World Health Organization

WHO Prequalification of Vector Control Products

Bioassay methods for insecticide-treated nets: Cone test

Factors which may affect validity of tests and studies using the cone test method:

- negative control mortality must not exceed 10% after 24 hours holding or 20% at extended holding times;
- no more or less than 5 mosquitoes should be used per cone;
- 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.

1. Purpose of the method

The purpose of the cone test is to investigate the biological activity of a material's surface (treated with active ingredient or not) under controlled laboratory conditions by means of observing relevant effects on test systems subjected to optimized exposure with the test material.

2. Considerations for use of the method

2.1. Classification as a bioassay

The cone test is best considered as a standardized bioassay which is useful in characterizing the biological availability and potency of active ingredients on the surface of a test material (e.g., an insecticide-treated net [ITN]). 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 use of cone tests within a study is valuable in characterizing the presence and bioavailability of active ingredients on the fabric surface, as well as the ability of the ITN fabric to replenish surface concentrations of active ingredients after washing.

The consistency of the method supports the analysis across samples prepared in different ways, thereby providing valuable information about the stability and consistency in behaviour of ITN fabrics through their intended useful life. However, the controlled laboratory setting 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.

The cone test is not an effective method for investigating the potential entomological efficacy of an ITN. The cone test can indicate the presence of insecticide on the surface of the ITN fabric, at a concentration which is sufficient to induce a biological effect under controlled laboratory conditions.

2.2. Use of the method

The cone test method can be used within a variety of **studies**. These may include:

- Regeneration study: Investigation of the time required for a) the surface and reservoir concentrations of AI(s) to reach equilibrium, and b) biological effects to be re-established, after depletion of the active ingredient from the surface.
- Wash resistance study: Investigation of the consistency and continuity of bioassay results over the intended useful life of the product by means of artificially ageing (washing) fabric samples
- Provide baseline/reference information about the characteristics of samples used in semi-field trials or long-term community studies.

It is important to note that the cone test alone cannot be used to quantify surface concentrations of AI (including absolute values or fractions of bioavailable/non-bioavailable forms) nor estimate the maximal bioavailable surface concentration on the fabric (yarns or coating). The sensitivity of the cone test is limited to identifying the presence of the active ingredient(s) in a bioavailable form at a concentration sufficient to induce the target effect on the exposed test system.

As the cone test 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. Test day is a significant contributor to variability, and therefore, where possible, studies using the cone test should be designed such that all cone tests are conducted on the same day.

The method may not be an appropriate choice for investigating products formulated with active ingredients whose mode of action may be inhibited by the parameters of the method, for example those active ingredients which may require the insect to be metabolically active, e.g., limited space for flight may not reflect the effects of pro-insecticides as the exposed insects are held close to the treated surface.

3. Materials

3.1. Cones

Standard test cones are made of translucent polyvinyl chloride (PVC). They are 12 cm in diameter, 9 cm in height, and have a volume of 180 cm^3. The vertex of the cone should have a hole 1.5 cm in diameter for the purpose of inserting mosquitoes into the cone. The hole should be sealable using a bung made from a material that discourages mosquito contact such as plastic.

3.2. Frame

The frame on which the cone test for ITNs is conducted should be made from a durable material that can withstand cleaning with polar solvents.

Four regularly spaced holes should be cut within the 25x25 cm squares allowing for four cone replicates to be conducted per sample of material. The holes should have a diameter between 11-12 cm. The purpose of the holes in the frame is to ensure that test mosquitoes are in contact with the test material and not the frame *(1)*.

