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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON D.C., 20460

OFFICE OF CHEMICAL SAFETY AND
POLLUTION PREVENTION

September 25, 2015

MEMORANDUM

SUBJECT: Science Review of a Laboratory Evaluation of Bite Protection from Repellent-Treated Clothing for the United States Military conducted with Human Subjects.

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ACTION REQUESTED

Conduct a science review of a completed laboratory study evaluating the bite protection of etofenprox treated military uniforms against mosquitoes. Determine the adequacy of the methods employed and the scientific validity of the reported data. These data are required to support the registration of EPA File Symbol 82392-G, Perimeter-Plus Insect Guard (0.9% etofenprox treated military uniform). The protocol used to conduct this study was previously reviewed and accepted by EPA and the HSRB on April 9, 2014. The protocol was amended to incorporate EPA and HSRB recommendations.

CONCLUSIONS

Scientific aspects of the special efficacy study to evaluate the bite protection efficacy of etofenprox-treated U.S. Military uniforms were assessed in terms of the recommendations of the EPA and of the EPA Human Studies Review Board. Study MRID 49684002 was conducted in accordance with Good Laboratory Practices as described in 40 CFR §160, and provides scientific data that are acceptable. The Human Studies Review Board will be asked to comment on this study.

SCIENCE REVIEW

Study objective: The objective of this study is to determine the bite protection level of etofenprox treated U.S. Military Fire Resistant Army Combat Uniforms (FRACUs) treated initially at an application rate of 0.9% etofenprox (weight/weight), and to assess the bite protection performance initially (0x) and after 20x, 50x, and 75x washes. This is a non-guideline study; therefore, it is not designed to fulfill the requirements of a specific OCSPP (formerly OPPTS) Guideline. This is considered a special study. This study was conducted in accordance with EPA, FIFRA (Federal Insecticide, Fungicide and Rodenticide Act), Good Laboratory Practice Standards (GLP); 40 CFR, Part 160 (October 1989). (§2.0, p. 14 of 285).

Identification of the test system: Replicate human subjects were used in this study to evaluate bite protection, which is a measure of the relative level to which a treated fabric prevents bites compared to the untreated control fabric. Two anthropophilic mosquito species (*Aedes aegypti* and *Anopheles albimanus*) were used as representatives for medically important mosquito species.

The fabric tested was U.S. Army Fire Resistant Army Combat Uniform (FRACU) treated with 0.9% etofenprox (wt/wt). This uniform is comprised of 65% Rayon, 10% Nylon and 25% *para*-aramid (Kevlar). For wearer comfort, this uniform is constructed with a more open weave, *i.e.* larger interstitial spaces between the fibers, which results in this uniform being the most difficult to repel mosquitoes from biting compared to all uniforms that the major U.S. military service branches have elected to treat.

Etofenprox treated samples, equivalent to the product being reviewed for registration, were sent from the manufacturing facility to the Natick Soldier Research, Development and Engineering Center (NSRDEC) for analysis and laundering in compliance with military standards. The widely accepted method of evaluating the efficacy of insecticide treated clothing includes laboratory aging of this treated clothing by laundering through standardized wash cycles per the American Association of Textile Chemists and Colorists (AATC) laundering protocol (See Appendix E, p. 202-203.). NSRDEC analytical evaluations were performed using Gas Chromatography (GC) according to the method described in Appendix IV (p. 237-239 of 285). Gas chromatograph (GC) testing confirmed an acceptable initial concentration of etofenprox in fabric samples, followed by a predictable decline in etofenprox content as the number of fabric washes increased (Table 1 on p.15 of 285). Appropriate samples of fabric were then sent to the

mosquito testing lab (CMAVE). Subsamples of unwashed fabrics selected for mosquito testing were sent to ADPEN Laboratories for independent analysis of etofenprox content. An independent analysis of unwashed, treated fabric was conducted by ADPEN Laboratories, Jacksonville, Florida (Appendix V). Analysis was conducted using HPLC and an ACE extractor and results (Table 2 on p.16 of 285) were comparable to those found by the NSRDEC (Table 1 on p.15 of 285). The fabric subsamples contained an average etofenprox content of 0.92% $\pm$ 0.05% (wt/wt), which was within the range of values acceptable to EPA for the nominal concentration of etofenprox in the proposed product.

