Designation: F2148 – 13

Standard Practice for Evaluation of Delayed Contact Hypersensitivity Using the Murine Local Lymph Node Assay (LLNA)[1]

This standard is issued under the fixed designation F2148; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This practice provides a methodology to use an in-situ procedure for the evaluation of delayed contact hypersensitivity reactions.

1.2 This practice is intended to provide an alternative to the use of guinea pigs for evaluation of the ability of a device material to stimulate delayed contact hypersensitivity reactions. This alternative is particularly applicable for materials used in devices that contact only intact skin. However, the guinea pig maximization test is still the recommended method when assessing the delayed hypersensitivity response to metals or when testing substances that do not penetrate the skin but are used in devices that contact deep tissues or breached surfaces. This practice may be used for testing metals, with the exception of nickel-containing metals, unless the unique physicochemical properties of the materials may interfere with the ability of LLNA to detect sensitizing substances.

1.3 This practice consists of a protocol for assessing an increase in lymphocyte proliferation within the nodes draining the site of administration on the ears of mice.

1.4 The LLNA has been validated only for low-molecular-weight chemicals that can penetrate the skin. The absorbed chemical or metabolite must bind to macromolecules, such as proteins, to form immunogenic conjugates.

1.5 This practice is one of several developed for the assessment of the biocompatibility of materials. Practice F748 may provide guidance for the selection of appropriate methods for testing materials for a specific application.

1.6 Identification of a supplier of materials or reagents is for the convenience of the user and does not imply a single source. Appropriate materials and reagents may be obtained from many commercial supply houses.

1.7 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.8 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

F619 Practice for Extraction of Medical Plastics

F720 Practice for Testing Guinea Pigs for Contact Allergens: Guinea Pig Maximization Test

F748 Practice for Selecting Generic Biological Test Methods for Materials and Devices

F750 Practice for Evaluating Material Extracts by Systemic Injection in the Mouse

2.2 *Other Documents:*[3]

ICCVAM NIH Publication No: 99-4494 The Murine Local Lymph Node Assay, 1999

ICCVAM NIH Publication NO: 11-7709 Usefulness and Limitations of the Murine Local Lymph Node Assay for Potency Categorization of Chemicals Causing Allergic Contact Dermatitis in Humans

3. Terminology

3.1 *Definitions:*

3.1.1 *AOO, n*—acetone olive oil solution (4:1 v/v) is a suitable nonpolar solvent.

3.1.2 *aqueous solvent, n*—in this assay refers to the polar solvent, saline.

3.1.3 *DMSO, n*—dimethylsulfoxide (nonaqueous, suitable organic solvent).

3.1.4 *DNCB, n*—2,4-dinitrochlorobenzene.

3.1.5 *formalin, n*—a 1/10 dilution of 37 to 39 % formaldehyde solution (formaldehyde) in PBS.

[1] This practice is under the jurisdiction of ASTM Committee F04 on Medical and Surgical Materials and Devices and is the direct responsibility of Subcommittee F04.16 on Biocompatibility Test Methods.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] Available from NICEATM, NIEHS, 79 Alexander Dr., Mail Drop EC-17, Research Triangle Park, NC 27709.

3.1.6 *ICCVAM, n*—Interagency Coordinating Committee on the Validation of Alternative Methods.

3.1.7 *nonaqueous solvent, n*—in this assay refers to the organic or nonpolar solvent, which shall be dimethylsulfoxide (DMSO) or acetone olive oil (AOO).

3.1.8 *PBS, n*—phosphate buffered saline, pH 7.2.

3.1.9 *positive control, n*—a substance capable of consistently stimulating lymphocyte proliferation.

3.1.10 *saline, n*—0.9 % sodium chloride (aqueous, polar solvent).

3.1.11 *TCA, n*—5 % trichloroacetic acid.

