Mosquito and Vector Control Association of California

Integrated Vector Management Committee

Mosquito Pesticide Resistance Monitoring Working Group

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Purpose

To provide recommendations, through the Integrated Vector Management Committee, to the membership of the Mosquito and Vector Control Association of California regarding the implementation of a standardized pesticide resistance monitoring program for mosquito populations in California.

Test Procedures for Insecticide Resistance Monitoring in Mosquitoes

Prepared by the Mosquito Pesticide Resistance Monitoring Working Group
Integrated Vector Management Committee
Mosquito and Vector Control Association of California

2016

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INTRODUCTION

Pesticides play an important role in mosquito and vector control programs. Currently there are only two classes of pesticides with different modes of action (organophosphates and pyrethroids) approved to control adult mosquitoes in California. The pesticide classes available to kill immature stages are also limited, and there is a concern that we may lose some of the products due to increased regulatory limitations. Costs of development and production of a new compound are extremely high and the prospects of alternative chemicals coming into the public health market soon are uncertain, making good stewardship of currently available insecticides an ever more important topic.

Over time mosquito populations have been exposed to pesticides not only from mosquito control operations, but also through residential, agricultural, and other applications. Strong selection pressures from multiple types of pesticide uses may contribute to the development of resistance, threatening the efficacy of current mosquito control programs and the ability to combat re-emergent or new vector-borne diseases.

Monitoring of pesticide resistance by periodically evaluating pesticide susceptibility of various populations should be an integral component of mosquito control programs. Currently, resistance is only monitored by some agencies in California, and methods used by these agencies may vary, making it difficult to assess and compare the data. This working group recommends the implementation of a standardized pesticide resistance monitoring program in California to ensure comparability of data obtained from different sources.

Testing Recommendations

The "*California Mosquito-Borne Virus Surveillance and Response Plan*", which can be found at http://westnile.ca.gov/resources.php, recommends that the evaluation of pesticide resistance in vector populations be conducted at least during the "normal season", or "Level 1". The need for additional testing will depend on a variety of factors, including the agency's usage of a particular pesticide, the general use of that class of pesticide for other pests besides mosquitoes and by other entities, field observations of

treatment failures, etc. The more frequently and widely a certain class of pesticide is used, the greater the selection pressure on the mosquito populations, even when the applications are not made with the objective to control mosquitoes. This group recommends an evaluation as soon as mosquitoes can be colleted in sufficient numbers from the field in the spring, and at least one additional evaluation from the same area at the end of the season. Mosquito populations should be collected from as many areas of concern as possible, since resistance can be a localized problem.

In order to make results comparable across the state, we recommend that all agencies involved in resistance testing adopt the same protocols and susceptible reference colonies. The reference colonies currently recommended are the *Culex tarsalis* BFS (Bakersfield Field Station) colony, and the *Cx. pipiens quinquefasciatus* CQ1 colony, and a list of the districts that currently have those colonies can be found in Appendix A. Districts interested in starting susceptible colonies for the purpose of resistance testing should make arrangements directly with the districts from that list to have mosquitoes shipped to them. For districts that would like to run their own resistance bioassays but can't maintain a colony, arrangements can be made to have mosquitoes from the susceptible colonies shipped to them at the time of their testing. That will need to be coordinated in advance between the district running the bioassays and a district that maintains susceptible colonies. Alternatively, but not ideally, bioassays may be run without a susceptible colony for comparison.

It is the recommendation of this working group that all agencies use the protocols outlined in this document to allow for comparison of the data across the state. In addition, this group recommends that districts's staff attend the workshops offered by Dr. T. Steven Su (benchtop cup bioassay – larvicides), Dr. Janet McAllister (CDC, bottle bioassays – adulticides), or by the Mosquito and Vector Control Association of California (MVCAC – Laboratory Technologies Committee), or consult with Districts with experience running the bioassays. This document is not meant to replace the formal, hands-on training offered by those workshops.

Reporting of Results

This group recommends that all data be entered into a database. Agencies can enter the resistance testing data directly into the CalSurv Gateway (Pesticide Resistance Component) at www.gateway.calsurv.org. Combining results statewide will allow for easier analysis and comparison of results, as well as mapping of resistance patterns. Some functions of the pesticide resistance component are still under development and should be online in the near future.

Additional Testing and Resistance Management

The recommended bioassays included in this document for adulticides and larvicides provide fast and inexpensive methods for estimates of increased tolerance to pesticides. Bioassay data generated by these methods are good indicators of the presence of resistance in mosquito populations, but they do not measure resistance mechanisms or gene frequency accurately or suggest the epidemiological impact of such resistance. Resistance mechanisms and gene frequency can be analyzed by the use of biochemical and molecular assays performed on individual mosquitoes.

If increased tolerance or resistance is suspected in a population, as determined by the bioassays, this group suggests that additional biochemical and molecular tests be performed to determine the mechanism(s) and extent of resistance. Currently a few districts in California are equipped and trained to performed such tests. In addition, the California Department of Public Health (CDPH) also offers *kdr* testing of *Culex pipiens* complex mosquitoes at a fee.

Finding resistance in a particular area does not necessarily justify an immediate change in policy or control program for that area. Pesticide resistance may occur at different levels. Depending on the severity of resistance and the mechanism(s), current measures may still be sufficient to suppress transmission or a pesticide rotation may be warranted. Results obtained from the bioassays recommended here represent preliminary results, and resistance and its impact on the efficacy of control programs should be investigated on a case by case basis before the adoption of corrective measures.

APPENDIX A

MOSQUITO COLONIES

District	Species	Susceptible colony	Resistant colony
Coachella Valley MVCD	*Culex tarsalis*	BFS	
	Culex pipiens quinquefasciatus	CQ1	
Contra Costa MVCD	*Culex tarsalis*	BFS	
Greater Los Angeles County VCD	*Culex pipiens quinquefasciatus*	GRLAVCD/SYL	
Placer MVCD	*Culex pipiens quinquefasciatus*	CQ1	
	Culex tarsalis	BFS	
Sacramento-Yolo MVCD	*Culex tarsalis*	BFS	
	Culex pipiens quinquefasciatus	CQ1	
San Joaquin MVCD	*Culex pipiens quinquefasciatus*	CQ1	
San Mateo MVCD	*Culex pipiens pipiens*	Sus_Colony	
	Culex pipiens pipiens var. molestus		Resis_Colony (permethrin)
	Aedes sierrensis	Sus_Colony	
Shasta MVCD	*Culex pipiens quinquefasciatus*	CQ1	
Sutter-Yuba MVCD	*Culex tarsalis*	Yuba/Yolo99	
West Valley MVCD	*Culex pipiens quinquefasciatus*	WVCQ	

**Updated June 2015*

APPENDIX B

GUIDELINES FOR SHIPMENT OF MOSQUITOES

One option for districts which do not maintain susceptible colonies, or do not have the expertise on site but would still like to test mosquito populations for resistance, is to pre-arrange with other districts and ship mosquitoes to them. Shipping and testing should be arranged in advance with the receiving districts to ensure that they have the time, materials, and colony mosquitoes available for the tests, and to determine whether there is a cost associated with the tests to be performed. Before shipping mosquitoes, districts should verify with the receiving district which life stage would be preferable and the number needed for the tests desired. Districts need to keep in mind that there may be mortality associated with shipping and must account for that in their calculations of number of mosquitoes to send.

Shipping Adults

Adult mosquitoes should be kept cool and humid while shipping during the summer months. This can be accomplished by placing one or two artificial or Blue Ice®-type ice packs in the bottom of an insulated container, such as an ice chest/cooler box, and covering them with a dry towel. Mosquitoes can be placed in smaller, partially screened cartons within the container. Mosquitoes should be supplied with 10% sugar water solution through cotton ball puffs previously soaked in the sugar solution. If the cotton balls are too wet or dripping, mosquitoes will stick to the drippings at the bottom of the cartons and die. A damp towel or paper towels should be placed over the mosquito cartons in order to keep them humid. Any extra space in the ice chest should be filled with packaging paper or bubble wrap to ensure the cartons of mosquitoes do not shift during shipping. The container should then be taped shut and shipped overnight.

Shipping Pupae

Pupae usually withstand overnight shipping well if shipped in good conditions. Place pupae in Ziploc®-type bags filled with water (at least ¾ of the bag), close bag seal tightly, and place bag in standing position inside an insulated container, such as an ice chest/cooler box. If needed, use paper or other shipping materials to hold bag upright inside the cooler. Place Blue Ice®-type ice packs on the top part of the lunch cooler. Insulated lunch boxes with two compartments work well for shipment, as ice packs can

be placed in the top compartment, separate from the compartment holding the bag. Overnight shipping is required.

Shipping Larvae

Young larvae typically withstand shipping the best. High mortality often occurs when shipping late instar larvae. Larvae can be shipped in an insulated Thermos®-type jug filled to about ¾ with water. If shipping wild larvae, water from which the larvae were collected in the field is preferred to tap water. The container should be closed tightly and then taped to prevent leaks. Number of larvae per container will depend on the size of the container, but in general, up to 200 larvae withstand shipping.

