SOP: Cone Bioassay

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Version Control[1]

[1] Historical versions of SOPs can be found on the I2I website (https://innovationtoimpact.org/)

Version	Date	Updated by	Description of update(s)
2	April – May 2022	Alex Wright, Natalie Lissenden	Purpose, materials & equipment, data collection sheet information, glossary of terms and references added.
3	August 2024	Annabel Murphy-Fegan	Background, materials & equipment, procedure updated.

Related documents

- I2I Best Practice SOP Library, 30 October 2020 (https://innovationtoimpact.org/)
- WHO Guidelines for laboratory and field testing of long-lasting insecticidal nets (WHO, 2013)

  WHO Prequalification of Vector Control Products. Bioassay methods for insecticide-treated nets: Cone test. (WHO, 2023)

1. Purpose (Owusu & Müller, 2016)

The World Health Organization (WHO) cone bioassay plays an integral role in the evaluation of the efficacy of long-lasting insecticidal nets (LLINs) as well as insecticides used in indoor residual spraying. This bioassay investigates the biological activity of a material's surface under standardised laboratory conditions, with observations made on the effects on mosquitoes, including knock down (KD) and mortality. The test is used on a variety materials including pieces of bed nets, mud, cement and wood that can be treated or untreated with an active ingredient (AI). This SOP details the process for conducting a cone bioassay in laboratory LLIN testing.

2. Background

Precisely five, susceptible, non-blood-bed, 3-5-day old, nulliparous female *Anopheles* (species to be stated in the test report) mosquitoes are exposed to each piece of netting

(25cm x 25cm or 30 x 30cm) for three minutes under standard WHO cones held at an angle of 45-60°.Dependent upon the study protocol, the mosquitoes are then held for a set period of time, either 24 hours or an extended time period (e.g. 72 hours is required for fertility measurements) with access to sugar solution. Knock-down is recorded 60 minutes after exposure and mortality at 24 hour time periods until the end of holding time. Additional sub-lethal measurements also recorded include: 1) Fertility (quantity of eggs laid per female) and 2) fecundity (the proportion of fertile females). One piece each from four different nets should be tested. Up to four cones at a time may be attached to a piece of netting, and precisely five mosquitoes at one time should be exposed in a cone. This procedure should be repeated until a total of 50 mosquitoes have been exposed to each piece. Results should be reported for each net tested and for the four nets (4 pieces x 10 cone tests x 5 mosquitoes = 200 mosquitoes). Mosquitoes exposed to untreated net pieces are used as controls; they should be tested each day, just before and just after testing treated netting material.

If the mortality in controls on any day is <10%, the results for that day should be adjusted by Abbott's formula. If the mortality in controls is >10% at 24 hours and 20% after extended holding periods beyond 24 hours, the results are considered invalid and should be discarded. Bioassays should be carried out at 27 ± 2°C and 80 ± 20% relative humidity (RH).

The definitions of mortality and knock-down are those recommended by WHO (WHO, 2013). Mosquitoes are alive if they can both stand upright and fly in a coordinated manner. Mosquitoes that are moribund or dead are classified and recorded as knocked down at 60 minutes and as dead at 24 hours. A mosquito is moribund if it cannot stand (e.g. has one or two legs), cannot fly in a coordinated manner or takes off briefly but falls immediately. A mosquito is dead if it is immobile, cannot stand or shows no signs of life.

3. Materials & Equipment

a. Preparation of test room
    i. Paper cups

ii. Marker pen
iii. 10% bleach
iv. Bench guard
v. Masking tape
vi. Humidifier
vii. Heater

b. Pre-exposure period
    i. Aspirator
    ii. Incubator (optional)
    iii. Clock/ Timer
    iv. Environmental conditions log sheet

c. Preparation for assay
    i. Gloves
    ii. Lab coat
    iii. Untreated net pieces
    iv. Rubber bands
    v. Marker pen/label
    vi. Aspirators
    vii. Acrylic frame
    viii. Stopwatch/timer
    ix. Masking tape
    x. Cup racks
    xi. Aluminum foil
    xii. Cone plug (plastic material required to reduce mosquito resting behaviour)
    xiii. Environmental conditions log sheet

d. Exposure
    i. 10% Glucose soaked cotton wool

e. Post- exposure
    i. +4°C refrigerator

ii. Decontamination spray (according to protocol, active ingredient)
iii. Biohazard bag

