Standard operating procedure for testing the susceptibility of adult sand flies to insecticides in WHO tube tests

World Health Organization

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Contents

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Other stakeholders:[1] Dr Susanne Stutz (BASF SE, Limburgerhof, Germany); Dr Sebastian Horstmann (Envu, Mosquito Management, Monheim, Germany); Dr Mark Hoppe (Syngenta, Basel, Switzerland); Dr John Lucas (Consultant, Sumitomo Chemical Co. Ltd, London, United Kingdom); Dr Barnabas Zogo (Sumitomo Chemical Co. Ltd, London, United Kingdom); Dr James W. Austin (BASF Corporation, Durham (NC), United States of America).

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1 The other stakeholders were representatives of the commercial entities who provided technical comments on the SOP with observer status. The stakeholders did not participate in finalization of the SOP.

Declarations of interest and confidentiality undertaking

WHO reported that it had received and reviewed declarations of interest and confidentiality undertakings from all the external contributors (i.e., experts who participated in the WHO consultation and peer review process) and had concluded that none could give rise to a potential or reasonably perceived conflict of interest related to the subjects discussed at the meetings.

1. Introduction, scope and purpose

This SOP describes the procedure for evaluating the susceptibility of adult sand fly vectors (*Phlebotomus* spp. and *Lutzomyia longipalpis*) to insecticides with the WHO tube test. The bioassay procedure described directly measures response to exposure, as sand fly mortality 24 h after exposure to a known standard concentration of an insecticide (e.g., the discriminating concentration, DC) for 1 h. This procedure should be followed to test the susceptibility of sand flies to insecticides that can be impregnated onto filter papers and not for insecticides that are unstable or cannot be impregnated on filter papers.

Instructions are provided on preparing materials, exposing sand flies and recording and interpreting test results. Filter papers impregnated with standard DCs of insecticides are used in this test. Filter papers impregnated with the validated discriminating concentrations of various insecticides and test kits for conducting WHO tube tests can be purchased using the WHO catalogue and order form available from the web site *(1)*.

The SOP was adapted from a similar SOP developed earlier by WHO for mosquitoes *(2)*. The initial draft of the SOP was prepared by Dr Vincent Corbel (IRD) and Dr Rajpal S. Yadav (WHO) and discussed at a WHO consultation on 29 June 2022 that was convened to review results of a WHO multi-centre study on determining discriminating concentrations of insecticides for monitoring resistance in sand flies *(3)*. Thereafter, a revised draft of the SOP incorporating the technical comments of study investigators, experts to WHO and other stakeholders listed above was peer reviewed. The peer reviewed SOP was finalized in consultation with the main contributors and the experts who had previously participated in the WHO consultation.

2. Equipment, reagents and consumables

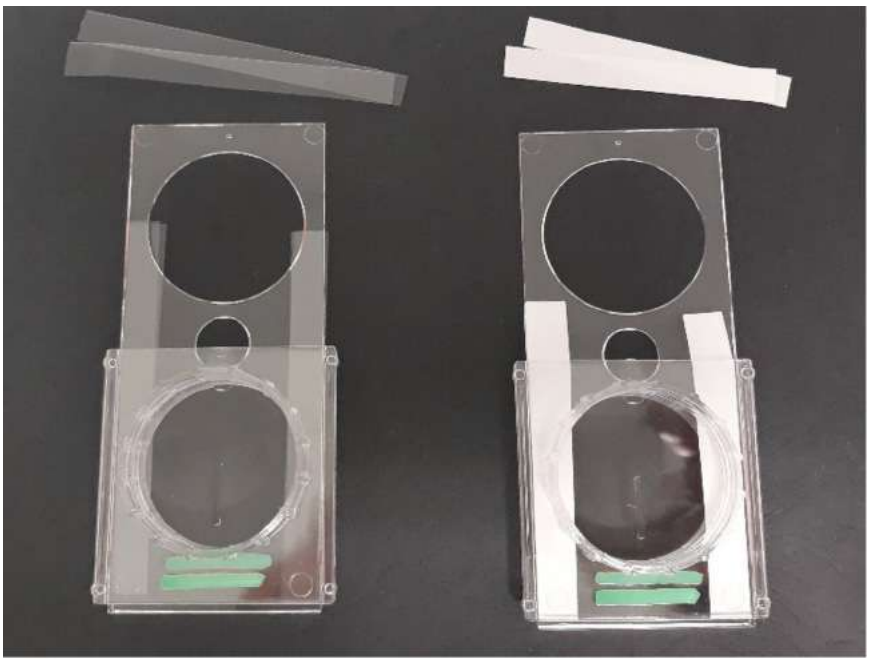
Most of the equipment required for this bioassay can be used many times; impregnated papers should be used a maximum of six times.