For testing against mosquitoes at CMAVE, fabric samples (trouser fabric and coat fabric) were formed into tightly-fitting sleeves, the sleeves were placed onto each subject's arms, and then the subject's arms were introduced into test cages for a 15-minute exposure period for each fabric and respective washing regime for each mosquito species. Test cages (~59,000 cm_3) contained an average of 195 female mosquitoes; this is equivalent to one mosquito per 303 cm^3. Following each test, mosquitoes from the test cages were collected with an aspirator, knocked down with carbon dioxide, and then transferred to a cold table and sorted. Mosquitoes with visible blood in their abdomen were counted as having taken a blood-meal and remaining mosquitoes were crushed on white paper to verify the absence of blood.

Experimental design: The basic experimental unit in this study is a sleeve test. Each test involved a subject exposing a (unwashed treated, untreated, and washed treated) fabric-sleeved arm into a cage of one species of mosquito for 15 minutes per hour for up to eight hours. The data obtained from each 15 minutes test with each experimental unit were counts of the number of blood-fed female mosquitoes and the total number of female mosquitoes in each test cage. The observed bite-through proportion (or 'rate') is the proportion of blood-fed female mosquitoes to the total number of mosquitoes in each test cage, which is expressed as percent bite protection. Bite-through rates in the etofenprox treatment will be corrected using Abbott's formula for 'background' bite-through rates in the control (untreated fabric sleeve). To increase testing precision, each subject served as their own treatment and control. Therefore, the experimental design consisted of five groups tested in the following order per mosquito species. The test groups were:

- 1 test with an untreated FRACU fabric-sleeve, which serves as the control.
- 1 test with treated unwashed (0x) FRACU fabric.
- 1 test with treated washed (20x) FRACU fabric.
- 1 test with treated washed (50x) FRACU fabric.
- 1 test with treated washed (75x) FRACU fabric.

FRACU fabric from coats (shirts/blouses) and trousers was tested as described in the tables below (Table 4, and Table 5). Each subject (8 subjects) tested sleeves from each group (coats/trousers) once for each mosquito species, for a total of 8 replicates per group per mosquito species, which resulted in 16 replicates per fabric group for this experiment as shown in Table 4. The study director added the 75x washes treatment to the protocol following a discussion with EPA and received WIRB approval before starting the study.

The unit of measure for determining repellent effects in this experiment (% bite protection based on the proportion of blood-fed to total mosquitoes in a cage) differs from skin applied repellent evaluations where the "Landings with Intent to Bite" measure is used and efficacy is measured as Complete Protection Time. A detailed justification for the test system was presented in the protocol (§2.0 p.47 of 285) and in the study (§3.0, p.14 of 285). These specifications are in-line with current U.S. Military specifications for treated uniforms.

As illustrated in Table 3, which was presented to the Human Studies Review Board on April 20, 2014, the precision of the overall bite protection value for a treated fabric depended on the true bite-through rate for the control fabric and the true level of bite protection. The precision of overall bite protection was predicted to increase as the control bite-through rate and/or the bite protection increased. In each case, the precision was also predicted to improve with the number of subjects, although the benefit per additional subject was predicted to significantly decrease after eight subjects.

Table 3. Predicted Precision of Overall Bite Protection.

True bite-through rate for control fabric (θC)	**50%**		**20%**	
True bite protection for treated fabric (βT)[1]	**80%**	**95%**	**80%**	**95%**
Number of Subjects	**Expected half-width of a 95% confidence interval for % bite protection[2]**			
3	5.2%	2.7%	8.8%	4.5%
4	4.5%	2.3%	7.5%	3.8%
5	4.0%	2.0%	6.7%	3.4%
6	3.7%	1.9%	6.0%	3.0%
7	3.4%	1.7%	5.6%	2.8%
8	3.2%	1.6%	5.2%	2.6%
9	3.0%	1.5%	4.9%	2.4%
10	2.8%	1.4%	4.7%	2.3%
15	2.3%	1.2%	3.8%	1.9%
20	2.0%	1.0%	3.3%	1.3%

[1] Bite incidence for treated fabric was calculated from bite protection as $\theta T = \theta C(1 - \beta T/100)$.

