PLEASE NOTE: This protocol is privileged and confidential, and is for the sole use of Dr. Scott Carroll, his agents and associates.

TITLE: TEST OF PERSONAL INSECT REPELLENTS
PROTOCOL NUMBER: C-L–001

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1. STUDY AIM/PURPOSE

This protocol describes the procedures used to evaluate the efficacy of personal repellents to biting arthropods. It pertains to repellents that are applied directly to the skin (e.g., sprays, creams, lotions, gels) or to clothing and is designed to test their efficacy against blood-sucking arthropods, such as mosquitoes (Culicidae), black flies (Simuliidae), biting gnats, (Ceratopogonidae), ticks (Ixodidae), and fleas (Pulicidae). In using the common term 'repellents', we recognize that the modes of action of materials that deter blood-feeding insects are largely unknown. In consequence, we use 'repellents' to include materials that may repel, deter biting, act as physical barriers to biting, or otherwise protect participants from bites.

This protocol applies to tests sponsored by government agencies or companies that serve consumer interest in personal protection from arthropods. Efficacy tests are required by the United States and State Environmental Protection Agencies (EPA) for registration and legal marketing. Our role is to provide quality scientific assessments of repellency, based on federal testing guidelines. The

specific intent of this protocol is to meet State of California Department of Pesticide Regulation (DPR) requirements for their oversight of pesticide exposure studies. It permits them to review and permit our procedures on an annual basis without having to review the details of each test undertaken (e.g., sponsor, formulation, exact test design information that may differ from test to test). Each individual sponsored test is subject to appropriate IRB review. We inform the California DPR of the planned conduct of such tests and may monitor them.

Tests are conducted in the laboratory with captive strains of arthropods, and in the field under natural conditions. Only new materials receiving low toxicity scores in dermal, ocular and ingestion studies, or established products with modified delivery systems, are candidates for testing. Low product toxicity is one part of an 18-part risk/discomfort minimization strategy. Procedures conform to the US EPA Pesticide Assessment Guidelines (subdivision G 95-1(d)(1); Nov. 1982), informed by the draft revised guidelines of 2000 (OPPTS 810.3700). Tests are partially randomized, subject-blinded experiments. Under supervision, measured doses are applied to test subject's forearms or lower legs, or to fabric then worn by those subjects. Participants or technicians record the time of any events that are used to determine failure (defined below), with exposure terminated at failure. Results are evaluated with respect to US EPA standards for efficacy (time to First Confirmed Bite, Complete Protection Time, biting rates in comparison to controls and comparison articles, and variation with time after application). Our proposed starting date is **6 April 2006**. End date will be one year after approval. Report submission will be within that time.

2. BACKGROUND

Efficacy testing is a basic step in the development of insect repellents. Our procedures are based on standard protocols in the field of insect repellents (e.g., American Society for Testing and Materials form E-939-94 (2005): “Standard Method of Field Testing Topical Applications of

Compounds as Repellents for Medically Important and Pest Arthropods". Consumer interest in non-deet products is considerable, and a number of alternative products are currently marketed without proper federal or state EPA registration. Our consulting group works as a subcontractor to sponsors that develop low toxicity (often botanical or "natural") insect repellents, normally with the direct intention of seeking EPA registration. The use of human subjects is indicated by the low toxicity of the products, the goal of producing products for human use, and US/*EPA* requirements for demonstrating product efficacy.

3. SIGNIFICANCE

In response to rapidly growing consumer and public health requirements, numerous companies are working to develop effective, alternative, low toxicity repellents. To insure consumer safety and satisfaction, it is important that alternative products be tested with procedures mandated by US/*EPA* and in a manner consistent with the testing of products already registered. In addition, because the mode of operation of developmental alternatives may be different from that of DEET, it is important that research be conducted in a manner sensitive to alternative evaluations of data, especially time series data.

Risk to participants is considered low. This evaluation is based on the low toxicity of the formulations to be tested in combination with our 18-part risk/discomfort minimization strategy Section G (below). The benefits of this study will accrue primarily to the public at large. Participants will receive a modest compensation, as well as the reward of participating in scientific study relevant to their personal and public lives. The scientific and public health communities will benefit from the evaluation of alternative insect repellents.

