

WHO PQT/VCP Implementation Guidance – Bioassay Methods for ITNs – 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 than 5 mosquitoes should be used per cone
- Identification of issues related to the health of test organisms
- Room conditions (e.g. temperature and humidity) at which the test is conducted or the mosquitoes are held for delayed mortality monitoring

Bioassay Methods for ITNs – Cone Test

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 organisms subjected to optimized exposure (forced contact) 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, such as 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 organism (test system e.g. mosquito) (Table 1)

The cone test is not necessarily an effective method for investigating the potential entomological efficacy of an ITN under user conditions. The cone test can indicate the presence of insecticide on the surface of the ITN fabric, at a concentration which is sufficient to induce an effect on test organisms (test systems) under highly controlled laboratory conditions. The utilization of cone tests within a study,

which relies on various preparations of samples, is valuable in characterizing the presence and bioavailability of active ingredients on the fabric surface.

The consistency of the method supports the analysis between samples, which have been subjected to different preparations, thereby providing valuable information about the stability and consistency in behavior 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.

2.2. Use of the method

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

- Regeneration time study - investigation of the time required for biological effects to be re-established after depletion of the active ingredient from the surface
- Wash resistance study (bioefficacy) – investigation of the consistency and continuity of bioassay results over the intended useful life of the product by means of artificially aging (washing) fabric samples

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 surface concentration on the fabric (yarns/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 organism.

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.

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 cm³. 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.

4 regularly spaced holes should be cut within the 25x25 cm squares allowing for 4 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.

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 organisms. 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 (Image / diagram).

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

The frame should be positioned at a 45° to 60° degree angle. Within a study which includes use of the cone method, the angle of the frames should be consistent and documented in the study protocol.

3.3. Aspirators

Aspirators should be 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 may be entered into the Perspex tube through aspiration either through 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 wing 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 for use prior to exposure procedures and post-exposure in cone test. The top must be an untreated netting which can be affixed to the receptacle. The top of the holding receptacle should have a 1.5-2 cm hole, sealable with cotton wool or another means, that allows the introduction or removal of test organisms by means of an aspirator.

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 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 controls) were obtained should be documented with certificate of conformity and batch numbers reported.

The test samples to be used in the method should be prepared as 25 x 25 cm squares. 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.

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, in which the cone test is being used. The purpose of the positive control is to validate the experimental procedures.

4. Test Room

Temperature should be maintained at $27 \pm 2^\circ \text{C}$.

Humidity should be maintained at $80 \pm 20\%$.

The light cycle should be in line with that of the insectaries and holding room.

Environmental conditions at which the test is conducted should be documented and reported.

5. Test Systems (Organisms)

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 as well as the purpose of the study in which the cone method is utilized.

5.2. Age and nutritional status of the mosquitoes

3-5 day old mosquitoes should be used. Mosquitoes should be nulliparous for evaluating knock down and mortality endpoints.

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

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 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 1 hour prior to testing.

6. Selection of Endpoints and Considerations

Table 2 provides information pertaining to the relevant endpoints which may be observed and measured by means of the cone test.

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. Tests with mortality exceeding these thresholds should be repeated.

8. Experimental Method

The timing of the cone 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.

8.1. Preparation of room and materials

Ensure that the room conditions have been stabilized at $27 \pm 2^\circ\text{C}$ and $80 \pm 20\%$.

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 labeled.

Ensure separate cone test boards, cones and 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 5 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 5 mosquitoes into the next cone.

Repeat this procedure until mosquitoes have been introduced into all cones.

3 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 have been tested and the exposure procedures are complete.

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 sugar source on top of the holding receptacle. Sugar source is 10% glucose or sucrose solution made with sterile water. If a different knock down is measured this should be justified.

Maintain the holding room/area at $27 \pm 2^{\circ}\text{C}$ and $80 \pm 20\%$ 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

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).

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 24-hour mortality (M-24) 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 documented to enable understanding of test conduct.

It is recommended that tables of full results are presented in an annex if figures are used in the report.

