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**Memorandum**

Date: March 2, 2010

TO : The Commission  
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SUBJECT : Staff Response to the ICCVAM Recommendations on Revisions to the Murine  
Local Lymph Node Assay, a Method for Determining Sensitizing Potential

This memorandum discusses the recommendations of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) regarding the murine Local Lymph Node Assay (LLNA) including: (1) updates to the test method protocol; (2) establishment of performance standards; and (3) a modified form of the assay, the reduced Local Lymph Node Assay (rLLNA). In addition, information is provided on whether these revisions are acceptable in the regulatory context for the purpose of classification for labeling under the Federal Hazardous Substances Act (FHSA) (15 U.S.C. 1261-1278).

I. Introduction

A. Background

The National Institutes of Health Revitalization Act of 1993 directed the National Institute of Environmental Health Science (NIEHS) to establish a method and criteria for the validation and regulatory acceptance of alternative testing methods (Public Law No. 103-43, Section 1301). To accomplish these goals, NIEHS created an ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) which was made permanent by the ICCVAM Authorization Act of 2000 (Public Law 106-545). The Committee is composed of representatives from 15 Federal regulatory and research agencies; these agencies generate, use, or provide information from toxicity test methods for risk assessment purposes. The duties of ICCVAM are to review, optimize, and validate new, revised, or alternative test methods that encourage the reduction, refinement, or replacement of the use of animals in testing. In addition, ICCVAM is to provide test recommendations to Federal agencies and other stakeholders to facilitate appropriate interagency and international harmonization of toxicological test protocols.

In 1998, the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) was established to assist ICCVAM in performing the activities necessary for the validation and regulatory acceptance of alternative test methods. ICCVAM submits test recommendations for a test method to Federal agencies that require or recommend acute or chronic toxicological testing. According to Public Law 106-545, these agencies should promote and encourage the development and use of alternatives to animal test methods for regulatory purposes, and ensure that any new or revised acute or chronic toxicity test method is valid for its proposed use under the mandate of the ICCVAM Authorization Act of 2000. Federal agencies have 180 days to identify any relevant test methods for which the ICCVAM test recommendations may be added or substituted, review such test recommendations, and notify ICCVAM if they will adopt the ICCVAM test recommendations.

ICCVAM forwarded three recommendations to the Commission for action: (1) an updated LLNA test method protocol, (2) LLNA test method performance standards, and (3) a modified version of the LLNA, the rLLNA. CPSC needs to determine if the proposed revisions to the LLNA test method, the inclusion of performance standards and the modified LLNA, the rLLNA, would be acceptable under the Federal Hazardous Substances Act (FHSA). The Commission needs to respond back to ICCVAM by March 22, 2010.

#### B. Validation of Alternative Methods

Validation of alternative methods is required before regulatory acceptance and utilization by Federal agencies. In general, for an alternative method to be considered valid it must be reliable (i.e., the toxicity predictions of test substances are repeatable within the same laboratory and reproducible across/among different laboratories) and relevant (i.e., the alternative test method is useful for measuring the biological effect of interest such as sensitization).

The reliability and relevancy of an alternative test method can be assessed from the statistical analysis of data. The relevance of an alternative test method can be determined by comparing the performance of the alternative test to the test that it is designed to replace. Performance is typically evaluated by calculating the accuracy<sup>1</sup>, false positive rate<sup>2</sup>, false negative rate<sup>3</sup>, sensitivity<sup>4</sup>, or specificity<sup>5</sup> of the alternative test method. The reliability of the alternative test method can be determined from the reproducibility of test method results within and among laboratories.

#### C. Federal Hazardous Substances Act Requirements

Precautionary labeling of hazardous household substances is mandated by the FHSA (the Act), 15 U.S.C. § 1261-1275. Under the FHSA, to be a hazardous substance, a product must present one or more of the hazards enumerated in the statute and it must have the potential to cause substantial personal injury or substantial illness during or as a result of any customary or reasonably foreseeable handling or use.

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<sup>1</sup> Accuracy - proportion of correct outcomes

<sup>2</sup> False positive rate - proportion of all negative substances that are falsely identified as positive

<sup>3</sup> False negative rate - proportion of all positive substances that are falsely identified as negative

<sup>4</sup> Sensitivity – the proportion of all positive substances that are classified as positive

<sup>5</sup> Specificity – the proportion of all negative substances that are classified as negative

FHSA “Strong Sensitizer”: “Strong sensitizers” are one of the seven hazards defined under the FHSA. The definition of “*strong sensitizer*” which appears in section 2(k) of the FHSA (15 U.S.C. §1262(k); restated in 16 CFR 1500.3(b)(9)) is:

*Strong sensitizer means a substance which will cause on normal living tissue through an allergic or photodynamic process a hypersensitivity which becomes evident on reapplication of the same substance and which is designated as such by the Commission. Before designating any substance as a strong sensitizer, the Commission, upon consideration of the frequency of occurrence and severity of the reaction, shall find that the substance has significant potential for causing hypersensitivity.*

Five substances have been identified in the FHSA as strong sensitizers<sup>6</sup>: (1) paraphenylenediamine and products containing it; (2) powdered orris root and products containing it; (3) epoxy resin systems containing in any concentration ethylenediamine, diethylenetriamine, and diglycidyl ethers of molecular weight less than 200; (4) formaldehyde and products containing 1 percent or more of formaldehyde; and (5) oil of bergamot and products containing 2 percent or more of oil of bergamot.

