World Health Organization

WHO Prequalification of Vector Control Products

Bioassay methods for insecticide-treated nets: tunnel test

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

- negative control mortality must not exceed 10% after 24 hours holding or 20% at extended holding times;
- blood feeding in the control must exceed 50%;
- no more or less than 50 mosquitoes should be used per tunnel *(1)*;
- 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 *(2)*.

1. Purpose of the method

The purpose of the tunnel 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 an exposure that is more representative of interaction with the test material while host seeking.

2. Considerations for use of the method

2.1. Classification as a bioassay

The tunnel 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 system** (e.g., mosquito [Table 1]).

The use of tunnel 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. It is especially useful for measuring the induced effect of the surface concentration 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 under laboratory conditions.

The consistency of the method supports the analysis across samples prepared in different ways, thereby providing valuable information about the stability and consistency in behavior of ITN fabrics through their intended useful life. However, the controlled laboratory setting, use of a non-human bait animal, 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 tunnel test is not an effective method for investigating the potential entomological efficacy of an ITN. The tunnel 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 tunnel test method can be employed 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 aging (washing) fabric samples.
- Provide baseline/reference information about the characteristics of samples used in semi-field trials or community studies.

It is important to note that the tunnel 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 tunnel 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 tunnel 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.

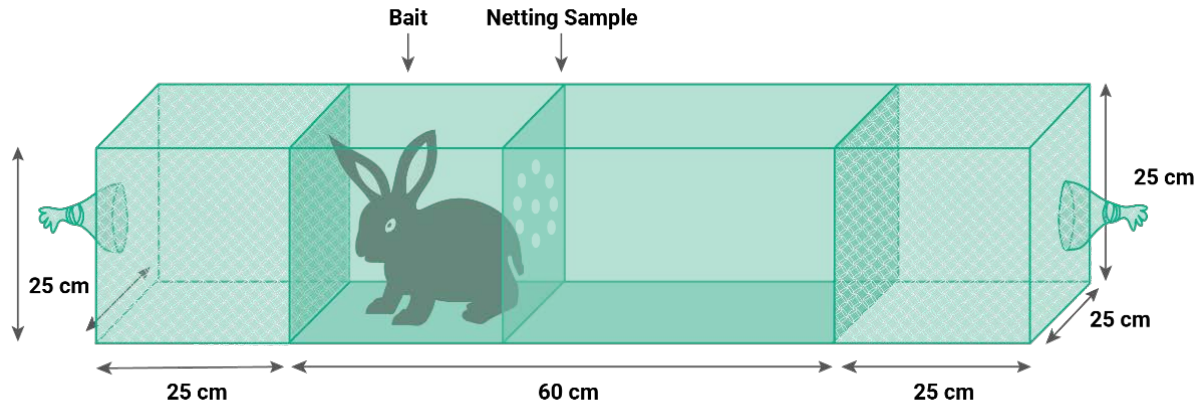
The method may not be an appropriate choice for investigating products formulated with active ingredients whose mode of action is rapid incapacitation after tarsal contact for which the cone test is recommended.

3. Materials

3.1. Tunnels

Standard test tunnels are made of glass or acrylic. The material needs to be clear and resistant to cleaning with polar solvents. The tunnel comprises three chambers (Fig. 1), 1) netted release chamber (25 x 25 x 25cm), 2) response chamber (60 x 25 x 25cm) and 3) netted collection chamber (25 x 25 x 25cm). The two mesh cages allow mosquitoes to rest and are important to minimise mortality from poor handling. The response chamber has a volume of 37,500cm^3. Mosquitoes are released and pass through a deliberately holed netting sample to reach the bait animal.

Fig. 1. Tunnel test



3.2. Bait animals

The tunnel test uses an animal bait. The way that the animals are cared for should be documented including the steps taken to ensure that the animal bait has not been treated with insecticides, e.g., for flea control or endectocides for worm control. Animals should be supervised by a veterinarian and fed on a diet free from pesticides. The animals should be gently restrained and are normally shaved on their back to allow the mosquitoes greater access to the host for blood feeding. Care should be taken so that the animal is calm and cannot injure itself during the test. The use of a restraint or sedation is advised for this means.

3.3. Aspirators

Aspirators that comprise a clear Perspex tube with an aperture of around 1 cm are recommended. 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.

