

**Streamlined durability monitoring of Insecticide-Treated Nets (ITNs) distributed during the [year] mass campaign in [Country]**

**Study Protocol**

[Month Year]

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# Background

## Evolution of ITN durability monitoring

Between 2010 and 2018 the proportion of households in sub-Saharan Africa with at least one insecticide-treated net[[1]](#footnote-2) (ITN) increased from 47% to 72%. The percentage of the population that could be protected by an ITN assuming each ITN in a household can be used by two people increased from 33% to 57% over the same period[[2]](#footnote-3). It is important to sustain coverage and monitor net durability and the average useful life of a net. WHO recommends that countries routinely monitor net durability following mass distribution campaigns[[3]](#footnote-4) and provided technical guidance on estimating physical survival and bio-efficacy in 2013[[4]](#footnote-5),[[5]](#footnote-6),[[6]](#footnote-7).

Since 2013, durability monitoring studies have been initiated and completed in 11 countries for 10 standard pyrethroid-only ITN brands (see Annex 1)[[7]](#footnote-8). Studies have measured the effect of normal daily use on: attrition (as measured by the loss of nets for any reason as well as due to wear and tear from households); physical durability (as measured by the number and size of holes in the net); and insecticidal efficacy (as measured by cone bioassay, tunnel test, and chemical content analysis, depending on type of net). Results from these studies suggest that the physical durability of similar products may vary significantly, between less than two years to four or more years, and that differences are largely driven by environmental and behavioral factors. Median all cause attrition for the same studies was estimated as 12.5% of ITNs in the first 6 months following distribution, most often driven by reallocation of nets to other households and family members. Between 6 and 36 months, median net attrition per month was 2.1%. Mean 24-hour mortality for deltamethrin ITNs ranged from 29-97%, while there was less variation among alpha-cypermethrin nets (64-85%).

While vector control has contributed substantially to the global reduction in malaria burden recorded since 2000, global progress towards malaria control and elimination has stalled in recent years and the long-term effectiveness of malaria vector control is threatened by the emergence and intensification of insecticide resistance in key mosquito populations. New types of ITNs that use more than one active ingredient and are effective against insecticide resistant mosquitoes have been developed, but large-scale uptake has been slow prior to 2020 and very limited durability monitoring data exists for these new products.

The United States President’s Malaria Initiative (PMI) has long supported ITN durability monitoring. To date, PMI has supported a full durability monitoring protocol to generate basic information on the physical integrity and bioefficacy of ITNs as well as information on ITN care and use. These studies generally compare two types of nets in one area or one type of net in two areas of a given country. More recently, PMI has developed a more streamlined protocol for durability monitoring for countries that have already generated considerable ITN durability data and have more focused questions, particularly around durability of new types of nets.

This protocol outlines the approach for streamlined ITN durability monitoring in [Country] for ITNs distributed as part of the [Year] mass campaign.

## Overview of [year] mass distribution campaign

*Describe the recent ITN mass campaign in terms of dates, coverage, characteristics of ITN brands deployed (netting material, denier, active ingredient(s)) and quantities distributed (or planned if the campaign is yet to take place).*

Table 1 Characteristics of [Country] [year] ITN mass campaign

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Geographic area** | **ITN brand** | **ITN type** | **Active Ingredients** | **Quantity** | **Distribution date** |
|  |  |  |  |  |  |
|  |  |  |  |  |  |
|  |  |  |  |  |  |

## Overview of streamlined ITN durability monitoring in [Country]

### Monitoring sites

*Name and describe the monitoring sites.*

### Characteristics of ITN brands monitored

*Name and briefly describe the ITN brand(s) included in the monitoring activity.*

*e.g., PermaNet 3.0 brand ITNs, manufactured by Vestergaard, are rectangular, white, with polyester sides and a polyethylene roof. The side yarn is incorporated with deltamethrin at 2.8 g/kg, while the roof yarn has deltamethrin at 4.0 g/kg combined with synergist piperonyl butoxide at 25 g/kg.*

### Activity timing

*Complete the table below to describe the planned timing of survey rounds.*

Table 2 Monitoring activity timing

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  |  |  |  | **Date of survey rounds** |
| **Study site** | **ITN brand** | **ITN type** | **Campaign date** | **Baseline** | **12-month** | **24-month** | **36-month** |
| *e.g. North* | *Brand A* | *PBO-synergist* | *March 2021* | *May ‘21* | *March ‘22* | *March ‘23* | *March ‘24* |
| *e.g. West* | *Brand B* | *Dual AI* | *March 2021* | *May ‘21* | *March ‘22* | *March ‘23* | *March ‘24* |
|  |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |

## Objectives

1. To assess the insecticidal efficacy of [brand or brands to be monitored] in [“one” or “multiple” as required] locations, as measured by cone bioassays, tunnel tests and chemical testing, over a three-year period of field use; and compare the insecticide effectiveness across these [“locations” or “brands” as appropriate] and identify major determinants of field performance (e.g. characteristics of net users, washing behaviors, etc.).
2. To monitor the physical integrity of the nets as measured by a net hole assessment and short questionnaire.
3. To estimate indirectly the level of attrition of the nets at each round.

## Expected Benefits and Value

The results of the proposed study will:

* Provide the NMCP, PMI, and Roll Back Malaria (RBM) partners with valuable information regarding the new ITN brands distributed during the mass campaign, e.g. insecticidal efficacy, and whether and how this varies by [“location” or “brand”, as appropriate].

# Methods

## Study design

The principle design for streamlined ITN durability monitoring is a prospective study using repeat cross-sectional visits to sampled clusters with randomly sampled ITNs withdrawn from selected households at each round.

Once ITNs have landed in country and before the ITN distribution campaign begins, 20 ITNs per site/brand will be sampled from the central stores to undergo bioassay and chemical residue testing. 10 ITNs from the 20 will be tested first and the second set of 10 nets only tested if preliminary results do not meet manufacturer specifications. Results from these tests will form the pre-distribution baseline against which study results will be compared.

Within a few months (ideally 1-3 months, but not more than six months) following the mass campaign, a representative sample of approximately 410 campaign nets from each study site will be identified through a cluster household survey with all campaign nets from consenting households forming the sampling frame. These nets will be labelled with a unique identifier. At baseline, a random sample of 30 campaign nets will be selected from the sampling frame and withdrawn for bioassays and physical integrity assessment; samples from these nets may undergo chemical residue testing, depending on the bioassay results. At each subsequent survey round (12-, 24- and 36-months) a random sample of 45 campaign nets will be selected from the sampling frame and withdrawn for bioassays, chemical residue testing and, physical integrity assessment. A household survey capturing household characteristics and net care behaviors will be administered at each household from which one or more campaign nets are withdrawn. Withdrawn nets will be replaced on a like-for-like basis with new ITNs, marked with the date so they are not sampled in future rounds. Prior to undergoing destructive insecticide effectiveness testing, the physical integrity of sampled ITNs will be measured at each round using the standard hole assessment approach[[8]](#footnote-9). At each survey round, ITN attrition will be indirectly estimated based on the number of randomly selected campaign nets that have to be replaced in the sample until the full contingent of 45 nets has been identified and withdrawn.

Figure 1 Overview of study design

## Study sites

*Edit the description below as appropriate depending on your study design (comparing different brands in similar locations or the same brand in different locations).*

The study will be carried out in [Location(s)] (Table 3).

Table 3 Study site characteristics

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Location 1** | **Location 2** | **Location 3** |  |
| Study site |  |  |  |  |
| ITN brand |  |  |  | **Source** |
| **Environment** |
| Elevation (m) |  |  |  |  |
| Annual precipitation (mm) |  |  |  |  |
| Mean max temperature (°C) |  |  |  |  |
| Mean min temperature (°C) |  |  |  |  |
| Climate classification (Koppen-Geiger) |  |  |  |  |
| Population main economic activity |  |  |  |  |
| **Demographics and health** |
| Population (Province) |  |  |  |  |
| Total fertility rate |  |  |  |  |
| Percentage of children under 5 years with fever in the past two weeks |  |  |  |  |
| **Malaria epidemiology** |
| Description of malaria transmission season(s) |  |  |  |  |
| Prevalence of anemia in children under 5 years |  |  |  |  |
| Percentage of households with at least one ITN for every 2 people (%) |  |  |  |  |
| Population access to ITN (%) |  |  |  |  |
| Population use of ITN (%) |  |  |  |  |
| Ratio of ITN use:access |  |  |  |  |
| Malaria prevalence among children under five, by RDT (%) |  |  |  |  |
| Malaria prevalence among children under five, by microscopy (%) |  |  |  |  |
| *Population access*: Proportion of population that would be able to use an ITN if each ITN in a household was used by two people. *Use:Access ratio*: Ratio of population use to population access |

Figure 2: Streamlined ITN durability monitoring sites

*Map of Streamlined ITN durability monitoring sites*

## ITN brands

*Complete the table below for each ITN brand included in the monitoring activity. Add or remove columns as required for the number of brands being monitored.*

Table 4 Characteristics of ITN brands

|  |  |  |  |
| --- | --- | --- | --- |
| **Brand** |  |  |  |
| **Type*Pyrethroid-onlyPBO-synergistDual AI*** |  |  |  |
| **Chemical content(g/kg and mg/m2)** |  |  |  |
| **Fabric** |  |  |  |
| **Denier** |  |  |  |
| **Shape** |  |  |  |
| **Manufacturer** |  |  |  |

## Sample size

Sample size determination for this activity is informed by the standard WHO guidance for field testing of mosquito nets[[9]](#footnote-10). Forty-five ITNs per location will be collected at each assessment round (12-, 24, 36-months). Logistically, these ITNs will be sampled as 3 ITNs from households in each of 15 clusters. Assuming, in the worst-case scenario, that the level of the main outcome measure of interest (which will vary based on ITN type) is 50% after three years, the sample will provide a precision of 12%-points in a one-sided analysis.

An analytic sample size of 45 ITNs at monitoring completion (the 36-month round) must be translated into an operational sample to inform the size of the sampling frame required at baseline. Secondary analysis of attrition data from 12 PMI-funded durability monitoring studies completed or initiated since 2015 identified the monthly estimates of attrition shown in Table 5[[10]](#footnote-11). The median values have been used in this analysis.

Table 5 Estimates of ITN attrition

|  |  |  |
| --- | --- | --- |
| **Time period** | **Monthly median attrition** | **Monthly upper-quartile attrition** |
| Baseline to 12-month | 2.0% | 3.5% |
| 12-month to 36-month | 2.1% | 3.0% |

Assuming that at 36-months it is desirable to have at least 100 ITNs present in the sampling frame in each location (from which to select the final 45 nets), then at baseline a minimum of 410 ITNs must be listed on the sampling frame. For consistency with the sampling approach used for standard durability monitoring, the study will work with 15 clusters in each location. The required number of households per cluster will depend on the mean household size, assuming the standard distribution strategy of 1 ITN for every 1.8 people was followed during distribution planning. To reduce intra-cluster correlation considering ITNs potentially sourced from the same household, while maintaining efficiency in study design, the number of ITNs included in the sampling frame from any one household is capped at 2. Table 6 provides a look-up for the number of households per cluster, based on the mean household size in a location. To use the table, household size in the study locations is rounded down to the nearest table entry.