3.1.12 *tritiated thymidine, n*—^{H3}methyl thymidine, specific activity 2 Ci/mM (in PBS) I^{125} IUDR-radioactive uridine.

3.1.13 *vehicle controls, n*—an aqueous, polar solvent and a non-aqueous, nonpolar solvent.

4. Summary of Practice

4.1 Test and control substances or extracts are applied to the ears of test mice. The draining lymph nodes are harvested and lymphocyte proliferation evaluated. Comparisons are made with the control and test specimens tested under identical conditions.

5. Significance and Use

5.1 The propensity of a material to stimulate delayed contact hypersensitivity must be assessed before clinical application of devices containing this material. Delayed hypersensitivity may occur anywhere in the body. Systemic delayed hypersensitivity may have a complex set of reactions and consequences depending on the actual tissue/organ site of reaction. Although the reactions are seldom life-threatening, severe tissue and organ damage my result over time. Skin is the usual test site to determine the propensity of a material to cause delayed hypersensitivity.

5.2 The standard historical test methods have involved the use of guinea pigs with a cutaneous application and observation of the reaction site. The use of the murine local lymph node assay results in a numerical quantitation of stimulation, rather than subjective evaluation and could be used to determine dose responses.

5.3 This practice may not be predictive of events occurring during all types of implant applications. The user is cautioned to consider the appropriateness of the method in view of the materials being tested, their potential applications, and the recommendations contained in Practice F748.

6. Preparation of Test Specimens

6.1 Specimens should be prepared in accordance with Practice F619. All solid materials shall be extracted. Extractions shall be done with an aqueous (polar) solvent and a nonaqueous (nonpolar or organic) solvent, either DMSO or AOO.

6.2 Liquid test articles and gels shall be used directly if they are not irritants. A liquid that is an irritant shall be diluted with an aqueous or nonaqueous solvent based on solubility of the liquid test article until the solution is non-irritating.

6.3 Wholly aqueous solutions are not suitable for application to the ear. Therefore, for use in the assay, add 0.05 g of hydroxyethyl cellulose[4] to each 10 mL of the aqueous vehicle control and test solutions to aid in holding the solution to the ear. One percent Pluronic L92 may also be used as an aqeuous vehicle.

6.4 The final specimen to be extracted should be prepared with a surface finish consistent with end-use application.

6.5 The specimen shall be sterilized by the method to be used for the final product.

6.6 Care should be taken that the specimens do not become contaminated during preparation and aseptic technique is recommended.

7. Preparation of Positive Controls

7.1 *Nonaqueous Positive Control*—The use of a moderate positive control as a substitute or in addition to a strong positive control should be considered.

7.1.1 *Moderate Positive Control*—Prepare a solution of 25 % hexyl cinnamic aldehyde (HCA) in an acetone:olive oil (4:1 v/v) solvent. Shake the flask until a homogenous solution is obtained.

7.1.2 *Strong Positive Control*—Weigh 0.025 g of DNCB and place in a flask. Add enough DMSO to dissolve all of the DNCB. Add more DMSO to bring the level up to 10 mL. Cap and shake the flask until a homogenous solution is obtained.

7.1.3 The dose level of the positive control should not produce systemic toxicity as evidenced by clinical observations.

7.2 *Aqueous Positive Control*—Neutral buffered formalin is commercially available. (Or dilute formaldehyde 1⁄10 in PBS. Place 1 mL of formaldehyde in a 10-mL flask. Add enough PBS to mix the two solutions. Add more PBS to bring the level up to 10 mL. Cap and shake the flask until a homogeneous solution is obtained.)

7.3 Aqueous solutions are not suitable for application to the ear. Therefore, for use in the assay, add 0.05 g of hydroxyethyl cellulose[4] to each 10 mL of the aqueous positive control to aid in holding the solution to the ear until absorbed. One percent Pluronic L92 may also be used as an aqueous vehicle.

