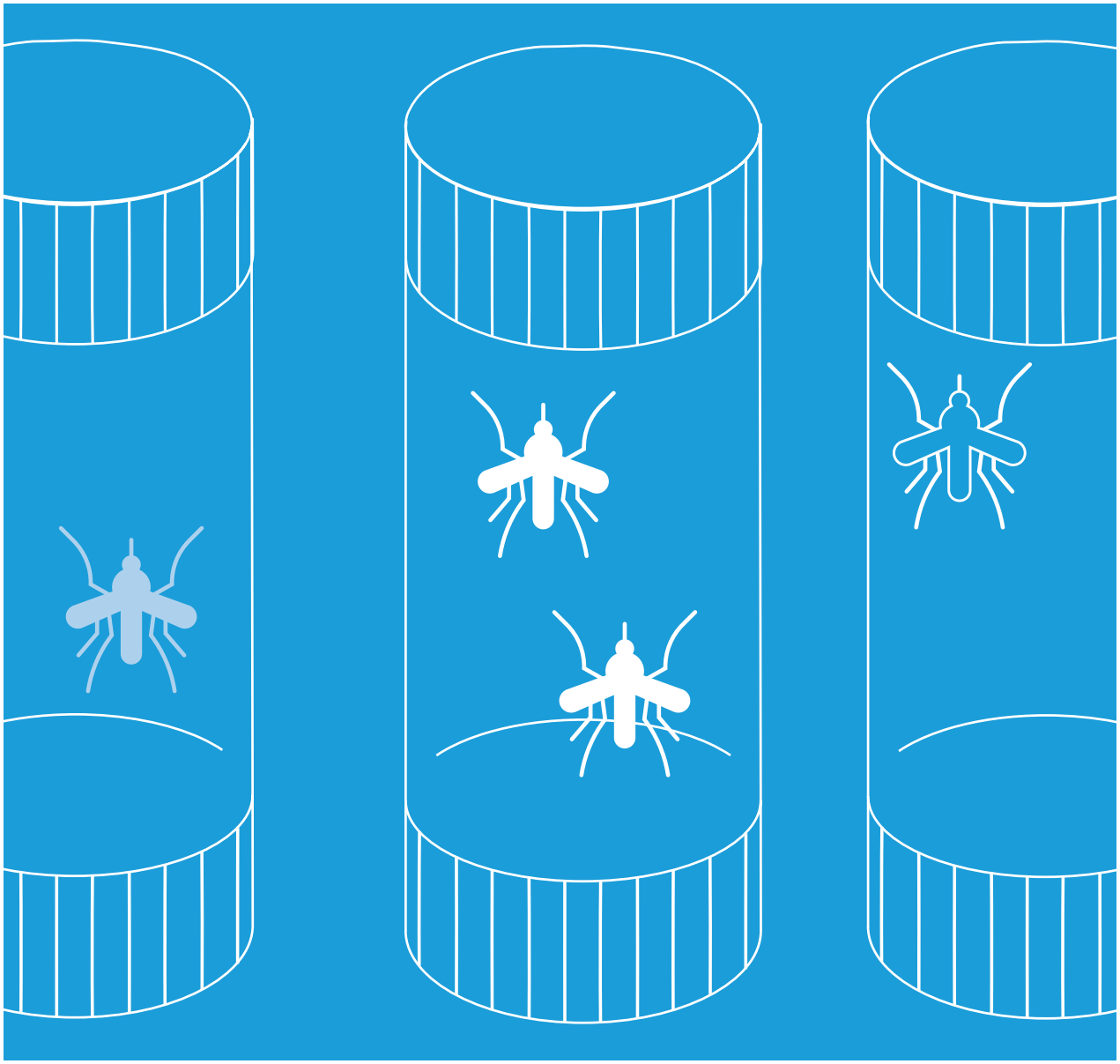
World Health Organization

Standard operating procedure

for determining the ability of PBO to restore susceptibility of adult mosquitoes to pyrethroid insecticides in WHO tube tests

