World Health Organization

Standard operating procedure

for evaluating the sterilizing properties of pyriproxyfen in adult female mosquitoes in WHO bottle bioassays

SOP version: PPXN-Bioassay/01/14 January 2022

1. Introduction, scope and purpose

Juvenile hormone mimics such as pyriproxyfen could potentially be used in vector control products with the aim of inhibiting/reducing the fertility and fecundity of adult female mosquitoes; in turn this may lead to a reduction in the density of the next generation of vectors (offspring). The purpose of this standard operating procedure (SOP) is to describe the process for evaluating the sterilizing properties of pyriproxyfen in adult female mosquito populations/strains.

This susceptibility bioassay is a response-to-exposure test, measuring the reduction of oviposition rate in adult female mosquitoes pre-exposed to a known standard concentration (e.g. the discriminating concentration) or to serial concentrations of pyriproxyfen for a fixed period of 1 hour.

This SOP describes the process of exposing mosquitoes to pyriproxyfen in glass bottles, chambering mosquitoes for oviposition after exposure, and recording and interpreting test results. The SOP complements the World Health Organization (WHO) SOP for testing insecticide susceptibility of adult mosquitoes in WHO bottle bioassays WHO Bottle-bioassay/01/14 January 2022.

2. Equipment

☐	250 mL Wheaton® bottles with screw caps (16 bottles). If reused, ensure that they have been cleaned properly by following the procedure for bottle washing described later.
☐	weighing spatula
☐	microbalance
☐	vortex mixer
☐	magnifying lens or binocular loupe
☐	2 amber glass bottles (volumes 50–100 mL). 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)
☐	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)
☐	mosquito cages (minimum dimensions 20 cm x 20 cm)
☐	racks
☐	appropriate personal protective equipment

3. Materials

☐	technical grade insecticide or stock solution
☐	acetone (reagent for analysis)
☐	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)
☐	paper cups for holding individual mosquitoes (100 mL capacity; 6 cm height x 6 cm width) (i.e. storage chamber for 1 test mosquito)
☐	narrow mesh netting in sufficient quantities to cover the paper cups and rubber bands to secure the netting pieces on the cups
☐	graduated syringes (10–30 mL)
☐	disposable plastic tips for micropipettes (e.g. 100 µL, 200 µL and 1000 µL)
☐	glass Pasteur pipettes of various volumes to count and remove larvae from paper cups
☐	deionized water
☐	medical grade cotton wool

☐	tally counters
☐	laboratory pads and bench protectors
☐	10% sugar solution (glucose dissolved in water at 10% w/v)
☐	aluminium foil
☐	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
☐	antibacterial cleaner such as 70% isopropyl alcohol or ethanol
☐	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

Both female mosquitoes collected in the field and mosquitoes from a susceptible laboratory colony are needed to conduct these bioassays (200 of each at least).

The key outcome of this test is the quantification of female oviposition inhibition, which is measured 7 days after exposure to pyriproxyfen in glass bottles. For this reason, and in contrast to the WHO tube test or the WHO bottle bioassay for other insecticides, it is essential that mosquito females be inseminated (or mated) and blood-fed before being exposed to pyriproxyfen and that the test mosquitoes survive for the 7 day post-exposure period and manipulations. To maximize insemination and produce fit mosquitoes capable of surviving the entire test period, it is essential to:

- have the test mosquitoes well nurtured and uncrowded during the larval and adult stages to reduce mortality in the control during the test;
- mix the test adult female mosquitoes with plenty of vigorous males in the days preceding blood feeding and insecticide testing; and
- use blood-fed females that are 5–7 days of age to avoid control mortality during the test and increase oviposition success after the bioassays.

6. Preparation of stock solutions of discriminating concentrations

In 2022, WHO adopted a discriminating concentration of **100 µg** of pyriproxyfen (active ingredient) per bottle for *Anopheles gambiae s.s.*, *An. funestus s.s.* and *An. stephensi (1).* Discriminating concentrations for other vector species have yet to be established.

Calculations for the amount of insecticide (active ingredient) and solvent (acetone) to prepare the stock solution are given in Annex 1 of this SOP.

