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# Topical Application of Insecticidal Active Ingredients

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The development of new insecticidal formulations is important to overcome the burgeoning hurdle of insecticide resistance in field populations of mosquitoes and other pest arthropods. In order to standardize the testing of current and future small molecule chemical agents as insecticides, specific protocols should be shared, broadly endorsed, and certified among public health entomologists to decrease the variability in toxicological endpoints observed between laboratories. As variability in toxicological data can result due to differences among sub-populations acquired from the field, experimenter, environment, etc., it is paramount to diminish the effect of factors that contribute to variability in scientific protocols, whenever possible. We propose the utilization of a single topical application protocol that will aid in the characterization of both current and future (in development) insecticidal active compounds. This protocol may be utilized to calculate the theoretical dose required to kill a specific percentage of a pest population (e. g. LD<sub>25</sub>, LD<sub>50</sub>, LD<sub>95</sub>) and to compare the toxicity of insecticides in a given pest population or sub-sample. Moreover, it may allow for the evaluation of insecticide resistance to be quantified compared to a given susceptible strain. The adoption of this protocol by numerous testing laboratories should decrease the variability in data observed between different researchers and increase the translatability of toxicological characterizations within the scientific community.

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Norris, E. J., A. D. Gross, B. M. Dunphy, S. Bessette, L. C. Bartholomay, J. R. Coats. Comparison of the insecticidal characteristics of commercially available plant essetnail oils against *Aedes aegypti* and *Anopheles gambiae* (Diptera: Culicidae). *J. Med. Entomol.* 52: 993-1002.

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In order to analyze toxicological dose-response data and obtain theoretical dose-response statistics, it is recommended that you choose 5 concentrations of your insecticidal agent that consistently produce between 10-90% mortality at 24 hr. This will allow for the PROBIT model to reliably and accurately calculate an LD<sub>50</sub> value. This is the most informative value for comparing insecticidal efficacy of different compounds and dose-level for calculating resistance ratios between various strains. These concentrations can be found by making a variety of doses (range-finding doses) and applying them to an initial set of mosquitoes. The low or high mortality produced by these formulations can be updated accordingly to produce toxicologically informative concentrations.

### MATERIALS

 **Acetone** Contributed by users

 **10 uL Gastight Syringe** Sigma

**Aldrich Catalog #20972**

 **Repeating Dispenser** Sigma

**Aldrich Catalog #20943**

 **Featherweight**

**forceps** BioQuip Catalog #4750

Insecticidal active ingredients can be highly toxic to laboratory personnel, household pets, and insect colonies. Handle these agents with caution and refer to the Safety Data Sheet (SDS) for any special working procedures. Organic solvents can also be dangerous if inhaled, or in contact with skin or eyes. Proper laboratory attire should be worn. These include, at a minimum, safety goggles, close-toed shoes, and nitrile lab gloves. A lab coat is recommended for particularly toxic substances in the event of

spills. Removing gloves, lab coats, etc. after working with insecticides will prevent cross contamination of insect rearing spaces, personnel, and personal spaces.

It is important to determine that your insecticidal active is soluble in the solvent you wish to apply as a vehicle. Acetone is recommended as it is relatively non-toxic at the concentrations applied in this protocol for a large number of model insects. If the insecticidal active ingredient is not soluble in acetone, other solvents must be explored. These may include, but are not limited to ethanol, methanol, ethyl acetate, DMSO, and water. Based on the physical and chemical properties of the insecticidal active ingredients, other solvents may be needed.

Check to see that your solvent choice is not considerably toxic to the mosquito species you will utilize in your protocol. High toxicity in your vehicle control will make analysis difficult.

### Anesthetize Mosquitoes

- 1 Take a container of all female mosquitoes and gently introduce carbon dioxide gas to anesthetize individuals. From a gas cylinder equipped with a regulator and a hose, slowly increase the rate of flowing CO<sub>2</sub> to a gentle stream. Flow CO<sub>2</sub> into container with mosquitoes (cover hand over container to ensure CO<sub>2</sub> concentration builds within container) for 30 seconds

### Transfer Anesthetized Mosquitoes

- 2 Place ice in another container and place two, 90-mm glass petri dish bottoms on ice. On the bottom surface of the petri dishes, place 2 90-mm filter papers to prevent the build up of excess condensation, as condensation may adversely affect some species of mosquito (*Anopheles* spp. are particularly sensitive). Carefully transfer anesthetized mosquitoes into petri dishes on ice. The ice will prevent mosquitoes from reanimating. Make sure to transfer less than 200 mosquitoes to the petri dish at a given time. This will allow you to complete testing on each mosquito and prevent each individual from remaining on the ice for too long. Mosquitoes left on ice for too long may not reanimate.

### Apply Insecticide Topically

- 3 Individually pick up each mosquito by the hind legs with featherweight forceps (Bioquip) and carefully apply 0.2 µL of insecticidal solution to the pronotum of each individual mosquito. This corresponds to one pump of the repeating dispenser. It is good to make sure the repeating dispenser is dispensing by pumping and tapping the tip of the syringe to a kimwipe to detect a solvent droplet. For each treatment group (i.e. insecticide concentration), treat a total of 25 mosquitoes and move treated mosquitoes to the next petri dish on ice. Transferring mosquitoes to the final observation container too soon may cause them to reanimate quickly and escape. Also, transferring mosquitoes quickly to a observation container may cause mosquitoes to stick to the cup, if the insecticidal agent is oily. The subsequent transfer to a secondary petri dish will prevent these issues from arising. After all 25 mosquitoes are treated with your insecticide concentration of choice, quickly transfer them to the observation

container. The observation container should be a small deli cup (8-ounce deli cup) with tulle placed over the top to allow adequate ventilation.

#### Maintain Mosquitoes in Incubator Overnight

- 4 Move mosquitoes (now in observation container) into an incubator that can maintain 28 C with approximately 80% relative humidity. Provide cotton pad moistened with 10% sucrose water to prevent mosquitoes from desiccating.

#### Record Knockdown and Mortality

- 5 Record percentage knockdown at 1 hr and percentage mortality at 24 hr. Toxicity endpoints may be changed to appropriately characterize insecticidal efficacy if necessary (e.g. 2-hr knockdown, 48-hr mortality). A minimum of 3 biological cohorts should be included in the data set (mosquitoes from different rearing groups) before analysis. Mortality should be defined as ataxia (inability to move) after prodding each individual with a camelhair brush. Knockdown is defined as the inability to fly or orient in the upright direction.

#### Run analysis using SAS

- 6 SAS code is featured below (be sure to modify the data to match your dose-response data):

##### **PROBIT ANALYSIS (for variable amount of insects per replicate)**

# "Enter data in Italicized section as **concentration\_total insect number\_number dead**"  
# 'Underscore (\_) corresponds to space'  
# 'Be sure to remove italicized data before copying into SAS software, as this font style may adversely affect the software analysis. It is used here to communicate where to enter data.'  
# This code may be used to calculate a  $KD_{50}$  using knockdown percentages instead of mortality.

```
data mgk;  
input dose number res;  
cards;  
0.0001 30 29  
0.00005 30 24  
0.00002 30 5  
0.00001 30 2  
0.000002 30 0  
0 30 0  
;  
proc probit data=mgk log10 optc plots=(all);  
model res/number=dose/inversecl lackfit;  
output out=new p=p_hat;  
run;  
ods graphics on;  
quit;
```