World Health Organization

WHO Prequalification of Vector Control Products

Bioassay and semi-field methods for insecticide-treated nets: Ifakara Ambient Chamber Test (IACT)

Factors which may affect validity of studies using IACT:

- negative control mortality must not exceed 10% after 24 hours holding or 20% at extended holding times.
- blood feeding in the control should be monitored.
- identification of issues related to the health of test systems.
- environmental conditions (e.g. temperature and humidity at which the test is conducted or the mosquitoes are held for delayed mortality monitoring).
- tests should be conducted in alignment with the test system's circadian rhythm.
- sample size needs to be calculated before each trial.
- mosquito phenotypic resistance should be characterized before or during each trial *(1)*.

1. Purpose of the method

The purpose of the IACT is to investigate the biological activity of a constructed insecticide-treated net (ITN) under controlled closed semi-field conditions by means of observing relevant effects on test systems subjected to exposure more representative of a natural interaction with the host and test material while host seeking than in a laboratory setting.

2. Considerations for use of the method

2.1. Classification as a bioassay

The IACT is best considered as a standardized bioassay which is useful in characterizing the entomological efficacy of a whole ITN under user conditions *(2)*. The method:

- can be conducted in a consistent manner across testing facilities;
- allows for variations in test **sample preparation** in order to investigate how ITN fabrics may change through their life stages, e.g. new, in/post storage, in-use and end-of-life;
- allows for the investigation of multiple observable endpoints in the **test system** (e.g., mosquito [Table 1]).

The IACT can be used to measure the efficacy of ITNs with active ingredients that are designed to kill; reduce blood-feeding, including those that sterilize mosquitoes. It is especially useful for measuring the induced effect of ITNs with active ingredients that are designed to reduce blood feeding (e.g. irritant pyrethroids), or that require mosquitoes to be metabolically active (host seeking) to be fully potent, e.g. pro-insecticides.

The consistency of the method supports the analysis across sample preparations and vector resistance profiles, thereby providing useful information about the changes in the biological performance of an ITN through their intended useful life against multiple vector profiles.

The IACT is used to investigate the entomological efficacy of an ITN under controlled closed semi-field conditions using constructed ITNs. The IACT is designed to indicate the presence and persistence of active ingredients and their interaction with fabric integrity, efficacy in relation to free-flying mosquito interaction with the ITN, and penetration of mosquitoes through holed samples. However, the closed nature of the method and conditions through which the exposure of mosquitoes to the test material is regulated may limit the extrapolation of findings to those conditions typically found in operational use of ITNs.

2.2. Use of the method

The use of the IACT method can be employed within a variety of **studies**. These may include:

- to investigate and define ITN product characteristics, including baseline/reference information about the samples used in semi-field or community studies in order to interpret the generated efficacy data;
- closed system semi-field studies: generation of data on the continued efficacy of ITNs under user conditions;
- As a substitute for tunnel tests.

Within each type of study, several ITNs and/or preparations can be tested simultaneously with positive and negative controls. The method is an appropriate choice for investigating products formulated with active ingredients which exhibit various modes of action proposed to have public health benefit for malaria control.

The sensitivity of the IACT is limited to identifying the efficacy of the ITN as a mosquito interacts with it under controlled user conditions. It provides information on how the constructed product performs including bioavailability of active ingredient(s) and in preparations with disrupted fabric integrity.

