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Common Protocol for Uniform Evaluation of Insecticides/ Bio-larvicides for use in Vector Control

Revised Draft

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REVISED DRAFT

COMMON PROTOCOL FOR UNIFORM EVALUATION OF INSECTICIDES/ BIOLARVICIDES FOR USE IN VECTOR CONTROL

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1. INTRODUCTION

Integrated vector management (IVM) is a universally accepted strategy for the control of vector borne diseases in an effective and sustained manner. National vector borne disease control programme (NVBDCP), the nodal agency of the Government of India for vector borne disease control, has also endorsed the strategy. Among the available vector control options, chemical control is decisively superior over environmental and biological control that have limited applicability especially in mitigating sporadic unpredictable outbreaks of vector borne diseases. Deployment of chemical control embraces the whole gamut of strategies that include indoor residual sprays (IRS), larvicides, insect growth regulators (IGRs), insecticide treated nets (ITN)/ long lasting insecticide nets (LLINs) and an ever-lengthening list of household insecticide formulations for personal protection measures. In India, vector control measures recommended and practiced by the NVBDCP largely rely on site-specific chemical control, using insecticides belonging to different groups. A major impediment to this strategy has been the development of resistance by vector species to the insecticides, which necessitates frequent replacement of the existing insecticides in the control programme with new insecticides showing adequate human and environmental safety. The NVBDCP has the ultimate responsibility to introduce new insecticides to the national control programme based on the results of scientific evaluation of the products. To facilitate this, industries (national and international), National Centre for Disease Control (NCDC), institutes of the Indian Council of Medical Research (ICMR) and NVBDCP have to organize both laboratory and field trials to evaluate the insecticide compounds for their bio-efficacy and effectiveness on target and non-target organisms to arrive at the decisions (regulatory aspects are given in Box 1). The products have to be evaluated in multi-centric mode at different sites with variable ecology to ascertain their adaptation for control in diverse ecological situations in the country. Further, it is mandatory that only the insecticides that are registered with the Central Insecticide Board (CIB) are to be used in the control programme. The WHO Pesticide Evaluation Scheme (WHOPES) is the only international programme aiming at promotion and evaluation of pesticides for public health use by providing technical assistance to the member countries and also encouraging the industries to develop more promising insecticides for the vector control programme.

1.1. Need for a common protocol for uniform evaluation

It has been the responsibility of the NVBDCP to select and introduce new insecticides for national vector control programme on the basis of their suitability and adaptability to Indian conditions assessed through multi-centric laboratory and field trials by the collaborating research organizations (ICMR/ NCDC). As a mandatory requirement for CIB registration and subsequently for the use under the NVBDCP, the trials should generate data on both entomological and epidemiological impact of the new compounds for countrywide use.

Although, general guidelines by WHOPES for evaluation of insecticides are available, past experiences with insecticide evaluation have shown discrepancies in the methodology adopted by different institutes and because of this a meaningful comparison of results generated at different sites becomes difficult. In order to avoid such difficulties, development of a common protocol for uniform evaluation of insecticides has become imperative. The trials conducted by

different institutions at different sites following the common protocol will minimise the discrepancies in the methodology and thereby their results could be compared more meaningfully to facilitate the NVBDCP to arrive at a decision. Keeping this in view and also to be in line with the WHOPES guidelines for insecticide evaluation, the common protocol jointly prepared by the National Institute for Malaria Research (NIMR), New Delhi and the Vector Control Research Centre (VCRC), Puducherry in the year 2000 has been revised/ updated by the Sub-Committee on revision of SOP and common protocol constituted by the DG, ICMR.

Box 1: Regulatory aspects for introduction of insecticides into the national programme (NVBDCP)

- Under the National Vector Borne Disease Control Programme insecticides used are based on certain epidemiological and entomological criteria. The programme uses insecticides for indoor residual spray, space spray and treatment of mosquito nets. Larvicides are also used for urban malaria, filaria and other vector borne diseases control.

- As per the Insecticides Act, only those insecticides are to be used in the country, which have the approval of the Central Insecticide Board (CIB).

- The trials are conducted by various research institutions to determine the safety, efficacy and cost-effectiveness of chemical larvicides and adulticides before introduction into the programme. Multi-centric trials through common protocols are encouraged. Once the results of the trial become available and indicate the potential use of particular insecticide(s) under the programme, these results are also discussed in a sub-committee of technical experts. Thereafter the findings of the trials and recommendations of the committee are deliberated in the Technical Advisory Committee (TAC), headed by the Director General of Health Services, Ministry of Health and Family Welfare, Government of India.

- Based on the details of the trial, national and international data available in respect of the product, approval of CIB through a valid registration is sought by the manufacturers. Before procurement of the products the specifications are approved by a Technical Committee headed by the Additional Director General, Directorate General of Health Services, Government of India. The TAC makes appropriate decision. Such TAC decisions are then taken up by the programme after the approval of the Ministry of Health and Family Welfare, Government of India for application and appropriate policy decision.

1.2. Procedure for evaluation of insecticides

The laboratory and field evaluations for testing of insecticides are performed under three Phases. Summary of activities under each phase is given in Table 1.

Phase I

Evaluation of the new technical products or their formulations is done on laboratory-bred arthropods. This phase includes studies on candidate insecticide's efficacy and persistence, and cross-resistance in vectors to other insecticides currently in use.

Phase II

Evaluation is carried out in the field on a small-scale under well-controlled conditions. This phase provides sufficient information on various aspects related to efficacy in field conditions including safety of the insecticide to operators and inhabitants. It is also an opportunity to verify the effect of the insecticide on non-target fauna. This phase is important, as this is the first field experiment with flexibility of testing different doses and different evaluation methods. This phase suggests the suitability of the candidate insecticide for testing in Phase III.

Phase III

Evaluation in this phase is on a large scale [village(s) scale] against disease vectors prevalent in the area. This phase includes entomological, epidemiological and safety evaluation.
Introduction into the programme depends on the results of this phase.

Table 1. Activities to be undertaken in each phase

Phase	Type of studies	Activities
I	Laboratory testing	Efficacy to target vector Cross-resistance to insecticides Persistence
II	Limited field trials/ simulated trials in the field	Efficacy under different ecological conditions Dose and method of application Persistence Safety observations
III	Field trials (moderate or large-scale)	Efficacy to target vectors Persistence Entomological studies Epidemiological studies Safety observations Acceptance and other social aspects Collateral effects/ benefits

1.3. Special instructions

- Phase I evaluation may be avoided for WHOPES passed insecticides
- Sponsoring industries (national/international) to provide data on human/mammalian toxicity and environmental safety
- Investigators may consult specifications of WHOPES (www.who.int/whopes/en/) and Bureau of Indian Standards (earlier ISI)
- Evaluations must be in multi-centric mode involving different ecological conditions, social strata, etc.
- Duration of the study has to be strictly adhered to
- It is important to keep the state and district health programme personnel apprised of the trial throughout and should be involved as investigators
- It is important to keep NVBDCP, Delhi be posted with the trials and their progress
- Products to be tested should have clearance from the ethical committee
- Informed consent should be obtained from the human volunteers associated with the evaluation

2. EVALUATION PROTOCOLS FOR INSECTICES

2.1. Indoor Residual Spraying

Indoor residual spraying (IRS) is one of the effective vector control tools. In India, it is extensively used for malaria and kala-azar control. Indoor resting (endophilic) mosquitoes effortlessly pick up lethal dosages of insecticide through tarsal contact which reduces the longevity of the vectors resulting in the interruption of disease transmission. IRS is spraying of synthetic chemical insecticides, mostly with wettable powder formulations, in the habitat of vector resting. The effectiveness of IRS depends on the following criteria:

- Endophily and endophagy of mosquitoes and also partial endophily of mosquitoes (mosquitoes which rest indoors for some time after blood meal)
- Adequate coverage of sprayable surfaces in the habitats such as walls, eaves, ceiling/ roof and other potential resting places of disease vectors
- Residual activity of the insecticide formulation throughout the transmission period

The insecticides which are evaluated for the first time (new insecticides), the standard specifications of the compound should be provided by the sponsoring agency. The sponsoring agency should also provide toxicological indices on safety for humans and non-target organisms especially against domestic pet animals. (In other words, the material safety data sheet of the new insecticide should be provided). Evaluation should be conducted in three Phases, I, II and III.

The new insecticides that showed promising activity in the laboratory trial (Phase I) will be considered for Phase II and Phase III evaluation. The insecticides passed by WHOPES will be taken directly for Phase II and Phase III evaluation.

2.1.1. Laboratory studies (Phase I)

Duration: 3 months

Objectives

- To determine the intrinsic activity of the given insecticide against the target vector species by determination of LD50 and LD90
- To determine the diagnostic concentration for monitoring resistance to the insecticide and cross-resistance to other insecticides in the field
- To assess irritant and excito-repellent properties of the insecticide by through determining (‘Time to first take off’) FT50 and FT90 after exposure to treated substrates.
- To assess efficacy and residual activity of the insecticide

2.1.1.1. Intrinsic activity

Objective

- To determine the intrinsic activity of an insecticide to a target species

This is done by the topical application of an active ingredient to isolate toxicity from confounding effects resulting from insect behavior (WHO/CDS/NTD/WHOPES/ GCDPP/ 2006.3).

Method of testing intrinsic insecticidal activity

- To prepare topical solutions, technical grade insecticide is dissolved in acetone, which is a highly volatile organic solvent and will remain on the insect cuticle for only a short period of time.
- The doses used in topical application are expressed in nanograms of active ingredient per mg of body weight of live mosquito.
- For this purpose, 50 non-blood-fed susceptible female mosquitoes are weighed initially to determine the average live-weight.
- A constant volume of 0.1 µl should be delivered to the pronotum using an appropriate hand-held or automatic pipetting device. Larger volumes may cause increased mortality due to solvent toxicity.
- After testing for mosquito mortality using wide range of concentrations, a narrow range of at least five concentrations yielding a mortality range from 5% to 99% (preferably 2–3 dosages below 50% and 2–3 above 50%) should be selected and used per test. A total of 50 susceptible, non-blood-fed, 2–5 day-old female mosquitoes are used at each concentration.
- The mosquitoes are lightly anaesthetized with CO_2 for 30 seconds and placed on a plate cooled to 4 °C to maintain anesthesia condition during the manipulations.
- For the treatment group, two batches of 25 mosquitoes are used for each test concentration of the insecticide.
- A volume of 0.1 µl of insecticide solution of the required concentration is deposited on the pronotum of the females using suitable applicator.
- Another two batches of 25 females treated with 0.1 µl of pure acetone serve as controls.
- After dosing, the females are transferred into clean holding cups and provided with 10% sugar solution on cotton wool and held for 24 hours at 27 $\pm$ 2 °C temperature and 80 $\pm$ 10% RH.
- Mortality is recorded 24 hours after the topical applications.
- The test is replicated three times using separately reared batches and the results are pooled for statistical analysis.
- Fresh insecticide dilutions should be prepared for each new test replicate.
- The relationship between dose and mortality is analyzed using log-dose probit regression (Finney, 1971). Commercial software is now available to compute estimates of the LD_{50} and other LD values and their 95% confidence limits. If mortality exceeds 20% in the control batch, the replicate is rejected. If mortality in the controls is between 5% and 20%, results with the treated samples are corrected using Abbott's formula:

$$\text{Mortality (\%)} = \frac{X - Y}{100 - Y} \times 100$$

Where

X = percentage mortality in the treated sample and
Y = percentage mortality in the control.

- It is possible to compare the probit mortality per log dose regressions for two insecticides by a parallelism test (WHO/CDS/NTD/WHOPES/ GCDPP/ 2006.3).

2.1.1.2. Diagnostic concentration

The diagnostic concentrations are generally used to detect or monitor the presence of resistance in the target vector species such as *Anopheles culicifacies*, *An. fluviatilis, An. minimus, An. stephensi* and *An. sundaicus* (malaria vectors); *Culex quinquefasciatus* (filariasis vector); *Phlebotomus argentipes* and *Ph. papatasi* (Kala-azar vectors) and *Aedes aegypti* and *Ae. albopictus* (dengue vectors) to the given insecticide.

The diagnostic concentrations recommended by the WHO for each group of vectors are chosen so that exposure for a standard period of 1 hour followed by 24 hours holding can be relied upon to cause 100% mortality of individuals of susceptible strains. To avoid false reporting of resistance in the field (where there is no true resistance), WHO sets the diagnostic concentration at twice the minimum concentration that causes 100% mortality. The prescribed dosage of insecticides (on impregnated papers) and time of exposure for different vectors are given in Table 2.

Preparation insecticide impregnated papers

- Diagnostic concentrations are determined by exposing the target mosquito species to a graded series of dosages of insecticide (technical grade) that are impregnated on filter-paper.

- Rectangular pieces of Whatman® No. 1 filter-paper (12 x 15 cm) are impregnated with 2 ml of solvent, generally acetone, and mixed with a non-volatile carrier such as silicon oil (e.g. BDH Dow Corning® 556) or Risella® (Shell) or olive oil according to the insecticide tested (the manufacturer should be consulted for both solvent and carrier selection).

- The carrier oil allows the production of a stable, thin and homogeneous layer of the active ingredient on the filter-paper and prevents crystallization of active ingredients that would be otherwise solid at room temperature.

- Since the acetone is volatile, the concentrations are normally expressed as the percentage of active ingredient per unit volume of silicon on the filter-paper.

- Filter-papers are impregnated with 3.6 mg/cm² of the carrier oil, i.e. 648 mg/paper or 0.66 ml/paper for silicon oil (taking into account that silicon oil has a density of 0.98). A filter-paper, impregnated at 1%, contains 6.6 mg of technical insecticide, or 367 mg/m².

- The filter paper is supported on a cardboard and the impregnation is done by pipetting the insecticide solution evenly on to the paper.

- The impregnated papers are air dried for 24 hours and used for exposure to the insects with WHO test kit.

- The insecticide impregnated papers, particularly should not be used more than five times (WHO, 1998). The WHO tubes for testing susceptibility of adult mosquitoes and the testing method are described in Box 2.

Determination of diagnostic concentration

Mosquitoes are exposed to different concentrations of the insecticide impregnated on papers. (The detail procedure of testing using the standard WHO tube method is given in Box 2). Concentrations should be chosen so that at least one concentration gives 100% mortality, at least two concentrations give between 50% and 99% mortality, and at least two give between 5% and 50% mortality.

The concentration/mortality relationship is determined on three replicate batches. The concentration/mortality results are then pooled to produce a log dose/ probit mortality regression line from which the LD_{99} can be estimated. As mentioned above, the diagnostic concentration corresponds to twice the minimum concentration that kills 100%. Dosages of 2 to 4 times of the minimum concentration that kills 100% of mosquitoes will be used to determine the dosage of application for residual efficacy trials.

2.1,1.3. Irritant or excito-repellent properties

The irritant effect of an insecticide is an important characteristic to be considered as it modifies the tarsal contact time with the treated surface. This is studied by releasing mosquitoes in to WHO cones placed to an insecticide treated surface and closed with polyethylene plugs. Mosquitoes do not prefer to rest on plastic cones or polyethylene plugs and thereby remain in contact with the insecticide treated surface.

The irritant property should be first assessed using filter paper impregnated with the diagnostic concentration of the given insecticide (technical grade). If there is any significant irritancy with the treated filter paper compared to the control, further tests are carried out with the relevant formulation of the insecticide on various substrates commonly used for making houses/ shelters (mud, cement, plywood, thatch).

The selected surfaces are sprayed with the recommended dosage (i.e. the lowest one causing ≥80% mortality for longer duration) of the insecticide. For each test, susceptible, non-blood fed, 2-3 days old female mosquitoes (50 numbers) are individually introduced in to the plastic cone. After a settling period of 60 seconds the time lapse between 'first landing' and the 'next take off' of the mosquito is recorded as FT. The mosquitoes are then grouped by classes of first take off time (0-1 s, >1-2 s, >2-4 s, >4-8 s, ……>128-256 s) and cumulative frequencies are used to calculate the FT_{50} and FT_{95} using probit analysis. Mosquitoes that do not take off at least once during the 256 seconds exposure (test period) are discarded. Wherever possible a positive control should be used with an insecticide well known for its irritant property (e.g. Permethrin).

Table 2. Diagnostic doses of different insecticides for different vectors

Insecticides	***Anopheles****	***Culex* spp.****	***Aedes* spp.**	***Phlebotomus* spp.**
Organochlorine				
DDT	4% 1 h	4% 4 h	4% 1 h	4% 1 h
Organophosphate				
Malathion	5% 1 h	5% 1 h	5% 1 h	5% 1 h
Fenitrothion	1% 2 h	1% 2 h	1% 2 h	1% 2 h
Carbamates				
Propoxur	0.1% 1 h			
Bendiocarb	0.1% 1 h			
Pyrethroids				
Deltamethrin	0.05% 1 h			
Cyfluthrin	0.15% 1 h			
Lambdacyhalothrin	0.05% 1 h			
Permethrin	0.75% 1 h	0.25% 3 h	0.25% 3 h	
Etofenprox	0.5% 1 h	0.025% 1 h	0.025% 1 h	

* WHO 1998; ** WHO 1981.

2.1.1.4. Residual activity

Residual activity is studied in laboratory. This is to assess the period of residual activity of the sprayed deposits. Residual action should have to be assessed on different pre-fabricated substrates such as mud, brick, thatched, cemented, tin, etc. The substrates are 1 m^2 in size prepared on wooden frames. A minimum of seven replicates for each substrate per dosage are prepared. All the seven replicates for each substrate are sprayed with the insecticide to make a homogenous residual deposit of the desired concentration (2-4 times of the minimum concentration that causes 100% mortality) of active ingredient per unit area using a Potter Spray Tower® (WHO, 2006). All substrates are stored unsealed under controlled temperature

Box 2: Adult susceptibility test (WHO tube method)

The test is essentially conducted using the WHO test kit and method (WHO 1998). The kit for conducting the test and insecticide impregnated papers could be obtained on payment from the Vector Control Research Unit, School of Biological Sciences, 11800 Universiti Sains Malaysia (USM), Penang, Malaysia (who makes on behalf of WHO).

Kit: The WHO tube test kit includes green dotted (holding tube) and red dotted (exposure tube) plastic tubes (of 125 mm in length and 44 mm in diameter), with each tube fitted at one end with a 16 mm mesh screen, slide-units with screw cap on either side with a large orifice to transfer the mosquitoes and a small orifice for introduction of mosquitoes by aspirator; copper and steel clips; instruction sheet; log-probit papers; report forms; glass aspirators with 60 cm rubber tubing and mouth piece; roll of adhesive tape and white paper sheets (12 x 15 cm).

Impregnated papers: Filter papers impregnated with WHO recommended diagnostic dosage or other dosages of insecticides could be obtained from the sponsoring agency or from the University of Science Malaysia, Penang, Malaysia on order and payment.

Method: Tubes with green dot should be used for holding of mosquitoes and for control exposures. Tubes with red dot should be used for insecticide exposures. The green dot tube should be lined from inside with a plain paper fastened with a steel clip and later fixed to the slide by threading into screw cap. As needed, the required number of green dot tubes are lined from inside with insecticide-control papers duly fastened with steel clips and red dot tubes lined with insecticide impregnated papers of the designated dosage and fastened with copper clip.

Tests should be performed preferably with 3-day old sugar fed females of laboratory strain or 3-day old sugar fed F1 female progeny of field-collected adults or females emerged from the immature collected from field. Where only field collected adults can be used, their physiological status (i.e. unfed, blood fed, semi-gravid, gravid) should be carefully recorded (WHO, 1998).

Batches of 25 non-blood-fed female mosquitoes, aged 2–5 days, are introduced into the holding tube (marked with a green dot) through the small orifice on the slide and closed and held for one hour at 25 °C ± 2 °C and 80% ± 10% RH to acclimatize. The holding tubes are appropriately labelled with locality, species tested, etc. and provided with glucose source. After the holding period to observe for injured and dead mosquitoes, green dot tubes with insecticide-control papers and red dot tubes with insecticide impregnated papers are screwed to the respective holding tubes. The mosquitoes are transferred by gentle blowing to the tubes with insecticide-control papers and with insecticide impregnated papers and the tubes are held vertically for one hour under subdued light. During the exposure time, glucose source should be removed. At the end of the exposure time, the mosquitoes are gently blown back in to the respective holding tubes which are placed vertically in a dark place for 24hours with sucrose solution at 25 °C ± 2 °C and 80% ± 10% RH. Dead mosquitoes are counted after 24 hours. A total of 100 mosquitoes (four replicates containing 25 mosquitoes each) are used for each test concentration and for the control. Results are expressed as percentage mortality after 24 hours and corrected for any control mortality.

After each exposure, the tube test kit should be washed with soap and clean water and dried.

conditions (30^{O}C $\pm$ 2 ^{O}C), relative humidity (80%), air circulation and ambient light cycles until ready for testing. Residual activity is determined by cone bioassays on four replicates for each substrate exposing the target vector species for 30 minutes and recording the mortality after 24 hours holding period, following the method given in section 1.1.1.5f. Three sprayed substrates will be used for chemical analysis. From each substrate three samples will be subjected to chemical assay. Cone bioassays should be carried out initially one week after the spraying and subsequently at fortnightly/ monthly intervals until the mosquito mortality drops below 80%. From this assessment, three to five best dosages will be selected for Phase II evaluation.

2.1.1.5. Cross resistance

To assess the cross resistance in mosquito vectors to other insecticides in use under vector control programme, susceptibility tests will be carried out using WHO tube test kit as described in section 2.1.1.2.

The susceptibility status of vector species should be categorised as per the WHO criteria: susceptible- 98 to100% mortality, verification required- 81 to 97% mortality, resistant- < 80% mortality. Data should be recorded in the format given in Table 3. The results of this test will indicate the susceptibility status of the vectors to the insecticide(s) tested.

Table 3. Insecticide susceptibility test (WHO tube method)

Village ..... Sub-centre .....PHC ..... District .....

Insecticide (%)..... Impregnation date .....No. of times paper used .....

Date of Test.....Temp: Min ..... Max .....Humidity: Min .....Max ……

Test species .....Lab/F1/Field collected species .....

Exposure time ..... Minutes .....

Replicate	**No. exposed****	**Number knocked down in 1 h**	**No. dead after 24 h**	**% mortality**	**Corrected % mortality#**	**number***
Test 1 Test 2 Test 3 Test 4 Control 1 Control 2						

*Separate row for each replicate; **15 to 20 mosquitoes/replicate; #After Abbott's formula when mortality in control replicates is between 5 and 20% (<5% no correction needed and > 20% test to be repeated).

$$\text{Corrected mortality (\%)} = \frac{\text{(\% Test mortality – \% Control mortality)}}{\text{(100 – \% Control mortality)}} \text{ X } 100$$

2.1.2. Small-scale field trial – Using Experimental Huts (Phase II)

Duration: 6 months (preferably during transmission season).

Objectives

General

To measure the efficacy and residual activity of insecticides on wild mosquito population (in villages huts or experimental huts)

Specific

- To assess the efficacy of the insecticide in terms of mortality (immediate and delayed) and residual effect
- To study the impact on the behaviour of the mosquitoes (deterrence, blood feeding inhibition and induced exophily)
- To determine the optimum application dosage of the insecticide to be used for Phase III evaluation.
- To record the ease of application and perceived side-effects during the application and use.

Determination of the efficacy of insecticides can be done only where entry and exit of mosquitoes are monitored and scavenging of knocked down or dead mosquitoes is prevented. Such conditions can be achieved only in experimental hut trials.

2.1.2.1. Experimental hut study design

The experimental hut consist of a single room with four windows; size of each window was 0.45 x 0.45 m, grilled with wooden planks fixed horizontally in tilted position one above the other leaving a gap of 1 cm between two planks through which mosquitoes could enter into the hut but could not exit. There are two windows on the front door side and one on each of the sides and a screened (using nylon mesh) verandah (verandah trap) at the backside. The dimensions of the huts resemble to those of the village huts (Length 3m, width 3 m and height 2.5 m) having brick walls with cement plastering and thatched roof, above which there are tin sheeted roofing for protecting the thatched roof. There is no space between the thatched ceiling and tin-roofs. The huts are constructed one foot above the ground level on a platform made up of brick and cement. The platform has a water-filled moat (6' depth x 6' breadth) all around to deter entry of scavenging ants. The moat is made at two feet away from the hut walls, except on the back side of the hut where it is at 1.5 ft away from the base of the verandah trap. At the centre of the hut, the roof is at a height of 2.5 m and near the wall the height is 2 m; this difference in height is to maintain a slope of the roof. The eave on the backside (facing towards east) has a gap of 1-2 cm and through this gap mosquitoes could exit, but those mosquitoes will be collected in the verandah trap. There is one wooden door of 0.75m x 1.5m facing towards west. The huts are maintained and kept under lock until the volunteers use them for sleeping.

Ideally, several huts are required to allow comparison of different treatments simultaneously. A minimum of four replicates (four huts) per treatment arm and an equal number of control huts are to be used.

2.1.2.2. Assessment prior to hut trial

The pre-trial assessment is necessary to ensure that the huts are comparable in their attractiveness to the target species and the target species is present in adequate numbers in the study area and also to ensure that the huts are not contaminated with insecticide.

For acclimatization and to attract mosquitoes into the experimental hut, an adult volunteer enrolled for this purpose will sleep (preferably under an untreated mosquito net) in each of the huts from dusk to dawn for a period of 15 days.

Subsequently, the suitability of the experimental huts will be assessed based on the following criteria over a period of one month prior to starting the hut-evaluation.

1. ***Indoor resting of mosquitoes*:** The target mosquito species will be collected from the experimental huts in the morning hours weekly twice, keeping equal intervals between the two successive collections. In parallel, mosquito collections will be carried out in randomly selected village huts (number may be equal to the number of experimental huts). The mosquitoes will be identified to species and counted. Per man-hour vector density (PMD) (number collected/man-hours spent) will be calculated for the experimental huts and village huts and compared between the two.

2. ***Tightness of huts (from recovery rate)*:** Around 75 (depending on availability) fully-fed field collected *An. fluviatilis* female mosquitoes will be released during one evening into each experimental hut (which will be closed). The following morning mosquitoes will be recaptured. At least 70% of the released mosquitoes should be recaptured. The recovery rate (number recaptured/total released X 100) will be assessed on a minimum of five occasions.

3. ***Absence of scavengers*:** To know the presence of scavengers inside the experimental huts, four batches of 25 dead mosquitoes will be kept on the floor including verandah (in four corners) of each hut in the evening and the number present in the next day morning will be recorded. Such observations will be made on eight occasions, twice a week during the four weeks.

Each experimental hut trial should have a negative control and, if possible, a positive control. For evaluating an insecticide for IRS, the negative control involves only a sleeper without insecticide treatment or with the formulation minus the active ingredient. This is relevant in situations, where the inert ingredient of the formulation or substrate may exert an effect by itself. For a positive control, an insecticide commonly used in the country at a recommended dosage will be selected.

It is highly necessary that all field staff including supervisors engaged in the trial be blinded to the allocation of treatments to avoid bias during the trial. Usually double-blinding of senior

investigators and implementers is desirable, if not, the minimal requirement is single blinding of field operatives and supervisors.

2.1.2.3. Rotation of sleepers

For IRS trials, treatments cannot be rotated, and hence it is necessary to demonstrate that there is little or no variation in the attractiveness of huts during pre-trial assessment (this also illustrates the importance of optimum positioning of huts during construction). The sleepers should be rotated between huts so that every sleeper is allocated to each hut-treatment an equal number of times. In practice, sleepers will have to be rotated daily between the huts.

2.1.2.4. Ethical considerations

The trial proposal should be submitted to the respective ethical institutions and authorities and clearance should be obtained before undertaking the trial. Informed consent should be obtained from the volunteers involved in the study (Annexure 1). Chemoprophylaxis should be administered wherever necessary as per the national guidelines, and volunteers should be medically supervised.

2.1.2.5. Implementation

Safety instructions and protective measures should be strictly observed. Antidote and instructions for treatment of intoxication should be present on site and made available to the responsible officer.

Experimental huts should be completely renovated before starting each new trial and carefully cleaned. The sprayed surfaces are replaced and absence of contamination has to be demonstrated by appropriate bioassay tests.

Operators must ensure that the insecticide formulation is safely and correctly applied as per the WHO guidelines (WHO, 2003). The treatments are conventionally applied to the walls, ceiling, eaves and doors, but this may be altered according to the nature of the treatment and the manufacturer's recommendations. As IRS causes a higher degree of contamination to the huts, it will be necessary to remove and replace the door, substrates and ceiling material between trials.

2.1.2.6. Assessment of the quality of treatment

To monitor the quality of spraying, four Whattman filter papers (each paper 5cm X 5cm) leveled properly were struck on the walls (one on each wall of the hut) of each experimental hut, before spraying, and removed at 24 h after spraying. The papers were wrapped in aluminum foils and subjected to analysis for insecticide content. Chemical analysis results are combined for each substrate to provide the average concentration of insecticide (expressed in mg/m^2).

2.1.2.7. Evaluation procedure

Determination of the dosage and residual activity

The residual activity of the target dosage is assessed by carrying out standard bioassay at regular intervals, preferably on day 1, day 7 post-spraying and thereafter weekly, using WHO cones. Batches of 10 non-blood-fed mosquitoes, 2–5 days old, are released in to each cone and exposed for 30 minutes on each of the walls of each hut and on ceiling. Wherever, it is not practical to use non-blood fed 2-5 days old (F1 females) mosquitoes for the assay, wild caught blood-fed female mosquitoes may be used for cone bioassay.

The number of weeks/months during which there is mortality above the "cut-off point" (80% mortality after 24 hours' holding) is recorded.

Safety considerations have to be taken into account when selecting the dosage to be tested.

Air-borne toxicity of insecticide

Some insecticides may have fumigant properties. This can be assessed by estimating, in comparison with an unsprayed hut (control), the mortality of mosquitoes placed in small cages (5 to 10 cages in each hut, 25 non blood-fed female mosquitoes in each cage) hanging from the ceiling, for 4–8 hours up to a maximum of 12 hours, at different distances from the sprayed surfaces. The mosquitoes are then kept for 24 hours' observation after being transferred to clean cages. The mortality in comparison to the controls is recorded to assess the air-borne toxicity.

Efficacy and impact on vector behaviour

During the experiment hut trials, adult volunteers should carefully follow the instructions of the trial supervisor. Sleepers should enter the huts at a standard time in the evening and remain inside until a standard time in the morning. From time to time, the supervisor should make an unexpected check at night to ensure that instructions are being followed by the volunteers sleeping in the huts. While the volunteers are inside the huts for sleeping, it has to be ensured that the windows are kept closed.

Mosquito collections

In the evening, before the sleeper occupying the hut, its room and verandah were cleaned and white cloths were spread on floor of the hut including verandah. The verandah trap was furnished with cotton pads soaked in 5% glucose solution to reduce the risk that unfed female mosquitoes exiting in the night would die of starvation.

The next morning, the windows are closed and the mosquitoes found dead on the floor sheet are picked up using forceps and placed in cups provided with moist cotton wool, and then the white cloths were removed from the floor. The resting alive mosquitoes are collected separately from the veranda, room and net (if present), with reliable records of location using

aspirators and flashlights. All mosquito specimens collected from each part of the hut are kept separately, brought to the laboratory, identified to species and classified according to their gonotrophic condition (unfed, fed, half gravid, gravid). The live-caught females are kept on observation for 24 h to record delayed mortality, if any. It may not be possible to control the conditions during holding as strictly as in Phase I studies. However, humidity and temperature should be controlled within tolerable limits by use of insulated containers or wet towels wrapped around the holding cages.

