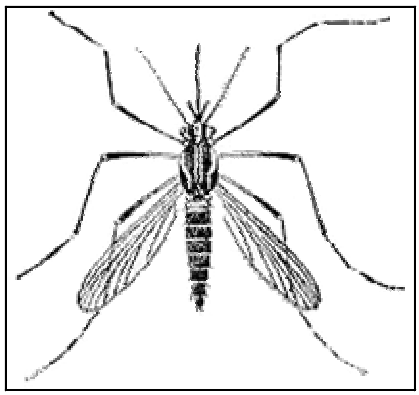
BEHAVIOR MODIFYING COMPOUNDS FOR DISEASE VECTOR CONTROL



TRAINING MANUAL
v. 4.0 February 2007

Supported by NIH Grant #: 5UO1AI05477
Developed by Nicole L. Achee

Disclaimer: This manual is intended to provide general guidelines only for conducting behavioral assays with the HTSS device. Study design details have intentionally been excluded because this will depend on several parameters to include species of mosquito being assayed and environmental testing conditions. For more information regarding the HTSS device and specific guidance for conducting CIA/SRA/TOX assays please contact Nicole L. Achee at nachee@usuhs.mil.

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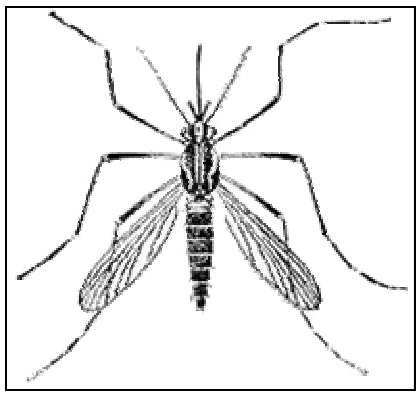

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ASSAY SCHEDULING

1. Calculate the total count of females available for assay testing from gallon containers.
2. Schedule assay testing according to the following guidelines:
    a. CIA: 10 females in each control and 10 females in each treatment per replicate. **One control chamber can be used for up to 2 treatment chambers for each replicate**.
    b. SRA: 20 females for each replicate.
    c. TOX: 20 females in each control and 20 females in each treatment per replicate. **One control chamber can be used for up to 2 treatment chambers for each replicate**.
3. After developing the weekly schedule, transfer daily schedules onto a calendar to be kept within the assay testing area. This schedule should include the number of replicates required for each type of assay (CIA, SRA, TOX) according to specific chemical and concentration. The configuration for testing (i.e. number of control chambers vs. treatment chambers) is designated using a coding system.

Coding Example: 1T1C- one Ttreatment chamber and one Control chamber.
2T1C- two Treatment chambers and one Control chamber.

Schedule Example:

Monday	Tuesday	Wednesday	Thursday	Friday
CIA: 0.25 Deet 3 Reps (1TIC)	CIA: 2.5 Deet 6 Reps (2T1C)	CIA: 25 Deet 6 Reps (1T1C)	CIA: 250 Deet 4 Reps (2T1C)	CIA: 500 Deet 5 Reps (1T1C)
SRA: 0.25 Deet 9 Reps (1T1C)	SRA: 2.5 Deet 6 Reps (2T2C)	SRA: 25 Deet 9 Reps (1T1C)	SRA: 250 Deet 7 Reps (2T2C)	SRA: 500 DDT 9 (20's) (1T1C)
TOX: 0.25 Deet 3 Reps (1T1C)	TOX: 2.5 Deet 3 Reps (2T1C)	TOX: 25 Deet 6 Reps (1T1C)	TOX: 250 Deet 5 Reps (2T1C)	TOX: 500 Deet 4 Reps (1T1C)

4. After developing the weekly schedule, write daily requirements of females by groupings of 10's or 20's (according to pint cartons) on a white eraser board and place in insectary. This board will be used on the following Monday when sorting females for assays.

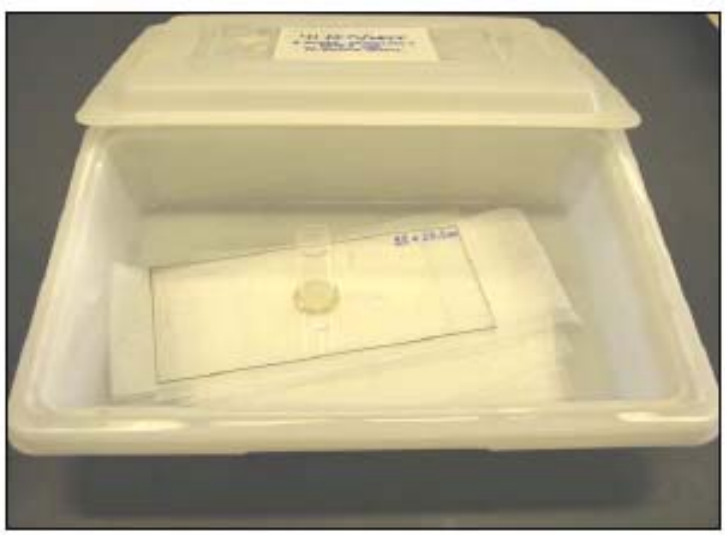
Sorting Example:

Monday	Tuesday	Wednesday	Thursday	Friday
CIA: 0.25 Deet 6 (10's)	CIA: 2.5 Deet 9 (10's)	CIA: 25 Deet 12 (10's)	CIA: 250 Deet 6 (10's)	CIA: 500 Deet 10 (10's)
SRA: 0.25 Deet 9 (20's)	SRA: 2.5 Deet 6 (20's)	SRA: 25 Deet 9 (20's)	SRA: 250 Deet 7 (20's)	SRA: 500 DDT 9 (20's)
TOX: 0.25 Deet 6 (20's)	TOX: 2.5 Deet 5 (20's)	TOX: 25 Deet 12 (20's)	TOX: 250 Deet 8 (20's)	TOX: 500 Deet 8 (20's)

PREPARATION OF NETTING STRIPS

1. Clean gloves should always be worn when handling netting *before* and after cutting into strips.
2. Clean tabletop with industrial soap and water and wipe completely dry with paper towels before netting is placed on top.
3. Use an 11 X 25 cm template and pencil to mark the outline of individual netting strips. Clean template with tap water to remove graphite residue after use.
4. Netting strips are then cut to size using designated scissors *(these scissors should not be used for other activities).*
5. Cut netting strips should then be immediately placed into a sealed container until used in assay testing.
6. Netting that has not been cut should be folded neatly and placed back into a sealed storage bin along with the template and scissors.

Tabletops should be wiped clean and new gloves worn before handling netting. A template made from Plexiglas can used to outline netting strips prior to cutting. Be sure to wash the template with soap and water after use to remove graphite residue from the pencil.



Cut netting strips should be stored in a sealed container until used in the assays.

GENERAL HTSS DEVICE DEFINITIONS

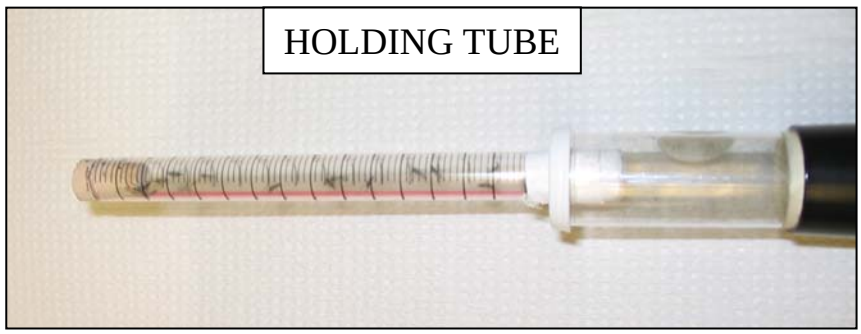
Netting: Material that is treated with either test chemicals or solvents alone.
Test Chamber: Metal compartment that is configured as either a "Treatment" or "Control" chamber based on the presence of treated or control netting.
Clear Chamber: Clear plastic compartment that contains no netting.
Chamber Unit: Complete HTSS device containing at least one test and one clear chamber.
Release Chambers: Plexiglas chambers designed to contain female mosquitoes once released from HTSS chambers upon completion of each CIA / SRA replicate.

MOSQUITO PREPARATION

1. Prior to assay testing females should be sorted into groups of 10's (for CIA) and 20's (for SRA and TOX) based on the weekly assay schedule (*see instructions for Assay Scheduling and Insectary Activity Descriptions*).
2. On each day of assay testing, the proper pint cartons are transported from the insectary to a sorting area where specimens are mechanically aspirated from each pint carton into individual miniaturized "holding tubes" (i.e., sections of plastic tubing) and a cork is placed on the open end. Be sure to use properly labeled holding tubes (control vs. specified chemical; CIA vs. SRA etc.). **Females should be counted prior to aspiration to ensure the accurate count for data sheets.**
    a. For example: If the cardboard carton was designated as containing ten mosquitoes but there is a count of nine, then label the holding tube with the number "9" using a piece of tape (extra sorted mosquitoes can also be used to adjust for mortality or miscounts).
3. Holding tubes are then placed into corresponding organizing trays to separate control vs. individual chemical treatment CIA, SRA and TOX. Keep organizing trays in the test area in which assay testing will occur to ensure temperature and humidity acclimation.
4. Knockdown and other signs of physical distress should be evaluated periodically within holding tubes. If needed, holding tubes can be stored in an insectary until testing. If tubes are stored in an insectary, make sure to allow mosquitoes to acclimate to testing area humidity and temperature for at least 10 minutes prior to use in an assay.
5. Control holding tubes and treatment holding tubes can be reused after washing with industrial soap and water (see *ASSAY CHAMBER/HOLDING TUBE/TRAY WASHING*). **If reusing holding tubes, label each tube according to chemical tested and store in labeled sealed bags. If disposing holding tubes used for chemicals that show behavioral responses, dispose according to biohazard waste guidelines after washing with soap and water.**

MOSQUITO PREPARATION

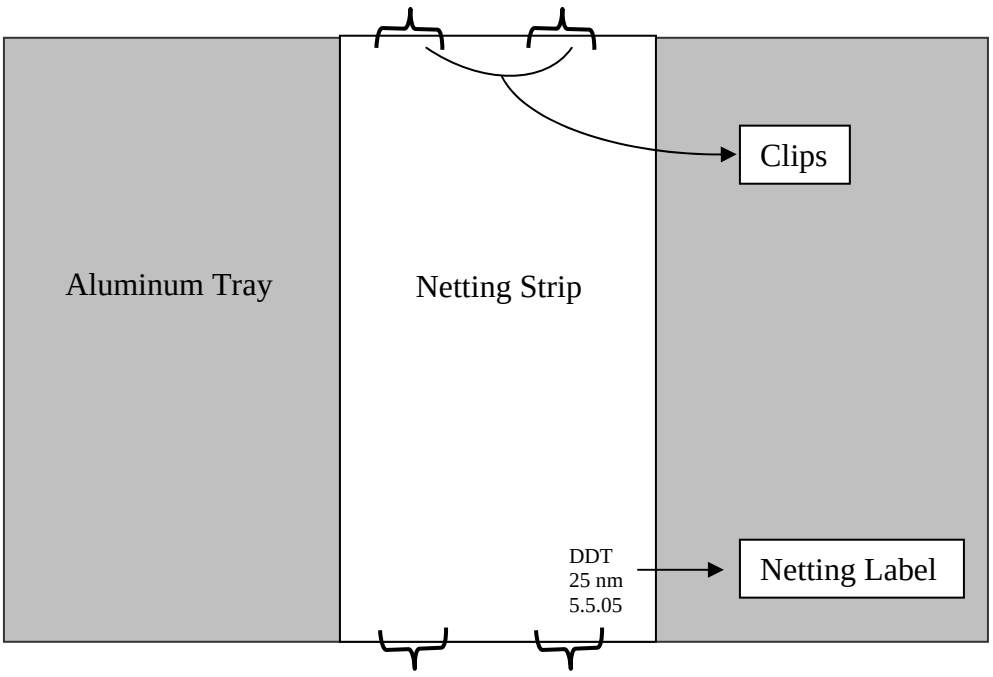
Designated mosquitoes are transferred from the insectary to a sorting area where they are recounted before mechanically aspirating into holding tubes. If the number of females is different than expected, a label is affixed to the tube to ensure proper data recording. If available, extra sorted females can also be used to adjust for miscounts or mortality.



