ICCVAM-Recommended Test Method Protocol: The Murine Local Lymph Node Assay: 2-Bromodeoxyuridine-ELISA Test Method (LLNA: BrdU-ELISA), a Nonradioactive Alternative Test Method for Assessing the Allergic Contact Dermatitis Potential of Chemicals and Products

Originally published as Appendix B of "ICCVAM Test Method Evaluation Report on the Murine Local Lymph Node Assay: BrdU-ELISA"
A Nonradioactive Alternative Test Method to Assess the Allergic Contact Dermatitis Potential of Chemicals and Products
NIH Publication No. 10-7552 – Published 2010
Available at: http://iccvam.niehs.nih.gov/methods/immunotox/llna-ELISA/TMER.htm

This page intentionally left blank

1.0 General Principle of Detection of Skin Sensitization Using the Nonradiolabelled Murine Local Lymph Node Assay: 2-Bromodeoxyuridine-ELISA Test Method (LLNA: BrdU-ELISA)

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 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 (^{3}H-methyl thymidine or ^{125}I-iododeoxyuridine) incorporation into the DNA of dividing lymphocytes, and assesses this proliferation in the draining lymph nodes proximal to the application site (see **Annex I**). Due to the use of radioactivity, the LLNA has limited use in regions where the acquisition, use, or disposal of radioactivity is problematic. The LLNA: BrdU-ELISA was therefore developed as a nonradioactive modification to the LLNA (Takeyoshi 2001), which uses nonradiolabelled 5-bromo-2-deoxyuridine (BrdU) (Chemical Abstracts Service Registry Number [CASRN] 59-14-3) with detection by an enzyme-linked immunosorbent assay (ELISA) to assess lymphocyte proliferation. The ability to detect skin sensitizers without the necessity of using a radioactive label for DNA eliminates the potential for occupational exposure to radioactivity and waste disposal issues. Similar to the LLNA, the LLNA: BrdU-ELISA studies the induction phase of skin sensitization and provides quantitative data suitable for dose-response assessment. Lymphocyte proliferation in test groups is compared to that in the concurrent vehicle-treated control group. The proliferation is proportional to the dose and to the potency of the applied allergen and provides a simple means of obtaining a quantitative measurement of sensitization. The LLNA: BrdU-ELISA assesses this proliferation as the proliferation in test groups compared to that in vehicle treated controls. The ratio of the proliferation in treated groups to that in concurrent vehicle treated controls, termed the stimulation index (SI), is determined, and should be $\geq$1.6 before a test substance can be considered as a skin sensitizer, with specific limitations for borderline positive results (i.e., SI between 1.6 and 1.9) as described in Section 3 of this Test Method Evaluation Report.

The methods, described here are based on the use of measuring BrdU content to indicate an increased number of proliferating cells in the draining auricular lymph nodes. BrdU is an analog of thymidine and is similarly incorporated into the DNA of proliferating cells. The incorporation of BrdU is measured by ELISA, which utilizes an antibody specific for BrdU that is also labeled with peroxidase. When the substrate is added, the peroxidase reacts with the substrate to produce a colored product that is quantified at a specific absorbance using a microtiter plate reader. A concurrent positive control is added to each assay to provide an indication of appropriate assay performance.

2.0 Description of the LLNA: BrdU-ELISA

2.1 Sex and strain of animals

The mouse is the species of choice for the LLNA: BrdU-ELISA assay. Validation studies were conducted exclusively with the CBA/JN strain, but other CBA substrains can be used. Young adult female mice (nulliparous and non-pregnant) are used because most data in the existing database were generated using mice of this gender.[1] At the start of the study, mice should be 8-12 weeks of age. All

[1] Male mice may be used if it is sufficiently demonstrated that these animals perform as well as female CBA mice in the LLNA: BrdU-ELISA.