Since its inception in 1972, CPSC has not designated any substances to be strong sensitizers. However, in 1986 the Commission issued a rule clarifying the FHSA’s “strong sensitizer” definition with supplemental definitions as recommended by a Technical Advisory Panel on Allergic Sensitization (TAPAS)<sup>7</sup>. The following supplemental definitions were intended to clarify the interpretation of the statutory definition for a “strong sensitizer”:

- *Sensitizer: A sensitizer is a substance that will induce an immunologically-mediated (allergic) response, including allergic photosensitivity. This allergic reaction will become evident upon re-exposure to the same substance. Occasionally, a sensitizer will induce and elicit an allergic response on first exposure by virtue of active sensitization.*
  
- *Strong: In determining that a substance is a “strong” sensitizer, the Commission shall consider the available data for a number of factors. These factors should include any or all of the following (if available):*
  - *Quantitative or qualitative risk assessment*
  - *Frequency of occurrence and range of severity of reactions in healthy or susceptible populations*
  - *The result of experimental assays in animals or humans (considering dose-response factors), with human data taking precedence over animal data*
  - *Other data on potency or bioavailability of sensitizers*
  - *Data on reactions to a cross-reacting substance or to a chemical that metabolizes or degrades to form the same or a cross-reacting substance*
  - *The threshold of human sensitivity*
  - *Epidemiological studies*
  - *Case histories*

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<sup>6</sup>16 CFR §1500.13

<sup>7</sup>16 CFR §1500.3(c)(5)

- Occupational studies
- Other appropriate in vivo and in vitro test studies

- *Severity of Reaction: The minimal severity of a reaction for the purpose of designating a material as a “strong sensitizer” is a clinically important reaction. For example, strong sensitizers may produce substantial illness, including any or all of the following:*

- physical discomfort
- distress
- hardship
- functional or structural impairment

*These may, but not necessarily, require medical treatment or produce loss of functional activities.*

- *Significant potential for causing hypersensitivity: “Significant potential for causing hypersensitivity” is a relative determination that must be made separately for each substance. It may be based on chemical or functional properties of the substance, documented medical evidence of allergic reactions obtained from epidemiological surveys or individual case reports, controlled in vitro or in vivo experimental assays, or susceptibility profiles in normal or allergic subjects.*

- *Normal living tissue: The allergic hypersensitivity reaction occurs in normal living tissues, including the skin and other organ systems, such as the respiratory or gastrointestinal tract, either singularly or in combination, following sensitization by contact, ingestion or inhalation.*

While the FHSA does not require manufacturers to perform any specific battery of toxicological tests to assess the potential risk of chronic hazards, the manufacturer is required to label a product appropriately according to the FHSA requirements; with the exception if the product is a toy or other article intended for use by children and is a hazardous substance, then the product is by definition a banned hazardous substance unless specifically exempted<sup>8</sup>. When determining if a consumer product, which is composed of a mixture of substances, is a hazardous substance, the mixture should be tested and not the individual components of the mixture because synergistic or antagonistic reactions may lead to erroneous determinations concerning the toxic, irritant, corrosive, etc. properties of the substance (16 CFR § 1500.5).

Sensitizers in Art Materials: Congress amended the FHSA in 1988 to include the Labeling of Hazardous Art Materials Act (LHAMA) requirements. The LHAMA requires a reviewing procedure for developing precautionary labels for all art materials. This amendment to the FHSA concerns chronic health hazards known to be associated with a product or product component when present in a physical form, volume or concentration that presents the potential to produce a chronic health hazard as determined by a toxicologist. Within the regulation under the Act, a “sensitizer” is defined as *a substance known to cause, through an allergic process, a chronic adverse health effect which becomes evident in a significant number of people on re-exposure to the same substance*<sup>9</sup>. To protect users from known sensitizers found within art

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<sup>8</sup>16 C.F.R. §1500.3(b)(15)(i)

<sup>9</sup>16 C.F.R. §1500.14(b)(8)(i)(B)(9)

materials, each label shall contain a list of those sensitizers present in sufficient amounts to contribute significantly to a known skin or respiratory sensitization.<sup>10</sup>

#### D. Past and Current Sensitization Testing

Data on the sensitization potential of some chemicals comes from studies using human volunteers, and the development of animal sensitization tests has been based on a comparison to the human tests performed with the same chemicals. Two approaches for predictive sensitization testing in humans that have been in use are the Human Maximization Test (HMT) and the Human Repeated Insult Patch Tests (HRIPT). These tests vary with regard to the number of induction patch tests, the placing of the patches and the use of a maximization step. The HMT is no longer in use due to ethical concerns about its potential health consequences. Contract laboratories have performed the vast majority of human sensitization tests and the scientific literature contains a limited number of publications giving results from tests with cosmetic ingredients as preservatives and fragrance chemicals.

Historically, the Guinea Pig Maximization Test (GPMT) and the Buehler Assay (BA) have been the primary animal assays used to determine the sensitizing ability of a chemical. The GPMT is a highly sensitive method using Freund's complete adjuvant as an immune enhancer. It includes both intra-dermal and topical induction treatments. The BA uses repeat closed topical applications. The GPMT is regarded as a more sensitive assay that may also, for certain substances, overestimate the sensitization hazard for the compound tested. The Buehler test is less sensitive and may underestimate the sensitization potential of a compound.

