Standard Operating Procedure for WHO Insecticide Susceptibility Test against Adult Sandflies (Tube Test method)

1. PURPOSE / SCOPE

The purpose of this Standard Operating Procedure (SOP) is to describe the process to follow in the evaluation of insecticide efficacy against sandfly strains using the WHO diagnostic kit. The susceptibility bioassay is a direct response-to-exposure test, measuring sandfly mortality to a known standard concentration of a given insecticide. The scope of this SOP covers the procedure for the performance of adult sandfly susceptibility using the WHO diagnostic kit.

2. TEST MATERIALS

The materials requires for this test are:

a. 6 green-dotted holding tubes
b. 6 red-dotted exposure tubes
c. 12 steel clips (rings)
d. 12 copper clips (rings)
e. 6 slide units (put colour dots before use)
f. 12 screw caps with grid (and using a piece of narrow mesh netting, preferably < 500µm)
g. 6 sheets of clean white papers (12 x 15 cm)
h. 6 sheets of insecticide-treated Whatman no. 1 filter papers (12 x 15 cm)
i. 2 sheets of oil-impregnated Whatman no. 1 filter papers as control (in case of Pirimiphos-methyl test use Whatman no. 1 treated with acetone alone)
j. 1 mouth aspirator/ mechanical aspirator (glass-made)
k. Non-blood fed, **2-7-days-old female sandfly** (max. 25 specimen per tube)
l. 10% glucose solution (dissolved in water at 10% w/v).
m. Cotton wool
n. Stopwatch/ laboratory clock
o. Calibrated, humidity and temperature monitors
p. Pencil, sticky label and a permanent ink pen
q. Data Recording sheet
r. Testing chamber 27^{o}C ± 2^{o}C and 80% ± 10% RH relative humidity (RH)

3. SAFETY

- Ensure that prior to using any chemical or biological compound the associated Risk Assessment (RA) and Material Safety Data Sheets (MSDS) have been read and understood.
- When working in the laboratory, always wear a lab coat, hand gloves and safety glasses.
- Clean the lab bench with ethanol and cover the working area with aluminium foil/blotting paper sheet.
- Use clean tubes and other materials.
- Ensure all working areas are cleared of materials except those needed for the experiment.
- Staff working in laboratories must have received laboratory induction training and documented in training files.
- Restrict sandfly escape from the holding containers and exposure tubes while working. An adequate sandfly cages (eg Bugdorm Insect Rearing ref. 4E4590 size 47.5 x 47.5 x 93cm) can be used during testing to avoid any sandfly escape.
- Dispose of waste materials appropriately following the bio safety rules of the lab.

4. TEST PROCEDURES AND PREPARATION OF TEST MATERIALS

4.1 Labelling the tubes

The WHO tube test kit consists of plastic tubes (125 mm in length, 44 mm diameter).Two types of tube come with WHO test kit (Figure 1):

a) The holding tube, into which **a blank piece of filter paper** is inserted, is identified with a **green dot.**

b) The exposure tube, into which the **oil/acetone treated paper (control) or the insecticide treated paper** are inserted are identified with a **red dot.**

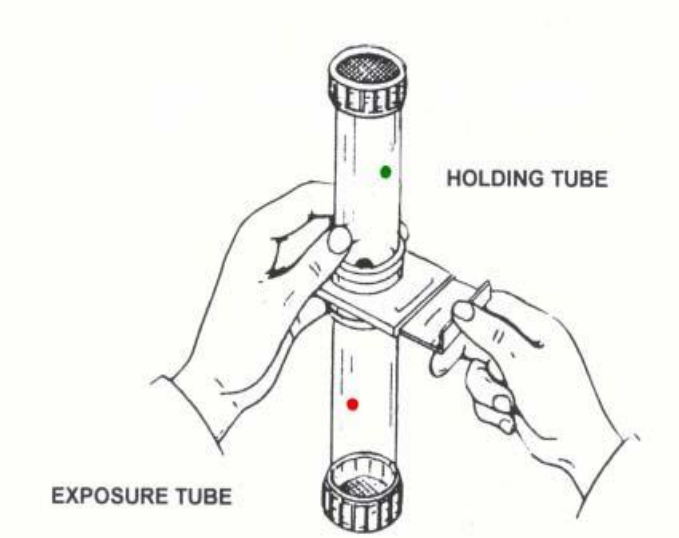


Figure 1 – The holding tube and the exposure tube of the WHO test kit

Label the green and red dotted tubes with a sticky label and write labels using a permanent ink pen as shown below (Figures 2 to 4).

Holding tube number:	1
Date of testing:	12MAY17
Operator initial:	JW

Figure 2 – Example holding tube sticker information

Exposure tube number:	1
Insecticide in use:	Permethrin
Insecticide concentration:	0.75%
Batch number:	PY123
Expiry date:	MAY18
Date of initial use:	12MAY17
Number of times used:	III
Operator initials:	JW

Figure 3 – Example exposure tube sticker information

4.2 Preparation of green-dotted holding tubes

- Take six white paper sheets (12 × 15 cm), roll into a cylinder shape, and insert one each into the six holding tubes. In each tube, fasten into position against the wall of the tube with 2 two steel rings (clips) one at the top end and one at the bottom (Figure 4).

