OPPTS 810.3700. Product Performance of Skin-Applied Repellents of Insects and Other Arthropods

(a) Scope and Applicability.

(1) Purpose. This guideline describes tests that should generally meet requirements of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq*.) for demonstrating product performance or effectiveness. This guideline recommends specific protocols for conducting product performance testing of insect and tick repellents intended for direct application to human skin, which may be formulated as lotions, liquids or pressurized sprays. It reflects the Agency's recommendations for minimum steps necessary to develop reliable data on repellent product performance. Deviations from this guideline are permissible, but should be fully explained and justified.

(2) Related Standards

(A) Any research conducted under this guideline is subject to FIFRA §12(a)(2)(P), which makes it unlawful "for any person to use any pesticide in tests on human beings unless such human beings (i) are fully informed of the nature and purposes of the test and of any physical and mental health consequences which are reasonably foreseeable therefrom, and (ii) freely volunteer to participate in the test."

(B) Research conducted under this guideline is likely to be subject to the requirements of EPA regulation at 40 CFR Part 26, particularly subparts K-Q. Note in particular the following provisions:

(i) 40 CFR 26.1203 provides:

". . . under no circumstances shall a person conduct or sponsor [covered] research that involves intentional exposure of any human subject who is a pregnant woman (and therefore her fetus) or child."

(ii) 40 CFR 26.1125 provides:

"Any person or institution who intends to conduct or sponsor human research covered by section 26.1101(a) shall, after receiving approval from all appropriate IRBs, submit to EPA prior to initiating such research all information relevant to the proposed research specified by section 26.1115(a), and, to the extent not already included [certain other information]."

(iii) 40 CFR 26.1303 provides:

Any person who submits to EPA data derived from human research covered by this subpart shall provide at the time of submission information concerning the ethical conduct of such research. To the extent available to the submitter and not previously provided to EPA, such information should include:

(a) Copies of all of the records relevant to the research specified by § 26.1115(a) to be prepared and maintained by an IRB.
(b) Copies of all of the records relevant to the information identified in § 26.1125(a) through (f).
(c) Copies of sample records used to document informed consent as specified by § 26.1117, but not identifying any subjects of the research.

(iv) 40 CFR 26.1705 provides:

" . . .EPA shall not rely on data from any research initiated after April 7, 2006, unless EPA has adequate information to determine that the research was conducted in substantial compliance with subparts A through L of this part, or if conducted in a foreign country, under procedures at least as protective as those in subparts A though L of this part."

(C) Good Laboratory Practice Standards (GLP) as defined in 40 CFR 160.1 to 160.195 apply to both laboratory and field studies of repellent efficacy. According to 40 CFR 160.17: "EPA may refuse to consider reliable for purposes of supporting an application for a research or marketing permit any data from a study which was not conducted in accordance with this part." 40 CFR 160.12(b) requires with any submitted research data "[a] statement describing in detail all differences between the practices used in the study and those required by this part."

(b) Definitions. The following definitions are of special importance in understanding this guideline:

(1) ***Bite*** means penetration of human skin by the mouthparts of an insect or other arthropod, associated with ingestion of blood and resulting abdomen swelling and color change.

(2) ***First confirmed bite (FCB)*** means a probe or bite followed within 30 minutes by a second, confirming, probe or bite.

(3) ***Landing*** describes the behavior of a flying or jumping insect that lands, but does not

probe or bite.

(4) ***Probe*** means penetration of human skin by the mouthparts of an insect or other arthropod, without ingestion of blood. Mosquitoes inject saliva during probing, to help locate capillaries and to block haemostatic responses of the host.

(5) ***Protection time (PT)*** means the time from application of a repellent until it is no longer effective. Depending on how repellency is measured, protection time may be expressed as the time to first confirmed bite (TFCB), or as the duration of the period of maximum Relative Protection (RP) compared to controls.

(6) ***Questing*** describes the behavior of a tick or chigger seeking a host; in the context of laboratory testing of repellency against ticks and chiggers, it means upward movement of the tick or chigger from the wrist toward the raised elbow.

(7) ***Relative Protection (RP)*** is a metric of repellent performance usually associated with claims to repel nuisance mosquitoes or flies, or ticks or chiggers. RP is expressed as a percentage reduction in probes or bites in treated subjects as compared to untreated controls. A landing and probe without biting, or a landing, probe and bite are each counted as a single event in calculating RP. It is calculated by the formula $RP = (1 - TB^t/TB^c) * 100$, where TB^t is the total number of probes/bites in the treatment group and TB^c is the total number of probes/bites in the control group.