Table 6 Required cluster sizes

|  |  |  |
| --- | --- | --- |
| **Mean household size in study sites is at least…** | **Campaign ITNs per household (assuming 1 per 1.8 people)** | **Required cluster size (households)** |
| 3.2 | 1.8 | 16 |
| 3.4 | 1.9 | 15 |
| 3.7 | 2.1 | 14 with net cap |
| 4 | 2.2 | 14 with net cap |
| 4.3 | 2.4 | 14 with net cap |
| 4.7 | 2.6 | 14 with net cap |
| 5.2 | 2.9 | 14 with net cap |
| 5.8 | 3.2 | 14 with net cap |
| 6.6 and above | 3.7 | 14 with net cap |

Mean household size in [Location(s)] is [mean household size] and so this study will sample 15 clusters with [number] households per cluster for each location.

## Sampling procedures

### Stage one: selection of clusters

The campaign ITN distribution registers for the study locations will be sourced from the National Malaria Control Program. A cluster will be defined as the lowest level geographic area used for campaign planning. Cluster selection will be done with probability proportional to size sampling (PPS), with the number of ITNs distributed per cluster as the measure of size. If registers are not available, census enumeration areas will be sampled using PPS with population as the measure of size.

### Stage two: selection of households and study net sampling frame

Within each selected cluster, [number] households will be selected using the following methodology: if the cluster is less than 200 households the field team will list or map all inhabited houses and the team leader will randomly sample the required number of households with equal probability using random number lists. In addition, half the number of required households (rounding up) will be selected as alternate households, which will be used if a sampled household reports never to have received any nets from the campaign. Following the household definition used in the ITN distribution campaign, the definition of a household will be “people eating from the same pot”.

If the cluster exceeds 200 households, an equal size section-approach will be used. With the help of local leaders, the cluster will be divided in sections of approximately equal size (80-100 households). One of these sections will be randomly selected by the team leader using a random number generator and within this section all households will be listed and/or mapped. Household selection will then proceed as above. The number of sections will be recorded by the team leader so that weights can be correctly applied for analysis. To facilitate household selection, the use of satellite images and building footprints to map households before fieldwork begins will be explored.

Sampled households will be screened to determine whether they participated in the ITN distribution campaign. Screening will consist of a brief introduction by the study team and a set of questions to determine respondent and household eligibility. If a household is not eligible for the study because they did not participate in the mass campaign or they participated but have no campaign ITNs remaining in the household they will be dropped and will not be included in the sampling frame. If a household confirms participation in the campaign and the presence of one or more campaign ITNs, information on the study will be given and oral consent sought using the consent script. The information sheet and consent form will be available in [“English”, “French” or “Portuguese”] and [Local written language(s)] and will be read to the respondent in their local language by a member of the field team fluent in that language. If the household does not give consent, it will be dropped, and one of the alternate households will be visited until the total of [number] households is reached.

For each consenting household, the GPS coordinates and the full name of the head of household will be recorded and entered in the *Net List and Sampling* form which will be used to identify households during annual follow-up visits.

Within each household, all campaign ITNs will be identified by the field team based on the net label, net characteristics, and confirmation of net source from the household members. Each campaign net will be labeled with a unique identifying number that will be used to create a master list of nets which will serve as the sampling frame at each assessment round.

### Stage three: selection of nets for analysis

Using the ITN sampling frame generated during stage two at baseline, 3 ITNs per cluster will be randomly selected and withdrawn for analysis at each assessment round. For the 12-, 24- and 36-month follow-up assessment rounds, 6 alternate ITNs per cluster will also be selected from the sampling frame. These alternate ITNs will be used in sequence in case any of the ITNs intended to be sampled are lost-to-follow-up or are otherwise not available during the visit (for example, if the identified ITN is not available on the day of visit). No alternate ITNs should be required at baseline as ITN selection and interviews will occur immediately following the creation of the sampling frame and all households and ITNs should be available. Oral consent will be sought from the head of household or their representative prior to proceeding, and respondents will be administered a shortened version of the standard ITN durability monitoring questionnaire. Households will receive a new ITN to replace the one withdrawn; the new ITN will be the same brand as the one withdrawn or, if this is not possible, have the same active ingredients. Study ITNs will be relabeled and packaged in individual plastic bags for transport to the laboratory.

## Questionnaires

The monitoring activity will use two questionnaires. The first, *Net Listing and Sampling*, will capture information on study clusters, selected household locations and tagged campaign ITNs at the time of screening. The second will capture information on the ITNs selected at each assessment round for analysis.

The *Net Listing and Sampling* questionnaire will be used to identify eligible households and list eligible households and their campaign ITNs in the sampling frame. It comprises sections for:

* Screening questions to determine household eligibility for listing (these will be administered as part of sharing study information with selected households; no information will be recorded for ineligible households or those that refuse to participate except for the outcome of the screening visit [ineligible/refusal]).
* Household identification, including GPS coordinates head of household’s full name.
* ITN identification, recording the number of campaign ITNs present and assigning each a unique ID.

The main monitoring questionnaire will be used to capture information associated with ITNs selected to undergo laboratory analysis at each round. It comprises sections for:

* Household characteristics (composition, assets and other factors potentially associated with insecticide deterioration)
* Selected campaign ITN handling and use patterns, and washing and drying habits
* Laboratory-based hole assessment

This study will field questionnaires using publicly available Open Data Kit (ODK) software to conduct electronic data collection (EDC). Additionally, at baseline, the *Net List and Sampling* form will be generated on paper to permit immediate random selection of ITNs for the first round of analysis. Questionnaires will be adapted from ODK durability monitoring questionnaires and tested repeatedly to ensure fully functioning versions are available at the start of field work. Questionnaires will incorporate skip patterns and filters, and internal consistency checks, range checks and logical checks to strengthen data quality. Questionnaires will be fielded using electronic data collection and will be available in [“English”, “French”, or “Portuguese”] and [Local written language(s)].

## Field procedures

### Preparatory phase

During the preparatory phase ITN campaign distribution registers will be sourced and cluster sampling in each study location will be completed. After experiences in other countries where the ITN brand found during data collection was not as anticipated, steps will be taken to verify the target nets were distributed in the monitoring locations through discussion and confirmation with the NMCP and staff closest to the locations. A detailed set of master training materials will be modified to match the country context. A visual aid for ITN brand identification will also be prepared in advance. This will be a laminated sheet with photographs of the campaign ITN brands with one photograph of the label and one of the net. Visual aids and tally sheets for the hole assessment will also be prepared in advance.

Working with [name of in-country implementing partner], job descriptions for monitoring team positions will be developed and competent staff and a laboratory capable of conducting the required bioassays identified. Chemical residue testing will be conducted by [Name of laboratory]. The same laboratory will be used for analysis from all data collection timepoints during the life of the activity.

### Field work

#### Teams and training

Each monitoring location will have its own implementation team, with two technicians and one driver [customize as required for country set-up]. It is estimated that the time needed at baseline to list/map households, generate the ITN sampling frame and interview households in each of the 15 clusters is 18 days per location, including travel to and from the study sites. This assumes that for each cluster, the household listing, sampling, and interviews can be completed in one day in addition to 3 days of travel. During follow-up rounds, each location can typically be completed by one team in 8 days (assuming passable road conditions, as travel from one cluster to another will be the determining factor for fieldwork time during follow-up rounds).

Technicians will be carefully selected so that they are culturally acceptable, have good knowledge of the local languages, have experience performing bioassays, and experience in entomological monitoring or household surveys. Prior to the baseline study round there will be a three-day training that will include the following components:

* Understanding the study design and procedures to generate the sampling frame
* General approach to ethics of field work (consent and interview)
* Introduction to and practical use of the data entry software
* Detailed study of interview with role play
* Labeling and packaging of ITNs withdrawn for analysis
* Practice hole assessment at laboratory using frame
* Overview of bioassay standard operating procedures (SOPs)

Just prior to each follow-up round, a two-day refresher training will be given, anticipating that the same technicians will be engaged during the life of the study. Exact training lengths will depend on the level of experience among technicians engaged in the activity. Baseline training will be led in-country by a team of local, regional and/or international experts in durability monitoring. Refresher trainings will be conducted remotely and led by the same team as at baseline using video conferencing software.

#### Logistics and administration

Partner laboratories or field team agencies will ensure sufficient administrative and logistics staff are budgeted to support fieldwork.

#### Sensitization

As soon as clusters are selected the local authorities and key influencers such as chiefs will be informed of the purpose and expected timing of the survey and their support sought. Communities within clusters will be sensitized to the study objectives and activities to obtain maximum cooperation for the surveys.

#### Household consent and interviews

During screening to create the ITN sampling frame, selected households will be visited and the head of household or their representative will be interviewed to determine whether the household qualifies for the study. Study information will be provided in [“English”, “French” or “Portuguese”] and [Local written language] and, if necessary, will be read to the respondent in their local language by a member of the field team fluent in that language. Written consent will be sought to include the household in the study sampling frame and capture GPS coordinates and the full name of the head of household. Each household will receive a unique identification number consisting of the cluster and the household’s number. If a selected household is not available for any reason (refusal or moved out of the cluster at follow-up rounds), the household will be dropped and one of the sampled alternates used instead.

At baseline and follow-up rounds, ITNs will be sampled for withdrawal from the sampling frame. Following ITN sampling, the respective households in a cluster will be visited. At follow-up rounds, study information will be provided again and – for all rounds – oral consent will be sought before the sampled ITN is withdrawn and the short questionnaire administered to the head of household or their representative. If a selected household is not available at the time of visit for any reason, the team will continue with selection of any remaining ITNs in the cluster before revisiting unavailable households. If all other ITNs have been sampled and selected households are still unavailable, these ITNs/households will be dropped and one or more alternate ITN(s) targeted for inclusion, from the predetermined list of alternate ITNs for the cluster.

#### Identification and labeling of campaign net

In order to identify an ITN as coming from the mass campaign and create the ITN sampling frame, technicians will inspect each net in households selected for the sampling frame and compare the label and net characteristics with the visual aids previously prepared. If the label matches the campaign net brand, respondents will be asked about the source and time of obtaining the net. If this information confirms the net as a campaign net, it will be tagged with a unique ID in indelible ink. Nets that cannot be verified as campaign nets will not be included in the sampling frame.

Prior to each follow-up round, the ITN sampling frame will be updated to remove nets withdrawn during the previous round and a new random selection of 45 nets per location will be performed. For each selected net, the net ID, associated household ID and name of the head of household will be printed by cluster. The field team will use these details to locate households and ITNs for follow-up. All paper forms will be destroyed by team leaders in the field as the teams complete data collection.

#### Net removal and replacement for analysis

Following receipt of oral consent, technicians will administer the questionnaire, remove the net, and pack it in an individual plastic bag for transport to the laboratory for hole assessment, bioassays, and onward shipping of samples for chemical residue testing. A like-for-like new replacement net will then be given to the household and marked with the date so they are not sampled in future rounds.

