Standard Operating Procedure for testing insecticide resistance with WHO tube tests

Effective Date: 20 November 2024

SOP #: IRT-2024

PacMOSSI
Pacific Mosquito Surveillance Strengthening for Impact

Scope

This SOP provides a detailed protocol for conducting insecticide resistance testing on *Aedes* or *Anopheles* mosquitoes to evaluate their susceptibility to various insecticides used in vector control programs. This SOP aims to standardize the testing process to ensure reliable and accurate results.

Overview

Description: This protocol follows the guidelines outlined by the WHO for testing the insecticide susceptibility of field-collected mosquitoes using tube tests (WHO 2022). The primary objective is to assess resistance to insecticide classes commonly used in public health vector control, including pyrethroids, organophosphates, and others.

Advantage: The equipment and supplies are inexpensive and portable.

Disadvantage: The bioassays require large numbers of adult mosquitoes that are standardised in age and condition, and representative of the field population. There is no direct correlation between bioassay results and expected efficacy of insecticidal interventions under normal usage conditions.

Data: Susceptibility bioassay results provide critical evidence to assess threats to the efficacy of insecticide-based interventions such as long-lasting insecticidal nets (LLINs) or indoor residual spraying (IRS).

Overall process for insecticide resistance testing

Table 1: Outline of steps for testing insecticide resistance from mosquito collection through to identification and storage.

Process	Further PacMOSSI resources	Location
1. Field collection of mosquitoes	SOP for conducting larval and pupal surveys for *Aedes*	SOP #: LAE-2021
	SOP for conducting larval and pupal surveys for *Anopheles*	SOP #: LAN-2021
	Ovitraps	SOP #: OVI-2021
	SOP for collecting resting mosquitoes with the Prokopack aspirator	SOP #: PRO-2021
2. Rearing mosquito specimens	SOP for maintaining *Aedes aegypti* and *Aedes albopictus* colony mosquitoes	SOP #: CAE-2022
	SOP for maintaining *Anopheles farauti s.s.* colony mosquitoes	SOP #: CAN-2022
3. WHO tube bioassays	Conducted using this SOP	
4. Morphological identification and storage	A morphological key to the common mosquito species in the Pacific including medically important vectors	
	SOP for vector surveillance, processing and storage	SOP #: MOS-2021

Mosquitoes

Sampling the field population:
Priority should be given to monitoring resistance of the species that play the greatest role in pathogen transmission. Mosquito field collections should span diverse and geographically separate sites to maximize genetic variability of the target species. For *Anopheles*, gravid females are usually collected and their offspring reared to adults for testing, alternatively larval collections can be utilised. For *Aedes*, egg and larval collections are usually the most efficient method for obtaining sufficient mosquito numbers. Rearing to adults for testing can be done in basic facilities. Samples should be pooled during rearing to minimize siblings within test groups.

Mosquitoes for resistance testing:
WHO tube tests should be conducted using either F0 (field-caught) or F1 (first laboratory generation) adult mosquitoes. Results are most comparable when tests are conducted with healthy adult mosquitoes of the same sex, age and physiological status. **Test using adult female mosquitoes that are 3–5 days old, non–blood fed and starved of sugar for at least 2 hours before the test.**

Each tube should contain 25 female mosquitoes, aiming for this exact number whenever possible. Do not exceed 25 to prevent overcrowding, and ensure a minimum of 20 mosquitoes per tube to maintain sufficient statistical power. Thus, a single round of bioassays with six tubes requires 150 mosquitoes.

