



SOP: Intrinsic Toxicity Testing

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Timeline

Version	Date	Reviewed by	Institution
1	12/07/23	Katherine Gleave	LSTM, I2I
2			

Version Control¹

Version	Date	Updated by	Description of update(s)
2	04/07/23	Annabel Murphy	Updated: Format and structure under sub-headings and footnotes. Added glossary of terms.

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

Related documents

- I2I Best Practice SOP Library, 30 October 2020
(<https://innovationtoimpact.org/>)
- The performance of Topically Applied Bioassays LITSOP056.
- Waste Disposal Procedure LITSOP008

1. Purpose

The objective of this test is to determine the intrinsic activity of the insecticide. This is done via topical application to the mosquito to isolate toxicity from confounding effects resulting from insect behavior or variable dosing.

2. Background

A minimum of five concentrations are required covering a range of mortality from 10% to 90%. Fifty insecticide susceptible, non-blood-fed, 2–5-day-old female mosquitoes are treated at each concentration and 50 treated with acetone alone which serve as negative controls. A minimum of three replications from separate cohorts are required for each test concentration (5 doses x 1 control x 50 mosquitoes and three replicates = 900 mosquitoes). Log Dose Probit analysis is then used to determine the Lethal Dosage (LD) of the experimental insecticide for 50% and 90% mortality (LD₅₀ and LD₉₀).

A batch of mosquitoes (the number of mosquitoes being dependent on the aptitude of the staff member) is anaesthetized with CO₂ for 15–30 seconds and placed on a plate cooled to 4 °C to maintain anesthesia. A volume of 0.1µl of insecticide solution is deposited on the pronotum. After dosing, the mosquitoes are returned to the clean holding cups, provided with 10% sugar solution on cotton wool and held for 24 hours at 27 ± 2 °C temperature and 80 ± 10% relative humidity (RH). Temperate species may have different environmental requirements.

Knock down of mosquitoes is assessed at 60 min, and mortality is assessed at 24 hours. A mosquito is recorded as being alive if it can both stand upright and fly in a coordinated manner. A mosquito is recorded as being 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 recorded as being dead if it is immobile, cannot stand or shows no signs of life. If control mortality is 10%, the results for that day are considered invalid and should be discarded.

Alongside the blank control the testing of a positive control, of a standard insecticide, is encouraged. The studies are performed against well-characterized laboratory reared mosquitoes. Where possible, all three mosquito genera (*Anopheles*, *Aedes* and *Culex*) should be tested. Finding the correct dose range can be difficult. Therefore, it is recommended to start with a lesser number of test mosquitoes and a large series of doses to find the appropriate range.

Solutions for topical applications are prepared by dissolving technical grade insecticide in acetone, a highly volatile organic solvent, which has the advantage of remaining on the insect cuticle for only a short period of time. The doses used in topical application are typically expressed in nanograms of active ingredient per mg of body weight of live mosquito.

3. Materials and equipment

3.1. General.

- A minimum of 900 non-blood-fed, 2–5-day-old female mosquitoes
- Chill table
- Micro Pipettes
- Paper towels and or filter papers
- Forceps
- Aspirator
- Holding cups, labels, and pens
- Cotton wool pads, 10% sugar water

- Scales, dilution glass wear, active ingredient, and diluent
- Amber vials
- CO₂, a sealed box with a sealed pipe entry from a CO₂ canister or dry ice²
- Gloves, lab coat, protective glasses
- Bench cover
- Magnifying light

4. Procedure

4.1. Preparation of test system

- Calculate average live weight of the target species by anesthetizing and weighing 50 non-blood fed susceptible female mosquitoes.
- Label holding cups to clearly identify each experimental compound, dose, species, and replicate.
- Aspirate mosquitoes into the labeled paper holding cups. The number per cup depends on how many the operator can treat in one run. Catch up an appropriate number of cages to provide the required 50 mosquitoes per dose.
- Choose female mosquitoes that are fit, appropriately sized, and able to fly consistently. (Parameters for the assessment of mosquito fitness (weight, wing length) must be defined by each test facility based on their knowledge of the species/strains of mosquito they will use for laboratory

² A separator in the box provides a uniform distribution of CO₂ and protection from freezing

studies. These parameters, and the method in which they will be assessed, must be described in a SOP.)

- Put cups in a humidity and temperature-controlled holding room set at $27 \pm 2^\circ \text{C}$ temperature and $80 \pm 10\%$ RH until the test room and materials are prepared. Place a clean bench guard on top of the bench.

4.2. Dilutions

- Put on lab coat, gloves and protective glasses. If possible, work in a fume cupboard- turn on air.
- Prepare the solutions and place each dose in a labeled amber glass container with a chemically resistant lid. Some compounds can be sensitive to light (amber glass) and acetone is highly volatile, so the lid reduces evaporative loss of the diluent.

4.3. Exposure

- Ensure the equipment is prepared and clean and put on personal protective equipment (PPE).
- Turn on the chill table and allow it to cool to 4°C . Have paper towels or filter paper available to keep moisture off the surface of the chill table: the mosquitoes will stick to the surface if it is not kept dry. In addition, the towel will reduce contamination of the chill table surface; change with every treatment or when wet (Figure 1.)



Figure 1: Chill table with magnifier

- Prepare the knockdown box: either connect the CO₂ canister to the box or place the dry ice inside an ice box. Place perforated separator to allow the CO₂ to be distributed evenly to the holding cups and to protect mosquitoes from freezing on the dry ice (Figure 2.)



Figure 2: Cooler separator

- A constant volume of 0.1 μ l should be delivered to the mosquito pronotum using a calibrated pipettor. The chemical should be applied with the lowest dose first and proceed with increasing concentrations. Change the pipette tip with each treatment (Figure 3.)



Figure 3: Dose being directly applied to mosquito using capillary action micropipette.

- Control cages should be exposed to acetone alone at the beginning of each dose range treatment.
- Once dosed, the mosquitoes are returned to the original labeled holding cup, provided with 10% sugar solution on cotton wool and held for 24 hours at 27 ± 2° C temperature and 80 ± 10% RH.

4.4. Post Exposure

- Decontaminate all insecticide-contaminated material according to the specific active ingredient decontamination instructions.
- Store or dispose of the chemicals as per the instructions from the manufacturer. Refer to waste disposal procedure LITSOP008 for safe disposal of waste.

4.5. Recording knock down and mortality

- Record knockdown 60 minutes after treatment application. A mosquito is classified as knocked down if it cannot stand (e.g., has one or two legs), lies on its back moving legs and wings but unable to take off, cannot fly in a coordinated manner or takes off briefly but falls immediately.
- Record mortality 24 hours after exposure. A mosquito is classified as dead if it is immobile, cannot stand or shows no signs of life. If control mortality is > 10%, the test must be repeated.

5. Additional data collection

Record time of testing.

6. Deviations from standard protocol

All deviations from the standard protocol should be noted in data collection sheets.

7. Glossary of terms

CO ₂	Carbon Dioxide
LD	Lethal dose
LD ₅₀	Lethal Concentration 50%
LD ₉₀	Lethal Concentration 90%
RH	Relative Humidity
PPE	Personal Protective Equipment
SOP	Standard Operating Procedure
µl	Microlitre

8. References