

Materials Supplied in Kit

This kit contains enough supplies for 24 replicates, but most materials are reusable, if caution is used to avoid contamination. The only exception are the wax-lined cups, which can be purchased at local grocery stores or pharmacies.

<i>Item</i>	<i>Number included in kit</i>
3 ml Plastic Pipettes	5
Wax-Lined Paper Cups (88 ml / 3 oz)	24
Soup Cups (16 oz)	24
Rubber Bands	24
Fine Mesh Fabric	24
Fish Food	1 Falcon Tube
Falcon Tubes (for measuring water)	4
Mason Jar 32 oz (for collecting water)	2
Bottles of Water 20 oz	2

B. Specimens

150 4th instar larvae per site (minimum, testing more improves accuracy)

Collecting Water from Target Sites

- 1) Prior to your larvicide application (preferably the day before), collect water from the target area using one of the **Mason Jars**. Label the jar 'control'.
- 2) Store the jar of pre-treatment water in a cool dark place or refrigerator. If water is stored in a refrigerator, make sure to allow it at least 2 hours to come back to room temperature before running your bioassay.
- 3) Following your larvicide application, use the second **Mason Jar** to collect water from the target area. Label this jar as 'treated water'.
 - a. METHOPRENE: Treated water should be collected immediately after the field application and transferred directly to bioassay cups as quickly as possible. No longer than 6 hours between water collection and the bioassay.

- b. *Bti* / *L. sphaericus*: Water can be collected and stored for up to 48 hours in a refrigerator. If water is stored in a refrigerator, make sure to allow it at least 2 hours to come back to room temperature before running your bioassay.

Rearing Mosquitoes

* Please refer to our *Aedes albopictus* and *Culex pipiens* collection kit guideline for instructions for collecting mosquitoes. These guidelines are posted on our website (<https://www.neregionalvectorcenter.com/resistance>).

- 1) Several days before the planned trials, collect specimens from the field.
 - a. *Cx. pipiens*: When collecting egg rafts, plan ahead to ensure that they are 3rd and 4th instar at the time of your application. You will want to collect egg rafts approximately 7 – 10 days before you want to conduct your bioassay. The timeframe will depend on the temperature of the room where you conduct the trials.
 - b. *Ae. albopictus*: Collect egg sheets ahead of time. These can be stored for up to 1 month before hatching (see *Ae. albopictus* guidelines). Hatch the eggs approximately 7 -to- 10 days before your planned larvicide application. The timeframe will depend on the temperature of the room where you conduct the trials.
- 2) Once eggs hatch, transfer larvae into **water bottles**, with ¼ of the water poured out. Add two **fish food** pellets to each bottle. Monitor the bottles over the next 4 -to- 5 days and add another pellet if there doesn't appear to be enough food in the water.
- 3) Once larvae are 3rd or 4th instar, they are ready to use in efficacy bioassays. If you see pupae in the bottle you should use the larvae immediately.
- 4) If you need to slow development to match your timeline, you can put the larvae in a cold room or adjust the room temperature.

Efficacy Bioassay Procedure*

* *The minimum number of containers needed for a sufficient sample size when testing a site for resistance is 10, which should contain treated water. An additional four containers will act as controls, two with the water collected from the field prior to the larvicide deployment in the field and two with water from water bottles.*

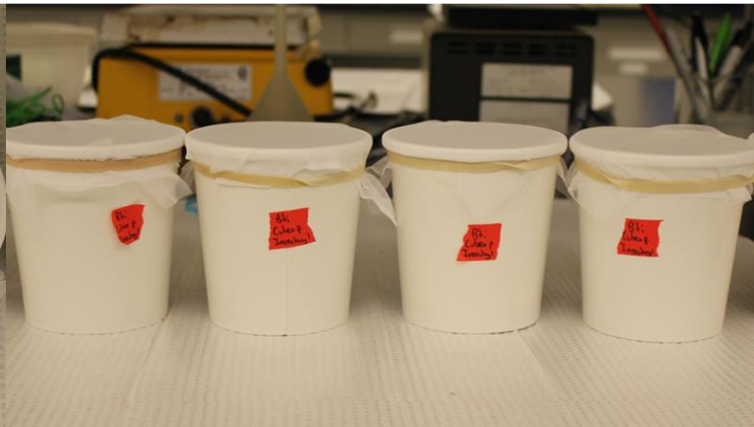
- 1) Clear a working area of approximately 1.5 ft² and lay out 10 of the **wax-lined paper cups** to be used for the trial. If water from the field has been stored in a refrigerator take it out so it can come to room temperature.

- 2) Add a quarter of a **fish food** pellet into each small wax-lined paper cup. In order to measure out a quarter of a pellet, crush it and then divide the powder into four roughly equal portions.
- 3) Place one small wax cup with fish food inside each of the **soup cups**. Label two containers as the ‘field controls’, two as ‘bottle water controls’, and the others with the pesticide that was deployed. Also include the species being tested and any other relevant information (e.g. site name / date) on the label.
- 4) For the field control cups, use one of the **3 ml plastic pipettes** to count 15 mosquito larvae into one of the **Falcon Tubes**. Once the larvae have been counted, fill the falcon tube to the 45 ml line with the pre-treatment water from the field. Pour the water and larvae into the smaller cup and then add an additional 30 ml to cup so that they each contain a total of 75 ml of water. Do the same for the two bottled water control cups.
- 5) For the treatment cups, use another **3 ml plastic pipettes** to count 15 mosquito larvae into another the **Falcon Tube**. Follow the same procedure described above until all cups contain water and larvae.)
- 6) Use the **rubber bands** to attach the **fine mesh fabric** to the top of the soup cups to prevent adults from escaping. Place the containers on a table away from sunlight in a temperature-controlled room.

(A)



(B)



(A) The cups should be covered in mesh (B) by carefully applying a rubber band. Be sure to cover the top completely so no adults can escape, particularly for the methoprene trials

- 7) Collecting data
 - a. **METHOPRENE**: Check the containers every 24 hours for the next 6 - 9 days and record the number of adults that have emerged in each container. Continue to monitor the trial until no more living pupae remain. If >10% of adults have emerged this may indicate an issue with field efficacy. To determine if your population is resistant you are encouraged to submit specimens to the NEVBD

Pesticide Resistance Monitoring Program for testing
(<https://www.neregionalvectorcenter.com/resistance>).

- b. *Bti* / *L. sphaericus* **TRIALS**: Count and record the number of larvae alive after 24 hours. If >10% of larvae are alive this may indicate a control issue in the field. To determine if your population is resistant you are encouraged to submit specimens to the NEVBD Pesticide Resistance Monitoring Program for testing (<https://www.neregionalvectorcenter.com/resistance>).
- 8) After the trial is over, discard the small cups, the rest of the materials can be reused. Be sure to wash the **mason jars** thoroughly. Rinse with soap and water at least three times and allow to dry for 48 hours. Before rinsing wash with acetone, if available.
- 9) If mortality in the control cup is > 20% discard this trial and start again.