Materials

Standard WHO tube assay kit includes the following:

- Insecticide-impregnated papers* (Refer to the tables at the end of this document for diagnostic insecticide concentrations)
- Oil-impregnated control papers*
- Bioassay tubes (6 **green dot**; 2 **yellow dot** and 4 **red dot**) and slide units
- 6 steel clips (rings) to hold white papers in the holding tubes
- 6 copper clips (rings) to hold impregnated or control papers in exposure tubes
- Sheets of clean white paper (12 x 15cm) for lining the holding tubes
- 1 roll of adhesive plastic tape
- Aspirators*

* Items can also be obtained separate to the kit from the supplier

Further materials for tube bioassays:

- Live female mosquitoes (3-5 days old)
- A4 paper
- 1 adult mosquito cage (for holding period)
- Cotton wool
- 10% sugar solution
- Adhesive labels
- Timer
- Temperature and humidity logger
- Towels for cage
- Aluminium foil
- Mosquito cups
- Gloves (latex or nitrile) and laboratory coat
- Alcohol for cleaning
- Data collection forms
- Stationary
- Data capture tablet

Storage and handling of papers

Impregnated and control papers must be stored sealed in their original plastic boxes in a refrigerator at 4–8 °C to maintain their shelf life. Where feasible, use temperature data loggers to monitor storage conditions, as significant temperature fluctuations can compromise paper efficacy and result in unreliable assay outcomes. Test papers should never be exposed to direct sunlight or to temperatures higher than 8°C, except for short periods during use or shipment.

Shelf life and usage: Unused impregnated papers have a shelf life of 2–5 years from manufacture dependent on correct storage and the insecticide class. Papers should not be used past their expiry date, which is indicated on the packaging. Each paper can be reused up to a maximum of six times if stored and handled correctly.

Preparation for testing: Before use, remove impregnated papers from the refrigerator at least 1 hour in advance to allow them to reach room temperature. This avoids condensation of water on the surface of the papers, which can hydrolyse the insecticide.

Storage between testing rounds: After use, label each paper with the date of use on the untreated side using a pencil. Reusable papers that have been used fewer than six times can be stored for future testing by separating them with aluminium foil and store it according to temperature guidelines.

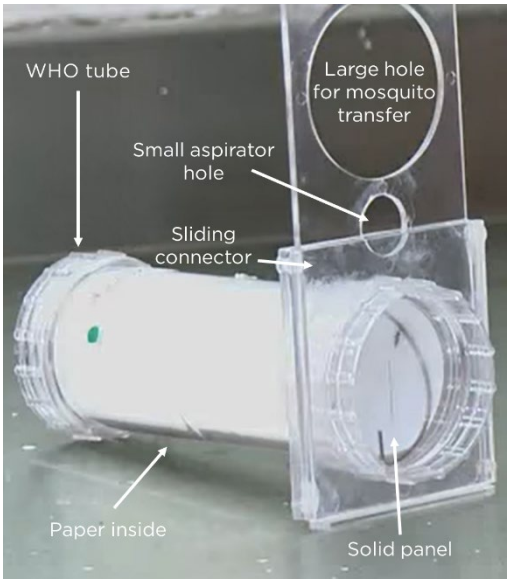
Storage between tests within the same round: For bioassays conducted over multiple days, impregnated papers can remain in exposure tubes to minimize handling and damage. Wrap each tube in aluminium foil and store it according to temperature guidelines. Before reuse, bring the wrapped tubes to room temperature for 1 hour before removing the foil.

Overview of insecticide resistance testing

Set up of bioassay tubes

Each insecticide concentration is tested using four replicate exposure tubes, with each batch of bioassays including two control tubes. A total of 150 non-blood-fed adult female mosquitoes (20-25 per tube) are required for a complete insecticide resistance test. The test tubes are distinguished by coloured dots as follows:

- **Red dot (Exposure Tubes):** Contain impregnated papers for testing the insecticide concentration. Four exposure tubes are used for each concentration.
- **Yellow dot (Control Tubes):** Contain control filter papers to validate the bioassay. Two control tubes are included in each batch of tests.
- **Green dot (Holding Tubes):** Contain clean white paper and are used to hold mosquitoes prior to testing. The number of holding tubes is usually six, equating to the total of the exposure and control tubes used (i.e. four for the exposure tubes and two for the control tubes).



