Appendix B

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

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Preface

The murine local lymph node assay (LLNA) is a test method developed to assess whether a chemical may induce allergic contact dermatitis (ACD) in humans. In 1998, the LLNA was submitted to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) for evaluation as a stand-alone alternative to the guinea pig sensitization tests accepted by U.S. regulatory agencies. In 1999, based on a comprehensive evaluation of the LLNA by an independent scientific peer review panel (Panel),[1] ICCVAM concluded that the LLNA is an acceptable alternative to the guinea pig test methods to assess the ACD hazard potential of most types of substances (Dean et al. 2001; ICCVAM 1999). The Panel also concluded that the LLNA offers animal welfare advantages compared to the traditional guinea pig test methods, in that it provides for animal use refinement (i.e., elimination of distress and pain) and reduces the total number of animals required.

An ICCVAM interagency Immunotoxicity Working Group (IWG) reviewed the 1999 Panel report (ICCVAM 1999) and developed recommendations applicable to the regulatory use of the LLNA. The interagency IWG then worked with the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) to produce a recommended test method protocol (ICCVAM 2001)[2] that would accurately reflect the ICCVAM and Panel recommendations. This current version of the ICCVAM-recommended test method protocol incorporates updates made in 2009, as described below, and an update made in 2010 to incorporate procedures for calculating the estimated concentration of a substance expected to produce a stimulation index of 3 (EC3), which is relevant to estimates of relative skin sensitization potency.

In March 2008 and April 2009, ICCVAM and NICEATM organized Panels to evaluate new versions and applications of the LLNA. The Panels provided conclusions and recommendations in their reports, many of which were applicable to the traditional LLNA test method protocol.[3] ICCVAM subsequently considered the Panels' conclusions and recommendations, as well as comments from the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) and the public, and updated the 2001 ICCVAM-recommended LLNA test method protocol (ICCVAM 2001). The updated LLNA test method protocol reduces animal numbers by 20% for each test by decreasing the minimum number of animals per dose group from five to four. A reduced LLNA procedure, which reduces animal numbers by 40% by using only one high-dose group for each test is also described. Further, improved guidance on dose selection and other procedures to improve assay accuracy and reproducibility are provided.

This updated ICCVAM-recommended LLNA test method protocol is based on evaluation of current experience and scientific data and is provided to Federal agencies for their consideration as a standardized test method protocol for generation of data for regulatory purposes. Prior to conducting an LLNA test to meet a regulatory requirement, the appropriate regulatory agency should be contacted for their current guidance on the conduct and interpretation of this assay. Users should be aware that the proposed test method protocol could be revised based on any additional optimization and/or validation studies that are conducted in the future. ICCVAM recommends that test method users consult the NICEATM-ICCVAM website (http://iccvam.niehs.nih.gov) to ensure use of the most current test method protocol. Additional information on the ICCVAM LLNA review process and deliberations of the Panel can also be found at the NICEATM-ICCVAM website (http://iccvam.niehs.nih.gov) or in the Panel reports (ICCVAM 2008, 2009b).

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[1] http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf
[2] http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/LLNAProt.pdf
[3] http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf
http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2009.pdf

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

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

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

2.0 Description of the Murine Local Lymph Node Assay

2.1 Sex and Strain of Animals

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

2.2 Preparation of Animals

The temperature of the experimental animal room should be 22°C (±3°C) and the relative humidity 30% to 70% (although the aim is for 50%-60%). Lighting should be artificial, the sequence 12 hours light, 12 hours dark. Mice should be provided with an unlimited supply of standard laboratory mouse diets and drinking water. The mice should be quarantined/acclimatized for at least five days before the test (ILAR 1996). Mice should be allocated to small groups by a stratified randomization or other appropriate method before the start of the study unless adequate scientific rationale for housing mice individually is provided (ILAR 1996). Four animals per cage is the recommended housing arrangement. The mice should be uniquely identified before being placed in the study. The mice should not be identified via the ear (e.g., marking, clipping, or punching). Colored marks on the tail or other appropriate methods should be used. All mice should be examined (e.g., clinical signs, body weights, observation of excrement) before the start of the test to ensure good health and the absence of skin lesions.

2.3 Preparation of Doses

Solid test substances should be dissolved or suspended in appropriate solvents/vehicles and diluted, if appropriate, before the mice are dosed. Liquid test substances may be dosed directly (i.e., applied neat) or diluted before dosing. Insoluble substances, such as those generally seen in medical devices, should be subjected to an exaggerated extraction in an appropriate solvent to reveal all extractable constituents for testing before dosing. Fresh preparations of the test substance should be prepared daily unless stability over the test period is demonstrated.

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

2.4 Test Conditions

2.4.1 Solvent/Vehicle

The selected solvent/vehicle should not interfere with or bias the test result and should be selected to maximize the test concentrations while producing a solution/suspension suitable for application of the test substance. Recommended solvents/vehicles are acetone: olive oil (4:1 by volume), *N*,*N*-dimethylformamide, methyl ethyl ketone, propylene glycol, and dimethyl sulfoxide. Others may be used (Kimber and Basketter 1992) if sufficient scientific rationale is provided. 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. Appropriate solubilizers (e.g., 1% Pluronic L92) should be incorporated, and wholly aqueous vehicles may need to be avoided. It may be necessary for regulatory purposes to test the substance in the clinically relevant solvent or product formulation.