Mosquitoes are collected in the huts, twice a week after spraying for a period until the density of the vector mosquitoes declined to a minimum level (based on the density of vectors in the control huts) due to seasonal effect. Data must be carefully reported on the record sheets by the local supervisor.

The compilation of data for each treatment allows determination of the four indicators of efficacy and mosquito behavior as described below.

2.1.2.8. Safety and operational issues

Spray men and other handlers of insecticides should be questioned about any perceived adverse effects. This can provide a useful indicator whether the given insecticide is suitable/ acceptable for testing at Phase III. Ease of application by the spraying operators should be reported (mixing, dilution of insecticide, spraying, impregnation). The sleepers are enquired regularly during the study period about their perceived side-effects, and the responsible officer is expected to pay special attention to spontaneous complaints, if any.

2.1.2.9. Data analysis

Indicators

Four indicators are used to assess the efficacy of a formulated insecticide sprayed on walls: deterrence, induced exophily, inhibition of blood-feeding and mortality. These indicators are calculated in comparison to the untreated control hut with respect to the following four criteria:

1. The entry rate, which is the total number of female mosquitoes found in the hut and verandah. A reduction of entry rate (deterrence) is observed with certain types of repellent insecticide, presumably because the insecticide vapour or dust is detectable by mosquitoes before they enter a treated hut.

2. The exit rate, which is the proportion of female mosquitoes found in the verandah trap compared to the total number found in the hut and verandah. The exit rate allows estimation of induced exophily or excito-repellency.

3. The blood-feeding rate, which is the proportion of blood-fed female mosquitoes compared to the total number found in the hut (room + verandah). The reduction in the number of blood-fed mosquitoes between treated and control hut allows an assessment of the blood-feeding inhibition caused by the insecticide.

4. The mortality rate, which is the proportion of female mosquitoes found dead in the hut immediately after spraying and 24 hours later. The difference in mortality between a control hut (natural mortality) and a treated hut allows estimation of the insecticide-induced mortality rate.

The personal protective effect of a treatment in an experimental hut study is determined by the reduction of the number of blood-fed mosquitoes in the treatment hut compared to the number blood fed in the control hut. It may be estimated using the following formula and expressed as a percentage:

Protective effect (feeding inhibition) = 100 x (Bc- Bt)/ Bc,

Where Bc is the total number blood-fed in the control hut and
Bt is the total number blood-fed in the treatment hut

The overall insecticidal effect of a treatment needs to take into account that significant numbers were deterred and not killed by the treatment. It can be estimated by the following formula and expressed as a percentage:

Overall insecticide effect = 100 x (Dt–Dc)/ Ec,

Where, Dt is the total number of mosquitoes dying in the treatment hut,
Dc is the total number dying in the control hut and
Ec is the total number entering the control hut.

2.1.2.10. Statistical analysis

Prior to treatment, a statistical test should be applied to ensure that there is no appreciable difference between huts in attractiveness to mosquitoes.

The number of female mosquitoes entering each hut is tabulated by species and day. It is likely that the distribution from day to day will be over dispersed and fit a Poisson distribution with variance equal to mean. Therefore, Poisson regression analysis or a non-parametric test such as Kruskal-Wallis or Wilcoxon rank-sum test should be used.

After the interventions have begun, the number of mosquitoes of each species entering the huts, the proportion of mosquitoes that exit early, the proportion that are killed within the hut and the proportion that successfully blood-feed may be compared by species and analyzed using Poisson regression for numeric data and logistic regression for proportional data (e.g. Stata 6 Software). The clustering of observations made in one hut-night, and controlling for any variation between huts and sleepers, needs to be controlled for. Comparisons between treatments are made by successively dropping treatments from the overall comparison. This process allows each treatment to be compared with every other one. As a less powerful but valid alternative, the numbers of blood-fed and dead mosquitoes and overall totals collected from each hut may be compared using the nonparametric Kruskal-Wallis test.

2.1.3. Small-scale field trial – Using Village Huts (Phase II)

Wherever, construction of experimental huts is not feasible, the phase II evaluation may be carried out in the existing village huts with minor modifications with the consent of the respective household heads.

2.1.3.1. Selection of dosages for application

From the residual activity assay results (Phase I laboratory trial) as described under section 2.1.1.4., three to five best dosages are selected for conducting phase II evaluation in the huts.

2.1.3.2. Selection of study area

Study area should be selected in consultation with respective State/ District health department and based on logistics. In case the study is proposed to be carried out in two or more villages, comparable villages in terms of vector density, eco-type, should be selected. Vector density should be ascertained prior to the evaluation to select comparable villages. The houses with considerable vector density should be selected for the trial.

2.1.3.3. Selection of houses

In the selected village(s), a minimum of 24 houses should be selected. These houses should be designated for interventions among four arms. Each house should be labelled with arm code and insecticide dosage code. The houses in each arm should be distributed equitably among the dosages of spraying and control (one house for each dose and one for control) as shown in Table 4. In case of a request for comparison with an insecticide currently in use, additional six houses (positive control) should be selected (total 30 houses) and distributed as shown in Table 5. Selection of houses should be done with the informed consent of the head of the family and written permission needs to be obtained to implement minor alterations of the structure of the house, if needed.

No other vector control intervention should be undertaken during this phase of trial in the selected villages. Alternatively, villages without insecticidal spray by the programme may be selected as controls. In case the ongoing spraying is suspended during the study, surveillance mechanism should be strengthened for providing protection from the disease to the villagers (as only a few houses are selected for spraying as per the evaluation criteria). Where feasible, hamlets or small villages having the required number of houses for the evaluation can be selected.

Table 4. Distribution of houses in arms with different doses

	Houses with spray of candidate insecticide			Houses without spray of insecticide
			Arm	
Replicates	**Dose 1**	**Dose 2**	**Dose 3**	**Control**
1	House-1	House-2	House-3	House-4
2	House-5	House-6	House-7	House-8
3	House-9	House-10	House-11	House-12
4	House-13	House-14	House-15	House-16
5	House-17	House-18	House-19	House-20
6	House-21	House-22	House-23	House-24

Table 5. Table showing distribution of houses in the arms with different doses with additional houses for comparison insecticide

	Houses with spray of candidate insecticide			Houses without spray	Houses with spray of comparison insecticide
			Arm		
Replicates	**Dose 1**	**Dose 2**	**Dose 3**	**Control-1**	**Control-2**
1	House-1	House-2	House-3	House-4	House-5
2	House-6	House-7	House-8	House-9	House-10
3	House-11	House-12	House-13	House-14	House-15
4	House-16	House-17	House-18	House-19	House-20
5	House-21	House-22	House-23	House-24	House-25
6	House-26	House-27	House-28	House-29	House-30

Spraying of insecticide should be conducted by the investigators in collaboration with the PHC Medical Officer and District Malaria Officer with intimation to other health personnel of the programme in the state. For Phase II trial, spraying of the candidate insecticide should be done in the selected villages during the period when routine spray operation is carried out in the other villages by the NVBDCP either during first or second round (Box 3).

2.1.3.4. Spraying of houses

This should be done with the standard equipment (knapsack lever operated or compression sprayer or stirrup pumps) following the norms of spray (Boxes 4 & 5, Table 6) in use in the routine vector control programme. The technique of spraying should be the same as that is in regular use in the area. Care should be taken to spray the houses for complete coverage and proper dose dispersion. Inhabitants should be informed in advance about the preparations for spraying and precautions to be taken after spraying. This can be better accomplished with the help of local panchayats, opinion leaders, school teachers, religious leaders/priests and others. Spray men should be given orientation on spraying technique before spray and necessary precautions to protect themselves and inhabitants from contamination. Salient safety measures to be ensured by the supervisory staff during the operation are given in Box 6. Good quality spray should be ensured by the supervisory staff (Box 7). Spraying should be carried out under the strict supervision of the staff and mopping up of spraying, if needed, should be done immediately in a day or two. Lapses in spray will seriously affect the results of the trial. Coverage of spray and other details should be recorded in the proforma given in Table 7.

2.1.3.5. Assessment of the quality of treatment

Refer to section 2.1.2.6. for assessment of the quality of spraying by chemical analysis.

2.1.3.6. Assessment of residual activity on different surfaces

Residual activity is determined using cone bioassays (WHO 1981a) on different wall surfaces available in the study area, viz. cement, mud, thatch, tin, etc. Houses sprayed with different doses of the insecticide, with different surfaces should be selected for the cone bioassays. Similarly, surfaces should be identified in control houses. On the selected surfaces, areas of 1 sq ft should be marked with pencil. At least 4 squares should be marked for a given dose of insecticide for each type of surface. Not more than 2 squares should be selected in one house for given type of surface. At least 2 squares for each type of surface should be marked on unsprayed surfaces for control. Care should be taken to mark the squares at different heights on the wall surfaces. The cone bioassay procedure is given under section 2.1.2.7. Bioassays should be carried out at weekly/fortnightly intervals in the above marked squares to assess the persistence. Inhabitants should be advised not to physically alter the marked areas, mud plaster, white wash, paint, etc.

Box 3: Scheduled dates of spray of insecticides in different states recommended by NVBDCP

Dates for First Round * Spray (Date/Month)	States
1/3	Andaman & Nicobar Islands,
15/3	Karnataka, Meghalaya, Tripura, Arunachal Pradesh, Nagaland, Assam, Manipur
16/4	Himachal Pradesh, Pondicherry
16/4	Sikkim
16/4	Tamil Nadu
16/4	Punjab
1/5	Daman and Diu
1/5	Andhra Pradesh, Bihar, Chandigarh, Goa, Gujarat, Jammu and Kashmir, Jharkhand, Madhya Pradesh, Orissa, Uttar Pradesh, Uttarakhand
15/5	Haryana, Dadra and Nagar Haveli
15/5	Rajasthan, West Bengal
1/6	Maharashtra
Focal spray	Delhi, Kerala, Lakshadweep

* Dates for subsequent rounds (II /III) should be finalised on the basis of residual activity of the insecticide in weeks and in consultation with local health programme personnel/ NVBDCP.

Box 4: General specifications of spray pump and spraying (NVBDCP)

- Pump: Stirrup pump or Hand Compression prayer Pump (ISI mark).
- Nozzle tip: stainless steel flat-fan type; discharge rate of 740–850 ml per minute [If more than 850 ml, nozzle tip should be replaced].
- Distance and angle of lance from wall: 45 cm and 60□.
- Swath width: 53 cm (21") with 3 inches overlap while spraying.
- Operation of stirrup pump plunger: 20–26 strokes per minute; 10–15 cm movement.
- 10 psi pressure at the nozzle tip
- Operation of HC pump: Above discharge rate should be attained a 40 psi
- Rate of coverage: 5 min per house with average sprayable surface of 150m^2

Box 5: Norms of spray of NVBDCP (Source: MAP 1995)

- To cover 1 million population, 52 squads of 5 members each are required for 5 months.
- Squad comprises 2 pump men; 2 spray men; 1 insecticide suspension supplier and a superior field worker to supervise and keep record.
- Every squad should cover 60–80 houses in plain area and 50–60 houses in hilly/foothill area each day.
- Each squad receives 2—Pumps; 2—Nozzle tips, 4—15 litre bucket; 1—5/10litre bucket; 3 m—Asbestos thread; 1— Measuring mug (500 g); 1m2—Straining cloth; 2—Pump washers; 3 x 3m—Plastic sheet.

Table 6. Requirement of insecticide per house (average sprayable area 50 m^2)

% formulation (a.i.)	Dosage (a.i.) /m^2	Insecticide	Insecticide for 10 litres
50%	1 g	300 g	1000 g
25%	1 g	600 g	2000 g
10%	25 mg	37.5 g	125 g
5%	25 mg	75 g	250 g
2.5%	25 mg	150 g	5 00g
*10 litres of suspension should be sufficient for 3 rural houses with sprayable surface area of ~ 500 m2.			

Table 7. Details of spray and insecticide consumption

Name of the village .....PHC .....District .....

State .....pray Squad No. ..... Name of the Sr.F.W./Supervisor .....

Insecticide and formulation..... Date of spray .....

S. No	H.No.	HOF	Houses			Rooms		%HC	%RC		Insecticide consumed in kg
			Sprayed	L	R	Targeted	Sprayed		CS	TS	
1.											
2.											
Total											

HOF: Head of family; L: Locked; R: Refused; CS: Cattle shed; TS: Temporary shed; % coverage = Number sprayed x 100/Number targeted; HC: House coverage; RC: Room coverage

Box 6: Safety measures (Source: Malaria Vector Control WHO/WHOPES/2002.5 and MAP 1995)

Spraymen

- Spraymen should have protective accessories and clothing, e.g. goggles, gloves, boots, and two sets of working clothes.
- Spray men should wash hands and face every time after insecticide is handled.
- Eating, drinking and smoking should be avoided while spraying.
- Spraymen should be advised to take bath after each day's work.

Inhabitants

- All food, cooking utensils, bedding, clothing, and portable furniture in the house should be removed from the house before spraying.
- Children and sick people should be temporarily shifted.
- Inhabitants should not enter the sprayed rooms until the spray is dry, and instructed to sweep the floors before allowing small children or indoor domestic animals into the rooms.

Any adverse effect of spray to inhabitants and spray men or accidental exposure to insecticide should be informed to the supervisor. Supervisory staff should know the first aid procedures for insecticide exposures and information on the nearest medical facility. The patient should be immediately moved to well ventilated area, remove contaminated clothes, loosen the clothes and taken to medical facility. Head should always be kept upright not to obstruct respiration while transportation. Supervisors should provide the label of the insecticide container to the medical officer for advice on antidote. Supervisors should be trained for giving resuscitation (artificial respiration).

Box 7: Supervision of spraying

Concurrent

- Date of spray, advance notification, scheduling, spray crew
- Discharge rates of nozzles, condition of pumps
- Preparation of suspension and supervision for technique, speed, coverage, safety, etc.

Consecutive

- Evidence of spray deposits
- Uniformity of spray
- Coverage, if less, reasons (refusals or locked premises or others)
- Mopping up of operations to spray unsprayed houses
- Extent of defacing of spray by mud-plastering, white wash, etc. and reasons.

The residual activity of the target dosage is assessed by carrying out standard bioassay at regular intervals, preferably on day 1 and day 7 post-spraying and thereafter weekly, using WHO cones. Batches of 10 non-blood-fed mosquitoes, 2–5 days old, are released in to each cone and exposed for 30 minutes on each of the walls of each hut and on ceiling. Wherever, it is not practical to use non-blood fed 2-5 days old (F1 females) mosquitoes for the assay, wild caught blood-fed female mosquitoes may be used for cone bioassay. After the exposure the mosquitoes are carefully removed and placed in plastic containers covered with nylon net fastened with rubber band. Mosquitoes are provided with 10% sucrose solution soaked in cotton wool. After 24 h of holding, percent mortalities are computed from the total number of alive and dead mosquitoes in the replicates for each type of surface and recorded in the format as given in Table 8.

The number of weeks/months during which there is mortality above the "cut-off point" (80% mortality after 24 hours' holding) is recorded. After each exposure the kit should be washed with soap and clean water, and dried for next use. Results are expressed as overall persistence against a given dose of insecticide.

Table 8. Cone bioassays for persistence studies

Village ..... Sub-centre..... PHC ..... District ..... Date of bioassay .....
Insecticide & Dose ..... Temperature: Exposure ..... Holding.....Humidity.....
Holding..... Date of last spray and round..... Type of surface:
Mud/cement/brick/thatch..... Test species..... Lab/F_1/Field collected.....

Replicates	House code	30 minute knock-down (no.)	24 h % mortality	Corrected % mortality	Remarks
Replicate 1					
Replicate 2					
Replicate 3					
Replicate 4					
Control 1					
Control 2					

2.1.3.7. Entomological evaluation

The entomological collections should be carried out on regular intervals, preferably on day 1 and day 7 post-spraying and thereafter weekly, concurrently with the assays conducted for residual activity. Evaluation for all indicators should be done in all sprayed and control houses on the same day. Data of each of the evaluation indicators should be entered in the respective proforma. The methods given below are for the evaluation of three dosages of a given insecticide (four replicates for each dosage) and equal number of controls. In case of comparison with another insecticide, additional houses should be selected.

2.1.3.7.1. Floor sheet collection

White cloth is spread on the preceding night on the entire floor of house before inhabitants retire to bed. Next morning, before the inhabitants resume their regular activities, the dead and morbid mosquitoes lying on the floor sheet should be picked up with forceps and scored. Other

dead insects lying on the floor should be separately collected and stored for monitoring. The inhabitants of the trial houses are asked not to physically damage the knocked-down mosquitoes. Precautions should be taken to protect the knocked-down mosquitoes from scavengers such as ants. These mosquitoes should be identified to species and abdominal (gonotrophic) condition recorded in the format given in Table 9. This indicator (floor sheet collection) provides comparative data on immediate mortality after contact with insecticide sprayed at different dosages.

2.1.3.7.2. Mosquito collection indoors

Following the floor sheet collection, resting mosquitoes in the house (indoor) are collected using aspirator. Care should be taken to collect all resting mosquitoes to the possible extent. The collections of mosquitoes are labelled according to the test arm and kept in 150 ml cups (10 individuals per cup), with 10% sucrose solution provided and maintained in a climatic chamber for 24 hours at 27°C $\pm$ 2°C and 80% $\pm$ 10% RH. The percent mortality after 24 hours is recorded. This indicator provides data on delayed mortality at different dosages tested.

Table 9. Floor sheet collection

Village..... Sub-centre..... PHC ..... District..... State.....
Date of collection.....Insecticide & Dose.....Type of structure: Mud/cement/brick/Thatch/ stone/.....Temperature: Min.....Max..... Relative humidity: Min ..... Max.....

Arm No. (House code and dose)*	Species	Males	Females				Total no. (dead + morbid)
			UF**	BF	SG	G	
Dose 1 Dose 2 Control 1 Control 2							

UF = Unfed; BF = Blood fed; SG = Semi-gravid; G = Gravid; * Separate row for each replicate; **: Unfed mosquitoes should be dissected for parity (Refer section 1.1.1.5g5).

2.1.3.7.3. Exit window trap collection

Exit window traps should be fixed in the evening hours before sunset in all the houses. Next morning all the mosquitoes from the exit traps should be collected. The dead mosquitoes should be placed in petri dishes lined with moist filter paper for species identification and scoring. The live mosquitoes should be transferred to a cage and brought to the laboratory wrapped in a wet towel. The cage should be kept preferably in an unsprayed room. During the holding period optimum conditions for survival should be provided, i.e. 27$\pm$ 2^{o}C temperatures, 80% $\pm$ 10% RH and a glucose source. Where it is not possible to maintain temperature and relative humidity a climate chamber can be used. The mortality of live mosquitoes is scored after 24 hours holding period. The collected mosquitoes are identified to species and their abdominal conditions recorded in the format given in Table 10. This indicator provides data on relative excito-repellency property of the insecticide at different dosages.

Table 10. Exit trap collection

Village ..... Sub-centre ..... PHC ..... District ..... State.....
Date of collection.....Insecticide & Dose..... Type of structure: Mud/cement/brick/ stone/.....Temperature: Min ..... Max..... Relative humidity: Min .....Max.....

(a) Dead mosquitoes								
Arm No. (House code & Dose)*	**Species**	**Male**	**Female**				**Total**	
			UF**	**BF**	**SG**	**G**		
Dose 1								
Dose 2								
Dose 3								
Control								
(b) Alive mosquitoes								
Arm No. (House code & Dose)*	**Species**	**Male**	**Female**				**Total**	**Mortality after 24 h**
			UF**	**BF**	**SG**	**G**		
Dose 1								
Dose 2								
Dose 3								
Control								
UF=Unfed; BF= Blood fed; SG= Semi-gravid; G= Gravid; * Separate row for each replicate; **Unfed mosquitoes should be dissected for parity (Refer section 1.1.1.5g5).								

Table 11. Hand catch collection

Village.....Sub-centre ..... PHC ..... District ..... State .....
Date of collection.....Insecticide & Dose..... Type of structure: Mud/cement/brick/ stone/.....Temperature: Min ..... Max..... Relative humidity: Min..... Max .....

Arm No. (House code & dose)	**Species**	**Male**	**Female**				**Total**	**Mortality after 24 h**
			UF*	**BF**	**SG**	**G**		
Dose 1								
Dose 2								
Dose 3								
Control 1								
Control 2								
UF=Unfed; BF= Blood fed; SG= Semi-gravid; G= Gravid; * Unfed mosquitoes should be dissected for parity (Refer section 1.1.1.5g5). MHD– No. of mosquitoes collected/No. of persons X time (h).								

2.1.3.7.4. Sibling species identification (where ever)

Where ever appropriate, the mosquito samples will be analyzed for sibling species composition using molecular assays.

2.1.3.7.5. Parity

All unfed mosquitoes collected by different methods should be dissected and tracheolar skeins are observed to assess parity. In nulli-parous mosquitoes the tracheolar skeins will be in coiled condition and in parous mosquitoes the skeins are distended (WHO 1975). Mosquitoes should be categorised as nulliparous and parous mosquitoes and recorded in the format given in Table 12. Reduced parity rate indicates the reduction of longevity of the mosquito population. The data should be expressed for each dosage and control separately.

Table 12. Parity rates

Village ..... Sub-centre ..... PHC..... District..... State.....
Insecticide & Dose.....

Species	Total dissected	No. nulliparous	No. parous	Percent parity
1.				
2.				
3.				
Parity rate = No. parous x 100 / No. dissected.				

2.1.3.7.6. Airborne toxicity (cage method)

Some insecticides may produce air-borne toxicity. This can be assessed by estimating, in comparison with an unsprayed hut (control), the mortality of mosquitoes placed in small cages (5 to 10 cages in each hut, 25 non blood-fed female mosquitoes in each cage) hanging from the ceiling, for 4–8 hours up to a maximum of 12 hours, at different distances from the sprayed surfaces. The mosquitoes are then kept for 24 hours' observation (at 27 ± 2^{o}C and 60–70% RH) after being transferred to clean cages. The mortality in comparison to the controls is recorded (as shown in Table 13) to assess the air-borne toxicity due to volatility of the insecticide.

Table 13. Airborne toxicity

Village ..... Sub centre .....PHC ..... District ..... State.....
Date of bioassay ..... Household No ..... Insecticide and dose.....
Temperature: Min ..... Max..... Relative humidity: Min .....Max.....
Test species ..... Lab/F_1/Field collected mosquito.....

Replicate * Cage No.	No. of mosquitoes	No. knocked down after 1 h	No. killed after 24 h holding	% mortality after 1h	% mortality after 24h
Replicate 1					
Replicate 2					
Control					

2.1.3.7.7. Significance of entomological indicators

- Floor sheet collections: Immediate mortality
- Aspirator collection indoors: Delayed mortality
- Exit trap collections: Induced exophily/ excito-repellency
- Parity: Reduction of parity rate indicates reduction of survival rate
- Sibling species composition: Differential responses among the species
- Air-borne toxicity (for volatile insecticides): Volatile effect

2.1.3.7.8. Assessment of impact of the insecticide on vector

Number of vector mosquitoes found dead on the floor sheet in the morning	(a)
Number found dead in exit traps	(b)
Number found alive in exit traps	(c)
Number found dead after 24 h holding among live mosquitoes	(d)
Total entry = a+b+c+d	(e)

Comparisons between various treatments and control should be made and the inferences should be drawn as under:

Immediate mortality= [a+b/e] X 100

Excito-repellency= Comparative exit to entry rate (b+c/e) X 100

Delayed mortality= Number dead after 24 h among the live mosquitoes [d/e] X 100

Overall mortality= [a+b+d/ e] X 100

2.1.3.8. Perceived side effects, acceptability by householders and collateral benefits

Information on these aspects will be collected by interviewing the inhabitants using structured questionnaire (Annexure 2).

2.1.3.9. Human safety

This should be accomplished by interviewing the subjects using structured questionnaire or by physically examining known inhabitants from both sprayed and unsprayed houses for certain health parameters, before and after spray to see any change in certain health parameters due to insecticidal spray (Annexure 3). A medical practitioner should be associated for collection of the data.

2.1.3.10. Criteria for selection of dose of insecticide for Phase III trial

The minimum dosage of the insecticide that gives has the potential to reduce human-mosquito contact; with at least 10–12 weeks residual efficacy and with minimum adverse effects should be selected for the Phase III trial.

2.1.4. Large-scale field trials (Phase III)

Duration: 12 months

The efficacy of the insecticide formulations that are found suitable for IRS in experimental hut or small-scale field trials (Phase II) should be evaluated in large-scale (at village level) against mosquito populations.

Objectives

- to establish the efficacy of insecticide formulations at the selected application rate(s) against the target vector species, when applied to all or most households in the community;
- To confirm residual activity and application intervals
- To study the impact on disease prevalence
- To assess community acceptability of the new insecticides or formulations and collateral benefits
- to observe the ease of application and handling of the insecticide product, and to record perceived side-effects, if any, on operators and households

2.1.4.1. Selection of villages and collection of baseline data

The Phase III trials are generally designed as cluster randomized trial. The unit of intervention for this phase of trial is the village because the effect of the intervention is to act upon the entire village/ community and population of mosquitoes within it, even though not all households may accept the intervention measure. The usual effect of insecticide used for IRS is to reduce the longevity, density and infectivity rate of the vectors.

Selection of villages should be done in consultation with the district/ state health programme personnel. It is essential that treatment and comparison (control) villages are eco-epidemiologically homogenous. Villages with an average API of 2 and above (in last 3–5 years) with a population of ~3000 (one or cluster of villages) should be selected for treatment. For comparison, village(s) with a similar population size located at a distance of about 5–10 km from the treatment village(s) should be selected. The distance is maintained to avoid infiltration of mosquitoes in to the treated area from outside. If known, the flight range of the vector species should be taken in to account while selecting the treated and control villages. Where selection of well separated treatment and control villages is not feasible, it may be possible to increase the size of the area to several villages for treatment and use its central part for evaluation, thus achieving a barrier of treated villages. Such a barrier should be wider than the known or expected flight range of the vector. For multi-centric evaluation, the villages selected should be from different ecotypes (at least three) having different vector species and three seasons are to be covered for evaluation.

The villages should be allocated to treatment or control arms at random in order to minimize the bias due to other risk factors and to permit a clear demonstration of the effect of the intervention (IRS). If villages are heterogeneous, it may be desirable to stratify them in terms of size, location (ecotype), types of breeding site, disease incidence rates (e.g. SPR or API), use

of household protection measures and entomological parameters (vector abundance). Collection of such baseline data in order to have comparable intervention and control groups may require a preparatory phase of a few months to a year, depending on the entomological and transmission patterns of the area. Within each stratum, villages are randomly allocated to the intervention or control arms.

Conducting IRS trials with negative controls is not acceptable for ethical reasons. A positive control, such as DDT or deltamethrin spraying, would be an acceptable alternative, but it would then be difficult to demonstrate a difference in efficacy between treatment and control arms. Therefore, as an alternative to a positive control, an equivalent forms of protection that has no effect on vector populations: chemoprophylaxis, for example could be used.

Matched pair designs are a special case in which the villages are stratified in pairs and one member is then randomly assigned to the treatment arm and the other to the control arm. Stratified designs are usually preferable to matched pair designs. Cluster-randomized trials with fewer than five clusters per arm are not advisable, because parametric tests may be unreliable with such small numbers. The number of entomological monitoring sites should be equal in each village, and will depend on the number of villages in each arm, the power of the study to detect an expected or minimum percentage impact and the available resources. Since houses may vary greatly in their attractiveness to mosquitoes, for practical reasons and consistency, the same entomological monitoring sites should be maintained throughout the study (WHO/CDS/NTD/WHOPES/ GCDPP/ 2006.3).

2.1.4.2. Census

In collaboration with the respective PHC and District Malaria Office, census and numbering of all houses should be carried out in the selected experimental and control villages prior to spraying. Census details are recorded using a format as given in Table 14.

Table 14. Record of census of households

Village ..... Sub-centre ..... PHC..... District.....

State..... Date of survey .....

S. No.	House No.	Name	Relation	Age/ Gender	Educa-tion	Profe-ssion	Type of Structure	No. of rooms	No. of cattle-sheds	No. of tempo-orary sheds	Sleeping habit inside/ outside
1.											
2.											
3.											
4.											
5.											
6.											

2.1.4.3. Ethical considerations

Ethical clearance should be obtained from the appropriate institutions/ authorities. This should include the informed consent form and the trial's information sheet to be provided to the households/ study communities.

In general, the following ethical rules need to be applied:

- The benefits of research (trial) should be equitable among the villages/ communities and individuals involved. Consent must be obtained from community leaders.
- The participants should be informed in clear, in the vernacular/ local language about the objectives, study protocol, and advantages and inconveniences. Participants should be told they have complete liberty to participate or refuse to participate.
- The content of an information sheet prepared in local language and as approved by the ethics committee should be made available to every participating member.
- Sharing of the trial's findings with the community and local, regional and national health officials needs to be assured.
- For village scale intervention trials, the community must decide collectively, although individuals do have the option to refuse at a household level. A village committee that can represent the interests of the community is required.

2.1.4.4. Spraying of villages

In the treatment villages, the candidate insecticide (the given formulation) is sprayed and in the control villages an equivalent forms of protection that has no effect on vector populations (e.g. chemoprophylaxis) should be used (refer to section 2.1.4.1). Spraying in the treatment villages is done in collaboration with the respective PHC Medical Officer and District Malaria Officer. Coverage of indoor residual spraying should be (i) total – all dwellings (human dwellings, mixed dwellings, cattle sheds, temporary sheds and other structures, if any) are sprayed, (ii) complete – all sprayable surfaces are treated, (iii) sufficient – ensuring an uniform application of the target dosage, and (iv) repeated spraying– if the duration of the trial is longer than the duration of effective action (residual activity) of the insecticide treatment. All the houses in the treated villages are numbered and entered in the trial database.

Spraying should be done with the technique, equipments in use in the routine vector control programme under strict supervision following national (NVBDCP) guidelines. Inhabitants of the selected villages should be informed in advance about spraying and benefits of getting their dwellings sprayed. Necessary precautions should be taken for the protection of spray men by providing protective clothing; goggles, gloves etc. (refer Box 6). Each house is marked on the wall indicating date and coverage of spray [Number of rooms sprayed/ number of rooms in the dwelling (e.g. Dwelling No. 5/5)]. Coverage of spray should be recorded and presented as indicated in the Phase II (refer to section 2.1.3.4.).

Spray men and supervisors should strictly follow the insecticide label recommendations and the safety instructions provided by the principal investigator. Safety should also be ensured during transport, storage and disposal of insecticides. Spray men should be informed of the adverse health effects of insecticides, including signs and symptoms of poisoning of the insecticides they are using.

During large-scale field trial, an experienced physician should monitor the workers, respond to any adverse health event, and recognize the signs and symptoms of different types of insecticides poisoning. Parents should be warned about risky situations involving children.

