



Standard Test Method for Evaluation of Delayed Contact Hypersensitivity¹

This standard is issued under the fixed designation E 993; 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 approval. A superscript epsilon (ϵ) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method determines whether a test substance will elicit delayed-contact hypersensitivity in guinea pigs. The test method is applicable to individual chemical compounds, simple and complex mixtures, and also finished products.

1.2 *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 Federal Standard:

OPPTS 870.2600 Health Effects Test Guidelines, Skin Sensitization. US Environmental Protection Agency Office of Prevention, Pesticides and Toxic Substances²

3. Terminology

3.1 *skin sensitization*—a delayed immunologically mediated cutaneous reaction to a substance as a result of prior exposure.

3.2 *induction concentration, n*—the amount of a substance, either suspended, dispersed or dissolved in an appropriate matrix, that will induce a threshold-level of irritation.

3.2.1 *Discussion*—In the case of non-irritating materials, the tested material is used without dilution.

3.3 *challenge dose, n*—the highest concentration of test substance that causes no irritation effects.

4. Summary of Test Method

4.1 The test material or a solution of the material is placed on the shaved backs of 20 guinea pigs under occlusive patches. Six hours later, the patches and test material are removed from the animals. This procedure is repeated at the same site once a week for two more weeks for a total of three 6-h exposures. These are called the induction exposures. (See **OPPTS 870.2600**)

¹ This test method is under the jurisdiction of ASTM Committee E35 on Pesticides and is the direct responsibility of Subcommittee E35.26 on Safety to Man. Current edition approved Nov. 1, 2005. Published November 2005. Originally approved in 1984. Last previous edition approved in 2001 as E 993 – 01.

² Available from the Environmental Protection Agency Ariel Rios Building 1200 Pennsylvania Avenue, N.W. Washington, DC 20460 (202) 260-2090

4.2 Two weeks after the last induction exposure, the test material is applied to a freshly clipped skin site on each test animal that has not been exposed previously. The same patching procedure for the induction exposures is used. Ten additional untreated animals are patched in the same manner to serve as controls. This treatment is called the primary challenge.

4.3 Twenty-four hours after the primary challenge application, the animals are depilated, and a minimum of 2 h later, the test sites are graded on a scale of zero to three. This grading is repeated 24 h later and is called a 48-h grade.

4.4 Grades of one or greater in the test group indicate sensitization, provided grades of less than one are noted on control animals.

4.5 Test animals may be rechallenged 7 to 15 days after primary challenge. Test material is applied to freshly clipped, previously unexposed skin sites (same procedure as challenge). Ten previously untreated animals serve as controls.

5. Significance and Use

5.1 This test method can be used to determine whether a test material will elicit skin sensitization or delayed contact hypersensitivity in guinea pigs.^{3,4,5,6}

5.2 The results of this test method are a good indication of the potential for a given test material to produce delayed contact hypersensitivity in human beings under the appropriate conditions.

5.3 This test method can be used as a screening technique before conducting a similar test with human subjects.

6. Reagents and Materials

6.1 *Bandage*, a 20 by 20 mm pad on a 37 by 40 mm adhesive square, or a chamber.

6.2 *Cream or Lotion Hair Remover*.

³ Buehler, E. V., *Arch. Dermat.*, Vol 91, 1965, pp. 171–177.

⁴ Griffith, J. F. and Buehler, E. V., “Prediction of Skin Irritancy and Sensitizing Potential with Animals and Man”, *Cutaneous Toxicity*, Academic Press, NY, 1977, pp. 162–173.

⁵ Ritz, H. L., and Buehler, E. V., “Planning, Conduct, and Interpretation of Guinea Pig Sensitization Patch Tests”, *Cutaneous Toxicity*, Academic Press, NY, 1980, pp. 25–40.

⁶ “Delayed-type Contact Sensitization”, *Principles for Evaluation of the Toxicity of Household Substances*, National Academy of Sciences, Washington, DC, 1977, pp. 36–44.

6.3 *Rubber Dam*, elastoplast, or other suitable materials.

6.4 *Small Animal Clipper*.

7. Test Animals

7.1 Young, adult Dunkin Hartley outbred guinea pigs are preferred. Usually 20 test animals, 10 control animals, and 4 to 8 animals for primary irritation are employed for each test substance.

7.2 Equal numbers of males and females in both test and control groups should be used.

8. Pretest Conditioning

8.1 Examine the animals on arrival for overt signs of disease, and condition them to the environment for a minimum of four days. Select animals that have not been used on any other tests.