In 1997, the LLNA was proposed to ICCVAM as a stand-alone alternative method to the GPMT and the BA for hazard identification. In 1999, based on the validation database and performance, ICCVAM recommended the LLNA as an alternative test method for assessing the skin sensitization potential of most types of substances. The consensus of the peer review panel was that the LLNA performed as well as the GPMT and BA for hazard identification of strong to moderate chemical sensitizing [dermal] agents but lacked strength in accurately predicting some weak sensitizers and some strong irritants. The LLNA provides several advantages compared to the guinea pig assays, including elimination of potential pain and distress, use of fewer animals, shorter test duration, a more objective end point, less test substance required, and the availability of dose-response information. United States regulatory agencies accepted the LLNA as a valid alternative test method for allergic contact dermatitis testing. The LLNA was adopted as a test guideline (test guideline [TG] 429) in 2002 by the Organization for Economic and Cooperative Development (OECD) after the ICCVAM validation of the assay.

In the intervening years, the National Toxicology Program (NTP) has extensively used the assay to study chemical hypersensitivity based upon its acceptance as a stand-alone alternative. The Environmental Protection Agency (EPA) indicates that the LLNA along with the GPMT and BA are acceptable test methods, with the LLNA as a preferred alternative method, where applicable, to the guinea pig tests. The Food and Drug Administration (FDA) in its Guidance for Industry indicates that the sensitizing potential of a drug should be screened using an appropriate test such

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<sup>10</sup>16 C.F.R. §1500.14(b)(8)(i)(E)(5)

as the GPMT, BA, LLNA, the guinea pig inhalation induction and challenge assay, or other appropriate alternative assays<sup>11</sup>.

## II. Alternative Tests for Sensitization, ICCVAM Recommendations

Currently, no *in vitro* or *in silico*<sup>12</sup> systems have undergone validation for determining sensitizing potential. Both approaches are evolving methodologies and are being pursued to reduce the numbers of expensive laboratory and animal experiments performed.

The remainder of Section II of this memo will describe each of the submitted ICCVAM recommendations, relevant validation and performance data, and ICCVAM conclusions.

### A. LLNA Test Method Protocol

#### 1. Background

The LLNA is a test method developed to assess the potential of a test substance to induce allergic contact dermatitis in humans. The basic principle underlying the LLNA is that sensitizers induce proliferation of lymphocytes in the lymph node draining the site of substance application. Under appropriate test conditions, this proliferation is proportional to the dose applied, and provides a means of obtaining an objective measurement of sensitization. The LLNA was the first test method evaluated and recommended by ICCVAM. As stated earlier, the advantages of this test method include that it uses fewer animals, provides dose-response information and eliminates pain and distress compared to the guinea pig assays. In 2001, following a comprehensive independent peer review of the LLNA, ICCVAM developed recommendations applicable to the regulatory use of the LLNA and prepared a recommended protocol. In March 2008, ICCVAM and NICEATM convened another independent scientific peer review panel (Panel) to evaluate new versions and applications of the LLNA. The Panel provided conclusions and recommendations in its report, many of which were applicable to the LLNA test method protocol. ICCVAM subsequently considered the Panel's conclusions and recommendations, as well as comments from the SACATM and public, and updated the 2001 ICCVAM recommended LLNA protocol.

#### 2. Validation and Performance

The four main areas of revision from the 2001 protocol are: (1) guidance within the test protocol for reductions in the number of positive control animals, including statistical analysis for reduction of animals (the newly added Annex II); (2) an extensive discussion concerning collection of individual animal data; (3) a detailed discussion regarding the recommended numbers of animals per dose group; and (4) detailed guidance on the evaluation of local irritation and systemic toxicity to ensure that the appropriate highest dose is tested (the newly added Annex III).

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<sup>11</sup> Guidance for Industry. Immunotoxicology Evaluation of Investigational New Drugs, October 2002, Center for Drug Evaluation and Research.

<sup>12</sup>*In silico* data is a computational approach using sophisticated computer models for the determination of a sensitizing potential.

### 3. Recommendations for the Updated Test Method Protocol

On March 3 through March 8, 2008, an international peer review panel composed of expert scientists from industry, academia and other scientific professionals organized by ICCVAM, in collaboration with NICEATM, convened to review and evaluate the validation status, make recommendations for revisions, and provide final comments on the usefulness and limitations of proposed modifications to the LLNA. The Panel stated that an updated LLNA test method should consider that:

- *“No skin reaction should be present, since presence of a skin reaction might indicate the onset of the elicitation phase of skin sensitization”.*
- *“Data should be collected at the level of the individual animal to allow for an estimate of the variance within control and treatment groups. Using this variance, a power analysis needs to be conducted to demonstrate that the modified method is utilizing a sufficient number of animals per treatment group to permit hazard identification with at least 95% power”.*
- *“Until sufficient data were collected to enable a reliable power calculation to be conducted to determine the optimal number of animals per dose group, at least five animals per dose group should be used. A minority opinion stated that if laboratories were operating under OECD guidance (OECD 2002) and a reliable validation dataset had been generated, then pooled data from at least four animals could be considered acceptable”.*
- *“A concurrent positive control should be included in each validation study to ensure that the test system was operating as expected and technical errors were not occurring. However, if a known sensitizer was being tested, a concurrent positive control might not be needed, thus reducing animal use”.*