2.1.4.5. Assessment of the quality of treatment

To achieve quality spraying (uniform application, adequate dosage), it is very important that spray-men are properly trained, well-maintained and calibrated equipment are used and spray operation is closely supervised. Papers (Whatman® No. 1) attached to the walls of randomly selected houses may be removed after the spray campaign and assayed for insecticide residue. Filter-papers are preferred to scrapings of sprayed mud surfaces because of difficulties of standardization.

2.1.4.6. Assay for residual activity

Residual activity should be assessed as per the procedure described in section 2.1.3.5.

2.1.4.7. Evaluation

Evaluation should be done in the sprayed and control villages at fortnightly interval in randomly selected dwellings representing the types of houses (cemented, stone, thatched, mud, etc.) and structures namely human dwellings, mixed dwellings (if present) and cattle sheds.

2.1.4.7.1. Efficacy

Several entomological parameters are relevant or required to estimate the entomological efficacy of a control intervention.

2.1.4.7.1.1. Vector density

Different methods of measuring population abundance may be used, each with advantages and limitations.

Indoor resting density: Hand catches of resting mosquitoes indoors in the dawn hours are one of the practical methods providing information on population density and also the favoured approach for estimating biting rates in regions where vectors are zoophilic and where human landing collection (HLC) yields low numbers of mosquitoes per night. A reduced proportion of gravid or semi-gravid mosquitoes may indicate insecticide-induced mortality or repellency. Indoor resting collections are indicative only of human biting rates if the proportion feeding on humans is established. Blood-meal identification of individual mosquitoes of a sample is carried out using precipitin or ELISA tests. From the product of indoor resting catch and the human blood index, an estimate of human biting rates may be derived. Assigning six houses per village for fortnightly indoor resting collections and exit trap catches gives meaningful data

on mosquito density, which is expressed as the number of vectors captured per room (or per person in the room) per unit time.

Additional information on exit rates or repellency of the insecticide may be obtained by attaching exit traps to windows of sprayed and unsprayed houses.

Pit-trap collections: Pit traps (pit shelters) dug in the ground, if attractive to the vector species, may provide information on outdoor resting behaviour if the vector commonly rests outdoors or is driven outdoors by the repellent activity of the insecticide. Pit traps are not used routinely, and many species will prefer to rest on other outdoor sites such as vegetation, root interstices, tree hollows.

Mosquito human landing collections (This may be done if feasible and on obtaining necessary clearance from human ethics committee): Vector density is traditionally monitored using human landing catches (HLC), which measure the number of landing mosquitoes per person per night. All night (dusk to dawn) mosquito landing collections should be made in one house in each treatment and control village at fortnightly intervals. Collections are required within and outside the house to assess indoor and outdoor biting rates (exo-endophagy). Persons volunteering to be baits should be informed about the experiment. Informed consent of the human volunteers involved in the study should be obtained prior to the collections (Annexure 4). The human volunteers may lie down or on a cot and can sleep as per their normal practice. The insect collectors, who will be catching the mosquitoes landing on the bait, are rotated every four hours to avoid bias and slackness. The sampling errors caused by variation in catcher efficiency or attractiveness may be reduced by increasing the number of capture sites per cluster. Hourly mosquito collections should be recorded in the format given in Table 15. Results are expressed as number of vectors landing per human bait per night or number of mosquitoes per bait per hour, if the collections are restricted to the hours of peak biting. The results provide information on biting rhythm and feeding habits of vector species in the areas.

Mosquito animal landing collections (This may be done if feasible and on obtaining necessary clearance from animal ethics committee): In regions where vectors are mainly zoophilic or present at low densities, HLC results in low capture rates and poor catcher efficiency. To establish more accurately the abundance of zoophilic vectors in a sprayed cluster when HLC gives limited data, catches are sometimes made from domestic animals (usually cattle) at fortnightly intervals. Landing collections on a cattle tied to a pole should be made from dusk to dawn at hourly interval for 10 min each hour. Data should be recorded in the format given in Table 16. Results are expressed as number of vectors landing per animal bait per night or number of mosquitoes per bait per hour, if the collections are restricted to the hours of peak biting. This will provide information on biting rhythm and feeding habits of the vector species in the area.

Light trap catches: Where a correlation between CDC light trap catches set beside occupied untreated nets and HLC has been established, light trap catches may be used as a surrogate method of collection. This method is much less labour-intensive than HLC because only a small, day-time working team is needed to collect and re-set several traps per day. In this

situation, CDC light traps provide a reliable alternative that overcome the ethical constraints and catcher variability associated with HLC. The traps are set during dusk hours indoors (human dwellings and animal sheds) as well as outdoors at fortnightly intervals in both treated and control villages. In the next morning, the trapped mosquitoes are collected, identified to species and recorded in the format given in Table 17.

Table 15. Mosquito landing collection (human)

Village..... Sub-centre..... PHC.....District ..... State.....

Date of collection.....Insecticide & Dose.....Temperature: Min.....

Max ..... Relative humidity: Min ..... Max .....

Time (Hrs)	**Malaria vector**					**Other anophelines**		***Cx. quinquefasciatus***		**Other culicines**	
	M	UF*	BF	SG	G	M	F	M	F	M	F
	T	NP	***P***								
1800											
1900											
2000											
2100											
2200											
2300											
2400											
0100											
0200											
0300											
0400											
0500											
0600											
Total											

M = Males; UF = Unfed females; BF = Blood fed females; SG = Semi-gravid females; G = Gravid females; P = Parous; NP = Nulliparous; T = Total dissected; *Unfed mosquitoes should be dissected for parity (see section 1.1.1.5g5).

Table 16. Mosquito landing/biting collection (animal)

Village..... Sub-centre….....PHC…….....
District……..... State….....…….....
Date of collection…….....Insecticide & Dose.....Temperature: Min.....
Max.....Relative humidity: Min …….....Max…….....….

Time (Hrs)	***An. culicifacies***					**Other anophelines**		***Cx. quinquefasciatus***		**Other culicines**	
	M	UF*	BF	SG	G	M	F	M	F	M	F
	T	NP	***P***								
1800											
1900											
2000											
2100											
2200											
2300											
2400											
0100											
0200											
0300											
0400											
0500											
0600											
Total											

M= Males; UF= Unfed females ; BF= blood-fed females, SG= Semi-gravid females, G= Gravid females; P = Parous; NP= Nulliparous; T= Total dissected; * Unfed mosquitoes should be dissected for parity (see section 1.1.1.5g5).

Table 17. Light trap collection

Village.....Date of collection .....Insecticide & Dose.....Collection site No & Type.....
Temperature: Min..... Max ..... Relative humidity: Min ..... Max .....

Trap No.	**Collection site No. and type**	***An. culicifacies***					**Other anophelines**		***Cx. quinquefasciatus***		**Other culicines**	
		M	**UF***	**BF**	**SG**	**G**	**M**	**F**	**M**	**F**	**M**	**F**
			T	**NP**	**P**							
1												
2												
3												

M= Males; F= Females; UF= Unfed females ; BF= blood-fed females, SG= Semi-gravid females; G= Gravid females; P = Parous; NP= Nulliparous; T= Total dissected; * Unfed mosquitoes should be dissected for parity (see section 1.1.1.5g5)

2.1.4.7.1.2. Vector longevity

The main effect of IRS is to reduce the longevity (survival) of vector mosquitoes and thereby the probability of transmitting disease (malaria). The simplest method of estimating mosquito longevity in the field is to measure the proportion parous of a sample of mosquitoes obtained from HLC or hand catches. The ovaries of unfed or freshly fed mosquitoes are dissected out to examine whether the tracheoles are coiled or uncoiled. Uncoiled tracheoles indicate that a female has developed and laid eggs at least once in her lifetime. The proportion of such parous females is an indirect measure of the probability of daily survival of mosquitoes in the population. If IRS with a given adulticide is effective, a marked reduction of the proportion parous females should be observed.

2.1.4.7.1.3. Infection and infectivity rates

Vector species collected from human landing and indoor resting hand catches are dissected out in 0.6% saline to examine mid-gut for the presence of oocysts and salivary glands for sporozoites using conventional (WHO 1975) or ELISA method (Wirtz *et al* 1985 &1992). With a successful IRS, few mosquitoes would survive the time required for sporozoites to mature, and so the sporozoite rate should be greatly reduced. In meso- or hypo-endemic areas where sporozoite rates may be less than 0.3%, samples of mosquitoes may be pooled into groups of 10 before the ELISA test with no loss of sensitivity. The overall numbers of mosquitoes tested may thus feasibly be increased to several thousand that would be required to detect a significant reduction as a result of IRS and to make meaningful comparisons between study arms. Results are recorded in a format as given in Table 18. Data should be represented for each insecticide and dosage separately.

Table 18. Vector infection and infectivity rates

Village….....Sub-centre….....PHC….....District…….....

Date of collection …… Insecticide & Dose.....

Species	Number dissected	Oocyst positive	Sporozoite positive	Oocyst rate	Sporozoite rate
1. 2. 3.					
Oocyst/sporozoite rate = $\frac{\text{No. found oocyst/sporozoite positive}}{\text{Total No. dissected}}$ X 100					

2.1.4.7.1.4. Entomological inoculation rate (EIR)

This is an important entomological indicator for measuring the epidemiological impact of a vector control intervention. EIR is the number of infective bites per person per night. It is estimated from the product of sporozoite rate multiplied by the human landing rate (number of mosquitoes per bait per night) or equivalent estimates of human biting rate if this has been

established for the vector species (e.g. light traps). Both components of EIR should be reduced by an effective insecticide.

EIR = Sporozoite rate (%) x No. of mosquitoes per bait per night in Human landing collection

2.1.4.7.2. Disease prevalence

Point prevalence of disease (malaria) through sample blood surveys (covering a minimum 10% of the total population in each of the study arms) should be assessed in treatment and control areas. The frequency and time of the survey should be decided based on the transmission pattern of the disease and in consultation with state health department. It is recommended that in case of two rounds of spraying, 1st sample blood survey should be conducted 15 days prior to first round of spraying, 2nd survey 30 days after first round of spraying, 3rd survey 30 days and 4th survey 70–80 days after 2nd round of spraying. Surveys may be carried out following systematic sampling method selecting every 4th or 5th house depending on the total number of households to be selected in each village, which will be proportionate to the population size (PPS) of the villages. Rapid diagnostic kits (RDKs)/ microscopic examination of stained blood smears will be used to screen the inhabitants of the selected houses for malaria infection. The test/ microscopic positive persons will be administered with anti-malaria drugs as per the national guidelines. The health workers of the respective PHC will be involved in the treatment of malaria positive persons. Sample survey data are recorded in the format given in Table 19 a&b. Further the data may be arranged according to the following age groups; 0–11 & 12–24 months, 2–4, 5–9, 10–14 years, and 15 years and above.

Table 19. Mass blood survey

a. Data collection sheet

Village…..... Sub-centre…..... PHC…..... District…….....…
Date of collection ……

S. No.	H. No.	HOF	Name of patient	Age	Sex	Blood smear No.	Fever history	Drugs given	Result of smear		
									Pv	*Pf*	Mix
1.											
2.											
3.											
4.											

b. Summary Sheet

Village…..... Sub-centre ..... PHC ..... District ..... State……
Date of collection …… Insecticide & Dose..... Date of spray/round .....

Part 1

S.No	Name of Village	Population					Total
		<1	1–4	5–8	9–15	>15	
1.							
2.							

Part 2

Blood smears collected						Drugs consumed			
<1	1–4	5–8	9–15	>15	Total	4 AQ	8AQ	Paracetamol	Other drugs

Part 3

Positives detected						% blood smears collected	Positives				Remarks
<1	1–4	5–8	9–15	>15	Total		*Pv*	*Pf*	Mixed	Total	

2.1.4.7.3. Significance of indicators

- **Hand catch:** Relative density of mosquitoes and other non-target insects
- **Human/animal bait catches:** Feeding preference, biting rhythm and man-mosquito contact
- **Light trap collections:** Where a correlation between light trap catches set beside occupied untreated nets and HLC has been established, light trap catches may be used as a surrogate method of collection.
- **Parity:** Indicates longevity/ survival of the vector species
- **Vector infection/ infectivity:** Intensity of transmission and role of different vectors
- **Entomological inoculation rate:** Indicator to measure transmission load and epidemiological impact
- **Disease prevalence:** Impact on incidence of malaria infection

2.1.4.7.4. Adverse effects, acceptability by householders and collateral benefits

These aspects will be assessed by interviewing the inhabitants using structured questionnaire (Annexure 2).

2.1.4.7.5. Human safety

This is accomplished by interviewing the subjects using structured questionnaire (Annexure 3). A medical practitioner should be associated for collection of data.

2.1.4.7.6. Operational acceptability

- Ease of application

- % Suspensibility of the wettable powder formulation should be within the limits mentioned in the technical data sheet
- difficulty in pumping, repeated clogging of nozzle, stability of suspension, maintenance need of equipment, nozzle corrosion, etc. should be ascertained from spraymen and supervisory staff
- Stability of insecticide suspension for sufficient time after mixing
- Stability of insecticide formulations in different storage conditions
- Safety to spraymen and inhabitants (as enquired and investigated in Annexure 3)
- Acceptability by community as determined by odour, effect on décor of premises, collateral benefits etc. (as enquired in Annexure 2)

2.1.4.7.7. Data analysis and interpretation

The primary unit of replication and analysis is the village. The preferred choice of statistical method will take into account the variation existing between villages. Multivariate analysis is therefore the preferred approach since it adjusts for such variation before estimating the effect of the treatment. Proportional data (e.g. parous rates, sporozoite rates, bioassay mortality) should be analyzed using logistic regression analysis (which is also used for evaluating experimental hut data). Numeric entomological data (e.g. mosquito resting density, human landing catches or light trap catches) are likely to be over-dispersed (i.e. not normally distributed between sites) and should be analyzed using Poisson regression or transformed using logs to a normal distribution before applying analysis of variance.

Analysis of the entomological parameters provides information on the probable epidemiological impact of the treatment on malaria transmission, as indicated by the estimates of EIR derived from these parameters, EIR, the product of the number of infective bites per unit period multiplied by the sporozoite rate, is increasingly being used to indicate the impact of vector control interventions. An overall analysis of entomological indicators will provide estimates of the efficacy of the treatment, while an analysis done by period may show changes in residual impact of the intervention over time. The residual activity of an insecticide is also shown by changes in the proportion killed using cone or wire-frame bioassays on sprayed surfaces.

2.2. Insecticide Treated Nets & Fabrics

Use of insecticide treated nets (ITNs) is a preventative method of control of malaria and kala-azar. Uses of ITNs are a very useful and practical way to prevent mosquito bites. ITNs are impregnated with insecticide that kill mosquito upon contact and its efficacy enhances when its use coincides with the seasonal abundance and biting rhythm of the mosquitoes and the sleeping time of the people who use them. . There are also collateral benefits in providing overall personal protection from other haematophagous insects. This vector control tool is eco-friendly as it minimises consumption of insecticides in the control programme.

ITN is at present available in two forms, one requires regular treatment with insecticides and the other is long-lasting insecticide treated nets (LLIN), which exert relatively permanent insecticidal activity for a period of about 4 years. For impregnation of nets with WHOPES passed insecticides, evaluation should be carried out in two Phases - Phase II and III, whereas for new insecticides three Phases, Phase I, II and III, of evaluation are required.

2.2.1. Nets requiring treatment (WHOPES passed insecticides)

2.2.1.1. Phase II evaluation (Field study with village huts)

Duration: 12 months (duration of the evaluation can be modified based on nature of insecticide used for impregnation; for permethrin effectiveness recorded 6 months & for alphacyno-pyrethorids 6-12 months after 1- 2 washings)

Objectives

- To determine the efficacy of the selected insecticide on a given fabric or different fabrics against mosquitoes (operational)
- To determine the optimum application dosage of insecticide in Phase III (technical) and its persistence
- To assess the safety aspects of impregnation and use (managerial)

Activities

- Baseline studies to assess the vector densities and vector behaviour for selection of study area
- Efficacy of insecticide on different net material using WHO cone bioassays with prior ensurement on proper dilution, verification of inspective manufacture/expiry date, dipping or treatment and storage
- Assessment of efficacy based on different entomological parameters
- Determination of effective dose of insecticide for impregnation
- Suitability of ITN for the area to ensure that ITN likely to reduce malaria in areas where vectors bite indoors and at night, rest indoors and bite humans preferentially
- Human safety evaluation and feasibility on social marketing
- Assessment of risk reduction (malaria) through usage of ITN

- Final review with collaborators and state health programme personnel

2.2.1.1.1. Selection of doses for application

For treatment of ITNs, WHO recommended insecticides (www.who.int/whopes/ quality/en/) can be used and minimum three dosages need to be selected for impregnation.

2.2.1.1.2. Selection of study area

Study area should be selected in consultation with respective State/District health department personnel to ensure that the area is free of ITN/LLIN use and does not receive other vector control measures also. If the study is to be carried out in two or more villages, care should be taken to select eco-epidemiologically homogenous villages. Surveys should be carried out to select villages with target vector mosquitoes in adequate numbers. Simultaneously studies should be undertaken in selected villages to examine biting rhythm of the vector species by mosquito landing collections (refer section 2.1.3.2.), resting behaviour of the vector species by light trap collections (refer section 2.1.4.7.1.1.) and susceptibility to different insecticides in the prevailing vectors (refer section 2.1.1.2, Box 2).

2.2.1.1.3. Selection of houses

In the selected village(s) 20 houses should be selected. These houses should be designated for interventions among four arms. Each house should be labelled with number of the arm and the dose of the insecticide. The houses in each arm should be distributed equitably among the dosages of impregnation and control - one house for each dosage and one for control as shown in Table 20.

In case of a request for comparison with another insecticide for impregnation an additional five houses should be selected (total 25 houses) and distributed as shown in Table 21. The selection of comparison-insecticide with the dosage of impregnation should be made in consultation with the NVBDCP, Delhi. Selection of houses should be with the written consent (Annexure 5) of the head of the family with permission to alter the structure with minor changes, if needed.

Table 20. Distribution of houses in different arms

Replicates	Houses with nets impregnated with candidate insecticide			Houses (negative controls)
	Arm Dose 1	Dose 2	Dose 3	
1	House-1	House-2	House-3	House-4
2	House-5	House-6	House-7	House-8
3	House-9	House-10	House-11	House-12
4	House-13	House-14	House-15	House-16
5	House-17	House-18	House-19	House-20

Table 21. Distribution of houses in different arms along with comparison insecticide

Arm	Houses with nets impregnated with candidate insecticide			Houses with Negative Control 1	Houses positive Control 2
	Dose 1	Dose 2	Dose 3		
1	House-1	House-2	House-3	House-4	House-5
2	House-6	House-7	House-8	House-9	House-10
3	House-11	House-12	House-13	House-14	House-15
4	House-16	House-17	House-18	House-19	House-20
5	House-21	House-22	House-23	House-24	House-25

In case the ongoing intervention is suspended, the surveillance mechanism should be strengthened for providing protection from the disease to the villagers. Where feasible, hamlets or small villages having required number of houses for the evaluation can be selected.

2.2.1.1.4. Treatment and distribution of nets

The exact number and sizes of nets to be distributed among the households should be determined after making census of the study area (Table 22). Assessment of requirement of nets should be made to protect all the inhabitants of the selected houses. Generally, it is recommended that for a family of five, two nets to be given. Impregnation of the nets should be done as given in Box 8.

Inhabitants should be educated on the use and storage of nets and instructed not to wash the nets. All the nets should be marked with a water-soluble ink to detect whether the nets are washed or not. Every time the team goes for data collection the marking on the nets should be checked and recorded. To ensure the regular use of nets by the inhabitants, volunteers from the village should be deputed to ascertain the compliance. Compliance of use of nets should be assessed fortnightly and the data should be recorded in the format given in Table 23.

Table 22. Record of census of households

Village……..... Sub-centre……..... PHC.....…….District.....……State….....

House No. ……..... Type of the structure…..…..Date

S. No.	Name	Age/ Gender	Education	Profe-ssion	No. of rooms	Sleeping habits – Inside/Outside	No. of nets required
HOF 1. 2. 3. 4.							

Box 8: Procedure for impregnation of nets and fabrics

Specification of mosquito nets would be polyester- polyamide nets with 75-100 denier*(weight in grams of 9,000 m yarn) is optimal , white or single colour, 156 mesh (range 120-200 mesh per sq.inch)'(minimum acceptable- mesh size 1.2 mmX 1.2mm in malarious areas; 0.6mm X 0.6 mm in kala-azare areas)(12 x 13 holes)/square inch. The doses (mg/ sq m) of impregnation for the study would be as follows:—

(1) Surface area of the net (sq m) = 2 [(length x height) + (height x width)] + (length x width)

(2) Volume of the water required for soaking one net = Initial volume of water – Volume of water remaining in the tub after complete soaking.

(3) Active ingredient (a.i.) of insecticide required for treatment of a net (mg) = Surface area in square metres x Target dose (mg/sq m)

(4) Volume of formulation required for a net = Weight in mg x 100/Concentration of the insecticide formulation.

The required volume of formulation should be added to cold water in a tub (15–20 litres capacity). Where feasible it is recommended that suspension required for 3 nets should be prepared and used for impregnation of two mosquito nets to ensure uniform impregnation. Repeated rubbing and squeezing should be carried out while the nets are in the tub (15 minutes). Remaining liquid in the tub should be discarded appropriately. The treated mosquito nets should be dried in shade on a plastic sheet. After drying, nets should be properly labeled with insecticide dose, date of impregnation on the cloth skirting at the bottom of the net with permanent marker. The nets should be placed in polyethylene bags and stored at room temperature safely.

- Same procedure is applicable for impregnation of nets/curtains of different fabrics
- For tablet formulations follow the instructions of the manufacturer

Table 23. Record of compliance of treated nets in households

Village……..Sub-centre……...PHC…….....District…….State……House No.....
Insecticide & Dose……Type of the structure ……Date of survey ...…….

House code (Arm No./ Insecticide and dosage)	Date and time of visit	Use of net	
		Yes	No
1.			
2.			
3.			

2.2.1.1.5. Persistent activity studies

Persistence is assessed by cone bioassays and ring-net bioassays and if the phase II trial is carried out in different ecological settings, persistence needs to be assessed for each area.

2.2.1.1.6. Cone bioassays

This will be assessed by performing WHO cone bioassays (WHO 1998) with slight modifications. For this bioassay, rim of the cone will be fixed to the treated nets fastened with rubber band. The cones will be fixed randomly on different flaps of the net of each dose. One net from each dose and one from control should be collected randomly. Preferably 3-day old sugar fed laboratory strain of vectors or F1 female progeny of field collected adults or females of mixed age (using females of same gonotrpohic condition) populations from unsprayed villages should be used for bioassays. Five mosquitoes should be introduced into each plastic cone through orifice and plugged with cotton wool. At least two replicates should be used on each of the five sections (roof and four sides) in each treated and control net. Thus, a total of 10 replicates of 5 mosquitoes in each replicate are used for each test sample, giving a total of 50 mosquitoes per sample. Number of mosquitoes knocked-down at the end of three minutes should be recorded and the mosquitoes are transferred to plastic containers with a nylon net fastened with rubber band. Mosquitoes are provided with sugar soaked cotton wool placed on the top of the net. The plastic containers should preferably be placed in an unsprayed room maintained at standard temperature (27 $\pm$ 2°C) and relative humidity (80% $\pm$ 10%); where it is not feasible to maintain such conditions, a moist chamber can be used as described by WHO (1981). Percent mortalities are calculated after 24 h of holding from the alive and dead mosquitoes and corrected to the control mortality, if it is between 5 and 20%, using Abbott's formula. Data should be recorded in the format given in Table 24. Results are expressed as overall persistence effect of treated net at a given dosage of insecticide.

Table 24. Cone bioassay

Village…… Sub-centre.....……..... PHC.....……. District…….State…….....
House No. .....…… Insectide and dose……Type of the structure……Date of survey…....……
Test species……..... Lab/ F_1/Field collected…….....

Net details	No. exposed	Number knocked-down after 3 minutes	No. dead after 24 h	% Mortality	Corrected % mortality*	(#10)
Replicate 1						
Replicate 2						
Replicate 3 - 10						
Control 1						
Control 2 Control 3-5						

$$\text{*Corrected \% mortality} = \frac{\text{\% Mortality in test replicates} - \text{\% Mortality in control}}{100 - \text{\% Control mortality}} \times 100$$

2.2.1.1.7. Ring-net bioassay (determination of median knock-down time)

Where, very high mortality rates are found, determination median knock-down time can be calculated using ring-net bioassay at fortnightly interval in the field following the procedure given by WHO (1998). A netting apparatus consisting of two intersecting circles of wires of 15 cm diameter, welded together to get sphere shape, should be wrapped with the treated mosquito net in such a way that a sleeve is left through which mosquitoes can be introduced and removed with an aspirator. Preferably laboratory reared, 3-day old, sugar fed, susceptible female of mosquito species should be used for the assay. One net from each dosage should be picked up randomly and one from control. Eleven mosquitoes should be introduced and the time required to knock-down 1st, 6th and 11th mosquito should be recorded. Maximum exposure of 2 h should be given. At least two ring-nets (two replicates) are used on each of the five sections (roof and four sides) in each net. The time required for the knock-down of 6th mosquito will indicate the median knock-down time. Data should be recorded in the format given in Table 25. Results are expressed for given insecticide and dose.

Table 25. Ring-net bioassay

Village…… Sub-centre..... PHC.....……District…….State…….....
House No. .....…… Insectide and dose……Type of the structure……Date of survey…....……
Test species……..... Lab/ F_1/Field collected…….....

Replicate number	Knock-down time (minutes		
	Ist	6th	11th
Replicate 1			
Replicate 2			
Control 1			
Control 2			

2.2.1.1.8. Entomological evaluation

Total number of mosquitoes in a structure should be measured every fortnight by the following methods in sequence mentioned below in 2.2.1.8a -2.2.1.8c). The main aspects to be covered during entomological evaluation:

Identification of vector(s) and sites of breeding

Biting site (indoor/outdoor) and time (indoor/outdoor or both)

Resting site (indoor/outdoor or both)

Host preferences (mainly human or animal or both)

ITN suitability (since ITN likely to reduce malaria transmission where vectors bite indoors at night when people are asleep, rest indoors and bite human preferentially)

2.2.1.1.8a. Floor sheet collection

For details see section 2.1.3.7.1.

2.2.1.1.8b. Exit window trap collection

For details see section 2.1.3.7.3.

2.2.1.1.8c. Hand catch

For details see section 2.1.3.7.2.

2.2.1.1.9. Significance of entomological indicators

Floor sheet collections: Immediate mortality
Exit trap collections: Excito-repellency
Hand catch: Relative density of mosquitoes and other non-target insects

2.2.1.1.10. Assessment of impact of ITN

Number of vector mosquitoes found dead on the floor sheets in the morning…(a)

Number collected alive by hand catch …(b)
Number found dead in exit traps …(c)
Number found alive in exit traps …(d)
Number found dead after 24 h holding among the alive mosquitoes …(e)
Total entry = a+b+c+d per room per night …(f)

Comparisons between various treatments and control should be made and the inferences should be drawn as under:

Immediate mortality = [a+c/f] X 100

Indoor resting density = a+b+c+d

Excito-repellency = exit to entry rate [(c+d)/ (e+f)] X 100

Delayed mortality = [Number dead after 24 hrs among the alive mosquitoes / (f)] X 100

Overall mortality = [Number dead on floor sheets, in traps & 24 h holding / (f)] X 100

2.2.1.1.11. Adverse effects, acceptability by community and collateral benefits

Information on these aspects will be generated by interviewing the inhabitants using structured questionnaires (Annexure 6).

2.2.1.1.12. Human safety

This should be accomplished by interviewing the inhabitants and applicators using structured questionnaire (Annexure 3). A medical practitioner should be associated for collection of data.

2.2.1.1.13. Criteria for selection of dose of insecticide for Phase III trial

The minimum dosage of the insecticide that has the potential to reduce human-mosquito contact as derived and revealed by experimental hut trial or small scale filed trials (Phase II) should be considered for further evaluation. While suggesting the insecticide due emphasis should be given on safety to human and environment.

2.2.1.2. Phase III trial

Duration: Three years

Objectives

- To assess the efficacy of selected application rate on target vector species
- To study the impact on disease prevalence
- To assess the persistence/application (impregnation) intervals of the net
- To assess the handling of ITN/acceptability and collateral benefits
- To study perceived side-effects to inhabitants
- To evaluate sustainability
- To assess environmental impact of old LLINs
- To assess public health impact of old LLINs

Activities (To cover community aspects, logistics and technical- local disposal & reuse)

- Assessment of net status in relation to time (used/damaged/ineffective/expired/retired)
- Multi-centric mode of evaluation in different ecotypes (or perennial transmission areas/high risk/low risk areas)
- Disease prevalence estimation
- Human safety evaluation/ communication strategy development/sustainability to keep coverage
- Technology transfer
- Study on social behaviour
- Effect of combination with IRS
- Impact assessment with other VBDs
- Assessing ethical issues/legal issues
- Final review with collaborators and state health programme personnel
- Operational impact to assess diseases incidence and to disregard development of possible resistance

SOCIOLOGICAL ACTIVITIES:

- Mass campaign for distribution
- Assessment of coverage and need for scaling up,
- Assessment of distribution strategy (free/ subsidized rate/through commercial marketing people)

2.2.1.2.1. Selection of villages

For selection of villages, the section 2.1.4.1 may be referred.

2.2.1.2.2. Census of selected villages

Census should be conducted prior to intervention in the selected experimental and control villages and houses should be numbered and recorded in the format given in Table 26. This is to estimate the number of nets actually required for the trial.

Table 26. Record of census of households

Village..... Sub-centre……..... PHC….....….District…….State…….....
House No. .....……Type of the structure……Date of survey…….....…….....

S. No.	House No.	Name	Relation	Age/ sex	Education	Profes-sion	No. of rooms	Sleeping habits inside/outside	No. of nets required
1.									
2.									
3.									
4.									

2.2.1.2.3. Treatment and distribution of nets

The required number of mosquito nets should be impregnated following the procedure described in Box 8. Informed consent should be obtained from the participants in the trial (Annexure 5). Inhabitants should be educated on the use and storage of nets and instructed not to wash the nets. All the nets should be marked with a water-soluble ink to detect whether the nets are washed or not. Every time the team goes for data collection the marking on the nets should be checked and recorded. To ensure the regular use of nets by the inhabitants, trained volunteers from the same village should be engaged. Compliance of use of nets should be assessed fortnightly and recorded in the format given in Table 27. Sample size for coverage and compliance is given in Table 28

Table 27. Record of compliance of treated nets in households

Village……..Sub-centre…….....PHC……..... District…….State…….
House No.....……Type of the structure……Date of survey……

House code (Arm No./ Insecticide dose)	Date and time of visit	Use of net	
		Yes	No
1.			
2.			
3.			
4.			
5.			