Using individual trays, holding tubes are organized according to control vs. individual chemical treatment and CIA,SRA and TOX.

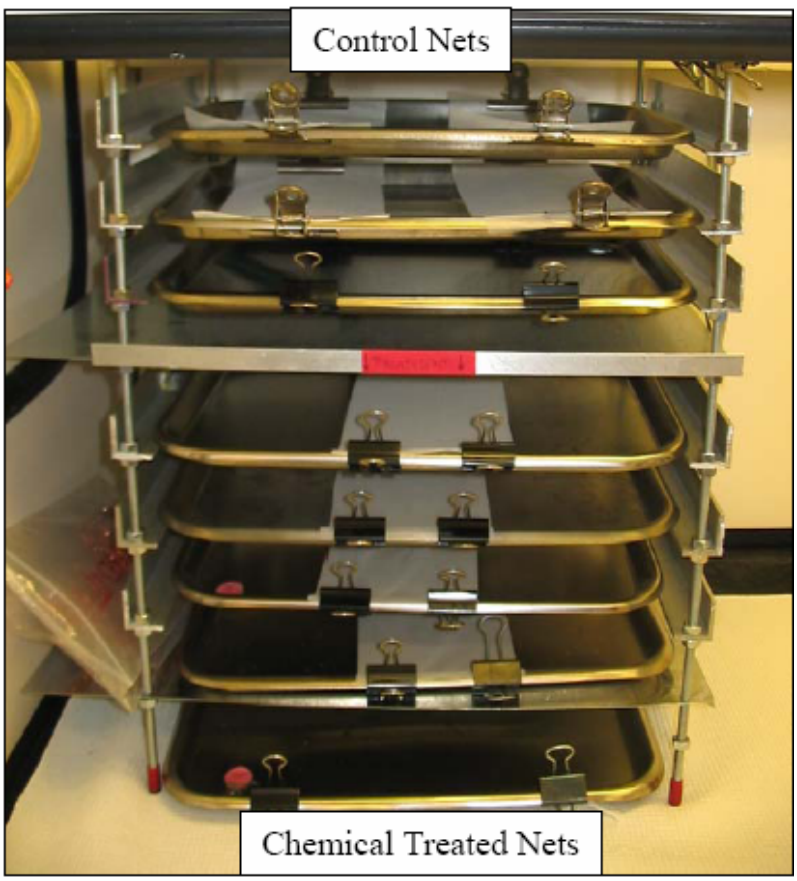
CHEMICAL APPLICATION TO NETTING

1. All netting should be treated underneath a chemical fume hood while wearing gloves and a lab coat. **Always wear gloves when handling netting *before* and after treatment.**
2. Netting strips should be labeled with chemical name/code, concentration and date treated in the lower right hand corner using a pencil prior to treatment (see diagram below).
3. After labeling, attach netting strips to aluminum trays using clips (see diagram below). Make sure that control netting is placed onto designated trays (i.e., control strips to control trays and treatment strips to treatment trays).
4. **Always treat the control netting first followed by treatment netting.** If the same chemical is being used to treat several netting strips, always start with the lowest concentration.
5. Using a 1000µl pipette, apply 1.5 ml of the proper solution to each netting strip in 0.750µl (2x) increments. Make sure the solution is spread evenly across the netting surface. **Separate pipettes should be used for control and treatment solutions.**
6. After each netting strip is treated, place the aluminum tray back on the drying rack and allow netting to dry for at least 15 minutes. Gloves should be disposed of according to biohazard protocols outlined for the testing laboratory.
7. While netting strips are drying, label metal test chambers with tape as either “control” or “treatment” including chemical name/code and concentration.
8. After 15 minutes, the dry netting is attached to the inner metal cylinder of corresponding metal test chambers using a magnet. **Always use gloves when handling netting and place control netting strips into test chambers before handling treatment netting strips. When handling treatment netting of the same chemical, start with the lowest concentration. Change gloves between handling each treatment netting when different chemicals are used**.
9. Any unused chemical should be returned to proper storage and empty bottles disposed of according to biohazard protocols outlined for the testing laboratory.
10. Trays are washed daily (see *Assay Chamber/Holding Tube/Tray Washing*).



CHEMICAL APPLICATION TO NETTING

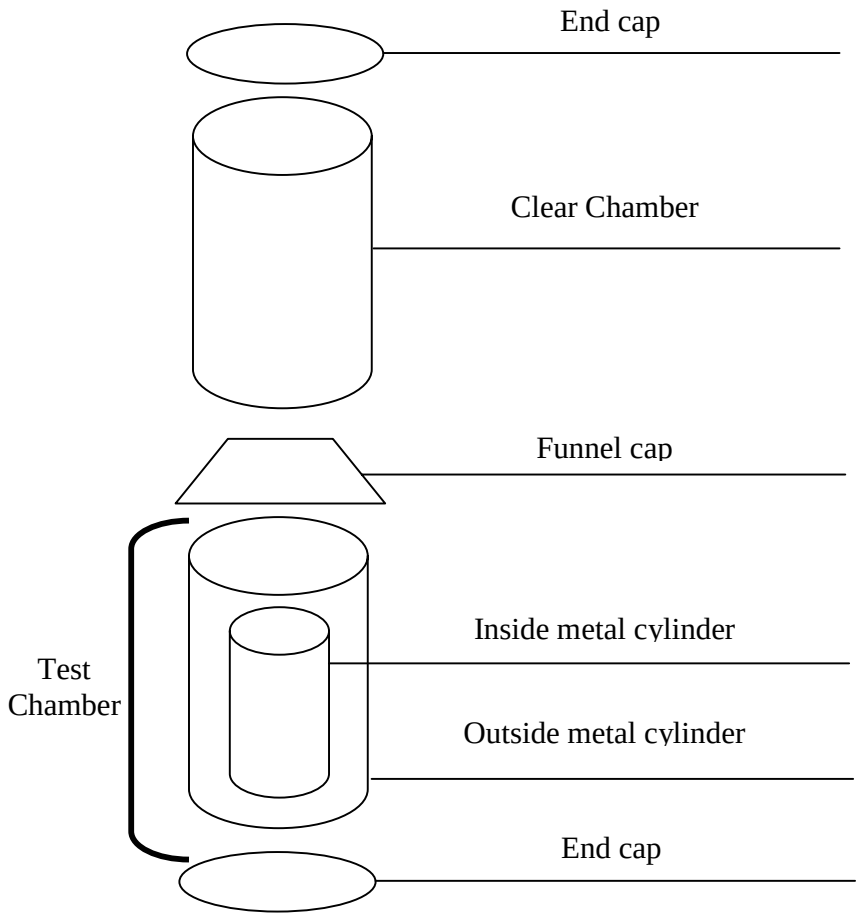
Chemical is applied to netting strips underneath a fume hood while wearing gloves and a lab coat. Gloves are worn when handling all netting *before* and after treatment. Labeled netting strips are attached to aluminum trays using clips prior to treating with chemicals. Control netting is treated first followed by treatment netting strips. Be sure to have a designated control pipette and tips and treatment pipette and tips.



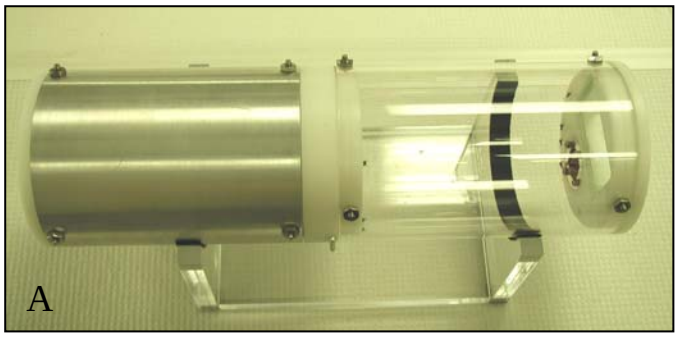
Immediately after treating each netting strip, the tray is placed on a drying rack keeping the control nets separate from those treated with chemical. After the last net has been treated, netting strips are left to dry for at least 15 min. before being used in the assays. Trays are washed daily according to standard washing protocols.

CONTACT IRRITANCY ASSAY (CIA)

1. HTSS Chamber Units are configured such that separate Treatment and Control Test Chambers are affixed to individual Clear Chambers and all Test Chamber gates are in the closed position. The flat side of funnel caps should be facing towards the Clear Chambers (see diagram below).
2. Felt is placed around each Clear Chamber as well as both viewing windows on the ends of each Chamber Unit.
3. Upon configuration completion, 10 female mosquitoes are transferred from individual holding tubes into each Test Chamber using mechanical aspirators and pressurized airflow. Check for mechanical KD due to transfer process.
4. After 30 seconds the gates of each Test Chamber are placed in the open position and a timer set for 10 minutes is immediately started.
5. After 10 minutes, the gates for each Test Chamber are placed into the closed position **(in the same direction in which they were opened)** and the number of mosquitoes within Treatment and Control Clear Chambers are recorded onto the CIA data sheet. Knockdown of females within all Test and Clear Chambers, temperature, humidity and time of day is also noted.
6. Upon completion of data recording, mosquitoes from Clear and metal Test Chambers are removed into Release Chambers and immediately killed using a vacuum water trap.
7. When all assays are completed for the day, chamber units are washed (see *ASSAY CHAMBER/HOLDING TUBE//TRAY WASHING*) and netting is stored or disposed as described in *STORAGE/DISPOSAL OF TREATED NETTING*.