☐	6.1. **Prepare the electronic balance:** Place a clean piece of weighing paper on the scale and set the scale to zero.
☐	6.2. **Prepare the bottle to store the stock solution:** Take a clean light-proof (amber coloured or aluminium foil-wrapped, if transparent) glass bottle of appropriate size and label it with the stock solution concentration and date.
☐	6.3. **Prepare the stock solution:** To prepare a test concentration of 100 µg AI for a 250 mL bioassay bottle (with a hypothetical degree of purity of 100%), using a clean spatula, weigh 10 mg of AI and dissolve it completely in 100 mL of acetone. Alternatively, to prepare a smaller volume of stock solution (e.g. 10 mL), add 1 mg AI to 10 mL of acetone. This stock solution will contain 100 µg of AI per mL of the solution.
☐	6.4. **Store the stock solution:** Solutions should be stored in a refrigerator at 4–8 °C for a maximum of 2 months.

7. Test procedure

A diagram representing the different phases of this procedure is shown in Fig. 1. This procedure should be conducted in parallel with wild-caught mosquitoes and those from a susceptible mosquito colony. A summary of the test conditions is given in Table 1.

Fig. 1. Phases of the WHO bottle assay for testing the susceptibility of adult mosquitoes to pyriproxyfen, an insect growth regulator

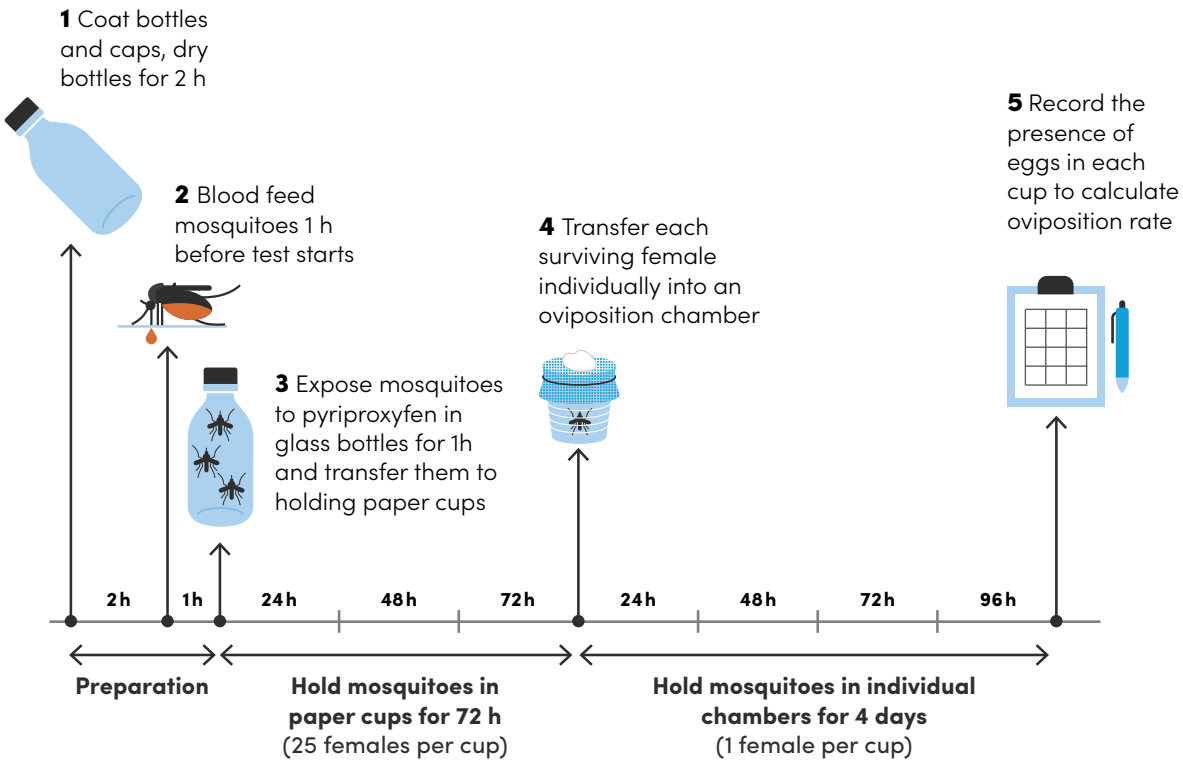


Table 1. Test conditions and number of mosquitoes required for testing the susceptibility of wild mosquitoes to a discriminating concentration of pyriproxyfen using WHO bottle bioassays

