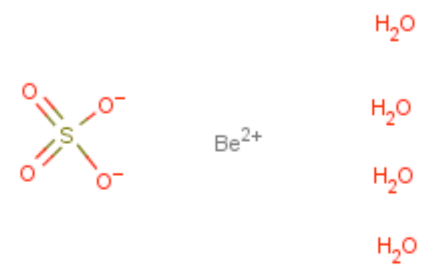
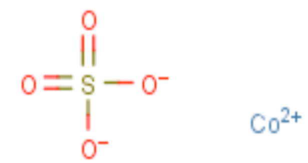
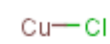
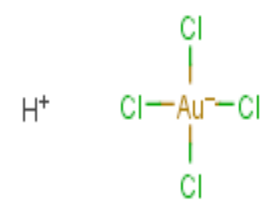
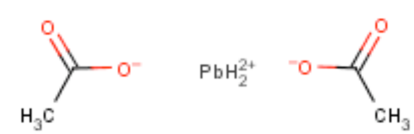
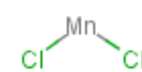
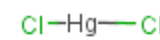
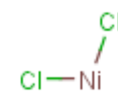
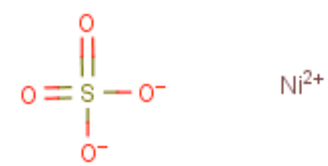
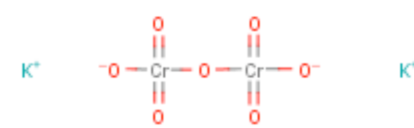
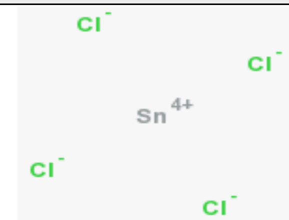
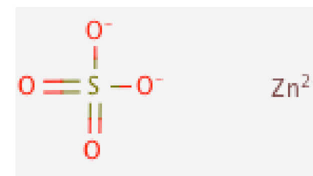
## Physico-Chemical Properties – Metals (Sorted Alphabetically)

| Substance Name | Synonyms | CASRN | Molecular Weight (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 tetrachloroplatinate***[5] | ***Ammonium platinous chloride, Ammonium chloroplatinate*** | ***13820-41-2*** | ***372.97*** | ***0.47*** | ***Solid*** | ***Inorganic Chemicals, Platinum Compounds*** |  |
| ***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; Salts | NA |

| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| Cobalt sulfate | Cobaltous sulfate | 10124-43-3 | 154.99 | 0.63 | Solid | Inorganic Chemicals; Metals; Salts |  |
| ***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; Metals; Salts*** |  |
| Manganese chloride | Manganese chloride, anhydrous | 7773-01-5 | 125.84 | 0.85 | Solid | Inorganic Chemicals, Manganese Compounds; Salts |  |

| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| ***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 (II) salts | NA | NA | NA | NA | Solid | Inorganic Chemicals; Metals; Salts | NA |
| ***Nickel sulfate*** | ***Nickel (II) sulfate*** | ***7786-81-4*** | ***154.76*** | ***-0.17*** | ***Solid*** | ***Inorganic Chemicals; Metals; Salts*** |  |
| ***Potassium dichromate*** | ***PDC*** | ***7778-50-9*** | ***294.18*** | ***-2.24*** | ***Solid*** | ***Inorganic Chemicals, Chromium Compounds; Inorganic Chemicals, Potassium Compounds*** |  |

| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| 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 represent the 11 metals reported in the original LLNA Evaluation Report (ICCVAM 1999).
Abbreviations: CASRN=Chemical Abstract Services Registry Number; g/mol=Grams per mole; Kow=Octanol-water partition coefficient; NA=Not available.
[1]$K_{ow}$ 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.

# Appendix C2

## Metals Tested in the LLNA - Comparative Data (Sorted Alphabetically)

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**Metals Tested in the LLNA - Comparative Data (Sorted Alphabetically)**

| 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] (Non-Aqueous Metals) | LLNA References | Guinea Pig Studies Outcome[1] (GPMT/BT) | Guinea Pig References | Human Outcome[1] | Human References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Aluminum chloride | 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. (1999a); Schneider and Akkan (2004) | + | Basketter et al. (1999a) | +[8,9] | Basketter et al. (1994); Kligman (1966); Basketter et al. (1999b) |
| | | 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 | + | | | | | | | | |

| 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] (Non-Aqueous Metals) | LLNA References | Guinea Pig Studies Outcome[1] (GPMT/BT) | Guinea Pig References | Human Outcome[1] | Human References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***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 | 7773-01-5 | 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 Scholes (1992); Basketter et al. (1999b); ICCVAM (1999) | + | 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 | - | | | | | | | | |
| Nickel (II) salts | NA | NA | NA | 1.40 | NA | + | + | NA | NA | Schneider and Akkan (2004) | NA | NT | +[8] | Kligman (1966); (Schneider and Akkan (2004) |

| 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] (Non-Aqueous 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 | 0.20 | 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 | + | | | | | | | | |
| | | 0.02, 0.1, 0.5 | 1.06, 1.04, 5.55 | 0.3 | 1% Pluronic L92 | + | | | | | | | | |
| | | 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 | + | | | | | | | | |
| | | 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) |

| 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] (Non-Aqueous Metals) | LLNA References | Guinea Pig Studies Outcome[1] (GPMT/BT) | Guinea Pig References | Human Outcome[1] | Human References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***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 | + | | | | | | | | |

Abbreviations: AOO = Acetone olive-oil (4:1); BT = Beuhler Test; CASRN = Chemical Abstracts Service Registry Number; Conc. = Concentration; DMF = Dimethylformamide; DMSO = Dimethyl sulfoxide; ETOH = Ethanol; GPMT = Guinea Pig Maximization Test; IDR = Insufficient Data Results; LLNA = Local Lymph Node Assay; NA = Not Available; NC = Not Calculated; SI = Stimulation Index.
[1] (+) = Sensitizer; (-) = Non-sensitizer
[2]Overall LLNA result based on "weight-of-evidence" with the majority and/or most severe result applicable to all chemicals except for nickel chloride.
[3]An aqueous vehicle is any vehicle containing at least 20% water. Conversely, a non-aqueous vehicle is any vehicle containing less than 20% water.
[4]Bold and italicized text represent the 11 metals that were recorded in the ICCVAM LLNA Evaluation Report (ICCVAM 1999).
[5]LLNA Project Report was provided by the European Crop Protection Association (ECPA).
[6]National Toxicology Program (NTP) data were provided by D. Germolec.
[7]Data obtained from the Human Patch Test Allergen.
[8]Data obtained from the Human Maximization Test.
[9]Data obtained from the Human Repeat Insult Patch Test.