7.4 For all specimens requiring extractions, prepare an aqueous and non-aqueous extract (DMSO or AOO are recommended but other permissible extractants are listed in the ICCVAM documents) following the procedures described in Practice F619.

8. Dosing of the Animals

8.1 Healthy, non-pregnant female CBA/Ca or CBA/j mice that are seven to twelve weeks of age shall be used. House the animals according to treatment group with five animals per cage.

[4] "Final Report on the Safety Assessment of Hydroxyethylcellulose, Hydroxypropylcellulose, Methylcellulose, Hydroxypropyl Methylcellulose, and Cellulose Gum," *J. Amer Coll Tox.*, Vol 5, No. 3, 1986, pp. 1-59.

8.2 *Day One*—Uniquely identify each mouse (ear tags or ear notches may not be used). Weigh each mouse to the nearest whole gram.

8.3 A minimum of five mice shall be used for each positive and negative control and each test sample. They shall be treated daily for three consecutive days by topical application of 25 µL of one of the solutions to the dorsal surface of both ears. For the aqueous groups only, the dorsal surface should be wiped with acetone just before treating to aid in absorption of the aqueous solution, although it will not be completely absorbed.

8.3.1 For testing, other than liquid test articles, the groups shall include: aqueous and nonaqueous positive controls, aqueous and nonaqueous vehicle controls, aqueous extract of the test sample, and nonaqueous extract of test sample.

8.3.2 For testing of liquid test articles, the groups shall include: aqueous and nonaqueous positive controls, the liquid test sample, and either an aqueous or a nonaqueous vehicle control appropriate for the nature of the liquid sample.

8.3.3 The extract shall be used within 24 h of preparation. The extract should be stored in a stoppered container at room temperature. The applications shall be performed at 24 ± 2 h intervals on Days 2 and 3. Table 1 describes the events for each day of the test.

8.3.4 Observe each mouse daily for signs of local irritation at the application site and for signs of systemic toxicity (see Practices F720 and F750). It may be advisable to pretest two mice if it is suspected that the material may be an irritant.

NOTE 1—The following steps through 9.3.3 until precipitation for 18 h take more than 8 h to complete and the laboratory needs to be prepared to accommodate this.

8.4 *Radiolabeled Tracer Preparation*—Prepare tritiated thymidine to a working concentration of 80 µCi/mL (v/v). The use of I^{125} I-UDR at 8 µCi/mL in PBS 10^{-5} *M* fluorodeoxyuridine is also acceptable. Each mouse will receive 250 µL of this. All standard precautions associated with using radioactive materials shall be adhered to. The laboratory shall be licensed to use radioactive material and all personnel shall be appropriately trained and certified.

8.4.1 To prepare the tritiated thymidine solution, add 0.8 mL of 1.0-mCi/mL tritiated thymidine (specific activity 2.0 Ci/mM) to a stoppered flask. Add sterile PBS to make 10 mL. Cap and mix well.

TABLE 1 LLNA Timetable

Day	Activity
<1	Prepare extracts to be <24 h old on Day 1 and plan for suitable extracts for Days 2 and 3
1	Weigh mouse, mark, and treat ears with samples
2	Observe mice and record any toxicity or irritation, treat ears
3	Observe mice and record any toxicity or irritation, treat ears
4	Observe mice and record any toxicity or irritation
5	Observe mice and record any toxicity or irritation
6	(or when appropriate) Prepare radioisotope 72 h ± 3 h after Day 3 application, observe mice for toxicity or irritation, weigh, and inject radioisotope IV 5 h ± 54 min after injection, euthanize mouse, and prepare lymph node cells precipitate the pellet for 18 h
7	Prepare the pellet count the radioactivity do the data analysis