Table 28. Sample size selection based on required confidence levels

Required Sample Size†

Population Size	Confidence = 95% Margin of Error				Confidence = 99% Margin of Error			
	5.0%	3.5%	2.5%	1.0%	5.0%	3.5%	2.5%	1.0%
10	10	10	10	10	10	10	10	10
20	19	20	20	20	19	20	20	20
30	28	29	29	30	29	29	30	30
50	44	47	48	50	47	48	49	50
75	63	69	72	74	67	71	73	75
100	80	89	94	99	87	93	96	99
150	108	126	137	148	122	135	142	149
200	132	160	177	196	154	174	186	198
250	152	190	215	244	182	211	229	246
300	169	217	251	291	207	246	270	295
400	196	265	318	384	250	309	348	391
500	217	306	377	475	285	365	421	485
600	234	340	432	565	315	416	490	579
700	248	370	481	653	341	462	554	672
800	260	396	526	739	363	503	615	763
1,000	278	440	606	906	399	575	727	943
1,200	291	474	674	1067	427	636	827	1119
1,500	306	515	759	1297	460	712	959	1376
2,000	322	563	869	1655	498	808	1141	1785
2,500	333	597	952	1984	524	879	1288	2173
3,500	346	641	1068	2565	558	977	1510	2890
5,000	357	678	1176	3288	586	1066	1734	3842
7,500	365	710	1275	4211	610	1147	1960	5165
10,000	370	727	1332	4899	622	1193	2098	6239
25,000	378	760	1448	6939	646	1285	2399	9972
50,000	381	772	1491	8056	655	1318	2520	12455
75,000	382	776	1506	8514	658	1330	2563	13583
100,000	383	778	1513	8762	659	1336	2585	14227
250,000	384	782	1527	9248	662	1347	2626	15555
500,000	384	783	1532	9423	663	1350	2640	16055
1,000,000	384	783	1534	9512	663	1352	2647	16317
2,500,000	384	784	1536	9567	663	1353	2651	16478
10,000,000	384	784	1536	9594	663	1354	2653	16560
100,000,000	384	784	1537	9603	663	1354	2654	16584
300,000,000	384	784	1537	9603	663	1354	2654	16586

2.2.1.2.4. Insecticide efficacy evaluation

Evaluation for insecticide efficacy should be carried out essentially following the procedure already described in the Phase II (2.2.1.1.6.). Fortnightly bioassays should be carried out throughout the duration of the Phase III. Nets for bioassays should be collected randomly and a minimum of four replicates should be used including control.

2.2.1.2.5. Entomological evaluation

The following evaluation should be done in the experimental and control villages at fortnightly intervals in randomly selected dwellings in corners and centre of the village.

2.2.1.2.5a. Floor sheet collection

For details see section 2.1.3.7.1.

2.2.1.2.5b. Exit window trap collection

For details see section 2.1.3.7.3.

2.2.1.2.5c. Hand catch

For details refer section 2.1.3.7.2.

2.2.1.2.5d. Human landing collection

(Human landing collection may be done if feasible and on obtaining necessary clearance from human ethics committee)

All night mosquito-landing collections are made 10 days prior to distribution of nets, in the 3rd and 6th month after distribution. Collections are made in two houses each in all the selected villages. Employing volunteer human baits inside the net mosquito landing collections are made from dusk to dawn both indoors and outdoors of the selected houses. Informed consent of the volunteers should be obtained before the experiment (Annexure 4). Hourly mosquito collections should be recorded separately in the format as given in Table 15. Results are expressed as number of vectors per bait per night. The observations provide information on biting rhythm and feeding habit of the vector species in the area.

2.2.1.2.5e. Infection and infectivity rates

For details refer to section 2.1.4.7.1.3.

2.2.1.2.5f. Entomological inoculation rate (EIR)

For details refer to section 2.1.4.7.1.4.

2.2.1.2.5g. Blood meal analysis

To determine the feeding preference of vector species, blood smears from abdomen of fed mosquitoes should be made on Whatman No. 1 filter paper. Blood samples numbered species-wise and structure-wise should be kept in plastic bags in desiccators before testing. Blood meal identification should be carried out by counter current immuno-electrophoresis (Bray *et al* 1984) with modifications of Collins *et al* (1986)/ ELISA. Results should be recorded in the following format given table Table 29.

Table 29. Record of blood meal source

Village……..... Sub-centre…..…..…..….. PHC.....….....….....….... District ...…….....
Date of collection ……..... House No. ..... Date of survey .....

S. No.	Date of collection	Species and code	Result of blood meal source*
1. 2. 3. 4. 5.			
*H = Human blood; B = Bovine blood.			

2.2.1.2.6. Disease prevalence

Data on point prevalence of malaria should be generated. Sample blood surveys should be carried out both in experimental and control villages. The frequency and period of the survey should be determined based on the transmission pattern of the disease and in consultation with state health programme department. It is recommended that the first survey should be conducted 30 days prior to the use of nets and subsequent surveys should be conducted every month. Surveys may be carried out following systematic sampling method selecting every 4^{th} or 5^{th} house depending on the total number of households to be selected in each village, which will be proportionate to the population size (PPS) of the villages. Rapid diagnostic kits (RDKs)/ microscopic examination of stained blood smears will be used to screen the inhabitants of the selected houses for malaria infection. The test/ microscopic positive persons will be administered with anti-malaria drugs as per the national guidelines. The health workers of the respective PHC will be involved in the treatment of malaria positive persons. Data should be recorded in the format given in Table 30 a & b.

Table 30. Mass blood survey

a. Data collection sheet

Village…..... Sub-centre…..... PHC…..... District…….....…

Date of collection ……

S.No.	H.No.	HOF	Name of patient	Age	Sex	Blood smear No.	Fever history	Drugs given	Result of smear		
									Pv	*Pf*	Mix
1. 2. 3. 4. 5.											

b. Summary Sheet

Village…..... Sub-centre .....….. PHC .....….. District .....…….State……
Date of collection …… Insecticide & Dose..... Date of spray/round .....….

Part 1

S.No/	Name of Village	HOF	Population					Total
			<1	1–4	5–8	9–15	>15	
1.								
2.								

Part 2

Blood smears collected						Drugs consumed			
<1	1–4	5–8	9–15	>15	Total	4 AQ	8AQ	Paracetamol	Other drugs

Part 3

Positives detected						% blood smears collected	Positives				Remarks
<1	1–4	5–8	9–15	>15	Total		*Pv*	*Pf*	Mixed	Total	

2.2.1.2.7. Significance of indicators

- Hand catch: Relative density of mosquitoes and other non-target insects
- Floor sheet collections: Immediate mortality
- Exit window trap collections: Excito-repellency of the insecticide
- Mosquito human landing collection: Man vector contact
- Infection and infectivity rates: Intensity of transmission
- Entomological inoculation rate: Force/ transmission load in the community
- Disease prevalence: Impact on incidence of malaria infection

2.2.1.2.8. Adverse effects, acceptability of community and collateral benefits

Collateral benefits of the insecticide treated nets should be assessed by interviewing the inhabitants using structured questionnaires for nuisance mosquitoes, bedbugs and human lice (Annexure 6).

2.2.1.2.9. Human safety

This should be accomplished by interviewing the subjects using structured questionnaire (Annexure 3). A medical practitioner should be associated for collection of data.

2.2.1.2.10. Operational acceptability and ease of impregnation

- The impregnated material should get into solution/suspension immediately and remain stable for sufficient period for proper impregnation
- The impregnated material should not cause irritation on contact with skin- Acceptability by community as determined by odour, effect on skin, etc. (as assessed from Annexure 6)
- Effect on non-target household pest insects and collateral benefits (as assessed from Annexure 6)
- Safety to applicators and inhabitants (as assessed from Annexure 3)

2.2.2. Evaluation of New insecticides for Impregnation of Nets

These are the insecticides evaluated for the first time. The specifications of the insecticide compound/ formulation should be provided by the manufacturer/ sponsoring agency together with material safety data sheet for toxicological indices on safety for humans and non-target organisms, particularly against domestic pet animals.

The evaluation should be conducted in three phases - I, II and III. Phase II and III evaluation should be similar as described earlier for WHOPES passed insecticides (For details refer to section 2.1.3 and 2.1.4). Evaluation methods for Phase I are given below.

2.2.2.1. Phase I

2.2.2.1.1. Duration: 6 months.

2.2.2.1.2. Objectives

- To assess efficacy and residual action of the insecticide on given net material
- To determine the effective dosages of the insecticide for net impregnation

2.2.2.1.3. Activities

- Chemical assay of the randomly sampled treated nets
- Cone bioassays on treated net to determine efficacy and residual action

2.2.2.1.4a. Chemical assay

This is required to confirm the target dosage of insecticide in the treated net. The samples for the assay need to be labelled, kept in separate aluminium foils and stored in a cool place, prior

to despatch for analytical laboratory. Care should be taken for correct measurement of area of netting. The weight of the insecticide per unit surface area (ex. Mg/m^2) needs to be calculated.

2.2.2.1.4b. Determination of dosage for impregnation

Dose for impregnation is essentially determined by exposing the laboratory reared 3-day old sugar fed female mosquitoes to treated nets using cone bioassays (Section 2.2.1.1.6.). To minimize the effect of overcrowding, in each cone only 5 females are to be introduced. Two square metre pieces of mosquito net of different fibres namely polyester, nylon, cotton and polypropylene are impregnated following the procedure described earlier (Box 8). These fabrics are impregnated with different concentrations - 5, 10, 15, 20, 25, 30, 40, 50 mg/m^2 (see Box 8). These impregnated fabrics are used to determine the susceptibility status of the vector species. At least two exposures should be made for each dose and a control. Results should be expressed as average of two exposures. Abbott's correction should be made if needed. Data should be recorded in the format given in Table 31. Percent mortalities observed after 24 h holding in exposures are regressed using probity analysis and from the dose mortality regression line dose for 99.9% mortality is determined for the given fabric. This dose can be used for assessing the residual action.

Table 31. Determination of dose for impregnation

Date of Exposure.....……Test species……..... Lab/ F_1/Field collected…….....

Dose (mg/m^2)	**No. exposed**	**Fabric**		**Average % mortality**	**Corrected % mortality**
		R1	**R2**		
5					
10					
15					
20					
25					
30					
40					
50					
Control 1					
Control 2					

2.2.2.1.5. Residual action on mosquitoes

Residual action is determined following the methods described earlier (section 2.2.1.1.6.). Two square metre of the given net/fabric is impregnated with the dose already determined (LC_{99}). Cone bioassays using the target vector species are conducted one day after impregnation and subsequently at fortnightly/ monthly intervals. The bioassays should be carried out during the entire period of study and further for a few more months, if needed. The period of efficacy should give $\geq$80 % mortality and that period should be considered as the minimum period of residual action for the given insecticide on the given fabric.

Phase II and III evaluations are to be performed as described for WHOPES passed insecticides for impregnation of nets (see sections 2.1.3. & 2.1.4.).

2.2.3. Long-lasting insecticide treated nets

The insecticide is incorporated into the fibres of the netting following two techniques (industry treatment), 1. The insecticide is incorporated into its fibres during the manufacturing process and the insecticide diffuses to the surface with temperature and 2. The insecticide impregnated to the net is protected by a chemical (resin) coating and thus these nets are called long-lasting insecticide treated nets (LLIN). The bioavailability of the insecticide on the surface of the net will be sufficient to be lethal to vector mosquitoes for extended periods (4- 5 years). The sponsoring agency should provide LLIN and its specifications.

Duration

Evaluation should be carried out for 20 WHO standard washes or for 3 years.

Objectives

- To evaluate the bio-efficacy of the net in relation to number of washes
- To study the impact of LLIN on disease prevalence
- To assess the collateral benefits of LLIN usage
- To test the social acceptability through coverage and utilization
- Human safety in accordance with increased coverage

2.2.3.1. Laboratory studies

Objective:

- To assess efficacy of LLINs in relation to 20 standard washing

2.2.3.1.1. Impact of washing on bio-efficacy of LLIN

Each net will be washed according to the standard WHO washing protocol in a non-plastic bowl (metal or aluminum) containing 10 litre of water (water from a well or de-chlorinated water with a maximum hardness of 5 dh) and containing 2g/l of soap (Savon de Marseille like). The nets will be subjected to a manual agitation for 3 minutes, left to soak for 4 minutes and re-agitated for 3 minutes (total 10 min). Agitation will be done by stirring the net with a pole at 20 rotations per minute. Nets will be thoroughly rinsed twice in fresh water (10 l per rinsing) and dried horizontally in the shade. Nets will be stored at ambient temperature during the length of the regeneration time (time between two successive washes) before the next wash. Regeneration time for the insecticide used in the LLIN will be provided by the industry/ sponsor.

The nets will be subjected to washing similarly up to for 20 times. Comparison of mosquito mortality should be made between washed LLIN, unwashed LLIN, washed plain net to evaluate the impact of washing on the efficacy of net.

2.2.3.1.2. Cone bioassays

The cone bioassays are to be performed on the nets (minimum four replicates) as per the procedure described in section 2.2.1.1.6 on the next day of washing and until the mortality drops below 80%. The number of washing up to which the mortality was 80% will be recorded. Same nets should be used for repeated washes and bioassays. The data are recorded in the format given in Table 32

2.2.3.1.3. Ring-net bioassays

The Ring-net bioassays will be carried out to determine the median knock down time following the procedure described in section 2.2.1.1.7. The frequency of the bioassay would be similar to the cone bioassays.

Table 32. Impact of washing on the bio-efficacy

Brand..... Date of evaluation..... Date of previous washing.....
Name of interviewer……..

Date*	No. of wash	Mortality after 24 h (cone bioassay)			Median knock-down time (Ring-net bioassay)		
		Washed LLIN	Unwashed LLIN	Washed plain net	Washed LLIN	Unwashed LLIN	Washed plain net
Replicate 1							
Replicate 2							
Replicate 3							
Replicate 4							
* Separate row for each replicate.							

2.2.3.2. Phase II evaluation (Experiment hut trial)

2.2.3.2.1. Objectives

The overall purpose is to determine the efficacy of LLIN washed 20 times relative to unwashed LLIN, conventionally treated net (with the same insecticide at WHO recommended dosage) washed to just before exhaustion and untreated net against susceptible mosquitoes in experimental huts that simulate local domestic habitations.

The primary outcomes measured in experimental huts will be:

- the deterrence (reduction in hut entry relative to the control huts provided with untreated nets);

- induced exophily (the proportion of mosquitoes that will be found in the veranda);
- blood feeding inhibition (the reduction in blood feeding compared with that in the control huts); and
- immediate and delayed mortality (the proportion of mosquitoes that are found killed of the total numbers that entered early morning and after 24 hrs holding alive mosquitoes).

The first three of these outcomes will be indicators of personal protection, and benefit individual users. The fact that blood-seeking females are killed will be also important because community-wide use of treated nets can, in some circumstances, produce a "mass population effect", i.e. a reduction in the density of infective mosquitoes in the area and, consequently, protection of the whole community, including those not using treated nets.

The personal protection effect of a treated net can be estimated by the calculation:

$$\% \text{ personal protection} = 100(B_u - B_t)/B_u$$

Where, B_u = is the total number blood-fed in the huts with untreated nets, and
B_t is the total number blood-fed in the huts with LLIN /treated nets.

The potential mass effect of a treatment can be estimated by the calculation:

$$\text{Mass killing effect } (\%) = 100(K_t - K_u)/T_u$$

Where, K_t is the number killed in the huts with LLIN treated nets,
K_u is the number dying in the huts with untreated nets, and
T_u is the total collected from the huts with untreated nets.

2.2.3.2.2. Methodology

The study will be carried out using the six experimental huts constructed in a village with abundance of the target vector species.

2.2.3.2.2.1. Design of experimental huts

The experimental huts are specially designed for recording the entering and exiting behavior of mosquitoes and for measuring response to insecticides/treated nets including mortality. At the end of the study, the huts can be renovated and used again. For the design of the experimental hut the section 2.1.2.1. may be referred. The number of huts to be constructed will be decided based on the number of arms (minimum four) included in the study and the number of replicates (four to six replicates) for each arm.

For acclimatization and to attract mosquitoes into the experimental hut, an adult volunteer will be enrolled for this purpose and he will sleep under an untreated mosquito net in huts from dusk to dawn for a period of 15 days. Clearance from ethical committee should be obtained to include human volunteers in the study.

2.2.3.2.2.2. Pre-hut trial assessment

Subsequently, the suitability of the experimental huts will be assessed based on the following criteria over a period of one month prior to starting the hut-evaluation.

i. Comparable indoor resting of mosquitoes with village huts:
ii. Tightness of huts (from recovery rate):
iii. Absence of scavengers:

Details of assessment of the three criteria are given in the section: 2.1.2.2.

2.2.3.2.2.3. Maintenance of the huts

The rotation of the arms (test nets) will be done every four to six days. On the 5^{th} or 7^{th} day the huts will be cleaned and ventilated to remove contamination from the nets previously used. The huts are surrounded by a water-filled moat to exclude ants and other scavengers.

2.2.3.2.2.4. Organization of trial

Time Schedule
Hut evaluation will be done over a period of 6 weeks (6 weeks x 6 days) or 12 weeks according to availability of the vector population following a preparatory period as detailed below:

Washing of nets:	05 months
Transportation to field site, acclimatization of experimental huts and assessment of their suitability:	02 months

Experimental Arms
Unwashed and **20 times washed LLINs** will be evaluated in experimental huts for their effects on free-flying, wild mosquitoes and for their ability to deter entry, repel or drive mosquitoes out of houses, induce mortality and inhibit blood-feeding.

Untreated nets will be used as a negative control. WHO approved any other LLIN **washed 0** and **20 times** shall be used as the positive controls. A **conventional ITN** washed to just before exhaustion shall act as a reference net. The point of exhaustion should be determined at the field site by washing the conventionally treated net using the Phase II protocol. WHO cone bioassays will be performed after each wash. The last wash for which the net still causes $\geq$80% mortality or $\geq$95% KD will be considered to be the number of washes required before exhaustion. Normally, the nets will be of the following size: 205-220cm long, 170-175cm wide, 150-155 cm high. The nets and the insecticide will be provided by the industry/ sponsor.

Preparation of nets (This is based on an example of six armed trial):
The nets will be coded (Codes X1, X2, X3, X4, X5 and X6 to indicate the six arms and letter code A, B, C, D, E, and F to indicate the six replicates of each arm, and Y and Z to indicate the additional nets of the arms, Y before any wash, Z washed until just before exhaustion or washed 20 times) by a member of the research team who will not directly be involved in the

evaluation of nets in experimental huts and the codes will not be communicated to the field supervisor and field workers.

Six replicate nets will be used per arm and each net will be tested one night per week; (e.g. X1A, X1B, X1C, X1D, X1E, X1F, in which X1 will be the experiment arm and A, B, C, D, E and F will be its replicates).

2.2.3.2.2.5. Washing:

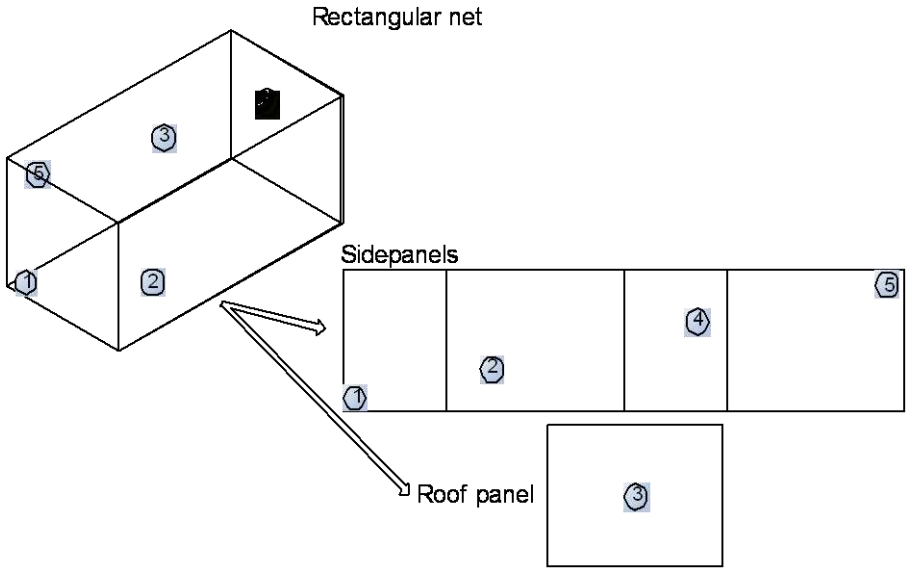
Refer to the section 2.2.3.1.1.

2.2.3.2.2.6. Bioassays

Bioassays and chemical analysis will be performed on the same nets on adjacent pieces of nets. Using the WHO prescribed cones; bioassays will be done on the nets with non-blood fed, susceptible vector mosquitoes. Cone bioassay before any wash and after washing 20 times or washing until just before exhaustion will be conducted. Also, one net per arm will be bio-assayed using non-blood fed susceptible vector species just before the field experiment (one randomly selected net out of the six replicate nets, A, B, C, D, E or F of each arm). One net per arm will be bio-assayed at the end of the field experiment with nets used in the huts (one randomly selected net out of the six replicate nets).

5 x 2 cone tests will be performed per net (on each section of the net: roof and 4 sides) (as shown in the figure below). Five female mosquitoes will be exposed per cone test

Figure 1. Pieces of nettings cut for chemical assay and bioassays



Exposure to net lasts for 3 minutes after which mosquitoes are held for 24 hours with access to sugar solution. KD is measured 60 minutes after exposure time and mortality after 24 hours. Results are pooled for the 50 mosquitoes tested per net

For baseline tests, results of the 5 locations on nets will be analyzed. After washing of nets, data of position 1 on the net will be considered separately and may have to be excluded since net at this position may have subjected to abrasion in routine use.

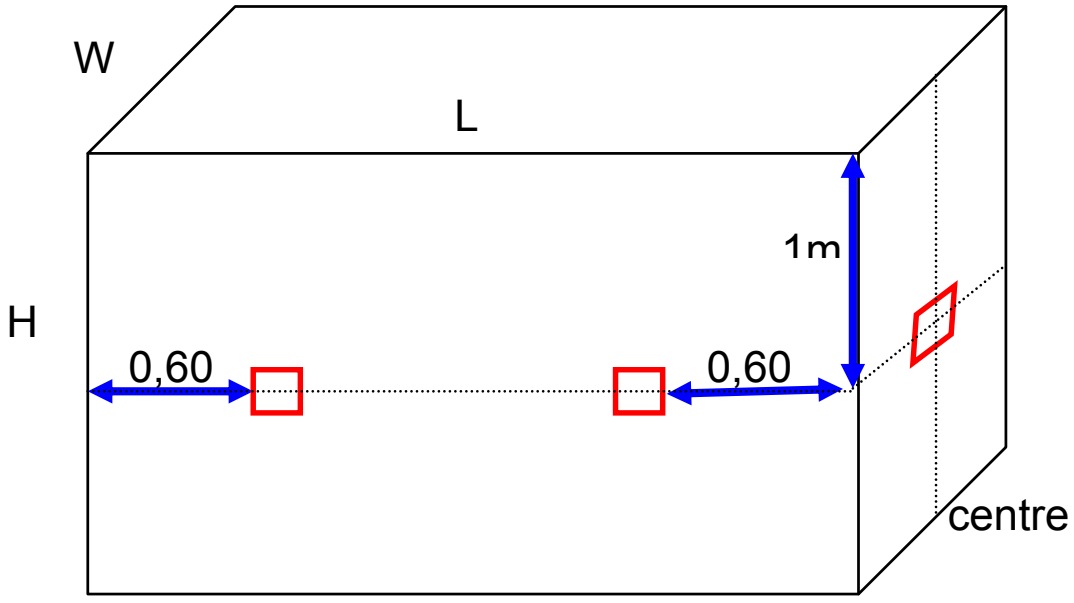
2.2.3.2.2.7. Chemical assays

Prior to any wash, 5 pieces of 30 X 30 cm nettings (i.e. one piece each from positions 1 to 5) will be taken from one of the two additional nets (X1-6Y) of each of the six arms (Figure 1). Similarly, net samples will be obtained after 20 washes or after washing until just before exhaustion. At the end of the experimental hut study, one used net will be sampled in the same ways as described above. The samples will be labeled and packed in aluminum foil and stored at +4^{o} C before sending them for chemical analysis. For chemical assay of washed nets, the net pieces cut from position number 1 will be analyzed separately as explained above.

2.2.3.2.2.8. Procedures for tests in huts

Tests will be done in the six experimental huts; in each hut one net will be used. Holes will be made in all nets of the six arms (six nets per arm) that will be used in the experimental huts to simulate the conditions of a torn net and to put emphasis on testing whether the insecticidal treatment, rather than the net, effectively prevents biting of the sleepers. Six holes (4 cm x 4 cm) will be made in each net, two each in long sides and one each in front and hind ends (Figure 2).

Figure 2. Holes made in nets tested in experimental huts



2.2.3.2.2.9. Procedure to be adopted by the volunteers sleeping under the nets inside the experimental huts

To sleep in the experimental huts, 12 volunteers, two (mostly husband and wife) per hut, will be selected in consultation with local village committee. The teams formed at the start of the study will not be changed unless any volunteer withdraws from the study.

The peripheral blood smears of the volunteers will be collected and examined in laboratory for the presence of malaria parasites. Those having malaria will be treated free of cost before they are included in the study.

The volunteers will be supplied with bedding set for sleeping.

The volunteers enter the experimental huts at 19.00 hours and remain inside until 05.30 hours. Inside the hut, they will sleep under the net assigned to that hut. Each volunteer will be compensated with some honorarium for their time spent in participating in the study. If a volunteer withdraws from the study, s/he will be paid for the period of his/her participation.

In the evening before the volunteers entered the hut for sleeping, white cloth sheets will be spread on the floor of the hut and verandah after cleaning them and the moat around the hut will be filled with water.

Volunteers will be asked not to smoke or to make fire inside the hut.

The treatment arms will be rotated among the huts each week according to a Latin square scheme, which will result in rotation of the sleepers each night to sleep under a different type of net. The volunteers will be asked to report any adverse events associated with use of any net as mentioned in the informed consent form for the net users (Annexure 3) and necessary medical care will be provided.

2.2.3.2.2.10. Rotation of treatments and volunteers

The nets and sleepers will rotate between huts using the Latin Square Rotation scheme (as shown below). The purpose of rotation is to minimize the variation caused by differences in attractiveness of huts (due to position) and sleepers. In practice, sleepers will rotate daily whereas experiment arms weekly.

In the morning, the nets, after collecting resting and dead mosquitoes, will be removed and stored in their corresponding labeled cotton bag.

At the end of each week nets will be removed from the hut. The huts will then be cleaned and ventilated to remove any contamination from the nets previously used. The mat and the beds (labeled according to treatment) will be rotated with the respective arms since they come in close contact with the treated net. The treatment is then rotated to a different hut. The trial should continue for a multiple of 6 weeks to ensure complete rotation through the huts. In most cases, one or two complete Latin squares should be long enough to obtain sufficient numbers of mosquitoes for adequate statistical analysis.

Latin Square ROTATION scheme for experiment arms, nets* and sleepers

Week	Day	Rotation of experiment arms						Rotation of teams					
		Hut H1	Hut H2	Hut H3	Hut H4	Hut H5	Hut H6	Hut H1	Hut H2	Hut H3	Hut H4	Hut H5	Hut H6
1	1	X5	X6	X3	X1	X4	X2	F	C	B	E	D	A
	2	X5	X6	X3	X1	X4	X2	A	E	D	F	C	B
	3	X5	X6	X3	X1	X4	X2	B	F	A	D	E	C
	4	X5	X6	X3	X1	X4	X2	D	B	F	C	A	E
	5	X5	X6	X3	X1	X4	X2	E	A	C	B	F	D
	6	X5	X6	X3	X1	X4	X2	C	D	E	A	B	F
	7	Ventilating, cleaning and washing of hut						No volunteers sleeping inside the hut					
2	8	X2	X4	X6	X3	X1	X5	A	B	E	C	D	F
	9	X2	X4	X6	X3	X1	X5	C	E	D	F	A	B
	10	X2	X4	X6	X3	X1	X5	F	C	A	D	B	E
	11	X2	X4	X6	X3	X1	X5	B	D	C	E	F	A
	12	X2	X4	X6	X3	X1	X5	D	A	F	B	E	C
	13	X2	X4	X6	X3	X1	X5	E	F	B	A	C	D
	14	Ventilating, cleaning and washing of hut						No volunteers sleeping inside the hut					
3	15	X6	X1	X4	X5	X2	X3	A	D	F	E	B	C
	16	X6	X1	X4	X5	X2	X3	D	B	A	C	F	E
	17	X6	X1	X4	X5	X2	X3	B	F	E	A	C	D
	18	X6	X1	X4	X5	X2	X3	E	C	D	B	A	F
	19	X6	X1	X4	X5	X2	X3	C	E	B	F	D	A
	20	X6	X1	X4	X5	X2	X3	F	A	C	D	E	B
	21	Ventilating, cleaning and washing of hut						No volunteers sleeping inside the hut					
4	22	X1	X3	X2	X4	X5	X6	E	C	B	F	A	D
	23	X1	X3	X2	X4	X5	X6	A	E	F	D	C	B
	24	X1	X3	X2	X4	X5	X6	F	B	E	C	D	A
	25	X1	X3	X2	X4	X5	X6	D	F	C	A	B	E
	26	X1	X3	X2	X4	X5	X6	C	D	A	B	E	F
	27	X1	X3	X2	X4	X5	X6	B	A	D	E	F	C
	28	Ventilating, cleaning and washing of hut						No volunteers sleeping inside the hut					
5	29	X3	X5	X1	X2	X6	X4	D	C	B	F	E	A
	30	X3	X5	X1	X2	X6	X4	C	E	A	B	D	F
	31	X3	X5	X1	X2	X6	X4	B	A	F	D	C	E
	32	X3	X5	X1	X2	X6	X4	F	B	E	C	A	D
	33	X3	X5	X1	X2	X6	X4	E	D	C	A	F	B
	34	X3	X5	X1	X2	X6	X4	A	F	D	E	B	C
	35	Ventilating, cleaning and washing of hut						No volunteers sleeping inside the hut					
6	36	X4	X2	X5	X6	X3	X1	B	E	A	C	F	D

37	X4	X2	X5	X6	X3	X1	D	C	F	A	E	B
38	X4	X2	X5	X6	X3	X1	F	B	D	E	A	C
39	X4	X2	X5	X6	X3	X1	C	A	E	B	D	F
40	X4	X2	X5	X6	X3	X1	A	F	C	D	B	E
41	X4	X2	X5	X6	X3	X1	E	D	B	F	C	A

NETS*:	SLEEPERS:
Arm X1, nets A,B,C,D,E,F**	A: Team A – two volunteers
Arm X2, nets A,B,C,D,E,F	B: Team B – two volunteers
Arm X3, nets A,B,C,D,E,F	C: Team C – two volunteers
Arm X4, nets A,B,C,D,E,F	D: Team D – two volunteers
Arm X5, nets A,B,C,D,E,F	E: Team E – two volunteers
Arm X6, nets A,B,C,D,E,F	F: Team F – two volunteers

2.2.3.2.2.11. Collection and processing of mosquitoes

Refer to sections 2.1.2.7 and 2.1.3.7.8.

2.2.3.2.2.12. Statistical analysis

For details of analysis, refer to section 2.1.2.10.

2.2.3.3. Phase II evaluation - (Village hut trial)

Wherever, construction of experimental huts is not feasible, the phase II evaluation may be carried out in the existing village huts with minor modifications with the consent of the respective household heads, following the same procedure as given under experimental hut trial, except that the rotation of sleepers may not be possible as is done in the case of experimental huts as the residents may raise objections to allow outsiders to sleep in their house.