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%-70% (although the aim is for 50%-60%). Lighting should be artificial, the sequence being 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 quarantined/acclimatized for at least five days prior to the start of the test (ILAR 1996). Mice should be allocated to small groups by a stratified randomization or other appropriate methods 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 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). 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) prior to the initiation 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, prior to dosing of the mice. Liquid test substances may be dosed directly (i.e., applied neat) or diluted prior to dosing. Insoluble materials, such as those generally seen in medical devices, should be subjected to an exaggerated extraction in an appropriate solvent to extract all extractable constituents for testing 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 solvent/vehicle should not interfere with or bias the test result and should be selected on the basis of maximizing the solubility in order to obtain the highest concentration achievable while producing a solution/suspension suitable for application of the test substance. Recommended vehicles are acetone: olive oil (4:1 v/v) (AOO), *N*,*N*-dimethylformamide (DMF), methyl ethyl ketone (MEK), propylene glycol, and dimethyl sulfoxide (DMSO) (Van Och et al. 2000; Kimber et al. 1994), but others may be used if sufficient scientific rationale is provided (Kimber and Basketter 1992). Particular care should be taken to ensure that hydrophilic materials are incorporated into a vehicle system that incorporates appropriate solubilizers (e.g., 1% Pluronic® L92) that wet the skin and does not immediately run off. Thus, wholly aqueous vehicles may need to be avoided. In certain situations, 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. 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.

Positive controls are used to demonstrate 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: BrdU-ELISA response at an exposure level expected to give an increase in the SI ≥ 1.6 over the negative control group. The positive control dose should be chosen such that the induction is reproducible but it does not cause excessive skin irritation or systemic toxicity. Preferred positive control substances are 50% hexyl cinnamic aldehyde (HCA; CASRN 101-86-0) and 50% eugenol (CASRN 97-53-0) in AOO. There may be circumstances in which, given adequate justification, other positive control substances meeting the above criteria may be used.

Although the positive control substance should be tested in the vehicle that is known to elicit a consistent response (e.g., AOO), there may be certain regulatory situations in which testing in a non-standard vehicle (clinically/chemically relevant formulation) will also be necessary. In such situations, the possible interaction of a positive control with this unconventional vehicle should be tested. 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 inclusion of a concurrent positive control group is recommended, there may be situations in which periodic testing (i.e., at intervals ≤6 months) of the positive control substance may be adequate for laboratories that conduct the LLNA: BrdU-ELISA regularly (i.e., conduct the LLNA: BrdU-ELISA at a frequency of no less than once per month) and have an established historical positive control database that demonstrates the laboratory's ability to obtain reproducible and accurate results with positive controls. Adequate proficiency with the LLNA: BrdU-ELISA 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 concurrent positive control group should always be included when there is a procedural change to the LLNA: BrdU-ELISA (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), 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.

Investigators should be aware that the decision to conduct a positive control on a periodic basis instead of concurrently has 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 may be questioned. Implications of these outcomes 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 and if the laboratory demonstrates, based on laboratory-specific historical data, that fewer mice can be used without substantially increasing the failure rate of the positive control (i.e., the rate at which SI < 1.6 and the frequency with which studies will need to be repeated due to positive control failure [Appendix A of ICCVAM 2009a]).

In instances where substances of a specific chemical class or range of responses are being evaluated, benchmark substances may be useful to demonstrate that the test method is functioning properly for detecting the skin sensitization potential of a test substance. Appropriate benchmark substances should have the following properties:

- Structural and functional similarity to the class of the substance being tested
- Known physical/chemical characteristics
- Supporting data from the LLNA: BrdU-ELISA

- Supporting data on known effects in animal models and/or from humans

2.5 Methodology

A minimum of four animals is used per dose group, with a minimum of three concentrations of the test substance, plus 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 assessment of interanimal variability and a statistical comparison of the difference between test substance and vehicle control group measurements. In addition, evaluating the possibility of reducing the number of mice in the positive control group is only feasible 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). 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. All 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) should be considered, where available, in selecting the three consecutive concentrations so that the highest concentration maximizes exposure while avoiding systemic toxicity and/or excessive local skin irritation (Kimber et al. 1994; OECD 2002). In the absence of such information, an initial prescreen test may be necessary (**Annex II**).

The LLNA: BrdU-ELISA experimental procedure is performed as follows:

Day 1. Individually identify and record the weight of each animal and any clinical observations. Apply 25 µL of the appropriate dilution of the test substance, the vehicle alone, or the concurrent positive control to the dorsum of each ear.

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

Day 4. No treatment.