# Appendix D1

## Physico-Chemical Properties and Chemical Classes of Substances Tested in Aqueous Solutions in the LLNA

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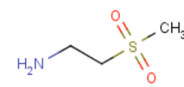
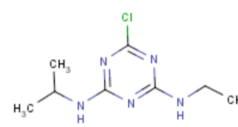
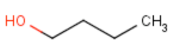
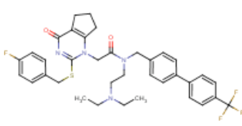
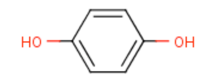
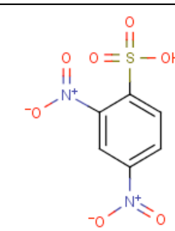
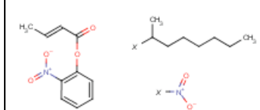
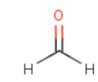
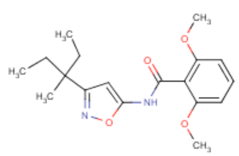
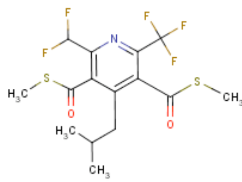
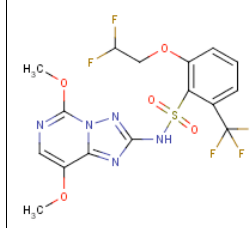
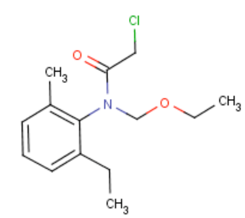
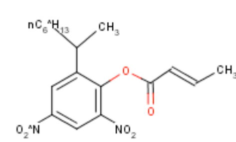
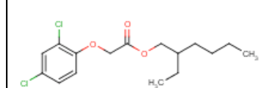
**Physico-Chemical Properties – Substances Tested in Aqueous Solution (Sorted Alphabetically)**

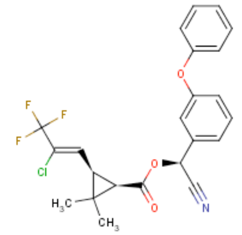
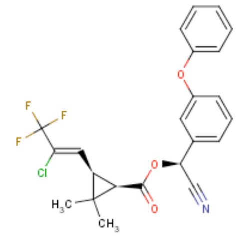
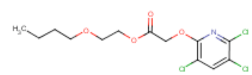
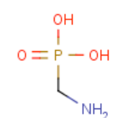
| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| AE F016382 00 TK71 A101 | NA | NA | NA | NA | NA | Formulation | NA |
| A SC600 | NA | NA | NA | NA | NA | Formulation | NA |
| 2-Aminoethyl-methylsulfone | Ethanamine, 2-(methylsulfonyl)- | 49773-20-8 | 159.63 | NA | Solid | Sulfur Compounds |  |
| Atrazine | Atrizine SC<br>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 |
| BASF #5 | NA | NA | NA | NA | Suspension | NA | NA |

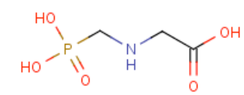
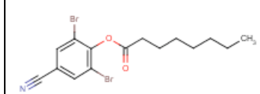
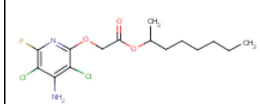
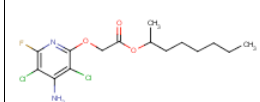
| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| BASF #6 | BAS 493 05 F | NA | NA | NA | Dispersion | NA | NA |
| BASF SC-1 | suspension concentrate 1 | NA | NA | NA | Emulsion | NA | NA |
| 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 |
| 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 |  |

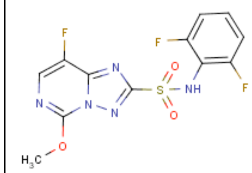
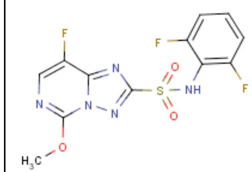
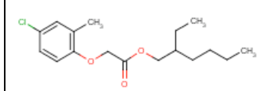
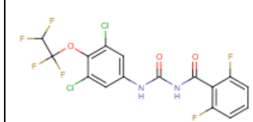
| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| 1,4-Dihydroquinone | Hydroquinone<br>p-hydroquinone | 123-31-9 | 110.11 | 1.17 | Solid | Phenols |  |
| 2.4-Dinitrobenzene sulfonic acid | 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<br>(9CI)<br>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 |

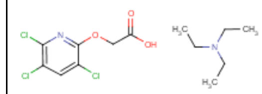
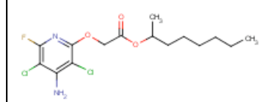
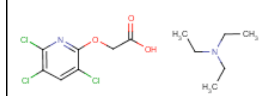
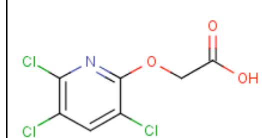
| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| FAR01060-00 | NA | NA | NA | NA | NA | Formulation | NA |
| F & Fo WG 50 + 25 | NA | NA | NA | NA | NA | Formulation | NA |
| Formaldehyde | 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 |  |

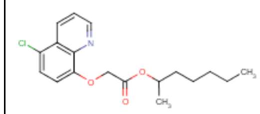
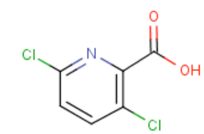
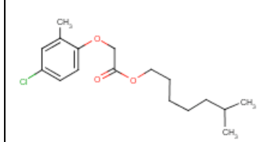
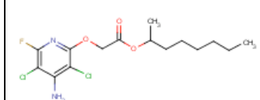
| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| Formulation 11 | 0.31 wt % penoxsulam, 84.2 wt % acetochlor | 219714-96-2<br>34256-82-1 | 483.37<br>269.77 | NA | Liquid | Formulation | <br> |
| Formulation 12 | 34.7% w/w 2,4-dinitro-6-(1-methylheptyl)phenyl crotonate<br>DE-126 | 6119-92-2 | 364.40 | NA | Liquid | Formulation |  |
| Formulation 13 | 87.6% w/w 2,4-Dichlorophenoxyacetic acid 2-ethylhexyl ester<br>2,4-D-2-ethylhexyl | 1928-43-4 | 333.25 | NA | Liquid | Formulation |  |