	Test conditions for glass bottle bioassays				
Test arm	**Bottle drying time after coating**	**Exposure time**	**No. of bottles per concentration or control[a]**	**No. of mosquitoes per bottle**	**Total no. of female mosquitoes exposed**
Wild mosquitoes exposed to DC[b] (100 µg AI/bottle)	2 h	1 h	4	25	100
Wild control mosquitoes (exposed to acetone only)	2 h	1 h	4	25	100
Susceptible colony exposed to DC[b] (100 µg AI/bottle)	2 h	1 h	4	25	100
Susceptible control mosquitoes (exposed to acetone only)	2 h	1 h	4	25	100

[a] The number of control bottles needs to be the same as the number of treated bottles (i.e. 4), unlike in other susceptibility bioassays where only 2 control bottles are needed.
[b] DC, discriminating concentration

Step 1: Coating of bottles

- ☐ 7.1. **Prepare clean and dry bottles:** Take 16 clean, empty 250 mL glass bottles with screw caps and dry them in an oven for 20 minutes or in open air for 1 to 2 hours, depending on the level of ambient humidity.
- ☐ 7.2. **If the stock solution and solvent (acetone) have been stored in a refrigerator, bring them to room temperature**: Remove them from the refrigerator and bring them to room temperature by letting them sit for 1 hour without opening the container cap.
- ☐ 7.3. **Label both the bottles and the caps** with the name, insecticide concentration (µg/bottle) and date of the test.

7.4. **Coat the interior and caps of the control and exposure bottles:** Coat the control bottles first and then the bottles with pyriproxyfen. Coat the bottles one by one following this process. Pictures illustrating the process are provided 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 bottles) and 1 mL of pyriproxyfen stock solution (for the exposure bottles). 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 the 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 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.

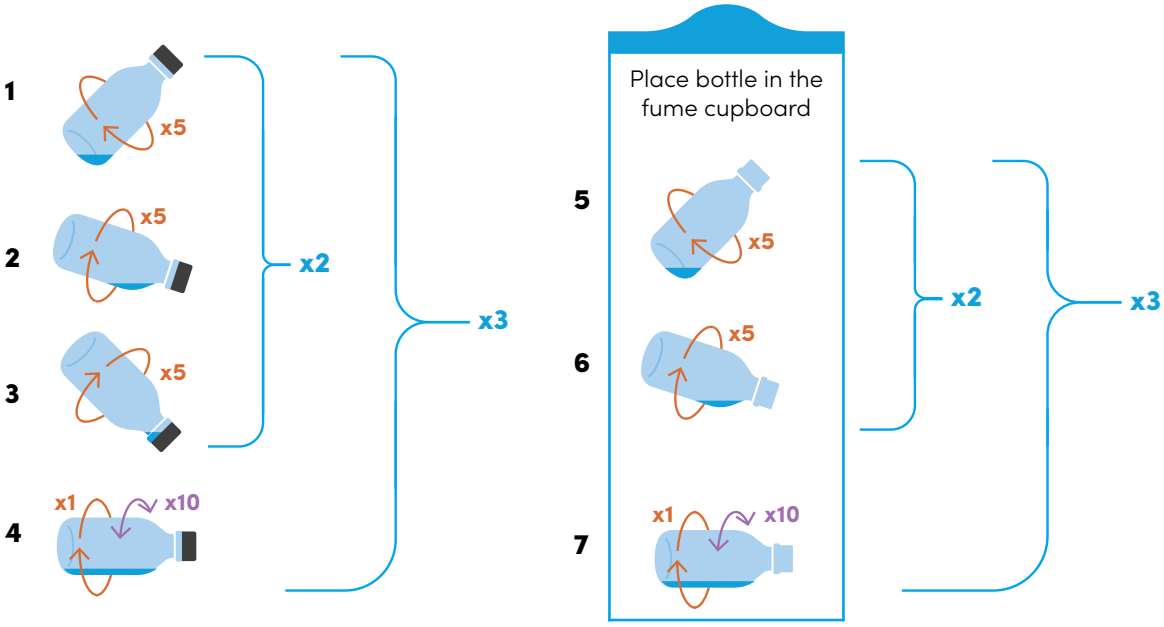
7.4.7. Hold the bottle at approximately a 45° angle with the neck of the bottle pointing upwards. Rotate at least 5 times to coat the base of the bottle (Fig. 2, step 5).

