Appendix B

ICCVAM-Recommended Protocol

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

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Preface

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

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

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

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

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

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

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

2.0 Description of the Local Lymph Node Assay

2.1 Sex and strain of animals

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

2.2 Preparation of animals

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

2.3 Preparation of doses

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

2.4 Test Conditions

2.4.1 Solvent/vehicle

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

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

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

2.4.2 Controls

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

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

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

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

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

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

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

2.5 Methodology

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

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

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

The LLNA experimental procedure is performed as follows:

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

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

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

Days 4 and 5. No treatment.

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

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

2.6 Observations

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

3.0 Calculation of Results

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

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

4.0 Evaluation and Interpretation of Results

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

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

5.0 Data and Reporting

5.1 Data

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

5.2 Test Report

The test report should contain the following information:

Test Substances and Control Substances

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

Solvent/Vehicle:

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

Test Animals:

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

Test Conditions:

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

Results:

- Signs of systemic toxicity and/or local irritation

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

Discussion of the Results

Conclusion

A Quality Assurance Statement for GLP-compliant Studies

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

6.0 References

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

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

Ehling G, Hecht M, Heusener A, Huesler J, Gamer AO, van Loveren H, et al. 2005. A European inter-laboratory validation of alternative endpoints of the murine local lymph node assay: first round. Toxicology 212:60-68.

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/opptsfrs/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Series/870-1200.pdf.

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

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 22:491-506.

Homey B, von Schilling C, Blumel J, Schuppe H-C, Ruzicka T, Jürgen AH, et al. 1998. An integrated model for the differentiation of chemical-induced allergic and irritant skin reactions. Toxicol Appl Pharmacol 153:83-94.

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 (ICCVAM) and the National Toxicology Program 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.

ICCVAM. 2001. Protocol: Murine Local Lymph Node Assay (LLNA). Purpose: Assessment of Allergic Contact Dermatitis Potential. Recommended by: ICCVAM Immunotoxicity Working Group – based on an Independent Expert Peer Review Panel Evaluation of the LLNA. Research Triangle Park, NC: National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna/LLNAProt.pdf.

ICCVAM. 2008a. 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.

ICCVAM. 2008b. Non-radioactive Murine Local Lymph Node Assay: Flow Cytometry Test Method Protocol (LLNA: BrdU-FC) Draft Background Review Document. Research Triangle Park, NC: National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/methods/immunotox/llna-BRD.htm.

ICCVAM. 2009. 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.

Institute of Laboratory Animal Research (ILAR). 1996. Guide for the Care and Use of Laboratory Animals. 7th ed. Washington, DC: National Academies Press.

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

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

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 103:63-73.

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 108:141-152.

OECD. 1987. Guideline For Testing of Chemicals – Test Guideline 402: Acute Dermal Toxicity. Paris: Organisation for Economic Co-operation and Development. Available: http://www.oecd.org/document/40/0,2340,en_2649_34377_37051368_1_1_1_1,00.html.

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

Reeder MK, Broomhead YL, DiDonato L, DeGeorge GL. 2007. Use of an enhanced local lymph node assay to correctly classify irritants and false positive substances. Toxicologist 96 (S-1):235.

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

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

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: 245-256.