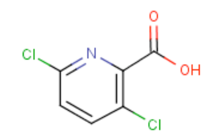
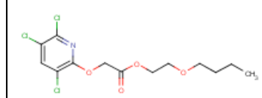
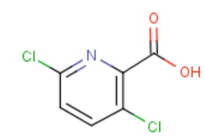
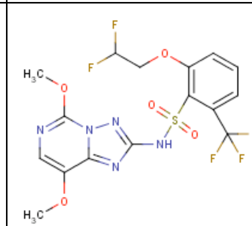
| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| Formulation 14 | 1.5 wt. % gamma-cyhalothrin<br>Nexide<br>Fentrol | 76703-62-3 | 449.85 | NA | Liquid | Formulation |  |
| Formulation 15 | 5.8 wt.% gamma-cyhalothrin<br>Nexide<br>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 dimethylammonium salt (active ingredient)<br>40.1% wt/wt glyphosate (acid equivalent)<br>8.3% w/w Geronol CF/AS 30 | 1066-51-9<br>1071-83-6 | 111.04<br>169.02 | NA | Liquid | Formulation |  |

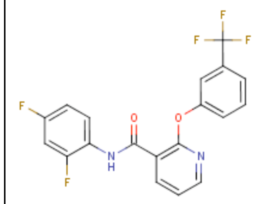
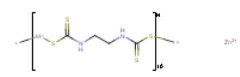
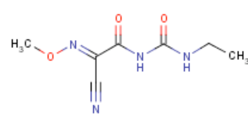
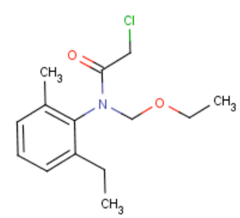
| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| | (ammonium adjuvant) | | | | | |  |
| Formulation 19 | 37.1 wt% Bromoxynil octanoate 9.23 wt% fluroxypyr-1-methylheptyl | 1689-99-2<br>81406-37-3 | 403.11<br>367.25 | NA | Liquid | Formulation | <br> |
| Formulation 2 | 14.2% w/w fluroxypyr -meptyl 0.22% w/w florasulam | 81406-37-3<br>145701-23-1 | 367.25<br>359.29 | NA | Liquid | Formulation |  |

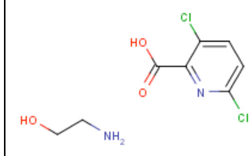
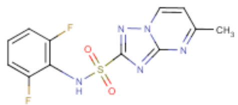
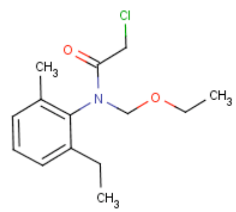
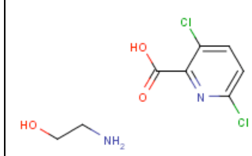
| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| | | | | | | |  |
| Formulation 20 | 0.39 wt% Florasulam 41.9 wt% 2-methyl-4-chlorophenoxyacetic acid 2-ethylhexyl ester (MCPA, 2-ethyl hexyl ester) | 145701-23-1<br>29450-45-1 | 359.29<br>312.84 | NA | Liquid | Formulation | <br> |
| 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 |  |

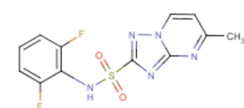
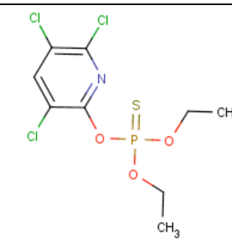
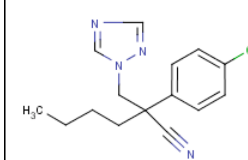
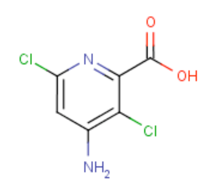
| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| Formulation 22 | 8.3 wt. % triclopyr triethylammonium 2.8 wt, % fluroxypyr-methyl heptyl ester | 57213-69-1<br>81406-37-3 | 357.66<br>367.25 | NA | Liquid | Formulation | <br> |
| Formulation 23 | 16.1 wt% Triclopyr -triethylammonium 11.6 wt% triclopyr acid | 57213-69-1<br>55335-06-3 | 357.66 | NA | Liquid | Formulation | <br> |

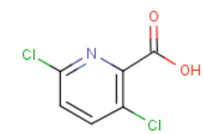
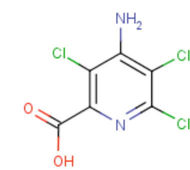
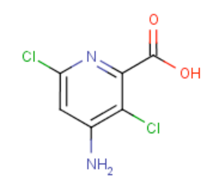
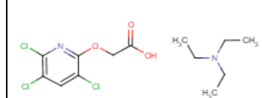
| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| Formulation 24 | 8.8 wt% Cloquintocet-mexyl | 99607-70-2 | 335.83 | NA | Liquid | Formulation |  |
| Formulation 25 | 2.2 wt.% clopyralid<br>37.7 wt.% MCPA-2-ethylhexyl ester<br>8.2 wt.% fluroxypyr -meptyl | 1702-17-<br>626544-20-7<br>81406-37-3 | 192.00<br>312.84/<br>367.25 | NA | Liquid | Formulation | <br><br> |

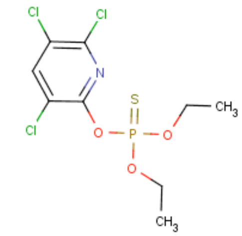
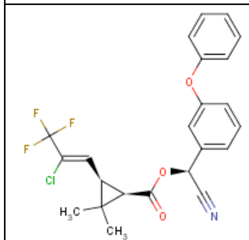
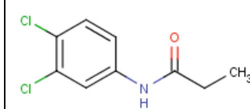
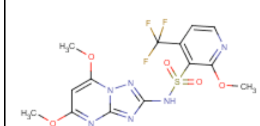
| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| Formulation 26 | 5.9 wt.% clopyralid<br>32.9 wt.% triclopyr-butotyl | 1702-17-6<br>64700-56-7 | 192.00<br>356.63 | NA | Liquid | Formulation | <br> |
| Formulation 27 | 45.2 wt% fluroxypyr-meptyl | 81406-37-3 | 192.00 | NA | Liquid | Formulation |  |
| Formulation 28 | 1.4 wt% penoxsulam<br>9.37 wt% diflufenican | 219714-96-2<br>83164-33-4 | 483.37<br>394.30 | NA | Liquid | Formulation |  |