4. Procedure

1. Preparation of test room and materials

a. Label paper cups with the protocol code, date of exposure, test item, exposure length, and replicate number
b. Ensure the cones, aspirators, frame and acrylic cone holding panel have all been cleaned in 10% bleach and rinsed twice with clean water.
c. Place a clean bench guard on top of the bench, and fix with masking tape.
d. An hour before the acclimation period, switch on the humidifier and heater as necessary to reach conditions of 27 ± 2°C and 80 ± 20% RH.

2. Acclimation (pre-exposure) period

a. Ensure testing occurs during the insectaries dark cycle to mimic the mosquitoes scotophase. Remove the glucose-soaked cotton wool from the mosquito cage one hour before starting all testing process. If blood feeding is required prior to the test (for investigation of e.g., fertility and fecundity) the time between blood feeding and initiation of the test should be within 6 hours and documented.
b. Remove or reduce if possible, males from the cage to be used for testing.
c. Transfer the cage of mosquitoes to be tested to the test room. The light cycle of the test room, insectaries and holding rooms must be the same. Acclimation can be either on the test bench or in the incubator depending on protocol specification.
d. Aspirate exactly five, 3-5 day old, nulliparous female mosquitoes into each paper cup. Choose mosquitoes that are fit, appropriately sized, and able to fly consistently. Do not choose mosquitoes that are small, missing one leg or wings, or that are unable to fly in a coordinated manner.

e. Allow mosquitoes to acclimatize for one hour. Record the temperature, humidity, logger ID number, and time acclimation started on the form.

3. Test material sample preparation

For preparation of net sampes use the following steps:

a. Place the net sample in a fume hood.
b. Test samples should be prepared as either 25 x 25cm or 30 x 30cm depending on the study. Use a plastic ruler to measure out the size of netting and draw the square(s) with a marker pen. Note: Netting samples should be cut from a panel part of the netting (i.e. not with any seam or attachment) unless stated in the protocol.
c. Use scissors to cut out the marked netting sample(s)
d. If the netting has been pre-cut for testing preparation, it must be wrapped appropriately in foil and stored according to the relevant risk assessment.
e. When cutting the test material, ensure that the material is not being stretched or compressed. Ensure that all prepared samples are labelled and stored appropriately in foil.

4. Preparation for the cone bioassay (The setup of the test is done during the acclimation period)

a. Put on gloves and lab coat
b. Cover the paper cups with the untreated net
c. Label paper cups (or disposable cups) with the protocol code, date of exposure, test item, exposure length, and replicate number.
d. Prepare the frames on the bench. Ensure there is one control frame.

e. Ensure the equipment is prepared and clean: Aspirators (1 for each active ingredient [AI], 1 for control), frame, stopwatch/timer, marker pens, masking tape, cup racks.
f. Net pieces to be tested should be packed individually in foil. Refrigerated samples must be allowed to reach room temperature before testing.
g. Prepare all the net pieces. Starting with the control, unwrap the net piece from the foil and position the net piece on the frame. Tape the net in place or use bulldog clips to hold it in place, ensuring that the test material is not being stretched or compressed Label the area on the frame with the corresponding net piece ID or protocol ID.
h. Set up the frame at 45-60° using a raised platform. Ensure that the angle is kept consistent throughout the study.
i. Plug the cone with plastic bung.
j. Record the temperature, humidity, logger ID, and time acclimation period ended on the form.

