



Designation: E 951 – 94 (Reapproved 2005)

Standard Test Methods for Laboratory Testing of Non-Commercial Mosquito Repellent Formulations On the Skin¹

This standard is issued under the fixed designation E 951; 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 These test methods apply to repellent compounds and formulations that can be appropriately diluted with ethanol, acetone, or a similar inert carrier for test purposes. The test methods described are not suitable for testing powders, sticks or other solid formulations, or for testing thixotropic or other fluids whose physical properties would be modified by dilution.

1.2 These test methods are designed and intended for use as a research standard to develop data on the efficacy of repellents applied to the skin of humans against laboratory-reared or field-collected mosquitoes. The use of these test methods will provide for the development of a data base whereby all investigators generate comparable data. Modifications of the equipment or procedures, or both, may be needed for tests against other kinds of biting arthropods.

1.3 The test methods are intended for use in testing materials that are in an advanced stage of development, for which human-use trials can be fully justified on scientific and ethical grounds. The test methods are not designed for the testing of commercial formulations where registration or advertising claims data are required.

1.3.1 A repellent should not be considered for testing on humans before its efficacy has been demonstrated in *in vitro*, animal, or other nonhuman test systems.

1.3.2 A repellent should not be applied to the skin before its safety has been established in appropriate toxicological tests on animals or other test organisms.

1.3.3 No repellent should be tested on humans without the written consent of the test subjects and prior approval of competent authority, as designated in the applicable laws and regulations governing experimentation on humans.

1.4 The values stated in inch-pound units are to be regarded as the standard. The values given in parentheses are for information only.

1.5 *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 Document

2.1 *ASTM Standards:*²

E 939 Test Method of Field Testing Topical Applications of Compounds as Repellents for Medically Important and Pest Arthropods (Including Insects, Ticks, and Mites): I Mosquitoes

2.2 *Other Documents:*

Directions for Abstractors and Section Editors of Chemical Abstracts³

Consolidated List of Approved Common Names of Insecticides and Other Pesticides⁴

Common Names of Insects and Related Organisms⁴

3. Apparatus

3.1 *Test Cage*—The following design and materials have been found suitable for construction of the mosquito cage (see Fig. 1):

3.1.1 The cage is rectangular in shape, length, width, and height is approximately 7.2 by 2 by 1.6 in. (18 by 5 by 4 cm). The top of the cage (5 by 18 cm) is made of metal or plastic mosquito screening, and the sides, ends, and floor are made of 1/8 in. (3.2 mm) clear acrylic plastic.

3.1.2 Five 1 1/8 in. (29 mm) circular openings are drilled in line on 1 3/8 in. (35 mm) centers in the floor of the cage.

3.1.3 The two sides and one of the ends of the cage are grooved and slotted to receive a flexible rectangular slide made of 0.012 in. (0.3 mm) cellulose acetate sheeting. The slide should move freely over the floor of the cage to open and close the five openings.

3.1.4 One end of the cage is fitted with a No. 3 stopper in a 1/2 in. (13 mm) hole for insertion of the test mosquitoes.

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 the American Chemical Society, 1155 16th St., N.W., Washington, D.C. 20036.

⁴ Available from the Entomological Society of America, College Park, MD 20740.

¹ These test methods are under the jurisdiction of ASTM Committee E35 on Pesticides and Alternative Control Agents and are the direct responsibility of Subcommittee 35.12 on Insect Control Agents.

Current edition approved Nov. 1, 2005. Published November 2005. Originally approved in 1983. Last previous edition approved in 2000 as E 951 – 94 (2000).

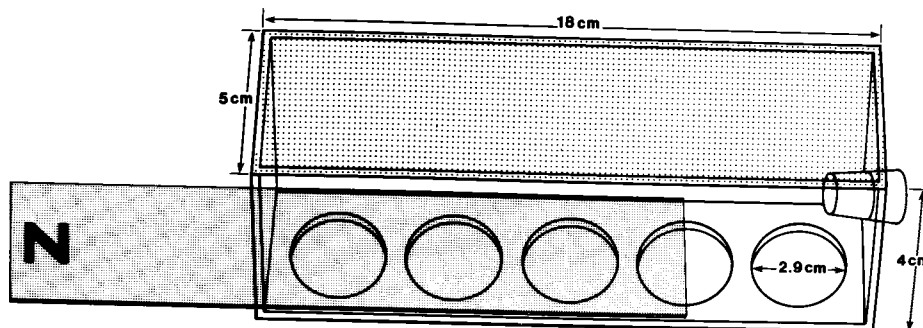


FIG. 1 Test Cage

3.2 *Harness*—Two belts are used to secure the test cage to the forearm during the test. These should be approximately 1 in. (2.5 cm) wide by 12 in. (30 cm) long. If an elastic material is used for the belts, snap or friction type fasteners should be provided for joining the ends. If an inelastic material is used, slides or buckles should be provided.

