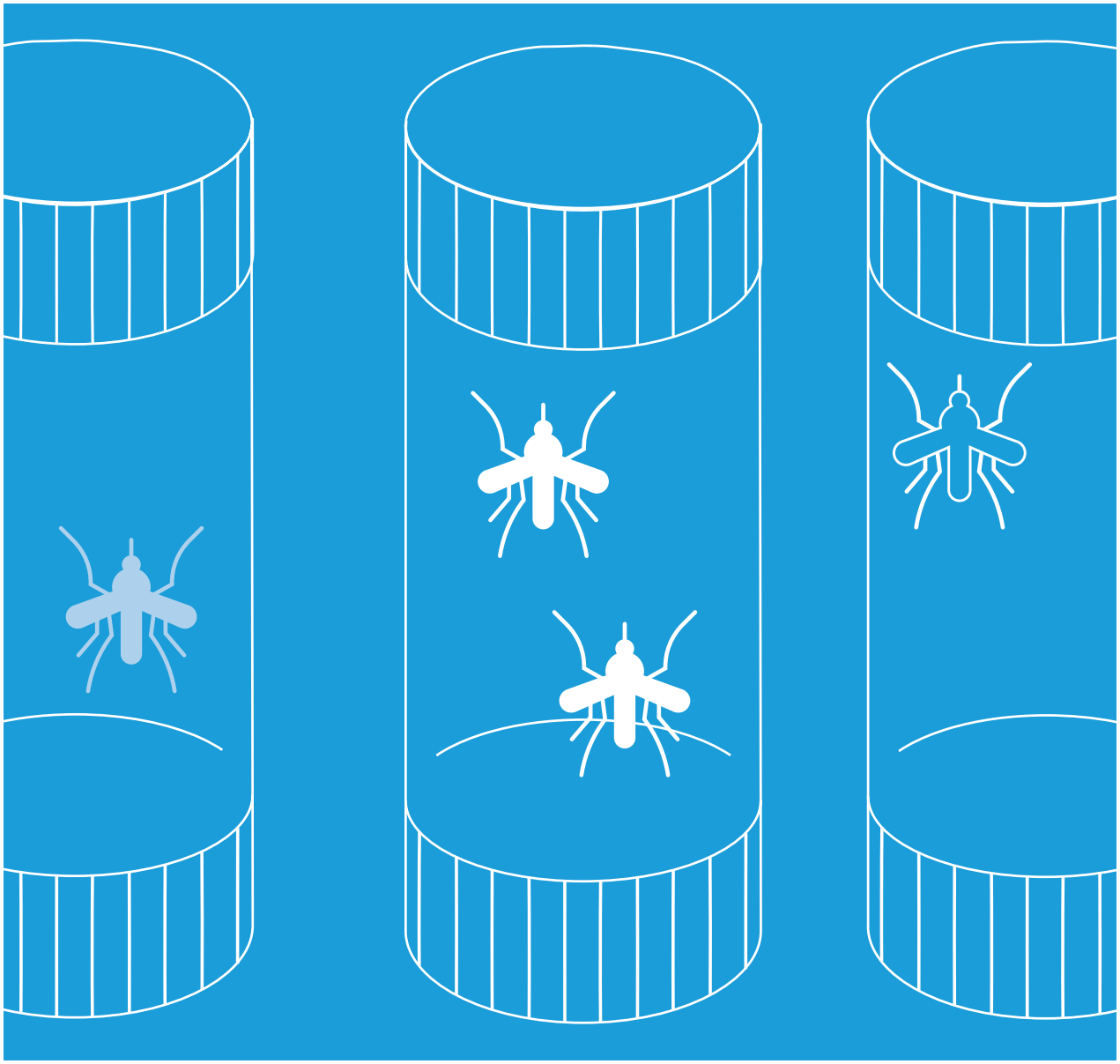
World Health Organization

Standard operating procedure for testing insecticide susceptibility of adult mosquitoes in WHO tube tests