8.2 Maintain the animals during pretest and test periods according to accepted laboratory practices for the care and handling of animals.^{5,6} Maintain room temperature at 65-75°F with a relative humidity between 30-70% and a 12-h dark / light cycle. It is essential that the guinea pigs receive an adequate amount of ascorbic acid.

8.3 House animals individually and identify each guinea pig with a cage card, listing animal and project number.

8.4 During acclimation, observe the animals for respiratory distress, diarrhea, emaciation, ocular and nasal discharges, skin lesions, and eye defects. Eliminate any animal demonstrating signs of spontaneous disease prior to the start of the study. Use only animals judged to be healthy. Animals on test should be segregated in different rooms both during and after acclimation. Guinea pig chow or the equivalent and water are to be available *ad libitum*.

9. Sample Preparation

9.1 The test substance should be applied in such a manner that a uniform contact with the skin is provided throughout the test. The application can be with undiluted test substance, with a solution, or with a uniform suspension. A slightly irritating dose is recommended for induction exposures. The concentration of test substance for primary challenge should be the highest nonirritating dose as determined by the primary irritation evaluation.

9.2 The criteria for the selection of the appropriate solvent should include consideration of the following:

- 9.2.1 Solubility characteristics of the test substance,
- 9.2.2 Compatibility of the test substance with the solvent,
- 9.2.3 Stability of the test substance in the solvent, and
- 9.2.4 Toxicity of the solvent.

9.3 The preferred solvents include the following:

9.3.1 *Polar Solvents*—water, aqueous ethanol (used for either induction or challenge, but not both), acetone, methanol.

9.3.2 *Nonpolar Solvents*—mineral oil, decane.

9.4 For solids or powders, apply in a manner consistent with the materials used and the anticipated human exposures. Use a sufficient amount of material to provide a uniform contact with the skin.

10. Patch Site Selection

10.1 Select the placement of patches with the test substance on the backs of the guinea pigs in accordance with the anticipated test program. A suggested arrangement of patch sites is presented in Fig. 1 for induction and challenge. See Fig. 2 for primary irritation.

11. Primary Irritation Evaluation

11.1 The day before exposure, clip the entire back and both sides of four previously unexposed animals.

11.2 For induction concentration screens, apply up to four patches containing different concentrations of the test substance in the same vehicle to each of the four animals in the following manner:

11.2.1 Apply 0.4 mL of the freshly prepared test substance or solution to a bandage, or 0.3 mL to chamber. Use the format for primary irritation studies given in Fig. 2 for the placement of the patches.

11.2.2 Alternate the different concentrations of the test substance on the various test sites among the animals to minimize the site-to-site variation in responsiveness.

11.3 Place the animals in a restrainer and occlude the patch with occlusive wrap pulled taut around the trunk of the animal and clipped to the restrainer.

11.4 Six hours later, take the animals from the restrainer, remove the occlusive wrap and the patches. Remove the residual substance by a gentle rinse with warm water (35 to 42°C), dry, and return the animals to their cages.

11.5 Grade the responses at 22 to 26 h and at 46 to 50 h; grade the test sites on a scale of 0 to 3 as follows:

- 0 = no reaction
- ± = slightly patchy erythema
- 1 = slight, but confluent erythema or moderate, patchy erythema
- 2 = moderate erythema
- 3 = severe erythema with or without edema

11.6 The highest nonirritating concentration is the concentration of test substance that induces responses not exceeding two ± grades in the group of four animals. The slightly irritating dose for induction is the concentration that induces responses not exceeding two grades of one.

11.7 The primary irritation screen for challenge concentration determination should use Format #2, Fig. 2 (that is no more than 2 patches per animal; 4 concentrations require 8 animals).

12. Induction of Sensitization

12.1 Clip the left shoulder area of each animal in the treatment group with a small animal clipper the day before exposure.

12.2 Apply a closed patch containing 0.4 mL of the freshly prepared test substance or solution. The patch should be applied to the clipped surface of each animal as quickly as possible after the test substance has been placed on the patch.

12.3 Place the animal in a restrainer and occlude the patch with occlusive wrap pulled taut around the trunk of the animal and clipped to the restrainer.