Shipping Egg Rafts

Culex egg rafts can be collected onto strips of filter paper by carefully dipping the filter paper into the water to pick up the egg raft or by carefully transferring each egg raft with a soft paintbrush to the filter paper. Excessively wet filter paper strips should be blotted against a dry paper towel to remove as much water as possible. The filter paper with the egg rafts should be placed on a plastic petri dish, covered with the petri dish lid, taped shut, and shipped overnight. Overnight shipping is required, as egg rafts need to get to the destination as soon as possible. Egg rafts that are in contact with excess water may hatch during shipping.

APPENDIX C

STANDARDIZED PROTOCOLS

APPENDIX C - 1

BENCHTOP CUP ASSAY

Mosquito Larvicide Bioassay

This bioassay was developed based on procedures from Dr. Mir Mulla's laboratory and the World Health Organization guidelines (WHO 2005), and modified by Dr. T. Steven Su.

Purpose

1. Monitor susceptibility of mosquito populations to larvicides;
2. Evaluate resistance management strategies;
3. Quality control of mosquito larvicide;
4. Evaluate impact of biotic and abiotic factors on activity of larvicides.

Materials

1. Larvicides: technical grade materials, such as primary powders (VectoBac TP, AquaBac PP OSF, VectoLex TP) or certain commercial products (VectoBac WDG, VectoBac 12AS, VectoLex WDG) for microbials (*Bacillus thuringiensis var israelensis* - Bti and *Lysinibacillus sphaericus* - Lsph); technical grade materials such as S-methoprene or some fast release formulations (Altosid SR-5, Altosid SR-20) for insect growth regulator (IGRs) (Juvenile hormone analogs - JHA); technical grade materials or commercial formulations such as Natular G30 (2.5% spinosad) or Natular 2EC (20.6% spinosad) for spinosad.
2. Mosquitoes: Laboratory-reared susceptible colonies (*Cx. tarsalis* BFS or *Cx. pipiens quinquefasciatus* CQ1 recommended) and field-collected mosquitoes - up to early 4th instars for Bti products, up to 3rd instars for Lsph products, late 4th instars for methoprene products, up to early 4th instars for spinosad.

3. General conditions and supplies: temperature-controlled room, pipette, pipette tips, 4-oz styrofoam/waxed paper cups, 20ml glass vials, analytical balance, shelves, trays.

Assay set-up

1. Test population should be concurrently assayed with reference colony
2. Room temperature: 80°F – 85°F (26°C to 29°C)
3. Concentrations: Microbial larvicides - based on whole products; IGR and spinosad larvicides – based on active ingredients; 4 – 5 concentrations being diagnostic to yield 5 – 95% mortality
4. Final volume: 100 ml/cup
5. Number of larvae/cup: 25
6. Number of replicates per concentration: at least 3
7. Larval food addition: Microbial larvicides - adequate food to support normal growth during test period; IGRs (JHA) - adequate food to support larvae to pupation

Results

1. Time to read results: Microbial larvicides – bioassay results should be read at 24 h for Bti and spinosad products and 48 h for Lsph products; IGRs (JHA) – bioassays should be read when all individuals die or emerge.
2. Criteria for reading results: Microbial larvicides (Bti, Lsph and spinosad) – dead larvae (total larvae introduced – total survivors, with moribund larvae counted as dead); IGRs (JHA) – inhibition of emergence (IE), which should be calculated as total larvae introduced – total FREE pupal exuviae.
3. Parameters: % larval mortality for microbials and %IE for IGRs (JHA).

A.I.	Test material	Larval instar	Exposure time (hours)	Mortality reading
B.t.i.	VectoBac TP, VectoBac 12AS, VectoBac WDG, AquaBac PP, AquaBac XT, AquaBac DF3000, Teknar HP-D, Teknar SC	3rd - early 4th	24	Larval mortality
L. sphaericus	VectoLex TP, VectoLex WDG, Spheratax PP	3rd	48	Larval mortality
Methoprene	Technical, Altosid liquid larvicide	late 4th	Complete adult emergence	Inhibition of adult emergence
Spinosad	Technical, Natular 2EC, Natular G30	3rd	24	Larval mortality

Data analysis

Ideally data should be analyzed by a probit analysis software. Alternatively, log paper (dose-response line) can be used, or Microsoft Excel charting log dose against mortality probit.

Commonly used parameters are LC50 (IE50), LC90 (IE90). Data heterogeneity will be affected by fitness of larvae used, handling stress, number of concentration replicates, number of larvae per cup, larval food used, and cross contamination.

Result interpretation and application

The Resistance ratio (RR) should be calculated based on the reference colony. Populations can be classified as susceptible, tolerant (RR < 5), or resistant (RR > 5).

RR = LC50 or LC90 of test population / LC50 or LC90 of reference colony

Set up methoprene assay

Material Altosid liquid larvicide (5% AI)* Lot _____

Mosquitoes *Cx. quinquefasciatus* colony (late 4th instars)

Dilutions 0.1% (400 ul of 5% ALL + 19.6 ml water), 0.01%,
0.001%, 0.0001%, 0.00001% (working suspensions, 3rd - 5th vials)

Treatments

Concentration (ppb)		0.25	1	5	25	Check
ul of 0.00001%		**250**	**1000**			**0**
ul of 0.0001%				**500**		
ul of 0.001%					**250**	
Larvae/cup	Rep. 1	25	25	25	25	25
	Rep. 2	25	25	25	25	25
	Rep. 3	25	25	25	25	25

Temperature 80 - 85ºF

Time to read results When all individuals have died or emerged

* or technical S-methoprene if available, acetone will be used as solvent

Read methoprene assay

Material Altosid liquid larvicide (5% AI)* Lot _____

Mosquitoes *Cx. quinquefasciatus* colony (late 4th instars)

Treatments

Concentration (ppb)		0.25	1	5	25	Check
ul of 0.00001%		**250**	**1000**			**0**
ul of 0.0001%				**500**		
ul of 0.001%					**250**	
Larvae/cup	Rep. 1	25	25	25	25	25
	Rep. 2	25	25	25	25	25
	Rep. 3	25	25	25	25	25

Temperature 80 - 85ºF

Time to read results When all individuals have died or emerged

Results (Exuviae)

Concentration (ppb)		**0.25**	**1**	**5**	**25**	**Check**
L/cup	Rep. 1					
	Rep. 2					
	Rep. 3					

Mortality

Concentration (ppb)		**0.25**	**1**	**5**	**25**	**Check**
L/cup	Rep. 1					
	Rep. 2					
	Rep. 3					
	Total					

* or technical S-methoprene if available, acetone will be used as solvent.

Data fitness estimate

Plotting dose-response line on log paper

Set up Lsph assay

Material VectoLex WDG (650 ITU/mg)* Lot _____

Mosquitoes *Cx. quinquefasciatus* colony (3rd instars)

Dilutions 1% (200 mg in 20 ml), 0.1%, 0.01%,
0.001% and 0.0001% (working suspensions, 4th and 5th vials)

Treatments

Concentration (ppm)		0.0015	0.0025	0.0100	0.0250	Check
ul of 0.0001%		**150**	**250**	**1000**		**0**
ul of 0.001%					**250**	
Larvae/cup	Rep. 1	25	25	25	25	25
	Rep. 2	25	25	25	25	25
	Rep. 3	25	25	25	25	25

Temperature 80 - 85ºF

Time to read results 48 hours post treatment

* or VectoLex TP (2,000 ITU/mg if available)

Read Lsph assay

Material VectoLex WDG (650 ITU/mg)* Lot _____

Mosquitoes *Cx. quinquefasciatus* colony (3rd instars)

Treatments

Concentration (ppm)		0.0015	0.0025	0.0100	0.0250	Check
ul of 0.0001%		**150**	**250**	**1000**		**0**
ul of 0.001%					**250**	
Larvae/cup	Rep. 1	25	25	25	25	25
	Rep. 2	25	25	25	25	25
	Rep. 3	25	25	25	25	25

Temperature 80 - 85ºF

Time to read results 48 hours post treatment

Results (# dead)

Concentration (ppb)		0.0015	0.0025	0.0100	0.0250	Check
L/cup	Rep. 1					
	Rep. 2					
	Rep. 3					

Mortality

Concentration (ppb)		0.0015	0.0025	0.0100	0.0250	Check
L/cup	Rep. 1					
	Rep. 2					
	Rep. 3					
	Total					

* or VectoLex TP (2,000 ITU/mg if available)

Data fitness estimate

Plotting dose-response line on log paper

Set up Bti assay

Material VectoBac WDG (3,000 ITU/mg)* Lot _____

Mosquitoes *Cx. quinquefasciatus* colony (3rd - early 4th instars)

Dilutions 1% (200 mg in 20 ml), 0.1%, 0.01% and 0.001% (working suspensions: 3rd and 4th vials)

Treatments

Concentration (ppm)		0.05	0.10	0.15	0.25	Check
ul of 0.001%		**500**	**1000**			**0**
ul of 0.01%				**150**	**250**	
Larvae/cup	Rep. 1	25	25	25	25	25
	Rep. 2	25	25	25	25	25
	Rep. 3	25	25	25	25	25

Temperature 80 - 85ºF

Time to read results 24 hours post treatment

* or VectoBac TP (7,000 - 10,0000 ITU/mg if available)

