# GUIDELINES FOR EFFICACY TESTING OF MOSQUITO REPELLENTS FOR HUMAN SKIN

**World Health Organization**

# GUIDELINES FOR EFFICACY TESTING OF MOSQUITO REPELLENTS FOR HUMAN SKIN



**CONTROL OF NEGLECTED TROPICAL DISEASES**
**WHO PESTICIDE EVALUATION SCHEME**

# CONTENTS

## ACKNOWLEDGEMENTS

# 1. INTRODUCTION

The purpose of these guidelines is to provide specific and standardized procedures and criteria for efficacy testing and evaluation of mosquito repellents for human skin. Their aim is to harmonize the testing procedures carried out in different laboratories and institutions in order to generate comparable data for registering and labelling such products by the national regulatory authorities. However, the requirements for registration of pesticides, including repellents, are determined by the national regulatory authorities.

These guidelines are an expanded and updated version of those recommended by the WHO Pesticide Evaluation Scheme (WHOPES) Informal Consultation on the evaluation and testing of insecticides, held at WHO headquarters in Geneva, Switzerland, on 7–11 October 1996 (1). These were reviewed and recommended by the WHO Consultation on testing and evaluation of public health pesticides, held at WHO headquarters on 23–27 February 2009.

The guidelines provide guidance and procedures on laboratory studies, field trials and evaluation of technical material used in mosquito repellent products and on the methods used to determine their application rate(s) and effectiveness. Guidance is also provided on the single-dose evaluation of formulated repellent products. With some modification, the guidelines can be used to determine the repellency of candidate compounds for other flying insects that blood-feed on humans.

Detailed treatment and analysis of repellent safety and toxicity data are beyond the scope of these guidelines, and it is assumed that preliminary human safety assessments have been undertaken before the material(s) are applied to human skin. Any side-effects and/or undesirable characteristics experienced in association with the application and use of repellents in laboratory studies and field trials should be recorded and reported. The protocol must include provision for medical care and the reporting of adverse events.

Products submitted for laboratory studies and/or field trials should be accompanied by the Material Safety Data Sheet, the labelling recommendation and the manufacturer's certification that the product is within the company's manufacturing specifications for that product. Independent physical and chemical assessment may be required before initiating the efficacy studies.

Biological tests are subject to the variation that accompany living organisms. Studies should therefore be conducted under the close supervision of personnel familiar with biological testing of repellents and with sound scientific and experimental procedures; the principles of good laboratory practice or other suitable quality schemes such as the International Organization for Standardization should be applied.

Studies on repellents should be undertaken in accordance with the applicable national ethical regulations. WHO guidelines for development of an informed consent form are provided in Annex 1.

Table 1 summarizes the sequence of stages for evaluating technical grade repellent materials and single-dose formulations of repellent products applied to human skin for personal protection.

**Table 1 Sequence of stages for evaluation of technical grade repellent materials and single-dose formulations of repellent products applied to human skin for personal protection**

|  |  | Parameters measured | Analysis suggested |
|---|---|---|---|
| Technical grade material | Laboratory studies | - effective dose | Probit–plane regression analysis |
|  |  | - complete protection time | Kaplan–Meier survival analysis |
|  | Field trials | - median effective dose;<br>- median effective time;<br>- repellent half-life | Probit–plane regression analysis |
|  |  | - complete protection time | Kaplan–Meier survival analysis |
| Formulated product | Laboratory Studies | - complete protection time | Kaplan–Meier survival analysis |
|  | Field trials | - complete protection time | Kaplan–Meier survival analysis |

## 2. LABORATORY STUDIES

The objective of laboratory studies is to estimate the effective dose of a repellent and the complete protection time provided by a repellent after application on the skin.

The specific aims of these tests are:

to estimate dose–response lines and effective doses (EDs) of a repellent corresponding to 50% ($ED_{50}$) and 99.9% ($ED_{99.9}$) protection from mosquito landing and/or probing;[1]

to estimate the complete protection time (CPT) of a repellent, which is the time between the application of the repellent and the first mosquito landing and/or probing.

Deet (*N,N*-diethyl-3-methylbenzamide) is the active ingredient of most commercially-available repellents and is recommended as the positive control (usually 20% in ethanol) against which the effectiveness of alternative mosquito repellents is judged.

Testing of repellents on human subjects is the method of choice as it utilizes the repellent end-user in the testing process and yields results that are relevant to the actual conditions of use. Use of laboratory animals or artificial membranes may inadequately simulate the situation in which repellents for use on human skin are intended to perform. Tests are carried out on adult human volunteers who may be selected from among candidates exhibiting mild or no sensitivity to mosquito bites. Equal numbers of male and female test volunteers are preferred.

---

[1] Landing and/or probing behaviour signifies the end-point of the repellent efficacy test. However, landing is not always associated with probing, and separate recordings of each behaviour may be needed. A repellent may provide efficacy by a reduction in biting activity but not in landing. Alternatively, it may provide efficacy in terms of landing, but those mosquitoes that land may all bite. Both scenarios may be important in determining the efficacy of a repellent.