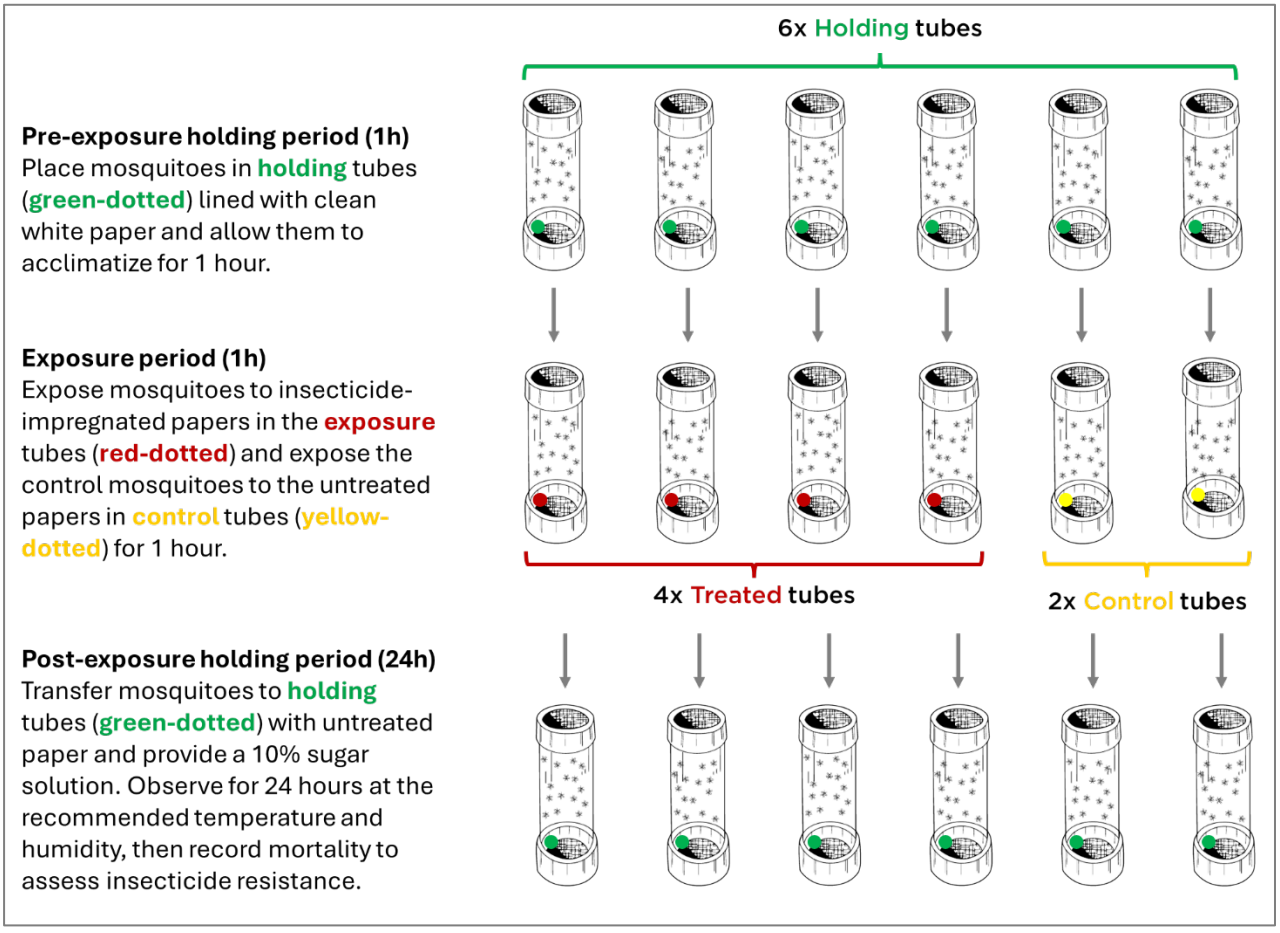
Testing conditions

The bioassay test, along with the adult mosquitoes maintained in cages, should be conducted at the recommended temperature of 27 °C ± 2 °C and a relative humidity of 75% ± 10%. Tests must be carried out in a building free from insecticidal contamination, with efforts to maintain stable conditions for temperature, humidity, and low illumination. Consistency in environmental conditions is crucial to ensure reliable results.