Read Bti assay

Material VectoBac WDG (3,000 ITU/mg)* Lot _____

Mosquitoes *Cx. quinquefasciatus* colony (3rd - early 4th instars)

Treatments

Concentration (ppm)		0.05	0.10	0.15	0.25	Check
ul of 0.001%		**500**	**1000**			**0**
ul of 0.01%				**150**	**250**	
Larvae/cup	Rep. 1	25	25	25	25	25
	Rep. 2	25	25	25	25	25
	Rep. 3	25	25	25	25	25

Temperature 80 - 85ºF

Time to read results 24 hours post treatment

Results (# dead)

Concentration (ppb)		0.05	0.10	0.15	0.25	Check
L/cup	Rep. 1					
	Rep. 2					
	Rep. 3					

Mortality (7/18)

Concentration (ppb)		0.05	0.10	0.15	0.25	Check
L/cup	Rep. 1					
	Rep. 2					
	Rep. 3					
	Total					

* or VectoBac TP (7,000 - 10,0000 ITU/mg if available)

Data fitness estimate

Plotting dose-response line on log paper

Set up Spinosad assay

Material Natular G30 (2.5% spinosad)* Lot _____

Mosquitoes *Cx. quinquefasciatus* colony (3rd - early 4th instars)

Dilutions 1% (1 g in 100 ml), **0.1%** (working suspension: 2nd vial)

Treatments

Concentration (ppm)		0.0025	0.00625	0.0125	0.025	Check
ul of 0.01%		**100**	**250**	**500**	**1000**	**0**
Larvae/cup	Rep. 1	25	25	25	25	25
	Rep. 2	25	25	25	25	25
	Rep. 3	25	25	25	25	25

Temperature 80 - 85ºF

Time to read results 24 hours post treatment

* or Natular 2EC (20.6% spinosad)

Read Spinosad assay

Material Natular G30 (2.5% spinosad)* Lot _____

Mosquitoes *Cx. quinquefasciatus* colony (3rd - early 4th instars)

Treatments

Concentration (ppm)		0.0025	0.00625	0.0125	0.025	Check
ul of 0.01%		**100**	**250**	**500**	**1000**	**0**
Larvae/cup	Rep. 1	25	25	25	25	25
	Rep. 2	25	25	25	25	25
	Rep. 3	25	25	25	25	25

Temperature 80 - 85ºF

Time to read results 24 hours post treatment

Results (# dead)

Concentration (ppb)		0.0025	0.00625	0.0125	0.025	Check
L/cup	Rep. 1					
	Rep. 2					
	Rep. 3					

Mortality (7/18)

Concentration (ppb)		0.0025	0.00625	0.0125	0.025	Check
L/cup	Rep. 1					
	Rep. 2					
	Rep. 3					
	Total					

* or Natular 2EC (20.6% spinosad)

Data fitness estimate

Plotting dose-response line on log paper

APPENDIX C - 2

BOTTLE BIOASSAY

Mosquito Adulticide Bioassay

The Centers for Disease Control and Prevention (CDC) has put together guidelines for using the bottle bioassay as a surveillance tool to detect resistance to insecticides in vector populations. The guidelines can be found at the CDC website or at (http://www.cdc.gov/malaria/resources/pdf/fsp/ir_manual/ir_cdc_bioassay_en.pdf). In this bioassay, mosquitoes are exposed to the insecticide by tarsal contact as they land and walk on the treated surfaces of the bottle. Time mortality data gathered from the bottle bioassays is a measure of the time it takes for a compound to penetrate the mosquito, traverse intervening tissues, and reach and act on the target site (Brogdon and McAllister 1998a, 1998b). With this bioassay, districts can evaluate different concentrations of an insecticide, which is very useful because different mosquito species may require the use of different discriminating doses. Ideally, discriminating doses should be calculated for each species using the chosen susceptible laboratory reared colonies. This working group recommends that California agencies participating in resistance testing use the *Culex tarsalis* BFS (Bakersfield Field Station) colony, and the *Cx. pipiens quinquefasciatus* CQ1 colony as susceptible reference populations when testing field collected *Cx. tarsalis* and *Cx. pipiens* complex mosquitoes. Doses have been calculated by the CDC for various species and populations (Table C-2.1) and may be used as guidelines or starting points. Doses specific for the *Culex tarsalis* BFS (Bakersfield Field Station) colony and the *Cx. pipiens quinquefasciatus* CQ1 colonies will be revised and updated in 2016-2017, and an addendum to this document will be submitted as new information becomes available. The procedures below are adaptated from the CDC bottle bioassay guidelines mentioned above.

Materials

- 250 ml Wheaton bottles with Teflon-lined screw caps
- Micropipettes and tips

- Acetone
- Chemical(s)/technical products to be tested
- Aspirator
- Timer
- Labels

Preparation

1. Start with clean bottles. See below for cleaning procedures. Make sure bottles are dry before using them. If an oven is available, place bottles only (no caps) in oven at 50°C for 15 to 20 minutes.

2. Mix stock solutions in a fume hood. If stock solutions were previously prepared and stored in the refrigerator, bring them to room temperature before use. Gently swirl the stock solution to mix it before use.

3. To make stock solutions, dilute the appropriate amount of technical grade insecticide in acetone. The final volume for the stock solution will depend on how many tests will be run (suggested amounts can be found in Table C-2.1). Once the stock solutions are prepared, most can be stored in the refrigerator in light proof (amber) bottles until needed. Some insecticides do not store well and should be prepared immediately before the bioassay is run, such as Naled and Resmethrin.

4. Label all bottles accordingly. Make sure to label the cap as well and keep individual bottles and their caps together. The entire bottle, including the inside of the cap, will be coated with acetone or an insecticide solution.

Dosing the bottle

1. Add acetone to each bottle (1 to 2 ml). Fasten the cap on so none of the acetone evaporates until the insecticide dose has been added to the bottle.

2. Add the appropriate amount (Table C-2.1) of the stock solution (mixed in section 2 above) to the acetone already in the bottle. Put the lid back on tightly. Since the acetone will later be allowed to evaporate, this is not a further dilution. Make sure to add the amount needed directly to the acetone in the bottle. This is important to ensure even distribution of the chemical on the surfaces of the bottle when rolling it and evaporating the acetone.

3. Be sure to make control bottles to run alongside the insecticide bottles. These bottles should have acetone added but no stock solution (pesticide).

4. Swirl the acetone and pesticide inside the bottle so that the bottom is coated. Invert the bottle and swirl to coat the inside of the cap. Place the bottle on its side for a moment to let the liquid pool. Gently rotate the bottle so that the sides are evenly coated. Roll the bottle on its side back and forth for a minute or two.

5. Remove the cap and continue rolling the bottle on its side until all acetone has evaporated, i.e. until all visible signs of any liquid are gone from inside.

6. Leave the bottle on its side and its cap in an inverted position. Bottles and caps can be covered with a towel to prevent light from prematurely breaking down any pesticide. For best results in high humidity, let the bottle dry with caps off for 2-6 hours. In a dry climate drying time is considerably shorter but at least 1/2 hour before use.

If you do not use the bottles right away, put the caps back on and place them in a dark place for storage. Depending on the insecticide used, prepared bottles can be stored from 12 h to 5 days in this manner. Resmethrin and Naled bottles do not store well. If you are unsure of whether a bottle is still good, you can “test” it by putting some known susceptible mosquitoes in the bottle. If they die in the expected time frame the bottle is still good to use.

Running the Assay

1. Ideally, 15 to 25 female mosquitoes will be introduced to each bottle. If mosquitoes are limited, fewer mosquitoes may be used, but it is not recommended to use less than 10-15 mosquitoes per bottle. Aspirating the mosquitoes can be very time-consuming. It is recommended to collect all of the mosquitoes needed for all the bottles and separate them into "holding tubes" so that they can be introduced into each bottle faster, with a gentle shake of the tubes inside the bottles. Holding tubes (one tube per bottle) can be made using glass or plastic tubing and closing one end with tule cloth and the other with a cork for easy access. Mosquitoes can be aspirated into these tubes ahead of time, usually a couple of hours before the assay, to save transferring time. Blowing into the tubes can introduce humidity into the bottle, which may contribute to mosquito mortality and confound results. Alternatively, shake the tube into the bottle while covering the mouth of the bottle with cotton or a hand being careful to not let mosquitoes escape. Mosquito transfer can be a critical step in the bioassay because handling can contribute to mosquito mortality, and potentially infected field collected mosquitoes must not be allowed to scape within the work area.

2. Use 3 to 4 replicates per insecticide concentration and controls. The susceptible reference colony (*Cx. tarsalis* BFS or *Cx. pipiens quinquefasciatus* CQ1) should ideally be tested at the same time as the field populations. The susceptible species used during the testing should be the same as the field-collected species. For a more sensitive test, the field and susceptible colonies can also be matched for gender, age, blood/sugar feeding or other conditions.

   To determine the number of bottles needed, multiply the desired replicates by the number of insecticides to be tested (field population plus reference colony). For example, if you are testing 3 insecticides against 2 mosquito populations plus your susceptible population for comparison, you will typically need:

4	*	4	*	3	=	48 bottles
(replications)		(3 insecticides + acetone only)		(2 field + 1 susceptible populations)		

Adjustments can be made to the calculation above depending on number of mosquitoes or bottles available. Although it is ideal to run 4 replications, 3 may be sufficient. Moreover, acetone controls can be run with fewer bottles.

