**DETAILED PROTOCOL:**

1. **Construct and Install Dual-Choice Chamber** (Figures 1, 2, 3)
2. Don a pair of nitrile gloves, a lab coat, and eye protection when handling mosquitoes at the assay station, throughout the chamber preparation and installation process, and duration of the assay.
3. Obtain metal gates (Figure 2A) and undo the screws to remove the shutter, spray paint shutter and interior rings white to reduce visual contrast and to provide a sealed surface to clean (Figure 2B).
4. Cut an 11 cm strip of non-hook side of Velcro and place vertically on the shutter side of the gate and hold in place with screws when the gate is reassembled (to prevent loss of small insects when the gate is opened).
5. Take an acrylic collar and fit over the outer ring of the gate, adding sections of aluminum tape (cut into 1 cm wide strips) so that the collar fits snuggly (Figure 2 C, D).
6. Take a glass tube and place in the other opening of the acrylic collar and add sections of aluminum tape so that the tube fits snuggly in the collar.
7. Prepare wire screen inserts by cutting a section of screen that is 16 x 22 cm. These screens will prevent direct contact by mosquitoes in the lumen of the tube to contact the test compounds placed outside the screen. **NOTE:** Wrap screen around the PVC tube and pinch so that the ends stand up and pull tightly and bend the screen to the right and left to form a fold. Using pliers, fold over to form a seam with the folded section about 1.3 cm wide. Use a hard metal edge and press the seam against the PVC tube. Slide the screen tube from PVC tube and gently pry one end wider to form a flange that will rest against the inner wall of the glass tube (Figure 2E). This will be placed on the end of the tube closest to the gate and will prevent mosquitoes from entering the space between the glass and the wire screen (Figure 2F).
8. Assemble the chamber with one glass tube (with the insect entry hole) between two gates (held in place with acrylic collars) and one glass tube (no entry hole) on each outer side of the gates (held in place with acrylic collars). Screens should be placed in the outer glass tubes such that the wider flange is on the end closest to the gate. The open ends of the glass tubes should be covered with netting held in place with elastic bands.
9. Attach strips of wiggle strips to plywood backing so that choice chambers are held in place during assays. Paint backing and strips white to provide uniform visual stimuli. Place assay chambers on supports so they are secure during testing (Figure 2G).

**2. Select** **responsive flying female mosquitoes** (Figure 3)

1. Use a draw box to collect the responsive test female mosquitoes from a cage.

 **NOTE:** The draw box is a clear acrylic box with a fan drawing air from a circular hole in the back of the box and circular port with a collar for air entry on the front of the box. An acrylic cylindrical holding tube that is screened at one end and with a screened funnel on the opposite end is placed into the circular port collar so that the screened funnel is inside the mosquito cage. A stopper attached to a wire is used to plug the funnel and capture the mosquitoes. When a hand is placed on the screened end of the holding tube, hand odors are drawn into the cage and responsive mosquitoes fly upstream.

**NOTE:** See Figure 3 for an image of the draw box with fan apparatus.

2. Place the cage with mosquitoes inside the draw box and close the door.

3. Untie the mesh sleeve of the mosquito cage and pull it through the circular port collar.

4. Place the cylindrical holding tube through the mesh sleeve of the cage with the plugged funnel opening facing inside the cage.

 4.1 Wrap a Velcro strap around the middle of the holding tube and the mesh sleeve then close the box door.

5. Turn on the fan, then gently pull out the stopper from the funnel end. Place the palm of a hand at the other end of the tube to attract flying females.

5.1 Keep hands hovering directly around the end of the tube. Wait until the required number of flying females have flown through the funnel and moved into the holding tube towards the hand.

6. Plug the funnel end of the tube with the stopper, switch off the fan, remove the tube containing mosquitoes, and tie up the mesh sleeve.

7. Empty the captured females into a temporary holding cage by unplugging the funnel and shaking them into the cage space. Bring the temporary cage to the assay station to begin preparing the chamber materials.

**3. Dual-Choice Chamber Preparation**

1. Label the outside of each chamber with a strip of tape to distinguish between the control and test chamber of each replicate (Figures 1, 2, 3).

1.1 Check that every replicate on the platform is alternating between control and test to avoid positional bias. Verify that six chambers are used per each control concentration and test concentration.

1. Close the metal gate connecting each chamber to prevent premature movement of mosquitoes when they are deposited into the system.

2.1 Using a battery powered aspirator, introduce twenty females from the temporary cage into the middle chamber of each replicate and tape the opening shut.

2.1.1. Let the mosquitoes acclimate inside the spaces for 30 min as remaining steps are prepared.

1. Treat every cotton pad underneath a chemical fume hood and allow them to air dry for 30 min.

3.1. For the control chemical, pipette 1 mL of acetone to each control cotton pad.

3.1.1. For the treatment chemical, prepare the essential oil to the desired concentrations in acetone. Pipette 1 mL/mg of the treatment chemical solution onto each cotton pad.

4. After 30 min, using forceps, add one cotton pad from step three underneath each interior screen (between the screen and the chamber).

4.1 Wrap a double layer of fine mesh netting across the open ends of the chambers and secure them in place with two elastic bands.

 5. Pack dry ice into 16 oz cardboard cartons and place one carton in front of each open end of the chamber system to act as the carbon dioxide activator.

6. Place black-out fabric over the chambers to minimize influence from laboratory lighting on mosquito movement.

**4. Dual-Choice Chamber Assay**

1. Open metal gates to initiate the assay and start a timer for 10 min.

 1.1 Record temperature and humidity data from a thermostat in the location where assay is occurring.

1. After 10 min, quickly shut every gate and remove fabric.
2. Immediately count and record the number of live mosquitoes within each control, middle, and test chamber.
3. Use a battery powered vacuum aspirator to recollect all live mosquitoes of the same treatment group from inside the chambers.

4.1 Deposit all mosquitoes of the same treatment group into a new cage to maintain for further analysis.

1. Provide the new cages with a 5-10 % sucrose solution.

5.1 Return the recovered mosquitoes to the same climate-controlled insectary rearing conditions.

**NOTE:** If there is no interest in maintaining any live mosquitoes for further analysis, they may be removed with the aspirator and discarded.

**5. Cleaning of Assay Components**

1. Don nitrile gloves and wear them when cleaning, washing, and drying the assay apparatus.

1.1 Clean the chambers and inner wire screens by coating every chamber and screen in 1-2 mL of acetone under a chemical fume hood.

1. Soak the pieces for a few minutes in hot water with detergent powder to remove lingering traces of the test compound.

2.1 Rinse each piece thoroughly under the faucet until soap is flushed out.

1. Add the chambers and screens to a gravity convection oven to be dried under heat (100 °C) overnight.
2. Spray the collars and metal gates with 70 % ethanol and set aside.

4.1 Set aside all finished pieces to be reassembled for another assay. This cleaning protocol is proven to prevent contamination in the next cohort of mosquitoes used in other Choice Chamber Assays.