WHO SOP version: PBO-insecticide synergist bioassay/01/14 January 2022



1. Introduction, scope and purpose

Piperonyl butoxide (PBO) is an inhibitor of detoxifying enzymes, primarily mono-oxygenases, that confer vector resistance to pyrethroids. PBO synergizes the effects of pyrethroid insecticides against mosquitoes presenting this resistance mechanism and is currently used in combination with pyrethroids to impregnate mosquito nets (known as pyrethroid-PBO nets), which are designed to mitigate the impact of this resistance mechanism on the effectiveness of insecticide treated mosquito nets.

The purpose of this standard operating procedure (SOP) is to describe the process for evaluating the ability of PBO to restore the susceptibility of mosquitoes to pyrethroids in pyrethroid-resistant vector populations. This bioassay provides an indirect assessment of the involvement of mono-oxygenases in conferring pyrethroid resistance, and results are meant to contribute towards decision-making on the deployment of pyrethroid-PBO nets.

This bioassay is a direct response-to-exposure test that compares mosquito mortality after exposure to a pyrethroids insecticide in one test group to mosquito mortality after pre-exposure to 4% PBO-treated papers followed by exposure to the same pyrethroid in the second test group. Its goal is to determine the level of mosquito susceptibility to pyrethroids restored by the PBO. This bioassay should be conducted with papers impregnated with the pyrethroids currently or previously used for IRS or on ITNs in the field. The bioassay should only be conducted with mosquito vector populations that are confirmed to be resistant to that pyrethroid.

While WHO has established a PBO discriminating concentration of 4% to be used in bioassays against *Anopheles* spp. mosquitoes, discriminating concentrations have not yet been established for determining the ability of PBO to restore pyrethroid susceptibility among *Aedes* spp. or other vectors.

This SOP provides instructions on the preparation of materials, mosquito exposure, recording and interpretation of test results. Filter papers impregnated with 4% PBO and standard discriminating concentrations of pyrethroid insecticides are needed for this test and are available for purchase from the Vector Control Research Unit, Universiti Sains Malaysia (USM).[1]

1 For procurement, download the catalogue and the order form from the WHO website (https://cms.who.int/teams/control-of-neglected-tropical-diseases/vector-ecology-and-management/vector-control/insecticide-resistance) or the USM website (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 be procured as needed by the testing laboratory. The contents include the following:

☐	Impregnated papers: Each box contains 8 sheets of insecticide-impregnated papers (see standard discriminating concentrations in Table 1).

☐	Control papers: Each box contains 8 sheets of carrier oil-impregnated papers to be used as the control.

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

☐	netting pieces
☐	1 cage to hold female mosquitoes (400 ideally)
☐	40 sheets of 15 cm x 12 cm white paper for lining the holding tubes
☐	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 discriminating concentrations (DCs) of pyrethroid insecticides and PBO for testing the ability of PBO to restore insecticide susceptibility in *Anopheles* mosquitoes

Insecticide class	Insecticide	Species for which DCs are validated[a]	DC for 1 h exposure %	Carrier oil
Synergist	Piperonyl butoxide (PBO)	Species-specific data unavailable	4%	Silicone oil
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. aculatus, 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. aculatus* and *An. stephensi*	0.50%	Silicone oil
	Permethrin	*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
	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	0.01%	Silicone oil

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

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 procedure requires 400 non-blood-fed adult female mosquitoes aged 3–5 days that need to be starved for 2 hours before the test. The mosquitoes are divided into 4 groups of 100 mosquitoes as follows:

- **Control group:** will be exposed to papers impregnated with a carrier oil;
- **PBO-only group:** will only be exposed to PBO-impregnated papers;
- **Insecticide-only group:** will only be exposed to insecticide-impregnated papers;
- **PBO + insecticide group:** will be exposed first to PBO-impregnated papers and then to insecticide-impregnated papers.