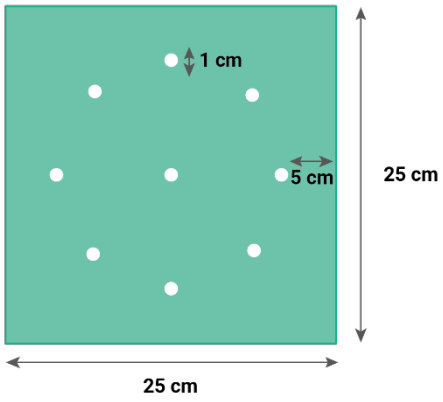
Since the tunnel test is a method for understanding the quality characteristics of an ITN, the history of and conditions to which test samples have been exposed are critical pieces of information for the interpretation of the resulting data. The means by which samples (including reference items) were 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 materials 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.

In each cut sample piece, cut nine holes each of 1 cm in diameter: one hole is located at the centre of the square, and the other eight are equidistant and located 5 cm from the border using a hole punch or scissors, taking care that the holes are standardized as if they are larger or smaller than standard this may invalidate the results (Fig. 2).

Fig. 2. Test material



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 tunnel 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.

3.5.4. Affixing test material to the tunnel frame

The test sample is attached to a 25 x 25cm frame to be slotted into the WHO tunnel. This needs to be attached securely to prevent mosquitoes from bypassing the netting sample via gaps between the frame and the tunnel. The means of attaching the sample to the frame should either use a disposable means such as a cardboard frame to which the sample is stapled or a reusable means in which the sample is attached through sandwiching it between two frames so it is firmly held in place. Reusable frames must be constructed of a material that can be cleaned to remove any pesticide residues. Care must be taken to ensure that the tunnels are securely attached in close contact to the board to prevent accidental trapping or escape of test insects.

Ensure that samples are adequately labelled.

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 that is 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 as well as the purpose of the study in which the tunnel method is applied.

5.2. Age and nutritional status of 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 testing 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

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

At the end of the starvation period, 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 through using a closed receptacle to transport holding cups or holding cages.

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 tunnel 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. Tunnel test endpoints

Endpoint	Time it is measured	Purpose and definition	Considerations
Mortality at 24 hours (M24)	24 hours after the 15-hour tunnel 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 tunnel test for measuring mortality is 12-15 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. 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 tunnel test is 24 hours. Control mortality should not exceed 10% after 24 hours. 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 (e.g., Mortality at x hours after exposure – Mx). Control mortality should not exceed 20% after extended holding times. Otherwise, the test is invalidated.
Blood feeding inhibition (BFI)	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.	Control blood feeding should exceed 50%. Otherwise, the test is invalidated.
Fertility – Eggs per female	Blood fed mosquitoes are held for 72 hours after 15 hours exposure in a tunnel 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.	Blood fed female mosquitoes are provided with an egg laying substrate for oviposition. The standard exposure time in the tunnel test for measuring fertility is 15 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 tunnel test for fertility measurements is 72 hours. Control mortality must not exceed 10% after

Endpoint	Time it is measured	Purpose and definition	Considerations
			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 Christopher's stage 5 eggs when dissected. Blood fed mosquitoes are held for 72 hours after 12-15 hours exposure in a tunnel test.	The measurement of proportion of fertile females in a tunnel 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 tunnel test for measuring fecundity is 12-15 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 tunnel 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 tunnel test with adequate justification.		

7. Test reproducibility

Adequate replication is an essential component of tunnel 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 tunnel 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 tunnel test is normally conducted during the dark phase, for example, between 18.00 and 09.00. 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 tunnels, aspirators 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 aspirators are prepared and labelled for the negative control, positive control, and each treatment to avoid 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 50 female mosquitoes from the holding receptacle into the negative control tunnel release cage and close the sleeve or cage opening.

Once all of mosquitoes are in the tunnel cover it with a black cloth to keep the animal bait calm and note the start time on the data sheet.

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

Continue until all the replicates for that testing day 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 tunnel 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 mortality and blood feeding status, 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 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 feeding success is less than 50% the test should be discarded and repeated. If M24 exceeds 5% then mortality should be control corrected using Abbotts Formula.

The number of tunnel 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 tunnel tests

Variability in tunnel 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 tunnel tests.

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. Kamande, D.S., Odufuwa, O.G., Mbuba, E., Hofer, L., Moore, S.J. Modified World Health Organization (WHO) tunnel test for higher throughput evaluation of insecticide-treated nets

(ITNs) considering the effect of alternative hosts, exposure time and mosquito density. Insects. 2022; 13:562 (https://doi.org/10.3390/insects13070562, accessed 30 August 2023).

2. Balmert, N.J., Rund, S.S.C., Ghazi, J.P., Zhou, P., Duffield, G.E. Time-of-day specific changes in metabolic detoxification and insecticide resistance in the malaria mosquito *Anopheles gambiae*. J Insect Physiol. 2014; 64:30-39 (https://doi.org/10.1016/j.jinsphys.2014.02.013, accessed 30 August 2023).