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 the 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 pointed upwards, and wait to see if any solvent pools at the base of the bottle.

Fig. 2. Procedure for evenly coating bottles with acetone



Source: redesigned from a diagram courtesy of the Liverpool School of Tropical Medicine, United Kingdom of Great Britain and Northern Ireland

☐ 7.5. **Let the bottles and caps dry for 2 hours:** Wrap the bottles in aluminium foil to protect the contents from light. Leave the open bottles and caps in an undisturbed fume hood (or separate room with ventilation) for 2 hours. After drying for 2 hours, check whether the inside surface of the bottles has dried completely.

Note: The bottles and caps should be drying for 2 hours, so the time interval between the bottle coating and bioassay is 2 hours (not 24 hours as recommended for other insecticides). While the bottles and caps are drying, the fume hood must remain switched on and should not be cleaned or used by other operators. Equipment, e.g. pipettes, may be cleaned and removed from the fume hood, but cleaning must not interfere with the bottles and the caps being dried in the fume hood.

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

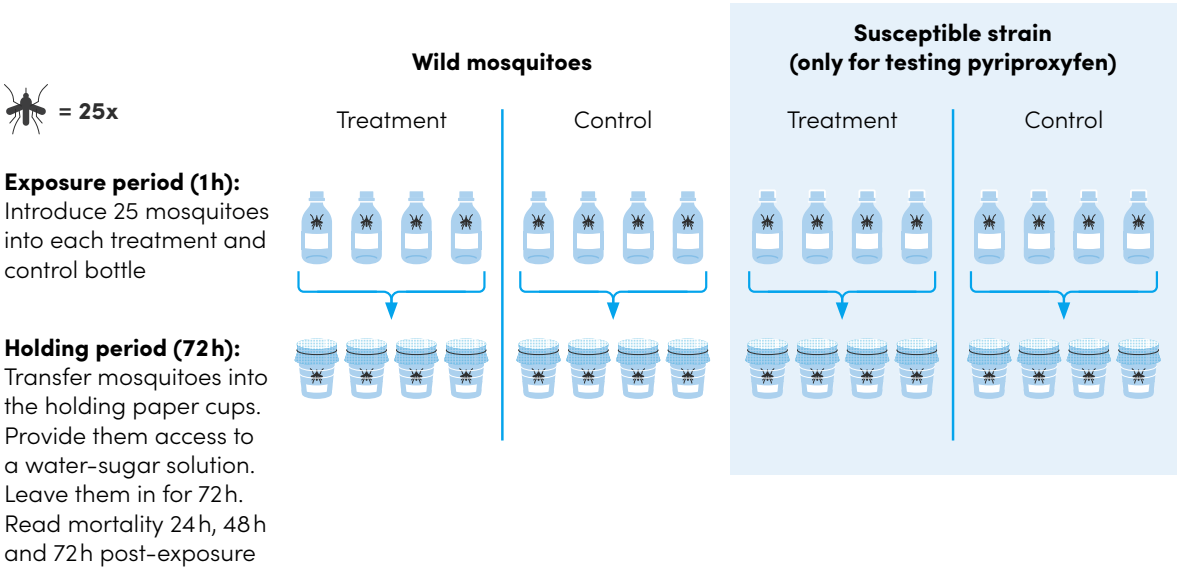
Note: The procedure for uniformly coating the bottles can be practiced initially by coating 1 or more bottles with a mixture of acetone and some colouring agent (e.g. a dye or ink).

Step 2: Blood feeding of mosquitoes

☐ 7.7. Provide test mosquitoes (both wild collected and susceptible colony) with access to a blood meal 1 hour before starting the exposure to pyriproxyfen.