4. METHODS

A. General Study Design

Most of our field tests are day-long affairs conducted on weekends or holidays at public or private sites with which we have arrangements. Laboratory tests are often briefer. Under the supervision

of the Study Director, measured dosages of test or comparison materials are applied to forearms and/or lower legs. The treated areas are protected from rubbing. Areas are exposed to test arthropods. Continuous or periodic exposure is used to evaluate the duration of efficacy relative to a negative control.

B. Methods of Data Analysis

Field and lab tests are conducted with large populations of arthropods. This permits the analysis of the replicates (data by subject) as independent values. Complete protection time (CPT) is measured the time from first exposure to the first confirmed failure (i.e., an alightment by a biting fly or mosquito with an attempt to feed) or crossing of a repellent barrier (ticks). A confirmed failure is a failure followed by another failure within 30 minutes. For example, a failure at 10 minutes followed by another at 55 minutes is not confirmed, but the third failure at 65 minutes would confirm the bite at 55 minutes, giving a CPT of 55 min.

Descriptive statistics include the mean and standard deviation of CPTs, failure rates and the numbers of failures. In addition, two general classes of statistical analyses may be applied. The first class compares the efficacy of test article(s) to the control and the comparison article(s). The percentage reduction in mean total bites or crossings is calculated as [1 - Mean comparator/Mean Untreated]100. The second class tests for uniformity over time in each treatment group to test the hypothesis that repellency does not decline with time. The distribution of scores and number of participants will determine whether parametric or analyses are employed. The results of these analyses are discussed with reference to the efficacy of each product, to the feeding biology of the pest species and to the context of product application.

C. Subject Selection

1) Who and Why. Most of our test subjects are undergraduate and graduate life sciences students enrolled at the University of California at Davis and other local state colleges and universities. As a group, college students possess the flexible schedules, physical vigor and enthusiasm for unusual

experiences that makes them ideal candidates for efficacy studies. The second subgroup of participants consists of staff and faculty members of such institutions and other college-educated adults from northern California who are similarly interested in scientific research also participate. A third group consists of mosquito and vector control professionals.

2) Total Number/Number per Group. Following US/*EPA* guidelines, a minimum of 6 subjects tests each test article, a minimum of 2 subjects tests each comparison article, and a minimum of 1 subject serves as untreated control. Because individuals vary in their attractiveness to biting insects and protection level from repellents (Carroll, in press), our tests sometimes use substantially more subjects than the US/*EPA* minimum, as arranged with the sponsor. Tests utilize similar numbers of males and females. A maximum of 35 subjects are engaged for any one test. Up to 350 subjects may be enrolled in a given year.

3) Inclusion/Exclusion Criteria. Participants are a minimum of 18 years old. Communication is conducted in spoken and written English. Participants must be able to read, write, understand and speak English at approximately the University of California college level. Subjects are excluded if they 1) report a hypersensitivity to insect bites or sensitivity to commercial insect repellents, eucalyptus or citrus oils or their components, or to plant-derived fragrances or related constituents commonly encountered in cosmetics, 2) express a strong aversion to the risk of being bitten by mosquitoes or other small-bodies biting flies, or to the handling of ticks, 3) are not confident of their ability to remain attentive during an approximately two to eight hour period of data collection, 4) are lactating or shown to be pregnant or 5) do not agree to sign a Consent Form after having been fully introduced to the study plan, the activities they are to perform, the risks involved, the compensation they will receive for their participation, and their rights as study participants. Pregnancy will be self-checked by each female volunteer on the morning of the repellent test using an OTC test kit provided by the Study Director. Results of each such test will be immediately verified by direct inspection by a female technician trained to make that assessment. Only volunteers scored as nonpregnant will be allowed to participate.

D. Participant Recruitment

1) Source(s): Participants are recruited by verbal networking through our academic and personal communities of friends, neighbors and scientists in Davis California. Individuals are recruited from the community specifically for each study. Studies are not conducted with individuals from particular employers or agencies.
2) Initial Contact Method: Initial contact is through word-of-mouth and telephone contact of individuals in our Volunteer Data Base.
3) Follow up Contact Method: Telephone interview, personal interview with the Study Director conducted at the Carroll-Loye Biological Research Offices.

E. Consent Process and Documentation

Interested individuals contact us by telephone to schedule a preliminary phone interview where the study and products are described and their questions are addressed. Exclusion criteria are exercised at this time. Risks of participation and rewards for participation are described.
All candidate participants take part in a mandatory 1.0 hour briefing before testing. Risks of participation are detailed, and test procedures (protocol, behavior to be maintained during the test, garments to wear, identification of arthropods, etc.) are explained.