SOP version: WHO Tube test/01/14 January 2022



1. Introduction, scope and purpose

This standard operating procedure (SOP) describes the process to follow for evaluating the susceptibility of adult mosquito vectors to insecticides using the World Health Organization (WHO) tube test. This bioassay is a direct response-to-exposure test, measuring mosquito mortality 24 hours after exposure to a known standard concentration of an insecticide (e.g. the discriminating concentration) for a period of 1 hour. This procedure should be followed for testing mosquito susceptibility to insecticides that can impregnate filter papers. For insecticides that are unstable on filter papers or that cannot be impregnated on filter papers, the WHO bottle bioassay procedure should be followed instead (see SOP Bottle-bioassay/01/14 January 2022).

This SOP provides instructions on preparation of materials, mosquito exposure, recording and interpretation of test results. Filter papers impregnated with standard discriminating concentrations (DCs) of insecticides are needed for this test. Filter papers impregnated with the validated discriminating concentrations of various insecticides, as well as test kits for conducting WHO tube tests can be purchased from the Universiti Sains Malaysia (USM) – Vector Control Research Unit (http://www.inreskit.usm.my).

2. Equipment, reagents and consumables

Equipment required for this bioassay can be used multiple times except for the impregnated papers, which should be used a maximum of 6 times.

A standard mosquito (adult) diagnostic test kit from USM includes the following items, although items can also be procured separately as needed:

☐	6 green-dotted holding tubes
☐	2 yellow-dotted control exposure tubes
☐	4 red-dotted exposure tubes
☐	6 steel clips (rings) to hold white papers in the holding tubes
☐	6 copper clips (rings) to hold insecticide- or oil-impregnated (control) papers in the exposure tubes
☐	6 slide units
☐	40 sheets of 15 cm x 12 cm white paper for lining the holding tubes

☐	2 aspirators with glass tubes, straight
☐	2 pieces of rubber tubing, 60 cm long
☐	2 glass mouthpieces for aspirators
☐	1 roll of adhesive tape
☐	1 label

Additionally, impregnated papers can can also be procured as needed from the Universiti Sains Malaysia (USM). The contents include the following:

☐	Impregnated papers: each box contains 8 sheets of insecticide-impregnated papers (see standard discriminating concentration in Table 1 and Table 2).
☐	Control papers: each box contains 8 sheets of carrier oil-impregnated papers to be used as the control. (For testing pirimiphos-methyl, use Whatman no. 1 filter paper treated with acetone alone.)

The following materials should be procured locally by the testing laboratory:

☐	netting pieces
☐	1 cage to hold female mosquitoes (25 mosquitoes per tube)
☐	6 sheets of clean white paper (12 cm x 15 cm)
☐	1 mouth aspirator/mechanical aspirator
☐	glucose to prepare 10% (w/v) sugar solution in water
☐	medical grade cotton wool

☐	timer (stopwatch)
☐	calibrated, traceable humidity and temperature monitors/data loggers
☐	permanent marker pens for labelling the tubes
☐	testing chamber or a climate-controlled insectary at 27 °C ± 2 °C and 75% ± 10% relative humidity
☐	appropriate personal protective equipment (e.g. laboratory coat, latex gloves)
☐	acetone or alcohol (for cleaning glassware and working table)
☐	antibacterial cleaner such as 70% isopropyl alcohol or ethanol