Figure 4 – Green dotted holding Tube with clips (rings)

- Screw in place a cap with <500µm mesh size net onto each holding tube (figure 5).
- Attach a slide unit to each of the holding tubes by screwing in place at the open end.

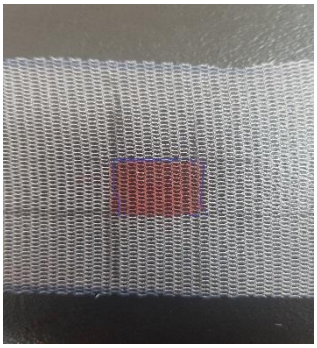


Figure 5. Example of a piece of a narrow mesh netting (one cm^2 area of the netting has 10 x 14 = 140 complete holes).

4.3 Preparation of 2 red-dotted control tubes

- Wear disposable gloves.
- Insert **2 oil impregnated paper** (control) into 2 red-dotted tubes (one per tube).
- Fasten the control paper with 2 copper rings and close the tube with a screw cap at one end.
- Remove the gloves and dispose of them in a biohazard bag.

4.4 Preparation of 4 red-dotted treated tubes

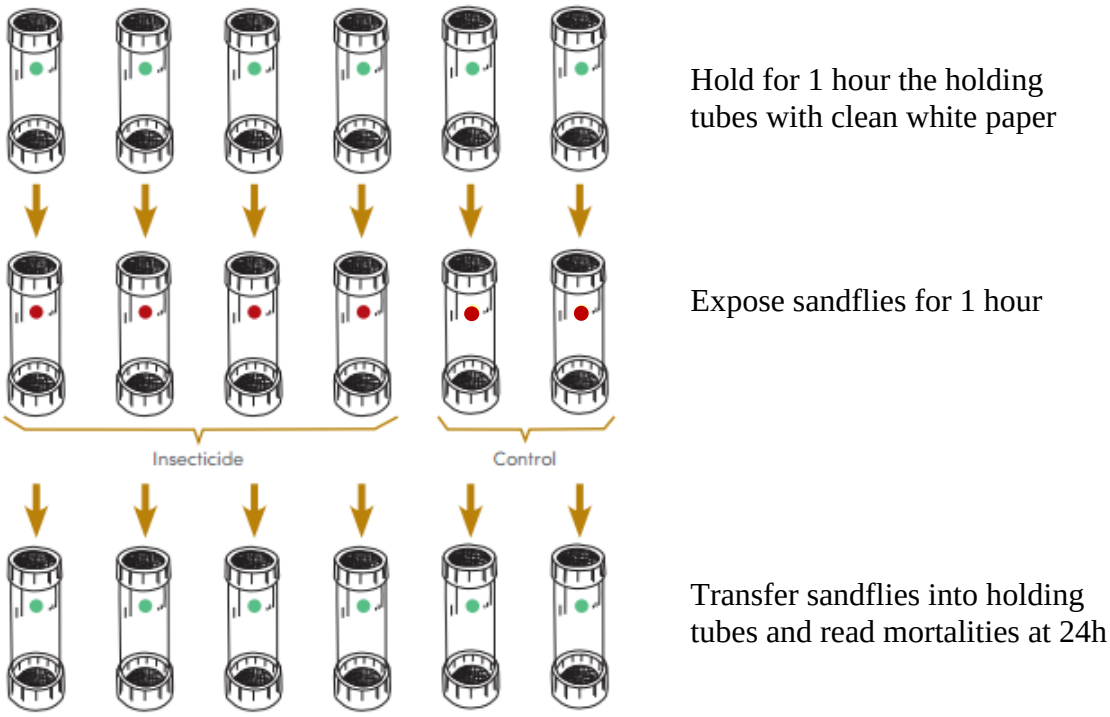
- Wear disposable gloves.
- Insert 4 **insecticide-treated papers** into 4 red-dotted tubes (one per tube). Ensure the label of paper is readable through the tube.

- Fasten the insecticide-treated paper with copper rings and close the tube with a screw cap.
- Remove the gloves and dispose of them in a biohazard bag.