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/ synergist.

5. Test procedures

Step 1: Labelling the tubes

The WHO tube test kit consists of plastic tubes. Three types of tubes come with a WHO test kit (Fig. 2):

- The holding tube, into which clean white paper is placed, 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 insecticide- or PBO-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 information on the type of paper contained in the tube and the exposure pathway in which the tube will participate ("PBO only", "PBO + pyrethroids", "Pyrethroids only" or "Solvent control") (Fig. 2). The batch number, paper impregnation date and expiry date are written on the filter paper boxes if procured from USM and should be written on the package or container if prepared locally in the test laboratory. Use a permanent marker pen to write the label. This information is important to ensure that the papers are not expired and have not exceeded the maximum number of uses.

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 pathway:
Test operator's initials:

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

Date of test (dd/mm/yyyy):
Control tube no.:
Test pathway:
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 exposure **to pyrethroid insecticide tube with a red dot:**

Date of test (dd/mm/yyyy):
Exposure tube no.:
Test pathway:
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:

d) For exposure **to PBO tube with a red dot:**

Date of test (dd/mm/yyyy):
Exposure tube no.:
Test pathway:
Synergist and concentration:
Batch # of the paper:
Paper impregnation date (dd/mm/yyyy):
Expiry date of papers (dd/mm/yyyy):
Date of first use of papers (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 four sheets of clean white paper (size 12 cm × 15 cm) rolled into a cylinder shape and insert one into each of the 4 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 close to tube by screwing the cap into place (Fig. 1).

5.2.3. Attach a slide unit to the other side of 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 1 control paper into a cylinder shape and insert it 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 using 2 copper rings, 1 at the top end and 1 at the bottom end.

5.3.3. Place a 16-mesh gauze on each holding tube and close the tube by screwing the cap into place.

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

☐

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

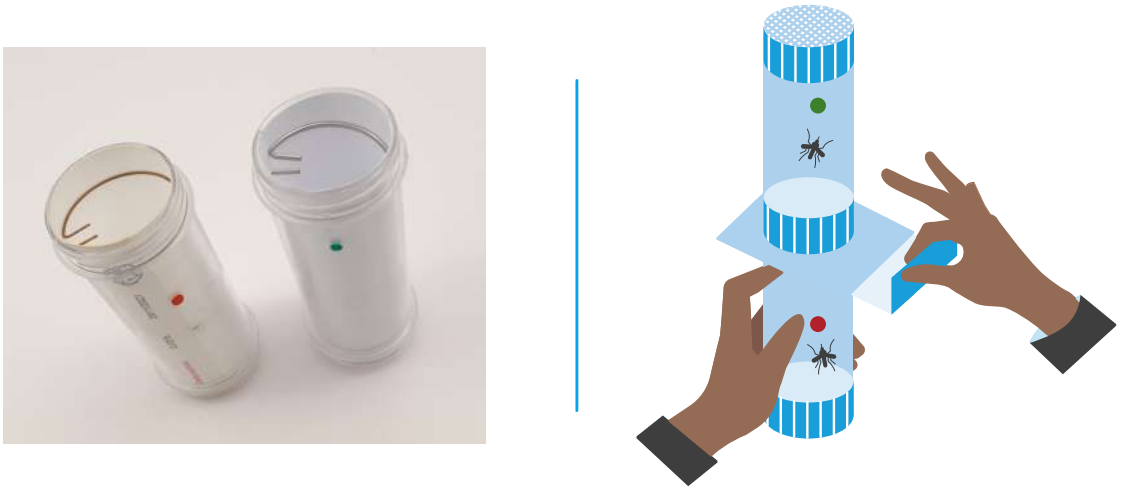
5.4.1. Wearing disposable gloves, roll 2 synergist-impregnated papers and 2 insecticide-impregnated papers into a cylinder shape and insert each of them into a red-dotted tube, ensuring that the stamped label on the paper is on the outer side and is readable through the transparent tube (Fig. 1).