Table 1. Insecticide DCs for WHO tube tests with *Anopheles* spp. mosquitoes

Insecticide class	Insecticide	Species for which DCs are validated	DC for 1 h exposure[a] (%)	Carrier oil/ solvent
Pyrethroids	Alpha-cypermethrin	*An. funestus* s.s., *An. gambiae* s.s. and *An. minimus*	0.05%	Silicone oil
		An. albimanus and *An. stephensi*	0.30%	Silicone oil
	Cyfluthrin	*An. aconitus, An. albimanus, An. arabiensis, An. dirus, An. freeborni, An. gambiae s.s., An. maculatus, An. minimus* and *An. stephensi*	0.15%	Silicone oil
	Deltamethrin	*An. aconitus, An. albimanus, An. arabiensis, An. dirus, An. freeborni, An. gambiae s.s., An. maculatus, An. minimus* and *An. stephensi*	0.05%	Silicone oil
	Etofenprox	*An. aconitus, An. albimanus, An. arabiensis, An. dirus, An. freeborni, An. gambiae s.s., An. maculatus* and *An. stephensi*	0.50%	Silicone oil
	Lambda-cyhalothrin	*An. aconitus, An. albimanus, An. arabiensis, An. dirus, An. freeborni, An. gambiae s.s., An. maculatus, An. minimus* and *An. stephensi*	0.05%	Silicone oil
		An. sacharovi	0.10%	Silicone oil
	Permethrin 40:60	*An. aconitus, An. albimanus, An. arabiensis, An. dirus, An. freeborni, An. gambiae s.s., An. maculatus, An. minimus* and *An. stephensi*	0.75%	Silicone oil
Carbamates	Bendiocarb	Species-specific data unavailable	0.10%	Olive oil
	Carbosulfan	Species-specific data unavailable	0.40%	Olive oil
	Propoxur	Species-specific data unavailable	0.10%	Olive oil
Organochlorines	DDT	Species-specific data unavailable	4.00%	Risella oil
	Dieldrin	Species-specific data unavailable	4.00%	Risella oil
		An. sacharovi	4.00%	Risella oil
Organophosphates	Fenitrothion	Species-specific data unavailable	1.00%[b]	Olive oil
	Malathion	Species-specific data unavailable	5.00%	Olive oil
	Pirimiphos-methyl	*An. albimanus, An. stephensi, An. minimus* and *An. funestus s.s.*	100 mg/m^2[c]	Acetone only
		An. gambiae s.s.	170 mg/m^2[c]	Acetone only

[a] These DCs are based on historical WHO recommendations, except for the DCs of alpha-cypermethrin and pirimiphos-methyl, which were validated in a recent WHO multi-centre study *(1)*.

[b] Fenitrothion requires 2 hour exposure period, instead of 1 hour

[c] These DCs are expressed as mg/m^2, as no carrier oil is used to treat papers with pirimiphos-methyl.

Table 2. Insecticide DCs for WHO tube tests with *Aedes* spp. mosquitoes

Insecticide class	Insecticide	Species for which DCs are validated	DC for 1 h exposure[a] (%)	Carrier oil/solvent
Pyrethroids	Alpha-cypermethrin	*Ae. aegypti*	0.05%	Silicone oil
		Ae. albopictus	0.08%	Silicone oil
	Deltamethrin	*Ae. aegypti* and *Ae. albopictus*	0.03%	Silicone oil
	Lambda-cyhalothrin	*Ae. aegypti*	0.05%	Silicone oil
		Ae. albopictus	0.08%	Silicone oil
	Permethrin 40:60	*Ae. aegypti* and *Ae. albopictus*	0.4%	Silicone oil
Carbamates	Bendiocarb	*Ae. aegypti* and *Ae. albopictus*	0.20%	Olive oil
Organophosphates	Chlorpyrifos-ethyl	*Ae. aegypti* and *Ae. albopictus*	1.00%	Olive oil
	Pirimiphos-methyl	*Ae. aegypti* and *Ae. albopictus*	60 mg/m^2 [b]	Acetone only
	Malathion	*Ae. aegypti*	1.5%	Olive Oil
		Ae. albopictus	5%	Olive Oil

[a] These DCs are based on validation in a recent WHO multi-centre study *(1)*.

[b] These DCs are expressed as mg/m^2, as no carrier oil is used to treat papers with pirimiphos-methyl.