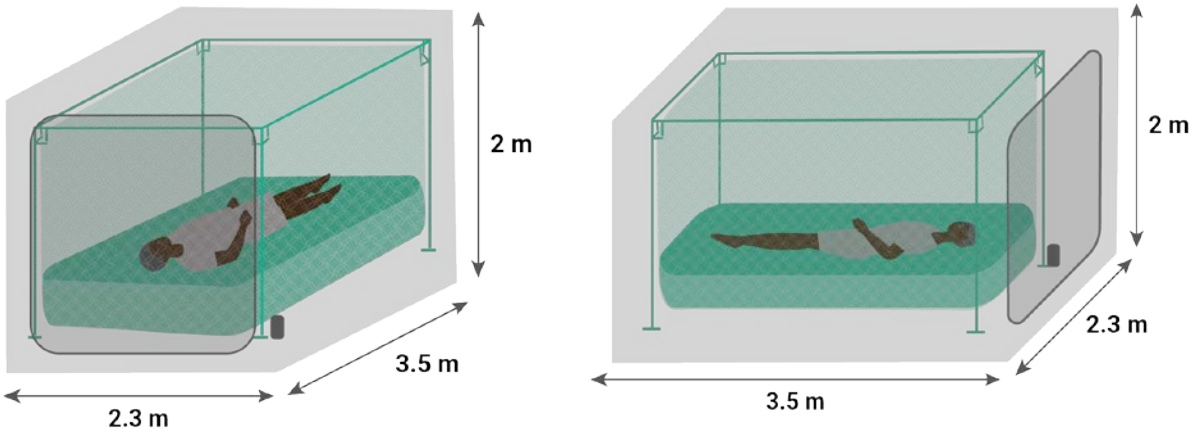
As the IACT method is a bioassay, it is subject to inherent variability that should be controlled for through the consistent rearing of test mosquitoes, careful preparation and handling of test samples, control of environmental conditions during test conduct and post-exposure holding, and conduct of adequate replicates to precisely estimate the selected endpoints for a given test.

3. Materials

3.1. Chambers

The IACT chamber measures 3.5 m x 2.3 m and is 2m high. Chambers are made of white netting on the roof and three sides with a white cloth floor and one white cloth side that opens to allow entry of people and equipment and seals with a zip to ensure retention of mosquitoes. The netting material needs to be of a mesh size <1.2mm to prevent mosquito escape and of a durable material to withstand regular use without tearing. The chambers are set up within an outer chamber made of fiberglass netting with high density polyurethane doors to separate chambers and ensure independence of observations. Ant prevention measures including an ant channel, ant bait and regular cleaning of chambers are required to prevent predation of mosquitoes by scavenging ants. An ITN is set up over a mattress on a frame on which a volunteer sleeps under the ITN (Fig. 1).

Fig. 1. IACT chamber with ITN



3.2. Human volunteers

The IACT estimates ITN efficacy under user conditions and therefore uses a human volunteer. This volunteer, who is medically supervised, is not permitted to smoke, drink alcohol or use perfumed skin care products for the duration of their involvement in the study as these factors can affect human attractiveness to mosquitoes. If female volunteers are involved, provision should be made to ensure that no pregnant women are involved in the study due to the complications of malaria in pregnancy.

Institutional ethical approval for the study must be sought from the local Ethical Review Board. Written informed consent must be obtained from each volunteer sleeper prior to their participation in the study. The consent form is explained to each volunteer in her/his local language by an interpreter. Sleepers should be monitored for possible adverse effects from mosquitoes or the ITNs and excluded if they experience any discomfort. In malaria endemic areas sleepers should be regularly monitored for malaria parasitemia to ensure that no individual carrying parasites participates in the study.

3.3. Aspirators

Aspirators that comprise a clear Perspex tube with an aperture of around 1cm are recommended. Mosquitoes are entered into the Perspex tube through aspiration through either mouth or mechanical aspiration. If mouth aspiration is conducted then it is recommended that a HEPA filter is used to prevent technical staff inhaling mosquito scales, dust or pesticide residues.

Mechanical aspirators such as the Prokopack are often preferred for collecting mosquitoes from larger areas including floors, walls and ceilings. The use of a 6-volt battery may be considered to minimise mechanical damage to resting mosquitoes that are to be held for delayed mortality monitoring.

3.4. Holding receptacle

Paper cups or other inert holding receptacle lined with paper that allows mosquitoes to comfortably rest are used prior to exposure procedures and during post-exposure monitoring. The top must be an untreated netting which can be affixed to the receptacle. The holding receptacle has a 1.5-2 cm hole sealable with cotton wool or another means that allows removal or introduction of test insects using an aspirator. It is recommended that no more than one mosquito per $20cm^3$ is retained in the cups to minimize mosquito mortality though overcrowding.