In preparation for the laboratory studies, the test area of the volunteer's skin should be washed with unscented soap and rinsed with water, then rinsed with a solution of 70% ethanol or isopropyl alcohol in water and dried with a towel. Given the possibility that various factors may alter a person's attractiveness to mosquitoes, and that this may in turn affect the outcome of repellency assays, test volunteers should avoid the use of fragrance and repellent products for 12 hours before and during testing. Volunteers should preferably not be tobacco users, or at least to have refrained from tobacco use for 12 hours prior to and during testing.

Standardized mosquito rearing and laboratory testing conditions are essential to ensure the reliability and reproducibility of data. Mosquitoes should be reared, maintained and tested (in a separate space or room) at 27 ± 2 C temperature, ≥80 ± 10% relative humidity, and a 12:12 (light:dark) photoperiod. Temperate mosquito species may require modifications to rearing conditions. Stock populations of adult mosquitoes should have access to sugar solution but not have been blood-fed. Observations of repellency should be made using female mosquitoes starved for the preceding 12 hours and, where practical, during times in the diel period that correspond with biting activity by that species.

Mosquito repellency tests should be conducted with three or more of the more anthropophilic *Aedes* (preferably *Aedes aegypti*), *Culex* (preferably *Culex quinquefasciatus*) and *Anopheles* (preferably *An. stephensi*, *An. gambiae* or *An. albimanus*) species. The test species, strain and age should be reported. Mosquitoes should be contained during testing using a cage (suggested metal frame for ease of decontamination, size: 35 –40 cm per side) with a solid bottom and top, screen or netting on the back, a clear acrylic sheet (for viewing) on the right and left sides, and a fabric sleeve for access on the front. Female mosquitoes should be collected from a stock population cage in which both sexes have been maintained to allow mating to occur. They should be host-seeking, of uniform age, preferably 5–7 days post-emergence (use different ages of mosquitoes when it is more suitable for a particular species and

justify such use in the study report). Active host-seeking females should be selected to ensure a good response from the test mosquitoes using an aspirator or an appropriate airflow apparatus.

## 2.1  Estimation of effective dose (technical material)

Serial dilutions of repellent are made with ethanol or another suitable diluent and tested to identify an effective dose range. Dosages giving responses between 10% and 90% are used for this analysis, preferably 2–3 dosages that give <50% repellent response and 2–3 dosages that give >50%.

Each volunteer uses incremental doses on the test forearm so that at least five successive applications of increasing dose are used by each volunteer. A single test comprises continuous use of the same mosquitoes by the same volunteer and is completed in one day. Replicate tests repeat this process using different batches of mosquitoes over several days. It is recommended that a minimum of three replicates be conducted per volunteer, with the number of volunteers sufficient to allow for statistical analysis.

One mL of ethanol or the same diluent used in the preparation of the test repellent is applied evenly using a pipette to ≈600 cm$^2$ of the forearm skin between the wrist and elbow (Annex 2) and allowed to dry (approximately 1 min for ethanol). Before insertion of the arm into the cage containing 50–100 female mosquitoes, the hands are protected by gloves made of material through which the mosquitoes cannot bite. The first step is to insert the forearm applied with diluent into the cage and to count the number of mosquitoes that land on and/or commence to probe the skin during a 30-second period. During testing, the volunteer should avoid movement of the arm. For the test to proceed, the biting rate must be ≥10 landings and/or probings in the 30-second period. The control forearm is carefully withdrawn and this arm is then treated with the lowest dose of repellent in 1 mL diluent and allowed to dry. The treated arm is placed in the cage for another 30-second period and

observed for mosquito landings and/or probings. This procedure is repeated for each additional incremental repellent dose. Successive tests should be carried out one after the other without delay and the repellent dose at each test calculated as the sum of the doses applied to arrive at the cumulative dose for each test (Table 2).

Table 2 **Example of successive doses applied to arrive at a cumulative dose for a sample experiment**

| Application sequence | Repellent solution concentration to be applied in 1 mL (mg/mL) | Cumulative amount of repellent (mg/600 cm$^2$ area) |
|---|---|---|
| Left-arm control | Pre-treated with alcohol[a] only | – |
| Left-arm dose 1 | 1 | 1 |
| Left-arm dose 2 | 1 | 2 |
| Left-arm dose 3 | 2 | 4 |
| Left-arm dose 4 | 4 | 8 |
| Left-arm dose 5 | 8 | 16 |
| Right-arm control | Pre-treated with alcohol[a] only | – |

[a] Alcohol or the same diluent as that used in the preparation of the repellent solution.

If, at any time, the landing and/or probing rate is too high to accurately count the number of mosquitoes landing and/or commencing to probe the skin, the mean landing and/or probing rate for the test should be calculated from a series of three readings, each five seconds long, and the sum multiplied by two to estimate the landings and/or probings that would occur in a 30-second period. Testing should not proceed when the

mosquito landing and/or probing rate on the exposed forearm is <10 females in 30 seconds.