Table 1

Endpoint	Time it is measured	Purpose/Definition	Considerations
Mortality at 24 hours after 3 minutes exposure (M24-3min)	24 hours after the 3-minute cone test exposure has ended	<p>The measurement of mortality in a cone test is an indicator of the lethal effects of test sample.</p> <p>Mortality is observed by the following indicators:</p> <ul style="list-style-type: none"> • No sign of life; immobile; cannot stand. • Moribund mosquitoes are also classified as dead after 24 hours of holding as it is unlikely that they would survive in nature i.e. 	<p>The standard exposure time in the cone test for measuring mortality is 3 minutes. Augmentation of the exposure time, or inclusion of multiple exposure times as different treatments, must be clearly declared and scientifically justified in the context of the product being tested and study being conducted.</p> <p>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.</p> <p>Extension of the exposure time must be declared and scientifically justified in the context of the product being tested and study being conducted.</p> <p>e.g.</p> <p>Mortality at 48 hours after 3 minutes exposure – M48-3min</p> <p>Mortality at 72 hours after 3 minutes exposure – M48-3min</p> <p>Mortality at x hours after 3 minutes exposure – Mx-3min</p> <p>Control mortality must not exceed 20% after extended holding times. Otherwise, the test is invalidated.</p> <p>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.</p>

<p>Knockdown at 60 minutes after 3 minutes exposure (KD60-3min)</p>	<p>60 minutes after the 3-minute cone test exposure has ended</p>	<p>The measurement of knockdown in a cone test is an indicator of sublethal effects (incapacitation) of a test sample. A knockdown effect has the potential to prevent mosquitoes from blood feeding on those individuals sleeping under an ITN.</p> <p>Knockdown is observed by the following indicators:</p> <ul style="list-style-type: none"> • 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 • No sign of life; immobile; cannot stand 	<p>The standard exposure time in the cone test for measuring knockdown is 3 minutes. Augmentation of the exposure time, or inclusion of multiple exposure times as different treatments, must be declared and scientifically justified based on the context/intent of the product being tested and the particular study being conducted.</p> <p>Regardless of the intended entomological effect of the product, KD60 should be observed and documented for the purpose of monitoring the experimental controls and thereby experimental acceptability.</p>
<p>Fertility - Eggs per female</p>	<p>72 and 96 hours after 3 minute exposure in a cone test</p>	<p>The measurement of eggs per female is an indicator of fertility.</p> <p>Fertility is observed by the following indicators:</p> <ul style="list-style-type: none"> • Number of eggs laid / live females at each period of observation • Reduction of fertility is determined by means of comparing the results 	<p>The standard exposure time in the cone test for measuring fecundity is 3 minutes. Augmentation of the exposure time, or inclusion of multiple exposure times as different treatments, must be clearly declared and scientifically justified in the context of the product being tested and study being conducted. The standard holding time post exposure in the cone test is 96 hours. Control mortality must not exceed 10% after 24 hours, nor 20% after 96 hours. Otherwise, the test is invalidated.</p>

		of test samples and the negative control	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 and 96 hours after 3 minute exposure in a cone test	<p>The measurement of proportion of fertile females in a cone test is an indicator of reduction of fecundity.</p> <p>Fecundity is observed by the following indicators:</p> <ul style="list-style-type: none"> • 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. • Reduction of fecundity is determined by means of comparing the results of test samples and the negative control 	<p>The standard exposure time in the cone test for measuring fecundity is 3 minutes. Augmentation of the exposure time, or inclusion of multiple exposure times as different treatments, must be clearly declared and scientifically justified in the context of the product being tested and study being conducted. The standard holding time post exposure in the cone test is 96 hours. Control mortality must not exceed 10% after 24 hours, nor 20% after 96 hours. Otherwise, the test is invalidated.</p> <p>Extension of the exposure time must be declared and scientifically justified in the context of the product being tested and study being conducted.</p>
Other	Applicants may propose other endpoints to be measured by means of the cone method with adequate justification		