Figure 6 – Red dotted exposure tube with rings

4.5. Exposure of Sandflies

- Aspirate about 150 active adult sandflies (in batches) from a cage into the six green-dotted holding tubes through the filling hole in the slide, to give six replicate samples of 25 sandflies per tube (Figure 7).
- After transferring sandflies into the six holding tubes, close the slide unit and set holding tubes in an upright position **for 1 hour.** At the end of this time, any moribund sandflies (i.e. those unable to fly) and dead one's have to be removed.
- One by one, attach the empty exposure tubes to the vacant position on the slides attached to the holding tubes.
- Gently slide the slide-unit to the open position and gently blow the sandflies from holding tubes into the exposure tubes.
- Once all the sandflies are in the exposure tubes, close the slide unit and place a cotton wool plug into the hole to lock the slide.
- Detach the green-dot holding tubes from the exposure tubes and set them aside.
- Sandflies are kept in the exposure tubes, which are set in a vertical position with the mesh-screen end uppermost, **for a period of 1 hour**. The tubes are placed in an area of reduced lighting or covered with cardboard discs to reduce light intensity and to discourage test sandflies from resting on the mesh-screen lid.
- At the end of the 1 hour exposure period, the sandflies are gently blown back into the holding tubes by reversing the procedure outlined above. The exposure tubes are detached from the slide units.



Hold for 1 hour the holding tubes with clean white paper

Expose sandflies for 1 hour

Transfer sandflies into holding tubes and read mortalities at 24h

Figure 7 – Steps to perform the WHO insecticide susceptibility test

- Place a piece of cotton wool soaked in ~10% sugar solution onto the mesh-screen end of the holding tubes. To soak the cotton wool in sugar solution follow these steps:
    - Pour some ~10% sugar solution out into a clean container
    - Take a piece of cotton wool of roughly 1 cm x 1 cm size
    - Submerge this is the 10% sugar solution
    - Remove the cotton wool and squeeze the cotton wool just enough to ensure it isn't dripping
    - Place it flat on the top of the holding tube to feed and hydrate the sandflies
    - Pour away remaining sugar solution and rinse out the container with tap water

- Sandflies are maintained in the holding tubes for 24 hours. Place the holding tubes of sandflies into the testing chamber at 25-27°C and 80% ± 10% RH.
- Fill out a status card for the holding cups and place in the testing chamber alongside the tubes.
- Record temperature and humidity during the recovery period.
- At the end of recovery period (24 hours post-exposure), the number of dead sandflies is counted and recorded.

- An adult sandfly is considered to be alive if it is able to hop. Any knocked down sandflies, whether or not they have lost legs or wings, are considered moribund and are counted as dead. A sandfly is classified as dead or knocked down if it is immobile or unable to stand or take off. Refer to Table 1 for scoring results.

Table 1 – Guidelines for knockdown / mortality of sandflies post test

Alive	**Knock down after 60 minutes or dead after 24 hours of exposure**	
	Moribund	**Dead**
Can both stand and hop in a coordinated manner	• Any sandfly that cannot hop in a coordinated manner • Any sandfly that lies laterally on its dorsal side, moving legs and wings but unable to take off • Any sandfly that can stand and take off briefly but falls down immediately	No sign of life; immobile; cannot stand

Taken and adapted from the 15th WHOPES WG report (2012):
http://apps.who.int/iris/bitstream/10665/75304/1/9789241504089_eng.pdf

5. RECORDING RESULTS:

Record results in a standard bioassay form (see Annex A).

6. STORING TEST PAPERS

⚠ Special Instruction for Multicenter study

Do not re-used two times the same paper. For different replicates, a new batch of impregnated papers must be used.

7. SCORING KD AND MORTALITY

- The assessment of KD and mortality (i.e. a count of the number of dead sandflies in both the exposure and the control tubes) is made at the end of the specified post-exposure periods.
- The mortality of the test sample is calculated by summing the number of dead sandflies across all exposure replicates and then expressing this as a percentage of the total number of exposed sandflies.

- A similar calculation should be made in order to obtain a value for the control mortality. If the control mortality is ≥20%, the tests must be discarded. When control mortality is >5%, mortality should be corrected be using Abbott formula as follows;

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

- If the control mortality is <5% (i.e. one dead sandfly out of 25), no correction of test results is necessary.
- Dose-response analysis will be performed using a log-probit statistical model (SPPS, IBM) to estimate lethal doses (LD_{50} and LD_{99}) and their confidence intervals (95%).
- **Crude data should be supplied to WHO/IRD after completion of each step of testing for all compounds in an appropriate format (eg excel file) for analysis.**

8. CLEANING

- Decontamination of tubes: soaking overnight in the decontaminant 20% of an alkaline solution (TFD4 or Decon90) if the equipment is in direct contact with the insecticide (exposure tubes, copper clips...) and 10% solution for the equipment used for the handling (holding tubes, steel clips…). The following day, rinse three times with tap water and dry at room temperature.
- Clean the bench and fixed equipment with ethanol.

NOTE: Decontaminant solution at 20% and 10% should be change at least once per month, or more if necessary.

ANNEX A – WHO data recording sheet for Bioassay results (source IRD)

WHO SUSCEPTIBILITY TESTS

Test n°	Date test	Place	Contact time
Species	Date impregnation	Insecticide	Observation time
Strains	Nbe use of papers	Concentration	Temperature
Femeles age	Operator		Humidity
Remarks			

Time / Concentration	N°	Time								Mortality after 24 h	Total
	Tot										
	%										
	Tot										
	%										
	Tot										
	%										