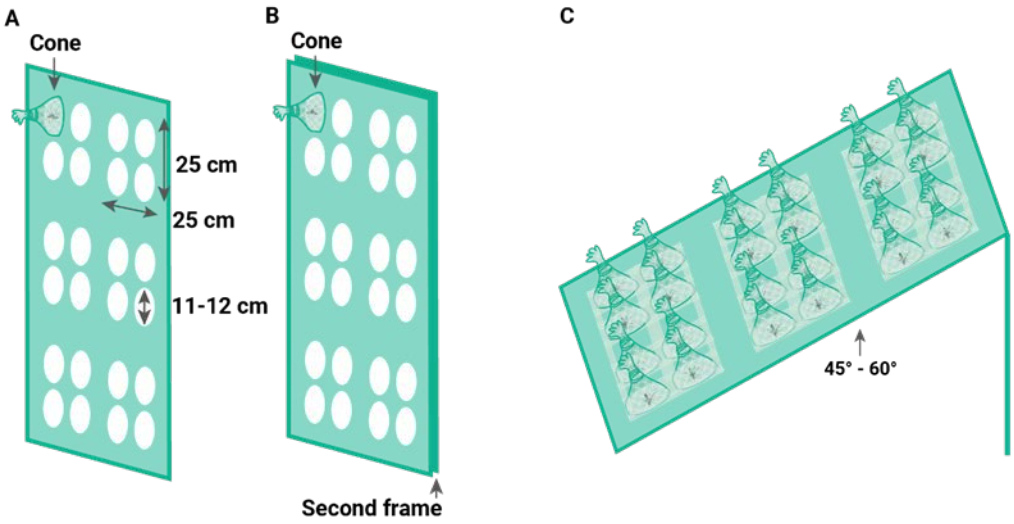
Use tape, clips, or other option to affix the test material and cone to the frame. Ensure that the test material is not being stretched nor compressed. Care must be taken to ensure that cones are securely attached in close contact to the board to prevent accidental trapping or escape of test systems. Alternatively, two frames can be used. The bottom frame is overlaid with the test sample and a cone over which the second frame is laid, and the two frames are held together so that the test samples and cones can be affixed between the two frames (Fig. 1).

Ensure that test samples are adequately labelled when attached to the frame.

The frame should be positioned at a 45°-to-60° degree angle *(2)*. The angle of the frames should be kept consistent within a study.

Fig. 1. Cone test frames and laboratory set up

A. Single frame design B. Double frame design C. Laboratory set-up with test samples in place



Cone frame with clusters of four holes within 25cm x 25cm space

3.3. Aspirators

Aspirators should comprise a clear Perspex tube with an aperture of around 1 cm that will fit into the cone that has an opening of 1.5 cm. Mosquitoes are entered into the Perspex tube 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.

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.Test samples

It is possible that test samples may be taken from source fabrics (prior to construction of the ITN), constructed ITNs, or received by the investigator as pre-cut fabric pieces as defined in certain study protocols.

In situations where test samples are being cut from constructed ITNs, the product specific sampling plan must be considered to ensure that differing fabrics are adequately represented. The total number of samples required is dependent upon the study and the product.

Since the cone test is a method for understanding the quality characteristics of an ITN, the history of and conditions to which test samples 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.

3.5.1. Test material sample preparation

The test samples to be used in the method should be prepared as either 25 x 25 cm or 30 x 30 cm squares, depending on the intent of the study (refer to study guidance documents for further details). When cutting the test material ensure that the material is not being stretched nor compressed.

Ensure that all prepared samples are adequately labelled and stored appropriately. Improper storage may impact the results of the test and invalidate the study.

Refrigerated samples must be allowed to reach room temperature before testing.

3.5.2. Washing

Where test samples require washing (artificial ageing) prior to use in the cone test method, equivalence between the various arms in the study/trial should be maintained. Thus, washing in each treatment arm should be completed at the same time, which may require the commencement of sample preparation for those samples with longer wash intervals weeks or months earlier than those with shorter wash intervals.

3.5.3. Sampling procedure

Sampling procedure for ITNs is dependent on the design and construction of the ITN, including the presence of multiple fabrics in the ITN design. Sampling schemes are described in the relevant study guidance documents and are designed to ensure that any differing fabrics in the ITN are adequately represented. The total number of samples required is dependent upon the study and the product.

3.6. Reference items (controls)

The purpose of the reference items (positive and negative controls) is to validate the experimental procedures. 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 and the intended effect of the product under investigation.

4. Test room environmental conditions

- Temperature should be maintained at 27 ± 2° C.
- Humidity should be maintained at 80 ± 20%.
- The light cycle should be in line with that of the insectaries and holding room.
- All environmental conditions at which the tests are conducted should be documented and reported.