Overview of mosquito exposure process

During WHO tube bioassays, adult female mosquitoes are exposed to insecticide-treated filter papers in the **red-dotted exposure** tubes for a standard duration of **60 minutes**. Control mosquitoes, which are not exposed to insecticide, are placed in **yellow-dotted control** tubes, where they are exposed to untreated filter papers. After the exposure period, mosquitoes from both the **exposure** and **control** tubes are transferred to clean **holding** tubes (**green-dotted**) lined with untreated paper and provided with a 10% sugar solution. They are then observed for 24 hours under recommended temperature and humidity conditions to monitor for delayed mortality. Mortality rates are recorded at the end of the 24 holding period to assess the presence of insecticide resistance or susceptibility in the mosquito population.

Figure 1: Overview of the insecticide resistance testing procedure using WHO tube bioassays



Prepare for bioassay testing

1. Decontaminate equipment:
    - Soak and thoroughly clean washable items (e.g., tubes, clips, aspirators) with detergent. Rinse thoroughly with running water, then place on a clean surface to dry under sunlight.
    - Perform this step the day before the insecticide resistance testing begins.

2. Set up workspace:
    - Clean all work surfaces with 70% ethanol before testing and cover with clean white A4 paper.

3. Prepare cups for transferring mosquitoes. These are used before the test to transfer mosquitoes from cages to the holding tubes (pre-exposure), and afterwards for sorting knocked-down or alive mosquitoes (post-exposure).
    - The number of cups required will depend on the number of tubes used.
    - Label each cup.
    - Keep pre-exposure cups clean by never placing post-exposure mosquitoes inside them.

4. Remove insecticide and control papers from the refrigerator (where they will be stored in the original plastic package or wrapped in aluminium foil for re-use) and allow them to reach room temperature for 1 hour on a clean surface.

5. Prepare bioassay tubes by completing the following steps on the clean bench:
    - Use masking tape to label all tubes with their replicate number, insecticide or control, test start and hold times, and date.
    - Place a 16-mesh gauze on each holding tube and screw the cap into place. Note for *Aedes* mosquitoes, use double mesh wire or substitute with untreated mosquito net cloth to prevent escape.
    - Position holding tubes and exposure tubes in parallel, with 4 **holding** tubes facing 4 **exposure** tubes and 2 **holding** tubes facing 2 **control** tubes.

6. Prepare **holding** tubes:
    - Wear clean gloves.
    - Insert rolled sheets of clean white paper into each **holding** tube (n = 6).
    - Fasten the paper into position against the wall of the tube with 2 steel clips, 1 at the top and 1 at the bottom.
    - Attach a slide unit on the opposite side and return the tubes to their designated positions on the bench.

7. Prepare **control** tubes:
    - Change gloves.
    - Insert rolled control paper into each **control** tube (n = 2), ensuring that the stamped label is on the outer side of the paper and readable through the transparent tube.
    - Fasten the paper into position with 2 copper rings, 1 at the top and 1 at the bottom.
    - Return the tubes to their designated positions on the bench.

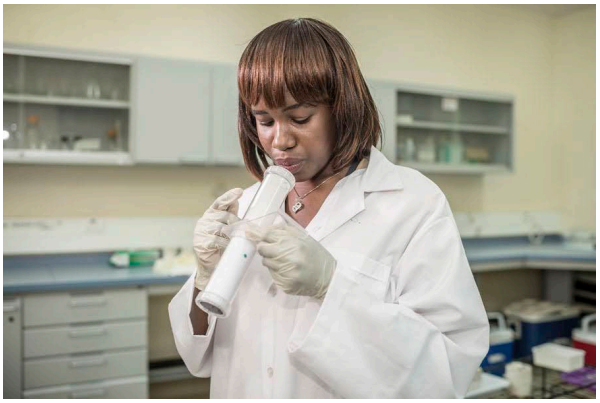
8. Prepare **exposure** tubes:
    - Change gloves.
    - Insert rolled insecticide papers into each **exposure** tube (n = 4), ensuring that the stamped label is on the outer side of the paper and readable through the transparent tube.
    - Fasten the paper into position using 2 copper rings in each tube, 1 at the top and one at the bottom.
    - Return the tubes to their designated positions on the bench.