2.2.3.4. Phase III evaluation

2.2.3.4.1. Objectives

- To evaluate the insecticidal activity and fabric integrity of LLIN over 36 months in comparison with conventionally treated mosquito nets using the same insecticide and under the same field conditions;

- To assess washing mode and washing habits of LLIN and ITN by the householders, and

- To assess the community acceptability of LLIN versus conventionally treated mosquito nets.

2.2.3.4.2. Methodology

This will be a prospective study with nets as the unit of observation as well as randomization. Two types of nets can be compared, i.e., LLIN and conventionally treated net of same fabric with the same insecticide (ITN) at WHO recommended dosage.

Insecticidal efficacy, wash resistance and fabric integrity and washing practices of the householders, as well as their perception on adverse effects, if any, will be assessed during the study. The ITNs will be evaluated for at least one year or until they fail to meet the cut-off efficacy criteria[1]. The efficacy of LLIN distributed in the villages will be monitored up to three years of continuous use under the field conditions.

2.2.3.4.2.1. Selection of villages

The LLINs and ITNs to be evaluated will be randomly distributed in 10-12 villages selected on the basis of population size, malaria incidence, accessibility, community use of ITNs/nets and multiple ethnicities in consultation with the State/District Health Authorities. In total, 440 households shall be included in the whole study. Of these, 300 households shall be given one coded LLIN each, and another 140 households shall be given one coded ITN each. This means, 300 LLINs and 140 ITNs shall be distributed in the beginning. All remaining persons in the households shall be given non-coded LLINs. The sample size calculation has been made based on the following criteria according to the study protocol:

The selection of multiple ethnicities for inclusion in the study shall be made in such a way that either the whole community fits in the study size with at least 60 households (30 with each type of net) included in each of the communities; or if the population size in a given community is large, a stratified sampling approach shall be used to select clusters of 60 adjacent households

2.2.3.4.2.2. Census and baseline household survey

A census will be carried out in all the selected villages. Enumeration of all houses will be done and detailed census with the name, age and sex of every family member will be recorded in registers**.** A baseline household survey will be carried out in all the selected villages using a structured questionnaire. Information will be collected on size of the family, educational status, occupation, average family income, type of house, number of sleeping places in a house, availability of nets/ITNs, their usage pattern, washing practices etc. Respondents will be heads of households or their spouses or their representatives. The data recorded in community registers and questionnaires in the field will be transferred into a computer data file in an MS Access format.

2.2.3.4.2.3. Community education and informed consent procedure

[1] *WHO cut off criteria: The conventionally treated nets are considered effective as long as at least 80% of them meet the cut-off criteria for the WHO cone bioassay test, i.e., if mortality in the WHO cone bioassays remains >80% and/or knockdown >95%.*

As part of the community entry activities, the assistance of community leaders and local health workers in the selected villages will be sought; (1) to obtain permission to use the community as a study site, (2) to inform the community members about the purpose of the study, consequent sampling procedure and replacement of sampled net with new ones and (3) to seek community acceptance for use. In addition, community level meetings shall be organized to educate all the people in the selected villages on the adverse consequences of malaria, the benefits of using treated/long-lasting nets, correct handling and use of nets in line with WHO recommendation[2] and the need for reporting any adverse events, if any, and to seek their support in successful conduct of the study.

Written Informed consent will be obtained from all heads of households to be enrolled in the study at the time of census survey when all potential households will be visited by a team of investigators before distribution of LLINs/ITNs (refer section 2.2.3.4.2.2.). A draft consent form is attached vide (Annex 5). To obtain informed consent of illiterate people, the informed consent form shall be read and explained by a member of the investigating team in local language in the presence of a community witness. Upon their consent, such people will be asked to mark a thumb impression on the form and the witness will be asked to sign. The participants shall be informed of possible benefits of sleeping under treated nets. They will also be made aware of expected adverse events during the initial few days of using such nets and which may include one or more of the following: itching of skin, facial burning/tingling, paraesthesia (numbness or a loss of physical sensation and/or tingling of skin), sneezing, liquid discharge from nose, feeling of headache, nausea, eye irritation and tears, experience of bad smell, body rashes etc. They will be told that based on previous experiences such events are usually transient in nature, however, they may contact a member of the research team or a physician at a local health facility just in case there is a need for medical attention. They will be advised to report on all such events to a team member for record.

Any potential participant who refuses to participate in the study will be advised to seek medical care at the nearest health facility upon observing any signs or symptoms of malaria. Alternative participants will be recruited in the study. Further, the initial sample size of the study will be adequate enough to take care of the mid-course withdrawals.

2.2.3.4.2.4. Withdrawal of participants

If at any point of time during the study a participant decides not to participate any further, he/she will be allowed to do so. All such participants withdrawing from the study will be allowed to retain their net.

Record of all such participants will be kept separately and their data will be eliminated in the final analysis of study outcome.

The requirement of nets will be estimated considering the number of nets needed for baseline assays, replacements at the time of withdrawal of these nets for bioassays/ chemical assays,

[2] *WHO (2002). Instructions for treatment and use of insecticide-treated mosquito nets. WHO Document WHO/CDS/WHOPES/GCDPP/2002.4 available on WHO Internet at http://whqlibdoc.who.int/hq/2002/WHO_CDS_RBM_2002.41.pdf*

and as an exit strategy for the ITN group (i.e., to provide LLIN to all ITN households whose nets have worn out or lost).

The nets shall not be pre-washed for their conventional treatment.

Volunteer adult males will be recruited locally for the study for treatment of mosquito nets. They will be given training on appropriate method of impregnation of nets and observance of safety precautions. Their informed consent shall be obtained using an informed consent form developed following the WHO guidelines (Annexure 1). They will also be told that proper use of safety equipment will prevent/minimize adverse events during impregnation of nets as mentioned in section 2.2.3.4.2.3. and that the project will ensure medical care should such a need arises. They will be provided with adequate protective equipment (gloves, face mask, goggles) and bath soap for washing and bathing at the end of day's work.

Treatment of nets will be supervised by one of the investigators. Nets will be treated individually in basins using the required quantity of the insecticide at the recommended dosage. The net to be treated will be unfolded and put into the treatment basin with the insecticide solution that has been prepared. The dipped net will be turned thoroughly in the solution for at least 2 minutes, removed and allowed to drip over the basin but not wrung. The treated nets will be dried lying flat on the ground.

2.2.3.4.2.5. Distribution of LLINs/ITNs

A random list of ID numbers required for the study according to the sample size will be produced by the statistician in SPSS to allocate to LLIN or conventional ITN. After random allocation of ID number to the two groups of nets (LLIN and ITN), the ID numbers would be written with wash-resistant ink on a piece of polyester band fixed on each net. In addition, these bands on each net will also be marked with a water-soluble ink as a quality control for the assessment of washing. After the ID numbers had been fixed on nets, all nets will be re-sorted in ascending order of ID number making it impossible to identify the type of nets by the field workers or study participants. Only the Principal Investigator will have the net master list to ensure that neither the distribution team nor the participant villagers know whether the net is an LLIN or ITN. The net master list will inter alia include the following information: ID number, type of net, household number, and dates of net distribution, sampling and replacement, if any. LLIN or a conventional ITN will then be distributed to households in each of the selected villages. This information that the nets have been marked with water-soluble ink as well as the purpose of such marking will also be provided to the participants in the interest of transparency. People will be asked not to remove the ID labels from the nets. The number of nets for each household will depend on the household size and sleeping places in order to obtain full community coverage with an LLIN/ITN and obviate the need of other vector control interventions in the village. The nets will be given free of charge to all households.

At the time of distribution of ITN/LLIN, every headperson of the household will be informed about the need for reporting adverse effects, if any, after using the nets, as well as their appropriate use and maintenance. This procedure will be repeated every time a net was

withdrawn for laboratory assays and replaced with a new one, as well as at the end of the study during the dissemination meetings.

2.2.3.4.2.6. Sampling of LLINs/ITNs

A. Chemical assays

Samples of the LLIN, as well as samples of the conventionally treated nets, will be subjected to chemical residue analysis in a recognized laboratory. To ensure that the target dose of the insecticide has been achieved, netting pieces will be cut at the beginning of the trial for baseline assays. Thereafter, netting pieces will be cut when ITNs get exhausted or at the end of one year, whichever is earlier, while pieces of LLIN will be cut at the end of year 3 or when they fail to meet WHOPES criteria.

Thirty nets from each research arm will be destructively sampled randomly at base line (at week 1) for chemical residue analysis and at the end of the study. This will require selecting 30 each of ID numbers from LLIN and ITN groups plus 2-4 extra numbers as possible replacements in case the selected participant could not be reached or the net had been lost to follow-up since the last visit. For the chemical assay, sampling should be performed according to the scheme given in Figure 1.

Thus, from each of the 30 sampled nets, four rectangular pieces of 30 cm x 30 cm size will be cut from positions 2, 3, 4 and 5 using sharp scissors.[3] The sub-samples will be rolled up and placed in new, clean and labelled aluminium foil for storage at $+4^0$C temperature prior to dispatch to the laboratory for the chemical assay. In the testing laboratory, the four sub-samples of each net will be assembled as one sample for chemical analysis. The results will provide the average target AI content of the insecticide in the LLINs or ITNs under evaluation.

B. Biological assays

Insecticide susceptibility tests of wild mosquito samples

Blood fed and gravid females of the target vector species will be collected from houses in the study sites using aspirators and maintained in the laboratory to lay eggs that will be used for rearing F_1 progeny for susceptibility tests. The laboratory reared 2 to 5 days old; non-blood fed F_1 females will be used for the insecticide susceptibility test at diagnostic concentrations to determine susceptibility level using the standard WHO kits (tubes) (WHO 1998) for the candidate insecticide.

Insecticide efficacy evaluation

[3] *For chemical assays, the sampling will be done from positions 2, 3, 4 and 5 only. Sample from position 1 will not be taken since netting fabric at this position is subjected to excessive abrasion in routine use (this portion of net is frequently manipulated while tucking the nets under the bed/mattress).*

The standard WHO procedure (cone bioassay) will be used for evaluation of insecticidal effect of ITN/LLINs (WHO, 2005a). Accordingly, at the start of the studies and every 6 months thereafter, 30 nets of each type (LLINs/ITNs) will be randomly drawn from the net master list by the principal investigator and used by the research team for collection of samples for cone bioassays.

To obtain a good representation from each net, five samples (25 cm x 25 cm) will be cut from each of the 30 randomly selected LLINs/ ITNs from positions 1 to 5 as shown in Figure 1 and used for the bioassays. Pieces will be cut as rectangles using sharp scissors. Bioassays will be done using cones on all the five pieces. On each netting sample, standard WHO cone will be placed and held in place using a plastic manifold. Five laboratory-bred 2 to 5 days old; non-blood fed F1 progeny of adults (fully susceptible to the candidate insecticide) collected from the study sites will be introduced into each cone and exposed for 3 minutes. The test will be done twice on each of the five netting samples cut from a net the same or the next day. Thus, in all 50 mosquitoes will be exposed on each net. Thus, altogether 1500 mosquitoes (30 nets x 5 positions per net x 5 mosquitoes per piece x 2 replicates) will be exposed.

After the exposure, the mosquitoes will be removed gently from the cones and kept separately in plastic cups provided with cotton-wool moistened with 10% glucose solution. Knockdown will be recorded after 60 minutes and mortality after 24 hours. Mosquitoes exposed to untreated nets will be used as controls. The bioassays will be done at 27±2 °C and 80±10% RH. Data will be recorded in a structured form for further analysis.

The bioassays will be done once at the start of the study as explained above, and every 6 months thereafter up to 3 years. Bioassays on ITNs will be done up to 1 year or until the biological activity declines below the recommended level (this will require a number of bioassays within the first year to determine when nets give knockdown rate of <95% and or mortality <80%). Nets selected randomly and withdrawn from a household for destructive sampling will be replaced with a new LLIN and the household will be excluded from the study for future sampling. Data for position 1 will be analyzed separately from those of other positions (no. 2 to 5) considering that netting at position 1 is subject to excessive abrasion in routine use. If there are significant variations in bioassay results, mean results of positions 2 to 5 will be used.

LLINs or ITNs which cause a knockdown rate of <95% and a bioassay mortality of <80% will be subjected to a tunnel test.

Tunnel test - The tunnel test will be carried out in the laboratory, by releasing non-blood fed female anopheline mosquitoes, aged 5–8 days, in a tunnel (square section 25 cm x 25 cm) made of glass, 60 cm length (WHO, 2005b). At each end of the tunnel, a 25-cm square mosquito cage covered with polyester netting is fitted. At one third of the length, a disposable cardboard frame is placed with the LLIN netting sample. The surface of netting "available" to mosquitoes is 400 cm^2 (20 cm x 20 cm), with nine holes each 1 cm in diameter: one hole is located at the centre of the square; the other eight are equidistant and located at 5 cm from the border. In the shorter section of the tunnel, a small rabbit used as bait will be placed, which will be unable to move but available for biting. In the cage at the end of the longer section of

the tunnel, 100 female mosquitoes will be introduced at 18:00. The following morning at 09:00, the mosquitoes will be removed by using a suction glass tube and counted separately from each section of the tunnel and mortality and blood feeding rates will be recorded Annexure 7).

During the test, tunnel will be placed in a place maintained at 27 °C ± 2 °C and 80% ± 10% relative humidity under subdued light. Several tunnels will be used simultaneously, one tunnel with untreated netting always being used as a negative control. Blood feeding inhibition will be assessed by comparing the proportion of blood-fed females (alive or dead) in treated and control tunnels. Overall mortality will be measured by pooling the mortalities of mosquitoes from the two sections of the tunnel.

Results of the cone and tunnel tests will be considered together to judge on LLIN performance. A candidate net will be deemed to meet the requirements of a LLIN if at the end of the study period of 3 years, at least 80% of sampled nets will retain bio-efficacy based on WHO cone bioassay and/or the tunnel test as detailed in the WHO guidelines.[4] If mortality in mosquitoes exposed to such 3 year old LLINs in the WHO cone bioassays falls below 80% and knockdown falls below 95%, nets will be tested using the tunnel test.

As blood-feeding in controls has a considerable impact on mortality in the presence of treated samples (i.e. the host-seeking behaviour increases the chance of contact with treated fabric), a minimum cut-off value of blood-feeding rate in controls will be established for tunnel tests.

2.2.3.4.2.7. Impact on disease prevalence

Refer to section 2.2.1.2.6.

2.2.3.4.2.8. Physical inspection of nets

The physical integrity of the net (e.g. size and number of holes) to be sampled will be determined by draping the nets over a frame and counting the number of holes and estimating their sizes according to the location on the net (top, upper side, lower side). The data will be recorded as per the questionnaire given in Annexure 8.

2.2.3.4.2.9. Assessment of community acceptance and practices

A team of experienced field staff will be trained on administering the questionnaire survey. Following assessments will be made:

a. An assessment of adverse effects, if any, of ITNs among the net impregnators will be made using a questionnaire (Annexure 9). All the LLIN and conventional net impregnators will be

[4] *Bio-efficacy criteria:*
Cone bioassay: criteria for acceptance- mortality in mosquitoes >80% and/or
Knock down >95%.
Tunnel Test: mortality in mosquitoes $\geq$80% and blood feeding inhibition $\geq$95%.

interviewed at the end of the day of impregnation of mosquito nets, again in the following day morning and one week after completing the impregnation work.

b. An assessment of adverse effects, if any, among ITN/LLIN users will be made using a questionnaire given in Annexure 10 during the periodic surveys. The Principal Investigator will select 30 ID numbers from LLIN group and another 30 from the ITN group from their respective master lists using a random selection procedure. The list of selected nets will be re-sorted in ascending order and given to the field team who will visit the study area one week and one month after distributing the nets to record perception of the participant users and to record any adverse effects. In addition, any such events reported proactively by the participants to the research team shall also be recorded and analyzed.

c. At the end of months 1, 6, 18 and 30, an adult householder in 30 each of selected households will be interviewed by door-to-door visit to assess net utilization pattern/frequency of use (including early morning observations), method and number of washes and type of detergent used and physical integrity of the net (size and number of holes) as per the questionnaire vide Annexure 8. Since interview assessment of washing frequency may not always be reliable, another net in the household, or a net in a neighboring household, will be marked with a water-soluble marker and the household revisited one month later to obtain a more accurate assessment of the washing frequency in the community.

b. Annual surveys (at the end of months 12, 24 and 36) of all the included households with remaining code LLINs/ITNs will also be conducted by visiting them door-to-door to record physical presence/absence and fabric integrity of the nets, where applicable, to estimate the annual attrition rate, besides information on people's perceptions and practices as mentioned above including any adverse effects observed. Questionnaires given in Annexure 8 and 10 will be used for these surveys too.

2.2.3.4.2.10. Interpretation of results / termination of the study

Each year, a formal report will be prepared and reviewed to take a decision on whether or not to continue the study for the next year. The decision will be made based upon the performance of the product in the field. If mortality in the WHO cone bioassays fall below 80% and or knockdown falls below 95%, nets will be tested in the tunnel test[5]. If mortality in the tunnel test falls below 80% and blood feeding inhibition falls below 95%, the net will be considered to have failed to meet WHOPES criteria. If >20% of the nets sampled fail to meet WHOPES criteria, the study will be stopped.

2.2.3.4.2.11. Ethical clearance and considerations

The study will involve the ethical issue of protecting people's rights, possible inconveniences caused to them and protecting infringement of privacy of women during the study and more specifically during census and sociological surveys. The survey teams will include a

[5] *Bioefficacy criteria: Cone bioassay: criteria for acceptance- mortality in mosquitoes ≥80% and/or knockdown ≥95%.*
Tunnel Test: mortality in mosquitoes ≥80% and blood feeding inhibition ≥95%.

sociologist and a woman health worker to ensure that no infringement on human right occurs during the survey.

The study will not involve experimental use of animals. If it is necessary to conduct tunnel test to assess inhibition of mosquito feeding through LLIN/ITNs, rabbits will be used and they will be given due care as per standard practices. Also, the necessary ethical clearance will be obtained from the Ethics Committee of the respective institution.

Note: Any household who withdraws from the study would be allowed to keep their LLIN/ITN.

2.2.3.4.2.12. Data entry and analyses

Data entered into the computer will have only codes of households and not names such that they are not easily identifiable to the study subjects by the data entry clerks. Furthermore, questionnaire data will be analyzed by inferential statistics (e.g. chi-square) to compare variables obtained. All information related to the participants will be kept confidential. The identity of the individual participant will not be revealed in any reports or publications resulting from the study.

Using the data obtained through questionnaire, community acceptance of LLINs (use rate, perceived benefits in malaria control, any adverse effects, washing and upkeep practices) and attrition rate will be assessed.

Data on adverse effects reported by the impregnators and users of LLINs and ITNs shall be separately analyzed and reported.

Results of the insecticide susceptibility tests (bioassay tests using WHO tube test and cone tests) will be analyzed for dose/response relationships (probit analysis) by the Maximum Likelihood method (Finney 1971). The differences in mortalities will be compared between LLIN and conventional ITN using the χ^2 statistic.

Using data of the tunnel test, blood feeding inhibition will be assessed by comparing the proportion of blood fed females (alive or dead) in treated and control tunnels. Overall mortality will be measured by pooling the immediate and delayed (24-hour) mortalities of mosquitoes from the two sections of the tunnel and the data will be interpreted using the criteria mentioned in section 4.2.10.

Data on chemical analysis will be analyzed to give average target concentration of the insecticide in each LLIN/ITN.

2.2.4. Insecticide incorporated plastic sheetings & fabrics

Recently many products are being developed for protection from mosquito bites in complex emergencies. The insecticide is incorporated into the fibres of the fabric/plastic sheeting and the insecticide constantly diffuses out. The bioavailability of the insecticide on the surface of the fabric/sheet will be sufficient to be lethal to vector mosquitoes for extended periods. These are generally used in temporary habitations (tents) such as refugee camps, rehabilitation colonies, temporary shelters, etc. as tents.

Duration

Total duration of the trial is 12 months.

Objectives

- To evaluate the bio-efficacy of the fabric/plastic sheet
- To study the impact of net/sheet on protection from mosquito bites
- To assess the collateral benefits
- To test the social acceptability
- Human safety and usage safety

The evaluation should be done in the laboratory and field simultaneously.

2.2.4.1. Laboratory evaluation

Assessment of efficacy to vectors is to be done by cone bioassay tests. Cone bioassays should be performed on Day 1 and at fortnightly intervals following the procedure described in section 2.2.1.1.6. Plain fabric/plastic sheeting of the given material is to be taken as control.

2.2.4.2. Field evaluation

2.2.4.2.1. Duration: 18 months preferably during transmission seasons.

2.2.4.2.2. Objectives

- To assess the efficacy on prevailing disease vectors
- To study the impact on disease prevalence
- To assess the efficacy/bio-availability of the fabric
- To assess the acceptability and collateral benefits
- To study safety of the fabric/sheeting (human safety and fire safety)

2.2.4.2.3. Activities

The activities are similar as described for LLIN in 2.3.2.1

2.2.4.2.4. Selection of localities and preparation of tents

Three localities having good vector productivity such as temporary habitations, slums, rehabilitation colonies, tribal hamlets, cantonment bases in remote areas should be selected. A minimum of six tents (6 x 5 x 2 m) should be laid at a distance of 5 metres with the given fabric/plastic sheeting. Control tents should also be prepared in the same locality or a different locality near-by with plain sheeting/fabric of the same material. Exit traps should be fixed to the nets.

2.2.4.2.5. Entomological evaluation

Evaluation should be done in the experimental and control tents at fortnightly intervals in randomly selected tents. Following evaluations should be done.

2.2.4.2.5a. Floor sheet collection

For details see section 2.1.3.7.1.

2.2.4.2.5b. Exit trap collection

For details see section 2.1.3.7.3.

2.2.4.2.5c. Hand catch collection

For details see section 2.1.3.7.2.

2.2.4.2.6. Disease prevalence

Disease prevalence studies should be carried as mentioned in section 2.2.1.2.6. of ITN.

Hand catch: Relative density of mosquitoes and other non-target insects

Disease prevalence: Impact on incidence of malaria infection

Interpretation of data should be made as described in previous sections

2.2.4.2.7. Significance of indicators

Hand catch: Relative density of mosquitoes and other non-target insects

Floor sheet collection: Immediate mortality
Exit window trap collection: Excito-repellency
Disease prevalence: Impact on incidence of malaria infection

2.2.4.2.8. Adverse effects, acceptability by the community and collateral benefits

These will be assessed by interviewing the inhabitants using structured questionnaires for nuisance mosquitoes, bedbugs, human lice, etc. (Annexure 6).

2.2.4.2.9. Human safety

This should be accomplished by interviewing the subjects using structured questionnaire (Annexure 6 two sets of questionnaire, one during the time of distribution and another at the end of third year of LLIN distribution). A medical practitioner should be associated for collection of data.

Experimental hut

2.3. Space spraying

Space spraying is the dissemination of small particles (less than 30 μm) that will remain airborne sufficiently long (usually not more than 30 minutes) to make contact with the target species (WHO, 2010). The aim of space treatments is to rapidly reduce populations of flying insect pests and vectors. Because this type of treatment is not intended to leave a residual deposit, it involves a very low dosage of insecticide, but more frequent applications are usually needed to control the emerging adult populations. Space spraying is one of the options for the control of vectors, especially of dengue and malaria and also it is commonly used in public health pest control programmes against nuisance mosquitoes and flies.

The common protocol, revised based on the WHO Guidelines (WHO, 2009), provides precise and standardized procedures and criteria for testing efficacy and evaluation of insecticides for indoor and outdoor space spray applications against vectors and pests of public health importance. The aim of the common protocol is to ensure a uniform evaluation of products for space spraying by different laboratories and institutions in order to generate comparable data for registering and labeling such products by national regulatory authorities, who however, determine the requirements for registration of pesticides (WHO, 2009).

The protocol for evaluation of space spraying (indoors as well as outdoors) provides procedures on laboratory studies (assessment of efficacy), small scale field testing (dosage determination for field trials) and operational trials (evaluation of effectiveness in operational settings). Although, most examples provided refer to mosquitoes, with some modifications the protocol can be used to determine efficacy against other flying vectors and pests of public health importance. Any operational trial of products should be preceded by laboratory studies and small-scale field testing that have been conducted in a field or room, depending on whether the treatment is intended for outdoor or indoor use (WHO, 2009).

Although, the studies proposed to be conducted following the common protocol may provide some information on safety and toxicity, it is presumed that preliminary eco-toxicity and human safety assessments have been undertaken before any field study is carried out; the protocol does not have provision to generate and analyze any such additional data in a detailed manner. However, any perceived side-effects observed during application and use in laboratory studies and field trials should be reported.

Material Safety Data Sheet (MSDS), the labeling recommendation and the manufacturer's certification that the product is within the company's manufacturing specifications for that product should be made available with the products submitted for laboratory and/or field testing. Independent physical and chemical assessments may be required before initiating the efficacy studies (WHO, 2009).

National ethical regulations should be followed while conducting trials on insecticides, and, if applicable, the necessary human and animal ethical clearance should be obtained from the respective authorities/ Institutional Committees before undertaking any such trial. The WHO guidelines for development of an informed consent form are provided in Annexure 1.

NVBDCP recommends ULV fogging both indoors and outdoors to mitigate mosquitoes and other disease vectors. It has the potential to be effective against peri-domestic breeding vectors. The effectiveness of fogging depends on dosage, droplet size of the aerosol [1–50 µm volume mean diameter (VMD)], and flight activity of the mosquito.

2.3.1. Phase I trial (laboratory studies)

Objectives

General

- To determine the intrinsic activity of the insecticide
- To study its efficacy and
- To assess cross-resistance with commonly used insecticides.

Well-characterized susceptible and/or resistant strains of laboratory-reared mosquitoes are used under standardized and controlled conditions to characterize the inherent properties of the insecticide, mainly by using technical material, and to determine its efficacy for comparative purposes as well as for planning field studies (WHO, 2009).

Specific

- To establish dose–response line(s) and determine the lethal dosage (LD) of the insecticide for 50% and 90% mortality (LD_{50} and LD_{90}) that allow assessment of the intrinsic activity of the insecticide against adult females of susceptible mosquito species
- To determine the lethal concentration (LC) of the insecticide (technical material and formulations) for 50% and 90% mortality (LC_{50} and LC_{90}), as determined by contact with insecticide spray
- To establish a diagnostic concentration for monitoring resistance to the insecticide in the field
- To assess cross-resistance with commonly used insecticides.

Requirements / stipulations

- Standardized mosquito rearing and laboratory testing facility (generally temperature 27 + 2 Co, relative humidity (RH) 80+10%, and photoperiod 12:12 hours- light: dark) to ensure reliability and reproducibility of data
- Adults are maintained on sugar solution (10% on cotton wool) and are non-blood-fed.
- Representative of all three mosquito genera (Anopheles, Aedes and Culex) are desirably to be tested, but in particular Aedes aegypti and Culex quinquefasciatus.
- Strains of test species may have different responses to given insecticides and should therefore initially be characterized by the intrinsic insecticidal activity to determine the level of response.

- Inclusion of a positive control, i.e. a standard insecticide with historical data, is also desirable.

2.3.1.1. Intrinsic insecticidal activity

2.3.1.1.1. Objective

- To determine the intrinsic activity of an insecticide to a target species

This is done by the topical application of an active ingredient to isolate toxicity from confounding effects resulting from insect behaviour.

For detailed methodology and data analysis the 'WHO Guidelines for efficacy testing of insecticides for indoor and outdoor ground-applied space spray applications (WHO/HTM/NTD/WHOPES/2009.2)' may be referred.

2.3.1.1.2. Method of testing intrinsic insecticidal activity

- To prepare topical solutions, technical grade insecticide is dissolved in acetone, which is a highly volatile organic solvent and has the advantage of remaining on the insect cuticle for only a short period of time.
- The doses used in topical application are expressed in nanograms of active ingredient per mg of body weight of live mosquito.
- Usually, 50 non-blood-fed susceptible female mosquitoes of the target species are weighed initially to determine the average live-weight.
- A constant volume of 0.1 µl should be delivered to the pronotum using an appropriate hand-held or automatic pipetting device. Larger volumes may cause increased mortality due to solvent toxicity.
- A total of 50 susceptible, non-blood-fed, 2–5 day-old female mosquitoes are used at each concentration, with at least five concentrations covering a range of mortality from 10% to 90%.
- A few mosquitoes at a time are lightly anaesthetized with CO_2 for 15–30 seconds and placed on a plate cooled to 4 °C to maintain anesthesia during the manipulations.
- A volume of 0.1 µl of insecticide solution of the calculated concentration is deposited on the pronotum as described above.
- Two batches of 25 females are used for each concentration of insecticide. Two batches of 25 females treated with 0.1 µl of pure acetone serve as controls.
- After dosing, the females are transferred into clean holding cups and provided with 10% sugar solution on cotton wool and held for 24 hours at 27 $\pm$ 2 °C temperature and 80 $\pm$ 10% RH.
- Mortality is recorded 24 hours after the topical applications.
- Three replicates from separately reared batches are tested and the results pooled for statistical analysis. Thus a minimum of 900 mosquitoes are required for this study.
- Fresh insecticide dilutions should be prepared for each new test replicate.
- The relationship between dose and mortality is analyzed using log-dose probit regression. Dosages giving responses between 10% and 90% are used for this analysis,

preferably 2–3 dosages below 50% and 2–3 above 50%. Commercial software is now available to compute estimates of the LD_{50} and other LD values and their 95% confidence limits (Annex 2). If mortality exceeds 20% in the control batch, the replicate is rejected. If mortality in the controls is between 5% and 20%, results with the treated samples are corrected using Abbott's formula:

$$\text{Mortality (\%)} = \frac{X - Y}{100 - Y} \times 100$$

Where

X = percentage mortality in the treated sample and
Y = percentage mortality in the control.

- A log–probit analysis should be performed for candidate and control insecticides and their slopes compared using a chi-squared parallelism test.
- Results of two series of assays are considered as not significantly different if the slopes of their log–probit lines are the same (i.e. null hypothesis of the parallelism test is not rejected) and the confidence intervals of their LD_{50} overlap.

2.3.1.2. Insecticidal activity of active ingredient(s) used as space sprays

2.3.1.2.1. Objective

- To assess the efficacy (LC_{50} and LC_{90}) of the insecticide or its formulation against the target vector/ pest

In this test, the target species is exposed to the test concentrations of the atomized insecticide in a 'Wind tunnel'.

The methodology follows the WHO Guidelines (WHO/HTM/NTD/WHOPES/2009.2)

- A total of 50 susceptible, non-blood-fed, 2–5 day-old female mosquitoes are used at each concentration, with at least five concentrations causing a range of mortality from 10% to 90%. A minimum of 900 mosquitoes are required for this test.

- In each application, duplicate cages of 25 non-blood-fed female mosquitoes are exposed to one of the test concentrations of the atomized insecticide in a **wind tunnel** (see Annex 3 for equipment specifications, maintenance and procedural details).

2.3.1.2.2. Description of 'Wind tunnel'

The apparatus consists of a cylindrical tube (15.2 cm in internal diameter) through which a column of air moves at 2.9 m/s. The mosquitoes are confined in a rimless cylindrical screen cage (mesh openings 1.22x1.60 mm and 0.28 mm diameter wire) made to the exact interior measurements of the wind tunnel. The cage is inserted into an opening 91.4 cm from the wind tunnel entrance; a flexible clear plastic sheet is used to close the opening (WHO, 2009).