	Probes/Bites in each post-application hour				
	1	2	3	4	5
Control Group	8	11	7	12	10
Treatment Group	0	0	1	2	4
RP by period	1-(0/8) 100%	1-(0/11) 100%	1-(1/7) 86%	1-(2/12) 83%	1-(4/10) 60%
Cumulative RP	1-(0/8) 100%	1-(0/19) 100%	1-(1/26) 96%	1-(3/38) 92%	1-(7/48) 85%

In this hypothetical example, which assumes equivalent exposure time and equal numbers of subjects in the treated and untreated groups, the test repellent shows relative protection of 100% in the first and second hours post-treatment, 86% in the third hour, and 83% in the fourth hour. It shows cumulative relative protection of 100% for two hours, 96% for three hours, and 92% for four hours.

(8) ***Repellency*** means the capacity of a product to drive or keep insects away from human skin to which repellent has been applied. Repellency of mosquitoes, biting flies, and fleas means that no insects land and probe or bite treated human skin. Repellency of ticks and chiggers means no questing ticks or chiggers crawl onto treated human skin.

(8) ***Repellent*** means a pesticide product that drives or keeps insects or other arthropods away from treated human skin.

(9) ***Time to First Confirmed Bite (TFCB)*** is a metric of repellent performance usually associated with claims to provide protection from insects which may transmit diseases, such as West Nile virus. TFCB is calculated as the average duration for all treated subjects from application of the repellent to the first landing with probe or bite which is confirmed within 30 minutes by a second, confirming probe or bite.

(c) General Considerations. The following general points apply to all testing of repellency against insects, ticks or mites addressed by this guideline.

(1) Scientific Considerations.

(A) Test substance. The end-use product as proposed for registration—the formulation in the final package–should be used for all repellent efficacy testing. Spray formulations should be applied directly from spray containers. Data from testing of active ingredients in different formulations or concentrations from the end-use product proposed for registration, or against pests other than those stated on the proposed label, is generally not acceptable to support registration. The test material should be stored at ambient temperature and humidity before the test.

(B) Subjects. At least six adult subjects aged 18-55—preferably equal numbers of men and women—should be used for each test. Subjects should avoid alcohol, tobacco, and fragrance products (perfume, cologne, hair spray, lotion, scented soap, etc.) for at least twelve hours before and throughout the test. Subjects should wear light-colored clothing, should avoid exertion which might increase perspiration, and avoid abrading, rubbing, touching, or wetting treated skin.

(C) Treatments per subject. To avoid cross-contamination and potential confounding interference of different repellents, each subject should normally receive no more than one treatment per test. All dermally applied repellents repel insects in their vapor form; thus multiple formulations may interact, making results unreliable. Multiple treatments per subject are acceptable only when testing substantially similar formulations containing identical active ingredient(s) at the same concentration. In this limited case it is particularly important that the test subject not rub or contaminate the repellent to keep treatments independent from each other.

(D) Skin test area size and preparation. The recommended treatment area is the subject's forearm, wrist to elbow, or lower leg, knee to ankle. The area should be washed with unscented soap, rinsed first with water and then with a solution of at least 50% ethanol or isopropyl base rubbing alcohol in water, and dried with a

clean towel. The surface area (in cm^2) of each test subject's forearm or lower leg should be calculated and reported. You may estimate surface area by measuring the circumference of the arm at the wrist and elbow (or the circumference of the leg at the ankle and knee) and at three or four equally spaced intermediate points; then by multiplying the average of these measures of circumference by the distance from the wrist to elbow (or from the ankle to the knee.) Adjacent areas above and below the treated test area should be covered with a light-colored material a proboscis cannot penetrate. Hands may be covered with latex gloves, and feet with shoes.

(E) Controls. Negative (untreated) controls are needed to establish the attractiveness of subjects to pests, and to establish and confirm biting or questing pressure. The preferred negative control is the untreated limb of a treated subject, but another untreated subject may be used as a control instead. Positive controls treated with a registered repellent of established efficacy are also recommended to calibrate the test system.

(F) Amount of repellent applied. The amount of repellent applied should be the typical dose applied by consumers. The Agency recommends using a 95% confidence limit around the mean as the most appropriate statistical measurement of a typical consumer dose.

(G) Data analysis. Statistical testing should examine variability among subjects and among multiple test sites or test sessions. Means should be reported with the associated 95% confidence interval and standard deviation. Statistical methods used to analyze test results should be fully described and explained.

(2) Ethical Considerations. The sponsor and investigators should reflect the following ethical considerations in the design and conduct of the research:

(A) Selection of Subjects. In selecting participants for both laboratory and field testing of repellents, special care must be taken to exclude subjects from vulnerable populations, including children, the elderly, people of limited mental capacity, and pregnant or nursing women.

(B) Minimization of risks to subjects. Without compromising the reliability of the data, it is important to minimize the risks to subjects through the design of the research. Research subjects may experience a toxic or allergic response to the repellent itself, or may experience the discomfort of insect bites or contract a vector-borne disease either as a consequence of the failure of the repellent to work, or by serving as an untreated control subject. Investigators should minimize risks of all these three types in the research design. Untreated control subjects may be exposed intermittently during testing to confirm continued pest pressure rather than being continuously exposed. When possible, threshold efficacy should be

established in the laboratory, using laboratory-bred insects known to be free of disease, before initiating field testing. Risks may also be reduced by pre-exposure vaccination (when possible) against diseases transmitted by an arthropod vector, by providing for emergency medical care, and by providing for continuing medical care to any subject who contracts a disease—such as West Nile virus or Lyme disease—transmitted by an arthropod vector against which the repellent is being tested.