Table 3. Insecticide DCs for WHO tube tests with *Culex quinquefasciatus* mosquitoes

Insecticide class	Insecticide	DC for exposure[a] (%)	Exposure time	Carrier oil
Organochlorines	DDT	4%	4 h	Risella oil
Pyrethroids	Deltamethrin	0.025%	1 h	Silicone oil
	Lambda-cyhalothrin	0.025%	1 h	Silicone oil
	Permethrin	0.25%	3 h	Silicone oil
Carbamates	Propoxur	0.10%	1 h	Olive oil
Organophosphates	Fenitrothion	1%	2 h	Olive oil
	Malathion	5%	1 h	Olive oil

[a] These DCs are based on historical WHO recommendations.

3. Health, safety and environmental protection

✓	Before using any chemical compound, laboratory staff should read and understand the risk assessment, material safety data sheets and the control of substances hazardous to health assessment for each chemical used.
✓	Appropriate personal protective equipment must be worn at all times when handling insecticides, including laboratory coat, gloves, safety glasses and a face mask when weighing out chemicals.
✓	Ensure all working areas are clear of other materials and cleaned prior to performing the test.
✓	All staff working in the laboratory must have received laboratory induction training and the training must be documented in the individual's training file.
✓	Dispose of all waste materials appropriately following the national/ institutional safety guidelines.
✓	When working with mosquitoes, minimize mosquito escape by keeping all doors and windows shut. If any mosquitoes escape, immediately use an electric bat to electrocute them.

4. Mosquitoes

This bioassay requires 150 non-blood-fed adult female mosquitoes aged 3–5 days. Mosquitoes need to be starved for 2 hours before using them in the test.

During rearing, mosquitoes need to be well nurtured and maintained in uncrowded trays during the larval stages, and in uncrowded cages during the adult stage. This is important to minimize mortality due to causes other than exposure to the insecticide.

During the exposure period, it is important that the number of mosquitoes per tube be 25 or as close to 25 as possible, but it should not exceed 25 to avoid crowding within the bottle.

5. Test procedure

Step 1: Labelling the tubes

The WHO tube test kit consists of plastic tubes. Three types of tubes come with a WHO test kit:

- The holding tube, into which clean white paper is inserted, is identified by a green dot.
- The control tube, into which oil-/acetone-treated filter paper is inserted, is identified by a yellow dot.
- The exposure tube, into which the insecticide-treated paper is inserted, is identified by a red dot.

☐ 5.1. **Label each green-, yellow- and red-dotted tube** with a sticky label containing the information shown below in the examples of labels for each type of tube. Write the label with a permanent marker pen. This information is important to ensure that the papers are within their shelf-life period (i.e. not expired) and have not exceeded the maximum number of uses.

Note: The batch number, paper impregnation date and expiry date are written on the filter paper boxes if they are procured from USM. If the papers are treated in the testing laboratory, the impregnation date and expiry date should have been written manually on a label and pasted on the paper wrap or storage container.

Examples of labels for tubes are given below. Investigators may want to use a subset of these depending on their testing set-up.

a) For a **holding tube with a green dot:**

Date of test (dd/mm/yyyy):
Holding tube no.:
Test operator's initials:

b) For a **control tube with a yellow dot:**

Date of test (dd/mm/yyyy):
Control tube no.:
Control in use:
Batch # of the control paper:
Paper impregnation date (dd/mm/yyyy):
Paper expiry date (dd/mm/yyyy):
Date of first use (dd/mm/yyyy):
No. of times this paper was previously used:
Test operator's initials:

c) For an **exposure tube with a red dot:**

Date of test (dd/mm/yyyy):
Exposure tube no.:
Insecticide & concentration:
Batch # of the paper:
Impregnation date (dd/mm/yyyy):
Expiry date (dd/mm/yyyy):
Date of first use (dd/mm/yyyy):
No. of times this paper was previously used:
Test operator's initials:

☐ 5.2. **Prepare holding tubes (green-dotted)**

5.2.1. Take 6 sheets of clean white papers (size 12 cm × 15 cm) rolled into a cylinder shape, and insert one into each of the 6 holding tubes. Fasten the sheet into position against the wall of the tube using 2 steel rings (clips), 1 at the top end and 1 at the bottom end (Fig. 1).