Day 5. Inject 0.5 mL (5 mg/mouse) of 10 mg/mL BrdU in physiological saline intraperitoneally.

Day 6. Record the weight of each animal and any clinical observations. Approximately 24 hours (24 h) after BrdU injection, humanely kill the animals. To further monitor the local skin response in the experimental study, 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.

Excise both bilateral draining auricular lymph nodes from each mouse ear (see diagram and description of dissection in **Annex I**) and store in a 1.5 mL centrifuge tube at -20^{o}C until BrdU is measured by ELISA.

For BrdU measurement, a single-cell suspension of lymph node cells (LNC) from each mouse is prepared by adding a small volume of physiological saline (approximately 0.3 mL) to the excised lymph nodes, crushing the lymph nodes with a disposable plastic pestle, and passing through a #70 nylon mesh or another acceptable technique for mechanical disaggregation (e.g., passing through 200 micron-mesh stainless steel gauze) to generate a single-cell suspension. The procedure for preparing the LNC suspension is a critical step of this assay; it is most important to crush the lymph node and suspend the LNC completely. Every technician should establish the skill in advance. The lymph nodes in negative control animals are small, so careful operation is required to avoid an artificial effect on SI values.

In each case, the target volume of the LNC suspension should be adjusted to a pre-determined optimized volume (approximately 15 mL) based on achieving a mean absorbance of the negative control group within 0.1-0.2. Because this absorbance depends on the assay apparatus and the target volume of cell suspension, every laboratory should decide their own optimal volume of LNC suspension in advance.

The incorporation of BrdU into lymph node cells should be determined using a commercial cell proliferation assay kit (Roche Diagnostics GmbH, Roche Applied Science, 68298 Mannheim, Germany; Cat. No. 11 647 229 001) after they are crushed and suspended in physiological saline. The absorbance is defined as the BrdU labeling index. Follow the instructions in the assay kit. Briefly, 100 μL of the LNC suspension is added to the wells of a flat-bottom microplate in triplicate. After fixation and denaturation of the LNC, anti-BrdU antibody is added to each well and allowed to react. Subsequently the anti-BrdU antibody is removed by washing and the substrate solution is then added and allowed to produce chromogen. Absorbance at 370 nm with a reference wavelength of 492 nm is then measured.

2.6 Reduced LLNA

Using this test method protocol, there is also the opportunity to perform a reduced LLNA: BrdU-ELISA (rLLNA: BrdU-ELISA). Use of the rLLNA: BrdU-ELISA has the potential to reduce the number of animals by omitting the middle and low dose groups from the LLNA: BrdU-ELISA (Kimber et al. 2006; ESAC 2007; ICCVAM 2009b). This is the only difference between the LLNA: BrdU-ELISA and the rLLNA: BrdU-ELISA. Thus, the test substance concentration evaluated in the rLLNA: BrdU-ELISA should be the maximum concentration that does not induce overt systemic toxicity and/or excessive local skin irritation in the mouse (**Annex II**). The rLLNA: BrdU-ELISA should be used for the hazard classification of skin sensitizing substances if dose-response information is not needed, provided there is adherence to all other LLNA: BrdU-ELISA protocol specifications.

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 II**). Weighing mice prior to treatment and at the time of necropsy will aid in assessing systemic toxicity. All observations are systematically recorded with records maintained for each individual mouse. Animal monitoring plans should include criteria to promptly identify those mice exhibiting systemic toxicity or excessive irritation, or corrosion of skin for euthanasia (OECD 2000).

3.0 Calculation of Results

Results for each treatment group are expressed as the mean SI. The SI is derived by dividing the mean BrdU labeling index/mouse within each test substance group and the concurrent positive control group by the mean BrdU labeling index for the solvent/vehicle control group. The average SI value for vehicle treated controls is then equal to one.

The BrdU labeling index is defined as:

BrdU labeling index = $(ABS_{em} - ABS\ blank_{em}) - (ABS_{ref} - ABS\ blank_{ref})$

where ABS = absorbance, em = emission wavelength and ref = reference wavelength.