#### Data collection, management and safety

For data collection, electronic devices will be used that allow a detailed programming of skip patterns and internal controls to ensure that all necessary data is collected and consistent. Depending on local conditions, data from each interview will be uploaded to a secure, web-based database at the end of each day, or as soon as a data connection can be established thereafter.

From the data, an ITN master list (the ITN sampling frame) will be created and updated after each assessment round (in addition to the cross-sectional data collected as part of the round).

The ITN master list will include the campaign ITN ID, GPS coordinates for the household, full name of head of household, a record of whether the net has previously been sampled and withdrawn, and a record of whether ITNs or whole households are known to no longer be available for sampling. Between surveys, this file will be password encrypted and safely kept on a fixed and secure data storage device (server), also with password protection. Access will only be available to monitoring study investigators. The ITN master list file(s) containing names and GPS coordinates will be deleted immediately following the final data collection at 36-months.

It is important to record precise geolocation of durability monitoring. To allow sufficient accuracy for geographic analysis of results, but not permit the location of any study households, GPS coordinates will be modified by introducing a small random error during data cleaning. Full name data will be removed during cleaning so that analytical data files will contain no personal identifiers.

#### Supervision and field support

Daily reports, including unforeseen challenges, will be shared with the study PI for discussion and resolution. External quality control will be provided by [Implementing partner / role] by monitoring data collection remotely in real-time and providing feedback to local teams via WhatsApp, email, and phone calls.

## Laboratory activities

Sampled ITNs will be sent to the study laboratory partner for hole assessment, bioassay testing, and sample preparation for shipping to [Name of laboratory].

### Net hole assessment

Prior to conducting bioassays and cutting samples for chemical residue testing each net will be assessed for physical integrity and signs of repair using a frame to hang the net in the laboratory. Separately inspecting each side and roof of the net, technicians will count and categorize present holes into four different sizes based on the WHO guidelines: 0.5 to <2 cm, 2 to <10cm, 10 to <25 cm and larger than 25 cm in diameter. Measuring rulers and hole templates will be used to ensure accurate measurement. The presence and number of repaired holes will be noted, but these will not be counted as holes. Data will be entered using electronic data collection in a copy of the main questionnaire for the selected ITN.

### Laboratory analyses

Insecticidal effectiveness testing will be performed according to modified WHO guidelines for pyrethroid-only nets and standard operating procedures developed in collaboration with PMI for testing PBO-synergist and dual AI nets. Adjacent net samples will be cut from each net panel for bioassays and chemical residue testing. Table 7 provides an overview of the methods used for new types of ITN for which standard approaches exist; a brief description of laboratory methods then follows. Full SOPs are provided in Sections 6 (PBO) and 7 (dual AI). SOPs for testing dual AI nets that include the insect growth regulator, pyriproxyfen, will be added when available.

Table 7 Overview of bioassay procedures

|  |  |  |  |
| --- | --- | --- | --- |
| **Type of ITN** | **Target ingredient** | **Cone assays** | **Tunnel tests** |
| Standard pyrethroid-only  | Alpha-cypermethrin, deltamethrin, permethrin | Susceptible strain on field samples and negative control |  |
| PBO-synergist with PBO only on the roof | Pyrethroid | Susceptible strain on roof and side field samples and negative control |  |
| Piperonyl butoxide (PBO) | Pyrethroid-resistant strain on roof of field samples, on positive PBO-synergist control, on pyrethroid-only positive control, and on negative control |  |
| PBO-synergist with PBO on all netting surfaces | Pyrethroid | Susceptible strain field samples and negative control |  |
| Piperonyl butoxide (PBO) | Pyrethroid-resistant strain on field samples, on positive PBO-synergist control, on pyrethroid-only positive control and on negative control |  |
| Dual AI | Alpha-cypermethrin | Susceptible strain on field samples and negative control |  |
| Chlorfenapyr |  | Pyrethroid-resistant strain on field samples, on dual AI positive control, on positive control of pyrethroid-only precursor to dual AI net (where this exists) and on negative control. |

### Cone bioassays

*Summarize the SOPs for cone bioassays and tunnel tests, depending on the ITN brands included in the monitoring activity. Text is included below as an example.*

#### Net preparation

For each sampled net, 30cm × 30cm pieces will be cut from standard positions 2, 3, 4 and 5 (standard position 1 will be included for pre-distribution baseline testing but excluded from post-distribution testing as it may be exposed to excessive abrasion in routine use through tucking and untucking under the bed or sleeping surface). Four total pieces will be cut for nets with the same active ingredients on all surfaces (pyrethroid-only, some PBO-synergist, dual AI nets). For nets with PBO only on the roof such as PermaNet 3.0 and Tsara Plus, 6 net pieces will be cut: side positions 2, 4 and 5 and 3 pieces cut in the roof position 3 (numbered 3.1, 3.2 and 3.3). For pyrethroid-only nets, PBO-synergist nets with PBO on all surfaces, and Interceptor G2 nets, tests will be conducted on four net pieces from positions 2, 3, 4, and 5. For PBO-synergist nets with PBO only on the roof, tests will be conducted on six net pieces from positions 2, 4, 5, and 3.1, 3.2 and 3.3. Netting samples will be labeled with the net ID and the cutting position and will be stored in a cool, dry place at 4 degrees Celsius until the tests start.

#### Testing residual efficacy of pyrethroids

To test the residual efficacy of pyrethroids, insectary-raised, 2-5-day old, unfed females of a pyrethroid-susceptible strain will be used. For each net piece tested (see 2.8.3.1 above), five mosquitoes at a time will be introduced into two WHO cones (10 mosquitos per piece in total). The total number of mosquitoes tested will be 40 or 60, depending on the ITN brand. The same cutting procedure will be followed for a sample of untreated netting as a negative control, and the same number of mosquitoes tested against the negative control. Control net results can be shared by all bioassays done the same day. If control mortality is over 10%, the test will be repeated. The knock-down effect will be measured 60 minutes after exposure (KD60) and mortality will be recorded after 24 hours.

#### Testing the overall impact of PBO nets on resistant mosquitoes

To evaluate the overall impact of PBO nets on resistant mosquitoes, a pyrethroid-resistant strain will be used in cone bioassays. Strains will be characterized before bioassays begin following standard SOPs. Cone bioassays on field samples will follow the same procedures as described in 2.8.3.2 above. Thus, the same number of mosquitos will be tested against the same net pieces from the same locations for both susceptible and resistant strains. In addition, the following controls will be used when testing PBO ITNs: one untreated netting negative control, five new PBO-brand nets as positive controls and 1 new ITN with the same pyrethroid active ingredient (e.g. PermaNet 2.0 when testing PermaNet 3.0).

### Tunnel tests

#### Testing residual efficacy of chlorfenapyr

Four tunnels will be used for each net piece to be evaluated using a pyrethroid-resistant strain. The first will be a tunnel with an untreated control net piece; the second will have the IG2 field sample to be evaluated; the third will have a piece of a new Interceptor net; the fourth will be from a new IG2 net. The IG2 field sample tested will be selected at random from among the four pieces used for the cone assay with a pyrethroid-susceptible strain. If more than 4 tunnels per night can be run, only one set of positive and negative controls need to be run, and the results can be shared amongst all the IG2 field samples being tested. A minimum of 10 positive IG2 control ITNs will be used for comparison during the test, given the high variation in mortality recorded in tests on new IG2 nets recorded in Burkina Faso.

One hundred resistant nulliparous female mosquitoes, aged 5-8 days and sugar-starved for 6 hours, will be introduced into the end opposite the bait at 18:00. The lights of the room will be turned off, and only turned on when the tunnel test is finished the following morning at 7:00. The overall exposure period should be 12-15h. The environmental conditions in the room during the night should be 27 ± 2 °C and 75% ± 10% relative humidity.

At 7:00, a narrow insert will be slid down between the two compartments of the tunnel, to prevent mosquitoes from moving between the compartments. All mosquitoes will be carefully collected from the tunnel, noting the compartment in which the mosquitoes were collected (initial compartment/animal compartment), the blood-feeding status (fed/unfed), and mortality (living/dead).

Surviving mosquitoes from the tunnels will be put into cups covered with untreated netting, and cotton wool soaked in sugar solution will be placed on top of the cups, allowing mosquitoes to feed ad libitum. The mortality will be recorded at 18:00 (24 hours after the tunnel test started), and then again at 48 and 72 hours. Data on blood feeding will also be recorded to estimate the corrected blood-feeding inhibition due to chlorfenapyr.

### Chemical residue

For the analysis of insecticidal content, four or six 10cm x 10cm samples will be cut from each sampled net at locations adjacent to those cut for the bioassays. These will be labeled with the net ID number, packaged in aluminum foil envelopes per net and shipped to the [Name of laboratory] for chemical residue testing using the ISO 17025 accredited analytical method RESMM002.

## Outcome measures

### Bioassay results

The primary outcome of insecticidal efficacy will be based on the bioassay results using the following criteria.

For **pyrethroid-only ITNs** and **PBO-synergist ITNs**, the proportion of ITNs achieved optimal and minimal effectiveness will be estimated at each assessment round.

Optimal Effectiveness is defined as:

* KD60 ≥ 95% or 24-hour mortality ≥ 80% by the cone assay

Minimal Effectiveness is defined as:

* KD60 ≥ 75% or 24-hour mortality ≥ 50% by the cone assay, or
* Mortality ≥ 80% or blood-feeding inhibition ≥ 90% by the tunnel test (where conducted)

For **PBO-synergist ITNs**, additional efficacy criteria will be quantified using the definition:

* The proportion of samples that are within 10% of the positive control values of KD60 or 24-hour mortality

As no agreed bioefficacy thresholds exist for **dual AI ITNs**, outcomes will be described in terms of the residual efficacy of active ingredients by comparing mortality among susceptible and resistant mosquito strains to field samples. Where tunnel tests are performed, proportion blood-fed and blood-feeding inhibition will also be presented. Field sample results will be compared to positive and negative controls.

### Chemical residue

Two metrics will be used to report the chemical residue test results:

1. The mean insecticide content across the overall sample and for each location
2. The proportion of nets with a g/kg value for each active ingredient that falls within the approved level defined by the WHO Pre-Qualification specification, or manufacturer specification.

### Net integrity

Net integrity will be measured at each round using the proportionate Hole Index (pHI) as recommended by WHO. Data from the net hole assessment will be transformed into the proportionate Hole Index (pHI) for each net in the following way:

*pHI= # size 1 holes + (# size 2 holes x 23) + (# size 3 holes x 196) + (# size 4 holes x 576)*

Based on the pHI each net is then categorized as “serviceable” or “torn”, with a subset of serviceable nets categorized as “good”, as follows [2-3]:

 Serviceable: total hole surface area ≤ 0.1 m² or pHI ≤ 642

Torn: total hole surface area > 0.1m² or pHI > 642

Good: total hole surface area < 0.01m² or pHI ≤ 64

### Net attrition rate

Net attrition will be indirectly estimated at each assessment round based on the number of alternate ITNs in the sample list that must be sought before all 45 required ITNs are identified and withdrawn. Lower attrition will be associated with a higher proportion of sampled ITNs still present in households and available to be withdrawn.

