World Health Organization

Standard operating procedure for testing insecticide susceptibility of adult mosquitoes in WHO bottle bioassays

Version: WHO Bottle-bioassay/01/14 January 2022

1. Introduction, scope and purpose

This standard operating procedure (SOP) describes the process for evaluating the susceptibility of adult mosquito vectors to insecticides using the World Health Organization (WHO) bottle bioassay. The bottle bioassay should be used to evaluate vector susceptibility to insecticides that are unstable or cannot impregnate usual Whatman no. 1 filter papers. This SOP is for testing susceptibility to these insecticides and should not be used for insecticide growth regulators that impact mosquito fecundity/fertility. Although WHO bottle bioassays are also used to test vector susceptibility to insect growth regulators, certain additional steps are required to measure the insect growth regulator's sterilizing properties (i.e. oviposition inhibition and reduction in fecundity) post-exposure. The procedure for testing insect growth regulators is presented in SOP PPXN-Bioassay/01/14 January 2022. For insecticides that can impregnate filter papers, the SOP for WHO tube tests should be followed (see SOP WHO Tube test/01/14 January 2022).

The WHO bottle bioassay is a modified version of the United States Centers for Disease Control and Prevention (CDC) bottle bioassay and has been developed to harmonize the test end-points with those of the WHO tube test, so that mosquito mortality is evaluated at the same time point post-exposure *(1)*. The WHO bottle bioassay is a direct response-to-exposure test, measuring mosquito mortality after 1 hour of exposure to a known standard concentration (e.g. the discriminating concentration) of an insecticide. The mortality of test mosquitoes is recorded at 24 hours (or 72 hours) after the 1-hour exposure period.

This SOP provides instructions on bottle preparation and coating, mosquito exposure, recording and interpretation of test results.

2. Equipment

☐	250 mL Wheaton® bottles with screw caps – 6 bottles per insecticide compound to be tested (12 for chlorfenapyr). If the bottles are washed and reused, ensure they have been cleaned properly by following the "Bottle washing" procedure described later.
☐	weighing spatula
☐	microbalance (electronic balance)
☐	vortex mixer
☐	magnifying lens or binocular loupe
☐	2 amber glass bottles (volumes 50–100 mL each). Alternatively, any clean glass bottle wrapped in aluminium foil can be used.

☐	a set of calibrated micropipettes (e.g. 100 µL, 200 µL and 1000 µL)
☐	timer (stopwatch)
☐	calibrated, traceable humidity and temperature monitors
☐	a 20 L plastic container or a 20 L sink
☐	aspirators for collecting mosquitoes (battery-powered apparatus should be used to avoid any exposure to insecticides or inhalation of allergens)
☐	mosquito cages (minimum dimensions 20 cm x 20 cm)
☐	racks
☐	appropriate personal protective equipment (laboratory coat, latex gloves)
☐	testing chamber or climate-controlled insectary at 27 °C ± 2 °C and 75% ± 10% relative humidity

3. Reagents and consumables

☐	technical grade insecticide (active ingredient [AI]) or its stock solution
☐	acetone (reagent for analysis)
☐	appropriate surfactant, if needed (e.g. MERO® i.e. 81% rapeseed oil methyl ester, manufactured by Bayer)[1]
☐	paper cups for holding adult mosquitoes (440 mL; 10 cm height x 9.5 cm width) (i.e. a storage chamber for a lot of 25 test mosquitoes)
☐	narrow mesh netting in sufficient quantities to cover the cups/bowls and rubber bands to secure the netting pieces on the paper cups/bowls
☐	disposable plastic tips for micropipettes (e.g. 100 µL, 200 µL and 1000 µL)

[1] An alternative surfactant, but still under evaluation, is Span 80.

☐	disposable glass pipettes of various volumes
☐	medical grade cotton wool
☐	10% sugar solution (glucose dissolved in water at 10% w/v)
☐	aluminium foil
☐	butter paper for weighing insecticide (or aluminium micro cups)
☐	adhesive tape
☐	permanent marker pens for labelling the bottles, caps and pipettes
☐	data recording sheet (Annex 2), pens and pencils for recording data
☐	disposable gloves
☐	acetone (for cleaning)
☐	ethanol (for cleaning work benches)
☐	TFD4 or Decon 90 (for cleaning equipment in contact with chemical compounds)

4. 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.
✓	All staff using this procedure must be trained in the safe operation of chemical fume hoods.
✓	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.