8.4.2 Confirm the concentration of this dilution. Dilute 0.08 to 200 mL with water using a 200-mL flask. Cap and mix well by inverting several times. Remove two 1-mL samples and place in scintillation vials. Add 10 mL of scintillation fluid to each vial, mix so that a vortex is formed, and "count" in a beta scintillation counter. Count each vial three times and calculate the mean. Calculate the concentration. First convert counts per minute (cpm) to disintegrations per minute (dpm) as follows:

$$\frac{\text{cpm}}{\text{decimal counter efficiency}} = \text{dpm}$$

For verification of the working tritiated thymidine solution, determine the closeness of the concentration to 80 µCi/mL. The final diluted solution contains 0.032 µCi. Since 1.0 µCi = 2 220 000 dpm, then 0.032 µCi = 71 040 dpm. Therefore:

$$\text{the µCi of the working solution} = \frac{\text{mean dpm}}{71\,040\ \text{dpm}} \times 80\ \text{µCi/mL}$$

Make adjustments to the solution as needed. Make similar verifications if I^{125} IUDR is used.

8.5 *In-Situ Labeling (Day 6)*—(72 ± 3 h after the last treatment was applied to the ears).

8.5.1 Record the weight of the mouse to the nearest gram. Inject the mouse intravenously with 250-µL sterile PBS containing 20 µCi of tritiated thymidine or 2 µCi I^{125}IUDR via the lateral tail vein using a 1.0-mL syringe and a needle no larger than 25 gage. The tail veins may be dilated for easier intravenous injection by placing the mice under a heat lamp.

9. Lymph Node Collection and Lymph Node Cell Preparation

NOTE 2—All equipment and solutions from this point should be treated as radioactive with appropriate precautions.

NOTE 3—If the investigators are not familiar with the location of the lymph nodes, refer to the diagram in the ICCVAM documents, consult a mouse anatomy book, or use a dye in trial mice to learn to locate the appropriate nodes. One suggested procedure is to inject 0.1 mL of 2 % Evan's blue dye intradermally into the tissue of the ear and then euthanize the mice after 5 to 10 min. Dissect the mice to expose the nodes.

9.1 Euthanize the mice 5 h ± 45 min after injection of the radioisotope.

9.2 Excise the draining (auricular) lymph node of each ear.

9.3 Prepare a single-cell suspension of lymph node cells (LNC) for each mouse.

9.3.1 Pool the nodes from both the left and right side of a single mouse in a labeled test tube containing 1 to 3 mL of PBS. Snip the nodes from a single mouse into small pieces with small scissors or transfer the lymph nodes directly onto a 200-mesh screen/cell dissociation cup. Gently mash the pieces through the screen directly into a 15-mL tube. Wash the mesh with 2 to 6 mL of PBS to facilitate the transfer of cell debris into the tube. Bring the volume in the centrifuge tube up to 10 mL.

9.3.2 Repeat this procedure for each animal.

9.3.3 Centrifuge the samples for 10 min at 190 to 200 xg at 2 to 8°C. Remove each supernatant by aspiration, leaving 1 to 2 mL of supernatant above each pellet. Gently agitate each pellet then bring up to 10 mL with PBS and resuspend by

vortexing. Beginning with the centrifugation step identified immediately above, in this subsection, repeat this washing procedure two more times. After the final wash, remove the supernatant above each pellet and resuspend in 3 mL cold 5 % TCA. Allow to precipitate at 2 to 8°C for 18 ± 1 h.

9.4 *Measurement of Radioactivity (Tritiated Thymidine):*

9.4.1 Centrifuge the precipitated cell suspension at 190 to 200 xg for 10 min at 2 to 8°C. Remove the supernatant above each pellet and add 1-mL of fresh 5 % TCA to the pellet and mix so that a vortex is formed to resuspend.

9.4.2 Transfer the suspension to a labeled scintillation vial containing 10 mL of scintillation fluid, cap, and mix well by shaking vigorously.

9.4.3 Prepare two reagent blanks to measure background tritium levels. Add 10-mL of scintillation fluid and 1-mL 5 % TCA to the scintillation vial and mix well.