5. Exposure

a. Aspirate exactly five female mosquitoes from a cup into the negative control cone and quickly plug the cone with the plastic bung or cotton wool. Ensure that correctly labelled aspirator is used to avoid cross-contamination.
b. Once all of mosquitoes are in the first cone, start the timer.
c. Wait for one minute and aspirate exactly five mosquitoes into the next cone.
d. Repeat this procedure until mosquitoes have been introduced into all cones, changing gloves and aspirator if exposing mosquitoes to different treatments.
e. Three minutes after mosquitoes have been introduced into the first cone, aspirate the mosquitoes back into the labelled paper cup (or disposable cup).
f. Write the time at which the exposure was ended for each cone and read the knock down 60 minutes later (see section 6 for knockdown assessment criteria).
g. Continue until all the samples have been tested

h. Place glucose-soaked wool on top of the cups after recording knockdown and place in racks.

6. Post Exposure

a. Return net pieces to corresponding aluminium foil, wrap and put in the +4°C refrigerator or designated storage following testing.
b. Decontaminate all insecticide-contaminated material according to the relevant SOP.
c. Dispose of bench guard in a biohazard bag.

7.Recording knock down and mortality

a. Blood fed mosquitoes should not be used in the measurement of mortality (blood feeding prior to the cone test will reduce the observed mortality).
b. Knock down. Mosquitoes are scored as being alive if they can both stand upright and fly in a coordinated manner. Mosquitoes that are moribund or dead are classified and recorded as knocked down at 60 minutes.
c. Mortality. Mosquitoes that are moribund or dead are classified and recorded as dead at 24 hours (or later time point for treatments giving delayed mortality).
d. A mosquito is moribund if it cannot stand (e.g. has one or two legs), cannot fly in a coordinated manner or takes off briefly but falls immediately. A mosquito is dead if it is immobile, cannot stand or shows no sign of life.

8.Collection and reporting of data

Data analysis

a. If knockdown and mortality in the negative control is < 5%

$$\text{KD60 (\%)} = \frac{\text{Total number of knocked down mosquitoes}}{\text{Total number of mosquitoes tested}} \times 100$$

$$\text{Observed mortality (\%)} = \frac{\text{Total number of dead mosquitoes}}{\text{Total number of mosquitoes tested}} \times 100$$

If knockdown and mortality in the negative control is >5% but <10% correct using Abbott's formula. Control mortalities of <5% require no correction.

Abbott's formula:

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

Control mortality must not exceed 10% after 24 hours and 20% after prolonged holding periods. The results must be discarded in this instance.

b. Ensure the following data is recorded in the data collection sheets.
    a. Date of exposure
    b. Protocol code
    c. Test item ID
    d. Test system species and strain
    e. Age of mosquito
    f. Abdominal status
    g. Exposure conditions
        i. Day or night
        ii. Time length of test
    h. Outcome measures
        i. Knock down time
        ii. Mortlity time
    i. Environmental conditions for
        i. Acclimation
            1. Start and End
                a. Data logger ID number

b. Temperature (C)
c. Humidity (%)
d. Time
e. Initials of staff

ii. Exposure
1. Start and End
a. Data logger ID number
b. Temperature (C)
c. Humidity (%)
d. Time
e. Initials of staff

iii. Post-exposure holding
1. 60 minutes, 24 hours, or an extended holding time
a. Data logger ID number
b. Temperature (C)
c. Humidity (%)
d. Time
e. Initials of staff

j. Scoring
i. Test item code
ii. Start time of exposure
iii. Insecticide code
iv. Replicate number
v. Number of mosquitoes tested
vi. 60 minute KD
vii. 24 hour mortality (or extended if needed)

k. Study Director signature and date

5. Glossary of terms

AI	Active ingredient
KD	Knock down
LLIN	Long-lasting insecticidal net
RH	Relative humidity
SOP	Standard Operating Procedure
WHO	World Health Organization
WHOPES	World Health Organization Pesticide Evaluation Scheme

6. References

Owusu, H. F., & Müller, P. (2016). How important is the angle of tilt in the WHO cone bioassay? *Malaria Journal*, *15*(1), 1–10. https://doi.org/10.1186/S12936-016-1303-9/FIGURES/6

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