CONTACT IRRITANCY ASSAY

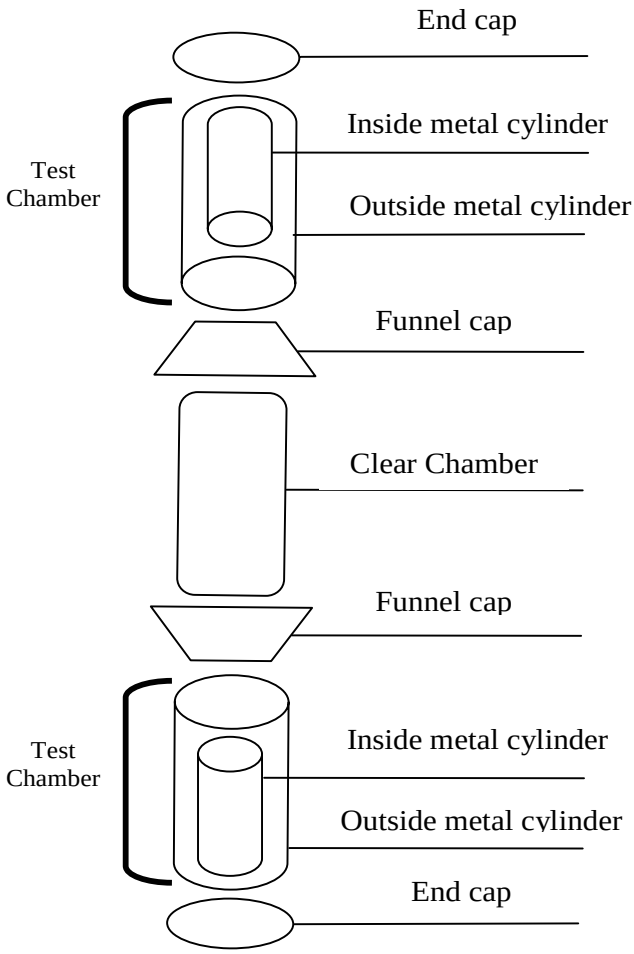


The configuration for the Contact Irritancy Assay (A) uncovered and (B) covered.

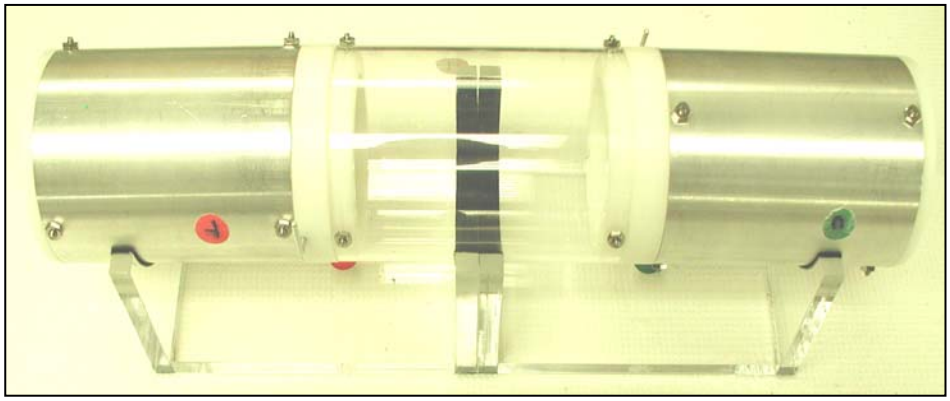
Mosquitoes are transferred from individual holding tubes into metal Test Chambers using mechanical aspirators and pressurized airflow. Once all the Test Chambers have mosquitoes, a 30 second “resting period” is initiated before the assay begins.

After each 10 minute replicate, the number of mosquitoes remaining in the Test Chambers and exiting in the Clear Chambers are recorded along with the knockdown response. When all the data is recorded, mosquitoes are removed from Clear and metal Test Chambers into Release Chambers.

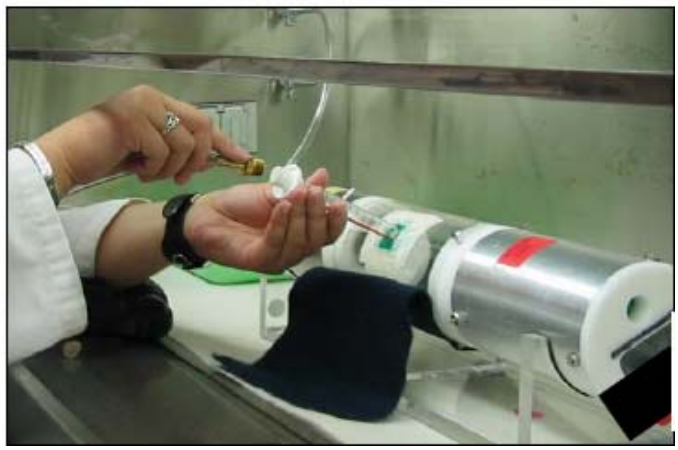
1. HTSS Chamber Units are configured such that both a Treatment and Control Test Chamber are affixed to one center Clear Chamber with both Test Chamber gates set in the closed position. The flat side of funnel caps should be facing towards the metal Test Chambers (see diagram below).
2. Felt is placed over the viewing windows of both metal Test Chamber end caps only.
3. Upon configuration completion, 20 female mosquitoes are transferred from an individual holding tube into the center Clear Chamber using mechanical aspiration and pressurized airflow. Check for mechanical KD due to the transfer process.
4. After 30 seconds the gates of each Test Chamber are placed in the open position and a timer set for 10 minutes is immediately started.
5. After 10 minutes, the gates for Test Chambers are set in the closed position **(in the same direction in which they were opened)** and the number of mosquitoes within both metal chambers as well as the center Clear Chamber are recorded. Knockdown of females within all chambers, temperature and humidity is also noted.
6. Mosquitoes are then vacuum-aspirated from the center Clear Chamber. Specimens within metal chambers are removed into Release Chambers and immediately killed using a vacuum water trap.
7. Once all the mosquitoes have been removed, the Control Test Chamber funnel cap is removed and both the Control Test Chamber and Clear Chamber are placed horizontally for 3 minutes to allow aeration of potential chemical vapors.
8. When all assays are completed for the day, chamber units are washed (see *ASSAY CHAMBER/HOLDING TUBE/TRAY WASHING*) and netting is stored or disposed as described in *STORAGE/DISPOSAL OF TREATED NETTING*.



SPATIAL REPELLENCY ASSAY



The configuration for the Spatial Repellency Assay.



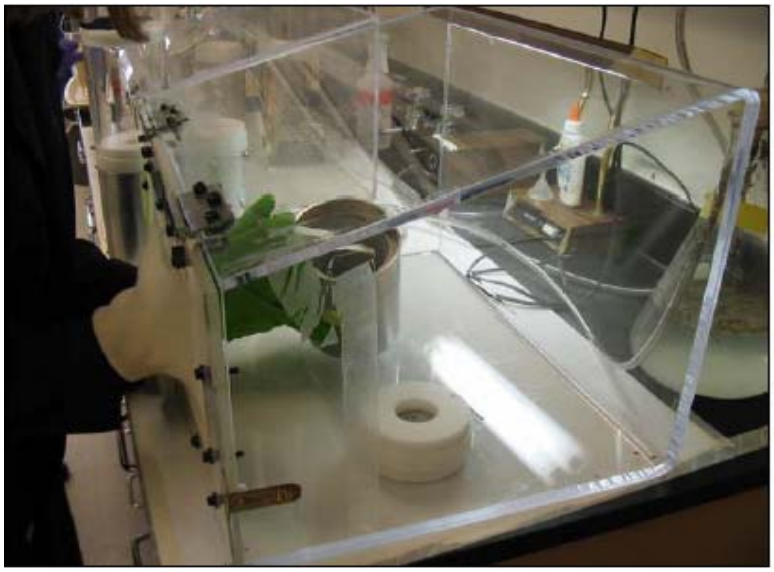
Mosquitoes are transferred from individual holding tubes into the center Clear Chamber using a mechanical aspirator and pressurized airflow. Viewing windows on end caps are covered.

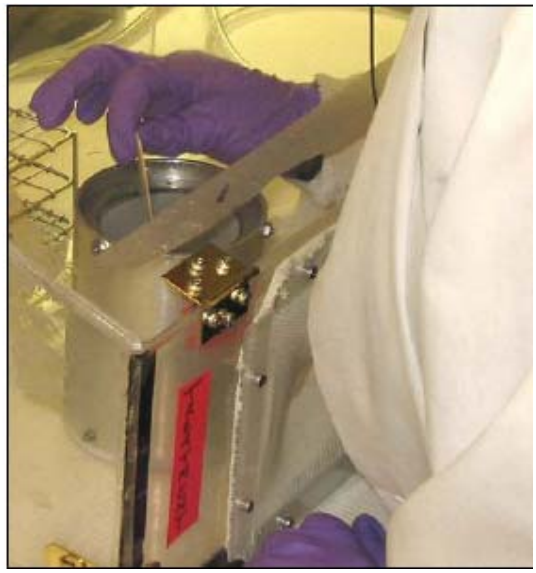
After each 10 min. replicate, the number of mosquitoes within the center Clear Chamber and both metal Test Chambers are recorded along with the knockdown response in all three chambers.

After data recording, mosquitoes are aspirated from the center Clear Chamber using a vacuum tube. Females within each metal Test Chamber are then removed in Release Chambers.

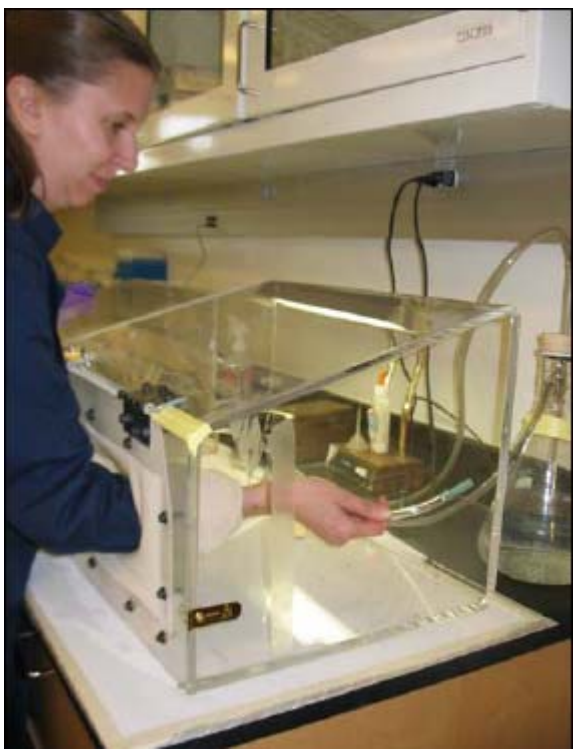
CIA / SRA RELEASE CHAMBERS

After each CIA and SRA replicate, mosquitoes within metal Test Chambers are removed into separate Control and Treatment Release Chambers. Gloves are worn when disassembling the chambers to prevent contact with treated netting.