2.4.2 Controls

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

Positive controls are used to demonstrate the appropriate performance of the assay by responding with adequate and reproducible sensitivity to a sensitizing substance for which the magnitude of the response is well characterized. Inclusion of a concurrent positive control is recommended because it demonstrates competency of the laboratory to successfully conduct each assay and allows for an assessment of intra- and interlaboratory reproducibility and comparability. The positive control should produce a positive LLNA response (i.e., a stimulation index [SI] $\geq$ 3). The positive control dose should be chosen such that the induction is reproducible but does not cause excessive skin irritation or systemic toxicity (i.e., SI > 20). Preferred positive control substances are 25% hexyl cinnamic aldehyde (HCA; Chemical Abstracts Service Registry Number [CASRN] 101-86-0) in acetone: olive oil (4:1 by volume) or 5% mercaptobenzothiazole (CASRN 149-30-4) in *N*,*N*-dimethylformamide. There may be circumstances where, given adequate justification, other positive control substances may be used.

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

While a concurrent positive control group is recommended, there may be situations in which periodic testing (i.e., at intervals $\leq$6 months) of the positive control substance may be adequate for laboratories that conduct the LLNA regularly (i.e., at least once per month) and that have an established history and a documented proficiency for obtaining reproducible and accurate results with positive controls. Adequate proficiency with the LLNA can be successfully demonstrated by generating consistent positive results with the positive control in at least 10 independent tests conducted in less than one year.

A concurrent positive control group should be included whenever there is a procedural change to the LLNA (i.e., change in trained personnel, change in test method materials and/or reagents, change in test method equipment, change in source of test animals, etc.). Such changes should be documented in laboratory reports. Consideration should be given to the impact of these changes on the adequacy of

the previously established historical database. It may be necessary to establish a new historical database to document consistency in the positive control results.

Investigators should be aware that the decision to include a positive control only periodically, instead of concurrently, will affect the adequacy and acceptability of negative study results generated without a concurrent positive control during the interval between each periodic positive control study. For example, if a false negative result is obtained in the periodic positive control study, all negative test substance results obtained in the interval between the last acceptable periodic positive control study and the unacceptable periodic positive control study may be questioned. Implications of these outcomes should be carefully considered when determining whether to include concurrent or periodic positive controls.

Consideration should also be given to using fewer animals in the concurrent positive control group when this is scientifically justified and if the laboratory demonstrates, based on laboratory-specific historical data,[5] that fewer mice can be used without substantially increasing the frequency with which studies will need to be repeated due to positive control failure. An example of how to reduce the number of mice in the concurrent positive control group is provided in **Annex II**.

Benchmark substances help demonstrate that the test method is functioning properly for detecting the skin sensitization potential of substances of a specific chemical class or a specific range of responses. Appropriate benchmark substances should have the following properties:

- Structural and functional similarity to the class of the substance being tested
- Known physicochemical characteristics
- Supporting data from the LLNA
- Supporting data on known effects in animal models and/or in humans

2.5 Methodology

A minimum of four animals per dose group is recommended, with at least three concentrations of the test substance, a concurrent negative control group treated only with the vehicle for the test substance, and a concurrent positive control. The processing of lymph nodes from individual mice allows for the identification of any individual animal responses that are outliers, in accordance with statistical tests such as Dixon's test (Dixon and Massey 1983). This will help to avoid false negative results for weaker sensitizers. That is, if an outlier is not identified and excluded, substances that normally would induce an SI just above 3 might be incorrectly classified as negative due to a low outlier value because the resulting mean SI may be less than 3. Individual animal measurements allow assessment of interanimal variability and a statistical comparison of the difference between test substance and vehicle control group measurements. Finally, reducing the number of mice in the positive control group is feasible only when individual animal data are collected.

Test substance treatment dose levels should be based on the recommendations given in Kimber and Basketter (1992) and in the ICCVAM Panel report (ICCVAM 1999). At least three consecutive doses are normally selected from an appropriate concentration series such as 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc. Adequate scientific rationale should accompany the selection of the concentration series used. The maximum concentration tested should be the highest achievable level that does not cause excessive local irritation and/or overt systemic toxicity (**Annex III**). Efforts should be made to identify existing toxicological information (e.g., acute toxicity and dermal irritation) and structural and physicochemical information on the test material of interest (and/or structurally related test materials) that may aid in selecting the appropriate maximum test substance dose level. In the absence of such information, an initial prescreen test may be necessary (**Annex III**).

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

The LLNA experimental procedure is performed as follows:

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

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

Days 4 and 5. No treatment.

Day 6. Record the weight of each mouse. Inject 250 μL of sterile phosphate-buffered saline (PBS) containing 20 μCi of tritiated (^{3}H)-methyl thymidine or 250 μL PBS containing 2 μCi of ^{125}I-iododeoxyuridine (^{125}IU) and 10^{-5} M fluorodeoxyuridine into each mouse via the tail vein (Kimber et al. 1995; Loveless et al. 1996). Five hours later, humanely kill each mouse and collect the draining (auricular) lymph nodes of both ears and place in PBS (one container per mouse). Both bilateral draining lymph nodes must be collected (see diagram and description of dissection in **Annex I**). To further monitor the local skin response, additional parameters such as scoring of ear erythema or ear thickness measurements (obtained either by using a thickness gauge, or ear punch weight determinations at necropsy) may be included in the study protocol.