This procedure should be used consistently throughout the experiment. The trained volunteer will record the number of landings and/or probings. At the conclusion of the dose–response experiment, 1 mL alcohol/diluent is applied on the other forearm and allowed to dry. This forearm is inserted in the cage for 30-seconds to verify that the number of landings and/or probings is approximately ≥10 per 30 seconds, as was observed at the beginning of the experiment. If the rate is <10 females in 30 seconds, the results of this experiment should be discarded.

Protection ($p$) is expressed as a proportion of the number of mosquito landings and/or probings on the treated arm ($T$) in relation to the number of landings and/or probings on the control arm ($C$) of the same individual:

$$p = 1 - ( T / C ) = ( C - T ) / C \qquad\qquad \text{(i)}$$

where C is the average of the landings/probings on the two untreated arms (the diluent-applied test arm before repellent-treatment and the other arm at the end of the experiment). Data are analysed using probit-plane regression analysis from which the $ED_{50}$ and $ED_{99.9}$ and their confidence limits can be estimated (2).

## 2.2 Estimation of complete protection time (technical material and formulated product)

The complete protection time, or CPT, of a repellent can be determined in one of two ways. Preferably, the $ED_{99.9}$ dose should be estimated using the procedures outlined in section 2.1; 1 mL of the repellent is then tested at the $ED_{99.9}$ level against 1 mL of the standard 20% ethanolic deet. Alternatively, 1 mL of the 20% ethanolic deet solution can be compared with the same amount (weight/weight) of the candidate repellent on the other arm. In both cases, treatments are applied to

≈600 cm$^2$ area (Annex 2) of the forearm skin between the wrist and elbow.

Two mosquito cages (size: 35–40 cm per side) each containing 200–250 non-blood-fed females are normally used. One cage is designated for testing the candidate repellent and the other for the positive control (ethanolic deet). During testing, the hands are protected by gloves made of material through which the mosquitoes cannot bite while the volunteer avoids movement of the arm.

Initially, the readiness of mosquitoes to land and/or probe must be assessed by inserting an untreated (alcohol- or diluent-treated) arm into a cage for 30 seconds or until 10 landings/probings are counted. The procedure is repeated with the other arm in the second cage. If this level of landing and/or probing is not achieved in either cage, the experiment should be discarded.

Before testing commences, 1 mL of the candidate repellent prepared in alcohol/diluent solution is applied to one arm and 1 mL of the deet standard solution is applied to the other arm. After 30 minutes, the repellent-treated arm is inserted into the appropriate cage and exposed for 3 minutes to determine landing and/or probing activity. Next, the deet-applied arm is exposed to determine landing and/or probing activity. This procedure is repeated at 30- or 60-minute intervals and should be used consistently throughout the experiment. The occurrence of one landing and/or probing in a 3-minute test interval concludes the test for that repellent dose. Complete protection time is calculated as the number of minutes elapsed between the time of repellent application and the first mosquito landing and/or probing. Most repellent studies of technical material are completed in 8 hours or less.

The number of volunteers included in the test should be sufficient to allow for statistical analysis. The median CPT and confidence interval can be estimated from the Kaplan–Meier Survival Function (Annex 3).

## 3. FIELD TRIALS

The objective of field trials is to extend the results of laboratory testing to estimate the optimum application dose, persistence and efficacy of a repellent material, in terms of repellency and protection time, against one or more mosquito vectors and/or pest species in different ecological and/or geographical settings. A minimum of two field tests are recommended, one each in different ecological and or geographical settings suitable for the target mosquito species where the human exposure occurs.

Assessment is made by human volunteers collecting mosquitoes landing and/or probing on one or more bare limbs (knee to ankle; elbow to wrist), depending upon the biting behaviour of the mosquito species. The volunteers should be: (i) from the same settings where the test is conducted, such that they are not exposed to unusual risk of infection; (ii) if appropriate and applicable, protected by chemoprophylaxis and/or vaccination; and/or (iii) where possible, at sites where there is no disease transmission but high abundance of target mosquito species.

The determination of proportional end-points of repellent efficacy and persistence is recommended. These allow concurrent estimation of EDs, effective period of time for a range of doses, repellent half-life and the CPT (*3*).

### 3.1 Efficacy and persistence of technical material

Collections are performed by volunteers skilled in the use of aspirators (blow-type or mechanical) or collecting tubes to catch all mosquitoes landing on an exposed limb before the mosquitoes commence to probe. Aspirated mosquitoes are transferred into holding cups that are labelled and changed every half-hour or, if collected in tubes, transferred to labelled holding cups that are changed every half-hour. Inclusion of equal numbers of male and female volunteers in the test is recommended.

Human landing catches are performed during the period of biting activity of the target mosquito species and indoors and/or outdoors depending upon mosquito behaviour. Where targeted species have short duration of main biting activity, tests with repellent material should begin such that the expected end-points as described above occur within the biting period of such species.

Volunteers should avoid using fragrance products 12 hours prior to and during the test. Volunteers should preferably not be tobacco users or at least have refrained from tobacco use for 12 hours prior to and during testing.