[2] Average half-width from 1,000 simulated datasets. Each dataset consisted of S subjects testing a pair of fabrics (control and treated). For each pair the total number of mosquitoes (M) was a Poisson (200) random variable, and the number of blood-fed mosquitoes was simulated as a binomial (θ,M) random variable. Subject-subject differences were simulated by adding a subject-specific normal (0,0.3) random variable to the logit of the true incidence for both control and treatment fabrics. For each simulated dataset a binomial generalized linear model was fit to the data using the GENMOD procedure in SAS. The model specified fixed effects for both subject and test material and used a log-link. Bite protection confidence intervals were then obtained by back-transforming the intervals for the contrast log(θT)-log(θC).

Treated clothing sets were evaluated at specific standardized wash intervals: unwashed (0x), 20x washes, 50x washes, and 75x washes (Table 4). Separate fabric specimens for each wash interval were tested, similar to that described in U.S. military GL/PD specifications (Appendix I). Two species of mosquitoes, *Aedes aegypti* and *Anopheles albimanus*, were tested separately. Only two species were tested because the main determinant factor upon receiving accurate information is tied into the anthropophilic nature of the mosquito species and their response in laboratory assays. As such, prior studies of this nature have shown little difference between these species in their bite protection results. Addition of a third species (*e.g.*, *Culex* spp.) would not have contributed sufficiently distinct data to offset the burden to subjects from participation in this kind of study. Culex spp. tend to also have much lower bite-through rates.

Table 4. Experimental Design

Fabric and Treatment Condition[1]	Number of Fabric Specimens	Number of Subjects	Number of Species[2]	Total Replicates per Fabric Type
Coat Untreated Unwashed Control[3]	1	8	2	16
Coat Treated Washed 75x	1	8	2	16
Coat Treated Washed 50x	1	8	2	16
Coat Treated Washed 20x	1	8	2	16
Coat Treated Unwashed (0x)	1	8	2	16
Trouser Untreated Unwashed Control[3]	1	8	2	16
Trouser Treated Washed 75x	1	8	2	16
Trouser Treated Washed 50x	1	8	2	16
Trouser Treated Washed 20x	1	8	2	16
Trouser Treated Unwashed (0x)	1	8	2	16

[1] Fabric treatment conditions are either untreated and unwashed (Control) or treated and unwashed (0x), treated and washed 20 times (20x) or treated and washed 50 times (50x) or treated and washed 75 times (75x)

[2]The test species are *Aedes aegypti* or *Anopheles albimanus.*

[3]Each subject serves as their own control for the bite protection calculation.

Laboratory-reared 6 $\pm$ 1 day old mosquitoes (expressed as days since pupation) were used for the bite protection assay. Because various mosquito species have differing behavior and levels of aggressiveness, females of two of the more aggressive and anthropophilic species were tested. One of these selected species was *Aedes aegypti*, a vector of yellow fever and dengue fever that is found commonly in Asia and South America. The second species was *Anopheles albimanus*, a tropical mosquito that is a highly aggressive biter, one of the most difficult species to repel, and is a competent vector for malaria transmission. Testing started within 15 min of loading cages with mosquitoes. The control cages were used only for controls and washed after the completion of a set of sleeves with a subject.

Test procedure. All testing for each subject per mosquito species was conducted within an eight hour time period. All test subjects elected to test both arms (one with coat fabric and the other with trouser fabric) at the same time to reduce their time commitment. Subjects were exposed to each treatment type for 15 minutes. Subjects removed their arms after each testing interval and were permitted to take a break at their discretion. Testing continued once a subject was ready. The total time commitment was approximately two hours per each mosquito species. Tests were closely monitored by trained staff. No safety concerns or unexpected allergic reactions or physical distresses were observed.

Control sleeves were tested first, followed by fabrics that were laundered. The testing process was repeated until the control sleeves and the four different sets of treated sleeves (75x washes, 50x washes, 20x washes, and 0x washes) were tested for each mosquito species. Following each exposure period, mosquitoes from the test cages were collected with an aspirator by trained laboratory staff (Figure 5, p.23 of 285). Once collected, the mosquitoes were then transferred to a knockdown table (Figure 6, p.23 of 285) and counted. Once mosquitoes were removed, the bottom of each test cage was wiped with cleaning solution to avoid contamination.