After calculating the number of bottles to be used in the bioassay, the number of mosquitoes needed can be estimated (for example, 16 bottles for each population would necessitate 320 female mosquitoes if you plan on using 20 per bottle). If there are not enough mosquitoes from each population, the number of insecticides tested, number of mosquitoes per bottle, or number of replicates per insecticide may be adjusted.

3. Examine the bottle to be sure all mosquitoes survived the transfer process. If you find one or two “crushed” or dead mosquitoes, make note of them. This is count “0”. Mosquitoes dead at zero minutes should be excluded from mortality calculations.

4. Start a timer and record how many mosquitoes are dead or alive (whichever is easier to count) every 15 minutes until all are dead. Mosquitoes are counted “dead” if they are moribund, lying on their sides or back and are unable to stand up or fly (even though their legs may be moving). “Spinners” – mosquitoes on their backs that are flapping their wings and going in circles are also counted as dead. Make sure everyone involved in resistance testing is in agreement with what constitutes a “dead” mosquito. Differences in counting dead mosquitoes can be a great source of variability when testing multiple populations and when more than one person is involved in the bioassay.

5. At the end of the assay, count the total number of mosquitoes in each bottle and calculate percent mortality for each time interval. Final counts to obtain the total

number of mosquitoes in each bottle can be facilitated by freezing bottles prior to counting.

6. Plot percent mortality (y axis) against time (x axis). Ideally mortality in the control bottles should be zero. In that case, percent mortality can be calculated by using the formula:

    Percent mortality = (# dead * 100) / total #

If mortality in the control bottles is greater than 10%, it is advisable to repeat the experiment, since mortality in those bottles should be very close to zero. It is advisable to adjust for control mortality using Abbott's formula. Percent mortality can be calculated as:

Percent mortality = [(# dead - # dead at 0 min)/(total # - # dead at 0 min)]*100

To clean bottles triple rinse them with acetone, disposing of the rinsate in an appropriately marked hazardous waste container and follow regulations for disposal of contents. Wash the bottles and caps with warm, soapy water, triple rinse with water, and then rinse with acetone, disposing of the acetone as mentioned above. Invert the bottles and caps and allow to air dry on the dish drain rack. If available, use an oven to make sure they are completely dry before using them again.

Table C-2.1 - Suggested stock solutions and bottle dosages to start with (Janet McAllister, CDC, 2014)		
Stock* (Techgrade AI + Acetone)	**Amount of Stock added to bottle**	**Final concentration/bottle****
Insecticides		
Chlorpyrifos 10 mg/ml	2 µl	20 µg/bottle
Etofenprox 5 mg/ml	2.5 µl	12.5 µg/bottle
Fenthion 10 µl/ml	80 µl	800 µg/bottle
Malathion 10 µl/ml	40 µl	400 µg/bottle
Naled 2 µl/50 ml	56.25 µl	2.25 µg/bottle
Permethrin 10 mg/ml	4.3 µl	43 µg/bottle
Prallethrin 10 mg/100ml	0.5 µl	0.05 µg/bottle
Resmethrin 10 mg/ml	3 µl	30 µg/bottle
Sumithrin 10 µl/ml	2 µl	20 µg/bottle
Temephos 10 mg/ml	85 µl	850 µg/bottle
Synergists		
DEF 10 µl/ml	12.5 µl	125 µg/bottle
Diethyl maleate 10 µl/ml	8 µl	80 µg/bottle
PBO 10 µl/ml	40 µl	400 µg/bottle

*Example: To make the stock for malathion (10 µl/ml), add 10 µl technical grade malathion to 990 µl acetone. The total volume is now 1 ml.

** Final concentrations are based on a density of 1 for the technical grade insecticide. If insecticide is purchased as a solid instead of a liquid, then the proper amount can be weighed to come to a final concentration in µg/bottle. Example: 10 mg AI dissolved in 1 ml acetone gives 10 µg/µl.

Running the assay with the actual product versus technical grade

This group recommends that technical grade is always used for the bioassays. Studies have been conducted comparing using technical grade insecticides and products not containing synergists and found that, in some instances, a different mortality curve is obtained even when adjustments for the dose are made based on the amount of technical grade. That may be due to the presence of other ingredients in the product. Procedures have been developed by Dr. Jack Petersen at Florida A&M University, and PHEREC (Public Health Entomology Research and Education Center) (Petersen 2004) for preparing test solutions from formulated pesticide products instead of technical grade. It is important to note that, if using formulated product, the susceptible colony/colonies will need to be used to calibrate the discriminating doses for each product to be used prior to performing bioassays on the field populations. Moreover, if testing a product containing a synergist in its formulation, the bioassay may not be able to detect very low levels of resistance.

Calibrating the bottles

The concentrations listed in the table above were developed at CDC for different susceptible colonies. Because each mosquito species may react to insecticides differently, this group recommends a revision of the doses recommended above, using the CQ1 and BFS colonies recommended for use as control. These doses will be updated as they become available. To determine bottle reference doses, the procedure below may be used (adapted from the CDC bioassay guidelines, http://www.cdc.gov/malaria/resources/pdf/fsp/ir_manual/ir_cdc_bioassay_en.pdf). The diagnostic dose should be the dose that kills 100% of the susceptible mosquitoes sometime between 30 and 60 minutes and is below the saturation point.

1. Prepare bottles as above making several sets of bottles with a range of different concentrations.

2. Run assays on known susceptible mosquitoes. It may be necessary to run additional sets narrowing the ranges of concentrations until the optimal diagnostic dose is determined.

3. A graph of the results should show that with increasing concentration the line gets straighter, steeper and moves toward the Y axis (left). If doses tested are in the correct range, the line will reach the "saturation point", which is the point where increasing the concentration does not change the line. Increasing the concentration does not cause the insecticide to penetrate the mosquito and get to the target site any quicker.

For example, increments of 10 µg may be used, starting with 10 µg/bottle and going to 100 µg/bottle. If there is not a clear saturation point it may be necessary to run additional bottles with < 10 µg /bottle or > 100 µg/bottle. If a saturation point is evident, values can be further refined by running more bottles at smaller increments near the break.

Alternatively serial dilutions may be run following published protocols that base the diagnostic dose on LC90 or LC95. Some assume the discriminating dose to be 3*LC90 or 2*LC95.

Scoring sheet

The sheet below is provided as an example and can be modified to incorporate any additional information, such as person running the bioassay, supplier information, lot number, date of bottle preparation, etc.

Bottle Bioassay

Pesticide/Active ingredient: _____Test Date: _____

Collection location: _____

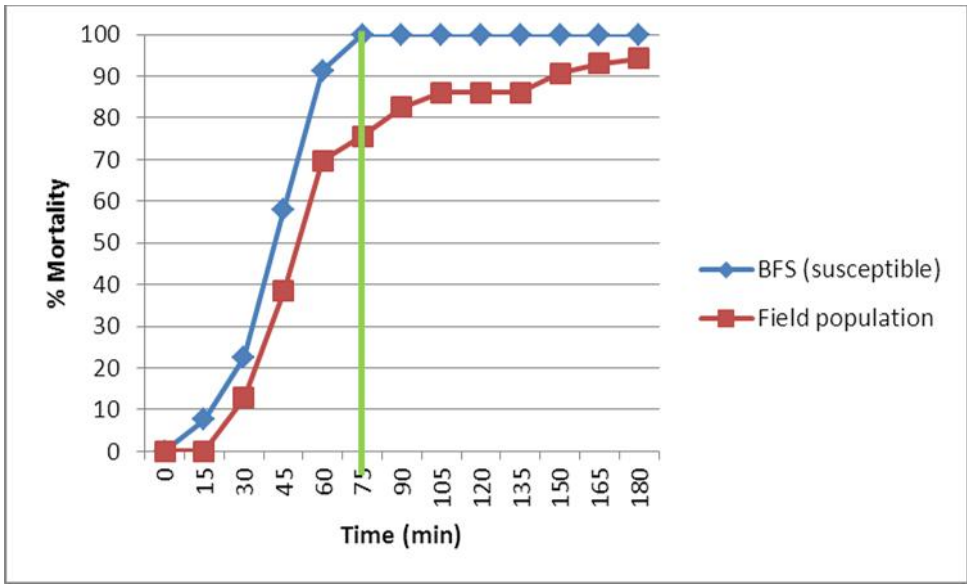
Species: _____Age (days): _____

Dose per bottle (micrograms): _____Air temp. (ºF): _____

Elapsed Time (min)	# dead				# alive				Total Dead	Percent Mortality
	R1	R2	R3	R4	R1	R2	R3	R4		
0										
15										
30										
45										
60										
75										
90										
105										
120										
135										
150										
165										
180										
# mosq in each bottle:					**Total # of mosq:**					

Interpreting bottle data

This group recommends, where possible, running bioassays of the field populations side by side with the susceptible colony for meaningful comparisons. Alternatively, but not ideally, bioassay results may be compared to a known response range of a susceptible reference colony, previous data generated from the same site over time, or within a geographic area. A resistance threshold for each insecticide can be determined by drawing a straight line down from the point at which 100% mortality was obtained in the susceptible colony. If any of the mosquitoes survived beyond this threshold, the data may be interpreted to mean that these survivors represent that proportion of the population that has something allowing them to delay the insecticide from reaching the target site and acting or, in other words, they have some degree of resistance. In the example below all mosquitoes that died before 75 minutes were susceptible. Any test mosquitoes that were to survive beyond the 75 minute threshold would be interpreted to have some degree of resistance. It is important to note that resistance detected with this bioassay may not necessarily translate into control failure. This test is much more sensitive in detecting the development of resistance than relying on observing failure of control in the field to detect resistance. The bioassay allows early detection of resistance in a population before the loss of the use of a product, informing management decisions in a timely manner to preserve susceptibility to the chemical.



