Designation: E 939 – 94 (Reapproved 2000)

Standard Test Method of Field Testing Topical Applications of Compounds as Repellents for Medically Important and Pest Arthropods (Including Insects, Ticks, and Mites):I Mosquitoes[1]

This standard is issued under the fixed designation E 939; 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 test method is used to evaluate the repellency of promising compounds that have undergone primary laboratory studies and have been approved for skin application for secondary testing.

1.2 This test method is designed for the study of mosquito repellents, but with some modifications this test method can be used to determine the repellency of candidate compounds for other flying insects that attack humans.

1.3 *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. Terminology

2.1 *Description of Term Specific to This Standard:*

2.1.1 *complete protection time (CPT)*—the time from application of the repellent to the time of the first confirmed bite (a second bite by the same species within 30 min of the first). This permits any number of unconfirmed bites during the CPT.

3. Summary of Test Method

3.1 A measured amount of the candidate material is applied to the forearm or sometimes the lower leg. These areas are then protected from rubbing and are continuously exposed to mosquitoes in the field to determine the length of time the treatment provides either complete protection or a high level of protection.

4. Significance and Use

4.1 This test method is an important part of the final phase of study in the development of mosquito repellents for personal use.

4.2 This test method is primarily designed to simulate a situation in which a person treated with a repellent is exposed to natural populations of attacking mosquitoes.

4.3 The simplicity of the test offers flexibility under a relatively wide range of circumstances and geographical locations. By following this test method, international testing with a variety of vector mosquito populations is no more difficult to accomplish than tests with various domestic species.

4.4 A number of people test topical applications of a repellent for the following reasons:

4.4.1 To determine how long the repellent is effective;

4.4.2 To establish the effective dosage range;

4.4.3 To establish the range of effectiveness on several mosquito genera and species in a number of geographical areas; and

4.4.4 To identify the material in terms of odor, staining capability, plasticizing effect, and oiliness or greasiness.

4.5 No repellent should be tested on humans without the written consent of the test volunteers (hereafter referred to as test subjects) and prior approval of competent authority, as designated in applicable laws and regulations governing experimentation on humans.

5. Apparatus

5.1 *Insect collection vials*.

5.2 *Aspirator*.

5.3 *Stereoscope* (optional).

5.4 *Standard References for Mosquito Identification*, for determining species present in the field (optional).

5.5 *Temperature and Humidity Reading Equipment*—Ideally, a continuous recording device such as a hygrothermograph should be used to record conditions during tests. If such equipment is not available, readings should be made immediately before, midway during, and immediately after the tests are made, using a sling psychrometer.

5.6 *Air Speed Indicator and Light Meter*, optional but preferred if equipment is available.

5.7 *Watch*.

5.8 *Headnets*.

5.9 *Cotton Gloves*.

5.10 *Battery-Operated Head Lamps*, with red filters for tests with nocturnally active species.

5.11 *Notebook, Test Sheets, and Pencils*, for recording species, test data, date, and locality of the test. A sample work sheet is attached with recorded results (see Annex A1.).

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[1] This test method is under the jurisdiction of ASTM Committee E35 on Pesticides and is the direct responsibility of Subcommittee E35.12 on Insect Control Agents.

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5.12 *Water Supply, Mild Soap, and Paper Towels*, for washing treated skin.

5.13 *Clothing*, should be appropriate to the season and geographical area.

NOTE 1—There are differences of opinion as to whether the clothing worn should be uniform in color and type, however, data available do not reject or confirm this contention.

5.14 *List of Chemical Names*, identifying the compounds or mixtures, or both, to be tested.

NOTE 2—In the event of a medical emergency, the chemical list along with pertinent toxicological data may be required.

6. Reagents and Materials

6.1 All test solutions are formulated on a weight to volume basis (usually with 95% ethanol).

6.2 Test chemicals in 25% ethanol solutions.

6.3 Test standard deet (*N,N*-diethyl-meta-toluamide) in a 25% ethanol solution.

6.4 For final development studies, formulations of lotion, liquids, creams, solids, or pressurized aerosols containing ingredients for field evaluation are included.

7. Sampling

7.1 Each candidate repellent is paired with each other repellent or a standard on the arms of a subject and exposed simultaneously to the same insect population (See Annex A1).