5.2.2. Place a 16-mesh gauze on each holding tube and screw the cap into place.

5.2.3. Attach a slide unit to each of the holding tubes by screwing it in place at the open end, as shown in Fig. 1.

☐ 5.3. **Prepare control tubes (yellow-dotted)**

5.3.1. Wearing disposable gloves, roll 2 control papers into a cylinder shape and insert each one into a yellow-dotted tube, ensuring that the stamped label on the paper is on the outer side and is readable through the transparent tube.

5.3.2. Fasten the control papers in each tube using 2 copper rings, 1 at the top end and 1 at the bottom end. Close the tube with a screw cap at one end (the bottom end).

5.3.3. Remove the gloves and dispose of them in a biohazard bag.

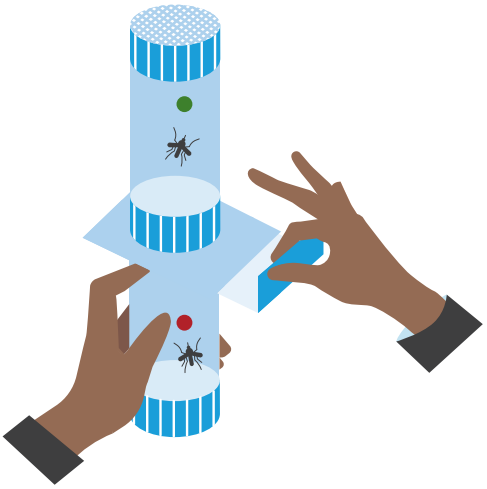
☐ 5.4. **Prepare exposure tubes (red-dotted)**

5.4.1. Wearing disposable gloves, roll 4 insecticide-treated papers into a cylinder shape and insert each one into a red-dotted tube, ensuring that the stamped label on the paper is on the outer side and is readable through the transparent tube.

5.4.2. Fasten the insecticide-treated paper in each tube using 2 copper rings, 1 at the top end and 1 at the bottom end. Close the tube with a screw cap at one end (the bottom end).

5.4.3. Remove the gloves and dispose of them in a biohazard bag.

Fig. 1. Test tubes. Left: A holding tube (green dot) with a steel clip (ring) and an exposure tube (red dot) with a copper clip. Right: A holding tube (green dot) connected to an exposure tube (red dot) with a slide unit



Source: photo courtesy of Institut de Recherche pour le Développement, Montpellier, France

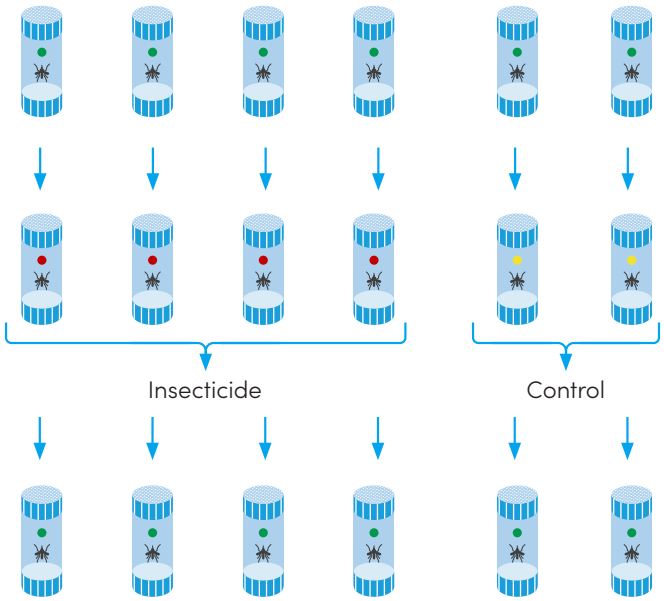
Step 2: Mosquito exposure

Fig. 2. Mosquito exposure and post-exposure processes

Pre-exposure holding period (1h): Hold mosquitoes in holding tubes lined with clean white paper.