The standard mosquito (adult) susceptibility test kit from Universiti Sains Malaysia is recommended for testing the susceptibility of sand flies to insecticides. It contains the following items, which can also be procured separately as necessary.

☐	6 green-dotted holding tubes
☐	2 yellow-dotted control exposure tubes
☐	4 red-dotted exposure tubes
☐	12 steel clips (rings) to hold white papers in the holding tubes

- [ ] 12 copper clips (rings) to hold insecticide- or oil-impregnated or acetone-impregnated (control) papers in the exposure tubes

- [ ] 6 slide units



Note: The test kits (equipment) used for testing susceptibility of sand flies are the same as for mosquitoes. However, as sand flies are much smaller than mosquitoes, they can escape through the gap between the body of the slide and the moveable shutter. A strip of thin transparent plastic (photo on left) or a strip of white paper (photo on right) can be fixed on the sides the slide (moveable shutter) to prevent escape of sand flies. WHO is improving the design of the tubes to solve this problem.

Source: Photo courtesy Stéphane Duchon, of Institut de Recherche pour le Développement, Montpellier, France

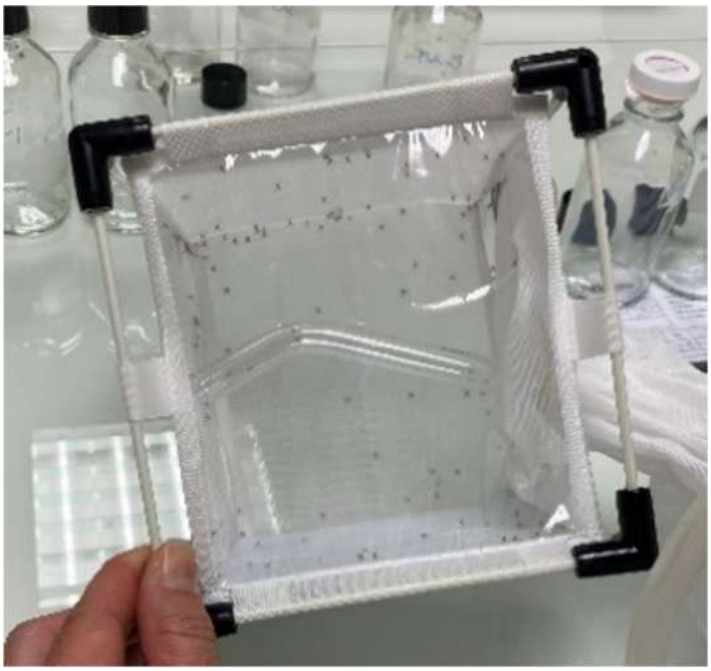
- [ ] Impregnated papers: Each box contains eight sheets of insecticide-impregnated papers (see standard DCs for sand flies in Table 1).

- [ ] Control papers: Each box contains eight sheets of carrier oil-impregnated papers to be used as control (Note: for testing pirimiphos-methyl, use Whatman No. 1 filter paper treated with acetone alone).

- [ ] 2 aspirators with glass tubes and mouthpieces, straight for collecting sand flies (Note: battery-powered aspirators should be used to avoid any exposure to insecticides or inhalation of allergens) (Note: use glass aspirators to prevent generation of static electricity).