The surface area of the skin of the limbs of each volunteer is measured (Annex 2) and the volume of repellent material needed to treat the area at a specific dose is determined. To do this, the technical material is diluted in ethanol (or any other suitable diluent specified by the manufacturer) in sufficient quantities to remain effective for the duration of the study. The appropriate dose of test repellent should be applied uniformly to a limb of the volunteer using a pipette and allowed to dry before the start of the first collection period. A blind test, in which volunteers are not aware of the nature of the treatment, is preferred.

A negative control volunteer should be used in the test, to whom the corresponding volume of alcohol or a suitable diluent is applied to the skin in the same manner as the repellent treatment. A positive control (20% ethanolic deet) may also be used.

A preliminary assessment of the suitability of collection sites by performing human landing catches prior to the test is recommended to minimize variation in the number of mosquitoes landing and/or probing among sites. This will result in the selection of sites that yield the most homogeneous densities of mosquitoes and with suitable biting rates.

Within each collection site, volunteers should be placed singly and separated from each other by $\geq$20 metres. A range of four

doses is recommended to be tested for each repellent and may be approximated from laboratory studies (see section 2.1). The doses are selected to produce a comprehensive range of protection rates at the end of the exposure period. Normally, the effective dose that provided 99.9% protection in laboratory studies is used as a guide to establish the dosages for field trials.

A completely randomized design is used. The number of test volunteers and the number of collection sites are equal to the number of doses to be tested plus the untreated control. If a positive control (20% deet in ethanol) is used, an extra collection site and volunteer will be required. A single (1-day) test comprises rotation of each volunteer at random among all collection sites at 1-hour intervals (Table 3). Each treatment combination of dose, negative and/or positive control and volunteer should be tested for a suitable period on two or more dates. Replicate tests should each be similarly randomized. The mosquito collection at each collection site for the repellent treated volunteers and the negative and/or positive controls should be chosen to be suitable for the target species. For example, under high biting pressure, this could be one minute each beginning at minute 15, 30, and 45 into the observation period, with the first measurement in the initial hour of observation period (0–1 hours) starting at minute 15 (i.e. minute 15–16) after repellent application.

Mosquitoes landing and/or probing on the skin should be collected with an aspirator for accurate counting and species identification. Species composition should be reported. Tests should not be conducted in windy conditions, but records should be made throughout each test of wind speed, temperature, relative humidity and precipitation amount(s) for possible later consideration and/or analysis.

At the conclusion of the experiment, the number of mosquitoes collected within each observation period (i.e. at 15–16 min, 30–31 min and 45–46 min), at each dose, is averaged for each replicate.

<u>Protection (efficacy)</u> afforded by the repellent ($p$) at each test period and for each dose is calculated as:

$$p = 1 - ( T / C ) = ( C - T ) / C \qquad\qquad \text{(ii)}$$

where $T$ is the number of mosquitoes collected from the treatment volunteer and $C$ is the number of mosquitoes collected from the negative (or positive) control volunteer.

The <u>persistence</u> of the repellent efficacy is assessed by the <u>median effective dose</u> ($ED_{50}$) and the repellent half-life. These end-points are estimated by fitting the data to a probit–plane regression model, which relates the protection afforded by the repellent to the test period and the natural logarithm (ln) of the dose of repellent applied:

$$\ln [p / (1 - p)] = a + b_1(D_0) + b_2 t_1 \qquad\qquad \text{(iii)}$$

where $p$ is the protection afforded by the repellent, $D_0$ is the dose calculated as the natural logarithm of the dose applied (ln [dose]), $t$ is the time post-treatment in hours and $a$, $b_1$ and $b_2$ are coefficients estimated using the probit–plane regression model.

The $ED_{50}$ corresponds to the application dose providing 50% protection ($p = 0.5$) at the time of repellent application ($t = 0$). Taking the value of 0.5 for $p$ and 0 for $t$ in equation iii (above), and solving for $D_0$, provides an estimate of the $ED_{50}$ (see Example A).

Other percentiles can be estimated by setting $t = 0$ and calculating $p$ for a specific application dose. For example, to estimate the $ED_{90}$ using the coefficients provided in Example A (above), put $t = 0$ and $p = 0.90$ in equation iii and calculate (see Example B).

The <u>median effective time</u> ($ET_{50}$) and other percentiles, such as the $ET_{99}$ (see Example C), for a specified application dose ($D_0$) can also be estimated from equation iii.

The <u>repellent half-life</u> can be estimated using equation iii as under:

$$\ln [1 / 2]b_1 / b_2 \hspace{3cm} \text{(iv)}$$

The <u>complete protection time</u> for a given dose is estimated from the time elapsed up to the first mosquito landing and/or probing in each replicate. The median CPT and its confidence interval can be estimated using the Kaplan–Meier survivor function procedure (see Annex 3).