5. Mosquito rearing and preparation

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 for the number of mosquitoes per bottle to be 25 or as close to 25 as possible, but it should not exceed 25 to avoid crowding within the bottle.

6. Preparation of stock solutions of discriminating concentrations

Some insecticides can be dissolved in acetone alone, but others (e.g. clothianidin, flupyradifurone, imidacloprid) tend to crystallize if dissolved in acetone alone. The uptake of a crystallized insecticide by the insect's body is very low. Therefore, a surfactant such as MERO® should be added to the acetone before preparing the stock solution in order to prevent crystallization of the insecticide.

Process for preparing and storing the stock solution

☐ 6.1. **Check the insecticide discriminating concentration and surfactant:** Insecticide discriminating concentrations and suitable quantities of MERO® to be used with different compounds are given in Tables 1 and 2 for *Anopheles* and *Aedes* spp., respectively. Annex 1 provides calculations for the amount of insecticide, solvent and surfactant to prepare the stock solution.

☐ 6.2. **Prepare the glass bottle to store the stock solution:** Take a clean light-proof glass bottle (amber coloured or aluminium foil-wrapped, if transparent) of appropriate volume and label it with the stock solution concentration and date.

☐ 6.3. **Prepare the stock solution:**

- Prepare electronic balance: Place a piece of clean weighing paper (or aluminium micro cup) on the scale and set the reading to zero.
- **With acetone as solvent:** To prepare a test concentration of 25 µg of AI for a 250 mL glass bottle, weigh 25 mg of AI using a clean spatula and dissolve it completely in 1 L of acetone (solvent) or any other solvent/surfactant mixture recommended by the manufacturer (e.g. MERO® + acetone, see procedure below). Alternatively, to prepare a small volume of the stock solution (e.g. 5 mL), put 125 µg of AI in the bottle and add 5 mL of solvent/surfactant. This stock solution will contain 25 µg of AI per mL of stock solution.
- **With acetone + MERO® (1500 ppm concentration) for testing *Aedes* spp**: Pipette out 0.17 mL (170 µL) of Mero® and put it in 100 mL of acetone in a bottle.
- **With acetone + MERO® (800 ppm concentration) for testing all *Anopheles* spp. except *An. albimanus*:** Pipette out 89 µL of Mero® and put it in 100 mL of acetone in a bottle or, for a smaller volume, pipette 8.9 µL of Mero® with 10 mL of acetone.
- **With acetone + MERO® (concentration 200 ppm) for testing *An. albimanus*:** For *An. albimanus*, the concentration of MERO® for the bottle bioassay is lower than that for other *Anopheles* spp *(1)*. To obtain a concentration of 200 ppm, pipette out 22 µL of Mero® and put it in 100 mL of acetone in a glass bottle or, for a smaller volume, pipette 2.2 µL of Mero® with 10 mL of acetone.

Table 1. Insecticide discriminating concentrations for WHO bottle bioassays with susceptible *Anopheles* spp. mosquitoes

Insecticide class	Insecticide	Species	Discriminating concentration (1 h exposure)	Holding time	Solvent or solvent + surfactant
Pyrethroids	Transfluthrin	*An. albimanus*, *An. stephensi*, *An. funestus s.s.*, *An. minimus*, *An. gambiae s.s.*	2 µg/bottle	24 h	Acetone
Neonicotinoids	Clothianidin	*An. albimanus*, *An. stephensi*	10 µg/bottle	24 h	Acetone + MERO[a] 200 ppm Acetone + MERO[a] 800 ppm
		An. funestus s.s., *An. gambiae s.s.*	4 µg/bottle	24 h	Acetone + MERO[a] 800 ppm
		An. minimus	6 µg/bottle	24 h	Acetone + MERO[a] 800 ppm
Butenolides	Flupyradifurone	*An. albimanus*	500 µg/bottle	24 h	Acetone + MERO[a] 200 ppm
		An. stephensi, *An. gambiae s.s.*	60 µg/bottle	24 h	Acetone + MERO[a] 800 ppm
		An. funestus s.s., *An. minimus*	100 µg/bottle	24 h	Acetone + MERO[a] 800 ppm
Pyrroles	Chlorfenapyr	*An. gambiae s.s.*, *An. stephensi*, *An. funestus s.s.*, *An. albimanus*	100 µg/bottle	72 h	Acetone