12.4 Six hours later, take the animal from the restrainer, remove the occlusive wrap and the patches. Remove remaining

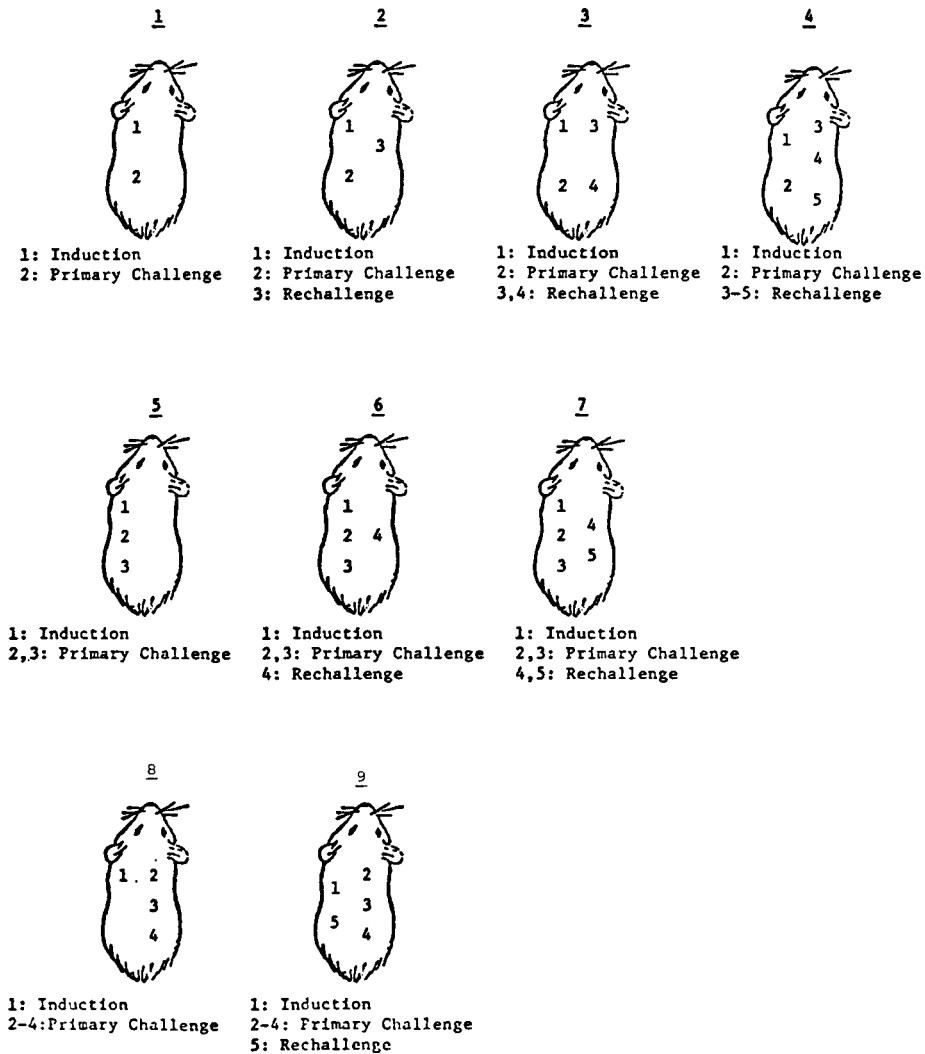


FIG. 1 Format for Sensitization Studies

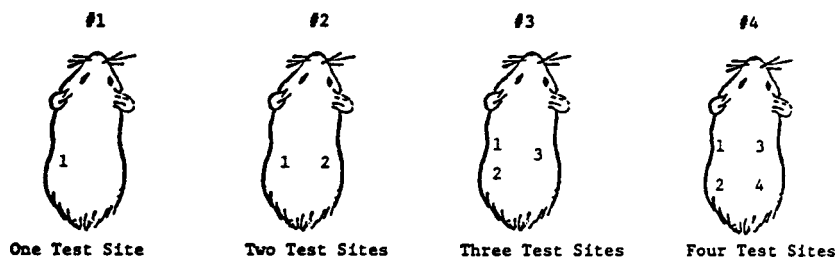


FIG. 2 Format for Primary Irritation Studies

substance by a gentle rinse with warm water (35 to 42°C). Dry the animals, and return them to their cages.

12.5 Repeat the application procedure at the same site once a week for the next two weeks for a total of three 6-h exposures (the interval between induction exposures may vary from five to nine days).

12.6 After the last induction exposure, leave the animals untreated for approximately two weeks (12 to 16 days) before conducting the primary challenge.