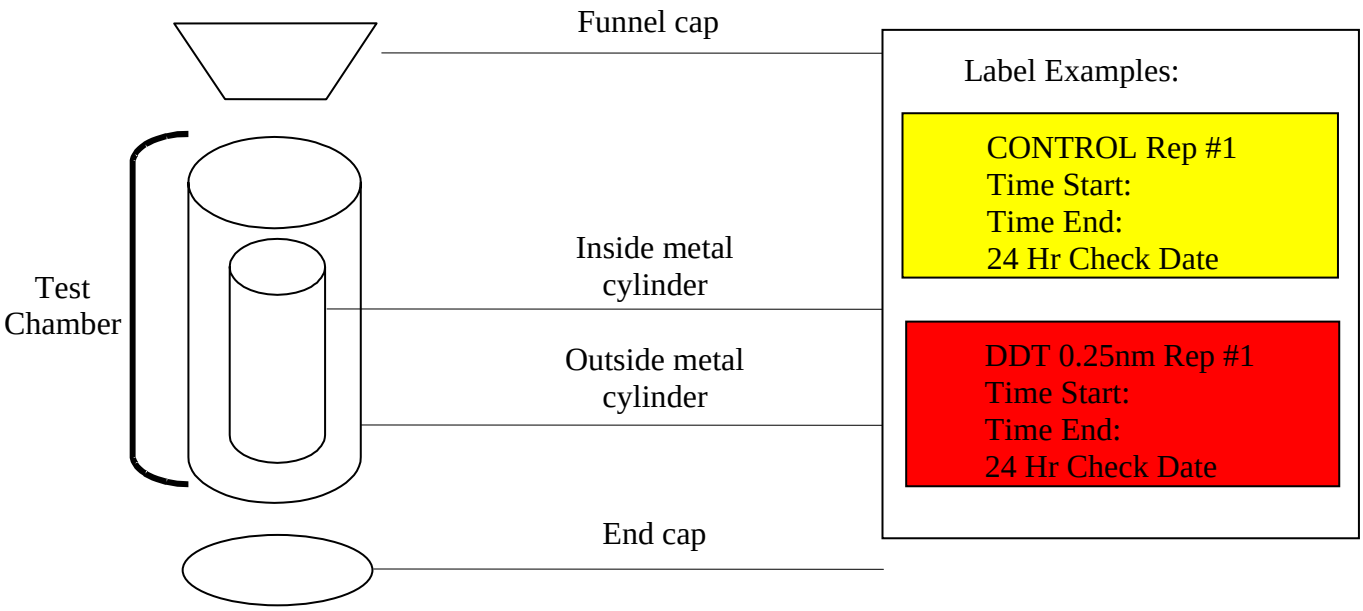
Once Test Chambers are disassembled, designated wooden applicator sticks are used to initiate flight of mosquitoes inside the metal Test Chambers. Separate applicator sticks are used for specific chemicals and/or concentrations.



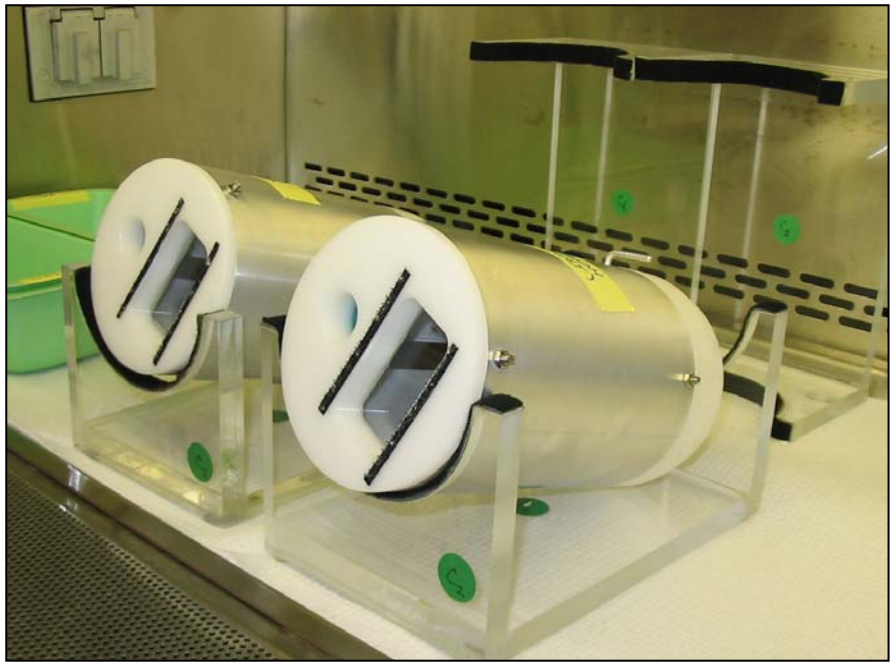
Mosquitoes within Release Chambers are then removed and killed using a vacuum water-trap.

TOXICITY ASSAY (TOX)

1. HTSS Chamber Units are configured such that separate Treatment and Control Test Chambers are fixed with an end and funnel cap and all Test Chamber gates are in the closed position. The flat side of funnel caps should be facing towards the outside of Test Chambers (see diagram below). The magnet should be at the top of the chamber.
2. Upon configuration completion, 20 female mosquitoes are transferred from individual holding tubes into Test Chambers using mechanical aspiration and pressurized airflow. Check for mechanical KD due to the transfer process.
3. Female mosquitoes are held in the Test Chambers for 1 hour.
4. After 1 hour, the number of knockdown females in Treatment and Control Test Chambers are recorded onto the TOX data sheet.
5. All mosquitoes are then transferred into properly labeled modified plastic cartons using a funnel and pressurized airflow.
6. Cartons are labeled with the following: Chemical name, Concentration, Replicate number, Time assay was started (ST), Time assay ended (ET), Time at 24hr and Date for checking mortality (see label examples below).
7. Place a cotton ball moistened with sugar water on top of each pint carton and then transfer cartons into separate Control and Treatment sealable containers.
8. Tape an index card on top of each sealed TOX container that lists the 24hr times in which each carton should be inspected and include columns designated for the number of females within each carton and mortality.
9. TOX containers are then transferred to a temperature (28°C) and humidity (80%) controlled insectary for 24 hours. At 24 hours the number of dead females are recorded for both control and treatment populations.
10. When all assays are completed for the day, chamber units are washed (see *ASSAY CHAMBER/HOLDING TUBE/TRAY WASHING*) and netting is stored or disposed as described in *STORAGE/DISPOSAL OF TREATED NETTING*.
11. After each use, all plastic cartons are washed using industrial soap and water. Netting on plastic cartons are briefly soaked in a diluted bleach solution then rinsed and air-dried. Cartons can be reused but should be separated for control vs. treatment use.



TOXICITY ASSAY



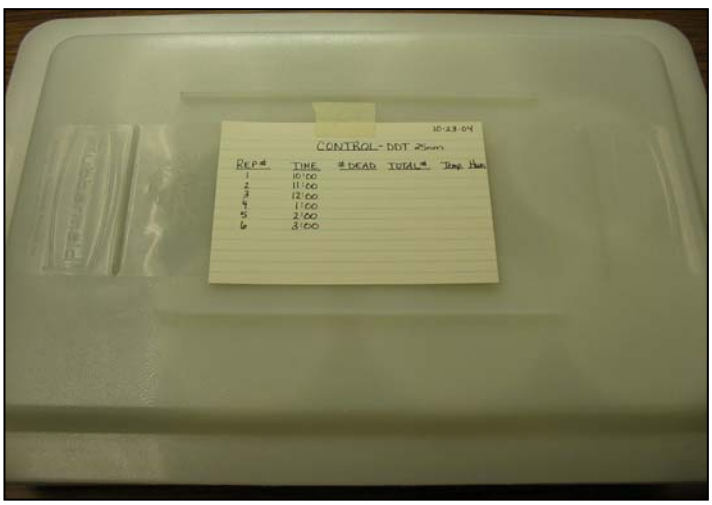
Mosquitoes are transferred into metal Test Chambers using a mechanical aspirator and pressurized airflow. Mosquitoes remain in chambers for a 1 hr exposure period.

After data recording, the gate to the test chamber is placed in the open position and mosquitoes are removed using a vacuum. Specimens are then transferred to individual plastic cups using pressurized airflow.

TOXICITY ASSAY- MOSQUITO PROCESSING

After each replicate for the TOX assay, mosquitoes are transferred into properly labeled plastic cartons.

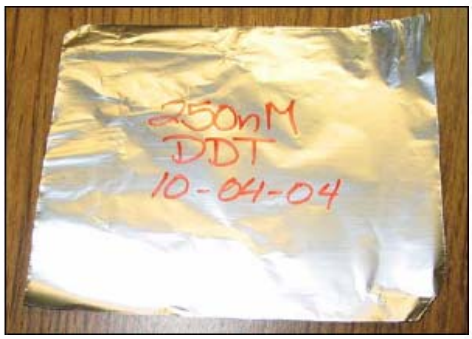
The plastic cartons are then placed into designated TOX sealable containers and transferred to an insectary. Sugar pads soaked in 10% sucrose solution is placed on top of each carton. Sugar pads should be remoistened when all laboratory activities are finished for the day.



An index card can be taped to the sealed containers that lists the “24 hr” times that each replicate should be checked the following day. Include columns for mortality and total number of females in each carton.

STORAGE OF TREATED NETTING

1. Always wear gloves and a lab coat when handling treated netting.
2. **Always remove control netting from corresponding chambers before handling treated netting**. **When handling treated netting always start with the lowest concentration. Change gloves between handling treated netting when different chemicals are used.**
3. Upon completion of assay testing for the day, individual netting is removed from metal Test Chambers underneath a chemical fume hood, folded and wrapped in aluminum foil labeled with the chemical name, concentration and date using a permanent marker.
4. Foil squares are placed into designated ziplock bags (i.e. control or specific treatment chemical) and stored at 4°C.

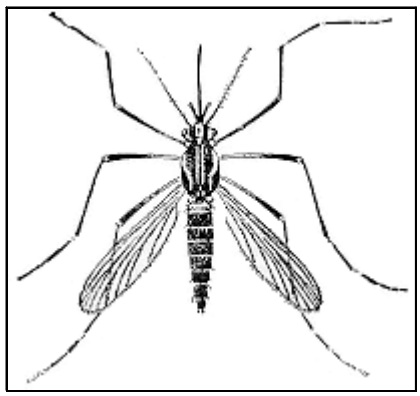



Label a piece of foil with the chemical name, concentration and date treated. Netting strips are then removed from the metal Test Chambers underneath a chemical hood using gloves and placed into designated foil pieces. Foil squares are then placed into sealable plastic bags labeled by chemical. Bags should be stored at 4°C.