- The technical insecticide in an acetone solution (0.5 ml total volume) is atomized through a nozzle (that takes approximately 3 seconds) to produce droplets with a Dv0.5 (the Dv0.5 represents the point at which half the volume of droplets is smaller and was formerly designated as the volume median diameter (VMD); see Annex 9 for a full description) of 15 ± 2 µm at the position of the cage.
- Mosquitoes are left in the wind tunnel for a further 5 seconds. After each test, the mosquitoes are lightly (15–30 seconds) anaesthetized with CO_2 and transferred immediately into clean holding cups provided with 10% sugar solution on cotton wool and held for 24 hours at 27 ± 2 °C temperature and 80 ± 10% RH. Mortality is recorded after 24 hours.
- With each insecticide test, control testes are conducted using acetone alone as the diluent, always at the beginning of the test. Tests should begin with the lowest dose and then proceed in turn with increasing concentrations.
- The wind tunnel is cleaned with a 0.5 ml spray of acetone between each series of concentrations.
- Three replicates from separately reared batches are tested and the results pooled where appropriate (to a total of three duplicated applications per concentration) for statistical analysis.
- The relationship between concentration and mortality is analysed using log-dose probit regression, as discussed above, and the LC_{50} and LC_{90} values are reported.
- Ideally, five concentrations producing mortality range between 10% and 90% are needed for analysis, 2–3 below 50% and 2–3 above 50%. If mortality exceeds 20% in the control batch, the test is rejected. If mortality in the controls is between 5% and 20%, the results with the treated samples are corrected using Abbott's formula.
- In addition to assessing the efficacy of an insecticide, the same procedure can be used to test formulations developed for space treatments. However, a different nozzle may be required to achieve the appropriate droplet size. Wind tunnel testing is not suitable for most high volume thermal fog formulations.

2.3.1.3. Diagnostic concentration for monitoring resistance

It is essential to determine the diagnostic concentrations (or dosages) (also referred to as discriminating concentrations/ dosages) of an insecticide for detecting or monitoring the presence of resistance in field populations of mosquitoes.

- It is determined by exposing mosquitoes (tarsal contact) to insecticide deposits on filter-paper.
- The WHO-recommended diagnostic concentrations for each group of vectors are chosen so that exposure for a standard period of time (usually 1 hour) followed by a 24-hour holding period can be relied upon to cause 100% mortality of individuals of susceptible mosquito strains.

- To avoid spurious reporting of resistance in the field where none may exist, WHO sets the diagnostic concentration at twice the minimum concentration that kills 99.9% of mosquitoes of the susceptible strain.

Determination of diagnostic concentrations is done with a graded series of dosages of insecticide (technical grade) applied to sheets of filter-paper. For detailed methodology to determine diagnostic concentration, refer to the WHO Guidelines (WHO/HTM/NTD/WHOPES/ 2009.2).

WHO-recommended discriminating concentrations of insecticides for adult mosquitoes are available on the WHO homepage on the Internet at http://www.who.int/whopes/resistance/en/.

2.3.1.4. Cross-resistance to other active ingredients/ insecticides

Cross-resistance (normally required for novel active ingredient(s)) of an active ingredient against known resistance mechanisms should be tested against well-characterized susceptible and resistant strains.

The resistant strains should preferably be homozygous for the selected resistance mechanism. If homozygosity cannot be achieved, periodic selection is usually necessary to prevent natural selection for susceptibility alleles from causing decline of resistance (WHO, 2009).
Reference strains should be monitored at least twice a year by bioassays or biochemical and/or molecular assays so that any reversion in resistance can be detected, assessed and corrected by selection.

Comparison of the values obtained with a susceptible mosquito strain with those from distinct resistant strains (particularly the LD_{50}) gives a good estimation of the existence and level of cross-resistance of the new candidate insecticide (resistance ratio RR_{50} and RR_{95}). Cross-resistance is indicated if the LC_{50}/LD_{50} or LC_{95}/LD_{95} of a strain carrying a particular resistance mechanism is significantly greater than that of the corresponding susceptible strain (WHO, 2009).

2.3.2. Phase II trial (small-scale evaluation)

The specific objective of the small scale trials is to determine the efficacy and optimum application dosage of the formulated space spray product under relatively controlled and comparable settings (WHO, 2009).

- Small-scale field testing and evaluation of insecticides for space spraying are done by observing the mortality of susceptible strains of laboratory-reared non-blood-fed female mosquitoes confined in cylindrical cages (see Annex 6 in the WHO Guideline, 2009 for details).
- For indoor applications, cages are placed at several locations indoors and, for outdoor applications, at downwind distances of up to 100 m.
- The time of spray application, as well as the meteorological conditions during spraying (e.g. temperature, humidity, wind speed and direction) should be recorded throughout the trial and reported.
- The delivery characteristics such as discharge rate, vehicle speed, nozzle angle and pressure should also be standardized and reported.

- Optimal conditions for outdoor spray trials usually occur at or near sundown, throughout the night, and up to an hour after sunrise when there is an increase in temperature with height above ground level, i.e. inversion, when stable conditions help to keep the small droplets from rising above the target zone.
- Space spraying outdoors should not be done when wind speed exceeds 15 km/hour or falls below 3 km/hour, or during rain.
- Equipment should be calibrated to deliver droplets with a Dv0.5 of 15 ± 2 µm, unless otherwise indicated as a result of rapid volatility or other physical characteristic of the test material, which could expand the range to between 10 µm and 40 µm.

2.3.2.1. Outdoor applications

Small scale outdoor trial is carried out with two primary objectives:

- To determine the dosage of active ingredient per hectare that achieves at least 90% mortality and
- To physically characterize the spray (mainly by assessing the droplet size).

Efficacy is assessed in an open field by observing the mortality of susceptible laboratory-reared mosquitoes confined in screen cages (see Annex 6 and section 3 in the WHO Guideline, 2009 for general specifications) suspended 1.5 m above ground level, at 25, 50, 75 and 100 m downwind of the spray vehicle.

2.3.2.1.1. Physical characterization (WHO, 2009)

- Coated transparent collectors, e.g. slides, are placed on rotators located adjacent to selected cages to physically characterize the spray (see Annexes 7 and 8 in the WHO Guideline, 2009).
- The rotator is switched on immediately before spraying and switched off and the collectors removed 15 minutes post-spraying (at the same time as removal of the bioassay cages).
- The collectors are transferred to the laboratory in a protective holder for droplet size assessment as soon as possible (depending on the volatility of the formulation).
- Collectors should also be run with the control cages concurrently, with application in an unexposed area, to detect the presence of natural environmental droplets (e.g. oils, plant sap) and discriminate them from spray droplets.
- Collectors coated with silicone or Teflon is suitable for non-volatile, oil-based insecticide formulations.
- For other types of formulations (e.g. water-based), droplets can be detected on magnesium oxide coated surfaces provided a tracer dye is added to the spray, e.g. fluorescent tracer.
- The purpose of the tracer is to make the smallest droplets visible under an ultraviolet light microscope, as a distinct crater may not be formed.
- The use of a tracer may also enable fluorometric and volumetric evaluation and discrimination between insecticide and other droplets.

- The craters in magnesium oxide, or non-volatile droplets on silicone- or Teflon-coated slides, are then examined under a microscope and the parameters (Dv0.5 and Dv0.9) are calculated, taking into account any spread factor (see Annex 8 in the WHO Guideline, 2009). *(Magnesium oxide coated surfaces are not suitable for measuring non-dyed droplet sizes of <10 µm.)*
- A minimum of 200 droplets should be measured for each collector, reading across the width of the collector as many times as necessary.
- A record should be kept of the number of traverses needed to count 200 or more droplets, being sure to read each traverse completely. A minimum of one traverse should be used. This information is used to determine the droplet density on the slide.

2.3.2.1.2. Dosage determination (WHO, 2009)

- In small-scale field trials, a range of dosages of the insecticide is used based on label instructions and laboratory studies.
- Dosages are selected to produce a range of efficacy that includes >95% mortality and at least one between 80% and 95%, in order to demonstrate a dosage that is effective but not excessive.
- The sequence of dosages should be randomized and the spray apparatus flushed with diluents or acetone between applications.
- A minimum of three lines, with 1–2 cages at each of the four distances (25, 50, 75 and 100 m), and a minimum of three replicates over time for each dosage are used.
- Cages are placed in the field for the standard exposure period of 15 minutes.
- For each replicate, there is a concurrent unsprayed control located at least 50 m upwind in which there are a minimum of two cages and a rotating collector.
- Use of a standard insecticide as a positive control is highly desirable.

2.3.2.1.3. Stipulations

- The trial should be conducted in open areas with no vegetation taller than short grass to impede the movement of the spray cloud through the sample line.
- The line of travel of the spray machine should be perpendicular to the wind.
- Tests should not be conducted when wind directions are in excess of 30° from the sample line (see Figure 3.1), because this excessively increases the distance between the spray line and the collection stations.
- When the wind direction is 15° off-perpendicular, the distance is increased by 3.5% and at 30° by 15.4%.
- The spray machine is turned on at a minimum of 100 m before reaching the test area and turned off at a minimum of 100 m beyond the test area.
- A total of 25 insecticide-susceptible, non-blood-fed, 2–5 day-old laboratory-reared female mosquitoes of the target species are introduced into each cage.
- Fifteen minutes after completion of the spraying, the mosquitoes from each cage are transferred by gentle blowing or rapid light CO2 anaesthesia into separately marked, clean holding cups.

- The cups are provided with sugar solution on cotton wool and placed in a protective container, with coolant if necessary, for transport to the laboratory.
- It is recommended that observation of knockdown at 60 minutes post-treatment (including the 15 minutes exposure time) is recorded. *To prevent excess exposure to the insecticide residue in the cage, sufficient field personnel should be on hand to rapidly collect and transfer the mosquitoes to holding cups after the exposure period.*
- The mosquitoes are held at 27 +2^{o}C temperature and 80 + 10% RH for 24-hour mortality observations.
- If replicates are carried out on the same day, there should be a minimum of 30 minutes interval between each replicate, using mosquitoes from different rearing cohorts.
- Where the formulation requires dilution before application, fresh dilutions should be prepared for each new replicate.
- If mortality is above 20% in the concurrent controls, the entire test is rejected. If mortality in the controls is between 5% and 20%, the results from the treated samples are corrected using Abbott's formula (see section 2.1 in the WHO Guideline, 2009).
- Average mortality is reported for each distance, i.e. sampling stations downwind and for each dosage.
- For vehicle-mounted equipment, a dosage that produces an average mortality of >90% over all four stations is desirable or at least comparable with the standard.
- For portable spray equipment, the same level of mortality should be obtained at 25 m.

The average mortality and standard deviation are determined for each dosage and compared using a suitable statistical test such as ANOVA. The minimum dosage that gives at least 90% mortality is identified and is used as the lowest test dosage for operational trials.

Table 34. Toxicity evaluation

Village.....Sub-centre.....PHC.....District……Date of bioassay.....
Temperature: Min ..... Max..... Relative humidity: Min .....Max.....
Test species……Lab/F_1/Field collected…….....……

Replicate* Cage No.	**No. of mosquitoes in cage**	**No. knocked-down after 1 h**	**No. killed after 24 h**	**% Mortality after 1h**	**% Mortality after 24h**
Replicate 1					
Replicate 2					
Replicate 3					
Replicate 4					
Replicate 5 (indoors)					
Control 1					
Control 2					
*Separate row for each replicate.					

2.3.2.2. Indoor applications

The objective of indoor application is to determine the dosage of active ingredient per cubic metre (m^3) for space treatments that achieve at least 90% mortality.

- Efficacy of indoor spraying of insecticides should be studied in an empty room, with minimum volume of 30 m3, by observing mosquito mortality in the test cages.
- Test conditions may be met by construction of experimental rooms provided they can be ventilated and adequately decontaminated.
- A total of 10 cages (see Annex 6 in the WHO Guideline, 2009) each containing 25 2–5 day-old non-blood-fed susceptible female mosquitoes are placed 25 cm from each corner at ceiling and floor levels and two at mid height near the centre of the room.
- The insecticide is released, without ventilation, through an opening located at mid height in the centre of the wall at one end of the room and the nozzle directed towards the centre of the room.
- Equipment must be calibrated before carrying out spraying to ensure a Dv0.5 of 15 ± 2 µm.
- At least three dosages of the insecticide and a minimum of three replicates for each dosage are conducted, using insects from different rearing cohorts and fresh dilutions (if the formulation needs to be diluted) for each replication.
- Mosquitoes are exposed for 60 minutes. The cages are then removed using appropriate personal protective measures.
- The mosquitoes from each cage are rapidly transferred into separately marked, clean holding cups each with a 10% sugar solution on cotton wool and, if necessary, placed in a protective container, containing coolant where appropriate, and transported to the laboratory.
- At the time of collection, observation of knockdown may be recorded.
- The mosquitoes are held at 27 + 2 °C temperature and 80 + 10% RH for 24-hour mortality observations.
- The room must be adequately ventilated between successive dosages of the same compound to remove all traces of the previous treatment.
- Each insecticide application requires a control. This is obtained prior to the application by exposing mosquitoes in cages for 60 minutes at each of the cage positions stated above.
- Rooms should be declared contaminated or unsatisfactory for use when test insects held in a cage in the room (without ventilation) for one hour show knockdown or mortality in excess of 10%.
- The average mortality and standard deviation are determined for each dosage and compared using a suitable statistical test, such as ANOVA.
- The minimum dosage that gives at least 90% mortality is identified and is used as the lowest test dosage for operational trials.
- If mortality exceeds 20% in the concurrent controls, the entire test is rejected. If mortality in the controls is between 5% and 20%, the results from the treated samples are corrected using Abbott's formula (see section 2.1 in the WHO Guideline, 2009).

Table 35. Observed mortality of mosquitoes at different doses

Type*	No. in cages	No. dead in cages	Total		Mortality	Corrected % mortality
			Dead	Alive		
Dose 1						
Dose 2						
Control						
* Separate row for each dose.						

2.3.2.3. Human safety

Data regarding perception of the staff involved in fog generation and collection of mosquitoes should be recorded in the pre-structured questionnaire (Annexure 3).

2.3.3. Phase III trial (Operational trial)

Based on the results of small scale trials, the effectiveness of the space spray product/ formulation has to be assessed in operational settings against field populations of the target species. The operational trial should be carried out in different situations (e.g. urban/rural and indoor/outdoor settings) and the test sites should be representative of the target species' habitat and the intended control areas. The trial should be conducted minimum in three sites for a period of three years. In addition to meteorological conditions (temperature, humidity, wind speed and direction) and delivery characteristics of the insecticide (discharge rate, vehicle speed, nozzle angle and pressure), configuration of buildings, dwellings, rooms (including significant furniture, wall hangings and clothing) and vegetation characteristics in the test sites should be surveyed and recorded during the application and sampling periods. Any other relevant observations such as time of sunset, sunrise, cloud cover and target species activity periods should be documented.

The initial dosage for operational trials should be determined on the basis of the dosage(s) recommended on the manufacturer's label or that found to cause at least 90% mortality in small-scale trials. Higher dosages may be required for assessment of effectiveness under operational conditions (increased obstructions and harborages in the natural habitats may require application of higher concentrations). Outdoor applications are based on dosage per hectare while indoor applications are based on dosage per cubic metre. The susceptibility of the target species to the insecticide must be verified beforehand (WHO, 2009).

The manufacturer's instructions or the description on operation and calibration of the equipment given in product label as well as WHO guidelines on *Space spray application of insecticides for vector and public health pest control* (WHO, 2003) should be followed. Machines must be calibrated to deliver a spray droplet distribution with a Dv0.5 of 15 ± 2 μm, unless rapid volatility or other characteristics of the test material indicate the need for larger or smaller droplets.

As indicated under small scale trials, spraying should not be carried out when wind speed exceeds 15 km/hour or falls below 3 km/hour or during rainfall. For outdoor spraying, optimal conditions prevail when there is an increase in temperature with height. Favourable

meteorological conditions usually occur at or near sundown, throughout the night, and up to an hour after sunrise (WHO, 2009).

2.3.3.1. Outdoor trials

2.3.3.1.1. Objectives

1. To cause and measure the reduction of adult density of the target vector species
2. To identify and confirm the effective dosage (required to achieve minimum average of 90% control) under operational settings and
3. To assess the impact of space spraying on disease

2.3.3.1.2. Evaluation *(The guidelines and the methodology describe hereunder are according to WHO Guideline (WHO, 2009)*

- The guidance is only general and needs to be adapted to local situations considering the characteristic features of trial areas and behavioural differences among mosquito/ vector species, but focusing on the target species population.
- Trial design must take into consideration the flight range and behaviour of the target species (e.g. endophily and/or exophily) as well as the method of insecticide application.
- To minimize the impact of re-invasion of the target species from unsprayed areas or infiltration from other areas, a sufficiently large area should be sprayed so that entomological assessment can be restricted to the central zone.
- In order to avoid the confounding influences of such immigration or recruitment from on-site larval habitats, pre- and post-monitoring of target species should be conducted shortly before, and as soon as possible after, the treatment at times appropriate to the sampling method.
- The optimal operational outdoor trial design is random allocation of comparable treated and untreated areas.
- To allow for statistical analysis and meaningful conclusion, the trial should be sufficiently replicated (normally three times) in space or preferably in time.
- The methods of collecting adult mosquitoes must be appropriate to the target species and conducted in multiple representative habitat sites within the test and control areas (e.g. indoors or outdoors, or both).
- Collection methods may include the use of light traps, baited traps, and oviposition traps, gravid traps, landing counts on subjects protected from bites, suction traps and vacuum devices (aspirators) for resting mosquitoes.
- Sampling sites and collection methods should be configured in a manner that maximizes the suitability for statistical analysis of data.

2.3.3.1.3. Evaluation using sentinel cages

(For the evaluation procedure using sentinel cages, WHO Guidelines 2009 on Space Spraying may be referred to).

2.3.3.2. Indoor trials

Evaluation methods

- Space spraying indoors is assessed by selecting several houses, each with multiple rooms. Kitchens should be avoided.
- In each room at least one cage (see Annex 6 in the WHO Guideline, 2009) should be located near the centre at 1.5 m height as a standard. A minimum of two additional cages should be placed adjacent to typical mosquito resting sites within each room. (*The use of spinners to assess spray droplet penetration into rooms is optional).*
- Twenty five (mixed age) female mosquitoes are released in each cage and exposed to insecticide application for 60 minutes in both experiment and control houses (*A room fogged with only solvent is treated as control*). Wild caught mosquitoes can be released in to the cages on the day of treatment. Alternatively, wild mosquitoes are reared and the F1 progeny used for testing.
- Prior to the application, external doors and windows, where appropriate, should be closed. In multi-room houses, the insecticide is applied beginning with the room furthest from the entrance and progressively moving towards the entrance. The application should be carried out to ensure that the spray is directed to all parts of each room and at the target dosage.
- A minimum of three replicates are carried out on different occasions at the optimal dosage required for 90% control as determined in small-scale trials or at the label recommended dosage using fresh dilutions (if the formulation needs to be diluted) for each replication. If 95% cage mortality is not achieved, a higher dosage is similarly tested ensuring that the manufacturer's labeling constraints are not exceeded.
- After exposure of mosquitoes for 60 minutes, the cages are removed from the house (*protective clothing has to be used while handling the cages*). The house is ventilated by opening doors and windows before allowing re-entry of unprotected individuals into the house. The mosquitoes from each cage are then rapidly transferred into separately marked, clean holding cups, each with a 10% sugar solution on cotton wool, and transferred to a container, containing coolant if necessary, and transported to the laboratory. At the time of collection, observation of knockdown may be recorded. The mosquitoes are held at 27 + 2 °C and 80 + 10% RH for 24-hour mortality observations.

2.4. Repellents

Repellents are either synthetic chemical or plant-based compounds. They are used for personal protection against mosquitoes and other haematophagous insects. Candidate compounds should be evaluated simultaneously in laboratory and field.

2.4.1. Laboratory evaluation

2.4.1.1. Objectives

- To estimate the effective dose
- To test the repellent activity of the candidate compound against mosquitoes
- To determine the average protection time rendered by the repellent
- To test the safety to volunteers

2.4.1.2. Repellency against mosquitoes

These studies will be carried out in the laboratory maintained at 27$\pm$ 2^{O}C and 60–70% relative humidity using laboratory-reared strains of vectors in cloth cages (2 cu ft). Studies will be carried out against each individual species in replicates of three. In each of the replicate cage, one hundred 3 to 5-day old sugar-fed female mosquitoes will be held. Mosquitoes that are pre-starved for - 12 hr h or more prior to testing will be used for the experiment. These conditions and format of experiment given below need standardisation for different species. Using a known repellent (DEET) as positive control is optional.

In the cage, five plastic bowls with sugar-soaked cotton (10% in water) should be placed at four opposing corners and one in the middle. These bowls should be treated separately with three different specified concentrations of the formulation, one specified concentration of DEET (known synthetic repellent compound as positive control) and with only sugar-soaked cotton (negative control). The four treated bowls will be placed in four opposing corners while the untreated negative control in the middle on the floor of the cage. Five minute landing counts will be made at 0, 1, 2, 4 and 6 h. The cups will be removed between the exposure intervals. Mean percent repellency for each percent formulation and species will be calculated based on the data of the three replicates at the given times of observation. Percent repellency will be calculated using the formula given below.

2.4.1.3. Determination of effective dose of repellency and protection time

Testing should be done for *Anopheles, Aedes* and *Culex* mosquitoes and other disease vectors. Evaluations should be carried out in the laboratory maintained at standard temperature and humidity. Tests are carried out on adult human volunteers who may be selected from among candidates exhibiting mild of or no sensitivity to mosquito bites. Equal numbers of male and female test volunteers are preferred. About (~) 600 cm^2 area of one forearm skin between wrist and elbow of human volunteer should be marked with marker and test repellent dissolved in a suitable solvent should be applied on the marked portion. . In preparation for the laboratory studies, the test area of the volunteer's skin should be washed with unscented soap and rinsed

with water, then rinsed with the solution of 70% ethanol or isopropyl alcohol in water and tried with towel. Given the possibility that various factors may alter a person's attractiveness to mosquitoes this in turn may affect the outcome of repellency assays, test volunteers should avoid the use of fragrance and repellent products for 12 hours before and during testing. Volunteers should preferably not to be tobacco users, or at least to have refrained from tobacco use for 12 hours prior to and during testing. Female mosquitoes should be collected from a stock population cage in which both sexes have been maintained to allow mating to occur. They should be host seeking, of uniform age, preferably 5-7 days, post emergence. Active host seeking females should be selected to ensure good response from the test mosquitoes. Hands are put into cages containing approximately 50-100 female mosquitoes in a 2 x 2 x 2 ft cloth cage. Observation of repellency should be made using female mosquitoes starved for the preceding 12 hours and, where practical, during times in the diel period that correspond with biting activity by that species. The stock population of adult mosquitoes should have access to sugar solution but not have been blood fed.

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

Each volunteer uses incremental doses on the test forearm so that at least five successive application of increasing dose are used by each volunteer. A single test comprises continuous use of the same mosquitoes by the same volunteer and is completed in one day. Replicate tests repeat this process using different batches mosquitoes over several days. It is recommended a minimum of three replicates be conducted per volunteer, with the number of volunteers sufficient to allow for statistical analysis; preferably, 2–3 dosages that give <50% repellent response and 2–3 dosages that give >50%. One ml of ethanol or the same diluents used in the preparation of the test repellent is applied evenly using pipette of the forearm skin and allowed to dry. Before the insertion of the arm into the cage containing 50-100 female mosquitoes, the hands are protected by gloves made of material through which the mosquitoes cannot bite. The first step is to insert the forearm applied with diluents into the cage and to count the number of mosquitoes that land on and/or commence to probe the skin during a 30-second period. During testing, the volunteer should avoid movement of the arm. For the test to proceed, the biting rate must be ≥10 landings and/or probing in the 30-second period. The control forearm is carefully withdrawn and this arm is then treated with the lowest dose of repellent in 1 mL diluent and allowed to dry.

The treated arm is placed in the cage for another 30-second period and observed for mosquito landings and/or probing. This procedure is repeated for each additional incremental repellent dose. Successive tests should be carried out one after the other without delay and the repellent dose at each test calculated as the sum of the doses applied to arrive at the cumulative dose for each test at any time, the landing and/or probing rate is too high to accurately count the number of mosquitoes landing and/or commencing to probe the skin, the mean landing and/or probing rate for the test should be calculated from a series of three readings, each five seconds long, and the sum multiplied by two to estimate the landings and/or probing that would occur in a

30-second period. Testing should not proceed when the mosquito landing and/or probing rate on the exposed forearm is <10 females in 30 seconds.

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

Protection (p) is expressed as a proportion of the number of mosquito landings and/or probing on the treated arm (T) in relation to the number of landings and/or probing on the control arm (C) of the same individual: $p = 1 - (T / C) = (C - T) / C$ (i) where C is the average of the landings/probing on the two untreated arms (the diluent-applied test arm before repellent treatment and the other arm at the end of the experiment). Data are analysed using probit-plane regression analysis from which the ED50 and ED99.9 and their confidence limits can be estimated.

The experiments on humans should be attempted only on toxicologically safe compounds. The complete protection time, or CPT, of a repellent can be determined in one of two ways. Preferably, the ED99.9 dose should be estimated using the procedures outlined in section 2.1; 1 mL of the repellent is then tested at the ED99.9 level against 1 mL of the standard 20% ethanolic deet. Alternatively, 1 mL of the 20% ethanolic deet solution can be compared with the same amount (weight/weight) of the candidate repellent on the other arm. In both cases, treatments are applied to ≈600 cm2 area (Annex 2) of the forearm skin between the wrist and elbow. Two mosquito cages (size: 35–40 cm per side) each containing 200–250 non-blood-fed females are normally used. One cage is designated for testing the candidate repellent and the other for the positive control (ethanolic deet). During testing, the hands are protected by gloves made of material through which the mosquitoes cannot bite while the volunteer avoids movement of the arm.

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

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

repellent application and the first mosquito landing and/or probing. Most repellent studies of technical material are completed in 8 hours or less. The number of volunteers included in the test should be sufficient to allow for statistical analysis. The median CPT and confidence interval can be estimated from the Kaplan–Meier Survival Function.

The time between application of the repellent and the second successive bite should be recorded as the protection time. The dose of repellent providing at least 6–8 h of protection in mosquito cage experiments may be considered to be an ideal compound for use in field as repellent for field evaluation. The experiment will have two positive controls with known synthetic repellents, e.g. DEET, and negative control only solvent that is used as base for preparing the test repellent. Data should be recorded in the format given in Table 36.

Table 36. Laboratory evaluation of repellents on humans (Cage method)

Name of the repellent……..... Date…….....Time …….....…....
Temp……..... Relative humidity.....……Dose of application……..…..…

Replicate number*	Time of first bite	Time of confirmatory bite	Protection time	Percent protection
Dose 1				
Dose 2				
Dose 3				
Dose 4				
Positive control				
Negative control				
* Separate row for each replicate.				

2.4.1.4. Human safety

A semi-structured questionnaire should be used to record the perceptions of human volunteers about the positive and/or negative side effects of the repellents (Annexure 3).

2.4.2. Field evaluation

The objective of field trials is to extend the results of laboratory testing to estimate the optimum application dose, persistence and efficacy of a repellent material, in terms of repellency and protection time, against one or more mosquito vectors and/or pest species in different ecological and/or geographical settings. Human subjects should be selected randomly without bias. Prior informed consent should be obtained for participation in the evaluation (Annexure 4). The effective dose determined in the laboratory should be used in the field evaluation. Use of known repellent compound and is optional. The experiments on human subjects should be attempted on toxicologically safe compounds. A minimum of two field tests are recommended, one each in different ecological and or geographical settings suitable for the target mosquito species where the human exposure occurs. Assessment is made by human volunteers collecting mosquitoes landing and/or probing on one or more bare limbs (knee to ankle; elbow to wrist), depending upon the biting behaviour of the mosquito species. The volunteers should be: (i) from the same settings where the test is conducted, such that they are

not exposed to unusual risk of infection; (ii) if appropriate and applicable, protected by chemoprophylaxis and/or vaccination; and/or (iii) where possible, at sites where there is no disease transmission but high abundance of target mosquito species. The determination of proportional end-points of repellent efficacy and persistence is recommended. These allow concurrent estimation of EDs, effective period of time for a range of doses, repellent half-life and the CPT.

Positive control (with DEET) and negative control (solvent alone) should be included for assessing the repellency of the candidate repellent. Five volunteers, two for test repellent, two for positive control and one for negative control should be employed for the evaluation.). Human volunteers should be placed at least 10 metres away from each other. Mosquitoes landing on the volunteers should be collected from dusk to dawn and examined for species identification. Insect collectors should be rotated every four hours to avoid slackness and bias. Both complete protection and percent repellency should be determined as described in laboratory evaluation. Data should be recorded in the format given in Table 37.

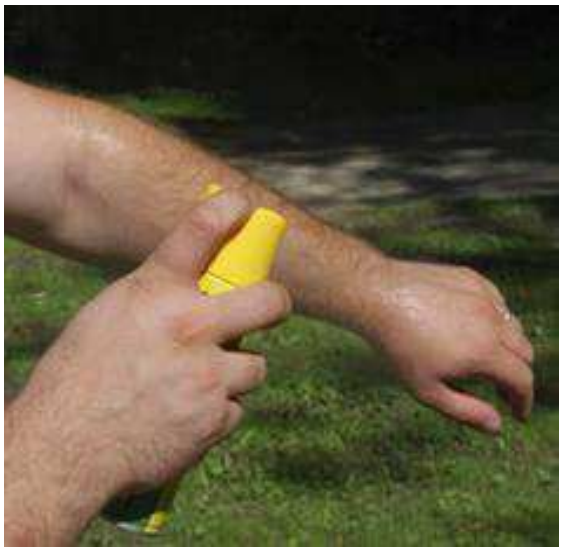
Table 37. Mosquito landing collections

Name of the repellent.....Date..... Time….. .....Temp.....

Relative humidity.....…Repellent and concentration.....Species.....

Time (Hrs)	**Number of bites**					**Percent protection**	**Average protection time**
	Test 1	**Test 2**	**Positive control 1**	**Positive control 2**	**Negative control**		
1800 1900 2000 2100 2200 2300 2400 0100 0200 0300 0400 0500 0600							
Total							

Herbals used as mosquito repellents



2.5. Chemical larvicides, Bio-larvicides and Insect Growth Regulators

Larvicides are used as a source reduction method in Urban Malaria Control Programme. This method is also effective against vectors of filariasis and dengue. Evaluation is carried out both for WHOPES passed and new insecticides. New insecticides are evaluated in 3 Phases— I, II and III, while for WHOPES passed insecticides only Phase II and III trials are recommended.

2.5.1 Phase I: Laboratory studies

2.5.1.1. Chemical larvicides

2.5.1.1.1. Duration

Evaluation should be carried out for 3-4 months.