Prepare a single-cell suspension of lymph node cells (LNC) excised bilaterally for each individual mouse. Prepare the single-cell suspension in PBS by either gentle mechanical separation through a 200-mesh stainless-steel gauze or another acceptable technique for generating a single-cell suspension. Wash LNC twice with an excess of PBS, and precipitate the DNA with 5% trichloroacetic acid (TCA) at 4°C for approximately 18 hours.

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

For the ^{125}IU method, transfer the 1 mL TCA pellet directly into gamma-counting tubes. Incorporation of ^{125}IU is determined by gamma counting and also expressed as dpm/mouse.

2.6 Reduced LLNA

This test method protocol includes a reduced LLNA (rLLNA) procedure that requires only the high-dose group. This reduction from three dose groups to one is the only difference between the multidose LLNA and the rLLNA (ESAC 2007; ICCVAM 2009a; Kimber et al. 2006). Like the multidose LLNA, the test substance concentration evaluated in the rLLNA should be the maximum concentration that does not induce overt systemic toxicity and/or excessive local skin irritation in the mouse. The rLLNA test method should be routinely used for the hazard identification of skin-sensitizing substances, except when a test article is expected to produce a positive result and dose-response information is needed. Because the rLLNA further decreases animal use by 40%, use of the multidose LLNA should be accompanied by appropriate scientific rationale.

2.7 Observations

Mice should be carefully observed at least once daily for any clinical signs, either of local irritation at the application site or of systemic toxicity (**Annex III**). Weighing mice before treatment and at the time of necropsy will help to assess systemic toxicity. Systematically record all observations and maintain records for each individual mouse. Animal monitoring plans should include criteria to promptly identify for euthanasia those mice exhibiting systemic toxicity, excessive irritation, or corrosion of skin (OECD 2000).

3.0 Calculation of Results

Results for each treatment group are expressed as the mean SI. Each SI is the ratio of the mean dpm/mouse within each test-substance treatment group or the concurrent positive-control-treated group against the mean dpm/mouse for the solvent/vehicle-treated control group. The average SI for the solvent/vehicle-treated control group is then 1. The decision process regards a result as positive when $SI \geq 3$.

The estimated concentration of a substance expected to produce an SI of 3 (i.e., the EC3) can also be calculated for positive LLNA results as an indicator of relative skin sensitization potency. The method for determining the EC3 is a simple linear interpolation of the points in the dose-response curve that lie immediately above and below SI = 3.

$$EC3 = c + \left[\frac{(3-d)}{(b-d)}\right] \times (a-c)$$

Coordinates:

(a = dose concentration immediately above SI = 3, b = SI immediately above 3)

(c = dose concentration immediately below SI = 3, d = SI immediately below 3)

When there are no data points that fall below SI = 3, a more complex log-linear extrapolation may be applied as described in Ryan et al. (2007) in which the two lowest test concentrations from the dose-response curve are used, provided the lowest SI value approaches the value of 3 and that a linear dose-response exists.

$$EC3_{ex} = 2^{\wedge}\left\{\log_2(c) + \frac{(3-d)}{(b-d)} \times \left[\log_2(a) - \log_2(c)\right]\right\}$$

Coordinates:

(a = dose concentration for next to lowest SI above 3, b = next to lowest SI above 3)

(c = dose concentration for lowest SI above 3, d = lowest SI above 3)

An example of how to calculate the EC3 using both the linear interpolation and the log-linear extrapolation approaches is provided in **Annex IV**.

In addition to an assessment of the magnitude of the SI, a statistical analysis for presence and degree of dose response may be conducted, which is possible only with the use of individual animals. Any statistical assessment should include an assessment of the dose-response relationship as well as suitably adjusted comparisons of test groups (e.g., pair-wise dosed group versus concurrent solvent/vehicle control comparisons). Statistical analyses may include, for instance, linear regression, Williams' test to assess dose-response trends, or Dunnett's test for pairwise comparisons. In choosing an appropriate method of statistical analysis, the investigator should be aware of possible inequality of variances and other related problems that may necessitate a data transformation or a nonparametric statistical analysis. In any case, the investigator may need to carry out SI calculations and statistical analyses with and without certain data points (outliers).

4.0 Evaluation and Interpretation of Results

In general, when the SI for any single treatment dose group is ≥3, the test substance is regarded as a skin sensitizer (Basketter et al. 1996; ICCVAM 1999; Kimber et al. 1994), and a test substance that does not meet this criterion is considered a nonsensitizer in the LLNA. However, the magnitude of the observed SI should not be the sole factor used to determine the biological significance of a skin sensitization response. Additional factors that could be considered include the outcomes of statistical analyses, the strength of the dose-response relationship, chemical toxicity, and solubility. For

instance, a quantitative assessment may be performed by statistical analysis of individual mouse data and may provide a more complete evaluation of the test substance's ability to act as a sensitizer (see **Section 3.0**). Equivocal results (e.g., the SI does not reach 3, but it is near 3 and there is a positive dose-response relationship) should be clarified by performing statistical analyses and by considering structural relationship to known skin sensitizers, available toxicity information (including observations of excessive local skin irritation and/or systemic toxicity in the LLNA study), and consistency of the positive control and solvent/vehicle control responses.