Candidates are encouraged to withdraw for any reason should they be so inclined. It is explained that we have recruited an excess number of candidates, such that any individual who wished to withdraw will not compromise the quality of the study. A copy of the Experimental Subjects Bill of Rights (California Department of Pesticide Regulation) is given to each candidate and read aloud by the Study Director. Candidates may then review the Bill of Rights individually, and the Study Director addresses any additional questions at the time. Then, Consent Forms are distributed to the candidates, and the Study Director reads the form aloud. Questions are addressed at this time, and continue to be addressed as the candidates then individually review the Consent Forms. Candidates are asked to decline to participate or to provide a signed Consent Form within

48 hr. Examples of the Consent Form and the Experimental Subjects' Bill of Rights are included herein.

F. Procedures

1) Study Procedures

For each candidate repellent, trials are performed during natural periods of activity of the arthropods to be tested. For example, mosquitoes may be tested in early morning and late evening, while ticks may be tested at any time.

A standard distribution for a single test article is 6 subjects treated with the candidate repellent, 2 treated with a 20% commercial deet product as a comparison article, and 1 negative to evaluate biting rate at frequent intervals (e.g., one minute exposure each 15 min) throughout the trial. The carrier of the topical product, lacking the active ingredient, may be the appropriate control article in some tests.

Application of test materials may involve the distribution of multiple repellents among forearms and lower legs; in some cases it is also desirable that participants serve as internal controls (e.g., one limb is untreated). However, a basic approach is to have repellent applied to only one limb per participant. Before application, limbs are cleaned with a mild fragrance-free detergent and water, rinsed with 30% isopropyl alcohol, and dried with a clean towel.

Dosage depends on the active ingredient, its concentration, and the delivery system. A rate of approximately 1.0 - 3.0 ml/ 600 cm^2 of skin or fabric surface is a commonly used, industry standard range. Treatment areas are, for the forearm, from the bend of the elbow to the wrist, and for the lower leg from the bend of the knee to the narrowest portion of the leg above the ankle. The surface area of forearm and/or lower leg or covering fabric is used to compute the amount of repellent used. The area is computed as the average of three evenly spaced circumferences for the arm, or five for the leg, multiplied by the length of treatment area.

Trials are performed for approximately 2 to 8 hr, with individual exposures terminated when a confirming failure occurs. If the label of the compound being tested suggests reapplication (e.g., "reapply when effectiveness diminishes") a second application may be tested after the confirming bite. The area is retested in the same manner as above.

During (and before, as pertinent) field tests (and laboratory tests as appropriate), all participants are directed to observe the following mandatory procedures:

a) avoid use of perfumed products (such as soap, deodorant, after-shave) after 2100 hrs the evening before;

b) wear headnet, coveralls and gloves (as provided by the Study Director for field tests) and loose-fitting, tightly woven clothing of neutral color;

c) treated skin is exposed on a specified schedule and kept from contact with other surfaces;

d) participants stay at least 1 m apart and in areas of insect activity;

e) participants perform intermittent light activities (standing, walking, raising arms);

f) participants abstain from drinking alcoholic beverages and smoking during the test;

In addition, the following data are collected and procedures are followed during the test:

a) environmental conditions are recorded; sample test arthropods are collected;

b) for mosquitoes, a minimum estimated biting rate of 30 bites/hour must be observed on the control participant(s) before the initiation of a trial, and maintained during the entire period. If not maintained, the trial will be stopped and the results discarded;

c) at least one qualified supervisor will be present during the entire trial to ensure that the protocol is followed, to note any potential deviations and uncontrolled variables, to confirm biting rates and perform on-site identification of the trial insects;

d) environmental events likely to influence the test results (e.g., rain, strong wind) will automatically cancel the trial.

2) Time: Tests require up to approximately 8 hours (‘1 day’) of a participant’s time.

3) Study Sites(s): Field sites for tests are in California, chosen for their concentration of actively feeding mosquitoes or flies and their proximity our base in Davis. Laboratory tests are conducted at Carroll-Loye Biological Research (letterhead address).

G. Risks/Discomforts

In addition to the informed consent procedures described above, risks from exposure to arthropods are minimized by a combination of 18 other facts and approaches.

1) A qualified supervisor is present during all phases of the efficacy test.

2) Test repellents incorporate US EPA-registered or deregulated active ingredients in new delivery forms, or are patented developmental biologicals considered to be of reduced risk for topical application by the US/*EPA* or the US Food and Drug Administration.