Subsequent to the Panel meeting, the ICCVAM Immunotoxicity Working Group (IWG) along with European Centre for the Validation of Alternative Methods (ECVAM) and Japanese Center for the Validation of Alternative Methods (JaCVAM) liaisons, considered the Panel’s conclusions and recommendations as well as those from the public and the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM). A series of meetings were convened throughout the summer concluding in an international harmonization meeting on September 23 through September 24, 2008, with the goal to harmonize the test method and performance standards. During these discussions the following was recommended for the test method protocol:

- The requirement for the number of animals per group was reduced to a minimum of four (from five). Statistical analysis of data from 83 LLNA studies (275 dose groups) demonstrated no diminished accuracy in the test method when the number of animals per dose group were reduced from five to four. Furthermore, the test method protocol in the performance standards was modified to allow for collection of data from individual animals or pooled cells.
- Considerations were provided for reducing the number of animals used in the concurrent positive control group. Inclusion of a positive control with each test is recommended, however periodic testing (i.e., at intervals  $\leq 6$  months) of the positive control substance may be considered in laboratories that conduct the LLNA regularly

(i.e., at a frequency of no less than once per month) and that have a history and a documented proficiency for obtaining consistent results with positive controls.

#### 4. ICCVAM Conclusion

In October 2008, ICCVAM finalized its conclusions and recommendations for updating the LLNA test protocol and endorsed the revisions and inclusions into the LLNA performance standards. Based upon these activities, a draft proposal was submitted to the OECD in 2009 for an update to its test guideline 429.

### B. LLNA Performance Standards

#### 1. Background

The purpose of performance standards is to provide the scientific basis for showing which new test methods have sufficient accuracy and reliability for a specific testing purpose. When ICCVAM evaluated the LLNA in 1999, the concept of performance standards had not yet been developed. In 2003 ICCVAM defined and described a process for the development of performance standards. In 2007 when CPSC nominated several modified versions of the LLNA for evaluation by ICCVAM, this necessitated the development of performance standards allowing for comparison of performance of the modified versions to that of the traditional LLNA. Therefore, ICCVAM is now providing performance standards for the LLNA so that modified versions that are mechanistically and functionally similar can be effectively and efficiently evaluated for their validity. The updated ICCVAM-recommended test method protocol addressed previously is the key reference used for establishing these performance standards and is found in Appendix A of the performance standard.

#### 2. Validation and Performance

Modified method protocols are expected to achieve a level of performance that is equivalent to or exceeds the accuracy and reliability of the traditional LLNA for identifying sensitizers. These performance standards are not proposed for evaluating other alternative test methods for measuring skin sensitization (e.g., *in vitro* methods) nor for any other *in vivo* test method.

The three elements of performance standards are: (1) essential test method components; (2) a minimum list of reference substances; and (3) accuracy and reliability values.

Essential test method components consist of essential structural, functional, and procedural elements of the validated test method that should be included in the protocol of a proposed modified method. Essential test method components include unique characteristics of the test method (i.e., application topically to both ears, collection of lymphocytes from the draining lymph nodes), critical procedural details (collection of lymphocytes must be during the induction phase, highest non-irritating dose should be selected, a minimum of 4 animals per dose group) and quality control measures (i.e., inclusion of positive and vehicle controls).

A minimum list of reference substances are used to assess the reliability and accuracy of a proposed similar test method. For the LLNA, 18 substances were selected along with 4 optional reference substances to represent a subset of those used to demonstrate the reliability and accuracy of the validated test method and are the minimum number that should be used to evaluate the proposed modified method. This list of reference substances was chosen from a database of 211 substances to represent the range of responses that the validated test method is capable of measuring (non-sensitizers to extreme sensitizers). This reference list substances that had well-defined chemical structures and forms, represented a range of chemical classes, were readily available from commercial sources, and that had consistent and high quality data from guinea pig tests and human studies (when possible).

Accuracy and reliability values are the standards that the proposed test method should meet or exceed when evaluated using the minimum list of reference substances. For these performance standards, a proposed modified method should have accuracy characteristics that meet or exceed that of the traditional LLNA. Therefore, with the 18 reference substances the proposed method should result in the correct classification based on a “yes/no” decision. Test method reliability is the degree to which a test method can be performed reproducibly within and among laboratories over time (intra-laboratory repeatability, intra-laboratory and inter-laboratory reproducibility). Internationally harmonized reproducibility standards were recommended.