5. Test systems

5.1. Species/strain selection

The selection of mosquito species/strains should be informed by the intended entomological mode of action of the product under investigation, and the study purpose in which the cone method is used.

5.2. Age and nutritional status of the test systems

Three-to-five-day old, nulliparous mosquitoes should be used.

Mosquitoes should be maintained on sugar solution prior to the test (up to one hour prior to the test) to minimize control mortality.

For investigation of certain endpoints, e.g., fertility and fecundity, blood feeding may be required prior to the test. In such cases, the time between blood feeding and initiation of the test should be within 6 hours and documented. Blood feeding prior to the cone test will reduce the observed mortality. Therefore, blood fed mosquitoes should not be used in the measurement of mortality.

5.3. Preparation and handling

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.

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 by using a closed receptacle to transport holding cups or holding cages *(3)*.

Allow mosquitoes to acclimatize for 1 hour prior to testing.

6. Selection of endpoints and considerations

Table 1 provides information pertaining to the relevant endpoints which may be observed and measured by means of the cone test. 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. WHO cone test endpoints

Endpoint	Time it is measured	Purpose and definition	Considerations
Mortality at 24 hours (M24)	24 hours after the 3-minute cone test exposure has ended.	The measurement of mortality in a cone test is an indicator of the lethal effects of test sample. Mortality is observed by the following indicators: o No sign of life; immobile; cannot stand. o Moribund mosquitoes are also classified as dead after 24 hours of holding as it is unlikely that they would survive in nature, i.e.: – any mosquito that cannot stand (e.g., has 1 or 2 legs); – any mosquito that cannot fly in a coordinated manner; – a mosquito that lies on its back, moving legs and wings but unable to take off; – a mosquito that can stand and take off briefly but falls down immediately.	The standard exposure time in the cone test for measuring mortality is 3 minutes. 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. Extension of exposure time beyond three minutes is considered to be a change in the methodology, and, as such, **validation data** must be collected and submitted with the product dossier to justify the change. The standard holding time post-exposure in the cone test is 24 hours. Control mortality must not exceed 10% after 24 hours. 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 3 minutes exposure – Mx). Control mortality must not exceed 20% after extended holding times. Otherwise, the test is invalidated.
Knockdown at 60 minutes (KD60)	60 minutes after the 3-minute cone test exposure has ended.	The measurement of knockdown in a cone test is an indicator of sublethal effects (incapacitation) of a test sample. Knockdown is observed by the following indicators: 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; o no sign of life; immobile; cannot stand.	The standard exposure time in the cone test for measuring knockdown is 3 minutes. Extension of the exposure time, or inclusion of multiple exposure times, must be declared and scientifically justified based on the context/intent of the product being tested and the particular study being conducted.

Endpoint	Time it is measured	Purpose and definition	Considerations
Fertility – Eggs per female	72 hours after 3-minute exposure in a cone test.	The measurement of eggs per female is an indicator of fertility. Fertility is observed by the following indicators: o number of eggs laid/live females at each period of observation; o reduction of fertility is determined by means of comparing the results of test samples and the negative control.	The standard exposure time in the cone test for measuring fertility is 3 minutes. 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 cone test for fertility 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.
Fecundity – Proportion of fertile females	Number of females with stage 4 and 5 eggs when dissected 72 hours after 3-minute exposure in a cone test.	The measurement of proportion of fertile females in a cone test is an indicator of reduction of fecundity. Fecundity is observed by the following indicators: o 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; o reduction of fecundity is determined by means of comparing the results of test samples and the negative control	The standard exposure time in the cone test for measuring fecundity is 3 minutes. 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 cone test 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 cone method with adequate justification.		

7. Test reproducibility

Adequate replication is an essential component of cone tests to ensure precise estimates of outcomes. This is dependent on the ITN preparation and mosquito strain used. Control mortality should not exceed 10% after 24 hours and 20% after prolonged holding periods. Should control mortality exceed these limits, the test day should be repeated.

8. Experimental method

The timing of the cone 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 time at which at tests were conducted and any variation among test days should be included in the study report.

8.1. Preparation of room and materials

Ensure that the room conditions have been stabilized at 27 ± 2°C and 80 ± 20%.

