



Designation: F 2148 – 07

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

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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. The guinea pig maximization test should be used for these 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 F 748 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 single source. Appropriate materials and reagents may be obtained from many commercial supply houses.

1.7 *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

F 619 Practice for Extraction of Medical Plastics

F 720 Practice for Testing Guinea Pigs for Contact Allergens: Guinea Pig Maximization Test

F 748 Practice for Selecting Generic Biological Test Methods for Materials and Devices

F 750 Practice for Evaluating Material Extracts by Systemic Injection in the Mouse

2.2 *Other Document:*

ICCVAM NIH Publication No: 99-4494 The Murine Local Lymph Node Assay, 1999³

3. Terminology

3.1 *Definitions:*

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

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

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

3.1.4 *DNCB*—2,4-dinitrochlorobenzene.

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

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

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

3.1.8 *PBS*—phosphate buffered saline pH 7.2.

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

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

3.1.11 *TCA*—5 % trichloroacetic acid.

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.

3.1.12 *tritiated thymidine*—^{H3} methyl thymidine, specific activity 2 Ci/mM (in PBS) I¹²⁵ IUDR-radioactive uridine.

3.1.13 *vehicle controls*—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 may 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 F 748.

6. Preparation of Test Specimens

6.1 Specimens should be prepared in accordance with Practice F 619. 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 an irritant. Liquids that are an irritant shall be diluted with an aqueous or nonaqueous solvent based on solubility of the liquid test article until the solution is nonirritating.

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 cellulose⁴ to each 10 mL of the aqueous vehicle control and test solutions to aid in holding the solution to the ear.

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*—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 homogeneous solution is obtained. 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 cellulose⁴ to each 10 mL of the aqueous positive control to aid in holding the solution to the ear until absorbed.

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 document) following the procedures described in Practice F 619.

8. Dosing of the Animals

8.1 Female CBA/Ca or CBA/j mice that are healthy and nonpregnant and seven to twelve weeks of age shall be used. House the animals according to treatment group with five animals per cage.

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 will 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 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, 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

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.