3.5. ITN preparation

The test materials to be used in the method should be prepared as constructed ITNs. Ensure that all prepared samples are adequately labelled and stored appropriately, as improper storage may impact the results of the test and invalidate the study. In between testing it is advisable to keep the products out of sunlight, e.g. wrapped in foil and in a temperature-controlled room. The environmental parameters of the storage room should be recorded.

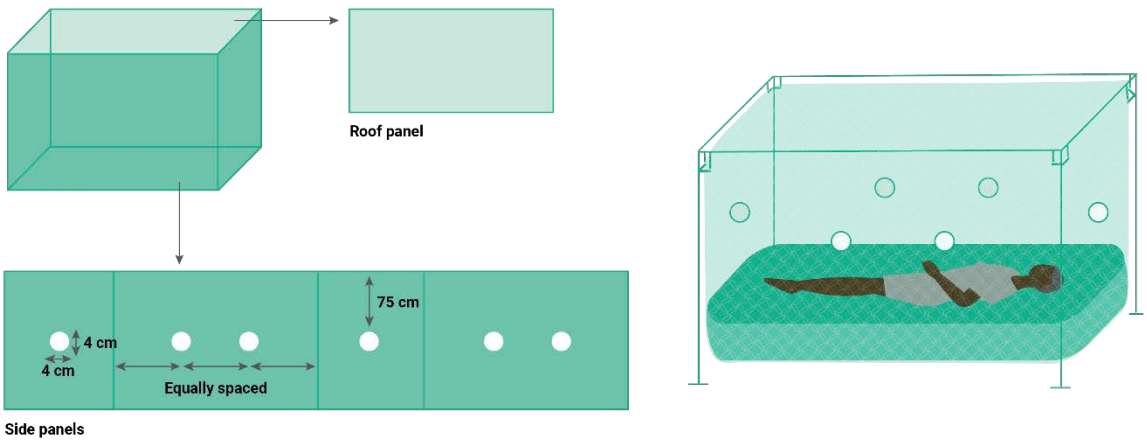
3.5.1. Damage replication

For standard evaluations of ITNs using either unwashed or artificially aged nets, the nets are deliberately holed to simulate operational ageing, using 4cm x 4cm holes. Studies that are conducted using operationally aged ITNs do not require additional holes to be cut.

Cut six holes in each net. Each hole should measure 4cm x 4cm in length and width. One hole is cut on each of the short sides of the ITN and two holes are cut on the longer sides of the ITN. Holes are placed 75 cm from the top of the net (Fig. 2). On the long sides, the holes are equally spaced, i.e., the first hole is made at one third of the distance and the second at two thirds of the distance from the edge of the side panel.

When cutting the test material ensure that the material is not being stretched nor compressed. To ensure standardization of holing the ITN should be placed over a frame and holes cut using a 4x4cm template. Larger or smaller holes than standard may invalidate the results.

Fig. 2. Damage replication in ITNs



3.5.2. Washing

Preparation of nets may take several weeks or even months for ITNs that have a long wash interval and must be artificially aged. To maintain equivalence between the various treatments in the trial, washing in each treatment arm should be completed at the same time, which means starting the washing of nets with longer wash intervals weeks or months earlier than those with shorter wash intervals. A minimum of six test nets are used for each preparation to measure between net heterogeneity.

Nets are washed in aluminium bowls containing 10 litres of water with a maximum hardness of 5dH and containing 2g/litre of soap (pH 10-11). Nets should be washed for a maximum of ten minutes and be agitated for a total of six minutes within the ten minutes (three minutes agitation, four minutes soaking, three minutes agitation), using manual agitation of 20 rotations per minute. Rinsing is conducted twice using 10 litres of clean water each time. Nets should be dried horizontally in the shade before being wrapped in foil and stored in labelled plastic bags at 25°C ±5 between washes. Ensure that nets are completely dry before storage or further washing.

The time between washes should correspond to the wash interval determined in the wash regeneration study.

Washed ITNs should be allowed to rest for two weeks between the completion of washing and the IACT commencement.

3.6. Storage of test and reference materials

Since the IACT is a method for understanding efficacy of an ITN, the history of and conditions to which test materials have been exposed are critical pieces of information for the interpretation of the resulting data. The means by which samples (including reference items) were obtained and the storage conditions prior to testing should be documented, with certificate of conformity and batch numbers reported.