3.3 *Template*—A template made of 1/8 in. (3.2 mm) acrylic plastic to match exactly the floor of the test cage is used as a guide to outline the five circular treatment areas on the forearm.

4. Reagents and Materials

4.1 The diluent used in the test will ordinarily be ethanol. However, for some repellents (for example, water-based or lanolin-based formulations) dilution in ethanol may be inappropriate. In such cases, the appropriate diluent will be used instead.

5. Sampling

5.1 Take a bulk sample and a laboratory sample as directed in any applicable material specifications. In the absence of such specifications, take a laboratory sample believed to be representative of the lot to be tested. In the case of a suspension, emulsion or similar formulation, thoroughly mix the material to be sampled before the sample is taken.

6. Test Specimen and Sample

6.1 Take test specimens by pipet as required (see 10.4 and 16.1.1). Take a new test specimen for each trial and replicate needed in the test. The number of trials and replicates needed depends on the variability of the results obtained and the degree of precision required. See 13.1 and 13.2.

6.2 In the case of a suspension, emulsion, or similar formulation, the sample must be thoroughly mixed before the test specimen is taken.

7. Conditioning

7.1 The mosquitoes used in the test should be conspecific females maintained on 10 % sucrose solution prior to testing. Laboratory-reared mosquitoes should be mated nullipars in the age-range of 5 to 15 days. Field-collected mosquitoes should be held in the laboratory for 48 h or longer prior to testing to allow for transport mortality and accommodation to laboratory conditions. During the period the mosquitoes should be provided with an appropriate substratum for oviposition.

NOTE 1—The age-range specified for the test mosquitoes is arbitrary and is intended only as an aid in standardization of the test among users. Users have reason to adopt a different age-range for testing should report the age-range actually used (12.3).

7.2 Where adequate facilities are available, rearing and testing conditions should be controlled and standardized at levels appropriate for the species under test.⁵ In areas or localities where suitable laboratory facilities are not available, rearing and testing should be carried out, if possible, in a building free of extremes of temperature, humidity, illumination, and wind. Where possible, all comparative tests should be made under similar conditions.

7.3 In the case of the four-hour ED50 test (14.1-19.1), the forearm should not be washed, rubbed, scratched, or otherwise treated in such a way as to nullify the repellent treatments during the four-hour test period. However, the test participants should be normally active during the test period to ensure that the factors of perspiration and abrasion are incorporated in the test.

7.4 Nothing in this section should be construed to mean that special test conditions may not be adopted for tests having a special purpose. For example, the wear resistance of the test repellent can be measured by abrading the treated forearm in a controlled manner before the test mosquitoes are applied to bioassay. In such special-purpose tests, the applicable parts of the standard should be followed as closely as possible.

7.5 Since test conditions may vary to some extent under this test method, it is essential that full information on all variables relating to the test repellent, test mosquitoes, procedures, and test conditions be made part of the final report (12.1-12.3).

TEST METHOD E50 (A)

8. Summary of Test Method

8.1 Five circular test areas are outlined on the flexor region of the forearm and treated with the diluent (as the control) and four serial dilutions of the test repellent (the repellent treatments). A cage having matching cutouts in its floor and containing ten mosquitoes is then applied to the forearm, and the numbers of mosquitoes feeding on the control and the repellent treatments is recorded. In subsequent trials, the range of dosages applied to the forearm is adjusted to bracket the

⁵ Gerberg, E. J., *Manual for Mosquito Rearing and Experimental Techniques*, Amer. Mosquito Control Assoc., Fresno, CA, 1970, 109 pp.

median effective dosage (ED₅₀) of the test repellent. The test is replicated at that range of dosages until a valid estimate of the ED₅₀ can be obtained. The data are analyzed by established methods of biological assay.

9. Significance and Use

9.1 The ED₅₀ test provides an estimate of the amount of repellent that must be applied to the skin to produce a given level of effectiveness against the mosquito test population. The levels of effectiveness that are usually of interest are the 50 % level (for comparative purposes) and the 95 % level (for practical purposes). The ED₅₀ test method is used to determine the effectiveness of a repellent against different kinds of mosquitoes or to compare the effectiveness of different repellents against any particular kind of mosquito. It may also be used to establish the dosages needed to provide protection under special conditions of climate, weather, activity, etc.

10. Procedure

10.1 Load the test cage with 10 to 20 female mosquitoes from the population to be tested.

NOTE 2—This number of mosquitoes is intended as a limitation on the number of bites received by the test subject. In tests against species having characteristically low laboratory feeding rates, larger numbers of mosquitoes should be used.