- [ ] 2 pieces of rubber tubing, each 60 cm long

- [ ] 40 sheets of 15 cm x 12 cm of clean white paper for lining the holding tubes (Note: 6 sheets of clean white paper are required for each test)

☐	12 labels (6 for green-dotted holding tubes); 4 for red-dotted exposure tubes; 2 for yellow-dotted control tubes)
☐	1 roll of adhesive tape
☐	narrow mesh netting (< 500 µm hole size) to prevent escape of sand flies
☐	sand fly cages (minimum dimensions 20 cm length x 20 cm width x 20 cm height)  *Source:* Photo courtesy Stéphane Duchon, Institut de Recherche pour le Développement, Montpellier, France
☐	glucose to prepare 10–50% (w/v) sugar solution in water (Note: different laboratories use different concentrations of sugar solution. Select a concentration that causes the lowest mortality rate in sand flies in control replicates within the range of acceptable mortality as per WHO guidelines).
☐	testing chamber or a climate-controlled insectary at 27 °C ± 2 °C and 75% ± 10% relative humidity
☐	medical-grade cotton wool
☐	a timer (stopwatch)
☐	calibrated, traceable humidity and temperature monitors and data loggers

☐	data recording sheet, pens and pencils
☐	permanent marker pens for labelling tubes
☐	appropriate personal protective equipment (e.g., laboratory coat, disposable latex gloves, safety glasses, face masks)
☐	acetone or alcohol (for cleaning glassware and work table)
☐	antibacterial cleaner, such as 70% isopropyl alcohol or ethanol
☐	a 20-L plastic container or a 20-L sink to wash tubes after test
☐	TFD4 or Decon 90 (for cleaning equipment in contact with chemical compounds including AIs)

Table 1. Insecticide discriminating concentrations (DCs) for WHO tube tests with *Phlebotomus* spp. and *Lutzomyia longipalpis* sand flies

Insecticide class	Insecticide active ingredient	Species for which DCs are validated[a]	DC for 1-h exposure[a] (%)	Carrier oil or solvent
Pyrethroids	Alpha-cypermethrin	*Phlebotomus papatasi*	0.1%	Silicone oil
		Phlebotomus argentipes	0.1%	
		Lutzomyia longipalpis	0.1%	
		Phlebotomus dubosqui	0.02%	
		Phlebotomus longipes	1%	
	Deltamethrin	*Phlebotomus papatasi*	0.05%	Silicone oil
		Phlebotomus argentipes	0.05%	
		Phlebotomus dubosqui	0.02%	
		Phlebotomus longipes	0.05%	
		Lutzomyia longipalpis	0.05%	
	Permethrin (40:60 cis:trans isomer ratio)	*Phlebotomus papatasi*	1%	Silicone oil
		Phlebotomus argentipes	1%	
		Phlebotomus dubosqui	0.5%	
		Phlebotomus longipes	1%	
		Lutzomyia longipalpis	0.5%	
Carbamates	Bendiocarb	*Phlebotomus papatasi*	0.05%	Olive oil
		Phlebotomus argentipes	0.1%	
		Phlebotomus dubosqui	0.05%	
		Phlebotomus longipes	0.4%	
		Lutzomyia longipalpis	0.15%	
Organophosphates	Malathion	*Phlebotomus papatasi*	5%	Olive oil
		Phlebotomus argentipes	5%	
		Phlebotomus dubosqui	5%	
		Phlebotomus longipes	5%	
		Lutzomyia longipalpis	2%	
	Pirimiphos-methyl[b]	*Phlebotomus papatasi*	100 mg/m^2	Acetone only
		Phlebotomus argentipes	100 mg/m^2	
		Phlebotomus dubosqui	50 mg/m^2	
		Phlebotomus longipes	100 mg/m^2	
		Lutzomyia longipalpis	100 mg/m^2	

[a] These DCs are based on a recent WHO multi-centre study on sand flies *(3)*.
[b] DC is expressed as mg ai/m^2, as no carrier oil is used to treat papers with pirimiphos-methyl.

3. Health, safety and environmental protection

✓	Before using any chemical compound, laboratory staff should read and understand the risk assessment, material safety data sheets and the control of substances hazardous to health 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.
✓	Laboratory staff/technicians should ensure that all working areas are clear of other materials and cleaned before 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.
✓	Laboratory staff/technicians dispose of all waste materials appropriately according to national and institutional safety guidelines.
✓	When working with sand flies, minimize sand fly escape by keeping all doors and windows shut. If any sand flies escape, immediately use an electric bat to electrocute them.