[a]MERO® (81% rapeseed oil methyl ester; manufactured by Bayer CropScience)

Table 2. Insecticide discriminating concentrations for WHO bottle bioassays with susceptible *Aedes* spp. mosquitoes

Insecticide class	Insecticide	Species	Discriminating concentration (1 h exposure)	Solvent or solvent + surfactant
Pyrethroids	Transfluthrin	*Ae. aegypti* and *Ae. albopictus*	3 µg/bottle	Acetone
	Metofluthrin	*Ae. aegypti* and *Ae. albopictus*	1 µg/bottle	Acetone
	Prallethrin	*Ae. aegypti* and *Ae. albopictus*	30 µg/bottle	Acetone
Neonicotinoids	Clothianidin	*Ae. aegypti*	20 µg/bottle	Acetone + MERO[a] 1500 ppm
		Ae. albopictus	10 µg/bottle	Acetone + MERO[a] 1500 ppm
Butenolides	Flupyradifurone	*Ae. aegypti* and *Ae. albopictus*	80 µg/bottle	Acetone + MERO[a] 1500 ppm

[a]MERO® (81% rapeseed oil methyl ester; manufactured by Bayer CropScience)

6.4. **Store and use the stock solution**

- Keep the stock solution bottle tightly closed to avoid evaporation of the acetone and store the bottle in a refrigerator at 4–8 °C for a maximum of 2 months.
- To use the stock solution, take the bottle out of the refrigerator, bring it to room temperature (usually for 30 minutes to 1 hour), pipette out the required volume, immediately close the bottle and put it back in the refrigerator for future use.

  Note: If the bottle containing the stock solution is opened without bringing it to room temperature, water vapour may condense in the bottle and hydrolyse the insecticide, or the coating solution in the bottle may not dry properly due to the presence of water molecules.

7. Bottle bioassay procedure

Note: For testing chlorfenapyr, the following procedures will need to be carried out in parallel with both female mosquitoes collected in the field and with mosquitoes from a susceptible laboratory colony.

Step 1: Coating of bottles

7.1. **Prepare clean and dry bottles:** Take clean, empty 250 mL glass bottles with screw caps in the required numbers for the bottle assay and dry them in an oven for 20 minutes or in open air for 1 to 2 hours depending on the level of humidity (Fig. 1).

Fig. 1. Open bottles and caps being dried horizontally in a dark place

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

☐ 7.2. **If the stock solution and surfactants have been stored in a refrigerator:** Take the bottles out of the refrigerator and bring them to room temperature by letting them sit for 30 minutes to 1 hour without opening the container cap.

☐ 7.3. **Label both the bottles and the caps with the insecticide name, insecticide concentration (µg/bottle) or control, and date of the test.**

☐ 7.4. **Coat the interior and cap of the control and exposure bottles:** Coat the control bottles first and then the bottles with insecticide. Coat the bottles one by one following the steps described below and shown in Fig. 2.

7.4.1. Hold the bottle at approximately a 45° angle with its mouth pointing upwards. Add 1 mL of acetone (for the control bottle) and 1 mL of insecticide stock solution (for the exposure bottle) using a pipette. Put the cap on the bottle and rotate the bottle at least 5 times to coat the base of the bottle (Fig. 2, step 1).

7.4.2. Tilt the bottle at a slight angle with the cap facing downwards, and rotate the bottle at least 5 times to coat the top portion of the bottle and the cap (Fig. 2, step 2).

7.4.3. Tilt the bottle to approximately a 45° angle with the lid pointing downwards, and rotate at least 5 times to coat the lid and neck of the bottle (Fig. 2, step 3). Repeat steps 7.4.1 to 7.4.3 at least 1 more time.

7.4.4. Lay the bottle on its side on a flat surface. Rock the bottle back and forth at least 10 times before flipping once and rocking it an additional 10 times or more to ensure that all sides are coated (Fig. 2, step 4).

7.4.5. Repeat steps 7.4.1 to 7.4.4 at least 3 times. Place the bottle in a fume hood and turn the power on to start the evacuation fan.

7.4.6. Remove the bottle cap to evaporate the acetone. Place the cap of the bottle face up inside the fume hood with the inner side of the cap exposed to the air.

Note: Acetone evaporation might build some pressure in the bottle. This is a normal condition, so be cautious when removing the cap from the bottle.