The decision process regards a result as positive when SI $\geq$ 1.6 (see **Section 3** of this Test Method Evaluation Report). However, the strength of the dose response, chemical toxicity, solubility, and, where appropriate, statistical significance should be considered together with SI values to arrive at a final decision (Basketter et al. 1996; ICCVAM 1999; EPA 1998; Kimber et al. 1998).

Collecting data at the level of the individual mouse will enable a statistical analysis for presence and degree of dose response in the data. Any statistical assessment could include an evaluation 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 or Williams's test to assess dose-response trends, and Dunnett's test for pairwise comparisons. In choosing an appropriate method of statistical analysis, the investigator should maintain an awareness of possible inequalities 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 (sometimes called "outliers").

4.0 Evaluation and Interpretation of Results

Consideration should be given to the possibility of borderline positive results when SI values between 1.6 and 1.9 are obtained. This is based on the validation database of 43 substances using an SI $\geq$ 1.6 for which the LLNA: BrdU-ELISA correctly identified all 32 LLNA sensitizers, but incorrectly identified two of 11 LLNA nonsensitizers with SI values between 1.6 and 1.9 (i.e. borderline positive) (see Section 3.0 of this Test Method Evaluation Report). If an SI value between 1.6 and 1.9 is obtained, other available information such as the nature of the dose-response, evidence of systemic toxicity or excessive local skin irritation, and, where appropriate, statistical significance together with SI values should be considered to confirm that such borderline positive results are potential skin sensitizers (see Section 3.0 of this Test Method Evaluation Report). Consideration should also be given to various properties of the test substance, including whether it has a structural relationship to known skin sensitizers. These and other considerations are discussed in detail elsewhere (Basketter et al. 1998).

Employing the optimized assay condition described previously, the mean SI value for the positive control group (50% HCA) should be equal to or greater than 1.6. If not, data derived from the experiment should not be used for evaluation.

5.0 Data and Reporting

5.1 Data

Data should be summarized in tabular form showing the individual animal BrdU labeling index values, the group mean BrdU labeling index/animal, its associated error term (e.g., standard deviation [SD], standard error of the mean [SEM]), and the mean SI value 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 (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 (CASRN; purity; concentration, where appropriate; volume used)

- Justification for choice of vehicle

Test Animals

- Source of CBA mice, housing conditions, diet, etc.
- Microbiological status of the animals, when known
- Number and age of animals

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 substance applied
- Details of food and water quality (including diet type/source, water source)
- Details of treatment and sampling schedules
- Methods for measurement of toxicity
- Criteria for considering studies as positive or negative
- Details of any protocol deviations and an explanation on 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 BrdU labeling indices and SI values for each treatment group
- Mean and associated error term (e.g., SD, SEM) for BrdU labeling index/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
- Dose response relationship
- Statistical analysis, where appropriate

Discussion of the Results

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

Conclusion

A Quality Assurance Statement for GLP-compliant Studies

- Indicate all inspections made during the study and the dates any results were reported to the Study Director; 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.

Basketter DA, Gerberick GF, Kimber I. 1998. Strategies for identifying false positive responses in predictive sensitisation tests. Food Chem Toxicol 36:327-33.

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.

ESAC. 2007. Statement on the Reduced Local Lymph Node Assay (rLLNA). European Commission Directorate General, Joint Research Centre, Institute for Health and Consumer Protection, European Centre for the Validation of Alternative Methods. April 2007. Available: http://ecvam.jrc.it/ft_doc/ESAC26_statement_rLLNA_20070525-1.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. 2009a. Recommended Performance Standards: Murine Local Lymph Node Assay. NIH Publication No. 09-7357. Research Triangle Park, NC: National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/docs/immunotox_docs/llna-ps/LLNAPerfStds.pdf.

ICCVAM. 2009b. 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 at: http://iccvam.niehs.nih.gov/methods/immunotox/LLNA-LD/TMER.htm.

ICCVAM. 2009c. Nonradioactive Murine Local Lymph Node Assay: Flow Cytometry Test Method Protocol (LLNA: BrdU-FC) Revised Draft Background Review Document. Research Triangle Park, NC: National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/methods/immunotox/fcLLNA/BRDcomplete.pdf.