Initially, eight subjects (four males and four females) were tested using each fabric type and mosquito species combination. Generally, influences related to the subject's individual attraction level, the host-seeking response of the test population, and corrections for the bite-through rate of untreated fabric are compensated for by testing a control sleeve. However, at the conclusion of testing on the initial eight, it was determined the tests with one female subject (subject #3) resulted in low control bite-through counts for both mosquito species so that test system could not be adequately evaluated. Rather than retest this subject against both species, another female (subject #4) was selected from the remaining alternates. This subject had one set of sleeves with very low bite-through amounts for the controls also. It was determined that this was caused by the use of mosquitoes that were incorrectly maintained and therefore did not respond as avidly to humans. Specifically, adequate sources of sugar and sucrose were inadvertently excluded from the rearing cage of a single batch of *An. albimanus.* The bite count analysis following the exposure of these specimen to test fabric indicated the population was compromised at the time of testing. Furthermore, the bite count analysis in subsequent testing of the same subject with *Ae. aegypti* indicated the subject was attractive to mosquitoes even though *An. albimanus* bite counts were aberrant. At the conclusion of retesting of this volunteer, the test system was considered to be fully and appropriately tested - for a total of four females and four males with "reliable" data - and a total of nine subjects in the experiment. These changes are reported as "Amendments #1 and #2" (Appendix II, p. 225-227 of 285). Each subject was tested with a single mosquito species on a given day; thus each test subject was exposed to mosquitoes on different days.

Protocol amendments and deviations. The approved protocol was dated May 21, 2015. **There were two amendments to the protocol** (Appendix II, p. 224-227). These amendments were discussed above and were considered necessary when a ninth subject, a female alternate (subject #4), had to be tested because the approved protocol was not explicit about how and when data collected from alternates would be used. In addition, **one more amendment should have been added** to the approved protocol: change in the test substance - use of a 0.9% etofenprox treated

fabric instead of 1.0% etofenprox treated fabric in mosquito testing. The 0.9% etofenprox treatment was a lower concentration of etofenprox than proposed in the original protocol, and very close to the certified limit for the nominal concentration of etofenprox in the original protocol. This change did not present any risk to the subjects and did not impact study results.

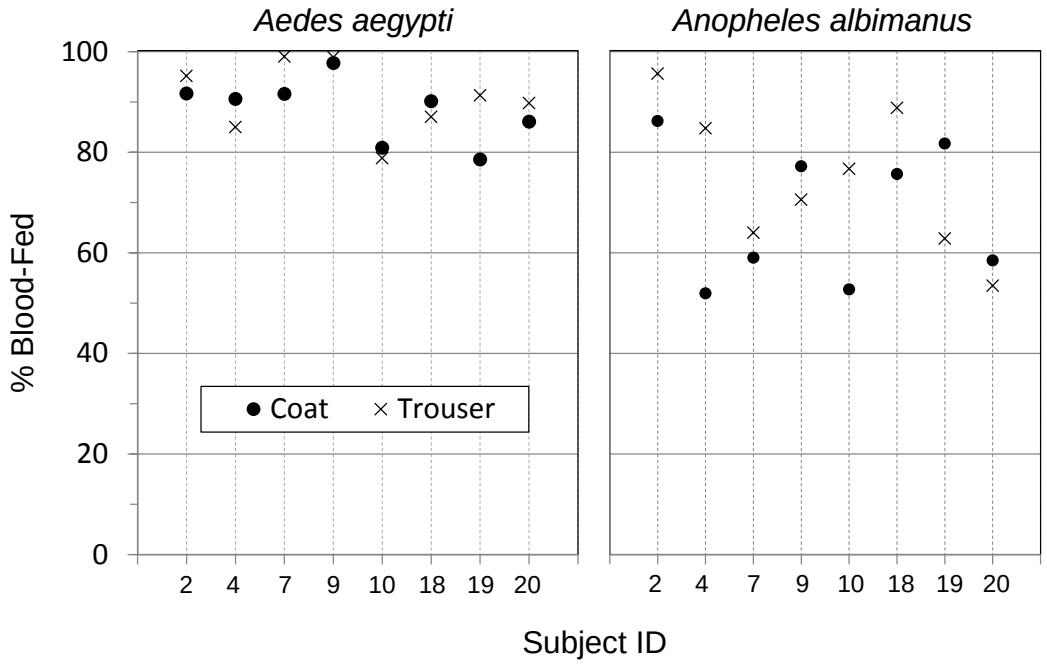
Eight protocol deviations occurred during the study (Appendix II, p. 228-230). Seven of the deviations did not impact the study, but one deviation – use of a different statistical analysis – will be discussed below in the data analysis section.

Results and analyses.

Test sleeves. The fabric subsamples that were tested at NSRDEC and ADPEN laboratories contained an average etofenprox content of 0.92% $\pm$ 0.05% (wt/wt), which is within the range of values acceptable to EPA for the nominal concentration of etofenprox in the proposed product.