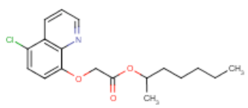
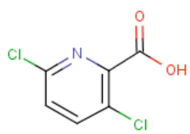
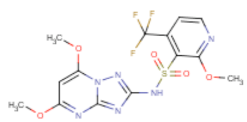
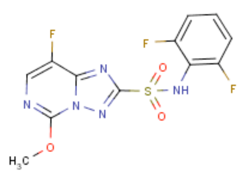
| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| | | | | | | |  |
| Formulation 29 | 35.6% mancozeb,<br>4.92% cymoxanil | 8018-01-7<br>57966-95-7 | 541.1<br>198.18 | NA | Liquid | Formulation | <br> |
| Formulation 3 | 455 g/L acetochlor<br>47 g/L clopyralid-olamine<br>14 g/L flumetsulam | 34256-82-<br>157754-85-5<br>98967-40-9 | 269.77<br>253.08<br>325.30 | NA | Liquid | Formulation |  |

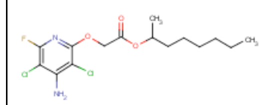
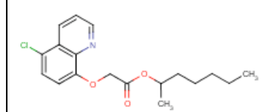
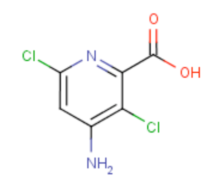
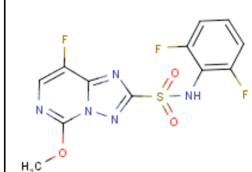
| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| | | | | | | |   |
| Formulation 30 | 455 g/L acetochlor<br>47 g/L clopyralid-olamine<br>14 g/L flumetsulam | 34256-82-1<br>57754-85-5<br>98967-40-9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

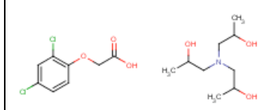
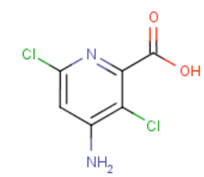
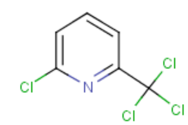
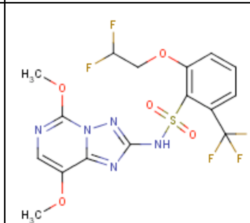
| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| | | | | | | |  |
| Formulation 31 | 18.7 wt.% chlorpyrifos | 2921-88-2 | 350.59 | NA | Liquid | Formulation |  |
| Formulation 32 | 11.2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

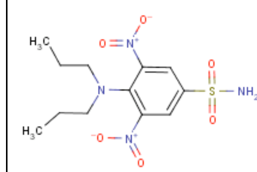
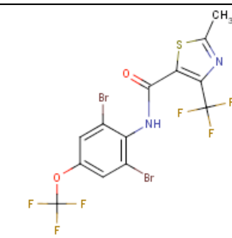
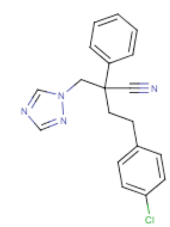
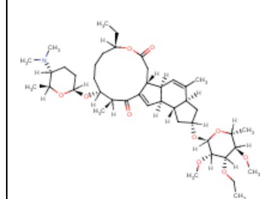
| **Substance Name** | **Synonyms** | **CASRN** | **Molecular Weight (g/mol)** | **Log Kow[1]** | **Physical Form** | **Chemical Class[2]** | **Structure[3]** |
|---|---|---|---|---|---|---|---|
| | 3.5 wt% aminopyralid<br>20.6 wt% clopyralid<br>7.0 wt% picloram | | | | | | <br> |
| Formulation 34 | 3.0 wt% aminopyralid | 150114-71-9 | | NA | Liquid | Formulation |  |
| Formulation 35 | 2.15 wt% aminopyralid-triisopropanolammonium<br>16.0 wt% triclopyr-triethylammonium | 566191-89-7<br>57213-69-1 | NA<br>357.66 | NA | Liquid | Formulation | NA<br> |

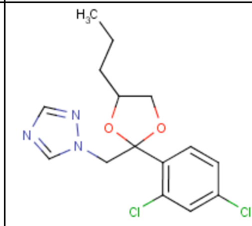
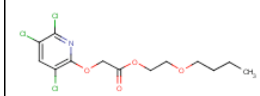
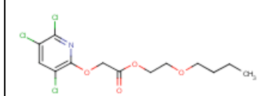
| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| Formulation 37 | 30.6 wt.% chlorpyrifos<br>0.54 wt% gamma-cyhalothrin | 2921-88-2<br>76703-62-3 | 350.60<br>449.85 | NA | Liquid | Formulation | <br> |
| Formulation 38 | 44.4 wt.% propanil | 709-98-8 | 218.08 | NA | Liquid | Formulation |  |
| Formulation 39 | 4.2 wt%<br>Pyroxsulam<br>8.7 wt%<br>Cloquintocet mexyl | 422556-08-9<br>99607-70-2 | 434.35<br>335.83 | NA | Liquid | Formulation |  |

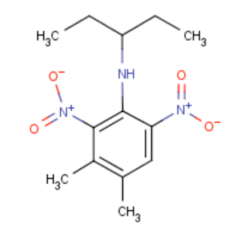
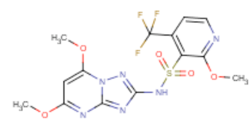
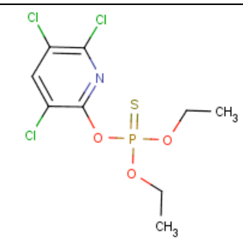
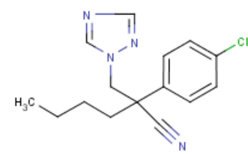
| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| | | | | | | |  |
| Formulation 4 | 100 g/L clopyralid mono-ethanolamine salt) | 1702-17-6 | 192.00 | NA | Liquid | Formulation |  |
| Formulation 40 | 1.2 wt% Pyroxsulam 0.21 wt% Florasulam 11.8 wt% Fluroxypyr-meptyl 3.6 wt% 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 |   |