3.7. Reference items (controls and comparator products)

The purpose of the reference items (positive and negative controls, and comparator products in the case of comparative efficacy studies) is to validate the experimental procedures. In comparative efficacy studies, the reference items serve a further purpose, as the results from reference items are used in statistical analyses to determine the non-inferiority of the ITN under investigation to the reference item.

The means by which reference items were obtained and the storage conditions prior to testing should be documented, with certificate of conformity and batch numbers reported.

Negative control samples should be untreated netting made of polyethylene or polyester. Positive controls should be selected in accordance with the study that is being conducted. All positive controls should be prequalified products that have an entomological mode of action and combination of AI(s) (if relevant) consistent with the intended entomological mode of action of the product that is under investigation.

4. Environmental conditions

4.1. Environmental monitoring

The environmental conditions of the ITN storage area, the ambient chambers, and the test room for post-exposure monitoring, should be continuously monitored and reported.

4.2. Post-exposure holding room environmental conditions

The temperature and humidity of the post-exposure holding room should be maintained at 27 ± 2° C and 80 ± 20%, respectively.

All environmental conditions at which the tests are conducted should be documented and reported.

4.3. Test conditions

It is recommended that the IACT is conducted in temperature-controlled settings (maintaining optimal environmental conditions through the use of heater, air conditioner and humidifier), maintaining the temperature at 27 ± 2° C and humidity at 80 ± 20%.

If the chamber is used under ambient settings, then temperature and humidity should be recorded as temperatures out of range may influence the observed results and impact interpretability of the resulting data.

The light cycle should be in line with that of the insectaries and holding room. IACT are conducted during the dark phase.

5. Test systems

5.1. Species/strain selection

The selection of test systems for use in the IACT should be informed by the:

- intended entomological mode of action of the product under investigation;
- purpose of the study in which the IACT method is applied.

5.2. Age and nutritional status of the mosquito test systems

Five-to-eight-day old, nulliparous mosquitoes should be used.

Mosquitoes should be maintained on sugar prior to the test (up to the beginning of the starvation period) to minimise control mortality. Mosquitoes should be starved before exposure to make sure that they are likely to blood feed. The length of starvation is strain dependent and should be determined at the facility to be the time required that ensures that test parameters are met in the control (i.e., 50% blood feeding success and <10% mortality after 24 hours and <20% mortality after 72 hours).

5.3. Preparation and handling

The timing of the IACT method tests within a study should be aligned with the circadian rhythm of the test system and be consistent when tests are conducted across multiple days/sample periods. The IACT is normally conducted during the dark phase.

Remove the sugar source from the mosquito cage at the start of the starvation period (usually 6-12 hours) before initiating testing process.

If mosquitoes need to be transferred from the insectaries to the test room ensure to minimize stress caused by sudden changes of temperature, humidity, sunlight, or wind through using a closed receptacle to transport holding cups or holding cages.

Select mosquitoes for testing. Aspirate female mosquitoes into a holding receptacle, e.g. test cages or cups. Do not choose mosquitoes that are small, missing legs or wings, or that are inactive.

Allow mosquitoes to acclimatize for one hour prior to testing.

6. Sample size

The number of mosquitoes released per replicate and the number of strains used is dependent on study design. However, a minimum of 20 mosquitoes per strain per replicate is recommended.

7. Selection of endpoints and considerations

Table 1 provides information pertaining to the relevant endpoints which may be observed and measured when using IACT. The endpoint to be used for decision-making purposes must be selected based on the intended entomological mode of action of the product under investigation and be used consistently across all laboratory studies, semi-field studies and semi-field supplemental bioassays. Justification for the selection of the decision-making endpoint must be presented in the study report.

Regardless of the intended entomological effect of the product, M24 should be observed and documented for the purpose of monitoring the experimental controls and thereby experimental acceptability.