Employing the optimized assay condition described previously, the mean SI value for the positive control group (25% HCA or 5% mercaptobenzothiazole) should be ≥3. If not, data derived from the experiment should not be considered for evaluation.

5.0 Data and Reporting

5.1 Data

Data should be summarized in tables showing the individual animal dpm values, the group mean dpm/mouse, the group's associated error term (e.g., standard deviation [SD], standard error of the mean [SEM]), and the mean SI value for each treated group, positive control group, and solvent/vehicle control group.

5.2 Test Report

The test report should contain the following information:

Test Substances and Control Substances:

- Identification data (e.g., CASRN, if available; source; purity; known impurities; lot number)
- Physical nature and physicochemical properties (e.g., volatility, stability, solubility, physicochemical properties relevant to the conduct of the study)
- Composition and relative percentages of components, if formulation

Solvent/Vehicle:

- Identification data (e.g., CASRN, if available; purity; concentration, where appropriate; volume used)
- Justification for choice of solvent/vehicle

Test Animals:

- Strain of mice used
- Source of mice, housing conditions, diet, etc.
- Number, age, and sex of mice
- Microbiological status of the mice, when known

Test Conditions:

- Details of test substance preparation and application
- Justification for dose selection (including results from prescreen test, if conducted)
- Vehicle and test substance concentrations used, and total amount of test substance applied
- Details of food and water quality (including diet type/source, water source)
- Detailed description of treatment and sampling schedules
- Methods for measurement of toxicity
- Criteria for considering studies as positive, negative, or equivocal

- Details of any protocol deviations and an explanation of how the deviation affects the study design and results

Reliability Check:

- Summary of results of latest reliability check, including information on substance, concentration, and vehicle used
- Concurrent and/or historical positive and negative (solvent/vehicle) control data for testing laboratory
- Date and laboratory report for the most recent periodic positive control and a report detailing the historical positive control data for the laboratory justifying the basis for not conducting a concurrent positive control, if a concurrent positive control was not included

Results:

- Individual weights of mice at start of dosing and at scheduled kill, as well as mean and associated error term (e.g., SD, SEM) for each treatment group
- Time course of onset and signs of toxicity, including dermal irritation at site of administration, if any, for each animal
- Table of individual mouse dpm values and SI values for each treatment group
- Mean and associated error term (e.g., SD, SEM) for dpm/mouse for each treatment group and the results of outlier analysis for each treatment group
- Calculated SI and an appropriate measure of variability that takes into account the interanimal variability in both the test substance and control groups
- Calculated EC3 value (for positive LLNA results)
- Dose-response relationship
- Statistical analyses, where appropriate, and method applied

Discussion of the Results and Conclusion:

- A brief commentary on the results, the dose-response analysis, and the statistical analyses, where appropriate, with a conclusion as to whether the test substance should be considered a skin sensitizer

Quality Assurance Statement for GLP-Compliant Studies:

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

6.0 References

Basketter DA, Gerberick GF, Kimber I, Loveless SE. 1996. The local lymph node assay: a viable alternative to currently accepted skin sensitization tests. Food Chem Toxicol 34(10): 985-997.

Basketter DA, Lea LJ, Dickens A, Briggs D, Pate I, Dearman RJ, et al. 1999. A comparison of statistical approaches to the derivation of EC3 values from local lymph node assay dose responses. J Appl Toxicol 19(4): 261-266.

Dean JH, Twerdok LE, Tice RR, Sailstad DM, Hattan DG, Stokes WS. 2001. ICCVAM evaluation of the murine local lymph node assay: II. Conclusions and recommendations of an independent scientific peer review panel. Reg Toxicol Pharmacol 34(3): 258-273.

Dixon WJ, Massey FJ. 1983. Introduction to Statistical Analysis. 4th ed. New York, NY:McGraw Hill.

Ehling G, Hecht M, Heusener A, Huesler J, Gamer AO, Van Loveren H, et al. 2005. An European inter-laboratory validation of alternative endpoints of the murine local lymph node assay: 2nd round. Toxicology 212(1): 69-79.

EPA. 1998. Health Effects Test Guidelines: OPPTS 870.1200 - Acute Dermal Toxicity. EPA 712–C–98–192. Washington, DC:U.S. Environmental Protection Agency. Available: http://www.epa.gov/ocspp/pubs/frs/publications/Test_Guidelines/series870.htm [accessed 29 November 2010].

ESAC. 2007. ESAC Statement on the Reduced Local Lymph Node Assay. Ispra, Italy:European Commission Directorate General, Joint Research Centre, Institute for Health and Consumer Protection, European Centre for the Validation of Alternative Methods. Available: http://ecvam.jrc.it/ft_doc/ESAC26_statement_rLLNA_20070525-1.pdf [accessed 6 July 2010].

Hayes BB, Gerber PC, Griffey SS, Meade BJ. 1998. Contact hypersensitivity to dicyclohexylcarbodiimide and diisopropylcarbodiimide in female B6C3F1 mice. Drug Chem Toxicol 21(2): 195-206.

Hayes BB, Meade BJ. 1999. Contact sensitivity to selected acrylate compounds in B6C3F1 mice: Relative potency, cross reactivity, and comparison of test methods. Drug Chem Toxicol 22(3): 491-506.