ICCVAM. 2009d. 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. 1998. Assessment of the skin sensitization potential of topical medicaments using the local lymph node assay: An interlaboratory exercise. J Toxicol Environ Health 53:563-79.

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 54:181-185.

OECD. 1987. Guideline For Testing of Chemicals – Test Guideline 402: Acute Dermal Toxicity. Paris:OECD. Available: http://www.oecd.org/document/40/0,2340,en_2649_34377_37051368_1_1_1_1,00.html.

OECD. 2000. Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation. Environmental Health and Safety Monograph Series on Testing and Assessment No. 19. Paris:OECD. Available: http://www.oecd.org/document/30/0,3343,en_2649_34377_1916638_1_1_1_1,00.html

OECD. 2002. Test Guideline 429. Skin Sensitisation: Local Lymph Node Assay, adopted April 24, 2002. In: OECD Guidelines for Testing of Chemicals. Paris:OECD. Available: http://titania.sourceoecd.org/vl=7033968/cl=16/nw=1/rpsv/cw/vhosts/oecdjournals/1607310x/v1n4/contp1-1.htm

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.

Takeyoshi M, Yamasaki K, Yakabe Y, Takatsuki M, and Kimber I. 2001. Development of non-radio isotopic endpoint of murine local lymph node assay based on 5-bromo-2'-deoxyuridine (BrdU) incorporation. Toxicology Letters 119:203-208.

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

Van Och FMM, Slob W, De Jong WH, Vandebriel RJ, Van Loveren H. 2000. A quantitative method for assessing the sensitising potency of low molecular weight chemicals using a local lymph node assay: employment of a regression method that includes determination of uncertainty margins. Toxicology 146:49-59.

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.

This page intentionally left blank

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

1.0 Background

Although minimal technical training of the LLNA: BrdU-ELISA 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: BrdU-ELISA 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: BrdU-ELISA.

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.

Evan's Blue Dye treatment:

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

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 AOO vehicle. 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 three 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-I-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-I-1**). The draining node ("auricular") will be positioned adjacent to the masseter muscle and proximal to and slightly above the jugular bifurcation.

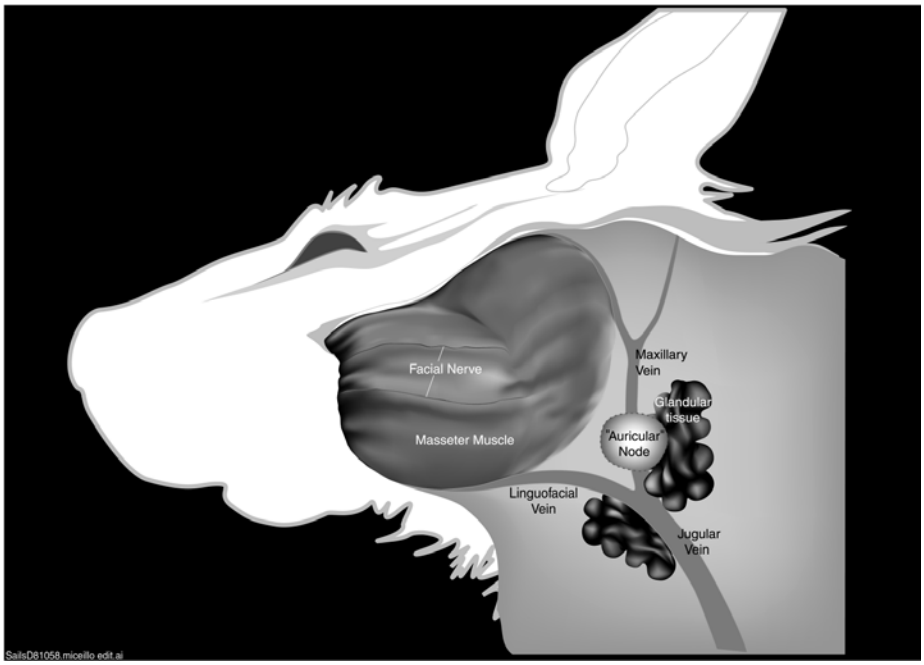
3.2 Ventral Dissection (Figure B-I-2)