If desired, a survival analysis can be conducted using Weibull distribution or the Kaplan-Meier survival analysis. A logarithmic extrapolation of time to death would not apply because mortality may never occur in some mosquitoes. Because reading of mortality is done at predefined points rather than exact times of death, time of mortality functions as a dependent variable versus continuing scoring of mortality and the Weibull distribution may be used (Pinder III et al 1978, Wagner et al 1984, Ames 2011). Alternatively, the knockdown curves for all mosquito populations can also be analyzed using the Kaplan-Meier survival analysis (Therneau and Grambsch 2000).

Using Inhibitors/Synergists

One of the great advantages of the bottle bioassay is the ability to investigate resistance mechanisms by adding synergists/inhibitors to the bottle if resistance is suspected. Piperonyl butoxide (PBO) suppresses oxidase activity, S.S.S-tributlyphosphorotrithioate (DEF) suppresses esterase activity, and diethyl maleate (DEM) suppresses glutathione-S-transferase (GST) activity. If a population is found or suspected to be resistant, and the mechanism is based on an elevated level of oxidases, esterases, or GSTs, by adding the inhibitors mentioned above and observing the

consequent changes in that population's bioassay response, it is possible to determine which mechanism(s) is (are) contributing to the resistance. For example, if permethrin resistance were detected in a population, and a subsequent set of bottles that contained permethrin + PBO revealed results an apparent recovery to full susceptibility, this finding would suggest that an oxidase mechanism may be responsible for the permethrin resistance in this population. If no change in susceptibility were observed, it would suggest that oxidase may not be the mechanism. If partial recovery were observed, it would suggest that oxidases are involved but some other mechanism is also playing a role in the resistance observed in the population. It is very common to have multiple mechanisms contributing to resistance. Knowing the mechanisms can help guide any resistance management decision. In the example above, if oxidases are determined to be the major mechanism involved, products containing PBO could be selected to overcome the oxidase resistance, potentially achieving better control and managing resistance in that population.

APPENDIX C-3

MICROPLATE ASSAYS

Modified levels of detoxification enzymes that metabolize insecticides can be common mechanisms of resistance. Enhanced levels or modified activity of esterases, oxidases, and/or glutathione-S-transferases (GSTs) may prevent the insecticide from reaching its site of action (Brogdon and McAllister 1998). Microplate or enzymatic assays are used to measure increased levels of these enzymes, protein, and the presence of the altered target site for organophosphates (McAllister et al 2012). The protocols below have been provided by Dr. Janet McAllister. It is recommended that any person receive hands-on training prior to performing the assays described below.

General guidelines:

- Assays should be run in triplicate on microplates.
- Mosquitoes to be tested should be knocked down by freezing or tested fresh. If frozen, mosquitoes should be kept in a freezer (-80°C) before testing.
- All solutions should be prepared before starting the tests, and directions can be found in the protocols below. Following the directions, there should be enough material for 3 or 4 biochemical assays and the protein assay run in triplicate from one mosquito. Adjust quantities according to the number of mosquitoes and populations to be tested. Note that some solutions have much shorter shelf-life than others.

MOSQUITO PREPARATION

Standard:

1. Homogenize 1 mosquito (adult or larva) in 100 µl of buffer (KPO_4)

2. Dilute to 1 ml with 900 µl of additional buffer

3. Follow the directions for each assay you wish to perform, and dilute controls following standard dilution steps

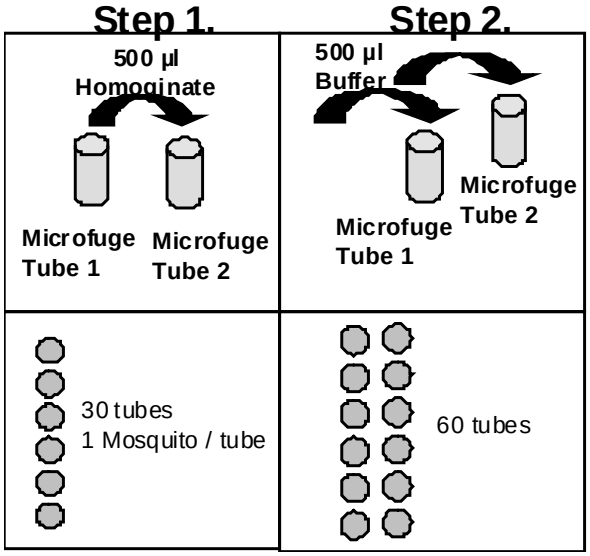
To increase the number of tests to run: dilute mosquito homogenate

Additional:

1. Remove 500 µl of mosquito homogenate and pipette into separate tube

2. Dilute to 1 ml with 500 µl of additional buffer to each tube

3. Double incubation times for elevated non-specific α-esterase, elevated non-specific β-esterase, and oxidase assays, and dilute controls following additional dilution steps



POTASSIUM PHOSPHATE (KPO_4) BUFFER

Mix: 6.6 g dibasic potassium phosphate
1.7 g monobasic potassium phosphate
1000 ml with dH_2O

Adjust pH to 7.2 with hydrochloric acid (HCl) and sodium hydroxide (NaOH)

This buffer is used for most of the assays and can be stored indefinitely at room temperature.

0.25m SODIUM ACETATE (NaOAc) BUFFER

Mix: 83 ml of 3M Sodium Acetate (NaOAc) with 900 ml dH_2O

Adjust pH to 5 with glacial acetic acid and sodium hydroxide (NaOH)

Adjust final volume to 1 liter

This buffer is used with the oxidase assay and can be stored indefinitely on the shelf at room temperature.

3M NaOAc can be purchased or made by dissolving 408.1 g of NaOAc in 800 ml of water. Once dissolved, adjust the final volume to 1 liter.

ELEVATED NON-SPECIFIC α-ESTERASE ASSAY

This assay measures levels of non-specific α-esterases present.

Make α-naphthyl acetate:

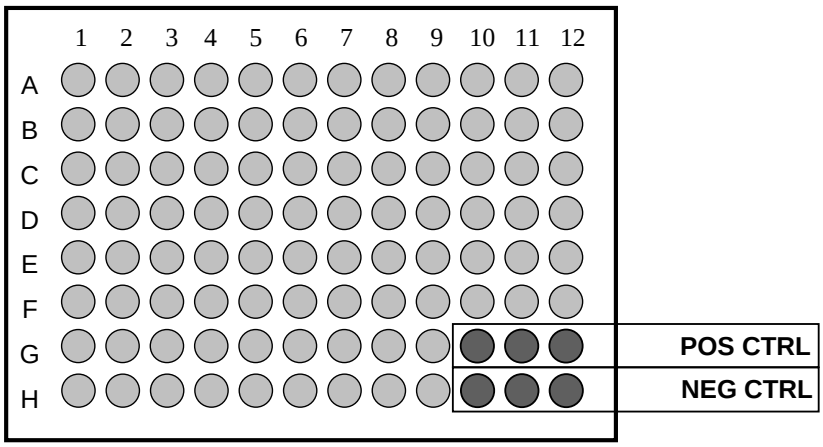
Dissolve:	56 mg α-naphthyl acetate
	20 ml acetone
Add:	80 ml KPO_4 buffer

Solution can be stored at 4°C.

Make dianisidine immediately before use or it will color-degrade. If desired, it can be prepared ahead of time and stored for several weeks at 4°C in a **light proof** bottle. Check color of dianisidine before use. Color should be pale yellow. If color is amber, discard and make fresh.

Weigh out 100 mg 0-dianisidine tetrazotized. Add 100 ml dH_2O immediately before use.

1. Homogenize mosquitoes as outlined in Mosquito prep
2. Put 100 µl mosquito homogenate in each well
3. Add 100 µl α-naphthyl acetate to each well
4. Incubate at room temperature for 10 min.
5. Add 100 µl Dianisidine to each well
6. Incubate 2 min and read using the 540 nm filter



POS CTRL = α-naphthol
NEG CTRL = Buffer (KPO_4)

ELEVATED NON-SPECIFIC β-ESTERASE ASSAY

This assay measures levels of non-specific β-esterases present.