Table 1. IACT endpoints

Endpoint	Time it is measured	Purpose and definition	Considerations
Mortality at 24 hours (M24)	24 hours after the 9-hour IACT exposure has ended	The measurement of mortality in a cone test is an indicator of the lethal effects of the net. Mortality is observed by the following indicators: No sign of life; immobile; cannot stand. Moribund mosquitoes are also classed as dead after 24 hours of holding as it is unlikely that they would survive in nature, i.e.: o any mosquito that cannot stand, e.g. has 1 or 2 legs; o any mosquito that cannot fly in a coordinated manner; o a mosquito that lies on its back, moving legs and wings but unable to take off; o a mosquito that can stand and take off briefly but falls down immediately.	The standard exposure time in the IACT for measuring mortality is 9 hours. Extension of the exposure time, or inclusion of multiple exposure times must be declared and scientifically justified in the context of the product being tested and study being conducted. The standard holding time post-exposure in the IACT is 24 hours for neurotoxins and 120 hours for pro-insecticides to ensure complete conversion of the parent molecule to the active metabolite even if ambient temperatures are below 27 degrees. Control mortality should not exceed 10% after 24 hours or 20% after extended holding times. Otherwise, the test is invalidated. Extension of the post-exposure holding time must be declared and scientifically justified in the context of the product being tested and study being conducted, e.g. Mortality at x hours after exposure – Mx.
Blood feeding inhibition	At the end of the exposure period	The proportion of unfed females. Blood fed includes partially or fully blood engorged mosquitoes. Blood feeding inhibition is the proportion of mosquitoes that are not fed. Blood feeding inhibition induced by the intervention may also be calculated as follows: 1) calculate average blood feeding in the control (C) arm, 2) calculate the blood feeding for each observation for each intervention (T) relative to the average blood feeding rate in the control using the formula 100x(C-T/C), 3) calculate the mean blood feeding inhibition (% and 95% CI) from all the observations in each arm.	Control blood feeding should exceed 50%. Otherwise, the test is invalidated.

Endpoint	Time it is measured	Purpose and definition	Considerations
Fertility – Eggs per female	Blood fed mosquitoes are held for 72 hours after 9 hours exposure in the IACT.	The measurement of eggs per female is an indicator of fertility. Fertility is observed by the following indicators: Number of eggs laid/females live at each period of observation.	Blood fed female mosquitoes are provided with an egg laying substrate for oviposition.
Fecundity –Proportion of fertile females	Number of females with Christopher's stage 5 eggs when dissected. Blood fed mosquitoes are held for 72 hours after 9 hours exposure in IACT.	The measurement of proportion of fertile females in an IACT test is an indicator of reduction of fecundity. Fecundity is observed by the following indicators: Number of females that have fully developed (viable eggs) at each period of observation measured using the Christopher's classification. Stage 4 and Stage 5 eggs are classified as viable.	The standard exposure time in the IACT for measuring fecundity is 9 hours. Extension of the exposure time, or inclusion of multiple exposure times must be clearly declared and scientifically justified in the context of the product being tested and study being conducted. The standard post-exposure holding time in the IACT for fecundity measurements is 72 hours. Control mortality must not exceed 10% after 24 hours, nor 20% after extended holding periods. Otherwise, the test is invalidated. Extension of the exposure time must be declared and scientifically justified in the context of the product being tested and study being conducted.
Other	Applicants may propose other endpoints to be measured by means of the IACT with adequate justification.		

8. Experimental method

Before each 9-hour exposure period:

- Clean each compartment to remove predators such as ants and spiders.
- Ensure that a clean sheet is in place over the mattress in each chamber.
- The equipment needed for each sleeper should be assembled. This includes mattress, sheet and pillow as well water, food and a chamber pot for comfort and to minimize the risk of sleepers leaving the chamber during the night.
- The aspirators and any other equipment must be cleaned in 10% bleach or appropriate cleaning agent and rinsed well with clean water. Separate aspirators must be prepared and labelled for the negative control, positive control, and each treatment. Each individual must have a separate aspirator to avoid contamination of samples and to safeguard participants from respiratory diseases.
- Mosquito holding receptacles are prepared and adequately labeled.

8.1. ITN allocation and hanging

Ensure all samples are adequately labelled.

The study supervisor will allocate the ITN to each chamber. The test sample is hung from a frame at a height such that the ITN can be tucked under the mattress and the holes are approximately halfway between the mattress and the roof of the net. Five cm of the net is tucked under the mattress to standardize the surface area of ITN available to the mosquitoes.