5.4.2. Fasten the impregnated papers using 2 copper rings, 1 at the top end 1 one at the bottom end (Fig. 1).

5.4.3. Place a 16-mesh gauze on each holding tube and close the tube by screwing the cap (Fig. 1).

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

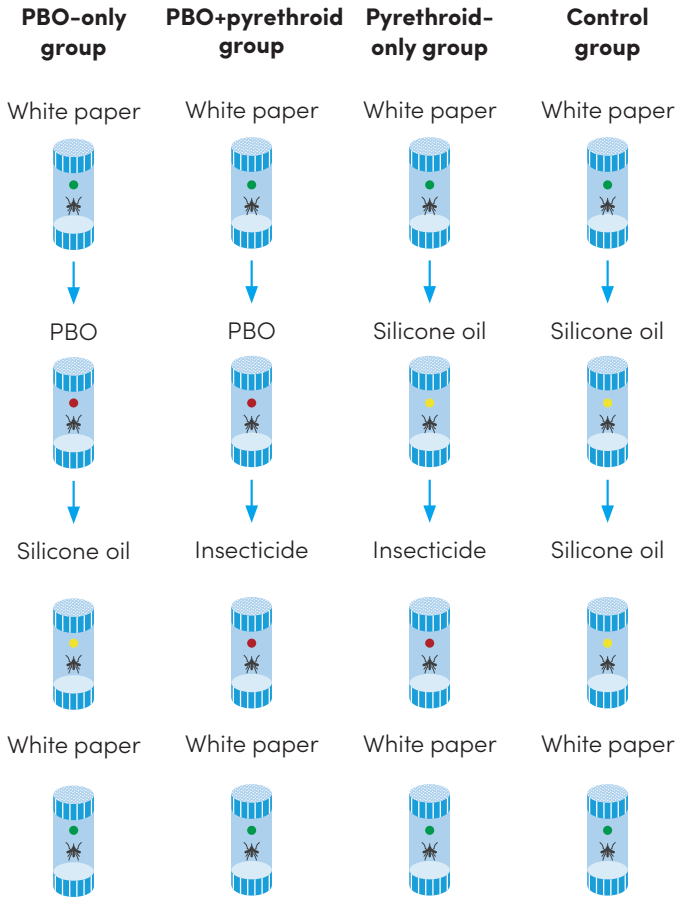
Fig. 1. Test tubes. Left: a holding tube (green dot) with a steel clip (ring) and an exposure tube (red dot) with a copper clip. Right: a holding tube (green dot) connected to an exposure tube (red dot) with a slide unit



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

Step 2: Mosquito exposure

Fig. 2. Mosquito exposure process, to be repeated 4 times until 100 mosquitoes have followed each test pathway



Pre-exposure holding period (1h): Hold mosquitoes in holding tubes lined with clean white paper (25 mosquitoes per holding tube).

Exposure to PBO (1h): Transfer 50 mosquitoes to 4% PBO-impregnated papers (2 tubes). Transfer the other 50 to oil-impregnated papers (2 tubes).

Exposure to insecticide (1h): Transfer 50 mosquitoes to insecticide-impregnated papers (2 tubes) and 50 to oil impregnated papers (2 tubes).

Post-exposure holding (24h): Transfer mosquitoes back into the holding tubes, provide them with access to cotton wool pads soaked in 10% sugar solution and hold for 24h. Read mortality at the end of the 24h holding.

Note: The following exposure procedures are repeated 4 times so that 100 mosquitoes follow each test pathway. Make sure that each paper can be used at least 4 additional times before starting the test or prepare to change the papers in the tubes between rounds.