7.4.7. Hold the bottle at approximately a 45° angle with the neck of the bottle pointing upwards. Rotate at least 5 times to coat the base of the bottle (Fig. 2, step 5).

7.4.8. Tilt the bottle at a slight angle with the neck of the bottle facing downwards, ensuring not to drain any remaining liquid, and rotate at least 5 times to coat the top portion of the bottle (Fig. 2, step 6). Repeat steps 7.4.7 and 7.4.8 at least twice.

7.4.9. Lay the bottle on its side in the fume hood. Rock the bottle back and forth at least 10 times before flipping and rocking it an additional 10 times or more to ensure that all sides are coated (Fig. 2, step 7).

7.4.10. Repeat steps 7.4.7 to 7.4.9 at least 3 times until the solvent has evaporated and is no longer visible. Check for solvent by holding the bottle at approximately a 45° angle with the neck pointing upwards, and wait to see if any solvent pools at the base of the bottle.

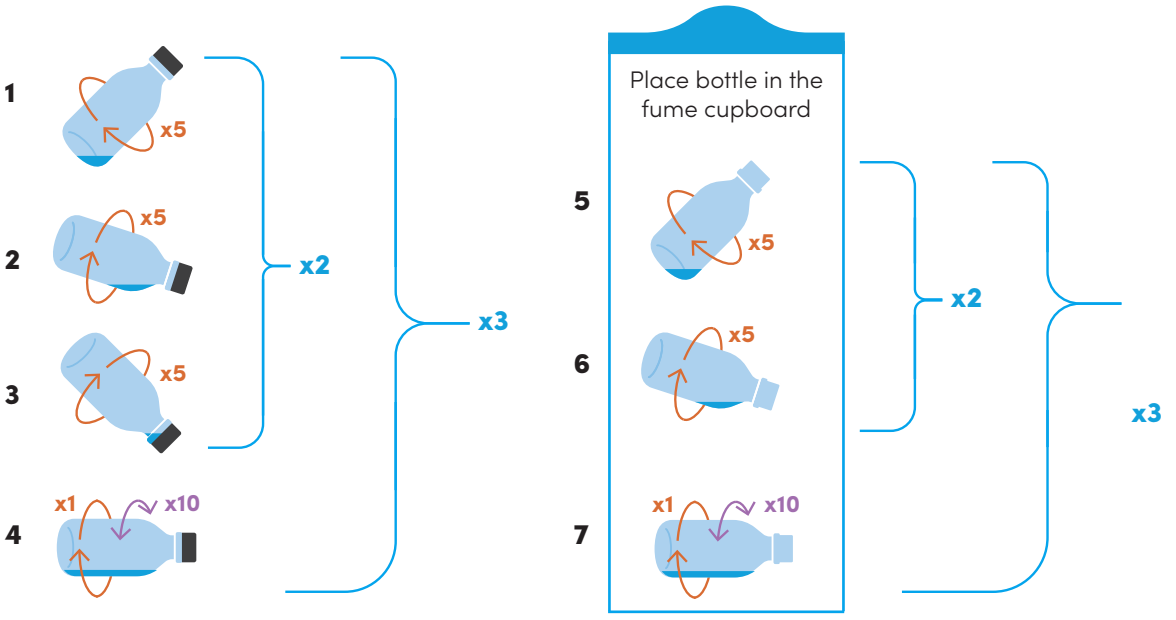
☐ 7.5. **Let the bottle dry for 24 hours:** Wrap the bottle in aluminium foil to protect the contents from light. Leave the open bottle and the cap in an undisturbed fume hood (or separate room with ventilation) for 24 hours. After 24 hours of drying, check whether the inside surface of the bottle has dried completely.

Note: While the bottles and caps are drying, the fume hood must remain switched on and should not be cleaned or used by other operators. Other equipment and glassware, e.g. pipettes, may be cleaned and removed from the fume hood, but cleaning must not interfere with the bottles drying in the fume hood.

☐ 7.6. **Store the bottles:** Put the caps on the bottles and keep them in a dark, cool, dry place until use.

Note: The procedure for uniformly coating the bottles can be practiced initially by coating 1 or more bottles with a mixture of acetone and some colouring agent (e.g. a dye or ink).