After each collection night, each ITN should be taken down and stored in its original packaging or wrapped in foil in a suitable storage area out of direct sunlight. The environmental conditions of the storage area should be continuously monitored with a data logger and should not exceed 32°C. Incorrect handling and storage of test items will invalidate the method.

8.2. Preparation of room and materials

Ensure that the room conditions have been stabilized at 27 ± 2°C and 80 ± 20% and a temperature logger is in place. The environmental conditions in experimental chambers (temperature and humidity) should be continuously monitored with a data logger.

Ensure that all test materials are hung correctly in the correct chamber for the study replicate.

Ensure that all the volunteers are under the net in the correct chamber.

Ensure all mosquitoes have been placed in holding receptacles in each chamber.

Check that each chamber is correctly closed to avoid mosquito escape from the chamber.

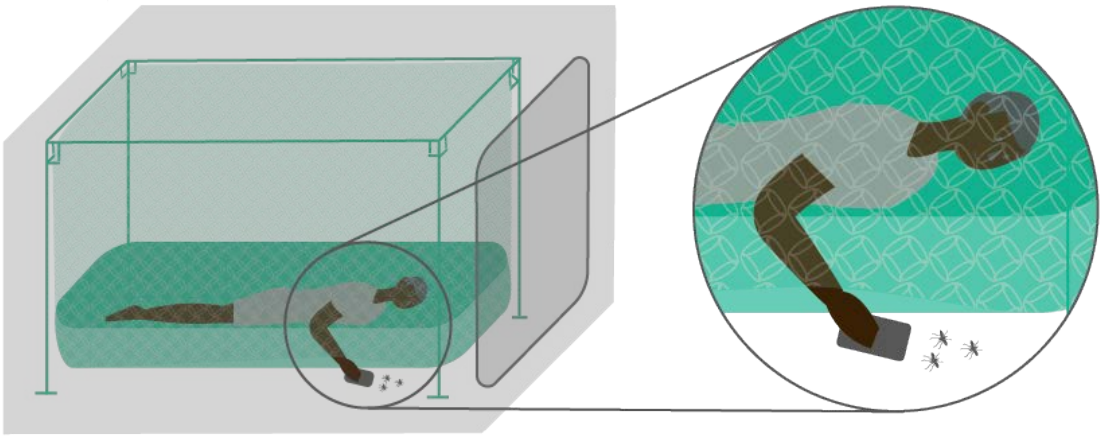
8.3. Exposure

Each volunteer releases female mosquitoes from the holding receptacle into their respective chamber after a signal. The mosquitoes are released outside of the ITN by removing the netted lid of the holding receptacle and gently shaking the holding receptacle to encourage the mosquitoes to leave (Fig. 3).

Once all of volunteers confirm that they have released their mosquitoes note the start time on the data sheet.

The timing of the IACT method tests within a study should be aligned with the circadian rhythm of the test organism and be consistent when tests are conducted across multiple days/sample periods. The test is normally conducted overnight between 21:00 and 06:00 hours.

Fig. 3. Mosquito release in IACT



8.4. Mosquito collections

At a specified time, mosquitoes are collected by the sleeping volunteers in a systematic way to ensure that no mosquitoes are missed or crushed as the volunteer goes about the collection. Dead and live mosquitoes are first collected from inside the ITN, taking care to check around the mattress for knocked down or dead mosquitoes. Dead mosquitoes are collected from the floor before collections of resting mosquitoes from the walls and ceiling are conducted. Collections should be performed in a systematic way, e.g. starting consistently in the left corner of the net floor or chamber and working slowly clockwise searching up and down with a torch.

The collection period should be for a consistent number of minutes each day and carefully supervised to ensure that mosquitoes are not missed. Correct training and high motivation of staff is key to conducting good quality evaluations.

8.5. Transit to post-exposure holding areas

Holding receptacles containing live mosquitoes should be labelled with the chamber number, test item identifier and the date of collection.

Transfer of collected mosquitoes to the post-exposure holding area should be done as soon as practically possible and in a manner that minimizes stress caused by sudden changes of temperature, humidity, sunlight, or wind through using closed receptacles to transport holding cups or holding cages.