Table 3 **Example of the allocation of four doses (*D*) of the candidate repellent, a negative control (*CN*) and a positive control (*CP*) to six volunteers (*V*) among six collection sites in a completely randomized design**

| Beginning of test period (hours) | Observation period (hours)[2] | Collection sites | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| 0 | 0–1 | V5CN | V4D4 | V6D2 | V1D3 | V2CP | V3D1 |
| 1 | 1–2 | V4D4 | V6D3 | V5D1 | V2D2 | V1CN | V3CP |
| 2 | 2–3 | V2D1 | V4CN | V1D4 | V3D2 | V6CP | V5D3 |
| 3 | 3–4 | V1D2 | V5CN | V2D3 | V6D1 | V4D4 | V3CP |
| 4 | 4–5 | V2D3 | V5CN | V1D1 | V6D2 | V3D4 | V4CP |
| 5 | 5–6 | V4CP | V3D2 | V5D4 | V2D3 | V1CN | V6D1 |
| 6 | 6–7 | V4D1 | V3D3 | V5D2 | V2CN | V6CP | V1D4 |

[2] In this example, within each observation period, three measurements (at 15, 30 and 45 minutes) are taken at each collection site.

Table 3 (continued) **Example of the allocation of four doses (*D*) of the candidate repellent, a negative control (*CN*) and a positive control (*CP*) to six volunteers (*V*) among six collection sites in a completely randomized design**

| Beginning of test period (hours) | Observation period (hours)[3] | Collection sites | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| 7 | 7–8 | V1CN | V2D1 | V4D2 | V6D3 | V5D4 | V3CP |
| 8 | 8–9 | V5D4 | V4D1 | V3D2 | V1D3 | V6CN | V2CP |
| 9 | 9–10 | V3D2 | V6CN | V5CP | V1D3 | V2D4 | V4D1 |

---

[3] In this example, within each observation period, three measurements (at 15, 30 and 45 minutes) are taken at each collection site.

## Example A

The following coefficients were estimated using the probit–plane regression model based on protection against *Anopheles gambiae s.l.* by deet (*4*):

$$a = 8.160$$
$$b_1 = 2.209$$
$$b_2 = -0.532.$$

The $ED_{50}$ is calculated as:

(1)     $\ln [p/(1 - p)] = a + b_1(D_0) + b_2 t_1$
(2)     $\ln [0.5/(1 - 0.5)] = 8.160 + 2.209(D_0) + -0.532 \,(0)$
(3)     $\ln (1) = 8.160 + 2.209(D_0) + 0$
(4)     $\ln (1) = 0 = 8.160 + 2.209(D_0)$
(5)     $-8.160/2.209 = D_0$
(6)     $-3.694 = D_0$
(7)     $\ln (ED_{50}) = D_0$
(8)     $ED_{50} = \exp (D_0)$
(9)     $ED_{50} = 2.7183^{(-3.694)}$
(10)    $ED_{50} = 0.025 \text{ mg/cm}^2.$

## Example B

The $ED_{90}$ is calculated as:

(1)     $\ln [p/(1 - p)] = a + b_1(D_0) + b_2 t_1$
(2)     $\ln [0.9/(1-0.9)] = 8.160 + 2.209 \,(D_0) + -0.532 \,(0)$
(3)     $\ln [9.0] = 8.160 + 2.209 \,(D_0)$
(4)     $2.197 - 8.160 = 2.209 \,(D_0)$
(5)     $-5.963 = 2.209 \,(D_0)$
(6)     $-5.963/2.209 = D_0$
(7)     $-2.699 = D_0$
(8)     $\ln (ED_{90}) = D_0$
(9)     $ED_{90} = \exp (D_0)$
(10)    $ED_{90} = 2.7183^{(-2.699)}$
(11)    $ED_{90} = 0.067 \text{ mg/cm}^2$

**Example C**

A volunteer applies a dose of 0.50 mg/cm$^2$ to the arm (i.e. $D_0$ = ln [0.50] mg/cm$^2$). Determine the ET$_{99}$ (using the same coefficients as provided in Example A):

(1)          ln $[p/(1 - p)]$ = $a + b_1(D_0) + b_2t$
(2)          ln $[0.99/(1.00 - 0.99)]$ = $a + b_1(D_0) + b_2t$
(3)          ln (99) – 8.160 – (2.209 × –0.693) = (–0.532)$t$
(4)          4.595 – 8.160 – (1 1.531) = –0.532$t$
(5)          –2.034 = –0.532$t$
(6)          $t$ = (–2.034/–0.532) = 3.8 hours after application

## 3.2    Efficacy and persistence of formulated products

The single-dose evaluation of formulated repellent products should be performed under field conditions at a minimum of two ecologically and geographically distinct locations. A preliminary assessment of collection sites by performing human landing catches prior to the test is recommended to minimize variation in the number of mosquitoes collected among such sites. This will include a selection of sites that yield the most homogeneous densities of mosquitoes and with suitable biting rates. The appropriate number of volunteers trained in mosquito aspiration will be required for the study, including individuals serving as positive controls and individuals serving as untreated test subjects (for ongoing monitoring of mosquito biting pressure). A minimum number of volunteers necessary to demonstrate statistical significance should only be included in the testing for ethical reasons.