## Data analysis and reporting

Electronic data files will be available immediately following the completion of household data collection. Hole assessment results will be added to ITN-specific data files in the laboratory. Completed data files will be exported from the secure online database and imported to Stata. Standard Stata do files adapted from previous durability monitoring studies will be used to apply consistent cleaning, management, and analysis steps. Staff from [Name of implementing partner] or a suitably qualified consultant will review and clean the data, doing further consistency checks and preparing files for analysis. Personal identifiers will be dropped from final data sets that may be shared, on request, outside the study team. All processes will be documented using Stata do files so that any interested partner can repeat the steps on their own copy of the data set.

Bioassay testing data will be entered in standard Excel worksheets, capturing information on number of mosquitoes tested, number of mosquitoes knocked down: 3 min, number of mosquitoes knocked down: 60 min, number of mosquitoes dead: 24 hours, number of mosquitoes alive: 24 hours. Summary data from these worksheets will be produced in Excel and exported to Stata for standard data cleaning and analysis. Final analysis will follow the previously defined outcome measures (see above). Excel sheets will be used until durability monitoring data is added to VectorLink Collect (expected 2021).

Results will be shared and discussed among partners after each assessment round and a summary report issued for each round. Once the final report is completed, a dissemination meeting will be organized to present findings and recommendations to malaria vector control stakeholders and partners in the country. Following the final study round, anonymized study data will be shared with PMI/USAID for archiving on the U.S. Government’s open data portal, [www.data.gov](http://www.data.gov). Final reports and anonymized study data will also be posted to the website, www.durabilitymonitoring.org.

# Ethical Considerations

The proposed study will be conducted according to the principles of the Declaration of Helsinki and the International Guidelines for Ethical Review of Epidemiological Studies.

This study has been determined to be “research with human subjects” and will be initiated only after receiving written approval from a recognized local ethics review board and [Name of implementing partner] Institutional Review Board (IRB). Those implementing this study will comply with all policies and procedures of all reviewing boards. Informed consent will be sought for all participants in this study.

This study has been designed to address the following ethical principles: respect for persons, beneficence, and justice. Efforts are made to protect individual autonomy, minimize harm, and maximize benefits and equitably distribute risks and benefits by using procedures which are consistent with sound research designs that take these issues into consideration. Since this is an exclusive interview survey without taking of samples of any kind no harm is expected to the participants.

## Informed Consent

Respondents in households selected to form the sampling frame will be informed about the purpose and nature of the study, what participation in the study requires and possible risks and benefits. Written consent will be obtained from the head of household or their representative. Respondents in households selected from which to withdraw an ITN for analysis will be informed as above and oral consent will be obtained before withdrawing and replacing the ITN and administering the study questionnaire.

Participants will be informed of all risks and protections through the study information sheet. Participants will also be informed of their right to withdraw from the study and to not answer any questions they do not feel comfortable answering. Respondents will be provided contact information for the PI and co-investigators who will be available to answer any questions about the study.

Information sheets and consent forms will be written in [“English”, “French” or “Portuguese”] and [Local written language] and, if necessary, will be read to the respondent in their local language by a member of the field team fluent in that language.

## Respect for persons and individual autonomy

*Potential Risks*

Potential risks to subjects are breach of confidentiality.

*Breach of Confidentiality*

The most significant risk is a breach of confidentiality. In this study, a breach of confidentiality could occur if private information from the surveys could be linked to an individual research respondent and this information was obtained by person(s) outside of the research project or something a participant says will be heard or found out by someone else.

*Strategies to Address Risks*

Steps will be taken to protect participants against potential risks posed by their participation in this research. Participants will be encouraged to contact the local co-investigators at any time to discuss any concerns they might have. All data and other information will be maintained confidentially and anonymous to the greatest extent possible. The following steps will be taken to protect against breaches of confidentiality.

*Identification of data sources*

None of the information registered is sensitive. Household GPS location and household members full names for the sampling frame will be recorded in raw data files. The master lists of ITNs will maintain a record of these identifiers so that follow-up visits can be made. This list will be an electronic data file only and will be kept in a secure location with password protection and access only by the PI and co-investigators. This database will be destroyed after the final assessment round.

GPS coordinates will be modified by introducing a small random error during data cleaning. This will permit sufficient accuracy for geographic analysis of results but will not permit the location of any study households. Full name data will be removed during cleaning so that analytical data files will contain no personal identifiers. Anonymized files are the only ones which will be shared publicly, for example with the [www.data.gov](http://www.data.gov) repository.

Written consent documents will be stored in locked containers and destroyed at the conclusion of the final assessment round.

*Data reporting*

All results of the study will be reported anonymously.

*Staff ethical training*

All research staff including interviewers and supervisors will be trained in human subject’s protection, especially the importance of protecting privacy and confidentiality.

## Beneficence (maximizing benefits and minimizing harm)

There is unlikely to be any direct benefit to participants themselvesat the time of the study or after the study. However, the proposed study may result in knowledge that can be applied to the design of future interventions to promote net use, and net care to increase net durability. Primary study results will support public health donors, governments and ITN manufacturers to better design, plan and implement ITN products and campaigns based on field results on insecticide effectiveness.

# Implementation timeline and study personnel

## Roles and Responsibilities

Study implementation will be conducted jointly by the National Malaria Control Program, [Name of implementing partner], [Name of any additional laboratories] and PMI. While all partners will give input into the study tools, analysis, and interpretation, [Name of lead implementing partner], with technical support from other partners, will be responsible for quality assurance of the design, implementation, and analysis of the study.

## Timeline

The proposed study timeline for the first 12 months of implementation is shown below (covering 2 data collection rounds).

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Pre-distribution** |  | **Post-distribution (months)** |
| **Activity** | **4** | **3** | **2** | **1** | **0** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |
|  | **Set-up** |
| Protocol drafted and shared with PMI and NMCP for review | X | X |   |   |  |   |   |   |   |   |   |   |   |   |   |   |   |
| Protocol and tools for IRB submission finalized |    |  | X |  |  |   |   |   |   |   |   |   |   |   |   |   |   |
| ITN campaign distribution |    |   |   |  | X |   |   |   |   |   |   |   |   |   |   |   |   |
|  | **Baseline round (6 months)** |
| Ethical review by [Name of implementing partner] |  | X | X |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Ethical review by [country] IRB |  |  | X | X |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Baseline training |  |  |  |  |  |  |  | X |  |  |  |  |  |  |  |  |  |
| Baseline fieldwork |  |  |  |  |  |  |  | X |  |  |  |  |  |  |  |  |  |
| Baseline hole assessment |  |  |  |  |  |  |  |  | X |  |  |  |  |  |  |  |  |
| Baseline bioassays |  |  |  |  |  |  |  |  | X | X |  |  |  |  |  |  |  |
| Baseline chemical residue testing |  |  |  |  |  |  |  |  | X | X | X |  |  |  |  |  |  |
| Baseline data cleaning and analysis |  |  |  |  |  |  |  |  |  |  | X | X |  |  |  |  |  |
| Draft ITN Durability Monitoring Baseline Report submitted to PMI |  |  |  |  |  |  |  |  |  |  |  |  | X |  |  |  |  |
|  | **12-month round** |
| Draw 12-month sample and program tools |  |  |  |  |  |  |  |  |  |  |  |  |  | X |  |  |  |
| Revise training materials |  |  |  |  |  |  |  |  |  |  |  |  |  | X |  |  |  |
| Submit for continuing review from [country] IRB |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  | X | X |
| 12-month training |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  | X |
| 12-month fieldwork |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  | X |

The timeline for the follow-up rounds (12-, 24- and 36-month rounds) are shown below taking as an example the 12-month round

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Activity** | **M****9** | **M****10** | **M****11** | **M****12** | **M****13** | **M****14** | **M****15** | **M****16** | **M****17** |
| **Follow-up round (12-month)** |
| Draw 12-month sample and program tools | X |   |   |   |   |   |   |   |   |
| Revise training materials | X |   |   |   |   |   |   |   |   |
| Submit for continuing review from [country] IRB |  |  | X | X |  |  |  |  |  |
| 12-month training |  |  |  | X |  |  |  |  |  |
| 12-month fieldwork |   |   |   | X |   |   |   |   |   |
| Follow-up hole assessment |  |  |  |  | X |  |  |  |  |
| Follow-up bioassays |   |   |   |   | X | X |  |   |   |
| Follow-up chemical residue testing |   |   |   |   |  X | X | X |  |   |
| Follow-up data cleaning and analysis |   |   |   |   |   |   | X | X |  |
| Draft ITN Durability Monitoring Follow-up Report submitted to PMI |   |   |   |   |   |   |   |   | X |

## Study personnel

The following personnel are named investigators on this study.

Principal Investigator:

Co-investigator:

# Bibliography

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#

# Annex 1: List of durability monitoring completed since 2013

Source: www.durabilitymonitoring.org

|  |  |  |
| --- | --- | --- |
| **Country** | **Product** | **Monitoring Areas** |
| Burma2016-2019 | DawaPlus 2.0PermaNet 2.0 | Tamu Township |
| DRC2016-2019 | DuraNet/MAGNetDawaPlus 2.0 | Sud-Ubangi / Mongala |
| Guinea2016-2019 | PermaNet 2.0 | Boffa / Dinguiraye |
| Tanzania (Zanzibar)2016-2019 | OlysetPermaNet 2.0 | Wete / North B |
| Malawi2016-2019 | YorkoolRoyal Sentry | Mangochi / Kasungu |
| Nigeria2015-2018 | DawaPlus 2.0 | Zamfara / Ebonyi / Oyo |
| Mozambique2015-2018 | MAGNetRoyal Sentry | Inhambane / Tete / Nampula |
| Madagascar2015-2017 | PermaNet 2.0 | Nosy / Varika / Maintirano / Tulear II / Ankazobe |
| Zimbabwe2015-2019 | DawaPlus 2.0DuraNet | 13 malaria endemic districts in Mashonaland Central and West Provinces |
| Ethiopia2015-2018 | PermaNet 2.0MAGNet | Oromia / Tigray / SNNP / Amhara |
| Benin2014-2017 | PADNETLifeNetPermaNet 3.0 | Oueme |
| Madagascar2013-2015 | NetProtectYorkoolRoyal Sentry | Ambanja / Morondava / Diego-Suarez / Mandoto / Sakaraha / Toamasina II, Randriamaherijaona |
| Nigeria2012-2014 | PermaNet 2.0DawaPlus 2.0 | Zamfara / Nasarawa / Cross River |
| Madagascar2011-2015 | YorkoolRoyal SentryNetProtect | Toamasinall/Morondava / Mandoto/Sakaraha / Ambanja/Diego |

# Annex 2: Bioassays to determine residual efficacy of piperonyl butoxide (PBO) and pyrethroids on PBO synergist nets

|  |  |  |  |
| --- | --- | --- | --- |
|  | Full name | Signature | Date (dd/mm/yyyy) |
| Author | PMI VectorLink |  |  |
| QA Reviewer |  |  |  |
| Approved by |  |  |  |

**Document history:**

|  |  |  |  |
| --- | --- | --- | --- |
| **Supersede version / issue date**  | **Revisions & reason for change** | **Current** **version & version date** | **Reviewer’s name**  |
|  |  |  |  |
|  |  |  |  |
|  |  |  |  |

1. **Purpose**

This SOP outlines the procedures for conducting bioassays to evaluate the insecticidal bioefficacy of piperonyl butoxide (PBO) and pyrethroids on PBO synergist insecticide treated nets (ITNs). PBO is added to nets due to its ability to inhibit the activity of oxidase enzymes which break down pyrethroids. However, there are concerns that the PBO may not remain effective on the net as long as the pyrethroid. To assess the residual bio-efficacy of both PBO and the pyrethroid, it is necessary to have procedures that separate the actions of these two compounds through testing with pyrethroid susceptible and resistant malaria vectors.