4. Sand flies

For monitoring the resistance of a wild population of sand flies, F1 females (progeny of wild-caught adults) of the same age should be used. The bioassay requires a minimum of 150 non-blood-fed adult female sand flies aged 2–7 days.[1] Sand flies should be starved for 2 h before testing.

During rearing, sand flies must be well nurtured and maintained in uncrowded pots 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 1-h exposure period, the number of sand flies per tube should be 25 or as close to 25 as possible; it should not exceed 25 to avoid crowding in the tube. Ideally, 150 active sand flies are aspirated (in batches) from a sand fly cage into the six green-dotted holding tubes through the filling hole in the slide, to give six replicate samples of 25 sand flies per tube.

1 The WHO-recommended age for mosquitoes used in bioassays is generally 3–5 days *(4)*. For practical reasons and in view of the difficulty of capturing large numbers of synchronized sand flies, the tube test procedures for sand flies were developed and validated with 2–7-day-old, non-blood fed females in a WHO coordinated multi-centre study involving eight participating laboratories *(3)*.

5. Test procedure

Step 1: Labelling the tubes

The WHO tube test kit consists of plastic tubes. A WHO test kit contains three types of tube:

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

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

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

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

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

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

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

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

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

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

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

5.2.1 Wearing disposable gloves, take six sheets of clean white paper (size 12 cm × 15 cm) rolled into a cylindrical shape, and insert one paper into each of the six holding tubes. Fasten the sheet into position against the wall of the tube with two steel rings (clips), one at the top and one at the bottom (Fig. 1A).

5.2.2 Place a mesh gauze (≤ 500 µm) on each holding tube, and screw the cap into place.

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

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

5.3.1 Wearing disposable gloves, roll two control papers into a cylindrical shape and insert one paper into each of the two yellow-dotted tubes, ensuring that the stamped label on the control paper is on the outside and is readable through the transparent tube.

5.3.2 Fasten the control papers in each tube with two copper rings, one at the top and one at the bottom. Close the tube with a screw cap at the bottom end.

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

5.4.1 Wearing disposable gloves, roll four insecticide-treated papers into a cylindrical shape and insert one paper into each of the four red-dotted tubes, ensuring that the stamped label on the paper is on the outside and is readable through the transparent tube.

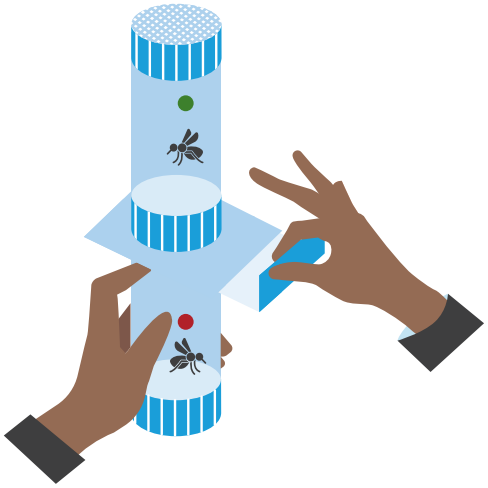
5.4.2 Fasten the insecticide-treated paper in each tube with two copper rings, one at the top and one at the bottom. Close the tube with a screw cap at the bottom end.

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

Fig. 1. Test tubes. A green-dotted holding tube with a steel ring (clip), a yellow-dotted control tube with a copper ring and a red-dotted exposure tube with a copper ring (A); a green-dotted holding tube connected to a red-dotted exposure tube with a slide unit (B)

A

B



Source: A: Photo courtesy of Stéphane Duchon, Institut de Recherche pour le Développement, Montpellier, France; B: WHO photo from reference *2*