Homey B, Von Schilling C, Blumel J, Schuppe HC, Ruzicka T, Ahr HJ, et al. 1998. An integrated model for the differentiation of chemical-induced allergic and irritant skin reactions. Toxicol Appl Pharmacol 153(1): 83-94.

ICCVAM. 1999. 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 and the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). NIH Publication No. 99-4494. Research Triangle Park, NC:National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/llnarep.pdf [accessed 21 October 2010].

ICCVAM. 2001. ICCVAM Immunotoxicology Working Group Recommended Protocol for the Murine Local Lymph Node Assay (LLNA): Testing of Chemicals for Contact Sensitizing (Allergic Contact Dermatitis [ACD]) Potential. Research Triangle Park, NC:National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/LLNAProt.pdf [accessed 29 November 2010].

ICCVAM. 2008. Independent Scientific Peer Review Panel Report. Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products. Research Triangle Park, NC:National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2008.pdf [accessed 29 November 2010].

ICCVAM. 2009a. ICCVAM Test Method Evaluation Report. The Reduced Murine Local Lymph Node Assay: An Alternative Test Method Using Fewer Animals to Assess the Allergic Contact Dermatitis Potential of Chemicals and Products. NIH Publication No. 09-6439. Research Triangle Park, NC:National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNA-LD/TMER.pdf [accessed 21 October 2010].

ICCVAM. 2009b. Independent Scientific Peer Review Panel Report: Updated Validation Status of New Versions and Applications of the Murine Local Lymph Node Assay: A Test Method for

Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products. Research Triangle Park, NC:National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/docs/immunotox_docs/LLNAPRPRept2009.pdf [accessed 15 December 2010].

ICCVAM. 2009c. Report on the ICCVAM-NICEATM/ECVAM/JaCVAM Scientific Workshop on Acute Chemical Safety Testing: Advancing *In Vitro* Approaches and Humane Endpoints for Systemic Toxicity Evaluations. Research Triangle Park, NC:National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/methods/acutetox/toxwksp-rpt.htm [accessed 8 July 2010].

ICCVAM. 2009d. Revised Draft Background Review Document: Non-radioactive Murine Local Lymph Node Assay: Flow Cytometry Test Method Protocol (LLNA: BrdU-FC). Research Triangle Park, NC:National Institute of Environmental Health Sciences. Available: iccvam.niehs.nih.gov/methods/immunotox/fcLLNA/recs.pdf [accessed 24 November 2010].

ILAR. 1996. Guide for the Care and Use of Animals. 7th ed. Washington, DC:National Academies Press.

Kimber I, Basketter DA. 1992. The murine local lymph node assay: a commentary on collaborative studies and new directions. Food Chem Toxicol 30(2): 165-169.

Kimber I, Dearman RJ, Betts CJ, Gerberick GF, Ryan CA, Kern PS, et al. 2006. The local lymph node assay and skin sensitization: a cut-down screen to reduce animal requirements? Contact Dermatitis 54(4): 181-185.

Kimber I, Dearman RJ, Scholes EW, Basketter DA. 1994. The local lymph node assay: developments and applications. Toxicology 93(1): 13-31.

Kimber I, Hilton J, Dearman RJ, Gerberick GF, Ryan CA, Basketter DA, et al. 1995. An international evaluation of the murine local lymph node assay and comparison of modified procedures. Toxicology 103(1): 63-73.

Loveless SE, Ladics GS, Gerberick GF, Ryan CA, Basketter DA, Scholes EW, et al. 1996. Further evaluation of the local lymph node assay in the final phase of an international collaborative trial. Toxicology 108(1-2): 141-152.

OECD. 2000. Guidance Document No. 19: Recognition, Assessment, and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation. Paris:OECD Publishing. Available: http://www.oecd.org/officialdocuments/displaydocumentpdf/?cote=env/jm/mono(2000)7&doclanguage=en [accessed 29 November 2010].

Patterson RM, Noga E, Germolec D. 2007. Lack of evidence for contact sensitization by Pfiesteria extract. Environ Health Perspect 115(7): 1023-1028.

Reeder MK, Broomhead YM, DiDonato L, DeGeorge GL. 2007. Use of an enhanced local lymph node assay to correctly classify irritants and false positive substances. Society of Toxicology Annual Meeting. Charlotte, NC.

Ryan CA, Chaney JG, Gerberick GF, Kern PS, Dearman RJ, Kimber I, et al. 2007. Extrapolating local lymph node assay EC3 values to estimate relative sensitizing potency. Cutan Ocul Toxicol 26(2): 135-145.

Tilney NL. 1971. Patterns of lymphatic drainage in the adult laboratory rat. J Anat 109: 369-383.

Vohr H, Jürgen AH. 2005. The local lymph node assay being too sensitive? Arch Toxicol 79(12): 721-728.

Woolhiser MR, Hayes BB, Meade BJ. 1998. A combined murine local lymph node and irritancy assay to predict sensitization and irritancy potential of chemicals. Toxicol Meth 8(4): 245-256.