Step 3: Exposure of mosquitoes to pyriproxyfen in bottles and holding for 72 hours post-exposure

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



7.8. **Prepare 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.

Aspirate and insert mosquitoes into the bottles: Insert mosquitoes first into all the control bottles and then into the pyriproxyfen-coated bottles. Use separate aspirators for the control and treated bottles. **Note**: Adult female mosquitoes of 5–7 days of age should be blood fed 1 hour before introducing them to the bottles. To introduce mosquitoes to each bottle:

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

7.8.2. 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.

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

7.8.4. Place the bottle in a vertical position and start the stopwatch to record the exposure time.

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

☐	7.10. **During the exposure time, prepare the holding paper cups**: Place a piece of netting over the opening of each 440mL paper cup and fasten it using a rubber band.
☐	7.11. **Transfer mosquitoes to the holding paper cups and hold them for 72 hours after the 1 hour of exposure**: Gently suck the mosquitoes out of each bottle, preferably using a mechanical aspirator, and release them into a net-covered adult holding cup. Place a piece of cotton wool soaked in a 10% sugar solution on the netting cover and hold the cups at 27 °C ± 2 °C and 75% ± 10% relative humidity for 72 hours.
☐	7.12. **Record mosquito knockdown:** Right after transferring the mosquitoes to the cups, record the number of mosquitoes that have been knocked down, as per the definition in Table 3.
☐	7.13. **Record mortality every 24 hours:** Count and record the number of mosquitoes found dead and alive (as per the definitions provided in Table 3) at 24 hours, 48 hours and 72 hours after 1 hour of exposure and both in treated and control cups. Enter the data in the recording sheet (Annex 2) or electronic data collection system.

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

Step 4: Chambering mosquitoes for oviposition

☐ 7.14. **Before the end of the holding period, prepare the individual mosquito oviposition chambers**:

7.14.1. Take 1 paper cup (100 mL) for each mosquito that remains alive in the treatment and control holding cups, cut round pieces of netting, and prepare a syringe and deionized water (Fig. 4, step 1).

7.14.2. Label each cup as "treatment" or "control". Number them and organize them taking into account the number of live blood-fed mosquitoes in the treatment and control holding cups (e.g. control 1, control 2, control 3, control 4, treatment 1, treatment 2, treatment 3, etc.) (Fig. 4, step 2).

7.14.3. Using a graduated pipette/syringe, add 30 mL deionized water to each 100 mL cup to provide an aquatic medium for the females to lay eggs (Fig. 4, step 3).

7.14.4. Cover each cup with a piece of netting with a round hole and fasten it using a rubber band (Fig. 4, step 4).

☐ 7.15. **Transfer the control mosquitoes to the oviposition chambers (i.e. 100mL paper cups**) (Fig. 4, step 5): Using an aspirator, transfer each live blood-fed mosquito one by one from the control holding cups to a paper cup labelled "control" (e.g. control 1, control 2, control 3, etc.) by introducing the mosquito through the opening in the netting. Only 1 mosquito should be placed in each paper cup. Put a small cotton wool pad soaked in a 10% glucose solution on top of the netting hole; replace the pads with freshly made sugar solution every morning for the subsequent 4 days.

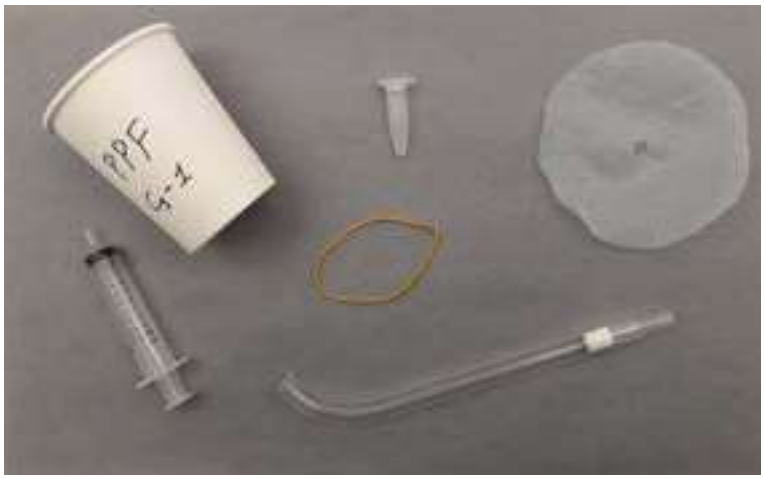
☐ 7.16. **Transfer the treated mosquitoes to the oviposition chambers (i.e. paper cups):** Use a separate aspirator and similarly transfer the mosquitoes one by one from the holding cups with treated females to the chambers labelled "treatment". Put a small cotton wool pad soaked in a 10% glucose solution on top of the netting hole; replace the pads with freshly made sugar solution every morning for the subsequent 4 days.