DISPOSAL OF TREATED NETTING

Follow designated guidelines for disposal of biohazard material regulated by the establishment where assay testing is being conducted.

CLEANING PROTOCOLS



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ASSAY CHAMBER/HOLDING TUBES/ TRAY WASHING PROTOCOL

Acetone Washing

1. Use a chemical hood and always wear a lab coat and gloves when washing with acetone.
2. Use separate metal pans for washing control and treatment components.
3. Pour acetone into enamel wash pans (control vs. treatment) filling the bottom of each pan to approximately ½ inch.
4. Wash components in proper order (*see Acetone Washing Order instructions below*).
5. Leave washed components under chemical hood to dry overnight or for at least 1 hour.
6. Place waste solution from wash pan into a designated acetone waste bottle using a funnel and tighten cap well.
7. Replace funnel, enamel wash pan and all acetone bottles to proper storage areas when finished.

Acetone Washing Order:

In control washing pan:

1. Trays used to apply acetone/ethanol to control netting (make sure to tilt tray such that acetone washes over clips used to hold netting strips).
2. Inside metal cylinders.

In treatment washing pan:

1. Inside metal cylinders. **Change acetone solution after washing each inside metal cylinder.**
2. Trays used to apply test chemical to treatment netting (make sure to tilt tray such that acetone washes over clips used to hold netting strips). **Change acetone solution after washing each trays.**

Industrial Soap Washing

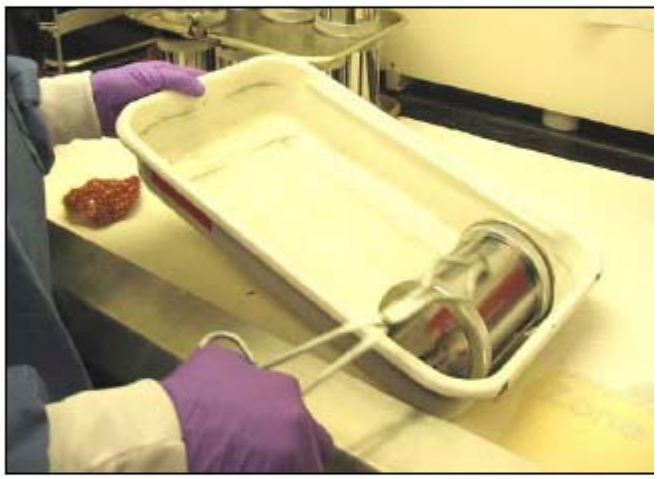
1. Use separate plastic washbasins for washing control and treatment components.
2. **Treatment end caps and treatment funnel caps should be washed in a separate metal pan.**
3. Dilute a capful of industrial laboratory equipment soap with tap water into all washbasins.
4. Using gloves, wash and rinse components well with designated sponges and tap water.
5. Leave washed components in drying trays and/or racks overnight or until dry.

Industrial Soap Washing Order:

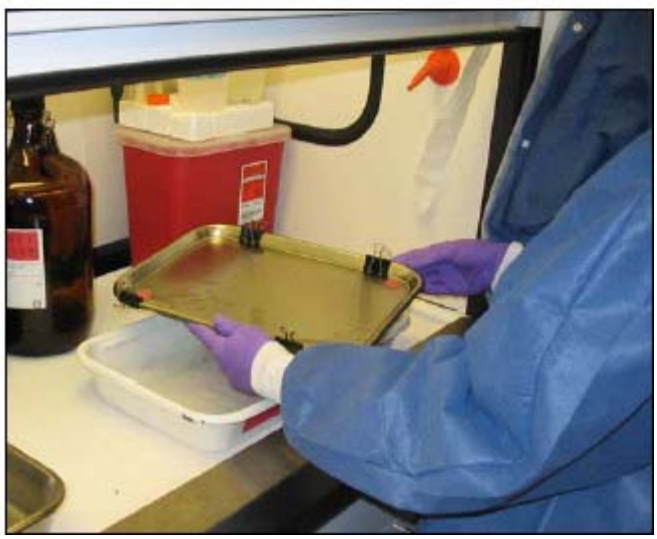
1. All control outer metal cylinders/end caps/holding tubes designated for soap washing.
2. All treatment outer metal cylinders/end caps/holding tubes designated for soap washing.

ASSAY CHAMBER/HOLDING TUBES/ TRAY WASHING PROTOCOL

Holding tubes, end caps, Clear Chambers and outer metal cylinders are washed using industrial soap and water. Make sure to keep control components separate from treatment components. After washing, rinse with tap water and allow to dry overnight.

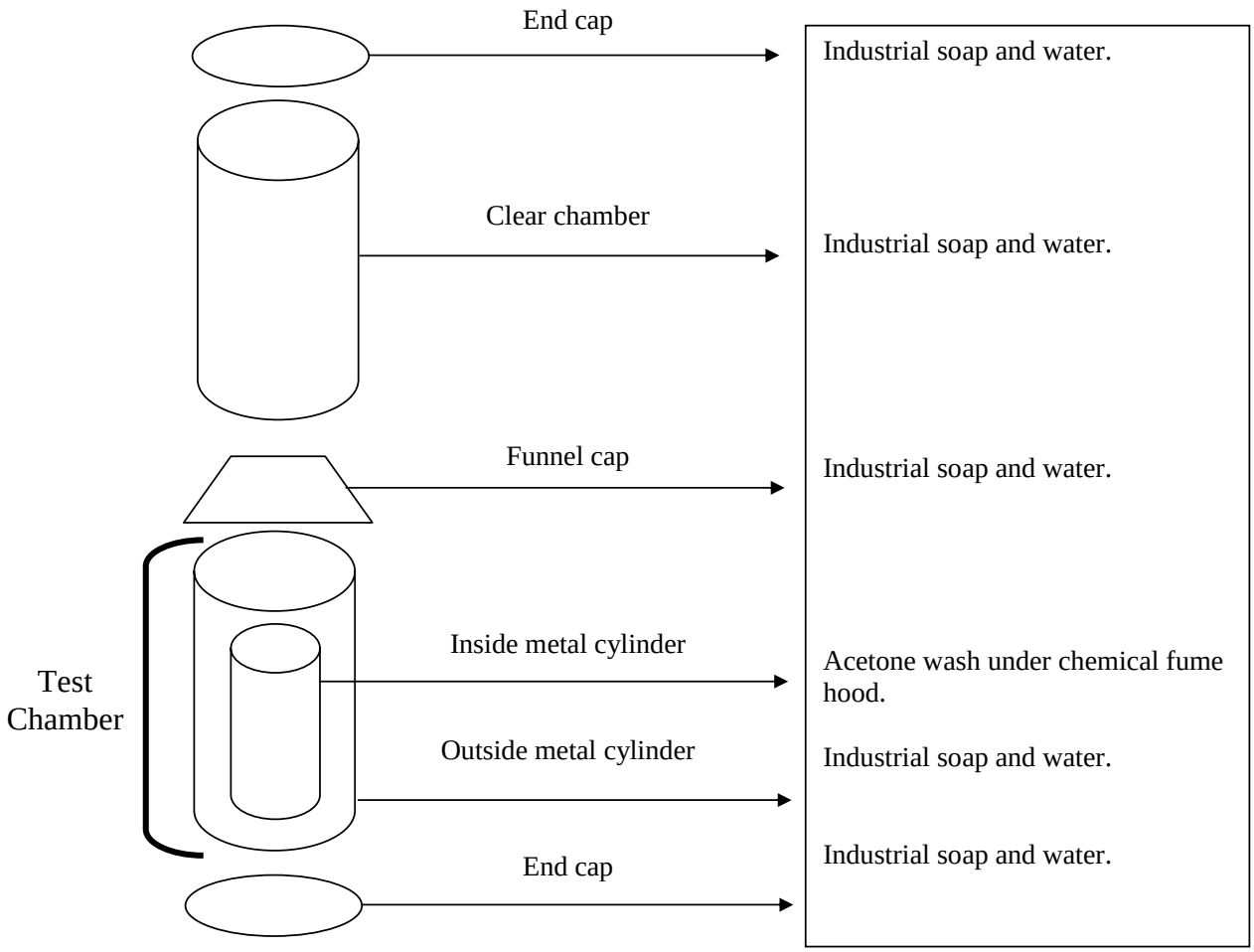


Inner metal cylinders are washed using acetone underneath a chemical fume hood. Be sure to wear a lab coat and gloves. Control and treatment cylinders are washed in separate metal pans. After each treatment cylinder is washed, liquid waste is disposed of properly and fresh acetone is added to the washing pan. After the last treatment cylinder is washed, the pan is rinsed with fresh acetone then waste disposed of properly.