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

1.0 Background

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

2.0 Training and Preparation for Node Identification

2.1 Identification of the Draining Node – Dye Treatment

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

2.1.1 Evan's Blue Dye Treatment:

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

2.1.2 Colloidal Carbon and Other Dye Treatments:

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

2.2 Identification of the Draining Node – Application of Strong Sensitizers

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

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

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

3.0 Dissection Approach

3.1 Lateral Dissection (Figure B-1)

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

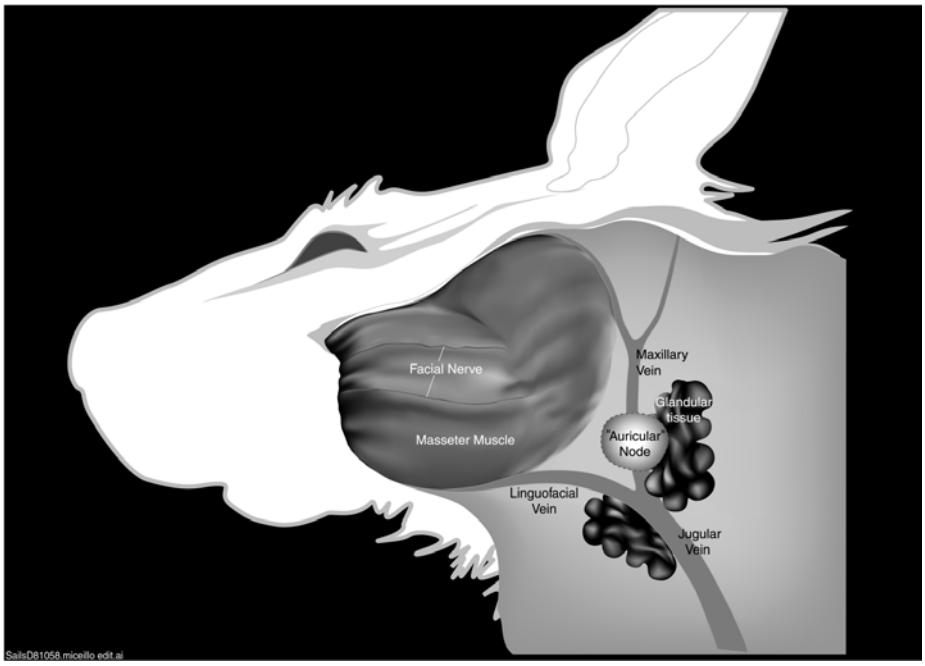
3.2 Ventral Dissection (Figure B-2)

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

4.0 Accuracy in Identification

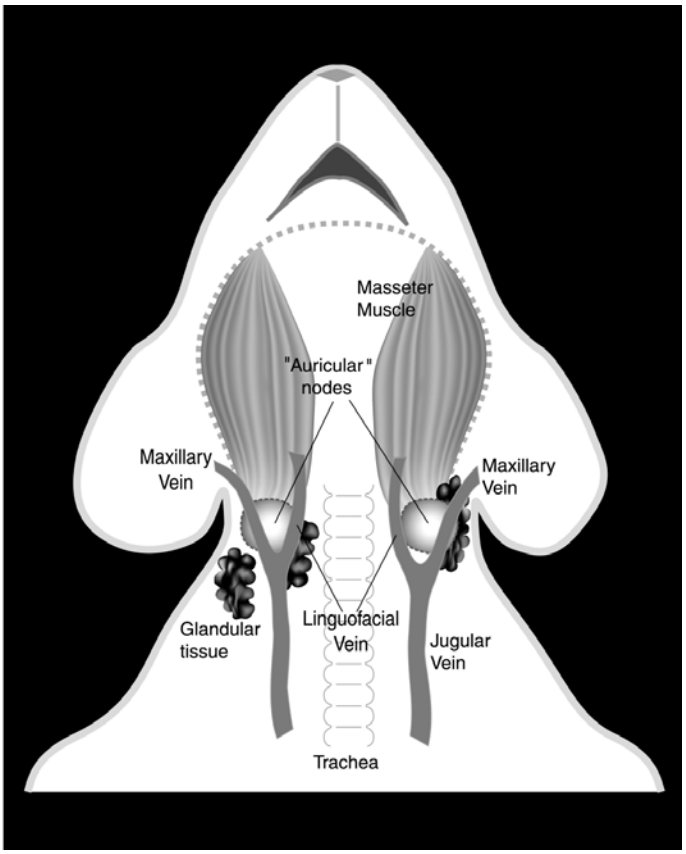
The nodes can be distinguished from glandular and connective tissue in the area by the uniformity of the nodal surface and a shiny translucent appearance. Application of sensitizing agents (especially the strong sensitizers used in training) will cause enlargement of the node. If a dye is injected for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

Figure B-1 Lateral Dissection



Credit: Dee Sailstad, U.S. EPA

Figure B-2 Ventral Dissection



Credit: Dee Sailstad, U.S. EPA

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

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

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

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

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

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

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

control and any concurrent test substances. Each laboratory is encouraged to determine the lowest number of animals to use in the positive control group based on the highest failure rate considered acceptable by the laboratory.

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

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

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

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

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

As noted in the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) murine local lymph node assay (LLNA) test method protocol, the maximum dose tested should be the maximum possible concentration that does not produce systemic toxicity and/or excessive local skin irritation after topical application in the mouse. In the absence of information to determine this concentration (e.g., acute toxicity and dermal irritation data, and/or structural and physicochemical information on the test material and/or structurally related test materials), a prescreen test should be performed using three dose levels of the test substance in order to define the appropriate dose to test in the LLNA. The maximum dose tested should be 100% of the test material for liquids or the maximum possible concentration for solids or suspensions.