In a single (1-day) test, at location 1, the study investigator should apply the formulated product to an exposed limb of each volunteer at the rate of 1 mL of formulated product (or 1 mL of 20% deet in ethanol for the positive control) on ≈600 cm$^2$ area. The limb is allowed to dry and the time recorded of application to each volunteer. Volunteers are individually assigned at random to collection sites arranged ≥20 metres apart. The

exposure time to mosquito populations at each collection site for the repellent treated volunteers and the positive control should be chosen to be suitable for the target species. For example, under high biting pressure, this could be 1-minute each at 15, 30 and 45 minutes into the observation period, with the first measurement in the initial observation period (0–1 hours) starting at minute 15 (i.e. minute 15–16) after repellent application. A suitable number of additional volunteers (untreated) should monitor biting pressure throughout the study. All mosquitoes landing on the exposed limb of each volunteer are collected by aspiration and transferred to a holding container for later counting and species identification. A single (1-day) test comprises rotation of each test volunteer, the positive control and an untreated control at random among all collection sites at 1-hour intervals.

Human landing catches should be performed during the period of biting activity of the target mosquito species. This may require treatment with repellent well in advance to ensure the expected end-point occurs within the biting period of the target species. The study should be otherwise conducted until the time to first landing and/or probing by a mosquito on a volunteer. Complete protection time is calculated as the number of minutes elapsed between the time of repellent application and the first mosquito landing and/or probing. The resulting data can be analysed using the Kaplan–Meier survivor function (Annex 3) and the median CPT and corresponding standard errors reported.

Percentage repellency (%$p$) in field trials can be determined for each hour of the test as:

$$\%p = ((\ C - T\ )\ /\ C) \times 100 \qquad\qquad\qquad (v)$$

Where $T$ is the average number of mosquitoes collected from the treatment volunteer(s) in a given hour of a test and $C$ is the average number of mosquitoes collected from the untreated or positive control volunteer in the same hour of the test. The calculation is repeated for each hour to result in a profile of hourly change in % repellency over the 12-hour test period.

# 4. REFERENCES

1. *Report of the WHO informal consultation on the evaluation and testing of insecticides. WHO, Geneva, 7– 11 October 1996*. Geneva, World Health Organization, 1996 (CTD/WHOPES/IC/96.1).

2. Rutledge LC. Mathematical models of the effectiveness and persistence of mosquito repellents. *Journal of the American Mosquito Control Association*, 1985, 1:56–62.

3. Rutledge LC et al. Efficacy of diethyl methylbenzamide (deet) against *Aedes dorsalis* and a comparison of two end points for protection time. *Journal of the American Mosquito Control Association*, 1989, 5:363–368.

4. Costantini C et al. Field evaluation of the efficacy and persistence of insect repellents DEET, IR3535, and KBR 3023 against *Anopheles gambiae* complex and other Afrotropical vector mosquitoes. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 2004, 98:644–652.

# ANNEX 1.
# GUIDELINES FOR DEVELOPMENT OF THE INFORMED CONSENT FORM

For: [name the group of individuals for whom this consent is written]

Name of principal investigator:
Name of organization:
Name of sponsor:
Name of proposal:

## PART I: Information sheet

**This sheet is a suggestion or example that can be modified according to the national rules and guidelines**

**1.    Introduction**
*State briefly who you are and explain to the participants that you are inviting them to take part in research that you are doing*.

**2.    Purpose of the research**
*Explain in lay terms why you are doing the research.*

**3.    Type of research intervention**
*State briefly the type of intervention that will be undertaken.*

**4.    Participant selection**
*State why this participant has been chosen for this research (adult males and females will preferably be recruited among the inhabitants of the study site, after having announced in the district, through oral advertisements, that the project is looking for volunteers. The selection will ensure that equal opportunities are provided to everybody. Report how many volunteers are expected to participate in the study. Criteria for inclusion and exclusion of volunteers needs to be reported.*

### 5. Voluntary participation

*Indicate clearly that volunteers can choose to participate or not. State that they will still receive all the services they usually do whether they choose to participate or not.*

### 6. Information on the repellent [name of the repellent]

*Explain to the participant why you are testing a repellent product. Provide as much information as is appropriate and understandable about the repellent product, such as its manufacturer or location of manufacture, and the reason for its development. Explain the known experience with this repellent product. Explain comprehensively, if any, all the known side-effects or toxicity of this repellent product.*

### 7. Participant protection against malaria or other vector-borne diseases

*Explain to each participant the safeguards that will be provided (e.g. chemoprophylaxis, where relevant) to protect them from malaria or other vector-borne diseases and, if necessary, their treatment.*

### 8. Description of the process, procedures and protocol

*Describe or explain to the participant the exact procedures that will be followed on a step-by-step basis and the tests that will be done.*

### 9. Duration

*Include a statement about the time commitments of the research for the participant, including the duration of the research and volunteer follow-up.*

### 10. Side-effects

*Potential participants should be told if there are any known or anticipated side-effects and what will happen in the event of a side-effect or an unexpected event. State who will provide medical care should a volunteer be injured in the study, and who will provide funds for treatment.*

### 11. Risks

*Explain and describe any possible or anticipated risks. Describe the level of care that will be available in the event that harm does occur, who will provide it and who will pay for it.*

### 12.    Discomforts
*Explain and describe the type and source of any anticipated discomforts that are in addition to the side-effects and risks discussed above.*