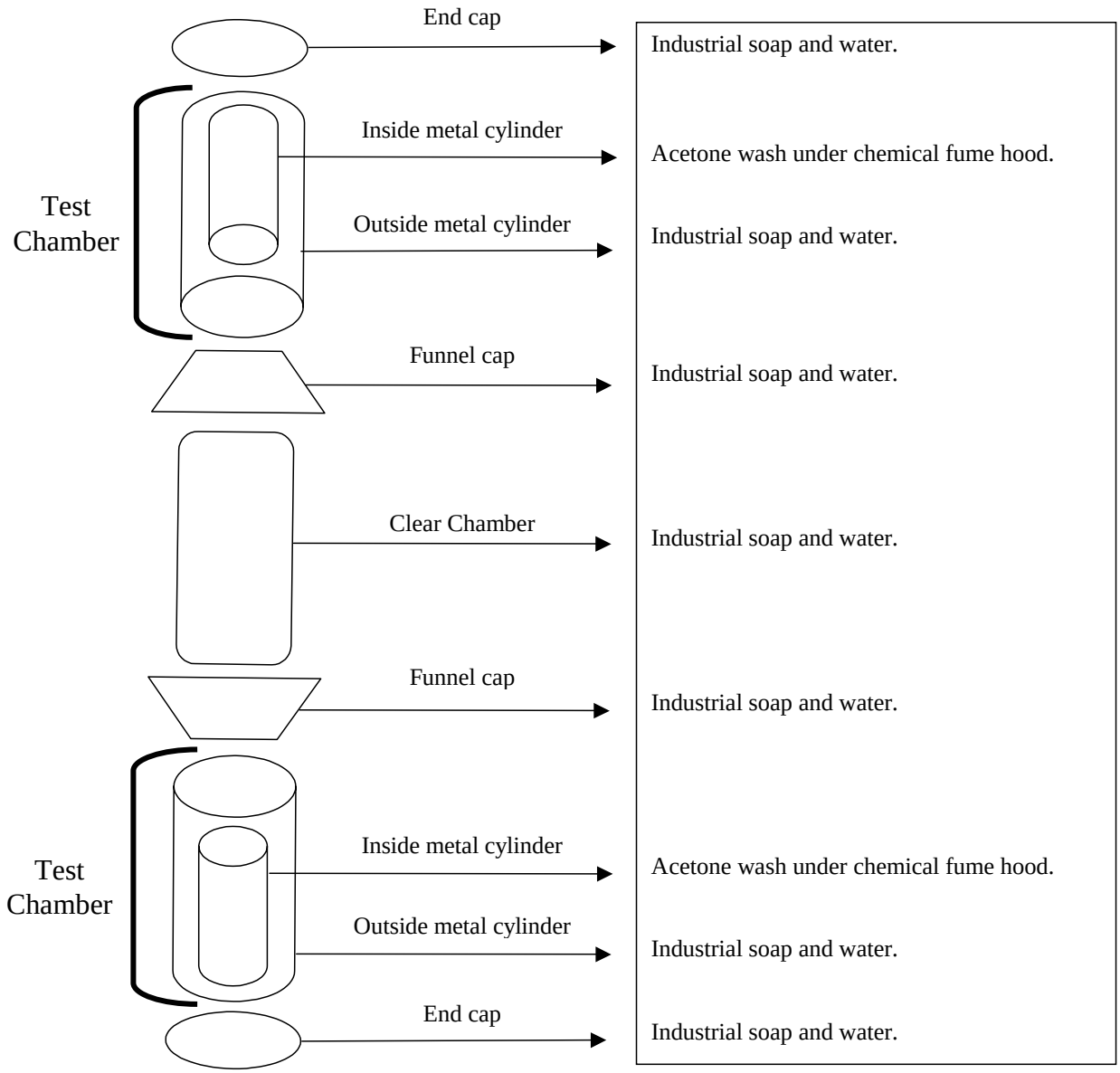
Metal trays and clips used to apply chemicals to netting strips, are also washed using acetone underneath a chemical fume hood. Control and treatment trays are washed in separate metal pans. After each treatment tray is washed, waste is disposed of properly and fresh acetone is added to the washing pan. After the last tray is washed, the pan is rinsed with fresh acetone then waste disposed of properly.

CHAMBER WASHING: CONTACT IRRITANCY ASSAY (CIA)

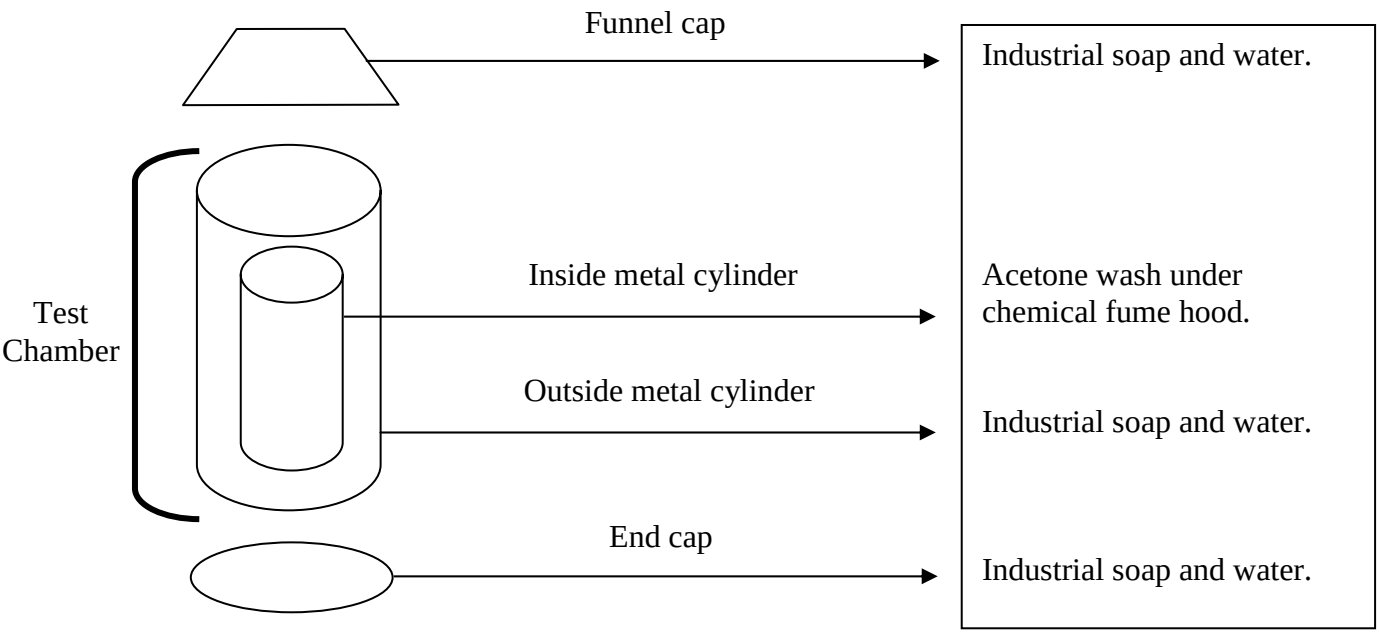


After washing, all components should be allowed to dry overnight (OR AT LEAST 1 HR) then placed into designated storage bins.

CHAMBER WASHING:
SPATIAL REPELLENCY ASSAY (SRA)



CHAMBER WASHING: TOXICITY ASSAY (TOX)



After washing, all components should be allowed to dry overnight (OR AT LEAST 1 HR) then placed into designated storage bins.

GENERAL LABORATORY CLEANING

A. RELEASE CHAMBERS:
1. Upon completion of assay testing for each day, all female mosquitoes remaining in the Release Chambers should be vacuumed-killed into a water trap.
2. Wearing gloves, discard any application sticks that were used to remove mosquitoes from assay metal Test Chambers.
3. Use a 10% bleach dilution to clean the inside of each release chamber and open to air dry overnight.
4. *WATER TRAPS SHOULD BE CLEANED EVERY FRIDAY*

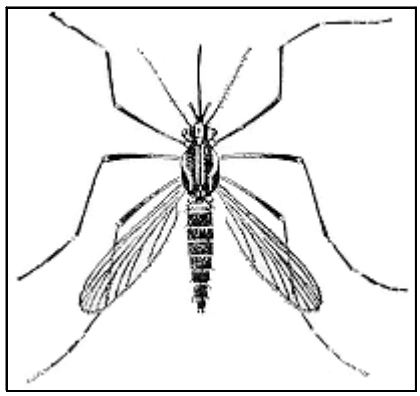
B. ACETONE WASTE DISPOSAL:

WASTE DISPOSAL WILL BE SITE-SPECIFIC, PLEASE REFER TO DESIGNATED SAFETY OFFICERS FOR PROPER DISPOSAL PROTOCOLS

C. BENCH TOP and CHEMICAL HOOD PAPER:
1. Paper covering all bench tops in the assay testing area and underneath chemical hoods should be replaced every Friday.

*** PAPER COVERING THE WORKING AREA WITHIN ALL CHEMICAL HOODS SHOULD BE IMMEDIATELY REPLACED IF CHEMICAL HAS SPILLED WHILE TREATING NETTING PIECES***

INSECTARY PROTOCOLS



N.Achee, Version 4.0 Feb. 07, NIH Grant #: 5UO1AI054777

Insectaries should be maintained at 28℃ and 80% Relative Humidity

Aedes aegypti (THAI STRAIN) ASSAY REARING SCHEDULE:

Activity	Monday	Tuesday	Wednesday	Thursday	Friday
Make Sugar Solution	When needed				
Moisten Sugar Pads		X Thus. Assay/ Gallons	X Fri. Assay/Gallons	X Gallons	X Gallons
Hatch Eggs		X WRAIR=12,000 eggs			
Separate Larvae			X WRAIR=226 cups (37 TRAYS)	X	X (as needed/ hatch)
Feed Larvae	X* Pellet (As needed/cup)		X CHOW (All)	X CHOW (All)	X PELLET (All)
Refresh Larval Water	X (As needed/cup)				
Pick/Sort Pupae	X	X	X	X	X
Sort Females for Assays	X				
Starve Females for Assays		X (For Wed's)	X (For Thu's)	X (For Fri's)	

Aedes aegypti (THAI STRAIN) COLONY REARING SCHEDULE:

Activity	Monday	Tuesday	Wednesday	Thursday	Friday
Make Sugar Pads		X			X
Starve for Blood Feeding	X			X	
Blood Feeding		X			X
Egg Harvest		X			
Replace Oviposition Cups		X			
Hatch Eggs		X** USU=2,000 eggs			
Separate Larvae			X USU=40 cups	X	
Feed Larvae	X* PELLET (As needed/cup)		X CHOW (All)	X CHOW (All)	X PELLET (All)
Refresh Larval Water	X (As needed/cup)				
Pick/Sort Pupae	X	X	X	X	X
Set Up New Adult Cages		X*** 3 Cages			

ASSAY INSECTARY ACTIVITY DESCRIPTIONS

Make Sugar Solution: Make a 10% sugar solution in the designated glass beaker using tap water (500ml) and nectar food (50 g) making sure sugar has dissolved. Cover and place into small refrigerator.

Moisten Sugar Pads (Cotton Balls):

Sugar Pads in Gallon Cartons: Remove sugar pads from 1-gallon plastic cartons and remoisten with fresh sugar water. Cotton balls should be moist to touch. **Monday throw away all sugar pads from assay gallon containers after sorting females-do not reuse.**

Sugar Pads on Assay Pint Cartons: Remoisten sugar pads by lightly squirting sugar water onto pads using the 10% sugar water squirt bottle.

Hatch Eggs: Cut a dried oviposition egg strip into 1 inch pieces making sure the necessary number of eggs needed to fulfill larval requirements for assay testing (~12,000 larvae; see cheat sheet) is being hatched. Make sure to cut away excess paper towel that does not contain eggs prior to placing into hatching cup. Place egg papers into white plastic rearing cups containing ~450 ml of tap water making sure the egg strip is submerged under water. Label each cup with "egg harvest system": Date Harvested (DH), generation and Date Hatched:
ex. DH: 1/1/07-F4 Hatch: 2/1/07. **Place one scoop (using red-tagged pipette scooper) of finely crushed fish pellets into each egg hatch cup.** On the first day after hatch transfer egg papers into newly labeled larval cups and leave without food overnight.

Separate Larvae: The first day after egg hatch sort 50 larvae into individual larval rearing cups and fill the cup with ~450 ml of tap water (first beveled line from bottom). Place sorted cups onto trays and label shelves according to egg harvest system from egg hatch (can use the label on hatching cups). Record the final number of larvae sorted onto the Larval Eclosion datasheet to estimate % egg hatch.