13. Primary Challenge

13.1 The day before exposure, clip the left posterior quadrant of the side and back of each animal in the treatment group and the previously untreated control animals.

13.2 Apply patch(es) having 0.4 mL of the test substance or solution at the maximum nonirritating concentration or 0.4 g of a solid to a freshly clipped skin site that has not been exposed previously.

13.3 Place the animal in a restrainer and occlude the patch with occlusive wrap pulled taut around the trunk of the animal and clipped to the restrainer.

13.4 Six hours later, take the animal from the restrainer, remove the occlusive wrap and the patches. Remove remaining substance by a gentle rinse with warm water (35 to 42°C). Dry the animals, and return them to their cages.

13.5 Twenty-four to thirty hours after initiation of the primary challenge, depilate all animals with cream or lotion hair remover. Place the depilatory on the test sites and surrounding areas and leave on for *no more than 30 min*.

13.6 Thoroughly wash off the depilatory with a stream of warm, running water. Dry the animals with a towel, and return them to their cages.

14. Grading Patch Sites

14.1 A minimum of 2 h after depilation, grade the test sites on a scale of zero to three (see 11.5). Record the grade for each animal.

14.2 Repeat the grading of all patch sites 24 h later. (These are called the “48-h grades”).

15. Rechallenge

15.1 Seven to 15 days after challenge consider a rechallenge of all test animals to confirm any reactions, to examine components of the test mixture, or to look at dose response characteristics.

15.2 The concentration of all substances for the rechallenge must be at their maximum nonirritating concentration. If this concentration is not known for one or more test materials, conduct a primary irritation test. If more than one material is to be used for rechallenge, confirm irritation by patch testing materials together.

15.3 Clip an area on the back of each animal that is appropriate for the number of samples to be involved in the rechallenge (see Fig. 1 for possible patch arrangements) the day before exposure.

15.4 Apply the appropriate patches to the treatment animals that are considered to be sensitized and to another group of previously untreated control animals. Rotate patch sights (see 11.2.2).

15.5 Continue the test as described in Sections 13 and 14. With regard to depilation, grade skin site at 24 and 44 h without depilation (that is through the fur). Sites are then depilated and scored again at 48 h. This procedure is designed to minimize sensitization due to the depilatory agent which contains a weak sensitizer (sodium calcium thioglycollate).

16. Method Validation

16.1 Periodically, validate the test method by using a positive sensitizer.

16.2 Dissolve dinitrochlorobenzene (DNCB) in 80% ethanol at a concentration of 0.1% for the induction doses and 0.02 % for the challenge dose. Conduct the test in the same manner as the standard test with the number of animals, dosing methodology and evaluation of the skin the same. Positive control studies do not need to be run with each group of test compounds, but should be preformed at least once a year to validate the test methodology and responsiveness of the test species. In the event that no sensitization studies have been run within the past twelve months, a positive control group must be run concurrently with the first set of test compounds.

17. Interpretation of Results

17.1 If skin grades of one or higher are obtained on two or more of the test animals, provided skin grades of less than one are seen on control animals, the test substance is considered to have induced skin sensitization.

17.2 If several of the control animals display skin grades of one or greater, a rechallenge test or retest should be considered using a lower concentration of test material.

18. Report

18.1 Name of investigator(s), laboratory address, and allocation of the raw data.

18.2 Description of the test facilities and housing conditions, including cages, humidity and temperature.

18.3 Detailed description of the test substances including the chemical name, Chemical Abstract Services (CAS) number, synonyms, structure, purity, source batch, lot number, physical/chemical properties.

18.4 The report should include how the study was conducted, the dates the study was initiated and terminated, and the results of both the primary challenge and the rechallenge in terms of incidence and severity of responses.

18.4.1 *Incidence*—The number of animals in each group showing skin grades of one or greater at either 24 or 48 h divided by the total number of animals tested in that group (for example, 10/20). Results are recorded separately for each time period as well as the total for all time periods.

18.4.2 *Severity*—The sum of the test grades divided by the total number of animals tested in a given group determined for both 24 and 48 h (for example 0.8 to 0.7). Grades of \pm are equal to 0.5 for calculation of severity indices. All average grades are to be rounded off to the nearest tenth of a unit.

19. Keywords

19.1 delayed contact hypersensitivity; erythema; guinea pigs; hypersensitivity; induction; patches; primary challenge; sensitization; skin

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