Exposure period (1h): Expose mosquitoes to insecticide-impregnated papers (red tubes) or control papers treated with oil or acetone alone (yellow tubes).

Post-exposure holding period (24h): Transfer mosquitoes into the holding tubes. Provide them access to a water-sugar solution. Read mortality at the end of the 24h.



- [ ] 5.5. **Aspirate and insert mosquitoes into the tubes:** Using an aspirator, aspirate 25 adult female mosquitoes from a mosquito cage into each of the 6 green-dotted holding tubes through the filling hole on the tube sliding door. A total of 150 mosquitoes is needed to fill the 6 tubes. Close the slide unit and set the holding tubes in an upright position (Fig. 2).

- [ ] 5.6. **Leave the mosquitoes in the holding tubes for 1 hour.**

- [ ] 5.7. **Transfer the mosquitoes from the holding tubes to the test tubes (2 control yellow-dotted and 4 exposure red-dotted tubes):** Only live mosquitoes should be transferred to the test tubes. Any moribund (i.e. those unable to fly) or dead mosquitoes should be removed.

    5.7.1. One by one, attach the empty exposure and control tubes to the vacant position on the sliding units attached to the holding tubes.

    5.7.2. Carefully slide the units open and gently blow the mosquitoes from the holding tubes into the exposure tubes.

    5.7.3. Once all the mosquitoes are in the exposure tubes, close the slide unit and place a cotton wool plug into the hole to lock the slide.

    5.7.4. Record the exact number of live mosquitoes exposed. Some mosquitoes could have died during the holding or transfer processes.

    5.7.5. Detach the green-dotted holding tubes from the exposure tubes and set them aside.

    5.7.6. Set the tubes in a vertical position with the mesh screen facing up. Place them in an area of reduced lighting, or cover them with cardboard discs to reduce light intensity and to discourage test mosquitoes from resting on the mesh screen lid.

- [ ] 5.8. **Leave the mosquitoes in the treatment (red-dotted) and control (yellow-dotted) tubes for 1 hour**.

☐ 5.9. **Transfer the mosquitoes back to the holding tubes at the end of the 1-hour exposure period.**

5.9.1. Gently blow the mosquitoes back into the green-dotted holding tubes by reversing the procedure outlined above.

5.9.2. Detach the exposure tubes from the slide units.

5.9.3. Place the tubes in an upright position with the mesh screen facing up.

5.9.4. Place a piece of cotton wool soaked in a 10% sugar solution on the mesh screen of the holding tubes. To soak the cotton wool in sugar solution, follow these steps:

- Pour some 10% sugar solution into a clean container.
- Take a piece of cotton wool that is roughly 5 cm x 5 cm in size.
- Submerge this in the sugar solution.
- Remove the cotton wool and squeeze it just enough to ensure that it is not dripping.
- Place the soaked cotton wool flat on the top of the holding tube to enable the mosquitoes to sugar-feed and/or hydrate.
- Pour away any remaining sugar solution into a sink and rinse out the container with tap water.

☐ 5.10. **Record the number of knocked down mosquitoes**, as per the definition in Table 4.

☐ 5.11. **Hold the mosquitoes in the holding tubes (green-dotted) for 24 hours** at 27 °C ± 2 °C and 75% ± 10% relative humidity.