3) All test materials have received low or lowest toxicity scores in oral, dermal and ocular toxicity test, as indicated by data provided by sponsors. Accordingly, risk from application to the skin is considered minor.

4) In field tests for registration, efficacy is normally demonstrated in prior laboratory tests by sponsors using caged mosquitoes. Accordingly, many participants typically receive no arthropod bite attempts, and few ever receive more than 5 bite attempts. No bite attempts occur during tick tests due to tick behavior (below).

5) Most tests are conducted only once. As a result, most participants are exposed to a particular formulation, or particular active, only once or twice by our testing program on an annual or multiple-year basis. Note also that tests with treated fabric will result in little direct contact of test formulae with subject skin.

6) Test participants are typically college-educated, vigorous individuals with field experience and training in the life sciences. A substantial portion of test participants has research experience and expertise in statistical thinking that aids in personal risk evaluation.

7) Participants are provided with and required to wear protective clothing and safety equipment.

8) Participants are instructed as to how to detect the initiation of biting by mosquitoes or other flies. Participants are directed to quickly remove arthropods before being bitten.

9) Participants work in pairs in field tests and are charged with monitoring for alighting arthropods on their partner throughout the test.

10) Participants in field tests are provided with hand-held electric entomological aspirators to quickly remove any mosquitoes that alight, before biting.

11) All test participants are orally informed of the risks of disease contraction in a clear and objective manner by the Study Director in advance of signing Consent Form, during pre-test meetings, immediately prior to the test, and during the conduct of the test.

a) Mosquitoes: Mosquito-vectored pathogens such as Western Equine Encephalitis and West Nile Virus are rare all test sites. State and local (mosquito abatement district) vector-borne disease data are monitored in advance of test date in the event that a true risk is ever indicated. Tests will not be performed if there have occurred more than one conversion in the nearest sentinel chicken flocks within one month of a test date. For laboratory testing, we rear disease free arthropods or obtain them from commercial and academic sources that specialize in their production for biomedical research. In lab tests with mosquitoes, individuals expose treated arms for no more than one minute at intervals of fifteen minutes.

b) Ticks and fleas: We conduct tick studies with unfed nymphal and adult ticks. At intervals of fifteen minutes, each participant places a tick on the hand with an artist's paintbrush. Ticks are observed as they approach the adjacent treated area of the arm, which begins at the wrist. Ticks are scored as crossing the repellent barrier if they move at least 2 cm into the treated area (toward the elbow) within three minutes. During this period, ticks are "questing" rather than feeding; feeding takes over an hour to initiate after site attachment, such that ticks in our study present no risk of biting. Ticks are contained in snap cap vials before and after testing, following the protocol of CDC. For fleas, exposures are10 seconds, after which they are quickly counted and brushed off, minimizing their time to penetrate the skin.

12) Untreated control individuals are always Study Directors or experienced management personnel.

13) Participants may withdraw from testing at any time for any reason without penalty, and they are informed of this fact in advance and during the test.

14) Individuals that display unanticipated dermal sensitivity to any bites inadvertently received during testing are removed from further exposure without penalty to their compensation for participation in that test.

15) At the discretion of the Study Director, any participant not adhering to test and safety guidelines during the test will be withdrawn from participation.

16) A complete first aid kit is maintained and carried by the Study Director. Participants are informed of its presence and location. Contact information for the nearest emergency medical facility (field: Rideout Hospital, 726 4th Street, Marysville, CA, telephone 530-749-4300; lab: (Sutter Davis Hospital, 2000 Sutter Place – Covell Blvd at Hwy 113, Davis 530-756-6440) is distributed to participants and posted at the study site during each test.

17) The Study Director carries a cellular telephone to contact emergency officials as needed.

18) Lastly, the risk of boredom and its associated problems such as distracting or detrimental behavior is lessened by the recruitment of groups of associated individuals who enjoy being together and who are aware of the importance of doing a good job (e.g., graduate students studying medical entomology).

H. Treatment and Compensation for Injury

In case of minor allergic responses to inadvertent insect bites or repellents, participants are informed in advance that the first aid kit contains antihistamines (diphenhydramine (50 mg tablets), chlorpheniramine maleate (3 mg tablets)), as well as topical steroid cream, that may be dispensed by the Study Director. Participants are kept well-informed before, during and after testing about relevant health issues, such as options for the treatment of skin irritations should they develop after the test. There is no plan for compensation for injury due to the low levels of risk involved in participation in this study.