Ensure that all test materials are acclimated to room temperature. If they are wrapped in foil, unwrap them so that they can acclimate completely.

Ensure the cones, aspirators, frame and any other equipment have all been cleaned in 10% bleach or appropriate cleaning agent and rinsed well with clean water.

Ensure that mosquito holding receptacles are prepared and adequately labelled.

Ensure separate aspirators are prepared and labelled for the negative control, positive control, and each treatment to avoid cross-contamination of samples.

Ensure that an appropriate bench guard or surface protector is in place.

8.2. Exposure

Ensuring that the correct aspirator is used, aspirate five female mosquitoes from the holding receptacle into the negative control cone and quickly plug the cone with the plastic bung.

Once all of mosquitoes are in the first cone, start the exposure timer.

Wait for 1 minute and aspirate five mosquitoes into the next cone.

Repeat this procedure until mosquitoes have been introduced into all cones, ensuring the correct aspirators are used for each treatment.

Three minutes after mosquitoes have been introduced into the respective cone, aspirate the mosquitoes back into the correct labelled holding receptacle.

Continue until all the replicates for that testing day have been tested and the exposure procedures are complete.

It is important to use only five mosquitoes in the cone as increasing the density will increase mosquito mortality.

Changes to the exposure duration are considered an alteration to the cone test method and require validation prior to the study commencement. Validation data should be included in the dossier submission.

8.3. Post-exposure

The post-exposure holding time should begin, and be documented, at the time mosquitoes are removed from the cone and returned to the holding receptacle.

Transfer the holding receptacles to the holding room/area. If mosquitoes need to be moved between rooms, they should be transferred in a closed receptacle to minimize stress caused by sudden changes of temperature, humidity, sunlight, or wind.

After recording initial 60-minute knockdown, place a 10% sugar source on top of the holding receptacle. Sugar source is 10% glucose or sucrose solution made with sterile water.

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.

9. Results

9.1. Considerations for the presentation of results

Considerations for the presentation of results is based on 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; median and interquartile range for count data, e.g. number of eggs. Data for the control arm/s 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 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 M24 exceeds 5% then mortality should be control corrected using Abbotts Formula.

The number of cone tests conducted per sample or per ITN should be presented.

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

Tables of full results should 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				
Knockdown at 60 minutes	Control				

9.2. Sources of variability in cone tests

Variability in cone tests is strongly related to test systems. Insect rearing is a critical consideration and measures to monitor mosquito fitness, for example, average mosquito weight and wing length, are a requirement to ensure consistent results. Mosquito fitness data should be presented in study reports, including the acceptable range(s) for that species/strain in the testing facility.

The time of conduct of tests should be consistent as the upregulation of enzymes occurs around the start of the dark phase of mosquito circadian rhythm and can strongly impact results.

Operator bias may impact the results and for this reason the control mortality is a critical measurement to ensure that improper mosquito handling is not an influence on mosquito mortality. Training and operator motivation are critical factors in the correct conduct of cone tests.

Test day is a significant contributor to variability, and therefore, where possible, studies using the cone test should be designed such that all cone tests are conducted on the same day.

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 – Supporting data considerations for novel bioassays

11. References

1. Koinari, M., Bubun, N., Amos, B., Kiari, K., Lahu, D., Karl, S. WHO cone bioassay boards with or without holes: relevance for bioassay outcomes in long-lasting insecticidal net studies. Malar J. 2022; 21:389 (https://doi.org/10.1186/s12936-022-04412-2, accessed 15 October 2023).
2. Owusu, H.F., Müller, P. How important is the angle of tilt in the WHO cone bioassay? Malar J. 2016; 15:243 (https://doi.org/10.1186/s12936-016-1303-9, accessed 15 October 2023).
3. Mbwambo, S.G., Bubun, N., Mbuba, E., Moore, J., Mbina, K., Kamande, D., Laman, M., Mpolya, E., Odufuwa, O.G., Freeman, T., Karl, S., Moore, S.J. Comparison of cone bioassay estimates at two laboratories with different *Anopheles* mosquitoes for quality assurance of pyrethroid insecticide-treated nets. Malar J. 2022; 21: 214 (https://doi.org/10.1186/s12936-022-04217-3, accessed 15 October 2023).