Step 2: Exposure of sand flies

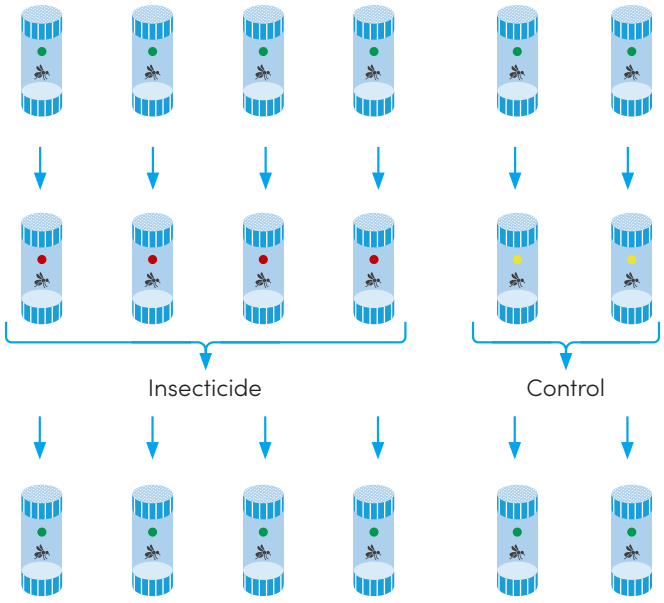
☐	5.5 **Prepare a cage with female sand flies:** Just before performing a bioassay, prepare a cage with only adult female sand flies to avoid inclusion of males in the tubes. This step is important because it is more difficult to identify and pick up female sand flies directly from a rearing cage than mosquitoes because of their smaller size and their rapid movement.
☐	5.6 **Aspirate and insert sand flies into the tubes:** Using an aspirator, aspirate 25 adult female sand flies in batches of 5 sand flies per aspiration from a cage into each of the six green-dotted holding tubes through the filling hole on the tube sliding door. A total of 150 sand flies is needed to fill the six tubes. Close the slide unit, and set the holding tubes in an upright position (Fig. 2).
☐	5.7 **Leave the sand flies in the holding tubes for 1 h.**

Fig. 2. Sand fly exposure and post-exposure processes

Pre-exposure holding period (1 h): Hold sand flies in holding tubes lined with clean white paper (green-dotted tubes).

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

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



☐ 5.8 **Transfer the sand flies from the holding tubes to the test tubes (two control yellow-dotted and four exposure red-dotted tubes):** Only live sand flies should be transferred to the test tubes. Any moribund (i.e., those unable to hop) or dead sand flies should be counted and removed.

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

5.8.2 Carefully slide the units open, and gently blow the sand flies from the holding tubes into the exposure tubes.

5.8.3 Once all the sand flies are in the exposure tubes, close the slide unit, and place a cotton-wool plug into the smaller hole to lock the slide.

5.8.4 Record the time of starting the exposure.

5.8.5 Record the exact number of live sand flies transferred to each exposure tube, as some might have died during holding or transfer.

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

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

☐ 5.9 **Leave the sand flies in the treatment (red-dotted) and control (yellow-dotted) tubes for 1 h**.

☐ 5.10 **Transfer the sand flies back to the holding tubes at the end of the 1-h exposure period.**

5.10.1 Gently blow the sand flies back into the green-dotted holding tubes by reversing the procedure outlined above (section 5.8).

5.10.2 Detach the exposure tubes from the slide units.

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

☐ 5.11 **Record the number of knocked down sand flies**, as per the definition in Table 4.

☐ 5.12 Place a piece of cotton wool soaked in a 10–50% sugar solution on the mesh screen of the holding tubes. To soak the cotton wool in sugar solution:

- Pour the sugar solution into a clean container.
- Submerge a piece of cotton wool roughly 1 cm x 1 cm in the sugar solution.
- Remove the cotton wool, and squeeze it just enough to ensure that it is not dripping.
- Place the soaked cotton wool flat on the top of the holding tube to enable the sand flies to sugar-feed and/or hydrate.
- Pour away any remaining sugar solution into a sink, and rinse out the container with tap water.

5.13 **Hold the sand flies in the holding tubes (green-dotted) for 24 h** at 27 °C ± 2 °C and 75% ± 10% relative humidity (note: record the actual temperature and relative humidity during the exposure and holding periods).