The prescreen test is conducted under conditions identical to the main LLNA study, except there is no assessment of lymph node cell proliferation and fewer animals per dose group can be used. One or two animals per dose group are suggested. All mice are observed daily for any clinical signs of systemic toxicity or local irritation at the application site. For example, observations might occur before and after treatment on Days 1, 2, and 3. Body weights are recorded before testing and before termination (Day 6). Both ears of each mouse are observed for erythema and scored using **Table B-3**. Ear thickness measurements are taken using a thickness gauge (e.g., digital micrometer or Peacock Dial thickness gauge) on Day 1 (predose), Day 3 (approximately 48 hours after the first dose), and Day 6 (termination). Additionally, on Day 6, ear thickness can be determined by ear punch weight determinations, which should be performed after the animals are humanely killed.

Excessive local irritation is indicated by an erythema score ≥3 and/or an increase in ear thickness of ≥25% on any day of measurement (ICCVAM 2009d; Reeder et al. 2007). The highest dose selected for the main LLNA study will be the next lower dose in the prescreen concentration series that does not induce systemic toxicity and/or excessive local skin irritation.

Table B-3 Erythema Scores

Observation	Value
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to eschar (i.e., piece of dead tissue that is cast off from the surface of the skin) formation preventing grading of erythema	4

A statistically significant difference from control animals has also been used to delineate irritants from nonirritants in the LLNA (Ehling et al. 2005; Hayes et al. 1998; Hayes and Meade 1999; Homey et al. 1998; Patterson et al. 2007; Vohr and Jürgen 2005; Woolhiser et al. 1998). While these statistical differences often occur when the increase in ear thickness is less than 25%, they have not been associated specifically with excessive irritation (Ehling et al. 2005; Patterson et al. 2007; Vohr and Jürgen 2005; Woolhiser et al. 1998).

Test guidelines for assessing acute systemic toxicity recommend a number of clinical observations for assessing systemic toxicity (EPA 1998). The following clinical observations, which are based on test guidelines and current practices (ICCVAM 2009c), may indicate systemic toxicity when used as part of an integrated assessment and, therefore, may indicate the maximum dose level to use in the main LLNA:

- Changes in nervous system function (e.g., piloerection, ataxia, tremors, and convulsions)
- Changes in behavior (e.g., aggressiveness, change in grooming activity, marked change in activity level)
- Changes in respiratory patterns (i.e., changes in frequency and intensity of breathing, such as dyspnea, gasping, and rales)
- Changes in food and water consumption
- Lethargy and/or unresponsiveness
- Any clinical signs of more than slight or momentary pain and distress
- Reduction in body weight >5% from Day 1 to Day 6
- Mortality

Moribund animals or animals obviously in pain or showing signs of severe and enduring distress should be humanely killed (OECD 2000).

Annex IV:
Procedures for Calculating the Estimated Concentration of a Substance Expected to Produce a Stimulation Index of 3 (EC3) in the Murine Local Lymph Node Assay

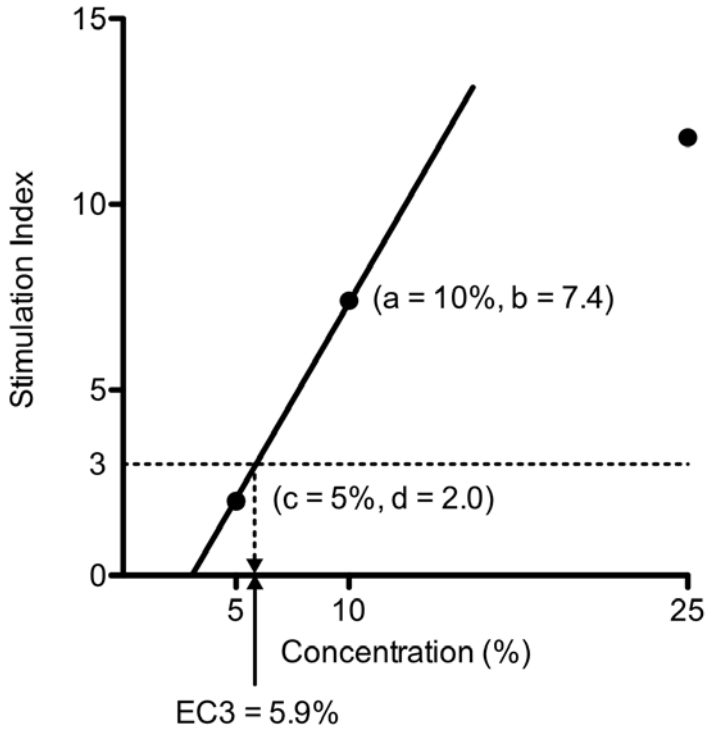
As mentioned in **Section 3.0** of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) murine local lymph node assay (LLNA) test method protocol, the estimated concentration of a substance expected to produce a stimulation index (SI) of 3 (i.e., the EC3) is the metric for determining relative skin sensitization potency using the LLNA. The method for determining the EC3 is a simple linear interpolation of the points in the dose-response curve that lie immediately above and below SI = 3, the classification threshold for sensitizers in the LLNA. This method was chosen from an evaluation of a variety of statistical approaches to derive EC3 values from LLNA dose-response data (Basketter et al. 1999). An example of how to calculate the EC3 using linear interpolation is provided below using the LLNA data in **Table B-4**. **Figure B-3** illustrates this data.