Feed Larvae: After sorting larvae into groups of 50, grind several chow pellets using the mortar/pestle. Add a scoop (using red-tagged pipette scooper) of finely crushed fish pellets into all larval cups. On Day 3 and 6 post-hatch, place one chow pellet into each larval cup. ***If > 5-10 pupae/ cup on Monday do not feed larvae.**

Refresh Larval Water: Dump water from all larval cups until ~1inch remains at the bottom making sure not to lose any larvae. Refill to 450 ml using tap water.

Pick/Sort Pupae: Remove pupae from larval rearing cups and separate female/male specimens by size using a mechanical separator. Once separated, conduct a visual inspection to be sure accidental males are removed. Sort female pupae into groups of no more than 250 (using the pupae estimator) and place each 250 group into a larval rearing cup. The larval rearing cup is then placed inside a 1-gallon plastic carton labeled with pupation date, female count and generation: ex: P: 1/1/07 250ct. F4. Place two sugar pads (moistened cotton balls) INSIDE each carton using a latex glove. Kill all male pupae collected during the sorting process using bleach.

Sort Females for Assays:

1. Mechanically aspirate females from gallon cartons and group into modified pint cartons (10's or 20's) according to the assay schedule for that week (*see Assay Scheduling description*).
2. Group cartons by daily requirements and place into green transporting trays. Place a cotton pad soaked in 10% sugar solution on top of each pint carton.
3. Properly label all transporting trays with the day and date the pint cartons will be used, age and generation of mosquitoes. EX: Thursday 1/4/07; 6 Day Old; F4.

Starve Females for Assays: Remove sugar pads from designated pint cartons. Store sugar pads in the refrigerator and use these pads in the gallon containers holding pupae picked the same week. **Females should be starved 24 hrs. prior to use in assay test system.**

COLONY INSECTARY ACTIVITY DESCRIPTIONS

Make Sugar Pads: Moisten two cotton balls with fresh 10% sugar solution and place into a small petri dish. Insert one petri dish into each 1-gallon plastic cartons holding adult mosquitoes. Sugar pads should be moist to touch.

Starve for Blood Feeding: Remove sugar pads from 1-gallon plastic cartons. Throw away cotton and clean petri dishes well with hot tap water.

Blood Feeding: See *Artificial Blood Feeding Protocol*.

Egg Harvest: Remove oviposition cups from adult containers and extract the paper towels containing eggs. Place the paper towels egg-side up on a clean surface and allow water to evaporate in a humidified room for no less than ~1 hour). Estimate the number of eggs on each strip and record on the Egg Harvest datasheet. After drying, cut away excess paper towel, remove any dead adult mosquitoes then place egg strips into individual ziplock bags labeled with "egg harvest system" and egg amount: EX: DH: 1/1/07 F4 ~8,000. Where DH=date of harvest and F4=generation of eggs.

Replace Oviposition Cups: Place a strip of paper towel along the inside of an oviposition cup (20ml plastic beaker) and fill half way with tap water. Make sure to cut away excess paper so that the paper towel DOES NOT hang over the sides of the oviposition cup. Place one oviposition cup into each adult 1-gallon container.

Hatch Eggs: Cut a dried oviposition egg strip into 1 inch pieces making sure the necessary number of eggs needed to fulfill larval requirements for colony maintenance is being hatched (~2,000 larvae). **Hatching order of egg strips should be from the oldest date of harvest to the newest.** Make sure to cut away excess paper towel that does not contain eggs prior to placing into hatching cup. Place egg papers into larval rearing cups containing ~450 ml of tap water making sure the egg strip is submerged under water. Label each cup with "egg harvest system" (see Egg Harvest above) and date hatched: EX: DH: 1/1/07 F4 Hatch: 1/4/07. **Place one scoop (using pipette scooper) of finely crushed fish pellets into each egg hatch cup.** On the first day after hatch transfer egg papers into newly labeled larval cups and leave without food overnight.

****Hatching Dates for Colony are on the Calendar in "C" Insectary at USU**

Separate Larvae: The first day after egg hatch sort 50 larvae into individual larval rearing cups and fill the cups with ~450 ml of tap water (first beveled line from bottom). Label the incubator shelf according to egg harvest system used from egg hatch (can use the label on cups) EX: DH: 1/1/07 F4 Hatch: 1/4/07.

Feed Larvae: After sorting larvae into groups of 50, grind several chow pellets using the mortar/pestle. Add a scoop (using pipette scooper) of finely crushed fish pellets into all larval cups. On Day 3 and 6 post-hatch, place one chow pellet into each larval cup. ***If > 5-10 pupae on Monday do not feed larvae.**

Refresh Larval Water: Dump water from all larval cups until ~1inch remains at the bottom making sure not to lose any larvae. Refill to 450 ml using tap water.

Pick/Sort Pupae: Remove pupae from larval rearing cups using a plastic pipette and place into a new rearing cup containing ~100 ml of tap water. Place all pupae into designated 1-gallon plastic containers labeled **with date of pupae and adult generation**. Place two sugar pads (cotton balls) INSIDE each gallon container.

Set Up New Adult Cartons: Kill all old adult mosquitoes from 1-gallon plastic cartons by allowing to freeze overnight. Remove dead mosquitoes and clean the inside of plastic containers with soapy water. Use a dilute bleach solution to clean netting. If necessary, the dilute bleach solution can also be used for cleaning the 1-gallon plastic cartons. Cartons can be immediately re-used once dry.

Artificial Membrane Blood-Feeding Protocol

BLOOD PREPARATION (To be done the day of feeding):

1. Using gloves and a lab coat, fill a 50cc plastic tube with expired human blood (10ml/feeder).

2. Measure out ATP using a scale and weigh boat, **the ratio of ATP:blood should follow: 0.025g ATP/10 ml blood.** After weighing, pour the ATP into the 50cc plastic tube and shake vigorously until dissolved.

FEEDING INSTRUCTIONS:

1. Attach ~ 3 in. by 3 in. square of synthetic membrane tightly across the bottom of each glass feeder using a rubber band. **Make sure the "bumpy" part of the membrane is on the outside and covers all bottom edges of the glass feeders.**

2. Place one feeder on top of each adult feeding cage.

3. Attach tubing from the water bath to all glass feeders.

4. Turn the water bath on and place the 50cc plastic tube of blood into the water bath chamber to warm for at least 15 min. **The plastic vial should be warm to the touch prior to placing the blood in the glass feeders.**

5. Once the blood is warmed, add 10 ml of blood into each glass feeder using a designated disposable pipette while wearing gloves and a lab coat.

6. When all feeders are filled, cover the feeding cages with a dark plastic sheet.

7. Clean the 50 cc tube, its cap and the disposable pipette well using tap water. Leave the 50cc tube uncovered to dry.

8. **Allow females to feed for 1 hour**.

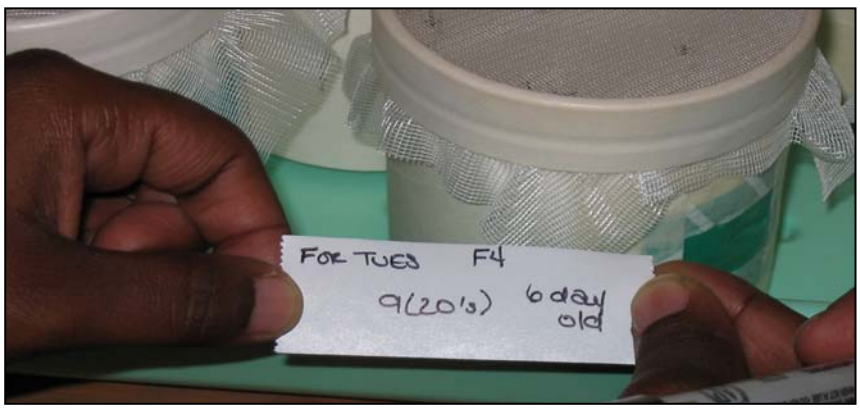
9. After 1 hour, turn off the circulating water bath, remove and store tubing. **Make sure to drain each tube piece prior to removal from feeder.**

10. Wearing gloves and a lab coat remove membrane pieces from each glass feeder and allow excess blood to drain into a pan containing bleach solution. Dip the used membrane into the bleach solution then place in a biohazard container for proper disposal.

11. Once all membrane pieces have been removed, gently clean feeders using diluted bleach solution and paper towels. Store feeders over clean paper towels in a larval pan.

SORTING FEMALES FOR ASSAYS

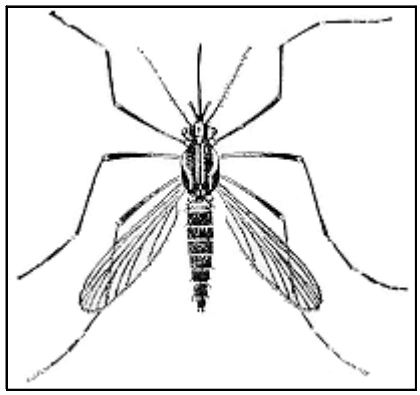
Mosquitoes are mechanically aspirated from gallon containers and sorted into groups of 10's or 20's within individual pint cartons according to the weekly assay schedule. A cotton ball soaked with a 10% sugar solution is placed on top of each carton.



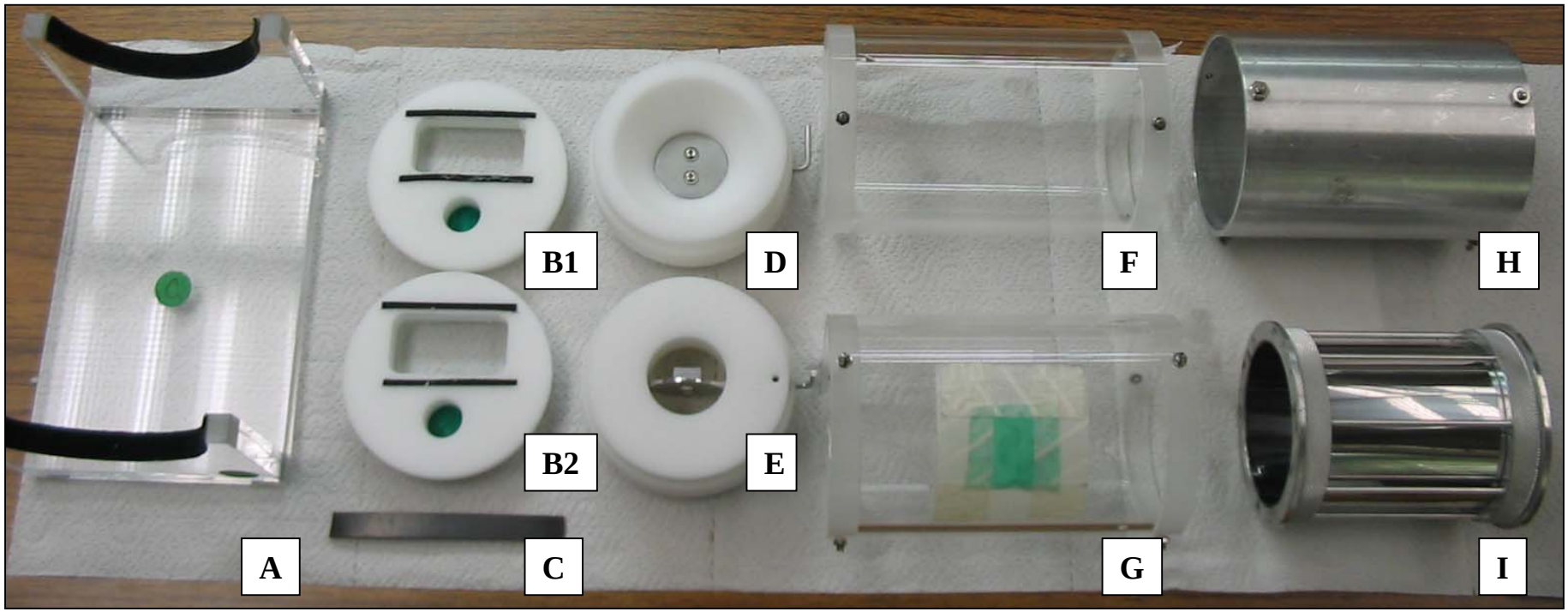
Pint cartons are then placed into individual trays which are properly labeled by date of use, the generation and age of females and the number of 10 or 20 groupings.