**Equipment and Materials**

* 1. WHO cones
	2. Plastic frame with holes the diameter of WHO cones

30cm x 30cm, with holes to allow mosquitoes in cones access to nets (see SOP 0× https://pmivectorlink.org/resources/tools-and-innovations/).

* 1. Aspirators with HEPA filter (separate aspirators for each insecticide, as well as separate aspirators for each technician)
	2. Plastic/paper cups
	3. Rubber bands
	4. Untreated netting (for covering cups)
	5. Cotton wool
	6. Timers
	7. Plastic frame, 30cm x 30cm
	8. Plastic frame, 30cm x 30cm, with 4 holes the same size as the base of a WHO cone.
	9. Binder/bulldog clips
	10. Holding board (for holding plastic frames at 45° angle)
	11. Sugar or honey
	12. Distilled water
	13. Stapler
	14. Paper labels
	15. Permanent marker pens
	16. Laboratory coat
	17. Laboratory gloves
	18. Temperature/humidity, max/min reader with digital display
	19. Data forms
	20. Pens
	21. WHO tubes
	22. PBO papers (4%) (from USM)
	23. 30x30x30 mosquito cage
	24. Untreated mosquito netting
	25. New PBO nets (positive control)
	26. New pyrethroid nets (positive control)
	27. Field sampled PBO nets

**MOSQUITOES NEEDED**

|  |  |
| --- | --- |
| **Strain** | **Characteristics** |
| Susceptible | Higher than 98% mortality when tested in standard WHO tube tests with papers treated with the pyrethroid of interest at the diagnostic dose. |
| Resistant | Either a well characterized (please see SOP16 on how to characterize) pyrethroid-resistant insectary strain or wild mosquitoes collected from the field. Wild mosquitoes should only be used if a resistant insectary strain is not available. For the purposes of these assays, resistant mosquitoes must have less than 70% mortality when tested in a WHO tube test with the pyrethroid of interest at the diagnostic dose. Additionally, the pre-exposure to PBO should result in an absolute increase in mortality of at least 20% (e.g. 30% to 50%). Ideally a malaria vector species should be used, but a well characterized *Aedes* or *Culex* species may also be used. |

**Control nets NEEDED**

|  |  |
| --- | --- |
| **Net type** | **Purpose** |
| Untreated netting | Negative control that must be tested each day, just before and just after testing treated netting material. If mortality in the untreated control is >10% all bioassays conducted on field nets on that day must be repeated. |
| New PBO net | Positive control that will be used to compare with field used nets. Therefore, the new PBO net must be the same brand as the field sampled PBO nets.  |
| New pyrethroid only net | Positive control that should be used if the PBO net has PBO on all surfaces. This is not necessary for synergist nets with PBO only on the roof as the pyrethroid sides will be used for comparison. Standard pyrethroid nets should have the same insecticide as the PBO net, for example, if Olyset Plus from the field was being tested, this would be compared to new Olyset Plus and new Olyset nets. |

1. **Safety**

|  |
| --- |
| **HAZARDS** |
| 1. *Hazard –* Dermal exposure to insecticide treated netting and insecticide papers.
 |

|  |
| --- |
| **RISK CONTROL** |
| 1. *Risk control –* Wear a lab coat and gloves at all times when handing ITNs and insecticide treated papers.
 |

* 1. **Glossary**

**Table 1. Classification of adult mosquitoes as alive, knocked down or dead in WHO Cone bioassays**

|  |  |  |
| --- | --- | --- |
| ***Alive*** | ***Knockdown (recorded 60 minutes after exposure)*** | ***Dead (recorded 24 hours after exposure)*** |
| -Can both stand and fly in a coordinated manner | -Any mosquito that cannot stand (e.g. has 1 or 2 legs due to exposure to insecticide) -Any mosquito that cannot fly in a coordinated manner -A mosquito that lies on its back, moving legs and wings but unable to take off -A mosquito that can stand and take of briefly but falls down immediately  | -No sign of life: immobile; cannot stand -A mosquito that lies on its back, moving legs and wings but unable to take off  |

* 1. **Background information**

As of September 2020, there were six PBO synergist ITNs that have WHO PQ listing for malaria prevention[[11]](#footnote-12) (Table 2). All six have a pyrethroid insecticide on the sides and roof of the net. Four have PBO on the sides and the roof of the net, while two have PBO only on the roof. The procedures are different for PBO synergist nets with PBO only on the roof compared to those with PBO on the sides and roof of the net. Note that PBO net panels may contain different levels of pyrethroid (e.g., PermaNet 3.0 sides have 2.8g/kg of deltamethrin on the walls (75 denier) compared with 4g/kg on the roof; Tsara Plus has 2.5g/kg of deltamethrin on the walls, compared with 3g/kg on the roof). These differences should be considered when interpreting the data.

**Table 2. WHO Pre-qualified PBO Synergist ITNs**

|  |  |  |  |
| --- | --- | --- | --- |
| **Net name** | **Type of pyrethroid** | **PBO location** | **Pyrethroid & PBO content** |
| DuraNet Plus | Alpha-cypermethrin | Sides & roof | * 6.0 g/kg (270 mg/m2) alpha‐cypermethrin
* 2.2 g/kg (99 mg/m2) PBO
 |
| VEERALIN | Alpha-cypermethrin | Sides & roof | * 6.0 g/kg (216 mg/m2) alpha‐cypermethrin
* 2.2 g/kg (79 mg/m2) PBO
 |
| PermaNet 3.0 | Deltamethrin | Roof | Roof:* 4 g/kg deltamethrin
* 25 g/kg PBO

Sides (deltamethrin only):2.8 g/kg for 75 denier2.1 g/kg for 100 denier |
| Tsara Boost | Deltamethrin | Sides & roof | * 12% (120mg/m2) deltamethrin
* 44% (440mg/m2) PBO
 |
| Tsara Plus | Deltamethrin | Roof | Roof: * 3g/kg (120mg/m2) deltamethrin
* 11g/kg (440mg/m2) PBO

Sides: * 2.5g/kg (100mg/m2) deltamethrin
 |
| Olyset Plus | Permethrin | Sides & roof | * 2% permethrin; 1% PBO
 |

1. **Procedures**

Figure 1 shows an overview flow chart of activities when conducting bioassay testing of PBO synergist nets.

**Figure 1. Flow chart showing nets required, pieces to be cut, mosquito strains to be tested and nets to be tested by mosquito strain.**

  

**2.1 Cutting Net Pieces**

2.1.1 Always wear gloves while handling and cutting the nets. Change gloves when handling different insecticides to avoid contamination.

2.1.2 If the net has PBO on all surfaces, cut a total of four netting pieces: 3 from the sides and 1 from the roof as shown in Figure 2.

2.1.3 If the net has PBO only on the roof (i.e. PermaNet 3.0 or Tsara Plus), cut a total of 6 netting pieces: 3 from the sides and 3 from the roof as shown in Figure 3.

2.1.3a For pre-distribution testing, a sample from position 1 is also taken. This position is omitted from tests conducted on field nets following distribution.

2.1.4 Label the individual netting pieces using paper labels stapled onto the corner of a net. Store each individual netting piece separately in aluminium foil in a refrigerator (4±3°C) when not in use. The net label and outside of the aluminium foil should also be labelled with the net brand, net code, netting piece number and age of net (e.g. PermaNet 3.0/B102/P3/12mo).

2.1.5 The same cutting and labelling procedure should also be followed for the positive controls (new PBO nets and new pyrethroid nets) and the negative control (untreated netting).

2.1.6 Additional pieces of netting should be cut for chemical analysis. For every position cut for bioassay an additional piece should be cut in an adjacent position for chemical analysis.

**Figure 2: Recommended positions from which netting pieces should be taken for post-distribution testing if PBO is on all surfaces of the net.**



**Figure 3: Recommended positions from which netting pieces should be taken for post-distribution testing if PBO is only on the roof of the net (i.e. PermaNet 3.0, Tsara Plus).**

**2.2 Mosquitoes to be used in bioassays**

* + 1. Bioassays will be done with both an insectary-reared pyrethroid susceptible strain (e.g. *An. gambiae* Kisumu) and a pyrethroid resistant strain or wild collected *Anopheles.*
		2. It is essential that for the pyrethroid resistant strain at least part of the resistance should be due to elevated p450 oxidases.
		3. Characterization of both susceptible and resistant mosquitoes must be done before net bioassays begin according to VectorLink SOP16 (Characterization of pyrethroid susceptible and resistant *Anopheles* for use in PBO synergist and dual active ingredient ITN bioefficacy monitoring).

**2.3 Bioassay Procedures**

2.3.1 Clean all testing surfaces with bleach solution or soapy water and dry using paper towel.

2.3.2 Prepare the paper cups by covering them with pieces of untreated netting and secure the netting with a rubber band. Cut a small slit in the netting in the center of the cup to allow the end of the aspirator to pass through the netting and plug this hole with a small wad of cotton wool.

* + 1. Wash hands and then prepare a 10% sugar/honey solution e.g., by adding 50g of sugar to 500ml of distilled water or 50ml of honey to 450ml of distilled water. Mix well with a spoon.
		2. Take the printed data forms ‘ITN Cone Bioassay Test Record Form’ (Form 2) and complete ‘Section 1: WHO ITN Cone Bioassay Location Details’, before starting tests.
		3. Gloves should be worn at all times during bioassays and should be changed when handling nets with different insecticides.

2.2.7 Place the solid plastic frame on the bench top and then place the netting on top of the frame. Place the cones on top of the netting and secure them by placing the plastic plate with 4 holes over the cones. Note that we will only use 2 cones per netting piece. Use binder clips to secure the frames to each other. The frames should then be placed at a 45° angle on the holding board so that mosquitoes rest on the netting and clamp the plates into place (Figure 4).

**Figure 3: Cone bioassay of mosquito net pieces.**

Courtesy of Dr Vincent Corbel, Institut de Recherche pour le Développement, Montpellier, France. Taken from ‘WHO Guidelines for Laboratory and field testing of long-lasting insecticidal nets.

2.2.8 Temperature and humidity should be monitored with a data logger or manually by recording the max/min during the testing and holding periods and recorded on the data forms.