Bite protection. Mosquitoes that visibly had blood in their abdomen were counted as having taken a blood-meal. All other mosquitoes were subjected to a crush test to determine if they had fed. The laboratory technician recorded mosquito counts until the same count of blood-fed mosquitoes was recorded twice. The results are summarized below.

Observed Feeding (bite-through) Rates, Untreated Controls. (Figure 7, p. 31 of 285)



Analysis of Bite Protection Experiments
Mean and Range of Control Blood-Feeding Rates (p. 265 of 285)

Species	Nr Obs	Mean PctFed	Min PctFed	Max PctFed
Aedes aegypti	16	89.5241	78.5714	99.0385
Anopheles albimanus	16	71.2327	51.9048	95.6311

Percent Bite Protection (Tables 6, 7 and 8; p. 31-33 of 285)

Ae. aegypti bite protection means for each fabric type and washing condition are given in Table 6. Established bite protection reference values applicable to fabric washed 0, 20, and 50 times are shown **(there is no DoD reference level established for 75 washings).** All bite protection means exceeded their respective DoD % bite protection reference levels.

Table 6. Percent Bite Protection, *Aedes aegypti*.

Number of Washes	Reference % Protection (DoD)	Fabric Type	Percent Bite Protection				
			Mean	Std. Error	2-sided 95% Confidence Interval	1-sided Confidence Interval	
						95% Lower Bound	Confidence of Exceeding Reference
0	85	Coat	92.7	1.8	88.6 - 96.9	89.4	>99%
		Trouser	88.3	3.1	80.9 - 95.7	82.4	84%
20	80	Coat	95.2	1.5	91.6 - 98.8	92.3	>99%
		Trouser	95.0	1.3	91.8 - 98.1	92.4	>99%
50	70	Coat	96.1	1.2	93.4 - 98.9	93.9	>99%
		Trouser	94.4	1.8	90.2 - 98.6	91.0	>99%
75	Not applicable	Coat	92.9	2.1	88.1 - 97.8	89.0	Not applicable
		Trouser	93.2	1.9	88.8 - 97.6	89.7	

Although the DoD reference bite protection levels apply only to observed means, it is also of interest to note that in only one instance did the confidence limits extend slightly below the DoD reference level. EPA prefers 90% as a reference level. The coat and trouser are made of the same fabric and averaging these values resulted in > 90% bite protection for all wash cycles.

Table 7 provides an analogous summary of the *An. albimanus* bite protection results and Table 8 does the same for analyses of bite protection averaged over both species. In all cases, the mean bite protection for every treatment combination exceeded 90% and any appropriate reference

level. In addition, the lower bounds of both types of confidence interval either equaled or exceeded the bite protection reference levels.

Table 7. Percent Bite Protection, *Anopheles albimanus*.

Number of Washes	Reference % Protection (DoD)	Fabric Type	Percent Bite Protection				
			Mean	Std. Error	2-sided 95% Confidence Interval	1-sided Confidence Interval	
						95% Lower Bound	Confidence of Exceeding Reference
0	85	Coat	93.4	2.5	87.4 - 99.3	88.6	>99%
		Trouser	93.6	2.2	88.3 - 98.9	89.4	>99%
20	80	Coat	96.4	1.1	93.8 - 98.9	94.3	>99%
		Trouser	96.4	0.9	94.2 - 98.5	94.6	>99%
50	70	Coat	95.9	1.0	93.4 - 98.4	93.9	>99%
		Trouser	95.2	1.9	90.7 - 99.7	91.6	>99%
75	Not applicable	Coat	95.8	1.6	92.0 - 99.6	92.7	Not applicable
		Trouser	96.4	1.2	93.6 - 99.2	94.2	

Table 8. Percent Bite Protection, Both Mosquito Species.