9.4.4 Wipe the outside of all vials and place in the scintillation counter. Allow the vials to dark adapt for 30 min. Count each vial three times.

10. Results

10.1 Incorporation of tritiated thymidine is measured by beta scintillation counting as counts per minute for each mouse and expressed as cpm/mouse. I^{125}IUDR is counted in a gamma counter. The cpm are converted to dpm by dividing by the counting efficiency for tritium. Calculate the arithmetic mean of the three counts in dpm per vial for each mouse. Subtract the mean dpm calculated for the reagent blank from the mean dpm calculated for each mouse. Express data as dpm per mouse. See 8.4.2.

$$\text{dpm} - \text{background} = \text{true dpm}$$

10.2 Calculate the arithmetic mean and standard deviation of the dpm for each group of five mice. Calculate the stimulation index (SI) by dividing the mean dpm of the test article by the mean dpm of the negative control.

11. Interpretation of Data and Test Report

11.1 Statistical methods shall be used as an aid in evaluation of test results. The Grubbs test may be used to determine outlying observations that are permissible to delete from the analysis. The Student's *t*-test is suitable for parametric data. The Mann-Whitney Rank Sum Test is suitable for non-parametric data. Groups differing from the negative control at the level of $p < 0.05$ are considered to be significantly different.

11.2 *Test Validity*—The study shall be considered valid if the following criteria are met:

11.2.1 The stimulation index for each positive control must be ≥3 and must be significantly greater than that of the vehicle control.

11.2.2 The average positive control value must be statistically significant compared to the negative control.

11.3 The test report shall include the weight data for the individual mice, the dpm values for the individual mice, the mean and standard deviation for each group, and the stimulation index for each group compared to the vehicle blank. The values statistically significantly different from the vehicle control shall be identified.

11.4 For a test substance to be considered a sensitizer, the stimulation index shall be ≥3. Statistical methods (*t*-test or Mann-Whitney Rank Sum Test) shall be used as an aid in the evaluation of test results.

11.5 Where choices are given in the performance of the test method (for example, extractant used or radioisotope used), the choice selected shall be indicated.

11.6 The weight of each mouse at the beginning and the end of the experiment shall be analyzed and any change in weight shall be noted and included in the analysis of the results especially in evaluating the toxicity of the extract.

12. Precision and Bias

12.1 The precision and bias of this practice is being established by ICCVAM.

13. Keywords

13.1 biocompatibility; contact hypersensitivity; local lymph node assay; LLNA; mouse; sensitization

APPENDIX

(Nonmandatory Information)

X1. RATIONALE

X1.1 Materials, components of materials, processing aids, or residuals from cleaning or sterilization may be capable of stimulating a hypersensitivity response. The use of the murine LLNA test provides objective data that can be subjected to statistical analysis. In addition, the murine LLNA test detects the first (inductive) stage of the sensitization processes whereas guinea pig testing detects the later (elicitation) stage.

X1.2 This practice is presented as a screening procedure for determining the propensity of a material to stimulate local lymph node responses. The test material generally used in this type of biocompatibility testing is an undiluted extract prepared according to specimen size and volume of extractant defined in Practice F619. If the results of this test procedure indicate a stimulation of lymph node proliferation and consequently that

the test material is a contact sensitizer, it is permissible to use this practice for determining dose responses. The results of the dose response studies may be used in risk assessment analysis.

X1.3 This practice is based on the test procedure described in the ICCVAM document. However, only single chemicals have been used in the validation study.

X1.4 The use of this assay for evaluating biocompatibility of medical devices is still undergoing validation. Therefore, whenever additional data are obtained using the guinea pig maximization test, comparison of the results of both test procedures will help in the interpretation of the appropriateness of these test methods.

X1.5 The use of radioisotopes is called for in this practice. The search for alternatives to the use of radioisotopes is encouraged, and when such procedures are validated, this practice may be revised to reflect the new procedures.