* + 1. For each of the net pieces testing should be done with 5 mosquitoes per cone using 2 cones, resulting in 10 mosquitoes per net piece. Therefore, for nets with PBO on all surfaces the total number of mosquitoes tested (four net pieces) is 40 and for nets with PBO only on the roof it is 60 (six net pieces).
		2. Mosquitoes exposed to untreated net pieces are used as controls; they should be tested at the start and end of each day. If the mortality in controls on any day is < 10%, the results for that day should be adjusted by Abbott’s formula. If the mortality in controls is > 10% on a given day, the results for that day are considered invalid and should be discarded.
		3. Before adding mosquitoes to the cones, set the timer for 3 minutes. For each cone, 5 mosquitoes should be introduced by aspirator into the cone and the cone blocked with a piece of cotton. The timer should be started as soon as all mosquitoes are in the cone. Ideally a separate timer should be used for each cone.
		4. Once the timer reaches 3 minutes, the mosquitoes should be aspirated gently from the cone and into the paper cup through the slit cut in the netting. Cover the slit with cotton wool after the mosquitoes are in the cup.
		5. Provide mosquitoes with honey/sugar solution by moistening a piece of cotton wool, squeezing it to remove excess solution, and placing it on top of the cup. Before handling sugar solution hands should be washed with soapy water to prevent contamination through handling treated netting.
		6. Knock-down of mosquitoes should be recorded 60 minutes after the end of the cone bioassay and mortality 24 hours after bioassay, according to the definitions in the glossary (Table 1).
	1. **Data management and interpretation**
		1. All cone bioassay data will be recorded using the ‘ITN Cone Bioassay Test Record Form’. Note that there are two form versions, one for PBO on the roof only and another for PBO on the sides and roof (See Form 2)
		2. Data will subsequently be entered into an excel database titled ‘PBO Synergist Cone Bioassay Database’ (file template to accompany this SOP).
		3. Efficacy criteria will be quantified using two definitions:

1) Exceeds WHO thresholds of 95% KD60 or 80% 24-hour mortality.

2) Is within 10% of the new PBO ITN KD60 or 24-hour mortality.

* 1. **Optional method for testing the residual efficacy of piperonyl butoxide**

The previous methods evaluate the bioefficacy of pyrethroids alone, and pyrethroids in combination with PBO. If PBO net efficacy has declined, it may be useful to determine whether the loss of efficacy is due to loss or unavailability of PBO. A method for assessing the residual efficacy of PBO is listed here. This method can be conducted at time points when mean mortality of PBO synergist nets has dropped below 80% using pyrethroid resistant mosquitoes.

2.4.1 PBO pre-exposure will be done using WHO tube tests and PBO papers (4%) or alternatively CDC bottle bioassays could be used with 100µg PBO/bottle). Appropriate untreated controls will also be tested.

2.4.2 Prior to the cone tests, at least 50 pyrethroid resistant mosquitoes should be exposed to PBO treated papers (4%) in a WHO tube test or a CDC bottle (treated at 100μg/bottle) for 60 minutes. After 60 minutes, the mosquitoes should be released into a cage with sugar solution before being used in cone bioassays.

2.4.3 Cone bioassays of netting pieces should be conducted as described earlier (2.3). If the net has PBO on all sides, 2 cones using 5 mosquitoes can be conducted on each of the four pieces using the PBO-pre-exposed mosquitoes, and 2 cones using 5 mosquitoes can be conducted on each of the four pieces using unexposed mosquitoes.

2.4.4 If the net has PBO on the roof only, 3 cones using 5 mosquitoes can be conducted on each of the three roof pieces using the PBO-pre-exposed mosquitoes, and 3 cones using 5 mosquitoes can be conducted on each of the three pieces using unexposed mosquitoes. The control will be done with 5 pieces and two cones conducted on each piece.

2.4.5 The results of the bioassays will be recorded using the ‘ITN Cone Bioassay Test Record Form’ which has tick boxes in Section 3 to record whether pre-exposure with PBO was conducted.

2.4.6 The data can be interpreted by dividing the percentage mortality in the resistant mosquitoes tested without pre-exposure by the percentage mortality in the resistant mosquitoes that were pre-exposed to PBO.

1. **QUALITY CONTROL**
	1. As described above, the susceptible and pyrethroid resistant strains should be characterized within two weeks before netting bioassays are conducted.
	2. Negative control bioassays using untreated netting will be conducted at the start and end of each day.
2. **SOP COPY CONTROL LOG**

**Purpose:** The log records the number of certified copies of this SOP printed and where they were distributed.

**When:** Whenever the SOP is reviewed: annually or more often when necessary.

**By whom:** By QA staff / designee

|  |  |
| --- | --- |
| **Distribution Date: 18th September 2020** | **Total number of certified copies** (including Master Copy)**: NA** |
| **SOP Distribution (location and number of certified copies)** |
|  |
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|  |  |  |  |

1. **FORMS**

**Form 1: SOP Training Log for Personnel Training Files**

|  |  |  |  |
| --- | --- | --- | --- |
| Date: | SOP Number and Title | Employee Signature | Supervisor Initials |
|  |  |  |  |
|  |  |  |  |
|  |  |  |  |
|  |  |  |  |

**Form 2. ITN Cone Bioassay Test Record Form (PBO on roof only)**

On following pages.

**Form 3. ITN Cone Bioassay Test Record Form (PBO on roof and sides)**

On following pages.

|  |  |
| --- | --- |
| **Name of Person Completing Form: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**(Surname, First Name) | **Date of WHO ITN Cone Bioassay Test: \_\_ \_\_ -\_\_ \_\_ - \_\_ \_\_ \_\_ \_\_** (DD-MM-YYYY) |
| **Section 1: WHO ITN Cone Bioassay Location Details** |
| **Country:** \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ **Province:** \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ **District:** \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ **Village:** \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ |
| **Household GPS Coordinates** (of net collection) (in decimal degrees)**:****Latitude** \_\_\_|\_\_\_|.\_\_\_|\_\_\_|\_\_\_|\_\_\_|\_\_\_º (i.e. 17.92412º)  **Longitude** \_\_\_|\_\_\_|.\_\_\_|\_\_\_|\_\_\_|\_\_\_|\_\_\_º (i.e. 25.85723 º) **Altitude**(in meters)**:** \_\_\_\_\_\_\_ |
| **GPS accuracy**(in meters)**:** \_\_\_\_\_\_(values >10 meters should be retaken) | **GPS data source:**[ ] GPS Device [ ] Phone [ ] Tablet [ ] Other  | **Specify GPS data source**(e.g. name/make of device; application; software; etc.)**:**  \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ |
|  |
| **ITN Code:**\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ |
| **ITN Brand**(*Please choose one option that best fits)*:[ ] Untreated Net (Control) [ ] TSARANet [ ] TSARABoost [ ] TSARAPlus [ ] TSARASoft [ ] DawaPlus 2.0 [ ] DawaPlus 3.0 [ ] DawaPlus 4.0 [ ] Duranet [ ] Interceptor [ ] Interceptor G2 [ ] MiraNet [ ] MAGNet [ ] OLYSET Net [ ] OLYSET Plus [ ] Panda Net 2.0 [ ]  PermaNet 2.0 [ ] PermaNet 3.0 [ ] Royal sentry [ ]  SafeNet [ ] VEERALIN [ ]  Yahe LN [ ] Yorkool LN [ ] Unknown [ ] Other **If other, specify**\_\_\_\_\_\_\_\_\_\_\_\_ |
| **Age of ITN** (*Please choose one)*:[ ] Baseline (0-6 Months) [ ]  12 Months [ ] 18 Months [ ]  24 Months [ ] 36 Months [ ] Unknown [ ] Other **If other, specify**\_\_\_\_\_\_\_\_\_\_\_[ ] New **If new, specify date of production and batch number:** \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ |

|  |
| --- |
| **Section 2: Mosquito Details** |
| **Mosquito Origin** (*Please choose one)*: [ ] Susceptible colony[ ] F0: Reared from wild larvae[ ] F1: Reared from eggs of wild adults[ ] F2: Reared from F1 adults[ ] Collected adults[ ] Pyrethroid Resistant Colony | If mosquito origin was F0, F1, F2, or Collected Adults, please specify details on where mosquitos were originally sourced:**Country:** \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ **Region:** \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ **District:** \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ **Village:** \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ |
| **Mosquito Species Tested** (*Please choose one):* [ ] A*n. gambiae* (Kisumu) [ ] *An. arabiensis* (susceptible colony) [ ] *An. funestus* s.l. [ ] *An. gambiae* s.l. [ ] *An. coluzzii VKPER (resistant colony)*[ ] *An. coluzzii (susceptible colony)* [ ] Other **If other, specify**\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ |
| **Mosquito Age (days)** (*Please choose one):* [ ] 2-5 days [ ] Other age (days) [ ] Unknown **If other, specify**\_\_\_\_\_\_\_\_\_\_ |
| **Section 3: WHO Cone Bioassay Test Details and Results**  |
| **Temperature** (○ Celsius): Exposure period: Max\_\_\_\_\_\_\_(Celsius) Min\_\_\_\_\_\_(Celsius) Holding period: Max\_\_\_\_\_\_\_(Celsius) Min\_\_\_\_\_\_(Celsius) | **Relative humidity** **(%):** Exposure period: Max \_\_\_\_\_\_ % Min \_\_\_\_\_\_% Holding period: Max\_\_\_\_\_\_% Min\_\_\_\_\_\_% |
| **Optional test:** was 60 minutes PBO pre-exposure conducted before net bioassay(*Please choose one):* [ ]  Yes (4% PBO paper) [ ]  Yes (100µg PBO/bottle) [ ]  No  |