(C) Independent Ethics Oversight. Protocols and supporting materials should be reviewed and approved by an Institutional Review Board or equivalent ethics review committee, independent of the investigators and sponsors of the research, before submission to EPA for review.

(D) Informed, Voluntary Consent of Subjects. Subjects should be fully informed, and able to demonstrate understanding of the procedures and risks involved during testing. In addition to the minimum elements of informed consent defined in 40 CFR 26.1116, subjects should be told what active ingredient(s) and product they will be exposed to, whether it is registered by EPA, and information regarding the potential hazard of the product—e.g., its toxicity, irritation potential, and allergenicity. Subjects should be given information on the symptoms and on the risks of contracting West Nile virus, Lyme disease, or other arthropod-vectored diseases that may occur in the area where the products are field-tested. The procedure for soliciting the fully informed and fully voluntary consent of the subjects should provide for verification by the investigators that subjects understand the information given to them.

(3) Data collection and reporting. The following information should be recorded and reported for all types of study described in this guideline. Further recommendations specific to each type of study are included in the appropriate sections below.

(A) Identification: Title, sponsor, investigators, name and location of the testing laboratory or field site, dates of study.

(B) Purpose of study.

(C) Materials

(i) Test substance: Identification of end product used (active ingredient(s), chemical formula, Chemical Abstracts number, manufacturer code name,) concentration or dilution, date of manufacture and how prepared; prior storage conditions, physical-chemical and biological properties

(ii) Directions for use of repellent tested.

(iii) Test subjects: Number, individual age, race, sex, reproductive or nursing status (if female), and other pertinent demographic data.

(iv) Test organisms: Genus and species, as well as subspecies or strain if that information is available.

(v) Additional information about test organisms used in laboratory studies: Development stage, age and sex of insects; rearing technique; preparation of insects for test (feeding/starving); methods for establishing freedom of test organisms from disease or disease organisms; insect density and biting pressure in each cage.

(vi) Test cages/chambers: Full description including construction material, size, sleeve description.

(D) Test conditions: Temperature, relative humidity, ambient lighting, air flow

(E) Test procedures.

(i) Preparation of subjects: Training, obtaining informed consent, determining attractiveness to insects, description of clothing, calculation of area of skin to be treated, washing, treatment.

(ii) Dose of repellent applied expressed as mass per unit area of treated skin. Explain how a typical consumer dose was calculated.

(iii) Number and body location of treatments applied to each subject;

(iv) Time of start and end of testing on each subject, including untreated controls;

(v) Exposure time to insects for each test for each subject;

(vi) Any deviations from the protocol and their impact on results of test;

(F) Results for each insect species tested.

In general, each landing, probe, and bite (or landing and probe) should be reported as a single event, for each treated subject and untreated control, for each period of exposure. More specific recommendations for reporting results are included in the discussion of each type of test below.

(G) Discussion

(H) Conclusions.

(I) Certification.

(J) References.

(K) Appendices.

(i) Protocol
(ii) Recruiting materials
(iii) Informed Consent materials
(iv) Risk/Benefit assessment
(v) Documentation of IRB Approval
(vi) All correspondence with IRB

(4) Record Retention Requirements.

(A) The record-keeping requirements in 40 CFR 26.1115 apply to Institutional Review Boards (IRBs) that review human research conducted under EPA's regulation.

(B) The record-keeping requirements of 40 CFR 169.2(j) apply to investigators who conduct pesticide research with humans subject to FIFRA §12(a)(2)(P).

(C) The record-keeping requirements of 40 CFR 169.2(k) apply to any person who submits the results of research to EPA in support of a petition for a tolerance or tolerance exemption or in support of registration or an application for registration.

(d) Specific Guidance for Testing Repellents of Mosquitoes and Biting Flies.

(1) Measurement of repellency.

(A) When the objective is to estimate protection from mosquito or biting fly nuisance, product performance may be expressed as Relative Protection (RP) over a given time as compared to untreated controls. Unless the product is 100% effective, relative protection compared to untreated controls is not appropriate to estimate protection from disease transmission.

(B) When the objective is to estimate protection from mosquito-borne disease, product performance should be expressed as Time to First Confirmed Bite (TFCB) or probe. In protecting against the possibility of disease transmission, a probe is as important as a bite. Several studies (Putman and Scott 1995; Kelly and Edman 1992; Matsuoka et al 2002; Vanderberg and Frevert 2004; Ho and Lavoipierre 1975) (see references in paragraph (g) of this guideline) have shown that pathogens can be transmitted during probing, and that probing behavior increases

in infected mosquitoes. A landing and probe is thus treated as equivalent to a landing, probe and bite in defining the first bite or the confirming bite.