Table B-4 LLNA Data Used for Calculating the EC3 by Linear Interpolation

Concentration	Mean SI
5%	2.0
10%	7.4
25%	11.8

Abbreviations: EC3 = estimated concentration of a substance expected to produce an SI of 3, the threshold value for a substance to be considered a sensitizer in the LLNA; LLNA = murine local lymph node assay; SI = stimulation index.

Figure B-3 Example of LLNA Data Above and Below Stimulation Index = 3



As shown in **Figure B-3**, the point in the dose-response curve lying immediately above SI = 3 corresponds to the concentration of 10% and the mean SI of 7.4. The point in the dose-response curve lying immediately below SI = 3 corresponds to the concentration of 5% and the mean SI of 2.0. Applying the following equation for linear interpolation to this data set results in EC3 = 5.9% as shown:

$$EC3 = c + \left[\frac{(3-d)}{(b-d)}\right] \times (a-c)$$

↓

Coordinates :

(a = 10% [dose concentration immediately above SI = 3], b = 7.4 [SI immediately above 3])

(c = 5% [dose concentration immediately below SI = 3], d = 2.0 [SI immediately below 3])

↓

$$EC3 = 5 + \left[\frac{(3-2)}{(7.4-2.0)}\right] \times (10-5) = 5.9\%$$

When there are no data points that fall below SI = 3, a more complex log-linear extrapolation may be applied as described in Ryan et al. (2007) in which the two lowest test concentrations from the dose-response curve are used, provided the lowest SI value approaches the value of 3 and that a linear dose-response exists. An example of how to calculate the EC3 using log-linear extrapolation is provided below using the LLNA data in **Table B-5** and **Figure B-4** provides a graphical illustration of this data.

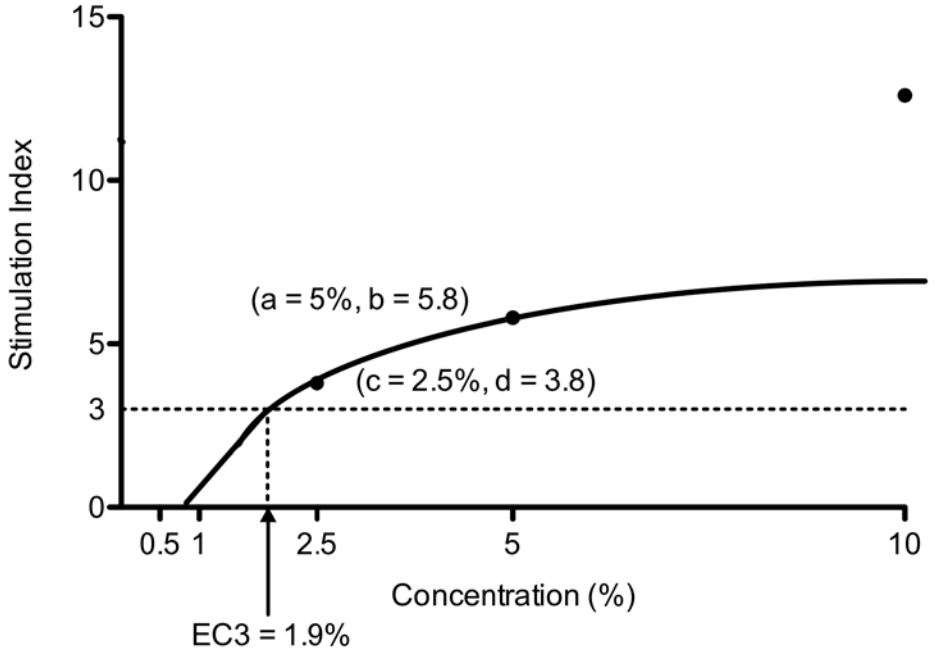
Table B-5 LLNA Data Used for Calculating the EC3 by Log-linear Extrapolation

Concentration	Mean SI
2.5%	3.8
5%	5.8
10%	12.6

Note: The data used in this example was adapted from Ryan et al. (2007).

Abbreviations: EC3 = estimated concentration of a substance expected to produce an SI of 3, the threshold value for a substance to be considered a sensitizer in the LLNA; LLNA = murine local lymph node assay; SI = stimulation index.

Figure B-4 Example of LLNA Data All Above Stimulation Index = 3



As shown in **Figure B-4**, all the points in the dose-response curve are above SI = 3. The two lowest SI values above 3 are 3.8 and 5.8 and their corresponding concentrations are 2.5% and 5%, respectively. Applying the following equation for log-linear extrapolation to this data set results in EC3 = 1.9% as shown:

$$EC3_{ex} = 2^{\wedge}\left\{\log_2(c) + \frac{(3-d)}{(b-d)} \times \left[\log_2(a) - \log_2(c)\right]\right\}$$

↓

Coordinates:

(a = 5% [dose concentration for next to lowest SI above 3], b = 5.8 [next to lowest SI above 3])

(c = 2.5% [dose concentration for lowest SI above 3], d = 3.8 [lowest SI above 3])

↓

$$EC3_{ex} = 2^{\wedge}\left\{\log_2(2.5) + \frac{(3-3.8)}{(5.8-3.8)} \times \left[\log_2(5) - \log_2(2.5)\right]\right\} = 1.9$$