### 3. Recommendations for using the LLNA Performance Standards

Draft performance standards were made public on September 2007. In March 2008, the international peer review panel convened to review, evaluate and comment on the usefulness and limitations of the performance standards. They concluded the following with regard to the performance standards:

- *“The Panel was asked what criteria should be used to evaluate the equivalence of a radioactive or non-radioactive LLNA method to the traditional LLNA, if one were proposed with a “major” change... (e.g., different mouse strain or use of male mice, change in the schedule for test article administration, change in schedule for lymph node excision, etc.). The Panel commented that the idea of what is a ‘major’ and a ‘minor’ change should be re-considered. The final version of the performance standards should be adequate to evaluate any protocol modifications”.*
- *“The Panel was asked if a new set of performance standards would be required for a modified version of the LLNA that incorporated one or more ‘major’ protocol changes. Based on the above response, the Panel concluded that a new set would not be required”.*
- *“The Panel was asked to comment on how many reference substances might be considered adequate for evaluating the validity of a modified version of the LLNA with a ‘major’ protocol change; specifically, if the 18 minimum reference substances ... would be sufficient.... The Panel concluded that a proposed modified LLNA should be evaluated with all 22 substances (including false negatives and false positives) and accuracy statistics calculated so that accuracies can be compared between the modified test method and the traditional LLNA. To the extent possible, rationale for any discordant results should be provided.... If the goal is to evaluate a specific applicability domain,*

*additional test substances might be needed.... However, the most potent sensitizers (e.g., DNCB) should always be identifiable. Considerable weight should be given to the balance between animal welfare and human safety when considering the adequacy of test method accuracy”.*

- *“The Panel was asked to comment, regardless of the number of reference substances, whether the alternative LLNA with a ‘major’ change should be required to obtain the same ‘call’ (and potency for sensitizers) as the traditional LLNA for the 18 minimum reference substances.... The Panel reiterated that an assay that is equivalent to the traditional LLNA is desired, but with the small number of reference substances available, clearly establishing equivalence will be extremely difficult”.*

Subsequent to the Panel meeting, the ICCVAM’s IWG along with ECVAM and JaCVAM liaisons, considered the Panel’s conclusions and recommendations as well as those from the public and SACATM. A series of meetings were convened throughout the summer concluding in an international harmonization meeting on September 23<sup>rd</sup>-24<sup>th</sup>, 2008, with the goal to harmonize the performance standards into one international standard. During these discussions the following was recommended for incorporation into the LLNA performance standards:

- The performance standards were applicable only to proposed methods with “minor” modifications that vary only by using non-radioactive methods for assessing lymphocyte proliferation. All other protocol modifications (e.g., mouse strain, timing of exposures, site of exposures) were considered “major” modifications.
- A harmonized list of 10 essential test components.
- An internationally harmonized list of reference chemicals (six substances were changed from the original ICCVAM list; rationale for their exclusion and replacement is provided in Appendix C2 of the performance standards).
- For test method accuracy, the proposed modified method should result in the correct sensitizer/non-sensitizer classification for each of the 18 required reference substances, but a misclassification of one weak sensitizer could be allowed.
- Intra-laboratory and inter-laboratory reliability measures of reproducibility were harmonized. Inter-laboratory reproducibility would be indicated by each of at least 3 laboratories obtaining ECt<sup>13</sup> values for the two designated substances within 0.5x to 2.0x of the historical provided EC3 concentration.

#### 4. ICCVAM Conclusion

In October 2008, ICCVAM finalized its conclusions and recommendations and endorsed the LLNA Performance standards.

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<sup>13</sup> An EC3 value is an estimated concentration of chemical necessary to elicit a 3-fold increase in lymph node cell proliferative activity. This 3-fold increase is used to discriminate between sensitizers and non-sensitizers in the traditional LLNA assay. Some proposed modified methods have decision criteria different from an EC3 (e.g., EC2), thus the performance standards have the designation “ECt” for those criteria which may not be EC3 and would be method specific.

## C. Reduced LLNA test method

### 1. Background

In 2007, CPSC requested that ICCVAM evaluate several modifications of the traditional LLNA, including the “reduced LLNA” (rLLNA), also referred to as the “cut-down” or “limit dose” LLNA. In the traditional LLNA, three dose levels of each test substance are evaluated. The rLLNA evaluates only the highest dose of the test substance along with concurrent vehicle- and positive-control groups. The highest concentration, as it is for the traditional LLNA, is defined as the maximum soluble concentration that does not induce excessive local irritation and/or overt systemic toxicity. Since the rLLNA differs from the traditional LLNA solely in the use of a single maximal dose instead of three doses, the test method protocol and performance standards for the traditional LLNA would remain unchanged (other than the single dose treatment) for the rLLNA. Furthermore, all of the testing limitations that apply to the traditional LLNA apply to the rLLNA as well. For example, the rLLNA may not be suitable for use with certain types of test substances, such as nickel salts, mixtures, high-molecular weight compounds that cannot penetrate the stratum corneum, strong dermal irritants, or chemicals whose pharmacodynamic activity is to release dermal cytokines that cause local lymph node proliferation.

### 2. Validation and Performance

Because the criteria for choosing the highest dose in the traditional LLNA and in the rLLNA are the same, the maximum dose level tested in the traditional LLNA and that tested in the rLLNA should be the same. Thus, the accuracy and reliability of the rLLNA test method should be similar for the same substances tested in the traditional LLNA.

Accuracy: for this performance analysis, the ability of the rLLNA to identify potential skin sensitizers was compared to that of the traditional LLNA by evaluating data from 471 traditional LLNA studies. In the 471 studies, 211 substances were from the 1998 ICCVAM validation of the traditional LLNA (ICCVAM 1999), and 246 were received from peer-reviewed literature and submissions to NICEATM in response to the May 17, 2007, Federal Register request for comments (72 FR 27815).