(2) Laboratory tests: Mosquitoes and stable flies. Although laboratory tests are not always required to verify the performance of mosquito and stable fly repellents, these tests may be valuable to establish preliminary threshold levels of repellency to mosquitoes before initiating field testing.

(A) Test species. Laboratory tests of mosquito repellency should be conducted with at least three genera of human biters; *Aedes aegypti*, an *Anopheles* sp., and a *Culex* sp. Stable fly tests should be conducted with *Stomoxys calcitrans*. Test insects should be identified by subspecies or strain if that information is available.

(B) Stage, age, and sex. Mosquitoes should be adult females 5 to 10 days old. Stable flies should be 3 days old. Report the age or age range of the test insects.

(C) Rearing techniques. Larvae should be reared under optimal conditions for the species. As a general guide, most species should be reared at 27±3°C, relative humidity 80±10%, and photoperiod 16:8 hours (light:dark). Alternative rearing methods may also be acceptable (see, for example, Gerberg and Barnard 1998). Use of alternative rearing techniques should be fully explained and justified. Adults should be fed 10% sucrose and no blood meal before being used in a test.

(D) Preparation of Insects for Testing. Test insects should be starved for 12 to 24 hours immediately before the test. Test insects should be established to be free of disease (e.g., malaria or West Nile virus); methods to ensure they are disease-free should be reported. Test insects should be used for only one test and should be destroyed immediately after the trial.

(E) Test cage and testing conditions. Cages should be at least 226,535 cm^3 (8 cubic feet), square or rectangular, with one sleeved opening for the subject's arm. Each cage should be used for only one subject and one treatment at a time. The temperature during the test should be kept at 22°C to 27°C, and relative humidity should be kept at 50% to 80%. Testing conditions should attempt to duplicate the preferred feeding time of day for the test species. Lights should be turned on for species that feed during the day (e.g., *Aedes aegypti*), and turned off or dimmed for night feeders (e.g., *Anopheles* spp. and *Culex* spp.). Investigators and subjects should avoid exhaling into the test cage; introduction of CO_2 could bias insects towards biting.

(F) Insect density. There should be at least one mosquito for each 1,133 cm^3 and at least 200 mosquitoes in each test cage. There should be at least one stable fly for each 5,034 cm^3 and at least 45 stable flies in each test cage.

(G) For tests of Time to First Confirmed Bite:

(i) Controls: A negative (untreated) control is necessary to verify biting pressure. The preferred negative control is the untreated forearm of each treated subject, but another untreated subject may be used as a control instead. Control forearms should be washed, rinsed, and dried exactly like treated forearms. Before testing with the treated arm, subjects should expose their untreated forearm to the mosquitoes or stable flies in the test cage to establish their attractiveness. At least 10 mosquito landings or probes within 30 seconds, or 5 stable fly landings or probes in 60 seconds, are needed to qualify as a subject. The control forearm may be removed from the test cage as soon as it has received the necessary number of landings. Before each exposure of a treated forearm, an untreated control forearm should be inserted through the sleeve into the cage and exposed to mosquitoes for up to 30 seconds or to stable flies for up to 60 seconds to verify biting pressure. A positive control treated with a registered repellent formulation of established efficacy is also recommended.

(ii) Exposure period: Thirty minutes after treatment with the repellent and immediately after verifying biting pressure as described above, the subject's treated forearm is inserted through the sleeve into the cage of insects for five minutes. After the five-minute exposure period the subject's forearm should be removed from the sleeve, taking care to minimize abrasion of the treated area of skin. Every 30 minutes the sequence is repeated: first exposing the untreated control arm to confirm biting pressure, then exposing the treated arm for five minutes. This cycle should continue so long as biting pressure lasts—that is, until the untreated control arm no longer receives 10 mosquito landings in 30 seconds or 5 stable fly landings in 60 seconds. Subjects may then continue the test using another cage with fresh insects until the repellent fails. Subjects should minimize rubbing the repellent-treated area of their arm when putting it into the cage, and between exposure periods.

(iii) Data Collection: During each exposure cycle the number of landings received on each untreated control and the number of seconds required to receive the requisite number of landings to confirm biting pressure should be recorded, as well as the number of bites or probes received by each treated subject during each five-minute exposure period. A landing and probe without biting, or a landing, probe, and bite on a treated subject are each recorded as a single event. Landings on treated subjects without probing or biting need not be recorded.