I. Alternatives

All participation is voluntary.

J. Costs to Participants

There are no costs to the participants.

K. Payment to participants

The participants are paid an hourly rate of $14.00 for their participation.

L. Confidentiality of Records

Research records and Consent Forms containing participant names are archived at the Offices of Carroll-Loye Biological Research. All data collected will remain confidential and names will not be used in analysis. Absolute confidentiality cannot be guaranteed, since research documents are not protected from subpoena.

5. QUALIFICATIONS OF INVESTIGATORS

Appended.

6. REFERENCE TO SPECIAL REQUIREMENTS AND ATTACHMENTS

Documents are attached at the end of this protocol. In order, they are 1) Participant Consent Form, 2) Experimental Subject's Bill of Rights, 3) Qualifications of Investigators

7. BIBLIOGRAPHY

American Society of Testing and Materials. 2005. 939-94 (2005).

Carroll S.P. In press. Topical insect repellents and factors that affect their performance. In: *Insect Repellents* (Debboun M, Frances S, and Strickman D, eds). CRC Press, Boca Raton, FL.

US/*EPA*. 1999. OPPTS.3700. Insect repellents for human skin and outdoor premises. Public Draft.

8. State of California Department of Pesticide Regulation

EXPERIMENTAL SUBJECT'S BILL OF RIGHTS

The rights below are the rights of every person who is asked to be in a research study. As an experimental subject I have the following rights:

1. To be told what the study is trying to find out.

2. To be told what will happen to me and whether any of the procedures pesticides or devices is different from what would be used in standard practice.

3. To be told about the frequent and/or important risks, side effects, or discomforts of the things that will happen to me for research purposes.

4. To be told if I can expect any benefit from participating, and, if so what that benefit might be.

5. To be told the other choices I have and how they may be better or worse than being in the study.

6. To be allowed to ask any questions concerning the study both before agreeing to be involved and during the course of the study.

7. To be told what sort of medical treatment is available if any complications arise.

8. To refuse to participate at all or to change my mind about participation after the study is started. This decision will not affect my right to receive the care I would receive if I were not in the study.

9. To receive a copy of the signed and dated consent form.

10. To be free of pressure when considering whether I wish to agree to be in the study.

9. CV of Study Director

Scott Carroll

Education

1991 Ph.D. in Biology, University of Utah
1983 M.S. in Zoology (with Distinction) University of Oklahoma
1980 B.S. in Ecology (with Honors) University of Minnesota

Professional Experience

2004- Faculty in Tropical Medicine, Lake Atitlan Medical Project
2003-04 Senior Fulbright Scholar, Australian-American Fulbright Commission.
1998- Associate, Department of Entomology, University of California, Davis.
1997-98 Assistant Professor, Department of Biology, University of New Mexico.
1994- Director, Carroll-Loye Biological Research
1991-96 Postdoctoral Associate, Center for Population Biology, University of California, Davis.
1985-97 NSF- and NIH-funded researcher.
1985 Research Fellow, Smithsonian Tropical Research Institute, Panama.

Research

Botanical Insect Repellents
Insect Behavior and Evolution
Ecology and Conservation Biology

Consulting- insect repellency testing

Selected Sponsors: Avon Inc., L'Oreal/Cosmair Inc., Safe Solutions, Primavera Laboratories, Inc., Cosmederm, Inc., Merck (EMD) Inc., Wisconsin Pharmacal Inc., Agraquest, Hartz Mountain, Inc.

Research Reports (1989-2006)

Approximately 100 reports to industry and federal regulatory agencies, focusing on tests, scientific testing strategies and development for natural products developed to control and repel mosquitoes, ticks, fleas, and lice in humans and domestic animals.

Publications (1985-2006)

Approximately 45 peer-reviewed journal papers and book chapters.

Science Advisory Service

2006-08 Associate Editor, *Functional Ecology* (British Ecological Society).
2005-06 Advisory affiliate, Insect Repellents, Armed Forces Pest Management Board.
2005-06 Advisory affiliate, Insect Repellents, Walter Reed Army Institute of Medical Research.
1998-00 Advisor, Insect Repellent Testing Guidelines, US Environmental Protection Agency.

10. Protocol Approval Signature

30 March 2006

Scott P. Carroll
Sponsor and Study Director

Date