☐ 7.17. **Let the mosquitoes oviposit**: Maintain the adult females in the paper cups at 27 °C ± 2 °C and 75% ± 10% relative humidity for 4 days. On each day, record the total number of mosquitoes dead on the data recording sheet (Annex 2) or electronic data collection system.

Step 5: Recording the oviposition rate

☐ 7.18. **At the end of the 7-day period (i.e. 3 days of holding and 4 days of chambering), inspect the control and treatment cups (chambers) for the presence of eggs:** Record the numbers of positive (with eggs) and negative (no eggs) chambers on the recording sheet (Annex 2) or electronic data collection system.

Fig. 4. Preparation of mosquito chambers and release of mosquitoes



Step 1. Materials needed for the test

Step 2. Label on an individual adult holding cup (chamber)

Step 3. Adding 30 mL of water to a 100 mL cup

Step 4. Netting cover with a hole fastened on a cup (chamber)

Step 5. Transferring 1 blood-fed female to a cup (chamber)

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

8. Criteria for test rejection

The test should be discarded if one of the following conditions is met:

- The mortality in control mosquitos of the susceptible laboratory strain or the wild collected sample is >20% at 72 hours post-exposure.
- The oviposition rate in the control mosquitos of the susceptible laboratory strain or the wild collected sample is ≤30% at the end of day 7 after 1 hour of exposure to pyriproxyfen.
- The oviposition inhibition in the susceptible laboratory strain at the end of day 7 after 1 hour of exposure to pyriproxyfen is <98%.

9. Data recording and estimation of end-points

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.[1]

The end-point of the test is the inhibition of oviposition among wild mosquitoes 7 days after they are exposed to the insect growth regulator, i.e. 3 days of initial holding plus 4 days of keeping the mosquitoes in individual chambers. To estimate the oviposition inhibition, it is first necessary to estimate the oviposition rates in the control and the treatment for both the susceptible colony and the wild mosquitoes.

- The oviposition rate is the proportion of females that lay eggs out of the total females chambered after the 72-hour holding period:

$$\text{Oviposition (\%)} = \frac{\text{Number of females that laid eggs}}{\text{Total number of females initially chambered}} \times 100$$

- The reduction in oviposition rate (oviposition inhibition) is calculated by dividing the percent reduction in oviposition rate in treated females by the percent reduction in oviposition rate in control females:

$$\text{Oviposition inhibition (\%)} = \left[1 - \left[\frac{\text{Oviposition \% in treatment}}{\text{Oviposition \% in control}}\right]\right] \times 100$$

Control mortality 72 hours post-exposure, which is needed to validate the test, is calculated by summing the number of dead control mosquitoes across all control replicates and then expressing this as a percentage of the total number of control mosquitoes following this formula:

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

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

10. Interpretation of test results

- **Confirmed resistance:** A wild vector population is considered to be resistant to pyriproxyfen if oviposition inhibition is <90% at the end of day 7 after 1 hour of exposure to the discriminating concentration of the insect growth regulator and oviposition inhibition in the susceptible mosquito strain (tested in parallel) is ≥98%.
- **Possible resistance:** If oviposition inhibition is ≥90% but <98% at the end of day 7 after 1 hour of exposure to the discriminating concentration of the insect growth regulator and oviposition inhibition in the susceptible mosquito strain (tested in parallel) is >98%, the presence of resistance in the wild vector population is possible but not confirmed. Test results should be confirmed by repeating the test with a new sample from the same wild mosquito population. (Note: avoid using F1 of the tested mosquitoes.) If 2 tests consistently show that oviposition inhibition is <98% in the wild collected sample while oviposition inhibition is ≥98% in the susceptible laboratory strain (tested in parallel), then resistance is confirmed.
- **Susceptibility:** A wild vector population is considered to be susceptible to pyriproxyfen if oviposition inhibition after 1 hour of exposure to the discriminating concentration is ≥98% at the end of day 7 and oviposition inhibition in the susceptible laboratory mosquito strain (tested in parallel) is ≥98%.