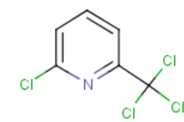
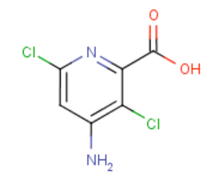
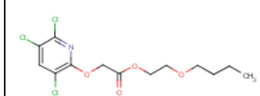
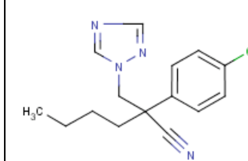
| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| | | | | | | | <br> |
| Formulation 41 | 1.10 wt% Aminopyralid potassium salt<br>0.47 wt% Florasulam | 150114-71-9<br>145701-23-1 | 207.02<br>359.29 | NA | Liquid | Formulation | <br> |

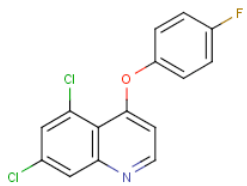
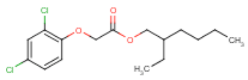
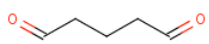
| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| Formulation 42 | 31 wt % 2,4-D-triisoproanolamine<br><br>1.52 wt % Aminopyralid triisopropanolammonium | 18584-79-7<br>150114-71-9 | 412.31<br>207.2 | NA | NA | Formulation | <br> |
| Formulation 43 | 17.9 wt % Nitrapyrin | 1929-82-4 | 230.91 | NA | NA | Formulation |  |
| Formulation 44 | 0.12 wt % Penoxsulam<br><br>40.38 wt % Oryzalin | 219714-96-2<br>19044-88-3 | 483.37<br>346.36 | NA | NA | Formulation |  |

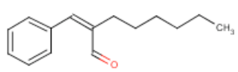
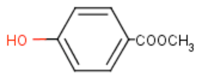
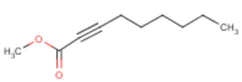
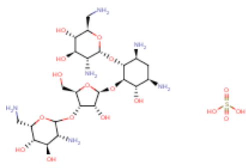
| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| | | | | | | |  |
| Formulation 45 | 7.53 wt % Thifluzamide<br><br>9.42 wt % Fenbuconazole | 130000-40-7<br>114369-43-6 | 528.06<br>336.82 | NA | NA | Formulation | <br> |
| Formulation 46 | 5.87 wt % Spinetoram | 187166-15-0 | 760.02 | NA | NA | Formulation |  |

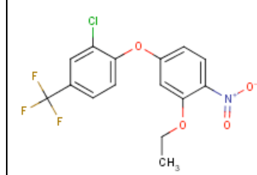
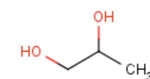
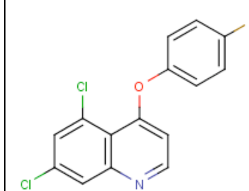
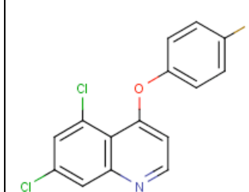
| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| Formulation 47 | 14.56 wt % propiconazole | 60207-90-1 | 342.22 | NA | NA | Formulation |  |
| Formulation 49 | 23.7 WT% Triclopyr BEE | 64700-56-7 | 356.63 | | Liquid | Formulation |  |
| Formulation 5 | 3,5,6-trichloro-2-pyridyloxyacetic acid, butoxy ethyl ester<br>Triclopyr-butotyl<br>triclopyr BEE | 64700-56-7 | 356.63 | | Liquid | Formulation |  |
| Formulation 50 | Glyphosate dimethylamine salt<br>glyphosate dimethylammonium salt | 34494-04-7<br>NA | NA | NA | Liquid | Formulation | NA |

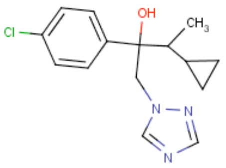
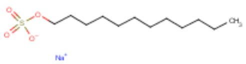
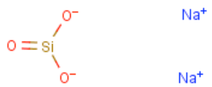
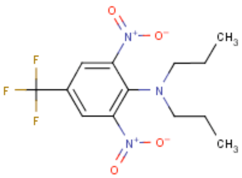
| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| Formulation 51 | 29.6 wt% Pendimethalin 0.51 wt% Pyroxsulam | 40487-42-1 422556-08-9 | 281.31 434.35 | | Liquid | Formulation |   |
| Formulation 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 |
| Formulation 55 | 4.6 wt% Myclobutanil | 88671-89-0 | 288.78 | NA | Liquid | Formulation |  |

| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| 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<br>64700-56-7 | 207.02 | NA | Liquid | Formulation | <br> |
| Formulation 7 | 45 g/L myclobutanil + 45 g/L quinoxyfen) | 88671-89-0<br>124495-18-7 | 288.78<br>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-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 |
| Fx + Me EW 69 | NA | NA | NA | NA | NA | Formulation | NA |
| Glutaraldehyde | Glutaral | 111-30-8 | 100.12 | NA | Liquid | Aldehydes |  |

| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| 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/<br><br>H-1,2,4-Triazole-1-ethanol, alpha-(4-chlorophenyl)- | 124495-18-7<br>113096-99-4 | 308.14<br>291.78 | 5.69<br>3.25 | Liquid | Heterocyclic Compounds |  |

| Substance Name | Synonyms | CASRN | Molecular Weight (g/mol) | Log Kow[1] | Physical Form | Chemical Class[2] | Structure[3] |
|---|---|---|---|---|---|---|---|
| | alpha-(1-cyclopropylethyl)- | | | | | |  |
| Saturated diglycerin | NA | NA | NA | NA | NA | NA | NA |
| 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 | 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, based on CASRN, were obtained from ChemID available at: http://chem.sis.nlm.gov/chemidplus/chemidheavy.jsp.