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

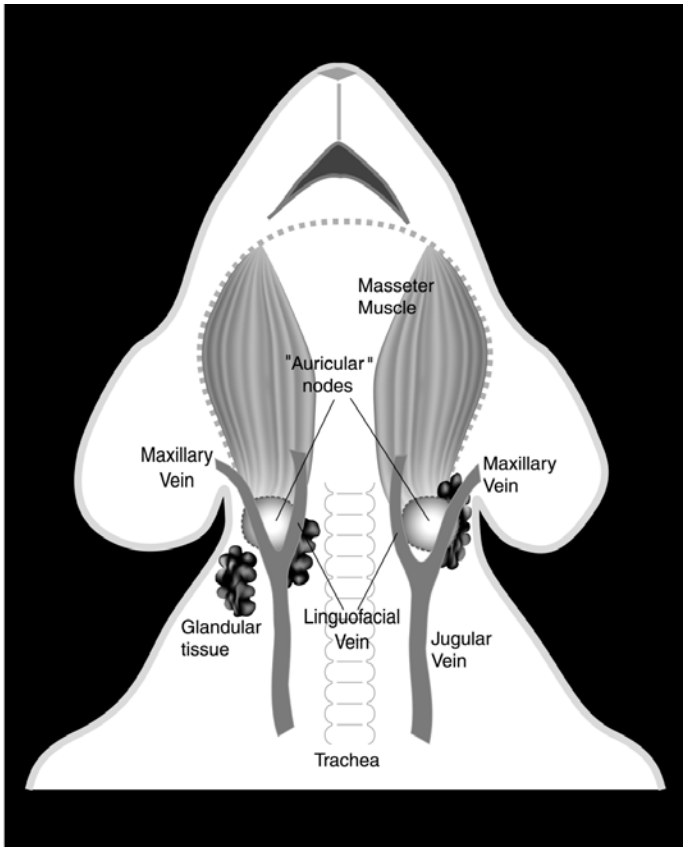
The nodes can be distinguished from glandular and connective tissue in the area by the uniformity of the nodal surface and a shiny translucent appearance. Application of sensitizing agents (especially the strong sensitizers used in training) will cause enlargement of the node size. If a dye is injected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

Figure B-I-1 Lateral Dissection



Credit: Dee Sailstad, U.S. EPA

Figure B-I-2 Ventral Dissection



Credit: Dee Sailstad, U.S. EPA

This page intentionally left blank

Annex II:
Evaluating Local Irritation and Systemic Toxicity in the LLNA: BrdU-ELISA

As noted in the ICCVAM LLNA: BrdU-ELISA test method protocol, the maximum dose tested should be the maximum possible concentration that does not produce systemic toxicity or excessive local 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: BrdU-ELISA.

The prescreen test is conducted under identical conditions as the main LLNA: BrdU-ELISA study, except there is no assessment of lymph node cell proliferation. The maximum dose tested should be 100% of the test material for liquids or the maximum possible concentration for solids or suspensions. One or two animals per dose group are suggested. All mice will be observed daily for any clinical signs of systemic toxicity or local irritation at the application site. 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-II-1**. 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 could be determined by ear punch weight determinations, which must 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 (Reeder et al. 2007; ICCVAM 2009c). The highest dose selected for the main LLNA: BrdU-ELISA 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-II-1 Erythema Scores

Observation	Value
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema (beet redness)	3
Severe erythema (beet redness) to eschar formation preventing grading of erythema	4

In addition to a 25% increase in ear thickness (Reeder et al. 2007; ICCVAM 2009c), a statistically significant increase in ear thickness in the treated mice compared to control mice has also been used to identify irritants in the traditional 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). While statistically significant increases can occur when ear thickness is less than 25%, they have not been associated specifically with excessive irritation (Woolhiser et al. 1998; Hayes and Meade 1999; Ehling et al. 2005; Vohr and Jürgen 2005; Patterson et al. 2007).

Test guidelines for assessing acute dermal toxicity recommend a number of clinical observations for assessing systemic toxicity (OECD 1987; EPA 1998). The following clinical observations, which are

based on test guidelines and current practices (ICCVAM 2009d), 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: BrdU-ELISA:

- 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 showing signs of severe pain and distress should be humanely killed (OECD 2000).