Fig. 2. Procedure for evenly coating bottles with the acetone/surfactant mixture



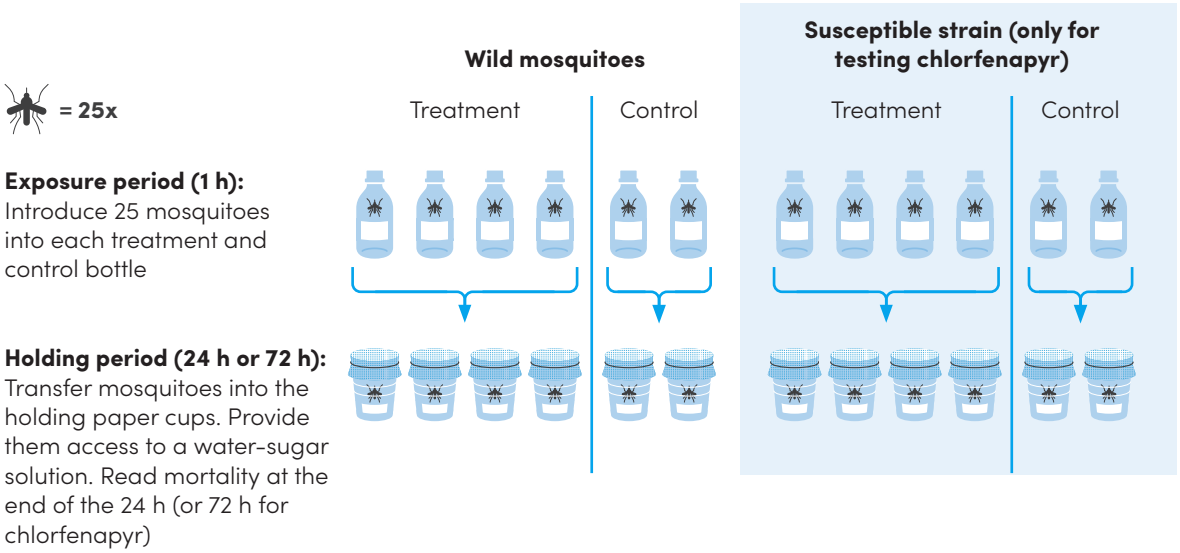
Source: Institut de Recherche pour le Développement, Montpellier, France

Step 2: Mosquito exposure (Fig. 3)

7.7. **Prepare the bottles for mosquito introduction:** Line up the bottles with their caps screwed on loosely so that it will be easy to introduce the mosquitoes. Place a piece of netting over the mouth of each bottle and fasten it with a rubber band.

Note: Adult female mosquitoes 3–5 days of age should be starved for 2 hours prior to testing.

Fig. 3. Diagram showing the process of mosquito exposure in bottles



7.8. **Aspirate and insert mosquitoes into the bottles**: Insert mosquitoes first into all the control bottles and then into the insecticide-coated bottles. Use separate aspirators for the control and insecticide-coated bottles.

7.8.1. Using preferably a mechanical aspirator, collect 25 non-blood-fed mosquitoes to be released in each bottle.

7.8.1. Insert the aspirator with 25 mosquitoes into the bottle through the hole in the netting piece that covers the mouth of the bottle. Position the aspirator's opening in the central part of the bottle and gently tap the mosquitoes into the bottle.

7.8.1. Quickly place the cap on the bottle to prevent mosquitoes from escaping.

7.8.1. Place the bottle in a vertical position and start the stopwatch to record an exposure time of 1 hour.

7.9. **Leave the mosquitoes in for 1 hour of exposure**.

☐	7.10. **During the exposure time, prepare the 6 holding paper cups:** Place a piece of netting over the opening of each of the 440 mL paper cups and fasten it using a rubber band.
☐	7.11. **Transfer mosquitoes to the holding paper cups and hold them for 24 hours (or 72 hours for chlorfenapyr)**: Gently suck the mosquitoes out of the bottle, preferably using a mechanical aspirator, and release them into the net-covered holding cups.[2] Place a piece of cotton wool soaked in a 10% sugar solution on the netting cover. **To ensure consistency of the results, especially with compounds that have a particular mode of action (e.g. chlorfenapyr), hold the treatment and control cups strictly at 27 °C ± 2 °C and 75% ± 10% relative humidity throughout the test**.
☐	7.12. **Record mosquito knockdown:** Right after transferring the mosquitoes to the paper cups, record the number of mosquitoes that have been knocked down, as per the definition in Table 3.
☐	7.13. **Record mortality after 24 hours (or 72 hours for chlorfenapyr):** Count and record the number of mosquitoes found dead and alive 24 hours post-exposure (or 72 hours for chlorfenapyr), as per the definitions provided in Table 3. Enter the data in the recording sheet or electronic data collection system.