(H) For tests of Relative Protection:

(i) Controls: A negative (untreated) control is necessary to establish a basis for comparison. The preferred negative control is the untreated forearm of each treated subject, but another untreated subject may be used as a control instead. Control forearms should be washed, rinsed, and dried exactly like treated forearms. Before beginning the test subjects should expose their untreated forearm to the mosquitoes or stable flies in the test cage to establish their attractiveness. At least 10 mosquito landings or probes within 30 seconds, or 5 stable fly landings or probes in 60 seconds, are needed to qualify as a subject. The control forearm may be removed from the test cage as soon as it has received the necessary number of landings. A positive control treated with a registered repellent formulation of established efficacy is also recommended.

(ii) Exposure Period: Once subject attractiveness has been established, exposures of treated and untreated forearms should be of equal duration. Thirty minutes after treatment with the repellent the subject's untreated forearm is inserted through the sleeve into the cage of insects for five minutes. After the five-minute exposure period the subject's untreated forearm should be removed from the sleeve, and the treated forearm should be inserted for five minutes. After the five-minute exposure period the subject's treated forearm is withdrawn from the sleeve. Every 30 minutes the sequence is repeated: first exposing the untreated control arm for five minutes, then exposing the treated arm for five minutes. This cycle should continue so long as biting pressure lasts—that is, until the untreated control arm no longer receives 10 mosquito landings in 30 seconds or 5 stable fly landings in 60 seconds. Subjects may then continue the test using another cage with fresh insects until the repellent fails. Subjects should minimize rubbing their arms when putting them into the cage, and between exposure periods.

(iii) Data Collection: The number of landings received on each untreated control should be recorded, as well as the number of bites or probes received by each treated subject, during each five-minute exposure period. A landing and probe without biting, or a landing, probe, and bite on a treated subject are each recorded as a single event. Landings on treated subjects without probing or biting need not be recorded.

(2) Field tests: Mosquitoes, black flies (gnats, southern buffalo gnats), ceratopogonids (no-see-ums, punkies, biting midges), sand flies, tabanids, and stable flies. Due to the prevalence of West Nile virus, it is strongly advised that sponsors and investigators conducting field tests employ protocols that minimize exposure of human subjects to mosquitoes in the field.

(A) Test species. Tests should be conducted with species that occur in the United

States. If tests are conducted in other countries or territories (e.g., Canada or Puerto Rico) the study report should justify the relevance of the test as a measure of repellency in the United States. Species should be collected by aspirating insects into a vial before the test, while determining biting pressure, and periodically throughout the test. The aspirated insects should be taken to the laboratory for subsequent identification, and should be described in the study report by genus and species, and if possible by subspecies or strain.

(B) Test sites. Field tests should be conducted in at least two areas. Tests for mosquito repellency should be conducted in environmentally distinct habitats (forest, grassland, salt marsh, wetland, beach, barns, urban environments) containing different species. They need not be in different states. Field tests should be conducted in areas where the West Nile virus or outbreaks of other arthropod-vectored diseases have not been detected. Repellency against black flies, stable flies, and other organisms that typically occur in only one habitat should be tested in two separate areas or in the same area on two separate days.

(C) Environmental conditions. The time of day (beginning and ending), and weather conditions during testing should be reported, including temperature, relative humidity, cloud cover, precipitation, light intensity, and wind speed during 90 seconds of observation for each exposure period. Repellent testing should not continue when wind speed exceeds ten miles per hour.

(D) Subject placement and behavior. During field tests, subjects should engage in normal outdoor activities, such as walking, standing, squatting, sitting, and raising and lowering their arms, but staying apart from other subjects.

(E) Establishing biting pressure. To ensure reliable data, it is recommended that biting pressure be established before treatment and confirmed at least every hour during the test. The preferred untreated control is an untreated forearm or lower leg of a treated subject. The Agency recommends that field testing not be conducted unless biting pressure on untreated controls is at least at the level of five landings or probes by mosquitoes or black flies during 5 minutes, or one landing or probe by stable flies, ceratopogonids, or tabanids during 5 minutes. Testing when biting pressure is below these minimum levels is unlikely to provide reliable results. Insects landing on untreated controls should be aspirated into a vial for subsequent identification. In tests of time to First Confirmed Bite, as soon as enough landings or probes to establish or confirm biting pressure have occurred, the subject's untreated limb may be covered. In tests of Relative Protection untreated controls must be exposed for the same period as treated subjects.

(F) Exposure period. Either continuous exposure throughout the testing period or intermittent exposure at fixed intervals during the testing period is acceptable. Reliable results may be obtained either by applying repellent to all subjects at

once, and then exposing all subjects together to coincide with arthropod activity periods, or by applying repellent at different times to different subjects (e.g., 1, 2, 4, 8, or 12 hours before exposure) and then exposing all subjects at once. The exposure parameters should be fully described in the study report.

(G) Data collection and reporting. Landings and probes/bites during the exposure period should be recorded by someone other than the subject. Recording may be by the investigator, an associate, or another subject working in a "buddy system." The number and time of landings received on each untreated control is recorded, as well as the number and time of each bite/probe received by each treated subject. A landing and probe without biting, or a landing, probe, and bite are each recorded as a single event. Landings on treated subjects without probing or biting need not be recorded.