### 13.    Benefits
*Mention only those activities that will be actual benefits (as an additional protection from mosquito bites) and not those to which they are entitled regardless of participation.*

### 14.    Incentives
*State clearly what you will provide the participants with as a result of their participation. WHO does not encourage incentives. However, it recommends that reimbursements for expenses incurred as a result of participation in the research be provided.*

### 15.    Confidentiality
*Explain how the research team will maintain the confidentiality of data, especially with respect to the information about the participant, which would otherwise be known only to the Principal Investigator/physician but would now be available to the entire research team. State any other groups that will have access to individually identifiable information collected in this study.*

### 16.    Sharing the results
*Where relevant, your plan for sharing the findings with the participants should be provided.*

### 17.    Right to refuse or withdraw
*This is a reconfirmation that participation is voluntary and includes the right to withdraw.*

### 18.    Whom to contact
*Provide the name and contact information of someone who is involved, informed and accessible (a local person who can actually be contacted). State also that the proposal has been approved, and how.*

**This proposal has been reviewed and approved by [name of the local ethical committee], whose task is to make sure that research participants are protected from harm. If you wish to find out about more the Local Ethical Committee, please contact [name, address and telephone number].**

## Part II: Certificate of Consent

This section can be written in the first person. It should include a few brief statements about the research and be followed by a statement similar to the one in bold below. If the participant is illiterate but gives oral consent, a witness must sign. A researcher or the person checking the informed consent must sign each consent form.

***I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it, and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate as a participant in this research and understand that I have the right to withdraw from the research at any time without in any way affecting my medical care.***

**Print name of participant:** _____

**Signature of participant:** _____

**Date:** _____
         **day / month / year**

*If illiterate*
A literate witness must sign (if possible, this person should be selected by the participant and should have no connection to the research team).

***I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.***

**Print name of witness: _____       AND
Thumb print of participant**

**Signature of witness:** _____

**Date:** _____
       **day  /  month  /  year**


*I have accurately read or witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.*

**Print name of researcher:** _____

**Signature of researcher:** _____

**Date:** _____
       **day  /  month  /  year**

**A copy of this Informed Consent Form has been provided to participant _____ (initialled by the researcher/assistant).**

## ANNEX 2.
## MEASUREMENT OF THE SURFACE AREA OF THE SKIN OF LIMBS

The surface area of skin used on a limb for repellent testing can be approximated on the basis of the surface area of a cylinder. Three dimensions are required for this purpose: the length of the treatment area, and the circumference of the limb at the proximal and distal boundaries of the treatment area.

The repellent should be applied evenly over the total surface area around the arm from wrist to elbow (A). The treatment area can be approximated by measuring the circumference (cm) of the wrist (B), the circumference (cm) at the elbow-cubital fossa (C) when the arm is extended, and the distance (cm) from the elbow to the wrist (D) (Figure 1).
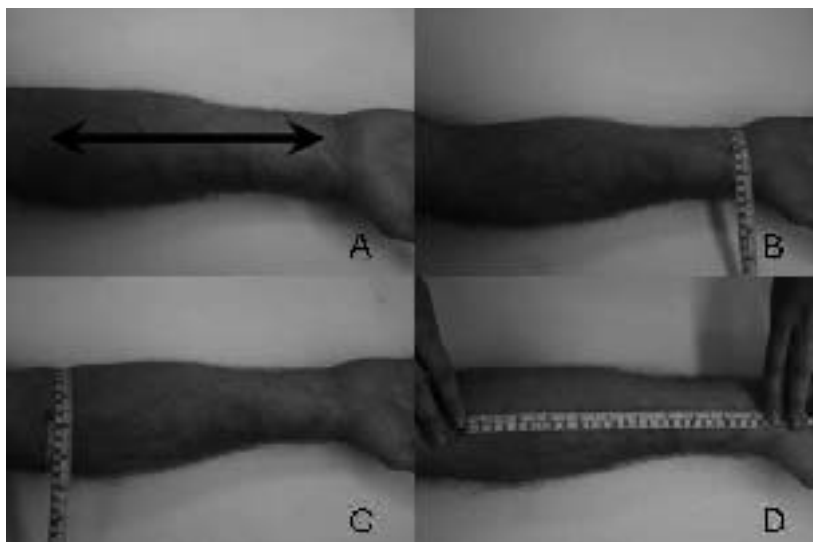


Figure 1. **Treated area (A) and estimation of forearm surface area by measurement of circumference of wrist (B), elbow cubital fossa (C) and length of arm (D)**

The surface area (in cm$^2$) of skin is then calculated by:

Area= ½ ($c_w$ + $c_e$) $D_{we}$                                    (iv)

where $c_w$ is the circumference of the wrist in cm, $c_e$ is the elbow-cubital fossa circumference in cm, and $D_{we}$ is the distance in cm between $c_e$ and $c_w$.