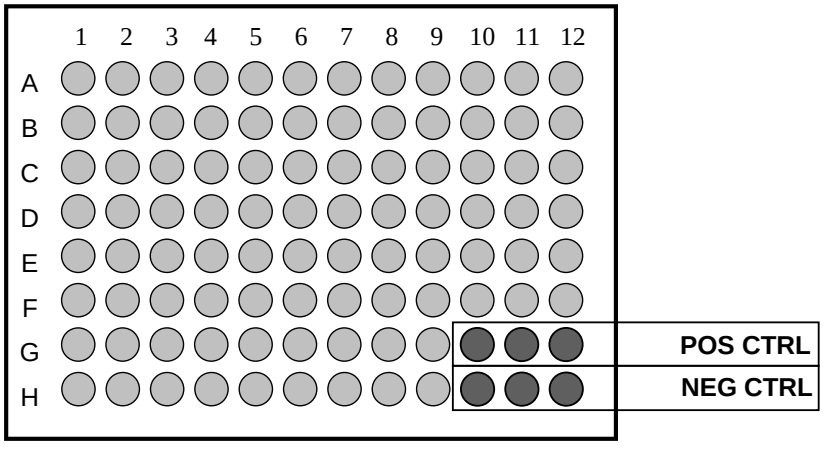
Make β-naphthyl acetate:

Dissolve:	56 mg β-naphthyl acetate
	20 ml acetone
Add:	80 ml KPO_4 buffer

Solution can be stored at 4°C. Make sure it is brought back to room temperature before use by placing in warm water bath (it may come partially out of solution when cold). Make dianisidine immediately before use or it will color-degrade. If desired, solution can be prepared ahead of time and stored for several weeks at 4°C in a **light proof** bottle. Check color of dianisidine before use. Color should be pale yellow. If color is amber, discard and make fresh.

Weigh out 100 mg 0-dianisidine tetrazotized. Add 100 ml dH_2O immediately before use.

1. Homogenize mosquitoes as outlined in Mosquito prep.
2. Put 100 µl mosquito homogenate in each well.
3. Add 100 µl β-naphthyl acetate to each well.
4. Incubate at room temperature for 10 min.
5. Add 100 µl Dianisidine to each well.
6. Incubate 2 min and read using the 540 nm filter.



POS CTRL = β-naphthol
NEG CTRL = Buffer (KPO_4)

OXIDASE REACTIONS

This assay measures heme peroxidase levels.

Make TMBZ:

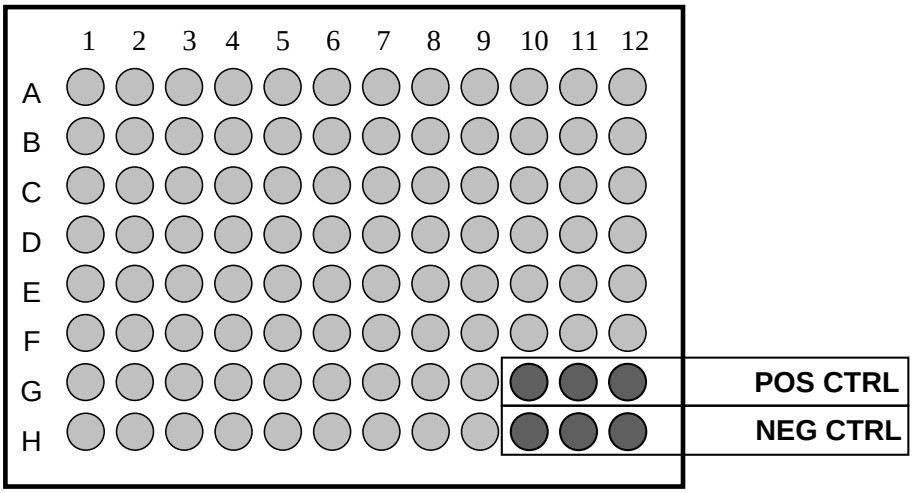
Dissolve:	50 mg 3,3',5,5'-Tetramethyl-Benzidine Dihydrochloride*(TMBZ [2HCL] or TMBZ)in 25 ml Methanol
Add:	75 ml 0.25M Sodium Acetate buffer**

Solution can be stored for a few days at 4°C. If this reagent has turned light blue, discard and make a fresh batch.

1. Put 100 µl mosquito homogenate sample in appropriate wells
2. Add 200 µl TMBZ
3. Add 1 drop (25 µl 3% hydrogen peroxide [H_2O_2])
4. Incubate for 5 min. then read plate with a microplate reader using a 620 nm filter

*TMBZ [2HCl] will dissolve if left to sit for a few minutes. TMBZ will dissolve if swirled under hot water out of the tap. **DO NOT** heat with an open flame or on a hot pad. **DO NOT** shake vigorously to aid in dissolving.

**It is very important the pH is exactly 5 for the 0.25M Sodium acetate buffer. Use acetic acid to adjust the pH.



POS CTRL = Cytochrome-C
NEG CTRL = Buffer (KPO_4)

PROTEIN ASSAY

This assay measures the amount of total protein present. When compared to a standard curve, can be used to quantify the size of each mosquito.

Make Protein Dye Reagent

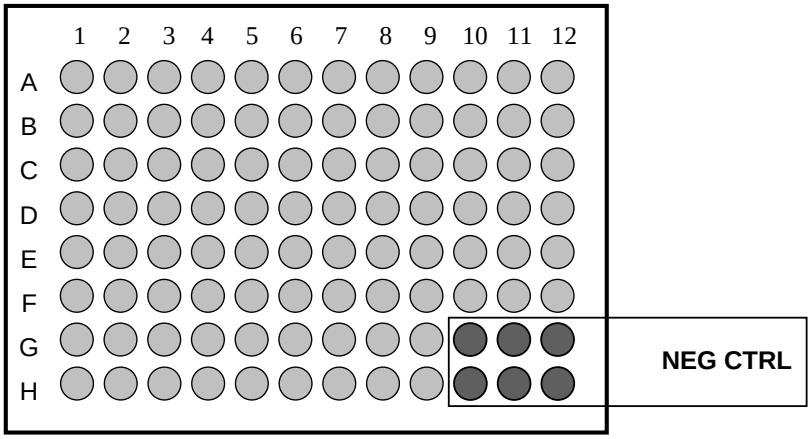
Mix: 20 ml Protein dye concentrate

80 ml dH_2O

Reagent can be stored indefinitely at 4°C in a **light proof** bottle.

1. Put 20 µl mosquito homogenate samples to each well
2. Add 80 µl KPO_4 buffer
3. Add 200 µl Protein dye reagent
4. Read plate immediately (T_0) with microplate reader using 620 nm filter

Assay can be run with bovine serum albumin to make a standard curve, which will relate the observed absorbance to the amount of protein present in a mosquito.



NEG CTRL = Buffer (KPO_4)

GLUTATHIONE-S-TRANSFERASE

This assay measures the level of Glutathione S-Transferase present.

Make reduced glutathione:

Mix: 61 mg reduced glutathione

100 ml KPO_4 buffer

Reagent can be stored for 3-4 d at 4°C.

Make cDNB:

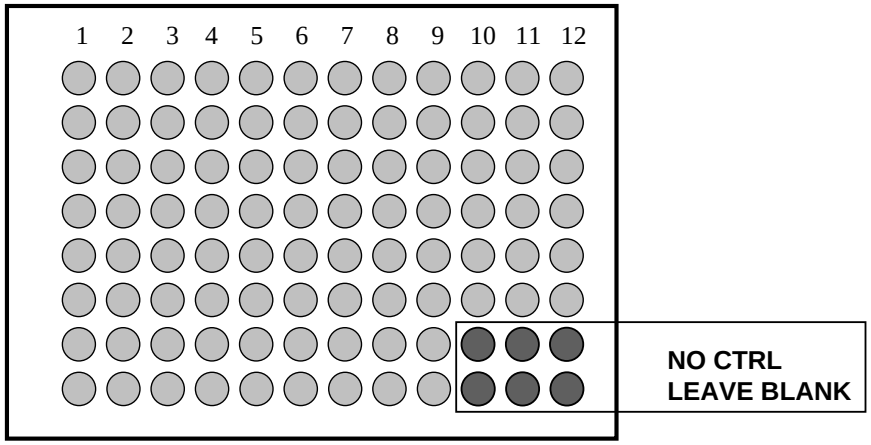
Dissolve: 20 mg 1-chloro-2,4'-dinitrobenzene

10 ml Acetone

Add: 90 ml KPO_4 buffer

Reagent can be stored for 3-4 d at 4°C.

1. Put 100 µl mosquito homogenate sample in appropriate wells
2. Add 100 µl reduced glutathione
3. Add 100 µl cDNB
4. Read plate immediately (T_0) with microplate reader using 340 nm filter
5. Read plate at 5 min. (T_5) with microplate reader using 340 nm filter
6. Subtract the T_0 reading from the T_5 reading and use this for your statistical analysis



ACETYLCHOLINE ESTERASE ASSAY

This assay measures the amount of acetylcholine esterase present.

Make ATCH:

Dissolve: 75 mg Acetylthiocholine iodide (ATCH)

10 ml Acetone

Add: 90 ml KPO_4 buffer

It can be stored for 3-4 d at 4°C.