Table 4. WHO definitions of knockdown and mortality of mosquitoes post-test

Mosquitoes considered alive after 1 h of exposure, or 24 h, 48 h or 72 h after exposure	Mosquitoes considered knocked down after 1 h of exposure or dead at 24 h, 48 h or 72 h after exposure
Can both stand and fly in a coordinated manner	• No sign of life; immobile; cannot stand • Any mosquito that cannot stand (e.g. has 1 or 2 legs) • Any mosquito that cannot fly in a coordinated manner • Any mosquito that lies on its back, moving legs and wings but is unable to take off • Any mosquito that can stand and take off briefly but falls down immediately

Source: page 77 of the *Report of the fifteenth WHOPES working group meeting (2).*

Step 3: Record mortality results

☐ 5.12. **Record mortality 24 hours after 1 hour of exposure:** Count and record the number of mosquitoes found dead and alive 24 hours after 1 hour exposure, as per the definitions provided in Table 4. Enter the data in the recording sheet or electronic data collection system.

6. Use and storage of impregnated papers

Do not use the same impregnated paper more than 6 times (equivalent to exposing 150 mosquitoes in a tube). Before reusing the papers in a new test, the sealed plastic box should be brought to room temperature (~ 25 °C) unopened for 1 hour. Test papers should never be exposed to direct sunlight.

When bioassays are conducted over a few days, impregnated papers can be retained in the exposure tubes, provided the tubes are individually wrapped in aluminum foil after each use and kept at 4–8 °C temperature.

Between insecticide resistance testing cycles, reusable papers (i.e. used less than 6 times) should be kept in their original plastic box, sealed with tape and stored in a cool container or refrigerator at 4–8 °C. At these temperatures, their shelf-life ranges from 2 to 5 years depending on the insecticide (Table 5).

Table 5. Shelf-life at optimal storage conditions and in storage at accelerated temperature conditions

Class	Insecticide	Shelf-life at optimum cold storage condition (4–8 °C)	Accelerated storage stability (54 ± 2 °C for 2 weeks or 40 ± 2 °C for 8 weeks)
Organochlorine	p,p'-DDT	5 years	Stable
Organophosphates	Malathion	3 years[a]	Stable
	Pirimiphos-methyl	3 years[a]	-
Carbamates	Bendiocarb	3 years[a]	-
	Propoxur	3 years[a]	-
Pyrethroids	Alpha-cypermethrin	2 years	Stable
	Cyfluthrin	2 years	Stable
	Deltamethrin	2 years	Stable
	Etofenprox	2 years	Stable
	Lambda-cyhalothrin	2 years	Stable

[a] Tentative (needs reconfirmation)

7. Criteria for test rejection

If the control mortality is >20%, the tests must be discarded and repeated.

8. Data recording and calculation of test results

During the test, data should be entered in paper-based or digital data recording forms. A paper template is provided in Annex 1 of this SOP. Digital DHIS2-base forms are available from the WHO Global Malaria Programme website.[1]

The end-point of the test is mosquito mortality 24 hours after 1 hour of exposure to the insecticide. Mosquito mortalities should be calculated separately for the 2 groups of tubes: treatment and control.

Treatment mortality is calculated by summing the number of dead mosquitoes across all replicates with insecticide impregnated papers and then expressing this as a percentage of the total number of mosquitoes in such replicates. Mortality in the control is calculated similarly:

$$\text{Treatment mortality (\%)} = \frac{\text{Number of treated female mosquitoes dead}}{\text{Total number of treated female mosquitoes}} \times 100$$

$$\text{Control mortality (\%)} = \frac{\text{Number of control female mosquitoes dead}}{\text{Total number of control female mosquitoes}} \times 100$$

- If the control mortality is <5%, no correction of test results is necessary.
- When control mortality is ≥5% and ≤20%, the test mortality should be corrected with the control mortality using Abbott's formula as follows:

$$\text{Corrected mortality} = \frac{(\text{\% exposed mortality} - \text{\% control mortality})}{(100 - \text{\% control mortality})} \times 100$$

9. Interpretation of test results

- **Confirmed resistance:** A vector population is considered to be resistant to an insecticide if mortality within the treatment group (corrected using the Abbott's formula, if necessary) is <90%, provided that at least 100 mosquitoes of each species were tested.
- **Possible resistance**: If the observed mortality within the treatment group (corrected using Abbott's formula, if necessary) is ≥90% but <98%, the presence of resistance is possible but not confirmed. Test results should be confirmed by repeating the test with a new sample from the same mosquito population. (Note: Avoid using F1 of the tested mosquitoes.) If 2 tests consistently show mortality within the treatment group <98%, then resistance is confirmed.
- **Susceptibility:** A vector population is considered to be susceptible to an insecticide if the mosquito mortality within the treatment group (corrected using the Abbott's formula, if necessary) is ≥98%.