*Example:*

A repellent study is conducted using the arm. For the volunteer, the researcher measures the circumference of the wrist as 18 cm and an elbow region circumference of 27 cm, where the treatment area ends. The distance from wrist to elbow-cubital fossa is measured as 26 cm. Using the equation above:

Area = ½ (18 cm + 27 cm) x 26 cm
Area = ½ (45 cm) x 26 cm
Area = 22.5 cm x 26 cm
Area = 585 cm$^2$

# ANNEX 3.
# ESTIMATION OF MEDIAN AND CONFIDENCE INTERVAL OF COMPLETE PROTECTION TIME USING THE KAPLAN– MEIER SURVIVOR FUNCTION

The Kaplan–Meier function is a non-parametric statistic that can be used to calculate the median complete protection time (CPT) of a repellent and its corresponding confidence interval. To do this, the CPT "survivor function", which is based on the complete protection times recorded during testing, must be determined. The data required for this purpose are:

The CPT in minutes ($t$) for each replication ($i$) of a test with $n$ replications (i.e. $t_i$ [$i = 1, 2,... n$]).

The number of replications ($r$) of the test sharing the same CPT ($j$) (where $r \leq n$ (i.e., $tj$ [$j = 1, 2, ... r$])).

The number of replications of the test not sharing the same CPTs (i.e. [$n - r$]).

To construct the table of survivor function estimates, the $t_j$ times are arranged in ascending order. For each $t_j$, there will be $n_j$ observations with a CPT greater than $t_j$. The number of repellent failures (CPTs) recorded between the time interval $t_j$ and $t_{j+1}$ is denoted by $d_j$. The Kaplan–Meier statistic ($S(t)$) estimates the probability of survival (or, inversely, the time to repellent failure) through this interval as $(n_j – d_j)/ n_j$. The standard error of the survivor function estimate at time $t$ is designated as $s.e.\{S(t)\}$.

The median complete protection time $t(50)$ is that beyond which 50% of the CPTs are recorded (i.e. $S\{t(50)\}$ =0.5). The estimated median CPT is the smallest observed CPT for which $S(t) \leq 0.5$. The 95% confidence interval of this estimate is given by: $t(50) \pm 1.96$    $s.e.\{t(50)\}$.

**Example**
In a hypothetical 10-hour test of the efficacy of a repellent applied on human skin, CPTs were recorded in 48 replications (Table 1). In 11 of the replications, no landings and/or probings

were received prior to the end of the study period (10 hours) and the CPT for these 11 replications is unknown.

Table 1 **Summary of the frequency of complete protection time (CPT) of a candidate repellent applied on human skin**

| CPT (min) | 120 | 150 | 210 | 390 | 420 | 450 | 480 | 510 | 600 |
|-----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Frequency ($d_j$) | 1 | 3 | 3 | 10 | 7 | 4 | 4 | 2 | 3 |

Using the data from this test, estimates of $(n_j - d_j)/n_j$ and $S(t)$ and their corresponding standard error are constructed, as presented in Table 2.

Table 2 **Estimates of $(n_j - d_j)/n_j$ and $S(t)$ and their corresponding standard error[4]**

| Time interval ($t_j$) in min | $n_j$ | $d_j$ | $(n_j - d_j)/n_j$ | $S(t)$ | Standard error $S(t)$ |
|---|---|---|---|---|---|
| 0– | 48 | 0 | 1.0000 | 1.0000 | 0.0000 |
| 120– | 47 | 1 | 0.9787 | 0.9787 | 0.0210 |
| 150– | 44 | 3 | 0.9318 | 0.9120 | 0.0420 |
| 210– | 41 | 3 | 0.9268 | 0.8453 | 0.0538 |
| 390– | 31 | 10 | 0.6774 | 0.5726 | 0.0798 |
| 420– | 24 | 7 | 0.7083 | 0.4056 | 0.0776 |
| 450– | 20 | 4 | 0.8000 | 0.3245 | 0.0719 |
| 480– | 16 | 4 | 0.7500 | 0.2434 | 0.0643 |
| 510– | 14 | 2 | 0.8571 | 0.2086 | 0.0597 |
| 600 | 11 | 3 | 0.7273 | 0.1517 | 0.0516 |

---

[4] Data processing software for calculation of these variables is available on the Internet. Search key word: Kaplan Meier survival function online calculator.

<u>Median complete protection time</u> is the smallest CPT for which $S(t) \leq 0.5$. In this case, at $t_j = 390$, $S(t) = 0.5726$, and at $t_j = 420$, $S(t) = 0.4056$. Our best estimate of the median CPT in this example is $t(50) = 420$ min.

<u>To calculate the 95% confidence interval</u> we set $S\{upper(50)\} = 390$ and $S\{lower(50)\} = 420$. The probability density function at $t(50)$ is estimated as:

$$f\{t(50)\} = \frac{S(390) - S(420)}{420 - 390} = \frac{0.5726 - 0.4056}{30} = 0.005567$$

The standard error of the survivor function at $t(50)$ is 0.0776 and the standard error of the median CPT is estimated by:

$$s.e.\{t(50)\} = \frac{1}{0.005567} \times 0.0776 = 13.94$$

The 95% confidence interval for the estimated median CPT for the repellent in this example has boundaries of $420 \pm 1.96 \times 13.94$ minutes. On this basis, in 95 out of 100 observations, we would expect the median CPT to lie between 393 to 447 minutes.