Table 3. WHO guidelines for 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: adapted from page 77 of the *Report of the fifteenth WHOPES working group meeting (2)*

8. Criteria for test rejection

For all insecticides, the test must be discarded and repeated if the control mortality is >20%. For chlorfenapyr, the bioassay should be discarded if mortality in the control group of the wild mosquito sample or in control group of the susceptible colony sample is >20%, or if temperature during the bioassay is outside of the range 27 °C ± 2 °C.

[2] An alternative suggestion made in the WHO consultation October 2021 to review the results of the multi-centre laboratory study to determine the insecticide concentrations of insecticides for monitoring of resistance *(1)*, was to first release mosquitoes from a bottle into a release cage and then collect mosquitoes from the cage. This has been reported to improve survival rate of control mosquitoes. This method however was not used in the WHO study.

9. 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 2 of this SOP. Digital DHIS2-base forms are available from the WHO Global Malaria Programme website.[3]

The end-point of the test is mosquito mortality at 24 hours (or 72 hours for chlorfenapyr) after 1 hour of exposure to the insecticide. Therefore, the number of dead mosquitoes is counted 24 hours (or 72 hours) after the 1 hour exposure period. Mosquito mortalities should be calculated separately for the treatment bottles and control bottles, and in the case of chlorfenapyr, separately for wild mosquitoes and the susceptible colony. Therefore, for chlorfenapyr, mortalities should be calculated in each of these 4 mosquito groups: wild control, wild treated, susceptible colony control and susceptible colony treated.

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.
- If the control mortality is ≥5% and ≤20%, the test mortality should be corrected with the control mortality using Abbott's formula as follows:

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

10. Interpretation of test results

For all insecticides, except for chlorfenapyr:

- **Susceptibility:** A vector population is considered to be susceptible to an insecticide if mosquito mortality (corrected using the Abbott's formula) is ≥98%.
- **Possible resistance**: If the observed mortality (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 <98%, then resistance is confirmed.
- **Confirmed resistance:** A vector population is considered to be resistant to an insecticide if mortality (corrected using the Abbott's formula) is <90%.

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

For chlorfenapyr:

WHO bottle bioassays with chlorfenapyr have shown some interlaboratory variability in test results due to the strong influence of testing conditions (especially temperature during bioassays). Therefore, to confirm resistance to chlorfenapyr in a wild vector population, at least 3 WHO bottle bioassays need to be conducted with the same vector population. Furthermore:

- temperature should be kept within 27 °C ± 2 °C and relative humidity within 75% ± 10% during all 3 tests;
- the mortality of test mosquitoes 72 hours post-exposure should be <90% in all 3 tests; and
- the mortality in the susceptible laboratory colony, tested in parallel to the wild mosquitoes, should be ≥98% in all 3 tests.

11. Bottle washing procedure

Below, we provide 2 different options for washing the bottles, depending on the availability of washing agents.

Generic wash method using Decon or TFD4

1. Remove any adhesive labels from the bottles before washing.
2. Add approximately 10 mL of acetone to each bottle to be washed and close with a cap.
3. Shake the bottles vigorously one by one.
4. Discard the acetone.
5. Prepare a 2–5% Decon solution (or equivalent product e.g. 10% alkaline detergent TFD4) in hot water in a 20 L open container or sink.
6. Submerge the acetone-rinsed bottles and caps in the Decon solution overnight.
7. The next day, remove the bottles and caps from the solution, scrub every bottle and cap vigorously with the Decon solution using a cleaning brush, and rinse them thoroughly 3 times with tap water.
8. Submerge the bottles and caps in clean tap water in a container or sink for 24 hours.
9. Remove the bottles, rinse them with clean tap water and dry them for 6–8 hours upside down on a rack (as shown in Fig. 4), or dry the bottles and caps in an oven at 50 °C for 20–30 minutes. Increase the drying time if moisture is still visible in the bottle.