# Appendix D2

## Substances in Aqueous Solutions Tested in the LLNA - Comparative Data (Sorted Alphabetically)

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**Substances in Aqueous Solutions Tested in the LLNA - Comparative Data (Sorted Alphabetically)**

| Substance Name | CASRN | Formulation Type | LLNA Conc. Tested (%) | LLNA Sis | LLNA $EC_3$ (%) | 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 | Submitted by: E. Debruyne, Bayer Crop Science | 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 | Submitted by: E. Debruyne, Bayer Crop Science | NA | NT |
| 2-Aminoethyl-methylsulfone | 49773-20-8 | | 10, 25, 50 | 0.4, 0.3, 0.3 | NC | 0.5% Tween 80/$H_2O$ | | - | 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 | 0.3 | 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 |
| 1-Butanol | 71-36-3 | | 5, 10, 20 | 1.6, 1.2, 1.4 | NC | $H_2O$ | | - | 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 | 1.3 | ACE/saline (1:1) | | + | | | | | | | |
| 2.4-Dinitrobenzene sulfonic acid | 89-02-1 | | 1, 10, 20 | 1.7, 1.5, 4.4 | 15.2 | $H_2O$ | | + | Ryan et al. (2002) | + | NA | NA | NT | NA | NT |
| | | | 1, 10, 20 | 0.9, 4.4, 11.6 | 6.4 | 1% Pluronic L92/$H_2O$ | | + | | | | | | | |
| 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 | + | | | | | | | |
| | | | 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/CaOlaHsd | + | | | | | | | |
| 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, 5.3 | 64.9 | 1% L92 | CBA/J | + | Bayer Crop Science, submitted by: E. Debruyne, | + | - | BT | Bayer Crop Science, submitted by: E. Debruyne, | NA | NT |
| F & Fo WG 50 + 25 | | WG | 2.5, 5.0, 10.0, 25.0 | 11.7, 12.6, 14.4, 15.2 | 0.0 | 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 | Formulation Type | LLNA Conc. Tested (%) | LLNA Sis | LLNA $EC_3$ (%) | 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 | $H_2O$ | | + | ECPA LLNA Project Report; Ryan et al. (2002) | + | NA | NA | ECPA LLNA Project Report; Andersen et al. (1984); Wahlberg and Boman (1985) | NA | ECPA LLNA Project Report |
| | | | 1, 10, 20 | 2, 4.8, 8.8 | 4.2 | 1% Pluronic L92/$H_2O$ | | + | | | | | | | |
| | | | 1, 5, 20 | 1.1, 3.8, 10.6 | 3.8 | 1% Pluronic L92/$H_2O$ | | + | | | | | | | |
| | | | 1, 5, 20 | 1, 2.2, 6.2 | 8 | 1% Pluronic L92/$H_2O$ | | + | | | | | | | |
| | | | 1, 5, 20 | 1.6, 2.6, 12 | 5.6 | 1% Pluronic L92/$H_2O$ | | + | | | | | | | |
| | | | 1, 5, 20 | 1.1, 2.5, 4.8 | 8.2 | 1% Pluronic L92/$H_2O$ | | + | | | | | | | |
| | | | 1, 5, 20 | 0.8, 1.3, 4.8 | 12.3 | 1% Pluronic L92/$H_2O$ | | + | | | | | | | |
| 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 | NA | NT |
| Formulation 10 | | EW | 2, 10, 50 | 1, 1, 5.2 | 29.0 | 1% L92 | BALB/c | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | 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 | Submitted by Dow AgroSciences | NA | NT |
| Formulation 12 | | EC | 0.2, 1, 5 | 1.2, 3, 11.6 | 1.00 | 1% L92 | BALB/c | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | 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 | Submitted by Dow AgroSciences | 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 | Submitted by Dow AgroSciences | 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 | Submitted by Dow AgroSciences | 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 | Submitted by Dow AgroSciences | 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 | Submitted by Dow AgroSciences | NA | NT |
| Formulation 19 | | EC | 1, 10, 25, 50 | 4.9,7.9,20,50.5 | 0.0 | 1% L92 | BALB/c | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | NA | NT |
| Formulation 2 | | SE | 5, 20, 80 | 2, 3.4, 15.8 | NC | 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 | 0.4 | 1% L92 | BALB/c | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | 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 | Submitted by Dow AgroSciences | NA | NT |
| Formulation 22 | | ME | 5, 25, 100 | 1.2, 1.4, 5.8 | 0.5 | 1% L92 | BALB/c | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | NA | NT |
| Formulation 23 | | SL | 5, 25, 100 | 0.8, 1, 1 | NC | 1% L92 | BALB/c | - | Submitted by Dow AgroSciences | - | NA | NA | Submitted by Dow AgroSciences | NA | NT |
| Formulation 24 | | OD | 2, 10, 50 | 1.4, 4.1, 11.7 | 0.1 | 1% L92 | BALB/c | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | NA | NT |
| Formulation 25 | | EC | 1, 5, 25 | 1.8, 2.6, 14.7 | 0.1 | 1% L92 | BALB/c | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | NA | NT |
| Formulation 26 | | EC | 1, 5, 25 | 1, 1, 4 | 0.2 | 1% L92 | BALB/c | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | NA | NT |
| Formulation 27 | | EC | 1, 5, 25 | 2.3, 2.5, 11.2 | 0.1 | 1% L92 | BALB/c | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | NA | NT |
| Formulation 28 | | SC | 5, 25, 100 | 1, 1, 1.1 | NC | 1% L92 | BALB/c | - | Submitted by Dow AgroSciences | - | NA | NA | Submitted by Dow AgroSciences | 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 | Submitted by Dow AgroSciences | 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 | 0.1 | 1% L92 | CBA/J | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | NA | NT |
| 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 | NA | NT |
| Formulation 32 | | EC | 5, 25, 100 | 6.5, 44.7, 69.3 | 0.0 | 1% L92 | CBA/J | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | 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 | Submitted by Dow AgroSciences | 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 | Submitted by Dow AgroSciences | 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 | Submitted by Dow AgroSciences | NA | NT |
| Formulation 37 | | EC | 1, 5, 15 | 1.4, 2.7, 7.5 | 0.1 | 1% L92 | CBA/J | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | NA | NT |
| Formulation 38 | | EC | 5, 25, 100 | 1.1, 4.6, 12.7 | 0.2 | 1% L92 | CBA/J | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | NA | NT |
| Formulation 39 | | OD | 1, 5, 25 | 1.7, 2.5, 3.3 | 0.2 | 1% L92 | CBA/J | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | NA | NT |
| Formulation 4 | | SL | 5, 25, 100 | 1.4, 1.1, 1.2 | NC | 1% L92 | BALB/c | - | Submitted by Dow AgroSciences | - | NA | NA | Submitted by Dow AgroSciences | NA | NT |
| Formulation 40 | | OD | 1, 5, 25 | 1.8, 2.8, 5.7 | 0.1 | 1% L92 | CBA/J | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | NA | NT |
| Formulation 41 | | SE | 5, 25, 100 | 1.9, 1.9, 4.7 | 0.5 | 1% L92 | CBA/J | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | NA | NT |
| Formulation 42 | | SL | 10, 50, 100 | NA | 1.0 | 1% L92 | CBA/J | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | NA | NT |