2.5.1.1.2. Objectives

The objectives of laboratory studies are:

- to establish dose-response line(s) against susceptible vector species;
- to determine the lethal concentration (LC) of the larvicide for 50% and 90% mortality (LC_{50} and LC_{90}). In case of insect growth regulators, to determine the concentration of IGR for 50% and 90% inhibition of adult emergence (IE_{50} and IE_{90});
- to establish a diagnostic concentration for monitoring susceptibility to the mosquito larvicide in the field; and
- to assess the cross-resistance with the commonly used insecticides

2.5.1.1.3. Determination of biological activity

Evaluation of biological activity of larvicides should be done on laboratory colonized larvae of known age or F_1 larvae of the field collected mosquitoes of *Anopheles stephensi, An. culicifacies*, *Culex quinquefasciatus, Aedes aegypti* and *Ae. albopictus* to the candidate insecticide and other insecticides which are currently in use in the programme. Standard WHO procedure has to be followed for bioassays (WHO/CDS/WHOPES/GCDPP/2005-13). The highest test concentration should not generally exceed 1 ppm or 1 mg/litre.

2.5.1.1.4. Preparation of stock solutions and test concentrations

The technical grade insecticides are insoluble in water. For the preparation of stock solutions, these materials have to be dissolved in organic solvents (acetone or ethanol). When a test is carried out using formulated materials distilled water is used in the preparation of the 1% stock solution and serial dilutions.

The volume of stock solution should be 20 ml of 1%, obtained by weighing 200 mg of the technical material and adding 20 ml solvent to it. It should be kept in a screw-cap vial, with aluminium foil over the mouth of the vial. Shake vigorously to dissolve or disperse the

material in the solvent. The stock solution is then serially diluted (ten-fold) in ethanol or other solvents (2 ml solution to 18 ml solvent). Test concentrations are then obtained by adding 0.1 – 1.0 ml (100-1000 µl) of the appropriate dilution to 100 ml or 200 ml chlorine-free or distilled water (Table 38). For other volumes of test water, aliquots of dilutions added should be adjusted according to Table 38. When making a series of concentrations, the lowest concentration should be prepared first. Small volumes of dilutions should be transferred to test cups by means of pipettes with disposable tips. The addition of small volumes of solution to100 ml, 200 ml or greater volumes of water should not cause noticeable variability in the final concentration.

Table 38. Aliquates of various strength solutions added to 100 ml water to yield final concentration (WHO/CDS/WHOPES/GCDPP/2005-13)

Initial solution		Aliquot (ml)	Final concentration (PPM) in 100 ml
%	PPM		
1.0	10 000.0	1.0	100.0
		0.5	50.0
		0.1	10.0
0.1	1000.0	1.0	10.0
		0.5	5.0
		0.1	1.0
0.01	100.0	1.0	1.0
		0.5	0.5
		0.1	0.1
0.001	10.0	1.0	0.1
		0.5	0.05
		0.1	0.01
0.0001	1.0	1.0	0.01
		0.5	0.005
		0.1	0.001
0.00001	0.1	1.0	0.001
		0.5	0.0005
		0.1	0.0001

2.5.1.1.5. Laboratory bioassays

Initially the mosquito larvae are exposed to a wide range of test concentrations and a control to find out the activity range of the materials under test. After determining the mortality of larvae in this wide range of concentrations, a narrower range (of 4-5 concentrations, yielding between 10% and 95% mortality in 24 h or 48 h) is used to determine LC_{50} and LC_{90} values.

Batches of 25 early fourth instars larvae are transferred by means of strainers, screen loops or droppers to small disposable test cups or vessels, each containing 100-200 ml of water. Small,

unhealthy or damaged larvae should be removed and replaced. The depth of the water in the cups or vessels should remain between 5 cm and 10 cm; deeper levels may cause undue mortality.

The appropriate volume of dilution is added (Table 38) to 100 ml or 200 ml water in the cups to obtain the desired target dosage, starting with the lowest concentration. Four or more replicates are set up for each concentration and an equal number of controls are set up simultaneously with tap water, to which 1 ml alcohol (or the organic solvent used) is added. Bioassays should be repeated at least three times on different days, using new solutions or suspensions and different batches of larvae each time. For long exposures, larval food should be added to each test cup, particularly if high mortality is noted in control. The test containers are held at 25-28°C and preferably a photoperiod of 12 h light followed by 12 h dark (12L:12D).

After 24 hours, percent mortality is determined scoring the dead and moribund larvae in test replicates. Moribund larvae are those incapable of rising to the surface or not showing the characteristic diving reaction when the water is disturbed. The results are recorded on the form provided (Table 39, where LC_{50} and LC_{90} values, and slope and heterogeneity analysis are also noted).

Larvae, that have pupated during the test should be discarded and counted for calculation of mortality. The experiment should be repeated if more than 10% larvae pupated or when more than 20% larval mortality occurs in the controls. If the larval mortality in control is between 5% and 20%, the mortalities of treated groups should be corrected according to Abbott's formula:

$$\text{Mortality (\%)} = \frac{X - Y}{X} 100$$

Where X = Percentage survival in the untreated control and
Y = Percent survival in the treated sample.

2.5.1.1.6. Data analysis

Data from all replicates should be pooled for analysis. LC_{50} and LC_{90} values are calculated form a log dosage-probit mortality regression line using computer software programs, or estimated using log-probit paper. Standard deviation or confidence intervals of the means of LC_{50} values are calculated and recorded on a form (Table 39). A test series is valid if the relative standard deviation (or coefficient of variation) is less than 25% or if confidence limits of LC_{50} overlap (Significant level at $P < 0.05$). The potency of the chemical against the larvae of a particular vector and strain can then be compared with the LC_{50} or LC_{90} values of other insecticides.

2.5.1.2. Bacterial larvicides

The laboratory bioassay procedures for bacterial products are the same as those for chemical larvicides, except in the preparation of stock suspensions.

2.5.1.2.1. Determination of the bio-potency of the bacterial larvicide products

The bio-potency of the material is first examined by comparing mosquito larval mortality produced by the product under test with the mortality produced by the corresponding reference standard or other technical or formulated product. The toxicity of preparations based on *Bacillus thuringiensis* subsp. *israelensis* (*B. thuringiensis* subsp. *israelensis*) can be determined against a standard product that has been calibrated using Aedes aegypti (*Ae. aegypti*) larvae. The potency of products tested is determined by the following formula:

$$\text{Potency of product "X"} = \frac{\text{Potency standard (ITU) x LC50 (mg)/1) standard}}{\text{LC50 (mg/l) of "X"}}$$

When the international reference standard is used, potency is expressed in International Toxic Units per milligram (ITU/mg). The bio-potency of products based on *B. thuringiensis* subsp. *israelensis* is compared with a lyophilized reference powder (IPS82, strain 1884) of this bacterial species using early fourth instar larvae of *Ae. aegypti* (strain Bora Bora). The potency of IPS82 has been arbitrarily designated as 15000 ITU/ mg powder against this strain of mosquito larva.

The bio-potency of products based on *Bacillus sphaericus* (*B. sphaericus*) is determined against a lyophilized reference powder (SPH88, strain 2362) of this bacterial species using early fourth instar larvae of *Culex pipiens pipiens* (*C. pipiens pipiens*) or *Culex quinquefasciatus*. The potency of SPH88 has been arbitrarily set at 1700 ITU/mg of powder against this mosquito strain.
The use of other bacterial larvicide reference powders and/or alternative strains of mosquito in this test are possible but must be approached warily, because it is inevitable that different results will obtain. Such alternatives must be the subject of careful cross-calibration with the reference powders and strains identified above. Ideally, such cross-calibration should be conducted by a group of independent expert laboratories. The alternative powders or strains, and the cross-calibration data that support them, should be made available to anyone who wishes to use, or check, the test.

In general, it is not necessary to calibrate with or test against the standard if comparing the activity of a bacterial product with other larvicide products. Bioassay results providing LC_{50} and LC_{90} values of products are sufficient to enable comparison among different products.

2.5.1.2.2. Preparation of reference standard suspensions for calibration of the bioassays

To prepare a "stock suspension", weigh 200 mg or 1000 mg of the solid product, place in a vial (30 ml) or volumetric flask, and add 20 ml or 100 ml distilled water, yielding 1% stock

suspension, or 10 mg/l. Most powders do not need blending or sonication. Vigorous shaking or stirring should facilitate suspension. If placed in tubes, the stock suspension can be frozen for future bioassays. Frozen aliquots must be homogenized thoroughly before use, because particles agglomerate during freezingFrom the "stock suspension", any necessary subsequent dilutions (see Table 38) are prepared by serial dilution. Plastic or paper cups are filled with 100 ml de-ionized water. Twenty-five late third or early fourth instar larvae of *Ae. aegypti* or *Cx. quinquefasciatus* (depending on the bacterial species to be tested: *Ae. aegypti* or *An. stephensi* larvae for *B. thuringiensis* subsp. *israelensis* and *An. stephensi* or *Cx. quinquefasciatus* larvae for *B. sphaericus*) are added to each cup. Using micropipettes, 400 µl, 300 µl, 200 µl, 100 µl, 80 µl, and 50 µl of a given suspension (see Table 38) are added to the cups and the solutions mixed to produce final concentrations of 0.04 mg/l, 0.03 mg/l, 0.02 mg/l, 0.01 mg/l, 0.008 mg/l and 0.005 mg/l, respectively, of the reference standard powder. Four or more replicate cups are used for each concentration and the control, which is 100 ml de-ionized water.

2.5.1.2.3. Preparation of suspensions of the product to be tested

For bioassays of technical (solid or liquid) products of unknown potency, an initial homogenate is made simply by mixing without reducing particle size. For assays of liquid formulations, 20 ml water is added to 200 mg in a vial. Serial dilutions are made and cups and larvae are prepared as described in the previous section. Range-finding bioassays are preformed using a wide range of concentrations of the product to determine its approximate toxicity. The results are then used to determine a narrower and more refined range of concentrations for precise bioassay.

2.5.1.2.4. Laboratory bioassays

To prepare a valid dose-response curve, only concentrations giving values between 10 % and 95% mortality should be used. A minimum of two concentrations above and two below the LC_{50} level must be used. Each bioassay series should involve at least four concentrations; and each concentration should be tested in four replicates of 25 early fourth instar larvae per replicate. Larvae are added to the cups prior to the addition of bacterial suspensions. No food is added to larval vessels when the exposure period is 24 h. Food may be required if the exposure period is longer. Mortality is scored at 24 h for *B. thuringiensis* subsp. *Israelensis* and 48 h for *B. sphaericus* by counting the live larvae remaining. The results are entered on the form (Table 39).

2.5.1.2.5. Data analysis

Mortality in control in the range of 5 to 20% should be corrected using Abbott's formula. If mortality in control is more than 20%, tests should be repeated. In case of control tests with more than 10% pupation should be repeated. LC99.9 of the candidate and standard biolarvicide preparations should be calculated from mortality regression lines by probit analysis Section: 5.1.1.6). Doses for application in Phase II are determined by multiplying the observed LC99.9 value with a factor of 2 or 3.

2.5.1.3. Insect Growth Regulators

The testing methods for insect growth regulators (juvenile hormone (JH) analogues and chitin synthesis inhibitors) differ in some aspects. JH analogues interfere with the transformation of late instar larvae to pupae and then to adult, whereas chitin synthesis inhibitors inhibit cuticle formation and affect all instars and stages of the mosquito. The effect of both types of insect growth regulators (IGRs) on mosquito larvae is expressed in terms of the percentage of larvae that do not develop into successfully emerging adults or inhibition of adult emergence (IE%).

2.5.1.3.1. Preparation of stock solution or suspension

The preparation of test solutions or suspensions and bio-assay set ups are the same as for the chemical larvicides (Sections 5.1.1.4 and 5.1.1.5).

2.5.1.3.2. Laboratory bioassays

Early third instar larvae are used for testing IGRs (JH analogues and chitin synthesis inhibitors). The accurate initial count of larvae is essential because of the cannibalistic or scavenging behaviour of larvae during the long exposure period. The long duration of the test also means that the larvae have to be provided with a small amount of food at a concentration of 10 mg/l at two-day intervals until mortality counts are made. The larvae in the control are fed in the same manner as those in the treated batches. If necessary, all the test and control cups should be covered with netting to prevent successfully emerged adults from escaping into the environment. Mortality or survival is counted every other day or every three days until the complete emergence of adults. The test containers are held at 25-28°C and preferably for a photoperiod of 12L: 12D.

At the end of the observation period, the impact is expressed as IE% based on the number of larvae that do not develop successfully into viable adults. In recording IE% for each concentration, moribund and dead larvae and pupae, as well as adult mosquitoes not completely separated from the pupal case, are considered as "affected". The number of successfully emerged adults may also be counted from the empty pupal cases. The experiment stops when all the larvae or pupae in the controls have died or emerged as adults. Data are entered on a form (Table 40). Any deformities or morphogenetic effects that occur in either the moulting immature mosquitoes or the emerging adults are also recorded.

2.5.1.3.3 Data analysis

The data from all replicates of each concentration should be combined. Total or mean emergence inhibition can be calculated on the basis of the number of third stage larvae exposed. The overall emergence of adults reflects activity. IE% is calculated using the following formula:

IE (%) = 100 – (T x 100)/C,

Where T= percentage survival or emergence in treated batches and

C= percentage survival or emergence in the control.

If adult emergence in the control is less than 80%, the test should be discarded and repeated. Where the percentage is between 80% and 95%, the data are corrected using Abbot's formula. IE values obtained at each concentration should be subjected to probit regression analysis to determine IE50 and IE90 values (using computer software programs or estimated from log-probit paper). The data analysis procedures stated in Section 5.1.1.6 should be followed.

2.5.1.4. Determination of diagnostic concentration

Diagnostic dose is determined by multi-plying the upper fiducial limit of $LC_{99.9}$ with a factor of 2 or 3 for routine susceptibility tests. For Phase II trials the doses for application in field should be in the range of 2 to 10 fold of the calculated diagnostic dose.

2.5.1.5. Cross-resistance assessment

To assess the cross-resistance to other insecticides currently in use in the programme, bioassays should be done using the diagnostic dose of the candidate and other insecticides. Data should be recorded in the format given in Table 39.

Table 39: Laboratory evaluation of the efficacy of larvicides against mosquito larvae (WHO/CDS/WHOPES/GCDPP/2005-13)

Experiment No: _____ Investigator: _____ Location:_____ Treatment date: _____
Material: _____ Formulation: _____ Temp.: _____ Lighting: _____
Species: _____ Larval Instar : _____ Larvae/cup: _____
Water: Tap/distilled Volume of water: _____ Food: _____ Date of stock solution made: _____

Date	Replicate	No. of dead larvae at various Conc. (mg/l) post exposure (hr.)													
		24 hr							48 hr						
		0.00							0.00						
	1														
	2														
	3														
	4														
	5														
	6														
	7														
	8														
	9														
	10														
	11														
	12														
	Total														
	Ave.														
	% mortality														

LC50 (CL 95%): _____
LC90 (CL 95%): _____
LC99 : _____

Slope: _____ Heterogeneity: _____

LC50 (CL 95%): _____
LC90 (CL 95%): _____
LC99: _____

Slope: _____ Heterogeneity: _____

Table 40: Laboratory evaluation of the efficacy of Insect growth regulators against mosquito larvae
(WHO/CDS/WHOPES/GCDPP/2005-13)

Experiment No: _____ Investigator: _____ Location:_____ Treatment date: _____
Material: _____ Formulation: _____ Sampling technique: _____
Species: _____ Larval Instar : _____ Larvae/cup: _____ Setting date: _____

Cumulative number of dead / alive mosquitoes after treatment (date or days pre or post treatment or setting) L: Larvae, P: Pupae, A: adults

Date														Grand total	
Conc.		Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
(mg/l)	Replicate	L P A	L P A	L P A	L P A	L P A	L P A	L P A	L P A	L P A	L P A	L P A	L P A	L P A	L P A
0.0	1														
	2														
	3														
	4														
	Total														
	Mean														
T1	1														
	2														
	3														
	4														
	Total														
	Mean														
T2	1														
	2														
	3														
	4														
	Total														
	Mean														
T3	1														
	2														
	3														
	4														
	Total														
	Mean														

Table 41: Small-scale field testing and evaluation of larvicides against mosquito larvae
(WHO/CDS/WHOPES/GCDPP/2005-13)

Experiment No: ____ Starting date: ________ Location: _______ Investigator: ________
Assessment date: ______ Pre or days post treatment: ______ Type of habitat: _____ Species: ______

Live larvae (L3-4) and pupae (P)/sample							
Treatment Dosage(__)	Sample	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Grand total
		L3-4* P*					
Control	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	% red						
	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	% red						
	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	% red						
	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	% red						
	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	% red						

Table 42: Small-scale field testing and evaluation of insect growth regulators against mosquito larvae (WHO/CDS/WHOPES/GCDPP/2005-13)

Experiment No: _______ Starting date: ________ Location: _______ Investigator: ________
Assessment date: ______ Pre or days post treatment: ______ Type of habitat: _____ Species: ______

Live larvae (L3-4) and pupae (P)/sample							
Treatment Dosage(__)	Sample	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Grand total
		L3-4* P*					
Control	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	% red						
	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	% red						
	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	% red						
	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	% red						
	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	% red						

2.5.2. Phase II: Small-scale field trials

Larvicides that show promise in laboratory studies (Phase I) are subjected to small-scale field testing (Phase II). The formulations are tested at three –five concentrations and Phase I studies will guide the dosages chosen for use in Phase II trials.

2.5.2.1. Duration

Evaluation should be carried out for 6 months.

2.5.2.2. Objectives

The objectives of small-scale field trials are:

- To determine the efficacy, residual activity of the formulations against target vector species in different breeding sites and ecological settings.
- To determine the optimum field application dosage (s);
- To monitor the abiotic parameters that may influence the efficacy of the product; and
- To record qualitative observations on the non-target biota cohabitating with mosquito larvae, especially predators.

2.5.2.3. Trials in natural breeding sites

The field efficacy of the larvicide under various ecological conditions is determined by selecting representative natural breeding habitats of the target species. For *Anopheles* spp. (*An. stephensi*, *An. culicifacies*) cement tanks, drums, garden pits, pools, rice plots, river/stream bed pools, water fountains and disused wells; for *Cx. quinquefasciatus* stagnant drains, cesspits, cesspools and disused wells and for *Aedes* spp. cement tanks, drums, peri-domestic water storage containers, air coolers and water fountains are best suited. A minimum of 3 replicates of each type of habitat should be randomly selected for each dosage of the formulation, with an equal number of controls. The size of the plot/habitat should be recorded, taking account of surface area and depth. As far as possible, the plots selected should be similar and comparable. Each of the confined breeding sources or containers can be considered as a discrete plot or replicate. Habitats such as drains and canals may be divided into sectors of 10 m^2 length and replicated for treatment and control.

Prior to application of larvicidal formulations, larval and pupal densities should be monitored for a week on at least two occasions before treatment. Habitats from each type with comparable pre-treatment densities should be assigned to either treatment or control groups.

Determine the pre-treatment larval and pupal density by dipping method using a standard dipper (300 ml capacity with 9 cm diameter) for pits, ponds, tanks, drains, drums etc., and bucket (3-liter capacity) for wells. Number of samples to be taken from each habitat should be decided on the basis of type and size of the habitat. For small habitats such as drums, pits, pools, tanks and wells, 3 to 5 dips per site, while for stagnant drain dips at a distance of 5 m are recommended.

The larval instars and pupae collected from each dipper sample are counted stage wise and returned back to the habitats.

About 3-5 dosages of the larvicide should be applied to the breeding habitats. Larvicide formulations (such as emulsifiable concentrate, suspension concentrate, liquids etc.) should be applied to the breeding habitats through a Knapsack sprayer/hand compression sprayer which should be calibrated prior to use and the rate of application be expressed per unit area.

The following formula is used to determine the application rate:

$$\text{Rate of application (ml/m}^2\text{)} = \frac{\text{Flow rate (ml/min)}}{\text{Width of swath (m) x walking speed (m/min)}}$$

The required concentration of larvicide suspension is calculated as follows:

$$\text{Concentration of larvicide (Emulsion/suspension)} = \frac{\text{Dosage to be applied (g/m}^2\text{)}}{\text{Application rate (ml/m}^2} \times 100$$

Other formulations such as granules, pellets, tablets and briquettes can be manually broadcast or thrown in the water.

Pos-treatment immature densities (all stages) should be monitored on 1st, 2nd, 3rd and 7th day post-treatment and then weekly twice until the density of fourth instar larvae (or pupae in case of IGRs) in the treated habitats reaches a level comparable to that in the control. Temperature and pH of habitat water should be recorded on each day of observation. Data are recorded on the form (Table 40). Dissolved oxygen, dissolved and total solids in the habitats should also be determined at two points of time during the trial period.

The assessment of an IGR's efficacy is based on the level of inhibition of emergence of adults and the percentage reduction in larval and pupal densities. Larvae and pupae are sampled as described above. Adult emergence can be monitored directly in the field by floating sentinel emergence traps in treated and untreated habitats (Table 42), by pupal isolation, or by sampling and counting pupal skins. Adult emergence may also be assessed by collecting pupae (20-40 per replicate) and bringing them to the laboratory in glass containers with the water from the respective habitats, then transferring them to small cups inside the holding cages. Dead larvae and pupae found in the cups should be removed and any morphological abnormalities recorded.

When monitored directly in the field, the pre-treatment and post-treatment data on adult emergence in treated and untreated habitats are analyzed for IE%. The following expression is used to calculate IE% values:

$$\text{IE (\%)} = 100 = (C1/T1) \times (T2/C2) \times 100$$

Where C1 is the number of adults emerged in control habitats before treatment, C2 the number of adults emerged in control habitats at a given interval after treatment, T1 the number of adults emerged in treated habitats before treatment and T2 the number of adults emerged in treated habitats after treatment.

When adult emergence is monitored in the laboratory using pupae collected from treated and untreated habitats, IE% is calculated using the following formula, on the basis of determining adult emergence from the number of pupae isolated (see also Section 2.1.1.3);

IE (%) = (C-T/C) x 100,

Where C = percentage emerging or living in control habitats and T = percentage emerging or living in treated habitats.

2.5.2.3.1 Data analysis

The mean number of pupae and larvae collected per dip is calculated for each sampling day and for each replicate. The reduction of larval and pupal densities or the IE% on post-treatment days will be estimated by comparing the pre- and post-treatment densities in the treated habitats with the corresponding densities in the untreated habitats using Mulla's formula.

$$\% \text{ Reduction} = 100 - \frac{C1 \times T2}{T1 \times T2} X\ 100$$

Where,

C_1 = Pre-treatment immature density in control sites
C_2 = Post-treatment immature density in control sites
T_1 = Pre-treatment immature density in treated sites
T_2 = Post-treatment immature density in treated sites

The differences between the dosages can be compared by two-way ANOVA with dosage and day as the main factors after transforming percentage reduction to arcsine values. The interaction effect of dosage and day is used to compare the effect of treatment over days. Pair wise comparison of dosages is done using the pos-hoc test based on least significant difference (LSD). The mean arcsine values should be back transformed to percentage values. The post-treatment day up to which 80% or 90% reduction is observed for each treatment or dosage will then be compared to determine the optimum application dosage and the residual effect (effective duration). (The optimum field application dosage is to be determined on the basis of effective duration for each dosage. The effective duration is the post-treatment day up to which the lower limit of the 95% CI for the mean % reduction of density will be >80%).

2.5.2.4 Simulated field trials

These trials are conducted for the mosquito species breeding in domestic and peri-domestic habitats in clean water. Trials should be carried out in containers (drums, jars, buckets, tubs, etc.). For *Cx. quinquefasciatus* field trials are not undertaken in simulated condition.

The efficacy of the larvicidal formulations is tested against laboratory reared *Aedes aegypti, A. albopictus* and *Anopheles stephensi* larvae under simulated field conditions. Cement tubs with 100 L or 200 L capacity (used commonly by households) are used for the trial. The diameter of the tubs at the water surface should be 75 cm.

Prior to testing, the cement tubs are decontaminated by filling them fully with water and setting them open in the sun. The tubs are then emptied, scrubbed, rinsed thoroughly with water and dried in the sun or shade for a day or two. The new tubs filled with water are allowed to sit for a week or two before the treatments. The tubs are placed under a roof, where the area is open on all sides, simulating field conditions. The placement of the tubs is configured in a block design form to equally distribute positional effects. The tubs are filled with domestic tap water (100 L or 200 L) up to the top. The tubs are covered with nylon mesh screen to prevent other mosquitoes or other insects from laying eggs and to protect the water from falling debris.

Two regimens of water can be used: In the first regimen, tubs are kept full for the duration of the experiment without removing water; and in the second regimen, half of the water in the tubs is removed and replenished weekly with fresh tap water to simulate water use conditions. The same two regimens are used for control as well as treatment.

To assess the efficacy, a batch of 50-100 laboratory-reared late third instar larvae of *A. aegypti* / *A. albopictus* is released into each cement tub or replicate. Before adding first cohort of larvae, 0.5 g ground up larval food is added to each tub initially and weekly thereafter. After 2-3 h of larval acclimation, the tubs are treated with the larvicidal formulation at 3-5 dosages in a randomized manner. In each water regimen, a minimum of four replicates of each dosage and four controls should be used. The water level in the tubs must be sustained.

For fast acting chemical larvicides, all the containers are examined after 48 h and live larvae are counted to score post-treatment larval mortality. For slow acting compounds, such as IGRs, the survival of larvae, pupae and pupal skins is assessed 2 days or more after treatment. The pupal skins provide the best gauge of final or overall effectiveness. To test residual activity, the treatments are challenged with new cohorts of larvae (third instar) of the same mosquito species weekly and larval food is added on alternate days or weekly. Larval survival is assessed 48 h post addition, and pupal skins are counted 2 days or more after addition. This process continues until no mortality is observed. Data are recorded on the form in Table 41.

For the IGRs under test, pupae are removed from the treated and control containers every other day and put into vials or cups with water from the respective containers, then placed in cages and adult emergence is recorded. Another precise method of assessing emergence is to count and remove pupal skins from containers (Table 42). Adults not freed from pupal skins are considered dead. The test is terminated when there is no statistically significant residual activity in terms of larval and pupal mortality or inhibition of emergence when comparing the treated batches and the untreated controls. Values of pH and water temperature should be recorded throughout the evaluation.

2.5.2.4.1 Data analysis:

Efficacy and residual activity of the larvicide at different dosages are determined from the post-treatment counts of live larvae and pupae in treated and control replicates as compared to the pre-treatment counts. The criteria for determining the level of effectiveness of a candidate larvicide should be >80% reduction in the pre-treatment counts (% reduction is calculated using the Mulla's formula).The method given in Section 5.2.3.1 should also be used to analyse data collected under simulated trials. However, since the denominator is known for simulated trials, a probit or logistic regression analysis is more suitable than ANOVA and is described below.

The data on the number of live and dead larvae and pupae from all replicates of each dosage on one day should be combined and percentage mortality calculated. Logistic or probit regression of the percentage mortality on dosage and number of post-treatment days should be used to determine the post-treatment day (and its 95% CI) up to which 80% or 90% (the desired level of control) is achieved for a given dosage. This analysis should be done using appropriate statistical software packages.

2.5.3. Phase III: Large-scale field trials

The efficacy of larvicides found to be suitable in small-scale field trials (Phase II) should be validated in larger scale field trials against natural vector populations in natural breeding habitats. In this phase, the larvicide is applied to the breeding sites of the target mosquito at the optimum field dosage(s) selected in the small-scale field trials using appropriate application equipment, depending on the formulation. The trials are to be conducted at least in three different eco-epidemiological settings.

2.5.3.1. Duration

Evaluation should be carried out for a period of one year covering three different seasons.

2.5.3.2 Objectives

The objectives of the trial are:

- To confirm the efficacy of the larvicide at the selected field application dosage(s) against the target vector when applied to large-scale plots in natural breeding sites;
- To confirm residual activity and application intervals;
- To record observations on the ease of application and dispersal of the insecticide;
- To observe community acceptance;
- To record any perceived side-effects on operators; and
- To observe the effect of the treatment on non-target organisms.

2.5.3.3 Selection of study sites

A locality of one square kilometer of urban area should be selected for treatment and a locality of similar type and size for control. All the types of breeding habitats should be surveyed to ascertain the breeding of target species in order to decide the suitability of the locality for the trail. A selected locality should have a minimum of 25 percent larval habitat positivity for the target vector species. Just as for the small-scale trials, each confined habitat can be considered as an individual replicate; larger habitats can be subdivided into replicates of about 10 m^2.

2.5.3.4 Assessment of pretreatment density

Pretreatment larval and pupal densities (and adult emergence in the case of IGRs) in the treatment and control habitats should be carried out for a week on at least two occasions before treatment. The immature population and adult emergence should be estimated in different types of larval habitat by using appropriate sampling devices (as in the small-scale field trials with natural populations).

2.5.3.5 Application of larvicide

All the breeding sites within the unit should be treated at the optimum field application dosage determinate in Phase II, using equipment that is appropriate to the formulation and its operational use. The optimum dosage for the major or most important larval habitat of the target species in the area can be used for all the habitats. Where small-scale trials found wide variation between optimum dosages for each type of habitat, the specific optimum dosage should be applied to each type of habitat. The habitats will be re-treated either at weekly or fortnightly intervals depending on the residual activity determined for the larvicide/IGRs for each type of habitat in phase II trial.

2.5.3.6 Assessment of post-treatment density

The impact of larvicidal treatments on the larvae and pupae of mosquitoes (and the inhibition of adult emergence) should be evaluated by sample collection at 48 h and then at weekly intervals using a fixed number of dips. Sampling procedures are similar to those followed for small-scale trials conducted in natural breeding habitats. Data should be recorded on the relevant form (Tables 5.4 and 5.5). Observations will be made for a minimum of three applications/treatments for each trial. The trials should be repeated at least three times on different seasons.

2.5.3.7 Impact on adult density

In addition to estimation of larval density, adult mosquito density in the localities should be monitored fortnightly by total catch method. At least 8 structures randomly selected should be used for the estimation of density per structure in treated and control areas. This will provide information on the trend in the reduction of target mosquito species.

2.5.3.8 Effect on non-target organisms

Specific, separate trials have to be carried out to assess the impact of larvicides on non-target organisms. However, during the large-scale trial, and where appropriate, non-target organisms

cohabitating with mosquito larvae can be counted and examined for impact of treatments while sampling mosquito larvae. Larvivorous fish, snails, polychaetes, shrimps, cray fish, crabs, mayfly naiads, copepods, dragonfly naiads, coleopterans and heteropterans, ostracods and amphipods are some of the non-target organisms that coexist with mosquito fauna.

2.5.3.9 Operational and community acceptability

During the trial, observations should be made on the ease of storage, handling and application of the insecticide formulation on the breeding sites, and of the effects of the insecticide formulation on the proper functioning of application equipment such as nozzle tips and gaskets, rotors, blowers, etc.

Observations are also recorded on the acceptability of the insecticide treatments to the residents of the area, particularly on domestic and peri-domestic breeding sites.

2.5.3.10 Data analysis

The mean number of pupae or larvae or non-target organisms collected per dip on each day of observation is calculated for each replicate in treatment and control. The statistical analysis to determine residual efficacy- including the number of post-treatment days over which the desired level of control is achieved at the selected dosage- is carried out following the method described in Section 5.2.3.1.

2.6. Monomolecular films

2.6.1. Phase I trial

2.6.1.1. Duration

Total duration of the trial is 3 months.