- ☐ 5.5. **Aspirate and insert mosquitoes into the holding tubes:** Using an aspirator, aspirate 25 adult female mosquitoes from a mosquito cage into each of the 4 green-dotted holding tubes through the filling hole on the tube sliding door. A total of 100 mosquitoes is needed to fill the 4 holding tubes. Close the slide unit and set the holding tubes in an upright position.
- ☐ 5.6. **Leave the mosquitoes in the holding tubes for 1 hour.**

☐ 5.7. **Transfer the mosquitoes from the holding tubes to the PBO and control tubes:** Only live mosquitoes should be transferred. Any moribund (i.e. those unable to fly) or dead mosquitoes should be removed.

5.7.1. One by one, attach the 2 tubes containing a PBO-impregnated paper and labelled for test pathways "PBO only" and "PBO + pyrethroid" and the 2 tubes with silicone oil papers labelled for test pathways "Pyrethroid only" and "Solvent control" to the vacant position on the 2 sliding units of 2 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 PBO and silicone oil tubes, close the slide unit and place a cotton wool plug into the hole to lock the slide.

5.7.4. Detach the empty holding tubes.

5.7.5. Record the exact number of live mosquitoes exposed in each tube. Some mosquitoes could have died during the holding or transfer processes.

☐ 5.8. **Leave the mosquitoes in the tubes for 1 hour.**

☐ 5.9. **Transfer the mosquitoes in the PBO and control tubes to the insecticide and control tubes:** Only live mosquitoes should be transferred. Moribund (i.e. those unable to fly) or dead mosquitoes should be removed.

5.9.1. Attach the red-dotted tube containing an insecticide-impregnated paper and labelled "PBO + pyrethroid" to the vacant position of the tube with PBO-impregnated paper and labelled "PBO + pyrethroid".

5.9.2. Attach the yellow-dotted tube containing a silicone oil paper and labelled "PBO only" to the vacant position of the tube with PBO-impregnated paper and also labelled "PBO only".

5.9.3. Attach the red-dotted tube containing insecticide-impregnated paper and labelled "Pyrethroid only" to the vacant position of the tube with silicone oil paper and labelled "Pyrethroid only".

5.9.4. Attach the yellow-dotted tube containing a silicone oil paper and labelled "Control" to the vacant position of the tube with silicone oil paper and labelled "Control".

5.9.5. Carefully slide the units open and gently blow the mosquitoes from the PBO and silicone oil tubes into the insecticide and new silicone oil tubes.

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

5.9.7. Detach the empty PBO and silicone oil tubes.

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

☐ 5.10. **Leave the mosquitoes in the tubes for 1 hour.**

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

5.11.1. Attach the 4 green-dotted tubes to each of the tubes with mosquitoes.

5.11.2. Gently blow the mosquitoes back into the green-dotted holding tubes.

5.11.3. Detach the insecticide and silicone oil tubes from the sliding units.

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

5.11.5. 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 the 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 10% 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.

☐ 5.12. **Record the number of knocked down mosquitoes**, as per the definition in Table 2.

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

Table 2. WHO definitions of knockdown and mortality of mosquitoes post-test

Mosquitoes considered alive after 1 h of exposure or 24 h after exposure	Mosquitoes considered knocked down after 1 h of exposure or dead at 24 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

☐ 5.14. **Record mortality 24 hours post-exposure:** Count and record the number of mosquitoes found dead and alive 24 hours post-exposure, as per the definitions provided in Table 2. 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 3).

Table 3. 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)
Pyrethroids	Alpha-cypermethrin	2 years	Stable
	Cyfluthrin	2 years	Stable
	Deltamethrin	2 years	Stable
	Etofenprox	2 years	Stable
	Lambda-cyhalothrin	2 years	Stable
Synergist	Piperonyl butoxide	3 years	Stable

[a] Tentative (needs reconfirmation)

7. Criteria for test rejection

The bioassay should be discarded when either the mortality in the "Control group" is >20% or when the mortality in the "PBO-only" group is >10%.