8.6. Mosquito scoring

Mosquitoes should be scored by species and collection location as dead unfed, dead blood fed, alive unfed and as alive blood fed. After recording initial mortality and blood feeding status, place sugar source on top of the holding receptacle. Sugar source is 10% glucose or sucrose solution made with sterile water.

8.7. Post-exposure

The post-exposure holding time should begin, and be documented, at the time mosquitoes are removed from the ITN and chamber returned to the labelled holding receptacle using a mouth aspirator. Live mosquitoes should be placed in holding receptacles, given sugar solution, and held at 27 ± 2 °C and 75 ± 10% relative humidity.

Maintain the holding room/area at 27 ± 2°C and 80 ± 20% for the entire holding period and ensure that the light dark cycle of the holding room is the same as that of the insectary from which the mosquitoes were acquired. Holding conditions should be continuously monitored with a data logger. Control mortality should not exceed 10% for up to 24 hours and 20% for up to 168-hour holding. High control mortality is generally linked to poor mosquito handling or suboptimal holding conditions (too hot, not sufficiently humid or poor cleanliness) and will invalidate the test.

- Delayed mortality of mosquitoes should be assessed every 24 hours up to the specified holding time dependent on the chemical mode of action of the active ingredient(s) on the ITN, depending on the claims of the manufacturer. If reduction in fecundity is a claim of the manufacturer, blood fed mosquitoes should be held in individual glass or plastic tubes with oviposition pads. The proportion of mosquitoes that lay eggs and the total number of eggs per female should be monitored after 4 days or mosquitoes are dissected at 72 hours and the proportion with mature eggs (Christopher's stage 5) is measured.

9. Results

9.1. Considerations for the presentation of results

Considerations for presentation of results is based on the study in which the method is used. All endpoints should be presented with an appropriate measure of centrality and dispersion (e.g. arithmetic mean % and 95% confidence intervals for percentages) and median and interquartile range for count data (e.g. number of eggs). Data for the control arm provides critical information needed to appraise the quality of study conduct and should always be presented.

Mosquito mortality at each holding period should be presented by arithmetic mean % mortality and 95% confidence intervals. Negative (untreated) control mortality and feeding success should also be reported at each post exposure holding time. If mortality at 24 hours exceeds 10% the test should be discarded and repeated. If 24-hour mortality (M24) exceeds 5% then mortality should be control corrected using Abbotts Formula.

The number of replicates conducted per arm or per ITN preparation should be presented.

Presentation of test environmental conditions is useful to enable understanding of test conduct.

Tables of full results must be presented in an annex if figures are used in the report.

Suggested summary data presentation for critical endpoints. Note that detailed guidance for presenting results from studies is given in the relevant study guidance document.

Outcome	Intervention and preparation	N	Mean (95% CI)	Odds ratio (95% CI)	p-value
Mortality at 24 hours	Control				
Blood feeding	Control				

10. Related documents

- WHO PQT/VCP Implementation guidance – Regeneration study for ITN fabric
- WHO PQT/VCP Implementation guidance – Wash resistance study for ITN fabric
- WHO PQT/VCP Implementation guidance – Semi-field studies for ITNs
- WHO PQT/VCP Implementation guidance – Considerations for the selection of controls for use in ITN studies
- WHO PQT/VCP Implementation guidance – Bioassay methods for ITNs: Cone test

11. References

1. Manual for monitoring insecticide resistance in mosquito vectors and selecting appropriate interventions. Geneva: World Health Organization; 2022 (https://iris.who.int/bitstream/handle/10665/356964/9789240051089-eng.pdf?sequence=1, accessed 20 November 2023).
2. Massue DJ, Lorenz LM, Moore JD, Ntabaliba WS, Ackerman S, Mboma ZM, Kisinza WN, Mbuba E, Mmbaga S, Bradley J, Overgaard HJ, Moore SJ. Comparing the new Ifakara Ambient Chamber Test with WHO cone and tunnel tests for bioefficacy and non-inferiority testing of insecticide-treated nets. Malar J. 2019;18(1):153. (doi: https://doi.org/10.1186/s12936-019-2741-y., accessed 15 October 2023).