2.6.1.2. Objective

- To assess the effective dose and efficacy of MMF

2.6.1.3. Determination of the dose for treatment and its efficacy

The monomolecular films (MMF) of organic compounds can act as larvicide by reducing the surface tension of the aqueous surface and subsequently killing the immature by interfering with spiracular opening at the water interface and preventing tracheal respiration. Because of this property this can be used for source reduction. In laboratory trials monomolecular film should be tested against all instars and pupae. Monomolecular films are effective only on clean water surface.

Different doses (1 mL/m^2) should be applied using pipette. Effective dose is one, which forms a no molecular layer over the entire surface of water, which as determined in laboratory. Rectangular enamel trays (45 x 30 cm) or (90 x 60 cm) should be filled with known volume of water (2 to 5 litres) and MMF should be applied in 6 different doses in separate trays. The effective dose is the lowest dose that covers the entire surface of the water. This can be ascertained by putting rice husk or coloured powder supplied by the manufacturer as indicator for spreading.

To determine the efficacy of the selected dose, 100 laboratory colonised I/II (1-3 days old) instar larvae and III/IV (4-8 days old) instar larvae should be released in individual trays with parallel control. For each instar a minimum of 3 replicates should be set and mortality of larvae should be recorded based on the larval stage till getting 100% death. (after 24, 48, 72 h and up to 2 weeks. Data should be recorded in the format given in Table 43. Assessment should be ascertained by counting number of larvae dead. In general, control of 1st to 4th instar mosquito larvae is relatively slower than control of pupae, and control of younger instars (1st to 3rd) is slower than 4th instars. Pupicidal effect is determined releasing 15 pupae in plastic cup holding 40 mL water. Based on the recommended dose the surface area of the cup is calculated and the cup was treated. The number of dead pupae was counted after every 15 minutes for 72 hours. Four replicates are to be performed. A pupa is considered dead if it did not show the characteristic stretching reaction n slight dipping.

Table 43. Observations on the MMF (to be revised)

No. exposed	Mortality after	Pupal /Adult emergence				
	24 h	48 h	72 h	1 week	2 week	3 week
I/II Instar III/IV Pupae						

2.6.2. Phase II trial

2.6.2.1. Duration

Evaluation should be carried out for 12 months.

2.6.2.2. Objectives

- To evaluate the efficacy of MMF different natural habitats or in simulated habitats
- To assess the persistence of the larvicide/ pupicide efficacy in different breeding habitats of the target vector species
- To determine the effective dose and frequency of application for Phase III trial

2.6.2.3. Efficacy in field

Natural breeding habitats of the target species have to be selected for the evaluation. For *Anopheles* spp. cement tanks, drums, pits, pools, water fountains and disused wells; for *Cx. quinquefasciatus* stagnant drains, pits, pools and disused wells and for *Aedes* spp. tanks, drums, discarded tyres, peri-domestic water storage containers, coolers and water fountains are best suited. Selected habitats should have a minimum density of 5 to 10 larvae per dip. A minimum of 3 replicates should be used for each type of habitat and dose. Each habitat should have at least 2 controls. Temperature, pH and water quality (polluted or clean) should be recorded.

Determine pre-treatment larval population density by dipping method using a standard dipper (300 ml capacity with 9 cm diameter) for pits, ponds, tanks, drains, drums, etc., and bucket well net (22.5 cm top diameter, 15.5 cm bottom diameter and 22.5 cm height) for wells. Number of samples to be taken from each habitat should be decided on the basis of type and size of the habitat. For small habitats such as drums, pits, pools, tanks and wells, 3 to 5 dips per site, while for stagnant drain dips at a distance of 5 m are recommended. MMF formulation should be applied 0.3-0.4 ml/m^2 with non pressurized plastic hand sprayer along the upwind edge and around the vegetative perimeter of the pond/ habitat and allowed to spread over the entire surface of the water/ or the breeding habitats. Spreading action of the MMF formulation is checked by the movement of rice Krispies placed on the water surface before application. Based on the size of the habitats the formulation is applied manually with a 10 ml measuring cylinder. The rate of application is expressed per unit area.

Larval sampling of treated and control habitats are made a day before the application of the MMF formulation for assessing the pre-treatment densities. Sampling after application is made at 24, 48 and 72 h and later at weekly intervals (minimum for 6–8 weeks) using the above mentioned methods. Larvae collected from the habitats should be categorized into I/II and III/IV instars and larvae and pupae recorded in the format given in a Table 44. The larval density per dip is calculated by dividing the total number of larvae collected by the number of dips taken. Natural predators if any should be scored.

Percent reduction in III/IV instars larvae and pupae should be calculated using the Mulla's formula.

$$\% \text{ Reduction} + 100 - \frac{C1xT2}{C2xT1} \times 100$$

Where,

C_1 = Pre-treatment immature density in control sites
C_2 = Post-treatment immature density in control sites
T_1 = Pre-treatment immature density in treated sites
T_2 = Post-treatment immature density in treated sites

Persistence of the MMF formulation in different breeding habitats of the target species is determined from the post-treatment density of larvae and pupae in treated and control sites as compared to the pre-treatment density. Achievement of >80% reduction in the treated habitats is considered as an effective dose for field application. Using this norm efficacious persistence period in the field can be calculated.

2.6.2.4. Simulated field trial:

These trials are conducted for the mosquito species breeding in domestic and peri-domestic habitats in clean water. Trials should be carried out in containers (drums, tanks, etc.). For Cx. quinquefasciatus field trials are not undertaken in simulated condition. For anophelines, cement tanks, each having a capacity of 100 litres filled with 40 to 50 litres of potable water with different concentrations of insecticides should be used. In each tank 1000 to 2000 first instar larvae of the target species should be released at weekly intervals for a period of 4 weeks.

Trials for *Ae. aegypti* should be carried out in 10 to 20 litre capacity drums with 5–10 litres of potable water. 100 to 200 larvae of Aedes should be introduced into the treated drums at weekly intervals for a period of 4 weeks. The tanks and drums should be covered with specially designed traps (dome shaped) to score adult emergence and prevent oviposition by other mosquito species/insects.

Water level in the tanks/drums should be maintained and finely ground larval food (yeast powder and dog biscuit mixture in a ratio of 60:40), should be provided daily until the completion of the experiment. Pre-treatment immature density should be determined by taking 3–5 dips covering the entire surface area and instar-wise data should be recorded. The live immature stages should be released back into the respective tanks/ drums.

The MMF formulation, at three selected dosages within the recommended range of doses of application should be applied with non pressurized plastic hand sprayer along the upwind edge and around the vegetative perimeter. Each dose should be applied to a minimum of 3 tanks/drums and control replicate should be left untreated.

Larval sampling of treated and control habitats are made at 24, 48, 72 h and later at weekly intervals for 6–8 weeks using dipping method. Initial and long-term efficacy should be assessed on the basis of the observed larval and pupal density. Data should be entered in the proforma (given below).

Efficacy and residual activity of the larvicide are determined from the post-treatment counts of larvae and pupae in treated and control replicates as compared to the pre-treatment counts. The criteria for determining the level of effectiveness of a candidate MMF formulation should be >80% reduction in the pre-treatment counts (% reduction is calculated using the Mulla's formula).

2.6.3. Phase III trial

2.6.3.1. Duration:

Evaluation should be carried out for 24 months.

2.6.3.2. Objectives

- To evaluate efficacy of MMF against larvae/ pupae in a locality
- To assess the persistence of the larvicide/ pupicide
- To determine the operational dose and its frequency of use

2.6.3.3. Study area

A locality of one square kilometre of urban area should be selected for treatment and a locality of similar type and size for control. All the types of breeding habitats should be surveyed to ascertain the breeding of target species in order to decide the suitability of the locality for the trial. A selected locality should have a minimum of 25 percent larval habitat positivity and 5–10 larvae per dip per habitat.

Evaluation of MMF formulation: The dose and frequency of application determined in Phase II trial will be employed for Phase III trials. The MMF application initially should cover all the habitats and subsequent applications should be undertaken either at weekly or fortnightly intervals depending on the persistence determined for the MMF for each type of habitat in Phase II trial. The MMF formulation is applied with non pressurized plastic hand sprayer along the upwind edge and around the vegetative perimeter. Spreading action of the MMF formulation is checked by the movement of rice Krispies placed on the water surface before application.

Estimation of larval density should be done at weekly intervals from different habitats on the basis of a fixed number of dips. Standard dipper should be used. Sampling should be made at a distance of 5 metre for larger habitats and 1 metre for smaller habitats. Care should be taken to cover all the sides. Data should be recorded (as given below format). Thus the impact of larvicide application is monitored by sampling and calculation of efficacy of the larvicide using Mulla's formula (As given above).

In addition to estimation of larval density, adult mosquito density in the localities should be monitored fortnightly by total catch method. At least 8 structures randomly selected should be used for the estimation of density per structure in treated and control areas. This will provide information on the trend in the reduction of target mosquito species.

Table 44. Field evaluation of MMF formulation against Anopheles / Culex / Aedes larvae/ pupae in (Type of habitat)

Duration after Treatment	Mean no. of 5 dips		
	I and II Instar larvae	III and IV instar larvae	Pupae
0 day			
1 day			
2 day			
3 day			
4 day			
1 week			
2 week			
2 week			
...			
8 week			

Table 45. Percentage reduction of larvae/ pupae in MMF formulation treated habitat (Type of habitat)

Habitat	Dosage	Hours/ Day Post-treatment	% reduction Larvae	% Reduction pupae	Total
Pond	Dose I	0 day			
		1 day			
		2 day			
		3 day			
		4 day			
		1 week			
		2 week			
		3 week			
		8 week			

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4. ANNEXURES

Annexure 1

GUIDELINES FOR DEVELOPMENT OF INFORMED CONSENT FORM
(WHO/HTM/NTD/WHOPES/2009.2)

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

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

PART I: Information sheet

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

1. Introduction

State briefly who you are, and explain to participants that you are inviting them to take part in research that you are doing.

2. Purpose of the research

Explain in lay terms why you are doing the research.

3. Type of research intervention

State briefly the type of intervention that will be undertaken.

4. Participant selection

State why this participant or household has been chosen for this research. The selection will ensure that equal opportunities are provided to everybody.

5. Voluntary participation

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

6. Information on the test product [name of the test product]

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

7. Description of the process, procedures and protocol

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

8. Duration

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

9. Side-effects

Potential participants should be told if there are any known or anticipated side-effects and what will happen in the event of a side-effect or an unexpected event.

10. Risks

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

11. Discomforts

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

12. Benefits

Mention only those activities that will be actual benefits and not those to which they are entitled regardless of participation.

13. Incentives

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

14. Confidentiality

Explain how the research team will maintain the confidentiality of data, especially with respect to the information about the participant, which would otherwise be known only to the physician but would now be available to the entire research team.

15. Sharing the results

Where relevant, your plan for sharing the findings with the participants should be provided.

16. Right to refuse or withdraw

This is a reconfirmation that participation is voluntary and includes the right to withdraw.

17. Whom to contact

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

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

PART II: Certificate of Consent

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

Print name of participant: _____

Signature of participant: _____

Date: _____

day / month / year

Annexure 2

Assessment of perceived benefits, side-effects and collateral benefits of Indoor Residual Spraying

Date of spraying ……..Date of interview/discussion …….....

1. Name of respondent: (Optional).....
2. Age:.....
3. Sex:.....
4. Education status:.....
5. Village name:.....
6. Do you know that insects transmit diseases ?.....
7. If you know, name the diseases .....
8. Do you protect yourself and family against these diseases ?.....
9. If so, how..... Indigenous.....Commercial .....
10. Are you aware whether something was sprayed in your house?
    If yes, when and why.....
11. Generally how many people sleep in the sprayed rooms(s)?.....
12. Do you sleep in sprayed room? .....
13. How does it smell?.....
14. Do the sleepers feel suffocated?.....
15. Have you allowed spraying in all rooms?: ..... If no, reasons .....
16. Does the insecticide leave stains on walls? .....
17. Any fear of poisoning:.....
18. Observations/perceptions of the effect of insecticide
    - on mosquito bites
    - on bed bugs
    - on head lice
    - on body lice
    - on domestic animals
    - any other
19. Do you agree to use insecticide spray in future? YES/NO

Reasons .....

Signature or LTI of inhabitant Signature of Interviewer

Place/Date:

(This format should be translated into respective local language(s) in the study area and provided to the householder and read to him. A copy of the signed consent form should be given to the householder).

Annexure 3

Human safety observations after insecticide exposure

(Medical Practitioner should fill this proforma)

Project Title:..... Institute:.....

Part A. Medical case history form

1. Spray man/Volunteer S.No.:.....
2. Name:.....
3. Age(yr): .....
4. Gender:.....
5. Occupation: .....
6. Address:.....
7. Past history:.....
   a. IlLLINess: Yes/No b. Poisoning: Yes/No c. Allergy: Yes/No
8. Exposure to pesticides (mention compound, duration of exposure etc.):
9. Family History:
   a. Allergy: Yes/No b. Mental IlLLINess: Yes/No c. Hemorrhagic disorders: Yes/No
10. Personal history
    a. Protective clothing: Complete/ Partial/ None
    b. Ablutions (washing/bathing/cloth changing): Good/ Fair/ Poor
    c. Personal habits: Smoking/ Alcohol/ Other addictions
11. Weather conditions: Temperature: Min.....Max.....
    Relative humidity (%): Min..... Max.....
12. Clinical profile (sign & symptoms) pre- and post-exposure:

(a) Vital signs	Pre-exposure (/ /2000)
Pulse rate	
Respiratory rate/minute	
Depth of respiration	
Temperature oF	
Chest tightness	
(b) General	Pre-exposure
Weakness	
Fatigue	
Sleep	
Urination	
Sweating	

(Contd.....)

(c) Gastro-intestinal					
	Pre-exposure	1 h	24 h	48 h	72 h
Nausea					
Vomiting					
Appetite					
Taste					
Abdomen pain					
Diarrhoea					
Siallorrhoea					
(d) Neuro-muscular					
	Pre-exposure	1 h	24 h	48 h	72 h
Headache					
Dizziness					
Irritability					
Pain					
Twitchings					
Tremors					
Convulsions					
Parasthesia					
Hallucinations					
Unconsciousness					
(e) Cardio-respiratory					
	Pre-exposure	1 h	24 h	48 h	72 h
Nasal discharge					
Wheeze					
Cough					
Expectoration					
Chest tightness					
Dyspnea					
Palpitation					
Heart conserveness					
Cyanosis					
Tachycardia					
(f) Eye					
	Pre-exposure	1 h	24 h	48 h	72 h
Miosis					
Lacrimation					
Double vision					
Blurred vision					
(g) Psychological					
	Pre-exposure	1 h	24 h	48 h	72 h
Temperament					
Judgement					
Nervousness					
Insomnia					

X = No; N = Normal; NAD = Nothing abnormal detected; Skin (Dermal reaction/Irritation/Allergic reaction):

13. Human toxicology proforma for liver and kidney function tests

Liver function tests

1. Serum bilirubin
2. SGO
3. SGPT
4. Serum alkaline phosphatase
5. Serum protein

Kidney function tests

1. Blood urea
2. Serum creatinine

Signature of Medical Officer/Physician

Date: (Seal)

Place:

Part B. Nerve conduction studies in spraymen

1. Time of recording and sample size—Study should be on at least 5 spraymen exposed to insecticide spray at the following frequency:
   – Before spray
   – Second study to be done three days after insecticide exposure
   – Third study to be done after five days of insecticide exposure

2. Nerves to be studied (on the right side of the subjects):
   – Median (Motor)
   – Lateral popliteal (Motor)
   – Facial nerve
   – Median- Orthrodromic sensory
   – Sural- Antidromic sensory
   – Blink response-early Phase

3. Suggested machine for the study—MEDLEC MSA Machine

4. Proforma for clinical diagnosis:
   Clinical Reg. No.....Date:.....
   Name: ..... Age: ..... Sex:.....

Nerve conduction study

1. Right/Left MEDIAN (Motor): THENAR MUSCLES: SURF. ELE.

   Wrist..... Elbow.....Supraclavicular.....
   Amp.....
   Latency..... msec..... msec..... msec.....m.v.
   Distancy .....cm ..... cm ..... cm
   Conduction velocity .....metres/sec. (Wrist to elbow)
   Conduction velocity .....metres/sec. (Elbow to supraclavicular region)

2. Right/Left ULLINar (Motor): Hypothemar muscles: Surf..... Ele.....
   Wrist..... Elbow..... Supraclavicular.....Amp.....
   Latency..... msec.....msec..... msec.....m.v.
   Distancy .....cm ..... cm ..... cm
   Conduction velocity ..... metres/sec (Wrist to elbow)
   Conduction velocity ..... metres/sec (Elbow to supraclavicular region)

3. Right/Left Lateral Popliteal: Ext. Dig. BR. : Surf. Ele.

   Ankle..... Knee.....Amp.....
   Latency..... msec.....msec..... msec.....m.v.
   Distance .....cm ..... cm .....cm
   Conduction velocity ..... metres/sec.

4. Right/Left Sural nerve (Antidormic-Sensory): Neelle Ele.

   Amplitude .....uv
   Latency ..... msec
   Distance .....cm
   Conduction velocity …….. m/sec

5. Right/Left Median (Orthrodromic Sensory)
   Stimulation- digital nerves-index finger
   Recording at wrist: Needle Ele./Surf. Ele.
   Amplitude .....uv.
   Latency .....msec

6. Right/Left ULLINar (Orthrodromic Sensory)
   Stimulation-digital nerves-index finger
   Recording at wrist: Needle Ele./Surf. Ele.
   Amplitude ..... uv.
   Latency ..... msec

7. Right/Left Facial nerve

Muscles Latency.....Amplitude.....Distance.....
Orb. oris .....msec ..... mv/uv ..... cm
Frontails..... msec ..... mv/uv ..... cm
Orb. oculi .....msec ..... mv/uv ..... cm

8. Needle Electromyography

(i). Fibrillations..... Fasciculations .....Insertional activity .....
Mystonic .....Interference pattern .....Amplitude of motor units .....

(ii). Fibrillations ..... Fasciculations ..... Insertional activity.....
Mystonic .....Interference pattern .....Amplitude of motor units .....
(iii). Fibrillations ..... Fasciculations .....Insertional activity.....
Mystonic .....Interference pattern.....Amplitude of motor units .....

9. Blink response study
Needle electrode (Conc.): Right Orb. Occuli.

Stimulation.....
Latency.....
Early Response ..... msec.
Late Response ..... msec.

Signature of Medical Practitioner

Date : (Seal)
Place :

Annexure 4

Informed consent form for human volunteers participating as bait in the insecticide evaluation studies

Project Title:.....

Name of the Institute and Address:.....

Names of the responsible Investigators:.....

I understand that I have been asked to take part in the trial of a new insecticide in our village. I have been told that this study is being done to control mosquitoes/sand-flies. I understand that I will be required to act as bait for the studies to assess the impact of the IRS/ITN/LLIN/repellents. I also understand that I will be employed both as active and passive bait for the insects. The study will be conducted during night usually from dusk to dawn.

I am informed that the agent being used in the trial will not cause risk or discomfort to human beings at the recommended dose.

I also understand that the Principal Investigator can exclude me from the study without my consent at any time. However, I am also free to withdraw from the study without assigning any reason and without any implications thereof.

I have gone through the contents in the consent form, understood and agree to abide by them. I am briefed about the precautions that will be taken during the experiment and also assured of against any liability or risk and I agree to participate voluntarily.

If I have any question about the study, I should contact (Name of the Principal Investigator) or (Name of the Investigator) for reporting any discomfort or for immediate medical help (if needed).

Signature/thumb impression of the volunteer Date: /

House No.:..... Village:..... PHC/CHC:..... District:.....State:.....

Signature of Principal Investigator/Investigator

(This format should be translated into respective local language (s) in the study area and provided to the householder and read to him. A copy of the signed consent form should be given to the householder).

Annexure 5

Informed consent from householders (ITN and LLIN)

Project Title:.....

Name of the Institute and Address: .....

Names of the responsible Investigators:.....

I understand that my family and I have been asked to take part in the trial of a new insecticide in our village. I have been told that this study is being done to control mosquitoes/sand-flies and that my house should be provided with insecticide treated nets and my family should be asked to sleep inside the net. The period of study is ..... months.

I am informed that the insecticide treated net causes no considerable risk or discomfort to human beings, if used at the recommended dose. I and all the adult members of the family are given necessary instructions about the safe use for self and children and proper storage of these nets.

I am told that my house/room might be modified to fit mosquito traps at the cost of the Investigators, but would be restored at the end of the study. I have been informed not to wash the net (s) supplied to us. During the study period I understand that the teams (Name of the Investigating Institute) may also visit our house even at odd hours for collection of mosquitoes and doing other tests on the nets.

I also understand that the Principal Investigator of the study can exclude me or my house from the study without my consent. However, I am also free to withdraw from the study without assigning any reason and without any cost implications thereof.

I have gone through the contents in the consent form, understood and agree to abide by them. I shall also apprise the members in my family of the contents in consent form and assure needed cooperation and precautions for the completion of the trial.

If I have any question about the study, I should contact (Name of the principal investigator) or (Name of the Investigator) at (Address of the Office/Institute) or for immediate medical help to the Medical Officer In charge (Name of the Medical Officer) , Primary Health Centre...../CHC..... in need.

I and members of my family agree to participate in the study voluntarily and not under duress or pressure or for remuneration.

Householder's signature/thumb impression Date:.....

House No.:.....Village:.....PHC/CHC:.....District:.....State:.....

Signature of Principal Investigator/Investigator

Place:

Date:

(This format should be translated into respective local language(s) in the study area and provided to the householder and read to him. A copy of the signed consent form should be given to the householder).

Annexure 6

Assessment of community perceptions on adverse effects and collateral benefits of insecticide treated nets (ITN) and long-lasting insecticide-treated nets (LLIN)

Date of supply…….. and interview/discussion ……..

1. Name of respondent: (Optional)
2. Age
3. Sex
4. Education status
5. Village name
6. Do you know why mosquito nets are used?
7. Do you use nets for protection for yourself/members of the family?
8. What are the other methods you use for protection?
9. Do you use any indigenous method for protection?
10. Are you aware whether something was provided for personal protection in your house? If yes, when and why?
11. Generally how many people sleep inside the net(s)?
12. Do you sleep inside the net?
13. How does it smell?
14. Do you feel any of the following?
    Skin irritation Nausea Vomiting Itching Headache
    Drowsiness Eye irritation Difficulty in breathing Any other
15. Do the sleepers complain about suffocation?
16. Any fear of poisoning:
17. Observations/perceptions of the effect of insecticide-treated bed net or LLIN
    - on mosquito bites
    - on bed bugs
    - on head lice
    - on body lice
    - on domestic animals
    - Any other

18. Do you recommend use of the new insecticide-treated net in future? Yes/No
    Reasons

Signature of Interviewer

Place:
Date:

(This format should be translated into respective local language/s in the study area and provided to the householder and read to him. A copy of the signed consent form should be given to the Householder).

Annexure 7

Form for tunnel bio-assay

Name of person doing bioassays: ……...

Date of test: LN Code: Temp:…….. °C RH: ……. %

Test mosquito species and strain:

Age of mosquitoes: ……..days

Test start time (hour/min): End time:

		Blood-fed females		Unfed females		Total	
		Alive	Dead	Alive	Dead	Alive	Dead
Control	Compartment 1[a]						
	Compartment 2[b]						
	Total						
Treatment (LN)[c]	Compartment 1						
	Compartment 2						
	Total						

[a]Compartment 1 refers to the long section of the tunnel where mosquitoes are released.

[b]Compartment 2 refers to the section between the test netting and the animal bait

[c]Additional treatment rows to be added to record data when more than one sub-sample of the same net or netting samples from other nets are run in parallel.

Annexure 8

Questionnaire for community acceptability, physical integrity and washing methods of nets

Title of the project:

Name of Principle Investigator:
Name of Organization:
Name of Sponsor:

Five digit survey code (first two digits country; one digit village; two digits for sample: -------

Country …… State: ……. District …….

Village …….. Nearest town ……

Date of survey (DD/MM/YY): ……/……/…….

1. Net usage and acceptance

Information on net usage provided by:
1) User of this net
2) Caretaker of those using the net
3) Head of household
4) Other (specify) ……

Information on net usage:
1) Year-round and every night.
2) Year-round but occasionally.
3) Seasonally but every night.
4) Seasonally and occasionally

How is the net used?
1) Hanging over the bed
2) Hanging over sleeping mat/mattress on the ground
3) Other (specify) ……

Does sleeping under the net have any adverse or beneficial effect on you?
1) Yes 2) No

If Yes, describe the effect. ……

When was the last time you washed the net? …….. (month)

How frequently you wash the net? ……. (month)

How many times have you washed the net?

How was the net last washed?

Water:

1) cold …. …..2) warm …….. 3) hot …….

With or without soap……

Soap:

1) Village (local)-made soap
2) Commercial bar
3) Commercial powder
4) Mix of soap and powder

Rubbing against rocks/stone:

1) Yes …. 2) No ….

Where was the net dried after washing?

1) Inside 2) Outside under shade 3) Outside under the sun

2. Physical inspection of nets

2.1 Does net have holes?

1) Yes … 2) No …

If yes, use the following code for sizes of holes

1) hole smaller than will allow a thumb to pass through
2) a larger hole, but will not allow a closed fist to pass through
3) hole bigger than a closed fist

Total number of holes per net:

…….. size 1
…….. size 2
…….. size 3

Total number of holes:

…….. lower half of the net
…….. upper half of the net
…….. roof

Total number of open/failed seams using the size coding provided above:

…….. total size 1
…….. total size 2
…….. total size 3

Total number of repairs:

….. with stitches
….. with knots
….. with patches

Total number of holes due to burns? # ……

Aspect of net:

1) clean
2) a bit dirty
3) dirty
4) very dirty

3. Assessment of attrition rate

1. Number of nets of each size provided to the household in the beginning:

2. Number of nets physically present on the day of visit:

3. If a net is found lost to follow up, give main reason for loss of each net (s):

(Ask openly what happened to the nets and depending on the answer probe for other possibilities, e.g. lost, sold, given to relation or friend, worn out, burnt, and eaten by rats)
Record the number of nets remaining in the house and for each one record the number/size of holes and tears to give an indication of the rate of wear and tear[6]

Name of investigator ……
Signature ……

[6] *This is a check on the truthfulness for the reasons given for the loss. We need to distinguish loss due to wear and tear (true attrition) from loss due to misdemeanour (e.g. selling on). If the remaining nets are quite holed, loss due to wear and tear would appear genuine.*

Annexure 9

Assessment of adverse effects, if any, among impregnators of nets

Title of the project:

Type of treatment: …….

1. Code # of the respondent impregnator:

2. Age of the respondent (years):

3. Education status:

4. Date(s) of net impregnation (dd/mm/yy): 5. No. of nets treated:

6. Date(s) of side-effects survey (dd/mm/yy):

7. Following treatment of nets did you observe any of the following effects (record past and present experience)

		Yes	No	If yes, duration
a	Itching of your skin			
b	Facial burning/tingling			
c	numbness or a loss of physical sensation and/or tingling of your skin (paraesthesia)			
d	Sneezing			
e	Liquid discharge from your nose			
f	Feeling of headache			
g	Symptom of nausea			
h	Eye irritation			
i	Tears coming from your eyes			
j.	Experience bad smell during use of nets			
k.	Body rashes			

Signature of interviewer
Date (dd/mm/yy):

Annexure 10

Assessment of adverse effects, if any, among ln/treated net users

Title of the project:

1. Household Code #: Net Code:

2. Date(s) of receipt of ITN/LN (dd/mm/yy):

3. Number of nets provided by project:

4. Number of coded-net users in your house:

5. Following the use of nets did you observe any of the following effects (record past and present experience)

		Yes	No	If yes, duration
a	Itching of your skin			
b	Facial burning/tingling			
c	numbness or a loss of physical sensation and/or tingling of your skin (paraesthesia)			
d	Sneezing			
e	Liquid discharge from your nose			
f	Feeling of headache			
g	Symptom of nausea			
h	Eye irritation			
i	Tears coming from your eyes			
j.	Experience bad smell during use of nets			
k.	Body rashes			

l. Any other symptoms, please specify?

m. Your overall experience and whether you will use your net regularly?

n. If the respondent answers positive to any of the questions mentioned above, ask if he/she had reported to a physician for medical attention

Signature of interviewer
Date (dd/mm/yy):

5. APPENDIX

Calculation of Doses

1. Measurement of sprayable surface area of a room

Formula = (L x W + W x H + H x L) x 2– W x L

Example: L: Length of wall = 12' W
Width of wall = 10'
Height = 8'

Area = (12 x 10+ I0 x 8+8 x 12) x 2–10 x 12
(l20+80+96) x 2–120 = 296 x 2 –120
592 – 120
472 ft^2 = 0.472 m^2

Note: For measuring artificial surfaces only length and width should be calculated.

2. Requirement for the preparation of spray suspension from wettable powders

Amount of wettable powders (WP) or water-dispersible power (WDP) required for the preparation of approximately 10 litres of spray suspension.

The general formula followed

X= A x B x D / C
X = amount of water-dispersible powder required
A = percentage concentration desired
B = quantity of spray desired
C = percentage concentration of water-dispersible power
D = 1 (when X and B are expressed in kg and litres

3. Requirement for the preparation of spray suspension from dust

The general formula followed

$$X = \frac{A \times 100}{B}$$

X = amount of dust required
A = dosage (kg/ha)
B = percentage concentration of dust

4. Measurement of surface area of mosquito breeding waters

(a) Rectangular/square area

Formula = L x W = Surface area

Example = 4.5 m x 3m = 13.5 m^2

Volume of water = Surface area x Depth*

(*The dose of Temephos or Fenthion may be doubled or tripled in case water bodies having more than 50 cm depth)

(b) Measurement of round surface area

Formula $=\pi r^2$ r^2 or 22/7 x r x r

e.g. Diameter of well/pit = 3 m
Radius of well/pit = 1.5 m
Area = 22/7 x 1.5 m x 1.5 m
= 3.1 x 1.5 x 1.5 = 6.97 m^2

(c) Measurement of volume of water in circular pit/well

Formula = π r^2 x depth

e.g. Diameter of well/pit = 3 m
Radius of well/pit = 1.5 m
Depth of well/pit = 0.30 m
π r^2 x depth = 3.1 x 1.5 x 1.5 x 0.30 m
= 2.092 m^2

Volume of water = 2.092 litres of water
or
π r^2 x depth = 3.1 x 150 cm. x 150 cm x 30 cm
= 2092500 cm^2

Volume of water = 2092500/1000
= 2092 litres of water

(d) Measurement in number of hectares in areas of different linear dimensions

$$\text{Area (hectares)} = \frac{\text{Length (m) x Width (m)}}{10000}$$

e.g., Length of breeding water = 1600 m
Width of breeding water = 25 m

$$\text{Area} = \frac{1600 \times 25}{10000} = \frac{40000}{10000} = 4 \text{ hectares}$$

or

$$\text{Area (acres)} = \frac{\text{Length (ft) x Width (ft)}}{43560}$$

e.g., Length of breeding water = 3600 ft
Width of breeding water = 500 ft

$$\text{Area} = \frac{3600 \times 500}{43560} = \frac{1800000}{43560} = 41.3 \text{ acres}$$

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