Of the 471 traditional LLNA studies, 318 results were positive for sensitizers and 153 were negative. Compared to the traditional LLNA, the rLLNA has an accuracy of 98.7% (465/471), a sensitivity of 98.1% (312/318), a specificity of 100% (153/153), a false positive rate of 0% (0/153), and a false negative rate of 1.9% (6/318).

Reliability: the extent of agreement among laboratories (inter-laboratory reproducibility) in assigning the same sensitization classification by the rLLNA was assessed with traditional LLNA data for five substances tested independently in the same vehicle at two or three laboratories. Those five substances were dinitrochlorobenzene (DNCB), hexyl cinnamic aldehyde (HCA), linalool alcohol, methyl salicylate, and potassium dichromate. There was 100% concordance among all studies for classifying DNCB, methyl salicylate,

and potassium dichromate as sensitizers or non-sensitizers. HCA and linalool alcohol, which were tested independently in two laboratories, were classified as sensitizers by one traditional LLNA study and as non-sensitizers by the other study. Review of these two studies indicates that the discordant results were due to differences in the highest dose levels tested. However, because the rLLNA and traditional LLNA use identical protocols and the data sets used to evaluate their accuracy are similar, the intra- and inter-laboratory reliability of the rLLNA was deemed to be similar by ICCVAM to that of the traditional LLNA.

### 3. Recommendations for Using the rLLNA

In March 2008, the international peer review panel convened to review, evaluate and comment on the usefulness and limitations of the proposed modified LLNA along with other modified LLNA methods. The peer review panel concluded the following with regard to the rLLNA test method:

- *“The rLLNA, which normally allows for testing at one dose level, should be routinely recommended for hazard identification, when used for testing purposes which do not require dose-response information, because it would offer time, cost, throughput and logistical benefits as well as using fewer animals. In instances when a necessity to measure relative skin sensitization potency for the purpose of risk assessment was present, then the traditional LLNA should be used in order to generate dose-response information. Still, the rLLNA should be used as the initial testing procedure to identify sensitizers and non-sensitizers before conducting the traditional LLNA even when dose-response information is required since if the test substance were negative in the rLLNA, it would not be necessary to conduct a multiple-dose traditional LLNA test. The benefits of screening out the negatives, which do not require dose-response information, are clear; however, the animal welfare gains will depend on the proportion of test substances in any class that turn out to be non-sensitizers. The possible consequences of delays from another round of testing of those materials identified as sensitizers should also be considered”.*
- *“The stimulation index (SI) based on the ratio of 3.0 as the cutoff value is indicative of a response that was sufficiently greater than the control and would be considered an immunologically relevant response, but the Panel recommended that statistical analyses be used to definitively establish that a response induced by a test substance is significantly different from the vehicle control. The Panel agreed that the LLNA protocol recommended by ICCVAM should be the standard protocol for all future rLLNA studies. Based on power calculations provided as supplemental information, the Panel agreed that five animals per dose group is an appropriate number to recommend for rLLNA studies following the traditional LLNA protocol”.*
- *“The Panel agreed that it was appropriate to assume that the intra-and inter-laboratory reproducibility of the rLLNA and the traditional LLNA would be similar, because reproducibility is more dependent on the method than on the number of dose groups. However, reducing the number of test substances dose groups from three to one might reduce the sensitivity of the assay. The traditional LLNA may have a greater chance of correctly identifying a sensitizer even in the presence of one or more technical errors since data from three dose groups are being considered and an  $SI \geq 3.0$  at any dose group would result in the substance being classified as a sensitizer. However, for the*

*purpose of adopting an assay that uses fewer animals and provides increased throughput for testing purposes, these hypothetical considerations are not a sufficient reason to argue against use of the rLLNA”.*

On the basis of Panel comments, ICCVAM updated the traditional LLNA test method protocol to provide guidance on identifying the appropriate maximum dose for testing. ICCVAM also recommended additional studies to further characterize and potentially improve the usefulness and applicability of the rLLNA for identifying potential skin sensitizers. These recommendations included that:

- Additional efforts should be made to understand the basis for abnormal dose responses for the six substances in this evaluation that would have resulted in false negative results using the rLLNA compared to the traditional LLNA. This information should help identify ways to improve the accuracy of the rLLNA compared to the traditional LLNA. Efforts should also be made to identify data from guinea pigs and humans for substances that exhibit abnormal dose responses in the traditional LLNA.
- All future traditional LLNA and rLLNA studies should collect individual animal data. This will allow detection of outliers and avoidance of false negative results that can occur from pooling data that include one or more abnormally low values. Existing LLNA studies using data pooled from all animals in a dose group, such as four of the six false negative rLLNA results in this evaluation, should be evaluated further with data obtained from individual animals within each dose group to determine if pooling of data may have led to false negative outcomes.

Subsequent to the Panel meeting, ICCVAM’s IWG along with ECVAM and OECD liaisons, considered the Panel’s conclusions and recommendations. A series of meetings were convened throughout the summer and winter with the goal to internationally harmonize the recommendations for use of the rLLNA. In order to reach consensus, it has been recommended that the rLLNA be considered an optional procedure that could provide an animal savings benefit.

#### 4. ICCVAM Conclusion

ICCVAM concluded that the scientific validity of the rLLNA has been adequately evaluated and that the performance of the rLLNA, when conducted in accordance with the updated ICCVAM-recommended LLNA protocol, is sufficient to distinguish between skin sensitizers and non-sensitizers in cases that do not require dose-response information. If dose-response information is required for a substance that, after consideration of all available information, is also suspected of having the potential to produce allergic contact dermatitis, it should be evaluated initially using the traditional LLNA.