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

1.0 Background

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

2.0 Training and Preparation for Node Identification

2.1 Identification of the Draining Node – Dye Treatment

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

2.1.1 Evan's Blue Dye treatment:

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

2.1.2 Colloidal carbon and other dye treatments:

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

2.2 Identification of the Draining Node – Application of Strong Sensitizers

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

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

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

3.0 Dissection Approach

3.1 Lateral Dissection (Figure B-1)

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

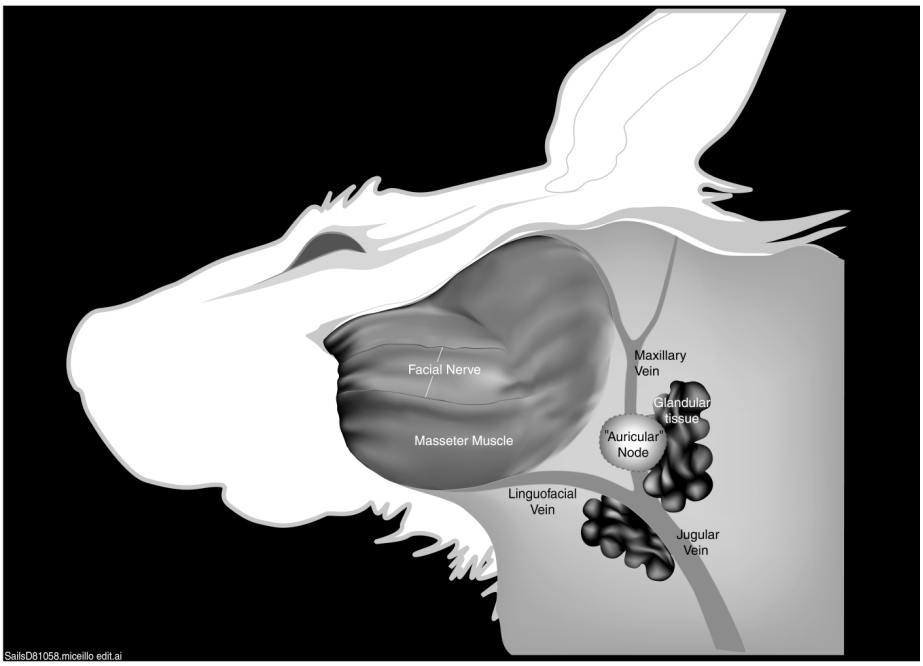
3.2 Ventral Dissection (Figure B-2)

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

4.0 Accuracy in Identification

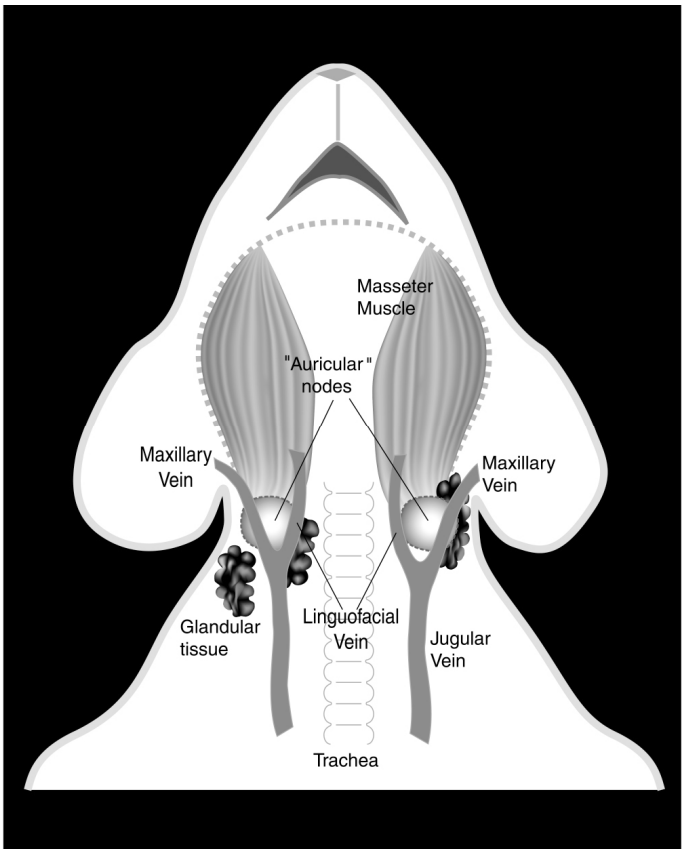
The nodes can be distinguished from glandular and connective tissue in the area by the uniformity of the nodal surface and a shiny translucent appearance. Application of sensitizing agents (especially the strong sensitizers used in training) will cause enlargement of the node size. If a dye is injected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

Figure B-1 Lateral Dissection



Credit: Dee Sailstad, U.S. EPA

Figure B-2 Ventral Dissection



Credit: Dee Sailstad, U.S. EPA

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Table B-3 Erythema Scores

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

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

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

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

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