| Substance Name | CASRN | Formulation Type | LLNA Conc. Tested (%) | LLNA SIs | LLNA $EC_3$ (%) | 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 | Submitted by Dow AgroSciences | NA | NT |
| Formulation 44 | | SC | 5, 25, 100 | NA | NC | 1% L92 | CBA/J | - | Submitted by Dow AgroSciences | - | NA | NA | Submitted by Dow AgroSciences | NA | NT |
| Formulation 45 | | SC | 5, 25, 100 | NA | NC | 1% L92 | CBA/J | - | Submitted by Dow AgroSciences | - | NA | NA | Submitted by Dow AgroSciences | 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 | NA | 0.4 | 1% L92 | CBA/J | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | NA | NT |
| Formulation 49 | | AL | 5, 25, 100 | 0.7, 1.4, 4.7 | 0.6 | 1% L92 | CBA/J | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | NA | NT |
| Formulation 5 | | EC | 3, 10, 30 | 1.4, 4, 11.5 | 0.1 | 1% L92 | BALB/c | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | NA | NT |
| Formulation 50 | | SL | 5, 25, 100 | 1.2, 1.2, 14.7 | 0.4 | 1% L92 | CBA/J | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | NA | NT |
| Formulation 51 | | OD | 5, 25, 100 | 1.6, 4.5, 2.9 | 0.1 | 1% L92 | CBA/J | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | NA | NT |
| Formulation 53 | | EW | 2.5, 7.5, 15 | 1.5, 3.2, 6.7 | 0.1 | 1% L92 | CBA/J | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | 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 | Submitted by Dow AgroSciences | NA | NT |
| Formulation 55 | | EW | 5, 25, 100 | 1.5, 2.5, 3.7 | 0.6 | 1% L92 | CBA/J | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | NA | NT |
| Formulation 56 | | SL | 5, 25, 100 | 3.3, 6.1, 3.9 | 0.0 | 1% L92 | CBA/J | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | NA | NT |
| Formulation 6 | | EW | 5, 20, 80 | 1.3, 2.7, 11.6 | 0.2 | 1% L92 | BALB/c | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | NA | NT |
| Formulation 7 | | SC | 20, 80, 100 | 1, 1.9, 3.2 | 1.0 | 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 | 0.7 | 1% L92 | BALB/c | + | | | | | | NA | NT |
| Formulation 8 | | EC | 1, 5, 25 | 0.9, 1.1, 7.3 | 0.1 | 1% L92 | BALB/c | + | Submitted by Dow AgroSciences | + | NA | NA | Submitted by Dow AgroSciences | 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 | Submitted by Dow AgroSciences | 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/$H_2O$ (l/l) | | + | Gerberick et al. (1992) | + | NA | NA | NT | NA | NT |
| Hexyl cinnamic aldehyde | 101-86-0 | | 3, 10, 30 | 1.2, 4.6, 18 | 6.7 | 1% Pluronic L92/$H_2O$ | | + | ECPA LLNA Project Report | + | NA | NA | NT | NA | NT |
| | | | 3, 10, 30 | 1.9, 4.2, 9.2 | 7 | 1% Pluronic L92/$H_2O$ | | + | | | | | | | |
| | | | 3, 10, 30 | 1.9, 2.2, 10.3 | 12 | 1% Pluronic L92/$H_2O$ | | + | | | | | | | |
| | | | 3, 10, 30 | 1.1, 2.5, 15.6 | 10.8 | 1% Pluronic L92/$H_2O$ | | + | | | | | | | |
| | | | 3, 10, 30 | 1.3, 2.2, 4.3 | 17.6 | 1% Pluronic L92/$H_2O$ | | + | | | | | | | |
| 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 | +[8] | Ryan et al. (2000); Basketter et al. (2005) |
| | | | NA | NA | 2.5 | 80% ETOH | | + | | | | | | | |
| 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) | +[8,9] | 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 | 30.8 | 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 | $H_2O$ | | - | Ryan et al. (2002) | - | NA | NA | NT | NA | NT |
| Propylene glycol | 57-55-6 | | 50, 100 | 1.2, 1.6 | NC | $H_2O$ | | - | 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) | +[9] | 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 |

| Substance Name | CASRN | Formulation Type | LLNA Conc. Tested (%) | LLNA Sis | LLNA $EC_3$ (%) | 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 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 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: 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 | | | | | | |
| Saturated diglycerin | NA | | 25, 50, 100 | 1.4, 2.1, 1.9 | NC | ETOH/$H_2O$ | | - | TNO Report[5] | - | NA | NA | NT | NA | NT |
| Sodium lauryl sulfate | 151-21-3 | | 5, 10, 25 | 3.0, 4.8, 8.5 | 4.9 | 1% Pluronic L92/$H_2O$ | | + | BGIA Project FP251[6] | + | 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[7] | - | 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 | 7.0 | 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 Nod Assay; OD = Oil dispersion; ME = Micro-emulsion; NA = Not Available; NC = Not Calculated; NT = Not Tested; PG = Propylene glycol; SC = Suspension concentrate; SE = Suspo-emulsion; SI = Stimulation Index; SL = Soluble concentrate; TK = Technical concentrate

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

[2]BT or GPMT result.

[3]Data from GlaxoSmithKline (GSK) were submitted by M.J. Olson.

[4]The LLNA Project Report was submitted by the European Crop Protection Association (ECPA).

[5]Netherlands Organisation for Applied Scientific Research (TNO) Report submitted by the Comite Europeen des Agents de Surface et de Leurs Intermediaires Organiques (European Committee of Surfactants and Their Organic Intermediates) submitted by K. Skirda.

[6]Berufsgenossenschaftliches Institut fur Arbeitsschutz (BGIA - German Institute for Occupational Safety and Health) Report submitted by H.W.Vohr.

[7]National Toxicology Program (NTP) data submitted by D. Germolec.

[8]Data obtained from the Human Repeat Insult Patch Test.