Mosquito exposure

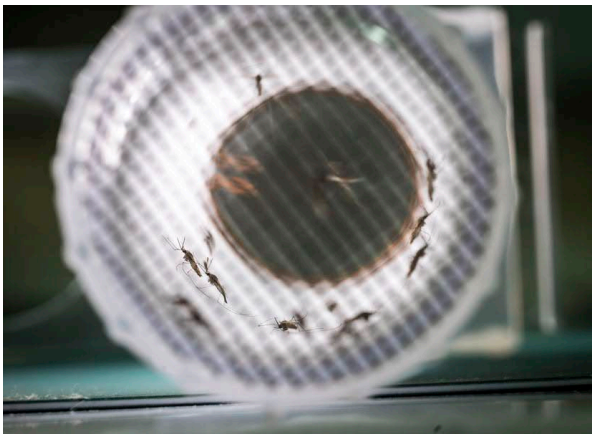
1. **Set up:**
   Transfer mosquitoes from cages to pre-exposure holding cups:
   - Mosquitoes should be non-blood fed and starved of sugar for 2 hours before use
   - Use 6 pre-exposure cups, and into each aspirate 20-25 adult female mosquitoes (total of 120-150 mosquitoes).
   - To separate the females from the males in the cage, place a warm hand and/or breathing on the side of the cage to attract the females to one side before aspirating them out of the cage.
   - For the pre-exposure and exposure steps below, only active and healthy mosquitoes should be used. Any moribund (i.e. unable to fly) or dead mosquitoes should not be used.

2. **Pre-exposure hold (1 hour)**:
   - Select a **holding** tube (start with the tubes that correlate with controls, then exposure tubes) and open the slide to expose the aspirator hole.
   - Aspirate all 20-25 mosquitoes from one pre-exposure cup and into the aspirator, ensuring no mosquitoes escape by placing your thumb over the end. Gently blow through the aspirator to transfer the mosquitoes into the **holding** tube. Then close the slide unit.
   - Return each tube to its designated position on the bench with the mesh screen facing up.
   - Repeat for all **holding** tubes and hold the mosquitoes in these tubes for **1 hour** (use a timer).

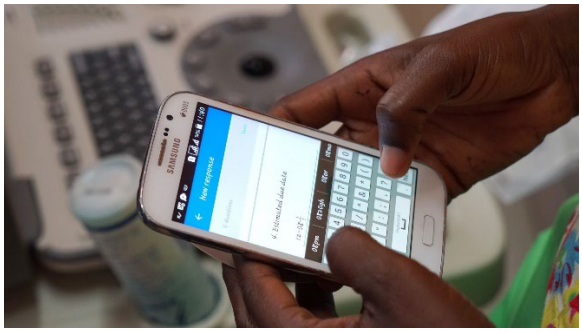
3. **Exposure (1 hour)**:
   - Select one set of corresponding **holding** and test tubes (**control** or **exposure**). Holding one in each hand and fasten them together with the sliding units.
   - Open the slide unit using your fingers and gently blow all of the mosquitoes from the **holding** tube into the test tube (**control** or **exposure**). Then close the slide unit and lock the slide by with a cotton wool plug. Note: you will need to work quickly and carefully.
   - Detach the **holding** and test tubes (**control** or **exposure**) and place them back in their designated positions with the mesh screen facing up.
   - Repeat for all **control** tubes and then the **exposure** tubes.
   - Reduce light intensity in the workspace or cover the tubes with cardboard discs or a sheet of paper to discourage mosquitoes from resting on the mesh screen.
   - Hold the mosquitoes in these tubes for **1 hour** (use a timer). (Note that for fenitrothion testing with *Anopheles* the exposure period is 2 h).