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Net Position** | **Replicates/Cones** | **Number of mosquitoes tested** | **Number of mosquitoes knocked down: 60 min** | **Number of mosquitoes dead: 24 hours** |
| **1 (side)** | **1** |  |  |  |
| **2** |  |  |  |
| **2 (side)** | **1** |  |  |  |
| **2** |  |  |  |
| **3.1 (roof)** | **1** |  |  |  |
| **2** |  |  |  |
| **3.2 (roof)** | **1** |  |  |  |
| **2** |  |  |  |
| **3.3 (roof)** | **1** |  |  |  |
| **2** |  |  |  |
| **4 (side)** | **1** |  |  |  |
| **2** |  |  |  |
| **5 (side)** | **1** |  |  |  |
| **2** |  |  |  |

|  |  |
| --- | --- |
| **Name of Person Completing Form: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**(Surname, First Name) | **Date of WHO ITN Cone Bioassay Test: \_\_ \_\_ -\_\_ \_\_ - \_\_ \_\_ \_\_ \_\_** (DD-MM-YYYY) |
| **Section 1: WHO ITN Cone Bioassay Location Details** |
| **Country:** \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ **Province:** \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ **District:** \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ **Village:** \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ |
| **Household GPS Coordinates** (of net collection) (in decimal degrees)**:****Latitude** \_\_\_|\_\_\_|.\_\_\_|\_\_\_|\_\_\_|\_\_\_|\_\_\_º (i.e. 17.92412º)  **Longitude** \_\_\_|\_\_\_|.\_\_\_|\_\_\_|\_\_\_|\_\_\_|\_\_\_º (i.e. 25.85723 º) **Altitude**(in meters)**:** \_\_\_\_\_\_\_ |
| **GPS accuracy**(in meters)**:** \_\_\_\_\_\_(values >10 meters should be retaken) | **GPS data source:**[ ] GPS Device [ ] Phone [ ] Tablet [ ] Other  | **Specify GPS data source**(e.g. name/make of device; application; software; etc.)**:**  \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ |
|  |
| **ITN Code:**\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ |
| **ITN Brand** (*Please choose one option that best fits)*:[ ] Untreated Net (Control) [ ] TSARANet [ ] TSARABoost [ ] TSARAPlus [ ] TSARASoft [ ] DawaPlus 2.0 [ ] DawaPlus 3.0 [ ] DawaPlus 4.0 [ ] Duranet [ ] Interceptor [ ] Interceptor G2 [ ] MiraNet [ ] MAGNet [ ] OLYSET Net [ ] OLYSET Plus [ ] Panda Net 2.0 [ ]  PermaNet 2.0 [ ] PermaNet 3.0 [ ] Royal sentry [ ]  SafeNet [ ] VEERALIN [ ]  Yahe LN [ ] Yorkool LN [ ] Unknown [ ] Other **If other, specify**\_\_\_\_\_\_\_\_\_\_\_\_ |
| **Age of ITN** (*Please choose one)*:[ ] Baseline (0-6 Months) [ ]  12 Months [ ] 18 Months [ ]  24 Months [ ] 36 Months [ ] Unknown [ ] Other **If other, specify**\_\_\_\_\_\_\_\_\_\_\_[ ] New **If new, specify date of production and batch number:** \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ |

|  |
| --- |
| **Section 2: Mosquito Details** |
| **Mosquito Origin** (*Please choose one)*: [ ] Susceptible colony[ ] F0: Reared from wild larvae[ ] F1: Reared from eggs of wild adults[ ] F2: Reared from F1 adults[ ] Collected adults[ ] Pyrethroid Resistant Colony | If mosquito origin was F0, F1, F2, or Collected Adults, please specify details on where mosquitos were originally sourced:**Country:** \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ **Region:** \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ **District:** \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ **Village:** \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ |
| **Mosquito Species Tested** (*Please choose one):* [ ] A*n. gambiae* (Kisumu) [ ] *An. arabiensis* (susceptible colony) [ ] *An. funestus* s.l. [ ] *An. gambiae* s.l. [ ] *An. coluzzii VKPER (resistant colony)*[ ] *An. coluzzii (susceptible colony)* [ ] Other **If other, specify**\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ |
| **Mosquito Age (days)** (*Please choose one):* [ ] 2-5 days [ ] Other age (days) [ ] Unknown **If other, specify**\_\_\_\_\_\_\_\_\_\_ |
| **Section 3: WHO Cone Bioassay Test Details and Results**  |
| **Temperature** (○ Celsius): Exposure period: Max\_\_\_\_\_\_\_(Celsius) Min\_\_\_\_\_\_(Celsius) Holding period: Max\_\_\_\_\_\_\_(Celsius) Min\_\_\_\_\_\_(Celsius) | **Relative humidity** **(%):** Exposure period: Max \_\_\_\_\_\_ % Min \_\_\_\_\_\_% Holding period: Max\_\_\_\_\_\_% Min\_\_\_\_\_\_% |
| **Optional test:** was 60 minutes PBO pre-exposure conducted before net bioassay(*Please choose one):* [ ]  Yes (4% PBO paper) [ ]  Yes (100µg PBO/bottle) [ ]  No  |

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Net Position** | **Replicates/Cones** | **Number of mosquitoes tested** | **Number of mosquitoes knocked down: 60 min** | **Number of mosquitoes dead: 24 hours** |
| **1 (side)** | **1** |  |  |  |
| **2** |  |  |  |
| **2 (side)** | **1** |  |  |  |
| **2** |  |  |  |
| **3 (roof)** | **1** |  |  |  |
| **2** |  |  |  |
| **4 (side)** | **1** |  |  |  |
| **2** |  |  |  |
| **5 (side)** | **1** |  |  |  |
| **2** |  |  |  |

# Annex 3: Bioassays to determine residual efficacy of alphacypermethrin and chlorfenapyr on mosquito nets

1. **Purpose**

This SOP outlines the procedures for conducting bioassays to evaluate the residual efficacy of alphacypermethrin and chlorfenapyr on mosquito nets such as the Interceptor G2 brand nets. The Interceptor G2 long-lasting insecticidal net (BASF, Ludwigshafen) is a multifilament polyester net produced with a proprietary polymer system. The net is coated with 200mg/m2 of chlorfenapyr and 100mg/m2 alphacypermethrin. Little is known about the bioefficacy of these two insecticides on Interceptor G2 nets that have been used in field conditions. To assess the residual bio-efficacy of both alphacypermethrin and chlorfenapyr, it is necessary to have a protocol that separates the actions of these two compounds.

Standard cone bioassays with standard susceptible strains will not allow a separation of the effects of alphacypermethrin and chlorfenapyr. In general, alphacypermethrin will have a rapid action whereas chlorfenapyr typically takes a longer time to affect the mosquitoes. In 3-minute cone bioassays on nets treated with chlorfenapyr at 200mg/m2 (the same dose present on the Interceptor G2), knockdown of *An. gambiae* s.s. Kisumu strain was 0% when measured one hour after the bioassay, and mortality was only 2% at 24 hours (WHO 2017). N’Guessan et al. (2007) found less than 20% mortality at 24 hours, which rose to nearly 80% at 72 hours. Other studies have found a more significant impact at 24 hours; Mosha et al. (2008) found increases of only 15-25% between mortalities recorded at 24 and 72 hours for the doses of 100, 250, and 500mg/m2. For alphacypermethrin, Tungu et al. (2016) found that Interceptor ITNs (with 200mg/m2 of alphacypermethrin) resulted in 100% knockdown of *An. gambiae* Kisumu at baseline. A similar result was found for *An. gambiae* Kisumu exposed to netting treated with alphacypermethrin at 100mg/m2 which resulted in 98% knockdown (WHO 2017c). We propose that the residual efficacy of alphacypermethrin on Interceptor G2 nets be assessed using the knockdown of pyrethroid-susceptible mosquitoes at 60 minutes after a 3-minute exposure to netting.

To measure the efficacy of the chlorfenapyr on netting will require more effort and the use of alphacypermethrin-resistant strains of mosquitoes. Oxborough et al. (2015) found that standard 3-minute cone bioassays conducted during the day were not reflective of the impact of chlorfenapyr in experimental hut studies. However, when nets were attached to filter papers and tested in WHO cylinders and the testing time was extended to 30 minutes at a temperature of 27°C (testing and holding), the results at 72 hours post-test were more representative of results in experimental huts (complete mortality).

Another option, described here, is the use of tunnel tests. As with the 30-minute cylinder bioassays, when tunnel tests were used, the results were much more reflective of the hut results, perhaps because the insecticide was available at night when mosquitoes were more metabolically active. Insecticide resistant *Culex quinquefasciatus* were used in tunnel tests to determine the regeneration time of Interceptor G2 nets (WHO 2017c), and the same principles can be used for determining the bioefficacy of these nets.

**Equipment and Materials**

* 1. WHO cones
	2. Aspirators (separate aspirators for introduction and removal of mosquitoes from the cone)
	3. Plastic cups
	4. Rubber bands
	5. Untreated netting
	6. Cotton
	7. Timers
	8. Plastic plates, 30cm x 30cm, solid
	9. Plastic plates, 30cm x 30cm, with 4 holes 10 cm in diameter
	10. Binder clips
	11. Sugar or honey solution (5-10%)
	12. Stapler
	13. Paper for labels
	14. Permanent marker
	15. Laboratory coat
	16. Glass tunnel
	17. Rabbit / guinea pig
	18. Latex-free gloves
	19. Data recording form
	20. Tiny Tag Data logger

**MOSQUITOES NEEDED**

|  |  |
| --- | --- |
| **Strain** | **Characteristics** |
| Susceptible | Higher than 98% mortality when tested in standard WHO tube tests with alphacypermethrin-treated papers (0.05%) |
| Resistant | Either an alphacypermethrin-resistant insectary strain or wild mosquitoes collected from the field that have less than 70% mortality when tested in a tunnel test with a new Interceptor net (200mg/m2 alphacypermethrin). Ideally a malaria vector species should be used, but a well characterized *Aedes* or *Culex* species might also be used. |

1. **Safety**

|  |
| --- |
| **HAZARDS** |
| List items that are risks, e.g., manual handling, sharps, chemical, biological, radiation1. *Hazard –* Insecticide and hazardous reagents (Insecticide treated netting)
2. *Hazard –* Handling of animals may result in bites to humans or injury to animals.
 |

|  |
| --- |
| **RISK CONTROL** |
| List what controls are put in place to minimize or lower the risk level, e.g., PPE, restrict use of item/chemical to trained persons, specific training and induction processes, designated waste disposal guidelines etc.1. *Risk control –* Wear lab coat and gloves at all times when handing insecticide and other reagents.
2. *Risk control –* Protocols in place to safely handle animals before, during, and after tunnel tests
 |

**Table 1. Classification of adult mosquitoes as alive, knocked down or dead in Phase I WHO Cone bioassays**

|  |  |  |
| --- | --- | --- |
| ***Alive*** | ***Knockdown (recorded 60 minutes after exposure)*** | ***Dead (recorded 24 hours after exposure)*** |
| -Can both stand and fly in a coordinated manner | -Any mosquito that cannot stand (e.g. has 1 or 2 legs) -Any mosquito that cannot fly in a coordinated manner -A mosquito that lies on its back, moving legs and wings but unable to take off -A mosquito that can stand and take of briefly but falls down immediately  | -No sign of life: immobile; cannot stand -A mosquito that lies on its back, moving legs and wings but unable to take off  |

1. **Procedures**

**Testing the residual efficacy of alphacypermethrin:**

**Preparing for cone bioassays**

* 1. Prepare the plastic cups by covering them with pieces of untreated netting and secure the netting with a rubber band. Cut a small slit in the netting in the center of the cup to allow the end of the aspirator to pass through the netting and plug this hole with a small wad of cotton.
	2. Prepare sugar/honey solution by adding 20g of sugar or 20ml of honey to 180ml of water. Mix well.
	3. Cut the four pieces of netting from the nets of interest. Be sure to wear gloves while handling the nets and cut 30x30cm pieces of the net. This should be done according to WHO protocols (2013) as shown in Figure 1. Position 1 is included when testing samples prior to distribution. Exclude position 1 for testing following distribution as it may be exposed to excessive abrasion from being tucked under the bed. Label the netting immediately using paper labels stapled onto the corner of a net. Store in aluminium foil when not in use in refrigerator (4°C±3°).