11. Bottle washing procedure

Below, we provide two 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. 5), 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. 5), 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. 5. 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) and solvent (acetone) 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 solution with acetone 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.

	Final concentration of AI (µg/bottle)	Final volume of acetone (mL)**	AI concentration of the initial stock solution (µg/bottle)	Volume to take from the initial stock solution (mL)	Volume of acetone 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

* Initial stock solution is prepared by weighing adequate AI amount and adjusting required volume of solvent (acetone)

** 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 with pyriproxyfen

World Health Organization

Data collection form – WHO bottle bioassay for evaluating the sterilizing properties of pyriproxyfen in adult female 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>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:</td><td colspan="2">End time of exposure:</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>

Mortality per bottle during the 72-hour holding period post-exposure to pyriproxyfen

Test arm	Bottles	Insecticide concentration	Number of knocked down mosquitoes after 1 h exposure	24 h (Day 1)		48 h (Day 2)		72 h (Day 3)	
				No. dead 24 h after exposure	Mortality 24 h after exposure %	No. dead 48 h after exposure	Mortality 48 h after exposure %	No. dead 72 h after exposure	Mortality 72 h after exposure %
Wild mosquitoes exposed to DC (100 µg AI/ bottle)	Bottle w1								
	Bottle w2								
	Bottle w3								
	Bottle w4								
Wild control mosquitoes (exposed to acetone only)	Bottle control w1								
	Bottle control w2								
	Bottle control w3								
	Bottle control w4								
Susceptible mosquitoes exposed to DC (100 µg AI/bottle)	Bottle s1								
	Bottle s2								
	Bottle s3								
	Bottle s4								
Susceptible control mosquitoes (exposed to acetone only)	Bottle control s1								
	Bottle control s2								
	Bottle control s3								
	Bottle control s4								

Total knockdown and mortality rates during the 72-hour holding period post-exposure to pyriproxyfen (all bottles)

	Knockdown %	Mortality %		
	At the end of 1 h exposure	24 h (day 1)	48 h (day2)	72 h (day 3)
Wild mosquitoes exposed to DC (100 µg AI/bottle)				
Wild control mosquitoes (exposed to acetone only)				
Susceptible mosquitoes exposed to DC (100 µg AI/bottle)				
Susceptible control mosquitoes (Exposed to acetone only)				

Mortality and oviposition rate during the 4-day oviposition period after the end of the 72-hour holding period

Test arm		120 h (Day 4)	144 h (Day 5)	168 h (Day 6)	192 h (Day 7)
Wild mosquitoes exposed to DC (100 µg AI/bottle)	Total no. chambered on day 4				
	No. dead				
	Mortality %				
	No. laid eggs				
	Oviposition rate %				
Wild control mosquitoes (exposed to acetone only)	Total no. chambered on day 4				
	No. dead				
	Mortality %				
	No. laid eggs				
	Oviposition rate %				
Susceptible mosquitoes exposed to DC (100 µg AI/bottle)	Total no. chambered on day 4				
	No. dead				
	Mortality %				
	No. laid eggs				
	Oviposition rate %				

Susceptible control mosquitoes (exposed to acetone only)	Total no. chambered on day 4				
	No. dead				
	Mortality %				
	No. laid eggs				
	Oviposition rate %				

Final test results

Test arm	Oviposition inhibition %
Wild control mosquitoes (exposed to acetone only)	
Susceptible mosquitoes exposed to DC (100 µg AI/bottle)	

The test mosquitoes are _____ (susceptible/resistant) to pyriproxyfen

Comments, if any:

_____

_____

_____

_____

_____

Verified by Study Director: _____ Date: _____

Standard operating procedure for evaluating the sterilizing properties of pyriproxyfen in adult female mosquitoes in WHO bottle bioassays

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