4. **End of exposure:**
   End the exposure period after 1 hour.
   - Starting with the **exposure** tubes, followed by the **control** tubes. Reattach each **holding** tube to the test tube.
   - Unlock the slide unit using your fingers and gently blow the mosquitoes from the test tube (**exposure** or **control**) into the **holding** tube.
   - Detach the **holding** and test tubes (**exposure** or **control**) and place them back in their designated positions with the mesh screen facing up.
   - Place a piece of cotton wool soaked in 10% sugar solution on the mesh screen of the **holding** tubes.
   - Repeat for all replicates.



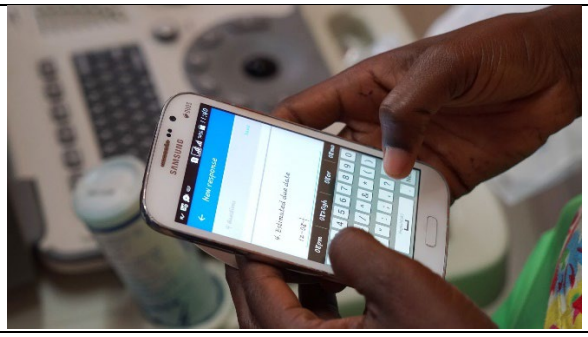
5. **Post-exposure knockdown reading:**
Immediately record the 60 minute knock-down results:
   - Fill in the data entry forms, including information about the insecticide and concentration.
   - Ensure the unique identifier for the suite of replicate tubes is physically labelled on the tests and recorded on the data form.
   - Record the number of knocked-down and alive mosquitoes (see definitions below).



6. **Post-exposure holding period (24 hours):**
   - Hold the mosquitoes in the **holding** tubes for **24 hours** after the end of exposure (use a timer).

7. **Post-exposure mortality reading**:
After 24 hours, sort the mosquitoes in each tube:
   - Selecting one **holding** tube at a time, remove the dead mosquitoes. To do this, place the tube on a clean surface and unlock the slide. Allow all of the dead mosquitoes to fall out onto the surface, and then close the slide (to prevent the alive mosquitoes to escape).
   - Carefully tilt the **holding** tube backwards and aspirate the dead mosquitoes into a labelled sample tube.
   - Place the holding tube in the freezer to knock down the alive mosquitoes. Remove them into a labelled sample tube.
   - Morphologically identify the specimens to species using a basic key.
   - Repeat for each replicate.

8. **Record the 24 hour mortality results:**
   - Count the mosquitoes and record the number of dead and alive mosquitoes for each species and sex.



9. **Pack up:**
   - Clean down workstation and pack away all equipment
   - Store the impregnated papers for reuse according the the specifications outlined above.

Note: it is always important to document any problems encountered when running the bioassays. Note if any mosquitoes escape from the bioassay tube during the exposure or holding periods (especially when tubes are old or cracked in the slide, and the mesh wire holes are big, old or broken).

Criteria for test rejection: If the control mortality is >20%, the test results must be discarded and the test repeated for all replicates.

Definitions for mosquito state

After the exposure period, mosquitoes are categorized as alive, knocked-down (KD), or dead as follows:

- **Alive:** Mosquitoes can stand and fly in a coordinated manner.
- **Knocked-down (KD) is recorded after 1 h and mortality after 24 h:** Mosquitoes are considered knocked-down or dead if:
    - They show no sign of life, are immobile, or unable to stand (e.g. has 1 or 2 legs).
    - Mosquitoes that cannot fly in a coordinated manner.
    - Mosquitoes that are on their back, moving their legs or wings but unable to take off.
    - Mosquitoes that can briefly take off but falls down immediately after.