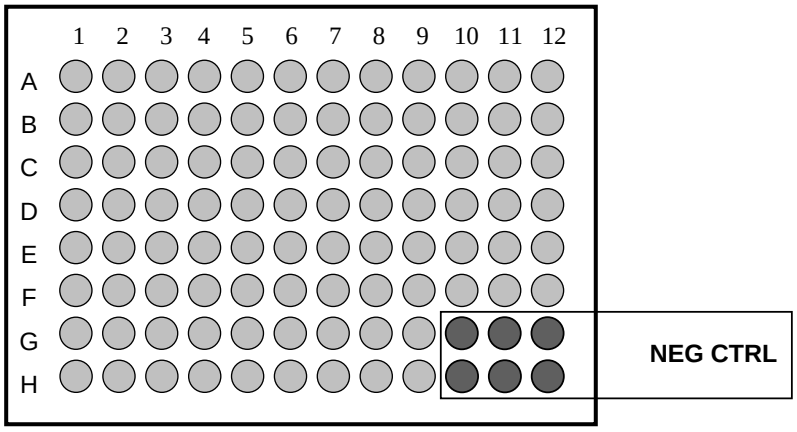
Make DTNB:

Mix: 13 mg Dithio-bis-2-nitrobenzoic acid (DTNB)

100 ml KPO_4 buffer

It can be stored for 3-4 d at 4°C.

1. Put 100 µl mosquito homogenate samples in appropriate wells
2. Add 100 µl ATCH to each well
3. Add 100 µl DTNB to each well
4. Read plate immediately (T_0) with microplate reader using 414 nm filter
5. Read plate at 10 min. (T_{10}) using 414 nm filter
6. Subtract the T_0 reading from the T_{10} reading and use this for your statistical analysis



NEG CTRL = Buffer (KPO_4)

INSENSITIVE ACETYLCHOLINE ESTERASE ASSAY

This assay determines if altered acetlycholine site is present. If very light yellow; no altered site, homozygous dominant; very dark yellow; altered target site, homozygous recessive; intermediate yellow; heterozygous individuals are present.

Make ATCH:

Dissolve: 75 mg Acetylthiocholine iodide (ATCH)
21 mg propoxur* (Baygon)
10 ml Acetone

Add: 90 ml KPO_4 buffer

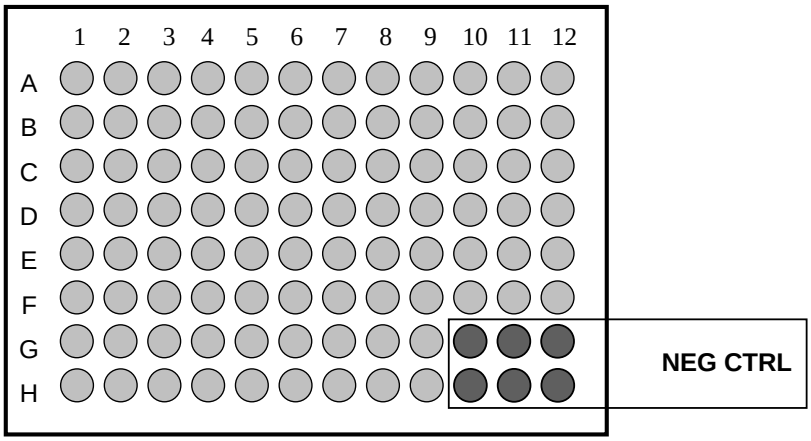
It can be stored for 3-4 d at 4°C. *Propoxur won't dissolve until KPO_4 buffer is added.

Make DTNB:

Mix: 13 mg Dithio-bis-2-nitrobenzoic acid (DTNB)
100 ml KPO_4 buffer

It can be stored for 3-4 d at 4°C.

1. Put 100 µl mosquito homogenate samples in appropriate wells
2. Add 100 µl ATCH to each well
3. Add 100 µl DTNB to each well
4. Read plate immediately (T_0) with microplate reader using 414 nm filter
5. Read plate at 10 min. (T_{10}) using 414 nm filter
6. Subtract the T_0 reading from the T_{10} reading and use this for your statistical analysis



NEG CTRL = Buffer (KPO_4)

CONTROLS

Stock for Oxidase: 10 mg Cytochrome-C (from bovine heart) dissolved in 100 ml Na Acetate buffer, pH 5 (same buffer as used for mixing TMBZ)***

Stock for α-Esterase: 50 mg α-naphthol dissolved in 10 ml acetone. Add 90 ml KPO_4 buffer

Stock for β-Esterase: 50 mg β-naphthol dissolved in 10 ml acetone. Add 90 ml KPO_4 buffer

1. Place 1-1.5 ml aliquots of stocks in microfuge tubes and freeze. Use light proof storage containers
2. When ready to use on a plate, thaw one tube of either stock, depending on test

Standard

Dilute the oxidase stock 1:55 (i.e. 22 µl cytochrome-C stock, 1.2 ml KPO_4 buffer)

Dilute the α-esterase stock 1:35 (i.e.35 µl α-naphthol stock, 1.2 ml KPO_4 buffer)

Dilute the β-esterase stock 1:35 (i.e.35 µl β-naphthol stock, 1.2 ml KPO_4 buffer)

Additional

Dilute the oxidase stock 1:110 (i.e. 11 µl cytochrome-C stock, 1.2 ml KPO_4 buffer)

Dilute the α-esterase stock 1:70 (i.e.17.5 µl α-naphthol stock, 1.2 ml KPO_4 buffer)

Dilute the β-esterase stock 1:70 (i.e.17.5 µl β-naphthol stock, 1.2 ml KPO_4 buffer)

Add 100 µl of the appropriate control to each of 3 wells on each plate you run as a positive control.

3. You can re-freeze the unused stocks as long as you have kept them protected from light.

*** Cytochrome-C is very photo labile. Make sure it is the last thing added to the oxidase plates before adding the TMBZ and H_2O_2.

The purpose of the "positive" controls is to allow plate to plate comparisons. The same amount of enzyme is added to each plate in the control and should give the same absorbance reading. A significant change in absorbance reading may mean some of the chemicals used are contaminated, out of date or were not mixed properly. A "negative" control is also recommended to add to each microplate. The negative control used by CDC is straight buffer with no mosquito ground in it.

The protein microplate assay measures the amount of total proteins in each mosquito. It can be used to correct for size when comparing different species or different "broods" of the same species. Use of this assay allows for the "correction" for larger mosquitoes possibly having higher enzyme levels due to their size. It also gives you a measure of the size of the mosquito by comparing them to a standard curve. The standard curve can be determined using bovine serum albumin. Procedures for how to do this come in a booklet provided by the manufacturer of the protein detection system we use.

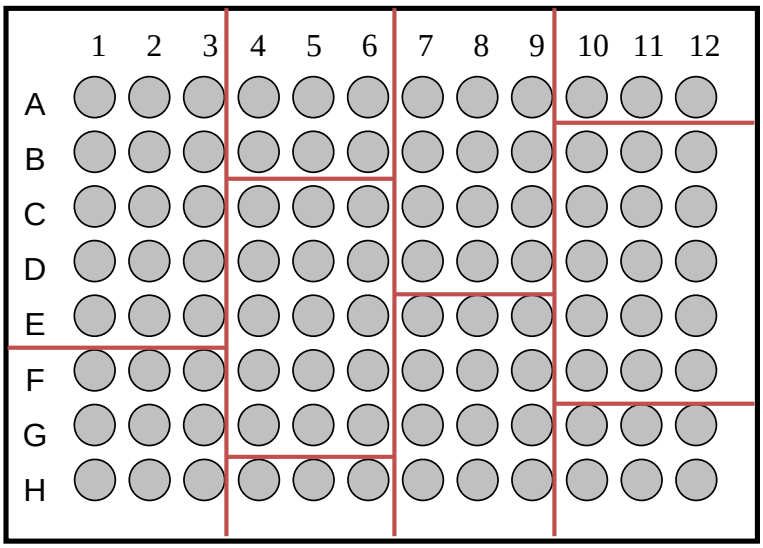
Tips and Considerations

Loading a microplate:

When loading a microplate in triplicate for a single enzyme assay, load the first three wells across (A 1-3) with homogenate from the first mosquito. Load the next mosquito in the wells directly below the first (B 1-3). Continue down the plate until you reach the bottom, move to the right and begin at the top again. Wells A 4-6 should contain your ninth mosquito if you have followed this pattern. The last 6 wells on the plate should be loaded with your positive and negative controls.

Example: Mosquito # 1 would go into wells A1, A2, A3

Mosquito # 2 would go into wells B1, B2, B3



Handling Data:

There are several ways you can handle your data collection. If it is not needed to quantify the amount of enzymes present, a known standard can be loaded to the plate that will give you an absorbance reading of your resistance threshold. The plate can then be scored as lighter or darker than the standard. By the same token, a color dot on a card can be created and used for comparison. In order to quantify how much enzyme is present, either a microplate reader (the best method by far) needs to be used or a scanner to put an image into a computer, then a software to measure the density of each well.

Determining a Resistance Threshold:

To determine a resistance threshold, run microplate assays on a susceptible population. The upper range limit of absorbance values for the susceptible population is the resistance threshold. Any absorbance higher than this limit can be considered resistant. The data collected with the microplate assay and bottle bioassay should correlate for populations with resistance. That is to say if 10 % of the population has resistance in it according to the bottle bioassay, then if a single mechanism is involved, such as elevated oxidase levels, 10% of the population should also have absorbance values above the resistance threshold. This may not be the case where multiple mechanisms are involved. Some resistance mechanisms, *kdr* for example, are not detected directly by using a microplate assay.