7.2 A round-robin or paired test is used in the experiments, usually based on the number of experimental materials being evaluated.

7.3 Treatments are exposed to the mosquito population for as long as the repellents are effective, and the biting activity continues.

8. Procedure

8.1 Determine the identity of species of mosquito in the test area prior to the test. Determine the time to begin and end tests each day by the activity of the species to be tested. Diurnal nocturnal or other patterns of mosquito behavior will govern the scheduling of tests.

8.2 For field tests, make the initial studies with a 25% ethanol solution of the candidate repellents (250 mg AI/mL). A compound or formulation may be retested at reduced or increased concentrations, or at full strength, if warranted.

NOTE 3—If the complete protection times (CPTs) of repellents are too long for the available testing period or if the CPTs are too short for evaluation, adjust the concentrations accordingly. The termination of an evaluation before a confirmed bite occurs is termed a "plus out" and should be avoided.

8.3 Spread 1 mL of the repellent formulation or repellent solution evenly over the forearm of the subject and compare directly with another repellent of the same concentration on the other arm (see Note 4). The behavior of some species necessitates the use of the legs instead of the arms as treatment sites. This can be determined by observation before the tests begin.

NOTE 4—The concentration of a compound being tested is not as important as the assurance that it is paired with another compound of equal concentration.

8.4 If legs are used as treatment sites, apply 1.5 mL on the skin between the ankle and knee.

8.5 Determine the surface area of the limbs of each test subject so that treatment rates of candidate repellents and the standard are uniformly applied. Adjust the application rates for differences in arm or leg size of different subjects.

8.6 Expose the treated arms or legs continuously to natural populations of the mosquito species being tested.

8.7 The duration of effectiveness of the repellent is indicated by the CPT.

8.8 Employ a balanced incomplete block (BIB) experimental design (round robin) when three to five chemicals are to be tested. With this design, test each repellent in the series on opposite arms of a given number of subjects.

NOTE 5—Ideally, the number of subjects should equal the number of candidate compounds, excluding the standard. Thus, if four compounds are to be tested, including the standard, three test subjects would be required. To illustrate, the pairings would be: AB, AC, AD, BC, BD, and CD. Subject 1 would test AB and CD; Subject 2, AC and BD, and Subject 3, AD and BC.

8.9 Because of their variability in attractiveness, assign repellents to test subjects in a randomized and balanced fashion so that each subject does not wear the same combination of materials more than once in a single BIB test series. For example, if compounds A, B, C, D, and E are tested with a standard F, then Subject 1 will test AB, CD, and EF; Subject 2 will test AC, BE, and DF; Subject 3 will test AD, BF, and CE; Subject 4 will test AE, BC, and CF; and Subject 5 will test AF, BC, and DE.

NOTE 6—If four or five compounds, excluding the standard, are tested in a BIB series, one replication of the BIB is sufficient for a statistical analysis. If three compounds are to be tested in this fashion, two replications of the round robin will be necessary.

8.10 A direct comparison of the candidate versus the standard repellent deet is used when fewer than three chemicals are to be tested. For these tests, four or more replications of tests with each chemical on at least three different subjects are necessary.

8.11 Express the results obtained by paired tests or BIB comparisons at CPT in minutes or hours.

8.12 During the experiment, subjects shall avoid contact with the treated skin surfaces. Touching, rubbing, or abrasive action on the treated skin can affect the results. Avoid *undue* sweating or wetting of the treated skin except in special tests designed to study the durability of repellents under adverse conditions. Loss due to evaporation and absorption is, of course, unavoidable but should be the only known reason for loss in these tests, if the above precautions are taken.

8.13 Test each species with its biting behavior in mind. For example, if a test subject remains immobile, some diurnally active mosquito species become less aggressive and biting pressure on the repellent treated skin will be reduced.

8.14 Intermittent walking, standing, and squatting, as well as raising and lowering the arms periodically, are very effective in attracting many of the daytime biting species. Test supervisors and subjects need to be aware of these behavioral differences so they will find the most effective means of testing the repellents against each of the various mosquito species

studied. To be successful, tests should be designed to *accommodate the test species* rather than the investigator.

8.15 Protect exposed parts of the body, such as the hands and face, by gloves and headnet in order for biting pressure to be concentrated on the treated skin and for the comfort of the subjects.