Calculating mortality rates

During the test, data should be recorded using either paper-based or digital forms. The primary endpoint is mosquito mortality, assessed 24 hours after a 1-hour exposure to the insecticide. Mortality calculations should be conducted separately for test mosquitoes (those exposed to the insecticide) and control mosquitoes.

To calculate mortality, determine the total number of mosquitoes that died in each group and divide this by the total number of mosquitoes initially exposed. The result is expressed as a percentage. This approach ensures accurate and separate assessment of mortality rates for treated and control groups.

Mortality for mosquitoes exposed to treated papers (test) is:

$$\textit{Treatment mortality}\ (\%) = \frac{\textit{Number of test mosquitoes that died}}{\textit{Total number of test mosquitoes}} \times 100$$

Mortality for mosquitoes exposed to untreated papers (control) is:

$$\textit{Control mortality}\ (\%) = \frac{\textit{Number of control mosquitoes that died}}{\textit{Total number of control mosquitoes}} \times 100$$

Correcting for mortality in the controls

Control mosquitoes, which are not exposed to insecticide, play a critical role in interpreting test results. Their mortality must be carefully assessed during analysis:

- **High control mortality** (above 20%) indicates potential test bias, rendering the results invalid. In such cases, the test must be discarded and repeated.
- **Low control mortality** (below 5%) allows for direct analysis of the results without correction.
- **Moderate control mortality** (between 5% and 20%) requires adjustment using Abbott's formula to account for natural mortality in the control group.

Abbott's formula:

$$\textit{Corrected treatment mortality}\ (\%) = \frac{(\%\ \textit{treatment mortality} - \%\ \textit{control mortality})}{(100 - \%\ \textit{control mortality})} \times 100$$

Checks when unexpected results are obtained

If unexpectedly high numbers of survivors are observed after exposure to an insecticide known to cause full mortality (based on local vector knowledge and control interventions):

- **Verify paper quality:** If the papers were obtained from USM, Malaysia, contact them to confirm the quality using their stored quality control samples.
- **Conduct susceptibility testing:** If feasible, organize for papers to be tested against a susceptible laboratory strain of mosquitoes in a research laboratory following standard procedures and ensuring the use of an optimal number of mosquitoes.

Interpretation of bioassay results

Interpret the observed mortality results according to the WHO guidance:

- **Confirmed resistance:** Mortality (corrected, if applicable) is <90%, provided at least 100 mosquitoes were tested. This indicates that the vector population is resistant to the insecticide.
- **Possible resistance:** Mortality (corrected, if applicable) is ≥90% but <98%, suggesting potential resistance. Confirm by repeating the test with a new sample from the same population (not the F1 of previously tested mosquitoes). Resistance is confirmed if two independent tests show mortality consistently below 98%.
- **Susceptibility:** Mortality (corrected, if applicable) is ≥98%, indicating that the vector population is susceptible to the insecticide.

Discriminating concentrations

Discriminating concentrations are predetermined insecticide doses used in bioassays to detect resistance by assessing mortality in a vector population after a standard exposure period.

Table 2. Insecticide discriminating concentrations and exposure times used from WHO tube bioassays against *Anopheles* mosquitoes

Insecticide class	Active ingredient	Discriminating concentration	Exposure period	Holding period	Carrier oil/ solvent
Pyrethroids	Alpha-cypermethrin	0.05%	1 h	24 h	Silicone oil
	Cyfluthrin	0.15%	1 h	24 h	Silicone oil
	Deltamethrin	0.05%	1 h	24 h	Silicone oil
	Etofenprox	0.50%	1 h	24 h	Silicone oil
	Lambda-cyhalothrin	0.05%	1 h	24 h	Silicone oil
	Permethrin	0.75%	1 h	24 h	Silicone oil
Carbamates	Bendiocarb	0.10%	1 h	24 h	Olive oil
	Carbosulfan	0.40%	1 h	24 h	Olive oil
	Propoxur	0.10%	1 h	24 h	Olive oil
Organochlorines	DDT	4.00%	1 h	24 h	Risella oil
	Dieldrin	4.00% / 0.40%	1 h	24 h	Risella oil
Organophosphates	Fenitrothion	1.00%	2 h	24 h	Olive oil
	Malathion	5.00%	1 h	24 h	Olive oil
	Pirimiphos-methyl	100 mg/m^2	1 h	24 h	Acetone only
Synergist	Piperonyl butoxide	4.00%	1 h	24 h	Silicone oil

Source: World Health Organization 2022. Note that the discriminating concentrations were validated against species found outside of the Pacific.