(i) Reporting Time to First Confirmed Bite. If repellency is expressed as time to first confirmed bite, the following data points should be reported:

(a) Time post-treatment of each probe or probe/bite experienced by each treated subject (or treated limb)
(b) Time of each landing on each control subject (or untreated limb)
(c) Total bites experienced by each subject in each exposure period
(d) Time to first bite for each subject
(e) Time to first confirming bite for each subject
(f) The calculated mean time to first confirmed bite for all treated subjects, with the associated 95% confidence limits, standard deviation, and standard error.

(ii) Reporting Relative Protection. If repellency is expresses as relative protection, the following data points should be reported:

(a) Time post-treatment of each probe or probe/bite experienced by each treated subject (or treated limb)
(b) Time of each probe or probe/bite received by each control subject (or untreated limb)
(c) Total probes + probes/bites received by each subject in each exposure period
(g) Total probes + probes/bites received by all control subjects in each exposure period
(h) Total probes + probes/bites received by all treated subjects in each exposure period
(i) The ratio of probes + probes and bites for treated subjects to probes + probes and bites for controls in each exposure period

(e) Specific Guidance for Testing Flea Repellents.

(1) Laboratory tests: Fleas

(A) Test species. All tests should be conducted using the cat flea, *Ctenocephalides felis*.

(B) Stage, age, and sex. Fleas should be adult males and/or females five to ten days old. Report the age or age range of the test insects.

(C) Rearing techniques. Fleas should be reared at 27±3°C, relative humidity 50-80%, and photoperiod 16:8 h (light:dark). Any alternative rearing techniques should be justified in the study report. Adults should not be blood fed for at least 24 hours pre-test. Fleas should be used for only one test and should be destroyed immediately after the trial.

(D) Test cage and testing conditions. Cages should be at least 226,535 cm^3 (8 cubic feet) in volume; square, circular, or rectangular; plastic or glass; with an opening on the top to insert the subject's arm. Cages should have a rough floor such as clean sand. Replications should be limited to one subject and treatment at a time for each cage. The temperature during the test should be kept at 22-27°C, relative humidity at 50-80%, and the lights should remain on.

(E) Flea density. There should be at least one flea per ~566 cm^3 and at least 100 fleas in each test cage.

(F) Treated test area. The subject's forearm, wrist to elbow, is recommended as the test area. Areas above and below the forearm on both treated and control subjects should be covered with a material the flea's mouthparts cannot penetrate.

(G) For tests to First Confirmed Bite:

(i) Controls. Negative or untreated controls are needed to establish and confirm biting pressure. The preferred negative control is the untreated forearm of a treated subject, but a different untreated subject is also acceptable. Control forearms should be washed, rinsed, and dried exactly like treated forearms. Before treatment the subject should expose his or her forearm to the fleas in the test cage to establish attractiveness to the fleas. At least 10 landings within 30 seconds are needed to qualify as a subject. Immediately before each exposure of a treated forearm, an untreated control forearm should be inserted through the sleeve into the cage and exposed to the fleas for up to 30 seconds to confirm biting pressure. As soon as ten landings have occurred the control forearm may be removed from the test cage. If fewer than ten landings occur within 30 seconds,

additional fleas should be added to the cage and another untreated forearm should be inserted into the cage for up to 30 seconds, until ten landings or probes occur within 30 seconds. A positive control treated with a registered repellent formulation of established efficacy is also recommended.

(ii) Exposure period. Within thirty minutes after treatment, and at intervals of no more than thirty minutes thereafter, the subject's untreated forearm should be inserted through the sleeve into the cage of fleas for up to 30 seconds to confirm biting pressure, and then removed. Then the treated forearm should be inserted through the sleeve into the cage of fleas for five minutes. Subjects should repeat this cycle, exposing an untreated control arm followed by a treated arm, until the untreated control arm no longer receives ten flea landings within 30 seconds. Subjects may then continue the test using another cage with fresh fleas until the repellent fails. Subjects should minimize rubbing the repellent-treated area when putting their arms into the cage and between exposure periods.

(iii) Data collection and reporting. The number of fleas landing on the treated forearm in each exposure period should be recorded. Flea repellency is expressed as the duration of repellent protection from application of the repellent until a failure of repellency. The mean time of repellent protection for all treated subjects and the associated 95% confidence interval, standard deviation, and standard error should be reported for each test.

(H) For tests of Relative Protection:

(i) Controls. Negative or untreated controls are needed to establish a basis for comparison. The preferred negative control is the untreated forearm of a treated subject, but a different untreated subject is also acceptable. Control forearms should be washed, rinsed, and dried exactly like treated forearms. Before treatment the subject should expose his or her forearm to the fleas in the test cage to establish attractiveness to the fleas. At least 10 landings within 30 seconds are needed to qualify as a subject. The control forearm may be removed from the test cage as soon as it has received the necessary number of landings. A positive control treated with a registered repellent formulation of established efficacy is also recommended.