[9]Data obtained from the Human Maximization Test

# Appendix D3

## Medical Device Eluates Tested in Aqueous Solutions in the LLNA

## Comparative Data

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**Medical Device Eluates Tested in Aqueous Solutions in the LLNA - Comparative Data**

| 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[4] (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 | + |
| 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 = disinterations 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 L 92
[3]Eluate spiked with 20% dinitrobenzenesulfonic acid (DNBS) (1:1)
[4] (+) = sensitizer; (-) + non-sensitizer
[5]Positive control is 20% DNBS

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# Appendix E

## Supplementary Analysis of Pesticide Formulations in the LLNA

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# 1.0 TESTING OF PESTICIDE FORMULATIONS

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

For the 22 formulations that had associated GP data for the formulation itself, 14% (3/22) were classified as sensitizers and 86% (19/22) as non-sensitizers according to the GP results (**Figure E-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 non-sensitizers (**Figure E-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 non-sensitizers in the GP test (**Table E-1**). There were no comparative human data with which to determine the actual human sensitization potential.

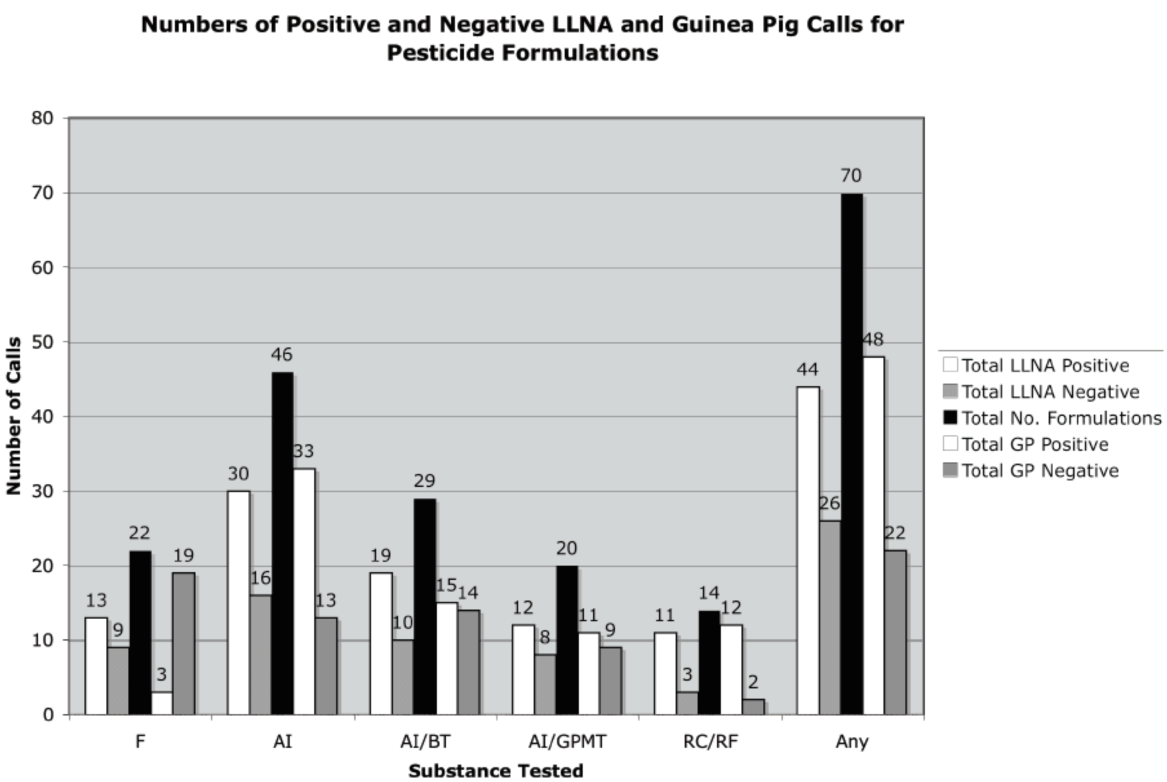
## 1.2 Testing of Pesticide Formulations: LLNA vs. GP with Any Available Reference Data for Relevant Substances

Of the 70 formulations, 69% (48/70) were classified as sensitizers and 31% (22/70) as non-sensitizers on the basis of various types of GP data (**Figure E-1**). To assign these classifications, a most conservative approach was used; i.e., 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 non-sensitizers (**Figure E-1**). Sixty-five percent (31/48) of the pesticide formulations classified as sensitizers by a GP test, based on the

[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%).

criteria given above would also have been classified as sensitizers in the LLNA (**Table E-1**). The LLNA also identified an additional 14 formulations as sensitizers that would have been classified as non-sensitizers by a GP test based on these criteria. However, the LLNA failed to identify an additional 36% (17/48) formulations as sensitizers, which would have been classified as such by a GP test, based on the criteria given above.

**Figure E-1 Numbers 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 E-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]) | 22 | 54 | 12/22 | 100 | 3/3 | 53 | 10/19 | 47 | 9/19 | 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 versus 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.

### 1.3 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 non-sensitizers 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 non-sensitizers (**Figure E-1**). Seventy-six percent (25/33) of the pesticide formulations identified as sensitizers based on a GP test of an active ingredient were identified as sensitizers in the LLNA (**Table E-1**). The LLNA also identified an additional five substances as sensitizers that were classified as non-sensitizers 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 E-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 E-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 non-sensitizers. By comparison, LLNA results with the complete formulation for each of these products identified 66% (19/29) as sensitizers and 34% (10/29) as non-sensitizers (**Figure E-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 E-1**). The LLNA also identified an additional eight substances as sensitizers that would have been classified as non-sensitizers 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 non-sensitizers. The proportion of formulations classified as sensitizers was similar that classified as sensitizers by the BT done 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 non-sensitizers. 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 E-1**). The LLNA also identified an additional five formulations as sensitizers that would have been classified as non-sensitizers by GPMT on an active ingredient. However, the LLNA failed to identify 36% (4/11) formulations as sensitizers that would have been classified as such by a GPMT based on an active ingredient (**Table E-1**).

### 1.4 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 non-sensitizers 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 non-sensitizers (**Figure E-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 E-1**). The LLNA also identified an additional two formulations as sensitizers that would have been classified as non-sensitizers by a GP test on a substance related to an active ingredient, or a related formulation.. However, the LLNA failed to identify an additional three formulations as sensitizers that would have been classified as by a GP test on a substance related to an active ingredient, or a related formulation (**Table E-1**).

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