APPENDIX C - 4

MOLECULAR ASSAY

Knockdown resistance (*kdr*) mechanism in *Culex pipiens* complex

The insect voltage-gated sodium channel (VGSC) is the target of pyrethroid insecticides. Certain mutations in the VGSC gene are associated with resistance development. The genetic mutation of the nucleotide base from A to T (L1014F) that converts wildtype amino acid Leucine (L) to phenylalanine (F) at codon 1014 is the most prevalent pyrethroid-associated resistance mechanism. This mutant gene is termed knockdown resistance (*kdr*) and was originally discovered in houseflies. The L1014F mutation is the most widely distributed mutation around the world conferring *kdr* resistance. The purpose of this assay is to test *Culex pipiens* and *Cx. quinquefasciatus* for the L1014F mutation in the VGSC associated with *kdr*-mediated pyrethroid resistance. The method described below is based on Chen et al. 2010.

Mosquitoes

Field caught *Culex pipiens* complex mosquitoes can be preserved in 70-90% ethanol, frozen at -80°C or tested fresh. Samples can be mixed sex adults or 4th instar larvae. It is recommended that individuals be tested to determine specific genotype profiles, but pools can also be used as a screening tool.

DNA Isolation

DNA can be isolated using common DNA isolation methods (such as Life Technologies MagMAX or Qiagen DNeasy Tissue kit). DNA is usually eluted at 30-50 µL volume per mosquito.

It is recommended that the abdomens of female mosquitoes be removed to prevent genotype cross referencing due to the possible genotype contamination from sperm in the spermatheca.

Assay

Primers and probes:

Forward primer: 5’- GTG TCC TGC ATT CCG TTC TT - 3’

Reverse primer: 5’- TTC GTT CCC ACC TTT TCT TG - 3’

Probe L1014F: 5’- FAM - CAC GAC AAA ATT TC - MGB - 3’

Probe Wildtype: 5’- VIC - CTC ACG ACT AAA TTT C - MGB - 3’

Reagents:

Taqman kit: *TaqMan® Universal Master Mix II with UNG* (Life Technologies) or other Taqman QPCR assay kits that are suitable for corresponding platform (i.e., SsoFast™ Probes Supermix, Bio-Rad).

Platform:

Applied Biosystems 7500 or other QPCR machines

Reaction Setup:

For 25 μL reaction volume, combine the following:

Components	Working Concentration	Vol / rxn (µL)
Molecular grade DI Water		8
Universal Master Mix II or equivalent	2X	10
Forward primer	900 nM	0.25
Reverse primer	900 nM	0.25
Probe L1014F	200 nM	0.25
Probe Wildtype	200 nM	0.25
Genomic DNA Template	5 ng	5.00
Total Volume		**25.00**

Thermal Cycling Conditions:

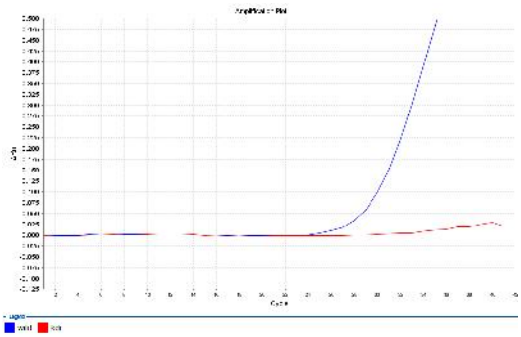
50°C/2 min X 1 cycle (for kits with UNG and SsoFast Probe Supermix)

95°C/10 min X 1 cycle

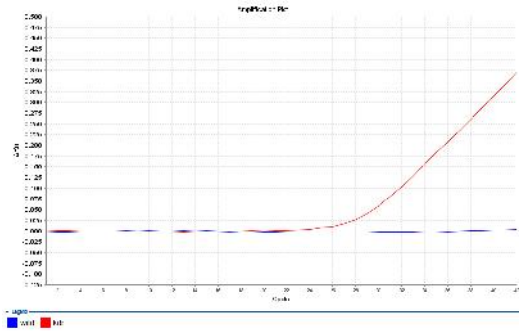
(95°C/10sec, 60°C/1 min) X 40 cycles

Result Interpretation and Examples

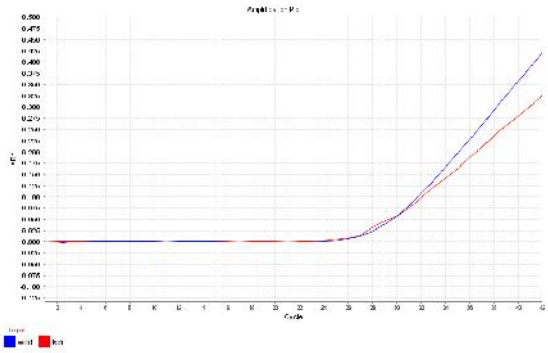
Using data analysis software associated with your QPCR machine platform, score each well for specific probe dye amplification. The *kdr* assay uses a wildtype allele specific probe labeled with VIC dye and a *kdr* specific probe labeled with FAM dye. In the assay results, a substantial increase in VIC fluorescence indicates a homozygous wildtype (SS), a substantial increase in FAM fluorescence indicates a homozygous *kdr* mutant (RR), and a, usually intermediate, increase in both signals indicates a heterozygote (RS) (Figure C-4.1).



(A). A substantial increase in VIC fluorescence indicates a homozygous wildtype (SS).



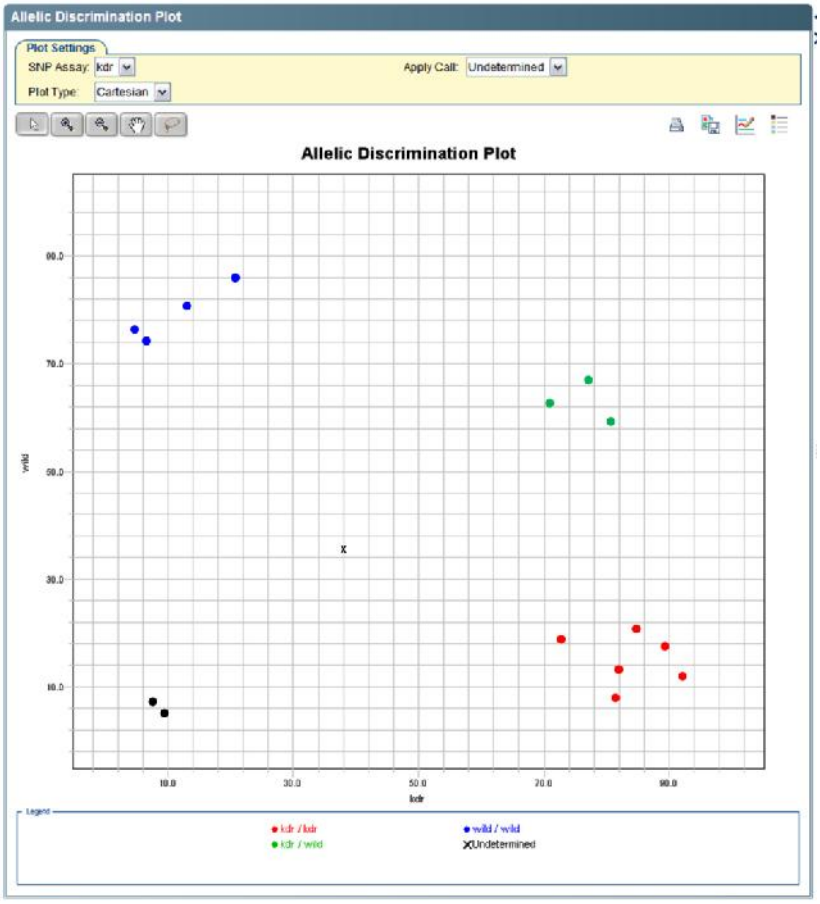
(B). A substantial increase in FAM fluorescence indicates a homozygous *kdr* mutant (RR).



(C) An intermediate-substantial increase in both VIC and FAM fluorescence indicates a heterozygote (RS).

Figure C-4.1. Taqman assay showing fluorescence increase for each genotype.

Most QPCR machines come with software that can perform allelic description experiments in that genotypes are automatically called and plotted against each other in bi-directional scatter plots (Figure C-4.2). The plot is useful to examine frequencies of each alleles of a given population. If using this method, be sure to check the raw data to assure the program properly assigns the sample to the correct allele quadrant.



Blue dots -homozygous wildtype (SS) individuals

Red dots -homozygous *kdr* individuals (RR)

Green dots -heterozygous individuals (RS)

Black dots -NTC (water only) controls

A black X means the machine is not able to call the genotype due to amplification quality issues.

Figure C-4.2. Allelic discrimination plot showing allelic distribution of 14 *Cx. pipiens* complex mosquitoes

The method described above is currently used by the California Department of Public Health (CDPH) and service is provided for a fee. Districts interested in testing mosquitoes for *kdr* are encouraged to contact CDPH to arrange for shipping and testing. Dr Shaoming Huang, of San Joaquin MVCD, has recently optimized this protocol for the *Culex pipiens* complex populations in California and this document will be updated with the optimized protocol as soon as it becomes available.

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