(ii) Exposure period. Once subject attractiveness has been established, exposures of treated and untreated forearms should be of equal duration. Thirty minutes after treatment with the repellent the subject's untreated forearm is inserted through the sleeve into the cage of fleas for five minutes. After the five-minute exposure period the subject's untreated forearm should be removed from the sleeve, and the treated forearm should

be inserted for five minutes. After the five-minute exposure period the subject's treated forearm is withdrawn from the sleeve. Every 30 minutes the sequence is repeated: first exposing the untreated control arm for five minutes, then exposing the treated arm for five minutes. This cycle should continue so long as biting pressure lasts—that is, until the untreated control arm no longer receives 10 landings in the first 30 seconds. Subjects may then continue the test using another cage with fresh fleas until the repellent fails. Subjects should minimize rubbing their arms when putting them into the cage, and between exposure periods.

(iii) Data collection and reporting. The number of fleas landing on each untreated and treated forearm in each exposure period should be recorded. Flea repellency is expressed as the duration of repellent protection from application of the repellent until a failure of repellency. The mean time of repellent protection for all treated subjects and the associated 95% confidence interval, standard deviation, and standard error should be reported for each test.

(2) Field tests: Fleas. Field tests are not routinely required for flea repellents, but may be conducted and submitted voluntarily. If field tests are conducted, re-application time in the label "Directions for Use" should reflect field results.

(f) Specific Guidance for Testing Tick and Chigger Repellents.

(1) Laboratory tests: Ticks and Chiggers.

(A) Test species. Tests should be conducted on the tick species the label claims to repel. Common tick species in the United States include: the blacklegged tick (deer tick, *Ixodes scapularis*), western blacklegged tick (deer tick, *Ixodes pacificus*), lone star tick *(Amblyomma americanum*), American dog tick *(Dermacentor variabilis*), and relapsing fever tick (softbacked tick, *Ornithodoros turicata*). Preferred test species for chiggers tested include the *Trombiculidae* family; *Eutrombicula splendens;* or *E. cinnabarrs*. Test animals should be identified by genus and species, and if possible by subspecies or strain.

(B) Stage and age. When testing with blacklegged (deer tick), lone star, and softbacked ticks, testing is appropriate with either adult or nymphal life stages. When testing with American dog ticks, the adult tick is recommended since the nymphs do not feed on humans. Tests with chiggers should use immature chiggers. Report the age or age range of all test animals.

(C) Rearing techniques. Test organisms should be reared at 22±3°C, relative humidity 50-80%, and photoperiod 16:8 (light:dark). Any alternative rearing techniques should be justified in the study report. Tick species should be

established to be free of disease, and methods used to ensure they are disease-free should be reported. Ticks or chiggers should be used for only one test and should be destroyed immediately after the trial.

(D) Number of ticks or chiggers. It is recommended that at least five ticks or chiggers be exposed to the treated forearm in each exposure period.

(E) Testing conditions. The temperature should be kept during the test at 22°C to 27°C, relative humidity at 50% to 80%, and the lights should remain on.

(F) Skin test area size and preparation. The test area is the forearm from the wrist to the elbow. The arm above and the hand below the test area of both treated and control subjects should be covered with a material that tick or chigger mouthparts cannot penetrate.

(G) Controls. A negative or untreated control is recommended to verify subject attractiveness and to establish and confirm active questing behavior. The negative control should be the untreated forearm of a treated subject. The control forearm should be washed, rinsed, and dried exactly like the treated forearm and then be exposed to the test organism to verify attractiveness and establish questing behavior. The test organism should be picked up carefully to prevent damaging its body or forelegs (e.g., with a soft artist's paint brush, forceps, or a cotton swab), and placed on the forearm of the control subject, near the wrist. Active questing behavior is established or confirmed if the test organism moves vertically at least 2 cm toward the elbow within 2 minutes.

(H) Exposure cycle. Within thirty minutes after treatment with the repellent, each subject should expose his or her untreated control arm to the test organisms to confirm questing behavior, and then expose the treated arm to the organisms as described in paragraph (I) below. Every 30 minutes, the sequence of exposing the untreated control arm followed by the treated arm should be repeated, so long as questing pressure lasts—that is, so long as the test organisms move vertically at least 2 cm toward the elbow of the untreated control arm within 2 minutes—and until the repellent fails to repel the test organisms.

(H) Test procedure. Subjects place their fingertips on a flat surface with the palm raised above the surface and the forearm held vertically, perpendicular to the surface. Using a suitable instrument (e.g, artist's paintbrush, forceps, or a cotton swab) the investigator should place at least five ticks or chiggers, one at a time, on the subject's forearm near the wrist, approximately 2 cm from the edge of the treated area of the forearm. Each tick or chigger should be guided gently (e.g., with paint brush, forceps, or cotton swab) in the direction of the treated area. Ticks or chiggers should be allowed five minutes, beginning from their first movement toward the treated area, to cross the boundary onto the treated area.