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.[2].

The end-point of the test is the comparison of mosquitoes exposed to the insecticide only and those exposed to a synergist and then to an insecticide in terms of mortality 24 hours post-exposure. The mortalities of control mosquitoes and synergist-only mosquitoes 24 hours post-exposure are needed to validate and adjust test results. Therefore, the 24-hour post-exposure mortalities should be calculated separately for the mosquitoes in each of the 4 exposure pathways: "PBO + pyrethroid", "Pyrethroid only", "PBO only" and "Control".

Mortality is calculated by summing the number of dead mosquitoes across all exposure replicates of the same exposure pathway and then expressing this as a percentage of the total number of mosquitoes exposed in that pathway:

$$\text{PBO+pyrethroid mortality (\%)} = \frac{\text{Number of female mosquitoes exposed to PBO+pyrethroid dead}}{\text{Total number of females mosquitoes exposed to PBO+pyrethroid}} \times 100$$

$$\text{Pyrethroid only mortality (\%)} = \frac{\text{Number of female mosquitoes exposed to pyrethroid only dead}}{\text{Total number of females mosquitoes exposed to insecticide only}} \times 100$$

$$\text{PBO only mortality (\%)} = \frac{\text{Number of female mosquitoes exposed to PBO only dead}}{\text{Total number of females mosquitoes exposed to PBO only}} \times 100$$

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

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

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

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

9. Interpretation of test results

Once mortalities have been corrected using Abbott's formula (if necessary), the mortality of the group exposed to "PBO + pyrethroid" can be compared to the mortality of those exposed to "Pyrethroid only" in order to evaluate the ability of PBO to restore mosquito susceptibility to pyrethroids. If the mosquito mortality in "insecticide only" replicates is ≥90% the effect of PBO cannot be reliability assess. If it is <90%, the results are categorized as follows:

- **Full restoration of susceptibility:** if the mortality in the "PBO + pyrethroid" group is ≥98%.
- **Partial restoration of susceptibility:** if the mortality in the "PBO + pyrethroid" group is <98% but at least 10% greater than the mean mortality in the "Pyrethroid only" group.
- **No restoration of susceptibility**: if the mortality in the "PBO + pyrethroid" replicates is equal to or less than the mortality in the "Pyrethroid only" replicates, or if the mortality in the "PBO+pyrethroid" group is greater than the mortality in the "Pyrethroid only group" but by less than 10%.

10. Cleaning process

☐	10.1. Soak the tubes overnight in a 20% alkaline solution (TFD4 or Decon 90) for the equipment in direct contact with the insecticide (exposure tubes, copper clips, etc.) or in a 10% solution for the equipment used for handling and holding mosquitoes (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

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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 determining the ability of PBO to restore vector susceptibility to pyrethroids in WHO tube tests

World Health Organization

Data collection form – determination of PBO ability to restore vector susceptibility to pyrethroids in WHO tube tests

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:**	
Pyrethroid tested and concentration:	**Date of pyrethroid paper impregnation (dd/mm/yyyy):**	**No. of times the pyrethroid papers have been used before:**
Synergist tested and concentration	**Date of synergist paper impregnation (dd/mm/yyyy):**	**No. of times the synergist 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:	

	Number of mosquitoes introduced	Number of knocked down mosquitoes after 1 h exposure time	No. of dead and alive mosquitoes 24 h after 1 h exposure			
			No. dead	No. alive	Mortality	Abbott's corrected mortality
PBO-only group						
Control group						
Pyrethroid-only group						
PBO + insecticide group						

Final result

Difference between mortality with PBO only and PBO + pyrethroid (after Abbott's correction, if required)	
Restoration of susceptibility to the pyrethroid: i.e. Full/Partial/None (see section 9)	

Comments, if any:

Verified by Supervisor: _____ Date: _____

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