10.2 Using the plastic template as a guide, outline five circular test areas on the flexor region of the test subject's forearm with a fine-tipped felt pen. Label the test areas "A" through "E", beginning with the test area nearest the elbow.

10.3 Set up four test tubes in a test tube rack and label them "1" through "4", from left to right.

10.4 Make up approximately 1 mL of 0.41 % of the test repellent in Tube 1 (Notes 3 and 4). Mix the preparation with a vortical mixer.

NOTE 3—The diluent used will ordinarily be ethanol, but in some cases it may be appropriate to use a different material. See 4.1.

NOTE 4—This strength (0.41 %) is calculated to provide a dosage of 0.016 mg of repellent per cm² of skin surface when 0.025 mL of the solution is spread over a 1 1/8 in.-(29 mm) diameter circular test area. The calculation assumes that the specific gravity of the test repellent is equal to one. If the actual specific gravity of the test repellent is known, a correction to the nominal dosage of 0.016 mg/cm² can be calculated.

10.5 Put 0.5 mL of diluent into each of tubes, 2, 3, and 4.

10.6 Transfer 0.5 mL of material from tube 1 to tube 2. Mix with the vortical mixer.

10.7 Transfer 0.5 mL of material from tube 2 to tube 3. Mix with the vortical mixer.

10.8 Transfer 0.5 mL of material from tube 3 to tube 4. Mix with the vortical mixer.

NOTE 5—Steps described in 10.5-10.8 are a serial dilution procedure. The solutions contained in Tubes 1 to 4 will provide repellent dosages of 0.016, 0.008, 0.004, and 0.002 mg/cm² when 0.025 mL of solution are applied to the test areas on the forearm. See Note 4.

10.9 Assign the control and the four repellent treatments (tubes 1 to 4) to the five test areas (A to E) at random.

NOTE 6—A new randomization is required for each trial and replicate of the test. Randomization can be accomplished by computer or with a die or table of random numbers. It is convenient to make enough randomizations

at one time for use in a number of trials.

10.10 Apply 0.025 mL of diluent with a micropipet to the test area designated as the control. Spread evenly with the tip of a glass rod.

10.11 Apply 0.025 mL of the material in Tube 4 to the test area designated for that treatment. Spread evenly.

10.12 Apply 0.025 mL of the material in Tube 3 to the test area designated for that treatment. Spread evenly.

10.13 Apply 0.025 mL of the material in Tube 2 to the test area designated for that treatment. Spread evenly.

10.14 Apply 0.025 mL of the material in Tube 1 to the test area designated for that treatment. Spread evenly.

10.15 After 4 min, fit the five cutouts in the floor of the test cage to the five test areas outlined on the test subject's forearm, and secure the test cage to the forearm with the two belts provided.

10.16 After one additional minute, withdraw the slide from the test cage to expose the test areas on the forearm to the mosquitoes.

10.17 At 1.5 min after the slide is withdrawn, record the numbers of mosquitoes feeding on each of the five test areas.

10.18 In subsequent trials, adjust the range of dosages to bracket the ED₅₀ of the test repellent by successively doubling or halving the concentration of repellent made up in Tube 1 (10.4).

NOTE 7—The general equivalents calculated in accord once with Note 4 are as follows: (1) A concentration of 1.0 % exactly repellent in Tube 1 provides a dosage of 0.03898 mg/cm² on the forearm. (2) A concentration of 25.65 % repellent in Tube 1 provides a dosage of 1.000 mg/cm² on the forearm. These figures may be used as conversion factors where needed. However, modification of the equipment or procedures described herein may necessitate recalculation of the conversion factors.

10.19 When the appropriate range of dosages has been determined (10.18), replicate the test as necessary to obtain an acceptably precise estimate of the ED₅₀ of the test repellent. If a low dilution of repellent is being applied, it may tend to spread beyond the boundary of the test area. This can often be prevented by applying undiluted repellents at the desired rate with an adjustable pipet. However, the amount of a repellent that can be applied to a given area of skin will ultimately be limited by runoff. The dose at which runoff occurs is a function of the viscosity of the repellent. The number of trials and replicates needed depends on the variability of the results obtained and the degree of precision required. See 13.1 and 13.2.

NOTE 8—It is best to limit the test participants to not more than one or two trials per day on each forearm. If the same subject is used in subsequent trials to bracket the median effective dose, care must be taken to cleanse completely the skin where the repellent had previously been applied. Otherwise, residues from the initial repellent application might compound the effects and results. Since there is reason to believe that the test repellent performs unequally on different test subjects, the replicates of the test should be divided equally among two or more subjects.

11. Calculation

11.1 Obtain the totals, over all replicates, of the feeding counts made on the control and each of the four repellent treatments. Convert the totals for the repellent treatments to percentages of the total for the control, and subtract each from