Number of Washes	Reference % Protection (DoD)	Fabric Type	Percent Bite Protection				
			Mean	Std. Error	2-sided 95% Confidence Interval	1-sided Confidence Interval	
						95% Lower Bound	Confidence of Exceeding Reference
0	85	Coat	93.0	1.4	89.8 - 96.3	90.5	>99%
		Trouser	91.0	2.6	84.8 - 97.1	86.0	97%
20	80	Coat	95.8	1.1	93.1 - 98.5	93.6	>99%
		Trouser	95.7	0.9	93.4 - 97.9	93.9	>99%
50	70	Coat	96.0	1.0	93.6 - 98.4	94.1	>99%
		Trouser	94.8	1.7	90.9 - 98.7	91.6	>99%
75	Not applicable	Coat	94.3	1.6	90.6 - 98.1	91.4	Not applicable
		Trouser	94.8	1.2	91.9 - 97.7	92.5	

The blood-feeding (or fabric bite-through) rates on non-treated fabric were very high, exceeding the 50% level for all human test subjects, although there were differences between the two mosquito species. These results indicated that the untreated FRACU fabric provided a reasonable worst-case scenario for intrinsic bite protection provided by the content and weave of non-treated fibers.

In all but one species-fabric-washing combination, the mean bite protection exceeded 90% (the lone exception was *Ae. aegypti* vs. unwashed treated trouser fabric (88% bite protection). With

only one exception, the lower limit of 95% confidence intervals met or exceeded the reference bite protection levels established for observed means.

In all cases, the mean bite protection provided by etofenprox-treated fabric against both mosquito species (as well as for the average of both species) was always greater than the standard reference level for FRACUs in specifications from the Department of Defense, regardless of the number of washes. The etofenprox treated fabric provided >90% bite protection against both species through 75x washes. At 0x washes the reason the bite-through rates appear slightly lower is because some treated fabrics require washing to make the active ingredient fully available for bite protection due the binders used and textile finishes applied over the fabric surface.

Statistical Analysis.

The statistical methods used to analyze the data collected in this study are listed as the eighth protocol deviation in this study (p. 230 of 285)

> **Summary of statistical description from USDA-ARS protocol from April 2014 HSRB meeting and on May 21, 2015 (p. 52-52 of 285): "**The primary objective of the data analysis is to estimate the overall (or 'mean') level of bite protection and associated 95% confidence interval for different 'treatments' (i.e., different combinations of fabric type, number of washes, and mosquito species). Subject-specific bite protection values will be calculated for each treatment using Abbott's formula as described in §8.5. These values will be averaged over all subjects to obtain mean observed bite protection values that can be used as a check on any model-based bite protection estimates.
>
> The numbers of blood-fed and total female mosquitoes found with treated and control fabric for each subject will be analyzed as binomial distributed data in a generalized linear model (GLiM) using a log link. A subject term will be added as a fixed effect in the model to adjust for subject-subject differences. (Alternatively, subjects could be treated as a random effect and the within-subject correlation accommodated using either generalized estimating equations or a mixed effect GLiM. The decision on how to analyze the collected data needs to be finalized.) Use of the log link makes it possible to obtain an estimate and confidence interval for the ratio of the treatment and control bite-through rates. The estimates and confidence intervals for percent bite protection are obtained from the relationship:
>
> $$\text{Percent Bite Protection} = [1 - (\text{treatment rate}) / (\text{control rate})] \times 100\%$$
>
> The GLiM model-based bite protection estimates could be obtained by analyzing multiple models each with just a single treatment group and the matched control group. However, it may also be of interest to compare the bite protections of different types of treated fabric, number of washes, or mosquito species. In this case, it would be necessary to include all of the treatments (and species) of interest in the same model. Because the GLiM uses a log link, hypothesis tests concerning ratios of bite protection can be formulated as linear contrasts in the GLiM."

Comments from the EPA review (March 21, 2014) of the USDA-ARS protocol presented to the HSRB in April 2014:

"The statistical analysis must be finalized. Two approaches to GLiM use are discussed in the present version (V1: 21-22). 'Recommendations for Data Analysis' expands on this discussion in V3: 13. The protocol should be amended to include the selected analysis before the study is executed."
(The reference to Volume 3 (V3) is: *Volume 3: Sielken, Jr., R. L., and L. R. Holden. 2014. Statistical Methods: Supplemental Information, Sielken & Associates Consulting, Inc.* A copy has been attached to this review.)

EPA response based on this study: The statistical analysis was not revised and finalized in the protocol used for conducting this study. Instead, the study director made a decision on the statistical methods after protocol approval and reported the selected method as a protocol deviation with no effect on the study results and conclusions. The deviation is shown below.

Protocol Deviation:

"Date: 27-Jul

Deviation: Bite protection data was not analyzed using a generalized linear model (GLiM) with a log link.