Wash procedure in places where Decon or TFD4 is not available

1. Remove any adhesive labels from the bottles before washing.

2. Rinse the bottles with acetone, if available, as described above.

3. Prepare a 10% soap solution in hot water in a 20 L open container or sink.

4. Submerge the bottles and caps in the soapy water for 24 hours.

5. Remove them from the soapy water, and either i) scrub every bottle and cap vigorously with soap solution using a cleaning brush and rinse them thoroughly 3 times with tap water, or ii) wash them in a washing machine with hot water.

6. Submerge the bottles and caps in clean tap water in a container or sink for 24 hours.

7. Remove the bottles, rinse them with clean tap water and dry them for 6–8 hours upside down on a rack (as shown in Fig. 4), or dry the bottles and caps in an oven at 50 °C for 20–30 minutes. Increase the drying time if moisture or droplets are still visible in the bottles.

To ensure that no insecticide residues are left in the washed bottles, it is recommendable to check the wash quality by selecting some dry bottles at random and exposing 5 mosquitoes in each, recording their knockdown at the end of 1 hour of exposure and mortality at 24 hours of holding post-exposure.

Fig. 4. Method of drying glass bottles upside down on raised steel rods

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

12. Acknowledgements

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13. 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).

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Annex 1. Calculations for preparing solutions for WHO bottle assays

A. Calculations for the amount of active ingredient (AI), solvent and surfactant to prepare the initial stock solution and subsequent dilutions for serial concentrations for coating bottles

The Excel calculation spreadsheet is available online at: https://cdn.who.int/media/docs/default-source/ntds/vector-ecology-mangement/calculation-tables-paper-impregnation-bottles-17jan2022-locked.xlsx

In the Excel spreadsheet, you will enter values in the green cells and the values in the white cells will be automatically calculated.

											Calculation for AI weight in g adjusted for purity of AI					Calculation for AI weight in mg adjusted for purity of the AI		
Insecticide class group	Surfactant	Insecticide	Targeted concentration of AI in the bottles (µg / bottle)	No. of bottles to be coated	Amount of surfactant per bottle* (ppm or µg)	Total weight of surfactant needed (mg)	Density of surfactant	Volume of surfactant (mL)	Volume of acetone (mL)	Total volume of coating solution (mL)	Amount of AI to weigh for coating bottles (g)	Purity of insecticide AI (%)	Adjusted amount of AI to weigh (g)*	Exact weight of AI (g)**	Total adjusted volume of coating solution (mL)***	Amount of AI to weigh (mg)**	Exact weight of AI (mg)***	Total adjusted volume of coating solution (mL)***
			a	b	c	d = (b × c)/1000	e	f = (d/e) / 1000	g = (b × 1) - f	h= f + g	i = (a x b) / 106	j	k = i x (100 / j)	l	m = (l × h) / k	n = k x 1000	o	p = (o × h) / n
	e.g. MERO[a]			1	800	0.8	0.900	0.001	1.00	1	0.000000	99.2	0.000000		#DIV/0	0.00	1	#DIV/0
		Control		1	800	0.8	0.900	0.001	1.00	1								
	None[b]			1					1.00	1	0.000000	99.8	0.000000		#DIV/0	0.00	1	#DIV/0
		Control		1					1.00	1								

AI, active ingredient
MERO, 81% rapeseed oil methyl ester
* Considering purity of the insecticide AI
** Exact weight of AI shown on the electronic balance
*** This is the volume adjusted for exact weight of AI; use 1 mL solution to coat a 250 mL bottle
[a] For example, clothianidin
[b] With acetone alone as a solvent; for example, chlorfenapyr and pyriproxyfen

B. Dilutions of stock solutions with acetone or acetone + surfactant to prepare serial concentrations of insecticides for coating bottles

The Excel calculation spreadsheet is available online at: https://cdn.who.int/media/docs/default-source/ntds/vector-ecology-mangement/calculation-tables-paper-impregnation-bottles-17jan2022-locked.xlsx

In the Excel spreadsheet, you will enter values in the green cells and the values in the white cells will be automatically calculated.

a. With acetone alone (i.e. without a surfactant; examples – chlorfenapyr and pyriproxyfen)