Those that cross at least 2 cm onto the treated area (toward the elbow) should be reported as 'not repelled'. Those that fail to cross the boundary, or that proceed less than 2 cm onto the treated area in five minutes, should be reported as "repelled". Ticks or chiggers which have been used in a test should be destroyed. A new group of ticks or chiggers should be exposed to the treated area every 30 minutes.

(I) Data reporting. Tick and chigger repellency is expressed by the duration of repellent protection for each test organism for each subject, based on percent reduction in the number of ticks crossing the repellent treated zone compared to a negative control.

(2) Field tests: Ticks and Chiggers. The Agency recommends laboratory testing for ticks and chiggers since reliable field tests have not been developed. The Agency is investigating potentially appropriate field tests that may be recommended in the future. Although field tests are not routinely conducted for testing performance of skin repellents against ticks and chiggers, if such tests are conducted, re-application time under label "Directions for Use" should reflect field results.

(g) References. Consult the following references for additional background information on repellent efficacy testing.

(1) Barnard, D.R. 1998. Mediation of deet repellence in mosquitoes (Diptera: Culicidae) by species, age, and parity. J. Med. Entomol. 35(3): 340-343.

(2) Frances, S.P. 1994. Response of a chigger, *Eutrombicula hirsti* (Acari: Trombiculidae) to repellent and toxicant compounds in the laboratory. J. Med. Entomol. 31(4): 628-630.

(3) Frances, S.P., N. Eikarat, B. Sripongsai, and C. Eamsila. 1993. Response of *Anopheles dirus* and *Aedes albopictus* to repellents in the laboratory. J. Am. Mos. Con. Assoc. 9(4): 474-476.

(4) Frances, S.P., A.E.T. Yeo, E.W. Brooke, and A.W. Sweeney. 1992. Clothing impregnations of dibutylphthalate and permethrin as protectants against a chigger mite, *Eutrombicula hirsti* (Acari: Trombiculidae). J. Med. Entomol. 29(6): 907-910.

(5) Gerberg, E. and D. Barnard. 1998. Manual for mosquito rearing and experimental techniques. AMCA Bull. 5.

(6) Ho, B.C., and M. M. Lavoipierre. 1975. Studies on filariasis. IV. The rate of scape of the third-stage larvae of *Brugia pahangi* from the mouthpart of *Aedes togoi* during the blood meal. J. Helminthol. 49 (1): 65-72.

(7) Kelly, R. and J.D. Edman. 1992. Multiple transmission of *Plasmodium gallinaceum* (Eucoccida: Plasmodiidae) during serial probing by Aedes aegypti (Diptera: Culicidae) on several hosts. J. Med. Entomol. 29 (2): 329-31.

(8) Klun, J.A. and M. Debboun. 2000. A new module for quantitative evaluation of repellent efficacy using human subjects. J. Med. Entomol. 37(1): 177-181.

(9) Matsuoka, H., S. Yoshida, M. Hirai, and A. Ishii. 2002. A rodent malaria, *Plasmodium berghei* is experimentally transmitted to mice by merely probing of infective mosquito, *Anopheles stephensi.* Parasitology Int. 51 (1): 17-23.

(10) Mount, G.A. and E.L. Snoddy. 1983. Pressurized sprays of permethrin and Deet on clothing for personal protection against the Lone Star tick and the American dog tick (Acari: Ixodidae). J. Econ. Entomol. 76: 529-531.

(11) Putnam, J.L., and T.W. Scott. 1995. The effect of multiple host contacts on the infectivity of dengue-2 virus-infected *Aedes aegypti*. J. Parasitol. 81(2): 170-4

(12) Rutledge, L.C., and R.K. Gupta. 1999. Variation in the protection periods of repellents on individual human subjects: an analytical review. J. Am. Mosq. Cont. Ass. 15(3) 348-355.)

(13) Schreck, C.E. Posey, K, and D. Smith. 1978. Durability of permethrin as a potential clothing treatment to protect against blood-feeding arthropods. J. Econ. Entomol. 71: 397-400.

(14) Sholdt, L.L. 1989. Effectiveness of permethrin treated military uniform fabric against human body lice. Military Medicine 154: 90-93.

(15) Smith, C.N. 1955. Insect repellents. Quarterly Report, Entomological Research. Entomology Research Branch, U.S. Department of Agriculture. 8pp.

(16) Soto J., Medina F., and J. Berman. 1995. Efficacy of permethrin treated uniforms in the prevention of malaria and leishmaniasis. Clinical Infectious Diseases 21: 599-602.

(17) Verwey, R.E. 1996. Laboratory method for testing insect repellents on human test subjects against chiggers in the laboratory. S.C. Johnson & Sons, Inc. Racine, WI. 3 pp.