Table 4. WHO definitions of knockdown and mortality of sand flies after a test[a]

Considered to be alive after 1 h of exposure or 24 h after exposure	Considered knocked down after 1 h of exposure or dead at 24 h after exposure
• Can both stand and hop in a coordinated manner	• No sign of life; immobile; cannot stand • Any sand fly that cannot hop in a coordinated manner • Any sand fly that lies on its back, moving legs and wings but unable to take off • Any sand fly that can stand and hop briefly but falls down immediately

[a] *Source*: Adapted from p. 78 of reference *5*

Step 3: Record mortality results

☐ 5.14 **Record the mortality rate 24 h of holding after 1 h of exposure:** Count and record the number of sand flies found dead and alive 24 h after 1 h of exposure, as per the definitions in Table 4. Enter the data on the recording sheet or the electronic data collection system.

6. Use and storage of impregnated papers

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

When bioassays are conducted over a few days, impregnated papers can be retained in the exposure tubes provided the tubes are individually wrapped in aluminium foil after each use, kept at 4–8 °C and brought to room temperature 1 h before the use.

Between cycles of testing for insecticide resistance, reusable papers (i.e., used fewer than six times) should be kept in their original plastic box, sealed with tape and stored in a cool container or a refrigerator at 4–8 °C.

7. Criteria for test rejection

If the mortality rate of controls is > 20%, the tests must be discarded and repeated.

8. Data recording and calculation of test results

During the test, data should be entered on a paper or digital data recording form (Annex 1).

The end-point of the test is sand fly mortality 24 h after 1 h of exposure to the insecticide. **Sand fly** mortality rates should be calculated separately for treatment and control tubes.

Mortality after treatment is calculated by summing the numbers of dead **sand flies** in all replicates with insecticide impregnated papers and expressing them as a percentage of the total number of **sand flies** in the replicates. The mortality rate of the control is calculated similarly.

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

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

- If the control mortality rate is < 5%, the test is valid.

- If the mortality rate after treatment is ≥ 5% and ≤ 20%, it should be corrected with the control mortality rate and Abbott's formula, as follows:

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

9. Interpretation of test results

The interpretations for classifying resistance below are adapted from the criteria for mosquitoes *(4)*.

- **Susceptibility:** A vector population is considered to be susceptible to an insecticide if the mortality rate of **sand flies** in the treatment group (corrected with Abbott's formula if necessary) is ≥ 98%.
- **Possible resistance**: If the observed mortality rate in the treatment group (corrected with Abbott's formula if necessary) is ≥ 90% but < 98%, resistance is possible but not confirmed. Results should be confirmed by repeating the test with a new sample from the same **sand fly** population. If two tests consistently show a mortality rate in the treatment group < 98%, resistance is confirmed.
- **Confirmed resistance:** A vector population is considered to be resistant to an insecticide if the mortality rate in the treatment group (corrected with the Abbott's formula if necessary) is < 90%, provided that at least 100 **sand flies** were tested.

10. Cleaning

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

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For further information, comments or suggestions, please contact: VVE@who.int

Annex. Data collection form for testing susceptibility to insecticides of adult sand flies in WHO tube tests

World Health Organization

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

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

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

Results per tube

Test arm	Tube	Number of sand flies introduced	Number of knocked down sand flies after 1-h exposure	No. of dead and alive sand flies at 24 h after 1-h exposure		Mortality at 24 h after 1-h exposure (%)
				No. dead	No. alive	
No. of sand flies exposed to papers impregnated with the DC[a] of the insecticide	Tube 1					
	Tube 2					
	Tube 3					
	Tube 4					
No. of sand flies exposed to control papers	Control tube 1					
	Control tube 2					

Final results (all tubes)

	Knocked down after 1-h exposure (%) (i.e., at the end of 1-h exposure)	Mortality at 24 h after 1-h exposure (%)	Abbott's corrected mortality, if required (%)
Sand flies exposed to the DC[a] of the test insecticide			

[a]DC: discriminating concentration.

Test result

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

Comments, if any:

_____

_____

Signed by the technician: _____

Verified and signed by the supervisor: _____

Date (dd/mm/yyyy): _____


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