Table 3. Insecticide discriminating concentrations and exposure times used from WHO tube bioassays against *Aedes* mosquitoes

Insecticide class	Active ingredient	Species used for validation	Discriminating concentrations for 1 h exposure	Holding period	Carrier oil/ solvent
Pyrethroids	Alpha-cypermethrin	*Ae. aegypti*	0.05%	24 h	Silicone oil
		Ae. albopictus	0.08%	24 h	Silicone oil
	Deltamethrin	*Ae. aegypti, Ae. albopictus*	0.03%	24 h	Silicone oil
	Lambda-cyhalothrin	*Ae. aegypti*	0.05%	24 h	Silicone oil
		Ae. albopictus	0.08%	24 h	Silicone oil
	Permethrin (40:60)	*Ae. aegypti, Ae. albopictus*	0.40%	24 h	Silicone oil
Carbamates	Bendiocarb	*Ae. aegypti, Ae. albopictus*	0.20%	24 h	Olive oil
	Propoxur	*Ae. aegypti*	0.10%	24 h	Olive oil
Organophosphates	Chlorpyrifos-ethyl	*Ae. aegypti, Ae. albopictus*	1.00%	24 h	Olive oil
	Pirimiphos-methyl	*Ae. aegypti, Ae. albopictus*	60 mg/m^2	24 h	Acetone only
	Malathion	*Ae. aegypti*	1.50%	24 h	Olive Oil
		Ae. albopictus	5.00%	24 h	Olive Oil

Source: World Health Organization 2022.

Safety

Wear appropriate personal protective equipment

Appropriate personal protective equipment must be worn at all times when handling insecticides, including laboratory coat, gloves and safety glasses.

Waste disposal

Dispose of all waste materials appropriately following relevant national and/or institutional guidelines.

Data form

Test date		Temperature	
Technician name		Humidity	
Mosquito collection location		Age of test females	
Mosquito species		Feeding status	
Insecticide tested		Exposure start time	
Insecticide concentration		Exposure end time	

Results for individual tubes		Observed number of mosquitoes				% dead
		Introduced into each tube	Knocked down at 1 hour after exposure	Dead at 24 hours after 1 hour exposure	Alive at 24 hours after 1 hour exposure	Mortality at 24 hours after 1 hour exposure
	Tube	#	#	#	#	%
Exposed to insecticide-treated papers in tubes	Tube 1					
	Tube 2					
	Tube 3					
	Tube 4					
Exposed to untreated papers in tubes	Control 1					
	Control 2					

Final results for all tubes		Knocked down at 1 hour after exposure	Mortality at 24 hours after 1 hour exposure	Abbott's corrected mortality
		%	%	%
Exposed to insecticide-treated papers in tubes	All tubes			

Test result

The vector population tested is _____ to the insecticide tested

Verified by supervisor _____

Date _____

References

WHO. Standard operating procedure for testing insecticide susceptibility of adult mosquitoes in WHO tube tests. Geneva: World Health Organization, 2022.
https://www.who.int/publications/i/item/9789240043831

Acknowledgements

Content was drafted by Amanda Murphy, Tessa Knox and Tanya Russell from James Cook University. We thank Stephen Gideon and Stephan Karl for providing the PNGIMR protocol that was used as reference material when developing this protocol. Where the image source is not noted, the images were provided by the authors.

Suggested citation

PacMOSSI consortium. (2024) 'Standard Operating Procedure for testing insecticide resistance with WHO tube tests.'