	Final concentration of AI (mg/m^2)**	Final volume of acetone (mL)*	AI concentration of the initial stock solution (mg/m^2)**	Volume to take from the initial stock solution (mL)	Volume (mL) of acetone to add
	a	b	c	d = (a x b)/c	e = b - d
Stock solution*			**100**		
Serial dilution no. 1	**50**	5	**100**	2.50	2.50
Serial dilution no. 2	**30**	5	**100**	1.50	3.50
Serial dilution no. 3	**20**	5	**100**	1.00	4.00
Serial dilution no. 4	**10**	5	**100**	0.50	4.50
Serial dilution no. 5	**5**	5	**100**	0.25	4.75

b. With acetone and a surfactant (e.g. MERO) - example of clothianidin

	Final concentration of AI (µg/ bottle)	Final volume of acetone + surfactant (mL)**	AI concentration of the initial stock solution (µg/bottle)	Volume to take from the initial stock solution (mL)	Volume of solution acetone + surfactant to add (mL)
	a	b	c	d = (a x b)/c	e = b - d
Stock solution*			**100**		
Serial dilution no. 1	**50**	5	**100**	2.50	2.50
Serial dilution no. 2	**30**	5	**100**	1.50	3.50
Serial dilution no. 3	**20**	5	**100**	1.00	4.00
Serial dilution no. 4	**10**	5	**100**	0.50	4.50
Serial dilution no. 5	**5**	5	**100**	0.25	4.75

AI, active ingredient
MERO, 81% rapeseed oil methyl ester

* Initial stock solution is prepared by weighing adequate AI amount and adjusting required volume of solvent with or without surfactant

** Prepare final volume according to the number of bottles to coat (e.g. 5 mL volume is needed to coat 4 bottles to account for any procedural loss of some solution)

Annex 2. Data collection form for WHO bottle bioassays

World Health Organization

Data collection form – WHO bottle bioassay for testing insecticide susceptibility of adult mosquitoes

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

<table>
<tr><td>Bioassay date (dd/mm/yy):</td><td colspan="2">Technician's name:</td></tr>
<tr><td>Country/place of mosquito collection:</td><td colspan="2">Coordinates

Latitude: Longitude:</td></tr>
<tr><td>Period of mosquito collection:

Start date:

End date:</td><td colspan="2">Collection method:</td></tr>
<tr><td rowspan="2">Insecticide tested and concentration:</td><td>Date of coating of the bottles (dd/mm/yy):</td><td>Number of times coated/control bottles have been previously used:</td></tr>
<tr><td colspan="2">Bottle storage temperature

Max: Min:</td></tr>
<tr><td>Mosquito species:</td><td colspan="2">Mosquito strain:</td></tr>
<tr><td>Age of females (days):</td><td colspan="2">Feeding status:

(unfed; sugar-fed and starved; other, specify)</td></tr>
<tr><td>Start time of exposure (hh/mm):</td><td colspan="2">End time of exposure (hh/mm):</td></tr>
<tr><td>Temperature during exposure + holding period (°C):

Max: Min:</td><td colspan="2">Relative humidity during exposure + holding period (%):

Max: Min:</td></tr>
</table>

Results per bottle

Test arm	Bottle	No. of mosquitoes introduced	No. of knocked down mosquitoes after 1 h exposure time	No. dead 24 h after 1 h exposure	Mortality 24 h after 1 h exposure	[For chlorfenapyr] No. dead 48 h after 1 h exposure	[For chlorfenapyr] No. dead 72 h after 1 h exposure	[For chlorfenapyr] Mortality 72 h after 1 h exposure
Wild mosquitoes exposed to DC[a] of the insecticide	Bottle w1							
	Bottle w2							
	Bottle w3							
	Bottle w4							
Wild control mosquitoes	Bottle control w1							
	Bottle control w2							
[For chlorfenapyr] **Susceptible mosquitoes exposed to DC[a] of the insecticide**	Bottle s1							
	Bottle s2							
	Bottle s3							
	Bottle s4							
[For chlorfenapyr] **Susceptible control mosquitoes (exposed to acetone)**	Bottle control s1							
	Bottle control s2							

[a] DC, discriminating concentration

Final results (all bottles)

	Knockdown %	Mortality %		Abbott's corrected mortality %	
	At the end of 1 h exposure	24 h	72 h (for chlorfenapyr)	24 h	72 h (for chlorfenapyr)
Wild mosquitoes exposed to DC of the insecticide					
Wild control mosquitoes					

Test result

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

Comments, if any:

_____

_____

_____

_____

_____

Verified by Supervisor: _____ Date: _____

Standard operating procedure for testing insecticide susceptibility of adult mosquitoes in WHO bottle bioassays

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