There is a small possibility of a false negative result (1.9%) in the rLLNA compared to the traditional LLNA. This information should be considered when evaluating results from the rLLNA, and negative results should always prompt a weight-of-evidence evaluation of supplemental information (e.g., possibility of downturn in response at the

high dose, test results with similar substances, peptide-binding activity, molecular weight, other testing data). If false negative results are suggested, confirmatory testing in the traditional LLNA or another accepted skin sensitization test method should be considered.

ICCVAM also concluded that, compared to the traditional LLNA, the rLLNA will reduce animal use by 40% for each test.

On October 29, 2008, ICCVAM endorsed the Test Method Evaluation Report (TMER) for the rLLNA test method, which includes the rLLNA background review document, the updated LLNA test method protocol and the LLNA performance standards.

### III. Related Events Regarding Sensitizer Testing

The GHS (Globally Harmonized System) is an internationally-harmonized approach to classification and labeling for all chemicals, and mixtures of chemicals. CPSC is a member of the U.S. Federal interagency work group participating in the development and possible implementation of GHS. The issue of sensitizers is addressed by the GHS in chapter 3.4. Health Sciences (HS) staff are part of an OECD expert group which was formed to develop the revised GHS approach on these issues.

In March, 2008, the OECD sensitization expert group met at CPSC to continue work on the proposal for revising the GHS chapter for skin sensitizers with respect to strong versus weak sensitizers (GHS chapter 3.4 addresses both respiratory and skin sensitizers). At its April 2008 meeting, the OECD Task Force on Harmonisation of Classification and Labelling agreed to the proposed revisions. The revised sensitizer chapter was submitted to the UN Subcommittee of Experts on the GHS as a formal proposal and was accepted.

HS staff believes that the proposed GHS approach for classifying and labeling chemicals that are sensitizers will generally be compatible with the revisions to the FHSA "strong sensitizer" supplemental definition staff has proposed (see attached staff technical report).

One of the issues that arose from discussions with the OECD expert group was that of sensitizer potency and tests that can be used to determine potency of chemicals that might be sensitizers. European scientists favored the sole use of the LLNA for the determination of sensitizer potency. The criteria recently adopted by the GHS to distinguish strong sensitizers from other sensitizers, is based on human, guinea pig, and LLNA data. Substances with positive responses in the human maximization test (HMT) or human repeat insult patch test (HRIPT) at induction thresholds  $\leq 500 \mu\text{g}/\text{cm}^2$  are classified as strong sensitizers. Similarly, LLNA EC3 values  $\leq 2\%$  are proposed to categorize substances as strong sensitizers and LLNA EC3 values  $> 2\%$  to categorize substances as "other sensitizers". Because of concerns about the scientific validity of this approach, CPSC staff nominated the LLNA test method, for determination of sensitization potency, to ICCVAM for its review. ICCAM was requested, in particular, to review the validation status of the use of the LLNA as a stand-alone assay for the determination of potency. In order to evaluate the accuracy of the LLNA for identifying strong sensitizers as defined by human data, NICEATM and ICCVAM used a database of 112 substances with both LLNA and human data to calculate human potency classification categories (strong vs. other than strong).

The ICCVAM Peer Review Panel (3/3-3/7/08) recommended that the LLNA should be used as part of a weight-of-evidence approach for potency determinations, not as a stand-alone assay. As a result, CPSC staff was able to persuade their European counterparts on the OECD expert panel to agree that the revisions to the GHS sensitization chapter embrace the use of the LLNA as part of a weight-of-evidence approach, not as a stand-alone test.

#### IV. ICCVAM Recommendations

On October 28<sup>th</sup> 2008, ICCVAM finalized its conclusions and recommendations for updating the LLNA test method protocol. It endorsed the updates to the test protocol and its inclusion into the newly developed LLNA performance standards. Based upon these activities, a draft proposal was submitted in 2009 to the OECD for an update to test guideline 429 (TG429), the test guideline for the LLNA. ICCVAM recommends utilizing the rLLNA test method, a modified method of the traditional LLNA, for identifying substances as sensitizers in cases that do not require dose-response information. If dose-response information is required for a substance that, after consideration of all available information, is also suspected of having the potential to produce allergic contact dermatitis, it should be evaluated initially using the traditional LLNA. Due to the small possibility of a false negative result in the rLLNA, ICCVAM recommends that negative results should always prompt a weight-of-evidence evaluation of supplemental information. If false negative results are suggested, confirmatory testing in the traditional LLNA or another accepted skin sensitization test method should be considered.

#### V. Discussion by CPSC Staff

Staff agrees with the revisions in the updated LLNA test method protocol. These revisions address animal welfare considerations by providing clear guidance and statistical support for reducing the number of animals used per treatment and positive control groups. Furthermore, the guidance on selection of the maximal concentration dose level tested and determination of local irritation and systemic toxicity should further reduce potential pain and distress associated with the method.