8.16 One responsible person records data and provides leadership to ensure the accuracy and uniformity of the experiments.

8.17 Use the number of bites per unit of time to indicate biting pressure. Determine biting pressure by exposing an untreated arm (or leg) and counting the number of each species biting in 1 min.

8.18 Make biting counts before, periodically during, and after each day of testing. Designated control subjects may be used to take biting rate counts. Participants in tests may make counts between tests when test repellents have been removed from the skin after failure to repel.

8.19 Record whether the test chemical or repellent formulation has any of the objectionable characteristics mentioned in 4.4.

NOTE 7—Identification of the species biting after dark is very difficult; therefore, in addition to a head lamp, data sheet, and collection vials, each test subject has a folding seat and battery-powered aspirator. Seats are positioned at approximately 10 m apart with the test subjects backs to the prevailing wind.

8.20 To determine biting rate and species present, each treated subject sits at his or her respective location and, at onset of mosquito activity at dusk and at 1 h intervals thereafter, aspirates mosquitoes biting the untreated lower legs from knee to ankle for 1 min.

8.21 Tests begin with the individuals instructed to use the vials to collect each mosquito biting their repellent-treated forearms. Vials containing mosquitoes are placed sequentially in a compartmented flat so that later they can be matched with a record of the treatment and time of bite kept by each test subject.

8.22 The procedure makes possible the determination of species biting, total numbers of bites during the exposure period, percent protection provided by a repellent as compared with no treatment, and duration of protection against particular species.

$$[(\text{bites on untreated leg} - \text{bites on treated arm}) \div \text{bites on untreated leg} \times 100]$$

9. Calculation

9.1 For the balanced incomplete-block experiments, calculate an analysis of variance using methods described in *The Design and Analysis of Experiments*.[2]

9.2 From this analysis of variance, determine the least significant difference (5% level) between any two repellents.

9.3 Compute an adjusted average protection time that compensates for variation between hosts and testing conditions.

NOTE 8—The following equation may be used to compute the adjusted average protection.[3]

$$\text{Average } T_i = \frac{2\ T_i - B_i}{rn} + M$$

where:

T_i = the total CPTs for all tests with repellent i,
B_i = the total CPT for both repellents in all pairs in which the repellent i occurred,
r = the number of times each pair was replicated,
n = the number of repellents, and
M = the grand mean of all CPTs in the series.

Due to the nature of the statistical analysis, the adjusted mean may occasionally fall outside the CPT range and at times be a negative value. Such data occur only when the candidate repellent has a very low CPT and thus is ineffective.

9.4 In the direct comparison tests between a candidate repellent and a standard, use a paired *t*-test to determine if there are significant differences (5% level) between the standard and the candidate.

9.5 Express the relative duration of effectiveness of the experimental repellent to that of the standard as the ratio of the CPT.

9.6 The ratio is a more reliable index than the actual CPT which may vary considerably between hosts and different populations of mosquitoes.

10. Precision and Bias

10.1 No precision data is available for this test method, however, the committee is interested in conducting an interlaboratory test program and encourages interested parties to contact the Committee E35 staff manager at ASTM Headquarters.

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[2] Kempthorn, O., *The Design and Analysis of Experiments*, John Wiley and Sons, Inc., New York, NY, 1952, Section 26.4, p. 532.

[3] Gilbert, I. H., Gouck, H. K., and Smith, C. N., "New Mosquito Repellents," *Journal of Economic Entomology*, Vol 48, No. 6, December 1955, pp. 741–743.

ANNEX

(Mandatory Information)

A1. SAMPLE WORK SHEET

A1.1 The sample work sheet contains simulated field test data to show how information is recorded and the CPT is calculated. See Fig. A1.1. A separate work sheet is needed for each species of mosquito tested against. Compound C is ineffective against *Aedes taeniorhynchus*, but it may not be true for another mosquito species, therefore, Compound C should be retained for further study.