Reason for Deviation: GLiM-based confidence intervals are inappropriate when there is subject-to-subject variation. A t-distribution confidence interval was used instead because it provided more accurate confidence intervals.

Effect on Study: None."

The full statistical analysis report is presented in Appendix VI (p. 248-285) of the study and is summarized below.

The primary purpose of the statistical analysis was to quantify the uncertainty in estimates of mean bite protection obtained for various treatments involving etofenprox-treated fabric. In particular for each treatment, T, the following questions were examined:

1. What was the mean bite protection and its standard error?
2. What was the range of values expected to contain the true bite protection (β_T) with 95% confidence?
3. What was the smallest value of true bite protection consistent with the data, with 95% confidence?
4. What level of confidence was associated with the true bite protection being at or above a reference value associated with a particular treatment?

These questions were addressed for each species of mosquito separately as well as for bite protection averaged over both species.

For each mosquito species separately, the simple mean bite protection over the 8 replicate subjects and its associated standard error were calculated for each of the 8 fabric-washing combinations (*i.e.*, 2 fabric types and 4 levels of washing).

A slightly modified approach was used to obtain the corresponding average bite protections for both species combined. The 16 'replicate' bite protection values could be used to obtain a pooled mean bite protection. However, because there is expected to be same-subject correlation in bite-protection for the two species, the 16 pooled values would not provide a valid estimate of the standard error (and associated degrees of freedom). Therefore, the 2 bite protection values were first averaged over species for the same subject to obtain 8 two-species average bite protections. This resulted in 8 independent bite protection 'averages'. Their average and an associated standard error were then calculated for each fabric type and washing condition separately as if there were 8 independent replicates. Because every subject provided a bite protection value for both species, the average of the eight 2-species averages was identical with the average of all 16 individual bite protections.

Two-sided and one-sided confidence intervals are used to examine the uncertainties associated with the mean bite protection estimates. Individual bite protection values are a function of individual counts, and, in theory, are unlikely to be purely normally distributed with equal variances. This limits the use of a single statistical model for the entire dataset. However, as long as attention is focused only on means of individual sets of 8 replicate bite protection values, simple (and robust) confidence interval methods using the t-distribution are reasonable. Such intervals only assume that the mean of 8 replicate bite protection values approximate a normal distribution. In addition, t-based intervals are fairly robust to deviations from this assumption. Simulations of bite protection datasets indicated that under a large variety of conditions, the t-based confidence intervals will have accurate coverage as long the majority of replicate subjects have less than 100% bite protection (*i.e.,* no blood-feeding at all). In this dataset, 100% bite protection was fairly uncommon and was not found in a majority of replicates for any combination of fabric type and washing condition.

Therefore, CL was calculated as the probability (×100%) that a t random variable with 7 degrees of freedom was less than or equal to Q. The t-distribution probabilities can be computed within most statistical software or, more simply, by just using the T.DIST function in EXCEL. Although is not theoretically a probability, CL can still be intuitively interpreted as "the confidence we have that a mean bite protection calculated from thousands of subjects would exceed RL."

Version 9.2 of SAS was used for all of the above calculations. This included calculation of individual blood-feeding rates and bite protection values from blood-fed mosquito counts.

HSRB Comments and Science Recommendations from the April 2014 Meeting Report Dated June 21, 2104

Science

"EPA's science review resulted in multiple comments. The Board reiterated several of EPA's recommendations:

- The HSRB agrees that the statistical analysis must be finalized prior to initiation of the study.

  EPA comment: The final study protocol dated May 21, 2014 did not include a finalized statistical analysis. The statistical methods use to analyze the results were reported as a protocol deviation.

- The Board agrees that at least 20 qualified subjects should be identified and that participants be selected at random from that larger group.

  EPA comment: The final protocol described recruitment of 20 subjects (§5.5, p. 19 of 285).

- The HSRB is concerned that two alternate participants is an insufficient number due to potential for dropout.

  EPA comment: The protocol was revised to include the random selection of eight subjects for testing while identifying the reaming 12 subjects from the pool of 20 as alternates (§5.5, p. 19 of 285).

- The Board agrees that the study should not be conducted until a product-specific fabric irritancy study is completed.

  EPA comment: The dermal irritation study was completed before the study began. Dermal irritation did not occur in the toxicology study or in the efficacy study.

The HSRB made the following additional comments:

1. Statistical Design. The proposed protocol is generally well designed from a statistical standpoint. Because the desired scope of inference is beyond the subjects in this study, each subject should be treated as a random effect (not as a fixed effect).