Staff agrees with the establishment of performance standards for the LLNA. Performance standards should bring greater consistency in the utilization of the traditional LLNA and therefore, more uniformity and confidence in the data. This is important since alternative *in vitro*, *in silico* or *in vivo* methods for determining sensitization, which are in development, most likely will be compared and validated to the LLNA. These performance standards provide in a clear and succinct manner, the basis by which new test methods will be determined to have sufficient accuracy and reliability for classifying whether a substance is a sensitizer or not. Furthermore, these LLNA performance standards have been harmonized internationally into one performance standard with Europe and Japan.

In 1984, the Commission adopted a policy to reduce the number of animals tested and to minimize the pain and suffering associated with testing (49 FR 22522). In addition, the utilization of laboratory animals is recommended in a tiered and sequential approach to testing.

In a tiered-testing strategy, the test substance is tested *in vivo* if the appropriate hazard determination cannot be made from physicochemical characteristics, expert opinion, prior human experience or animal testing. Staff agrees that the rLLNA has an applicability that is very specific; the rLLNA provides the option of significant animal savings benefit where dose-response information is not needed, especially where substances are expected to produce negative results. Under the FHSA, the determination of whether a substance is a “strong sensitizer” or not is based upon a weight-of-evidence approach. In the staff proposed revisions to the FHSA “strong sensitizer” supplemental definition (Attached staff technical report), it is written:

“Before designating any substance as a “strong sensitizer”, the Commission shall find that the substance has significant potential for causing hypersensitivity. *Significant potential for causing hypersensitivity* is a relative determination that must be made separately for each substance. It may be based on chemical or functional properties of the substance; documented medical evidence of hypersensitivity reactions upon subsequent exposure to the same substance obtained from epidemiological surveys or individual case reports; controlled *in vitro* or *in vivo* experimental studies; and, susceptibility profiles (e.g., genetics, age, gender, atopic status) in non-sensitized or allergic subjects.

In determining whether a substance is a “strong” sensitizer, the Commission shall consider the available data for a number of factors, following a weight-of-evidence approach. The following factors (if available), ranked in descending order of importance, should be considered:

- Well-conducted clinical and diagnostic studies
- Epidemiological studies, with a preference for general population studies over occupational studies
- Well-conducted animal studies
- Well-conducted *in vitro* test studies
- Cross-reactivity data
- Case histories”

Therefore, the rLLNA would fit into a weight-of-evidence evaluation under the FHSA. Staff believes that the draft test method recommendations for the rLLNA adequately addressed the low false negative rate by giving cautionary and weight-of-evidence consideration to the negative substances (and any possible false positive results). In terms of continuing international harmonization of test methods, current REACH legislation identifies the rLLNA as a validated method in the available “toolbox” of methods for identifying potential skin sensitizers. Furthermore, the ECVAM Scientific Advisory Committee (ESAC) concluded in 2007 “that the peer reviewed and published information is of a quality and nature to support the use of the rLLNA within tiered-testing strategies to reliably distinguish between chemicals that are skin sensitizers and non-sensitizers...”

Staff agrees with ICCVAM that the updated LLNA test method protocol, the LLNA performance standards and the alternative test method, rLLNA, are based on sound science and are scientifically valid for their proposed uses.

## VI. Options

The Commission can vote to:

1. Accept the ICCVAM recommendations and instruct staff to draft a letter to ICCVAM indicating acceptance of its recommendations.
2. Reject the ICCVAM recommendations and instruct staff to draft a letter to ICCVAM indicating rejection of its recommendations.

## VII. Recommendations by CPSC Staff

Staff recommends accepting the ICCVAM recommendations. Thus, staff recommends utilizing the updated LLNA test method for hazard identification of substances that could be sensitizers. Staff also recommends that there is applicability for use of the rLLNA when dose-response information is not needed and when it is used in a weight-of-evidence approach. Staff also recommends acceptance of the internationally harmonized LLNA performance standards since these standards provide support for the development of improved versions of the method as well as provide consistency in utilization of the assay, an assay which will be used as the gold standard for validation of alternative *in vitro*, *in silico*, or *in vivo* methods for determining sensitization.

Labeling of a consumer product regarding the hazards associated with that product is required by the FHSA. In order to determine the appropriate cautionary labeling for “strong sensitizers”, animal testing may be necessary. However, the Commission supports minimizing the number of animals used and reducing the pain or suffering associated with animal testing and encourages the development and use of alternatives to animal test models. Thus the staff recommends that the Commission accept the ICCVAM recommendations because the revised LLNA test method protocol, the LLNA performance standards, and the alternative rLLNA test method encourage the reduction, refinement, or replacement of animals in testing and the data indicate that the methods are scientifically valid methods. Further, the FHSA requires a weight-of-evidence approach. In this context, the revised LLNA protocol, performance standards, and rLLNA test method would result in additional data that could be used to make a determination if a neat chemical or a mixture is a “strong sensitizer”.

Staff will draft a letter to ICCVAM indicating the Commission’s actions with regard to the ICCVAM recommendations. The ICCVAM website (<http://iccvam.niehs.nih.gov/home.htm>) will link to the Commission website where we will post our acceptance or non-acceptance of the three recommendations. In the section of the ICCVAM website, Pertinent Regulations, Guidelines and Laws (<http://iccvam.niehs.nih.gov/agencies/regs.htm>), there will be an announcement of the Commission’s action on the acceptance or non-acceptance of the three ICCVAM recommendations. Once ICCVAM receives responses from all the agencies, it will publish a Federal Register notice announcing all the agencies responses.

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