If a humidified insectary is not available, a beaker of tap water can be placed with the pint cartons and the trays wrapped in plastic bags to maintain humidity levels.

APPENDICES



ASSAY CHAMBER SYSTEM COMPONENTS



A: Cradle
B1/B2: End caps with velcro strips used to attach felt pieces
C: Magnet used to hold netting strip to inside metal cylinder
D: Funnel cap-beveled side up with gate in closed position
E: Funnel cap-flat side up with gate in open position
F: Clear Chamber used in the CIA (no center hole)
G: Clear Chamber used in the SRA (contains center hole)
I: Inside metal cylinder of Test Chamber
H: Outside metal cylinder of Test Chamber

Test Date: (mm/dd/yy) **Tested By:** **Chemical Name:** **Date Received:**

Conc. (nm/cm2)	REP	Control		Treatment		CCNT	CM KD		TCNT	TM KD		TEMP	HUM	TIME OF DAY	GEN/ AGE	COMMENTS
		CNT	CBR	CNT	CBR		Metal	Clear		Metal	Clear					
25nm	1		C1		T1											
	2		C1		T1											
	3		C1		T1											
	4		C1		T1											
	5		C1		T1											
	6		C1		T1											
25nm	1		C1		T2											
	2		C1		T2											
	3		C1		T2											
	4		C1		T2											
	5		C1		T2											
	6		C1		T2											
25nm	1		C2		T3											
	2		C2		T3											
	3		C2		T3											
	4		C2		T3											
	5		C2		T3											
	6		C2		T3											
25nm	1		C2		T4											
	2		C2		T4											
	3		C2		T4											
	4		C2		T4											
	5		C2		T4											
	6		C2		T4											
25nm	1		C3		T5											
	2		C3		T5											
	3		C3		T5											
	4		C3		T5											
	5		C3		T5											
	6		C3		T5											

CONTACT IRRITANCY ASSAY (CIA)

Test Date: (mm/dd/yy) **Tested By:** **Chemical Name:** **Date Received:**

Conc. (nm/cm2)	Chamber (control #/treatment #)	REP	N	CCNT	TCNT	KD CLEAR	KD CONT	KD TRT	TEMP	HUM	TIME	GEN/AGE	KD PRIOR	COMMENTS
25nm	C1:T1	1												
	C1:T1	2												
	C1:T1	3												
	C1:T1	4												
	C1:T1	5												
	C1:T1	6												
	C1:T1	7												
	C1:T1	8												
	C1:T1	9												
25nm	C2:T2	1												
	C2:T2	2												
	C2:T2	3												
	C2:T2	4												
	C2:T2	5												
	C2:T2	6												
	C2:T2	7												
	C2:T2	8												
	C2:T2	9												
25nm	C3:T3	1												
	C3:T3	2												
	C3:T3	3												
	C3:T3	4												
	C3:T3	5												
	C3:T3	6												
	C3:T3	7												
	C3:T3	8												
	C3:T3	9												
25nm	C4:T4	1												
	C4:T4	2												
	C4:T4	3												
	C4:T4	4												
	C4:T4	5												
	C4:T4	6												
	C4:T4	7												
	C4:T4	8												
	C4:T4	9												

SPATIAL REPELLENCY ASSAY (SRA)

Test Date: (mm/dd/yy) **Tested By:** **Chemical Name:** **Date Received:**

TRT (nmoles/ cm2)	REP	Control CNT CBR		Treatment CNT CBR		C1HR KD	T1HR KD	1 HR TEMP	1 HR HUM	TIME ENDED	C24HR MORT	T24HR MORT	24 HR TEMP	24 HR HUM	GEN	Adult age
25nm	1		C1		T1											
	2		C1		T1											
	3		C1		T1											
	4		C1		T1											
	5		C1		T1											
	6		C1		T1											
25nm	1		C2		T2											
	2		C2		T2											
	3		C2		T2											
	4		C2		T2											
	5		C2		T2											
	6		C2		T2											
25nm	1		C3		T3											
	2		C3		T3											
	3		C3		T3											
	4		C3		T3											
	5		C3		T3											
	6		C3		T3											
25nm	1		C4		T4											
	2		C4		T4											
	3		C4		T4											
	4		C4		T4											
	5		C4		T4											
	6		C4		T4											
25nm	1		C5		T5											
	2		C5		T5											
	3		C5		T5											
	4		C5		T5											
	5		C5		T5											
	6		C5		T5											

TOXICITY ASSAY (TOX)

LABORATORY SUPPLIES						
Vendors Name	**Phone**	**Description**	**Qty**	**Unit**	**Unit Price**	**Catalog No.**
VWR	800-932-5000	Versi-Dry Lab Table Soakers, NALGENE	1	case	$158.89	52857-120
VWR	800-932-5000	GLV, BXD NTRLE AMBI, SM, 100 PK	1	cs	$268.48	40101-344
VWR	800-932-5000	Timer, Triple Display, VWR	3	ea	$28.41	62344-588
VWR	800-932-5000	Trace Jumbo, Ther/Hum Meter, VWR	2	ea	$61.11	61161-378
VWR	800-932-5000	Tape, Lab, Red VWR RL 1X500IN	2	ea	$5.69	36427-062
VWR	800-932-5000	Tape, Lab, wht VWR RL 1X500IN	2	ea	$7.18	36425-067
VWR	800-932-5000	Tape, Lab, Yel VWR RL 1X500IN	2	ea	$7.18	36426-060
Fisher Scientific	800-766-7000	Gen. PRP Tips 101-1000ul, PK1000	1	pk	$24.50	S63213
Standard Office Supplies	301-652-6922	Avery Write-On Tabs, Assorted Colors	10	pack	$3.73	AVE-16141
G Street Fabrics	301-231-8998	Nylon Organdy, 100% Nylon, White, 45 inches wide, SKU 4	160	yrd	$6.98	5200-0001
Standard Office Supplies	301-652-6922	Duracell AAA batteries	1	pack	$19.52	DUR-MN24VR16Z
Standard Office Supplies	301-652-6922	Duracell AA batteries	1	pack	$19.52	DUR-MN15VR16Z
Standard Office Supplies	301-652-6922	Procell Batteries D	2	box	$14.76	DUR-PC1300
VWR	800-932-5000	Tongue Depressors	2	pack	$16.68	62505-007
McMaster-Carr	732-329-3200	Low height, NSF Certified Type 304 Stainless steel pan	5	each	$25.08	4189T1
Walmart	1-800-925-6278	Sunbeam Personal Ultrasonic Humidifier	1	each	$27.96	4060552
INSECTARY SUPPLIES						
Vendors Name	**Phone**	**Description**	**Qty**	**Unit**	**Unit Price**	**Catalog No.**
Trenton Mills, LLC	731-855-1760	Orthopedic Stockinette, 6" x 25 yard roll	4	rl	$41.20	
A. Panza & Son, LTD	732-225-1314	Container pint paper white, 500/case	1	cs	$69.34	5678
A. Panza & Son, LTD	732-225-1314	Lid plastic for pint carton, 500/cs	1	cs	$66.95	5681
Interstate Blood Bank	901-525-7462	Diagnostic Whole Human Blood (CPD-A), Type O, 500ml	1	bag	$155.00	NA
WL Enterprises, Inc.	973-624-0100	Neptune Cans-Pints with Lids	1	case	$245.19	300
Hausherr's Machine Works	732-349-1319	Aspirator, battery powered 1.5 volt flashlight design	8	each	$39.00	NA
Container & Packing Sup.	800-473-4144	16oz Natural Tub	500	ea	$0.19	T012
Park's Gardens	800-213-0076	Perma-Nest Plant Trays 22x11x2 3/4	10	ea	$4.99	6116
Petsmart	888-839-9638	Hikari Cichlid Gold Food, 8.8 oz Large	20	pack	$5.29	601470
Petsmart	888-839-9638	Perky Pet 6 pack original flavor nectar	10	6 pack	$6.99	590128
Thomas Scientific	800-345-5232	Dynalon polyethylene jars, 165oz, 7 3/4 x 8 1/4	2	case	$67.10	6185U35
Mcmaster-Carr	732-329-3200	extruded acrylic hollow rod	40	ea	$1.71	8532K13
T&K Machine and Prepare	301-473-5699	Larval / Pupal Separator	1	unit	$345.00	NA
Standard Office Supplies	301-652-6922	Acclaim Natural Paper Towels	2	case	$25.98	GEP23504
BioQuip	310-667-8800	Green Polyester Netting	20yds	yd	$2.90	7250B
Mcmaster-Carr	732-329-3200	Tapered Cork Plug Size 4, 5/8" Large End OD, 15/32" Smal	1	each	$9.30	9566k18
Mcmaster-Carr	732-329-3200	EPDM Black Rubber Plug, Fits 5/16"-1/2" OD	3	each	$6.58	6448K65
CLEANING SUPPLIES						
Vendors Name	**Phone**	**Description**	**Qty**	**Unit**	**Unit Price**	**Catalog No.**
VWR	800-932-5000	Cellulose Sponges, Fine Pore	2	6 sponges	$19.91	58540-069
VWR	800-932-5000	Liqui-Nox Phosphate-Free Liquid Detergent	5	qt.	$11.96	21837-005
Staples	800-378-2753	CLOROX Bleach, 96 oz. bottle	20	each	$2.60	COX02490
Quality Biological Inc	800-443-9331	Acetone	1	case	$277.00	9006-03