1 https://www.who.int/teams/global-malaria-programme/prevention/vector-control/dhis-data-collection-and-collation-tools

10. Cleaning process

☐	10.1. Soak the tubes overnight in a 20% alkaline solution (TFD4 or Decon 90) for equipment in direct contact with the insecticide (e.g. treatment tubes with copper clips, etc.) or in a 10% solution for equipment that did not come in contact with the insecticide but was used for handling the mosquitoes (e.g. holding tubes, steel clips, etc.). **Note**: The 20% and 10% decontaminant solutions should be changed at least once per month, or more often if necessary.
☐	10.2. On the following day, rinse the equipment 3 times with tap water and dry at room temperature.
☐	10.3. Clean the bench and fixed equipment with acetone.

11. Acknowledgements

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12. References

1. Determining discriminating concentrations of insecticides for monitoring of resistance in mosquitoes: report of a multi-centre laboratory study with recommendations of WHO consultations. Geneva: World Health Organization; 2021 (in press).

2. Report of the fifteenth WHOPES working group meeting. WHO/HQ, Geneva, 18–22 June 2012. Geneva: World Health Organization; 2012 (http://apps.who.int/iris/bitstream/10665/75304/1/9789241504089_eng.pdf, accessed 3 January 2022).

For further information, please contact: vectorsurveillance@who.int

Annex 1. Data collection form for testing insecticide susceptibility of adult mosquitoes in WHO tube tests

World Health Organization

Data collection form – WHO tube test for testing insecticide susceptibility of adult mosquitoes

To be completed in black or blue ink only. Do not use pencil or correction fluid.

Date (dd/mm/yyyy):	**Technician's name:**	
Location of mosquito collection:	**Coordinates** Latitude: Longitude:	
Period of mosquito collection: Start date (dd/mm/yyyy): End date (dd/mm/yyyy):	**Collection method:**	
Insecticide tested and concentration:	**Date of paper impregnation (dd/mm/yyyy):**	**No. of times the same papers have been used before:**
Mosquito species:	**Mosquito stage and origin:** F0 adults (from wild larvae), F0 adults (wild collected), F1 adults (from wild larvae), F1 adults (progeny of wild adults)	
Age of females (days):	**Feeding status:** unfed; sugar-fed and starved; other, specify	
Start time of exposure (hh:mm):	**End time of exposure (hh:mm):**	
Temperature during exposure + holding period (°C): Max: Min:	**Relative humidity during exposure + holding period (%):** Max: Min:	

	Tube	Number of mosquitoes introduced	Number of knocked down mosquitoes 1 h after exposure	No. of dead and alive mosquitoes at 24 h after 1 h exposure		Mortality at 24 h after 1 h exposure
				No. dead	No. alive	Mortality (%)
Wild mosquitoes exposed to DC[a] of the insecticide	Tube 1					
	Tube 2					
	Tube 3					
	Tube 4					
Wild control mosquitoes	Control tube 1					
	Control tube 2					

Final results (all tubes)

	Knocked down after 1 h exposure (%) (at the end of 1 h exposure)	Mortality % (at 24 h)	Abbott's corrected mortality % (24 h)
Wild mosquitoes exposed to the DC[a] of a test insecticide			

[a]DC, discriminating concentration

Test result. The vector population is _____ (susceptible/resistant/possibly resistant) to the insecticide

Comments, if any:

Verified by Supervisor: _____ Date: _____

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