Skin Repellent Test No. 1

Field Species: *Aedes taeniorhynchus*

Location: *New Smyrna Beach, FL*
Date: 9-20-79

Chemical percent concentration[A]		Time							Protection time, min
		Subject	Arm, L/R	Treatment	First bite	Second bite			
(1) Chemical A	25 %	JD	L	8:00 AM	10:00 AM	10:15 AM			120
Deet standard	25 %		R	8:05 AM	1:05 PM	1:08 PM			300
(2) Chemical B	25 %	CS	L	8:10 AM	9:10 AM	—	12:10 PM	12:20 PM	240
Deet standard	25 %		R	8:15 AM	3:15 PM	3:30 PM			420
(3) Chemical C	25 %	KP	L	8:20 AM	8:30 AM	8:50 AM			10
Deet standard	25 %		R	8:25 AM	4:25 PM	4:54 PM			480
(4) Chemical A	25 %	KP	L	8:26 AM	9:59 AM	10:09 AM			93
Chemical B	25 %		R	8:28 AM	11:02 AM	11:29 AM			154
(5) Chemical B	25 %	JD	L	8:29 AM	11:45 AM	11:55 AM			196
Chemical C	25 %		R	8:31 AM	8:37 AM	8:57 AM			6
(6) Chemical C	25 %	CS	L	8:34 AM	8:54 AM	9:06 AM			20
Chemical A	25 %		R	8:38 AM	8:58 AM	9:42 AM	10:58 AM	11:09 AM	140

[A] In ethanol unless otherwise specified.

Laboratory Species: _____ Age: ____
No./Cage _____ Temperature: ___ Relative Humidity:___

Avidity-No. Bites/min before test: 76
No. Bites/min during test: 42
No. Bites/min after test: 38

FIG. A1.1 Sample Data Sheet

A1.2 *Explanation of the Data in Items 1 through 6 on the Sample Work Sheet:*

A1.2.1 *Sample Test Comparing Compound A with a Deet Standard*—Both materials tested at 25% concentration in ethanol; applied at 8:00 and 8:05 a.m. and complete protection at 10:00 a.m. and 1:05 p.m., respectively. Loss of CPT is confirmed when the first bite is followed by a second bite within 30 min. Deet is shown to be 2.5 times more effective than chemical A in this test.

A1.2.2 In this test, Chemical B sustains an initial bite at 9:10 a.m., but no confirming bite occurs within 30 min. At 12:10 p.m. a bite is recorded and is followed by a second bite 10 min later, thus confirming the first bite within 30 min. The CPT is then calculated from time of application, 8:10 a.m., to the first confirmed bite at 12:10 p.m., or CPT of 240 min.

A1.2.3 This test shows Compound C to be virtually ineffective against *Aedes taeniorhynchus* mosquitoes (CPT 10 min) while the deet standard has provided 8 h of protection without a bite.

A1.2.4 The first three examples were pairings with the deet standard. The last three pairings complete all possible combinations of the three candidate repellents in the BIB. It should be noted that different subjects are assigned different chemicals in each pairing as discussed in 8.9. In this example, Compound B appears to be more effective than Compound A.

A1.2.5 This test and the following (A1.2.6) confirm that

A1.2.6 In this example, Compound A receives a bite at 8:58 a.m., but 41 min transpires before a second bite occurs at 9:42 a.m. At 10:58 a.m., a third bite is received but it occurs 76 min after the second. Finally, a confirming bite is received at 11:09 a.m., 11 min later. Complete protection time is then calculated from 10:58 a.m., or the first *confirmed* bite. Though not a common occurrence, the example is given to avoid possible confusion when calculating CPT.

TABLE A1.1 Tabulation of Test Results[A]

Chemical 25 % in ethanol	Protection time	
	Range	Mean
Deet	300–480	400
B	154–240	197
A	93–140	118
C	6–20	12

[A] Results are ranked in order of effectiveness.

TABLE A1.2 Table of Ratios

Chemical	Concentration (MG/CM^2)	Ratio[A]
A	0.388	0.30
B	0.388	0.49
C	0.388	0.03
DEET (standard)	0.388	1.00

[A] The ratio is obtained using a standard as the denominator and the nonstandard(s) as the numerator. Above the mean CPT for *A* is 118 min, the mean CPT for DEET (the standard) is 400 min, and the ratio for *A* is 118/400 or 0.30.

A1.3 Based on the data given in the sample work sheet (see

Fig. A1.1), Table A1.1 is a tabulation of the results, ranked in order of effectiveness, before statistical analysis.

A1.4 A table of ratios should be generated as mentioned in 9.5 and 9.6. Based on the data given in the sample work sheet, a table of ratios is shown in Table A1.2.