**Figure 1: Recommended positions from which netting pieces should be taken for post-distribution testing (WHO 2013).**



* 1. Use the plastic plates to fix the cones onto the netting to be tested in place. Place the solid plastic plate on the bench top and then place the netting on top of the plate. Place the 4 cones on top of the netting and secure them by placing the plastic plate with 4 holes over the cones. Use binder clips to secure the plates to each other. The plates should then be placed at a 60° angle that allow access of the mosquitoes to the netting and clamp the plates into place.
	2. For each cone, 5 susceptible mosquitoes should be introduced into the cone and the cone blocked with a piece of cotton. The timer can be started as soon as all mosquitoes are in the cone. Ideally a separate timer should be used for each cone.
	3. Once the timer reaches 3 minutes, the mosquitoes should be aspirated gently from the cone and into the plastic cup through the slit cut in the netting. Cover the slit with cotton wool after the mosquitoes are in the cup. Provide mosquitoes with honey/sugar solution by moistening a piece of cotton wool, squeezing it to remove excess solution, and placing it on top of the cup.
	4. Knockdown of mosquitoes should be read by observing the number of mosquitoes unable to stand or fly at 60 minutes after the end of the cone bioassay.
	5. For each of the four net pieces tested, 10 mosquitoes (2 cones) should be completed, resulting in 40 mosquitoes used for testing all four net pieces (plus 50 mosquitoes exposed to control (untreated) netting in 3-minute cone tests to ensure mortality is due to the insecticide). See Figure 2. One set of controls can be used for all tests conducted in a day.

**Figure 2: A matrix for recording bioassay results, each empty cell should be filled with the results (number responding/number tested)**.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **KNOCKDOWN** | Cone 1 | Cone 2 | Cone 3 | Cone 4 | Cone 5 |
| Piece 2 (side) |   |   |   |   |   |
| Piece 3 (side) |   |   |   |   |   |
| Piece 4 (side) |   |   |   |   |   |
| Piece 5 (roof) |   |   |   |   |   |
| Control 1 |   |   |   |   |   |
| Control 2 |  |  |  |  |  |

**Testing the residual efficacy of chlorfenapyr:**

**Determination of a resistant strain**

* 1. The testing of chlorfenapyr bioefficacy is challenging and requires either a wild resistant strain or laboratory-reared resistant strain. The use of a resistant strain introduces considerable variability into the bioassay, as even laboratory resistant strains can vary in their degree of resistance. For this reason, before each series of tunnel tests, the resistant strain should be tested in a tunnel test.
	2. Tunnel tests consist of 60x25x25cm glass or plexiglass containers. At each end of the tunnel, a 30x30cm mosquito cage is fitted. The LN netting sample, held in a disposable cardboard frame, is placed at one third the length of the glass tunnel. The surface of netting available to the mosquitoes is 400 cm2 (20 cm x 20 cm), with nine holes 1 cm in diameter; one hole is located at the center of the square, and the other eight are equidistant and located 5 cm from the border. In the shorter section of the tunnel, a suitable bait (e.g. guinea pig or rabbit) is placed, which is unable to move and is available for mosquito biting (WHO 2013).
	3. Prior to commencing any tunnel tests, ensure that the temperature and humidity can be monitored throughout the testing and holding periods. This can be done using the TinyTag data loggers or similar loggers.
	4. To determine the resistant strain, two tunnels will be used. The first will have a piece of netting from a new Interceptor (alphacypermethrin 200mg/m2) (aired for a few days before use) and the second will have a piece of untreated netting.
	5. One hundred nulliparous female mosquitoes, aged 5-8 days and sugar-starved for 6 hours, will be introduced into the end of each tunnel opposite the bait at 18:00. The lights of the room will be turned off, and only turned on when the tunnel test is finished the following morning at 7:00. According to WHO guidelines (2013) the overall exposure period should be 12-15h. The environmental conditions in the room during the night should be 27 ± 2 °C and 75% ± 10% relative humidity
	6. At 7:00, a narrow insert will be slid down between the two compartments of the tunnel, to prevent mosquitoes from moving between the compartments. All mosquitoes will be carefully collected from the tunnel, noting the compartment in which the mosquitoes were collected (initial compartment/animal compartment), the blood-feeding status (fed/unfed), and mortality (living/dead). The results can be recorded as shown in Figure 3.
	7. The following formula will used to be assess mortality in the tunnel tests:

Let X = the percent living in the control tunnels

Let Y = the percent living in the alphacypermethrin tunnels

The corrected mortality due to alphacypermethrin will be (X-Y)/X × 100

If control mortality is greater than 20%, the test should be repeated.

* 1. If the resistant strain shows a mortality of less than 70% in a tunnel with a new Interceptor net, then it can be used for testing of chlorfenapyr in Interceptor G2 testing. If this threshold is not met, either another field strain should be used after testing, or the net pieces should be tested at a laboratory that maintains a suitable strain of resistant mosquitoes. Note that it is not necessary that the vector strain be used, as the tests are meant to assess the quantity of insecticide on the netting, not the susceptibility of the mosquitoes. Many field sites may have easier access to *Culex quinquefasciatus* or *Aedes aegypti* that meet these criteria and use of these strains are encouraged. Once a suitable resistant strain has been found, it can be used in tunnel tests.

**Figure 3: Table for recording data from tunnel tests**

**Tunnel tests**

* 1. The resistant strain should be used for the four tunnels described here.
	2. Prior to commencing any tunnel tests, ensure that the temperature and humidity can be monitored throughout the testing and holding periods. This can be done using the TinyTag data loggers or similar logger.
	3. Four tunnels will be used for each net piece to be evaluated. The first one will be a tunnel with an untreated control net piece. The second will have the G2 piece to be evaluated. The piece of the G2 to be used (from amongst the four pieces cut above) will be determined using a random number generator. The third will have a piece of a new Interceptor net. The fourth will be from a new G2 net. Note that if there is the possibility to run more than 4 tunnels per night, only one control, one new G2, and one new Interceptor tunnels need to be run, and the results can be shared amongst all of the field G2s being tested.
	4. One hundred resistant nulliparous female mosquitoes, aged 5-8 days and sugar-starved for 6 hours, will be introduced into the end opposite the bait at 18:00. The lights of the room will be turned off, and only turned on when the tunnel test is finished the following morning at 7:00. According to WHO guidelines (2013) the overall exposure period should be 12-15h. The environmental conditions in the room during the night should be 27 ± 2 °C and 75% ± 10% relative humidity
	5. At 7:00, a narrow insert will be slid down between the two compartments of the tunnel, to prevent mosquitoes from moving between the compartments. All mosquitoes will be carefully collected from the tunnel, noting the compartment in which the mosquitoes were collected (initial compartment/animal compartment), the blood-feeding status (fed/unfed), and mortality (living/dead). The results can be recorded as shown in Figure 4.
	6. The living mosquitoes from the tunnels will be put into cups covered with untreated netting, and cotton wool soaked in sugar solution will be placed on top of the cups, allowing mosquitoes to feed *ad libitum*. The mortality will be recorded at 18:00 (24 hours after the tunnel test started), and then again at 48 and 72 hours.
	7. The following formula will used to be assess mortality in the tunnel tests:

Let X = the percent living in the alphacypermethrin (Interceptor) tunnels

Let Y = the percent living in the G2 (field collected) tunnels

The corrected mortality due to chlorfenapyr will be (X-Y)/X × 100

Note that the equation above does not adjust for any mortality in the control tests. If control mortality is greater than 20%, the test should be repeated. The results of the field G2 net will be considered looking at the results of the four tests together.

* 1. And for analysis of blood-feeding inhibition in the tunnel tests, the following formula will used:

Let X = the percent feeding in the alphacypermethrin tunnels

Let Y = the percent feeding in the G2 tunnels

The corrected blood-feeding inhibition due to chlorfenapyr will be

(X-Y)/X × 100

Note that the formula above does not adjust for any lack of blood-feeding in the control tests. If control blood-feeding is less than 50%, the tests should be repeated

* 1. The cutoff for G2 nets will be defined at a later point after consideration of preliminary data.

**Figure 4: Table for recording data from tunnel tests.**



1. **QUALITY CONTROL**
	1.
	2. As described above, the susceptible strains should be characterized within 2 weeks of the bioassays. The resistant strain characterization should also occur within 2 weeks of testing to ensure that the current resistance status of the mosquito strain is being captured.
	3. The net pieces from the new Interceptor (Step 3.12) and new Interceptor G2 (Step 3.19) should be cut from new unused nets. Since the insecticide concentration should be the same throughout the net, any 30x30cm square piece can be used for the controls. Each piece can be used up to 10 times before a new piece should be used. The number of times the net piece has been used should be recorded on the cardboard frame of the net or a notebook.

1. Long-lasting insecticidal nets (LLINs) have played an important role in malaria prevention since 2000. The term LLIN is reserved for WHO prequalified vector control products. As several new insecticide treated nets (ITNs) are currently in trial but not yet prequalified by WHO, we use the broader term ITN rather than LLIN in this protocol. [↑](#footnote-ref-2)
2. World malaria report 2019. Geneva: World Health Organization; 2019. [↑](#footnote-ref-3)
3. WHOPES: **Guidelines for monitoring the durability of long-lasting insecticidal mosquito nets under operational conditions**, WHO/HTM/NTD/WHOPES/2011.5 <http://whqlibdoc.who.int/publications/2011/9789241501705_eng.pdf> [↑](#footnote-ref-4)
4. World Health Organization: **WHO guidance note for estimating the longevity of long-lasting insecticidal nets in malaria control.** Geneva: 2013. <http://www.who.int/entity/malaria/publications/atoz/who_guidance_longevity_llins/en/index.html>. [↑](#footnote-ref-5)
5. World Health Organization: **Estimating functional survival of long-lasting insecticidal nets from field data**. Vector Control Technical Expert Group Report to MPAC September 2013. <http://www.who.int/malaria/mpac/mpac_sep13_vcteg_llin_survival_report.pdf>. [↑](#footnote-ref-6)
6. WHO: **Guidelines for laboratory and field testing of long‐lasting insecticidal nets.** Geneva 2013, WHO/HTM/NTD/WHOPES/2013.3 <http://www.who.int/iris/bitstream/10665/80270/1/9789241505277_eng.pdf?ua=1> [↑](#footnote-ref-7)
7. Benin, Burma, DRC, Ethiopia, Guinea, Madagascar, Malawi, Mozambique, Nigeria, Tanzania (Zanzibar), Zimbabwe (Source: www.durabilitymonitoring.org) [↑](#footnote-ref-8)
8. World Health Organization: **WHO guidance note for estimating the longevity of long-lasting insecticidal nets in malaria control.** Geneva: 2013. [↑](#footnote-ref-9)
9. WHO: **Guidelines for laboratory and field testing of long‐lasting insecticidal nets.** Geneva 2013, WHO/HTM/NTD/WHOPES/2013.3 <http://www.who.int/iris/bitstream/10665/80270/1/9789241505277_eng.pdf?ua=1> [↑](#footnote-ref-10)
10. Burkina Faso, Burma, Burundi, DRC, Ghana, Kenya, Liberia, Madagascar, Mozambique, Niger, Nigeria, Tanzania (Zanzibar). [↑](#footnote-ref-11)
11. <https://www.who.int/pq-vector-control/prequalified-lists/en/> (Accessed 16th September, 2020) [↑](#footnote-ref-12)