Maintaining the sample size of 8 is a concern to the Board. This number does not allow for the possibility of dropouts; the HSRB recommended that additional participants be recruited so that a pool of alternates is available to ensure that the desired number of 8 completed tests is achieved. Questions were raised about how dropout data would be handled; *e.g.*, would it be analyzed or eliminated?

EPA comment: There were no dropouts in the study. One subject was discarded and replaced by an alternate due to low untreated control bite-through numbers. The data of the alternate was included in the data analysis.

Furthermore, if one arm of each participant is used as a control, then double the sample size would be needed. To reduce the burden of testing on each participant, another option would be to have 16 participants on whom the tests for only one species are conducted.

EPA comment: The study director retained eight test subjects in the study.

Additionally, the number of participants by gender could be restricted, rather than left to chance, in the enrollment process.

If the treatment could be randomized instead of ordered by dose, the study design would be stronger. The Board asked the Agency to reconsider how the treatments will be assigned to participants.

EPA comment: Subject selection was randomized but order of washes was retained from untreated to 75x to 50x to 20x to avoid any possibility of etofenprox carryover that might impact results.

2. Fabric sleeves. The Board believes that provision of a single sleeve size is inappropriate given the possible relationship between snugness of fit and bite protection. A range of sleeve sizes should be available corresponding to at least a portion of the uniform sizes available to soldiers; one-to-one correspondence is not required. However, each subject should be provided sleeves corresponding to their normal garment size. It is desirable to avoid rejecting either large subjects who cannot fit their arms into the "one sleeve size," or small subjects for whom the sleeves will be too loose. The Board heard that most soldiers do not wear their clothing tight; therefore, the HSRB recommended that the standard test conditions be as representative as possible of use conditions in the field. Characterization of the tightness of the sleeve should be recorded to enable comparing test outcomes and thereby gain insights for future protocols.

EPA comment: The protocol was revised to accommodate size differences in subjects' forearms. Three different sleeve sizes were made: small, medium and large (§5.8, p. 20 of 285)

4. Bite pressure. The Board noted that "bite pressure" is not clearly defined in this proposed protocol. The HSRB recommended that the term be clarified before the study begins.

EPA comment: The protocol and study defines 'biting pressure' in terms of cage density with an average density of one mosquito per 303 cm^3 of mosquito cage volume. The average number of mosquitoes per cage was 195.

5. Carryover. The proposed protocol assumes that each individual will conduct 8 two-arm trials on a single day. These studies would be conducted sequentially using each of two mosquito species. Each within-species protocol would be ordered as follows: untreated sleeves, 50x washed sleeves, 20x washed sleeves, 0x washed sleeves. This sequence minimizes, to the extent possible, carryover effects in within-species testing. However, the 0x sleeves from the first mosquito species will precede the untreated sleeve trials for the second species. Arms are to

be washed in between, but the efficacy of washing is unknown. A more conservative strategy would be separation of species trials by sufficient time (days) to allow excretion of absorbed etofenprox.

EPA comment: The protocol was revised to address the Board's concern. Subjects were only tested against one species per day (p. 50 of 285).

6. Additional comments. The HSRB raised concerns about the possibility of treatment failure. One way to address this concern would be to test repellency effectiveness on one or two participants before the proposed study begins.

EPA comment: Treatment failure did not occur.

Additional comments continued: The scientific justification for conducting human research was articulated during the Board meeting but is not clearly documented. The Board recommended that the rationale for doing this research on humans be fully built and explicitly stated in the proposal.

EPA comment: The study director summarized the need to conduct human research as follows (p. 77 of 285).

"15.0 ALTERNATIVES TO HUMAN USE

There are no viable alternatives to human use for this type of study. The objective of this study is to evaluate the bite protection performance of insecticide/repellent treated specimens of military clothing. The mosquito species that are to be used in this study are attracted to a number of cues, including heat and chemical compounds that are exuded by the host. Because the nature of these attractants is not fully understood, use of a non-human model is unlikely to deliver representative data."

Conclusion: The methods employed in this study were adequate to produce scientifically reliable data. They were based on the study protocol as amended before testing began. Etofenprox treated fabric provided a high degree of bite protection against mosquitoes. The reported protocol deviations was non-substantive in nature and did not affect the conduct of the research or the resulting data.

EPA Recommendation: The study is scientifically sound and acceptable.